

Content and Volume Overview

Bernard Jégou, Inserm, EHESP-School of Public Health, Rennes, France

Michael K Skinner, Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, USA

© 2018 Elsevier Inc. All rights reserved.

Summary

Overview of Volume 1 of Male Reproduction topics involves the sections of testis, development, endocrinology, male reproductive tract, male reproductive physiology, comparative mammalian male reproduction, and environmental toxicology.

Overview

Volume 1 is focused on male reproduction in mammals and has the correlated male reproduction in nonmammal species outlined in Volume 6 on Comparative Reproduction. The first section involves Testis Biology, as the testis is the location of spermatogenesis and source for the germline or spermatozoa, which allows propagation of the species. An overview and history of testis biology is presented followed by overviews of the structure and cell types in the testis. The detailed endocrinology associated with testis function and development is presented. The following section is on Testis Cell Biology that provides the cell and molecular biology aspects of the Sertoli cell that physically support the developing spermatogenic cells and Leydig cells that provide the source for androgens/testosterone in the male for testis function and a large number of other tissues in the male. The seminiferous tubules that are surrounded by peritubular cells and testicular macrophages in the interstitial between the tubules along with vascular cells are also reviewed. The spermatogenic cells in regard to structure, cell biology, and various cell–cell interactions are reviewed. The developmental aspects of the testis are reviewed in the male gonadogenesis and male sex determination chapters. The critical phenotypic and molecular aspects of the sex determination are reviewed. The subsequent pubertal developmental period is then presented. The adult testis physiology topics that focus on spermatogenesis, the associated syncytium, seminiferous cycle, spermiation, and the blood testis barrier are also reviewed. Methodologic reviews of testis cell and organ cultures and germ cell transplantation are presented. Finally, the physiological impacts of castration are reviewed.

The next major section deals with Male Endocrinology. The hypothalamus, pituitary, and testis axis is reviewed with subsequent chapters on the cell and molecular aspects of pituitary cells. Specific chapters on hormones, including Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH), Inhibin, Activin, and Androgen, are presented. The genomic and nongenomic actions of androgens, as well as dihydroxytestosterone (DHT), are reviewed. The associated androgen actions on somatic cells, brain programming, and aggressive behavior are then reviewed.

The Male Reproductive Tract section has a series of chapters for each organ and developmental process. The male reproductive tract fetal development is reviewed with a focus on Wolffian duct development. In the adult, the spermatozoa produced in the testis first enter the rete testis and travel through the efferent ducts to enter the epididymis. The structure, function, and endocrinology of these organs are reviewed. Epididymal sperm maturation is then reviewed considering the ability of sperm to develop the capacity for motility and the associated structural and molecular aspects of epididymis function. The epididymal sperm then move to the vas deferens for storage prior to ejaculation. The prostate is a glandular organ that produces components of the semen, and the structure, molecular, and endocrine aspects of prostate biology are reviewed. The seminal vesicle is the next male reproductive tract organ that also contributes components to semen, and the structure, secretion, and endocrinology of this organ are reviewed. The final male reproductive tract organ reviewed is the penis, and the structure, erection, endocrinology, and abnormalities are reviewed. The ejaculation and semen constituents are reviewed in the final chapter of this section.

The Male Reproductive Physiology section presents a number of key topics associated with male reproduction. This includes male pubertal development in regard to reproductive organ development and endocrinology. Male fertility in regard to sperm numbers, motility, and male reproduction senescence is reviewed. The roles of circannual and circadian rhythms are presented. The energetics, immunology, and nutritional factors in mammalian reproduction are reviewed. The regulatory roles of the pineal gland and vomeronasal organ in male reproduction are reviewed. Finally, the current knowledge of male contraception is presented.

The Comparative Mammalian Male Reproduction section is focused on a variety of different species. The nonmammalian species are presented in Volume 6 to provide a more complete comprehensive reproduction presentation. Volume 1 contains the major mammalian male reproduction chapters. An overview and then separate chapters on the cow, pig, horse, sheep, rodent, dog, cat, primate, and human are presented. The unique and conserved aspects of mammalian reproduction are reviewed. Finally, the assisted reproduction technology (ART) and contraceptive aspects in mammalian species are presented. A more human focus on ART and contraception are presented in Volumes 4 and 5.

The final section of Volume 1 involves the Environmental Toxicology of Mammalian Male Reproduction. The impacts and actions of testis toxicants are reviewed. Environmental factors such as temperature, metals, and steroids are discussed. The various endocrine steroids and associated antiandrogens or antiestrogens are discussed. This includes endocrine disruptor toxicants like the plastics derived, bisphenol A (BPA) and phthalates, and pesticides. The influence of these factors on paternal exposures and preconception exposures on offspring are reviewed. The epigenetic transgenerational inheritance impacts of environmental factors in male reproduction is presented. Overall, how these environmental factors impact the male reproduction is discussed and reviewed.

The breadth, quality, and educational impact of Volume 1 of the Encyclopedia of Reproduction is solely due to the chapters' authors who are internationally recognized authorities on the topics and contributed significantly to the scientific advances in the field. All are active researchers who have made major contributions in the area of Male Reproduction. The contributors to this volume are very much appreciated.

References

- Hafez, B., & Hafez, E. S. E. (2016). *Reproduction in Farm Animals*. John Wiley & Sons.
- Russell, L. D., Ettlin, R. A., Hikim, A. P., & Clegg, E. D. (1990). *Histological and Histopathological Evaluation of the Testis*. Cache River Press.
- Skinner, M. K., & Griswold, M. D. (2005). *Sertoli Cell Biology*. San Diego, CA: Elsevier -Academic Press.

TESTIS

Historic Considerations in Male Reproduction

Michael K Skinner, Washington State University, Pullman, WA, United States

Bernard Jégou, University of Rennes, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

The objective of this article is to provide a number of early historic observations and theories that influenced the development of the field of Male Reproductive Biology. The use of castration by ancient man to domesticate animals indicate the testis was the first organ studied in regards to its impact on male reproductive physiology. The differences between males and females also lead to the first biological theories on why these differences exist. One of the first uses of the microscope was the examination of sperm and the initial theories developed that impeded the initial theory of fertilization. The basis for sex determination was also one of the first biological considerations of Aristotle which impacted the biological sciences for over 2000 years. This article will present a number of historic considerations (**Table 1**) that were for the most part inaccurate and misleading, but have significantly impacted the progression of the field of reproductive biology.

Historic Castration (Testis) Considerations

Ancient civilizations used the removal of testis (i.e., castration) to domesticate animals including pigs, cattle, and sheep. The origins of castration, most likely developed at the time of the emergence of farming (i.e., neolithic period ~10,000 BC), either accidentally or intentionally are unknown but had a significant impact on ancient civilizations ([Diamandopoulos et al., 2005](#)). Therefore, this biological manipulation is the first documented physiological observation on the impacts of a specific organ on the biology of an organism. The first writings on castration was around 1500 BC by Assyrians in a tablet relating castration as punishment for sexual crimes. Then, the Greek medical writer Hippocrates in the 5th—4th century BC elaborated on the concept requiring the two parents. Physical traits on voice and feminization in eunuchs (i.e., human castrations) were documented by Aristotle in the 4th—3rd centuries BC ([Hippocrates, 1915](#)). He commented on the effects of castration in the rooster and man and gave a detailed description

Table 1 Historic considerations impacting male reproduction

<i>Reproductive topic</i>	<i>Observation</i>	<i>Period</i>
Castration	Testis first organ interest Domestication animals Eunuch characteristics	Prehistoric/neolithic Assyrian/egyptian/greek societies
Reproduction theory	Theory male provide seed (testis involved) <i>active</i> role and female provide soil <i>passive</i> role (prevailing theory 2000 years)	Aristotle (384–322 BC)
Environmental sex determination	Theory environmental factors such as heat (fire) and cold (water) promotes sex determination for male or female	Aristotle (384–322 BC)
Spermist theory/animalculism	One of the two theory of preformationism for which sperm head contained preformed offspring (homunculus) and female provide passive environment for development	Nicolaas Hartsoeker 1600s
Ovism	The other theory of preformation for which “the embryo is preformed in the ovum (egg) while the sperm only provides an aura seminalis,” that is a vital essence	1600s–1700s
Female ovary and egg role reproduction	Egg produced in ovary and has active role reproduction with sperm	William Harvey and Bishop Niels Stenson 1600s
Male reproductive tract organs anatomy	Detailed anatomy of male reproductive tract organs and specialized roles	Vesale 1543s and De Graaf 1600s
Genetic sex determination theory	The role of genetics followed by gonadal followed by phenotypic sex in mammals	Alfred Jost (1940s and 1950s)
Male reproduction endocrine theory	The role of endocrine hormones in male reproduction integrating the testis, brain and other reproductive organs	Berthold 1879; Alfred Jost (1940s and 1950s); Mc Cullagh (1930s and 1940s)

of the genital tract in mammals. Aristotle also developed early theories or speculations on the role of the testis in male biology (Hippocrates, 1962). Over the next 1000 years debates and theories on the impacts of the testis on male biology developed ranging from the impact of temperature on the testis to the effects of the testis on the heart, brain, and muscle development (Diamandopoulos et al., 2005). One of the more interesting speculations was the role of a testis derived liquid on the males biology (Diamandopoulos et al., 2005; Galen, 1964). Although the majority of these theories and speculations were neither accurate or based on specific observations, Aristotle's concepts had significant impacts for centuries on our approach and progress of scientific advances (Table 1).

The use of castrations in ancient societies in humans to create eunuchs was reported in Assyrian, Egyptian, and Greek cultures. Observations of the physiological impacts of castration were documented in all these societies. This included infertility, brain behavioral effects, voice changes, weight, and metabolism alterations and more. These observations led to a variety of early theories and speculations. A prominent link between the testis and the brain was discussed by Aristotle and others throughout history, which was the first speculations of the brain and gonadal axis today known to be endocrine based. Although these early speculations and theories were not completely accurate, they laid the foundation for much of the early male reproduction research in the last century.

Reproduction and Sperm Theory

One of the first recorded reproduction theories or speculations was proposed by Aristotle (Table 1). Observations with castration and role of the testis to create semen Aristotle proposed the male provided the seed having an active role in reproduction and the female provided the soil having a passive role in reproduction. Although this reproduction theory was based on observations at the time, this Aristotle concept persisted for nearly 2000 years and impacted the majority of reproductive biology research and society. A classic example involving one of the first observations following the development of the single lens microscope in the 1600s by Leewenhock (1678) was made on his own sperm which lead him to describe the presence and vigor of "animalcules," the name that he gave to spermatozoa. Under the name of "homunculus" the speculation was made by Hartsoeker (1694) that the spermatozoon contained the entire preformed human (homunculus)/animal (animalcules) with a number of supporting microscopic observations (Hartsoeker, 1696). The concept named "animalculism" was that the sperm provided the seed that contained the preformed animal (Fig. 1) and that the female provided the soil to allow its growth. Therefore, Aristotle's reproduction theory was supported.

This preformation sperm theory was debated for the next 100 years. The work of the Reverent Lazzaro Spallanzani (1717, 1780) lead to the description of the mobility of spermatozoa (the "small worms" as he wrote) and he also provided the first artificial insemination using a dog (Inza, 1964). Although all of Spallanzani's work supported the role of the sperm in reproduction, he believed in ovism and the role of a preformed person in the egg. The very long standing controversy about the role of spermatozoa in reproduction really ended with Prevost and Dumas in 1824 who rediscovered after Spallanzani the relationship between the male gamete and reproductive abilities. The named "spermatozoa" was first coined by Van Baer soon after (1827), and it was van Kolliker in 1841 who introduced the fact that spermatozoa derive from germ cells in the testis.

During the same period, it was William Harvey (1578–1657), who discovered circulation of blood, but he also studied the ovary anatomy making major observations on the histology of the ovary and anatomy of the female reproductive system. Observations led Harvey to the proposal that ovulation and the ovary had a critical role in reproduction (Harvey, 1651). Harvey did not discover the egg as during the same period Bishop Niels Stensen (1638–86) using fish ovaries to discover the egg and later using the mule, horse and donkey described the role of the egg in reproduction (Volker, 1989). The debate that the female and egg played an active role in reproduction was contested during the 1600s and 1700s period due in part to the predominant influence of the early Aristotle concepts. The active role of the female in reproduction slowly became accepted, however, this provides an example of how early scientific theories can develop into dogma and paradigms that can persist and sometimes impair the progression of science.

Male Reproductive Tract Organ and Anatomy

Anatomy was one of the first biological fields to develop and was documented by artists and scientists from early Egyptian, Greek, and Roman periods through the modern era. All those societies had documentation of testis and ovary which suggested their

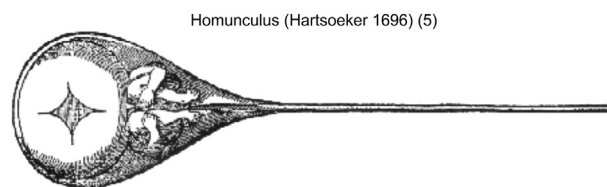


Fig. 1 Homunculus involving preformed human/animal in the head of the sperm to then allow growth in the female From Hartsoeker, N. *Principes de Physique*. Paris, 1696.

understanding of the link with reproduction. Later artists such as Leonardo Di Vinci (1452–1519) created drawings of reproductive systems and organs such as the male reproductive tract. This included an anatomical representation of coitus of a hemisected man and woman, and is best known for his analysis of fetal developmental anatomy (O'Malley, 2003). Many artists and scientists documented the anatomy of many reproductive organs, such as the work of Vesale in 1513, but one of the most influential and informative scientists was Regneri de Graaf in the 1600s (de Graaf, 1677). The most complete and insightful male and female reproductive tract anatomy was provided by de Graaf and detailed the specific organs, connections and hypothetical functions. An example of his detailed drawings of male reproductive tract anatomy is presented in Figs. 2 and 3. In addition to the testis, rete testis and epididymis, seminal vesical, vas deferens and penis are presented in detail (de Graaf, 1677). The work of early anatomists such as de Graaf set the ground work for our modern understanding of male reproduction. The development of the microscope in the late 1600s allowed more detailed anatomy and histology to be performed of male reproductive tract tissues. This analysis advanced the concepts of the "Cell Theory" which suggested tissues and organs had specific cells that allowed them to function. One of the first tissues studied was the testis due to its importance for reproduction and having more understanding than most tissues. Following the understanding sperm was produced within the seminiferous tubules, the first

Testis and Epididymis Anatomy (De Graaf, 1677) (10)

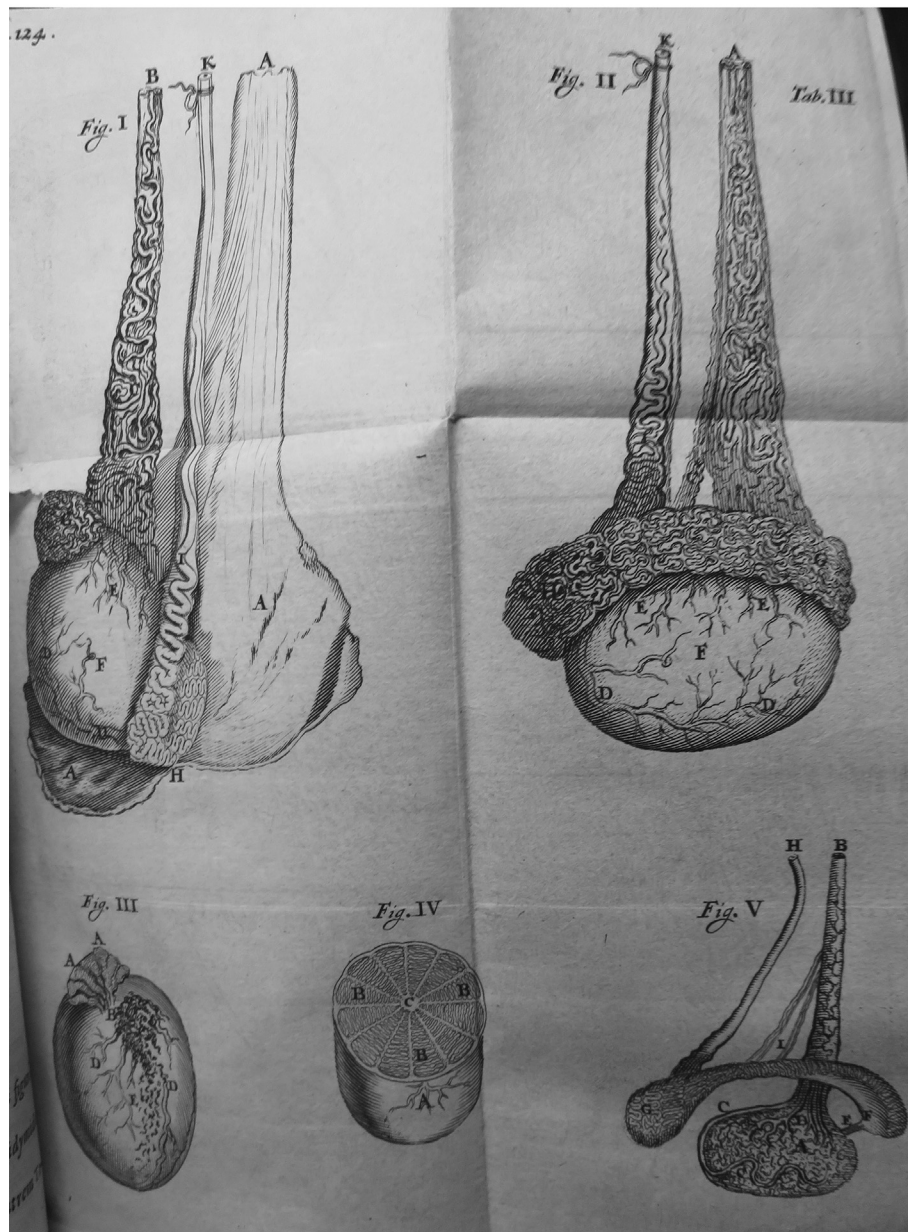


Fig. 2 Testis and epididymis male reproductive tract anatomy drawing From de Graaf, R. Opera Omnia: Lugd. Batav. Ex Officina Hackiana, 1677.

Seminal Vesicle Anatomy (De Graaf, 1677) (10)

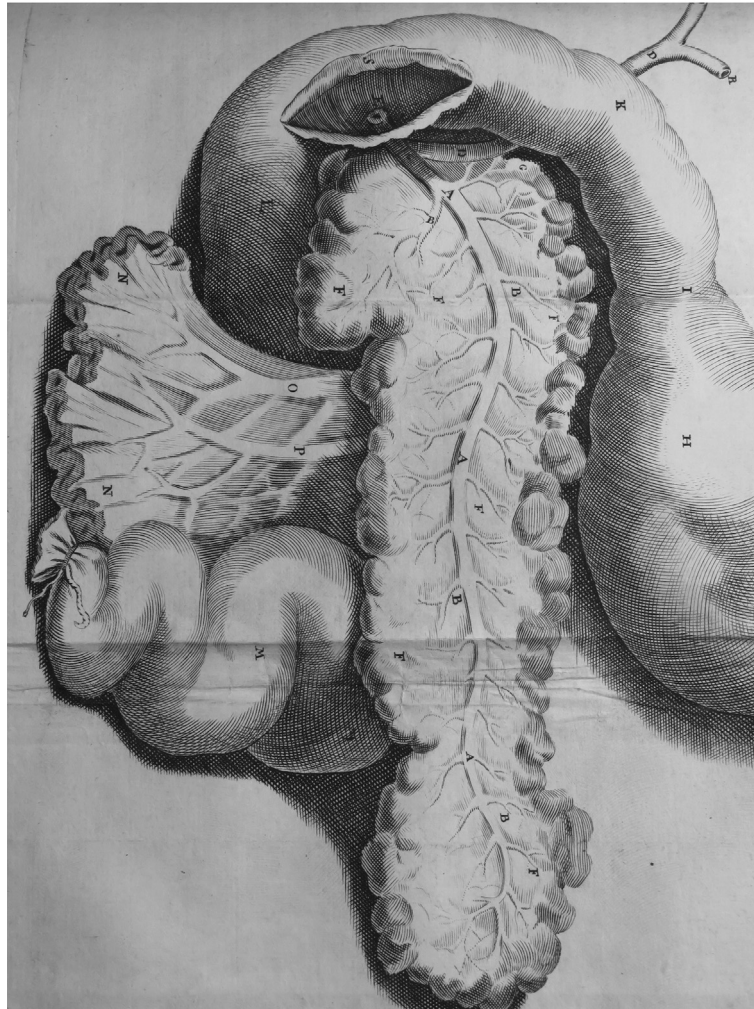


Fig. 3 Seminal vesicle and male reproductive tract anatomy drawing From de Graaf, R. *Opera Omnia*: Lugd. Batav. Ex Officina Hackiana, 1677.

somatic cell identified at this level was the Sertoli cell. Enrico Sertoli was an Italian physician that used the early microscope to describe the structure and histology of this critical cell type. Sertoli elaborated the crucial concept that Sertoli cells are the “nurse cells” of germ cells (Anon, 1987). His early diagrams of these cells that now have his name are shown in Fig. 4 (Sertoli, 1865). The various structures of the Sertoli cell and its role in the development of the seminiferous tubule are described from this 1865 observation (Sertoli, 1865). This is simply the first of many different observations by many scientists to describe specific cell types in various male reproduction tract organs. Clearly the organ anatomy and cellular histology of the 1600s–1800s help establish the initial building blocks for current reproduction research.

Early Sex Determination Theory

The origins of males versus females through the process of sex determination was also considered in early societies, but the first documented theory or speculation was provided by Aristotle (Table 1). The concept was that environment promoted sex determination and Aristotle suggested heat initiated male development and cold initiated female development (Fig. 4). Although this does exist in some organisms such as turtles, this is now known not to be a factor in mammals and most organisms. This theory has persisted even into the modern era. An example is in the 1980s when the prince of England was about to have a child the fact he had been a helicopter pilot was determined by the popular press to indicate he would have a male child. Therefore, even today the concept environment can impact sex determination persists from these early Aristotle concepts.

This environmental impact on sex determination persisted until the mid-1900s. The first modern theory of sex determination was provided by Alfred Jost, University of Paris in the 1940s and 1950s (Jost, 1945, 1970). The development of genetics in the early 1900s led to the identification of sex chromosomes that then through experiments by Jost on early fetal sex determination led to his

Sertoli Cell Structure (E. Sertoli, 1865) (12)

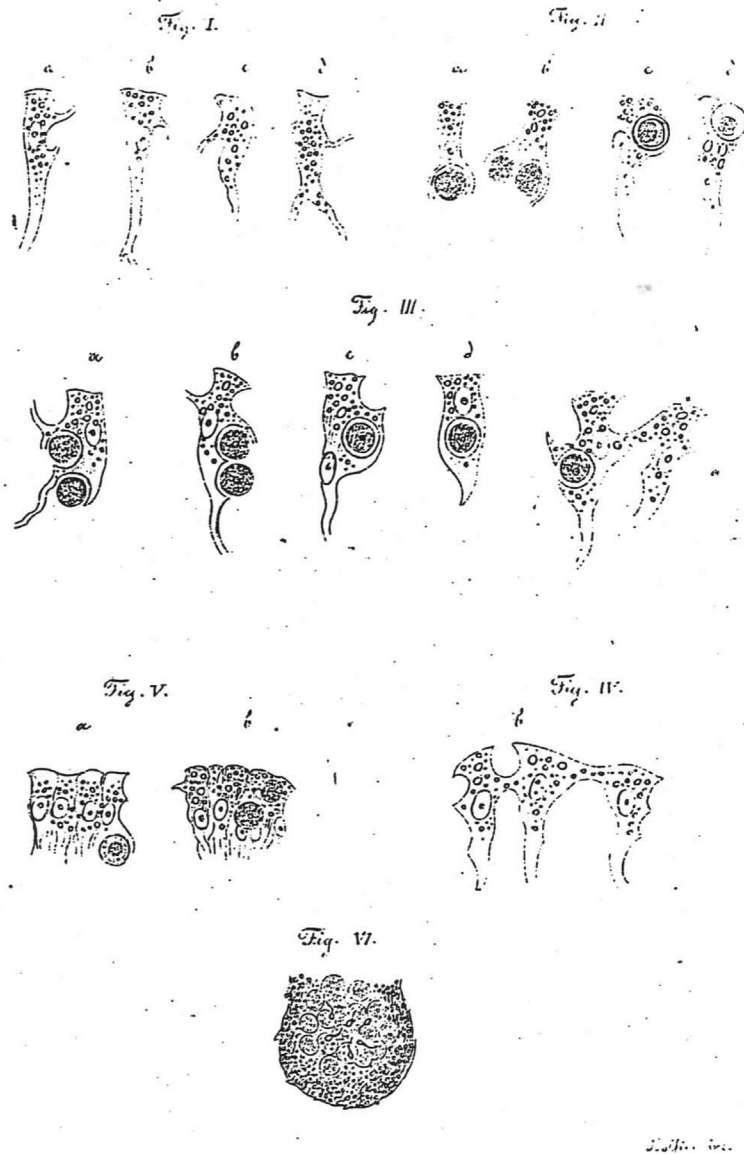


Fig. 4 Aristotle sex determination theory. The relative preponderances of the four elements and their qualities in males and females. The greater innate heat of the male allows for a greater degree of concoction of the nourishment and hence a more concentrated white semen is formed. From Sertoli E. (1865). Dell'esistenza di particolari cellule ramificate nei canalicoli seminiferi del testicolo umano. Morgagni 7, 31–40.

theory for sex determination ([Table 1](#)). This involved the theory that genetic sex (sex chromosomes) promote gonadal sex (testis or ovary) that then promotes phenotypic sex (male or female) ([Jost, 1945, 1970](#)), [Fig. 5](#). This sex determination theory displaced the early Aristotle concept of environmental sex determination, but surprisingly Aristotle's theory was predominant for nearly 2000 years. Another example of how early historic concepts can impede the progression of science by development of strong dogma or paradigms.

Early Endocrine Observations

The development of early anatomy and cell histology in the 1600s and 1700s established an understanding of the complexities of male reproduction in a number of different species. The first major advance to the next level was made by Claude Bernard in the 1850s with the advent of the field of physiology. He is considered the father of the field through his insight and experiments that different organ systems interact to influence their functions. He described the concept of cybernetic systems between organs and tissues through the internal milieu as the basis of how the organism functions ([De La, 1872](#)). Although he did not focus on

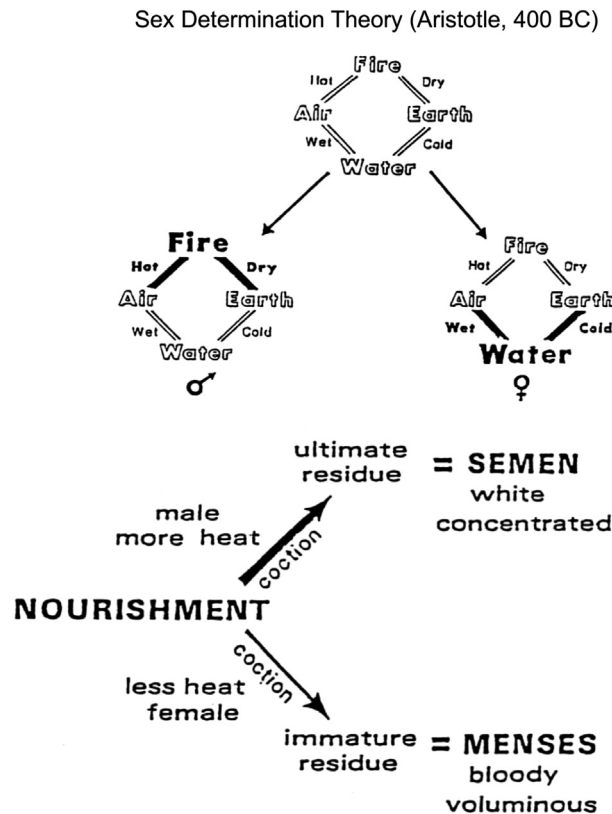


Fig. 5 Jost sex determination theory. Role of genetic sex (sex chromosomes) in promoting gonadal sex (testis or ovary) that promote male or female phenotypic sex characteristics (Jost, 1945).

reproduction, the concepts and theory initiated the fields of physiology, endocrinology and cell biology. One of the systems he did consider was the brain-gonadal interaction. Therefore, Bernard set the ground work for reproduction physiology and indirectly led to the field of endocrinology.

With this in mind and the summary of the main milestones concerning the testis itself, the seminiferous tubules and spermatozoa (see above) it is interesting to note that the first demonstration of the role of a “gland,” was provided by Berthold in 1849 when he showed that while castration was followed by regression of the cockscomb, transplantation of the testes to the castrated cocks restored its size. Very soon afterward Leydig (1850) provided the first microscopic description of the interstitial cells of the testis which were named the Leydig cells and are now known to produce the hormones named androgens, and Insulin-like factor 3 the latter being responsible for testicular descent in mammals.

The early 1900s involved a large number of physiology experiments to examine how organ systems influence each other and identified substances that mediate those interactions. Some of the first hormones identified were shown to be produced by the gonads and impact a wide variety of tissues (Table 1). This involved the identification of testosterone being produced by the testis (McCullagh, 1948). In addition, one of the first protein hormones inhibin being produced by the testis and impacting the brain hypothalamus (McCullagh, 1948). One of the prominent scientists involved was (McCullagh, 1948). This initiated in the following decades an increasing number of endocrine focused experiments to help understand male reproduction. Another example is related to the work of Alfred Jost, Fig. 6, who during his work on sex determination identified a testis determining factor that we now know is a specific gene (SRY), as well as a hormone anti-Müllerian hormone (AMH) which is critical in both male and female fetal sex determination and in the adult (Jost, 1945, 1970). Over the past 100 years the advances have been far more rapid than in previous centuries.

Summary

The review of ancient and early history has revealed that early history concepts for science are often not completely accurate, but they do have an important role in the progression of science. The advantage is that a theory is developed that can be tested through experiment and during that process new observations can be made to progress science. The disadvantage is that the theory developed can turn into dogma and a strong paradigm that becomes so ingrained that it can impede the progression of science. In considering the history and concepts of male reproduction, there have been good examples of both. Although the insight and

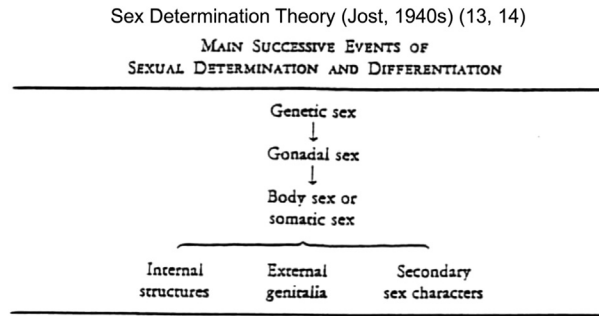


Fig. 6 Sex determination theory. From Jost, A. (1970). Hormonal factors in the sex differentiation of the mammalian foetus. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* **259**(828), 119–130; Jost, A. (1945). Sur l'action de divers androgenes dans la differentiation embryonnaire du sexe. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales* **139**, 670–672.

theories of Aristotle were surprising, the development of dogma around these concepts clearly impeded the progression of science in subsequent centuries. We have displaced many of these concepts, but even today we have our paradigms. The future will need to assess these paradigms and not resist the paradigm shifts required to advance science.

Considering the male reproduction historic concepts can assist in our understanding of the origins of current science and theories. Perhaps the biggest value of this historic understanding is to put the current activities in perspective, which will only help the advances of male reproductive sciences in the future.

References

- Anon. (1987). Historical tribute: On the existence of specialized branching cells in the seminiferous canaliculi of the human testis. By Enrico Sertoli. 1865. *Human Reproduction*, **2**(2), 83–84.
- De La, B. C. (1872). *Physiologie Generale* (p. 339). Paris: Librairie Hachette Et Cie.
- Diamandopoulos, A., Goudas, P., & Patsy, D. (2005). The ancient and medieval Greek writer's perceptions concerning the relationship between sexual characteristics and testicular volume. *Hormones (Athens, Greece)*, **4**(2), 117–120.
- Galen. (1964). De semine libri ii. In C. C. Kohn (Ed.), *Claudii galeni opera omnia* (p. 583). Hildesheim: Olms. Chapter 4.
- de Graaf, R. (1677). *Opera Omnia: Lugd. Batav. Ex Officina Hackiana*.
- Hartsoeker N. 1696. *Principes de Physique*. Paris.
- Harvey, W. (1651). Exercitationes de generatione animalium. In I. O. Pulleyn (Ed.), *Auibis accedunt quaedam de partu; de membranis ac humoribus uteri; & de conceptione*. Londini: Typid Du-Gardianis.
- Hippocrates, P. (1915). In H. Diels (Ed.), *Galen in hippocratis prorrheticum i commentaria iii* (p. 608). Leipzig: Corpus medicorum Graecorum. Chapter 16.
- Hippocrates, A. (1962). In E. Litte (Ed.), *Oeuvres completes d'hippocrate*. Amsterdam: Publication Hakkert. Chapter 6, Section 28.
- Inza, R. (1964). Monography on lazaro spallanzani. *La Semaine Medicale*, **124**, 622–630.
- Jost, A. (1945). Sur l'action de divers androgenes dans la differentiation embryonnaire du sexe. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales*, **139**, 670–672.
- Jost, A. (1970). Hormonal factors in the sex differentiation of the mammalian foetus. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, **259**(828), 119–130.
- McCullagh, E. (1948). Testicular dysfunction. *Bulletin of the New York Academy of Medicine*, **24**(6), 341–363.
- O'Malley, C. D. (2003). *Leonardo da Vinci on the human body: The anatomical, physiological, and embryological drawings of Leonardo da Vinci*. New York: Gramercy.
- Sertoli, E. (1865). Dell'esistenza di particolari cellule ramificate nei canalicoli seminiferi del testicolo umano. *Morgagni*, **7**, 31–40.
- Volker, A. (1989). Neils Stensen (1638–1686) in the medicine of his era. *Zeitschrift Fur Die Gesamte Innere Medizin*, **44**(7), 215–221.

Structure/Cells Overview

David M de Kretser, Monash University and Hudson Institute, Melbourne, VIC, Australia
Peter Stanton and Liza O'Donnell, Institute of Medical Research, Clayton, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Development of Testes

The testes are located in the scrotum since sperm production requires a temperature that is cooler than that of the abdomen (de Kretser, 2016). They develop in the embryo just distal to the kidneys and descend into the scrotum shortly after birth through the inguinal canal. This canal, found on both sides in the region of the groin, is formed by the attachments of one of the muscles of the abdominal wall. The canal extends downwards and medially in the groin and links the abdominal cavity with the scrotum (de Kretser, 2016; de Kretser et al., 1982; Clermont and Huckins, 1961; Roosen-Runge and Holstein, 1978; Hutson et al., 1990).

Descent of the Testes

In some males, the inguinal canal does not close and the testes may retract from the scrotum into the abdominal cavity for brief periods. The descent of the testis is important because the temperature of the scrotum is lower than the intra-abdominal temperature and the germ cells require a lower temperature for their survival (Hutson et al., 1992). In some males, the inguinal canal which normally closes after the testes descend, remains patent and the testes may retract for varying periods causing damage to the germ cells because of the higher intra-abdominal temperature. Descent of the testis begins in the fetus at about 28 weeks of gestation and should be complete by birth and it is controlled by a Leydig cell secreted protein called insulin-like protein 3 which is a member of the insulin-like protein super family (de Kretser et al., 1982; Bowles and Koopman, 2007).

Failure of the testes to descend should be diagnosed as soon possible as spermatogenic damage and infertility may result. Surgery can be undertaken to close off the inguinal canal so that the testes are permanently located in the scrotum. The testes are ovoid in shape and in adults, their volume ranges from 15 to 35 mL. At birth they are about 1–3 mL in volume grow rapidly during pubertal development. The availability of an orchidometer, a range of spheres from 1 to 3 mL in progressively increasing volumes to 35 mL, is every helpful in determining testicular size (Fig. 1).



Fig. 1 *Orchidometer*. This set of models of differing testicular size helps the physician in assessing the size of the testes in patients with delayed puberty, infertility or potential testicular tumors.

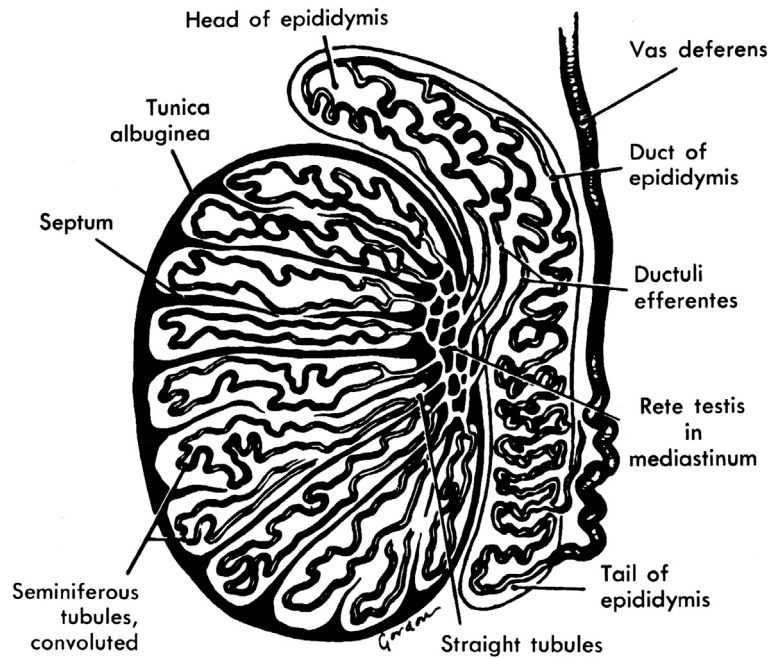


Fig. 2 The anatomical features of the testis, epididymis, vas deferens and the pampiniform plexus of veins, that surround the testicular artery, represent the venous drainage of the testis and epididymis.

The testes are covered by thick fibrous tissue that forms the tunica albuginea and should be smooth on their anterior and lateral surfaces. These surfaces are also covered by a serous membrane called the tunica vaginalis. Posteriorly, in the region under the epididymis, the tunica albuginea is thickened and projects into the parenchyma of the testes to form the mediastinum of the testes. A series of tubules traverse the mediastinum of the testis to link the seminiferous tubules to the efferent ducts that form the head of the epididymis (Roosen-Runge and Holstein, 1978) (Fig. 2).

In prepubertal boys, the germ cells in the testes are called gonocytes which are centrally placed in the seminiferous cords, that are the precursors of the seminiferous tubules. The other cellular components of the cords are the immature Sertoli cells that extend from the basement membrane of the tubule to the lumen of the seminiferous tubules. As development proceeds, the gonocytes move to the periphery of the cords to lie on the basement membrane of the cords and commence dividing by mitosis to give rise to the spermatogonial stem cells a process that requires retinoic acid and Oct 4 (de Rooij and Russell, 2000; de Rooij and Grootegeed, 1998; Dann et al., 2008; Bowles and Koopman, 2007). Continuation of spermatogonial mitosis requires the action of Foxo1 and the spermatogonial stem cells have the capacity for pluripotency, a characteristic marker of stem cells in many tissues (de Rooij and Russell, 2000; de Rooij, 2001; Goertz et al., 2011). When this occurs at the time of puberty, the basally placed Sertoli cells form specialized tight junctions just central to the gonocytes thereby preventing inter-cellular transport of substances and creating a blood–testis barrier (Dann et al., 2008). External to the basement membrane of the seminiferous tubules, there is a layer of myofibroblasts that can contract and increase the intra-tubular pressure. This facilitates the movement of sperm and the fluid produced by the Sertoli cells in to the rete testis (Simoni et al., 1999).

Functions

The testes have three functions, the production of sperm, the secretion of the steroid hormone, testosterone, after puberty and the production and secretion of protein hormones inhibin, activin, and follistatin. In addition, insulin and IGF1 are important in the control of Sertoli cell proliferation (Pitetti et al., 2013). Testosterone is synthesized and secreted by the Leydig cells of the testis that lie close to blood vessels found in the inter-tubular region of the testis. The Leydig cells have the characteristics of steroid secreting cells, namely a well developed smooth endoplasmic reticulum and mitochondria which have tubular cristae unlike “conventional” mitochondria in which the inner mitochondrial membrane forms “plate-like” cristae (de Kretser, 1967).

There are also lymphatic vessels in the inter-tubular compartment of the testes and these join abdominal lymphatics that also transport testosterone into the chest where they join the thoracic duct, the common duct of all lymphatic vessels in the body. The thoracic duct joins the venous system at the junction of the left subclavian vein and the left internal jugular vein (Stanton, 2016) (Fig. 2).

Spermatogenesis

The testes produce sperm in tubules termed seminiferous tubules by a process called spermatogenesis. These tubules are composed of germ cells and the supporting network of Sertoli cells. The tubules are surrounded by a layer of basement membrane, external to which lies a plate-like layer of peritubular cells which are contractile effectively “squeezing” the seminiferous tubules (Holstein et al., 1996; Simoni et al., 1999). Thereby they assist in moving sperm, released into the lumen of the seminiferous tubules, toward an irregular network of tubules located at the posterior and superior pole of the testis called the rete testis.

The rete testis is connected with the duct of the epididymis, a coiled tube that lies at the posterior aspect of the testis and is divided into the head, body and tail, the latter continuing as the vas deferens (Johnston and Whillis, 1954). In the epididymis, the sperm, which are still not motile, are moved by muscular contractions from the head to the tail of the epididymis. They acquire mobility as they pass through the epididymis to enter the vas deferens (Baker, 1989). The latter delivers the sperm during ejaculation to enter the prostatic urethra and pass through the penile urethra.

The cellular components in the seminiferous tubules are the germ cells that are the precursors of sperm and also the Sertoli cells. The latter are named after the person who first described them and they are a critical component of the seminiferous epithelium. They extend from the basement membrane of the tubule to the lumen and send projections between the surrounding germ cells not unlike the branches of a tree from the trunk. These projections contain microfilaments that provide a structural framework for the epithelium given that the germ cell components migrate from the basally placed spermatogonia to the centrally placed spermatids and their final product, the spermatozoa (Fig. 3).

In prepubertal boys, the germ cells in the testes are called gonocytes and they are centrally placed in the seminiferous cords that comprise the testis (Clermont and Huckins, 1961; de Rooij and Grootegoed, 1998; de Rooij and Russell, 2000). The other cells comprising the cords are the immature Sertoli cells that extend from the basement membrane of the cords surrounding the gonocytes. At the commencement of puberty, the gonocytes move to the periphery of the cords and the Sertoli cells form specialized cell junctions central to the gonocytes which will progress to give rise to the population of spermatogonia, the precursors to the subsequent stages of spermatogenesis (Johnston and Whillis, 1954). These changes, under the influence of the pubertal increase in FSH, act through Foxo1 and Oct 4 (Dann et al., 2008).

Where adjacent Sertoli cell projections meet basally, they form specialized tight cell junctions that prevent inter-cellular transport creating a blood–testis barrier (Dym and Fawcett, 1970; Russell, 1977). These tight junctions are placed at such a position in the seminiferous epithelium that only the spermatogonia are in contact with the basement membrane of the seminiferous tubules. All other germ cells lie central to the blood–testis barrier and are thus dependent on the Sertoli cells for transport of materials for optimal germ cell function and can be considered to “nurse” germ cells central to these inter-Sertoli cell junctions.

The inter-Sertoli cell junctions must open centrally to enable the progeny of spermatogonia, the primary spermatocytes, to leave the basal compartment and enter the luminal compartment. The inter-Sertoli cell junctions reform basally below the primary spermatocytes that now lie within adluminal compartment of the seminiferous tubule (Stanton, 2016).

Studies have shown that the number of Sertoli cells can affect the magnitude of sperm production. One of the important factors that controls Sertoli cell numbers is activin A which stimulates proliferation and inhibits differentiation of Sertoli cells (Baker, 1989; Kreuger et al., 1974). Increasing systemic levels of activin A using an adeno-associated virus expressing activin A (Russell, 1977) stimulated proliferation and prevented differentiation of Sertoli cells in mice. This was associated with disruption of the blood testis barrier formed by the inter-Sertoli cell junctions and resulted in a 23.5% decrease in testis weight due to diminished spermatogenesis linked to disordered Sertoli cell function. The latter was associated with increase in markers of juvenile Sertoli cells and a decrease in claudin-11, a marker of mature Sertoli cells. These data are consistent with studies of the levels of activin A in mice during normal post-natal development which established that activin A levels are elevated at birth but decline rapidly after day 4 postpartum (Meehan et al., 2000).

Other studies using treatment with FSH or thyroxine, a hormone secreted by the thyroid gland, can enhance Sertoli cell proliferation and thus increase sperm output.

In part, the action of FSH on spermatogenesis is exerted directly via spermatogonia which are the only germ cells that have FSH receptors (Simoni et al., 1999). Germ cells also do not have androgen receptors and thus the requirement of testosterone for successful spermatogenesis is dependent on the presence of androgen receptors on the Sertoli cells.

Since in the human, testicular sperm production continues from puberty throughout life, there is clearly a need for a population of stem cells to produce the precursor cells that develop into sperm. The cells forming this stem cell population are the spermatogonia that undergo several mitotic divisions and have 46 chromosomes as do all other cells in organs throughout the body (Amory et al., 2011). They develop from the gonocyte population found in the testes of prepubertal boys. The gonocytes are initially placed in the centre of the seminiferous cords and migrate to lie between the precursors of the Sertoli cells and, as with the gonocytes, the spermatogonia are basally placed in contact with the basement membrane of the seminiferous tubules. These cells undergo several stages of development and are designated by their cytological features before commencing meiosis.

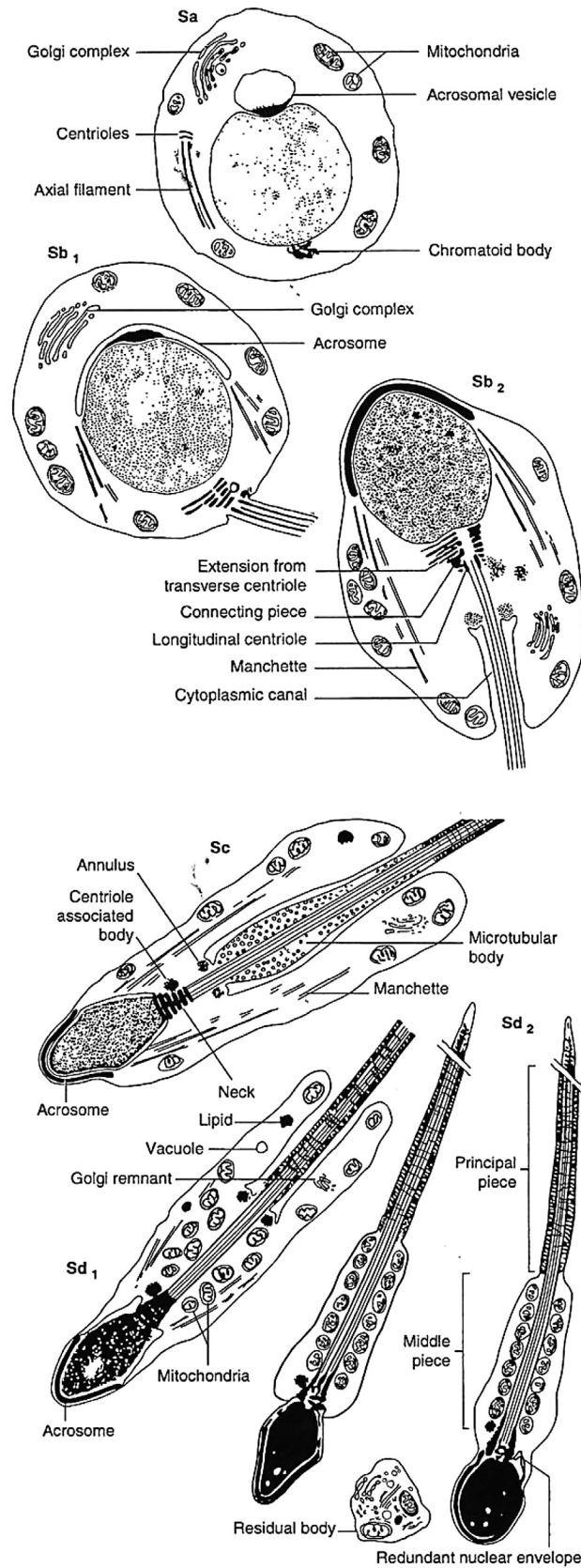


Fig. 3 The efferent ducts draining from the rete testis to form the head of the epididymis is illustrated together with the distal regions of the epididymis termed the body and tail.

The spermatogonia lose their contact with the basement membrane of the tubule when they begin another process of cell division called meiosis by which the chromosome numbers in these cells are reduced from 46 to 23. These cells are called primary spermatocytes.

During meiosis, the homologous chromosomes, derived originally from the fertilization of the egg, one provided by the mother, and the other member of the pair, provided by the father via his sperm, pair and replicate their DNA (deoxyribonucleic acid). The primary spermatocytes thus have nuclear features which enable the identification of the stages as the cells undergo the first meiotic division during which, one of each homologous chromosome pair, moves to the opposite pole of the cell. These cell types are named by the stage of meiosis that they have reached and can be identified by the chromatin pattern in their nucleus associated with the chromosome replication. Leptotene, zygotene, pachytene, diplotene, and diakinesis stages can be identified and, unlike cell division in somatic cells, these germ cells remain connected by intercellular bridges that link the cytoplasm of these cells. These bridges enable the development of the “chains” of germ cells and remain in place in the primary and secondary spermatocyte populations. This process requires the involvement of retinoic acid and androgens to proceed to completion. (Amory et al., 2011).

The completion the first meiotic division gives rise to cells called secondary spermatocytes that have half the number of chromosomes, 23, termed the haploid number, in contrast to their diploid precursor which had 46. The secondary spermatocytes then divide by mitosis to give rise to a further population of cells called round spermatids that are still connected by the cytoplasmic bridges.

Spermiogenesis

The round spermatids do not divide further but are transformed by a complex series of changes into a sperm, the process being called spermiogenesis (de Kretser, 1969). The basic changes in the developing spermatids during spermiogenesis are common to many mammalian species but the resulting sperm vary in their morphology especially in the shape of the head of the resulting sperm. The structure of the sperm tail however has many features in common across species. In the round spermatids the nucleus, which is centrally placed in the cell, is “capped” at one pole by a series of vesicles from the Golgi complex that coalesce to form a “cap” that is called the acrosome and is applied to that part of the nucleus closest to the acrosome. The acrosome covers approximately 30%–50% of the nuclear surface.

The nucleus, in the region of the acrosome, comes into close apposition with the cell membrane but remains separated from the nucleus by the acrosome.

Subsequently, as spermatid development continues, the nuclear chromatin undergoes a progressive condensation forming electron dense granules associated with stabilization of the DNA (Sassone-Corsi, 2002). That process involves the replacement of lysine-rich histones with transitional proteins, subsequently replaced by arginine-rich proteins called protamines. The nuclear chromatin granules condense as spermiogenesis progresses and it becomes more difficult to identify individual granules (Fig. 4).

At the pole of the nucleus opposite to the acrosome, a pair of centrioles, that participate in the development of the flagellum, lodge in a small fossa or indentation that still lies external to the nuclear membrane. This whole complex is called the connecting piece. The centriole closest to the nucleus, called the proximal centriole, lies at right angles to the plane of the distal centriole which gives rise to the core of the sperm tail called the axoneme. The axoneme is composed of a core of microtubules which forms the basis of the sperm tail sometimes called the axial filament (Fawcett, 1975).

The axial filament comprises nine pairs of doublet microtubules which surround two centrally placed single microtubules, a structure that is identical to the structure of cilia which also exhibit motility similar to the sperm tail.

A second set of nine outer dense fibers surround the axial filament distal to a dense ring termed the annulus. The annulus marks the distal end of the mid-piece and its mitochondrial sheath and defines the commencement of the fibrous sheath. The annulus marks the commencement of the region of the sperm tail called the principal-piece and the axonemal core, distal to the termination of the fibrous sheath, is termed the end-piece.

The final step, before sperm are released from the epithelium by a process called spermiation, is a movement of mitochondria, that up to this point have been distributed around the periphery of the spermatid cell membrane, to surround the mid-piece to form a “mitochondrial sheath” distal to the connecting-piece and ending at the annulus.

Spermiation, involves the release of sperm from the seminiferous epithelium. At this stage, the cytoplasm of the spermatid has migrated to a caudal position around the tail. Projections of Sertoli cells invaginate this caudal cytoplasmic collection to “literally” pull the residual cytoplasm off the spermatid, thereby releasing it into the lumen of the seminiferous tubule.

The residual bodies within the Sertoli cells, that contain the “unwanted” cellular components of the spermatids, are moved toward the base of the Sertoli cells and progressively “digested” by lysosomes. There is some data to suggest that these cellular components signal to the Sertoli cell that a “generation” of sperm have been released from that region of the epithelium.

The spermatozoa, that are released from the Sertoli cells are still immotile. They, together with fluid secreted by the Sertoli cells into the lumen of the seminiferous tubules, are moved toward the rete testis by the contractions of the peritubular myoid cells and enter the epididymis.

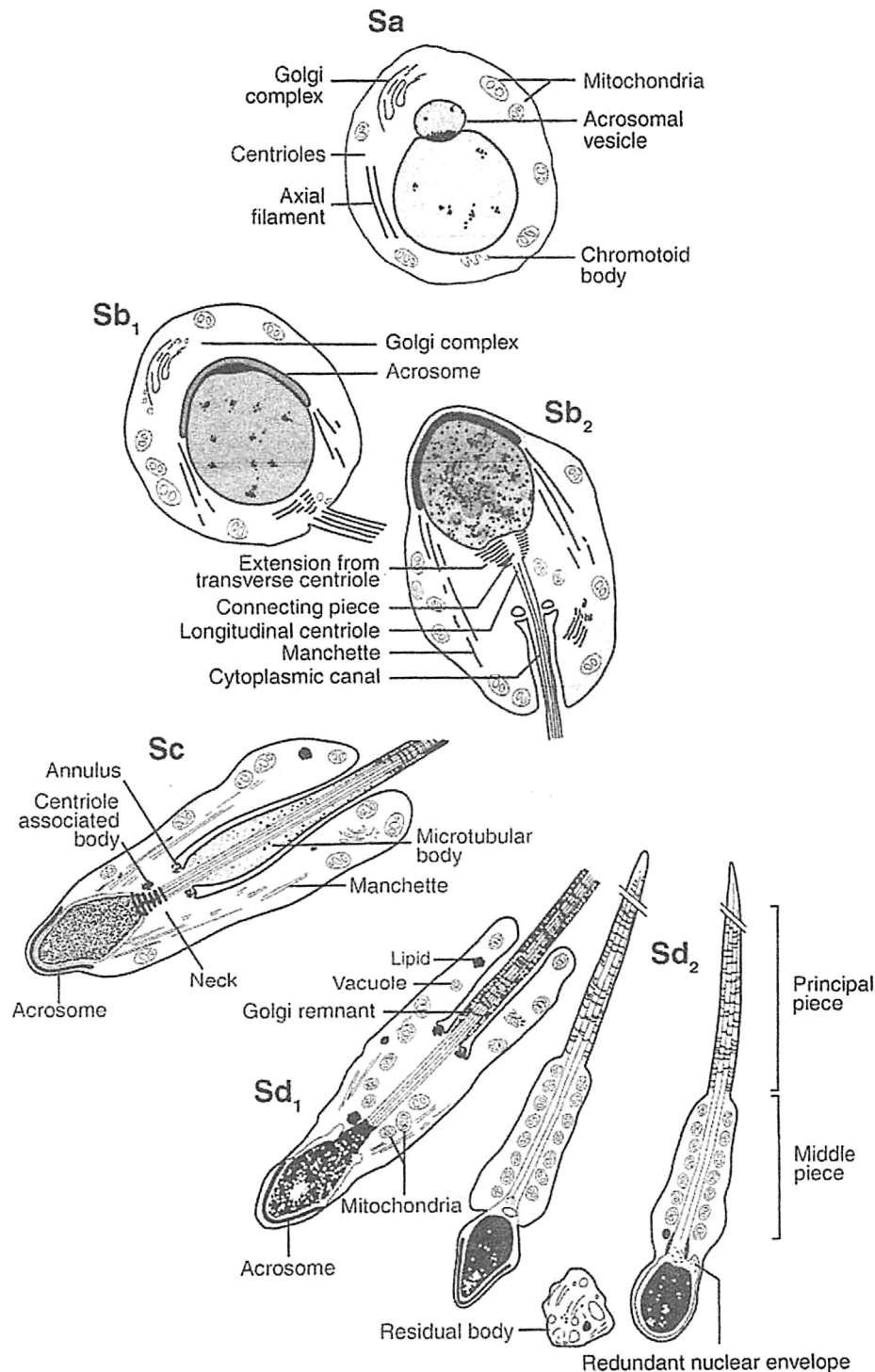


Fig. 4 The specific stages of spermiogenesis, the process by which a round spermatid (Sa) is transformed into a spermatozoon (Sd2) are illustrated. Reproduced with permission from de Kretser, D.M. (1969). Ultrastructural features of human spermiogenesis. *Zeitschrift für Zellforschung* **98**, 477–505.

References

- Amory, J. K., Muller, C. H., Shimshoni, J. A., et al. (2011). Suppression of spermatogenesis by bischloroacetyldiamines is mediated by the inhibition of testicular retinoic acid biosynthesis. *Journal of Andrology*, *32*, 111–119.
- Baker, H. W. G. (1989). Clinical evaluation and management of testicular disorders in the adult. In H. G. Burger, & D. M. de Kretser (Eds.), *The testis* (2nd ed., pp. 419–440). New York: Raven Press.

- Bowles, J., & Koopman, P. (2007). Retinoic acid, meiosis and germ cell fate in mammals. *Development*, 134, 3401–3411.
- Clermont, Y., & Huckins, C. (1961). Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male albino rats. *The American Journal of Anatomy*, 108, 79–97.
- Dann, C. T., Alvarado, A. L., Molyneux, L. A., Denard, B. S., Garbers, D. L., & Forteus, M. H. (2008). Spermatogonial stem cell self-renewal requires OCT4, a factor down regulated during retinoic acid-induced differentiation. *Stem Cells*, 26, 2928–2937.
- Dym, M., & Fawcett, D. W. (1970). The blood–testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biology of Reproduction*, 3, 308–326.
- Fawcett, D. W. (1975). The mammalian spermatozoon. *Developmental Biology*, 44, 394–436.
- Goertz, M. J., Wu, Z., Gallardo, T. D., Hamra, F. K., & Castrillon, D. H. (2011). Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. *The Journal of Clinical Investigation*, 121, 3456–3466.
- Holstein, A. F., Maekawa, M., Nagano, T., et al. (1996). Myofibroblasts in the lamina propria of human seminiferous tubules are dynamic structures of heterogeneous phenotype. *Archives of Histology and Cytology*, 59, 109–125.
- Hutson, J. M., Williams, M. P. L., Fallat, M. E., & Attah, A. (1990). Testicular descent, new insights into its hormonal control. *Developmental and Reproductive Biology*, 12, 1–56.
- Hutson, J. M., Baker, M. L., Griffiths, A. L., et al. (1992). Endocrine and morphological perspectives in testicular descent. *Reproductive Medicine Review*, 1, 165–177.
- Johnston, T. B., & Whillis, J. (1954). The male genital organs. In *Gray's Anatomy* (31st edn., pp. 1462–1473). London: Longmans Green & Co.
- de Kretser, D. M. (1967). Changes in the fine structure of the human testicular interstitial cells after treatment with human gonadotrophin. *Zeitschrift für Zellforschung*, 83, 344–358.
- de Kretser, D. M. (1969). Ultrastructural features of human spermiogenesis. *Zeitschrift für Zellforschung*, 98, 477–505.
- de Kretser, D. M. (2016). *Endocrinology adult and pediatric* (7th edn., vol. 2). Philadelphia: Elsevier Saunders.
- de Kretser, D. M., Temple-Smith, P. D., & Kerr, J. B. (1982). Anatomical and functional aspects of the male reproductive organs. In K. Bandhauer, & J. Frick (Eds.), *Handbook of urology* (vol. 16, pp. 1–131). Berlin: Springer Verlag.
- Kreuger, P. M., Hodgen, G. D., & Sherins, R. J. (1974). New evidence for the role of the Sertoli cell and spermatogonia in feedback control of FSH secretion in male rats. *Endocrinology*, 95, 955–962.
- Meehan, T., Schlatt, S., O'Bryan, M. K., de Kretser, D. M., & Loveland, K. L. (2000). Regulation of germ cell and Sertoli cell development by activin, follistatin and FSH. *Developmental Biology*, 220, 225–237.
- Pitetti, J. L., Calvel, P., Zimmermann, C., et al. (2013). An essential role for insulin and IGF1 receptors in regulating Sertoli cell proliferation, testis size and FSH action in mice. *Molecular Endocrinology*, 27, 814–827.
- de Rooij, D. G. (2001). Proliferation and differentiation of spermatogonial stem cells. *Reproduction*, 121, 347–354.
- de Rooij, D. G., & Grootegoed, J. A. (1998). Spermatogonial stem cells. *Current Opinion in Cell Biology*, 10, 694–701.
- de Rooij, D. G., & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology*, 21, 776–798.
- Roosen-Runge, E. C., & Holstein, A. F. (1978). The human rete testis. *Cell and Tissue Research*, 189, 409–433.
- Russell, L. D. (1977). Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. *The American Journal of Anatomy*, 148, 313–328.
- Sassone-Corsi, P. (2002). Unique chromatin remodelling and transcriptional regulation in spermatogenesis. *Science*, 296, 2176–2178.
- Simoni, M., Gromoll, J., Hoppner, W., Kamischke, A., Krafft, T., Stahle, D., & Nieschlag, E. (1999). Mutational analysis of the FSH receptor in normal nad infertile men: Identification and characterization of two discrete FSH receptor isoforms. *The Journal of Clinical Endocrinology and Metabolism*, 84, 751–755.
- Stanton, P. G. (2016). Regulation of the blood–testis barrier. *Seminars in Cell and Developmental Biology*, 59, 166–173.

Further Reading

- Barakat, B., O'Connor, A. E., Gold, E., de Kretser, D. M., & Loveland, K. L. (2008). Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. *Reproduction*, 136, 345–359.
- Cortes, D., Muller, J., & Skaakebaek, N. E. (1987). Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. *International Journal of Andrology*, 10, 589–596.
- Hogarth, C. A., Mitchell, D., Evanoff, R., Small, C., & Griswold, M. (2011). Identification and expression of potential regulators of the mitotic-to-meiotic transition. *Biology of Reproduction*, 84, 34–42.
- de Kretser, D. M., Kerr, J. B., & Paulsen, C. A. (1981). Evaluation of the ultrastructural changes in the human Sertoli cell in testicular disorders and their relationship to the changes in the levels of serum FSH. *International Journal of Andrology*, 4, 124–144.
- Nicholls, P. K., et al. (2012). Activin signalling regulates Sertoli cell differentiation and function. *Endocrinology*, 153, 6065–6077.

Endocrinology Overview RSS

Fiona Yuen, Christina Wang, and Ronald S Swerdloff, Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute, Torrance, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

DHT 5 Alpha-dihydrotestosterone.

GABA Gamma-aminobutyric acid.

GnRH Gonadotropin releasing hormone.

HPT Hypothalamic-pituitary-testis.

KISS1R Kisspeptin receptor 1 (previously known as GPR54).

KNDy Kisspeptin/neurokinin B/dynorphin.

Introduction

The hypothalamic-pituitary-testis axis (HPT axis) regulates function of the testis that is comprised of two main components: synthesis of testosterone and production of sperm in the male at various stages of life. Disorders of the testes or HPT axis may result in abnormal development, including ambiguous genitalia in infancy; failure to develop male sexual characteristics at puberty; and hypogonadism and infertility in adults. This article intends to provide a brief overview of the endocrinology of the male reproductive system including the HPT axis, testosterone biosynthesis, effects of testosterone, and aberrations in testosterone secretion and action. Spermatogenesis will also be discussed in brief.

Regulation of Steroidogenesis—HPT Axis

Anatomy

The hypothalamus sits at the base of the third ventricle of the brain; it has a specialized vascular system. The superior hypophyseal arteries branch from the internal carotid artery form the primary capillary plexus that carries blood to the median eminence. The blood is drained from the median eminence via the hypophyseal portal veins into the secondary venous plexus, which supplies the anterior pituitary gland.

The hypothalamus is divided into three zones: the lateral zone, medial zone, and periventricular zone. These zones are further subdivided into regions, and each region contains distinct nuclei (Crosby and Woodburne, 1940). Kisspeptin neurons can be found in the infundibular nucleus in humans (homologous to the arcuate nucleus of rodents), as well as the rostral preoptic area. In rodents, the kisspeptin neurons in the rostral area are located in the anteroventral periventricular nucleus and periventricular nucleus, whereas in humans, the kisspeptin neurons are scattered within the preoptic region. The neurons that release gonadotropin-releasing hormone (GnRH) are found from the preoptic area to the infundibular nucleus (Skorupskaitė et al., 2014).

The neurons that produce GnRH extend axons from the preoptic region to the median eminence at the base of the brain. The axon terminals release GnRH into the primary plexus capillaries, which then flows to the anterior pituitary via the hypophyseal portal system. The anterior pituitary contains the gonadotrophs that secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH circulate in the systemic arterial system to act on the Leydig cells and Sertoli cells in the testes.

The Leydig cells in the interstitium of the testes are responsible for producing sex steroids. The Sertoli cells within the seminiferous tubules are embedded in the germinal epithelium; they form the blood-testis barrier and are involved in the initiation and maintenance of spermatogenesis. They also produce sex hormone binding globulin (SHBG), anti-müllerian hormone, activin A, inhibin B, and other factors (Fig. 1).

Secretion of GnRH

GnRH is secreted in pulses by the GnRH neurons in the hypothalamus. Secretion surges in infancy producing an increase in LH secretion (mini-puberty in boys). There is another surge in puberty, again with increased LH secretion. Higher frequency GnRH pulses preferentially stimulate LH release, whereas lower frequency pulses preferentially stimulates FSH release (Thompson and Kaiser, 2014).

The GnRH neurons receive many excitatory and inhibitory signals from neuromodulators. Kisspeptin/neurokinin B/dynorphin (KNDy) neurons and kisspeptin neurons in the hypothalamus synchronize GnRH release (Fig. 2) (Pinilla et al., 2012). Kisspeptin acts directly on GnRH neurons to enhance GnRH secretion, via activation of phospholipase C, which affects calcium release and

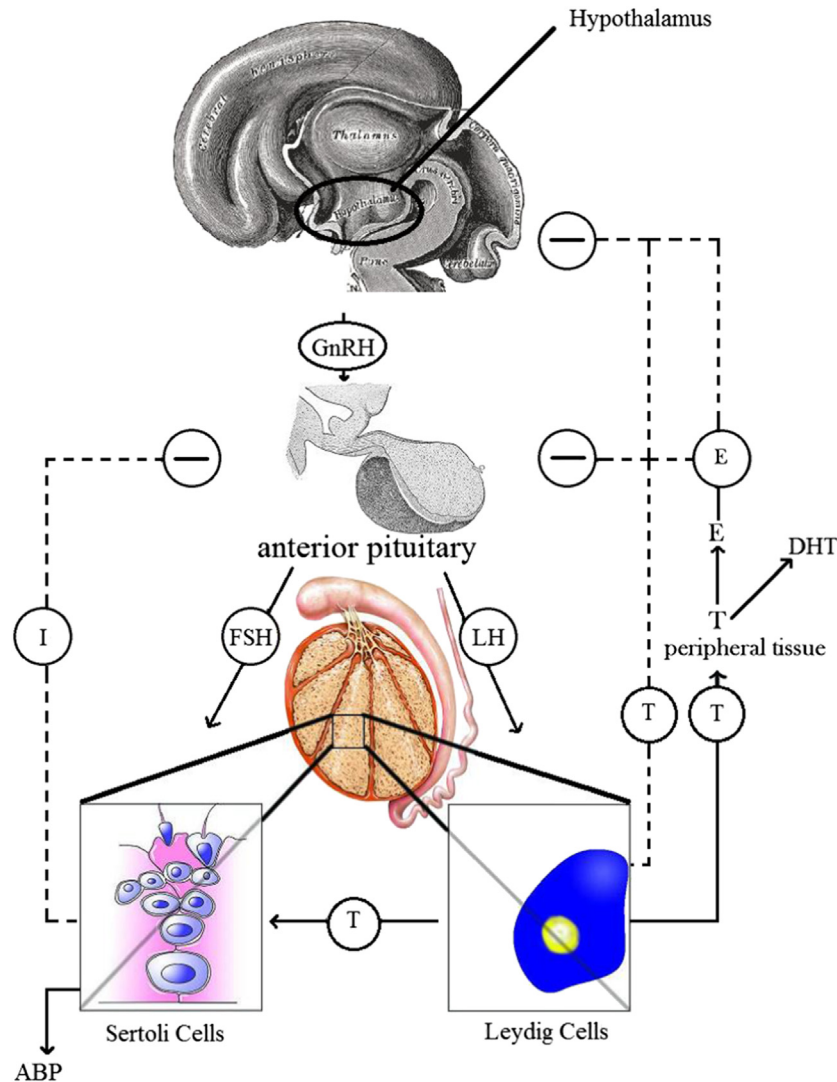


Fig. 1 Hypothalamic—Pituitary—Testis showing the negative feedback of the sex steroids on the hypothalamus and pituitary. Adapted with permission from Center for Male Reproductive Medicine and Microsurgery—Cornell, and Gray's Anatomy Plates from public domain.

activates protein kinase C. Kisspeptin and GnRH neurons have axo-somatic, axo-dendritic, and axo-axonal connections. The KNDy neurons are inter-connected into a neuronal network. Inside the KNDy neuronal network, neurokinin B (a tachykinin) initiates each GnRH pulse and dynorphin inhibits KNDy neural activity to terminate each pulse (Skorupskaitė et al., 2014).

Kisspeptin and its G-protein coupled receptor KISS1R (previously known as GPR54) are involved in the onset and progression of puberty. During puberty, kisspeptin secretion increases, and kisspeptin pulse frequency increases. Puberty can be postponed with administration of a kisspeptin antagonist. Mutations in kisspeptin or Kiss1R can lead to delayed or absent puberty and hypogonadotropic hypogonadism, or precocious puberty, depending on the type of mutation. Similarly, inactivating mutations of neurokinin B also cause hypogonadotropic hypogonadism (Skorupskaitė et al., 2014; Terasawa et al., 2013).

Other neurotransmitters, including glutamate and GABA, also have an effect on GnRH secretion. GABA has an inhibitory effect on GnRH prior to puberty, and during pubertal onset, GABAergic inhibition is reduced (Terasawa et al., 2013).

Multiple factors affect kisspeptin expression, including testosterone and progestogens, metabolic factors, and medications. KNDy neurons have ER alpha, progesterone, and androgen receptors. Importantly, GnRH neurons do not express androgen receptors or estrogen receptors, thus androgens and estrogens must act on other cells that affect GnRH secretion. KNDy neurons are an integral part of the mechanism by which sex steroids inhibit GnRH release. In the infundibular nucleus, estrogen and testosterone suppresses the secretion of kisspeptin and neurokinin B and stimulates the secretion of dynorphin, leading to decreased secretion of GnRH (Skorupskaitė et al., 2014). In women, during the late follicular phase there is also estrogen positive feedback to the kisspeptin neurons to induce the GnRH/LH surge during ovulation (Fig. 2). The mechanism behind the positive estrogen feedback is species specific.

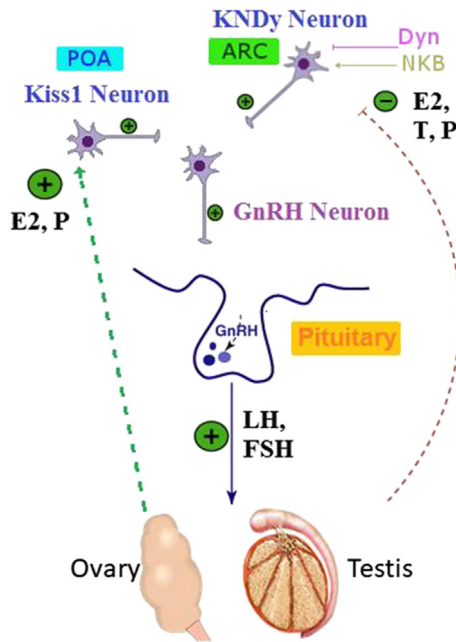


Fig. 2 Regulation of GnRH secretion by Kisspeptin and KNDy neuron. Modified from Roa J, Tena-Sempere M. (2010). Energy balance and puberty onset: Emerging role of central mTOR signaling. *Trends in Endocrinology and Metabolism* 21(9):519–528.

Environmental factors also affect kisspeptin expression. Stress increases corticotropin-releasing hormone, which inhibits GnRH release. Nutritional status affects leptin levels, and both caloric restriction and obesity cause hypogonadotropic hypogonadism. Leptin receptors are present on kisspeptin neurons, and in the setting of caloric restriction, leptin levels fall leading to a decrease in kisspeptin. Exogenous kisspeptin administration has been shown to improve the low gonadotropin levels and estrogen levels of malnourished rats (Castellano et al., 2005). Medications such as opioids inhibit GnRH release via the KNDy neurons, decreasing LH more strongly than FSH. There have been numerous studies on opioid-induced endocrinopathy, of which hypogonadism is one aspect.

FSH, LH, and the Testes

GnRH stimulates secretion of the gonadotropins LH and FSH. LH and FSH are two of four glycoprotein hormones that have identical alpha subunits but different beta subunits; the other two members of the family are TSH and hCG. Due to the differences in glycosylation, the half-life of LH is approximately 20 min, whereas the half-life of FSH is approximately 3–4 h (Fry et al., 1987).

FSH stimulates Sertoli cells to increase production of proteins including androgen binding protein, anti-Müllerian hormone, and inhibin B. It also increases the responsiveness of the Sertoli cell to testosterone, via effects on the androgen receptor. FSH receptors in men are only found in the testis, primarily on the Sertoli cells. Androgen binding protein (ABP) in rats is similar to SHBG in humans. They are glycoproteins with the same amino acid sequence but different sugar moieties. ABP does not appear in the rodent circulation, where in the human SHBG is secreted by the liver and binds circulating testosterone and estradiol. ABP binds to testosterone to create high levels of testosterone within the seminiferous tubules which is essential for spermatogenesis. Inhibin B inhibits FSH secretion at the level of the pituitary gland; it does not affect the hypothalamus. Activin A is another paracrine regulator of spermatogenesis and a stimulator of FSH secretion at the level of the pituitary gland. Anti-Müllerian hormone (AMH) is important during embryogenic regulation of sexual differentiation.

LH binds to the luteinizing hormone/chorionic gonadotropin (LH/CG) receptor on Leydig cells in the testis. This leads to increase in protein kinase A levels in the cell, upregulation of the steroidogenic acute regulatory protein (StAR) and other protein complexes. This will be discussed in detail on the section on testicular steroidogenesis.

In the testis, testosterone has paracrine effects; testosterone, along with FSH, stimulates initiation and maintenance of spermatogenesis. Testosterone has systemic effects, both as testosterone and via conversion to dihydrotestosterone (DHT) and estradiol (E2). Testosterone and its metabolites are part of a negative feedback loop at the level of the pituitary and the hypothalamus. In the central nervous system, aromatase converts testosterone into estradiol. As mentioned previously, testosterone and estradiol suppress GnRH release at the level of the hypothalamus, in part through suppression of KNDy neurons which leads to inhibition of GnRH release (Skorupskaite et al., 2014) (Fig. 2). Sex steroids also affect FSH and LH secretion at the level of the pituitary gland (Fig. 1). Testosterone has some direct effects on the gonadotrophs in the anterior pituitary, but its main effect is via intra-pituitary conversion to estradiol. Together, estradiol and testosterone make the anterior pituitary less responsive to GnRH by decreasing GnRH receptor binding (Bagatell et al., 1994; Heber et al., 1982).

Spermatogenesis

Spermatogenesis is tightly linked to the endocrinology of the testis. Spermatogenesis begins in the seminiferous tubules of the testes. As mentioned above, the Sertoli cells are stimulated by FSH and high testosterone concentrations within the testes. In men there are three types of spermatogonia: types Ad, Ap, and B. Some of the stem cells (Ad) are kept in reserve and rarely divide, whereas other stem cells (Ap) are actively dividing. These actively dividing spermatogonia may become Ap spermatogonia again, in a process of self-renewal, or they may become type B spermatogonia (diploid, 2n), which divide and differentiate to become primary spermatocytes (diploid, 4n) via mitosis. Each primary spermatocyte divides into two secondary spermatocytes (2n), via meiosis I. The secondary spermatocytes each divide via meiosis II again, forming four spermatids (1n) per primary spermatocyte. The spermatids then undergo changes in shape and structure in a process called spermiogenesis. Without testosterone or without FSH, spermatogenesis cannot be completed (Zirkin et al., 1989).

Gonadal Androgens

Testicular Steroidogenesis

Testosterone is the main androgen produced by the testes. Cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane by a complex of proteins including the steroidogenic acute regulatory protein (StAR) (Stocco et al., 2017), Translocator Protein (TSPO) (Papadopoulos et al., 2015) and voltage-dependent anion channel amongst others. In the adrenals, and to a smaller extent in the testes, liver, and brain, cholesterol is converted to pregnenolone via CYP11A (also known as cholesterol side-chain cleavage enzyme or P450_{sc}) located in the inner mitochondrial membrane (Miller, 1988). Pregnenolone is converted to androstenedione via CYP17A1 (17 α hydroxylase/17,20 lyase) and 3 β -HSD (delta 5–4 isomerase), via the delta-5 pathway and the delta-4 pathway. Androstenedione is converted to testosterone by 17 β -hydroxysteroid dehydrogenase (17 β -HSD). For a more detailed description, see Chapter Leydig Cell Androgen Synthesis (Fig. 3).

In men, over 90%–95% of testosterone comes from the testes, and the remaining testosterone is produced by the adrenal glands or from extraglandular conversion of dehydroepiandrosterone (DHEA) and androstenedione. A small amount of the testosterone produced in the testes is converted to estradiol via aromatase. The testes are a minor direct secretory source of circulating estradiol; however, the majority of the estradiol produced in men is from peripheral conversion of testosterone to estradiol (Raven et al., 2006). Examples of tissues with high levels of aromatase are adipose tissue, skin, bone, brain, pituitary, testis, liver, intestine, and breast stromal cells. Estradiol is important for skeletal growth during puberty, and high estradiol levels at the end of puberty causes fusion of the epiphyseal plates. Estradiol is important for maintaining bone health and estrogen deficiency leads to osteoporosis in men. Estradiol is also important in the regulation of the hypothalamic-pituitary-gonadal axis as described above.

Testosterone is also converted to dihydrotestosterone (DHT) via 5-alpha reductase. DHT is 3–5 times as potent as testosterone in some tissues. DHT has a higher affinity for the androgen receptor, and dissociates from the receptor about three times more slowly. Examples of tissues with high levels of 5-alpha reductase are the prostate, seminal vesicles, epididymis, liver, brain, skin, and hair follicles. The serum levels of DHT are much lower than the serum levels of testosterone, but in tissues such as the prostate, the local levels of DHT are 5–10 $\times$ higher than the level of testosterone (Swerdlhoff et al., 2017). DHT is not converted to an estrogen with aromatase, and thus does not have estrogenic effects (Liao et al., 1973).

Approximately 45% of serum testosterone in men is bound to SHBG, 50%–60% is bound to albumin, and 2% is free. Testosterone binds more strongly to SHBG than to albumin. Bioavailable testosterone includes albumin-bound testosterone plus free testosterone (Emadi-Konjin et al., 2003). According to the free hormone hypothesis, the “intracellular hormone concentrations” (and therefore biologic activity) “are dependent on the concentration of free rather than protein-bound hormone in the plasma” (Mendel, 1989). The level of free testosterone correlates to clinical symptoms of hypogonadism more closely than the level of total testosterone.

Testosterone and DHT are converted to inactive metabolites primarily by the liver. In the liver, oxidoreductases such as 3 α - and 3 β -hydroxysteroid dehydrogenases perform oxidoreduction, and the metabolites are then conjugated by glucuronidases. The inactivated metabolites are excreted into urine and bile (Gao et al., 2005).

Testosterone and Its Receptors

Humans have estrogen, progesterone, androgen, glucocorticoid, and mineralocorticoid receptors. These receptors have a hinge region, a hormone-binding domain, a DNA-binding domain, and a variable domain. The nuclear localization signal, which helps the receptor enter the nucleus, is located by the hinge region and is blocked by heat shock proteins (Gao et al., 2005). There are several isoforms of androgen receptors. Androgen receptors are primarily found in the cytoplasm. Free testosterone diffuses into the cell. If 5-alpha-reductase is present, the testosterone is converted into dihydrotestosterone. In the cytoplasm, the androgen is bound by the androgen receptor. The heat shock proteins dissociate from the androgen receptor, and the receptor is dimerized, phosphorylated, and translocated to the nucleus. In the nucleus, the androgen receptor dimer acts as a transcription factor to regulate gene expression via binding to the hormone response element (Gao et al., 2005). The androgen receptor also interacts with other transcription coregulators.

There are also nongenomic, rapid effects of androgens that is mediated via membrane androgen receptors. Even if the androgen is prevented from entering the cell, it can still cause changes in intracellular calcium, via membrane androgen receptors such as

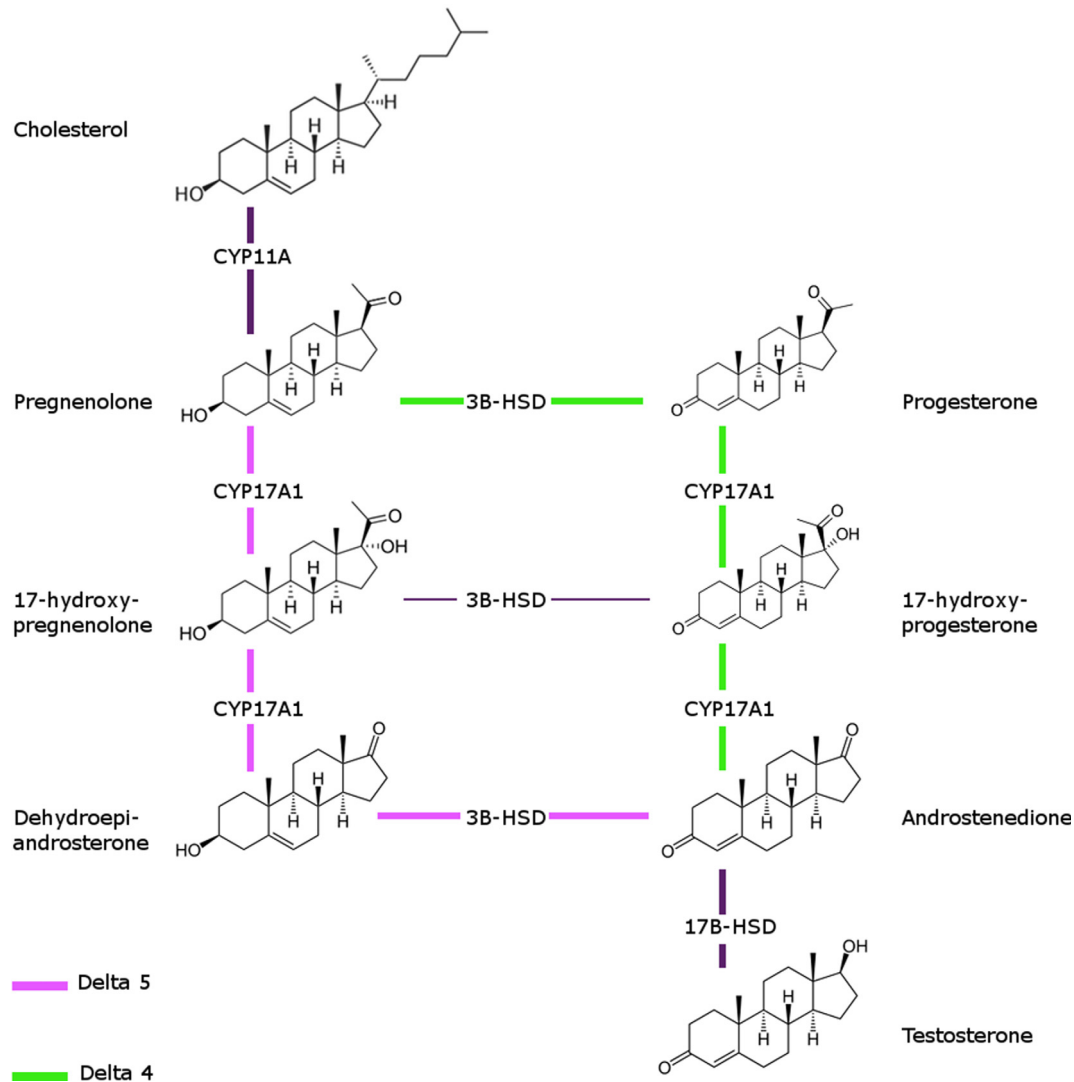


Fig. 3 Testicular steroidogenesis.

GPRC6A and ZIP9. These membrane androgen receptors affect ion transport channels via signal transduction proteins such as PI3k/Akt, Src, and Protein Kinase C (Hatzoglou et al., 2005).

Polymorphisms in the androgen receptor can alter the transcriptional activity of the androgen receptor. Androgen receptor genes with longer CAG repeat sequences are correlated with lower transcriptional activity. Certain polymorphisms have been linked to osteoporosis, infertility, prostate cancer, and androgenic alopecia. Because of diminished androgen feedback, patients with longer CAG repeats may have higher serum testosterone concentrations (Zitzmann and Nieschlag, 2003).

Effects of Testosterone

Human male and female gonads are the same until 6–7 weeks of fetal age. During the 7th week, differentiation begins. The precursors of the Leydig cells produce androgens as early as 6–7 weeks of gestation, preceding the secretion of LH by the pituitary. The Leydig cells reach their peak development and maturation around 17–21 weeks of gestation (Svechnikov et al., 2010). Testosterone is responsible for the development of the Wolffian duct and the structures such as the epididymis, prostate, seminal vesicles. The development of the external genitalia (including formation of the glans penis and corpora cavernosa), scrotal thinning and formation of rugae is dependent upon the presence of DHT.

During puberty, testosterone is required for development of secondary sexual characteristics (virilization) and spermatogenesis. Testosterone leads to growth of the penis, scrotum, prostate, and seminal vesicles. It also leads to the development of male pattern hair, increased sebum production (which may lead to acne), and deepening of the voice. It stimulates IGF-1 and growth hormone secretion. Testosterone and estradiol are involved in skeletal growth and increase in bone mineral density. Testosterone is also

involved in stimulation of libido, increase in skeletal muscle mass, redistribution and decrease of body fat, and decrease in HDL. During adulthood, testosterone maintains adult male characteristics, sexual function, and spermatogenesis. It is also involved in the stimulation of erythropoiesis.

As mentioned above, dihydrotestosterone is required in the formation of the male external genitalia. It also leads to facial and body hair, acne, male pattern baldness, and prostate growth. It is the primary androgen in the prostate, seminal vesicles, skin, and hair follicles in adults. In the setting of congenital 5-alpha-reductase 2 deficiency, the male genitalia and prostate are underdeveloped, but during puberty the testosterone levels rise normally and they develop male musculature. Persons with 5-alpha-reductase deficiency have lower levels of pubic and body hair, and do not develop androgenic alopecia.

With aging, testosterone levels tend to fall, thus there tends to be a loss of lean muscle mass and an increase in fat mass. It is controversial whether this decline is pathologic and whether it benefits from treatment, although the positive effects of testosterone on libido, muscle mass, and bone mineral density have been shown (Snyder et al., 2016; Wu et al., 2010).

Hypogonadism and Its Effects

Hypogonadism has various effects depending on the stage of development of the patient. In fetal development, androgen insensitivity syndrome or congenital defects in testosterone synthesis leads to ambiguous genitalia. In prepubertal children, androgen deficiency causes eunuchoidism, signs of which include small testes (<4 mL), infantile genitalia, high-pitched voice, and disproportionately long arms and legs relative to height, which may manifest as arm span greater than height or increased lower-to-upper body ratio. Adults who develop hypogonadism may have changes in their mental status, including lower motivation, decreased energy, depressed mood or irritability, and decreased concentration or memory. There is a decrease in libido/sexual function and fertility. They may have hyperlipidemia and insulin resistance. On examination of their semen, they may have sperm with abnormal morphologies (teratozoospermia), sperm with reduced motility (asthenozoospermia), low sperm count (oligozoospermia), or no sperm cells in the ejaculate (azoospermia). With longstanding androgen deficiency, there may be loss of androgen-dependent hair, reduced muscle mass, development of fine facial wrinkles, gynecomastia, reduction in prostate size, decreased bone mineral density, and decrease in testes volume. If the development of testosterone deficiency is rapid, they may have hot flashes and sweats.

Hypogonadism can be primary (primary testicular failure) or secondary (central hypogonadism or hypogonadotrophic hypogonadism) (Fig. 1). Primary testicular failure can be differentiated from hypogonadotrophic hypogonadism by measurement of FSH, LH, and testosterone levels. In both primary and secondary hypogonadism, testosterone levels are low. However, in primary hypogonadism, FSH and LH levels are elevated, whereas in secondary hypogonadism, FSH and LH are low. Causes of primary testicular failure include Klinefelter syndrome, testicular injury, and chemotherapeutic agents and irradiation. Causes of hypogonadotrophic hypogonadism include pituitary tumors, hemochromatosis, injury to the pituitary, Kallmann's syndrome, acute and chronic illnesses, medications including opioids, and obesity.

Conclusion

The hypothalamic-pituitary-testis axis controls testosterone production, and can be affected by internal and external factors. Recent studies have elucidated more details on the KNDy neurons and kisspeptin in the control of GnRH secretion and the mechanism of GnRH pulse generation. Pulsatile GnRH secretion results in the production and secretion of LH and FSH by the pituitary regulating testicular function. The testes are responsible for spermatogenesis and the production of androgens mainly, testosterone. Testosterone and its metabolites estradiol and DHT have paracrine and systemic effects, and defects in testosterone biosynthesis or testosterone receptor function can lead to hypogonadism.

References

- Bagatell, C. J., Dahl, K. D., & Bremner, W. J. (1994). The direct pituitary effect of testosterone to inhibit gonadotropin secretion in men is partially mediated by aromatization to estradiol. *Journal of Andrology*, 15(1), 15–21.
- Castellano, J. M., Navarro, V. M., Fernández-Fernández, R., Nogueiras, R., Tovar, S., Roa, J., Vazquez, M. J., Vigo, E., Casanueva, F. F., Aguilar, E., Pinilla, L., Dieguez, C., & Tena-Sempere, M. (2005). Changes in hypothalamic KiSS-1 system and restoration of pubertal activation of the reproductive axis by kisspeptin in undernutrition. *Endocrinology*, 146(9), 3917–3925.
- Crosby, E. C., & Woodburne, R. T. (1940). The comparative anatomy of the preoptic area and the hypothalamus. *Research Publications—Association for Research in Nervous and Mental Disease*, 20, 52–169.
- Emadi-Konjin, P., Bain, J., & Bromberg, I. L. (2003). Evaluation of an algorithm for calculation of serum “bioavailable” testosterone (BAT). *Clinical Biochemistry*, 36(8), 591–596.
- Fry, R. C., Cahill, L. P., Cummins, J. T., Bindon, B. M., Piper, L. R., & Clarke, I. J. (1987). The half-life of follicle-stimulating hormone in ovary-intact and ovariectomized Booroola and control merino ewes. *Journal of Reproduction and Fertility*, 81, 611–615.
- Gao, W., Bohl, C. E., & Dalton, J. T. (2005). Chemistry and structural biology of androgen receptor. *Chemical Reviews*, 105(9), 3352–3370.
- Hatzoglou, A., Kampa, M., Kogia, C., Charalampopoulos, I., Theodoropoulos, P. A., Anezinis, P., Dambaki, C., Papakonstanti, E. A., Stathopoulos, E. N., Stourmaras, C., Gravanis, A., & Castanas, E. (2005). Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells in vitro and in vivo. *The Journal of Clinical Endocrinology and Metabolism*, 90, 893–903.

- Heber, D., Dodson, R., Stoskopf, C., Peterson, M., & Swerdloff, R. S. (1982). Pituitary desensitization and the regulation of pituitary gonadotropin-releasing hormone (GnRH) receptors following chronic administration of a superactive GnRH analog and testosterone. *Life Sciences*, 30(26), 2301–2308.
- Liao, S., Liang, T., Fang, S., Castañeda, E., & Shao, T. C. (1973). Specificities involved in the receptor binding & nuclear retention of various androgens. *The Journal of Biological Chemistry*, 248(17), 6154–6162.
- Mendel, C. (1989). The free hormone hypothesis: A physiologically based mathematical model. *Endocrine Reviews*, 10, 232–274.
- Miller, W. L. (1988). Molecular biology of steroid hormone synthesis. *Endocrine Reviews*, 9(3), 295–318.
- Papadopoulos, V., Aghazadeh, Y., Fan, J., Campoli, E., Zirkin, B., & Midzak, A. (2015). Translocator protein-mediated pharmacology of cholesterol transport and steroidogenesis. *Molecular and Cellular Endocrinology*, 408, 90–98.
- Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P., & Tena-Sempere, M. (2012). Kisspeptins and reproduction: Physiological roles and regulatory mechanisms. *Physiological Reviews*, 92, 1235–1316.
- Raven, G., de Jong, F. H., Kaufman, J. M., & de Ronde, W. (2006). In men, peripheral estradiol levels directly reflect the action of estrogens at the Hypothalamo-pituitary level to inhibit gonadotropin secretion. *The Journal of Clinical Endocrinology and Metabolism*, 91(9), 3324–3328.
- Skorupskaitė, K., George, J. T., & Anderson, R. A. (2014). The kisspeptin-GnRH pathway in human reproductive health and disease. *Human Reproduction Update*, 20(4), 485–500.
- Snyder, P. J., Bhasin, S., Cunningham, G. R., Matsumoto, A. M., Stephens-Shields, A. J., Cauley, J. A., Gill, T. M., Barrett-Connor, E., Swerdloff, R. S., Wang, C., Ensrud, K. E., Lewis, C. E., Farrar, J. T., Cella, D., Rosen, R. C., Pahor, M., Crandall, J. P., Molitch, M. E., Cifelli, D., Dougar, D., Fluharty, L., Resnick, S. M., Storer, T. W., Anton, S., Basaria, S., Diem, S. J., Hou, X., Mohler, E. R., 3rd, Parsons, J. K., Wenger, N. K., Zeldow, B., Landis, J. R., & Ellenberg, S. S. (2016). Effects of testosterone treatment in older men. *The New England Journal of Medicine*, 374, 611–624.
- Stocco, D. M., Zhao, A. H., LN, T., Morohaku, K., & Selvaraj, V. (2017). A brief history of the search for the protein(s) involved in the acute regulation of steroidogenesis. *Molecular and Cellular Endocrinology*, 441, 7–16.
- Svechnikov, K., Landreh, L., Weissner, J., Izzo, G., Colón, E., Svechnikova, I., & Söder, O. (2010). Origin, development and regulation of human Leydig cells. *Hormone Research in Paediatrics*, 73, 93–101.
- Swerdloff, R. S., Dudley, R. E., Page, S. T., Wang, C., & Salameh, W. A. (2017). Dihydrotestosterone: Biochemistry, physiology, and clinical implications of elevated blood levels. *Endocrine Reviews*, 38(3), 220–254.
- Terasawa, E., Guerriero, K. A., & Plant, T. M. (2013). Kisspeptin and puberty in mammals. *Advances in Experimental Medicine and Biology*, 784, 253–273.
- Thompson, I. R., & Kaiser, U. B. (2014). GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression. *Molecular and Cellular Endocrinology*, 385, 28–35.
- Wu, F. C., Tajar, A., Beynon, J. M., Pye, S. R., Silman, A. J., Finn, J. D., O'Neill, T. W., Bartfai, G., Casanueva, F. F., Forti, G., Giwercman, A., Han, T. S., Kula, K., Lean, M. E., Pendleton, N., Punab, M., Boonen, S., Vanderschueren, D., Labrie, F., & Huhtaniemi, I. T. (2010). Identification of late-onset hypogonadism in middle-aged and elderly men. *The New England Journal of Medicine*, 363, 123–135.
- Zirkin, B. R., Santulli, R., Awoniyi, C. A., & Ewing, L. L. (1989). Maintenance of advanced Spermatogenic cells in the adult rat testis: Quantitative relationship to testosterone concentration within the testis. *Endocrinol*, 124(6), 3043–3049.
- Zitzmann, M., & Nieschlag, E. (2003). The CAG repeat polymorphism within the androgen receptor gene and Maleness. *International Journal of Andrology*, 26(2), 76–83.

TESTIS CELL BIOLOGY

Sertoli Cell

Bernard Jégou and Antoine D Rolland, Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

In 1865 at the age of 23 years, the Italian scientist Enrico Sertoli described for the first time the “cellule ramificata” (Sertoli, 1865; Setchell, 1993) within the human testis. His Austrian contemporary Victor von Ebner 13 years after the description called the cell a Sertoli cell, which has now been accepted for more than a century as the Sertoli cell (von Ebner, 1888). From the start Sertoli had made the assertions that the Sertoli cells which cover the wall of the seminiferous tubules in mammals were (i) individual cells; and (ii) linked to the production of spermatozoa within the seminiferous tubules. Then for decades, the Sertoli cells fascinated the histologists, but it is only with the advent of the electron microscope in the second half of the 20th century that Fawcett and Burgos (1956) ended the controversy about the possible syncytial status of the Sertoli cells by establishing that there are indeed individual cells.

Description of the Sertoli Cell

The Sertoli cell presents complex shapes (the “cellule ramificata”) which vary according to the different stages of spermatogenesis and extends from the basal lamina of the seminiferous tubules toward the lumen of these tubules (Fig. 1). This organization and polarity of the Sertoli cells is reflected by junctions between neighboring Sertoli cells and germ cells at different stages of differentiation, as well at the level of the wall the seminiferous tubules between the Sertoli cells and the extracellular matrix. The discovery and description of the cellular structural devices within the seminiferous tubules was essential in deciphering the extremely complex Sertoli cell–germ cell communication network (Fig. 1).

The structure and function of the structural devices from Sertoli cells and other tubular cells have been extensively reviewed (Russell, 1993; Jégou, 1991, 1992, 1993; Mruk and Cheng, 2015). We can classify them into three categories: (i) devices that are implicated in cell attachment, shape, and movement. These include the desmosome-like and ectoplasmic specializations; (ii) devices which combine their involvement in the functions described just before (i), but also which are involved in the transfer of cellular materials and molecules from Sertoli cells to germ cells or vice-versa: the spermatogonial processes, the tubulobulbar complexes, the spermatid processes and gap junctions; (iii) devices only involved in the transfer of materials such as the residual bodies which are formed from the elongated spermatids and detach in the lumen of the tubules when spermatozoa are released. Residual bodies are phagocytosed by the Sertoli cells.

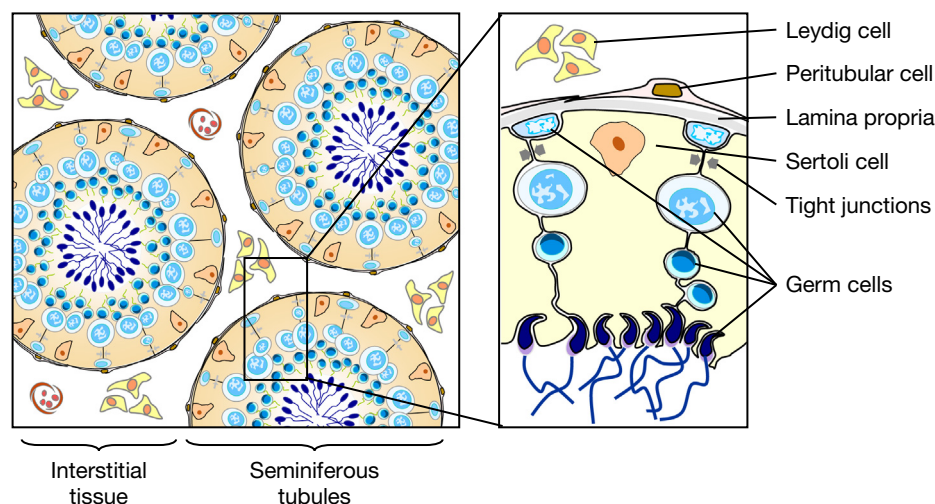


Fig. 1 Schematic representation of the intratesticular organization. Adapted from Rolland, A. D., Jégou, B. and Pineau, C. (2008). Testicular development and spermatogenesis: Harvesting the postgenomics bounty. *Advances in Experimental Medicine and Biology* **636**, 16–41. doi: 10.1007/978-0-387-09597-4_2.

Over the last decades, progress in deciphering the molecular bases of structural devices and the spatio-temporal regulation of molecules interconnecting the Sertoli cells and the germ cells has been instrumental to understand better how the testis functions and assumes its role in producing spermatozoa and various hormones important for spermatogenesis, and in men's health more generally.

How Do Sertoli Cells Control Spermatogenesis?

The Sertoli Cell Barrier

This structure was defined by as lying within the specialized Sertoli cell junctional complexes located in the basal segment of the seminiferous epithelium (Ploën and Setchell, 1992). This barrier is selective and divides the seminiferous epithelium into a basal compartment, that comprises the premeiotic germ cell categories, and an adluminal compartment, that encompasses meiotic and post-meiotic germ cells, the latter being the haploid spermatids at all steps of their development. Of note is that the Sertoli cell barrier is a crucial component of the so-called blood–testis barrier, which also comprises the blood vessels and the peritubular cells.

The Sertoli cell barrier is essential to (i) assume the cytoarchitecture of the Sertoli cell; (ii) produce the tubular fluid and create and maintain the micro-environment which is essential to meiosis and spermiogenesis (i.e., differentiation of the spermatids); (iii) establish distinct domains of the Sertoli cell plasma membrane such as the presentation of the FSH membrane receptors at the basal pole of the cell (Fawcett and Burgos, 1956); implement the immunological barrier which prevent antigens from the meiotic and post-meiotic germ cells to generate immune reactions which would be deleterious to fertility. According to several authors, FSH and testosterone are most likely involved in the formation, control, and maintenance of the Sertoli cell barrier (de Kretser and Burger, 1972; Kerr et al., 1993).

The Products of the Sertoli Cells Destinated to Germ Cells

It is now well established that the Sertoli cell produce the tubular fluid, peptides, proteins, and steroids (Bardin et al., 1988; Griswold, 1988; Jégou, 1992, 1993). This justifies its ancestral name of “nurse cell,” that is the cell which provides a crucial assistance for spermatogenesis to occur. While the products of the Sertoli cells were historically thought to be addressed toward the lumen of the tubules where the fluid transports freshly shed and nonmotile spermatozoa, it is now known that the Sertoli cell products necessary to early germ cells, peritubular myoid cells, and Leydig cells are secreted and directed basally, while those required for meiosis and spermiogenesis are likely to be directed apically (Gunsalus and Bardin, 1991; Byers et al., 1993).

The Hormonal Activity of the Sertoli Cells

Among the proteins produced by the Sertoli cells are the hormones named inhibin and activin which play a key role in the negative and positive retrocontrols on the production of FSH, respectively. Another key protein hormone which is exclusively produced by the Sertoli cell is the anti-Müllerian Hormone (AMH). AMH induces the resorption of the Müllerian ducts to favor the masculinization of the urogenital tract during fetal life. Of note is that AMH is still produced at all ages of life, but its paracrine role (i.e., within the seminiferous tubule) as well as its endocrine function (through the blood circulation) are largely unknown in the adult male. Last but not least, whether the estrogens produced by the immature Sertoli cells exert a hormonal control (i.e., outside the seminiferous tubules) remains to be established.

How Do Germ Cells Control Sertoli Cell Function?

Evidence for a Germ Cell Control

The qualification of the Sertoli cell as a “nurse cell” has occulted for a long time the fact that germ cells which represent about 70%–80% of the volume of the seminiferous tubule exert an essential feedback on it. Thus, the incredible plasticity of the seminiferous epithelium all along spermatogenesis exerts enormous cyclic constraints on the Sertoli cell cytoskeleton, illustrating the axiom that “structure is the function.” Sertoli cell function is for a large part dictated by the changing number, nature, and needs of the germ cell complements (Jégou, 1991, 1992, 1993). This has been experimentally established using (i) in vivo approaches by the implementation of various exposures (radiation, moderate heat, chemical agents) able to induce selective loss of a (or a limited) germ cell category (ies), and thereby inducing changes in the morphology and function of the Sertoli cell which can be observed and measured; (ii) in vitro, thanks to the culture and co-culture of isolated Sertoli cells and various categories of germ cells (Jégou, 1992, 1993). The microdissection of rat seminiferous tubules at the different stages of spermatogenesis owing to the transillumination of the tubules has also allowed demonstrating the spatio-temporal expression and production of a number of Sertoli cell products (Parvinen, 1993).

Mechanisms of the Germ Cell Control

We have hypothesized that there are different pathways by which germ cells exert their action on the Sertoli cells. These pathways represent a combination of (i) morphoregulatory mechanisms and membrane molecules (Gérard et al., 1995; Byers et al., 1993; Mruk and Cheng, 2015); (ii) the transfer of enormous amounts of germ cell materials all along the spermatogenetic process (Jégou and Sharpe, 1993); and (iii) soluble proteins from germ cell even though the extreme fragility and lability of isolated germ cells have hampered their intensive identification and study (Jégou, 1991, 1992, 1993).

The Sertoli Cell–Peritubular Cell Cross Talk

A lot of mystery remains on how the cells that border the tubules—the peritubular myoid cells—and the Sertoli cells actually dialogue. It remains that this dialogue is no doubt important from fetal life to adulthood to ensure the polarity and full functionality of Sertoli cells (Skinner, 1991; Jégou and Sharpe, 1993). This involves the co-production of extracellular matrix of the laminae propria, the production of molecules, the receptivity vis-à-vis different hormones (testosterone, FSH) and metabolic cooperations.

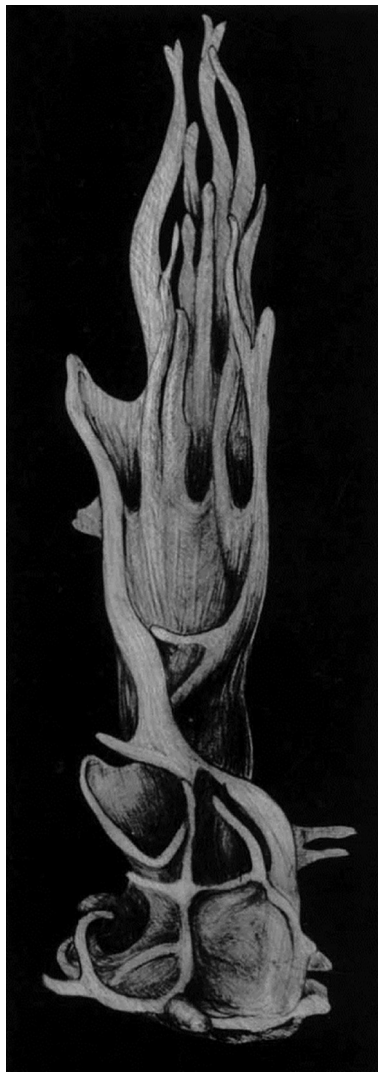


Fig. 2 Drawing from a three-dimensional reconstructed stage V rat Sertoli cell. From Wong, V. and Russell, L. D. (1983). Three-dimensional reconstruction of a rat stage V Sertoli cell: I. Methods, basic configuration, and dimensions. *The American Journal of Anatomy* **167**, 143–161.

The Sertoli Cell–Leydig Cell Cross Talk

The fact that the testis is divided in two compartments, the seminiferous tubules where Sertoli cells reside and the interstitial tissue where the Leydig cells are situated is not antinomic to the existence of a finely tuned and essential dialogue between Sertoli cells and Leydig cells for testicular function and men's health to be optimum. The dialogue between these two somatic cell types involve the interstitial fluid and the steroids (oestradiol and testosterone) as well as peptides and protein factors, the latter being far from having all been identified. It also involves the extracellular matrix and aspects of the mediation assumed by the peritubular cells which also remain largely unexplored.

Conclusion

The late Lonnie Russell who has remarkably studied and reviewed the “form, dimensions, and cytology of mammalian Sertoli cells” used to insist on their extraordinary well developed cytoskeleton, involving the classical actin and intermediate filaments as well as microtubules (Russell, 1993) (Fig. 2). This cytoskeleton displays all possible properties of plasticity to adapt to the cyclicity of spermatogenesis and its regulation. No doubt that the development of new imaging technologies, and the input of all aspects of other technologies which have irrigated the field of biology and medicine over the last decades will allow to fill the numerous gaps which still represent an obstacle to understand better the intimacy of the Sertoli cell and thereby of the physiology of the testis.

References

- Bardin, C. W., Cheng, C. Y., Musto, N. A., & Gunsalus, G. L. (1988). The Sertoli cell. In E. Knobil, J. Neill, et al. (Eds.), *The physiology of reproduction* (vol. 1, pp. 933–974). New York: Raven Press.
- Byers, S. W., Jégou, B., MacCalman, C., & Blaschuk, O. (1993). The Sertoli cell–cell and cell–substratum adhesion and the collective organization of the testis. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell* (pp. 431–446). Clearwater, FL: Cache River Press.
- Ebner von, V. (1888). Zur Spermatogenese bei den Säugetieren. *Archiv für Mikroskopische Anatomie*, 31, 236–292.
- Fawcett, D. W., & Burgos, M. H. (1956). The fine structure of Sertoli cells in human testis. *The Anatomical Record*, 124, 401–420.
- Gérard, N., Corlu, A., Kneip, B., Kercret, H., Rissel, M., Guguen-Guillouzo, C., & Jégou, B. (1995). Liver-regulating protein (LRP) is a plasma-membrane protein involved in cell contact-mediated regulation of Sertoli cell function by primary spermatocytes. *Journal of Cell Science*, 10, 917–925.
- Griswold, M. D. (1988). Protein secretion of Sertoli cells. *International Review of Cytology*, 100, 133–156.
- Gunsalus, G. L., & Bardin, C. W. (1991). Sertoli–germ cell interactions as determinants of bidirectional secretion of androgen-binding protein. *Annals of the New York Academy of Sciences*, 637, 327–339.
- Jégou, B. (1991). Spermatids are regulators of Sertoli cell function. In B. Robaire (Ed.), *Annals of the New York Academy of Sciences: 637. The male germ cell spermatogonium to fertilization* (pp. 340–353). New York: New York Academy of Sciences.
- Jégou, B. (1992). The Sertoli cell. In D. M. de Kretser (Ed.), *Baillière's clinical endocrinology and metabolism: 6. The testis* (pp. 273–311). London: Baillière Tindall.
- Jégou, B. (1993). The Sertoli–germ cell communication network. *International Review of Cytology*, 147, 25–96.
- Jégou, B., & Sharpe, R. M. (1993). Paracrine mechanisms in testicular control. In D. M. de Kretser (Ed.), *Molecular biology of the male reproductive system* (pp. 271–310). San Diego: Academic Press. Inc.
- Kerr, J. B., Millar, M., Maddocks, S., & Sharpe, R. M. (1993). Stage-dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. *The Anatomical Record*, 235(4), 547–559.
- de Kretser, D. M., & Burger, H. G. (1972). Ultrastructural studies of the human Sertoli cell in normal men and males with hypogonadotropic hypogonadism before and after gonadotrophic treatment. In B. B. Saxena, et al. (Eds.), *Gonadotrophins* (pp. 640–656). New York: Wiley Interscience.
- Mruk, D. D., & Cheng, C. Y. (2015). The mammalian blood–testis barrier: Its biology and regulation. *Endocrine Reviews*, 36, 564–591.
- Parvinen, M. (1993). Cyclic functions of Sertoli cells. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell* (pp. 331–347). Clearwater, FL: Cache River Press.
- Ploën, L., & Setchell, B. P. (1992). Blood–testis barriers revisited. An homage to Lennat Nicander. *International Journal of Andrology*, 15, 1–4.
- Russell, L. D. (1993). Form, dimensions, and cytology of mammalian Sertoli cells. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell* (pp. 1–37). Clearwater, FL: Cache River Press.
- Sertoli, E. (1865). Dell' esistenza di particolari cellule ramificate nei canaliculi seminiferi del testicolo umano. *Morgagni*, 7, 31–39.
- Setchell, B. P. (1993). Foreword. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell* (pp. v–vi). Clearwater, FL: Cache River Press.
- Skinner, M. K. (1991). Cell–cell interactions in the testis. *Endocrine Reviews*, 12, 45–77.

Further Reading

- Wong, V., & Russell, L. D. (1983). Three-dimensional reconstruction of a rat stage V Sertoli cell: I. Methods, basic configuration, and dimensions. *The American Journal of Anatomy*, 167, 143–161.

Molecular Biology of Sertoli Cells

Michael D Griswold, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Over 150 years ago Enrico Sertoli first suggested that the cell which now bears his name was a “sustentacular” or nurse cell. This suggestion was made because of the observed morphological relationships with the developing germ cells. The morphology of Sertoli cells has been described in some detail but even after years of research with new and better technological tools many of the complex functions of the Sertoli cells remain obscure. It has been shown that it is the expression of genes in embryonic Sertoli cells encoding transcription factors such as Sry and Sox9 that result in the formation of the testis (for a review see [Svingen and Koopman, 2013](#)). Gene expression in Sertoli cells is very dynamic beginning early in embryogenesis, has a role during the onset of spermatogenesis and throughout the reproductive lifetime of the organism.

The initial molecular studies on Sertoli cells began in the 1970s and 1980s when the techniques for primary culture were described ([Dorrington et al., 1975](#); [Steinberger et al., 1975](#)). The availability of enriched primary cultures of Sertoli cells and specific antibodies combined with improvements in microscopy have revealed several gene products that are required for the function of Sertoli cells. In the early 1980s it was shown the Sertoli cells secreted metal transport proteins such as transferrin and ceruloplasmin ([Skinner and Griswold, 1980](#)). It was initially proposed that the transport of metals by Sertoli cell gene products to developing germ cells was an example of the “nurse cell function.” This idea has been modified as the components of the iron transport pathway in the testis were described ([Leichtmann-Bardoo et al., 2012](#)). It was proposed that there was an iron cycle in the testis that was orchestrated by the Sertoli cells. It was proposed that iron was delivered by the Sertoli cells to the developing germ cells to support mitosis, meiosis, and mitochondriogenesis through the elongated spermatid stage. The bulk of the iron was then recycled back to the Sertoli cells in the ingested residual bodies where it could again be delivered to another stage of developing germ cells. In this proposed model, the nurse cell function of the Sertoli cells involved the provision and delivery of iron but also the removal and recycling of the potential toxic accumulation of iron in the residual bodies. It is likely that there are several such mechanisms that involve delivery and recycling of critical components for germ cell development.

Sertoli cells products can inhibit immune reactions and provide structural features under a broad classification of “bioprotective and structural” functions ([Franca et al., 2016](#)). Adjacent Sertoli cells synthesize the components of the tight junction complexes and contribute to the basement membrane and participate in the formation of desmosomes, gap junctions and some unique forms of junctions with germ cells. The nature of spermatogenesis results in the production of enormous numbers of gametes and therefore these structural elements must be extremely dynamic and involve the spatiotemporal expression of many genes.

A key molecular event in the Sertoli cells is the response to FSH and testosterone and the subsequent production of paracrine factors that regulate germ cell development ([Griswold et al., 1988](#)). Both FSH and testosterone act through Sertoli cells to alter gene expression throughout development and with the stage of the cycle of the seminiferous epithelium. Another key signaling molecule in spermatogenesis is vitamin A in the form of retinoic acid. The onset of germ cells committing to meiosis requires retinoic acid and the source of retinoic acid to initiate spermatogenesis are the Sertoli cells ([Hogarth and Griswold, 2010](#)).

The in vitro differentiation of germ cells into functional gametes has been reported for several species. These reports would suggest that the progression of stem germ cells into functional gametes is an autonomous program. However, even optimistically accepting the results from the most successful of these reports it is clear that germ cells in the absence of Sertoli cells are extremely inefficient at sperm production when compared to spermatogenesis in the testis. Clearly gene products from Sertoli cells are required for the efficient formation of functional gametes. Gene products from Sertoli cells provide the environment, the signaling, the immunoprotection and the structural components to promote the efficient differentiation of male germ cells into spermatozoa. While germ cells must have an autonomous program, Sertoli cells appear to be “permissive” in nature ([Russell and Griswold, 1993](#)).

One of the major issues surrounding studies on the molecular biology of Sertoli cells is the complexity of the testis histology and the close association of many different cell types. The ability to culture relatively pure Sertoli cell preparations allowed for some molecular studies but the removal of Sertoli cells from the testis and the in vitro culture can alter the results. A recent innovation is the use of the RiboTag mouse that allows the genetic tagging of polysomes in Sertoli cells in vivo. This technology has resulted in the ability to analyze mRNA associated with polysomes in vivo in a single cell type amongst many cell types in a complex tissue ([Sanz et al., 2013](#)). The use of the RiboTag mouse has revealed some genes that are overexpressed in Sertoli cells relative to the other cell types in the testis and provide clues as to specific functions within the cell. Genes involved in glutathione metabolism, cytochrome P450 enzymes, drug metabolism pathways, peptidase and enzyme inhibitor pathways were relatively overexpressed by adult Sertoli cells in vivo ([De Gendt et al., 2014](#)). These results emphasize the importance of the detoxifying role of Sertoli cells.

When examined in the context of testis biology, the molecular details of the function of Sertoli cells must be well controlled. It appears that Sertoli cells participate in multiple aspects of spermatogenesis with gene expression varying with stages of the cycle of the seminiferous epithelium and as a result of different endocrine signals. In addition, this variable gene expression must be present throughout the reproductive lifetime of the organism.

References

- De Gendt, K., Verhoeven, G., Amieux, P. S., & Wilkinson, M. F. (2014). Genome-wide identification of AR-regulated genes translated in Sertoli cells in vivo using the RiboTag approach. *Molecular Endocrinology*, 28, 575–591.
- Dorrington, J. H., Roller, N. F., & Fritz, I. B. (1975). Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. *Molecular and Cellular Endocrinology*, 3, 57–70.
- Franca, L. R., Hess, R. A., Dufour, J. M., Hofmann, M. C., & Griswold, M. D. (2016). The Sertoli cell: One hundred fifty years of beauty and plasticity. *Andrology*, 4, 189–212.
- Griswold, M. D., Morales, C., & Sylvester, S. R. (1988). Molecular biology of the Sertoli cell. *Oxford Reviews of Reproductive Biology*, 10, 124–161.
- Hogarth, C. A., & Griswold, M. D. (2010). The key role of vitamin a in spermatogenesis. *The Journal of Clinical Investigation*, 120, 956–962.
- Leichtmann-Bardoogo, Y., Cohen, L. A., Weiss, A., Marohn, B., Schubert, S., Meinhardt, A., & Meyron-Holtz, E. G. (2012). Compartmentalization and regulation of iron metabolism proteins protect male germ cells from iron overload. *American Journal of Physiology. Endocrinology and Metabolism*, 302, E1519–30.
- Russell, L. D., & Griswold, M. D. (Eds.). (1993). *Morphological and functional evidence for Sertoli-germ cell relationships*. Clearwater, FL: Cache River Press.
- Sanz, E., Evanoff, R., Quintana, A., Evans, E., Miller, J. A., Ko, C., Amieux, P. S., Griswold, M. D., & McKnight, G. S. (2013). RiboTag analysis of actively translated mRNAs in Sertoli and Leydig cells in vivo. *PLoS One*, 8, e66179.
- Skinner, M. K., & Griswold, M. D. (1980). Sertoli cells synthesize and secrete transferrin-like protein. *The Journal of Biological Chemistry*, 255, 9523–9525.
- Steinberger, A., Elkington, J. S., Sanborn, B. M., & Steinberger, E. (1975). Culture and FSH responses of Sertoli cells isolated from sexually mature rat testis. *Current Topics in Molecular Endocrinology*, 2, 399–411.
- Svingen, T., & Koopman, P. (2013). Building the mammalian testis: Origins, differentiation, and assembly of the component cell populations. *Genes & Development*, 27, 2409–2426.

Leydig Cells

Ilpo Huhtaniemi, Imperial College London, London, United Kingdom; and University of Turku, Turku, Finland
Katja Teerds, Wageningen University, Wageningen, The Netherlands

© 2018 Elsevier Inc. All rights reserved.

Introduction

Leydig cells (LC) belong to the somatic cell complement of the testis and are located in the interstitial tissue, that is, the space between seminiferous tubules (**Fig. 1**). Their role is to produce sex steroids, in particular testosterone (T) for the paracrine regulation of spermatogenesis within the testis, and for the various systemic endocrine effects, androgenic and anabolic, outside the testis. Next to T, LC produce another hormone, insulin-like 3 (INSL3), that plays a pivotal role in fetal life in the regulation of testicular descent. Testicular endocrine activity starts in utero, when T produced by fetal LC (FLC), and its metabolite 5 α -dihydrotestosterone (5 α -DHT), induce masculinization of the male genital structures. LC activity declines after birth and becomes reactivated just before puberty when most FLC have disappeared and replaced by adult LC (ALC). These ALC continue to produce T, though a slight decline in their endocrine activity occurs with aging (**Huhtaniemi, 2014**). The purpose of this chapter is to present an updated summary of salient features of the differentiation, regulation and function of LC, as well as their clinical aspects, along a male's lifespan. The main emphasis will be on two model species, i.e. the human and rodent (rats and mice).

Leydig Cell Development

Fetal Development

Following activation of the SRY gene located on the Y chromosome, the undifferentiated gonad differentiates into a testis. Within the testis two distinct compartments can be identified, that is, the testicular cords and the interstitium. Rodent studies have shown that the cells populating the interstitium form a diverse pool. Among these cells are Wilm's tumor 1 (WT1)+/Hairy and enhancer of

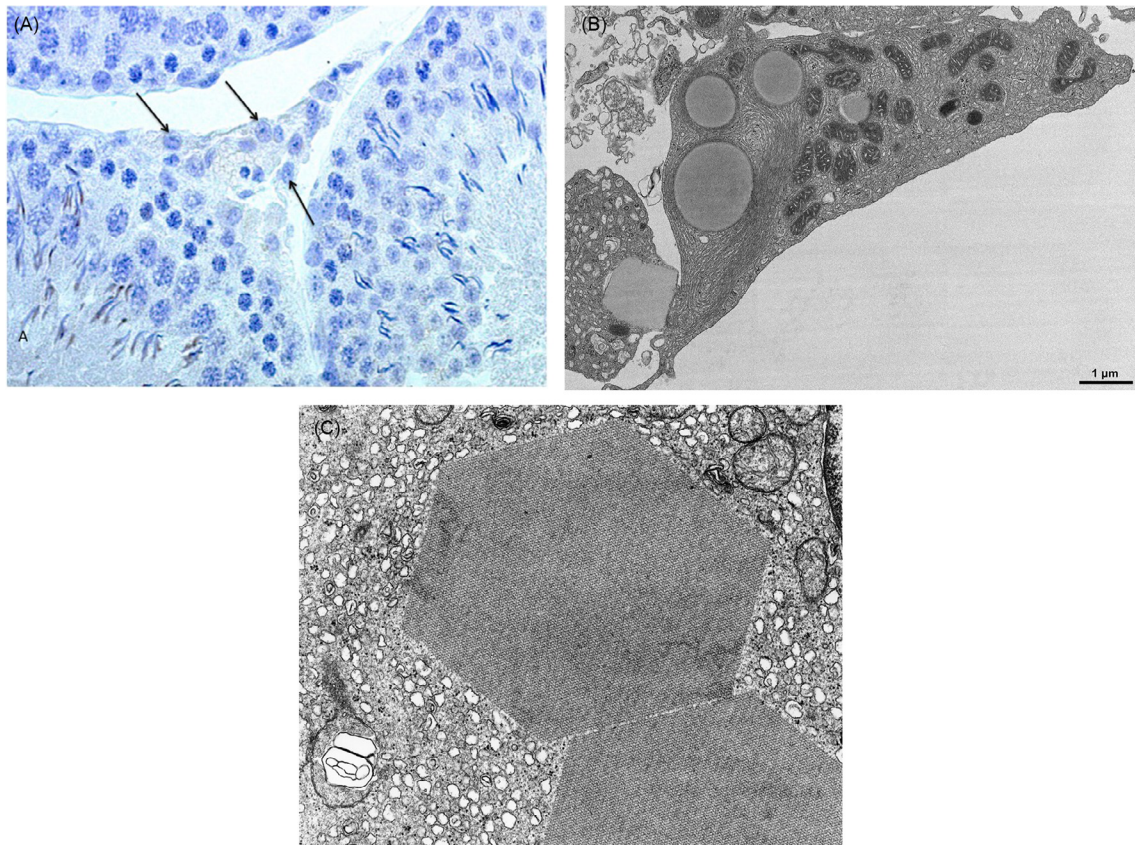


Fig. 1 (A) Light-microscopic picture of rat testis, showing the location of LC (arrows) in the interstitial tissue; (B) electron-microscopic (EM) picture of a mouse LC showing the main organelles (lipid droplets, mitochondria, and SEM); (C) EM picture of a monkey LC showing Reinke crystals. Panels B and C are compliments of Dr. H. Jastrow and Prof. H. Wartenberg (Univ. of Mainz).

split 1 (HES1)- and WT1 +/HES1 + cells, which are considered to be the progenitors from which the FLC population develops, implicating that the FLC population originates from two different lineages (Liu et al., 2016). During embryonic development a dramatic increase in the number of FLC occurs to provide sufficient amounts of androgens to virilize the male embryo. The increase in the size of the FLC population takes place mainly through recruitment from the progenitor pool, as differentiated FLC are mitotically inactive (reviewed in Teerds and Huhtaniemi, 2015).

Neonatal Development

The fate of the FLC after birth is dependent on the species. In rodents the FLC population regresses after birth and concomitant androgen production decreases until the development of the new population of ALC is initiated.

In humans on the other hand, most FLC already undergo involution during the second half of fetal life, when placental hCG production decreases. Some FLC escape involution and continue to be steroidogenically active throughout pregnancy. In the first few months after birth, neonatal activation of the hypothalamic-pituitary-testicular (HPT) axis occurs ("minipuberty"), through the elimination of negative feedback of placental steroids, leading to the establishment of a neonatal population of LC, originating from the surviving FLC as well as through differentiation of stem LC (SLC). This process is unique to primates and leads to the development of a highly active neonatal LC population, which may promote the imprinting of various cell types in androgen dependent organs, including the brain (reviewed in Teerds and Huhtaniemi, 2015). The neonatal activation of the HPT axis is only temporary and followed after about 6 months of life by a prolonged period of inactivity of pituitary gonadotropin secretion and concomitant steroidogenic quiescence until just before puberty.

Pubertal-Adult Development

Although there has been no doubt that, like the FLC population, ALC arise from stem cells that have their origin in the fetal testis, it was not until recently through lineage-tracing that the origin of this stem cell was brought to light. Rodent studies have revealed that part of the interstitial WT1 +/HES1 + progenitor cells lose WT1 expression between E10.5 and E12.5 but retain HES1, and acquire the expression of Glioma associated gene 1 (GLI1), a transcription factor involved in the hedgehog (Hh) signaling pathway. These WT1 –/HES1 +/GLI1 + expressing cells do not acquire CYP17A1 expression and thus steroidogenic capacity. They are thought to give rise to the ALC population that develops around puberty. Lineage-tracing studies further reveal that activation of the desert hedgehog (Dhh) signal transduction pathway plays an essential role in FLC and ALC development, but also reveal that FLC and ALC arise from different stem cells (Liu et al., 2016).

After birth in the neonatal rodent testis expression of lineage specific markers such as luteinizing hormone receptor (Lhr) and steroidogenic enzymes remains absent in SLC. Like FLC, postnatal SLC are characterized by the expression of platelet-derived growth factor receptor α (Pdgfra), GATA binding protein 4 (Gata4) and stem cell markers such as c-Kit and leukemia-inhibiting factor receptor (Lifr). In vitro experiments have disclosed that factors like LIF, stem cell factor (Scf), epithelial growth factor (Egf), fibroblast-like growth factor 2 (Fgf2), and platelet-derived growth factor α (Pdgfa) are important regulators of SLC proliferation. Differentiation of these cells into steroid producing progenitor LC (PLC), the second stage of ALC development, is induced by culturing SLC among others in the presence of LH, insulin-like growth factor 1 (Igf1), and thyroid hormone (reviewed in Teerds and Huhtaniemi, 2015).

Further proliferation and maturation of PLC into immature LC (ILC) and mature ALC is highly dependent on the presence of thyroid hormone and LH and will be discussed in more detail later.

Old Age

Deciphering the effect of aging on LC function is complicated by the concomitant acquisition in many men of obesity and chronic diseases, whose effects on HPT function differ from those of aging. The isolated aging effect in human males includes a decrease in the average total number of Leydig cells per testis by 44% between 20–48 and 50–76 years of age (Neaves et al., 1984). The reduced LC mass is able to maintain T production within the normal range of eugonadism in most aging men. In some men it initially leads to compensated primary hypogonadism (normal T, high LH) and subsequently to primary hypogonadism (low T, high LH) (Huhtaniemi, 2014). In rodents, the age-related reduction in T is predominantly due to unresponsiveness of the LC to LH (Wang et al., 2017). The decrease in T is more pronounced if the aging man is obese or has chronic diseases, in which case the gonadotropin compensation does not occur.

Leydig Cell Morphology

Fetal Leydig Cells

Mature FLC are characterized by a round nucleus, an abundant network of smooth endoplasmic reticulum (SER), mitochondria with tubulovesicular cristae, a small to moderately sized Golgi apparatus, numerous large lipid droplets and finger-like cell surface protrusions; the cells are often arranged in clusters in the interstitium.

In contrast to rodents where FLC undergo regression after birth, in the human testis the majority of FLC undergoes regression during the second trimester of pregnancy. The involution of the FLC population is associated with a decrease in cell volume, and the appearance of electron-dense cells (Haider, 2004).

Adult Leydig Cells

The morphological differences between FLC and ALC are relatively small (Fig. 1B). In rodents the most striking difference between these two types of LC is less abundant lipid droplets in the cytoplasm of mature ALC (Haider, 2004). This is in contrast to the human and primate testis where lipid droplets continue to be part of the cytoplasm of mature ALC. Another typical morphological characteristic of primate ALC is the presence of Reinke crystals in the cytoplasm (Fig. 1C). Although this structure has been studied extensively, no function has been attributed to it. Reinke crystals are not unique to the human testis but have been identified in Leydig cells of a few other species including the guinea pig (Prince, 2007).

Aged Leydig Cells

Ultrastructural analysis of the aging testis has revealed that many LC preserve their normal morphological appearance. Other LC acquire increased numbers of cytoplasmic or intranuclear Reinke crystals or paracrystalline inclusions (primate Leydig cells), multiple vacuoles, lipofuscin granules and lipid-laden cytoplasm, and show signs of dedifferentiation and involution with poorly developed endoplasmic reticulum and mitochondria, as well as presence of two or three nuclei (reviewed by Perheentupa and Huhtaniemi, 2009). These morphological alterations support the observations of decreased LC functionality in the aged testis. There is apparent correlation between the amount of deformed LC and the decrease in T production. Decreased testicular perfusion caused by atherosclerotic alterations in testicular arterioles may be the primary reason for the observed degenerative changes in LC and their reduced steroidogenic capacity during aging.

Leydig Cell Functions

Steroid Hormone Production

The most important steroid hormones produced by LC are androgens, in particular T. Steroidogenesis occurs under tropic regulation of the pituitary gonadotropin LH, and its action is modulated by numerous other endocrine, paracrine and autocrine factors (see below). Most details of the synthesis, secretion and metabolism of testicular steroid hormones have been unraveled in the 1950s and 1960s. Newer information about androgens concerns the identification and characterization of the steroidogenic enzyme genes and proteins, the mechanisms of cholesterol supply for steroid biosynthesis (see below), as well as the mechanisms of androgen action.

Androgens are essential for all masculine functions of the body, including sexual differentiation, development of secondary sex characteristics, spermatogenesis, masculine features of the muscle–bone apparatus, and male sexual behavior.

Canonical pathway of steroidogenesis

The synthesis of steroid hormones starts from cholesterol, for which LC have multiple sources: (a) de novo synthesis from acetyl coenzyme A, (b) stored cholesteryl esters, (c) exogenous lipoprotein-supplied cholesterol, and (d) plasma membrane-derived cholesterol (Tremblay, 2015). The most utilized source is the plasma low- and/or high-density lipoprotein–cholesterol complexes, endocytosed by receptor-mediated mechanisms. Cholesterol is thereafter esterified and stored in intracellular lipid droplets.

The next step is the trans-cytoplasmic transport of cholesterol from lipid droplets to the outer mitochondrial membrane by yet poorly understood mechanisms. The subsequent transfer of cholesterol from the outer to the inner mitochondrial membrane has been better characterized. This rapid step (within minutes) is rate-limiting and critically dependent on LH stimulation. A crucial role here is played by functional interaction of at least two proteins, that is, the steroidogenic acute regulatory protein (StAR) and the 18 kDa translocator protein (TSPO) (Rone et al., 2009). Inactivating mutations of the *StAR* gene lead to congenital lipoid adrenal hyperplasia, which condition is in principle lethal and characterized by a near complete blockade of steroidogenesis in adrenal cortex and gonads.

The next step in steroid biosynthesis, at the mitochondrial inner membrane, is the conversion of cholesterol to pregnenolone, catalyzed by the cytochrome P450 cholesterol side chain cleavage enzyme (P450_{sc}, CYP11A1) and auxiliary electron transferring proteins (Fig. 2). For the following steps, pregnenolone has to translocate from the mitochondria to the SER. Depending on the order of enzymatic reactions, two alternative pathways, Δ^5 or Δ^4 , are employed (Fig. 2). The preferred pathway depends on age and species, but the Δ^5 pathway is predominant in humans and the Δ^4 pathway in rodents. In the former, pregnenolone is first converted by the same cytochrome P450c17 (CYP17A1) enzyme via 17-hydroxypregnenolone (17-hydroxylation step) to dehydroepiandrosterone (lyase step). The following steps, in alternate order, are either the conversion of the 3 β -hydroxy-5-ene structure of dehydroepiandrosterone by 3 β -hydroxysteroid dehydrogenase/isomerase (HSD3B2) to the 3-keto-4-ene structure of androstenedione, or the reduction of dehydroepiandrosterone by 17 β -hydroxysteroid dehydrogenase (HSD17B3) to 5-androstene-3 β ,17 β -diol, with final formation of T after the two reactions taking place in alternate order. In the Δ^4 pathway, the first metabolic step after pregnenolone is its HSD3B2-catalyzed conversion to progesterone, after which the metabolism

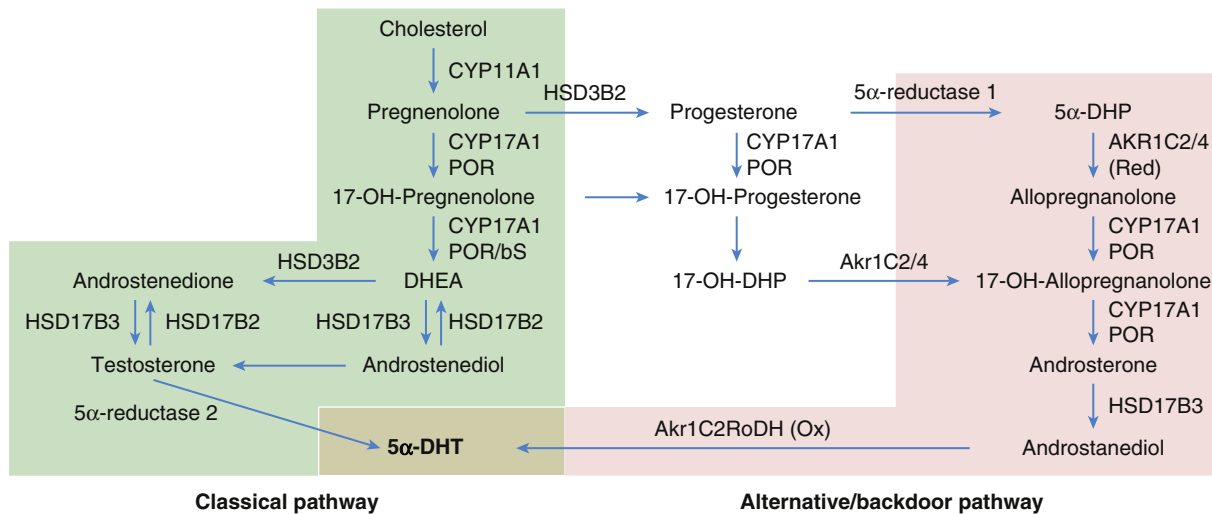


Fig. 2 The “classical” and alternative/backdoor pathways of androgen synthesis in LC. The factors functional in the classic pathway are CYP11A1 (cholesterol side-chain cleavage enzyme, P450_{scc}), CYP17A1 (17 α -hydroxylase/17,20-lyase, P450_{c17}), HSD3B2 (3 β -hydroxysteroid dehydrogenase, type 2), HSD17B3 (17 β -HSD3 [17 β -hydroxysteroid dehydrogenase, type 3]) and 5 α -reductase, type 2 (5 α -reductase 2, encoded by SRD5A2). The alternative/backdoor pathway uses the following additional enzymes: 5 α -reductase, type 1 (5 α -reductase 1, encoded by SRD5A1), AKR1C2 3 (3 α -reductase, type 3) and possibly AKR1C4 (3 α -reductase, type1) and RoDH (3-hydroxyepimerase, encoded by HSD17B6). The trivial names and abbreviations of the steroids are: 17-hydroxydihydroprogesterone (17OH-DHP), 5 α -pregnane-17 α -hydroxy-3,20-dione; 17-hydroxy-allopregnanolone (17OH-allo), 5 α -pregnane-3 α ,17 α -dihydroxy-20-one; 5 α -dihydroprogesterone (5 α -DHP), 5 α -pregnane-3,20-dione; allopregnanolone, 3 α -hydroxy-5 α -pregnan-20-one.

proceeds further through 3-keto-4-ene intermediates to T (Fig. 2). Except for the 17 β -HSD step all conversions are irreversible. An interesting detail about FLC is that their androgen production stops at androstenedione, and the last conversion of its 17-keto to 17 β -hydroxy group of T, catalyzed by HSD17B3 (Fig. 2), occurs in Sertoli cells in the seminiferous tubules (Fig. 1).

The daily production of T in a male is 6–7 mg, of which about 95% originates from the testes and the rest through peripheral conversion of testicular and adrenal androgenic precursors into T. LC produce also other steroid hormones, most of these are Δ^4 and Δ^5 intermediates of T synthesis. Some have weak androgenic activity (e.g., androsterone) or will be metabolized further in the testis or peripheral tissues. High concentrations of human testicular steroids are stored and secreted as functionally inert sulfate conjugates. The testis also produces small amounts of 5 α -DHT, which is the active molecular form in many extra-testicular actions of androgens (e.g., hair follicle and prostate). Likewise, a small number of androgens are aromatized to estrogens through action of CYP19 (P450_{arom}) within the testis tissue or in periphery (adipose and brain tissue).

Alternative pathway of androgen synthesis

While T is the main androgen produced by FLC, its conversion to the more bioactive androgen 5 α -DHT is important for masculinization of the external male genitalia. 5 α -DHT was formerly considered to be solely metabolized from T by the action of 5 α -reductase type 2 (SRD5A2) in genital skin, because males with inactivation of this gene have ambiguous genitalia, that is, disorder of sexual differentiation (DSD). Surprisingly, recent studies on specific cases of DSD have revealed that a significant proportion of fetal 5 α -DHT is produced from progesterone using the alternative or “backdoor” metabolic pathway with 5 α -dihydroprogesterone, allopregnanolone, 17-hydroxyallopregnanolone, androsterone, and androstanediol as intermediates (Fig. 2) (Flück and Pandey, 2014). The key enzymes in this pathway, not employed by the classical steroidogenic pathway, are the α -keto-reductases AKR2 and AKR4, of which the former converts androstanediol to 5 α -DHT. Because human males with SRD5A2 and AKR2/4 deficiency present with ambiguous genitalia, also the backdoor pathway is needed for normal male fetal masculinization.

INSL3

A nonsteroidal hormone produced by LC is insulin-like 3 (INSL3), which is a member of the insulin-relaxin family of peptides, acting through a G-protein coupled receptor relaxin/insulin-like family peptide receptor 2 (RXFP2) (Ivell et al., 2017). Its production starts in rodent and human FLC around the same stage of fetal development and is largely autonomous. It is expressed in both mature FLC and ALC, and its only clearly established function so far is its role, along with androgens, in the trans-abdominal testicular descent in fetal life. Evidence for this role of Insl3 and its receptor Rxfp2 comes from knockout mice, which present with a high degree of intraabdominal cryptorchidism.

There are also data on the role of INSL3 in the modulation of bone metabolism, as a regulator of steroid production and as sperm cell survival factor within the testis. Its concentration in serum correlates best with the number of functional LC in the testes (Ivell et al., 2017).

Intratesticular Hormone Levels

The intratesticular concentration of T in the human is about $2.5 \mu\text{mol L}^{-1}$ and although somewhat less in rodents, it is still in general 50–100-fold higher than in the peripheral circulation (Table 1). This high intratesticular concentration of T is considered critical for the maintenance of spermatogenesis. A drastic reduction in intratesticular T concentrations during treatment with gonadotropin-releasing hormone (GnRH) analogues or T, suppressing LH and FSH secretion, causes a blockage of spermatogenesis. Therefore, T treatment seems a promising hormonal male contraceptive (see below).

It remains an enigma why the intratesticular T concentration is so high to maintain spermatogenesis, as it by far exceeds the concentration needed to activate androgen receptors. A banal explanation, supported by recent experimental data, is that the concentration is so high only because the testis is the site of T synthesis (Oduwole et al., 2014); a much lower threshold concentration may be sufficient for spermatogenesis to proceed.

Leydig Cell Hormone Secretion

The secretion of steroid hormones from LC is considered a passive process due to their lipid solubility and ease of transit through cell membranes. The main steroid hormones produced and secreted by the human testis are listed in Table 1. T and the sulfate conjugates of pregnenolone, dehydroepiandrosterone and 5-androstene- 3β , 17β -diol are quantitatively most abundant. There is no intratesticular storage of bioactive steroid hormones, and the regulation of circulating steroid hormone concentrations occurs mainly at the level of their biosynthesis. Variation in testicular blood flow may be a key factor regulating this process. Since only about $30 \mu\text{g}$ of T is stored in the normal testes (Leinonen et al., 1981), the total T content has to turn over 200 times per day in order to produce the daily 6 mg needed of this hormone. There is diurnal variation in testicular steroid secretion, due to evening/night-time accentuation of LH secretion. Secretion pulses of serum T after the peaks of LH release (average interval of 2 h) are inconsistent, apparently due to the sluggish acute response of human testicular steroidogenesis to gonadotropin stimulation, and to the buffering effect of binding of steroid hormones to plasma transport proteins, in particular sex-hormone-binding globulin (SHBG). Whereas rodent experiments show dramatic (over 10-fold) acute responses of T secretion to LH/human chorionic gonadotropin (hCG) stimulation, the response of human testicular T, within 1–2 h, is only in the order of 30%–50%. A somewhat clearer twofold T response is seen with a 2–4 days' delay.

Regulation of Leydig Cells

Development

Four separate phases have been identified in the formation of the ALC population, based on developmental changes in cell morphology and steroidogenic capacity. The formation of the ALC population is initiated with the differentiation of stem cells. These SLC have an indifferent mesenchymal-like morphology, do not express lineage specific markers such as Lhr and steroidogenic enzymes, and are mainly found in close vicinity of the seminiferous tubules, although there are also reports of a perivascular next to a peritubular localization. The presence of Pdgfra and more recently Cluster of differentiation 90 (CD90) are considered to be important characteristics of SLC and used to isolate and culture these stem cells to study the factors involved in the induction of their differentiation along the LC lineage (reviewed in Teerds and Huhtaniemi, 2015; Li et al., 2017). In vitro studies have revealed that LH, Igf1, Pdgfa, and the active thyroid hormone tri-iodothyronine (T3) contribute to the differentiation of SLC. Recent in vitro evidence shows that next to these factors Dhh is essential for the early commitment of stem cells into the LC lineage, as in the absence of Dhh stem cells fail to differentiate into Lhr positive, steroid producing LC (Li et al., 2017).

Table 1 The mean testicular, spermatic vein and peripheral vein concentrations (in nmol L^{-1}) of the key testicular steroids in man (Leinonen et al., 1981)

Steroid	Testis	Spermatic vein	Peripheral vein
Pregnenolone sulfate	2600	430	90
Progesterone	130	23	0.8
17-Hydroxyprogesterone	690	45	3.2
Dehydroepiandrosterone	680	35	8.2
Dehydroepiandrosterone sunphate	2000	1400	1000
5-Androstene- 3β , 17β -diol	820	590	500
Androstenedione	740	45	2.5
Testosterone	2600	720	20
Testosterone sulfate	1400	150	13
5 α -Dihydrotestosterone	50	14	1.5
Estradiol	15	0.4	0.1

Further support for the role of Igf1 and T3 in SLC differentiation comes from in vivo studies. A targeted deletion of the *Igf1* gene delays the differentiation of SLC into PLC, but does not completely inhibit this process. Daily administration of T3 to newborn rat pups advances the differentiation of SLC into PLC, while a severe reduction in the availability of endogenous T3 leads to a delay in PLC development (reviewed in [Teerds and Huhtaniemi, 2015](#)).

Studies in *Lhr* knockout (LuRKO) mice have shown that beside these factors, LH is of the utmost importance in the differentiation of SLC into PLC. Initially it was reported that inactivation of the *Lhr* completely inhibits the formation of ALC by preventing the differentiation of SLC into PLC. More recently, in vitro studies using SLC isolated from adult rats have shown that in the absence of LH Dhh alone can induce to some extent the formation of T producing PLC ([Li et al., 2017](#)), implicating that limited differentiation of SLC in the absence of LH can occur.

Other factors have been implicated in the process of LC differentiation as well, such as the orphan nuclear receptor chicken-ovalbumin upstream promoter transcription factor II (*Coup-tfII* or NR2F2) and anti-Müllerian hormone (Amh). Time-specific deletion of *Coup-tfII* during late embryonic development does not prevent PLC formation but delays this process, similar to the response to *Amh* overexpression and the previously discussed effects of reduced T3 levels ([Teerds and Huhtaniemi, 2015](#); [Ye et al., 2017](#)).

Depending on the strain of rats, the first PLC develop between day 10 and 13 postpartum, while in primates this process is initiated just before puberty. These cells express *Lhr* and are steroidogenically active, though in contrast to mature ALC, the main androgens produced by PLC are androsterone and androstenedione ([Teerds and Huhtaniemi, 2015](#)).

Pulse chase experiments with [³H]-thymidine have shown that PLC undergo several cycles of proliferation before they differentiate into ILC. Studies on expression levels of marker genes for cell proliferation, for example, proliferating cell nuclear antigen (*Pcna*), *Cyclin a2* and *Cyclin d3* support these observations. Gene cluster analyses further extend these studies, showing high expression of cell cycle regulating genes such as Cell division cycle 25 (*Cdc25*) and *Cdc20* and genes encoding growth factor receptors like *Pdgfra* and *Interleukin-6-receptor* (*Cd126*). The expression of these genes decreases with the transition of PLC into ILC.

With the differentiation of PLC into ILC the cellular characteristics and the position of the cells in the interstitium changes. In the rat model by day 28 postpartum more and more LC translocate to the center of the interstitium. The cytoplasm of the cells contains now a well-developed Golgi apparatus and a large number of tubulovesicular mitochondria and small lipid droplets in which cholesterol is stored to serve as substrate for steroid production. With the transition from PCL to ILC, steroid production switches from androsterone and androstenedione to androstenediol. In contrast to PLC, ILC have only a limited proliferative capacity. Concomitantly, the mRNA and protein levels of *Pcna* and *Cyclins a2* and *d3* decrease while the levels of the cell cycle inhibitor *Cyclin g1* increases ([Teerds and Huhtaniemi, 2015](#); [Ye et al., 2017](#)).

In the rat model by day 50 postpartum ILC have transformed into mature ALC. The latter cells are large and mainly located in the center of the interstitium. The Golgi and mitochondrial complexes have further extended in size and the cytoplasm now contains an abundant SER network. Lipid droplets are no longer part of the cytoplasm, and consequently ALC have switched to another source of cholesterol as steroid precursor, which is now obtained by de novo synthesis or from serum lipoproteins. T has become the major androgen produced by mature ALC ([Teerds and Huhtaniemi, 2015](#)).

Despite the fact that the expression of genes involved in cell proliferation is low in ALC, interstitial cells in the adult testis have not completely lost their potency to proliferate. Pulse-chase experiments and gene cluster analysis have shown that the interstitial cells in the adult testis continue to have a limited capacity to proliferate.

Most studies on the sequence of events that occur along the LC lineage have been performed in rodents. Limited data is available on changes in gene expression and function along the LC lineage in the primate testis (reviewed in [Teerds and Huhtaniemi, 2015](#)).

Endocrine, Para- and Autocrine Regulation

The most important endocrine regulator of LC function is LH, and details of its actions are available in Chapter LH and the male. We describe here the effects of other regulators of LC function.

Several other peripheral hormones have been implicated to participate in the development of the ALC population, some of which have already been discussed, such as the thyroid hormone T3. It is at present not clear whether T3 affects LC development directly or indirectly by binding to thyroid hormone receptor α (Thra) in Sertoli cells.

FSH is another hormone that has been suggested to influence the transition of SLC into PLC, as treatment of prepubertal, hypophysectomized rats with highly purified FSH or recombinant FSH leads to the formation of small numbers of PLC/ILC. These effects of FSH are however indirect as Sertoli cells are the only cell type in the testis to express *Fshr* ([Teerds and Huhtaniemi, 2015](#)).

A considerable number of Sertoli cell-derived factors have been implicated to participate in the development of the ALC population. At present Dhh and *Pdgfra* are considered to be the most important locally produced factors involved. In vivo ablation of Dhh leads to a complicated testicular phenotype. ALC development is negatively affected, but this may in part be due to the fact that Sertoli cell polarity and seminiferous cord formation is severely disturbed ([Clark et al., 2000](#)). In vitro studies suggest that in the absence of LH the effect Dhh on the transition of SLC into PLC is relatively small, while the combined administration of LH and Dhh has a profound effect on the transition of SLC into PLC ([Li et al., 2017](#)). In the *Pdgfra* ablated testis there are indications that some transition of SLC into PLC occurs but that proliferation of the latter cells is severely hampered leading to a nearly complete absence of LC at day 42 postpartum ([Gnessi et al., 2000](#)). In vitro experiments support this assumption as addition of PDGFA to SLC increased their proliferation.

In addition to the factors described above there is a considerable number of paracrine factors mainly of Sertoli cell origin that in concert with LH, and to a lesser extent FSH, affect the process of SLC and PLC growth and differentiation. Among these factors are *Lif*, *Scf*, *Egf*/Transforming growth factor α (*Tgfa*), *Igf1*, and *Fgf2*. To keep the process of LC development in abeyance, locally produced inhibitors of this developmental process have also been identified, including *Amh*. As indicated, proliferation of PLC contributes largely to the final size of the mature ALC population. Next to the already mentioned factors, *TGFB* has been identified as an inhibitor of PLC proliferation. The stimulatory effects of LH on PLC development are thought to be indirect and autocrine by the stimulation of PLC *IGF1* production. LC proliferation is inhibited in *Igf1* knockout mice, despite the presence of LH.

SLC expression of the transcription factor *Coup-tfII* has been implicated to be required for LC lineage commitment. *Coup-tfII* further promotes the maturation of PLC into ILC (reviewed in [Teerds and Huhtaniemi, 2015](#)). The final differentiation of ILC into mature ALC is highly dependent on the presence of LH. Whether LH directly influences this process or via stimulating *Igf1* gene expression and synthesis, is at present not clear.

Clinical Aspects

Hypogonadism

Suppressed testicular, that is, LC production of T causes a condition termed hypogonadism. Its symptoms entail those of androgen deficiency, including reduced (oligozoospermia) or absent (azoospermia) spermatogenesis, poor sexual function, and reduced muscle mass, hair growth and bone mass (osteoporosis). Hypogonadism can be due to failure of the testis tissue (primary) or to failure at the hypothalamic-pituitary level to secrete gonadotropins (secondary). It may appear at different times of life ranging from fetal life until old age, and it is usually caused by a distinct organic failure in function at some level of the HPT axis, for example, mutation of genes regulating hypothalamic function, gonadotropin secretion or testicular steroidogenesis. Milder functional failure of testicular function in older age (andropause, late-onset hypogonadism) is a contentious topic, because it does not fulfill many diagnostic criteria of genuine organic hypogonadism, and opinions vary about its clinical significance and treatment options.

Aging of Leydig Cells

There is a slight gradual decline in LC function upon aging, which is caused combined by a defect in the gonadotropin control of LC function and by intrinsic alterations of LC. In aging males there is a reduction in LC number, LH efficacy to stimulate steroidogenesis, LC sensitivity to LH, all of them implicating a reduced effectual LH drive as cause for decreased T levels. Whether this decline is an inevitable physiological phenomenon or a pathophysiological response to aging-associated health problems is still poorly understood. In support of the latter are the findings that old men in perfect physical condition show marginal decline in serum T, and that the reversal of obesity in aging men reverses T suppression ([Huhtaniemi, 2014](#)). Aging per se is considered to cause primary LC hypofunction. Secondary hypofunction of the testis, regularly reported in older men (low T and inappropriately normal or low LH levels), is largely independent of age and associated with obesity and comorbidities.

In many strains of rodents the age-related decrease in T production is mainly due to reduced GnRH release from the hypothalamus, attenuating pituitary LH release, as well as reduced LC response to LH ([Wang et al., 2017](#)). Reproductive aging in the Brown Norway rat, in contrast to most other rodent species, is solely of gonadal origin, which makes it a good model of human male aging. Detailed analysis of this model has shown that decreased LH-stimulated cAMP production and cholesterol transport into the mitochondria are of particular importance in the decreased T production of aged LC. One key mechanism in LC aging is apparently the metabolic generation of free radicals ([Wang et al., 2017](#)), whereby aging cells are in a chronic state of oxidative stress as a consequence of an age-related imbalance. In line with these observations it has also been shown that decreased steroidogenesis is associated with a deficiency in autophagy of aged rat LC.

Leydig Cell Tumors

LC hyperplasia is common in various disorders of testicular function but its mechanism is still poorly understood, although excessive LH stimulation may be involved ([Rajpert-De Meyts et al., 2000](#)). A term “LC adenoma” is used when the nodule size exceeds several-fold the diameter of a seminiferous tubule. LC hyperplasia and adenomas can be induced in rodents by estrogens, gonadotropins and many chemical compounds. LC tumors account for 1%–3% of testicular neoplasms in humans, and about 20% of them occur between 5 and 10 years of age, in conjunction with precocious T production and puberty. Under these conditions, androgen secretion is gonadotropin independent, and therefore LH and FSH remain low in spite of external signs of puberty. In adults with LC tumors, gynecomastia is a common finding (caused by T conversion to estrogen), but the tumor’s excessive androgen secretion has rarely notable effects. LC tumors are always benign in children, whereas in adults 10%–15% of them are malignant. The latter are usually hormonally inactive and resistant to conventional chemotherapy and irradiation.

A special case of LC tumors is formed by excessive adrenocorticotropin (ACTH) secretion in poorly controlled 21-hydroxylase deficiency (congenital adrenal hyperplasia, CAH) or Nelson syndrome (postadrenalectomy status), where the direct ACTH stimulation may lead to development of hyperplastic LC nodules, termed testicular adrenal rests.

Mutations Affecting LC Function

Several mutations and polymorphisms have been discovered in the key genes regulating LC development and function in humans, and their phenotypes have been confirmed in genetically modified mice (reviewed in [Teerds and Huhtaniemi, 2015](#); [Tremblay, 2015](#)). Inactivating mutations of the *LH β -subunit* (*LHB*) cause hypogonadotropic hypogonadism with LC hypoplasia and lack of sexual maturation after birth. *LHCGR* mutations cause a more severe phenotype with complete lack of masculinization (46, XY DSD) and pubertal development in a genetic male, because in this case FLC fail to respond to hCG stimulation. Mutations in *INSL3* and its receptor gene are rare causes of cryptorchidism ([Ivell et al., 2017](#)). Another tropic hormone whose mutation causes LC hypofunction is osteocalcin. *Androgen receptor* mutations are detrimental for the maturation of ALC and several steroidogenic enzyme genes. Concerning steroidogenesis, mutations of *StAR* and most of the genes involved in T biosynthesis have been found as causes of LC hypofunction, DSD, testicular dysgenesis and hypogonadism. Furthermore, there is plethora of transcription factors whose mutations cause LC hypoplasia and hypoandrogenism, such as *SF1* (*NR5A1*), *DAX-1*, *GATA4*, *ARX*, *COUP-TFII*, *C/EBP β* , *NUR77*, *DHH*, *HHAT*, and *MAMLD1*. Somatic mutations of regulatory genes have been found in LC tumors.

Leydig Cells as a Target for Male Contraception

Despite the efforts for decades a modern male contraceptive is still not available ([Chao and Page, 2016](#)). The task is formidable because of multiple reasons, including the obstacles imposed by male biology, minimal tolerance for side-effects, societal attitudes and poor funding opportunities. The most advanced form today is the hormonal approach, where inhibition of LC production of T and suppression of the high intratesticular concentration of T, necessary for the maintenance of spermatogenesis, are the targets. The goal can be achieved by treatment with T (alone or in combination with progestin), whereby the circulating concentration of T becomes slightly elevated, increasing consequently the negative feedback inhibition of gonadotropin secretion. The suppression of gonadotropins, in particular of LH, brings about cessation of LC T production and the androgen support of spermatogenesis. Numerous clinical studies have been carried out on this regimen with encouraging results. Azoospermia has been achieved in many studies with over 90% efficacy. Unfortunately, the development of this method is currently at near standstill because of expected and unexpected side-effects (depression, altered libido, acne, weight gain), lack of public funding and the reluctance of pharma industry to invest in further research.

Leydig Cell Toxicology

The function of FLCs, including humans, has recently received increased attention because of the fears of these cells being a sensitive target for demasculinizing effects of endocrine disrupting chemicals (EDCs) through interference with their androgen production and action during pregnancy ([Wang et al., 2017](#)). While there is a plethora of studies in vivo and in vitro on antiandrogenic and/or estrogenic inhibitory effects of EDCs (mostly phthalates and bisphenol A) on FLCs in various animal models, there are large species differences in their actions. The findings of EDC effects on rat, mouse and human FLC T and INSL3 production are rather conflicting, and it is difficult to decipher salient conclusions from the existing data. Interestingly, the mechanisms whereby EDCs affect LC function appear to be very similar to the aging-related alterations of LC function, that is, decreased mitochondrial cholesterol transport and reduced cAMP response to LH.

Besides the conflicting results between various animal models, another caveat in the field of EDC research is whether positive findings have been made using doses that have relevance for real-life exposure from the environment. The existence of EDCs as mixtures in the environment further complicates the situation. The field is currently polarized between proponents and skeptics with respect to the importance of EDC health hazards. An interesting conclusion in a review on EDCs ([vom Saal and Hughes, 2005](#)) was that harmful effects are likely to be found in research sponsored by governmental funding, whereas no harm is found when the funding came from industrial sources! Much more research is undoubtedly needed before the issue of harmful EDC effects will be settled.

Conclusions

- Distinct populations of LC exist in the testis during a male's life span, including fetal, neonatal (in humans), and adult LC.
- The differentiation and function of human and rodent FLC is initially independent of LH/CG stimulation.
- Human FLC become subsequently highly dependent on hCG for T production, while the rodent FLC are, besides pituitary LH, responsive to a plethora of other hormones and paracrine factors.
- The complex regulation of LC from stem through PLC and ICL to mature LC has been unraveled by recent research.
- The main function of LC at all ages is to produce androgens (and INSL3) for the maintenance of spermatogenesis and extragonadal androgenic and anabolic effects, mainly through tropic stimulation of LH, but also by numerous auto- and paracrine factors.
- LC function slightly decreases during aging, but most aging men remain eugonadal.
- Clinically significant LC hypofunction results in hypogonadism which involves suppressed spermatogenesis and extragonadal androgen actions (e.g., sexual dysfunction).

- LC function and sexual maturation are affected by rare mutations of genes regulating LC functions.
- LC may give rise to benign and malignant tumors.
- LC and sexual development and function may be targets of deleterious effects of EDCs.

References

- Chao, J. H., & Page, S. T. (2016). The current state of male hormonal contraception. *Pharmacological Therapy*, 163, 109–117.
- Clark, A. M., Garland, K. K., & Russell, L. D. (2000). Desert hedgehog (Dhh) gene is required in the mouse testis for formation of adult-type Leydig cells and seminiferous tubules. *Biology of Reproduction*, 63, 1825–1838.
- Flück, C. E., & Pandey, A. V. (2014). Steroidogenesis of the testis—New genes and pathways. *Annals of Endocrinology (Paris)*, 75, 40–47.
- Gnessi, L., Basciani, S., Arizzi, M., Spera, G., Wang, C., et al. (2000). Leydig cell loss and spermatogenesis arrest in platelet-derived growth factor (PDGF)—A deficient mice. *Journal of Cell Biology*, 149, 1019–1026.
- Haider, S. G. (2004). Cell biology of Leydig cells in the testis. *International Review of Cytology*, 233, 181–241.
- Huhtaniemi, I. (2014). Late-onset hypogonadism: Current concepts and controversies of pathogenesis, diagnosis and treatment. *Asian Journal of Andrology*, 16, 192–202.
- Ivell, R., AgoulNIK, A. I., & Anand-Ivell, R. (2017). Relaxin-like peptides in male reproduction—A human perspective. *British Journal of Pharmacology*, 174, 990–1001.
- Leinonen, P., Ruokonen, A., Kontturi, M., & Vihko, R. (1981). Effects of estrogen treatment on human testicular unconjugated steroid and steroid sulfate production *in vivo*. *Journal of Clinical Endocrinology and Metabolism*, 53, 569–573.
- Li, X., Wang, Z., Jiang, Z., Guo, J., Zhang, Y., et al. (2017). Regulation of seminiferous tubule-associated stem Leydig cells in adult rat testes. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 2666–2671.
- Liu, C., Rodriguez, K., & Yao, H. H.-C. (2016). Mapping lineage progression of somatic progenitor cells in the mouse fetal testis. *Development*, 143, 3700–3710.
- Neaves, W. B., Johnson, L., Porter, J. C., Parker, C. R., Jr., & Petty, C. S. (1984). Leydig cell numbers, daily sperm production, and serum gonadotropin levels in aging men. *Journal of Clinical Endocrinology and Metabolism*, 59, 756–763.
- Oduwole, O. O., Vydra, N., Wood, N. E., Samanta, L., Owen, L., et al. (2014). Overlapping dose responses of spermatogenic and extragonadal testosterone actions jeopardize the principle of hormonal male contraception. *FASEB Journal*, 28, 2566–2576.
- Perheentupa, A., & Huhtaniemi, I. (2009). Aging of the human ovary and testis. *Molecular and Cellular Endocrinology*, 299, 2–13.
- Prince, F. P. (2007). The human Leydig cell. In A. H. Payne, & M. P. Hardy (Eds.), *The Leydig cell in health and disease* (pp. 71–89). Totowa, NJ: Humana Press.
- Rajpert-De Meyts, E., Skakkebaek, N. E., & Toppari, J. (2000). Testicular cancer pathogenesis, diagnosis and endocrine aspects. In L. J. De Groot, G. Chrousos, K. Dungan, K. R. Feingold, A. Grossman, et al. (Eds.), *Endotext [Internet]*. South Dartmouth (MA): MDText.com, Inc.
- Rone, M. B., Fan, J., & Papadopoulos, V. (2009). Cholesterol transport in steroid biosynthesis: Role of protein–protein interactions and implications in disease states. *Biochimica et Biophysica Acta*, 1791, 646–658.
- Teerds, K. J., & Huhtaniemi, I. T. (2015). Morphological and functional maturation of Leydig cells: From rodent models to primates. *Human Reproduction Update*, 21, 310–328.
- Tremblay, J. J. (2015). Molecular regulation of steroidogenesis in endocrine Leydig cells. *Steroids*, 103, 3–10.
- von Saal, F. S., & Hughes, C. (2005). An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. *Environmental Health Perspectives*, 113, 926–933.
- Wang, Y., Chen, F., Ye, L., Zirkkin, B., & Chen, H. (2017). Steroidogenesis in Leydig cells: Effects of aging and environmental factors. *Reproduction*, 154, R111–R122.
- Ye, L., Li, X., Li, L., Chen, H., & Ge, R. (2017). Insights into the development of the adult Leydig cell lineage from stem Leydig cells. *Frontiers in Physiology*, 8, Article 430.

Leydig Cells: Fetal to Aged Testes

Martine Culty and Vassilios Papadopoulos, University of Southern California, Los Angeles, CA, United States
Barry Zirkin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

In mammals, two distinct populations of Leydig cells have been identified: fetal Leydig cells (FLCs) and adult Leydig cells (ALCs). These cells appear sequentially from development through adulthood, with the appearance of FLCs preceding that of ALCs (Fig. 1). The high levels of androgens produced by the fetal testis are required for the proper development of the male reproductive system, brain masculinization, and the expression of key enzyme activities later in life. In the rat, testosterone production declines with the loss of the FLCs, reaching a nadir at 2–4 weeks postpartum, and then gradually increases to high levels with the development of ALCs from Leydig stem cells present in the neonatal testis (Fig. 2). Once formed, the ALCs rarely divide or die. With aging, there is a progressive decline in testosterone production by ALCs, resulting in reduced serum testosterone levels. Androgens are involved in many systems in the male in addition to reproduction, including cardiovascular, skeletal, and muscular. Consequently, it is critical to understand the regulation of Leydig cell formation and the causes and consequences of their aging.

Fetal Leydig Cells

During mouse gestation, fetal progenitor cells that express steroidogenic factor 1 (SF-1) have been identified as the cells that give rise to the FLCs (Fig. 1). FLC differentiation is promoted by platelet-derived growth factor A (PDGFA) and desert hedgehog (DHH), and suppressed by the Notch pathway (Barsoum et al., 2013). Knockout of DHH has been shown to result in the development of reduced numbers of FLCs, with the adults lacking Leydig cells. These results suggest a possible relationship between the FLCs and ALCs. However, the observations that FLCs and ALCs have different gene expression profiles and appear sequentially, and that few FLCs are seen in the adult testis, suggest that FLCs and ALCs represent distinct populations of cells arising from distinct types of fetal progenitor cells (Barsoum et al., 2013).

In the rat, FLCs produce testosterone by gestational day (GD) 15.5, with production increasing markedly thereafter and reaching a peak just prior to birth (Fig. 2). LH is not required either for the development of FLCs or for their initial testosterone production. The FLCs ultimately express LH receptor and respond to LH stimulation. The testosterone produced by the FLCs and its metabolite dihydrotestosterone (DHT) are required for the morphogenesis of organs of the urogenital system, including the epididymis, vas deferens, seminal vesicles, prostate, penis and scrotum, and later in gestation for penile growth and testicular descent into the scrotum. Testosterone also regulates testis development. Testosterone and its metabolites DHT and estradiol have programming effects on neural function, driving brain masculinization. Though some FLCs persist in the adult testis, it is unlikely that they contribute significantly to testosterone production in the adult (Barsoum et al., 2013).

Development of Adult Leydig Cells From Stem Cells

ALCs develop from stem cells (stem Leydig cells; SLCs) present in the early neonatal testis (Fig. 1). Four distinct stages of ALC development have been identified: SLCs, progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and ALCs

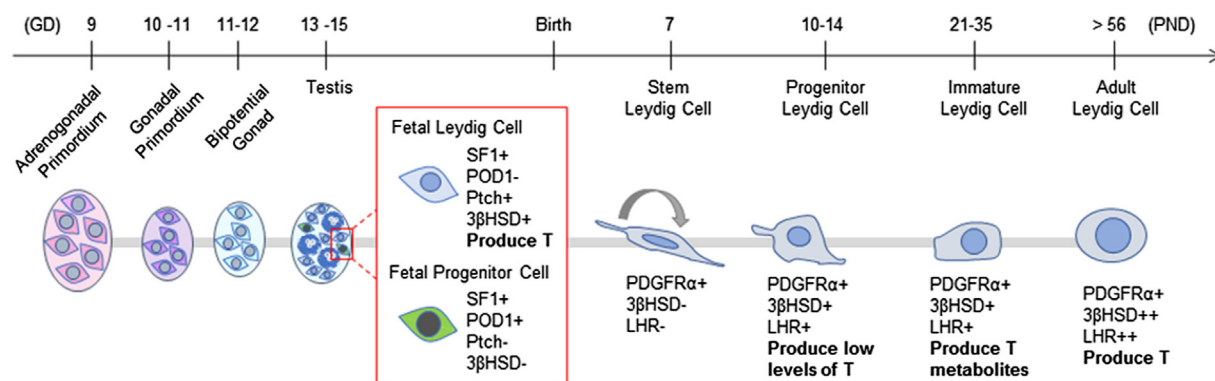


Fig. 1 Developmental timelines of testicular Leydig cells. Cells from the coelomic epithelium in the urogenital ridge form a common adrenogonadal primordium at gestation day (GD) 9. Between GD10–11, gonad and adrenal primordia separate. Between GD10.5 and 12.5, SRY expression drives Sertoli cell differentiation and male gonad formation. Between GD12.5 and GD13.5, fetal Leydig cells are formed from SF1+/POD1- progenitor cells, with increasing numbers seen from GD12.5 to 15.5. SF1+ cells that remain undifferentiated due to POD1 expression are believed to be the precursors of the stem Leydig cells that ultimately differentiate to progenitor, immature and ultimately adult Leydig cells.

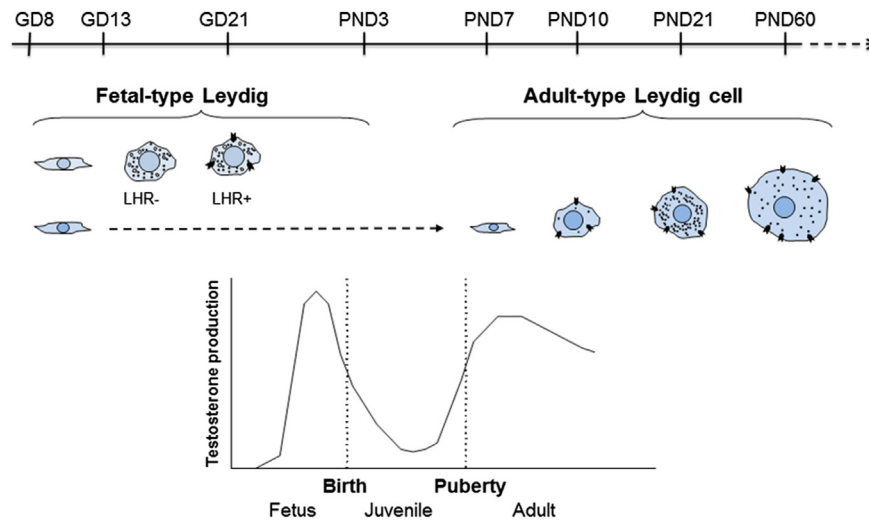


Fig. 2 Diagram of increasing testosterone production in the fetus by the fetal Leydig cells, reduction testosterone production postnatally with the loss of the FLCs, and then increasing testosterone production with the formation of ALCs from SLCs after birth.

(Ge et al., 2006). The SLCs are spindle-shaped cells surrounding the seminiferous tubules and also associated with the vasculature in the interstitial compartment of postnatal 7 rat testes. The cells were isolated and purified by virtue of their expression of PDGFR- α but not LHR or the steroidogenic enzyme 3β -hydroxysteroid dehydrogenase (3β -HSD). In vitro, the SLCs cells were found able to maintain their phenotype and to self-renew indefinitely, stimulated to do so by DHH, FGF2, PDGFBB, activin, and PDGFAA, and inhibited by TGF β (Chen et al., 2017). When cultured in medium containing LH, PDGF, thyroid hormone and IGF-1, SLCs were induced to differentiate into cells that expressed 3β -HSD and ultimately produced testosterone. The SLCs also were shown to be capable of differentiating into 3β -HSD-expressing Leydig cells in vivo after their injection into the interstitial compartment of adult rat testes from which Leydig cells had been eliminated by injection of the Leydig cell toxicant ethane dimethanesulfonate (Ge et al., 2006).

By postnatal day 14, some among the SLCs give rise to spindle-shaped PLCs in vitro. In contrast to the undifferentiated SLCs, PLCs express the Leydig cell markers 3β -HSD and LHR, and produce low levels of testosterone. By postnatal day 28, the PLCs form a population of ILCs that increases to ~ 13 – 14 million, accounting for half of the ALCs in the adult rat testis. The testosterone metabolite 5α -androstane- 3α , 17β -diol rather than testosterone is the major steroid produced of ILCs. The ILCs undergo one to two divisions and further differentiate, producing the full complement of ~ 25 million ALCs by postnatal day 56. The ALCs are terminally differentiated cells that are characterized by their production of high levels of testosterone (Fig. 2). At 90 days, Leydig cell testosterone production is 150 times greater than that produced by PLCs, and 5 times greater than by ILCs (Ge et al., 2006).

Adult Leydig Cell Function and Aging

The primary function of Leydig cells in the adult is to produce testosterone. Leydig cell testosterone production involves cholesterol transport into mitochondria, pregnenolone formation in the mitochondria, and conversion of pregnenolone into testosterone by enzymes of the smooth endoplasmic reticulum. Luteinizing hormone (LH), secreted by the pituitary gland in response to gonadotropin-releasing hormone (GnRH) from the hypothalamus, initiates this process by stimulating Leydig cell cAMP production which, in turn, stimulates cholesterol transfer into the mitochondria. The latter is the rate-determining step in testosterone formation. Cholesterol transport involves the actions of steroidogenic acute regulatory protein (STAR), translocator protein (18 kDa; TSPO), and other proteins of the transduceosome (Miller and Bose, 2011; Rone et al., 2012).

In both humans and rodents, serum testosterone levels decline progressively with aging (Chen et al., 1994; Wang et al., 1999). Age-related decreases in serum testosterone levels result from reduced testosterone production by aging Leydig cells, not from a reduction in cell numbers. Reduced serum testosterone, whether in young or older men, is associated with a number of metabolic and quality-of-life changes, including decreased lean body mass, bone mineral density, muscle mass, libido and sexual function, increased adiposity, cardiovascular disorders, and altered mood (Bhasin and Basaria, 2011; Hwang et al., 2011). In hypogonadal men in whom there are deficiencies in central stimulation (hypogonadotropic hypogonadism, Kallman's syndrome), serum testosterone can be elevated by administering LH or hCG, or indirectly with clomiphene or aromatase inhibitors. However, hypogonadism in most patients is not the result of central deficiencies, but rather decreased responsiveness of the Leydig cells to LH. In these men, attempts to increase Leydig cell testosterone production and thus serum testosterone levels through LH stimulation typically are not effective.

Testosterone replacement therapy (TRT) often is used to raise serum testosterone levels (Gruenewald and Matsumoto, 2003). The availability of methods by which to elevate testosterone levels has made TRT accessible and palatable to men. However, recent

studies suggest increased risk of cardiovascular disease resulting from TRT (Xu et al., 2013). There also are reports suggesting that TRT might increase the risk of prostate cancer (Bosland, 2014). Additionally, TRT in most men will suppress LH, resulting in reduced intratesticular testosterone concentrations and thus in spermatogenesis suppression, making this an inadvisable approach for men wishing to father children (Hwang et al., 2011; Ramasamy et al., 2011).

In Brown Norway rats, as in men, testosterone levels decrease with age despite unchanging LH levels (Chen et al., 1994). The culture of Leydig cells isolated from old rats with LH was shown to result in testosterone production that was reduced significantly below its production by LH-stimulated young cells. However, culturing the old cells with dibutyryl cAMP, thus bypassing LH receptor-G protein stimulation, resulted in elevated testosterone production that in fact was comparable to its production by LH-stimulated young cells. These studies suggest that reduced testosterone production by aging Leydig cells results from the relative insensitivity of the cells to LH caused by deficient signal transduction. Knowledge of the steps and mechanisms in testosterone formation has made it possible to consider the use of pharmacological means to increase serum (and intratesticular) testosterone by stimulating the Leydig cells themselves. TSPO, involved in translocating cholesterol to the inner mitochondrial membrane, comprises 2% of the outer mitochondrial membrane (Culty et al., 2002). TSPO drug ligands have been used in clinical studies to increase neurosteroids in men with neurological and psychiatric disease symptoms (Northdurfter et al., 2012). We observed that activation of TSPO by specific drug ligands resulted in increased T production by aged Leydig cells both in vitro and in vivo, and thus in elevated serum T levels (Chung et al., 2013). Such studies hold promise for providing new means by which to increase serum testosterone levels without administering LH-suppressive testosterone.

Summary

Fetal Leydig cells produce the high levels of testosterone in the fetus that are required for the establishment of the male reproductive system and for subsequent steroidogenic and reproductive functions of the adult. Fetal Leydig cells are lost beginning early in postnatal life, and as a consequence testosterone production declines. Testosterone levels begin to increase as adult Leydig cells develop from the stem Leydig cells of the neonatal testis. There is progressive decline in testosterone production by the adult Leydig cells as they age, associated with a number of metabolic and quality-of-life changes. TRT is available and widely used to elevate serum testosterone levels, but is not without risk. Knowledge of the mechanisms involved in testosterone formation has made it conceivable to use pharmacological means to increase serum (and intratesticular) testosterone by stimulating the Leydig cells themselves, thus avoiding TRT.

References

- Barsom, I. B., Kaur, J., Ge, R. S., Cooke, P. S., & Yao, H. H. (2013). Dynamic changes in fetal Leydig cell populations influence adult Leydig cell populations in mice. *The FASEB Journal*, 27, 2657–2666.
- Bhasin, S., & Basaria, S. (2011). Diagnosis and treatment of hypogonadism in men. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 25, 251–270.
- Bosland, M. C. (2014). Testosterone treatment is a potent tumor promoter for the rat prostate. *Endocrinology*, 155, 1–5.
- Chen, H., Hardy, M. P., Huhtaniemi, I., & Zirkin, B. R. (1994). Age-related decreased Leydig cell testosterone production in the brown Norway rat. *Journal of Andrology*, 15, 551–557.
- Chen, H., Wang, Y., Ge, R., & Zirkin, B. R. (2017). Leydig cell stem cells: Identification, proliferation and differentiation. *Molecular and Cellular Endocrinology*, 445, 65–73.
- Chung, J. Y., Chen, H., Midzak, A., Burnett, A. L., Papadopoulos, V., & Zirkin, B. R. (2013). Drug ligand-induced activation of translocator protein (TSPO) stimulates steroid production by aged brown Norway rat Leydig cells. *Endocrinology*, 154, 2156–2165.
- Culty, M., Luo, L., Yao, Z. X., Chen, H., Papadopoulos, V., & Zirkin, B. R. (2002). Cholesterol transport, peripheral benzodiazepine receptor, and steroidogenesis in aging Leydig cells. *Journal of Andrology*, 23, 439–447.
- Ge, R. S., Dong, Q., Sottas, C. M., Papadopoulos, V., Zirkin, B. R., & Hardy, M. P. (2006). In search of rat stem Leydig cells: Identification, isolation, and lineage-specific development. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 2719–2724.
- Gruenewald, D. A., & Matsumoto, A. M. (2003). Testosterone supplementation therapy for older men: Potential benefits and risks. *Journal of the American Geriatric Society*, 51, 101–115.
- Hwang, K., Walters, R. C., & Lipshultz, L. I. (2011). Contemporary concepts in the evaluation and management of male infertility. *Nature Reviews. Urology*, 8, 86–94.
- Miller, W. L., & Bose, H. S. (2011). Early steps in steroidogenesis: Intracellular cholesterol trafficking. *Journal of Lipid Research*, 52, 2111–2135.
- Nothdurfter, C., Rammes, G., Baghai, T. C., Schule, C., Schumacher, M., Papadopoulos, V., & Rupprecht, R. (2012). Translocator protein (18 kDa) as a target for novel anxiolytics with a favourable side-effect profile. *Neuroendocrinology*, 24, 82–92.
- Ramasamy, R., Stahl, P. J., & Schlegel, P. N. (2011). Medical therapy for spermatogenic failure. *Asian Journal of Andrology*, 14, 57–60.
- Rone, M. B., Midzak, A. S., Issop, L., Rammouz, G., Jagannathan, S., Fan, J., Ye, X., Blonder, J., Veenstra, T., & Papadopoulos, V. (2012). Identification of a dynamic mitochondrial protein complex driving cholesterol import, trafficking, and metabolism to steroid hormones. *Molecular Endocrinology*, 26, 1868–1882.
- Wang, C., Sinha Hikim, A. P., Lue, Y. H., Leung, A., Baravarian, S., & Swerdloff, R. S. (1999). Reproductive aging in the Brown Norway rat is characterized by accelerated germ cell apoptosis and is not altered by luteinizing hormone replacement. *Journal of Andrology*, 20, 509–518.
- Xu, L., Freeman, G., Cowling, B. J., & Schooling, C. M. (2013). Testosterone therapy and cardiovascular events among men: A systematic review and meta-analysis of placebo-controlled randomized trials. *BMC Medicine*, 11, 108.

Peritubular Myoid Cells in Testis

Ryan P Thompson, Eric E Nilsson, and Michael K Skinner, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Background

In men of good health, there are layers of flattened cells and extracellular matrix (ECM) proteins that make up the wall of the seminiferous tubules. The cells located along this wall are peritubular myoid cells, which have smooth muscle-like properties (Mayerhofer, 2013). Peritubular myoid cells fall under the classification of a mesenchymal/stromal cell type. They reside immediately exterior to the extracellular matrix (ECM) that is at the basal surface of the Sertoli cells in seminiferous tubules (Skinner, 1989). Peritubular cells have been found in all mammalian species looked at thus far, although there are differences in the numbers of layers of peritubular cells (Skinner and Griswold, 2005).

Peritubular myoid cells possess the ability to contract and use this feature to transport immotile sperm. Peritubular cells produce growth factor receptors that respond to endothelins (ET), vasopressin and platelet-derived growth factor beta (PDGF β), in turn leading to contractility of the seminiferous tubule (Skinner, 2005). Human testicular peritubular cells (HTPCs) manufacture extracellular matrix components such as the proteoglycan decorin, thought to influence paracrine signaling between testicular cells. Peritubular cells along with extracellular matrix proteins form the walls of the seminiferous tubules. There are distinct differences in peritubular cells between humans and rodents. In a human, there are several layers of stretched out, thin, spindle-shaped peritubular cells and extracellular matrix (ECM) proteins that form the wall of the seminiferous tubule. However, in a laboratory rodent this same wall is constructed of a single peritubular cell layer with a minimal extracellular matrix (ECM). No matter the model, mature peritubular cells express markers of smooth muscle-like cells. This expression of smooth muscle actin is induced around the age of sexual maturity in primates primarily by androgens. Androgens are thought to play a critical role in the differentiation process that occurs in peritubular cells. After expression of smooth muscle actin is evoked, removing the androgen stimulation appears to have no effect (Mayerhofer, 2013). Peritubular myoid cells (PMCs) also provide structural support to seminiferous tubules. It is worth noting that the seminiferous tubular wall compartment in men who are subfertile or infertile with impaired spermatogenesis is often abnormal. Testicular tubular fibrosis, which is easily diagnosed by thickened deposits of extracellular matrix (ECM), is known as a clear sign of male infertility. Testicular tubular fibrosis and morphological alterations to peritubular cells, such as hypertrophy, likely suggest that particular capacities of peritubular cells, such as hormonal and contractile functions, are modified. Figs. 1 and 2 clearly illustrate the differences between a healthy layer of peritubular cells in comparison to that in an infertile man (Mayerhofer, 2013).

Peritubular cells continue to divide throughout a lifetime and therefore need growth regulation even after adulthood (Skinner, 1989). Early in embryonic development during the time of testis determination, peritubular cells originate from mesenchymal cells within the mesonephros next to the developing gonad. Mesonephric cells travel into the gonad and drive seminiferous cord formation along with Sertoli and primordial germ cell aggregates to form precursor peritubular cells that are part of the outer layer of what will become the seminiferous tubules (Skinner, 2005).

Sertoli Cell–Peritubular Cell Interactions

Previous studies suggest both peritubular cells and Sertoli cells produce Transforming growth factor α (TGF- α), which is capable of acting as an EGF (epidermal growth factor)-like substance to trigger peritubular cell growth. The assumption is made that TGF- α possibly has an important role in controlling testis cell growth and mediates a peritubular cell–Sertoli cell interaction. Conversely, transforming growth factor- β (TGF- β) is a growth factor that can act as an inhibitor in these interactions. Transforming growth factor- β (TGF- β) is a family of gene products that promote cell differentiation and inhibit cell division, in particular in TGF α responsive cells. TGF- β originates from peritubular cells as well as Sertoli cells. TGF- β was found to have no major effects on Sertoli cell function or growth. However TGF- β blocked the ability of TGF- α to promote peritubular cell proliferation. TGF- β was also found to induce peritubular cells to form colonies in cell culture and heighten the production of extracellular matrix components. As a result, TGF- β is thought to likely play a part as a chemotactic agent for peritubular cells and impact seminiferous tubule morphogenesis (Skinner, 1989).

Peritubular cells express activin, a product that has the ability to act on Sertoli cells to influence prepubertal Sertoli cell proliferation. Peritubular cells express heregulins also known as neu differentiation factors (NDF), such as NDF α and NDF β , which have the ability to act through receptors located on Sertoli cells (Skinner, 2005). In addition to these factors, peritubular cells express leukemia inhibitory factor (LIF), which has the ability to act on early-stage spermatogenic cells and likely also Sertoli cells (Piquet-Pellorce et al., 2000). Several other potential paracrine factors produced by peritubular cells have the ability to influence Sertoli cell function (Table 1). The connection between Sertoli cells and peritubular cells and their ability to regulate one another was discovered as a result of androgens not being able to promote peritubular cell differentiation on their own, rather, the presence of gonadotrophin-stimulated Sertoli cells was necessary as well. A combination of Leydig cell and peritubular cell interactions with Sertoli cells have been noted as being essential to Sertoli cell development and function (Skinner, 2005).

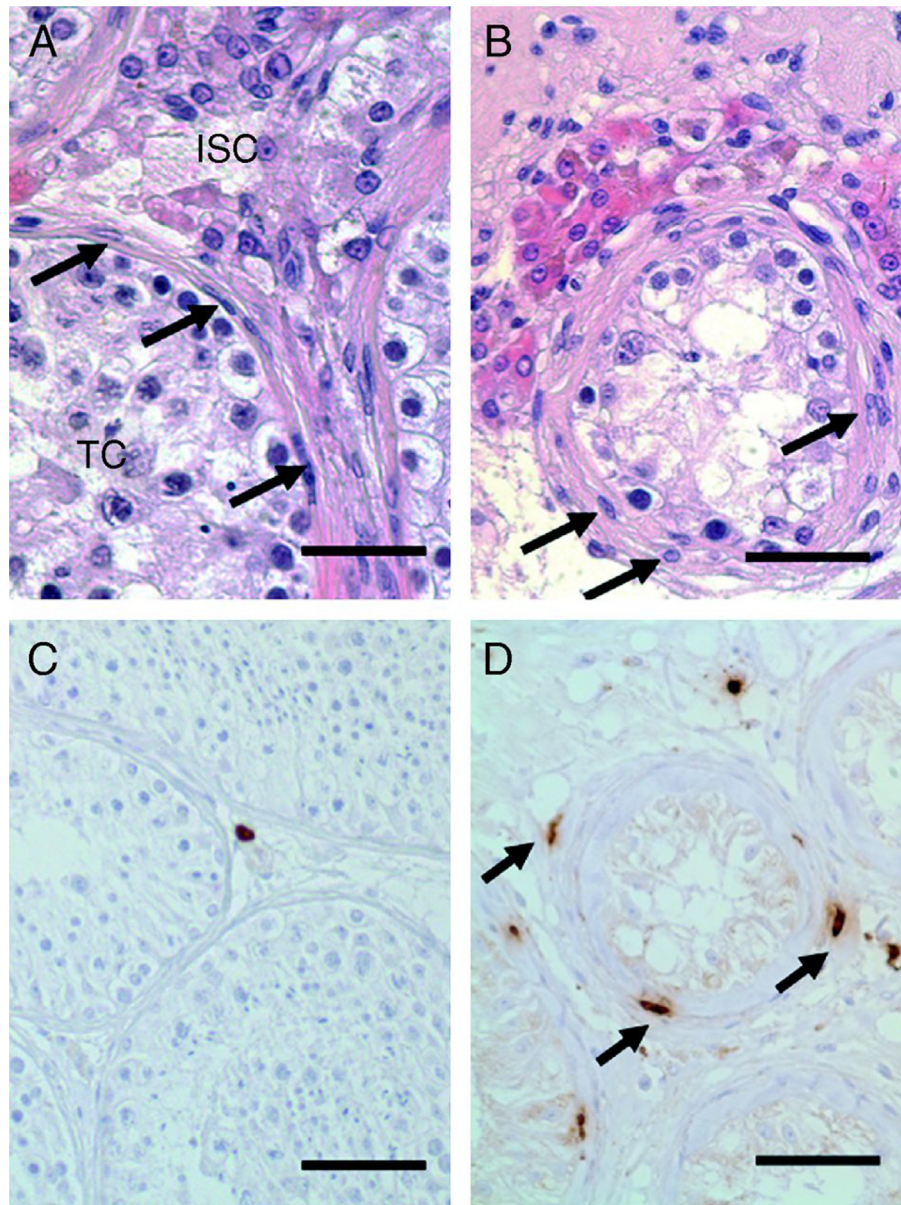


Fig. 1 Testicular morphology and composition of the tubular wall in the adult human testis in health (normal spermatogenesis; A and C) and disease (infertility B and D). (A) The tubular wall (*arrows*) is normally an inconspicuous compartment, in which only few nuclei of peritubular cells are visible in hematoxylin and eosin (H&E)-stained sections. Note the close proximity of the tubular wall to the tubular compartment (TC) containing Sertoli cells and germ cells and to the interstitial compartment (ISC) with Leydig cells. Bar: $\sim 30\ \mu\text{m}$. (B) In samples from infertility patients, the tubular wall is often enlarged, deposits of ECM are seen, and both elongated and round peritubular cell nuclei (*arrows*) are seen in the thickened wall. H&E stain. Bar $\sim 30\ \mu\text{m}$. (C and D) Immunohistochemical identification of mast cells containing tryptase, in a normal testis (C) and in a testicular biopsy of an infertility patient (D). Note that in the section of the normal testis, only one mast cell is present in the interstitial compartment (C). In contrast, in the section from the infertility patient, several mast cells are seen and accumulate in the wall of seminiferous tubules (*arrows* in D) Bar $\sim 60\ \mu\text{m}$. From Mayerhofer, A. (2013). Human testicular peritubular cells: More than meets the eye. *Reproduction*, 145, R107–16.

Studies have shown that both Sertoli cells and peritubular myoid cells express androgen receptors (Norton et al., 1994). It was found that the effect of androgens on Sertoli cells was greater in Sertoli cell aggregates in co-cultures containing peritubular cells as opposed to Sertoli cell preparations without the presence of peritubular cells. Therefore, it's postulated that the stimulatory effects of peritubular cells on functions of Sertoli cells in co-culture, specifically to sustain the production of androgen binding protein (ABP), are affected at least moderately by component(s) manufactured by peritubular cells and secreted into the medium. Peritubular cells accumulate androgens and react to testosterone *in vivo* in addition to *in organ culture*. Peritubular cells in culture, when stimulated by androgens, secrete substances that have the capability to maintain elevated rates of androgen binding protein production by Sertoli cells. As postulated, an experiment confirmed basal rates of androgen binding protein were raised by the presence of peritubular

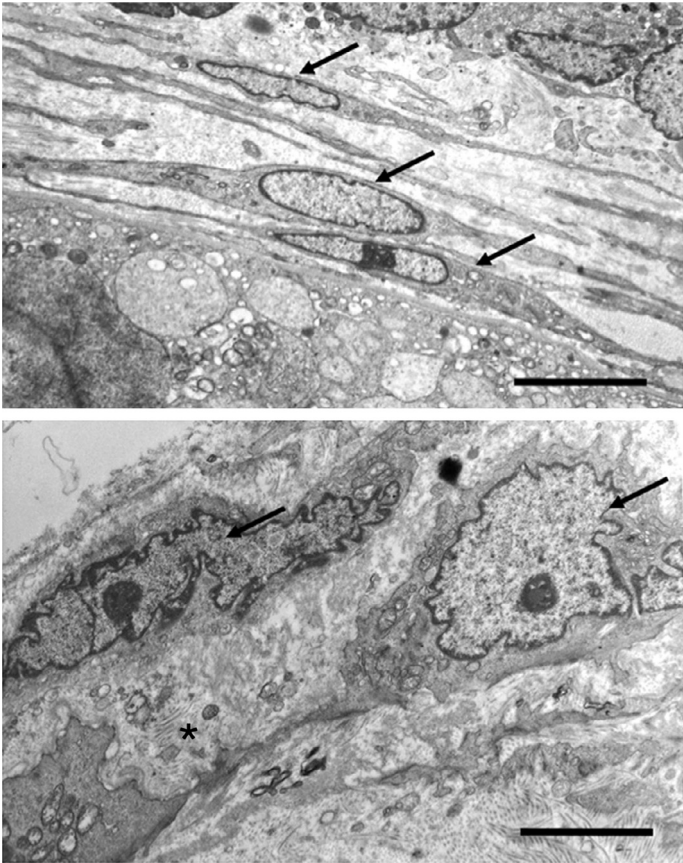


Fig. 2 Electron microscopy of human peritubular cells in the wall of seminiferous tubules. (Top) In the healthy adult, several layers of thin, peritubular cells (*arrows* at nuclei) and ECM form the wall of seminiferous tubules. Bar: ~3 μm . (Bottom) The morphology of the tubular wall of an infertility patient reveals cellular hypertrophy (*arrow* at nucleus) and increased ECM (*asterisk*). Bar: ~4 μm . These micrographs are taken with permission from Fig. 2 of our previous paper (Schell et al., 2010). Copyright 2010, reproduced with permission from The Endocrine Society. From Mayerhofer, A. (2013). Human testicular peritubular cells: More than meets the eye. *Reproduction*, 145, R107–16.

Table 1 Major Sertoli cell and peritubular cell paracrine regulatory products

<i>Potential paracrine factors</i>	<i>Site production</i>	<i>Site action</i>	<i>Action/proposed function</i>
PModS	Peritubular	Sertoli	Paracrine regulatory agent
TGF- α	Peritubular	Sertoli	Growth stimulation EGF-like
TGF- β	Both	Both	Growth inhibition
TGF-I	Both	Both	Maintenance cell growth/differentiation
NDF α /NDF β	Peritubular	Sertoli	Increase differentiation
LIF	Peritubular	Gonia/Sertoli	Growth stimulation
Activin	Peritubular	Sertoli	Growth stimulation
NT3	Sertoli	Peritubular	Embryonic chemotactic factor
bFGF	Sertoli	Peritubular	Growth stimulation

cells. When peritubular cell levels were raised to 30%, a nearly proportional increase in androgen binding protein production was noted, leading scientists to speculate that the increase was connected to the heightened levels of peritubular cell factors released. It is important to note that co-culture of Sertoli cells with peritubular cells assists in maintaining the production of androgen binding protein (ABP) with or without the presence of follicle stimulating hormone (FSH). Peritubular cells lack the ability to synthesize androgen binding protein (Skinner and Fritz, 1985).

After Sertoli cell ablation in an adult mouse testis, a single peritubular myoid cell layer with a standard appearance was found to encompass the majority of seminiferous tubules up to 1 year following ablation. After 1 year, there was loss of integrity in the

connective tissue of either the peritubular or interstitial regions. These findings indicate that seminiferous tubule remodeling occurs over a long period of time. It is postulated that the remodeling process incorporates several different cell types such as peritubular myoid and interstitial cells. Findings appear to indicate that, although the presence of the basement membrane is able to preserve peritubular myoid cell fate for many months following Sertoli cell ablation, the Sertoli cells are vital to maintaining routine peritubular myoid cell function. Findings indicate that peritubular myoid cells are largely responsible in regulating immune cell access to the seminiferous epithelium. It was noted that where macrophage invasion of the tubule transpired, peritubular myoid cell morphology was changed, however, it is not known whether this was responsible for causing the macrophage invasion or just a repercussion (Rebourcet et al., 2014).

Studies have revealed that when Sertoli cells were not present the peritubular myoid cells were not able to form a functional barrier to biotin. This result points towards Sertoli cells being able to support the barrier properties of peritubular myoid cells. Findings also indicate that when Sertoli cells are not present, the peritubular myoid cells and underlying extracellular matrix are adequate for keeping macrophages out of most tubules. Therefore, it is thought that peritubular myoid cells mediate the immune privilege of the seminiferous tubules (Rebourcet et al., 2014).

Developmental studies have revealed that during fetal and neonatal life Sertoli cells are crucial to maintaining seminiferous tubular structure and the preservation of peritubular myoid cell phenotype. However, as an animal enters puberty Sertoli cells are not necessary to retain seminiferous tubule structure as well as peritubular myoid cell differentiated function, since at this stage the basement membrane is capable of supporting these functions. Previous developmental studies have indicated that Sertoli cells are fundamental at all ages to the upkeep of all germ cell populations, including spermatogonia (Rebourcet et al., 2014).

Signaling Pathways

Peritubular cells generate the paracrine factor known as PModS that is responsible for mediating mesenchymal–epithelial interactions and regulating Sertoli cell functions necessary for the process of spermatogenesis. PModS has been observed to stimulate cGMP levels in Sertoli cells and sustain heightened cGMP levels in culture for as long as 5 days. There are two different isoforms of PModS that have been isolated, PModS (A) and PModS (B), although it appears there is little difference between the two and they share the same apparent biological activities. Findings suggest that PModS (A) is a processed form of PModS (B). PModS has the ability to affect tyrosine phosphorylation of Sertoli cell proteins, and blockage of tyrosine phosphorylation puts an end to the activity of PModS on Sertoli cells. The gonadotrophin follicle stimulating hormone (FSH) and the testicular paracrine factor PModS promote actions affiliated with a more differentiated state of the Sertoli cell, such as the secretion of androgen-binding protein and transferrin. PModS has been shown to raise Sertoli cell cGMP levels, however, cGMP does not directly influence the actions of PModS. Signaling mechanisms such as cAMP levels, calcium uptake, and phosphatidyl inositol hydrolysis, were not affected by the paracrine factor PModS. Findings suggest that PModS potentially shapes an important phosphorylation event. PModS has been observed to have an impact on the tyrosine phosphorylation of a Sertoli cell protein(s) following a 72-h treatment. It is not yet determined whether these tyrosine phosphorylations are correlated with the increased differentiation of the cell and/or influence the actions of PModS (Norton et al., 1994).

Androgen Receptors

Peritubular cells react to androgens by heightening the production of PModS (Anthony and Skinner, 1989). Mice with a targeted disruption of the androgen receptor gene (Ar) in peritubular myoid cells saw a continuous loss of spermatogonia. This finding led researchers to suggesting that peritubular myoid cells depend on testosterone (T) action to generate factors influencing the spermatogonial stem cell in its niche. The addition of testosterone to adult mouse peritubular myoid cells in vitro was found to result in considerable spikes in the amount of glial cell line-derived neurotrophic factor (GDNF) mRNA and protein. The findings revealed that testosterone has the ability to control the production of glial cell line-derived neurotrophic factor by peritubular myoid cells in vitro and support the hypothesis that peritubular myoid cells have a major effect on the microenvironment of the niche and spermatogonial stem cell maintenance (Chen et al., 2014).

Lgr4 Role in the Maintenance of Spermatogenesis

A study in mice found that Lgr4 is selectively expressed in peritubular myoid cells. Knockout of Lgr4 leads to germ cells going into arrest at meiosis I, followed by apoptosis. The levels of androgen receptor, alpha-smooth actin and extracellular matrix proteins were substantially lowered in Lgr4 mutant (Lgr4^{1-/-}) mice. Sertoli cell nucleus position as well as functional protein expression differed in Lgr4^{1-/-} mice. Wnt/β-catenin signaling was disabled in the peritubular myoid cells of Lgr4^{1-/-} mice. After Wnt/β-catenin was reactivated through breeding with Apc^{min/+} mice or by Gsk3β inhibitor treatment, the deficiency of Lgr4 was incompletely rescued. These findings as a whole indicate that peritubular myoid cell Lgr4 signaling by means of Wnt/β-catenin is critical to spermatogenesis (Qian et al., 2013).

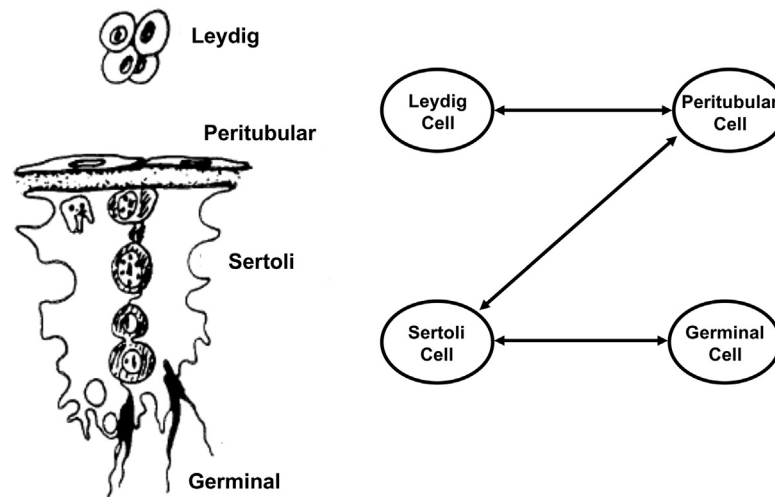


Fig. 3 Diagram of testis cell associations and the interactions between cells. From Skinner, M. K. (1989). Peritubular myoid cell–Sertoli cell interactions which regulate testis function and growth. *Perspectives in Andrology*. 175–182.

Connection to Male Fertility

Spermatogonial stem cells (SSCs) are essential for lifelong spermatogenesis in men. In men, human testicular peritubular myoid cells (HTPCs) are thought to partly responsible for regulating the overall testicular microenvironment through the actions of secreted proteins. Findings support the concept of human peritubular cells playing a critical role in spermatogonial stem cell (SSC) niches in men. Human peritubular cells have been shown to supply several neurotrophic factors, such as glial cell line derived neurotrophic factor (GDNF). GDNF is essential for the renewal of spermatogonial stem cells (SSCs) throughout species. GDNF is a factor that comes from both peritubular cells and Sertoli cells. Due to the proximity of peritubular cells to spermatogonial stem cells, peritubular cells by the way of GDNF may regulate spermatogonial stem cells in a paracrine manner. Findings have contributed to the idea that peritubular cells of the seminiferous tubule wall are important to the spermatogonial stem cells (SSC) niche in the human testis. Peritubular cells are also thought to be a part of the general regulation of testicular functions by producing elements which have the ability to affect Sertoli cells and Leydig cells (Wen et al., 2016). Fig. 3 indicates the relationship between three of the major cell types found in the testis (Skinner, 1989). In summary, peritubular myoid cells have a critical function for testis biology and it is the cellular interactions of all cell types that is required.

References

- Anthony, C. T., & Skinner, M. K. (1989). Cytochemical and biochemical characterization of testicular peritubular myoid cells. *Biology of Reproduction*, 40, 811–823.
- Chen, L. Y., Brown, P. R., Willis, W. B., & Eddy, E. M. (2014). Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. *Endocrinology*, 155, 4964–4974.
- Mayerhofer, A. (2013). Human testicular peritubular cells: More than meets the eye. *Reproduction*, 145, R107–16.
- Norton, J. N., Vigne, J. L., & Skinner, M. K. (1994). Regulation of Sertoli cell differentiation by the testicular paracrine factor PMoDS: Analysis of common signal transduction pathways. *Endocrinology*, 134, 149–157.
- Piquet-Pellorce, C., Dorval-Coiffec, I., Pham, M. D., & Jegou, B. (2000). Leukemia inhibitory factor expression and regulation within the testis. *Endocrinology*, 141, 1136–1141.
- Qian, Y., Liu, S., Guan, Y., Pan, H., Guan, X., Qiu, Z., Li, L., Gao, N., Zhao, Y., Li, X., Lu, Y., Liu, M., & Li, D. (2013). Lgr4-mediated Wnt/beta-catenin signaling in peritubular myoid cells is essential for spermatogenesis. *Development*, 140, 1751–1761.
- Rebourcet, D., O'shaughnessy, P. J., Monteiro, A., Milne, L., Cruickshanks, L., Jeffrey, N., Guillou, F., Freeman, T. C., Mitchell, R. T., & Smith, L. B. (2014). Sertoli cells maintain Leydig cell number and peritubular myoid cell activity in the adult mouse testis. *PLoS One*, 9, e105687.
- Skinner, M. K. (1989). Peritubular myoid cell–Sertoli cell interactions which regulate testis function and growth. *Perspectives in Andrology*, 175–182.
- Skinner, M. K. (2005). Sertoli cell–somatic cell interactions. In GRISWOLD/SKINNER (Ed.), *Sertoli cell biology*. San Diego, CA: Elsevier-Academic Press.
- Skinner, M. K., & Fritz, I. B. (1985). Androgen stimulation of Sertoli cell function is enhanced by peritubular cells. *Molecular and Cellular Endocrinology*, 40, 115–122.
- Skinner, M. K., & Griswold, M. D. (2005). *Sertoli cell biology*. San Diego, CA: Elsevier-Academic Press.
- Wen, Q., Wang, Y., Tang, J., Cheng, C. Y., & Liu, Y. X. (2016). Sertoli cell Wt1 regulates peritubular Myoid cell and fetal Leydig cell differentiation during fetal testis development. *PLoS One*, 11, e0167920.

Vascular Cells

Sarah Romereim, UNL Biological Systems Engineering, Lincoln, NE, United States

Andrea Cupp, UNL Animal Science, Lincoln, NE, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cadherin Transmembrane proteins that participate in cell-cell adhesion.

Coelomic epithelium The inner layer lining the body cavity and is immediately adjacent to/covering the gonad.

Coelomic vessel The embryonic precursor to the main testicular artery, located on the side of the gonad adjacent to the coelomic epithelium and farthest from the mesonephros.

Days post coitus (dpc) Embryonic day of development with the timing of conception designated as day 0.

Elastin An extracellular protein that is highly elastic and allows tissues to resume their shape after stretching/contracting.

Extracellular matrix A broad category of molecules secreted from cells into the intercellular space to provide structural and biochemical support to the tissue.

Filopodia A cellular structure made of thin projections of cytoplasm supported by bundles of actin filaments.

Germ cell A cell that gives rise to gametes (i.e., sperm and oocytes).

Lumen The cavity/channel within a tube.

Mesonephric vascular plexus The network of small blood vessels present within the mesonephros during embryonic development.

Mesonephros The temporary kidney of the mammalian embryo which is replaced by the metanephros.

Morphogenesis The biological process of changing the shape of an organism or tissue.

Seminiferous tubules Curved tubules within the lobes of the testis where meiosis and spermatogenesis occur.

Somatic cell Any cell other than a germ cell or gamete.

Splice variants The product of alternative splicing in which different combinations of RNA exons can be used to yield a variety of protein products from a single gene.

Testis cords The male embryonic sex cords that are the precursor to seminiferous tubules.

Undifferentiated A cell that is still multi-potent and has not attained its final cell identity.

Vascular endothelial cells The cells that make up the inner lining of all blood vessels in the cardiovascular system.

Introduction

Creating a functional mammalian testis is a very involved procedure with many critically important steps. One of the most crucial events during this process is the migration of vascular endothelial cells (i.e., cells that make up the inner lining of blood vessels) into the developing gonad. Without the migration of endothelial cells into the gonad from the nearby mesonephros (i.e., embryonic kidney) there would be no patterning or reorganization to form the seminiferous tubule structures that are necessary for testis functionality (Buehr et al., 1993). This article will take you through the sequence of events involved in this remarkable migratory event that shapes the testis and will use rodents as the main examples. As you will see, these vascular cells follow a series of actions. The migrating endothelial population waits for an invitation, stays on the designated paths as it moves, communicates with its hosts, and is necessary for testis tissue functionality.

The migration of cells from one organ to another can be discussed as three main events. (1) Cells receive a signal creating an impetus to pack up and move. They lose their adhesion to the surrounding cells and change their cytoskeletal dynamics so that they now have the ability to migrate. (2) Cells actively migrate into a new tissue. In the case of endothelial cells migrating into the testis, they do so along specific paths to section the testis into avascular domains. (3) The cells must then integrate into their new home and begin to function as part of the tissue as a whole. This occurs as the disparate vascular cells reconnect and form new blood vessels. Throughout this process, the cells of the gonad secrete signaling molecules telling the endothelial cells when and where to migrate. The endothelial cells also secrete signaling molecules and communicate with the developing testis. Together, they control testis morphogenesis during a critical period of development (Fig. 1).

The Nature of the Vascular Endothelial Cell

Blood vessels are multilayered structures. They contain an inner layer of endothelial cells surrounding the lumen through which blood passes. These endothelial cells are connected to each other primarily via a transmembrane adhesion protein called

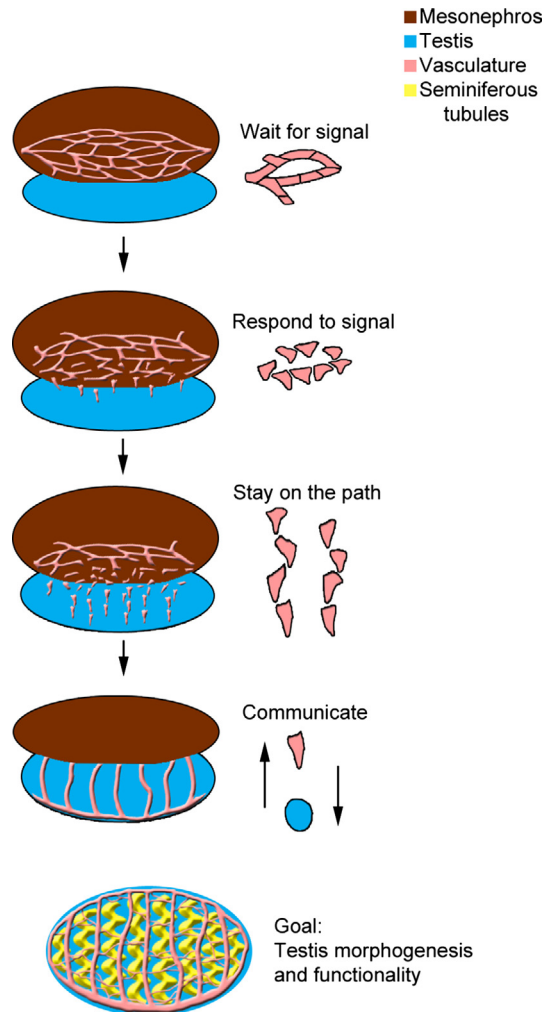


Fig. 1 Vascular endothelial cells follow specific steps during migration from the mesonephros into the developing testis.

VE-cadherin (vascular endothelial cadherin) in structures called adherens junctions (Lampugnani et al., 1995). The smallest blood vessels, capillaries, are made of single endothelial cells wrapped around the lumen and a surrounding basement membrane (secreted extracellular matrix). Larger blood vessels contain a one-cell-thick layer of multiple connected endothelial cells, thicker layers of extracellular matrix (for stability), layers of elastin (for flexibility in response to blood pressure changes), and smooth muscle cells (to control the diameter of the vessel). Based on the types of cell-cell adhesion molecules besides VE-cadherin that are expressed in the endothelial cells, the inner lining of some blood vessels will have more/larger gaps in between cells and thus greater permeability than in other vessels. The endothelial lining of blood vessels is thus crucial in controlling what molecules and cells are exchanged between the blood and surrounding tissues. As you will see, these vascular endothelial cells can also be a driving force in developmental processes and provide crucial cell-cell communication.

Setting the Stage for Vascular Migration Into the Testis

Prior to sex determination, the mammalian gonads have the potential to become either ovaries or testes. The earliest developmental events are the same for both male and female embryos. First, an area of undifferentiated tissue (coelomic epithelium) thickens and forms the urogenital ridge, which will give rise to both the reproductive and urinary tracts (Fig. 2). However, this newly formed gonad precursor only includes somatic cells, not germ cells (the future sperm and oocytes). The primordial germ cells then begin to migrate into the early gonad from the hindgut region of the embryo. These changes occur before the critical window when the sex determination switch is flipped to either testis or ovary development. That critical time is at approximately 10.5 *dpc* (days post coitus, i.e., day of embryonic development) in most mouse lines that have been researched or 12 *dpc* in the rat. The human window of sex determination is approximately between days 41–44 post ovulation (Hanley et al., 2000). As you can see in Fig. 2, however, the migration of the germ cells into the gonad continues to occur during and after sex determination.

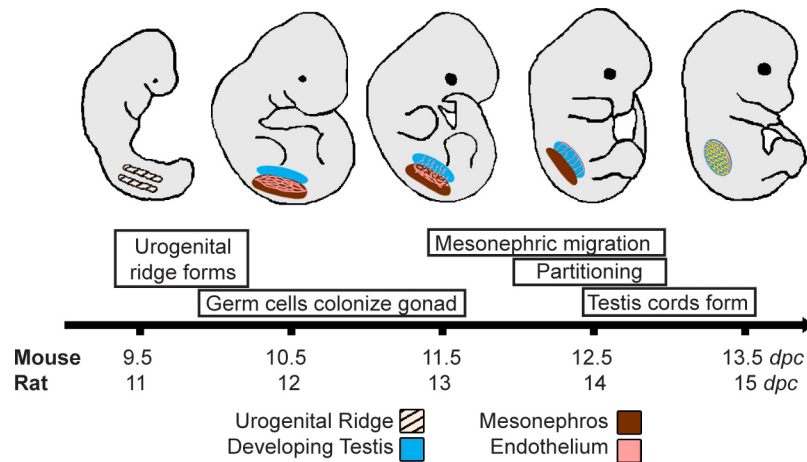


Fig. 2 Migration of mesonephric endothelial cells into the testis occurs immediate after the critical sex determination window.

The distinction between male and female development is made by the presence or absence of a Y chromosome containing the *SRY* gene (Sex-Determining Region of Y). The expression of *SRY* during a brief but critical window activates a complex network of gene expression and cell-to-cell signaling that sets the events in motion to cause male reproductive tract development and to suppress female tract development. The main genetic players in this network are well conserved between humans and mammalian research models like the mouse. The initiation of the male signaling network is a prerequisite for the vascular endothelial cell migration that is so important in forming the appropriate testis structures (Capel et al., 1999; Martineau et al., 1997; Raymond et al., 1999).

Once the male gene expression patterns have been established in the somatic cells of the testis, another gene is turned on to produce a secreted signaling molecule: Vascular Endothelial Growth Factor A (VEGFA). This protein is involved in creating blood vessels in a wide variety of biological contexts including the development of various organs and tumor growth. The VEGFA protein is produced and released from somatic cells in the early testis and diffuses away until it makes contact with its receptor (i.e., KDR; Kinase Insert Domain Receptor) on endothelial cells in the neighboring mesonephros tissue. This is a well-studied event in rodents, but embryonic development studies in humans have not been undertaken; however, somatic cells of adult human testis biopsies do secrete VEGFA. While there are variations (splice variants) of VEGFA of different sizes with that can have differences in function, the main result of VEGFA binding to its receptor on an endothelial cell is to promote cell proliferation and migration (Bott et al., 2006; Baltes-Breitwisch et al., 2010; Ergün et al., 1997).

Endothelial Cell Migration

When VEGFA from the developing testis first makes contact with endothelial cells within the mesonephros, those vascular endothelial cells are fully connected to each other in a network of capillaries called the mesonephric vascular plexus. These existing blood vessels break apart in response to the VEGFA so that individual cells can migrate into the nearby gonad tissue. In an embryo that is XX and therefore developing a female reproductive tract, the mesonephric vascular plexus remains intact and endothelial cells do not migrate into the ovary during this window in development (11.5–13.5 dpc in the mouse). Instead, the ovary gains vasculature by promoting branching and extension of nearby vessels into the ovarian tissue rather than dissolving intact vessels as in the XY embryo. This major sex-specific vascularization event of the testis is distinct in that it requires the dissolution of blood vessels, the migration of separated cells, and then the reassembly of blood vessels from those cells within the testis. However, the population of endothelial cells does not migrate all at once. Vascular cells trickle into the developing testes over a period of approximately 24 h using the rodent developmental timeline (Fig. 2) (Coveney et al., 2008).

The mechanics of this migration are similar to actively migrating cells in other contexts. First, the cells lose their connections (cellular adhesions) to each other and change from a flattened, extended shape to a more amorphous shape with multiple long extensions called filopodia (Fig. 3). These cell shape changes have been directly observed by fluorescent microscopy in endothelial cells within mesonephros and gonad tissue in culture dishes. The way that the cells actually migrate has not been directly studied within the mesonephros/testis context, but studies of other types of endothelial cells have shown that VEGFA can directly affect the cytoskeleton (Waltenberger et al., 1994; Li et al., 2005). In order to achieve active migration in a specific direction, the cytoskeleton (especially actin fibers) on the side of the cell that is closest to the source of the VEGFA has to be stabilized and extended. The cytoskeleton on the side farthest from the source of the VEGFA is less stable and the actin fibers will be depolymerized more quickly. In this way, the cell effectively “crawls” towards the target tissue by gradually extending the “leading edge” and retracting the “trailing edge.”

One fascinating and not yet fully understood aspect of the vascular endothelial cell migration is that the cells stay on specific paths as they travel through the testis. The general direction of migration (towards the far side of the testis) is established by a diffusion gradient of VEGFA, but the way that this broad signal is narrowed to separate paths through the tissue is not known. However it

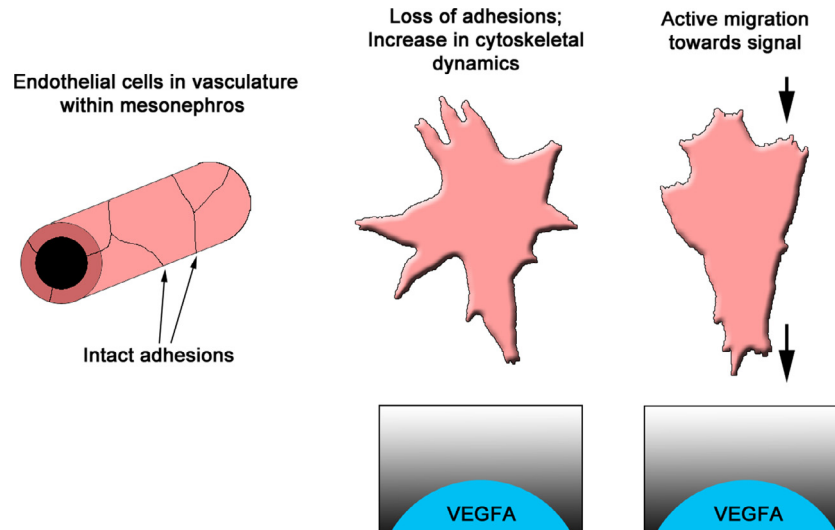


Fig. 3 Endothelial cell migration requires changes in cell-cell adhesion and cytoskeleton dynamics in response to a diffusion gradient of a signaling molecule such as VEGFA.

is accomplished, it is a well-established observation that the endothelial cells travel through specific parts of the testis and not others, separating the testis into approximately 10 avascular domains (Coveney et al., 2008).

Partitioning the testis into domains separated by newly re-formed blood vessels made of the immigrated endothelial cells is a key step in developing testis morphology. If the vascular endothelial cell migration occurs but the re-formation of blood vessels in the testis is experimentally prevented by inhibiting the vascular cell-cell adhesions, then testis cords (the precursors to seminiferous tubules) cannot form at all. The formation of testis cords requires the reorganization of the cells of the testis so that the germ cells are surrounded by layers of somatic cells (Sertoli cells and peritubular myoid cells) with areas between the testis cords containing other interstitial cells (including the Leydig cells and blood vessel branches). If VEGFA signaling is inhibited, the migratory event does not take place at all and testis cords do not form (Bott et al., 2006; Baltés-Breitwisch et al., 2010). This vascular endothelial cell migration event is thus important not only for supplying circulation to the testis but also for initiating structural changes that must occur for future testis functionality. This migration and the testis changes that result from it cannot be directly studied in human embryos, so knowledge of these events depends on rodent embryonic studies. However, given the strong similarities between human and rodent testis morphology and the conservation of the genes involved in testis development, it is reasonable to suspect that these developmental events occur in a similar manner (Combes et al., 2009; Bott et al., 2006; Tilmann and Capel, 1999).

Integration Into the Testis Community

The re-formation of blood vessels from migratory endothelial cells to separate the avascular domains of the testis occurs between 12.25 *dpc* and 12.5 *dpc* in the mouse (Combes et al., 2009). So, the reestablishment of endothelial cell-cell adhesion is coordinated and relatively quick. Further growth and development of the testis is accompanied by blood vessel branching and extension of vessels into the interstitial space between testis cords. This ensures adequate circulation to all areas of the testis.

A key role that the vascular endothelial cells play once they have arrived in the testis is to communicate with the surrounding tissue by secreting a signaling protein called platelet-derived growth factor (PDGF). This signaling protein from the endothelial cells interacts with somatic cells in the testis to promote their proliferation. If PDGF signaling is experimentally inhibited, testis cords do not form and vascularization of the testis can actually be inhibited even if endothelial cell migration occurs. Thus, the mere presence of vascular endothelial cells is not sufficient for testis cord morphogenesis. Instead, it is the cell-to-cell interactions between the endothelial cells and the somatic testis cells that are important (Cool et al., 2011; Uzumcu et al., 2002).

An additional cell-cell communication role that the endothelial cells must play is to recruit macrophages from outside the testis. Fetal macrophage migration into the newly forming major testicular artery (known as the coelomic vessel) starts as early as 10.5 *dpc* in the mouse and is dependent on endothelial cell presence. These macrophages appear to promote tissue remodeling to aid in testis vascularization and testis cord formation (DeFalco et al., 2014).

Finally, beyond the vital contributions to testicular development and morphogenesis, the importance of vasculature to the adult testis cannot be overlooked. The reproductive capabilities of the adult male will depend on the circulation of nutrients and oxygen into the testis, thermoregulation via the blood, incoming signals from the pituitary gland to regulate hormone and sperm production, and output of steroid hormones to interact with the hypothalamus and pituitary gland. All of these functions require adequate vascularization of the testis (Fig. 4).

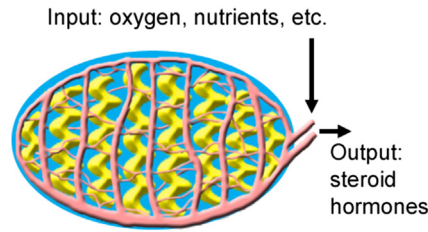


Fig. 4 Role of vasculature in adult testis.

Conclusion

While it is just one cell type among many involved in testis development and function, the vascular endothelial cell is a very necessary contributor. This population of cells begins as a vascular network in the mesonephros and then migrates into the testis to establish an entirely different vascular network. The endothelial cell migration depends on the signal, VEGFA, coming from the somatic cells of the early testis. Endothelial cells respond to the signal by disengaging their adhesions with each other and changing their cytoskeleton so that they can actively migrate towards the source of the signal. As they migrate into the testis, they do so along designated paths through an as-yet-unknown mechanism. They form the main future testicular artery (the coelomic vessel) on the far side of the testis, and also partition the testis into approximately 10 avascular domains. This partitioning is required for testis cord formation. However, as the endothelial cells are migrating they are simultaneously secreting their own signal: PDGF. This protein interacts with the somatic cells of the testis in order to promote their proliferation and coordinately establish the testis cords. Without either VEGF signaling or PDGF signaling, the testis cords cannot form. Additionally, the vascular endothelial cells recruit macrophage migration into the testis to further assist in testis cord formation. All of these efforts together result in testis morphogenesis and vascularization. The vasculature itself will then be crucial in adult testis physiology to circulate nutrients and signaling molecules into and out of the tissue.

References

- Baltes-Breitwisch, M., et al. (2010). Neutralization of vascular endothelial growth factor antiangiogenic isoforms or administration of proangiogenic isoforms stimulates vascular development in the rat testis. *Reproduction*, 140(2), 319–329. Available at <http://www.reproduction-online.org/content/140/2/319.short> (Accessed August 19, 2014).
- Bott, R. C., et al. (2006). Vascular endothelial growth factor and kinase domain region receptor are involved in both seminiferous cord formation and vascular development during testis morphogenesis in the rat. *Biology of Reproduction*, 75, 56–67.
- Buehr, M., Gu, S., & McLaren, A. (1993). Mesonephric contribution to testis differentiation in the fetal mouse. *Development (Cambridge, England)*, 117(1), 273–281.
- Capel, B., et al. (1999). Migration of mesonephric cells into the mammalian gonad depends on Sry. *Mechanisms of Development*, 84, 127–131.
- Combes, A. N., et al. (2009). Endothelial cell migration directs testis cord formation. *Developmental Biology*, 326(1), 112–120.
- Cool, J., DeFalco, T. J., & Capel, B. (2011). Vascular-mesenchymal cross-talk through Vegf and Pdgf drives organ patterning. *Proceedings of the National Academy of Sciences of the United States of America*, 108(1), 167–172.
- Coveney, D., et al. (2008). Four-dimensional analysis of vascularization during primary development of an organ, the gonad. *Proceedings of the National Academy of Sciences of the United States of America*, 105(20), 7212–7217.
- DeFalco, T., et al. (2014). Yolk-sac-derived macrophages regulate fetal testis vascularization and morphogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 111(23), E2384–E2393.
- Ergün, S., et al. (1997). Vascular endothelial growth factor and its receptors in normal human testicular tissue. *Molecular and Cellular Endocrinology*, 131(1), 9–20.
- Hanley, N., et al. (2000). SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. *Mechanisms of Development*, 91(1), 403–407.
- Lampugnani, M., et al. (1995). The molecular organization of endothelial cell to cell junctions: Differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). *The Journal of Cell Biology*, 129(1), 203–217. Available at <https://www.ncbi.nlm.nih.gov/pubmed/7698986>.
- Li, Z. D., et al. (2005). VEGF induces proliferation, migration, and TGF-beta1 expression in mouse glomerular endothelial cells via mitogen-activated protein kinase and phosphatidylinositol 3-kinase. *Biochemical and Biophysical Research Communications*, 334(4), 1049–1060.
- Martineau, J., et al. (1997). Male-specific cell migration into the developing gonad. *Current Biology*, 7(12), 958–968.
- Raymond, C. S., et al. (1999). A region of human chromosome 9p required for testis development contains two genes related to known sexual regulators. *Human Molecular Genetics*, 8(6), 989–996.
- Tilman, C., & Capel, B. (1999). Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. *Development (Cambridge, England)*, 126(13), 2883–2890.
- Uzumcu, M., Dirks, K. A., & Skinner, M. K. (2002). Inhibition of platelet-derived growth factor actions in the embryonic testis influences normal cord development and morphology. *Biology of Reproduction*, 66(3), 745–753.
- Waltenberger, J., et al. (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *Journal of Biological Chemistry*, 269(43), 26988–26995.

Further Reading

- Brennan, J., & Capel, B. (2004). One tissue, two fates: Molecular genetic events that underlie testis versus ovary development. *Nature Reviews Genetics*, 5(7), 509–521.
- Ferrara, N., Gerber, H.-P., & LeCouter, J. (2003). The biology of VEGF and its receptors. *Nature Medicine*, 9(6), 669–676.
- Hoch, R. V., & Soriano, P. (2003). Roles of PDGF in animal development. *Development (Cambridge, England)*, 130(20), 4769–4784.

- Lamallice, L., Le Boeuf, F., & Huot, J. (2007). Endothelial cell migration during angiogenesis. *Circulation Research*, 100(6), 782–794.
- Olsson, A.-K., et al. (2006). VEGF receptor signaling—in control of vascular function. *Nature Reviews. Molecular Cell Biology*, 7(5), 359–371.
- Piprek, R. P. (2010). Molecular and cellular machinery of gonadal differentiation in mammals. *International Journal of Developmental Biology*, 54(5), 779–786.
- Ridley, A. J., et al. (2003). Cell migration: Integrating signals from front to back. *Science (New York, N.Y.)*, 302(5651), 1704–1709.
- Romereim, S. M., & Cupp, A. S. (2016). Mesonephric cell migration into the gonads and vascularization are processes crucial for testis development. In R. P. Piprek (Ed.), *Molecular mechanisms of cell differentiation in gonad development*. Heidelberg, Berlin: Springer.
- Sargent, K. M., et al. (2015). Vascular endothelial growth factor A: Just one of multiple mechanisms for sex-specific vascular development within the testis? *Journal of Endocrinology*, 227(2), R31–50.
- Svingen, T., & Koopman, P. (2013). Building the mammalian testis: Origins, differentiation, and assembly of the component cell populations. *Genes and Development*, 27(22), 2409–2426.

Spermatogenic Cells—Structure

Jacques Auger, Université Paris Descartes, Paris, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

The male reproductive capacity of adult mammals depends on the ability of their testes to produce viable spermatozoa, as well as, an adequate level of several hormones including testosterone necessary for the development of libido and reproductive functions. The formation of the male gamete within the seminiferous tubules is a highly complex process, in preparation from embryonic life (migration of primordial germ cells), fetal, neonatal and prepubertal (production of gonocytes, prespermatogonia) which develops from puberty and continues throughout life through the renewal and division of spermatogonia stem cells. Differentiating germ cells are distributed in a highly organized manner as they move between adjacent Sertoli cells, from the basal to the adluminal compartments of the seminiferous tubules following the successive developmental stages: (i) A-spermatogonia, (ii) B-spermatogonia, (iii) primary spermatocyte, (iv) secondary spermatocyte, (v) spermatid and, (vi) spermatozoon (Fig. 1).

The general organization of spermatogenesis is essentially identical in all mammals and can be divided into three main phases, each of which leads to the production of a distinct category of germ cells:

(1) the mitotic phase which corresponds to the proliferation of spermatogonia. During this phase, spermatogonia produce two types of diploid cells: stem cells, necessary for the renewal of their own stock, and spermatogonia that proliferate in cascade to generate differentiated spermatogonia. These latter generate in their final phase primary (preleptotene) spermatocytes,

(2) the meiotic phase characterized by two successive cell divisions without intermediate DNA synthesis and which results in the formation of haploid spermatids. In all species, this phase is divided into five successive stages called leptotene, zygotene, pachytene, diplotene and diacinese,

(3) the spermiogenic phase which corresponds to the metamorphosis of each spermatid into a spermatozoon.

Table 1 summarizes the main cellular types and morphological transformations along the spermatogenic process. At the end of these three phases, the spermatozoa are released into the lumen of the seminiferous tubules, a process called spermiation. The morphology features of germ cells and somatic cells of the seminiferous epithelium, tissular organization and synchronic distribution along spermatogenesis have been evaluated over the years in many species (see notably, Clermont, 1963; De Kretser and Kerr, 1988; Russell and Ettlin, 1990). The main studies involving human spermatogenesis were performed around the 60s by Clermont and colleagues, who described GCs, spermatogonial kinetics and cell renewal. This article will concentrate on the morphological and structural features of the male germ cells in mammals with special reference to humans.

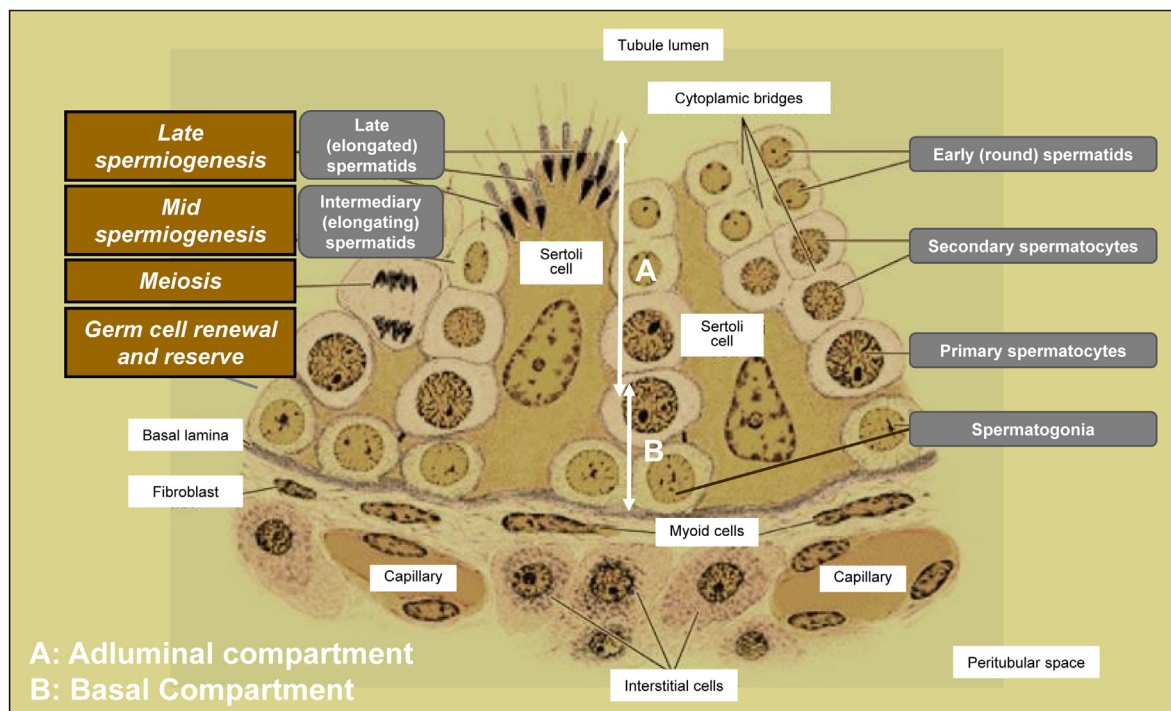


Fig. 1 Germ cell organization within a seminiferous tubule (transverse section) with corresponding spermatogenic phases.

Table 1 Summary of the mammalian germ cell types along the spermatogenic process**Mitotic phase**

A proportion of spermatogonia enter mitosis and, after five mitosis, become type B spermatogonia, which divide into primary spermatocytes

A1 Spermatogonia → A2 Spermatogonia → A3 Spermatogonia → A4 Spermatogonia
→ (Intermediate spermatogonia) → B Spermatogonia → Primary spermatocytes

Meiotic phase

Primary spermatocytes enter meiosis. The first meiotic division leads to secondary spermatocytes and the second division to the haploid round spermatids

Primary spermatocytes ($2n$ chr/ $4n$ DNA) → Secondary spermatocytes ($2n$ chr/ $2n$ DNA) → Round spermatids ($1n$ chr/ $1n$ DNA)

Spermiogenesis

The spermatogenic step where spermatids differentiate into spermatozoa:

- DNA is associated with the protamines nuclear basic proteins and become highly condensed
- The acrosome, tail and the midpiece containing mitochondria are formed
- Most of the cytoplasm is eliminated

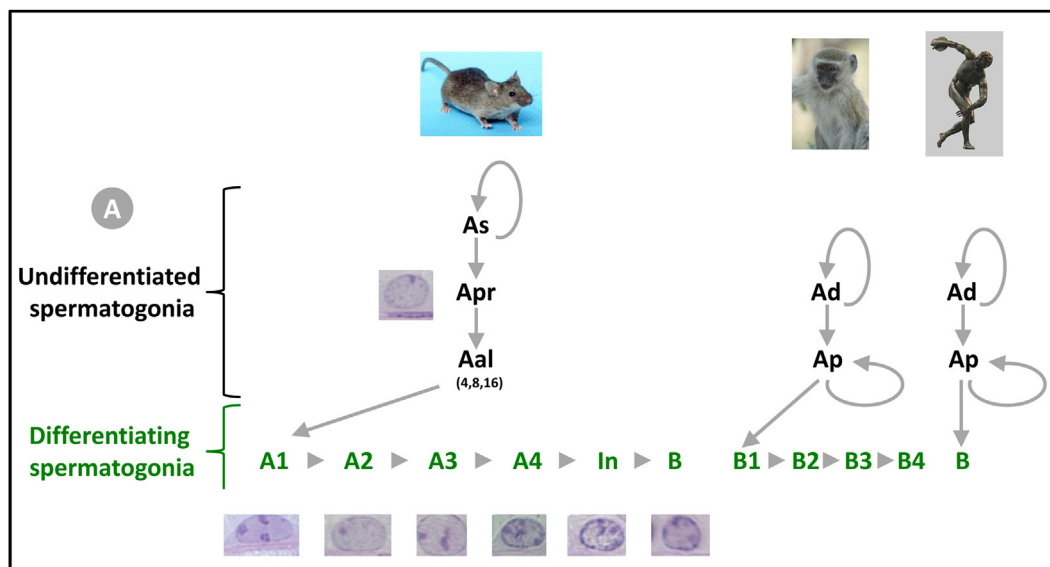
Round spermatids → Elongating spermatids → Elongated spermatids → Spermatozoa

Spermatogonia

In all mammals, spermatogonia are defined as constituting the mitotic compartment of spermatogenesis including stem, undifferentiated and differentiating cell types, possessing distinct morphological and molecular characteristics. Even though the real nature of the spermatogonial stem cell and its regulation is still debated the general consensus holds that in steady-state spermatogenesis the stem cell compartment needs to balance differentiation versus self-renewal.

Spermatogonia derived from the differentiation of gonocytes. In adult mammals, spermatogonia are very few in number and located in the periphery of the seminiferous tubules, leaning against the *lamina propria*, in contact with the Sertoli cells. In primates, such as monkey and man, there are three morphologically distinct subpopulations of spermatogonia: dark type A (Ad spermatogonia), pale type A (Ap spermatogonia) and type B (B spermatogonia). In humans, Ad spermatogonia represent the reserve of stem cell, and Ap spermatogonia correspond to a population of cells intended to ensure the renewal of the stock of the progenitor cells of the spermatogenic process. The Ap and Ad spermatogonia are considered to be the counterpart of the mouse undifferentiated spermatogonia and type B that of the mouse differentiating spermatogonia (Fig. 2). Ad spermatogonia does not divide under normal conditions of testicular function. On the other hand, Ap spermatogonia divide by mitosis to maintain their own cellular stock and for each of them, generate 2 B spermatogonia, which themselves divide by mitosis and enter into meiosis. In humans, only one generation of B spermatogonia has been characterized, whereas there are four in the macaque.

Indeed, if the general pattern of spermatogonial organization and their differentiation (from stem cell to differentiated spermatogonia) is globally identical in mammals, the spermatogonial system presents many differences from one species to another in primates, and between primates and rodents (Ehmcke and Schlatt, 2006; Boitani et al., 2016; see Fig. 2).

**Fig. 2** A schematic diagram of different cell subtypes within the spermatogonial compartment in the mouse, monkey and man.

The ability to distinguish different classes of spermatogonia is critically dependent on the morphological characteristics of the nuclei as revealed on stained preparations of transverse sections of seminiferous tubules by conventional light microscopy.

In humans, from a morphological point of view:

- The Ad nuclei contain a deeply stained dust-like chromatin with a characteristic large central vacuole-like cavity, which, at the electron microscopic level, appears as a chromatin rarefaction zone; close to the nuclear membrane one or more nucleoli are found.
- In contrast, the ovoid Ap nuclei contain a fine, homogenous and weakly stained chromatin, one or two nucleoli lying close to the nuclear membrane and no vacuoles.
- B spermatogonia exhibit the features described for other species, though the human cells are somewhat smaller. Type B spermatogonia are characterized by large clumps of intensely stained condensed chromatin under the nuclear membrane of an ovoid nucleus contrasting with a fine granulated chromatin in the rest of the nucleus.

Spermatogonia as observed by conventional light microscopy have a poorly stained cytoplasm. Studies of whole-mounts of seminiferous tubules have demonstrated that they remain connected by intercellular bridges such that large numbers are effectively linked together. Using the PAS reaction, glycogen is found in Ad spermatogonia.

At the electron microscope level (observations by transmission electron microscopy, TEM), the basal position of spermatogonia within the epithelium and their extensive contact with the basal membrane of the tubule are clearly evident. The extent of this contact decreases in B spermatogonia which will eventually lose all contacts with the basal membrane to become preleptotene primary spermatocytes (see below). All types of spermatogonia are characterized by a relatively electron-lucent cytoplasm and a paucity of cytoplasmic organelles. Because of incomplete cytokinesis during mitosis, spermatogonia remain connected by intercellular bridges, 2–3 μm in width usually not containing organelles or microtubules.

Attempts have been made to classify spermatogonia at the TEM level, notably in humans. Most investigators agree that it is possible in humans to identify the type A dark, type A pale and type B spermatogonia, though intermediate forms may occur. The classification is based on: (i) the nuclear and nucleolar features, (ii) the presence of aggregations of mitochondria, (iii) the presence or absence of glycogen granules, and, (iv) the presence of crystalloids (in human spermatogonia).

- Ad spermatogonia: oval shaped nucleus with electron translucent region compatible with the nuclear vacuole observed by light microscopy, small nuclei adjacent to the nuclear membrane, mitochondria lying close to the nucleus with a cristae generally extending transversally across the matrix, abundant rough endoplasmic reticulum, Golgi complex poorly developed, presence of glycogen granules, often aggregated, presence of crystalloids (=collections of fibrils and tubules aligned along the long axis of the structure up to $\sim 3 \mu\text{m}$ in length).
- Ap spermatogonia: less contact with the basal membrane, finely granulated homogeneous chromatin, nuclei without vacuoles, amorphous peripherally placed nucleoli, paucity of organelles, few mitochondria generally isolated or by pairs, glycogen granules rarely observed, presence of crystalloids.
- B spermatogonia: the spermatogonia having the least contact with the basal membrane, peripherally placed chromatin less prominent than by light microscopy (due to the thin sections), nucleolus centrally placed, mitochondria scattered without the intermitochondrial material observed in type A spermatogonia.

In both nonhuman and human primates, some authors suggest the existence of so-called A transition spermatogonia, having no unequivocal morphological characteristics of either Ad or Ap spermatogonia. In the rat, Type A isolated (Ais) is believed to be the stem cell, whereas in humans, it is unclear which Type A spermatogonia is the stem cell.

Spermatocytes

The cells in the spermatogenic process that are involved in meiosis are the primary and secondary spermatocytes (Fig. 3). Spermatocytes are the cells originating from the last spermatogonial division (B spermatogonia) from which spermatids, then, spermatozoa with half the chromosome complement of the original progenitor cell will be generated following the meiotic phase of spermatogenesis. The process of meiosis involves two cell divisions, the first involves primary spermatocytes generating after the diakinesis secondary spermatocytes which in turn are involved in the second meiotic division producing the haploid spermatids. Because of the long duration of the prophase of the first division (for example, 24 days in humans), primary spermatocytes are found on tubule sections, in every stage of spermatogenesis (and, for a given stage different types of spermatocytes may be observed). By contrast, because the second meiotic division progresses rapidly (lasting about 5 h in humans), secondary spermatocytes are rarely observed on tubule sections.

Primary Spermatocytes

The primary spermatocytes arising from B spermatogonia lose contact with the basal membrane of the seminiferous tubule and migrate in direction of the adluminal compartment by transiently breaking the tight junction complexes of the Sertoli cells. The primary spermatocytes exhibit the features of the first meiotic division in terms of nuclear morphology. As the spermatocytes pass through meiotic prophase they become larger and the appearance of the nuclear chromatin alters, reflecting the condensation

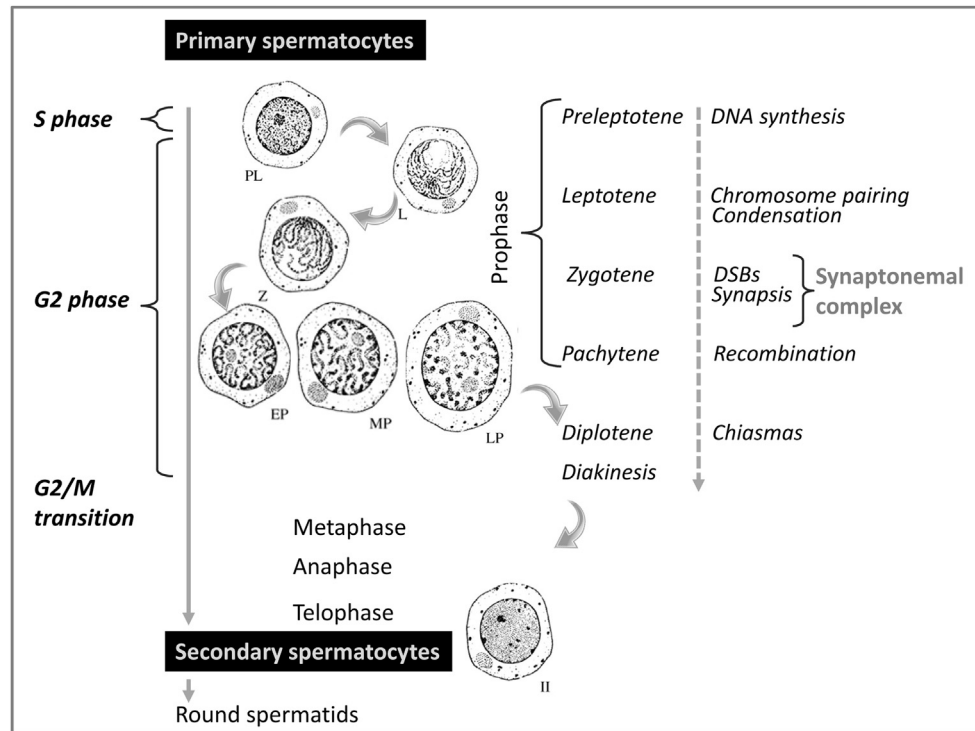


Fig. 3 Summary of the meiotic stages with the corresponding cellular types and subtypes.

and movement of the chromosomes as they prepare for meiotic division. The meiosis phase involves several types of spermatocytes ranging in size (from small, for example, preleptotene, to very large, for example, late pachytene) and presenting typical morphological features for each of the five stages of the prophase of the first meiotic division, preleptotene, leptotene, zygotene, pachytene, and diplotene which are clearly distinguished under the optical microscope. Peripherally located nucleoli are frequently seen in primary spermatocytes. TEM reveals they consist of extremely electron dense aggregations of granules. The cytoplasm of primary spermatocytes is more electron-dense than that of spermatogonia and contains evenly scattered polysomes and ribosomes. Features of rough and smooth endoplasmic reticulum are rare. However, during the progression of the prophase, the Golgi complex close to the nucleus gradually enlarges. Thanks to its high resolution power, TEM has revealed the existence of a subcellular structure, the synaptonemal complex in the nuclei of primary spermatocytes form a number of species. The synaptonemal complex consists of two lateral elements that appears as electronic dense fibrils equidistant from a central element consisting of a delicate linear region of increased electron density (its appearance and evolution is presented below with the different cellular and subcellular morphological features at each stage of the prophase). The number of synaptonemal complexes is equal to the number of bivalents. Though the synaptonemal complex provides the framework necessary for synapsis, it is thought that the recombination modules are the vital components in the crossing-over of genetic material.

Primary spermatocytes are joined to each other by intercellular bridges similar to those found between spermatogonia. They are separated from adjacent Sertoli cells by distinct intercellular spaces that are modified in some regions by desmosome-like structures.

Preleptotene stage

They present a spherical nucleus with an aspect similar to those of B spermatogonia, though smaller. Notably, they lack any chromosomal elements. They are termed preleptotene spermatocytes in reference to the S phase, the preleptotene phase of DNA synthesis, the process by which each chromosome when it condenses is composed of a pair of chromatids, the total of DNA content representing twice the diploid content.

Leptotene stage

During this stage, the chromosomes become apparent as single fine threads. TEM has revealed they are attached at each extremity to the nuclear membrane (giving the “bouquet” aspect) by “attachment plaques” which in fact are the lateral element of the synaptonemal complexes assembling and appearing at this stage. Though at this stage the chromatid pairs are present, they are not visible and will not do so until later in prophase. At this stage, the X and Y chromosomes appear associated with the dense body of the sexual vesicle, a special condensation of chromatin fibrils.

Ovoid mitochondria aggregated in groups of two or three, with an electron dense material similar to that seen in spermatogonia are observed at this stage (and at the subsequent zygotene stage).

Zygotene stage

This stage is characterized by the thickening of the chromosomal elements that begin the process of pairing termed synapsis. Pairing of the elements of the synaptonemal complexes begins at this stage.

Pachytene stage

The long pachytene stage begins with the completion of synapsis and is associated with further thickening and shortening of chromosomes. During this stage, exchanges of chromosome material between maternal and paternal homologous chromosomes occur by crossing over. At the points of crossing over, bridges (chiasmata) are seen in variable numbers. Depending on the chiasmata, different aspects of the chromosomes can be observed. Nuclear and cytoplasmic growth of pachytene spermatocytes result in these cells becoming the larger of all germ cells. Fully formed synaptonemal complexes are observed by TEM at this stage.

Diplotene stage

Desynapsis occurs during this phase. The paired chromosomes partially separate (jointly to the synaptonemal complexes disassembly) but remain joined at their chiasmata. Subsequently, the chromosomes further shorten, then detach from the nuclear membrane during the diakinetin phase, the stage where the two chromatids of each chromosome can be seen.

Diakinesis to telophase

Diakinesis is rapidly followed by the disappearance of the nuclear membrane, the appearance of the spindle and the alignment of the homologous chromosome pair with the metaphase plate, followed by the migration of each part of the chromosome pair toward the opposite poles of the spindle during the anaphase. The telophase completely separates the chromosomes and produces the secondary spermatocytes.

Secondary Spermatocytes

These are the cells that undertake the second meiotic division which begins after a very short interphase without DNA replication takes place. The phases are typical of any cell division (pro-, meta- ana and telophase 11) in that one chromatid from each pair (making up each chromosome) migrates to each daughter nucleus. The secondary spermatocytes have the haploid number of chromosomes, though their DNA content is still diploid so that when they complete meiosis, the resulting spermatids have both an haploid chromosomal content and DNA content.

Secondary spermatocytes are round cells with a size ranging between the primary spermatocyte and the round spermatid size and a greater nucleo-cytoplasmic ratio. They are situated close to the lumen of the tubule (see figure) and their nucleus contains an homogeneous chromatin network throughout which large globular chromatin masses are dispersed. Nucleoli centrally positioned are often observed. Light and electron microscopy reveal the presence of intercellular bridges between adjacent secondary spermatocytes similar with those found for other germ cell categories. TEM reveals scattered features of endoplasmic reticulum arranged concentrically around the nucleus, a prominent Golgi complex containing vesicles with electron-dense granules and dispersed mitochondria with dilated intracristal spaces.

Spermatids

Spermatids are the products of the second meiotic division and lack the ability to divide. Their progressive transformation takes a unique, complex, and lengthy process (for example, 23 days in humans) of morphological and functional differentiation termed spermiogenesis which sees the early spermatids with a usual cellular aspect (a round cell with a central nucleus surrounded by homogeneously distributed organelles) at the time of their formation to be metamorphosed into highly specialized polarized cells: the spermatozoa. Under physiological conditions, the transmission of the paternal genome to the oocyte at the time of fertilization requires the development of the acrosomal system, the rearrangement of the nucleus and perinuclear structures, the assembly of the flagellar structures, the elimination of the vast majority of the cytoplasm concomitant with a reorganization of cell organelles. These changes will be briefly reviewed using human spermiogenesis as a model. The major morphological features of spermiogenesis are common to all species but, details in the morphogenetic process and the resulting sperm morphological characteristics vary for each species since different morphological features may depend on different species-specific genetic determinants. The mammalian sperm morphogenesis is a continuum sequence of complex morphological changes which has been subdivided for descriptive purposes into successive steps, the number of which varies according to the species (rat: 19, mouse:16, ram, bull, boar: 15, baboon: 10). In humans, six steps are distinguished according to the shape of the nucleus, the aspect of the chromatin and the general morphology of the cell (**Fig. 4**): Sa, Sbl (round/early spermatids), Sb2, Sc (elongating/intermediary spermatids) Sd1, Sd2 (elongated/mature/late spermatids).

The first step corresponds to the small round spermatid produced by the second division of meiosis. The round spermatid, smaller than the secondary spermatocyte presents a central spherical nucleus with a diffuse chromatin containing one or more nucleoli lacking a clear fibrillary center (subsequently, in spermatids in elongation, the nucleolus fragments before disappearing completely), a well developed Golgi apparatus, adjacent centrioles, dispersed mitochondria lying peripherally close to the plasma membrane and microtubules (the future components of the axoneme) visible at the periphery of the cytoplasm.

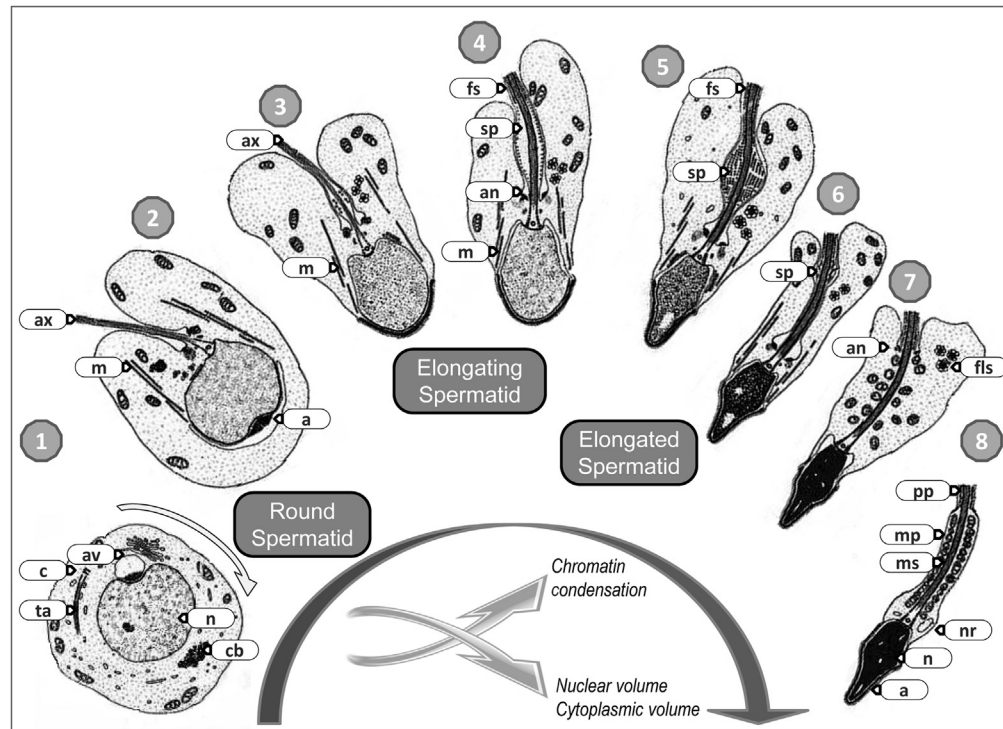


Fig. 4 Spermiogenesis steps in humans. Modified from Holstein, A. F. (1976). Ultrastructural observations on the differentiation of spermatids in man. *Andrologia* 8 (2), 157–165.

In addition the chromatid body, a regular electron-dense mass, can be observed adjacent to the Golgi complex. During the evolution of the round spermatid, the nucleus and the Golgi vesicle attached to it rotate to a position opposite to that of the developing flagellum.

Spermatids are located in the adluminal compartment of the seminiferous tubule, in close contact with Sertoli cells. Spermatids at different steps of differentiation may be observed in the same tubule cross section (see Fig. 1).

Formation of the Acrosome

The evolution of the acrosomal material, as revealed by PAS staining, has been one of the criteria used to identify the steps of the spermatid differentiation by light microscopy. The acrosome morphogenesis is generally described according to four subsequent distinct morphological phases which can be easily distinguished by TEM: the Golgi, the cap, the acrosome and the maturation phases. These four phases exhibit a general pattern but display variations in different species. Soon after the formation of the early round spermatid, the Golgi apparatus assembles the elements necessary for the formation of the acrosome: several small secretory-like granules rich in glycoproteins form on the trans face of the Golgi stacks. These proacrosomic granules coalesce to give a single larger acrosomic vesicle which associate with the nuclear membrane and tightly adheres to it to become an acrosome. This early acrosome show an electron-dense core and a less dense cortex by TEM. The Golgi apparatus continues to contribute glycoproteins to the acrosome by means of numerous small carrier vesicles. The lighter peripheral portion of the acrosome expands at the surface of the nucleus taking the shape of a cape covering at this step ~half of the nuclear surface. The tight adhesion of the acrosome membrane to the nuclear membrane is due to the presence of proteinaceous substance that later condense to form part of a rigid capsule or perinuclear theca that covers the nucleus of spermatozoa. With the displacement of most of the cytoplasm toward the caudal pole of the spermatid, the Golgi apparatus separates from the acrosome, regresses and finally disappears. At later steps of spermiogenesis and as the nucleus takes its species-specific shape, the acrosome also condenses and conforms to the changing form of the apex of the nucleus shape. The final size and shape of the acrosome varies from one species to another (for example, small in man and important in the guinea pig for example and paddle-shaped in human vs. hook-shaped in rodents).

Nuclear Elongation and Chromatin Condensation

During spermiogenesis, the central nuclei of the round/early spermatids elongate and condense their chromatin to become the main polarized component of the head compartment with an acquired species-specific shape (piriform in man,

flattened or paddle-shaped in ram, bull, and dog, falciform in rodents). A remodeling of the chromatin from its nucleosomal form to a thread-like filamentous form is first observed, then, the chromatin filaments thicken, become coarse and aggregate into compact masses that coalesce to form a dense chromatin mass (as illustrated in Fig. 4). It is established that the first morphological transformation is associated with histones modifications and loss, the second being linked to the presence of TP1 and TP2 transition proteins. Late in spermiogenesis, when the chromatin condensation has occurred and the definitive shape of the nucleus has been acquired, the transition proteins are replaced by arginine and cysteine-rich protamines conferring to the nucleus an extreme compaction protecting the genome. Several studies have indicated a specific arrangement of chromosomes in round and intermediate spermatids which could be involved in the formation of the male pronucleus.

Assembly of the Flagellar Structures

During the early steps of spermiogenesis, the pair of centrioles migrates toward the nucleus. One out of the two centrioles triggers the formation of the axoneme consisting of a bundle of microtubules, arranged in a 9 + 2 pattern (the intracytoplasmic axoneme is observed precociously from step 2 in humans). As the centrioles move toward the nucleus, they pull with them the plasma membrane, which then forms a double-wall sleeve around the growing axoneme. In the elongated spermatid, electron-dense material is deposited around the centrioles and forms a striated collar that characterizes the neck portion of the tail. The sperm tail is not similar to other flagellum or cilium since it associates a periaxonemal component including two specific cytoskeletal elements, the outer dense fibers and the fibrous sheath which are deposited along the central axoneme soon after it terminates its growth. The mitochondria peripherally close to the plasma membrane in round spermatids migrate toward the forming tail as the manchette appears in elongating spermatids. Following the sliding of the annulus (which derives in part from the chromatoid body) along the axoneme and the periaxonemal structures, the mitochondria line up in an orderly manner along the dense fiber between the neck and the annulus. Later during spermiogenesis, the mitochondria condense and dispose themselves side by side and tip to tip in a spiral manner. This mitochondrial sheath delimitates the midpiece of the length whose length is species-specific.

Cytoplasm Elimination

The gradual elimination of the cytoplasm from early spermiogenesis until the formation of mature spermatids is an evident observation in seminiferous tubule sections by light microscopy and TEM. In the most advanced phases of spermiogenesis, nearly 90% of the spermatid cytoplasm is eliminated. This progressive elimination takes place in two stages: (i) the first is continuous via the tubulobulbar complexes, the cytoskeleton-related membrane structures developing at sites of intercellular attachment in the mammalian seminiferous epithelium, a component of the sperm release mechanism (at apical junctions between Sertoli cells and spermatids the structures internalize adhesion junctions and evaginations of the elongating spermatids penetrating inside the Sertoli cells), (ii) the second occurs at the time of spermiation when the main part of the cytoplasm that remains after the reduction of tubulobulbar complexes is eliminated in the form of corpuscles called residual bodies, which are captured and phagocytosed by Sertoli cells. These residual bodies contain most of the spermatid organelles, lipids and polyribosomes, and their phagocytosis probably plays is known to play an important role in regulating the functioning of the Sertoli cell and in synchronizing the cycle of the seminiferous epithelium.

Transitory Organelles and Structures

Spermiogenesis is characterized by the presence of transient organelles whose role is unknown or not fully elucidated. Cytoplasmic microtubules anchored on the nuclear ring posterior to the acrosome constitute the manchette. The manchette displaces part of the cytoplasm with its organelles behind the caudal part of the nucleus and the nuclear ring also moves caudally. It is visible in the elongating spermatids and disappears well before the end of spermiogenesis. It consists of microtubules which form a sort of mitotic half-spindle arranged around the caudal region of the nucleus. The manchette, connected by a filamentary tract to the nuclear envelope and to the chromatin, could exert an external stress on the nuclear form, sliding gradually toward the base of the nucleus (Lehti and Sironen, 2016). The spindle-shaped body.

The chromatoid body located in the cytoplasm of round spermatids appears like a cluster of electron dense granules that presumably derives from the nuclear membrane, although its significance remains unknown. It contains actin, long half-life RNA, basic proteins, ribonucleoprotein particles as well as polysaccharides and cations.

References

- Boitani, C., Di Persio, S., Esposito, V., & Vicini, E. (2016). Spermatogonial cells: Mouse, monkey and man comparison review. *Seminars in Cell and Developmental Biology*, 59, 79–88.
- Clermont, Y. (1963). The cycle of the seminiferous epithelium in man. *American Journal of Anatomy*, 112, 35–51.
- De Kretser, D. M., & Kerr, J. B. (1988). The cytology of the testis. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction*. New York: Raven Press.

Ehmcke, J., & Schlatt, S. (2006). A revised model for spermatogonial expansion in man: Lessons from non-human primates. *Reproduction*, 132, 673–680.

Lehti, M. S., & Sironen, A. (2016). Formation and function of the manchette and flagellum during spermatogenesis. *Review Reproduction*, 151, R43–R54.

Russell, L. D., & Ettlin, R. (1990). *Histological and histopathological evaluation of the testis*. St. Louis: Cache River Press.

Further Reading

Oko, R., & Clermont, Y. (1998). Spermiogenesis. In *Encyclopedia of reproduction*. San Diego: Academic Press.

Energetics of Spermatogenic Cells

Michael D Griswold, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Energy metabolism in the testis is governed by the unique structural and productivity issues of germ cell development (Griswold, 1993). First, since haploid gene expression can trigger immune responses the testis is an immune privileged tissue by virtue of tight cellular junctions between adjacent Sertoli cells. This barrier is one of the most restrictive in all of biology and results in the formation of a basal and an adluminal compartment. Transfer of metabolites required for energy production must negotiate this barrier. Second, the net outflow of cellular components into the germ cells and ultimately out of the organism is very high. Estimates are that human males produce 1000–1500 sperm per second throughout their reproductive lifetime. Third, Sertoli cells extend from the basement membrane to the lumen of the seminiferous tubules and encompass stacks of developing germ cells. The apical end of a Sertoli cell and germ cells near that location are too far from a capillary blood supply to be satisfied by a diffusion process. Finally, nearly all metabolic determinations made on a single cell type in the testis have been made in vitro where the environment is vastly different than when cells are in situ.

It appears from published research on isolated cells that the metabolic requirements of Sertoli cells and germinal cells are quite different. Cultured Sertoli cells from immature rats metabolize added glucose primarily to lactate that is externalized into the culture medium. These results have led to the concept that one of the “nurse cell” functions of the Sertoli cells is to predigest glucose to lactate for use by germ cells. RNA synthesis, protein synthesis and oxygen consumption in cultured germ cells is stimulated by added lactate. In vivo evidence for this concept is lacking and it has been demonstrated that many cells in culture may have an impaired citric acid cycle and produce copious amounts of lactate. Cultured Sertoli cells contain large lipid droplets and utilize oxidation of lipids as a major source of energy. In addition, cultured Sertoli cells can utilize glutamine and leucine as source of intermediates for the citric acid cycle. Sertoli cells metabolism is greatly affected by hormones such as FSH, testosterone and retinoic acid. These external agents act on Sertoli cells in cycles and modify the metabolism (Alves et al., 2013).

Germ cells appear to be primarily dependent on anerobic and aerobic carbohydrate metabolism. The spermatogonia that reside in the basal compartment of the testis have access to the blood supply and can use glucose as a source of ATP. Spermatocytes and spermatids reside in the adluminal compartment of the testis and can be some distance from the blood supply. These cells appear to rely on glycolysis and possibly lactate as a substrate. The metabolism of spermatozoa is better established since spermatozoa can be examined in their natural state as isolated cell types. Sperm have a very high glycolytic activity and require glucose for hyperactivated motility, capacitation and in vitro fertilization (Boussouar and Benahmed, 2004). Several genes encoding sperm specific forms of enzymes in the glycolytic pathway are expressed only in spermatids and spermatozoa. Sperm also have mitochondria confined to the mid-piece and therefore also undergo aerobic respiration and lipid oxidation (Rato et al., 2012).

The metabolic activities of the testicular cells and the relationships of these activities to cellular interactions still have many open questions. The problems associated with studies of metabolic pathways and their regulation in whole testis tissue are complex because of the multiple cell types. Since placing isolated cells in culture introduces other barriers to interpretation many questions remain unanswered. Fertility is clearly affected by energy production and reserves but the cellular mechanisms that link ATP production to spermatogenic cell development are not well understood. Questions relating to how Sertoli cells handle problems associated with high metabolic demand and low oxygen tension, or why and how germ cell metabolism appears to be changing during development remain (Griswold, 1993).

References

- Alves, M. G., Rato, L., Carvalho, R. A., Moreira, P. I., Socorro, S., & Oliveira, P. F. (2013). Hormonal control of Sertoli cell metabolism regulates spermatogenesis. *Cellular and Molecular Life Sciences*, 70, 777–793.
- Boussouar, F., & Benahmed, M. (2004). Lactate and energy metabolism in male germ cells. *Trends in Endocrinology and Metabolism*, 15, 345–350.
- Griswold, M. (1993). Unique aspects of the biochemistry and metabolism of Sertoli cells. In M. GRISWOLD (Ed.), *RUSSELL, L. Cache River Press: The Sertoli cell*. Clearwater FL.
- Rato, L., Alves, M. G., Socorro, S., Duarte, A. I., Cavaco, J. E., & Oliveira, P. F. (2012). Metabolic regulation is important for spermatogenesis. *Nature Reviews. Urology*, 9, 330–338.

Spermatozoa and Sperm Structure

Jacques Auger, Paris Descartes University, Paris, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

During spermiogenesis, haploid round spermatids displaying classical cellular anatomy—typically, a round cell with a central rounded nucleus surrounded by the usual cellular organelles—gradually turn into testicular spermatozoa, which, in the simplest terms, are made of a head and a flagellum composed of a proximal midpiece and a distal principal piece or tail. This morphogenetic process is characterized by complex biochemical and structural transformations of the cellular components required in order the sperm cells may fulfill their physiological function: moving progressively to transport a copy of the haploid genome to, and to interact with, the oocyte, thereby delivering its genetic information—along with a pool of proteins and RNAs—through fertilization. The vast majority of mammalian mature spermatozoa have a uniform morphology. By contrast, human spermatozoa, as observed on a stained semen smear, exhibit noticeable morphological variations in size or shape of the head and/or midpiece and/or tail. Those are the morphological and biometric characteristics of the spermatozoa retrieved from the uterine cavity or in the vicinity of oocytes, and thus those having a good fertility potential which have led to the definition of a morphologically normal human spermatozoon (World Health Organization, 2010).

This article will concentrate on the normal structure and substructures of physiological mammalian spermatozoa, using primarily the normal human spermatozoa as a model.

General Features of the Mammalian Spermatozoon

As it can be easily observed on stained and smeared semen samples by light microscopy, mammalian spermatozoa have a polarized organization following three major subcellular regions: the sperm head, the midpiece and the principal piece of the flagellum that can be typically observed by various microscopical approaches (Fig. 1). However, sperm cells from different mammalian species exhibit modifications of this basic structural pattern. For example, the shape of the head varies from one species to another (e.g., slightly flattened in humans and falciform in rodents). Also, the overall length of mammalian spermatozoa is species specific and varies considerably, for example $\sim 50\text{ }\mu\text{m}$ in boar $\sim 60\text{--}70\text{ }\mu\text{m}$ in human and $\sim 90\text{ }\mu\text{m}$ in bull. Its volume is very reduced compared to that of the oocyte, for example, $30\text{ }\mu\text{m}^3$ in the bull, that is, $1/20000$ of the volume of the cow's egg.

The main morphological features of the ejaculated sperm cell are established at the end of the spermiogenetic process so that ejaculated spermatozoa are morphologically identical to testicular spermatozoa though only the ejaculated sperm are functionally mature following the biochemical and molecular alterations undergone during their transport through the epididymal duct. The spermatozoon, like other cells, is totally enclosed in a lipoprotein plasma membrane containing glycoprotein particles and covered by a glycoprotein cell coat. The lipid and glycoprotein components are distinct according to each sperm compartment delimitating surface domains that are crucial for sperm functions. For example, the equatorial segment of the sperm plasma membrane is involved in the sperm oocyte interaction.

Morphologically normal (= potentially competent) human spermatozoa observed by conventional light microscopy on a smear properly stained have a regular oval head outline with a major axis measuring $3.7\text{--}4.7\text{ }\mu\text{m}$ and a minor axis measuring $2.5\text{--}3.2\text{ }\mu\text{m}$ with a major axis to minor axis ratio in the range $1.3\text{--}1.8$ (World Health Organization, 2010). The distinct acrosomal region (corresponding to both acrosomal and underlying nuclear structures) covers $40\%\text{--}70\%$ of the head surface, has a regular outline and a homogeneous texture. The postacrosomal region of the head has a regular outline and a homogeneous texture. The midpiece under conventional microscopy has a major axis $3.3\text{--}5.2\text{ }\mu\text{m}$ microns long in the prolongation of the major axis of the head, a regular contour, a homogeneous texture and a diameter of $0.5\text{--}0.7\text{ }\mu\text{m}$. The presence of residual cytoplasm of minimal size (one-third of the sperm head size) is not considered abnormal. The principal piece has a regular contour and a homogeneous appearance and it develops in the prolongation of the midpiece, measuring approximately $45\text{ }\mu\text{m}$ (about 10 times the length of the head) with a diameter decreasing gradually from the proximal part ($\sim 0.4\text{ }\mu\text{m}$) to the distal extremity of the flagellum.

Morphological and Structural Features of a Normal (Potentially Fertilizing) Human Spermatozoon as Revealed by Transmission Electron Microscopy (TEM)

The analysis of sperm morphology by conventional light microscopy on sperm smears is rather simple, but it has obvious technical limitations in term of resolving power and the ability to describe the internal substructures: the best looking spermatozoon by optical microscopy is not necessarily free of cellular or subcellular defects. By contrast, transmission electron microscopy (TEM), due to its resolving power and application on ultrathin sections, enables a fine and precise examination of all sperm substructures (Moretti et al., 2016) including the sperm internal organization (typically, the various substructures of the head compartment are well observed with a $\times 10,000\text{--}20,000$ magnification while fine observations of the axonemal components in a transverse section of the tail may necessitate $\times 100,000$ magnification and more).

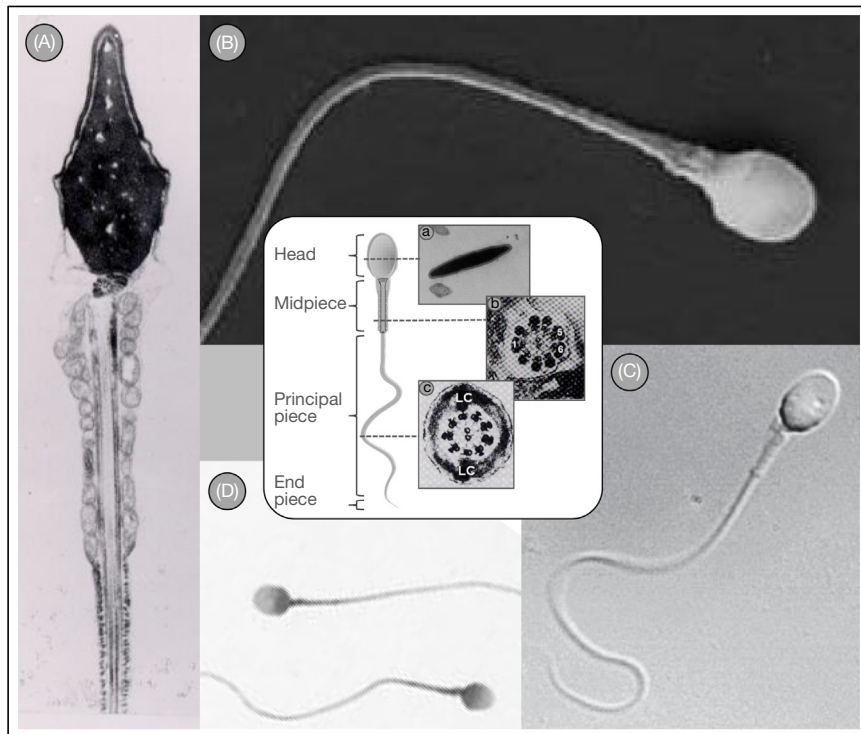


Fig. 1 Morphologically normal human spermatozoa by various microscopic techniques. (A) Ultra-thin longitudinal and sagittal section of the half proximal part of a normal spermatozoon by transmission electron microscopy (TEM) showing a piriform profile of the head compartment, a well delineated acrosome covering most of the anterior part of the highly condensed nucleus containing some vacuoles, a transverse section of the proximal centriole and a segmented column in the neck region, a clear longitudinal image of the mitochondrial sheath delimiting the midpiece, and a less clear image of the axis of the flagellum containing the axoneme and the outer dense fibers and from the end of the midpiece, the peripheral fibrous sheath. (B) A scanning electron microscopy (SEM) image showing the typical regular paddle-shaped head compartment; (C) image obtained by the MSOME technique using light microscopy with Nomarski differential interferential contrast and $\times 6300$ final magnification showing clearly the limits of the head, midpiece and principal piece compartments and revealing the presence of a “vacuole” whose exact origin is still debated; (D) observation by optical microscopy of two morphologically normal spermatozoa with a pale homogeneous acrosome region (Shorr stained smear observed at $\times 1000$ final magnification). Central figure: a schematic drawing of a normal human spermatozoon with its different head and tail compartments and associated images of transverse sections by TEM, at the head level (highly condensed and regular nucleus surrounded by an acrosome with a less dense texture; a), midpiece level (axoneme in central position with the ODFs associated to the peripheral doublets surrounded by mitochondria; b), and principal piece (from center to periphery: central pair and peripheral doublets of the axoneme, associated ODFs and the fibrous sheath surrounded by the plasma membrane, LC: longitudinal; c: column).

Sperm Head

At the end of the spermiogenetic process the late/mature/elongated spermatid has acquired a fully developed acrosome usually covering the anterior part of the head where it caps the nucleus. It has lost most of its cytoplasm essentially in the head region and presents a noticeably reduced nuclear volume with a highly condensed nuclear material. The subsequent exfoliated testicular spermatozoon as the ejaculated mature spermatozoon presents an acquired species-specific head shape (i.e., piriform in humans, flattened or paddle shape in ram, bull and dog, and falciform in many rodents). The vast majority of the sperm head volume corresponds to the nucleus whose shape in most mammalian species follows the shape of the sperm head. The shape of the head/nucleus is species-specific and usually flattened dorsoventrally; the outline tends to be oval, ensiform or falciform. The remaining volume of the head is occupied by the acrosome, cytoskeletal elements and a residual cytoplasm. It is generally admitted that the acquisition of a “hydrodynamic” head shape with a highly condensed nuclear material may facilitate passive and active sperm transport in the male and female genital tract while the compaction of the chromatin compensating for the lack of DNA repair enzymes, allows safe transport of the male genome. At the end of the sperm journey in the female genital tract, the acrosome and nucleus, with the head part of the sperm plasma membrane represent the more important sperm head structures in the processes of sperm–oocyte interaction and fertilization.

Acrosome

Mammalian sperm acrosomes perform two principal exocytosis-dependent functions: (i) to serve as a site for the sequestration and release of proteins required for binding to and penetration of the zona pellucida and, (ii) to enable the sperm to fuse with the

oolemma. These functions are dependent upon the acrosome structure. The acrosome is a Golgi-derived secretory organelle with a cap-like structure covering the anterior part of the sperm head of most animal sperm. It is formed during an early stage of spermiogenesis and resembles the cellular lysosome, a bag-like structure that normally functions in intracellular digestive and defensive mechanisms. In mammals, the size and shape of the acrosome varies from species to species and depends on the morphology of the sperm head, it is determined, in part, by the underlying nuclear morphologies. The shape of the acrosome is quite variable between species, ranging from skull-cap/paddle-shaped (spatulate) in several larger mammals, including humans, to sickle-shape in rodents. In most mammalian species, the sac-like structure of the acrosome basically consists of two parts, the anterior and equatorial segments (Fig. 2). It is surrounded by an inner acrosomal membrane (IAM) that is laminated to the nuclear foundation and a fusogenic outer acrosomal membrane (OAM) that is closely apposed to the plasma membrane over the acrosome. The inner and outer acrosomal membranes surround the electron-dense content of the acrosome. The equatorial segment forms the posterior acrosomal margin where the IAM and OAM meet. The detailed structural composition, organization and the functions of the mammalian acrosome have been recently reviewed (Fléchon, 2016).

Nucleus

The nucleus contains the condensed chromatin carrying paternal genetic information with its typical homogeneous and dense aspect as observed on smears either by conventional microscopy or on sperm sections by TEM. This last technique also reveals small areas of uncondensed chromatin in the neck region and the presence of nuclear vacuoles—probable local defects of chromatin condensation—of irregular outline and size depending on the species, without surrounding membranes distributed at random in the dense nuclear material, particularly frequent and large in human spermatozoa. The homogeneous and dense textural characteristic of the mammalian sperm chromatin is linked with the histone–protamine exchange during the elongating phase of spermiogenesis which drastically modifies the structure and spatial organization of the chromatin. At the end of this process, the sperm chromatin is ~7 times more condensed than in the nucleus of any somatic cells. Ward and Coffey (1991) have proposed a model for the coiling of the DNA loop domain in sperm nuclei of rodent and human spermatozoa (Fig. 3).

In this model, the protamine-bound DNA is coiled, with a very slight bend in the protamine DNA complex, into concentric circles. These circles, of one loop, then collapse into a toroid-shaped structure, termed the DNA loop doughnut, in which the neutral DNA protamine complexes are tightly packed together by Van der Waal's forces. Each doughnut represents one DNA loop domain attached to the sperm nuclear matrix. The sperm nuclear matrix is not observed on standard sperm preparations and its observation required chromatin to be relieved of protamine compaction. In addition to the nuclear matrix, sperm nuclei contain a unique structure, termed the sperm nuclear annulus, located at the implantation fossa. When the nuclear matrix is disrupted during decondensation, the DNA remains anchored to this structure. Although the sperm nucleus is a highly ordered and well conserved structure from one sperm cell to another as well as from one individual to another, the organization of the mature sperm nucleus appears to be species-specific, limiting the ability to translate findings from mouse or bovine to human. Despite the near complete packaging of the sperm genome as protamine-associated DNA, it is increasingly clear that specific regions retain a somatic-like structure: 10%–15% histones are retained in spermatozoa in humans, but only ~1% in mice. Several studies have indicated a specific arrangement of the mammalian sperm chromosomes which could be involved in the formation of the male pronucleus and the first steps of the embryonic development.

The nucleus is surrounded by an unusual nuclear membrane closely applied to the nucleus: unlike somatic cells where numerous pores are scattered over most of the nuclear envelope surface, pores are absent in the nuclear sperm membrane except in the most

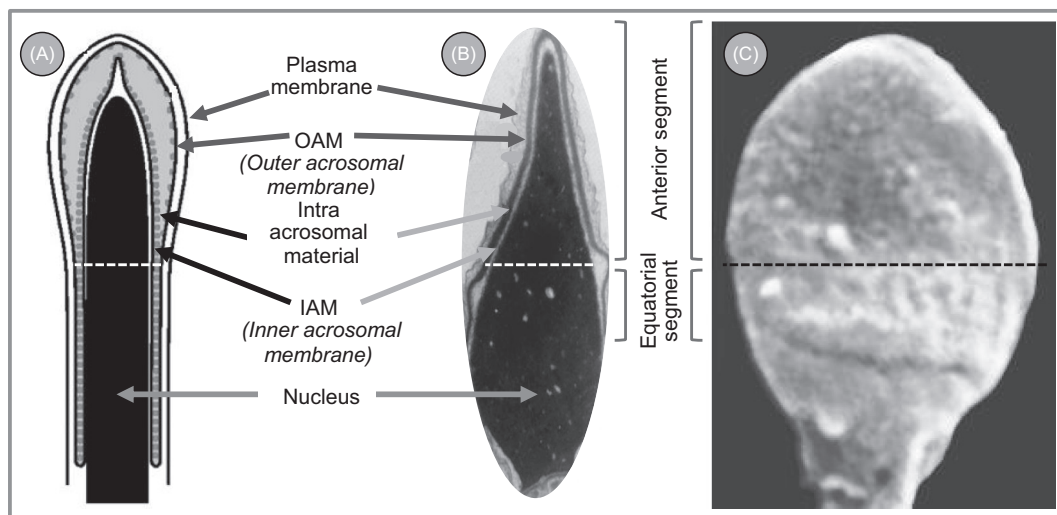


Fig. 2 Morphology and subcellular components of a normal human acrosome. (A) Schematic drawing. (B) Corresponding longitudinal and sagittal section observed by TEM. (C) Frontal view by SEM.

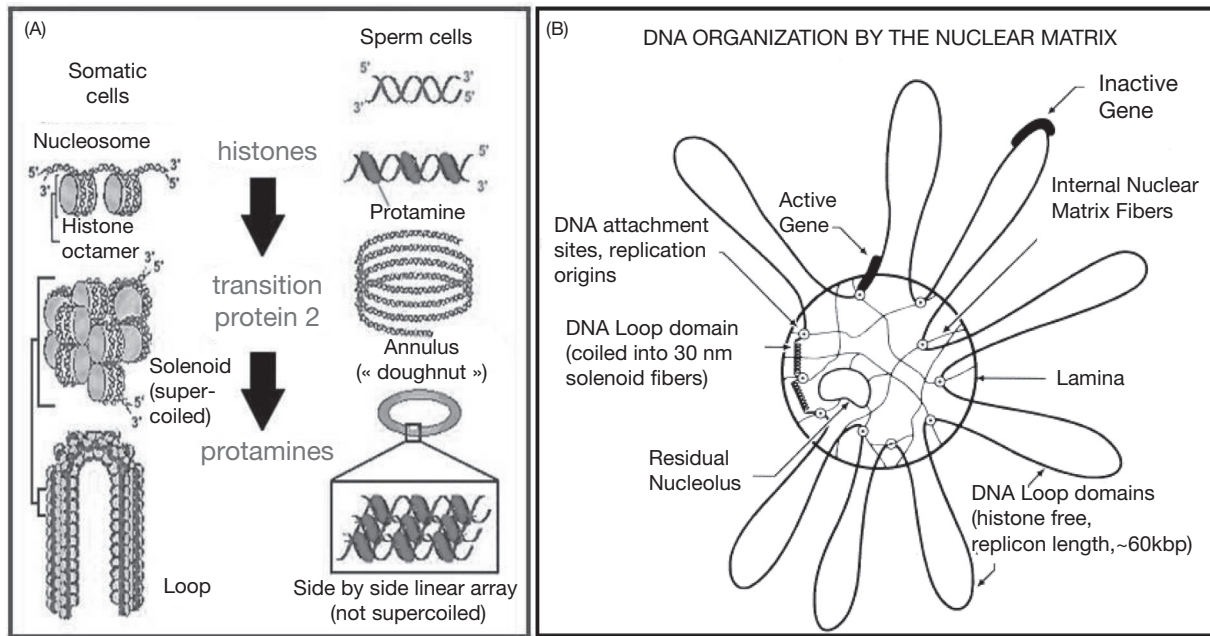


Fig. 3 Human sperm nucleus: structure and DNA organization. (A) DNA packaging in somatic versus sperm nuclei. (B) Diagram of the organization of the DNA in the nucleus of the human spermatozoa after extraction of the protamines by SDS and 2 M NaCl. The nuclear matrix retains the initial form of the nucleus and the DNA deploys outwards in the form of loops attached by their base to the matrix. Adapted from Ward, W.S. and Coffey, D.S. (1991). DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. *Biology of Reproduction* **44**, 569–574.

posterior region of the head where the nuclear envelope has many folds/projections around the neck (redundant nuclear envelope), filling the posterior nuclear space. The nuclear membrane and the plasma membrane coalesce to form the posterior ring. The outer surface of the nuclear membrane thickens to constitute the basal plate at the implantation fossa of the tail.

Neck

The neck or connecting piece is a short segment between the sperm head and tail with a basic structural organization in all mammalian species despite species-specific fine variations. It has the shape of a truncated cone whose large base is a curved, electron-dense, leaflet called the capitulum facing the basal plate, a thickened, electron-dense zone of the nuclear membrane situated on the cephalic side and, the small base in the distal position in close juxtaposition with the initial part of the flagellum. At a high TEM magnification, many filaments arranged perpendicularly traverse this zone suggesting their role in the attachment of the flagellum to the head. The neck region comprises a proximal centriole, having a distinctive structure composed of nine microtubule triplets, surrounded by pericentriolar material, which lies in the implantation fossa and is oriented obliquely to the axis of the flagellum, in the dorso-ventral plane of the spermatozoon and, a modified distal centriole extending to the proximal part of the axoneme. It is flanked by nine segmented/cross-striated columns losing their individuality in their proximal portion where they fuse with the capitulum and developing distally where they dispose themselves in the continuation of the dense fibers at the level of the modified distal centriole. The distal centriole forms the axoneme, whereas the proximal centriole is associated with the formation of capitulum. Thus, the connecting piece originates from both the proximal centriole and the distal centriole. The sperm centriole is a key structure because, after fertilization, the egg polarity resets through the formation of the centrosome from the proximal centriole associated with the sperm head. The centrosome assembles the first mitotic spindle of the zygote.

The Flagellum or Tail

The tail is the longest part of the sperm cell, consisting of midpiece, principal piece, and end piece. The tail surrounded by a plasma membrane contains (Fig. 4):

- a centrally placed axoneme, composed of nine microtubule doublets surrounding a central doublet, (9 + 2 structure) resembling a cilium in structure which extends from the neck region to the end piece of the tail, surrounded by outer dense fibers (ODFs),
- a tight helix of wrapped mitochondria around the dense fibers which provide the energy (ATP) for cellular function and movement, extending from the neck to the principal piece, in the proximal part of the tail (the middle piece or midpiece),
- a fibrous sheath (FS) surrounding the dense fibers in the principal piece,
- an end piece where the axonemal 9 + 2 structure is lost, the nine doublets forming 18 single microtubules, the central tubule pair often lacks there and, ODFs and FS are absent.

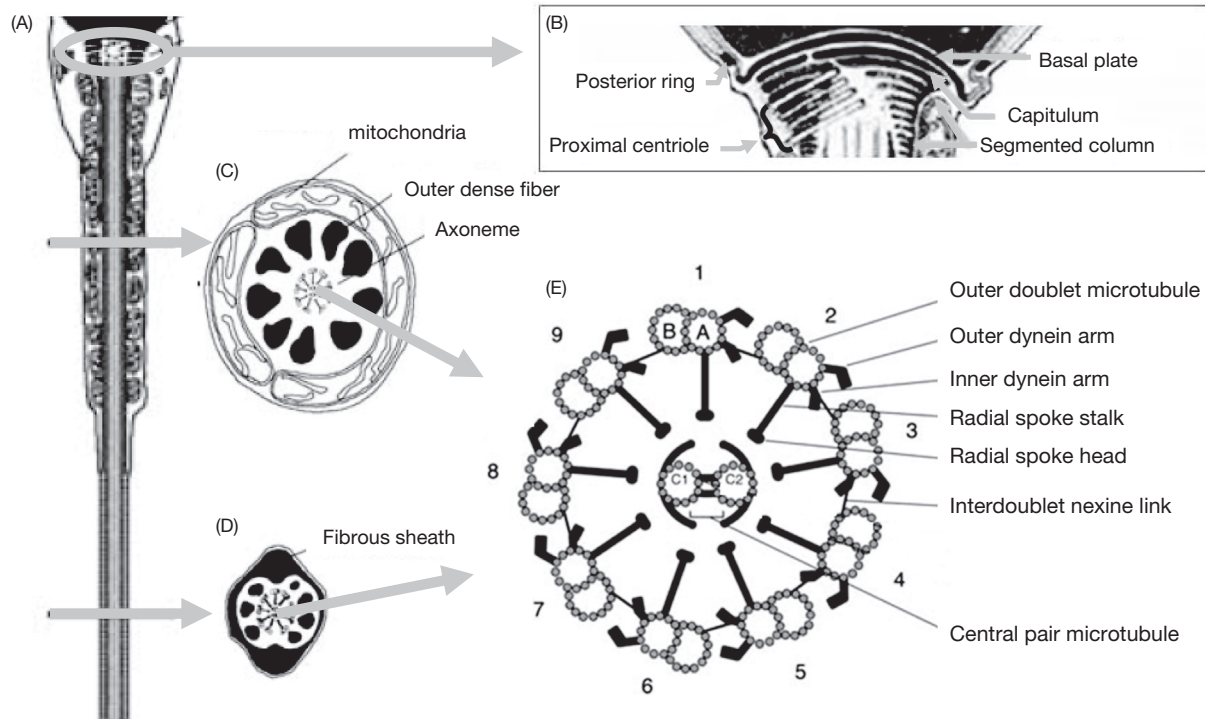


Fig. 4 Neck and tail morphology and corresponding subcellular structures for a normal human spermatozoon. (A) Drawing of the aspect of the neck and half part of the tail in a longitudinal section observed by TEM. (B) Details of the structures of the neck region or connecting piece. (C) Drawing of a transversal section of the midpiece as observed by TEM. (D) Drawing of a transversal section of the principal piece as observed by TEM. (E) Schematic organization and components of the axoneme.

The periaxonemal structures, ODF and FS, are two unique cytoskeletal elements along the central axoneme which distinguish the sperm tail from simple flagella or cilia. Furthermore, ODF is a characteristic structure of the species with internal fertilization.

Axoneme

The mammalian axoneme (Fig. 4) present in all parts of the flagellum shares its basic structure with axoneme of most cilia and flagella in plants and animals. A central pair of microtubules is encircled by 9 microtubules doublets (configuration 9 + 2). Each doublet consists of one complete microtubules exhibiting 13 protofilaments (subunit A) and one incomplete, C-shaped, microtubule apposed to the wall of the complete microtubule (subunit B). The two subunits of the doublet are composed of tubulin protofilaments. From each subunit A, two dynein arms extend in the direction of the subunit B of the neighboring doublet. Dynein is a structural protein and an ATPase using ATP as a source of energy to generate flagellum movement. Each pair of peripheral microtubules is connected to the adjacent pair by nexine links, and connected to the central pair by nine radial spokes.

The flagellar movement results from the sliding of the microtubules by successive cycles of attachment and detachment of the internal and external dynein arms onto the adjacent microtubules. The radial spokes could intervene in the curvature of the flagellum.

Mitochondrial Sheath and Annulus

The midpiece contains the axoneme surrounded by the mitochondria wrapped helically, generally, end to end, around the outer dense fibers. The nature and organization of the helices including the number of mitochondria, the number of coils and finally, the overall length of the midpiece are species specific. The mitochondria deliver the ATP necessary for the autonomous displacement of the sperm cells.

At the caudal end of the mitochondrial sheath, the annulus or Jensen's ring, marks the limit between the midpiece and principal piece. At low magnification, the annulus appears as a dense homogeneous structure, it appears to consist of closely packed circumferentially oriented filamentous subunits. The flagellar membrane is firmly adherent to the annulus. The annulus is supposed to have the mechanical role of preventing a caudal displacement of the mitochondria during tail movement.

Outer Dense Fibers (ODFs)

ODFs are nine thin cylindrical cross-striated structures surrounding the peripheral doublets and associated with them (see the central caption in Figs. 1 and 4). ODFs extend from their junction with the striated columns to different parts of the principal piece.

They have a petal-like shape, a dense, inhomogeneous structure and a grossly rounded outline on transverse sections, while with variations in shape among them. In some species, satellite fibrils are visible between the outer fibers and doublets. They are unequal in size and length (in both humans and rats, ODFs extend to about 60% of the total length of the principal piece). All ODFs are thickest in the proximal part of the middle piece and progressively diminish in diameter from base to tip of tail. In humans, several ODFs have a larger section than others and these ODFs are the longest. ODFs are not thought to play an active role in axonemal or flagellar movement. Rather, cross-linked by extensive disulfide bridges, these fibers may serve to stiffen the tail and/or provide elastic properties.

Fibrous Sheath

The fibrous sheath develops on the entire length of the principal piece and lies subjacent (but not attached) to the plasma membrane. This structure could be unique to mammalian species and some birds. It is organized into two longitudinal columns connected by circumferentially oriented half circle ribs. These ribs thicken at their crossover to the columns and form broad bands in some species by interconnecting with each other. At high magnification, the columns are less closely packed than the subunits of the ribs. The ribs of the fibrous sheaths are separate elements except at their ends, occasionally they even bifurcate in tangential section. FS abruptly ends at the junction of the principal piece and end piece of the tail. FS is partly responsible for the specific wave movement of the sperm tail. Besides its role in sperm motion, the fibrous sheath works as a scaffold for proteins in signaling pathways, so it might be involved in regulation of sperm maturation, motility, capacitation, hyperactivation and/or AR.

References

- Fléchon, J. E. (2016). The acrosome of eutherian mammals. Review. *Cell and Tissue Research*, 363, 147–157.
- Moretti, E., Suter, G., & Collodel, G. (2016). The importance of transmission electron microscopy analysis of spermatozoa: Diagnostic applications and basic research. *Systems Biology in Reproductive Medicine*, 62, 171–183.
- Ward, W. S., & Coffey, D. S. (1991). DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. *Biology of Reproduction*, 44, 569–574.
- World Health Organization. (2010). *WHO Laboratory manual for the examination and processing of human semen* (5th edn.). Geneva, Switzerland: WHO Press.

Further Reading

- García-Vázquez, F. A., Gadea, J., Matás, C., & Holt, W. V. (2016). Importance of sperm morphology during sperm transport and fertilization in mammals. *Asian Journal of Andrology*, 18, 844–850.
- Johnson, G. D., Lalancette, C., Linnemann, A. K., Leduc, F., Boissonneault, G., et al. (2011). The sperm nucleus: Chromatin, RNA and the nuclear matrix. *Reproduction*, 141, 21–36.
- Lindemann, C. B., & Lesich, K. A. (2016). Functional anatomy of the mammalian sperm flagellum. Review. *Cytoskeleton*, 73, 652–669.

Cell–Cell Interactions—Structural

Nathália de Lima e Martins Lara, Gleide Fernandes de Avelar, Guilherme Mattos Jardim Costa, and Samyra Maria dos Santos Nassif Lacerda, Federal University of Minas Gerais, Belo Horizonte, Brazil

Rex A Hess, University of Illinois at Urbana-Champaign, Urbana, IL, United States

Luiz Renato de França, Federal University of Minas Gerais, Belo Horizonte, Brazil; and National Institute for Amazonian Research, Manaus, Brazil

© 2018 Elsevier Inc. All rights reserved.

Introduction

Interactions between cells are required for the maintenance or alterations/changes of cellular function, differentiation and growth, being therefore crucial for the normal physiology of tissues and organs. A great array of cellular interactions are required for the coordination of reproductive processes, at the structural and molecular levels. As millions or even billions of spermatozoa are daily produced in the testis, in a very active and complex process named spermatogenesis that occurs in the seminiferous epithelium, this naturally requires a very coordinated and organized interactions among testicular cells. For instance, since seminiferous cords/tubules formation, extensive interactions between the germ and Sertoli cells are needed for the development/self-renew and differentiation of germ cells, particularly in relation to the structural and nutritional aspects. In addition, the formation of the blood-testis or Sertoli cell barrier (SCB) between adjacent Sertoli cells, which creates a specialized microenvironment, is crucial for the development of more mature germ cells. In order to provide an optimal androgen milieu, in the intertubular compartment there is also a set of complex interactions and communications between the steroidogenic Leydig cells and other important interstitial cells. In this article, the main structural cellular interactions present in the testis (summarized in [Table 1](#)) is discussed, focusing particularly on its composition and importance for spermatogenesis.

Tubular Compartment

Sertoli–Sertoli Cell

During spermatogenesis, especially when germ cells are undergoing meiosis they show a different profile of surface antigens that are usually detected by the immune system and lead to an autoimmune reaction. In order to protect the development of these germ cells, Sertoli cells form the Sertoli cell barrier (SCB) (the most important component of the so-called blood-testis barrier), which helps to create an immune privileged environment inside the seminiferous epithelium, where foreign or autoimmunogenic antigens can be tolerated without inducing a detrimental immune response, leading ultimately to male infertility ([França et al., 2012](#)).

Initial evidence for the existence of the SCB came from different sources. Firstly, it was observed that the composition of the fluid present in the lumen of the seminiferous tubule is different from blood plasma or lymph. This difference would not exist if the tubule walls were permeable to molecules from the intertubular and basal compartments. Other evidence came from the use of tracers and dyes, determining the entry rate of different fluids and the relationship of a SCB to the fluid composition and solubility. Therefore, it was concluded that there must be a mechanism for controlling the entry of substances into the seminiferous tubule.

Table 1 Summary of the main junctions present in the testis and the cell types involved

<i>Junction</i>	<i>Cells involved</i>
Tight junctions	Sertoli–Sertoli cell (SCB ^a)
Adherens junctions	Sertoli cell-spermatogonia
	Leydig–Leydig cell
Gap junctions	Sertoli–Sertoli cell (SCB)
	Sertoli–germ cells
	Leydig–Leydig cell
Desmosomes	Sertoli–Sertoli cell (SCB)
	Sertoli cell-spermatocytes/early spermatids
Hemidesmosomes	Sertoli cell-basement membrane
Tubulobulbar complex	Sertoli–Sertoli cell (SCB)
	Sertoli cell-elongated spermatid (around spermiation)
Ectoplasmic specialization (ES)	Sertoli–Sertoli cell (SCB; basal ES)
	Sertoli cell-elongated spermatid (apical ES)
Intercellular bridge	Germ–germ cell
	Leydig–Leydig cell (Fetal)
Digitations	Leydig cell-macrophage

^aSCB, Sertoli cell barrier.

The SCB is formed by specialized junctional complexes between adjacent Sertoli cells, near the basement membrane, dividing the seminiferous epithelium into two compartments: basal and adluminal. Spermatogonia and early primary spermatocytes (preleptotene/leptotene stage) are located in the basal compartment. Spermatocytes must move from basal to the adluminal compartment, through a transient, intermediate compartment, without damaging the barrier structure. This intermediate compartment is created by a newly formed barrier, basal to the migrating cell, separating it from the basal compartment. The old junctional complex, luminal to the spermatocyte, is then dismantled and these cells enter the adluminal compartment. In this way, more advanced spermatocytes (from leptotene/zygotene onward) and the round and elongated spermatids are located in the protected adluminal compartment (Cheng and Mruk, 2012).

The main junctional constituent of the SCB is the tight junction, but desmosomes, gap junctions, tubulobulbar complexes and basal ectoplasmic specialization (the last two are testis-specific types of adherens junctions and will be discussed in more detail in the next section) are also present and collectively maintain this morphological immune barrier of the testis. The proteins involved include occludins, claudins, cadherins, catenins, and connexins, usually anchored by actin or vimentin filaments. This particular arrangement gives strength to the SCB, making it one of the tightest barriers in mammalian tissues.

Beyond strength and immunological protection, the barrier also provides for communication between the adjacent Sertoli cells, which is essential for coordination and signaling during the different stages of the seminiferous epithelial cycle. In addition, the junctional complexes restrict and control the transit of molecules from blood and interstitial spaces to the seminiferous tubule (and vice-versa), effectively limiting the immune system's access to the adluminal compartment of the seminiferous epithelium. The specialized microenvironment created is enhanced by immunomodulatory factors produced locally that cause an immunosuppressive response, instead of a possible destructive proinflammatory reaction. Sertoli cell polarity is also conferred by the presence of the barrier, illustrated by the usual basal location of the nucleus and an uneven distribution of cytoplasmic organelles within the Sertoli cell. This asymmetric organization is very important for the support of spermatogenesis, as physiological and cellular events take place in the corresponding cellular compartments in a stage-specific manner (Fig. 1).

In mammals, the establishment of the SCB occurs when the Sertoli cells stop proliferating and terminally differentiate, coinciding with the production of primary spermatocytes, tubular fluid secretion and lumen formation. As observed in the SCARKO mice model (mice with a selective ablation of the androgen receptor in Sertoli cells), germ cells are also important for the establishment and effectiveness of the barrier, as the meiotic phase of spermatogenesis can only be initiated in the presence of a competent SCB. Permeability and structure of the barrier is highly dynamic. As spermatogenesis proceeds, different molecules are transported to and from the interior of the tubule, depending on the spermatogenic cycle phase and the needs of the developing germ cells. Due to the presence of the barrier, hormones, such as FSH and LH for example, may act indirectly in germ cells, via Sertoli cells, which allows for a higher level of control by these supporting somatic cells over the spermatogenic process (França et al., 2016).

Sertoli–Germ Cell

The interactions between the Sertoli and germ cells in the seminiferous epithelium must be tightly regulated, because Sertoli cells provide the main control over spermatogenesis, providing nutritional and structural support for all developing germ cells. This Sertoli/germ cell interaction occurs through different types of junctions, such as adherens junctions, desmosomes, and gap junctions, depending on the stage of the seminiferous epithelium cycle and germ cell-specific requirements. For example, adherens junctions are usually detected between Sertoli and spermatogonia located in the stem cell niche, being important for stem cell homing and colonization. Desmosomes, in turn, are the major anchoring junction between spermatocytes/early spermatids and the Sertoli cells. Additionally, gap junctions provide crucial communications between adjacent germ cells, which is essential for the coordination of germ cell development throughout the different stages of differentiation (Fig. 2).

The testis forms a unique type of adherens junction, called ectoplasmic specialization (ES), which is not found in any other epithelium and is usually considered as one of the strongest anchoring junctions in the mammalian body (Cheng and Mruk, 2015). Despite participating in the SCB (basal ES), apical ectoplasmic specialization is also found at the Sertoli cell–elongated spermatid interface, conferring spermatid adhesion and polarity. Spermatid polarity involves the elongating heads pointing perpendicularly toward the basement membrane of the seminiferous tubule, allowing for maximum packing of developing spermatids in the seminiferous epithelium. This polarity and the very well-coordinated spermatogenic process allows the usually very high sperm output observed during spermatogenesis. Loss of spermatid adhesion and early release of the spermatids to the lumen of the seminiferous tubule is often caused by the disruption of cell polarity. In this context, it has also been shown that DICER (involved in the miRNA biosynthesis pathway) is an important player in the regulation of these Sertoli/germ cell junctions (Korhonen et al., 2015).

Structurally, the ectoplasmic specializations consist on bundles of actin microfilaments sandwiched between flattened cisternae of the Sertoli cell endoplasmic reticulum, and are present in the Sertoli cell membrane that faces the spermatid. This structure is closely associated with the microtubule network of the Sertoli cell, and is probably responsible for the movement of elongating spermatids across the seminiferous epithelium during the stages of the cycle. Injection of a microtubule stabilizer (taxol) lead to the retention of spermatids deep within the Sertoli cell crypts, while disruption of both ES and microtubules trapped the spermatids inside the epithelium, blocking their release at spermiation, until they were phagocytosed by the Sertoli cells. The molecular composition of the ES is constituted by nectin-, cadherin- and integrin-based protein complexes. A polarity protein complex, composed of

partitioning-defective3/partitioning-defective6/atypical protein kinase C (Par3/Par6/aPKC), is also an integrated component of the apical ectoplasmic specialization.

The apical ectoplasmic specialization appears in step 8 spermatids, replacing gap junctions and desmosomes as the only anchoring device between the spermatid and the Sertoli cell. Around the time of spermiation (steps 18–19 of spermiogenesis in rats, and steps 15–16 in mice), the apical ES undergoes degeneration, through the formation of structures similar to endocytic vesicles, which initiates the elimination of cytoplasmic debris resulting from the release of the spermatids. These vesicular structures are called tubulobulbar complexes. They form narrow tubular projections of the plasma membrane of the elongating spermatid head, penetrating an invagination of the Sertoli cell plasma membrane and forming a bulbous end. The tubular portion of the complex is surrounded by actin filaments, while the bulb is more associated with cisternae of the endoplasmic reticulum. The tubulobulbar complex facilitates the internalization of the disassembled apical ES junctions, in preparation for the release of sperm cells during spermiation (Vogl et al., 2013). Steroids, especially androgens, may be involved with the regulation of tubulobulbar complex formation, as it occurs concomitant with the occurrence of androgen-dependent stages of the seminiferous epithelium cycle (Fig. 3).

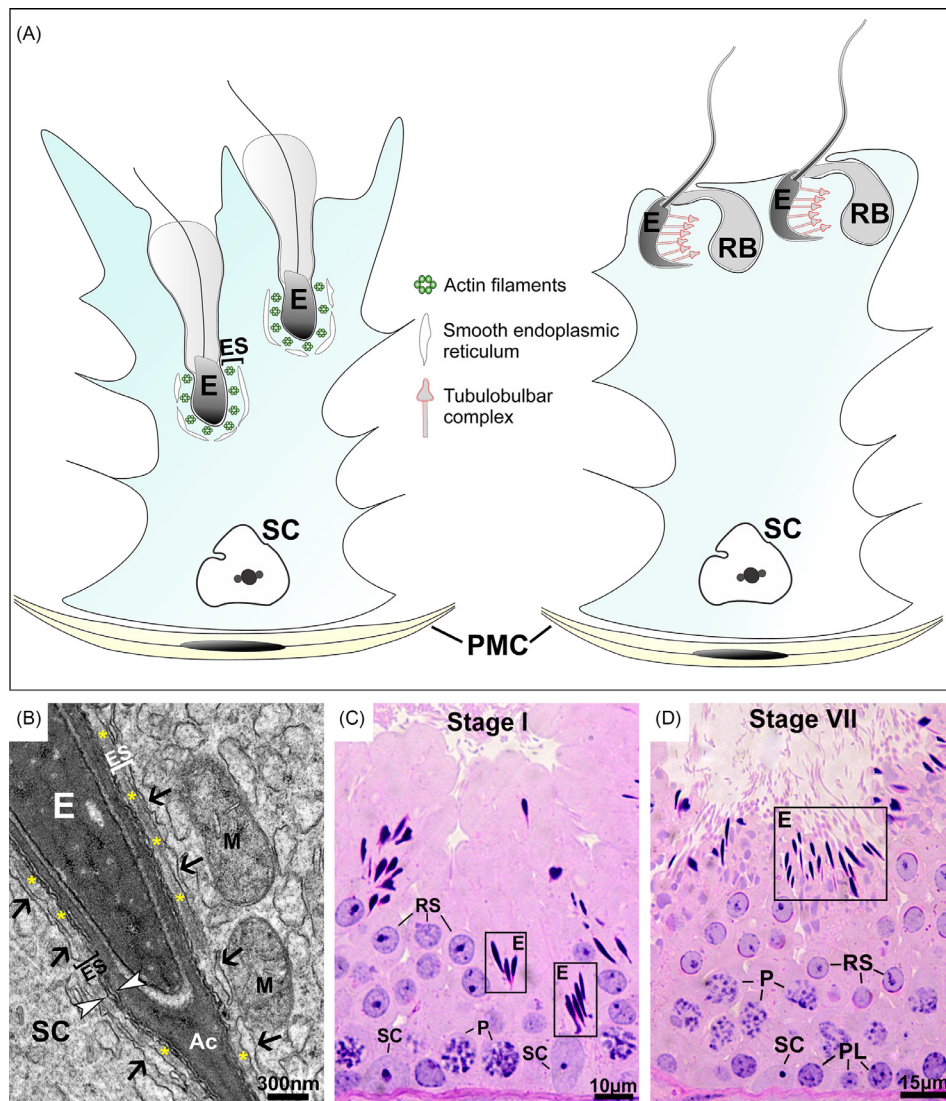


Fig. 3 Ectoplasmic specialization (ES) and tubulobulbar complex in elongating spermatids. (A) Schematic representation of the ectoplasmic specialization (*left*) and the tubulobulbar complex (*right*) and the relationship between the elongating spermatids and the Sertoli cell. (B) Electron microscopy of an elongating spermatid showing the ES structure, composed by actin filaments (*yellow asterisks*) sandwiched between smooth endoplasmic reticulum cisternae (*arrows*) and the apposing plasma membranes of the Sertoli and the germ cell (*opposing white arrowheads*). (C, D) Cross-section of mouse seminiferous tubules illustrating the intimate relationship between the Sertoli cell and the elongating spermatids delimited by the rectangle. (C) Stage I tubule showing the elongating spermatids deep within the cripts of the Sertoli cell. (D) Stage VII tubule, showing elongated spermatids at the edge of the seminiferous epithelium, close to the lumen, in preparation for spermiation. ES, Ectoplasmic specialization; SC, Sertoli cell; Ac, Acrosome; M, Mitochondria; PL, Preleptotene spermatocytes; P, Pachytene spermatocytes; RS, Round spermatids; E, Elongating spermatids; RB, Residual body; PMC, Peritubular myoid cell.

Sertoli Cell–Tunica Propria

The exterior wall of the seminiferous tubule—the tunica propria—is composed mainly by the peritubular myoid cells, separated from the Sertoli cells by a basement membrane. This cell layer is responsible for structural integrity and contraction of the seminiferous tubule and regulatory interactions between the interstitial and tubular compartments. The peritubular myoid cells are in constant interaction with the nearby Sertoli cells and participate actively in the SCB (Skinner, 1993) and establishment of the stem cell niche. Their interaction is mainly mediated by the extracellular matrix of the basement membrane present between them, produced cooperatively by both peritubular and Sertoli cells. The peritubular myoid cells are capable of influencing the Sertoli cell function and vice-versa, changing the cellular synthetic profile, proliferation, differentiation and hormone responsiveness. Some paracrine factors have been shown to be involved in this regulation, such as activin, PModS, and growth factors (TGF α , TGF β , IGF-1), for example, but no structural interaction is described directly between these cells. Therefore, although not in direct contact, the Sertoli and peritubular myoid cells have a close association that involves a number of cell–cell interactions, mainly paracrine, in order to maintain Sertoli cell function, retention of peritubular myoid cell phenotype/activity, and spermatogenesis (Verhoeven et al., 2000).

Additionally, recent reports discuss the existence of a local apical ES-blood-testis barrier-basement membrane functional axis, mainly mediated by polarity proteins that could coordinate the events of junction restructuring in the seminiferous epithelium during spermatogenesis. This can be illustrated by proteins at the Sertoli cell–extracellular matrix interface, such as integrins, which can provide a feedback regulatory mechanism to modulate barrier integrity. In addition, proteins released during spermiation (e.g., fragments of laminins from the apical ES) can collaborate with polarity proteins to regulate SCB remodeling, directly or indirectly, via hemidesmosomes in the basement membrane. Experimental manipulation of hemidesmosomes integrins can disrupt the barrier function. These findings are supported by the fact that three cellular events that involve cellular interactions but at opposite ends of the Sertoli cell epithelium, namely (1) spermiation; (2) transit of primary spermatocytes and SCB restructuring; and (3) germ cell-cycle progression through meiosis that occurs concomitant in time. Therefore, it seems logical to assume that a regulatory loop would coordinate these cellular events (Wong and Cheng, 2009).

Germ–Germ Cell

During spermatogenesis, the germ cells go through successive cell divisions but cytokinesis is usually incomplete, resulting in daughter cells that are connected by cytoplasmic bridges. This process forms a syncytium of clonal cells derived from one spermatogonial stem cell. These bridges are maintained throughout spermatogenesis, even when germ cells migrate from the basal to the adluminal compartment through the SCB, and are eliminated only at the final stage of spermatid development, along with the residual cytoplasmic bodies. The presence of this syncytium is responsible for the synchronous development of germ cells by sharing essential signals, proteins, mRNA and organelles, which helps to coordinate mitosis, meiosis, differentiation and apoptosis of these cells (Smith and Braun, 2012).

The intercellular bridges are large and stable cytoplasmic channels, with an electron dense wall composed mainly of actin. Some proteins were shown to participate in these intercellular bridges, with Tex14 (Testis-Expressed Gene 14) being the most studied. This protein forms the mitotic spindle matrix and its absence in mice leads to spermatogenesis interruption at the meiotic phase and no formation of intercellular bridges. Tex14 blocks the complex centralspindlin that is formed by the proteins MKLP1 (Mitotic Kinesin-Like Protein 1) and MgcRacGAP (Male Germ Cell Rac GTPase-activating protein). This complex recruits abscission effector proteins, such as Cep55 (Centrosomal Protein 55-KDa), that are responsible for the completion of cytokinesis (Greenbaum et al., 2011).

In summary, the presence of cytoplasmic bridges between germ cells is a unique mode of intercellular communication, essential for fertility. However, the specific roles of the intercellular bridges for stem cell self-renewal, proliferation and differentiation of more advanced germ cells still need to be elucidated. The study of the germ cell's ability to inactivate abscission during cytokinesis could provide relevant information leading to the ability to regulate this cell division event in abnormal cells found in cancers (Fig. 4).

Intertubular Compartment

Leydig cells are the main component of the intertubular (interstitial) compartment of the testis. Among other important functions, these steroidogenic cells are responsible for synthesizing and secreting androgens, which are essential hormones for spermatogenesis and development of male sex characteristics. Other important components of the interstitial compartment include macrophages, fibroblasts, lymphocytes, mast cells, blood, and lymphatic vessels. The interaction between these components and the Leydig cells is very important for their development and differentiation and for the correct function of the testis. In this context, the most important and studied cell to cell structural interaction is between Leydig cells and macrophages, while less is known about the interactions with the other interstitial cells. It is worth mentioning that, although anatomically separated, Sertoli and Leydig cells highly modulate each other's development and function through paracrine interactions and secreted factors, such as androgens, inhibin, interleukin-1, growth factors, and estrogen.

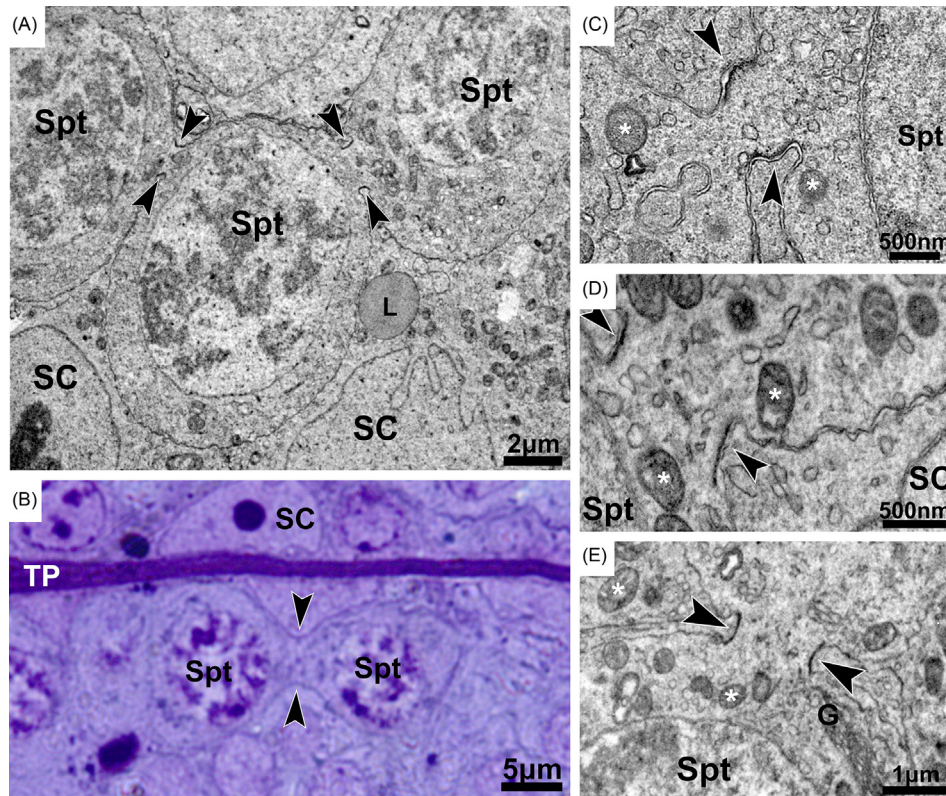


Fig. 4 Intercellular bridges between germ cells. (A) Electron microscopy section of the seminiferous epithelium, showing three pachytene spermatocytes (Sp) connected by intercellular bridges (opposing *arrowheads*). (B) Semithin cross-section of an intercellular bridge between pachytene spermatocytes. (C, E) Higher magnification of the intercellular bridges observed by electron microscopy. Note that, due to the large size of the bridge, the cells form a syncytium, where signals, proteins, mRNA and even organelles can be shared. SC, Sertoli cell; Spt, Spermatocyte; L, Lipid droplet; TP, Tunica propria; *, Mitochondria; G, Golgi complex.

Leydig–Leydig Cell

Each Leydig cell is coupled to another Leydig cell through adherens and gap junctions and these connections are present in both fetal and adult populations. The presence of these junctions allows the exchange of ions and small molecules, besides controlling androgen secretion. Various proteins are involved in this communication, such as connexins (especially connexin-43, which abundance is dependent on LH levels) and cadherins, cell adhesion molecules (You et al., 2000; Poutis et al., 2010). Intercellular bridges with continuous cytoplasm can also be observed between some fetal Leydig cells (FLC).

In the adult population of Leydig cells (ALC), the gap junctions form before the increase in secretory activity of these cells, which is associated with puberty, when this population is established. The structural diversity and presence of these junctions increase along with the maturity of the adult Leydig cell and, in general, the ALC tend to form more gap junctions in comparison to the FLCs (Haider et al., 2007). Some of the gap junctions have a distinctive annular appearance, as they are formed between a cell process and the main cytoplasmic region of the other cell. However, the reason for this characteristic is still unknown. The presence of gap junctions between a group of Leydig cells strongly suggests a coordinated activity, which could be related to the variation in androgenic activity of Leydig cells depending on the stage of the spermatogenic cycle, as well as proximity of these Leydig cell groups to the tunica propria and the seminiferous tubule itself (Fig. 5).

Leydig-Immune Cells

In most species, Leydig cells are the most prevalent cell type in the testicular interstitium, while macrophages are the second most frequent cell. Testicular macrophages and Leydig cell numbers increase proportionately throughout development, as changes in their morphology are coordinated. This association suggests a functional relationship between both cells, but much remains to be learned. From studies mainly based on rats, it is estimated that there is approximately one macrophage for every three to five Leydig cells in the testis. The macrophages are usually in direct contact with adjacent Leydig cells through membrane associations or “digitations.” These interactions appear as long microvillus-like Leydig cell processes inserted within coated membrane invaginations into the macrophage cytoplasm. The digitations probably function as anchoring sites and for the exchange of signaling factors between both cells, as multiple coated vesicles are seen near these structures (Hutson, 2006). This interaction is

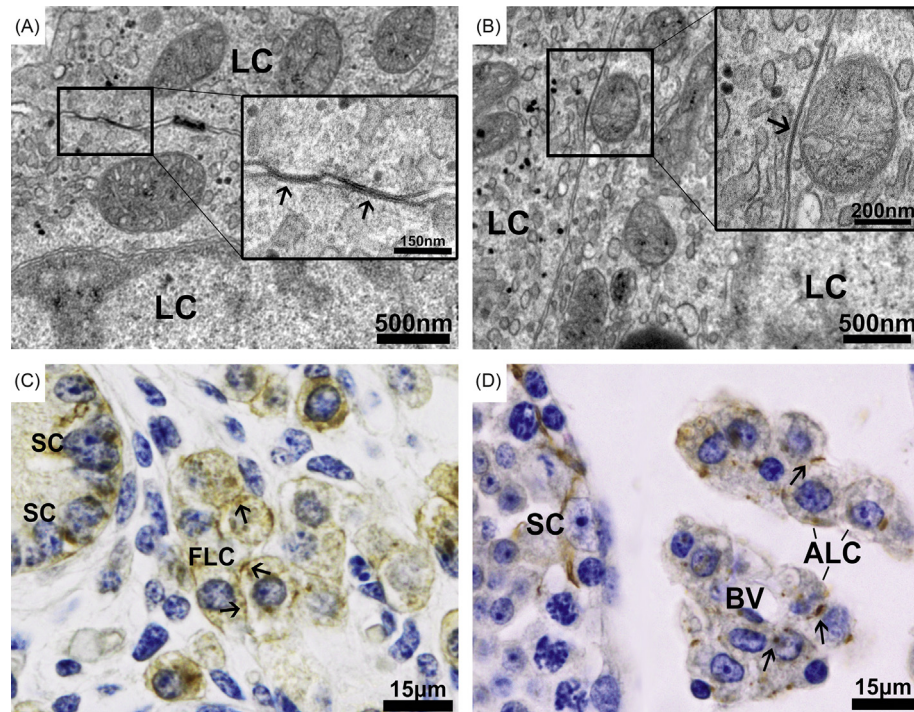


Fig. 5 Gap junctions between Leydig cells. (A, B) Electron microscopy images of the contact between two Leydig cells. The insert shows the gap junctions in higher magnification. (C, D) Immunohistochemistry for Connexin-43 in the mouse testis, between fetal Leydig cells (FLC; embryonic day 18.5) (C) and adult Leydig cells (ALC; 70 postnatal day) (D). Observe that the staining is also present between the Sertoli cells in the seminiferous cord/tubule. LC, Leydig cell; SC, Sertoli cell; BV, Blood vessel; Arrows, Gap junctions.

a remarkable characteristic of testicular macrophages, as there are no other examples of specialized areas of contact between macrophages and other cell types outside of the immune system. Another interesting aspect is that aging has a negative effect on steroidogenesis and one of the reasons could be the loss of these digitations between macrophages and Leydig cells, interrupting their communication and the exchange of molecules, including 25-hydroxycholesterol. This type of cholesterol is transferred from macrophages to Leydig cells under normal conditions and enter the steroidogenic pathway (Fig. 6).

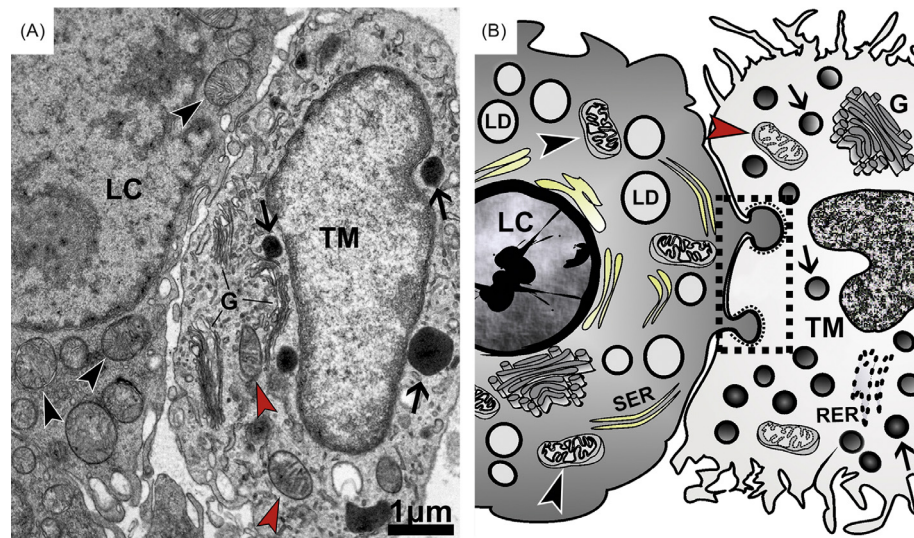


Fig. 6 Relationship between Leydig cells and testicular macrophages. (A) Electron microscopy image of the Leydig cell contacting a macrophage in the adult mouse testis. (B) Schematic representation of the micrograph in (A), including the digitations (dotted rectangle), the structural interaction that exists between Leydig cells and macrophages. LC, Leydig cell; TM, Testicular macrophage; G, Golgi complex; RER, Rough endoplasmic reticulum; SER, Smooth endoplasmic reticulum; LD, Lipid droplet; black arrowheads, Leydig cell mitochondria (tubular cristae); red arrowheads, macrophage mitochondria (lamellar cristae); arrows, lysosomes.

The macrophages are capable of influencing Leydig cell proliferation, differentiation, and testosterone secretion. It was shown that, after Leydig cell destruction by a cytotoxic drug (EDS; ethane 1,2-dimethane sulphonate), testicular macrophages were responsible for the phagocytosis of the apoptotic Leydig cells. There is a wave of proliferative activity when new Leydig cells repopulate the interstitium and a concomitant increase in the number of resident macrophages. In addition, the lack of macrophages during the proliferation wave disrupts Leydig cell recovery. A mouse model with reduced numbers of macrophages (osteopetrotic mouse; *op/op*) presents poor fertility, reduced testosterone levels and abnormal Leydig cells. Regarding testosterone production, the macrophages may have positive or negative effects, depending on the stimulus and the mediators produced. It has also been suggested that the testicular macrophages could metabolize Leydig cell secreted steroids, as peritoneal macrophages are able to convert androstenedione to testosterone and dihydrotestosterone (Hales, 2007).

It has also been suggested that Leydig cells could mediate the local regulation of the testicular leukocyte populations and thus provide an additional source of immunoregulatory polypeptides. Leydig cells have specific receptors and recognition molecules encoded on their surface and thus have the ability to interact with lymphocytes. As such, Leydig cell are the only highly differentiated endocrine cells that spontaneously form rosettes with lymphocytes, macrophages or eosinophils. This complex relationship is probably involved in the maintenance of the testis as an immunologically protected site, where immune cells appear to have restricted proinflammatory activity and immune responses are usually limited, possibly due to immunomodulatory factors secreted by Leydig cells. Androgens are also known to be immunosuppressive; therefore, it is also possible that immunological suppression is related to the high levels of intratesticular testosterone (Diemer et al., 2003).

Leydig-Endothelial Cells

Vascular and lymphatic endothelial cells are abundant in the well vascularized interstitial/intertubular compartment of the testis and their proximity to Leydig cells suggests their functional interaction. The diffusion of testosterone to blood circulation requires the communication between Leydig cells and the microvasculature, although the mechanism for this hormone transport is not completely known and may occur through passive diffusion. In this regard, cell contacts between Leydig and closely associated vascular endothelium have been described. A paracrine interdependence between Leydig and endothelial cells has been reported to possibly involve hormone transcytosis and vascular endothelial growth factors produced by the Leydig cells (Haider et al., 2007). Vascular endothelial cells produce the peptide endothelin, which was shown to stimulate testosterone production in the rat. It is also noted that the lymphatic endothelium associates with the peritubular wall and Leydig cell clusters. Endothelial cells of the testis are connected to each other by tight junctions and, in general, the capillaries are unfenestrated. These characteristics provide a supplemental mechanism of immunoprotection by controlling and protecting the testicular microenvironment, in addition to the SCB. However, further investigation is needed to clarify the cell interactions involved in this relationship (Martin, 2016).

References

- Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implications for male contraception. *Pharmacological Reviews*, 64, 16–64.
- Cheng, C. Y., & Mruk, D. D. (2015). Biochemistry of Sertoli cell/germ cell junctions, germ cell transport, and spermiogenesis in the seminiferous epithelium. In M. D. Griswold (Ed.), *Sertoli cell biology* (2nd edn., pp. 333–383). Oxford: Elsevier Academic Press.
- Diemer, T., Hales, D. B., & Weidner, W. (2003). Immune-endocrine interactions and Leydig cell function: The role of cytokines. *Andrologia*, 35, 55–63.
- França, L. R., Auharek, S. A., Hess, R. A., Dufour, J. M., & Hinton, B. T. (2012). Blood-tissue barriers: Morphofunctional and immunological aspects of the blood-testis and blood-epididymal barriers. In C. Y. Cheng (Ed.), *Biology and regulation of blood tissue barriers* (1st edn., pp. 237–259). New York: Landes Bioscience and Springer Science.
- França, L. R., Hess, R. A., Dufour, J. M., Hofmann, M. C., & Griswold, M. D. (2016). The Sertoli cell: One hundred fifty years of beauty and plasticity. *Andrology*, 4, 189–212.
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3, a005850.
- Haider, S. G., Servos, G., & Tran, N. (2007). Structural and histological analysis of Leydig cell steroidogenic function. In A. H. Payne, & M. P. Hardy (Eds.), *Leydig cell in health and disease* (1st edn., pp. 33–45). New Jersey: Humana Press.
- Hales, D. B. (2007). Regulation of Leydig cell function as it pertains to the inflammatory response. In A. H. Payne, & M. P. Hardy (Eds.), *Leydig cell in health and disease* (1st edn., pp. 305–321). New Jersey: Humana Press.
- Hutson, J. C. (2006). Physiologic interactions between macrophages and Leydig cells. *Experimental Biology and Medicine*, 231, 1–7.
- Korhonen, H. M., Yadav, R. P., Da Ros, M., Chalmel, F., Zimmermann, C., Toppari, J., Nef, S., & Kotaja, N. (2015). DICER regulates the formation and maintenance of cell–cell junctions in the mouse seminiferous epithelium. *Biology of Reproduction*, 93, 139.
- Martin, L. J. (2016). Cell interactions and genetic regulation that contribute to testicular Leydig cell development and differentiation. *Molecular Reproduction and Development*, 83, 470–487.
- Pointis, G., Gilleron, J., Carette, D., & Segretain, D. (2010). Physiological and physiopathological aspects of connexins and communicating gap junctions in spermatogenesis. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 365, 1607–1620.
- Skinner, M. K. (1993). Sertoli cell-peritubular myoid cell interactions. In M. D. Griswold, & L. D. Russell (Eds.), *The Sertoli cell* (1st edn., pp. 477–484). Clearwater: Cache River Press.
- Smith, B. E., & Braun, R. E. (2012). Germ cell migration across Sertoli cell tight junctions. *Science*, 338, 798–802.
- Verhoeven, G., Hoeben, E., & De Gendt, K. (2000). Peritubular cell–Sertoli cell interactions: Factors involved in PmodS activity. *Andrologia*, 64, 42–45.
- Vogl, A. W., Young, J. S., & Du, M. (2013). New insights into roles of tubulobulbar complexes in sperm release and turnover of blood-testis barrier. *International Review of Cell and Molecular Biology*, 303, 319–355.
- Wong, E. W. P., & Cheng, C. Y. (2009). Polarity proteins and cell–cell interactions in the testis. *International Review of Cell and Molecular Biology*, 278, 309–353.
- You, S., Li, W., & Lin, T. (2000). Expression and regulation of connexin43 in rat Leydig cells. *Journal of Endocrinology*, 166, 447–453.

Cell–Cell Interactions—Molecular

Antoine D Rolland and Bernard Jégou, Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et travail) - UMR_S 1085, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

The testicular homeostasis relies on external stimuli, such as endocrine cues from the pituitary gland, as well as on a complex and permanent communication network between the different cell types within the testis. The latter is indeed necessary for the different cells to properly differentiate during early life. It is also responsible for the maintenance of all the different aspects of testicular functions in adulthood, notably androgen secretion by Leydig cells and continuous, daily production of spermatozoa within seminiferous tubules. To do so, Leydig cells, peritubular myoid cells, Sertoli cells, and germ cells at various stages of differentiation converse altogether by using several devices: locally-produced hormones, secreted paracrine factors such as growth factors, cytokines/chemokines or various organic compounds, as well as direct physical cell–cell interactions. According to a pioneer of the electronic microscopy, the anatomical complexity of the seminiferous epithelium is entirely unique (Fwacett, 1975). Therefore, studying cell–cell communications within the testis has always been very challenging.

The fundamental importance of this intercellular crosstalk has, for a part, been clearly illustrated by difficulties of germ cell cultures, especially the most differentiated ones (i.e., meiotic spermatocytes and postmeiotic spermatids), for which isolation from their micro- and macro-environments followed by culture alone inexorably leads to cell death. This absolute germ cell requirement for support from somatic cells, at both the physical and nutritional levels, also sustains the long-standing view that full spermatogenesis could not be achieved *in vitro* (for review, see Galdon et al., 2016). Besides, not only the germ cells require signals from somatic cells: the interactions between somatic cells are very numerous, and of many kinds, while germ cells also influence somatic cell functions (for review, see Jégou, 1993).

In addition to morphological/anatomical studies, dissecting cellular communication pathways has classically involved approaches where interactions were first disrupted, prior to eventual reconstruction. These include *in vitro* experiments in which primary testicular cells are isolated prior to culture (i) together with a second cell type; (ii) with the conditioned medium from a second cell type; or (iii) alone with a defined medium containing a potential paracrine factor or combination of factors to be tested. *In vivo* experiments have also been used to investigate cell communication within the testis, for instance through chemical treatments to perturb endocrine function or, even more drastically, by using chemical or physical treatments (irradiation, temperature) to completely ablate a specific cell type (for reviews, see Jégou, 1993; Smith et al., 2015). More recently, the democratization of high-throughput expression technologies (i.e., transcriptomics and proteomics) has allowed identifying promising candidate factors (for review, see Calvel et al., 2010) while functional genomics, especially in the mouse, has enabled the functional assessment of specific signaling pathways.

Altogether, these different and often complementary approaches have uncovered some crucial communication pathways within the testis, though many remain to be identified or require further investigations to be fully understood. In this article, rather than providing an exhaustive review of the literature, several exemplary signaling pathways will be used to illustrate the many cell relationships as well as the diversity of communication devices at play within the adult testis.

Paracrine and Autocrine Actions of Intratesticular Testosterone

Endocrine regulation is arguably the mechanism that has been the most intensively studied from the beginning of our attempts to understand testis physiology, that is, from the beginning of the 20th century. While some aspects of these regulatory pathways are beyond the scope of this article, it is worth mentioning that deciphering the local actions of testosterone has long consisted in trying to discriminate those from FSH action. Indeed, while *in vivo* manipulation of androgen levels has provided us with many insights into this crucial signaling pathway, feedback loops ultimately impact gonadotropin release (i.e., LH and FSH), making it almost impossible to ascertain the respective roles of testosterone and FSH. Furthermore, testosterone can be aromatized into estradiol, the latter being able to signal within the testis where estrogen receptors are also expressed. However, male mice lacking estrogen-receptor beta (ESR2) are fertile (Krege et al., 1998) while infertility in mice lacking estrogen-receptor alpha (ESR1) is indirect and results from impaired reabsorption of the luminal fluids in efferent ducts (Hess et al., 1997). Finally, mostly the use of *in vitro* models (e.g., primary testicular cell cultures) and the generation of mutant mice for the androgen receptor (AR) have finally helped us to precisely address this question.

Respective Role of Testicular Cells Mediating Testosterone Action

Testosterone classically mediates its effects through binding to AR, a nuclear receptor that will then modify the expression of specific target genes. Within the testis, Leydig cells, peritubular cells, Sertoli cells as well as vascular endothelial and smooth muscle cells represent potential mediators of the effects of testosterone as they all express AR. The first mutant mouse models for AR have thus proved to be somehow poorly adapted to precisely investigate testosterone signaling in the testis as their drastic phenotypes reflected the cumulative effects of AR ablation in all these somatic cell types. Still, they all pointed out towards an important

regulatory role of testosterone on meiosis completion and/or spermiogenesis, with spermatogenesis arrested at the pachytene spermatocyte stage in many cases. Cell type-specific ablation of the AR on the other hand has allowed a more precise dissection of testosterone effects on each of these cell types (for review, see [Chang et al., 2013](#)).

First, while the expression of AR in germ cells has been a matter of controversy, the absence of testicular phenotype in mutant mice with germ cell-specific AR ablation (GC-ARKO) demonstrated that the direct action of testosterone on germ cells was not essential for spermatogenesis ([Tsai et al., 2006](#)) and did not account for the germ cell defects observed in all other models. Similarly, the specific ablation of AR in vascular endothelial (VE) cells (VE-ARKO mice; [O'Hara and Smith, 2012](#)) or in vascular smooth muscle (VSM) cells (VSM-ARKO; [Welsh et al., 2010](#)) proved androgen signaling in these cells to be dispensable for spermatogenesis, even though testis weight of VSM-ARKO mice was reduced as a result of impaired vasomotion and subsequent Leydig cell function alterations.

Unfortunately, conclusions on how and to which extent AR signaling in Leydig cells directly influences spermatogenesis remain difficult to make. The Leydig cell-specific AR ablation (LC-ARKO mice) results in male infertility, with atrophied testes, spermatogenesis arrested at the round spermatid stage and a complete absence of epididymal sperm ([Xu et al., 2007](#)). In this model, however, mutant mice also exhibit primary hypogonadism, with low testosterone levels and high LH and FSH levels, which are likely to subsequently alter Sertoli and peritubular cell functions. Furthermore, the specificity of the Amhr2-Cre used for this knockout is dubious, as AR was not eliminated in all Leydig cells and, on the contrary, was lost in some Sertoli cells.

A similar issue of Cre recombinase specificity/characterization has prevented conclusions from a first peritubular cell-specific AR knockout model (PMC-ARKO; [Zhang et al., 2006](#)). Hopefully, a second, more robust model was subsequently generated (PTM-ARKO mice; [Welsh et al., 2009, 2012](#)). PTM-ARKO mice appeared normal at birth but became infertile during adulthood with a reduction in all types of germ cells and no sperm production. The progressive loss of spermatogonia, abnormal basement membrane, and altered secretion by Sertoli cells observed in these mice suggest that AR signaling in peritubular cells is essential for appropriate development and/or maintenance of the spermatogonial stem cell niche. Leydig cell functions were also found altered: testosterone circulating levels were normal but intratesticular testosterone and LH levels were increased, indicative of compensatory Leydig cell failure.

Finally, the investigation of Sertoli cell-specific AR knock-out mice (SC-ARKO) has provided important and consistent data ([Chang et al., 2004](#); [De Gendt et al., 2004](#); [Holdcraft and Braun, 2004](#)). Testes from mutants indeed display drastic weight reduction with spermatocytes blocked at the diplotene stage. While altered levels of testosterone and LH have sometimes been described, all models point toward defective maturation of Sertoli cells, disruption of the blood–testis barrier (BTB), compromised adhesion of round spermatids and spermiation failure as the primary causes for the observed germ cell defects. It can thus be concluded that AR signaling in Sertoli cells is essential for regulating seminiferous tubule remodeling in order to support meiosis completion and spermatid differentiation.

Identification of Androgen-Receptor Target Genes and Downstream Effective Pathways

While the AR signaling in Sertoli cells was found to be essential for spermatogenesis, the underlying mechanisms remain poorly understood. Many transcriptomic studies have been conducted in various models of testosterone depletion/restoration or in ARKO mice to identify testosterone-regulated genes in the testis (for review, see [Verhoeven et al., 2010](#)). The information provided by these experiments appeared somehow limited as no obvious genes important for spermatogenesis could be highlighted. Still, all these studies pointed out genes involved in cell–cell junction and adhesion, demonstrating further that testosterone mediates its effects by regulating the structure of seminiferous tubules and the BTB integrity.

In addition to the AR-mediated nuclear mechanism, two nonclassical pathways may also underlie the action of testosterone in the testis (for review, see [Rahman and Christian, 2007](#)). First, the binding of testosterone on a subset of AR induces their localization near the plasma membrane where they bind and activate Src tyrosine kinase. Src then phosphorylates EGFR, which triggers activation of the MAP kinase cascade, ultimately leading to the activation of the CREB transcription factor. In vitro experiments further demonstrated that the AR-dependent activation of Src and ERK signaling pathways regulates adhesion of germ cells to Sertoli cells, sperm release, as well as BTB dynamics. Second, through activation of an unidentified G-protein coupled receptor, testosterone induces Ca^{2+} influx in Sertoli cells, which is likely to alter many cellular processes. However, this pathway being AR-independent, it does account for the phenotypes observed in the different ARKO models.

Extrinsic Factors Regulating Spermatogonial Stem Cells

Our understanding of spermatogonial stem cell (SSC) biology has long been hampered because of (i) their low abundance within a highly complex tissue, (ii) the virtual absence of specific markers, and (iii) the lack of appropriate functional assay to assess and unequivocally identify these cells. The development of the SSC transplantation techniques in the mid-1990s ([Brinster and Avarbock, 1994](#); [Brinster and Zimmermann, 1994](#)) finally allowed to evaluate the stem cell activity of enriched cell populations and to correlate this activity with the expression of potential markers for subpopulations of undifferentiated spermatogonia. Together with the setting up of appropriate cell culture conditions, this powerful tool has allowed rapid improvement of our knowledge regarding the functional characteristics of SSC and how their microenvironment controls the balance between self-renewal and differentiation.

The GDNF Signaling Pathway

Our understanding of SSC biology has greatly progressed thanks to the identification of glial cell-line neurotrophic factor (GDNF), a distant member of the TGF β family, as a crucial regulator of SSC maintenance. *Gdnf*-null mice die shortly after birth, thus preventing from the evaluation the role of this factor during spermatogenesis. Heterozygous mice for *Gdnf*, on the other hand, are viable and fertile. They however display age-related spermatogenesis defects, with tubules often containing only Sertoli cells as a result of progressive SSC pool depletion. Consistently, the overexpression of *Gdnf* resulted in the exact opposite phenotype, that is, the accumulation of undifferentiated spermatogonia, clearly demonstrating the important role of GDNF in regulating SSC maintenance (Meng et al., 2000) (Fig. 1).

Since then, SSC cultures typically involve addition of GDNF in the medium, allowing to expand these cells for weeks/months while maintaining their stem cell activity. Such cultures have also been used to decipher the intracellular networks that mediate GDNF action in SSC: GDNF first binds to GFR α 1, a GPI-anchored transmembrane molecule, which in turn activates the RET tyrosine kinase, both proteins being present at the membrane of undifferentiated spermatogonia. Upon autophosphorylation, RET next activates several kinases of the Src family (e.g., Src, Yes, Lin, and Fin), the PI3/Akt signaling pathway as well as the Ras/ERK1/2 pathway. The activation of these intracellular pathways together with the subsequent transcription of downstream gene

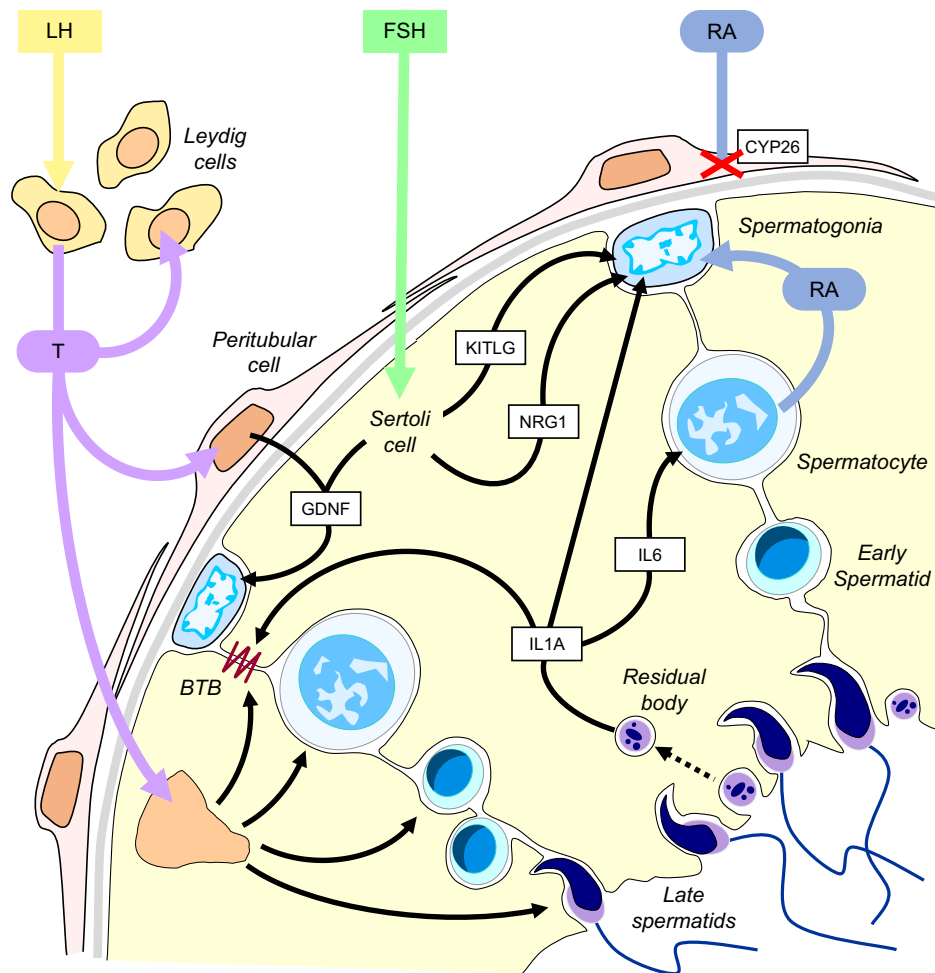


Fig. 1 Molecular cell-cell interactions within the testis. A nonexhaustive view of molecular factors involved in cell-cell communication between testicular cell types is presented. Leydig cell stimulation by LH triggers testosterone (T) secretion which acts in an autocrine manner on Leydig cells or in a paracrine manner on Sertoli cells and peritubular cells. T signaling in Sertoli cells regulates the blood-testis barrier (BTB), meiosis completion by spermatocytes, spermatid differentiation as well as germ cells adhesion and spermiogenesis. In peritubular cells, T notably upregulates GDNF that controls spermatogonial stem cell maintenance. FSH on the other hand only signals through Sertoli cells leading to upregulation of GDNF, KITLG, and NRG1. Both KITLG and NRG1 subsequently promotes differentiation of spermatogonia. The entry of retinoic acid (RA) within the seminiferous tubules is prevented by CYP26A1-C1 activity in peritubular cells. Instead, RA is synthesized by spermatocytes and acts on spermatogonia by promoting their differentiation as well as by triggering their commitment to meiosis. The phagocytosis of residual bodies (i.e., remaining cytoplasm from elongated spermatids as they detach from Sertoli cells) by Sertoli cells induces IL1A secretion in these cells, and IL1A subsequently induces IL6 in an autocrine manner. IL1A finally stimulates spermatogonial cell proliferation while IL6 inhibits meiotic DNA synthesis. Protein factors are indicated within a rectangle while organic compounds (i.e., RA and T) are within an oval.

targets modify cell cycle progression of SSC. Importantly, an early microarray experiment aiming at identifying GDNF-regulated genes compared isolated SSC cultured with or without this factor (Oatley et al., 2006) and pointed out several transcription factors (Bcl6b, Etv5, Id4, Lhx1, Pou3f1, and T) which were all later on demonstrated to be important for SSC fate.

GDNF is expressed in Sertoli cells and peritubular cells. However, the paracrine regulation of SSC by GDNF is most of times attributed to Sertoli cells only. Since SSC lie on the basement membrane of seminiferous tubules, it can easily be imagined that GDNF from nearby peritubular cells may influence SSC as well. More, the specific ablation of *Gdnf* in peritubular cells actually results in complete absence of germ cells in the adulthood following progressive loss of SSC after the first spermatogenetic cycle. While it is tempting to speculate that GDNF from Sertoli cells is also important for SSC maintenance, a Sertoli cell-specific knockout for GDNF will be required to draw such conclusion. Besides, the potential interaction between Sertoli cells and peritubular cells through this signaling pathway will require further investigations since GDNF was also shown to increase Sertoli cell proliferation. Interestingly, *Gdnf* is upregulated by testosterone and FSH in peritubular cells and Sertoli cells, respectively (Chen et al., 2014; Tadokoro et al., 2002) (Fig. 1). Furthermore, activation of NOTCH in Sertoli cells by JAG1 from spermatogonial cells leads to *Gdnf* downregulation (Garcia et al., 2017). A complex interplay of endocrine and paracrine factors thus controls GDNF levels in order to regulate the delicate balance between self-renewal and differentiation of SSC.

Other Factors Controlling SSC Self-Renewal

The maintenance of SSC allowed by addition of GDNF in culture media has also enabled to demonstrate in vitro the roles for several other testicular factors in self-renewal and/or survival of SSC, most of which being dependent on, or acting downstream of GDNF itself. These include IGF1 (Huang et al., 2009) produced by Leydig cells, LIF (Dorval-Coiffec et al., 2005; Guan et al., 2006) produced by peritubular cells, FGF2 (Takashima et al., 2015), CXCL12 (Yang et al., 2013), CCL9 (Simon et al., 2010), and WNT5A (Yeh et al., 2011) produced by Sertoli cells, CSF1 (Oatley et al., 2009) produced by Leydig cells, macrophages, perivascular cells and/or peritubular cells, as well as a few factors that are produced by differentiated germ cells, such as VEGFA₁₆₄ (Caires et al., 2012), BMP8A (Zhao et al., 1998), and BMP8B (Zhao et al., 1996).

Factors Involved in Spermatogonial Differentiation

It is only over recent years that our knowledge on SSC self-renewal has rapidly progressed. On the other hand, one of the first identified factor regulating spermatogonial fate—Kit ligand (KITLG, also called KITL, steel factor or stem cell factor)—triggers their differentiation (for review, see Zhang et al., 2011b). In the adult testis, KITLG is expressed and secreted by Sertoli cells. Upon binding to KITLG, the KIT proto-oncogene receptor tyrosine kinase (KIT, C-Kit or CSFR) transiently activates PI3K/AKT and ERK1/2 signaling pathways, ultimately promoting G1/S transition and DNA synthesis in A1–A4 differentiating spermatogonia (Fig. 1). Indeed, KIT is present at the surface of differentiated spermatogonia, but not of SSC. The drastic reproductive phenotypes of several mutant mice at the *white spotting* (W) and *steel* (Sl) loci, which contain KIT and KITLG, respectively, clearly demonstrate the indispensable role of this signaling pathway. For instance, a single point mutation in the PI3K-binding site of KIT induces male fertility with complete arrest of spermatogenesis at the spermatogonial stage (Blume-Jensen et al., 2000). Additionally, a truncated form of KIT, tr-KIT, is specifically expressed in spermatids, suggesting that KIT signaling may also be involved during spermiogenesis and/or fertilization. Importantly, FSH, but not testosterone, upregulates KITLG in Sertoli cells, providing a first mean to control this pathway (Fig. 1). Fine-tuning regulation of KIT expression in spermatogonial cells is also necessary to ensure appropriate balance between self-renewal and differentiation (for review, see Rossi, 2013). Transcription factors SOHLH1/2 have been shown to directly activate the transcription of KIT in spermatogonia, while the transcriptional repressor PLZF switches it off as meiosis is initiated. Most importantly, retinoic acid (RA) was found to render spermatogonia responsive to KITLG by upregulating KIT at both the transcriptional and translational levels (Busada et al., 2015; Raverdeau et al., 2012). RA therefore appears a critical signaling pathway upstream of KITLG/KIT (see below, section **Extrinsic Factors Regulating Spermatogonial Stem Cells**).

Additional paracrine factors involved in spermatogonial differentiation have also recently been identified. The Sertoli cell-specific ablation of NRG1 demonstrated this member of the epidermal growth factor (EGF) to be crucial for type A spermatogonia differentiation as well as for transition from type B spermatogonia to preleptotene primary spermatocytes. Further in vitro experiments evidenced that, like KITLG, NRG1 is a downstream effector of RA-mediated spermatogonial differentiation and is upregulated by FSH in Sertoli cells. NRG1 however signals through the EGF receptor tyrosine kinase ERBB2 in spermatogonia, independently of the KITLG/KIT signaling pathway (Chapman et al., 2015; Zhang et al., 2011a). Additionally, BMP4 produced by germ cells at various developmental stages, was demonstrated to enhance spermatogonial differentiation in vitro by upregulating KIT in a RA-dependent manner (Pellegrini et al., 2003). Similarly, INHBA from Sertoli cells and germ cells was found to promote SSC differentiation in vitro (Nagano et al., 2003). Finally, while VEGFA₁₆₄ was proved to promote SSC self-renewal (see above), VEGFA_{165b} on the contrary induces their differentiation (Caires et al., 2012).

Retinoic Acid Pathway and Male Germ Cell Differentiation

One of the most important breakthrough in the field of reproductive biology has been the identification of retinoic acid (RA) as the substance triggering the germ cells to undergo meiosis. Strikingly, while it was known for almost a century that vitamin A, the precursor for the synthesis of RA, was essential for spermatogenesis (Wolbach and Howe, 1925), the fact that RA actually is

the active metabolite of this pathway was only recently discovered thanks to studies on fetal gonads: fetal male germ cells were indeed found to enter meiosis (which they normally do only after birth, from puberty onwards) in mice lacking the retinoid-degrading enzyme CYP26B1 (Bowles et al., 2006). In this mouse fetal gonad model, RA thus constitutes the ovarian “meiosis-inducing substance” and CYP26B1, which is specifically expressed in the fetal testis, corresponds to the “meiosis-preventing factor” (Byskov and Saxén, 1976). Subsequently ALDH1A1 was identified as the enzyme providing a source of RA in the mouse fetal ovary (Bowles et al., 2016).

The situation in the juvenile and adult testes, however, appears somewhat more complicated: RA does not only seem to drive meiosis onset through the upregulation of *Stra8*. More, its primary function appears to regulate spermatogonial differentiation by turning on KIT signaling (Fig. 1). Either vitamin-A-deficient models or animals lacking vitamin A metabolizing or signaling proteins have indeed demonstrated that removing RA prevents male germ cells from developing further than undifferentiating spermatogonia. Additionally, the RA signaling pathway in the adult testis requires an incredibly fine-tuned regulation at both the spatial and temporal levels so germ cells within the testis do not differentiate synchronously, and rather go through continuous spermatogenesis and sperm production. The initiation of spermatogonial differentiation, that is, the A to A1 transition, occurs precisely at stages VII–VIII of the mouse seminiferous epithelium cycle while the transition from type B spermatogonia to leptotene spermatocyte initiates slightly earlier, at stage VI. This timing and coordination of germ cell differentiation was recently proposed to result from oscillations or pulses of RA along the seminiferous epithelium, with peak levels at stage VII–VIII (Hogarth et al., 2013). More, this RA gradient, by promoting spermatogonial differentiation in a reoccurring manner, has been proposed to both initiate and maintain the cycle of the seminiferous epithelium (for review, see Griswold, 2016).

The fine-tuning of RA levels within the testis is achieved thanks to the cellular compartmentalization of enzymes and proteins involved in RA synthesis, degradation, uptake, and storage as well as by developmentally-regulated expression of these factors (Sugimoto et al., 2012; Vernet et al., 2006). Peritubular cells express all three RA-degrading enzymes (CYP26A1, B1, and C1), thus constituting a catabolic barrier that prevents RA, either circulating or synthesized by Leydig cells, to enter seminiferous tubules. The source of RA within the tubules therefore relies on the expression of ALDH1A1 and ALDH1A2, two of the three RA synthesizing enzymes, by Sertoli cells and spermatocytes, respectively (Fig. 1). The dogma for retinoid signaling in the testis was therefore that retinol was uptaken by Sertoli cells and transformed into RA, which would finally act on undifferentiated spermatogonia through RA receptors to trigger differentiation and subsequent commitment to meiosis. This simplistic view has however been challenged recently both in terms of RA origin and receptivity. First, testes from neonatal mutant mice with Sertoli cell-specific ablation of the three RA synthesizing enzymes (*Aldh1a1-3^{ser}-/-*) only contain spermatogonia blocked in an undifferentiated state, demonstrating that RA from Sertoli cells was indeed indispensable for spermatogonial differentiation during the first wave of spermatogenesis. Further culture experiments of these mutant testes however demonstrated that RA was used in an autocrine manner to activate RAR α in Sertoli cells. This suggests that the RA-induced spermatogonial differentiation in the juvenile testis is indirect and that a yet unidentified paracrine signal from Sertoli cells is involved in this process (Raverdeau et al., 2012). Second, when these same *Aldh1a1-3^{ser}-/-* mice were injected with a single RA dose, not only spermatogonia could differentiate up to preleptotene spermatocytes, but full spermatogenesis could even be observed several weeks after the injection. These results implies that the RA required for the A to A1 transitions after the first round of spermatogenesis is not provided by Sertoli cells, but rather by leptotene/pachytene spermatocytes thanks to ALDH1A2 activity. Furthermore, mutant mice lacking RAR γ in spermatogonia display normal progression of the first spermatogenetic cycle but exhibit an age-related progressive spermatogenetic decline as spermatogonia do not differentiate anymore (Gely-Pernot et al., 2012). This model therefore demonstrated that, unlike in the juvenile testis where RA-induced A to A1 transition involves paracrine signaling from Sertoli cells, the spermatogonial differentiation beyond the first wave of spermatogenesis involves the direct RA-signaling activation through RAR γ in undifferentiated spermatogonia (Fig. 1).

Influence of Germ Cells Upon Somatic Cells

Germ cell differentiation during spermatogenesis relies on both intrinsic factors specific to the germline and on extrinsic signals from surrounding somatic cells. While germ cells appears dispensable for testis differentiation during fetal life, germ cell products, such as the above-mentioned RA or BMP4, are likely to influence somatic cells in order to induce and/or maintain somatic cell functions after birth. Due to their intimate location within the seminiferous epithelium, Sertoli cells which surround and support germ cells all along their differentiation process represent a preferential target for such signals.

In agreement with this hypothesis, the appearance of new germ cell types during the first spermatogenetic wave coincides changes in Sertoli cell biology: they stop proliferating, establish cell–cell junctions in order to create the BTB and start secreting the seminiferous fluid (evidenced by the appearance of a lumen) while acquiring a mature molecular phenotype (e.g., loss of AMH and KRT18 the gain of AR and GATA1) (for review see, Sharpe et al., 2003). Conversely, the disappearance of specific germ cell types, either induced experimentally in animals or under pathological conditions in humans, results in abnormal Sertoli cell activity, suggesting again the potential involvement of differentiated germ cells in maintaining adult Sertoli cell functions. To address such mechanisms, a number of in vitro experiments were conducted where immature Sertoli cells were cultured together with enriched germ cell preparations or their-conditioned media. These experiments notably demonstrated that germ cell and/or their secreted products indeed influence the secretion of Sertoli cells (e.g., transferrin, ABP, inhibin B, and estradiol) and promote the formation of the BTB (Clifton et al., 2002; Le Magueresse and Jégou, 1986; Le Magueresse et al., 1988). Co-culture experiments and germ cell depleted models were also used to demonstrate gene expression regulation by germ cells, providing one of the means

by which cyclical gene expression in the Sertoli could be established and/or maintained (O'Shaughnessy et al., 2008; Syed et al., 1999).

In this context, one of the very few pathways investigated into details is the specific regulation of the interleukin 1 alpha (IL1A) by late spermatids. Residual bodies released from spermatozoa as they detach from the seminiferous epithelium are rapidly phagocytosed by Sertoli cells, which triggers the production and secretion of IL1A. Subsequently, IL1A induces the secretion of IL6 by Sertoli cells in an autocrine, lipoxygenase-dependant mechanism (Syed et al., 1995). Importantly, IL1A and IL6 were found in vitro to stimulate spermatogonial cell proliferation (Pöllänen et al., 1989) and to inhibit meiotic DNA synthesis (Hakovirta et al., 1995), respectively. Furthermore, IL1A was also shown to regulate the BTB remodeling (Lie et al., 2011). Taken together, these data suggest that residual bodies and their phagocytosis by Sertoli cells could constitute an important signal controlling both germ cell differentiation and the dynamics of the seminiferous epithelium (Jégou, 1991).

The regulation of somatic cells by germ cells has caught relatively little attention as compared to other cell–cell interactions within the testis. The extent to which germ cells indeed account in the overall regulation of testis homeostasis therefore remains relatively unclear. More, the molecular factors that mediate these effects, either secreted by or present at the membrane of germ cells, are also largely unknown.

Conclusions

As illustrated throughout this article, the extreme morphological entanglement of testicular cells is reflected by a systematic communication network between Sertoli cells and germ cells, somatic cells within each of the two main anatomical compartments of the testis, as well as between these compartments. The communication between testicular cells is absolutely crucial to ensure normal reproductive functions in the male. Describing in a systematic way our current knowledge of these complex and many cellular interactions would definitely exceed the length constraint of the present article. Therefore, many molecular pathways at play within the testis could not be detailed here. These include notably the prostaglandin pathway, the endocannabinoid system or the signaling by bile acids, the three of them emerging as important regulators of male fertility (for reviews, see du Plessis et al., 2015; Frungieri et al., 2015; Sèdes et al., 2017).

Some fundamental aspects of testis physiology that are highly relevant in terms of cell–cell interactions were also voluntarily omitted as they are reviewed elsewhere within this encyclopaedia. For instance, the BTB constitutes a crucial immunological barrier which continuous remodeling, to allow the transport of preleptotene spermatocytes, results from the combination of direct cell–cell interactions as well as of paracrine and endocrine regulations. Additionally, the immune privilege provided by the testicular environment, to ensure protection of differentiated germ cells from the host immune response, also relies on locally-produced factors, including androgens, cytokines and bioactive lipids.

While many paracrine pathways that regulate testis physiology are now being well-characterized, much work is still needed in order to clarify how they may account for the etiology of many cases of unsolved male infertility. Noteworthy is that a better understanding of these delicate regulatory networks is also likely to help identifying relevant targets for male contraceptives.

References

- Blume-Jensen, P., Jiang, G., Hyman, R., Lee, K.-F., O'Gorman, S., & Hunter, T. (2000). Kit/stem cell factor receptor-induced activation of phosphatidylinositol 3'-kinase is essential for male fertility. *Nature Genetics*, 24(2), 157–162. <https://doi.org/10.1038/72814>.
- Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., & Koopman, P. (2006). Retinoid signaling determines germ cell fate in mice. *Science*, 312(5773), 596–600. <https://doi.org/10.1126/science.1125691>.
- Bowles, J., Feng, C.-W., Miles, K., Ineson, J., Spiller, C., & Koopman, P. (2016). ALDH1A1 provides a source of meiosis-inducing retinoic acid in mouse fetal ovaries. *Nature Communications*, 7, 10845. <https://doi.org/10.1038/ncomms10845>.
- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91(24), 11303–7. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7972054>
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91(24), 11298–11302. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7972053>.
- Busada, J. T., Chappell, V. A., Nidenberger, B. A., Kaye, E. P., Keiper, B. D., Hogarth, C. A., & Geyer, C. B. (2015). Retinoic acid regulates kit translation during spermatogonial differentiation in the mouse. *Developmental Biology*, 397(1), 140–149. <https://doi.org/10.1016/j.ydbio.2014.10.020>.
- Bykov, A. G., & Saxén, L. (1976). Induction of meiosis in fetal mouse testis in vitro. *Developmental Biology*, 52(2), 193–200. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12194432>.
- Caires, K. C., de Avila, J. M., Cupp, A. S., & McLean, D. J. (2012). VEGFA family isoforms regulate spermatogonial stem cell homeostasis in vivo. *Endocrinology*, 153(2), 887–900. <https://doi.org/10.1210/en.2011-1323>.
- Calvel, P., Rolland, A. D., Jegou, B., & Pineau, C. (2010). Testicular postgenomics: Targeting the regulation of spermatogenesis. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 365(1546), 1481–1500. <https://doi.org/10.1098/rstb.2009.0294>.
- Chang, C., Chen, Y.-T., Yeh, S.-D., Xu, Q., Wang, R.-S., Guillou, F., & Yeh, S. (2004). Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. *Proceedings of the National Academy of Sciences*, 101(18), 6876–6881. <https://doi.org/10.1073/pnas.0307306101>.
- Chang, C., Lee, S. O., Wang, R.-S., Yeh, S., & Chang, T.-M. (2013). Androgen receptor (AR) physiological roles in male and female reproductive systems: Lessons learned from AR-knockout mice lacking AR in selective Cells1. *Biology of Reproduction*, 89(1), 21. <https://doi.org/10.1095/biolreprod.113.109132>.
- Chapman, K., Medrano, G., Chaudhary, J., & Hamra, F. (2015). NRG1 and KITL signal downstream of retinoic acid in the germline to support soma-free syncytial growth of differentiating spermatogonia. *Cell Death Discovery*, 1, 15018. <https://doi.org/10.1038/cddiscovery.2015.18>.
- Chen, L.-Y., Brown, P. R., Willis, W. B., & Eddy, E. M. (2014). Peritubular Myoid cells participate in male mouse Spermatogonial stem cell maintenance. *Endocrinology*, 155(12), 4964–4974. <https://doi.org/10.1210/en.2014-1406>.
- Clifton, R. J., O'Donnell, L., & Robertson, D. M. (2002). Pachytene spermatocytes in co-culture inhibit rat Sertoli cell synthesis of inhibin beta B-subunit and inhibin B but not the inhibin alpha-subunit. *The Journal of Endocrinology*, 172(3), 565–574. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11874705>.

- De Gendt, K., Swinnen, J. V., Saunders, P. T. K., Schoonjans, L., Dewerchin, M., Devos, A., & Verhoeven, G. (2004). A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. *Proceedings of the National Academy of Sciences of the United States of America*, 101(5), 1327–1332. <https://doi.org/10.1073/pnas.0308114100>.
- Dorval-Coiffec, I., Delcroix, J.-G., Hakovirta, H., Toppari, J., Jégou, B., & Piquet-Pellorce, C. (2005). Identification of the leukemia inhibitory factor cell targets within the rat testis. *Biology of Reproduction*, 72(3), 602–611. <https://doi.org/10.1095/biolreprod.104.034892>.
- Frungieri, M. B., Calandra, R. S., Mayerhofer, A., & Matzkin, M. E. (2015). Cyclooxygenase and prostaglandins in somatic cell populations of the testis. *Reproduction*, 149(4), R169–R180. <https://doi.org/10.1530/REP-14-0392>.
- Fwacett, D. W. (1975). Ultrastructure and function of the Sertoli cell. In D. W. Hamilton, & R. O. Greep (Eds.), *Endocrinology: vol. 5. Handbook of physiology. Male reproductive system*. Baltimore: Waverley Press. Section 7.
- Galdon, G., Atala, A., & Sadri-Ardekani, H. (2016). In vitro spermatogenesis: How far from clinical application? *Current Urology Reports*, 17(7), 49. <https://doi.org/10.1007/s11934-016-0605-3>.
- Garcia, T. X., Parekh, P., Gandhi, P., Sinha, K., & Hofmann, M.-C. (2017). The NOTCH ligand JAG1 regulates GDNF expression in Sertoli cells. *Stem Cells and Development*, 26(8), 585–598. <https://doi.org/10.1089/scd.2016.0318>.
- Gely-Pernot, A., Raverdeau, M., Célébi, C., Dennefeld, C., Feret, B., Klopstein, M., & Mark, M. (2012). Spermatogonia differentiation requires retinoic acid receptor γ . *Endocrinology*, 153(1), 438–449. <https://doi.org/10.1210/en.2011-1102>.
- Griswold, M. D. (2016). Spermatogenesis: The commitment to meiosis. *Physiological Reviews*, 96(1), 1–17. <https://doi.org/10.1152/physrev.00013.2015>.
- Guan, K., Nayernia, K., Maier, L. S., Wagner, S., Dressel, R., Lee, J. H., & Hasenfuss, G. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature*, 440(7088), 1199–1203. <https://doi.org/10.1038/nature04697>.
- Hakovirta, H., Syed, V., Jégou, B., & Parvinen, M. (1995). Function of interleukin-6 as an inhibitor of meiotic DNA synthesis in the rat seminiferous epithelium. *Molecular and Cellular Endocrinology*, 108(1–2), 193–198. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7758835>.
- Hess, R. A., Bunick, D., Lee, K.-H., Bahr, J., Taylor, J. A., Korach, K. S., & Lubahn, D. B. (1997). A role for oestrogens in the male reproductive system. *Nature*, 390(6659), 509–512. <https://doi.org/10.1038/37352>.
- Hogarth, C. A., Evanoff, R., Mitchell, D., Kent, T., Small, C., Amory, J. K., & Griswold, M. D. (2013). Turning a spermatogenic wave into a tsunami: Synchronizing murine spermatogenesis using WIN 18,446. *Biology of Reproduction*, 88(2), 40. <https://doi.org/10.1095/biolreprod.112.105346>.
- Holdcraft, R. W., & Braun, R. E. (2004). Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. *Development*, 131(2), 459–467. <https://doi.org/10.1242/dev.00957>.
- Huang, Y.-H., Chin, C.-C., Ho, H.-N., Chou, C.-K., Shen, C.-N., Kuo, H.-C., & Ling, T.-Y. (2009). Pluripotency of mouse spermatogonial stem cells maintained by IGF-1- dependent pathway. *The FASEB Journal*, 23(7), 2076–2087. <https://doi.org/10.1096/fj.08-121939>.
- Jégou, B. (1991). Spermatids are regulators of Sertoli cell function. *Annals of the New York Academy of Sciences*, 637, 340–353. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/1785779>.
- Jégou, B. (1993). The Sertoli-germ cell communication network in mammals. *International Review of Cytology*, 147, 25–96. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8225836>.
- Krege, J. H., Hodgin, J. B., Couse, J. F., Enmark, E., Warner, M., Mahler, J. F., & Smithies, O. (1998). Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proceedings of the National Academy of Sciences of the United States of America*, 95(26), 15677–15682. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9861029>.
- Le Mageres, B., & Jégou, B. (1986). Possible involvement of germ cells in the regulation of oestradiol-17 beta and ABP secretion by immature rat Sertoli cells (in vitro studies). *Biochemical and Biophysical Research Communications*, 141(2), 861–869. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/3026395>.
- Le Mageres, B., Pineau, C., Guillou, F., & Jégou, B. (1988). Influence of germ cells upon transferrin secretion by rat Sertoli cells in vitro. *The Journal of Endocrinology*, 118(3), R13–R6. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/3183569>.
- Lie, P. P.-Y., Cheng, C. Y., & Mruk, D. D. (2011). Interleukin-1 is a regulator of the blood–testis barrier. *The FASEB Journal*, 25(4), 1244–1253. <https://doi.org/10.1096/fj.10-169995>.
- Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., & Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287(5457), 1489–1493. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/10688798>.
- Nagano, M., Ryu, B.-Y., Brinster, C. J., Avarbock, M. R., & Brinster, R. L. (2003). Maintenance of mouse male germ line stem cells in Vitro1. *Biology of Reproduction*, 68(6), 2207–2214. <https://doi.org/10.1095/biolreprod.102.014050>.
- O'Hara, L., & Smith, L. B. (2012). Androgen receptor signalling in vascular endothelial cells is dispensable for spermatogenesis and male fertility. *BMC Research Notes*, 5(1), 16. <https://doi.org/10.1186/1756-0500-5-16>.
- O'Shaughnessy, P., Hu, L., & Baker, P. J. (2008). Effect of germ cell depletion on levels of specific mRNA transcripts in mouse Sertoli cells and Leydig cells. *Reproduction*, 135(6), 839–850. <https://doi.org/10.1530/REP-08-0012>.
- Oatley, J. M., Avarbock, M. R., Telaranta, A. I., Fearon, D. T., & Brinster, R. L. (2006). Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proceedings of the National Academy of Sciences*, 103(25), 9524–9529. <https://doi.org/10.1073/pnas.060332103>.
- Oatley, J. M., Oatley, M. J., Avarbock, M. R., Tobias, J. W., & Brinster, R. L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development*, 136(7), 1191–1199. <https://doi.org/10.1242/dev.032243>.
- Pellegrini, M., Grimaldi, P., Rossi, P., Geremia, R., & Dolci, S. (2003). Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: A potential role of BMP4 in spermatogonia differentiation. *Journal of Cell Science*, 116(16), 3363–3372. <https://doi.org/10.1242/jcs.00650>.
- du Plessis, S. S., Agarwal, A., & Syriac, A. (2015). Marijuana, phytocannabinoids, the endocannabinoid system, and male fertility. *Journal of Assisted Reproduction and Genetics*, 32(11), 1575–1588. <https://doi.org/10.1007/s10815-015-0553-8>.
- Pöllänen, P., Söder, O., & Parvinen, M. (1989). Interleukin-1 alpha stimulation of spermatogonial proliferation in vivo. *Reproduction, Fertility, and Development*, 1(1), 85–87. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/2496437>.
- Rahman, F., & Christian, H. C. (2007). Non-classical actions of testosterone: An update. *Trends in Endocrinology and Metabolism*, 18(10), 371–378. <https://doi.org/10.1016/j.tem.2007.09.004>.
- Raverdeau, M., Gely-Pernot, A., Feret, B., Dennefeld, C., Benoit, G., Davidson, I., & Ghyselinck, N. B. (2012). Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *Proceedings of the National Academy of Sciences*, 109(41), 16582–16587. <https://doi.org/10.1073/pnas.1214936109>.
- Rossi, P. (2013). Transcriptional control of KIT gene expression during germ cell development. *The International Journal of Developmental Biology*, 57(2–4), 179–184. <https://doi.org/10.1387/jdb.130014pr>.
- Sèdes, L., Martinot, E., Baptissart, M., Baron, S., Caira, F., Beaudoin, C., & Volle, D. H. (2017). Bile acids and male fertility: From mouse to human? *Molecular Aspects of Medicine*, 56, 101–109. <https://doi.org/10.1016/j.mam.2017.05.004>.
- Sharpe, R. M., McKinnell, C., Kvinn, C., & Fisher, J. S. (2003). Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. *Reproduction*, 125(6), 769–784. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/12773099>.
- Simon, L., Ekman, G. C., Garcia, T., Carnes, K., Zhang, Z., Murphy, T., & Hofmann, M.-C. (2010). ETV5 regulates Sertoli cell chemokines involved in mouse stem/progenitor Spermatogonia maintenance. *Stem Cells*, 28(10), 1882–1892. <https://doi.org/10.1002/stem.508>.
- Smith, L. B., O'Shaughnessy, P. J., & Rebourcet, D. (2015). Cell-specific ablation in the testis: What have we learned? *Andrology*, 3(6), 1035–1049. <https://doi.org/10.1111/andr.12107>.

- Sugimoto, R., Nabeshima, Y., & Yoshida, S. (2012). Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mechanisms of Development*, 128(11–12), 610–624. <https://doi.org/10.1016/j.mod.2011.12.003>.
- Syed, V., Stéphan, J. P., Gérard, N., Legrand, A., Parvinen, M., Bardin, C. W., & Jégou, B. (1995). Residual bodies activate Sertoli cell interleukin-1 alpha (IL-1 alpha) release, which triggers IL-6 production by an autocrine mechanism, through the lipoxigenase pathway. *Endocrinology*, 136(7), 3070–3078. <https://doi.org/10.1210/endo.136.7.7789334>.
- Syed, V., Gomez, E., & Hecht, N. B. (1999). Messenger ribonucleic acids encoding a serotonin receptor and a novel gene are induced in Sertoli cells by a secreted factor(s) from male rat meiotic germ cells. *Endocrinology*, 140(12), 5754–5760. <https://doi.org/10.1210/endo.140.12.7194>.
- Tadokoro, Y., Yomogida, K., Ohta, H., Tohda, A., & Nishimune, Y. (2002). Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mechanisms of Development*, 113(1), 29–39. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11900972>.
- Takashima, S., Kanatsu-Shinohara, M., Tanaka, T., Morimoto, H., Inoue, K., Ogonuki, N., & Shinohara, T. (2015). Functional differences between GDNF-dependent and FGF2-dependent mouse Spermatogonial stem cell self-renewal. *Stem Cell Reports*, 4(3), 489–502. <https://doi.org/10.1016/j.stemcr.2015.01.010>.
- Tsai, M.-Y., Yeh, S.-D., Wang, R.-S., Yeh, S., Zhang, C., Lin, H.-Y., & Chang, C. (2006). Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells. *Proceedings of the National Academy of Sciences*, 103(50), 18975–18980. <https://doi.org/10.1073/pnas.0608565103>.
- Verhoeven, G., Willems, A., Denolet, E., Swinnen, J. V., & De Gendt, K. (2010). Androgens and spermatogenesis: Lessons from transgenic mouse models. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 365(1546), 1537–1556. <https://doi.org/10.1098/rstb.2009.0117>.
- Vernet, N., Dennefeld, C., Rochette-Egly, C., Oulad-Abdelghani, M., Chambon, P., Ghyselinck, N. B., & Mark, M. (2006). Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology*, 147(1), 96–110. <https://doi.org/10.1210/en.2005-0953>.
- Welsh, M., Saunders, P. T. K., Atanassova, N., Sharpe, R. M., & Smith, L. B. (2009). Androgen action via testicular peritubular myoid cells is essential for male fertility. *The FASEB Journal*, 23(12), 4218–4230. <https://doi.org/10.1096/fj.09-138347>.
- Welsh, M., Sharpe, R. M., Moffat, L., Atanassova, N., Saunders, P. T. K., Kilter, S., & Smith, L. B. (2010). Androgen action via testicular arteriole smooth muscle cells is important for Leydig cell function, vasomotion and testicular fluid dynamics. *PLoS One*, 5(10), e13632. <https://doi.org/10.1371/journal.pone.0013632>.
- Welsh, M., Moffat, L., Belling, K., de França, L. R., Segatelli, T. M., Saunders, P. T. K., & Smith, L. B. (2012). Androgen receptor signalling in peritubular myoid cells is essential for normal differentiation and function of adult Leydig cells. *International Journal of Andrology*, 35(1), 25–40. <https://doi.org/10.1111/j.1365-2605.2011.01150.x>.
- Wolbach, S. B., & Howe, P. R. (1925). Tissue changes following deprivation of fat-soluble a vitamin. *The Journal of Experimental Medicine*, 42(6), 753–777. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/19869087>.
- Xu, Q., Lin, H.-Y., Yeh, S.-D., Yu, I.-C., Wang, R.-S., Chen, Y.-T., & Chang, C. (2007). Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells. *Endocrine*, 32(1), 96–106. <https://doi.org/10.1007/s12020-007-9015-0>.
- Yang, Q.-E., Kim, D., Kaucher, A., Oatley, M. J., & Oatley, J. M. (2013). CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. *Journal of Cell Science*, 126(4), 1009–1020. <https://doi.org/10.1242/jcs.119826>.
- Yeh, J. R., Zhang, X., & Nagano, M. C. (2011). Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. *Journal of Cell Science*, 124(14), 2357–2366. <https://doi.org/10.1242/jcs.080903>.
- Zhang, C., Yeh, S., Chen, Y.-T., Wu, C.-C., Chuang, K.-H., Lin, H.-Y., & Chang, C. (2006). Oligozoospermia with normal fertility in male mice lacking the androgen receptor in testis peritubular myoid cells. *Proceedings of the National Academy of Sciences*, 103(47), 17718–17723. <https://doi.org/10.1073/pnas.060856103>.
- Zhang, J., Eto, K., Honmyou, A., Nakao, K., Kiyonari, H., & Abe, S.-i. (2011a). Neuregulins are essential for spermatogonial proliferation and meiotic initiation in neonatal mouse testis. *Development*, 138(15), 3159–3168. <https://doi.org/10.1242/dev.062380>.
- Zhang, L., Tang, J., Haines, C. J., Feng, H., Lai, L., Teng, X., & Han, Y. (2011b). *c-kit* and its related genes in spermatogonial differentiation. *Spermatogenesis*, 1(3), 186–194. <https://doi.org/10.4161/spmg.1.3.17760>.
- Zhao, G. Q., Deng, K., Labosky, P. A., Liaw, L., & Hogan, B. L. (1996). The gene encoding bone morphogenetic protein 8B is required for the initiation and maintenance of spermatogenesis in the mouse. *Genes & Development*, 10(13), 1657–1669. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/8682296>.
- Zhao, G. Q., Liaw, L., & Hogan, B. L. (1998). Bone morphogenetic protein 8A plays a role in the maintenance of spermatogenesis and the integrity of the epididymis. *Development*, 125(6), 1103–1112. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/9463357>.

DEVELOPMENT

Formation of the Testis Primordium

Emmi Rotgers and Humphrey H-C Yao, National Institute of Environmental Health Sciences (NIEHS/NIH), Research Triangle Park, NC, United States

© 2018 Elsevier Inc. All rights reserved.

Formation of the Testis From the Bipotential Gonad

The testes form from a primitive undifferentiated gonad or genital ridge that has the potential to become either testes or ovary. In this article, we will describe the earliest events in testis development, when gonadogenesis, or formation of the gonads, occurs. Because most of the experimental evidence comes from studies using the mouse as a model organism, the morphological and molecular events during gonad formation are discussed mostly with reference to mouse development.

A functional gonad requires the presence of germ cells and somatic cells in order to produce gametes and hormones. The somatic cells of the gonads originate from the coelomic epithelium, a thin layer of epithelial cells that covers the gonad. While the primitive gonad is establishing, germ cell precursors, or primordial germ cells, migrate into the gonad from elsewhere in the embryo (discussed in the second part of this article). Key steps in forming the gonad are (1) proliferation of the coelomic epithelial cells, (2) ingression of the coelomic epithelium into the gonad proper, and (3) subsequent expansion and sexual differentiation of the somatic cells (Fig. 1). This process is controlled by a group of transcription factors and signaling molecules. At the onset of gonadogenesis, the coelomic epithelial cells that are destined to form the gonad become positive for the transcription factor Wilms' tumor 1 (WT1) (Kreidberg et al., 1993). Without *Wt1*, thickening of the coelomic epithelium is reduced dramatically (Kreidberg et al., 1993). The coelomic epithelium proliferates in an anterior to posterior fashion. The anterior-posterior thickening of the coelomic epithelium and the subsequent establishment of the gonad require the action of another transcription factor GATA binding protein 4 (GATA4) (Hu et al., 2013). GATA4 expression in the anterior part of the gonad precedes the initial thickening of the coelomic epithelium, and without *Gata4*, proliferation of the coelomic epithelial cells, and subsequent formation of the gonad fail to occur (Hu et al., 2013). The thickening of the coelomic epithelium is followed by a breakdown of its underlying basement membrane and ingression of the coelomic epithelial cells to form the gonad proper. In addition to WT1 and GATA4, the coelomic epithelial cells also express steroidogenic factor 1 (SF1), an orphan nuclear receptor that is important for gonad formation (Hoivik et al., 2010). Without functional SF1, the gonad does not expand properly and the gonadal cells eventually die (Luo et al., 1994). The early SF1 expression in the coelomic epithelium is promoted by GATA4 and two homeodomain proteins SIX1 and SIX4 (sine oculis homeobox homologs 1 and 4) (Hoivik et al., 2010; Fujimoto et al., 2013).

After its initial thickening, the coelomic epithelium continues to proliferate and provides progenitors to the developing gonad. This proliferation of the coelomic epithelial cells is promoted by Lim homeobox 9 (LHX9) and insulin signaling. Loss of *Lhx9* or insulin receptor and IGF type I receptor (*Igf1r*) result in stunted gonad development due to decreased proliferation of gonadal somatic cells (Birk et al., 2000; Pitetti et al., 2013). What the coelomic epithelial cells become after proliferation is determined by the polarity of the cells. The coelomic epithelium proliferates asymmetrically, where the progenitor cells remain as a part of the coelomic epithelium and maintain an undifferentiated epithelial cell fate. The daughter cells, on the other hand, ingress to the gonad proper, and acquire mesenchymal characteristics. During the ingression process, epithelial cells lose their cell polarity and cell-cell connections, and migrate through the basement membrane. Once they pass through the basement membrane, the ingressing epithelial cells form clusters that become the future somatic cells in the testis (Fig. 1). Multiple factors and pathways are involved in the invagination of the coelomic epithelium. Homeodomain transcription factor EMX2 and possibly SIX1/SIX4 are critical for the proliferation and the subsequent migration of the coelomic epithelial cells through the basement membrane. Without *Emx2*, the coelomic epithelial cells accumulate on the gonadal surface and fail to migrate and differentiate (Kusaka et al., 2010). A tight regulation of Notch signaling pathway is also necessary to maintain the asymmetric divisions and proper balance between progenitor and differentiated cell populations. A defect in the Notch signaling pathway results in abnormal accumulation of the progenitors and a failure of them to differentiate into the somatic cells in the testis (Potter et al., 2016; Lin et al., 2017). The coelomic epithelial cells that enter the gonad during sex determination become Sertoli cells. Proliferation and ingression of coelomic epithelial cells continue after sex determination, but cells ingressing at this time contribute to fetal and adult Leydig cell populations in the interstitium (Karl and Capel, 1998).

After the gonad has formed, it is important to ensure that new somatic cell progenitor cells survive and proliferate to form the testes. The survival of gonadal cells is controlled by SF1 and WT1. In the absence *Sf1* and *Wt1*, the coelomic epithelium begins to thicken during the earliest steps of gonad development, but then these structures gradually disappear through apoptosis of the gonadal somatic cells (Luo et al., 1994; Kreidberg et al., 1993). In addition to survival, sufficient proliferation of gonadal somatic cells is necessary for testis development (Schmahl and Capel, 2003). Without proliferation of the somatic cells in this early phase, such as in knockout mouse models for *Pbx1* and *Cbx2*, the resulting gonads are very small and poorly formed (Schnabel et al., 2003; Katoh-Fukui et al., 1998).

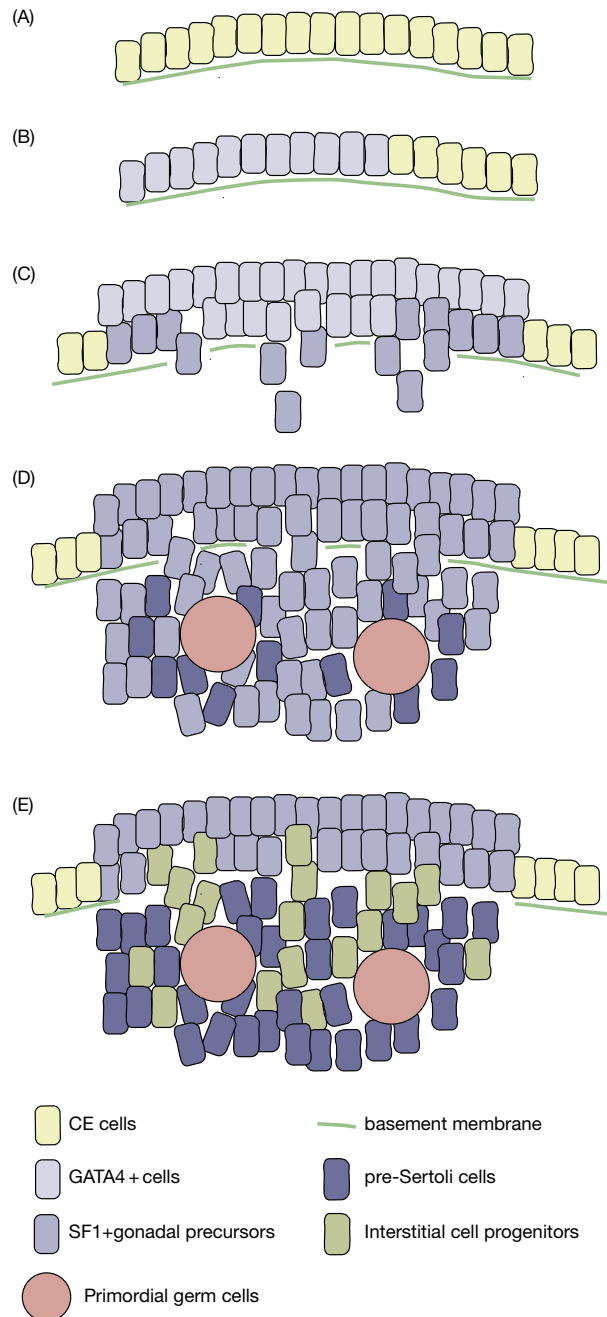


Fig. 1 Formation of the gonad. (A) The coelomic epithelium (CE), which lies on top of the future gonad, has an intact basement membrane prior to gonad formation. (B) Coelomic epithelial cells begin to express GATA4, prior to proliferation and thickening of the coelomic epithelium. (C) The cells that are destined to become gonadal somatic cells begin to express SF1. The basement membrane disintegrates and cells begin to ingress into the gonad. (D) Supporting cell progenitors (pre-Sertoli cells) continue to enter the gonad and proliferate. Primordial germ cells arrive to the gonad and they are surrounded by the pre-Sertoli cells. (E) The proliferating precursor cells from the coelomic epithelium contribute also to interstitial cells. Modified from Piprek, R. P., Kloc, M., and Kubiak, J. Z. 2016. Early development of the gonads: Origin and differentiation of the somatic cells of the genital ridges. *Results and Problems in Cell Differentiation* **58**, 1–22.

Development of the genital ridges lays the foundation for testicular development by ensuring that sufficient numbers of Sertoli cell precursors are established. Once the testis primordium is established, testicular development continues with migration of interstitial cell progenitors, vascular cells and macrophages into the gonad, which is covered in detail in other articles.

Primordial Germ Cell Formation and Migration

While the gonad is forming, primordial germ cells that migrate from the hindgut begin to enter the gonad. Primordial germ cells first gain their identity as a few cells in the epiblast, an embryonic structure next to the embryonic ectoderm (Fig. 2A). Primordial germ

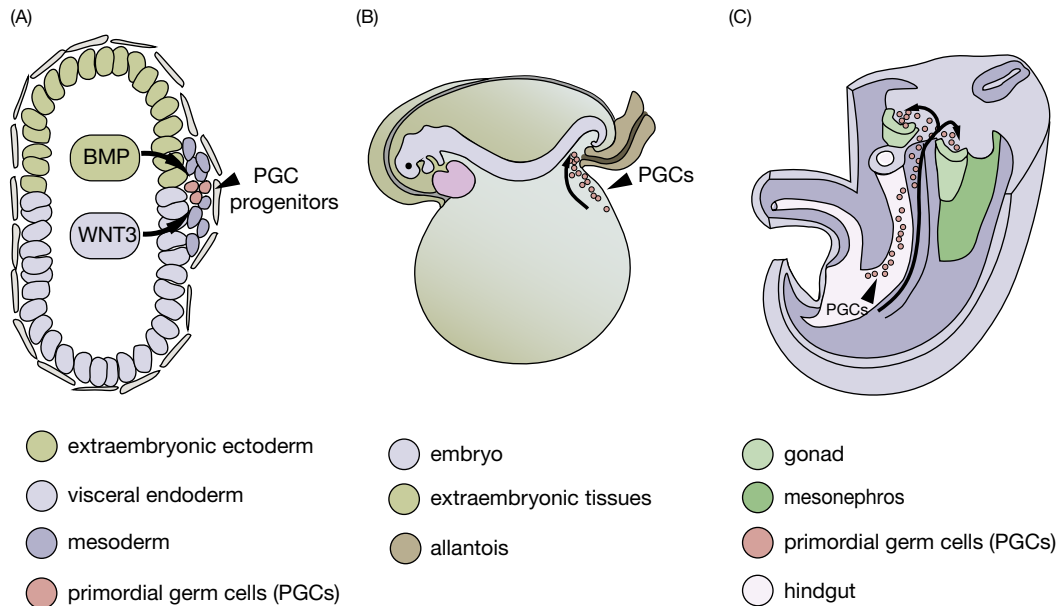


Fig. 2 Specification and migration of primordial germ cells. (A) Primordial germ cells are first specified in the epiblast. This process is controlled by BMP from the extraembryonic ectoderm and WNT3 signaling from the visceral endoderm. (B) As the embryo develops the primordial germ cells migrate to the base of the allantois. (C) When primordial germ cells begin their long migration toward the gonads, they leave the allantois and enter the hindgut. They migrate through the hindgut and mesentery until they reach the gonads. Arrows denote direction of migration. Modified from Tang, W. W., Kobayashi, T., Irie, N., Dietmann, S., and Surani, M. A. 2016. Specification and epigenetic programming of the human germ line. *Nature Reviews. Genetics* **17**, 585–600; Rosen, M. P., and Cedars, M. I. 2011. Female reproductive endocrinology and infertility, In D. G. Gardner and D. Shoback (eds.), *Greenspan's basic & Clinical endocrinology*, 9th edn. (New York, NY: The McGraw-Hill Companies). Chapter 13.

cell specification is directed by cues from the adjacent structures: bone morphogenetic protein (BMP) signaling from the extraembryonic ectoderm and Wnt family member 3 (WNT3) from the visceral endoderm (Tang et al., 2016). These signals are sensed by the presumptive primordial germ cell progenitors, and activate genes that distinguish them from somatic cells.

As the embryo develops, primordial germ cells relocate to the extraembryonic region at the base of the allantois, a membrane that is important for the development of the umbilical cord and placenta. From the allantois, primordial germ cells migrate through the hindgut and mesentery toward the gonads (Fig. 2B). Several factors promote primordial germ cell survival during the long migration process, such as DND microRNA-mediated repression inhibitor 1 (DND1), and Kit ligand (KitL) and stromal cell derived factor 1 (SDF1, also known as CXCL12) (Youngren et al., 2005; Gu et al., 2009; Molyneaux et al., 2003).

There are two theories on the migration process of the primordial germ cells: active migration of the primordial germ cells toward the gonads, which is induced by signals secreted by cells along the migratory path, or passive movement of primordial germ cells due to physical changes of the embryonic structure (Harikae et al., 2013). According to the theory of active migration, primordial germ cells sense chemoattractant signals secreted by somatic cells in the gonad, adjacent mesonephros, and mesentery. In response to these signals with the specific receptors, primordial germ cells actively migrate through the hindgut toward the gonads. Examples of chemoattractant signalling pathways are Kit ligand and c-Kit receptor (also known as Steel and KIT, respectively), and SDF1 and its receptor CXCR4 (chemokine (CXC motif) receptor 4). Kit pathway is involved in primordial germ cell proliferation, survival, and migration throughout the migration period (Gu et al., 2009). Without Kit ligand or its receptor, mice have reduced proliferation of migrating primordial germ cells during fetal life and are sterile as adults. Kit ligands elicit their action within a short range, meaning that the target cell has to be close to the cell secreting the Kit ligand. Interestingly, cells that produce Kit ligand form a path which migrating primordial germ cells follow. Kit ligand-expressing cells surround primordial germ cells that are already in the allantois right after primordial germ cells have become specified (Gu et al., 2009). As migration begins, Kit ligand is expressed both in the gonads and the midline area through which primordial germ cells migrate. Later on, when primordial germ cells reach the gonads, Kit ligand expression is switched off in the migratory path outside the gonads, but maintained in the gonads. Kit ligand not only promotes primordial germ cell survival and proliferation, but also influences their motility. Without Kit ligand, primordial germ cells migrate to the correct direction but at a lower rate than normal, resulting in fewer primordial germ cells reaching the gonads (Gu et al., 2009).

There are several similarities between the roles of Kit/Kit ligand and the SDF1/CXCR signaling pathways in controlling primordial germ cell migration. If SDF1 or its receptor CXCR4 are deleted in mice, very few germ cells reach the gonads and the adult mice are sterile (Ara et al., 2003; Molyneaux et al., 2003). SDF1 also promotes primordial germ cell survival and is expressed in the somatic cells along the migratory path of primordial germ cells in a similar fashion as Kit ligand. However, unlike Kit ligand, SDF1 is involved in establishing the direction of primordial germ cell migration. If ectopic SDF1 is present in embryonic organs other than the gonads, primordial germ cells begin to migrate toward that region erroneously (Molyneaux et al., 2003). Also, in

contrast to Kit ligand which is necessary for primordial germ cell migration from start to finish, SDF1 is dispensable for the early stages of migration, but required for the later stages of migration through the mesentery and hindgut to the gonads (Molyneaux et al., 2003; Ara et al., 2003). In conclusion, KIT and SDF1/CXCR4 signaling pathways play complementary roles in primordial germ cell proliferation, survival, and migration.

The second theory for primordial germ cell migration is the passive theory: when primordial germ cells are first incorporated into the hindgut endoderm, proliferation, and expansion of the hindgut passively propels primordial germ cells forward and toward the gonad (Harikae et al., 2013). If the hindgut does not expand, primordial germ cells are stuck at the hindgut entrance. It has been proposed that both of the active and passive theories are involved: the initial long-range migration of the primordial germ cells is promoted by passive translocation, but the survival and final steps of migration before reaching the gonads are promoted by chemoattractants secreted from the gonads. Interestingly, migration of primordial germ cells to the correct site still occurs even though formation of the gonads is compromised (Hu et al., 2013; Kreidberg et al., 1993). An explanation for this could be that production of chemoattractants in not only the gonads but also the adjacent mesentery and mesonephros is sufficient to guide the migrating primordial germ cells to their final destination.

After primordial germ cells reach the gonads, they cease migration and begin to form cell-to-cell associations with the somatic cells. It is hypothesized that reaching the site of highest concentration of the chemoattractants prompts primordial germ cells to stop migrating (Richardson and Lehmann, 2010). In addition, gonadal somatic cells likely suppress primordial germ cell migration, but the molecular mechanisms are not known. In the fetal testis, primordial germ cells become surrounded by Sertoli cells, which are responsible for the formation of testis cords. While they are enclosed in the testis cords, germ cells undergo rapid proliferation, form aggregates called germ cell cysts, and then cease mitosis (Lei and Spradling, 2013). Germ cell development that follows formation of fetal testes is discussed in detail in the following article on spermatogonial development and spermatogenesis.

References

- Ara, T., Nakamura, Y., Egawa, T., Sugiyama, T., Abe, K., Kishimoto, T., Matsui, Y., & Nagasawa, T. (2003). Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proceedings of the National Academy of Sciences of the United States of America*, 100, 5319–5323.
- Birk, O. S., Casiano, D. E., Wassif, C. A., Cogliati, T., Zhao, L., Zhao, Y., Grinberg, A., Huang, S., Kreidberg, J. A., Parker, K. L., Porter, F. D., & Westphal, H. (2000). The LIM homeobox gene *Lhx9* is essential for mouse gonad formation. *Nature*, 403, 909–913.
- Fujimoto, Y., Tanaka, S. S., Yamaguchi, Y. L., Kobayashi, H., Kuroki, S., Tachibana, M., Shinomura, M., Kanai, Y., Morohashi, K., Kawakami, K., & Nishinakamura, R. (2013). Homeoproteins *Six1* and *Six4* regulate male sex determination and mouse gonadal development. *Developmental Cell*, 26, 416–430.
- Gu, Y., Runyan, C., Shoemaker, A., Surani, A., & Wylie, C. (2009). Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development*, 136, 1295–1303.
- Harikae, K., Miura, K., & Kanai, Y. (2013). Early gonadogenesis in mammals: Significance of long and narrow gonadal structure. *Developmental Dynamics*, 242, 330–338.
- Hoivik, E. A., Lewis, A. E., Aumo, L., & Bakke, M. (2010). Molecular aspects of steroidogenic factor 1 (SF-1). *Molecular and Cellular Endocrinology*, 315, 27–39.
- Hu, Y. C., Okumura, L. M., & Page, D. C. (2013). *Gata4* is required for formation of the genital ridge in mice. *PLoS Genetics*, 9, e1003629.
- Karl, J., & Capel, B. (1998). Sertoli cells of the mouse testis originate from the coelomic epithelium. *Developmental Biology*, 203, 323–333.
- Katoh-Fukui, Y., Tsuchiya, R., Shiroishi, T., Nakahara, Y., Hashimoto, N., Noguchi, K., & Higashinakagawa, T. (1998). Male-to-female sex reversal in *M33* mutant mice. *Nature*, 393, 688–692.
- Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D., & Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell*, 74, 679–691.
- Kusaka, M., Katoh-Fukui, Y., Ogawa, H., Miyabayashi, K., Baba, T., Shima, Y., Sugiyama, N., Sugimoto, Y., Okuno, Y., Kodama, R., Iizuka-Kogo, A., Senda, T., Sasaoka, T., Kitamura, K., Aizawa, S., & Morohashi, K. (2010). Abnormal epithelial cell polarity and ectopic epidermal growth factor receptor (EGFR) expression induced in *Emx2* KO embryonic gonads. *Endocrinology*, 151, 5893–5904.
- Lei, L., & Spradling, A. C. (2013). Mouse primordial germ cells produce cysts that partially fragment prior to meiosis. *Development*, 140, 2075–2081.
- Liu, Y. T., Barske, L., DeFalco, T., & Capel, B. (2017). *Numb* regulates somatic cell lineage commitment during early gonadogenesis in mice. *Development*, 144, 1607–1618.
- Luo, X., Ikeda, Y., & Parker, K. L. (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell*, 77, 481–490.
- Molyneaux, K. A., Zinszner, H., Kunwar, P. S., Schaible, K., Stebler, J., Sunshine, M. J., O'Brien, W., Raz, E., Littman, D., Wylie, C., & Lehmann, R. (2003). The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. *Development*, 130, 4279–4286.
- Pitetti, J. L., Calvel, P., Romero, Y., Conne, B., Truong, V., Papaioannou, M. D., Schaad, O., Docquier, M., Herrera, P. L., Wilhelm, D., & Nef, S. (2013). Insulin and IGF1 receptors are essential for XX and XY gonadal differentiation and adrenal development in mice. *PLoS Genetics*, 9, e1003160.
- Potter, S. J., Kumar, D. L., & DeFalco, T. (2016). Origin and differentiation of androgen-producing cells in the gonads. *Results and Problems in Cell Differentiation*, 58, 101–134.
- Richardson, B. E., & Lehmann, R. (2010). Mechanisms guiding primordial germ cell migration: Strategies from different organisms. *Nature Reviews. Molecular Cell Biology*, 11, 37–49.
- Schmahl, J., & Capel, B. (2003). Cell proliferation is necessary for the determination of male fate in the gonad. *Developmental Biology*, 258, 264–276.
- Schnabel, C. A., Selleri, L., & Cleary, M. L. (2003). *Pbx1* is essential for adrenal development and urogenital differentiation. *Genesis*, 37, 123–130.
- Tang, W. W., Kobayashi, T., Irie, N., Dietmann, S., & Surani, M. A. (2016). Specification and epigenetic programming of the human germ line. *Nature Reviews. Genetics*, 17, 585–600.
- Youngren, K. K., Coveney, D., Peng, X., Bhattacharya, C., Schmidt, L. S., Nickerson, M. L., Lamb, B. T., Deng, J. M., Behringer, R. R., Capel, B., Rubin, E. M., Nadeau, J. H., & Matin, A. (2005). The *Ter* mutation in the *dead end* gene causes germ cell loss and testicular germ cell tumours. *Nature*, 435, 360–364.

Further Reading

- Piprek, R. P., Kloc, M., & Kubiak, J. Z. (2016). Early development of the gonads: Origin and differentiation of the somatic cells of the genital ridges. *Results and Problems in Cell Differentiation*, 58, 1–22.
- Rosen, M. P., & Cedars, M. I. (2011). Female reproductive endocrinology and infertility. In D. G. Gardner, & D. Shoback (Eds.), *Greenspan's basic & clinical endocrinology* (9th edn). New York, NY: The McGraw-Hill Companies. Chapter 13.

Male Sex Determination—Phenotypic

Gabriel Livera, CEA – INSERM – Universities Paris 7 and Paris 11, Fontenay-aux-Roses, France

© 2018 Elsevier Inc. All rights reserved.

The male sex determination is a sequential process that starts during fetal life in mammals. The building of the testis from the undifferentiated bipotential gonad is the process central to establish the male characteristics in the embryo and later. The somatic cells of the male gonad will impose the spermatogenic fate to the neighboring germ cells and act on distant tissues through hormone production to virilise the reproductive tract and other organs. The formation of testes in the embryo is thus key for the establishment of maleness. During fetal life, the proper differentiation of the testis is not only mandatory for allowing the sperm production later, in adulthood, but also to permit the masculinization of the fetus with the development of appropriate reproductive organs both intern and extern.

Testicular Differentiation

Before the testes acquire their adult oval-shape, side by side with the epididymides, in a scrotal position they undergo a developmental process starting with two long fine threads that occupy most of the abdominal cavity, the undifferentiated embryonic gonads. The many cell types of the testis are all formed early during development with a fast and sequential sequence of differentiation (Svingen and Koopman, 2013). In mammals, this sequence is usually initiated during the first third of the gestation. The testis comprises various somatic cells and germ cells and all have to acquire a male fate during development. However, it is only the testicular somatic cells, more specifically the Sertoli and Leydig cells, that are required to properly masculinize the embryo (Fig. 1).

The gonad appears first as an anlage on the ventral face of the mesonephros, a primitive kidney present during embryonic development in mammals. The thickening of it due to cell proliferation and migration of various cell types, including the primordial germ

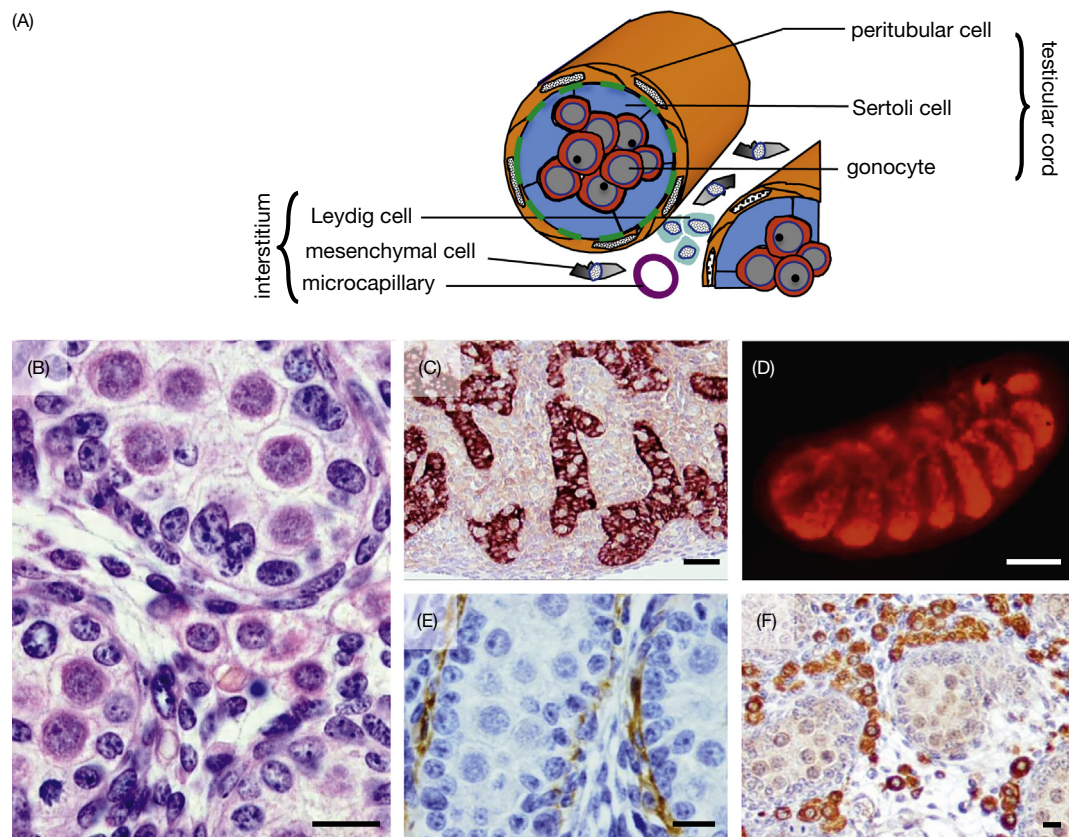


Fig. 1 Testicular differentiation. (A) Various testicular cell types appear during fetal life and form the testicular cords and the interstitium. (B) Histological section from a 15.5 dpc mouse fetal testis. (C) Sertoli cells stained with AMH from a 8 wpf human testis. (D) Gonocytes stained with DDX4 in a 13.5 dpc mouse testis. (E) Peritubular myoid cells stained with α -SMA in a 15.5 dpc mouse testis. (F) Leydig cells stained with 3- β HSD in a 17.5 dpc mouse testis. Scale bars: 10 μ m, B, E and F; 30 μ m, C; 100 μ m, D. AMH, anti-Müllerian Hormone; DDX4, Dead box polypeptide 4/VASA homolog; α -SMA, alpha smooth muscle actin; 3- β HSD, 3-beta hydroxy steroid deshydrogenase.

cells, will result in an undifferentiated bipotential gonad. Such can be observed at 11.5 days post-conception (dpc) in the mouse and 5 weeks post fertilization (wpf) in humans. The gonads then contain the bipotential precursors of somatic and germ cells.

Shortly after, in XY embryo, testicular cords form (Nel-Themaat et al., 2009). This is the first morphological event that allows distinguishing a testis from an ovary. It relies on the differentiation of the Sertoli cells. The first Sertoli cells can be observed as early as 12.0 dpc in the mouse testis and about 6 wpf in humans. Embryonic Sertoli cells are the conductor of the male sex determination. These cells will provoke the rapid appearance of the steroidogenic testicular cells, the Leydig cells, as soon as 12.5 dpc. They also trigger the differentiation of the peritubular myoid cells surrounding the cords and impose the male fate to the germline.

Testicular Cords

The gonadal anlage results from a thickening of the coelomic epithelium due to cell proliferation and migration. These cells are believed to be the precursors of the somatic lineages in the genital crest. Interestingly the XY embryonic gonad grows slightly thicker than its XX counterpart from then on.

Sertoli cells are the first to differentiate within the future testis (Magre and Jost, 1991). These cells provide support and protection to the male germline and are thus termed supporting cell lineage. In the mouse embryo, around 12.5 dpc, Sertoli cells that have differentiated polarize and aggregate to surround groups of germ cells. The tube-like structures then created are the testicular cords, the rudiments of the seminiferous tubules that will be observed in the adult testis. The simultaneous formation of several cords has been demonstrated to be a two step process: first, a large bundle of Sertoli and germ cell formed, second it is partitioned in cord-forming units due to its fractionation by cells migrating from the mesonephros toward the coelomic epithelium (Cool et al., 2012). Sertoli cells do proliferate actively during the fetal life and cease progressively multiplying during post-natal life. In the mouse, Sertoli cell proliferation is almost null around 15 days postpartum (dpp) (Vergouwen et al., 1991). Thus the fetal and post-natal multiplication of Sertoli cell is key to establish the testicular size. Indeed it is accepted that each Sertoli cell can only support a given number of germ cells in the adult testis.

Myoid cells surround the testicular cords, forming an additional outer layer. These peritubular cells appear at 13.5 dpc in the mouse and aggregate around Sertoli cells. Then, they rapidly elongate and flatten. At 14.5 dpc, peritubular cells start expressing markers of smooth muscular cells such as alpha smooth muscle actin (α -SMA). In the human, peritubular cells are morphologically recognizable at 12 weeks of gestation. Together with Sertoli cells they will produce a basement membrane or basal lamina (Merchant-Larios and Taketo, 1991). In the adult peritubular myoid cells display a contractile function needed to allow sperm progression in the tubules.

The Sertoli cells develop tight junctions between adjacent cells. This is part of what is called the blood testis barrier (BTB). Such BTB is a structure required for the proper progression of spermatogenesis. In the mouse the BTB is set up between 15 and 20 dpp. This coincides with the period when a lumen appears in the testis cords; then these are not anymore cords but tubules. This transformation of testis cords in seminiferous tubules is a mark of their terminal differentiation, albeit these do not produce yet sperm cells. The tubular diameter will progressively increase to accommodate the developing seminiferous epithelium until adulthood.

Vascularization

The embryonic testis displays very early a specific vasculature. The embryonic gonads contain numerous microcapillaries and in the case of the embryonic testis a blood vessel becomes visible very early on the ventral part of the gonad just beneath the epithelium (Brennan et al., 2002). This male-specific coelomic vessel will be the future spermatic artery. It likely originates from the cells that migrated from the mesonephros as these bear endothelial cell markers and are thus believed to form the vasculature of the gonadal primordium. The distinctive coelomic vessel can be observed in the male fetal gonad by 13.5 dpc. The specific arterial system set up in the male embryonic is believed to facilitate the proper exportation of male hormones.

Leydig Cells

The interstitial tissue, in-between the cords, contains the Leydig cells that are responsible for the production of androgens. Fetal Leydig cells form a population distinct from the adult ones. Fetal Leydig cells appear just after initial sex differentiation and induce masculinization of the fetus by producing androgens (Habert et al., 2001). Leydig cell number increases throughout fetal life from about 200 at 12.5 dpc up to more than 10,000 in the new-born mouse. At the same time, those cells progressively increase in size and aggregate forming clumps often near or around blood vessels. Leydig cells produce testosterone as early as they differentiate and testosterone production peaks about 17.5 dpc in the mouse, and 14 wpf in humans (Livera et al., 2006). These androgens will be mandatory for the masculinization of the fetus. Of interest, fetal Leydig cells differ from the Leydig cell population observed in the adult testis. Indeed, at puberty a new population of adult Leydig cells will replace earlier populations. These later have small and relatively fewer cytoplasmic lipid droplets in comparison to fetal Leydig cells (Haider et al., 1995). Fetal Leydig cells in rodents can produce testosterone independently of luteinizing hormone stimulation (LH), while adult Leydig cells heavily depend upon LH stimulation. This appears untrue in the case of human fetal Leydig cells that are literally bathed in chorio-gonadotropin (LH analog). Perhaps the best criterion differentiating fetal Leydig cells from their adult counterpart is their ability to respond to multiple LH-stimulation without desensitization. This feature likely accounts for their formidable androgen production. Of note, fetal Leydig cells also produce another hormone required for proper masculinization (INSL-3).

Germ Cells

During fetal life, the testis contains exclusively mitotic germ cells, the meiotic and post-meiotic cells will only be formed after birth with meiotic onset at puberty. The germ cells are not formed within the embryonic gonad but originate from outside. The primordial germ cells (PGC) migrate from a region quite distant from the gonads, being at the base of the allantois at 6.5 dpc. After a long journey, germ cells finally reach the genital crests around 10 dpc. At that time they are termed post-migratory PGCs, they actively proliferate and are often observed with patches of condensed chromatin in their nuclei. In the rodents, once included in testis cords, germ cells will progressively stop dividing. Then they are often referred as “gonocytes” (Culty, 2009). The exact reason for this mitotic arrest is unknown though it has been hypothesized that it may facilitate epigenetic events occurring then. The quiescence phase of gonocytes starts from 14.5 or 15.5 dpc and lasts up to birth in the mouse. At that time gonocytes are easily recognized due to their large spherical nuclei containing fine chromatin granules and often two globular nucleoli. In the human testis no quiescence phase has been reported possibly due to the lack of synchronicity of the human germline during development. During the beginning of postnatal life, in rodents, the gonocytes relocate progressively from the center of the cords to the basement membrane. This will also be the place homing the future spermatogonia that derived from the gonocytes. The transition from gonocyte to a prespermatogonial type in contact with the basement membrane occurs before birth in humans.

Masculinisation of the Reproductive Tract

Internal and external genitalia develop after the testicular differentiation. The internal reproductive organs are—apart the testes—the epididymis, the vas deferens and the accessory glands (seminal vesicles, prostate and bulbourethral glands). The epididymis is a long duct connected to the seminiferous tubules in its anterior part (head) and in which sperm will mature. The vas deferens is the portion of tube connected to the posterior end of epididymis (cauda). The accessory glands provide fluids to lubricate the duct system and sustain the sperm. This forms the reproductive tract that passes the sperm from testes up to the ejaculation. The external genital organs are the penis and the scrotum, a pouch-like structure holding the testes below the body wall.

Internal Genitalia

Unlike the gonads, the male and female reproductive tracts originate from different primordia. Prior to sex determination, embryos contain two complete sets of ductal structures. The male genital tract arises from the Wolffian ducts and the female one from Müllerian ducts (Rey et al., 2000). Those ducts are also termed respectively mesonephric and paramesonephric ducts due to their position in the mesonephros bordering the gonad. In the male embryo, the primordium of the female tract, the Müllerian ducts, will disappear while the primordium for male tract will develop. The regression of the Müllerian duct is due to the anti-Müllerian hormone (AMH) production by Sertoli cells during development. Androgens produced by fetal Leydig cells actively maintain Wolffian ducts, which give rise to the epididymis, vas deferens and seminal vesicles. The external genitalia differentiate from the urogenital sinus. External genitalia define the sex for the civil status in the human species.

The two pairs of ducts co-exist in the mesonephroi in both sexes at the beginning. The Wolffian duct appears as a continuous tube that runs the length of the urogenital ridge. Thus the Wolffian duct is present all along the mesonephros; it is nearby the Müllerian duct. Wolffian ducts open in the urogenital sinus at the most posterior end of the embryo.

The first event evidencing a male differentiation of the reproductive tract is the regression of the Müllerian ducts. This regression is observable at 14 dpc in the mouse male embryo and at 8 wpf in human ones (Dyche, 1979). These have almost completely disappeared by 9 wpf. Second, shortly after, due to the development of the definitive kidney (metanephros) and under the action of androgens the Wolffian ducts will give rise to the male reproductive tract. This witnesses a functional change from urinary to reproductive function. Testosterone allows the stabilization and the differentiation of Wolffian ducts (Welsh et al., 2007). The main organ resulting from this differentiation is the epididymis. The formation of the epididymis involves a complex program to achieve the formation of a long tube with a complex pattern of coiling, formation of septae, and regional functionalization. The cranial part of the duct become connected to the testis cords forming the rete testis and the portion of the duct just below tremendously elongate and becomes convoluted to form the epididymis. The caudal part of the duct will develop a muscular envelope and form the vas deferens. In the most distal part of each duct, just prior the junction with the sinus, a lateral diverticulum will grow and produce the seminal vesicle. Thus the main internal accessory reproductive organs are all derived from the Wolffian ducts. The prostate and bulbourethral gland originate from the differentiation of the urogenital sinus (Table 1).

Pioneer studies by Alfred Jost evidenced that in utero castration of rabbit fetuses prior to sexual differentiation systematically triggered a female-type development of the genitalia (Jost, 1953). This demonstrated that only testicular hormones are key for both the regression of the Müllerian ducts (AMH) and the development of Wolffian ducts (testosterone).

External Genitalia

The external genital differentiation is also profoundly stroke by the presence of testicular androgens (mostly dihydro-testosterone in this case). The external genitalia differentiate from a bipotential anlage composed of the genital tubercle, folds and outer swellings that remain sexually undifferentiated until 9 wpf in human embryos (Yamada et al., 2003). Then male specific change will appear

Table 1 Chronology of the main events during male differentiation

	<i>Mouse</i>	<i>Human</i>
Formation of the gonadal primordium	10 dpc	4 wpf
Colonization by the primordial germ cells	10.5–11.5 dpc	4–5 wpf
Formation of testicular cords	12.5 dpc	7 wpf
Differentiation of Leydig cells	12.5 dpc	8 wpf
Regression of Müllerian ducts	14 dpc	8 wpf
Masculinization of external genitalia	16 dpc	10 wpf
Appearance of seminal vesicle	16 dpc	10 wpf
Appearance of prostatic buds	17 dpc	10 wpf
Increased anogenital distance	16 dpc	11–13 wpf
Fully descended testes	25 dpp	30 wpf

dpc, days post-conception; *dpp*, days post-partum; *wpf*, weeks post-fertilization.

Modified from Saint-Dizier M. & Chastand-Maillard S: *La reproduction animale et humaine*. (2014) Chapter 1: p. 19–39. Quae, Versailles, France.

with a near-complete male phenotype observable at the beginning of the second trimester (14 wpf). The genital tubercle elongates and grows to form the penis. The folds located below, on each side of the urethral groove, will close over the groove. The swellings on the outer sides of the folds, also referred as labio scrotal ridges, will form a pouch to later accommodate the testes, the scrotum. As the scrotum forms, testes descend down the posterior abdominal wall, through the inguinal canal. This descent is a long process starting around 10 wpf and usually completed around 30 wpf. Testicular descent also depends on androgen and on another Leydig cell secretion the insulin-like 3 (INSL-3). Testicular descent is decomposed in a two phases process with INSL-3 mediating the first part, trans-abdominal, and androgens mediating the second part, inguino-scrotal (Klonisch et al., 2004). In mice, testicular descent is mostly post-natal and completed by 25 dpp. This extra-abdominal position of the testes provides them with a temperature lower than the body temperature, a difference mandatory for proper spermatogenesis. However, one should keep in mind that not all mammals have descended testes.

Male Determination of Other Tissues

The development of male-specific features is obviously not only restricted to the gonads and reproductive tract, albeit studies and consensus remain scarce in other tissues. The perineal growth and the digit ratio are indicators that have been promoted as markers of virilisation. The brain evidently is also primed differently in males and females. Though this is far from exhaustive other parameters have not yet received detailed attention.

The development of the genital tubercle and genital swellings into, respectively, the penis and scrotum is accompanied by an increase in perineal growth and thus a longer anogenital distance (distance from the anus to the posterior base of the genital tubercle). This distance is longer in males than in females in most mammals (twice as long in rodents) (Welsh et al., 2008). As this feature is easy to observe at birth, it is often used as a marker of the proper exposure to androgens and proper masculinisation during fetal life.

The ratio of the lengths of the index and ring finger (2D,4D digit ratio) has been proposed as another marker of androgen action during development in mice and humans (McIntyre, 2006). Males have generally a longer ring finger (digit 4) relative to the length of index finger (digit 2) than females. This parameter is quite attractive as it is easy to measure though it remains debated.

Sexual dimorphism of brain structures has been reported in various species. This sexual differentiation of the brain induces neuroanatomical changes that are believed to impact behavior. However many environmental factors also shape the brain and this complexity has limited a clear view of what is due to a direct hormonal effect. Maybe one of the best examples that male and female brains differ at the anatomical level is the existence of a nucleus in the rat brain with a different size according to the sex, the “sexually dimorphic nucleus of the preoptic area” of the hypothalamus (Gorski et al., 1978). In the male rat the volume of this structure is about three to eight times larger than in females and this sexual difference is mostly established during post-natal development. This structure is implicated in sexual behavior in animals. The volume or neuron number has since been reported to differ in numerous other areas of the brain albeit many variations in species exist. Lastly, early differences observed between the XX and XY embryonic brains (prior testicular steroidogenesis) also point toward likely cell-autonomous events possibly related to the chromosomal constitution. This possibility that some male features might rely on cell-autonomous events (not being the consequence of having testes) is similar to what has been reported in marsupials, where the formation of a scrotum, or a pouch, is a consequence of the absence or presence of two X chromosomes.

Anecdotally, the mammary gland development differs according to the sex in some mammals but not in others. Though in humans, at birth, roughly similar mammary structures are observed in boys and girls, in rats and mice, the post-natal mammary structures appear strikingly different. In the rat fetus, the mammary anlage shows a male-specific atrophy of the rudimentary buds. This suppresses nipple formation during post-natal life, another parameter often used to monitor proper masculinisation, albeit exclusively in rodents.

In conclusion, the testicular development is key to maleness acquisition. The hormonal secretions of the developing testis ensure that the development of the gonad is properly coordinated with that of the rest of the organism to ensure the complete establishment of the male reproductive potential once adult. Testicular androgens are the main endocrine factors promoting masculine phenotypes. A period of high testosterone production in males during the fetal life drives the differentiation of primary reproductive tissues. Nevertheless, the particular pattern of testosterone production varies among vertebrate species and differences in potential target tissues are widely debated.

References

- Brennan, J., Karl, J., & Capel, B. (2002). Divergent vascular mechanisms downstream of Sry establish the arterial system in the XY gonad. *Developmental Biology*, 244, 418–428.
- Cool, J., DeFalco, T., & Capel, B. (2012). Testis formation in the fetal mouse: Dynamic and complex de novo tubulogenesis. *Wiley Interdisciplinary Reviews: Developmental Biology*, 1, 847–859.
- Culty, M. (2009). Gonocytes, the forgotten cells of the germ cell lineage. *Birth Defects Research. Part C, Embryo Today*, 87, 1–26.
- Dyche, W. J. (1979). A comparative study of the differentiation and involution of the Mullerian duct and Wolffian duct in the male and female fetal mouse. *Journal of Morphology*, 162, 175–209.
- Gorski, R. A., Gordon, J. H., Shryne, J. E., & Southam, A. M. (1978). Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Research*, 148, 333–346.
- Habert, R., Lejeune, H., & Saez, J. M. (2001). Origin, differentiation and regulation of fetal and adult Leydig cells. *Molecular and Cellular Endocrinology*, 179, 47–74.
- Haider, S. G., Laue, D., Schwochau, G., & Hilscher, B. (1995). Morphological studies on the origin of adult-type Leydig cells in rat testis. *Italian Journal of Anatomy and Embryology*, 100(Suppl 1), 535–541.
- Jost, A. (1953). Fetal intersexuality induced by methylandrostenediol in the rat; with reference to a probably similar clinical observation. *Comptes Rendus des Seances de la Societe de Biologie et de Ses Filiales*, 147, 1930–1933.
- Klonisch, T., Fowler, P. A., & Hombach-Klonisch, S. (2004). Molecular and genetic regulation of testis descent and external genitalia development. *Developmental Biology*, 270, 1–18.
- Livera, G., Delbes, G., Pairault, C., Rouiller-Fabre, V., & Habert, R. (2006). Organotypic culture, a powerful model for studying rat and mouse fetal testis development. *Cell and Tissue Research*, 324, 507–521.
- Magre, S., & Jost, A. (1991). Sertoli cells and testicular differentiation in the rat fetus. *Journal of Electron Microscopy Technique*, 19, 172–188.
- McIntyre, M. H. (2006). The use of digit ratios as markers for perinatal androgen action. *Reproductive Biology and Endocrinology*, 4, 10.
- Merchant-Larios, H., & Taketo, T. (1991). Testicular differentiation in mammals under normal and experimental conditions. *Journal of Electron Microscopy Technique*, 19, 158–171.
- Nel-Themaat, L., Vadakkan, T. J., Wang, Y., Dickinson, M. E., Akiyama, H., & Behringer, R. R. (2009). Morphometric analysis of testis cord formation in Sox9-EGFP mice. *Developmental Dynamics*, 238, 1100–1110.
- Rey, R., Jossio, N., & Racine, C. (2000). In L. J. De Groot, G. Chrousos, K. Dungan, et al. (Eds.), *Sexual differentiation*. South Dartmouth (MA): Endotext.
- Svingen, T., & Koopman, P. (2013). Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. *Genes & Development*, 27, 2409–2426.
- Vergouwen, R. P., Jacobs, S. G., Huiskamp, R., Davids, J. A., & de Rooij, D. G. (1991). Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. *Journal of Reproduction and Fertility*, 93, 233–243.
- Welsh, M., Saunders, P. T., & Sharpe, R. M. (2007). The critical time window for androgen-dependent development of the Wolffian duct in the rat. *Endocrinology*, 148, 3185–3195.
- Welsh, M., Saunders, P. T., Fiskin, M., Scott, H. M., Hutchison, G. R., Smith, L. B., & Sharpe, R. M. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *The Journal of Clinical Investigation*, 118, 1479–1490.
- Yamada, G., Satoh, Y., Baskin, L. S., & Cunha, G. R. (2003). Cellular and molecular mechanisms of development of the external genitalia. *Differentiation*, 71, 445–460.

The Molecular Control of Testis Determination

Abigail Harris and Andy Greenfield, MRC Harwell Institute, Oxfordshire, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

SRY: The Y-Linked Mammalian Testis-Determining Gene

Primary sex determination describes the process by which the embryonic gonad develops as a testis or ovary. Subsequent to this, the phenotypic sex of the fetus, including the anatomy of the internal and external genitalia, is established by hormonal products of the gonads (or the absence thereof). In mammals, the Y chromosome is a dominant male determinant. *SRY* is the gene found on almost all mammalian Y chromosomes that is responsible for initiating testis determination during embryonic development. *SRY*, which encodes an HMG box transcription factor, is expressed in somatic supporting cell precursors and initiates a pathway of gene expression that results in the differentiation of Sertoli cells (Larney et al., 2014). Thus, sex determination is a matter of controlling the fate of the supporting cell lineage in a sexually dimorphic fashion. Other morphogenetic processes required for testis development, such as the formation of testis cords and associated vasculature, and the differentiation of Leydig cells, are thought to depend on Sertoli cell signals. In the absence of the Y chromosome, ovary development ensues due to pro-ovarian pathways of gene expression.

In the mouse, the mammalian species in which the molecular mechanisms underlying gonad development have been most intensively studied, *Sry* expression exhibits exquisite control. It is first detected at around 10.5 days *post coitum* (dpc) in somatic cells, approximately 24 h after the formation of the gonadal primordium. Expression is initiated at the center of this long thin organ and by 11.5 dpc, at its peak, expression has spread to the gonadal poles. By 12.5 dpc, *Sry* expression is extinguished. This dynamic spatiotemporal profile is related to its function: *SRY* is only required at early stages of gonad development to initiate Sertoli cell differentiation, partly by repressing ovarian pathways of development (see sections **Sox9: Conserved Master Regulator of Testis Determination** and **FGF9: Propagating the Testis-Determining Signal**); but it is not required to maintain Sertoli cell identity. That role is performed by another HMG box transcription factor encoded by *SRY*'s principal target gene, *SOX9* (see section **SOX9: Conserved Master Regulator of Testis Determination**) and another transcription factor, *DMRT1* (section **The "Battle of the Sexes": A Life-Long Struggle**).

A number of studies in the mouse reveal that, depending on genetic background, a significant delay in the expression of *Sry* can result in complete or partial gonadal sex reversal, that is, XY ovary or ovotestis development (Carre and Greenfield, 2014). These observations underline the requirement for timely expression of *SRY* to oppose ovarian developmental pathways: a 'window of opportunity' exists for *SRY* and it must not miss it (Hiramatsu et al., 2008). A delay to, or reduction in, *Sry* expression at this critical time-point can arise following mutation in some key regulatory genes, such as *Insr/Igf1r*, *Wt1*, *Gata4/Fog2*, *Cbx2*, *Six 1/4*, *Jmjd1a*, *Map3k4*, and *Gadd45g*. Such genes may act to directly enhance the transcription of *Sry*, or promote proliferation of *SRY*-positive somatic cells, or both. Mutation in human genes required for normal *SRY* expression may account for a large number of cases of 46,XY partial or complete gonadal dysgenesis (CGD), namely XY female development (Bashamboo and McElreavey, 2015). Mouse mutant models offer a way of investigating molecular mechanisms that underlie such human disorders of sex development (DSDs) (Table 1). However, the mouse and human sex-determining networks are not identical. *GADD45γ* is a key regulator of mouse *Sry*, through its activation of *MAP3K4*, but no human *GADD45g* mutations are reported (Bogani et al., 2009; Warr et al., 2012). However, whilst no human *MAP3K4* mutations have been reported in 46,XY DSD, mutations in the related gene, *MAP3K1*, do disrupt human testis determination (Pearlman et al., 2010).

In the absence of *SRY*, in XX embryonic gonads, ovary development ensues. Ovarian development is characterized by the absence of Sertoli cells and testis cords; instead, pregranulosa cells (the homologous ovarian supporting cell lineage) form and, along with germ cells, organize into ovigerous cords at around 13.5 dpc in the mouse. At around birth these ovigerous cords form primary follicles, containing a single oocyte surrounded by layers of granulosa cells and then theca (steroidogenic) cells. Alternative genetic pathways predominate in the XX gonad and direct development towards an ovarian fate. Canonical WNT/ β -catenin signaling is one major pro-ovarian pathway, requiring genes such as *RSPO1*, *WNT4*, and *CTNNB1* (Chassot et al., 2014). This pathway is implicated in ovary development in mice and humans. A key pro-ovarian transcription factor is *FOXL2* (Pannetier et al., 2016). Again, this factor is implicated in ovarian development and function in humans, mice and goats (see section **The 'Battle of the Sexes': A Life-long Struggle**).

SOX9: Conserved Master Regulator of Testis Determination

A wealth of genetic data in humans and mice has established an over-arching role for *SOX9* in testis determination (Jakob and Lovell-Badge, 2011; Warr and Greenfield, 2012). Indeed, evidence exists implicating *SOX9* in testis development in vertebrates more broadly. Human patients with campomelic dysplasia and 46,XY gonadal dysgenesis, resulting in sex reversal, were the first cases reported to have mutations in *SOX9* (Foster et al., 1994). Mouse studies then showed that inactivation of *Sox9* by gene targeting caused complete XY gonadal sex reversal (Barriónuevo et al., 2006). Moreover, forced expression of *Sox9* in XX gonadal somatic cells at around the time of sex determination resulted in testis development. These data suggest that *SOX9* activity is both necessary and sufficient for testis determination. *SOX9* is a transcription factor that has been shown to be required for the

Table 1 Mouse and human gonadal phenotypes associated with mutations in testis-determining genes

Gene name/symbol	Mouse mutant phenotype	Human clinical phenotype	References (PMID)
Sex determining region of Chr Y (<i>SRY</i>)	Complete XY gonadal sex reversal in deletion mutant	Point mutations cause 46,XY complete gonadal dysgenesis (CGD)—sex reversal	2401216 2247149
<i>SRY</i> (sex determining region Y)-box 9 (<i>SOX9</i>)	Knockout heterozygotes die around birth; conditional deletion causes complete XY gonadal sex reversal	Loss of <i>SOX9</i> functionality by mutation results in campomelic dysplasia and XY CGD.	15056615 16207837 7990924
Fibroblast growth factor 9 (<i>FGF9</i>); Fibroblast growth factor receptor 2 (<i>FGFR2</i>)	On the sensitized C57BL/6 J background the loss of <i>FGF9</i> by knockout causes fetal XY complete gonadal sex reversal; conditional deletion of <i>FGFR2</i> , or point mutation, also causes partial or complete sex reversal	One report of an individual with craniosynostosis and 46,XY CGD with an <i>FGFR2</i> mis-sense mutation	11290325 17940049 18155190 24956260 26362256
Doublesex and mab-3 related transcription factor 1 (<i>DMRT1</i>)	<i>DMRT1</i> knockout causes infertility and postnatal somatic cell reprogramming of Sertoli cell to granulosa cell-like fate	A report of one individual with 46,XY CGD and a mis-sense mutation in <i>DMRT1</i>	11040213 21775990 26005864
Nuclear receptor subfamily 0, group B, member 1 (<i>NROB1</i>)	Loss of <i>NrOb1</i> (<i>Dax1</i>) on sensitized background causes XY gonadal sex reversal; <i>NrOb1</i> over-expression on sensitized background disrupts testis determination	Altered <i>NROB1</i> dosage thought to be responsible for X-linked dosage-sensitive sex reversal (<i>DSS</i>)	12679814 18633137 9486644 8570694
Growth arrest and DNA-damage-inducible 45 gamma (<i>GADD45g</i>)	Loss of <i>Gadd45g</i> causes complete XY gonadal sex reversal in the fetus and adult	No reports of <i>GADD45g</i> mutations in DSD	23102581 23102580 23516551
Mitogen-activated protein kinase kinase 4, 1 (<i>MAP3K4</i> , <i>MAP3K1</i>)	<i>Map3k4</i> knockout and point mutation cause fetal XY gonadal sex reversal; <i>Map3k4</i> over-expression rescues <i>Tas</i> sex reversal phenotype	No reports of <i>MAP3K4</i> mutations, but a large number of mutations reported in related gene, <i>MAP3K1</i> , in 46,XY DSD	19753101 24452333 21129722 21559298 23102580
Mitogen-activated protein kinase 11, 14 (<i>MAPK11</i> , <i>MAPK14</i>) Chromobox 2 (<i>CBX2</i>)	Double knockout of <i>Mapk11</i> (p38a MAPK) and <i>Mapk14</i> (p38b MAPK) causes fetal XY gonadal sex reversal <i>Cbx2</i> (<i>M33</i>) knockout causes fetal XY sex reversal	No human mutations reported <i>CBX2</i> mutations reported in case of 46,XY CGD	9641679 22186409 19361780
Sine oculis-related homeobox 1, 4 (<i>SIX1</i> , <i>SIX4</i>) Nuclear receptor subfamily 5, group A, member 1 (<i>NR5A1</i>)	<i>Six1/4</i> double knockout causes fetal XY sex reversal Loss of <i>Nr5a1</i> (<i>Sf1</i> , <i>Flz-F1</i>) causes gonadal agenesis; SF1 acts along with <i>SRY</i> to up-regulate <i>Sox9</i>	No human mutations reported <i>NR5A1</i> point mutations found in variety of DSD and infertility cases	23987514 8187173 18454134 22028768 27378692 20887963
Wilms tumor 1 homologue (<i>WT1</i>)	Targeted deletion of the WT1(+KTS) isoform causes complete XY gonadal sex reversal through disruption to <i>Sry</i> expression	<i>WT1</i> mutations reported in Frasier Syndrome patients with sex reversal	11509181 19549635 12932885 24009392
Lysine (K)-specific demethylase 3A (<i>KDM3A</i>)	Loss of <i>KDM3A</i> (<i>JMJD1A</i>) on a sensitized background causes XY gonadal sex reversal	No reports of human mutations	
GATA binding protein 4 (<i>GATA4</i>)	Loss of <i>GATA4</i> causes XY gonadal sex reversal due to disruption to <i>Sry</i> and <i>Sox9</i>	<i>GATA4</i> mutations reported to disrupt human testis determination	17848526 21385577 21220346
Zinc finger protein, multitype 2 (<i>ZFPM2</i>)	<i>ZFPM2</i> (<i>FOG2</i>) acts together with <i>GATA4</i> to control testis determination	<i>ZFPM2</i> mutations reported to disrupt human testis determination	17540364 21385577 24549039
Desert hedgehog (<i>DHH</i>)	<i>Dhh</i> knockout on a mixed genetic background causes infertility and failure of Leydig cell specification	A number of reports of <i>DHH</i> mutations in 46,XY DSD, including CGD	12050120 25927242

expression of a number of genes in the developing testis, including *Amh*, *Vanin-1*, *Ptgsd*, and *Cyp26l1*. In fact, genome-wide chromatin immunoprecipitation experiments (ChIP-seq) indicate that SOX9 binds thousands of target genes, including most of those already implicated in testis determination (Rahmouni et al., 2017). It remains unclear how many of these are positive targets, up-regulated by SOX9, and how many are negative targets. Nor is it known whether SOX9 functions at all regions to which it is recruited. As with many genome-wide approaches, subsequent focused functional studies will be needed, in this case to determine precisely which SOX9 targets function in testis development. It will also be important to understand the relationship between SOX9 and other SOX proteins that have been implicated in testis development, such as SOX8, SOX10, and SOX3. The compensatory actions of such orthologues might complicate the mechanistic insights offered by the study of knockout phenotypes.

In the mouse, SOX9 can be detected in somatic cells from early stages of gonad development, initially in both XX and XY gonads. It is then down-regulated in the XX gonad and up-regulated in XY gonad, such that by 11.5 dpc expression is effectively restricted to the XY gonad. The hypothesis that SRY might regulate the expression of *Sox9* is supported by a number of observations, including co-expression of both genes in the nuclei of supporting cell precursors, their shared function in testis determination and, crucially, the fact that *Sry* expression precedes that of *Sox9*. Chromatin immunoprecipitation of SRY in mouse embryonic gonads identified a region around 15 kb from the transcriptional start-site of *Sox9* that was bound by SRY, known as TES (Sekido and Lovell-Badge, 2008). The core region of this putative enhancer element, termed TESCO, is capable of driving expression of a *lacZ* reporter gene in an XY-specific fashion in transgenic embryonic gonads. In cell transfection assays the activity of the enhancer is enhanced by the joint recruitment of both SRY and SF1 (NR5A1), a transcriptional co-factor known to be important in gonad formation and subsequent differentiation. These data and other reports support a model in which SRY and co-factors bind to the TESCO enhancer from around 10.5 dpc, resulting in up-regulation of its expression. In the absence of SRY, no such up-regulation occurs and ovary development ensues. Data also support a role for SOX9 in binding to its own gonadal enhancer and maintaining its own expression and effectively 'locking in' Sertoli cell identity. SOX9 remains a marker of Sertoli cells in the testis throughout the life of an individual.

The phenotypic consequences of genetically ablating TES and TESCO have been examined using CRISPR/Cas9-mediated genome editing (Gonen et al., 2017). Loss of either element does not lead to a loss of *Sox9* expression and XY gonadal sex reversal as a consequence. Rather, *Sox9* expression is down-regulated: to 62% of wild-type levels at 14.5 dpc in embryos lacking TESCO, and 44% wild-type levels in those lacking TES at 13.5 dpc. Only in the context of another genetic insult does the loss of either element overtly disrupt the phenotypic course of testis development. These data suggest that another *Sox9* gonadal enhancer (or enhancers) exist that can operate adequately in the absence of TES/TESCO. The identity of these is currently unknown, although alternative regions upstream of the TSS, putatively required for expression of human SOX9, have been reported (Gonen et al., 2017). It is a well-founded prediction that any other functional *Sox9* enhancers will also be bound by SRY, but this will need to be tested in vivo.

FGF9: Propagating the Testis-Determining Signal

The role played by certain genes in sex determination is occasionally identified by serendipitous discovery in a mouse knockout, rather than on the basis of an inviting expression profile or a case of human sex reversal. Embryos lacking the fibroblast growth factor FGF9 exhibit severe lung and limb defects. They also exhibit complete gonadal sex reversal when bred onto the C57BL/6J genetic background (Colvin et al., 2001). This genetic background is known to be sensitized to disruptions to testis determination. Careful examination of gonads from FGF9-deficient embryos revealed that whilst *Sox9* was initially up-regulated as normal in the mutant XY gonad, high levels of *Sox9* were not maintained and expression was gradually lost by 12.5 dpc (Kim et al., 2006). Loss of the pro-testis program of gene expression results in activation of the ovarian program. Interestingly, expression of *Fgf9* was also shown to be lost in XY gonads lacking SOX9. This observed mutual dependence resulted in a model in which SOX9 acts to positively regulate *Fgf9* expression and then FGF9 in turn acts, through its receptor FGFR2, to positively enforce *Sox9* expression: a positive feedback loop. As predicted, loss of FGFR2 itself also causes XY gonadal sex reversal in mice.

The details of this model were called into question when gonad development was examined in mutants lacking both FGF9 and the pro-ovarian signaling molecule, WNT4 (Jameson et al., 2012a). Mutual antagonism between WNT4 and FGF9 had been established by studying gonad development in mutants lacking either gene: XY gonads lacking FGF9 ectopically express high levels of *Wnt4*; XX gonads lacking WNT4 exhibit high levels of *Fgf9* (Kim et al., 2006). These data suggest that in the absence of one of these proteins, sex reversal occurs due to the loss of inhibitory signals that normally oppose the other pathway. When XY embryos were generated lacking both proteins, testes developed. This observation suggests that the primary role of FGF9 in the XY gonad is to repress the anti-testis activity of WNT4; when WNT4 is absent, FGF9 is not required for SRY/SOX9 to initiate and maintain testis development. It is worth noting that the relationship between FGF9 and WNT4 is not, however, symmetric: loss of both proteins in XX gonads does not rescue the partial female-to-male sex reversal phenotype of WNT-deficient XX gonads. Moreover, the molecular nature of this genetically-defined opposition between FGF9 and WNT4 is unclear.

FGF9 is a secreted growth factor that acts in a paracrine fashion to reinforce the cell-autonomous activities of the transcription factors SRY and SOX9. Studies of testis development in vitro, using organ culture methodologies, have been invaluable in dissecting the pathways required for testis determination and have permitted the identification of the cellular process of mesonephric endothelial cell migration as fundamental for the development of testis cords and associated vasculature. Two questions that arise are: how does the testis-determining signal initiated by SRY expression at the center of the gonad spread to the poles, and why is there no appreciable delay in the formation of testis cords at the poles versus the center? Independent culture of XY gonadal

segments (center and poles) in vitro reveals that *Sox9* expression is not maintained in polar tissue when separated from the central segment and testis cord formation is disrupted as a consequence (Hamamatsu et al., 2010). Reconstruction and partition experiments showed that rescue of these defects is not dependent on cellular movement from the center of the gonad; rather, the critical role of the central domain can be substituted by the supply of exogenous FGF9. *Fgf9* expression itself is first detected in the center of the gonad at the 15–16 tail somite stage. These data suggest that FGF9 acts as a rapidly-acting, poleward diffusible factor in XY gonads that promotes testis cord formation via its positive effects on *Sox9* expression. Interestingly, despite FGF9's established role in opposing the pro-ovarian activity of WNT4, reducing levels of WNT4 in XY gonads, using heterozygous *Wnt4* mutant gonads, did not alter the reduced tubulogenic capacity of polar segments.

In acting to support and reinforce the expression of SOX9, FGF9 is functioning in a similar way to another molecule, prostaglandin D2 (PGD2). PGD2 also amplifies SOX9 expression in Sertoli cells—and acts to promote recruitment of cells to the Sertoli lineage—and this hints at the existence of back-up gene networks that exist to buffer the testis-determining signal from disruption (Wilhelm et al., 2005). Along with the antagonistic roles that testis-determining genes play, this buffering likely reflects the potential peril associated with initiating testis or ovary development in a single bipotential primordial, one in which there is expression of molecules from both the testis- and ovary-promoting pathways at early stages. Functional redundancy is also a familiar theme in the study of sex determination, and the roles of certain molecules are sometimes only revealed when other compensating family members are inactivated simultaneously. These genetic mechanisms ensure the canalization of male or female gonad development from a bipotential primordial.

The “Battle of the Sexes”: A Life-Long Struggle

Despite the observation of mutual antagonism between individual pro-testis and pro-ovary gene pairs, such as in the case of FGF9 and WNT4, and SOX9 and RSPO1, it is perhaps best to see the testis- and ovary-determining gene regulatory networks as opposed at multiple points (Fig. 1). Indeed, it is by the very nature of a sex-determining gene that when it is inactivated, complete or partial sex reversal is caused by the ectopic activation of the alternate pathway, establishing the normal inhibitory role of the inactivated gene. There is a sense in which all testis-determining genes that have been characterized are also anti-ovary genes: it is this that accounts for male-to-female gonadal sex reversal. In the case of ovary-promoting genes such as *Wnt4*, *Rspo1* and *Foxl2*, the sex reversal that arises after deletion is only partial, suggesting a greater complexity in the pathways, molecular and cellular, that establish ovarian identity. The molecular basis of the opposition between SRY–SOX9–FGF9 and RSPO1–WNT4–FOXL2 is not well understood, and much research remains to be done to delineate all the points of contact between these pathways. It is highly likely that this will also require the identification of novel testis- and ovary-determining genes.

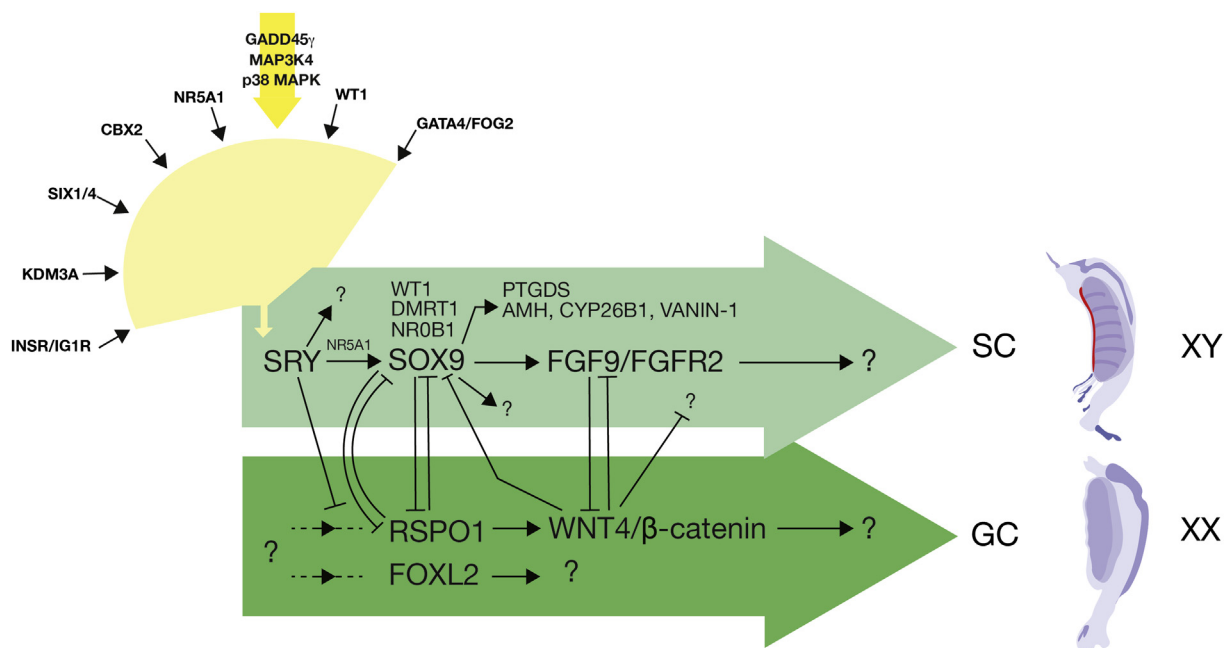


Fig. 1 Mutual antagonism between the testis- and ovary-determining networks. The testis-determining gene regulatory network (SRY–SOX9–FGF9/FGFR2) is shown in the upper (light green) arrow; the ovarian network (RSPO1/WNT4/β-catenin–FOXL2) is shown below in the darker green arrow. Arrows indicate positive regulatory relationships, whilst hammered lines indicate negative interactions. Question marks indicate knowledge gaps, some of which are addressed in the main text. In XY gonadal somatic cells, the testicular pathway results in Sertoli cell (SC) differentiation, which drives testis morphogenesis. This pathway is initiated by SRY activity: *Sry* expression is itself regulated by a set of genes (at the edge of the yellow segment). In the absence of SRY, the pro-ovarian pathway drives granulosa cell (GC) differentiation and ovary formation.

The antagonistic actions of the testis- and ovary-determining gene networks were at one point thought to be required only to establish the fate of the developing testis or ovary during the fetal period. However, conditional gene targeting experiments in mouse adult gonads indicate that the requirement to antagonize opposing pathways in sex identity operates even in adult gonadal lineages. The ubiquitous deletion of FOXL2 in the adult XX ovary at 8-weeks of age results in granulosa cell reprogramming consistent with sex reversal around 3 weeks after ablation (Uhlenhaut et al., 2009). Typical follicular structure is lost and seminiferous tubule-like structures replace them. Cells with the morphological characteristics of Sertoli cells appear and express familiar markers such as *Sox9* and *Dmrt1*. These cells are associated with nearby Leydig-like cells that produce testosterone. ChIP data show that FOXL2 can bind to the *Sox9* enhancer, TESCO, and in vitro transfection assays suggest that FOXL2 can repress TESCO activity. Overall, these data suggest that maintenance of ovarian identity requires continuous expression of FOXL2, which actively represses *Sox9* expression. It is perhaps not surprising that the loss of the pro-testis factors DMRT1 and SOX9 in the adult testis can likewise result in male-to-female sex reversal. Loss of DMRT1 results in up-regulation of FOXL2 and reprogramming of Sertoli cells to a granulosa-like cell identity. In this environment, theca cells appear that produce oestrogen and germ cells become feminized (Matson et al., 2011). It will be important to determine whether this interesting and unexpected plasticity of the adult supporting cell lineage is unique and, if so, whether it simply reflects the common embryonic origin of testicular and ovarian somatic cells or some other property or function of this lineage.

Studying the Transcriptome and Epigenome of Gonadal Somatic Cells

This review has so-far adopted a very gene-centric view of the process of sex determination: we have focused on individual testis-determining genes, defined as such by the gonadal sex reversal caused by their inactivation; and we have examined their roles, especially their inhibitory relationships with known ovary-determining genes. There are numerous genes and proteins with a role in gonad development that have not been discussed, mainly due to the limitations of space, but also due to their roles being somewhat 'downstream' of sex-determination, in cellular differentiation and physiology, or due to a role in establishing the fate of lineages other than the supporting cells. In this section, we will take a genome-wide view of the molecular genetics of sex determination: what is known about the transcriptomes of the developing supporting cells at distinct stages, and the epigenomes that underlie them?

Researchers have used expression profiling in the developing mouse gonad for many years, focusing on identifying transcripts exhibiting sexually dimorphic expression, as a means of discovering new candidate sex-determining genes. Experiments have grown in sophistication since early glass-slide microarray experiments that assayed a few thousand genes and newer technologies permit the examination of whole transcriptomes of distinct gonadal cell lineages over several days of development (Jameson et al., 2012b). Such studies are key to understanding the contribution made by specific cell-types to sex determination and the disruption caused by gene mutation or other insults. It is likely that, in the near future, the analysis of single-cell transcriptomes will shed light on the dynamism and heterogeneity of transcriptional control during gonadogenesis.

The molecular basis of differential transcription is attributed to control of the epigenome: the totality of chemical modifications to DNA and its associated histone proteins and RNA molecules that is thought to determine the accessibility of chromatin to transcription factors and the basal transcriptional machinery. Our knowledge of the relationship between dynamic epigenomic events, including long-range interactions between enhancers and promoters, is growing, as is our understanding of how epigenomic marks are deposited, read and erased in distinct cell-types. However, the technologies used to study such events are still in their relative infancy: our ability to determine the epigenomic characteristics of limiting numbers of cells, or in some cases an individual cell, is highly restricted. In this sense, epigenomics is lagging behind transcriptomics. But single-cell approaches in epigenomics promise to shed light on cellular heterogeneity and integration during the establishment of organ fate and this promise is likely to drive the technology forward.

The importance of epigenomic marks to sex determination is clear from XY gonadal sex reversal phenotypes associated with the deletion in mice of two chromatin-associated proteins: the polycomb group protein CBX2 (M33) and the histone demethylase KDM3A (JMJD1A). Sex-reversal in both cases is associated with disrupted *Sry* expression. CBX2 is a component of the polycomb-repressive complex 1 (PRC1), which functions in regulation of gene expression through recognition of methylated histones and methylation of histones. XY gonadal sex reversal in the *Cbx2* KO mouse embryo is rescued by forced expression of an *Sry* transgene, suggesting that other events downstream of *Sry* expression are not disrupted by the loss of CBX2 (Katoh-Fukui et al., 2012). KDM3A, a histone demethylase, is reported to be recruited to the *Sry* locus. Loss of KDM3A on a sensitized genetic background results in XY gonadal sex reversal associated with a significant increase in levels of the inhibitory epigenetic mark H3K9me2 at *Sry* (Kuroki et al., 2013). The authors propose that KDM3A-mediated H3K9 demethylation may induce deheterochromatinization of *Sry*, allowing access to H3K4 methyltransferase and additional transcription factors/co-factors that drive transcription.

Genome-wide characterization of the epigenome of developing gonadal cell lineages is an area of increasing interest, partly because it is now a more tractable task due to improvements in methodologies using very limiting cell numbers. One of the first attempts at such a study reported the analysis of fetal Sertoli cells by DNase1-seq and ChIP-seq for the acetylated histone, H3K27Ac, a mark of active enhancers (Maatouk et al., 2017). Regions of open chromatin, that are putative regulatory sites, are DNase1-sensitive, and DNase1-seq identifies such sites in an unbiased fashion. Sertoli cells purified from the gonad at 15.5 dpc, using FACS, were subjected to DNase1-seq, which identified over 81,000 DNase1 hypersensitive sites (DHSs) at various positions

with respect to nearby genes. One such DHS was found at the *Sox9* gonadal enhancer, TESCO. Similar experiments were also performed at 13.5 dpc and were compared with data from other tissues to identify Sertoli cell-specific DHSs. Interestingly, Sertoli cell DHSs were associated with Sertoli cell- and pregranulosa cell-expressed genes, whilst, as expected, H3K27Ac was enriched near Sertoli cell-expressed genes only. Open Sertoli cell chromatin in the vicinity of pro-ovarian genes may reflect binding of transcription factors that preserve a poised chromatin state or of repressive factors that maintain the Sertoli cell state. The dual use of such regulatory sites to act as repressors in the Sertoli lineage and enhancers in granulosa cells might also underlie the plasticity of the supporting cell lineage discussed earlier. A similar dual-use model applies to TESCO, which is bound by SOX9 in Sertoli cells and FOXL2 in granulosa cells. Further studies, particularly of the chromatin state of pre-granulosa cells, will be required to investigate the generality of this model. Overall, such studies offer a rich resource for identifying novel regulatory elements required for sex determination, some of which will mediate the mutual antagonism of the male and female pathways.

Knowledge Gaps and Future Research

Here, we have reviewed the central role of the testis-determining axis of SRY–SOX9–FGF9, but studies of individuals with unexplained cases of DSDs, including 46,XY CGD and 46,XX testicular DSD, suggest that other genes remain to be identified that play an important role in the specification of the gonadal supporting cell lineage. Moreover, we have not had the space to discuss other molecules whose precise place in the hierarchy of testis determination remains unclear despite a role having been established by knockout studies in the mouse. These include NR0B1 (DAX1), WT1 and GATA4/FOG2. In addition, we have not had space to discuss what is known about the origin and specification of other important testicular cell lineages, such as the interstitial peritubular myoid and Leydig cells. The fate of germ cells in the developing testis and ovary is also an area of intensive investigation.

Finally, novel technologies are likely to transform how we study mammalian testis-determining genes. Genome editing, whole genome/transcriptome/epigenome analyses, the development of cell and organoid models in mice and humans, single cell approaches and mathematical modeling should all allow more rapid progress to be made in our understanding of how genes, gene products and cells interact in space and time in this fascinating multi-lineage organ in order to specify gonadal fate and sexual identity.

Acknowledgments

We wish to thank Rebecca Muir for assistance in the production of Fig. 1. We apologize to colleagues whose research was omitted due to space constraints. Research in the Greenfield laboratory at Harwell is funded by the UK Medical Research Council (MC_U142684167).

References

- Barriónuevo, F., Bagheri-Fam, S., Klattig, J., Kist, R., Taketo, M. M., Englert, C., & Scherer, G. (2006). Homozygous inactivation of *Sox9* causes complete XY sex reversal in mice. *Biology of Reproduction*, 74, 195–201.
- Bashamboo, A., & McElreavey, K. (2015). Human sex-determination and disorders of sex development (DSD). *Seminars in Cell & Developmental Biology*, 45, 77–83.
- Bogani, D., Siggers, P., Brixey, R., Warr, N., Beddow, S., et al. (2009). Loss of mitogen-activated protein kinase kinase 4 (MAP3K4) reveals a requirement for MAPK signalling in mouse sex determination. *PLoS Biology*, 7, e1000196.
- Carre, G. A., & Greenfield, A. (2014). Characterising novel pathways in testis determination using mouse genetics. *Sexual Development*, 8, 199–207.
- Chassot, A. A., Gillot, I., & Chaboissier, M. C. (2014). R-spondin1, WNT4, and the CTNNB1 signaling pathway: Strict control over ovarian differentiation. *Reproduction*, 148, R97–110.
- Colvin, J. S., Green, R. P., Schmahl, J., Capel, B., & Ornitz, D. M. (2001). Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell*, 104, 875–889.
- Foster, J. W., Dominguez-Steglich, M. A., Guioli, S., Kwok, C., Weller, et al. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. *Nature* 372, 525–530.
- Gonen, N., Quinn, A., O'Neill, H. C., Koopman, P., & Lovell-Badge, R. (2017). Normal levels of *Sox9* expression in the developing mouse testis depend on the TES/TESCO enhancer, but this does not act alone. *PLoS Genetics*, 13, e1006520.
- Hiramatsu, R., Matoba, S., Kanai-Azuma, M., Tsunekawa, N., Katoh-Fukui, Y., et al. (2008). A critical time window of *Sry* action in gonadal sex determination in mice. *Development*, 136, 129–138.
- Hiramatsu, R., Harikae, K., Tsunekawa, N., Kurohmaru, M., Matsuo, I., & Kanai, Y. (2010). FGF signaling directs a center-to-pole expansion of tubulogenesis in mouse testis differentiation. *Development*, 137, 303–312.
- Jakob, S., & Lovell-Badge, R. (2011). Sex determination and the control of *Sox9* expression in mammals. *The FEBS Journal*, 278, 1002–1009.
- Jameson, S. A., Lin, Y. T., & Capel, B. (2012a). Testis development requires the repression of *Wnt4* by *Fgf* signaling. *Developmental Biology*, 370, 24–32.
- Jameson, S. A., Natarajan, A., Cool, J., DeFalco, T., Maatouk, D. M., Mork, L., Munger, S. C., & Capel, B. (2012b). Temporal transcriptional profiling of somatic and germ cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS Genetics*, 8, e1002575.
- Katoh-Fukui, Y., Miyabayashi, K., Komatsu, T., Owaki, A., Baba, T., et al. (2012). *Cbx2*, a polycomb group gene, is required for *Sry* gene expression in mice. *Endocrinology*, 153, 913–924.
- Kim, Y., Kobayashi, A., Sekido, R., DiNapoli, L., Brennan, J., et al. (2006). *Fgf9* and *Wnt4* act as antagonistic signals to regulate mammalian sex determination. *PLoS Biology*, 4, e187.
- Kuroki, S., Matoba, S., Akiyoshi, M., Matsumura, Y., Miyachi, H., et al. (2013). Epigenetic regulation of mouse sex determination by the histone demethylase *Jmjd1a*. *Science*, 341, 1106–1109.
- Larney, C., Bailey, T. L., & Koopman, P. (2014). Switching on sex: Transcriptional regulation of the testis-determining gene *Sry*. *Development*, 141, 2195–2205.
- Maatouk, D. M., Natarajan, A., Shibata, Y., Song, L., Crawford, G. E., Ohler, U., & Capel, B. (2017). Genome-wide identification of regulatory elements in Sertoli cells. *Development*, 144, 720–730.

- Matson, C. K., Murphy, M. W., Sarver, A. L., Griswold, M. D., Bardwell, V. J., & Zarkower, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature*, *476*, 101–104.
- Pannetier, M., Chassot, A. A., Chaboissier, M. C., & Pailhoux, E. (2016). Involvement of FOXL2 and RSP01 in ovarian determination, development, and maintenance in mammals. *Sexual Development*, *10*, 167–184.
- Pearlman, A., Loke, J., Le Caignec, C., White, S., Chin, L., et al. (2010). Mutations in *MAP3K1* cause 46, XY disorders of sex development and implicate a common signal transduction pathway in human testis determination. *American Journal of Human Genetics*, *87*, 898–904.
- Rahmoun, M., Lavery, R., Laurent-Chaballier, S., Bellora, N., Philip, G. K., et al. (2017). In mammalian foetal testes, SOX9 regulates expression of its target genes by binding to genomic regions with conserved signatures. *Nucleic Acids Research*. <https://doi.org/10.1093/nar/gkx328>.
- Sekido, R., & Lovell-Badge, R. (2008). Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. *Nature*, *453*, 930–934.
- Uhlenhaut, N. H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., et al. (2009). Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell*, *139*, 1130–1142.
- Warr, N., & Greenfield, A. (2012). The molecular and cellular basis of gonadal sex reversal in mice and humans. *WIREs Developmental Biology*, *1*, 559–577.
- Warr, N., Carre, G. A., Siggers, P., Faleato, J. V., Brixey, R., et al. (2012). Gadd45gamma and Map3k4 interactions regulate mouse testis determination via p38 MAPK-mediated control of Sry expression. *Developmental Cell*, *23*, 1020–1031.
- Wilhelm, D., Martinson, F., Bradford, S., Wilson, M. J., Combes, A. N., Beverdam, A., Bowles, J., Mizusaki, H., & Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Developmental Biology*, *287*, 111–124.

Pubertal Development

Lindsey A Loomba-Albrecht, University of California Davis Medical Center, Sacramento, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Adipose tissue Fat tissue; main role is to store energy for the body.

Bone accretion The process of growth of bone, typically by gradual increase over time.

Bone mineral density The amount of bone mineral (such as calcium) within bone tissue.

Estrogen The primary female sex hormone; also plays important roles in male development, including major effects on growth.

Lean body mass The portion of body weight that is not fat (including bone, water, and muscle tissue).

Protein synthesis The process by which protein is made; fundamental aspect of body growth.

Sexual dimorphism A difference in size or appearance between males and females.

Testosterone The primary male sex hormone; plays a critical role in the development of male reproductive tissues, promotes secondary sexual development and influences changes in growth and body composition.

Introduction

Puberty is the period of human development during which sexual maturation occurs. Boys normally begin puberty between 9 and 14 years of age, with an average age of onset of 10 years. In boys, puberty begins when hormonal signals from the hypothalamus and pituitary gland in the brain stimulate the testes to begin producing the hormone testosterone. Testosterone levels rise, leading ultimately to a rapid increase in height, the development of secondary sexual characteristics, and other physical changes. Pubertal progression can be described using standards proposed by Tanner (known as Tanner stages or sexual maturity rating stages). In boys, genital changes are described in five stages, from prepubertal (Tanner stage 1), to fully mature (Tanner stage 5) (Marshall and Tanner, 1970).

The developmental changes that occur during puberty are governed largely by hormonal factors, though environment and genetic factors are also important. The end result of puberty is a striking sexual dimorphism between mature males and females. In addition to gender differences in sexual characteristics, mature males possess more lean body mass and less body fat than females. Males are taller, have greater bone mass and have different body proportions. An understanding of normal pubertal developmental change is important in order to understand when a disease state may be present.

Linear Growth

After a period of relatively stable growth velocity in mid childhood, a significant increase in growth velocity occurs during puberty. This is known as the pubertal growth spurt, and it results in a gain of about 28 cm of height in males (Marshall and Tanner, 1970; Kelch, 1994). In boys, the pubertal growth spurt occurs toward the end of puberty, whereas in girls the growth spurt is an early pubertal event. Thus, boys have a longer period of prepubertal growth than girls, and a boy who is in early puberty likely has significant growth remaining. The difference in adult height between men and women in the United States of 12 cm (roughly 5 in.) is due partly to heights attained prior to puberty (since boys have a longer time period for growth), and partly due to total height gained during the pubertal growth spurt. In boys, peak height velocity is about 11.3 cm/year and occurs at an average age of 13.7 years (compared with 12.1 years and 9.8 cm/year in girls) (Granados et al., 2015). There may be considerable individual variation in these figures as overall health and nutrition, body weight, adiposity, hormonal abnormalities, chronic disease, and family patterns may all influence timing and degree of linear growth.

The onset and control of the pubertal growth spurt depends on a number of hormonal factors (see Table 1). Rising testosterone levels occurring during the time of puberty lead to higher estradiol levels in boys through peripheral conversion of testosterone to estrogen. Testosterone and estrogen (known as “gonadal steroids” since they are steroid hormones produced in the gonads) play a critical role in stimulating growth hormone production. Growth hormone is a major regulator of linear growth, and works primarily by increasing insulin like growth factor-1 (IGF-1) levels. IGF-1 has growth-promoting effects on many tissues throughout the body, including cartilage, bone, and skeletal muscle. Thus, GH and IGF-1 levels increase in puberty in response to rising levels of gonadal steroids. In addition, circulating thyroid hormone produced by the thyroid gland plays an important role in linear growth. Deficiency in any of these hormones may decrease or eliminate the pubertal growth spurt.

The epiphyseal plate (also known as the growth plate) is the portion of the long bone where growth occurs and length is added. Each long bone typically has two growth plates (one on each end of the bone) which are composed primarily of cartilage. Cartilage

Table 1 Hormonal effects on male pubertal growth

<i>Hormone</i>	<i>Primary source</i>	<i>Major effects</i>
Growth hormone (GH)	Pituitary gland	• Promotion of linear growth
IGF-1	Liver	• Stimulation of IGF-1 production
		• Increased protein synthesis
		• Cell growth and proliferation
Testosterone	Testes	• Increased cartilage and skeletal growth
		• Development of secondary sexual characteristics
Estrogen	Converted from testosterone in peripheral tissues	• Promotion of linear growth
		• Bone maturation and increased bone density
		• Cessation of linear growth
Thyroid hormone	Thyroid gland (anterior neck)	• Linear growth and bone maturation
		• Brain development
		• Increased protein synthesis

Styne, D.M. 2016. Disorders of growth, In Styne, D.M. *Pediatric endocrinology: A clinical handbook*. Cham: Springer International, 47–89; Styne, D.M. 2016. Disorders of puberty, In Styne, D.M. *Pediatric endocrinology: A clinical handbook*. Cham: Springer International, 189–232.

cells, or chondrocytes, in the growth plate divide rapidly and ultimately are calcified and converted to bone. This process stops and growth cessation occurs once the chondrocytes undergo programmed cell death under the influence of estrogen. Completed growth is observed radiographically on an X-ray as an epiphyseal line (showing closure of the epiphysis).

Linear growth is accompanied by advancement of skeletal development. This is measured clinically by a study known as a bone age, which looks at development of specific bones in the hand and wrist. As a child progresses through puberty, the bones in the hand and wrist change in a predictable manner; this provides an estimate of skeletal age. The further the skeletal age is from the age of epiphyseal fusion (a bone age of about 17 years in boys), the more growth that remains. For example, a healthy boy with short stature but a delayed bone age likely has significant growth remaining. Alternately, a short boy with a significantly advanced bone age may have very little remaining growth potential.

Body Composition

Progression through puberty is marked by gender specific changes in body composition, which refers to the amount or distribution of fat, bone, water, and muscle in the body. Body composition in a given individual is determined by many factors, and is particularly influenced by nutrition and physical activity. Pubertal changes in body composition are regulated largely by hormonal processes and ultimately result in significant differences between men and women. In early childhood, boys and girls have similar fat mass and fat free mass and prepubertal boys have only slightly more skeletal mass and bone mineral density than prepubertal girls. By the end of puberty, however, boys have significantly more lean body mass and skeletal mass and females have significantly greater fat mass (see [Table 2](#)).

Measurement

A variety of modalities are used to measure body composition. The technique of choice is determined by ease of use, need for accuracy, expense, and degree of radiation exposure.

Field techniques are easy to use and low risk, but generally suffer in terms of accuracy. Body mass index (BMI) is a calculation commonly made in clinical practice, but has major limitations for assessing adiposity in children. Skinfold thickness, measured

Table 2 Overview of pubertal changes in body composition

<i>Trait</i>	<i>Males</i>	<i>Females</i>	<i>Sexual dimorphism by early adulthood</i>
Fat mass	Modest increase	Increases	F > M
Body fat percentile	Stable	Substantially increases	F > M
Muscle mass	Substantial increase	Increases	M > F
Bone mass	Increases	Increases	M > F

M, male; F, female.

Veldhuis, J.D., Roemmich, J.N., Richmond, E.J., et al. 2005. Endocrine control of body composition in infancy, childhood, and puberty. *Endocrine Reviews* **26**(1), 114–146; Lombar-Albrecht, L.A., Styne, D.M. 2009. Effect of puberty on body composition. *Current Opinion in Endocrinology, Diabetes and Obesity* **16**(1), 10–15; Wells, J.C. 2007. Sexual dimorphism of body composition. *Best Practice & Research: Clinical Endocrinology & Metabolism* **21**(3), 415–430; Hills, A.P., Byrne, N.M. 2010. An overview of physical growth and maturation. *Medicine and Sport Science* **55**, 1–13.

using a tool called a caliper, can provide an estimate of adiposity. This is an inexpensive and readily available technique, but is not highly accurate owing to differences in examiner technique and subject variability in age, ethnicity, and gender. Bioelectrical impedance analysis (BIA) is commercially available, noninvasive and portable, and provides a better estimate of adiposity than skinfold thickness. BIA measures electrical impedance (opposition to flow of current through the body), which provides an estimate of total body water. This estimate is then used to determine fat free mass and body fat.

More accurate techniques include dual energy X-ray absorptiometry (DXA), which is highly available and has little radiation exposure. DXA provides a good estimate of bone mineral content and can additionally estimate fat free mass and fat mass. Computed tomography (CT) and magnetic resonance imaging (MRI) allow for accurate calculation of adipose tissue and fat free mass; CT is not routinely used due to concerns over radiation exposure in children. Other techniques are even more accurate, but are reserved for research settings given the specialized equipment and operating expenses required.

Skeletal Development and Bone Density

Total bone mass increases during childhood, with rapid increases in bone mass occurring during puberty. The average age of peak calcium accretion (the time where calcium is laid down into the skeleton most rapidly) is age 14.1 years for boys (Bailey et al., 2000), which occurs about 6 months after peak height velocity in males. Approximately 25%–40% of adult calcium is laid down during the 2 years surrounding the pubertal growth spurt (Bailey et al., 2000; Bailey et al., 1999; Baxter-Jones et al., 2011), making this time period critical for lifelong skeletal health. The major increase in bone mass accumulation occurs in later pubertal stages (Zemel, 2013); males ultimately reach peak bone mass in early adulthood (Berger et al., 2010). Young adult males have greater bone mass than adult females due to overall larger body size.

Many factors influence skeletal development during puberty, including hormonal influences and interplay with other aspects of physical growth. Estrogen plays a critical role in bone maturation and the attainment of normal bone mineral density in both sexes, with effects on new bone formation, bone mineralization, epiphyseal maturation, and establishment of normal skeletal proportions. Other hormones, including androgens, growth hormone, and IGF-1 also contribute to peak bone mass.

Lean body mass also affects bone mineral content, via favorable effects of weight-loading and mechanical strain on bone accretion. Weight bearing, as well as forces generated by muscle contraction, cause bones to adapt their shape and increase density in response to such loads. Fat free mass, as opposed to fat mass, seems to have the major effect on bone development. Obese children have stronger bones, and this appears to be due to effects of increased fat free mass, not their increased adiposity (Sioen et al., 2016). Of note, peak lean body mass accretion appears to occur slightly before peak calcium accretion in boys.

Evaluation of bone mineralization in children is primarily performed clinically through DXA scanning. It is imperative that correct standards for age are used, as comparison to normal adult standards will result in inaccurate reports suggesting low bone density (as young children and adolescents have not yet reached peak bone mass). Pediatric reports utilize z-scores to quantify bone density. These are standard deviation scores which describe how far a given patient's bone mineral density is from matched control values. Caution must be used in interpreting the results, as inattention to the patient's age, gender, ethnicity, and maturity level will result in erroneous values.

Lean Body Mass

Skeletal muscle comprises the majority (>50%) of lean body mass and plays a critical role in movement and locomotion, thermoregulation and metabolism. In early childhood, boys appear to have slightly greater muscle mass than girls, with one study showing 10% greater musculature around the spine in prepubertal boys as compared with girls (Arfai et al., 2002). However, during puberty, this difference becomes pronounced. Boys gain considerable amounts of lean mass and relatively little fat mass during the pubertal years; at maturity, men have about 1½ times the lean body mass (and skeletal mass) of women. Regional differences are also apparent with increased muscularization of the upper body in men compared to women. Men also tend to have greater mass of thigh muscles in front of the femur (as opposed to behind it) compared to women.

Increasing muscular growth in pubertal boys occurs largely as the result of rising testosterone levels. An increase in testosterone stimulates muscle protein synthesis and muscle fiber growth, as well as increased fat breakdown (called lipolysis). Strength is related to muscle size; strength relative to muscle mass is nearly the same in men and women.

Fat Mass

Total fat mass is similar in males and females until just before puberty. During puberty, boys do not significantly change their absolute fat mass. Their percent body fat declines throughout puberty as a result the relatively stable fat mass and increasing fat free mass. By the end of puberty, percent body fat approximates the adult male value of 10%–15% (versus 25% in adult females) (Veldhuis et al., 2005). During puberty, males develop an inverted triangle body shape, with broadening of the shoulders and chest. Fat distribution may become more android in nature, with fat distributed mainly in the abdominal area. This is in contrast to the gynecoid shape (with fat distributed primary on the hips and thighs), which may become more pronounced in females during the pubertal years. Sexual dimorphism in shape is greatest in young adults, and declines with age (Wells, 2007).

Leptin is a metabolically active molecule which acts to regulate appetite and energy expenditure. It is derived from adipose tissue and circulating leptin levels correlate with total fat mass. In prepubertal boys and girls, leptin levels are similar. However, during

puberty, leptin levels decline in males at least partially as a result of increasing testosterone secretion. In females, leptin levels appear to rise under the influence of estradiol. Leptin levels thus exhibit striking sexual dimorphism and are affected by both gonadal steroids and fat mass.

Obesity will result in taller stature in childhood and earlier pubertal development; adult height is not affected. On a population level, the obesity epidemic appears to be leading to early puberty in overweight boys and may be leading to delayed puberty in obese boys. The norms for pubertal timing in males have not yet changed as a result, however.

Brain Development

The brain changes both structurally and functionally during adolescence. The gray matter of the brain, the portion containing the cell bodies, begins a period of increased growth just prior to puberty. During this time period, an increased number of connections form which are ultimately pruned back later in adolescence. This maturation has been described as an inverted-U shaped pattern of development. In the prefrontal cortex, an area of the brain located behind the forehead, this process appears to contribute to increasing impulse control and better judgment in older adolescents as compared with younger ones. The frontal and parietal lobes of the brain attain peak gray matter levels around age 12 in boys (and age 11 in girls), prior to undergoing thinning into adulthood (Giedd et al., 1999). The white matter of the brain is composed of bundles of myelinated axons, which connect gray matter areas and carry nerve signals. White matter volume increases steadily during childhood and adolescence, with males showing greater white matter increases than females (Perrin et al., 2009). Additionally, certain regions of the brain have been shown to develop sexual dimorphism during the adolescent years. Pubertal hormones appear to contribute to this process.

Additional Physical Changes

Primary and Secondary Sexual Characteristics

The first physical change in male puberty is enlargement of the testes. Further growth of the penis and testes, as well as pubic hair development, occur under the influence of gonadal and adrenal hormones. Primary sexual characteristics are present at birth and include the testes and penis in the male. Secondary sexual characteristics are features that develop during puberty but are not directly involved in reproduction. These characteristics may distinguish males from females and arise for the most part due to the influence of pubertal hormones.

Other Changes

In pubertal boys, the larynx, cricoid cartilage and laryngeal muscles increase in size (and the “Adam’s apple” is formed). Vocal cords lengthen and the voice deepens, with abrupt changes in vocal quality occurring between Tanner stage 3 and 4 (Harries et al., 1997). Growth of body hair, including facial, underarm, abdominal, chest, and pubic hair occurs. Sebaceous glands enlarge and sweat production increases due to increased activity of sweat gland. Facial morphology changes, and the male face becomes more square, with more angular features and a relatively larger nose and mandible (jaw bone). Hands and feet become relatively larger as well. Enlargement of the penis and testes occur as noted above.

Conclusion

Puberty is a time of major developmental change in males. Changes in body composition, linear growth and sexual development all result in significant sexual dimorphism by the end of puberty. These changes are largely regulated by endocrine factors.

References

- Arfaei, K., Pitukcheewanont, P. D., Goran, M. I., et al. (2002). Bone, muscle, and fat: Sex-related differences in prepubertal children. *Radiology*, 224(2), 338–344.
- Bailey, D. A., Martin, A. D., McKay, H. A., Whiting, S., & Mirwald, R. (2000). Calcium accretion in girls and boys during puberty: A longitudinal analysis. *Journal of Bone and Mineral Research*, 15(11), 2245–2250.
- Bailey, D. A., McKay, H. A., Mirwald, R. L., Crocker, P. R., & Faulkner, R. A. (1999). A six-year longitudinal study of the relationship of physical activity to bone mineral accrual in growing children: The university of Saskatchewan bone mineral accrual study. *Journal of Bone and Mineral Research*, 14(10), 1672–1679.
- Baxter-Jones, A. D., Faulkner, R. A., Forwood, M. R., Mirwald, R. L., & Bailey, D. A. (2011). Bone mineral accrual from 8 to 30 years of age: An estimation of peak bone mass. *Journal of Bone and Mineral Research*, 26(8), 1729–1739.
- Berger, C., Goltzman, D., Langsetmo, L., et al. (2010). Peak bone mass from longitudinal data: Implications for the prevalence, pathophysiology, and diagnosis of osteoporosis. *Journal of Bone and Mineral Research*, 25(9), 1948–1957.
- Giedd, J. N., Blumenthal, J., Jeffries, N. O., et al. (1999). Brain development during childhood and adolescence: A longitudinal MRI study. *Nature Neuroscience*, 2(10), 861–863.
- Granados, A., Gebremariam, A., & Lee, J. M. (2015). Relationship between timing of peak height velocity and pubertal staging in boys and girls. *Journal of Clinical Research in Pediatric Endocrinology*, 7(3), 235–237.
- Harries, M. L., Walker, J. M., Williams, D. M., Hawkins, S., & Hughes, I. A. (1997). Changes in the male voice at puberty. *Archives of Disease in Childhood*, 77(5), 445–447.

- Kelch, R. P. (1994). Adolescent sexual development. In M. S. Kappy, R. M. Blizzard, & C. J. Migeon (Eds.), *The diagnosis and treatment of endocrine disorders in childhood and adolescence* (4th ed., pp. 193–234). Springfield: Charles C. Thomas Publisher.
- Marshall, W. A., & Tanner, J. M. (1970). Variations in the pattern of pubertal changes in boys. *Archives of Disease in Childhood*, 45(239), 13–23.
- Perrin, J. S., Leonard, G., Perron, M., et al. (2009). Sex differences in the growth of white matter during adolescence. *NeuroImage*, 45, 1055–1066.
- Sioen, I., Lust, E., De Henauw, S., Moreno, L. A., & Jimenez-Pavon, D. (2016). Associations between body composition and bone health in children and adolescents: A systematic review. *Calcified Tissue International*, 99(6), 557–577.
- Veldhuis, J. D., Roemmich, J. N., Richmond, E. J., et al. (2005). Endocrine control of body composition in infancy, childhood, and puberty. *Endocrine Reviews*, 26(1), 114–146.
- Wells, J. C. (2007). Sexual dimorphism of body composition. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 21(3), 415–430.
- Zemel, B. (2013). Bone mineral accretion and its relationship to growth, sexual maturation and body composition during childhood and adolescence. *World Review of Nutrition and Dietetics*, 106, 39–45.

Further Reading

- Binkovitz, L. A., Henwood, M. J., & Sparke, P. (2008). Pediatric DXA: Technique, interpretation and clinical applications. *Pediatric Radiology*, 38, S227–S239.
- Blakemore, S. J., Burnett, S., & Dahl, R. E. (2010). The role of puberty in the developing adolescent brain. *Human Brain Mapping*, 31(6), 926–933.
- Hills, A. P., & Byrne, N. M. (2010). An overview of physical growth and maturation. *Medicine and Sport Science*, 55, 1–13.
- Lomba-Albrecht, L. A., & Styne, D. M. (2009). Effect of puberty on body composition. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 16(1), 10–15.
- Styne, D. M. (2016a). Disorders of growth. In D. M. Styne (Ed.), *Pediatric endocrinology: A clinical handbook* (pp. 47–89). Cham: Springer International.
- Styne, D. M. (2016b). Disorders of puberty. In D. M. Styne (Ed.), *Pediatric endocrinology: A clinical handbook* (pp. 189–232). Cham: Springer International.
- Wells, J. C., Treleaven, P., & Cole, T. J. (2007). BMI compared with 3-dimensional body shape: The UK national sizing survey. *The American Journal of Clinical Nutrition*, 85(2), 419–425.

TESTIS PHYSIOLOGY

Testis Physiology—Overview and Histology

Nathália LM Lara, Guilherme MJ Costa, Gleide F Avelar, and Samyra MSN Lacerda, Federal University of Minas Gerais, Belo Horizonte, Brazil

Rex A Hess, University of Illinois at Urbana-Champaign, Urbana, IL, United States

Luiz R de França, Federal University of Minas Gerais, Belo Horizonte, Brazil; and National Institute for Amazonian Research, Manaus, Brazil

© 2018 Elsevier Inc. All rights reserved.

Functional Organization of the Testis

Testis is the male reproductive gonad. It yields two main functions: the production of testosterone, the male sexual hormone, and sperm. These functions are crucial for the maintenance of male characteristics, but also for the preservation of species, which will be further described in this article. The mammalian testis is covered by a protective fibrous capsule, the tunica albuginea, composed mainly of collagen with some fibroblastic and smooth muscle elements. The thickness, composition and amount of connective tissue shows tremendous variation among species and its percentage in the testis ranges from ~4% in mice to ~20% in humans. The capsule extends from the tunica inward, forming the testicular parenchyma, but also delineates the rete testis and the mediastinum. Besides its protective function, this capsule is also involved in regulating blood flow, temperature, pressure and sperm movement (Russell and França, 1995).

Functionally, the testis parenchyma is organized into two major compartments: tubular and intertubular, also called the interstitium (Fig. 1). The tubular compartment is composed by seminiferous tubules that can be subdivided from its exterior wall to its center into tunica propria, seminiferous epithelium and tubular lumen. The tunica propria contains the peritubular smooth muscle-like myoid cells, that form one (ex: mice and rat) to several layers (ex: humans and marsupials) according to the species, containing also collagen and laminin fibers. The seminiferous epithelium is a highly organized tubular lining that is integrated with germ cells and the somatic, supporting Sertoli cells, which also secrete the fluid responsible for tubular lumen formation and sperm transport. No blood vessels, nerves or other cell types are present in the seminiferous epithelium. The tubular diameter shows high variation among mammals (Table 1), ranging from ~160 to ~450 μm in mammals. In the intertubular compartment, the steroidogenic Leydig cells are usually the most frequent cell type present. Besides these cells, blood and lymphatic

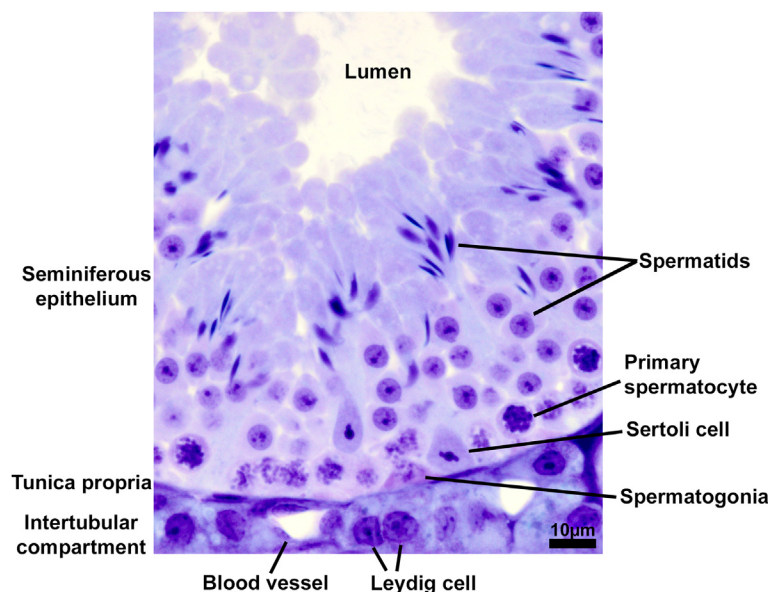


Fig. 1 Testis parenchyma composition in adult mouse, depicting the two main compartments of the testis: tubular and intertubular. The tubular compartment is constituted by the tunica propria, constituted by peritubular myoid cells and basal lamina, and seminiferous epithelium, composed by Sertoli cells and germ cells in different stages of development, with a central tubular lumen. In the intertubular compartment, Leydig cells are found adjacent to blood vessels, lymphatic vessels (not shown) and fibers and cells of connective tissue (not shown).

Table 1 Comparative parameters related to testis stereology and spermatogenic events in mammalian species

	GSI ^a (%)	Tubular diameter (μm)	Seminiferous tubule (%)	LC (%)	LC volume (μm ³)	LC per testis gram (× 10 ⁶)	SC per testis gram (× 10 ⁶)	SC efficiency ^b	DSP per testis gram (× 10 ⁶)
Mouse	0.55–0.76	216	91–93	3.7–5.3	1021–1450	29–49	39–41	10.5–11.5	45–48
Rat	0.76	354	89	2.8	1207	12.6	27	8–10.3	17–24
Hamster	2.13	275	92.5	2.7	1092	55	44.5	8.2	24
Capybara	0.12	205	50	32.1	2029	186	19	5.6	10
<i>Akodon montensis</i>	1.1	233	96.6	1.1	807	14.6	54.7	10.3	62
Collared peccary	0.21	255	77.4	12.8	1170	120	28	11.1	23.4
Boar	0.4	224	83–85	7–19	1100–2200	60–120	16–39	12.4	20–27
Cat	0.078	223	88	6	2044	30	32	5.1	16
Dog	0.07–0.33	209–237	85–90	4–9	756–1064	46–111	33–41	6–8	14–22
Horse	0.09	230	61–73	18.4	4300	21–24	28	8.7–10.5	16–21
Marmoset	0.36	256	92.4	2.0	1416	13.9	35	8	18
Human	0.08	280	62	6.4	3000–4300	9	49	3	4–4.5

LC, Leydig cell; SC, Sertoli cell; DSP, daily sperm production.

^aGonadosomatic index = testis mass divided by body mass.

^bRound spermatids per Sertoli cell (SC).

vessels, nerves, mast cells, macrophage and dendritic cells, as well as fibroblasts and connective tissue fibers are found in the interstitium, and their amount varies significantly according to the species. Although spermatogenic and steroidogenic activities occur in two distinct functional compartments of the testis, their full accomplishments require very complex cellular and physiological interactions between both, an important aspect that is advanced in other chapters.

As shown in **Table 1**, although the testis compartments are similarly organized among the mammalian species already investigated, high variation is noted for several representative parameters, such as the gonadosomatic index (testis mass/body mass), the percentage occupied by seminiferous tubules and Leydig cells in testicular parenchyma, tubular diameter, Leydig and Sertoli cell numbers, Sertoli cell efficiency and, as a consequence, spermatogenic efficiency (daily sperm production per testis gram). For instance, human spermatogenic efficiency is very low, particularly when compared to rodents. Large variation is also observed for the percentage of Leydig cells, ranging from ~1% in *Akodon montensis* to ~30% in capybara, the biggest rodent worldwide, with a predominant investment in male dominance through androgens, instead of sperm production.

Fig. 2 illustrates testis histology in some mammalian species depicted in **Table 1**. In this figure, it is easy to recognize the large area occupied by connective tissue within the intertubular compartment in human testis, whereas the percentage of interstitial space taken by Leydig cells in capybaras can reach up to 80% in some males. In the collared peccary, these steroidogenic cells present a unique and peculiar arrangement in the testis parenchyma, forming dark stripes around the seminiferous tubules lobes, due to the high lipid content in the cytoplasm of these cells. It is considered that these functional differences among species are related, for instance, to their reproductive strategies. Besides that, the knowledge of these peculiar characteristics makes a given species an interesting model for the investigation of testis function. For example, studies were developed in our laboratory using the collared peccary to evaluate Leydig cell influence on the spermatogonial niche and spermatogonial differentiation (Campos-Junior et al., 2012), an aspect that will be presented in more detail in the last section of this book chapter.

Spermatogenesis

Spermatogenesis is a highly organized process that occurs in the seminiferous epithelium and is orchestrated by Sertoli cells. Based mainly on morphological and functional criteria, this process has been classically divided into three well-characterized phases, named spermatogonial (proliferative), spermatocytary (meiotic) and spermiogenic (differentiation). However, due to several recent findings related to spermatogonial stem cell biology and regulation, the spermatogonial phase could be subdivided into two functionally different categories or subsets, comprising the undifferentiated and differentiated spermatogonial pools (**Fig. 3**). Germ cells are not randomly distributed in the seminiferous epithelium, but organized in distinct and regular cellular associations known as stages of the seminiferous epithelial cycle (Hess and França, 2007). In this article, the germ cell types as well as spermatogenic phases and the basic Sertoli cell functions will be briefly described.

Germ Cell Syncytium and Apoptosis

Incomplete cytokinesis during mitosis is a typical feature of germ cells and spermatogenesis. This morphofunctional phenomenon forms an intercellular communication by creating stable intercellular bridges, also called cytoplasmic bridges, which are

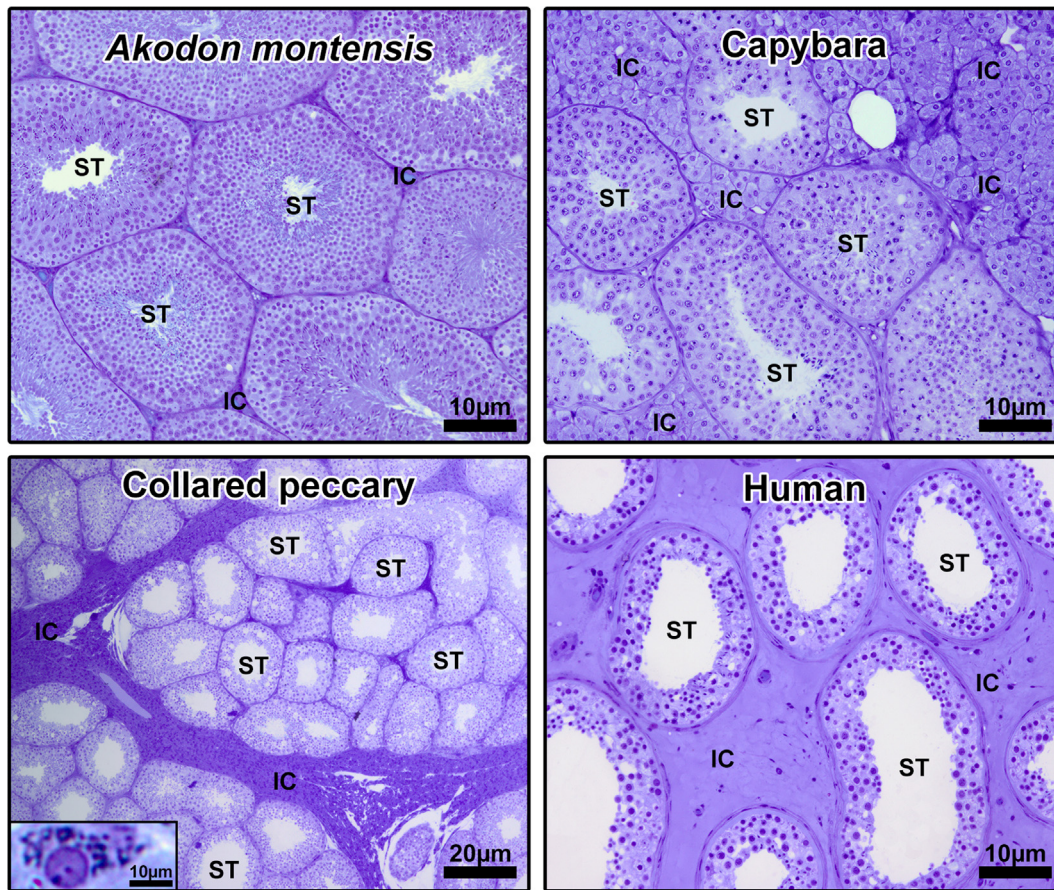


Fig. 2 Comparative testis histology of four representative mammalian species: *Akodon montensis*, capybara, collared peccary and human. Observe the differences in volume density of the two testis compartments—the seminiferous tubules (ST) and intertubular compartments (IC). Leydig cell volume density (%) is much higher in capybara, whereas very few Leydig cells are observed in the wild rodent *Akodon montensis*. In the peccary, these steroidogenic cells present a unique and peculiar arrangement in the testis parenchyma, forming dark stripes around the seminiferous tubules lobes, due to the high lipid content in their cytoplasm (insert). In man, connective tissue of the intertubular compartment is quite extensive.

maintained throughout spermatogenesis. The intercellular bridge is large enough to allow the passage and sharing of cell organelles, such as mitochondria, and several molecular signals. The type A single (As) spermatogonium is considered the only dividing germ cell without intercellular bridges. It was shown that *Tex14* knockout mice present defects in the intercellular bridges formation and, consequently, the germ cells were unable to complete meiosis, making these rodents infertile. As reviewed by [Greenbaum et al. \(2011\)](#), several roles for mammalian intercellular bridges in spermatogenesis have been proposed, including the following:

- sharing products of both sexual chromosomes, so haploid cells can remain “phenotypically diploid” even after meiosis;
- allowing the transference of cytoplasmic signals, important to maintain the synchrony in cell divisions; and
- producing a uniform population of sperm, detecting possible errors during germ cell development and facilitating the elimination of the entire syncytium by apoptosis.

Germ cell loss is normally observed in the adult testis, contributing therefore to the maintenance of healthy spermatogenesis and determining the total sperm output. Thus, an intricate homeostatic control of germ cell renewal, proliferation and apoptosis is necessary to maintain normal sperm production. In this regard, significant germ cell loss occurs during the spermatogonial phase, mainly regulating the density of these cells, which possibly represents an early mechanism to limit the number of germ cells that can be supported by each Sertoli cell. Apoptosis is also frequently observed during meiosis, probably serving as a defense mechanism for the preservation of the species and as quality control for monitoring chromosomal damage in the spermatocytes. The extent and purpose of spermatid apoptosis is less clear, but may also be associated with quality control, especially as related to DNA integrity after chromatin condensation ([Fig. 4](#)) ([Murphy and Richburg, 2014](#)), or represent a mechanism related to the finite number of germ cells that each individual Sertoli cell is capable of supporting. The rate of apoptosis, the most affected cell types affected, as well as the pathways involved in this process vary significantly among species.

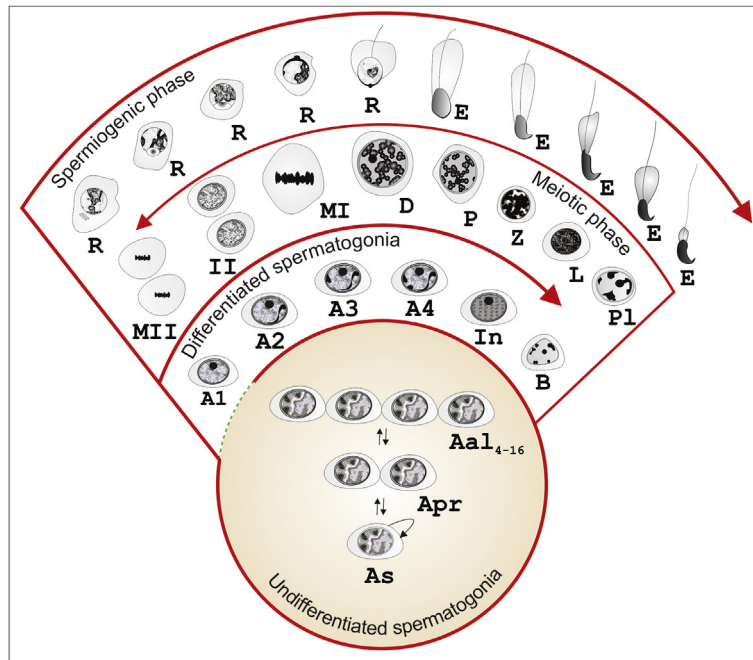


Fig. 3 Schematic representation of the mouse spermatogenic process: spermatogonial, meiotic and spermiogenic phases. Spermatogonial phase can be subdivided into two distinct functional categories, that is, undifferentiated and differentiated spermatogonia. The type A single spermatogonium (As) is considered the true stem cell, while type A paired (Apr) and A aligned (Aal₄₋₁₆) are considered amplifying stem cells. There is a fine balance between differentiation and self-renewal in the undifferentiated spermatogonial pool before the final differentiation (*dotted green line*) to type A1 spermatogonium (A1). After that, these cells are considered definitively committed to sperm production, going through meiotic and spermiogenic phases. Spermatogonial cells: type A undifferentiated [single (As), paired (Apr) and aligned (Aal₄₋₁₆)] and differentiated (A1–4); intermediate (In); and type B (B) spermatogonia. Primary spermatocytes: preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P) and diplotene (D) spermatocytes. Meiotic divisions (MI and MII). Secondary spermatocyte (II). Round (R) and elongated spermatids (E).

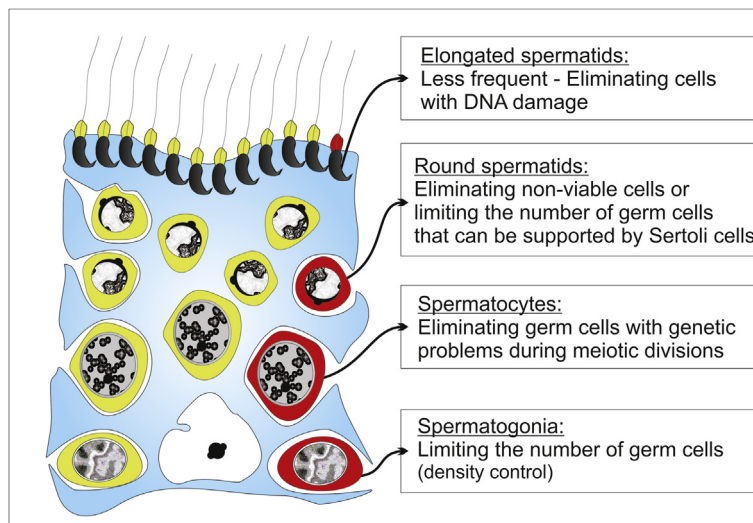


Fig. 4 Schematic illustration of germ cell loss (*red*) during spermatogenesis and the potential reasons for apoptosis of these cells in each phase of the spermatogenic process.

Spermatogonial Phase

Undifferentiated spermatogonia

This category or subset, as mentioned above, is responsible for maintaining the balance between self-renewal and differentiation, which is fundamental for ensuring continuous sperm production in mammals. This category is comprised of single (As), paired (Apr) and aligned (Aal) type A spermatogonia. In the past, single spermatogonia were considered the true stem cell among germ cells. However, because Apr and Aal spermatogonia have the ability to return to a more undifferentiated state through

syncytial/clonal fragmentation in a steady state, several recent studies have demonstrated that these cells are considered as both progenitors and amplifying cells of the spermatogenic process. This characteristic is very important in regulating the number of cells that can progress and be sustained throughout the next steps of spermatogenesis. These undifferentiated cells are normally present in all stages of the seminiferous epithelial cycle and the size of the syncytium/clones (Apr, Aal 4, 8, 16) in mice determines the type and prevalence of the membrane receptors involved in self-renewal and/or differentiation (Yoshida, 2012).

Several studies, mainly in mice, have shown that As and Apr spermatogonia present higher expressions of GFRA1 (GDNF family receptor alpha-1) and Nanos2 (Nanos homolog 2), which are factors involved in self-renewal. As soon as these cells start to differentiate and become Aal spermatogonia (Al 4, 8, 16), a gradual reduction of these factors is observed, while the expression of Neurogenin-3 and RAR (retinoic acid receptor) increases, concomitantly with the spermatogonial syncytium/clones enlargement. Lower expression of RAR on As and Apr spermatogonia ensures that these cells do not differentiate at the stages nearing spermiation, when the levels of retinoic acid (which stimulates germ cell differentiation) are elevated. This control prevents the depletion of spermatogonial stocks for the next cycle, ensuring continuous sperm production. Aal spermatogonia normally express the RAR and receive retinoic acid stimuli, which starts the expression of a classic stem cell factor receptor, known as c-kit, and is considered crucial for spermatogonial proliferation and differentiation (Fig. 5).

Differentiated spermatogonia

This category of spermatogonia is observed after the differentiation of As, Apr or Aal spermatogonial types, under retinoic acid influence. Because the newly formed cells are committed to sperm production, losing their reversibility characteristic is a functional process crucial for spermatogenesis. The differentiation process occurs concomitantly with spermiation and type B spermatogonia division to preleptotene spermatocytes, and this germ cell composition in the seminiferous epithelium determines the quantity of retinoic acid available to promote spermatogonial differentiation. After differentiation, spermatogonia pass through successive mitotic divisions until the formation of preleptotene cells. The number of mitotic divisions is related to the number of differentiated spermatogonial generations in a species, which seems to be phylogenetically determined and highly correlated with the magnitude of sperm production. For example, due to the fact that most rodents present five more generations of differentiated spermatogonia than humans, the expected number of sperm produced could be up to 32-fold higher (Fig. 6). In contrast to the previous subset (undifferentiated spermatogonia), the presence of differentiated spermatogonia occurs in specific germ cell associations, called stages of the seminiferous epithelial cycle, which presents germ cells at different states of differentiation (please see below) (Hess, 1990; Hess and França, 2007). It is worth mentioning that the characterization of differentiated spermatogonial cell types is based on the presence of heterochromatin, whereas their generation number is based on the presence of mitotic figures and the quantification of spermatogonial cells per seminiferous tubule cross-section.

Meiotic Phase

Similar to the transition of undifferentiated to differentiated spermatogonia, due to the capacity of type B spermatogonia to express retinoic acid receptors and yield preleptotene spermatocytes, the transition from mitosis to meiosis also depends of retinoic acid. Thus, *Stra8* (stimulated by retinoic acid gene 8) is activated after the direct action of retinoic acid in the spermatogonium and triggers the expression of important transcription factors, assuring DNA replication and the subsequent events of meiotic prophase.

During meiosis, pairing, synapsis, crossing-over and segregation of homologous chromosomes are observed. This complex and crucial process starts with the division of the type B spermatogonia, generating preleptotene spermatocytes (Pl) (Fig. 7). Pl are the first meiotic cells to be formed and are actively involved in DNA synthesis, being usually the last germ cells to synthesize this molecule. Pl are followed by leptotene spermatocytes, when the chromosomes become more apparent as long strands and form a peculiar arrangement called bouquet. Chromosomal pairing and synaptonemal complex formation start in zygotene and are

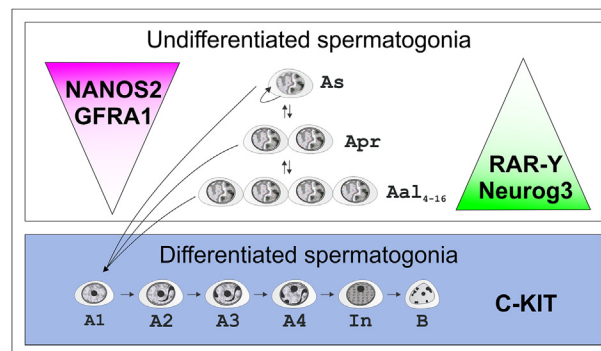


Fig. 5 Molecular expressions that are characteristic of undifferentiated and differentiated spermatogonial cells. Single (As) and paired (Apr) spermatogonia express high levels of self-renewal factors (GFRA1 and Nanos2), while aligned (Aal₄₋₁₆) spermatogonia show higher expression of proteins involved in differentiation (RAR-Y and Neurog3). After differentiation, the type A1, A2, A3, A4, Intermediate (In) and B spermatogonia predominantly express a protein (c-kit) involved in germ cell proliferation and differentiation.

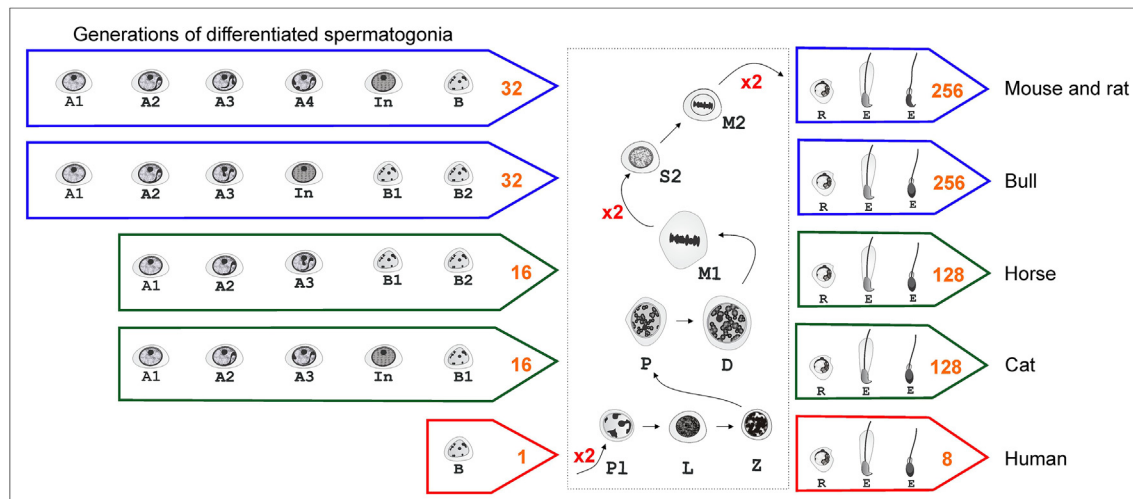


Fig. 6 Number of differentiated spermatogonia generations and the magnitude of sperm production. Contrasting with the number of cellular divisions (two) that are constant during meiosis, the number of differentiated spermatogonial generations, which seems to be considered phylogenetically determined, significantly varies among the mammalian species; directly influencing therefore the magnitude of sperm production. Spermatogonial phase: type A (A1–4); intermediate (In); and type B (B) spermatogonia. Meiotic phase: preleptotene (P1), leptotene (L), zygotene (Z), pachytene (P) and diplotene (D) spermatocytes; meiotic divisions (M1 and M2); secondary spermatocyte (S2). Spermiogenic phase: Round (R) and elongated spermatids (E). The number present in the tip of the bar represents the possible number of cells yield, whereas $\times 2$ illustrates the cell divisions after the differentiated spermatogonial phase.

completed in pachytene spermatocytes. In this last step, thickening and shortening of the chromosomes and exchange of chromosomal material occur, in a process called crossing-over, which is responsible for genetic variability among individuals within the same species. The end of synaptonemal complex occurs in the diplotene spermatocyte, when the chromosomes are partially separated. Diakinesis involves the separation of chromosomes into two sister chromatids. This first meiotic prophase is followed by metaphase, when the paired chromatids concentrate at the equator of the germ cell. Anaphase involves the movement of paired chromatids towards the cell poles, and telophase gives rise to daughter cells called secondary spermatocytes. Different from primary spermatocytes, these cells have a very short life span, with haploid number of chromosomes, although they have duplicated DNA content. The secondary spermatocytes pass through the second meiotic division very quickly, forming the round spermatids, which present a haploid content of chromosomal DNA (Fig. 7).

Spermiogenic Phase

The spermiogenic phase is a long process in which haploid, round spermatids pass through striking morphological changes until spermatozoa formation. The structural and functional changes that occur in the developing spermatids are usually subdivided into steps. During this phase, the following cellular events can be observed: (a) formation of new organelles, including the chromatoid body composed by RNA; (b) nuclear chromatin compaction; (c) acrosome formation by the Golgi apparatus; (d) flagellum formation; (e) development of ectoplasmic specializations and tubulobulbar complexes at the interface between Sertoli cells and elongating/elongated spermatids; (f) elimination of cytoplasm by spermatids (residual body); and (g) release of spermatids from the seminiferous epithelium (spermiation). Therefore, during spermiogenesis, many important genes/proteins have specific and crucial roles in the development of spermatozoa.

This spermatogenic phase is highly dependent on androgens (steroids) and the presence of androgen binding proteins (ABP) located in the Sertoli cell processes promotes high androgen concentrations near the elongated spermatids. Through the maintenance of spermatid adhesions to Sertoli cells, androgens induce elongation of the final steps of spermatids. Furthermore, several studies have demonstrated that testosterone is able to sustain complete spermatid differentiation by itself. In addition, follicle-stimulating hormone (FSH) has also an important role in sperm cytostructural modifications, acting on round spermatid flagellum extrusion and in the replacement of histones by protamines during spermiogenesis, assuring therefore the correct condensation of spermatid nuclei (Smith and Walker, 2014). Recent studies have also shown estrogen pathways to be involved in the preparation of elongating spermatids for release during spermiation (Kumar et al., 2017).

Sertoli Cell

Sertoli cells orchestrate germ cells development and differentiation, being therefore crucial for the completion of spermatogenesis. Among the innumerable Sertoli cells functions the following should be mentioned: (a) delivery of nutrients and growth factors for spermatogenic cells; (b) physical support of germ cells; (c) release of the spermatids towards the tubular lumen during spermiation;

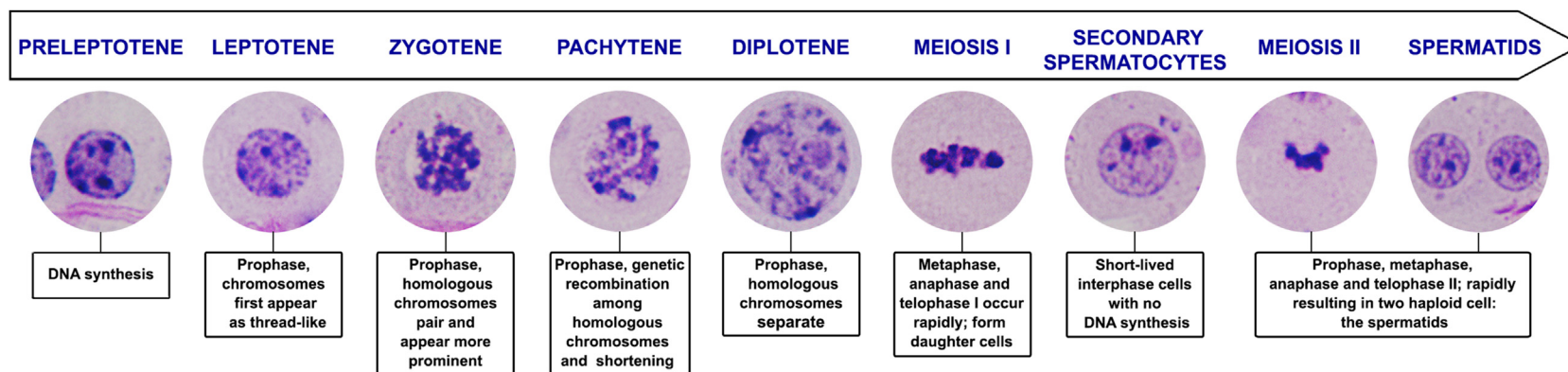


Fig. 7 Morphology and characterization of mouse spermatocytes at different stages of the meiotic phase of spermatogenesis. During meiosis, pairing, synapsis, crossing-over and segregation of homologues chromosomes are observed. The main characteristics/events presented in each cellular phases or steps (preleptotene, leptotene, zygotene, pachytene, diplotene, meiosis I, secondary spermatocytes, meiosis II and spermatids) are listed in the boxes placed below each considered spermatocyte type.

(d) secretion of tubular fluid; (e) phagocytosis of cytoplasm (the residual body) derived from late spermatids; and (f) phagocytosis of apoptotic germ cells. In addition, due to the presence of FSH, androgens, estrogens and thyroid hormones receptors, Sertoli cells also intermediate most of the hormonal regulation of spermatogenesis (Fig. 8A). Sertoli cells are able to accomplish these numerous, and sometimes simultaneous, functions because each cell is tall and extends through the entire height of the epithelium, from basement membrane to the lumen (Hess and Vogl, 2015). Cytoplasmic arms of each Sertoli cell is capable of extending around four different generations of developing germ cells, as it forms intricate membrane organelles. Its surface area can reach $16,000 \mu\text{m}^2$. Sertoli cells change dramatically in morphology and physiology over the course of the seminiferous epithelial cycle, with the translocation of numerous organelles, expression of numerous different proteins for specific functions, and changes in the location of these proteins to meet different needs of the changing germ cell associations.

In mammals, Sertoli cells are known to proliferate actively during the fetal period, presenting, at least in well-investigated laboratory rodents (mice and rats), a peak in proliferation just before birth. Postnatally, its proliferation extends for around 2–3 weeks

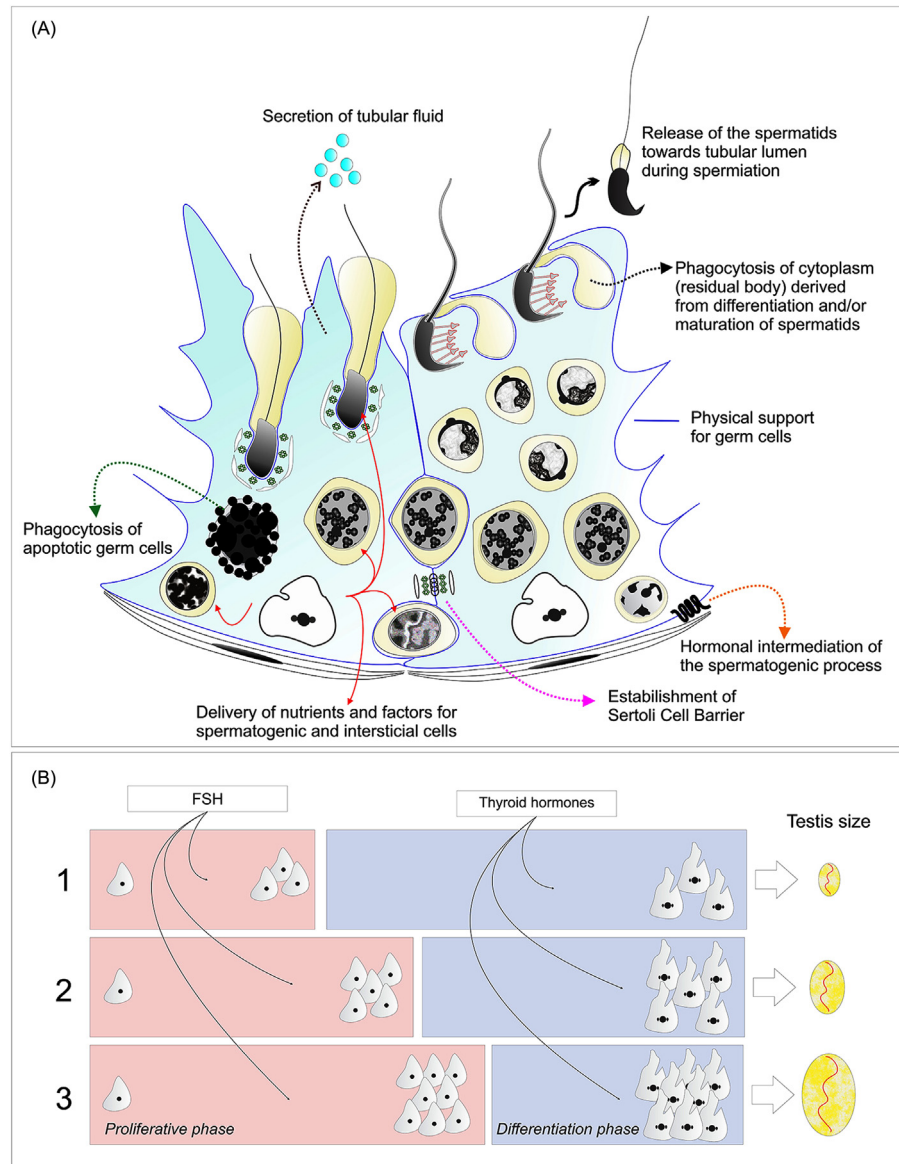


Fig. 8 Main Sertoli cell functions and the balance between Sertoli cell proliferation and differentiation. (A) Schematic illustration of the key factors related to the Sertoli cell functions. As it can be observed, the Sertoli cell is involved in crucial spermatogenic events, such as germ cell nutrition and support, signaling, hormonal intermediation, luminal fluid secretion, endocytosis and spermiation. (B) Length of the Sertoli cell proliferation window (pink boxes) is crucial to the determination of their final number. The major factors controlling proliferation and differentiation phases of Sertoli cells are FSH and thyroid hormones, respectively. In laboratory rodents, experimental manipulation of these factors can shorten (B1) or prolong (B3) Sertoli cell proliferation, resulting respectively in smaller and larger testis/sperm production. (B2) Animals under normal conditions, exhibit an equilibrium between proliferative and differentiation phases.

after birth in rats and 10–15 days in mice, and the end of its mitotic phase is correlated with the initiation of primary spermatocyte differentiation, Sertoli cell-barrier development and fluid secretion/lumen formation. In humans, Sertoli cells proliferate during the perinatal and neonatal periods, and it is believed that this somatic cell becomes quiescent for several years, followed by a second proliferation peak in response to increased gonadotrophins at puberty. A similar pattern is observed in pigs, but the second proliferation peak occurs in a matter of months and not years, because puberty in pigs occurs around 4 months of age. Except for the transitional area between the seminiferous tubules and the *rete testis*, in adulthood the number of Sertoli cells in the testis is rather stable. However, recent *in vitro* and *in vivo* studies, in laboratory animals, seasonal animals (hamsters) and humans, suggest that there is a fine modulation of Sertoli cell differentiation. Therefore, these cells seem to be able to return to a more immature state or, additionally, a specific population of Sertoli cells can keep their immature/stemness state, which could be the situation for Sertoli cells located in the transitional area between seminiferous tubules and the *rete testis* (Figueiredo et al., 2016).

Although the mechanisms that regulate Sertoli cell proliferation are not yet completely elucidated, it is strongly suggested that FSH is the main factor involved in this process. However, other factors such as for instance androgens, estrogens, activin, TGF- β , BMP2, BMP7, IL1 and TNF α , probably play important roles on Sertoli cell proliferation. In contrast to FSH, thyroid hormones are responsible for the differentiation/maturation of Sertoli cells, changing them functionally from a mitotic to a non-mitotic state. As each Sertoli cell is capable of supporting a relatively fixed number of germ cells, in a species-specific manner, experimental manipulation of the Sertoli cell population will affect the spermatogonial niche functionality and, ultimately, the magnitude of sperm production (Fig. 8B–D).

As already mentioned, the stabilization of Sertoli cell proliferation and their functional maturation coincide with the development of the blood–testis or Sertoli cell-barrier (SCB). The anatomical basis of SCB consists of the establishment of tight junctions between Sertoli cells, in addition to the presence of basal ectoplasmic specializations, gap and adherens junctional proteins, which also contribute to this structural and functional junctional complex. Therefore, at the molecular level, the SCB provides a physical barrier to segregate the cellular events of spermatogenesis, particularly controlling and restricting the paracellular transport of several substances (paracrine factors, hormones and biological molecules) across the seminiferous epithelium. Additionally, the SCB also helps to establish an immunoprivileged microenvironment for the development of the spermatogenic process, which is especially important as meiotic germ cells and developing spermatids can produce several immunogenic autoantigens. Furthermore, studies have shown that the SCB is also involved in different cellular events during spermatogenesis; for example, spermatogonial differentiation, initiation of meiosis, or reinitiation of spermatogenesis after a toxic injury (França et al., 2016).

Stages and Cycle of the Seminiferous Epithelium and Spermatogenic Wave

Stage classification methods are very useful and enable studies involving toxicological and comparative analyses, making it possible to follow specific cellular alterations caused by the administration of drugs that promote changes in spermatogenesis. In a tubular cross-section, it is observed that the seminiferous epithelium is composed of Sertoli and germ cells that are closely associated. It can also be noted that, differing from Sertoli cells, germ cells are organized in a well-defined manner in concentric layers, where the most immature germ cells (spermatogonia and early spermatocytes) are located at the base of the seminiferous epithelium, adjacent to the basement membrane, while more advanced germ cells (leptotene/zygotene to spermatids) occupy successive layers towards the tubular lumen.

In 1952, Leblond and Clermont described a classical method in which they accurately defined the germ cell associations present in seminiferous tubules of rats and found that these associations, although different, were constant and with a specific time duration. Analyzing the development of the spermatid acrosomal system, these scientists classified rat spermatogenesis into 14 cellular associations, called stages. In rodents and most non-primate species, one stage occupies a cross section of a seminiferous tubule. Based on the same methodology, 12 different stages were described in mice (Fig. 9) and 6 in man. At the same time, another method for stage classification, named the tubular morphology system, was already described. In the tubular method, only eight stages of the seminiferous epithelium cycle are described for any species evaluated, but these stages include all cells and steps seen with the acrosomal system of classification. This latter classification, used for several mammalian species, takes into account the spermatid nuclear morphology and localization, their association with other germ cell types (i.e., differentiated spermatogonia and spermatocytes) and the presence of meiotic figures. More recently, another classification that could integrate both acrosomal and tubular morphology systems was developed. In this simpler and useful classification, the spermatogenic process was divided into six representative phases: (I) before meiosis; (II) after meiosis; (III) presence of acrosomal granule; (IV) presence of acrosome; (V) pre-spermiation; and (VI) post-spermiation. A novel immunohistochemical approach was also recently introduced, in which the stages are visualized using immunolocalization of proteins that identify specific germ cells and Sertoli cells and label the acrosomal formation (Nakata et al., 2015). As more and more antibodies become available, this latter method of classification may become more popular, as it provides a powerful method for tracing gene/protein expressions through the cycle of spermatogenesis. Independent of the classification criteria used, in humans and some other primate species more than one stage is observed per cross section of the seminiferous tubule. The functional meaning for this finding is still unknown but might be related to the germ cell clonal size, an aspect that surely deserves an accurate investigation.

Development of the stages is not related to any movement in the longitudinal axis of the seminiferous tubule. However, there is a distinct order of cellular associations (stages) along the length of the tubule, forming linear segments. The continuity of these segments is usually observed in a descending sequence from the proximal connections to the *rete testis* to the approximate center of the tubule, where the stage sequence is reversed (Nakata et al., 2017); thus, stage VII would be adjacent to stage VI, and so on. This

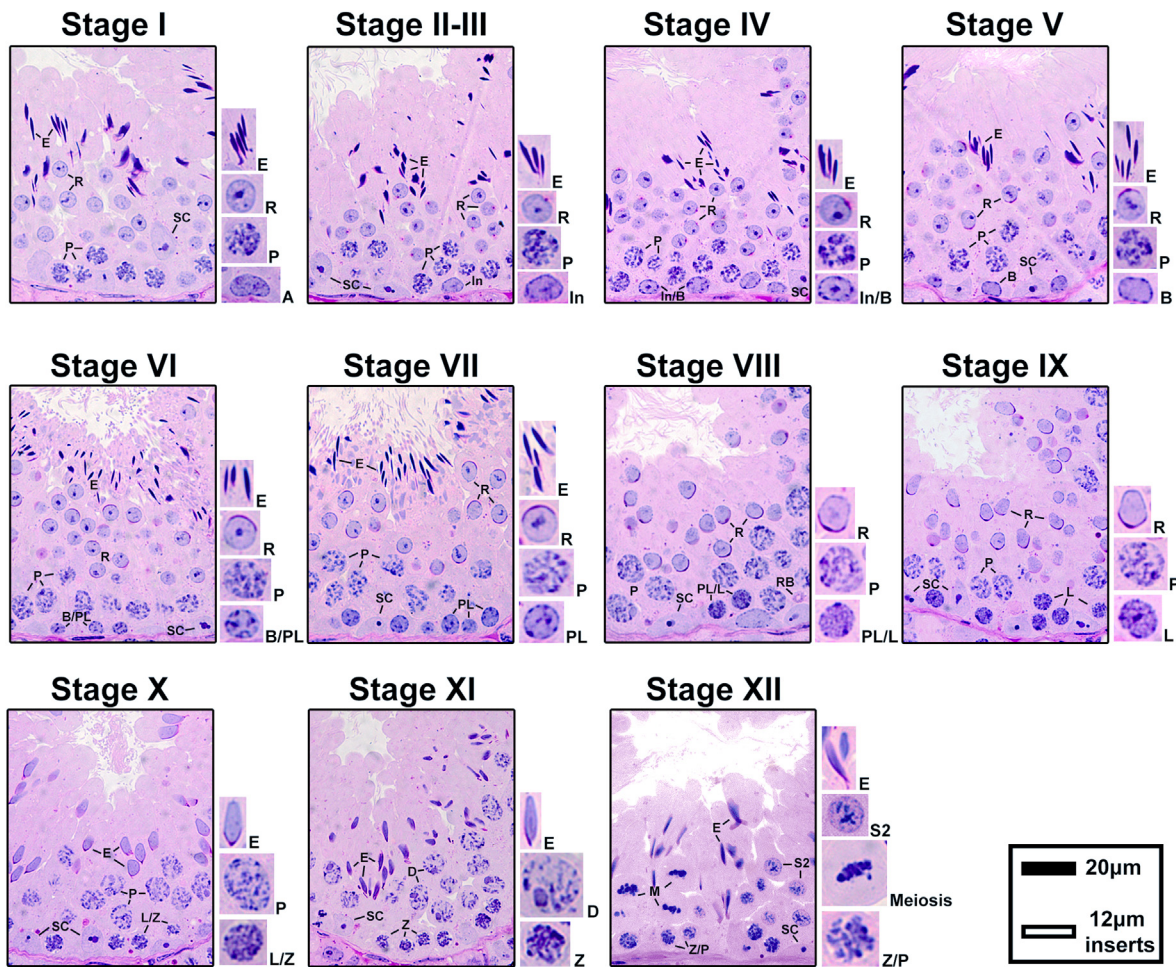


Fig. 9 Stages I–XII of the seminiferous epithelial cycle in the mouse, according to the development of the acrosomic system. The individual germ cell nuclei shown in the right column represent the germ cells found in association at each particular stage. A, type A differentiated spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; PL, preleptotene; L, leptotene; Z, zygotene; P, pachytene; D, diplotene spermatocytes; meiosis, meiotic figure; S2, secondary spermatocyte; R, round spermatids; E, elongating/elongated spermatids; SC, Sertoli cells.

special arrangement will eventually lead to a complete series of stages, defined as a spermatogenic wave. However, unfortunately, very few species were investigated in this particular aspect, making the spermatogenic wave still a not very well understood functional aspect of spermatogenesis. Nevertheless, it is considered that the importance of wave is a continuous commitment of germ cells to the cycle, as well as the release of sperm along the seminiferous tubules in a steady way in non-seasonal breeders, ensuring therefore constant sperm production and maturation (Griswold, 2016).

In association with the concept of spermatogenic phases, the cycle of the seminiferous epithelium was defined as a series of transformations that occur, within the same stage, leading to the differentiation of germ cells in a vertical way. It means that one cycle can be represented by the differentiation of spermatogonia into spermatocytes and spermatocytes into spermatids at the same stage. Thus, the cycle of the seminiferous epithelium was characterized as a temporal and sequential event (time and space) of changes in cellular associations in a given area of the seminiferous tubule. Several studies have suggested that Sertoli cells cyclically change their functions, coordinating the differentiation of germ cells. One of the most important candidates to play a role in this regulation is the retinoic acid, which acts as a stimulator of the periodic germ cells differentiation. For instance, it was shown that an increase in retinoic acid signaling is able to induce type A spermatogonia differentiation and influence Sertoli cell gene expression, modulating their action on the other germ cells (Hess and França, 2007; Griswold, 2016).

Another very important functional aspect of spermatogenesis is duration of the seminiferous epithelium cycle. Although controlled by the germ cell genotype (França et al., 1998), it is considered species-specific and does not have a conserved feature among species or orders. Nevertheless, several studies, mainly from our research group, have demonstrated that phylogeny is usually associated with stage frequencies, particularly when considering the combination of stages in pre-meiotic and post-meiotic phases among closely related species. Therefore, when the frequencies of these two combined phases are considered, three patterns can be recognized among species: (1) a relative equilibrium between pre-meiotic and post-meiotic phase frequencies (e.g., marsupials); (2) pre-meiotic phase corresponding to two thirds of the spermatogenic cycle (e.g., ruminants); and (3) pre-meiotic

Table 2 Frequencies of pre-meiotic, meiotic and post-meiotic stages of the seminiferous epithelium cycle and the duration of each spermatogenic cycle and the total spermatogenesis in several mammalian species

	Pre-meiotic (%) ^a	Meiotic (%) ^b	Post-meiotic (%) ^c	Spermatogenic cycle length (days)	Total duration of spermatogenesis (days)
Mouse	18–23	9–11	66–71	8.6–8.9	38.7–40.1
Rat	24	5	71	12.9	58
Hamster	26–29	5.5–7.5	65–66	9–17	39–76
<i>Akodon montensis</i>	28	8.2	63.8	8.9	40
Capybara	44.8	14.6	40.6	11.9	53.5
Collared peccary	38	11	51	12.6	55.1
Boar	28–31	12	56–60	9	40.5
Opossum	48.9	4.8	46.3	17.3	77.9
Cat	45.5	17.6	36.9	10.4	46.8
Dog	37–41	7–11	50–57	12.6–13.8	56–62
Horse	35	15.8	49.2	12.2	54.9
Bovine	64.7	8.8	26.5	14	63
Buffalo	64.8	9.2	26.0	8.6	38.7
Goat	58.7	9.4	31.9	10.6	47.7
Marmoset	61	8	31	15.4	69.1
Human	45–75	5–7	20–50	16	72

^aAfter spermiation and prior to metaphase.

^bMeiosis I through meiosis II.

^cAfter completion of meiosis until spermiation.

frequency lasting about one-quarter of the entire spermatogenic cycle (e.g., laboratory rodents) (Table 2). As also observed in Table 2, in most mammalian species investigated up-to-date the total duration of spermatogenesis is situated in the range of ~40 to ~60 days. It means that, from undifferentiated spermatogonia, usually less than 2 months are required for the formation and release (spermiation) of sperm from the seminiferous epithelium.

Leydig Cells and Steroidogenesis

As previously noted in Table 1, the steroidogenic Leydig cell is the most frequent cell type observed in the intertubular compartment. The Leydig cell exhibits huge variations in individual volumes among mammalian species. For instance, in humans and horses the Leydig cell individual volume reaches more than 4000 μm^3 , whereas in *Akodon montensis* this value is around 800 μm^3 , being only 400 μm^3 in rams. Interestingly, in a comparative study not yet published from our laboratory, involving 10 different breeds of dogs, we observed that the more aggressive breeds (pitbull and pincher) have a much greater occupancy of Leydig cells, compared to other breeds such as the poodle, whose testis has a much larger tubular compartment and spermatogenic efficiency. These findings illustrate the importance of evaluating the testis compartments, in order to have a better understanding of reproductive strategies and animal behavior.

The pattern of distribution and organization (cytoarchitecture) of Leydig cells in the intertubular compartment is highly variable among the mammals species already investigated (see Fig. 2) and probably represents important functional aspects that are yet unknown or have not been clearly elucidated. The last comprehensive review of this subject was published more than four decades ago (Fawcett et al., 1973). A recent publication from our laboratory showed that in the collared peccary Leydig cells present a unique cytoarchitecture, forming a cord-like structure surrounding the seminiferous tubules lobes, and only very few Leydig cells are present among the seminiferous tubules. This pattern markedly differs from those already described by Fawcett and colleagues in 1973. Taking advantage of this knowledge, we developed studies regarding spermatogonial stem cell biology and niche in this species, in which we found that, in contrast to differentiated type A spermatogonia, the undifferentiated spermatogonial cells were located closer to the intertubular compartment areas without Leydig cells (Campos-Junior et al., 2012). In another investigation, we observed that, during postnatal testis development in pigs, and somewhat similar to horses, seminiferous tubule maturation and lumen formation follow an asymmetric pattern, being more advanced in the intermediate areas distant from the tunica albuginea and closer to the central area, where the rete testis and the testis mediastinum are located (Avelar et al., 2010). Interestingly, individual Leydig cell volume in the intermediate areas was always much larger at all investigated ages, maintaining this functional pattern even in sexually mature pigs. As similar results are now being observed for the seminiferous tubules in rodents, having the rete testis as a reference point, these striking finding open new venues for testis function investigation and, once more, reinforces the functional interactions between the two different testis compartments.

Regarding androgen production in the testis, it has been suggested that testosterone levels are more related to mitochondria and smooth endoplasmic reticulum present in the cytoplasm than in the Leydig cells number and size. In addition, in comparison to the plasma levels, intratesticular testosterone concentration is strikingly higher, meaning that normal spermatogenesis demands a rather high amount of androgens. As already mentioned, testis steroidogenesis is crucial for maintenance of androgen levels, being

essential for the completion of full spermatogenesis and for the development and preservation of sexual male characteristics. Overall, androgen controls spermatogenesis through Sertoli cell intermediation, as germ cells per se do not express receptors for these hormones. Androgens and probably other steroids, including estrogen, are especially necessary for spermiogenesis and sperm release.

The production of androgens and steroids in general begins as early as gonadal differentiation takes place and it is maintained up to senescence, when its levels decrease. In this context, it has been demonstrated that Leydig cell capacity for producing testosterone varies along with the different phases of testis development and is associated with the enzymatic composition of these cells. Hence, studies in the literature reported that during fetal life a particular Leydig cell population is present in the testis, producing androgen as an intermediate product. At this phase, it was recently shown that Sertoli cells are remarkably necessary for completion of testosterone synthesis, once these supporting somatic cells express 17 β -hydroxysteroid dehydrogenase 3 (*Hsd17b3*), the last enzymatic step necessary for testosterone production. *Hsd17b3* is not detected in Fetal Leydig cells. Thus, the androgen produced by Fetal Leydig cell is delivered to the Sertoli cell that will provide the final conversion to testosterone (Shima and Morohashi, 2017). Postnatally, the Leydig cell population changes dramatically and the Adult type Leydig cell develops the enzymatic machinery necessary to accomplish testosterone production. Concomitantly, *Hsd17b3* gene is silenced in Sertoli cells and, in this respect, the only source of testosterone in post pubertal testis is the Leydig cell (Ye et al., 2017). In this context, the rather substantial interactions between Leydig cells and macrophages and the possibility of the coexistence of distinct Leydig cells populations in the testis of some species represent an interesting aspect of testis function that might be taken into consideration in future studies.

References

- Avelar, G. F., Oliveira, C. F. A., Soares, J. M., Silva, I. J., Dobrinski, I., Hess, R. A., & França, L. R. (2010). Postnatal somatic cell proliferation and seminiferous tubule maturation in pigs: A non-random event. *Theriogenology*, 74, 11–23.
- Campos-Junior, P. H. A., Costa, G. M. J., Lacerda, S. M. S. N., Rezende-Neto, J. V., de Paula, A. M., Hofmann, M. C., & França, L. R. (2012). The spermatogonial stem cell niche in the collared peccary (*Tayassu tajacu*). *Biology of Reproduction*, 86, 155–165.
- Fawcett, D. W., Neaves, W. B., & Flores, M. N. (1973). Comparative observations on intertubular lymphatics and the organization of the interstitial tissue of the mammalian testis. *Biology of Reproduction*, 9, 500–532.
- Figueiredo, A. F. A., França, L. R., Hess, R. A., & Costa, G. M. J. (2016). Sertoli cells are capable of proliferation into adulthood in the transition region between the seminiferous tubules and the rete testis in Wistar rats. *Cell Cycle*, 15, 2486–2496.
- França, L. R., Ogawa, T., Avarbock, M. R., Brinster, R. L., & Russell, L. D. (1998). Germ cell genotype controls cell cycle during spermatogenesis in the rat. *Biology of Reproduction*, 59, 1371–1377.
- França, L. R., Hess, R. A., Dufour, J. M., Hofmann, M. C., & Griswold, M. D. (2016). The Sertoli cell: One hundred fifty years of beauty and plasticity. *Andrology*, 4, 189–212.
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3, a005850.
- Griswold, M. D. (2016). Spermatogenesis: The commitment to meiosis. *Physiological Reviews*, 96, 1–17.
- Hess, R. A. (1990). Quantitative and qualitative characteristics of the stages and transitions in the cycle of the rat seminiferous epithelium: Light microscopic observations of perfusion-fixed and plastic-embedded testes. *Biology of Reproduction*, 43, 525–542.
- Hess, R. A., & França, L. R. (2007). Spermatogenesis and cycle of the seminiferous epithelium. In C. Y. Cheng (Ed.), *Molecular mechanisms in spermatogenesis* (1st edn, pp. 1–15). New York: Springer.
- Hess, R. A., & Vogl, A. W. (2015). Sertoli cell anatomy and cytoskeleton. In M. Griswold (Ed.), *Sertoli cell biology* (2nd edn, pp. 1–55). Oxford: Elsevier Academic Press.
- Kumar, A., Dumasia, K., Deshpande, S., & Balasinar, N. H. (2017). Direct regulation of genes involved in sperm release by estrogen and androgen through their receptors and coregulators. *The Journal of Steroid Biochemistry and Molecular Biology*, 171, 66–74.
- Leblond, C. P., & Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Annals of the New York Academy of Sciences*, 55, 548–573.
- Murphy, C. J., & Richburg, J. H. (2014). Implications of Sertoli cell induced germ cell apoptosis to testicular pathology. *Spermatogenesis*, 4, e979110.
- Nakata, H., Wakayama, T., Takai, Y., & Iseki, S. (2015). Quantitative analysis of the cellular composition in seminiferous tubules in normal and genetically modified infertile mice. *The Journal of Histochemistry and Cytochemistry*, 63, 99–113.
- Nakata, H., Sonomura, T., & Iseki, S. (2017). Three-dimensional analysis of seminiferous tubules and spermatogenic waves in mice. *Reproduction*, 154, 569–579.
- Russell, L. D., & França, L. R. (1995). Building a testis. *Tissue and Cell*, 27, 129–147.
- Shima, Y., & Morohashi, K.-I. (2017). Leydig progenitor cells in fetal testis. *Molecular and Cellular Endocrinology*, 445, 55–64.
- Smith, L. B., & Walker, W. H. (2014). The regulation of spermatogenesis by androgens. *Seminars in Cell and Developmental Biology*, 30, 2–13.
- Ye, L., Li, X., Li, L., Chen, H., & Ge, R.-S. (2017). Insights into the development of the adult Leydig cell lineage from stem Leydig cells. *Frontiers in Physiology*, 8, 430.
- Yoshida, S. (2012). Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. *Reproduction*, 144, 293–302.

Spermatogonial Stem Cell and Niche

Nathan C Law and Jon M Oatley, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Spermatogonial stem cells (SSCs) are the cornerstone of the entire male germ cell lineage in postnatal life. Indeed, the billions of sperm produced throughout the reproductive lifespan of a male are derived from a pool of SSCs. Continuous spermatogenesis, therefore, requires a robust SSC pool that not only self-renews to maintain the stem cell pool, but also generates large numbers of progenitors that will ultimately form spermatozoa. Thus, complex regulatory systems delicately balance fate decisions of SSCs between self-renewal and progenitor formation, which are thought to be driven by factors from a specified microenvironment, also known as the *niche*. Somatic cells, systemic circulation, and other germ cells are thought to all contribute to niche composition and ultimately communicate with SSCs to maintain continuous spermatogenesis. In this article, we will briefly discuss the current breadth of knowledge in SSC niche composition and the known factors that regulate SCC function.

The Stem Cell Niche

The term *niche* was first coined by Schofield in 1978 to describe a specialized anatomical microenvironment that controls stem cell dynamics and function (Schofield, 1978). In general, niches are regulatory units that control the capacity of tissue-specific stem cells to either self-renew or produce progenitors through cell division. Tissue-specific stem cells are thought to divide by one of three modes: (1) symmetrical division generating two identical stem cells, (2) symmetrical division producing two progenitors, or (3) asymmetrical division yielding one stem cell and one committed progenitor. SSCs are thought to divide either symmetrically forming two identical SSCs or asymmetrically to form an SSC and a progenitor; although this model has only been observed in *Drosophila melanogaster* (Tulina and Matunis, 2001) and *Caenorhabditis elegans* (Kimble and White, 1981), it remains to be determined in mammals. Regardless, dynamic changes in the SSC niche microenvironment control the fate decisions of SSCs toward either self-renewal or progenitor formation.

Stem cell niches are primarily comprised of a growth factor milieu, extracellular matrix elements, and other contributions from somatic support cells. Importantly, the composition of a stem cell niche is not static. For example, niches change during the course of development. As a stem cell lineage is establishing, niche composition drives fate decisions of stem cells that typically favor symmetrical division and self-renewal as the pool grows. Once steady-state conditions are reached, stem cells are thought to rarely self-renew and instead, periodically give rise to progenitors that amplify in numbers to populate a tissue and maintain homeostasis. In times of stress and regeneration, the stem cell niche also changes to support rebuilding of the cell lineage. Thus, there is plasticity in the niche environment based on the demands of the stem cell pool. In studies with mice, Shinohara et al. showed that SSCs derived from pups or adults have relatively equal stem cell capacity when transplanted into adult recipients, but colonization occurs 9.4 times more often and more expansively in recipient pups versus adults (Shinohara et al., 2001). These data suggest that although SSCs derived from pups or adults have similar stem cell capacity, the niche in pups is drastically different from adults and likely reflects remodeling that occurs over time as steady-state spermatogenesis is reached.

A_{single} Model

Classic morphological studies conducted by Huckins, Oakberg, and de Rooij in the 1970s utilizing rodent models paved the way for much of what we understand regarding the male germ cell lineage. In rodents, SSCs reside within a heterogeneous population of spermatogonia, composed of type A, Intermediate, and type B spermatogonia. The type A spermatogonia can be further divided into undifferentiated and differentiating subpopulations. SSCs are a rare subset of undifferentiated spermatogonia, with the remaining undifferentiated population being nonstem cell progenitors. In the prevailing “A_{single}” model of spermatogonial differentiation, SSCs are thought to predominantly exist as single type A undifferentiated spermatogonia (A_{single} or A_s). Recent studies suggest that the A_s population is a heterogeneous pool composed of both true SSCs and SSCs transitioning to a nonstem cell progenitor phenotype (Lord and Oatley, 2017; de Rooij, 2017). Progenitors form paired (A_{paired} or A_{pr}) and aligned (A_{aligned} or A_{al}) chains of 2–32 undifferentiated spermatogonia persistently connected by intracellular bridges formed after incomplete cytokinesis. These intracellular bridges facilitate transfer of protein and mRNA between the progenitor spermatogonia and are thought to synchronize the process of differentiation. Retinoic acid (RA) triggers the terminal differentiation of A_{pr} and A_{al} undifferentiated spermatogonia which transition to an A1 differentiating state. A series of proliferation events lead to A2, A3, A4, Intermediate, and type B spermatogonia before meiosis initiates forming haploid spermatids and ultimately spermatozoa (Fig. 1).

In primates and humans, much less is known about the organization of the male germline. The undifferentiated population consists of two subtypes of A_s spermatogonia, A_{dark} (or A_d) and A_{pale} (or A_p), named for their differences in chromatin architecture and nuclear appearance through hematoxylin staining in histological studies. A_d spermatogonia are believed to constitute slow-cycling SSCs and A_p are the immediate nonstem cell progenitors that enter differentiation to form type B spermatogonia. However,

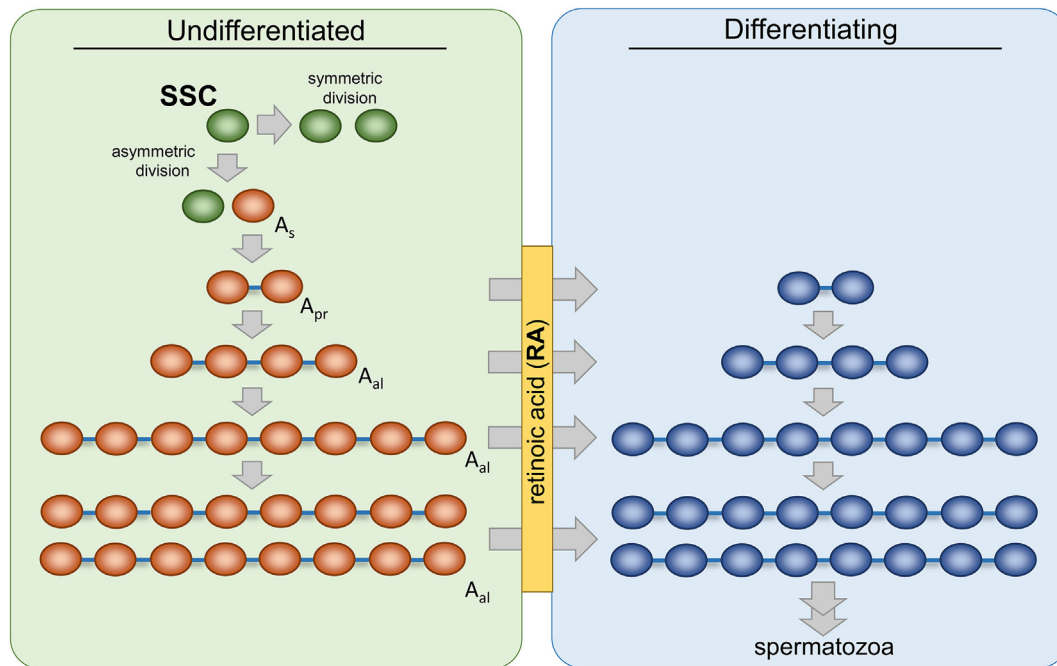


Fig. 1 Schematic of the “A_{single}” model of spermatogonial differentiation. The undifferentiated pool of spermatogonia consists of rare spermatogonial stem cells (SSCs) and type A undifferentiated progenitors arranged as single (A_s), paired (A_{pr}), or aligned chains (A_{al}) of spermatogonia. SSCs divide either symmetrically to self-renew or asymmetrically producing undifferentiated progenitors that transition to a differentiating phenotype in response to retinoic acid (RA). Differentiating spermatogonia proceed through a program of terminal differentiation to ultimately for spermatozoa.

it has been suggested that differences in nuclear morphology between A_d and A_p spermatogonia may simply be attributed to differences in cell cycle status among human and primate A_s spermatogonia (Hermann et al., 2009). Interestingly, human and primate spermatogonia lack intracellular bridges and upon induction of differentiation, transition directly to type B spermatogonia as opposed to type A differentiating and Intermediate spermatogonia as in rodents. Ultimately, these subtle differences in the organization of spermatogenesis between humans, primates, rodents, and other species is believed to underlie differences in total daily sperm output (Hermann et al., 2009).

Origin of the SSC Pool

Primordial germ cells (PGCs) are the earliest known embryonic precursors to the germ cell lineage for both males and females. PGCs arise from the proximal epiblast around embryonic day 6.5 (E6.5) in rodents and 2–3 weeks gestation in humans in response to bone morphogenetic proteins 4 and 8b (BMP4 and BMP8b) secreted by the extraembryonic ectoderm (Gunesdogan et al., 2014). PGCs then undergo a remarkable migration along the primitive hindgut to ultimately colonize the genital ridges adjacent the mesonephros, structures that will eventually form the gonads and kidneys, respectively. PGCs express C–X–C motif chemokine receptor 4 (CXCR4), a receptor that directs chemotactic migration toward the developing gonad via C–X–C chemokine motif ligand 12 (CXCL12) secreted from the genital ridges (Richardson and Lehmann, 2010). Likewise, the receptor c-Kit expressed by PGCs also participates in active migration—although more in the capacity of cell survival—in response to Kit ligand (Richardson and Lehmann, 2010). Thus, during the early stages of the SSC lineage, BMP4/8b, CXCL12, and Kit ligand are critical factors present in a dynamic niche environment.

While migrating to the genital ridges, PGCs rapidly proliferate and undergo genome-wide demethylation to prime the cells for later sex-specific remethylation. PGCs are initially bipotential, or capable for forming either male or female lineages. After colonizing the embryonic gonad, PGCs undergo sex determination and associate with somatic cells that regulate meiotic progression in a sex-specific manner. In females, PGCs form oogonia that enter meiosis in response to RA produced by the mesonephros. Oogonia then arrest in prophase I until shortly before ovulation in postnatal life. In males, PGCs transition to prospermatogonia but do not enter meiosis because associated Sertoli cells break down local RA via high levels of the RA-degrading enzyme Cyp26a1 (Koubova et al., 2006). Thus, RA is a critical niche component that regulates sex-specific meiotic progression of PGCs in the developing gonad.

Following sex determination, prospermatogonia begin reestablishing methylation imprints around E15 (in rodents) and proliferate until E16.5, at which point they enter a phase of mitotic arrest that persists until shortly after birth. Prospermatogonia inhabit the luminal cavity of seminiferous tubules (or seminiferous cords) during embryonic development. During the first

3 days of postnatal life, prospermatogonia migrate to the periphery of seminiferous tubules and colonize along the basement membrane adjacent Sertoli cells. Those prospermatogonia that colonize the basement membrane undergo a change in morphology and eventually re-enter the cell cycle around postnatal day 3. Prospermatogonia transition to SSCs during a defined window in postnatal development, which is believed to occur around postnatal day 6 in rodents and several months in primates and livestock.

Rodents are unique from higher order mammals such as primates and humans in that prospermatogonia that colonize the basement membrane become one of two different populations, either spermatogonia that directly enter differentiation to form the “first round of spermatogenesis” or SSCs that ultimately support subsequent rounds of spermatogenesis (Yoshida et al., 2006; Kluin and de Rooij, 1981). The first round of spermatogenesis in murine species is thought to have evolved from selective pressure to reach reproductive age faster; primates and humans lack this unique first round of sperm production. Otherwise, the sequence of events involved in the maturation of PGCs to SSCs appears to be conserved across most mammals, albeit with subtle differences in timing and/or duration.

SSC Homing and Transplantation

Homing is a term that describes the process by which stem cells migrate to and colonize niches. The ability of PGCs, prospermatogonia, or SSCs to home to a niche is essential for the establishment of continuous spermatogenesis and is also the basis for transplantation of SSCs into recipient testes. Tissue-specific stem cells, including SSCs, possess two definitive characteristics: (1) the ability to self-renew and maintain the entire cell lineage, and (2) the capacity to regenerate the cell lineage. Thus, transplantation is the most unequivocal measure of SSC content because transplanted cells must not only home to and colonize available niches, but also regenerate and sustain the spermatogenic lineage.

Transplantation of SSCs was first developed by Brinster and colleagues in 1994 (Oatley and Brinster, 2006; Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). The procedure involves dispensing cell suspensions containing SSCs through the efferent duct of recipient testes using a microinjection needle. The cell suspension fills the rete testis which feeds into the seminiferous lumen of each tubule. SSCs dispersed into the lumen migrate through the blood–testis barrier (BTB) to the basement membrane and occupy niches that will ultimately lead to repopulation of the testis, in a manner similar to what occurs during postnatal establishment of the SSC pool (discussed above). Transplantation efficiency is estimated to be between 5% and 12%, with niche availability and homing through the BTB being the limiting factors in SSC colonization.

From a research standpoint, transplantation is used as a functional assay to assess the stem cell capacity of the injected cell suspension (Helsel and Oatley, 2017). To evaluate only the experimental cell population, recipients are depleted of endogenous germ cells either experimentally using radiotherapy or various chemotherapeutics such as busulfan, or genetically where mice carry mutations in the c-Kit receptor (termed W/W^v). In the absence of a functional c-Kit receptor, PGCs do not colonize the gonad (discussed above) and thus produce mice devoid of a mature germline. Labeling of the injected cell population through the use of report transgenes such as *LacZ* or *Gfp*, enables quantification of donor-derived spermatogenic colonies. Each colony is derived from clonogenic expansion and differentiation from a single SSC (Dobrinski et al., 1999; Zhang et al., 2003; Kanatsu-Shinohara et al., 2006). Thus, one colony equates to one successful SSC transplanted. Therefore, transplantation can be used as a quantitative and qualitative measure of stem cell capacity. Lineage tracing is an experimental technique complimentary to transplantation for the qualitative assessment of stem cell potential, which involves marking stem cells with a constitutively-expressed reporter in such a way that all progeny derived from each stem cell will inherit the mark. Unfortunately, lineage tracing has limited quantitative value due to the limitations of chemically-driven labeling techniques (for more information, lineage tracing is reviewed in Kretzschmar and Watt, 2012). To date, transplantation is the most unequivocal functional measure of an SSC and a quantitative measure of SSC content from donor cell suspensions.

From a clinical standpoint, SSC transplantation offers a means of fertility restoration for men afflicted by certain types of infertility. Approximately 10% of couples are infertile worldwide, with half of all cases attributed to male factors (Chandra et al., 2013; Matzuk and Lamb, 2008). Impaired SSC function remains a leading cause of male infertility and transplantation could restore fertility in these men. Furthermore, SSCs are sensitive to radiotherapy and chemotherapeutic treatments (Holoch and Wald, 2011). While sperm cryopreservation overcomes the adverse effects of cancer therapies in adult men, prepubertal boys have yet to produce viable sperm and are thus susceptible to life-long infertility. Approximately 1 in 450 individuals are survivors of adolescent cancer, with both the incidence and survival of adolescent cancer continually rising (Gracia and Ginsberg, 2007). Thus, current research efforts are focusing on the isolation of SSCs and improving the efficiency of SSC transplantation.

Experimental transplantation in rodents has been used to understand much of what we know regarding SSC homing. For example, signaling through the CXCR4 expressed in SSCs is necessary for their homing to niches following transplantation and maintenance of the population in vivo during postnatal life (Yang et al., 2013; Kanatsu-Shinohara et al., 2012). CXCL12 secreted by Sertoli acts through CXCR4 on SSCs to regulate proliferation and inhibit RA-induced differentiation (Yang et al., 2013). Also, SSCs express $\alpha 6$ - and $\beta 1$ -integrin (Shinohara et al., 2000), a complex that forms the laminin receptor, and laminin is the primary component of the seminiferous basement membrane. Transplantation studies have shown that $\beta 1$ -integrin expressed by SSCs and Sertoli cells is necessary for retention of SSCs to their cognate niches (Kanatsu-Shinohara et al., 2008). Furthermore, $\beta 1$ -integrin is necessary for attachment of cultured spermatogonia to laminin-coated plates in vitro (Kanatsu-Shinohara et al., 2008). E-Cadherin (also known as Cadherin 1 or Cdh1) is also preferentially expressed by undifferentiated

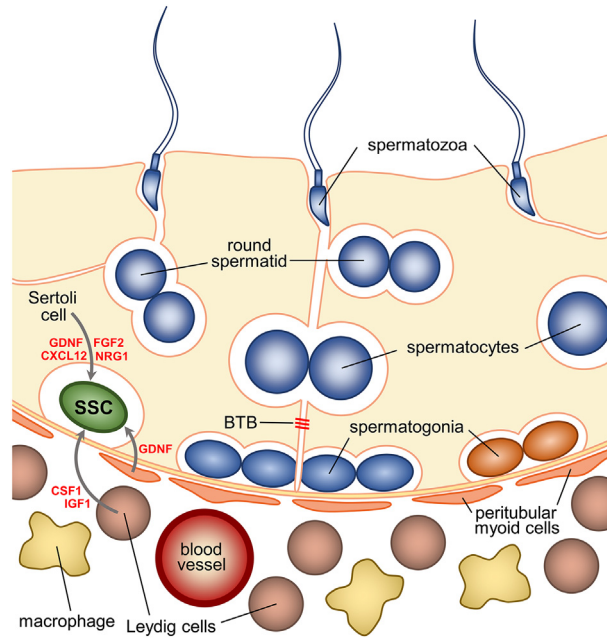


Fig. 2 Model of the seminiferous tubule and SSC niche composition. SSCs nestled along the seminiferous membrane and below the blood-testis-barrier (BTB) are exposed to glial cell-derived neurotrophic factor (GDNF), fibroblast growth factor 2 (FGF2), C-X-C motif chemokine ligand 12 (CXCL12), and neuregulin 1 (NRG1) produced by the adjacent Sertoli cells. Peritubular myoid cells also produce GDNF. Leydig cells contribute colony stimulating factor 1 (CSF1) and insulin-like growth factor 1 (IGF1). Together, these factors as well as others yet to be identified regulate SSC survival, proliferation, self-renewal, and progenitor formation.

A_s , A_{pr} , and A_{al} spermatogonia and is likely a component of the SSC niche (Tokuda et al., 2007). However, $\alpha 6/\beta 1$ -integrin and E-cadherin are not solely expressed by SSCs. Therefore, further studies are necessary to identify niche-specific adhesion molecules that participate in SSC homing (Fig. 2).

Sertoli Cell Contributions to the SSC Niche

Sertoli cells are generally considered key contributors to the SSC niche. For example, experimentally increasing the number of Sertoli cells by approximately 50% in mice leads to a greater than threefold increase in the number of niches occupied by SSCs (Oatley et al., 2011). Sertoli cells are closely associated with spermatogonia. This association is first established with the formation of seminiferous cords during development and remains in mature seminiferous tubules into adulthood. Because SSCs reside in the basal compartment of the seminiferous epithelium, they are exposed to factors secreted by the interstitial cells and derived from the systemic circulation. Thus, the anatomical location of SSCs along the basement membrane and beneath the BTB is a defining characteristic of the mature SSC niche. Sertoli cells are also capable of acting as intermediates relaying systemic signals to the SSC niche. For example, the pituitary-derived gonadotropin follicle-stimulating hormone (FSH) acts on Sertoli cells leading to the secretion of growth factors that support the SSC niche.

FSH stimulates Sertoli cell production of one of the most well-known regulators of SSC function, glial cell line-derived neurotrophic factor (GDNF) (Tadokoro et al., 2002; Ding et al., 2011). Progressive exhaustion of spermatogenesis occurs in mouse models with a *Gdnf* null allele in a dosage-dependent manner, indicating that GDNF is necessary for SSC self-renewal and maintenance in vivo (Meng et al., 2000). Conversely, overexpression of *Gdnf* leads to the accumulation of undifferentiated spermatogonia (Meng et al., 2000). In primary cultures of mouse spermatogonia, GDNF is necessary for long-term survival as well as maintenance of regenerative capacity as measured via transplantation (Nagano et al., 2003; Kubota et al., 2004). GDNF is also necessary for the proliferation and survival of cultured spermatogonia from rats (Ryu et al., 2005), rabbits (Kubota et al., 2011), cattle (Oatley et al., 2016), and humans (Sadri-Ardekani et al., 2009; He et al., 2010), suggesting that GDNF is likely a conserved factor necessary for the maintenance and function of SSCs across most mammalian species. However, further transplantation studies are necessary to truly assess the requirement of GDNF in the maintenance of stem cell capacity in many of these species. GDNF signals through a complex of the RET receptor and GDNF family receptor $\alpha 1$ (GFR $\alpha 1$) present on SSCs as well as other A_s , A_{pr} , and A_{al} spermatogonia. Therefore, while GDNF is unequivocally necessary for survival and proliferation of SSCs and other undifferentiated spermatogonia, growth factors that specifically direct self-renewal or differentiation decisions by SSCs alone have yet to be determined.

Fibroblast growth factor 2 (FGF2) is another Sertoli-cell derived growth factor that is necessary for SSC maintenance. Co-supplementation of mouse primary spermatogonia cultures with FGF2 and GDNF leads to the long-term expansion of SSCs in vitro (Kubota et al., 2004; Kanatsu-Shinohara et al., 2005). Likewise, epidermal growth factor (EGF) has been shown to augment the actions of GDNF-induced proliferation and survival in cultures of spermatogonia (Kanatsu-Shinohara et al., 2005). However, neither FGF2 nor EGF can support SSCs alone; co-supplementation with GDNF is still necessary to maintain spermatogonial cultures (Kubota et al., 2004).

Interestingly, Sertoli cells also express factors that promote the formation of progenitors from SSCs. Neuregulin 1 (NRG1) is expressed by Sertoli cells in mice (Zhang et al., 2011) and peritubular myoid cells in rats (Zhang et al., 2011), and conditional inactivation of NRG1 in mice leads to the accumulation of undifferentiated spermatogonia, indicating a block in formation of differentiating spermatogonia (Zhang et al., 2011). Furthermore, rat germ cells express the NRG1 receptor ERBB3 and supplementation of culture media with NRG1 leads to the formation of spermatogonial chains, thus indicating progenitor formation (Hamra et al., 2007).

Interstitial Cells and Circulatory Contributions to the SSC Niche

The interstitial spaces of the testis are predominantly populated with Leydig cells, peritubular myoid cells, macrophages, and a network of blood vessels, all of which provide vital contributions to the SSC niche. Leydig cells are the predominant source of testosterone in the testis and do so in response to the pituitary-derived luteinizing hormone. Peritubular myoid cells line the seminiferous tubule as a smooth muscle-like layer to provide peristaltic action and structural support. Macrophages are the immune cells of the testis, separated from the advanced germ cells by the BTB. In addition to these primary functions, research suggests that testicular interstitial cells and the vasculature may play important roles in SSC function.

Leydig and some peritubular myoid cells—but not Sertoli cells—produce the SSC regulator colony stimulating hormone 1 (CSF1) (Oatley et al., 2009). Addition of CSF1 to cultures of spermatogonia also treated with GDNF and FGF2 leads to increased SSC concentration as measured by transplantation without altering spermatogonial proliferation, overall suggesting that CSF1 regulates SSC self-renewal activity specifically (Oatley et al., 2009). Also, *Csf1r* expression is enriched in SSCs (Helsel et al., 2017b), supporting the conclusion that CSF1 promotes SSC self-renewal.

Insulin-like growth factor 1 (IGF1) is another Leydig cell-derived factor that regulates the proliferation of numerous testicular cells, including SSCs. Also, the testicular vasculature is likely another source of IGF1. Addition of IGF1 to cultures of spermatogonia sustained with GDNF leads to the expansion of SSCs via proliferation (Kubota et al., 2004). The IGF1 receptor (IGF1R) is expressed by spermatogonia including SSCs and promotes DNA synthesis and proliferation through the G₂/M checkpoint (Potter and DeFalco, 2017).

In addition to Sertoli cells, peritubular myoid cells also produce GDNF (Chen et al., 2016). The mouse seminiferous epithelium can be roughly categorized into 12 sequential stages of germ cell development. Sertoli cells produce GDNF during stages IX through I, and peritubular myoid cells produce GDNF during stages II through IV (Potter and DeFalco, 2017).

Other Factors That Promote Progenitor Formation

Numerous studies agree that RA is the principal trigger for terminal differentiation of spermatogonia in the postnatal/adult testis. However, recent studies indicate that RA acts only on undifferentiated, nonstem cell spermatogonia, and the niche protects SSCs from local RA exposure (Lord et al., 2017). Thus, what factors promote asymmetrical or symmetrical formation of progenitors by SSCs? Studies utilizing primary cultures of murine undifferentiated spermatogonia have begun to answer this question. First, supplementation of spermatogonia cultures with either BMP4 or Activin A decreases SSC number and promotes the differentiation of spermatogonia (Pellegrini et al., 2003). Additionally, (as discussed above) NRG1 produced by the feeder cells in spermatogonial cultures leads to the formation of chains indicative of progenitor formation. Further studies are necessary to understand the SSC-specific responses to BMP4, Activin A, and NRG1 that promote progenitor formation while preventing exhaustion of the SSC pool.

Based on the aforementioned studies with cultured spermatogonia, progenitor formation is likely regulated through (1) temporal secretion of pro-differentiation factors by somatic or other germ cells, (2) the dynamic response to factors regulated intrinsically by SSCs, or (3) a combination of both. Recent studies have shed light on complexity of this regulation. Wingless-type MMTV integration site (WNT) signaling promotes the proliferation of progenitor spermatogonia in culture (Yeh et al., 2011) and WNTs are expressed in a stage-specific manner by Sertoli and interstitial cells that coincides with progenitor formation (Tokue et al., 2017). Interestingly, the WNT inhibitor SHISA6 has confers resistance to WNT signaling by a subset of spermatogonia believed to constitute SSCs (Tokue et al., 2017). Thus, the temporal regulation of pro-progenitor factors in combination with intrinsic control of SSCs to these responses, likely underscores the fate of some spermatogonia to form progenitors. Whether additional pro-progenitor or pro-self-renewal factors participate in this combinatorial regulation has yet to be determined.

Summary and Conclusions

As research continues to elucidate the composition of the SSC niche, our knowledge regarding the function and behavior of SSCs continues to broaden. This knowledge will continue to identify underlying causes of male infertility and generate new means of rectifying such ailments. Future studies focused on changes in the concentration of niche factors will illuminate the chemical, environmental, genetic, and physical factors that impact male fertility. For example, mice and humans normally begin to experience reproductive senescence and declining fertility with advanced age. Previous studies in mice found that serial transplantation at 3 month intervals maintained spermatogenesis in young recipients, suggesting that SSC self-renewal persists beyond the lifespan of mice and that niche breakdown likely contributes to the decline in spermatogenesis with age (Ryu et al., 2006). Thus, premature niche breakdown could underlie the clinical presentation of infertility among many men. Also, changes in niche factor concentration could help combat the detrimental side effects chemotherapeutics in men. For example, GDNF expression is elevated in Sertoli cells following busulfan treatment of mice (Ryu et al., 2006; Zohni et al., 2012). Therefore, additional growth factor supplementation could protect SSCs from depletion with cancer treatment.

Even with advances in our understanding of the SSC niche, our knowledge is still rudimentary, as evidenced by the fact that self-renewal of spermatogonial cultures can only be sustained for approximately 6 months (Helsel et al., 2017a). Therefore, current studies are actively searching for factors that sustain spermatogonia from several species with the hope of stably expanding an SSC pool for research and/or clinical application with techniques such as transplantation. In addition to pro-proliferative and pro-survival factors necessary for all undifferentiated spermatogonia like GDNF, FGF2, EGF, and IGF1, there are likely other SSC-specific growth-supporting factors yet to be identified. Likewise, the search continues for SSC-specific factors that regulate self-renewal and/or progenitor formation as well as the continued in vivo studies of previously identified factors such as CXCL12, CSF1, NRG1, BMP4, and Activin A.

With the great breadth of knowledge that has been gleaned from studies of the last few decades, several unanswered questions remain, so our understanding of SSC niche composition and SSC function will continue to evolve into the future.

References

- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91(24), 11303–11307.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91(24), 11298–11302.
- Chandra, A., Copen, C. E., & Stephen, E. H. (2013). Infertility and impaired fecundity in the United States, 1982–2010: Data from the National Survey of Family Growth. *National Health Statistics Report*, 67(1–18), 11. following 19.
- Chen, L. Y., Willis, W. D., & Eddy, E. M. (2016). Targeting the Gdnf gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. *Proceedings of the National Academy of Sciences of the United States of America*, 113(7), 1829–1834.
- de Rooij, D. G. (2017). The nature and dynamics of spermatogonial stem cells. *Development*, 144(17), 3022–3030.
- Ding, L. J., Yan, G. J., Ge, Q. Y., et al. (2011). FSH acts on the proliferation of type A spermatogonia via Nur77 that increases GDNF expression in the Sertoli cells. *FEBS Letters*, 585(15), 2437–2444.
- Dobrynski, I., Ogawa, T., Avarbock, M. R., et al. (1999). Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. *Molecular Reproduction and Development*, 53(2), 142–148.
- Gracia, C. R., & Ginsberg, J. P. (2007). Fertility risk in pediatric and adolescent cancers. *Cancer Treatment and Research*, 138, 57–72.
- Gunesdogan, U., Magnusdottir, E., & Surani, M. A. (2014). Primordial germ cell specification: A context-dependent cellular differentiation event [corrected]. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 369(1657).
- Hamra, F. K., Chapman, K. M., Nguyen, D., et al. (2007). Identification of neuregulin as a factor required for formation of aligned spermatogonia. *The Journal of Biological Chemistry*, 282(1), 721–730.
- He, Z., Kokkinaki, M., Jiang, J., et al. (2010). Isolation, characterization, and culture of human spermatogonia. *Biology of Reproduction*, 82(2), 363–372.
- Helsel, A. R., & Oatley, J. M. (2017). Transplantation as a quantitative assay to study mammalian male Germline stem cells. *Methods in Molecular Biology*, 1463, 155–172.
- Helsel, A. R., Oatley, M. J., & Oatley, J. M. (2017a). Glycolysis-optimized conditions enhance maintenance of regenerative integrity in mouse Spermatogonial stem cells during long-term culture. *Stem Cell Reports*, 8(5), 1430–1441.
- Helsel, A. R., Yang, Q. E., Oatley, M. J., et al. (2017b). ID4 levels dictate the stem cell state in mouse spermatogonia. *Development*, 144(4), 624–634.
- Hermann, B. P., Sukhwani, M., Simorangkir, D. R., et al. (2009). Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. *Human Reproduction*, 24(7), 1704–1716.
- Holoch, P., & Wald, M. (2011). Current options for preservation of fertility in the male. *Fertility and Sterility*, 96(2), 286–290.
- Kanatsu-Shinohara, M., Miki, H., Inoue, K., et al. (2005). Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. *Biology of Reproduction*, 72(4), 985–991.
- Kanatsu-Shinohara, M., Inoue, K., Miki, H., et al. (2006). Clonal origin of germ cell colonies after spermatogonial transplantation in mice. *Biology of Reproduction*, 75(1), 68–74.
- Kanatsu-Shinohara, M., Takehashi, M., Takashima, S., et al. (2008). Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. *Cell Stem Cell*, 3(5), 533–542.
- Kanatsu-Shinohara, M., Inoue, K., Takashima, S., et al. (2012). Reconstitution of mouse spermatogonial stem cell niches in culture. *Cell Stem Cell*, 11(4), 567–578.
- Kimble, J. E., & White, J. G. (1981). On the control of germ cell development in *Caenorhabditis elegans*. *Developmental Biology*, 81(2), 208–219.
- Kluin, P. M., & de Rooij, D. G. (1981). A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. *International Journal of Andrology*, 4(4), 475–493.
- Koubova, J., Menke, D. B., Zhou, Q., et al. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103(8), 2474–2479.
- Kretzschmar, K., & Watt, F. M. (2012). Lineage tracing. *Cell*, 148(1–2), 33–45.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101(47), 16489–16494.

- Kubota, H., Wu, X., Goodyear, S. M., et al. (2011). Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. *The FASEB Journal*, 25(8), 2604–2614.
- Lord, T., & Oatley, J. M. (2017). A revised Asingle model to explain stem cell dynamics in the mouse male germline. *Reproduction*, 154(2), R55–R64.
- Lord, T., Oatley, M. J., & Oatley, J. M. (2017). Testicular architecture is critical for mediation of retinoic acid responsiveness by undifferentiated spermatogonial subtypes in the mouse. *Stem Cell Reports*, 2018.
- Matzuk, M. M., & Lamb, D. J. (2008). The biology of infertility: Research advances and clinical challenges. *Nature Medicine*, 14(11), 1197–1213.
- Meng, X., Lindahl, M., Hyvonen, M. E., et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287(5457), 1489–1493.
- Nagano, M., Ryu, B. Y., Brinster, C. J., et al. (2003). Maintenance of mouse male germ line stem cells in vitro. *Biology of Reproduction*, 68(6), 2207–2214.
- Oatley, J. M., & Brinster, R. L. (2006). Spermatogonial stem cells. *Methods in Enzymology*, 419, 259–282.
- Oatley, J. M., Oatley, M. J., Avarbock, M. R., et al. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development*, 136(7), 1191–1199.
- Oatley, M. J., Racicot, K. E., & Oatley, J. M. (2011). Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. *Biology of Reproduction*, 84(4), 639–645.
- Oatley, M. J., Kaucher, A. V., Yang, Q. E., et al. (2016). Conditions for long-term culture of cattle undifferentiated Spermatogonia. *Biology of Reproduction*, 95(1), 14.
- Pellegrini, M., Grimaldi, P., Rossi, P., et al. (2003). Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: A potential role of BMP4 in spermatogonia differentiation. *Journal of Cell Science*, 116(Pt 16), 3363–3372.
- Potter, S. J., & DeFalco, T. (2017). Role of the testis interstitial compartment in spermatogonial stem cell function. *Reproduction*, 153(4), R151–R162.
- Richardson, B. E., & Lehmann, R. (2010). Mechanisms guiding primordial germ cell migration: Strategies from different organisms. *Nature Reviews. Molecular Cell Biology*, 11(1), 37–49.
- Ryu, B. Y., Kubota, H., Avarbock, M. R., et al. (2005). Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. *Proceedings of the National Academy of Sciences of the United States of America*, 102(40), 14302–14307.
- Ryu, B. Y., Orwig, K. E., Oatley, J. M., et al. (2006). Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. *Stem Cells*, 24(6), 1505–1511.
- Sadri-Ardekani, H., Mizrak, S. C., van Daalen, S. K., et al. (2009). Propagation of human spermatogonial stem cells in vitro. *JAMA*, 302(19), 2127–2134.
- Schofield, R. (1978). The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells*, 4(1–2), 7–25.
- Shinohara, T., Orwig, K. E., Avarbock, M. R., et al. (2000). Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proceedings of the National Academy of Sciences of the United States of America*, 97(15), 8346–8351.
- Shinohara, T., Orwig, K. E., Avarbock, M. R., et al. (2001). Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *Proceedings of the National Academy of Sciences of the United States of America*, 98(11), 6186–6191.
- Tadokoro, Y., Yomogida, K., Ohta, H., et al. (2002). Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mechanisms of Development*, 113(1), 29–39.
- Tokuda, M., Kadokawa, Y., Kurahashi, H., et al. (2007). CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. *Biology of Reproduction*, 76(1), 130–141.
- Tokue, M., Ikami, K., Mizuno, S., et al. (2017). SHISA6 confers resistance to differentiation-promoting Wnt/beta-catenin signaling in mouse Spermatogenic stem cells. *Stem Cell Reports*, 8(3), 561–575.
- Tulina, N., & Matunis, E. (2001). Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. *Science*, 294(5551), 2546–2549.
- Yang, Q. E., Kim, D., Kaucher, A., et al. (2013). CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. *Journal of Cell Science*, 126(Pt 4), 1009–1020.
- Yeh, J. R., Zhang, X., & Nagano, M. C. (2011). Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. *Journal of Cell Science*, 124(Pt 14), 2357–2366.
- Yoshida, S., Sukeno, M., Nakagawa, T., et al. (2006). The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development*, 133(8), 1495–1505.
- Zhang, X., Ebata, K. T., & Nagano, M. C. (2003). Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. *Biology of Reproduction*, 69(6), 1872–1878.
- Zhang, J., Eto, K., Honmyou, A., et al. (2011). Neuregulins are essential for spermatogonial proliferation and meiotic initiation in neonatal mouse testis. *Development*, 138(15), 3159–3168.
- Zohni, K., Zhang, X., Tan, S. L., et al. (2012). The efficiency of male fertility restoration is dependent on the recovery kinetics of spermatogonial stem cells after cytotoxic treatment with busulfan in mice. *Human Reproduction*, 27(1), 44–53.

Spermatogenic Cell Syncytium

Juho-Antti Mäkelä and Jorma Toppari, University of Turku, Turku, Finland; and Turku University Hospital, Turku, Finland

© 2018 Elsevier Inc. All rights reserved.

Introduction

Connections of male germ cells by intercellular processes were described already in the 19th century by von La Valette-St. George (1865), Sertoli (1878), and von Ebner (1888). Transmission electron microscopy and live-cell imaging studies have provided a detailed description of intercellular bridge (ICB) assembly, changes in its structure in different phases of spermatogenesis and evidence for intercellular movement of molecules, granules, vesicles, and organelles via ICBs (Burgos and Fawcett, 1955; Fawcett et al., 1959; Dym and Fawcett, 1971; Huckins, 1978a; Weber and Russell, 1987; Ventelä et al., 2003). The bridge channel, that connects the syncytial cells, is no open space but it is filled by microtubules, endoplasmic reticulum, filaments, ribosomes, mitochondria, vesicles, and granules (Dym and Fawcett, 1971; Weber and Russell, 1987; Ventelä et al., 2003; Clermont and Rambourg, 1978). Their presence in the ICB channel suggests that cellular material is actively being transported between the interconnected cells.

The cells that are connected by ICBs are called a syncytium. Male germ cells enter and complete spermatogenesis in syncytia (Fig. 1). Cell fate decisions within the syncytium are shared and the whole clone either proceeds according to the strict spermatogenic program or undergoes apoptosis (Huckins, 1978b). Cytoplasmic contiguity, that the ICBs enable, is considered a prerequisite for synchronous spermatogenic progress of the clone. Compared to gap junctions, that connect cells in numerous tissues, ICBs (0.5–3 µm) are huge openings in the cell membrane that provide the basic framework to establish and maintain cytoplasmic homogeneity within the syncytium (Weber and Russell, 1987).

The current understanding is that ICB formation is a distinctive form of cytokinesis (Greenbaum et al., 2011). Cytokinesis can be divided into three phases: selection of the site for cell division, cleavage furrow ingression and formation of a contractile ring, and abscission of the midbody (Greenbaum et al., 2011; Fededa and Gerlich, 2012). From a germ cell point of view, the last step is the most interesting one. While midbodies and ICBs are involved in every cell division, in somatic cells they are short-lived, whereas in germ cells ICBs are stabilized, which is a prerequisite for syncytial development, spermatogenesis, and male fertility. Development of a syncytium has been conserved in the male germ-line for over a billion years, from invertebrates to humans (Burgos and Fawcett, 1955; Rasmussen, 1973). While lack of spermatogenic syncytia results in meiotic failure and infertility, mutations in cytokinetic proteins can cause formation of multinucleated germ cells that are able to develop to postmeiotic phase emphasizing the importance of intercellular connection for spermatogenesis (Holstein and Eckmann, 1986; Carmena et al., 1998; Brill et al., 2000; Greenbaum et al., 2006).

Due to their wide diameter, ICBs, at least on theoretical level, enable exchange and equal distribution of cytoplasmic components and organelles or their structural components, granules, and vesicles (cytomixis). In nondividing germ cells the nuclear envelope prohibits intracolon exchange of nuclear material via ICBs. In prometaphase to late telophase, when the nuclear membrane is lacking or is just being reassembled, a specific structure called bridge-partitioning complex preserves genomic integrity and transiently closes ICBs to avoid the drift of chromatin to neighboring cells (Miething, 2003).

Many hypotheses have been proposed as to address the function and significance of germ cell intercellular bridges (reviewed by Guo and Zheng, 2004). According to the “cytoplasm sharing” hypothesis passage of cytoplasmic constituents via ICBs ensures synchronous spermatogenesis within the syncytium. Another hypothesis suggests that intercellular traffic via ICBs is needed to even out stochastic and gene content-derived (after meiotic segregation) differences in the transcriptome and permit genotypically haploid cells to function in a phenotypically diploid manner (Braun et al., 1989). Two examples will clarify the point: (1) some genes that are essential for postmeiotic spermatogenesis are located in the sex chromosomes. Haploid spermatids possess just one sex chromosome (say X), which makes them dependent on sharing of gene products derived from the other sex chromosome (Y); (2) Not all alleles are good and sharing of the gene products encoded by normal alleles might help to shelter from the undesired effects. According to the “critical coordination” hypothesis rapid ICB-mediated signal transfer is needed at critical steps (such as A₁ transition, discussed below) during spermatogenesis to coordinate the process. “Gamete equivalence” hypothesis emphasizes the need to create gametes that are uniform in structure and morphology (similar acrosomes, for instance Ventelä et al., 2003) as gamete heterogeneity associates with fertility problems. Equivalence and high overall sperm quality would result from cytoplasm sharing and elimination of heterogeneity that the ICBs allow for. Finally, LeGrand sees ICBs as a means for the germ cells to monitor the internal state of their sister cells for emergence of self-promoting genes, and, if needed, induce apoptosis of the whole cyst (LeGrand, 2001).

All these hypotheses provide a plausible explanation for the existence of ICBs in the germ-line but it deserves to be mentioned that ICBs only provide wide channels and do very little per se to contribute to the outcomes discussed above. For cytomixis to occur, the interconnected cells need to build the cytoskeletal infrastructure and molecular motors to make the cargo moving. To ensure even but not random distribution of cytoplasmic constituents the task needs to be assigned to specialized macromolecules, such as the chromatoid body, that are active at the ICB interface (Ventelä et al., 2003).

This article provides a concise review of our current knowledge about the formation male germ cell syncytium and its significance for spermatogenesis. It will focus mainly on mouse, rat and *Drosophila* where the most study has been completed.

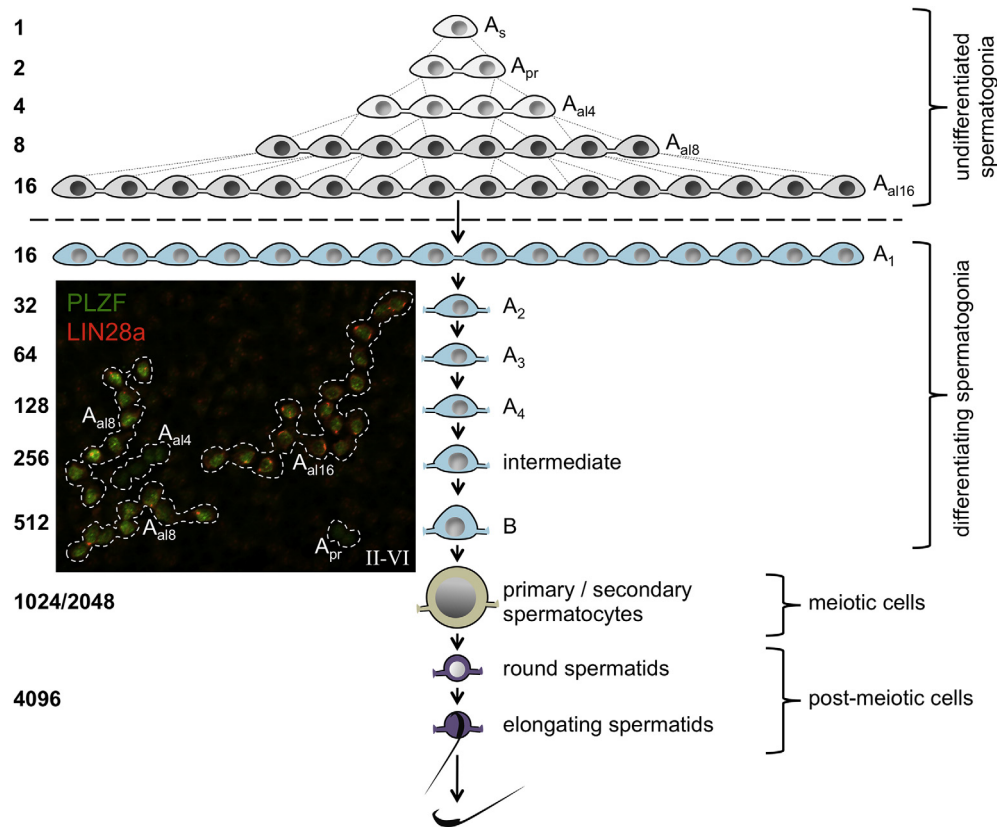


Fig. 1 Spermatogenesis and formation of spermatogenic cell syncytium in the mouse. The only postnatal germ cell type lacking a cytoplasmic connection is the A-single (A_s) spermatogonium. Division of A_s produces A-paired (A_{pr}), a cyst of two cells. Cytokinesis in the male germ-line is incomplete and each cell division therefore doubles the syncytial length. Accordingly, mitosis of A_{pr} gives rise to A-aligned4 (A_{al4}), a 4-cell cyst, then A_{al8} and A_{al16}. These cells are called undifferentiated spermatogonia (gray cells), the stem and progenitor cells of the adult mouse testis. They transit into A₁, the first subpopulation of differentiating spermatogonia (blue cells) and enter spermatogenesis as a cyst of 16 cells (shown) or as A_s or shorter cysts. A₁ spermatogonia undergo six mitotic divisions before the onset of meiosis as primary spermatocytes (khaki cells). Each division doubles the cyst length (column on the left). Meiotic divisions quadruple the syncytial length, give rise to spermatids (purple cells) and bring the theoretical maximum size for a spermatogenic cyst to 4096 cells. In practice, such lengths are not achieved and the cyst occasionally splits. This color key is maintained throughout this article. Inset shows whole-mount staining of adult mouse seminiferous tubule at stages II–VI of the seminiferous epithelial cycle. PLZF (promyelocytic leukemia zinc finger, green) is expressed in A_{pr} and A_{al4-16} undifferentiated spermatogonia, whereas LIN28a (red) stains strongly only A_{al8-16}. Low expression of both is seen in intermediate/type B spermatogonia on the background.

Ring Canals in *Drosophila*

Germ cell division in the *Drosophila* germ-line is incomplete and results in the formation of a 16-cell cyst in which the diploid germ cells are interconnected by stable cytoplasmic bridges called ring canals (RC). Formation of RCs results from cessation of contractile ring constriction and consequent cleavage furrow arrest (Hime et al., 1996; Robinson and Cooley, 1996; Huynh and St Johnston, 2004). Further development of the germ cell syncytium is sex-dependent, and in the female, one of the syncytial cells is selected as the oocyte and the rest (15 cells) will become nurse cells that eventually transfer most of their cytoplasmic material to the oocyte (Huynh and St Johnston, 2004). In the male, the cyst undergoes meiotic divisions to give rise to a syncytium of 64 haploid germ cells (Fig. 2) (Huynh and St Johnston, 2004). A characteristic feature of the *Drosophila* germ-line is the fusome, a specialized and highly vesiculated cytoplasmic structure that connects the cells in the germ cell cyst via RCs. The fusome originates from a spectro-some, which is a spherical structure present in germ-line stem cells (Lin et al., 1994). The function of the fusome is not well understood but it may have a role in coordination of cell division during *Drosophila* gametogenesis (Lin et al., 1994). Interestingly, in the female germ-line the fusome is disintegrated upon oocyte specification but in the male it persists until late spermiogenesis and might serve to align spermatid flagella within a cyst (Hime et al., 1996; Lin et al., 1994). To our knowledge, an equivalent structure does not exist in mammals.

Despite the nomenclature, RCs of the two sexes are structurally different and mature male and female RCs share only a few structural components, including MKLP1 (mitotic kinesin-like protein 1; named Pavarotti in *Drosophila*), a key component in mammalian midbodies and ICBs, too (Eikenes et al., 2013). Besides the germ-line, RCs also connect some populations of somatic cells in

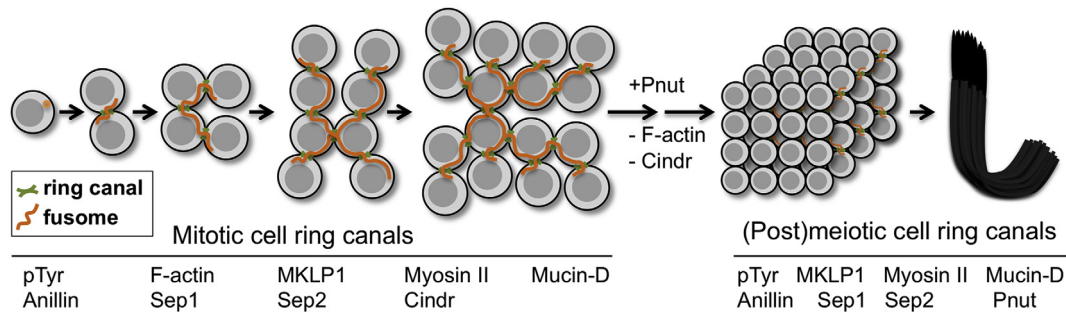


Fig. 2 Spermatogenesis and formation of the spermatogenic cyst and the fusome in *Drosophila*. The gonialblast (on the left) divides four times to produce a 16-cell spermatogonial cyst that enters meiosis. The meiotic divisions give rise to a cyst consisting of 64 spermatids. Ring canals (RCs, green) are formed as a result of cleavage furrow arrest and consequential incomplete cytokinesis. Fusome (orange) connects all the cells in a cyst via RCs. It is a derivative of the spectrosome, a spherical structure located in the cytosol of germ-line stem cells (predecessors of gonialblasts). RC composition is temporally regulated and while many proteins are ubiquitously present in RCs (pTyr, MKLP1, Myosin II, Anillin, Sep1, Sep2, Mucin-D), some are lost (F-actin and Cindr) and others are introduced (Pnut) during spermatogenesis.

Drosophila. Somatic RCs between ovarian follicle cells and more restrictedly in larval wing imaginal discs allow intercellular diffusion of cytoplasmic proteins (McLean and Cooley, 2013).

RCs in *Drosophila* are tyrosine phosphorylated, and emergence of phosphotyrosine (pTyr) epitopes in the late telophase is considered the first step in the RC assembly (Robinson and Cooley, 1996). Proteins containing pTyr accumulate in the ring canal that already contains contractile ring F-actin and myosin II, scaffold protein Anillin and septins (Sep1, Sep2), that can self-assemble into rings (Robinson and Cooley, 1996; Kinoshita et al., 2002). Src64 kinase has been proposed to play a key role in controlling tyrosine phosphorylation at the site of RC formation and in definition of RC diameter (Eikenes et al., 2015). However, Src64 deficient male flies are fertile, which implies that other proteins might compensate for loss of Src64 (Dodson et al., 1998). Upon maturation F-actin and scaffold protein Cindr are removed from the RC, and the contractile ring is consequently disassembled. This is probably a key step in incomplete cytokinesis and stabilization of RCs, and it coincides with the mitosis to meiosis shift in both sexes (Eikenes et al., 2013). Both Cindr and F-actin are translocated to the fusome and they are vitally important proteins for its expansion (Eikenes et al., 2013). The mature postmitotic RC of *Drosophila* male germ-line consists of pTyr-containing proteins, Anillin, septins (Sep1, Sep2, Pnut), MKLP1, Zipper (nonmuscle myosin II heavy chain), and glycoprotein Mucin-D (Fig. 2) (Carmenta et al., 1998; Hime et al., 1996; Eikenes et al., 2013; Cooley, 1998; Kramerova and Kramerov, 1999). Interestingly, RCs of the *Drosophila* male germ-line are structurally similar to somatic RCs. Despite their relatively small diameter—when compared to oogenesis-associated RCs (up to 10 μm)—they permit intercellular movement of cytoplasmic particles to equilibrate protein content among transcriptionally mosaic cells, and are essential for fertility (Cooley, 1998; McLean and Cooley, 2014). Fragmentation of spermatogonial cysts via abscission of intercellular connections result in dedifferentiation of differentiation-committed germ cells—a mechanism reminiscent of replenishment of mammalian testis stem cell niche, as discussed below (Brawley and Matunis, 2004).

Intercellular Bridges in Mammals

Spermatogenic Cell Expansion and Stabilization of the Intercellular Bridge

Complete cytokinesis is an exception in the mammalian male germ-line under steady state conditions. The only germ cell type in the adult testis that is not connected to another cell by a cytoplasmic connection is the A-single (A_s) spermatogonium (Fig. 1). According to the A_s theory from 1971 by Huckins and Oakberg, the A_s spermatogonia function as spermatogonial stem cells during steady state spermatogenesis (Huckins, 1971; Oakberg, 1971; de Rooij and Russell, 2000). More recent research has complicated the situation a bit and among other things it has shown that (1) considerations about whether A_s divisions are symmetrical (dividing into similar cells) or asymmetrical (dividing into dissimilar cells) are obsolete as division of A_s is in ~95% of the cases incomplete and the daughter cells are connected by a cytoplasmic bridge (A-paired, A_{pr}), that (2) A_s can commit to differentiate without preamplification, and that (3) undifferentiated type A (A_{undiff} , A_s and cysts of up to 16 cells, Fig. 1) spermatogonia retain the ability to function as stem cells after fragmentation of the cysts under steady state spermatogenesis and especially during repopulation of the seminiferous epithelium after cytotoxic stress or transplantation (Nakagawa et al., 2007, 2010; Hara et al., 2014). Formation of an ICB between the progeny of an A_s division (A_{pr}) would lead to exhaustion of the A_s population were it not replenished from alternative sources. Indeed, fragmentation of A_{pr} and A_{al4} (a cyst of four cells) is relatively common and gives rise to new A_s spermatogonia, whereas their division is always incomplete and doubles the syncytial length (Fig. 3) (Hara et al., 2014). The mechanisms that control stability and breakdown of ICBs and consequential fragmentation of A_{undiff} syncytia have not been well characterized.

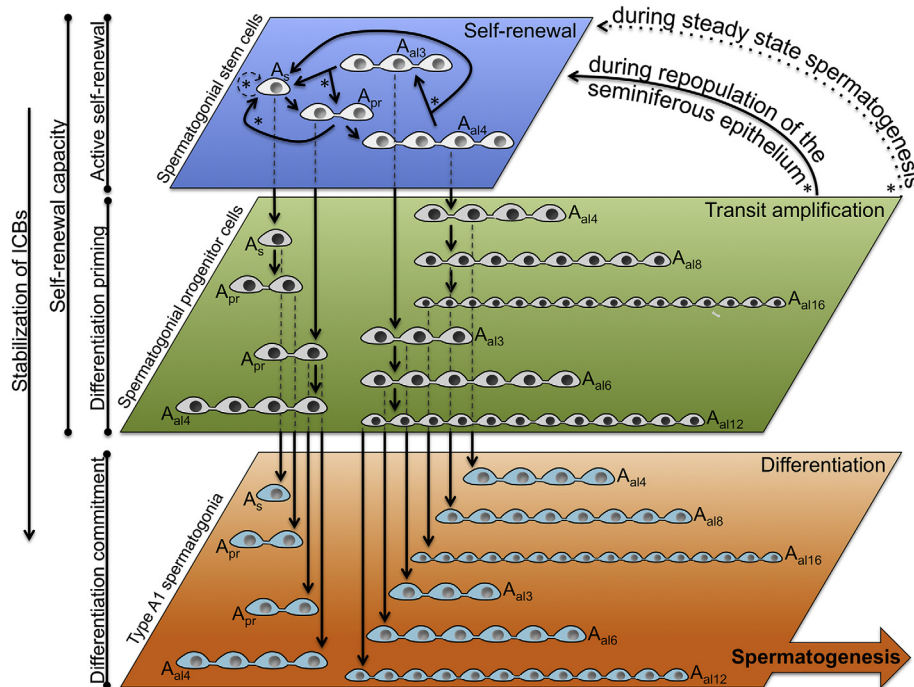


Fig. 3 Dynamics of the stem and progenitor spermatogonia during mouse spermatogenesis. GFRa1-positive (GDNF family receptor alpha 1) stem cells (blue plane) actively interconvert between short syncytial and single cell states through incomplete cytokinesis and cyst fragmentation (*) and maintain the self-renewing spermatogonial population, or are primed to differentiate and enter the transit-amplifying progenitor state (green plane). Under steady state spermatogenesis, the progenitor cells are bound to enter spermatogenesis but they retain the capacity for self-renewal via fragmentation in case of a need to repopulate the seminiferous epithelium. Once entered spermatogenic process as A₁ spermatogonia (orange plane), germ cells are committed to differentiate according to a rigid time-scaled program.

Differentiation propensity within the A_{undiff} population is associated with chain length. Increased A_{undiff} cyst length is typically connected with a higher likelihood to differentiate with a concomitant upregulation of a group of markers (such as Ngn3 [neurogenin 3], Sox3 [Sry-box 3], and RARγ [retinoic acid receptor gamma], and downregulation of others (most notably glial cell-line derived neurotrophic factor [GDNF] receptor GFRa1 [GDNF family receptor alpha 1]) (Nakagawa et al., 2010; Raverot et al., 2005; Ikami et al., 2015). As described above, GFRa1-expressing A_s, A_{pr}, and A_{al} spermatogonia comprise a single stem cell pool, in which cells continually and reversibly interconvert between singly isolated and short syncytial states through incomplete cell division and fragmentation of syncytia. Ngn3 positive cells are recruited from this pool by differentiation-priming activity of the Wnt/beta-catenin pathway (Fig. 3) (Nakagawa et al., 2010; Hara et al., 2014; Takase and Nusse, 2016; Tokue et al., 2017). While these Ngn3-positive progenitor spermatogonia retain ability for self-renewal, they are destined to differentiate under steady state conditions (Nakagawa et al., 2007, 2010). Therefore, similar to *Drosophila*, stabilization of the ICB can be regarded as a prerequisite for differentiation commitment in the mammalian male germ-line (Brawley and Matunis, 2004). Acquisition of RARγ expression equips the Ngn3-positive progenitor spermatogonia with a competence to respond to differentiation-inducing stimuli, most notably retinoic acid (RA) (Ikami et al., 2015). RA-induced transition of A_{undiff} spermatogonia into type A₁ differentiating spermatogonia is considered the onset of spermatogenesis due to its irreversible nature (Fig. 3). A_{undiff} to A₁ transition is characterized by notable changes in spermatogonial transcriptome and induction of Kit and DNA methyltransferases that act on the committing cell epigenome (Schrans-Stassen et al., 1999; Shirakawa et al., 2013). It is not known whether architecture of the ICB changes as a result of spermatogonial differentiation commitment but stabilization of its structure might contribute to inability of differentiating spermatogonia to revert to the naïve state.

Having transitioned into a type A₁ spermatogonium, the destiny of a male germ cell is fixed and it undergoes spermatogenic differentiation according to a rigid time-scaled program, or perishes by apoptosis (Fig. 1). In the mouse, A₁ spermatogonia first divide six times mitotically (A₁ → A₂ → A₃ → A₄ → intermediate → B → spermatocyte) before entering meiosis as preleptotene spermatocytes. Meiotic divisions quadruple the number of germ cells resulting in a theoretical 4096-fold amplification in cell number during the course of spermatogenesis (Fig. 1). The progeny of spermatogenic cell mitotic and meiotic divisions are connected by ICBs. However, syncytia of these cells occasionally split and the maximal observed size for a spermatogenic cell syncytium (spermatids in this case) is 650 cells (Ren and Russell, 1991). Cell fate decisions within a syncytium are shared and all the cells in the cyst coordinately progress on the spermatogenic pathway.

Germ cells are fully dependent on somatic Sertoli cells for survival, maintenance and differentiation (Franca et al., 2016). As spermatogenesis progresses the germ cells translocate from the basal lamina of the seminiferous epithelium, between the Sertoli

cells to the lumen of the seminiferous tubule. Having detached from the basal lamina as leptotene primary spermatocytes, the germ cells are engulfed by Sertoli cells and they need to traverse the Sertoli cell barrier (SCB, also known as the blood–testis barrier, BTB) to the immune-privileged adluminal side of the seminiferous epithelium in order to continue maturation. The translocation through the Sertoli cell cytoplasm and SCB is a remarkable effort when keeping in mind that the germ cells do not traverse SCB as solitary cells but as a sieve-like syncytium that can consist of at least some tens and maybe hundreds of cells (theoretically 1024 cells), and the clone spans a number of Sertoli cells. The junctions that form the SCB remain functional during the spermatocyte translocation and maintain the integrity of the SCB. This requires the formation of a transient intermediate compartment where the leptotene spermatocytes are bound by basal and apical tight junctions (Smith and Braun, 2012). The cytoplasmic connection between maturing germ cells provided by the ICB is maintained until the end of spermatogenesis and mature elongated spermatozoa are disengaged from the seminiferous epithelium as single cells (spermiation). ICBs are lost upon spermiation and they are not observed to connect the residual bodies, the excess cytoplasm of spermatozoa, that are phagocytosed by the Sertoli cells (Weber and Russell, 1987).

ICBs are dynamic and there are rearrangements in their structure at various steps during spermatogenesis, especially in spermiogenesis (Weber and Russell, 1987). This reflects to ICB dimensions that vary considerably in different phases of spermatogenesis. While ICB length generally fluctuates between 0.5 and 1 μm during the course of rat spermatogenesis, the ICB diameter undergoes spermatogenic cell type dependent changes. In differentiating type A spermatogonia the ICB diameter ranges from 0.5 to 1 μm ; in intermediate and type B it is about 1.3 μm . The ICBs that connect spermatocytes are slightly wider. At the onset of spermiogenesis, ICBs of step 1 spermatids are approximately 1.8 μm in diameter. There is a gradual increase in diameter of spermiogenic cell ICBs and by step 18 they reach width of 3.0 μm . At the last step of spermiogenesis (step 19) ICBs constrict to about 2.0 μm in diameter (Weber and Russell, 1987).

ICBs are not characteristic only to postnatal germ cells but they also exist in embryonic germ cells. Upon arrival to the bipotential gonad on embryonic day 10.5 (E10.5) most of the primordial germ cells (PGCs) exist as single cells and only 2% are found in groups of more than two cells (Pepling and Spradling, 1998). In the following days they proliferate actively to form cysts and enter mitotic quiescence at around E14.5 (McLaren, 1984; Tam and Snow, 1981). These cells, called gonocytes, are connected by ICBs but these bridges are weak and the gonocyte cysts partially fragment by birth (Greenbaum et al., 2009; Lei and Spradling, 2013). Partial fragmentation leads to appearance of single cells and short cysts (Lei and Spradling, 2013). Independent of the germ cell identity (embryonic, stem/progenitor, mitotic, meiotic or postmeiotic) formation of ICBs depends on one protein preferentially expressed in germ cells: TEX14 (testis-expressed gene 14) (Greenbaum et al., 2006, 2007, 2009; Iwamori et al., 2012).

Mechanism of Germ Cell ICB Formation

Formation of an intercellular bridge is an elemental part of every cell division event. However, in somatic cells, ICB is rapidly cleared at the end of cytokinesis and cytoplasmic contiguity between the nascent daughter cells is lost. Formation of a stable ICB as seen in germ cells can be regarded as a modification of normal cell division that involves stabilization of the midbody, a bulge that lies in the middle of ICB. The midbody matrix consists of numerous protein complexes, including the centralspindlin complex (Fededa and Gerlich, 2012). Centralspindlin is a heterotetramer of MLKP1 and RacGAP1 (Rac GTPase-activating protein 1, also known as MgcRacGAP or CYK-4) and it interacts with the cytoskeleton and is involved in recruitment of the midbody abscission machinery as discussed below (White and Glotzer, 2012). Binding of centralspindlin to CEP55 (centrosomal protein 55), a dimeric coiled-coil protein, is considered the first step toward midbody abscission (Zhao et al., 2006). CEP55 is a protein scaffold that brings centralspindlin in contact with ESCRT-I (endosomal sorting complexes required for transport) complex via interaction with ALIX (ALG-2 interacting protein X) and TSG101 (tumor susceptibility gene 101) subunit (Morita et al., 2007; Carlton and Martin-Serrano, 2007). This, in turn, results in recruitment of the abscission machinery in the form of ESCRT-III complex (Fig. 4A) (White and Glotzer, 2012). MLKP1 and RacGAP1 colocalize with TEX14 in the germ cell ICB in mouse and man (Greenbaum et al., 2007; Naud et al., 2003). The mechanism of TEX14 action to block midbody abscission and stabilize the ICB is to prevent recruitment of ESCRT-I. This is achieved by physical inhibition of CEP55 interaction with ALIX and TSG101 (Fig. 4B) (Kim et al., 2015). TEX14 is indispensable for germ cell ICB formation (Greenbaum et al., 2006).

TEX14, ALIX, and TSG101 all carry an AxGPPx3YxPP (Ala793, Gly795, Pro796, Pro803, and Pro804) motif that binds to EABR (ESCRT and ALIX-binding region) sequence of CEP55 (Kim et al., 2015). The affinity of CEP55-EABR for the TEX14 AxGPPx3YxPP motif is 3–10 times higher than toward the same motif in ALIX or TSG101. In addition, interaction of TEX14 with CEP55-EABR is more stable than that of ALIX. To further promote TEX14-CEP55 interaction, TEX14 is recruited to the midbody before CEP55, whereas ALIX and TSG101 enter midbody later than CEP55 during somatic cell cytokinesis. On top of higher affinity and more stable interaction, this mechanism gives TEX14 a competitive advantage for CEP55-EABR interaction over ALIX and TSG101 (Kim et al., 2015; Iwamori et al., 2010). As a result ALIX1 is displaced from the midbody, ESCRT-I and ESCRT-III will not get recruited and TEX14-CEP55 interaction stabilizes the cytoplasmic connection, and tethers the daughter cells. New proteins are then recruited to the nascent stable ICB (Fig. 4B).

In spermatogonial and spermatocyte early bridges TEX14 forms an inner ring in the midbody matrix and is surrounded by an outer ring of centralspindlin and septins 2, 7, and 9 (putative orthologs of *Drosophila* Sep1, Pnut and Sep2, respectively) (Greenbaum et al., 2007; Pan et al., 2007). TEX14 is able to interact with itself—a characteristic, which probably facilitates the formation of a ring-like structure (Iwamori et al., 2010, 2011). As the ICB matures, the TEX14 ring starts to expand and it eventually merges with the outer ring (Greenbaum et al., 2007). There is a concomitant increase in ICB diameter, and removal of septins leaves

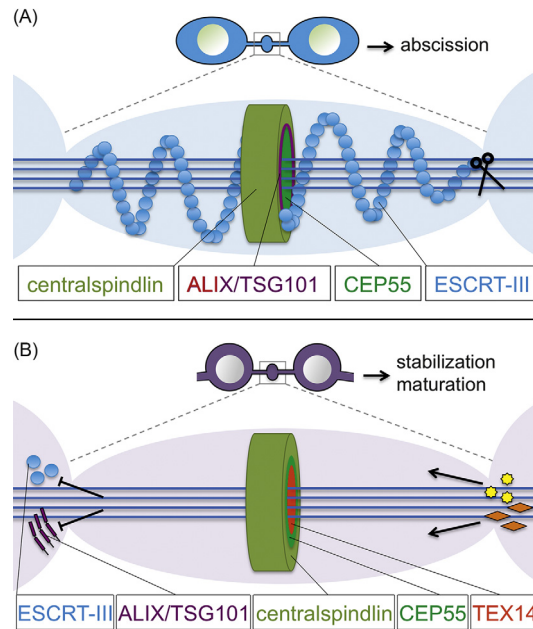


Fig. 4 Midbody abscission vs. stabilization of the intercellular bridge. (A) In somatic cells (or TEX14 deficient germ cells) ESCRT-III is recruited into the midbody via centralspindlin-CEP55-ALIX-TSG101 interaction, and the midbody is abscised resulting in complete cytokinesis and separation of the two daughter cells. (B) In germ cells, TEX14 interacts with the CEP55 EABR sequence, that is needed for ALIX/TSG101 dependent ESCRT-III recruitment. As a result ALIX/TSG101 and ESCRT-III subunits never enter the midbody, the abscission is blocked, and new proteins (*star* and *diamond*) required for ICB stabilization and maturation are recruited. The mitotic spindle (blue lines) is disassembled later on.

TEX14, CEP55, MKLP1, RacGAP1, pericentrin, delta-tubulin, and actin the role as the structural framework of mature ICBs (Greenbaum et al., 2006, 2007; Chang et al., 2010; Kato et al., 2004; Russell et al., 1987). Maturation of ICBs is associated with involvement of new proteins in spermatocytes and spermatids, such as keratin 5, HSF2 (heat shock factor 2), protocadherin-a3, and plectin (Fig. 5) (Tres et al., 1996; Alastalo et al., 1998; Johnson et al., 2004; Guttman et al., 1999). Unlike in *Drosophila*, Anillin never becomes a part of the stabilized ICB and it is removed from the midbody as TEX14 enters it (Greenbaum et al., 2007). RBM44, an RNA-binding protein with a hypothesized function in RNA transport between germ cells via ICBs, is transiently expressed in mature spermatocyte ICBs and colocalizes with TEX14 (Iwamori et al., 2011). RBM44 is lost from ICBs upon completion of meiosis and it is not needed for ICB formation; neither is its action essential for spermatogenesis. RBM44 deficient mice develop ICBs normally, they are fertile and, for an unknown reason, produce significantly higher number of sperm than wild-type control males (Iwamori et al., 2011). Gonocyte ICBs are typical early bridges with clear outer and inner rims of MKLP1 and TEX14, respectively (Greenbaum et al., 2009).

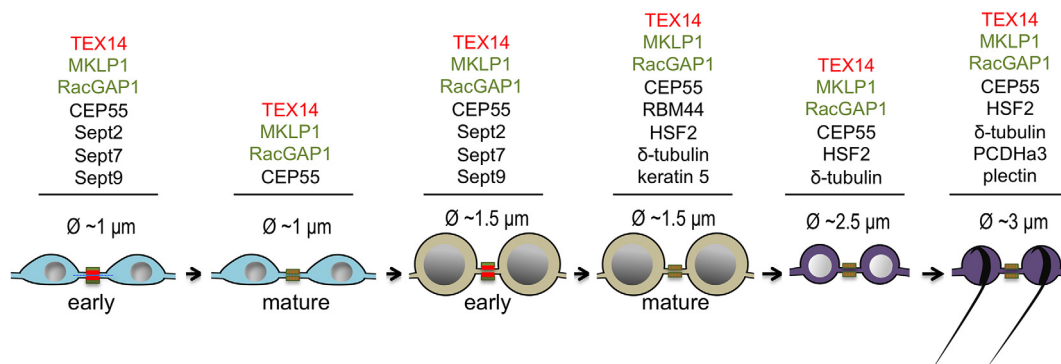


Fig. 5 Maturation of the intercellular bridges. As a result of ICB stabilization the mitotic spindle (dotted blue line) is disassembled. In early spermatogonial and spermatocyte ICBs there are two discernible rings: inner ring of TEX14 (red) and an outer centralspindlin ring (green, MKLP1 + RacGAP1). As these bridges mature, septins are lost and the inner ring expands and merges with the outer ring. In general, the ICB diameter grows as spermatogenesis proceeds and reaches a width of 3 μm in elongating spermatids. ICBs undergo constant reorganization in their structure and while some proteins are universally present in ICBs (TEX14, centralspindlin, CEP55, and actin) some are more specific: HSF2 and δ-tubulin localize only to late meiotic and postmeiotic ICBs, RBM44, and keratin 5 in mature spermatocyte ICBs, and protocadherin-a3 and plectin are exclusively present in late spermiogenic ICBs.

Germ cell cytokinesis is considered a deviation from normal (somatic) cytokinesis and, apart from the fate of the midbody, it is mechanistically highly if not completely similar to it. In TEX14 deficient mice midbody formation takes place normally in germ cells, which indicates that it is not a TEX14-dependent process (Greenbaum et al., 2007). The mechanism of its abscission in TEX14-deficient germ cells has not been characterized in detail but it is likely that, when TEX14 is not present, ESCRT III is recruited via an CEP55-ALIX-TSG101-dependent mechanism and cytokinesis is completed in a somatic cell manner (Fig. 4) (Greenbaum et al., 2007; Kim et al., 2015). While TEX14 is indispensable for ICB formation in mammalian germ cells, ectopic expression of TEX14 in somatic cells did not initially result in stabilization of ICBs (Greenbaum et al., 2007). However, overexpression of TEX14 leads to establishment of stable ICBs in somatic cells indicating that TEX14 is the only germ cell specific protein needed to block midbody abscission (Iwamori et al., 2010).

Intercellular Bridges and Fertility

There are two knock-out mouse models that illustrate the crucial connection between ICBs and fertility: TEX14 and germ cell specific RacGAP1 null mice both lack ICBs and fail to sire offspring (Greenbaum et al., 2006; Lores et al., 2014). In TEX14 null testes spermatogenesis proceeds to spermatocyte stage but germ cells fail to complete meiosis and no postmeiotic cells exist (Greenbaum et al., 2006). A closer look at the meiotic cells reveals that most early primary spermatocytes undergo apoptosis and very few pachytene spermatocytes are observed (Greenbaum et al., 2006). Interestingly, undifferentiated and differentiating spermatogonial populations look overtly normal regardless of loss of ICBs (Greenbaum et al., 2006). Accordingly, and due to relative enrichment of spermatogonia because of severe depletion of meiotic and postmeiotic cells, genes associated with undifferentiated spermatogonia (with the peculiar exception of GDNF target genes) and early differentiating germ cells markers are upregulated in TEX14 KO testes, whereas pan-germ cell marker Vasa is expressed at a lower level than in control testis (Iwamori et al., 2012). It is possible, however, that lack of ICBs in premeiotic germ cells interferes with a process that is needed to prepare the germ cells for meiosis, and therefore contributes to meiotic failure in TEX14 null testis and partially explains the TEX14 null phenotype.

Conversely, conditional RacGAP1 knock-out mice show impaired spermatogenesis and infertility. The phenotype is, however, more complex and the severity of spermatogenesis defect ranges from normal-looking tubule to Sertoli cell only phenotype probably due to incomplete Cre-mediated recombination (Lores et al., 2014). Interestingly, lack of RacGAP1 does not only lead into lack of ICBs but appearance of multinuclear germ cells suggesting that there is a problem in centralspindlin formation, and accordingly in cytoplasmic constriction (Lores et al., 2014). The reproductive phenotype therefore originates to a certain extent from inability of midbody establishment, not instability of the nascent ICB. Since most germ cells in the RacGAP1 null testis contain just one nucleus, it is likely that in most cases the cleavage furrow fully ingresses and the midbody is abscised. Variable penetrance of the used Cre promoter and inconsistent outcome of germ cell cytokinesis, however, make the results challenging to interpret. For these reasons, TEX14 null mice provide a better model to address the question about ICB significance for spermatogenesis and fertility. An important conclusion can still be drawn: establishment, maintenance, and stabilization of intercellular bridges are crucial for fertility.

Instability of ICBs and Maintenance of Spermatogonial Stem Cells

According to a model proposed by Shosei Yoshida and coworkers (discussed in detail above) the stem cell potential within the testis resides primarily in GFR α 1-positive single cells and short syncytia (stem), and latently in Ngn3-positive undifferentiated spermatogonia that preferably exist as longer syncytia (progenitor) (Fig. 3) (Nakagawa et al., 2007, 2010; Hara et al., 2014). The former population actively interconverts and exists as single cell or short syncytial states, whereas Ngn3-positive do not usually fragment, and under steady state conditions instable ICBs are therefore observed principally within the GFR α 1-positive A_{undiff} subset (Hara et al., 2014). From this point of view one might consider that loss of ICB formation ability (as observed in TEX14 null mice) would result in stem cell enrichment. Quite on the contrary, however, expression of GDNF-regulated genes is downregulated in TEX14 deficient testis. Despite these findings, there is not a stem cell maintenance defect in the TEX14 knock-out testis (Greenbaum et al., 2006). Interestingly, maintenance of TEX14 null SSPCs (spermatogonial stem and progenitor cells, the in vitro term for A_{undiff}) is compromised in vitro (Iwamori et al., 2012). This is probably due to a proliferation defect in these cells because their transcriptome is nearly identical to control SSPCs (Iwamori et al., 2012). Unfortunately, it is not known if TEX14 deficient SSPCs are transplantable and whether their capacity to home into the testis stem cell niche and form nascent colonies is normal. Despite poor proliferation in vitro, maintenance of spermatogonia at least up to 1 year of age in vivo suggests that TEX14 null A_{undiff} are able to self-renew for an extended period of time. ICBs also function as a cytoskeletal organizer and in TEX14 deficient SSPCs the architecture of the cytoskeleton has altered dramatically, which is bound to affect cell physiology (Iwamori et al., 2012).

While complete loss of ICBs is deleterious to fertility, increased instability of A_{undiff} syncytia, their consequent fragmentation and higher incidence of A_s spermatogonia is associated with improved life-time fertility, as observed in JMJD3 (Jumonji domain containing-3) deficient mice (Iwamori et al., 2013). JMJD3, a H3K27 (histone 3 lysine 27) demethylase, is an epigenetic modifier and it regulates stem cell fate and differentiation in a number of cell lineages (reviewed by Burchfield et al., 2015). JMJD3 is not needed for differentiation in the male germ-line, but it plays a role in the maintenance of ICBs and reduction of *Jmjd3* is considered to increase the number of spermatogonial stem cells via an unknown, allegedly TEX14-independent, mechanism (Iwamori et al., 2013). In cultured SSPCs, knock-down of *Jmjd3* leads to increased number of nascent colonies and alteration of colony morphology

from a grape-like to compact cluster-like appearance. The former finding can be explained by higher instability of ICBs or improved stemness of SSPCs (probably two sides of the same coin), but the latter one is intriguing because a similar morphological change is associated with SSPC reprogramming into a pluripotent state (Kanatsu-Shinohara et al., 2004). Enlarged testis size and prolonged fertility in JMJD3 deficient mice are likely secondary to enhanced self-renewal of A_{undiff} —an untested hypothesis supported by the in vitro findings, too. It can be concluded from the above studies, that formation of short-lived unstable syncytia and interconversion between single cell and short syncytial states is a dynamic feature of testis stem cell niche, and probably required for normal fertility.

Concluding Remarks

While CEP55-TEX14 interaction is indispensable for formation and maintenance of stable ICBs in mammals, the molecular mechanisms responsible for structural rearrangements in and around the nascent ICB are largely unknown. As discussed above, the dimensions and architecture of early intercellular bridges typical for gonocytes and spermatogonia differs remarkably from the mature ones that are seen in spermatids. What is more, spermiogenic ICBs are under constant process of remodeling but the identities of the proteins involved in these events are not known (Weber and Russell, 1987). Postformation functions of TEX14 are obscure but it has been speculated that it might work as a protein scaffold to recruit other bridge components (Greenbaum et al., 2006). Interestingly, despite obvious similarity between kinase-like domain of TEX14 and kinase domain of Src64, a kinase that is needed to induce RC formation in *Drosophila* (see above), the kinase activity of TEX14 is poor and TEX14-mediated phosphorylation of tyrosine or threonine residues therefore hardly plays a role in the ICB development (Greenbaum et al., 2006; Eikenes et al., 2015). Controlled instability of ICBs within the testis stem cell niche is probably required for maintenance of life-long male fertility—another hypothesized function assigned to germ cell ICBs. What are the factors that destabilize the A_{undiff} ICBs and how they mechanistically contribute to fragmentation of short GFRa1+ cysts await identification and clarification. Although germ cell ICBs have been studied for over 150 years, very little is known about their formation and function at the molecular level. There is a dire need for more research on these unique and fascinating intercellular connections.

References

- Alastalo, T. P., Lonnstrom, M., Leppa, S., et al. (1998). Stage-specific expression and cellular localization of the heat shock factor 2 isoforms in the rat seminiferous epithelium. *Experimental Cell Research*, 240(1), 16–27.
- Braun, R. E., Behringer, R. R., Peschon, J. J., Brinster, R. L., & Palmiter, R. D. (1989). Genetically haploid spermatids are phenotypically diploid. *Nature*, 337(6205), 373–376.
- Brawley, C., & Matunis, E. (2004). Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. *Science*, 304(5675), 1331–1334.
- Brill, J. A., Hime, G. R., Scharer-Schuksz, M., & Fuller, M. T. (2000). A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis. *Development*, 127(17), 3855–3864.
- Burchfield, J. S., Li, Q., Wang, H. Y., & Wang, R. F. (2015). JMJD3 as an epigenetic regulator in development and disease. *The International Journal of Biochemistry & Cell Biology*, 67, 148–157.
- Burgos, M. H., & Fawcett, D. W. (1955). Studies on the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (*Felis domestica*). *The Journal of Biophysical and Biochemical Cytology*, 1(4), 287–300.
- Carlton, J. G., & Martin-Serrano, J. (2007). Parallels between cytokinesis and retroviral budding: A role for the ESCRT machinery. *Science*, 316(5833), 1908–1912.
- Carmena, M., Riparbelli, M. G., Minestrini, G., et al. (1998). *Drosophila* polo kinase is required for cytokinesis. *The Journal of Cell Biology*, 143(3), 659–671.
- Chang, Y. C., Chen, Y. J., CH, W., YC, W., Yen, T. C., & Ouyang, P. (2010). Characterization of centrosomal proteins Cep55 and pericentrin in intercellular bridges of mouse testes. *Journal of Cellular Biochemistry*, 109(6), 1274–1285.
- Clermont, Y., & Rambourg, A. (1978). Evolution of the endoplasmic reticulum during rat spermiogenesis. *The American Journal of Anatomy*, 151(2), 191–211.
- Cooley, L. (1998). *Drosophila* ring canal growth requires Src and Tec kinases. *Cell*, 93(6), 913–915.
- de Rooij, D. G., & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology*, 21(6), 776–798.
- Dodson, G. S., Guarnieri, D. J., & Simon, M. A. (1998). Src64 is required for ovarian ring canal morphogenesis during *drosophila* oogenesis. *Development*, 125(15), 2883–2892.
- Dym, M., & Fawcett, D. W. (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biology of Reproduction*, 4(2), 195–215.
- Eikenes, A. H., Brech, A., Stenmark, H., & Haglund, K. (2013). Spatiotemporal control of cindr at ring canals during incomplete cytokinesis in the *drosophila* male germline. *Developmental Biology*, 377(1), 9–20.
- Eikenes, A. H., Malerod, L., Lie-Jensen, A., et al. (2015). Src 64 controls a novel actin network required for proper ring canal formation in the *drosophila* male germline. *Development*, 142(23), 4107–4118.
- Fawcett, D. W., Ito, S., & Slautterback, D. (1959). The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. *The Journal of Biophysical and Biochemical Cytology*, 5(3), 453–460.
- Fededa, J. P., & Gerlich, D. W. (2012). Molecular control of animal cell cytokinesis. *Nature Cell Biology*, 14(5), 440–447.
- Franca, L. R., Hess, R. A., Dufour, J. M., Hofmann, M. C., & Griswold, M. D. (2016). The sertoli cell: One hundred fifty years of beauty and plasticity. *Andrology*, 4(2), 189–212.
- Greenbaum, M. P., Yan, W., Wu, M. H., et al. (2006). TEX14 is essential for intercellular bridges and fertility in male mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103(13), 4982–4987.
- Greenbaum, M. P., Ma, L., & Matzuk, M. M. (2007). Conversion of midbodies into germ cell intercellular bridges. *Developmental Biology*, 305(2), 389–396.
- Greenbaum, M. P., Iwamori, N., Agno, J. E., & Matzuk, M. M. (2009). Mouse TEX14 is required for embryonic germ cell intercellular bridges but not female fertility. *Biology of Reproduction*, 80(3), 449–457.
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3(8), a005850.
- Guo, G. Q., & Zheng, G. C. (2004). Hypotheses for the functions of intercellular bridges in male germ cell development and its cellular mechanisms. *Journal of Theoretical Biology*, 229(1), 139–146.
- Guttman, J. A., Mulholland, D. J., & Vogl, A. W. (1999). Plectin is concentrated at intercellular junctions and at the nuclear surface in morphologically differentiated rat sertoli cells. *The Anatomical Record*, 254(3), 418–428.

- Hara, K., Nakagawa, T., Enomoto, H., et al. (2014). Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell*, 14(5), 658–672.
- Hime, G. R., Brill, J. A., & Fuller, M. T. (1996). Assembly of ring canals in the male germ line from structural components of the contractile ring. *Journal of Cell Science*, 109(Pt 12), 2779–2788.
- Holstein, A. F., & Eckmann, C. (1986). Multinucleated spermatocytes and spermatids in human seminiferous tubules. *Andrologia*, 18(1), 5–16.
- Huckins, C. (1971). The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *The Anatomical Record*, 169(3), 533–557.
- Huckins, C. (1978a). Spermatogonial intercellular bridges in whole-mounted seminiferous tubules from normal and irradiated rodent testes. *The American Journal of Anatomy*, 153(1), 97–121.
- Huckins, C. (1978b). The morphology and kinetics of spermatogonial degeneration in normal adult rats: An analysis using a simplified classification of the germinal epithelium. *The Anatomical Record*, 190(4), 905–926.
- Huynh, J. R., & St Johnston, D. (2004). The origin of asymmetry: Early polarisation of the drosophila germline cyst and oocyte. *Current Biology*, 14(11), R438–49.
- Ikami, K., Tokue, M., Sugimoto, R., et al. (2015). Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. *Development*, 142(9), 1582–1592.
- Iwamori, T., Iwamori, N., Ma, L., Edson, M. A., Greenbaum, M. P., & Matzuk, M. M. (2010). TEX14 interacts with CEP55 to block cell abscission. *Molecular and Cellular Biology*, 30(9), 2280–2292.
- Iwamori, T., Lin, Y. N., Ma, L., Iwamori, N., & Matzuk, M. M. (2011). Identification and characterization of RBM44 as a novel intercellular bridge protein. *PLoS One*, 6(2), e17066.
- Iwamori, N., Iwamori, T., & Matzuk, M. M. (2012). Characterization of spermatogonial stem cells lacking intercellular bridges and genetic replacement of a mutation in spermatogonial stem cells. *PLoS One*, 7(6), e38914.
- Iwamori, N., Iwamori, T., & Matzuk, M. M. (2013). H3K27 demethylase, JMJD3, regulates fragmentation of spermatogonial cysts. *PLoS One*, 8(8), e72689.
- Johnson, K. J., Zecevic, A., & Kwon, E. J. (2004). Protocadherin alpha3 acts at sites distinct from classic cadherins in rat testis and sperm. *Biology of Reproduction*, 70(2), 303–312.
- Kanatsu-Shinohara, M., Inoue, K., Lee, J., et al. (2004). Generation of pluripotent stem cells from neonatal mouse testis. *Cell*, 119(7), 1001–1012.
- Kato, A., Nagata, Y., & Todokoro, K. (2004). Delta-tubulin is a component of intercellular bridges and both the early and mature perinuclear rings during spermatogenesis. *Developmental Biology*, 269(1), 196–205.
- Kim, H. J., Yoon, J., Matsuura, A., et al. (2015). Structural and biochemical insights into the role of testis-expressed gene 14 (TEX14) in forming the stable intercellular bridges of germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, 112(40), 12372–12377.
- Kinoshita, M., Field, C. M., Coughlin, M. L., Straight, A. F., & Mitchison, T. J. (2002). Self- and actin-templated assembly of mammalian septins. *Developmental Cell*, 3(6), 791–802.
- Kramerova, I. A., & Kramerov, A. A. (1999). Mucinoprotein is a universal constituent of stable intercellular bridges in drosophila melanogaster germ line and somatic cells. *Developmental Dynamics*, 216(4–5), 349–360.
- LeGrand, E. K. (2001). Genetic conflict and apoptosis. *Perspectives in Biology and Medicine*, 44(4), 509–521.
- Lei, L., & Spradling, A. C. (2013). Mouse primordial germ cells produce cysts that partially fragment prior to meiosis. *Development*, 140(10), 2075–2081.
- Lin, H., Yue, L., & Spradling, A. C. (1994). The drosophila fusome, a germline-specific organelle, contains membrane skeletal proteins and functions in cyst formation. *Development*, 120(4), 947–956.
- Lores, P., Vernet, N., Kurosaki, T., et al. (2014). Deletion of MgcRacGAP in the male germ cells impairs spermatogenesis and causes male sterility in the mouse. *Developmental Biology*, 386(2), 419–427.
- McLaren, A. (1984). Meiosis and differentiation of mouse germ cells. *Symposia of the Society for Experimental Biology*, 38, 7–23.
- McLean, P. F., & Cooley, L. (2013). Protein equilibration through somatic ring canals in drosophila. *Science*, 340(6139), 1445–1447.
- McLean, P. F., & Cooley, L. (2014). Bridging the divide: Illuminating the path of intercellular exchange through ring canals. *Fly (Austin)*, 8(1), 13–18.
- Miething, A. (2003). The formation and dissolution of the bridge-partitioning complex in intercellular bridges of dividing germ cells in the testis of the golden hamster. *Journal of Submicroscopic Cytology and Pathology*, 35(3), 271–276.
- Morita, E., Sandrin, V., Chung, H. Y., et al. (2007). Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. *The EMBO Journal*, 26(19), 4215–4227.
- Nakagawa, T., Nabeshima, Y., & Yoshida, S. (2007). Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Developmental Cell*, 12(2), 195–206.
- Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R. E., & Yoshida, S. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science*, 328(5974), 62–67.
- Naud, N., Toure, A., Liu, J., et al. (2003). Rho family GTPase Rnd2 interacts and co-localizes with MgcRacGAP in male germ cells. *The Biochemical Journal*, 372(Pt 1), 105–112.
- Oakberg, E. F. (1971). Spermatogonial stem-cell renewal in the mouse. *The Anatomical Record*, 169(3), 515–531.
- Pan, F., Malmberg, R. L., & Momany, M. (2007). Analysis of septins across kingdoms reveals orthology and new motifs. *BMC Evolutionary Biology*, 7, 103.
- Pepling, M. E., & Spradling, A. C. (1998). Female mouse germ cells form synchronously dividing cysts. *Development*, 125(17), 3323–3328.
- Rasmussen, S. W. (1973). Ultrastructural studies of spermatogenesis in drosophila melanogaster meigen. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 140(1), 125–144.
- Raverot, G., Weiss, J., Park, S. Y., Hurley, L., & Jameson, J. L. (2005). Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Developmental Biology*, 283(1), 215–225.
- Ren, H. P., & Russell, L. D. (1991). Clonal development of interconnected germ cells in the rat and its relationship to the segmental and subsegmental organization of spermatogenesis. *The American Journal of Anatomy*, 192(2), 121–128.
- Robinson, D. N., & Cooley, L. (1996). Stable intercellular bridges in development: The cytoskeleton lining the tunnel. *Trends in Cell Biology*, 6(12), 474–479.
- Russell, L. D., Vogl, A. W., & Weber, J. E. (1987). Actin localization in male germ cell intercellular bridges in the rat and ground squirrel and disruption of bridges by cytochalasin D. *The American Journal of Anatomy*, 180(1), 25–40.
- Schrans-Stassen, B. H., van de Kant, H. J., de Rooij, D. G., & van Pelt, A. M. (1999). Differential expression of c-kit in mouse undifferentiated and differentiating type a spermatogonia. *Endocrinology*, 140(12), 5894–5900.
- Sertoli, E. (1878). Sulla struttura dei canalicoli seminiferi dei testicoli studiata in rapporto allo sviluppo dei nemaspermi. *Archivio per le Scienze Mediche*, 2, 107–146.
- Shirakawa, T., Yaman-Deveci, R., Tomizawa, S., et al. (2013). An epigenetic switch is crucial for spermatogonia to exit the undifferentiated state toward a kit-positive identity. *Development*, 140(17), 3565–3576.
- Smith, B. E., & Braun, R. E. (2012). Germ cell migration across sertoli cell tight junctions. *Science*, 338(6108), 798–802.
- Takase, H. M., & Nusse, R. (2016). Paracrine wnt/beta-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. *Proceedings of the National Academy of Sciences of the United States of America*, 113(11), E1489–97.
- Tam, P. P., & Snow, M. H. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *Journal of Embryology and Experimental Morphology*, 64, 133–147.
- Tokue, M., Ikami, K., Mizuno, S., et al. (2017). SHISA6 confers resistance to differentiation-promoting wnt/beta-catenin signaling in mouse spermatogenic stem cells. *Stem Cell Reports*.

- Tres, L. L., Rivkin, E., & Kierszenbaum, A. L. (1996). Sak 57, an intermediate filament keratin present in intercellular bridges of rat primary spermatocytes. *Molecular Reproduction and Development*, 45(1), 93–105.
- Ventelä, S., Toppari, J., & Parvinen, M. (2003). Intercellular organelle traffic through cytoplasmic bridges in early spermatids of the rat: Mechanisms of haploid gene product sharing. *Molecular Biology of the Cell*, 14(7), 2768–2780.
- von Ebner, V. (1888). Zur spermatogenese bei den säugethieren. *Archiv für Mikroskopische Anatomie*, 31, 236–292.
- von La Valette-St. George, A. (1865). Über die genese der samenkörper. *Mitt Archiv für Mikroskopische Anatomie*, 1, 403–414.
- Weber, J. E., & Russell, L. D. (1987). A study of intercellular bridges during spermatogenesis in the rat. *The American Journal of Anatomy*, 180(1), 1–24.
- White, E. A., & Glotzer, M. (2012). Centralspindlin: At the heart of cytokinesis. *Cytoskeleton (Hoboken)*, 69(11), 882–892.
- Zhao, W. M., Seki, A., & Fang, G. (2006). Cep55, a microtubule-bundling protein, associates with centralspindlin to control the midbody integrity and cell abscission during cytokinesis. *Molecular Biology of the Cell*, 17(9), 3881–3896.

Seminiferous Cycle

Juho-Antti Mäkelä and Jorma Toppari, University of Turku, Turku, Finland; and Turku University Hospital, Turku, Finland

© 2018 Elsevier Inc. All rights reserved.

The Seminiferous Epithelial Cycle and the Stages of the Seminiferous Epithelium

The development of male germ cells in the seminiferous epithelium can be divided into three phases: (1) mitotic phase, characterized by mitotic divisions of spermatogonia; (2) meiotic phase, including genetic recombination and two meiotic divisions that quadruple the cell number; and (3) spermiogenic phase, the complex differentiation cascade of postmeiotic haploid germ cells that eventually gives rise to mature spermata with an acrosome and a flagellum. The sequential progress of these phases is strictly regulated in time and space and spermatogenic cell differentiation through these phases is coordinately controlled within and between the germ cell syncytia present at a specific tubular area.

Histology of testicular tissue and seminiferous tubules may seem complex at first. Germ cells of varying morphology are situated inside the seminiferous tubule in a complex organization that does neither display any obvious hierarchy among the intermingled populations of germ cells nor a repetitive pattern of layering. However, the germ cells are organized in a strict order. Cross-sections of individual tubules within one histological specimen include areas that look similar and host the same populations of germ cells with a specific morphology. These recurring cellular associations, also known as the stages of the seminiferous epithelial cycle, are in constant progress as the germ cells are continually developing and their characteristics (cell shape, size, ploidy) change over time (Fig. 1). If the tissue were alive one could follow the gradual development of the cell associations and notice that after a specific interval the cross-section has reached the original appearance, and is said to have completed a cycle of the seminiferous epithelium (used interchangeably with “spermatogenic cycle,” “seminiferous cycle,” and “seminiferous epithelial cycle” in the literature) (Leblond and Clermont, 1952; Oakberg, 1956).

In rodents with a high number of premeiotic germ cell mitoses, the stages follow each other in a numerical order along the length of the seminiferous tubule, and form the spermatogenic wave, or the wave of the seminiferous epithelium. In human and primates, this kind of segmental arrangement of stages is not observed due to low amplification of differentiation-committed spermatogonial cells. This makes histological studies of human spermatogenesis considerably harder as a cross-section of the seminiferous tubule typically contains two to four stages, whereas in rodents it is uncommon to have more than one stage per cross-section (Amann, 2008). In most species, including man and mouse, the seminiferous cycle is subdivided into 12 stages (marked with Roman numerals I–XII) (Fig. 2) (Leblond and Clermont, 1952; Muciaccia et al., 2013). The stage classification is based primarily on gradual development of the acrosome in spermatids, the postmeiotic germ cells. In rodents, the segmental arrangement of the stages enables identification and isolation of stages based on their light absorption characteristics (Parvinen, 1982; Toppari and Parvinen, 1985; Kotaja et al., 2004).

It needs to be stated clearly that the morphological characteristics of the germ cells present at a given stage, or the number of stages are arbitrary and defined by morphology. The stages do neither have any biological relevance to the progression of the spermatogenic process per se, nor do they represent stagnant checkpoint-like developmental states but are persistently progressing

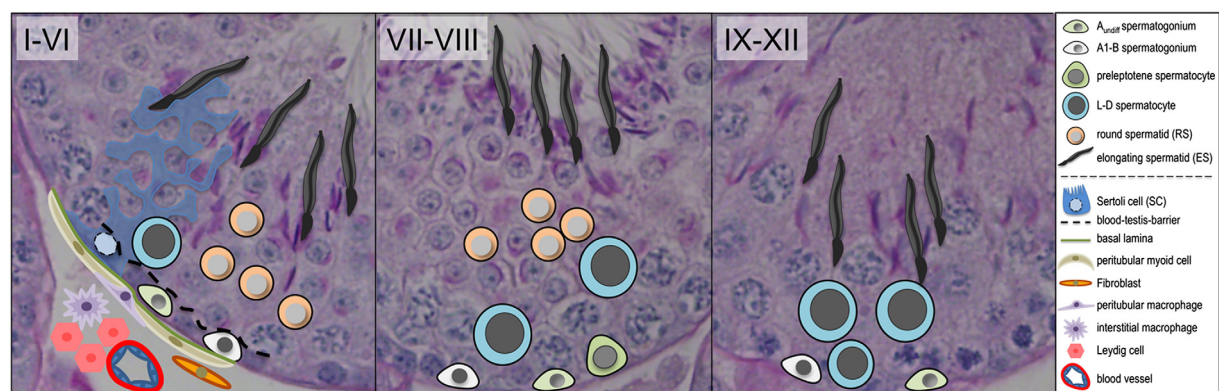


Fig. 1 Histology of the mouse testis—cross-sections of adult seminiferous tubules at different stages of spermatogenesis. During spermatogenesis germ cells at different phases of maturation form cellular associations of constant composition. Twelve (I–XII) associations, or stages of the seminiferous cycle, have been designated for most mammalian species, including man and mouse (shown in figure). For practical purposes stages I–VI, VII–VIII, and IX–XII are often shown as one “pooled” stage. Undifferentiated (A_{undiff}) and differentiating spermatogonia (A_1 to type B) and early meiotic preleptotene spermatocytes dwell on the basal lamina of the seminiferous epithelium. Leptotene to diakinetid (L–D) and secondary spermatocytes plus postmeiotic spermatids do not have a contact to the basal lamina but are engulfed by Sertoli cells and intermingled on the adluminal side of the blood–testis barrier. Peritubular myoid cells and peritubular macrophages encase the seminiferous tubules. Testicular blood vessels run in the interstitium, a home to testosterone-producing Leydig cells, interstitial macrophages, and fibroblasts. The color key used for different cell types is used throughout this article. Micrographs courtesy of Dr. Noora Kotaja.

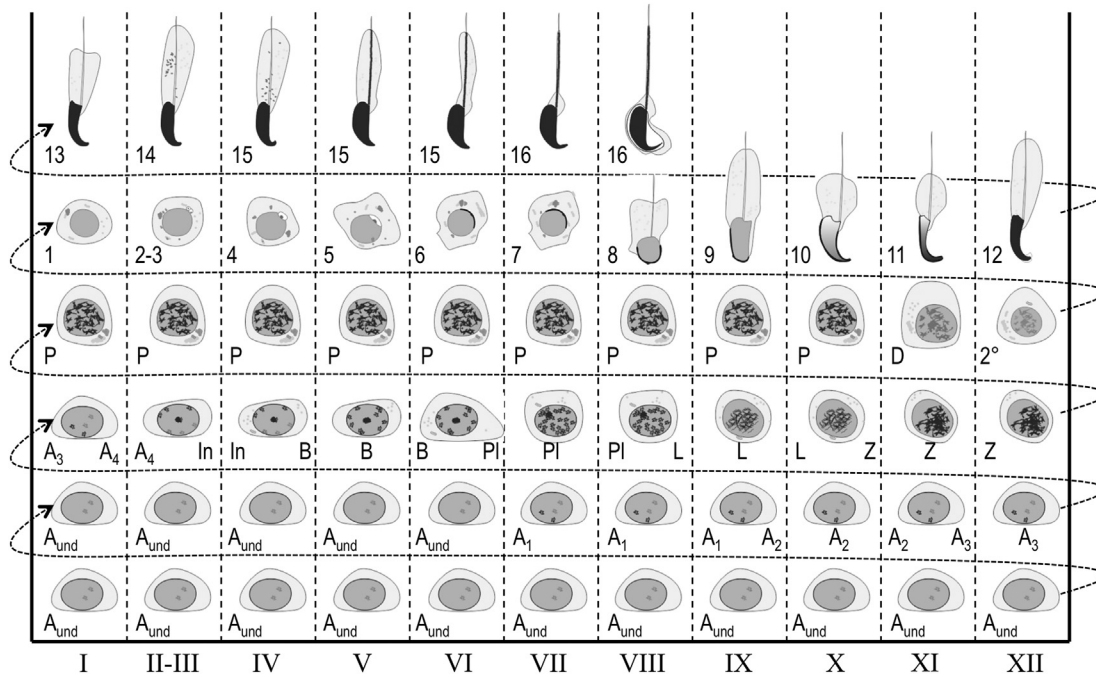


Fig. 2 Seminiferous epithelial cycle map of mouse spermatogenesis. Stages of the seminiferous epithelium are depicted in the vertical columns (Roman numerals I–XII). The most immature germ cells are placed at the bottom, whereas the most mature are at the top making it a hierarchical map and not necessarily implying to their location within the epithelium. In order to follow the progress of spermatogenesis and gradual development of male germ cells, one has to move from left to right, and from bottom to top. A cycle is a complete series of stages that follow one another in a logical order. Arabic numerals refer to steps of spermatid maturation, that is, spermiogenesis. A_{und} , undifferentiated spermatogonia; A1–4, type A1–A4 spermatogonia; In, intermediate spermatogonia; B, type B spermatogonia; PI, preleptotene spermatocytes; L, leptotene spermatocytes; Z, zygotene spermatocytes; P, pachytene spermatocytes; D, diplotene spermatocytes; 2°, secondary spermatocytes plus meiotic divisions. Drawn by MSc Sheyla Estefani Cisneros-Montalvo.

as different cohorts of germ cells at a specific area synchronously undergo spermatogenic differentiation. Staging of the seminiferous cycle is merely a tool that makes spermatogenesis easier to study, analyze, and comprehend. During the course of one cycle a certain area in the tubule undergoes all the stages. A spermatogenic unit of a Sertoli cell and associated germ cells is like a water molecule in the ocean. Its position in space does not change as a result of the passing spermatogenic wave, but the wave does affect the developmental state of spermatogenic cells within the unit and lifts them one level higher in the differentiation hierarchy (Fig. 2).

The duration of the seminiferous cycle varies between different species and even within a species between the different strains (Russell et al., 1990). The cycle length in man is 16 days, whereas in the mouse it takes only 8.6 days to complete a cycle (Oakberg, 1956; Heller and Clermont, 1963). Approximately four cycles are needed by germ cells to undergo spermatogenesis from a differentiating spermatogonium to a mature elongated spermatid with a flagellum and an acrosome. This brings the duration of spermatogenesis to 74 and 35 days in man and mouse, respectively (Fig. 3A) (Heller and Clermont, 1964; Clermont, 1972). The cycle length and therefore the duration of spermatogenesis are fundamental characteristics of a species. Creation of knock-out animal models and naturally occurring mutations have shown that spermatogenesis can go wrong at many different steps and is not even initiated if undifferentiated spermatogonia fail to commit. However, up to date it has not been reported that in any conditions either due to loss of gene function or adverse environment the seminiferous cycle length would have been altered. However, in rodents the first round of spermatogenesis represents a distinctive program of germ cell differentiation, and it differs from the following ones in many aspects, including the cycle length (Yoshida et al., 2006; Kluin et al., 1982). The reason or the underlying mechanisms for shortening of the cycle in peripubertal rodents are not known. It has been hypothesized that the first cycle is started from fetal gonocytes that would follow a distinct developmental rhythm, whereas all the following cycles start from the spermatogonial stem cell pool.

The Onset and Cycle-Dependent Coordination of Spermatogenesis

In the mouse, spermatogenesis starts when the differentiation-primed subpopulation of type A undifferentiated spermatogonia (A_{undiff}) transition into type A1 differentiating spermatogonia (A_1) at stages VII–VIII of the seminiferous cycle (Fig. 3B). This transition is an irreversible step and A_1 spermatogonia are committed to differentiate into haploid elongated spermatids over the course of 35 days (Clermont, 1972). A_{undiff} become sensitive to differentiation-inducing stimuli (1) by upregulating the expression of retinoic acid receptor gamma (RAR γ), (2) by becoming mitotically quiescent by stage II of the seminiferous epithelial cycle,

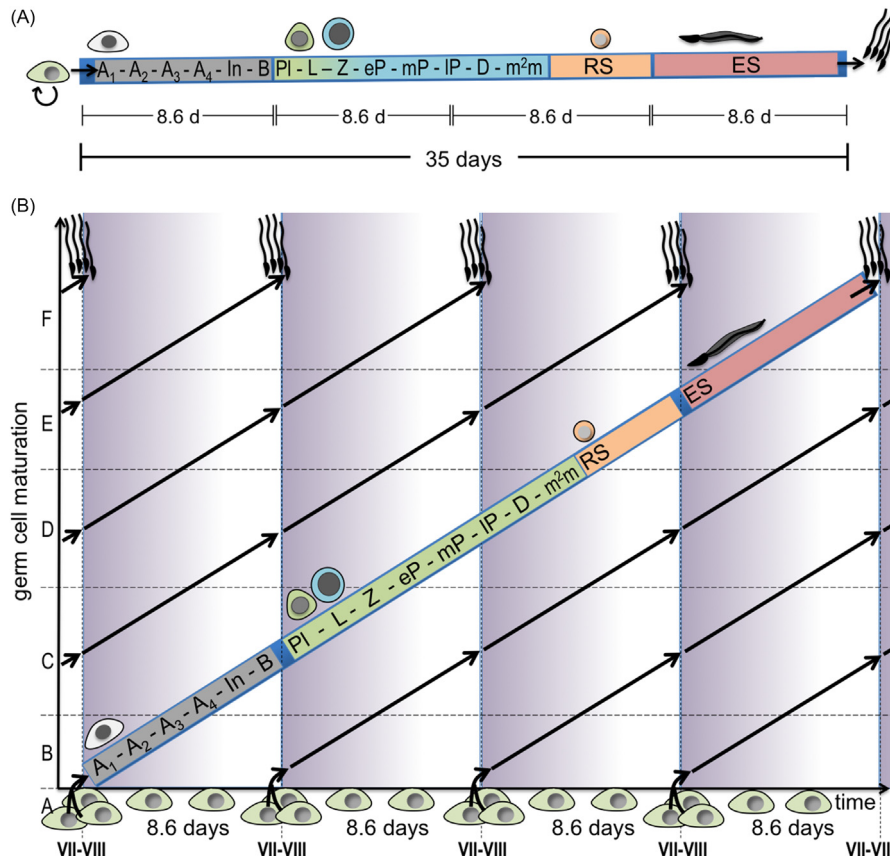


Fig. 3 Kinetics of spermatogenesis. (A) Timeline of sperm production in the mouse. Self-renewing undifferentiated spermatogonia transit into type A₁ differentiating spermatogonia upon entering spermatogenesis. Spermatogenic cells undergo the mitotic (gray rectangle), meiotic (green to blue), and spermiogenic (orange and red) phases to give rise to mature spermatids 35 days, or roughly four 8.6-day seminiferous cycles, later. eP-mP-IP, early-mid-late pachytene spermatocytes. (B) Schematic presentation of germ cell maturation at a specific locus in the seminiferous tubule as a function of time. Spermatogenesis starts as A_{undiff} spermatogonia differentiate into A₁ spermatogonia at stages VII–VIII of the seminiferous epithelial cycle. The next generation of differentiating spermatogonia is formed after one cycle of the seminiferous epithelium, that is, 8.6 days later, and spermatids are released after 35 days. Asynchronous onset of spermatogenesis along the length of the seminiferous tubule ensures continuous sperm release. At a specific locus, sperm release is pulsatile (once in 8.6 days). Purple shading on the background depicts cyclic action of Sertoli cells during the spermatogenic cycle. Hierarchically stratified layers of germ cells (A–F as described in Fig. 2) are continually recreated as spermatogenesis proceeds. Abbreviations as described in Figs. 1 and 2.

and (3) by being exposed to differentiation-priming activity of the Wnt/beta-catenin signaling pathway (de Rooij, 2001; Ikami et al., 2015; Takase and Nusse, 2016; Tokue et al., 2017). A_{undiff} are a heterogeneous population of cells, and despite having a capacity to self-renew (stem cells), only a subset of them actually does so, while the majority are destined to differentiate (progenitor cells). Differentiation propensity within the A_{undiff} spermatogonia is associated with increased expression of neurogenin3 (Ngn3) and Sox3 (SRY-box 3), and decreased expression of GFRa1 (GDNF family receptor alpha 1) (Ikami et al., 2015; Raverot et al., 2005). A_{undiff} at stages VII–IX are exposed to the pulsatile secretion of retinoic acid that stimulates expression of Stra8 (stimulated by retinoic acid gene 8), promotes entry into S phase of the cell cycle and induces directly and indirectly the expression of Kit, a hallmark of spermatogonial differentiation commitment, in the differentiation-primed A_{undiff} (Pellegrini et al., 2008; Zhou et al., 2008; Carlomagno et al., 2010; Barrios et al., 2012; Suzuki et al., 2012; Yang et al., 2013; Chen et al., 2016; Hogarth et al., 2015). The expression of DNA methyltransferases 3a and 3b (Dnmt3a/b) plus a number of other genes are also stimulated in the nascent committed spermatogonia (Shirakawa et al., 2013; Evans et al., 2014). Activation of Dnmt3a/b results in modification of the committing germ cell epigenome and is probably responsible for the irreversibility of the event (Shirakawa et al., 2013).

While stem and progenitor spermatogonia divide in an at least somewhat random fashion (restricted to stages X–II, though), the mitoses of differentiating spermatogonia are rigidly timed and bound to the progress of the seminiferous cycle (Fig. 2) (de Rooij and Russell, 2000). A₁ spermatogonia divide at stage IX to produce A₂ spermatogonia. A₃ spermatogonia are derived from mitosis of A₂ at stage XI. A₃ spermatogonia give rise to A₄ at stage I. A₄ spermatogonia divide at stage II to form intermediate spermatogonia (In), which undergo mitosis at stage IV to produce type B spermatogonia. The last mitotic division in the male mouse germ-line takes place at stage VI. The progeny of the division are meiotic preleptotene spermatocytes, and retinoic acid has a vital role in the meiotic entry of germ cells (Anderson et al., 2008). Spermatocytes undergo two meiotic divisions about 1.5 cycles later at stage

XII, and the haploid round spermatids appear at stage I. Their cellular morphology starts to change from round to elongated at stage VIII (step 8) and fully matured step 16 elongated spermatids are disengaged from the epithelium (spermiation) one cycle later. Differentiation of spermatids (spermiogenesis) is an intricate process and takes approximately 1.5 cycles to complete independent of the species. The duration of different stages is known and there is some biological variation in the duration of particular stages among individuals of the same species (Muciaccia et al., 2013). The seminiferous cycle in man differs somewhat from the mouse. Most remarkably, meiotic entry and spermiation take place at earlier stages and the spermatogenic starting point, that is, transition from progenitor to differentiating spermatogonial cell (A_{undiff} to A_1 in the mouse), is not known (Muciaccia et al., 2013).

Thousands of coding and noncoding RNAs are transcribed during the spermatogenic cycle (Laiho et al., 2013). The expression of many of these transcripts is confined to a specific cell type and/or specific stage of the seminiferous cycle. These genes are said to have a stage-dependent pattern of expression. Transillumination-assisted microdissection method has made it possible to analyze stage-specific gene and protein expression in vitro (Toppari and Parvinen, 1985; Parvinen et al., 1986). Many key regulatory factors, such as inhibins and activins (Bhasin et al., 1989; Kaipia et al., 1991, 1992, 1993, 1994), testicular interleukin-1 (Soder et al., 1991) and stem cell factor/Kit-ligand (Yan et al., 1999; Hakovirta et al., 1999) are expressed cyclically, as well as plasminogen activators that are hypothesized to act on structural dynamics of the seminiferous epithelium (Penttilä et al., 1994; Toppari et al., 1986, 1988; Vihko et al., 1987). However, not all regulatory factors are cyclically expressed, for example, GATA 4 and 6 (Ketola et al., 1999).

Coordination of individual steps on the spermatogenic pathway is vital for its successful completion. Meiotic checkpoints ensure genetic integrity of gametes and provide the system with a quality control mechanism, and a way to dispose of the flawed germ cells (Subramanian and Hochwagen, 2014). Apoptosis is no stranger to spermatogenesis and spermatogenic cells can undergo apoptotic cell death at many different phases of differentiation (Huckins, 1978; Allan et al., 1992; Shaha et al., 2010). It is estimated that only one fourth of spermatogonial progeny makes it to mature spermatids (Russell et al., 2002). In spite of massive apoptosis, the spermatogenic machinery of the testis is a highly efficient system and fertile men produce about 100 million sperm per day (Johnson et al., 1980). Synchronous spermatogenesis of different cohorts of germ cells at four hierarchical layers of differentiation (differentiating spermatogonia, spermatocytes, round spermatids, elongating spermatids—not all present at every stage, possibly two different generations present at a particular stage) provides the system with a possibility to coordinate the development of spermatogenic cells at different hierarchical layers of development, and it also saves space (Figs. 2 and 3B). However, it also sets unprecedented requirements for the somatic supporting cells of spermatogenesis, especially the Sertoli cells.

The Sertoli Cell and Regulation of the Seminiferous Epithelial Cycle

Sertoli cells (SCs) are the only somatic cells located within the seminiferous tubules. SCs in the adult are a large highly specialized postmitotic epithelial cell type that spans from the basal lamina of the seminiferous epithelium to the tubular lumen, and as one among its many functions provides a physical scaffold for the developing germ cells (Fig. 1). The main function of SCs is to generate a specialized microenvironment for each stage of the seminiferous cycle that enables germ cells to differentiate, divide, and survive. This does not apply just to a single subpopulation of germ cells but due to presence of up to six different generations of germ cells (including stem and progenitor A_{undiff} spermatogonia) at a particular stage, this is a considerable effort (Figs. 2 and 3B). SCs display unparalleled plasticity in terms of function and gene expression across the cycle of the seminiferous epithelium.

Germ cells do not express receptors for androgens or gonadotropins FSH (follicle-stimulating hormone) or LH (luteinizing hormone), but it is the Sertoli cells that are responsible for transducing endocrine signals and other cellular cues into paracrine regulation of spermatogenesis. FSH and testosterone are the key regulators of spermatogenesis and SCs therefore have a central role in the maintenance of the epithelial cycle. The effect of these two factors on SCs is stage-dependent. The highest levels of androgen receptor (AR) are expressed in stages VI–VII, whereas FSH acts on Sertoli cells most strongly in early stages of the seminiferous epithelial cycle (Zhou et al., 2002; Kangasniemi et al., 1990a). FSH and testosterone regulate the expression of hundreds of genes in Sertoli cells (McLean et al., 2002; De Gendt et al., 2014). Due to disparities in their windows of action (in the late-to-early or mid-to-late stages for FSH and testosterone, respectively) they regulate different aspects of spermatogenesis (Parvinen, 1993). The concerted action of both of them is required for normal spermatogenesis. The Sertoli cell functional cycle not only involves cyclical changes in gene expression but also in cell morphology and biochemical activity (Parvinen, 1993; Morales and Clermont, 1993). The importance of cyclic gene expression in SCs for normal spermatogenesis has been taken as given. This is understandable because a gene that is expressed in Sertoli cells often regulates transcription of other genes in germ cells. Thus stage-dependent gene expression in SCs reflects to stage-dependent expression of a germ cell gene needed to complete a specific step along the differentiation pathway, for instance. Stage-dependent expression of receptors (AR, FSHR, etc.) for ligands that are continually available (testosterone, FSH, etc.) is one of the main mechanisms that is needed for maintenance of the seminiferous cycle. In the absence of gonadotropins, apoptosis starts in pachytene spermatocytes and spermatids at stages VII–VIII (Toppari et al., 1989).

Sertoli cell functional cycle coincides with the seminiferous epithelial cycle. They are not the same thing, however, and the phase of the Sertoli cell cycle is dictated by the stage of seminiferous cycle. In undisrupted testis, due to the intimate relationship of SCs and associated spermatogenic cells, the opposite is equally true and full manifestation of the characteristics of either of the cycles is not possible in the absence of the other. There are three different mechanisms that have been suggested to control the Sertoli cell cycle and therefore the seminiferous epithelial cycle, too: (1) Stage-dependent expression of receptors for ligands that are continually available (such as testosterone and FSH), (2) paracrine and juxtacrine factors whose availability is limited to (a) specific stage(s) (such as retinoic acid as discussed below), and (3) Sertoli cell intrinsic program that drives stage-dependent gene expression

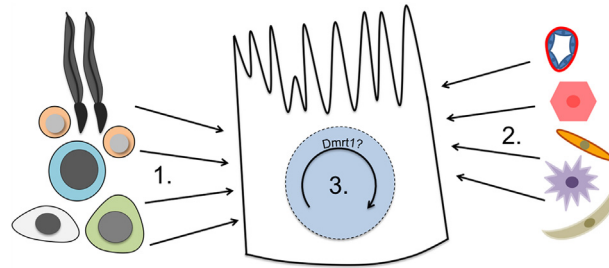


Fig. 4 The regulation of the Sertoli cell cycle. The cyclic function of the Sertoli cell (in the middle, colored blue) is regulated by three different mechanisms: (1) Stage-dependent availability of paracrine and juxtacrine factors produced by spermatogenic cells, (2) Stage-dependent expression of receptors for endocrine and paracrine factors, and (3) Sertoli cell intrinsic program that controls cyclic gene expression independent of germ cells.

independent of germ cells probably via a *Dmrt1*-dependent mechanism (Fig. 4) (Timmons et al., 2002; Matson et al., 2011). Germ cells are not, however, the puppets of SCs and their dictatorial paracrine control mechanisms. Xenotransplantation of germ cells from rat testis to mouse seminiferous tubules recapitulates rat spermatogenesis in an alien environment, and rat spermatogenic cells differentiate according to their intrinsic 12.9-day cycle instead of following the host's seminiferous cycle of 8.6 days (Russell and Brinster, 1996; Franca et al., 1998). The cycle length thus seems to be an inherent property of the germ cells, which just make use and take benefit from the surrounding somatic environment to accomplish their task of spermatogenic differentiation. Moreover, experiments where differentiating spermatogonia were killed with X-irradiation (Kangasniemi et al., 1990b), which resulted in a specific lack of succeeding spermatogenic cells over time, revealed a strong influence of the germ cells on Sertoli cell cyclic function (Kaipia et al., 1990, 1991; Kangasniemi et al., 1990c).

Re-establishment of the spermatogenic wave after allogeneic transplantation takes half a year in the mouse (Ventelä et al., 2002). For the first 2–3 months posttransplantation the colonies derived from single stem cells represent just one stage, even after fusion and having reached the length of >1 cm (Ventelä et al., 2002). Synchronization of the cycle within a colony suggests that the inducer is the transplanted cell and its progeny. Synchronization of germ cell differentiation in the fused colonies is an unexpected finding and the mechanism responsible for cycle shift in one or more of the merging colonies is not known. In these colonies, spermatogenic differentiation only takes place in the middle parts and spermatogonial proliferation is seen in the periphery. It might be that lack of a barrier function elicited by differentiating germ cells allows diffusion of a stage-synchronizing factor to neighboring regions along the seminiferous tubule resulting thus in the observed outcome. Spermatogenesis would need to be reinstated over a specific threshold for this barrier to be established and this would take about 6 months in the discussed context.

While pieces of data suggest that germ cells have superior control on the seminiferous epithelial cycle over Sertoli cells, SCs display at least some aspects of their cyclic function in testis devoid of germ cells: in a congenitally germ cell deficient testis, in case of a block in spermatogenic entry due to vitamin A or RA signaling deficiency, or after germ cell depletion (Timmons et al., 2002; Sugimoto et al., 2012). Developmental heterogeneity is first observed in testicular somatic cells and a report suggests that the seminiferous epithelial cycle is prefigured in SCs during fetal development, latest mouse embryonic day 18 (Timmons et al., 2002). The data from xenogeneic transplantation studies nonetheless indicate that germ cells are capable of overriding the SC intrinsic clock and adjust it according to their own needs (Franca et al., 1998; Clouthier et al., 1996; Ogawa et al., 1999a,b). The molecular mechanisms responsible for resetting the SC clock have yet to be fully uncovered but it is obvious that paracrine and juxtacrine signaling is involved. There is one potent factor, though, that might have a crucial role in the process—retinoic acid.

The Onset and Maintenance of the Seminiferous Cycle—The Role of Retinoic Acid

Retinoic acid is a potent short-lived lipophilic paracrine and autocrine factor. In the testis it is synthesized from blood-borne retinol (fat-soluble dietary vitamin A) by the action of retinol and retinaldehyde dehydrogenases (RDH, RALDH), and actively degraded by CYP26 (cytochrome P450 family 26) enzymes (Rhinn and Dolle, 2012). The levels and distribution of RA in the testis are tightly controlled. Besides degradation, the bioavailable levels of RA can be regulated by storage of retinol in lipid droplets after esterification (Chung and Wolgemuth, 2004). Circulating retinol is bound to a carrier protein.

There are three receptors (RARα, RARβ, and RARγ) that bind RA. They form heterodimeric complexes with retinoid X receptors (RXRα, RXRβ, RXRγ). Interestingly, at many genomic loci the nuclear function of RAR/RXR heterodimer depends on ligand availability. Upon ligand binding the complex associates with co-activator proteins and stimulates transcription of RA responsive genes, whereas if the ligand is not available, RAR/RXR heterodimers adopts a conformation that enables interaction with co-repressors and results in downregulation of RA responsive gene expression (Rhinn and Dolle, 2012).

The data supporting the role for RA in the induction and maintenance of the seminiferous epithelial cycle is solid. Despite recent advances in understanding the role of RA in molecular detail, the crucial importance of RA for spermatogenesis has been known for nearly a century (Wolbach and Howe, 1925). Loss of RA signaling due to vitamin A deficiency, for instance, leads to azoospermia due to a block in the spermatogenic entry and accumulation of A_{undiff} spermatogonia. Administration of RA or its precursors restores the seminiferous epithelial cycle and spermatogenesis (Morales and Griswold, 1987; van Pelt and de Rooij, 1990). RA availability

needs to be tightly regulated developmentally and during steady state spermatogenesis as ectopic RA would result in premature meiotic entry both in prepubertal testis and during spermatogenesis (Sugimoto et al., 2012; MacLean et al., 2007). Differentiation-primed A_{undiff} spermatogonia at stages II–VIII are sensitive to RA stimulus but the transition into A_1 only takes place at stages VII–VIII (Hogarth et al., 2015). Testis must therefore actively employ mechanisms that limit bioavailability of RA at stages II–VI. The highest RA levels are recorded at stages VIII–IX but RA stays at a relatively high concentration throughout stages VII–XII (Hogarth et al., 2015; Sugimoto et al., 2012; Hasegawa and Saga, 2012). Due to short half-life of RA, it is likely that RA is actively produced at these stages, while only residual levels of RA are measured at stages I–VI. This stage VII–IX specific RA pulse coincides with induction of Stra8 expression in A_1 spermatogonia and preleptotene spermatocytes and beginning of Kit expression in A_1 spermatogonia, which is considered a hallmark of spermatogonial differentiation commitment (Pellegrini et al., 2008; Zhou et al., 2008). Retinoic acid has also been shown to affect gene expression in seminiferous epithelium in a stage-specific manner in vitro (Vihko et al., 1987; Toppari et al., 1986).

Sertoli cell-derived RA is needed for the efficient initiation of the first cycle of the seminiferous epithelium in the prepubertal rodent (Raverdeau et al., 2012; Tong et al., 2013). However, the subsequent cycles may partly or fully depend on RA from alternative sources. The most likely candidates would be the preleptotene spermatocytes that appear one cycle (8.6 days) later, or late, stage VII–VIII, pachytene spermatocytes (Raverdeau et al., 2012; Davis et al., 2013; Vernet et al., 2006a; DeFalco et al., 2015). Both these cell types strongly express proteins that are needed for RA synthesis (Raverdeau et al., 2012; DeFalco et al., 2015; Vernet et al., 2006b). Round spermatids of stages II–VI limit availability of retinol for RA synthesis by expressing genes that are needed for its esterification and storage (Sugimoto et al., 2012; Vernet et al., 2006b). However, postmeiotic germ cells might also produce RA or its precursors (DeFalco et al., 2015; Vernet et al., 2006b). Interestingly, late round spermatids and elongating spermatids express *Bcmo1* (beta-carotene oxygenase 1), a gene needed to produce RA precursor retinaldehyde from beta-carotene (Vernet et al., 2006b). Additionally, testicular macrophages have been suggested to play a role in testicular RA metabolism and creation of spermatogonial niche, and the RA producing machinery is also present in Leydig cells (DeFalco et al., 2015; Vernet et al., 2006b). The significance of Leydig cell and macrophage-derived RA for spermatogenesis can be justifiably questioned because peritubular myoid cells, which encase the seminiferous tubules, strongly express Cyp26 isoforms, the enzymes needed for RA degradation (MacLean et al., 2007; Vernet et al., 2006b). One of the functions of peritubular myoid cells would therefore be the degradation of extratubular RA originating from the interstitium, vasculature or neighboring tubules. The patchy location of a subset of testicular macrophages at the peritubulus might still enable these cells to play role in the intratubular RA metabolism (DeFalco et al., 2015).

Based on the available data (see above), the following model for the role of RA in the initiation and maintenance of the seminiferous cycle is proposed (Fig. 5): RA is actively degraded in the embryonic testis. In prepubertal rodents, Sertoli cell-derived RA induces the onset of the first round of spermatogenesis along the whole length of the seminiferous tubule in a temporally controlled manner, and as a result a subset of nascent spermatogonia transition into differentiating spermatogonia. The subsequent cycles depend on RA produced by differentiating germ cells and acts on differentiation-primed A_{undiff} spermatogonia. In the adult, preleptotene spermatocytes appearing once during every cycle, after 8.6-day intervals, and late pachytene spermatocytes synthesize and secrete RA at stages VII–IX of the seminiferous cycle. RAR γ expressed by the spermatogonial progenitor cells respond to this RA pulse and commit to differentiate. The progeny of these cells then takes part in producing the next pulse in 8.6 days and so on. This mechanism would be the basis of seminiferous epithelial cycle and Sertoli cell functional cycle and reset the Sertoli cell “clock” to stage VII once during every cycle. Esterification and storage of RA precursors by early step 2–6 round spermatids (at stages II–VI) would limit the availability of RA and prevent premature A_{undiff} to A_1 transition and untimely meiotic entry of differentiating spermatogonia.

RA signaling is not vitally important only for the onset and maintenance of spermatogenesis and the seminiferous epithelial cycle but also for the Sertoli cells. Lack of RAR α in SCs results in uniform expression of stage-dependent transcripts in adult SCs, which suggests that RA signaling regulates the SC cyclic gene expression and therefore the Sertoli cell cycle (Fig. 5). However, ablation of RAR α in SCs does not immediately abolish the seminiferous cycle, and the data suggest that the seminiferous epithelial cycle can proceed even after loss of SC cyclicity (Vernet et al., 2006a). This raises two questions: for how long and at what cost? Indeed, SC specific deficiency of RAR α results primarily in spermatogenic defects and ultimately in SC only phenotype where seminiferous tubules are devoid of germ cells. These data provide another piece of evidence for the interdependency of the seminiferous and Sertoli cell cycle.

The Origin of the Seminiferous Cycle and Its Spatially Asynchronous Onset

The RA-induced transition of differentiation-primed A_{undiff} to A_1 spermatogonia at stages VII–VIII sets the stage for male germ cell differentiation. Its spatio-temporally asynchronous implementation along the length of the seminiferous tubule ensures continual supply of sperm and guarantees that mature elongated spermatids are always available for release from a specific proportion of seminiferous epithelial surface (Fig. 3B). Were it not asynchronous, sperm would be released in a pulsatile manner, that is, every 16th day in man, for instance. It is obvious that this would greatly reduce the capacity of a male to fertilize an oocyte. Conversely, in species where sperm is released just once per a reproductive season (many species of fish, e.g.) synchronous spermatogenesis is a viable reproductive strategy (Billard, 1986).

In the mouse and rat, the seminiferous cycle can be synchronized in time and space. This happens after injection of RA or its precursor into animals that have been on a vitamin A deficient diet, or to early neonatal mice, and results in loss of spermatogenic

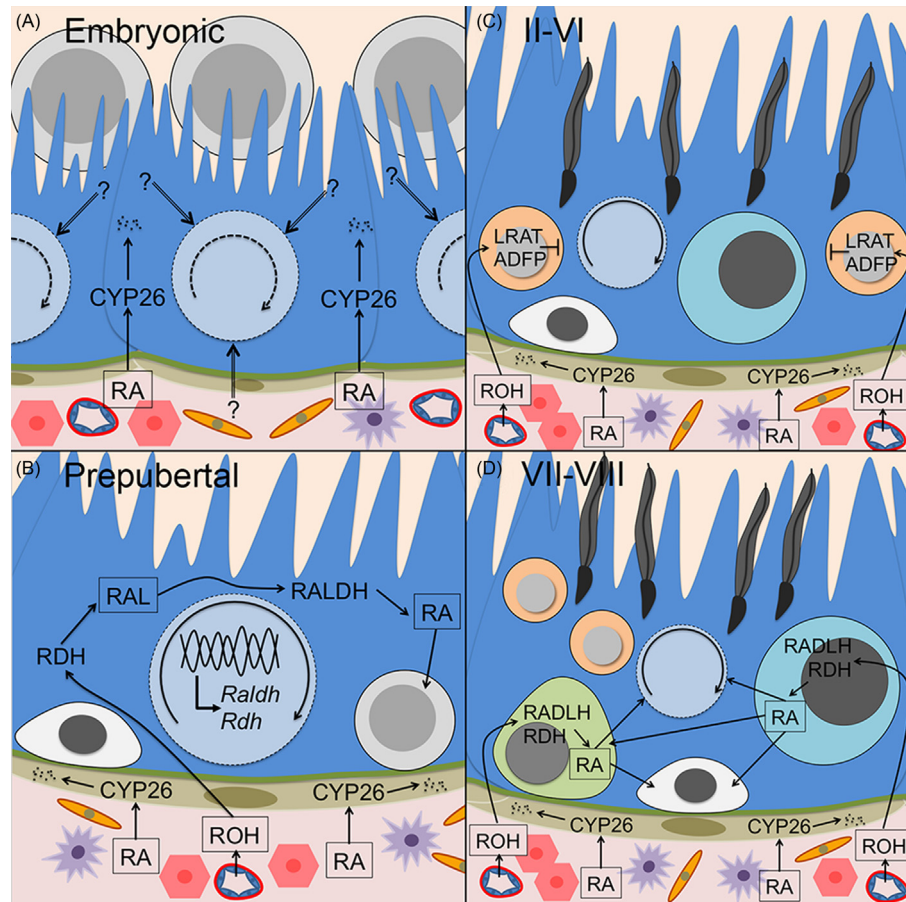


Fig. 5 A model for the role of RA in the induction and maintenance of the seminiferous cycle. (A) In the developing embryo, testicular somatic cells strongly express CYP26 enzymes that degrade RA in order to prevent premature meiotic entry of the gonocytes (*round gray cells*). Unidentified factors (*question marks*) act on Sertoli cells to prefigure the seminiferous epithelial cycle in the SC epigenome. (B) RA production is induced in prepubertal Sertoli cells in a patch-like fashion. Blood-borne retinol (ROH) is converted first into retinaldehyde (RAL) by retinol dehydrogenase (RDH), and then into RA by RALDH (retinaldehyde dehydrogenase). Spatio-temporally controlled synthesis and release of RA results in an asynchronous spermatogenic entry of gonocyte-derived nascent spermatogonia (*round gray cells*). RA does not affect all germ cells but some are unresponsive to RA and form the spermatogonial stem cell pool (*flattened gray cells*). (C) Round spermatids of stages II–VI express LRAT (lecithin-retinol acyltransferase) and ADFP (adipose differentiation related protein) whose action leads to esterification and storage of retinol and consequent low RA synthesis and availability. This mechanism prevents the untimely spermatogenic entry of stage II–VI undifferentiated spermatogonia. (D) At stage VII–VIII late pachytene spermatocytes express proteins that are needed for RA synthesis (RDH and RALDH). RA induces meiotic entry of type B spermatogonia/preleptotene spermatocytes that also produce RA. RA derived from these sources (so called RA pulse) forces differentiation-primed A_{undiff} spermatogonia (*flattened gray cells*) to commit and also affects Sertoli cell cyclic function. Appearance of a new generation of RA producing cells in 8.6 days is the key mechanism that drives and coordinates the cycle of the seminiferous epithelium. Peritubular myoid cells strongly express CYP26 and degrade RA derived from Leydig cells, interstitial macrophages, circulation, and neighboring seminiferous tubules.

wave due to synchronous differentiation of germ cells along the whole length of the seminiferous tubule and existence of just a few stages at a given time (Morales and Griswold, 1987; van Pelt and de Rooij, 1990; Davis et al., 2013). As discussed above, this happens because unavailability of RA leads to accumulation of stem and differentiation-primed A_{undiff} spermatogonia, and a sudden burst of RA results in synchronous spermatogenic entry at all locations along the tubule and near simultaneous completion of spermatogenesis, and pulse-like release of sperm that is repeated in every 8.6 and 12.9 days in mouse and rat, respectively (Morales and Griswold, 1987; van Pelt and de Rooij, 1990). The synchronizing effect of RA on spermatogenesis depends on pretreatment availability of endogenous RA (Hogarth et al., 2015; Sugimoto et al., 2012; Davis et al., 2013). If the testis has never experienced normal cyclical RA, the cycle can be permanently synchronized (Hogarth et al., 2015; Davis et al., 2013). However, the seminiferous epithelial cycle is progressively desynchronized if spermatogenesis had started in the treated animals (Bartlett et al., 1990).

The ultimate trigger for asynchronous spermatogenesis and entry of nascent spermatogonia into the first cycle of seminiferous epithelium and consequent first round of spermatogenesis is yet to be identified. The earliest signs of asynchronous germ cell development are observed during early postnatal life as on postnatal day 2–3 RA signaling is activated in a patch-like fashion in a subset of nascent spermatogonia (Fig. 5B) (Snyder et al., 2010, 2011). This does not provide an answer about the nature of inducing factor and how does it mechanistically contribute to the asynchronous onset of spermatogenesis, though, but merely suggests that it is probably of fetal or embryonic descent. The cellular origin of the inducing signal has not been demonstrated,

but SCs are the most likely candidate due to early heterogeneity within this cell population (Fig. 5A) (Timmons et al., 2002). It is also possible, however, that a subset of gonocytes is set aside early on during prenatal development and it is these cells that enter spermatogenesis in the prepubertal rodent. One thing is evident, however, that is, the need for more research on the developmental origin of the seminiferous epithelial cycle.

The Sertoli Cell “Clock”

The conclusion from a bulk of data discussed above is that cyclic gene and protein expression and function of adult-type Sertoli cell is regulated by “a clock” and retinoic acid sets the clock but differentiating meiotic and postmeiotic germ cells are needed for its fine-tuning. The idea of differentiating germ cells having an effect on Sertoli cell cyclic function is not a new one but its molecular identity has awaited validation (Parvinen, 1993; de Rooij et al., 1985; Kaipia et al., 1991). What is then the nature of the Sertoli cell “clock” and the pace maker of the seminiferous epithelial cycle (Fig. 6)? Ability of RA to reset the Sertoli cell cycle provides a hint as to how the cyclic function of SCs and the seminiferous cycle are controlled. There might be other spermatogenic cell-derived paracrine factors in addition to RA that fine-tune the function of SCs and contribute to its characteristic seminiferous cycle dependency. SCs would only be required for the onset of the seminiferous epithelial cycle and thereafter the gradual appearance of more advanced populations of spermatogenic cells would be in charge of keeping the spermatogenic process and the seminiferous cycle going. In this model the seminiferous cycle-inducing capacity of Sertoli cells via secretion of RA would not be lost upon SC maturation because transplantation and consequent repopulation of the seminiferous epithelium would not take place in the absence of it. These considerations would make the spermatogenic program encoded into germ cell genomes the pace maker of spermatogenesis and the ultimate controller of Sertoli cell functional cycle, and would leave SCs a role in the initiation of the seminiferous epithelial cycle during the first (and second) round of spermatogenesis and later in life if needed. However, after allogeneic transplantation the cell density of A_{undiff} might also play a role and the differentiation-inducing stimulus (RA) might arise from the A_{undiff} population itself when sufficient cell density is reached (Ventelä et al., 2002). Identification and characterization of significant seminiferous epithelial cycle modifiers or those that are able to reset the cycle in a RA manner (unlikely to exist) are yet to be achieved.

The availability of data showing the significance of RA for human spermatogenesis is limited but surprisingly it may not have an equally central role in primates as in rodents. Long-term administration of RA synthesis inhibitors results in near or complete absence of sperm suggesting that RA signaling is vital for human spermatogenesis, as well (Heller et al., 1963). However, there were defects only in postmeiotic germ cell differentiation but the treatment had no effect on spermatogonia and spermatocytes. Just a glimpse at the stage map of human spermatogenesis suggests that a similar hot spot of cell fate decisions as stage VII–VIII is in rodents (A_{undiff} to A_1 , meiotic entry, start of spermatid elongation and release of matured spermatids), does not exist in man (Muciaccia et al., 2013). Either the RA pulse in man is multiphasic, which is unlikely, or RA has a somewhat different role in human spermatogenesis. Further studies about the role of RA in human spermatogenesis are in high demand.

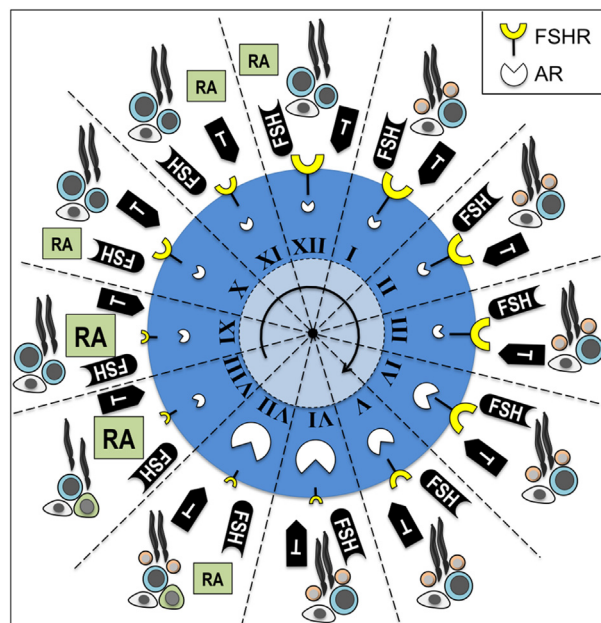


Fig. 6 The Sertoli cell clock. During the course of the seminiferous cycle, the Sertoli cell is exposed to varying environment and phase of the Sertoli cell clock (I–XII) is determined by paracrine and endocrine factors, composition of the interacting germ cell population and the intrinsic Sertoli cell cycle. Untimely and ectopic availability of an adjusting agent, or lack of it, will affect the Sertoli cell clock and depending on the identity and intensity of the signal may reset (like RA) or adjust it. Size of the icon (FSHR, AR, RA) corresponds to the abundance of the specific molecule.

References

- Allan, D. J., Harmon, B. V., & Roberts, S. A. (1992). Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. *Cell Proliferation*, 25(3), 241–250.
- Amann, R. P. (2008). The cycle of the seminiferous epithelium in humans: A need to revisit? *Journal of Andrology*, 29(5), 469–487.
- Anderson, E. L., Baltus, A. E., Roepers-Gajadien, H. L., et al. (2008). Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 105(39), 14976–14980.
- Barrios, F., Filippini, D., Campolo, F., et al. (2012). SOHLH1 and SOHLH2 control kit expression during postnatal male germ cell development. *Journal of Cell Science*, 125(Pt 6), 1455–1464.
- Bartlett, J. M., Weinbauer, G. F., & Nieschlag, E. (1990). Stability of spermatogenic synchronization achieved by depletion and restoration of vitamin A in rats. *Biology of Reproduction*, 42(4), 603–612.
- Bhasin, S., Krummen, L. A., Swerdloff, R. S., et al. (1989). Stage dependent expression of inhibin alpha and beta-B subunits during the cycle of the rat seminiferous epithelium. *Endocrinology*, 124(2), 987–991.
- Billard, R. (1986). Spermatogenesis and spermatology of some teleost fish species. *Reproduction Nutrition Development*, 26(4), 887–920.
- Carlomagno, G., van Bragt, M. P., Korver, C. M., Repping, S., de Rooij, D. G., & van Pelt, A. M. (2010). BMP4-induced differentiation of a rat spermatogonial stem cell line causes changes in its cell adhesion properties. *Biology of Reproduction*, 83(5), 742–749.
- Chen, Y., Ma, L., Hogarth, C., Wei, G., Griswold, M. D., & Tong, M. H. (2016). Retinoid signaling controls spermatogonial differentiation by regulating expression of replication-dependent core histone genes. *Development*, 143(9), 1502–1511.
- Chung, S. S., & Wolgemuth, D. J. (2004). Role of retinoid signaling in the regulation of spermatogenesis. *Cytogenetic and Genome Research*, 105(2–4), 189–202.
- Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiological Reviews*, 52(1), 198–236.
- Clouthier, D. E., Avarbock, M. R., Maika, S. D., Hammer, R. E., & Brinster, R. L. (1996). Rat spermatogenesis in mouse testis. *Nature*, 381(6581), 418–421.
- Davis, J. C., Snyder, E. M., Hogarth, C. A., Small, C., & Griswold, M. D. (2013). Induction of spermatogenic synchrony by retinoic acid in neonatal mice. *Spermatogenesis*, 3(1), e23180.
- De Gendt, K., Verhoeven, G., Amieux, P. S., & Wilkinson, M. F. (2014). Genome-wide identification of AR-regulated genes translated in sertoli cells in vivo using the RiboTag approach. *Molecular Endocrinology*, 28(4), 575–591.
- de Rooij, D. G. (2001). Proliferation and differentiation of spermatogonial stem cells. *Reproduction*, 121(3), 347–354.
- de Rooij, D. G., & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology*, 21(6), 776–798.
- de Rooij, D. G., Lok, D., & Weenk, D. (1985). Feedback regulation of the proliferation of the undifferentiated spermatogonia in the chinese hamster by the differentiating spermatogonia. *Cell and Tissue Kinetics*, 18(1), 71–81.
- DeFalco, T., Potter, S. J., Williams, A. V., Waller, B., Kan, M. J., & Capel, B. (2015). Macrophages contribute to the spermatogonial niche in the adult testis. *Cell Reports*, 12(7), 1107–1119.
- Evans, E., Hogarth, C., Mitchell, D., & Griswold, M. (2014). Riding the spermatogenic wave: Profiling gene expression within neonatal germ and sertoli cells during a synchronized initial wave of spermatogenesis in mice. *Biology of Reproduction*, 90(5), 108.
- Franca, L. R., Ogawa, T., Avarbock, M. R., Brinster, R. L., & Russell, L. D. (1998). Germ cell genotype controls cell cycle during spermatogenesis in the rat. *Biology of Reproduction*, 59(6), 1371–1377.
- Hakovirta, H., Yan, W., Kaleva, M., et al. (1999). Function of stem cell factor as a survival factor of spermatogonia and localization of messenger ribonucleic acid in the rat seminiferous epithelium. *Endocrinology*, 140(3), 1492–1498.
- Hasegawa, K., & Saga, Y. (2012). Retinoic acid signaling in sertoli cells regulates organization of the blood–testis barrier through cyclical changes in gene expression. *Development*, 139(23), 4347–4355.
- Heller, C. G., & Clermont, Y. (1963). Spermatogenesis in man: An estimate of its duration. *Science*, 140(3563), 184–186.
- Heller, C. H., & Clermont, Y. (1964). Kinetics of the germinal epithelium in man. *Recent Progress in Hormone Research*, 20, 545–575.
- Heller, C. G., Flageolle, B. Y., & Matson, L. J. (1963). Histopathology of the human testes as affected by bis(dichloroacetyl)diamines. *Experimental and Molecular Pathology. Supplement*, 2, 107–114.
- Hogarth, C. A., Arnold, S., Kent, T., Mitchell, D., Isoherranen, N., & Griswold, M. D. (2015). Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. *Biology of Reproduction*, 92(2), 37.
- Huckins, C. (1978). The morphology and kinetics of spermatogonial degeneration in normal adult rats: An analysis using a simplified classification of the germinal epithelium. *The Anatomical Record*, 190(4), 905–926.
- Ikami, K., Tokue, M., Sugimoto, R., et al. (2015). Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. *Development*, 142(9), 1582–1592.
- Johnson, L., Petty, C. S., & Neaves, W. B. (1980). A comparative study of daily sperm production and testicular composition in humans and rats. *Biology of Reproduction*, 22(5), 1233–1243.
- Kaipia, A., Toppari, J., Mali, P., et al. (1990). Stage- and cell-specific expression of the ornithine decarboxylase gene during rat and mouse spermatogenesis. *Molecular and Cellular Endocrinology*, 73(1), 45–52.
- Kaipia, A., Parvinen, M., Shimasaki, S., Ling, N., & Toppari, J. (1991). Stage-specific cellular regulation of inhibin alpha-subunit mRNA expression in the rat seminiferous epithelium. *Molecular and Cellular Endocrinology*, 82(2–3), 165–173.
- Kaipia, A., Penttilä, T. L., Shimasaki, S., Ling, N., Parvinen, M., & Toppari, J. (1992). Expression of inhibin beta A and beta B, follistatin and activin-A receptor messenger ribonucleic acids in the rat seminiferous epithelium. *Endocrinology*, 131(6), 2703–2710.
- Kaipia, A., Parvinen, M., & Toppari, J. (1993). Localization of activin receptor (ActR-11B2) mRNA in the rat seminiferous epithelium. *Endocrinology*, 132(1), 477–479.
- Kaipia, A., Penttilä, T. L., & Toppari, J. (1994). Follicle-stimulating hormone regulation of inhibin alpha-subunit mRNA in staged rat seminiferous tubules. *European Journal of Endocrinology*, 131(3), 323–329.
- Kangasniemi, M., Kaipia, A., Mali, P., Toppari, J., Huhtaniemi, I., & Parvinen, M. (1990a). Modulation of basal and FSH-dependent cyclic AMP production in rat seminiferous tubules staged by an improved transillumination technique. *The Anatomical Record*, 227(1), 62–76.
- Kangasniemi, M., Veromaa, T., Kulmala, J., Kaipia, A., Parvinen, M., & Toppari, J. (1990b). DNA-flow cytometry of defined stages of rat seminiferous epithelium: Effects of 3 Gy of high-energy X-irradiation. *Journal of Andrology*, 11(3), 312–317.
- Kangasniemi, M., Kaipia, A., Toppari, J., Mali, P., Huhtaniemi, I., & Parvinen, M. (1990c). Cellular regulation of basal and FSH-stimulated cyclic AMP production in irradiated rat testes. *The Anatomical Record*, 227(1), 32–36.
- Ketola, I., Rahman, N., Toppari, J., et al. (1999). Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. *Endocrinology*, 140(3), 1470–1480.
- Kluin, P. M., Kramer, M. F., & de Rooij, D. G. (1982). Spermatogenesis in the immature mouse proceeds faster than in the adult. *International Journal of Andrology*, 5(3), 282–294.
- Kotaja, N., Kimmins, S., Brancorsini, S., et al. (2004). Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. *Nature Methods*, 1(3), 249–254.
- Laiho, A., Kotaja, N., Gyenesei, A., & Sironen, A. (2013). Transcriptome profiling of the murine testis during the first wave of spermatogenesis. *PLoS One*, 8(4), e61558.

- Leblond, C. P., & Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Annals of the New York Academy of Sciences*, 55(4), 548–573.
- McLean, G., Li, H., Metzger, D., Chambon, P., & Petkovich, M. (2007). Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. *Endocrinology*, 148(10), 4560–4567.
- Matson, C. K., Murphy, M. W., Sarver, A. L., Griswold, M. D., Bardwell, V. J., & Zarkower, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature*, 476(7358), 101–104.
- McLean, D. J., Friel, P. J., Pouchnik, D., & Griswold, M. D. (2002). Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat sertoli cells. *Molecular Endocrinology*, 16(12), 2780–2792.
- Morales, C., & Clermont, Y. (1993). Structural changes of the sertoli cell during the cycle of the seminiferous epithelium. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell* (pp. 305–329). Clearwater, FL: Cache River Press.
- Morales, C., & Griswold, M. D. (1987). Retinol-induced stage synchronization in seminiferous tubules of the rat. *Endocrinology*, 121(1), 432–434.
- Muciaccia, B., Boitani, C., Berloco, B. P., et al. (2013). Novel stage classification of human spermatogenesis based on acrosome development. *Biology of Reproduction*, 89(3), 60.
- Oakberg, E. F. (1956). Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *The American Journal of Anatomy*, 99(3), 507–516.
- Ogawa, T., Dobrinski, I., Avarbock, M. R., & Brinster, R. L. (1999a). Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. *Biology of Reproduction*, 60(2), 515–521.
- Ogawa, T., Dobrinski, I., & Brinster, R. L. (1999b). Recipient preparation is critical for spermatogonial transplantation in the rat. *Tissue & Cell*, 31(5), 461–472.
- Parvinen, M. (1982). Regulation of the seminiferous epithelium. *Endocrine Reviews*, 3(4), 404–417.
- Parvinen, M. (1993). Cyclic function of sertoli cells. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell* (pp. 331–347). Clearwater, FL: Cache River Press.
- Parvinen, M., Vihko, K. K., & Toppari, J. (1986). Cell interactions during the seminiferous epithelial cycle. *International Review of Cytology*, 104, 115–151.
- Pellegrini, M., Filippini, D., Gori, M., et al. (2008). ATRA and KL promote differentiation toward the meiotic program of male germ cells. *Cell Cycle*, 7(24), 3878–3888.
- Penttilä, T. L., Kaipia, A., Toppari, J., Parvinen, M., & Mali, P. (1994). Localization of urokinase- and tissue-type plasminogen activator mRNAs in rat testes. *Molecular and Cellular Endocrinology*, 105(1), 55–64.
- Raverdeau, M., Gely-Pernot, A., Feret, B., et al. (2012). Retinoic acid induces sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *Proceedings of the National Academy of Sciences of the United States of America*, 109(41), 16582–16587.
- Raverot, G., Weiss, J., Park, S. Y., Hurley, L., & Jameson, J. L. (2005). Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Developmental Biology*, 283(1), 215–225.
- Rhinn, M., & Dolle, P. (2012). Retinoic acid signalling during development. *Development*, 139(5), 843–858.
- Russell, L. D., & Brinster, R. L. (1996). Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules. *Journal of Andrology*, 17(6), 615–627.
- Russell, L. D., Ettlin, R. A., Sinha-Hikim, A. P., & Clegg, E. D. (1990). *Histological and histopathological evaluation of the testis*. Clearwater, FL: Cache River Press.
- Russell, L. D., Chiarini-Garcia, H., Korsmeyer, S. J., & Knudson, C. M. (2002). Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. *Biology of Reproduction*, 66(4), 950–958.
- Shaha, C., Tripathi, R., & Mishra, D. P. (2010). Male germ cell apoptosis: Regulation and biology. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 365(1546), 1501–1515.
- Shirakawa, T., Yaman-Deveci, R., Tomizawa, S., et al. (2013). An epigenetic switch is crucial for spermatogonia to exit the undifferentiated state toward a kit-positive identity. *Development*, 140(17), 3565–3576.
- Snyder, E. M., Small, C., & Griswold, M. D. (2010). Retinoic acid availability drives the asynchronous initiation of spermatogonial differentiation in the mouse. *Biology of Reproduction*, 83(5), 783–790.
- Snyder, E. M., Davis, J. C., Zhou, Q., Evanoff, R., & Griswold, M. D. (2011). Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. *Biology of Reproduction*, 84(5), 886–893.
- Soder, O., Syed, V., Callard, G. V., et al. (1991). Production and secretion of an interleukin-1-like factor is stage-dependent and correlates with spermatogonial DNA synthesis in the rat seminiferous epithelium. *International Journal of Andrology*, 14(3), 223–231.
- Subramanian, V. V., & Hochwagen, A. (2014). The meiotic checkpoint network: Step-by-step through meiotic prophase. *Cold Spring Harbor Perspectives in Biology*, 6(10), a016675.
- Sugimoto, R., Nabeshima, Y., & Yoshida, S. (2012). Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of sertoli cells in mouse seminiferous epithelium. *Mechanisms of Development*, 128(11–12), 610–624.
- Suzuki, H., Ahn, H. W., Chu, T., et al. (2012). SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. *Developmental Biology*, 361(2), 301–312.
- Takase, H. M., & Nusse, R. (2016). Paracrine wnt/beta-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. *Proceedings of the National Academy of Sciences of the United States of America*, 113(11), E1489–97.
- Timmons, P. M., Rigby, P. W., & Poirier, F. (2002). The murine seminiferous epithelial cycle is pre-figured in the sertoli cells of the embryonic testis. *Development*, 129(3), 635–647.
- Tokue, M., Ikami, K., Mizuno, S., et al. (2017). SHISA6 confers resistance to differentiation-promoting wnt/beta-catenin signaling in mouse spermatogenic stem cells. *Stem Cell Reports*.
- Tong, M. H., Yang, Q. E., Davis, J. C., & Griswold, M. D. (2013). Retinol dehydrogenase 10 is indispensable for spermatogenesis in juvenile males. *Proceedings of the National Academy of Sciences of the United States of America*, 110(2), 543–548.
- Toppari, J., & Parvinen, M. (1985). In vitro differentiation of rat seminiferous tubular segments from defined stages of the epithelial cycle morphologic and immunolocalization analysis. *Journal of Andrology*, 6(6), 334–343.
- Toppari, J., Vihko, K. K., Rasanen, K. G., Eerola, E., & Parvinen, M. (1986). Regulation of stages VI and VIII of the rat seminiferous epithelial cycle in vitro. *The Journal of Endocrinology*, 108(3), 417–422.
- Toppari, J., Tsutsumi, I., Campeau, J. D., Ahmad, N., & diZerega, G. S. (1988). Plasminogen activator secretion in the rat seminiferous epithelium after hypophysectomy and gonadotropin treatment. *Archives of Andrology*, 20(3), 219–227.
- Toppari, J., Tsutsumi, I., Bishop, P. C., et al. (1989). Flow cytometric quantification of rat spermatogenic cells after hypophysectomy and gonadotropin treatment. *Biology of Reproduction*, 40(3), 623–634.
- van Pelt, A. M., & de Rooij, D. G. (1990). Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biology of Reproduction*, 43(3), 363–367.
- Ventelä, S., Ohta, H., Parvinen, M., & Nishimune, Y. (2002). Development of the stages of the cycle in mouse seminiferous epithelium after transplantation of green fluorescent protein-labeled spermatogonial stem cells. *Biology of Reproduction*, 66(5), 1422–1429.
- Vernet, N., Dennefeld, C., Guillou, F., Chambon, P., Ghyselinck, N. B., & Mark, M. (2006a). Prepubertal testis development relies on retinoic acid but not retinoid receptors in sertoli cells. *The EMBO Journal*, 25(24), 5816–5825.
- Vernet, N., Dennefeld, C., Rochette-Egly, C., et al. (2006b). Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology*, 147(1), 96–110.
- Vihko, K. K., Toppari, J., & Parvinen, M. (1987). Stage-specific regulation of plasminogen activator secretion in the rat seminiferous epithelium. *Endocrinology*, 120(1), 142–145.
- Wolbach, S. B., & Howe, P. R. (1925). Tissue changes following deprivation of fat-soluble vitamin. *The Journal of Experimental Medicine*, 42(6), 753–777.

- Yan, W., Linderborg, J., Suominen, J., & Toppari, J. (1999). Stage-specific regulation of stem cell factor gene expression in the rat seminiferous epithelium. *Endocrinology*, 140(3), 1499–1504.
- Yang, Q. E., Racicot, K. E., Kaucher, A. V., Oatley, M. J., & Oatley, J. M. (2013). MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. *Development*, 140(2), 280–290.
- Yoshida, S., Sukeno, M., Nakagawa, T., et al. (2006). The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development*, 133(8), 1495–1505.
- Zhou, Q., Nie, R., Prins, G. S., Saunders, P. T., Katzenellenbogen, B. S., & Hess, R. A. (2002). Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *Journal of Andrology*, 23(6), 870–881.
- Zhou, Q., Nie, R., Li, Y., et al. (2008). Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: An in vivo study in vitamin A-sufficient postnatal murine testes. *Biology of Reproduction*, 79(1), 35–42.

Spermiation

Liza O'Donnell and Peter G Stanton, The Hudson Institute of Medical Research and Monash University, Clayton, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Disengagement complex An integrin-based adhesion junction that mediates the final release of sperm from the Sertoli cell.

Ectoplasmic specialization A Sertoli cell-derived actin-based adhesion junction that enables developing spermatids to adhere to Sertoli cells as they elongate.

Seminiferous epithelium The epithelium within the seminiferous tubules of the testis: it is comprised of the Sertoli cells and germ cells at different stages of development.

Sertoli cell A large complex somatic cell that is required for male germ cell development and sperm production.

Spermatogenesis The entire process by which immature precursor male germ cells develop into spermatozoa within the seminiferous tubules.

Spermiogenesis The maturational process by which round spermatids transform into spermatozoa.

Tubulobulbar complex A novel endocytic-type machinery present in Sertoli cells: it consists of an actin-associated tubule portion terminating in a bulbous vesicle portion that can bud off to become an endocytic vesicle.

Introduction

Spermiation is the process by which elongated spermatids, at the end of spermatogenesis, undergo their final remodeling and are released in the lumen of the seminiferous tubules prior to their passage to the epididymis. This process is long, taking more than 80 h in rats (O'Donnell et al., 2011), and is multi-faceted, involving various cytoskeletal structures within the adjacent Sertoli cells. The goal of spermiation is to release the mature spermatid from the Sertoli cell, however this process also involves remodeling of the spermatid to produce a streamlined spermatozoon.

The morphological and ultrastructural events associated with spermiation are well described and are conserved across mammalian species including rodents and humans (O'Donnell et al., 2011; Russell, 1993). The molecular regulation of spermiation is less well understood but involves signaling and adhesion molecules (Chapin et al., 2001) as well as the regulation of unique cytoskeletal structures.

Spermiation is an important determinant of sperm content in the ejaculate. Spermiation is vulnerable to various insults including hormone suppression and toxicant exposure, and the failure of sperm release can have a major impact on testicular sperm output (O'Donnell et al., 2011; Russell, 1991).

Initiation of Spermiation

Prior to spermiation, elongated spermatids have an extensive cytoplasm located around the flagellum (Figs. 1 and 2). The spermatid nuclei are located deeper within crypts between Sertoli cells (O'Donnell et al., 2011; Russell, 1993) and associate with the Sertoli cells via a unique actin-associated adhesion junction called the ectoplasmic specialization, or ES (Vogl et al., 2000). This is a Sertoli cell adhesion junctional structure that forms opposite spermatids as they commenced elongation some weeks prior, and it confers tight adhesion between these cells as the spermatids proceed through the elongation phase of spermiogenesis. Spermiation begins when the elongated spermatid nuclei are rapidly translocated to the luminal edge of the epithelium (Figs. 1 and 2); this upwards translocation is facilitated by the ES associating with motor proteins that move along tracks of microtubules within Sertoli cells (Guttman et al., 2000).

At the beginning of spermiation, the extensive spermatid cytoplasm projects out into the tubule lumen, but the spermatid remains tightly associated with the Sertoli cell via an extensive ES structure that surrounds the spermatid nucleus, see Fig. 2 and (Russell, 1993). The successful release of sperm necessitates the removal of this complex. To accomplish this, a testis-specific structure called the Tubulobulbar Complex (TBC) forms between the Sertoli cell and the spermatid (Figs. 2 and 3, reviewed in Vogl et al., 2014). TBCs are a modified form of clathrin-based endocytic machinery. These first form in the plasma membrane of the Sertoli cell in regions of ES disassembly, first appearing as a coated pit on the cytoplasmic side of the Sertoli cell plasma membrane (Fig. 3). This coated pit elongates into a tubular structure, facilitated by the polymerization of dendritic actin filaments, terminating in a bulbous region surrounded by endoplasmic reticulum (Vogl et al., 2014). The mature TBCs are comprised of both spermatid and Sertoli cell plasma membranes (Fig. 3). Once TBCs are fully formed, the bulbous region buds off to become an endocytic vesicle which is then processed by the Sertoli cell. Therefore TBCs are large endocytic structures that likely internalize extensive regions of

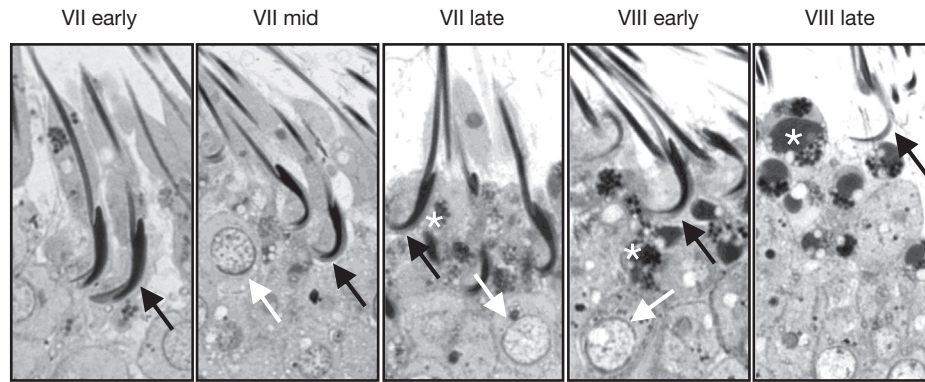


Fig. 1 Light microscopy of the progression of spermiation in the rat. The stages of the spermatogenic cycle are indicated along the top of the micrographs. The elongated spermatid nucleus is indicated by black arrows, whereas white arrows indicate the developing round spermatids in the epithelium below. The white asterisk indicates the condensing cytoplasm of the spermatid as it is gradually removed by the Sertoli cell. Spermiation commences at the beginning of stage VII in rats and mice, as the spermatid nuclei line up along the luminal edge. Spermiation ends with the disengagement of sperm towards the end of stage VIII, with the whole process taking ~80 h to complete. Figure modified from O'Donnell et al. (2011).

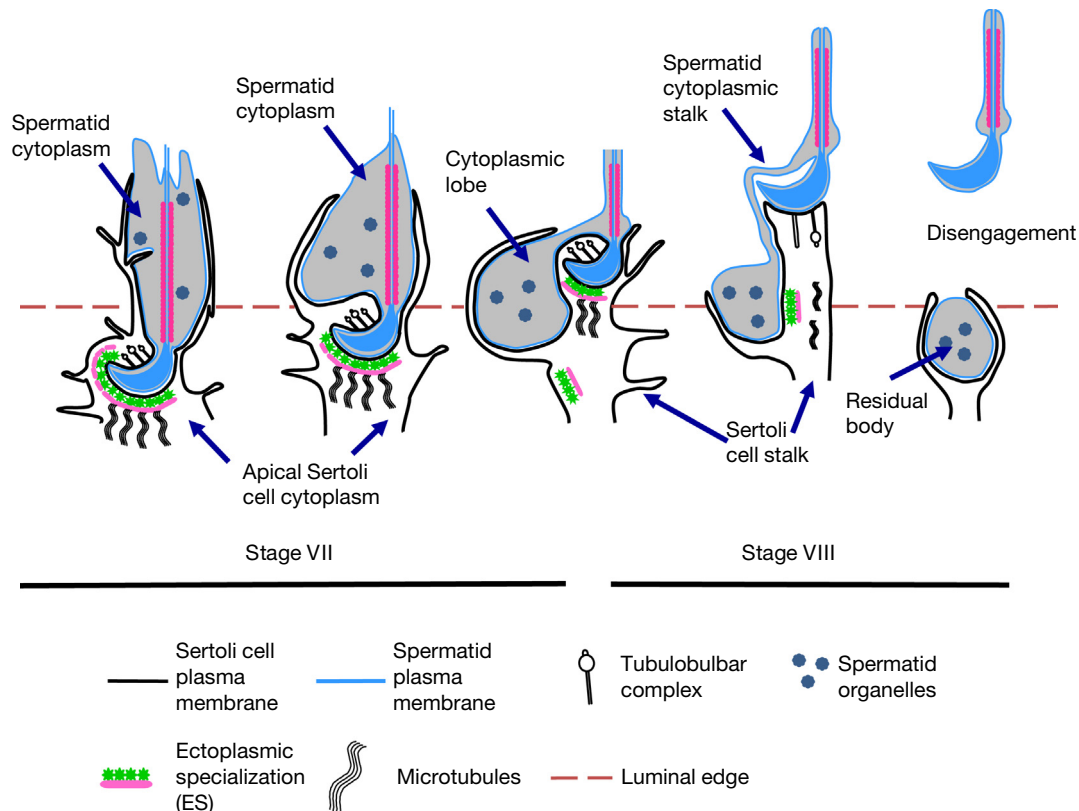


Fig. 2 Diagram of the major processes involved in spermiation. The diagram is based on rat spermatogenesis and is modified from O'Donnell et al. (2011). The diagram summarizes various morphological and ultrastructural events as described in Russell (1993). Note the relationship of the spermatid nucleus with the luminal edge of the seminiferous epithelium (indicated by the *dashed line*), as well as the changes in the shape of the Sertoli cell cytoplasm (*white*) around the spermatid and the remodeling of the spermatid cytoplasm (*gray*). The diagram summarizes how the spermatid nucleus is gradually extended out into the tubule lumen by the elongation of the Sertoli cell cytoplasmic stalk, and how the spermatid cytoplasm remains anchored to the Sertoli cell and is gradually stripped off as the spermatid is extended further into the lumen.

both Sertoli cell and spermatid plasma membranes, together with their associated proteins. The functions of TBCs will be further discussed below, however the fact that components of the ES and adhesion molecules are present within TBCs, highlights their role in ES removal and the remodeling of adhesion junctions (Vogl et al., 2014; O'Donnell et al., 2011; Upadhyay et al., 2012).

Another event that occurs at the beginning of spermiation is the clustering of integrin molecules along the outside curvature of the spermatid nucleus (Beardsley et al., 2006; O'Donnell et al., 2011). This clustering could be triggered by the spermatid being

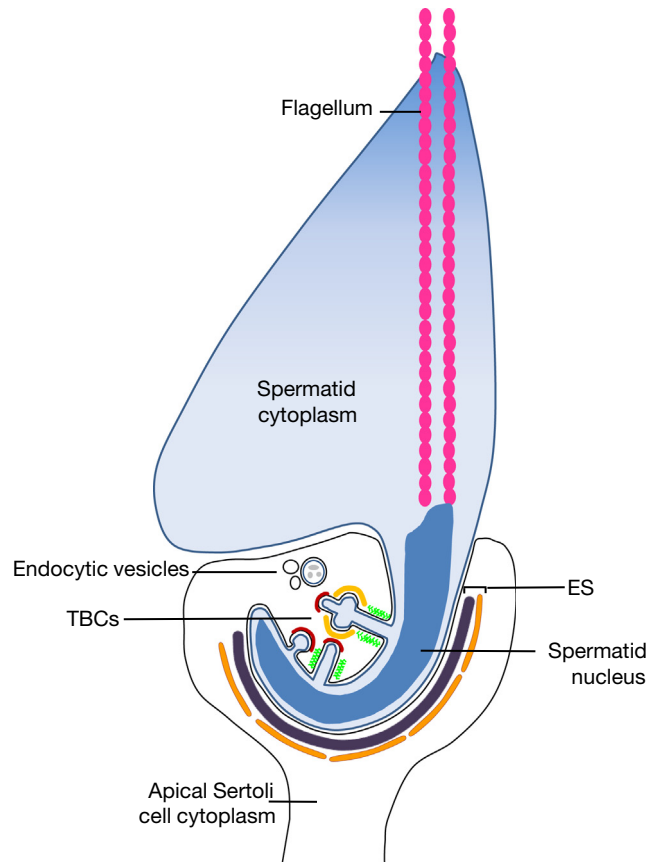


Fig. 3 Diagram of the Tubulobulbar complexes (TBCs) forming in the ventral curvature of the spermatid nucleus, in sites of the Sertoli cell deficient in ectoplasmic specialization (ES). The diagram has been modified from O'Donnell et al. (2011). The TBCs first form as a small clathrin-coated pit (clathrin indicated in red) and then elongate into a mature tubular structure with a round bulbous structure towards one end; this bulbous structure is surrounded by endoplasmic reticulum (indicated in yellow). The maturation of the TBCs appears to be facilitated by the polymerization of dendritic actin (indicated in green). This bulbous structure eventually buds off to form endocytic vesicles in the apical Sertoli cell cytoplasm. The spermatid cytoplasm is indicated in blue shading; note that some of the cytoplasm could potentially flow down towards TBCs and be present within them, as described by Russell (Russell, 1993).

subjected to shear forces from the flow of seminiferous tubule fluid through the lumen, necessitating the formation of an extremely tight adhesion junction that is capable of “holding on” to the spermatid nucleus as it is extended out into the lumen (O'Donnell et al., 2011). This integrin-based junction is likely a focal adhesion type junction (Beardsley et al., 2006, O'Donnell et al., 2011).

The Progression of Spermiation

Once spermiation is initiated, the spermatid and its associated adhesion junctions with the Sertoli cell undergo extensive remodeling over many hours. The entire process takes more than 80 h in the rat (O'Donnell et al., 2011), but is shorter in the mouse: likely around 40 h, based on the duration of the spermiation stages (Russell et al., 1990). Throughout the entire spermiation process, $\alpha 6 \beta 1$ integrins cluster along the Sertoli cell membrane opposite the outer curvature of the spermatid nucleus where it contacts the Sertoli cell, presumably allowing the Sertoli cell to tightly grip the spermatid as the ES is removed (Beardsley et al., 2006). The ligand on the spermatid that binds to Sertoli cell $\alpha 6 \beta 1$ integrin is likely composed of several laminin chains (Yan and Cheng, 2006).

As spermiation proceeds, the volume of the spermatid cytoplasm is greatly reduced, around 50% in rats (Russell, 1993). TBCs may facilitate cytoplasmic reduction by enveloping portions of spermatid cytoplasm and filtering out organelles, allowing a gradual reduction in the volume of the cytoplasm, see Figs. 1, 2 and 3 and O'Donnell et al. (2011), Russell (1993), Upadhyay et al. (2012). As the spermatid cytoplasm reduces in volume, it appears to gradually flow downwards, appearing around the spermatid nucleus where it is termed the cytoplasmic lobe (Figs. 1 and 2). In fact, the cytoplasm itself does not move but instead remains anchored to the Sertoli cell while the apical portion of the Sertoli cell, called the Sertoli cell stalk, extends and gradually pushes the spermatid

head out into the lumen (see Fig. 2). In this way, the Sertoli cell essentially “strips off” the spermatid’s cytoplasm to reveal its streamlined form. This process is obviously important, as any remaining cytoplasm would impede sperm morphology and motility. While the Sertoli cell appears to be responsible for removing the spermatid cytoplasm, there are circumstances where an abnormality within the spermatid itself, such as genetic defects that impact on the structure of the spermatid cytoplasm, prevent the Sertoli cell from successfully removing the cytoplasm (O’Donnell et al., 2011). Such abnormalities are usually associated with impaired sperm release and/or impaired sperm motility as well as infertility (O’Donnell et al., 2011).

During the long process of spermiation, there is extensive remodeling of adhesion junctions and multiple generations of TBCs form, mature and are dissolved. At this time, the Sertoli cell cytoplasm surrounding the spermatid nucleus is rich in endoplasmic reticulum (ER), which changes as spermiation progresses. In the early stages of spermiation, the Sertoli cell apical process surrounding the spermatid head contains so-called flattened ER which transitions into a tubular arrangement as spermiation proceeds (Clermont et al., 1980). Some of this ER associates with the bulbar portion of the TBCs and with the ES as it is dismantled. The ER at this site seems likely to be involved in the active remodeling of adhesion junctions and of TBC formation and dissolution.

Many adhesion, cytoskeletal and signaling proteins are present at the site of the spermiation and undergo changes in localization over the course of the entire process, highlighting the fact that there are marked changes in adhesion junction composition during spermiation (O’Donnell et al., 2011; Chapin et al., 2001). As discussed earlier, the ES is the major adhesion junction between spermatids and Sertoli cells at the beginning of spermiation, however this structure is gradually removed as spermiation progresses. The timing of ES removal appears to vary between species, as in the opossum the ES is lost very soon after the initiation of spermiation, whereas in the rat and mouse ES removal is more gradual (Russell, 1993). Small portions of ES appear to be dismantled at focal sites, followed by the appearance of a coated pit that will form into a mature TBC (Fig. 3) (Russell, 1993); this observation suggests that focal sites of ES disassembly are required for the initial formation of TBCs. However, TBCs appear to be responsible for much of the ES removal that subsequently occurs, as various ES-associated molecules are found within TBCs, and the appearance of TBC correlates with ES loss (Vogl et al., 2014; Vogl et al., 2013). Additionally, the observation of fragments of Sertoli cell and spermatid plasma membranes within TBCs confirms their role in adhesion junction removal (Vogl et al., 2013). Interestingly, early electron microscopy studies suggested that ES structures could be recycled away from the spermatid and translocated downwards to face the newly elongating spermatids below (Russell, 1993) and some observations suggest that TBCs could be involved in this recycling via long-loop recycling endosomes (Vogl et al., 2013).

Late Spermiation

Towards the end of spermiation but prior to spermatid release, the elongated spermatids are at the very edge of the lumen, only remaining in contact with a small proportion of the Sertoli cell cytoplasm (Figs. 1 and 2). The Sertoli cell stalk extends the spermatid out into the lumen while the spermatid’s cytoplasm is now concentrated and situated below the level of the spermatid’s nucleus (Figs. 1 and 2). At the same time, the round spermatids below have just begun the process of elongation, with their nuclei polarized to one side and oriented towards the base of the epithelium (see Fig. 1), and an extensive newly-formed Sertoli cell ES can be seen opposite their acrosomes (Beardsley et al., 2006).

At this point in spermiation, which corresponds to late stage VIII in the rodent, the traditional Sertoli cell ES is not seen in association with the elongated spermatid nucleus (Beardsley et al., 2006; O’Donnell et al., 2011) and therefore is no longer likely involved in spermatid-Sertoli cell adhesion. Instead, a focal-adhesion type junction containing $\alpha 6 \beta 1$ -integrin and a phosphorylated form of Focal Adhesion Kinase (FAK) remains concentrated at the remaining portion of the Sertoli cell that adheres to the spermatid (Beardsley et al., 2006; O’Donnell et al., 2011). This adhesion complex, termed the disengagement complex, appears to form at the beginning of spermiation and persists throughout ES removal (O’Donnell et al., 2011), and likely adheres to the spermatid via specific surface laminins (Yan and Cheng, 2006). TBCs are also still observed at this time, however some observations suggest their morphology may change somewhat, and in particular they often lack the bulbous portion (Russell, 1993). This could suggest that the TBCs at this stage are no longer removing junction components; perhaps instead these “remnant” TBCs could facilitate anchorage of the spermatid to the Sertoli cell (Russell, 1993).

The term “spermiation machinery” is a term used to encompass all of the adhesion and related structures (including TBCs and the ES) that are present between the spermatid and the Sertoli cell during spermiation (O’Donnell et al., 2011). The spermiation machinery is highly dynamic as spermiation progresses (O’Donnell et al., 2011) and facilitates a major redistribution and probably recycling of adhesion and signaling proteins as the spermatid-Sertoli cell junctions are remodeled prior to sperm release (Chapin et al., 2001; O’Donnell et al., 2011). TBCs are an important part of this machinery, removing a variety of adhesion and ES-associated proteins (Vogl et al., 2013). Adhesion-related signaling proteins can be seen in focal “dots” within the Sertoli cell cytoplasm as they are presumably relocated from the Sertoli cell-spermatid interface (Beardsley and O’Donnell, 2003; Chapin et al., 2001). The final disengagement complex that remains likely includes non-bundled actin, various signaling proteins and kinases, as well as integrins and associated cytoskeletal molecules (O’Donnell et al., 2011).

Also at this time, the spermatid cytoplasm has condensed into what will become known as the residual body when the sperm is released. This cytoplasm is now highly condensed and contains various organelles (see Fig. 1), and remains attached to the spermatid by a long cytoplasmic stalk (Fig. 2; O’Donnell et al., 2011).

Disengagement

The final moment of spermiation occurs when the spermatids are released into the tubule lumen; after their release, the spermatids are now referred to as sperm or spermatozoa. This release process is termed disengagement, as the sperm appear to rapidly disengage from the Sertoli cell in an apparent instantaneous loss-of-adhesion event (Russell, 1993). In fact, disengagement is so rapid that it has been rarely observed in histological sections (Russell, 1993). All spermatids in one part of the epithelium are released simultaneously and are rapidly swept away in the tubule fluid towards the rete testis and epididymis, likely propelled by actin-based contractility of the myoid cells surrounding the tubule (O'Donnell et al., 2011; Russell, 1993). In sections of normal testis, released sperm are rarely observed in the lumen of the tubule, as they are swiftly transported to the rete testis. Because of the arrangement of the spermatogenic cycle in “waves” in the seminiferous tubules, the disengagement of sperm occurs in pulses along the length of the tubules, allowing sperm to be constantly produced.

The signaling mechanism that causes the actual disengagement of spermatids from Sertoli cells is not defined, but it seems clear that the Sertoli cell determines when to release the sperm (O'Donnell et al., 2011, Russell, 1993). In vitro models of spermiation have revealed that disengagement can be inhibited by various pharmacological agents including inhibition of integrin function and protein phosphorylation (Chapin et al., 2001; O'Donnell et al., 2011). It is generally considered that coordinated signals within Sertoli cells promote the disengagement complex to release the sperm, as the spermatids are rather inert at this stage due to their lack of cytoplasm (O'Donnell et al., 2011; Russell, 1991). However, the triggers for these signals, and how all Sertoli cells in the same area of tubule release sperm at a similar time, remains unknown. The loss-of-adhesion event is likely associated with changes in adhesive status of the $\alpha 6 \beta 1$ -integrins within the disengagement complex (Chapin et al., 2001, O'Donnell et al., 2011); this could be likened to the integrin-mediated loss-of-adhesion events that occur as cells migrate along extracellular matrix (O'Donnell et al., 2011).

As the sperm are released, the cytoplasmic stalk attaching the spermatid to its cytoplasm stretches and breaks and the subsequent residual body remains anchored to the Sertoli cell (O'Donnell et al., 2011, Russell, 1993). Very soon after, the Sertoli cell completely envelopes and phagocytoses the residual bodies. These large circular vesicles contain various remnant structures, vacuoles, lipid droplets, condensed mitochondria and likely a multitude of proteins, surrounded by the spermatid plasma membrane and by the Sertoli cell plasma membrane (Morales and Clermont, 1993). The residual bodies are then rapidly transported towards the base of the seminiferous epithelium. During migration, they fuse with secondary lysosomes within the Sertoli cell cytoplasm and are transformed and ultimately phagocytosed by the Sertoli cell (Morales and Clermont, 1993). Residual bodies can modulate Sertoli cell functions and could therefore could regulate aspects of spermatogenesis such as blood-testis-barrier function, see (O'Donnell et al., 2011). Intriguingly, recent studies suggest that lipid-like particles derived from residual bodies could be eliminated into the interstitial space (Xiao et al., 2017). Even more intriguingly, the elimination of sperm-specific proteins into the interstitium via residual bodies has been suggested to promote peripheral tolerance of the immune system (Tung et al., 2017). Thus residual bodies may be a way of consistently delivering sperm proteins, which would otherwise be recognized as foreign by the immune system, to immune cells within the interstitium to actively promote immune system tolerance to sperm (Tung et al., 2017).

Spermiation Failure

Spermiation is highly vulnerable to disruption. Defects in the spermiation process can be seen in response to many different pharmacological agents and reproductive toxicants as well as in various transgenic mouse models (O'Donnell, 2014; O'Donnell et al., 2011; Russell, 1993; Russell, 1991). Disruptions to spermiation are often an early feature of spermatogenic damage: the failure of sperm to be released often precedes other morphological abnormalities in spermatogenesis. Given that spermiation is largely controlled by the Sertoli cell, it seems reasonable to assume that many pharmacological agents and toxicants target Sertoli cell signaling that in turn inhibits sperm release. Spermiation failure is often a sign that “all is not well” (Russell, 1993, Russell, 1991) leading to the hypothesis that Sertoli cells could prevent sperm release, and thus restrict fertility, when the environmental conditions are unfavorable (O'Donnell et al., 2011).

The inherent vulnerability of spermiation is likely due to the fact that it is a multistep process, involving many different adhesion, cytoskeletal and signaling molecules as well as formation of endocytic structures: the disruption of any one of proteins involved in these events could conceivably impact on spermiation. It is also important to note that spermiation fails at multiple steps, depending on when the process is disrupted. For example, spermatids can fail to commence spermiation, and instead of being translocated to the luminal edge via microtubule-based motors, remain within the Sertoli cells where they are phagocytosed (O'Donnell, 2014; O'Donnell et al., 2011). Spermatids can be prematurely released before their cytoplasm has been properly removed, or they can fail to disengage at the very end of spermiation (O'Donnell, 2014, O'Donnell et al., 2011).

Spermiation is clearly responsive to hormones. Spermatogenesis is regulated by androgens, produced locally within the testis in response to pituitary LH stimulation, and by FSH released by the pituitary, and both hormones have independent, additive and synergistic effects on testicular sperm production (O'Donnell et al., 2017). Spermiation is impaired by the suppression of androgen and/or FSH (O'Donnell et al., 2011), and by elevated estradiol (Kumar et al., 2015 and references therein). Failure of the final disengagement of spermatids is seen in rodents, monkeys and men after androgen and/or FSH suppression, highlighting that this final loss-of-adhesion event is regulated by endocrine signals targeting the Sertoli cells (O'Donnell, 2014, O'Donnell et al., 2011). Retinoic acid signaling, which regulates multiple aspects of spermatogenesis (O'Donnell et al., 2017), is also required for normal spermiation, reviewed in (O'Donnell et al., 2011).

Because sperm release from the testis ultimately influences the sperm output from the testis, spermiation failure can contribute to reduced sperm numbers in the ejaculate. Thus the success of spermiation is an important determinant of male fertility. This is best characterized in models of hormone-based male contraception, where androgen and FSH suppression leads to spermiation failure initially, but ultimately disrupts earlier germ cell development, reviewed in (O'Donnell et al., 2011). In this setting, the early induction of spermiation failure in some men undergoing hormone-based contraception can lead to azoospermia (zero sperm in the ejaculate), despite the fact that their testes continue to produce spermatids. The degree of spermiation failure induced by hormone-based contraception can influence whether an individual achieves azoospermia or continues to produce sperm in the ejaculate (O'Donnell et al., 2001). Therefore the induction of spermiation failure can influence the efficacy of hormone-based contraception.

While spermiation is a determinant of sperm counts and fertility, it can also presumably influence sperm quality. Other than determining whether sperm are released or not, spermiation also accomplishes the removal of the spermatid cytoplasm to ensure its streamlined form that is necessary for normal motility. The failure to properly remove cytoplasm results in abnormal spermatids in the epididymis and usually infertility, as demonstrated in a number of transgenic mouse models, reviewed in (O'Donnell et al., 2011). However the contribution of spermiation failure to conditions of human male infertility is not well defined. It is known that excess cytoplasm on human spermatozoa (which likely arises from defective spermiation) is associated with increased generation of reactive oxygen species (ROS) which can induce DNA damage in the sperm and decrease its reproductive potential (Aitken and De Iuliis, 2010).

Conclusion

In conclusion, spermiation is a long, complex and multistep process, where the final goal is to release a streamlined spermatozoon. The process involves removal of the extensive cytoplasm from the mature elongated spermatid, and the removal of the ES junction that has enabled the Sertoli cell to tightly adhere to the spermatid during its elongation and development. The spermatid is pushed out into the lumen as its cytoplasm is “stripped” off by the Sertoli cell. As the ES junction is removed, another integrin-based focal adhesion-like junction maintains tight adhesion of the spermatid to the Sertoli cell. The remodeling of ES junctions during spermiation, and perhaps some aspects of cytoplasm removal, is mediated by TBCs which are specialized endocytic structures in Sertoli cells. The final release of sperm from the Sertoli cells is termed disengagement, and involves an instantaneous loss-of-adhesion event likely mediated by signaling pathways in the Sertoli cell. Because of its multi-faceted nature and the involvement of numerous adhesion and signaling molecules, spermiation is highly vulnerable to disruption by various environmental insults and the suppression of hormones. Spermiation is a key determinant of sperm content, and possibly sperm quality, in the ejaculate and thus the success of this process is important for male fertility.

References

- Aitken, R. J., & De Iuliis, G. N. (2010). On the possible origins of DNA damage in human spermatozoa. *Molecular Human Reproduction*, 16, 3–13.
- Beardsley, A., & O'Donnell, L. (2003). Characterization of normal spermiation and spermiation failure induced by hormone suppression in adult rats. *Biology of Reproduction*, 68, 1299–1307.
- Beardsley, A., Robertson, D. M., & O'Donnell, L. (2006). A complex containing alpha6beta1-integrin and phosphorylated focal adhesion kinase between Sertoli cells and elongated spermatids during spermatid release from the seminiferous epithelium. *The Journal of Endocrinology*, 190, 759–770.
- Chapin, R. E., Wine, R. N., Harris, M. W., et al. (2001). Structure and control of a cell-cell adhesion complex associated with spermiation in rat seminiferous epithelium. *Journal of Andrology*, 22, 1030–1052.
- Clermont, Y., McCoshen, J., & Hermo, L. (1980). Evolution of the endoplasmic reticulum in the Sertoli cell cytoplasm encapsulating the heads of late spermatids in the rat. *The Anatomical Record*, 196, 83–99.
- Guttman, J., Kimel, G., & Vogl, A. W. (2000). Dynein and plus-end microtubule-dependent motors are associated with specialized Sertoli cell junction plaques (ectoplasmic specializations). *Journal of Cell Science*, 113, 2167–2176.
- Kumar, A., Dumasia, K., Gaonkar, R., et al. (2015). Estrogen and androgen regulate actin-remodeling and endocytosis-related genes during rat spermiation. *Molecular and Cellular Endocrinology*, 404, 91–101.
- Morales, C., & Clermont, Y. (1993). Structural changes of the Sertoli cell during the cycle of the seminiferous epithelium. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell*. Clearwater, FL: Cache River Press.
- O'Donnell, L. (2014). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis*, 4, e979623.
- O'Donnell, L., Narula, A., Balourdos, G., et al. (2001). Impairment of spermatogonial development and spermiation after testosterone-induced gonadotropin suppression in adult monkeys (*Macaca fascicularis*). *The Journal of Clinical Endocrinology and Metabolism*, 86, 1814–1822.
- O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., et al. (2011). Spermiation: The process of sperm release. *Spermatogenesis*, 1, 14–35.
- O'Donnell, L., Stanton, P., & de Kretser, D. M. (2017). Endocrinology of the Male Reproductive System and Spermatogenesis. In L. J. De Groot, G. Chrousos, K. Dungan, K. R. Feingold, A. Grossman, J. M. Hershman, C. Koch, M. Korbonits, R. McLachlan, M. New, J. Purnell, R. Rebar, F. Singer, & A. Vinik (Eds.), *Endotext*. South Dartmouth (MA): MDText.com, Inc.
- Russell, L. (1993). Role in spermiation. In L. D. Russell, & M. D. Griswold (Eds.), *The Sertoli cell*. Clearwater, FL: Cache River Press.
- Russell, L. D. (1991). The perils of sperm release—'let my children go. *International Journal of Andrology*, 14, 307–311.
- Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P., & Clegg, E. D. (1990). *Histological and histopathological evaluation of the testis*. Place: Cache River Press.
- Tung, K. S., Harakal, J., Qiao, H., et al. (2017). Egress of sperm autoantigen from seminiferous tubules maintains systemic tolerance. *The Journal of Clinical Investigation*, 127, 1046–1060.
- Upadhyay, R. D., Kumar, A. V., Ganeshan, M., & Balasinar, N. H. (2012). Tubulobulbar complex: cytoskeletal remodeling to release spermatozoa. *Reproductive Biology and Endocrinology*, 10, 27.
- Vogl, A. W., Du, M., Wang, X. Y., & Young, J. S. (2014). Novel clathrin/actin-based endocytic machinery associated with junction turnover in the seminiferous epithelium. *Seminars in Cell & Developmental Biology*, 30, 55–64.

- Vogl, A. W., Pfeiffer, D. C., Mulholland, D., et al. (2000). Unique and multifunctional adhesion junctions in the testis: ectoplasmic specializations. *Archives of Histology and Cytology*, 63, 1–15.
- Vogl, A. W., Young, J. S., & Du, M. (2013). New insights into roles of tubulobulbar complexes in sperm release and turnover of blood-testis barrier. *International Review of Cell and Molecular Biology*, 303, 319–355.
- Xiao, C. Y., Wang, Y. Q., Li, J. H., et al. (2017). Transformation, migration and outcome of residual bodies in the seminiferous tubules of the rat testis. *Andrologia*.
- Yan, H. H., & Cheng, C. Y. (2006). Laminin alpha 3 forms a complex with beta3 and gamma3 chains that serves as the ligand for alpha 6beta1-integrin at the apical ectoplasmic specialization in adult rat testes. *The Journal of Biological Chemistry*, 281, 17286–17303.

Testis Cell and Organ Culture

Ingrid Sadler-Rigglesman and Michael K Skinner, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Testis Cell Isolation and Culture

The testis, the male gonad in animals, produces male germ cells and sexual hormones. It contains several primary cell types. Germ cells, Sertoli cells and peritubular cells are located within the seminiferous tubules. Germ cells are developed in the tubules supported by the two types of somatic cells, Sertoli and peritubular cells. In between tubules (interstitium) Leydig cells are present. Leydig cells are the location of steroidogenesis and synthesize hormones essential for germ cell differentiation (Christensen and Mason, 1965) (Fig. 1).

The procedures for isolating individual testis cell populations for culture are very similar across the animal spectrum. In 1969, Hall et al. were able to look at interstitial versus tubular cell types by manually dissecting the testis. Welsh and Wiebe (Welsh and Wiebe, 1975) introduced in 1975 an isolation method using the enzyme collagenase, which worked well for digesting the tissue without hurting viability of the isolated cells, and Dornington et al. (1975) published a similar method in the same year that used the enzymes trypsin, desoxyribonuclease (DNase) and collagenase. Tung et al. (1984) improved the enzymatic digestion method by adding hyaluronidase which helped to reduce cross contamination of cell types. This method of using progressive steps of enzymatic digests has been used with slight modifications for isolation of individual testicular cell fractions from a wide range of species. The three enzymes trypsin, collagenase and hyaluronidase together with DNase are used for most protocols, but the order and combination in which they are used may change depending on the animal species and cell type to be isolated.

The following is a technique used for the isolation of testis cells from immature rats (around 20 days of age). In 20 d rats the Sertoli cells cease to divide and comprise a large fraction of the testicular cell types because spermatogenesis has not started yet and so the proportion of germ cell contamination in the Sertoli cell fraction is low (Fig. 2).

Sertoli Cell and Peritubular Cell Isolation and Purification (Tung et al., 1984 With Modifications)

The rats are sacrificed and testes removed from each rat. After rinsing the testes several times in sterile HBSS (Hank's Buffered Salt Solution) they are detunicated (the tunica albuginea encloses the testis) and minced finely. The initial enzymatic digest uses trypsin (protein hydrolysis) and DNase. The addition of DNase is necessary at every purification step for removal of the DNA released from damaged cells, otherwise the cells would clump together into a gelatinous mass and be impossible to separate. The trypsin digest will separate the tubules, and interstitial cells (Leydig cells etc.) will be freed and can be separated from the tubules by gravity sedimentation. The tubules will settle to the bottom of the tube and the supernatant can be discarded. Several washes of the tubules using gravity sedimentation are needed to guarantee that most of the interstitial cells are removed. The next step in the procedure involves collagenase (serine protease) digestion, again with DNase added. This step will break the tubules into smaller pieces and release a large percentage of the peritubular cells. The tubule pieces (with Sertoli cells attached) can be separated from the peritubular cells by gravity sedimentation. The tubules will settle to the bottom and peritubular cells can be removed with the supernatant. The peritubular cells can be washed to remove cellular debris and enzymatic solution by pelleting the supernatant gently in a centrifuge, and then resuspending the peritubular cells in a physiological buffer. After several washes, they can be plated in appropriate medium for further study in cell culture. The remaining tubules need to be washed again several times to remove as many contaminating peritubular cells as possible and will then be further digested with hyaluronidase and DNase. This step will

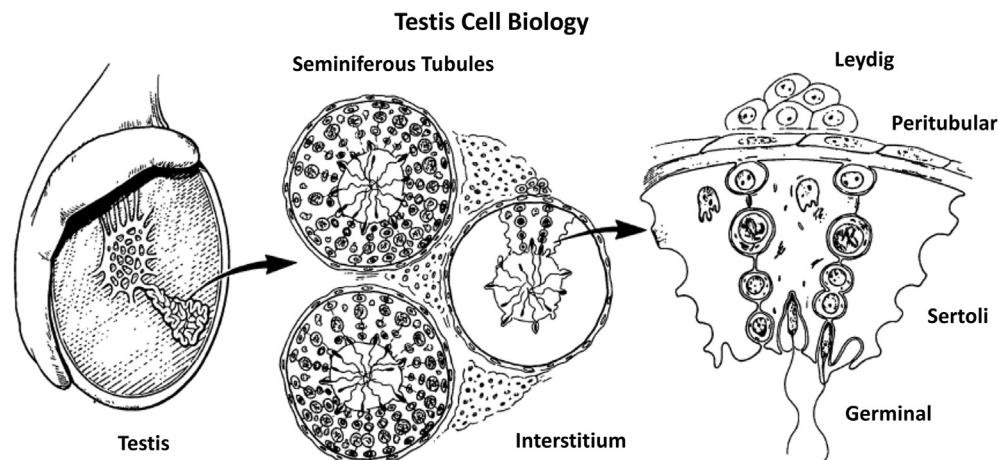


Fig. 1 Cell types in the testis. Modified from Skinner, M. K. (1991). Cell-cell interactions in the testis. *Endocrine Reviews* 12, 45–77.

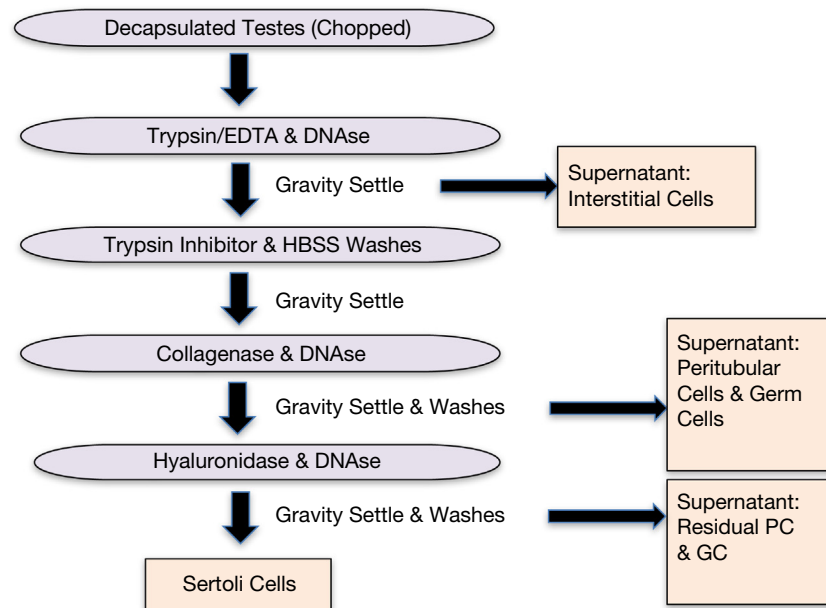


Fig. 2 Flowchart for isolation of different cell types from the testis.

further break down the tubules, releasing the germ cells and remaining peritubular cells. In a microscopic check the Sertoli cells can be seen as small aggregates and after several washes using gravity sedimentation of the Sertoli cell aggregates, they can be plated in the appropriate medium. If further dissociation is needed before plating they can be homogenized by shearing using a hypodermic needle with small needle size and passaged through a cell strainer which will result in a single cell suspension. Also, if needed, a hypotonic shock (using a 1:10 dilution of Hank's Balanced Salt Solution—HBSS with water) can be added after preparation of Sertoli cells which will not damage the Sertoli cells but will burst and get rid of remaining germ cells (Galdieri et al., 1981). This is especially important in the preparation of Sertoli cells from adult testes since spermatogenesis is present at that time and contamination with germ cells could pose a problem in the cell culture. In addition germ cells will not attach to the culture plate like Sertoli cells and will die after a few days in culture. So, the purity of Sertoli cells will increase after a few days in culture.

Leydig Cell Isolation and Purification (Risbridger and de Kretser, 1986; Klinefelter et al., 1987 With Modifications)

The testis interstitium contains a mix of Leydig cells, macrophages, epithelial cells, and fibroblasts. As mentioned before, Leydig cells synthesize hormones important for sexual development and germ cell differentiation and are needed for study in that context. Testes are prepared as described above and digested with collagenase until the testes start to dissociate into tubules, thus releasing the interstitial cells. Over-digestion needs to be avoided in order to keep the tubules intact and avoid contamination of the Leydig fraction with tubular cells. The interstitial cells will be in the supernatant after letting the tubules settle out and are removed for further processing. Density gradients such as Percoll gradients are used to separate Leydig cells from other interstitial cells. The interstitial mix will be loaded on a Percoll gradient and spun at low g. The Leydig cells will concentrate at a certain Percoll density and can be isolated as a pure fraction. The Percoll needs to be washed off before plating the cells for cell culture.

Testicular Macrophage Isolation and Purification (Yee and Hutson, 1983)

Testicular macrophages are the main immune cells of the mammalian testis. There are two different populations which differ in morphology and localization (in the interstitium or on the surface of seminiferous tubules). They are involved in classical immune functions but also in organogenesis, spermatogenesis and male hormone production.

The protocol in the original paper describing isolation of testicular macrophages from mammalian testis (Yee and Hutson, 1983) is still being used with minor modifications. After decapsulating the testes and washing them in PBS, the testes are minced and then incubated with a digestive enzyme like trypsin or collagenase with DNase added. The seminiferous tubules are allowed to sediment and the supernatant is collected and centrifuged. The pellet containing a mixture of interstitial cells is resuspended in serum free medium or PBS and plated on plastic cell culture dishes. Under these conditions the macrophages will attach, but not the other cells. After only a short incubation period of about 20–30 min the non-adherent cells are washed off and the remaining—adherent—cells constitute the macrophage fraction which has a purity of over 95% (Winnall et al., 2011).

Germ Cell Isolation and Purification (Bellvé, 1979 With Modifications)

Germ cells at different stages of spermatogenesis can be isolated depending on the age of the animal used for preparation. But especially for mammals it is difficult to grow germ cells in culture by themselves, with the exception of spermatogonial stem cells. Sertoli cells are essential for the structural support and paracrine control (through for example testosterone and FSH) of germ cells during spermatogenesis. Therefore Sertoli cells are often used in a co-culture with germ cells to study the stages and regulation of spermatogenesis. Still, it is difficult to achieve complete spermatogenesis in cell culture, but it can be done in organ cultures using testis fragments as described below in section Testis Organ Culture.

Isolation of germ cell fractions follows essentially the same enzymatic digestion protocol as used for somatic testis cells. An initial enzymatic digest with trypsin or collagenase will release interstitial cells and the majority of peritubular cells, which will stay in the supernatant during gravity sedimentation and can be discarded while tubule fragments and Sertoli cell/germ cell clusters will be in the pellet. Further enzymatic digestion with trypsin/EDTA and DNase will fully dissociate the seminiferous tubules and release Sertoli and germ cells.

A germ cell fraction can be isolated different ways. Panning is an efficient method for crude separation of germ cells from Sertoli cells and relies on the fact that Sertoli cells will stick to a culture plate while germ cells will not. So, after plating and a 2–3-day incubation the germ cells can be removed from the top of the plate leaving the Sertoli cells behind. Germ cells can then be further purified based on their density using a gradient made with BSA (bovine serum albumin) or Percoll. Another approach is to remove Sertoli cell clusters with a cell strainer which will result in a single cell germ cell fraction in the filtrate. Again, this fraction can be further purified using a gradient.

Sometimes it is necessary to isolate germ cells at a specific developmental stage and this can be done using a Sta-put ([Romrell et al., 1976](#); [McCarrey et al., 1992](#)) apparatus, which is a velocity sedimentation cell separator allowing the isolation of cell fractions based on their differences in gravity sedimentation rate. Germ cells at different stages of development have different sedimentation rates.

Cell culture of germ cells can be accomplished by co-culturing them with feeder cells like Sertoli cells. This co-culture can be improved by growing the cells on specific substrates like Matrigel, collagen or in a soft-agar culture. Also, growth in 3-D culture systems providing “scaffolding” for the germ cells has shown promise in improving germ cell viability and growth ([Hunter et al., 2012](#); [Stukenborg et al., 2009](#)).

Testis Organ Culture (Dores et al., 2012)

Cell culture systems are very helpful because they reduce a complex structure into smaller parts and therefore help one to understand complicated processes by examining and manipulating the single parts of the system. An in vitro cell culture system is indispensable for studying cell–cell interactions, roles of hormones and growth factors, gene expression and morphological changes. Some processes however, for example spermatogenesis, need a more intact system to function properly in order to fully finish their developmental events. So far it has not been possible to produce functioning spermatozoa using an in vitro cell culture system alone.

Organ culture on the other hand preserves the proper niche of testicular structure and paracrine exposures which are needed for proper spermatogenesis. Organ culture of testis fragments can be maintained for several weeks. Testis organ culture’s main use has been to study spermatogenesis with clinical implications for help with reproductive issues.

Organ culture protocols often use the air–liquid interface method ([Trowell, 1954](#)) in which a filter is carefully set on top of the appropriate cell culture medium into a well of a multi-well cell culture plate ([Levine et al., 2000](#)). It is important that the filter float on top of the medium and is not submerged. A small piece of testicular tissue or a whole embryonic testis are placed on the filter floating on cell culture medium. The testis piece or embryonic whole testis will be covered with a drop of medium to prevent it from drying out. This places the organ culture close to the atmosphere in the incubator, and allows for higher oxygen concentrations in the cultured organs. Instead of just using liquid medium, other supporting substrates like agarose can also be used ([Stukenborg et al., 2008](#)). These more solid substrates can be saturated with medium with the testis piece placed on top. Incubation of the organ culture in a CO₂ cell culture incubator can continue from a few days to several weeks with frequent medium changes.

Human testicular pieces have been cultured this way for about 2 weeks with decreasing germ cell numbers throughout this time ([Roulet et al., 2006](#)). [Nakamura et al. \(2017\)](#) could maintain a mouse testis organ culture with spermatogenesis for over 2 months. One of the most important features to maintain a fully functioning organ culture seems to be the proper medium and additives. For example, a change in the medium from serum to serum replacements like GlutaMax® and AlbuMax® made a big difference in successfully maintaining organ cultures for longer periods of time and achieving full spermatogenesis. These experiments have been mostly done with mammalian tissue, but with appropriate culture conditions could probably be extended to other species.

Summary

Cell and organ culture of testis cells or testis tissue from a variety of species is advancing the understanding of processes in the testis on both a molecular and whole system level. Cell and organ culture together provide means in basic and applied research to investigate this complex organ in detail providing information on organogenesis, male germ cell production, the specific cell types and their ways of interacting, and the signaling pathways controlling testis function.

This knowledge is invaluable for finding out more about the mechanisms of spermatogenesis, which provides the means to develop new diagnostic tools and therapies for restoring male fertility and for assisted reproduction. It will also be helpful, for example, for preservation of gametes, maintenance of livestock and species conservation. Organ cultures in particular are needed for research where the intact testis structure is required, as in toxicological studies and for in vitro maturation of sperm cells.

References

- Bellvé, A. R. (1979). The molecular biology of mammalian spermatogenesis. *Oxford Reviews of Reproductive Biology*, 1, 159–261.
- Christensen, A. K., & Mason, N. R. (1965). Comparative ability of seminiferous tubules and interstitial tissue of rat testes to synthesize androgens from Progesterone-4-14c in vitro. *Endocrinology*, 76, 646–656.
- Dores, C., Alpaugh, W., & Dobrinski, I. (2012). From in vitro culture to in vivo models to study testis development and spermatogenesis. *Cell and Tissue Research*, 349, 691–702.
- Dorrington, J. H., Roller, N. F., & Fritz, I. B. (1975). Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. *Molecular and Cellular Endocrinology*, 3, 57–70.
- Galdieri, M., Ziparo, E., Palombi, F., Russo, M. A., & Stefanini, M. (1981). Pure Sertoli cell cultures: A new model for the study of somatic–germ cell interactions. *Journal of Andrology*, 2, 249–254.
- Hall, P. F., Irby, D. C., & De Kretser, D. M. (1969). Conversion of cholesterol to androgens by rat testes: Comparison of interstitial cells and seminiferous tubules. *Endocrinology*, 84, 488–496.
- Hunter, D., Anand-Ivell, R., Danner, S., & Ivell, R. (2012). Models of in vitro spermatogenesis. *Spermatogenesis*, 2, 32–43.
- Klinefelter, G. R., Hall, P. F., & Ewing, L. L. (1987). Effect of luteinizing hormone deprivation in situ on steroidogenesis of rat Leydig cells purified by a multistep procedure. *Biology of Reproduction*, 36, 769–783.
- Levine, E., Cupp, A. S., & Skinner, M. K. (2000). Role of neurotrophins in rat embryonic testis morphogenesis (cord formation). *Biology of Reproduction*, 62, 132–142.
- Mccarrey, J. R., Berg, W. M., Paragioudakis, S. J., Zhang, P. L., Dilworth, D. D., Arnold, B. L., & Rossi, J. J. (1992). Differential transcription of P_{gk} genes during spermatogenesis in the mouse. *Developmental Biology*, 154, 160–168.
- Nakamura, N., Merry, G. E., Inselman, A. L., Sloper, D. T., del Valle, P. L., Sato, T., Ogawa, T., & Hansen, D. K. (2017). Evaluation of culture time and media in an in vitro testis organ culture system. *Birth Defects Research*, 109, 465–474.
- Risbridger, G. P., & De Kretser, D. M. (1986). Percoll-gradient separation of Leydig cells from postnatal rat testes. *Journal of Reproduction and Fertility*, 76, 331–338.
- Romrell, L. J., Bellvé, A. R., & Fawcett, D. W. (1976). Separation of mouse spermatogenic cells by sedimentation velocity. A morphological characterization. *Developmental Biology*, 49, 119–131.
- Roulet, V., Denis, H., Staub, C., Le Tortorec, A., Delaleu, B., Satie, A. P., Patard, J. J., Jegou, B., & Dejuicq-Rainsford, N. (2006). Human testis in organotypic culture: Application for basic or clinical research. *Human Reproduction*, 21, 1564–1575.
- Stukenborg, J. B., Wistuba, J., Luetjens, C. M., Elhija, M. A., Huleihel, M., Lunenfeld, E., Gromoll, J., Nieschlag, E., & Schlatt, S. (2008). Coculture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. *Journal of Andrology*, 29, 312–329.
- Stukenborg, J. B., Schlatt, S., Simoni, M., Yeung, C. H., Elhija, M. A., Luetjens, C. M., Huleihel, M., & Wistuba, J. (2009). New horizons for in vitro spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. *Molecular Human Reproduction*, 15, 521–529.
- Trowell, O. A. (1954). A modified technique for organ culture in vitro. *Experimental Cell Research*, 6, 246–248.
- Tung, P. S., Skinner, M. K., & Fritz, I. B. (1984). Fibronectin synthesis is a marker for peritubular cell contaminants in Sertoli cell-enriched cultures. *Biology of Reproduction*, 30, 199–211.
- Welsh, M. J., & Wiebe, J. P. (1975). Rat sertoli cells: A rapid method for obtaining viable cells. *Endocrinology*, 96, 618–624.
- Winnall, W. R., Muir, J. A., & Hedger, M. P. (2011). Rat resident testicular macrophages have an alternatively activated phenotype and constitutively produce interleukin-10 in vitro. *Journal of Leukocyte Biology*, 90, 133–143.
- Yee, J. B., & Hutson, J. C. (1983). Testicular macrophages: Isolation, characterization and hormonal responsiveness. *Biology of Reproduction*, 29, 1319–1326.

Further Reading

- Skinner, M. K. (1991). Cell–cell interactions in the testis. *Endocrine Reviews*, 12, 45–77.

Blood-Testis Barrier

Baiping Mao, Ming Yan, Linxi Li, and C Yan Cheng, Population Council, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The blood-testis barrier (BTB), also known as the Sertoli cell epithelial barrier, is one of the tightest blood-tissue barriers in the mammalian body (Setchell, 2008; Franca et al., 2012; Cheng, 2012; Cheng and Mruk, 2012; Pelletier, 2011; Stanton, 2016; Mital et al., 2011). In 1964, the term BTB was first coined by Chiquoine in a report, examining the morphological events during cadmium-induced testis injury (Chiquoine, 1964), which is known to cause male sterility when administered at acute doses in rodents (Parizek and Zahor, 1956; Parizek, 1960). However, the concept of the BTB was first reported more than 100 years ago when dyes administered to rodents, which stained virtually all organs except the brain and seminiferous epithelium of the testis (Setchell, 2008; Setchell and Waites, 1975), illustrating there is a unique blood-tissue barrier in the testis and the brain to protect these two vital organs in mammals, and subsequently called the blood-testis barrier and the blood-brain barrier, respectively. These findings also illustrate that microvessels in the interstitial space, located at the intersections of seminiferous tubules in the interstitium, contribute virtually no barrier function to confer the BTB function in the testis. Interestingly, in rodents, peritubular myoid cells form a single cell layer behind the basement membrane and type I collagen layer versus several layers of myoid cells in primates around the seminiferous tubule (Setchell and Breed, 2006). This peritubular myoid cell layer in rodents has been shown to be an effective barrier in blocking the passage of electron dense markers such as colloidal carbon or lanthanum across the tunica propria since ~85% of tubules were capable of blocking electron dense substances from entering the seminiferous epithelium (Fawcett et al., 1970; Dym and Fawcett, 1970). These findings thus illustrate that peritubular myoid cell layer is a considerably important contributor to the BTB function in rodents. This conclusion was reached because the myoid cell layers in primates that wrap around the seminiferous tubules are not effective in blocking the passage of electron dense markers across seminiferous epithelium when administered via i.v. (Dym, 1973). In short, the BTB in primates and possibly humans is solely dependent on the Sertoli cells for the provision of the needed barrier function to support spermatogenesis. As such, the BTB is also known as the Sertoli cell epithelial barrier in the testis.

In the mammalian testis, the BTB is located near the basement membrane, which also segregates the seminiferous epithelium into the basal and the adluminal (apical) compartment (Fig. 1). Due to the presence of the BTB, Sertoli cells and germ cells at different stages of their development are the only cell types found in the seminiferous epithelium, whereas Leydig cells, residual macrophages, and microvessels are found in the interstitium. Spermatogonial stem cells (SSC), also referred to as (or including) A_{single} (A_s) in rodents vs. A_{dark} in humans reside in the stem cell niche (de Rooij, 2009; Ehmcke et al., 2006); undifferentiated spermatogonia including A_{paired} (A_{pr}) and A_{aligned} (A_{al}) vs. A_{pale} in humans; and differentiated spermatogonia including $A1-4$, $A_{\text{intermediate}}$ (A_{in}) and type B spermatogonia in rodents vs. B spermatogonia in humans (Cheng and Mruk, 2012; Ehmcke and Schlatt, 2006) are all residing in the basal compartment. Once type B spermatogonia transform to preleptotene spermatocytes in the basal compartment in both rodents and humans, these are the only germ cells that are being transported across the BTB, at stage VIII of the epithelial cycle in rodents vs. stage III in humans (Hess and de Franca, 2008; Parvinen, 1982; Amann, 2008; Chen et al., 2017a) while differentiating into leptotene spermatocytes. As such, zygotene and pachytene transform to diplotene spermatocytes, preparing for meiosis I/II in stage XIV vs. XII tubules in the rat and mouse testis (and stage VI tubules in humans), respectively, to form haploid spermatids (step 1). Step 1 spermatids in turn undergo extensive differentiation and transformation including 19 or 16 steps in rat or mouse testes (and 6 steps in humans), respectively, to become elongated spermatids via spermiogenesis. As such, fully developed spermatids (i.e., spermatozoa) line-up at the adluminal edge against the tubule lumen to prepare for their eventual release at spermiation at stage VIII of the epithelial cycle in both rat and mouse testes (and stage II in humans). Thus, for spermatids, all the cellular events pertinent to the post-meiotic development take place strictly in the adluminal compartment, behind the blood-testis barrier, suggesting that BTB may be crucial to support meiosis and post-meiotic spermiogenesis. In fact, studies in rodents have shown that the assembly of a functional BTB is crucial for the completion of meiosis I/II. For instance, treatment of neonatal rats with diethylstilbestrol (DES, a synthetic nonsteroidal estrogen) that delayed the establishment of a functional BTB by 4-weeks also delayed the first wave of spermiogenesis, such as meiosis I/II by 4-weeks, since pachytene spermatocytes failed to enter meiosis but underwent degeneration (Toyama et al., 2001), leading to meiotic arrest. On the other hand, exposure of rodents to acute doses of toxicants, such as cadmium or glycerol known to induce irreversible BTB damage, also induced infertility in which spermatocytes failed to enter meiosis (Hew et al., 1993; Wiebe et al., 2000; Setchell and Waites, 1970; Siu et al., 2009). Collectively, these findings illustrate the significance of the BTB to support spermatogenesis.

It is of interest to note that the testis is an immune-privileged organ (Kaur et al., 2013, 2014). Immune privilege is a concept referring to a specific site in the mammalian body, such as the testis, the brain, and the eye which are known immune privilege sites since they can protect grafted tissue (e.g., when skin is transplanted to one of these organs) from rejection by the host animals (Medawar, 1948; Brent, 2016). There are two different aspects of immune privilege, namely privileged sites and privileged tissues. A privileged site refers to a site in which foreign tissue grafted to that site can survive longer, or even indefinitely, than in a nonprivileged site. A privileged tissue is a tissue that resists rejection when grafted into a nonprivileged site. The testis is a unique organ because it is having both aspects of the immune privilege (Fijak and Meinhardt, 2006). Studies have shown that Sertoli cells in the testis are responsible to confer the testis as an immune privileged organ (Kaur et al., 2014), possibly through its secretory

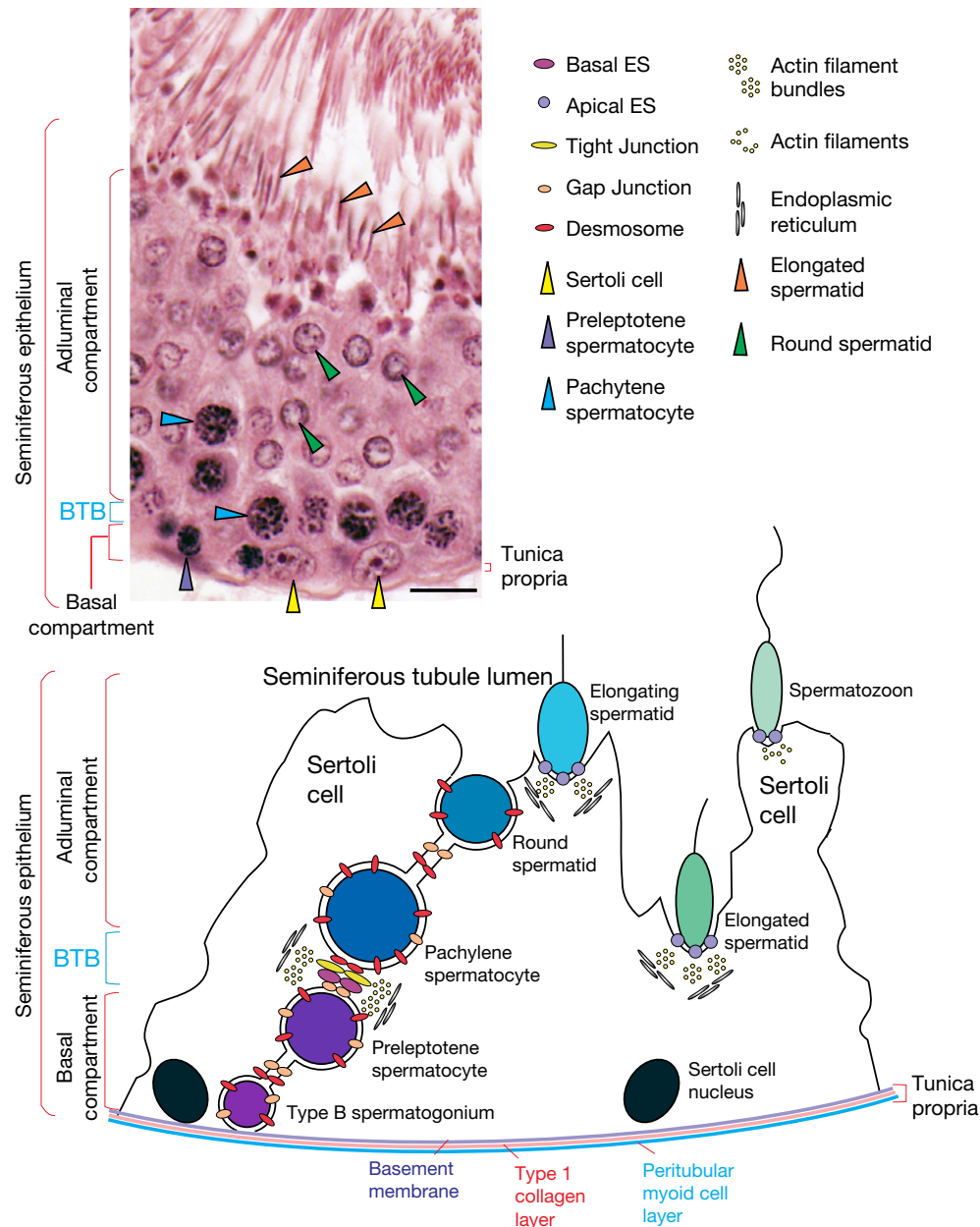


Fig. 1 A schematic drawing illustrates the relative location and morphological features of the blood-testis barrier (BTB) in relation to other cell junctions in the seminiferous epithelium of adult rat testes. The top panel is the cross-section of a typical adult rat testis, showing an early stage VIII seminiferous tubule, illustrating different germ cell types across the seminiferous tubule. The relative location of the BTB that segregates the seminiferous epithelium into the basal and the adluminal compartments is also noted. The bottom panel is a schematic drawing of the seminiferous epithelium. The BTB is constituted by both actin-based tight junction (TJ), basal ectoplasmic specialization (basal ES), and gap junction (GJ), as well as the intermediate filament-based desmosome. It also segregates the epithelium into the basal and the adluminal compartments. The most notable morphological feature of the BTB is the two arrays of actin filament bundles that are sandwiched in-between the apposing Sertoli cell plasma membranes and the cisternae of endoplasmic reticulum. At the interface of all other germ cell types (except step 8–19 spermatids) and Sertoli cells, they are supported by GJ and desmosome. However, there is only one anchoring junction type found between Sertoli cells and step 8–19 spermatids, namely the apical ES. The apical ES is morphologically similar to the basal ES except that only one array of actin filament bundles is noted since no unique morphological feature is contributed by the elongating/elongated spermatids when examined by electron microscopy. Scale bar, 60 μm.

biomolecules including both cytokines (e.g., interleukins, interferons) and immunosuppressive biomolecules whose identities remain to be elucidated. In fact, recent studies have shown that Sertoli cells are unique components of successful xenografts in treating different diseases such as diabetes that is prone to tissue rejection when pancreatic islet cells from one species are grafted to another species (Luca et al., 2015, 2016; Bistoni et al., 2012).

Herein, we summarize findings regarding the biology of the BTB on topics that have not recently been reviewed. Thus, many of the background information on BTB is not covered in details herein since these topics have been discussed in several recent reviews (Franca et al., 2012; Pelletier, 2011; Stanton, 2016; Kaur et al., 2014; Mruk and Cheng, 2015; Li et al., 2016). Readers are encouraged to seek the needed information from these reviews.

Blood-Testis Barrier (BTB)—Structural and Morphological Features

As noted above, the BTB is not conferred by endothelial cells of the microvessels in the interstitium, except that in rodents (but not primates), the peritubular myoid cell layer in the tunica propria located behind the basement membrane and the type I collagen layer contribute some of the barrier function. Instead, the BTB is created by adjacent Sertoli cell junctions near the base of the seminiferous epithelium as noted in Fig. 1. The major actin-based cell-cell anchoring junction type that is responsible for the BTB integrity, similar to other blood-tissue barriers, is the tight junction (TJ) between adjacent Sertoli cells which create a belt-like structure near the base of the seminiferous epithelium (Fig. 2). However, unlike TJs found in other blood-tissue barriers, the TJ at the Sertoli cell BTB coexists with another testis-specific actin-based adherens junction (AJ) called the basal ectoplasmic specialization (basal ES), as well as the gap junction (GJ), which collectively contribute to the belt-like structure that encircle the base of the seminiferous epithelium (Fig. 2). These junctions, namely TJ, basal ES and GJ together with the intermediate filament-based desmosome constitute the BTB (Fig. 2). As noted in Fig. 1, the basal ES is the most notable junction at the BTB that confers the unusual adhesive strength of the immunological barrier. The basal ES is typified by the presence of two arrays of actin filament bundles, one each on the cytoplasm of the two adjacent Sertoli cells that are sandwiched in-between the apposing Sertoli cell–cell plasma membranes and cisternae of the endoplasmic reticulum (Fig. 1). Interestingly, basal ES coexists with TJ, thereby the unique actin filament bundles confer strong adhesive function to the BTB, whereas the GJ provides the needed intercellular communications to fine-

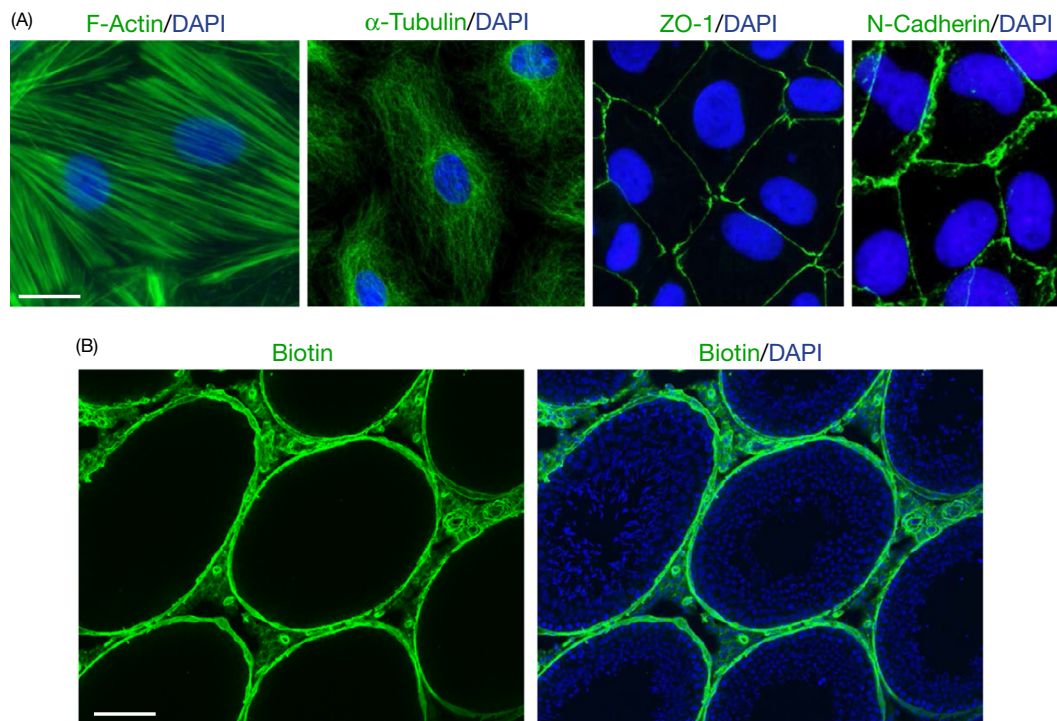


Fig. 2 Morphological features of the Sertoli cell BTB in vitro and in vivo. Sertoli cells are known to establish a functional TJ-permeability barrier when cultured in vitro which mimics the Sertoli cell BTB in vivo. In (A), Sertoli cells cultured in vitro for ~2–3 days established a functional TJ-barrier, which is supported by the F-actin (green fluorescence) and microtubules (MT; shown herein is the α -tubulin, green fluorescence, with together with the β -tubulin are the building blocks of MTs) that stretched across the Sertoli cell cytosol. These cytoskeletons thus support the establishment of the Sertoli cell TJ-barrier as noted by the precise localization of TJ adaptor protein ZO-1 (zonula occludens 1) and basal ES integral membrane protein N-cadherin at the Sertoli cell cortical zone, as these adhesion proteins utilize actin for attachment. Scale bar, 40 μ m, which applies to other micrographs in this panel. In (B), this is the typical result of an in vivo BTB integrity assay wherein functional BTB is located near the base of the each seminiferous tubule that blocks the diffusion of a membrane impermeable biotin (such as sulfo-NHS-LC-biotin from Thermo Fisher Scientific, green fluorescence when biotin is visualized with Alexa Fluor 488-streptavidin as described (Gao et al., 2016)) across the immunological barrier to enter the adluminal compartment. In short, biotin (green fluorescence) is restricted to the interstitial space and the basal compartment. Scale bar, 300 μ m.

tune various junction types at the BTB to maintain its homeostasis to support the barrier function throughout the epithelial cycle of spermatogenesis (Li et al., 2012, 2010). Altogether, these junctions create a unique blood-tissue barrier in the testis in which the BTB is capable of blocking the diffusion of even a small membrane impermeable molecular probe, sulfo-NHS-LC-biotin (Mr 556.59), from crossing the barrier to enter the adluminal compartment as noted in Fig. 2, which illustrates the tightness of this immunological barrier based on a typical in vivo BTB integrity assay.

Functions of the BTB

The BTB has multiple functions in the testis to support spermatogenesis. First, the BTB creates a unique microenvironment in the adluminal compartment of the testis to support meiosis and spermiogenesis by segregating serum proteins, electrolytes, mineral salts, water, and virtually all other somatic cells including immune cells from entering the space behind the barrier (Figs. 1–2). Second, the BTB also determines the fluid composition and microenvironment of the seminiferous tubule since studies have shown that the levels of proteins, electrolytes, hormones (e.g., testosterone), mineral salts, cytokines and other biomolecules found in the seminiferous tubule fluid or in the rete testis compartment are considerably lower from the testicular lymph and serum (Setchell and Waites, 1975; Turner et al., 1984; Setchell et al., 1978; Setchell, 1980). This function of the BTB is achieved through its role in regulating the paracellular (between adjacent Sertoli cells) and transcellular (through intracellular transport pathway, such as receptor or endocytic vesicle-mediated transport) transport of substances across the barrier. Due to the presence of the actin microfilament bundles of the basal ES which coexists with the TJ, virtually no substances including water, electrolytes, nutrients (e.g., glucose), hormones and biomolecules can penetrate the BTB unless specific receptors are present on the surface of Sertoli cells (e.g., FSH receptors) (Setchell, 2008; Bardin et al., 1988; Griswold, 1998). However, the BTB is not entirely impenetrable since it allows the passage of certain (including proteins and biomolecules) but not all molecules (e.g., proteins), illustrating its selectivity function (Cheng and Mruk, 2012; Tung et al., 2017). For instance, a recent report has shown that some meiotic germ cell antigens (MGCA) expressed on sperm are being sequestered behind the BTB, however, some non-sequestered MCGA are transported outside the BTB as cargoes and discarded (Tung et al., 2017). However, the mechanism(s) that regulate this type of selectivity remains unknown. For instance, the biomolecule(s) and the molecular mechanism(s) that trigger and determine this selectivity remain to be elucidated.

Regulation of the BTB—An Emerging Concept of Regulation

The topic on the regulation of BTB function is the subject of several recent reviews which include the involvement of mTOR (mammalian target of rapamycin) (Li and Cheng, 2016; Mok et al., 2013), FAK (focal adhesion kinase) (Li et al., 2013; Cheng and Mruk, 2009; Wan et al., 2014), Src family kinases (e.g., c-Src, c-Yes) (Li et al., 2016; Xiao et al., 2012; Chojnacka and Mruk, 2015), ICAMs (intercellular adhesion molecules, including ICAM-1 and ICAM-2) (Xiao et al., 2013; Mruk, 2016), cytokines (Lie et al., 2012; Lui and Cheng, 2007; Xia et al., 2005), MAPKs (Li et al., 2009) and cytoskeletons (Tang et al., 2016; Lie et al., 2010, 2011). Thus, these topics are not covered herein to avoid redundancy. Instead, we summarize findings based on a series of recent reports, depicting an emerging concept regarding the presence of an autocrine-based local regulatory axis that modulates BTB function.

F5-Peptide—A Peptide Derived From Laminin- γ 3 Chains at the Apical ES That Regulates BTB Dynamics

During stage VIII of the epithelial cycle, fully developed spermatids transformed to spermatozoa begin to line-up in the adluminal compartment against the tubule lumen to prepare for their release at spermiation in late stage VIII tubules. Laminin- γ 3 chain, one of the constituent proteins of the apical ES that forms a bona fide adhesion protein complex (Yan and Cheng, 2006) with α 6 β 1-integrin (Palombi et al., 1992; Salanova et al., 1995), is cleaved at its domain IV, likely through the action of MMP2 (metalloprotease 2) which was shown to be highly expressed in stage VIII tubules (Siu and Cheng, 2004a), to release a biologically active peptide called F5-peptide (Yan et al., 2008; Su et al., 2012). Studies have shown that this 50-amino acid peptide is capable of inducing remodeling of the Sertoli cell BTB both in vitro (Yan et al., 2008) and in vivo (Su et al., 2012; Gao et al., 2016). More important, the disruptive effects of the F5-peptide on BTB integrity is highly reversible (Su et al., 2012; Gao et al., 2016), possibly because this BTB regulator is produced endogenously to support spermatogenesis. Furthermore, F5-peptide is also able to potentiate further breakdown of apical ES, thereby facilitating spermiation (Gao et al., 2016). In short, these findings also illustrate its potential of serving as a naturally occurring reversible male contraceptive peptide if it can be delivered to the testis at an appropriate dose. Studies have shown that laminin- γ 3 chain is not an integrated membrane protein since it is lacking a transmembrane domain (Yan and Cheng, 2006; Koch et al., 1999). Thus, it likely adheres onto the spermatid cell surface to form a bona fide adhesion complex with the Sertoli cell specifically expressed α 6 β 1-integrin (Palombi et al., 1992; Salanova et al., 1995). When domain IV of the laminin- γ 3 chain is cleaved by MMP2, the F5-peptide either needs to be transported inside the Sertoli cell if it is internalized to exert its biological action via an “inside-out” signaling cascade or it exerts its effects through an “outside-in” signaling cascade (Durrant et al., 2017; Patsoukis et al., 2017). Indeed, F5-peptide was found to utilize the peptide

transporter 1 (also known as solute carrier family 15 member 1, SLC15A1) expressed in Sertoli cells to enter Sertoli cells to exert its effects (Su et al., 2015). On the other hand, signaling studies have shown that F5-peptide exerts its biological effects through either “inside-out” or “outside-in” signaling (Su et al., 2012), illustrating the testis is equipped with a highly sophisticated signaling network to regulate BTB dynamics. Presently, the receptor that works in concert with F5-peptide in the testis remains unknown, however, studies have shown that laminins and/or fragments of laminin chains usually serve as the ligand to activate integrin-based signaling pathway (Pozzi et al., 2017; Combes et al., 2015; Yan et al., 2007). It is likely that an integrin-based receptor expressed at the basal ES/BTB to serve as the putative receptor for the F5-peptide. Recent studies have shown that F5-peptide exerts its disruptive effects through changes in actin- or microtubule (MT)-binding and regulatory proteins, which in turn perturbs the organization of actin- and MT-based cytoskeletons (Gao et al., 2016). For instance, following overexpression of F5-peptide in the testis in vivo using a mammalian expression vector pCI-neo vs. empty vector alone (to serve as a control) for transfection with a transfection efficiency of ~60%, F-actin organization across the entire seminiferous epithelium in the treatment group was considerably disrupted (Su et al., 2012; Gao et al., 2016). These disruptive changes include: (i) the prominent track-like structures surrounding elongating spermatids that are being transported across the epithelium in stage V tubules were grossly disrupted, appearing as truncated actin filaments; (ii) F-actin no longer localized robustly at the apical ES, appearing as bulb-like structures at the concave side of spermatid heads found in control testes, instead, F-actin was diffusely localized; (iii) the F-actin at the basal ES/BTB was no longer tightly localized at the site to support BTB function, thereby perturbing the BTB integrity as noted in an in vivo BTB integrity assay (Gao et al., 2016). More important these changes were the result of disruptive spatiotemporal expression of the actin barbed end capping and bundling protein Eps8 and also the branched actin polymerization-inducing protein Arp3. As such, the F-actin network at the ES favored the configuration of a branched/unbundled organization (Gao et al., 2016), no longer supporting apical and basal ES function. Furthermore, the MT-conferred tracks that were robustly expressed across the seminiferous epithelium and were aligned almost perpendicular to the basement membrane noted in control testes were also found to be grossly disrupted following overexpression of F5-peptide in the testis (Gao et al., 2016). They were either truncated and/or mis-aligned, by laying parallel to the basement membrane, thereby failing to support the transport of spermatids and also organelles (e.g., endosomes, phagosomes, residual bodies) across the epithelium. As such, groups of elongated spermatids were trapped deep inside the epithelium in late stage VIII, IX, and even X or XI tubules (Gao et al., 2016). The significance of these findings is that there is a local autocrine-based functional axis that links the apical ES and the basal ES/BTB across the seminiferous epithelium, known as the apical ES-basal ES/BTB axis. In short, F5-peptide released at the apical ES site at spermiation during stage VIII of the epithelial cycle can potentiate apical ES degeneration but also induces basal ES/BTB remodeling to support the transport of preleptotene spermatocytes across the immunological barrier. As such, the two cellular events that take place at the opposite end of the seminiferous epithelium, namely spermiation and BTB remodeling, are coordinated in stage VIII tubules.

NC1 (Non-Collagenous 1) Domain Peptide—A Peptide Derived From the Basement Membrane Collagen α 3(IV) Chain That Regulates BTB Dynamics

Earlier studies have shown that using antibodies prepared against the collagen chains found in the basement membrane were capable of perturbing the Sertoli cell TJ-permeability barrier function (Siu et al., 2003), illustrating there is a potential functional axis between the basement membrane and the BTB to modulate BTB function to support spermatogenesis. Subsequent studies have shown that the C-terminal noncollagenous (NC1) domain of ~230 amino acid residues derived from collagen α 3 (IV) chain (i.e., type IV collagen α 3 chain, a major constituent component of the basement membrane) when cleaved by the action of MMP9 in the basement membrane (Siu and Cheng, 2004b), is capable of inducing Sertoli cell TJ-permeability barrier function (Wong and Cheng, 2013). Similar observations are obtained either by using purified recombinant NC1 domain protein (Wong and Cheng, 2013) or through the use of a cDNA clone encoding NC1 domain for its overexpression using the pCI-neo mammalian expression vector (Chen et al., 2017b). It was also noted that the NC1 domain generated at the basement membrane was transported across the seminiferous epithelium to reach the apical ES site using an MT-dependent transport mechanism (Chen et al., 2017b). This conclusion is reached based on the finding that the use of Taxol (a drug that specifically stabilizes MTs by preventing MT depolymerization, thereby disrupting MT dynamics) was found to block the transport of NC1 domain across the seminiferous epithelium by perturbing MT organization, and Taxol had no apparent effect on F-actin organization in the epithelium (Chen et al., 2017b). More important, NC1 domain is capable of inducing BTB integrity in the testis in vivo through disruptive changes in the organization of F-actin and also MTs across the epithelium (Chen et al., 2017b). Besides its effects at the BTB, NC1 domain was also found to induce apical ES degeneration by perturbing the spatiotemporal expression of actin regulatory proteins such as Arp3 and Eps8, thereby perturbing F-actin organization (Chen et al., 2017b). Since adhesion protein complexes, such as laminin-integrin and nectin-afadin, are actin-based apical ES proteins, a disruption of F-actin thus perturbs their localization, causing apical ES degeneration that leads to spermatid exfoliation from the epithelium, mimicking spermiation. Taking these data collectively, NC1 domain is another autocrine-based peptide generated at the basement membrane during the epithelial cycle to modulate BTB remodeling to support spermatogenesis. These findings also confirm the notion that there is a local apical ES-BTB-basement membrane functional axis in the testis to support spermatogenesis through its effects on the BTB. In this context, it is of interest to note that both F5-peptide and NC1 domain peptide exert their effects similarly by promoting BTB remodeling, causing the Sertoli cell TJ-barrier disruption.

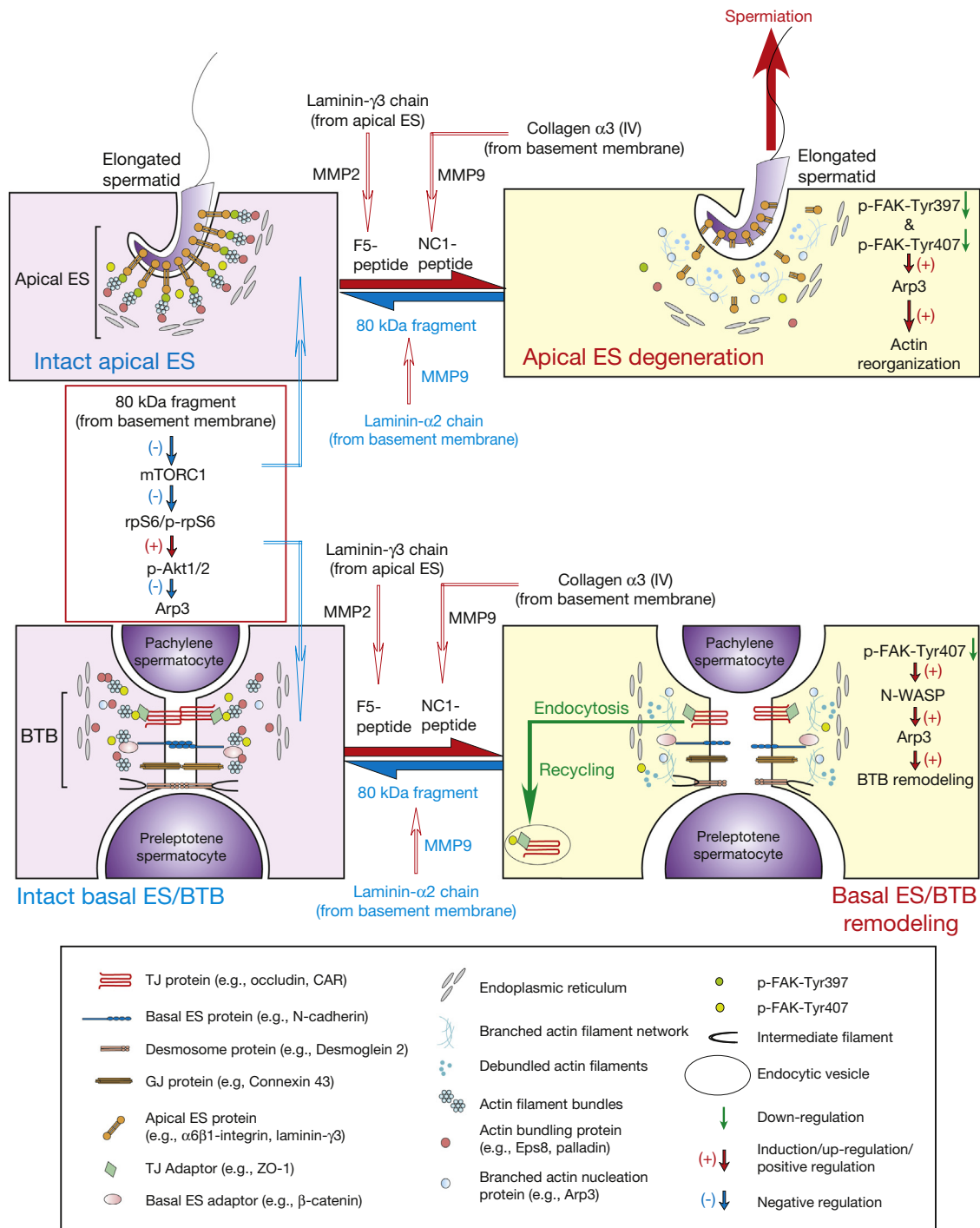


Fig. 3 A schematic drawing illustrates the regulatory biomolecules in the apical ES-BTB/basal ES-basement membrane functional axis in the adult testis that modulate the integrity of the basal ES/BTB and apical ES. As detailed in text, biologically active F5-peptide released from the laminin-γ3 chain at the apical ES (at spermiation, via the action of MMP2) and also NC1 domain peptide from the collagen α3(IV) chain in the basement membrane (via the action of MMP9) have been shown to be crucial regulators of ES function. First, these peptides induce further breakdown of the apical ES to facilitate the release of sperm at spermiation. Second, these peptides are also capable of inducing basal ES/BTB remodeling to facilitate the transport of preleptotene spermatocytes across the immunological barrier. Since these events take place concurrently across the seminiferous epithelium at stage VIII of the epithelial cycle, they thus serve as important regulators to coordinate these cellular events during spermatogenesis. Furthermore, an 80 kDa biologically active generated at the basement membrane (via the action of MMP9) also modulates the basal ES/BTB function (and possibly the apical ES function) by promoting the integrity of the ES. In short, the action of the F5-peptide or NC1 domain is in sharp contrast to the 80 kDa fragment, making these biomolecules as molecular switches to promote ES restructuring and integrity (or stability), respectively.

80 kDa Fragment—A Peptide Derived From the Basement Membrane Laminin $\alpha 2$ Chain That Regulates BTB Dynamics by Promoting BTB Integrity

Since both F5-peptide and NC1 domain peptide are endogenously generated biologically active peptides to induce BTB remodeling which are also capable of potentiating apical ES degeneration to support spermiation, we seek to investigate if there is another functional loop that supports BTB function and also apical ES integrity instead of causing their disruption. As anticipated, it was shown that laminin- $\alpha 2$ chain, also a constituent component of the basement membrane, likely to be its 80 kDa fragment residing at its C-terminal region, was involved in maintaining BTB integrity (Gao et al., 2017a, b) (Fig. 3). This conclusion is reached based on the findings that a knockdown of laminin- $\alpha 2$ chain by RNAi using shRNA was capable of inducing Sertoli cell TJ-permeability barrier disruption (Gao et al., 2017b), illustrating its physiological function under normal conditions was to maintain BTB integrity. This 80 kDa fragment of laminin- $\alpha 2$ chain derived from the basement membrane appears to be transported to the apical ES site via a MT-dependent mechanism (Gao et al., 2017b). More important, the disruptive effects of the laminin- $\alpha 2$ shRNA on the BTB function were shown to be mediated through the mTORC1-p-rpS6 signaling pathway involving p-Akt1/2 by up-regulating p-rpS6 expression with a concomitant down-regulation of p-Akt1/2 expression (Gao et al., 2017a) (Fig. 3). Earlier studies have shown that the mTORC1-p-rpS6-Akt1/2 signaling pathway modulates BTB dynamics by disrupting BTB integrity (Mok et al., 2012, 2014) through disruptive changes in the organization of F-actin in Sertoli cells, which is mediated by a surge and a reduction in p-rpS6 and p-Akt1/2 expression, respectively (Mok et al., 2012, 2015). Collectively, these findings illustrate the presence of another feedback loop at the basement membrane-BTB axis in which collagen- $\alpha 2$ -derived 80 kDa fragment modulates BTB dynamics by making the Sertoli cell barrier “tighter” vs. the disruptive effects of the F5-peptide and the NC1-domain peptide which make the Sertoli cell barrier “leaky”. Thus, the combined effects of this 80 kDa laminin- $\alpha 2$ chain fragment vs. the F5-peptide and the NC1 domain peptide provide an efficient system to support BTB dynamics, such as to facilitate the transport of preleptotene spermatocytes across the immunological barrier at stage VIII of the epithelial cycle as depicted in the hypothetical model shown in Fig. 3.

Concluding Remarks and Future Perspectives

As briefly reviewed herein, it is likely that through these contrasting effects of different autocrine-based peptides at the apical ES-BTB/basal ES-basement membrane axis, the homeostasis of the apical ES and the basal ES/BTB can be precisely regulated at different stages of the epithelial cycle to support spermatogenesis, even though these ultrastructures are located at the opposite ends of the seminiferous epithelium. Since the two ultrastructures are linked underneath by both the F-actin and MT networks, their disassembly and reassembly can be easily coordinated through changes in the corresponding F-actin (e.g., Arp3, formin 1, Eps8, palladin) and MT (e.g., EB1, MARK4) binding and regulatory proteins. Fig. 3 thus summarizes results of these findings, depicting the role of the F5-peptide derived from laminin- $\gamma 3$ chain at the apical ES, and NC1 domain peptide derived from collagen $\alpha 3$ (IV) chain and 80 kDa fragment derived from laminin- $\alpha 2$ chain at the basement membrane on apical ES and basal ES/BTB integrity and function. However, some crucial questions have yet to be addressed. First, what is the upstream signaling molecule(s) that determines the activation of which functional axis, namely apical ES-basal ES/BTB, basal ES/BTB-basement membrane vs. apical ES/basement membrane axis, should be activated? Is that a cytokine or a defined stage of germ cells? How are these functional axis link to the known regulators of BTB dynamics, such as FAK, mTORC1, mTORC2, and SFKs (Src family kinases). Nonetheless, the hypothetical model shown in Fig. 3 provides many of the information necessary to design functional experiments in future studies.

Acknowledgment

This work was supported by grants from the National Institutes of Health (NICHD R01 HD056034 to C.Y.C.; U54 HD029990 Project 5 to C.Y.C.).

References

- Amann, R. P. (2008). The cycle of the seminiferous epithelium in humans: A need to revisit? *Journal of Andrology*, 29, 469–487.
- Bardin, C. W., Cheng, C. Y., Musto, N. A., & Gunsalus, G. L. (1988). The Sertoli cell. In E. Knobil, J. D. Neill, L. L. Ewing, G. S. Greenwald, C. L. Markert, & D. W. Pfaff (Eds.), *The physiology of reproduction* (pp. 933–974). New York: Raven Press.
- Bistoni, G., Calvitti, M., Mancuso, F., Arato, I., Falabella, G., Cucchia, R., et al. (2012). Prolongation of skin allograft survival in rats by the transplantation of microencapsulated xenogeneic neonatal porcine Sertoli cells. *Biomaterials*, 33, 5333–5340.
- Brent, L. (2016). Transplantation tolerance—A historical introduction. *Immunology*, 147, 267–268.
- Chen, H., Mruk, D. D., Xiao, X., & Cheng, C. Y. (2017a). Human spermatogenesis and its regulation. In S. J. Winters, & I. T. Huhtaniemi (Eds.), *Male hypogonadism, contemporary endocrinology* (pp. 49–72). New York: Springer International Publishing AG. https://doi.org/10.1007/978-3-319-53298-1_3.
- Chen, H., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2017b). Regulation of spermatogenesis by a local functional axis in the testis: Role of the basement membrane-derived noncollagenous 1 domain peptide. *The FASEB Journal*, 31, 3587–3607.
- Cheng, C. Y. (2012). *Biology and regulation of blood-tissue barriers* (pp. 1–361). Landes Bioscience/Springer Science+Business Media, LLC.
- Cheng, C. Y., & Mruk, D. D. (2009). Regulation of blood-testis barrier dynamics by focal adhesion kinase (FAK). An unexpected turn of events. *Cell Cycle*, 8, 3493–3499.
- Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implication in male contraception. *Pharmacological Reviews*, 64, 16–64.
- Chiquoine, D. (1964). Observations on the early events of cadmium necrosis of the testis. *The Anatomical Record*, 149, 23–36.

- Chojnacka, K., & Mruk, D. D. (2015). The Src non-receptor tyrosine kinase paradigm: New insights into mammalian Sertoli cell biology. *Molecular and Cellular Endocrinology*, 415, 133–142.
- Combes, A. N., Davies, J. A., & Little, M. H. (2015). Cell-cell interactions driving kidney morphogenesis. *Current Topics in Developmental Biology*, 112, 467–508.
- Durrant, T. N., van den Bosch, M. T., & Hers, I. (2017). Integrin $\alpha_5\beta_3$ outside-in signaling. *Blood*. <https://doi.org/10.1182/blood-2017-03-773614>.
- Dym, M. (1973). The fine structure of the monkey (*Macaca*) Sertoli cell and its role in maintaining the blood-testis barrier. *The Anatomical Record*, 175, 639–656.
- Dym, M., & Fawcett, D. W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biology of Reproduction*, 3, 308–326.
- Ehmcke, J., & Schlatt, S. A. (2006). Revised model for spermatogonial expansion in man: Lessons from non-human primates. *Reproduction*, 132, 673–680.
- Ehmcke, J., Wistuba, J., & Schlatt, S. (2006). Spermatogonial stem cells: Questions, models and perspectives. *Human Reproduction Update*, 12, 275–282.
- Fawcett, D. W., Leak, L. V., & Heidger, P. M. (1970). Electron microscopic observations on the structural components of the blood-testis barrier. *Journal of Reproduction and Fertility*, (Suppl. 10), 105–122.
- Fijak, M., & Meinhardt, A. (2006). The testis in immune privilege. *Immunological Reviews*, 213, 66–81.
- Franca, L. R., Auharek, S. A., Hess, R. A., Dufour, J. M., & Hinton, B. T. (2012). Blood-tissue barriers: Morphofunctional and immunological aspects of the blood-testis and blood-epididymal barriers. *Advances in Experimental Medicine and Biology*, 763, 237–259.
- Gao, Y., Mruk, D. D., Lui, W. Y., Lee, W. M., & Cheng, C. Y. (2016). F5-peptide induces aspermatogenesis by disrupting organization of actin- and microtubule-based cytoskeletons in the testis. *Oncotarget*, 7, 64203–64220.
- Gao, Y., Chen, H., Lui, W. Y., Lee, W. M., & Cheng, C. Y. (2017a). Basement membrane laminin $\alpha 2$ regulation of BTB dynamics via its effects on F-actin and microtubule (MT) cytoskeletons is mediated through mTORC1 signaling. *Endocrinology*, 158, 963–978.
- Gao, Y., Mruk, D., Chen, H., Lui, W. Y., Lee, W. M., & Cheng, C. Y. (2017b). Regulation of the blood-testis barrier by a local axis in the testis: Role of laminin $\alpha 2$ in the basement membrane. *The FASEB Journal*, 31, 584–597.
- Griswold, M. (1998). The central role of Sertoli cells in spermatogenesis. *Seminars in Cell & Developmental Biology*, 9, 411–416.
- Hess, R. A., & de Franca, L. R. (2008). Spermatogenesis and cycle of the seminiferous epithelium. *Advances in Experimental Medicine and Biology*, 636, 1–15.
- Hew, K. W., Heath, G. L., Jiwa, A. H., & Welsh, M. J. (1993). Cadmium *in vivo* causes disruption of tight junction-associated microfilaments in rat Sertoli cells. *Biology of Reproduction*, 49, 840–849.
- Kaur, G., Mital, P., & Dufour, J. M. (2013). Testis immune privilege—Assumptions versus facts. *Animal Reproduction*, 10, 3–15.
- Kaur, G., Thompson, L. A., & Dufour, J. M. (2014). Sertoli cells—Immunological sentinels of spermatogenesis. *Seminars in Cell & Developmental Biology*, 30, 36–44.
- Koch, M., Olson, P. F., Albus, A., Jin, W., Hunter, D. D., Brunken, W. J., et al. (1999). Characterization and expression of the laminin $\gamma 3$ chain: A novel, non-basement membrane-associated, laminin chain. *The Journal of Cell Biology*, 145, 605–618.
- Li, N., & Cheng, C. Y. (2016). Mammalian target of rapamycin complex (mTOR) pathway modulates blood-testis barrier (BTB) function through F-actin organization and gap junction. *Histology and Histopathology*, 31, 961–968.
- Li, M. W. M., Mruk, D. D., & Cheng, C. Y. (2009). Mitogen-activated protein kinases in male reproductive function. *Trends in Molecular Medicine*, 15, 159–168.
- Li, M. W. M., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2010). Connexin 43 is critical to maintain the homeostasis of blood-testis barrier via its effects on tight junction reassembly. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 17998–18003.
- Li, M. W. M., Mruk, D. D., & Cheng, C. Y. (2012). Gap junctions and blood-tissue barriers. *Advances in Experimental Medicine and Biology*, 763, 260–280.
- Li, S. Y., Mruk, D. D., & Cheng, C. Y. (2013). Focal adhesion kinase is a regulator of F-actin dynamics: New insights from studies in the testis. *Spermatogenesis*, 3, e25385.
- Li, N., Tang, E. I., & Cheng, C. Y. (2016). Regulation of blood-testis barrier by actin binding proteins and protein kinases. *Reproduction*, 151, R29–41.
- Lie, P. P. Y., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2010). Cytoskeletal dynamics and spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 365, 1581–1592.
- Lie, P. P. Y., Cheng, C. Y., & Mruk, D. D. (2011). The biology of the desmosome-like junction: A versatile anchoring junction and signal transducer in the seminiferous epithelium. *International Review of Cell and Molecular Biology*, 286, 223–269.
- Lie, P. P. Y., Cheng, C. Y., & Mruk, D. D. (2012). The biology of interleukin-1: Emerging concepts in the regulation of the actin cytoskeleton and cell junction dynamics. *Cellular and Molecular Life Sciences*, 69, 487–500.
- Luca, G., Mancuso, F., Calvitti, M., Arato, I., Falabella, G., Bufalari, A., et al. (2015). Long-term stability, functional competence, and safety of microencapsulated specific pathogen-free neonatal porcine Sertoli cells: A potential product for cell transplant therapy. *Xenotransplantation*, 22, 273–283.
- Luca, G., Arato, I., Mancuso, F., Calvitti, M., Falabella, G., Murolo, G., et al. (2016). Xenograft of microencapsulated Sertoli cells restores glucose homeostasis in db/db mice with spontaneous diabetes mellitus. *Xenotransplantation*, 23, 429–439.
- Lui, W. Y., & Cheng, C. Y. (2007). Regulation of cell junction dynamics by cytokines in the testis—A molecular and biochemical perspective. *Cytokine & Growth Factor Reviews*, 18, 299–311.
- Medawar, P. B. (1948). Immunity to homologous grafted skin; the fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *British Journal of Experimental Pathology*, 29, 58–69.
- Mital, P., Hinton, B. T., & Dufour, J. M. (2011). The blood-testis and blood-epididymis barriers are more than just their tight junctions. *Biology of Reproduction*, 84, 851–858.
- Mok, K. W., Mruk, D. D., Silvestrini, B., & Cheng, C. Y. (2012). pS6 regulates blood-testis barrier dynamics by affecting F-actin organization and protein recruitment. *Endocrinology*, 153, 5036–5048.
- Mok, K. W., Mruk, D. D., & Cheng, C. Y. (2013). Regulation of blood-testis barrier (BTB) dynamics during spermatogenesis via the “Yin” and “Yang” effects of mammalian target of rapamycin complex 1 (mTORC1) and mTORC2. *International Review of Cell and Molecular Biology*, 301, 291–358.
- Mok, K. W., Mruk, D. D., & Cheng, C. Y. (2014). pS6 regulates blood-testis barrier dynamics through Akt-mediated effects on MMP-9. *Journal of Cell Science*, 127, 4870–4882.
- Mok, K. W., Chen, H., Lee, W. M., & Cheng, C. Y. (2015). pS6 regulates blood-testis barrier dynamics through Arp3-mediated actin microfilament organization in rat Sertoli cells. An *in vitro* study. *Endocrinology*, 156, 1900–1913.
- Mruk, D. D. (2016). Emergent roles for intercellular adhesion molecule-1 in the restructuring of the blood-testis barrier during spermatogenesis in the mammal. *Histology and Histopathology*, 31, 159–166.
- Mruk, D. D., & Cheng, C. Y. (2015). The mammalian blood-testis barrier: Its biology and regulation. *Endocrine Reviews*, 36, 564–591.
- Palombi, F., Salanova, M., Tarone, G., Farini, D., & Stefanini, M. (1992). Distribution of $\beta 1$ integrin subunit in rat seminiferous epithelium. *Biology of Reproduction*, 47, 1173–1182.
- Parizek, J. (1960). Sterilization of the male by cadmium salts. *Journal of Reproduction and Fertility*, 1, 294–309.
- Parizek, J., & Zahor, Z. (1956). Effect of cadmium salts on testicular tissue. *Nature*, 177, 1036–1037.
- Parvonen, M. (1982). Regulation of the seminiferous epithelium. *Endocrine Reviews*, 3, 404–417.
- Patsoukis, N., Bardhan, K., Weaver, J. D., Sari, D., Torres-Gomez, A., Li, L., et al. (2017). The adaptor molecule RIAM integrates signaling events critical for integrin-mediated control of immune function and cancer progression. *Science Signaling*, 10. <https://doi.org/10.1126/scisignal.aam8298>.
- Pelletier, R. M. (2011). The blood-testis barrier: The junctional permeability, the proteins and the lipids. *Progress in Histochemistry and Cytochemistry*, 46, 49–127.
- Pozzi, A., Yurchenko, P. D., & Iozzo, R. V. (2017). The nature and biology of basement membranes. *Matrix Biology*, 57–58, 1–11.
- de Rooij, D. G. (2009). The spermatogonial stem cell niche. *Microscopy Research and Technique*, 72, 580–585.
- Salanova, M., Stefanini, M., De Curtis, I., & Palombi, F. (1995). Integrin receptor $\alpha 6\beta 1$ is localized at specific sites of cell-to-cell contact in rat seminiferous epithelium. *Biology of Reproduction*, 52, 79–87.
- Setchell, B. (1980). The functional significance of the blood-testis barrier. *Journal of Andrology*, 1, 3–10.
- Setchell, B. P. (2008). Blood-testis barrier, functional and transport proteins and spermatogenesis. *Advances in Experimental Medicine and Biology*, 636, 212–233.

- Setchell, B. P., & Breed, W. G. (2006). Anatomy, vasculature and innervation of the male reproductive tract. In J. D. Neill (Ed.), *Physiology of reproduction* (3rd edn., pp. 771–825). Elsevier: Amsterdam.
- Setchell, B. P., & Waites, G. M. H. (1970). Changes in the permeability of the testicular capillaries and of the “blood-testis barrier” after injection of cadmium chloride in the rat. *The Journal of Endocrinology*, 47, 81–86.
- Setchell, B. P., & Waites, G. M. B. (1975). The blood-testis barrier. In D. W. Hamilton, & R. O. Greep (Eds.), *Male reproductive system: Vol V. The handbook of physiology section 7* (pp. 143–172). Washington, D.C.: American Physiological Society.
- Setchell, B., Davies, R., Gladwell, R., Hinton, B., Main, S., Pilsworth, L., et al. (1978). The movement of fluid in the seminiferous tubules and rete testis. *Annales de Biologie Animale, Biochimie, Biophysique*, 18, 623–632.
- Siu, M. K. Y., & Cheng, C. Y. (2004a). Interactions of proteases, protease inhibitors, and the $\beta 1$ integrin/laminin $\gamma 3$ protein complex in the regulation of ectoplasmic specialization dynamics in the rat testis. *Biology of Reproduction*, 70, 945–964.
- Siu, M. K. Y., & Cheng, C. Y. (2004b). Dynamic cross-talk between cells and the extracellular matrix in the testis. *BioEssays*, 26, 978–992.
- Siu, M. K. Y., Lee, W. M., & Cheng, C. Y. (2003). The interplay of collagen IV, tumor necrosis factor- α , gelatinase B (matrix metalloprotease-9), and tissue inhibitor of metalloprotease-1 in the basal lamina regulates Sertoli cell-tight junction dynamics in the rat testis. *Endocrinology*, 144, 371–387.
- Siu, E. R., Mruk, D. D., Porto, C. S., & Cheng, C. Y. (2009). Cadmium-induced testicular injury. *Toxicology and Applied Pharmacology*, 238, 240–249.
- Stanton, P. G. (2016). Regulation of the blood-testis barrier. *Seminars in Cell & Developmental Biology*, 59, 166–173.
- Su, L., Mruk, D. D., Lie, P. P. Y., Silvestrini, B., & Cheng, C. Y. (2012). A peptide derived from laminin- $\gamma 3$ reversibly impairs spermatogenesis in rats. *Nature Communications*, 3, 1185. <https://doi.org/10.038/ncomms2171>.
- Su, L., Zhang, Y., Cheng, C. Y., Lee, W. M., Ye, K., & Hu, D. (2015). Slc15a1 is involved in the transport of synthetic F5-peptide into the seminiferous epithelium in adult rat testes. *Scientific Reports*, 5, 16271.
- Tang, E. I., Mruk, D. D., & Cheng, C. Y. (2016). Regulation of microtubule (MT)-based cytoskeleton in the seminiferous epithelium during spermatogenesis. *Seminars in Cell & Developmental Biology*, 59, 35–45.
- Toyama, Y., Ohkawa, M., Oku, R., Maekawa, M., & Yuasa, S. (2001). Neonatally administered diethylstilbestrol retards the development of the blood-testis barrier in the rat. *Journal of Andrology*, 22, 413–423.
- Tung, K. S., Harakal, J., Qiao, H., Rival, C., Li, J. C., Paul, A. G., et al. (2017). Egress of sperm autoantigen from seminiferous tubules maintains systemic tolerance. *The Journal of Clinical Investigation*, 127, 1046–1060.
- Turner, T. T., Jones, C. C., Howards, S. S., Ewing, L. L., Zegeye, B., & Gunsalus, G. L. (1984). On the androgen microenvironment of maturing spermatozoa. *Endocrinology*, 115, 1925–1932.
- Wan, H. T., Mruk, D. D., Tang, E. I., Xiao, X., Cheng, Y. H., Wong, E. W., et al. (2014). Role of non-receptor protein tyrosine kinases in spermatid transport during spermatogenesis. *Seminars in Cell & Developmental Biology*, 30, 65–74.
- Wiebe, J., Kowalik, A., Gallardi, R., Egeler, O., & Clubb, B. (2000). Glycerol disrupts tight junction-associated actin microfilaments, occludin, and microtubules in Sertoli cells. *Journal of Andrology*, 21, 625–635.
- Wong, E. W. P., & Cheng, C. Y. (2013). NC1 domain of collagen $\alpha 3(\text{IV})$ derived from the basement membrane regulates Sertoli cell blood-testis barrier dynamics. *Spermatogenesis*, 3, e25465. PMID:23885308; PMCID:PMC3710226 <https://doi.org/10.4161/spmg.25465>.
- Xia, W., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2005). Cytokines and junction restructuring during spermatogenesis—A lesson to learn from the testis. *Cytokine & Growth Factor Reviews*, 16, 469–493.
- Xiao, X., Mruk, D. D., Cheng, F. L., & Cheng, C. Y. (2012). c-Src and c-Yes are two unlikely partners of spermatogenesis and their roles in blood-testis barrier dynamics. *Advances in Experimental Medicine and Biology*, 763, 295–317.
- Xiao, X., Mruk, D. D., & Cheng, C. Y. (2013). Intercellular adhesion molecules (ICAMs) and spermatogenesis. *Human Reproduction Update*, 19, 167–186.
- Yan, H. H. N., & Cheng, C. Y. (2006). Laminin $\alpha 3$ forms a complex with $\beta 3$ and $\gamma 3$ chains that serves as the ligand for $\alpha 6\beta 1$ -integrin at the apical ectoplasmic specialization in adult rat testes. *The Journal of Biological Chemistry*, 281, 17286–17303.
- Yan, H. H. N., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2007). Ectoplasmic specialization: A friend or a foe of spermatogenesis? *BioEssays*, 29, 36–48.
- Yan, H. H. N., Mruk, D. D., Wong, E. W. P., Lee, W. M., & Cheng, C. Y. (2008). An autocrine axis in the testis that coordinates spermiogenesis and blood-testis barrier restructuring during spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 8950–8955.

Castration

Christine Aurich, Vetmeduni Vienna, Vienna, Austria

© 2018 Elsevier Inc. All rights reserved.

Methods for Castration

Castration includes any action by which an individual loses the use of his testes. This can be achieved either by surgical removal of the testes or by in situ destruction of testicular function. Methods comprise surgical, chemical, immunological, and endocrine approaches. Castration usually causes final loss of testicular functions with regard to both, the production of spermatozoa and testicular hormones. However, depending on the aim of castration, occasionally only one or the other effect may be desired. Sometimes, reversible castration might be required because of possible future reproduction of the respective male. In addition to surgical removal of the testes and epididymis, a variety of approaches for castration have been developed with the aim to offer cheaper, safer, or more diverse opportunities of controlling testicular function. Surgical castration is usually bilateral orchiectomy, that is, removal of both testes and epididymis, but may also include other surgical measures that result in loss of testicular function. Alternatively, chemical, hormonal, or immunological approaches for modulation of testicular function can be considered (Table 1). Chemical castration comprises the local, intratesticular use of pharmaceutical drugs and substances that cause coagulation and subsequent degeneration of testicular tissue. Exogenous substitution of hormones may result in interruption of the hypothalamus–pituitary–gonadal axis resulting in loss of testicular function. Immunological castration aims at the systemic production of specific antibodies reducing the release or action of hormones involved in the regulation of testicular function like GnRH or LH.

Reasons for Castration

The primary objective of castration is the control of reproduction. On a population basis, this aims at avoiding general overpopulation and limiting the transmission and spread of diseases (e.g., in free roaming dogs, cats, horses, or in wildlife populations). Castration on an individual basis is done to prevent for examples males with low genetic potential, carriers of congenital diseases (usually nonlicensed bulls or stallions) or bull calves in commercial beef systems from unwanted mating. It is also used in animals belonging to owners that do not want to produce any offspring for example, in pets or performance horses. Besides, many other reasons for castration apply and may differ considerably among species. Sexual behavior in male individuals—irrespective of species—often includes some degree of aggression or is related to unmanageable sexual drive or activity. Sexual behavior depends at least to a certain degree on testosterone production. Castration may therefore aim at modifying behavior and reducing behavioral problems. It will usually improve the manageability of males by humans. The handling of males especially in large domestic animal species like cattle, pigs, or horses can represent a serious safety concern. Male sexual behavior also often complicates animal husbandry by for example destruction of fences or enclosures when attempting to approach nearby oestrous females. Interestingly, also in humans, castration has been used as a—voluntary—measure for sex offenders, but the effectiveness and ethics of such treatments has been heavily discussed. Recently a similar discussion on castration of animals has started. Without medical reasons, castration of male animals is increasingly perceived as ethically questionable. In consequence, in some Scandinavian countries, gonadectomy of companion animals without medical reasons is no longer allowed. While in horses and mules, castration of nonlicensed stallions was for centuries routine because of an improved manageability of the castrated males, the percentage of stallions among riding and performance horses has recently considerably risen because an increasing amount of owners prefers intact male horses nowadays.

Table 1 Reasons for castration of males

- | |
|--|
| <ul style="list-style-type: none">• Control of reproduction<ul style="list-style-type: none">○ Prevention of overpopulation○ Prevention of unwanted reproduction• Control of male behavior<ul style="list-style-type: none">○ Reduction of reproductive behavior○ Reduction of aggressive behavior• Improvement of meat production in food producing animal species<ul style="list-style-type: none">○ Increase in weight gain○ Improvement of carcass quality○ Reduction of unpleasant meat flavor and odor (pig, goat, sheep)• Treatment of reproductive diseases<ul style="list-style-type: none">○ Benign prostatic hyperplasia (human, canine)○ Prostatic cancer (human, canine)○ Testicular neoplasma○ Equine viral arteritis shedder status |
|--|

In meat producing animals, castration affects carcass quality and prevents undesired sex-related taste and odor of heated meat in boars (also called boar taint) and male small ruminants. Boar taint is caused by skatole, a product of tryptophane breakdown in the gut, and by 16-androstene steroids (mainly androstenone) from the testis. It occurs in some, but not all postpubertal boars. In male small ruminants, a “sweaty” meat flavor has been attributed to the production of volatile acids but it has been proposed that accumulation of androstenone in adipose tissue could also contribute to the unpleasant odor of ram mutton. Nevertheless, the prevalence of castration in meat-producing animals varies with location: Castration as a measure to prevent boar taint involves 90% of male piglets in most European countries; however, in countries like the United Kingdom, Ireland, Spain, Portugal, and Australia, castration of male piglets is only rarely performed. Instead, male pigs are slaughtered at an earlier age, that is, before starting to present the boar taint (Rault *et al.*, 2011).

Castration is also a medical treatment for different reproductive pathologies in males. Benign hyperplasia of the prostate gland frequently occurs in aging men and dogs. The disease is androgen-dependent with overproduction of dihydrotestosterone within the prostate being the primary mediator. In dogs not used for breeding, castration is the most effective treatment. In men, application of a synthetic steroid type II 5 α reductase inhibitor is a successful treatment and also efficient in dogs for which castration is no option. Another disease with similarities between men and male dogs is prostate cancer which in men is usually treated by radical surgery and/or radiation therapy. Androgen deprivation is used with locally advanced or metastatic disease. The tumor is initially hormone-response, but in most cases develops into an androgen-independent neoplasm. Such a pathogenesis has also been suggested for the development of canine prostate carcinoma. However, many subtypes of prostate carcinoma with the exception of prostate adenocarcinoma are also found in castrated dogs. There is evidence that castration in dogs does not initiate the development of prostatic carcinoma, but supports tumor progression (Teske *et al.*, 2002). Therefore, castration is neither a preventative nor an effective treatment for the condition. Testicular neoplasia may occur in men and many domestic animal species, but in animals is mainly diagnosed in stallions and dogs. It will usually result in surgical removal of the affected testis. Equine arteritis virus causes abortion in mares and thus infection may result in considerable financial loss. Stallions persistently shedding the virus via semen form the main reservoir for the disease. After acute infection, the virus may persist in the accessory sexual glands of intact male horses for weeks or even years. Transient reduction of testicular androgen production by immunocastration will often terminate persistence of equine arteritis virus and is the treatment of choice for the condition in breeding stallions where surgical castration is no option.

Welfare Aspects

Castration of livestock but also of horses and companion animals is often done in the field with minimum financial expenditure although the procedure may have severe impact on animal welfare. It causes not only acute pain but also chronic discomfort during the postoperative period. This may also impair the animals’ performance with regard to feed intake and body weight gain. Pain and discomfort are related to traumatic injury, subsequent inflammation or tissue degeneration and necrosis. The degree of pain and discomfort may differ considerably with regard to species and age, castration methods as well as peri- and postoperative care. Responses to castration are considerably reduced by application of local anesthetics or nonsteroidal anti-inflammatory drugs at surgery (Webster *et al.*, 2013; White *et al.*, 1995). While such treatment is not an issue for humans, companion animals or valuable horses, castration of young livestock is traditionally performed without anesthesia or postoperative analgesia. Even today, there are only few European countries where the use of a local or general anesthetic for castration is mandatory (e.g., in Norway, Switzerland, and the Netherlands). However, pain management in association with castration is under discussion in many countries. The situation is often complicated by the fact that analgesic drugs may not be approved for pain relief in food producing animals in some countries. The pig industry argues against castration with anesthesia because of an increase in costs and workload, but also doubts the efficiency of such a treatment (Tuytens *et al.*, 2012). On the other hand, if managed properly the positive impact of castration of males on animal welfare should not be underestimated. Adult stallions and bulls will often be housed in single stalls without any social contact to conspecifics to avoid fighting, injury, or unwanted mating. In valuable breeding sires, this situation has to be accepted to a certain degree when refinement of their environment and proper care help to improve it. Similarly, in companion animals, uncastrated males are sometimes difficult to keep because their sexual drive and inappropriate behavior may annoy the owner. Indoor confinement or segregation from oestrous females is not always possible. Castration may also reduce the potential to show aggressive behavior in dogs. Castration will therefore help to provide these animals with a more pleasant life and reduce the risk that they end up in animal shelters. In animals that have already been sheltered, gonadectomy often improves adoptability, ensures that the animals will not repopulate shelters with their offspring, and increases the chance for retention in their adoptive homes (Root Kustritz, 2014). There is evidence that castrated dogs and cats in general have a longer lifespan than their intact conspecifics. Finally, it has to be taken into consideration that castration may affect not only the phenotype of a male but also general health of an individual because besides reproductive function, gonadal steroid hormones are involved in the regulation of several body systems. In humans, androgen deprivation therapy is effective in relieving symptoms of metastatic prostate cancer and increasingly used in men with non-metastatic prostate cancer. However, androgen deprivation therapy contributes to a decline in quality of life not only because of impaired sexual functions, but also because of physical function. There is evidence of loss in muscle strength and functional abilities. Effects on cognitive functions have been suggested but are less certain (reviewed by Alibhai *et al.*, 2006). In dogs, a role of castration in the development of cancer has been suggested. While some tumor types may be increased in castrated male dogs, others are decreased. In conclusion, in animals with a considerable life expectancy like companion

animals and horses, side effects of castration on general health and life quality cannot be excluded. This contributes to an ongoing discussion whether castration as a measure to improve manageability of animals can still be accepted. The risks and benefits of castration of an individual animal therefore have to be critically considered and discussed.

Surgical Castration

Orchiectomy is the oldest available and most common technique for castration of a male. However, it is a traumatic procedure, requires a qualified person and careful follow-up of the individual animal during the postoperative period to prevent complications. Main complications include excessive hemorrhage due to inappropriate crushing of the spermatic blood vessels, eventration (mainly prolapse of omentum or small intestine), inflammation, infection, and also myiasis. Secondary complications like funiculitis, sepsis, peritonitis, phimosis, or paraphimosis may occur.

In veterinary medicine, “open” and “closed” castration procedures are performed: “open” castration refers to a technique in which the inguinal tunic is incised for removal of the testis and epididymis but not sutured thereafter while “closed” castration includes procedures with suturing the inguinal tunic after removal of the organs. In animals, primary closure is rarely recommendable for castrations performed in the field. In contrast, when castration is performed aseptically in a surgical facility, advantages of primary closure include faster wound healing and thus earlier return to work (e.g., in male dogs and horses). Depending on size and age of the animals, castration may be performed with the animal standing while sedated and local anesthesia applied (e.g., in stallions) or in lateral or dorsal recumbency. In large animal species the spermatic cord is crushed with “emasculators” or similar instruments that help crushing and transecting the spermatic cord and *musculus cremaster* and thus reduce the risk of subsequent hemorrhage.

To avoid creation of an open wound, alternative “bloodless” surgical procedures causing loss of testicular function have been developed. Such approaches very much depend on the anatomy of the male genital tract in the respective species. In ruminants, the bottle-like shape of the scrotum and location of the testes in a relatively large distance from the abdominal wall in comparison to other species allow for castration by crushing the spermatic cords with clamps (e.g., the Burdizzo clamp) or application of rubber rings at the scrotal neck. These will dramatically reduce the blood supply of the scrotal organs resulting in tissue degeneration (Bretschneider, 2005). Because gonadal steroids stimulate growth and increase feed efficiency in ruminants, there is some interest in males that are infertile but still produce testicular steroid hormones. Such a situation exists in cryptorchids and has been imitated surgically by shortening the scrotum to an extent that the testes are located in a near-inguinal position. Short scrotum bulls and rams, due to the anabolic properties of androgens (Fig. 1), are more similar to intact than castrated males in growth rate, carcass characteristics, and meat tenderness but have the advantage that their infertility simplifies management (Glimp et al., 1971). Nevertheless, with all these alternative surgical approaches, failure may occur and at least some animals do not become completely infertile. Due to inappropriate inhibition of blood supply, testicular and epididymal degeneration is sometimes incomplete or due to insufficient increase of temperature within the testes, spermatogenesis is maintained at least in parts of the testes (Fig. 2A and B). Such failure also depends on the age of animals at surgery with earlier intervention being usually more successful with the Burdizzo technique.

Chemical Castration

Chemical castration (chemocastration, chemosterilization) offers the advantages of a single, low-cost, and permanent treatment resulting in loss of testicular function. This may be an alternative to surgical castration in some circumstances, that is, in regions

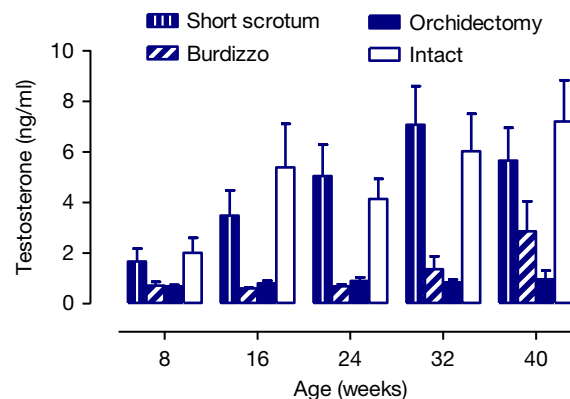


Fig. 1 Plasma testosterone concentration (mean + SEM) from 8 to 40 weeks of age in male cattle after resection of the scrotum (SR), Burdizzo castration (BZ), orchidectomy (OR), or left intact as controls (CO; $n = 10$ per group). Significant differences BZ and OR vs. SR and CO at all times ($P < 0.05$), significant difference over time ($P < 0.05$). Adapted from Pieler, D., Wohlsein, P., Peinhopf, W., et al. (2014). Endocrine testicular function and spermatogenesis persist in calves after partial scrotal resection but not Burdizzo castration. *Theriogenology* 80, 1300–1306.

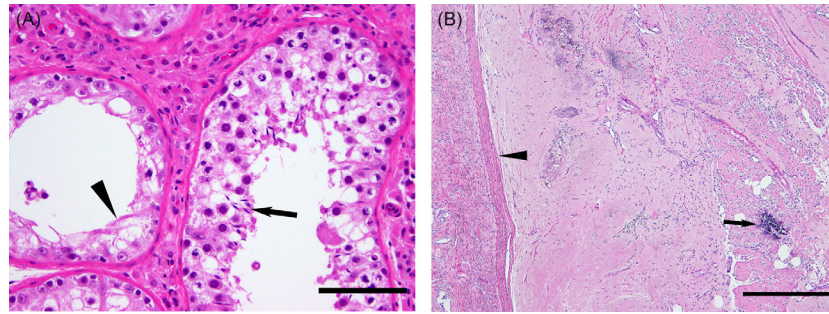


Fig. 2 Testicular tissue of male cattle after Burdizzo castration. Tissue was collected at 40 weeks of age, Burdizzo castration was performed at 8 weeks of age. (A) Testicular tissue with heterogeneous atrophy with some tubules exhibiting full spermatogenesis including elongated spermatids (arrow). In other tubules variable degrees of atrophy were present (arrowhead). HE, bar = 100 µm. (B) Complete loss of testicular tissue and replacement by fibrous tissue with few mineralized foci (arrow). This tissue is surrounded by a fibrous capsule most likely representing the tunica albuginea (arrowhead). HE, bar = 100 µm. From Pieler, D., Wohlsein, P., Peinhopf, W., et al. (2014). Endocrine testicular function and spermatogenesis persist in calves after partial scrotal resection but not Burdizzo castration. *Theriogenology* 80, 1300–1306.

where cutaneous myiasis can complicate surgical treatment. Other complications like herniation or excessive hemorrhage are also avoided by this technique. The procedure requires only light or no sedation, pre-surgical preparation, and postoperative care. Chemical castration is therefore advantageous in regions where financial resources for population control are limited. A variety of chemical substances has been used for chemocastration. These include cadmium, calcium chloride, chlorhexidine, formalin, lactic acid, ethanol, zinc, and many others. These substances are injected into the testes and will induce tissue destruction, sclerosis, and subsequent loss of testicular function. The response may sometimes be incomplete. Also, considerable pain and discomfort in the post-operative period have been reported and scrotal fistulae may develop. It has also to be taken into consideration that chemicals like formalin or cadmium are not acceptable for the treatment of food producing animals. Recently, chemical castration in cattle has been performed with sodium chloride (20% and 30% solutions) to induce destruction of testicular cells by osmotic processes. This procedure was successful in calves at 20 days of age, but ineffective in calves near 5 months of age. Only minor signs of stress and inflammation were recorded (Oliveira et al., 2017). A variety of experiments on chemocastration by intratesticular injection of zinc-based formulations and calcium chloride in several species have been reported (reviewed by Cavalieri, 2017). Eventually, zinc gluconate has received US FDA approval as chemosterilant for use in male dogs in the United States and also some other countries (product name Zeuterin in the US respective EsterilSol in South America). Evidence of pain on injection as well as injection site reactions requiring treatment were reported in approximately 5% of cases. The underlying causes included necrotizing orchitis and scrotal ulceration. Treatment with zinc gluconate induced azoospermia in 80%–90% of treated dogs and 70% of cats, but for example not in adult bears. The great variation in the response rate to chemosterilants has been associated with differences in dose rates, concentrations, diluents, and injection techniques. The injection of excessive doses as well as inadequate restrained of animals results in loss of fluid from the testis into the scrotal space increasing the incidence of scrotal necrosis. Optimizing the procedure of injection may contribute to better effectiveness and reduction of side effects. However, effectiveness of treatment very much depends on testicular size and age of animals with the response being less successful in animals with larger testes because larger testicular size will prevent effective contact of chemosterilants with the majority of testicular tissue (reviewed by Cavalieri, 2017).

Hormone-Based Immunological Castration (Immunocastration)

Immunocastration is the stimulation of endogenous antibody production against hormones which are obligatory for successful reproduction by vaccination. The vaccinated individual becomes infertile for as long as sufficient antibody titers are present. The effect may be reversible or permanent. Hormones which have been targeted for immunocastration in males are gonadotrophin releasing hormone (GnRH) and follicle stimulating hormone (FSH). In humans, the development of contraceptive vaccines was initiated because of the sustainable rise in the worldwide population providing additional family planning options. In animals, immunocastration vaccines have been developed for limiting fertility in feral, livestock, and also companion animals (Bradley et al., 1999). It has to be considered that for the limitation of fertility in populations, the demands for efficacy, and controllability of such a treatment are lower than for the management of individual fertility. For free-ranging species, a long-lasting immune response in a high percentage of the target population is desirable. Present vaccines are unlikely to simulate a sufficient immune response in every individual and the duration of infertility is highly variable. These problems question the application of immunovaccination for management of individual fertility where precise predictability of treatment efficiency and maintenance is highly desirable. In humans, the potential use of GnRH vaccines for immunocastration is further complicated because subsequent supplementation of androgens is necessary for maintenance of secondary sex characteristics and libido. Nevertheless, immunization against GnRH has been considered an alternative for the treatment of androgen-dependent prostatic cancer and benign prostatic hypertrophy in men. A vaccine (Gonadimmune) has been developed but has not been commercialized so far.

In domestic animal species, active immunization against GnRH has recently gained practical significance. Despite the fact that passive immunization might be more selective, reproducible, and precisely timed, the method is at present not applicable for use in animal production systems because of the short maintenance of antibody titers. GnRH itself is too small to be immunogenic. Active GnRH vaccination therefore involves the injection of a GnRH analog which is conjugated to a foreign protein (carrier protein) and combined with an adjuvant. Vaccination induces the formation of anti-GnRH antibodies which inhibit the action of endogenous GnRH. The immune reaction may differ considerably among species and also among individuals within a given species. These may at least in part be overcome by adaptation of the vaccination scheme and of the adjuvant. Adverse effects such as excessive wound swelling or the development of persistent granulomas at the injection site or even pyrexia may occur but also seem to be associated with the adjuvant.

In bulls, active immunization against GnRH has been applied with the aim to reduce aggressive behavior. Because the carcass quality of bulls is superior to that of castrated males, it was also determined if GnRH immunization may be advantageous in comparison to surgical castration with regard to weight gain and meat quality not only in bulls but also in rams and bucks. In small ruminants, the reduction of male odor in meat has also been considered. Different vaccines and vaccination schemes have been used. GnRH immunization generally reduced LH secretion, but effects on testosterone concentration, behavior, and carcass characteristics were not consistent (reviewed in [Bonneau and Enright, 1995](#)). Therefore, GnRH immunization may be an interesting approach for castration of ruminants, but a more predictable response would be desirable. Nevertheless, for immunization of heifers, a GnRH vaccine (Vaxstrate) has been commercialized.

In young intact male pigs, GnRH immunization is by now routinely used as an effective method for inhibition of genital tract development, reduction of gonadotrophin and testosterone release, decreasing fat androstendione levels and the incidence of boar taint ([Bonneau and Enright, 1995](#)). GnRH vaccines for pigs have already been commercialized. Improvac, a vaccine against boar taint which has been developed in Australia and produced by Zoetis is approved in > 60 countries. A modification of the vaccine, Equity (Zoetis) has been commercialized as an oestrus control vaccine for horses in Australia and New Zealand; however, it has also been used for the immunocastration of adult stallions. Both vaccines, Improvac and Equity, differ with regard to the adjuvant. They both have to be injected twice with an interval of at least 4 weeks before efficient antibody titers are developed. However, in pigs, immunocastration is usually performed at an early age and a short-lasting effect is sufficient. The first injection of Improvac is usually given at an age of 17–18 weeks or earlier, the second at 21–22 weeks and animals are slaughtered when 26 weeks old. This vaccination scheme results in an efficient inactivation of endogenous GnRH and elimination of boar taint, results are considered very consistent among individuals at slaughtering. In contrast, in stallions, where vaccination is mainly performed at a postpubertal or mature stage, the outcome with regard to suppression of male behavior, semen quality, and fertility is quite variable ([Janett et al., 2009](#)). If Equity is not available as is the case in Europe, Improvac can also be injected in horses. Both vaccines may result in a severe soft tissue reaction at the injection site and also general side effects like increased body temperature. More importantly, effects on future fertility of an individual stallion are not predictable and infertility may persist. For the transient castration of stallions with future perspectives as breeding sires, the treatment with Equity or Improvac is therefore questionable.

Interruption of the Hypothalamus–Pituitary–Gonadal axis

Exogenous steroid hormones inhibit GnRH and subsequent LH and FSH release by making use of the negative feedback mechanism of the hypothalamus–pituitary–gonadal axis. The supplementation of exogenous hormones can therefore suppress fertility. Also, sustained exposure to GnRH, its analogues or antagonists reduces GnRH-stimulated gonadotrophin secretion through GnRH receptor down-regulation, internalization, and signal uncoupling. These processes can be summarized as interruption of the hypothalamus–pituitary–gonadal axis. They are considered reversible and may result in transient reduction of testicular function in males; however, species-specific variation has to be taken into consideration.

In many species, such as dogs and horses, treatment of males with high doses of exogenous progestins impairs testicular function and semen quality through a negative feedback inhibition of LH release. Nevertheless, interruption of spermatogenesis does not reliably occur. Therefore, such treatment has not gained much practical consideration so far. In dogs, administration of exogenous androgens is more effective and results in a significant but transient decline in daily sperm output and sperm motility. Treatment with anti-androgens has less pronounced effects on spermatogenesis ([Kutzler and Wood, 2006](#)), but is an effective treatment for benign prostate hyperplasia in this species.

The development of GnRH analogues for transient suppression of fertility in domestic and wildlife animals has recently gained increasing interest. Eventually, a slow releasing implant containing 4.7 or 9.4 mg of the GnRH analogue deslorelin has been licensed in Europe for the induction of temporary infertility in adult male dogs and ferrets and has been commercialized (Suprelorin, Virbac). The implant has many advantages such as ease of use, biocompatibility, safety as well as long-term release of sufficient concentrations of the drug. In male dogs, the 4.7 mg deslorelin implant is efficient for suppressing fertility for approximately 6 months, the 9.4 mg implant for 12 months, however, with considerable variation among individual dogs. This variation is at least in part influenced by body weight. Repeated use is possible and provides a continuous period of suppression of gonadal function ([Trigg et al., 2006](#)). The implant is also effective for the treatment of benign prostate hyperplasia in dogs. For population control in wildlife canids such as for example coyotes, the suppression of testicular function of implants available at present is not long-lasting enough to be attractive. In other companion animal species, results were less convincing than in dogs. In adult male cats, treatment with the 9.4 mg deslorelin implant resulted in complete sterility not before 72 days after application ([Romagnoli et al., 2016](#)), but was not effective at all in rabbits.

The horse has long been suggested to be resistant against down-regulation of GnRH receptors at the pituitary level. Recently, prolonged release of deslorelin (2.2 mg implants) has been associated with increased interovulatory intervals in mares leading to the assumption that potent GnRH agonists may be capable of down regulating GnRH receptors in this species. Treatment of adult stallions with 4.7 mg deslorelin implants after a transient increase finally reduced testosterone concentration for 6 weeks (Falomo et al., 2013). Reversible down-regulation of the hypothalamic-pituitary-gonadal axis in mature stallions was also reported after treatment with the GnRH antagonist acyline for 50 days (Davolli et al., 2016). However, these treatments never resulted in complete infertility of stallions and only minor effects on male sexual behavior were reported. Nevertheless, the development of effective treatment protocols with GnRH agonists or antagonists may offer attractive approaches for transient suppression of testicular function in stallions.

References

- Alibhai, S. M. H., Gogov, S., & Allibhai, Z. (2006). Long-term side effects of androgen deprivation therapy in men with non-metastatic prostate cancer: A systematic literature review. *Critical Reviews in Oncology/Hematology*, 60, 201–215.
- Bonneau, M., & Enright, W. J. (1995). Immunocastration in cattle and pigs. *Livestock Production Science*, 42, 193–200.
- Bradley, M. P., Eade, J., Penhale, J., & Bird, P. (1999). Vaccines for fertility regulation of wild and domestic species. *Journal of Biotechnology*, 73, 91–101.
- Bretschneider, G. (2005). Effects of age and method of castration on performance and stress response of beef male cattle: A review. *Livestock Production Science*, 97, 89–100.
- Cavalieri, J. (2017). Chemical sterilization of animals: A review of the use of zinc- and CaCl₂ based solutions in male and female animals and factors likely to improve responses to treatment. *Animal Reproduction Science*, 181, 1–8.
- Davolli, G. M., Ball, B. A., Esteller-Vico, A., et al. (2016). Reversible downregulation of the hypothalamic-pituitary-gonadal axis in stallions with a novel GnRH antagonist. *Theriogenology*, 86, 2272–2280.
- Falomo, M.E., Normando, S., Zanibellato, E., Romagnoli, S. (2013). Sexual behavior and serum testosterone concentration in stallions treated with slow-release implants of deslorelin acetate. *Journal of Veterinary Behavior* 8, 278–284.
- Glimp, H. A., Dikeman, M. E., Tuma, H. J., Gregory, K. E., & Cundiff, L. V. (1971). Effect of sex condition on growth and carcass traits of male Hereford and Angus cattle. *Journal of Animal Science*, 33, 1242–1247.
- Janett, F., Stump, R., Burger, D., & Thun, R. (2009). Suppression of testicular function and sexual behavior by vaccination against GnRH (Equity) in the adult stallion. *Animal Reproduction Science*, 115, 88–102.
- Kutzler, M., & Wood, A. (2006). Non-surgical methods of contraception and sterilization. *Theriogenology*, 66, 514–525.
- Oliveira, F. C., Ferreira, C. E. R., Haas, C. S., et al. (2017). Chemical castration in cattle with intratesticular injection of sodium chloride: Effects on stress and inflammatory markers. *Theriogenology*, 90, 114–119.
- Rault, J. L., Lay, D. C., Jr., & Marchant-Ford, J. N. (2011). Castration induced pain in pigs and other livestock. *Applied Animal Behavior Sciences*, 135, 214–225.
- Romagnoli, S., Baldan, A., Righetti, C., Milani, C., Mollo, A., & Stellaletta, C. (2016). Semen quality and interval to sterility in tom cats treated with a 9.4 mg deslorelin implant. *Journal of Feline Medicine and Surgery*, 1–6.
- Root Kustritz, M. V. (2014). Pros, cons, and techniques of pediatric neutering. *Veterinary Clinics of North America, Small Animals Practice*, 44, 221–233.
- Teske, D., Naan, E. C., van Dijk, E. M., Van Garderen, E., & Schalken, J. A. (2002). Canine prostate carcinoma: Epidemiological evidence of an increased risk in castrated dogs. *Molecular and Cellular Endocrinology*, 197, 251–255.
- Trigg, T. E., Doyle, A. G., Walsh, J. D., & Swangchn-uthai, T. (2006). A review of advances in the use of the GnRH agonist Deslorelin in control of reproduction. *Theriogenology*, 66, 1507–1512.
- Tuytens, F. A. M., Vanhonacker, F., Verhille, B., De Brabander, D., & Verbeke, W. (2012). Pig producer attitude towards surgical castration of piglets without anaesthesia versus alternative strategies. *Research in Veterinary Sciences*, 92, 524–530.
- Webster, H. B., Morin, D., Jarrell, V., et al. (2013). Effects of local anesthesia and flunixin meglumine on the acute cortisol response, behavior, and performance of young dairy calves undergoing surgical castration. *Journal of Dairy Science*, 96, 6285–6300.
- White, R. G., DeShazer, J. A., Tressler, C. J., et al. (1995). Vocalization and physiological responses of pigs during castration with or without local anesthetic. *Journal of Animal Science*, 73, 381–386.

Further Reading

- Junaidi, A., Williamson, P. E., Martin, G. B., et al. (2009). Dose-response studies for pituitary and testicular function in male dogs treated with the GnRH superagonist, Deslorelin. *Reproduction in Domestic Animals*, 44, 725–734.
- Oliveira, F. C., Ferreira, C. E. R., Haas, C. S., et al. (2017b). Chemical castration in cattle with intratesticular sodium chloride: Effects on stress and inflammatory markers. *Theriogenology*, 90, 114–119.
- Smith, A. N. (2014). The role of neutering in cancer development. *Veterinary Clinics of North America: Small Animal Practice*, 44, 965–975.
- Smith, J. (2008). Canine prostatic disease: A review of anatomy, pathology, diagnosis, and treatment. *Theriogenology*, 70, 375–383.
- Squires, E. L., Badzinski, S. L., Amann, R. P., McCue, P. M., & Nett, T. M. (1997). Effects of altrenogest on total scrotal width, seminal characteristics, concentrations of LH and testosterone and sexual behavior of stallions. *Theriogenology*, 48, 313–328.
- Stout, T. A. E. (2005). Modulating reproductive activity in stallions: A review. *Animal Reproduction Science*, 89, 93–103.
- Thompson, D.L. Jr. (2000). Immunization against GnRH in male species (comparative aspects). *Animal Reproduction Science* 60-61, 459–469.
- Zamaratskaia, G., & Kroyer Rasmussen, M. (2015). Immunocastration of pigs—Situation today. *Procedia Food Science*, 5, 324–327.
- Delves, P. J. (2004). How far from a hormone-based contraceptive vaccine? *Journal of Reproduction and Immunology*, 62, 69–78.
- Pieler, D., Wohlsein, P., Peinhopf, W., et al. (2014). Endocrine testicular function and spermatogenesis persist in calves after partial scrotal resection but not Burdizzo castration. *Theriogenology*, 80, 1300–1306.

Germ Cell Transplantation

Hiroshi Kubota, Kitasato University, Aomori, Japan

Ralph L Brinster, University of Pennsylvania, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

All stem cells share a common property, the ability to self-renew and the production of daughter cells that undergo terminal differentiation, by which the function of the tissue and organ are maintained. Both these characteristics must be demonstrated to unequivocally identify a cell as a stem cell; therefore, to assess the biological function of a cell of interest, cell transplantation to a recipient is required. A large number of studies on hematopoietic stem cells and pluripotent stem cells, including embryonal carcinoma cells, embryonic stem (ES) cells, and induced pluripotent stem (iPS) cells, have been carried out since the 1950s, because these cells had functional assay systems based on cell transplantation to assess their biological functions. Until the early 1990s, most other tissue stem cells could not be unequivocally identified because a transplantation system was not available to evaluate their ability to self-renew and terminally differentiate.

The role of germ cells is to transmit genetic information to the next generation for continuity of the species. The process of male germ cell production, spermatogenesis, is a complex and highly productive system, because after puberty spermatozoa are continuously produced in the testis from SSCs that are the foundation of this highly productive system. A germ cell transplantation technique that allows identification of SSCs was developed in 1994, which was a major breakthrough for basic research and for translational/clinical use of male germ cells. In this article, the basic procedure of germ cell transplantation and application of the germ cell transplantation technique, including a quantitative SSC assay are described. In the last section, potential clinical applications using the transplantation technique and SSCs are discussed.

Basics of Germ Cell Transplantation Method

Outline of Germ Cell Transplantation Method

A technique for the transplantation of testicular germ cells into seminiferous tubules of recipient males was developed using mice (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). The basic procedure consists of three parts: preparing a recipient male mouse prior to transplantation, preparing a testis cell suspension containing SSCs from a fertile male mouse (Fig. 1A and B), and microinjecting the cell suspension into the seminiferous tubules of an infertile recipient mouse (Fig. 1C). Two months after transplantation, donor-derived spermatogenesis is reconstituted from the transplanted SSCs, which continue to produce spermatozoa throughout the remaining life of the recipient (Fig. 1D). The recipient males will become fertile if sufficient SSCs are transplanted, and the males will generate progeny with donor haplotype following mating to wild type females (Fig. 1E and F). Long-term maintenance of spermatogenesis can be achieved only by SSCs capable of undergoing self-renewal and constant generation of daughter cells that differentiate into spermatozoa. Therefore, the germ cell transplantation technique established a mechanism to transfer SSCs from one male to another and to generate functionally normal spermatozoa derived from donor SSCs, resulting in production of progeny carrying the donor haplotype.

Preparation of Recipients

For efficient colonization of donor cells, endogenous germ cells of recipient mice must be depleted. It is important for the SSC niche, which is the microenvironment supporting self-renewal of SSCs (Oatley and Brinster, 2012), to be empty for efficient donor SSCs colonization of the recipient testis seminiferous tubule basement membrane. Appropriate recipient mice can be prepared by injection of Busulfan, which is an alkylating agent and used as an anti-cancer chemotherapy drug, to eliminate SSCs and other endogenous germ cells. One month following Busulfan injection most endogenous germ cells have disappeared, and these mice can be used as recipients. Mice with a congenital defect in germ cell development are an alternative to Busulfan-injected mice. The white spotting (*W*) mouse that has a mutation in the *Kit* gene, which encodes a receptor tyrosine kinase essential for primordial germ cell (PGC) proliferation and spermatogonial differentiation, is one of the best recipients for germ cell transplantation (Brinster and Avarbock, 1994). In addition, immature testes before forming the blood-testis barrier of Sertoli cells, which occurs between 10 and 16 days post-partum in the mouse, showed a higher efficiency of colonization of donor SSCs. The blood-testis barrier of Sertoli cells consists of tight junctions between Sertoli cells that allows passage of only small molecules and separates the seminiferous tubule into the adluminal compartment containing the differentiated stages of germ cells from the SSCs, which in contrast are exposed to all blood and lymph constituents. Other congenital germ cell-defective mice caused by spontaneous, targeted, or induced mutations can be used as a recipient if they have no defect in the microenvironment for spermatogenesis, such as in the SSC niche, secretion of differentiation factors, or the architecture of seminiferous tubules (Brinster, 2002). A drawback to the use of mutant mice is that they are often more difficult to maintain in sufficient numbers for recipients.

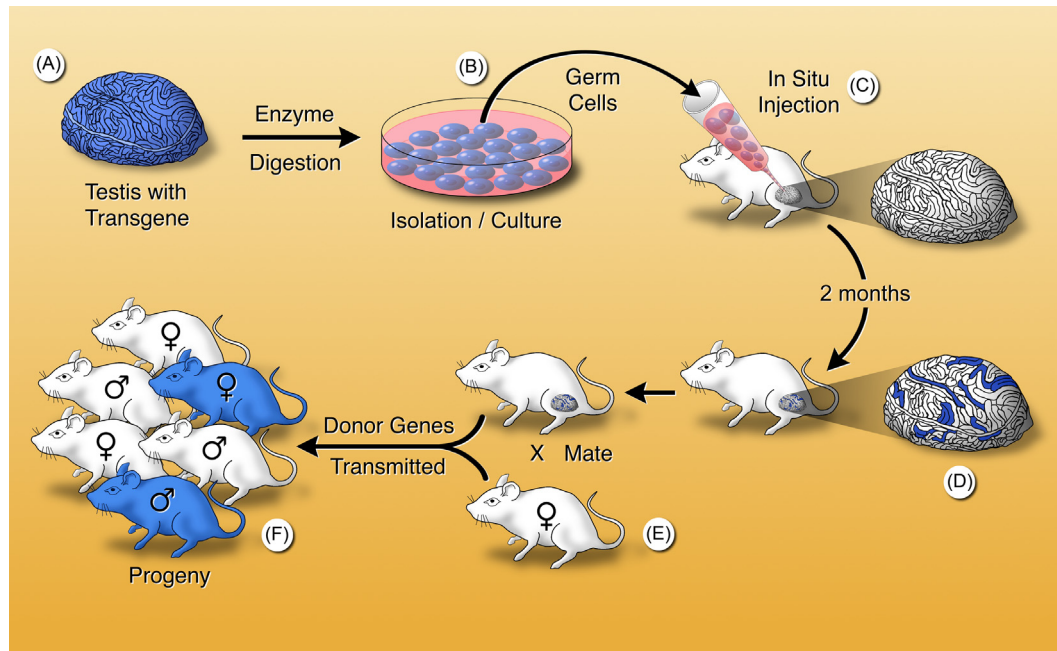


Fig. 1 Outline of germ cell transplantation method. (A) A single cell suspension is prepared from the testes of a fertile male. (B) The cells can be cultured with appropriate conditions. (C) Cells are microinjected into the seminiferous tubules of an infertile recipient male. (D) SSCs colonize the basement membrane of the seminiferous tubules and generate donor-cell-derived spermatogenesis. When donor cells carry a reporter transgene (e.g., β -gal), colonies can be identified as blue stretches of tubule. (E) The recipient male mates to a wild-type female. (F) Progeny with the donor haplotype are produced. Modified from Brinster, R.L. (2002). Germline stem cell transplantation and transgenesis. *Science* 296, 2174–2176. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Preparation of Donor Cells

The donor cell sample should be prepared as a single cell suspension by enzymatic digestion of testis tissues and then introduced into the seminiferous tubules through a micropipette (Brinster and Zimmermann, 1994). Testis tissue from late fetal stages to old adults can be used as donor tissue/cell sources. PGCs and gonocytes, also called prospermatogonia or prespermatogonia, which are precursors of SSCs in embryonic, fetal and neonatal testes, can colonize recipient testis following transplantation, differentiate to SSCs, and reconstitute donor-derived spermatogenesis. Donor cells derived from transgenic mice that express a reporter gene, such as *lacZ* for β -galactosidase (β -gal) or *gfp* for green fluorescent protein (GFP), are useful because donor cells can be unequivocally identified in recipient testes by visualizing the reporter gene products (Fig. 1).

Cell-Injection Methods

Three different methods to introduce donor cells into seminiferous tubules of recipient mice were developed (Ogawa et al., 1997). The first method is the injection of cells directly into the seminiferous tubule using a micropipette (Fig. 2Aa). This method is the most direct way to introduce germ cells into seminiferous tubules. Donor germ cells are forced into the rete testis from the injected tubule and can then enter other seminiferous tubules because all seminiferous tubules are connected by the rete testis, from which spermatozoa normally are transmitted to the epididymis and then to the vas deferens for ejaculation. The cell suspension entering the rete testis can fill several other seminiferous tubules by retrograde flow. The second method is to insert a micropipette directly into the rete testis and fill the seminiferous tubules (Fig. 2Ab). The third method is to insert a micropipette into one of the efferent ducts, which connect the rete testis to the epididymis, and thread the pipette into the rete testis (Fig. 2Ac). This method is the most accurate for controlling the injection volume because less cell suspension leaks from the insertion site of the micropipette, and a larger number of tubules are filled with the injection suspension. Addition of a small amount of trypan blue to the donor cell suspension facilitates monitoring tubule filling (Fig. 2B). Normally, 70%–90% of the seminiferous tubules can be filled by injecting 4–10 μ L of cell suspension depending upon the size of recipient testes. All three methods have been used for mice and resulted in successful reconstitution of donor spermatogenesis.

Regeneration Process of Donor Spermatogenesis

The reconstitution process of donor-derived spermatogenesis by transplanted SSCs can be investigated using transgenic mice expressing β -gal (Nagano et al., 1999). When donor testis cells from these mice are microinjected into the lumen of the seminiferous

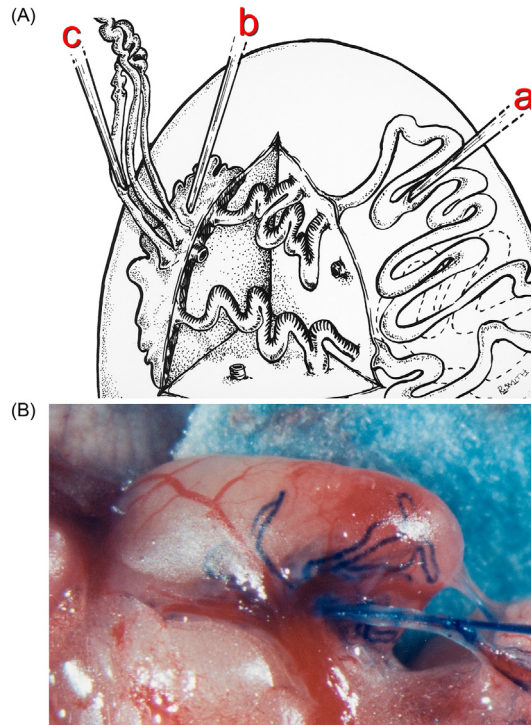


Fig. 2 Three cell-injection methods into a recipient testis. (A) The micropipette can be inserted directly into the seminiferous tubules (a), into the rete testis (b), or into an efferent duct (c). (B) A micropipette was inserted through an efferent duct. Recipient seminiferous tubules were beginning to fill by injecting donor cells with dye (trypan blue). Modified from Ogawa, T., Arechaga, J.M., Avarbock, M.R. and Brinster, R.L. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. *The International Journal of Developmental Biology* 41, 111–122. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tubules of an infertile recipient male, some donor germ cells reach the basement membrane passing through the blood-testis barrier of Sertoli cells in the opposite direction of normal spermatogenesis (Fig. 3A). After colonization, the donor cells begin proliferating laterally on the basement membrane during the first month and then gradually differentiate toward the lumen (Fig. 3B). By 1 month following transplantation, donor-derived spermatocytes appear in the adluminal compartment of the seminiferous tubules. By 2 months after transplantation, donor germ cells fill the tubules, and spermatozoa begin to appear (Fig. 3C and D). Although the process of spermatogenesis from spermatogonia to mature spermatozoa in mice is reported to be 35 days, nearly twice as long is necessary to produce donor spermatozoa following transplantation (Brinster, 2002). The reasons for the difference of 35 days versus 2 months remains unclear, but migration from the lumen to the basement lamina, entering the SSC niche, and proliferation laterally, are likely involved in the time to produce mature spermatozoa following germ cell transplantation. In addition, the 35-day spermatogenesis time reported is measured from A1 spermatogonia, and the time for differentiation from an SSC to A1 spermatogonia is unknown and may account for a significant part of the additional 1 month for transplanted SSCs to produce spermatozoa. Following the first initiation of spermatogenesis at 2 months post-transplantation, the area of donor-derived spermatogenesis continues to extend laterally along recipient seminiferous tubules for a variable time but does not generally fill the entire length of the tubule (Fig. 3C and D).

Application of Germ Cell Transplantation Technique for Stem Cell Research

Functional Assay for Spermatogonial Stem Cells

The germ transplantation procedure has established a quantitative assay for identification of SSCs. When donor cells from a transgenic mouse expressing any visible reporter gene, such as β -gal, GFP, or other fluorescent protein are transplanted into recipient testes, donor-derived spermatogenesis is unequivocally identified 2 months after transplantation. When β -gal-expressing germ cells were transplanted, donor-derived spermatogenesis was identified as blue colonies after staining with X-gal, which identifies β -gal activity (Fig. 3D). These colonies are each derived from a single donor cell; hence, the number of blue colonies represents the number of SSCs in the cell suspension microinjected that could find an SSC niche and produce spermatogenesis. The colonization efficiency of SSCs transplanted into adult recipient testes is approximately 10% (Nagano, 2003); therefore, the actual SSC number in a donor cell suspension has been suggested to be 10-fold greater than the colony number. The factors responsible for the low colonization efficiency are not clear, but the necessity to migrate in the opposite direction of normal spermatogenesis between Sertoli cells, spontaneous differentiation before reaching the SSC niche, and passing through the blood-testis barrier are possible reasons.

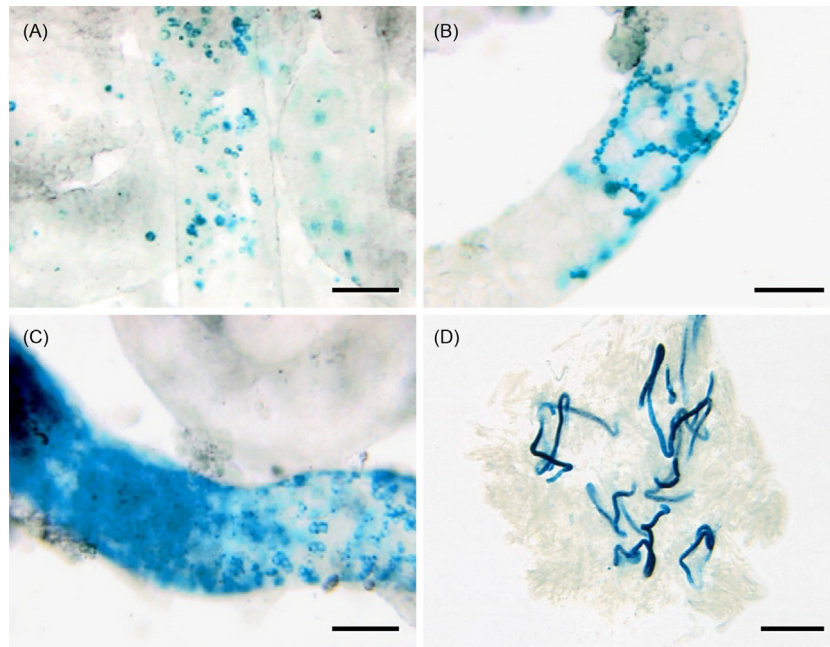


Fig. 3 Regeneration process of spermatogenesis derived from donor SSCs expressing β -gal. (A) One week after transplantation, some blue cells were observed on the basement membrane. (B) Two weeks after transplantation, spermatogonial spreading with chain formation was observed. (C) Two months after transplantation, dark blue colonies with full spermatogenesis were identified. Donor cell-derived colonies continued to grow at the extremities. (D) Two months after transplantation, spermatogenic colonies derived from donor SSCs were identified as blue stretches of tubules under lower magnification. Each blue spermatogenic colony in the recipient testis is developed from a single donor SSCs. The testes were stained with X-gal. Scale bars: (A–C) 100 μ m, (D) 2 mm. Modified from Nagano, M., Avarbock, M.R. and Brinster, R.L. (1999). Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biology of Reproduction* 60, 1429–1436. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Although the original estimate for the number of SSCs in an adult mouse testis was approximately 20,000–35,000 based on morphometric criteria (Kubota and Brinster, 2006; Ogawa et al., 1997), the functional SSC number estimated by the transplantation assay is 3000, which represents 0.01% of all seminiferous tubule cells (Nagano, 2003).

When fluorescent protein-expressing cells are used for the donor source, repopulated donor SSCs can be recovered for further biological investigation by noninvasively identifying and recovering the spermatogenic colonies in the recipients. In addition, donor-derived post-meiotic germ cells can be used for round spermatid injection (ROSI) or intra-cytoplasmic sperm injection (ICSI) to produce offspring carrying the donor haplotype (Yanagimachi, 2005). Development of the functional assay system has resulted in enormous advances in understanding SSC biology and germ cell differentiation. Furthermore, the transplantation technique provides a powerful approach to study the SSC niche (Oatley and Brinster, 2012).

Functional Assay for Spermatogonial Stem Cell Precursors

In addition to providing a powerful means to identify SSCs in any donor cell suspension, the transplantation technique can identify precursor cells that are able to differentiate into SSCs. Gonocytes, which are the direct precursor of SSCs, are located in the center of seminiferous tubules of fetal and newborn testes and are mitotically inactive until the onset of spermatogenesis. In mice, gonocytes begin to proliferate and move to the periphery of the seminiferous tubules a few days after birth, and then they differentiate to SSCs. When gonocytes from fetal and newborn testes are transplanted into seminiferous tubules of mature testes, donor-derived spermatogenesis occurs, indicating that the donor gonocytes differentiated to SSCs in recipient mature testes. Furthermore, when PGCs, the precursor of gonocytes, and epiblast cells, the precursor of PGCs, were transplanted into immature testes, but not mature testes, before forming the blood-testis barrier of Sertoli cells, donor-derived spermatogenesis was reconstituted (Chuma et al., 2005). Therefore, the transplantation technique can be used for not only a functional assay to identify SSCs, but also to assess developmental potential of other cell types to produce SSCs. This ability has been applied to generate functionally normal spermatozoa from pluripotent cell lines, such as ES cells or iPS cells, without making chimeras. In a mouse model, epiblast-like cells were initially induced from pluripotent cells, and then further induced to form PGC-like cells. Following purification of the PGC-like cells, they were transplanted into the seminiferous tubules of immature *W* mouse testes. Donor-derived spermatozoa in the recipient testes were produced, and normal offspring were generated from the donor-derived spermatozoa after ICSI (Hayashi et al., 2011). To obtain functional gametes from pluripotent cells, the germ cell transplantation technique constitutes a final critical step and can be used as a functional assay to evaluate the quality of precursors of SSCs induced from pluripotent cells in culture.

Application of Germ Cell Transplantation to Various Mammalian Species

Application to Non-Rodent Species

The basic methods for spermatogonial transplantation in the mouse have been followed for all mammalian species, and the technique has been extended to many species, including other laboratory rodents, farm animals, companion animals, and primates (Brinster, 2007; González and Dobrinski, 2015) (Table 1). The procedure for non-mouse rodents, such as rat or hamster, is essentially the same as for mouse; however, some modifications for non-rodents are necessary because of species-specific anatomy of the testis, particularly size and testis rete structure. While there are three methods to inject donor germ cells as described above, donor SSCs in non-rodent species are introduced into the rete testis by ultrasound guidance or surgical dissection. Although Busulfan injection can be used for recipient preparation in non-rodent species, appropriate injection timing and doses for Busulfan must be determined in each species and is logistically difficult and inconvenient. Alternatively, local irradiation of testes has been shown to be effective for ablation of endogenous germ cells. Complete removal of endogenous germ cells is not essential, and residual endogenous spermatogenesis may be helpful to maintain a healthy testicular microenvironment to enhance donor spermatogenesis. In addition, immature males without pretreatment can be used as recipients of farm animals. In this situation, recipients with persistent endogenous spermatogenesis will produce both donor-derived and recipient-derived progeny; therefore, genotyping is necessary to determine the sire of each individual offspring. Furthermore, several experiments have indicated that donor germ cells from several species of farm animals were not rejected in MHC-incompatible recipients of the same species even without immunosuppressive treatments. This is strikingly different from laboratory rodents, which require immunosuppression when allogenic donor cells are transplanted. In goat and sheep, progeny with donor SSC-haplotype were successfully generated from immunologically incompatible recipients (González and Dobrinski, 2015) (Table 1).

Germline Modification Using Germ Cell Transplantation

An enormously valuable application of SSCs is for germline modification, also known as transgenesis (Brinster, 2002). The first transgenic animal using SSCs was created by transduction of a retrovirus vector containing the β -gal gene into mouse SSCs (Nagano et al., 2001). Although retroviral transduction was used in the initial approach, subsequent development of a long-term culture system now allows a variety of techniques to select successful modifications, resulting in generation of not only knock-out mice by homologous recombination (Kanatsu-Shinohara et al., 2006), but also gene-edited mice using the CRISPR/Cas9 system (Wu et al., 2015). In the mouse system, several methods for germline modification, including microinjection of DNA into the pronucleus, transplantation of pluripotent stem cells into blastocysts, viral injection into early embryos, and somatic cell nuclear transfer to enucleated eggs, have been developed (Behringer et al., 2014). Although pronuclear injection and pluripotent stem cell transplantation have been routinely used in the mouse until recently, germline modification in animals other than mouse was extremely difficult and inefficient. However, the development of the CRISPR/Cas9 system has enormously facilitated genetic manipulation of fertilized eggs and resulted in large numbers of transgenic animals in many species. In the CRISPR/Cas9 system with fertilized eggs, selection of correctly modified-embryos and avoidance of mosaicism containing both modified and

Table 1 Germ cell transplantation in various mammalian species^a

Donor	Recipient	Colonization	Spermatogenesis	Offspring	Transgenesis
Mouse	Mouse	+	+	+	+
Rat	Rat	+	+	+	+
Mouse	Rat	+	+	—	—
Rat	Mouse	+	+	+	—
Hamster	Mouse	+	+	—	—
Rabbit	Mouse	+	—	—	—
Cat	Mouse	+	—	—	—
Dog	Mouse	+	—	—	—
Pig	Mouse	+	—	—	—
Cattle	Mouse	+	—	—	—
Horse	Mouse	+	—	—	—
Baboon	Mouse	+	—	—	—
Macaque	Mouse	+	—	—	—
Human	Mouse	+	—	—	—
Dog	Dog	+	+	—	—
Goat	Goat	+	+	+	+
Sheep	Sheep	+	+	+	—
Pig	Pig	+	+	—	—
Cattle	Cattle	+	+	—	—
Macaque	Macaque	+	+	—	—

^aSee text for references.

un-modified cells are major drawbacks. In contrast to fertilized eggs, SSCs can self-renew and replicate for long periods in vitro (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004), which makes possible evaluation of both the correct genetic modification and expansion of the corrected cell type. In addition, SSCs can be cryopreserved for long periods (Avarbock et al., 1996), thereby permanently capturing and storing any genetic modification. Hence, genetic manipulation of SSCs by the CRISPR/Cas9 system with germ cell transplantation enables robust techniques for transgenesis in many species of animals without obvious drawbacks. In fact, the CRISPR/Cas9 system has now been applied to generate transgenic rats as well as mice (Chapman et al., 2015). The feasibility of correcting existing mutations by the CRISPR/Cas9 based-gene editing of murine SSCs has already been demonstrated (Wu et al., 2015). Similar approaches will be applied for a wide range of species not only to generate transgenic animals, but also to treat genetic disorders (Table 1).

Xenotransplantation

Following transplantation of rat SSCs into the seminiferous tubules of infertile immunocompromised mice, rat spermatogenesis was established in recipient testes (Clouthier et al., 1996) (Fig. 4A and B), and the rat spermatozoa in the mouse testes were functionally normal. The differentiation period from type A1 spermatogonia to mature spermatozoa is 52 days in rats and 35 days in mice. However, rat spermatogenesis occurring in mouse seminiferous tubules progressed at the rate found for rat spermatogenesis in rat testes indicating that the genotype of the germ cell, not the genotype of Sertoli cells or other environmental factors, controls the specific timing of spermatogenesis characteristic of each species. The result of rat to mouse transplantation experiments suggested that xenogeneic spermatogenesis might be reconstituted by transplantation of SSCs from different species into immunocompromised mice. However, when germ cells from various mammalian species, including rabbits, dogs, cats, pigs, cattle, horses, baboon, macaques, and humans, were transplanted into infertile immunocompromised male mice, complete donor-derived spermatogenesis did not occur, but primitive spermatogonia from all these species colonized and proliferated for 1–12 months on the basement membrane of the seminiferous tubules in immunocompromised mice (Brinster, 2002, 2007; Dobrinski et al., 2000; González and Dobrinski, 2015; Nagano et al., 2002) (Fig. 4C and D, Table 1). At present, the colony-forming spermatogonia in immunocompromised mice are believed to be SSCs and perhaps early undifferentiated spermatogonia. The remarkable results of xenogeneic transplantations indicate that factors produced in the mouse SSC niche can support proliferation of SSCs from many species.

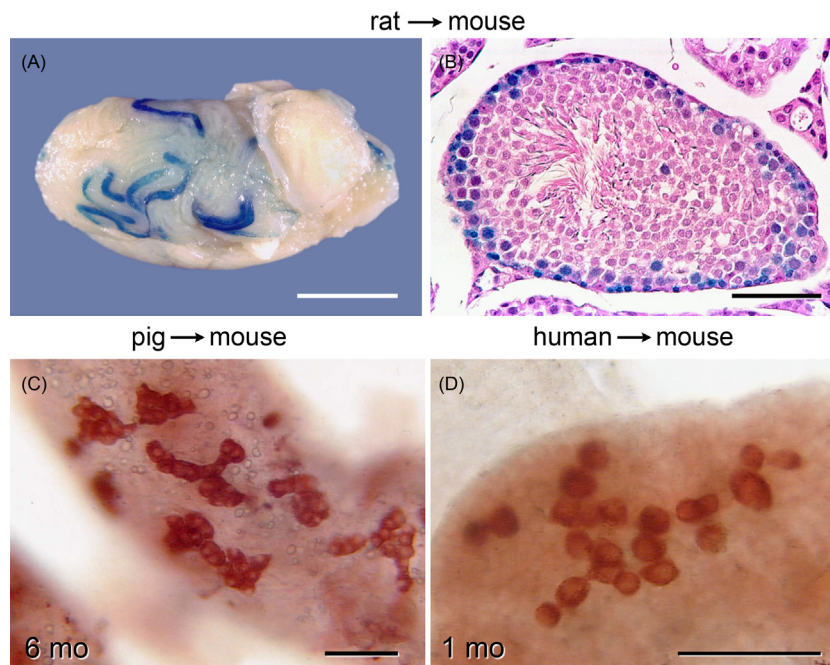


Fig. 4 Xenogeneic spermatogenesis and spermatogonial colonization in nude mouse testes. (A) Four months after transplantation of rat testis cells expressing β -gal, rat spermatogenesis was reconstituted in the recipient mouse testis (blue stretches of tubules). The testis was stained with X-gal. (B) Histological analysis of rat spermatogenesis in the nude mouse testis 4 months after transplantation. The testis was stained with X-gal plus hematoxylin and eosin. (C) Six months after transplantation of porcine testis cells, clusters of porcine spermatogonia were observed. (D) One month after transplantation of human testis cells, clusters of human spermatogonia were observed. Germ cells in C and D were identified by species specific antibody staining and appear red. Scale bars: (A) 2 mm, (B–D) 50 μ m. Modified from Clouthier, D.E., Avarbock, M.R., Maika, S.D., Hammer, R.E. and Brinster, R.L. (1996). Rat spermatogenesis in mouse testis. *Nature* 381, 418–421, Dobrinski, I., Avarbock, M.R. and Brinster, R.L. (2000). Germ cell transplantation from large domestic animals into mouse testes. *Molecular Reproduction and Development* 57, 270–279, and Nagano, M., Patrizio, P. and Brinster, R.L. (2002). Long-term survival of human spermatogonial stem cells in mouse testes. *Fertility and Sterility* 78, 1225–1233. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Because the characteristics of the process of spermatogenesis are well conserved among mammalian species, conservation of self-renewing factors for SSCs and perhaps initial replication must be conserved as well. However, species variation in factors necessary for differentiation during spermatogenesis must exist among mammalian species. Sertoli cell transplantation from the same species as the donor germ cells may overcome the block in differentiation of xenogeneic donor germ cells in mouse testes. Transplantation of Sertoli cells, in addition to SSCs from xenogeneic species, might reestablish the necessary niche and differentiation factors for foreign species spermatogenesis in immunodeficient mice. It is extremely important to develop functional assays for non-rodent SSCs in which both self-renewal and differentiation can be evaluated.

Preservation of the Germline and Future Directions

Cryopreservation of Germline

Cryopreservation of SSCs from many species has been successful, and mouse SSCs can be cryopreserved for at least 14 years and, after thawing and transplantation, will regenerate spermatogenesis that produces functional spermatozoa (Avarbock et al., 1996; Wu et al., 2012). Thus, cryopreservation of SSCs provides an effective method by which to preserve the germline of individual males for long periods providing a potentially immortal lifespan for male germlines. Although semen cryopreservation is commonly used to preserve the germline of certain economically, biologically, or scientifically valuable males, including livestock breeds or endangered animal species, semen cryopreservation methods are complex and must be independently developed for each species. Thus, semen preservation techniques have only been developed for a limited number of species, but SSCs of many species are readily cryopreserved using procedures for somatic cells. Moreover, the number of SSCs can be increased in culture, and long-term culture techniques are currently available for several species (Kubota et al., 2011). Culture techniques for many valuable species, including human, livestock, and endangered animals will be developed in the future (Kubota and Brinster, 2006, 2008).

Clinical Applications of Germ Cell Transplantation

Functional spermatozoa can be obtained by transplantation of precursor cells of SSCs from immature males before puberty or even from fetuses, as well as from mature and aged males. Thus, an important, potential clinical application for human SSC transplantation is in prepubertal boys undergoing chemotherapy or radiation treatment for cancer (Brinster, 2007; Kubota and Brinster, 2006) (Fig. 5). Approximately 80% of childhood cancers can be cured, but SSCs and other germ cells are extremely sensitive to chemotherapeutic agents and radiation, resulting in nearly 1 in 5000 male cancer survivors of reproductive age being infertile or

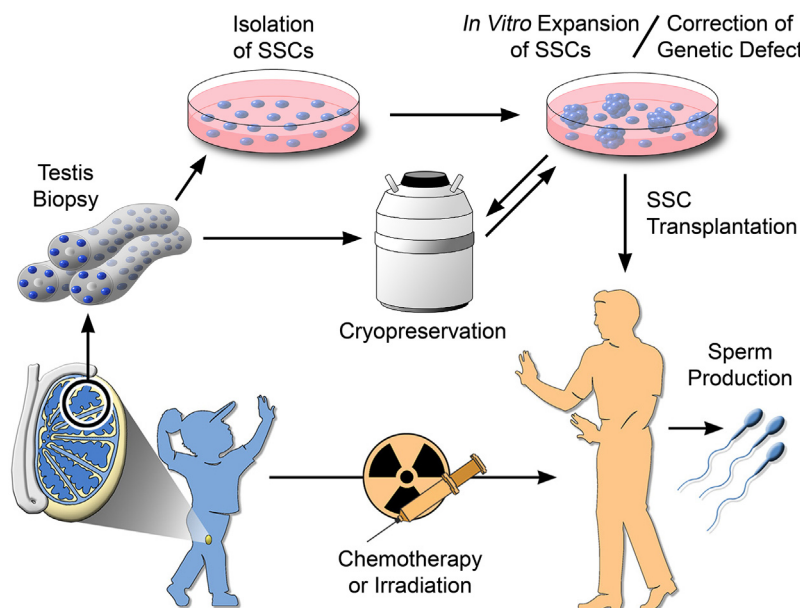


Fig. 5 Clinical application of SSCs by germ cell transplantation. In prepubertal patients with cancer, SSCs could be isolated by testis biopsy before treatment with chemotherapy or irradiation and expanded in culture. The biopsy containing SSCs or expanded SSCs could be cryopreserved. After successful treatment, the SSCs would be transplanted to the patient's testes to restore fertility. For a patient carrying a genetic defect, the defective gene could be corrected in SSCs during culture. The SSCs with the corrected gene could be transplanted into the testes of the patient. Modified from Kubota, H. and Brinster, R.L. (2006). Technology insight: In vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature Clinical Practice Endocrinology and Metabolism* 2, 99–108 and Brinster, R.L. (2007). Male germline stem cells: From mice to men. *Science* 316, 404–405.

extremely sub-fertile. While adults can cryopreserve semen before germ cell destroying therapies for future use in artificial insemination or in vitro fertilization, this option is not available for prepubertal boys because complete spermatogenesis has not been established. For prepubertal boys, cryopreservation of a testicular biopsy can be employed for future autologous transplantation into the seminiferous tubules following successful cancer treatment. The biopsy contains SSCs, which have the potential to colonize and restore spermatogenesis following transplantation. However, efficient culture methods to allow in vitro expansion of human SSCs are needed to increase the number of SSCs and eliminate potential cancer cells before transplantation of SSCs (Fig. 5).

Many genetic mutations causing human disease have been identified, and in the future, germline gene-editing may be considered for therapeutic correction. Once cultivation techniques of human SSCs become available, controversy will inevitably arise regarding the ethics of research designed to generate spermatozoa from gene-corrected human SSCs.

Concluding Remarks

Germ cell transplantation has revolutionized the study of the male germline of not only research species, but of all mammals, including companion animals, farm animals, endangered species and primates. Although the germ cell transplantation technique was originally developed in the mouse and allowed extensive characterization of murine SSCs, future studies will focus on a large range of species, including humans. Development of an efficient in vitro culture system supporting self-renewal and expansion of human SSCs and feasible germ cell transplantation protocols are critical to accelerate the advance of new medical applications and germ cell-based therapies.

Acknowledgements

We thank James Hayden, RBP, FBCA, for assistance in preparation of figures.

References

- Avarbock, M. R., Brinster, C. J., & Brinster, R. L. (1996). Reconstitution of spermatogenesis from frozen spermatogonial stem cells. *Nature Medicine*, 2, 693–696.
- Behringer, R., Gertsenstein, M., Nagy, K. V., & Nagy, A. (2014). *Manipulating the mouse embryo: A laboratory manual*. New York: Cold Spring Harbor Laboratory Press.
- Brinster, R. L. (2002). Germline stem cell transplantation and transgenesis. *Science*, 296, 2174–2176.
- Brinster, R. L. (2007). Male germline stem cells: From mice to men. *Science*, 316, 404–405.
- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11303–11307.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Chapman, K. M., Medrano, G. A., Jaichander, P., Chaudhary, J., Waits, A. E., Nobrega, M. A., Hotaling, J. M., Ober, C., & Hamra, F. K. (2015). Targeted germline modifications in rats using CRISPR/Cas9 and spermatogonial stem cells. *Cell Reports*, 10, 1828–1835.
- Chuma, S., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Hosokawa, M., Nakatsuji, N., Ogura, A., & Shinohara, T. (2005). Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. *Development*, 132, 117–122.
- Clouthier, D. E., Avarbock, M. R., Maika, S. D., Hammer, R. E., & Brinster, R. L. (1996). Rat spermatogenesis in mouse testis. *Nature*, 381, 418–421.
- Dobrynski, I., Avarbock, M. R., & Brinster, R. L. (2000). Germ cell transplantation from large domestic animals into mouse testes. *Molecular Reproduction and Development*, 57, 270–279.
- González, R., & Dobrynski, I. (2015). Beyond the mouse monopoly: Studying the male germ line in domestic animal models. *ILAR Journal*, 56, 83–98.
- Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., & Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell*, 146, 519–532.
- Kanatsu-Shinohara, M., Ikawa, M., Takehashi, M., Ogonuki, N., Miki, H., Inoue, K., Kazuki, Y., Lee, J., Toyokuni, S., Oshimura, M., et al. (2006). Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 8018–8023.
- Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., & Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biology of Reproduction*, 69, 612–616.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 16489–16494.
- Kubota, H., & Brinster, R. L. (2006). Technology insight: In vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature Clinical Practice Endocrinology and Metabolism*, 2, 99–108.
- Kubota, H., & Brinster, R. L. (2008). Culture of rodent spermatogonial stem cells, male germline stem cells of the postnatal animal. *Methods in Cell Biology*, 86, 59–84.
- Kubota, H., Wu, X., Goodyear, S. M., Avarbock, M. R., & Brinster, R. L. (2011). Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. *The FASEB Journal*, 25, 2604–2614.
- Nagano, M., Avarbock, M. R., & Brinster, R. L. (1999). Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biology of Reproduction*, 60, 1429–1436.
- Nagano, M., Brinster, C. J., Orwig, K. E., Ryu, B. Y., Avarbock, M. R., & Brinster, R. L. (2001). Transgenic mice produced by retroviral transduction of male germ-line stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 13090–13095.
- Nagano, M., Patrizio, P., & Brinster, R. L. (2002). Long-term survival of human spermatogonial stem cells in mouse testes. *Fertility and Sterility*, 78, 1225–1233.
- Nagano, M. C. (2003). Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biology of Reproduction*, 69, 701–707.
- Oatley, J. M., & Brinster, R. L. (2012). The germline stem cell niche unit in mammalian testes. *Physiological Reviews*, 92, 577–595.

- Ogawa, T., Arechaga, J. M., Avarbock, M. R., & Brinster, R. L. (1997). Transplantation of testis germinal cells into mouse seminiferous tubules. *The International Journal of Developmental Biology*, 41, 111–122.
- Wu, X., Goodyear, S. M., Abramowitz, L. K., Bartolomei, M. S., Tobias, J. W., Avarbock, M. R., & Brinster, R. L. (2012). Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. *Human Reproduction*, 27, 1249–1259.
- Wu, Y., Zhou, H., Fan, X., Zhang, Y., Zhang, M., Wang, Y., Xie, Z., Bai, M., Yin, Q., Liang, D., et al. (2015). Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. *Cell Research*, 25, 67–79.
- Yanagimachi, R. (2005). Intracytoplasmic injection of spermatozoa and spermatogenic cells: its biology and applications in humans and animals. *Reproductive Biomedicine Online*, 10, 247–288.

MALE ENDOCRINOLOGY

Hypothalamic Pituitary Testis Axis

David Morritz de Kretser, Hudson Institute and Monash University, Melbourne, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Understanding testicular function and the pathological states that result in male infertility or androgen deprivation requires knowledge of the hormonal control of the testis. The regulation of reproduction is under the control of the hypothalamic-pituitary gonadal axis. As early as 1930, the importance of the pituitary gland in controlling reproduction as well as many other functions was recognized (Smith, 1930).

Clear evidence for the involvement of this system also emerges from the seasonal influence on reproductive processes in females and males from many species but there is less evidence for a role of season in the control of human reproduction (Popa and Fielding, 1931; Harris, 1948; Green and Harris, 1951). These observations indicate that the changing day length is detected via the eyes and result in a change in reproductive behavior. In addition, the effect of psychological stress and marked weight loss associated with anorexia are known to influence the regularity of menstrual cycles. Further, as early as 1930 the need for replacement therapy following the removal of the pituitary was recognized (Smith, 1930).

The neural pathways involved result in stimulating the hypothalamus to send signals that act to alter the secretion of hormones from the pituitary gland that influence testicular and ovarian function (Popa and Fielding, 1931; Harris, 1948; Green and Harris, 1951).

As with all endocrine glands in the body, the endocrine control of the testis is exerted via the hypothalamic-pituitary testicular axis. The pituitary gland is formed by two components, one of epithelial origin from the developing pharynx which forms the anterior pituitary or adenohypophysis and a neural down growth from the hypothalamic region of the brain that forms the neurohypophysis (Figs. 1–4); (Popa and Fielding, 1931). The latter is responsible for the secretion of oxytocin and vasopressin.

Those signals are transmitted by peptide hormones that utilize a portal system of blood vessels to carry them from the hypothalamus to the anterior part of the pituitary gland (Fig. 4). In contrast, the posterior pituitary is composed of nerve fibers arising from nerve cells which release their products into blood vessels thus entering the circulation and transporting those entities to their sites of action (Green and Harris, 1951). Harris, one of the pioneers in this field articulated the basis of our current understanding of the physiology of the hypothalamo-hypophyseal system. He used electrical stimulation to demonstrate the neural link to the pituitary gland (Green and Harris, 1951).

The mechanisms involved are the secretion of gonadotropin releasing hormone (GnRH), a decapeptide secreted by hypothalamic neurones into the hypothalamo-hypophyseal portal vascular system that conveys the secreted GnRH to stimulate the gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH) secretion by the gonadotrophin secreting cells in the anterior pituitary gland (Guillemin, 1964; Gersh, 1938). In turn, FSH and LH circulate in the blood stream to stimulate spermatogenesis and testosterone secretion by the testis in the male, and ovulation and estradiol secretion by the ovary in the female.

The pulsatile nature of LH secretion clearly supports the pulsatile nature of the GnRH stimulus but the pulsatility is less evident from the pattern of FSH secretion since the longer half-life of FSH obscures the pulsatility. The pulse frequency and amplitude is set

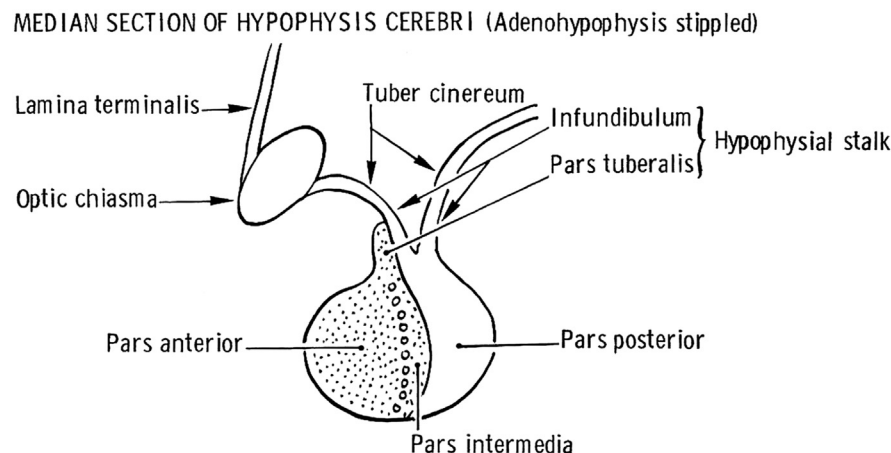


Fig. 1 A diagram of a median section of the pituitary gland.

FLOOR OF THIRD VENTRICLE: FROM BELOW

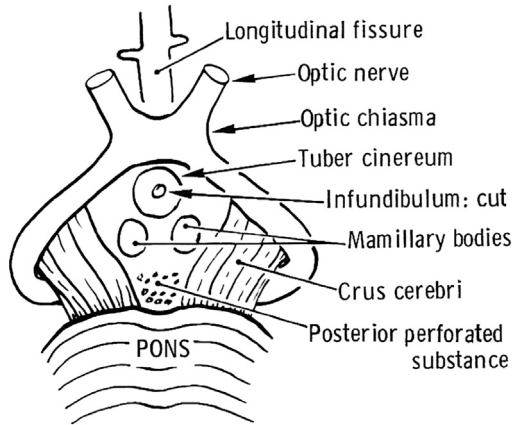


Fig. 2 A diagram of the region of the third ventricle from below.

MEDIAN SECTION: BODY OF SPHENOID & HYPOPHYSIS

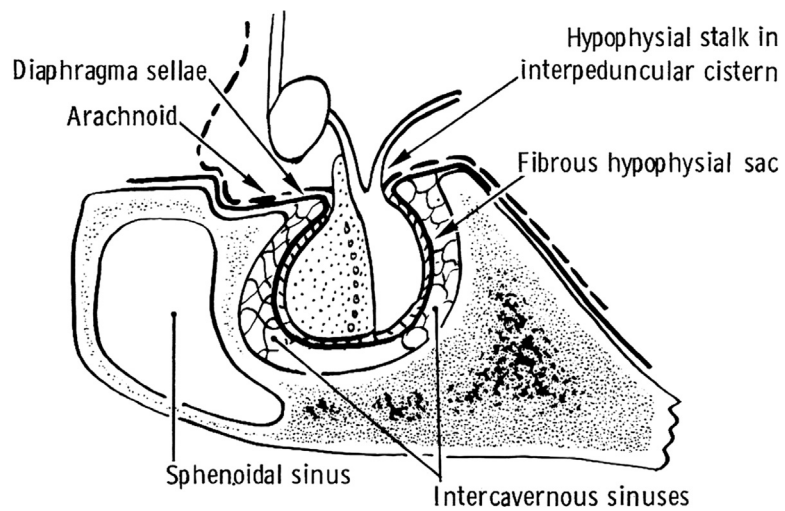


Fig. 3 A diagram of a median section through the pituitary and the body of the sphenoid bone.

CORONAL SECTION: HYPOPHYSIS CEREBRI

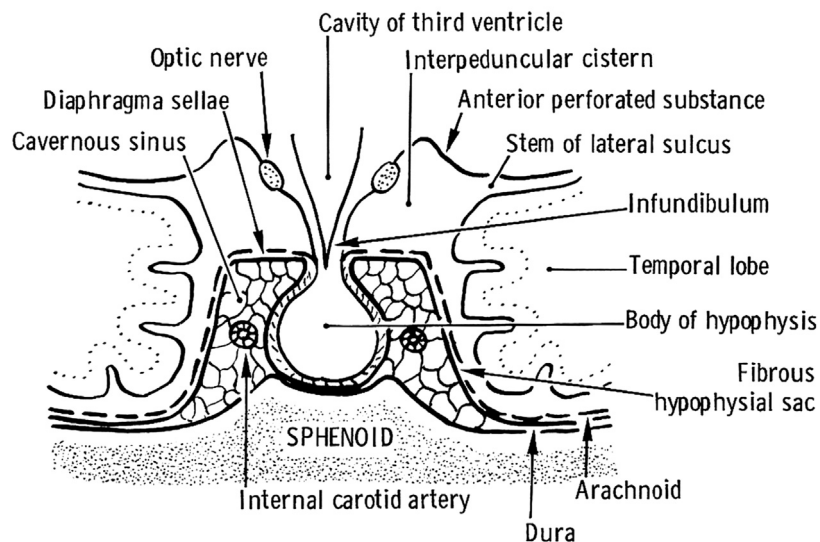


Fig. 4 A diagram of a coronal section of the pituitary gland and adjacent structures.

by signals from a collection of nerve cells in the hypothalamus called the arcuate nucleus. Thus external stimuli, such as the changes that occur due to seasonal alterations in photoperiod, are detected by the eyes and relayed to the hypothalamus. Therein, these visual stimuli are transduced into neural signals that are transmitted to pathways that affect the GnRH pulsatility. The latter, in turn, influence the secretion of FSH and LH by the pituitary. The early onset of pulsatile GnRH secretion results in precocious puberty whereas the failure to initiate pulsatile GnRH results in a delay or a failure to commence puberty in both males and females.

Modifications in the peptide structure of GnRH has resulted in some analogues having a higher affinity and high potency whereas other structural changes can result in agents that can bind to the receptor but fail to induce downstream signaling and can thus act as contraceptive agents.

As with most hormonal systems, the target tissue responds to the hormone and also sends feedback signals that modulate the bioactivity concerned. Thus the steroid hormones produced by the ovary and testis, estradiol and testosterone, exert a negative feedback action on the secretion of FSH and LH.

FSH also stimulates the ovary and the testis to secrete inhibin, a protein hormone that acts as its feedback regulator (Robertson et al., 1987). It is composed of two subunits termed α and β and specifically suppresses FSH and the DNA sequences for the encoding genes were cloned for bovine inhibin (Forage et al., 1986). A second protein that specifically suppresses FSH was also isolated (Robertson et al., 1987) and given the name FSH suppressing protein. The same protein was also isolated independently and given the name, follistatin, the name by which it is now known (Ying et al., 1987). Other agents such as neuropeptide Y enhance GnRH binding to its receptors and augment LH pulses. Immuno-neutralization of NPY attenuates the LH surge at ovulation. Similarly, the actions of estradiol on GnRH are complex, suppressing the magnitude in the first 36 h of exposure and then augmenting the response after 48 h.

Since the release of GnRH by the hypothalamus is pulsatile, the subsequent FSH and LH release by the pituitary is also pulsatile. The pulsatility is more evident for LH which has a shorter half-life than FSH. Through GnRH, which enables neural inputs into the regulation of the gonadotrophins, external factors such as photoperiod, nutrition, stress, infection, and inflammation can affect their secretory patterns. The arcuate nucleus, a collection of nerve cells or neurones in the hypothalamus, is responsible for setting the pulse frequency and amplitude of GnRH secretion. Thus the early onset of pulsatile secretion initiates precocious puberty and a delay in the onset results in delayed puberty. Some mutations in the GnRH receptor can negate the pulses of GnRH from influencing FSH and LH secretion and thus acting as a cause of the failure to initiate puberty.

The pathway controlling GnRH pulse frequency involves kisspeptin acting on the GPR-54 receptor on GnRH secreting neurones. In general kisspeptin and glutamate increase GnRH release and endogenous opioid peptides inhibit GnRH secretion. The actions of estrogenic steroids such as estradiol are complex with increasing levels being initially inhibitory and later stimulatory, in part dependent on the pulse frequency. As indicated above, the onset of FSH and LH secretion determines the timing of puberty in that an early onset results in precocious puberty and a failure or delay in the onset results in delayed puberty. In some instance, mutations in the genes encoding the GnRH receptor are the cause of the failure of FSH and LH secretion or result in low levels of these gonadotrophins causing delayed puberty or infertility. Mutations in the gene encoding this receptor are known to cause hypogonadotrophic hypogonadism which presents in males and females as the failure of pubertal onset. NPY is a 36 amino acid protein member of the pancreatic polypeptide family and enhances GnRH binding to gonadotrophs in the pituitary gland and augments the LH response to GnRH. In the testis, NPY expressing nerve fibers are confined to the capsule of the testis and capsular blood vessels (Allen et al., 1989).

Given that FSH and LH are the agents that circulate via the blood to the ovary and the testis, they are critical components that regulate spermatogenesis in males and oogenesis and ovulation in females. In turn, the ovarian and testicular steroid hormones, estradiol and testosterone, act predominantly as negative regulators of FSH and LH. In addition, FSH stimulates the gonads to produce a hormone, inhibin that acts as a specific inhibitor of FSH secretion. There are two forms of inhibin which are dimers of two subunits, alpha(α) and beta(β_A) and beta (β_B). Inhibin A ($\alpha\beta_A$) and Inhibin B ($\alpha\beta_B$) both suppress FSH secretion. Castration of males or females results in a rapid decrease in the serum inhibin levels indicating that the gonads are the source of these proteins. In the male, the Sertoli cells of the testes are the site of production of inhibin. There is yet another protein that regulates FSH and that is called follistatin which is structurally unrelated to the activins and inhibins (Meinhardt et al., 1998). In contrast to inhibin, the levels of follistatin did not change after castration, indicating that it was also produced at other sites.

There are also proteins that exert a stimulatory influence on FSH levels. These are dimers of the inhibin β_A and β_B units which can form proteins called activin A ($\beta_A\beta_A$) and activin B ($\beta_B\beta_B$) and stimulate FSH secretion (Vale et al., 1986; Ling et al., 1986).

Follistatin acts by binding the activins with high affinity and in vivo, the follistatin-activin complex is targeted to a lysosomal degradation pathway. Follistatin also binds to heparin sulfate proteoglycans that form part of the basement membranes of many tissues throughout the body. In addition to binding and inactivating the activins, follistatin also binds another protein called myostatin which, as its name suggests, is a "repressor" of muscle mass. Thus mutations of myostatin in cattle, which render myostatin inactive, have very large muscles as seen in the Belgian Blue breed of cattle.

The amino acid sequence of the activins and follistatin from rodents and humans is very highly conserved such that they are virtually identical. This degree of conservation across such a wide range of animal species suggests that these proteins have important functions.

Although, the activins are involved in the control of FSH, they are also regulators of our body's response to infection. Within 30 min of a challenge using lipopolysaccharide (LPS), a component of the *Salmonella* bacterium, activin A increases significantly (Jones et al., 2007). Other data available suggests that activin A is a major regulator of our body's inflammatory response. For instance, the levels of serum activin A and activin B are markedly elevated in patients admitted to intensive care units with acute

respiratory failure caused by a variety of agents (de Kretser et al., 2013) Should they remain elevated, they are indicators of a very poor outcome not just in the acute phase of the infection but also for up to a year after that hospital admission.

The outcome of inflammation in many organs is fibrosis or scarring and activin is responsible for the fibrosis and follistatin can be used to attenuate the degree of fibrosis.

While this section focuses on the male, these proteins have important regulatory functions in the female, covered in another section of this Encyclopedia of Reproduction.

Acknowledgment

The figures are based on illustrations from Regional Anatomy Illustrated, Churchill Livingstone 1983, a company that does not exist any longer. It was formed from a merger of E&S Livingstone (Edinburgh, Scotland) and J&A Churchill (London). This company, originally owned by Harcourt and Pearson, is now integrated into Elsevier's Health Science Division.

References

- Allen, L. G., Wilson, F. J., & MacDonald, G. J. (1989). Neuropeptide Y containing nerve fibres in rat gonads: Sex difference and development. *Biology of Reproduction*, 1989(40), 371–378.
- de Kretser, et al. (2013). Serum activin A and B levels predict outcome in patients with acute respiratory failure: A prospective cohort study. *Critical Care*, 17, R263.
- Forage, R. G., Ring, J. M., Brown, R. W., McInerney, B. V., Cobon, G. S., Gregson, R. P., Robertson, D. M., Morgan, F. S., Hearn, M. T., Findlay, J. K., & de Kretser, D. M. (1986). Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proceedings of the National Academy of Sciences of the United States of America*, 83, 3091–3095.
- Gersh, I. (1938). Relation of histological structure to the active substances extracted from the posterior lobe of the hypophysis. *Research Publications - Association for Research in Nervous and Mental Disease*, 17, 433.
- Green, J. D., & Harris, G. W. (1951). The neurovascular link between the neurohypophysis and adenohypophysis. *The Journal of Endocrinology*, 5, 136–144.
- Guillemin, R. (1964). Hypothalamic factors releasing pituitary hormones. *Recent Progress in Hormone Research*, 20, 89–130.
- Harris, G. W. (1948). Neural control of the pituitary gland. *Physiological Reviews*, 28, 139–145.
- Jones, K. L., Mansell, A., Patella, S., Scott, B. J., Hedger, M. P., de Kretser, D. M., & Phillips, D. J. (2007). Activin is a critical component of the inflammatory response and its binding protein follistatin reduces mortality in endotoxemia. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 16239–16244.
- Ling, N., Ying, S. Y., Ueno, N., Shimasaki, S., Esch, F., Hotta, M., & Guillemin, R. (1986). Pituitary FSH is released by a heterodimer of β subunits from the two forms of inhibin. *Nature*, 321, 779–782.
- Meinhardt, A., O'Bryan, M. K., McFarlane, J. R., Lovelad, K. L., Mallidis, C., Foulds, L. M., Phillips, D. J., & de Kretser, D. M. (1998). Localisation of follistatin in the testis. *Journal of Reproduction and Fertility*, 112, 233–241.
- Popa, G., & Fielding, U. (1931). A portal circulation from pituitary to hypothalamic region. *Journal of Anatomy*, 65, 88–90.
- Robertson, D. M., Klein, R., de Vos, F., McLachlan, R. I., Wettenhall, R. E., Hearn, M. T., Burger, H. G., & de Kretser, D. M. (1987). The isolation of polypeptides with FSH suppressing activity from bFF which are structurally different to inhibin. *Biochemical and Biophysical Research Communications*, 149, 744–749.
- Smith, P. E. (1930). Hypophysectomy and replacement therapy. *American Journal of Anatomy*, 45, 205–207.
- Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., Woo, W., Karr, D., & Spies, J. (1986). Purification and characterisation of an FSH releasing protein from porcine ovarian fluid. *Nature*, 321, 776–779.
- Ying, S. Y., Becker, A., Swanson, G., Tan, P., Ling, N., Esch, F., Ueno, N., Shimasaki, R., & Guillemin, R. (1987). Follistatin specifically inhibits pituitary follicle stimulating hormone release in vitro. *Biochemical and Biophysical Research Communications*, 149, 133–139.

Further Reading

- Robertson, D., Foulds, L., Leversha, L., Morgan, F. J., Hearn, M. T., Burger, H. G., Wettenhall, R. E., & de Kretser, D. M. (1985). Isolation of inhibin from bovine follicular fluid. *Biochemical and Biophysical Research Communications*, 126, 220–226.

Pituitary Cell and Molecular

Pauline Campos, Matan Golan, and Ombeline Hoa, University of Montpellier, Montpellier, France
Tatiana Fiordeliso, Universidad Nacional Autónoma de (UNAM), CDMX, México
Patrice Mollard, University of Montpellier, Montpellier, France

© 2018 Elsevier Inc. All rights reserved.

The Gonadotropic System

The Gonadotropes

Gonadotropes are endocrine cells secrete LH and FSH located in the anterior pituitary. In the adult, they constitute 7%–15% of the anterior pituitary population (Moriarty, 1976). Importantly, studies have revealed 70% of the gonadotropes are bihormonal while 15% and monohormonal. These percentages can shift under different physiological conditions, such as castration or oestrous cycle (Moriarty, 1976).

Distribution of the Gonadotropes

Cells of the adult mammalian anterior pituitary were primarily described as part of the “diffuse endocrine system” (initially coined by Feyrter in 1938), with an appearing heterogeneous distribution of the different hormone-producing cells, however, studies over the last decade have challenged this concept in mouse models and demonstrated that the anterior pituitary consists of intermingled and highly-organized 3D networks of both endocrine and non-endocrine cells, including gonadotropes (Mollard et al., 2012). In the embryo, it appears that developing gonadotropes are initially organized in cell clusters in the central medio-lateral region of the anterior pituitary, with some isolated cells evident in more lateral regions (Budry et al., 2011). At birth the network of gonadotropes is organized as strings of cells extending from the ventral surface towards the center of the gland, with a second wave of gonadotropes appearing with the first 2 weeks postnatally on the dorsal surface, again organized as cell strings. At puberty and later on in adulthood, the network of gonadotropes is in close proximity with the pituitary microvasculature which ensures both the distribution of incoming GnRH towards gonadotropes and collection of secreted LH and FSH (Budry et al., 2011).

The Gonadotropins

The gonadotropins, LH and FSH are dimeric pituitary glycoprotein hormones have a common alpha and distinct beta subunit. The beta subunit confers unique biological activity on these hormones. LH and FSH appear to be packaged into different secretory granules when localized in bihormonal gonadotropes (Childs et al., 1986). After synthesis in the endoplasmic reticulum and passage through the Golgi apparatus, hormones are delivered to the plasma membrane through a constitutively or regulated secretory pathway; in the latter, fusion of secretory vesicles to the plasma membrane is arrested waiting for specific signals to be secreted (Durán-Pastén and Fiordeliso, 2013). The biosynthesis and secretion of LH and FSH is tightly regulated by counterbalancing positive inputs from the hypothalamus (GnRH) and negative feedback from the gonads (steroid and peptide hormones) as well as paracrine factors such as inhibins, activins, and follistatin (Durán-Pastén and Fiordeliso, 2013). Integration of these different regulatory signals by the gonadotropes results in the coordinated control of synthesis, packaging, and differential secretion of gonadotropins to accurately respond and control sexual maturation and normal reproductive function (Durán-Pastén and Fiordeliso, 2013).

GnRH Regulation of LH/FSH Secretion

Gonadotropins are released under the control of gonadotropin-releasing hormone (GnRH) (Iida et al., 1991; Leong and Thorner, 1991). This neurohormone is a decapeptide secreted in the portal blood by the GnRH neurons which are located in the hypothalamus. GnRH binds to its receptor that is located at the surface of gonadotropes and coupled to a G-protein system.

Several studies have shown that there is an absolute necessity of a pulsatile GnRH stimulus to sustain physiologic gonadotrope function (Belchetz et al., 1978). It is indeed possible to restore normal gonadotropin secretion with pulsatile GnRH administration in castrated monkeys bearing hypothalamic lesions rendering them hypogonadotropic, whereas continuous infusion of the peptide is completely ineffective (Belchetz et al., 1978). Similarly, in humans, continuous infusion of GnRH in normal subjects for prolonged periods first raises plasma levels of LH and FSH but this response attenuates after about 5 h and is completely lost after a few days (Gossage and Duncan, 1985). Those results indicate that continuous GnRH can result in a desensitization or down regulation of the processes responsible for gonadotropin release (Belchetz et al., 1978). The loss of the GnRH response with continuous treatment is known to be due to rapid uncoupling of the GnRH receptor from its intracellular signaling molecules (Adams et al., 1986; Tsutsumi and Webster, 2009). It has been showed that on one hand, continuous GnRH stimulation causes a transient increase in Gs/cAMP signaling that rapidly returns to baseline whereas Gq/11/DAG/Ca²⁺ signaling remains elevated during the entire period of GnRH stimulation causing a downregulation of receptor number. Pulsatile GnRH, on another hand, causes matching pulses of Gs/cAMP signaling of constant amplitude over time and Gq/11/DAG/Ca²⁺ signaling shows an initial pulse matching

but then feedback on itself so no further pulses are seen after 2 h, allowing a time locked regulation of LH pulses in response to GnRH pulses (Tsutsumi and Webster, 2009). Pulsatile stimulation with GnRH agonists can therefore be used to stimulate gonadotropin secretion, whereas sustained treatment ultimately reduces gonadotropin secretion, and this underlies GnRH agonist efficacy against steroid hormone-dependent cancers (Conn and Crowley, 1994).

Pulsatile Secretion of LH

In both males and females, secretion of LH and FSH is episodic, with secretory bursts that are mediated by a concordant episodic release of GnRH. The intervals between LH pulses in vivo range from 30 to 60 min in the gonadectomized rat (between 30 and 40 min), sheep (about 60 min), and monkey (50 min), with longer intervals reported in intact animals (Levine et al., 1982; Clarke and Cummins, 1985; Levine and Duffy, 1988; Terasawa et al., 1988; Goodman et al., 1995). Pulses of LH are characterized by their specific asymmetric shape with a fast increase immediately followed by a slower decrease, that is due to the clearance rate of LH from the blood. Overall, the intricate relationship between pulsatile GnRH release and secretory capacity of the pituitary gonadotropes generates the cyclic fluctuations in LH secretion.

Role of Calcium in GnRH-Stimulated Gonadotropin Secretion

Calcium Signaling in Gonadotrope Cells

Exocytosis in excitable cells is dependent on intracellular Ca^{2+} concentration. Gonadotropes, as other pituitary endocrine cells, display spontaneous intracellular Ca^{2+} transients dependent on changes in the membrane electrical activity (Leong and Thorner, 1991; Zemkova et al., 2006; Durán-Pastén and Fiordelisio, 2013). However, these membrane potential oscillations are small and do not produce the necessary intracellular Ca^{2+} increase to generate hormonal secretion (Tse et al., 1997; Stojilkovic et al., 2005). As a result, without extrinsic stimulus, basal secretion is low (Stojilkovic, 2006; Stojilkovic et al., 2005). In gonadotropes, the cytosolic Ca^{2+} levels regulate several maturation steps that secretory vesicles must undergo prior to fusion, like priming of secretory vesicles (Martin, 2003) but an entirely different phenomenon occurs when the intracellular Ca^{2+} concentration rises abruptly, and promotes the fusion of docked secretory vesicles with the plasma membrane. In contrast to nerve synapses, where intracellular Ca^{2+} influx is primarily responsible for this abrupt rise, exocytosis in gonadotropes is triggered to a large extent by calcium released from intracellular stores. Indeed, once GnRH binds its receptor, the α subunit of the G α q/11 protein dissociates and activates PLC- β , resulting in the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP2) and the production of two second messengers: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (InsP3). InsP3 binds to its receptor in the endoplasmic reticulum leading to oscillatory Ca^{2+} release and Ca^{2+} -dependent modulation of membrane electrical activity. Each Ca^{2+} elevation is in phase with membrane hyperpolarization, resulting in the opening of Ca^{2+} dependent SK-type K $^{+}$ channels (Kukuljan et al., 1997) and also Ca^{2+} channels (predominantly high-voltage-activated L-type calcium channels), allowing Ca^{2+} influx. Thus GnRH stimulates rapid Ca^{2+} mobilization from internal IP3-sensitive stores, followed by external Ca^{2+} influx. In most cases, the latest does not contribute to general Ca^{2+} elevation or gonadotropin secretion, but is crucial for refilling the intracellular Ca^{2+} pools (Stojilkovic et al., 1993).

Three different types of Ca^{2+} responses can be elicited in single gonadotropes depending on the concentration of GnRH: sub-thresholds, baseline oscillations and biphasic responses (Naor, 2009; Durán-Pastén and Fiordelisio, 2013). Subthreshold GnRH concentrations produce either small monophasic Ca^{2+} transients or irregular small Ca^{2+} spikes. With higher GnRH concentrations, large oscillatory Ca^{2+} transients are produced. Eventually these Ca^{2+} spikes fuse into an biphasic Ca^{2+} response which comprises two variants; biphasic oscillatory and biphasic non-oscillatory, also known as spike-plateau. It has been clear that different Ca^{2+} release patterns observed with increasing doses of GnRH underlie the dose dependent increase of gonadotropin secretion, but these different patterns also encode other cell functions. For instance, spike-plateau Ca^{2+} responses are associated to LH secretion and oscillatory Ca^{2+} responses to the synthesis of LH β -subunits (Naor, 2009). Whereas LH secretion is recreated in a pulsatile manner depending on GnRH stimulation; FSH secretion is mainly constitutive, thereby protein sorting domains in the β subunit of gonadotropins and the association with certain proteins may be responsible for differential sorting and packaging of LH and FSH into different secretory granules (Anderson, 1996).

Gonadotropin Exocytosis

Secretory granules containing LH and FSH must undergo a well-defined series of events: recruitment, tethering at the plasma membrane, priming, and vesicle fusion with the plasma membrane. Granules secretion in gonadotropes, as in other secretory cells, is a mechanism that uses the secretory vesicle synaptotagmin as a Ca^{2+} sensor and is mediated by SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins as effectors. Practically, synaptotagmin detects Ca^{2+} elevation and allows fusion of the gonadotropin-containing-vesicles to the membrane. Using electron microscopy, it has been showed that vesicles containing LH and FSH docks in a SNARE-depending manner into dedicated areas with diameter ranging from 100 to 500 nm whereas additional pores of 20–40 nm diameter have also been found, probably representing the constitutive pathway of gonadotropins (Savigny et al., 2007).

Transcriptional Regulation of Gonadotropins

Regulation of gonadotropins also occurs at the transcriptional level. The gonadotropins are composed of three subunits: a common alpha subunit that is similar in both LH and FSH, and a unique beta subunit that confers the biological specificity. The three subunits are encoded by different genes that are located on separate chromosomes and can therefore be differentially regulated. GnRH plays a major role in the control of the synthesis of the two gonadotropins, but it is by no means a sole factor. A complex interplay exists with other components of the HPG axis through feedback loops. Together, these combined factors allow the fine tuning of the transcription levels of the three subunits and hence form the basis of the ability of the pituitary to match its output to various reproductive states.

The Effect of GnRH

The transcription of the three subunits is highly sensitive to changes in the GnRH pulse frequency, and, to a lesser extent, the amplitude of the pulse. In males, rapid pulses (a pulse every 30 min) increase expression in both beta subunits, and more significantly of LH β , whereas slow frequencies (a pulse every 2–4 h) favor expression of the FSH β . Only the expression of the LH β is sensitive to changes in amplitude of GnRH pulses. The alpha subunit seems to be less responsive to changes in GnRH pulse frequency. The increase in transcript levels occurs within minutes after a GnRH pulse, but in order to sustain high transcription levels, subsequent pulses need to be present at the appropriate frequency (high frequencies will favor LH β transcription whereas low frequencies will enhance the transcription of FSH β).

The ability of the transcription machinery to respond to the changing pulse frequencies is mediated through the intracellular signal transduction pathways. Binding and activation of the GnRH receptor induces an increase of intracellular calcium as well as activation of protein kinase C (PKC). Both factors will act downstream through intermediate messengers to affect the synthesis of the subunit genes.

The calcium concentration in gonadotropes increases following activation of the GnRH receptor, both due to entrance of calcium through the plasma membrane and release from internal stores. This increase in intracellular calcium stimulates gonadotropin subunit transcription in a frequency-dependent manner, with higher frequencies increasing the expression of LH β and slower frequencies selectively enhancing FSH β transcription. These effects are mediated through the activation of calmodulin by the increase in intracellular calcium which in turn activates various second messengers that act at the nucleus to increase the transcription of the gonadotropin subunits in a frequency dependent manner (Melamed et al., 2012).

Calcium, along with PKC, also affect transcription through the mitogen-activated protein kinase (MAPK) pathway. The binding of the GnRH receptor activates PKC which, along with an increase in intracellular calcium, induces the activation of MAPK family of proteins that phosphorylate transcription factors that induce gonadotropin subunit synthesis. The activation of MAPK proteins is highly sensitive to pulsatile GnRH application and can therefore mediate the frequency of the GnRH pulsatility to the nucleus.

Gonadal Steroids

The gonadal steroids act as a crucial link between the gonad and the higher levels of the HPG axis, providing another pathway to differentially affect the synthesis of the gonadotropin subunits. The canonical pathway of steroid hormone action is through binding to steroid receptors which are then translocated to the nucleus and bind specific responsive elements in the promotor of genes, thus exerting their effect on gene transcription. In the male, circulating androgens affect the transcription of all three gonadotropin subunits. In general androgen can affect gonadotropin expression both through direct action on gonadotropes and through indirect action on the pulsatile secretion of GnRH. Androgens inhibit the expression of the alpha and LH β subunits. The exact mode of this inhibition is unclear as specific androgen-receptor responsive elements have not been identified in all LH β promoters studied and it is possible that the action is mediated by interactions with other transcription factors. Interestingly, in rodent models androgen directly interacts with the FSH β gene to up-regulate its expression.

Activin, Inhibin, and Follistatin

The FSH β gene is also under tight regulation by the activating-inhibin and follistatin hormones. While the full descriptions of these hormones are described in other chapters, it is important to mention them here briefly as they form part of the differential regulation mechanism of LH β and FSH β by exerting a specific control over the expression of the FSH β gene. Activins are critical regulators of FSH β transcription. They bind to specific receptors on the gonadotrope membrane and ultimately activate SMAD elements at the nucleus that specifically enhance the synthesis of FSH β . Follistatin, produced both by the pituitary and in the gonads binds activin, preventing the interaction to its receptor and thus acts as an inhibitor of FSH β expression. Inhibins, produced by the gonads, interfere with the binding of activin to its receptor and therefore inhibit its ability to induce FSH β expression.

Gonadotrope Interactions and Plasticity at the Level of the Gland

Apart from the intracellular level, the gonadotropes interact between each other within their 3D cell network organization. This highly organized structure suggests homotypic cell–cell interactions. This communication between gonadotropes is mediated

through gap-junctions (Göngrich et al., 2016) pore-forming proteins that can act to connect adjacent cells by allowing passage of small molecules such as calcium and other cellular messengers. This mode of information transfer along the gonadotrope network allows the pituitary to mount its typical large-scale hormonal response to hypothalamic stimuli.

Moreover, it has been demonstrated that both immortalized cell lines and cells in pituitary slices extend cellular processes and increase cell movement after GnRH stimulation (Bliss et al., 2007), and it is possible that this type of dynamic reorganization of the gonadotrope exists in vivo. In fact, this network remodeling may have a role in modifying the response of gonadotropes by changing the relationship of the gonadotrope network with the vasculature (Tibolt and Childs, 1985; Hodson et al., 2012) as gonadotropes were found to change their position relative to the vasculature in response to gonadal steroids (Alim et al., 2012).

References

- Adams, T. E., Cumming, S., & Adams, B. M. (1986). Gonadotropin-releasing hormone (GnRH) receptor dynamics and gonadotrope responsiveness during and after continuous GnRH stimulation. *Biology of Reproduction*, 35(4), 881–889. Available at <http://www.ncbi.nlm.nih.gov/pubmed/3028518>.
- Alim, Z., et al. (2012). Gonadotrope plasticity at cellular and population levels. *Endocrinology*, 153(10), 4729–4739.
- Anderson, L. (1996). Intracellular mechanisms triggering gonadotrophin secretion. *Reviews of Reproduction*, 1(3), 193–202.
- Belchetz, P., et al. (1978). Hypophysial responses to continuous and intermittent delivery of hypothalamic gonadotropin-releasing hormone. *Science*, 202(4368), 631–633. Available at <http://www.sciencemag.org/cgi/doi/10.1126/science.100883>.
- Bliss, S. P., et al. (2007). Signaling complexes associated with the type I gonadotropin-releasing hormone (GnRH) receptor: colocalization of extracellularly regulated kinase 2 and GnRH receptor within membrane rafts. *Molecular Endocrinology (Baltimore, Md.)*, 21(2), 538–549. Available at <http://www.ncbi.nlm.nih.gov/pubmed/17068198>.
- Budry, L., et al. (2011). Related pituitary cell lineages develop into interdigitated 3D cell networks. *Proceedings of the National Academy of Sciences of the United States of America*, 108(30), 12515–12520.
- Childs, G. V., et al. (1986). Cytochemical evidence for different routes of gonadotropin-releasing hormone processing by large gonadotropes and granulosa cells. *Endocrinology*, 119(3), 1329–1338.
- Clarke, I. J., & Cummins, J. T. (1985). Increased gonadotropin-releasing hormone pulse frequency associated with estrogen-induced luteinizing hormone surges in ovariectomized ewes. *Endocrinology*, 116(6), 2376–2383.
- Conn, P. M., & Crowley, W. F., Jr. (1994). Gonadotropin-releasing hormone and its analogs. *Annual Review of Medicine*, 45, 391–405. Available at: <https://doi.org/10.1146/annurev.med.45.1.391>.
- Durán-Pastén, M. L., & Fiordelisio, T. (2013). GnRH-induced Ca^{2+} signaling patterns and gonadotropin secretion in pituitary gonadotrophs. Functional adaptations to both ordinary and extraordinary physiological demands. *Frontiers in Endocrinology*, 4, 1–13.
- Göngrich, C., et al. (2016). Electrotonic coupling in the pituitary supports the hypothalamic-pituitary-gonadal axis in a sex specific manner. *Frontiers in Molecular Neuroscience*, 9, 1–12.
- Goodman, R. L., et al. (1995). Endogenous opioid peptides control the amplitude and shape of gonadotropin-releasing hormone pulses in the ewe. *Endocrinology*, 136(6), 2412–2420.
- Gossage, A., & Duncan, S. (1985). The role of gonadotrophin releasing hormone in the investigation and treatment of hypogonadism. *Postgraduate Medical Journal*, 61(713), 195–200.
- Hodson, D. J., et al. (2012). Existence of long-lasting experience-dependent plasticity in endocrine cell networks. *Nature Communications*, 3, 605.
- Iida, T., et al. (1991). Spontaneous and agonist-induced calcium oscillations in pituitary gonadotrophs. *Molecular Endocrinology (Baltimore, Md.)*, 5(7), 949–958. Available at <http://www.ncbi.nlm.nih.gov/pubmed/1944300>.
- Kukuljan, M., Vergara, L., & Stojilkovic, S. S. (1997). Modulation of the kinetics of inositol 1,4,5-trisphosphate-induced $[\text{Ca}^{2+}]_i$ oscillations by calcium entry in pituitary gonadotrophs. *Biophysical Journal*, 72(2 Pt 1), 698–707.
- Leong, D. A., & Thorner, M. O. (1991). A potential code of luteinizing hormone-releasing hormone-induced calcium ion responses in the regulation of luteinizing hormone secretion among individual gonadotropes. *Journal of Biological Chemistry*, 266(14), 9016–9022.
- Levine, J. E., & Duffy, M. T. (1988). Simultaneous measurement of luteinizing hormone (LH)-releasing hormone, LH, and follicle-stimulating hormone release in intact and short-term castrate rats. *Endocrinology*, 122(5), 2211–2221.
- Levine, J. E., et al. (1982). Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanesthetized, ovariectomized sheep. *Endocrinology*, 111(5), 1449–1455. Available at <http://www.ncbi.nlm.nih.gov/pubmed/6751794>.
- Martin, T. F. J. (2003). Tuning exocytosis for speed: fast and slow modes. *Biochimica et Biophysica Acta – Molecular Cell Research*, 1641(2–3), 157–165.
- Melamed, P., et al. (2012). Gonadotrophin-releasing hormone signalling downstream of calmodulin. *Journal of Neuroendocrinology*, 24(12), 1463–1475.
- Mollard, P., et al. (2012). A tridimensional view of pituitary development and function. *Trends in Endocrinology and Metabolism*, 23(6), 261–269.
- Moriarty, G. C. (1976). Immunocytochemistry of the pituitary glycoprotein hormones. *Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*, 24(7), 846–863. Available at: <https://doi.org/10.1177/24.7.60435>.
- Naor, Z. (2009). Signaling by G-protein-coupled receptor (GPCR): Studies on the GnRH receptor. *Frontiers in Neuroendocrinology*, 30(1), 10–29.
- Savigny, P., Evans, J., & McGrath, K. M. (2007). Cell membrane structures during exocytosis. *Endocrinology*, 148(8), 3863–3874.
- Stojilkovic, S. S. (2006). Pituitary cell type-specific electrical activity, calcium signaling and secretion. *Biological Research*, 39(3), 403–423. PMID 17106574.
- Stojilkovic, S. S., et al. (1993). Mechanism of agonist-induced $[\text{Ca}^{2+}]_i$ oscillations in pituitary gonadotrophs. *Journal of Biological Chemistry*, 268(11), 7713–7720.
- Stojilkovic, S. S., Zemkova, H., & Van Goor, F. (2005). Biophysical basis of pituitary cell type-specific Ca^{2+} signaling-secretion coupling. *Trends in Endocrinology and Metabolism*, 16(4), 152–159.
- Terasawa, E., et al. (1988). Norepinephrine is a possible neurotransmitter stimulating pulsatile release of luteinizing hormone-releasing hormone in the rhesus monkey. *Endocrinology*, 123(4), 1808–1816.
- Tibolt, R. E., & Childs, G. V. (1985). Cytochemical and cytophysiological studies of gonadotropin-releasing hormone (GnRH) target cells in the male rat pituitary: differential effects of androgens and corticosterone on gnRH binding and gonadotropin release. *Endocrinology*, 117(1), 396–404.
- Tse, F. W., et al. (1997). Local Ca^{2+} release from internal stores controls exocytosis in pituitary gonadotrophs. *Neuron*, 18(1), 121–132.
- Tsutsumi, R., & Webster, N. J. G. (2009). GnRH pulsatility, the pituitary response and reproductive dysfunction. *Endocrine Journal*, 56(6), 729–737. Available at <http://joi.jlc.jst.go.jp/JST.JSTAGE/endorj/K09E-185?from=CrossRef>.
- Zemkova, H., et al. (2006). Roles of purinergic P2X receptors as pacemaking channels and modulators of calcium-mobilizing pathway in pituitary gonadotrophs. *Molecular Endocrinology (Baltimore, Md.)*, 20(6), 1423–1436. Available at <http://www.ncbi.nlm.nih.gov/pubmed/16543406>.

Follicle Stimulating Hormone (FSH) and Male Reproduction in Mammals

Peter J O'Shaughnessy, University of Glasgow, Glasgow, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Activin Protein hormones/growth factors that are important in development and adult function. Member of the transforming growth factor-beta (TGFβ) family.

Apoptosis Tightly regulated cell suicide also called programmed cell death.

Azoospermia Lack of sperm in the ejaculate.

Hypophysectomy Surgical procedure to remove the pituitary gland.

Inhibin Protein hormone/growth factor related to activin.

Leydig cells Androgen-producing cells found in the testis interstitium.

Microarray Method for measuring the levels of large numbers of different mRNA transcript species.

Mitogen Factor that stimulates mitosis.

Oligospermia Low sperm concentrations in the ejaculate.

Sertoli cells Somatic cells which make up part of the epithelium of the seminiferous tubule and which regulate testis development and spermatogenesis.

Spermatid Male germ cell type that undergoes a differentiation process (called spermiogenesis) to form a spermatozoon.

Spermatocyte Male germ cell type that undergoes meiosis to form a spermatid.

Spermatogenesis Production of spermatozoa from spermatogonial stem cells.

Spermatogonia Basic male germ cell type which undergoes mitosis and is the engine driving the large number of germ cells formed in the testis. Differentiates into a spermatocyte during spermatogenesis.

Transcription factor Protein that controls the rate of DNA transcription to mRNA.

Introduction

Follicle stimulating hormone (FSH) is a protein hormone made up of two separate protein subunits, an alpha subunit (which is common to three different hormones: FSH, luteinising hormone (LH), and thyroid stimulating hormone (TSH)) and a beta subunit which is specific to FSH. The hormone is secreted into the blood by the gonadotrope cells of the anterior pituitary gland which is found at the base of the brain. Release of FSH is in response to stimulation of the pituitary by gonadotropin releasing hormone (GnRH) which is released from a regulatory part of the brain called the hypothalamus. In the adult female FSH is critical for ovarian follicle growth and without appropriate FSH stimulation an individual would not be fertile. FSH and LH (which is also released by gonadotrope cells) have evolved as separate hormones from a common ancestral hormone and it is likely that it was the needs of ovarian function in mammals that drove the evolution of two separate hormone (Huhtaniemi, 2015). In males, LH is known to be critical for the regulation of testis function and for fertility but the role that FSH plays is less straightforward and our understanding of the topic has undergone considerable revision over the years. This article will provide an examination of our current knowledge of FSH action largely derived from animal studies (rodents in particular) but also using examples from human and non-human primate research where relevant.

To understand the role of FSH in the male a knowledge of the cell biology of the testis is essential. Briefly, the testis is divided into two component parts (1) the seminiferous tubules (*red asterisk in Fig. 1A*) where germ cells develop from spermatogonia into mature sperm in a process called spermatogenesis and (2) the interstitial tissue (*green asterisk in Fig. 1A*) which lies between the seminiferous tubules. The interstitial tissue contains the Leydig cells (*red arrows in Fig. 1B*) which act primarily to secrete androgens (mainly testosterone). In addition to germ cells undergoing development (*black arrowheads in Fig. 1B*), the seminiferous tubules also contain the Sertoli cells (*blue arrows in Fig. 1B*) which have a primary role in testis development and function and act to maintain spermatogenesis (Rebourcet et al., 2014a,b, 2016; Smith et al., 2015). Spermatogenesis itself is divided into three phases; a proliferative phase where spermatogonia undergo mitotic division to increase their numbers and become spermatocytes, a meiotic phase in which the spermatocytes undergo a specialized type of cell division to become spermatids followed by a final maturation phase (called spermiogenesis) in which the post-meiotic spermatids undergo differentiation to form the mature sperm.

From a historical perspective, a role for FSH in male reproduction was first postulated in the 1930s by Roy Greep and colleagues although, at around the same time, it was also reported that LH could act to stimulate testis function and spermatogenesis. Subsequently, it was shown that LH acts on the Leydig cells of the testicular interstitial tissue to stimulate testosterone synthesis which, in turn, travels to the seminiferous tubules and stimulates spermatogenesis through actions on the Sertoli cells (Fig. 1C). The essential

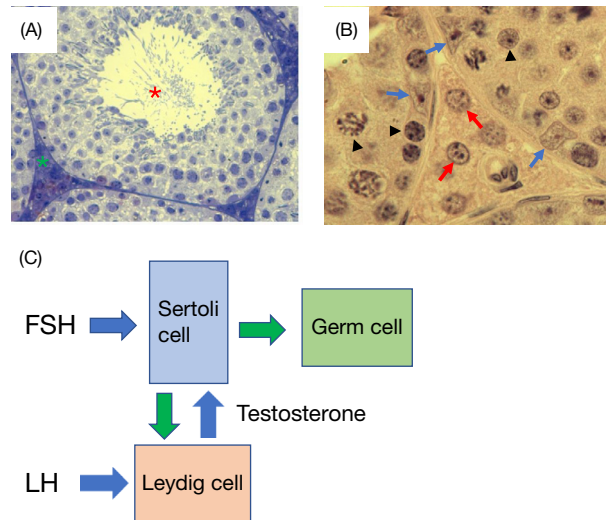


Fig. 1 Testis morphology and interactions between different testicular cell types. (A) Low power micrograph showing a section through a mammalian testis. A cross section through a seminiferous tubule is labeled with a red asterisk while a representative section of interstitial tissue, found between different seminiferous tubules, is labeled with a green asterisk. (B) Higher power image showing interstitial tissue and the walls of different seminiferous tubules. Representative Leydig cells are shown with red arrows while Sertoli cells are shown with blue arrows. There are many different germ cell types in the walls of the seminiferous tubules and representative cells are identified with black arrowheads. (C) Schematic diagram showing interactions between the main somatic and germ cell types in the testis and how these interactions are regulated by the gonadotropins LH and FSH. The Leydig cells are stimulated by LH to produce testosterone and both FSH and testosterone act to stimulate the Sertoli cells. The Sertoli cells, in turn, regulate Leydig cells activity and stimulate germ cell proliferation and differentiation. Blue arrows show stimulation by known factors while green arrows show that the stimulatory factors are not known.

role of LH and testosterone in the control of spermatogenesis has been confirmed many times since these early studies but there is still debate about the role of FSH in testicular development and in adult testis function. In part this is because the importance of FSH may change as the animal or individual develops through puberty but, also, there are species differences in the significance of FSH in male reproduction. Finally, uncertainty about the role of FSH also arises because spermatogenesis is a complex process that is dominated by androgen action and it can be difficult to pick apart the effects of FSH from those of androgen. What is known is that FSH acts through specific receptors on the Sertoli cells, that it stimulates Sertoli cell function (at least at some points in development) and that it probably acts to optimize spermatogenesis and the number of mature sperm produced by the testis.

Control of FSH Secretion

In the male FSH secretion is regulated by GnRH from the hypothalamus, by locally produced activins and by androgens and inhibin secreted by the testis. Principal control is by GnRH which is released in pulses by the hypothalamus and so FSH (and LH) secretion is also pulsatile. This secretion from the pituitary is then modulated by the other factors. Activins produced in the pituitary act to stimulate FSH secretion but activin action is inhibited by testicular inhibin which prevents activin acting at its receptor. Inhibin is secreted by the Sertoli cells under the regulation of FSH and so this is a negative feedback mechanism that acts to maintain steady state (albeit pulsatile) levels of the hormone. Finally, testicular androgen acts to inhibit GnRH secretion from the hypothalamus and thus reduces FSH secretion by the gonadotrophs. FSH does not act directly to regulate androgen secretion by the testis but this is another pathway by which the gonads can regulate FSH secretion.

The FSH Receptor

FSH acts by binding to the FSH-receptor (FSHR) which is only found on the Sertoli cells in the testis and so all effects of FSH on spermatogenesis or on Leydig cell function must be mediated through the Sertoli cells. The FSHR is located on the plasma membrane and is a transmembrane G protein-coupled receptor with both an extracellular and intracellular component. Binding of FSH to the extracellular component of the receptor causes conformational changes which activate the intracellular part of the receptor. This activation leads to stimulation of the enzyme adenyl cyclase which also sits within the plasma membrane of the Sertoli cell. Activation of adenyl cyclase, in turn, catalyzes formation of the nucleoside cyclic AMP (cAMP) from ATP. This cAMP acts as a "second messenger" which causes further stimulation of intracellular signaling pathways and changes in cell activity and gene transcription (Smith and Walker, 2015).

FSH Effects on the Sertoli Cell

At a molecular level, changes in cAMP levels in the Sertoli cell caused by FSH binding to its receptor act to regulate up to five different signaling pathways in the Sertoli cells (PKA, ERK/MAPK, PI3K, Ca^{2+} , and phospholipase A_2 (PLA_2) pathways) leading to altered activation of gene transcription in the cells (Smith and Walker, 2015) (Fig. 2). Changes in gene transcription are brought about largely as a result of changes in the expression and activity of a number of transcription factors including CREB (cAMP response element binding) (Fig. 2). Activation of PLA_2 by FSH also acts to stimulate formation of prostaglandins in the cells which can act as intracellular signals or may be secreted by the cells (Fig. 2).

On a broader level, studies of how FSH regulates Sertoli cell activity have used a variety of methods including Sertoli cell cultures, treatment of animals lacking gonadotrophins (e.g., hypophysectomised rats which have had their pituitary gland removed or hypogonadal (*hpg*) mice which lack GnRH through a mutation) and, more recently, transgenic mice in which either the FSHR or the FSH β subunit has been knocked out (generating FSHRKO and FSH β KO mice respectively). In the FSHRKO and FSH β KO transgenic models, loss of the receptor or loss of the FSH β subunit means that there is no FSH stimulation of the Sertoli cell. Using these models a number of studies have tried to determine what effect FSH has on the Sertoli cell beyond stimulation of second messengers. Results show that, before puberty, FSH acts to increase general Sertoli cell activity by increasing cellular gene transcription and by regulating protein translation. In addition, FSH appears to increase the synthesis of a number of specific proteins such as androgen-binding protein, inhibin, androgen receptor, lactate dehydrogenase (LDH), kit ligand (KL), and glial cell line-derived neurotrophic factor (GDNF). Several of these proteins are involved in spermatogenesis (LDH controls the synthesis of the major fuel source for the germ cells, KL is involved in the survival and proliferation of the spermatogonia, transferrin transfers iron to the germ cells and GDNF is required for the self-renewal and survival of the spermatogonial stem cells in the seminiferous tubules) and this may be one of the ways in which FSH regulates spermatogenesis.

In addition to stimulating Sertoli cell activity, other studies using FSHRKO and FSH β KO mice have shown that FSH is essential for ensuring that the normal number of Sertoli cells form during development. Sertoli cell proliferation occurs primarily during fetal development in all mammalian species with a more species-specific pattern of development between birth and puberty. Near to puberty the Sertoli cells undergo a final differentiation stage in many species and cell proliferation stops. The number of Sertoli cells that form during development and that are present in the adult is important as it dictates both the number of germ cells that the testis can support and, probably, the number of Leydig cells which can form/survive in the adult (Rebourcet et al., 2014a,b). A decrease in either germ cell or Leydig cell number is likely to affect adult fertility and may have an effect on overall adult health through reduced blood levels of testosterone. The increase in Sertoli cell number during fetal/post-natal development is regulated primarily through androgen action and insulin/IGF signaling with the stimulatory effects of FSH, in mice at least, confined largely to the period between birth and puberty. These post-natal effects of FSH on Sertoli cell proliferation in the mouse may be mediated through the ERK/MAPK pathway and appear to require active insulin/IGF stimulation of the cells. While the role of FSH in

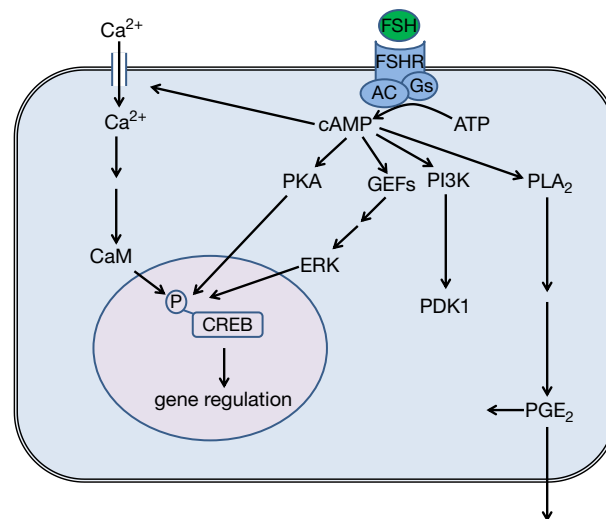


Fig. 2 FSH-signaling pathways in the Sertoli cell. Binding of FSH to its receptor causes the receptor-coupled G protein (Gs) to activate adenyl cyclase (AC) leading to the formation of cyclic AMP (cAMP) from ATP. Increased levels of cAMP in the cell can then activate a number of different pathways leading to gene regulation and prostaglandin formation in the cell. These include (i) activation of protein kinase A (PKA) which can directly lead to increased phosphorylation and activation of transcription factors such as cAMP response element binding (CREB) protein, (ii) action through guanine nucleotide exchange factors (GEFs) to activate CREB via extracellular signal-regulated kinase (ERK) (iii) induction of phospholipase A_2 (PLA_2) and formation of prostaglandin E_2 (PGE_2). FSH stimulation also leads to Ca^{2+} influx into the Sertoli cells and increased intracellular Ca^{2+} can activate multiple downstream paths including phosphorylation of CREB. Adapted from (Walker, W.H. and Cheng, J. (2005) FSH and testosterone signaling in Sertoli cells. *Reproduction* **130**, 15–28. (c) Society for Reproduction and Fertility (2005). Reproduced by permission.

determining the final, adult Sertoli cell number may be relatively limited in the mouse, evidence from other species is less clear and it is possible that FSH is of greater importance. There is evidence, for example, that in the rat and human FSH may act to stimulate Sertoli cell proliferation during fetal development (Allan and Handelsman 2005).

Control and timing of the final Sertoli cell maturation phase around puberty is important as it leads to the cessation of cell proliferation and determines the final number of cells that will be present in the adult. A decline in FSH action may be involved but it is also clear that the process requires thyroid hormone (TH) and in animals or humans with low TH levels there is a delay in maturation and continued Sertoli cell proliferation. Conversely, high TH levels lead to early Sertoli cell maturation, a reduced period of cell proliferation and fewer cells present in the adult testis. Overall, the number of Sertoli cells that form during development depends on the actions of androgen, FSH, insulin/IGF, TH and, possibly, local activin production.

As the Sertoli cells undergo the final, pubertal maturation phase of development they appear to become less sensitive and less responsive to the effects of FSH. This decline is associated with an increase in levels of the enzyme phosphodiesterase which acts to degrade cAMP and, thereby, limit intracellular signaling. There may also be more specific changes to the different intracellular pathways (such as a decrease in the ERK/MAPK path) mediated, perhaps, by changes in Sertoli cell adhesion to the wall of the seminiferous tubule. Whatever the mechanism, it has been suggested that the loss of FSH-sensitivity in the Sertoli cells is linked to initiation of final differentiation—that is, a decline in the ERK/MAPK pathway and increased cAMP degradation leads to a cessation in proliferation and a commitment to differentiated function although what induces this change in sensitivity is not known.

FSH effects on spermatogenesis

During Development

Spermatogenesis in the adult is a continuous process with germ cells at all stages of development present in the seminiferous tubules at all times (Fig. 1). Males are born, however, with only immature germ cells present in the testis and during post-natal development, therefore, there is a stage at which the first complete progression of spermatogenesis takes place from spermatogonia through to mature spermatozoa (also called a spermatogenic wave or cycle). This first wave, which completes after the onset of puberty, differs in comparison to later waves in that many of the developing germ cells undergo apoptosis (regulated cell death) at the spermatocyte stage and this appears to be an essential event which allows subsequent spermatogenic waves to occur normally. Studies have shown that one of the effects of FSH is to limit the extent of germ cell apoptosis during the first wave and if FSH levels are reduced there is a significant increase in germ cell death which may have knock-on effects to reduce germ cell number in the adult.

In the Adult

While there is good evidence that FSH is required for normal initiation of spermatogenesis it is less clear whether it plays a major role in the adult. Study of FSHRKO and FSH β KO mice shows that in the absence of FSH stimulation complete spermatogenesis takes place in the testes but the total number of germ cells is reduced (O'Shaughnessy, 2014). In comparison, there is failure to complete spermatogenesis leading to infertility in animals lacking androgen receptors on the Sertoli cells. In both FSHRKO and FSH β KO mice there is a 50%–60% loss of germ cells with all three categories of cells (spermatogonia, spermatocytes, and spermatids) showing a similar drop in numbers (O'Shaughnessy et al., 2012) (Fig. 3). This suggests that FSH is acting early in the process of spermatogenesis, during the proliferative stage of the spermatogonia, and that the reduced numbers of the more mature cells in FSHRKO and FSH β KO mice is a consequence of reduced spermatogonial number. Proliferative rates of spermatogonia *per se* are reported to be normal in FSHRKO mice and so the effect of withdrawal of FSH action appears to be an increase in apoptosis of the developing cells. Further evidence for this comes from *hpg* mutant mice (animals lack both LH and FSH) which have been crossed with animals lacking androgen receptors in the Sertoli cells (to create *hpg*.SCARKO mice). These animals are particularly useful because it is known that FSH can indirectly stimulate androgen production by the Leydig cells which can make interpretation of the effects of FSH complex. In *hpg*.SCARKO mice, however, androgens cannot affect Sertoli cell activity and so we can study FSH effects directly. Treatment of these animals with FSH increases spermatogonial and spermatocyte numbers without inducing spermatid formation (this would be predicted as completion of meiosis requires androgen action) consistent with results from the FSHRKO and FSH β KO mice. Overall, data from knockout mice and earlier *in vitro* studies suggest that the effect of FSH in the adult is to increase Sertoli cell number in the prepubertal period, the total number of germ cells in the testis and the number of germ cells associated with each Sertoli cell. These effects of FSH are achieved by increasing the number of spermatogonia in the testis and by increasing the number of these cells that enter meiosis (Abel et al., 2008). Overall, the likelihood is that FSH acts as a survival factor for premeiotic germ cells, limiting apoptotic cell death, rather than as a mitogen which stimulates cell proliferation (Huhtaniemi, 2015).

While FSH is clearly required to optimize spermatogenesis in the adult, what is less certain, however, is whether the loss of germ cells in adult FSHRKO and FSH β KO mice is due to loss of FSH action in the adult or to abnormal germ cell death during the first spermatogenic wave with consequential effects for subsequent spermatogenesis. Early studies on the effects of FSH administration to rats deprived of LH and FSH as adults (e.g., through hypophysectomy) would suggest that FSH is required into adulthood to maintain spermatogonial numbers and that FSH effects are not limited to the first spermatogenic wave. There are, however, limitations to these types of study as it is difficult to achieve normal physiological concentrations of the hormones through hormone

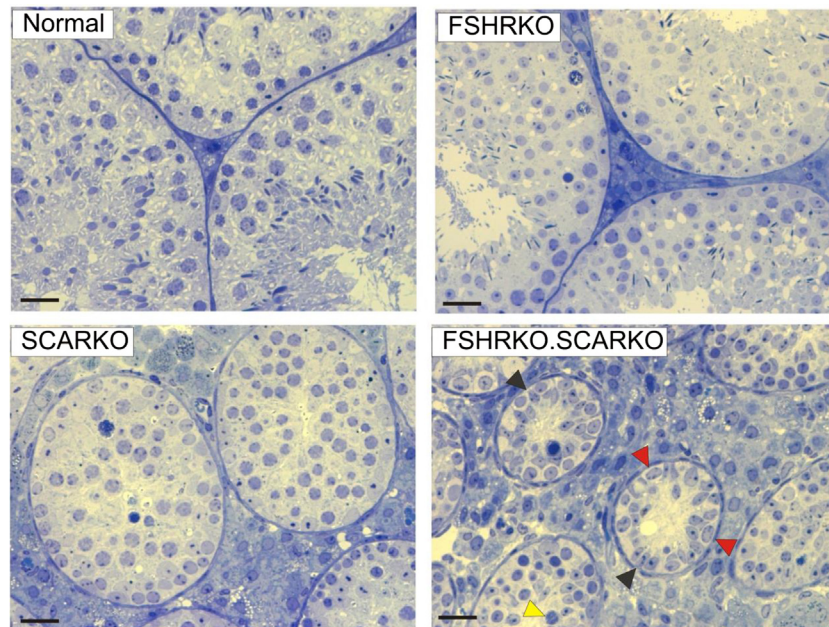


Fig. 3 Cross sections from testes of adult normal, FSHRKO, SCARKO, and FSHRKO·SCARKO mice. Testes from the FSHRKO mice contained all stages of spermatogenesis, although germ cell number was reduced. In SCARKO mice, there was progressive loss of spermatocytes as spermatogenesis progressed and few spermatids were present indicating a block in meiosis. In FSHRKO·SCARKO mice, the tubules were of a much smaller diameter with Sertoli cells (black arrowheads) and smaller numbers of spermatogonia (red arrowheads) seen in the tubules. Spermatogonia entered meiosis but development stopped early in meiosis in most cells (yellow arrowhead). From (Abel, M.H., Baker, P.J. and Charlton, H.M. *et al.* (2008) Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. *Endocrinology* **149**, 3279-3285 with permission from the Endocrine Society.

treatment regimes. One interesting study which has not yet, as far as I am aware, been carried out would be to determine whether FSH-treatment of FSH β KO mice restores normal spermatogenesis although, as above, achieving a normal FSH profile would be difficult and it is not clear whether androgen levels would change. At a cellular level, the effects of FSH on spermatogonial numbers are likely to be mediated through stimulated synthesis of specific proteins in the Sertoli cells (such as GDNF).

With respect to FSH and spermatogenesis, data from the rat and the mouse appear to apply generally across mammalian species, but there is one notable exception, the Djungarian Hamster. This animal is a seasonally breeding rodent in which daylength affects gonadotropin secretion causing the testes to go through cycles of activity and relative quiescence. Out of season, spermatogenesis is suppressed but treatment with FSH alone will induce full recovery without stimulating androgen secretion from the Leydig cells (Lerchl *et al.*, 1993). Testosterone will synergise with FSH and increase sperm production in these animals but the predominant hormone regulating spermatogenesis is FSH in a mirror image of other species.

Interactions With Androgen

As discussed, data from FSHRKO, FSH β KO, and SCARKO mice show that in the adult male androgens are the major drivers of spermatogenesis with FSH required mainly to increase the number of germ cells going through the process. Data from mice lacking Sertoli cell receptors for *both* FSH and androgen (FSHRKO·SCARKO mice) suggest, however, that there are interactions between the effects of the two hormones and that these effects can be additive, synergistic or redundant (Abel *et al.*, 2008). This applies to the effects of FSH on both spermatogenesis and Sertoli cell activity. With respect to spermatogenesis, the total number of germ cells in the testis is more affected by the loss of both hormone receptors than either hormone receptor on its own. This effect appears to be synergistic, rather than just additive, as very few germ cells develop in the double knockouts (Fig. 3). The primary failure in animals lacking both FSHR and the androgen receptor is an inability to generate spermatocytes from spermatogonia indicating that both hormones are needed to optimize these early stages of spermatogenesis. In addition, there is some evidence to suggest that both FSH and testosterone act to stimulate spermiation which is the process by which mature spermatids are released from the Sertoli cells. Overall, it is thought that both FSH and androgen act as survival factors to maintain the appropriate balance between germ cell division and germ cell death during spermatogenesis (Huhtaniemi, 2015). The two hormones act together to ensure that optimal numbers of germ cells are able to complete the spermatogenic process.

Synergistic effects between FSH and androgens are also seen in the expression of some Sertoli cell mRNA transcripts. As an example, Musashi RNA-binding protein 1 (MSI1), which plays a central role in post-transcriptional gene regulation (ie regulation of mRNA transcript levels after transcription has occurred), is specifically expressed in the Sertoli cells in the testis and its expression

is unaffected by knockout of either FSHR or AR. When both are knocked out, however, there is a significant drop of more than 50% in transcript expression (Abel et al., 2008). In this case it means that both hormones act to stimulate MSI1, perhaps because of the importance of its expression, and an effect is only seen when the actions of both hormones are prevented.

FSH Effects on Androgen Secretion

Androgen levels in the male are predominantly controlled by the action of LH on the Leydig cells (Fig. 1). Nevertheless, there is also considerable evidence to suggest that FSH is able to stimulate androgen secretion by the Leydig cells, at least under some circumstances. Since FSHR are only found on the Sertoli cells, however, the effects of FSH on the Leydig cells must be indirect and mediated through the Sertoli cells. Initial reports of FSH action on the Leydig cells came from immature hypophysectomised rats (i.e., rats lacking LH and FSH) which showed an increase in testosterone production by the testes in response to treatment with FSH. A similar effect was subsequently confirmed in adult *hpg* mice treated with FSH and microarray studies showed that the effects of FSH on Leydig cell function could be seen within a few hours of the first treatment. This means that there is an acute responsiveness to FSH in these animals as the hormone must act (presumably) to stimulate Sertoli cell secretion of an active factor which then acts directly on the Leydig cells. The factor would be expected to be secreted as there is no direct contact between Sertoli cells and Leydig cells and a number of potential candidates have been described which can stimulate Leydig cell function directly (e.g., atrial natriuretic peptide (ANP), epidermal growth factor (EGF), and corticotropin-releasing factor (CRF)). It is not known, however, whether any of these factors are secreted by the Sertoli cells in response to FSH. To add to the uncertainty, the Sertoli cell-secreted factor also appears to act primarily to increase steroidogenic enzyme levels without stimulating androgen production directly and so factors which act directly on Leydig cell androgen production may not be of relevance. Finally, it is unclear what role this interaction between FSH, Sertoli cells, and Leydig cells plays in the normal control of Leydig cell function since effects of FSH are generally only seen in the absence of LH. It is likely that this will only be resolved once the identity of the Sertoli cell-derived factor that stimulates Leydig cell function is finally established. Other studies have shown that the Sertoli cells in the adult animal are essential for maintaining the Leydig cell population and, while this effect is unlikely to be directly dependent on FSH, it does emphasize the importance of the Sertoli cells in regulating Leydig cell action. It is clear, therefore, that identification of the active factors involved Sertoli cell/Leydig cell communication must be a priority for future studies of testis biology.

Role of FSH in Primates

Data described in the studies above comes mainly from rodent models because of the relative ease with which hormone levels can be manipulated in these animals. It is likely that the results are broadly applicable to other species (with the notable exception of the Djungarian hamster) and there have been a number of studies in the human and other primates that have highlighted the similarities, but also the differences, to the rodent models. For example, a number of human patients have been described with inactivating mutations in either the *FSHR* or *FSHB* genes. Men with inactivation of *FSHR* have reduced testis size and are subfertile but they are not azoospermic (i.e., they produce sperm) (Huhtaniemi, 2015). This phenotype is similar to the *FSHRKO* mouse suggesting that FSH in both humans and mice is not required for ongoing spermatogenesis but acts to ensure production of optimal germ cell number. Somewhat confusingly, however, three men with inactivating *FSHB* mutations have all been shown to be azoospermic (Huhtaniemi, 2015). The possibility exists that some of these men had other abnormalities which caused, or exacerbated, the lack of sperm production and so further study with more cases will be required to allow us to establish the role of FSH in human spermatogenesis. Evidence from other primates is also complex (Plant and Marshall, 2001) but favors the concept that FSH effects on spermatogenesis in man and monkeys are similar to those in rodents with the hormone acting as a survival factor to premeiotic germ cells.

Use of FSH to treat oligospermia in humans has produced variable results and may only be effective if patients are pre-screened to identify those who are responsive to FSH (Huhtaniemi, 2015). Interestingly, it has also been suggested that the levels of FSH currently used to treat oligospermic patients are not high enough and that using four times the standard dose may be able to stimulate full spermatogenesis (Huhtaniemi, 2015). This would suggest that full-stimulation of the Sertoli cells by either FSH or androgen is sufficient to ensure normal spermatogenesis and that this is achieved normally through a combined effect of FSH and androgen with androgen the more potent agonist.

What We Don't Know

We have learned much about the role of FSH in reproductive biology since its discovery in the 1930s. This is especially true of its effects in the ovary but we also now understand a great deal more about what FSH does in the male, particularly since the generation of transgenic mouse models. Nevertheless, it is clear from what has been described above that a number of questions about the effects of FSH and its mechanism of action in the male remain outstanding and a number of these are highlighted here:

*Is the loss of germ cells in the adult *FSHβKO* or *FSHRKO* mouse due to loss of FSH action in the adult?* One problem with conventional transgenic animals is that the animal develops as a fetus and neonate in the absence of the gene and protein of interest. As described

above, reduced germ cell number in the adult knockout mice may, therefore, be due to disruption to the first wave of spermatogenesis which occurs at puberty. What is needed is a transgenic mouse model in which the FSH β subunit or FSHR can be deleted specifically in the adult. There are transgenic techniques available now which allow the timing of gene knockout to be regulated using an external inducing factor and the most widely used of these factors is tamoxifen. Unfortunately, tamoxifen acts through interaction with the estrogen receptor and so, by itself, has marked effects on mouse testis structure and development. This means that these inducible systems are far from optimal for studies into testis function and, as yet, there are no animal models available which allow specific, adult knockout of FSH or its receptor.

Why do the Sertoli cells become refractory to FSH at puberty? After puberty Sertoli cells continue to express FSHR but their response to FSH is attenuated. Some of the potential intracellular mechanisms involved in this process are known (eg increased phosphodiesterase activity) but how and why the process is induced at all is unknown. Loss of sensitivity to FSH during this maturation phase may be important in limiting the potency of FSH as a spermatogonial survival factor.

How does FSH act as a spermatogonial survival/proliferation factor? Since FSH cannot act directly on the spermatogonia it must induce release of active factors from the Sertoli cells. A number of FSH-dependent Sertoli cell proteins have been identified but the nature of these factors remains unknown.

How does FSH act on Leydig cells and is it an important mechanism? As with the previous question, the identity of the FSH-dependent Sertoli cell factor or factors that regulate Leydig cell activity remains unknown as does its potential role in normal regulation of Leydig cell function.

What is the role of FSH in male primates? This question is discussed in some detail above but it is worth re-stating that, while most evidence suggests that FSH effects in primates are similar to those in rodents, there is sufficient contradictory data to mean the question remains unanswered at present.

References

- Abel, M. H., Baker, P. J., Charlton, H. M., et al. (2008). Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. *Endocrinology*, 149, 3279–3285.
- Allan, C. M., & Handelsman, D. J. (2005). In vivo FSH actions. In M. K. Skinner, & M. D. Griswold (Eds.), *Sertoli cell biology* (pp. 171–197). San Diego, London: Elsevier Academic Press.
- Huhtaniemi, I. (2015). A short evolutionary history of FSH-stimulated spermatogenesis. *Hormones*, 14, 468–478.
- Lerchl, A., Sotiriadou, S., & Behre, H. M. (1993). Restoration of spermatogenesis by follicle-stimulating hormone despite low intratesticular testosterone in photoinhibited hypogonadotropic Djungarian hamsters (*Phodopus sungorus*). *Biology of Reproduction*, 49, 1108–1116.
- O'Shaughnessy, P. J. (2014). Hormonal control of germ cell development and spermatogenesis. *Seminars in Cell and Developmental Biology*, 29, 55–65.
- O'Shaughnessy, P. J., Monteiro, A., & Abel, M. (2012). Testicular development in mice lacking receptors for follicle stimulating hormone and androgen. *PLoS One*, 7, e35136.
- Plant, T. M., & Marshall, G. R. (2001). The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. *Endocrine Reviews*, 22, 764–786.
- Rebourcet, D., O'Shaughnessy, P. J., Monteiro, A., et al. (2014a). Sertoli cells maintain Leydig cell number and peritubular myoid cell activity in the adult mouse testis. *PLoS One*, 9, e105687.
- Rebourcet, D., O'Shaughnessy, P. J., Pitetti, J. L., et al. (2014b). Sertoli cells control peritubular myoid cell fate and support adult Leydig cell development in the prepubertal testis. *Development*, 141, 2139–2149.
- Rebourcet, D., Wu, J., Cruickshanks, L., et al. (2016). Sertoli cells modulate testicular vascular network development, structure, and function to influence circulating testosterone concentrations in adult male mice. *Endocrinology*, 157, 2479–2488.
- Smith, L. B., O'Shaughnessy, P. J., & Rebourcet, D. (2015). Cell-specific ablation in the testis: What have we learned? *Andrology*, 3, 1035–1049.
- Smith, L. B., & Walker, W. H. (2015). Hormone signalling in the testis. In T. M. Plant, & A. J. Zeleznick (Eds.), vol. 1. *Knobil and Neill's Physiology of Reproduction* (pp. 637–690). Amsterdam: Elsevier.

Further Reading

- O'Donnell, L., Stanton, P., de Kretser, D.M. (2017) Endocrinology of the male reproductive system and spermatogenesis. In De Groot L.J., Chrousos G., Dungan, K. et al. (eds) South Dartmouth: Endotext.
- O'Shaughnessy, P. J. (2015). Testicular development. In T. M. Plant, & A. J. Zeleznick (Eds.), vol. 1. *Knobil and Neill's physiology of Reproduction* (pp. 567–594). Amsterdam: Elsevier.
- Walker, W. H., & Cheng, J. (2005). FSH and testosterone signaling in Sertoli cells. *Reproduction*, 130, 15–28.

LH and the Male

Ilpo T Huhtaniemi, Imperial College London, London, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

The pituitary gonadotropin luteinizing hormone (LH) is the crucial stimulator of testicular steroidogenesis, in particular of testosterone (T) production, in the Leydig cells present in testicular interstitial tissue. LH and T form the functional backbone of the hypothalamic–pituitary–testicular (HPT) axis (Fig. 1), a regulatory circuit where LH stimulates T production, which reciprocally regulates LH secretion by negative feedback action. LH functions by binding to the LH receptor (LHR), a G-protein coupled receptor (GPCR), located on Leydig cell plasma membranes. LH secretion and T production start in fetal life, and the latter is responsible for masculinization of the male fetus. In humans, instead of pituitary LH, placental choriongonadotropin (hCG), an LH analogue, is responsible for fetal testicular stimulation. Following a short postnatal active period (minipuberty), prepubertal quiescence follows, and the HPT activity reawakens at puberty and persists for the rest of life, slightly diminishing in old age. We will review in this article the salient features the human and rodent HPT axis throughout an individual's life-span.

LH and hCG

LH and hCG, the third gonadotropin follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) form the family of glycoprotein hormones. They are structurally similar dimeric molecules consisting of a common α -subunit (CGA) [92 amino acids (aa)] and a hormone-specific β -subunit (121 aa in LH β [LHB] and 145 aa in hCG β [CGB]) (Fig. 2). LH is produced by gonadotrope cells of the anterior pituitary gland and hCG by placental trophoblasts. LH and hCG have some differences in their aa sequence, in addition CGB has a 24 aa long C-terminal extension and is more heavily glycosylated than LHB (30% vs. 15%). The differences provide hCG with longer half-life (12–24 h vs. 20 min) and higher bioactivity than LH.

The CGA gene is located on chromosome 6q14.3 and the cluster of one *LHB* and six *CGB* genes and pseudogenes on chromosome 19q13.32. The crystal structure of deglycosylated hCG reveals the so-called “cysteine knot structures” that reminds growth factor-type signaling molecules, such as TGF β . Both LH/hCG subunits have an elongated shape, with two β -hairpin loops on one side of the central cysteine knot and a long loop on the other side (Fig. 2). Noncovalent interactions keep the subunits together, and the dimer is stabilized by a segment of the β -subunit that extends like a “seatbelt” around the α -subunit and is “locked” by a disulfide bridge (Fig. 2).

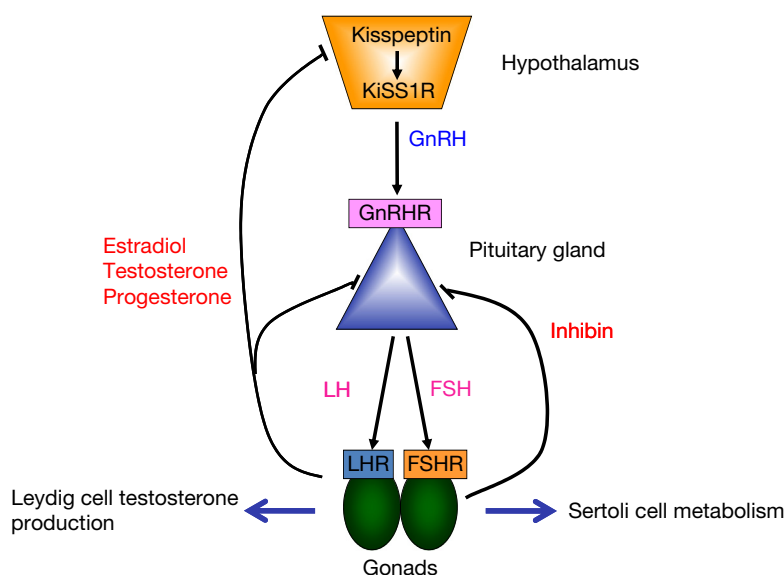


Fig. 1 The hypothalamic–pituitary–testicular (HPT) axis. The main hormones functioning in the HPT axis are depicted, including the effects of kisspeptin on GnRH secretion, GnRH on LH and FSH secretion, and their effects on testicular function, followed by negative feedback effects of testicular sex steroids and inhibin. *GnRH*, gonadotropin-releasing hormone; *KiSS1R*, kisspeptin receptor; *LH*, luteinizing hormone; *FSH*, follicle-stimulating hormone; *R*, receptor.

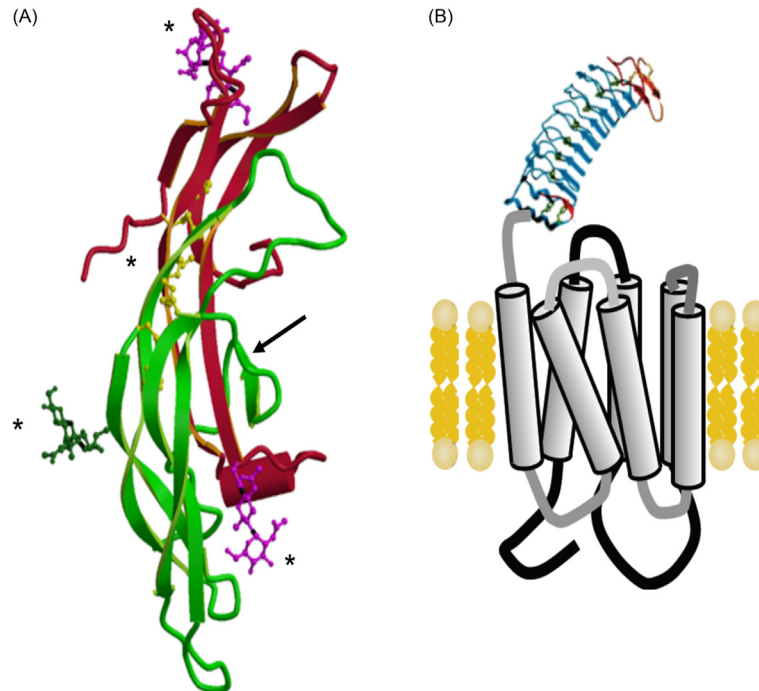


Fig. 2 Schematic 3D structures of LH (panel A) and LHR (panel B). In (panel A) *red color* depicts the α -subunit (CGA) and *green color* the β -subunit (LHB). The arrow points to the “seat belt” structure of the β -subunit, which stabilizes the dimeric structure of the hormone. The asterisks depict the carbohydrate moieties attached to the hormone molecule. In (panel B), the structure in the top part depicts the leucine rich repeats of the extracellular receptor domain, which forms the primary binding site of the hormone. The cylindrical structures in the middle of the picture depict the seven transmembrane domains of the receptor molecule. (Courtesies of Drs. J. Dias and E. Reiter).

LH Receptor (LHR)

Gonadotropin receptors (and TSHR) are sialoglycoproteins with structural similarity, belonging to class A of the large family of GPCRs (**Fig. 2**). The single-chain LHR of approximately 75 kDa size is composed of 675 amino acids and is subdivided into a long extracellular domain, a transmembrane domain crossing the plasma membrane as seven α -helices that are connected by three extracellular and three intracellular loops, followed by a short C-terminal intracellular tail. Multiple leucine-rich repeats in the extracellular domain form the site of ligand binding. Both LH and hCG bind in humans to the same LHCGR [the term luteinizing hormone/choriogonadotropin receptor (LHCGR) signifies that in humans both LH and hCG bind to the same receptor. An appropriate term in rodents is LHR because they do not produce chorionic gonadotropin (CG)].

The *LHR* gene is localized on chromosome 2p21. The first 10 exons of *LHR* encode the extracellular receptor domain, and the last long 11th exon the rest of the receptor molecule. Transcription of the *LHR* gene is subject to silencing and activation through epigenetic mechanisms and phosphorylation and dephosphorylation events, a variety of signal transduction pathways, and derecruitment of inhibitors ([Dufau et al., 2010](#)). The TATA-less and GC-rich minimal *LHR* promoter is about 175 bp long, and upstream there are several inhibitor regions. Several hormones regulate *LHR* expression, including FSH, prolactin, growth hormone, and IGF-1.

LHR Signaling

LH and hCG binding to the extracellular domain of LHR alters the tertiary structure of the transmembrane receptor domain locking it into an active conformation. It will thereafter be coupled to a stimulatory G protein (Gs) and activates adenylyl cyclase, and enzyme that converts ATP to cyclic adenosine-3',5'-monophosphate (cAMP), which forms the primary intracellular second messenger of LH action (**Fig. 3**) ([Ascoli et al., 2002](#); [Tremblay, 2015](#)). Thereafter cAMP activates several protein kinases (PK), the best known of them being PK-A. This and other activated protein kinases lead, through a cascade of regulatory events to functional responses including increased expression of steroidogenic genes. As information has accumulated, the simple cAMP and PK-A mediated Leydig cell activation, and termination of LH action by phosphodiesterase-catalyzed degradation of cAMP, has evolved into a complex network involving interplay of multiple ligands, receptors, signaling cascades, and transcription factors, as summarized in **Fig. 3**.

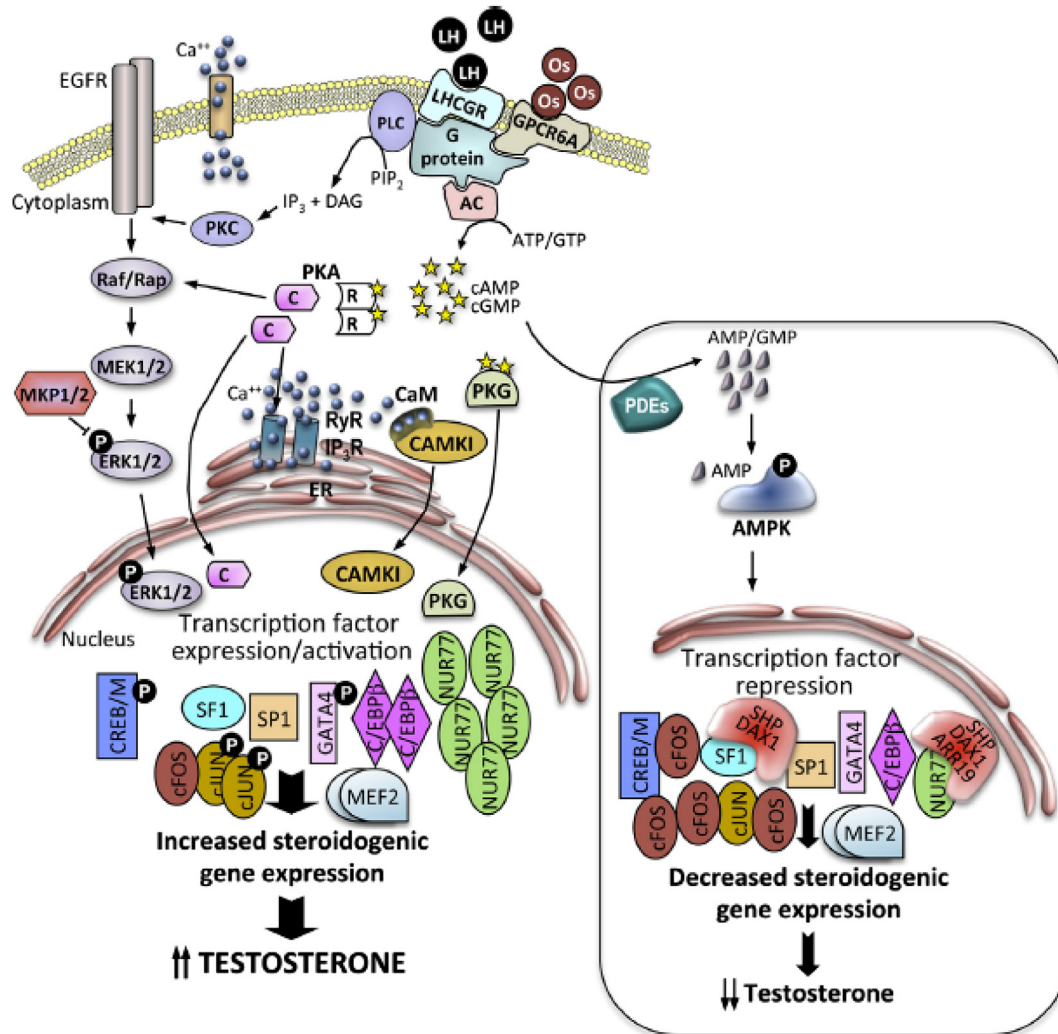


Fig. 3 Schematic presentation of the mechanisms involved in LH-stimulated steroid production in Leydig cells. Binding of LH and osteocalcin (Os) to their G protein-coupled receptors (LHCGR and GPCR6A, respectively) activates adenyl cyclase (AC) leading to cAMP/cGMP production (yellow stars). Intracellular calcium (blue circles) concentration also increases following activation of the ryanodine receptor (RyR) and the inositol triphosphate receptor (IP₃R) located in the endoplasmic reticulum (ER). Increases cAMP, cGMP, and Ca²⁺ levels activate various kinases including protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), extracellular signal-regulated kinases 1 and 2 (ERK1/2), Ca²⁺/calmodulin (CaM) kinase I (CAMKI). These kinases are then involved in the activation of several transcription factors (CREB/CREM, GATA4, cJUN). Expression levels of some transcription factors (NUR77, cJUN, C/EBP β) are also increased. This induces expression of genes involved in steroid hormone synthesis and leading to increased T secretion. Within the Leydig cells, the stimulatory pathways are inhibited via phosphatases such as MKP1 and MKP2, which dephosphorylate ERK1/2, and via the repression mechanism active in steroidogenic cells (box on the right). To prevent overstimulation, cAMP is degraded by phosphodiesterases (PDEs) into AMP, which in turn activates the AMP-activated protein kinase (AMPK). Activated-AMPK represses the expression of transcription factors known to stimulate steroidogenesis (NUR77 and cJUN) and activates the expression of repressors of steroidogenesis (cFOS and DAX1). Other repressors are also involved including SHP and ARR19. All together, these events lead to a reduction in the expression of steroidogenic genes and in steroid hormone production. From Tremblay, J.J. (2015). Molecular regulation of steroidogenesis in endocrine Leydig cells. *Steroids* 103, 3–10, with permission.

Leydig Cell Steroidogenesis

The cascade of steroidogenesis (Fig. 4) starts with phosphorylation of the steroidogenic acute regulatory (StAR) protein in mitochondria. Basal StAR expression is low, but it quickly responds to LH, stimulating transfer of cholesterol from the mitochondrial outer to inner membrane, a rate-limiting step in steroidogenesis. The first step of steroidogenesis is the conversion of cholesterol to pregnenolone in mitochondria, catalyzed by the cholesterol side chain cleavage enzyme (CYP11A1). Thereafter the steroid molecule is transferred to smooth endoplasmic reticulum, where it is metabolized further. In humans, androgen synthesis proceeds mainly through the Δ^5 pathway (pregnenolone $\rightarrow$ 17-hydroxypregnenolone $\rightarrow$ dehydroepiandrosterone $\rightarrow$

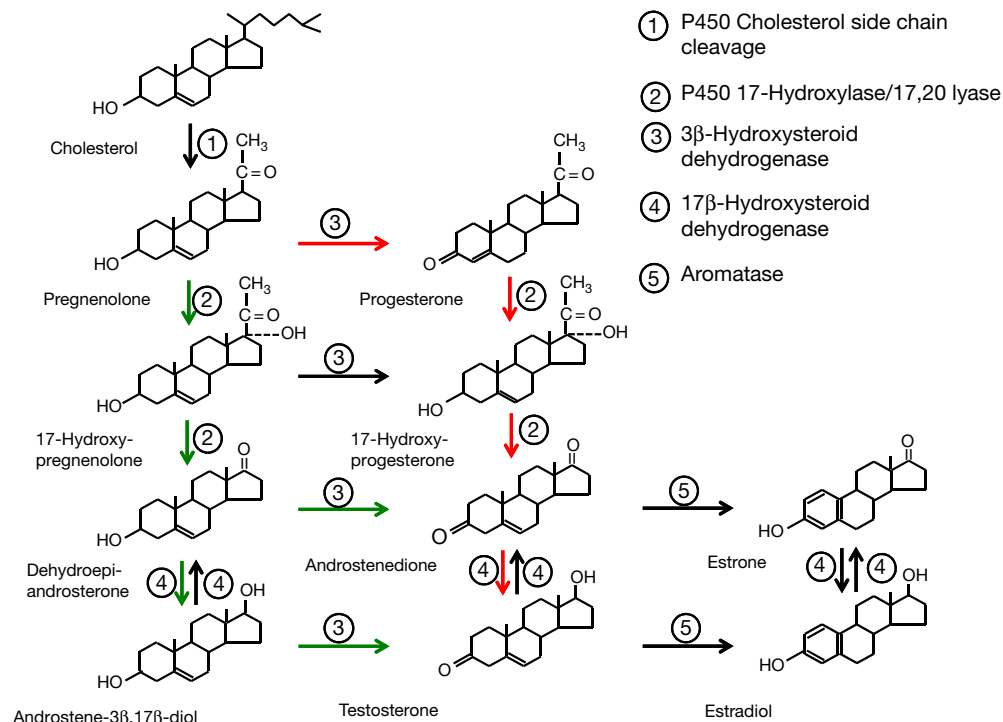


Fig. 4 The key steroid metabolic steps in the ovary and testis leading to formation of T and estradiol. The Δ^5 pathway, used by the human testis, is marked with *green arrows*, and the Δ^4 pathway, more important in rodents, with *red arrows*. The same enzyme with dual function, P450 17-hydroxylase/17,20-lyase catalyzes both 17-hydroxylation and ring D side chain cleavage in pregnenolone and progesterone. Apart from the interconversion of 17-keto and 17-hydroxy steroids all other reaction in the steroid metabolic pathway are irreversible. Multiple isoform of 17-hydroxysteroid dehydrogenase (enzyme 4), with differing substrate specificities, catalyze these reactions.

androstenediol $\rightarrow$ T), whereas rodent testes use the Δ^4 pathway (pregnenolone $\rightarrow$ progesterone $\rightarrow$ 17-hydroxyprogesterone $\rightarrow$ androstenedione $\rightarrow$ T).

The magnitude of the steroidogenic response to LH/hCG stimulation differs greatly between humans and rodents. Whereas hCG injection stimulates an acute 10–20-fold increase in rat and mouse serum T levels within 1 h, a similar injection to humans brings about a sluggish 30%–50% acute increase and a delayed somewhat higher twofold response 2–4 days later. The main reason for the difference is apparently the nature of T in circulation. In rodents it is mainly in free form or loosely bound to plasma proteins (albumin) and therefore rapidly eliminated and metabolized, whereas in humans about half of T is bound to (and buffered with) high-affinity to sex hormone-binding globulin (SHBG).

Feedback Regulation in the HPT Axis

Gonadotropin secretion, stimulated by hypothalamic gonadotropin-releasing hormone (GnRH), is kept in homeostatic balance by negative feedback effects from the testis (T and inhibin-B) (Fig. 1) (Goodman, 2015; Herbison, 2015). In primates, and possibly humans, androgens suppress LH synthesis primarily through action on the GnRH pulse generator, but inhibitory effects are also found at the level of pituitary gonadotropes, which effect requires conversion of T to estradiol. The feedback inhibition at pituitary level is more prominent in rats. The hypothalamic level inhibition of GnRH secretion in men can be achieved with both androgen and estrogen. Because GnRH neurons do not express androgen or estrogen receptors, their negative feedback action has to be indirect. Indeed, evidence is mounting that hypothalamic kisspeptin neurons do express these receptors and mediate the sex steroid suppression. The function of the male HPT axis is tonic with only negative feedback effects from the testes, as opposed to negative and positive ovarian feedback in females.

LH Action in the Male Throughout Life

The HPT axis has three active periods throughout life (Fig. 5). The first is in fetal life, the second after birth (these two phases are merged together in rodents), and the third phase starts at puberty and continues until old age. Two distinct populations of Leydig cells exist in the testes, that is, the fetal and adult populations (Teerds and Huhtaniemi, 2015). The former appear in fetal life and

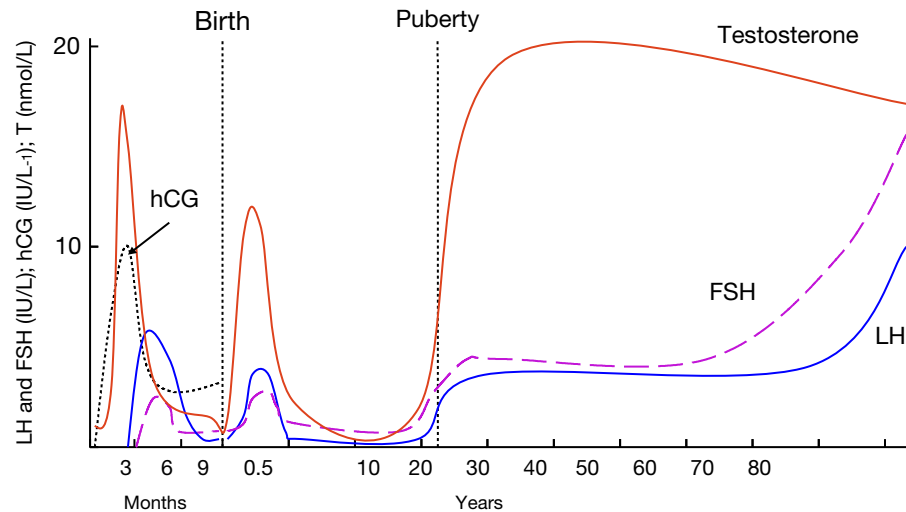


Fig. 5 Function of the HPT axis throughout the life-span of a human male. The fetal and postnatal activity of T production is due to a fetal population of Leydig cells. Adult Leydig cells appear to the testis at puberty and persists with somewhat decreasing activity throughout the rest of life. In rat and mouse, the fetal and neonatal peaks of T production are merged together with peak activity a few days before birth.

disappear after birth. The latter appear at puberty and persist thereafter for the rest of life. We will next review the special features of HPT function throughout life.

Fetal Life

Fetal Leydig cell T production apparently begins autonomously, or at least independently of LH/hCG action, because T can be detected in human and rodent fetal testes before the onset of LHR expression (O'Shaughnessy et al., 2006; Teerds and Huhtaniemi, 2015). However, the peak of fetal Leydig cell T production, occurring on gestation week 13–14 in humans and embryonic (e) days 18–18.5 in mice and rats, is clearly under influence of gonadotropin action, that is, pituitary LH in rodents and placental hCG in humans. Although pituitary LH concentration starts rising in fetal circulation after gestation week 12, it is apparently of minor functional importance, because the concentration of hCG around the peak of T production is at least 10-fold higher (Fig. 5). Following the fetal peak of T and LH levels, they decline toward the end of gestation apparently reflecting the onset of negative feedback of placental steroid hormones.

The situation in rats and mice is different, because they do not produce choriongonadotropin. Furthermore, their masculinization in fetal life is normal in the absence of LH and LHR, which is explained by the promiscuity of rodent fetal Leydig cells to stimulation by numerous nongonadotropic endocrine and paracrine factors (Teerds and Huhtaniemi, 2015). Hence, while human fetal Leydig cell T production is critically dependent on hCG/LHR activation, the rodent fetal Leydig cells are only LH responsive, but not LH dependent.

Minipuberty

The next phase of testicular endocrine activity in humans, minipuberty, occurs after birth from the first postnatal week until about 6 months of age, with a peak between 1 and 3 months (Fig. 5), when serum T in the baby boys is in the adult male range (Kuiri-Hänninen et al., 2014). The mechanism for this HPT activation is apparently the elimination of the negative feedback of placental steroids on gonadotropin secretion at birth.

The biological activity of T during minipuberty has been questioned, because it occurs concomitantly with a clear increase of SHBG, reducing the proportion of biologically active free T. In agreement with this, no postnatal increase exists in salivary T levels, considered to reflect the concentration of free hormone in circulation. However, there are several signs of increased androgen action in baby boys, such as increased testicular volume and penile length, and increase in the number of testicular Leydig, Sertoli, and germ cells.

Inhibition of the postnatal HPT activity in nonhuman primates by orchidectomy or GnRH analogue treatment reduced testicular weight and penis length. More prolonged effects include delayed puberty, reduced sexual, and aggressive behavior in adulthood as well as some metabolic effects. In rodents the fetal peak in T during the latter third of pregnancy declines gradually until the 2nd week of postnatal life, thus reminding of human minipuberty. Inhibition of the postnatal HPT activity in male rats brings about ejaculatory impotence in young adult age, which gradually subsides as the animals grow older.

To conclude, minipuberty most likely has a role in the development of reproductive capacity. The postnatal T peak may have a role in “stabilizing” the male genitalia, but it may also have anabolic effects on body growth and development, as well as on neural and psychosexual development.

Prepuberty

This dormant period of the HPT axis between minipuberty and puberty is viewed to result from function of a neurobiological break imposed on the GnRH pulse generator (Plant, 2015). However, this period is not completely quiet, because some activity is observed in the form of low night-time secretion pulses of gonadotropins and low peaks of T, associated with sleep onset. The prepubertal testis produces small amounts of T, and testicular T production at this age is highly responsive to hCG stimulation. Hence, the prepubertal HPT quiescence is primarily due to the lack of gonadotropin secretion, and the Leydig cells are ready to respond once LH secretion begins. The minor T production of prepubertal boys is functionally insignificant, as evidenced by the lack of sex difference of serum T concentrations in prepubertal children.

In rodents, there is a gradual decline of T production during the 2–3 postnatal weeks until the nadir at week 3 (Tapanainen et al., 1984; O’Shaughnessy et al., 2006). The decline is due to decreasing gonadotropin levels and gradual disappearance of the fetal Leydig cell population. Therefore, no distinct quiescent period of the HPT activity is observed.

Puberty

The onset of puberty is triggered by release of the neurobiological break imposed on the GnRH pulse generator, which results in reawakening of the HPT axis (Plant, 2015). At least a decline of inhibitory signals (GABA, opiates) and amplification of the excitatory inputs (glutamate, kisspeptin), are involved in the increased frequency and amplitude of GnRH, and consequently of gonadotropin pulses.

During pubertal maturation, the small night-time secretory pulses of LH (mean levels $<0.1\text{--}0.2\text{ IU} \times \text{L}^{-1}$ and peaks up to $1.0\text{ IU} \times \text{L}^{-1}$) begin to increase and start being accompanied by increased T secretion (Mitamura et al., 1999). Circadian rhythm of the very low LH and T concentrations already occurs in prepubertal boys 3–5 year of age, at least 3 year before the onset of puberty, and an increase in LH secretion starts about a year before the first signs of puberty, which require sufficient increase in T secretion. While higher night-time LH secretion persists, LH peaks spread to the whole 24-h period. The lag time between the LH and T pulse shortens as puberty progresses from about 2 h to 10–30 min. The consequences of increased LH-stimulated T production triggers gradually the physical secondary signs of puberty. Now also the feedback regulation of LH secretion begins, and increased T production starts inhibiting LH secretion by suppressing kisspeptin and GnRH secretion.

The sequence of events is similar in rodents as in humans, when the pubertal reactivation of their HPT axis occurs at around 3 weeks of postnatal life (Tapanainen et al., 1984; O’Shaughnessy et al., 2006).

Adult Age

The adult human HPT axis is set at the level of maintaining LH concentration at around $2\text{--}9\text{ IU} \times \text{L}^{-1}$ and T at $10\text{--}30\text{ nmol} \times \text{L}^{-1}$. Secretion pulses of serum T after the LH pulses (average interval of 1–2 h) are inconsistent, apparently due to the sluggish acute response of human testicular steroidogenesis to gonadotropin stimulation, and to the buffering effect of steroid hormone binding to plasma transport proteins. In rodents, in particular mice, serum T concentration shows major (up to 100-fold) variation between sequential samples, apparently due to its presence in protein-unbound form.

T secretion has diurnal variation with higher concentrations present in the morning hours and a trough in the evening (Mitamura et al., 1999). Data on circannual rhythm are inconclusive. The daily production of T in a male is 6–7 mg; about 95% originates from the testes and the rest from peripheral metabolism of adrenal androgen precursors. Besides T the human testes store and secrete intermediates and metabolites of the steroidogenic synthetic pathway, and in particular their sulfate conjugates. The latter have no known hormonal function, and apparently represent storage and secretory forms. T is also metabolized in Leydig cells further to the more active androgen metabolite $5\alpha\text{-DHT}$ and the female sex hormone estradiol (Fig. 4).

The secretion of steroid hormones is considered a passive process due to their lipid solubility and easy transit through cell membranes. It is noteworthy that the concentration of T in the testis tissue is about 100-fold higher than in peripheral circulation. This high intratesticular concentration has been considered crucial for the maintenance of spermatogenesis, although ultimately it may reflect the fact that the testis is the site of T synthesis (Oduwole et al., 2014).

In circulation, only about 2% of T appears in free, that is, nonprotein-bound form, 44% is bound to SHBG and 54% to albumin and other proteins. The plasma level of SHBG is under endocrine regulation, increasing clearly by estrogen and upon aging, and decreasing somewhat by androgen, and strongly by obesity. If the HPT axis functions normally, changes in SHBG levels do not affect the level of bioactive androgens, since they are quickly compensated for by changes in set-point of the feedback regulation.

Treatment of healthy men with T suppresses LH, FSH, and intratesticular T while maintaining peripheral T action. This leads to severe suppression of spermatogenesis—to the extent that the treatment provides a successful means of male contraception. If such men receive LH or hCG injections, their spermatogenesis recovers qualitatively, due to the restoration of intratesticular T levels. However, sperm counts remain by about 50% reduced, meaning that the reinitiation of spermatogenesis is possible with T alone,

but its quantitative recovery also needs FSH. When FSH was added to the treatment, spermatogenesis was fully restored. Treatment of T-suppressed men with purified FSH was able to stimulate spermatogenesis, though not quantitatively, suggesting that the effects of LH/T and FSH are, at least to some extent, complementary.

Aging

There is a modest decline of the HPT function with age, amounting to an annual decrease of 0.5%–1.5% of serum T concentration in most studies (Huhtaniemi, 2014). Opinions about the clinical importance of this modest decline vary. In some men the decline is deeper, and for them a new syndrome [male menopause, male climacterium, andropause, viropause, male midlife transition, late-onset hypogonadism (LOH), functional hypogonadism, etc.] has been coined. T treatment is being widely marketed and prescribed for its treatment, but still largely without evidence-based proof of efficacy over risks.

The aging-related decline in HPT function has been under intensive investigation (Wu et al., 2010; Huhtaniemi, 2014). One important question is whether the changes observed are due to biological age or to other concomitant alterations, and potentially treatable and reversible. Indeed, 75% of aging men with symptomatic hypogonadism (i.e., subnormal serum T with specific symptoms) are overweight or obese, and their T production improves after weight loss, lifestyle modification, and proper treatment of comorbidities. Obesity-related hypogonadism is of the secondary hypothalamic–pituitary type (low T and low or inappropriately normal LH), whereas the minority, caused by old age, is of the primary type due to testicular insufficiency (low T, high LH). SHBG levels increase with aging and decrease with obesity, and therefore the two conditions influence free T concentration to opposite directions.

Histological findings provide proof for the hormonal findings (low T, high LH) on primary testicular failure with aging. When compared between 20–48 and 50–76 years the average total number of Leydig cells per testis decreases by 44%, despite largely persistent normal ultrastructure of many Leydig cells. In most men, the decreased Leydig cell number and steroidogenic capacity are compensated for by elevated LH secretion. When this compensatory response is exhausted, T begins to decline and hypogonadism ensues. Animal experiments have shown that impaired LHR function, possibly due accumulating load of free oxygen radicals with age, is an important cause for the impairment of Leydig cell steroidogenesis. There is *in vivo* evidence in humans on impaired responsiveness of aged Leydig cells to LH/hCG stimulation as an additional cause for their weakened steroidogenesis.

Animal experiments indicate diminished outflow of GnRH as the cause of suppressed LH and T pulses in aging males with more frequent and smaller LH pulse. T-clamp experiments have demonstrated that the pituitary sensitivity to secrete LH in response to GnRH is not altered with age.

Causes of the gonadotropin suppression in obese men are not known in details. Peripheral and central insulin resistance may be involved, causing the suppression in SHBG and consequently lower total T levels. Other factors include hypothalamic inhibition by proinflammatory cytokines (TNF α and IL-6) originating from adipocytes, and central nervous system endocannabinoids that can downregulate hypothalamic function. In addition, adipocytokines such as leptin and adiponectin have been shown to modulate GnRH and gonadotropin secretion. Conversely, the suggested role of excess of fat tissue-produced estrogens in reducing the GnRH-gonadotropin secretion has not been confirmed in men.

To conclude, it seems that the primary hypogonadism specifically associated with aging is due to impaired Leydig cell function, which reduces gonadal feedback and consequently increases LH secretion, whereby the hypothalamic–pituitary function remains largely unaffected upon aging. The other and more common functional type of hypogonadism associated, but not caused, by aging is due to obesity, and it is of the secondary type caused by impairment of gonadotropin secretion.

Pathophysiology of LH/LHR Action in Men and Mice; Lessons From LH Subunit and Receptor Mutations and Knockouts

Human mutations and genetically modified mouse models have brought important novel information about the function of the HPT axis and its disturbances (Narayan, 2015; Huhtaniemi, 2017). All gonadotropin subunit mutations detected are inactivating, but both constitutively activating and inactivating mutations have been detected in gonadotropin receptor genes. Animal models for each type of mutation have been produced. The following text summarizes the key findings on these nature- and man-made experiments.

CGA and LHB

No human mutations in CGA have been detected, for the obvious reason that they would interfere with the production of hCG, which would be incompatible for the maintenance of pregnancy. However, CGA knockout (KO) mice have been produced, which is possible because the mouse genome does not contain a gene for choriongonadotropin. The CGA KO mice are hypothyroid and hypogonadal, due to lack of TSH, LH, and FSH, but male fetal development and differentiation are unaffected, because mouse fetal Leydig cells can produce T independent of gonadotropin stimulation (see above).

Human *LHB* mutations have been described in six men. The men are normally masculinized at birth, because hCG is able to maintain normal stimulation fetal Leydig cell T production. However, they completely lack normal pubertal maturation in the absence of pituitary LH and its stimulation of postnatal Leydig cell function.

The *LHB* KO mouse is normally masculinized at birth because of the aforementioned promiscuity of fetal mouse Leydig cells to nongonadotropic stimulation. However, this response is lost in adult Leydig cells that are unable to produce T without LH stimulation. Hence the *LHB* KO mouse is a complete phenocopy of the cognate human mutation, with complete lack of postnatal male sexual development.

Activating *LHR* Mutations

The first mutations in the HPT axis discovered were the activating *LHR* mutations because of their dramatic phenotype: an early onset (0.5–3 years) familial male-limited precocious puberty (Fig. 6). In these patients a point mutation in the transmembrane domain of *LHR* renders the receptor protein into constantly active conformation without ligand. Altogether > 10 such point mutations have been described today. If untreated, puberty of the boys progresses rapidly, resulting in premature epiphyseal closure, and compromised adult height. Various antihormonal therapies are used for the treatment (medroxyprogesterone, ketoconazole, cyproterone acetate, anastrozol, bicalutamide) to reduce the premature virilization.

An activating *LHR* knockin mouse model has been recently reported with precocious puberty, Leydig cell hyperplasia, and elevated T levels at very young age. Later on the mice develop Leydig cell adenomas and disturbances in sexual function and hyperplastic changes in genital structures.

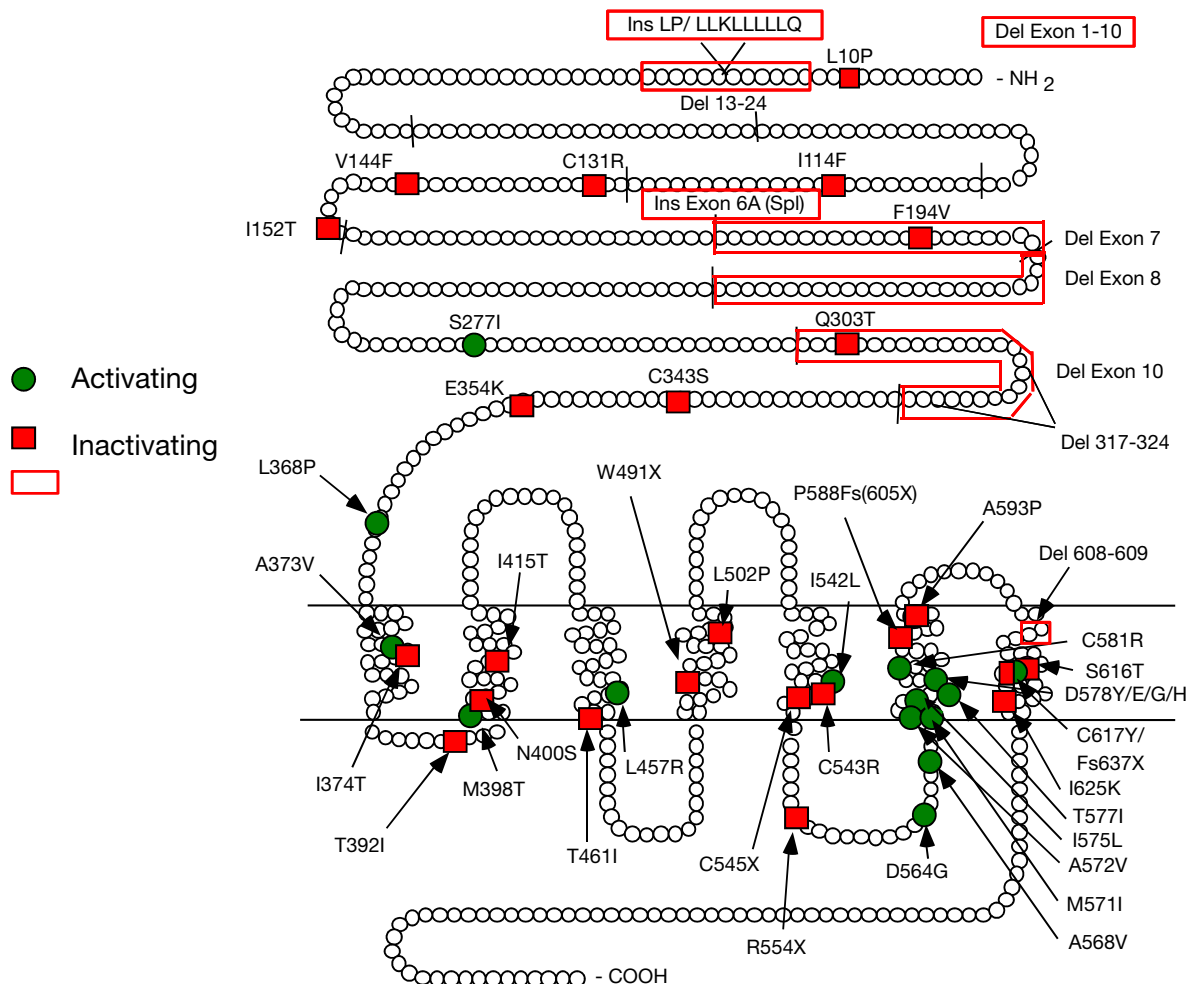


Fig. 6 Locations of the currently known mutations of human *LHR*. Red boxes indicate inactivating mutation and green circles activating mutations. The short lines across the peptide chain depict the exon boundaries. *Fs*, frame shift; *Ins*, insertion; *Del*, deletion; *Spl*, splice variants; *X*, premature stop codon. Huhtaniemi, I. (2017). Male hypogonadism resulting from mutations in the genes for the gonadotropin subunits and their receptors. In: Winters, S.J. and Huhtaniemi, I.T. (eds.) *Male hypogonadism: Basic, clinical and therapeutic principles*. 2nd edn., pp. 127–152. Cham: Springer.

Inactivating *LHR* Mutations

Because of multiple mechanisms of receptor inactivation, there is no preferred domain of inactivating mutations in *LHR*, which exist in all parts of the gene and include both point mutation and deletions (Fig. 6). The inactivating mutations vary in severity, with consequent variability of phenotype. The mildest form is hypospadias and/or micropenis (Leydig cell hypoplasia, LCH Type 2), and the total receptor inactivating form causes complete male to female sex reversal (LCH Type 1) due to near-total to total lack of Leydig cell T production and sexual differentiation both in utero and postnatally. The phenotype differs from the androgen insensitivity syndrome by the lack of pubertal breast development, which in the latter is possible through conversion of the normally produced T to estradiol. The testes of individuals with complete *LHR* inactivation are totally devoid of mature Leydig cells.

The *LHR* KO mouse phenocopies some, but not all, features of the respective human mutation. The differences in phenotype can be easily explained by differences in HPT function of the two species in early development. Unlike human males with *LHR* inactivation the KO males are normally masculinized at birth, because of the earlier mentioned mouse fetal Leydig cell promiscuity to nongonadotropic stimulation. This response is lost in adult Leydig cells, which explains why the *LHR* KO mice, as humans with similar mutation, lack pubertal maturation and testicular T production in adult age.

Concluding Remarks

The crucial role of LH stimulates T production in induction and maintenance of function of the male mammals is undisputed. Recent inactivating mutations in humans of *LHB* and *LHR*, as well as knockout models for the same genes, have strengthened this concept. Mouse models provide accurate phenocopies of human mutations and explain the mechanisms of species difference in the HPT function. As information about the role of LH in the regulation of testicular function increases it is expected that better diagnostic and therapeutic tools for male hypogonadism, as well as male contraception, will develop.

References

- Ascoli, M., Fanelli, F., & Segaloff, D. L. (2002). The lutropin/choriogonadotropin receptor, a 2002 perspective. *Endocrine Reviews*, 23, 141–174.
- Dufau, M. L., Liao, M., & Zhang, Y. (2010). Participation of signaling pathways in the derepression of luteinizing hormone receptor transcription. *Molecular and Cellular Endocrinology*, 314, 221–227.
- Goodman, R. L. (2015). Neuroendocrine control of gonadotropin secretion: Comparative aspects. In T. M. Plant, & A. J. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn., pp. 1537–1574). Waltham: Academic Press.
- Herbison, A. E. (2015). Physiology of the adult gonadotropin-releasing hormone neural network. In T. M. Plant, & A. J. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn., pp. 399–467). Waltham: Academic Press.
- Huhtaniemi, I. (2014). Late-onset hypogonadism: Current concepts and controversies of pathogenesis, diagnosis and treatment. *Asian Journal of Andrology*, 16, 192–202.
- Huhtaniemi, I. (2017). Male hypogonadism resulting from mutations in the genes for the gonadotropin subunits and their receptors. In S. J. Winters, & I. T. Huhtaniemi (Eds.), *Male hypogonadism: Basic, clinical and therapeutic principles* (2nd edn., pp. 127–152). Cham: Springer.
- Kuiri-Hänninen, T., Sankilampi, U., & Dunkel, L. (2014). Activation of the hypothalamic–pituitary–gonadal axis in infancy: Minipuberty. *Hormone Research in Paediatrics*, 82, 73–80.
- Mitamura, R., Yano, K., Suzuki, N., Makita, Y., & Okuno, A. (1999). Diurnal rhythms of luteinizing hormone, follicle-stimulating hormone, and testosterone secretion before the onset of male puberty. *Journal of Clinical Endocrinology and Metabolism*, 84, 29–37.
- Narayan, P. (2015). Genetic models for the study of luteinizing hormone receptor function. *Frontiers in Endocrinology*, 6, 152.
- O'Shaughnessy, P. J., Baker, P. J., & Johnston, H. (2006). The foetal Leydig cell—Differentiation, function and regulation. *International Journal of Andrology*, 29, 90–95.
- Oduwole, O. O., Vydra, N., Wood, N. E., et al. (2014). Overlapping dose responses of spermatogenic and extragonadal testosterone actions jeopardize the principle of hormonal male contraception. *FASEB Journal*, 28, 2566–2576.
- Plant, T. M. (2015). Neuroendocrine control of the onset of puberty. *Frontiers in Neuroendocrinology*, 38, 73–88.
- Tapanainen, J., Kuopio, T., Pelliniemi, L. J., & Huhtaniemi, I. (1984). Rat testicular endogenous steroids and Leydig cells between fetal period and sexual maturity. *Biology of Reproduction*, 31, 1027–1035.
- Teerds, K. J., & Huhtaniemi, I. T. (2015). Morphological and functional maturation of Leydig cells: From rodent models to primates. *Human Reproduction Update*, 21, 310–328.
- Tremblay, J. J. (2015). Molecular regulation of steroidogenesis in endocrine Leydig cells. *Steroids*, 103, 3–10.
- Wu, F. C., Tajar, A., Beynon, J. M., et al. (2010). Identification of late-onset hypogonadism in middle-aged and elderly men. *New England Journal of Medicine*, 363, 123–135.

Inhibin and Male

David M Robertson, Monash University, Clayton, VIC, Australia; and University of New South Wales, Sydney, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Structure

Inhibin A and B consist of two covalently linked subunits (α and either β A or β B subunits) which are produced as 50–60k precursor monomers to form covalently linked $\sim$ 110k dimers. Under the action of the protease, furan either intracellularly or following secretion, the α subunit is cleaved at one or two sites while β A/ β B subunits are cleaved at one site to form a range of processed and partially processed inhibin forms with molecular weights ranging from 30k to 60k. The α subunit in contrast to the β subunit is mono- or di-glycosylated. The β A/ β B subunits also form homo- and hetero-dimers called activin A,B and AB with the mature molecule ($\sim$ 30k) the major form secreted. Other activin forms are known (activin C,D,E) but the corresponding inhibin forms in mammals have not been identified.

The α and β B subunit genes are located on human chromosome 2 and the β A subunit gene on human chromosome 7. There is a high (85%) structural homology across species for each of the three subunits with 63% homology within species between β A and β B subunit sequences. Activin A in particular, shows a high degree of homology across species with a single amino acid difference across human, bovine, porcine, ovine and murine species (Walton et al., 2011).

Assay Methods

A number of biological and immunological methods have been used to quantitate inhibin. Inhibin was originally isolated employing in vitro bioassays using pituitary cells in culture with FSH release or cell content as the biological end point. Activins, conversely, stimulate FSH secretion from pituitary cell cultures but have also been found to exhibit mitogenic activity in a range of cell systems including the testis. Enzyme-linked immunoassays (ELISAs) have been developed for inhibin A and B using paired antibodies directed to the α and either β A/ β B subunits. ELISAs to the α subunit (using antibodies directed to NH₂ and COOH-terminal regions (Pro- α C) have been devised which detects primarily the free α subunit in serum as inhibin precursor levels in serum are low. Non-specific inhibin assays have also been devised which detect all α subunit containing inhibin forms. These ELISAs have been widely applied to serum, gonadal extracts and cell culture media in a range of species. Many of these ELISAs are commercially available (Walton et al., 2011).

Isolation and Native Forms

Inhibin A was originally isolated from ovarian follicular fluid as both mature ($\sim$ 32k) and partially processed (58k) forms consisting of a dimer of mature β A and either processed or partially processed α subunit. These forms are bioactive based on their FSH suppressing activity. Inhibin A has been isolated from ovine rete testis fluid with a mol wt $\sim$ 30k consisting of a α and ovine β A sequence.

Inhibin B is the most common or abundant form of inhibin found in males in humans, non-human primates and rodents while inhibin A predominates in sheep but is also found in addition to inhibin B in bovine and porcine species. The 30k mature and partially processed 50–60k inhibin forms show similar in vitro bioactivities, however the unprocessed precursor inhibin form is bioinactive due to its inability to binding to betaglycan, a cofactor essential for inhibin's inhibitory activity. However the unprocessed precursor levels in human serum are minimal. A comparison of the in vitro and in vivo bioactivities of inhibin A and B in rats showed that human 30k inhibin B was $4.2 \times$ and $1.45 \times$ more potent than 30k inhibin A. The α subunit is bioinactive based on its FSH suppressing activity.

An assessment of molecular weight forms of inhibin B in human male serum shows a combination of mature (26–30k) and partially processed (60–65k) dimeric (α and β B) forms and an absence of inhibin A. In healthy men or men with testicular disorders, inhibin B exists in plasma as 29% precursor, 8% free α subunit and the remainder as the mature 30k form. The free α subunit exists as either the full α subunit precursor (49k) or predominantly as a partially processed molecule (29k) termed Pro- α C (Makanji et al., 2011).

Location and Production by the Testis

Following castration, inhibin diminish to nondetectable levels indicating the testis is the major source for inhibin, while activins levels remain unchanged indicating nongonadal sources. Inhibin α subunit (as mRNA or protein) is produced by the Sertoli and Leydig cells but not by germ cells. The β A subunit, as mRNA and as detected by immunocytochemistry has been detected in Sertoli and peritubular cells while β B subunit has been identified in the Sertoli cell, peritubular cells, spermatogonia, primary spermatocytes and round spermatids. In the rat, mouse, monkey and human, inhibin B only is synthesized primarily from the Sertoli

cell while the Leydig cell produces free α subunit and possibly some inhibin B. Leydig cell stimulation with human chorionic gonadotropin (a hormone with high luteinizing hormone activity) in humans resulted in an increase in free α subunit but no increase in inhibin B. Considering the high levels of activin A produced by Sertoli cells in these species, it is surprising that substantial amounts of inhibin A are not also produced. This incongruity suggests that α subunit dimerisation with either β A or β B subunits by the Sertoli cell is likely to be a regulated process with a preference in the formation of inhibin B rather than inhibin A in some but not all species.

Inhibin α subunit and to a lesser extent, β B subunit production by Sertoli cells *in vitro* are stimulated by FSH through the protein kinase A pathway. Earlier studies failed to detect FSH stimulation of β B subunit in Sertoli cells but this was established with more sensitive nuclear run-on or real time PCR methods. The expression of activins by germ cells is not thought to contribute to inhibin synthesis or inhibin formation by Sertoli cells. Immature Sertoli cells also produce β A subunit, but it is not normally stimulated by FSH.

The close relationship between Sertoli cell number, sperm number and serum inhibin B has been observed in the rat, monkey and human suggesting that inhibin B is a marker of Sertoli cell number and hence sperm number based on their close relationship. In the rhesus monkey, serum inhibin B correlated with both Sertoli cell number and testicular round spermatid number. This association is supported by studies showing that administration of GnRH or FSH to monkeys or to men with acquired hypogonadotropic hypogonadism led to an increase in inhibin B from undetectable levels into the normal range in parallel with the recovery of spermatogenesis. However, administration of FSH to men with normal spermatogenesis showed no increase in inhibin B despite an increase in serum inhibin α subunit. Inhibin B levels remained unchanged following LH administration. Thus inhibin production is linked to Sertoli cells number and the closely linked sperm number ([Hedger and Winnall, 2012](#); [Ramaswamy and Plant, 2001](#); [Sharpe et al., 1999](#)).

Role of Testicular Germ Cells

The α subunit, β B subunit and inhibin B show cyclic behavior across the spermatogenic cycle in rats peaking in Stages XIII–I of the cycle with lowest levels in Stages VII–VIII. This pattern parallels changes in FSH sensitivity in tubule segment cultures across the cycle. The β A subunit peaks in Stages VII–XII. This variation across cycle in inhibin B and α , β subunits suggests that their expression is modified by the stage of spermatogenesis and thus by particular germ cell types. To examine this further, α and β B subunit levels were monitored in rats following vitamin A deficiency and replacement where stages of spermatogenesis can be specifically investigated. Under these conditions, α and β B subunit levels decreased following Vitamin A deficiency but recovered with Vitamin A replacement in parallel with the recovery of spermatogenesis. It was concluded that inhibin B expression was stimulated by the presence of early spermatocytes and round spermatids while mid-pachytene spermatocytes exerted an inhibitory effect. Using X-irradiation to deplete the testis of spermatogonia and all subsequent germ cell types, resulted in inhibin B levels decreasing to non-detectable levels. Other procedures modifying germ cell number (chemotherapy, androgen treatment) also led to decreases in inhibin B. These studies suggest that a germ cell factor is influencing Sertoli cell production of inhibin B. Co-cultures of Sertoli cells and isolated germ cells have identified inhibitory effects on inhibin B protein and message however the factor(s) has not yet been identified ([Hedger and Winnall, 2012](#)).

An inverse relationship has been observed between serum inhibin B and FSH in normal men and men with testicular pathologies. Following chemotherapy and testicular irradiation, spermatogenesis including spermatogonia and inhibin B levels decrease to non-detectable levels while FSH reaches castration levels. With androgen treatment (e.g., in contraceptive trials), despite sperm number as well as serum FSH/LH decreasing to near non-detectable levels, serum inhibin B decreases partially, in some cases minimally, suggesting that serum inhibin B is only partially under gonadotropin control. As an explanation, it should be noted that the process of spermatogenesis is largely unchanged prior up to the spermiogenic stage by androgen antagonist treatment. However, subsequent stages associated with sperm release are inhibited by androgen antagonist treatment leading to the premature loss of round spermatids from the epithelium and a reduction in sperm release into the reproductive tract. Hence inhibin B production under the influence of early stage spermatogenesis can continue despite absence of sperm in the ejaculate ([De Kretser et al., 2004](#)).

Collectively, these data indicate that FSH stimulates Sertoli cell proliferation and inhibin B secretion in the pre-pubertal testis. Once spermatogenesis has been initiated, FSH stimulation of Sertoli cell number and sperm number ceases. Thus changes in inhibin B levels as a result of testicular damage or endocrine manipulation in the adult are related more to changes in germ cell composition and their influence on Sertoli cell inhibin production. Reductions in inhibin B prior to puberty are a reflection of reduced Sertoli cell number.

Changes With Age

Both α and β B subunits are localized to fetal human and rat Sertoli cells. Inhibin A and B are detectable in human male serum at 14–16 weeks of fetal life. In male fetuses at mid trimester, inhibin B levels correlate directly with LH and indirectly with FSH. Following birth, inhibin B and FSH are low until puberty when both increase between Tanner stage G1 and G2. Inhibin B falls with age in men, by age 70–85y inhibin B has fallen 25% while serum FSH increased fourfold. In rats and rhesus monkeys there is a prepubertal peak in inhibin B prior to the subsequent post pubertal rise. In rats but not humans, serum inhibin A is detected at low levels post birth peaking at day 15 then decreasing thereafter ([Itman et al., 2006](#)).

Action on the Pituitary

Exogenous administration of purified preparations of inhibin A and B to rats, monkeys and sheep have shown a selective suppression of FSH but not LH while active immunoneutralisation using antisera raised to the inhibin α subunit showed a rise in FSH. Further evidence was derived from endocrine manipulations of gonadotrophin secretion (e.g., following hemicastration and suppression of gonadotrophins by steroid treatment) and by review of serum hormone patterns associated with human testicular disorders. Inhibin's endocrine role was underscored in studies in mice where inhibin A was inducibly expressed and secreted by the liver. In both short and long term studies in which physiologic and high inhibin A serum levels were maintained, serum FSH, testicular size and seminiferous tubule volume were reduced in strong support of the endocrine role of inhibin in regulating FSH (Pierson et al., 2000).

While inhibin has been shown to preferentially reduce pituitary FSH production and secretion, activin in contrast, has been shown to preferentially stimulate FSH β subunit synthesis and thus FSH. Activin's action is mediated by binding to two pituitary receptor proteins in sequence, Activin receptor Type II (ActRII) and Type I receptor (ALK4) which in turn phosphorylates specific second messenger intracellular proteins (SMAD2/3) which migrate to the nucleus. Other SMAD proteins (SMAD 4/7) either inhibit or facilitate this transfer or interaction with DNA. Activin B in addition to that seen with activin A is thought to involve an additional Type I receptor (ALK7) which is known to phosphorylate SMADs 1,2,5.

Studies exploring the possibility that inhibin may exert its inhibitory actions by inhibiting activin's action through a specific receptor had been unsuccessful. Binding proteins for inhibin were identified but failed to mediate inhibin's action. Inhibin was able to weakly suppress activin's stimulation of FSH production and secretion. However its inhibitory activity was increased dramatically with the identification of a cell surface proteoglycan called betaglycan which bound inhibin, creating a high-affinity complex blocking the ActRII receptor site limiting the stimulatory action of activin. Betaglycan also interacted with other members of the TGF β superfamily for example, TGF β II where it is essential for activity, and BMP2,4,7. As pituitary gonadotrophs express α , β B and β B subunits as well as betaglycan and activin type 1 and 2 receptors why cannot locally produced inhibin be responsible for inhibiting activin's action on the pituitary? While this is possible, the bulk of the evidence supports gonadally produced inhibin as the major inhibin source (Bilezikjian et al., 2006; Lewis et al., 2000).

To differentiate between the contributions of inhibin and the reproductive steroids, testosterone and estradiol as endocrine hormones in regulating FSH, *in vivo* studies in men were undertaken using aromatase inhibitors, to suppress the conversion of testosterone to estradiol (a key steroid in regulating FSH and LH), and ketoconazole which suppresses testosterone and estradiol production. Inhibin B levels were marginally decreased (16%) while FSH levels doubled following treatment. In contrast, in men with anorchia (prepubertal testis) where inhibin B levels were low, FSH was markedly elevated to approach castrate levels. It was concluded that inhibin B was able to regulate or stabilize FSH secretion under normal circumstances but that once inhibin B levels fell below normal range values, the underlying stimulatory effects of steroids on FSH becomes apparent. These authors concluded that in the human male, inhibin B is the principal gonadal feedback regulator of FSH secretion unless seminiferous tubule function is severely compromised (Boepple et al., 2008).

Inhibin B has been detected in seminal plasma showing a wide range of concentrations. It correlates with sperm number in fertile and infertile men and is absent following vasectomy. However semen and serum inhibin B levels do not always correlate, for example, in progestin male contraceptive studies, semen inhibin B levels were non-detectable while detectable in serum and vice versa. The reasons are unclear (Anderson, 2001).

Over Expression and Deletion of Inhibin Subunit Genes

Mice with deletions of the α and β B subunit genes showed a loss of inhibin B with a corresponding increase in serum FSH. Deletion of the α subunit results in the development of testicular tumors by 4 weeks post birth attributed to the concomitant rise in the production of the β A/ β B subunits as activins A/B which are potent mitogens. Prior to tumor formation normal spermatogenesis was observed although FSH was elevated twofold over controls. Embryogenesis appeared to be normal.

In contrast, over expression of the α subunit led to decreases in serum FSH, testicular size and sperm production but the mice remain fertile. These decreases are attributed to a decline in activin production and its mitogenic effects upon the testis as a result of an increase in α : β subunit dimerisation due to the increase in α subunit production.

Deletion of the β A subunit gene is lethal at birth while deletion of the β B subunit gene and thus a loss of inhibin B, showed negligible effects on the testis and fertility in adult male mice. Overall these studies show an inhibitory endocrine role of inhibin on FSH levels but are complicated by the trophic effects of excess activin when the α subunit is deleted (Chang et al., 2002).

Clinical Relevance

The observed correlation between serum inhibin B, Sertoli cell number and sperm count has suggested that inhibin B may be suitable as a biomarker of testicular function. During infancy and childhood, inhibin B is a clinically useful marker of Sertoli cell function in boys with various testicular disorders (anorchia, abdominal testis and gonadal dysgenesis). In addition, inhibin B is a useful marker in the diagnosis of primary and congenital central hypogonadism where inhibin B and FSH are low and in acquired central

hypogonadism where inhibin B is normal. Inhibin B is also a useful marker of spermatogenesis in cancer survivors following radiation therapy and chemotherapy. In particular serum inhibin B is a predictive marker of azoospermia in boys and adult males treated for testicular cancer (Valeri et al., 2013).

References

- Anderson, R. A. (2001). Clinical studies: Inhibin in the adult male. *Molecular and Cellular Endocrinology*, 180, 109–116.
- Bilezikjian, L. M., Blount, A. L., Donaldson, C. J., & Vale, W. W. (2006). Pituitary actions of ligands of the TGF-beta family: Activins and inhibins. *Reproduction*, 132, 207–215.
- Boepple, P. A., Hayes, F. J., Dwyer, A. A., et al., N., (2008). Relative roles of inhibin B and sex steroids in the negative feedback regulation of follicle-stimulating hormone in men across the full spectrum of seminiferous epithelium function. *The Journal of Clinical Endocrinology and Metabolism*, 93, 1809–1814.
- Chang, H., Brown, C. W., & Matzuk, M. M. (2002). Genetic analysis of the mammalian transforming growth factor- β superfamily. *Endocrine Reviews*, 23, 787–823.
- De Kretser, D. M., Buzzard, J. J., Okuma, Y., et al. (2004). The role of activin, follistatin and inhibin in testicular physiology. *Molecular and Cellular Endocrinology*, 225, 57–64.
- Hedger, M. P., & Winnall, W. R. (2012). Regulation of activin and inhibin in the adult testis and the evidence for functional roles in spermatogenesis and immunoregulation. *Molecular and Cellular Endocrinology*, 359, 30–42.
- Itman, C., Mendis, S., Barakat, B., & Loveland, K. L. (2006). All in the family: TGF-beta family action in testis development. *Reproduction*, 132, 233–246.
- Lewis, K. A., Gray, P. C., Blount, A. L., et al. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. *Nature*, 404, 411–414.
- Makanji, Y., Harrison, C. A., & Robertson, D. M. (2011). Feedback regulation by inhibins A and B of the pituitary secretion of follicle-stimulating hormone. *Vitamins and Hormones*, 85, 299–321.
- Pierson, T. M., Wang, Y., DeMayo, F. J., et al. (2000). Regulable expression of inhibin A in wild-type and inhibin alpha null mice. *Molecular Endocrinology*, 14, 1075–1085.
- Ramaswamy, S., & Plant, T. M. (2001). Operation of the follicle-stimulating hormone (FSH)–inhibin B feedback loop in the control of primate spermatogenesis. *Molecular and Cellular Endocrinology*, 180, 93–101.
- Sharpe, R. M., Turner, K. J., McKinnell, C., et al. (1999). Inhibin B levels in plasma of the male rat from birth to adulthood: Effect of experimental manipulation of Sertoli cell number. *Journal of Andrology*, 20, 94–101.
- Valeri, C., Schteingart, H. F., & Rey, R. A. (2013). The prepubertal testis: Biomarkers and functions. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 20, 224–233.
- Walton, K. L., Makanji, Y., Robertson, D. M., & Harrison, C. A. (2011). The synthesis and secretion of inhibins. *Vitamins and Hormones*, 85, 149–184.

Activins in Male Reproduction

Kate L Loveland, Hudson Institute of Medical Research, Clayton, VIC, Australia; and Monash University, Clayton, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Discovery and Physiological Context: Activins are Central to Reproductive Health

Although the testis produces factors that can maintain secondary sexual characteristics in the absence of ongoing spermatogenesis, the pituitary-derived hormone, follicle stimulating hormone (FSH), is required to establish and maintain fertility. FSH levels are widely used to assess the reproductive health of males (Kelsey et al., 2017), and the extent of FSH synthesis and secretion reflects endogenous and environmental factors that control fertility.

The search for important cellular and biochemical elements that control reproductive health led to the isolation of inhibins and activins in the 1980s. Independent teams discovered activin as a protein present in ovarian follicular fluid that stimulates pituitary cell FSH secretion, placing activin in an important reciprocal relationship with the newly described inhibitor of pituitary FSH production, inhibin. The excitement these discoveries generated was fuelled by recognition that inhibin and activin are in the TGF β superfamily of over 30 ligands that signal through a distinct receptor class. Activin B was purified in 1992 and identified as the pituitary-derived activin moiety most likely to be driving FSH β subunit gene transcription in gonadotropes (reviewed in Fortin et al., 2015).

Assays measuring circulating activins and inhibins are now commonly employed along with FSH measurements to ascertain hypothalamic–pituitary–gonadal (HPG) axis function within individuals. Activins function as both endocrine and paracrine factors, being synthesized and acting directly on cells within many different organs, such as gonads, brain, bone marrow, adrenals, intestinal tract, as well as circulating widely to coordinate organ growth and homeostasis. Despite the important, diverse roles of activins and inhibins in human clinical issues, most knowledge of activin biology has been gained from studying animal models, especially rodents in which genetic modifications and culture methods permit functional pathway analyses.

Structural Features of Activins, Members of the TGF β Superfamily of Ligands

Activins Are Dimeric Ligands

The activins are classified as TGF β superfamily members, based on common ligand structure and shared signaling machinery usage. Five activin genes encode distinct β subunits: activin β A (e.g., *INHBA* gene, may also be termed inhibin β A), B, C, D, and E, with D not present in the mammalian genome. These form and function as homo- and heterodimers, with two β A subunits forming activin A, activin β A, and β B subunits forming activin AB, and activin B a dimer of two β B subunits, and four of these subunits collectively forming the signaling moiety (Fig. 1). Individual activin subunit genes and proteins are highly conserved, with mouse and human activin A mature proteins 100% identical, and human activin A and B 64% identical (reviewed in Loveland and Hedger, 2015).

Subunit Domains and Factors Affecting Ligand Functionality

Each subunit is synthesized with a proregion that directs entry into the endoplasmic reticulum during translation for eventual secretion, and pro- and mature-protein domains. Formation of the overall tertiary structure of activin signaling molecules is initiated by dimerization through a single intermolecular disulfide bond within the mature region. Proteolytic processing separates the pro- and mature domains. The proregion remains closely associated until the ligand is secreted; this noncovalent association is relatively weak for activin, enabling ready availability for receptor binding upon secretion. Proregions mediate formation of three intramolecular disulfide bonds within each mature signaling molecule (Harrison et al., 2011). The inhibin α subunit is produced in a similar way, but does not form homodimers. Instead, an α subunit pairs with an activin β subunit to generate a potent inhibitor of activin binding to its receptor. Inhibin A forms as an α : β A dimer and inhibin B as α : β B (Fig. 1). The stoichiometry of α and β subunits produced by each cell determines the relative levels of either activin or inhibin generated. Additional factors affecting activin bioactivity are specific proteolytic enzymes that separate the pro- and mature domains, pro-convertases (e.g., PCSK3, also named furin, for activin A). The activin proregion also affects ligand stability and binding to the extracellular matrix (ECM), in addition to its attainment of the tertiary structure required for signaling. Selective binding of activin to the heparin sulfate proteoglycan side chains of perlecan or agrin (Wang et al., 2016) may also increase its local concentration or mediate its presentation to the receptor to initiate signaling.

Activin Signaling Mediators and Regulators

Activin Signaling

The TGF β superfamily ligands constitute a unique class of molecules that signal through cell surface receptors with intracellular serine/threonine kinase activity. Activins first bind a Type II receptor subunit which contains a constitutively active kinase, typically

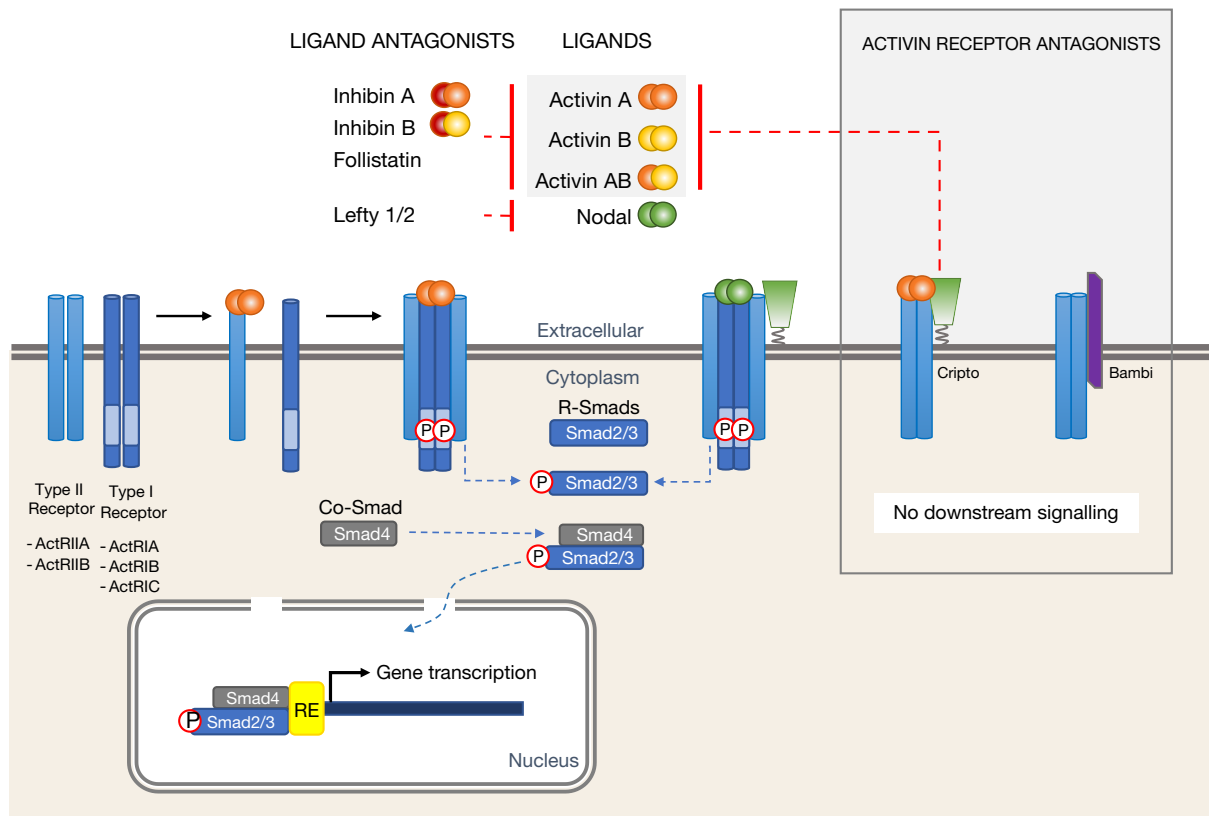


Fig. 1 Activin signaling machinery and inhibitors. Dimeric ligands (activin A, B, and AB) signal by binding to Type II then Type I receptor subunits. The heterotetrameric receptor subunits recruit and phosphorylate Smad2 and 3, which then bind Smad4 and translocate into the nucleus to activate target genes. Inhibitors include soluble and membrane-associated antagonists, as well as intracellular proteins.

ActRIIB (encoded by *ACVR2B*) and also ActRII (also termed ActRIIA, encoded by *ACVR2A*) (Fig. 1). This enables recruitment and activation via phosphorylation of a Type I receptor, typically ALK4 (ActRIIB) for activin A and ALK7 (ActRIC) for activin B.

The activated Type I receptor can recruit and phosphorylate SMAD2 and/or SMAD3 molecules. C-terminal phosphorylation of these regulatory Smad proteins leads two of them to associate with a single SMAD4 molecule. Termed the common SMAD, or co-SMAD, SMAD4 is recruited during signal transduction by nearly all TGF β ligands, while regulatory Smads (R-Smad; includes SMAD1, 2, 3, 5, and 8) and receptor subunit utilization profiles are ligand-specific. Nuclear import of the trimeric Smad complex is mediated by binding to importin proteins; SMAD2 and SMAD3 delivery into the nucleus allows them to bind DNA directly, and to interact with transcriptional cofactors to promote transcription from SMAD binding elements (SBEs) in target gene promoters (reviewed in Hill, 2016). Alternative splicing of SMAD2 transcripts predominantly yields a long isoform that does not bind DNA, while an isoform lacking exon 3 does. SBEs feature direct or inverted repeats of the AGAC consensus sequence near the transcription start site, but specific binding can also be determined by the complement of cell- and developmentally-regulated cofactors such as Forkhead box protein H1 (FOXH1, also known as FAST-1), c-Jun, c-Fos, NANOG, and OCT4 (pluripotency factors) and TAZ, YAP, and TEADs (Hippo pathway transcription factors). Some cofactors have suppressive effects, moderating signaling inputs from TGF β ligands. Posttranslational modification of SMADs via acetylation, poly-ADP-ribosylation and monoubiquitylation can influence their transcriptional activity (Hill, 2016). Thus, the specific cell and tissue context determines the outcomes of activin binding to its receptors.

Common and Distinct Machinery for Signal Transduction by TGF β Superfamily Ligands

Central to activin functions are the complex interactions with other TGF β superfamily ligands through shared and separate signaling pathways. The three prototypical superfamily members, TGF β 1-3, signal through Type I and Type II homodimeric receptors that are not used by other ligands, and TGF β 2 is uniquely reliant on a third nonsignaling receptor, TGFBR3 (also named betaglycan) to signal. Despite activating different receptors, activins and TGF β s both initiate signal transduction by inducing phosphorylation of SMAD2 and SMAD3. Important cell-specific roles for these SMADs have been delineated, for example with SMAD3 in proliferating Sertoli cells (see Section “Activins in Infant and Juvenile Testes”). The bone morphogenetic proteins (BMPs) also share signaling receptors with activin, but their binding triggers recruitment and phosphorylation of SMAD1, 5, and 8.

Although SMAD4 is common to the transduction cascade for each of these signals, target gene activation of is conveyed through unique binding to ligand-specific genomic response elements. For example, Nodal also signals through the same receptors as activin to phosphorylate SMAD2 and 3, however the relative intensity of activin A versus Nodal signaling can direct SMADs to different binding elements. In this manner, the relative abundance of each ligand and intracellular suite of coactivators determines the profile of target gene activation, mediating the diverse array of responses characteristic of this family.

Signaling Inhibitors, Competitors, and Enhancers

Bioactivity of activins and other TGF β superfamily ligands is controlled by a plethora of regulatory molecules with distinct and overlapping roles (Fig. 1). The synthesis of inhibin α and β C subunits within cells producing either activin β A or β B subunits leads to inhibitory dimer formation and decreases production of signaling-active activin. Potent and abundant extracellular, soluble regulators also include follistatin and follistatin-like3 (also termed FSTL3 and follistatin-related protein). The Nodal co-receptor, CRIPTO, impairs activin signaling by blocking its access to receptor subunits. The membrane anchored BAMBI shares structural homology with the Type 1 receptors, blocking downstream signal propagation due to the absence of an active intracellular kinase domain. Intracellular inhibitors of activin signaling include SMAD 7, SKI, and SKIL (previously termed SnoN), with SKIL synthesis induced by TGF β pathway activation. Synthesis profiles for many of these have been mapped during testis development and associate with distinct spermatogenic stages; physiological roles of many have been addressed by examination of mice lacking these genes (Itman et al., 2011).

Activin and Inhibin Synthesis: Links With Functional Roles

Inhibin Subunit Production

The testis is central to the complex and interdependent pathways and cellular sites of synthesis which maintain the activin bioactivity levels required for testis growth and ongoing spermatogenesis. The roles of activin in the seminiferous epithelium that include blood–testis barrier regulation and control of germline differentiation (Sections “[Activins in Infant and Juvenile Testes](#)” and “[Activin Roles in Adult Testes and in the Epididymis](#)”) highlight the importance of balanced and coordinated local inhibin and activin subunit production to fertility.

The inhibin α subunit, encoded by the *INHA* gene, appears to be synthesized solely within Sertoli cells in adults, triggered predominantly in response to FSH stimulation of its receptors. FSH-induced activation of the *INHA* promoter cyclic-AMP (cAMP) response element can be partly enhanced by androgens. In the highly polarized Sertoli cells of adults, both apical and basal secretion of inhibin have been demonstrated in vitro. The central role of circulating inhibin for hypothalamo-pituitary–gonad (HPG) axis regulation is established (Section “[Activin in the Hypothalamic–Pituitary–Gonadal Axis](#)”), though whether inhibin impacts directly on haploid germ cells in the lumen as originally proposed is unverified.

The potential for developmentally regulated control of inhibin synthesis throughout the Sertoli cell lifespan is not fully delineated; the stimulus for upregulation of *INHA* synthesis in fetal Sertoli cells after sex-determination is not understood (Young et al., 2015). In addition to FSH, other signaling moieties have been implicated from in vitro studies to modulate inhibin α subunit production, with results varying with the species and age of the originating animal from which the cells were derived. Factors that influence inhibin secretion in cultured cells include testosterone, estrogen, and epidermal growth factor; culture methods may also influence the outcomes, as culture densities influence Sertoli cell responses to FSH.

Activin Subunit Synthesis

In contrast to inhibin, activin subunit production occurs throughout the body. Activin β A subunit synthesis is stimulated by classic proinflammatory factors, such as those acting through the interleukin-1/Toll-like receptor (IL1/TLR) pathway via Myd88, tumor necrosis factor (TNF), lipopolysaccharide (LPS), reactive oxygen species (ROS), colony stimulating factor 2, IL13, CD40L, TGF β , and interferon γ (IFN γ) (Loveland and Hedger, 2015). This is central to the roles of activin A as a mediator of myeloid lineage cell (monocyte and macrophage) functions. Macrophages and dendritic cells can both produce and be affected by activin A. Both retinoic acid and glucocorticoids can downregulate monocyte activin A synthesis, while T cell production of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN γ may increase it (Chen and Ten Dijke, 2016). Activin A is produced by several testicular cell types, including gonocytes and peritubular myoid cells in the developing testis, and in spermatocytes, Leydig cells, mast cells, and macrophages in adults. Activin B subunit synthesis in spermatocytes and spermatids demonstrates the potential for multiple activin isoforms to be functioning within the seminiferous epithelium. Studies of cultured Sertoli cells highlighted differential control of the activin A and B subunits, with inflammatory cytokines inducing activin A but not activin B subunit synthesis. Coculture with pachytene spermatocytes suppressed Sertoli cell inhibin β B-subunit synthesis, highlighting the likelihood of complex, local modulation of activin bioactivity.

An important feature of activin production in adult testes is its controlled release at specific stages of the seminiferous epithelium cycle, linked with functional changes in the blood–testis barrier and spermiation. FSH exposure and intracellular cAMP elevation stimulates, and IL1/TNF inhibits, inhibin α -subunit production in Sertoli cells, and they have the opposite effect on β A-subunit synthesis. This reciprocal regulation underlies the highly stage-specific expression of inhibin B and activin A throughout the cycle,

with a decline in inhibin and an increase in activin A synthesis occurring during elongating spermatid release into the tubule lumen (termed spermiation). Sertoli cells phagocytose the residual germ cell cytoplasm, and inflammatory gene expression is increased in both germ and Sertoli cells. This part of the seminiferous epithelium cycle regulated by activin bioactivity is central to mediating release of spermatozoa in sufficient numbers for male fertility.

Similar to *INHBA*, synthesis of both *INHBA* and *INHBB* transcripts is upregulated in the fetal mouse testis during the week after sex determination (Young et al., 2015). Activin A levels peak as male germline cells in the fetal testis, termed gonocytes or prospermatogonia, transform into spermatogonia to initiate the first spermatogenic wave and form the stem cell population that persists throughout adulthood (Barakat et al., 2008). The important recent studies showing that adequate macrophage numbers within fetal and adult testes are crucial for normal testicular growth and for maintenance of spermatogonial stem cells indicate an as yet unexplored importance of activin A bioactivity for governing immune cell actions within the testis at different stages of development. How changes in activin and inhibin levels are initiated and regulated during testicular development remains to be determined, along with the mechanisms that govern their storage, secretion, and degradation.

Activin in the Hypothalamic–Pituitary–Gonadal Axis

Circulating levels of gonadal-derived activin and inhibin proteins are central to male reproductive function, as these provide reciprocal signals to mediate control of the HPG axis (Fig. 2). Gonadotrophin releasing hormone (GnRH) production within the hypothalamus is affected by several gonadal hormones, including estrogen, testosterone, progesterone, and activin bioactivity levels, with the latter determined locally by activins, inhibins, and follistatin. Pulsatile GnRH release acts on the pituitary gland to drive synthesis of FSH and leutinizing hormone (LH), which are comprised of a common α subunit and a distinct β subunit. Activin B signaling stimulates FSH β subunit synthesis in pituitary gonadotrophs and promotes FSH production; this is directly blocked by inhibin, which prevents receptor binding and activation. Activin A promotes LH β subunit synthesis, and is antagonized by testosterone. This highlights the physiological importance of the differing receptor engagement and distinct signaling pathway outcomes engaged by each activin subunit. Circulating FSH levels are directly linked in juveniles to testis growth and in adults to sperm production through its actions on Sertoli cells, while maintenance of LH levels is linked to Leydig cell development and function, including directly influencing testosterone production.

Dynamic and Developmentally Regulated Actions Within the Testis

Activins in the Fetal Testis

Sertoli cells form the essential niche for male germline development. They provide essential cues for the sexually indifferent primordial germ cells arriving at the gonadal ridge to enter the male differentiation pathway by mouse embryonic day 11.5 (E11.5), evoking changes in other somatic cells which result in formation of seminiferous cords within an appropriate milieu of interstitial

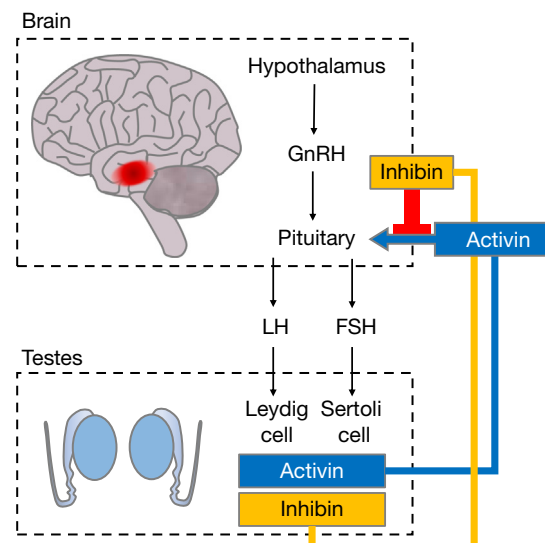


Fig. 2 Reciprocal control of the hypothalamic–pituitary–gonadal (HPG) axis by the endocrine actions of gonadal-derived activin and inhibin proteins. The hypothalamus produces gonadotrophin releasing hormone (GnRH), which in turn drives FSH synthesis in the pituitary. FSH production is facilitated by activin signaling in pituitary gonadotrophs to stimulate FSH β subunit synthesis. Activin signaling is directly blocked by inhibin, which prevents receptor binding and activation. Circulating FSH levels are directly linked in juveniles to testis growth and in adults to sperm production.

cells. Gonocytes proliferate for approximately 2 days, and by E13.5 become quiescent until shortly after birth in rodents. Crucial epigenetic reprogramming of the male germline occurs during this week of fetal life, featuring essential genome-wide demethylation and remethylation to prepare the naïve genome of the earliest sperm precursors (reviewed in [Young et al., 2015](#)). The extent of Sertoli cell proliferation in the fetus and neonate prior to puberty is a strong determinant of adult sperm output, because individual adult Sertoli cells can support simultaneous maturation of a finite number of germ cells.

Inhibin α , β A, and β B subunit mRNAs are elevated in male, but not female, gonads after sex determination. In contrast, upregulation of the *FST* transcript, encoding follistatin, in ovaries suppresses vasculature formation. Analysis of *Inhba* $-/-$ mice, lacking the activin A subunit, provided evidence that activin A drives fetal Sertoli cell multiplication. In these mice, initial events of testicular formation are normal as germ cells become enveloped within Sertoli cell cords. However, Sertoli cell proliferation and the cyclin D2 transcript (*Ccnd2*) were reduced in knockouts by E15.5, and Sertoli cells number at birth in was significantly lower. A subtle but inverse effect on germ cells was detected slightly earlier in *Inhba* $-/-$ testes: the *Cdkn1a* transcript, encoding the p21^{kip1} cell cycle inhibitor in gonocytes, was lower in heterozygous and knockout testes only at E13.5, and increased germ cell numbers were identified using stereological methods. TGF β has a similar inhibitory effect of on fetal gonocyte multiplication in vivo ([Mendis et al., 2011](#)). Because *Inhba* $-/-$ mice die at birth, this model cannot be used to examine activin A depletion in adults. However, selective *Inhba* removal from Leydig cells or of *Smad4* from Sertoli cells (rendering them nonresponsive to activin A and to other TGF β ligands) both yielded shorter, truncated testis cords ([Archambeault and Yao, 2010](#)). These results, extended by additional analyses of fetal testis development, demonstrated an important role for locally produced activin A, particularly from Leydig cells, in the crosstalk between somatic cells that drives the expansion and likely coiling of the testis cords late in fetal development, which ultimately facilitates normal testis development and function.

Immediately after sex determination and prior to the marked elevation of inhibin and activin transcripts, Nodal and its signaling regulators, Lefty1 and Cripto, are upregulated in germ cells. This can sustain germ cell pluripotency and may prevent the inappropriate germline entry into meiosis that occurs normally in female fetal gonads. Thus, a complex interplay of signals between several TGF β superfamily ligands contributes to overall testis growth, with each serving cell-specific roles and several likely to interact with other signaling pathways.

Activins in Infant and Juvenile Testes

Sertoli cell proliferation is maximal around birth in rodents and continues around 12 days in mouse and 16 days in rats. Loosely analogous to human puberty, at this time Sertoli cells transform into nonproliferative cells joined by complex junctions, forming the highly polarized seminiferous epithelium required to support meiotic and haploid phases of spermatogenesis.

The male germ cells present at birth in rodents are quiescent gonocytes, centrally positioned within seminiferous cords formed by proliferating Sertoli cells. The cords are surrounded by a dynamic extracellular matrix and proliferating peritubular cells within a loose interstitium of proliferating Leydig cells, fibroblasts, sparse immune cells, and lymphatic, and blood vessels. Within 1 day after birth in mice (3 days in rats), gonocytes resume mitosis and move radially to contact the cord basement membrane, becoming spermatogonia. At this initiation of spermatogenesis, approximately 50% of spermatogonia begin differentiation immediately to form the first sperm over the following 5–6 weeks; the remainder become the spermatogonial stem cells which self-renew to enable production of sperm throughout adult life. Cells that fail in this gonocyte-to-spermatonium transformation are removed by apoptosis. Disruptions to this may underpin emergence and/or maintenance of the “germ cell neoplasia in situ cells” (GCNIS cells) that precede emergence of testicular tumors in young men (Section “[Testis Cancer and Potential Links With Endocrine Disrupting Chemicals](#)”).

Fetal gonocytes synthesize the activin β A subunit transcript and protein; the transcript is absent at birth but the protein persists. The β A protein is absent from nascent spermatogonia, and the activin antagonists, Follistatin and Bambi are produced ([Fig. 1](#); reviewed in [Young et al., 2015](#)). Activin A sources in the juvenile testis include Leydig and peritubular cells and spermatocytes.

Tight regulation of activin signaling as spermatogonial differentiation commences is potentially linked with the effects of Nodal and bone morphogenetic protein BMP8A to promote spermatogonial proliferation, and the support of spermatogonial stem cell multiplication by the noncanonical family member, Glial cell-derived neurotrophic factor (GDNF). Additionally, BMP4, BMP8A, and BMP8B can also support spermatogonial differentiation. Because all signaling machinery elements are shared by the multiple TGF β superfamily ligands present, the local, tight regulation of their production and activity at the onset of spermatogenesis and during juvenile testis development is important to adult fertility.

Studies of mice lacking activin and inhibin subunits, most notably those generated by Matzuk and colleagues (reviewed in [Namwanje and Brown, 2016](#)), demonstrated effects of activin bioactivity on testes and many other organs. Some aspects of their chronic dysregulation on male reproduction are relatively subtle and may reflect biological redundancies and/or a moderating role for activin in mediating growth and homeostasis.

Inhibin α subunit absence (*Inha* $-/-$ mouse; [Matzuk et al., 1992](#)) does not grossly affect initiation of spermatogenesis, however the resulting high activin levels drive Sertoli cell proliferation past day 12. Sertoli cell-like tumors emerge at 4 weeks, with spermatogenesis progressively lost and adrenal tumors forming later. This identified inhibin as a tumor-suppressor that suppresses activin and allows p27^{kip1} inhibition of cyclinD2/E1 and Ck4/2 cell cycle complexes in Sertoli cells. Deletion of *Smad3* prevents this phenotype, while *Smad2* deletion has no effect; granulosa cell tumor formation in *Inha* $-/-$ females is reduced by *Smad2* deletion. In *Smad3* $-/-$ mice with normal activin/inhibin levels, Sertoli cell maturation is delayed, while Sertoli cells mature earlier in *Smad3* $+/-$ mice and support earlier germ cell maturation in juveniles. Such strict dependency on *Smad3* for Sertoli cell

proliferation coincides with a barrier to phosphorylated Smad2 nuclear entry that is absent from postmitotic cells. Distinct activin gene targets are identified for proliferating versus postmitotic Sertoli cells, linked to differences in activin exposures (Itman et al., 2009). The distinct expression profiles of individual Smads during Sertoli and germ cell development indicate they perform specific roles related to the many different TGF β signaling moieties for which they transduce and regulate gene transcription.

Fertility is unaffected in mice lacking the β B subunit gene. Because *Inhba* $-/-$ mice die at birth, to study activin A postnatal roles, the *Inhba*^{BK/BK} mouse with reduced activin bioactivity (~ 10 -fold lower at sites of β A activity) was generated by replacing the activin β A mature coding sequence with that of β B. These mice usually reach adulthood and can reproduce, but first sperm production occurs 9 days late. Newborn and 7 day old mouse testes contain more germ and Leydig cells compared to wild type littermates, and 7 day testes have fewer Sertoli cells. Similar to *Inhba* $-/-$ testes, the Sertoli to germ cell ratio is lower, indicative of reduced Sertoli cell proliferation in activin A insufficiency conditions. Germ cell differentiation markers do not follow normal patterns in juvenile *Inhba*^{BK/BK} mice, highlighting somatic and germ cell interdependency.

Other hormones interact with activin in juveniles. Sertoli cell proliferation is greatly enhanced by FSH in the second week of life, as shown in culture experiments and through neutralization of FSH bioactivity in *Inha* $-/-$ mice; in the latter, the outcome on Sertoli cell tumor development supports the understanding that this is developmentally-regulated. Activin A also regulates androgen receptor and androgen target gene production, and it modulates FSH-induced aromatase activity; *Inha* $-/-$ mice lacking a functional androgen receptor exhibit a slightly milder tumor phenotype. Thus, activin bioactivity is tightly regulated for appropriate testicular development. It directly influences Sertoli and germ cell maturation to determine the timing of pubertal onset through multiple mechanisms.

Activin Roles in Adult Testes and in the Epididymis

The blood–testis barrier (BTB) that forms from specialized junctions between adjacent Sertoli cells in the seminiferous epithelium is critical for adult fertility, restricting contact between postmitotic germ cells and the immune system and preventing rejection of spermatogenic cells that form after immune tolerance establishment. This complex structure changes throughout the seminiferous epithelium cycle, allowing adluminal movement of differentiated spermatogonia entering meiosis. Activin, along with TGF β , contributes to the cyclical loosening and reformation of this barrier.

The chronic absence of activins or inhibins, or reduction in their activity, has sequelae for male fertility which belie the complex integration of developmental cues affecting male fertility. Such outcomes are subtle and some differ with development. For example, while the testis, epididymis and vas deferens in 25 day *Inha* $-/-$ mice appear normal, the progressive loss of spermatogenic capacity at 8 weeks is accompanied by marked regression of Leydig and excurrent duct epithelial cells, and by a state of androgen insufficiency. *Inba* $+/-$ mice exhibit phenotypic variation throughout development, with adjacent tubules exhibiting advanced and depleted spermatogenesis at 16 days of age that is resolved by 4 weeks. By 8 weeks, *Inba* $+/-$ testes are larger and produce more sperm than littermates, and at 26 weeks they display reduced spermatogenesis, proliferating Sertoli cells and evidence of reduced Leydig cell steroidogenic capacity. As described in Section “Activins in Infant and Juvenile Testes,” substitution of the activin β A subunit with β B in the *Inhba*^{BK/BK} mouse does not result in structural abnormalities of either testis of epididymis at 8 weeks, nor does the absence of one *Inhba* allele impact grossly on fertility. These results indicate normal testis development and function can occur within a range of activin bioactivities, while close scrutiny identifies developmental windows and specific cell types which are affected by altered activin bioactivity.

Activin A in culture can support proliferation of rodent stem and progenitor Leydig cells and prevent their differentiation. A gradient of activin production along the epididymis, from high in the caput to low in the cauda, where follistatin levels increase, affect immune cell functions, duct coiling and no doubt other processes along this tract and into the vas deferens that are yet to be revealed. Importantly, each of these functions occurs within the context of signaling by other TGF β superfamily members and different signaling pathways, so that activin does not solely determine functional outcomes.

Clinical Applications and Links to Testicular Pathologies Affecting Male Reproductive Health

Activins and Inhibins as Biomarkers and Therapeutic Targets

Circulating inhibin B is an established measure of spermatogenesis and Sertoli cell functionality, correlating with testicular size and sperm count. Inhibin B levels may also predict adult fertility in juveniles with conditions affecting testicular function. Inhibin B is made in Sertoli cells and possibly spermatocytes, while both Sertoli and Leydig cells have been identified as producing the inhibin α subunit proregion peptide, pro- α C. The elevation of serum FSH levels in infertile men in which spermatogenesis is disrupted may arise from changes in Sertoli or Leydig cell function or reflect differing testicular cellularity. In adult men, inhibin B and FSH levels in serum are inversely correlated, and inhibin A is not detectable. Conditions which suppress spermatogenesis, such as GnRH or steroid treatment, chemotherapy or irradiation, can have mild to significant impacts on inhibin levels that reflect testicular function.

Production of activin within the testis does not appear to contribute significantly to circulating levels, including during systemic inflammation, when activin is elevated in serum. Seminal glands and epididymides are both potential sources of activin A found in seminal plasma, while the origin of activin B in human seminal fluid is unknown.

Expanding knowledge of immunomodulatory roles for activins (Chen and Ten Dijke, 2016) has led to investigations of its potential as a therapeutic target in conditions of testicular inflammation. In a model of autoimmune epididymo-orchitis, in which

mice are immunized with testis extracts and exhibit testicular immune cell infiltrations, testicular fibrosis and germ cell loss that mimic human disease, the peak of elevated *Inhba* expression was concurrent with active disease. Agents such as follistatin, soluble activin receptors and modified activin prodomains are under investigation as compounds that may mitigate the chronic effects of acute and ongoing inflammation to reduce damage to the testis and epididymis, and thereby preserve fertility.

Testis Cancer and Potential Links With Endocrine Disrupting Chemicals

The rate of testicular cancer continues to increase world-wide, associated with a spectrum of phenotypes referred to as the testicular dysgenesis syndrome, including hypospadias and cryptorchidism; each may impair male fertility and have potential comorbidities. Both genetic and environmental mechanisms may support GCNIS cell emergence and progression into seminomas or nonseminomas in young men. Disrupted androgen activities, through exposure to endocrine disrupting compounds, represent a likely component, potentially linked with altered TGF β superfamily activity.

Activin, Nodal and BMP signaling components have each been implicated in testicular germ cell tumors. The upregulation in testicular tumors of activin inhibitors, inhibin α , betaglycan, and Cripto, and of its competitor, Nodal, indicates activin blockade may be required to sustain these tumors. Circumstances leading to disruption of either or both BMP and activin signaling may enhance the opportunity for Nodal to sustain pluripotent germ cells into adulthood, where the dynamic milieu of the postpubertal seminiferous epithelium can support their expansion as seminomas and inappropriate differentiation as nonseminomas.

Because activin and inhibin functions affect all stages of testis development and are intimately linked with hormone production and function, the potential impact of endocrine disruptors on this pathway has been investigated. Exposure of mice to di-*n*-butyl phthalate (DBP) daily from day 4 to 14 exhibited reduced Sertoli cell multiplication and anogenital distance (indicating reduced androgen bioactivity), while increasing inhibin levels. Integration of knowledge about activin signaling, inhibin functions and endocrine disruptor actions may provide important information about how environmental signals affect reproductive health.

References

- Archambeault, D. R., & Yao, H. H. (2010). Activin A, a product of fetal Leydig cells, is a unique paracrine regulator of Sertoli cell proliferation and fetal testis cord expansion. *Proceedings of the National Academy of Sciences of the United States of America*, 107(23), 10526–10531.
- Barakat, B., O'Connor, A. E., Gold, E., de Kretser, D. M., & Loveland, K. L. (2008). Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. *Reproduction*, 136(3), 345–359.
- Chen, W., & Ten Dijke, P. (2016). Immunoregulation by members of the TGF β superfamily. *Nature Reviews Immunology*, 16(12), 723–740.
- Fortin, J., Ongaro, L., Li, Y., Tran, S., Lamba, P., Wang, Y., Zhou, X., & Bernard, D. J. (2015). Activin signaling in gonadotropes: What does the FOX say... to the SMAD? *Molecular Endocrinology*, 29(7), 963–977.
- Harrison, C. A., Al-Musawi, S. L., & Walton, K. L. (2011). Prodomains regulate the synthesis, extracellular localisation and activity of TGF- β superfamily ligands. *Growth Factors*, 29(5), 174–186.
- Hill, C.S. (2016) Transcriptional control by the SMADs. *Cold Spring Harbor Perspectives in Biology*. 8(10), pii: a022079.
- Itman, C., Small, C., Griswold, M., Nagaraja, A. K., Matzuk, M. M., Brown, C. W., Jans, D. A., & Loveland, K. L. (2009). Developmentally regulated SMAD2 and SMAD3 utilization directs activin signaling outcomes. *Developmental Dynamics*, 238(7), 1688–1700.
- Itman, C., Wong, C., Whiley, P. A., Fernando, D., & Loveland, K. L. (2011). TGF β superfamily signaling regulators are differentially expressed in the developing and adult mouse testis. *Spermatogenesis*, 1(1), 63–72.
- Kelsey, T. W., McConville, L., Edgar, A. B., Ungurianu, A. I., Mitchell, R. T., Anderson, R. A., & Wallace, W. H. B. (2017). Follicle stimulating hormone is an accurate predictor of azoospermia in childhood cancer survivors. *PLoS One*, 12(7), e0181377.
- Loveland, K. L., & Hedger, M. P. (2015). Activins and inhibins in Sertoli cell biology: Implications for testis development and function. In M. Griswold (Ed.), *Sertoli cell biology* (pp. 201–221). Atlanta, GA: Elsevier Science.
- Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J., & Bradley, A. (1992). Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature*, 360(6402), 313–319.
- Mendis, S. H., Meachem, S. J., Sarraj, M. A., & Loveland, K. L. (2011). Activin A balances Sertoli and germ cell proliferation in the fetal mouse testis. *Biology of Reproduction*, 84(2), 379–391.
- Namwanje, M., Brown, C.W. (2016) Activins and inhibins: Roles in development, physiology, and disease. *Cold Spring Harbor Perspectives in Biology*. 8(7), pii: a021881.
- Wang, X., Fischer, G., & Hyvönen, M. (2016). Structure and activation of pro-activin A. *Nature Communications*, 7, 12052.
- Young, J. C., Wakitani, S., & Loveland, K. L. (2015). TGF- β superfamily signaling in testis formation and early male germline development. *Seminars in Cell & Developmental Biology*, 45, 94–103.

Leydig Cell Androgen Synthesis

Lu Li, University of Southern California, Los Angeles, CA, United States

Barry R Zirkin, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States

Vassilios Papadopoulos, University of Southern California, Los Angeles, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Testicular Leydig cells are the primary source of androgens, which are indispensable for the development of the male reproductive system. Androgens synthesized by the interstitial Leydig cells include testosterone, 5 α -dihydrotestosterone, androstenedione, androstanediol, and androsterone (Payne and Hales, 2004; Teerds and Huhtaniemi, 2015). Among these, testosterone is the most prevalent male sex androgen. Testosterone induces masculinization before birth, drives the establishment of male secondary sexual characteristics after birth and supports spermatogenesis throughout life (Payne and Hardy, 2007).

There are two classes of enzymes involved in androgen biosynthesis: the cytochrome P450 heme-containing proteins and the hydroxysteroid dehydrogenases. These enzymes are located in mitochondria and smooth endoplasmic reticulum (SER), respectively, and they catalyze the conversion of cholesterol to androgens. Notably, the prevalent androgens produced by Leydig cells at each developmental stage are different due to the developmental, stage-specific expression of steroidogenic enzymes (Ye et al., 2017). Given that testosterone is considered to be the most important androgen for masculinization and spermatogenesis, we will mainly discuss testosterone biosynthesis in this article.

Source of Cholesterol

In Leydig cells, cholesterol is synthesized de novo from acetate. Besides de novo synthesis, plasma lipoproteins bring cholesterol to Leydig cells (Hu et al., 2010). The de novo pathway is preferably used in the rat and lipoproteins in humans (Payne and Hardy, 2007). Cholesterol can be stored in the form of cholesterol esters in lipid droplets or come from various membranes e.g. plasma membrane.

Acetate

Under normal physiological conditions, Leydig cells preferentially synthesize cholesterol de novo from acetate (Fig. 1). Among numerous enzymes required for the conversion of acetate to cholesterol, 3-hydroxy-3-methylglutaryl (HMG)-CoA-reductase (HMGCR) located in the SER is the rate-limiting enzyme that catalyzes the condensation of acetyl-CoA into mevalonate. De novo synthesis of cholesterol is also under the control of pituitary luteinizing hormone (LH). The newly formed cholesterol will be transferred from the endoplasmic reticulum to the plasma membrane. Excess cholesterol is converted to cholesterol esters by acetyl-CoA acetyltransferase (ACAT1) and packed into lipid droplets.

Plasma Lipoprotein and Intracellular Cholesterol Esters

Newly synthesized cholesterol esters are the source of cholesterol for androgen synthesis in response to trophic hormone stimulation. Depending upon conditions, however (e.g., LH-induced desensitization of the steroidogenic response), Leydig cells may utilize plasma lipoprotein as the source for cholesterol synthesis (Fig. 1). In this scenario, cholesterol may come either from plasma low-density lipoprotein (LDL) or high-density lipoprotein (HDL). Cholesterol esters released from LDL are delivered to the plasma membrane or to lipid droplets and mitochondria for androgen synthesis. Cholesterol esters from HDL are hydrolyzed and the released free cholesterol is used for androgen synthesis.

Plasma Membrane

In mammalian cells, free cholesterol is abundant in the plasma membrane (Maxfield and Wustner, 2002). The origin of this cholesterol could be from either de novo synthesis or lipoprotein import and processing. Hormone-treatment of Leydig cells induces rapid mobilization of free cholesterol from the inner leaflet of the plasma membrane to mitochondria for steroid synthesis (Freeman, 1989; Venugopal et al., 2016). This cholesterol constitutes the pool of free cholesterol used for steroidogenesis in response to LH stimulation until de-esterification of stored cholesterol provides large amounts to sustain steroidogenesis.

Biosynthesis of Androgens From Cholesterol

Mobilization of Cellular Cholesterol

As noted above, cholesterol is released from the plasma membrane and then lipid droplets upon trophic stimulation by LH or human chorionic gonadotropin (hCG). LH/hCG binds to a G protein-coupled receptor that activates the adenylate cyclase leading

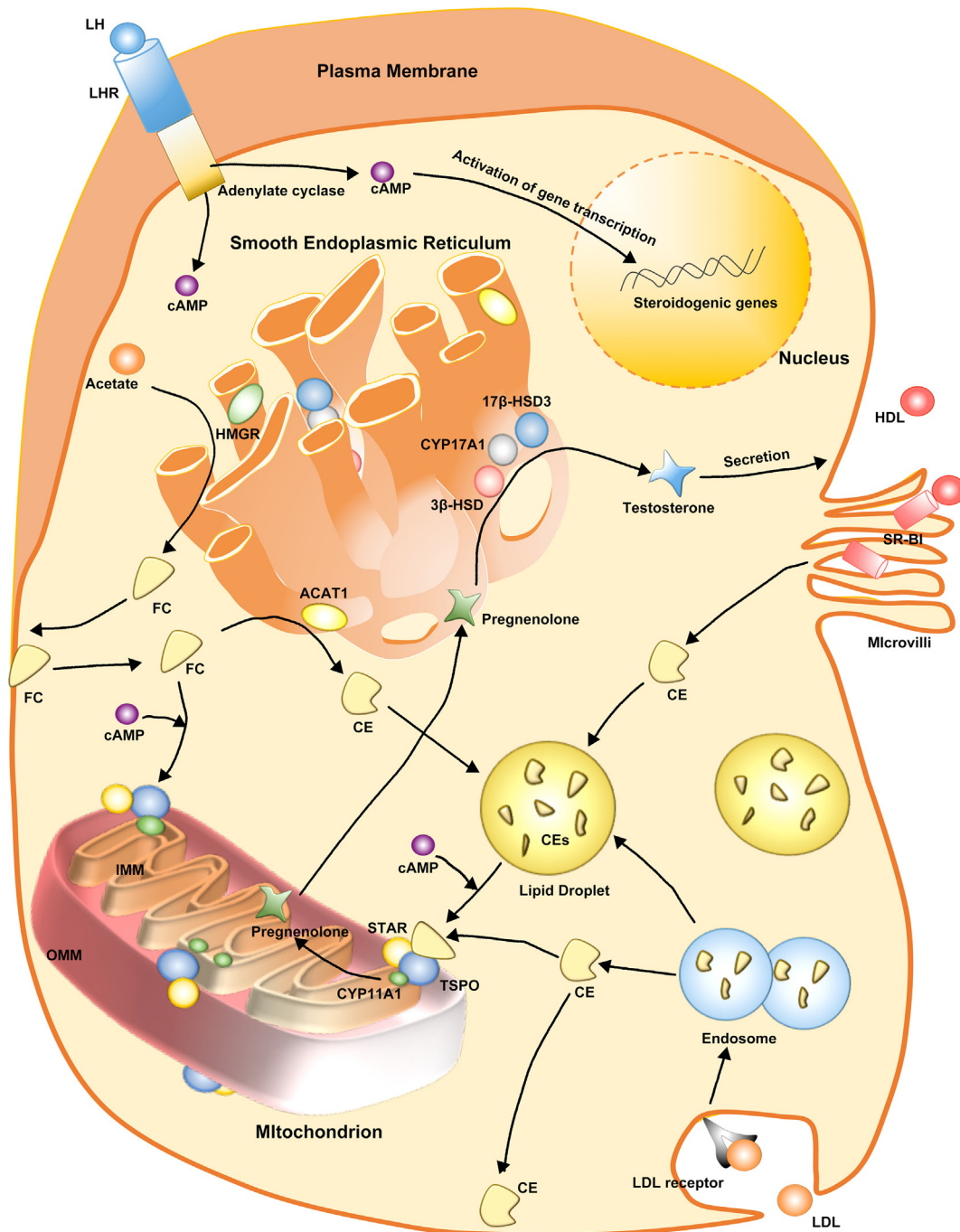


Fig. 1 Molecular and cellular events involved in testosterone biosynthesis in Leydig cells. In Leydig cells, androgens are derived from cholesterol. This figure depicts testosterone synthesis in Leydig cells. Cholesterol is either derived by de novo synthesis or from low-density lipoprotein (LDL) or high-density lipoprotein (HDL). Following this, cholesterol is delivered to mitochondria for the first step of testosterone biosynthesis. At the inner mitochondrial membrane (IMM), cholesterol is catalyzed to pregnenolone by CYP11A1. Pregnenolone is translocated from mitochondria to smooth endoplasmic reticulum, at which it is further processed to testosterone and other androgens. Then testosterone is secreted by Leydig cells. FC, free cholesterol; CE, cholesterol ester; SR-B1, scavenger receptor class B, type I.

to cyclic adenosine 3',5'-monophosphate (cAMP) production. cAMP-dependent protein kinase then is activated to initiate a series of events that includes the mobilization of free cholesterol in the plasma membrane and phosphorylation of neutral cholesteryl ester hydrolase (nCEH), leading to the mobilization of cholesterol esters.

Translocation of Cholesterol in Mitochondria

Steroidogenesis begins with the transfer of free cholesterol from the plasma membrane and intracellular stores into mitochondria—the rate-limiting step in androgen biosynthesis. This initial rapid response to LH, and its second messenger cAMP, is mediated

by hormone-sensitive protein synthesis, protein–protein interactions, and organelle communications. Chronic stimulation by LH is required for optimal expression of steroidogenic enzymes leading to sustainable steroid formation.

The first catalytic reaction in androgen biosynthesis occurs in mitochondria. Before this reaction, cholesterol must be translocated from its various locations in the cell to the outer mitochondrial membrane (OMM). This may occur through either a vesicular or non-vesicular transport pathway (Fig. 1). An important mechanism of vesicular transport is the delivery of cholesterol through protein–protein interactions. There is evidence that there is increased association of mitochondria with the endoplasmic reticulum in Leydig cells in response to hormone treatment mediated via AAA+ ATPase domain 3 (ATAD3) protein (Issop et al., 2015). It is also possible that the soluble *N*-ethylmaleimide-sensitive factor attachment receptor (SNARE) complexes facilitate the delivery of cholesterol by promoting functional protein–protein interactions between lipid droplets and mitochondria (Lin et al., 2016). Indeed, there is strong interorganelle interactions that facilitate free cholesterol exchange in response to LH in Leydig cells (Issop et al., 2013). Cholesterol also can be delivered through a non-vesicular transport pathway, mediated by high-affinity cholesterol-binding proteins including steroidogenic acute regulatory protein (STAR). In particular, STARD4 has high affinity with free cholesterol and is involved in cholesterol transport (Mesmin et al., 2011).

Cholesterol targeting into mitochondria is mediated by the formation of a mitochondrial scaffold, the transduceosome, created by protein–protein interactions of cytosolic and OMM proteins (Rone et al., 2009). After cholesterol is translocated to the OMM, it will be delivered to the inner mitochondrial membrane (IMM), where it is converted to pregnenolone by CYP11A1 (Fig. 1). Cholesterol is imported and metabolized by the 800-kDa bioactive metabolon, composed of the OMM/IMM translocator protein (TSPO), voltage-dependent anion channel (VDAC), ATAD3, and CYP11A1 (Rone et al., 2012). The rapid induction of STAR formation by LH and its subsequent targeting and insertion into OMM leads to accelerated cholesterol delivery to CYP11A1 (Rone et al., 2012; Papadopoulos and Miller, 2012).

Biosynthesis of Testosterone From Cholesterol

The process of testosterone biosynthesis from cholesterol involves four enzymes: cytochrome P450 family 11 subfamily A1 (CYP11A1), 3 β -hydroxysteroid dehydrogenase/ $\Delta^5 \rightarrow \Delta^4$ isomerase (3 β -HSD), CYP17A1, and 17 β -HSD3 (Miller and Auchus, 2011; Payne and Hales, 2004). CYP11A1 is located in mitochondria, 3 β -HSD is present in mitochondria but predominantly found in SER, and CYP17A1 and 17 β -HSD3 are found only in SER (Figs. 1 and 2).

CYP11A1

CYP11A1, which is generically termed cytochrome P450 (P450_{scc}), is a 56-kDa enzyme located in the IMM (Fig. 1). It catalyzes the conversion of cholesterol to pregnenolone (Fig. 2). This conversion includes three sequential oxidation reactions: hydroxylation of cholesterol at C22, hydroxylation of 22(R)-hydroxycholesterol at C20, and cleavage of the C20–22 bond of 20(R), 22(R)-dihydroxycholesterol to yield pregnenolone and isocaproaldehyde. All three reactions are catalyzed in the same site of CYP11A1. The mitochondrial environment is required for the catalytic activity of CYP11A1 since CYP11A1 is lacking mitochondrial leader and when targeted to the ER is inactive. During the conversion, CYP11A1, a monooxygenase, catalyzes oxidation by utilizing electrons from reduced nicotinamide adenine dinucleotide phosphate (NAPDH). A mitochondrial electron transport system consisting of ferredoxin reductase (FdR) and ferredoxin (FdX) facilitates the electron transport between CYP11A1 and NAPDH.

CYP11A1 determines the biosynthetic capacity of Leydig cells. The amount of CYP11A protein is primarily decided by transcription of the *CYP11A* gene, which can be regulated in a hormonally responsive fashion. The transcription of the *CYP11A* gene is induced by both cAMP and cAMP-dependent protein kinase. Upon LH stimulation, the activation of cAMP is necessary for the expression of involved enzymes in androgen synthesis. Steroidogenic factor 1 (SF1) also is essential for androgen synthesis, involved in the regulation of the expression of CYP11A and other steroidogenic enzymes.

3 β -HSD

Pregnenolone diffuses passively from the mitochondria to SER for further processing. Although 3 β -HSD is found in mitochondria, it is abundant in SER (Fig. 1) in proximity to CYP17A1 and 17 β -HSD3 so that each intermediate of testosterone synthesis can move directly to the next enzyme. Depending upon the species, Leydig cells use either the Δ^4 or Δ^5 pathway to produce testosterone (Fig. 2). Rodent Leydig cells preferentially follow the Δ^4 pathway while human and rabbit Leydig cells follow the Δ^5 pathway. In the Δ^4 pathway, pregnenolone is converted to Δ^4 steroids by 3 β -HSD. 3 β -HSD is a 42-kDa enzyme that converts pregnenolone to progesterone (Fig. 2). Additionally, it can also convert 17 α -hydroxypregnenolone to 17 α -hydroxyprogesterone (17OHP), dehydroepiandrosterone (DHEA) to androstenedione, and androstenediol to testosterone. Two chemical transformations are catalyzed by 3 β -HSD to stimulate these conversions (Fig. 2). First, the 3 β -hydroxyl group of the substrate is oxidized to the ketone group, when NAD⁺ is converted to NADH. Next, NADH activates the second activity of 3 β -HSD, the $\Delta^5 \rightarrow \Delta^4$ isomerase activity, i.e. catalyzing the isomerization of the double bond from carbon 5 and 6 to carbon 4 and 5, to yield Δ^4 steroid.

CYP17A1

Human and rabbit Leydig cells synthesize testosterone via the Δ^4 pathway, in which the first reaction is catalyzed by CYP17A1. CYP17A1 is a 57-kDa enzyme that expresses both 17 α -hydroxylase and 17, 20-lyase activities. In the Δ^5 pathway, CYP17A1 converts pregnenolone to 17 α -hydroxypregnenolone (Fig. 2). Then it converts 17 α -hydroxypregnenolone to DHEA by its 17, 20-lyase activity (Fig. 2). The presence of cytochrome *b*₅ increases the rate of lyase reaction more than 10-fold. In addition to the two-step conversion

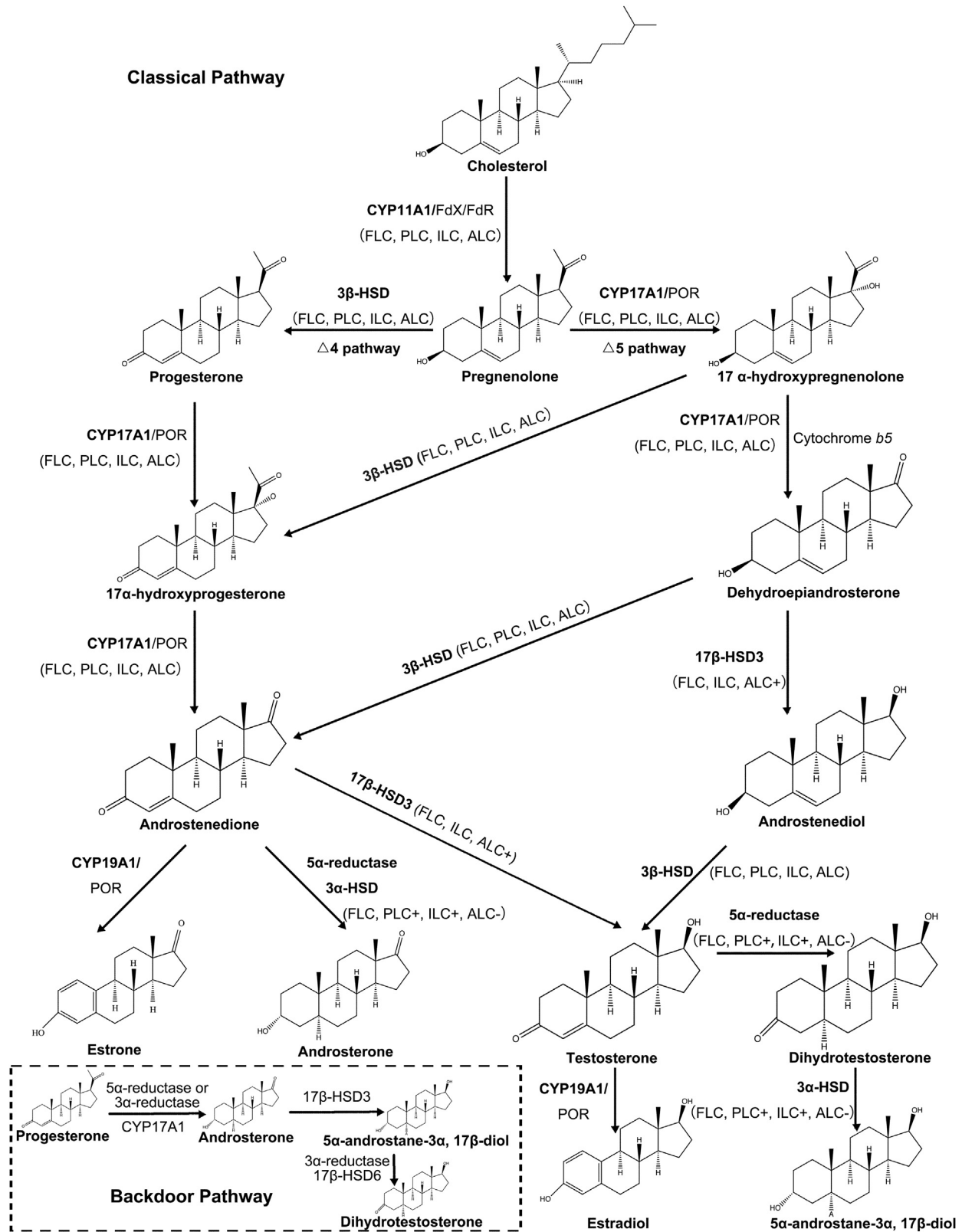


Fig. 2 Androgen biosynthetic and metabolic pathways in Leydig cells. (Top) In adult Leydig cells (ALCs), androgens are synthesized via the classical pathway. The major steps involved in the classical pathway include conversion of cholesterol to pregnenolone and synthesis of testosterone from pregnenolone by following two species-dependent pathways (Δ^4 and Δ^5). (Bottom left) Testosterone is further metabolized to dihydrotestosterone (DHT), 5α -androstane- 3α , 17β -diol and estradiol. In fetal Leydig cells (FLCs), using the backdoor pathway. The critical enzyme employed by the backdoor pathway is 3α -reductase, which converts 5α -androstane- 3α , 17β -diol to DHT. All the enzymes, co-enzymes, electron transport systems and direction of reactions are shown. Androgens are emphasized by larger structural images. The developmental stages during which key enzymes are expressed are included in parentheses. “+” and “-” represent significant and negligible expression levels of enzymes, respectively.

of pregnenolone to DHEA, CYP17A1 can also catalyze the transformation of progesterone to 17OHP. However, under normal conditions, 17OHP is not a critical precursor for androgen synthesis; the conversion of 17OHP to androstenedione catalyzed by CYP17A1 is 50-fold less efficient than the conversion of 17 α -hydroxypregnenolone to DHEA. The reaction catalyzed by CYP17A1 also requires the microsomal electron transport system termed flavoprotein P450 oxidoreductase (POR). POR is a single membrane-bound flavoprotein that receives two electrons from NADPH and delivers them at once to CYP17A1. In human cells, the interaction between POR and CYP17A1 is facilitated by cytochrome *b*₅ and the serine phosphorylation of CYP17A1. As the sole electron transfer system serving for all microsomal P450s, POR also delivers electrons to CYP19A1 for metabolism of androgens.

17 β -HSD3

17 β -HSD3 is a 34.5-kDa enzyme specifically expressed in the testis. It can catalyze DHEA transformation to androstenediol, and androstenedione to testosterone (Fig. 2). Androstenediol derived from DHEA may contribute to testosterone biosynthesis in Leydig cells.

Other Androgens and Estrogens in Leydig Cells

As noted above, in addition to testosterone, Leydig cells synthesize androgens that are intermediate products of testosterone biosynthesis, including DHEA, androstenediol and androstenedione (Fig. 2). Leydig cells also can metabolize androgens into dihydrotestosterone (DHT) and estrogens (Fig. 2).

DHT

DHT is a potent androgen for male sexual differentiation. It is derived from testosterone by a 30-kDa microsomal enzyme, 5 α -reductase. In the fetus, DHT and testosterone induce genital tubercle transformation into male external genitalia. In humans, two isoforms of 5 α -reductase have been identified. Type 1 5 α -reductase potentially contributes to pubertal virilization, as proven by the consequences of classic 5 α -reductase deficiency. Type 2 5 α -reductase, the more prevalent isoform in Leydig cells, is responsible for directing male sexual differentiation. Beyond converting testosterone to DHT, 5 α -reductase, especially the type I isoform, can also metabolize progesterone, 17OHP and related C₂₁ steroids. Moreover, type I isoform expressed in immature mouse Leydig cells participates in androstenediol synthesis via two distinct pathways (Fig. 2).

Estradiol and estrone

In addition to androgens, Leydig cells can also produce estrogens. 17 β -Estradiol derived from testosterone, is the main estrogen formed in Leydig cells. A microsomal enzyme, CYP19A1 (58-kDa), generically termed P450arom, catalyzes the aromatization of testosterone to estradiol. CYP19A1 aromatase also catalyzes the conversion of androstenedione to estrone (Fig. 2). In the male, androgen-derived estrogens contribute to numerous physiological processes, including skeletal maturation.

Developmental-Specific Expression of Enzymes

There are two distinct populations of Leydig cells identified in different stages of male sexual development. Fetal Leydig cells (FLCs) appear at the fetal stage, and a second population, the adult Leydig cells (ALCs), develop from stem cells after birth. The differentiation of ALCs can be divided into four stages: stem Leydig cells (SLCs), progenitor Leydig cells (PLCs), immature Leydig cells (ILCs), and mature adult Leydig cells (mature ALCs). The androgens formed at the various stages of Leydig cell development vary due to the differential expression of steroidogenic enzymes (Fig. 2) (Kotula-Balak et al., 2012; Ye et al., 2017).

FLCs express CYP11A1, 3 β -HSD, CYP17A1, 5 α -reductase, and 3 α -HSD. These enzymes continually increase during the fetal stage. The expression of 17 β -HSD3 is controversial and likely species-specific. In the mouse no 17 β -HSD3 has been detected in FLC; the enzyme is found in the tubular cells of the testis, indicating a possible cooperation between different testicular cells to produce androgens. However, the enzyme is present in rat FLC. At fetal day 15.5, the testis begins to produce testosterone. Its production gradually increases. DHT and androstenedione also are synthesized by FLCs. DHT is essential for the development of the external male genitalia. In FLCs, DHT is derived from progesterone by following the “backdoor” pathway (Fig. 2). Enzymes catalyzing this metabolic pathway include 5 α -reductase, 3 α -reductase, 17 β -HSD3 and 17 β -HSD6. The key enzyme of the “backdoor pathway,” 3 α -reductase, converts 5 α -androstane-3 α , 17 β -diol to DHT.

After the first postnatal week, testosterone declines due to reduced numbers of FLCs and their reduced steroidogenic activities. The level of testosterone is elevated again when ALCs start developing. In rats, ALC differentiation begins around postnatal day 14 and finishes around postnatal day 60. SLCs express no steroidogenic enzymes whereas PLCs express CYP11A1, 3 β -HSD, and CYP17A1 and therefore synthesize androstenedione. PLCs also express androgen-metabolizing enzymes, including 5 α -reductase and 3 α -HSD. These enzymes can metabolize androstenedione into androsterone, which is the main product of PLCs. When PLCs further differentiate into ILCs, CYP11A1, 3 β -HSD, and CYP17A1 are concomitantly increased. The appearance of 17 β -HSD3 enables ILCs to synthesize testosterone. However, due to the continuous presence of 5 α -reductase and 3 α -HSD, testosterone will be metabolized, leading to a high production of 5 α -androstane-3 α , 17 β -diol in ILCs. When ALCs become mature, testosterone production rises significantly as a consequence of increased numbers of LH receptors and thus elevated responsiveness to LH. At the same time, decline of 5 α -reductase activity also contributes to the accumulation of testosterone in mature ALCs. The

large quantity of testosterone secreted by mature ALCs is required for the initiation and the maintenance of spermatogenesis as well as the establishment of secondary sex characteristics in puberty and through adulthood.

Enzymes of Androgen Biosynthesis in Pathological Conditions

Every step of testosterone biosynthesis and the conversion of testosterone to DHT can be disturbed, leading to changes in androgen production and androgen balance. Disruption of androgen synthesis results in disorders of male sexual development (Miller, 2005; Miller and Auchus, 2011).

For example, STAR (STAR1) is a key protein regulating the transport of cholesterol into mitochondria. Mutations of the *STAR1* gene result in a severe genetic disorder of steroidogenesis called lipoid congenital adrenal hyperplasia (CAH). The loss of STAR in CAH leads to over 80% decline of steroids. The affected 46, XY fetuses CAH cannot undergo normal virilization and thus are born with female external genitalia. In patients with a less severe form of lipoid CAH, patients have 20%–25% of normal STAR activity. They have normal external male genitalia but mild hypergonadotropic hypogonadism. The patients with a *CYP11A1* mutation cannot be clinically and hormonally distinguished from those with lipoid CAH.

Mutations of 3 β -HSD1 have not been reported; such mutations would prohibit placental progesterone biosynthesis, leading to a spontaneous first-trimester abortion. The mutations of the *HSD3B2* gene cause 3 β -HSD2 deficiency and consequent decrease of testosterone production. The severely mutated form of 3 β -HSD2 results in lipoid CAH. In patients with this mutation, the level of Δ^5 steroids increases significantly while that of Δ^4 steroids increases slightly, leading to a high ratio of Δ^5 to Δ^4 steroids. Patients with the 17 β -HSD3 deficiency or 5 α -reductase 2 deficiency share the same clinical characteristics as those deficient for 3 β -HSD2; i.e. undermasculinization with hypoplastic-to-normal male internal genitalia, female external genitalia, and loss of the prostate. Additionally, 5 α -reductase 2 deficiencies may be associated with the male pattern baldness. Complete CYP17A1 deficiency includes the absence of both 17 α -hydroxylase and 17,20-lyase activities that inhibit the production of gonadal sex steroids. As a consequence, the affected 46, XY males present with the absence or abnormalities of male external genitalia. Selective deficiency of 17 α -hydroxylase and 17,20-lyase activities has also been identified.

Differences of Androgen Biosynthesis in Human and Rodent Leydig Cells

To date, most knowledge of androgen biosynthesis in Leydig cells has come from studies of rodent models, including steps of androgen synthesis and enzymes involved in this process. In contrast, we understand much less about androgen biosynthesis in humans in part because of the difficulty in obtaining samples. It remains unclear whether the factors identified in rodent Leydig cells play the same roles in human Leydig cells, and/or whether there are novel pathways in human Leydig cells that are involved in producing androgens. However, without doubt, differences of androgen biosynthesis between human and rodent Leydig cells do exist (Teerds and Huhtaniemi, 2015).

In contrast to rodents, three distinct populations of human Leydig cells, FLCs, neonatal Leydig cells (NLCs), and ALCs have been identified during male sexual development. NLCs appear between birth and the 6 months thereafter, concomitant with a unique peak of testosterone production in the human. This testosterone surge is thought to contribute to imprinting of many cell types of androgen-dependent organs. In rodents, no NLCs have been reported. There also are species-dependent differences in both the cholesterol source and the enzyme preference. Typically, Leydig cells utilize de novo synthesized cholesterol for androgen biosynthesis. However, in cases in which rodents use LDL or HDL as the cholesterol source for androgen biosynthesis, the Leydig cells heavily rely on the SR-BI/selective pathway. In contrast, human Leydig cells utilize the LDL/LDL-receptor endocytic pathway. Moreover, CYP17A1 in rodent Leydig cells acts on 17 α -hydroxyprogesterone (Δ^4), whereas in human Leydig cells it acts on 17 α -hydroxypregnenolone (Δ^5).

Summary

Testosterone biosynthesis is essential for the development of internal/external male genitalia, the establishment of secondary male characteristics, and spermatogenesis. Leydig cells are the primary source of testosterone in the testis. In addition to testosterone, Leydig cells produce other steroids that are essential for male development, including DHT and estradiol.

In Leydig cells, testosterone and other androgens are synthesized from cholesterol derived from different sources. The translocation of cholesterol from OMM to IMM is the hormone-sensitive and rate-limiting step in androgen biosynthesis. Four steroidogenic enzymes catalyze the biosynthesis of testosterone: CYP11A1, 3 β -HSD, CYP17A1, and 17 β -HSD3. After synthesis, testosterone can be further metabolized into DHT, estradiol, androsterone, and 5 α -androstenediol by three enzymes, 5 α -reductase, CYP19A1, and 3 α -HSD. Functionally different from ALCs, FLCs synthesize more DHT to support male sexual development in fetal life.

There are still many unknowns about Leydig cell androgen biosynthesis, particularly so in the human. For example, the origin of human Leydig cells, the identification of individual SLCs, and the cell fate of NLCs all remain to be clarified. Moreover, it is well known that the substrate specificity of cytochrome P450s is limited, and this suggests the presence of alternative unexplored yet substrates for androgen formation (Lieberman, 2008).

Investigating Leydig cell androgen biosynthesis is important in order to understand the underlying mechanisms of male sexual development and the origins of associated developmental defects. Moreover, reduced androgen formation is associated with aging.

Indeed, 20%–50% of men over age 60 are reported to have serum testosterone levels significantly below those of young men (Midzak et al., 2009). Whether in aging or young men, reduced serum testosterone is associated with a number of metabolic and quality-of-life changes, including infertility, erectile dysfunction, obesity, osteoporosis and cardiovascular disease. Greater understanding of the molecular, cellular and biochemical mechanisms of androgen formation could lead to the design of methods to induce and control androgen formation.

References

- Freeman, D. A. (1989). Plasma membrane cholesterol: removal and insertion into the membrane and utilization as substrate for steroidogenesis. *Endocrinology*, 124, 2527–2534.
- Hu, J., Zhang, Z., Shen, W. J., & Azhar, S. (2010). Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones. *Nutrition & Metabolism (London)*, 7, 47.
- Issop, L., Rone, M. B., & Papadopoulos, V. (2013). Organelle plasticity and interactions in cholesterol transport and steroid biosynthesis. *Molecular and Cellular Endocrinology*, 371, 34–46.
- Issop, L., Fan, J., Lee, S., Rone, M. B., Basu, K., Mui, J., & Papadopoulos, V. (2015). Mitochondria-associated membrane formation in hormone-stimulated Leydig cell steroidogenesis: role of ATAD3. *Endocrinology*, 156, 334–345.
- Kotula-Balak, M., Hejmej, A., & Bilińska, B. (2012). Hydroxysteroid dehydrogenases – localization, function and regulation in the testis. In R. A. Canuto (Ed.), *Dehydrogenases*. Rijeka: InTech.
- Lieberman, S. (2008). The generally accepted version of steroidogenesis is not free of uncertainties: other tenable and possibly superior renditions may be invented. *Journal of Steroid Biochemistry and Molecular Biology*, 109, 197–199.
- Lin, Y., Hou, X., Shen, W. J., Hanssen, R., Khor, V. K., Cortez, Y., Roseman, A. N., Azhar, S., & Kraemer, F. B. (2016). SNARE-mediated cholesterol movement to mitochondria supports steroidogenesis in rodent cells. *Molecular Endocrinology*, 30, 234–247.
- Maxfield, F. R., & Wustner, D. (2002). Intracellular cholesterol transport. *Journal of Clinical Investigation*, 110, 891–898.
- Mesmin, B., Pipalia, N. H., Lund, F. W., Ramlall, T. F., Sokolov, A., Eliezer, D., & Maxfield, F. R. (2011). STARD4 abundance regulates sterol transport and sensing. *Molecular Biology of the Cell*, 22, 4004–4015.
- Midzak, A. S., Chen, H., Papadopoulos, V., & Zirkin, B. R. (2009). Leydig cell aging and the mechanisms of reduced testosterone synthesis. *Molecular and Cellular Endocrinology*, 299, 23–31.
- Miller, W. L. (2005). Disorders of androgen synthesis—from cholesterol to dehydroepiandrosterone. *Medical Principles and Practice*, 14(Suppl. 1), 58–68.
- Miller, W. L., & Auchus, R. J. (2011). The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocrine Reviews*, 32, 81–151.
- Papadopoulos, V., & Miller, W. L. (2012). Role of mitochondria in steroidogenesis. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 26, 771–790.
- Payne, A. H., & Hales, D. B. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews*, 25, 947–970.
- Payne, A. H., & Hardy, M. P. (2007). *The Leydig cell in health and disease*. Totowa, NJ: Humana Press.
- Rone, M. B., Fan, J., & Papadopoulos, V. (2009). Cholesterol transport in steroid biosynthesis: role of protein-protein interactions and implications in disease states. *Biochimica et Biophysica Acta*, 1791, 646–658.
- Rone, M. B., Midzak, A. S., Issop, L., Rammouz, G., Jagannathan, S., Fan, J., Ye, X., Blonder, J., Veenstra, T., & Papadopoulos, V. (2012). Identification of a dynamic mitochondrial protein complex driving cholesterol import, trafficking, and metabolism to steroid hormones. *Molecular Endocrinology*, 26, 1868–1882.
- Teerds, K. J., & Huhtaniemi, I. T. (2015). Morphological and functional maturation of Leydig cells: from rodent models to primates. *Human Reproduction Update*, 21, 310–328.
- Venugopal, S., Martinez-Arguelles, D. B., Chebbi, S., Hullin-Matsuda, F., Kobayashi, T., & Papadopoulos, V. (2016). Plasma membrane origin of the steroidogenic pool of cholesterol used in hormone-induced acute steroid formation in Leydig cells. *Journal of Biological Chemistry*, 291, 26109–26125.
- Ye, L., Li, X., Li, L., Chen, H., & Ge, R. S. (2017). Insights into the development of the adult Leydig cell lineage from stem Leydig cells. *Frontiers in Physiology*, 8, 430.

Androgen Receptor

Iain J McEwan, University of Aberdeen, Aberdeen, United Kingdom

Lee B Smith, University of Edinburgh, Edinburgh, United Kingdom; and University of Newcastle, Callaghan, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

The importance of testosterone for masculinization of the male fetus was established in a series of experiments spanning 100 years from the mid 19th Century to the first half of the 20th century. Arnold Berthold conducted transplantation studies with testes and castrated rooster's, showing that a "secreted substance" restored male behavior and physiological activity (Freeman et al., 2001; Smith et al., 2013). Refining what this substance might be began with observations in freemartin cattle. In cases of twin fetuses with one male and one female fetus, the female fetus was noted to display signs of development of male sex organs, for example the seminal vesicles and epididymis. In two independent studies, Lillie and Keller and Tandler postulated that this resulted from the exchange of a testicular secretion to the female fetus from a male twin, via the placental vasculature (reviewed in Smith et al., 2013). The discovery that this factor was testosterone was made by Jost who in a series of studies between 1947 and 1953 showed that transplantation of a testis into a female rabbit resulted in masculinization, and then demonstrated that the same was true if a crystal of testosterone propionate was used instead, thus defining testosterone as the key hormone driving male development (Josso, 2008).

However, exactly how testosterone was able to mediate its impact on the body remained a mystery for the next three decades. Individuals that were genetically XY, but developed a female body form were well known. In these individuals, gender was classified as female at birth and the first presentation of symptoms tended to be failure of menstruation during puberty, at which time karyotype investigation revealed the presence of a Y chromosome, and further investigation revealed small, internal testes. These individuals produce high levels of testosterone, but for some reason their tissues are unable to respond to this stimulation. The condition was initially called testicular feminization, but more recently is termed complete androgen insensitivity syndrome (CAIS).

An important step on the road to defining why these individuals were unable to respond to testosterone was the identification of a mouse line in a colony at MRC Harwell, Oxfordshire in 1970 (Lyon and Hawkes, 1970). In this curious mouse line, a proportion of males born in the colony developed female sexual characteristics, including a small anogenital distance, nipples, and female sexual behavior. Internally these mice lacked many male reproductive tissues, possessing small testes, that had failed to descend into the scrotum, but lacking seminal vesicles, epididymides and a prostate; a phenotype very similar to CAIS individuals. The gene responsible for this was mapped to the X-chromosome in these mice and termed the Androgen Receptor, and the equivalent human X-chromosome gene was eventually identified and cloned in 1988 (Lubahn et al., 1988).

Androgen Receptor Gene and Protein

Gene Structure and Expression

The gene for the androgen receptor (AR) contains eight exons, spanning 150 kb, and is a single copy gene in men, while woman utilize one functional copy of the gene, due to X-inactivation. Exon one codes for the structurally flexible amino-terminal domain (NTD); exons 2 and 3 for the DNA binding domain (DBD); exon 4 a flexible hinge linking the DBD with the ligand binding domain (LBD) (exon 4–8). The protein is typically 920 amino acids in a length and has a molecular weight of 110 kDa (Fig. 1).

The promoter sequence lacks a typical TATA box and expression of the receptor gene is driven by the transcription factor Sp1, which binds upstream of the transcription start site and at multiple sites within the 5'UTR (reviewed in Hunter et al., 2017). The highest expression of the transcript is in reproductive tissues such a prostate and uterus, but high levels of the receptor mRNA have also been detected in liver, kidney, skeletal muscle and adipose tissue. Notably, the androgens testosterone and DHT have been found to negatively, or positively, auto-regulate expression of the receptor gene (Hunter et al., 2017). The androgen-dependent regulation of receptor levels is likely to contribute to the tissue-selective responses to testosterone.

Domain Organization

Ligand binding domain

The ligand binding domain (LBD) is located at the C-terminal half of the receptor protein and adopts a globular α -helical folded structure (Fig. 1). The hormones testosterone and dihydrotestosterone (DHT) bind with similar affinity to the largely hydrophobic ligand binding pocket, but demonstrate distinct dissociation rates that accounts for the increased potency of DHT (Askew et al., 2007; McEwan, 2013). Selectivity for androgenic steroids is mediated by a limited number of specific amino acid-steroid interactions, involving hydrogen bonding with residues asparagine 706, glutamine 712, arginine 753 and threonine 878 and hydrophobic interactions with leucine 705, methionine 746 and phenylalanine 747 (McEwan, 2013).

Hormone binding results in a conformational change in the LBD, leading to a more compact structure and the positioning of helix 12 to create a surface pocket important for intramolecular interactions with the amino-terminal domain (NTD) of the receptor and co-regulatory proteins (AF2, Fig. 1). A recent study characterized for the first time a surface on the LBD that leads

Human Androgen Receptor Gene



Human Androgen Receptor Protein

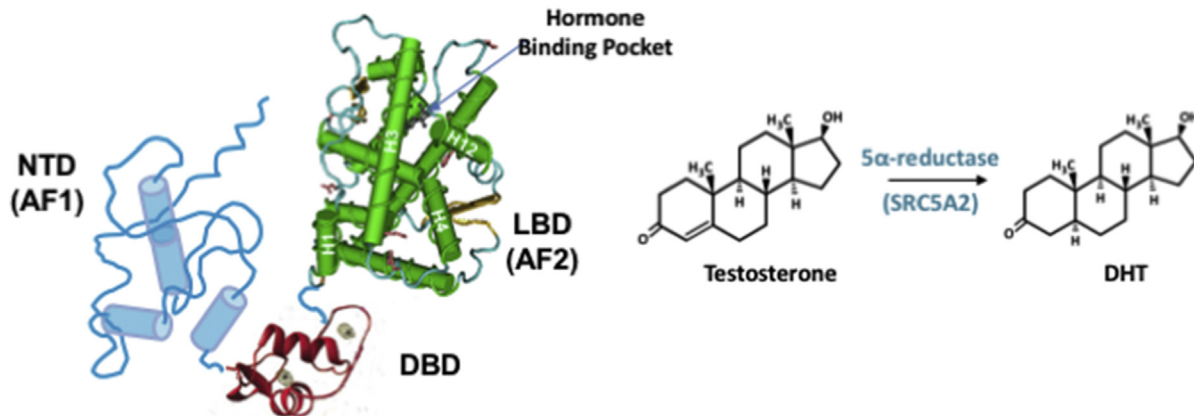


Fig. 1 Androgen receptor gene and protein structure. Top panel shows a schematic representation of the human androgen receptor gene. The protein codon exons (1–8) are colored to match the domain structure of the protein (bottom panel). The bent arrow represents the transcription start site (+1). Bottom panel shows the structural and functional domains of the receptor protein. LBD, ligand binding domain; DBD, DNA binding domain; and NTD, amino-terminal domain. Activation functions (AF) 1 and 2 are also indicated.

to homo-dimerization of the AR-LBD (Nadal et al., 2017). This involved amino acids in helices 5, 7–9 and intervening loops (Fig. 1). Of particular significance is that a number of receptor point mutations associated with androgen insensitivity syndrome (AIS) or prostate cancer map to this large dimerization surface (Nadal et al., 2017).

DNA binding domain/hinge region

The DNA binding domain (DBD) is a helical domain linked to the LBD by a flexible hinge of 40 amino acids. The latter contains a bipartite nuclear localization signal ($^{629}\text{RKLKK}^{633}$) that is necessary for translocation of the receptor into the nucleus (see below). Folding of this domain is dependent upon the coordination of two zinc ions and results in the formation of a “recognition helix” that fits into the major groove of DNA double helix and mediates specific receptor-DNA interactions. In addition, a sequence of five amino acid forms a loop and acts as a dimerization interface holding two receptor monomers together on the DNA.

N-terminal domain

The N-terminal domain (NTD) represents more than half the receptor protein and in contrast to the LBD and DBD has a flexible, intrinsically disordered, structure (McEwan, 2012). This region of the receptor has a propensity to form α -helical structure and is essential for receptor action by establishing multiple protein–protein interactions inside the cytoplasm and nucleus of the cell (see below).

Within the AR-NTD are a number of polymorphic repeats sequences coding for the amino acids glutamine (CAG), glycine (GGC) and proline (CCC,CCG,CCT). The largest poly-glutamine repeat varies between 9 and 38 in the normal population and expansion to greater than 40 residues is associated with reduced function, protein aggregation and results in Kennedy’s disease, a neurodegenerative condition (La Spada et al., 1991).

The importance of the AR-NTD has been further demonstrated by the recent identification of splice variants, lacking the LBD in castrate resistant prostate cancer (CRPC) (reviewed in McEwan, 2012). These receptor polypeptides contain the NTD and DBD, but have unique C-terminal sequences and arise from the splicing of cryptic exons within the AR gene.

Receptor Posttranslational Modifications

Posttranslational modification of proteins is a common feature in eukaryotic cells and an important mechanism for controlling protein function, localization and stability. The androgen receptor is subject to phosphorylation primarily on serine residues and acetylation, methylation, sumoylation or ubiquitination on lysine residues. The functional consequences of specific modification of individual amino acids is an ongoing area of research, but in general terms these changes can alter receptor intracellular trafficking, hormone-dependent transcription and protein turnover.

Classical Androgen Receptor Signaling

In the absence of hormone, the receptor is located in the cytosol as part of a multiprotein complex with chaperones (Hsp 90, Hsp 70) and cochaperones (FKBP51) (**Fig. 2**). This complex is critical for maintaining the AR in a “fit state” to bind the steroid hormones testosterone or the more potent DHT. The binding of hormone causes conformational changes in the receptor protein: “helix 12” in the LBD re-aligns, closing off the ligand binding pocket, often referred to as a mouse trap action; and the nuclear localization signal (sequence of basic amino acids) in the hinge is exposed, allowing interactions with the cell’s transport network (motor protein dynein, microtubules tracks, importin proteins) and translocation to the nucleus.

The binding of hormone also permits dimerization of the receptor. In the cytosol this is thought to involve inter-molecular interactions between an amino acid sequence, ²³FngLF²⁷, in the AR-NTD and the AF2 pocket on the surface of AR-LBD (**Fig. 2**) (**van Royen et al., 2007**). This is referred to as the N/C-terminal interaction, which maybe unique to the mechanism of action of the AR (**Langley et al., 1995**). Once in the nucleus receptor dimers can be stabilized through the recently described surface on the LBD and the D-box region in the DBD. Binding to DNA can disrupt the N/C-terminal interaction (**van Royen et al., 2007**) opening up regions of the AR-NTD for interactions with the cellular transcriptional machinery (**Fig. 2**).

Early in vitro studies defined two classes of AR DNA binding site: hormone response elements (HRE) that were also bound by the glucocorticoid and progesterone receptors and more selective androgen response elements (ARE) only functional with the AR DBD. In an in vivo model, the SPARKI (specificity-affecting AR Knock-in) mouse, where the AR DBD was swapped for the GR the impact on male development and gene expression and fertility were observed (**Schauwaers et al., 2007**). These elegant studies demonstrate a role for AR-selective AREs in the development of male reproductive tissues and fertility, but not in other tissues.

Genome wide studies, involving chromatin immunoprecipitation (ChIP) and ChIP-seq, have largely confirmed the 15 bp consensus binding site for the AR made up of 6 bp half sites: 5'-GGA/TACAnnnTGTTCT-3'. However, these studies have also revealed that the receptor can recognize and bind variations consisting of single or multiple half-sites. Importantly, these studies have also allowed the identification of key transcription factors that participate in androgen-dependent gene regulation, including GATA2, FOXO1, ETS1 and AP1 (**Bolton et al., 2007; Mills, 2014**). Interestingly, interactions with pioneering transcription factors may lead to reprogramming of AR gene targets in progression of prostate cancer to a hormone-resistant stage (**Mills, 2014**). Binding sites for the receptor are typically located within 1 Kbp of the transcription start site and often within the promoter sequences. However, some binding sites can be located in enhancer regions several kbp from the start site e.g., prostate specific antigen gene. Genome-wide expression (RNA-seq) experiments has also revealed networks of genes up- or down-regulated in response to hormone in different cells and tissue. These include genes involved in development, cell growth and cell cycle regulation and metabolism (**Bolton et al., 2007; Mills, 2014**).

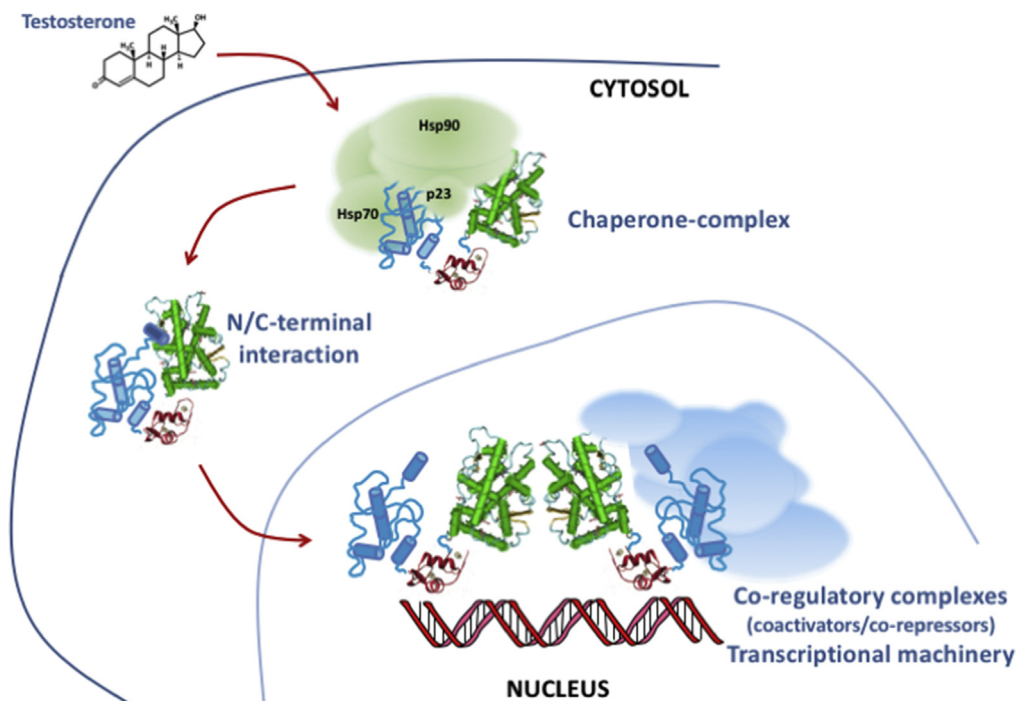


Fig. 2 Classical mechanism of androgen receptor action. In the absence of hormone, the androgen receptor is predominantly found in the cytosol complexed with molecular chaperones and associated proteins (Chaperone complex). Upon binding hormone, the receptor dissociates and the N/C-terminal interaction occurs between FngLF motif and AF2 pocket on the LBD, the hormone bound receptor then translocates to the nucleus and binds specific DNA response elements associated with target genes. The receptor complex can then recruit coregulator proteins, which modify chromatin structure or directly recruit RNA Polymerase II (Coregulator complex).

Mechanisms of transcriptional regulation can involve opening or closing of chromatin structure and/or the recruitment of coactivator or corepressor complexes and the cell's transcriptional machinery. The AR interacts with a large number of potential coregulatory proteins, including histone modifying enzymes (e.g., LSD1, Set9), coactivators/repressors (e.g., SRC-1,2, and 3/SMRT) and general transcription factors (e.g., TFIIF, TFIID) (Fig. 2) (Reviewed in McEwan, 2012).

Methods of Dissecting Androgen Receptor Function

One of the major complexities surrounding the dissection of AR function is the myriad of roles it plays throughout the body. The classic approach to dissecting gene function in physiology is to knock it out in mice and study the resulting phenotype. However, as we have seen with the testicular feminized mouse, and which was repeated when an *Ar* knockout mouse was produced (Yeh et al., 2002), the essential role of the AR in normal male development means that male tissues do not develop or are secondarily impacted, making it extremely difficult to study the specific roles for AR signaling in tissues and cells. This problem was successfully addressed in 2004, with the publication of mouse models in which conditional gene-targeting was exploited to knock-out AR in individual testicular cell-types (reviewed Smith and Walker, 2014).

To achieve this, scientists exploited the Cre-Lox system of conditional gene targeting. In this system, the target gene (androgen receptor) is genetically modified with the addition of two target loxP sites placed within introns of the gene. Under normal conditions these introns are naturally spliced out when an mRNA is produced, thus the animal develops and functions completely normally. However, these target sequences are recognized by a Cre Recombinase protein, which causes recombination between the two target sites, essentially snipping out the intervening DNA sequence. By placing these target sequences either side of a key exon, and then controlling which cell-type expressed the Cre Recombinase (by placing the gene for this protein downstream of a cell-specific promoter) researchers were able to selectively knockout the androgen receptor in specific cell or tissue types. To date at least five different alleles of androgen receptor have been targeted for deletion in this way (reviewed in Smith and Walker, 2014), and these have been used to determine the cell-specific role of androgen receptor in all cell types of the testis and male reproductive tissues, as well as many peripheral tissues.

Androgen Receptor Signaling in the Testis

AR is widely expressed in testis tissues, present in all somatic cell types, yet absent from germ cells. This means that the impact of testosterone, which we know to be essential for spermatogenesis, must be mediated by signaling in somatic cells. Selective AR knockout from somatic cell-types has permitted dissection of these roles and attribution of impacts of AR signaling on each cell-type. AR signaling functions in Sertoli cells to support postmeiotic germ cell development. Given their close proximity and the role of Sertoli cells as "nurse cells" this is perhaps predictable, however what was surprising was the finding that loss of Sertoli cell AR reduced the final Leydig cell populations size by 40% (De Gendt et al., 2005). Further targeting studies demonstrated that AR signaling in Peritubular myoid cells supported all stages of spermatogenesis, but also, Sertoli cell function and Leydig cell development, whilst Leydig cell AR signaling was essential for maturation of the Leydig cell population and function in adult life. Together these separate reductionist approach studies uncovered a far more complex and intertwined network of androgen interactions in the testis, than previously thought.

One of the caveats of this approach is that the timing of AR knockout is dependent upon the onset of Cre Recombinase expression. In the case of Sertoli cells for example, use of the *Amh*-Cre line which is active from embryonic day 13 means that Sertoli cells develop having never been exposed to androgen receptor signaling. This makes it challenging to dissect developmental from adult function effects. To address this a variation on this approach, encoding the Cre Recombinase gene within a lentivirus which is injected into the efferent ducts has allowed the induction of AR knockout in Sertoli cells only in adulthood. This has confirmed that AR action inside Sertoli cells in adulthood is essential for spermatogenesis and male fertility (Willems et al., 2015). Additional research on the role of androgen receptor in other cells of the testis in adulthood is ongoing.

To further our understanding of AR action, ectopic activation of AR expression has also been used to enhance our understanding of its normal role. Prematurely expressing androgen receptor in mouse Sertoli cells from fetal life (it normally switches on at post-natal day 4), leads to premature maturation of the Sertoli cell (and Leydig cell) populations, inextricably linking androgen receptor to a role in testicular maturation (Hazra et al., 2013). Together these approaches, selective ablation, or activation of AR, have provided, so we thought, a complete dissection of the role of AR signaling in both reproductive and other tissues, however, a recent observation has shown us we have much yet to learn.

Nonclassical Androgen Receptor Signaling

Because AR functions as a transcription factor, our molecular understanding of the role of testosterone has been established through identification of AR target genes, identified due to the presence of androgen response elements (ARE) within their promoters. Changes in gene transcription can take hours to demonstrate a physiological response, and yet in 2004, isolated Sertoli cells were shown to rapidly respond to testosterone stimulation within a minute, by upregulating activity of MAP-kinase signaling, and that this required androgen receptor.

This paradigm shifting observation suggested that AR is able to act in a nonclassical manner to affect physiological change, however whether testosterone acts via this pathway in vivo remained a mystery until 2016. Ironically, although we have learnt much from the cell-specific ablation models of AR in mice, the very nature of how this system works disrupts both classical and nonclassical signaling pathways simultaneously. In a groundbreaking study, (Toocheck et al., 2016) utilized specific activators and inhibitors of either classical or nonclassical AR signaling in Sertoli cells in mice to show that both pathways share overlapping functions, yet also distinct roles during spermatogenesis, and that nonclassical AR signaling (in addition to classical AR signaling) is essential for male fertility. This work identified, that a meiotic block in sperm development occurs if the nonclassical pathway is specifically inhibited, even if the classical AR signaling pathway is working. This finding alone has changed the way we view AR signaling. It is highly likely that this pathway is active in many cell-types of the body, with possible implications not only under normal conditions, but possibly for our understanding of AR signaling in disease.

Summary

In conclusion, since it was first cloned in 1988, our understanding of the critical roles of androgen receptor in both reproductive and nonreproductive tissues has continued to expand. The recent confirmation of novel modes of action of androgen receptor means there is still much yet to learn. Future studies will exploit this advance in our fundamental understanding to inform our knowledge and suggest treatment options in situations of pathology, for example disorders of sexual development and testis cancer and prostate cancer.

References

- Askew, E. B., Gampe, R. T., Jr., Stanley, T. B., Faggart, J. L., & Wilson, E. M. (2007). Modulation of androgen receptor activation function 2 by testosterone and dihydrotestosterone. *The Journal of Biological Chemistry*, 282(35), 25801–25816.
- Bolton, E. C., So, A. Y., Chaivorapol, C., Haqq, C. M., Li, H., & Yamamoto, K. R. (2007). Cell- and gene-specific regulation of primary target genes by the androgen receptor. *Genes & Development*, 21(16), 2005–2017.
- De Gendt, K., Atanassova, N., Tan, K. A., de Franca, L. R., Parreira, G. G., McKinnell, C., Sharpe, R. M., Saunders, P. T., Mason, J. I., Hartung, S., Ivell, R., Denolet, E., & Verhoeven, G. (2005). Development and function of the adult generation of Leydig cells in mice with Sertoli cell-selective or total ablation of the androgen receptor. *Endocrinology*, 146(9), 4117–4126.
- Freeman, E. R., Bloom, D. A., & McGuire, E. J. (2001). A brief history of testosterone. *The Journal of Urology*, 165(2), 371–373.
- Hazra, R., Corcoran, L., Robson, M., McTavish, K. J., Upton, D., Handelsman, D. J., & Allan, C. M. (2013). Temporal role of Sertoli cell androgen receptor expression in spermatogenic development. *Molecular Endocrinology*, 27(1), 12–24.
- Hunter, I., Hay, C. W., Esswein, B., Watt, K., & McEwan, I. J. (2017). Tissue control of androgen action: The ups and downs of androgen receptor expression. *Mol Cell Endocrinol*. <https://doi.org/10.1016/j.mce.2017.08.002>.
- Josso, N. (2008). Professor Alfred Jost: The builder of modern sex differentiation. *Sexual Development*, 2(2), 55–63.
- La Spada, A. R., Wilson, E. M., Lubahn, D. B., Harding, A. E., & Fischback, K. H. (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature*, 352(6330), 77–79.
- Langley, E., Zhou, Z. X., & Wilson, E. M. (1995). Evidence for an anti-parallel orientation of the ligand-activated human androgen receptor dimer. *The Journal of Biological Chemistry*, 270(50), 29983–29990.
- Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S., & Wilson, E. M. (1988). Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science*, 240(4850), 327–330.
- Lyon, M. F., & Hawkes, S. G. (1970). X-linked gene for testicular feminization in the mouse. *Nature*, 227(5264), 1217–1219.
- McEwan, I. J. (2012). Intrinsic disorder in the androgen receptor: Identification, characterisation and drugability. *Molecular BioSystems*, 8(1), 82–90.
- McEwan, I. J. (2013). Androgen receptor modulators: A marriage of chemistry and biology. *Future Medicinal Chemistry*, 5(10), 1109–1120.
- Mills, I. G. (2014). Maintaining and reprogramming genomic androgen receptor activity in prostate cancer. *Nature Reviews. Cancer*, 14(3), 187–198.
- Nadal, M., Prekovic, S., Gallastegui, N., Helsen, C., Abella, M., Zielinska, K., Gay, M., Vilaseca, M., Taules, M., Houtsmuller, A. B., van Royen, M. E., Claessens, F., Fuentes-Prior, P., & Estebanez-Perpina, E. (2017). Structure of the homodimeric androgen receptor ligand-binding domain. *Nature Communications*, 8, 14388.
- Schauwaers, K., De Gendt, K., Saunders, P. T., Atanassova, N., Haelens, A., Callewaert, L., Moehren, U., Swinnen, J. V., Verhoeven, G., Verrijdt, G., & Claessens, F. (2007). Loss of androgen receptor binding to selective androgen response elements causes a reproductive phenotype in a knockin mouse model. *Proceedings of the National Academy of Sciences of the United States of America*, 104(12), 4961–4966.
- Smith, L. B., & Walker, W. H. (2014). The regulation of spermatogenesis by androgens. *Seminars in Cell & Developmental Biology*, 30, 2–13.
- Smith, L. B., Mitchell, R. T., & McEwan, I. J. (2013). *Testosterone: From basic research to clinical applications*. New York: Springer.
- Toocheck, C., Clister, T., Shupe, J., Crum, C., Ravindranathan, P., Lee, T. K., Ahn, J. M., Raj, G. V., Sukhwani, M., Orwig, K. E., & Walker, W. H. (2016). Mouse spermatogenesis requires classical and nonclassical testosterone signaling. *Biology of Reproduction*, 94(1), 11.
- van Royen, M. E., Cunha, S. M., Brink, M. C., Mattern, K. A., Nigg, A. L., Dubbink, H. J., Verschure, P. J., Trapman, J., & Houtsmuller, A. B. (2007). Compartmentalization of androgen receptor protein-protein interactions in living cells. *The Journal of Cell Biology*, 177(1), 63–72.
- Willems, A., Roesl, C., Mitchell, R. T., Milne, L., Jeffery, N., Smith, S., Verhoeven, G., Brown, P., & Smith, L. B. (2015). Sertoli cell androgen receptor signalling in adulthood is essential for post-meiotic germ cell development. *Molecular Reproduction and Development*, 82(9), 626–627.
- Yeh, S., Tsai, M. Y., Xu, Q., Mu, X. M., Lardy, H., Huang, K. E., Lin, H., Yeh, S. D., Altuwajiri, S., Zhou, X., Xing, L., Boyce, B. F., Hung, M. C., Zhang, S., Gan, L., & Chang, C. (2002). Generation and characterization of androgen receptor knockout (ARKO) mice: An in vivo model for the study of androgen functions in selective tissues. *Proceedings of the National Academy of Sciences of the United States of America*, 99(21), 13498–13503.

Nongenomic Androgen Actions in Male Reproductive Tissues

Peter Thomas, University of Texas at Austin, Austin, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Nongenomic Steroid Actions

The classic genomic mechanism of steroid action entails diffusion of steroids into target cells where they bind to intracellular receptors belonging to the nuclear receptor superfamily, causing receptor dimerization and translocation to the nucleus. The steroid-bound receptor dimer acts as a transcription factor by binding to specific DNA-sequences called hormone response elements on the regulatory regions of genes and recruiting co-regulator proteins which results in alterations in the rates of gene transcription. This genomic mechanism of steroid action, involving new mRNA and protein synthesis, is relatively slow with functional responses typically occurring over a period of several hours to days. In addition, there is substantial evidence that steroids also bind to steroid receptors on the cell surface or inside the cell membrane to rapidly activate intracellular signaling pathways resulting in fast hormonal responses. These rapid responses are nongenomic because they are not blocked by inhibitors of transcription and translation and involve activation of second messengers (Levin, 2011; Thomas, 2012). Rapid changes in signaling involving adenylyl cyclase (AC), epidermal growth factor receptor (EGFR), mitogen-activated protein kinases (MAPkinases), extracellular-regulated kinases 1 and 2 (ERK1/2), phosphatidylinositol 3-kinase (PI3K), Akt, Ca^{2+} , and diacylglycerol (DAG) have been reported after steroid treatments. For example, testosterone treatment increases Ca^{2+} levels in Sertoli cells within 20 s (Rahman and Christian, 2007), and progesterone hormones increase cyclic adenosine monophosphate (cAMP) levels in teleost sperm and induce sperm hypermotility within 1 min (Thomas, 2012). This induction of fish sperm hypermotility is through a nongenomic mechanism because it is too rapid to be mediated by a genomic mechanism. However, rapid steroid activation of second messenger signaling can also ultimately lead to genomic responses. Testosterone activation of MAPkinase in Sertoli cells causes rapid phosphorylation of the transcription factor, cAMP response element binding protein (CREB), and upregulation of several CREB-regulated genes (Rahman and Christian, 2007). Similarly, testosterone upregulation of proapoptotic genes in prostate cancer cells through activation of a cell membrane receptor is mediated through MAPkinase activation (Thomas et al., 2014). Since all these nonclassical steroid actions are initially mediated through activation of second messenger signaling pathways the term nongenomic will be used in this article to describe them.

Receptors Mediating Nongenomic Steroid Actions

Nuclear Receptors

Nongenomic steroid actions are mediated by membrane receptors or binding proteins located on the cell surface or by receptors tethered to the inside of the cell membrane and at other extranuclear locations (Levin, 2011). Nuclear receptors for all the major classes of steroids have been shown to be intermediaries in nongenomic steroid actions initiated at these extranuclear locations. Ligand binding to nuclear androgen, estrogen and progesterone receptors (ARs, ERs, and PRs) in a variety of reproductive tissues enables them to associate with Src (intracellular tyrosine kinase) resulting in activation of Src kinase and EGFR dimerization and activation (transactivation) which in turn initiates MAPkinase signaling. On the other hand, epidermal growth factor (EGF) phosphorylates ER in breast and prostate cancer cells through EGFR and Src to stimulate the formation of a signaling complex between EGFR, AR/ER, and Src (Migliaccio et al., 2006). Thus, rapid, nongenomic steroid signaling may involve complex interactions between nuclear receptors and between EGF/EGFR and the nuclear steroid receptor/Src complex. Nuclear steroid receptors have also been detected on the surface of cells using specific antibodies and shown to be activated at the cell surface using steroid-protein complexes that cannot enter cells. Palmitoylation of ERs and ARs increases their association with caveolin-1 resulting in transport of a small proportion of the receptors to the plasma membrane where they are localized in caveolae (Levin, 2011). Membrane-associated nuclear receptors have also been shown to transduce steroid signals intracellularly by the same mechanism as members of the G protein-coupled receptor (GPCR) superfamily, through activation of G proteins. For instance, the membrane-bound ER ($\text{ER}\alpha$) induces rapid intracellular signaling pathways and nitric oxide production in rodent vascular cells through activation of an inhibitory G-protein. In addition, truncated forms of ERs such as $\text{ER}\alpha 46$ and $\text{ER}\alpha 36$ have shown been to mediate nongenomic steroid signaling.

Membrane Receptors

Rapid steroid activation of intracellular signaling pathways resulting in nongenomic responses has also been demonstrated in cells that lack nuclear steroid receptors. In addition, many nongenomic steroid actions in vertebrate cells are not modulated by specific nuclear steroid receptor agonists and antagonists. Furthermore, the biochemical binding characteristics of steroid receptors detected on the plasma membranes of numerous cell types differ from those of the nuclear steroid receptors. Taken together, these findings suggest the existence of distinct steroid membrane receptors unrelated to nuclear steroid receptors. Different steroid membrane receptors for estrogens, androgens and progestins have been identified in the past 15 years. A 7-transmembrane progesterone receptor that mediates progesterone induction of oocyte maturation by a nongenomic mechanism, named membrane progesterone receptor alpha

(mPR α), was discovered in 2003 in fish ovaries (Thomas, 2012). Altogether, five closely-related membrane progesterin receptors (mPR- α , - β , - γ , - δ , - ϵ) have been identified in vertebrate tissues. Although the mPRs are coupled to G proteins they do not belong in the GPCR superfamily and instead are members of the progesterin adipoQ receptor (PAQR) family. Progesterin activation of G proteins through mPRs results in alterations of intracellular signaling through various pathways including those involving cAMP, Ca²⁺, PI3K, MAPkinase, and EGFR. The mPRs mediate a wide variety of progesterin reproductive actions including initiation of sperm hypermotility, oocyte meiotic maturation, inhibition of cell death and apoptosis of ovarian follicle cells, and reproductive behaviors (Thomas, 2012). In 2005, the orphan 7-transmembrane GPCR, GPR30, was shown to have all the characteristics of an estrogen membrane receptor in an ER-null breast cancer cell line. GPR30 is coupled to G proteins and stimulates similar intracellular signaling pathways to those activated through the mPRs. The functions of GPR30, now known as G protein-coupled estrogen receptor (GPER), have been studied extensively and implicated in a broad range of physiological and pathological actions of estrogens and xenoestrogens including proliferation of numerous cell types, and tumorigenesis. GPER has an important role in the maintenance of oocyte meiotic arrest in fish. On the other hand, only a few reproductive functions of GPER such as regulation of GnRH secretion and spermatogonial cell proliferation have been identified in mammals and GPER knockout mice are fertile and display few reproductive abnormalities. A third novel steroid membrane receptor, the membrane androgen receptor, ZIP9, has been recently discovered in fish ovaries. ZIP9 is a 7–8 transmembrane protein belonging to the ZIP (ZRT- and Irt-like Protein, SLC39A) family that transports zinc across the cell membrane and from organelles into the cytoplasm to regulate zinc homeostasis. ZIP9 displays all the binding characteristics of a novel androgen receptor in both fish ovarian follicle cells and in breast and prostate cancer cells and is coupled to G proteins (Thomas et al., 2017). The occupied receptor induces apoptosis in both ovarian and cancer cells through the intrinsic pathway, involving upregulation of Bax, P53, JNK, and Caspase 3. Interestingly, testosterone induces both MAPkinase activation and zinc influx through ZIP9 in a G protein-dependent manner and testosterone's apoptotic action is dependent on both of these responses. Thus, ZIP9 acts as a membrane androgen receptor and a zinc transporter, with both these functions being required for the androgen-dependent apoptotic response.

Other Steroid Binding Proteins

A diverse variety of other proteins, including steroid hormone binding globulins (SHBG), ion channels, receptors for other hormones and adaptor proteins have also been proposed to have important roles in rapid steroid signaling. For example, androgens bound to SHBG can activate the SHB receptor on the cell surface of Sertoli cells leading to G protein and AC activation. Moreover, androgens have been shown to antagonize the activation of the eicosanoid receptor by its natural ligand in prostate cancer cells. The CatSper channel mediates the progesterone-induced Ca²⁺ increase in human sperm. In addition, progesterone membrane component one (PGRMC1) has an important adaptor protein function in nonclassical progesterone signaling through regulation of mPR expression on the plasma membrane.

Nongenomic Androgen Actions in Male Reproduction

Nongenomic actions of estrogen and progesterone have been studied extensively in a wide variety of cell types. Moreover, the nuclear and membrane receptors that mediate many of these rapid estrogen and progesterone effects have been identified and are the subject of several recent reviews. In comparison, relatively few studies have been conducted on nongenomic androgen actions in vertebrate cells and the identity of an androgen membrane receptor was unknown until a few years ago. Recently, convincing evidence has been obtained for nongenomic androgen actions and androgen-dependent signaling in male reproductive tissues (Rahman and Christian, 2007; Walker, 2010). In addition, the novel membrane androgen receptor, ZIP9, was recently discovered and identified in both the male and female reproductive systems (Thomas et al., 2014). Androgens have an indispensable role in the regulation of male reproduction and are essential for development of the male reproductive system, normal spermatogenesis and fertility. Here rapid androgen signaling and nongenomic actions in the male reproductive system and the receptors mediating these responses will be briefly reviewed.

Sertoli Cells

Androgens, produced in the testis by Leydig cells, are released into the circulation and also diffuse into the seminiferous tubules, the site of spermatogenesis. The Sertoli cells, which surround the germ cells and provide nourishment for their development, perform several of critical functions during spermatogenesis that are regulated by androgens, including maintenance of the blood–testis barrier, meiosis, attachment of Sertoli cells to spermatids, and sperm release. Studies with AR mutant mice that lack the androgen response element (ARE) specific for androgens have shown that classic genomic androgen actions through selective AREs are required to support complete spermatogenesis and for the regulation of numerous genes in Sertoli cells. However, the results of recent studies suggest several rapid, nongenomic androgen actions on Sertoli cells involving a variety of signaling pathways and receptors also contribute to germ cell development.

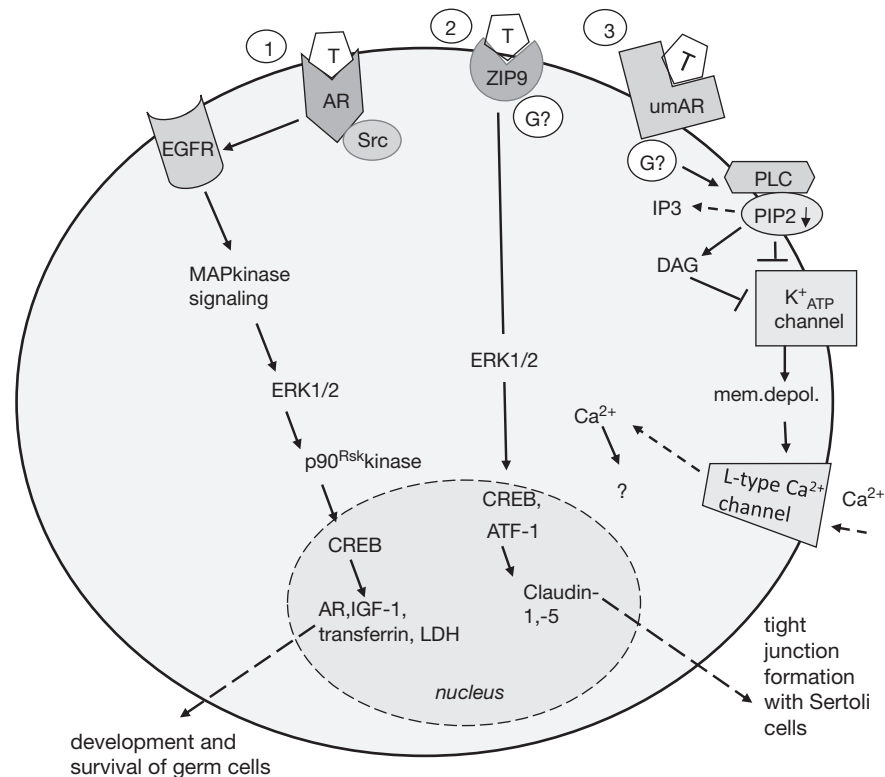


Fig. 1 Nongenomic androgen signaling pathways in Sertoli cells. (1) Testosterone signaling through a Src/EGFR/MAPkinase/p90^{Rsk}kinase/CREB pathway mediated by a membrane-associated AR resulting in upregulation of genes critical for the development and survival of germ cells. LDH: lactic dehydrogenase. (2) Testosterone signaling through a G protein/ERK1/2 pathway mediated by ZIP9 resulting in upregulation of claudin genes essential for tight junction formation and maintenance of the blood–testis barrier. (3) Testosterone signaling through a G protein/PLC-PIP2/K⁺_{ATP}/L-type Ca²⁺ channel mediated by an unidentified membrane androgen receptor (umAR) resulting in Ca²⁺ influx. *Mem.depol.*: membrane depolarization; *solid lines*: regulatory pathways; *hatched lines*: biological responses; *?*: downstream target unknown.

Mediated through membrane-associated AR

Testosterone binding to a subcellular fraction of the AR located near the cell membrane of Sertoli cells causes the receptor to associate with and activate Src tyrosine kinase which in turn phosphorylates the EGFR resulting in its dimerization and activation (transactivation) and the initiation of MAPkinase signaling. ERK1/2 phosphorylation causes activation of p90^{Rsk} kinase which leads to CREB phosphorylation and CREB-regulated transcription. CREB activates a variety of genes in Sertoli cells involved in the development and survival of germ cells, including the AR, insulin-like growth factor 1 (IGF-1), transferrin, and lactic dehydrogenase. The finding that the AR antagonist flutamide blocked CREB phosphorylation suggests this rapid action is mediated through the AR. Furthermore, studies using cultured Sertoli cells with an AR mutant that can only activate rapid androgen signaling show that this signaling pathway participates in the adhesion of Sertoli cells to germ cells which is important for the maintenance of spermatogenesis (Walker, 2010; Fig. 1, pathway 1).

Mediated through ZIP9

The results of recent studies suggest testosterone activation of rapid, nongenomic signaling in Sertoli cells is also mediated through the novel membrane androgen receptor, ZIP9, which also functions as a zinc transporter. Evidence was obtained that ZIP9 is an intermediary in androgen maintenance of the blood–testis barrier. The blood–testis barrier is indispensable for spermatogenesis by providing an immune-privileged microenvironment for the completion of meiosis. Androgen regulation of claudin production and tight junction formation by Sertoli cells are required for preservation of the blood–testis barrier. This testosterone action is not mediated through the AR because it can also be demonstrated in an AR-deficient Sertoli 93RS2 cell line. Low concentrations of testosterone caused rapid phosphorylation of ERK1/2, and the transcription factors CREB and ATF-1 in the 93RS2 cells which was accompanied by increased claudin-1 and -5 expression and tight junction formation. Interestingly, all these androgen actions were blocked after knockdown of the expression of ZIP9 with siRNA (Buldan et al., 2016; Fig. 1, pathway 2). Thus, this study provides the first clear evidence for an important role of ZIP9 as an intermediary in androgen regulation of the blood–testis barrier which has important implications for completion of spermatogenesis and male fertility. However, the physiological role of androgen-mediated zinc signaling through ZIP9 in Sertoli cells and possible cross-talk with AR-dependent signaling have not been investigated.

Mediated through unidentified membrane androgen receptor

A third rapid androgen-dependent pathway involves a rapid influx of Ca^{2+} into rat Sertoli cells and increased input resistance and depolarization through an androgen receptor on the cell membrane (Loss et al., 2004; Fig. 1, pathway 3). The identity of this receptor remains unresolved and has been proposed to be the AR (Rahman and Christian, 2007), or an unidentified receptor (Walker, 2010), based on positive or negative results with AR-specific ligands, respectively. Experiments using pharmacological tools have shown these androgen effects are dependent on activation of a pertussis toxin-sensitive G protein, blockage of the $\text{K}^{+}_{\text{ATP}}$ channel and activation of phospholipase C (PLC). PLC activation hydrolyses phosphoinositol (PIP2) to inositol triphosphate (IP3) and DAG. The depletion of PIP2 stores (also likely the increase in DAG) stimulates closure of the $\text{K}^{+}_{\text{ATP}}$ channels resulting in increased input resistance and depolarization which in turn opens voltage-dependent L-type Ca^{++} channels, enabling Ca^{++} influx (Loss et al., 2004). Currently, the physiological significance of this androgen signaling pathway is unclear.

Prostate Cells**Mediated through membrane-associated AR splice variant**

A wide variety of nongenomic androgen actions and signaling pathways activated through the AR and membrane androgen receptors have been reported in prostate cancer cells. Androgens also have critical functions in the prostate gland, regulating its development and maintenance. In addition, androgens have a major role in promoting the initial stages of prostate cancer. Progression from the androgen-sensitive to the castration-resistant stage of prostate cancer is regulated by the AR and its splice variants. The ineffectiveness of AR antagonist therapy during this castration-resistant stage severely limits the therapy options during the advanced stages of prostate cancer. An AR splice variant cloned from prostate cancer tissues, AR8, lacks a DNA binding domain and is primarily localized on the plasma membrane. Overexpression of AR8 in LNCaP prostate cancer cells has been shown to cause association of AR and Src with EGFR in response to EGF treatment and increase AR phosphorylation. In contrast, these EGF responses are blocked by knockdown of AR8 expression in prostate cancer cells and are accompanied by decreases in cell proliferation and increases in apoptosis of cells cultured in an androgen depleted medium. Collectively, these results suggest that the AR splice variant AR8 located on the plasma membrane of prostate cancer cells is involved in castration resistance by promoting AR proliferative and survival responses to growth factors and hormones (Yang et al., 2011).

Mediated through membrane-associated AR

Growth factors such as EGF interact with nuclear steroid receptors to promote ligand-independent steroid receptor-regulated transcription resulting in the growth and proliferation of cells and breast and prostate cancer progression. However, the crosstalk is bidirectional because activation of nuclear steroid receptors greatly increases EGF signaling through a nongenomic mechanism in both breast and prostate cancer cells. EGF stimulation of cytoskeletal changes and DNA synthesis are blocked by treatment with ER and AR antagonists in LNCaP cells, which suggests both ERs and AR are components of these EGF-mediated actions. Interestingly, a small proportion of the ER β (ER β) and AR are associated with each other under these prostate cancer cells under unstimulated conditions. EGF promotes phosphorylation of tyrosine residues on ER and stimulates the formation of a complex between EGFR, AR, ER β , and Src tethered to the cell membrane which is required for Src activity, EGFR phosphorylation and the growth stimulatory effects of EGF (Migliaccio et al., 2006). Taken together these results show reciprocal regulation of EGF- and nuclear steroid receptor/Src-mediated signaling in prostate cancer cells through a multiple nuclear steroid receptor/Src/EGFR protein complex. Another study showed that the scaffold protein MNAR (modulator of nongenomic activity of estrogen receptor) co-precipitates with the AR and Src which suggests it may be part of the EGFR/ER/AR/Src signaling complex.

Nongenomic androgen activation of ERK1/2 signaling in AR-positive LNCaP cells is mediated through matrix metalloproteinase (MMP) regulation of EGFR transactivation. Knockdown studies with paxillin siRNA have indicated an essential role for this multi-domain adaptor protein in androgen- and EGF-induced ERK1/2 signaling in LNCaP and in AR-null PC-3 cells, respectively, and also in the proliferation, migration and invasion of these cancer cells. Evidence was obtained that EGFR transactivation stimulates Src-induced phosphorylation of specific paxillin tyrosine residues which in turn promotes ERK1/2-mediated phosphorylation of several paxillin serine residues and the proliferation response. The finding that ERK1/2-mediated transcription in these cells is dependent on paxillin phosphorylation suggests that paxillin may enhance ERK1/2-mediated proliferation in these cancer cells by stimulating ERK1/2-regulated transcription (Sen et al., 2010).

A subpopulation of the AR is localized to the lipid raft compartment in LNCaP cells where it interacts with Akt1. This interaction is increased by androgen treatment resulting in elevated Akt1 activity, whereas it is blocked by the AR-antagonist, bicalutamide. Interestingly, this rapid increase in Akt1 activity does not involve PI3K signaling. The results suggest lipid rafts function to transmit nongenomic androgen signals through the AR and Akt pathway in these cancer cells.

Mediated through unidentified membrane androgen receptors unrelated to AR

Androgens also induce nongenomic actions in prostate cancer cells through membrane receptors unrelated to nuclear steroid receptors. Androgen binding to the cell surface of LNCaP cells has been detected by confocal microscopy using the membrane impermeable testosterone conjugate, T-BSA-FITC. Although testosterone-BSA phosphorylates ERK1/2 in LNCaP cells, it does not significantly activate two other MAPkinases, p38 MAPK and JNK. Treatment with the AR antagonist cyproterone did not blunt the ERK1/2 phosphorylation response to testosterone-BSA which suggests this nongenomic action is not mediated through the AR but instead through an unidentified membrane receptor (Wang et al., 2008). Activation of ERK1/2 is associated with an

increase in the concentration of the c-Fos protein which is a constituent of the AP1 transcription factor that regulates the growth and transformation of cells, and therefore, may have a major role in prostate cancer development.

Rapid androgen signaling initiated at the cell surface resulting in actin cytoskeleton reorganization, inhibition of cell proliferation, motility, and adhesion, as well as induction of apoptosis has been described in LNCaP (AR positive) and DU145 (AR-deficient) prostate cancer cells. This nongenomic androgen action is clearly not mediated by the AR in LNCaP cells because it is not altered by treatment with AR antagonists such as flutamide or by treatment with AR siRNA (Hatzoglou et al., 2005; Papadopoulos et al., 2009). Androgen activation of an unidentified membrane androgen receptor in LNCaP cells triggers a signaling pathway involving activation of focal adhesion kinase (FAK), PI3K, rho small GTPases, and Rho-associated protein kinase (ROCK) which results in actin polymerization and ultimately apoptosis. Although small GTPases (Rho A, Rho B and Cdc42) and ROCK signaling were also shown to regulate actin reorganization in DU145 cells, the results suggest that it is not mediated by activation of the FAK/PI3K pathway. Instead, down-regulation of the pro-survival FAK/PI3K pathway was observed 12–24 h after testosterone treatment (Papadopoulos et al., 2009). The resulting apoptosis in DU145 cells is likely mediated by an intrinsic pathway because it is associated with increased Caspase 3 activity. Testosterone-mediated PI3K and ROCK signaling in DU145 cells also upregulates serum- and glucocorticoid-inducible kinase (SGK1) which in turn activates Na^+/H^+ exchangers and increases intracellular pH (Chatterjee et al., 2014). The finding that treatment with an inhibitor of Na^+/H^+ exchanger activity blocked actin polymerization in response to testosterone treatment suggests an involvement of these exchangers in actin reorganization mediated by a membrane androgen receptor.

Androgens also induce rapid and transient L-type calcium channel-dependent increases in intracellular Ca^{2+} levels in LNCaP cells through a receptor on the cell surface associated with a pertussis toxin-sensitive G protein, suggesting it is coupled to an inhibitory G protein. This androgen action was not blocked with the AR antagonist, cyproterone acetate, which suggests it is mediated through an unidentified membrane receptor unrelated to the AR (Sun et al., 2006).

Mediated through ZIP9

Nongenomic androgen signaling mediated by the membrane androgen receptor ZIP9 has been clearly demonstrated in AR-null prostate cancer (PC-3) and breast cancer cell lines (MDA-MB-231 and MDA-MB-468) as well as in AR-positive LNCaP cells (Thomas et al., 2014, 2017). Gain of function studies conducted with PC-3 and MDA-MB-231 cells overexpressing ZIP9, as well as loss of function experiments in MDA-MB-468 and LNCaP cells in which ZIP9 expression was knocked down with siRNA show that membrane androgen receptor binding, and androgen signaling and apoptotic actions in these cells are dependent on cell surface expression of the ZIP9 protein. The ZIP9 protein displays high affinity, specific androgen binding to the plasma membranes of these cells with characteristics typical of membrane androgen receptors. ZIP9 has low binding affinities for several AR agonists and antagonists. Testosterone treatment (10–20 nM) specifically phosphorylates ERK1/2 and increases intracellular zinc concentrations through ZIP9 in these cancer cells, both of which are required for an apoptotic response. Interestingly, both androgen-dependent MAPkinase and zinc signaling are mediated through activation of a G protein. Several lines of evidence suggest ZIP9 is coupled to an inhibitory G protein (Gi) in these cancer cells. Firstly, pertussis toxin pretreatment blocks MAPkinase and zinc signaling as well as apoptosis in response to testosterone treatment. The finding that testosterone treatment causes decreased cAMP production in ZIP9-transfected cells is also consistent with activation of a Gi protein. Finally, an in situ proximity ligation assay revealed a close association of ZIP9 with a Gi in ZIP9-transfected PC-3 cells. Testosterone induces ZIP9-mediated apoptosis in prostate and breast cancer cells through upregulation of components of the intrinsic apoptotic pathway, Bax (Bcl-2-associated X protein) and P53 (tumor protein 53), as well as JNK (c-Jun N-terminal kinases) which is involved in both the intrinsic and extrinsic pathways. These increases in gene expression were blocked by pretreatment with either a zinc chelator or a MAPkinase inhibitor, indicating both signaling pathways are required for upregulation of these proapoptotic members. The finding that the concentrations of Bax, cytochrome C and Caspase 3 proteins as well as Caspase 3 activity are also increased after testosterone treatment are consistent with activation of the intrinsic apoptotic pathway (Fig. 2). It is concluded from these studies that ZIP9, a member of the zinc transporter ZIP family, functions as a specific membrane androgen receptor coupled to an inhibitory G protein in prostate cancer cells through which testosterone activates MAPkinase and zinc signaling pathways regulating apoptosis. The discovery that ZIP9 expression is upregulated in invasive malignant cells in prostate cancer biopsy samples further suggests that ZIP9 is a potential therapeutic target for treating prostate cancer.

Spermatogenic Cells

Mediated through ZIP9

A recent study has shown that testosterone rapidly activates ERK1/2, CREB, and ATF-1 in spermatogenic GC-2 cells, through an AR-independent mechanism (Shihan et al., 2015). These androgen-dependent actions are blocked by silencing ZIP9 and the G protein, $\text{G}\alpha 11$. A ligation proximity assay showed a close association between ZIP9 and $\text{G}\alpha 11$, which suggests the G-protein is directly coupled to the receptor. Taken together these results suggest that rapid androgen signaling in the GC-2 spermatogenic cells is mediated through a ZIP9/ $\text{G}\alpha 11$ pathway. The physiological significance of this nongenomic androgen pathway in spermatogenic cells is currently unknown.

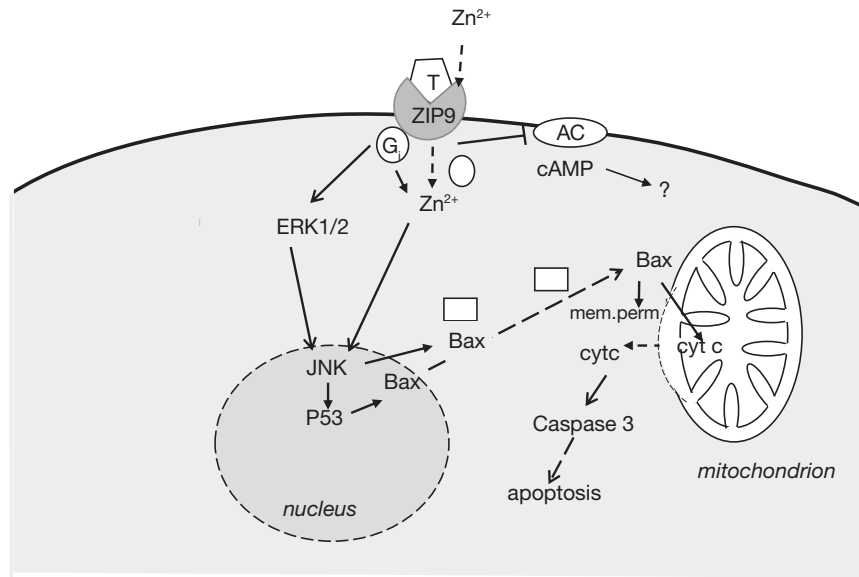


Fig. 2 Nongenomic androgen signaling mediated by ZIP9 coupled to an inhibitory G protein that induces apoptosis through the intrinsic pathway in prostate and breast cancer cells. *Mem.perm.*: Membrane permeability increase; *cyt c*: cytochrome *c*; *solid lines*: regulatory pathways; *hatched lines*: biological responses; *?*: downstream target unknown. Note: ZIP9 is also localized on mitochondrial and nuclear membranes (see Thomas et al., 2017).

GnRH-Producing Hypothalamic Cells

Mediated through membrane-associated AR

Androgens have also been shown to activate intracellular signaling pathways through a cell surface-mediated mechanism in neuroendocrine cells. The plasma membranes of immortalized GnRH-secreting murine GT1-7 cells display androgen binding activity and treatment with dihydrotestosterone (DHT) attenuates forskolin upregulation of cAMP production. This inhibitory DHT action is blocked by treatment with hydroxyflutamide and pertussis toxin which suggests it is mediated through activation of the AR and an inhibitory G (G_i) protein, respectively. Western blot analysis with an AR antibody confirmed the presence of an immunoreactive band of the correct size for the AR in plasma membrane fractions of GT1-7 cells. Treatment with testosterone, DHT, or testosterone conjugated to BSA also significantly increased intracellular Ca^{2+} concentrations within 3 min and increased GnRH secretion twofold after 1 h (Shakil et al., 2002). Whereas 24- and 36-h treatments with testosterone and DHT resulted in downregulation of GnRH mRNA levels as reported previously, similar treatments with testosterone-BSA were ineffective which suggests differential androgen regulation of GnRH secretion and mRNA expression through cell-surface mediated nongenomic and intracellular AR-mediated genomic mechanisms, respectively.

Summary

A bewildering diversity of rapid androgen signaling pathways has recently been identified in cells in the male reproductive system, particularly in Sertoli and prostate cells. However, in many cases their physiological significance remains unclear because the functions of these signaling pathways identified in cell lines have not been confirmed by conducting more physiologically relevant studies in tissues and in whole organisms. Clearly, more research in a variety of physiological contexts is required to identify the reproductive functions of these androgen signaling pathways. In addition, knowledge of the potential interactions between these androgen signaling pathways as well as their possible crosstalk with genomic androgen actions will be required for a clearer understanding of the roles of nongenomic steroid actions in male reproductive physiology. Research over the past few years has shown that the novel membrane receptor, ZIP9, is an intermediary in nongenomic androgen actions in Sertoli, prostate, and spermatogenic cells. The involvement of ZIP9 in other rapid androgen actions not mediated through the AR remains to be investigated. This exciting recent progress on nongenomic androgen actions provides a sound basis for future research in this area.

References

- Buldan, A., Dietz, R., Shihan, M., & Scheiner-Bobis, G. (2016). Non-classical testosterone signaling mediated through ZIP9 stimulates claudin expression and tight junction formation in Sertoli cells. *Cellular Signalling*, 28, 1075–1085.
- Chatterjee, S., Schmidt, S., Pouli, S., Honisch, S., Alkahtani, S., Stourmarar, C., & Pang, F. (2014). Membrane androgen receptor sensitive NA/H exchanger activity in prostate cancer cell. *FEBS Letters*, 588, 1571–1579.

- Hatzoglou, A., Kampa, M., Kogia, C., Charalampogoulos, I., Theodoropoulos, P. A., Anezinis, P., Dambaki, C., Papakonstanti, E. A., Stathopoulos, E. N., Gravanis, A., & Castanas, E. (2005). Membrane androgen receptor activation induces apoptotic regression of human prostate cancer cells *in vitro* and *in vivo*. *Journal of Clinical Endocrinology and Metabolism*, 90, 893–903.
- Levin, E. R. (2011). Minireview: Extracellular steroid receptors: Roles in modulation of cell functions. *Molecular Endocrinology*, 25, 377–384.
- Loss, E. S., Jacobsen, M., Costa, Z. S. M., Jacobus, A. P., Borelli, F., & Wassermann, G. F. (2004). Testosterone modulates K⁺ ATP channels in Sertoli cell membrane by a PLC–PIP2 pathway. *Hormone & Metabolism Research*, 36, 519–525.
- Migliaccio, A., Casoria, G., Di Domenico, M., Ciociola, A., Lombardi, M., De Falco, A., Nanayakkara, M., Bottero, D., De Stasio, R., Varricchio, I., & Auricchio, F. (2006). Crosstalk between EGFR and extranuclear steroid receptors. *Annals of the New York Academy of Sciences*, 1089, 194–200.
- Papadopoulou, N., Papakonstanti, E. A., Kallergi, G., Alevizopoulos, K., & Stourmas, C. (2009). Membrane androgen receptor activation in prostate and breast tumor cells: Molecular signaling and clinical impact. *IUBMB Life*, 61, 56–61.
- Rahman, F., & Christian, H. C. (2007). Non-classical actions of testosterone: An update. *Trends in Endocrinology and Metabolism*, 18, 371–378.
- Sen, A., O'Malley, K., Wang, Z., Raj, G. V., DeFranco, D. B., & Hammes, S. R. (2010). Paxillin regulates androgen- and epidermal growth factor-induced MAPK signaling and cell proliferation in prostate cancer cells. *Journal of Biological Chemistry*, 285, 28787–28795.
- Shakil, T., Hogue, A. N. E., Husain, M., & Belsham, D. D. (2002). Differential regulation of gonadotropin-releasing hormone secretion and gene expression by androgen: Membrane versus nuclear receptor activation. *Molecular Endocrinology*, 16, 2592–2602.
- Shihhan, M., Chan, K.-H., Konrad, L., & Scheiner-Bobis, G. (2015). Non-classical testosterone signaling in spermatogenic GC-2 cells is mediated through ZIP9 interacting with Gn α 11. *Cellular Signalling*, 27, 2077–2086.
- Sun, Y.-H., Gao, X., Tang, Y.-J., Xu, C.-L., & Wang, L.-H. (2006). Androgen induce increase in intracellular calcium via a G protein-coupled receptor in LNCaP prostate cancer cells. *Journal of Andrology*, 27, 671–678.
- Thomas, P. (2012). Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *General and Comparative Endocrinology*, 175, 367–383.
- Thomas, P., Pang, Y., Dong, J., & Berg, A. H. (2014). Identification and characterization of membrane androgen receptors in the ZIP9 zinc transporter subfamily: II. Role of human ZIP9 in testosterone-induced prostate and breast cancer cell apoptosis. *Endocrinology*, 155, 4250–4265.
- Thomas, P., Pang, Y., & Dong, J. (2017). Membrane androgen receptor characteristics of human ZIP9 (SLC39A) zinc transporter in prostate cancer cells: Androgen-specific activation and involvement of an inhibitory G protein in zinc and MAPkinase signaling. *Molecular and Cellular Endocrinology*, 447, 23–34.
- Walker, H. W. (2010). Non-classical actions of testosterone and spermatogenesis. *Philosophical Transactions of the Royal Society B*, 365, 1557–1569.
- Wang, Z., Liu, L., Hou, J., Wen, D., Yan, C., Pu, J., Ouyang, J., & Pan, H. (2008). Rapid membrane effects of testosterone in LNCaP cell. *Urologia Internationalis*, 81, 353–359.
- Yang, X., Guo, Z., Li, W., Shimells, H., Chen, M., Brodie, A. M. H., Chen, H., Xiao, Z., Veenstra, T. D., & Qiu, Y. (2011). Novel membrane-associated androgen receptor splice variant potentiates proliferative and survival response in prostate cancer cells. *Journal of Biological Chemistry*, 286, 36152–36160.

Androgen Receptor Ligands: Agonists and Antagonists

Folake A Orafidiya, Queen's University, Belfast, United Kingdom

Amy E Monaghan, University College London, London, United Kingdom

Iain J McEwan, University of Aberdeen, Aberdeen, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Agonist A ligand, usually a small molecule, which binds and activates a receptor protein.

Antagonist A ligand, usually a small molecule, that can bind and switch off receptor activity. Traditional antagonists act as competitive inhibitors for agonist binding.

Anabolic Process of growth and differentiation of cells and increased body mass.

Cachexia Wasting syndrome, associated with loss of weight, muscle atrophy, weakness and tiredness.

SARD Selective androgen receptor degrader.

SARM Selective androgen receptor modulator.

Introduction

The androgen receptor (AR) is a member of the steroid receptor subfamily of nuclear receptors. The binding of the hormones testosterone or the 5 α -reduced metabolite, DHT activate the receptor protein present in the cell cytosol. Binding of hormone leads to conformational changes in the receptor protein, reorganization of bound molecular chaperones and nuclear translocation and receptor dimerization. The precise order and molecular details of these events remain active areas of research and debate. What is less ambiguous is that transport of the receptor to nucleus permits binding to specific DNA sequences in the genome and regulation of androgen target genes. Thus, the AR, primarily acts as a ligand-activated transcription factor, although rapid signaling by hormone can result in recruitment of the receptor protein to the plasma membrane and activation of protein-kinase signaling pathways.

Androgen Receptor Structure–Function

The AR has the structural and functional domain organization typical of members of the nuclear receptor superfamily (**Fig. 1**). The ligand binding domain is made up of 300 amino acids and makes up the C-terminal third of the protein. This domain is highly α -helical and globular in structure and the binding of agonists causes conformation rearrangement of helix 12 and compacting of the structure (**Fig. 1**). The binding of the hormones testosterone or DHT occurs in the ligand binding pocket (LBP) which is buried in the LBD and is lined with hydrophobic residues, reflecting the chemical properties of steroid hormones, and a relatively small number of amino acids that make ligand discriminating interactions including threonine 877 and asparagine 705 with the hydroxyl group at C17 and glutamine 711 and arginine 752 with the oxygen at C3 (**McEwan, 2013**). Binding of agonist results in the formation of pockets on the LBD surface, notably AF2 which is important for protein-protein interactions and a region, BF3, which can bind small molecules and is allosterically linked to AF2 (**Fig. 1**) (**McEwan, 2013**). More recently, a large surface area of 1000 Å² has been characterized, involving helices 5, 7, 8 and 9, strand 1 and intervening loops, which mediates homo-dimerization of the LBD (**Nadal et al., 2017**).

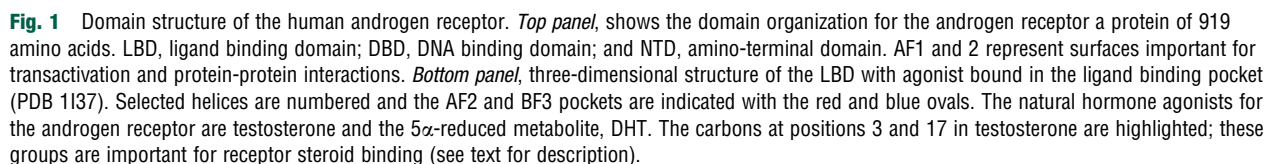
Agonists

Agonists of the Androgen Receptor

Any molecule capable of binding and altering a receptor from its resting state to an activated form, and consequently resulting in a biological response is referred to as an agonist. Agonists can either be endogenous and exogenous.

Endogenous agonists for the AR include the hormones testosterone and DHT (**Fig. 1**) which are naturally synthesized in the body in the gonads and peripheral tissues respectively. As early as the first trimester of fetal development, androgens start to shape the male phenotype by regulating the development of the prostate, scrotum, testes and related organs. After birth, the secretion of androgens ensures the maintenance of these male reproductive organs in addition to facilitating the production of sperm and differentiation of secondary male sex characteristics at puberty and adulthood.

Aside from the reproductive systems, other organs of the body such as the brain, liver, hair follicles, skin, kidney, bone, are also responsive to the effects of androgens. In the brain, behavioral patterns such as mood changes, aggression and cognitive abilities can



Exogenous agonists are synthetic molecules which can be introduced into the body topically, orally or by injection (**Table 1**). Mesterolone, which is used as a replacement therapy to treat males with androgen deficiency or infertility, is an example of an exogenous AR agonist. In addition to androgenic actions, AR agonists can be classed as selective androgen receptor modulators (SARMs) or anabolic androgenic steroids (AAS) (see **Table 1**). Clinically, AR agonists are used to treat osteoporosis ([Snyder et al., 2017](#)), anemia ([Paustian et al., 2016](#)), human immunodeficiency virus wasting syndrome ([Sardar et al., 2010](#)), and hypogonadism in males ([Darby and Anawalt, 2005](#)) (**Table 1**).

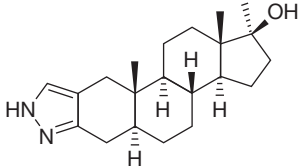
Importance of Intracrinology

The gonads, testes and ovaries, are the main sites of testosterone biosynthesis with the weak androgen, dehydroepiandrosterone (DHEA) produced by the adrenal gland. Testosterone (or DHEA) is then transported to target tissue via the blood stream, bound to carrier proteins such as sex hormone binding globulin (SHBG). However, there is increasing evidence for the expression of steroid biosynthetic enzymes in peripheral tissues, such as the prostate, and the importance of intracrinology in the production of testosterone. Inactive precursors such as DHEA, DHEA-sulfate, androstenedione can be converted to testosterone and DHT via tissue specific enzymes such as 3 β -hydroxysteroid dehydrogenase (3 β -OHSD), 17 β -hydroxysteroid dehydrogenase (17 β -OHSD), and 5 α -reductase. The DHT formed is used strictly for intracellular and local actions. About 50% of androgens are made in the prostate while the other 50% are synthesized in the testes (Labrie et al., 2005). Aside from the prostate, the intra-tissue conversion of DHEA to testosterone also takes place in the brain where it affects mood and behavior.

Table 1 Selected synthetic agonists of the androgen receptor

Agonist	Structure	Relative Binding	Action	Uses
7 α -methyl-19-nortestosterone (Trestolone)		260% DHT	ASS	Potential male contraception
Andarine		6% R1881	SARM	Muscle wasting Osteoporosis Performance enhancement
Danazol (Danatrol)		2% DHT	Androgenic	Anaemia Endometriosis
Fluoxymesterone (Halotestin, Ultandren)		0.4% R1881	ASS	Hypogonadism Anaemia (men) Breast cancer (woman)
Mesterolone		25% R1881	ASS	Hypogonadism
Methyltrienolone (R1881)		100 %	ASS	Laboratory studies of AR action
19-nortestosterone (Nandrolone)		32% DHT 60% R1881	ASS	Cachexia Anaemia Osteoporosis
Oxymetholone (Androl, Anapolon)		0.3% R1881	ASS	Osteoporosis Anaemia Cachexia
Oxandrolone (Anavar, Lonavar)		0.3% R1881	ASS	Hypogonadism Osteoporosis Anaemia Cachexia

Table 1 Selected synthetic agonists of the androgen receptor—cont'd

Agonist	Structure	Relative Binding	Action	Uses
Stanozolol (Winstrol, Stromba)		0.8% R1881	ASS	Angioedema Performance enhancement

Relative binding affinity is expressed as percentage of high affinity reference androgen in bracket (DHT or R1881).

These extra gonadal mechanisms of androgen synthesis are of importance in diseases such as prostate cancer where the growth and continued survival of the cancer is highly dependent on the presence of androgens, hence, a knowledge of all the sources of androgens is extremely crucial in informing the choice of treatment of this cancer. Although surgical and medical castration decrease the levels of serum testosterone from about 500 ng/dL to less than 50 ng/dL, androgens are still present within the prostate after castration, hence the use of other interventions such as antiandrogens, 5 α -reductase inhibitors to minimize the levels of androgens (Nishiyama, 2014).

Binding Affinities of Agonists

Ligands of the AR elicit their effects by binding to the ligand binding pocket (LBP) of the ligand binding domain (LBD) of the receptor. The structure of the LBD has been well characterized through crystallography (Fig. 1) which has led to the engineering of many compounds that can interact with the AR as either agonists or antagonists (McEwan, 2013) (Tables 1 and 2). The amino acid residues in the LBP are quite flexible and they make up the interface for the interaction of ligands and the receptor. The hydrophobic residues of the LBP adopt variable positions to fit in the steroid core via Van der Waals interactions, while polar amino acids form hydrogen bonds with polar atoms of the steroids. Collectively these interactions lead to stabilization, specificity and selectivity in agonist binding (McEwan, 2013). Testosterone and DHT bind to the AR with similar binding affinity ($K_D = 0.3\text{--}0.8\text{ nM}$), but the latter ligand dissociates about three to fourfold slower. The binding affinity of an AR ligand is often reported as a relative binding affinity (RBA) which is the measure of the ability of the ligand to competitively displace a high affinity ligand such as DHT or metribolone (R1881). The binding affinities for a selection of synthetic agonists of the AR are shown in Table 1.

Antagonists

Inhibition of the AR has multiple therapeutic indications. AR inhibitors may be employed in the treatment of prostate cancer, acne, seborrhea, hirsutism, androgenic alopecia, polycystic ovarian syndrome, endometrial cancer, and epithelial ovarian cancer. Clinical trials for the efficacy of antiandrogen therapy in triple negative metastatic breast cancer and spinal bulbar muscular atrophy (SBMA) are ongoing (<https://clinicaltrials.gov>). The structures of the clinical antiandrogens outlined below are included in Table 2, alongside their mechanism of action and applications.

Steroidal AR Inhibitors

AR inhibitors, or antiandrogens, can be broadly categorized as steroidal or non-steroidal. A third class of therapeutics, often misnamed antiandrogens, are those which reduce androgen biosynthesis or secretion through effects on the gonads, hypothalamus or pituitary gland, including finasteride, dutasteride and abiraterone, amongst others. Steroidal AR inhibitors often display mixed agonist-antagonist activities, with additional activities at estrogen, progesterone and glucocorticoid receptors mediating an array of side effects.

The first antiandrogen to be used clinically was cyproterone acetate (CPA; Table 2), a competitive AR inhibitor (McEwan, 2013) with additional inhibitory effects on the synthesis of androgens and spermatogenesis. Side effects of CPA are mediated primarily through progesterone and glucocorticoid receptor binding—including decreased basal cortisol and ACTH, and reduced adrenal response to insulin induced hypoglycaemia. CPA is not suitable for pregnant women due to the risk of feminisation of a male fetus, as well as the risk of secondary adrenal insufficiency. Adrenal insufficiency is also a risk in rapid withdrawal due to glucocorticoid receptor mediated suppression of ACTH.

Other steroidal antiandrogens include trimethyltrienolone (RU2956), developed as a derivative of the anabolic-androgenic steroid metribolone (R1881, Table 1). However the coincidental development of non-steroidal antiandrogen flutamide (Table 2) prevented progression of this compound to the clinic. Other steroidal therapies with antiandrogen activity include chlormadinone acetate (CMA), a steroidal progestin with antiandrogen and antigonadotrophic effects and spironolactone, a steroidal antimineralocorticoid with antiandrogen and progesterone effects. Unlike the CPA, these ligands are weak partial agonists of the AR, capable of

Table 2 Structures and mechanisms of androgen receptor inhibitors

Antagonist	Structure	Action	Uses
Apalutamide (ARN-509)		Competitive, non-steroidal antiandrogen	Under development; Phase III clinical trials CRPC
ASC-J9		Hsp90 inhibitor Selective androgen receptor degrader	Experimental inhibitor of androgen receptor signalling
Bicalutamide		Competitive, non-steroidal antiandrogen	Prostate cancer
Cyproterone Acetate		Competitive, steroidal antiandrogen	Prostate cancer
EPI-001		Androgen receptor amino-terminal domain antagonist	Under development; Phase I clinical trials CRPC
Enzalutamide		Second generation, competitive, non-steroidal antiandrogen	Prostate cancer, CRPC
Flutamide		Competitive, non-steroidal antiandrogen	Prostate cancer
Galeterone		Selective androgen receptor degrader Inhibitor of CYP17A1	Under development for prostate cancer; Phase III clinical trials
Nilutamide		Competitive, non-steroidal antiandrogen	Prostate Cancer

competing with testosterone for AR binding and subsequently eliciting a much weaker effect than the endogenous hormone. CMA is also a competitive inhibitor of 5 α -reductase, preventing conversion of testosterone to the more potent DHT. These drugs have never been marketed clinically for diseases driven by the AR.

Non-Steroidal Antiandrogens

Nonsteroidal antiandrogens compete with circulating testosterone and DHT for AR binding, blocking downstream AR dependent gene regulation, but also negative feedback of testosterone action at the hypothalamus and pituitary. This can result in a moderate increase in the baseline levels of plasma testosterone and oestrogens, which in prostate cancer may eventually lead to resistance. Following development of resistance in prostate tumors, antiandrogen withdrawal may result in a short term regression in disease, due to a reduction in negative feedback in the HPA-axis and a subsequent drop in serum androgen concentrations.

The first of the clinically approved non-steroidal antiandrogens, flutamide, was tested preclinically in 1973, showing competitive antagonism of the AR via binding to the ligand binding domain (LBD), and entered clinical trials in 1975. However, due to hepatotoxicity, clinical preference for prostate cancer is now bicalutamide (see below). Derivatives of flutamide include hydroxyflutamide and nilutamide, both of which competitively inhibit binding of testosterone to the AR. The antiandrogen nilutamide (Table 2) was approved for use in Europe in 1987. However its clinical use has been limited by its toxicity profile, most notably a high incidence of interstitial pneumonitis. Another non-steroidal antiandrogen was also described in 1973, but never marketed; DIMP (*N*-(3,5-dimethyl-4-isoxazolylmethyl)phthalimide) was a weakly competitive AR inhibitor structurally related to thalidomide.

Bicalutamide (Table 2) was developed as a more selective antiandrogen, capable of selectively inhibiting peripheral AR with reduced effects on negative feedback loops in the hypothalamus and pituitary, possibly due to reduced blood brain barrier penetration. The affinity of bicalutamide for the AR was found to be four times greater than that of hydroxyflutamide, and in vivo dosing did not cause an increase in serum testosterone. Bicalutamide progressed to the clinical trials under the brand name Casodex in 1989 and received approval for use by the FDA in 1995. Bicalutamide remains a first line therapy for the treatment of metastatic prostate cancer. Since its introduction, point mutations have been identified, in patients and cell lines, which promote resistance to bicalutamide, including a well characterized mutation at codon 742 in the AR LBD.

Second Generation Antiandrogens

Enzalutamide (Table 2) was first described in 2009 as a second-generation antiandrogen with higher affinity for the AR than bicalutamide, and capable of retaining activity in the presence of increased AR expression, often seen in metastatic PCa. Enzalutamide also prevented nuclear translocation of the AR and impaired binding of the AR DNA binding domain to hormone response elements in androgen responsive genes (McEwan, 2013). Since its identification, enzalutamide has entered the clinic for therapeutic use in several developed countries. Despite its increased efficacy compared to bicalutamide, research has still unveiled mechanisms of resistance in patients, including an inactivating F868L mutation in the AR LBD (Lallous et al., 2016). Resistance is also conferred by the expression of AR splice variants lacking the LBD (Antonarakis et al., 2014). A long, non-coding RNA *Malat1* has also been shown to drive enzalutamide mediated selection of AR-v7 expression and downregulation of this splice variants can be achieved by siRNA and the degradation enhancer ASC-J9 (Wang et al., 2017).

The F868L mutation has also been shown to confer resistance to apalutamide (ARN-509, Table 2) which is currently in phase III clinical trials for castrate-resistant prostate cancer (Balbas et al., 2013). Although apalutamide was reported to have greater efficacy than enzalutamide in animal models of PCa, alongside absence of AR nuclear localisation or DNA binding, it remains to be seen how the drug will compare to enzalutamide in a large-scale trial.

Non-Competitive Antiandrogens

Unlike the LBD, the AR amino terminal domain is critical for receptor function and is structurally intrinsically disordered. Development of resistance to antiandrogens in prostate cancer, driven by mutated and splice variant isoforms of the AR which lack the LBD, provides a new opportunity for researchers to develop therapies that deviate from classical competitive inhibition.

The bisphenol A derivative EPI-001 (Table 2) was identified by preclinical screening of marine sponge extracts and shown to be capable of binding to and inhibiting the AR via an interaction in the amino terminal domain of the receptor, which is required for recruitment of co-regulatory proteins and proper receptor function (Andersen et al., 2010). EPI derivatives were capable of inhibiting the AR and reducing tumor burdens in animal models and entered phase I/II clinical trial as EPI-506 in 2016. Naphetenone B and Sinkotamides A–E are other marine compounds reported to mediate antiandrogen activity by binding to the AR amino terminal domain. However naphetenone B also has activity at the glucocorticoid receptor, and these small molecules remain the subject of preclinical research.

Selective Androgen Receptor Degradors (SARDs)

ASC-J9 (Table 2) was first described as an AR inhibitor in spinal-bulbar muscular atrophy (SBMA or Kennedy's disease). Unlike previously described antiandrogens, ASC-J9 is proposed to selectively enhance degradation of the AR by disrupting the interactions

between the AR and co-regulatory proteins such as ARA-70. This degradation is not dependent on the presence of testosterone, and ASC-J9 is capable of degrading wild-type AR in addition to the enzalutamide resistant F868L mutant receptor, and the splice variant AR-v7 (Wang et al., 2017). Several mechanisms have been proposed for ASC-J9's action in prostate, renal and bladder cancers, including: modulation of inflammatory cytokine signaling; inhibition of cancer stem cell proliferation; and modulation of hypoxia inducible factor and vascular endothelial growth factor signaling. ASC-J9 has been tested clinically as a topical therapy for acne (www.clinicaltrials.gov).

Galeterone (Table 2) has been shown to reduce AR expression by increasing degradation, reducing expression of both full length AR and AR-v7. Galeterone is also active against the T878A and F868L mutant ARs. Phase I and II clinical trials have been undertaken with galeterone, showing that the drug is well tolerated and has measureable effects on PSA levels. Further trials are ongoing (www.clinicaltrials.gov).

Natural products have also been identified as a potential source of SARDs, including piperlongumine, a naturally occurring alkaloid of the long pepper (*Piper longum*); berberine, an isoquinoline alkaloid from the *Berberis* genera; and isosilybin B, a *Silybum marianum* or "milk thistle" extract.

Rationally designed SARDs have also been developed preclinically. Based on the AR ligand arylthiohydantoin RU59063, SARD279, and SARD033 are linked to a hydrophobic adamantyl group which is effective at causing selective degradation of the target protein (Gustafson et al., 2015). Other steroid conjugates include PROTACS: proteolysis targeting chimeric molecules, in which an AR ligand is attached to a recognition element for ubiquitin-protein ligase, and specific and non-genetic inhibitor of apoptosis protein (IAP) dependent protein erasers, or SNIPERs, which recruit IAP ubiquitin ligases to the AR (Shibata et al., 2017).

Mutations in the Androgen Receptor Altering Ligand Binding Properties

Mutations in the AR LBD have been associated with failure of testosterone and DHT to act resulting in androgen insensitivity syndrome, which is a partial or complete failure of male development in XY individuals. Point mutations in the receptor can disrupt hormone binding affinity, increase dissociation rate or alter binding specificity (<http://androgendb.mcgill.ca/>).

Conversely, the failure of antagonists such as bicalutamide and enzalutamide is correlated with prostate cancer progression to a castrate resistant stage (<http://androgendb.mcgill.ca/>). The mutation of threonine 878, to alanine, found in the prostate cell line LNCaP, directly disrupts the binding to the C17 group and broadens the types of steroid that can be accommodated in the LBP and is associated with loss of antagonism by hydrox-flutamide. The mutation results in an altered positioning of Helix 12 switching the activity of the antiandrogen to an agonist. Mutation of tryptophan 742 to cysteine and phenylalanine 868 to leucine, also part of the LBP, have been associated with resistance to the antiandrogens bicalutamide and enzalutamide respectively (Lallous et al., 2016).

Conclusions

The activity of the full-length AR is finely regulated by the binding of agonists or antagonists to activate or blunt the receptor response. The binding of the natural hormones testosterone or DHT to the LBD causes local structural rearrangements, receptor dimerization and allosteric regulation of receptor function. These events can, to a greater or lesser extent, also be achieved through various synthetic agonists. Conversely, switching off AR activity with antagonists is important in the treatment of a range of hormone-dependent diseases from acne to cancer. However, the therapeutic use of agonists and antagonists of the receptor is not without difficulties and side-effects, which can limit the effectiveness of such treatments. There is therefore a continued need for research into the mechanisms underpinning tissue-selective androgen action and receptor expression. Such investigations will be fundamental to the development of new molecules to target AR signaling in a more tissue-specific manner and thereby increase the effectiveness of androgen replacement or ablation treatments.

References

- Andersen, R. J., Mawji, N. R., Wang, J., Wang, G., Haile, S., Myung, J. K., Watt, K., Tam, T., Yang, Y. C., Bañuelos, C. A., Williams, D. E., McEwan, I. J., Wang, Y., & Sadar, M. D. (2010). Regression of castrate-recurrent prostate cancer by a small-molecule inhibitor of the amino-terminus domain of the androgen receptor. *Cancer Cell*, 17(6), 535–546.
- Antonarakis, E. S., Nakazawa, M., & Luo, J. (2014). Resistance to androgen-pathway drugs in prostate cancer. *The New England Journal of Medicine*, 371(23), 2234.
- Balbas, M. D., Evans, M. J., Hosfield, D. J., Wongvipat, J., Arora, V. K., Watson, P. A., Chen, Y., Greene, G. L., Shen, Y., & Sawyers, C. L. (2013). Overcoming mutation-based resistance to antiandrogens with rational drug design. *eLife*, 2, e00499.
- Chenu, C., Adlanmerini, M., Boudou, F., Chantalat, E., Guihot, A. L., Toutain, C., Raymond-Letron, I., Vicendo, P., Gadeau, A. P., Henrion, D., Arnal, J. F., & Lenfant, F. (2017). Testosterone prevents cutaneous ischemia and necrosis in males through complementary estrogenic and androgenic actions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 37, 909–919.
- Christou, P. A., Markozannes, G., Tsatsoulis, A., Mastorakos, G., & Tigas, S. (2017). Effects of anabolic androgenic steroids on the reproductive system of athletes and recreational users: A systematic review and meta analysis. *Sports Medicine*. <https://doi.org/10.1007/s40279-017-0709-z> (Epub ahead of print).
- Darby, E., & Anawalt, B. D. (2005). Male hypogonadism: An update on diagnosis and treatment. *Treatments in Endocrinology*, 4(5), 293–309.
- Gustafson, J. L., Neklesa, T. K., Cox, C. S., Roth, A. G., Buckley, D. L., Tae, H. S., Sundberg, T. B., Stagg, D. B., Hines, J., McDonnell, D. P., Norris, J. D., & Crews, C. M. (2015). Small-molecule-mediated degradation of the androgen receptor through hydrophobic tagging. *Angew. Chem. Int. Ed. Engl.*, 54(33), 9659–9662.

- Inoue, T., Miki, Y., Kakuo, S., Hachiya, A., Kitahara, T., Aiba, S., Zouboulis, C. C., & Sasano, H. (2014). Expression of steroidogenic enzymes in human sebaceous glands. *Journal of Endocrinology*, 222(3), 301–312.
- Labrie, F., Belanger, A., Luu-The, V., Labrie, C., Simard, J., Cusan, L., Gomez, J., & Candas, B. (2005). Gonadotropin-releasing hormone agonists in the treatment of prostate cancer. *Endocrine Reviews*, 26(3), 361–379.
- Lallous, N., Volik, S. V., Awrey, S., Leblanc, E., Tse, R., Murillo, J., Singh, K., Azad, A. A., Wyatt, A. W., LeBihan, S., Chi, K. N., Gleave, M. E., Rennie, P. S., Collins, C. C., & Cherkasov, A. (2016). Functional analysis of androgen receptor mutations that confer anti-androgen resistance identified in circulating cell-free DNA from prostate cancer patients. *Genome Biology*, 17, 10.
- Lolli, F., Pallotti, F., Rossi, A., Fortuna, M., Caro, G., Lenzi, A., Sansone, A., & Lombardo, F. (2017). Androgenetic alopecia: A review. *Endocrine*, 57, 9–17.
- McEwan, I. J. (2013). Androgen receptor modulators: A marriage of chemistry and biology. *Future Medicinal Chemistry*, 5(10), 1109–1120.
- Nadal, M., Prekovic, S., Gallastegui, N., Helsen, C., Abella, M., Zielinska, K., Gay, M., Vilaseca, M., Taulès, M., Houtsmuller, A. B., van Royen, M. E., Claessens, F., Fuentes-Prior, P., & Estébanez-Perpiñá, E. (2017). Structure of the homodimeric androgen receptor ligand-binding domain. *Nature Communications*, 8, 14388.
- Nishiyama, T. (2014). Serum testosterone levels after medical or surgical androgen deprivation: A comprehensive review of the literature. *Urologic Oncology*, 32(1), 38.e17–28.
- Paustian, L., Chao, M. M., Hanenberg, H., Schindler, D., Neitzel, H., Kratz, C. P., & Ebell, W. (2016). Androgen therapy in Fanconi anemia: A retrospective analysis of 30 years in Germany. *Pediatric Hematology and Oncology*, 33(1), 5–12.
- Rosario, E. R., Chang, L., Head, E. H., Stanczyk, F. Z., & Pike, C. J. (2011). Brain levels of sex steroid hormones in men and women during normal aging and in Alzheimer's disease. *Neurobiology of Aging*, 32(4), 604–613.
- Sardar, P., Jha, A., Roy, D., Majumdar, U., Guha, P., Roy, S., Banerjee, R., Banerjee, A. K., & Bandyopadhyay, D. (2010). Therapeutic effects of nandrolone and testosterone in adult male HIV patients with AIDS wasting syndrome (AWS): A randomized, double-blind, placebo-controlled trial. *HIV Clinical Trials*, 11(4), 220–229.
- Shibata, N., Nagai, K., Morita, Y., Ujikawa, O., Ohoka, N., Hattori, T., Koyama, R., Sano, O., Imaeda, Y., Nara, H., Cho, N., & Naito, M. (2017). Development of protein degradation inducers of androgen receptor by conjugation of androgen receptor ligands and inhibitor of apoptosis protein ligands. *Journal of Medicinal Chemistry*. <https://doi.org/10.1021/acs.jmedchem.7b00168> (Epub ahead of print).
- Snyder, P. J., Kopperdhal, D. L., Stephens-Shield, A. J., Ellenberg, S. S., Cauley, J. A., Ensrud, K. E., Lewis, C. E., Barrett-Connor, E., Schwartz, A. V., Lee, D. C., Bhasin, S., Cunningham, G. R., Gil, L. T. M., Matsumoto, A. M., Swerdloff, R. S., Basaria, S., Diem, S. J., Wang, C., Hou, X., Cifelli, D., Dougar, D., Zeldow, B., Bauer, D. C., & Keaveny, T. M. (2017). Effect of testosterone treatment on volumetric bone density and strength in older men with low testosterone a controlled clinical trial. *JAMA Internal Medicine*, 177(4), 471–479.
- Wang, R., Sun, Y., Li, L., Niu, Y., Lin, W., Lin, C., Antonarakis, E. S., Luo, J., Yeh, S., & Chang, C. (2017). Preclinical Study using Malat1 Small Interfering RNA or Androgen Receptor Splicing Variant 7 Degradation Enhancer ASC-J9^R to Suppress Enzalutamide-resistant Prostate Cancer Progression. *European Urology*. S0302-2838(17)30285–3 <https://doi.org/10.1016/j.eururo.2017.04.005> (Epub ahead of print).

Aggressive Behavior

Cecilia Jalabert, University of British Columbia, Vancouver, BC, Canada

Kathleen M Munley and Gregory E Demas, Indiana University, Bloomington, IN, United States

Kiran K Soma, University of British Columbia, Vancouver, BC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Aggression is one of the most important social behaviors. It is displayed by virtually all animals and serves a wide range of adaptive functions. Aggressive behaviors enable the acquisition and defense of limited valuable resources, such as mates, territories, shelters, and food. However, aggressive encounters are also a costly investment in terms of time, energy, predation risk, and physical injury. Animals evaluate the costs and benefits of competing for resources in search of maximal fitness payoffs to make the decision to fight or give up. In this way, aggressive behaviors mediate the establishment of dominance and subordination relationships among competitors that enable access to a given limited resource. In the context of choosing a mate, for example, the contenders evaluate strength and establish dominance in front of a potential mate. In solitary or territorial species, aggression implies exclusive use of a resource, but in gregarious species, aggression sets relations of dominance and establishes hierarchies. Consequently, aggressive interactions have a direct impact on the survival and reproductive success of individuals.

Historically, studies on aggression have focused on male–male competition. However, aggression is not exclusive to males and occurs not only during competition for a mate. Female–female contests, although less studied, are also seen in nature. Just like in males, preferential access to a resource is advantageous for females, which drives this behavior. Aggression has been defined in a variety of ways. Aggression has traditionally been defined as an overt behavior with the intention of inflicting physical damage upon another individual. Different classifications of aggressive interactions have been proposed, although they have only more recently been defined around the context in which they occur: 1- Territorial aggression; 2- Disputes over food; 3- Aggression to establish relationships of dominance; 4- Parental aggression; 5- Aggression for sexual competition; 6- Antipredator aggression; 7- Irritable aggression.

The neuroendocrine mechanisms of aggressive behavior are conserved in vertebrates and have been principally studied during the breeding season. These studies have focused on male–male interactions and on the role of gonadal steroid hormones, predominantly testosterone (T), as the primary factor regulating aggression. The objective of this article is to provide a brief overview of the endocrine mechanisms modulating aggressive behavior, describing the role of both gonadal and nongonadal steroids. In addition, we discuss the neural circuits that underlie aggression and the role of androgens in promoting aggressive behaviors in primates.

Endocrine Mechanisms of Aggression

Gonadal Steroids

Traditionally, blood-borne gonadal steroids have been the primary focus of neuroendocrine studies on aggression (**Fig. 1**). The potential role of androgens in aggressive behavior was first explored in roosters in the mid-19th century by Arnold Berthold. In his classic “ablate and replace” study, Berthold removed the testes of sexually immature male chickens and found that castrated male chickens, called capons, exhibited a decrease in the development of certain secondary sex characteristics, such as wattles and a prominent comb; and a decrease in male-typical behaviors, including aggression and mating behaviors. However, when testes were transplanted into capons, the capons exhibited normal behavioral and morphological development. Berthold also observed via dissection that the testes had developed additional vasculature following transplantation, suggesting that a blood-borne factor secreted by the testes is essential for proper behavioral, physiological, and morphological development in roosters. This compound, which Berthold termed “productive verhältnis der hoden,” is now known as the androgen T. Berthold’s experiment was the first to provide evidence that gonadal T regulates aggression in males.

Since the publication of Berthold’s work, numerous studies have provided evidence that circulating gonadal steroids, such as T and estradiol (E₂), promote aggression by binding directly to androgen receptors (AR) and estrogen receptors (ER) in the brain, modulating neural pathways relevant to aggressive behavior. Sex steroid secretion is particularly important in facilitating aggression during the breeding season, when aggressive behavior may be crucial to acquiring a mate and maintaining territorial boundaries. In seasonally-breeding vertebrates, the gonads grow before the breeding season and regress following the termination of breeding. Circulating sex steroid concentrations fluctuate alongside gonadal growth and regression, and high levels generally occur during the breeding season and basal or nondetectable levels occur during the nonbreeding season.

While gonadal steroid secretion is important in modulating aggressive behaviors, recent work indicates that these behaviors are not exclusively regulated by gonadal steroids. There is now strong evidence that the brain is capable of metabolizing circulating gonadal steroids and precursors (such as dehydroepiandrosterone, DHEA) and even synthesizing these steroids *de novo* from cholesterol (**Fig. 1**). Additional steroidal and nonsteroidal hormones play a role in modulating aggression, including glucocorticoids, a class of steroids known for their antiinflammatory and immunosuppressive effects.

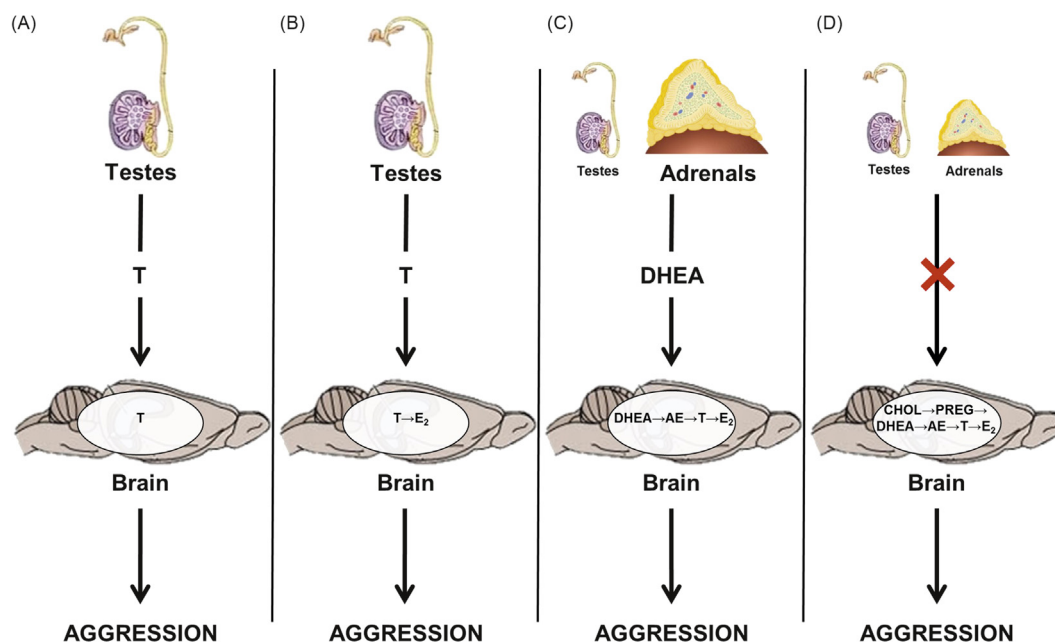


Fig. 1 Pathways by which steroids could affect aggression. (A) Gonadal testosterone (T) acts directly on the brain; (B) gonadal T is converted locally to estradiol (E_2); (C) adrenal dehydroepiandrosterone (DHEA) is converted locally to T and/or E_2 ; (D) neurosteroids are produced locally de novo from cholesterol (CHOL) via conversion to pregnenolone (PREG), DHEA, androstenedione (AE), T, and E_2 , in the absence of gonadal and adrenal steroid production.

Nongonadal Steroids

DHEA is an inert androgen precursor that can be metabolized in tissues that express the appropriate steroidogenic enzymes. DHEA is secreted by the adrenal glands and can travel in the circulation as DHEA-S, the sulfated form of DHEA. Once DHEA has reached a target organ or tissue, it can bind with very low affinity to several different types of hormone receptors, including AR, ER, and glucocorticoid receptors. Alternatively, DHEA can be locally converted to active steroid hormones, such as T and E_2 , which can then bind with high affinity to AR and ER, respectively.

The role of DHEA in modulating aggressive behavior has mostly been studied in vertebrates that exhibit year-round aggression, particularly some birds and rodents. Both males and females of these species exhibit territorial aggression outside of the breeding season, despite the fact that the gonads are regressed and circulating androgen levels are low. In fact, the aggressive behaviors observed in nonbreeding some birds are qualitatively and quantitatively similar to those displayed during the breeding season, while Syrian and Siberian hamsters actually show increased aggression toward conspecifics when they are not in breeding condition. Interestingly, these animals also consistently display elevated plasma, gonadal, and adrenal DHEA levels, suggesting that the adrenals and gonads are secreting DHEA into the circulation during the nonbreeding season. Moreover, there is emerging evidence that nonbreeding birds and rodents can display locally elevated DHEA and androgen levels and increased AR and ER in brain regions associated with aggression. Thus, seasonally-breeding animals use two different neuroendocrine mechanisms to sustain year-round territorial aggression: breeding animals rely on circulating gonadal steroids to promote aggressive behaviors, whereas reproductively quiescent animals use circulating DHEA and DHEA synthesized de novo in the brain as an alternative source of androgens during the nonbreeding season.

Because seasonally-breeding vertebrates rely on an alternative mechanism to maintain aggression year-round, physiological indicators of season are critical in signaling animals to shift from one mechanism to another throughout the year. In particular, the hormone melatonin plays an essential role in initiating the physiological and endocrine changes necessary for this "seasonal switch." Melatonin is secreted by the pineal gland, a structure located between the cerebellum and cerebral cortical hemispheres of the brain. Melatonin secretion is high at night and low during the day; therefore, changes in day length, or photoperiod, result in changes in the pattern and duration of melatonin secretion. For example, some species of seasonally-breeding rodents, such as Siberian hamsters, breed during the summer, when there is a longer photoperiod than the winter. During the summer, the duration of melatonin secretion is short compared to the winter. Circulating melatonin binds to melatonin receptors on the gonads and adrenals, resulting in a decrease in gonadal steroid synthesis and an increase in adrenal DHEA synthesis (Fig. 2).

While the adaptive value of this alternative neuroendocrine mechanism of aggression is still being investigated, there are several potential benefits of utilizing DHEA as a source of androgens during the nonbreeding season. For example, maintaining high levels of aggression year-round may benefit individuals by allowing them to gain access to limited food resources and to defend their territories. Furthermore, the regulation of aggressive behaviors via this mechanism is far less energetically costly than sustaining high levels of circulating sex steroids. During the breeding season, a significant amount of energy is devoted to reproductive

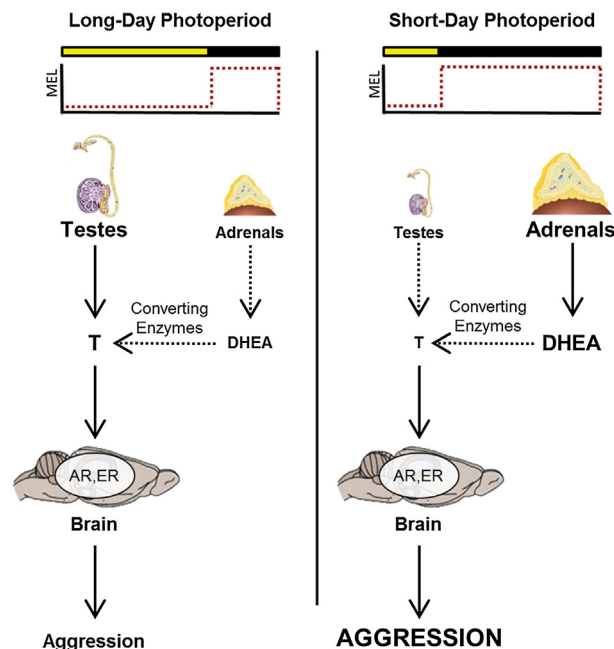


Fig. 2 Theoretical model describing the relationships among melatonin (MEL) secretion, seasonal changes in serum testosterone (T) and dehydroepiandrosterone (DHEA) levels, and aggression in male Siberian hamsters. During the summer, long-day photoperiods (LD) result in a short duration of MEL secretion compared to the short-day photoperiods (SD) of the winter. During LD, T is secreted from the testes, whereas during SD, DHEA is secreted by the adrenal glands. Dotted lines and smaller font sizes symbolize less abundant levels of hormones than solid lines and larger font sizes. T can either directly bind to neural androgen receptors (AR) or can be locally converted to estradiol and bind to neural estrogen receptors (ER). Differences in AR and ER binding and/or abundance in the brain result in changes in aggression, and in hamsters, there is higher aggression during SD than LD.

physiology, such as maintaining the gonads, often at the expense of other physiological processes. Thus, by using an inert precursor (e.g., DHEA), which can be locally and quickly converted to androgens, an individual can exhibit year-round aggression without incurring the energetic costs of maintaining high gonadal steroid levels.

Neural Circuits of Aggression

The regulation of social behavior, such as aggression, depends on neural circuits, including the social behavior network (SBN). These well-studied neural circuits consist of reciprocally connected brain regions, or nodes, located in the forebrain, midbrain, and hindbrain. In mammals, the SBN consists of six nodes: the extended medial amygdala [the medial amygdala and the medial bed nucleus of stria terminalis (BnST)], the lateral septum (LS), the preoptic area (POA), the anterior hypothalamus, the ventromedial hypothalamus (VMH), and the periaqueductal gray. Each of these nodes regulates multiple forms of social behaviors, including sexual behavior, parental behavior, and aggressive behavior. Thus, this network works as a whole, where each region of the network responds to several social stimuli, rather than working independently in the regulation of distinct types of social behavior. In this way, the SBN receives and evaluates external stimuli, integrates them with internal physiological information, and produces an appropriate response with a distinctive circuit activity pattern, biasing behavior that is adaptive for a specific context. For example, across brain nodes, there is a particular pattern of neural activation for sexual behavior, and the same brain nodes show a different pattern of neural activation for aggressive behavior.

Social behavior emerged early in animal evolution, playing a key role in determining the survival and fitness of individuals. Therefore, its neural control is under strong evolutionary pressures. Neuroanatomical and functional studies that assessed the distribution, connectivity, and neurochemistry of the brain nodes of the SBN have found that the neural circuits that regulate social behavior are highly conserved across vertebrates and play similar roles in the regulation of these behaviors. Similarly, despite the diversity of social behaviors regulated by this network (i.e., aggression or paternal care), the neural circuits and hormones involved in modulating these behaviors have been highly conserved in all vertebrate lineages. Birds, reptiles, amphibians, and teleost fish all contain nodes of the SBN that are homologous with the mammalian counterparts described above and have similar activation patterns in similar social contexts. The conservation of these nodes enables comparative studies in different species to establish general principles among vertebrates.

More recent work suggests that a broader social decision-making (SDM) network regulates adaptive social behaviors in response to different context or stimuli. The SDM consists of both the classic SBN nodes and also the mesocorticolimbic reward system. The mesocorticolimbic reward system is composed of several interconnected nodes, including the ventral tegmental area (VTA) and

nucleus accumbens. The VTA contains dopamine-producing neurons that project to the nucleus accumbens and other forebrain regions. Signaling from these dopaminergic neurons regulates several different behaviors, including motivational aspects of motivation, preparatory behavior, and appetitive behavior. Additionally, this system is involved in positive reinforcement associated with stimuli, a fundamental aspect for the expression of appetitive-approach behaviors. The SDM network appeared early in evolution and is also highly conserved among vertebrates.

Every node of the SBN and SDM expresses sex steroid receptors, indicating that these brain regions are sex steroid-sensitive and that hormones play key roles in modulating the activity of these networks and regulating social behaviors. Sex steroids, such as androgens and estrogens, act on the central nervous system by binding to their intracellular receptors (e.g., AR and ER). Bound receptors act as transcription factors to regulate gene expression in brain cells. This genomic mechanism of action allows steroid hormones to modulate the activity of the different brain regions and act indirectly, but not deterministically, on the execution of a behavior. For instance, sex steroids can change a stimulus response threshold, such as the response threshold toward a competitor, but do not typically trigger aggression itself. The genomic effects of steroid hormones on the central nervous system require several hours or days to develop and produce persistent changes in physiology and behavior.

In addition, sex steroids can exert short-term (within 30 min) nongenomic effects, which are often mediated by plasma membrane receptors or by the allosteric modulation of neurotransmitter receptors. For example, ER, such as ER α and ER β , can be associated with the plasma membrane and rapidly regulate intracellular signaling pathways. Another membrane-associated ER is the G-protein-coupled estrogen receptor-1 (GPER-1), which is also present in the brain. Estrogens rapidly influence aggressive behaviors in birds and mammals via intracellular signaling in the SBN and SDM. These rapid effects are more prominent during short photoperiods in both mice and song sparrows. Interestingly, E₂ administration has been shown to rapidly (within 30 min) increase aggression exclusively during the nonbreeding season in both deer mice and song sparrows. These data suggest that steroids act via nongenomic mechanisms to maintain nonbreeding aggression in mammals and birds.

Furthermore, androgens and estrogens produced by the testes and ovaries are under neural influence via the hypothalamic-pituitary-gonadal axis, or HPG axis. Within the HPG axis, the hypothalamus integrates neuroendocrine input and environmental stimuli to regulate the release of gonadotropin-releasing hormone (GnRH). In turn, GnRH secretion stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland, which then stimulate gonadal secretion of sex steroids. Circulating sex steroids reach virtually every cell in the body, including cells in the central nervous system, where they can bind to sex steroid receptors and regulate cells in the SBN and SDM.

In addition, the brain itself is capable of locally synthesizing sex steroids. The brain possesses the necessary enzymes for steroidogenesis and can synthesize active sex steroids called neurosteroids, either from inert androgen precursors or de novo from cholesterol (Fig. 1). Neurosteroids can regulate behavior via actions on the SBN and SDM. For example, in male song sparrows, aromatase, the enzyme that converts androgens into estrogens, is critical for the expression of aggression in the breeding season, but also in the nonbreeding season when circulating sex steroids are nondetectable. Several brain regions, such as the POA, VMH, and BnST, contain elevated levels of aromatase year-round. In contrast, aromatase activity decreases in the nucleus taeniae, which is similar to the mammalian medial amygdala, during the molt, when song sparrows also show a decrease in aggressive behaviors. Similarly, the activity of brain 3 β -hydroxysteroid dehydrogenase/ Δ 5- Δ 4 isomerase (3 β -HSD), an enzyme that catalyzes the conversion of DHEA to an active androgen, changes with season and social context. In song sparrows, 3 β -HSD activity is higher during the nonbreeding season in multiple brain areas, and an aggressive social stimulus rapidly increases enzymatic activity in the brain. Together, these data provide evidence of neural steroidogenesis and suggest that local neurosteroid production occurs predominantly during the nonbreeding season.

Androgens and Primate Aggression

Aggression has been a central topic of human and nonhuman primate research for many years. Considerable research indicates the importance of environmental and social influences on the development and expression of primate aggressive behavior. Dominance is the most well-known social influence on aggression, and dominance hierarchies are a major organizing principle in many primate societies. Although the hormonal mechanisms of primate aggression have been studied, much less is known compared with rodents. Most recent studies on the neuroendocrine regulation of primate aggression measure androgens in feces or urine. While collection of such samples can be performed noninvasively, excreted levels frequently represent a fraction of plasma levels. In general, high rates of aggression are positively correlated with elevated androgen concentrations in nonhuman primates. This correlation is observed in seasonal changes in androgens and aggression, sex differences in aggression, and increased aggression at puberty.

Many primates reproduce seasonally and have provided information on correlations between androgens and aggression. Seasonality in testicular activity, with peak circulating T concentrations during the mating season, has been reported in many primates. Although exceptions exist, the majority of research suggests that androgens do not necessarily cause aggression in nonhuman primates. In Japanese macaques, seasonal increases in circulating androgens precede seasonal increases in aggression by several months, suggesting that these hormones do not affect aggression in a simple causal way. Further, experimental elevations of T do not reliably increase aggression. In rhesus monkeys, injections of human chorionic gonadotropin stimulated T production, but had no consistent effect on rates of aggression. Rather, increased circulating T levels were associated with an intensification of existing behavior, which did not disrupt group stability. In contrast, injections of T propionate increased aggression in dominant male long-tailed macaques (*M. fascicularis*), but increased submission in subordinates. When male marmosets (*Callithrix jacchus*) were castrated as neonates and tested as adults, they displayed high rates of aggression with female partners and low rates with

male partners. Moreover, the effects of neonatal castration on aggressive behavior were reversed by T treatment in adulthood. Several studies have found that castration of adult males has little effect on their aggressive behavior, suggesting that gonadal T is not required to maintain aggression in adults.

Some studies in nonhuman primates also suggest that aggressive behavior can be unrelated to fecal T levels, but related to adrenal androgens. Although the adrenal gland can secrete high levels of DHEA and DHEA-S, the role of adrenal DHEA and DHEA-S in primate aggression is largely unknown. One study assessed circulating DHEA-S levels in a population of wild baboons and found high DHEA-S concentrations in both male and female baboons, showing marked age-related decreases in both sexes. DHEA-S levels were not compared with aggressive behaviors, however.

Although the study of DHEA as a regulator of aggression in humans has received some limited experimental attention, much less is known about this mechanism compared to nonprimates. However, alterations in DHEA and DHEA-S have been implicated in a range of psychiatric disorders in humans. Circulating levels of DHEA-S are generally 1000-fold higher than levels of DHEA in humans. Studies from rodents and birds suggest that androgens, such as DHEA, may regulate aggression in situations where aggression seems otherwise T-independent. Adrenal DHEA appears to play a role in human aggression, as indicated by studies on "conduct disorder," typically defined as a collection of symptoms, including aggression directed toward people or animals, destruction of property, theft, and serious violations of rules. Prepubertal boys with conduct disorder were found to have higher levels of plasma DHEA-S, but not T, than normal control boys. Also, DHEA-S concentrations were correlated with the intensity of aggression as rated by parents and teachers. In another study, plasma DHEA-S concentrations were found to be higher in boys with conduct disorder than in boys with attention-deficit/hyperactivity disorder (ADHD) or normal controls. Recent research has examined circulating levels of cortisol, DHEA and DHEA-S in delinquent adolescent boys diagnosed with conduct disorder compared with healthy controls. Hormone levels were correlated with aggression as determined by the Child Behavior Checklist and the Overt Aggression Scale. Delinquent boys had higher DHEA-S levels than control boys, but did not show any differences in either DHEA or cortisol. Collectively, these data suggest a relationship between DHEA-S and aggression, at least in male adolescents diagnosed with clinically-relevant psychiatric conditions.

Adrenal androgen precursors may also contribute to the regulation of aggression in human females. Adolescent and adult females with congenital adrenal hyperplasia who were exposed to high levels of adrenal androgen precursors in the prenatal and early postnatal periods were found to have greater self-reported aggression ratings than were control females. More recently, adrenal androgen precursor levels have been determined in adolescent girls diagnosed with conduct disorder. Specifically, blood samples were drawn from adolescent girls with either conduct disorder or no psychiatric disorder, and samples were assessed for DHEA, DHEA-S, cortisol, and gonadal androgens and estrogens. Girls with conduct disorder scored higher on a clinical aggression scale and demonstrated significantly lower cortisol to DHEA ratios, but did not differ from control girls on any other hormone measurement. Furthermore, girls diagnosed with aggressive conduct disorder had lower cortisol to DHEA ratios than those with nonaggressive conduct disorder.

In addition to regulating aggression in adolescents, DHEA also modulates aggressive behaviors in adults. In a study of alcohol withdrawal, serum levels of DHEA-S and cortisol, as well as DHEA-S response to treatment with an exogenous steroid, dexamethasone, were determined in adult alcohol-dependent or healthy control males. Alcohol-dependent subjects displayed reduced basal and dexamethasone-induced levels of DHEA-S compared with control subjects in late alcohol withdrawal. When alcohol-dependent subjects were separated into high and low aggression groups, lower basal DHEA-S levels were seen only during early alcohol withdrawal in high aggression individuals, whereas DHEA was lower only during late withdrawal in low aggression individuals relative to control subjects. In contrast, dexamethasone-induced decreases in DHEA-S were observed during both early and late alcohol withdrawal, whereas lower DHEA levels were only seen during early withdrawal. While the meaning of these results is not entirely clear, these data suggest an important link between DHEA and aggressive behavior, at least under conditions of drug withdrawal. Whether a link between DHEA, DHEA-S and aggressive behavior exists in healthy adult and adolescent males and females remains to be determined. Regardless, it is clear that considerably more research on DHEA and human aggression is needed.

Conclusions

Aggression is displayed by virtually all organisms and allows males and females to compete for access to limited resources. Many studies have examined the endocrine and neural regulation of aggression, and these studies highlight the importance of steroid hormones, such as testosterone, and brain circuits, such as the social behavior network. Aggression occurs in a wide variety of physiological and environmental contexts, and the neuroendocrine regulation of aggression varies across contexts as well. For example, in the breeding season, gonadal sex steroids play important roles in the modulation of aggressive behavior, but in the nonbreeding season, neurally-synthesized sex steroids are critical. Furthermore, sex steroids affect aggressive behavior via both genomic and nongenomic mechanisms, and the balance between these two mechanisms of action depends on the physiological and environmental contexts. Despite the variability of aggressive behaviors across species, the relevant neural circuits and neuroendocrine mechanisms are generally well conserved. Therefore, comparative studies in different vertebrate taxa can shed light on common mechanisms and establish general principles in the control of aggression.

Further Reading

- Adkins-Regan, E. (2005). *Hormones and Animal Social Behavior* (1st Edn.). Princeton: Princeton University Press.
- Demas, G. E., Polacek, K. M., Durazzo, A., & Jasnow, A. M. (2004). Adrenal hormones mediate melatonin-induced increases in aggression in male Siberian hamsters (*Phodopus sungorus*). *Hormones and Behavior*, 46, 582–591.

- Goldman, B. D. (2001). Mammalian photoperiodic system: Formal properties and neuroendocrine mechanisms of photoperiodic time measurement. *Journal of Biological Rhythms*, 16, 283–301.
- Goodson, J. L., & Kabelik, D. (2009). Dynamic limbic networks and social diversity in vertebrates: From neural context to neuromodulatory patterning. *Frontiers in Neuroendocrinology*, 30, 429–441.
- Heimovics, S. A., Trainor, B. C., & Soma, K. K. (2015). Rapid effects of estradiol on aggression in birds and mice: the fast and the furious. *Integrative and Comparative Biology*, 55(2), 281–293.
- Jasnow, A. M., Huhman, K. L., Bartness, T. J., & Demas, G. E. (2000). Short-day increases in aggression are inversely related to circulating testosterone concentrations in male Siberian hamsters (*Phodopus sungorus*). *Hormones and Behavior*, 38, 102–110.
- King, J. A. (1973). The ecology of aggressive behavior. *Annual Review of Ecology and Systematics*, 4, 117–138.
- Laredo, S. A., Landeros, R. V., & Trainor, B. C. (2014). Rapid effects of estrogens on behavior: Environmental modulation and molecular mechanisms. *Frontiers in Neuroendocrinology*, 35(4), 447–458.
- Maney, D. L., & Goodson, J. L. (2011). Neurogenomic mechanisms of aggression in songbirds. *Advances in Genetics*, 75, 83–119.
- Moyer, K. E. (1968). Kinds of aggression and their physiological basis. *Communications in Behavioral Biology*, 2, 65–87.
- Nelson, R. J. (2005). *Biology of Aggression* (1st ed.). New York: Oxford University Press.
- Newman, S. W. (1999). The medial extended amygdala in male reproductive behavior: a node in the mammalian social behavior network. *Annals of the New York Academy of Sciences*, 877, 242–257.
- O'Connell, L. A., & Hofmann, H. A. (2011). The vertebrate mesolimbic reward system and social behavior network: a comparative synthesis. *The Journal of Comparative Neurology*, 519, 3599–3639.
- Quiring, D. P. (1944). Transplantation of testes (by A. A. Berthold). *Bulletin of the History of Medicine*, 16, 399–401.
- Rose, R. M., Gordon, T. P., & Bernstein, I. S. (1972). Plasma testosterone levels in the male rhesus: Influences of sexual and social stimuli. *Science*, 178, 643–645.
- Soma, K. K., Rendon, N. M., Boonstra, R., Albers, H. E., & Demas, G. E. (2015). DHEA effects on brain and behavior: Insights from comparative studies of aggression. *The Journal of Steroid Biochemistry and Molecular Biology*, 145, 261–272.
- Svare, B. B. (1983). *Hormones and Aggressive Behavior* (1st Edn.). New York: Plenum.
- Van Goozen, S. H. M., Matthys, W., Cohen-Kettenis, P. T., Thijssen, J. H. H., & van Engeland, H. (1998). Adrenal androgens and aggression in conduct disorder prepubertal boys and normal controls. *Biological Psychiatry*, 43(2), 156–158.
- Wingfield, J. C., Ball, G. F., Dufty, A. M., Hegner, R. E., & Ramenofsky, M. (1987). Testosterone and aggression in birds. *American Scientist*, 75, 602–608.
- Wingfield, J. C., Hegner, R. E., Dufty, A. M., Jr., & Ball, G. F. (1990). The "challenge hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *The American Naturalist*, 136(6), 829–846.

MALE REPRODUCTIVE TRACT

Male Reproductive Tract: Development Overview

Diya B Joseph and Chad M Vezina, University of Wisconsin-Madison, Madison, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Human male reproductive tract development is described in this chapter. The male reproductive tract consists of organs involved in the storage, maintenance and transportation of male reproductive cells. The major structures of the male reproductive tract originate from the endoderm-derived cloaca and the mesoderm-derived Wolffian duct. Male reproductive tract and urinary system development are closely linked and together their structures comprise the urogenital system. All human developmental ages mentioned in this chapter are counted from the day of fertilization.

Developmental Origins of the Male Reproductive Tract

Gastrulation gives rise to three germ layers: the endoderm, ectoderm and mesoderm. Male reproductive tract development begins early in gestation and involves cells from all three germ layers (Fig. 1). The cloaca is a transient pouch-like structure at the terminal

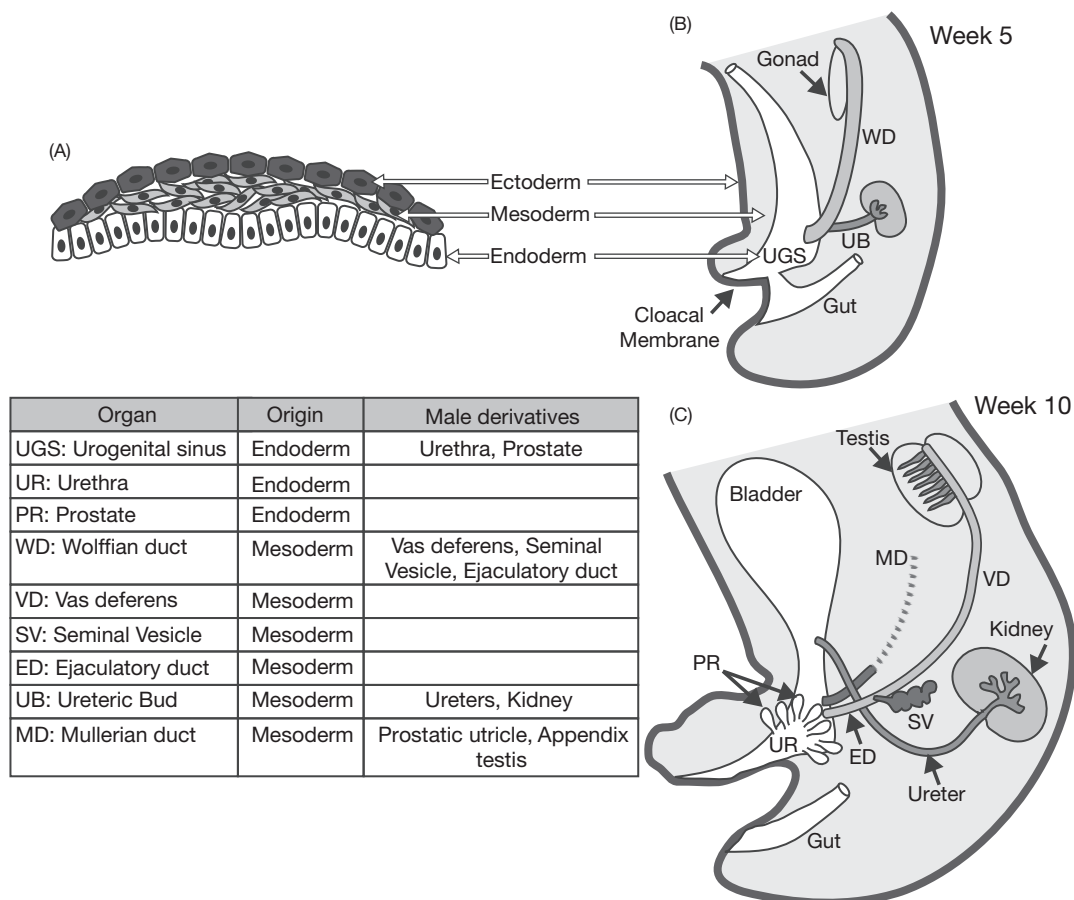


Fig. 1 Germ cell layer of origin of male reproductive tract structures. (A) Cross sectional view of a gastrulated embryo showing the three germ layers: endoderm (white), mesoderm (light gray) and ectoderm (dark gray). (B) Sagittal view of the male reproductive tract in a week 5 embryo during the bi-potential stage. (C) Sagittal view of the male reproductive tract in a week 10 embryo after the onset of sexual differentiation. Structures of the reproductive tract are colored according to germ layer of origin.

portion of the endoderm-derived hindgut in the early embryo. The cloaca (Latin for “sewer”) differentiates into the urogenital sinus, bladder, urethra and prostate in males. Paired epithelial Wolffian ducts derive from the mesoderm and insert into the cloaca. Testicular factors including testosterone masculinize male reproductive structures and drive cellular differentiation within them. The Wolffian duct gives rise to the epididymis, vas deferens, seminal vesicle and ejaculatory duct. In the mature adult, sperm from the testes are transported through the epididymis, vas deferens and ejaculatory duct to the urethra. At the urethra, the seminal vesicle and prostate contribute secretions to the ejaculate that promote sperm health. The urethra is the conduit for deposition of sperm during reproduction (Moore and Persaud, 2003).

Urogenital Sinus, Urethra, and Prostate

Growth and re-positioning of mesenchymal tissue divides the cloaca into the urogenital sinus ventrally and the anorectal sinus dorsally by week 7 of gestation.

Urogenital sinus

The ventral portion of the cloaca gives rise to the urogenital sinus (UGS). The UGS is a transient structure comprised of a simple epithelial tube featuring a balloon-shaped central cavity (Fig. 1). Paired Wolffian ducts insert into the UGS around week 4 of gestation. The UGS is divided into three parts:

- The cranial or upper part of the UGS differentiates into the urinary bladder.
- The middle or pelvic portion develops into the pelvic urethra and prostate.
- The caudal or lower part develops into the phallic urethra.

Urethra

The urethra develops from the middle and caudal regions of the UGS. The pelvic urethra extends from the bladder to the body wall. The phallic urethra extends from the body wall to the tip of the external genitalia. Urethral glands (Littre’s glands) and bulbourethral glands (Cowper’s glands) emerge as urethral outgrowths around week 12 of gestation.

Lineage tracing studies following the fate of endodermal cells within the developing urogenital tract show that the entire urethral epithelium derives from endoderm (Seifert et al., 2008). The developing urethra forms a stratified epithelium comprised of basal, intermediate, and superficial layers. Epithelial–mesenchymal interactions are crucial for urethral morphogenesis. Sonic hedgehog (SHH) peptide is secreted from epithelium and activates GLI transcription factors in nearby mesenchyme. GLI transcription factors drive bone morphogenetic protein 4 (BMP4) transcription and mesenchymal differentiation. Molecular mapping studies in the mouse embryo reveal a multilayered pelvic urethral mesenchyme consisting of lamina propria, muscularis mucosa, submucosa, and muscularis propria (Abler et al., 2011). Male urethra morphogenesis is guided by androgens, principally by androgen-induced signals from the male urethral mesenchyme that drive urethral epithelium differentiation and remodeling.

Prostate

The prostate is a male accessory sex gland positioned at the base of the bladder and surrounding the pelvic urethra. Prostate secretions contribute to the ejaculate and promote sperm health. Prostate ductal development initiates in utero as solid epithelial buds deriving from UGS epithelium. The epithelial buds elongate, branch, and canalize to form a complex ductal system draining into the urethral lumen. Most studies on early prostate development have been carried out in rodents. The early development program of prostate budding is remarkably conserved between rodents and humans, even though their prostates differ anatomically at sexual maturity.

In the human fetus, prostate development occurs in response to testosterone production by testicular Leydig cells around week 7 of gestation. Testosterone acts on androgen receptor (AR)-expressing UGS mesenchymal cells. AR activation increases the abundance of UGS mesenchymal steroid 5 alpha reductase type 2 (SRD5A2). SRD5A2 converts testosterone to the more potent dihydrotestosterone (DHT). DHT binding to AR amplifies androgen signaling in the UGS mesenchyme, which evokes paracrine signaling mechanisms that instruct UGS epithelium to form prostatic buds. Prostate bud outgrowth begins around week 10 of gestation and continues until week 24.

Studies in mice have shown that prostatic bud number and location are precisely controlled. The Nk-3 transcription factor locus-1 (NKX3-1) is the earliest marker of prostate specified UGS epithelial cells. Although prostatic bud formation cannot occur in the absence testosterone, several other factors including SHH, SOX9, HOXB13, and WNT5A are also required for prostatic bud formation and subsequent prostate ductal development.

Prostate formation is dependent on complex epithelial–mesenchymal interactions. Tissue recombination experiments have shown that androgen signaling deriving from UGS mesenchyme not UGS epithelium directs prostate bud formation. However, epithelial AR is required for prostate epithelial cell differentiation and secretory protein production. UGS mesenchyme is organized into distinct zones, and some zones serve as signaling centers to guide prostate morphogenesis. UGS mesenchymal condensations (mesenchymal pads) lie on the UGS periphery, are characterized by *FGF10* mRNA expression, and guide directional outgrowth of prostatic buds (Thomson and Cunha, 1999). Epigenetic mechanisms including DNA methylation and histone acetylation regulate expression of key genes involved in prostate development. DNA methylation controls E-cadherin (CDH1) and AR abundance,

which in turn control prostate bud elongation and timing of bud formation respectively (Keil et al., 2014a,b). Histone acetylation controls BMP2 expression to regulate prostatic ductal branching (Keil et al., 2015).

In later stages, the prostate undergoes branching, canalization and differentiation to form a pseudo-stratified epithelium consisting of basal cells, secretory luminal cells and rare neuroendocrine cells. The prostate increases in size following an upsurge in testosterone production during puberty. The study of early prostate development is receiving renewed interest as the reawakening of embryonic processes has been implicated in the pathogenesis of prostate cancer and benign prostatic hyperplasia.

Rete Testis, Epididymis, Vas Deferens, Seminal Vesicle

The intermediate mesoderm lies between paraxial and lateral plate mesoderm of the fetus and gives rise to ductal structures of the urogenital system. Crests of intermediate mesoderm called urogenital ridges form near the midline and along the cranio-caudal body axis. Early in week 4 of gestation, a non-functional, transient excretory organ called the pronephros develops within the urogenital ridge at the position of the thorax. The rudimentary tubular structures of the pronephros feed into the pronephric duct, which joins the cloaca at approximately week 4 of gestation. The pronephric duct is formed by mesenchymal to epithelial transition of intermediate mesoderm. The pronephros undergoes degeneration through an apoptotic program. The mesonephros forms caudal to the degenerating pronephros late in week 4 of gestation. The mesonephros contains glomeruli and mesonephric tubules that function as temporary kidneys until week 10 of gestation, when the metanephros permanently assumes kidney function. The mesonephric tubules open into mesonephric ducts (also known as Wolffian ducts) which drain into the cloaca. The mesonephros degenerates in the cranial to caudal direction around week 8, leaving a few residual tubules that will give rise to efferent ductules of the testes (Rao and Burnett, 2013).

The Wolffian ducts are precursors for ductal structures of the male reproductive and urinary tracts. The ureteric bud emerges as a Wolffian duct outgrowth near its insertion into the cloaca (Fig. 1). The ureteric bud undergoes branching and differentiation within a specialized mesenchyme called the metanephric blastema to form the metanephros or permanent kidneys. The ureteric bud is also the precursor for the ureters, which connect the kidneys to the bladder. The caudal portion of the Wolffian duct between the ureteric bud and the insertion site into the UGS is called the common nephric duct. Common nephric duct apoptosis positions the ureters at their final insertion site within the bladder, spatially separating the ureteral and Wolffian duct openings to the lower urinary tract. Ureter separation from the Wolffian duct is completed by week 7 of gestation. Paired box 2 (PAX2) expression marks the intermediate mesoderm early after gastrulation. Lineage tracing studies show that the PAX2 expressing intermediate mesoderm gives rise to Wolffian ducts, the ureteric bud and the metanephric blastema (Bouchard et al., 2002).

During the ambisexual stage, two sets of paired genital ducts are present in male and female embryos. The paramesonephric or Müllerian ducts extend in the craniocaudal direction and lie lateral to the Wolffian ducts. Müllerian duct formation occurs between week 6 and 7 of gestation. Lineage tracing studies in chick and mouse models demonstrate that Müllerian ducts develop from the coelomic epithelial layer, which derives from lateral plate mesoderm. Although Wolffian ducts lie in close apposition to and stimulate Müllerian duct formation, the Wolffian ducts do not contribute epithelial cells to the Müllerian ducts (Guioli et al., 2007; Orvis and Behringer, 2007).

Sexual differentiation of the male reproductive tract begins around week 7 of gestation. Müllerian inhibiting substance (MIS) produced by Sertoli cells and testosterone produced by the interstitial Leydig cells of the fetal testis act on the reproductive tract to induce male differentiation. MIS is a glycoprotein of the TGF- β family of growth factors which causes irreversible regression of the Müllerian ducts in males. Testicular MIS production commences by week 8 of gestation and Müllerian duct regression occurs between weeks 8 and 10. The prostatic utricle near the UGS and the appendix testis near the male gonads are Müllerian duct remnants in males. Female reproductive structures including the uterus, vagina and oviducts form in the absence of MIS. Testosterone-mediated AR activation supports Wolffian duct survival in males. Reproductive tract structures derived from the Wolffian ducts are formed between week 9 and 13 of gestation. Regional expression of homeobox (HOX) genes drives segmental differentiation of the Wolffian duct into the epididymis, vas deferens and seminal vesicle. The Wolffian ducts regress in females due to insufficient testosterone to support cell survival (Rao and Burnett, 2013).

Rete testis

After the mesonephros undergoes regression, the remaining mesonephric tubules form the efferent ductules. The seminiferous tubules, which contain sperm cells, are connected to the efferent ductules by a maze-like network of interconnecting tubes called the rete testis. The ciliated cells lining the rete testis guide sperm into the efferent ductules.

Epididymis

The efferent ductules, which receive sperm from the testis, drain into the epididymis. The epididymis is a convoluted series of tubules that derives from the portion of the Wolffian duct adjacent to the testis. The epididymis stores and transports sperm.

Vas deferens

The medial Wolffian duct segment forms the vas deferens. Androgens drive Wolffian duct differentiation into the vas deferens around week 12 of gestation. Smooth muscle surrounding the vas deferens contracts during ejaculation to propel sperm from the epididymis to the urethra.

Seminal vesicle

The seminal vesicles develop as lateral outgrowths from the caudal Wolffian duct segment. The seminal vesicles form around week 10 of gestation, after the onset of testosterone synthesis by the fetal testis. Seminal vesicles contribute secretions to the ejaculate. The most caudal Wolffian duct portion, positioned between the seminal vesicle and the urethra, is called the ejaculatory duct. Ejaculatory ducts drain the contents of the seminal vesicle and vas deferens into the urethra.

External Genitalia

Internal fertilization requires specialized male and female external reproductive organs. Lateral plate mesoderm, endoderm, and surface ectoderm cells contribute to the external genitalia. The initial phase of external genitalia development is essentially the same in males and females. In the later phase, androgens drive masculinization of the external genitalia in males (Blaschko et al., 2012; Yamada et al., 2003).

Early phase: Formation of ambisexual external genitalia

The early phase of external genitalia development, which occurs between 4 and 7 weeks of gestation, is essentially the same in males and females. This phase occurs independently of androgen action as it happens before the onset of testicular testosterone production. The cloacal membrane, formed by direct contact of the endoderm and surface ectoderm, is intact at 4 weeks of gestation. Proliferating lateral plate mesenchymal cells form paired lateral swellings above the cloacal membrane. These swellings fuse at the midline to form the genital tubercle. The genital tubercle is a bi-potential structure which is the precursor of the penis in males and clitoris in females. Paired mesenchymal swellings called urogenital folds and labio-scrotal swellings form on either side of the cloacal membrane. After cloacal septation around week 7, the cloacal membrane becomes the urogenital membrane ventrally and the anal membrane dorsally. The cloacal membrane ruptures at two sites to form the urethral orifice and the anal opening. The urogenital membrane is bounded by urogenital folds and lies within a temporary indentation on the ventral genital tubercle surface called the urethral groove. The urethral groove is lined by a solid cord of endodermal cells comprising the urethral plate epithelium. The urethral plate epithelium is the region of the phallic urethra distal to the UGS.

Studies in mice have shown that early patterning of the genital tubercle does not depend on androgens but does require Sonic hedgehog (SHH) signaling. Mice harboring inactivating mutations in the SHH gene fail to form external genitalia. SHH signaling from the urethral plate epithelium coordinates cell movements during external genitalia development (Perriton et al., 2002).

Later phase: Sexual differentiation of the external genitalia

Male testicular androgens induce genital tubercle differentiation into the penis. In females, the genital tubercle fails to elongate and forms the clitoris in the absence of androgens. Testosterone synthesis begins by week 7 and maximal concentrations in the fetus are achieved between weeks 10 and 15 of gestation. Early signs of sexual differentiation in the external genitalia can be detected by week 9 and complete differentiation is achieved by weeks 12–13.

The steroid hormone dihydrotestosterone (DHT) masculinizes the external genitalia. Testosterone is converted to DHT by the action of steroid metabolizing enzymes like SRD5A2 which are expressed in genital tubercle mesenchymal cells. DHT initiates androgen signaling in androgen receptor expressing mesenchymal cells. Interactions between androgen activated mesenchyme and urethral plate epithelium initiate male external genitalia differentiation.

Androgen exposure elongates the genital tubercle into the penis. Early in sexual differentiation, the proximal phallic urethra is a closed hollow tube but the distal portion comprising the urethral plate epithelium remains a solid mass of cells. The urogenital folds lining the penile ventral surface guide midline fusion of the urethral plate, forming a hollow urethral tube that extends the whole length of the penis. The fusion of urogenital folds occurs in a proximal to distal direction, positioning the urethral orifice at the tip of the penis. Hypospadias, a common birth defect, occur from defective fusion of urogenital folds. Specialized mesenchyme induces the differentiation of the distal urethral into a stratified squamous epithelium. The labio-scrotal swellings fuse in the midline to form the scrotum. The skin covering the developing penis is derived from surface ectoderm. Specialized structures of the penis, the corpus cavernosa and the corpus spongiosum, derive from the proliferation and differentiation of mesoderm derived cells.

The Genitourinary Development Molecular Anatomy Project (GUDMAP) website provides curated information on reproductive tract anatomy, histology, mRNA and protein expression over developmental time (www.gudmap.org).

Signaling Pathways in Male Reproductive Tract Development

Studies in rodents have greatly advanced our knowledge of signaling pathways involved in male reproductive tract development. Table 1 provides a parallel chronology of the major events in male reproductive tract development in human and mouse.

Sonic Hedgehog Signaling

Sonic hedgehog (SHH) peptide is a developmental morphogen secreted by epithelial cells. The secreted SHH peptide relieves the repression of smoothened (SMO) by binding to its inhibitor patched (PTC1). SMO activation initiates transcription of GLI transcription factors which are involved in several developmental and morphogenetic processes. SHH is expressed in

Table 1 Chronology of male reproductive tract development in human and mouse

Human	Internal reproductive tract	External genitalia	Mouse
Week 3	Gastrulation; cloacal membrane forms		E6-E6.5
Week 4	Nephrogenic cord forms pronephros forms cloaca develops from hindgut mesonephros forms Wolffian duct fuses with cloaca (day 26)	Genital tubercle forms	E8.5 E9 E9.5 E9.5–E11.5 E9.5 E11–E11.5
Week 5	Ureteric bud forms (day 28) Cloacal septation begins Common nephric duct apoptosis		E10.5–E11.5 E10.5 E11–E12
Week 6	Müllerian duct forms	Urogenital and labio-scrotal folds form	E12–E13 E14
Week 7	Cloacal septation complete Ureters join bladder Onset of testosterone synthesis	Cloacal membrane rupture	E13–E13.5 E13–E14 E13–E13.5
Week 8	Müllerian ducts reach UGS		E13.5
<i>Onset of sexual dimorphism</i>			
Week 9	Müllerian duct degeneration Wolffian duct differentiation		E16.5 E16.5–P1
Week 10	Müllerian duct degeneration complete Seminal vesicle forms Prostate forms Wolffian duct differentiation		E16.5 E16.5 E16.5–E18.5 E16.5–P1
Week 11	Wolffian duct differentiation		E16.5–P1
Week 12	Wolffian duct differentiation		E16.5–P1
Week 13		Urethral tube closure	E16.5
2nd Trimester		Growth of external genitalia Inguinal descent (week 23)	E15.5–P1 E15.5–E17.5
3rd Trimester		Growth of external genitalia Scrotal descent (weeks 24–34)	E15.5–P1 E17.5–P20

Provides a parallel chronology of male internal reproductive tract and external genitalia development in the human and mouse. Developmental age in humans is depicted here in weeks from the time of fertilization. Developmental age in the mouse embryo is depicted as days (E) from the time of fertilization.

hindgut-derived structures including the cloacal epithelium, where it patterns the surrounding mesenchyme. SHH induces mesenchymal GLI2, which regulates epithelial and mesenchymal proliferation and apoptosis during cloacal development. SHH regulates cloacal septation by promoting proliferation of mesenchymal cells in the urorectal septum (Seifert et al., 2009). SHH signaling is required for prostate formation and external genitalia development. Treatment with SHH inhibitors impairs prostate ductal growth and morphogenesis (Podlasek et al., 1999). SHH knockout mice show a complete absence of external genitalia (Haraguchi et al., 2001).

WNT-Beta Catenin Signaling

WNT ligand binding to cell surface Frizzled receptors stabilizes and activates the transcription factor beta-catenin. WNT signaling is involved in several aspects of urogenital development including the septation of the cloaca into the urogenital and anorectal sinuses. Disruption of WNT signaling results in rectourethral fistulas (abnormal connection between the urethra and rectum). Epithelial beta-catenin is also required for prostate development and growth (Mehta et al., 2013). The WNT-beta catenin pathway acts downstream of SHH signaling to regulate external genitalia development (Miyagawa et al., 2009).

Bone Morphogenetic Proteins

Bone morphogenetic proteins (BMPs) are growth factors belonging to the TGF-beta superfamily. BMPs bind to serine threonine kinase receptors and initiate intracellular signaling through SMAD proteins. BMP7 expression in the urorectal septum is required for cloacal septation. In addition, BMP7 expression maintains proliferation and cell survival in the cloacal epithelium (Xu et al., 2012). Expression of BMP7 in the UGS mesenchyme restricts prostate ductal budding and prevents excessive branching of elongating ducts (Grishina et al., 2005).

Fibroblast Growth Factors

Fibroblast growth factors are a family of secreted growth factors that signal through tyrosine kinase fibroblast growth factor receptors to regulate proliferation, differentiation and morphogenesis during embryonic development. FGF signaling is required for cell survival during the early stages of genital tubercle outgrowth. FGF signaling in the ectoderm is required for urethral tube formation. Deletion of FGF10 or its receptor FGFR2 results in severe hypospadias (Harada et al., 2015). FGFR2, a critical regulator of prostate development, is required for branching and growth of prostate buds. FGF10 is a paracrine mediator of epithelial to mesenchymal signaling during prostate bud formation. FGF10 knockout mice fail to form prostate, seminal vesicle, bulbourethral glands and caudal vas deferens (Thomson and Cunha, 1999).

PAX Genes

Paired box (PAX) genes are tissue specific transcription factors that determine lineage specification in the early embryo. PAX2 and PAX8 are required for Wolffian duct formation from the intermediate mesoderm. In PAX2 and PAX8 double mutant mice, the intermediate mesoderm fails to undergo the mesenchymal to epithelial transition required for Wolffian duct formation (Bouchard et al., 2002). GATA3, a downstream effector of PAX2, regulates Wolffian duct growth and caudal extension which is required for fusion with the cloaca and formation of definitive kidneys (Grote et al., 2006).

EPH Receptors/Ephrins

EPH receptors are a family of receptor tyrosine kinases with plasma membrane bound ligands called ephrins. EPH receptor/ephrins are involved in the maintenance of cell–cell adhesion and communication between similar or different cell types during developmental processes. The ephrin receptors EPHA4 and EPHA7 are expressed in the mesenchyme surrounding the cloaca and Wolffian duct where they mediate Wolffian duct fusion with the cloaca (Weiss et al., 2014). Signaling from EPHA4 and EPHB2 is required for apoptosis of the common nephric duct for proper separation of ureters from Wolffian ducts (Peuckert et al., 2016). EphrinB1 expressed by prostatic mesenchyme regulates prostate growth and branching (Ashley et al., 2010).

Vitamin A/Retinoic Acid Signaling

Retinoic acid, a derivative of vitamin A (retinol), binds to nuclear retinoic acid receptors to activate transcriptional programs for differentiation and organogenesis. The spatial expression of retinaldehyde dehydrogenases, which convert retinaldehyde to retinol, is tightly regulated in a tissue specific manner. Apoptosis induced by retinoic acid signaling is required for ureter separation from the Wolffian duct and proper positioning in the bladder (Batourina et al., 2005). Retinoic acid is a powerful inducer of prostate budding (Vezina et al., 2008a). Retinoic acid signaling regulates external genitalia formation by maintaining SHH and BMP4 expression in the genital tubercle (Liu et al., 2012).

Müllerian Inhibiting Substance

Müllerian inhibiting substance (MIS) or anti-Müllerian hormone (AMH) is a gonadal hormone secreted by Sertoli cells of the developing testis. The secreted glycoprotein MIS belongs to the TGF-beta family of transcription factors. MIS acts through AMH Type II receptors expressed by Müllerian duct mesenchyme to initiate apoptosis and degeneration of the Müllerian duct (Behringer, 1995; Abler et al., 2011).

Androgens, INSL3

Testosterone synthesis initiates from fetal Leydig cells during week 7 of gestation. Testosterone is converted to the more potent dihydrotestosterone (DHT) by the enzyme SRD5A2. DHT acts on AR expressing cells to initiate androgen-dependent transcriptional programs. DHT regulates prostate and seminal vesicle formation, external genitalia masculinization and formation of the vas deferens and epididymis. The Leydig cell specific insulin-like peptide INSL3 binds to relaxin/insulin like family peptide receptor 2 (RXFP2) to promote testicular descent into the scrotum (Barsoum and Yao, 2006).

Endocrine Disruptors

Environmental toxins with the capability of interfering with endocrine signaling are known as endocrine disruptors (Prusinski et al., 2016). In utero exposure to endocrine disruptors adversely affects hormone-dependent development and increases risk of adulthood disease. Maternal exposure to low doses of Bisphenol A, an estrogenic compound found in plastics, has been shown to increase prostate size in rodent models (Gupta, 2000; Dolinoy et al., 2007). Early exposure to Bisphenol A can also increase the risk of prostate cancer in rodent models of estrogen-induced carcinogenesis by inducing long-term changes to the DNA methylome (Cheong et al., 2016). The anti-androgenic endocrine disruptor vinclozolin found in fungicides, can induce

hypospadias in mice (Buckley et al., 2006). In utero exposure to persistent environmental pollutants called 2,3,7,8 tetrachlorodibenzo-*p*-dioxin impairs reproductive function of male and female rodents (Gray and Ostby, 1995; Bjerke and Peterson, 1994). In utero dioxin exposure disrupts mouse prostate formation (Vezina et al., 2008b) and sensitizes mice to hormone-mediated urinary dysfunction (Ricke et al., 2016).

Congenital Anomalies of the Male Reproductive Tract

Congenital anomalies of the male reproductive tract can reduce fertility. Hypospadias are the most common congenital anomaly of the male reproductive tract, with an occurrence of 1 in 250 live male births. Hypospadias occur when the urethral opening is not at the tip of the penis, but instead on the ventral surface or scrotal region. Defects in urethral tube closure result in hypospadias (Baskin and Ebbes, 2006). Genetic, endocrine and environmental factors have been implicated in the occurrence of hypospadias. Epispadias, which occur when the urethral opening is on the dorsal surface of the penis, is a much rarer condition (affecting 1 in 117,000 males) (Gearhart and Jeffs, 1992). Unlike hypospadias, epispadias result from defects in the cloacal membrane (Suzuki et al., 2017). Another congenital anomaly called chordee is associated with increased curvature of the penis. A congenital condition called posterior urethral valves is associated with the occurrence of flaps of urethral tissue that obstruct urine flow and impair reproductive function (Agarwal, 1999).

Persistent Müllerian duct syndrome is a rare anomaly of the reproductive tract in which Müllerian duct derivatives (uterus and oviduct) persist in males. The persistence of Müllerian duct derivatives can be due to insufficient production of Müllerian inhibiting substance (MIS) or insensitivity of the Müllerian duct to MIS (Elias-Assad et al., 2016).

Congenital anomalies of Wolffian duct derivatives include ectopic insertion of the ureter into the urethra, seminal vesicle, ejaculatory duct or vas deferens. Ectopic ureters are a result of abnormal ureteric bud formation and abnormal separation from the Wolffian duct during kidney development. Ectopic ureter insertion into the seminal vesicle results in the development of congenital seminal vesicle cysts. Seminal vesicle anomalies on their own do not contribute to male infertility. However, these defects are often observed with other Wolffian duct defects that affect fertility (Kroovand and Perlmutter, 1981).

Defects in vas deferens development are a major cause of male infertility. Congenital bilateral absence of the vas deferens results in male infertility from obstructive azoospermia (lack of sperm in semen). This condition is highly prevalent in males who have abnormal mucus production from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Abnormal mucus production in affected individuals results in obstruction and destruction of the vas deferens, leading to infertility in later life (Stuhrmann and Dork, 2000). Other congenital anomalies of Wolffian duct derivatives include agenesis of the epididymis, epididymal cysts with loss of continuity, agenesis of the seminal vesicle and agenesis of the ejaculatory duct.

References

- Abler, L. L., Keil, K. P., Mehta, V., Joshi, P. S., Schmitz, C. T., & Vezina, C. M. (2011). A high-resolution molecular atlas of the fetal mouse lower urogenital tract. *Developmental Dynamics*, 240, 2364–2377.
- Agarwal, S. (1999). Urethral valves. *BJU International*, 84, 570–578.
- Ashley, G. R., Cathal Grace, O., Vanpoucke, G., & Thomson, A. A. (2010). Identification of Ephrinb 1 expression in prostatic mesenchyme and a role for Ephb–Ephrinb Signalling in prostate development. *Differentiation*, 80, 89–98.
- Barsoum, I., & Yao, H. H. (2006). The road to maleness: From testis to Wolffian duct. *Trends in Endocrinology and Metabolism*, 17, 223–228.
- Baskin, L. S., & Ebbes, M. B. (2006). Hypospadias: Anatomy, etiology, and technique. *Journal of Pediatric Surgery*, 41, 463–472.
- Batourina, E., Tsai, S., Lambert, S., Sprengle, P., Viana, R., Dutta, S., Hensle, T., Wang, F., Niederreither, K., McMahon, A. P., Carroll, T. J., & Mendelsohn, C. L. (2005). Apoptosis induced by vitamin A signaling is crucial for connecting the ureters to the bladder. *Nature Genetics*, 37, 1082–1089.
- Behringer, R. R. (1995). The Mullerian inhibitor and mammalian sexual development. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 350, 285–288. discussion 289.
- Bjerke, D. L., & Peterson, R. E. (1994). Reproductive toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in male rats: Different effects of in utero versus Lactational exposure. *Toxicology and Applied Pharmacology*, 127, 241–249.
- Blaschko, S. D., Cunha, G. R., & Baskin, L. S. (2012). Molecular mechanisms of external genitalia development. *Differentiation*, 84, 261–268.
- Bouchard, M., Souabni, A., Mandler, M., Neubuser, A., & Busslinger, M. (2002). Nephric lineage specification by Pax2 and Pax8. *Genes & Development*, 16, 2958–2970.
- Buckley, J., Willingham, E., Agras, K., & Baskin, L. S. (2006). Embryonic exposure to the fungicide Vinclozolin causes Virilization of females and alteration of progesterone receptor expression in vivo: An experimental study in mice. *Environmental Health*, 5, 4.
- Cheong, A., Zhang, X., Cheung, Y. Y., Tang, W. Y., Chen, J., Ye, S. H., Medvedovic, M., Leung, Y. K., Prins, G. S., & Ho, S. M. (2016). DNA Methylome changes by estradiol benzoate and bisphenol A links early-life environmental exposures to prostate cancer risk. *Epigenetics*, 11, 674–689.
- Dolinoy, D. C., Huang, D., & Jirtle, R. L. (2007). Maternal nutrient supplementation counteracts bisphenol A-induced DNA Hypomethylation in early development. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 13056–13061.
- Elias-Assad, G., Elias, M., Kanety, H., Pressman, A., & Tenenbaum-Rakover, Y. (2016). Persistent Mullerian duct syndrome caused by a novel mutation of an anti-Mullerian hormone receptor gene: Case presentation and literature review. *Pediatric Endocrinology Reviews*, 13, 731–740.
- Gearhart, J. P., & Jeffs, R. D. (1992). Exstrophy of the bladder, Epispadias, and other bladder anomalies. In P. C. Walsh, A. B. Retik, T. A. Stamey, & E. D. Vaughan, Jr. (Eds.) (6th ed), vol. 2. *Campbell's urology*. Philadelphia: W.B. Saunders Co.
- Gray, L. E., Jr., & Ostby, J. S. (1995). In utero 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. *Toxicology and Applied Pharmacology*, 133, 285–294.
- Grishina, I. B., Kim, S. Y., Ferrara, C., Makarenkova, H. P., & Walden, P. D. (2005). Bmp7 inhibits branching morphogenesis in the prostate gland and interferes with notch signaling. *Developmental Biology*, 288, 334–347.

- Grote, D., Souabni, A., Busslinger, M., & Bouchard, M. (2006). Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. *Development*, 133, 53–61.
- Guioli, S., Sekido, R., & Lovell-Badge, R. (2007). The origin of the Mullerian duct in Chick and Mouse. *Developmental Biology*, 302, 389–398.
- Gupta, C. (2000). Reproductive malformation of the male offspring following maternal exposure to estrogenic chemicals. *Proceedings of the Society for Experimental Biology and Medicine*, 224, 61–68.
- Harada, M., Omori, A., Nakahara, C., Nakagata, N., Akita, K., & Yamada, G. (2015). Tissue-specific roles of Fgf signaling in external genitalia development. *Developmental Dynamics*, 244, 759–773.
- Haraguchi, R., Mo, R., Hui, C.-C., Motoyama, J., Makino, S., Shiroishi, T., Gaffield, W., & Yamada, G. (2001). Unique functions of sonic hedgehog signaling during external genitalia development. *Development*, 128, 4241–4250.
- Keil, K. P., Abler, L. L., Laporta, J., Altmann, H. M., Yang, B., Jarrard, D. F., Hernandez, L. L., & Vezina, C. M. (2014a). Androgen receptor DNA methylation regulates the timing and androgen sensitivity of mouse prostate ductal development. *Developmental Biology*, 396, 237–245.
- Keil, K. P., Abler, L. L., Mehta, V., Altmann, H. M., Laporta, J., Plisch, E. H., Suresh, M., Hernandez, L. L., & Vezina, C. M. (2014b). DNA methylation of E-cadherin is a priming mechanism for prostate development. *Developmental Biology*, 387, 142–153.
- Keil, K. P., Altmann, H. M., Abler, L. L., Hernandez, L. L., & Vezina, C. M. (2015). Histone acetylation regulates prostate ductal morphogenesis through a bone morphogenetic protein-dependent mechanism. *Developmental Dynamics*, 244(11), 1404–1414.
- Kroovand, R. L., & Perlmutter, A. D. (1981). Congenital anomalies of the vas deferens and epididymis. In S. J. Kogan, & E. S. E. Hafez (Eds.), *Pediatric Andrology*. Dordrecht, Netherlands: Springer.
- Liu, L., Suzuki, K., Nakagata, N., Mihara, K., Matsumaru, D., Ogino, Y., Yashiro, K., Hamada, H., Liu, Z., Evans, S. M., Mendelsohn, C., & Yamada, G. (2012). Retinoic acid signaling regulates sonic hedgehog and bone morphogenetic protein Signaling during genital tubercle development. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, 95, 79–88.
- Mehta, V., Schmitz, C. T., Keil, K. P., Joshi, P. S., Abler, L. L., Lin, T. M., Taketo, M. M., Sun, X., & Vezina, C. M. (2013). Beta-catenin (Ctnnb1) induces bmp expression in urogenital sinus epithelium and participates in prostatic bud initiation and patterning. *Developmental Biology*, 376, 125–135.
- Miyagawa, S., Moon, A., Haraguchi, R., Inoue, C., Harada, M., Nakahara, C., Suzuki, K., Matsumaru, D., Kaneko, T., Matsuo, I., Yang, L., Taketo, M. M., Iguchi, T., Evans, S. M., & Yamada, G. (2009). Dosage-dependent hedgehog signals integrated with Wnt/B-catenin signaling regulate external genitalia formation as an appendicular program. *Development*, 136, 3969–3978.
- Moore, K. L., & Persaud, T. V. N. (2003). *The developing human: Clinically oriented embryology* (7th ed.). Philadelphia: Elsevier.
- Orvis, G. D., & Behringer, R. R. (2007). Cellular mechanisms of Müllerian duct formation in the mouse. *Developmental Biology*, 306, 493–504.
- Perriton, C. L., Powles, N., Chiang, C., Maconochie, M. K., & Cohn, M. J. (2002). Sonic hedgehog signaling from the urethral epithelium controls external genital development. *Developmental Biology*, 247, 26–46.
- Peuckert, C., Aresh, B., Holenya, P., Adams, D., Sreedharan, S., Porthin, A., Andersson, L., Pettersson, H., Wolf, S., Klein, R., Oxburgh, L., & Kullander, K. (2016). Multimodal Eph/Ephrin signaling controls several phases of urogenital development. *Kidney International*, 90, 373–388.
- Podlasek, C. A., Barnett, D. H., Clemens, J. Q., Bak, P. M., & Bushman, W. (1999). Prostate development requires sonic hedgehog expressed by the urogenital sinus epithelium. *Developmental Biology*, 209, 28–39.
- Prusinski, L., Al-Hendy, A., & Yang, Q. (2016). Developmental exposure to endocrine disrupting chemicals alters the epigenome: Identification of reprogrammed targets. *Gynecology and Obstetrics Research: Open Journal*, 3, 1–6.
- Rao, P. K., & Burnett, A. L. (2013). Development of the male reproductive system. In P. K. Kavoussi, R. A. Costabile, & A. Salonia (Eds.), *Clinical urologic endocrinology: Principles for Men's health*. London: Springer London.
- Ricke, W. A., Lee, C. W., Clapper, T. R., Schneider, A. J., Moore, R. W., Keil, K. P., Abler, L. L., Wynder, J. L., Lopez Alvarado, A., Beaubrun, I., Vo, J., Bauman, T. M., Ricke, E. A., Peterson, R. E., & Vezina, C. M. (2016). In utero and Lactational Tcd exposure increases susceptibility to lower urinary tract dysfunction in adulthood. *Toxicol. Sci*, 150(2), 429–440.
- Seifert, A. W., Harfe, B. D., & Cohn, M. J. (2008). Cell lineage analysis demonstrates an endodermal origin of the distal urethra and perineum. *Developmental Biology*, 318, 143–152.
- Seifert, A. W., Bouldin, C. M., Choi, K. S., Harfe, B. D., & Cohn, M. J. (2009). Multiphasic and tissue-specific roles of sonic hedgehog in cloacal Septation and external genitalia development. *Development*, 136, 3949–3957.
- Stuhrmann, M., & Dork, T. (2000). Cfr gene mutations and male infertility. *Andrologia*, 32, 71–83.
- Suzuki, K., Matsumaru, D., Matsushita, S., Murashima, A., Ludwig, M., Reutter, H., & Yamada, G. (2017). Epispadias and the associated Embryopathies: Genetic and developmental basis. *Clinical Genetics*, 91, 247–253.
- Thomson, A. A., & Cunha, G. R. (1999). Prostatic growth and development are regulated by Fgf10. *Development*, 126, 3693–3701.
- Vezina, C. M., Allgeier, S. H., Fritz, W. A., Moore, R. W., Strerath, M., Bushman, W., & Peterson, R. E. (2008a). Retinoic acid induces prostatic bud formation. *Developmental Dynamics*, 237, 1321–1333.
- Vezina, C. M., Allgeier, S. H., Moore, R. W., Lin, T. M., Bemis, J. C., Hardin, H. A., Gasiewicz, T. A., & Peterson, R. E. (2008b). Dioxin causes ventral prostate agenesis by disrupting Dorsoventral patterning in developing mouse prostate. *Toxicological Sciences*, 106, 488–496.
- Weiss, A. C., Airik, R., Bohnenpoll, T., Greulich, F., Folk, A., Trowe, M. O., Rudat, C., Costantini, F., Adams, R. H., & Kispert, A. (2014). Nephric duct insertion requires EphA4/EphA7 signaling from the Pericloacal mesenchyme. *Development*, 141, 3420–3430.
- Xu, K., Wu, X., Shapiro, E., Huang, H., Zhang, L., Hickling, D., Deng, Y., Lee, P., Li, J., Lepor, H., & Grishina, I. (2012). Bmp7 functions via a polarity mechanism to promote cloacal Septation. *PLoS One*, 7, e29372.
- Yamada, G., Satoh, Y., Baskin, L. S., & Cunha, G. R. (2003). Cellular and molecular mechanisms of development of the external genitalia. *Differentiation*, 71, 445–460.

Wolffian Duct Development

Barry T Hinton, University of Virginia School of Medicine, Charlottesville, VA, United States

Maria Christina W Avellar, Federal University of São Paulo-Paulista School of Medicine, São Paulo, Brazil

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cryptorchidism Failure of one or both testes to descend into the scrotum.

Epididymis An elongated cord-like structure along the posterior border of the testis; its coiled duct provides for storage, transit, and maturation of spermatozoa and is continuous with the vas deferens (ductus deferens).

E2f4/E2f5 Is a member of the E2F family of transcription factors that contain a series of conserved domains including a DNA binding domain, a dimerization domain, a transactivation domain, and a tumor suppressor protein associated domain embedded within the transactivation domain.

Emx2 Empty spiracles homeobox 2 gene, is a transcription factor.

Foxc1/Foxd1 Forkhead box gene, is a transcription factor.

Gata3 GATA-binding factor 3, transcription factor 3 that binds to the DNA sequence "GATA."

Lim1 Transcription factor that binds to the LIM domain, a cysteine-rich zinc binding domain.

Mesoderm Layer of cell within the developing embryo between the ectoderm and endoderm. Divides into: (1) Paraxial mesoderm that gives rise to bone, cartilage and dermis. (2) Intermediate mesoderm that gives rise to the reproductive tract, lower urinary tract and the kidney. (3) Lateral plate mesoderm that gives rise to adipose, blood, endothelium, heart, smooth muscle, and spleen.

Mesonephric duct The duct of the mesonephros that persists in the male as the duct system leaving the testis including the epididymis, vas deferens, seminal vesicle, and ejaculatory duct.

Pax2 Paired-box containing gene, is a transcription factor.

Six1 Sineoculis homeobox homolog 1, is a transcription factor.

Testosterone Male sex hormone produced by the Leydig cells within the testis responsible for maintaining male secondary sex characteristics.

Transcription factor Proteins involved in the process of transcribing DNA into messenger RNA by binding to a specific DNA sequence.

Wolffian duct Eponym for the mesonephric duct. So named after Caspar Friedrich Wolff, who described the ducts in chick embryos.

Wt-1 Wilms tumor-1 gene, is a transcription factor.

Migration of Wolffian Duct Cells and the Formation of the Duct

During development the embryonic mesoderm splits into three divisions, the paraxial mesoderm, the intermediate mesoderm, and the lateral plate mesoderm. The mesonephros, from which the Wolffian and Müllerian ducts are derived, is formed from the intermediate mesoderm. The mechanism(s) by which the Wolffian duct begins at the rostral end of the embryo and then elongates to join the cloaca, the primitive bladder, is/are not well known. However, studies using the chick embryo have shown that once cells have undergone specification in the rostral end of the embryo, they migrate toward the cloaca under the influence of a gradient of fibroblast growth factor 8 (FGF8) and its signaling transduction pathways ([Atsuta and Takahashi, 2015](#)). So long as the cells are kept surrounded by high concentrations of FGFs, they maintain a migratory mesenchymal-like phenotype. The cells that are more distal, and therefore removed from the high concentrations of FGF8 undergo a mesenchymal-epithelial transition and form a lumen. The manner by which the cells undergo specification at the beginning of this event is not known. Several transcription factors are also required for Wolffian duct formation and maintenance including *Gata3*, *Lim1*, *Pax2*, *Wt-1*, *Six1*, *Emx2*, and *Foxc1/Foxd1* (see reviews by [Murashima et al., 2015b](#); [McMahon, 2016](#)). Null mutations of each of these genes results in varying degrees of malformation of the Wolffian duct, for example, *Gata3* null mutant mice fail to complete migration to the cloaca ([Grote et al., 2006](#)). In addition to transcription factors, the surface ectoderm close to the mesonephros also plays a role during the formation of the mesonephric duct ([Obara-Ishihara et al., 1999](#)).

Induction of Mesonephric Tubules

As the mesenchymal cells migrate toward the cloaca, they induce the adjacent mesenchyme to form S- or J-shaped epithelial tubules, the mesonephric tubules. However, many regress and the remaining few, those that lie more cranially and are opposite the testis

eventually form the efferent ducts. However, studies by [Sainio et al. \(1997\)](#) suggest that the efferent ducts maybe derived, in part, by the mesonephric duct. The formation of the distinct arrangement of the efferent ducts is unclear but may involve mesonephric tubule branching morphogenesis and/or fusion of individual tubules. During this process it is clear that a specific number of efferent ducts and their patterning are established for each species, and via mechanisms that are not known, the cells of the mesonephric duct migrate toward a single point on the testis and fuse/join/contribute to the rete testis. Sonic hedgehog (*Shh*; [Murashima et al., 2014](#)) has been shown to be a major regulator of some these events, and the transcription factors *Wt-1* and *Six1*, which are expressed in the mesenchyme, regulate the mesonephric tubule number in the cauda/distal region of the mesonephric duct. This data provides evidence that two types of mesonephric tubules, cranial-type, and cauda-type, develop and are differentially regulated ([Sainio et al., 1997](#)).

The number and patterning of the human efferent ducts differs considerably from other species. In addition, in the human, the classical “caput” region comprises mostly efferent ducts, and has very limited epididymal duct contribution, whereas in other species, the caput, including the initial segment, region is only epididymal duct. Therefore, it is important to realize that it is very difficult to compare functions of the human caput with that of other species.

Epididymal disjunction in humans is a condition in which the efferent ducts fail to fuse with the testis. More severe testicular-epididymal fusion anomalies such as complete separation of the testis and epididymis with epididymal atrophy can also occur. Epididymal disjunction is seen in approximately 2%–4% of males but increases significantly to approximately 25% in males that were cryptorchid at birth. Very often these anomalies were only discovered when the adult male underwent surgical procedures for exploration of the tunica vaginalis, congenital hernia repair, and emergencies involving testicular torsion ([Caterino et al., 2014](#)). Therefore, it is important that epididymal abnormalities be detected at orchidopexy or otherwise male infertility, which may be classified as idiopathic, will result.

Elongation and Coiling of the Wolffian Duct

Elongation and coiling are not trivial events in that mouse epididymis elongates from 1 mm to over 1 m, whereas the rat and human epididymis elongates to 3 m and 6 m respectively. It is difficult to examine the development of the human epididymis, and therefore animal models, for example gene knockouts in mice, are used instead. These models have provided clues as to the mechanisms of elongation and coiling, which are probably similar to the mechanisms that occur in the human. At embryonic day 14.5 (E14.5) in the mouse and E17.5 in the rat, the Wolffian duct is straight, and then 2 days later coiling is observed in the most proximal region (putative initial segment region). Coiling gradually progresses along the duct with time, and at E18.5 in the mouse, three-dimensional coiling is initiated (see reviews by [Joseph et al., 2009](#); [Hinton et al., 2011](#)). Elongation occurs via two major events, epithelial cell proliferation and epithelial cell rearrangements, for example, medio-lateral intercalation (convergent-extension; [Xu et al., 2016](#)). Coiling and elongation continue during the postnatal period but now a series of septa divide the duct into a number of segments (10 for the mouse and 19 for the rat). Interestingly, the number and position of septa in the human epididymis is variable. Although there are several mediators of elongation and coiling (see below), it is not clear as to the mechanisms that dictate length and shape. The epididymis must be of the correct length or male infertility will result ([Fig. 1](#)).

Congenital unilateral/bilateral absence of the vas deferens (CUAVD, CBAVD): CBAVD is found in approximately 1%–2% of infertile men and is often associated with mutations of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, whereas CUAVD is less common and is not associated with CFTR gene mutations ([Ong et al., 2017](#)). However, it should be noted that not all patients that carry mutations in the CFTR gene have an absence of the vas deferens. Commonly, in patients carrying CFTR mutations, it is only the caput region, presumably the efferent ducts, that remains, whereas the more distal regions of the epididymis and the vas deferens are absent. This would suggest that the mesonephric duct had either failed to migrate to the cloaca and/or it did migrate but underwent atrophy soon after fusion to the cloaca. The latter suggestion seems plausible because the efferent ducts/mesonephric tubules were present, and were presumably induced to form by the migrating mesonephric duct. The importance of the CFTR gene toward Wolffian duct development is unclear, although since fluid is present in the lumen during development, any changes in fluid secretion resulting in an increase in viscosity would cause luminal blockage and epithelial atrophy.

Regulators of Stabilization, Elongation, and Coiling

The stabilization and differentiation of the Wolffian duct are dependent on several factors, which include sex hormones and growth factors. In the female fetus, the lack of androgens in the hormonal milieu prevents the stabilization of the Wolffian duct, resulting in its regression. In the male fetus, androgens are necessary for Wolffian duct stabilization and later for the highly coordinated morphogenic program that will transform this duct into its adult derivatives ([Welsh et al., 2008](#)).

Androgen Regulation

Androgens are steroid hormones that exert their effects through activation of its cognate androgen receptor (AR), a member of the superfamily of ligand-activated steroid hormone receptor. The AR binds both testosterone and its 5- α -reduced metabolite

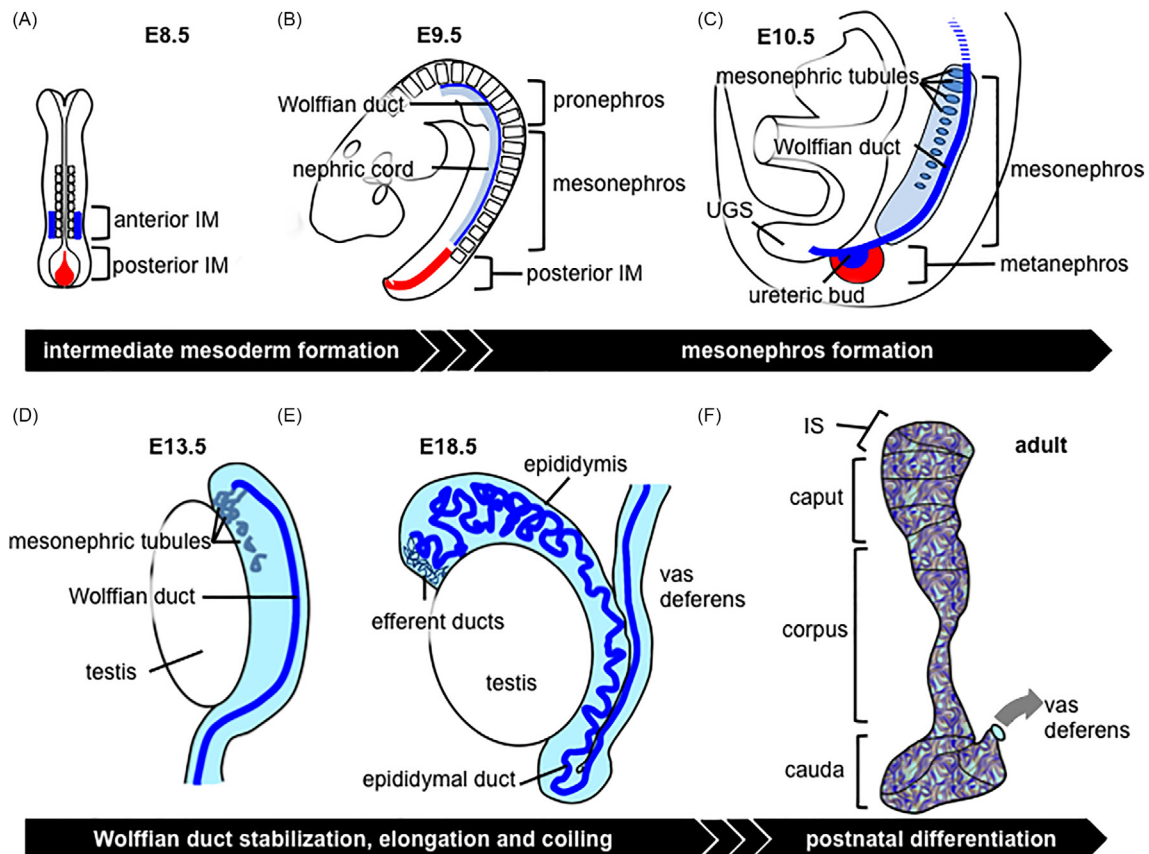


Fig. 1 Schematic diagram of mouse Wolffian/epididymal duct development. (A–C) The origin of the epididymis is the intermediate mesoderm. Spatiotemporally distinct intermediate mesoderm at E8.5 gives rise to the Wolffian duct (WD) and metanephric mesenchyme. The anterior intermediate mesoderm gives rise to the pronephros and the whole WD, whereas the posterior intermediate mesoderm gives rise to the metanephric mesenchyme. The WD begins to form from the anterior intermediate mesoderm at E8.5 and grows posteriorly reaching the urogenital sinus at E9.5. Concomitantly, the pronephros regresses through apoptosis. The WD induces the formation of mesonephric tubules from the mesenchyme (nephric cord) adjacent to the WD in a cranio-caudal manner. At the caudal end of the WD, the metanephros is initiated by ureteric bud formation through the interaction between WD epithelia and the metanephric mesenchyme at E10.5. (D) After gonadal sexual differentiation begins, the WD in the female embryo regresses from cranial to caudal and the WD in the male embryo is stabilized. The cranial set of mesonephric tubules connected to the WD is stabilized while the caudal set of mesonephric tubules regresses via apoptosis. (E) In the male embryo, the stabilized WD begins to coil from the cranial portion at E15.5. The duct continues to elongate and coil throughout development. (F) Ductal elongation and coiling continue after birth. The single-layered ductal epithelia undergo differentiation between P15 and P44. During this time, the regions of the epididymis, initial segment, caput, corpus and cauda, and the septa become morphologically distinct. Sperm transport through the duct begins at approximately P35. IM, intermediate mesoderm; UGS, urogenital sinus; IS, initial segment. Modified from Murashima, A., Xu, B. and Hinton, B.T. (2015b). Understanding normal and abnormal development of the Wolffian/epididymal duct by using transgenic mice. *Asian Journal of Andrology* **17**, 749–755; and reproduced by kind permission from the Asian Journal of Andrology.

dihydrotestosterone (DHT), a more potent androgen. AR activity is essential for Wolffian duct development because severe mutations that completely disrupt the AR activity in XY males are associated with the absence of epididymis/vas deferens, whereas patients with milder mutations that allow residual AR activation display normal Wolffian duct development (see review by Shaw and Renfree, 2014). Consistent with this clinical observation, Wolffian duct derivatives are absent in AR knockout (ARKO) mice (Welsh et al., 2009). Testosterone itself, but not DHT, has been shown to orchestrate a dominant role in the AR-dependent Wolffian duct development. In fact, DHT is not present in the Wolffian duct until epididymal differentiation is complete, and 5- α -reductase-deficient patients (which prevents conversion of testosterone to DHT) show normal Wolffian duct differentiation. Since AR acts as a ligand-dependent transcription factor, it is likely that regulation of expression of specific target genes (classical genomic androgen signaling) plays an important role in AR-dependent Wolffian duct development. Role of several androgen-targets in cell proliferation/elongation and coiling during the embryonic period include factors such as *inhibin beta A* (*Inhba*), *Wnt5a*, *Fgf8*, *Dusp6*, *Hoxa10*, various growth factors, among others (see review by Murashima et al., 2015a,b). However, the responsible downstream events and molecular and cellular determinants of androgen action are still unclear.

A schematic representation of the timeline key events for androgen action in Wolffian duct development in male Wistar rat fetuses is shown in Fig. 2. Wolffian duct development induced by androgens has been proposed as a biphasic

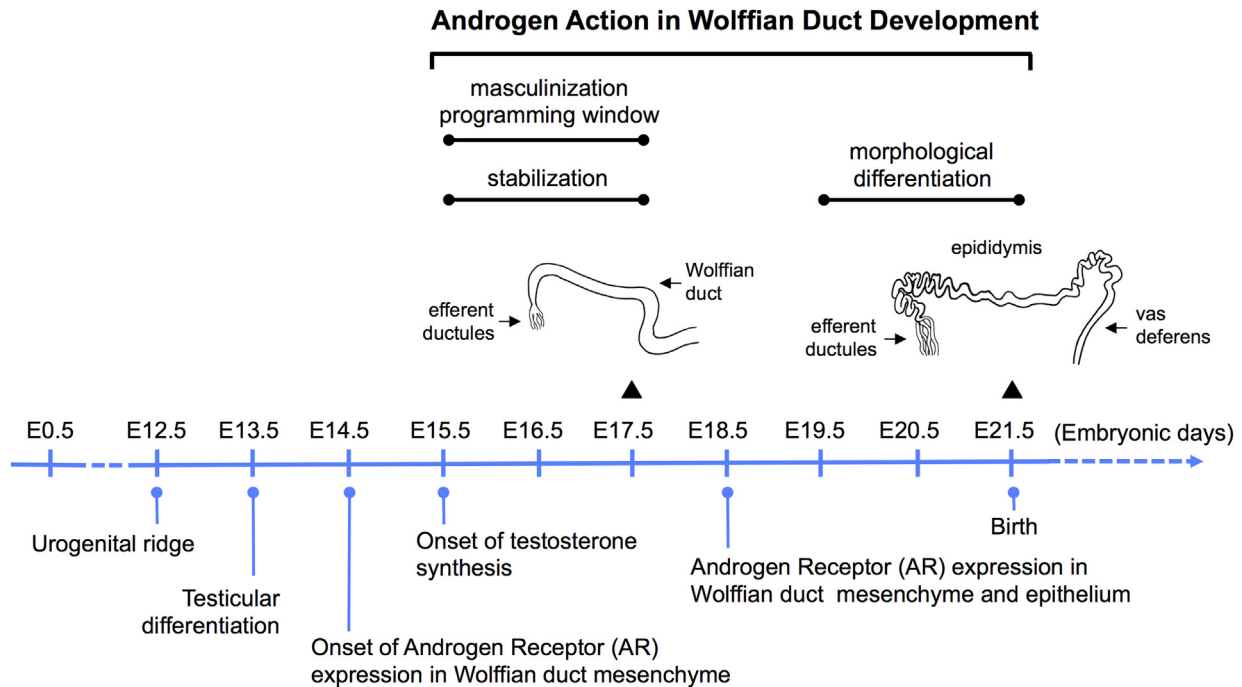


Fig. 2 Schematic diagram representing key events of androgen action during Wolffian duct development in male Wistar rat fetuses. Each embryonic age (E0.5–E21.5 days) is indicated in the timeline. The presence of spermatozoa in vaginal smears the following morning was considered evidence of mating and the time was defined as E0.5. The male programming window, as well as the periods during which the Wolffian duct is undergoing stabilization and morphological differentiation to originate a fully functional adult epididymis, vas deferens, and seminal vesicle is indicated. As a reference, the isolated Wolffian duct at both ages E17.5 (straight duct) and E21.5 (differentiated/developed duct) is shown. In the rat, testis differentiates at E13.5 and begins to secrete testosterone at E15.5, 1 day after the initial detection of AR protein in the mesenchymal cells of the Wolffian duct. Then, at E18.5 a peak in the testosterone plasma concentration is observed and AR is localized in both mesenchymal and epithelial cells. Wolffian duct development induced by androgens has been proposed as a biphasic process: testosterone first stabilizes the duct in males and then the duct undergoes morphological differentiation into the epididymis, vas deferens and seminal vesicle (not shown). The morphological differentiation of the Wolffian duct, which starts at E19.5, is programmed between E15.5 and E17.5 in the rat. The parallel in human timeline of the rat masculinization programming window is likely 8–14 weeks of gestation.

process: testosterone first stabilizes the duct in males and then morphologically differentiates it into the epididymis, vas deferens, and seminal vesicle. The morphological differentiation of Wolffian duct, which starts at E19.5, is programmed between E15.5 and E17.5 in the rat (Fig. 2). In humans, this rat masculinization programming window is likely to occur between 8 and 14 weeks of gestation (Welsh et al., 2008). Interestingly the spatio-temporal patterns of Wolffian duct morphogenesis in rat and mouse are heterochronic and the initial coiling of the future epididymis observed in the rat at E19.5 is seen earlier at E16.5 in the mouse (see review by Hinton et al., 2011).

Different experimental approaches, including the use of AR pharmacological tools and genetic mutant models, have demonstrated that dynamic mesenchymal–epithelial interactions under control of AR/testosterone signaling drives androgen-dependent mesenchyme-derived molecules that act as Wolffian duct epithelial paracrine regulators during the Wolffian duct morphogenesis. Androgens exert their effects on the Wolffian duct epithelium indirectly by androgen-dependent mesenchymal-derived factors and just after E18.5, as well through AR that is now also found in the WD epithelium. Mesenchymal ARs are needed for Wolffian duct stabilization, growth and coiling, while epithelial ARs are needed for later development and adult function (see reviews by Shaw and Renfree, 2014; Murashima et al., 2015a). Thus, Wolffian duct morphogenesis requires direct androgen action on the mesenchyme to maintain its survival and it is mesenchyme–epithelium crosstalk that determines the regional specialization of the epithelium. The complex signaling network involving molecules of mesenchymal origin which instruct epithelial growth under control of androgen action, however, is still poorly understood.

Androgen action during the masculinization programming window, which occurs several days prior any sign of Wolffian duct morphological differentiation (Fig. 2), has been proposed as sufficient to fully promote Wolffian duct development. Genetic mutations in AR or treatment with AR antagonists to block androgen signals during the male programming window result in regression of the Wolffian duct and an absence of the epididymis in adult males. Thus, disturbances during this period have the potential to lead to clinical pathologies with long-lasting consequences throughout life (see review by Skakkebaek et al., 2016). Disorders in androgen synthesis and/or action have been linked to atypical development of the male reproductive tract. At least 50 different congenital abnormalities of urogenital formation are recognized by the medical term of disorders of sex development (DSDs). Recently, it has been proposed that reproductive problems such as cryptorchidism, hypospadias, testicular germ cell cancer, low

sperm count, and primary hypogonadism have a common origin in fetal life as a result of impaired testosterone synthesis or action during the masculinization programming (more recently been hypothesized to be testicular dysgenesis syndrome, TDS) (Welsh et al., 2008; van den Driesche et al., 2017). In addition to genetic causes, environmental exposure of fetal testes to androgens or antiandrogen endocrine disrupting chemicals is recognized as important factor to TDS susceptibility.

Hox Genes

Hox genes are members of the homeotic transcription factor family that play a key role in controlling the body plan along the cranio-caudal axis (also referred to as anterior–posterior), and specify segment identity of tissues within the embryo. Mutations in *Hox* genes result in homeotic transformation of that tissue, that is, one part of that tissue develops into another part of the tissue. *Hox* genes are highly expressed within the developing Wolffian duct, particularly *Hoxa9*, *Hoxd9*, *Hoxa10*, *Hoxd10*, *Hoxa11*, *Hoxa13*, and *Hoxd13* (Snyder et al., 2010). Mice expressing null mutations of *Hoxa10* and *Hoxa11* resulted in the homeotic transformation of the vas deferens in which where the straight proximal region was transformed into a highly coiled segment, typical of the cauda region (Branford et al., 2000). However, the mechanisms by which *Hox* genes regulate Wolffian duct morphogenesis and segmentation are not known.

Inhibin Beta A

This gene is present in proximal Wolffian duct mesenchyme and mesonephros from both male and females before E12.5 in rat, but later its expression becomes androgen dependent. *Inhibin beta A* knockout mice (Tomaszewski et al., 2007) show pronounced Wolffian duct coiling defects. Cell division was lower in the knockout compared to wild-type but not zero, thereby suggesting that coiling and elongation via cell proliferation are separate events. Apoptosis was not observed in either the wild-type or knockout Wolffian ducts. In situ hybridization experiments showed the presence of *Inhibin beta A* in the mesenchyme whereas its downstream signaling components, SMAD2/3, were localized within the epithelial cells. Therefore, the data would suggest that factors within the mesenchyme, for example, *Inhibin beta A*, regulate the coiling and partially the cell proliferation rate during embryonic development of the Wolffian duct. There is also evidence that the effects of *inhibin beta A* in coiling control can be at least in part mediated by upregulation of AR in the epididymal epithelium.

Growth Factors

Different growth factors participate in the regulation of gene expression, especially through epithelial–mesenchymal, acting as androgen-regulated mediators in the developing Wolffian duct (see review by Murashima et al., 2015a). In addition to FGF8 signaling, FGF ligands can also bind and activate alternatively-spliced forms of four tyrosine kinase FGF receptors (FGFRs 1–4). FGFR1 is expressed in the mesenchyme, while FGFR2 is in the epithelium maintaining the Wolffian duct and mesonephric mesenchyme. FGFR2 in the epithelia is suggested to maintain the caudal part of the WD by regulating cell proliferation. Epidermal growth factor (EGF) and its receptor (EGFR) signaling are also active during the developing Wolffian duct. While it has been suggested that EGF can maintain development of isolated Wolffian ducts in culture in the absence of a testis, antibodies against EGF and EGFR can prevent ductal development in the presence of a testis. The association of EGF and antiandrogens can prevent Wolffian duct stabilization, suggesting that EGF-induced effects in the developing duct are under androgen regulation. AR can either directly regulate expression/activity of growth factors, or indirectly through other androgen-regulated target molecules.

Protein Tyrosine Kinase 7 (Ptk7)

Ptk7 is a component of the noncanonical Wnt/planar cell polarity and the *Wnt* canonical signaling pathway. In a number of tissues the protein functions in cell migration, cell adhesion, cell polarity, cell proliferation, and the organization of the actin cytoskeleton. During the development of the Wolffian duct, evidence suggests that Ptk7 regulates epithelial planar cell polarity and epithelial cell rearrangements such as medio-lateral intercalation (convergent extension) and radial intercalation of the mesenchymal cells closely surrounding the duct (Xu et al., 2016). Further studies revealed that planar polarized cells also expressed polarized distribution of active myosin II, which is necessary for cell to migrate and intercalate correctly. Loss of Ptk7 resulted in a shortened epididymis, which in turn resulted in loss of sperm motility. Therefore, an epididymis that is not of the correct length will result in male infertility.

Epithelial Canonical Wnt/ β catenin

Studies have provided evidence that the canonical Wnt/ β catenin signaling pathway plays a crucial role during Wolffian duct morphogenesis (Kumar et al., 2016). Wnt/ β catenin signaling, as shown by using Wnt reporter mice, is active in the duct during the embryonic period and later in the adult, to a limited region in the caput (segments 2–4). Loss of signaling of this pathway, whether by using pharmacological reagents and mouse null mutations, results in a duct that fails to undergo elongation through a reduction in cell proliferation and increase in cell apoptosis, and coiling.

β -Defensin SPAG11C (Sperm-Associated Antigen 11C)

SPAG11 is a member of the β -defensin multigenic family of epithelial-secreted peptides that present both antimicrobial and immunomodulatory properties, as well as ability to regulate sperm function. Its expression drastically changes during Wolffian duct/epididymal development, since it is observed mainly in mesenchymal cells in the developing rat Wolffian duct, in contrast to its predominant localization in epithelial cells of the postnatal and adult epididymis. Manipulation of the androgen status in rat Wolffian duct organotypic cultures showed SPAG11C as a downstream target of AR signaling. In addition, incubation of isolated Wolffian ducts with recombinant SPAG11C protein impaired duct morphological differentiation as a result of reduced epithelial cell proliferation (Ribeiro et al., 2017). These observations implied a putative role for SPAG11C in epididymal morphogenesis. The contribution of other β -defensins in shaping Wolffian duct morphogenesis in response to different developmental cues is still under investigation (Ribeiro et al., 2016).

Lumicrine Fluid Factors

Although testicular luminal fluid factors, or lumicrine factors, play a role in the regulation of adult initial segment in the epididymis, lumicrine factors do not appear to play a role in Wolffian duct elongation and coiling. When E14.5 mouse Wolffian ducts are cultured *in vitro*, they elongate and coil without the testis, but androgens are necessary (Hinton et al., 2011). However, in the postnatal Wolffian duct/epididymis, lumicrine factors are crucial for maintenance of cell proliferation.

References

- Atsuta, Y., & Takahashi, Y. (2015). FGF8 coordinates tissue elongation and cell epithelialization during early kidney tubulogenesis. *Development*, 142, 2329–2337.
- Branford, W. W., Benson, G. V., Ma, L., Maas, R. L., & Potter, S. S. (2000). Characterization of Hoxa-10/Hoxa11 transheterozygotes reveals functional redundancy and regulatory interactions. *Developmental Biology*, 224, 373–387.
- Caterino, S., Lorenzon, L., Cavallini, M., Cavaniglia, D., & Ferro, F. (2014). Epididymal-testicular fusion anomalies in cryptorchidism are associated with proximal location of the undescended testis and with widely patent process vaginalis. *Journal of Anatomy*, 225, 473–478.
- Grote, D., Souabni, A., Busslinger, M., & Bouchard, M. (2006). Pax 2/8-regulated Gata 3 expression is necessary for morphogenesis and guidance of the nephric duct in the developing kidney. *Development*, 133, 53–61.
- Hinton, B. T., Galdamez, M. M., Sutherland, A., Bomgardner, D., Xu, B., Abdell-Fattah, R., & Yang, L. (2011). How do you get six meters of epididymis inside a human scrotum? *Journal of Andrology*, 32, 558–564.
- Joseph, A., Yao, H., & Hinton, B. T. (2009). Development and morphogenesis of the Wolffian/epididymal duct: More twists and turns. *Developmental Biology*, 325, 6–14.
- Kumar, M., Syed, S. M., Taketo, M. M., & Tanwar, P. S. (2016). Epithelial Wnt/ β catenin signaling is essential for epididymal coiling. *Developmental Biology*, 412, 234–249.
- McMahon, A. P. (2016). Chapter three—Development of the mammalian kidney. *Current Topics in Developmental Biology*, 117, 31–64.
- Murashima, A., Akita, H., Okazawa, M., Kishigami, S., Nakagata, N., Nishinakamura, R., & Yamada, G. (2014). Midline-derived Shh regulates mesonephric tubule formation through the paraxial mesoderm. *Developmental Biology*, 386, 216–226.
- Murashima, A., Kishigami, S., Thomson, A., & Yamada, G. (2015a). Androgens and mammalian male reproductive tract development. *Biochimica et Biophysica Acta*, 1849, 163–170.
- Murashima, A., Xu, B., & Hinton, B. T. (2015b). Understanding normal and abnormal development of the Wolffian/epididymal duct by using transgenic mice. *Asian Journal of Andrology*, 17, 749–755.
- Obara-Ishihara, T., Kuhlman, J., Niswander, L., & Herzlinger, D. (1999). The surface ectoderm is essential for nephric duct formation in intermediate mesoderm. *Development*, 126, 1103–1108.
- Ong, T., Marshall, S. G., Karczeski, B. A., Stern, D. L., Cheng, E., & Cutting, G. R. (2017). Cystic fibrosis and congenital absence of the vas deferens. In M. P. Adam, H. H. Ardinger, R. A. Pagon, S. E. Wallace, et al. (Eds.), *Gene Reviews [Internet]*. Seattle: University of Washington.
- Ribeiro, C. M., Silva, E. J., Hinton, B. T., & Avellar, M. C. (2016). β -Defensins and the epididymis: Contrasting influences of prenatal, postnatal, and adult scenarios. *Asian Journal of Andrology*, 18, 323–328.
- Ribeiro, C. M., Ferreira, L. G., Thimoteo, D. S., Smith, L. B., Hinton, B. T., & Avellar, M. C. (2017). Novel androgen-induced activity of an antimicrobial β -defensin: Regulation of Wolffian duct morphogenesis. *Molecular and Cellular Endocrinology*, 442, 142–152.
- Sainio, K., Hellstedt, P., Kreidberg, J. A., Saxen, L., & Sariola, H. (1997). Differential regulation of two sets of mesonephric tubules by WT-1. *Development*, 124, 1293–1299.
- Shaw, G., & Renfree, M. B. (2014). Wolffian duct development. *Sexual Development*, 8, 273–280.
- Skakkebaek, N. E., Rajpert-De Meyts, E., Buck Louis, G. M., Toppari, J., Andersson, A. M., Eisenberg, M. L., Jensen, T. K., Jørgensen, N., Swan, S. H., Sapra, K. J., Ziebe, S., Priskorn, L., & Juul, A. (2016). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*, 96, 55–97.
- Snyder, E. M., Small, C. L., Bomgardner, D., Xu, B., Evanoff, R., Griswold, M. D., & Hinton, B. T. (2010). Gene expression in the efferent ducts, epididymis, and vas deferens during embryonic development of the mouse. *Developmental Dynamics*, 239, 2479–2491.
- Tomaszewski, J., Joseph, A., Archambeault, D., & Yao, H. H. (2007). Essential roles of inhibin Beta a in mouse epididymal coiling. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 11322–11327.
- van den Driesche, S., Kilcoyne, K. R., Wagner, I., Rebouret, D., Boyle, A., Mitchell, R., McKinnell, C., Macpherson, S., Donat, R., Shukla, C. J., Jørgensen, A., Meyts, E. R., Skakkebaek, N. E., & Sharpe, R. M. (2017). Experimentally induced testicular dysgenesis syndrome originates in the masculinization programming window. *JCI Insight*, 2, e91204.
- Welsh, M., Saunders, P. T., Fisk, M., Scott, H. M., Hutchison, G. R., Smith, L. B., & Sharpe, R. M. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *The Journal of Clinical Investigation*, 118, 1479–1490.
- Welsh, M., Sharpe, R. M., Walker, M., Smith, L. B., & Saunders, P. T. (2009). New insights into the role of androgens in Wolffian duct stabilization in male and female rodents. *Endocrinology*, 150, 2472–2480.
- Xu, B., Washington, A., Domeniconi, R. F., Ferreira-Souza, A. C., Lu, X., Sutherland, A., & Hinton, B. T. (2016). Protein tyrosine kinase 7 is essential for tubular morphogenesis of the Wolffian/epididymal duct. *Developmental Biology*, 412, 219–233.

Further Reading

- Becnel, L. B., Darlington, Y. F., Ochsner, S. A., Easton-Marks, J. R., Watkins, C. M., McOWiti, A., Kankanamge, W. H., Wise, M. W., DeHart, M., Margolis, R. N., & McKenna, N. J. (2015). Nuclear receptor signaling atlas: Opening access to the biology of nuclear receptor signaling pathways. *PLoS One*, *10*, e0135615.
- Georgas, K. M., Armstrong, J., Keast, J. R., Larkins, C. E., et al. (2015). An illustrated anatomical ontology of the developing mouse lower urogenital tract. *Development*, *142*(10), 1893–1908.
- Harding, S. D., Armit, C., Armstrong, J., Brennan, J., Cheng, Y., Haggarty, B., Houghton, D., Lloyd-MacGilp, S., Pi, X., Roochun, Y., Sharghi, M., Tindal, C., McMahon, A. P., Gottesman, B., Little, M. H., Georgas, K., Aronow, B. J., Potter, S. S., Brunskill, E. W., Southard-Smith, E. M., Mendelsohn, C., Baldock, R. A., Davies, J. A., & Davidson, D. (2011). The GUDMAP database—An online resource for genitourinary research. *Development*, *138*, 2845–2853.
- Sainio, K. (2003). Development of the mesonephric kidney. In P. D. Vize, A. S. Woolf, & J. L. Bard (Eds.), *The kidney: From normal development to congenital disease* (pp. 75–86). Cambridge, MA: Academic Press.
- Smith, L. B., Mitchell, R. T., & McEwan, I. J. (2013). *Testosterone: From basic research to clinical applications*. London: Springer.
- Staack, A., Donjacour, A. A., Brody, J., Cunha, G. R., & Carroll, P. (2003). Mouse urogenital development: A practical approach. *Differentiation*, *71*, 402–413.

Rete Testis: Structure, Cell Biology and Site for Stem Cell Transplantation

Rex A Hess, University of Illinois, Urbana, IL, United States

Louis Hermo, McGill University, Quebec, Canada

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blood–testis barrier The barrier formed between Sertoli cells, which divides the seminiferous epithelium into basal and adluminal compartments and consists of a morphological tight junctional component that regulates the entry and exit of molecules and passage of early spermatocytes through the barrier and an immunological competence that results in an immune-privileged site for the protection of differentiating germ cells.

Efferent ductules Small tubules that connect the external rete testis and epididymis and support an epithelium consisting of ciliated and absorptive non-ciliated cells.

Extratesticular rete testis A type that consists of flattened irregular-shaped anastomosing sacs and channels lined by a cuboidal to squamous epithelium and located adjacent to the tunica albuginea, which is found in rodents.

Mediastinal rete testis A type that has irregular-shaped anastomosing channels lined by a cuboidal to squamous epithelium and extending from the tunica albuginea into the testicular core, which is found in primates, including man and all large mammals.

Primary cilium A single non-motile cilium, with a 9 + 0 axonemal complex, found on nearly every cell at one point in development, which serves unique functions such as chemical sensation, signal transduction, cell cycle, and cellular growth.

Seminiferous tubule The testicular structure lined by an epithelium consisting of non-dividing Sertoli cells and germ cells, which undergo the process of spermatogenesis to produce sperm that are released into the lumen and transported into the rete testis.

Transitional region The short, narrow segment where seminiferous tubules open into the rete testis, and which are lined by tall, modified Sertoli cells, called transitional cells that protrude into the lumen in such a manner as to form a plug or valve like structure angled towards the rete testis and a subset of cells may serve as stem cells capable of proliferation.

Tubulus rectus Only present in some species, this is the straight tubule that connects the transitional region to the rete testis anastomosing spaces, often considered the proximal part of the rete, and in some species the epithelial cells may differ from those in the rete proper by containing an abundance of glycogen.

Abbreviations

FSH Follicle stimulating hormone

LacZ Beta-galactosidase

LH Luteinizing hormone

Notch1 Notch gene homolog 1

SOX-9 SRY-box containing gene 9

WT1 Wilms tumor 1

Organization of the Rete Testis

The rete testis is a continuous chamber of flattened or irregular-shaped anastomosing channels into which seminiferous tubules empty their luminal contents of spermatozoa, proteins, water, and other substances (Leeson, 1962; Dym, 1976; Roosen-Runge and Holstein, 1978; Setchell, 1978). Newly released spermatozoa and fluids are transported rapidly from this flattened cavernous space through the tunica albuginea into the numerous efferent ductules that connect to the head of the epididymis. There are two basic designs observed for rete testis organization (Fig. 1): (a) mediastinal and (b) extratesticular.

Mediastinal Rete Testis

Mediastinal rete testis is robust and surrounded with considerable amounts of dense connective tissue (Figs. 1 and 2). It is found in primates, including man and all large mammals, such as dogs and bulls (Wrobel, 2000). These irregular-shaped anastomosing channels extend from the tunica albuginea at the hilus through connective tissue septa in a fan-like manner into the testicular core, approximately 1/3 of the testis in man, providing a secure attachment. The rete epithelium of larger mammals is surrounded

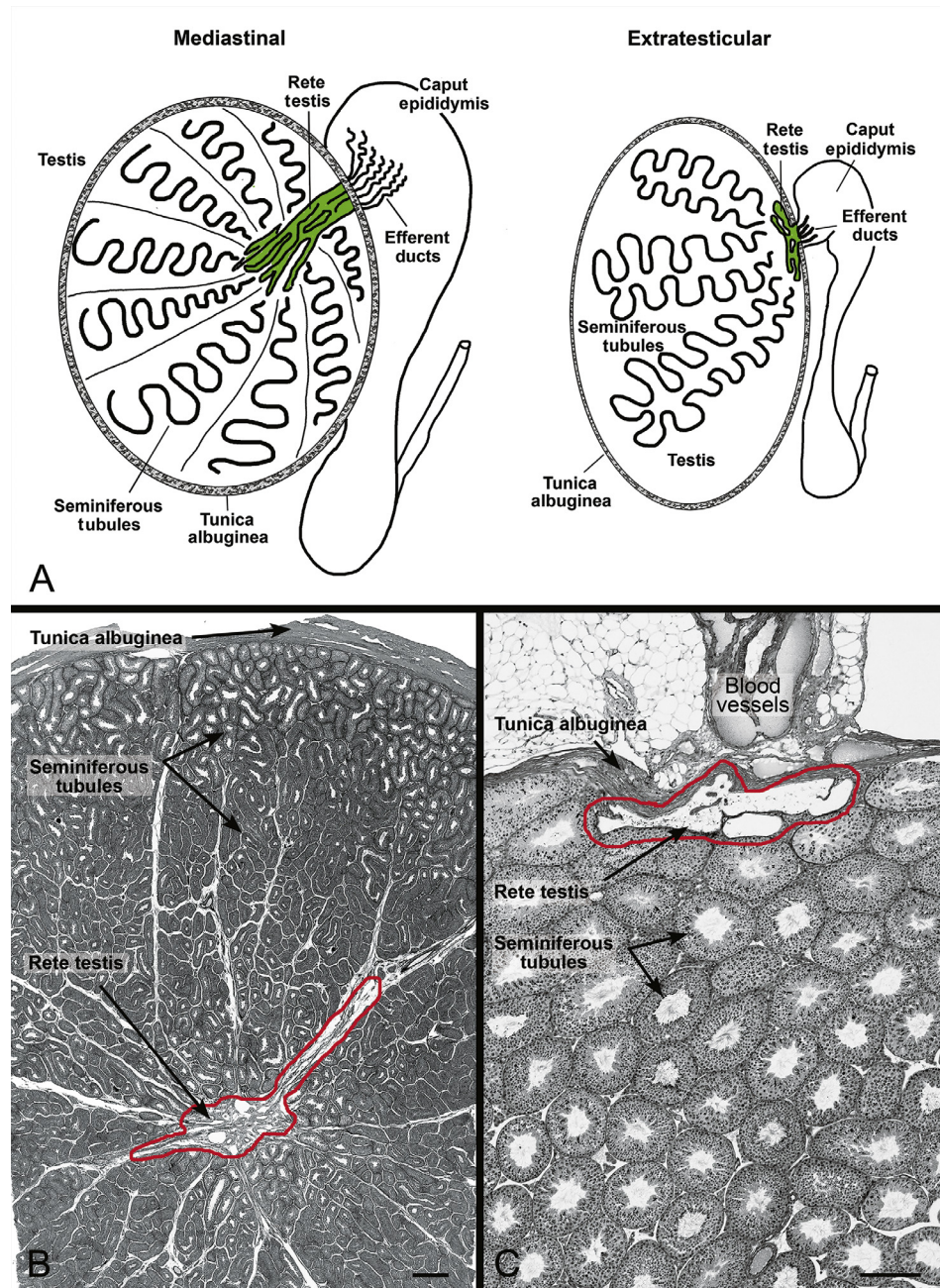


Fig. 1 (A) Illustrations showing the mediastinal and extratesticular rete testes (green). Mediastinal rete is found in primates, including man and all large mammals. Extratesticular rete is found in rodent species, such as mice and rats. Seminiferous tubules open into the rete testis, which collects the sperm and tubular fluid and channel them through the tunica albuginea into the efferent ducts that merge into the caput epididymis. (B) LM of the mediastinal rete testis outlined in red, which extends into the center of the dog testis. Seminiferous tubules extend as lobules from the rete testis to the tunica albuginea connective tissue capsule that surrounds the outside of the testis. Bar = 700 μ m. (C) LM of the extratesticular rete testis of the mouse outlined in red. It is located at the edge of the testis, with seminiferous tubules underneath and tunica albuginea on the outside. Rete testis exits at the testicular hilus, where blood vessels also penetrate the capsule. Bar = 250 μ m.

mostly by thick bundles of collagen and interspersed with the blood vasculature, lymphatic vessels and contractile myoid cells or bundles of smooth muscle.

Extratesticular Rete Testis

The entire extratesticular rete testis is located adjacent to the tunica albuginea and is found in rodents (Figs. 1 and 2). This type of rete extends only a short distance into the testis, but penetrates the tunica albuginea and extends briefly outside the testicular capsule (Hess, 2014). Smooth muscle or myoid cells do not line the base of this epithelium, in contrast to the efferent ductules and

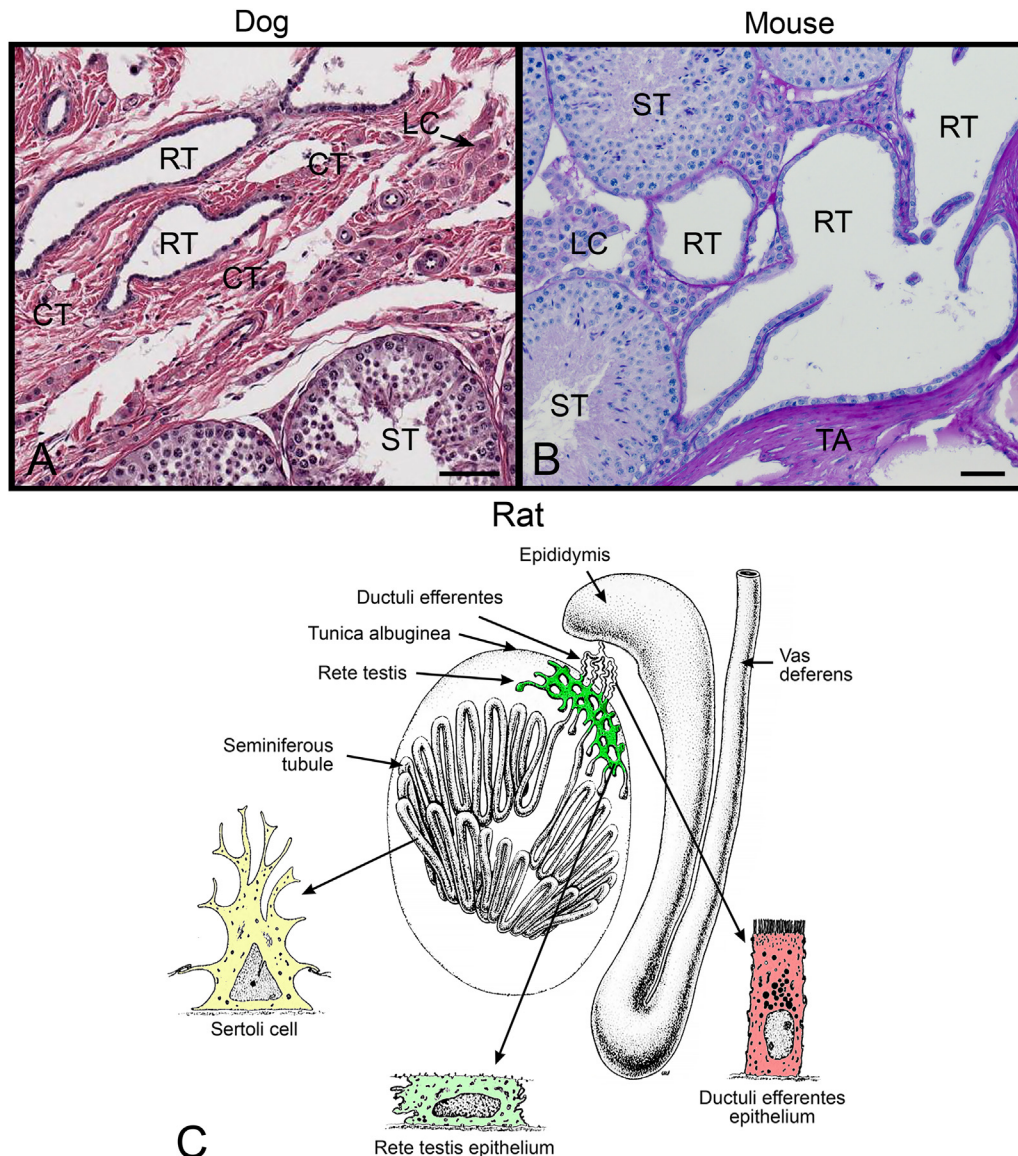


Fig. 2 (A) LM of the rete testis of the dog consists of flattened chambers surrounded by collagen connective tissue bundles (CT) and adjacent seminiferous tubules (ST). Leydig cells (LC) are found between the seminiferous tubules and the rete testis. The rete epithelium is low cuboidal to squamous. Bar = 100 μ m. (B) LM of the rete testis (RT) of the mouse consists of anastomosing chambers located adjacent to the tunica albuginea capsule (TA) of the testis. There is very little connective tissue surrounding the chambers that are bulging against the seminiferous tubules (ST), with Leydig cells (LC) in between. Bar = 100 μ m. (C) Schematic representation of the rat rete testis (green) is shown in an illustration modified from a drawing taken from the archives of Dr. Yves Clermont, McGill University. The two ends of a seminiferous tubule open into the rete cavernous spaces that are located at the hilus region, which extends into the testis and exits through the tunica albuginea and empties into the small ductuli efferentes tubules that continue to the epididymis. The rete testis epithelium is short cuboidal to squamous, compared to the taller stellate shaped Sertoli cells of the seminiferous epithelium and columnar epithelium of the ductuli efferentes.

epididymis. As in the case of the mediastinal rete, the extratesticular rete opens into a varying number of tubes of small diameter, the efferent ductules, depending on the species.

Transitional Region

Seminiferous tubules of the testis are long narrow and convoluted tubules ($\sim 250 \mu$ m in diameter) that terminate with a hairpin-like turn that opens at each end into the rete testis through short, narrow segments called the transitional region or zone (Dym, 1974; Hermo and Dworkin, 1988; Nakata et al., 2015). These segments (Fig. 3), unlike the seminiferous tubule proper, are lined by tall, modified Sertoli cells (also called transitional cells). The latter show tight junctions apically, and their free apical processes often protrude into the lumen in such a manner as to form a plug or valve like structure angled towards the rete testis (Hermo and Dworkin, 1988). Spermatozoa are not produced in this region, which shows rare germ cells, although in hamsters

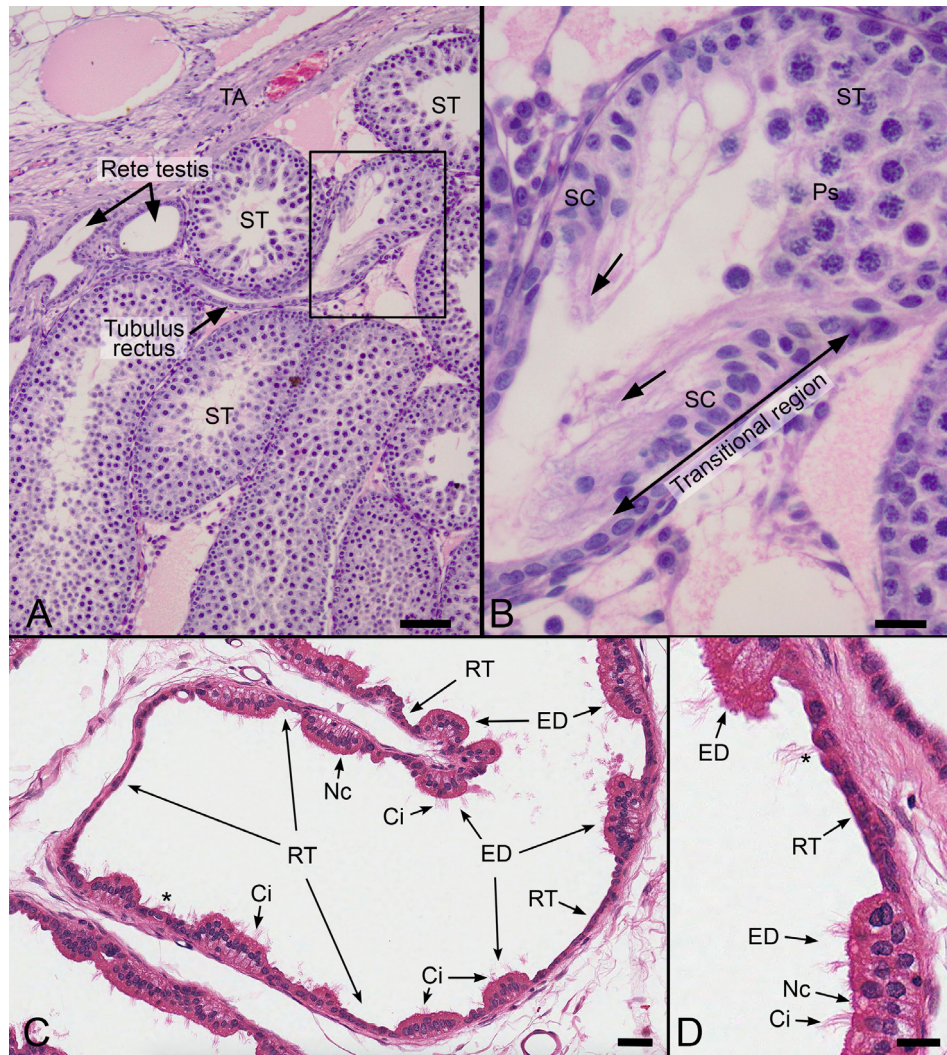


Fig. 3 (A) Low power LM photo of the rat rete testis junction with the seminiferous tubule (ST). The boxed area of the transitional region is magnified in (B). The rete testis is located adjacent to the tunica albuginea (TA) capsule. A short, straight tubule, or *tubulus rectus*, can be seen between the transitional region (in the *black box*) and the rete testis sacs. Bar = 100 μ m. (B) Higher magnification of the transitional region (outlined by *arrows*) between the seminiferous tubule (ST) and rete testis. Germ cells including pachytene spermatocytes (Ps) are present in the seminiferous tubule epithelium, but are missing in the transitional region, where only Sertoli cells (SC) are present and whose apical processes protrude into the lumen in the direction of the rete. Bar = 25 μ m. (C) A lower LM photo of the mouse rete testis/efferent ductule junction, which is located outside of the tunica albuginea. The rete testis epithelium (RT) is squamous, while the efferent ductule epithelium is short columnar in height and contains nonciliated (Nc) and ciliated (Ci) cells. At the junction, the epithelium changes abruptly from squamous to columnar, but there are pockets of ductuli efferentes epithelium interspersed between the rete epithelium. Occasional ciliated cells (*) are seen within the rete epithelium, but only in this junctional region. Bar = 50 μ m. (D) LM higher magnification of the mouse rete testis (RT)/efferent ductule junction showing the squamous epithelium, with one ciliated cell (*) in the rete testis section between the pockets of efferent ductule epithelium (ED). The efferent ductule has ciliated cells with motile cilia (Ci) and nonciliated cells (Nc) showing endocytic vesicles. Bar = 30 μ m.

spermatogonial stem cells are present in higher concentration (Aiyama et al., 2015). Transitional epithelial cells have adult Sertoli cell-like features such as microtubules in the apical cytoplasm and an elaborate network of endoplasmic reticulum, as well as phagocytotic activity and active endocytosis. However, other features suggest these cells are different, such as interdigitations of their lateral plasma membranes, apically located tight junctions and a unique thick and convoluted basement membrane structure. Immunostaining with SOX-9, WT1 and proliferation markers reveals that these cells are undifferentiated Sertoli cells capable of proliferation in the adult testis (Aiyama et al., 2015; Figueiredo et al., 2016; Kulibin and Malolina, 2016).

Rete Testis Epithelium

The seminiferous tubule transitional region changes abruptly from a tall columnar epithelium into a low cuboidal epithelium to form the characteristic structural appearance of the rete testis (Figs. 2 and 3). In some species, the initial connection with the rete is

a narrow, straight tubule that has been called *tubulus rectus* (Osman and Plöen, 1979) and is considered to be an initial region of the rete, but sometimes having modified epithelial cells. Rete testis epithelial cells show some species variation, with some cells being more squamous and others short columnar. In all species, the nuclei, with deep infoldings of the nuclear envelope, lie elongated and parallel to the basement membrane. The epithelial cells show numerous lateral plasma membrane folds and interdigitations, but the apical membrane is fairly smooth revealing only a few short microvilli and occasional small invaginations or pits of the clathrin-coated and uncoated variety. A long, single primary cilium is at times observed extending from the epithelial cells into the lumen (Dym, 1976). The cytoplasm shows components of an active endocytic apparatus (Morales and Hermo, 1983; Hermo and Dworkin, 1988), including endosomes, multivesicular bodies (MVBs) and few lysosomes. Fluid-phase and adsorptive endocytosis have been demonstrated along with receptor-mediated endocytosis. The cytoplasm also contains cytoskeletal elements, scattered mitochondria, with some aggregating in the basal region, Golgi apparatus, and endoplasmic reticulum. Lipid inclusions are also observed, and in some species the rete epithelium can be loaded with glycogen inclusions (Fawcett and Dym, 1974).

Efferent Duct Connection

Rete testis penetrates the thick tunica albuginea proximally as flattened channels but opens distally into expanded spaces (Hess, 2014), where the squamous epithelium meets the openings of a varying number of efferent ductules (Figs. 2 and 3). At this merging point there is a sudden change from the cuboidal to squamous rete testis epithelium to the columnar epithelium of the efferent ducts, which is identified by the projection of long cilia into the lumen, characteristic of ciliated cells. The junctional area often consists of efferent duct epithelium interspersed between rete testis squamous epithelia (Fig. 3).

Rete Testis Function

Rete testes have an appearance consistent with a space that is used for the collection of seminiferous tubular fluids and spermatozoa that are to be distributed into the numerous separate openings of the emerging efferent ductules. In rats, transport through the long efferent ductules is rapid (45 min) (Dym, 1976). It is also assumed that sperm are transported through the rete in a rapid manner. There is limited evidence that rete testis is active in secretion of proteins into the lumen, but rather its epithelium participates in fluid-phase, adsorptive and receptor mediated endocytosis (Hinton and Keefer, 1983; Morales and Hermo, 1983). The lack of water protein channels, aquaporins 1 and 2 in the rat, supports the conclusion that the rete testis does little to increase the concentration of sperm in the lumen. In fact, this is a major function of the efferent ductules amply decorated with numerous aquaporins, which remove about 90% of the luminal fluids (water) coming from the testis and derived from Sertoli cells (Hermo and Smith, 2011). The flow of fluid through the rete testis is unaffected by LH, FSH, prolactin, and the prostaglandins (Hinton and Setchell, 1993). Rete testis fluid, except for some modifications, is similar to seminiferous tubular fluid, and both fluids differ significantly from blood plasma. Rete testis differs from seminiferous tubule fluid by being isotonic with blood plasma, possibly due to the ability to move some ions such as sodium and chloride from the surrounding vasculature (Setchell, 1978).

Considerable evidence suggests that a major function of the rete epithelium is the endocytosis of proteins, as suggested by the higher concentration of total protein in the seminiferous tubule fluid as compared to that of the rete fluid (Setchell, 1978). Such proteins are internalized by coated pits and end up in lysosomes where they are degraded. The secreted proteins of Sertoli cells, namely androgen binding protein, sulfated glycoprotein-2/clusterin and sulfated glycoprotein-1, have all been demonstrated to be endocytosed by the rete epithelial cells (Morales and Hermo, 1983; Hermo et al., 1991, 1992, 1998). These proteins appear to be directly or indirectly related to late spermatids forming in the testis, where they may have a functional role. However, as future sperm leaving the seminiferous tubules, all three of these proteins dissociate from sperm and appear free in the rete lumen to be endocytosed and degraded. However, the rete epithelium is also involved in transcytosis, an event that allows selective proteins to be taken up by small uncoated pits and vesicles of the apical cell surface which are then delivered to the lateral plasma membrane. After fusion with this membrane the contents of these vesicles are released into the intervening lateral intercellular spaces allowing them to be free to access the blood vessels of the underlying connective tissue areas (Morales and Hermo, 1983). The nature and functions of these proteins has yet to be documented, but this is an event also noted for the epithelial cells of the vas deferens (Hermo and de Melo, 1987).

Of particular note, the long primary cilium found extending from rete epithelial cells (Dym, 1976) into the lumen may serve as chemical sensory detectors, but their signaling pathways have not been uncovered. Although the blood–testis barrier, at the basally located Sertoli–Sertoli cell junctional complexes of seminiferous tubules (Dym and Fawcett, 1970; Hinton and Keefer, 1983), provides innate resistance to autoimmune reactions, the apical Sertoli–Sertoli complexes of the transitional zone is susceptible. This segment contains resident intraepithelial lymphocytes and macrophages, which make up approximately 20% of the interstitial cells providing a region capable of rapid autoimmune responses (Takahashi et al., 2007).

Interestingly in rodents and mammals, seminiferous tubules, efferent ductules and the epididymis are enveloped beneath the basement membrane by smooth muscle cells referred to as myoid cells (Oliveira et al., 2016). The latter form distinct layer(s) enveloping these tubules. As sperm emanating from the seminiferous tubules are immotile, myoid cells contract and move sperm into the lumen of rete testis. However, in rodents the rete testis lacks myoid cells. Hence it is proposed that the role of the efferent ductules is to propel sperm out of the rete lumen. A major function of the epithelial cells of the efferent ductules is to absorb water (90%), via a number of aquaporin protein channels located at the apical pole of their epithelial cells, from the lumen into the underlying connective tissue spaces (Hermo and Smith, 2011). The volume and rapid uptake of water through this epithelium

must generate a suction-like pressure in the rete whereby the sperm located there would be propelled out of the rete lumen into the efferent ductules.

Another caveat to this story is the relevance of the plug (valve) in the transition zone. As sperm are moved out of the seminiferous tubules by myoid cells, they enter the transition zone. Here myoid cells move sperm to the rete testis, and as sperm leave the transition zone they would push aside the plug oriented towards the rete lumen. This plug or valve-like structure would however prevent regurgitation of sperm and substances from the rete lumen back into the lumen of seminiferous tubules (Hermo and Dworkin, 1988). Its presence is of relevance in today's stem cell transplantation studies as will be explained in the next section.

Rete Testis Site for Stem Cell Transplantation

In the mid-1970s, Martin Dym identified a specific area where the rete testis was positioned in the gross structure of the adult rat testis (Dym, 1976). It was seen as a small opaque spot or window at the upper pole of the testis, and at the point where the efferent ductules emerged. It was also adjacent to where the vascular channels penetrated the testis, that is, the hilus of the testis. Interestingly in rodents, the efferent ductules and vascular channels are surrounded by a large fat pad and care has to be taken in separating one from the other. This is particularly relevant in studies related to efferent duct ligation. Once this is done, the oval window can also be found and origin of the efferent ductules located. This window has become functionally important over the years, as it is the site where various substances (electron dense tracers), for example, ferritin and thorotrast, could be injected into the lumen of the rete testis, which is accomplished by introducing these tracers with a 30-gauge needle attached to a syringe and the use of a dye to visualize their entry into the rete lumen (Dym, 1976; Morales and Hermo, 1983).

From personal observations (Hermo and Morales, unpublished) the needle is administered into the rete lumen at the window, and with gentle pressure, the tracers and dye in a fluid medium are injected into the rete. It should be noted that just the tip of the needle has to puncture the rete and that a deep entry is fatal; in this case the tracers enter the interstitial space of the testis. However, when introduced into the rete lumen these tracers also enter the lumen of the efferent ductules. This is in fact the normal route and flow of substances, that is, from seminiferous tubules into the rete lumen and hence into the efferent ductules. Administration of electron dense tracers into the rete lumen has been profitable for definition of the endocytic organelles of both the rete testis epithelium and that of the efferent ductules, and is visualized by electron microscopy (Hermo and Morales, 1984; Morales et al., 1984; Hermo et al., 1991). In various time course studies, the internalization of these tracers by the rete and efferent ductule epithelial cells was evaluated. At early time points, tracers appear in coated and uncoated pits of the cell surface, small subsurface vesicles and endosomes (1–5 min), followed by MVBs (15–30 min) and lysosomes (1 h). Thus a fortuitous site for injection of various substances resides in the rete testis and this can be visualized with the naked eye.

This approach also has permitted the analysis of endocytic activity by Sertoli cells (Morales et al., 1985). However, for tracers injected into the rete lumen to be able to enter the seminiferous tubule lumens, one has to apply an ample amount of pressure to penetrate the plug formed by the Sertoli cells of the transitional zone. After repeated trials and errors, and the proper positioning of the needle into the rete lumen, which is purely a chance thing, one can be successful and enter the rete allowing the entry of tracers directly into the seminiferous tubule lumen. This takes a steady hand and a lot of practice. However, once the plug is penetrated and the tracers and dye are administered properly, they can be seen to flow in a retrograde manner filling the lumen of the seminiferous tubules. The flow occurs on its own without any added force on the needle, and with time each tubule in the testis becomes progressively filled with tracer and dye. It should be pointed out that in many cases (50% of the time), the tracers do not enter the seminiferous tubule lumen but enter the interstitial space of the testis. In such cases there is no flow of fluids and thus one has to re-try to position the needle into the rete lumen. In fact, the rete lumen is a flattened space and attempting to get the needle into this invisible space is highly problematic. Thus practice makes perfect but once perfected, the method can provide solid results.

The rete testis injection site became clinically relevant over the past decades, as Ralph Brinster and colleagues began to harvest germline stem cells from normal animals and introduce them into infertile and germ cell-depleted male, lacking the capability to produce sperm (Brinster, 2002). Using a reporter (LacZ) transgene in the donor animal it was possible to show that true stem cells were transplanted, as only spermatogonial stem cells would be capable of sustained spermatogenesis in the recipient testis. The donor stem cells were injected into the seminiferous tubule via the rete testis of recipient males and the stem cells appeared to migrate through the seminiferous epithelium to reside on the basement membrane of the tubules, where they subsequently underwent the process of spermatogenesis.

Another possible function of the rete could be to prevent damage to the blood–testis barrier after efferent ductule blockage. It has been observed that in male transgenic (Tg) mice constitutively expressing Notch1, the epithelial cells of the Tg tubuli recti and rete testis were not abnormal, but the rete testis lumen was highly dilated and contained numerous spermatozoa, suggesting a blockage downstream (Lupien et al., 2006). Data revealed a blockage of the efferent ducts at their interface with the rete, as also noted by absence of spermatozoa in the epididymis of all adult Tg mice, without seriously affecting spermatogenesis. Hence in abnormal situations of efferent duct blockage, the rete testis is capable of temporary dilation to accommodate the adverse situation and thus prevent the possible disruption of blood–testis barrier function (Nykänen and Korman, 1978; Hess, 2014).

Conclusion

The rete testis is a continuous chamber of flattened and irregular-shaped anastomosing channels lined by squamous to cuboidal epithelial cells adjacent to the tunica albuginea (rodents) and extending centrally into the testis of larger mammals including

man. The rete testis serves as a conduit and point of exit for newly released spermatozoa and fluids emanating from seminiferous tubules. The epithelial cells are highly active in endocytosis, with several proteins derived from Sertoli cells and sperm emanating from seminiferous tubules. Thus while a seemingly passive chamber, the rete has important roles in fine-tuning the lumen for sperm as they enter the efferent ducts. Of clinical significance, the rete testis has over many decades been used as an appropriate site to inject stem cells into the lumen of seminiferous tubules to restore a deficient or reduced spermatogenesis. It may also serve in obstructions of the efferent ducts to expand and prevent disruption of the blood–testis barrier.

References

- Aiyama, Y., Tsunekawa, N., Kishi, K., Kawasumi, M., Suzuki, H., Kanai-Azuma, M., Kurohmaru, M., & Kanai, Y. (2015). A niche for GFRalpha1-positive spermatogonia in the terminal segments of the seminiferous tubules in hamster testes. *Stem Cells*, 33(9), 2811–2824.
- Brinster, R. L. (2002). Germline stem cell transplantation and transgenesis. *Science*, 296(5576), 2174–2176.
- Dym, M. (1974). The fine structure of monkey Sertoli cells in the transitional zone at the junction of the seminiferous tubules with the tubuli recti. *The American Journal of Anatomy*, 140(1), 1–25.
- Dym, M. (1976). The mammalian rete testis—a morphological examination. *The Anatomical Record*, 186(4), 493–523.
- Dym, M., & Fawcett, D. W. (1970). The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biology of Reproduction*, 3(3), 308–326.
- Fawcett, D. W., & Dym, M. (1974). A glycogen-rich segment of the tubuli recti and proximal portion of the rete testis in the guinea-pig. *Journal of Reproduction and Fertility*, 38(2), 401–409.
- Figueiredo, A. F., Franca, L. R., Hess, R. A., & Costa, G. M. (2016). Sertoli cells are capable of proliferation into adulthood in the transition region between the seminiferous tubules and the rete testis in Wistar rats. *Cell Cycle*, 15(18), 2486–2496.
- Hermo, L., & de Melo, V. (1987). Endocytic apparatus and transcytosis in epithelial cells of the vas deferens in the rat. *The Anatomical Record*, 217(2), 153–163.
- Hermo, L., & Dworkin, J. (1988). Transitional cells at the junction of seminiferous tubules with the rete testis of the rat: their fine structure, endocytic activity, and basement membrane. *The American Journal of Anatomy*, 181, 111–131.
- Hermo, L., & Morales, C. (1984). Endocytosis in nonciliated epithelial cells of the ductuli efferentes in the rat. *The American Journal of Anatomy*, 171(1), 59–74.
- Hermo, L., & Smith, C. E. (2011). Thirsty business: cell, region, and membrane specificity of aquaporins in the testis, efferent ducts, and epididymis and factors regulating their expression. *Journal of Andrology*, 32(6), 565–575.
- Hermo, L., Wright, J., Oko, R., & Morales, C. R. (1991). Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). *Biology of Reproduction*, 44(6), 1113–1131.
- Hermo, L., Morales, C., & Oko, R. (1992). Immunocytochemical localization of sulfated glycoprotein-1 (SGP-1) and identification of its transcripts in epithelial cells of the extratesticular duct system of the rat. *The Anatomical Record*, 232(3), 401–422.
- Hermo, L., Barin, K., & Oko, R. (1998). Androgen binding protein secretion and endocytosis by principal cells in the adult rat epididymis and during postnatal development. *Journal of Andrology*, 19(5), 527–541.
- Hess, R. A. (2014). Disruption of estrogen receptor signaling and similar pathways in the efferent ductules and initial segments of the epididymis. *Spermatogenesis*, 4(2), e979103.
- Hinton, B. T., & Keefer, D. A. (1983). Evidence for protein absorption from the lumen of the seminiferous tubule and rete of the rat testis. *Cell and Tissue Research*, 230(2), 367–375.
- Hinton, B. T., & Setchell, B. P. (1993). Fluid secretion and movement. In L. D. Russell, & M. D. Griswold (Eds.), *The sertoli cell* (pp. 249–267). Clearwater, FL: Cache River Press.
- Kulibin, A. Y., & Malolina, E. A. (2016). Only a small population of adult Sertoli cells actively proliferates in culture. *Reproduction*, 152(4), 271–281.
- Leeson, T. S. (1962). Electron microscopy of the rete testis of the rat. *The Anatomical Record*, 144, 57–67.
- Lupien, M., Dievert, A., Morales, C. R., Hermo, L., Calvo, E., Kay, D. G., Hu, C., & Jolicœur, P. (2006). Expression of constitutively active Notch1 in male genital tracts results in ectopic growth and blockage of efferent ducts, epididymal hyperplasia and sterility. *Developmental Biology*, 300(2), 497–511.
- Morales, C., & Hermo, L. (1983). Demonstration of fluid-phase endocytosis in epithelial cells of the male reproductive system by means of horseradish peroxidase-colloidal gold complex. *Cell and Tissue Research*, 230(3), 503–510.
- Morales, C., Hermo, L., & Clermont, Y. (1984). Endocytosis in epithelial cells lining the rete testis of the rat. *The Anatomical Record*, 209(2), 185–195.
- Morales, C., Clermont, Y., & Hermo, L. (1985). Nature and function of endocytosis in Sertoli cells of the rat. *The American Journal of Anatomy*, 173(3), 203–217.
- Nakata, H., Wakayama, T., Sonomura, T., Honma, S., Hatta, T., & Iseki, S. (2015). Three-dimensional structure of seminiferous tubules in the adult mouse. *Journal of Anatomy*, 227(5), 686–694.
- Nykänen, M., & Kormano, M. (1978). Early effects of efferent duct ligation on the rat rete testis. *International Journal of Andrology*, 1(1), 225–234.
- Oliveira, R. L., Parent, A., Cyr, D. G., Gregory, M., Mandato, C. A., Smith, C. E., & Hermo, L. (2016). Implications of caveolae in testicular and epididymal myoid cells to sperm motility. *Molecular Reproduction and Development*, 83(6), 526–540.
- Osman, D. I., & Plöen, L. (1979). Fine structure of the modified sertoli cells in the terminal segment of the seminiferous tubules of the bull, ram and goat. *Animal Reproduction Science*, 2(4), 343–351.
- Roosen-Runge, E. C., & Holstein, A. F. (1978). The human rete testis. *Cell and Tissue Research*, 189(3), 409–433.
- Setchell, B. P. (1978). *The mammalian testis*. Ithaca, NY: Cornell University.
- Takahashi, K., Naito, M., Terayama, H., Qu, N., Cheng, L., Tainosho, S., & Itoh, M. (2007). Immunomorphological aspects of the tubuli recti and the surrounding interstitium in normal mice. *International Journal of Andrology*, 30(1), 21–27.
- Wrobel, K. H. (2000). Morphogenesis of the bovine rete testis: the intratesticular rete and its connection to the seminiferous tubules. *Anatomy and Embryology (Berlin)*, 202(6), 475–490.

Efferent Ductules: Structure and Function

Rex A Hess, University of Illinois, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blind-ending tubules Small-diameter tubules arising from developmental anomalies that grow out from rete testis, efferent ducts or the epididymis on one end but do not connect on the other end and appear to have undeveloped epithelia.

Common efferent ducts The terminal region where the tubule joins the epididymis, as a single tubule in rodents but as individual tubules in larger mammals, with both types having a narrow lumen.

Conus efferent ducts The central region between rete testis and epididymis, where the highly coiled tubules begin to merge and the lumen becomes more narrow.

Fluid reabsorption Rapid transport of fluids, including ions, water, selected proteins and other molecules, by the ductal epithelium in an isosmotic equilibrium from the lumen into the interstitial space containing the vascular system.

Motile cilia Finger-like projections on the apical surface of specific epithelial cells, with a 9 + 2 axonemal complex, capable of coordinated movement that helps to stir the luminal fluids.

Primary cilium A single non-motile cilium, with a 9 + 0 axonemal complex, found on nearly every cell at one point in development, which is involved in unique functions such as chemical sensation, signal transduction, cell cycle and cellular growth.

Proximal efferent ducts The region originating at the rete testis as individual, straight tubules with a wider lumen and more dilute concentrations of sperm.

Rete testis An irregularly-shaped chamber of anastomosing channels, lined by a cuboidal to squamous epithelium, extending from the tunica albuginea and connecting to the separate efferent ductules.

Nomenclature

AR Androgen receptor

ESR1 Estrogen receptor 1

ESR2 Estrogen receptor 2

AQP1 and 9 Aquaporin 1 and 9

SLC9A3 Na⁺/H⁺ exchanger-3

CAR2 Carbonic anhydrase 2

CFTR Cystic fibrosis transmembrane conductance regulator

SGP-1 and 2 Sulfated glycoproteins 1 and 2

E2 17β-Estradiol

*Esr1*KO Estrogen receptor 1 knockout

*Ar*KO Aromatase knockout

T Testosterone

DHT Dihydrotestosterone

DES Diethylstilbestrol

Organization of the Efferent Ductules

Efferent ductules, previously called vasa efferentia, are small, delicate and parallel tubules that connect the rete testis with the epididymal duct (Illo and Hess, 1992; Hess, 2002). They begin as a series of individual straight ducts originating from the rete testis in all mammalian species, but as enlarged chambers with folded mucosa in avian species. As the ductules extend toward the epididymis they become highly convoluted and progressively smaller in diameter. Efferent ducts are organized into two basic designs, depending on the species (Fig. 1): (1) a funnel and (2) parallel coils. The funnel type of ductule is formed by merging of the proximal ducts, arising separately from the rete testis, until they form a single, very small common duct that enters the head or initial segment of the epididymis. This formation is typically observed in smaller mammalian species, such as rodents. The parallel coil formation is found in larger mammals and birds (Illo and Hess, 1994; Hess, 2002). This design consists of numerous, adjacent coils of efferent ductules arising from the rete testis, with most of these ducts remaining independent, rather than merging, and thus providing multiple entries into the initial region of the epididymis. In man, the more caudal efferent ducts (referring to their position as

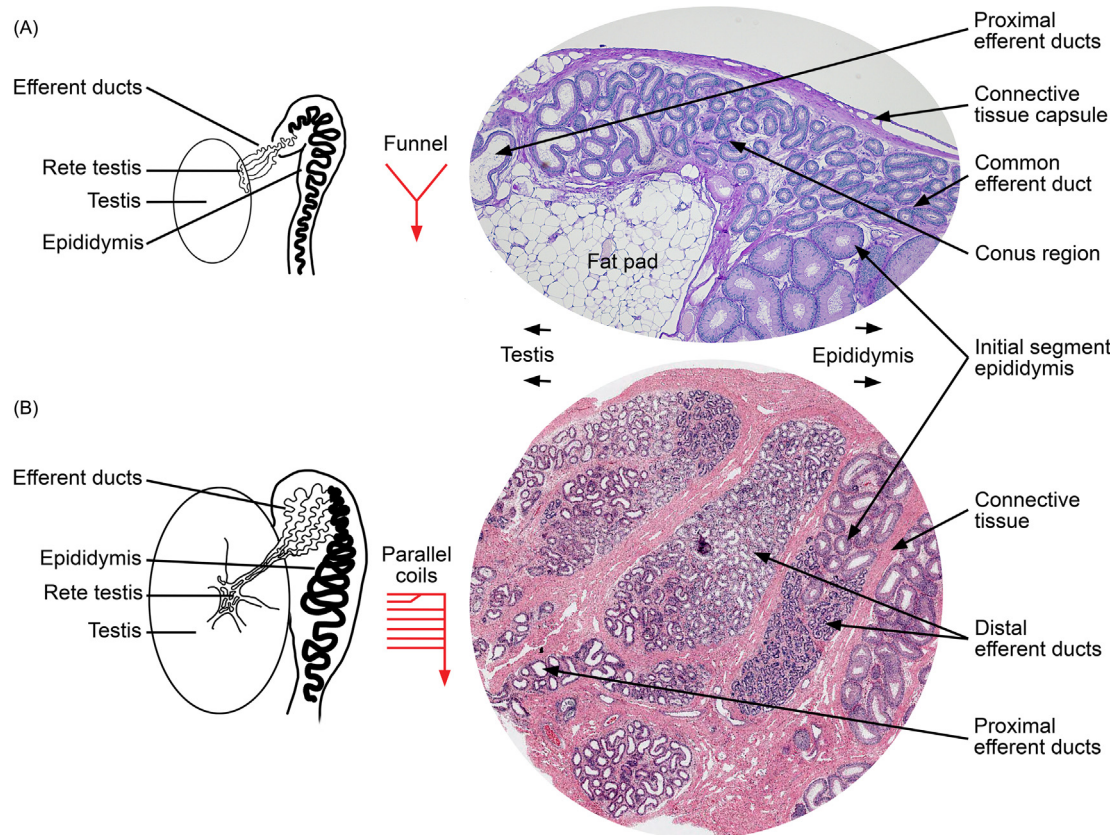


Fig. 1 Two basic types of efferent ductule organization are illustrated. (A) Funnel-like formation that is seen in smaller mammals. Individual proximal or initial region ductules, arising from the rete testis, merge to form a single, common duct that enters the head of the epididymis. Histology of this design shows the proximal ducts having a wider lumen than those in the conus and common duct regions, near the initial segment of the epididymis. A connective tissue capsule is seen on the outer edge but the epididymal fat pad is seen underneath. (B) Formation of parallel coils is found in larger mammals and man. Most of the individual proximal efferent ducts, connected to the rete testis, tend to stay separated, with only a few ducts merging in the cephalic region of the epididymis. Thus, there are numerous separate entries of the coiled efferent ducts into the caput epididymis. Histology of this organization shows the separate ducts surrounded by thick areas of connective tissue. The proximal ducts have a wider lumen than ducts in the distal region, near the initial segment of the epididymis.

they exit the testis) tend to join the epididymis independently, without merging. However, many of the more cranial ducts show some looping and merging to form a very short common duct (Yeung et al., 1991). With both types of organization, there can be numerous proximal efferent ductules arising from the rete testis. However, considerable variation in the number of proximal ductules is found, even within a species. For example, the rat has between 2 and 8 proximal ductules and in man the number ranges from 6 to 15.

The proximal efferent ductules are typically straight and have a wider lumen than those found in the more distal regions, closer to the epididymis. The coiled region of ductules is referred to as the conus vasculosus or "conus". In rodents, the ducts begin to merge in the conus to form a single tubule called the "common duct" that penetrates the connective tissue capsule of the caput epididymis. In man and larger mammals, the coiled ductules form several different types of junctions with the epididymis, as some exhibit end-to-end and others end-to-side junctions (Yeung et al., 1991). In larger mammals and birds, the efferent ducts are embedded entirely in thick bundles of connective tissue that are shared with the head of the epididymis and occupy a major portion of the caput (Yeung et al., 1991). The thick bundles of collagen attach the epididymal head to the hilus of the testis, where blood vasculature enters and the rete testis exits. In rodents, the common duct only shares a very small portion of the caput epididymis, adjacent to the initial segment, and the remainder of the efferent ducts are found in loose connective tissue and an extensive epididymal fat pad that extends between the testis and head of the epididymis, which allows for easy isolation of the individual ducts (La et al., 2012).

Development

Efferent ductules originate from the mesonephros and associated embryonic kidney tubules (Hess, 2002). During development, these ductules are the first site of epithelial androgen receptor (AR) expression in the male reproductive tract (Cooke et al., 1991).

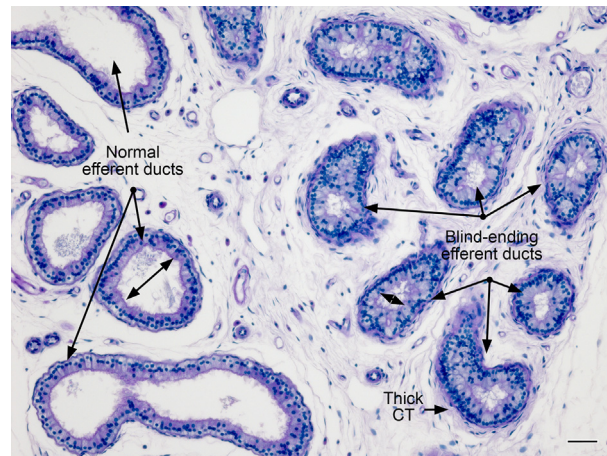


Fig. 2 Blind-ending efferent ducts are recognized by their smaller luminal diameters, a thicker coat of surrounding connective tissue and a more regressed epithelium. These aberrant ductules are typically segregated from the normal ducts. Bar = 40 μ m.

Estrogen receptors are also expressed first in this region's epithelium (Hess, 2002) and disruption of estrogen receptor 1 (ESR1) results in the formation of an increased number of blind ending tubules (Hess et al., 2000) and abnormal epithelial morphology (Hess et al., 1997b; Lee et al., 2009). Interestingly, loss of AR during perinatal development also produces abnormal rete testis and efferent ducts, similar to the loss of ESR1 alone (Rivas et al., 2002). Ciliated cells appear in the early postnatal period in mice and by day 28 in rats (Ilio and Hess, 1994). Differentiation of the conus epithelium is delayed until about day 32 in rats. In men, ductuli efferentes formation first begins at about 6 weeks of fetal development and lumen canalization begins during the eighth week, but complete formation within the head of the epididymis does not occur until between 20 and 28 weeks. Nonciliated cells begin to differentiate even in fetal life, as aquaporin-1 (AQP-1) is expressed in the apical brush border of the epithelium. Expression of other proteins and cellular organelles associated with nonciliated cell physiology begins as early as day 7 postnatally (see review: Hess, 2002), but lysosomes and a fully developed endocytic apparatus are not found until near early puberty.

A common developmental anomaly in efferent ducts is the formation of blind-ending tubules (Fig. 2), which are quite common in rodents. These abnormal tubules are also seen in larger mammals and man (Guttroff et al., 1992). The aberrant tubules can arise from many regions: directly from the rete testis, branching from any region of the efferent ductules and growing blindly from the epididymis. In larger mammals and man, blind-ending ducts are associated with sperm granulomas and epididymal cysts and thus contribute to infertility and pathology in the caput epididymis (Ilio and Hess, 1994). However, in the rodent aberrant tubules do not accumulate sperm or form granulomas, and the epithelium is underdeveloped and contains little cytoplasm, resulting in relatively small tubules that are surrounded by dense connective tissue.

Efferent Duct Epithelium

There is an abrupt change from the squamous to cuboidal/columnar epithelium at the histological junction of the rete testis and efferent ductules (Fig. 3), whose epithelium is composed of ciliated and nonciliated cells, with some species also showing basal cells and intraepithelial lymphocytes or macrophages (Hess, 2002). The efferent ductule epithelium sits on a typical basement membrane that is surrounded by a thin smooth muscle layer. There is considerable variation in cell morphology along the length of the ductules, as well as species variations (Ilio and Hess, 1994). Regional differences are principally the presence or absence of lysosomal granules, vacuoles and endocytic vesicles in nonciliated cells. It is unclear whether these morphological differences represent the same cell type in different phases of function or if the cells permanently express varying morphologies along the efferent ducts. In some regions, particularly proximal, the epithelium is fairly uniform, but in the conus and common duct areas there can be significant variations in epithelial height, with large folds occurring in some species such as in birds and man. In the rat, unique grooves penetrating nearly to the basement membrane can be found running longitudinally along sections of the ductal epithelium (Fig. 4), but they are inconsistent in location (Hess, 2002). These grooves are lacking in most species but are seen in some regions of human efferent ductules (Yeung et al., 1991). The transition from efferent ductule to initial segment of the epididymis is typically abrupt, as the epithelium suddenly changes from short columnar to tall, pseudostratified columnar cells with thin, branched microvilli (also called stereocilia). However, in humans, the transition appears to be gradual, with islands of epididymal cells first seen interspersed with efferent ductule epithelium (Yeung et al., 1991).

Ciliated Cells

A noteworthy characteristic found in efferent ductules is the presence of motile (kinetic) cilia (Fig. 5), an organelle also present in the female oviduct. Nonciliated cells are present in greater proportions in the proximal zone and numbers of ciliated cells are

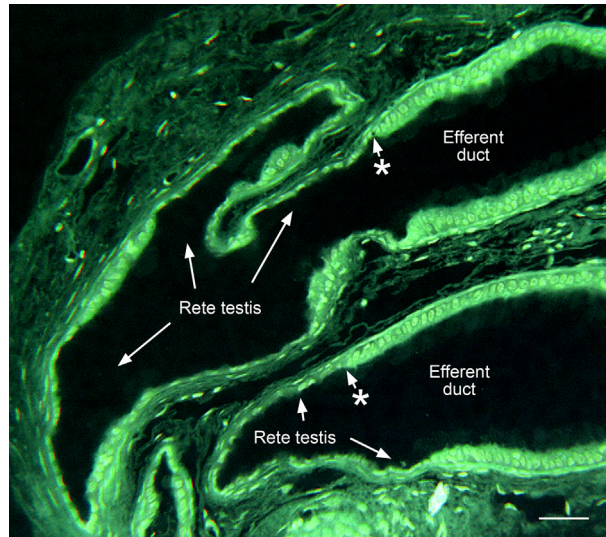


Fig. 3 Rete testis to proximal efferent ductule transition. The image has been inverted in photoshop to emphasize the abrupt transition (*) of the squamous epithelium of rete testis into the columnar epithelium of efferent ductules. Bar = 40 μm .

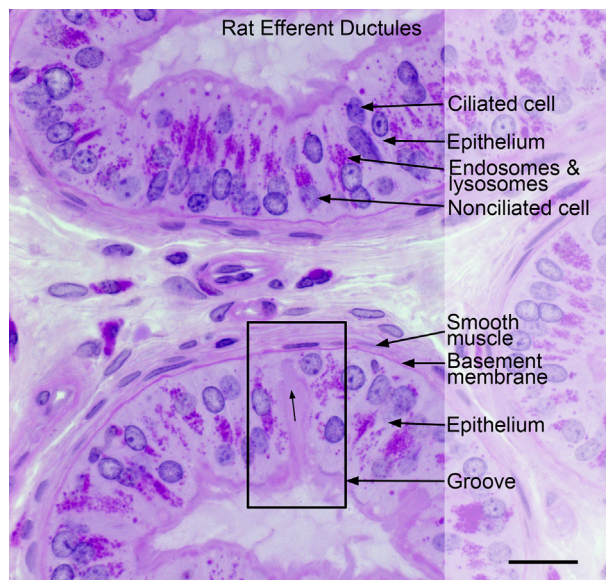


Fig. 4 Rat efferent ductule cross sections stained with periodic acid/Schiff and hematoxylin to show the carbohydrate rich areas of endosomes and lysosomes that are involved in fluid reabsorption in nonciliated cells. Ciliated cell nuclei are located near the lumen. The cross section on the bottom shows an epithelial groove (*boxed area*), where adjacent epithelial cell membranes abruptly penetrate the epithelium (*arrow*) until nearly touching the basement membrane. Bar = 20 μm .

increased in zones nearest the epididymis, but is also species variable (Ilio and Hess, 1994). Ciliated cells can occupy up to 80% of the epithelium (Hess, 2015). Cilia extend as parallel finger-like structures from basal bodies into the lumen (Fig. 5) and bend together in the lower half, but are more random near their tips. Surprisingly, the cilia have a rotational twist and random beat, rather than a coordinated wave (Hess, 2015). It has been suggested that the cilia's primary function is to stir the luminal fluids to ensure homogeneous fluid reabsorption by nonciliated cells (Hess, 2003, 2015). Across all species, ciliated cells have a typical appearance, with an abundance of apical mitochondria to provide energy for the motile cilia. The nucleus of ciliated cells has a consistently apical position in the cytoplasm near the lumen in rodent and avian species, but in man and other large mammals nuclear position varies by region. The nuclear membrane often exhibits deep indentations (Fig. 5). The cell is also recognized by examining the apical cell surface with high resolution to observe a row of basal bodies that support the extended ciliary structures.

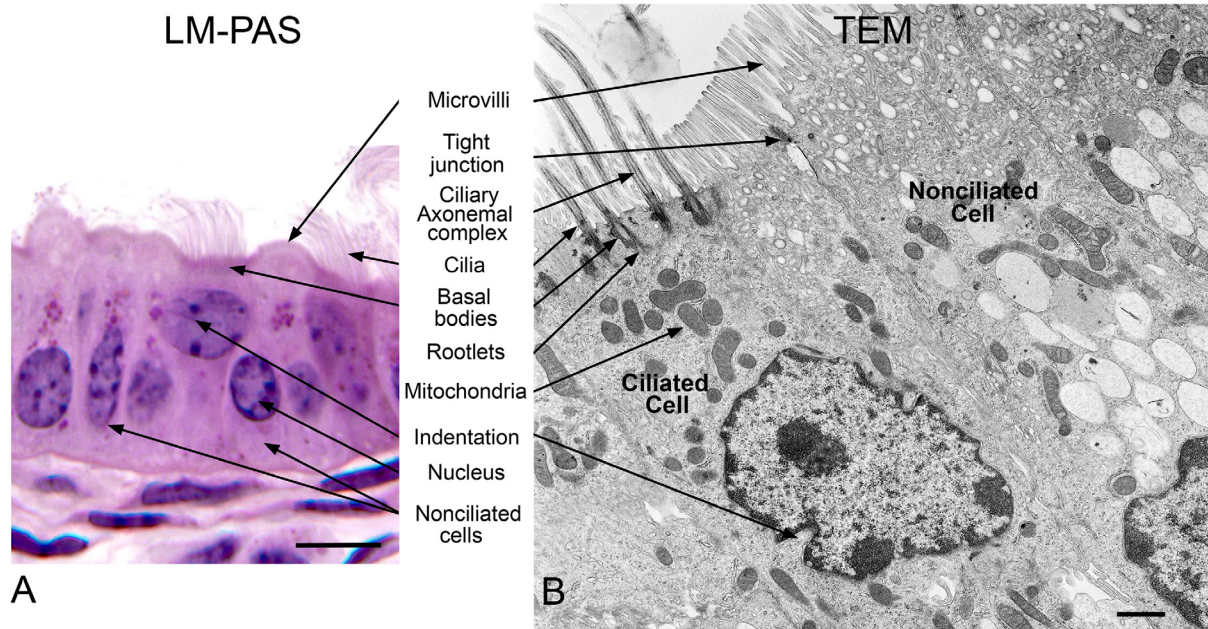


Fig. 5 Light microscopy (LM) with periodic acid/Schiff staining (PAS) and transmission electron microscopy (TEM) of the efferent duct epithelium. (A) LM of mouse epithelium illustrating the ciliated cells with motile cilia extending from a row of basal bodies. A nuclear membrane indentation can sometimes be observed with light microscopy. Nuclei of nonciliated cells are located in the basal cytoplasm. Bar = 10 μm. (B) TEM of rat epithelium illustrating both ciliated and nonciliated cells. The ciliated cell has prominent cilia protruding into the lumen between microvilli and resting on basal bodies and rootlets that extend into the apical cytoplasm. Numerous mitochondria reside under the basal bodies to provide energy for the motile cilia. Bar = 1 μm.

Nonciliated Cell

The major cell type lining the efferent ductule lumen is the nonciliated cell (Fig. 6), whose absorptive function is critical for efferent ductule function. Their reabsorption of luminal fluids increases the concentration of sperm several fold (Clulow et al., 1998). In most species, the ratio of nonciliated to ciliated cells is about 3–5:1, but in the common duct of rats, ciliated cells are increased. Only bulls have a greater proportion of ciliated cells. Nuclei of nonciliated cells are always located basally near the basement membrane and sometimes show considerable indentation of the nuclear membrane. Also consistent with the function of fluid reabsorption is the presence of a highly folded plasmalemma in the basal aspects of the cell. In some regions, at least in rats, large lipid droplets are observed along the basement membrane, but cryomicroscopy and special stains are usually required for visualization (Ilio and Hess, 1994).

The nonciliated cell is characterized by an apical brush border of microvilli that connect the lumen to its apical cytoplasm via a labyrinth of canaliculi, vesicles and apical tubules at the base of the microvilli. Ultrastructural features associated with endocytosis in this cell type reveal large dilated tubular elements between the bases of microvilli and tubular coated pits that extend into the apical cytoplasm. Time course studies of luminal tracers suggest that the apical tubules are part of the elaborate endocytic/lysosomal system (Hermo et al., 1994). Approximately 30% of the apical tubules are recycled back to the apical plasma membrane, but the remaining tubules are involved in the formation of endosomes, multivesicular bodies and finally secondary lysosomes. The endocytic apparatus in the apical cytoplasm of rats (Fig. 6) contains more endosomes and lysosomes than in mice (Hess, 2002). The endocytic apparatus is apparent in the apical cytoplasm with transmission electron microscopy, but with light microscopy it is best visualized using periodic acid/Schiff staining of the associated carbohydrates (Fig. 4). In all species, there are regional differences in staining characteristics, with the terminal zone (common duct in rodents) showing more intense staining for endocytic activity.

Nonciliated cells have two additional distinctive features that are frequently overlooked. First is the presence of apocrine secretion of the apical cytoplasm, which is usually region specific and present only in larger mammals and birds (Ilio and Hess, 1994; Hermo and Jacks, 2002). Secondly, similar to rete testis epithelium, nonciliated cells of efferent ductules also possess a primary cilium that protrudes like a finger into the lumen, above the row of short microvilli (Hess, 2015). Primary cilia have recently been promoted as organelles that help to coordinate cell signaling pathways (Satir et al., 2010). Although nothing is known about this organelle in the male reproductive tract, we can speculate that it has an important signaling activity related to luminal fluid flow and possibly sperm concentration. In kidney, bending of the primary cilium in the direction of fluid flow in proximal tubules is associated with calcium signaling. Changes in the ciliary length are associated with occlusions and the repair of renal tubules (Christensen et al., 2012) and thus also may be important for efferent duct function.

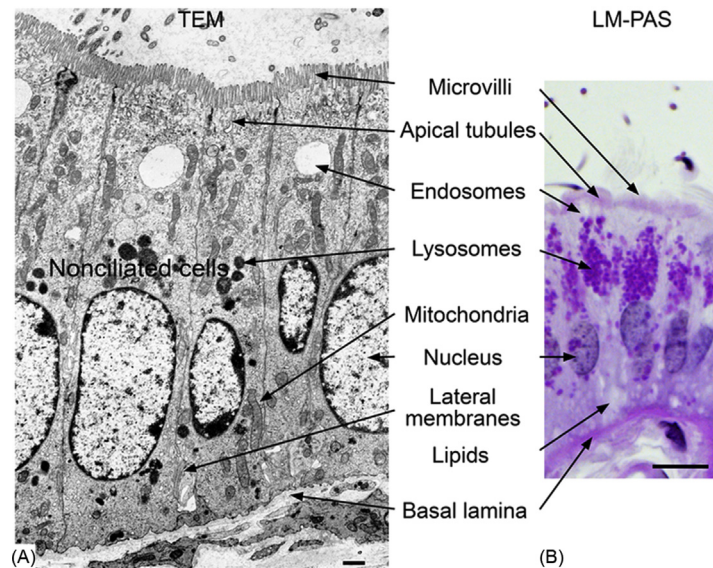


Fig. 6 Transmission electron microscopy (TEM) and light microscopy (LM) with periodic acid/Schiff staining (PAS) of the nonciliated cell in the efferent duct epithelium. (A) TEM of the mouse epithelium with several nonciliated cells showing a luminal brush border of microvilli. Apical tubules extend from the bottom of adjacent microvilli and are involved in endocytosis of luminal fluids through the endosomal/lysosomal apparatus. Long, thin mitochondria are scattered in the cytoplasm and the large nuclei are located near the basal lamina (basement membrane). Lateral plasma membranes are folded near the basal region. Bar = 1 μm . (B) LM of rat nonciliated cells stained with PAS. The microvillus border is less distinct than with TEM. An abundance of endosomes and lysosomes are present in the apical cytoplasm and are PAS+. In some cells, lipid droplets or vacuoles are present near the basement membrane. Bar = 10 μm .

Function of Efferent Ductules

Efferent ductules transport sperm from the rete testis to the epididymis in approximately 45 min in the rat. However, they are more than sperm conduits, as the major function of efferent ductules is a kidney-like reabsorption of luminal fluid, which increases sperm concentration 28-fold as they enter the epididymis (Hess et al., 1997b; Clulow et al., 1998). It is reported that between 50% and 96% of luminal fluids are removed in efferent ducts as sperm transit this region, with most reabsorption occurring in the proximal zone, soon after leaving the rete testis (Clulow et al., 1998; Hess 2002; Hansen et al., 2004). Fluid reabsorption involves multiple activities, including solute transport, passive water permeability, endocytosis and secretion (Hermo et al., 1994; Clulow et al., 1998; Hansen et al., 1999; Hess, 2002). The epithelium is considered to have leaky tight junctions, which allows rapid equilibration of ions between lumen and blood (Hansen et al., 1999). Some of the more important proteins involved include SLC9A3 (Na^+/H^+ exchanger-3), AQP1 and 9 (aquaporins), CAR2 (carbonic anhydrase 2), CFTR (cystic fibrosis transmembrane conductance regulator), and Na^+/K^+ ATPase (Ilio and Hess, 1992; Hansen et al., 1999; Lee et al., 2001; Leung et al., 2001; Joseph et al., 2010).

The endocytosis pathway depends upon an elaborate morphological network of microvilli, coated pits, apical tubules, endosomes and lysosomes (described above). It has been calculated that approximately 50%–90% of the total protein leaving the testis is reabsorbed in the efferent ductules (see review Hess, 2002). Endocytosis may also provide an alternative route for water movement, because the aquaporin-1 (AQP1) knockout mouse did not show fluid accumulation in the lumen comparable to the *Esr1* knockout mouse (Hess et al., 1997b; Zhou et al., 2001). Endocytosis of the sulfated glycoproteins SGP-1 and SGP-2, Sertoli cell secretory proteins found among the luminal macromolecules in efferent ducts, is a classic example of protein removal from luminal fluid by endocytosis. SGP-2 is also called clusterin and apolipoprotein J (Morales et al., 1996). These proteins are bound to sperm in rete testis fluid but released from the plasma membrane of sperm in the efferent ductal lumen.

Physiology of the efferent duct epithelium depends on the presence of Na^+/K^+ -ATPase along the basolateral membranes and a Na^+/H^+ exchanger (SLC9A3) in the apical microvillus membrane (Zhou et al., 2001), which is consistent with Na^+ transport in the kidney (Hansen et al., 1999). About 70% of the fluid reabsorption is dependent on the apical SLC9A3 exchanger, which is higher than that of the kidney. Na^+ transport across the basal membranes is coupled with the movement of other ions such as Cl^- and HCO_3^- (Clulow et al., 1998). In the proximal region, bicarbonate is secreted into the lumen but there is nearly complete reabsorption of bicarbonate in the distal common duct. Thus, efferent ducts have the highest luminal pH (see review Clulow et al., 1998). Overall, the fluid movement model involves active Na^+ transport, luminal flow-dependent reabsorption, adrenal-independent fluid flow, electroneutral ion flux and rapid isosmotic reabsorption that equilibrates with blood plasma (Hansen et al., 2004). Water movement from lumen to interstitium includes both paracellular and transcellular pathways, with differential regulation of water channels in efferent duct epithelial membranes (Oliveira et al., 2005; Ruz et al., 2006; Hermo and Smith, 2011).

Regulation of Efferent Ductule Function

Regulation of efferent ductal function is beginning to be understood, but like in the kidney, fluid reabsorption is under multiple mechanisms of control and thus complex. Basically, there is direct physiological control, as well as an integrated endocrine regulation of fluid reabsorption in efferent ductules. Na^+/H^+ exchange at the apical membrane is most important in regulation of fluid reabsorption (Clulow et al., 1998; Hansen et al., 1999), but molecules involved in this process are regulated by steroid hormone receptor activity. However, direct control of reabsorption is provided by the luminal flow rate, which allows for the fine tuning of fluid reabsorption and careful regulation of sperm concentrations entering the epididymis (Clulow et al., 1998). This flow-dependence may be associated with the presence of motile and primary cilia lining the ductal epithelium (Hess, 2002, 2015), as cAMP has been shown to abolish fluid reabsorption (Man et al., 2003).

Efferent duct epithelium expresses multiple steroid receptors (Hess et al., 2011). AR is present, similar to other regions of the male reproductive tract; however, AR is not the most abundant receptor. Instead, ESR1 is the predominant steroid receptor (Fig. 7) and is expressed in efferent ductules at levels higher than the female uterus (Hess et al., 1997a, 2011). Therefore, for many years it was assumed that 17 β -estradiol (E2), the natural ligand of ESR1 would be the predominant hormone required for regulating efferent duct function. However, such reasoning is incorrect. The steroid estrogen is not essential for efferent duct morphology and function, but its receptor, ESR1, is indispensable.

Esr1 knockout mice (*Esr1*KO) were infertile and showed dramatic rete testis dilation (Eddy et al., 1996), which was subsequently shown to result from the loss of ESR1 activity in the efferent duct epithelium (Hess et al., 1997b). In *Esr1*KO mice, efferent ducts were also distended with luminal fluids and epithelial height was reduced due to the loss of microvilli and endocytic apparatus. Accumulation of luminal fluids produced back pressure within the testis, resulting in dilation of rete testis and seminiferous tubules and long-term testicular atrophy, while androgen levels were elevated. Because the *Esr1*KO mouse was a developmental model, the receptor was also inactivated in adult males using a pure antiestrogen compound and results recapitulated the *Esr1*KO phenotype (Oliveira et al., 2002, 2003; Cho et al., 2003). Thus, blocking the estrogen receptor, without lowering androgens or removing estrogens, completely disrupted efferent duct physiology and morphology. Expression of many proteins involved in fluid reabsorption (SLC9A3, AQP1, 9, CAR2, SLC4A4, CFTR, SLC26a3, ATP1a1) are altered by the loss of ESR1 activity (Lee et al., 2001, 2008; Zhou et al., 2001; Toda et al., 2008; Joseph et al., 2010).

The *Esr1*KO mouse data clearly indicate that the receptor is required for efferent duct morphology and function; therefore, a similar response was expected following blockage of endogenous estrogen production. Aromatase, the enzyme that converts androgens to estrogens, was eliminated in aromatase knockout mice (ArKO), but the males were basically normal at first with an initial fertility and no evidence of fluid accumulation in the testis, although with aging there was a progressive disruption of spermatogenesis (Robertson et al., 2002). These data seemed inconsistent with the *Esr1*KO mouse and at first obfuscated our understanding of estrogen and efferent duct function. However, further studies showed that ESR1 expression is constitutive in efferent ducts and not reduced after castration or ductal ligation (Oliveira et al., 2004). E2, dihydrotestosterone (DHT) and 5- α -androstane-3- β -17- β -diol (a DHT metabolite) stimulated efferent ductal epithelium following castration-induced regression (Oliveira et al., 2004; Picciarelli-Lima et al., 2006). Thus, endocrine regulation of efferent ducts may involve both estrogen and androgen receptors, with or without the natural ligand in a ligand-independent manner or through the binding of a DHT metabolite or by the interaction of AR/ESR1 complexes with specific DNA response elements.

The only way to decrease efferent duct ESR1 has been to treat males with high doses of E2 or to block the receptor with a potent antiestrogen (Oliveira et al., 2003). However, it is interesting that perinatal treatment with the potent synthetic estrogen diethylstilbestrol (DES), with or without the blockade of androgen production, had no effect on ESR1, but did induce disappearance of AR, and resulted in abnormal rete testis and efferent ducts nearly identical to the *Esr1*KO mouse (Rivas et al., 2002). The DES study supports the hypothesis that androgen/estrogen balance, especially developmentally, may be important. Another study also

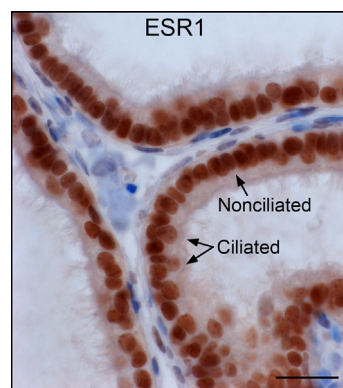


Fig. 7 Mouse efferent ductules immunostained for estrogen receptor, ESR1. Staining is intense in the nuclei of the epithelium. The ciliated cell nucleus appears to have somewhat lighter staining. Light staining is also seen in the cytoplasm. Bar = 20 μm .

suggested a role for AR and ESR1 interaction in the efferent ducts, as exogenous testosterone supplementation increased fluid reabsorption, but an anti-androgen (flutamide) and an anti-estrogen (tamoxifen) gave the same result, while E2 caused a decrease (Clulow et al., 1998). Unfortunately, these early studies did not examine changes in AR and ESR1 expression, making interpretation difficult, as E2 treatment alone downregulates ESR1 and AR (Oliveira et al., 2004). A recent study showing that membrane ESR1, in addition to nuclear presence of ESR1, is required for normal efferent duct morphology (Nanjappa et al., 2016) raises new questions regarding mechanisms that coordinate the regulation of this male reproductive tract region.

Conclusion

The efferent ductules provide an essential connection between the testis and epididymis and their normal function is required for male fertility. In numerous species, efferent ducts occupy a major portion of the head of the epididymis. This kidney-like organ displays a dynamic cellular morphology reflecting its major function in the reabsorption of over 90% of the luminal fluid, which results in a 28-fold increase in sperm concentration. The inaccessible location of these ductules, buried in thick connective tissue in larger mammals or the opaque epididymal fat pad in rodents, has resulted in their morphological and physiological contributions being easily overlooked. The epithelium has a unique presence of motile cilia, which function to stir the luminal fluids, but also primary cilia extending from the surface of nonciliated cells, possibly involved in the complex physiology associated with ion transport and water movement. Regulation of efferent ducts involves complex interactions of both androgens and estrogens, with and without natural ligands, and also requires the presence of at least the membrane estrogen receptor. An understanding of the mechanism of hormonal balance that is required for efferent duct function remains elusive, but it is well established that ESR1 expression is abundant in this region and it stays intact under multiple experimental conditions.

References

- Cho, H. W., Nie, R., Carnes, K., Zhou, Q., Sharief, N. A., & Hess, R. A. (2003). The antiestrogen ICI 162,780 induces early effects on the adult male mouse reproductive tract and long-term decreased fertility without testicular atrophy. *Reproductive Biology and Endocrinology*, 1(1), 57.
- Christensen, S. T., Clement, C. A., Satir, P., & Pedersen, L. B. (2012). Primary cilia and coordination of receptor tyrosine kinase (RTK) signalling. *The Journal of Pathology*, 226(2), 172–184.
- Clulow, J., Jones, R. C., Hansen, L. A., & Man, S. Y. (1998). Fluid and electrolyte reabsorption in the ductuli efferentes testis. *Journal of Reproduction and Fertility. Supplement*, 53, 1–14.
- Cooke, P. S., Young, P., Hess, R. A., & Cunha, G. R. (1991). Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs. *Endocrinology*, 128(6), 2874–2879.
- Eddy, E. M., Washburn, T. F., Bunch, D. O., Goulding, E. H., Gladen, B. C., Lubahn, D. B., & Korach, K. S. (1996). Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology*, 137(11), 4796–4805.
- Gutroff, R. F., Cooke, P. S., & Hess, R. A. (1992). Blind-ending tubules and branching patterns of the rat ductuli efferentes. *The Anatomical Record*, 232(3), 423–431.
- Hansen, L. A., Clulow, J., & Jones, R. C. (1999). The role of Na⁺–H⁺ exchange in fluid and solute transport in the rat efferent ducts. *Experimental Physiology*, 84(3), 521–527.
- Hansen, L. A., Dacheux, F., Man, S. Y., Clulow, J., & Jones, R. C. (2004). Fluid reabsorption by the ductuli efferentes testis of the rat is dependent on both sodium and chloride. *Biology of Reproduction*, 71(2), 410–416.
- Hermo, L., & Jacks, D. (2002). Nature's ingenuity: Bypassing the classical secretory route via apocrine secretion. *Molecular Reproduction and Development*, 63(3), 394–410.
- Hermo, L., & Smith, C. E. (2011). Thirsty business: Cell, region, and membrane specificity of aquaporins in the testis, efferent ducts, and epididymis and factors regulating their expression. *Journal of Andrology*, 32(6), 565–575.
- Hermo, L., Oko, R., & Morales, C. R. (1994). Secretion and endocytosis in the male reproductive tract: A role in sperm maturation. *International Review of Cytology*, 154, 106–189.
- Hess, R. A. (2002). The efferent ductules: Structure and functions. In B. Robaire, & B. Hinton (Eds.), *The epididymis: From molecules to clinical practice* (pp. 49–80). New York: Kluwer Academic/Plenum Publishers.
- Hess, R. A. (2003). Estrogen in the adult male reproductive tract: A review. *Reproductive Biology and Endocrinology*, 1(1), 52.
- Hess, R. A. (2015). Small tubules, surprising discoveries: From efferent ductules in the turkey to the discovery that estrogen receptor alpha is essential for fertility in the male. *Animal Reproduction*, 12(1), 7–23.
- Hess, R. A., Gist, D. H., Bunick, D., Lubahn, D. B., Farrell, A., Bahr, J., Cooke, P. S., & Greene, G. L. (1997a). Estrogen receptor (alpha and beta) expression in the excurrent ducts of the adult male rat reproductive tract. *Journal of Andrology*, 18(6), 602–611.
- Hess, R. A., Bunick, D., Lee, K. H., Bahr, J., Taylor, J. A., Korach, K. S., & Lubahn, D. B. (1997b). A role for oestrogens in the male reproductive system. *Nature*, 390(6659), 509–512.
- Hess, R. A., Bunick, D., Lubahn, D. B., Zhou, Q., & Bouma, J. (2000). Morphologic changes in efferent ductules and epididymis in estrogen receptor-alpha knockout mice. *Journal of Andrology*, 21(1), 107–121.
- Hess, R. A., Fernandes, S. A., Gomes, G. R., Oliveira, C. A., Lazari, M. F., & Porto, C. S. (2011). Estrogen and its receptors in efferent ductules and epididymis. *Journal of Andrology*, 32(6), 600–613.
- Ilio, K. Y., & Hess, R. A. (1992). Localization and activity of Na⁺/K⁺-ATPase in the ductuli efferentes of the rat. *The Anatomical Record*, 234(2), 190–200.
- Ilio, K. Y., & Hess, R. A. (1994). Structure and function of the ductuli efferentes: A review. *Microscopy Research and Technique*, 29(6), 432–467.
- Joseph, A., Hess, R. A., Schaeffer, D. J., Ko, C., Hudgin-Spivey, S., Chambon, P., & Shur, B. D. (2010). Absence of estrogen receptor alpha leads to physiological alterations in the mouse epididymis and consequent defects in sperm function. *Biology of Reproduction*, 82, 948–957.
- La, D. K., Creasy, D. M., Hess, R. A., Baxter, E., Pereira, M. E., Johnson, C. A., Vinken, P., & Snook, S. S. (2012). Efferent duct toxicity with secondary testicular changes in rats following administration of a novel leukotriene a4 hydrolase inhibitor. *Toxicologic Pathology*, 40(5), 705–714.
- Lee, K. H., Finnigan-Bunick, C., Bahr, J., & Bunick, D. (2001). Estrogen regulation of ion transporter messenger RNA levels in mouse efferent ductules are mediated differentially through estrogen receptor (ER) alpha and ERbeta. *Biology of Reproduction*, 65(5), 1534–1541.
- Lee, K. H., Bunick, D., Lamprecht, G., Choi, I., & Bahr, J. M. (2008). Differential expression of genes important to efferent ductules ion homeostasis across postnatal development in estrogen receptor-alpha knockout and wildtype mice. *Asian-Australasian Journal of Animal Sciences*, 21(4), 510–522.
- Lee, K. H., Park, J. H., Bunick, D., Lubahn, D. B., & Bahr, J. M. (2009). Morphological comparison of the testis and efferent ductules between wild-type and estrogen receptor alpha knockout mice during postnatal development. *Journal of Anatomy*, 214(6), 916–925.

- Leung, G. P., Gong, X. D., Cheung, K. H., Cheng-Chew, S. B., & Wong, P. Y. (2001). Expression of cystic fibrosis transmembrane conductance regulator in rat efferent duct epithelium. *Biology of Reproduction*, 64(5), 1509–1515.
- Man, S. Y., Clulow, J., & Jones, R. C. (2003). Signal transduction in the ductuli efferentes testis of the rat: Inhibition of fluid reabsorption by cyclic adenosine 3',5'-monophosphate. *Biology of Reproduction*, 69(5), 1714–1718.
- Morales, C. R., Igdoura, S. A., Wosu, U. A., Boman, J., & Argraves, W. S. (1996). Low density lipoprotein receptor-related protein-2 expression in efferent duct and epididymal epithelia: Evidence in rats for its in vivo role in endocytosis of apolipoprotein J/clusterin. *Biology of Reproduction*, 55(3), 676–683.
- Nanjappa, M. K., Hess, R. A., Medrano, T. I., Locker, S. H., Levin, E. R., & Cooke, P. S. (2016). Membrane-localized estrogen receptor 1 is required for normal male reproductive development and function in mice. *Endocrinology*, 157(7), 2909–2919.
- Oliveira, C. A., Zhou, Q., Carnes, K., Nie, R., Kuehl, D. E., Jackson, G. L., Franca, L. R., Nakai, M., & Hess, R. A. (2002). ER function in the adult male rat: Short- and long-term effects of the antiestrogen ICI 182,780 on the testis and efferent ductules, without changes in testosterone. *Endocrinology*, 143(6), 2399–2409.
- Oliveira, C. A., Nie, R., Carnes, K., Franca, L. R., Prins, G. S., Saunders, P. T., & Hess, R. A. (2003). The antiestrogen ICI 182,780 decreases the expression of estrogen receptor- α but has no effect on estrogen receptor- β and androgen receptor in rat efferent ductules. *Reproductive Biology and Endocrinology*, 1(1), 75.
- Oliveira, C. A., Mahecha, G. A., Carnes, K., Prins, G. S., Saunders, P. T., Franca, L. R., & Hess, R. A. (2004). Differential hormonal regulation of estrogen receptors ER α and ER β and androgen receptor expression in rat efferent ductules. *Reproduction*, 128(1), 73–86.
- Oliveira, C. A., Carnes, K., Franca, L. R., Hermo, L., & Hess, R. A. (2005). Aquaporin-1 and -9 are differentially regulated by estrogen in the efferent ductule epithelium and initial segment of the epididymis. *Biology of the Cell*, 97, 385–395.
- Picciarelli-Lima, P., Oliveira, A. G., Reis, A. M., Kalapothakis, E., Mahecha, G. A., Hess, R. A., & Oliveira, C. A. (2006). Effects of 3- β -diol, an androgen metabolite with intrinsic estrogen-like effects, in modulating the aquaporin-9 expression in the rat efferent ductules. *Reproductive Biology and Endocrinology*, 4(1), 51.
- Rivas, A., Fisher, J. S., McKinnell, C., Atanassova, N., & Sharpe, R. M. (2002). Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: Evidence for importance of the androgen–estrogen balance. *Endocrinology*, 143(12), 4797–4808.
- Robertson, K. M., O'Donnell, L., Simpson, E. R., & Jones, M. E. (2002). The phenotype of the aromatase knockout mouse reveals dietary phytoestrogens impact significantly on testis function. *Endocrinology*, 143(8), 2913–2921.
- Ruz, R., Gregory, M., Smith, C. E., Cyr, D. G., Lubahn, D. B., Hess, R. A., & Hermo, L. (2006). Expression of aquaporins in the efferent ductules, sperm counts, and sperm motility in estrogen receptor- α deficient mice fed lab chow versus casein. *Molecular Reproduction and Development*, 73(2), 226–237.
- Satir, P., Pedersen, L. B., & Christensen, S. T. (2010). The primary cilium at a glance. *Journal of Cell Science*, 123(Pt 4), 499–503.
- Toda, K., Okada, T., Hayashi, Y., & Saibara, T. (2008). Preserved tissue structure of efferent ductules in aromatase-deficient mice. *The Journal of Endocrinology*, 199(1), 137–146.
- Yeung, C. H., Cooper, T. G., Bergmann, M., & Schulze, H. (1991). Organization of tubules in the human caput epididymidis and the ultrastructure of their epithelia. *The American Journal of Anatomy*, 191(3), 261–279.
- Zhou, Q., Clarke, L., Nie, R., Carnes, K., Lai, L. W., Lien, Y. H., Verkman, A., Lubahn, D., Fisher, J. S., Katzenellenbogen, B. S., & Hess, R. A. (2001). Estrogen action and male fertility: Roles of the sodium/hydrogen exchanger-3 and fluid reabsorption in reproductive tract function. *Proceedings of the National Academy of Sciences of the United States of America*, 98(24), 14132–14137.

Endocrinology and Pathology of Rete Testis and Efferent Ductules

Rex A Hess, University of Illinois, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Efferent ducts Delicate and small, coiled tubules, lined by an epithelium of ciliated and nonciliated cells that reabsorb over 95% of the luminal fluids and concentrate sperm that are transported from rete testis to the epididymis.

Funnel organization Efferent ductules found in rodents show this type of formation. Numerous individual ducts arising from the rete testis eventually merge until there is only one small tubule that enters the head of the epididymis, thus funneling all sperm into a more concentrated space.

Faslodex® A pure antiestrogen drug (ICI-182,780) that blocks estrogen binding to estrogen receptors and destabilizes the receptors leading to their degradation and preventing their nuclear transcriptional activation. This compound causes an increase in testosterone.

Granuloma An inflammatory reaction in a tissue, when there is an attempt to wall off the foreign substance. In rete testis and efferent ducts, the foreign substances are sperm, but also immature spermatids that can be sloughed into the seminiferous tubular lumen.

Parallel coils organization Efferent ductules found in larger mammals and man have this type of formation. Most of the numerous individual ducts arising from the rete testis do not merge, but eventually coil and then enter separately into the head of the epididymis.

Rete testis An irregularly-shaped chamber of anastomosing channels, lined by a cuboidal to squamous epithelium, extending from the tunica albuginea and connecting to the separate efferent ductules.

Spermatocele A cyst found in rete testis, efferent ductules or head of the epididymis, which retains a watery fluid and sperm. These can form as abnormal diverticula during development or after an inflammatory response.

Nomenclature

AR Androgen receptor

AQP1 and 9 Aquaporin 1 and 9

cAMP Cyclic adenosine monophosphate

Cftr Cystic fibrosis transmembrane conductance regulator

DES Diethylstilbestrol

DHT Dihydrotestosterone

ESR1 Estrogen receptor-1 (ER α)

*Esr1*KO Estrogen receptor-1 knockout

E2 17 β -estradiol

FGF8 Fetal growth factor-8

OPN Osteopontin

RTF Rete testis fluid

SLC9A3 Na⁺/H⁺ Exchanger-3

T Testosterone

VDR Vitamin D receptor

Endocrinology of Rete Testis and Efferent Ducts

Rete testis and efferent ductules are responsible for the transport of sperm from testis to the head of the epididymis. This transit route for sperm involves an important modification of the luminal fluids, primarily in efferent ductules, where over 95% of the fluid is reabsorbed, thus concentrating sperm prior to storage and maturation in the epididymis. Our knowledge of endocrine regulation of these two, sequential organ chambers has been limited due to the fact that most studies have emphasized the testis and epididymis and because it has been difficult to isolate and work specifically with the smaller and delicate tissues of rete testis and efferent ducts. There is no lack of steroid hormones in the luminal seminal fluids that arrive from seminiferous tubule secretions and steroid receptors are plentiful in both the rete testis and efferent duct epithelium, but direct evidence of functional regulation is found only for the efferent ducts, which appear to depend more on estrogen receptor- α (ESR1) than androgen receptor (AR), the

recognized male hormone receptor. Much of what we have learned has come indirectly from experimental models and various knockout mice, but a pattern has emerged suggesting that hormonal regulation of this region in the male reproductive tract depends on the balance of androgens to estrogens, rather than the specific concentration of either hormone alone, particularly during development.

Hormone Receptors

Androgen receptor (AR) is abundant throughout the male reproductive tract epithelium and is strongly expressed in rete testis and efferent ductules (Table 1). In contrast, reports of ESR1 expression have been inconsistent for rete testis, depending on species and age. On the other hand, ESR1 expression in efferent ductule epithelium has been very strong in all species studied and at all developmental stages. Data for the ciliated cells of the ductal epithelium have been inconsistent, with some being negative for ESR1 in some species and in others being weak to strongly positive. It is noteworthy that the nonciliated epithelial cells of efferent ductules express an abundance of several nuclear and cytoplasmic steroid receptors in the same cell (Fig. 1). However, the way in which multiple steroid receptors interact and sort their use of overlapping cofactors in the regulation of transcription remains to be determined. Nevertheless, the efferent ductule data highlight the importance of an androgen/estrogen balance in its physiology.

Regulation of Function

Fluid entering the rete testis from seminiferous tubules contains an abundance of steroid hormones and metabolites of both androgens and estrogens, as well as progesterone. Information regarding their direct regulation of adult rete testis function is lacking because most studies were focused on testicular effects, which were often determined by ligation of the proximal efferent ducts and collection of the accumulated fluids. Early studies reported large differences between seminiferous tubule fluid and rete testis fluid (RTF), but subsequent studies using better techniques found that sperm and inositol concentrations are similar in both regions, but differences in potassium, chloride and protein concentrations are significant and rete secretions provide some dilution to the luminal contents. Major changes in the luminal fluids occur in the efferent ductules, where sperm concentration is increased 28-fold. Treatment with LH does increase androgen concentration in RTF, but direct effects on rete testis are not known.

Table 1 Hormone receptors found in rete testis and the efferent duct epithelium

Receptor ^a	Rete testis	Efferent duct
AR	+++	+++
ESR1	+/-	++++
ESR2	++	++
PR	-	+/-
VDR	ND	++
THRA	ND	++
FSH-R	ND	+

^aAR: Androgen receptor; ESR1, 2: estrogen receptors α and β ; PR: progesterone receptor; VDR: vitamin D receptor; THRA: thyroid hormone receptor α ; FSH-R: follicle stimulating hormone receptor; ND: not determined.

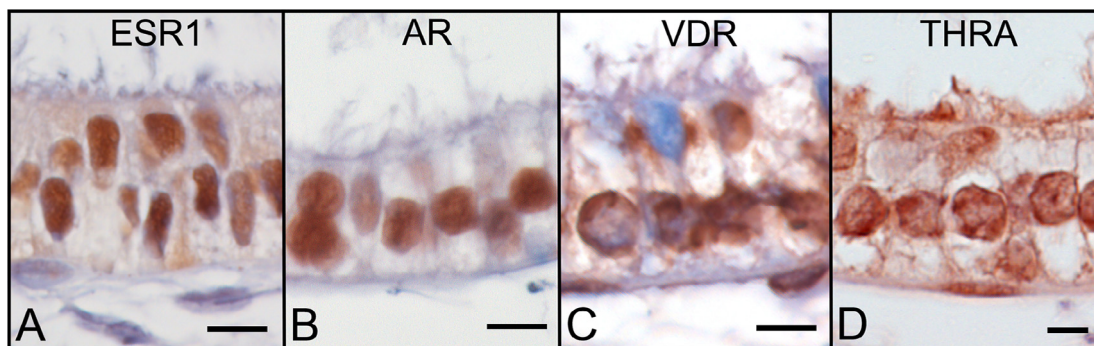


Fig. 1 Immunohistochemical staining for nuclear steroid receptors located in the epithelium of hamster efferent ductules. (A) ESR1 (estrogen receptor- α) showing nuclear staining in both ciliated and nonciliated cells. (B) Androgen receptor (AR) is positive in both epithelial cell types. (C) Vitamin D receptor (VDR) is positive in all epithelial cell types but some ciliated cells are negative (blue nucleus). (D) Thyroid hormone receptor- α (THRA), is positive in all epithelial cell types. Bars = 10 μ m. Previously published by Hess, R.A., Fernandes, S.A., Gomes, G.R., Oliveira, C.A., Lazari, M.F. and Porto, C.S. (2011). Estrogen and its receptors in efferent ductules and epididymis. *Journal of Andrology* 32(6), 600–613 and used with permission.

Regulation of efferent ductule function has received more investigation, but the data paint a complex picture suggesting that multiple mechanisms are involved in the regulation of fluid reabsorption. Exogenous testosterone (T) treatment increased, but estradiol (E2) decreased fluid reabsorption in rat efferent ducts. Tamoxifen (an antiestrogen) and flutamide (antiandrogen) gave results similar to that of T. Testosterone in vitro also inhibits cAMP induced secretion of Cl^- , possibly working through a membrane receptor, and cAMP treatment alone inhibits overall fluid reabsorption. On the other hand, Faslodex[®] (a pure antiestrogen) treatment in the adult results in total blockage of fluid reabsorption and an upregulation of *Cfr*, which increases the movement of water into the lumen. Thus, it is possible that the estrogen pathway is responsible for the reabsorption, while T is responsible for the regulation of secretion, which provides a balance for the overall physiology that is proportional to hormonal concentrations found in the luminal fluids. Alterations in numerous transport-related genes have also been reported, as well as genes associated with extracellular matrix remodeling, which would account for the pathomorphological changes seen in efferent ductules of the *Esr1* knockout mouse (*Esr1KO*). In the *Esr1KO* mouse and Faslodex[®]-treated rodents, *ESR1* is lost but *AR* remains expressed. These data appear to be contradictory at times, but new studies have provided an explanation and emphasize the importance of determining the effects of treatment on the expression of steroid hormone receptors in these tissues, which was lacking in the earlier studies. In efferent ducts, exogenous administration of E2 causes the loss of both *AR* and *ESR1*, while T, as well as dihydrotestosterone (DHT), has no effect on either receptor. Thus, the interpretation of prior physiological studies must be reexamined, especially in light of data from the *Esr1KO* and adult males treated with Faslodex[®], which demonstrated that blocking the estrogen receptor alone, without altering T was sufficient to disrupt the primary physiological function of efferent ductules, reabsorption of over 95% of the luminal fluid.

ESR1 expression is constitutive in efferent duct epithelium and is undisturbed by castration or ligation at the rete testis junction. However, castration and ligation decreased the luminal diameter (due to the loss of rete testis fluids) and reduced epithelial height, even with *ESR1* expression. After castration, *AR* expression was lost. Treatment after castration with E2, T or DHT increased the epithelial height but not to normal levels. The best response was with the combined treatment of E2 and T. Some genes expressed in efferent ductules contain both estrogen and androgen response elements. For example, *Slc9a3* message (the Na^+/H^+ exchanger) was reduced nearly sixfold in *Esr1KO* and antiestrogen-treated mice, but in castrated males, T increased the expression. *SLC9A3* is located along the microvillus border of nonciliated cells and is the major protein responsible for fluid reabsorption. Thus, the complexity of efferent ductule regulation likely requires dual involvement of androgens and estrogens for growth and maintenance of epithelial cell morphology and the physiology of reabsorption and secretion. However, future studies are necessary if we are to uncover the separate, as well as overlapping transcriptional activities in these unique ductules.

One clear example of dual regulation by estrogens and androgens was found in the regulation of aquaporin 9 (AQP9) expression in the microvillus border of the nonciliated cells. Castration, as well as ligation, caused the down-regulation of AQP9 in both efferent ductules and the initial segment epididymis. DHT, but not T, was able to restore AQP9 expression in both efferent ductules and initial segment region. E2 alone, as well as 5α -androstane- 3β - 17β -diol, also restored AQP9 immunostaining in the efferent ducts but not in the epididymis. The antiestrogen Faslodex[®] produced a dramatic reduction in AQP9, without any effect on efferent ductule expression of the *AR*. Therefore, a differential regulation of AQP9 expression was seen in efferent ductules and the epididymis, but only in the efferent ducts was its regulation dependent on both *AR* and *ESR1*. Careful interpretation of these studies is required, as it has been shown that Faslodex[®] also decreases AQP1 expression in efferent ductules. However, with AQP1 it has been demonstrated that its disappearance is secondary, due to the morphological disruption of microvilli, while AQP9 downregulation occurs prior to the loss of the microvillus border. Osteopontin (OPN) is another protein that shows differential regulation between efferent ductules and epididymis. After castration, OPN is lost in the epididymis, but shows no change in the efferent duct epithelium. Unfortunately, the expression of VDR, a known regulator of OPN, was not evaluated, but it is possible that VDR expression is constitutive in efferent ducts, similar to *ESR1*, or that a dual regulation of OPN is provided by the two respective hormones.

The aromatase knockout mouse (*ArKO*) showed no effects on the efferent ductule epithelium and raised questions regarding the claim that estrogen was essential for male reproduction. However, there is continuous expression of *ESR1* in the *ArKO*, as seen after castration; therefore, efferent duct morphology and function is *ESR1* dependent, but may not require direct E2 binding. This conclusion has become more complex with the discovery that loss of the membrane *ESR1*, while retaining nuclear *ESR1*, produces the same efferent duct phenotype as seen in the global *Esr1KO*. These data suggest that the ligand-independent activation of *ESR1* may be important in the efferent duct epithelium, but also dependent on the ability of the membrane *ESR1* to coordinate the nuclear signaling required for fluid reabsorption, which also is known to be reliant on flow dynamics of the luminal fluids.

Pathology of Rete Testis and Efferent Ducts

A much greater understanding of rete testis and efferent ductule physiology has been gathered by the study of pathological lesions. Basically, there are three major ways to induce abnormalities in this region of the male reproductive tract (Fig. 2): (1) physical ligation; (2) luminal compaction or excessive reabsorption; and (3) inhibition of reabsorption. One of the earliest discoveries was that ligation of the efferent ductules leads to the accumulation of fluid from the seminiferous tubules, resulting in dilation of the lumen in both efferent ductules and rete testis. If the ligation is placed near the rete testis, fluid accumulates also in the testis, causing dilation of the tubules, increased testis weight and eventually testicular atrophy, which is called “back pressure atrophy.” A broad range of direct and indirect effects that involve rete testis and efferent ductule dysfunctions is listed and discussed in Table 2.

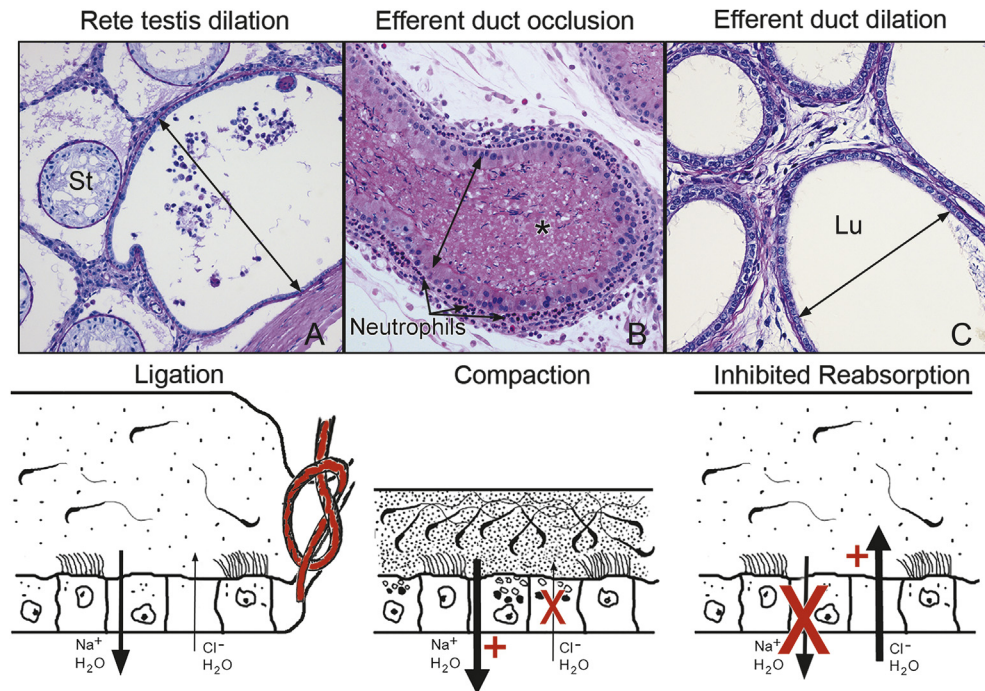


Fig. 2 The drawings represent three basic mechanisms that lead to efferent duct pathological responses: ligation, compaction, and inhibited reabsorption. Histological representations are shown above each drawing. (A) Rete testis dilation (double arrow) and excessive buildup of fluid occurs rapidly following ligation of efferent ductules, occlusions of any kind near the rete testis and inhibition of fluid reabsorption. After ligation, the resorption of Na^+ and H_2O and secretion of Cl^- remain normal but the fluid produced by seminiferous tubules exceeds the physiological limit and begins to accumulate. If the dilation continues unresolved for an extended period of time, seminiferous tubules also dilate and the testis shows a temporary increase in weight. The long-term consequence of rete dilation is seminiferous tubular atrophy (St), with the loss of the germinal epithelium. (B) Efferent ductule occlusion occurs following compaction of the luminal contents (double arrow). The center photo is from a rat treated with the fungicide benomyl, which induces sloughing of immature spermatids along with normal sperm that become compacted in the lumen of efferent ducts (*), due to the excess removal of water and other noncellular components, which produces ductal occlusion. Within hours of treatment, an inflammatory response of massive numbers of neutrophils (polymorphonuclear leukocytes) can be seen surrounding the ductal basement membrane, in an effort to ward off the luminal contents that are antigenic. It is not known if the inflammatory response caused an increased reabsorption of fluid or decreased epithelial secretion, but the end result is an occlusion, which eventually can lead to calcification, fibrosis, and scarring. Under certain conditions, the epithelium may enter mitosis and attempt to re-canal or grow around the occluded area. (C) Efferent ductule dilation (double arrow) occurs following the inhibition of fluid reabsorption and/or excessive secretion into the lumen, as well as blockage downstream near the common duct and initial segment epididymis. Inhibited reabsorption occurs in the *Esr1*KO mouse and following adult exposures to the antiestrogen Faslodex, and involves the downregulation of water channels and ion transport proteins, as well as the upregulation of *Cfr*, which results in the movement of water from the interstitial space into the lumen (Lu). This figure is modified from Hess, R.A. (2014). Disruption of estrogen receptor signaling and similar pathways in the efferent ductules and initial segments of the epididymis. *Spermatogenesis* 4(2), e979103, available online at <http://www.tandfonline.com/doi/full/10.4161/21565562.2014.979103>.

Developmental Pathology

Embryological development is a separate event for rete testis and efferent ductules, until they eventually join in the extratesticular region. Thus, developmental anomalies can result in separate pathological defects, but after the two organs join to form a patent lumen for the passage of sperm, lesions can begin to overlap. It has been shown that kidney malformations can be associated with efferent duct abnormalities, because there are specific genes associated with the formation of kidney proximal tubules (metanephros origin) that are also specific for the developing efferent ducts (mesonephros origin). However, it can be difficult to make interpretations when the rete testis shows cystic formations, because the cause could be the abnormal development of efferent ducts and subsequent luminal obstruction. There are several hormones and specific genes that are essential for normal development of efferent ductules. For example, loss of FGF8 (fetal growth factor-8) results in the failure to form efferent ductules and the *Lgr4* knockout results in less convoluted ducts. Both of these mutant abnormalities result in male infertility.

The effects of diethylstilbestrol (DES) exposure on male reproductive tract development are considered classical examples of estrogen-included developmental disorders. They include cryptorchidism, testicular atrophy, epididymal cysts, sperm abnormalities, sperm granulomas, adenocarcinoma, prostatic inflammation, and other changes that could affect fertility and male reproductive function. Of particular note, DES causes the formation of rete testis adenocarcinomas, luminal dilation of both rete testis and efferent ductules and significant reduction in epithelial cell height. These changes were first thought to be due to an early induction of estrogen receptor in the male tract, but further studies are now pointing to disruption in the androgen/estrogen balance, both in the hormones and their respective receptors.

Table 2 Direct and indirect lesions associated with dysfunctions of rete testis and the efferent ductules

<i>Lesion</i>	<i>Discussion</i>
Inflammation	It is not known if this is a cause or the result of ductal abnormalities; however, neutrophils induce considerable epithelial damage. Inflammatory cells can also enter the lumen and have been associated with sperm stasis and blockage of efferent ducts
Ductal occlusion	Causes may involve: (a) compaction due to excessive reabsorption; (b) epithelial necrosis; (c) luminal calcification; (d) varicocele vascular dilation; (e) blind-ending formations
Phagocytosis	This appears to be an epithelial response to sperm stagnation
Sperm granuloma	Formed in response to sperm stasis, ductal occlusion and inflammation and in larger mammals
Spermatocoele (cyst formation)	This may begin due to a developmental diverticulum of the rete testis or an overgrowth of the epithelium
Fibrosis	This is typically an end-stage result of overproduction of collagen in response to sperm stasis, inflammation and necrosis of the efferent ductule epithelium
Luminal calcification (lithiasis)	Unknown cause, but may involve osteopontin expression in efferent duct epithelium and an imbalance in ESR1 and/or VDR. In rete testis, these could be derived from microlithiasis granules in the seminiferous tubules
Epithelial hyperplasia	This can occur developmentally in the rete testis as a cystic overgrowth or cribriform pockets in the epithelium. It is also seen in efferent ductules after long-term occlusion, specific gene knockouts and in particular epididymal cystadenomas in men
Recanalization	This is a term used to describe growth of the efferent duct epithelium in an attempt to circumvent the luminal occlusion
Ciliary loss	Knockout of genes associated with cilia formation can result in the disruption of fluid reabsorption. It is unclear if the disruption is primary due to failure of fluid mixing or secondary due to indirect effects on the nonciliated cell
Desquamation	Certain toxicants cause necrosis of the epithelium; however, this can be secondary to the disruption in area blood flow
Lipid accumulation	Changes in cytoplasmic lipid vacuoles has been noted in some pathological conditions, but it is not known if this is the cause or a secondary accumulation due to disruption in the endocytosis/lysosomal pathways
Efferent duct luminal dilation	Inhibition of fluid reabsorption will cause dilation in rodent species. Occlusions only in the distal duct will cause dilation in the proximal ducts. Abnormal development of the ductal epithelium will show luminal dilation during the postnatal period
Rete testis luminal dilation	This is a classic or signature response due to efferent duct occlusion in all species, but also a response due to inhibited fluid reabsorption in the rodent species. It may be difficult to separate fluid accumulated induction of dilation from overgrowth, if the lesion has been present from birth. Rete dilation can also occur independent of efferent duct dysfunction in cases of blind-ending rete testis cyst formation
Testicular swelling	This accumulation of fluid occurs only if rete testis fluid cannot exit through the efferent ductules at a required rate of flow. It is caused by ductal occlusion near the rete testis or failure of reabsorption
Seminiferous tubular atrophy	There can be many causes of testicular atrophy that are independent of rete testis and efferent ductule function. However, sustained failure of adequate fluid exit through the rete testis can induce “back-pressure atrophy.” There are species differences in response and it appears the cranial seminiferous tubules are more susceptible to this pathology than are the tubules in the caudal region of the testis

Prenatal exposure to DES has no effect on ESR1, but instead causes the disappearance of AR, which then results in abnormalities in the rete testis and efferent ducts. However, treating with both DES and T did not fully restore the efferent duct lumen, but did restore the rete testis to normal diameter. DES also abolished AQP1 expression in the developing efferent ductal epithelium, but a GnRH antagonist (which would block the stimulation of Leydig cell production of T) produced no change in AQP1. These data are confusing when we consider the *Esr1*KO mouse, in which there is no effect on AR, but ESR1 is lacking in efferent ductules, and the phenotype is nearly identical to that produced by DES. The only conclusion that can be made is that during development a careful balance in the androgen/estrogen pathways is required and that both hormones overlap in as yet unexplained ways.

Adult Pathology

A large number of chemicals have been shown to induce rete testis and efferent ductal abnormalities in the adult male. Some lesions are direct causes of exposure, but sometimes it is difficult to be sure, as they may be indirect effects (Table 2). For example, excessive reabsorption of fluid may induce rete testis dilation but the same lesion is caused also by the inhibition of fluid reabsorption. The reabsorption of fluid at an excessive rate can leave the lumen compacted with sperm, clogging the exit and occluding the efferent ducts. If the occlusion occurs in the proximal efferent ducts, such pathology will also obstruct the rete testis, if the cause is not resolved rapidly. This pathologic response is most susceptible in rodent species that have the funnel organization of efferent ductules. Exposure to the fungicide benomyl, a microtubule poison, illustrates this pathological response (Fig. 2). Benomyl causes massive sloughing of germ cells from the seminiferous epithelium, but sloughing alone does not induce occlusions. Instead, excess removal of luminal fluid concentrates the cellular components, with as yet an unknown mechanism. However, one potential mechanism may involve an inflammatory response, as rapid recruitment of neutrophils has been noted in several cases of toxicant-induced occlusions and granuloma formations in efferent ducts and rete testis. For example, it is known that the tight junctions

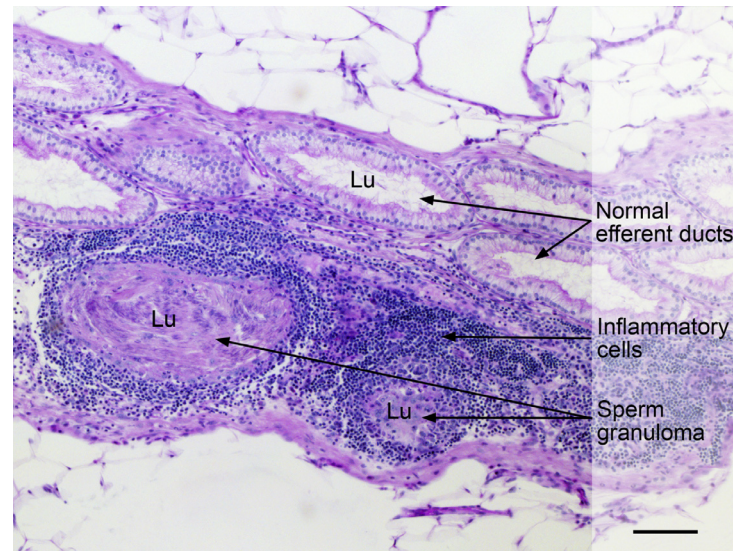


Fig. 3 An example of sperm granuloma formation adjacent to a normal patent efferent ductule. The lumen (Lu) is clear with cilia extending into the opening in the normal ducts, as few sperm are present in the ductules, until after fluid reabsorption. The occluded ducts are surrounded by thick layers of inflammatory cells, some of which have penetrated the epithelium. The lumen (Lu) has become compacted and the epithelium appears nearly destroyed by the process of forming the sperm granuloma. Bar = 100 μ m.

are leaky in this region of the male tract and in experimental allergic orchitis, inflammatory lesions are observed first in the rete testis. A wide range of chemicals induce occlusions, as well as a growing number of gene disruption models. In most cases, it appears that either a direct alteration in fluid reabsorption has occurred or there has been an induced leakage of sperm antigens from the lumen, which causes inflammation and eventually the formation of the granulomas (Fig. 3).

Dilation due to reabsorption failure in rete testis and efferent ducts is opposite to that of occlusion. The responses to both dilation and occlusion are now recognized as classical or signature lesions, in the sense that if blockage occurs near the rete testis, with lumens greatly expanded by excess fluid, the sequella will include a temporary increase in testis weight, seminiferous tubular dilation and subsequent atrophy. The primary cause of luminal dilation is the failure to reabsorb fluids at a required rate, particularly in the rodent species. The resulting excess fluid overwhelms the funnel organization found in the terminal segment of the efferent ducts. Species having numerous parallel coils of ductules entering independently into the epididymis have not shown this typical response, although individual ductules can show dilation, as well as regions of the rete testis. ESR1 expression is an absolute requirement for fluid reabsorption in efferent ductules, with or without the presence of E2 produced by aromatase activity. The pathological response to ESR1 loss or interference with its transactivation is a dramatic dilation of ductal lumens and loss of epithelial organelles required for fluid reabsorption, which also results in a dramatic decrease in epithelial height. This response is found early during development in the *Esr1* KO, but treatment of adult wild-type males with the potent antiestrogen Faslodex[®], and other chemicals that target ESR1, also result in a nearly identical pathology. Other causes of this pathological response include disruption of ciliary formation, loss of the sodium/hydrogen exchanger, carbonic anhydrase II, polycystin-1 and vasodilation of blood vessels near the rete testis.

Pathology in Men

There are numerous reports of rete testis and efferent ductule pathologies in men. It has been stated that 1/3 of human epididymides show evidence of one or more efferent ductules being occluded and pathological changes are more common in efferent ducts than in the epididymis and vas deferens. These reports primarily deal with various types of cyst formations, with several variations on this theme, including the following: cystadenoma, cystic ectasia, cystic dysplasia, congenital blind-ends, and spermatocele. These cysts can be associated with inflammation, as well as efferent duct occlusions, but in some cases the exit of sperm into the epididymis remains intact. At least five human diseases have been associated with abnormal functions of efferent ducts and rete testis due to developmental defects. These diseases include: von Hippel-Lindau disease, Young's syndrome, varicocele (dilated pampiniform veins), cystic fibrosis, and kidney disease or renal failure.

Conclusions

Pathological changes in rete testis and the efferent ductules are commonly associated with the endocrine regulation of these tubular organs. The complexity of these pathological changes appears to involve a disruption in the delicate balance between androgen and

estrogen signaling pathways, in an epithelium that expresses multiple nuclear steroid receptors in the same cell. The intricate coordination of this endocrinology is further complicated by the presence of kinetic ciliated cells in the efferent ducts and primary cilia in both the rete testis and efferent ducts. An understanding of the mechanisms required for maintenance of the hormonal balance will help to establish the primary versus secondary causes of reproductive pathology in this region of the male reproductive tract.

Further Reading

- Arao, Y., Hamilton, K. J., Goulding, E. H., Janardhan, K. S., Eddy, E. M., & Korach, K. S. (2012). Transactivating function (AF) 2-mediated AF-1 activity of estrogen receptor alpha is crucial to maintain male reproductive tract function. *Proceedings of the National Academy of Sciences of the United States of America*, 109(51), 21140–21145.
- Clulow, J., Jones, R. C., Hansen, L. A., & Man, S. Y. (1998). Fluid and electrolyte reabsorption in the ductuli efferentes testis. *Journal of Reproduction and Fertility. Supplement*, 53, 1–14.
- Cooke, P. S., Nanjappa, M. K., Ko, C., Prins, G. S., & Hess, R. A. (2017). Estrogens in male physiology. *Physiological Reviews*, 97(3), 995–1043.
- Free, M. J., & Jaffe, R. A. (1979). Collection of rete testis fluid from rats without previous efferent duct ligation. *Biology of Reproduction*, 20(2), 269–278.
- Ganjam, V. K., & Amann, R. P. (1976). Steroids in fluids and sperm entering and leaving the bovine epididymis, epididymal tissue, and accessory sex gland secretions. *Endocrinology*, 99(6), 1618–1630.
- Georgas, K. M., Chiu, H. S., Lesieur, E., Rumballe, B. A., & Little, M. H. (2011). Expression of metanephric nephron-patterning genes in differentiating mesonephric tubules. *Developmental Dynamics*, 240(6), 1600–1612.
- Hess, R. A. (2002). The efferent Ductules: Structure and functions. In B. Robaire, & B. Hinton (Eds.), *The epididymis: From molecules to clinical practice* (pp. 49–80). New York: Kluwer Academic/Plenum Publishers.
- Hess, R. A. (2014). Disruption of estrogen receptor signaling and similar pathways in the efferent ductules and initial segments of the epididymis. *Spermatogenesis*, 4(2), e979103.
- Hess, R. A. (2015). Small tubules, surprising discoveries: From efferent ductules in the turkey to the discovery that estrogen receptor alpha is essential for fertility in the male. *Animal Reproduction*, 12(1), 7–23.
- Hess, R. A., Fernandes, S. A., Gomes, G. R., Oliveira, C. A., Lazari, M. F., & Porto, C. S. (2011). Estrogen and its receptors in efferent ductules and epididymis. *Journal of Andrology*, 32(6), 600–613.
- Hess, R. A., & Nakai, M. (2000). Histopathology of the male reproductive system induced by the fungicide benomyl. *Histology and Histopathology*, 15(1), 207–224.
- Hoshii, T., Takeo, T., Nakagata, N., Takeya, M., Araki, K., & Yamamura, K. (2007). LGR4 regulates the postnatal development and integrity of male reproductive tracts in mice. *Biology of Reproduction*, 76(2), 303–313.
- Joseph, A., Shur, B. D., & Hess, R. A. (2011). Estrogen, efferent ductules, and the epididymis. *Biology of Reproduction*, 84(2), 207–217.
- Kohno, S. J., Munoz, A., Williams, T. M., Teuscher, C., Bernard, C. C., & Tung, K. S. (1983). Immunopathology of murine experimental allergic orchitis. *Journal of Immunology*, 130, 2675–2682.
- Lee, K. H., Bunick, D., Lamprecht, G., Choi, I., & Bahr, J. M. (2008). Differential expression of genes important to efferent ductules ion homeostasis across postnatal development in estrogen receptor-alpha knockout and wildtype mice. *Asian-Australasian Journal of Animal Sciences*, 21(4), 510–522.
- Leung, G. P., Cheng-Chew, S. B., & Wong, P. Y. (2001). Nongenomic effect of testosterone on chloride secretion in cultured rat efferent duct epithelia. *American Journal of Physiology. Cell Physiology*, 280(5), C1160–1167.
- Luedtke, C. C., McKee, M. D., Cyr, D. G., Gregory, M., Kaartinen, M. T., Mui, J., & Hermo, L. (2002). Osteopontin expression and regulation in the testis, efferent ducts, and epididymis of rats during postnatal development through to adulthood. *Biology of Reproduction*, 66(5), 1437–1448.
- Man, S. Y., Clulow, J., & Jones, R. C. (2003). Signal transduction in the ductuli efferentes testis of the rat: Inhibition of fluid reabsorption by cyclic adenosine 3',5'-monophosphate. *Biology of Reproduction*, 69(5), 1714–1718.
- Nanjappa, M. K., Hess, R. A., Medrano, T. I., Locker, S. H., Levin, E. R., & Cooke, P. S. (2016). Membrane-localized estrogen receptor 1 is required for normal male reproductive development and function in mice. *Endocrinology*, 157(7), 2909–2919.
- Newcombe, N., Clulow, J., Man, S. Y., & Jones, R. C. (2000). pH and bicarbonate in the ductuli efferentes testis of the rat. *International Journal of Andrology*, 23(1), 46–50.
- Nistal, M., Gonzalez-Peramato, P., Sousa, G., Garcia-Cabezas, M. A., Rodriguez, J. I., & Cajaiba, M. M. (2010). Cystic dysplasia of the epididymis: A disorder of mesonephric differentiation associated with renal maldevelopment. *Virchows Archiv*, 456(6), 695–702.
- Oliveira, C. A., Carnes, K., Franca, L. R., Hermo, L., & Hess, R. A. (2005). Aquaporin-1 and -9 are differentially regulated by estrogen in the efferent ductule epithelium and initial segment of the epididymis. *Biology of the Cell*, 97, 385–395.
- Oliveira, C. A., Mahecha, G. A., Carnes, K., Prins, G. S., Saunders, P. T., Franca, L. R., & Hess, R. A. (2004). Differential hormonal regulation of estrogen receptors ER alpha and ER beta and androgen receptor expression in rat efferent ductules. *Reproduction*, 128(1), 73–86.
- Pereira, M. F., Fernandes, S. A., Nascimento, A. R., Siu, E. R., Hess, R. A., Oliveira, C. A., Porto, C. S., & Lazari, M. F. (2014). Effects of the oestrogen receptor antagonist Fulvestrant on expression of genes that affect organization of the epididymal epithelium. *Andrology*, 2(4), 559–571.
- Picciarelli-Lima, P., Oliveira, A. G., Reis, A. M., Kalapothakis, E., Mahecha, G. A., Hess, R. A., & Oliveira, C. A. (2006). Effects of 3-beta-diol, an androgen metabolite with intrinsic estrogen-like effects, in modulating the aquaporin-9 expression in the rat efferent ductules. *Reproductive Biology and Endocrinology*, 4(1), 51.
- Piner, J., Sutherland, M., Millar, M., Turner, K., Newall, D., Sharpe, R. M., 2002. Changes in vascular dynamics of the adult rat testis leading to transient accumulation of seminiferous tubule fluid after administration of a novel 5-hydroxytryptamine (5-HT) agonist. *Reproductive Toxicology* {Piner, 2002 #3901} 16 (2), 141–150.
- Rivas, A., McKinnell, C., Fisher, J. S., Atanassova, N., Williams, K., & Sharpe, R. M. (2003). Neonatal coadministration of testosterone with diethylstilbestrol prevents diethylstilbestrol induction of most reproductive tract abnormalities in male rats. *Journal of Andrology*, 24(4), 557–567.
- Setchell, B. P., Davies, R. V., Gladwell, R. T., Hinton, B. T., Main, S. J., Pilsworth, L., & Waites, G. M. H. (1978). The movement of fluid in the seminiferous tubules and rete testis. *Annales de Biologie Animale, Biochimie, Biophysique*, 18(2B), 623–632.
- Toda, K., Okada, T., Hayashi, Y., & Saibara, T. (2008). Preserved tissue structure of efferent ductules in aromatase-deficient mice. *The Journal of Endocrinology*, 199(1), 137–146.
- Wang, Y. Y., Lin, Y. H., Wu, Y. N., Chen, Y. L., Lin, Y. C., Cheng, C. Y., & Chiang, H. S. (2017). Loss of SLC9A3 decrease CFTR protein and causes obstructed azoospermia in mice. *PLoS Genetics*, 13(4), e1006715.
- Yao, G., Hu, S., Yu, L., Ru, Y., Chen, C. D., Liu, Q., & Zhang, Y. (2017). Genome-wide mapping of in vivo ERα-binding sites in male mouse efferent ductules. *Endocrinology*, 158(11), 3724–3737.
- Yasuhara, F., Gomes, G. R., Siu, E. R., Suenaga, C. I., Marostica, E., Porto, C. S., & Lazari, M. F. (2008). Effects of the antiestrogen fulvestrant (ICI 182,780) on gene expression of the rat efferent ductules. *Biology of Reproduction*, 79(3), 432–441.

Cell Biology of the Epididymis

Robert Sullivan and Clémence Belleannée, Université Laval, Quebec City, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Glossary

Apocrine secretion Secretion of cellular components (e.g., proteins, peptides, nucleic acids) through blebbing of the apical membrane of cells and the subsequent release of membrane-bound vesicles into the lumen.

Blood–epididymis barrier Physical barrier formed at the apical pole of the epithelium of the epididymis to prevent equilibration between blood and epididymal fluid. This barrier maintains the unique composition of the latter that is important for sperm maturation and protection.

Epididymal fluid Fluid that bathes spermatozoa during their maturation in the epididymis. The composition of this fluid varies along the epididymal tubule as a result of strong secretion/reabsorption activities.

Epididymal sperm Spermatozoa that are found in the epididymis. Those entering the epididymis are immotile and unable to fertilize, while those stored in the distal region of the epididymis possess all the abilities required to fertilize an oocyte.

Epididymis A single convoluted tubule that transports spermatozoa from the testis to the *vas deferens*. This organ is responsible for the transport, maturation, and storage of spermatozoa.

Merocrine secretion The most common secretion pathway. Intracellular membrane-bound vesicles are carried to the cell membrane, and their contents (e.g., proteins) are secreted into the extracellular environment following the transient fusion of secretory vesicles with the outer cell membrane.

Posttesticular sperm maturation The process during which immature spermatozoa (i.e., nonmotile and devoid of fertilizing ability) acquire their capacity to move, to recognize and fertilize an oocyte. This occurs in the epididymis.

Evolution of the Epididymis in Vertebrates

The epididymis, from Greek *epi* for “on” and *-didymoi* for “twins” or “testicles,” is derived from the Wolffian duct. It connects the *rete testis* via multiple *vas efferens*, to the *vas deferens*. It is the hallmark of vertebrate phyla practicing internal fertilization such as cartilaginous fishes (elasmobranchs), reptiles, birds, and mammals. In species with scrotal testicles, the epididymis is located on the gonad surface whereas in species with inguinal or abdominal gonads, the distal portion of the epididymis with sperm storage functions is located more externally. In vertebrate phyla practicing external fertilization such as amphibians and teleostean fishes, the epididymis is not part of the male reproductive tract. In these species, fully functional spermatozoa are transported from the testis to the exterior via the spermatic duct (Jones, 2002).

In vertebrate species practicing internal fertilization, testicular sperm, even though fully differentiated, are nonfunctional and must transit the epididymis to acquire their fertilizing ability and forward motility. In these species, sperm release is not always synchronized with ovulation. It can be speculated that the epididymis generates heterogeneity among spermatozoa in such a way that subpopulations of male gametes deposited in the female genital tract will be functional at different time points in order to increase the fertility window of a given ejaculate.

Epididymis Anatomy

The epididymis is a long single highly convoluted tubule. The unraveled epididymal duct can reach a length of 50–60 m in large mammalian species and 3–5 m in humans. Sperm transit along the epididymis takes 3–12 days depending on the species and the length of the tubule. The diameter of the intraluminal compartment of the epididymis increases from the proximal to the distal region and the sperm concentration (spermatocrit) increases dramatically along the epididymis. Indeed, water reabsorption is one function performed by the epithelium bordering the intraluminal compartment of the epididymis and results in an increase in sperm concentration.

Based on gross anatomy, the epididymis is divided into three segments; the proximal caput (the head); an elongated middle corpus region and a distal, swollen cauda (Fig. 1). An additional proximal segment can be distinguished in rodent epididymis called the initial segment. Histology of the latter is characterized by epithelial cells with a cuboidal appearance when compared to the epithelial cells bordering the lumen of the caput epididymal segment. In large mammals and humans, the initial segment is not distinguishable. In fact, the proximal epididymal segment of the human epididymis is mainly formed by *vas efferens*. With regard to the male reproductive tract in general, the anatomy of the epididymis varies from one species to another (Yeung and Cooper, 2002; Sullivan and Mieusset, 2016). The abdominal, inguinal, and scrotal location of the testis also has an influence on the anatomy of the epididymis (Kleisner et al., 2010).

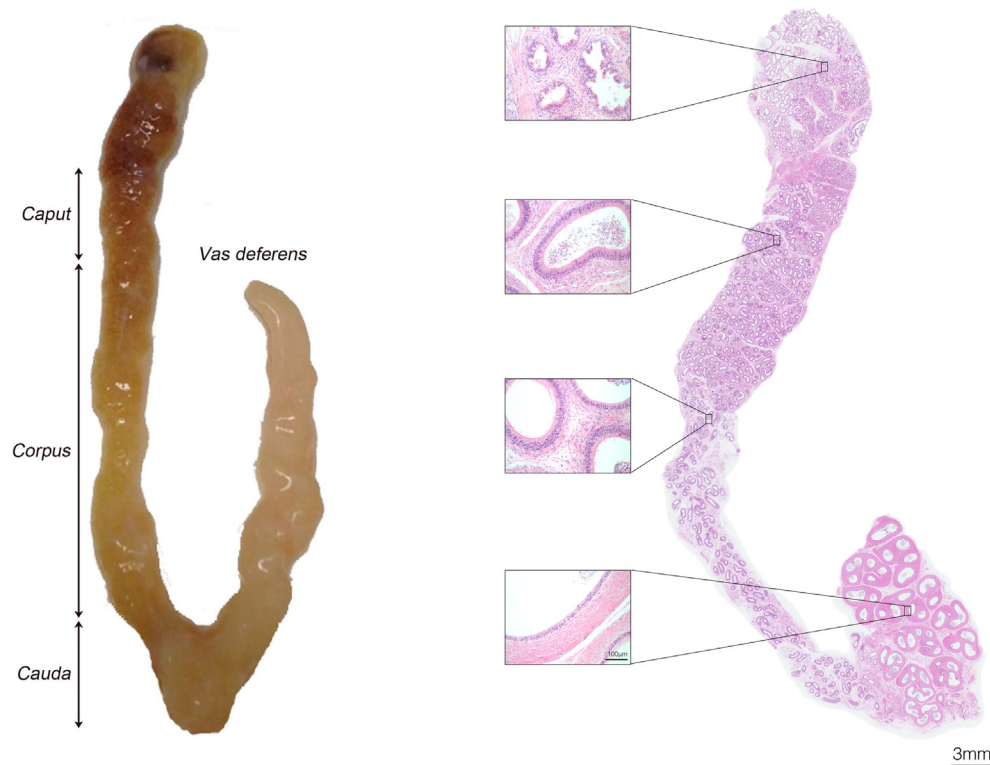


Fig. 1 The human epididymis. A photograph (*left*) and a longitudinal section together with cross sections (*right*) of a human epididymis. *Right panel* reproduces with permission from Sullivan, R., Belleannee, C. (2017). Role of the epididymis in sperm maturation. In de Jonge, C. J. and Barratt, C.L.R. (eds). *The sperm cell. Production, maturation, fertilization, regeneration*, 2nd edn. Cambridge: Cambridge University Press.

Connective tissue septa that separate the epididymis into segments have been described in laboratory rodents as well as in a limited number of species such as dogs and primates. These structures are constant from one individual to another and as such the epididymis can be divided into a fixed number of segments: 10 in the mouse and 19 in rats. It has been hypothesized that each of these segments responds differently to lumicrine factors controlling gene expression along the epididymis. In other mammalian species, including man, these connective tissues are not as well organized. Additional experimental work is necessary to understand the functionality of epididymal segmentation by connective tissue septa that has been described in some species (Turner et al., 2003).

Roles of the Epididymis: An Overview

With its location between the *rete testis* and the *vas deferens*, the epididymis is involved in *sperm transport* along the male reproductive tract. As testicular and epididymal spermatozoa are immotile, sperm transport depends on peristaltic movement of the epididymal tubule. Contraction of smooth muscle layers surrounding the tubule is also involved. Epididymal smooth muscle layers surrounding the epididymal tubule in the cauda segment and around the *vas deferens* are developed in humans and are involved in sperm progression at ejaculation.

As evidenced by pioneering studies performed on different mammalian species, the epididymis is of major importance to male fertility. Although spermatozoa collected from the caput epididymis are fully differentiated, they are immotile and unable to fertilize the egg (Orgebin-Crist, 1967). In vitro fertilization and artificial insemination performed with epididymal spermatozoa showed that sperm functionality increases during epididymal transit; the first sperm capable of fertilization appear in the corpus segment. Acquisition of both motility and fertilizing ability by spermatozoa transiting along the epididymis is collectively known as *sperm maturation*. Due to the extreme compaction of their DNA and the absence of many organelles such as Golgi/endoplasmic reticulum and ribosomes, spermatozoa leaving the testis are transcriptionally, and translationally inactive cells. Thus, they are incapable of controlling their own protein neosynthesis and function. Therefore, posttesticular sperm maturation exclusively depends on extracellular factors secreted by the epithelial cells surrounding the intraluminal compartment of the epididymis (Fig. 2). Proteins synthesized by the epididymal epithelium and secreted into the intraluminal milieu are essential for sperm maturation (Cornwall, 2009). Transcriptional as well translational activity of the epididymis are under androgen control: testosterone is secreted by the testis and dihydrotestosterone is synthesized locally through high epididymal alpha-reductase activity (Robaire and Hamzeh, 2011).

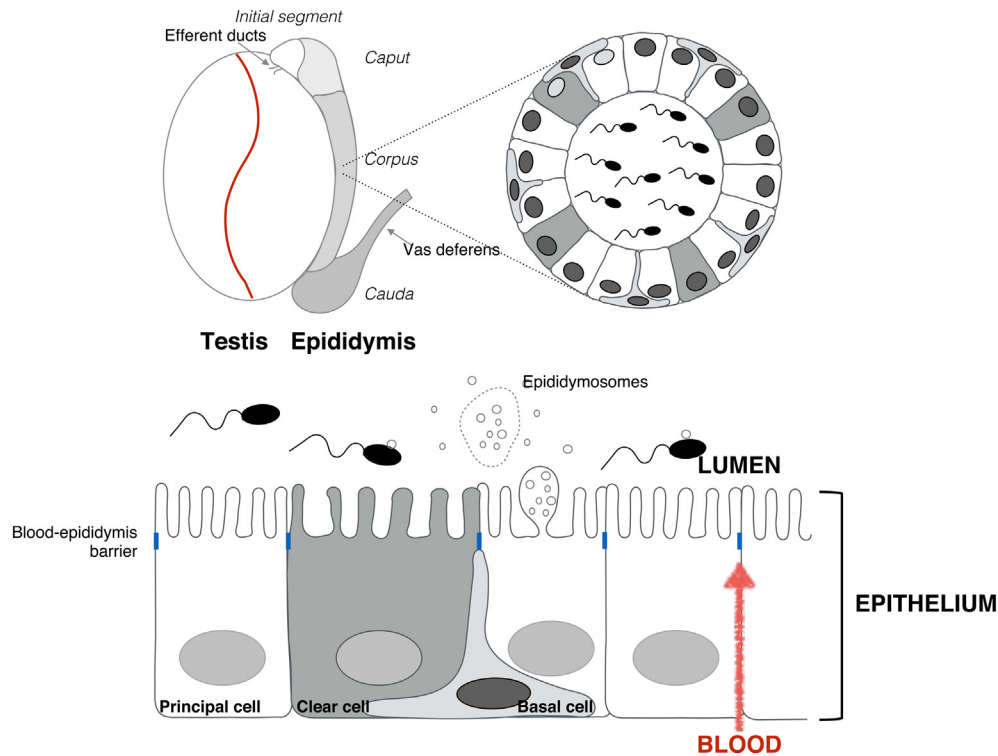


Fig. 2 Schematic diagram of the gross anatomy and the cellular organization of the epididymal epithelium.

Tight junctions between epithelial cells of the epididymal tubules allow maintenance of an *intraluminal milieu* that is distinct from other body fluids. In comparison to composition of the blood, the epididymal fluid calcium concentration is markedly lower, and phosphate and potassium concentrations are increased whereas sodium is decreased. The pH is acidic (pH around 6.5) and the osmolality is much higher than arterial blood. This peculiar inorganic salt composition of the epididymal fluid may contribute to dehydration of spermatozoa during epididymal transit. The tight junctions between epididymal epithelial cells form the *blood–epididymal barrier*, which, together with the blood–testis barrier, protect sperm against the immune system (Cyr et al., 2007). Spermatogenesis and the presence of sperm in the male reproductive tract occur long after the establishment of the immune defense of an individual. Tight junctions along the male reproductive tract ensure that the spermatozoa harboring specific autoantigens are isolated from the immune system. Together with this physical barrier, other factors are involved in the rather complex immune regulatory processes of the epididymis.

The distal cauda segment of the epididymis has a bulbous appearance. Compared to the upper epididymal segments, the thickness of the epithelium is reduced and the lumen diameter is greatly increased. The thinness of the epididymal epithelium in this segment correlates with low protein synthesis and secretory activities. In fact, the transcriptome of the cauda region reveals a much less complex gene expression pattern when compared to the caput and corpus epididymides. Whereas luminal secretions from caput and corpus epididymides are required for the acquisition of sperm motility and fertilizing ability, cauda epididymal secretions are important for the associated *sperm reservoir function*. The cauda epididymis has evolved to ensure that a sufficient number of spermatozoa will be available at ejaculation. The demand for sperm storage obviously depends on the reproductive behavior of each species. With respect to testis size, the cauda epididymis sperm reservoir capacity is important in species practicing polygynous or multimale/multifemale mating systems when compared to a monogamous species. In humans, the sperm reservoir capacity of the cauda epididymidis is estimated at 2–3 ejaculates; it can be as high as 10–15 ejaculates in large domestic animals. Experimental cryptorchidism in which the testis and/or the epididymis is relocated in the inguinal canal or in the abdominal cavity, clearly demonstrates that the cauda epididymis, and thus the sperm reservoir function, is markedly affected by temperature increase. From these observations, it was hypothesized that the cauda epididymis was the driving force for testicular descent. Once ejaculated, spermatozoa will survive 24–36 h in a physiological solution. In the cauda epididymis, these cells can survive up to 15 days. The sperm storage capacity of the cauda epididymis involves maintenance of sperm metabolic quiescence by preserving a low intraluminal pH and sodium concentration and a high fluid viscosity to inhibit flagellar motility. This is coupled with secretion of factors that inhibit sperm activation and capacitation. At ejaculation, these inhibitory factors are diluted allowing sperm activation (Bedford, 2015).

The epididymal functions of sperm transport, protection, maturation, and storage are complex. They depend on intricate interactions between the epididymal epithelium bordering the intraluminal compartment with lumicrine factors originating from the testis or from more proximal segments of the duct and/or with transiting spermatozoa. Epithelial cell populations found

in the epididymis exhibit specific gene expression patterns and exert independent functions to ensure proper posttesticular sperm processing in this organ. However, as evidenced by ex vivo/in vivo studies performed on whole epididymal tubules, epithelial cells also participate in a well-orchestrated intercellular communication system (Cornwall, 2009).

It is obvious that defects in these complex cell biology processes can impact male fertility even though the implication of the epididymis in the pathophysiology of sterility or infertility in males has been poorly investigated. On the other hand, these processes have been proposed as targets for novel male contraceptive strategies.

Despite its apparently simple organization, the epididymal epithelium is composed of different cell types that intercommunicate with each other (Fig. 2). This well-orchestrated cell organization is essential for optimal control of the sperm environment and epididymal functions. In the following sections, we will (i) portray the overall features of the epididymal epithelium, (ii) describe the unique features of each of the main cell types found in the epididymis, and (iii) summarize the principal functions ensured by each of these epididymal cell types.

Overall Features of the Epididymal Epithelium

The epididymis is formed by a two-layered pseudostratified epithelium (Fig. 2). The base of the epithelium is separated from the connective tissue by a basement membrane formed by a fibrous extracellular matrix. Smooth muscle and blood vessel endothelial cells populate the connective tissue. The apical pole of the epithelium is in direct contact with the sperm-bathing epididymal fluid and exposes microvilli, whose cytoskeleton is composed of actin filaments (as opposed to true stereocilia, which are microtubule-based structures). The composition of the intraluminal epididymal fluid is unique in terms of protein/ion content and, pH/osmolality parameters, and is important for sperm maturation. The presence of tight junctions between epididymal epithelial cells forms a blood–epididymis barrier, which controls this sperm-surrounding environment and discriminates it from blood. This junctional complex encompasses gap junctions that ensure communication and coordination between cells throughout the entire length of the epididymal tubule (Hermo and Robaire, 2002).

Epididymal Epithelial Cell Structures

Seven distinct cell populations are found in the different segments of the epididymis. While *clear cells* are found in the caput, corpus and cauda epididymidis, *narrow* and *apical cells* are exclusively present in the initial segment. *Principal cells*, which represent up to 80% of total epithelial cells, *basal cells*, *halo cells*, and *dendritic cells* are found along the entire tubule (Belleannee et al., 2012).

Principal cells

Principal cells are the major cell type found in the epididymis and exhibit a tall columnar shape in the proximal regions that transitions to a squared-off appearance in the distal regions. Principal cells are composed of efficient secretory and endocytic apparatus including endoplasmic reticulum, Golgi apparatus and secretory granules as well as coated pits, endosomes, multivesicular bodies, and lysosomes. The apical pole membrane of principal cells forms elongated microvilli that increase the surface area for exchange between epithelial cells and the epididymal fluid by up to 25 times. Principal cell microvilli measure on average 500 nm–1.0 μ m in length and 100 nm in width and are packed on the cell surface in large numbers to form the epididymal brush border (Hermo, 1995). This brush border allows for intraluminal components to be absorbed at a faster rate into principal cells through specific transporters. For instance, principal cells specifically express several channels and transporters such as: cystic fibrosis transmembrane conductance regulator (CFTR), aquaporin 9 (AQP9), bradykinin type 2 receptor, pannexins, chloride channel protein 3 (ClC3), membrane-associated carbonic anhydrases CAIV and CAII, and TRPV6 and TMEM16A that mediate the transport of elements important to epididymal physiology (i.e., ATP, chloride, calcium, water, and bicarbonate).

Epithelial principal cells are specialized in fluid secretion. At least three types of secretion exist in the epididymis: (1) secretion/reabsorption of ions, organic compounds, and water through transporters located at the apical pole of the cells, (2) merocrine, and (3) apocrine secretions. (1) *Secretion/reabsorption through transporters*. Epithelial cells lining the epididymis express transporters responsible for considerable changes to fluid composition throughout the epididymis. These changes include net water, Na^+ , Cl^- , and HCO_3^- reabsorption, K^+ secretion, carnitine secretion, and luminal acidification through aquaporins (water transporters), the $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter (NKCC1), cystic fibrosis transmembrane conductance regulator (CFTR, Cl^- , and HCO_3^- conducting ion channel), the organic cation/carnitine transporter (OCTN2), the Na^+/H^+ exchanger (NHE3), and the $\text{Cl}^-/\text{HCO}_3^-$ exchanger (AE2). These secretions into the lumen of the epididymis occur under the control of adrenergic and purinergic agonists and peptide hormones. (2) *Merocrine secretion*. Epididymal principal cells possess an extensive Golgi apparatus, which ensures the secretion of diverse glycoproteins tagged with a signal peptide through the classical merocrine secretion pathway. Exhaustive proteomic studies have shown that secretion/reabsorption of proteins is dynamic along the epididymal tubule and thereby creates distinct microenvironments in which spermatozoa sequentially acquire their fertilizing abilities. (3) *Apocrine secretion*. Apocrine secretion consists of the budding of the apical membrane of principal cells. Once released into the intraluminal environment, these blebs containing selected intracellular organelles of epithelial cells release small membranous vesicles referred to as epididymosomes (Machtinger et al., 2016) (Fig. 2). These extracellular vesicles play a role in the transfer of proteins to the sperm surface, including proteins necessary for sperm functionality such as those involved in recognition of the egg zona pellucida. Recent evidence shows that epididymosomes also transport small noncoding RNA as a mechanism of intercellular communication that can modulate gene expression along the epididymis (Sullivan and Saez, 2013).

Narrow cells

Narrow cells are only present in the initial segment of the adult epididymis in rodents. Their name refers to their characteristic elongated and narrow shape. As evidenced by electron microscopic studies, narrow cells contain numerous vesicles participating in endocytosis that are recycled to the apical plasma membrane, as well as numerous mitochondria. These cells express the proton pump V-ATPase subunits and are involved in proton secretion and acidification of the epididymal fluid in the rodent initial segments.

Apical cells

Similar to narrow cells, apical cells are found in the initial segment of the epididymis. Compared to other epididymal epithelial cells, apical cells display a spherical nucleus that is located at the apical pole of the epithelium. To date there is no evidence that these cells are in physical contact with the basal membrane of the epithelium. The fact that apical cells secrete regulated on activation, normal T cell expressed, and secreted (RANTES or Ccl5) chemokine suggests a role in the control of inflammatory responses in the epididymis. Whereas similar cells have been described in other mammalian species including humans, further investigation is required in order to characterize and define the functions of these cells.

Clear cells

Clear cells are characterized by an apical pole enriched with mitochondria and display a complete and functional endocytic apparatus. For instance, numerous vesicles, endosomes, coated pits, multivesicular bodies and lysosomes have been observed in clear cells by electron microscopy studies. These cells express the vacuolar proton pump ATPase (V-ATPase), whose activity contributes to luminal acidification in order to maintain spermatozoa in a quiescent state prior to ejaculation (Breton et al., 1996). As observed by high resolution technologies such as helium ion microscopy (HIM) the clear cell apical membrane features microvilli as well as relatively short membrane ridges called microplicae. These membrane protrusions contain a high density of proton-pump V-ATPase and elongate in the presence of several intraluminal factors, including bicarbonate, cAMP, and ATP to increase proton secretion efficiency. Under steady-state conditions, the V-ATPase is distributed between subapical vesicles and the apical plasma membrane of clear cells. However, in response to stimuli originating from the luminal environment (high pH, low bicarbonate concentration, angiotensin II, purinergic agonists), the V-ATPase is recruited into the plasma membrane, which elongates to form extended microvilli. Functional studies have shown that the increase in microvilli length is correlated with an increase in proton secretion into the lumen (Breton and Brown, 2013). Furthermore, clear cells are thought to be endocytic cells that remove proteins from the epididymal fluid in addition to cytoplasmic droplets that detach from spermatozoa during epididymal sperm maturation.

Basal cells

Basal cells are pyramidal-shaped cells located at the base of the epithelium that directly interact with neighboring principal and clear cells through gap junctions. It was recently established that these cells are capable of projecting dynamic cytoplasmic extensions called axiopodia in the initial segment of the rodent epididymis. Axio-podia extensions toward the intraluminal compartment and retractions follow an oscillatory pattern and are proposed to participate in a mechanism of epididymal fluid sampling. Although the role of basal cells remains uncertain, previous studies have reported the presence of enzymes such as the Yf subunit of glutathione-S-transferase (GST), CuZn-superoxide dismutase (SOD), and metallothionein that are involved in the protection against reactive oxygen species. In addition, basal cells were reported to display endocrine properties through the secretion of prostaglandins and to present similar features to adult stem cells. This latter study suggested that basal cells might be multipotent stem cells that have the capacity to differentiate in vitro and to potentially regenerate the epididymal epithelium.

Halo cells

Halo cells are found all along the epididymis, but are more abundant in the proximal regions. They represent a mixture of different immune cells such as helper T lymphocytes, cytotoxic T lymphocytes, and macrophages, and their number increases during aging. The existence of these immunocompetent cells is indisputable but their exact characteristics and their immunological functions remain to be clarified.

Mononuclear phagocytes

Mononuclear phagocytes comprise dendritic cells and macrophages that are in close proximity to the epididymal epithelium. These cells were discovered recently and have been characterized all along epididymides from transgenic mouse models by using different immune cell markers. They surround the base of the epithelium and extend long narrow dendrites between epithelial cells toward the lumen of the proximal epididymal regions. Dendritic cells are also retrieved from normal human epididymides, with specific subsets showing a significant increase under inflamed conditions. While basal cells possess dendritic cell-like features, these two populations are distinct in terms of protein marker expression and are proposed to play distinct functions in the epididymis. Their specific roles throughout the epididymis remain to be addressed.

Conclusion

In vertebrates, the epididymis is an elaborate tubule in which distinct epithelial cell types perform specific functions to ensure the production of functionally mature spermatozoa. While initial studies performed by pioneers shed light on the complexity of this

tissue organization and cell functions, new avenues of exploration are continually opened to uncover the complexity by which somatic and germinal cells collaborate to sustain male reproductive abilities. Unraveling the molecular and cellular processes that affect sperm function are essential for the development of new contraceptive strategies and to better understand the origin of male infertility.

References

- Bedford, J. M. (2015). Human spermatozoa and temperature: The elephant in the room. *Biology of Reproduction*, 93, 97.
- Belleannee, C., Thimon, V., & Sullivan, R. (2012). Region-specific gene expression in the epididymis. *Cell and Tissue Research*, 349, 717–731.
- Breton, S., & Brown, D. (2013). Regulation of luminal acidification by the V-ATPase. *Physiology (Bethesda)*, 28, 318–329.
- Breton, S., Smith, P. J., Lui, B., & Brown, D. (1996). Acidification of the male reproductive tract by a proton pumping (H⁺)-ATPase. *Nature Medicine*, 2, 470–472.
- Cornwall, G. A. (2009). New insights into epididymal biology and function. *Human Reproduction Update*, 15, 213–227.
- Cyr, D. G., Gregory, M., Dube, E., Dufresne, J., Chan, P. T., & Hermo, L. (2007). Orchestration of occludins, claudins, catenins and cadherins as players involved in maintenance of the blood–epididymal barrier in animals and humans. *Asian Journal of Andrology*, 9, 463–475.
- Hermo, L. (1995). Structural features and functions of principal cells of the intermediate zone of the epididymis of adult rats. *The Anatomical Record*, 242, 515–530.
- Hermo, L., & Robaire, B. (2002). Epididymal cell types and their functions. In B. Robaire, & B. T. Hinton (Eds.), *The epididymis, from molecules to clinical practice*. New York: Kluwer Academic/Plenum Publishers.
- Jones, R. C. (2002). Evolution of the epididymis. In B. Robaire, & B. Hinton (Eds.), *The epididymis from molecules to clinical practice. A comprehensive survey of the efferent ducts, the epididymis and the vas deferens*. New York: Kluwer Academic/Plenum Publishers.
- Kleisner, K., Ivell, R., & Flegr, J. (2010). The evolutionary history of testicular externalization and the origin of the scrotum. *Journal of Biosciences*, 35, 27–37.
- Machtinger, R., Laurent, L. C., & Baccarelli, A. A. (2016). Extracellular vesicles: Roles in gamete maturation, fertilization and embryo implantation. *Human Reproduction Update*, 22, 182–193.
- Orgebin-Crist, M. C. (1967). Sperm maturation in rabbit epididymis. *Nature*, 216, 816–818.
- Robaire, B., & Hamzeh, M. (2011). Androgen action in the epididymis. *Journal of Andrology*, 32, 592–599.
- Sullivan, R., & Mieusset, R. (2016). The human epididymis: Its function in sperm maturation. *Human Reproduction Update*, 22, 574–587.
- Sullivan, R., & Saez, F. (2013). Epididymosomes, prostasomes, and liposomes: Their roles in mammalian male reproductive physiology. *Reproduction*, 146, R21–35.
- Turner, T. T., Bomgardner, D., Jacobs, J. P., & Nguyen, Q. A. (2003). Association of segmentation of the epididymal interstitium with segmented tubule function in rats and mice. *Reproduction*, 125, 871–878.
- Yeung, C. H. & Cooper, T. (2002). Acquisition and development of sperm motility upon maturation in the epididymis. In: Robaire, B. & Hinton, B. T. (eds.) *The epididymis, from molecules to clinical practice*. New York: Plenum.

Further Reading

- Sullivan, R., Belleannee, C., & de Jonge, C. J. (2017). Role of the epididymis in sperm maturation. In C. L. R. Barratt (Ed.), *The sperm cell. Production, maturation, fertilization, regeneration* (2nd edn). Cambridge: Cambridge University Press.

Epididymis: Sperm Maturation and Motility

Gail A Cornwall, Texas Tech Health Sciences Center, Lubbock, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The formation of spermatozoa occurs in the testis in a process known as spermatogenesis. However, often overlooked is the fact that in mammals testicular sperm are nonfunctional. It is only during transit through the long highly convoluted tubule known as the epididymis that sperm undergo maturation and acquire progressive motility and the ability to fertilize an oocyte (Cornwall, 2008). Because spermatozoa are transcriptionally silent, maturation is induced by molecules that are released by the surrounding epididymal epithelium. The epididymis exhibits highly regionalized gene expression with different epididymal segments synthesizing and secreting unique subsets of proteins that can bind to the sperm surface and/or modify existing sperm proteins. This highly segment-specific pattern of gene and protein expression results in a luminal environment that is constantly changing along the length of tubule thereby creating unique microenvironments that are essential for sperm maturation. In addition to different proteins in the lumen, changes in lipids, sugars, and ions, also contribute to the unique segment-specific microenvironment. Thus as spermatozoa migrate through the epididymal tubule from the caput/head to the cauda/tail region, they are on a maturational assembly line, ultimately arriving in the cauda epididymis as a functionally mature cell (Fig. 1). In this article, we will discuss the acquisition of sperm motility and fertility during epididymal transit, modifications in spermatozoa that are important for their maturation, and mechanisms for delivery of maturation-associated molecules to spermatozoa. While most of the experimental work described herein was performed in animal models, parallels can be drawn with the human epididymis which similarly plays essential roles in sperm maturation (Sullivan and Miesusset, 2016).

Sperm Motility Maturation

Perpetuation of the species requires that a single spermatozoon binds and fuses with the oocyte during fertilization thereby delivering its genetic material essential for the formation of the embryo. To accomplish this, spermatozoa must exhibit robust progressive motility that propels the cell up the female tract and provides sufficient force for penetration of the zona pellucida that surrounds the oocyte. Indeed, poor sperm motility is often associated with male infertility. While the sperm head contains the nucleus with DNA, the sperm flagellum contains the motile apparatus that is essential for motility (Fig. 2). The sperm flagellum consists of a connecting piece that attaches the flagellum to the sperm head, midpiece containing mitochondria that provides the energy for motility, and the principal piece and endpiece that generate the waveform pattern of motility. The motility motor of the flagellum is the axoneme which runs from the connecting piece to the endpiece. Composed of microtubules in a 9 + 2 configuration, the axoneme creates the flagellar beat via sliding of a microtubule doublet in relation to adjacent microtubules through the action of dynein arms attached to the microtubules. Accessory components in the flagella include the outer dense fibers and fibrous sheath that assist with maintenance of flagellar structure and beat. Sperm motility also requires energy, namely in the form of ATP that is used by the dynein ATPases, and which is generated by oxidative phosphorylation in the mitochondria and glycolysis in the flagellum and head. More recently, AMP was also shown to modulate flagellar wave form (Vadnais et al., 2014).

Upon entering the initial segment region of the epididymis, spermatozoa possess all the necessary machinery for active motility but only exhibit a slight twitching motion. As spermatozoa progress along the tubule, they gradually acquire the capacity for progressive motility. This maturation is reflected by an increase in the percentage of spermatozoa that exhibit movement and qualitative changes in their motility patterns. Immature spermatozoa exhibit a high amplitude, low frequency flagellar beat and often swim in circles while mature epididymal spermatozoa exhibit a low amplitude, high frequency flagellar beat (Fig. 3). This pattern of motility produces the forward linear progression that propels spermatozoa in a straight line as they swim out of the seminal plasma after ejaculation. In the female tract, additional maturational changes in sperm motility occur that are necessary for egg penetration. It is important to note that the maturational changes in sperm motility that are described here were determined

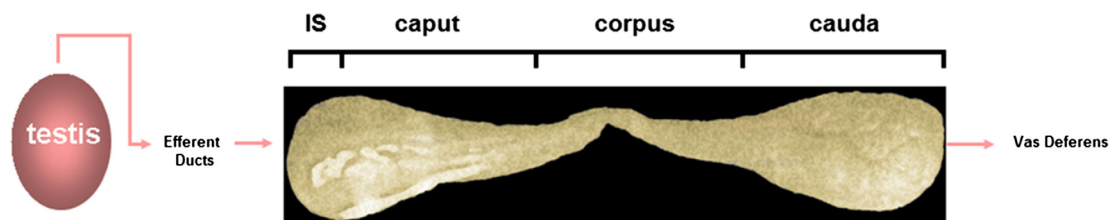


Fig. 1 Epididymal sperm maturation. Sperm migrate out of the testis into the efferent ducts and then enter the initial segment (IS) region of the epididymis. Spermatozoa then move distally into the caput (head), corpus (body), and then cauda (tail) region of the epididymis where they are stored until release into the vas deferens upon ejaculation.

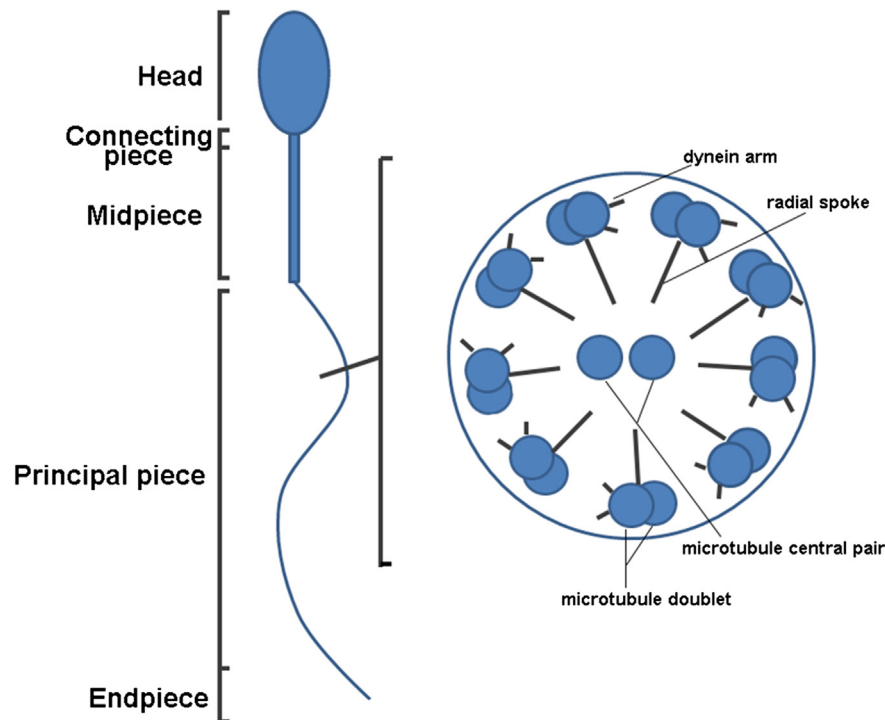


Fig. 2 Schematic diagram of a spermatozoon. The spermatozoon is composed of a head and flagella. The flagella includes a connecting piece, midpiece, principal piece, and endpiece. The axoneme, which runs within the flagella from the connecting piece to the endpiece, is composed of microtubules in a 9+2 configuration (*right diagram*).

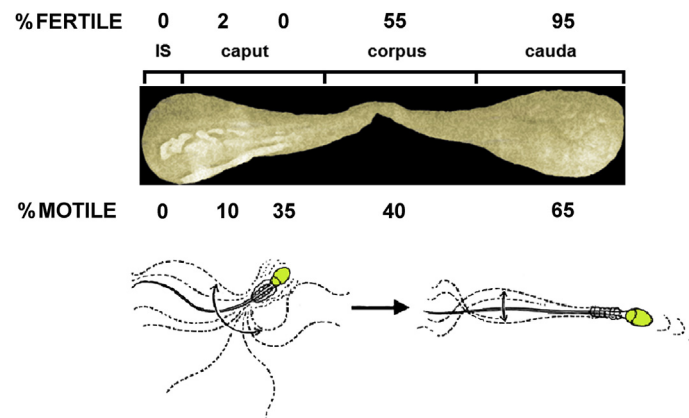


Fig. 3 Motility and fertility maturation in the rodent epididymis. As spermatozoa migrate from the caput to the cauda epididymis, there is an increase in the percentage of spermatozoa that fertilize oocytes and exhibit motility. The sperm flagellar pattern transitions from a high amplitude, low frequency pattern characteristic of immature spermatozoa to low amplitude, high frequency typical of mature spermatozoa. Data presented from Orgebin-Crist, M. C. (1967). Sperm maturation in rabbit epididymis. *Nature* **216**, 816–818; Hinton, B. T., Dott, H. M. and Setchell, B. P. (1979). Measurement of the motility of rat spermatozoa collected by micropuncture from the testis and from different regions along the epididymis. *Journal of Reproduction and Fertility* **55**, 167–172.

following dilution of epididymal spermatozoa into a physiological buffer. In situ, despite acquiring the capacity for motility, spermatozoa are stored in the cauda epididymis in a quiescent state. The mechanism by which cauda epididymal spermatozoa are kept immotile varies between species and includes the presence of immobilin, a protein that increases the viscosity of the epididymal fluid preventing sperm movement (Usselman and Cone, 1983) or acidic pH (Acott and Carr, 1984).

To date the precise mechanisms that are important for the initiation and maturation of sperm motility in the epididymis are not known. Early studies showed that motility could be induced in immature caput spermatozoa by increasing intracellular cAMP suggesting the soluble adenylyl cyclase/cyclic adenosine monophosphate/cAMP-dependent protein kinase signaling pathway is

important, possibly via cross-talk with other signaling pathways (Hoskins et al., 1975; Vadnais et al., 2014). Other signaling pathways important for sperm motility acquisition include phosphoprotein phosphatase I (PPP1) which plays a key role in suppressing sperm motility (Freitas et al., 2017). PPP1 activity is high in caput epididymal spermatozoa which are immotile while PPP1 is inactive in cauda spermatozoa which are motile. PPP1 activity is mediated by its interactions with PPP1R2, a PPP1 inhibitor, that when phosphorylated by glycogen synthase kinase 3 (GSK3) is unable to bind PPP1, making it active. The Wnt signaling pathway is also important for sperm motility maturation since it can mediate GSK3 activity through its binding and activation of low density lipoprotein receptor-related protein receptor (LRP6) which in turn induces GSK3 inhibition (Koch et al., 2015).

Other processes that are important for motility maturation include sperm disulfide bond formation. Immature caput epididymal spermatozoa are sulfhydryl-rich. However, during epididymal transit many sperm proteins are oxidized including those associated with chromatin and tail structures so that mature cauda spermatozoa are disulfide-rich and possess fewer free sulfhydryls (Calvin and Bedford, 1971). Disulfide bond formation stabilizes sperm structures which is an essential part of the maturation process. Indeed, immature caput epididymal spermatozoa induced to become motile by the addition of cAMP phosphodiesterase inhibitors exhibit flagellar angulation with the sperm head bent back on to its flagella (Cornwall et al., 1986). Addition of diamide, a sulfhydryl oxidant, prevented the flagellar angulation suggesting disulfide bond formation is important for maintaining flagellar straightness during motility acquisition (Cornwall et al., 1988). Subsequent studies suggested that adenylyl cyclase plays a role in regulating flagellar stabilization possibly by sulfhydryl oxidation of several sperm protein sulfhydryls including that of fatty acid binding protein 9 (Ijiri et al., 2014).

Acquisition of Fertilizing Ability

During epididymal transit, spermatozoa also gradually acquire the ability to fertilize oocytes in vitro and in vivo. Fertilization competence requires multiple biochemical and physiological changes that occur in spermatozoa and include acquiring the ability to undergo protein tyrosine phosphorylation, bind to the zona pellucida that surrounds the oocyte, undergo the acrosome reaction, fuse with the oocyte plasma membrane, and fertilize the oocyte (Gervasi and Visconti, 2017). Ultimately by acquiring these properties in the epididymis, spermatozoa are then fully capable of undergoing capacitation in the female tract, a last maturational event that is required prior to fertilization. The acquisition of sperm fertility in the epididymis appears to be independent from motility and involves different mechanisms since the site where these two events occur in the epididymis are distinct (Fig. 3). In all species examined, spermatozoa must migrate through the caput region to acquire an ability to fertilize. However, the region where the first fertilizing spermatozoa appear varies with rodent and human spermatozoa acquiring fertility in the proximal cauda epididymis. The acquisition of fertilizing ability also does not correspond with the production of viable offspring. Early studies by Orgebin-Crist (1967) showed that while spermatozoa from the rabbit corpus epididymis will fertilize oocytes, they do not result in viable offspring. In contrast, when ejaculated rabbit spermatozoa were used, fertilization and development proceeds normally and viable embryos are generated. Further studies indicated the loss of embryos that occurred with corpus epididymal spermatozoa was due to a delay in the fertilization process (Orgébin-Crist, 1968). Thus additional maturational changes distal to the corpus region are necessary for normal embryogenesis. To date the mechanisms of how spermatozoa acquire their fertilizing ability remains unclear. However, an infertile phenotype is associated with the loss or modification of many proteins that are acquired by spermatozoa during epididymal transit emphasizing not only a role for these proteins in fertilization but also the complexity of this process (Skerget et al., 2015).

Maturation-Associated Changes in Spermatozoa

As spermatozoa migrate through the epididymis they undergo many modifications including changes in protein, sugar, and lipid content that ultimately contribute to their maturation. Proteomic analyses of spermatozoa isolated from different epididymal regions from several species reveal a highly active remodeling of proteins including those associated with the sperm membrane and acrosome (Belleannee et al., 2011; Labas et al., 2015; Guyonnet et al., 2012). Studies in the mouse epididymis showed that over 700 proteins were added while over 1000 proteins were removed as spermatozoa migrated from the caput to the cauda epididymis (Skerget et al., 2015). Of these, gene ontology (GO) analyses showed that proteins added to spermatozoa during transit were enriched in several categories related to sperm function including those involved in sperm capacitation, sperm-egg recognition, motility, as well as those related to mitochondrial protein processes and high density lipoprotein particle clearance. GO analysis of proteins removed during epididymal transit indicated proteins involved in Golgi transport, ion transport and homeostasis, protein localization, and metabolic processes, possibly reflecting those that had performed remodeling functions during maturation but were no longer needed. Proteomic analyses also revealed maturational changes in the sperm acrosomal matrix during epididymal transit. While changes in proteins associated with the acrosomal membrane are expected, several epididymal secretory proteins also became associated with the acrosomal matrix, an insoluble component of the acrosome, as spermatozoa transited the epididymis (Guyonnet et al., 2012). The acrosome is an organelle associated with the sperm head that plays critical roles in fertilization and thus changes in its structure in the epididymis may be an important maturational process necessary for fertilization.

Posttranslational Modifications

In addition to the gain or loss of proteins, during epididymal transit many sperm-associated proteins undergo posttranslational modifications (PTM) which contribute to sperm maturation. PTM involve the modification of amino acids and the attachment of functional groups such as GPI anchors, acetyl, alkyl, phosphate or glycosyl groups, addition of peptides such as SUMO or ubiquitin, and structural changes such as disulfide bond formation or proteolytic cleavage. Phosphorylation and N-linked glycosylation are the most prevalent PTM as reported in the Swiss-Protein Knowledgebase. Because PTM can affect protein structure, changes in protein localization, interactions, activity, and stability can occur and thus PTMs are a powerful way to fine tune protein function.

Phosphorylation is the addition of a phosphate group to serine, threonine, tyrosine, or histidine amino acids. This requires the transfer of a phosphate group from ATP or GTP to the protein by action of specific kinases while dephosphorylation utilizes protein phosphatases. Both kinases and phosphatases are present in epididymal spermatozoa. Further, changes in protein phosphorylation status have been described in spermatozoa during epididymal transit with corresponding changes in protein localization or function suggesting this PTM plays an important role during sperm maturation. Proteomic studies by [Baker et al. \(2012\)](#), showed changes between caput and cauda sperm in phosphorylation status of different proteins. One protein of particular interest is IZUMO1, a protein essential for sperm-oocyte fusion, that changes localization in the sperm head following phosphorylation, allowing it to participate in fusion with the oocyte ([Inoue et al., 2005](#); [Sosnik et al., 2009](#)).

Glycosylation is an enzymatic process that attaches glycans/sugars to proteins, lipids, or other organic molecules. Changes in both N-linked and O-linked glycosylation have been described in epididymal spermatozoa and thus glycosylation is a significant PTM that modifies epididymal sperm surface molecules important for maturation. Indeed, carbohydrates linked to proteins, lipids, and other molecules form a glycocalyx on the sperm surface that is important for fertilization ([Schröter et al., 1999](#)). Sperm plasma membrane glycoproteins that contribute to the glycocalyx are modified during epididymal transit as shown by changes in lectin binding properties ([Srivastava and Olson, 1991](#)). Also, during epididymal transit spermatozoa acquire a net negative charge likely due to the increase in incorporation of negatively charged sialic acid ([Holt, 1980](#)). Proteomic analyses identified several N-linked sialylated glycopeptides in epididymal spermatozoa including ADAM family members, basigin, and testis-expressed protein 1 ([Villaverde et al., 2016](#)). The changes in the sialic content of these proteins in spermatozoa from different epididymal regions suggest a role in maturation.

Other PTM that occur in spermatozoa during epididymal transit include ubiquitination and protein processing. Ubiquitination is the modification of proteins by the covalent attachment of ubiquitin to lysine residues. Ubiquitination usually earmarks a protein for degradation by the proteasome pathway and indeed defective spermatozoa in the epididymis become ubiquitinated suggesting a mechanism for quality control ([Sutovsky et al., 2001](#)). Other studies suggest the ubiquitin–proteasome pathway may also play a role in fertilization ([Sutovsky, 2011](#)). Many sperm-associated proteins undergo protein processing during epididymal transit. This involves the breaking of peptide bonds between amino acids by specific peptidases and proteases. This can result in changes in protein localization which is thought to contribute to the formation of specific sperm domains ([Phelps et al., 1990](#)).

Lipid Modifications

Maturation in the epididymis is also associated with changes in the lipid composition of sperm membranes. Generally as spermatozoa move from the caput to the cauda region there is a decrease in the cholesterol: phospholipid ratio which correlates with an increase in membrane fluidity ([Jones, 2002](#)). There also is an increase in the ratio of polyunsaturated to saturated fatty acids which may also contribute to membrane fluidity changes. It is likely that increased membrane fluidity in cauda spermatozoa is required for fertilization events including ability to acrosome react and egg fusion.

Migration of Cytoplasmic Droplet

The cytoplasmic droplet is a remnant of germ cell cytoplasm that remains associated with spermatozoa following their release from the testis. During epididymal transit the cytoplasmic droplet migrates from the neck toward the midpiece by mechanisms not understood. In some species the cytoplasmic droplet is discarded by mature spermatozoa and it remains unclear whether retaining the cytoplasmic droplet is associated with infertility. It also is unknown if the cytoplasmic droplet plays a role in sperm maturation although roles in osmoregulation have been proposed ([Fetic et al., 2006](#)). The presence of several enzymes including lysosomal hydrolases and metabolic enzymes including those involved in ATP production in cytoplasmic droplets also suggests a role in the production of energy as part of the maturation process ([Yuan et al., 2013](#)).

Delivery of Maturation-Associated Molecules to Spermatozoa

During epididymal transit, spermatozoa acquire maturation-associated molecules by several different mechanisms. One such mechanism is by extracellular vesicles, including microvesicles and exosomes, which are released from the epididymal epithelium and carry noncoding RNA, lipids, and protein cargo to spermatozoa and function as a means of intracellular communication. Specifically, exosomes in the epididymis, known as epididymosomes, are small (40–100 nm) membrane bound vesicles that transport proteins with proposed functions in fertilization and sperm motility as well as in sperm remodeling to specific sperm domains

and therefore are thought to play important roles in sperm maturation (Rejraji et al., 2006; Sullivan, 2015). Proteomic analyses of isolated exosomes also revealed a number of proteins associated with the ubiquitin–proteasome pathway suggesting additional roles for exosomes in targeting sperm for degradation (Girouard et al., 2011). In support of this, distinct populations of epididymosomes were shown to preferentially bind to defective, dying sperm while other populations bound to viable spermatozoa (Frenette et al., 2010). Thus the diversity of extracellular vesicles in the epididymal lumen likely reflects the many roles they play in epididymal function.

Epididymosomes also carry microRNAs (miRNA) that are delivered to spermatozoa during epididymal transit. Moreover, studies in mouse suggest this process is highly selective as a distinct subset of miRNAs was found only in epididymosomes and spermatozoa and not in the surrounding epididymal epithelium (Reilly et al., 2016; Belleannée et al., 2013). While exosome cargo usually reflects that of its originating cell, the unique populations of miRNAs in epididymosomes and their transfer to spermatozoa led to the suggestion they may function at the time of fertilization where activation of RNA pathways are necessary (Reilly et al., 2016). Epididymosomes also deliver tRNA fragments, which can mediate gene expression, to spermatozoa during epididymal transit. Changes in the levels of tRNA fragments were detected in spermatozoa from mice that had undergone dietary restriction leading to the suggestion that they could affect embryogenesis and play a role in epigenetic inheritance (Sharma et al., 2016).

In addition to delivering cargo to spermatozoa, exosomes released from one epididymal region also have been shown to deliver cargo to downstream epididymal epithelial cells. Indeed, in vitro studies showed that miRNAs and proteins can be transferred from epididymosomes to epididymal epithelial cells in culture (Belleannée et al., 2013). Thus exosomes may be a mechanism by which the luminal fluid can communicate with and regulate epididymal functions. Finally, in addition to classic epididymosomes, the epididymal lumen also contains larger vesicles up to 1000–2000 nm suggesting unique structures/functions that have not yet been determined.

A second mechanism by which maturation associated proteins and perhaps other molecules are delivered to spermatozoa is by the interaction of membrane-less, dense bodies with the sperm surface. Studies by Asquith et al. (2005) showed that dense bodies containing chaperones hsp1 and gp96 were present in the epididymal lumen and that these proteins were later detected on cauda spermatozoa. It was proposed that dense bodies may be a means to deliver chaperones important for the remodeling of the sperm surface during fertilization.

In addition to interactions of extracellular vesicles and dense bodies with spermatozoa, some epididymal proteins are secreted into the lumen by their release from secretory granules in the classic secretory pathway. These soluble epididymal proteins then bind to the sperm surface. Finally, more recently high resolution helium ion microscopy showed direct interactions between spermatozoa in the lumen and the surrounding epididymal epithelial cells raising the possibility for a direct transfer of proteins by these interactions (Păunescu et al., 2014).

Summary

Taken together the maturation of spermatozoa in the epididymis is a complex yet poorly understood process. The transfer of molecules from the epididymal epithelium to spermatozoa is essential for maturation and is mediated by several different mechanisms including epididymosomes that can specifically target delivery to discrete sperm domains, dense bodies, and absorption of soluble proteins to the sperm surface. The delivery of maturation-associated molecules induces changes in sperm structure including the sperm surface via the addition, removal, or modification of existing proteins, lipids, and sugars, ultimately resulting in sperm acquiring motility and fertility.

References

- Acott, T. S., & Carr, D. W. (1984). Inhibition of bovine spermatozoa by caudal epididymal fluid: II. Interaction of pH and a quiescence factor. *Biology of Reproduction*, 30, 926–935.
- Asquith, K. L., Harman, A. J., McLaughlin, E. A., Nixon, B., & Aitken, R. J. (2005). Localization and significance of molecular chaperones, heat shock protein 1, and tumor rejection antigen gp96 in the male reproductive tract and during capacitation and acrosome reaction. *Biology of Reproduction*, 72, 328–337.
- Baker, M. A., Hetherington, L., Weinberg, A., Naumovski, N., Velkov, T., Pelzing, M., Dolman, S., Condina, M. R., & Aitken, R. J. (2012). Analysis of phosphopeptide changes as spermatozoa acquire functional competence in the epididymis demonstrates changes in the post-translational modification of Izumo1. *Journal of Proteome Research*, 11, 5252–5264.
- Belleannée, C., Belghazi, M., Labas, V., Teixeira-Gomes, A. P., Gatti, J. L., Dacheux, J. L., & Dacheux, F. (2011). Purification and identification of sperm surface proteins and changes during epididymal maturation. *Proteomics*, 1, 1952–1964.
- Belleannée, C., Calvo, É., Caballero, J., & Sullivan, R. (2013). Epididymosomes convey different repertoires of microRNAs throughout the bovine epididymis. *Biology of Reproduction*, 30, 1–11.
- Calvin, H. I., & Bedford, J. M. (1971). Formation of disulphide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *Journal of Reproduction and Fertility. Supplement*, 13, 65–75.
- Cornwall, G. A. (2008). New insights into epididymal biology and function. *Human Reproduction Update*, 15, 213–227.
- Cornwall, G. A., Smyth, T. B., Vindivich, D., Harter, C., Robinson, J., & Chang, T. S. K. (1986). Induction and enhancement of progressive motility in hamster caput epididymal spermatozoa. *Biology of Reproduction*, 35, 1065–1074.
- Cornwall, G. A., Vindivich, D., Tillman, S., & Chang, T. S. K. (1988). The effect of sulfhydryl oxidation on the morphology of immature hamster epididymal spermatozoa induced to acquire motility in vitro. *Biology of Reproduction*, 39, 141–155.
- Fetic, S., Yeung, C. H., Sonntag, B., Nieschlag, E., & Cooper, T. G. (2006). Relationship of cytoplasmic droplets to motility, migration in mucus, and volume regulation of human spermatozoa. *Journal of Andrology*, 27, 294–301.

- Freitas, M. J., Vijayaraghavan, S., & Fardilha, M. (2017). Signaling mechanisms in mammalian sperm motility. *Biology of Reproduction*, 96, 2–12.
- Frenette, G., Girouard, J., D'Amours, O., Allard, N., Tessier, L., & Sullivan, R. (2010). Characterization of two distinct populations of epididymosomes collected in the intraluminal compartment of the bovine cauda epididymis. *Biology of Reproduction*, 83, 473–480.
- Gervasi, M. G., & Visconti, P. E. (2017). Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology*, 5, 204–218.
- Girouard, J., Frenette, G., & Sullivan, R. (2011). Comparative proteome and lipid profiles of bovine epididymosomes collected in the intraluminal compartment of the caput and cauda epididymidis. *International Journal of Andrology*, 34, e475–e486.
- Guyonnet, B., Zabet-Moghaddam, M., SanFrancisco, S., & Cornwall, G. A. (2012). Isolation and proteomic characterization of the mouse sperm acrosomal matrix. *Molecular & Cellular Proteomics*, 11, 758–774.
- Holt, W. V. (1980). Surface-bound sialic acid on ram and bull spermatozoa: Deposition during epididymal transit and stability during washing. *Biology of Reproduction*, 23, 847–857.
- Hoskins, D. D., Hall, M. L., & Munsterman, D. (1975). Induction of motility in immature bovine spermatozoa by cyclic AMP phosphodiesterase inhibitors and seminal plasma. *Biology of Reproduction*, 13, 168–176.
- Ijiri, T. W., Vadrnais, M. L., Huang, A. P., Lin, A. M., Levin, L. R., Buck, J., & Gerton, G. L. (2014). Thiol changes during epididymal maturation: A link to flagellar angulation in mouse spermatozoa? *Andrology*, 2, 65–75.
- Inoue, N., Ikawa, M., Isotani, A., & Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature*, 434, 234–238.
- Jones, R. (2002). Plasma membrane composition and organization during maturation of spermatozoa in the epididymis. In B. Robaire, & B. Hinton (Eds.), *The epididymis: From molecules to clinical practice* (pp. 405–416). New York, NY: Kluwer Academic/Plenum Publishers.
- Koch, S., Acebron, S. P., Herbst, J., Hatiboglu, G., & Niehrs, C. (2015). Post-transcriptional Wnt signaling governs epididymal sperm maturation. *Cell*, 163, 1225–1236.
- Labas, V., Spina, L., Belleanne, C., Teixeira-Gomes, A. P., Gargaro, A., Dacheux, F., & Dacheux, J. L. (2015). Analysis of epididymal sperm maturation by MALDI profiling and top-down mass spectrometry. *Journal of Proteomics*, 113, 226–243.
- Orgebin-Crist, M. C. (1967). Sperm maturation in rabbit epididymis. *Nature*, 216, 816–818.
- Orgebin-Crist, M. C. (1968). Maturation of spermatozoa in the rabbit epididymis: Delayed fertilization in does inseminated with epididymal spermatozoa. *Journal of Reproduction and Fertility*, 16, 29–33.
- Păunescu, T. G., Shum, W. W., Huynh, C., Lechner, L., Goetze, B., Brown, D., & Breton, S. (2014). High-resolution helium ion microscopy of epididymal epithelial cells and their interaction with spermatozoa. *Molecular Human Reproduction*, 20, 929–937.
- Phelps, B. M., Koppel, D. E., Primakoff, P., & Myles, D. G. (1990). Evidence that proteolysis of the surface is an initial step in the mechanism of formation of sperm cell surface domains. *Journal of Cell Biology*, 111, 1839–1847.
- Reilly, J. N., McLaughlin, E. A., Stanger, S. J., Anderson, A. L., Hutcheon, K., Church, K., Mihalas, B. P., Tyagi, S., Holt, J. E., Eamens, A. L., & Nixon, B. (2016). Characterisation of mouse epididymosomes reveals a complex profile of microRNAs and a potential mechanism for modification of the sperm epigenome. *Scientific Reports*, 6, 31794.
- Rejz, H., Sion, B., Prensier, G., Carreras, M., Motta, C., Frenoux, J. M., Vericel, E., Grizard, G., Vernet, P., & Drevet, J. R. (2006). Lipid remodeling of murine epididymosomes and spermatozoa during epididymal maturation. *Biology of Reproduction*, 74, 1104–1113.
- Schröter, S., Osterhoff, C., McArdle, W., and Ivell, R. (1999). The glycocalyx of the sperm surface. *Human Reproduction Update* 5, 302–313.
- Sharma, U., Conine, C. C., Shea, J. M., Boskovic, A., Derr, A. G., Bing, X. Y., Belleanne, C., Kucukural, A., Serra, R. W., Sun, F., Song, L., Carone, B. R., Ricci, E. P., Li, X. Z., Fauquier, L., Moore, M. J., Sullivan, R., Mello, C. C., Garber, M., & Rando, O. J. (2016). Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. *Science*, 351, 391–396.
- Skerget, S., Rosenow, M. A., Petritis, K., & Karr, T. L. (2015). Sperm proteome maturation in the mouse epididymis. *PLoS One*, 10, e0140650.
- Sosnik, J., Miranda, P. V., Spiridonov, N. A., Yoon, S. Y., Fissore, R. A., Johnson, G. R., & Visconti, P. E. (2009). Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. *Journal of Cell Science*, 122, 2741–2749.
- Srivastava, A., & Olson, G. E. (1991). Glycoprotein changes in the rat sperm plasma membrane during maturation in the epididymis. *Molecular Reproduction and Development*, 29, 357–364.
- Sullivan, R. (2015). Epididymosomes: a heterogeneous population of microvesicles with multiple functions in sperm maturation and storage. *Asian Journal of Andrology*, 17, 726–729.
- Sullivan, R., & Mieusset, R. (2016). The human epididymis: its function in sperm maturation. *Human Reproduction Update*, 22, 574–587.
- Sutovsky, P. (2011). Sperm proteasome and fertilization. *Reproduction*, 142, 1–14.
- Sutovsky, P., Moreno, R., Ramalho-Santos, J., Dominko, T., Thompson, W. E., & Schatten, G. (2001). A putative, ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa in the mammalian epididymis. *Journal of Cell Science*, 114, 1665–1675.
- Usselman, M. C., & Cone, R. A. (1983). Rat sperm are mechanically immobilized in the caudal epididymis by “immobilin,” a high molecular weight glycoprotein. *Biology of Reproduction*, 29, 1241–1253.
- Vadrnais, M. L., Cao, W., Aghajanian, H. K., Haig-Ladewig, L., Lin, A. M., Al-Alao, O., & Gerton, G. L. (2014). Adenine nucleotide metabolism and a role for AMP in modulating flagellar waveforms in mouse sperm. *Biology of Reproduction*, 128, 1–14.
- Villaverde, A. I., Hetherington, L., & Baker, M. A. (2016). Quantitative glycopeptide changes in rat sperm during epididymal transit. *Biology of Reproduction*, 91, 1–13.
- Yuan, S., Zheng, H., Zheng, Z., & Yan, W. (2013). Proteomic analyses reveal a role of cytoplasmic droplets as an energy source during epididymal sperm maturation. *PLoS ONE*, 8, e7746.

Further Reading

- Hinton, B. T., Dott, H. M., & Setchell, B. P. (1979). Measurement of the motility of rat spermatozoa collected by micropuncture from the testis and from different regions along the epididymis. *Journal of Reproduction and Fertility*, 55, 167–172.

Gene Regulation in the Epididymis

Kenneth P Roberts and Theodore R Chauvin, Washington State University, Spokane, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Overview of Epididymal Function

The epididymis is a single convoluted tubule, through which sperm travel upon release from the testis. The function of the epididymis is to provide the microenvironment and molecular machinery required for the maturation of sperm. Sperm leave the testis morphologically mature, or nearly so, but are unable to swim progressively, or to penetrate and fertilize an oocyte. Both of these functional capacities are acquired in the epididymis in a process referred to as posttesticular sperm maturation. As sperm are largely, if not totally, transcriptionally and translationally inert, the maturation process must be completed using cellular processes that are present in the sperm upon leaving the testis, along with extracellular processes functioning in the luminal fluid of the epididymis. Since the contribution of the epididymal luminal microenvironment is required for sperm maturation, and due to the fact that the maturation process proceeds as the sperm transit the epididymis, the precise regulation of gene expression along the length of the epididymal tubule is critical to the sperm maturation process. Of particular importance is the regulation of genes encoding secreted proteins, as these may interact directly with sperm to facilitate maturation. The maturation produced by secreted proteins occurs either by modifying the sperm in some way (i.e., glycosidases, proteases, etc.), by becoming a component of the sperm surface, or, in some cases, becoming an intracellular sperm protein. In addition, many epididymal proteins are required for maintaining appropriate luminal conditions, such as pH and isotonicity, for sperm maturation and storage. Regulation of genes encoding these proteins is also very important to the maturation process. For a comprehensive review of epididymal development, structure, and function see [Robaire et al. \(2006\)](#).

Anatomy of the Epididymis

The epididymis has gross anatomical regions designated as the initial segment and caput (both in the proximal region), corpus (middle region), and cauda (distal region) [Fig. 1](#). The initial segment is present as an anatomically distinct region in mice and rats. The initial segment together with the caput region comprise the region of the epididymis most active in sperm maturation. The cauda region of the epididymis is where sperm are stored prior to ejaculation. Gene expression in the epididymis was shown many years ago to be carefully regulated in a region-specific manner ([Cornwall and Hann, 1995](#)). There are many genes expressed in the caput epididymis that are differentially regulated in the corpus and/or cauda epididymis. This makes

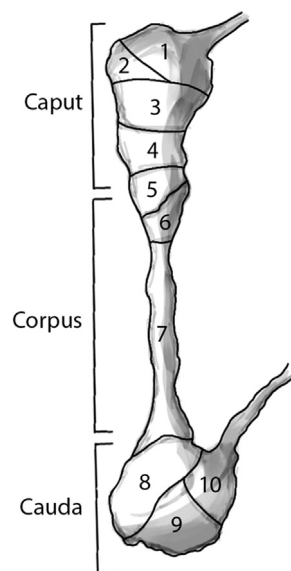


Fig. 1 Schematic representation of the mouse epididymis showing the caput (*proximal*), corpus (*middle*), and cauda (*distal*) anatomical regions, as well as the segmental pattern comprising each region. Each segment of the epididymis is separated from adjacent segments by a connective tissue septum, creating a unique interstitial environment in each segment. Segment 1 of the mouse epididymis is also denoted as the initial segment. This region is not anatomically distinct in the human epididymis. Drawing by Madeleine Roberts, based on descriptions in Turner, T. T., Johnston, D. S., Jelinsky, S. A., Tomsig, J. L. and Finger, J. N. (2007). Segment boundaries of the adult rat epididymis limit interstitial signaling by potential paracrine factors and segments lose differential gene expression after efferent duct ligation. *Asian Journal of Andrology* **9**, 565–573.

intuitive sense, given the differential function of each region. Upon closer examination, the epididymis is actually subdivided into a series of compartments, the number of which are species-specific, which are each divided by septa that isolate each segment; this creates a series of distinct microenvironments that have been shown to have specific patterns of gene expression (Fig. 1). Expression of many genes along the epididymis, in each of its segments, is effected by androgens and by factors in the luminal fluid that are products of the testis. These two sources of gene regulation are also interdependent for some genes. Specific examples of genes regulated in a segment-specific manner, and by androgens or luminal factors, are detailed below with discussion of what is known of the molecular mechanisms that control their expression.

Regional Gene Expression

Early studies on epididymal gene expression and protein production characterized the predominant secretory proteins of the epididymis. The major secretory products were first identified by gel electrophoresis and given letter designations (Proteins A, B/C, and D/E). This work was followed by the isolation and characterization of several proteins abundant in epididymal fluid (Cornwall and Hann, 1995). Some of these proteins were shown to be enriched in, or specific to, the epididymis. Most of these proteins were shown to have a regional pattern of expression along the epididymis. For instance, cystatin-related epididymal spermatogenic protein (CRES), protein B/C, and glutathione peroxidase 5 (GPX5) are reproductive proteins with expression in the caput epididymis, while sperm associated antigen 11 (Spag 11 or HE2) is expressed in the corpus, and WAP four-disulfide core domain 2 (Wfdc2 or HE4) and cysteine rich secretory protein 1 (Crisp1) are expressed primarily in the corpus and cauda. Even genes with well-characterized expression in other tissues, such as proenkephalin, CRBP, and HGF, have region-specific expression in the epididymis. This early molecular work supported the general hypothesis that regional gene expression patterns along the length of the epididymis are key to the function of the epididymis.

With the advent of microarray technology, it became possible to assay the majority of mRNA transcripts along the length of the epididymis, and to determine the overall patterns of regional gene expression. Using this technology, the mouse epididymis has been shown to express over 2500 genes in the caput, corpus, and cauda epididymis (Chauvin and Griswold, 2004). Of these, there was enriched expression of 162 genes in the caput, 55 genes in the corpus, and 133 genes in the cauda, providing further evidence for extensive region-specific gene expression in the epididymis.

Gross dissection of the epididymis into initial segment, caput, corpus, and cauda regions is sufficient to demonstrate region-specific gene expression. However, as noted above (Fig. 1), each of these regions can be further subdivided into segments of coiled epididymal tubule separated by distinct septal boundaries (Turner et al., 2007). The number of these boundaries is maintained by a connective tissue septum. Each of these segments is thought to create a unique microenvironment that supports segment-specific function, including gene expression. A powerful demonstration of the segment-specific function of the epididymis was provided by experiments that isolated segment 1 or segment 2 of the rat epididymis, followed by injection with growth factors epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), or vascular endothelial growth factor A (VEGFA) to stimulate phosphorylation of MAP kinase (MAPK) (Turner et al., 2007). These experiments showed that phosphorylation of MAPK was confined to the injected segment. Concomitant injection of collagenase, to break down the intersegmental septum, with the growth factors, resulted in stimulation of MAPK in the adjacent segment. Thus, it appears that the intersegmental septa provide a functional compartmentalization of the epididymis which contributes to region-specific gene regulation.

Microarray gene expression analysis of the individual segments of the epididymis confirms the hypothesis that these segments are individually regulated, and has allowed for detection of regulated genes that are not detectable when large numbers of segments are combined for analysis (Johnston et al., 2005, 2007). Johnston et al. have used this approach to study epididymal gene expression and have shown that 16,000–17,000 genes are expressed in the mouse and rat epididymis (Johnston et al., 2005, 2007). Of these, 77 and 110 transcripts were found to be epididymis-specific in the mouse and rat, respectively. Among these transcripts were several genes that are known to be specific to the epididymis, including lipocalin 5, glutathione peroxidase, and Adam 7. Using data from the mouse epididymis, over 6000 genes exhibited segment-specific regulation (at least twofold difference between two segments), and over 60 genes were differentially expressed by 100-fold or greater (Johnston et al., 2005). Examples of tightly regulated genes include a disintegrin and metalloproteinase domain (Adam 28), expressed only in segments 1–2, myomesin 2 (*Myom2*), expressed only in segments 6–7, and small muscle protein, X-linked (*Smpx*), expressed only in segments 8–10. There are also families of genes, such as the defensins, aquaporins, and lipocalins that are regionally expressed, with various members of the families expressed in different regions of the epididymis (Belleannee et al., 2012b).

Although the segment-specific control of gene expression is now well established in the epididymis, the mechanisms responsible for this regulation are not well understood. It seems likely that paracrine regulators in each segment play a role, as well as regulatory molecules found in the lumen of the epididymal fluid.

Regulation of Epididymal Gene Expression by Androgens and other Testicular Factors

Much of the male reproductive tract, including the epididymis, requires androgens for normal growth, development, and function. Testosterone, produced by the testicular Leydig cells, reaches the epididymis by two routes: peripheral circulation

and by transport in the luminal fluid. Testosterone transported in the luminal fluid is predominantly bound to androgen binding protein (ABP) and taken up in a receptor-mediated fashion by the epididymal epithelium. Testosterone is a sex steroid hormone that can be converted to estradiol by aromatization or reduced to 5-hydroxy-testosterone (DHT) by 5 α -reductase. DHT has an affinity for the androgen receptor that is approximately 10 times that of testosterone. Local conversion of testosterone to estradiol is of major regulatory importance in the efferent ducts, which are the excurrent tubules leading to the epididymis. DHT, on the other hand, is the predominant sex steroid required for normal function of the epididymis, supporting differential gene expression along the length of the epididymis (Robaire and Hamzeh, 2011). Early studies showed that androgen withdrawal, typically achieved by castration, results in profound changes in the epididymis including alterations in protein secretion and gene expression, and pronounced changes in cellular morphology in the epididymal epithelium (Robaire and Henderson, 2006; Robaire et al., 2007).

A simple model for androgen withdrawal is bilateral castration, effectively removing the source of androgens from the animal. Androgen can then be replaced by injections, or chronically using testosterone-filled silastic implants, to demonstrate the restoration of androgen-dependent functions of the epididymis. However, castration also removes all other contributions of the testis to epididymal fluid, including sperm. Therefore, this may also remove other potential regulatory factors that may be required for normal epididymal function, including some functions that are also dependent on androgen. To isolate the effects of luminal components from androgens on epididymal gene expression, the efferent ducts can be ligated, thus eliminating testicular luminal factors while keeping androgen levels normal. Likewise, unilateral castration leaves the ipsilateral epididymis free of testicular factors while still influenced by normal circulating testosterone levels. The combination of these approaches allows the separation of the effects of testicular factors in the luminal fluid from the effects of androgens. Using these techniques in combination, Garrett et al. showed that while proenkephalin, Protein B/C, and Protein D/E (Crisp1) are all decreased by castration, only Protein B/C and Protein D/E are restored with exogenous testosterone treatment. Proenkephalin requires testicular factors for normal levels of expression. Similar regulatory requirements have been shown for several other proteins as well (Hinton et al., 1998).

Comprehensive gene expression studies have demonstrated that the caput epididymis has several hundred genes that are regulated by androgens, with roughly half of them upregulated and half downregulated (Chauvin and Griswold, 2004; Robaire and Hamzeh, 2011). The effect of androgen withdrawal is progressive over several days, with over 1000 genes affected. The restoration of gene expression by exogenous androgen starts within hours of administration of androgen and continues over several days. However, gene regulation is not returned completely to normal by androgen administration, emphasizing the importance of luminal factors in the regulation of epididymal gene expression.

The families of genes controlled by androgen in the epididymis, as determined by transcriptional profiling experiments, included many different required cellular pathways such as metabolism, transport, proteolysis, and immune response, as well as cell signaling, signal transduction, and transcriptional regulation (Chauvin and Griswold, 2004; Robaire et al., 2007). Many of the transcripts induced by androgen replacement form parts of intact pathways that may shed light on the central action of androgen in the epididymal epithelium. Among the proteins increased by androgen is insulin-like growth factor 1 (IGF1), which is functionally linked to several other proteins that are also induced after 1 day of androgen treatment of castrated rats (Fig. 2) (Robaire and Hamzeh, 2011). These gene expression clusters, with IGF1 and EGF centrally located, suggest that these growth factors may be central to the normal gene expression profiles of the epididymis that are supported by DHT.

Mechanism of Androgen Gene Regulation

Dihydrotestosterone is the active form of androgen acting in the epididymis (Robaire and Henderson, 2006). The converting enzyme 5 α -reductase (5 α R) is found along the entire length of the epididymis and effectively converts testosterone to DHT in the epididymal epithelium. Many of the effects of androgen withdrawal by castration are mimicked by the administration of 5 α -reductase inhibitors, and sperm from rats treated with such inhibitors have decreased motility and abnormal morphology. There are two transcripts that encode 5 α -reductase in the epididymis. 5 α -reductase type 1 is expressed most highly in the initial segment, whereas the type 2 transcript is expressed throughout the length of the epididymis and most highly in the caput. Ligation of the efferent ducts results in a significant decrease in the expression of 5 α R in the epididymis, with the most pronounced effect in the proximal part. This requirement for luminal factors for the proper support of gene expression has been indicated for a number of other proteins in addition to 5 α R, including proenkephalin, CRES, and gamma-glutamyltransferase (GGT) (Hinton et al., 1998). Although the expression of 5 α R is dependent on luminal factors, the identity of these factors has not been determined. It has been proposed that ABP could be the luminal factor responsible, as it is taken up in a receptor-mediated fashion by the epididymal epithelium. Fibroblast growth factor 2 (FGF2) has also been proposed as a luminal regulator of epididymal gene expression. Regardless of the identity of the luminal factor(s) responsible, it seems clear that both luminal factors are required for normal androgen regulation of the epididymal epithelium.

The classic pathway for androgen regulation of gene expression involves binding of testosterone or DHT to the androgen receptor (AR), followed by translocation of the AR to the nucleus where it binds to androgen response elements (ARE) in the regulatory region of androgen-responsive genes. Additional levels of regulation are provided by co-activator proteins that bind to the AR and modulate its activity. These co-activators are often responsible for the differential expression of androgen-regulated genes in different tissues and cell types, and under varying physiological conditions. Such co-activators may explain, at least in part, the

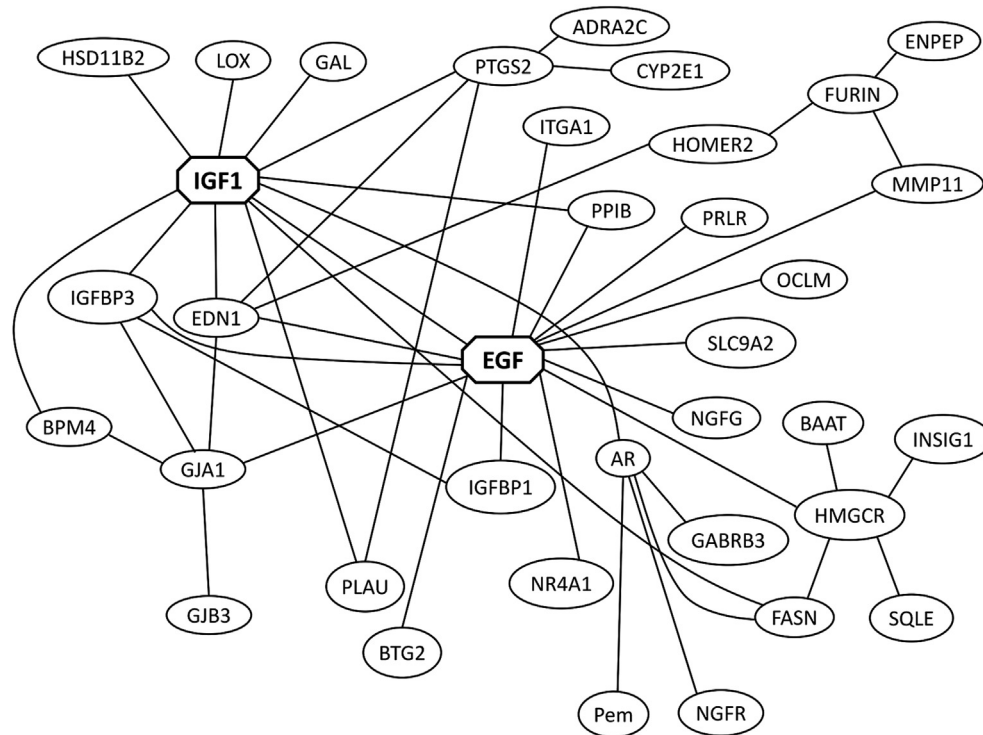


Fig. 2 A map of genes that respond within 24 h to administration of dihydrotestosterone in the androgen-deprived epididymis in vivo. This image shows only the genes that have been documented in the literature to have a primary interaction with one another. These interactions include effects on gene expression, regulation, protein modification and/or protein–protein binding. Note the central effect of DHT on gene networks involving IGF1 and EGF. Drawing based on information in Robaire, B. and Hamzeh, M. (2011). Androgen action in the epididymis. *Journal of Andrology* **32**, 592–599.

varying response to androgen by the same genes in an epididymal segment-specific fashion. To date, over 160 different co-activators of the androgen receptor have been described. The mechanism of androgen action via the androgen receptor mediated pathway is summarized in Fig. 3 and reviewed in (Robaire and Hamzeh, 2011).

In addition to the AR-mediated mechanism of androgen action, there appear to be additional pathways for androgen action in the epididymal epithelium that may contribute to androgen-regulated gene expression in the epididymis. Hamzeh and Robaire (2011) demonstrated, using an immortalized epididymal cell line (PC-1), that DHT stimulates a pathway involving the activation of steroid receptor coactivator (SRC) kinase (Hamzeh and Robaire, 2011). Within 1 min of exposure to DHT, phosphorylation of Mitogen-activated protein kinase 3 (MAP3K/ERK1) in PC-1 cells was significantly increased. This increase was blocked by hydroxy-flutamide, a selective antagonist against the androgen receptor. The phosphorylation of ERK was dependent on the activation of SRC, and was downstream of the activation of the IGF1 and EGF receptors. The phosphorylation of ERK results in the activation of CREB which is then responsible for the subsequent modification of gene expression (Fig. 3).

Mechanism of Epididymal Gene Regulation by MicroRNAs

Gene regulation in the epididymis is also under the control of the microRNA (miRNA) regulatory system. Deletion of the Dicer1 nuclease, a key enzyme for the processing of miRNA, in the caput epididymis results in the dedifferentiation of the epididymal epithelium, defects in lipid homeostasis, and an imbalance in sex steroid receptor expression (Bjorkgren and Sipila, 2015). This deletion results in mice that produce sperm with altered lipid composition and are infertile. Decreases in the expression of several epididymal genes, including lipocalin 8, cystatin 8, glutathione peroxidase 5, the estrogen receptors, and the androgen receptor, results from loss of Dicer1 (Bjorkgren et al., 2012). It is not known whether the effects of the Dicer1 knockout is direct on these genes, or secondary to the decreased levels of androgen receptor. However, microarray and deep sequencing experiments have identified expression of miRNAs that pair with potential mRNA targets expressed in the same regions of the epididymis. For instance, miRNAs predicted to target the cystic fibrosis transmembrane conductance regulator (*Cftr*), claudin-10, and GLI pathogenesis-related 1 like 1 (*Glpr1L1*) have been identified and found to be negatively correlated to their proposed target mRNA (Belleannet et al., 2012a). These observations suggest a direct posttranslational regulatory effect on mRNA levels in the epididymis.

MicroRNAs have also been proposed as mediators of segment-specific gene regulation in the epididymis. It is known that certain microRNAs are released from cells (extracellular miRNAs) and participate in cell-to-cell communication. Approximately 300

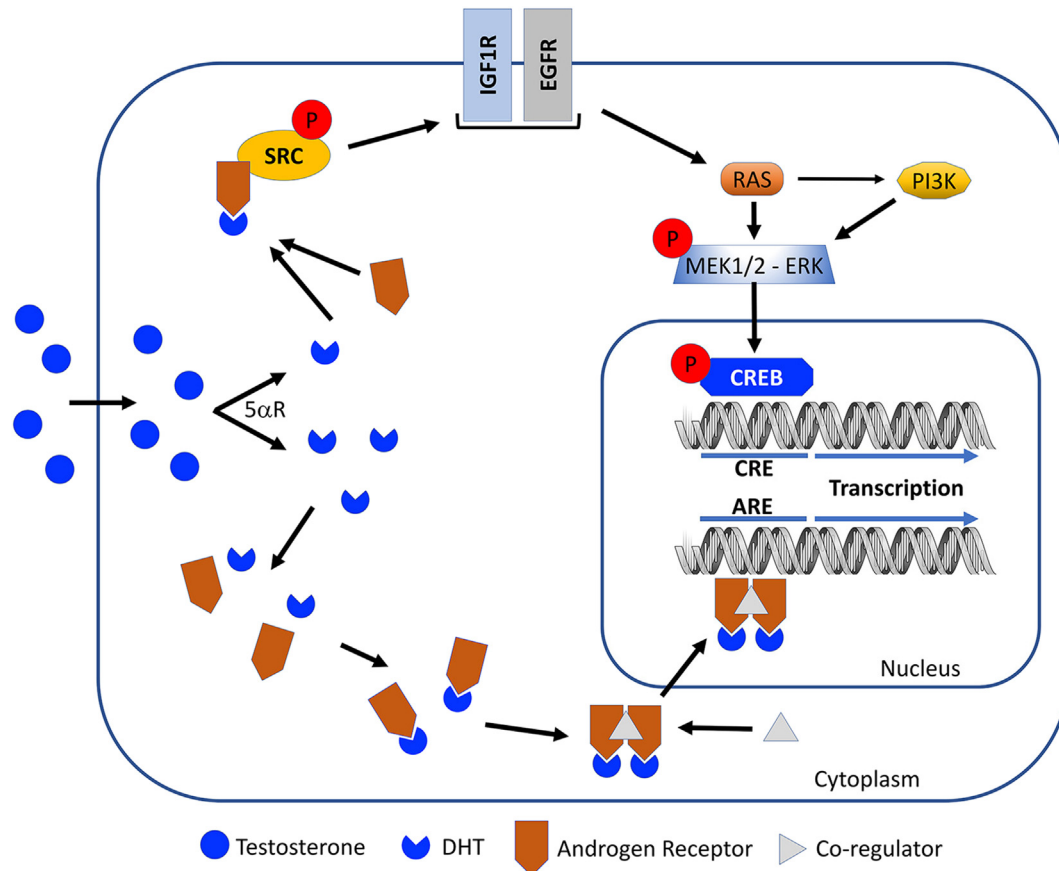


Fig. 3 Diagrammatic representation of the classic nuclear pathway for androgen-regulated gene expression in a target cell (lower half of figure) and nongenomic signal transduction by androgen via stimulation of the c-steroid receptor coactivator (SRC) tyrosine kinase (upper half of figure). Testosterone enters the cell by passive diffusion where it is converted to DHT, a more powerful androgen, by $5\alpha R$. DHT binds to the androgen receptor and the complex, including one or more co-regulators, is translocated to the nucleus where it interacts with the regulatory region of target genes to either stimulate or suppress their transcription. The androgen receptor—SRC complex signals through the epidermal growth factor receptor (EGFR) and the insulin-like growth factor-1 receptor (IGF1R) to stimulate the rat sarcoma viral oncogene (RAS), resulting in phosphorylation of mitogen activated protein kinase kinase 1/2 (MEK1/2), originally known as extracellular regulated MAP kinase (ERK), either directly or through activation of phosphatidylinositol 3 kinase (PI3K), resulting in activation of cAMP response element binding protein (CREB) which regulates transcription of target genes. Drawing based on information in Hamzeh, M. and Robaire, B. (2011). Androgens activate mitogen-activated protein kinase via epidermal growth factor receptor/insulin-like growth factor 1 receptor in the mouse PC-1 cell line. *The Journal of Endocrinology* **209**, 55–64.

microRNAs have been identified in the caput, corpus, and cauda regions of both human and mouse epididymis and a subset of these (5–28) are region-specific (Belleannee et al., 2012a; Nixon et al., 2015). These results are consistent with a region-specific gene regulatory activity for these microRNAs. In the epididymis, miRNAs are known to be packaged and secreted in epididymosomes, which are small membrane-bound vesicles of epithelial origin. Epididymosomes interact with spermatozoa, transferring both proteins and lipids to sperm (Belleannee, 2015). This same transfer could occur between epididymosomes and epithelial cells at some distance from the site of miRNA secretion. The population of miRNAs found in epididymosomes are distinct from the population isolated from epididymal epithelial cells in the same region of the epididymis, suggesting that a distinct population of miRNAs is secreted for the purpose of regulating genes in cells downstream in neighboring segments (Belleannee, 2015). Taken together, these observations suggest that miRNAs are a possible regulator of segment-specific gene regulation in the epididymis. The potential roles of miRNAs packaged into epididymosomes is summarized in Fig. 4.

Gene regulation by the androgen receptor, and regulation of the androgen receptor itself, is mediated through the direct AR regulation of microRNA29a (Ma et al., 2013). The levels of miR-29a are increased in the epididymis after castration and suppressed by subsequent administration of androgen, suggesting a role of this microRNA in androgen action. The control of miR-29a expression is direct, with the androgen receptor binding directly to an ARE in the miR-29a gene, suppressing its expression. When miR-29a is over-expressed, the resulting phenotype resembles androgen deprivation, with the epididymis being substantially reduced in size, and the expression of the androgen receptor diminished. The AR gene is not, however, a direct target of miR-29a. The suppressive effect of miR-29a on AR expression is mediated by the suppression of IGF1 expression by miR-29a, and also demonstrates that the expression of the AR is increased by IGF1.

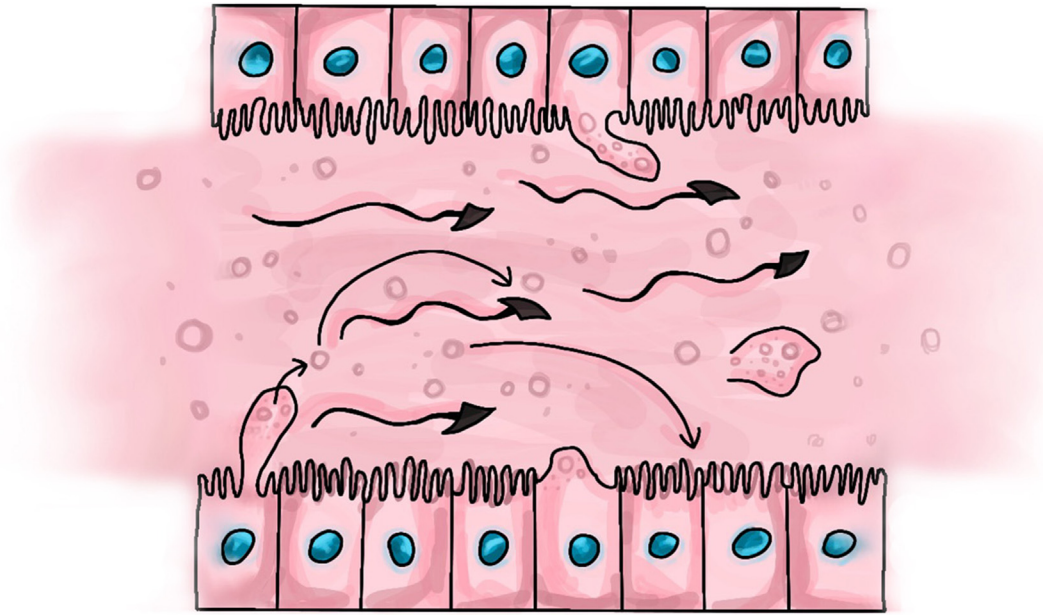


Fig. 4 Schematic representation of a segment of epididymal tubule showing apocrine secretion of extracellular vesicles (epididymosomes), packaged with microRNAs, interacting with sperm or with downstream epithelial cells. These extracellular vesicles have a population of microRNAs distinct from those found in adjacent epithelial cells. Some of the extracellular vesicles continue in the luminal fluid and contribute to the composition of the seminal plasma. Drawing by Madeleine Roberts, based on descriptions in Belleannee, C. (2015). Extracellular microRNAs from the epididymis as potential mediators of cell-to-cell communication. *Asian Journal of Andrology* **17**, 730–736.

Other Gene Regulatory Mechanisms

It is well known that the testis requires a temperature $\sim 2\text{--}3^\circ\text{C}$ lower than abdominal temperature for normal sperm production. The epididymis also requires this lower temperature for normal maintenance of sperm storage in the cauda epididymis. Surgical relocation of the epididymis to the abdomen, an experimental model for cryptepididymis that raises the temperature of the epididymis to that of the abdomen, results in altered expression of genes, including *Bcl2* and *bax*, which may be responsible for inducing apoptosis in the cauda epididymis (Jara et al., 2002).

In addition to temperature, normal fluid flow in the epididymis is also required for normal function. Gene expression microarray analyses have shown that vasectomies result in altered expression of several genes in the human epididymis (Thimon et al., 2008). Genes affected include *Crisp1* (the predominant protein secreted in the cauda epididymis), Beta-Defensin 126 (*Defb126*), Disintegrin and metalloproteinase domain-containing protein 7 (*ADAM7*), and epididymal sperm binding protein 1 (*ELSPBP1*). The cause of these gene expression alterations has not been determined but is most likely due to either increases in luminal fluid pressure or altered rates of luminal fluid flow.

Summary

The sole function of the epididymis is to provide the microenvironment necessary for the maturation of testicular sperm to sperm that are fully functional upon ejaculation. The epididymis has no known role in systemic physiology, and is only rarely the site of any disease process. Defects in epididymal function are likely to account for a significant percentage of idiopathic male infertility, especially in those cases where no testicular defect in sperm production can be found. The importance of epididymal function to normal fertility, combined with its specific role in reproduction, make it an attractive tissue to target for the development of male contraceptives. Precise control of gene expression is critical to the normal function of the epididymis and there are many different signaling pathways and cellular processes that control this gene expression. The many facets of carefully regulated gene expression discussed above suggest that there are promising diagnostics for male infertility, as well as points for contraceptive intervention, yet to be discovered in future studies of this critical reproductive tissue.

References

- Belleannee, C. (2015). Extracellular microRNAs from the epididymis as potential mediators of cell-to-cell communication. *Asian Journal of Andrology*, *17*, 730–736.
- Belleannee, C., Calvo, E., Thimon, V., Cyr, D. G., Legare, C., Garneau, L., & Sullivan, R. (2012a). Role of microRNAs in controlling gene expression in different segments of the human epididymis. *PLoS One*, *7*, e34996.
- Belleannee, C., Thimon, V., & Sullivan, R. (2012b). Region-specific gene expression in the epididymis. *Cell and Tissue Research*, *349*, 717–731.

- Bjorkgren, I., Saastamoinen, L., Krutskikh, A., Huhtaniemi, I., Poutanen, M., & Sipilä, P. (2012). Dicer1 ablation in the mouse epididymis causes dedifferentiation of the epithelium and imbalance in sex steroid signaling. *PLoS One*, 7, e38457.
- Bjorkgren, I., & Sipilä, P. (2015). The role of Dicer1 in the male reproductive tract. *Asian Journal of Andrology*, 17, 737–741.
- Chauvin, T. R., & Griswold, M. D. (2004). Androgen-regulated genes in the murine epididymis. *Biology of Reproduction*, 71, 560–569.
- Cornwall, G. A., & Hann, S. R. (1995). Specialized gene expression in the epididymis. *Journal of Andrology*, 16, 379–383.
- Hamzeh, M., & Robaire, B. (2011). Androgens activate mitogen-activated protein kinase via epidermal growth factor receptor/insulin-like growth factor 1 receptor in the mouse PC-1 cell line. *The Journal of Endocrinology*, 209, 55–64.
- Hinton, B. T., Lan, Z. J., Rudolph, D. B., Labus, J. C., & Lye, R. J. (1998). Testicular regulation of epididymal gene expression. *Journal of Reproduction and Fertility. Supplement*, 53, 47–57.
- Jara, M., Esponda, P., & Carballada, R. (2002). Abdominal temperature induces region-specific p53-independent apoptosis in the cauda epididymidis of the mouse. *Biology of Reproduction*, 67, 1189–1196.
- Johnston, D. S., Jelinsky, S. A., Bang, H. J., DiCandeloro, P., Wilson, E., Kopf, G. S., & Turner, T. T. (2005). The mouse epididymal transcriptome: Transcriptional profiling of segmental gene expression in the epididymis. *Biology of Reproduction*, 73, 404–413.
- Johnston, D. S., Turner, T. T., Finger, J. N., Owtscharuk, T. L., Kopf, G. S., & Jelinsky, S. A. (2007). Identification of epididymis-specific transcripts in the mouse and rat by transcriptional profiling. *Asian Journal of Andrology*, 9, 522–527.
- Ma, W., Hu, S., Yao, G., Xie, S., Ni, M., Liu, Q., Gao, X., Zhang, J., Huang, X., & Zhang, Y. (2013). An androgen receptor-microRNA-29a regulatory circuitry in mouse epididymis. *The Journal of Biological Chemistry*, 288, 29369–29381.
- Nixon, B., Stanger, S. J., Mihalas, B. P., Reilly, J. N., Anderson, A. L., Dun, M. D., Tyagi, S., Holt, J. E., & McLaughlin, E. A. (2015). Next generation sequencing analysis reveals segmental patterns of microRNA expression in mouse Epididymal epithelial cells. *PLoS One*, 10, e0135605.
- Robaire, B., & Hamzeh, M. (2011). Androgen action in the epididymis. *Journal of Andrology*, 32, 592–599.
- Robaire, B., & Henderson, N. A. (2006). Actions of 5alpha-reductase inhibitors on the epididymis. *Molecular and Cellular Endocrinology*, 250, 190–195.
- Robaire, B., Hinton, B. T., & Orgebin-Crist, M. C. (2006). *The epididymis*. New York: Elsevier.
- Robaire, B., Seenundun, S., Hamzeh, M., & Lamour, S. A. (2007). Androgenic regulation of novel genes in the epididymis. *Asian Journal of Andrology*, 9, 545–553.
- Thimon, V., Calvo, E., Koukoui, O., Legare, C., & Sullivan, R. (2008). Effects of vasectomy on gene expression profiling along the human epididymis. *Biology of Reproduction*, 79, 262–273.
- Turner, T. T., Johnston, D. S., Jelinsky, S. A., Tomsig, J. L., & Finger, J. N. (2007). Segment boundaries of the adult rat epididymis limit interstitial signaling by potential paracrine factors and segments lose differential gene expression after efferent duct ligation. *Asian Journal of Andrology*, 9, 565–573.

Vas Deferens

Ryan Flannigan and Marc Goldstein, Center for Male Reproduction and Microsurgery, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Development

The vas deferens (VD) is derived from the mesonephric or Wolffian ducts. Here, it is formed from the channel or linkage which forms between the mesonephros to the urogenital sinus. This occurs alongside the ureteric development. The VD then progressively lengthens as it descends along with the testis through the inguinal canal into the scrotum (Gier and Marion, 1970; Hannema and Hughes, 2007).

Function

The VD's primary function is to transport sperm during emission from the epididymis to the ejaculatory duct. This is performed through a rhythmic propulsion of fluid and sperm at the time of emission. The VD also functions to store sperm in conjunction with the epididymis and convoluted VD. The VD produces fluid that facilitates sperm transport, and resorbs dead sperm and sperm parts that aren't ejaculated.

Anatomy and Structure

Anatomy

The VD is between 30 and 35 cm in length, Fig. 1. The proximal 2–3 cm is convoluted in shape as it leaves the epididymis. It travels within the spermatic cord from the scrotum through the external and internal inguinal rings. It enters the pelvis lateral to the epigastric vessels, then courses anterior to the external iliac artery, medially along the pelvic sidewall before it crosses anterior to the ureter as it approaches the bladder and accompanies the seminal vesicle entering the ejaculatory duct at the base of the posterior prostate. The VD is categorized into 5 segments: (1) The paraepididymal segment or convoluted VD; (2) scrotal (straight) segment; (3)

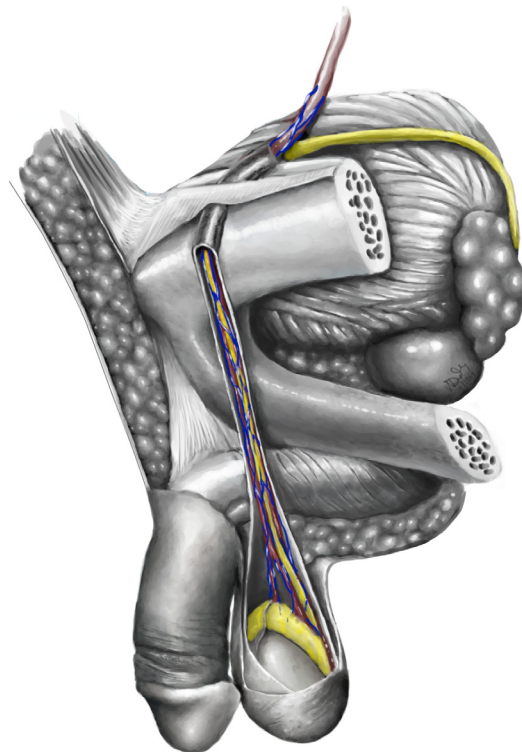


Fig. 1 Demonstrating the anatomical path of the vas deferens. It travels within the spermatic cord from the scrotum through the external and internal inguinal rings. It enters the pelvis lateral to the epigastric vessels, then courses anterior to the external iliac artery, medially along the pelvic sidewall before it crosses anterior to the ureter as it approaches the bladder and accompanies the seminal vesicle entering the ejaculatory duct at the base of the posterior prostate.

Inguinal canal segment; (4) Retroperitoneal segment; (5) Ampulla segment (also convoluted). In both the second and third segments, the VD travels within the spermatic cord, posteriorly between the internal and external spermatic fascias.

Vascular and Lymphatic Supply

The VD harbors an outer adventitia that occupies the primary neurovascular structures. The blood supply is derived from the inferior or superior vesical artery, which are branches of the internal iliac artery (AKA hypogastric artery), from which the deferential artery arises. The venous drainage occurs via deferential veins which drain into the pampiniform plexus toward the testicular end, and into the pelvic venous plexus once in the retroperitoneum. The lymphatic drainage extends into the internal and external iliac nodes.

Structure

The VD measures 1–2.7 mm in outer diameter while the lumen measures 0.2–0.7 mm. The outer most layer consists of the adventitia. Next, the muscularis layer, is composed of a helically oriented outer longitudinal layer, a medial thick circular layer and an inner longitudinal layer, **Fig. 2**. Each muscle cell is surrounded by 6–12 additional cells allowing electrical coupling and facilitates transduction of depolarization from cell to cell ([Koslov and Andersson, 2013](#)). The mucosal layer lining the lumen of the VD consists of pseudostratified columnar epithelium, with a transition of cell type from base to lumen of: principal cells, pencil cells, and mitochondria-rich cells. The thickness of the epithelium decreases from the testis end to the seminal vesicle end. The epithelium is configured into microvilli (steriocilia). Lymphocytes are frequently found among the epithelial cells. Basal cells form a discontinuous layer between the epithelium and the lamina propria which provides an interface to the muscular layers ([Dixon et al., 1998](#)).

Innervation

Autonomic innervation controls both motor and secretory function. Sympathetic nerves originate from preganglionic efferent nerves derived from thoracic and lumbar regions (T10–L2), which then pass through the hypogastric nerves, and convalesce in the pelvic ganglia. Here, the pelvic ganglia also consists of parasympathetic nerves which were derived from the splanchnic nerves consisting of sacral roots 2, 3, and 4. These nerves then proceed to innervate pelvic organs such as the VD. The main trunks exist in the perivascular adventitia around the vessels with the predominance of nerve fibers surrounding the arteries and minimal numbers surrounding the veins. These nerves are generally noradrenergic in nature, however, acetylcholinesterase (AChE) positive nerves also exist in this space. Noradrenergic nerves have been identified to be localized primarily to the lamina propria and are more plentiful near the distal, prostate portion of the VD. In humans, the ratio of axonal varicosities to smooth muscle cells is 1:30. These make up the dominant motor component of innervation for the VD. The noradrenergic nerves contain catecholamine-synthesizing enzymes such as tyrosine hydroxylase and dopamine beta hydroxylase in the majority of intramuscular fibers, and nearly 2/3 contain neuropeptide Y, while only small populations contain enkephalin (ENK), galanin (GAL), somatostatin (SOM), or nitric oxide synthase (NOS). These noradrenergic fibers mediate muscular contraction and transport of sperm. Cholinergic nerves however, localize more commonly within the inner muscle layers and are less plentiful. They contain vasoactive intestinal polypeptide (VIP) and NPY in the intramuscular fibers, and NPY, VIP, nitric oxide synthase (NOS) and acetylcholinesterase in the subepithelial fibers. Purine receptors have also been identified within the autonomic nerves of the VD, but of unknown significance. The sensory nerves transmit signals to the dorsal root ganglia ([Koslov and Andersson, 2013](#); [Dixon et al., 1998](#)).

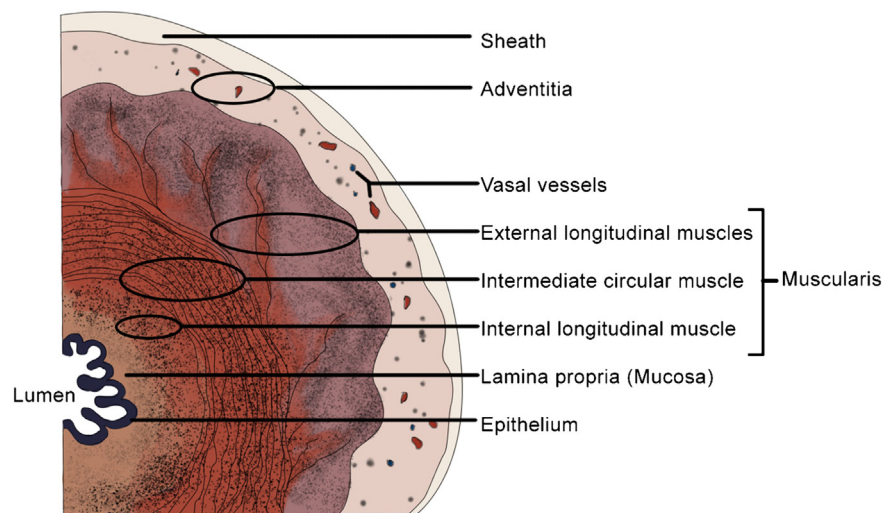


Fig. 2 Illustration of the cross section of the vas deferens. Outer adventitial layer; muscular layer consisting of outer longitudinal fibers, medial circular fibers, and inner longitudinal fibers; epithelial layer surrounding the lumen.

Clinical Considerations

Clinical Assessment of the Vas Deferens

The scrotal portion of the vas deferens is assessed clinically by palpation. It may also be identified by ultrasound as a tubular structure with the absence of Doppler flow. The pelvic portion of the vas deferens, as it enters the ejaculatory ducts, can be assessed by trans-rectal ultrasound (TRUS). However, neither test is able to determine patency. Thus, if obstruction is suspected by history and physical examination, the intraoperative vasography is necessary to confirm obstruction. Conventionally, formal vasography would be performed by creating a small hemi-vasotomy and injecting radio-opaque contrast into the abdominal lumen while taking an X-ray. However, this is typically reserved for cases that are difficult to diagnose the level of obstruction based upon history and physical. Otherwise, scrotal, inguinal and abdominal VD patency is generally assessed by injecting normal saline or lactated ringers toward the abdominal side. If resistance or reflux is met, then obstruction is likely. In this case, a formal vasogram is indicated. Another option, if obstruction in the inguinal portion of the VD from prior hernia repair is suspected, is to feed the tail of a 2-0 monofilament toward the abdominal side of the VD. The point at which the suture stops likely reflects the point of obstruction. This is a practical solution in many cases but does not have the precision of a formal vasogram when there is no prior hernia repair. An additional option for assessment of the abdominal end involves injecting indigo carmen in the lumen of the VD and determining if there is any collection of blue dye in the urine. This is practical for assessing unilateral obstruction, but not if the suspected obstruction is bilateral as the urine will be already blue in the event that the first side was patent. Assessing the testicular end of a VD requires examining the fluid from the vasal lumen or from a barbotaged specimen if the lumen is dry. This fluid is then examined under a bright-field microscope under 400 x magnification in the operating room. The presence of sperm or many sperm heads proves patency of the epididymis.

Congenital Bilateral Absence of the Vas Deferens

Congenital bilateral absence of the vas deferens (CBAVD) is characterized the absence of the corpora and cauda of the epididymis, absence of the vas deferens and absence of the seminal vesicles. The caput, or at least some of the caput, of the epididymis is usually present, with only the caput present in 21%, caput and corpora in 67%, and caput, corpora and cauda of the epididymis in 12% (Liu et al., 2017). This typically occurs bilaterally but can occur unilaterally. It carries a prevalence of 1% among infertile men and 25% among men with primary obstructive azoospermia (Yu et al., 2012). CBAVD is due to a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This gene encodes a salt homeostasis anion channel among epithelial cells. In the event of a CFTR gene mutation leading to loss of function of this gene, thickened secretions typically occur in lung and pancreas. These thick secretions are also thought to be present in the forming vas deferens during development, causing obstruction and subsequently resulting in abnormal development. Over 1950 CFTR mutations have been identified, but fortunately only a subset has been identified to cause disease in an autosomal recessive pattern. Severe, class 1, 2, and 3 mutations in both copies of the CFTR gene lead to cystic fibrosis (CF) which carries a prevalence of 0.03% among the general population but is the most frequently encountered lethal genetic diagnosis among Caucasians (Yu et al., 2012). Mild forms of the mutations, class 4 and 5, may be phenotypically normal or present with CBAVD. Among men with CBAVD, 78% carry at least 1 mutation, and 46% carry 2 mutations, termed compound heterozygotes. Most common alleles found to be mutated among Northern Europeans, include: 5T (25%), F508del (17%), and R117H (3%) (Yu et al., 2012).

Clinically, these men present with low volume, clear, watery semen and low semen pH due to atrophic seminal vesicle or obstruction of seminal vesicle ducts or ejaculatory ducts. In these men, the semen is comprised only of clear, acidic prostatic fluid. They are amenable to epididymal sperm extraction from the caput of the epididymal remnant. This may be performed by percutaneous epididymal sperm aspiration (PESA) Fig. 3A, or microsurgical epididymal sperm aspiration (MESA) Fig. 3B. Sperm is successfully retrieved using PESA in 61%–96% of patients, and yields thousands to millions (Esteves et al., 2011). MESA is the gold standard with successful sperm retrieval in 96%–100% of patients, yielding 15–95 million sperm (Bernie et al., 2013) and pregnancy and live delivery rates of up to 70%. IVF/ICSI is typically required in most cases. All patients presenting with CBAVD should receive CFTR testing for both the patient and their partner, and have an informed discussion with the clinician or a geneticist regarding risk of CF to the offspring. Men presenting with vasal agenesis should also receive an abdominal ultrasound to evaluate for renal agenesis, since this is present among 73.3% of men with unilateral absence of the vas deferens and 11.8% among CBAVD (Salwan and Abdelrahman, 2017).

Duplication Anomalies

Duplication of the vas deferens is exceedingly rare. Only 28 case reports have emerged in the literature. These have been found during orchidopexy, varicocelectomy, inguinal hernia repair, vasectomy and radical prostatectomy. It is thought to occur secondary to a transverse division or duplication of the central portion of the mesonephric (Wolffian) duct during organogenesis. These can be classified as: Type 1, multiple vas deferens without polyorchidism; type 2, multiple vas deferens with polyorchidism; type 3, false poly-vas, with ectopic ureter draining into the ejaculatory system (Breitinger et al., 2016).

Ectopic Vas Deferens

Ectopia of the vas deferens is rare. The ectopic VD may connect to the ureter or the bladder. During embryogenesis, the ureter arises from a bud of the Wolffian duct and connects to the urogenital sinus via a channel. This channel is assimilated with the vas deferens

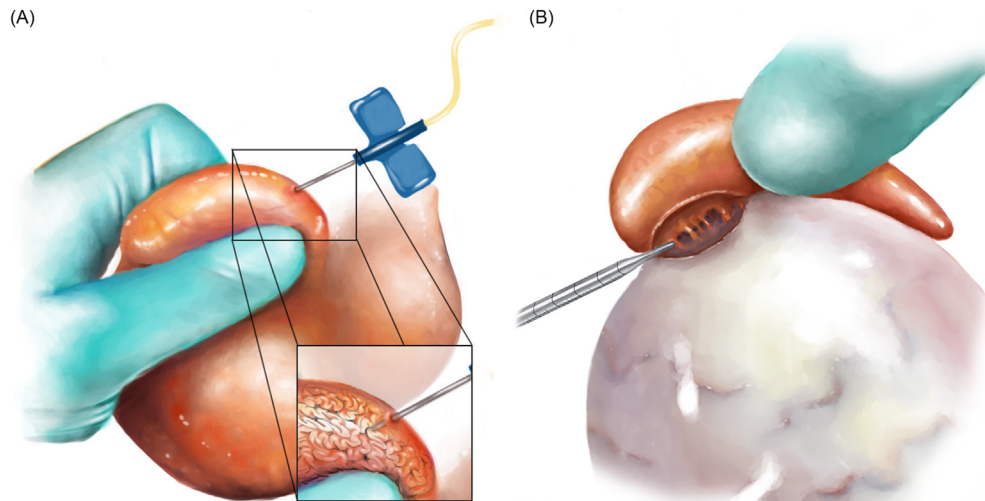


Fig. 3 (A) Percutaneous epididymal sperm aspiration (PESA). This is performed under a local anesthetic, and a 21–26-gauge needle is inserted into the caput of the epididymis and sperm is withdrawn. (B) Microsurgical epididymal sperm aspiration (MESA). This is performed under a general anesthetic. The testis is delivered, and the tunica vaginalis is opened. The caput of the epididymis is gently reflected and the tunical surface is incised exposing the 6–11 efferent ducts. One dilated duct is punctured and sperm is retrieved with a glass micropipette via capillary action.

arising also from the Wolffian duct and both enter the primitive bladder. Normally, the portion of the primitive bladder that the VD inserts into becomes the posterior urethra, namely the verumontanum; however, if failure of the primitive bladder migration occurs, then the VD remains inserted into the bladder. If the primitive bladder fails to obliterate the bridging tissue between the VD and the ureter, then the final position of the VD orifice inserts into the ureter or the ureter inserts into the VD. Ectopic VD may present with UUTI's, vesicovasal, or vesicoureterovasal reflux causing epididymitis, and possible scrotal abscess. Associated conditions include imperforate anus and renal dysplasia (Saifee and Modi, 2016).

Acknowledgement

Medical Illustrator: Vanessa Dudley.

Source of Funding: New York Section of the American Urological Association E. Darracott Vaughan MD, Research Scholar Award.

References

- Bernie, A. M., Ramasamy, R., Stember, D. S., & Stahl, P. J. (2013). Microsurgical epididymal sperm aspiration: Indications, techniques and outcomes. *Asian Journal of Andrology*, 15(1), 40–43.
- Breitinger, M. C., Roszkowski, E. H., Bauermeister, A. J., & Rosenthal, A. A. (2016). Duplicate vas deferens encountered during inguinal hernia repair: A case report and literature review. *Case Reports in Surgery*, 2016, 8324925.
- Dixon, J. S., Jen, P. Y., & Gosling, J. A. (1998). Structure and autonomic innervation of the human vas deferens: A review. *Microscopy Research and Technique*, 42(6), 423–432.
- Esteves, S. C., Miyaoka, R., & Agarwal, A. (2011). Sperm retrieval techniques for assisted reproduction. *International Brazilian Journal of Urology*, 37(5), 570–583.
- Gier, H. T., & Marion, G. B. (1970). Development of the mammalian testis. In *Development, anatomy, and physiology*, 1. New York: Academic Press.
- Hannema, S. E., & Hughes, I. A. (2007). Regulation of Wolffian duct development. *Hormone Research*, 67(3), 142–151.
- Koslov, D. S., & Andersson, K. E. (2013). Physiological and pharmacological aspects of the vas deferens—an update. *Frontiers in Pharmacology*, 4, 101.
- Liu, J., Wang, Z., Zhou, M., Li, M., & Zhan, W. (2017). Scrotal ultrasonic features of congenital bilateral absence of vas deferens. *Ultrasound Quarterly*, 33(2), 153–156.
- Saifee, Y., & Modi, P. (2016). Adult presentation of ectopic vas deferens with dysplastic kidney. *Journal of Endourology Case Reports*, 2(1), 6–7.
- Salwan, A., & Abdelrahman, A. (2017). Congenital absence of vas deferens and ectopic kidney. *International Journal of Surgery Case Reports*, 34, 90–92.
- Yu, J., Chen, Z., Ni, Y., & Li, Z. (2012). CFTR mutations in men with congenital bilateral absence of the vas deferens (CBAVD): A systemic review and meta-analysis. *Human Reproduction*, 27(1), 25–35.

Further Reading

- Goldstein, M., & Schlossberg, S. (1988). Men with congenital absence of the vas deferens often have seminal vesicles. *The Journal of Urology*, 140, 85–86.

Prostate—Overview

Takeshi Sasaki, Omar E Franco, and Simon W Hayward, NorthShore University HealthSystem, Evanston, IL, United States

© 2018 Elsevier Inc. All rights reserved.

General Description

The prostate is a secretory glandular organ located at the base of the urinary bladder, it surrounds the urethra and is found in most mammals. The structure of the prostate varies widely between species, in humans the gland is a compound structure composed of branched ducts lined with secretory epithelial cells surrounded by smooth muscle. In young men the prostate is described as approximately the size of a walnut. The prostate secretes a slightly alkaline fluid that in humans comprises around a third of the ejaculate, most of the balance being made up of seminal vesicle (SV) secretions. The alkaline nature of the prostatic secretions provides protection for sperm from the more acidic vaginal environment, contributing to increased sperm motility and survival, thus positively contributing to fertility. Development and adult function of the prostate is dependent upon androgens produced primarily in the testes, although in humans there is also a small, but significant, adrenal contribution.

Interest in the prostate stems mostly from the three common disease conditions that are associated with it, prostate cancer, benign prostatic hyperplasia (BPH) and prostatitis with associated chronic pelvic pain syndrome. These conditions are major causes of morbidity (suffering) and, in the case of prostate cancer, mortality (death) in the male population.

Development

The early embryo is composed of three tissues; ectoderm, which becomes the skin and skin derivatives such as sweat and mammary glands, endoderm which will form the gastro-intestinal tract and derivative organs such as the liver, and, the mesoderm which exists between the other two layers and gives rise to structures such as connective tissues, muscle and various mesodermally-derived epithelial organs such as the kidney. The prostate is derived from the cloaca representing the caudal (posterior) end of the embryonic gut. This is in contrast to the adjacent seminal vesicles and ureters, which are derived from the primitive Wolffian duct, a mesodermal derivative formed by condensation of mesenchyme into epithelium. Thus the SV and ureteral epithelium are of mesodermal origin while the bladder, prostate and urethra epithelia are all endodermal in origin.

In reptiles and birds the separation of the urogenital and gastro-intestinal tracts is internal with feces, urine, eggs and sperm all exiting (in most species) through the cloaca at the caudal end of the gut. Most birds do not have a penis, mating by touching their cloacae together, although some groups, such as ducks, do have a reproductively functional penis. During the embryonic development of mammals, however, the cloaca is subdivided by the formation of the urorectal septum, into the urogenital sinus (UGS) ventrally (towards the front) and the rectum and anal canal dorsally (behind the bladder and prostate). In humans this process starts around 4 weeks post conception and is completed by 6 weeks, in mice the corresponding period is embryonic days 12–14. As a result of this subdivision of the cloaca, mammals have a complete separation of the gastro-intestinal and genitourinary tracts, with discrete evacuation routes for feces (the anus) and urine (the urethra).

Following the subdivision of the cloaca the upper end of the UGS ultimately forms the urinary bladder in both males and females while the lower portion develops, under the influence of sex steroid hormones, to form the prostate in males and the upper portion of the vagina in females. Staack et al. presented a well described and beautifully illustrated paper showing this process in the mouse (Staack et al., 2003).

Development of the prostate begins with the growth of prostatic buds from the fetal UGS starting at about 10 weeks postconception in the human, or at 19 days of gestation in the rat and at 17 days in the mouse. Androgens produced in the testes act on androgen receptors (AR) in the urogenital sinus mesenchyme (UGM) to induce developmental programs including epithelial budding, proliferation, arborization and canalization giving rise to tubular structures lined with tall columnar secretory epithelium surrounded by smooth muscle that differentiates concurrently with the epithelial developmental program. Prostatic ducts differentiate in a proximal to distal direction (from the urethra to the ductal tip) in the growing prostate starting as a solid cord of epithelial cells the tips proliferate and as the cord grows luminal and basal cells differentiate. The epithelium undergoes ductal canalization at the point at which luminal and basal cells become distinguishable setting up cells that can respond to normal functional signals, notable testicular androgens, and initiate and maintain the expression of characteristic secretory markers such as the kallikrein serine protease, prostate specific antigen (PSA). In adulthood, androgens act upon prostatic smooth muscle (which expresses AR) to maintain a fully differentiated growth-quiescent epithelium, and on the prostatic epithelium to induce and maintain secretory function. These events are mediated by the actions of androgens on the stromal cells, with the role of the epithelial AR apparently being restricted to regulating secretory functions.

In humans and common laboratory animals such as mice, the structure of the prostate is relatively stable throughout the year. However in many seasonally breeding animals, the organ responds to changes in testicular size and androgen production that occurs during the breeding season. This allows the organ to grow and function when needed and then reduce in size through apoptosis (programmed cell death) reflecting the reduced testosterone levels in the rest of the year. This response is mirrored in some ways by the various forms of therapy designed to deprive the prostate of active androgens to induce shrinkage in BPH and proliferation and metastasis in prostate cancer.

Cell Types and Structure

Epithelial cells cover the various internal and external surfaces of the body. They have many forms that relate to specific functions. A familiar example is the keratinized squamous epithelium that forms the skin, providing a tough waterproof layer covering the body surface. There are many specialized epithelia including those of the intestine, where cells that secrete digestive enzymes and adsorb nutrients are found, and secretory cells in the breast and prostate that make and secrete the components of milk and prostatic fluid respectively.

The prostatic ducts empty into the urethra which is lined by a so-called transitional epithelium, a specialized lining that has evolved to separate urine from the rest of the body, incorporating a waterproof layer with the ability to cover surfaces of varying volume. Transitional epithelium covers the whole urinary tract from the renal calyces and pelvis, through the ureters and urinary bladder to the distal end of the urethra. In humans transitional epithelial cells cover the proximal 2 mm or so of the prostatic ducts. There is then an abrupt shift in cellular phenotype to a stratified columnar or stratified cuboidal phenotype with basal epithelial cells and secretory luminal cells.

The basal epithelial cells of the prostate, like those in many other organs are somewhat flattened and lie along the epithelial basement membrane (Fig. 1A and B). They express an array of characteristic markers including p63 and cytokeratins 5 and 14. However, unlike the basal epithelium of organs such as the mammary and salivary glands, prostatic basal cells are not myoepithelial in nature, meaning that these cells have no muscular contractile action. Milk and saliva are moved through the alveolar and ductal structures of the breast and salivary gland by contractions of the myoepithelium. In contrast the prostatic secretions are voided by the overall contraction of the smooth muscle of the prostate during ejaculation. The function of prostatic basal epithelium has been the subject of much debate, with no definitive conclusions. Possible roles include maintaining ductal structure—these cells have long processes that surround the ducts which may be suggestive of a structural role, and while prostatic development is possible, in their absence, at least in experimental models, such structures seem to be subject to extreme responses to hormonal changes (Kurita et al., 2004). Basal epithelial cells may also be involved in the transportation of lipids or nutrients to the luminal epithelium, since lipid droplets are noted in the basal cells and lipids are required for the generation of secretory granules in the luminal cells.

Luminal epithelial cells line the prostatic ducts, depending upon the region of the prostate these are cuboidal to tall columnar in shape, and are active secretory cells manufacturing and secreting the components of the prostatic fluid (Fig. 1A and B). Luminal epithelial cells characteristically express cytokeratins 8 and 18, they also almost uniformly express the androgen receptor, which functions to regulate the expression of a number of the characteristic proteins expressed by the prostate, notably including PSA and prostate-specific acid phosphatase.

The epithelial tissue also contains rare neuroendocrine cells, these are hard to identify visually but can be seen clearly using immunohistochemical staining for chromogranin A or synaptophysin. These cells are sparse throughout the prostate with individual cells seen occasionally in ducts and glands. The exact role of these cells in the prostate is unclear, however they are similar to networks seen in other organs allowing some speculation. The neuroendocrine cells have long branched dendritic processes and seem to communicate with the nonneuroendocrine prostatic epithelium through the use of neuroendocrine secretory granules correlating to a number of known products. The cells appear to be innervated and represent a potential communication network throughout the prostate allowing regulation of growth differentiation and secretory activity of the epithelium through paracrine and neurocrine mechanisms.

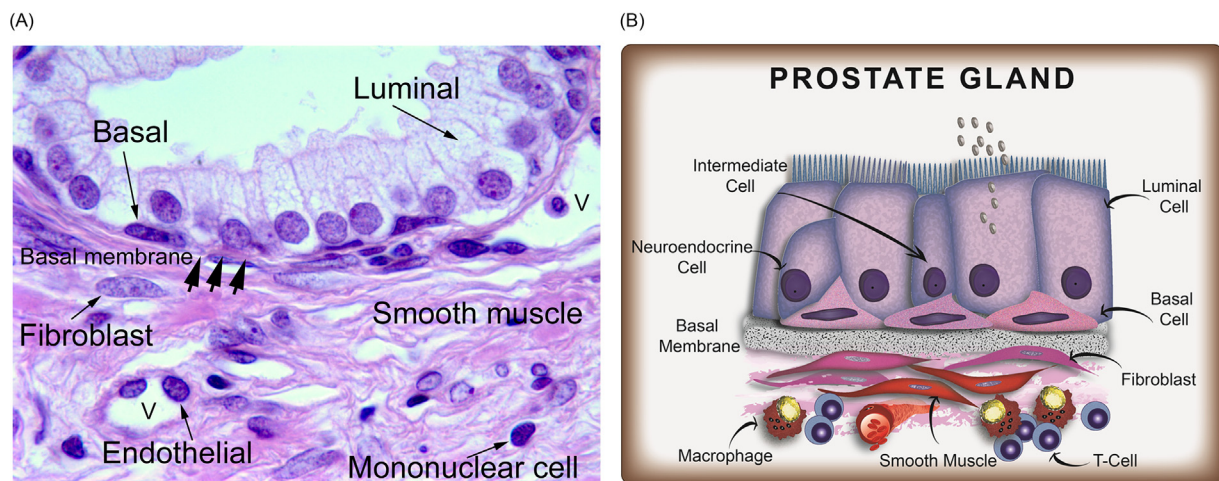


Fig. 1 Major cell types found in the prostate. (A) Representative images of hematoxylin and eosin staining of normal human prostate (original magnification $\times 1000$). V, vessel. (B) Stylized diagram depicting major cell types in the prostate. The epithelium is composed of secretory luminal cells, basal cells that line the basement membrane, neuroendocrine cells that are normally rare but can expand in advanced cancer, and intermediate cells associated with proliferation. The stroma surrounding the epithelial cells is a complex mix of smooth muscle, fibroblasts, endothelial cells and immune/inflammatory cells infiltrate along with blood vessels and nerves. Panel B modified from Strand, D. W. et al. (2016) *Differentiation* 91, 141.

The prostatic stroma, the nonglandular components of the organ, is predominantly smooth muscle and fibrous tissue (often described as a fibromuscular stroma) with associated blood vessels and nerves (Fig. 1A and B). The stroma typically makes up approximately half of the total volume of the prostate. Stromal cells play an important role in both the differentiation and development of the prostate as well as the support of normal prostatic architecture and function. The prostate is surrounded by a fibrous capsule that is penetrated by various nerves that can provide a route for the invasion of tumor cells through the capsule in prostate cancer. Extracapsular extension of a tumor is a quantifiable early negative prognostic marker in the progression of prostate cancer. The prostatic stroma also plays an important role in both the negative and positive regulation of prostate cancer growth and is a potential target for therapy in early stage prostate cancer patients.

Prostatic Anatomy

Over several decades a number of different animal species have been used to provide in vivo models of prostate development and disease. The most notable among these have been mice, rats, dogs, and various nonhuman primates. These species have inherent advantages and disadvantages. The principle disadvantage of most animal prostates as models for the human organ is the profound structural differences between the single lobed human organ and the multilobed organ found in most other species and especially obvious in laboratory rodents. This difference is reflected in the types of disease found in human and non-human prostates, with benign prostatic hyperplasia, which is the most common abnormality in the human prostate, being reported only in dogs and chimpanzees. Therefore while mice and rats, for example, are relatively cheap and easy to maintain, results obtained from them are not necessarily directly applicable to the human condition. In mice and rats the prostate is composed of four paired lobes (anterior, dorsal, lateral and ventral prostate) designated by their position around the urethra (Fig. 2). These lobes grow out into the surrounding peritoneal space and are not encapsulated in the manner of the human organ.

Dr. John McNeal, a pathologist working at Stanford University, developed the nomenclature that is now most commonly used to describe the structure of the human prostate (McNeal, 1984). McNeal divided the prostate into histologically distinct and anatomically separate areas. These are; nonglandular fibromuscular stroma surrounding the organ and the peripheral, transitional and central glandular zones containing a complex, yet histologically distinct ductal system (Fig. 2). The central zone is a wedge of glandular tissue making up most of the base of the prostate and surrounding the ejaculatory ducts. The peripheral zone comprises most of the remainder of the gland surrounding most of the central zone and extending to partially surround the distal portion of the urethra. McNeal also identified the transition zone a smaller, glandular region which surrounding the prostatic urethra.

The peripheral zone is the principal site of prostatitis and carcinoma of the prostate. The peripheral zone ducts exit directly laterally from the postero-lateral recesses of the urethral wall. The system consists of small, simple round to oval acinar structures emptying into long narrow ducts surrounded by a stroma of loosely arranged and randomly interwoven muscle bundles. This area is

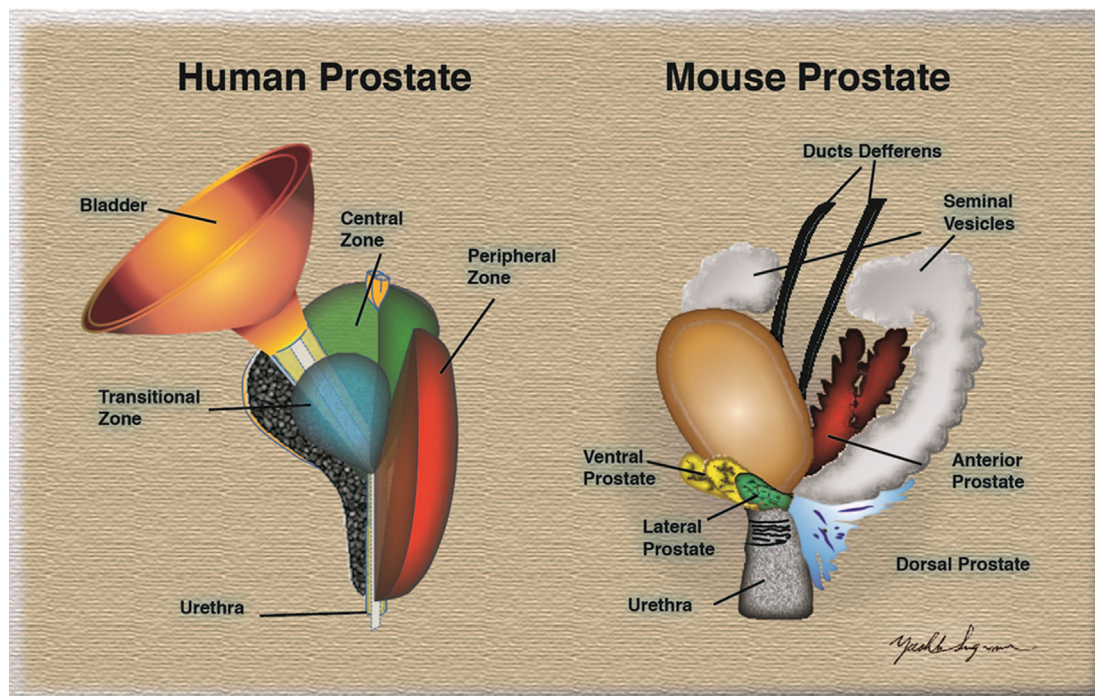


Fig. 2 Structure of human and mouse prostate. (Left) Diagram depicting the three major prostatic zones of the adult human prostate described by McNeal: central zone, peripheral zone, and transitional zone. (Right) Diagram depicting the four major prostatic lobes of the mouse prostate: lateral prostate, dorsal prostate, ventral prostate, and anterior prostate. Reproduced from Aaron, L., Franco, O.E., and Hayward, S. W. (2016) Review of prostate anatomy and embryology and the etiology of benign prostatic hyperplasia. *The Urologic Clinics of North America* 43, 279–288.

the principle site of prostatitis and carcinoma of the prostate although not of BPH. Within the peripheral zone is included the proximal urethral segment of the prostate. The central zone ducts closely follow the ejaculatory ducts. These ducts and acini are large and of irregular shape in cross section. The acini are polyhedral. The muscular stroma of the central zone is much more compact than in the peripheral zone. The transitional zone immediately surrounds the urethra between the bladder and the verumontanum. This is a small volume of the prostate, perhaps 5% in the normal organ, but is the principal site of BPH pathogenesis. Nodular expansion of this region of the prostate results in compression of the urethra and the partial bladder outlet obstruction associated with BPH.

Various authors have suggested a homology between the lobes of the rodent and zones of the human prostate. However such parallels are, at best, weak, the 2001 Bar Harbor Consensus meeting concluded that “there is no existing supporting evidence for a direct relationship between the specific mouse prostate lobes and the specific zones in the human prostate” (Shappell et al., 2004).

The prostate, like many visceral organs, is surrounded by an adipose depot known as periprostatic fat. This is a potential energy reserve, source of lipids, adipochemokines and inflammatory cells that may be significant in prostate cancer and BPH pathogenesis. At present there is little information available on the relationship between the prostate and this adjacent tissue.

Diseases of the Prostate

The source of much of the interest in such a small organ is its outsized propensity for diseases that cause extensive morbidity and mortality. Prostate cancer, BPH and prostatitis are the three major prostatic disease conditions and will be addressed briefly here.

Prostate Cancer

Prostate cancer is the second most commonly diagnosed non-skin malignancy in American men and the second leading cause of cancer deaths, with approximately 180,000 new cases and 26,000 deaths projected by the American Cancer Society in 2016. That being said, prostate cancer is a disease that far more men will die with, rather than from. Prostate cancer is commonly discovered following a routine blood test for serum PSA. This test has become controversial recently because its widespread use has resulted in the diagnosis of a large number of prostate cancers that would likely not have threatened the life of the patient had they remained undiagnosed. This has resulted in a high level of surgery for prostate cancer, with in excess of 40 prostates excised from patients for every life saved from the disease. Given the level of iatrogenic morbidity (suffering resulting from the intervention) from this surgery this was considered by many to be excessive overtreatment of the condition and as a result the PSA testing guidelines have been redrawn.

Risk factors for prostate cancer include race and family history. Certain inherited genetic mutations, for example in the BRCA1 and 2 genes, are also associated with increased risk of prostate cancer and with more aggressive disease. A patient with a raised PSA level and/or a suspicious digital rectal examination or transrectal ultrasound (TRUS) will normally undergo a prostatic biopsy. This involves the collection of a number of needle cores (typically a dozen or more) using a TRUS-guided approach through the rectum. These cores are then sent for examination by a pathologist who will diagnose cancer or other lesions using established grading scores. In prostate cancer the Gleason grading system (graded 1–5) is most commonly used with the two most common areas in the sample being assessed to give a combined Gleason score from 2 to 10. In general, and in the absence of other factors, areas of Gleason grade 3 or lower (and scores of 3 + 3 or less) are considered to be disease with a lower risk of progression. It should be noted that a positive biopsy always indicates the presence of cancer, but gives little more than an outline of how extensive the tumor might be. It should also be noted that there is a strong possibility of a false negative result as the chance of missing a small tumor even with a TRUS-guided needle biopsy is significant. Thus there may well be a need to re-biopsy a patient with a negative result at a later date.

Patients with high grade localized tumors (contained within the prostatic capsule, or locally invasive) will most likely undergo prostatectomy to remove their entire prostate. Historically this was an “open” operation but is now mostly performed robotically, a less invasive approach with better recovery times. Patients with disease that has characteristics representing a low risk of progression may be offered Active Surveillance (AS) as an alternative approach. Under an AS protocol patients are routinely monitored and surgical intervention may occur should their disease be found to progress, however in the interim, and for many patients this can be a successful long-term approach, this allows them to avoid an unnecessary surgery and possible iatrogenic complications that can include impotence and urinary incontinence.

Prostate cancer, at initial diagnosis, is almost always an adenocarcinoma. By definition, a carcinoma is a tumor derived from epithelial cells, and is a class that includes most common human cancers. An adenocarcinoma has glandular characteristics, often reflecting the organ of origin. Precursor lesions include prostatic intraepithelial neoplasia and prostatic inflammatory atrophy. Cancer arises from an accumulation of genetic alterations occurring in the epithelial cells of the tumor. This results in structural changes affecting the phenotype (defined in terms of cellular and nuclear shape and characteristics) of the epithelium and associated changes in the stromal cells resulting from the loss of normal signaling between epithelial and stromal tissues.

A patient whose disease fails surgical therapy, marked by an increase in serum PSA, a so called biochemical recurrence, or by the detection of metastatic lesions will undergo a series of medical interventions. These will most likely include some form, or possibly multiple forms of androgen ablation therapy aimed at suppressing the production and/or actions of androgens in the prostate. Over the last few years a large armamentarium of new drugs have been developed to tackle late stage hormone resistant prostate cancer. At present none of these is curative, however survival times continue to improve.

Benign Prostatic Hyperplasia (BPH)

BPH is not usually a life threatening condition in countries with well developed healthcare systems. This was not always the case and unrelieved urinary retention caused by BPH can have negative consequences on both the urinary bladder and kidneys, and can ultimately lead to death if not relieved.

BPH is found histologically in the vast majority of aging men. In 2007 there were 1.9 million diagnoses of the condition and 120,000 surgeries were performed to relieve symptoms in men who did not respond to medical interventions. The annual direct cost of the disease is several \$bn, and the incidence is increasing as the population ages.

The pathogenesis and progression of BPH is not well understood. It has long been known that the major risk factors were aging and the presence of functional testes. So BPH has long been considered to be a product of sex steroid action including changes in the ratio of testosterone to estrogen upon an aging prostate. However, this is a complex disease and multiple mechanisms are almost certainly active in many patients. A number of comorbidities that are associated with inflammation, including obesity, metabolic syndrome and type 2 diabetes appear to play an important role in disease progression. There is broad recognition that BPH might result from more than one initial cause. A complicating factor is that patients tend to present with a profile of lower urinary tract symptoms (LUTS) that can result from a number of causes. These symptoms are assessed by the International Prostate Symptom Score (IPSS). A diagnosis of BPH normally includes checking the volume of the prostate using TRUS to confirm that this is the cause of the problem. Three medical approaches to the disease have been developed, these are 5 α -reductase inhibitors (5ARIs), drugs that inhibit the conversion of testosterone to the biologically more active dihydrotestosterone, these drugs cause prostate cell death and reduce the size of the organ over time; α -adrenergic blockers target the dynamic muscular component of LUTS relaxing the smooth muscle and allowing better urine flow; Phosphodiesterase (PDE) inhibitors are a frontline treatment for erectile dysfunction that also show activity affecting smooth muscle tone in the prostate. PDE5 inhibitors can be combined with α -blockers to provide an additive effect. General practice is currently focused around the idea that patients with a large prostate benefit from the addition of a 5ARI to an α -adrenergic blockers as this will both shrink a large prostate over time and relax the prostatic muscle. Although many men have a prolonged response to existing agents, a significant number (20%–25%) have side effects severe enough that they do not remain compliant with the therapy and around a third progress to surgical intervention even in the face of treatment. This represents many surgeries in elderly men, who may not be optimal candidates for such procedure.

A clearer understanding of the specific manner in which specific comorbidities contribute to benign prostate growth would allow for the development of new medical approaches for patient subpopulations. Two classes of drugs for such a large patient population results in very limited personalization of therapy, and new approaches to this condition are certainly needed.

Prostatitis

Prostatitis is a symptomatic inflammation of the prostate. There are a number of forms of prostatitis that reflect the complexity of the condition. Bacterial prostatitis can be either acute or chronic in nature. The causative agents include bacterial organisms similar to those causing urinary tract infections and sexually transmitted diseases, notably *Neisseria gonorrhoeae*. These conditions cause local pain, edema, swelling and possibly fever. Acute bacterial prostatitis can usually be treated with antibiotics and will resolve, while the chronic condition can be more challenging to treat effectively.

The most common prostatitis diagnosis however is, chronic abacterial disease. There is no obvious infectious organism and the symptoms, dysuria and low grade pelvic pain do not respond to antibiotics and has become known as chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS). A role for bacteria in this condition has been largely discounted by the field and symptomatic links to interstitial cystitis suggest that the causes of the condition may relate to chronically activated pelvic nerves. The diagnosis of CP/CPPS is complex and not entirely agreed upon and the treatment can follow many different routes including α -adrenergic blockers, nonsteroidal antiandrogens, antidepressants, psychological therapy and physical therapy. This range of options to a large extent seems to reflect the fundamental lack of a deep understanding of the condition as it presents in many patients.

Acknowledgements

This work was funded by the US National Institutes of Health through grants 5U01 CA151924 and 5R01 DK103483 and through philanthropic support by the Rob Brooks fund.

References

- Kurita, T., Medina, R. T., Mills, A. A., & Cunha, G. R. (2004). Role of p63 and basal cells in the prostate. *Development (Cambridge, England)*, 131, 4955–4964.
- McNeal, J. E. (1984). Prostate Anatomy and BPH Morphogenesis. *Progress in Clinical and Biological Research*, 145, 27–54.
- Shappell, S. B., Thomas, G. V., Roberts, R. L., Herbert, R., Ittmann, M. M., Rubin, M. A., Humphrey, P. A., Sundberg, J. P., Rozengurt, N., Barrios, R., et al. (2004). Prostate pathology of genetically engineered mice: Definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. *Cancer Research*, 64, 2270–2305.
- Staack, A., Donjacour, A. A., Brody, J., Cunha, G. R., & Carroll, P. (2003). Mouse urogenital development: A practical approach. *Differentiation; research in biological diversity*, 71, 402–413.

Further Reading

- Aaron, L., Franco, O. E., & Hayward, S. W. (2016). Review of prostate anatomy and embryology and the etiology of benign prostatic hyperplasia. *The Urologic Clinics of North America*, 43, 279–288.

- Bushman, W. A., & Jerde, T. J. (2016). The role of prostate inflammation and fibrosis in lower urinary tract symptoms. *American Journal of Physiology-Renal Physiology*, 311, F817–F821.
- Eeles, R., Goh, C., Castro, E., Bancroft, E., Guy, M., Al Olama, A. A., Easton, D., & Kote-Jarai, Z. (2014). The genetic epidemiology of prostate cancer and its clinical implications. *Nature Reviews Urology*, 11, 18–31.
- Rodriguez-Nieves, J. A., & Macoska, J. A. (2013). Prostatic fibrosis, lower urinary tract symptoms, and BPH. *Nature Reviews Urology*, 10, 546–550.
- Roehrborn, C. G. (2016). Benign Prostatic Hyperplasia: Etiology, Pathophysiology, Epidemiology, and Natural History. In W. S. McDougal, A. J. Wein, L. R. Kavoussi, A. W. Partin, & C. A. Peters (Eds.) (11th edn., vol. 103. *Campbell-Walsh Urology* (pp. 2425–2462.e9). Philadelphia, PA: Elsevier Health Sciences. Review.
- Shen, M., Wang, X., Economides, K., Walker, D., & Abate-Shen, C. (2008). Progenitor cells for the prostate epithelium: Roles in development, regeneration, and cancer. *Cold Spring Harbor Symposia on Quantitative Biology*, 73, 529–538.
- Wang, X., Kruithof-de Julio, M., Economides, K. D., Walker, D., Yu, H., Halli, M. V., Hu, Y.-P., Price, S. M., Abate-Shen, C., & Shen, M. M. (2009). A luminal epithelial stem cell that is a cell of origin for prostate cancer. *Nature*, 461, 495–500.

Relevant Websites

- <https://www.cancer.gov/types/prostate>—The National Cancer Institute website provides a set of basic information on prostate cancer including information on treatment options and current research.
- <https://www.niddk.nih.gov/health-information/health-topics/urologic-disease/benign-prostatic-hyperplasia-bph/Pages/facts.aspx>—The NIDDK website provides a set of basic information on BPH including information on treatment options and current research.
- <https://www.niddk.nih.gov/health-information/health-topics/urologic-disease/prostate-problems/pages/facts.aspx>—The NIDDK website provides a set of basic information on prostatitis including information on treatment options and current research.
- <http://www.gudmap.org/index.html>—Readers interested in the molecular details of the development of the prostate, or other urogenital organs are referred to the GenitoUrinary Development Molecular Anatomy Project (GUDMAP) website.

Prostate Structure

William A Ricke, University of Wisconsin-Madison, Madison, WI, United States

Barry G Timms, University of South Dakota School of Medicine, Vermillion, SD, United States

Frederick S vom Saal, University of Missouri-Columbia, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The prostate is one of the male accessory glands that contributes to the male ejaculate by producing seminal fluid. In mice, removal of this organ reduces fertility (Pang et al., 1979). Our understanding of prostate development, maturation and anatomy has progressed steadily during the past century, but has been partially hindered by reliance on historical anatomical descriptions (Tisell and Salander, 1975; McNeal, 1980a,b; Timms, 1997). Lowsley's (1912) description of prostatic lobes has been replaced by the now widely accepted concept that the human prostate is better described as consisting of zones (McNeal, 1981) found within one discrete organ.

Recent advancements in computer technology allow organ structure to be visualized in 3-dimensions via digitized serial reconstruction. Three-dimensional reconstruction requires tracing, digitizing and axial alignment of identified objects (anatomical structures) within each section from a series of sequential sections. This provides a powerful tool for examining anatomical features, including those of the prostate. This technique has been particularly useful in understanding the complex pattern of ductal morphogenesis, an aspect that is extremely difficult to grasp when viewing 2-dimensional histological sections using a microscope (Timms et al., 1994; Timms, 1997). Additionally, the digital information used to reconstruct the prostatic features provides the basis for quantitative comparisons of experimental manipulations (Timms et al., 1994, 2005; vom Saal et al., 1997). Advanced prostatic imaging using magnetic resonance imaging (MRI) has also been used to build upon our knowledge of the prostate in both men (Bhavsar and Verma, 2014) and mice (McAuley et al., 2017), and will likely continue to provide insights into prostate homeostasis and pathogenesis.

The prostate is an exquisitely steroid hormone sensitive organ. Understanding hormone action during the different life stages of prostate organogenesis, puberty, and throughout adulthood and aging, is critical for understanding prostate anatomy and the different diseases of this organ. Indeed, the majority of men experience some form of prostate pathology during their lifetime. Our focus has been on examining the long-term impact of disruption of normal prostate development on structural and functional abnormalities related to specific types of prostate disease that often are not recognized until much later in life.

The prostate is important in fertility and general health. It is clinically significant because it often becomes diseased as evidenced by the frequency of prostatitis, benign prostatic hyperplasia, and prostate cancer. Prostate cancer (CaP) is one of the most common carcinomas in the Western world (Weir et al., 2003). Benign prostatic hyperplasia (BPH), which is sometimes referred to as benign prostatic hypertrophy, is a common condition beginning in middle age in men. Nearly half of middle-aged men can expect to develop urinary symptoms associated with BPH during their lifetime (Verhamme et al., 2002). Based on animal research, the potential exists for environmentally relevant concentrations of endocrine disrupting chemicals (EDCs) to impact human prostate pathogenesis (NTP-CERHR, 2008).

While there are no human data at this time to assess whether any aspect of prostate disease in men is related to developmental or adult exposures to EDCs, there is a marked similarity in effects in mice and humans due to exposure during sexual differentiation to high doses of the estrogenic drug diethylstilbestrol (DES). DES has been considered the prototypical EDC because there is a large literature about developmental exposure in humans and experimental animals. The conclusion from this literature is that the mouse is an important animal model for predicting the effects of developmental exposure to estrogenic chemicals and drugs in humans.

Historical Overview of the Prostate

Pathology of the prostate was attributed in 1685 by Samuel Collins to "indulgence in venery" that is, the pursuit of sexual pleasure. Collins recognized that prostate size was important with regard to urethral obstruction; this was also deliberated in 1769 by Giam-battista Morgagni (Zuckerman, 1936; Franks, 1954). Franks (1954) noted that Morgagni identified the site of origin of hyperplasia within the prostate and also examined the prevalence of this disease in aged men.

John Hunter observed in 1786 that the prostate (and other male accessory organs) underwent involution following castration. Zuckerman (1936) commented on the remarkable point that even though the implication of this observation would suggest that castration might serve as a treatment to relieve the effects of prostatic enlargement on urethral obstruction, which can be fatal, the implication of this observation was not grasped until many decades later. Castration as a treatment for enlargement of the prostate was finally proposed in the late part of the 19th century, after which it became the preferred method for treating the disease. This approach was soon abandoned because mortality associated with surgery was unacceptably high in that medical era. In addition, although it might seem surprising today, at the end of the nineteenth century there was still considerable controversy concerning the role of the testes in accessory reproductive organ function. At this time it was believed that any effects of the testes on organs such as the prostate were probably mediated via innervation, not secreted substances. This controversy is interesting in that it had been

reported in the mid 1800s that testicular grafts reversed the effects of castration in cockerels, which led to attempts to reverse impotence in aging men by means of xenografting testicular tissues. However, in 1927 procedures for extracting gonadal steroids were described, and testosterone was finally identified as the most potent of the testicular androgen in 1935. At this time, prostate growth in castrated male rats was routinely used as a bioassay for potency of testicular androgens (Burstein, 1955; Medvei, 1982).

Prostate Anatomy and Homology Among Species

Prostate Development and Anatomy

The prostate ducts begin forming as epithelial buds at about 70 days of gestation in humans, gestation day 16.5 in mice and 18.5 in rats (Takeda et al., 1986). These outgrowths begin as solid epithelial structures that branch during late fetal life in humans forming a compound tubulo-alveolar glandular structure (Marker et al., 2003). In mice, birth occurs within about 2 days of the beginning of prostate differentiation, while in rats birth occurs about 4 days after the initiation of gland genesis (Fig. 4). Widespread ductal branching occurs throughout infancy and adolescence. The adult structure is not achieved until approximately 7 weeks postnatally (Sugimura et al., 1986a,b,c). In humans, the pubertal increase in androgen secretion by the testes results in the prostatic glandular ducts forming an obvious lumen within the terminal acini. The epithelial lining becomes highly differentiated and structured, after which androgen-dependent secretory activity commences.

As shown in Fig. 1, the anatomy of the human prostate is described as consisting of zones (McNeal, 1981). The transition zone (TZ) is composed of short glandular ducts, which extend from the urethra above the intersection of the ejaculatory ducts. In the normal prostate the TZ consists of approximately 5% of prostate volume, but can increase in size significantly with age, mainly due to benign prostatic hyperplasia. The central zone (CZ) surrounds the transition zone just under the urinary bladder and is the zone through which the ejaculatory ducts pass and connect with the urethra. The CZ constitutes approximately 25% of the normal prostate and in general is not associated with benign prostatic hyperplasia. Another region that is less commonly described in men, is the anterior fibromuscular stroma. The peripheral zone (PZ) is located outside the posterior region of the central zone, and these ducts extend from the urethra below the intersection of the ejaculatory ducts (Lavoipierre, 2000). The PZ constitutes approximately 70% of the normal prostate volume and increases with age due primarily to the increased incidence of prostate cancer; the prostatic ducts in the PZ are the most prone to cancer, however the TZ has been reported to account for up to a quarter of prostate cancer cases (McNeal et al., 1988).

Interestingly, although BPH is commonly thought of as increased hyperplasia of the prostatic tissue, it is generally through increased proliferation of nodular growth and less so total prostatic proliferation. The structure of these nodules are spheroid, ranging in size from millimeters to centimeters in diameter. These nodular structures can be primarily glandular nodules (containing

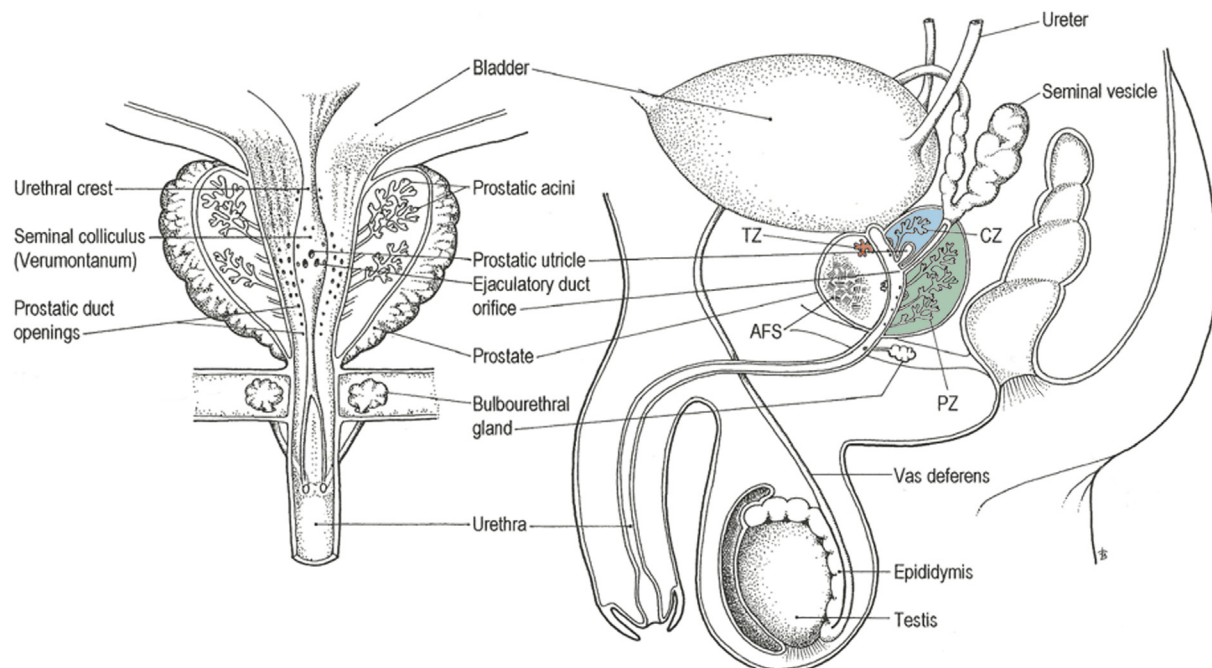


Fig. 1 Diagrams of frontal and sagittal sections of the male urogenital complex showing the anatomical position of the adult prostate and associated structures. The prostatic zones are: central zone (CZ), peripheral zone (PZ), and transition zone (TZ). The anterior fibromuscular stroma (AFS) is also shown. From: Timms, B.G. (1997). *Anatomical perspectives of prostate development*. In: Naz, R. K. (ed.). *Prostate: Basic and clinical aspects*. New York: CRC Press.

epithelium and stroma) or stromal nodules containing primarily stromal cells (e.g., fibroblast-like cells or smooth muscle-like cells). Nodules emanating from the TZ may run parallel and surround the prostatic urethra effectively decreasing urethral compliance and urinary flow, leading of obstructive voiding disorder (OVD). As such, descriptors of BPH include glandular (or epithelial), stromal, and mixed (glandular and stromal) nodules. Although prostatic nodules have long been implicated as structural changes of the prostate, new developments in defining BPH have recently surfaced including the role of fibrosis and collagen deposition (Gharaee-Kermani et al., 2012, 2013; Ma et al., 2012; Bauman et al., 2014; Hao et al., 2016), ultimately changing urethral compliance and general structure of the prostate and urethra.

The ejaculatory ducts form from the embryonic Wolffian ducts during development. The ejaculatory ducts enter the prostatic urethra caudal and lateral to the site of the utricle (a “blind pouch”), which is the remnant of the Müllerian ducts as they merge and enter the posterior urogenital sinus (Fig. 2). The utricle becomes enclosed in the central zone of the prostate in men. Each ejaculatory duct (extensions of the vas deferens) merges with the ipsilateral seminal vesicle duct, which differentiates during the start of the 4th month of embryonic life in humans from the Wolffian duct proximal to the urogenital sinus. The ejaculatory ducts lead into the prostatic urethra next to an enlarged portion of the urethral crest known as the verumontanum. The verumontanum (also referred to as the colliculus seminalis or in latin, mountain ridge), is the amalgamation of three distinct structures: prostatic ducts, ejaculatory ducts, and the utricle (Fig. 1).

The prostatic urethra can be subdivided into a proximal segment (from bladder neck to verumontanum) and distal segment (from verumontanum to external sphincter). This forms a 35–40 degree angle from the horizontal edge at the verumontanum. The proximal urethra is surrounded by circular smooth muscle, which is commonly referred to as the preprostatic sphincter that is the autonomic component of the control of urination and also serves to stop retrograde ejaculation into the urinary bladder, as it is stimulated by alpha-adrenergic neurons to contract during ejaculation.

In contrast to men, the mouse prostatic complex consists of four paired sets of prostate lobes, tissues that immediately surround the prostatic urethra, as well as the ducts that connect the lobes to the urethra (Fig. 3A). The lobes are labeled for their anatomical locations and include: anterior prostate (AP), ventral prostate (VP), dorsal prostate (DP), and lateral prostate (LP). The morphology of the mouse prostate has been described in a series of papers by Sugimura et al. (1986a,b,c). The dorsal and lateral prostates intermingle and are commonly assessed together as the dorsal-lateral prostate or more simply the dorsolateral prostate. The AP, also known as the coagulating gland, lies in apposition to the ipsilateral seminal vesicle, and these are the most cranial of the prostatic

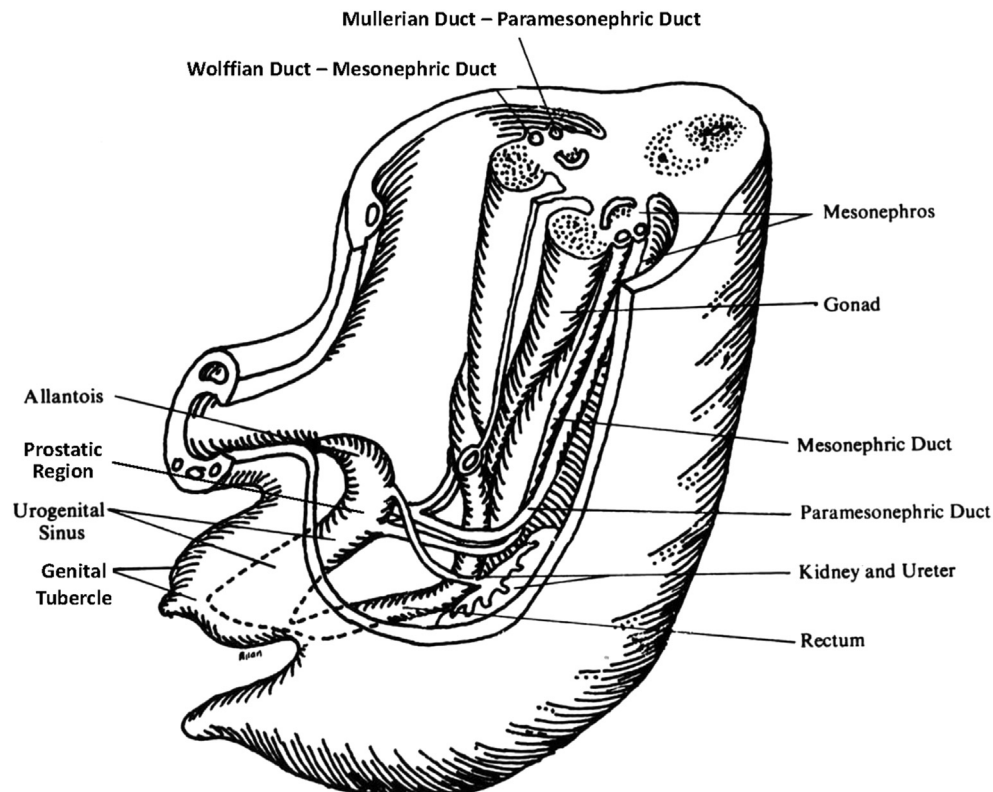


Fig. 2 Genital ducts prior to differentiation. Drawing of a human embryo at about 42 days of age with the upper half and left body wall cut away to demonstrate the gonads, associated Wolffian (mesonephric) and Müllerian (paramesonephric) ducts, and urogenital sinus (UGS). The prostate differentiates from the cranial UGS. The gut and its mesentery have been removed. Modified from: Allan, F.D. (1960). *Essentials of human embryology*. New York: Oxford University Press.

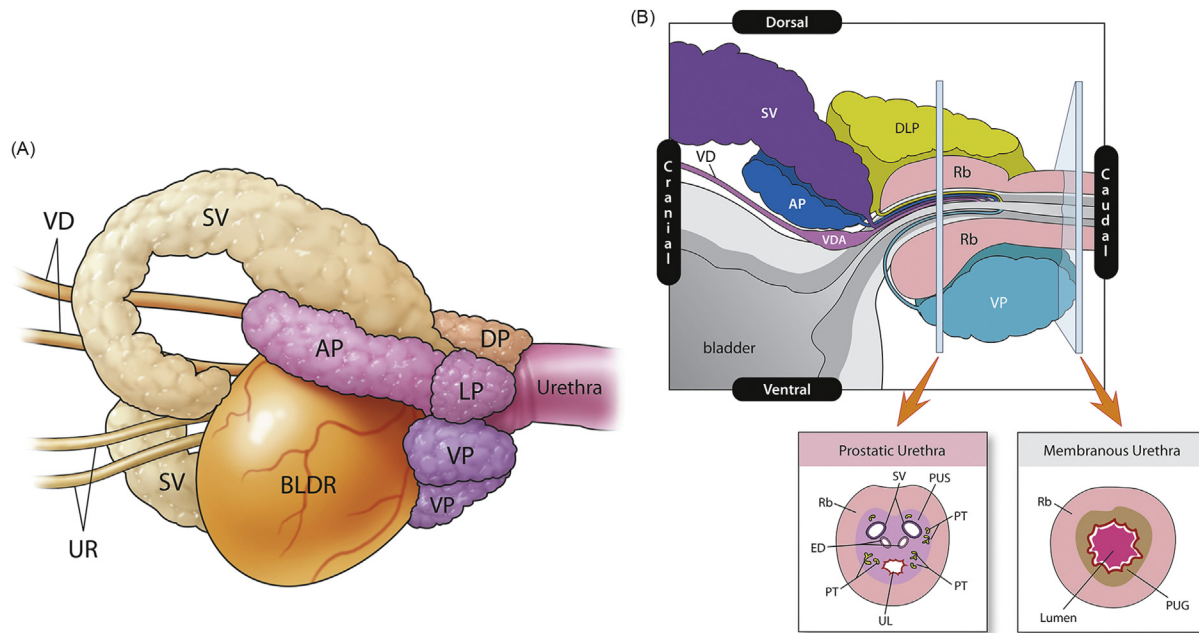


Fig. 3 Adult male mouse lower urinary tract. (A) The mouse has four paired prostatic lobes including: ventral prostate (VP), dorsal prostate (DP), lateral prostate (LP), and anterior prostate (AP) also known as the coagulating gland. Other structures include: urethra (U), urinary bladder (BLDR), ureters (UR), seminal vesicle (SV), and vas deferens (VD). (B) Schematic of a sagittal view of a adult male mouse lower urinary tract, prostatic complex, and urethra. The dorsal and lateral lobes are often grouped together as the dorsal-lateral prostate (DLP). Although the prostatic lobes surround the prostatic urethra, dorsal-lateral prostatic ducts connect to the urethra by entering into the rhabdosphincter (Rb) and run along the prostatic urethra from cranial to caudal ultimately entering the urethra near the verumontanum. These are a complex of tissues/ducts and not simply one duct/lobe. In contrast to the prostate, the seminal vesicles and ejaculatory ducts have one duct that connects to each SV and each vas deferens to the urethra. Caudal from the prostatic urethra, the membranous urethra enlarges in luminal volume. The lower left schematic is a transverse section of the prostatic urethra containing the periurethral space (PUS) which is made up of the urethral lumen (UL), prostatic tissues/ducts (PT), ejaculatory ducts (ED), SV ducts, all of which are surrounded by the Rb. The membranous urethra enlarges relative to the prostatic urethra and contains peri-urethral glands (PUG) surrounding the urethral lumen. The lower right schematic is a transverse section of the membranous urethra containing PUG surrounding the urethral lumen.

ducts. The DP ducts extend dorsally from the prostatic urethra, whereas the VP ducts extend ventrally, and LP ducts extend laterally from the prostatic urethra.

Within the prostate lobes prostatic fluids are produced that are a chief component of the seminal plasma. Prostatic secretions are primarily produced by the luminal cells within the prostate, which upon ejaculation, are transported to the urethra via the prostatic ducts (multiple ducts per prostate lobe) that enter through the rhabdosphincter and run parallel to the prostatic urethral lumen until entering the urethral lumen near the verumontanum (Nicholson and Ricke, 2011; Nicholson et al., 2012) (Fig. 3B). Prostatic glandular ducts extend from the prostatic urethra and branch into terminal ducts that are lined with pseudostratified columnar epithelium. In men, a continuous layer of basal cells underlies the luminal epithelium, while in rodents, basal cells are seemingly interspersed along the epithelial basement membrane (Hayward et al., 1998).

The prostatic epithelium and glandular ducts of the prostate are surrounded by varying degrees of smooth muscle. In humans, this layer of smooth muscle is much thicker than in rodents (Hayward et al., 1998), as such, the stroma to epithelium ratio is much higher in the human prostate compared to rodents. The function of this cell biological difference is unknown. However, in benign urinary diseases in men, alpha-adrenergic blockers, which act upon smooth muscle cells, are commonly used.

Evidence for Homology of the Rodent Dorsolateral and Human Prostate

Due to similarities and differences between rodents and human prostates, as well as the use of various mouse strains as the primary models of human disease, there has been interest in discerning whether zones of the human prostate are comparable to the different prostatic lobes in mice. In mice, prostate lobes can grow outwardly into the abdominal cavity without a restrictive fibromuscular “capsule” as found in humans. This lack of anatomical similarity has long been proposed as a reason for the mouse being an irrelevant functional model for the study of benign diseases like BPH, as decreased urinary function is considered unlikely. Contrasting the lobular prostate's role in disease, it has been proposed that experimental BPH in mice may be due to an increase in prostatic tissue and ducts surrounding the prostatic urethra (Nicholson et al., 2012), much like the new prostatic growth (i.e., BPH nodules) found in men, which can impinge upon the prostatic urethra decreasing urethral luminal area and hence cause an obstruction and urinary symptoms.

PROSTATE DEVELOPMENT IN RAT FETUSES

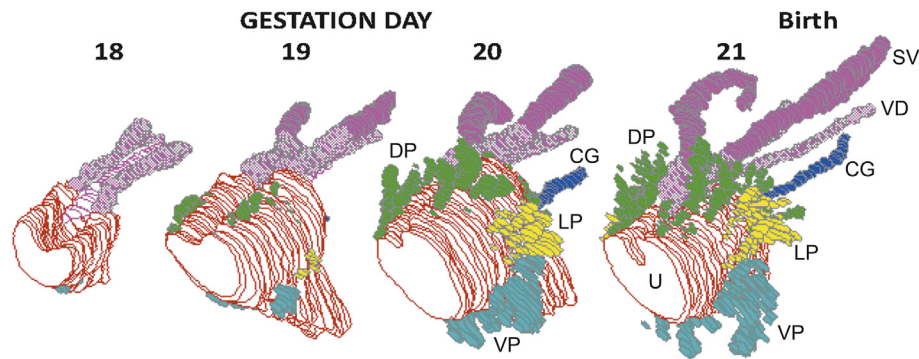


Fig. 4 Serial section reconstructions of the rat urogenital complex on gestation day (GD) 18, 19, 20, and 21, illustrating the stages of early prostate development. Prostate morphogenesis begins at GD 18 and by GD 19 the dorsal, ventral, lateral and dorsocranial (coagulating gland) buds are visible. On GD 21 (the day before birth), prostate budding from the UGS is essentially complete. *U*: urethra; *DP*: dorsal prostate; *LP*: lateral prostate; *VP*: ventral prostate; *CG*: coagulating [dorsocranial] gland; *SV*: seminal vesicle; *VD*: vas deferens. From: Timms, B.G., Peterson, R.E. and vom Saal, F.S. (2002). 2,3,7,8-Tetrachlorodibenzo-p-dioxin interacts with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses. *Toxicological Sciences* **67**, 264–274.

However, there remains controversy regarding whether the range of interspecies variations observed in the structure of the adult prostate gland reflects a diversity that makes it difficult to find suitable animal models for the study of human prostatic diseases (McNeal, 1990). Using a computer-assisted three-dimensional methodology to visualize the microanatomy of the developing prostate, we (Timms et al., 1994) have compared the ductal budding patterns during prostatic morphogenesis in rat, mouse, and human (Figs. 4 and 5). This reconstruction procedure revealed marked similarities in prostate development among rodents and humans.

When distinct species share comparable regulatory systems and common patterns of developing structures, this is taken as evidence that the structures are homologous between species, even though the final form may appear markedly different. A classic example is the wing of a bat, hand of a human, and fin of a dolphin. Thus, in evolutionary biology it is the structural and functional similarities in development, rather than appearance in adulthood, that is used as the basis for assessing whether structures such as the prostate are homologous between species. We proposed that the prostatic ducts that originate from similar regions of the urogenital sinus in rats, mice and humans are homologous structures (Timms et al., 1994). First, the dorsolateral ducts that develop in rodents show a pattern of budding similar to that in the human (Fig. 5). This finding is profound in that others (Price, 1963) observed that the relationship between prostate ductal openings into the urethra persists in the adult in the same relative position as found in the fetus. The major difference between rodents and humans is that, unlike rodents, the human prostate does not exhibit significant postnatal duct development in the ventral region of the developing urogenital sinus. The ventral region of the mouse and rat prostate may thus likely be less relevant for investigating factors that might impact the pathology of the human prostate, since ducts in this region are not found in men (Fig. 5C). To this end, it is the rodent dorsolateral prostate ducts that are homologous to ducts in the human prostate. Additionally, the anterior prostate ducts in the rodent, which are the most cranial ducts that develop from the fetal urogenital sinus, also appear homologous to the most dorsocranial (anterior prostate) ducts in humans.

Many animals, including dogs and cats, have prostates that show a similar developmental pattern to the peripheral zone in the human prostate, while the seminal vesicles and the central zone of the prostate are absent (McNeal, 1980a,b). The glandular tissue that forms the transition zone of the prostate, the zone that is prone to BPH in men, is thus absent in dogs (Hiraoka and Akimoto, 1987; Aumüller, 1989; McNeal, 1990). Nonetheless, adult male intact dogs can develop symptoms of BPH and have hyperplastic prostates. Moreover, experimentally, BPH can be induced in dogs by altering their hormonal milieu (Walsh, 1976; Coffey and Walsh, 1990; Johnston et al., 2000).

Homology also involves biochemical and molecular similarities, and a focus of ongoing research is on regulatory factors that mediate prostate development in humans and animal models (Adams et al., 2002; Thomson et al., 2002; Nicholson et al., 2015). Mesenchyme from the mouse prostate has been shown to produce the appropriate regulatory factors that were able to induce differentiation of human bladder transitional cell urothelium into prostatic epithelium expressing prostatic proteins (Abouseif et al., 1999), thus providing evidence for the similarity of regulatory factors required to identify homologous structures. As lobe-specific or zone-specific molecular determinants of development emerge, we will gain further insight into homology between species.

Environmental Impacts on Development of Prostate Ducts

Endocrine Disrupting Chemicals Alter Prostate Development

Endocrine disruptors such as dioxins, which act via the aryl hydrocarbon receptor (AhR) and also effect the estrogen-response pathway, have been implicated in prostate disease in a two-hit prostate disease model. We exposed fetal/neonatal male mice to

Region-Specific Ductal Morphogenesis of Mice and Men

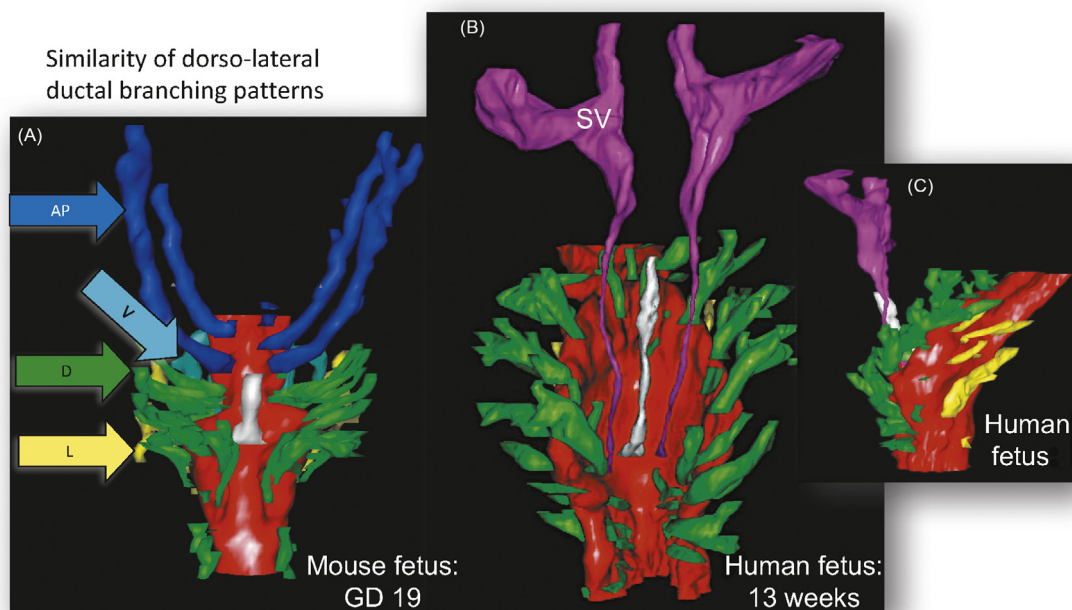


Fig. 5 Panel A: Dorsal view of a newborn mouse urogenital sinus (UGS) reconstruction showing primarily the dorsal (D) prostatic outgrowths aligned with the paired prostatic sulci. The seminal vesicles and ejaculatory ducts have been removed from this reconstruction to clearly illustrate that the most cranial ducts form the long paired anterior prosstate (AP), also referred to as the coagulating glands. These glands extend and attach by a membrane to the anterior surface of the mouse seminal vesicles during subsequent development. Also shown are a few of the lateral (L) and ventral (V) prostatic ducts. The uterine is the midline structure in white. Panel B: Dorsal view of a 13-week male human fetus showing a similar paired pattern of prostatic duct outgrowths along the UGS. The most cranial dorsal prostatic outgrowths correspond to the equivalent anatomical location of the mouse coagulating glands. At these stages of fetal growth, the mouse and human prostate budding patterns demonstrate striking similarities. The uterine is the midline structure in white, and also shown are the seminal vesicles (SV). Panel C is a lateral view of the same human UGS. Modified from: vom Saal, F.S. and Timms, B.G. (1999). The role of natural and manmade estrogens in prostate development. In: Naz, R. K. (ed.) *Endocrine disruptors: Effects on male and female reproductive systems*. pp. 307–327, Boca Raton, FL: CRC Press.

2,3,7,8-tetrachlorodibenzodioxin (TCDD) (a dioxin; Hit #1), and later in adulthood changed their androgen and estrogen hormonal milieu (Hit #2) to mimic that of aging men (a decrease in androgen and an increase in free estrogen). The dioxin exposed mice had more clinically relevant aggressive BPH and lower urinary tract dysfunction compared to controls (Ricke et al., 2016).

Estrogen: Prostate Development and Adult Prostate Morphology

Many questions surround the safety of environmental chemicals with estrogenic activity regarding their ability to alter prostate development and subsequent susceptibility to disease later in adulthood, including prostate cancer and BPH (Zuckerman, 1936; Franks, 1954; Ricke et al., 2006, 2008; Nicholson et al., 2012). A basis for this supposition is that developmental biologists recognized that the region of the developing urogenital sinus just caudal to the bladder, from which the prostatic ducts emerge during fetal life, develops into a portion of the vagina in females. Thus, it is reasonable to venture that, since in the female the vagina is an estrogen-responsive organ, the male organ that develops from the same embryonic tissues, that is, the prostate, might also be estrogen responsive. This led to speculation that estrogens may play a role in regulating prostate organogenesis, subsequent function, and pathology.

In contrast to the notion that estrogens may play a role in normal prostate development, there are numerous reports that prenatal or neonatal exposure to high doses of estrogens, such as estrogenic endocrine disrupting chemicals (including the drug DES), dramatically inhibit the normal development of prostate ducts during fetal/neonatal life in rodents (Prins, 1997). In sharp contrast, more recently there have been studies concerning the stimulatory effects of endogenous estrogens, as well as very low doses of synthetic and natural estrogenic chemicals on development of the prostate. Taken together, these findings suggested the possibility that very low versus very high doses of estrogens could have opposite effects on prostate gland genesis, since non-monotonic dose-response relationships are a common feature of hormones (Vandenberg et al., 2012).

We found that male mouse fetuses exposed in utero to a very small increase (0.1 pg/mL) in free serum estradiol (i.e., within a physiological range) resulted in an increase in the number of fetal prostatic glandular buds (40% more) and overall prostate

size, which was associated with a permanent increase in the number of prostatic androgen receptors (Nonneman et al., 1992; vom Saal et al., 1997; Timms et al., 1999). There was also a significant expansion of the utricule in male mouse fetuses produced by the 0.1 pg/mL increase in free serum estradiol (vom Saal et al., 1997); the utricular portion of the Müllerian duct differentiates into the dorsocranial portion of the vagina in females (vom Saal et al., 1992).

Male mouse exposure to xenoestrogens during fetal life has also been demonstrated to affect prostate structure (Timms et al., 2005). Utilizing the computer assisted 3-D reconstruction method, we discovered that feeding pregnant mice a low 0.1 µg/kg/day dose of DES, the same low dose of the drug ethinylestradiol, or a 10 µg/kg/day dose of the estrogenic chemical bisphenol A (BPA) also stimulated additional prostate duct formation, as well as increased duct size in male mice examined on gestation day 19, just prior to parturition (Fig. 6). Specifically, these estrogens produced an increase in dorsal-lateral duct numbers and duct volume. Assessment of prostate proliferation revealed a twofold increase in cellular proliferation in the dorso-lateral ducts, particularly in cytokeratin-5 positive putative basal cells. Fetal estrogen exposure also lead to urethral constriction near the bladder neck as well as marked malformation in the verumontanum. The implications of these findings are that early-life xenoestrogen exposure may lead to the development of prostate and/or urethral abnormalities found later in adulthood. The hypothesis is that BPH, cancer, and other pathologies may be associated with abnormal ductal development during fetal life. Hence, a foundation for prostatic structural diseases found in adulthood may be explained in part by the hormone environment during gland genesis (Prins et al., 2017). In this regard, the research area of developmental origins of health and disease (DOHaD) is an active area of intense interest, and likely plays an important role in the prostate.

Exposure to supplemental estrogen (in combination with androgen) in adulthood has been related to hyperplasia of the prostate in dogs (DeKlerk et al., 1979), neoplasia in Noble rats (Leav et al., 1988) and mice, as well as inducing metastatic human prostate cancer in a tissue recombination model (Ricke et al., 2006, 2008). In C57Bl/6 male mice, elevation of either androgens or estrogens alone fails to produce dysplasia or prostatic intraepithelial neoplasia, but treatment with the combination of androgens and estrogens resulted in prostatic dysplasia and prostatic intraepithelial neoplasia (Ricke et al., 2008). Hormone manipulation appears to be strain specific, because in Noble rats, neoplastic tumors can be induced to form in the dorsolateral prostatic lobes, while Sprague Dawley rats typically do not develop any prostatic lesions (Noble, 1982; Leav et al., 1988, 1989; Ofner et al., 1992). Although fewer than 1% of Noble rats spontaneously develop adenocarcinoma of the prostate, treatment with a combination of low doses of

EFFECT OF ESTROGENIC CHEMICALS ON PROSTATE DUCT NUMBER AND SIZE IN GD 19 MALE CD-1 MOUSE FETUSES

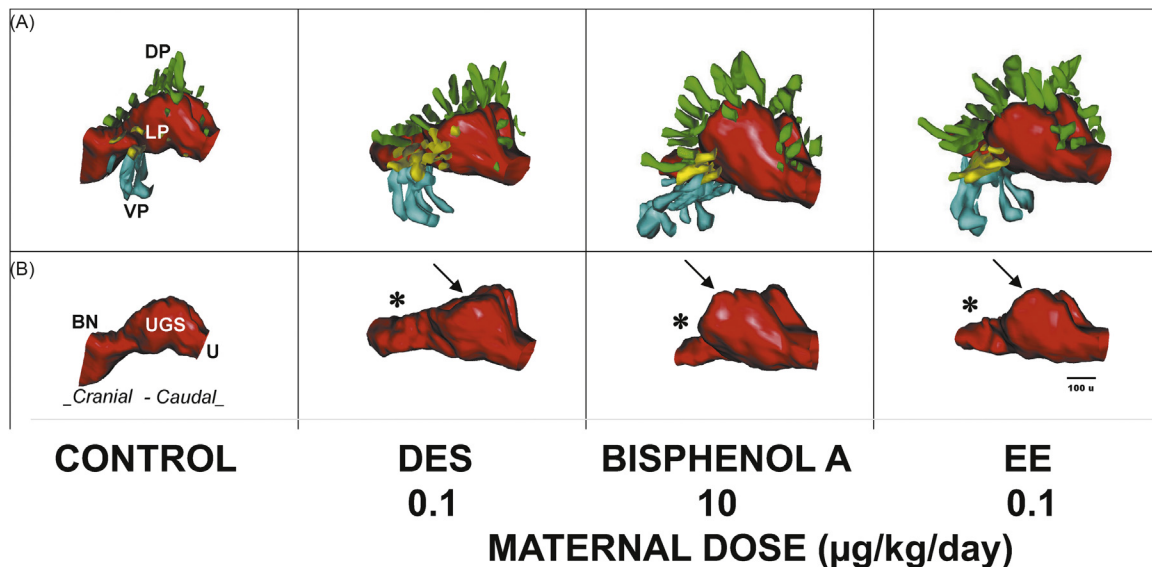


Fig. 6 Three-dimensional serial section reconstruction of the urogenital sinus (UGS) from GD-19 mice exposed to low doses of estrogenic chemicals: diethylstilbestrol (DES), bisphenol A and ethinylestradiol (EE), during fetal development. The UGS depicted for each treatment was closest to the group mean for prostate duct number and size. All images are viewed from a left-lateral perspective. The upper images (Panel A) illustrate the differences in patterns of prostate ductal development following fetal exposure to these chemicals compared to control, untreated animals. There is a significant increase in both the total number of ducts in estrogen treated animals with a corresponding increase in overall prostate volume, particularly in the dorsal (DP) and lateral (LP) regions. The lower images (Panel B) show the marked alteration in the shape of the urethra (U) in the region of the bladder neck (BN), which is markedly constricted (*) in the mice exposed to the estrogenic chemicals, compared to controls. In addition, the region of the UGS associated with the development of the dorsolateral buds (the prostatic sulcus or colliculus—arrow) is enlarged, particularly by bisphenol A, compared to controls. Ventral prostate (VP). Bar = 100 µm. From: Timms, B.G., Howdeshell, K.L., Barton, L., et al. (2005). Estrogenic chemicals in plastic and oral contraceptives disrupt development of the mouse prostate and urethra. *Proceedings of the National Academy of Sciences* 102, 7014–7019.

testosterone and estradiol-17 β for 4 months or longer leads to multifocal prostatic epithelial dysplasia (Ho et al., 1992; Ofner et al., 1992), and results in the transition from dysplasia to neoplastic tumors in about 20% of treated males after ~10 months. Histo-pathological examination of prostates in Noble rats treated with androgen and estrogen showed that the tumors primarily involved glandular epithelium, and metastases after transplantation into new hosts revealed differentiated epithelial components (Noble, 1982). Prostatic neoplastic development occurs primarily in specific regions of the peripheral zone of the human prostate gland (McNeal and Bostwick, 1986). Dysplasia in the dorsolateral lobe of testosterone and estradiol treated Noble rats are nearly identical to the premalignant lesions described in the human gland.

Estrogens in conjunction with androgens have also been implicated in BPH/lower urinary tract dysfunction in male mice (Nicholson et al., 2012). Although prostate lobe growth increased and prostatic urethral area decreased leading to a subsequent bladder volume increase, it is likely that periurethral prostatic growth, which also significantly increased, was a major contributor to bladder enlargement. In addition to prostate proliferation, changes in prostatic urethral stiffness due to collagen deposition has also been proposed as a important prostatic structural change involved in disease progression of the prostate associated with other lower urinary tract symptoms or LUTS (Ma et al., 2012; Bauman et al., 2014; Hao et al., 2016).

Lastly, with regard to estrogens serving as ligands for receptors to promote disease progression, we have uncovered that many of the effects induced by estrogens in the development of prostate carcinogenesis and BPH/lower urinary tract dysfunction are mediated through estrogen receptor alpha. In mice hormonally induced to develop BPH or prostatic carcinogenesis, loss of estrogen receptor alpha, but not estrogen receptor beta, prevented pathogenesis for both diseases (Ricke et al., 2008; Nicholson et al., 2015). Furthermore, treatment of mice manifesting BPH with a selective estrogen receptor alpha antagonist prevented the development of BPH, whereas treatment with a selective estrogen receptor beta antagonist had no effect on preventing the development of BPH/lower urinary tract dysfunction (Nicholson et al., 2015). Overall, this suggests that estrogen receptor alpha is a key mediator of estrogen ligands involved in prostate disease initiation and progression.

Summary

The prostate is a sex accessory gland found in male mammals that provides secretions that aid in reproductive success. The prostate is a hormone regulated gland that can be dramatically affected by sex hormones and endocrine disrupting chemicals that we and others have found to be associated with prostate pathogenesis, both morphological and functional. The development of computer assisted 3-D technology has provided the opportunity to examine factors that alter prostate gland genesis during fetal life and the relationship of these changes to disease onset later in life using rodent models. These studies have revealed that, in contrast to some prior predictions, there are homologous regions of the prostate in the mouse, rat and human that make rodents useful models for the study of human prostate disease.

Acknowledgements

We thank Ms. Sally Griffith-Oh for her help in making figures of the mouse lower urinary tract, and Dr. Ellen Shapiro for providing the human tissue. Funding was provided by grants to WAR (U54 DK104310), CAR from NIEHS (ES-11549), BGT from EPA R-827403, and FVS from NIEHS (ES11283).

References

- Abouseif, S., El-Sakka, A., Young, P., & Cunha, G. R. (1999). Mesenchymal reprogramming of adult human epithelial differentiation. *Differentiation*, 65, 113–118.
- Adams, J. Y., Leav, I., Lau, K. M., et al. (2002). Expression of estrogen receptor beta in the fetal, neonatal, and prepubertal human prostate. *Prostate*, 52, 69–81.
- Aumüller, G. (1989). Morphologic and regulatory aspects of prostatic function. *Anatomy and Embryology*, 179, 519–531.
- Bauman, T. M., Nicholson, T. M., Abler, L. L., et al. (2014). Characterization of fibrillar collagens and extracellular matrix of glandular benign prostatic hyperplasia nodules. *PLoS One*, 9, e109102.
- Bhavsar, A., & Verma, S. (2014). Anatomic imaging of the prostate. *BioMed Research International*, 2014, 728539.
- Burstein, S. R. (1955). The historical background of gerontology, iii, the quest for rejuvenation. *Geriatrics*, 10, 536–540.
- Coffey, D. S., & Walsh, P. C. (1990). Clinical and experimental studies of benign prostatic hyperplasia. *The Urologic Clinics of North America*, 17, 461–475.
- DeKlerk, D. P., Coffey, D. S., Ewing, L. L., et al. (1979). Comparison of spontaneous and experimentally induced canine prostatic hyperplasia. *The Journal of Clinical Investigation*, 64, 842–849.
- Franks, L. M. (1954). Benign nodular hyperplasia of the prostate: A review. *Annals of the Royal College of Surgeons of England*, 14, 92–106.
- Gharaee-Kermani, M., Kasina, S., Moore, B. B., et al. (2012). Cxcr-type chemokines promote myofibroblast phenocconversion and prostatic fibrosis. *PLoS One*, 7, e49278.
- Gharaee-Kermani, M., Rodriguez-Nieves, J. A., Mehra, R., et al. (2013). Obesity-induced diabetes and lower urinary tract fibrosis promote urinary voiding dysfunction in a mouse model. *Prostate*, 73, 1123–1133.
- Hao, L., Greer, T., Page, D., et al. (2016). In-depth characterization and validation of human urine metabolomes reveal novel metabolic signatures of lower urinary tract symptoms. *Scientific Reports*, 6, 30869.
- Hayward, S. W., Haughney, P. C., Rosen, M. A., et al. (1998). Interactions between adult human prostatic epithelium and rat urogenital sinus mesenchyme in a tissue recombination model. *Differentiation*, 63, 131–140.
- Hiraoka, Y., & Akimoto, M. (1987). Anatomy of the prostate from fetus to adult-origin of benign prostatic hyperplasia. *Urological Research*, 15, 177–180.

- Ho, S.-M., Yu, M., Leav, I., & Viccione, T. (1992). The conjoint actions of androgens and estrogens in the induction of proliferative lesions in the rat prostate. In J. J. Li, S. A. Li, & S. Nandi (Eds.), *Hormonal carcinogenesis*. New York: Springer-Verlag.
- Johnston, S. D., Kamolpatana, K., Root-Kustritz, M. V., & Johnston, G. R. (2000). Prostatic disorders in the dog. *Animal Reproduction Science*, 60–61, 405–415.
- Lavoipierre, A. M. (2000). Ultrasound of the prostate and testicles. *World Journal of Surgery*, 24, 198–207.
- Leav, I., Ho, S.-M., Ofner, P., et al. (1988). Biochemical alterations in sex hormone-induced hyperplasia and dysplasia of the dorsolateral prostates of noble rats. *Journal of the National Cancer Institute*, 80, 1045–1053.
- Leav, I., Merk, F. B., Kwan, P. W. L., & Ho, S.-M. (1989). Androgen supported-estrogen enhancement of epithelial proliferation in the prostate of intact noble rats. *Prostate*, 15, 23–40.
- Lowsley, O. S. (1912). The development of the human prostate gland with reference to the development of other structures at the neck of the urinary bladder. *The American Journal of Anatomy*, 13, 299–349.
- Ma, J., Gharaee-Kermani, M., Kunju, L., et al. (2012). Prostatic fibrosis is associated with lower urinary tract symptoms. *The Journal of Urology*, 188, 1375–1381.
- Marker, P. C., Donjacour, A. A., Dahiya, R., & Cunha, G. R. (2003). Hormonal, cellular, and molecular control of prostatic development. *Developmental Biology*, 253, 165–174.
- McAuley, E. M., Mustafi, D., Simons, B. W., et al. (2017). Magnetic resonance imaging and molecular characterization of a hormone-mediated murine model of prostate enlargement and bladder outlet obstruction. *The American Journal of Pathology*, 187(11), 2378–2387.
- McNeal, J. E. (1980a). The anatomic heterogeneity of the prostate. In D. Coffey, D. Merchant, & G. Murphy (Eds.), *Models of prostate cancer* (pp. 149–160). New York: Liss.
- McNeal, J. E. (1980b). Anatomy of the prostate: An historical survey of divergent views. *The Prostate*, 1, 3.
- McNeal, J. E. (1981). The zonal anatomy of the prostate. *Prostate*, 2, 35–49.
- McNeal, J. (1990). Pathology of benign prostatic hyperplasia. Insight into etiology. *The Urologic Clinics of North America*, 17, 477–486.
- McNeal, J. E., & Bostwick, D. G. (1986). Intraductal dysplasia: A premalignant lesion of the prostate. *Human Pathology*, 17, 64–71.
- McNeal, J. E., Redwine, E. A., Freiha, F. S., & Stamey, T. A. (1988). Zonal distribution of prostatic adenocarcinoma. Correlation with histologic pattern and direction of spread. *The American Journal of Surgical Pathology*, 12, 897–906.
- Medvei, V. C. (1982). *A history of endocrinology*. Lancaster, PA: MTP Press.
- Nicholson, T. M., & Ricke, W. A. (2011). Androgens and estrogens in benign prostatic hyperplasia: Past, present and future. *Differentiation*, 82, 184–199.
- Nicholson, T. M., Ricke, E. A., Marker, P. C., et al. (2012). Testosterone and 17beta-estradiol induce glandular prostatic growth, bladder outlet obstruction, and voiding dysfunction in male mice. *Endocrinology*, 153, 5556–5565.
- Nicholson, T. M., Moses, M. A., Uchtmann, K. S., et al. (2015). Estrogen receptor-alpha is a key mediator and therapeutic target for bladder complications of benign prostatic hyperplasia. *The Journal of Urology*, 193, 722–729.
- Noble, R. L. (1982). Prostate carcinoma of the nb rat in relation to hormones. *International Review of Experimental Pathology*, 23, 113–158.
- Nonneman, D. J., Ganjam, V. K., Welshons, W. V., & vom Saal, F. S. (1992). Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. *Biology of Reproduction*, 47, 723–729.
- NTP-CERHR (2008). Ntp-cerhr monograph on the potential human reproductive and developmental effects of bisphenol A, National Toxicology Program-Center for the Evaluation of Risks to Human Reproduction, NIH Publication No. 08–5994, September 2008. Accessed September 19, 2017, <http://ntp.niehs.nih.gov/ntp/ohat/bisphenol/bisphenol.Pdf>.
- Ofner, P., Bosland, M., & Vena, R. L. (1992). Differential effects of diethylstilbestrol and estradiol-17β in combination with testosterone on rat prostate lobes. *Toxicology and Applied Pharmacology*, 112, 300–309.
- Pang, S., Chow, P., & Wong, T. (1979). The role of the seminal vesicles, coagulating glands and prostate on the fertility and fecundity of mice. *Journal of Reproduction and Fertility*, 56, 129–132.
- Price, D. (1963). Comparative aspects of development and structure in the prostate. *National Cancer Institute Monograph*, 12, 1.
- Prins, G. S. (1997). Developmental estrogenization of the prostate gland. In R. K. Naz (Ed.), *Prostate: Basic and clinical aspects* (pp. 247–265). New York: C. R. C. Press.
- Prins, G. S., Ye, S. H., Birch, L., et al. (2017). Prostate cancer risk and DNA methylation signatures in aging rats following developmental bpa exposure: A dose-response analysis. *Environmental Health Perspectives*, 125, 077007.
- Ricke, W. A., Ishii, K., Ricke, E. A., et al. (2006). Steroid hormones stimulate human prostate cancer progression and metastasis. *International Journal of Cancer*, 118, 2123–2131.
- Ricke, W. A., McPherson, S. J., Bianco, J. J., et al. (2008). Prostatic hormonal carcinogenesis is mediated by in situ estrogen production and estrogen receptor alpha signaling. *The FASEB Journal*, 22, 1512–1520.
- Ricke, W. A., Lee, C. W., Clapper, T. R., et al. (2016). In utero and lactational tcdd exposure increases susceptibility to lower urinary tract dysfunction in adulthood. *Toxicological Sciences*, 150, 429–440.
- vom Saal, F. S., Montano, M. M., & Wang, M. H. (1992). Sexual differentiation in mammals. In T. Colborn, & C. Clement (Eds.), vol. 21. *Chemically induced alterations in sexual and functional development: The wildlife/human connection* (pp. 17–83). Princeton, New Jersey: Princeton Scientific Publishing Co.
- vom Saal, F. S., Timms, B. G., Montano, M. M., et al. (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proceedings of the National Academy of Sciences*, 94, 2056–2061.
- Sugimura, Y., Cunha, G. R., & Donjacour, A. A. (1986a). Morphogenesis of ductal networks in the mouse prostate. *Biology of Reproduction*, 34, 961–971.
- Sugimura, Y., Cunha, G. R., & Donjacour, A. A. (1986b). Morphological and histological study of castration-induced degeneration and androgen-induced regeneration in the mouse prostate. *Biology of Reproduction*, 34, 973–983.
- Sugimura, Y., Cunha, G. R., Donjacour, A. A., et al. (1986c). Whole-mount autoradiography study of DNA synthetic activity during postnatal development and androgen-induced regeneration in the mouse prostate. *Biology of Reproduction*, 34, 985–995.
- Takeda, H., Lasnitzki, I., & Mizuno, T. (1986). Analysis of prostatic bud induction by brief androgen treatment in the fetal rat urogenital sinus. *The Journal of Endocrinology*, 110, 467–470.
- Thomson, A. A., Timms, B. G., Barton, L., et al. (2002). The role of smooth muscle in regulating prostatic induction. *Development*, 129, 1905–1912.
- Timms, B. G. (1997). Anatomical perspectives of prostate development. In R. K. Naz (Ed.), *Prostate: Basic and clinical aspects*. New York: CRC Press.
- Timms, B. G., Mohs, T. J., & Didio, L. J. A. (1994). Ductal budding and branching patterns in the developing prostate. *The Journal of Urology*, 151, 1427–1432.
- Timms, B. G., Petersen, S. L., & vom Saal, F. S. (1999). Prostate gland growth during development is stimulated in both male and female rat fetuses by intrauterine proximity to female fetuses. *The Journal of Urology*, 161, 1694–1701.
- Timms, B. G., Howdeshell, K. L., Barton, L., et al. (2005). Estrogenic chemicals in plastic and oral contraceptives disrupt development of the mouse prostate and urethra. *Proceedings of the National Academy of Sciences*, 102, 7014–7019.
- Tisell, L.-E., & Salander, H. (1975). The lobes of the human prostate. *Scandinavian Journal of Urology and Nephrology*, 9, 185.
- Vandenberg, L. N., Colborn, T., Hayes, T. B., et al. (2012). Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*, 33, 378–455.
- Verhamme, K. M., Dieleman, J. P., Bleumink, G. S., et al. (2002). Incidence and prevalence of lower urinary tract symptoms suggestive of benign prostatic hyperplasia in primary care—The triumph project. *European Urology*, 42, 323–328.
- Walsh, P. C. (1976). Benign prostatic hyperplasia: Etiological considerations. *Progress in Clinical and Biological Research*, 6, 1–8.
- Weir, H. K., Thun, M. J., Hankey, B. F., et al. (2003). Annual report to the nation on the status of cancer, 1975–2000, featuring the uses of surveillance data for cancer prevention and control. *Journal of the National Cancer Institute*, 95, 1276–1299.
- Zuckerman, S. (1936). The endocrine control of the prostate. *Proceedings of the Royal Society of Medicine*, 29, 1557–1567.

Further Reading

Allan, F. D. (1960). *Essentials of human embryology*. New York: Oxford University Press.

vom Saal, F. S., & Timms, B. G. (1999). The role of natural and manmade estrogens in prostate development. In R. K. Naz (Ed.), *Endocrine disruptors: Effects on male and female reproductive systems* (pp. 307–327). Boca Raton, FL: CRC Press.

Timms, B. G., Peterson, R. E., & vom Saal, F. S. (2002). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin interacts with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses. *Toxicological Sciences*, 67, 264–274.

Prostate—Cell Biology and Secretion

Timothy D Gauntner and Gail S Prins, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The prostate gland is an accessory sex organ that contributes secretions into the seminal fluid, which aids in sperm delivery and fertilization capacity. Two basic types of cells comprise the prostate gland: epithelial cells, which produce exocrine secretions, and stromal cells, which provide structure to the gland and help expel secretions into the urethra. Within these two broad categories of cells are numerous subtypes, the cellular, and molecular biology of which are detailed below. Although much of what is known about prostate cell biology has important implications for benign and malignant human prostatic disease, the ultimate role of the prostate gland and its constituent cells is to contribute secretions to the seminal fluid for enhancement of sperm delivery. The chemical constituents of these prostatic secretions and their physiologic roles are discussed in depth below.

Cell Biology

The cells of the prostate gland can be broadly classified into two types of cells: the parenchymal epithelial cells, which form the acini and ducts of the gland and secrete prostatic fluid, and the stromal cells, which envelop the acini and ducts to provide support and structure to the gland. The organization of these cell types within the human prostate is shown in Fig. 1.

Epithelial Cells

The acini and ducts, or parenchyma, of the prostate gland are formed by three phenotypically and functionally distinct lineages of epithelial cells—basal, luminal, and neuroendocrine cells (McNeal, 1998). Prostate epithelial cell stem cells, discussed in-depth below, reside within the basal cell layer. A fibro-muscular stroma envelops the glandular epithelium and helps to expel prostatic secretions into the urethra during ejaculation (McNeal, 1998).

Luminal epithelial cells

The exocrine product of prostate glands is produced by secretory luminal cells, which line the lumen of the glandular acini. Secretory cells are cuboidal or pseudo-columnar and rest on a layer of basal cells over the basement membrane. They secrete a variety of proteins and products into the lumen of the glands at the apex of the cell (Forest, 1979). Secretory luminal epithelial cells in the human prostate can be identified via immunohistochemical staining for prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), androgen receptor (AR), the homeobox transcription factor NKX3.1, and cytokeratins CK8 and CK18

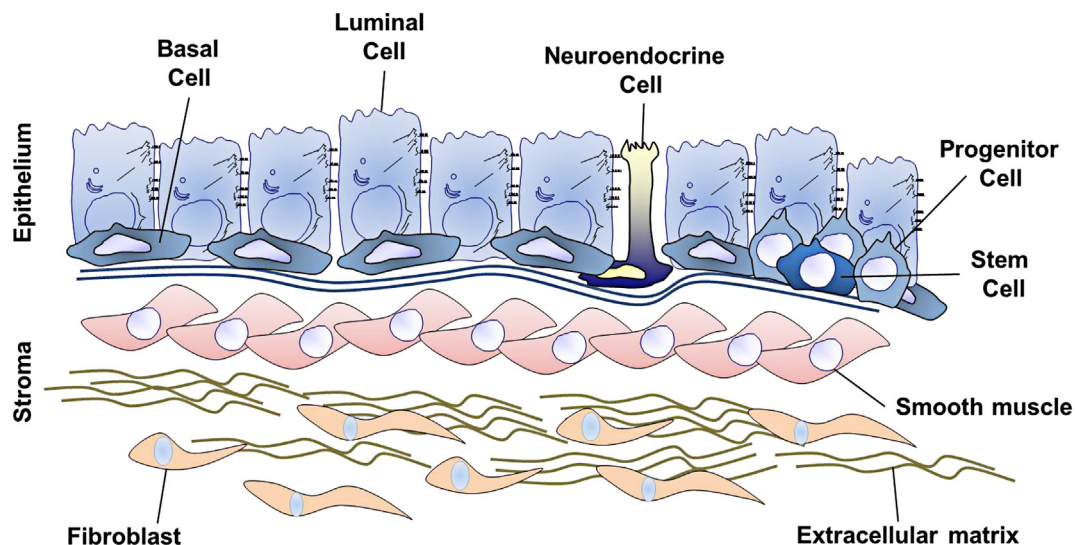


Fig. 1 Schematic depiction of the cell types and organization in the adult prostate gland. The epithelium has three types of differentiated cells; the secretory luminal cells, the basal cells along the basement membrane and a rare neuroendocrine cell. The rare stem cell resides in the basal compartment and upon asymmetric division, gives rise to a transient proliferative progenitor cell population that lineage commits to the differentiated cells. The stromal cells of the prostate include smooth muscle cells and fibroblasts.

(Wang et al., 2001; Sherwood et al., 1991; Bhatia-Gaur et al., 1999; Bonkhoff and Remberer, 1996; Epstein, 1993). Luminal cells express high levels of AR and depend on androgen signaling for their survival and secretory activity (Wright et al., 1996).

Basal epithelial cells

Basal cells are flat to cuboidal, nonsecretory epithelial cells. The human prostate has a continuous layer of basal cells with an approximate 1:1 ratio to secretory luminal cells. In other species, the basal cell layer is discontinuous and has an average ratio of 1:7 basal to secretory luminal cells (El-Alfy et al., 2000). Basal epithelial cells are distinguished from their luminal counterparts by expression of CK5, CK14, and the transcription factor p63, and by the absence of PSA or PAP expression (Wang et al., 2001; Sherwood et al., 1991; Bhatia-Gaur et al., 1999; Bonkhoff and Remberer, 1996; Epstein, 1993). Additionally, basal cells are mostly AR negative (Prins et al., 1991), although they may indirectly respond to circulating androgen via paracrine signaling with AR-expressing luminal cells or stromal cells. In addition to extracellular paracrine signaling, gap junction proteins between basal and luminal epithelial cells permit direct cytoplasmic signaling, suggesting that functions unique to each cell type are co-regulated by the other cell type. Furthermore, junction-like structures are found between cells in the basal layer, leading some to propose the existence of a blood-prostate barrier similar to that found in the testis (El-Alfy et al., 2000; Habermann et al., 2001). Such a barrier might explain the poor perfusion of systemically-administered antibiotics into the human prostate.

Neuroendocrine cells

Neuroendocrine cells represent a third, minor population of epithelial cells within the prostate gland. Although the functional significance of neuroendocrine cells is not well understood, they are known to secrete hormones and cell-signaling factors in response to neural stimulation. They are members of the diffuse amine precursor uptake and decarboxylation system, which is a part of the larger diffuse neuroendocrine system within the body. This classification, however, is merely based on histological and biochemical similarities among neuroendocrine cells found in various tissues throughout the body—cells of the diffuse neuroendocrine system do not necessarily share a common embryologic origin, nor are their functions coordinated by any known mechanism. Prostate neuroendocrine cells are found in the basal cell layer and exist in two distinct morphological variants: an open type, which has a single apical process that projects through the secretory cell layer to the glandular lumen, and a closed type, which has multiple dendritic processes that extend through the basal and luminal epithelial layers but do not reach the lumen. Neuroendocrine cells can be identified by their expression of chromogranin-A, synaptophysin, neuron-specific enolase, calcitonin, bombesin, and somatostatin (Bonkhoff et al., 1991; Abrahamsson et al., 2000). They do not express PSA or AR. While the precise physiologic role of neuroendocrine cells is unknown, they likely integrate neurohormonal signals to modulate development, proliferation, and secretory function of the prostate gland through paracrine mechanisms. Their embryologic origin is the subject of ongoing debate. The two morphological variants of neuroendocrine cells may even have different origins, as there is compelling evidence demonstrating both a neural crest origin of these cells, as well as an origin from a common prostate epithelial stem cell (Szczyrba et al., 2017).

Epithelial stem cells

Stem cells are responsible for repopulating the prostate epithelium throughout life. They reside in a stem cell niche within the basal epithelial layer, where they remain in a quiescent state until niche-derived signals stimulate their considerable proliferative potential. Much of what is known about prostate stem cells is derived from rodent models, however, knowledge of human prostate epithelial cells is rapidly increasing. The great impetus for studying prostate stem cell biology relates to the observation that cancer cells have many of the same characteristics as stem cells: the ability to divide nearly indefinitely, to resist apoptosis, to move within a tissue or even spread into other tissues, and acquisition of altered metabolic states, among others. Indeed, a central hypothesis in the fields of cancer and stem cell biology over the past decade is that stem cells are themselves oncogenic targets and tumor-initiating cells (Lang et al., 2009; Nikitin et al., 2007). Since stem cells are long-lived and quiescent, they may be susceptible to the accumulation of genetic mutations. Many scientists believe this is an important mechanism in prostate carcinogenesis.

There are three fundamental properties of a prostate stem cell: (1) slow growth rate/relative quiescence, (2) high replicative potential, and (3) ability to regenerate a prostate-like gland (Lang et al., 2009). During development of the prostate gland, the stem cells undergo relatively high proliferation in order to generate sufficient progeny to form the gland. In the adult gland, however, the stem cells remain in a relatively quiescent state due to both inherent growth-control mechanisms and cues from the niche. The signaling pathways that control this growth and the decisions to enter self-renewal are both autonomous, originating from the stem cells themselves, and extrinsic, originating from progenitor cells, stromal cells, and other cells within the niche. These pathways include the bone morphogenic peptide/transforming growth factor β (BMP/TGF β), wntless-integrated (Wnt)/ β catenin, fibroblast growth factor (FGF), Notch, Hedgehog, and Ephrin pathways (Prins and Putz, 2008).

At present, our knowledge of hierarchical relationships and regulatory pathways is largely based on adult stem cells, and it is possible that these relationships differ during early development (Xin et al., 2007; Leong et al., 2008; Goldstein et al., 2008; Ousset et al., 2012). Nonetheless, it is known that a precursor epithelial stem cell population is specified from the UGS endodermal lineage during molecular determination of the prostate prior to bud initiation. This process is programmed by a specific combination of WNTs, FGFs, other morphogens and prostate-specific transcription factors that commit the cells to a prostatic fate (Bieberich et al., 1996; Keil et al., 2012; Calderon-Gierszal et al., 2014). In the adult gland, this prostate-specified stem cell exists as a rare cell type that is relatively growth-quiescent and occasionally undergoes asymmetric cell division to give rise to either common or lineage-specific progenitor cell populations (linear versus bifurcated lineage models; see Fig. 2) (Kasper, 2008). These progenitor

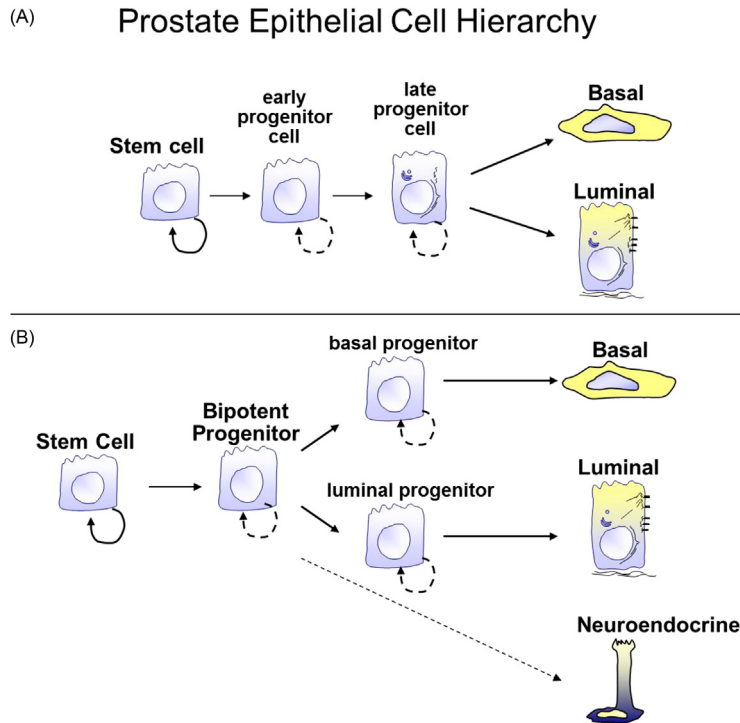


Fig. 2 Linear and bifurcated models for human prostate epithelial cell hierarchy. (A) A prostate pluripotent stem cell undergoes asymmetric self-renewal to repopulate itself and generate a bipotent progenitor cell that transiently amplifies, forming late stage progenitor cells (aka intermediate cells) that lineage commitment to either basal or luminal epithelial cells. (B) A bifurcated model proposes that the bipotent progenitor cell gives rise to unipotent basal progenitor cells, unipotent luminal progenitors as well as a rare neuroendocrine cell type. The unipotent basal and luminal progenitor cells, upon several divisions, give rise to differentiated basal and luminal cell populations, respectively, under physiologic conditions. Adapted from Kasper, S. (2008). Exploring the origins of the normal prostate and prostate cancer stem cell. *Stem Cell Reviews* 4, 193–201.

cells are expanded by transient amplification prior to terminal differentiation, into basal, luminal, and neuroendocrine cell types. The stem cell niche, or microenvironment, provides signals to the stem cell and progenitor cells that dictate decisions for self-renewal, lineage commitment, and differentiation (Blum et al., 2010). This niche includes the mesenchyme/stromal and resident immune cells that communicate to stem cells through paracrine factors, as well as neighboring epithelial cells that communicate through gap junctions and cell adhesion molecules. It is important to note that prostate epithelial stem and early progenitor cells are AR-negative (Hu et al., 2011; Kasper, 2009; Huang et al., 2014) and are therefore not directly regulated by androgen action. As a result, any effects of androgens on prostate epithelial stem cell homeostasis and differentiation must be mediated through indirect actions via the stem cell niche, which includes AR⁺ stromal and luminal epithelial cells (Berry et al., 2008). Recent studies show that human prostate stem/progenitor cells express the estrogen receptors ER α , ER β , and G-protein estrogen receptor (GPER) and exhibit self-renewal responses to estrogens, implicating their involvement in the maintenance of the stem/progenitor pool (Hu et al., 2011; Prins et al., 2014). Thus, multiple signaling pathways directly and indirectly modulate the equilibrium between self-renewal and differentiation in prostate epithelial stem cell populations (Prins and Hu, 2013).

Epithelial stem cell niche

The human prostate stem cell niche is incompletely understood in terms of its anatomical location, histologic characteristics, cellular constituents, and hierarchical differentiation. In the adult human, the prostate stem cells reside among the basal epithelial layer of cells. The fate of these stem cells is governed by intrinsic and extrinsic factors. Intrinsic factors control stem cell self-renewal and differentiation in a cell-autonomous manner and include intracellular signaling molecules, chromatin remodeling enzymes, transcription factors, and noncoding RNA such as miRNA and piRNA (Zon, 2008). Although intrinsic factors ultimately determine whether a stem cell remains quiescent, undergoes symmetric self-renewal, or differentiates via asymmetric self-renewal or committed differentiation, a network of extrinsic factors impinges upon the intrinsic network to integrate the needs of the tissue in regulating stem cell niche dynamics. Extrinsic factors include circulating hormones, locally produced paracrine factors, cellular adhesion molecules, extracellular matrix (ECM) molecules and a host of other factors. With the exception of circulating hormones, these factors are products of cells within the stem cell niche.

Lineage hierarchy

There are two hypotheses about how prostate stem cells form differentiated luminal and basal epithelial cells. The first hypothesis is that a single multipotent stem cell, upon asymmetric cell division gives rise to a multipotent progenitor cell, which, upon division,

generates basal- or luminal-specific progenitor cells. These lineage-specific progenitor cells ultimately give rise to the fully differentiated epithelia. The second hypothesis states that there are two separate populations of unipotent prostate stem cells—basal stem cells and luminal stem cells. Each of these divides to form lineage-specific differentiated basal and luminal cells. A diagram of both lineage models is shown in [Fig. 2](#).

Stromal Cells

The prostatic stroma is predominantly composed of smooth muscle and fibroblast cells, with intermixed endothelial cells, blood vessels, lymphatic vessels, nerves, adipose cells, circulating immune cells, and bone-derived mesenchymal stem cells. The stroma as a whole is known to play important roles in the development, structure and function of the prostate gland ([Nemeth et al., 1997](#)). Nevertheless, the precise origin and function of individual types of stromal cells are largely unknown. To this end, recent research efforts in prostate cell biology have focused on elucidating the specific roles of various stromal cell types, including the role of the extracellular matrix (ECM), which is secreted by stromal cells. One striking feature of the stromal compartment is its ability to change cellular phenotype and composition of ECM in response to cues from the microenvironment. In the past, these alterations in stromal phenotype and ECM composition had been considered merely reactive changes; however, it is now widely believed that these stromal changes may play active roles in benign and malignant prostate diseases.

Smooth muscle cells

Smooth muscle cells are immunohistochemically defined by expression of α -smooth muscle actin, myosin, desmin, vinculin, and vimentin. These cells provide the mechanical means by which prostatic secretions are expelled during emission ([Hayward et al., 1996](#)). The distribution of smooth muscle cells varies between species, with rodent smooth muscle found strictly in a periacinar distribution and human smooth muscle found in the interductal stroma as well as periacinar locations. Prostatic smooth muscle cells consistently express nuclear AR. In the mature prostate, smooth muscle AR is believed to stimulate paracrine signaling in response to androgens, which helps maintain fully differentiated growth-quiescent epithelium ([Prins et al., 1991, 1992](#); [Cunha et al., 1996](#)).

Fibroblasts

Fibroblasts are α -smooth muscle actin-negative and vimentin-positive. Classically, fibroblasts have been thought to be the primary cells responsible for deposition of ECM, however, it is now known that smooth muscle cells also contribute to the ECM. Beyond their role in providing structural support to the prostate gland, fibroblasts also serve as immune system regulators and mediators of the wound-healing response.

Stromal-epithelial interactions

In addition to providing structural support and contractile function to the gland, the stroma plays a fundamental role in the development and homeostasis of the prostate. Developmentally, androgen receptor-positive urogenital sinus mesenchyme (UGM) is necessary and sufficient to induce formation of prostatic buds from the urogenital sinus epithelium (UGE) ([Cunha, 1972](#)). Thus, UGM is instructive toward the epithelium, translating cues from circulating hormones into paracrine factors that direct proliferation and morphological development of the UGE. In the adult gland, stromal-epithelial interactions are also important in homeostasis of the normal gland, as well as in development of benign and malignant pathology.

It is believed that aberrant growth with benign prostatic hyperplasia is driven, in part, through reawakening of the embryonic growth potential of prostatic stroma. Likewise, evidence clearly shows a critical role for the stroma in driving prostate cancer progression ([McNeal, 1983](#)). Malignant epithelial cells induce an altered phenotype in the neighboring stroma, which are referred to as cancer associated fibroblasts (CAFs). It should be noted, however, that in vivo CAFs are composed primarily of smooth muscle cells with a minor fibroblast population. Nevertheless, CAFs have an altered transcriptional program that upregulates secretory products and engages in an exchange of factors and molecules that promote altered metabolism, survival, growth, and proliferation within the tumor ([Adisetiyo et al., 2014](#)).

The regional variations in epithelial proliferation and apoptosis along the prostatic ducts are associated with differing stromal organization. In the rodent prostate, the distribution of fibroblasts and smooth muscle cells varies along the length of a duct. In the distal regions, there is a discontinuous layer of fibroblasts between the smooth muscle cells, which surround the gland, whereas the stroma surrounding the proximal duct has multiple continuous layers of smooth muscle with only a few rare fibroblasts interspersed ([Nemeth et al., 1997](#)). Stromal growth factor expression mirrors the differing stromal organization along the prostatic ducts and is the basis for the variations in epithelial proliferation ([Nemeth et al., 1997](#)). It appears that, in general, fibroblastic signaling promotes epithelial proliferation, whereas smooth muscle signaling promotes cell death.

Extracellular matrix

The extracellular matrix (ECM) is produced by stromal cells and provides structural support and shape to the prostate gland. Beyond mere scaffolding, however, the ECM modulates cellular function and behavior through biophysical and biochemical cues ([Montano and Bushman, 2017](#)). Macromolecules such as collagen, elastin, laminin, fibronectin, fibrin, and perlecan are secreted by stromal cells in specific abundances and ratios that vary by tissue microenvironment and developmental stage. For instance, the basement membrane that surrounds epithelial cells and separates them from the stroma predominantly includes type IV

collagen, laminin, and heparan sulfate proteoglycan. The composition of this basement membrane shifts from fetal development through adulthood. Different macromolecular compositions of ECM provide different physical cues such as stiffness, tensile strength and pH. These function as signals to regulate epithelial cell proliferation, adhesion, and apoptosis to maintain appropriate homeostasis. The ECM is a particularly important component of the stem cell niche, where it is one of several extrinsic factors that regulate stem cell self-renewal. The ECM is also thought to play a very important role in prostate cancer.

Prostatic Secretions

The Prostate Gland Contributes Secretions to the Seminal Plasma

The primary function of the sex accessory tissues, which include the prostate, seminal vesicles, bulbourethral glands and glands of Littre, is the production of seminal plasma, the nongamete portion of the semen. The seminal plasma serves as a buffered, nutrient-dense transport medium for sperm as they are deposited into the female genital tract. Although individual components augment the fertilizing capacity of sperm, seminal plasma substances are not required for mature spermatozoa to successfully fertilize the egg. With a pH of 7.2–7.8, seminal plasma is slightly alkaline and acts to neutralize the acidic environment of the vagina. Prostaglandins in seminal plasma stimulate contraction of smooth muscle along the female genital tract, helping to propel sperm to the egg. Additional substances include zinc and IgA, which have important bacteriostatic properties, as well as an array of prostatic proteins that help prevent autoagglutination of sperm. Altogether, prostate gland secretions account for 25% of the ejaculate, which totals about 1–6 mL in a human male. Important components of the seminal plasma that are secreted by the prostate gland are listed in [Table 1](#).

Role of Prostatic Secretions in Coagulation/Liquefaction

Semen coagulates and forms a gelatinous mass soon after ejaculation due to clotting factors produced by the seminal vesicles. Although analogous to blood coagulation, the seminal coagulation process has a unique biochemistry that does not involve prothrombin, fibrinogen or the hematologic clotting factors. Furthermore, the seminal coagulation process is not inhibited by heparin or sodium citrate. Prostate-derived proteolytic enzymes, including seminin, plasminogen activators, and prostate-specific antigen (PSA), liquefy the coagulated semen after 15–60 min. This liquefaction frees the sperm to advance up the female reproductive tract. Defective or absent secretion of these liquefying factors from the prostate is one cause of reduced male fertility.

Regulation of prostatic secretion

Hormonal

The production of seminal plasma, including the prostatic component, is under hormonal control and driven by circulating androgens, which are always present in normal males. In the prostate, circulating testosterone is taken up by prostate cells, converted to dihydrotestosterone (DHT) which binds to luminal cell AR, dimerizes, and translocates to the nucleus. Nuclear AR binds response elements on androgen-regulated genes, initiating gene transcription with subsequent translation of secretory proteins or enzymes whose products are secreted into seminal fluid.

Neural

Prostatic secretions are also under neural control ([Ventura et al., 2002](#)). Both the epithelial and stromal compartments in the prostate are innervated by the autonomic nervous system.

Table 1 Prostate-secreted components of human seminal plasma

<i>Component</i>	<i>Function</i>
Prostate-specific antigen (PSA)	Liquefaction of semen
Citric acid	Inhibit premature capacitation of sperm
Spermine	Enhance sperm motility; Immunosuppressive actions
Cholesterol	Stabilize sperm membrane
Prostatic acid phosphatase (PAP)	Unknown
Phospholipids	Stabilize sperm membrane
Prostaglandins	Enhance sperm motility; modulate cervical mucous; Immunosuppressive
Zinc	Stabilize chromatin within sperm head
<i>Immunoglobulins</i>	
IgG	Antimicrobial
IgA	Antimicrobial

Components of Prostatic Fluid

Zinc

The prostate gland has the highest concentration of zinc of any organ within the human body (Bertrand and Vladesco, 1921). Prostatic secretions are responsible for the high concentration of zinc in the seminal plasma, which at $140 \mu\text{g mL}^{-1}$ is nearly 100-fold higher than the serum zinc concentration (Eliasson, 1977). The precise physiologic role of such high concentrations of zinc in the prostate gland and the seminal plasma is unknown. Indeed, while the prostate has a multitude zinc-containing metalloenzymes, the observed concentration of zinc far exceeds the theoretical concentration that would be necessary for such enzymes. Within the seminal plasma, zinc serves to stabilize chromatin within the sperm head. It also binds to semenogelin I and II in the seminal plasma, which serves to regulate liquefaction (Heathcote and Washington, 1973; Fair and Wehner, 1976; Jonsson et al., 2005). Finally, zinc also functions as an antibacterial factor in both seminal plasma and nonejaculatory prostatic secretions, with in vitro studies demonstrating activity against an array of gram-positive and gram-negative bacteria.

Citric acid

Citrate is a potent binder of metal ions and is one of the major anions in human seminal plasma. The mean concentration of citrate in the ejaculate is 376 mg/dL, which is 500–1000 times higher than that in blood plasma. Its molar concentration (20 mM) is comparable to that of the divalent metals together, 13.6 mM (calcium 7 mM; magnesium 4.5 mM; zinc 2.1 mM). Seminal plasma citrate predominantly originates from the prostate with a 100-fold less concentration found in seminal vesicle secretions (Zaneveld and Tauber, 1981). The high levels of citrate have been linked to the decreased ability of prostatic mitochondria to oxidize citrate, which results in an imbalance between citrate production and oxidation (Costello and Franklin, 1989; Costello and Franklin, 1994). Kavanagh (1994) measured citrate and isocitrate levels and found the prostate to have a 33:1 ratio, whereas other tissues demonstrated 10:1 ratios. This finding suggests decreased aconitase activity as the reason behind such high citrate levels in prostatic secretions.

Sodium, potassium, calcium, magnesium and chloride

Although seminal plasma contains calcium and magnesium in higher concentrations than other body fluids, the relative contribution of the prostate to these levels has not been established. It is known, however, that the concentration of sodium, potassium, calcium, magnesium, and chloride within prostatic fluid varies between individuals and states of wellness or disease (Kavanagh, 1985). This is in part a passive response to varied levels of citrate secretion in prostatic fluid during states of disease.

Spermine

Polyamines are small, positively-charged organic molecules that have two or more primary amino groups. These molecules are found in all eukaryotic cells, where they have pleiotropic effects on a variety of physiologic processes including cell proliferation. Spermine, so named as a polyamine that was first discovered in semen, is present in seminal plasma at concentrations of 50–350 mg/dL and imparts a unique odor to semen. The prostate gland has the highest concentration of spermine in the human body and is the primary source of this polyamine in seminal plasma. The enzyme ornithine decarboxylase (ODC) is the rate-limiting enzyme in the synthesis of polyamines and spermine. The expression of ODC is increased in BPH tissue, which suggests a possible role for polyamines in the pathogenesis of this disease.

At physiologic pH, spermine has four positive charges that bind strongly to acidic or negatively charged molecules such as phosphate ions, nucleic acids, or phospholipids. Spermine is generated via a series of enzymatic reactions that use other polyamines as substrates, proceeding from ornithine to putrescine to spermidine to spermine. Spermine is oxidized enzymatically by diamine oxidase, which is found in the seminal plasma. The aldehyde products from this reaction are very reactive and toxic to both sperm and bacteria (Folk et al., 1980; Williams-Ashman et al., 1972). This is one reason why prolonged exposure of sperm to seminal plasma will reduce its fertilizing capacity.

Prostaglandins

In 1934, von Euler coined the term prostaglandins to describe the active components in seminal plasma in the assumption that they were produced by the prostate. Over 20 years later, Eliasson determined that the majority of prostaglandins originate from the seminal vesicles, with the prostate gland's contribution being minimal (Eliasson, 1959). Nevertheless, the name stands to this day. Prostaglandins are expressed throughout the body, but in humans the highest concentrations by far are found in the seminal vesicles. The seminal plasma contains 15 different prostaglandins at a concentration of $100\text{--}300 \mu\text{g mL}^{-1}$. Prostaglandins are 20-carbon hydroxyl fatty acids containing a cyclopentane ring and two side chains. They are classified into four major groups (A, B, E, and F) based on the structure of the cyclopentane ring and given a number based on the structure of the side chain (e.g., PGE₃).

The E group of prostaglandins predominates in the male reproductive tract, while the F group predominates in the female. Prostaglandins have a variety of potent proposed physiologic effects in the male and are involved in erection, ejaculation, sperm motility, and testicular and penile contractions. Additionally, seminal fluid prostaglandins deposited in the vagina affect cervical mucus, vaginal secretions, uterine contractions.

Cholesterol and lipids

The human prostate contributes to the lipid fraction of seminal plasma, which contains 185 mg/dL of total lipids, 103 mg/dL of cholesterol, and 83 mg/dL of phospholipids. The phospholipids have been further characterized as 44% sphingomyelin, 12.3% ethanolamine plasmalogen, and 11.2% phosphatidylserine. This ratio of cholesterol to phospholipid helps stabilize the sperm against temperature and environmental shock (White, 1976; Poulos and White, 1973).

Immunoglobulins

Immunoglobulins are found in the seminal plasma at levels lower than in blood. Specifically, IgG is measured from 7 to 22 mg/dL and IgA from 0 to 6 mg/dL. IgM has also been detected in the seminal plasma at very low levels (Gahankari and Golhar, 1993). Given the lower levels of immunoglobulins, it is theorized that these may simply be exudates, however, IgA has been localized more so to the bulbourethral glands and suggests an immunologic component to the bulbourethral gland function. IgG and IgA are likely secreted from the prostate (Rumke, 1974).

Acid phosphatase

Acid phosphatase activity is more than 200 times more abundant in the prostate than any other tissue. Prostatic acid phosphatase (PAP) is a 102 kD glycoprotein dimer with seminal plasma levels of $0.3\text{--}1\text{ g L}^{-1}$. The substrate for PAP in the seminal plasma may be phosphorylcholine phosphate, which is rapidly hydrolyzed by the enzyme. However, the physiologic significance of the high levels of PAP and their function are not well understood (Chu et al., 1977; Lowe and Trauzzi, 1993). Serum acid phosphatase concentration was once widely measured as biomarker for metastatic prostate cancer; however, this has been usurped the more sensitive and specific assay for prostate specific antigen (PSA).

Prostate specific antigen (PSA)

Prostate specific antigen is a 33 kD glycoprotein that acts as a serine protease and is produced almost exclusively in prostate epithelial cells (Watt et al., 1986). PSA is involved in lysing the ejaculatory clot with semenogelin as its substrate. The PSA gene (*hKLK3*) is a member of the kallikrein family of genes, which are all located on chromosome 19 (Yousef and Diamandis, 2003). Expression of PSA is dependent upon androgens. Clinically, PSA is used as an important serum marker for prostate cancer and prostate disease since its presence in the circulation indicates a breakdown in the epithelial/basement membrane barrier. Unfortunately, while PSA is organ-specific, it is not cancer-specific and is found in the serum of patients with BPH as well as prostatitis. PSA is found in seminal plasma in concentrations from $0.5\text{ to }5\text{ mg mL}^{-1}$ whereas, PSA in serum of men 50–80 years of age without prostate disease is 1000-fold less at $1.0\text{--}4.0\text{ ng mL}^{-1}$ (Catalona et al., 1991). The serum level of PSA in men with prostate cancer is generally elevated ($>4.0\text{ ng mL}^{-1}$), but not until its level is $>10\text{ ng mL}^{-1}$ does this measurement suggest specificity for prostate cancer. As prostate cancer cells remain AR-dependent, even in the absence of androgens, circulating levels of PSA in patients with metastatic disease can reach into the hundreds and monitoring levels over time can be a useful indicator of progression and relapse after treatment failure (Partin et al., 1990; Oesterling et al., 1988).

References

- Abrahamsson, P. A., Dizzeyi, N., Alm, P., di Sant'agnese, P. A., Deftos, L. J., & Aumuller, G. (2000). Calcitonin and calcitonin gene-related peptide in the human prostate gland. *Prostate*, 44, 181–186.
- Adisetiyo, H., Liang, M., Liao, C. P., Jeong, J. H., Cohen, M. B., Roy-Burman, P., & Frenkel, B. (2014). Dependence of castration-resistant prostate cancer (CRPC) stem cells on CRPC-associated fibroblasts. *Journal of Cellular Physiology*, 229, 1170–1176.
- Berry, P. A., Maitland, N. J., & Collins, A. (2008). Androgen receptor signalling in prostate: Effects of stromal factors on normal and cancer stem cells. *Molecular and Cellular Endocrinology*, 288, 30–37.
- Bertrand, G., & Vladesco, R. (1921). Prostatic zinc concentration. *Proceedings of the Academy of Sciences*, 11, 176–179.
- Bhatia-Gaur, R., Donjacour, A. A., Scivolino, P. J., Kim, M., Desai, N., Young, P., Norton, C. R., Gridley, T., Cardiff, R. D., Cunha, G. R., Abate-Shen, C., & Shen, M. M. (1999). Roles for Nkx3.1 in prostate development and cancer. *Genes & Development*, 13, 966–977.
- Bieberich, C. J., Fujita, K., He, W. W., & Jay, G. (1996). Prostate-specific and androgen-dependent expression of a novel homeobox gene. *The Journal of Biological Chemistry*, 271, 31779–31782.
- Blum, R., Gupta, R., Burger, P. E., Ontiveros, C. S., Salm, S. N., Xiong, X., Kamb, A., Wesche, H., Marshall, L., Cutler, G., Wang, X., Zavadil, J., Moscatelli, D., & Wilson, E. L. (2010). Molecular signatures of the primitive prostate stem cell niche reveal novel mesenchymal-epithelial signaling pathways. *PLoS One*, 5, e13024.
- Bonkhoff, H., & Remberger, K. (1996). Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: A stem cell model. *Prostate*, 28, 98–106.
- Bonkhoff, H., Wernert, N., Dhom, G., & Remberger, K. (1991). Relation of endocrine-paracrine cells to cell proliferation in normal, hyperplastic, and neoplastic human prostate. *Prostate*, 19, 91–98.
- Calderon-Gierszal, E., Kajdacsy-Balla, A., Li, G., van Breemen, R. B., & Prins, G. S. (2014). In *Directed differentiation of human embryonic stem cells (hESC) to prostate: Novel models that verify bisphenol A effects on human prostate development* International Congress of Endocrinology/Endocrine Society Annual Meeting, Chicago, IL, USA.
- Catalona, W. J., Smith, D. S., Ratliff, T. L., Dodds, K. M., Coplen, D. E., Yuan, J. J., Petros, J. A., & Andriole, G. L. (1991). Measurement of prostate-specific antigen in serum as a screening test for prostate cancer. *The New England Journal of Medicine*, 324, 1156–1161.
- Chu, T. M., Wang, M. C., Kuciel, R., Valenzuela, L., & Murphy, G. P. (1977). Enzyme markers in human prostatic carcinoma. *Cancer Treatment Reports*, 61, 193–200.
- Costello, L. C., & Franklin, R. B. (1989). Prostate epithelial cells utilize glucose and aspartate as the carbon sources for net citrate production. *Prostate*, 15, 335–342.
- Costello, L. C., & Franklin, R. B. (1994). Effect of prolactin on the prostate. *Prostate*, 24, 162–166.
- Cunha, G. R. (1972). Epithelio-mesenchymal interactions in primordial gland structures which become responsive to androgenic stimulation. *Anatomical Record*, 172, 179.

- Cunha, G. R., Hayward, S. W., Dahiya, R., & Foster, B. A. (1996). Smooth muscle-epithelial interactions in normal and neoplastic prostatic development. *Acta Anatomica (Basel)*, 155, 63–72.
- El-Alfy, M., Pelletier, G., Hermo, L. S., & Labrie, F. (2000). Unique features of the basal cells of human prostate epithelium. *Microscopy Research and Technique*, 51, 436–446.
- Eliasson, R. (1959). Studies on prostaglandin; occurrence, formation and biological actions. *Acta Physiologica Scandinavica. Supplementum*, 46, 1–73.
- Eliasson, R. (1977). Seminal plasma accessory genital glands and fertility. In A. T. K. Crocett, & R. L. Urry (Eds.), *Male infertility: Workup, treatment, and research*. New York: Grune & Stratton.
- Epstein, J. I. (1993). PSA and PAP as immunohistochemical markers in prostate cancer. *The Urologic Clinics of North America*, 20, 757–770.
- Fair, W. R., & Wehner, N. (1976). The prostatic antibacterial factor: Identity and significance. *Progress in Clinical and Biological Research*, 6, 383–403.
- Folk, J. E., Park, M. H., Chung, S. I., Schrode, J., Lester, E. P., & Cooper, H. L. (1980). Polyamines as physiological substrates for transglutaminases. *The Journal of Biological Chemistry*, 255, 3695–3700.
- Forest, M. G. (1979). Plasma androgens (testosterone and 4-androstenedione) and 17-hydroxyprogesterone in the neonatal, prepubertal and peripubertal periods in the human and the rat: Differences between species. *Journal of Steroid Biochemistry*, 11, 543–548.
- Gahankari, D. R., & Golhar, K. B. (1993). An evaluation of serum and tissue bound immunoglobulins in prostatic diseases. *Journal of Postgraduate Medicine*, 39, 63–67.
- Goldstein, A. S., Lawson, D. A., Cheng, D., Sun, W., Garraway, I. P., & Witte, O. N. (2008). Trop2 identifies a subpopulation of murine and human prostate basal cells with stem cell characteristics. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 20882–20887.
- Habermann, H., Chang, W. Y., Birch, L., Mehta, P., & Prins, G. S. (2001). Developmental exposure to estrogens alters epithelial cell adhesion and gap junction proteins in the adult rat prostate. *Endocrinology*, 142, 359–369.
- Hayward, S., Baskin, L., Haughney, P., Foster, B., Ar, C., Dahiya, R., Prins, G., & Cunha, G. (1996). Stromal development in the ventral prostate, anterior prostate and seminal vesicle of the rat. *Acta Anatomica*, 155, 94–103.
- Heathcote, J. G., & Washington, R. J. (1973). Analysis of the zinc-binding protein derived from the human benign hypertrophic prostate. *The Journal of Endocrinology*, 58(3), 421.
- Hu, W. Y., Shi, G. B., Lam, H. M., Hu, D. P., Ho, S. M., Madueke, I. C., Kajdacsy-Balla, A., & Prins, G. S. (2011). Estrogen-initiated transformation of prostate epithelium derived from normal human prostate stem-progenitor cells. *Endocrinology*, 152, 2150–2163.
- Huang, C. K., Luo, J., Lee, S. O., & Chang, C. (2014). Androgen receptor differential roles in stem/progenitor cells including prostate, embryonic, stromal, and hematopoietic lineages. *Stem Cells*, 32(9), 2299–2308.
- Jonsson, M., Linse, S., Frohm, B., Lundwall, A., & Malm, J. (2005). Semenogelin I and II bind zinc and regulate the activity of prostate-specific antigen. *The Biochemical Journal*, 387, 447–453.
- Kasper, S. (2008). Exploring the origins of the normal prostate and prostate cancer stem cell. *Stem Cell Reviews*, 4, 193–201.
- Kasper, S. (2009). Identification, characterization, and biological relevance of prostate cancer stem cells from clinical specimens. *Urologic Oncology*, 27(3), 301.
- Kavanagh, J. P. (1985). Sodium, potassium, calcium, magnesium, zinc, citrate and chloride content of human prostatic and seminal fluid. *Journal of Reproduction and Fertility*, 75, 35–41.
- Kavanagh, J. P. (1994). Isocitric and citric acid in human prostatic and seminal fluid: Implications for prostatic metabolism and secretion. *Prostate*, 24, 139–142.
- Keil, K. P., Mehta, V., Abler, L. L., Joshi, P. S., Schmitz, C. T., & Vezina, C. M. (2012). Visualization and quantification of mouse prostate development by in situ hybridization. *Differentiation*, 84, 232–239.
- Lang, S. H., Frame, F. M., & Collins, A. T. (2009). Prostate cancer stem cells. *Journal of Pathology*, 217, 299–306.
- Leong, K. G., Wang, B. E., Johnson, L., & Gao, W. Q. (2008). Generation of a prostate from a single cell. *Nature*, 456, 804–808.
- Low, F. C., & Trauzzi, S. J. (1993). Prostatic acid phosphatase in 1993. Its limited clinical utility. *The Urologic Clinics of North America*, 20, 589–595.
- McNeal, J. E. (1983). The prostate gland: Morphology and pathobiology. In *Monographs in urology*, 4 pp. pp. 3–11). Burroughs Wellcome Company.
- McNeal, J. E. (1998). Anatomy and normal histology of the human prostate. In C. S. A. B. D. G. Foster (Ed.), *Pathology of the prostate*. Philadelphia: W.B. Saunders Co.
- Montano, M., & Bushman, W. (2017). Morphoregulatory pathways in prostate ductal development. *Developmental Dynamics*, 246, 89–99.
- Nemeth, J., Sensibar, J., White, R., Zelnar, D., Kim, I., & Lee, C. (1997). Prostatic ductal system in rats: Tissue-specific expression and regional variation in stromal distribution of transforming growth factor- β 1. *Prostate*, 33, 64–71.
- Nikitin, A. Y., Matoso, A., & Roy-Burman, P. (2007). Prostate stem cells and cancer. *Histology and Histopathology*, 22, 1043–1049.
- Oesterling, J. E., Chan, D. W., Epstein, J. I., Kimball, A. W., Jr., Bruzek, D. J., Rock, R. C., Brendler, C. B., & Walsh, P. C. (1988). Prostate specific antigen in the preoperative and postoperative evaluation of localized prostatic cancer treated with radical prostatectomy. *The Journal of Urology*, 139, 766–772.
- Oussel, M., van Keymeulen, A., Bouvencourt, G., Sharma, N., Achouri, Y., Simons, B. D., & Blanpain, C. (2012). Multipotent and unipotent progenitors contribute to prostate postnatal development. *Nature Cell Biology*, 14, 1131–1138.
- Partin, A. W., Carter, H. B., Chan, D. W., Epstein, J. I., Oesterling, J. E., Rock, R. C., Weber, J. P., & Walsh, P. C. (1990). Prostate specific antigen in the staging of localized prostate cancer: Influence of tumor differentiation, tumor volume and benign hyperplasia. *The Journal of Urology*, 143, 747–752.
- Poulos, A., & White, I. G. (1973). The phospholipid composition of human spermatozoa and seminal plasma. *Journal of Reproduction and Fertility*, 35, 265–272.
- Prins, G. S., Birch, L., & Greene, G. L. (1991). Androgen receptor localization in different cell types of the adult rat prostate. *Endocrinology*, 129, 3187–3199.
- Prins, G. S., Cooke, P. S., Birch, L., Donjacour, A. A., Yalcinkaya, T. M., Silteri, P. K., & Cunha, G. R. (1992). Androgen receptor expression and 5 α -reductase activity along the proximal-distal axis of the rat prostatic duct. *Endocrinology*, 130, 3066–3073.
- Prins, G. S., & Hu, W. Y. (2013). Prostate stem cells, hormones and development. In S. D. Cramer (Ed.), *Stem cells and prostate cancer*. Berlin: Springer LLC.
- Prins, G. S., Hu, W. Y., Shi, G. B., Hu, D. P., Majumdar, S., Li, G., Huang, K., Nelles, J. L., Ho, S. M., Walker, C. L., Kajdacsy-Balla, A., & Van Breemen, R. B. (2014). Bisphenol A promotes human prostate stem-progenitor cell self-renewal and increases in vivo carcinogenesis in human prostate epithelium. *Endocrinology*, 155, 805–817.
- Prins, G. S., & Putz, O. (2008). Molecular signaling pathways that regulate prostate gland development. *Differentiation*, 76, 641–659.
- Rumke, P. (1974). Origin of immunoglobulins in semen. *Clinical and Experimental Immunology*, 17, 287–297.
- Sherwood, E., Theyer, G., Steiner, G., Berg, L., Kozlowski, J., & Lee, C. (1991). Differential expression of specific cytokeratin polypeptides in the basal and luminal epithelia of the human prostate. *Prostate*, 18, 303–314.
- Szczyrba, J., Niesen, A., Wagner, M., Wandernoth, P. M., Aumüller, G., & Wennemuth, G. (2017). Neuroendocrine cells of the prostate derive from the neural crest. *Journal of Biological Chemistry*, 292, 2021–2031.
- Ventura, S., Pennefather, J. N., & Mitchelson, F. (2002). Cholinergic innervation and function in the prostate gland. *Pharmacology & Therapeutics*, 94, 93–112.
- Wang, Y., Hayward, S. W., Cao, M., Thayer, K., & Cunha, G. R. (2001). Cell differentiation lineage in the prostate. *Differentiation*, 68, 270–279.
- Watt, K. W., Lee, P. J., M'Timkulu, T., Chan, W. P., & Loo, R. (1986). Human prostate-specific antigen: Structural and functional similarity with serine proteases. *Proceedings of the National Academy of Sciences of the United States of America*, 83, 3166–3170.
- White, I. G., Darin-Bennett, A., and Poulos, A. 1976. Lipids of human semen. In: Hafez, E. S. E. (ed.) Human semen and fertility regulation in men. St. Louis: C. V. Mosby.
- Williams-Ashman, H. G., Janne, J., Coppoc, G. L., Geroch, M. E., & Schenone, A. (1972). New aspects of polyamine biosynthesis in eukaryotic organisms. *Advances in Enzyme Regulation*, 10, 225–245.
- Wright, A. S., Thomas, L. N., Douglas, R. C., Lazier, C. B., & Rittmaster, R. S. (1996). Relative potency of testosterone and dihydrotestosterone in preventing atrophy and apoptosis in the prostate of the castrated rat. *The Journal of Clinical Investigation*, 98, 2558–2563.
- Xin, L., Lukacs, R. U., Lawson, D. A., Cheng, D., & Witte, O. N. (2007). Self-renewal and multilineage differentiation in vitro from murine prostate stem cells. *Stem Cells*, 25, 2760–2769.

- Yousef, G. M., & Diamandis, E. P. (2003). An overview of the kallikrein gene families in humans and other species: Emerging candidate tumour markers. *Clinical Biochemistry*, 36, 443–452.
- Zaneveld, L. J., & Tauber, P. F. (1981). Contribution of prostatic fluid components to the ejaculate. *Progress in Clinical and Biological Research*, 75A, 265–277.
- Zon, L. I. (2008). Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. *Nature*, 453, 306–313.

Further Reading

- di Sant'Agnese, P. A., & Cockett, A. T. (1996). Neuroendocrine differentiation in prostatic malignancy. *Cancer*, 78, 357–361.

Prostate Disease Overview

Gail P Risbridger, Monash University, Clayton, VIC, Australia; and The University of Melbourne, Parkville, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

ADT	Androgen deprivation therapy
AR	Androgen receptor
BPH	Benign prostatic hyperplasia
CP/CPPS	Chronic prostatitis/chronic pelvic pain syndrome
CRPC	Castration resistant prostate cancer
CZ	Central zone
IPSS	International prostate symptom score
LUTS	Lower urinary tract symptoms
NOS	Nitric oxide synthase
PC	Prostate cancer
PDE5	Inhibitors phosphodiesterase-5 inhibitors
PSA	Prostate-specific antigen
PZ	Peripheral zone
TILs	Tumor infiltrating lymphocytes
TURP	Transurethral resection of the prostate
TZ	Transition zone

Introduction: Anatomy and Function of the Prostate Gland

The prostate gland is an accessory male reproductive organ, located at the base of the bladder, and surrounding the urethra. It produces prostatic secretions that contain zinc, citric acid, calcium, phosphates, and other enzymes essential for sperm health and motility. At ejaculation, semen passes through the ejaculatory ducts and mixes with secretions from the prostate gland (Fig. 1).

Prostatic fluids arise from the epithelial cells lining the ducts that are separated from surrounding stroma by a basement membrane. The epithelium consists of basal, neuroendocrine and secretory (luminal) cells and it is polarized so that the secretions arise at the luminal aspect of the epithelium including prostatic-specific antigen (PSA). Basally located epithelial cells secrete extracellular matrix (ECM) for the basement membrane and also include the stem and stem progenitor populations. The surrounding fibromuscular stroma consists of smooth muscle cells, fibroblasts, nerves, endothelial cells, immune cells, and blood vessels. The smooth muscle component provides contractility that is required when proteins are exuded from the glands (Fig. 2).

The prostate gland is divided into three anatomical zones in the human: the central (CZ), transition (TZ), peripheral (PZ), and anterior fibromuscular zones, with each zone having a unique morphology and physiology (Fig. 1). The zones of the prostate are especially relevant, as different prostatic diseases arise from different zones. In rodents the structure of the gland is slightly different and is divided in lobes: ventral, anterior, and dorsolateral.

Diseases of the Prostate Gland

The prostate is extremely prone to disease in older men, with the most common disorders being benign prostatic hyperplasia (BPH), prostatitis and prostate cancer. BPH is defined as the progressive hyperplastic growth of the stromal prostate cells, resulting in enlargement of the gland. Since the prostate surrounds the urethra, enlargement due to BPH restricts urethral flow and is often accompanied by debilitating low urinary tract symptoms (LUTS).

Prostatitis is defined as inflammation of the prostate. The majority of prostatitis cases are nonbacterial in origin (vs. nonbacterial) and are characterized by chronic discomfort and pain in the pelvic region. The condition of prostatitis can be chronic (chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS)) and men with a history of CP/CPPS have an increased risk of prostate cancer (PC). It is thought that chronic inflammation can promote tumorigenesis as it does in other tumors types.

Prostate cancer is characterized by uncontrolled cellular proliferation, epithelial dedifferentiation, and a gradual loss of ductal architecture. It is a progressive disease where benign glands generate premalignant lesions that eventually become cancerous foci. Prostate cancer is relatively slow growing but remains a lethal disease that is one of the most common causes of cancer death in men.

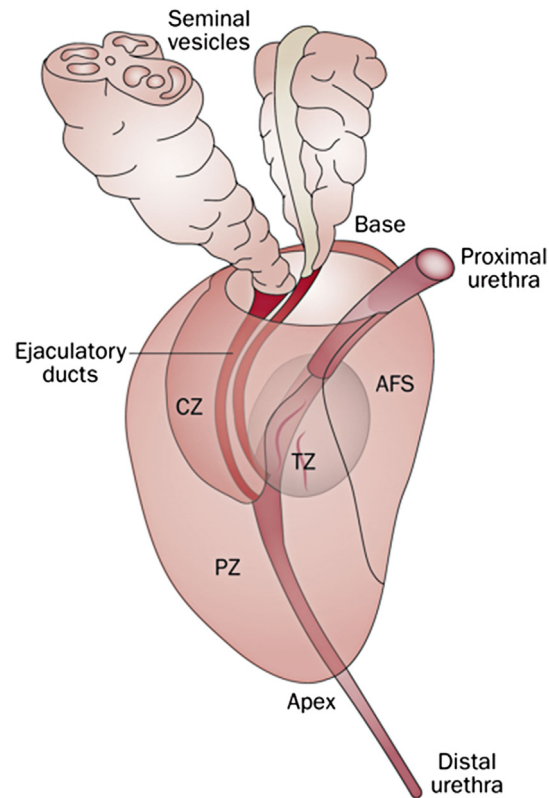


Fig. 1 Anatomical zones of the prostate. The prostate consists of four anatomical zones; the central (CZ), peripheral (PZ), and transition (TZ) zones, and the anterior fibromuscular stroma (AFS).

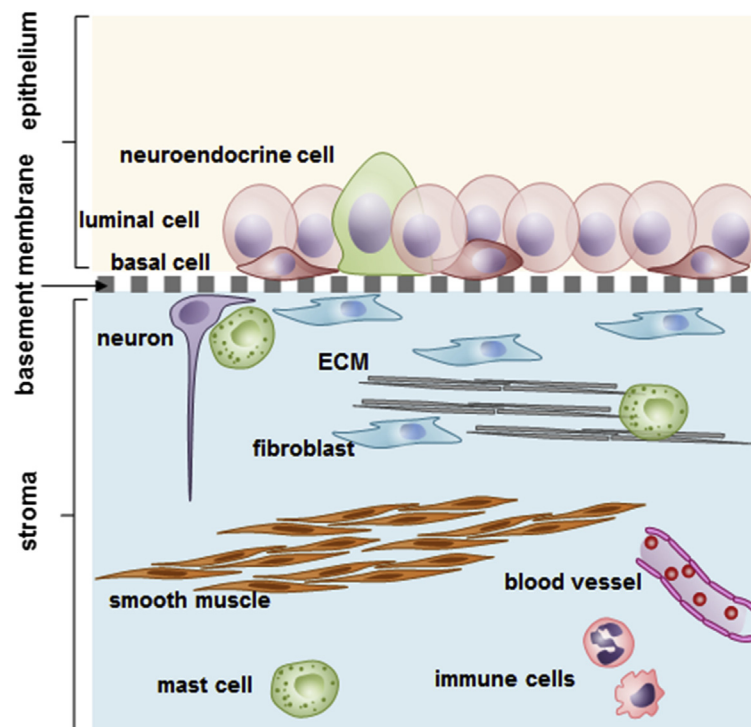


Fig. 2 Distribution of cell types within the prostate. The prostate is a glandular organ, consisting of an epithelial and stromal component, separated by a basement membrane.

Benign Prostatic Hyperplasia (BPH)

The zones of the prostate in the human gland are notable because different prostatic diseases arise from different zones. The transition zone, which is ~15%–30% of the prostate gland, is the site where BPH most commonly arises. Much of the following text will refer to BPH in men because there are only a few animal models of BPH; dogs spontaneously develop BPH but rodents do not. Although rats and mice are frequently used as experimental models for BPH, the difference between the anatomy of the prostate in men and rodents is limiting.

Benign prostatic hyperplasia may be defined histologically as a hyperplasia of the epithelium, stroma, or both in the periurethral transition zone of the prostate. The complex of symptoms that is often attributable to men with BPH are collectively referred to as lower urinary tract symptoms (LUTS). LUTS include frequency of urination, urgency, urinary incontinence, waking up multiple times at night to void (nocturia), weakened urinary stream, straining to void, and a sense of incomplete emptying of the bladder. LUTS are not specific to nor exclusive of BPH and there are many other potential causes of LUTS beside BPH, such as urethral stricture disease, neurologic bladder dysfunction, malignancies (including bladder and advanced prostate cancer), amongst others. Nevertheless, the prevalence of BPH increases in aging men, and is estimated as the 4th most common condition affecting men aged over 50 years old. By 80 years of age approximately 80% of men will have BPH (Roehrborn, 2008).

The Etiology of BPH

Men may have histological evidence for BPH without any symptoms and the size of the prostate gland does not correlate with the severity of symptoms. Human BPH is focal with distinct nodules of hyperplasia within the gland (McNeal, 1984, 1985; Deklerk et al., 1979). Focal growth of transition zone nodules compresses the urethra resulting in urinary retention which is widely believed to be the fundamental cause of the profile of symptoms which comprise LUTS due to BPH. However it is too simplistic to consider hyperplasia as the sole underlying cause of BPH. Instead the etiology of BPH is thought to be related to two components: a static component caused by anatomical obstruction and enlargement of the prostate gland plus a dynamic component which relates to the smooth muscle tone. Therefore current pharmacotherapies for this condition aim to reduce the size or to reduce the smooth muscle tone of the prostate. Overall BPH is a progressive condition and the natural history of the disease is believed to occur in three phases: (i) pathological BPH when there is evidence of tissue proliferation but no LUTS symptoms (ii) clinical BPH when LUTS develop and (iii) progressive disease when LUTS symptoms progress.

Treatment of BPH

Unquestionably, there is a role for hormones in development of BPH. The prostate gland is an androgen dependent organ and, with other sex steroids such as estrogens, hormones are essential for the development and maintenance of the gland. However, BPH does not occur in eunuchs and for over 100 years, castration was shown to reduce prostate size and in some patients led to the improvement of urinary symptoms (White, 1895). Contemporary treatments to reduce prostate size address the need to block androgen signaling. The most successful approach has been to block conversion of androgen to the more potent 5 α reduced metabolite, dihydrotestosterone using 5 α -reductase inhibitors. Beginning with finasteride, 5 α -reductase inhibitors are commonly used for the medical management of BPH. 5 α -reductase inhibitors act predominantly to reduce the size of the prostate gland and thus symptomatic changes will typically occur after 3–6 months.

An alternative pharmacotherapy approach to the treatment of BPH is the use of α 1-adrenergic receptor antagonists (α 1-blockers) to relax the prostate muscle and improve clinical symptoms. α 1-blockers do not reduce prostate growth, but act to block the binding of noradrenaline released from the sympathetic nervous system to the α 1-adrenergic receptor, which induces contraction in the smooth muscle of the prostate and bladder neck. As the α 1-adrenergic receptor is also expressed in blood vessels, the first class of drugs were relatively nonspecific α 1-blockers already in use for the management of hypertension, such as Terazosin, Alfuzosin, and Doxazosin. More recently, specific α 1A-adrenergic receptor antagonists are commonly used. Sildosin and Tamsulosin are the α 1A-receptor subtype-specific antagonists available clinically, yet the clinical responses are similar to nonreceptor specific antagonists. There are currently five α 1-blockers clinically available for the treatment of BPH (alfuzosin, doxazosin, silodosin, tamsulosin, and terazosin) with similar clinical efficacy. The α 1-blockers act relatively quickly, with results generally seen in 1–2 weeks, and generally act on any size prostate gland (large or small), but will not significantly change volume or serum PSA.

PDE5 inhibitors (phosphodiesterase-5 inhibitors) were recently added to the Australian Medicines Handbook as a treatment option for BPH. PDE5 inhibitors were initially approved for the treatment of erectile dysfunction and there is little proof-of-mechanism data about their role within the prostate. Alterations in the nitric oxide/cGMP signaling pathway were linked to the pathogenesis of BPH. A comparative analysis of mRNA from tissue collected from patients with and without BPH found that expression of the nitric oxide synthase gene was significantly lower in BPH diagnosed patients. The reduction in expression of nitric oxide synthase was positively correlated with increasing age and prostate volume. Clinical trials involving the use of PDE5 inhibitors for the treatment of LUTS associated with BPH found reduction in IPSS score was negatively correlated with age (Gacci et al., 2012). This supports the hypothesis that BPH pathogenesis results in a decrease in nitrergic innervation, leading to an increase in smooth muscle tone, and reduced clinical efficacy of PDE5 inhibitors in older men.

If medical management of BPH fails then surgery may be required. Transurethral resection of the prostate (TURP) removes the obstructing tissue using a resectoscope. TURP remains the predominant surgical approach in managing BPH. However the use of medical based therapies, specifically the α 1-adrenergic receptor antagonists and the 5 α -reductase inhibitors, has dramatically changed the clinical management of BPH. The widespread use of inexpensive and effective medical treatments as a first line of treatment has moved surgical intervention for BPH to a second line therapy.

Overall BPH is a very common disorder of the prostate gland increasing with increasing age. The etiology involves two components: the static and dynamic component. Medical management of BPH involves both reduction of prostate growth (static) using 5 α -reductase inhibitors, and change in smooth muscle contractility (dynamic) using α 1-adrenergic receptor antagonists and PDE5i. These treatments have side effects and there is a need to better understand the etiology of BPH and understand why patient responses are variable. Eventually this will lead to better tailoring of treatment and precision in the management of patients for this, as for other, diseases.

Prostatitis: Inflammation of the Prostate Gland

Prostatitis is a common condition related to inflammation within the prostate gland. There are four main classes of prostatitis, as described by [Krieger et al. \(1999\)](#). 90%–95% of reported cases of prostatitis are defined as chronic pain and discomfort from the pelvic region. The etiology of chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) is largely unknown requiring a complex treatment strategy ([Thakkinstian et al., 2012](#)). Bacterial and acute inflammation occurs less often and is most commonly treated with antibiotics.

Despite the unknown etiology of most cases of prostatitis, there is a general awareness that chronic inflammation may predispose or lead to pathologies of the gland, including both BPH and malignancy. The hypothesis is that chronic prostatic inflammation causes tissue damage and continuous wound healing, leading to prostatic enlargement. Patients with chronic inflammation and benign prostatic hyperplasia (BPH) have larger prostate volumes, more severe lower urinary tract symptoms (LUTS) and a higher probability of acute urinary retention than their counterparts without inflammation. Interestingly, chronic inflammation might be a predictor of poor response to BPH medical treatment ([Gandaglia et al., 2013](#)).

Chronic inflammation is also implicated in prostate cancer, as it is in other types of solid tumors. Increased levels of inflammatory factors, arising from changes to the tumor microenvironment, are thought to promote tumor progression. The presence of tumor infiltrating lymphocytes (TILs) also has prognostic relevance, similar to other types of solid tumors. In a recent study the immunological microenvironment of primary tumor was strictly correlated with patient outcome; specifically, infiltration of different TIL populations could predict poor outcome in men with locally advanced disease who relapse after surgery and radiotherapy. More work is warranted on this topic since the evidence provides a strong rationale for immunological treatment of prostate cancer ([Nardone et al., 2016](#)).

Prostate Cancer (PC)

PC is the most common noncutaneous cancer in men and the second leading cause of cancer death, constituting a global public health challenge. Unlike benign disease, prostate cancers usually arise in the peripheral zone of the gland and some men will have both BPH and prostate cancer. PC is a relatively slow growing disease that usually takes many years to progress. Most men are diagnosed with early stage localized prostate cancer and either considered to be at low risk of progressing and go onto active surveillance, or at high risk of progressing, requiring active treatment (removal of gland at radical prostatectomy or radiotherapy). About 1/3rd of men treated are not cured, the tumor recurs and the disease becomes advanced and/or metastatic. Men with advanced disease typically undergo androgen deprivation therapy via medical castration that initially reduces tumor burden and symptoms, but castrate-resistant prostate cancer (CRPC) develops resulting in death ([Fig. 3](#)).

The Challenges of Diagnosis and Treatment

A definitive diagnosis of prostate cancer is based on pathology and is most frequently adenocarcinoma and less frequently neuroendocrine. For decades the pathology of adenocarcinomas has been reported using the Gleason pattern to assign a score. The Gleason grade is an important tool for prediction of patient outcome and in making decisions for treatment. The system has evolved over time firstly with the calculation of a total Gleason Score that was calculated from the sum of the first and second most common patterns reflecting the fact that prostate cancer is heterogenous. Subsequently and more recently the Gleason Grade Grouping system has further simplified the system to provide better prediction of biochemical relapse (see Review [Kim and Andriole, 2015](#); [Epstein et al., 2016](#)).

At initial diagnosis most men are diagnosed with localized disease and the risk of progressing to more advanced disease is evaluated by PSA level, abnormalities upon digital rectal examination and Gleason Grade of tumor in needle biopsies (cT-stage). Those men with low-risk tumors are typically recommended for active surveillance but those men with intermediate or aggressive

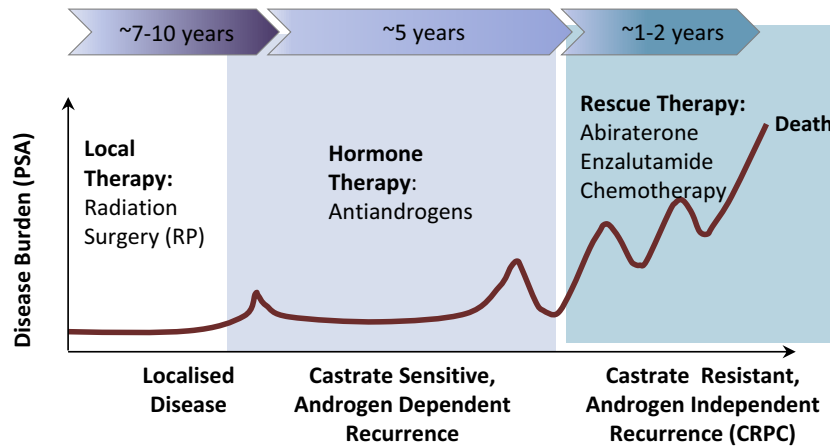


Fig. 3 Schematic of prostate cancer disease progression.

(high-risk) cancer are assigned to active treatment that commonly includes radical prostatectomy or radiotherapy. Men with neuroendocrine tumors are considered to be at high risk of progressing to advanced disease.

Although there is about a 95% 5 year survival for men with localized disease, some men will go on to progress to advanced disease. Since the seminal discoveries of Huggins (Huggins and Hodges, 1972) showing that prostate cancer is a hormone dependent cancer and relies upon androgens for growth, the universal standard of care is androgen deprivation therapy (ADT). ADT is effective when the tumors are castration sensitive, but inevitably the tumors become castration resistant and despite salvage therapies, these men will eventually succumb to their disease. Despite many years of scientific and clinical research, prostate cancer remains a lethal disease in 2017 and accounts for more than 25,000 deaths in the US each year. The challenges for treating men with localized disease and advanced disease are different and the clinical management and focus of research programs is varied.

For men with localized disease PSA screening, extended biopsy templates and enhanced imaging techniques have enhanced the ability to identify men with PC, but has also so increased over-detection of clinically insignificant disease. This potentially results in the over-treatment of men with tumors that were likely to remain indolent and hence the saying that some men will die with, but not of, their PC. The critical question here is to be able to identify and accurately predict at diagnosis, which tumors are likely to remain indolent (vs. those that will progress), so that over-treatment (and conversely under-treatment) is negligible.

There are important drawbacks to active surveillance. At diagnosis, it is possible to misclassify high-risk cancers and miss the “window of curability.” Up to 30% of men who meet eligibility criteria for active surveillance decide to undergo immediate surgery and RP, are reclassified with aggressive tumors on final pathology. During monitoring, repeated biopsies are a cost burden on the health system and pose risks to quality of life including septicaemia, rectal and urinary tract hemorrhage, urinary retention, haematospemia, anesthetic-complications, pain, anxiety and erectile dysfunction (Chang et al., 2013). A limited number of studies have attempted to identify gene signature of likely indolent versus aggressive tumors in men with moderate grade prostate cancer, but these are not in common clinical use and remain to be refined to achieve accuracy of prediction of patient outcome (Fraser et al., 2017) (Fig. 4).

For men who are at high risk of progressing to more advanced disease, surgery and radiotherapy are common treatments but if these fail, the disease requires further treatment. In 1940, Charles Huggins identified the role of androgens in prostate cancer progression and this discovery was a crucial breakthrough that established the principles of hormone suppressive therapy for advanced disease. The response to hormone withdrawal (androgen deprivation therapy, ADT) in men with castrate sensitive tumors is immediate and often dramatic, although the side effects are significant and the response is rarely sustained. The failure to remain responsive to ADT is because the tumors develop mechanisms to resist the systemic loss of hormone. One resistance mechanism

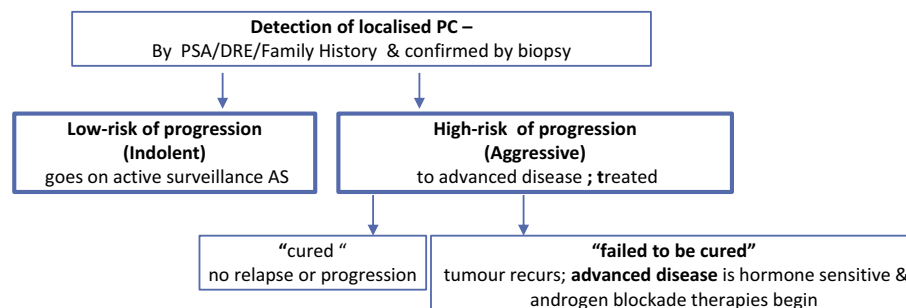


Fig. 4 A summary of steps in the progression of localized to advanced disease showing where some critical decision steps occur.

involves activation of local biosynthesis of androgens within the tumor itself thereby overcoming the loss of systemic androgens. Understanding how this happens and the need to block intratumoral androgen biosynthesis, led to development and use of Abiraterone to block CYP 17, 20 lyase enzymes that convert progestins to testosterone. (De Bono et al., 2011) Other mechanisms of resistance to ADT include progressive changes in androgen receptor signaling, such as AR gene amplification, AR gene mutations, hypersensitivity to androgens and altered expression of AR co-activators and co repressors. (Knudsen and Penning, 2010) The need to block progressive changes to AR signaling led to use of another 2nd line therapy, enzalutamide (Beer et al., 2014), that blocks the AR. Both Abiraterone and Enzalutamide are used in combination with chemotherapy for men who have failed conventional ADT treatment but the survival is extended by months and is not curative.

Tailoring Treatments for Patients With Prostate Cancer

Thus the need to find treatments for men who have (and are at high risk of) lethal PC remain an imperative for clinicians and scientists. Molecular subtyping of tumors from men with metastatic CRPC identified frequent aberrations of AR, ETS genes, TP53, and PTEN in about 40%–60% of cases (Robinson et al., 2015). Many genes and pathways changes were noted and about 89% of CRPC cases had clinically actionable aberrations, including 62.7% with aberrations in AR, 65% in other cancer-related genes. This information could impact on treatment decisions. Similar studies reported molecular subtyping of primary localized prostate cancers, although the overall burden of aberrations is lower than in CRPC tumors (Cancer Genome Atlas Research, 2015). Using this type of information where molecular changes are noted for individual (or groups) of patients, is crucial to determining how to tailor treatment to specific subsets of patients, as well as identifying markers of treatment response and resistance to therapies.

Whilst treating lethal disease as it emerges is a necessary approach, there has been a shift in thinking that maybe earlier treatments in men with high risk localized disease might give greater benefit rather than wait for incurable disease to occur. Known as upfront therapies, there are recent trials that suggest this approach may provide patient benefit. For example, in the trials known as STAMPEDE and CHAARTED both progression free survival and overall survival was improved when docetaxel chemotherapy was given in combination with androgen deprivation therapy rather than when ADT fails. In the STAMPEDE trial, six cycles of docetaxel at the beginning of ADT for metastatic prostate cancer resulted in significantly longer overall survival than that with ADT alone (Sweeney et al., 2015). Upfront therapies such as this might be an important option to consider for men who are fit enough to undergo chemotherapy when they have newly diagnosed metastatic prostate cancer and begin androgen deprivation therapy.

Summary

The prostate is a complex gland prone to disease especially in older men. Prostate cancer has received much attention in the lay, scientific and clinical arenas, but despite decades prostate cancer remains a lethal disease for some patients. BPH and prostatitis are also common conditions but do not generally receive the same degree of attention and there have been fewer research advances for patient benefit. With an aging population in many countries, there is an imperative and need to find curative options for men with benign and malignant prostate disease.

References

- Beer, T. M., Armstrong, A. J., Rathkopf, D. E., et al. (2014). Enzalutamide in metastatic prostate cancer before chemotherapy. *The New England Journal of Medicine*, 371, 424–433.
- Cancer Genome Atlas Research Network. (2015). The molecular taxonomy of primary prostate cancer. *Cell*, 163, 1011–1025.
- Chang, D. T., Challacombe, B., & Lawrentschuk, N. (2013). Transperineal biopsy of the prostate—is this the future? *Nature Reviews. Urology*, 10, 690–702.
- De Bono, J. S., Logothetis, C. J., Molina, A., et al. (2011). Abiraterone and increased survival in metastatic prostate cancer. *The New England Journal of Medicine*, 364, 1995–2005.
- Dekker, D. P., Coffey, D. S., Ewing, L. L., et al. (1979). Comparison of spontaneous and experimentally induced canine prostatic hyperplasia. *The Journal of Clinical Investigation*, 64, 842–849.
- Epstein, J. I., Zelefsky, M. J., Sjoberg, D. D., et al. (2016). A contemporary prostate cancer grading system: A validated alternative to the Gleason score. *European Urology*, 69, 428–435.
- Fraser, M., Sabelnykova, V. Y., Yamaguchi, T. N., et al. (2017). Genomic hallmarks of localized, non-indolent prostate cancer. *Nature*, 541, 359–364.
- Gacci, M., Corona, G., Salvi, M., et al. (2012). A systematic review and meta-analysis on the use of phosphodiesterase 5 inhibitors alone or in combination with alpha-blockers for lower urinary tract symptoms due to benign prostatic hyperplasia. *European Urology*, 61, 994–1003.
- Gandaglia, G., Briganti, A., Gontero, P., et al. (2013). The role of chronic prostatic inflammation in the pathogenesis and progression of benign prostatic hyperplasia (BPH). *BJU International*, 112, 432–441.
- Huggins, C., & Hodges, C. V. (1972). Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA: Cancer Journal for Clinicians*, 22, 232–240.
- Kim, E. H., & Andriole, G. L. (2015). Prostate cancer: A simplified prostate cancer grading system. *Nature Reviews. Urology*, 12, 601–602.
- Knudsen, K. E., & Penning, T. M. (2010). Partners in crime: Deregulation of AR activity and androgen synthesis in prostate cancer. *Trends in Endocrinology and Metabolism*, 21, 315–324.
- Krieger, J. N., Nyberg, L. Jr. & Nickel, J. C. 1999. NIH consensus definition and classification of prostatitis. *JAMA*, 282, 236–7.
- Mclean, J. E. (1984). Prostate anatomy and BPH Morphogenesis. *Progress in Clinical and Biological Research*, 145, 27–54.

- Mcneal, J. E. (1985). Morphology and biology of benign prostatic hyperplasia. In N. Bruchovsky, A. Chapdellaine, & F. Neumann (Eds.), *Regulation of androgen action*. Berlin: Congressdruck R. Bruckner.
- Nardone, V., Botta, C., Caraglia, M., et al. (2016). Tumor infiltrating T lymphocytes expressing FoxP3, CCR7 or PD-1 predict the outcome of prostate cancer patients subjected to salvage radiotherapy after biochemical relapse. *Cancer Biology & Therapy*, 17, 1213–1220.
- Robinson, D., van Allen, E. M., Wu, Y. M., et al. (2015). Integrative clinical genomics of advanced prostate cancer. *Cell*, 161, 1215–1228.
- Roehrborn, C. G. (2008). Pathology of benign prostatic hyperplasia. *International Journal of Impotence Research*, 20(Suppl. 3), S11–8.
- Sweeney, C. J., Chen, Y. H., Carducci, M., et al. (2015). Chemohormonal therapy in metastatic hormone-sensitive prostate cancer. *The New England Journal of Medicine*, 373, 737–746.
- Thakkinstian, A., Attia, J., Anothaisintawee, T., & Nickel, J. C. (2012). Alpha-blockers, antibiotics and anti-inflammatories have a role in the management of chronic prostatitis/chronic pelvic pain syndrome. *BJU International*, 110, 1014–1022.
- White, J. W. (1895). I. The results of double castration in hypertrophy of the prostate. *Annals of Surgery*, 22, 1–80.

Seminal Vesicle Gland—Overview

John J Bromfield, Laila A Ibrahim, and Jason A Rizo, University of Florida, Gainesville, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Epithelium Cell layer that is commonly found lining glandular structures and mucosal surfaces. Mostly secretory in nature.

Fertilization The process of haploid sperm and egg forming a new diploid cell.

Mesonephros Excretory organ similar to the kidney active during fetal development that forms part of the male reproductive tract.

Development and Structure

The development of the seminal vesicle glands is testosterone dependent and follows a similar pattern in most mammals. The formation of the seminal vesicle glands occurs in parallel with the development of the mesonephros, a primitive excretory organ of the early embryo. The embryonic mesonephros consists of numerous tubules that form the urogenital ridge. Each tubule connects a vascular glomerulus at one end, and opens into the Wolffian excretory duct at the other. The cranial tubules and glomeruli of the Wolffian duct undergoes atrophy and the corporal section develops into the epididymis (Sadler, 2012). By the thirteenth week of development in the human an invagination forms in the caudal portion of the Wolffian duct close to the urogenital sinus, dividing it into the deferent duct and the ejaculatory duct. This invagination constitutes the earliest form of the seminal vesicle gland which is at first a straight tube that branches into numerous convoluted ducts later in development.

In rodents, the formation of the seminal vesicle glands begins at the end of gestation, around day 19. At this point in development the seminal vesicle gland epithelium increases in height and forms a lumen (Fig. 1). The density of granular endoplasmic reticulum in the epithelium, important for secretion, increases (Aumuller and Riva, 1992). These changes constitute early fetal preparations for the main adult secretory activity of the epithelium. In human embryos, seminal vesicles differentiation is completed by the seventh month of gestation.

Fully developed seminal vesicle glands are paired structures consisting of convoluted and curved tubular glands with numerous lateral projections located posterior to the prostate and urinary bladder, superior to the distal ureter, and posterior to the rectum (Fig. 1). Laterally, the ampulla of the vas deferens and the veins of the prostatic venous plexus lie along its medial margins. The principal cells of the seminal vesicle glands are slender columnar epithelium, containing numerous mitochondria and a well-developed rough-endoplasmic reticulum important for protein secretion. Basal cells of the seminal vesicle gland are small stellate cells with a compact cytoplasm and few organelles (Aumuller and Riva, 1992).

The growth, development, and differentiation of the seminal vesicle glands from birth to the onset of puberty is slow. Before birth the seminal vesicle gland epithelium is a hollow tube surrounded by a layer of mesenchyme. Following birth the epithelium undergoes dramatic growth and branching (Settle et al., 2001) (Fig. 1). Buds from the epithelium grow laterally into the

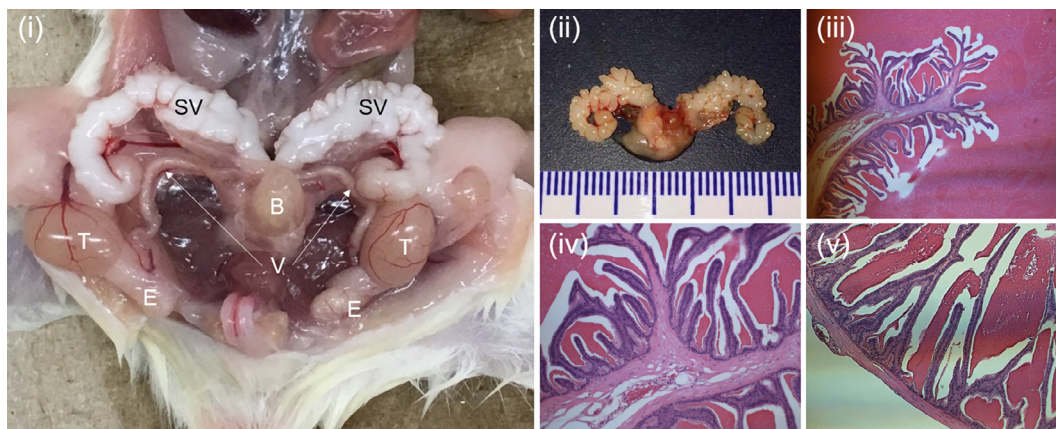


Fig. 1 Mouse seminal vesicle glands. Gross morphology of the male reproductive tract (i) showing the placement of the large seminal vesicle glands (SV; ii) above the bladder (B) and meeting at the juncture of the vas deferens (V). The relative size of the seminal vesicles is evident in comparison to the testicles (T) and tail of the epididymis (E). Photomicrographs showing the internal (iii, iv) and capsular (v) epithelium of the seminal vesicle gland at 10 $\times$ and 40 $\times$ magnifications. The columnar epithelium (purple) is evident against the dense seminal vesicle gland fluid (pink).

mesenchyme as systemic androgen concentration increases, the elongating buds bifurcate, and the epithelium folds upon itself. After puberty, maximal androgen levels provoke growth of the seminal vesicles that triggers functional differentiation of the secretory epithelium. Finally, the glands develop to form sac-like structures which, in humans, have a capacity to store a volume of about 3.4–4.5 mL (Aumuller and Riva, 1992).

In humans, the duct of the seminal vesicle and the vas deferens share a common ejaculatory duct that opens into the urethra (Hafez, 2000); however, the ejaculatory duct is not present in any of the domestic mammals, where each duct opens separately into the urethra (Setchell, 1991). Dogs and cats have no seminal vesicle glands, while boars are considered to have the largest seminal vesicle glands among domestic animals. In humans, there are some individual variations in size and storage capacity of the seminal vesicle glands; two to three ejaculations may deplete the seminal vesicle glands to such an extent that a refractory period of at least 2 days is required before the glands fully restore sufficient fluid for a normal ejaculate. Conversely, in the bull the seminal vesicle glands are capable of storing up to 50 mL of secretions, sufficient to provide fluid for up to a dozen ejaculates.

Secretion of Seminal Vesicles

The seminal vesicle glands are an androgen dependent organ that secretes a significant fraction of the fluid that eventually becomes semen (seminal fluid). In most species the major contribution to semen volume is provided by the seminal vesicle glands. For example, human seminal vesicle glands contribute between 70% and 85% of ejaculate volume, bull seminal vesicle glands produce approximately 50% of ejaculate volume, and in the mouse seminal vesicle glands produce approximately 90% of the ejaculate volume (Kierszenbaum and Tres, 2011). Seminal vesicle fluid is a thick, viscous, alkaline fluid, resulting in semen having a mild alkaline pH. The alkalinity of semen helps neutralize the acidic environment of the vagina to maintain sperm viability in the female reproductive tract.

Seminal vesicle gland secretions contain various proteins, enzymes, mucus, vitamins, amino acids, ions, minerals, flavins, and hormones. Seminal vesicle gland secretions are characterized by a high content of fructose. Fructose is the primary source of energy for spermatozoa within the ejaculate. Other carbohydrates, inositol, glucose, fucose, ribose and sorbitol are also present in small amounts in seminal vesicle gland secretions. The lipid hormone, prostaglandin is found in all accessory sex glands but have their highest concentration in the seminal vesicle glands. Four types of prostaglandin are present in seminal vesicle gland secretions; prostaglandin A, B, E, and F. The secretions of the seminal vesicle glands contain ions including high concentration potassium, lower concentration sodium, while chloride is almost absent. The role of these ions is unclear but may center on sperm motility or contraction of muscle in male and/or female reproductive tracts. Large scale proteomic studies have identified upwards of 2000 seminal vesicle derived proteins in humans (Duncan and Thompson, 2007). Proteins required for the coagulation and liquefaction of semen such as semenogelins I, II, and kallikrein are secreted from the seminal vesicle glands and prostate and are integral to the functionality of semen (Lilja et al., 1987). Seminal vesicle secretions of induced ovulatory species such as camelids (including the llama) contain specific ovulation-inducing factors like β -nerve growth factor (β -NGF), which acts at the level of the brain to induce ovulation (Tribulo et al., 2015).

It would seem that while the seminal vesicle glands secrete a complex fluid, the main role of this fluid is to facilitate sperm survival and transport through both male and female reproductive tracts to enable fertilization. However, the high number of compounds in seminal vesicle secretions may suggest secondary roles in communication between sire and dam at the time of conception.

Role of the Seminal Vesicle Gland in Reproduction

The primary role of the seminal vesicle gland is to produce a supportive fluid to maintain sperm viability and functionality during transport through the male reproductive tract at ejaculation and female reproductive tract to facilitate fertilization of the ovum. Seminal vesicle fluid is rich in energy substrates, buffering agents and antioxidants expressly for the purpose of maintaining the functional capacity of the sperm. As sperm utilize carbohydrate for their energy to traverse the female reproductive tract they produce large amounts of reactive oxygen intermediates which can potentially harm the valuable paternal DNA contained within the sperm nucleus. High concentrations of antioxidant present in seminal vesicle gland fluid help to reduce potential oxidative stress and subsequent DNA damage. A major role of seminal vesicle gland derived prostaglandins is smooth muscle contraction. It is surmised that prostaglandins in semen aid in sperm transport in both male and female reproductive tracts by stimulating contraction of the testicular capsule, seminiferous tubules, epididymis, vas deferens, cervix and uterine body (da Silva et al., 2016). As semen is a coagulatory fluid the process of liquefaction is a necessity to release sperm to become free swimming in the ejaculate. The process of liquefaction requires seminal vesicle gland derived proteases to allow sperm to pass through the cervix after vaginal deposition at coitus.

The role of seminal vesicle gland fluid may not be as simple as solely maintaining sperm viability and facilitating sperm transport. Invertebrates including mosquitoes, crickets and flies use seminal vesicle gland fluid as a means to alter reproductive characteristics in mated females. In mice, horses, pigs and humans, seminal vesicle gland fluid induces significant changes to the maternal cellular environment of the endometrium or cervix. Interestingly, seminal vesicle gland fluid is nearly indispensable in natural conception, however assisted reproductive technologies such as artificial insemination and in vitro fertilization prove that seminal vesicle gland fluid is not a requirement for fertilization or successful pregnancy outcomes. Nevertheless, evidence in

mice, cattle and women suggest exposure to seminal vesicle gland fluid may improve fertility and benefit pregnancy outcomes (Odhiambo et al., 2009, Bromfield et al., 2014, Tremellen et al., 2000).

While seminal vesicle gland fluid is no longer a requirement for fertilization due to new technologies it is interesting to consider its potential as a mediator of pregnancy success in a number of species.

References

- Aumuller, G., & Riva, A. (1992). Morphology and functions of the human seminal vesicle. *Andrologia*, 24, 183–196.
- Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J., & Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 2200–2205.
- Da Silva, B. F., Meng, C., Helm, D., Pachl, F., Schiller, J., Ibrahim, E., Lynne, C. M., Brackett, N. L., Bertolla, R. P., & Kuster, B. (2016). Towards understanding male infertility after spinal cord injury using quantitative proteomics. *Molecular & Cellular Proteomics*, 15, 1424–1434.
- Duncan, M. W., & Thompson, H. S. (2007). Proteomics of semen and its constituents. *Proteomics. Clinical Applications*, 1, 861–875.
- Hafez, E. S. E. (2000). Anatomy of male reproduction. In B. Hafez, & E. S. E. Hafez (Eds.), *Reproduction in farm animals* (7th edn.). Baltimore, MD: Lippincott Williams & Wilkins.
- Kierszenbaum, A., & Tres, L. (2011). Sperm transport and maturation. In Mosby (Ed.), *Histology and cell biology: An introduction to pathology*. MO Elsevier: St. Louis.
- Lilja, H., Oldbring, J., Rannevik, G., & Laurell, C. B. (1987). Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. *The Journal of Clinical Investigation*, 80, 281–285.
- Odhiambo, J. F., Poole, D. H., Hughes, L., DeJarnette, J. M., Inskip, E. K., & Dailey, R. A. (2009). Pregnancy outcome in dairy and beef cattle after artificial insemination and treatment with seminal plasma or transforming growth factor beta-1. *Theriogenology*, 72, 566–571.
- Sadler, T. W. (2012). *Langman's medical embryology*. Philadelphia, PA: Lippincott Williams & Wilkins.
- Setchell, B. P. (1991). Male reproductive organs and semen. In T. P. Cupps (Ed.), *Reproduction in Domestic Animals* (4th edn.). London: Academic Press, Inc.
- Settle, S., Marker, P., Gurley, K., Sinha, A., Thacker, A., Wang, Y., Higgins, K., Cunha, G., & Kingsley, D. M. (2001). The BMP family member Gdf7 is required for seminal vesicle growth, branching morphogenesis, and cytodifferentiation. *Developmental Biology*, 234, 138–150.
- Tremellen, K. P., Valbuena, D., Landeras, J., Ballesteros, A., Martinez, J., Mendoza, S., Norman, R. J., Robertson, S. A., & Simon, C. (2000). The effect of intercourse on pregnancy rates during assisted human reproduction. *Human Reproduction*, 15, 2653–2658.
- Tribulo, P., Bogle, O., Mapletoft, R. J., & Adams, G. P. (2015). Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology*, 83, 1394–1401.

Seminal Vesicle—Structure

Manabu Yoshida, The University of Tokyo, Miura, Kanagawa, Japan

Natsuko Kawano, Meiji University, Kawasaki, Kanagawa, Japan

Teruaki Iwamoto, Sanno Hospital, Tokyo, Japan

Kaoru Yoshida, Toin University of Yokohama, Yokohama, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Aspermia A medical condition in which semen is not produced.

Asthenozoospermia A medical condition in which sperm has reduced motility (<40% sperm motility).

Azoospermia A medical condition in which the semen contains no sperm.

Cryptozoospermia A medical condition in which the semen contains very little sperm in the pellet after centrifugation.

Hemospermia A medical condition in which blood is found in the semen.

Hypogonadism A medical condition in which there is diminished gonad activity (the testes in males).

Oligozoospermia A medical condition in which the semen contains reduced sperm (<15 × 10⁶ spermatozoa/mL).

Prostatitis Inflammation of the prostate.

Seminal vesiculitis Inflammation of the seminal vesicles.

Abbreviations

FSH Follicle stimulating hormone

ICSI Intracytoplasmic sperm injection

LH Luteinizing hormone

LOH Late-onset hypogonadism

SV Seminal vesicle

SVCA Seminal vesicle adenocarcinoma

TESE Testicular sperm extraction

Basics of the Seminal Vesicle

The seminal vesicles (SVs) are a pair of male accessory reproductive glands, located posteroinferior to the urinary bladder. The SVs are derived from the mesonephric duct (Wolffian duct), and develop as branches of the vas deferens at the junction of the vas deferens and the urethra. Thus, the excretory ducts of the SVs join with the ampulla of the vas deferens and the ejaculatory ducts merge with the verumontanum of the prostatic urethra. At ejaculation, the emission of SV secretions occurs dominantly through sympathetic action. Although there are a few exceptions, such as canines and felines, almost all mammalian species have SVs of varying sizes. In adult mice, the normal size of the SV is 1.5–2 cm in length and 0.3–0.5 cm in diameter (**Fig. 1**). In adult humans, the normal size is 5–10 cm in length and 3–5 cm in diameter (**Fig. 2**). Generally, secretions from the SVs account for 50%–80% of the volume of ejaculated semen and contain ions (Na⁺, K⁺, Cl[−]), sugars (fructose, glucose), peptides (glutathione), and, above all, are highly concentrated in protein (**Mata, 1995**). SV secretions partly act as a protectant of sperm in the female reproductive tract (**Araki et al., 2015**), and SV removal can severely reduce fertility (**Kawano et al., 2014**) as well as the metabolism of the next generation (**Bromfield et al., 2014**). In this article, we introduce the structural SV, focusing on three topics: the epithelial cells of the SVs, the human SV, and SV-derived diseases.

Epithelial Cells of the Seminal Vesicles

The SVs consist of a single layer of secretory epithelial cells, and an outer layer of smooth muscle (**Fig. 3**). The epithelia is essentially simple columnar in character, and the SV lumen, with partial diverticula, foldings, or outpouchings, may cause resemblance to pseudostratified columnar epithelia (**Fig. 3**). The protein concentration in the SV lumen can reach 200 mg/mL, indicating the high performance of SV epithelial cells in secretion and dewatering.

The secretory machinery of the SV epithelia requires well-developed rough endoplasmic reticulum, Golgi apparatus, and secretory vesicles to accommodate the massive production and secretion of SV proteins. In fact, numerous rough endoplasmic reticulum

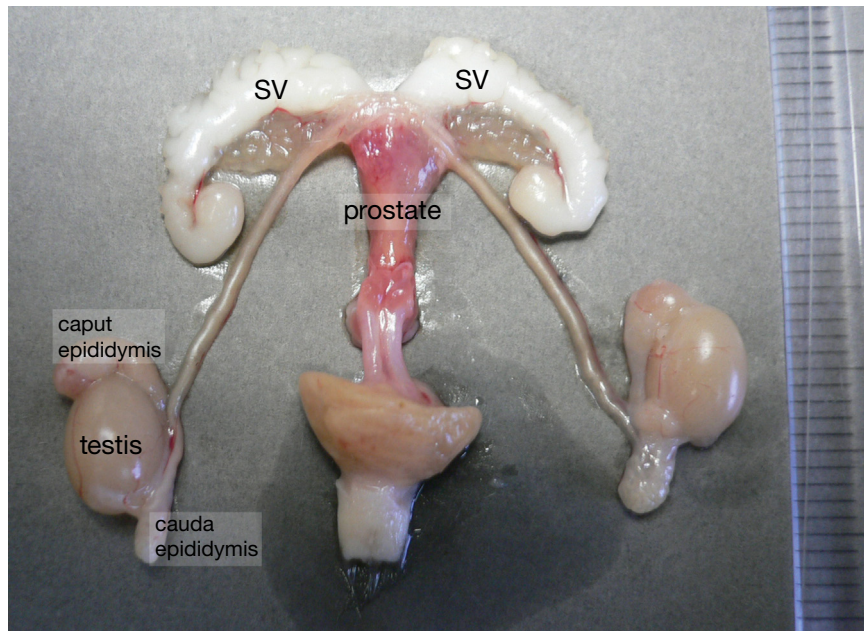


Fig. 1 SV and male reproductive organs of mouse.

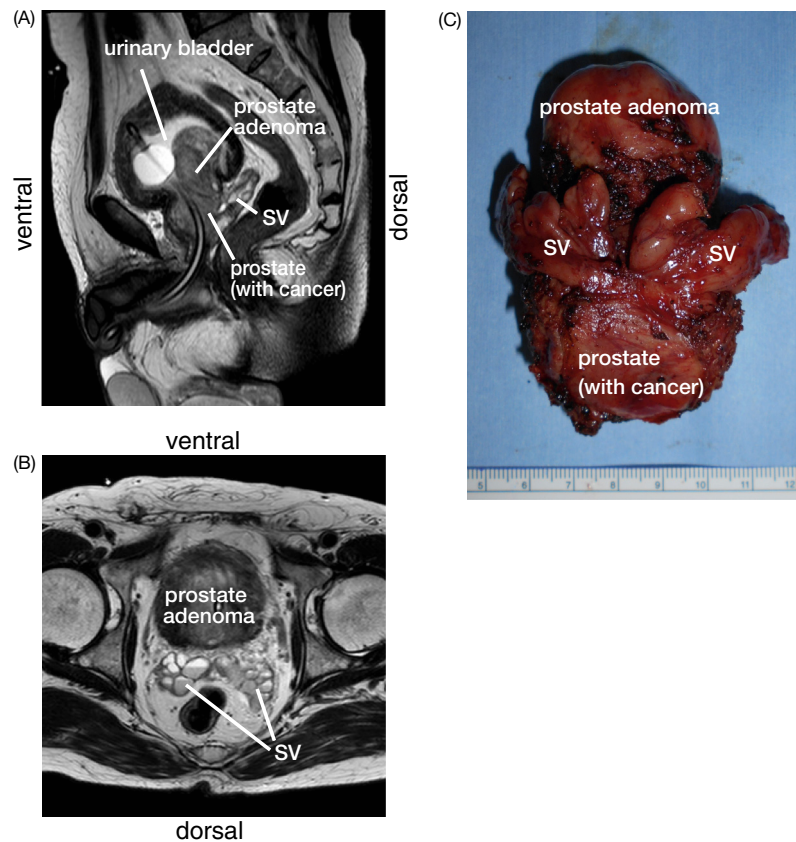


Fig. 2 Human SV of a patient with prostate cancer and adenoma protruding into the bladder. (A and B) MRI images of the patient: (A) sagittal section; (B) cross section of pelvic part. SV is located posterior side of urinary bladder. (C) SV with prostate cancer and an adenoma of the prostate excised from the patient. Figures were provided by Department of Urology, International University of Health and Welfare Hospital.

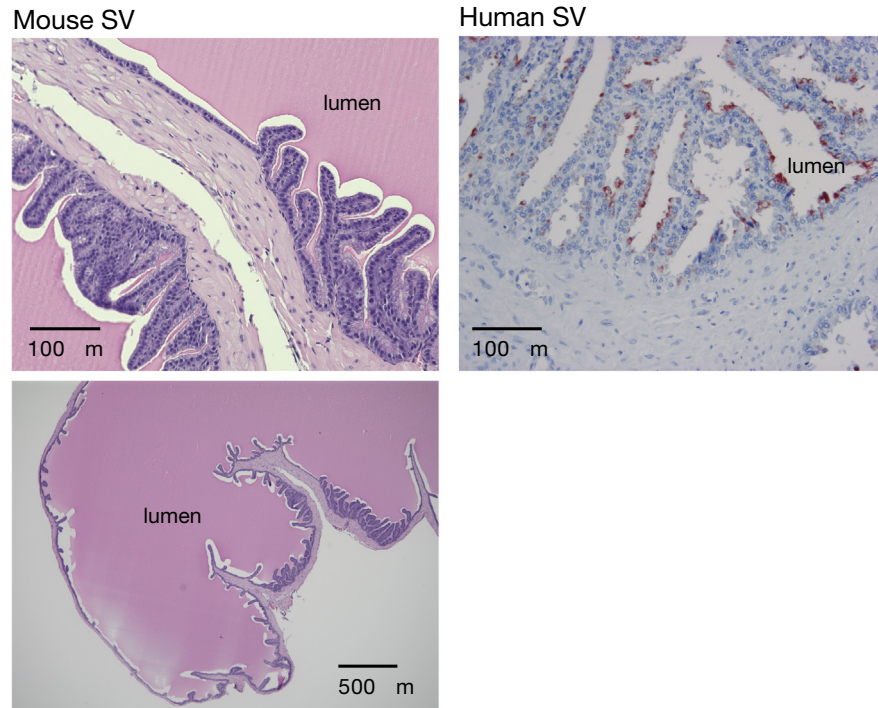


Fig. 3 Cross section of mouse and human SVs. (Left) Mouse SV. (Right) Human SV.

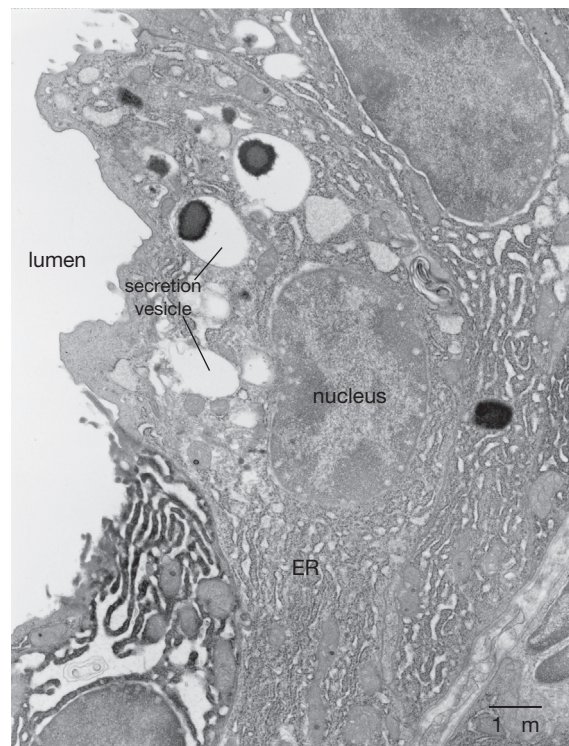


Fig. 4 Electron microscopic observation on an epithelial cell of mouse SV.

are a frequently observed characteristic of SV epithelial cells (**Fig. 4**). SV proteins aggregate easily, in order to aggregate the semen and form a copulatory plug; the experimental expression of SV proteins in *Escherichia coli* is difficult because of this propensity for aggregation. However, unaggregated proteins are required in the epithelia and male reproductive tract before ejaculation. It is likely that SV proteins are folded precisely in the endoplasmic reticulum.

Another mechanism behind the high concentration of proteins in the SV lumen may be dewatering. SV epithelial cells take up water and electrolytes (Levine and Kelly, 1980) and aquaporins are present in the apical and basolateral membranes of SV secretory cells (Brown et al., 1993). The secretory and dewatering functions of the SV epithelia enable the high concentration of proteins found in SV secretions, and furthermore, induce the multifunctional capability of seminal plasma (e.g., regulation of sperm motility, protection of sperm from the female immune system).

The Human Seminal Vesicle

The prostate is prone to serious tumors, and therefore this male accessory gland has been extensively studied. However, the SVs seldom develop such pathology, and when removed, do not impact viability. In fact, in the case of curative prostatectomy for prostate cancer, removal of the SVs is a standard procedure (see Fig. 2). As described above, the size of normal adult SV is 5–10 cm in length and 3–5 cm in diameter (Fig. 2). The average amount of semen ejaculated by normal healthy men ranges from 1.5 to 2.5 mL, and secretions from the SVs account for 50%–80% of the ejaculate. The physiological role of the SVs is not entirely known. Understanding of the sources of sperm components and the functions of the SVs has been enabled by the split ejaculation study. Mawson and Fischer (1953) collected ejaculates in three separate containers. They have assumed that the first fraction was a mixture of sperm, epididymal and prostatic secretions, and the second fraction was a mixture of prostatic and vesicular secretions. The third fraction was assumed to originate mainly in the SVs. While, semenogelin 1, a 52 kDa-protein expressed exclusively in the SVs, is a major component of the semen coagulum (Robert and Gagnon, 1999), and together with semenogelin 2, it inhibits sperm motility. Hydrolysis by the protease prostate-specific antigen induces the degradation of the coagulum, enabling the sperm to move (Yoshida et al., 2008). Therefore, clinical SV problems can be related to male infertility.

Obstructive Azoospermia, Cryptozoospermia, Oligozoospermia, Asthenozoospermia, and Aspermia Due to Seminal Vesicle Diseases

Azoospermia and aspermia are rarely caused by obstruction of ejaculatory ducts due to diseases of the SVs or prostate; however, it does occur in more than 5% of infertile patients (Modgil et al., 2016). Infertility case reports with semen analysis displaying cryptozoospermia or azoospermia, but normal spermatogenesis by testes histology, have been diagnosed with cystic dilatation of the SVs. It is assumed, in these cases, that the cause of the cryptozoospermia or azoospermia was partial or complete occlusion of the ejaculatory duct due to cystic dilatation of the SVs. Successful conception was achieved for one patient who underwent retrograde dilatation of the ejaculatory duct (Iwasaki et al., 1988).

Atrophy of the Seminal Vesicles Due to Hypogonadism

The SVs are androgen-dependent organs, and decreased androgen levels are accompanied by SV atrophy. This can result in “non-obstructive” azoospermia, aspermia, and oligozoospermia. Male hypogonadism is classified into two types: primary hypogonadism, in which the testis itself is impaired, and secondary hypogonadism, caused by defects in the hypothalamus or pituitary gland.

Primary hypogonadism: This is often a hypogonadotropic hypogonadism characterized by elevated FSH and LH in the blood and decreased testosterone. Primary hypogonadism is a symptom of Klinefelter syndrome, and also describes many azoospermia cases of unknown cause. Androgen deficiency symptoms are improved by testosterone replacement therapy, particularly decreased semen volume, and the atrophic SVs recover their function. However, azoospermia is not ameliorated. In this situation, sperm can be retrieved by microdissection-testicular sperm extraction (microdissection-TESE), enabling conception by intracytoplasmic sperm injection (ICSI).

Secondary hypogonadism: Secondary or hypogonadotropic hypogonadism is characterized by a decrease in blood FSH, LH, and testosterone. Typical diseases of secondary hypogonadism include Kallman syndrome and congenital gonadotropin deficiency. Proper hormone therapy can improve both the late-onset hypogonadisms (LOH) and spermatogenesis disorders with a high rate of success, particularly decreased semen volume. Although testosterone replacement therapy such as Testosterone enanthate is relatively inexpensive, simple, and effective against LOHs, it does not affect spermatogenesis. Combination therapy with recombinant human FSH (r-hFSH) and human chorionic gonadotropin (hCG), which is currently available as a self-injectable medication, has been successful in the treatment of hypogonadotropic hypogonadism. This therapy increases the testicular and seminal volumes, induces the appearance of sperm in ejaculate in azoospermia cases, returns seminal findings to normal levels, and enables natural conception.

Seminal Vesiculitis

Seminal vesiculitis, that is, inflammation of the SVs, is a rare disease that often occurs in combination with prostatitis. The main symptoms are hematospermia (blood in the semen) and lower abdominal and perineal pain. Patients often present with hematospermia without any other symptoms. Although the cause of seminal vesiculitis is not well understood, it often occurs through

nonspecific infection with tuberculosis or other atypical infections. One diagnosis method involves quantifying the leukocytes present in ejaculate. According to the World Health Organization (WHO) guideline, normal semen contains less than 1×10^6 leukocytes/mL (WHO, 2010). Moreover, it is possible to infer whether the leukocytes are from the SVs or prostate. As described above, the third fraction of ejaculated semen consists of mainly SV secretions. Therefore, analysis of the third fraction, through counting the leukocytes and obtaining bacterial cultures, can be useful for diagnosis of SV infections. However, a high leukocyte count in semen does not necessarily indicate seminal vesiculitis and/or prostatitis. Moreover, it is not clear whether a high semen leukocyte count leads to impaired spermatogenesis. Liu et al. used transurethral seminal vesiculoscopy for the diagnosis of seminal vesiculitis, and demonstrated that the dilation of SVs and retention of turbid solution by leukocytes in the SV secretions were effective for diagnosis (Liu et al., 2014). The ejaculatory ducts and SVs were visualized to confirm the diagnosis of seminal vesiculitis and to determine the cause of the disease.

Fragmented peptides from semenogelin 1 have antibacterial activity which is strictly zinc-dependent (Edström et al., 2008). Furthermore, abnormal expression of semenogelin 1 has been observed in the SV tissue of intractable seminal vesiculitis patients (Liu et al., 2016). Thus, study of semenogelin 1 and its fragmented peptides may help identify causes of urinary tract infection, and improve methods of sperm screening.

Adenocarcinoma of the Seminal Vesicles

The incidence of primary seminal vesicle adenocarcinoma (SVCA) is extremely low compared to prostate cancer: these are only 60 cases of SVCA reported in the global literature. SVCA tends to occur in patients over 50 years old. Diagnosis is typically delayed because there are no specific symptoms and no specific tumor markers, though some tumor markers have been suggested, such as serological cancer antigen 125, and cytokeratin subsets 7 and 20. A very rare case of primary mucinous adenocarcinoma arose from a seminal vesicle cyst, which was associated with an ectopic ureter opening and ipsilateral renal agenesis. In that case, the development of SVCA may have been due to chronic stimulation of the ectopic ureter's secretion (Lee et al., 2007).

Why is SVCA so rare compared to prostate cancer? In one study, the thymidine labeling index (TLI), which measures DNA amplification, of prostate adenocarcinomas averaged 0.90%, which was significantly higher than for either normal epithelium or the epithelium of nodular hyperplasia (Meyer et al., 1982). On the contrary, the TLI of SV epithelium averaged 0.02% (Meyer et al., 1982), suggesting that the proliferative rate of SV cells is quite low. This may account for the rarity of SVCA.

References

- Araki, N., Trencsenyi, G., Krasznai, Z. T., et al. (2015). Seminal vesicle secretion 2 acts as a protectant of sperm sterols and prevents ectopic sperm capacitation in mice. *Biology of Reproduction*, 92(1), 8, 1–10.
- Bromfield, J. J., Schjenken, J. E., Chin, P. Y., et al. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America*, 111(6), 2200–2205.
- Brown, D., Verbavatz, J. M., Valenti, G., et al. (1993). Localization of the CHIP28 water channel in reabsorptive segments of the rat male reproductive tract. *European Journal of Cell Biology*, 61(2), 264–273.
- Edström, A. M., Malm, J., Frohm, B., et al. (2008). The major bactericidal activity of human seminal plasma is zinc-dependent and derived from fragmentation of the semenogelins. *Journal of Immunology*, 181(5), 3413–3421.
- Iwasaki, A., Hirokawa, M., Hosaka, M., et al. (1988). Study of cystic dilatation of the seminal vesicles and its classification. *Nihon Hinyokika Gakkai Zasshi*, 79(8), 1385–1392.
- Kawano, N., Araki, N., Yoshida, K., et al. (2014). Seminal vesicle protein SVS2 is required for sperm survival in the uterus. *Proceedings of the National Academy of Sciences of the United States of America*, 111(11), 4145–4150.
- Lee, B. H., Seo, J. W., Han, Y. H., et al. (2007). Primary mucinous adenocarcinoma of a seminal vesicle cyst associated with ectopic ureter and ipsilateral renal agenesis: a case report. *Korean Journal of Radiology*, 8(3), 258–261.
- Levine, N., & Kelly, H. (1980). Control of reabsorption of water and electrolytes by guinea-pig seminal vesicle in vivo. *Journal of Reproduction and Fertility*, 58(2), 421–427.
- Liu, B., Li, J., Li, P., et al. (2014). Transurethral seminal vesiculoscopy in the diagnosis and treatment of intractable seminal vesiculitis. *Journal of International Medical Research*, 42(1), 236–242.
- Liu, B., Song, Z., Su, S., et al. (2016). Abnormal expression of Sg I is closely related to seminal vesiculitis. *Urology*, 88, 227 e9–227 e14.
- Mata, L. R. (1995). Dynamics of the seminal vesicle epithelium. *International Review of Cytology*, 160, 267–302.
- Mawson, C. A., & Fischer, M. I. (1953). Zinc and carbonic anhydrase in human semen. *Biochemical Journal*, 55(4), 696–700.
- Meyer, J. S., Sufrin, G., & Martin, S. A. (1982). Proliferative activity of benign human prostate, prostatic adenocarcinoma and seminal vesicle evaluated by thymidine labeling. *Journal of Urology*, 128(6), 1353–1356.
- Modgil, V., Rai, S., Ralph, D. J., & Muneer, A. (2016). An update on the diagnosis and management of ejaculatory duct obstruction. *Nature Reviews. Urology*, 13(1), 13–20.
- Robert, M., & Gagnon, C. (1999). Semenogelin I: a coagulum forming, multifunctional seminal vesicle protein. *Cellular and Molecular Life Sciences*, 55(6–7), 944–960.
- WHO. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th edn). Geneva: World Health Organization.
- Yoshida, K., Kawano, N., Yoshiike, M., et al. (2008). Physiological roles of semenogelin I and zinc in sperm motility and semen coagulation on ejaculation in humans. *Molecular Human Reproduction*, 14(3), 151–156.

Seminal Vesicle—Secretion

John E Schjenken, David J Sharkey, and Sarah A Robertson, The University of Adelaide, Adelaide, SA, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

The male accessory sex glands include the prostate, seminal vesicle, and bulbourethral glands. These secretory organs together produce seminal plasma, a viscous fluid with a mild alkaline pH which neutralizes the acidic environment of the vagina and contributes to maintenance of sperm viability following ejaculation. Seminal plasma has important roles in reproduction that are broadly conserved across mammalian species and even in invertebrates, despite differences between individual species in accessory gland structures, seminal plasma composition and biological actions (McGraw et al., 2015).

Seminal vesicles are the major contributing organ to seminal plasma. First described in 1521 by Berengario a Capri, they were originally and mistakenly regarded as a storage organ for semen. They are now recognized as a functional unit together with the ampulla of the deferent and ejaculatory ducts that contribute to producing the seminal fluid (Aumuller and Riva, 1992; Schjenken and Robertson, 2014). These glands are androgen dependent and form in parallel with the development of the mesonephros around the 13th week of development in the human and towards the end of gestation (day 19) in the mouse (Brandes, 1974). In humans, following birth and up until puberty the development of the seminal vesicle glands is slow, but with increased androgen levels around puberty, the glands expand in size and increase their secretory capacity (Aumuller and Riva, 1992).

The secretions of the seminal vesicle gland play a facilitating role in male reproductive biology, with the complex fluid containing factors which promote sperm survival and transport, and capacity to secure female reproductive investment and sire offspring. After intromission, seminal vesicle derived proteins stimulate physiological changes in the female reproductive tract to maximize the chances of reproductive success. Depending on the species, this can take the form of a physical structure called the “copulatory plug” that prevents sperm loss and block mating by other males. In invertebrates, seminal plasma factors act to promote egg release and block female receptivity to other males. In some mammals, seminal plasma agents also promote ovulation while in many others, the female immune response is suppressed from rejecting the foreign sperm and instead persuaded to promote immune tolerance of embryos and fetuses sired by the same but not other males. The extent to which seminal vesicle proteins are effective in executing these biological processes has an impact on the likelihood of conception, and also influences the developmental trajectory and phenotype of offspring (Schjenken and Robertson, 2014; Brandes, 1974).

The seminal vesicle thus supports a variety of important mechanisms to maximize the chances of the male successfully siring healthy offspring. This article provides an overview of the secretions of the seminal vesicle glands and their biological activity in promoting sperm viability and function, and other aspects of promoting male reproductive success. The focus is mainly on mammalian species, but it is important to acknowledge that seminal vesicle secretions exhibit important functions in a wide range of vertebrates and invertebrate species, whenever intromission at mating occurs (McGraw et al., 2015; Schjenken and Robertson, 2014).

Secretions of the Seminal Vesicle

The fluid secreted by the seminal vesicle is rich in a wide range of bioactive moieties ranging from inorganic salts and micronutrients such as potassium and zinc, carbohydrates particularly reducing sugars such as fructose, lipid derivatives including prostaglandins and phosphorylcholine, and a unique array of glycoproteins notably lactoferrin, proteinase inhibitors and cell–cell signaling molecules known as cytokines (McGraw et al., 2015). The factors secreted by the seminal vesicles have functional roles in semen coagulation, sperm motility and capacitation and female immune regulation.

There are limited studies aimed at assessing the proteomic composition of human seminal vesicle fluid, primarily due to difficulties associated with the collection of clinical samples. Some studies have utilized aspirated seminal vesicle fluid collected during surgery to identify the presence of a group of basic proteins termed seminal vesicle specific antigens (Lee et al., 1989; Rui et al., 1984). These proteins are produced by the glandular epithelium and are now identified as members of the Semenogelin family, which are amongst the most abundant seminal vesicle secreted proteins.

In rodents, these proteins play a crucial role in copulatory plug formation (Lee et al., 1989; Duncan and Thompson, 2007). Other copulatory plug proteins, including the seminal vesicle secretion 2 (SVS2) protein, fibronectin, lactoferrin, and kallikrein are also produced at high levels by the seminal vesicle (Kawano et al., 2014; Lilja et al., 1987). Transglutaminase IV is essential for normal plug formation in mice, and its deficiency leads to infertility despite normal sperm count, sperm motility, and reproductive morphology since without a plug, few sperm are retained in the female tract after mating (Dean, 2013). SVS2 has been shown to be essential for normal fertility in mice through a role in sustaining sperm survival in the uterus (Kawano et al., 2014).

Considered as markers of human seminal vesicle function by the World Health Organization, fructose and prostaglandins are secreted at high levels by the seminal vesicle (WHO, 2010). Fructose is the primary sugar produced by the seminal vesicles with the levels of fructose reflecting the rate of production of secretory exudate (WHO, 2010; Mann and Lutwak-Mann, 1976). Fructose is

consumed by sperm following ejaculation and is used as the primary source of energy by spermatozoa as they traverse the female tract (Gonzales, 2001).

Prostaglandins are present in the seminal plasma of most vertebrate species (Schjenken and Robertson, 2014; Gonzales, 2001). Prostaglandins are found in all accessory sex glands, but in human semen are predominantly derived from the seminal vesicles (Gerozissis et al., 1982). These hormones are thought to enhance sperm motility as well as play a role in immune regulation in the female reproductive tissues, with prostaglandins being present in the seminal plasma of a number of species (Schjenken and Robertson, 2014; Gonzales, 2001).

Other seminal vesicle secreted proteins, including MHS-5 and protein C inhibitor (PCI) have also been used to assess seminal vesicle function (Calderon et al., 1994; Kise et al., 2000). For example, PCI has been used as a marker of seminal vesicle obstruction or agenesis (Kise et al., 2000).

Similarities and Differences Between Species in Seminal Vesicle Biology

There is large variation in the size and structure of the male accessory sex glands between mammalian species, and the composition and balance of their secreted proteins and other bioactive factors. In most species the seminal vesicle are the major contributor to ejaculate volume (McGraw et al., 2015). For example in humans seminal vesicle secretions comprise between 70% and 85% of the ejaculate, while in mice the seminal vesicle glands contribute approximately 90% of the ejaculate (Kierszenbaum and Tres, 2011). In contrast, the most prominent accessory sex gland in boars are the bulbourethral glands, while canine and feline species appear to lack seminal vesicles altogether (McGraw et al., 2015). The extensive differences between even closely related species in accessory glands is consistent with the considerable variation seen in male sex organs more broadly. This is viewed as evidence of rapid evolution in these tissues and functional alignment with reproductive strategy. For example, polyandrous rodent species invest more resources and often evolve larger and more elaborate male reproductive organs than related species where between-male competition is lower (Eberhard, 2009).

Despite the broadly conserved function of seminal plasma constituents from insects to mammals, there is substantial variation in seminal vesicle fluid composition between species (McGraw et al., 2015; Poiani, 2006). For example, boar seminal vesicles secrete both fructose and citric acid which in the human, are made in both the prostate and seminal vesicle (Mann, 1964), while the BSP proteins which are produced in seminal vesicles in the bull and contribute to sperm capacitation and sperm storage are produced by the epididymis in mice and humans (Lefebvre et al., 2007; Suarez, 2008). In camels and likely other induced ovulatory species, seminal vesicle secreted beta nerve growth factor acts following coitus to stimulate ovulation (Ratto et al., 2012).

Function of Seminal Vesicle Secretions on Sperm Survival

A central function of seminal vesicle secretions is to support spermatozoa as they transit through the male genital tract and subsequently the female tract to fertilize the oocyte. This occurs through factors that provide nutrient and energy requirements, protect sperm from oxidative stress and immune destruction, enable capacitation, and facilitate storage when immediate fertilization is not possible.

One key sperm survival factor is fructose, produced by the seminal vesicles and present in high concentrations in male seminal fluid. Following ejaculation, motile spermatozoa utilize fructose as their primary energy source for metabolism and motility (Schoenfeld et al., 1979). For this reason, the concentration of fructose in human seminal fluid is used as a surrogate marker of seminal vesicle gland function, as recommended by WHO guidelines for the processing and evaluation of human semen (WHO, 2010).

Sperm motility is positively influenced by the presence of prostaglandins, particularly the 19-hydroxylated forms of prostaglandin E (19OH-PGE1 and 19OH-PGE2), which are present at extraordinarily high concentrations in human seminal fluid and produced predominantly by the seminal vesicles (Aitken and Kelly, 1985). Prostaglandins may assist in protecting the male gametes from oxidative stress and inflammatory damage. The abundance of antioxidants produced by the seminal vesicle glands also act to mitigate against potential damage to paternal DNA from oxidative stress. Additional substances indicated as able to stimulate sperm motility include potassium, magnesium, bicarbonate, and prolactin, all of which are secreted products of the seminal vesicle glands (Gonzales, 2001).

An important event to occur at ejaculation is coagulation, the process whereby ejaculated semen immediately forms a gelatinous state. This process is mediated largely by the cross-linking of the seminal vesicle produced proteins Semenogelin (SEMG) 1 and 2 (Yamasaki et al., 2017). Coagulation is thought to be important in ensuring prolonged contact of spermatozoa with other seminal fluid components, as well as in aiding the retention of seminal fluid in the female tract and preventing the premature capacitation of sperm. An additional role of coagulation is in transiently restricting sperm motility by trapping them in the gelatinous mesh network, a process believed to be mediated by a fragment of the degraded SEMGs, the seminal plasma motility inhibitor, SPMI (Yamasaki et al., 2017). Premature acquisition of fertilizing capacity outside of the female tract is prevented by the seminal vesicle proteins, seminal vesicle secretion (SVS) 2, 3, and 4 (Kawano et al., 2014). Some 20 min or so after ejaculation, coagulated seminal fluid undergoes the process of liquefaction, whereby seminal vesicle-derived proteases degrade SEMGs and their by-products (including SPMI).

Once sperm pass through the uterine cervix, they acquire the ability to fertilize the oocyte, through a maturation process known as capacitation. Capacitation confers to sperm both enhanced motility and the ability to undergo the acrosome reaction. Recent studies have identified soluble CD38 (sCD38), produced by the seminal vesicle gland and released into the seminal fluid, as a critical regulator of sperm capacitation in mice and humans (Kim et al., 2015a), mediating its effects through interacting with CD31 present on sperm.

Inorganic moieties released by the seminal vesicle that contribute to supporting sperm function include potassium, bicarbonate, magnesium, prolactin, ascorbic acid, and zinc ligands (Gonzales, 2001). Potassium, bicarbonate, magnesium and prolactin all act to enhance sperm motility (Gonzales, 2001) while ascorbic acid operates in association with antioxidant defenses to reduce injury mediated by oxygen radicals and preserve the functional competence of sperm (Lewis et al., 1997). Zinc ligands secreted by seminal vesicle fluid regulates chromatin stability by reducing sperm chromatin zinc content (Bjorn Dahl and Kvist, 1990).

Male to Female Signaling Factors Produced in the Seminal Vesicle

Male seminal fluid regulation of female reproductive physiology and behavior is well-described in the insect kingdom (Gillott, 2003). For instance in *Drosophila*, male factors stimulate egg release, induce antimicrobial protein synthesis, and influence brain function to decrease female receptivity to other males (Ram and Wolfner, 2007). Successful fertilization and transmission of the male germ line to the next generation depend on the male's capacity to induce an adequate response in the female. The female is an active participant in this exchange—the information provided in seminal fluid is subject to “cryptic female choice,” a process by which females interrogate the reproductive fitness of the prospective male partners and invest reproductive resources accordingly (Roldan et al., 1992). Substantial male resource investment is placed in promoting the male's chance of successful fertilization of females, and drives a high rate of evolution in male accessory gland function. This process ensures optimal female reproductive investment and maximal progeny fitness (Eberhard, 2009).

Less is known about the male to female communicating function of seminal fluid and its consequences in mammals, where seminal fluid has generally been considered to have one biological function—providing the male gametes to fertilize the oocyte at conception. However a wide range of studies now clearly demonstrate that seminal fluid activates female responses in humans, mice and other animals including pigs, dogs and sheep, and that the seminal vesicle is the source of the key signaling agents mediating this interaction (Gillott, 2003; Schjenken and Robertson, 2014).

A key function of the seminal vesicle immune-regulatory factors is to modulate the female immune response, so that foreign male histocompatibility antigens can be accommodated and not rejected. This suppresses generation of effector immunity against sperm antigens and induces the female immune system to tolerate the presence of male transplantation proteins, or “histocompatibility” antigens. Because these same histocompatibility antigens are also present on embryos fertilized by the male, the immune response to seminal fluid effectively “primes” the female immune response to maintain tolerance and facilitate survival of embryos sired by that male.

Seminal Vesicle Immune Regulatory Factors

Amongst the key factors that mediate immune regulatory function is transforming growth factor beta, which is synthesized in the seminal vesicles in a latent form and then becomes activated either when the accessory gland secretions mix at ejaculation (e.g., in the pig), or in the female reproductive tract following coitus (e.g., in human) (Robertson et al., 2002; Tremellen et al., 1998). The three isoforms of TGFB (TGFB1, TGFB2, and TGFB3) are produced by the seminal vesicle and released into the seminal plasma of mice (Tremellen et al., 1998), humans (Tremellen et al., 1998) and every mammalian species so far examined, at extremely high concentrations ranging from ~200–1000 ng/mL. This concentration is unmatched in any other biological fluid other than possibly colostrum, where potent immune-regulatory function is required to induce oral tolerance in infants (Robertson et al., 2002).

Additionally in humans, 19-OH PGE1 and 19-OH PGE2 are potent immune-regulatory seminal fluid factors, through direct effects on dendritic cells (Gerozissis et al., 1982). In mice, CD38 has been identified as a third key regulator of the female immune environment following coitus (Kim et al., 2015b) and it also acts to enhance sperm capacitation in humans (Kim et al., 2015a). Whether CD38 has a similar immune regulatory role in humans is not yet clear.

Actions of Seminal Vesicle Immune Regulatory Factors in Promoting Reproductive Success

In mammalian species, including rodents and humans, the permissive immune response to seminal fluid commences when seminal vesicle derived immune regulatory factors are deposited in the female tract at coitus. TGFB interacting with 19-OH PGE and other agents binds to receptors on cervical or uterine epithelial cells (depending on the species and site of seminal fluid deposition) and elicits an inflammation-like response within the female tissues. The seminal fluid contact induces dramatic changes in cytokine gene expression that cause rapid recruitment of immune cell populations into the local tissues.

This immune response has been extensively characterized in mice where seminal fluid exposure results in induction of maternal immune tolerance to paternal transplantation antigens later expressed by the developing fetus and placenta

(Robertson et al., 2009). Many of the cytokines released by epithelial cells lining the female tract also act to directly promote embryo development and facilitate successful implantation and robust placental development (Robertson and Sharkey, 2016). Some of the beneficial effects of seminal fluid exposure are mediated through the stimulation of an immune cell type known as regulatory T cells (Treg cells), which react with paternal transplantation antigens in seminal fluid and later expressed by the conceptus, ensuring optimal immune tolerance for pregnancy is established (Guerin et al., 2011).

Similar immune priming events are activated within the cervical tissues of women after the introduction of seminal fluid at coitus (Robertson and Sharkey, 2016). Early investigations into this response revealed an efflux of neutrophils into cervical mucus following donor insemination with whole semen (Pandya and Cohen, 1985; Thompson et al., 1992). Neutrophils recruited into the cervical lumen may be important for the clearance of non-fertilizing sperm and other cellular debris as well as for the prevention of infection by potentially harmful pathogens introduced at coitus. It was then demonstrated that vaginal intercourse initiates a similar response, extending into the epithelium and deeper stromal tissue of the ectocervix of periovulatory women (Sharkey et al., 2012a). The major cell types recruited into the cervical tissue were macrophages and dendritic cells, along with a smaller contingent of memory T cells. Leukocyte recruitment is regulated by seminal fluid induction of genes encoding cytokines including CSF2 (GM-CSF), IL6, and CXCL8. Importantly, seminal fluid contact was an absolute requirement for the initiation of the inflammatory response as no inflammatory response was observed in women who abstained from intercourse or when seminal fluid contact was prevented by the use of condoms (Sharkey et al., 2012a).

TGFB has been identified as the key mediator of male to female seminal fluid signaling in human (Sharkey et al., 2012b). In men, TGFB is produced mainly by the seminal vesicle and secreted into the seminal fluid at very high concentrations in a predominantly latent, inactive form. TGFB is then activated upon deposition into the female tract via mechanisms including the low pH of the vagina and endogenous protease activity. Once activated, seminal vesicle-derived TGFB induces expression of potent immune-regulatory and pro-tolerance genes CSF2 and IL6 within the cervical tissue (Sharkey et al., 2012b). Prostaglandins are also implicated as mediators of the postcoital inflammatory response in women through inducing the neutrophil regulator CXCL8 (Denison et al., 1999).

Impact of Seminal Vesicle Secretions on Offspring Phenotype

Whether a pregnancy is conceived in the presence of sufficient seminal fluid secretions may have a bearing not just on fertility, but on pregnancy outcome. Recent studies in mice show that if pregnancies are initiated in the absence of seminal vesicle secretions after surgical removal of the male seminal vesicle glands, fertility and fecundity is reduced and also the resultant offspring show signs of growth restriction and altered metabolic phenotype (Bromfield et al., 2014). This implies that the father's contribution to pregnancy goes far beyond the provision of male gametes at conception and that molecules carried by the male ejaculate contribute to fetal development and programming of lifelong health (Lane et al., 2014).

However, seminal plasma is not mandatory for pregnancy success, as pregnancies are initiated using assisted reproductive technologies like artificial insemination (AI) where seminal plasma is washed away, and in vitro fertilization (IVF) where conception may occur without any male contact. Rather, evidence suggests that the quality of pregnancy and health of offspring may be compromised if conception occurs in the absence of seminal plasma. Evidence for this can be seen in several species with recent experiments in mice and golden hamsters highlighting the importance of seminal vesicle secretions for reproductive outcomes (Bromfield et al., 2014; Sjoblom et al., 2005; Wong et al., 2007). In these studies, excision of the seminal vesicles profoundly affects fertility and health outcomes in offspring with reduced fertility, delayed embryo development, and evidence of metabolic dysfunction (Bromfield et al., 2014). The reduction in fertility in the absence of seminal vesicles can be partly explained by oxidative damage to sperm. However the detrimental programming effects are also attributed to a lack of priming by the seminal vesicle signaling agents, as embryos derived from females exposed to seminal vesicle fluid and transferred to females not exposed to seminal vesicle fluid exhibit similar phenotypes (Bromfield et al., 2014). Consistent with an impact of seminal vesicle secretions, excision of all accessory sex glands in the golden hamster leads to a similar reduction in offspring fitness to that observed in mice (Wong et al., 2007).

Further evidence for seminal vesicle secretions contributing to the quality of pregnancy and developmental programming are seen in other mouse studies. Exogenous TGFB administered at mating to boost seminal fluid can rescue pregnancy loss in abortion-prone CBA/J $\times$ DBA/2 mouse matings, and a mechanism involving Treg cells is demonstrated (Clark et al., 2008).

Seminal Vesicle Secretions and Pregnancy Disorders in Women

Seminal fluid contact prior to conception is associated with susceptibility to various pregnancy pathologies including preeclampsia and in utero growth restriction. Pregnancies conceived following longer periods of sexual cohabitation with the conceiving male partner are relatively protected from preeclampsia and in utero growth restriction. Strikingly, clinical studies show that a change in male partner or use of barrier methods of contraception elevates the risk of preeclampsia, implying that exposure to the conceiving partner's seminal fluid contributes to this protection (Kho et al., 2009).

Exposure to seminal plasma from the conceiving partner around the period of embryo transfer in women undergoing IVF treatment is demonstrated to improve conception rate (Crawford et al., 2015; Tremellen et al., 2000). While a direct causal link has been

demonstrated in humans between seminal vesicle immune regulatory molecules and the benefits of seminal fluid contact, it has been postulated that the mechanisms that link prepregnancy coital activity and improved pregnancy outcomes are associated with an immune priming response, consistent with the Treg cell response demonstrated in mice (Robertson and Sharkey, 2016).

Environmental Regulation of Seminal Vesicle Function

There is increasing evidence that environmental exposures can impact both male and female reproductive capacity and the health of offspring (Lane et al., 2014). Nutritional perturbations and exposure to environmental chemicals can all compromise the quality of the male ejaculate and appear to impart consequences for offspring. This may be caused by changes in genetic and epigenetic marks in sperm (Lane et al., 2014; Fullston et al., 2013; Rissman and Adli, 2014), but there is also a prospect of effects on the immune priming capacity of seminal fluid, and particularly the secretory capacity of the seminal vesicles and the composition of seminal plasma.

Very little is known about the impact of the paternal environment on the composition of seminal vesicle secretions. There are some studies which demonstrate variability amongst seminal vesicle derived components in the seminal plasma. In humans for example, seminal plasma TGFB varies considerably between individuals, correlating inversely with age and is markedly increased in individuals with chronic HIV infection (Sharkey et al., 2016; Kafka et al., 2012). Additionally, levels of the seminal vesicle marker fructose are positively correlated with BMI (Martini et al., 2010) while seminal plasma prostaglandin levels are related to sperm DNA fragmentation and infertility (Intasqui et al., 2016). There is also evidence of fluctuations within men over time in TGFB and other seminal plasma components (Sharkey et al., 2016, 2017). These studies imply that changing paternal environment including nutrition, chemical exposures and the reproductive tract microbiome can affect seminal vesicle secretions, but further studies are required to define the mechanisms through which this occurs.

Conclusions

The simplistic view that seminal plasma acts just as a transport medium for sperm is now redundant with studies demonstrating key roles for seminal plasma molecules to support sperm function prior to conception and to create a postcopulatory environment that influences fertilization, embryo and fetal development and the health of the offspring (Schjenken and Robertson, 2014). Amongst the accessory sex glands, the seminal vesicles are a major contributor to composition of seminal plasma in most species, including humans. Despite the critical role that seminal vesicle secretions play around the period of conception and throughout pregnancy, there is limited information on the secretome of the seminal vesicles and the specific contributions of the different seminal vesicle secreted molecules.

Studies into the secretory capacity of seminal fluid may identify environmental exposures that alter the composition of seminal vesicle secretions and thus impact male and female reproductive capacity. State of the art proteomic techniques to characterize the seminal vesicle secretome of various species are required to identify the key conserved factors in addition to the species specific factors that contribute to function. More detailed knowledge of how seminal vesicle secretions are regulated and their biological effects will advance understanding of this important area of biology and may reveal more key functions for this organ in male and female reproductive health.

References

- Aitken, R. J., & Kelly, R. W. (1985). Analysis of the direct effects of prostaglandins on human sperm function. *Journal of Reproduction and Fertility*, 73, 139–146.
- Aumuller, G., & Riva, A. (1992). Morphology and functions of the human seminal vesicle. *Andrologia*, 24, 183–196.
- Bjorndahl, L., & Kvist, U. (1990). Influence of seminal vesicular fluid on the zinc content of human sperm chromatin. *International Journal of Andrology*, 13, 232–237.
- Brandes, D. (1974). *Fine structure and cytochemistry of male accessory sex organs*. New York, NY: Academic Press Inc.
- Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J., & Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 2200–2205.
- Calderon, I., Barak, M., Abramovici, H., Gruener, N., Yavez, H., Paz, G., & Homonnai, Z. T. (1994). The use of a seminal vesicle specific protein (MHS-5 antigen) for diagnosis of agenesis of vas deferens and seminal vesicles in azoospermic men. *Journal of Andrology*, 15, 603–607.
- Clark, D. A., Fernandes, J., & Banwatt, D. (2008). Prevention of spontaneous abortion in the CBA x DBA/2 mouse model by intravaginal TGF-beta and local recruitment of CD4+8+ FOXP3+ cells. *American Journal of Reproductive Immunology*, 59, 525–534.
- Crawford, G., Ray, A., Gudi, A., Shah, A., & Homburg, R. (2015). The role of seminal plasma for improved outcomes during in vitro fertilization treatment: Review of the literature and meta-analysis. *Human Reproduction Update*, 21, 275–284.
- Dean, M. D. (2013). Genetic disruption of the copulatory plug in mice leads to severely reduced fertility. *PLoS Genetics*, 9, e1003185.
- Denison, F. C., Grant, V. E., Calder, A. A., & Kelly, R. W. (1999). Seminal plasma components stimulate interleukin-8 and interleukin-10 release. *Molecular Human Reproduction*, 5, 220–226.
- Duncan, M. W., & Thompson, H. S. (2007). Proteomics of semen and its constituents. *Proteomics. Clinical Applications*, 1, 861–875.
- Eberhard, W. G. (2009). Postcopulatory sexual selection: Darwin's omission and its consequences. *Proceedings of the National Academy of Sciences of the United States of America*, 106(Suppl 1), 10025–10032.
- Fullston, T., Ohlsson Teague, E. M., Palmer, N. O., DeBlasio, M. J., Mitchell, M., Corbett, M., Print, C. G., Owens, J. A., & Lane, M. (2013). Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *The FASEB Journal*, 27, 4226–4243.

- Gerozissis, K., Jouannet, P., Soufir, J. C., & Dray, F. (1982). Origin of prostaglandins in human semen. *Journal of Reproduction and Fertility*, 65, 401–404.
- Gillott, C. (2003). Male accessory gland secretions: Modulators of female reproductive physiology and behavior. *Annual Review of Entomology*, 48, 163–184.
- Gonzales, G. F. (2001). Function of seminal vesicles and their role on male fertility. *Asian Journal of Andrology*, 3, 251–258.
- Guerin, L. R., Moldenhauer, L. M., Prins, J. R., Bromfield, J. J., Hayball, J. D., & Robertson, S. A. (2011). Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. *Biology of Reproduction*, 85, 397–408.
- Intasqui, P., Camargo, M., Antonias, M. P., Cedenho, A. P., Carvalho, V. M., Cardozo, K. H. M., Zylbersztein, D. S., & Bertolla, R. P. (2016). Association between the seminal plasma proteome and sperm functional traits. *Fertility and Sterility*, 105, 617–628.
- Kafka, J. K., Sheth, P. M., Nazli, A., Osborne, B. J., Kovacs, C., Kaul, R., & Kaushic, C. (2012). Endometrial epithelial cell response to semen from HIV-infected men during different stages of infection is distinct and can drive HIV-1-long terminal repeat. *AIDS*, 26, 27–36.
- Kawano, N., Araki, N., Yoshida, K., Hibino, T., Ohnami, N., Makino, M., Kanai, S., Hasuwa, H., Yoshida, M., Miyado, K., & Umezawa, A. (2014). Seminal vesicle protein SVS2 is required for sperm survival in the uterus. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 4145–4150.
- Kho, E. M., McCowan, L. M., North, R. A., Roberts, C. T., Chan, E., Black, M. A., Taylor, R. S., Dekker, G. A., & Consortium, S. (2009). Duration of sexual relationship and its effect on preeclampsia and small for gestational age perinatal outcome. *Journal of Reproductive Immunology*, 82, 66–73.
- Kierszenbaum, A., Tres, L. Sperm transport and maturation. In: Mosby (ed.) *Histology and cell biology: An introduction to pathology*. St. Louis, MO, Elsevier; 2011: 624.
- Kim, B. J., Park, D. R., Nam, T. S., Lee, S. H., & Kim, U. H. (2015a). Seminal CD38 enhances human sperm capacitation through its interaction with CD31. *PLoS One*, 10, e0139110.
- Kim, B. J., Choi, Y. M., Rah, S. Y., Park, D. R., Park, S. A., Chung, Y. J., Park, S. M., Park, J. K., Jang, K. Y., & Kim, U. H. (2015b). Seminal CD38 is a pivotal regulator for fetomaternal tolerance. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 1559–1564.
- Kise, H., Nishioka, J., Satoh, K., Okuno, T., Kawamura, J., & Suzuki, K. (2000). Measurement of protein C inhibitor in seminal plasma is useful for detecting agenesis of seminal vesicles or the vas deferens. *Journal of Andrology*, 21, 207–212.
- Lane, M., Robker, R. L., & Robertson, S. A. (2014). Parenting from before conception. *Science*, 345, 756–760.
- Lee, C., Keefer, M., Zhao, Z. W., Kroes, R., Berg, L., Liu, X. X., & Sensibar, J. (1989). Demonstration of the role of prostate-specific antigen in semen liquefaction by two-dimensional electrophoresis. *Journal of Andrology*, 10, 432–438.
- Lefebvre, J., Fan, J., Chevalier, S., Sullivan, R., Carmona, E., & Manjunath, P. (2007). Genomic structure and tissue-specific expression of human and mouse genes encoding homologues of the major bovine seminal plasma proteins. *Molecular Human Reproduction*, 13, 45–53.
- Lewis, S. E., Sterling, E. S., Young, I. S., & Thompson, W. (1997). Comparison of individual antioxidants of sperm and seminal plasma in fertile and infertile men. *Fertility and Sterility*, 67, 142–147.
- Lilja, H., Oldbring, J., Rannevik, G., & Laurell, C. B. (1987). Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. *The Journal of Clinical Investigation*, 80, 281–285.
- Mann, T. (1964). *The biochemistry of semen and the male reproductive tract*. New York, NY: John Wiley and Sons, Inc.
- Mann, T., & Lutwak-Mann, C. (1976). Evaluation of the functional state of male accessory glands by the analysis of seminal plasma. *Andrologia*, 8, 237–242.
- Martini, A. C., Tissera, A., Estofan, D., Molina, R. I., Mangeaud, A., de Cuneo, M. F., & Ruiz, R. D. (2010). Overweight and seminal quality: A study of 794 patients. *Fertility and Sterility*, 94, 1739–1743.
- McGraw, L. A., Suarez, S. S., & Wolfner, M. F. (2015). On a matter of seminal importance. *BioEssays*, 37, 142–147.
- Pandya, I. J., & Cohen, J. (1985). The leukocytic reaction of the human uterine cervix to spermatozoa. *Fertility and Sterility*, 43, 417–421.
- Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioral Ecology and Sociobiology*, 60, 289–310.
- Ram, K. R., & Wolfner, M. F. (2007). Sustained post-mating response in *Drosophila melanogaster* requires multiple seminal fluid proteins. *PLoS Genetics*, 3, e238.
- Ratto, M. H., Leduc, Y. A., Valderrama, X. P., van Straaten, K. E., Delbaere, L. T., Pierson, R. A., & Adams, G. P. (2012). The nerve of ovulation-inducing factor in semen. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 15042–15047.
- Rissman, E. F., & Adli, M. (2014). Minireview: Transgenerational epigenetic inheritance: Focus on endocrine disrupting compounds. *Endocrinology*, 155, 2770–2780.
- Robertson, S. A., & Sharkey, D. J. (2016). Seminal fluid and fertility in women. *Fertility and Sterility*, 106, 511–519.
- Robertson, S. A., Ingman, W. V., O'Leary, S., Sharkey, D. J., & Tremellen, K. P. (2002). Transforming growth factor beta—A mediator of immune deviation in seminal plasma. *Journal of Reproductive Immunology*, 57, 109–128.
- Robertson, S. A., Guerin, L. R., Moldenhauer, L. M., & Hayball, J. D. (2009). Activating T regulatory cells for tolerance in early pregnancy—The contribution of seminal fluid. *Journal of Reproductive Immunology*, 83, 109–116.
- Roldan, E. R., Gomendio, M., & Vitullo, A. D. (1992). The evolution of eutherian spermatozoa and underlying selective forces: Female selection and sperm competition. *Biological Reviews of the Cambridge Philosophical Society*, 67, 551–593.
- Rui, H., Mevag, B., & Purvis, K. (1984). Two-dimensional electrophoresis of proteins in various fractions of the human split ejaculate. *International Journal of Andrology*, 7, 509–520.
- Schjenken, J. E., & Robertson, S. A. (2014). Seminal fluid and immune adaptation for pregnancy—Comparative biology in mammalian species. *Reproduction in Domestic Animals*, 49(Suppl 3), 27–36.
- Schoenfeld, C., Amelar, R. D., Dubin, L., & Numeroff, M. (1979). Prolactin, fructose, and zinc levels found in human seminal plasma. *Fertility and Sterility*, 32, 206–208.
- Sharkey, D. J., Tremellen, K. P., Jasper, M. J., Gemzell-Danielsson, K., & Robertson, S. A. (2012a). Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *Journal of Immunology*, 188, 2445–2454.
- Sharkey, D. J., Macpherson, A. M., Tremellen, K. P., Mottershead, D. G., Gilchrist, R. B., & Robertson, S. A. (2012b). TGF-beta mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. *Journal of Immunology*, 189, 1024–1035.
- Sharkey, D. J., Tremellen, K. P., Briggs, N. E., Dekker, G. A., & Robertson, S. A. (2016). Seminal plasma transforming growth factor-beta, activin a and follistatin fluctuate within men over time. *Human Reproduction*, 31, 2183–2191.
- Sharkey, D. J., Tremellen, K. P., Briggs, N. E., Dekker, G. A., & Robertson, S. A. (2017). Seminal plasma pro-inflammatory cytokines interferon-gamma (IFN) and C-X-C motif chemokine ligand 8 (CXCL8) fluctuate over time within men. *Human Reproduction*, 1–9.
- Sjoberg, C., Roberts, C. T., Wikland, M., & Robertson, S. A. (2005). Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology*, 146, 2142–2153.
- Suarez, S. S. (2008). Regulation of sperm storage and movement in the mammalian oviduct. *The International Journal of Developmental Biology*, 52, 455–462.
- Thompson, L. A., Barratt, C. L., Bolton, A. E., & Cooke, I. D. (1992). The leukocytic reaction of the human uterine cervix. *American Journal of Reproductive Immunology*, 28, 85–89.
- Tremellen, K. P., Seamark, R. P., & Robertson, S. A. (1998). Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. *Biology of Reproduction*, 58, 1217–1225.
- Tremellen, K. P., Valbuena, D., Landers, J., Ballesteros, A., Martinez, J., Mendoza, S., Norman, R. J., Robertson, S. A., & Simon, C. (2000). The effect of intercourse on pregnancy rates during assisted human reproduction. *Human Reproduction*, 15, 2653–2658.
- WHO. (2010). *WHO laboratory manual for the examination and processing of human semen*. Geneva: World Health Organization.
- Wong, C. L., Lee, K. H., Lo, K. M., Chan, O. C., Goggins, W., WS, O., & Chow, P. H. (2007). Ablation of paternal accessory sex glands imparts physical and behavioural abnormalities to the progeny: An in vivo study in the golden hamster. *Theriogenology*, 68, 654–662.
- Yamasaki, K., Yoshida, K., Yoshiike, M., Shimada, K., Nishiyama, H., Takamizawa, S., Yanagida, K., & Iwamoto, T. (2017). Relationship between Semenogelins bound to human sperm and other semen parameters and pregnancy outcomes. *Basic and Clinical Andrology*, 27, 15.

The Seminal Vesicles: Endocrinological Aspects

Sandro La Vignera, Rosita A Condorelli, Giorgio I Russo, Giuseppe Morgia, and Aldo E Calogero, University of Catania, Catania, Italy

© 2018 Elsevier Inc. All rights reserved.

Sex differentiation and growth of seminal vesicles (SVs) are highly dependent on androgen levels. In this context, the secretory activity of the seminal vesicles in men are dependent by the endogenous increase of serum testosterone. In a rat model it has been showed that the increase in serum testosterone or treatment with androgens was associated with greater secretory activity of the seminal vesicles and increased seminal vesicle weight (Higgins and Burchell, 1978). It is generally known that the seminal vesicles possess 5- α -reductase activity, which converts testosterone to dihydrotestosterone, the active hormone (Gonzales, 2001). The secretory activity of the seminal vesicle is also regulated by the nervous system and both the cholinergic and adrenergic neurons affect its function. Muscarinic stimulus (cholinergic) increases nitric oxide production (Gonzales, 2001). Seminal vesicle is a source of nitric oxide synthase in men and previous data showed that the increase in nitric oxide may be associate with better secretion of fructose by the gland (Ehren et al., 1997).

Since seminal vesicles are considered as androgen-dependent, their characteristics may be used as biological marker of androgenic activity. Gonzales has developed a method to assess the androgen activity at the reproductive tract level based on both the serum testosterone level and the corrected seminal fructose level under the basal and the post-clomiphene stimulation. Men received 100 mg clomiphene citrate daily for 5 days. The second blood and semen samples were obtained one day after the last administration of clomiphene. Levels of serum testosterone were more related to the corrected seminal fructose than to seminal fructose concentration. Seventy-one percent of subjects with low serum testosterone levels had low levels of corrected seminal fructose, whereas only 28% of the same subjects had low levels of seminal fructose (uncorrected fructose) (Gonzales and Villena, 2001). The results suggests that corrected seminal fructose may be used as a biological marker of androgen activity in the reproductive tract.

Based on these premises, corrected seminal fructose measurement may be considered as the best marker of seminal vesicle function (Gonzales, 2002). The combined measurement of the serum testosterone level and the level of corrected seminal fructose allows the diagnosis of hypoandrogenism and/or obstructive process at the reproductive tract (Gonzales, 2001). An increase in the serum testosterone after clomiphene stimulation without increase in the corrected or true corrected seminal fructose suggests an obstructive process at the reproductive tract level, whereas absence of increase in serum testosterone and corrected/true corrected seminal fructose levels may indicate hypogonadism (Gonzales, 2002).

The relationship between serum levels of testosterone and seminal fructose levels has been previously analyzed by Gonzales et al.

In fact, it can be found normal levels of serum testosterone and low values of seminal fructose in men with azoospermia due to obstruction of the ejaculatory ducts. It should be noted in fact that one third of azoospermia and severe oligozoospermia have an obstruction of the ejaculatory duct (Gonzales, 2002).

It should be also point out that about 10% of azoospermic subjects may have congenital absence of the seminal vesicles and vas deferens. In this case, the diagnostic criteria are: low seminal volume (<0.5 mL), low pH and low concentration of seminal fructose with normal serum FSH levels. Diagnosis is also possible through measuring the seminal level of the specific protein of the seminal vesicle (MHS-5) (Calderon et al., 1994). This protein is absent in agenesis of the seminal vesicle. This is another useful marker for agenesis of seminal vesicles and/or the vas deferens.

Despite that increased serum testosterone level is related to seminal vesicle function, treatment with high doses of testosterone propionate results in diminished levels of secretions of seminal vesicles and prostate, probably due to down regulation.

SVs are responsible for the production of ejaculate and the major component of this liquid are semenogelin I, Alkaline fluid, Fructose, Prostaglandins, Clotting factors.

The remaining volume of semen is made up of testicular spermatozoa, prostatic secretions and mucus from the bulbourethral gland (La Vignera et al., 2013; La Vignera et al., 2011).

Several evidences have demonstrated a close link between diabetes mellitus (DM) and seminal vesicles function. In particular, our previous study showed that SVs of infertile patients with type 2 DM and DN had particular characteristics on ultrasound, as increase in body antero-posterior diameter, fundus antero-posterior diameter and in fundus/body ratio. SV dysfunction is one of the factors thought to contribute to male infertility due to SV dysfunction may relay to inflammation (La Vignera et al., 2011b). In particular, the seminal vesicles of diabetic patients are characterized by reduced or absent modification of the anterior-posterior diameter (evaluated with ultrasound examination) after ejaculation; the same parameter in healthy men has an mean reduction of about 3–4 mm.

Considering the contribution of seminal vesicles to the total ejaculation volume, it is clear that from the endocrinological point of view, the differential diagnosis of the hypospermia (reduction of ejaculate volume <1.5 mL) concerns the following conditions: hypogonadism, obstruction of ejaculatory ducts and the retro-ejaculation (Roberts and Jarvi, 2009). In diabetic patients, these three conditions are frequent due to the high frequency of hypogonadism, male accessory gland infection and neuropathy (La Vignera et al. 2011a; Condorelli et al., 2014).

Take home messages

- Sex differentiation and growth of seminal vesicles (SVs) are highly dependent on androgen levels. In this context, the secretory activity of the seminal vesicles in men are dependent by the endogenous increase of serum testosterone;
- Corrected seminal fructose measurement may be considered as the best marker of seminal vesicle function;
- Several evidences have demonstrated a close link between diabetes mellitus (DM) and seminal vesicles function. In particular, our previous study showed that SVs of infertile patients with type 2 DM and DN had particular characteristics on ultrasound.

References

- Calderon, I., Barak, M., Abramovici, H., Gruener, N., Yavez, H., Paz, G., & Homonnai, Z. T. (1994). The use of a seminal vesicle specific protein (MHS-5 antigen) for diagnosis of agenesis of vas deferens and seminal vesicles in azoospermic men. *Journal of Andrology*, 15, 603–607.
- Condorelli, R. A., Vicari, E., Calogero, A. E., & La Vignera, S. (2014). Male accessory gland inflammation prevalence in type 2 diabetic patients with symptoms possibly reflecting autonomic neuropathy. *Asian Journal of Andrology*, 16, 761–766.
- Ehren, I., Sjostrand, N. O., Hammarstrom, M., & Wiklund, N. P. (1997). Is glandular formation of nitric oxide a prerequisite for muscarinic secretion of fructose in the guinea-pig seminal vesicle? *Urological Research*, 25, 433–438.
- Gonzales, G. F. (2001). Function of seminal vesicles and their role on male fertility. *Asian Journal of Andrology*, 3, 251–258.
- Gonzales, G. F. (2002). Basal serum testosterone as an indicator of response to clomiphene treatment in human epididymis, seminal vesicles and prostate. *Andrologia*, 34, 308–316.
- Gonzales, G. F., & Villena, A. (2001). True corrected seminal fructose level: a better marker of the function of seminal vesicles in infertile men. *International Journal of Andrology*, 24, 255–260.
- Higgins, S. J., & Burchell, J. M. (1978). Effects of testosterone on messenger ribonucleic acid and protein synthesis in rat seminal vesicle. *The Biochemical Journal*, 174, 543–551.
- La Vignera, S., Condorelli, R., Vicari, E., D'Agata, R., & Calogero, A. E. (2011a). Diabetes mellitus and sperm parameters. *Journal of Andrology*, 33, 145–153.
- La Vignera, S., Condorelli, R. A., Vicari, E., Tumino, D., Morgia, G., Favilla, V., Cimino, S., & Calogero, A. E. (2013). Markers of semen inflammation: supplementary semen analysis? *Journal of Reproductive Immunology*, 100, 2–10.
- La Vignera, S., Condorelli, R. A., Vicari, E., D'Agata, R., & Calogero, A. E. (2011b). Seminal vesicles and diabetic neuropathy: ultrasound evaluation in patients with couple infertility and different levels of glycaemic control. *Asian Journal of Andrology*, 13, 872–876.
- La Vignera, S., Vicari, E., Condorelli, R. A., D'Agata, R., & Calogero, A. E. (2011). Male accessory gland infection and sperm parameters (review). *International Journal of Andrology*, 34, e330–347.
- Roberts, M., & Jarvi, K. (2009). Steps in the investigation and management of low semen volume in the infertile man. *Canadian Urological Association Journal*, 3, 479–485.

Penis Structure

Geng-Long Hsu and Shih-Ping Liu, Puli Christian Hospital, Nantou County, Taiwan; and National Taiwan University Medical School, Taipei City, Taiwan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Buck's fascia A fibrous fascia encircling the corpora cavernosa and corpus spongiosum. It is regarded as the deeper layer (hence its Latin term, *fascia penis profunda*), and sandwiches erection-related vasculature (the dorsal nerve, dorsal artery, deep dorsal vein, cavernosal veins, para-arterial veins, and lymphatic vessels) against the tunica albuginea. In addition, Buck's fascia is imbued with smooth muscle from the tunica dartos arising from within the scrotum.

Cavernosal vein A specific drainage vein of the corresponding corpus cavernosum via emissary veins; it spans the entire length of the penis except for the penile crus.

Colles' fascia A fibrous layer encircling Buck's fascia and is in continuity with the superficial perineal fascia caudally and to Scarpa's fascia of the abdomen cranially. It further reinforces the integrity of the penile structure and encompasses several superficial dorsal veins, nerves, arteries, and lymphatic vessels.

Corpora cavernosa Two of the three major cylinders which comprise the major erectile bodies of the penis, the corpora cavernosa contain endothelial-lined sinusoids beginning at the mid-glanular level and ending approximately 0.2–0.5 cm away from the ischial tuberosity.

Corpus spongiosum The third cylinder (the other two being the corpora cavernosa) which comprises the major erectile bodies of the penis. Like the corpora cavernosa, the corpus spongiosum contains endothelial-lined sinusoids; however, it also contains the urethra (where urine passes through). It is continuous with the glans penis distally and the prostate gland proximally.

Deep dorsal vein Located at the dorsal groove formed by the corpora cavernosa, the DDV is the common drainage vein of the corpora cavernosa (via emissary veins) and the corpus spongiosum (via circumflex vein).

Distal ligament Grouped from the dorsal outer longitudinal layer of the tunica and extending into the glans penis, the distal ligament acts as a trunk to buttress the glans penis.

Dorsal artery The final branch of the pudendal artery, the dorsal artery is positioned dorsally along the corpora cavernosa. It is the major supplier of blood to the glans penis.

Dorsal nerve Arises from the pudendal nerve to innervate the glans penis, prepuce, and penile skin layer, from which sexual pleasure is derived.

Erectile dysfunction Defined as the inability to either attain or maintain a rigid erection for satisfactory coitus.

Erection-related veins A catch-all term for those veins which are located between the tunica albuginea and Buck's fascia, called as such because these veins are responsible for venous drainage during erection.

Hypospadias A congenital abnormality in which the opening to the urethra (the urethral meatus) is on the underside of the glans penis (distal hypospadias) or (more rarely) nearer the scrotum (proximal hypospadias).

Os penis Refers to the penis bone present in most mammals; humans technically lack an *os penis* but do possess an *os penis* analog: the distal ligament.

Para-arterial veins The veins that sandwich the dorsal artery, para-arterial veins are classified as either *lateral* and *medial* in accordance with their anatomical location.

Penile dysmorphism General term to describe a penis with a deviated or deformed erectile shape. It may be further classified as of a dorsal, ventral, lateral, or combined type.

Penile fibroskeleton Term describing the fibrous collagen tissue which builds up an erection's bone-like rigidity; the major ingredients all possess same histology type and include the tunica albuginea, the distal ligament, the ischiocavernosus muscles, and the bulbospongiosus muscles.

Phimosis A condition in which the penile foreskin cannot be retracted to free the glans.

Tunica albuginea The coat surrounding the corpora cavernosa, the tunica albuginea is the deepest fascia and is composed of inner circular and outer longitudinal collagen fibers in organized arrays interlaced with elastic fibers that form an irregular, latticed network on which the collagen fibers rest.

Embryology of the Penis

Origin of the Gonadal Embryogenesis

In embryogenesis, the gonadal ridge appears at approximately the 5th week of gestation and gives rise to the gonadal cords whereby gender differentiation of the external genitalia occurs between the 7th and 17th weeks. The Y-chromosome directs male

differentiation through the SRY gene, which in turn triggers testicular development from the gonadal cords. Thereafter, endogenous androgens begin to secrete.

Origin of the Penile Relevant Structure

In the cloacal membrane, the caudal region comprises the endoderm and ectoderm, which forms the urethra from urogenital folds whereby the precursor of the external genitalia develops; meanwhile, the cranial region is occupied by mesenchymal mesoderm with which the genital tubercle develops. It subsequently develops into the corpora cavernosa and glans penis under the influence of endogenous androgens such as dihydrotestosterone. On the caudal membrane, the endodermal portion is thought to be tabularized from proximal to distal and forms the urethra; whereas the ectodermal portion forms the penile skin and prepuce.

Human Penile Structure

The Fibroskeleton of the Human Penis

In the entire human body, the penis is uniquely gifted in its capacity to fluctuate between a spongy softness and a bony rigidity. Its fibroskeleton design is indispensable for this variability and requires that the tunica albuginea surrounding the corpora cavernosa play a pivotal role. When cavernosal sinusoids are filled with fluid blood (Fig. 1A), the existence of relevant structures such as the ischiocavernosus muscle, the bulbospongiosus muscle, and the rigid distal ligament in the glans penis are integral for maintaining an erection; absent these structures, the rigidity would most certainly be compromised. The tunica albuginea has been consistently, albeit incorrectly, described as a single layer structure with a uniform circumferential thickness; it is in actuality a bi-layered structure with a 360 degrees inner circular and a 300 degrees outer longitudinal layer, and is the major contributor of the fibroskeleton (Fig. 1A). The thinner collagen bundle of the inner layer is arranged circumferentially and the coarser collagen fibers of the outer

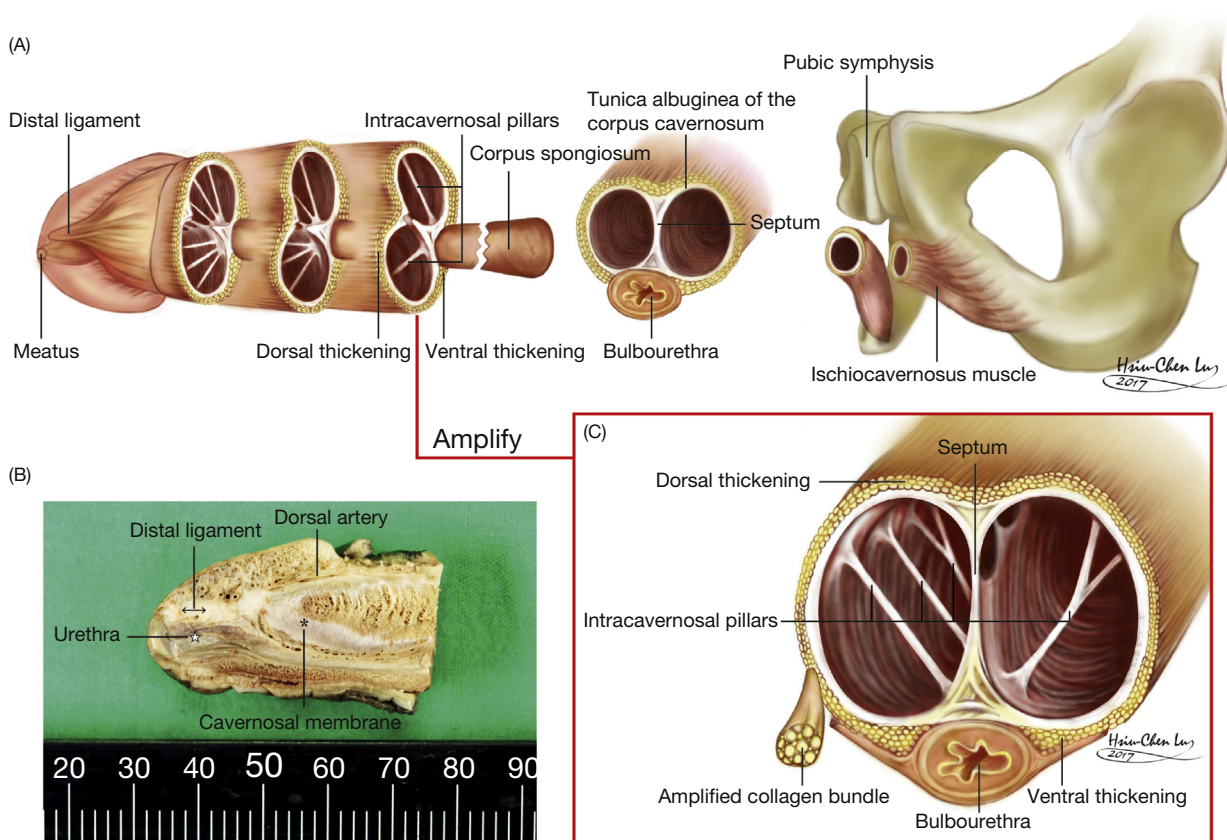


Fig. 1 Schematic illustration of the fibroskeleton of the human penis. (A) The tunica albuginea of the corpora cavernosa is a bi-layered structure in which a 360 degrees complete inner circular layer, together with the intracavernosal pillars, contains and supports the sinusoids, which are removed in this illustration. The inner layer and the 300 degrees incomplete outer longitudinal layer are a continuation of ischiocavernosus and bulbospongiosus muscles, respectively. Both layers are composed of collagen bundles with a longitudinal and circular orientation, respectively. There is an absence of outer layer bundles at the region between the 5 and 7 o'clock positions where the corpora cavernosa are in close contact with the corpus spongiosum. Distally, they are grouped into the glans penis forming the distal ligament, located at the 12 o'clock position of the distal urethra. This structure is indispensable for supporting the glans penis. The median septum is incomplete with dorsal fenestration at the pendulous portion of the penis and is complete where the penile crura are formed. (B) Photo of a distal segment of human penis, lateral sagittal section, presents the left dorsal artery, urethral meatus, inelastic distal ligament (*os penis* analog) and cavernosal membrane, which were not discovered until the 1990s. (C) An amplified illustration of the fibroskeleton in the distal penis which is markedly extensible in length when erect. Note the tunica albuginea and intracavernosal pillars form the major relay structure of the penile fibroskeleton.

layer arrayed longitudinally and extend from the ischiocavernosus and bulbospongiosus muscles. Interestingly, the 360 degrees inner layer encircles a cavernosal membrane (Fig. 1B) to completely contain the erectile sinusoids and—together with the intra-cavernosal pillars—support the cavernosal sinusoids. This membrane protects the delicate sinusoids in much the same way that an amnion protects a fetus.

There is a paucity of outer layer bundles at the region between the clockwise 5 o'clock and 7 o'clock positions. It is here that two structures form; these structures, termed the ventral thickening, are a continuation of the left and right bulbospongiosus muscles, respectively. There is close contact of the corpora cavernosa with the corpus spongiosum between them (Fig. 1C). Between the 11 and 1 o'clock positions on the dorsal aspect, there is also a dorsal thickening of the outer longitudinal tunica, a radiating aspect of the bilateral ischiocavernosus muscles. The continuation of the outer longitudinal layer of the tunica, located at the 12 o'clock position of the distal urethra, is grouped into the glans penis to form the distal ligament—an *os penis* analog. This structure is arranged centrally and acts as a trunk of the glans penis to maintain patency of the distal urethra and preserve the range of ejaculation. At the pendulous portion of the penis the median septum exhibits dorsal fenestration; the extent of fenestration is commensurate with the quantity of the intracavernosal pillars. In the distal penis, the pillars are most numerous where the septum is most incomplete, to allow for tensile capability.

Overall, in the human penis, the components of the skeletal muscles include the ischiocavernosus and bulbospongiosus muscles and their continuing fibroskeleton. The distribution of the tunica albuginea is, however, singularly dependent upon specific anatomical parts in accordance with which a functional requirement exists—take the corpora cavernosa and corpus spongiosum, for instance. Thus, the skeletal ischiocavernosus muscle, bulbospongiosus muscle, and a bi-layered tunica albuginea support and encircle the corpora cavernosa. A mono-layered tunica albuginea contains the corpus spongiosum while the skeletal bulbospongiosus muscle partially encloses it, thereby allowing ejaculation despite erectile rigidity (since tumescence, as opposed to rigidity, is conserved). This is a result of the paucity of the outer longitudinal layer. In the glans penis, however, the bony distal ligament is entrapped by the smooth muscle sinusoids and serves as a kind of trunk, allowing the glans to protect the reflexogenic erectile mechanism—thus rendering it essential to coitus.

Paired Corpora Cavernosa of the Human Penis

Among the three cylinders in the human penis, the most incredible is the paired corpora cavernosa, which contain endothelial-lined sinusoids beginning at the mid-glanular level (Fig. 2A) and ending approximately 0.2–0.5 cm away from the ischial tuberosity via a

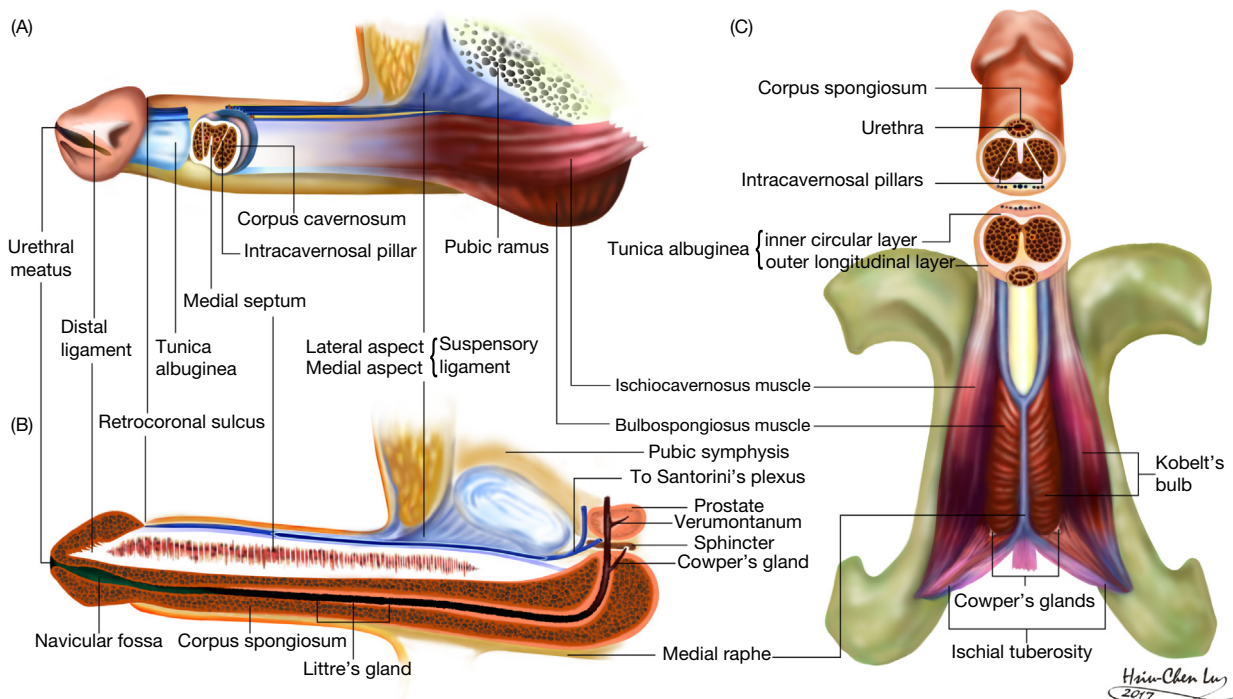


Fig. 2 Schematic illustration of a three-dimensional view of the human penis. (A) *Lateral aspect*. The penis leans upon the suspensory ligament which is an extension of the linea alba. It is capped by the glans penis. Proximally, the corpus spongiosum is held by the bulbospongiosus muscle, in which the fibers are mostly transverse. The corpora cavernosa are surrounded by the tunica albuginea, which is a bi-layered structure (an inner circular and an outer longitudinal layer with multiple sublayers). The intracavernosal pillars (which may be considerably larger distally) are a continuation of the inner circular layer. The corpus cavernosum is entrapped in the ischiocavernosus muscle with the muscle fiber aligned in the longitudinal direction. (B) *Medial aspect*. The distal ligament is aggregated from the collagen bundles of the outer longitudinal layer of the tunica albuginea. It is an inelastic fibrous structure which forms the trunk of the glans penis. The incomplete septum is dorsally fenestrated. The corpus spongiosum contains the urethra. (C) *Ventral aspect*. The three-dimensional structure of the human penis is evident. The ischiocavernosus muscle is paired and situated at the lateral boundary of the perineum. Each segment covers its ipsilateral penile crus. Meanwhile the anterior fibers of the bulbospongiosus muscle partially spread out to encircle the corpus cavernosum and mostly insert into the ventral thickening of the tunica.

skeleton-fibrous ligament. On the pendulous (visible) portion of the penis, the twin corpora communicate freely via a dorsally-fenestrated, incomplete septum; the septum becomes complete nearer the body and toward the penile crura, which are surrounded by ischiocavernosus muscle and relevant tendinous tissues. Elastic-rich intracavernosal pillars run between the 10 and 2 o'clock to 6 o'clock positions; the number of pillars increases distally and is inversely proportional to the completeness of the septum (Fig. 2B). At the beginning portion of each crus lies Kobelt's bulb of the cavernosum, which subsequently tapers toward the proximal end (Fig. 2C). Moreover, the corpora cavernosa are distally very near but proximally divergent, each eventually terminating near its respective ischial tuberosity.

Within the corpora cavernosa, the cavernosal sinusoids are highly trabeculated with fibroelastic, smooth muscle fibers and fibroblast. These fibers are thicker and stronger at the periphery of the cavernosum than at the center, which features diametrically larger cavernous spaces. Within each of the compartments formed by this crisscrossed pattern, numerous blood vessels and nerves flourish. These spaces are lined by flattened endothelial cells similar to those lining blood vessels. The cavernosal sinusoids are supplied with blood from the cavernosal and helicine arteries, which drain into the sinusoidal space via the venules starting at the peripheral sinusoids beneath the tunica albuginea. Free-flowing blood throughout the sinusoidal spaces then converge to emissary veins, which pass blood obliquely via the inner circular and outer longitudinal tunica to the deep dorsal vein (DDV), the cavernosal veins (CVs), and PAVs, which eventually drain to the larger circular system. Absent any veno-occlusive dysfunction, the corpora cavernosa is the most ideal milieu in the entire human body to test Pascal's law. The law states that pressure applied to any part of an enclosed fluid at rest is transmitted undiminished to every portion of the fluid and to the walls of the containing vessel.

Singular Corpus Spongiosum and Glans Penis

The third cylinder of the penis is the singular corpus spongiosum, which, like its counterparts, the two corpora cavernosa, also contains endothelial-lined sinusoids. Unlike the corpora cavernosa, however, the corpus spongiosum hosts the urethra, where it dilates to form the urethral bulb nearby Kobelt's bulb of the spongiosum proximally. The corpus spongiosum is ventral relative to paired corpora cavernosa, and it connects the glans penis distally and the prostate gland proximally (Fig. 2B). Also in contrast to the corpora cavernosa, the corpus spongiosum lacks an outer longitudinal tunica or intracavernosal pillars, thereby assuring low pressure during an erection. This design, consequently, allows for an extraordinarily cushioned milieu for the propulsion of urine and semen. The spongiosum body is full of coarser elastic fiber and does not contribute to penile erectile rigidity but does contribute significantly to penile swelling (tumescence). At the point, it lies intimately against the inferior fascia of the urogenital diaphragm, from which it receives a fibrous investment. The urethra enters the bulb closer to the upper than to the lower surface. On the lower surface, there is a median sulcus, from which a thin fibrous septum projects into the substance of the bulb and divides it imperfectly into two lateral lobes (Fig. 2C).

The glans penis is a conical vascular body forming the extremity of the penis and covered by a stratified squamous epithelium, and at its end is the urethral meatus, where a slit-like vertical external urethral orifice presents. It forms a round projecting border at its proximal limit; this border is called the *corona glandis* and overhangs the retrocoronal sulcus. The glans is buttressed with a distal ligament, whereby a surge in pressure can be transmitted and ejaculate emitted during coitus despite being hindered by vaginal wall compression. Immediately beneath the subcutaneous layer, there is a strong perisinusoidal fibroelastic shell which is responsible for its resilient nature and cushioning function, both being critical for coitus. The elastic fibers are thicker and denser in the glans sinusoids than elsewhere in the entire penis.

In summary, the sinusoids are specific to their respective containers: the corpora cavernosa, the corpus spongiosum and the glans penis; the conventional medical knowledge regarding the spongiosal and glans sinusoids ought to be updated. Before the penile hilum forms, the paired corpora cavernosa override the singular corpus spongiosum. The smooth muscle structure is found inside the vascular walls: the arterial, venous, and sinusoidal walls which are intertwiningly formed with smooth muscle cells and fibrous tissue structures throughout the glans penis, the single corpus spongiosum, and the paired corpora cavernosa. It is no stretch to say that the human penis mimics the structure of the human body, in which the skeletal muscles and skeleton encompass visceral organs and their containing smooth muscles. The health of the organs depends upon the integrity of its muscles, and such is the case in the structure of the penis as well.

Urethra

Another critical function of the penis is the passage of urination, which occurs via the urethra (Fig. 2). The male urethra, formed from the endodermal portion of the caudal membrane, runs through the corpus spongiosum and is composed of six parts: the bladder neck, the prostatic urethra, the membranous urethra, the bulbous urethra, the penile urethra, and the fossa navicularis. The urethra receives its blood supply from both proximal and distal directions. The bladder neck's internal sphincter is encircled by fibromuscular band covered with transitional cell epithelium. It is closed during ejaculation; a retrograde ejaculation occurs if it is otherwise dysfunctional. The prostatic urethra is the segment, 3–5 cm in size, which extends from the bladder neck to the deep perineal membrane. The verumontanum, (Latin: *colliculus seminalis*), is positioned in the middle of the prostatic urethra as well as the prostatic utricle, which is a remnant of the embryonic Müllerian duct. The ejaculatory ducts open on either side of it into the urethra. It serves as a landmark for acknowledging the external urinary sphincter just distal to it.

The membranous urethra is the shortest and narrowest part of the urethra, which measures only 1–2 cm, although it is susceptible to straddle trauma. It runs through the pelvic floor and is enclosed by the external urethral sphincter. The bulbar urethra

is widest and surrounded by the spongiosal body, and the penile urethra is the longest segment, accounting for some 15–20 cm. Cowper's gland presents in the proximal spongy urethra, whereas the urethral gland of Littre presents on the floor of the urethra and spans its entire length. Both glands are responsible for mucus secretion, which lubricates the urethra for ejaculate. The fossa navicularis represents the terminal portion of the spongy urethra, which increases in diameter just proximal to the meatus.

The wall of the urethra consists of mucosa with variable epithelium. The bladder neck and prostatic urethra contain transitional epithelium, the spongy urethra is covered with stratified columnar epithelium, and the fossa navicularis is covered with squamous epithelium, which is designed to withstand the stresses of abrasion. The submucosal layer contains connective tissue, glands, and some a very thin muscularis. The urethral lumen morphology varies throughout its length: it is crescentic in the prostatic urethra, stellate in the membranous urethra, trapezoidal in the bulb, horizontally configured along the penile urethra, and an inverted T-shape—vertically configured—in the meatus. This results in a spiral urination stream; it is hypothesized that this is advantageous for effective propulsion, and thereby the excretion of urine from the body, which has a cleansing effect on the meatus. Forking urinary streams or other urinary difficulties is a sign of urethral stricture of escalated severity.

Penile Arterial System

To meet the demands of a variably flaccid and rigid penis, the penile vascular system is unique: both the arterial supply and the venous drainage comprise a dual system which is appropriate for tissue metabolism and sinusoidal functions. The penis has a dual blood supply to the entire penile tissue and extensible sinusoids which are assorted into specific cavernosal, spongiosal, and glans entities. The superficial external pudendal branches of the femoral artery supply the tissue superficial to Buck's fascia, including the skin of the penis. These branches divide into dorsolateral and ventrolateral arteries, which provide collaterals across the midline, and contain a rich subdermal plexus. The arterial supply of the structures deeper than Buck's fascia ultimately arises from the anterior division of the internal iliac artery, which gives rise to the internal pudendal artery (Fig. 3).

The internal pudendal artery leaves the pelvis through the greater sciatic foramen below the piriformis. It travels in Alcock's canal (within the ischiorectal fossa) along the surface of the obturator internus. After it pierces the deep perineal membrane, it delivers blood to three additional penile arterial branches. The first branch is the bulbourethral artery, which runs medially in the deep perineal space to supply the corpus spongiosum and the urethra. It also anastomoses with the penile cavernosal and dorsal artery in the glans to ensure that the urethra has both an anterograde and retrograde vascular supply. The second branch is the cavernosal artery, which supplies blood to the penile crus and runs forward along the entire sinusoids of the corpora cavernosa via multiple helicine arteries and which plays a predominant role in erectile function. This is a typical example of an end artery, which is also expressed in the retina and kidney glomerulus. This end artery bears no anastomosis and is therefore susceptible to trauma.

The cavernosal arteries divide into branches after piercing fascial layers, especially the tunica albuginea in the penile hilum to supply the entire cavernosal sinusoids. They then divide into branches which enter, and are supported by, the sinusoidal wall. The dual arterial system is either forming a rich capillary network via many small arterial vessels, or forming the helicine arteries of Muller which are contracted and tortuous in the flaccid state, but dilated and straight in the erect state to erectile sinusoids. The vessels themselves give off tiny capillary structures which supply the trabecular tissue and are most abundant in the posterior aspect of the corpora cavernosa.

The paired dorsal arteries (DAs) run over the penile crus toward the midline where it pierces the suspensory ligament along with the DDV, the CV medially, and dorsal nerves laterally. This artery plays a minor role in supplying blood to the cavernosal sinusoids throughout its course along the entire corpora cavernosa (except for the penile crura). Furthermore, this artery supplies the glans penis, tissues superficial to Buck's fascia (including the Colles' fascia), the dermis, and skin.

Penile Venous System and Erection-Related Veins

There is a general rule that the ratio of number of veins to number of arteries is 2:1 in the tissues of the adult human body. A reverse 1:2 ratio exists in the fetal umbilicus and in the conventional penile venous anatomy. The venous system of the human penis has been widely studied and is generally categorized into superficial, intermediate, and deep venous systems, which are responsible for venous drainage of the superficial, intermediate, and deepest penile tissues, respectively. The superficial dorsal vein (SDV) is located between Buck's fascia and Colles' fascia for general venous drainage of superficial tissues, and a single DDV accompanied by a pair of DAs is positioned between the tunica albuginea and Buck's fascia for blood drainage of the corpora cavernosa, a chamber essential to facilitating a rigid erection. Finally, the deep vein system includes the crural and CVs for drainage of the deep tissues. Thus, according to the received medical wisdom, the penis, like the umbilical cord, constitutes a rare exception in the human anatomy in that its attendant arteries number fewer than its attendant veins do.

Recent serial studies, however, found that each of the DAs is sandwiched by a medial and lateral PAV and that the DDV is accompanied by CVs which are more deeply housed in their own perivascular sheath in accordance with the drain corresponding to the corpus cavernosum (Fig. 3). It is important to recognize that the DDV is not exclusive in having dedicated emissary and circumflex veins; the CVs and PAVs also possess them. The DDV is located in the median groove between paired corpora cavernosa and is confluent from five to seven veins with digital distribution emerging from the glans penis; it receives blood from the corpora cavernosa through emissary veins and from the corpus spongiosum through the circumflex vein. In other words, it remains the common drainage channel of the paired corpora cavernosa and the corpus spongiosum. It is flanked by a pair of CVs which are distributed along the entire length of corpora cavernosa, although these lie in a deeper position. Bilaterally, each DA is respectively

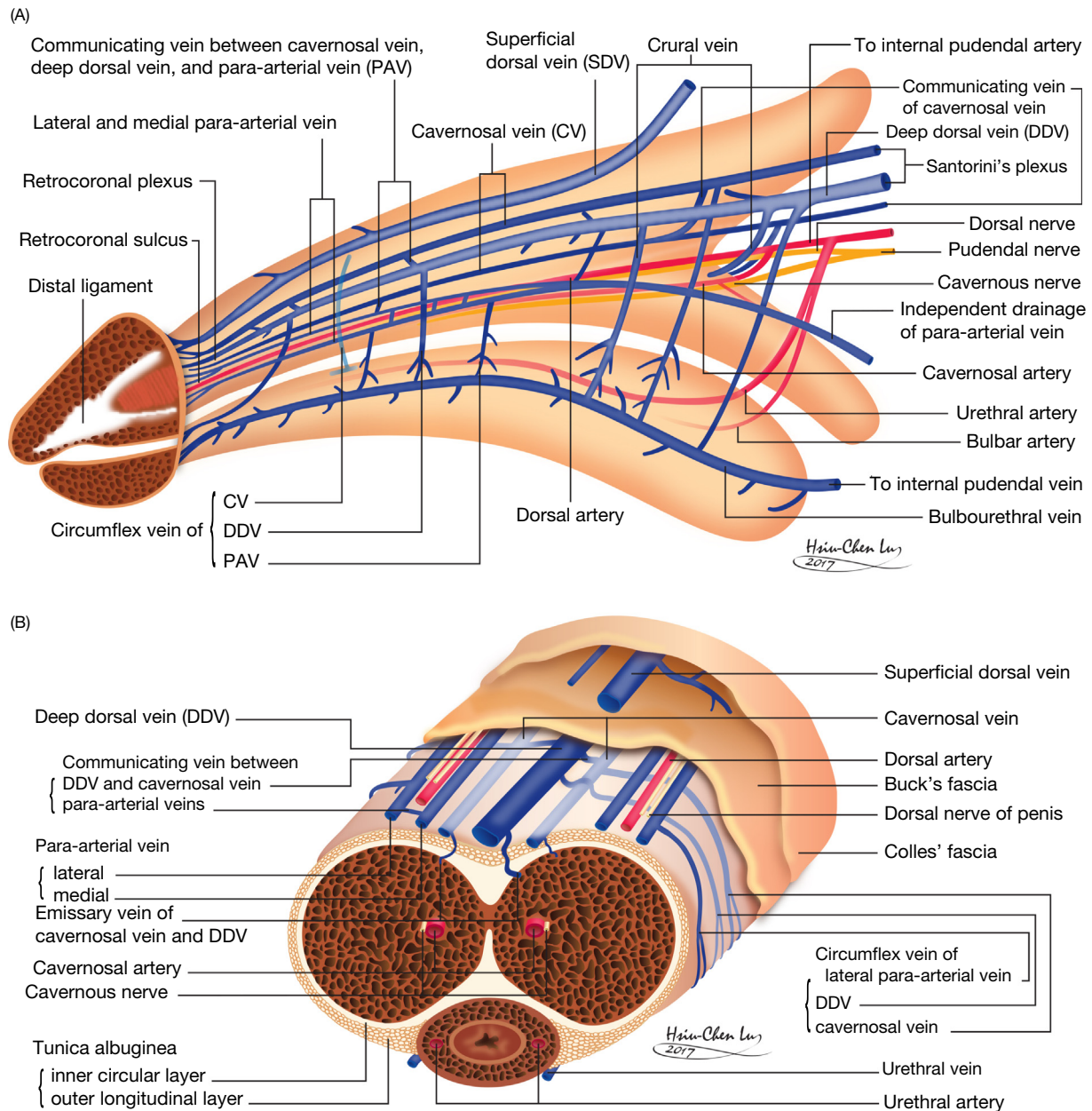


Fig. 3 Illustration of new penile vascular anatomy. (A) Lateral view: The glans penis is composed of specific sinusoids and a stout distal ligament (the *os penis* analog) for supporting the entire glans. The DDV is consistently in the median position and receives blood of the emissary veins from the corpora cavernosa and of the circumflex vein from the corpus spongiosum. The DDV is sandwiched between the cavernosal veins (CV), but these lie at a deeper position. Bilaterally, each dorsal artery is respectively sandwiched by its corresponding medial and lateral para-arterial veins (PAVs). Note that the lateral PAV merges with the medial vein proximally. The deeper color of the veins indicates the deepest part of the vasculature. The pudendal artery and nerve are distributed in a similar manner, but are relatively simple. (B) Cross section of the mid-penis: Note that the number of veins is seven (a larger count than the traditionally described singular vein), although it drops to four at the level of the penile hilum because each pair of the veins merges. Erection-related vasculature is arrayed in an imaginary arc on the dorsal aspect of the tunica albuginea, which is composed of multiple collagen bundles with a 360 degrees complete inner circular layer and a 300 degrees incomplete outer longitudinal coat. Thus, the penile vascular system still complies with the general rule in the body that the number of veins is normally higher than the number of arteries.

sandwiched by its corresponding medial and lateral PAVs. A slumped-looking communicating vein can clearly be identified between the two, acting as a kind of hammock or sling for the corresponding dorsal artery. The lateral PAV merges with the medial one proximally. Therefore, the number of veins between the tunica albuginea and Buck's fascia in the pendulous portion of the penis is seven, not one as was traditionally believed. (Although the number becomes four at the level of the penile hilum as a merger takes place in each pair of nomenclature veins.) Finally, all drainage veins account for six to eight independent channels which exit the cavernosal body and drain into the prostatic venous plexus of Santorini. Hence, the penile erection related vascular system still

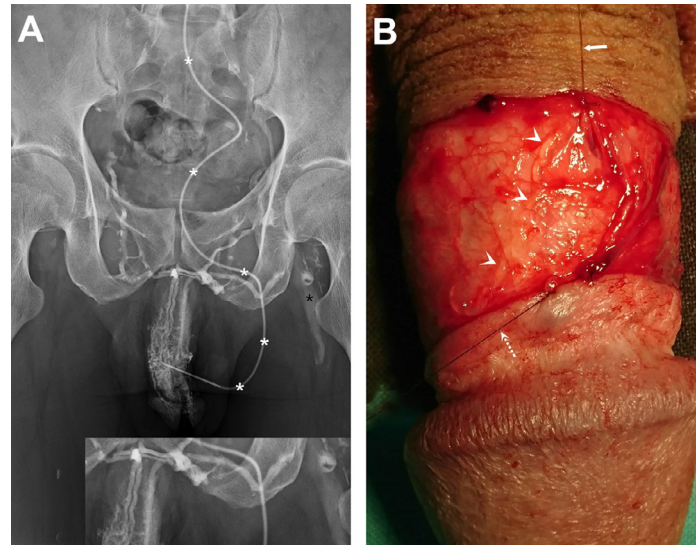


Fig. 4 Cavernosogram showing erection-related veins and photo demonstrating lymphatic vessel. (A) In a 29-year-old male, an A-P view cavernosogram shows the superficial dorsal vein, circumflex veins, and cavernosal veins are directly drained to the internal pudendal vein and femoral veins (*black asterisk*) via a #19 scalp needle (*white asterisk*). (B) This photo presents a transparent lymphatic vessel (*white arrow heads*), superficial veins (tagged by a black silk suture, *dotted arrow*) above the Colles' fascia and the deep dorsal trunk (tagged by a brown chromic suture, *arrow*) which is under Buck's fascia.

complies with the general anatomical rule that veins number more than arteries do. Erection-related veins are arrayed in an imaginary arc on the dorsal aspect of the corpora cavernosa.

The blood drainage of the corpus spongiosum is required given the excessive amount of spongiosal sinusoids in each erection occurrence. Bulbo-urethral veins drain the corpus spongiosum, the urethra and the glans penis. Those veins will join the internal pudendal vein, which will drain to the internal iliac vein. Most blood in the glans sinusoids will drain to DDV, CVs, and PAVs, and some will be conducted to the corpus spongiosum.

The relationship of the vasculature to the penile fibroskeleton is interesting, and the difference between the penile venous and arterial paths is substantial. The veins traverse an oblique path between the inner and outer layers of the tunica albuginea, whereas the arteries take a more direct route. This design is optimal for facilitating penile erection in that the venous vasculature is susceptible to being compressed. In this new understanding of the penile venous anatomy, the conventional crural vein or cavernosal is just a variant circumflex vein which exits from either the lateral or medial aspect of the penile crus. This concept can be well applied to cavernosography on patients (*Fig. 4A*) whose erection-related veins are directly communicated with the superficial venous system and not apparently with so-called "deep veins." For the penile venous anatomy, a categorization of metabolic veins and erection-related veins seems to be more appropriate.

Penile Nervous System

The penis is the most sensitive organ in the male body. In the central nervous system, the medial preoptic nucleus and paraventricular nucleus of the hypothalamus and the limbic system are responsible for stimulating the spinal autonomic centers to cause erections via the action of dopamine, melanocortin, and oxytocin. The sympathetic spinal center is located in T12-L2 while the parasympathetic spinal center is located in S2,3,4. In the peripheral nervous system, the pudendal nerve arises from S2,3,4 and the pelvic plexus, which provides autonomic control.

The pudendal nerves innervate somatic motor to bulbospongiosus and ischiocavernosus muscles, as well as sensory innervations to the penis (*Fig. 3*). Afferent nerve fibers travel from sensory receptors to the penile dorsal nerve and then from the pudendal nerve into the sacral spinal cord. From here they ascend in the spinothalamic or posterior column/medial lemniscus pathway. The sympathetic adrenergic nerve fibers and receptors are present in the cavernous trabeculae, and surround the penile cavernosal arteries. Noradrenaline is the major neurotransmitter controlling penile flaccidity and de-tumescence.

The cavernous nerves arise from the inferior hypogastric plexus, located posterolateral to the seminal vesicles and lateral to the prostate gland. In the membranous portion of the urethra, the nerve fibers are located at 3 and 9 o'clock, whereas at the distal bulb of penis they are located at 1 and 11 o'clock and then enter into the corpora cavernosa. This nerve is a combination of parasympathetic and visceral afferent fibers and provides the nerve supply to the cavernosal sinusoids (*Fig. 3*). It runs dorsomedial to the penile cavernosal arteries below Buck's fascia. Accompanying arterial distribution, the cavernous nerves branch to the crus and the main trunk innervates the corpora cavernosa. The autonomic nerve fibers innervate the helicine arteries. The cholinergic nerve endings stimulate the nitrous oxide-synthase and therefore the release of nitrous oxide which is the principal neurotransmitter causing penile erection via increasing the production of cyclic guanosine monophosphate causing cavernosal smooth muscle

relaxation. In the glans penis and prepuce, the free nerve-endings are rich in quantity; there are many Meissner's and Pacinian corpuscles, which helps explain heightened sensitivity to burning, pain, temperature, position change, pressure, light touch, itch, and pleasurable sexual sensations.

Lymphatic system

The penile lymphatics initiate from the prepuce and penile skin, which run proximally toward the presymphyseal plexus via lymphatic vessels, in which some coalesce and travel at the region of the frenulum or the corona (Fig. 4B) in the company of the SDV or DDV and DA. It then diverges into right and left trunks to join the lymphatics from the scrotum and perineum. They run along superficial external pudendal vessels into the superficial inguinal nodes, especially the superomedial group. Some lymphatic drainage occurs through the femoral canal into Cloquet's node. The lymphatic vessels from the glans and penile urethra drain into deep inguinal nodes, presymphyseal nodes and, occasionally, into external iliac nodes. The lymphatics of proximal urethra drains into the pelvic lymph nodes.

Penile Fascial Layers and Ligaments

Multiple fascial layers encircling the central sinusoids comprise the structural character of the human penis (Fig. 3B). The fascial layer has a sliding ability prerequisite for erectile function and is composed of five enveloping layers: these layers, in order from innermost to outermost, are the tunica albuginea, Buck's fascia, Colles' fascia, dermis and skin. (*ABCDs* is a simple mnemonic used to describe the human penile structure.) The tunica albuginea is the coat of the corpora cavernosa, which is the deepest fascia and is composed of inner circular and outer longitudinal collagen fibers in organized arrays interlaced with elastic fibers that form an irregular, latticed network on which the collagen fibers rest. The more distal the elastic fibers, the more abundant they are, enabling the extensibility of the distal penis during an erection.

Originating from the subtunical plexus, emissary veins travel between the inner and outer layers of the tunica and usually exit the outer layer in an oblique fashion. The outer layer of the tunica is lacking between the border of the corpora cavernosa and the corpus spongiosum and acts as the determinant structure for achieving erection rigidity, which results from sealing off the venous outflow. Subsequently, the Buck's fascia encircles the corpora cavernosa and corpus spongiosum in one package. This layer is regarded as the deeper layer—*fascia penis profunda*—and pushes erection-related vasculature against the tunica albuginea; this vasculature involves the dorsal nerve, dorsal artery, DDV, CVs, PAVs, and lymphatic vessels. In addition, the Buck's fascia is endorsed with smooth muscle from the tunica dartos arising from within the scrotum. This implies that this structure is indispensable to connecting the penile shaft and scrotum. A contracted scrotum is then normally seen in a rigid erection.

Colles' fascia further solidifies the integrity of the penile structure and encompasses several SDVs, unnamed nerves, arteries, and lymphatic vessels. This layer is in continuity with the superficial perineal fascia caudally and to Scarpa's fascia of the abdomen cranially. Therefore, this sound arrangement can restrict penile hematoma formation to the area between the inguinal ligament and the fascia lata and ventral to the perineal body of the pelvic floor.

The dermis layer presents as connective tissue enveloping the inner penile tissues. This thin, loose tissue, abundant in smooth muscle layer, is continuous at the root of the penis with connective tissue in the scrotal Dartos fascia. In the penis, Dartos fascia is loosely attached to the skin and Buck's fascia below, and contains superficial vascular, nervous plexus, and lymphatic vessels. In the perineum, Dartos fascia is continuous with the superficial perineal Colles' fascia. At the penile neck, it folds back on itself to form the foreskin (prepuce) which is directly continuous with the fibroelastic shell over the glans penis. Immediately proximal to the meatus, it folds again to fuse a median raphe forming the frenulum, located on the ventral side of the glans, and attaches the prepuce to the glans, where the frenular artery is covered and extraordinarily sensitive due to its numerous nerve-endings. All tissues are covered by a skin with a stratified squamous epithelium, but the deeper urethral meatus, where a number of small, highly sensitive papillae are covered by mucosa or skin. In summary, an integral penile structure (identified by the mnemonic *ABCDs*) comprises a highly complex anatomy, even though the penis appears, at least superficially, to be a simple organ. Additionally, the prepuce presents an inner and outer leaf and covers the glans of the flaccid penis to varied extent.

At the proximal end, the entire penile structure is attached firmly to the pelvic wall via the suspensory ligament superiorly, via the fundiform ligament laterally, and (remarkably tenaciously) via a skeleton-fibrous cavernosal ligament inferiorly. The fundiform ligament, without elastic component, extends from the inferior border of the rectus sheath and linea alba; it splits into two fasciculi which encircle the root of the penis. The richly elastic suspensory ligament passes caudally from the lower end of the linea alba, forming upper fibers, and from the symphysis pubis, forming lower fibers. Together they form a strong fibrous yet elastic component, which extends to the upper surface of the root of the penis, where it blends with the Colles' fascia. This characteristic is impressive because its collagen bundle look likes an elastic lace under direct observation by microscopy.

The importance is second to none on those relevant structures to penile reconstruction. Inside the glans penis, there is a distal ligament supporting the glans penis, and the ability to engage in coitus can be ruined if a penis loses an intact distal ligament—even though its erectile function may be otherwise normal. This unique anatomical arrangement may explain why the glans penis is strong enough to bear the buckling pressure of coitus, as well as how an erect penis is sufficiently rigid but never compresses the corpus spongiosum, which otherwise would present an obstacle to ejaculation. A strong glans protects the reflexogenic erectile mechanism which may be evoked in response to global contraction of the perineal muscles, which in turn compresses the veins and the erectile tissue to encourage penile rigidity and allow a rhythmic ejaculation. Overall, an integral penile anatomical structure is a fundamental requirement for healthy erectile function and as a conduit for urinary and seminal fluids.

Clinical Relevance of Penile Structure

Congenital Abnormality of the Penile Form

Congenital defects may occur if abnormality ensues during embryonic development. Aphallia is a rare (1 in 5.5 million births) congenital condition in which developmental failure of the genital tubercle causes non-formation of the phallus: the penis, including the corporeal bodies, is completely absent. The urethra may open at any point of the perineal midline from the pubis to the anus or anterior wall of the rectum. Even rarer (1 in 30 million births) is diphallia, which is duplication of the penis resulting from an incomplete fusion of the genital tubercle, in which two distinct forms of penile duplication are recognized. The most common form is associated with bladder exstrophy complex. The patient exhibits a penis resembling that of a bird's, which consists of two separated corpora cavernosa that are associated with two separate hemiglans. Rarer still is triphallia, a condition which is similar to diphallia except that, as its name indicates, describes the occurrence of three distinct phalluses.

Diseases in the Tunica Albuginea-Related Penile Dysmorphology

There are two forms of penile dysmorphology which may be categorized as congenital and acquired entities. Penile curvature results from asymmetrical development of the tunica albuginea whereby the outer longitudinal layer is the determinant structure for penile erectile shape. This structure is the determining structure for penile morphology and penile prosthesis protection and was not identified until the 1990s. Peyronie's disease is an inflammatory condition that is characterized by the formation of fibrous, noncompliant plaques within the tunica albuginea. A hypothesized pathophysiology is repeated tunical mechanical stress and microvascular trauma as well as abnormal wound healing. The Peyronie's plaques are most likely produced by tunical fibroblasts in response to cytokine stimulation.

Diseases of Penile Vascular System

Penile veno-occlusive dysfunction (also known as penile leakage) is the leading cause of erectile dysfunction and thus has attracted a great deal of medical attention. Similarly, penile arterial insufficiency has attracted medical attention for almost a century and is potentially a sign of cardiovascular disease. Given that approximately 30% of males with erectile dysfunction are disappointed with the results of phosphodiesterase-5 inhibitor agents, the demand for—and research into—alternative treatments has steadily increased. A more lasting treatment would require the complete occlusion of the leakage points where the emissary veins are the target.

Diseases in the Prepuce

Phimosis is frequently found and smegma is easily formed in an open sac formed by the preputial inner and outer leaf. A balanoposthitis results consequently from chronic inflammation, which an infant may suffer from if the foreskin cannot be retracted in order to free the glans penis; in older males, this may aggravate a retrograde urethritis and/or contribute to a urinary infection. A circumcision is indicated once the acute infection is mitigated.

Diseases in the Male Urethra

Hypospadias is diagnosed if the urethral opening presents somewhere besides the tip of the glans penis. Surgical repair (conducted as early as possible) is recommended.

Disease in the Penile Nervous System

Any condition that jeopardizes the penile innervation will compromise the integrity of penile health either centrally or peripherally, which will, in turn, result in erectile dysfunction because erectile tissues are unable to fill with blood. These include prostatectomy nerve injury, traumatic nerve injury, etc.

Disease in the Lymphatic System

Lymphedema is a condition of lymphatic blockage resulting from external or endogenic causes including infection; some foreign bodies are not uncommon to encounter such as paraffin, glass ball and hyaluronic acid, etc.

Further Reading

- Elhanbly, S., et al. (2004). Erectile dysfunction in smoker: a penile dynamic and vascular study. *Journal of Andrology*, 25(6), 991–995.
- Hsieh, C. H., & Hsu, G. L. (2016). Current role of vascular surgery (arterial and venous) in erectile dysfunction. In M. L. Djordjevic, & F. E. Martins (Eds.), *International book of erectile dysfunction* (pp. 129–157). New York: Nova Science.

- Hsieh, C. H., et al. (2015). Tunical outer layer plays an essential role in penile veno-occlusive mechanism evidenced from electrocautery effects to the corpora cavernosa in defrosted human cadavers. *Urology*, 86(6), 1129–1136.
- Hsu, G. L. (2006). The hypothesis of human penile anatomy, erection hemodynamic and their clinical applications. *Asian Journal of Andrology*, 8(2), 225–234.
- Hsu, G. L. (2011). Physiological approach to penile venous stripping surgical procedure for patients with erectile dysfunction. Google Patents; Patent No: US 8,240,313 B2 <http://www.google.com/patents/US20110271966>.
- Hsu, G. L., et al. (1992). The three-dimensional structure of the human tunica albuginea: anatomical and ultrastructural level. *International Journal of Impotence Research*, 4, 117–129.
- Hsu, G. L., et al. (1994). Anatomy and strength of the tunica albuginea: its relevance to penile prosthesis extrusion. *Journal of Urology*, 151(5), 1205–1208.
- Hsu, G. L., Chen, S. H., & Weng, S. S. (1997). Out-patient surgery for the correction of penile curvature. *British Journal of Urology*, 79(1), 36–39.
- Hsu, G. L., et al. (2003). Penile venous anatomy: an additional description and its clinical implication. *Journal of Andrology*, 24(6), 921–927.
- Hsu, G. L., Hsieh, C. H., Wen, H. S., Hsu, W. L., & Chen, C. W. (2004a). Anatomy of the human penis: the relationship of the architecture between skeletal and smooth muscles. *Journal of Andrology*, 25(3), 426–431.
- Hsu, G. L., et al. (2004b). Outpatient penile implantation with the patient under a novel method of crural block. *International Journal of Andrology*, 27(3), 147–151.
- Hsu, G. L., et al. (2005). Distal ligament in human glans: A comparative study of penile architecture. *Journal of Andrology*, 26(5), 624–628.
- Hsu, G. L., et al. (2006). Formulas for determining the dimensions of venous graft required for penile curvature correction. *International Journal of Andrology*, 29(5), 515–520.
- Hsu, G. L., et al. (2012). Penile veins are the principal component in erectile rigidity: a study of penile venous stripping on defrosted human cadavers. *Journal of Andrology*, 33(6), 1176–1185.
- Hsu, G. L., et al. (2013). Reconstructive surgery for idealizing penile shape and erectile functional restoration on patients with penile dysmorphology and erectile dysfunction. *Arab Journal of Urology*, 11(4), 375–383.

Penis Structure—Erection

Geng-Long Hsu, Hsu's Andrology and National Taiwan University, Taipei, Taiwan
Hsiu-Chen Lu, China Medical University, Taichung, Taiwan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Detumescence The inverse of tumescence, detumescence describes a state of the penile corpora cavernosa in which blood from erection-related veins drains back into the larger circulatory system.

Ejaculation An action of gushing fluid composed of semen and sperm, initiated by peristalsis from the semiferous tubule along the epididymis, seminal vesicle, ejaculation orifice, prostate urethra, external sphincter, membranous urethra and penile urethra to the urethral meatus.

Erection rigidity Refers to varying grades of erectile rigidity, with peak rigidity (a full erection) characterized by a bone-like hardness in the penis.

Intracavernosal pillars Fibroelastic tissues distributed between the 10 or 2 o'clock to 6 o'clock positions of the inner tunical layer of the corpora cavernosa. The pillars are intertwined with collagen bundles and elastic fibers.

Nitric oxide activities A key neurotransmitter to relax smooth muscle of the vascular endothelium within the sinusoidal wall of the paired corpora cavernosa, the lone corpus spongiosum, and the glans penis.

Parasympathetic activity The part of the autonomic nervous system that is responsible for regulating the penile sinusoids into tumescence, which results from increased penile arterial supply and decreased venous outflow when active; in the autonomic nervous system, it is the counterpart to sympathetic activity.

Penile arterial inflow An extent of penile artery supply to the sinusoids of the penis, it is largely routed to the corpora cavernosa where arterial supply speed varies from 2–3 mL/min to 60–80 mL/min.

Penile cavernosal-sinusoidal trapping A state of the penile sinusoids in which the penile arterial supply exceeds venous drainage, resulting in sinusoidal expansion.

Smooth muscles One of the muscle categories, the *smooth* muscles scatter along the vascular wall, gastrointestinal tract, reproduction tract, sinusoidal walls, etc.

Sympathetic tone The part of the autonomic nervous system that is responsible for regulating the penile sinusoids into detumescence resulting from increased venous outflow and decreased penile arterial supply; in the autonomic nervous system, it is the counterpart to parasympathetic activity.

Tunica outer longitudinal layer A tenacious collagen layer surrounding the corpora cavernosa, the collagen fibers in organized arrays interlaced with elastic fibers that form an irregular, latticed network on which the collagen fibers rest. This tissue determines drainage speed and the penile morphology and is the pivotal coat to protect an implanted prosthesis.

Tumescence Inverse of detumescence, tumescence describes a state of the penile corpora cavernosa characterized by sinusoidal expansion resulting from increased penile arterial supply and decreased venous drainage of erection-related veins.

Venous drainage A term that describes the return of sinusoidal blood back to the larger systemic circulatory system following an erection.

The Human Penile Structure and Erections

Comparative Anatomy in Mammals

In male animals, the os penis (the penis bone) exists in most species to achieve the best chance of paternity; this durable structure increases the coital frequency and often the duration as well. The penis bone, which is attached to the tip of the penis rather than the base, provides structural support for male animals that engage in prolonged intromission. The longest sexual intercourse was performed by a couple of rattlesnakes (*Crotalus L.*) that made love for 23 h and 15 min (Fig. 1). The coital duration is not proportionally correlated, though: for instance, while a male lion's os penis allows him to engage in an impressive 250 copulations in 4 days, each copulation can only last for a minute (which is still longer than that of a male rat, for whom copulation lasts only a few tenths of second). However, the male's hardy os penis makes it easy to get geared up for the next willing female shortly after his previous ejaculation. Although mounting is always required in mating behaviors, a male dog can swing its penis in a right-about direction after successful mating. This facility is particularly impressive, since there is neither a truly synovial joint nor a synarthrosis inside the canine penis. The answer might lie in the peculiar anatomy of the canine penis, which is composed of a long os penis in the front and a stout and minimally expandable corpora cavernosa at the rear.

Humans, woolly monkeys, and spider monkeys are the only primates lacking this hardy piece of anatomy. How do human males manage to function (sexually) without an os penis if, as conventional wisdom suggests, no os analog structure exists? It is a good question, but the human penis may have no need for an os penis given that erection rigidity is attained through a hydraulic

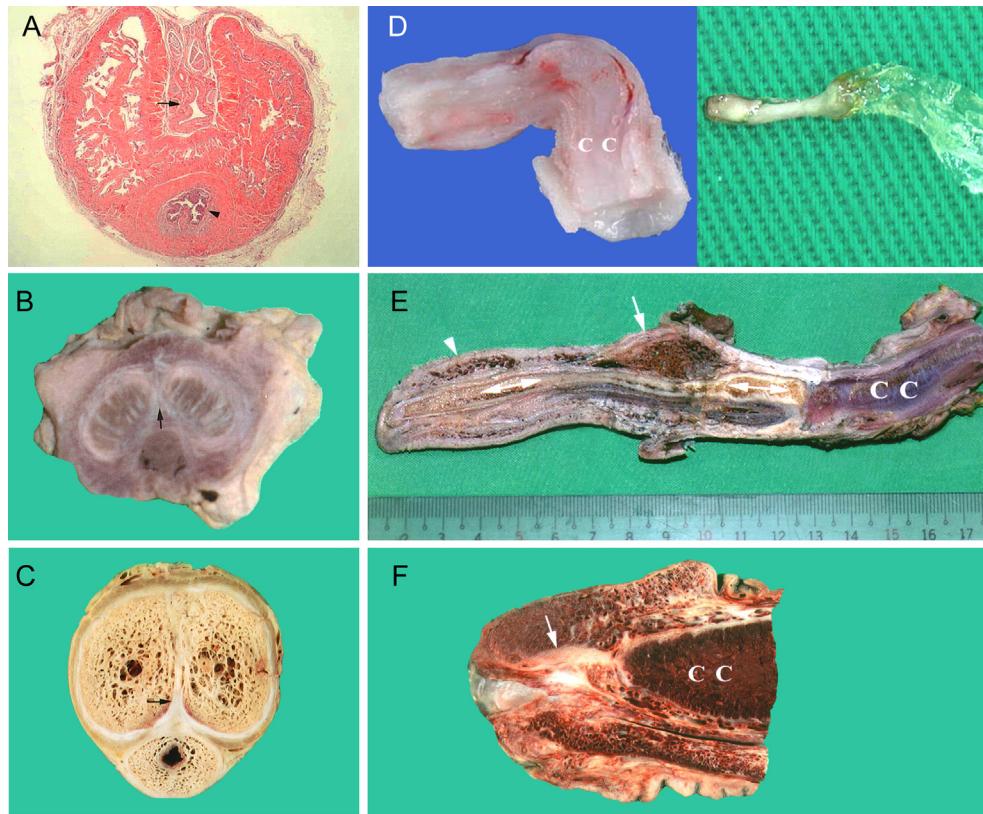


Fig. 1 Comparison of penile structure in different species, cross-sections of the corpora cavernosa and longitudinal aspect of the glans penis are from A to C and D to F, respectively. (A) In rats, the corpus cavernosum, devoid of the medial septum and intra-cavernosal pillars, is positioned between the deep dorsal vein (*arrow*) and the urethra (*arrowhead*) (H & E stain, reduced from 25 $\times$). (B) In dogs, a complete septum (*arrow*) and abundant intracavernosal pillars are obvious (1 $\times$). (C) In male humans, an intracavernosal pillar is not uncommonly encountered (not demonstrated). A septum (*arrow*) is significant, but incomplete and dorsally fenestrated. Note the clear delineation of the inner circular and outer longitudinal layers of the tunica albuginea (1 $\times$). (D) In rats, a short os penis is positioned between the glans penis and the corpus cavernosum (left panel, 7 $\times$). The amount of glanular tissue is scanty. The junction between the glans penis and the corpus cavernosum looks like a knee joint, which provides a flipping action during mating. The short os penis (right panel, 1 $\times$) can be better demonstrated after clearing and alizarin red S staining because only bony tissue can be preserved. (E) In dogs, the os penis (*double-headed arrow*) is enveloped with a unique glans penis of two compartments (*arrowhead* and *arrow*, respectively). Similar to the rat penis, the corpora cavernosa are not intromitted. However, they are reinforced with abundant intracavernosal pillars and a complete septum (1 $\times$). (F) In male humans, the distal ligament (*arrow*) within the glans penis is obvious and should be regarded as a ligamentous structure rather than a mere collection of sinusoids. The distal ligament is an aggregation of the outer longitudinal layer of the tunica albuginea and acts as a buttress of the glans penis (1 $\times$). Courtesy reproduction from *Journal of Andrology*, 26(5):624–628, 2005.

system rather than a bony structure. To know the relation of structure and peculiar function in the human penis, it is advisable to first know the comparative penile anatomy in varied species.

Recently a study was done on rat, dog, and human penises. In the rat's penis, although there is a short os penis, performing a flipping action with a rigid erection would be challenging, as neither a significant septum nor intracavernosal pillars is found in the corpus cavernosum (**Fig. 1A**). In dogs, with their long os penis, there is a complete septum between the paired corpora cavernosa as well as abundant intracavernosal pillars located behind the os penis (**Fig. 1B**). In human beings, for extraordinary hydraulic extensibility there is an incomplete septum with dorsal fenestration and moderately abundant intracavernosal pillars in the corpora cavernosa (**Fig. 1C**), which allows free communication between the two corpora cavernosa.

The anatomical structure of the glans penis varies between different species. In the glans penis of both rats (**Fig. 1D**) and dogs (**Fig. 1E**), there is a bony structure, i.e., the os penis. In these mammals, the os penis is the only supporting structure in the glans penis; there is no synovial joint in the species. In contrast to these os penises, the human glans penis contains an os analog, named the distal ligament (**Fig. 1F**). This newfound structure is inelastic and formed through an aggregation of the outer longitudinal layer of the tunica albuginea, in which neither a vascular component nor nerve tissue is seen. Moreover, many tributaries of supporting structures radiate directly from the distal ligament.

In histological sections, the os penis is composed mainly of type I collagens interlocked with type III collagens both in rats (**Fig. 2A**) and dogs (**Fig. 2B**) while connecting the os penis with its fibrous envelope. In humans, the distal ligament is composed of type I collagens (**Fig. 2C**), and neither osteocytes nor chondrocytes, characteristics of the bony structure, are found. The distribution of intracavernosal pillars is intermediate between the rat and dog; its pronounced wavy appearance results from

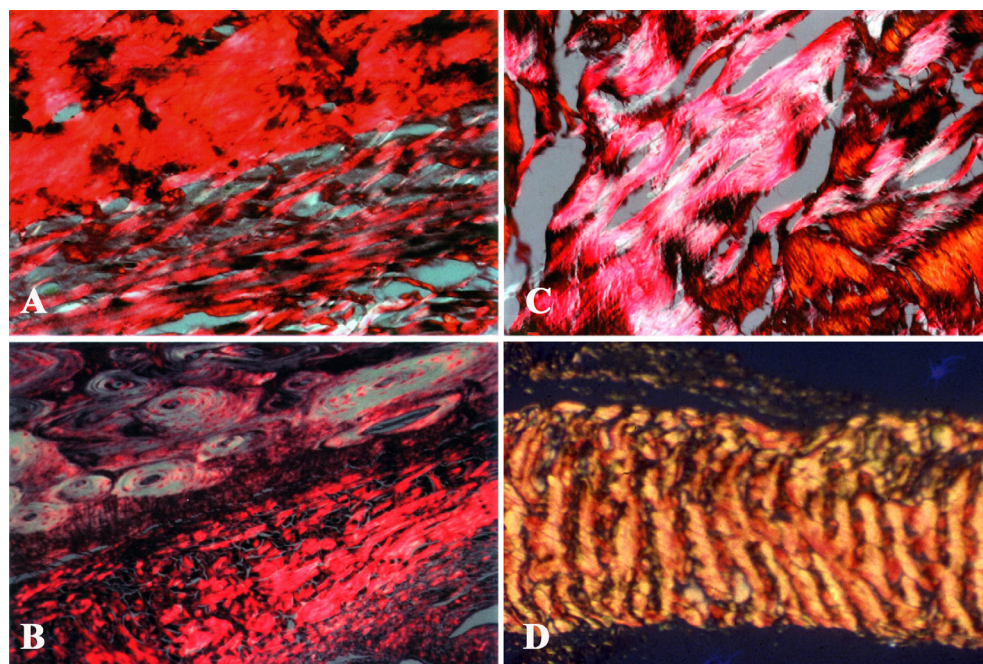


Fig. 2 Histology of the os penis, os analog, and intracavernosal pillars of the human penis. (A) In rats, the type I collagen with a reddish appearance is the major component of the os penis, which can be regarded as a woven bone (upper). The interlocking type III collagen with a greenish appearance is uncommon in the os penis proper, but substantial in its envelope (lower) (Picrosirius red stain, 400 $\times$). (B) In dogs, the appearance of a lacuna is characteristic. Osteocytes as well as chondrocytes are seen (Picrosirius red, 100 $\times$). (C) The os analog of human beings is similar to the os penis of a dog. Type I collagen is also the major component, with type III collagen as an interlocking ingredient. However, there are neither osteocytes nor chondrocytes (Picrosirius red, 400 $\times$). (D) In the human penis, the distribution of intracavernosal pillars is intermediate between the rat and dog (**Fig. 1B**); its pronounced wavy appearance results from an extraordinary extensibility via elastic component (Picrosirius red, 25 $\times$). Courtesy reproduction from *Journal of Andrology*, 26(5):624–628, 2005.

extraordinary extensibility via the elastic component (**Fig. 2D**). Moreover, in the tunica albuginea of the corpora cavernosa, elastic fibers are sparse in the canine penis, few in the murine penis, and abundant in the human penis.

During mating, only the long os penis, associated with its glans penis, is intromitted, but the corpora cavernosa are not because these are deeply buried in the male body and can only act as a support of the long os penis. Therefore, either the junction between the os penis and the corpora cavernosa or that of the os penis to the glans penis is extraordinarily strong.

In dogs, the overwhelmingly abundant intracavernosal pillars and a complete septum will enhance the strength of the corpora cavernosa. In rats, the mounting duration is so short that these enhancing micro-architectures are not necessary. The resulting insufficient rigidity may, in turn, facilitate the flipping movement of the intromitted penis for the removal the semen plug deposited by a previous male. In human beings, the corpora cavernosa are intromitted. The intracavernosal pillars and the septum may increase dispensability and may, therefore, be mandatory in order to establish sufficient rigidity by congestion of the sinusoids if longer coital time is required. This suggests that a man may be susceptible to premature ejaculation if his penis is erect but insufficiently rigid. A complete septum meets the requirement for establishing a pair of sufficiently strong corpora to buttress a long os penis such as that found in dogs, and the absence of this structure will be sufficient to support a short os penis whose mounting time is as short as a few of tenths second, as it is in rats. In human beings, the medium septum with its unique dorsal fenestration is observed, and the paired corpora cavernosa may be regarded as an ideal milieu to apply Pascals' law which states that pressure applied to any part of the enclosed fluid at rest is transmitted undiminished to every portion of the fluid and to the walls of the containing vessel. It appears to be enforced with intracavernosal pillars; sufficient erectile rigidity could not otherwise be reached. Not surprisingly, therefore, one donor artery is sufficient in attempting arterial reconstruction. Plenty of elastic components in the tunica albuginea of the corpora cavernosa thus increase the erectile capability of the penis. Overall, this implies that not only the increment of erectile length, but also the erectile girth of the human structure may, therefore, expand.

Essential Structures for Erection Rigidity in the Human Penis

Human males lack an os penis but instead possess an os analog—the distal ligament—located within the glans penis. It is complemented by the human penis's evolved hydraulic system. However, this particular evolution is not without its disadvantages because a hydraulic system is vulnerable to insufficient rigidity if veno-occlusive dysfunction exists. Unsurprisingly, it is common for physicians to encounter males who suffer from erectile dysfunction, which is defined as difficulty (or inability) to attain or maintain an erection of sufficient rigidity. It is also not uncommon for young boys to question their parents and ask why the penis is

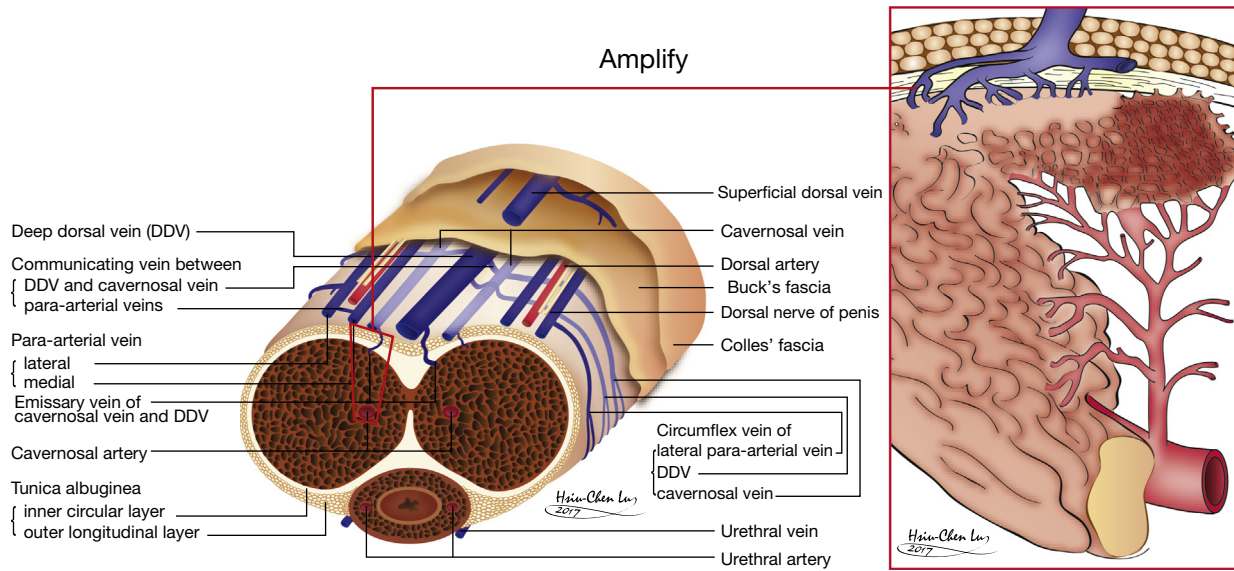


Fig. 3 An illustration of the relationship between sinusoids and emissary veins in human corpora cavernosa. Left: A cross-section of the human corpora cavernosa. Right: An amplified view of an emissary vein and its supplying sinusoids. Their anatomical relation is like a cluster of grapes, where the emissary veins are regarded as the grape stems and each sinusoid as the fruit.

sometimes soft like a worm and sometimes hard like a bone. Although a functional brain is a prerequisite for healthy penile sexual function, a rigid erection can be a purely mechanical event arising within penis itself—some males can sustain a full, rigid erection resulting from reflexogenic activity (originating between the 2nd and 4th vertebrae of the sacral spine) despite sustaining an injury to the upper spinal cord.

It is necessary to describe the indispensable structures for achieving erectile rigidity. There are six essential structures to reach this aim: healthy sinusoids, a functional tunica albuginea (especially the outer longitudinal layer), intact intracavernosal pillars, an integral distal ligament, and normal ischiocavernosus and bulbospongiosus muscles. The seamless interplay of the tunica albuginea and cavernosal sinusoids is the primary step of a penile erection (Fig. 3). The cavernous artery delivers blood to two types of branches: firstly, to the outer capillaries, which are responsible for supplying nutrition to the penis in the flaccid state as well as the smooth muscle and nerve fibers; secondly, to the inner helicine arteries (Fig. 1, left), which open directly into cavernous spaces without entering capillaries, which are then emptied into the post-cavernous venules. These inner arteries are for fulfilling the erectile function; they are shaped like corkscrews and allow the penis to elongate and expand without compromising flow. Multiple layers of smooth muscle surround the helicine branches. This muscle is contracted while flaccid, allowing only small amounts of blood into the lacunar spaces. After the appropriate stimulus, muscle relaxation occurs and the arteries dilate and straighten, increasing blood flow and dilating the lacunar spaces and, in turn, applying pressure against the outer longitudinal layer of the tunica albuginea to reduce the blood drainage, which eventually results in a rigid erection. Application of real world physics would assume that the corpora cavernosa comprise an ideal vessel in which to apply Pascal's law, which states that pressure applied to any part of an enclosed fluid at rest is transmitted undiminished to all walls of the containing vessel. The cavernosal sinusoidal expansion and decreased venous drainage result from tunical synchronizing limitation. The tunica albuginea is a bi-layered structure with a 360° inner circular and a 300° outer longitudinal layer which is the buttress against the sinusoidal wall to seal off the emissary veins which pass obliquely along the inner and outer longitudinal layer, an extension from the ischiocavernosus and bulbospongiosus muscles. The 360° inner layer encircles a cavernosal membrane to completely contain the erectile sinusoids and—together with the intra-cavernosal pillars—supports the cavernosal sinusoids. The distal ligament is an os penis analog and is arranged centrally and acts as a trunk of the glans penis to maintain patency of the distal urethra and preserve the range of ejaculation; it helps maintain the integrity of the entire penile structure while an intracavernosal pressure surge is conveyed distally. These structures, and their respective functions, are all indispensable for achieving penile erection and physiology.

Important Neuronal Center and Neurotransmitter for Erection

It is difficult, if not impossible, to enjoy a love life without a healthy working central nervous system. This is especially true for sexual activity because erection, emission, ejaculation, and orgasm are specifically coordinated by corresponding areas in the brain. The medial preoptic nucleus, paraventricular nucleus of the hypothalamus, and the limbic system, via the action of oxytocin, melanocortin and dopamine, are responsible for stimulating the spinal autonomic centers to cause erections. The sympathetic spinal center is located in the 12th thoracic vertebra to the 2nd lumbar vertebra and the parasympathetic spinal center is located in the 2nd to 4th vertebrae of the sacral spine. The baseline sympathetic tone originates from the intermediolateral gray matter of the thoracolumbar spinal cord in the 1st thoracic vertebra to 2nd lumbar vertebra. As a rule, a functional penis requires

a functional heart. Penile sympathetic stimulation flows through several pathways, including the sympathetic chain ganglia (in the 1st thoracic vertebra) which also supply such structures as the heart and vascular system via the splanchnic nerve in the 5th thoracic vertebra to the 12th lumbar vertebra. The autonomic nerve fibers innervate the helicine arteries. The cholinergic nerve endings stimulate the nitrous oxide-synthase (NOS) and therefore the release of nitrous oxide (NO), which is the principal neurotransmitter causing penile erection.

Adrenergic nerve fibers and receptors are present in the cavernous trabeculae and surround the deep penile arteries. Sympathetic contraction is thought to be mediated by activation of postsynaptic alpha-adrenergic receptors and modulated by presynaptic alpha-adrenergic receptors. Noradrenaline is the major neurotransmitter controlling penile flaccidity and tumescence. Acetylcholine is required for vascular smooth muscle relaxation, and cholinergic nerves have been demonstrated within the cavernosal smooth muscle and surrounding penile arteries.

Non-noradrenergic, non-cholinergic (NANC) neurons release NO, which in turn increases the production of cyclic guanosine monophosphate (cGMP), a second messenger, causing cavernosal smooth muscle relaxation. Other neurotransmitters, including vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), prostaglandins, and other peptides, are also thought to be involved in the erectile process. Therefore, it is understandable that both sexual stimulation such as sexual thoughts (psychogenic stimuli) and genital touch (reflexogenic stimuli) can produce a penile erection.

Human Erection Mechanism

Erection Physiology

The human erection results from a complex interplay of psychological, hormonal, neurologic, vascular, and cavernosal contributors with a neuronal and endothelial nitric oxide effect. A firm erection requires good arterial blood inflow, trapping of sinusoidal blood, and limiting venous outflow of erection-related veins. It is commonly agreed to use six phases for describing an erection cycle. In order to provide a comprehensive overlook, the Table 1 Summary details the entire erection process. The autonomic nervous system controls penile state, which varies between flaccid and erectile states; two system sub-branches, the sympathetic and parasympathetic, are located in the 12th thoracic to 2nd lumbar vertebra and in the 2nd to 4th sacral spine, respectively. The higher center for the autonomic system is in the thalamus, and, unsurprisingly, the sympathetic and parasympathetic systems are also appointed to the thoracolumbar and craniosacral systems. The penis is usually kept in a spongy-soft flaccid state (Fig. 4) by a baseline sympathetic tone, which precipitates release of norepinephrine from penile adrenergic nerves, resulting in tonic contraction of the smooth muscle cells of the sinusoidal trabecula and penile arterioles as well. The human penis itself is a hydraulic system which depends on the vascular system; thereafter, this smooth muscle contraction results in significant resistance to arterial blood inflow and prevents cavernosal sinusoids from expanding, thereby keeping the penis in the flaccid state (flaccid phase). Not surprisingly, norepinephrine is released when a man experiences pain or stress, which in turn fades an erection even if the penis is in a fully erect state. Norepinephrine also likely inhibits the release of nitric oxide, thereby further inhibiting stimulation of erections; consequently, the male penis is kept in a flaccid state. Its counterpart parasympathetic system plays a role in re-starting the erection process.

Whenever sexual arousal ensues—originating either from the brain or from peripheral stimulation (latent phase)—there is a rapid impulse increasing parasympathetic activity which overrides the basal sympathetic tone. Increased parasympathetic tones result in a decrease in norepinephrine levels and an increase in acetylcholine release, which in turn increases nitric oxide synthase activity; subsequently, nitric oxide releases from both non-adrenergic, noncholinergic neurons and endothelial cells of vascular tissue and cavernosa sinusoids. Then, nitric oxide enhances, or inhibits the degradation, of both intracellular second messengers—cGMP (cyclic guanosine monophosphate) and cAMP (cyclic adenosine monophosphate). A profound smooth muscle relaxation (especially in the cavernosal sinusoids of the corpora cavernosa) results in encouraging helicine artery supply, increasing sinusoidal expansion, and limiting venous drainage outflow of erection-related veins. It is well-elucidated that increasing nitric oxide neurotransmitters stimulates adenylate cyclase to elevate intracellular levels of cGMP. Both cGMP and cAMP are potassium channel openers that lead to hyperpolarization within the cells and, subsequently, cause closure of voltage-dependent calcium

Table 1 Summary of six phases for the erection process

Phases
Flaccid: Resting state with minimal arterial inflow at around 2–3 mL/min, which is good for physiological purposes
Latent: Sexual stimulation, either central or local, causes increase in arterial inflow which could elevate to 60–80 mL/min
Tumescence: Rapid elongation and girth expansion of penis resulting from sinusoidal expansion to limit the outflow of erection-related veins ^a
Full erection: Complete penile expansion characterized by high intra-corporeal pressure and decreased arterial inflow
Rigid erection: Ischiocavernosus and bulbospongiosus muscle contraction with further surge in intracavernosal pressure
Detumescence: Increased venous outflow and decreased arterial inflow until a flaccid state is resumed

^aPenile erection-related veins (PERV) include one deep dorsal vein, two cavernosal veins, and two pairs of para-arterial veins.

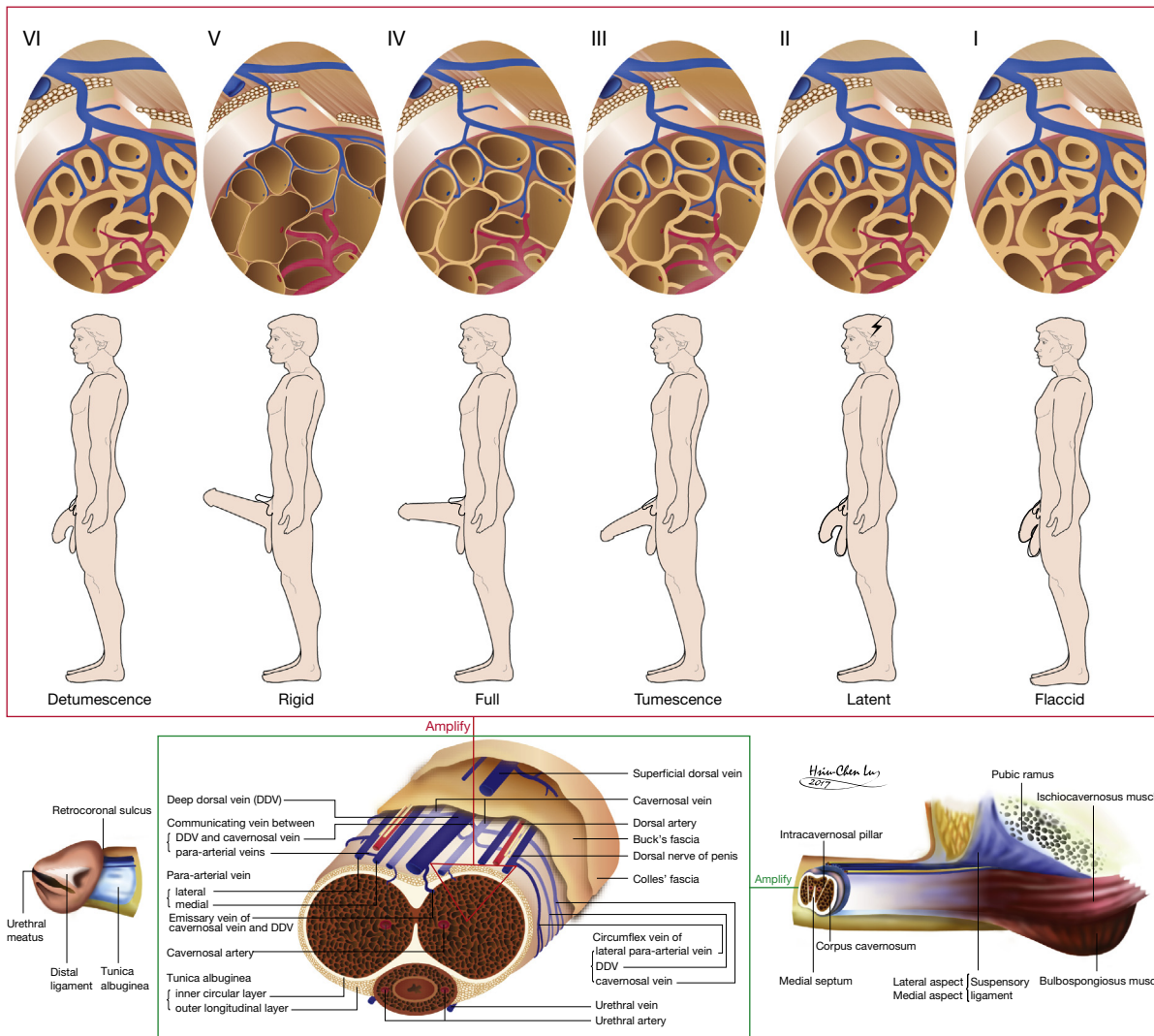


Fig. 4 A schematic illustration of the human erection process in six-phase cycle. Bottom: Lateral aspect. The penis leans upon the suspensory ligament, which is an extension of the linea alba. It is capped by the glans penis. Proximally, the corpus spongiosum is held by the bulbospongiosus muscle, in which the fibers are mostly transverse. The corpora cavernosa are surrounded by the tunica albuginea, which is a bi-layered structure consisting of an inner circular and an outer longitudinal layer with multiple sublayers. The intracavernosal pillars, which may be considerably larger distally, are a continuation of the inner circular layer. The corpus cavernosum is entrapped in the ischiocavernosus muscle, with the muscle fibers aligned in the longitudinal direction. Note the distal ligament within the glans penis. In the middle, a cross-section of the corpora cavernosa depicts the anatomy of the penile shaft. Uppermost: Pictures I–VI depict, step by step, that the penile helicine artery and cavernosal sinusoids dilate, thereby blocking the emissary veins; also depicted is the cavernosal sinusoids' response when a central sexual arousal ensues. The erection process is divided into six phases: flaccid, tumescence, latent, full, rigid, and detumescence, with the final step leading back to a flaccid state.

channels, which, along with intracellular organelle sequestration of calcium, leads to decreased intracellular calcium levels. An intracellular calcium drop causes the corporal smooth muscle relaxation, which enhances active dilation of the penile arteries, arterioles, and sinusoids, resulting in increased arterial inflow and sinusoidal expansion and in limited penile venous outflow of the erection-related veins. This ultimately leads to elevated intracavernosal pressure against the outer tunical layer of the corpora cavernosa.

Fulfilling effective erectile function requires a delicately synchronized cavernosal smooth muscle action, which is made possible by the presence of gap junctions located in the membranes of adjacent muscle cells. It is very likely that this synchronized erectile activity plays a pivotal role in the erection process because cavernosal smooth muscle tissue is only sparsely innervated by nervous tissue. These gap junctions allow the free exchange of a second-messenger molecules and larger ions such as calcium, which is indispensable for coordinated smooth muscle erectile activity. Active dilation of the cavernous and helicine arteries of the penis is the first event to occur during erection. This is closely followed by trabecular smooth muscle relaxation, which increases the compliance of the sinusoids, causing them to expand to accommodate the increased blood inflow. This expansion causes compression of the sub-tunical venous plexuses and emissary veins between the compliant venous sinusoids and the surrounding

noncompliant tunica albuginea—particularly the outer longitudinal layer—thereby reducing venous outflow from the penis (tumescence phase). The rapid increases in penile arterial inflow results in pressure elevation within the penis that is comparable to systemic arterial levels. These arterial inflows include the bulbourethral artery, the cavernosal artery, and the dorsal artery to supply the sinusoids in the corpus spongiosum, corpora cavernosa, and glans penis, respectively or jointly. An intracavernosal pressures exceeding 90 mmHg can be regarded as the starting point of erection, and then further rapid elevation in pressure will cause a full penile erection (Full erection phase); in particular, provided that the patient does not suffer from venous leakage, further contraction of the ischiocavernosus and bulbospongiosus muscles raises the pressure several hundred millimeters mercury just within the corpora cavernosa (rigid erection phase). However, the sinusoidal pressure of the corpus spongiosum and glans penis sustains much lower intra-sinusoidal pressure due to the virtual absence of outer tunical layer and intracavernosal pillars. This pressure difference between these anatomical structures guarantees a smooth ejaculation and also provides a “cushion” protection for a sexual partner.

Ejaculation is facilitated by rhythmic contractions of the bulbocavernosus muscles, which contribute into the ventral thickening of the tunica albuginea, positioned at around the 5 and 7 o'clock positions. An intracavernosal pressure surge can be detected because of this anatomical relationship. Ejaculation is a typical sympathetic tone climax which results in noradrenaline secretion surge, which in turn, causes smooth muscle contraction and of course detumescence, ultimately returning the penis back to a flaccid state (detumescence phase). The reciprocating cycle of penile erections described in the preceding paragraphs is perpetual unless and until a male suffers from erectile dysfunction.

Tunica Outer Longitudinal Layer Is a Key Structure for the Erection Mechanism

Although the human penile anatomy and erection physiology have been extensively studied, we may still not thoroughly understand this unique organ. In the last few decades we have endeavored to shed light on the penile tunical and venous anatomy with the discovery of a bi-layered tunica and an intriguing model of penile erection-related veins. The former substantiates a model of the tunica albuginea of the corpora cavernosa as a bi-layered structure with a 360° complete circular layer (smooth muscle component) and a 300° incomplete outer longitudinal coat (skeletal muscle component) in contrast to a single inner circular layer. (This anatomical discovery raises additional questions: How can the erection process be fulfilled if no outer longitudinal layer exists? How can emissary veins be sealed off when passing the tunica if there is just one single tunical layer?) The latter proves that one deep dorsal vein, two cavernosal veins, and two pairs of para-arterial veins (as opposed to one single deep dorsal vein) are situated between Buck's fascia and the tunica albuginea for corpora cavernosa drainage. Thus, the corpora cavernosa communicates with the larger circulatory system via the penile arterial and venous vasculature.

During the Second World War, a critically injured soldier exhibited a rigid postmortem erection following the administration of an intracavernous blood transfusion at 25 mL/min. This incident implies that the human erection could prove to be, at its core, a mechanical matter. While it is generally accepted that the human erectile mechanism involves an intricate interplay among hormonal, vascular, neurological, sinusoidal, pharmacological, and psychological factors, the mechanism itself and the relative contribution of each respective component remain somewhat unclear, and merit further study. To identify a research model for differentiating venogenic factors from psychogenic factors in the ED contributor list, cadavers were deliberately chosen. A dynamic cavernosometry was performed in 48 cadavers for various hemodynamic studies (Table 2). Between 2002 and 2003, five fresh cadavers were obtained for demonstrating the pivotal role of the penile erection-related veins in a penile erection. That study was criticized for using normal saline with a speed of 150 mL/min, which far exceeds the arterial inflow rate of 60–80 mL/Min in human penile physiology. A second criticism questioned whether and how we could prove that the sinusoidal cells were totally dead. Although normal saline is used for volume expansion in medical purposes, the viscosity coefficient is zero, and, consequently, the infusion rate will be abnormally high. We therefore use 10% colloid and defrosted cadavers to address the two concerns. We investigated the role of venous outflow to isolate the key determinant of erectile function. The dynamic infusion cavernosometry and cavernosography were conducted on 15 defrosted human cadavers, both before and after the meticulous removal and ligation of penile veins. Preoperatively, an infusion rate of more than 28.1 mL/min (14.0–85.0 mL/min) was required to induce a rigid erection. Following surgery, we obtained the same result at a rate of 7.3 mL/min (3.1–13.5 mL/min) across the entire sample. The rigid erection is a mechanical phenomenon contingent on venous competence, and penile veins are the principal component

Table 2 Summary of hemodynamic study on 48 valid human male cadavers in varied purposes from 2002 to 2013

Grouping	Cadaver		Infusion rate or volume			Purpose of study for penile erection-related veins ^b
	No.	Study time	Fluid	Preoperative ^a	Postoperative	
Fresh	5	2002–2003	Normal saline	1050 mL	89 mL	The pivotal role in erection
Defrosted	15	2009–2010	10% colloid	28.1 mL/min	7.3 mL/min	The principal component in erectile rigidity
Defrosted	7	2010–2012	10% colloid	30.2 mL/min	2.8 mL/min	Venous drainage of the corpora cavernosa
Defrosted	11	2011–2013	10% colloid			Role in penile veno-occlusive Mechanism
Total	48					

^aThe PERV stripping surgery.

^bPenile erection-related veins (PERV) include one deep dorsal vein, two cavernosal veins, and two and pairs of para-arterial veins.

for erectile rigidity. Similarly, seven cadavers were studied for exploring the venous drainage of the corpora cavernosa. Eventually, 11 valid defrosted cadavers were studied via electrocautery ($n = 6$) or ligation ($n = 5$); after exposing the defrosted cadaveric penises to electrocautery with varied intracavernous pressure, we were able to prove (indirectly) that the outer tunica acts as the determinant structure of the veno-occlusive mechanism.

Clinical Relevance of Penile Erection

Diseases of the Penile Vascular System

There are penile arterial insufficiency and penile venous occlusive dysfunction in this disease entity. The failure to attain and/or maintain a rigid erection is a common symptom of penile arterial insufficiency or veno-occlusive dysfunction. Poor penile arterial function is commonly regarded as a major cause of vasculogenic erectile dysfunction; however, penile veno-occlusive dysfunction may be the far more prevalent one. The former is frequently regarded as an early sign of cardiovascular disease. Either arterial reconstruction or endovascular treatment are feasible options. Generally, any measure that benefits cardiovascular health will potentially improve penile arterial function. The prevalence of penile veno-occlusive dysfunction may be more widespread than commonly believed. Most impotent males suffer from this problem; one set of treatment options is characterized by venous intervention. Penile venous stripping is superior among these options, which also includes conventional venous ligation, venous coil embolization, and venous sclerotherapy.

Diseases of the Penile Cavernosal Sinusoids

The corpora cavernosa comprise the most ideal milieu in the human body in which to apply the Pascal's law. It is therefore the structure that is most vulnerable to malfunction resulting from penile arterial insufficiency, cavernosal fibrosis, and excessive venous drainage. The cavernosal fibrosis may be the most ill-defined disease despite extensive research. In the natural physiology, penile cavernosal sinusoids are not vulnerable to the aging process because the sinusoidal walls are at rest most the time, which distinguishes them from other end artery organs such as the retina (which typically rests only at night) and the kidney (which works 24 h/day). Penile implants remain the gold standard if cavernosal fibrosis is so severe that all other medical treatments fail.

Diseases of the Penile Spongiosal Sinusoids

The corpus spongiosum is the structure for guarding the passage of urine and ejaculate. A tethered penile ventral deviation happens when a fibrosis of the corpus spongiosum is severe enough to constrain the elongation ability; such a deviation also potentially constrains the passage of ejaculate.

Diseases of the Glans Sinusoid

The glans penis should not be completely rigid when the penis is erect; such rigidity would diminish the "cushion" effect between the penile shaft and the vaginal wall and thereby, as well, the patency of urethral ejaculation. However, some patients present concerns about an excessively soft glans, and questions regarding this matter are frequently asked in clinical setting. The character of the glans sinusoids is specific and divergent from that of the sinusoids in the corpora cavernosa and corpus spongiosum, respectively. Excessive venous drainage should be ascribed if a patient complains his glans is too supple, although a psychogenic origin cannot be overlooked.

Diseases of the Tunical Albuginea

The erectile morphology is determined by the tunica albuginea, which is the major component of the penile fibroskeletal system. Penile dysmorphology may be due to congenital penile curvature and acquired Peyronie's disease. In addition, a severe ventral curvature is not uncommonly presented because a tethering force is generated from a fibrotic corpus spongiosum, e.g., one affected by urethral manipulation syndrome, which is a term that describes urethral fibrosis caused by insertion of a catheter or other endoscopic instrument. In the most severe cases, surgical intervention is still the best viable option.

Diseases of the Distal Ligament

Although the distal ligament—an os analog in the human penis—is a relatively newfound structure, it is deemed an indispensable structure of penile integrity. This structure is so paramount that a traumatic disruption, although rare, causes the inability to engage in penile coitus despite otherwise intact erectile function of the corpora cavernosa. A surgical repair is required if traumatic disruption occurs.

Priapism

Priapism describes a situation in which the human penis remains in a rigid erectile state for over 6 h despite the absence of sexual stimulation. It is not uncommon to encounter in a clinical setting. It is commonly categorized into ischemic, non-ischemic, and recurrent ischemic types. The most prevalent is ischemic priapism, and it occurs when blood does not adequately drain from the corpora cavernosa; it is generally painful, resulting in a corporeal rigidity but a pale and non-engorged glans penis. On the contrary,

in non-ischemic priapism—as the name suggests—the penile erection veins are not completely blocked. Non-ischemic priapism typically results from a shunt formed between the corpus spongiosum and an artery or disruption of the nerve of the parasympathetic nervous system, which enhances penile arterial inflow.

Medical history and physical examination is the first impression of the priapism which is further documented by sonography and cavernosal blood gas analysis. The most common cause of ischemic priapism is sickle cell disease followed by medication such as selective serotonin re-uptake inhibitors (SSRIs), cocaine, cannabis, and intracavernous injection agents such as papaverine or prostaglandin. Treatment strategy aims on terminating the underlying cause. In treating patients with ischemic priapism, it is typically advised to administer a nerve block (for pain relief) followed by aspiration of blood from the corpus cavernosum. A cavernosal measurement is advised by cold normal saline irrigation or, if that has no effect, then a phenylephrine injection. Non-ischemic priapism is often treated with cold packs and compression. Shunting surgery may be required if standard measures are not effective. Sequelae include erectile dysfunction and penile deviation.

Further Reading

- Anderson, K., & Stief, C. (2000). Penile erection and cardiac risk: pathophysiologic and pharmacologic mechanism. *The American Journal of Cardiology*, 86, 23–25.
- Elhanbly, S., Abdel-Gaber, S., Fathy, H., El-Bayoumi, Y., Wald, M., & Niederberger, C. S. (2004). Erectile dysfunction in smoker: a penile dynamic and vascular study. *Journal of Andrology*, 25, 991–995.
- Hsieh, C. H., Wang, C. J., Hsu, G. L., Chen, S. C., Ling, P. Y., Wang, T., et al. (2005). Penile veins play a pivotal role in erection: the hemodynamic evidence. *International Journal of Andrology*, 28, 88–92.
- Hsieh, C. H., Huang, Y. P., Tsai, M. H., Chen, H. S., Huang, P. C., Lin, C. W., et al. (2015). Tunical outer layer plays an essential role in penile veno-occlusive mechanism evidenced from electrocautery effects to the corpora cavernosa in defrosted human cadavers. *Urology*, 86, 1129–1136.
- Hsu, G. L. (2011). Physiological approach to penile venous stripping surgical procedure for patients with erectile dysfunction. Google Patents; Patent No: US 8,240,313 B2 <http://www.google.com/patents/US20110271966>.
- Hsu, G. L., Hsieh, C. H., Wen, H. S., Chen, Y. C., Chen, S. C., & Mok, M. S. (2003). Penile venous anatomy: an additional description and its clinical implication. *Journal of Andrology*, 24, 921–927.
- Hsu, G. L., Hsieh, C. H., Wen, H. S., Hsu, W. L., & Chen, C. W. (2004). Anatomy of the human penis: the relationship of the architecture between skeletal and smooth muscles. *Journal of Andrology*, 25, 426–431.
- Hsu, G. L., Lin, C. W., Hsieh, C. H., Hsieh, J. T., Chen, S. C., Kuo, T. F., et al. (2005). Distal ligament in human glans: a comparative study of penile architecture. *Journal of Andrology*, 26, 624–628.
- Hsu, G. L., Hung, Y. P., Tsai, M. H., Hsieh, C. H., Chen, H. S., & Molodysky, E. (2012). Penile veins are the principal component in erectile rigidity: a study of penile venous stripping on defrosted human cadavers. *Journal of Andrology*, 33, 1176–1185.
- Hsu, G. L., Molodysky, E., Liu, S. P., Chang, H. C., Hsieh, C. H., & Hsu, C. Y. (2013). Reconstructive surgery for idealizing penile shape and erectile functional restoration on patients with penile dysmorphology and erectile dysfunction. *Arab Journal of Urology*, 11, 375–383.
- Hsu, G. L., Huang, Y. P., Tsai, M. H., Chang, H. C., Liu, S. P., Molodysky, E., et al. (2013). The venous drainage of the corpora cavernosa in the human penis. *Arab Journal of Urology*, 11, 384–391.
- Lue, T. F. (1998). Physiology of penile erection and pathophysiology of erectile dysfunction and priapism. In P. C. Walsh, A. B. Retik, et al. (Eds.), *Campbell's urology* (pp. 1155–1174). Darien: Saunders.
- Nehra, A., Goldstein, I., Pabby, A., Nugent, M., Huang, Y. H., de las Morenas, A., Krane, R. J., Udelson, D., Saenz de Tejada, I., & Moreland, R. B. (1996). Mechanism of venous leakage: a prospective clinico-pathological correlation of corporeal function and structure. *The Journal of Urology*, 156, 1320–1329.
- Newman, H. F., Northrup, J. D., & Devlin, J. (1964). Mechanisms of the human penile erection. *Investigative Urology*, 1, 350–355.
- Wallach, S. J., & Hart, B. L. (1983). The role of the striated penile muscles of the male rat in seminal plug dislodgement and deposition. *Physiology & Behavior*, 31, 815–821.

Penis Endocrinology

Jing-yao Liang, Kuo General Hospital, Tainan, Taiwan; and Chang Jung Christian University, Tainan, Taiwan

Hong-Chiang Chang, National Taiwan University, Taipei, Taiwan

Geng-Long Hsu, Hsu's Andrology and National Taiwan University, Taipei, Taiwan

© 2018 Elsevier Inc. All rights reserved.

Glossary

ACTH (adrenocorticotrophic hormone) Also known as corticotropin, is a polypeptide tropic hormone produced and secreted by the anterior pituitary gland.

Adrenarche It is a term to describe an early stage of sexual maturation in many higher primates that in human occurs at some 10–11 years of age. In boys, the principal physical consequences of adrenarche are especially pubic hair growth, change of sweat composition that produces adult body odor, increasing oiliness of the skin and hair and mild acne may occur.

Aldosterone The main mineralocorticoid hormone is a steroid hormone produced by the zona glomerulosa of the adrenal cortex in the adrenal gland.

Androgen A category of natural or synthetic compounds, mostly steroid hormones that stimulate or control the development and maintenance of male characteristics.

Androgen receptors It is a type of receptor in the nucleus of a cell, which is activated by binding androgenic hormones such as testosterone and dihydrotestosterone in the cytoplasm and then conveyed into the nucleus. It plays mainly as a DNA-binding transcription factor that regulates gene expression.

CAH (congenital adrenal hyperplasia) Any of several autosomal recessive diseases resulting from mutations of genes for enzymes mediating the biochemical steps of production of mineralocorticoids, glucocorticoids or sex steroids from cholesterol by the adrenal glands (steroidogenesis).

DHEA (dehydroepiandrosterone) The primary precursor of natural androgens and estrogens. It is also named as dehydroisoandrosterone or dehydroandrosterone.

DHT (dihydrotestosterone) A metabolite of testosterone, and a more potent androgen than testosterone in terms of affinity toward androgen receptors.

Erectile dysfunction The inability to either attain or maintain a rigid erection for satisfactory coitus.

GH (growth hormone) It is also known as somatotropin, a peptide hormone that stimulates growth, cell reproduction, and cell regeneration in humans and other animals.

IGF-1 (insulin-like growth factor 1) A hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults.

Pubarche It is a term to describe the first appearance of pubic hair in a child. It usually results from rising levels of androgens from the testes or adrenal glands and is one of the prominent physical changes of puberty.

SHBG (sex hormone-binding globulin) or sex steroid binding globulin (SSBG) A glycoprotein that binds to the two sex hormones: androgen and estrogen.

SRY (sex-determining region Y protein) A DNA-binding protein (also known as gene-regulatory protein/transcription factor) encoded by the SRY gene that is responsible for the initiation of male sex determination in humans.

Testosterone The primary male sex hormone, an anabolic steroid, which plays the key role in the development of male reproductive tissues such as prostate, testicles and promoting secondary sexual characteristics such as hair growth and increasing muscle and bone mass.

Introduction to Androgens and Other Hormones That Affect the Penis

Androgens refer to a number of natural or synthetic compounds, mostly steroid hormones that stimulate or control the development and maintenance of male characteristics in vertebrates. They exert their action through binding to and activation of the androgen receptor which is a ligand-dependent nuclear transcription factor and a member of the steroid hormone nuclear receptor family. Given its widespread scattering in many tissues and specific cells, the androgen receptor has a diverse range of biological actions including pivotal roles in the development and maintenance of the cardiovascular, immune, neural, musculoskeletal, hemopoietic and reproductive systems.

Dehydroepiandrosterone (DHEA) is synthesized from cholesterol (**Fig. 1**). Although it functions as an endogenous precursor to more potent androgens such as testosterone and dihydrotestosterone (DHT), DHEA has been found to possess some degree of androgenic activity in its own right, acting as a low affinity ($K_i = 1 \mu\text{M}$), weak partial agonist of the androgen receptor. DHEA and DHEA-S, its sulfate metabolite, also act as a neurosteroid and neurotrophin. Although a relatively weak androgen, DHEA is responsible for the androgenic effects of adrenarche, such as early pubic and axillary hair growth, adult-type body odor, increased

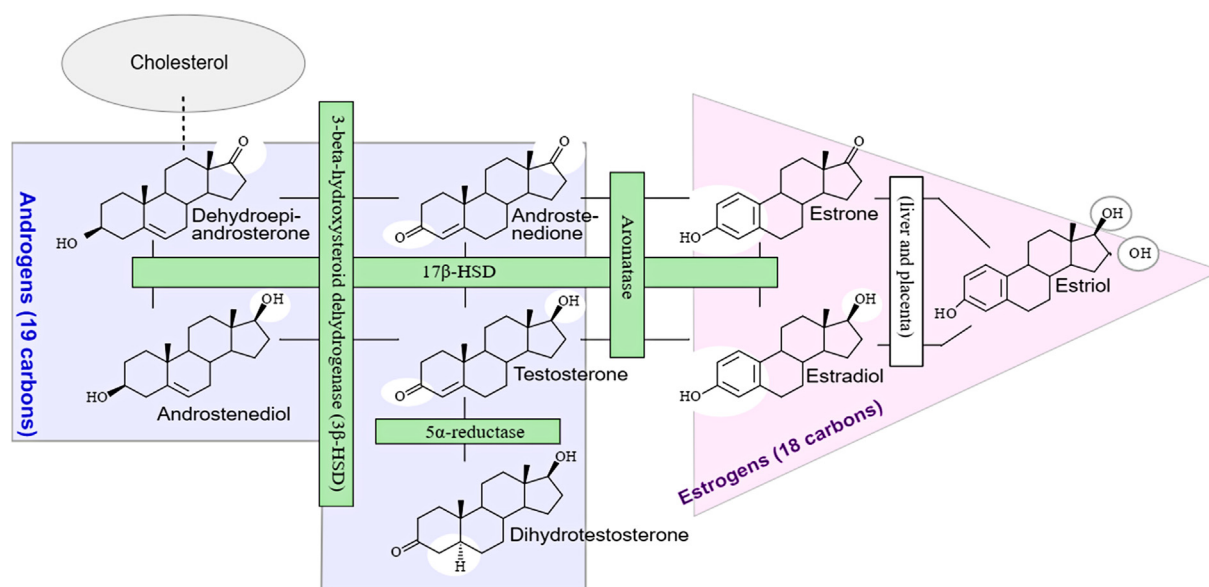


Fig. 1 Illustrative algorithms in human androgen production. Endogenous production of androgens and estrogens from the cholesterol which is the precursor. Originally from Häggström M, Richfield D (2014). "Diagram of the pathways of human steroidogenesis". WikiJournal of Medicine 1 (1). <https://doi.org/10.15347/wjm/2014.005>. ISSN 20024436.

oiliness of hair and skin, and mild acne. DHEA is also used orally as a medication and dietary supplement, to restore or increase DHEA and DHEA-S levels.

Testosterone is the primary and most well-known androgen. In men, testosterone plays a key role in the development of male reproductive tissues such as the testis and prostate, as well as promoting secondary sexual characteristics such as increased muscle and bone mass, and the growth of body hair. In addition, testosterone is involved in health and well-being, and the prevention of osteoporosis. Testosterone is biosynthesized in several steps from cholesterol and is converted in the liver to inactive metabolites. It is secreted primarily by the testicles of males and, to a lesser extent, the ovaries of females. Small amounts are also secreted by the adrenal glands. Testosterone is also an anabolic steroid, which is related to growth of muscle mass and strength, increased bone density and strength, and stimulation of linear growth and bone maturation.

Testosterone levels are at their highest during adolescence and early adulthood. The first physical signs of testosterone, or androgens, in the body are apparent during puberty. A boy's voice changes, his shoulders broaden, and his facial structure becomes more masculine. But as men get older, their testosterone levels decline about 1% per year after age 30 (Table 1).

Dihydrotestosterone (DHT), or 5α-dihydrotestosterone (5α-DHT), is an endogenous androgen. The enzyme 5α-reductase catalyzes the formation of DHT from testosterone in certain tissues including the prostate gland, seminal vesicles, epididymides, skin, hair follicles, liver, and brain. Relative to testosterone, DHT is considerably more potent as an agonist of the androgen receptor (AR). During male embryogenesis DHT has an essential role in the formation of the male external genitalia, while in the adult male DHT

Table 1 Demographic distribution of testosterone level in human

Male		Female	
Age	T level (ng/dL)	Age	T level (ng/dL)
0–5 months	75–400	0–5 months	20–80
6 months to 9 years	<7–20	6 months to 9 years	<7–20
10–11 years	<7–130	10–11 years	<7–44
12–13 years	<7–800	12–16 years	<7–75
14 years	<7–1200	17–18 years	20–75
15–16 years	100–1200	19+ years	8–60
17–18 years	300–1200		
19+ years	240–950		
Average adult male	270–1070	Average adult female	15–70

30+ years – 1% per year.

This chart breaks down the normal ranges of testosterone by age and gender, according to the Mayo Clinic.

acts as the primary androgen in the prostate gland, seminal vesicles, skin, and hair follicles. It is, therefore, notorious for the prostate hyperplasia which, in turn, causes urinary obstruction in geriatric males.

Growth hormone (GH), also known as somatotropin, is a peptide hormone that stimulates growth, cell reproduction, and cell regeneration in humans and other animals. After its synthesis in the anterior pituitary gland, GH is released into the blood stream and then stimulates the liver to produce insulin-like growth factor-1 (IGF-1). IGF-1 then stimulates systemic body growth and has growth-promoting effects on almost every cell in the body, especially skeletal muscle, cartilage, bone, liver, kidney, nerves, skin, hematopoietic cell, and lungs. An animal model showed that IGF-1 promotes proliferation and migration of cavernous smooth muscle cells. IGF-1 also stimulated vascular endothelial growth factor (VEGF) secretion from cavernosal smooth muscle cells. Some clinics attempt to induce a penile enlargement by injecting recombinant human GH or IGF-1 into the corpus cavernosum, but there is no solid evidence supporting such practice.

Aldosterone is a steroid hormone produced by the zona glomerulosa of the adrenal cortex in the adrenal gland. It is essential for sodium conservation in the kidney, salivary glands, sweat glands, and colon. It plays a central role in the regulation of the plasma sodium (Na^+), the extracellular potassium (K^+) and arterial blood pressure. An in-vitro study on isolated human penile corpus cavernosal tissue showed that aldosterone has no direct contractile action or a relaxant action on human penile cavernous tissue, but acts to significantly enhance the noradrenaline-induced contraction. The effect on the noradrenaline-induced contraction is probably caused by aldosterone enhancing the affinity of the alpha-receptors for noradrenaline in isolated human penile corpus cavernosal tissue. The study suggested that aldosterone acts to enhance contraction of corpus cavernosal tissue, and is one of the restraining factors for human penile erection.

How Hormones Affect the Penis in Different Stages of Life

Prenatal Stage

In the human being, sexual differentiation is the process of developing observable traits difference on reproduction tracts between males and females. This includes the development of different genitalia and the internal genital tracts, breasts, body hair, and plays a role in gender identification. At an early stage in embryonic development, both sexes possess equivalent internal structures. These are the mesonephric ducts and paramesonephric ducts. As females have two X chromosomes, and males have one Y chromosome in addition to an X chromosome. The presence of the SRY gene on the Y chromosome causes the development of the testes in males, and the subsequent release of hormones which cause the paramesonephric ducts to regress.

By 7 weeks, a fetus has a genital tubercle, urogenital groove and sinus, and labioscrotal folds. Males become externally distinct between 8 and 12 weeks, as androgens enlarge the phallus and cause the urogenital groove and sinus to fuse in the midline, producing an unambiguous penis with a phallic urethra, and a thinned, rugated scrotum. In this stage, the role of testosterone is far smaller than that of dihydrotestosterone (DHT), and the latter will differentiate the remaining male characteristics. This reminds us that DHT plays so important role that the external genitalia can form normally and so does the prostate hyperplasia at males' early and late life respectively.

Early Infancy

In the first weeks of life for male infants, testosterone levels rise. The levels remain in a pubertal range for a few months but usually reach the barely detectable levels of childhood by 4–6 months of age. However, in this stage, no significant changes have been identified in other parts of the body.

Before Puberty

Before puberty effects of rising androgen levels occur in both boys and girls. These include adult-type body odor, increased oiliness of skin and hair, acne, the appearance of pubic hair (i.e., pubarche), axillary hair (armpit hair), growth spurt, accelerated bone maturation, and facial hair. Erection of the penis is common for a prepubertal boy, but not until puberty does an "adult type" penis growth occur.

Puberty

Pubertal effect is commonly termed pubarche which begins to occur when androgen has been higher than normal adult female levels for months or years. In males, these are usual late pubertal effects and occur in women after prolonged periods of heightened levels of free testosterone in the blood. The effects include lots of events such as growth of spermatogenic tissue in testicles, male fertility, penis enlargement, increased libido and frequency of erection, remodeling of facial bone contours in conjunction with human growth hormone, completion of bone maturation and termination of growth, increased muscle strength and mass, shoulders become broader, rib cage expands, deepening of voice, growth of the Adam's apple, enlargement of sebaceous glands, and altered body hair growth such as pubic hair, facial hair, chest hair, periareolar hair, perianal hair, and armpit hair etc.

According to the National Center for Health Statistics, the upper 95th percentile in the United States for onset age of puberty for boys is 14 (an increase in testicular size being the first sign).

Reproductive Stage

Progressive genital enlargement lasts into the third decade of life and is the result of increasing testosterone production from the testes and androgens from the adrenals. The period is characterized by an increase in the size of the penis, erections, sexual hair development, increase in muscle mass, acne, seborrhea, and a decrease in body fat.

Aging Stage

Testosterone levels reach a peak level around the third or fourth decade of life, then gradually reduce as men age. This effect is sometimes referred to as andropause or late-onset hypogonadism (LOH). Low testosterone levels can cause changes in sexual function, including reduced sexual desire, or low libido, fewer spontaneous erections, erectile dysfunction; infertility, and shrinkage of a penis. LOH is also associated with emotional changes, such as low self-confidence or lack of motivation; and physical changes, like increased body fat, reduced muscle bulk and strength, and decreased bone density.

Diagnosis and Management of Endocrine-Related Penile Conditions

Late-Onset Hypogonadism (LOH)

LOH is a clinical and biochemical syndrome associated with advancing age and characterized by typical symptoms mentioned in the previous paragraph, and most importantly, confirmed by the evidence of a low serum testosterone level. This testosterone reduction and associated symptom complex have been variously referred to as andropause, thereafter, terms of ADAM (androgen deficiency in the aging male), and PADAM (partial androgen deficiency in the aging male) are introduced.

There is a circadian variation in serum total testosterone. The blood sample should be obtained in the morning for an accurate reading.

As a man ages, the penis tends to undergo an actual reduction in size. The reduction—in both length and thickness—typically isn't dramatic but may be noticeable. If a man's erect penis is 6 in. long when he is in his 30s, it might be 5 or 5-and-a-half inches when he reaches his 60s or 70s. Atherosclerosis and fibrosis of corpus cavernosa are likely two major causes of penile shrinkage (Fig. 2). Albeit this phenomenon is not beyond controversy, it is always complained by a male whose age exceed 60 years suffers from gradual onset of penile dysmorphology associated with erectile dysfunction.

There is a strong association among age, testosterone deficiency, cavernosal fibrosis, and erectile dysfunction. Age, testosterone deficiency and cavernosal fibrosis are potentially correctable factors of cavernosal fibrosis and organic erectile dysfunction. Testosterone replacement therapy (TRT) alone or in association with phosphodiesterase-5 (PDE5) inhibitors, may lower the risk of cavernosal fibrosis or decrease the severity the fibrosis in patients with erectile dysfunction. It may resort to penile venous stripping and tunical patching reconstruction if the disease fails to be free from deterioration chronologically.

Oral testosterone supplement, transdermal delivery, and injections are available routes of TRT. Only symptomatic subjects with documented evidence of low serum testosterone levels should consider having TRT, and the patient should be well informed about benefits, goals, and precautions of TRT. During replacement therapy, key parameters including testosterone, hematocrit, hemoglobin, prostatic specific antigen (PSA), blood pressure and liver function profile should be monitored.

Delayed Puberty

Delayed puberty in boys is defined clinically by the absence or incomplete development of secondary sexual characteristics by an age at which 95% of boys have initiated sexual maturation.

Constitutional growth delay (CGD) is the most common cause of short stature and pubertal delay. Other boys with delayed puberty usually result from hypogonadism (inadequate gonadal steroid secretion) which, in turn, is most often caused by a defective gonadotropin secretion from the anterior pituitary, due to defective production of gonadotropin-releasing hormone (GnRH) from the hypothalamus.

High serum concentrations of LH and FSH are associated with various causes of gonadal disease, called primary hypogonadism and/or defects in their receptors on the membrane of the gonadal cells. Low or normal serum LH and FSH concentrations are associated with diminished GnRH-induced gonadotropin secretion, called secondary hypogonadism. This defect can be because of hypothalamic dysfunction (either anatomic or functional), hypopituitarism, hypothyroidism, or hyperprolactinemia.

Hormone therapy is reserved for patients with confirmed organic gonadotrophin deficiency (hypogonadotrophic hypogonadism), for those with a gonadal abnormality (e.g., Turner's syndrome, Klinefelter's syndrome), and for those with constitutional delay who have an abnormal psychosocial adjustment. Typically, those with constitutional delay require only a short course of intramuscular testosterone to induce puberty.

Patients with organic delay require early diagnosis and treatment to achieve normal development and optimal height and avoid issues of psychosocial adjustment with peers.

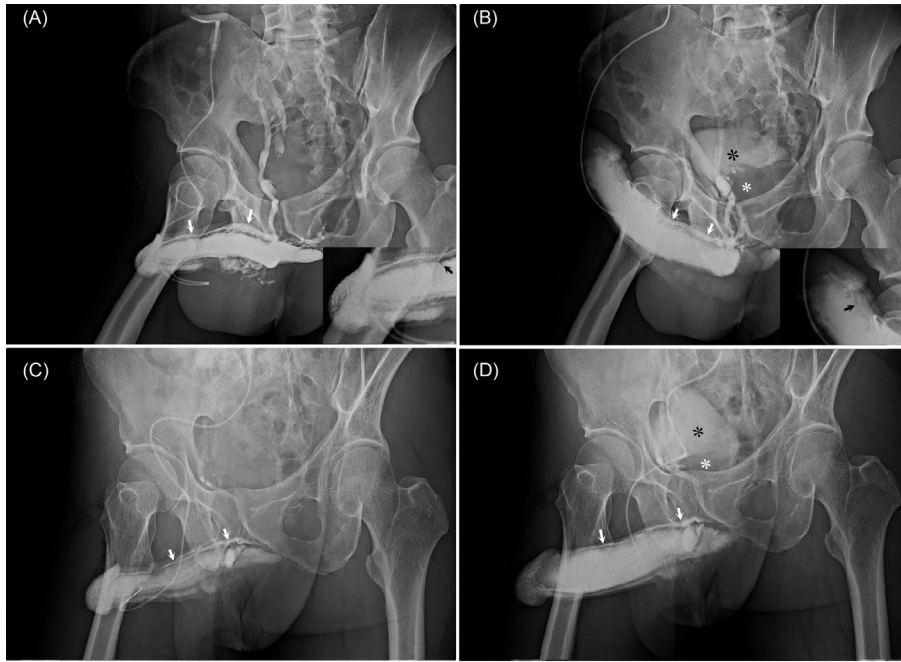


Fig. 2 Cavernosographic evidence of tunical fibrosis in geriatric males? (A) In a 65-year-old man, the plain cavernosogram (30° oblique views) was obtained when 20 mL diluted iohexol solution was injected into the corpora cavernosa via a no. 19 scalp needle. The deep dorsal vein (*white arrows*) was well demonstrated although the cavernosal veins were masked. Note that the radiolucent area (*black arrow*, inserted) was noted along the dorsal aspect of the distal corpora cavernosa. (B) An early phase of the pharmaco-cavernosograms was made 15 min after the 20 µg prostaglandin E1 was injected intra-cavernously via the same scalp needle. Again the deep dorsal vein (*white arrows*) was well shown despite the penis was in a rigid erection. No smooth lineation was noted along the distal tunica albuginea (*black arrow*, inserted) resulting from peripheral sinusoids was tethered by fibrotic tunica. Does the dorsal contour fail to preserve lineation resulting from tunical fibrosis? Note the urinary (*black asterisk*) and the enlarged prostate gland (*white asterisk*) which occupies the bladder base. (C) In a 67-year-old man, the plain cavernosogram (30° oblique views) was obtained when 20 mL diluted iohexol solution was injected into the corpora cavernosa via a no. 19 scalp needle. The deep dorsal vein (*white arrows*) was well noted although the cavernosal veins were masked. Note that the lineation of the dorsal aspect cannot be obvious. (D) Again an early phase of the pharmaco-cavernosograms was undertaken 15 min after the 20 µg prostaglandin E1 was injected intra-cavernously via the same scalp needle. The deep dorsal vein (*white arrows*) was well shown. The contour of the dorsal tunica was not lineated resulting from peripheral sinusoids was tethered by fibrotic tunica. Does the dorsal contour fail to preserve lineation resulting from tunical fibrosis? Note the urinary (*black asterisk*) and the enlarged prostate gland (*white asterisk*) which occupies the bladder base.

Congenital Adrenal Hyperplasia (CAH)

CAH refer to any of several autosomal recessive diseases resulting from mutations of genes for enzymes mediating the biochemical steps of production of mineralocorticoids, glucocorticoids or sex steroids from cholesterol by the adrenal glands (steroidogenesis).

There are six major forms of CAH, each caused by a mutation of one of the six enzymes required for the biosynthesis of cortisol. In each of these forms, the decrease in cortisol secretion results in a decrease in negative feedback at the level of the hypothalamus-pituitary. The resulting increase in adrenocorticotrophic hormone (ACTH) vs. corticotropin-releasing hormone (CRH) output attempts to return the cortisol secretion to normal if the mutation permits some degree of enzymatic activity. At the same time, the increased ACTH secretion results in a markedly elevated production of the cortisol precursors before the mutant block.

Some female infants with classic CAH have ambiguous genitalia due to exposure to high concentrations of androgens in utero. CAH due to 21-hydroxylase deficiency is the most common cause of ambiguous genitalia in genotypically normal female infants (46XX). They may be raised as boys due to enlarged clitoris mimicking penises. Less severely affected females may present with early pubarche. Young women may present with symptoms of polycystic ovarian syndrome which manifests oligomenorrhea, polycystic ovaries and hirsutism.

Males with classic CAH generally have no signs of CAH at birth. Some may present with hyperpigmentation, due to co-secretion with melanocyte-stimulating hormone (MSH), and possible penile enlargement.

Growth Hormone Deficiency and Related Conditions

Congenital GH deficiency is associated with aberrant androgen physiology and micropenis. GH replacement therapy alone can result in a catch-up in phallic size in patients with micropenis secondary to isolated congenital GH deficiency.

While for cases with GHRH (growth hormone releasing hormone) receptor deficiency, the patients have a normal penis size before puberty, whereas microphallus is typical of GH deficiency and GH receptor deficiency. Severe GH deficiency is associated with small penis size with normal penile growth at adolescence or with testosterone treatment in childhood. This is also true of GH receptor deficiency.

Adrenal Virilism or Adrenogenital Syndrome

Adrenal virilism is a syndrome in which excessive adrenal androgens cause virilization, i.e. masculinization. Diagnosis is clinical and confirmed by elevated androgen levels with and without dexamethasone suppression; determining the cause may involve adrenal imaging. There may be functioning adrenal tumors resulting in masculinization of prepubertal children. In boys, it produces a pseudo-precocious puberty. Sexual hair develops and the penis is enlarged to adult size, with frequent erections.

Treatment depends on the cause whereby adrenalectomy, removal of the causation tumor, or oral glucocorticoids for non-tumor hyperplasia is a specific option.

Congenital 5 α -Reductase Deficiency

This is an autosomal recessive intersex condition caused by a mutation in the 5 α reductase type II gene. This genetic mutation can result in pseudohermaphroditism. The condition typically presents with underdeveloped male genitalia and prostate. Males with this condition are often raised as girls due to their lack of conspicuous male genitalia. At the onset of puberty, although still in lack of DHT, their testosterone levels elevate normally. Their musculature develops like that of other male adults. After puberty, men with this condition have a large deficiency of pubic and body hair and reportedly no incidence of androgenic alopecia (pattern hair loss), and no incidence of prostate cancer.

Further Reading

- Brawer, M. K. (2004). Testosterone replacement in men with andropause: an overview. *Revista de Urología*, 6(Suppl. 6), S9–S15.
- Carrillo, A., et al. (2007). *Pediatric endocrinology* (5th edn, vol. 2, pp. 365–384). Wiley-Blackwell.
- Davey, R. A., & Grossmann, M. (2016). Androgen receptor structure, function and biology: from bench to bedside. *Clinical Biochemist Reviews*, 37(1), 3–15.
- Hsu, G. L., et al. (2007). Long-term result of an autologous venous grafting for penile morphological reconstruction. *Journal of Andrology*, 28(1), 186–193.
- Hsu, G. L., Molodysky, E., Liu, S. P., Chang, H. C., Hsieh, C. H., & Hsu, C. Y. (2013). Reconstructive surgery for idealizing penile shape and erectile functional restoration on patients with penile dysmorphism and erectile dysfunction. *Arab Journal of Urology*, 11(4), 375–383.
- Hughes, I. A. (2011). Minireview: sex differentiation. Available: <http://endo.endojournals.org/content/142/8/3281.full>.
- Iacono, F. (2012). Testosterone deficiency causes penile fibrosis and organic erectile dysfunction in aging men. Evaluating association among age, TDS and ED. *BMC Surgery*, 12(Suppl. 1), S24.
- Levy, J. B., & Husmann, D. A. (1996). Micropenis secondary to growth hormone deficiency: does treatment with growth hormone alone result in adequate penile growth? *Journal of Urology*, 156(1), 214–216.
- Liu, X., Lin, C. S., Spencer, E. M., & Lue, T. F. (2001). Insulin-like growth factor-I promotes proliferation and migration of cavernous smooth muscle cells. *Biochemical and Biophysical Research Communications*, 280(5), 1307–1315.
- Muguruma, H., et al. (2008). Effect of aldosterone on isolated human penile corpus cavernosum tissue. *BJU International*, 102(4), 500–503. <https://doi.org/10.1111/j.1464-410X.2008.07536.x>.
- Pescovitz, O. H., & Eugster, E. A. (2004). *Pediatric endocrinology: mechanisms, manifestations, and management* (pp. 991–995). Totowa, NJ: Humana Press Inc.
- Playmate, S. R. (1989). Circadian variation in testosterone, sex hormone binding globulin, and calculated non-sex hormone-binding globulin bound testosterone in healthy young and elderly men. *Journal of Andrology*, 10(5), 366–377.
- Rosenbloom, A. L. (2007). *Pediatric endocrinology* (5th edn, vol. 2, p. 75). Informa healthcare USA, Inc. Chapter 3.
- Young, H. K., & Kim, J. J. (2011). Testosterone replacement therapy for late-onset hypogonadism: current trends in Korea. *Asian Journal of Andrology*, 13(4), 563–568.

Erection Abnormality

Geng-Long Hsu, Hsu's Andrology and National Taiwan University, Taipei, Taiwan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acquired penile deviation A condition that penile curvature results from external tethering force to penile shaft or internal fibrosis of the fibroskeleton of the penis. This term is diverted from the congenital penile deviation which develops without fibrosis within the penis.

Congenital penile deviation A status that penile curvature develops along with penile growing up at adolescence. It is always seen without remarked cause after adult life.

Erectile dysfunction A condition of inability either to attain or maintain a rigid erection for satisfactory coitus. The etiologies vary from iatrogenic to natural course including arteiogenic, neurogenic, psychogenic, pharmacologic, endocrinologic, cavernosal, and venogenic contributions.

Full erection A situation to describe a human penis attaining a complete erection. Both girth widening and length elongation are seen in addition to a rigid erection which is sustainable to buckling pressure.

Hourglass deformity A condition that a penile narrowing portion occurs in the middle penile shaft is so severe that an erectile penis looks like an hour glass.

Partial erection A situation that a penis fails to attain a complete erection resulting from internal penile structure dysfunction or external tethering limitation to the corpora cavernosa.

Penile dysmorphology A situation of the penis with a deformed shape when erection. A convertible term is a penile curvature which is a result of area differences between the convex and concave surfaces of the corpora cavernosa if bisected. It could be categorized as dorsal, ventral, left lateral, right lateral and combined types.

Peyronie's disease A chronic inflammation of the tunica albuginea of the corpora cavernosa in the human penis and also named as induratio penis plastica. It may involve the cavernosal sinusoidal trabeculae along the intracavernosal pillars while fibrotic scar initiated from the tunica albuginea. A painful erection is pronounced at earliest 3 months followed by penile deformity. Spontaneous subside may happen, however surgical intervention may require if deformity persists and interfere normal coitus.

Priapism A condition that persistent penile erection ensues despite no sexual arousal or even painful feeling which always results in detumescence.

Veno-occlusive erectile dysfunction A situation of penile venous leakage which is named to describe an inability of penile erection related veins being sealed off and consequently, fail to limit sinusoidal blood draining from the corpora cavernosa to systemic circulation in each erection bout.

Soft glans syndrome A situation complained by many males who acknowledge their glans penis is too weak to coitus. It is a general term that male subjectively feels one's glans penis being too soft. It may be inadvertently ascribed resulting from excessive drainage of penile erection related veins.

Erection Norm in Human Penis

Anatomy Reason for Penile Erectile Function and Morphology

The human penis appears to be the most peculiar organ which owns its magic extensibility resulting from the hydraulic system which, consequently, attracts most attentions in the entire human body. Albeit human penis has been in its current anatomical form for 3000 centuries, its anatomy and the erection process are still not thoroughly depicted despite extensive studies had been made. Humans penis possess an os analogue associated with some proportionally large and extraordinarily extensible corpora cavernosa which are the ideal milieu to apply Pascal's law in the entire human body if no venous leakage exists. The law depicts that pressure applied to any part of the enclosed fluid transmitted undiminished to every portion of the fluid and to the walls of the containing vessel. The erectile capability of the human penis ensues then and largely depends on sinusoids in the corpus spongiosum, the glans penis within which a distal ligament, and the corpora cavernosa, which are also responsible for erectile rigidity and erectile penile shape. It seems that the human corpora cavernosa are destined to be vulnerable to erectile dysfunction if rigidity is essential for coitus, defined as inability either to attain or maintain a rigid erection for satisfactory coitus.

Penile fibroskeleton is a determinant structure for penile morphology (Fig. 1). Recent studies substantiate a model of the tunica albuginea of the corpora cavernosa as a bi-layered structure with a 360° complete inner circular layer and a 300° incomplete outer longitudinal coat spanning from the bulbospongiosus and ischiocavernosus proximally and extending continuously into the distal ligament within the glans penis. The above two muscles, the entire outer tunica and distal ligament of the penis could be collectively categorized as skeletal components. The inner layer contains and supports the sinusoids via intracavernous pillars, the

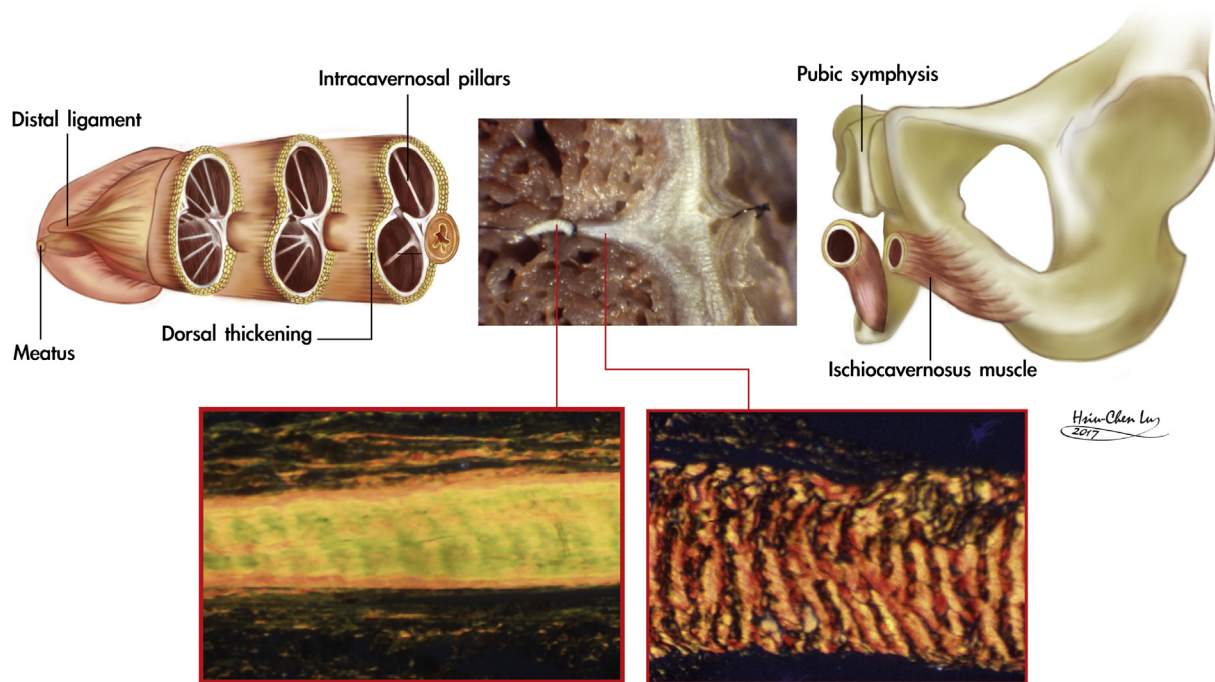


Fig. 1 Extensibility of the fibroskeleton in the human penis. A. The tunica albuginea of the corpora cavernosa is the principal structure of the fibroskeleton in the human penis. It is a bi-layered structure in which a 360 degrees complete inner circular layer, together with the intracavernosal pillars, contains and supports the sinusoids. The extensibility of the intracavernosal pillars (upper middle) is located from 2 or 10 to 6 O'clock positions to enhances the penile rigidity when erection. It can be stretched to a thinner and green appearance (Lower, left) while the collagen type 1 act as a steel-like supporter and the intertwined collagen type 3 shows off, which mimics the erection status. In contrast, simulating a flaccid status (lower, right), its pronounced wavy appearance results from elastic fiber relaxed and the collagen type 1 stands out. (Picrosirius red, 25 $\times$).

erection-related veins, and artery which could collectively be allocated as the smooth muscle components. The delicate interplay between the vascular structures and tunica albuginea results in an erection process which is a mechanical phenomenon. The tunica outer layer plays a pivotal role in penile morphology and erection rigidity. The intracavernosal pillar is distributed between 2 or 10 to 6 O'clock positions (**Fig. 1**) in the penile pendulous portion and is commensurately abundant distally until the distal ligament meet. An abundant elastic component is characteristic for extraordinary extensibility evidenced by the flattened status (Figure, right lower) while presents markedly wavy appearance (Figure, left lower) which correspond to being stretched (erection) and tension free status (flaccid) respectively. Thus penile elongation and girth expansion occur predominantly between the retrocoronal level and penile base, we, thereafter, can acknowledge why and how the flaccid penile shape is not a matter of concern, and only erectile penile morphology is significant because satisfactory coitus requires healthy erectile function which depends upon the sinusoidal expansion. An anatomy concept is interestingly for acknowledging penile rigid erection and morphology.

Although a rigid erection of the corpora cavernosa is initiated by either central or local sexual stimuli, a local penile response is a common endpoint. Therefore, we focus on the corpora cavernosa whereby a function speaks volume. Apart from the regular vascular system for nutrition via the capillaries, there is a system for erectile function in which sinusoids shunt directly from arterial to venous channels bypassing the capillaries. The main source of blood supply to the cavernosal sinusoids originates from the internal pudendal artery (**Fig. 2**) which is the end branch of the internal iliac artery, although accessory contributions may arise from the external iliac, obturator, vesical, and femoral arteries. The internal pudendal artery gives the first branch as the bulbourethral artery which supplies the sinusoids of the corpora spongiosum. The corpora cavernosa are primarily supplied by the cavernosal artery which is the second branch of the internal pudendal artery and most distally the dorsal artery which supplies the glans and the corpus spongiosum.

The sinusoidal blood drains directly to subtunica venous plexus, subsequently passing through the tunica albuginea via the emissary veins, and then to the deep dorsal veins, cavernosal veins and para-arterial veins independently, which are collectively called erection related veins because they drain the sinusoidal blood separately. This new understanding of penile venous anatomy is highly significant. Not only do we consider the traditional deep dorsal vein significant, but also the cavernosal vein, which distributes through the entire penile length and so does the para-arterial veins. The deep dorsal vein that lies consistently in the median position receives blood from the circumflex veins of the corpus spongiosum and from the emissary veins of the corpora cavernosa. Emissary veins (**Fig. 2**) run between the inner and outer layers of the tunica for a short distance, often piercing the outer layer bundles in an oblique manner. Therefore, and significantly, these emissary veins can be easily occluded by the shearing action elicited by the inner circular and outer longitudinal layers of the collagen bundles in the tunica albuginea.

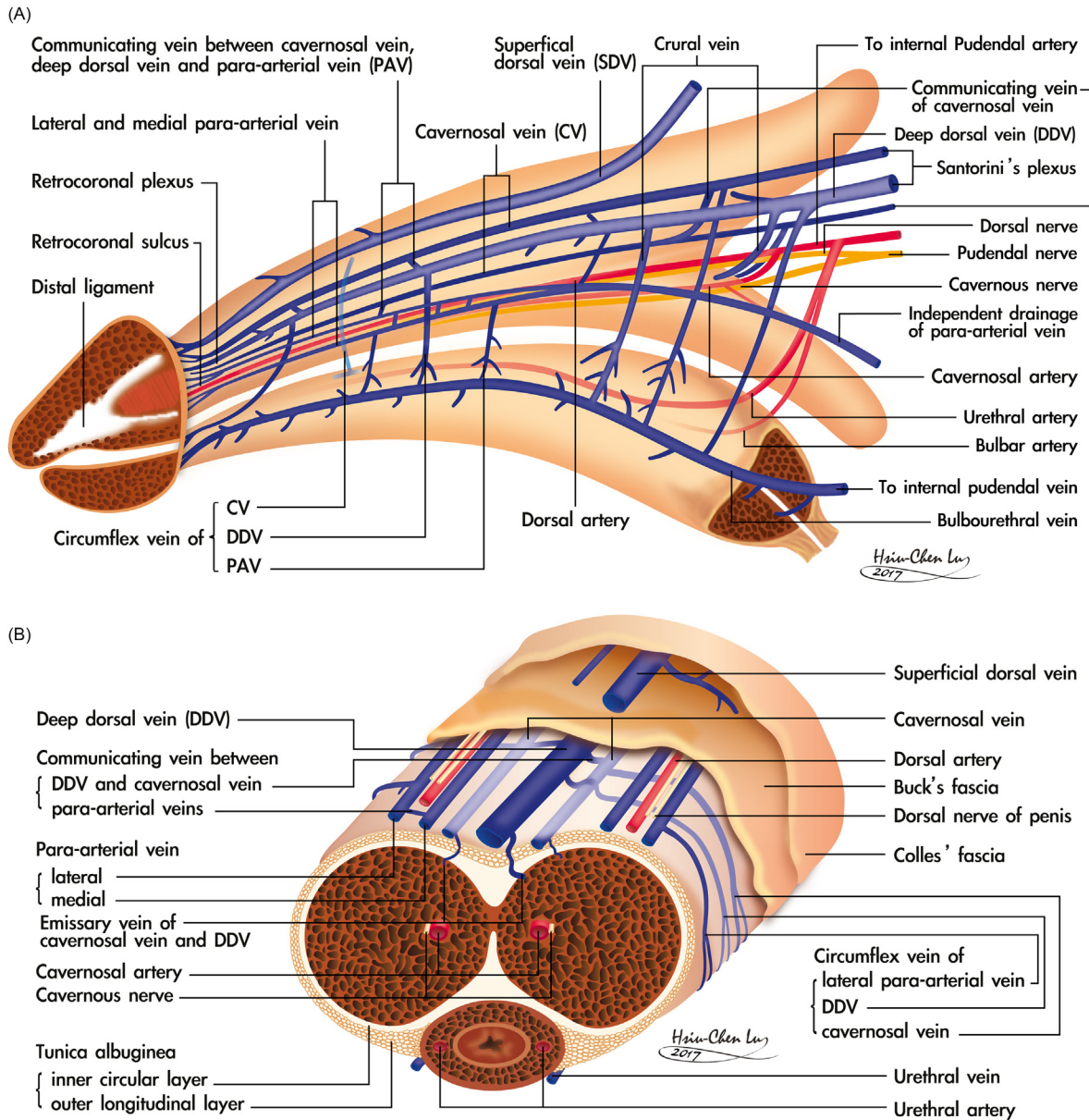


Fig. 2 Illustration of new penile vascular anatomy. (A) Lateral view: The glans penis is supported by a stout distal ligament (the *os penis* analogue) centrally just above the urethra. The DDV is consistently in the median position and receives blood of the emissary veins from the corpora cavernosa and of the circumflex vein from the corpus spongiosum. The DDV is sandwiched between the cavernosal veins (CV), but these lie at a deeper position. Bilaterally, each dorsal artery is respectively sandwiched by its corresponding medial and lateral para-arterial veins (PAVs). The pudendal artery and nerve are distributed in a similar manner but are relatively simple. (B) Cross section of the mid-penis. The corpora cavernosa is full with cavernosal sinusoids which are specific from that of the corpus spongiosum and glans penis. Note that the number of veins is seven (a larger count than the traditionally described singular vein), although it drops to four at the level of the penile hilum because each pair of the veins merges. The erection-related vasculature is arrayed in an imaginary arc on the dorsal aspect of the tunica albuginea. Thus, the penile vascular system still complies with the general rule in the body that the number of veins is normally higher than the number of arteries. Note the membranous urethra just connect the proximal end of the corpus spongiosum.

The deep dorsal vein is sandwiched by cavernosal veins that are coalesced to one at the penile base. Bilaterally each dorsal artery is sandwiched by the medial and lateral para-arterial vein respectively. Veins from the glans penis form a retrocoronal plexus that drains predominantly into the deep dorsal vein, the cavernosal veins, the para-arterial veins, the urethral veins and the corpus spongiosum respectively. The deep dorsal vein courses proximally in the midline between the paired corpora cavernosa and empties into the periprostatic plexus. The superficial dorsal vein drains the skin and the subcutaneous tissue superficial to Buck's fascia and may have direct shunts to the corpora cavernosa. This, in turn, drains into the superficial external pudendal vein. If and only if the erectile function of corpora cavernosa is healthy, the penis not only expands in its girth but also increases in its length.

Table 1 Demography of penile erection angle

Angle (degree)	Percent of population (%)
0–30	5
30–60	30
60–85	31
85–95	10
95–120	20
120–180	5

Erection Norm in Human Penis

Erection is a seamless interplay between psychologic, endocrinologic, vascular, neurologic, pharmacologic, and systemic metabolic health of the entire body. Implies a healthy erectile function depends upon the integral health of all systemic systems. However, it is not uncommon to encounter a disabled male with healthy erectile function, that is, the penile erectile function is free from disability in man suffered from spinal cord trauma. Whenever a sexual arousal ensues either from brain origin or from peripheral stimulation, a normal erection shall be subjected to a penis with healthy erectile function and ideal penile morphology. The former one is rudimentary while the latter issue is appropriate to engage in genital coitus. Therefore, we focus on the penis itself in this session. Although without a functional brain, it might be difficult to perceive sexual pleasure, in contrary, however, without a functional penis, the central order or coordination finds no effectors.

Regarding penile morphology, erection angle matters to fulfill natural coitus. Albeit sexual behavior is so individualized that there is no uniform, however, the missionary position is widely regarded as the most popular one which allows couple for close body contact and eye contact with kissing and talking throughout. Therefore, the erection angle may matter albeit it may not be a critical issue. This is the reason why there is a limited publication on penile erection angle which is more or less in normal distribution from 0 to 180 degrees (Table 1). Implies a mean may be in 90 degrees, however, clinically 120° may be more appropriate for coitus, otherwise, either a dorsal or ventral curvature should be considered. A severe ventral curvature is extraordinary awkward to do genital intercourse. Theoretically, most males are not in an ideal penile morphology. Erection angle may not be practical because it is simply a two-dimensional consideration.

Erection Dymorphology

Penile Dymorphology

An ideal penile shape is attractive to every male; however, it is very rare to encounter a perfectly ideal penile morphology in general population. Thus there is more or less a penile dymorphology which is somewhat diverted from a geometrically neutral shape. The curvature is a result of area differences (two dimensions) between the convex and concave surfaces of the corpora cavernosa if bisected. How much is the area difference in the penis with dymorphology? Given a dymorphological penis can be fixed to a perfect shape, an excision of excessive tunica or a patch to the shortage tunica is the policy of reconstruction. Excision or patching surgery results in postoperative penile shortage or elongation respectively. In order to predict how much is the area difference (Fig. 3), via a calculus or geometry method, we develop a formula which is $\pi r^2 \theta / 45^\circ$ (Fig. 3A), where r is the penile radius in centimeters, and θ is the deviation of the curvature in degrees. Clinically, r can be calculated from half of the measures between the 3 and 9 o'clock positions in centimeters when the penis is artificially erected, and θ is the estimated deviation angle in each patient. Once the two-dimensional amount is determined, the second one from goniometry, $2\pi r \theta' / \theta$ (Fig. 3B), is good to predict the length (one dimension) of area difference resulting in dymorphology. An engineering measurement can then be attainable.

Penile morphology is paramount in performing genital coitus. It is not uncommon to encounter tortured minds in many patients who had suffered from penile deformity resulting from acquired Peyronie's disease, congenital penile deviation and a variety of injury. Although the traditional anatomical paradigm of the tunica albuginea of corpora cavernosa has consistently overlooked the outer longitudinal layer which is the determined tissue of making penile morphology, the penile dymorphology must suffer victims along with human history. The penile dymorphology can be categorized to lateral, ventral and dorsal curvature in prevalent order, it can then be further assorted into left lateral, ventral, left ventrolateral, right lateral, left dorsolateral, right ventrolateral, dorsal and right dorsolateral curvature (Table 2). Thus left lateral deviation stands out followed by ventral, right lateral and dorsal curvature which is comparatively uncommon regardless it is resulting from acquired or congenital.

Penile Morphological Reconstruction

In general, the effectiveness of either medical intervention or natural morphology restoration is unpredictable although several clinical trials have been launched. Clinically varied surgical methods have been introduced in an attempt to establish an ideal penile shape in addition to erectile function restoration. The first corporoplasty was debuted in 1965. This surgical solution is followed by modified tunical excision, tunical patches and tunical plication in an attempt to make an ideal penile shape. Tunical fashion is deemed required but the plication procedure. As a rule, there is not necessary to receive corporoplasty surgery until a satisfactory

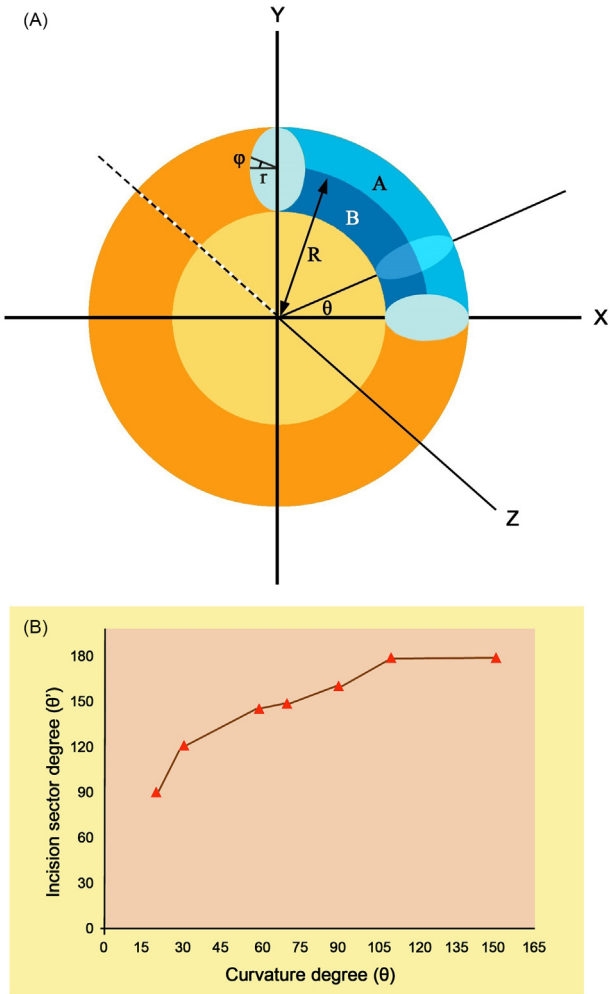


Fig. 3 Schematic illustration of discrepancy amount causing penile curvature. (A) A formula derived from calculus which is identical to that from geometry. Suppose that the center of a pipe revolves about the Z-axis by radius R as illustrated in the figure. Let S_1 and S_2 denote the outer and inner surfaces of the pipe, respectively. The difference in area between S_1 and S_2 is $S_1 - S_2 = \int_0^\pi 2\pi(R + r \sin \varphi) \cdot r d\varphi - \int_0^\pi 2\pi(R - r \sin \varphi) \cdot r d\varphi = 8\pi r^2$. Consequently, we can determine the difference in area for any degree, θ , and ΔS , which is independent of R , but dependent on the pipe radius, r , and θ : $\Delta S = \frac{S_1 - S_2}{360} \times \theta = \frac{\pi r^2 \theta}{45}$. (B) The relationship of the tunical incision sector and penile curvature degree. The incision sector (ϕ) and the curvature degrees (θ) is derived from goniometry. Seven curved penises of human cadavers with varied deviations of 20, 30, 60, 70, 90, 110, and 150 degrees respectively were collected. An 1–0 silk suture was put at the 12 o'clock position to serve as a guide of keeping the central line. The tunica was stepwise cut along the most curvilinear line until the penile shaft was straight. Subsequently, a curve of continuous parameters was then made with interpolation. The length of the patched veins required, consequently, was $2\pi r \theta' / \theta$. Note that a curved penis less than 110 degrees accounts for only approximation. Reproduction from Hsu, G. L. et al. (2006). Formulas for determining the dimensions of venous graft required for penile curvature correction. *International Journal of Andrology* 29(5), 515–520.

Table 2 The distribution of penile deformity

Direction of cure	Number (%)
Left lateral	54 (42.2)
Left ventrolateral	23 (18.0)
Left dorsolateral	5 (3.9)
Ventral	26 (20.3)
Dorsal	3 (2.3)
Right lateral	11 (8.6)
Right ventrolateral	4 (3.1)
Right dorsolateral	2 (1.6)
Total	128 (100)

coitus is severely interfered by penile dysmorphology when erection. Subsequently, tunical plication was popular owing to simplicity and reproducibility. However, postoperative outcomes were not beyond controversy and so does reliability because erectile dysfunction may result from tunical defect and dysmorphology resumption. Penile corporoplasty for mature Peyronie's disease might be a good option for this complicated disease entity. A postoperative penile shortage should be avoided by all means, which is a problem that can be solved through grafting (Fig. 4). Therefore graft surgery may be recommendable although technically challenging. Many kinds of resources have advocated, among them autologous venous material might be the material of choice for covering a corporotomy defect because it's functional and histological compatibility. Likewise, similar procedures are feasible to patients with congenital penile curvature undergoing penile corporoplasty. For anatomical consideration the deep dorsal vein and cavernosal veins were recommended although a controversy was addressed on its sufficiency, it appeared unequivocally sufficient after a scientific formula was developed, however (Fig. 3). This surgical intervention has become routine works in our practice since then. Relevant surgical solutions have been extended to use the autologous venous wall acting as the tunical inner layer (Fig. 4) and meanwhile, the porcine small intestinal submucosa functioning as the tunical outer longitudinal layer, which can achieve a prospective outcome. Of course, this challenging surgery warrants further research.

Erectile Abnormality

Pathophysiology of Erectile Dysfunction

The human erectile mechanism is an intricate interplay of hormonal, vascular, neurological, sinusoidal, pharmacological, and psychological factors, the relative influence of each respective component remains, however, somewhat unclear. What leads to an adequate erection is likely still not completely understood, with the current consensus remaining multifaceted and complex. There must be freed from a psychological disturbance, an intact neurological deficit, an imbalance hormonal profile, an adverse drug influence, a systemic disease, a dysfunctional artery and a fibrotic intracavernous tissue. Implies functional penis requires penile arterial sufficiency, normal sinusoidal tissue, and functional veins. Although there is no agreement on what would be the major contributor, there is a bias towards endothelial function due to the dramatic effects demonstrated by phosphodiesterase-5-inhibitors on erectile function.

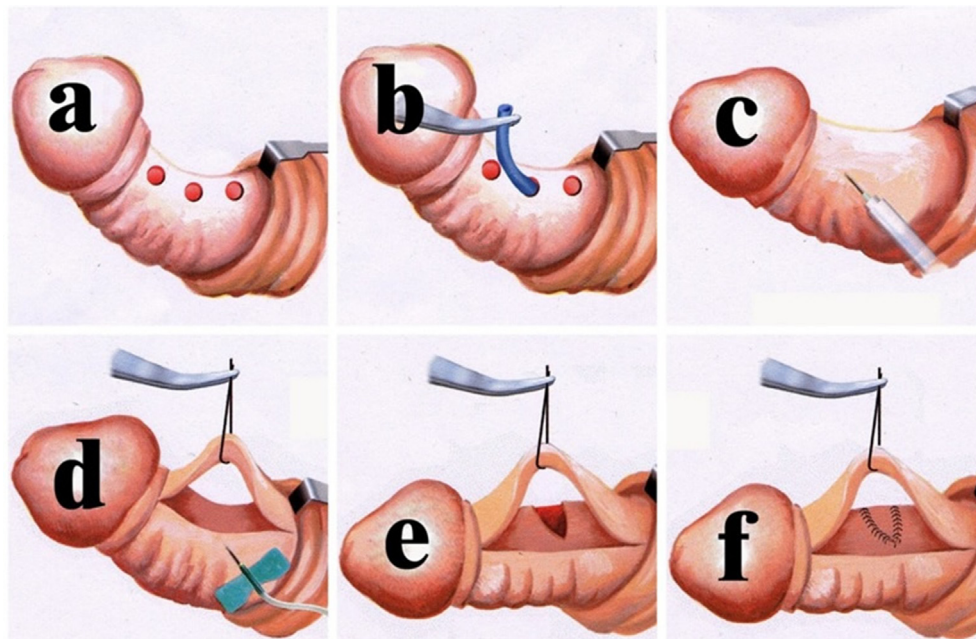


Fig. 4 Schematic illustration of penile autologous patched surgery. (A) A retrocoronal circumferential incision is made and the prepuce is degloved. The major branch of the deep dorsal vein is readily identified with a milking manipulation. Making an appropriate opening at the exits of the emissary veins, rather than making a complete opening on Buck's fascia, is done in order to perform the pull-through maneuver of the deep dorsal and cavernosal veins. (B) Completing the pull-through maneuver requires the surgeon to make 4–5 openings. The deep dorsal vein is stripped and doubly ligated with each emissary vein. This is preserved for patch material. (C) The hydro-dissection technique is used to facilitate the intact separation, the isolation, and the tag of the neurovascular bundle. (D) An artificial erection is generated, with normal saline via a 21G or 19G scalp needle, in order to determine where the depression center is, which is feasible for an incision. Using formulas for determining the dimensions of venous graft required for penile curvature correction. (E) An incision is made with a new, sharp surgical scalpel when the neurovascular bundle is well-protected until the penis is straightened. (F) The autologous venous grafting is fashioned to the tunical defect with a running suture of 6–0 nylon. It is then enforced at each centimeter. Eventually, an artificial small intestine submucosa has used to cover the patched area to enhance the tenacity which is sustainable to intracavernous pressure.

Contrary to this, however, are recent hemodynamic studies on fresh and defrosted human cadavers whereby rigid erections were unexceptionally reproduced despite the lack of endothelial extensibility. Constant low perfusion rate was used to mimic arterial inflow, and the staged removal of erection related veins produced increasingly more rigid erections. Could it signify that those veins may, in fact, be the predominant factor that underlies erectile rigidity? Certainly, and in the light of our increased understanding of the penile vasculature, it would at least warrant re-evaluation of the role of the venous contributor for erectile dysfunction.

On penile tissue as the integral organ, the corpora cavernosa, skeletal muscle components unite smooth muscle components to meet the requirements for the erection and only allow vascular and nervous tissue to communicate with the systemic circulation system. Their anatomical relation is seems like a cluster of grape when the emissary vein is regarded as the grape trunk and each sinusoid as a fruit (Fig. 2). The rigid erection of the corpora cavernosa overall depends on cooperation among healthy sinusoids, a normal tunica, functional arteries, and competent erection related veins. Thereafter vascular dysfunction is an important cause of male erectile dysfunction. It can be classified as veno-occlusive dysfunction, arterial insufficiency, or mixed. Accordingly, the best option for restoring penile erectile function may be competence of penile erection related veins, restoration of penile arterial function and sinusoid rejuvenation.

Arterial Insufficiency

Penile arterial supply is the initial contribution of erectile function. In 1923, it was Leriche who recognized a connection between arterial insufficiency and loss of erectile function when he observed that endarterectomy of an occluded bifurcation of aorta did improve blood flow in the lower extremities and simultaneously marked the improvement of erectile capability. A dramatic enhancement of the cavernosal arterial flow is the primary initial process in obtaining a sinusoidal expansion in the paired corpora cavernosa, which is a result of smooth muscle relaxation. Any arterial lesion that compromises arterial inflow to the penis can lead to erectile dysfunction; this is particularly true for lesions in specific arteries such as the cavernosal and dorsal artery.

It is commonly agreed that the prevalence of the arterial disease increases with age and subsequently is a major cause of organic erectile dysfunction in men over the age of 50. Consensus shows that erectile dysfunction seems to be a bellwether for a development of coronary artery disease in asymptomatic men, and both diseases share the same medical conditions and risk factors such as diabetes mellitus, hypertension, hypercholesterolemia, hyperlipidemia and smoking habit etc. Of course, erectile dysfunction can be due to atherosclerotic or traumatic arterial occlusive disease. Furthermore, in arteriogenic erectile dysfunction, the corpora cavernosa shows lower oxygen tension, which, in turn, may result in a decreased volume of sinusoidal smooth muscle and subsequent venous leakage, ensues. In an experimental animal model, rabbits with iatrogenic iliac atherosclerotic disease demonstrated alterations in their downstream penile arteries and a reduction in cavernosal smooth muscle content. The pathogenesis of erectile dysfunction in these patients is likely to be related to endothelial dysfunction. These alterations were associated with decreased nitric oxide synthase (NOS)- and NO-mediated relaxation of sinusoidal tissues in the corpora cavernosa. In those whose erectile dysfunction is owing to traumatic stenosis of pudendal or cavernous arteries has been noted in young men with long-distance cyclists or pelvic trauma, the ultimate goal of the arterial reconstruction is to provide an alternative arterial pathway to bypass arterial lesions that cause obstruction in the hypogastric-cavernous arterial bed, and offers an option for potential cure.

Veno-Occlusive Dysfunction

This may be specific to excessive venous drainage. Early detumescence and insufficient rigidity are the common symptoms. In 1873, the Italian Francesco Parona injected varicosity dorsal penile vein of an impotent young patient with hypertonic saline in order to cause sclerosis and subsequently reduce the excessive venous outflow. Subsequently, the concept that erectile disorders might be treated surgically by occluding venous channels from the penis was materialized as early as the turn of the 20th century such as Henry Raymond, James Duncan, Joe Wooten and Frank Lydston in 1895, 1895, 1902, and 1908 respectively. Exemplified in 1902, Joe Wooten suggested, as a justification for this type of surgery, the hypothesis of atonic impotence, claiming that it is the result of the loss of smooth muscle tonicity in the corpora cavernosa, resulting in dilated veins and sinusoids at that level. Later in 1908, Frank Lydston published his results on 100 venous ligation procedures reporting that 50% of his patients were definitely cured and that the rest had improved their sexual function. Lydston claimed, as an explanation for these good results that he ligated not only the superficial dorsal vein but also the deep dorsal vein of the penis, as well as collateral veins, courting penile tumescence, along with a sensation of enlargement of the penis which boosted the psychological confidence of patients in the effectiveness of this technique.

Beginning in the 1930's Oswald Swinney Lowsley combined simple dorsal vein plication with a surgically more advanced perineal crural technique in which he plicated the bulbocavernosus and ischiocavernosus muscles with several mattress sutures. After his initial report in 1935, he could follow-up 273 patients of the more than 1000 patients operated upon in his later publication from 1953. Afterward, as urologists embraced different techniques of vascular surgery and transplantation, attempts to resolve erectile dysfunction were directed towards the possibility of producing a greater blood supply through arterial revascularization techniques and meanwhile limiting venous drainage through venous ligation. These techniques of venous surgery disappeared from medical literature until in the 1980's the rebirth of venous surgery took place as a result of the new field of investigation of erectile physiology.

Unfortunately, a severe restraint of penile venous surgery for treating erectile dysfunction has been recommended since 1996 thus far. Every treatment modality must be somewhat effective; otherwise, repeated experiments would not be performed on the human body. This principle is applicable to all methods of penile venous surgery. It justly deserved to be condemned and abandoned if its erection restoration outcome is disappointing and it is seemingly unavoidable on adverse side effects, such as irreversible deformity and permanent penile numbness. Despite these drawbacks, our method of penile venous stripping with venous ligation is performed at the exits of emissary just closest to the outer tunica has been developed with the inspiration of patients' positive response in tandem with the advances of penile tunical and venous anatomy associated with the erection mechanism since 1986. We applied a refined penile venous stripping in many thousands of patients under acupuncture-assisted local anesthesia on an outpatient basis. It ultimately won the USPTO (The United States Patent and Trademark Office) patent on August 14, 2012. Although currently, we recommend penile venous stripping to patients who are nonresponders to phosphodiesterase-5 (PDE-5) inhibitors, we always fail to decline patients who are recommended by operated patients.

The problem is how to identify those offensively veins? Recently, computerized tomography-cavernosography is advised to be most advanced, however, after our chronic observation; it appears that the penile venous anatomy per se is only demonstrable via a pilot set of cavernosography and the veno-occlusive dysfunction physiology is appropriately documented via pharmacocavernosography (cavernosograms after working of intracavernously injected vaso-active agent such as prostaglandin E1). Those offensive veins can be enhanced visible once via squeezing manipulation after larger veins are shuttled down. Totally the ligation sites up to 132 positions. Albeit it seems technical challenging, this innovative method turns the venous surgery for treating erectile dysfunction from one that has been abandoned to a curable option because its prototype has been sustainable since 1986.

The current consensus of most urologists is that ED is due to the cavernosal factor, an inability to achieve a rigid erection is attributed to the loss of smooth muscle relaxation and fibrous compliance. We observed in our study, however, that reaching a rigid erection was actually a hemodynamic phenomenon, in which Pascal's law could be applied to be performed. We then shifted our focus to fresh cadavers with inspiration drawn from the trauma history gained during World War II by Newman HF. It was discovered then that the corpora cavernosa was easily accessible for the purpose of blood transfusion in emergency situations where no peripheral vein was available. Amazingly, a rigid erection ensued regardless of the absence of vital signs in a wounded soldier when the transfusion speed exceeded 25 mL/min. After penile venous stripping, the induction flow was notably less than prior to removal. The maintenance flow of most subjects was also much lower than the normal arterial in attaining a sufficient erection, thereafter, the role of venous surgery in treating erectile dysfunction deserves reevaluated? Although venous surgery for erection dysfunction has been criticized for not being durable, the recurrence of sexual inadequacy on operated patients, however, has not been supported by any evidence of regeneration of the venous vasculature. Accordingly our chronological research and clinical application for over three decades, we found that not only the veno-occlusive dysfunction is the predominant cause of erectile dysfunction, but also the penile venous stripping is categorically a viable option at a negligible complication at an ambulatory basis. In contrast, an obstructive penile venous drainage is also a severe medical problem. It is renowned as a condition of priapism which the penile venous drainage is significantly hindered resulting from sickle cell anemia or straddle injury, which will be illustrated more.

Clinical Relevance Erection Abnormality

Disease of Venous Drainage Blockage

In a normal situation, the penile detumescence occurs when cavernous blood dissipates via erection related veins after an erection bout. A priapism develops resulting from persistent, over 6 h, a penile erection occurs if venous drainage is compromised despite no sexual desire or even painful suffering. It may be induced by drug, trauma or disease such as leukemia and sickle cell anemia. It is commonly classified into ischemic, nonischemic and recurrent ischemic types. The most common one is ischemic priapism which happens when blood does not adequately drain away from the corpora cavernosa. The penile erection veins are not completely blocked in the nonischemic priapism which is typically resulting from a shunt formed between the corpus spongiosum and an artery or disruption of the nerve of parasympathetic nervous system.

Medical history and physical examination is the first impression of the priapism and then is further documented by sonography and cavernosal blood gas analysis. The most common cause of ischemic priapism is sickle cell disease followed by medication such as selective serotonin reuptake inhibitor, cocaine, and cannabis, intracavernous injection agent including papaverine and prostaglandin etc. Ischemic priapism is typically advised with a nerve block for pain relief followed by aspiration of blood from the corpus cavernosum. A cavernosal measurement is an advice by cold normal saline irrigation or phenylephrine injection if no obvious effectiveness, whereas nonischemic priapism is often treated with cold packs and compression. Shunting surgery may be required if usual measures are not effective. Priapism may lead to permanent fibrosis of the corpora cavernosa so severe that it sustains an erectile dysfunction or penile dysmorphology.

Disease of Penile Excessive Venous Drainage

Excessive penile venous drainage appears to be the most popular etiology resulting in erectile dysfunction. Penile veno-occlusive dysfunction is newly termed instead of venogenic erectile dysfunction or penile venous leakage, new treatment modality is limited but the first line usage of phospho-di-esterase-5 inhibitor. In young males younger than 40 years, it is unexceptionally noted to have

excessive venous drainage veins once psychological influence is ruled out. Unfortunately, they are always refractory to phosphodiesterase-5 inhibitors. Their symptoms include insufficient rigidity, early detumescence, loss of morning erection, position dependent impotence, soft glans syndrome and gradual onset of early ejaculation.

Since 1986 we have successfully treated many hundreds of young males who suffer from venous leakage, this denotes that venogenic erectile dysfunction is real and the current medical community should reevaluate this disease rather than just ignore this disease entity. The corpora cavernosa is nutritious with blood and susceptible to the rapid development of the venous drainage speed to override the penile arterial supply. Therefore it is understandable to acknowledge the controversial disease.

Disease of Penile Arterial Insufficiency

A functional penile artery is a prerequisite for erectile function. Similar to other kinds of cardiovascular disease, arterial vasculogenic causes of erectile dysfunction increase with age and are most prevalent in those with risk factors for atherosclerosis such as diabetes, hypertension, hyperlipidemia, smoking, and obesity. Traumatic events, which occur more often in younger men, are due to pelvic and/or perineal trauma. Arterial insufficiency can be detected using color Doppler ultrasonography, if the peak systolic velocity is less than 25 cm/s. Angiography, while being the benchmark for all arterial investigations, is reserved with the aim of concurrent arterial revascularization or angioplasty if a penile arterial stenosis is suspected. Recently a penile arterial stent is revisited with a controversial result.

Diseases of Corporeal Fibrosis

Corporal fibrosis usually occurs after Peyronie's disease, refractory low-flow priapism, the chronic intracavernous injection of vasoactive drugs, explantation of an infected penile prosthesis and severe penile trauma etc. Peyronie's disease is a connective tissue disorder which involves the tunica albuginea. It is commonly ascribed to repeated microtrauma on the tunica albuginea and sinusoidal tissues, which extends to involve the corporeal body. Specifically, scar tissue tethers sensation nerve will cause pain which may last for the first 3 months and then reach a mature stage in 1 year. It always present with erection dysfunction, loss of girth and penile length, penile dysmorphology and even hourglass deformity. A variety of treatments have been used, but none have been especially effective. Tunical patch surgery may be required if it is refractory.

Further Reading

- Azadzoi, K. M., et al. (1997). Relationship between cavernosal ischemia and corporal veno-occlusive dysfunction in an animal model. *The Journal of Urology*, 157(3), 1011–1017.
- Hsu, G. L., et al. (2007). Long-term result of an autologous venous grafting for penile morphological reconstruction. *Journal of Andrology*, 28(1), 186–193.
- Hsu, G. L., et al. (2006). Formulas for determining the dimensions of venous graft required for penile curvature correction. *International Journal of Andrology*, 29(5), 515–520.
- Hsu, G. L., et al. (2012). Penile veins are the principal component in erectile rigidity: A study of penile venous stripping on defrosted human cadavers. *Journal of Andrology*, 33(6), 1176–1185.
- Hsu, G. L., et al. (2006). Insufficient response to venous surgery: Is penile vein recurrent or residual? *Journal of Andrology*, 27(5), 700–706.
- Hublin, J. J., et al. (2017). New fossils from Jebel Irhoud, Morocco and the pan-African origin of *Homo sapiens*. *Nature*, 546(7657), 289–292.
- Levine, F. J., Greenfield, A. J., & Goldstein, I. (1990). Arteriographically determined occlusive disease within the hypogastric-cavernous bed in impotent patients following blunt perineal and pelvic trauma. *The Journal of Urology*, 144(5), 11147–11153.
- Leriche, R. (1923). Désoblitérations artérielles hautes (oblitération de la terminaison de l'aorte) comme cause des insuffisances circulatoires des membres inférieurs. *Bulletins et Mémoires de la Société de Chirurgie de Paris*, 49, 1404.
- Nehra, A., et al. (1998). Cavernosal expandability is an erectile tissue mechanical property which predicts trabecular histology in an animal model of vasculogenic erectile dysfunction. *The Journal of Urology*, 159(6), 2229–2236.
- Nesbit, R. M. (1965). Congenital curvature of the phallus: Report of three cases with description of corrective operation. *The Journal of Urology*, 93, 230–232.
- Newman, H. F., Northup, J. D., & Devlin, J. (1964). Mechanism of human erection. *Investigative Urology*, 1, 350–353.
- Sparling, J. (1997). Penile erections: Shape, angle and length. *Journal of Sex & Marital Therapy*, 23(3), 195–207.

Ejaculation/Semen

Pierre Clément, Université de Versailles-Saint Quentin en Yvelines, Montigny le Bretonneux, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

Ejaculation comes from the Latin word 'ejaculari' meaning to project, to throw forcefully. Then, ejaculation can be defined as the forceful propulsion of semen out of the body. Because ejaculation occurs in a sexual context in response to a combination of brain (visual, auditory, olfactory, and fantasy) and peripheral (penile and erogenous zones) stimuli, the term ejaculatory response is sometimes read in scientific/medical texts. The primary objective of ejaculation is to allow the male to deliver its gametes (i.e. spermatozoa) for fertilization of the female mature reproductive cell (i.e. ovum). Ejaculation appeared relatively early in the evolution of living species as an effective mean of reproduction and many aspects of the ejaculatory response are common to a number of animal species, from reptiles to mammals. The ejaculatory response is characterized by the synchronized succession of physiological events that form two distinct phases that are emission and expulsion. Emission is the secretion of the spermatozoa from testes (more exactly from epididymis) together with different components of the seminal fluid produced by accessory sex glands. Mixing of all the elements of the semen takes place in the part of the urethra surrounded by the prostate (i.e. prostatic urethra). Once emission phase is completed, expulsion is triggered that occurs in form of intense rhythmic contractions of several pelvic striated muscles that led to forceful expulsion of semen through the urethral meatus.

Semen

Semen is composed of spermatozoa and fluid synthesized and secreted by the sex glands that contribute to ejaculation. Semen contains a variety of enzymes, sugars, lipids, oligo-elements, and other substances which provide the male gametes with a nutritive and protective milieu. Due to the anatomical arrangement of the ejaculatory tract, the components of the seminal fluid are deposited into the prostatic urethra sequentially. As a result, ejaculate (i.e. expelled semen) is divided in portions characterized by differences in their composition and function. The first portion of the ejaculate consists of a small amount of fluid from the bulbourethral glands and is usually lacking spermatozoa. It contains mucus, acid phosphatase, and sialic acid that contribute to lubrication, semen coagulation, and neutralization of the acidity of urethral and vaginal environment. The second portion of the ejaculate is characterized by a low-viscosity opalescent fluid released from the prostate. A few spermatozoa are found in this fraction which is richer in sugars, organic acids, fibrinolysin, proteolytic enzymes, and zinc (chromatin stabilizer). Then the principal portion of the ejaculate is expelled which contains the highest concentration of spermatozoa, along with secretions from the epididymis, and vas deferens, as well as prostatic and seminal vesicle fluids. This fraction contains amino acids, vitamins, mucus, fructose (the main source of energy for spermatozoa), and prostaglandins (immunosuppressive action). A last fraction can be distinguished that consists of seminal vesicle secretions. In sexually mature men, the volume of seminal fluid varies between 0.1 and 10 mL and contains an average of 30.10^6 spermatozoa per mL. Semen volume depends on multiple factors and on the time elapsed from the previous ejaculation.

Anatomy

Detailed anatomical description of the genital tract is provided in other sections of this volume. Here, a brief overview of the organs and anatomical structures participating to the ejaculatory response (i.e. ejaculatory tract) is given. The organs that contribute to the emission phase of ejaculation synthesize, secrete, and/or discharge the different components of the seminal fluid.

- Epididymis: located posterior to the testis, the epididymis is the site where spermatozoa undergo final maturation and are stored prior to be transported onward into the urethra through the vas deferens.
- Vas (or ductus) deferens: as emission occurs, smooth muscle cells in the wall of the vas deferens strongly contract that allow spermatozoa to rapidly reach the prostatic urethra. Before joining the urethra, the vas deferens widens to form the ampulla of the vas deferens that lies close to the seminal vesicle.
- Seminal vesicles: composed of epithelial cells, which produce 50%–80% of the total volume of seminal fluid. Epithelium is surrounded by smooth muscle cells, which contraction during emission discharges the content of the gland into the prostatic urethra (Fig. 1).
- Prostate: single gland surrounding the urethra immediately below the bladder. It is composed of alveolar epithelium, which secretes 15%–30% of the total volume of seminal fluid, and fibromuscular tissue which contracts as emission occurs to pour prostatic fluid into the prostatic urethra. The openings of the ejaculatory ducts (ampulla of the vas deferens, ducts of the seminal vesicles and prostate) are found on the dorsal side of the urethra and form a particular structure, the veru montanum, which prevents seminal fluid to go backward into the ducts during the expulsion phase of ejaculation.

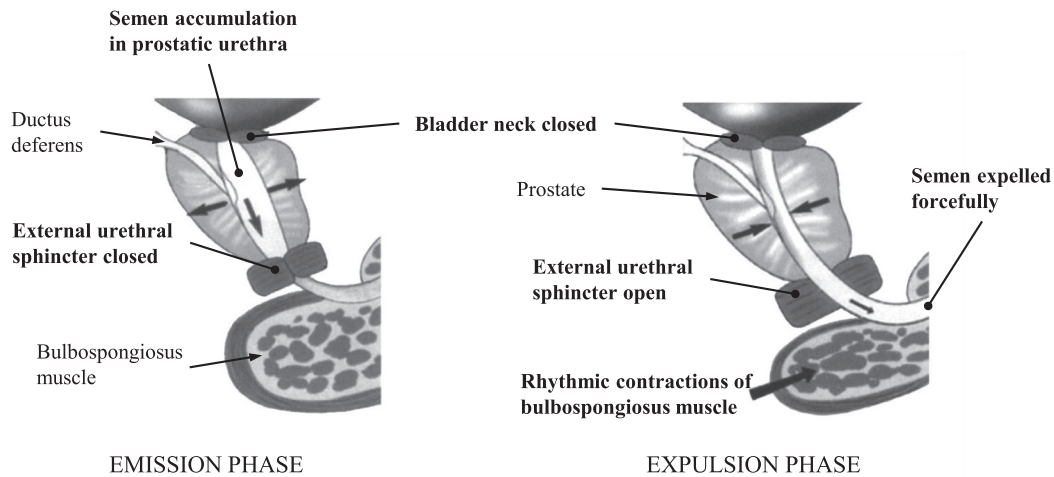


Fig. 1 Peripheral events of emission and expulsion phases of ejaculation. During emission, bladder neck and external urethral sphincter are closed and secretions from sex glands (seminal fluid + spermatozoa) are poured into the prostatic urethra where they accumulate. During expulsion, bladder neck remains closed, external urethral sphincter is opened, and rhythmic contractions of bulbospongiosus muscle forcefully propelled semen throughout the urethra.

- Bulbourethral glands (or Cowper's glands): pea size paired glands closely invested by a layer of striated muscle. They produce a very small amount of seminal fluid (<5% of the total volume) which is discharged into the penile portion of the urethra, downstream of the prostatic urethra.

Intense and rhythmic contractions of muscles and anatomical structures participating to expulsion led to forceful propelling of semen in anterograde direction, through the urethral meatus (**Fig. 1**).

- Bladder neck (or internal urethral sphincter): is composed of smooth muscle cells that firmly contract during expulsion to avoid semen to flow backward into the bladder.
- Urethra: the urethra epithelium is surrounded, over about half of its length, by circular striated muscle forming the external urethral sphincter (or rhabdosphincter). This sphincter rhythmically contracts during the expulsion phase to help semen to move forward.
- Pelvipereineal striated muscles: include levator ani, ischiocavernosus and bulbospongiosus muscles, with the latest having a preponderant role. As expulsion occurs, the rhythmic and intense contractions of these muscles are essential in forceful propelling of semen throughout the urethra and are responsible for saccadic expulsion of semen out of the body.

Peripheral Response

Innervation

Sensory afferents

Sensory receptors stimulated during coitus or masturbation are essentially located in the penile skin, prepuce, and glans. Sensory inputs are conveyed to the upper sacral and lower lumbar segments of the spinal cord via the dorsal nerve of the penis, a sensory branch of the pudendal nerve (**Fig. 2**). A relatively sparse sensory innervation of ductus deferens, prostate, and urethra has also been described which reaches the lumbosacral spinal cord via the pudendal nerve. A second afferent pathway is constituted by fibers traveling along the hypogastric nerve and, after passing through the paravertebral lumbosacral sympathetic chain, enters the thoracolumbar segments of spinal cord (**Fig. 2**). Sensory afferents terminate in the medial dorsal horn and the dorsal gray commissure of the spinal cord.

Motor efferents

Cell bodies of the preganglionic sympathetic neurons are located in the intermediolateral cell column and in the central autonomic region of the thoracolumbar segments of the spinal cord. The majority of sympathetic fibers relay in the paravertebral sympathetic chain and continue through the splanchnic nerve to reach the inferior mesenteric ganglia. Arising from the inferior mesenteric ganglia is the hypogastric nerve that forms, after joining the parasympathetic pelvic nerve, the pelvic plexus from which arise fibers innervating the ejaculatory tract (**Fig. 2**).

Cell bodies of the preganglionic parasympathetic neurons are located in the intermediolateral cell column of the lumbosacral segments of the spinal cord in an area referred to as the sacral parasympathetic nucleus. Neurons of the sacral parasympathetic nucleus send projections, via the pelvic nerve, to the postganglionic neurons located in the pelvic plexus (**Fig. 2**).

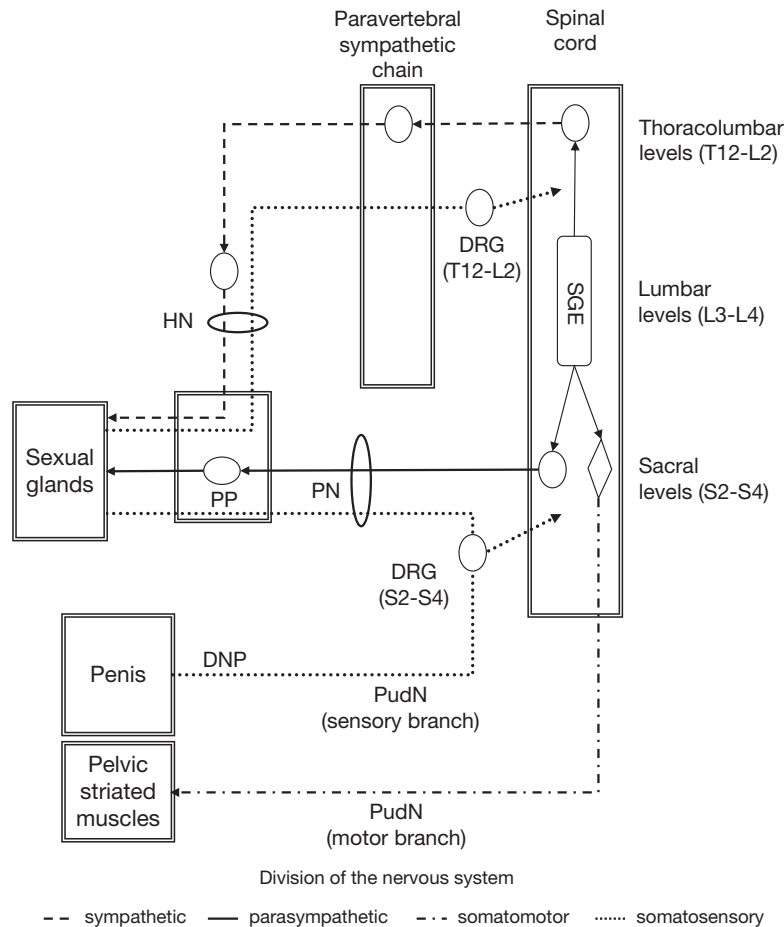


Fig. 2 Schematic view of the autonomic and somatic innervation of genitalia. Neural pathways involved in ejaculation are indicated. *DNP*, dorsal nerve of the penis; *DRG*, dorsal root ganglia; *HN*, hypogastric nerve; *PN*, pelvic nerve; *PP*, pelvic plexus; *PudN*, pudendal nerve; *SGE*, spinal generator of ejaculation. Reprinted from Handbook of Clinical Neurology, vol.130, P. Clement and F. Giuliano, Anatomy and physiology of genital organs – men, pp. 19–37, 2015, with permission from Elsevier.

Cell bodies of the motoneurons are found at the lumbosacral spinal level in the Onuf's nucleus, exit the ventral horn of the medulla and proceed via the motor branch of the pudendal nerve to the pelvic floor striated muscles, including bulbospongiosus-muscle (Fig. 2).

Physiology

Activation of sympathetic, parasympathetic, and somatic divisions of the nervous system occurs in synchrony during ejaculation. In addition, the non-adrenergic non-cholinergic (NANC) system also contributes to the control of ejaculation through modulation of sexual glands.

Based on the innervation of the male genitalia, it is proposed that parasympathetic tone controls epithelial secretion in sexual glands while sympathetic tone controls contractile activity of the smooth muscle cells composing sexual glands and related ducts. Clinical observations confirmed the essential role of sympathetic pathways supplying the urogenital region in men. Notably, disruption of sympathetic nerves in patients who underwent retroperitoneal lymphadenectomy for surgical treatment of testicular cancer suffer ejaculatory dysfunctions. Accordingly, surgical approaches that spare sympathetic innervation reduce the incidence of ejaculatory dysfunctions in these patients. Electrical stimulation of the hypogastric plexus, which harbors postganglionic sympathetic neurons innervating the urogenital area, triggers emission of semen in paraplegic men unable to ejaculate during sexual intercourse. Saccadic expulsion of semen is caused by synchronized contractions of pelvipereineal striated muscles and external urethral sphincter, with a key role for the bulbospongiosus muscle. Activity of these muscles is characterized by intense and short bursts of contraction that are not under voluntary control, explaining that expulsion is regarded as a reflexive event. Genital sensory innervation including sensory receptors in the penis (skin, prepuce, and glans) has a crucial role in ejaculation. In humans, different modes of penile stimulation can elicit contractions of bulbospongiosus muscle. This led to the development of clinical procedures for evaluation of neural pathways controlling ejaculation and for triggering ejaculation.

Neurochemistry

Noradrenaline and acetylcholine

There is a dense autonomic (both sympathetic and parasympathetic) innervation of the male genitalia. Sympathomimetic agents, which mimic in part the action of noradrenaline, have been shown to cause seminal tract and sexual glands contractions. The effect is mainly mediated by α -1 adrenoreceptor subtypes that are expressed by smooth muscle cells. Cholinomimetic agents, which partially reproduce the activity of acetylcholine, can also elicit contractions of sexual glands through activation of muscarinic receptor subtypes. Moreover, stimulation of muscarinic receptors triggers seminal fluid secretion from seminal vesicles.

Nitric oxide

Nitric oxide (NO) fibers are a major component of the NANC system and have been detected in the entire seminal tract in the man. In vitro functional investigations on human seminal vesicles indicate that NO exerts an inhibitory effect on smooth muscle contractile activity.

Oxytocin

Oxytocin (OT) is a hormone produced predominantly by the pituitary gland and also, in a lower amount, by testes and prostate. Oxytocin induces contraction of smooth muscle cells in sexual glands through direct and indirect mechanisms and was suggested to promote spermatozoa transport in the vas deferens (Filippi et al., 2002).

Sensory receptors

The crucial role of penile sensory stimuli in the ejaculatory response is best illustrated by the effect of topical anesthetics. Combination of lidocaine and prilocaine and extract from Asian natural herbs were reported to significantly increase ejaculation latency in men diagnosed with premature ejaculation following application on the penile glans (Xia et al., 2013).

Spinal Control

The Spinal Network of Ejaculation

A neuronal circuitry exists in the spinal cord that controls ejaculation. This circuitry includes groups of neurons lying in different spinal cord segments (Fig. 3). Sympathetic preganglionic neurons innervating the urogenital area are located in the intermediolateral cell column and the dorsal gray column of thoracolumbar (12th thoracic-2nd lumbar) spinal segments. Preganglionic

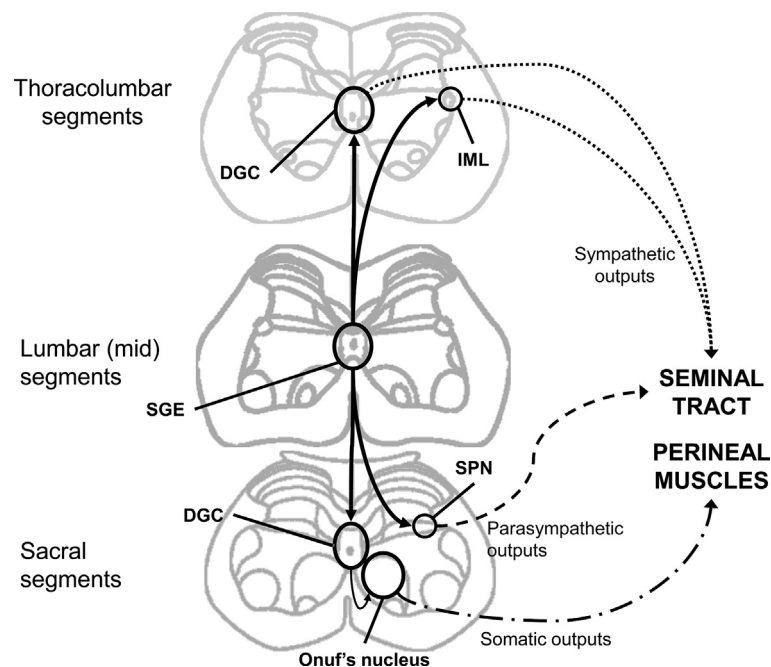


Fig. 3 Schematic view of the spinal network of ejaculation. The spinal generator of ejaculation (SGE) projects to: (i) thoracolumbar sympathetic centers including dorsal gray column (DGC) and intermediolateral nucleus (IML), (ii) lumbosacral parasympathetic centers including sacral parasympathetic nucleus (SPN), (iii) lumbosacral somatic centers including Onuf's nucleus. Those autonomic and somatic spinal centers control seminal tract and perineal striated muscles. Reprinted from Handbook of Clinical Neurology, vol.130, P. Clement and F. Giuliano, Anatomy and physiology of genital organs – men, pp. 19–37, 2015, with permission from Elsevier.

parasympathetic neurons sending projections to the urogenital area form the sacral parasympathetic nucleus that is located in the intermediolateral cell column of sacral segments (2nd sacral - 4th sacral). Somatic motoneurons supplying pelvic perineal striated muscles and urethral sphincter are found in a single nucleus (namely Onuf's nucleus) in the ventral horn of S2-S4 segments. Finally, a group of spinal interneurons has been identified to play a role of generator of ejaculation (Chehensse et al., 2017; Truitt and Coolen, 2002). Peripheral and cerebral sexually relevant stimuli are summated in the spinal generator that, once the excitatory threshold is reached, triggers synchronized activity of autonomic and somatic neurons belonging to the spinal network of ejaculation. A key component of the spinal generator of ejaculation (namely lumbar spinothalamic neurons) has been characterized around the central canal in lumbar (L3-L4) segments. Lumbar spinothalamic neurons project to preganglionic autonomic neurons innervating the sexual glands and to motoneurons innervating bulbospongiosus muscle (Fig. 3). Integrity of the spinal network is sufficient for ejaculation to occur as evidenced in rats with thoracic spinal segment sectioned (Borgdorff et al., 2008). In man, the existence of a spinal generator of ejaculation has recently been evidenced based on clinical and neurohistochemical investigations. It was reported that lesion of L3-L5 spinal segments is correlated with the inability of spinal cord injured patients to ejaculate during sexual intercourse (Chehensse et al., 2017).

Neurochemistry

GABA

The neurotransmitter γ -aminobutyric acid (GABA) is widely distributed in the central nervous system and virtually influences all centrally driven neurophysiological processes. Baclofen, an antagonist of GABA-B receptor subtypes, is delivered in the subarachnoid space to alleviate refractory spasticity in patients with spinal cord injury or multiple sclerosis. Clinical observations showed that intrathecal injection of baclofen altered the ability of these patients to ejaculate (Denys et al., 1998). Experimental investigations provided clarification on GABA mechanism of action by demonstrating that blockade of GABA receptors in the spinal generator of ejaculation inhibited ejaculation (Borgdorff et al., 2008).

Oxytocin

Neurons located in the hypothalamus send projections containing oxytocin onto the autonomic neurons (both sympathetic and parasympathetic) innervating the urogenital tract. Antagonists of OT receptor inhibit the ejaculatory response in a model of pharmacologically induced ejaculation (Clement et al., 2013). It is then postulated that descending oxytocin hypothalamic neurons modulate the spinal mechanisms of ejaculation.

Serotonin

Autonomic and somatic spinal ejaculatory centers receive multiple serotonin (5-HT) projections from several brain origins. The fact that various 5-HT receptor subtypes exist in the spinal ejaculatory centers with different cellular location (pre/postsynaptic, auto/heteroreceptors) and pharmacodynamic properties (ligands affinity, activatory/inhibitory) makes it difficult to understand the exact role of spinal 5-HT in the control of ejaculation. From a series of pharmacological studies using different experimental models and providing apparently conflicting results, it can be concluded that 5-HT exerts a multilevel and multimodal action in spinal mechanisms of ejaculation (triggering/facilitation of ejaculation, inhibition of expulsion; for details see Giuliano and Clement, 2012).

Substance P

The crucial role of genital sensory stimuli in ejaculation has been mentioned earlier in the text. One neurotransmitter involved in the transmission of sensory information from the periphery to the spinal cord is substance P which is produced by primary sensory neurons. The receptor for substance P (neurokinin-1; NK1) is expressed in the spinal generator of ejaculation. The selective neurotoxic lesion of NK1-expressing lumbar spinothalamic neurons resulted in suppression of ejaculation with no alteration of other sexual responses in male rat free to copulate (Truitt and Coolen, 2002).

Other neurotransmitters

Neurochemical characterization of the spinal ejaculatory network led to the identification of other factors including cholecystokinin octapeptide fragment, galanin, gastrin releasing peptide, and endogenous opioid peptide enkephalin (reviewed in Giuliano and Clement, 2012). However, functional evidence is scarce and the respective role of those factors is poorly understood. Of interest is the pharmacological study showing the pro-ejaculatory action of agonist of receptor for gastrin releasing peptide in the rat (Sakamoto et al., 2008).

Brain Control

The Brain Network of Ejaculation

The existence of a neuronal circuitry in the brain specifically controlling ejaculation has been demonstrated experimentally that is part of larger network involved in the regulation of the sexual response (Giuliano and Clement, 2012; Veening and Coolen, 2014). This cerebral network is composed of several interconnected groups of neurons distributed in several divisions of the brain in sensory/integrative, excitatory, and inhibitory areas (Fig. 4). The posteromedial division of the bed nucleus of the stria terminalis,

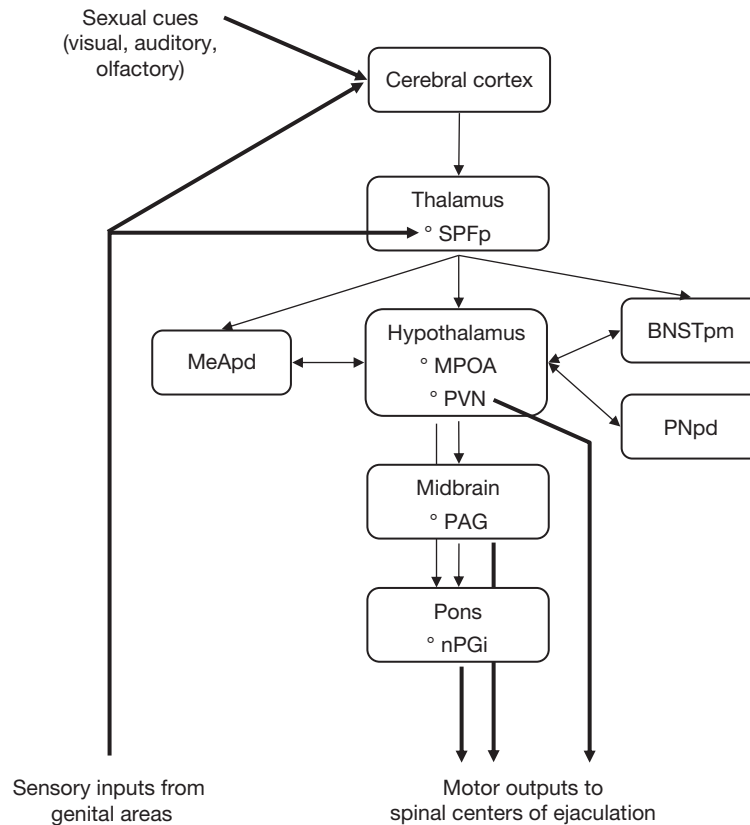


Fig. 4 Diagram of brain structures and central pathways involved in ejaculation. *BNSTpm*, posteromedial bed nucleus of stria terminalis; *MeApd*, posterodorsal medial amygdaloid nucleus; *MPOA*, medial preoptic area; *PAG*, periaqueductal gray; *nPGi*, paragigantocellular nucleus; *PNpd*, posterodorsal preoptic nucleus; *PVN*, paraventricular thalamic nucleus; *SPFP*, parvicellular part of the subparafascicular thalamus.

the posterodorsal area of the medial amygdala, the posterodorsal preoptic nucleus, and the parvicellular part of the subparafascicular thalamus contain group of neurons that were found activated concomitantly to ejaculation. In addition, the subparafascicular thalamus receives direct projections from lumbar spinothalamic neurons, the essential component of the spinal generator of ejaculation. The above brain structures are involved in the integration and processing of sexually-relevant stimuli including sexual cues and peripheral somatosensory stimuli. Brain structures containing neurons that send excitatory signals to the spinal network of ejaculation have been identified. One pathway includes neurons in the medial preoptic area that project onto neurons of the paraventricular hypothalamic nucleus which in turn send axons to autonomic neurons belonging to the spinal centers of ejaculation (Fig. 4). Another excitatory pathway consists in neurons located in the lateral hypothalamus and directly contacting lumbar spinothalamic neurons (Facchinetti et al., 2014). Finally, the source of inhibitory tone on the spinal command of ejaculation has been found in a number of nuclei in the ventral medulla (Fig. 4). Gigantocellular nuclei and ventral raphé nuclei directly project onto autonomic and somatic spinal ejaculatory centers as well as lumbar spinothalamic neurons. In view of the complexity of the brain ejaculatory network and the variety of descending pathways, a multilevel and multimodal control on spinal executive mechanisms of ejaculation is evident.

Non-invasive neuroimaging technologies allow the detection of brain areas that are activated or inhibited (in comparison with a standardized basal condition) in relation with a controlled experimental situation. A few studies conducted in healthy male volunteers led to the identification of intense neuronal activation in relation with ejaculation in the mesodiencephalic transition zone, in the thalamus in regions including medial, ventral, and subparafascicular thalamic subareas, as well as in the parietal cortex (Arnou et al., 2002; Holstege et al., 2003). The latter is considered as a site which processes sensory information. However, because ejaculation is a brief phenomenon and part of a global sexual response, the limited temporal resolution of functional imaging techniques makes it difficult to discriminate brain regions specifically activated during ejaculation.

Neurochemistry

Serotonin

The role of cerebral 5-HT in the control of the ejaculatory response has received particular attention. Selective 5-HT reuptake inhibitors (SSRIs) are widely prescribed as antidepressants and have long been associated with sexual side effects including delayed ejaculation. On this basis, new therapeutical approach of premature ejaculation was developed and eventually led to the registration

of the first agent (dapoxetine) in this indication. The blockade of 5-HT transporters by SSRIs results in increased 5-HT extracellular levels and, consequently, in enhanced 5-HTergic neurotransmission in the CNS. The increased 5-HT tone is suggested to be the mechanism by which SSRIs lengthens ejaculation latency (Giuliano and Clement, 2012). The mode of action of brain 5-HT in the control of ejaculation has been extensively studied in laboratory animals. An opposite action of 5-HT on sexual behavior and more particularly ejaculatory behavior was reported in male rats depending on the site where 5-HT is microinjected (Hull et al., 2004). Delivery of 5-HT into 5-HTergic projection areas (forebrain, medial preoptic area) delayed or inhibited ejaculation whereas delivery into sites containing 5-HTergic neuron cell bodies (raphe nuclei) facilitated ejaculatory behavior. As aforementioned, a comprehensive view of the role of 5-HT is complicated by the variety of receptor subtypes expressed in the CNS. Ligands of 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{2C} receptors have been tested in different animal paradigms (Giuliano and Clement, 2012; Hull et al., 2004). Stimulation of 5-HT_{1A} receptors enhanced ejaculatory behavior in the male rat. This receptor subtype is a somatodendritic autoreceptor (i.e. expressed on cell bodies of 5-HT neurons) which activation turns down firing of 5-HT neurons and, as a consequence, decreases 5-HT release. Then, pro-ejaculatory action of 5-HT_{1A} agonists may be explained by reduction of the 5-HTergic inhibitory tone on CNS ejaculatory mechanism. Stimulation of 5-HT_{1B} receptors resulted in inhibition of ejaculation in the male rat. Although, because these receptor subtypes can be presynaptic autoreceptors or postsynaptic heteroreceptors, the exact modality of 5-HT_{1B} involvement is still unclear. Finally, it was reported that stimulation of 5-HT_{2A/C} receptors inhibited ejaculatory behavior in the male rat.

Dopamine

Another neurotransmission system that has been widely explored is the dopaminergic system, and more particularly the incerto-hypothalamic pathway (Hull et al., 2004; Peeters and Giuliano, 2008). The dopaminergic incerto-hypothalamic pathway is composed of neurons which cell bodies are in the medial zona incerta and in the periventricular nucleus and which terminals are in the hypothalamus (including lateral and medial preoptic areas, and parvocellular division of the paraventricular nucleus), the thalamus and the midbrain. Dopamine has a pro-ejaculatory activity and the medial preoptic area is a key component of the incerto-hypothalamic pathway in mediating this effect. It has long been unclear which dopamine receptor subtypes were involved as availability of selective pharmacological ligands was limited. The dopamine D₂-like receptors, which include D₂, D₃, and D₄ subtypes, were found implicated but it is only recently that the crucial role of D₃ receptors was revealed in the male rat by the use of highly selective D₃ antagonist (Clement et al., 2009). Based on observations of anejaculation in schizophrenic patients treated with antipsychotics (antagonists of D₂-like receptors), the antipsychotic levosulpiride was tested in men with premature ejaculation and was reported to lengthen ejaculation latency.

Oxytocin

Oxytocinergic parvocellular neurons in the hypothalamic paraventricular nucleus project in multiple areas in the CNS, including brain structures belonging to the brain network of ejaculation (bed nucleus of the stria terminalis, medial amygdala, and medial preoptic area). Stimulation and blockade of brain oxytocin receptors respectively facilitated and inhibited ejaculatory behavior in the male rat (Arletti et al., 1985; Clement et al., 2013). Direct action on oxytocinergic pathway and modulation of 5-HT and dopamine neurotransmission are possible mechanisms mediating oxytocin receptor ligands effect on the ejaculatory response. In human however, a recent clinical trial failed to reveal a significant effect of oxytocin antagonist on delay to ejaculate in premature ejaculation patients.

Other neurotransmitters

Neuroanatomical and functional investigations have been conducted in order to define the role of other neurochemical factors participating to the cerebral control of ejaculation (Giuliano and Clement, 2012). Notably, evidence have been gathered in the male rat showing the inhibitory effect of endogenous opioids and agonists of opioid receptors (mainly μ subtypes). In man with premature ejaculation, the opioid analgesic tramadol has been reported to be an effective on-demand treatment. The efficacy of tramadol is certainly explained by a dual action on opioid receptors (preferential agonist for μ receptors) and 5-HT transporter (inhibitor). Finally, immunohistochemical studies have detected a number of neuropeptides in the rat brain circuitry of ejaculation. Among them, cholecystokinin, galanin, and tuberoinfundibular peptide 39 have been found in pivotal brain structures although their respective role in the control of the ejaculatory process remains to be clarified.

Conclusion

Ejaculation is a short-lasting physiological event and is part of a more global sexual response that includes sexual desire, arousal, and erection. The discrimination of the physiological and pharmacological mechanisms specifically related to ejaculation is thus challenging. A neurobiological approach has been developed that is based on animal models and imaging techniques leading to major advances in the understanding of the neurophysiopharmacology of ejaculation and physiopathology of ejaculatory disorders. Description of CNS circuits (including the spinal generator of ejaculation) specifically controlling the ejaculatory response is a major milestone although many questions remain. Continuing exploration of the CNS control of ejaculation and identification of more selective pharmacological targets in the coming years will undoubtedly provide further insights and open new therapeutical avenues.

References

- Arnov, B. A., Desmond, J. E., Banner, L. L., et al. (2002). Brain activation and sexual arousal in healthy, heterosexual males. *Brain*, 125, 1014–1023.
- Arietti, R., Bazzani, C., Castelli, M., & Bertolini, A. (1985). Oxytocin improves male copulatory performance in rats. *Hormones and Behavior*, 19, 14–20.
- Borgdorff, A. J., Bernabé, J., Denys, P., Alexandre, L., & Giuliano, F. (2008). Ejaculation elicited by microstimulation of lumbar spinothalamic neurons. *European Urology*, 54, 449–456.
- Chéhensse, C., Facchinetti, P., Bahrami, S., et al. (2017). Human spinal ejaculation generator. *Annals of Neurology*, 81, 35–45.
- Clément, P., Bernabé, J., Compagnie, S., et al. (2013). Inhibition of ejaculation by the non-peptide oxytocin receptor antagonist GSK557296: a multi-level site of action. *British Journal of Pharmacology*, 169, 1477–1485.
- Clément, P., Pozzato, C., Heidbreder, C., et al. (2009). Delay of ejaculation induced by SB-277011, a selective dopamine D3 receptor antagonist, in the rat. *Journal of Sexual Medicine*, 6, 980–988.
- Denys, P., Mane, M., Azouvi, P., et al. (1998). Side effects of chronic intrathecal baclofen on erection and ejaculation in patients with spinal cord lesions. *Archives of Physical Medicine and Rehabilitation*, 79, 494–496.
- Facchinetti, P., Giuliano, F., Laurin, M., Bernabé, J., & Clément, P. (2014). Direct brain projections onto the spinal generator of ejaculation in the rat. *Neuroscience*, 272, 207–216.
- Filippi, S., Vannelli, G. B., Granchi, S., et al. (2002). Identification, localization and functional activity of oxytocin receptors in epididymis. *Molecular and Cellular Endocrinology*, 193, 89–100.
- Giuliano, F., & Clément, P. (2012). Pharmacology for the treatment of premature ejaculation. *Pharmacological Reviews*, 64, 621–644.
- Holstege, G., Georgiadis, J. R., Paans, A. M. J., et al. (2003). Brain activation during human male ejaculation. *Journal of Neuroscience*, 23, 9185–9193.
- Hull, E. M., Muschamp, J. W., & Sato, S. (2004). Dopamine and serotonin: influences on male sexual behavior. *Physiology and Behavior*, 83, 291–307.
- Peeters, M., & Giuliano, F. (2008). Central neurophysiology and dopaminergic control of ejaculation. *Neuroscience and Biobehavioral Reviews*, 32, 438–453.
- Sakamoto, H., Matsuda, K., Zuloaga, D. G., et al. (2008). Sexually dimorphic gastrin releasing peptide system in the spinal cord controls male reproductive functions. *Nature Neuroscience*, 11, 634–636.
- Truitt, W. A., & Coolen, L. M. (2002). Identification of a potential ejaculation generator in the spinal cord. *Science*, 297, 1566–1569.
- Veening, J. G., & Coolen, L. M. (2014). Neural mechanisms of sexual behavior in the male rat: emphasis on ejaculation-related circuits. *Pharmacology, Biochemistry and Behavior*, 121, 170–183.
- Xia, J. D., Han, Y. F., Zhou, L. H., Chen, Y., & Dai, Y. T. (2013). Efficacy and safety of local anaesthetics for premature ejaculation: a systematic review and meta-analysis. *Asian Journal of Andrology*, 15, 497–502.

MALE REPRODUCTIVE PHYSIOLOGY

Male Puberty: Neuroendocrine Disruption of Reproduction

Julie Fudvoye, Jean-Pierre Bourguignon, and Anne-Simone Parent, University of Liège, Liège, Belgium; and University Hospital of Liège, Chênée, Belgium

© 2018 Elsevier Inc. All rights reserved.

Introduction: Peripheral Versus Central Effects of Endocrine Disrupting Chemicals on Reproduction

Reproductive functions involve the coordinated action of different central and peripheral structures including the hypothalamus, pituitary gland, gonads, uterus, vagina, breast, prostate, etc. Several studies have shown that exposure to endocrine disrupting chemicals (EDCs) is associated with reproductive disorders such as alteration of the timing of puberty, infertility, abnormal cyclicity, premature ovarian failure, testicular cancer or adverse pregnancy outcome (Gore et al., 2015). Some of those disorders have been shown to involve direct effects of EDCs on the gonads and other peripheral tissues. For instance, studies have reported that early exposure to bisphenol A altered fetal ovarian steroidogenic gene and microRNA expression in sheep (Veiga-Lopez et al., 2013; Rivera et al., 2011) and inhibited germ cell nest breakdown (Wang et al., 2014). Prenatal exposure to diethylstilbestrol (DES) leads to an increased expression of the homeobox A10 gene in the uterus through epigenetic mechanisms (Bromer et al., 2009). The testis is also directly targeted by EDCs during early human life, as indicated by intratesticular anomalies described in the testicular dysgenesis syndrome in human (Skakkebaek et al., 2001) which are very similar to testicular anomalies described in rodents after early exposure to phthalates (Sharpe et al., 1995). Central endocrine disruption initially received relatively less attention but increasing evidence during the past decade has shown that the neuroendocrine system is also targeted by EDCs (Gore, 2001; Bourguignon et al., 2010; Frye et al., 2012). This chapter will summarize the data illustrating the effects of EDCs on sexual differentiation of the brain as well as the hypothalamic control of puberty and reproduction.

Neuroendocrine Disruption of Sexual Differentiation of the Brain

Sex steroids such as estradiol or testosterone organize the sexual differentiation of the brain during critical developmental windows of development (Schwarz and McCarthy, 2008; Gore, 2008). This early organization at the hypothalamic level allows the coordination of sex appropriate reproductive physiology and sexual behavior. Sexual differentiation of the brain is mostly dependent on the action of fetal and maternal hormones on steroid hormone receptors, but others factors participate to the establishment of the sex differences. The aromatization of testosterone into estradiol by the enzyme p450 aromatase (present in specific brain regions during critical periods of perinatal development) is one of the mechanisms whereby testosterone acts to organize and sexually differentiate brain structures and chemistry in males (Roselli et al., 2009). Alpha fetoprotein is another factor involved in the sexual differentiation of the brain. This circulating protein is produced by the fetal liver and binds estrogens with high affinity. In addition to its known neuroprotective role, it specifically delivers estrogens to target brain cells to ensure female differentiation as described in rodents (Bakker and Baum, 2008). Although most of the sex differences in the brain are established during the prenatal and neonatal periods, it appears that pubertal hormones also contribute to the addition of new cells in the sexually dimorphic regions (Ahmed et al., 2008).

Two hypothalamic regions play a crucial role in the regulation of sexually dimorphic reproductive physiology: the anteroventral periventricular hypothalamic nucleus (AVPV) and the sexually dimorphic nucleus of the preoptic area (SDN-POA). The AVPV volume is sexually dimorphic: the region has been described as larger in female than in male rats. Testicular androgens, after aromatization into estrogens, appear to be responsible for this defeminisation/masculinization of the AVPV (Wright et al., 2010). AVPV neurons project onto and directly stimulate GnRH neurons in the preoptic area. In rodents, AVPV plays a well-established role in the control of the preovulatory LH surge (Wiegand et al., 1982; Ronnekleiv and Kelly, 1986). The SDN-POA is approximately fivefold larger in male than in female rats (Gorski et al., 1978, 1980). The volume of the SDN-POA is increased by estradiol aromatized from testicular androgens perinatally. It is thought to play a role in male reproductive behavior and mate choice (Rhees et al., 1999).

Because those nuclei express nuclear hormone receptors (Estrogen receptor alpha, Estrogen receptor beta, androgen receptor and progesterone receptor) (Chakraborty et al., 2003; Orikasa et al., 2002; Mann and Babb, 2005; Simerly et al., 1990; McAbee and DonCarlos, 1999; Quadros et al., 2002, 2007), they are potential direct targets of endocrine disrupting chemicals. Initial studies illustrated the effects of early exposure to phytoestrogens on sexually dimorphic nuclei in the hypothalamus. Phytoestrogens are naturally occurring plant compounds structurally or functionally similar to oestrogens. Isoflavones are the most hormonally active and most characterized phyto-oestrogens. They are found in soy beans but also in berries, wine, grain, nuts and soya-fortified foods (Kurzer and Xu, 1997). Most isoflavones bind nuclear estrogen receptors (ER) and act as partial agonists. They appear to have a higher affinity for estrogen receptor beta (ER β) (Kuiper et al., 1998) which could explain varying activity depending on the tissue

since ER β expression varies across cell types and is sexually dimorphic (Cao and Patisaul, 2011, 2013). Some isoflavones such as genistein have been shown to bind to GPR30 (Thomas and Dong, 2006). Recent data suggests that isoflavones can also alter DNA and histone methyltransferases as well as other modifiers of chromatin structure (Shanle and Xu, 2011; Li and Tollefsbol, 2010).

While not always in accordance, several studies have reported an effect of isoflavones which is consistent with an estrogenic effect with an increased volume of the POA in males. For instance, genistein administered prenatally until adulthood leads to an increased volume of the POA in exposed males but not females (Scallet et al., 2004). When the exposure is shorter, this increase is not observed or not maintained (Lewis et al., 2003).

Neonatal exposure to genistein increased tyrosine hydroxylase immunoreactivity in the male anteroventral periventricular nucleus (Patisaul et al., 2006, 2007), indicating that genistein leads to a demasculinization of the AVPV through interference with endogenous estrogens. Genistein has also been shown to decrease kisspeptin fiber density in the female anteroventral periventricular nucleus and arcuate nucleus (Losa et al., 2011).

Synthetic endocrine disrupting chemicals have been shown to affect sexual differentiation of the brain as well. Rubin et al. (2006) have shown that perinatal exposure to low doses of bisphenol A (BPA) reduced the sex differences in the population of tyrosine hydroxylase (TH) neurons in the AVPV, primarily through a decline in the number of labeled neurons in females. Another study from McCaffrey et al. showed a defeminization of the female AVPV (evaluated through a decrease of TH positive cell numbers) after perinatal exposure to a wide range of BPA doses (McCaffrey et al., 2013). Evidence of a demasculinization of the male SDN-POA (reduced volume as labeled by calbindin) was also observed after such exposure (McCaffrey et al., 2013). The volume of the sexually dimorphic nucleus of the preoptic area is larger in males exposed perinatally to low doses of BPA but not in female, suggesting a sex specific effect of BPA exposure on SDN-POA volume (He et al., 2012). However, a study from Kwon et al. has yielded discordant results since perinatal administration of BPA did not produce detectable effects on the SDN-POA volume (Kwon et al., 2000). Differences among results may be due to differences in the age of analyses, the timing of exposure, the route of exposure or the chosen doses of EDCs.

Neuroendocrine Disruption of Pubertal Timing

Secular Trend in Age at Puberty and Environment

Puberty is a life period characterized by the maturation of the hypothalamic-pituitary-gonadal axis. It involves both physiological and behavioral changes that ultimately lead to the achievement of reproductive capacity. Puberty results from the activation of a complex neuroendocrine machinery which leads to an increase in frequency and amplitude of gonadotropin-releasing hormone (GnRH) secretion in the hypothalamus (Herbison, 2016).

A reduction in menarcheal age has been reported between 1850 and 1960 in Scandinavian countries (Tanner, 1962) and further in many European countries and USA (reviewed in Parent et al., 2003, 2015). These observations were thought to be the result of the improvement in health and nutritional status with the changes in socio-economical conditions. After a constant advance between 1850 and 1960, it appears that, in several countries with relatively stable and uniform condition of life, menarcheal age has shown only minor progression during the past decades while breast development seems to still be advancing (Parent et al., 2015). Some studies have identified a secular trend towards early onset of puberty, irrespective of the level of BMI (Aksglaede et al., 2009), suggesting the involvement of other environmental influences, among which endocrine disruptors chemicals. Detailed analysis reveals that the pattern of age distribution is affected both in boys and girls. This justifies a revision of the belief according to which current changes correspond to an advancement of pubertal timing in females. Current variations in pubertal timing actually involve a trend towards earliness for initial pubertal stages and towards lateness for final pubertal stages. This data suggests a role for environmental influences including nutrition, stress and endocrine disruptors. This hypothesis is supported by some observations of association between early exposure to endocrine disrupting chemicals or other environmental factors and the age at onset of puberty as well as rodent data which will be described below.

Challenges in Demonstrating the Endocrine Disruption of Puberty Timing

Before discussing the available data linking exposure to EDCs and neuroendocrine disruption of pubertal timing, it is important to be aware of some challenges that we face in this area.

A growing body of evidence demonstrates that fetal and early postnatal life is a critical period of sensitivity to the effects of EDCs (Gore et al., 2015; Fudvoye et al., 2014). The capacity of environmental factors to shape the neuroendocrine system is thought to be maximal during fetal and early postnatal life and possibly less important when approaching the time of onset of puberty (Parent et al., 2015). However, most studies on the role of environmental factors in triggering onset of puberty focused on the period immediately preceding puberty (Parent et al., 2015). The current recommendations of the Organization of Economic Co-operation and Development (OECD) for testing effects of EDCs on rodent female puberty, define postnatal days 22–42 as the validated exposure period with timing of vaginal opening and increase in uterine weight as evidence of puberty. Those guidelines ignore the earlier period before 25 days that is critical for neuroendocrine maturation. The effects of environmental stressors on pubertal timing depend on the period of occurrence or exposure. For instance, prepubertal underfeeding may lead to delayed puberty (Biro et al., 2005; Dunger et al., 2006; Ong et al., 2004, 2006; Cheng et al., 2012; Himes, 2006; Roa and Tena-Sempere, 2010) whereas intrauterine growth restriction is associated with early puberty (Ibáñez et al., 2006, 2011). Likewise, psychosocial stress shortly

before or during puberty may cause delayed menarche or amenorrhea (van Noord and Kaaks, 1991; Tahirović, 1998) whereas advancement of puberty has been described in girls who had experienced such stress in early postnatal life or infancy (Moffitt et al., 1992; Wierson et al., 1993). Those data are consistent with the concept that environmental clues affect pubertal timing differently depending on the life period when they come into action. In the early phase of organization of adaptive mechanisms, adverse conditions can be interpreted centrally as a risk for species survival and are translated into need of early reproductive fitness. Reversely, in a period closer to puberty, similar adverse conditions can be interpreted as a risk for quality and outcome of pregnancy and are translated into a need for delayed reproductive fitness.

Another challenge comes from observations that are inconsistent with the classical toxicology perspective according to which there will be no effect of a chemical below a threshold of exposure. This principle implies that the dose-response relationship is linear. However, hormones have complex concentration-response patterns which lay the foundation for dose-response characteristics exhibited by EDCs (Gore et al., 2015). We have showed that neonatal exposure to BPA for 2 weeks leads to a delayed maturation of GnRH secretion after a low environmentally relevant dose and advanced maturation after exposure to a higher dose (Franssen et al., 2016). Opposing effects on male rat puberty have also been observed after exposure to lower or higher doses of phthalates (Ge et al., 2007; Saillenfait et al., 2008). In addition, low-dose mixtures, consistent with human exposure, can have effects not conforming to simple additive models (Kortenkamp, 2008; Christiansen et al., 2012). However, most animal and human studies so far have focused on one or a single group of EDCs.

EDCs exposure, by modifying the hormonal environment, can affect pubertal timing either through central mechanisms, acting at different levels of the hypothalamic-pituitary system or through interaction with the peripheral target tissues such as the breast, uterus, testis or ovaries. Peripheral mechanisms can coexist with central mechanisms or secondarily facilitate them. Such a concept is supported by the dissociation between advancement in age at onset of breast development in Denmark, the Netherlands and Belgium without parallel change in menarcheal age (Parent et al., 2015). A single pubertal event can be influenced by different endocrine pathways. For instance breast development can be due to ovarian estrogen secretion under the stimulation by pituitary gonadotropins and/or estrogenic effects of EDCs independently of hypothalamic-pituitary maturation. Moreover, EDCs can interfere with the physiological inhibitory feedback mechanisms of sex steroids at the hypothalamic-pituitary level while they can also stimulate neuroendocrine maturation of GnRH secretion (Rasier et al., 2006).

Disruption of the Hypothalamic Control of Puberty

Several animal studies have reported alterations of pubertal timing after exposure to EDCs. Some models allowed the identification of neuroendocrine mechanisms leading to such effects.

In females, the effects on EDCs on the timing of vaginal opening depend on the period of exposure (Parent et al., 2015). When the animals are exposed to BPA during pregnancy only, age at vaginal opening is either not affected (Tinwell et al., 2002; Murray et al., 2007) or early (Honma et al., 2002; Nikaido et al., 2004; Howdeshell et al., 1999). When exposure takes place between birth and 15 days of age, age at vaginal opening is either not affected (Adewale et al., 2009; Losa-Ward et al., 2012; Nagao et al., 1999; Yu et al., 2010) or early (Adewale et al., 2009; Losa-Ward et al., 2012; Fernández et al., 2009; Nah et al., 2011). A dose of 50 µg/kg causes advancement of puberty while 50 mg/kg has no effect, indicating a nonlinear dose-response relationship (Adewale et al., 2009; Losa-Ward et al., 2012). We also showed that a very low doses of 25 ng/kg/day from postnatal day 1 to 15 delayed maturation of GnRH secretion while a high dose of 5 mg/kg/day led to an accelerated maturation (Franssen et al., 2016). Taken as a whole, those studies show that BPA effects depend markedly on the window of exposure and the dose, with possible nonlinear dose-response relationship. Several studies have provided evidence of neuroendocrine changes after early exposure to BPA in the female rat. These will be discussed further in the mechanistic chapter below. Most studies investigating the consequences of neonatal exposure to genistein led to equivocal conclusions since age at vaginal opening was either normal with no dose-related effect (Nagao et al., 2001; Jefferson et al., 2005; Kouki et al., 2003) or early and dose- or duration-dependent (Lewis et al., 2003; Kouki et al., 2003) or even late and dose-dependent (Jefferson et al., 2009). Using phthalates, the period of exposure appears crucial since prenatal exposure accounts for late vaginal opening (Salazar et al., 2004) whereas postnatal exposure is associated with normal timing (Gray et al., 2006; Moral et al., 2007) or early vaginal opening irrespective of the dose (Hu et al., 2013; Ma et al., 2006). Polybrominated EDCs appear to cause either no change or delay in vaginal opening depending on the dose and irrespective of the period of exposure (Stoker et al., 2004; Lilienthal et al., 2006; Ceccatelli et al., 2006).

In male rodents, balano-preputial separation is commonly used as a marker of puberty. With the exception of early balano-preputial separation after exposure to phthalates (Ge et al., 2007; Saillenfait et al., 2008), EDCs appear to cause either no effect or a delay of sexual maturation in the male rodent. BPA does not show any effect whatever the dose and the window of exposure (Tinwell et al., 2002; Nagao et al., 1999; Tan et al., 2003; Ashby and Lefevre, 2000; Kato et al., 2006). Similarly, using a very low dose of BPA neonatally for 5 days, we did not see any effect of exposure on balano-preputial separation. However, when pulsatile GnRH secretion from hypothalamic explants is studied at 25 days in such exposed animals, GnRH pulse frequency is reduced, indicating a delayed maturation of GnRH secretion caused by BPA neonatally. This indicates that GnRH secretion could be extremely sensitive to disruption without being sufficient or lasting sufficiently to account for phenotypic changes. When similar doses of different EDCs are used at different periods of exposure in the male rodent, they are found to be effective in delaying puberty after postnatal (after weaning) exposure as opposed to no effects after prenatal exposure. Such findings are obtained using dichlorodiphenylchloroethylene (DDE), vinclozolin (Warner et al., 2004) or diethylstilbestrol (DES) (Warner et al., 2004; Mol et al., 2002) suggesting that the fetus is less sensitive than the juvenile animal. The newborn rat can also be more sensitive than the fetus as found after

exposure to DES (Warner et al., 2004). Such a conclusion was also drawn in a review of several studies on BPA effects in the female rat since gestational exposure has no effect on age at vaginal opening whereas neonatal exposure is followed by early puberty (Guo et al., 2004). Importantly, both periods of exposure fall into the so-called “programming window” indicating that, even within this particular period, there may be differences in sensitivity to endocrine disruption. The dose of EDC however plays a critical role since, when investigated at a given period of life, higher doses appear to be more effective such as shown after prenatal exposure for DDE (Leijs et al., 2008) and phthalates (Denham et al., 2005; Den Hond et al., 2002), or after lactational exposure for DDE, vinclozolin or DES (Warner et al., 2004) or following exposure after weaning for DDE (Grandjean et al., 2012; Warner et al., 2004), vinclozolin (Warner et al., 2004; Adgent et al., 2012), DES (Warner et al., 2004; Kim et al., 2011), phthalates (Deng et al., 2012; Strom et al., 2001) and polybrominated diphenyl ethers (PBDE) (Giampietro et al., 2004). It is noteworthy that in two studies using phthalates, opposing effects are observed since lower doses are associated with early puberty and higher doses with delay (Deng et al., 2012; Denham et al., 2005). Interestingly enough, we have reported similar opposing effects using DES neonatally in the female rat (Fernandez-Rhodes et al., 2013).

Neuroendocrine Disruption of Ovulation

Ovulation is under the control of GnRH secretion which is tightly regulated by the positive and negative feedbacks of gonadal steroids. In both males and females, GnRH secretion is suppressed by the negative feedback taking place at the level of the arcuate nucleus. The positive feedback of estrogens is mediated by the AVPV and potentiates the surge in GnRH and subsequently luteinizing hormone (LH) that precedes ovulation. In rodents, the sexual differentiation of this positive feedback takes place during the perinatal period and is particularly sensitive to disruption by hormones and EDCs. It has been shown that the administration of testosterone or estradiol benzoate during the neonatal period can alter the positive feedback by estradiol (Padmanabhan and Veiga-Lopez, 2014). Such data suggests that early exposure to EDCs could alter the programming of the preovulatory LH surge. In addition, exposure to EDCs during adulthood could alter the central control of ovulation. This article will summarize the effects of neonatal or adult exposure to EDCs on the hypothalamic control of ovulation. As discussed above for puberty, it is sometimes difficult to differentiate the effects of EDCs on the central versus the ovarian control of ovulation. Some animal models can help to shed light upon such aspects.

The sheep offers a good model for studying the effects of EDCs on the neuroendocrine control of ovulation thanks to the availability of noninvasive approaches to monitor follicular dynamics as well as surgical approaches to obtain hypophyseal portal blood (Padmanabhan and Veiga-Lopez, 2014). Prenatal exposure of female sheep to excess androgens has been shown to lead to adult reproductive disorders with LH excess, multifollicular ovarian morphology and corpus luteus dysfunction. Such early exposure to testosterone disrupted the estradiol negative feedback (Wood and Foster, 1998; Sharma et al., 2002), estradiol positive feedback (Wood and Foster, 1998; Sharma et al., 2002) and progesterone negative feedback (Robinson et al., 1999). Neonatal exposure to BPA leads to reproductive disorders similar to those found in testosterone exposed sheep with LH excess and severely dampened preovulatory LH surge (Savabieasfahani et al., 2006).

Neonatal exposure to BPA in rodents has been shown to disrupt pubertal timing but also leads to premature anestrus and ovarian anomalies. As described for the sheep, these anomalies could result from disrupted organization at the level of the hypothalamus/pituitary or at the level of the ovaries. Adewale et al. have shown that alterations of estrus cycle caused by a relatively low dose of BPA was not accompanied by a reduction of the number of GnRH neurons expressing the immediate early gene *c-fos* suggesting that steroid-positive feedback was not defeminized by BPA. However, this study did not rule out the possibility that other regulation of the GnRH neurons could have been altered (Adewale et al., 2009). Other studies suggest that neonatal exposure to BPA decreased basal and GnRH induced LH secretion and increased GnRH pulsatility in adult rats, suggesting a permanent effect on GnRH pulsatility and pituitary signaling (Fernández et al., 2009). There is relatively less information on the neuroendocrine disruption of ovulation caused by EDCs other than BPA. Exposure to TCDD (2,3,7,8-tetrachlorodibenzo-para-dioxin) has been associated with abnormal ovulation and premature ovarian failure (Gregoraszczyk and Ptak, 2013). While some studies showed that TCDD disrupted estradiol secretion in human granulosa cells (Baldridge et al., 2015), others have reported alteration of the hypothalamic-pituitary axis. Takeda et al. (2014) have showed that exposure of pregnant rats to TCDD led to decreased expression of gonadotropins in pups and LH and FSH levels appeared to be decreased in virgin female mice (Huang et al., 2011). Tributyltin chloride is used as a biocide in antifouling paints which exposure has been associated with obesity. It has recently been shown to alter estrus cyclicity and cause decreased surge LH levels and GnRH expression (Sena et al., 2017). Very few data is available regarding the effects of phthalates on estrus cyclicity and ovulation. One study showed that exposure to DEHP during adult life led to increased duration of estrus in mice (Hannon et al., 2014).

Mechanisms of EDCs Effects on the Overall Neuroendocrine Control of the Reproductive System

The hypothalamic-pituitary-gonadal axis is responsible for the development of puberty and acquisition of the reproductive function. A subset of neurons in the anterior hypothalamus secrete gonadotropin-releasing hormone (GnRH) which, through its pattern of release, controls all aspects of reproductive function throughout life. The secretory activity of GnRH neurons depends on trans-synaptic and glial inputs mediated by neurotransmitters and cell-cell signaling molecules. New methods have provided more

information regarding the dynamic coordination of network contributing to the central control of the pubertal process and reproduction (Ojeda and Lomniczi, 2014). When studying the central mechanisms potentially involved in the neuroendocrine disruption of puberty or ovulation, GnRH neurons appear to be a potential target of exposure to endocrine disruptors. Using a murine model of neonatal exposure to dichlorodiphenyltrichloroethane (DDT), our team has confirmed the involvement of neuroendocrine disruption in the alteration of pubertal timing. Pulsatile GnRH secretion was accelerated after early exposure to DDT and this effect was followed by early sexual maturation (Rasier et al., 2007). Further studies have indicated that such effects involve the estrogen receptor, the orphan dioxin (AhR, arylhydrocarbon) receptor and a subtype of glutamate receptor (Rasier et al., 2008). Another study of our group showed that exposure to DES (10 µg/kg/day) during the first 5 days of life was associated with early vaginal opening while a dose of 1 µg/kg/day was associated with delayed vaginal opening and delayed developmental increase in GnRH pulse frequency (Franssen et al., 2014). DES is a potent synthetic estrogen which was prescribed to prevent miscarriages during the 1950s and 1960s. Its prescription is now because of the increased risk of vaginal cancer after in utero exposure. DES is still used as a paradigmatic EDC in animal models. Our observations counteract the classical hypothesis that EDCs cause sexual precocity. Although precocity is indeed observed in several studies and possibly reflects both peripheral and central mechanisms, delayed female puberty after neonatal exposure to a potent estrogenic EDC is a new finding that is likely to be of neuroendocrine origin. In addition, it appears that prenatal food restriction could have additive effects to neonatal DES exposure on the neuroendocrine effects of leptin. When females were exposed in utero to prenatal food restriction and postnatally to DES, the stimulatory effect of leptin on GnRH secretion in vitro was completely blunted (Franssen et al., 2014). This reinforces the demonstration of a neuroendocrine disruption of GnRH secretion and illustrates the potential additive effects of different environmental stressors.

Other studies have shown effects of EDCs on GnRH secretion. Fernandez et al. demonstrated an increase of GnRH pulsatility in infantile rats after neonatal exposure to BPA (Fernández et al., 2009). Interestingly, GnRH pulsatility remained disrupted in adult rats neonatally exposed to BPA (Fernández et al., 2009). Losa-Ward et al. (2012) have reported morphological alteration of RFamide-related peptide-3 (RFRP3) neurons known to inhibit GnRH neuron activity after neonatal exposure to BPA. They showed reduced RFRP3 perikaria, fiber density and contacts on GnRH neurons, suggesting that BPA-induced premature puberty could result from decreased inhibition of GnRH neurons. GnRH neurons response to steroid-positive feedback, however, was not affected by neonatal exposure to BPA in the study from Adewale et al. (2009). More recently, our group has reported that the developmental increase in frequency of GnRH release at the time of puberty is delayed in female rats exposed neonatally to a low dose of BPA (25 ng/kg/day). Interestingly, the effects were opposite with a high dose of BPA (5 mg/kg/day). The mechanism involved respectively increased or reduced tone of γ -aminobutyric acidergic (GABAergic) neurotransmission which is known to play an inhibitory control on GnRH secretion (Franssen et al., 2016).

Neuroendocrine disruption of reproduction can act through interference with the production or the action of brain neuropeptides which regulate GnRH secretion. Kisspeptin, encoded by the KISS1 gene, is known to stimulate GnRH secretion and plays a crucial role in the onset of puberty (Seminara et al., 2003; de Roux et al., 2003). Navarro et al. have shown a reduction in hypothalamic expression of KISS1 mRNA at puberty after neonatal exposure to a high dose of BPA as it was observed after exposure to estradiol benzoate (Navarro et al., 2009). Similarly, Patisaul et al. found a reduction in kisspeptin immunoreactivity (estimated in term of fibers density) in the arcuate nucleus in adult female rats (Patisaul et al., 2009) after neonatal exposure. Regarding the earlier vaginal opening that occurs after exposure to a high dose of BPA (Franssen et al., 2016), those observations were unexpected since Kiss1 is considered as a major gatekeeper of puberty onset. The reduction in kisspeptin expression could then be part of a reactive mechanism to BPA exposure. Similarly, a study performed in the sheep showed a decrease of Kiss1 mRNA levels at the rostral, mid and caudal regions of the hypothalamus in foetus exposed in utero to a complex mixture of EDCs (Bellingham et al., 2009). Such effects were not observed after adult exposure, supporting the hypothesis that the period of exposure is critical for such regulatory processes. Xi et al. (2011) showed that perinatal exposure to a high dose of BPA resulted in the up-regulation of the expression levels of KISS-1 and GnRH in both male and female pups. This observation was not replicated after postnatal exposure to BPA, highlighting once again the critical perinatal period for BPA affecting reproductive neural circuits in hypothalamus (Xi et al., 2011). Acute infusion of BPA in the stalk-median eminence of mid to late pubertal female rhesus monkeys led to a suppression of GnRH and kisspeptin secretion for the highest doses while lower doses did not have any apparent effects (Kurian et al., 2015).

GnRH neurons themselves express ER β but also G protein coupled receptor 30 (GPR30) and estrogen-related receptor γ , all potential receptors for endocrine disrupting chemicals. Thus GnRH neurons could be directly targeted by EDCs. A recent study has shown that BPA significantly decreased calcium activity in GnRH neurons in explants. Blockade of GABAergic and glutamatergic input did not abrogate the inhibitory effect of BPA, suggesting a direct effect of BPA on GnRH neurons (Klenke et al., 2016). Other studies in fish suggest that early exposure to BPA ou bisphenol S could increase the number of GnRH3 neurons during development which suggests a broader effect of BPA and BPS on neurodevelopment (Qiu et al., 2016).

Conclusion and Perspectives

The hypothalamus provides a unique site for finely tuned integration of endogenous and environmental factors influencing the control of puberty and reproduction. While initial studies focused on the effects of EDCs on peripheral organs, recent data have shown that the hypothalamic-pituitary control of reproduction can be targeted by environmental pollutants. While such effects are extremely difficult to study in humans, animal models play a crucial role in deciphering the neuroendocrine mechanisms of

action of such compounds. Neuroendocrine effects of EDCs can be opposing and involve different mechanisms depending on the dose or whether they take place early, during fetal and neonatal life or late during prepubertal life.

These last few years, the knowledge of molecular and genetic mechanisms controlling the pubertal process has accelerated considerably. Puberty results from coordinated changes in a multiplicity of genes organized into functional networks. Recent evidence suggests that a dual mechanism of epigenetic regulation affecting the transcriptional activity of neurons involved in stimulating GnRH release plays a fundamental role in the timing of puberty (Ojeda and Lomniczi, 2014; Lomniczi et al., 2015). In addition, a multilayered microRNA-operated switch appears to be involved in the increase in GnRH expression during the prepubertal period (Messina et al., 2016). Epigenetic repression and activation of gene transcription might be a mechanism by which environmental factors affect pubertal development and reproduction. Because EDCs have been recently shown to alter DNA methylation, histone modifications and microRNA expression (Gore et al., 2015), further studies will be needed in order to explore the involvement of such mechanisms in the neuroendocrine disruption of reproduction.

References

- Adewale, H. B., Jefferson, W. N., Newbold, R. R., & Patisaul, H. B. (2009). Neonatal bisphenol-A exposure alters rat reproductive development and ovarian morphology without impairing activation of gonadotropin-releasing hormone neurons. *Biology of Reproduction*, 81, 690–699.
- Adgent, M. A., Daniels, J. L., Rogan, W. J., Adair, L., Edwards, L. J., Westreich, D., Maisonet, M., & Marcus, M. (2012). Early-life soy exposure and age at menarche. *Paediatric and Perinatal Epidemiology*, 26, 163–175.
- Ahmed, E. I., Zehr, J. L., Schulz, K. M., Lorenz, B. H., DonCarlos, L. L., & Sisk, C. L. (2008). Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions. *Nature Neuroscience*, 11, 995–997.
- Aksglaede, L., Juul, A., Olsen, L. W., & Sørensen, T. (2009). Age at puberty and the emerging obesity epidemic. *PLoS One*, 4(12), e8450.
- Ashby, J., & Lefevre, P. A. (2000). The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators. *Journal of Applied Toxicology*, 20, 35–47.
- Bakker, J., & Baum, M. J. (2008). Role for estradiol in female-typical brain and behavioral sexual differentiation. *Frontiers in Neuroendocrinology*, 29, 1–16.
- Baldrige, M. G., Marks, G. T., Rawlins, R. G., & Hutz, R. J. (2015). Very low-dose (femtomolar) 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) disrupts steroidogenic enzyme mRNAs and steroid secretion by human luteinizing granulosa cells. *Reproductive Toxicology*, 52, 57–61.
- Bellingham, M., Fowler, P. A., Amezcua, M. R., Rhind, S. M., Cotinot, C., Mandon-Pepin, B., Sharpe, R. M., & Evans, N. P. (2009). Exposure to a complex cocktail of environmental endocrine-disrupting compounds disturbs the kisspeptin/GPR54 system in ovine hypothalamus and pituitary gland. *Environmental Health Perspectives*, 117, 1556–1562.
- Biro, F. M., Khoury, P., & Morrison, J. A. (2005). Influence of obesity on timing of puberty. *International Journal of Andrology*, 29, 272–277.
- Bourguignon, J. P., Rasier, G., Lebrethon, M. C., Gerard, A., Naveau, E., & Parent, A. S. (2010). Neuroendocrine disruption of pubertal timing and interactions between homeostasis of reproduction and energy balance. *Molecular and Cellular Endocrinology*, 324, 110–120.
- Bromer, J. G., Wu, J., Zhou, Y., & Taylor, H. S. (2009). Hypermethylation of Homeobox A10 by in utero diethylstilbestrol exposure: An epigenetic mechanism for altered developmental programming. *Biology of Reproduction*, 80, 79–85.
- Cao, J., & Patisaul, H. B. (2011). Sexually dimorphic expression of hypothalamic estrogen receptors α and β and Kiss1 in neonatal male and female rats. *The Journal of Comparative Neurology*, 519, 2954–2977.
- Cao, J., & Patisaul, H. B. (2013). Sex-specific expression of estrogen receptors α and β and Kiss1 in the postnatal rat amygdala. *The Journal of Comparative Neurology*, 521, 465–478.
- Ceccatelli, R., Faass, O., Schlumpf, M., & Lichtensteiger, W. (2006). Gene expression and estrogen sensitivity in rat uterus after developmental exposure to the polybrominated diphenylether PBDE 99 and PCB. *Toxicology*, 220, 104–116.
- Chakraborty, T. R., Hof, P. R., Ng, L., & Gore, A. C. (2003). Stereologic analysis of estrogen receptor alpha (ER alpha) expression in rat hypothalamus and its regulation by aging and estrogen. *The Journal of Comparative Neurology*, 466, 409–421.
- Cheng, G., Buyken, A. E., Shi, L., Karaolis-Danckert, N., Kroke, A., Wudy, S. A., Degen, G. H., & Remer, T. (2012). Beyond overweight: Nutrition as an important lifestyle factor influencing timing of puberty. *Nutrition Reviews*, 70, 133–152.
- Christiansen, S., Kortenkamp, A., Axelstad, M., Boberg, J., Scholze, M., Jacobsen, P. R., Faust, M., Lichtensteiger, W., Schlumpf, M., Burdorf, A., & Hass, U. (2012). Mixtures of endocrine disrupting contaminants modelled on human high end exposures: An exploratory study in rats. *International Journal of Andrology*, 35, 303–316.
- Deng, F., Tao, F. B., Liu, D. Y., Xu, Y. Y., Hao, J. H., Sun, Y., & Su, P. Y. (2012). Effects of growth environments and two environmental endocrine disruptors on children with idiopathic precocious puberty. *European Journal of Endocrinology*, 166, 803–809.
- Denham, M., Schell, L. M., Deane, G., Gallo, M. V., Ravenscroft, J., DeCaprio, A. P., & Akwesasne Task Force on the Environment. (2005). Relationship of lead, mercury, mirex, dichlorodiphenyldichloroethylene, hexachlorobenzene, and polychlorinated biphenyls to timing of menarche among Akwesasne Mohawk girls. *Pediatrics*, 115, e127–34.
- Den Hond, E., Roels, H. A., Hoppenbrouwers, K., Nawrot, T., Thijs, L., Vandermuelen, C., Winneke, G., Vanderschuren, D., & Staessen, J. A. (2002). Sexual maturation in relation to polychlorinated aromatic hydrocarbons: Sharpe and Skakkebaek's hypothesis revisited. *Environmental Health Perspectives*, 110, 771–776.
- de Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L., & Milgrom, E. (2003). Hypogonadotropic hypogonadism due to loss of function of the Kiss1-derived peptide receptor GPR54. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 10972–10976.
- Dunger, D. B., Ahmed, M. L., & Ong, K. K. (2006). Early and late weight gain and the timing of puberty. *Molecular and Cellular Endocrinology*, 25, 140–145.
- Fernández, M., Bianchi, M., Lux-Lantos, V., & Libertun, C. (2009). Neonatal exposure to bisphenol A alters reproductive parameters and gonadotropin releasing hormone signaling in female rats. *Environmental Health Perspectives*, 117, 757–762.
- Fernandez-Rhodes, L., Demerath, E. W., Cousminer, D. L., Tao, R., Dreyfus, J. G., Esko, T., Smith, A. V., Gudnason, V., Harris, T. B., Launer, L., McArdle, P. F., Yerges-Armstrong, L. M., Elks, C. E., Strachan, D. P., Kutalik, Z., Vollenweider, P., Feenstra, B., Boyd, H. A., Metspalu, A., Mihailov, E., Broer, L., Zillikens, M. C., Oostra, B., van Duijn, C. M., Lunetta, K. L., Perry, J. R., Murray, A., Koller, D. L., Lai, D., Corre, T., Toniolo, D., Albrecht, E., Stöckl, D., Grallert, H., Gieger, C., Hayward, C., Polasek, O., Rudan, I., Wilson, J. F., He, C., Kraft, P., Hu, F. B., Hunter, D. J., Hottenga, J. J., Willemssen, G., Boomsma, D. I., Byrne, E. M., Martin, N. G., Montgomery, G. W., Warrington, N. M., Pennell, C. E., Stolk, L., Visser, J. A., Hofman, A., Uitterlinden, A. G., Rivadeneira, F., Lin, P., Fisher, S. L., Bierut, L. J., Crisponi, L., Porcu, E., Mangino, M., Zhai, G., Spector, T. D., Buring, J. E., Rose, L. M., Ridker, P. M., Poole, C., Hirschhorn, J. N., Murabito, J. M., Chasman, D. I., Widen, E., North, K. E., Ong, K. K., & Franceschini, N. (2013). Association of adiposity genetic variants with menarche timing in 92,105 women of European descent. *American Journal of Epidemiology*, 178, 451–460.
- Franssen, D., Ioannou, Y. S., Alvarez-real, A., Gerard, A., Mueller, J. K., Heger, S., Bourguignon, J. P., & Parent, A. S. (2014). Pubertal timing after neonatal diethylstilbestrol exposure in female rats: Neuroendocrine vs peripheral effects and additive role of prenatal food restriction. *Reproductive Toxicology*, 44, 63–72.
- Franssen, D., Gérard, A., Hennuy, B., Donneau, A. F., Bourguignon, J. P., & Parent, A. S. (2016). Delayed neuroendocrine sexual maturation in female rats after a very low dose of bisphenol A through altered GABAergic neurotransmission and opposing effects of a high dose. *Endocrinology*, 157(5), 1740–1750.

- Frye, C. A., Bo, E., Calamandrei, G., Calzà, L., Dessi-Fulgheri, F., Fernández, M., Fusani, L., Kah, O., Kajta, M., Le Page, Y., Patisaul, H. B., Venerosi, A., Wojtowicz, A. K., & Panzica, G. C. (2012). Endocrine disruptors: A review of some sources, effects, and mechanisms of actions on behaviour and neuroendocrine systems. *Journal of Neuroendocrinology*, 24, 144–159.
- Fudvoye, J., Bourguignon, J. P., & Parent, A. S. (2014). Endocrine-disrupting chemicals and human growth and maturation a focus on early critical windows of exposure. *Vitamins and Hormones*, 94, 1–25.
- Ge, R. S., Chen, G. R., Dong, Q., Akingbemi, B., Sottas, C., Santos, M., Sealfon, S. C., Bernard, D. J., & Hardy, M. P. (2007). Biphasic effects of postnatal exposure to diethylhexylphthalate on the timing of puberty in male rats. *Journal of Andrology*, 28(4), 513–520.
- Giampietro, P. G., Bruno, G., Furcolo, G., Casati, A., Brunetti, E., Spadoni, G. L., & Galli, E. (2004). Soy protein formulas in children: No hormonal effects in long-term feeding. *Journal of Pediatric Endocrinology & Metabolism*, 17, 191–196.
- Gore, C. (2001). Environmental toxicant effects on neuroendocrine function. *Endocrine*, 14, 235–246.
- Gore, A. C. (2008). Developmental programming and endocrine disruptor effects on reproductive neuroendocrine systems. *Frontiers in Neuroendocrinology*, 29(3), 358–374.
- Gore, A. C., Chappell, V. A., Fenton, S. E., Flaws, J. A., Nadal, A., Prins, G. S., Toppari, J., & Zoeller, R. T. (2015). Executive summary to EDC-2: The Endocrine Society's second scientific statement on endocrine-disrupting chemicals. *Endocrine Reviews*, 36, 593–602.
- Gorski, R. A., Gordon, J. H., Shryne, J. E., & Southam, A. M. (1978). Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Research*, 148, 333–346.
- Gorski, R. A., Harlan, R. E., Jacobson, C. D., Shryne, J. E., & Southam, A. M. (1980). Evidence for the existence of a sexually dimorphic nucleus in the preoptic area of the rat. *The Journal of Comparative Neurology*, 193, 529–539.
- Grandjean, P., Grønlund, C., Kjær, I. M., Jensen, T. K., Sørensen, N., Andersson, A. M., Juul, A., Skakkebaek, N. E., Budtz-Jørgensen, E., & Weihe, P. (2012). Reproductive hormone profile and pubertal development in 14-year-old boys prenatally exposed to polychlorinated biphenyls. *Reproductive Toxicology*, 34(4), 498–503.
- Gray, L. E., Laskey, J., & Ostby, J. (2006). Chronic di-*n*-butyl phthalate exposure in rats reduces fertility and alters ovarian function during pregnancy in female long Evans hooded rats. *Toxicological Sciences*, 93, 189–195.
- Gregoraszczuk, E. L., & Ptak, A. (2013). Endocrine-disrupting chemicals: Some actions of POPs on female reproduction. *International Journal of Endocrinology*, 2013, 828532.
- Guo, Y. L., Lambert, G. H., Hsu, C. C., & Hsu, M. M. (2004). Yucheng: Health effects of prenatal exposure to polychlorinated biphenyls and dibenzofurans. *International Archives of Occupational and Environmental Health*, 77, 153–158.
- Hannon, P. R., Peretz, J., & Flaws, J. (2014). Daily exposure to di(2-ethylhexyl) phthalate alters estrous cyclicity and accelerates primordial follicle recruitment potentially via dysregulation of the phosphatidylinositol 3-kinase signaling pathway in adult mice. *Biology of Reproduction*, 90, 136.
- He, Z., Paule, M. G., & Ferguson, S. A. (2012). Low oral doses of bisphenol A increase volume of the sexually dimorphic nucleus of the preoptic area in male, but not female, rats at postnatal day 21. *Neurotoxicology and Teratology*, 34, 331–337.
- Herbison, A. E. (2016). Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nature Reviews. Endocrinology*, 12, 452–466.
- Himes, J. H. (2006). Examining the evidence for recent secular changes in the timing of puberty in US children in light of increases in the prevalence of obesity. *Molecular and Cellular Endocrinology*, 254–255, 13–21.
- Honma, S., Suzuki, A., Buchanan, D. L., Katsu, Y., Watanabe, H., & Iguchi, T. (2002). Low dose effect of in utero exposure to bisphenol A and diethylstilbestrol on female mouse reproduction. *Reproductive Toxicology*, 16, 117–122.
- Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenberg, J. G., & vom Saal, F. S. (1999). Exposure to bisphenol A advances puberty. *Nature*, 401, 763–764.
- Hu, J., Du, G., Zhang, W., Huang, H., Chen, D., Wu, D., & Wang, X. (2013). Short-term neonatal/prepubertal exposure of dibutyl phthalate (DBP) advanced pubertal timing and affected hypothalamic Kisspeptin/GPR54 expression differently in female rats. *Toxicology*, 314, 65–75.
- Huang, L., Huang, R., Ran, X. R., Liu, H. Y., Zhang, Y., Dai, L. J., & Li, B. (2011). Three-generation experiment showed female C57BL/6J mice drink drainage canal water containing low level of TCDD-like activity causing high pup mortality. *The Journal of Toxicological Sciences*, 36, 713–724.
- Ibáñez, L., Jiménez, R., & de Zegher, F. (2006). Early puberty-menarche after precocious pubarche: Relation to prenatal growth. *Pediatrics*, 117, 117–121.
- Ibáñez, L., López-Bermejo, A., Díaz, M., & Marcos, M. V. (2011). Endocrinology and gynecology of girls and women with low birth weight. *Fetal Diagnosis and Therapy*, 30, 243–249.
- Jefferson, W. N., Padilla-Banks, E., & Newbold, R. R. (2005). Adverse effects on female development and reproduction in CD-1 mice following neonatal exposure to the phytoestrogen genistein at environmentally relevant doses. *Biology of Reproduction*, 73, 798–806.
- Jefferson, W. N., Doerge, D., Padilla-Banks, E., Woodling, K. A., Kissling, G. E., & Newbold, R. (2009). Oral exposure to genistin, the glycosylated form of genistein, during neonatal life adversely affects the female reproductive system. *Environmental Health Perspectives*, 117, 1883–1889.
- Kato, H., Furuhashi, T., Tanaka, M., Katsu, Y., Watanabe, H., Ohta, Y., & Iguchi, T. (2006). Effects of bisphenol A given neonatally on reproductive functions of male rats. *Reproductive Toxicology*, 22, 20–29.
- Kim, J., Kim, S., Huh, K., Kim, Y., Joung, H., & Park, M. (2011). High serum isoflavone concentrations are associated with the risk of precocious puberty in Korean girls. *Clinical Endocrinology*, 75, 831–835.
- Klenke, U., Constantin, S., & Wray, S. (2016). BPA directly decreases GnRH neuronal activity via a noncanonical pathway. *Endocrinology*, 157, 1980–1990.
- Kouki, T., Kishitake, M., Okamoto, M., Oosuka, I., Takebe, M., & Yamanouchi, K. (2003). Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: Estrous cycle and lordosis. *Hormones and Behavior*, 44, 140–145.
- Kortenkamp, A. (2008). Low dose mixture effects of endocrine disruptors: Implications for risk assessment and epidemiology. *International Journal of Andrology*, 31(2), 233–240.
- Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B., & Gustafsson, J. A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*, 139, 4252–4263.
- Kurian, J. R., Keen, K. L., Kenealy, B. P., Garcia, J. P., Hedman, C. J., & Terasawa, E. (2015). Acute influences of bisphenol A exposure on hypothalamic release of gonadotropin-releasing hormone and Kisspeptin in female rhesus monkeys. *Endocrinology*, 156, 2563–2570.
- Kurzer, M. S., & Xu, X. (1997). Dietary phytoestrogens. *Annual Review of Nutrition*, 17, 353–381.
- Kwon, S., Stedman, D. B., Elswick, B. A., Cattley, R. C., & Welsch, F. (2000). Pubertal development and reproductive functions of Crl:CD BR Sprague-Dawley rats exposed to bisphenol A during prenatal and postnatal development. *Toxicological Sciences*, 55, 399–406.
- Leijds, M. M., Koppe, J. G., Olie, K., van Aalderen, W. M., Pd, V., Vulsma, T., Westra, M., & ten Tusscher, G. W. (2008). Delayed initiation of breast development in girls with higher prenatal dioxin exposure; a longitudinal cohort study. *Chemosphere*, 73, 999–1004.
- Lewis, R. W., Brooks, N., Milburn, G. M., Soames, A., Stone, S., Hall, M., & Ashby, J. (2003). The effects of the phytoestrogen genistein on the postnatal development of the rat. *Toxicological Sciences*, 71, 74–83.
- Li, Y., & Tollefsbol, T. O. (2010). Impact on DNA methylation in cancer prevention and therapy by bioactive dietary components. *Current Medicinal Chemistry*, 17, 2141–2151.
- Lilienthal, H., Hack, A., Roth-Harer, A., Grande, S. W., & Talsness, C. E. (2006). Effects of developmental exposure to 2,2,4,4,5-pentabromodiphenyl ether (PBDE-99) on sex steroids, sexual development, and sexually dimorphic behavior in rats. *Environmental Health Perspectives*, 114, 194–201.
- Lomicz, A., Wright, H., & Ojeda, S. R. (2015). Epigenetic regulation of female puberty. *Frontiers in Neuroendocrinology*, 36, 90–107.
- Losa, S. M., Todd, K. L., & Sullivan, A. W. (2011). Neonatal exposure to genistein adversely impacts the ontogeny of hypothalamic kisspeptin signaling pathways and ovarian development in the peripubertal female rat. *Reproductive Toxicology*, 31, 280–289.
- Losa-Ward, S. M., Todd, K. L., McCaffrey, K. A., Tsutsui, K., & Patisaul, H. B. (2012). Disrupted organization of RFamide pathways in the hypothalamus is associated with advanced puberty in female rats neonatally exposed to bisphenol A. *Biology of Reproduction*, 87, 28.

- Ma, M., Kondo, T., Ban, S., Umemura, T., Kurahashi, N., Takeda, M., & Kishi, R. (2006). Exposure of prepubertal female rats to inhaled di(2-ethylhexyl)phthalate affects the onset of puberty and postpubertal reproductive functions. *Toxicological Sciences*, 93, 164–171.
- Mann, P. E., & Babb, J. A. (2005). Neural steroid hormone receptor gene expression in pregnant rats. *Brain Research. Molecular Brain Research*, 142, 39–46.
- McAbee, M. D., & DonCarlos, L. L. (1999). Regulation of androgen receptor messenger ribonucleic acid expression in the developing rat forebrain. *Endocrinology*, 140, 1807–1814.
- McCaffrey, K. A., Jones, B., Mabrey, N., Weiss, B., Swan, S. H., & Patisaul, H. B. (2013). Sex specific impact of perinatal bisphenol a (BPA) exposure over a range of orally administered doses on rat hypothalamic sexual differentiation. *Neurotoxicology*, 36, 55–62.
- Messina, A., Langlet, F., Chachlaki, K., Roa, J., Rasika, S., Jouy, N., Gallet, S., Gaytan, F., Parkash, J., Tena-Sempere, M., Giacobini, P., & Prevot, V. (2016). A microRNA switch regulates the rise in hypothalamic GnRH production before puberty. *Nature Neuroscience*, 19, 835–844.
- Moffitt, T. E., Caspi, A., Belsky, J., & Silva, P. A. (1992). Childhood experience and the onset of menarche: A test of a sociobiological model. *Child Development*, 63, 47–58.
- Mol, N. M., Sørensen, N., Weihe, P., Andersson, A. M., Jorgensen, N., Skakkebaek, N. E., Keiding, N., & Grandjean, P. (2002). Spermaturation and serum hormone concentrations at the age of puberty in boys prenatally exposed to polychlorinated biphenyls. *European Journal of Endocrinology*, 146, 357–363.
- Moral, R., Wang, R., Russo, I. H., Mallo, D. A., Lamartiniere, C. A., & Russo, J. (2007). The plasticizer butyl benzyl phthalate induces genomic changes in rat mammary gland after neonatal/prepubertal exposure. *BMC Genomics*, 8, 453.
- Murray, T. J., Maffini, M. V., Ucci, A. A., Sonnenschein, C., & Soto, A. M. (2007). Induction of mammary gland ductal hyperplasias and carcinoma in situ following fetal bisphenol a exposure. *Reproductive Toxicology*, 23, 383–390.
- Nah, W. H., Park, M. J., & Gye, M. C. (2011). Effects of early prepubertal exposure to bisphenol a on the onset of puberty, ovarian weights, and estrous cycle in female mice. *Clinical and Experimental Reproductive Medicine*, 38, 75–81.
- Nagao, T., Saito, Y., Usumi, K., Kuwagata, M., & Imai, K. (1999). Reproductive function in rats exposed neonatally to bisphenol a and estradiol benzoate. *Reproductive Toxicology*, 13, 303–311.
- Nagao, T., Yoshimura, S., Saito, Y., Nakagomi, M., Usumi, K., & Ono, H. (2001). Reproductive effects in male and female rats of neonatal exposure to genistein. *Reproductive Toxicology*, 15, 399–411.
- Navarro, V. M., Sánchez-Garrido, M. A., Castellano, J. M., Roa, J., Garcia-Galiano, D., Pineda, R., Aguilar, E., Pinilla, L., & Tena-Sempere, M. (2009). Persistent impairment of hypothalamic KiSS-1 system after exposures to estrogenic compounds at critical periods of brain sex differentiation. *Endocrinology*, 150, 2359–2367.
- Nikaido, Y., Yoshizawa, K., Danbara, N., Tsujita-Kyutoku, M., Yuri, T., Uehara, N., & Tsubura, A. (2004). Effects of maternal xenoestrogen exposure on development of the reproductive tract and mammary gland in female CD-1 mouse offspring. *Reproductive Toxicology*, 18, 803–811.
- Ojeda, S. R., & Lomniczi, A. (2014). Puberty in 2013: Unravelling the mystery of puberty. *Nature Reviews. Endocrinology*, 10, 67–69.
- Ong, K. K., Potau, N., & Petry, C. J. (2004). Opposing influences of prenatal and postnatal weight gain on adrenarche in normal boys and girls. *The Journal of Clinical Endocrinology and Metabolism*, 89, 2647–2651.
- Ong, K. K., Ahmed, M. L., & Dunger, D. B. (2006). Lessons from large population studies on timing and tempo of puberty (secular trends and relation to body size): The European trend. *Molecular and Cellular Endocrinology*, 254–255, 8–12.
- Orikasa, C., Kondo, Y., Hayashi, S., McEwen, B. S., & Sakuma, Y. (2002). Sexually dimorphic expression of estrogen receptor beta in the anteroventral periventricular nucleus of the rat preoptic area: Implication in luteinizing hormone surge. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 3306–3311.
- Padmanabhan, V., & Veiga-Lopez, A. (2014). Reproduction symposium: Developmental programming of reproductive and metabolic health. *Journal of Animal Science*, 92, 3199–3210.
- Parent, A. S., Teilmann, G., Juul, A., Skakkebaek, N. E., Toppari, J., & Bourguignon, J. P. (2003). The timing of normal puberty and the age limits of sexual precocity: Variations around the world, secular trends, and changes after migration. *Endocrine Reviews*, 24, 668–693.
- Parent, A. S., Franssen, D., Fudvoye, J., Gérard, A., & Bourguignon, J. P. (2015). Developmental variations in environmental influences including endocrine disruptors on pubertal timing and neuroendocrine control: Revision of human observations and mechanistic insight from rodents. *Frontiers in Neuroendocrinology*, 38, 12–36.
- Patisaul, H. B., Fortino, A. E., & Polston, E. K. (2006). Neonatal genistein or bisphenol-A exposure alters sexual differentiation of the AVPV. *Neurotoxicology and Teratology*, 28, 111–118.
- Patisaul, H. B., Fortino, A. E., & Polston, E. K. (2007). Differential disruption of nuclear volume and neuronal phenotype in the preoptic area by neonatal exposure to genistein and bisphenol-A. *Neurotoxicology*, 28, 1–12.
- Patisaul, H. B., Todd, K. L., Mickens, J. A., & Adewale, H. B. (2009). Impact of neonatal exposure to the ERalpha agonist PPT, bisphenol-A or phytoestrogens on hypothalamic kisspeptin fiber density in male and female rats. *Neurotoxicology*, 30, 350–357.
- Quadros, P. S., Pfau, J. L., Goldstein, A. Y., De Vries, G. J., & Wagner, C. K. (2002). Sex differences in progesterone receptor expression: A potential mechanism for estradiol-mediated sexual differentiation. *Endocrinology*, 143, 3727–3739.
- Quadros, P. S., Pfau, J. L., & Wagner, C. K. (2007). Distribution of progesterone receptor immunoreactivity in the fetal and neonatal rat forebrain. *The Journal of Comparative Neurology*, 504, 42–56.
- Qiu, W., Zhao, Y., Yang, M., Farajzadeh, M., Pan, C., & Wayne, N. L. (2016). Actions of bisphenol A and bisphenol S on the reproductive neuroendocrine system during early development in zebrafish. *Endocrinology*, 157, 636–647.
- Rasier, G., Toppari, J., Parent, A. S., & Bourguignon, J. P. (2006). Female sexual maturation and reproduction after prepubertal exposure to estrogens and endocrine disrupting chemicals: A review of rodent and human data. *Molecular and Cellular Endocrinology*, 254–255, 187–201.
- Rasier, G., Parent, A. S., Gérard, A., Lebrethon, M. C., & Bourguignon, J. P. (2007). Early maturation of gonadotropin-releasing hormone secretion and sexual precocity after exposure of infant female rats to estradiol or dichlorodiphenyltrichloroethane. *Biology of Reproduction*, 77, 734–742.
- Rasier, G., Parent, A. S., Gérard, A., Denooz, R., Lebrethon, M. C., Charlier, C., & Bourguignon, J. P. (2008). Mechanisms of interaction of endocrine-disrupting chemicals with glutamate-evoked secretion of gonadotropin-releasing hormone. *Toxicological Sciences*, 102, 33–41.
- Rhees, R. W., Al-Saleh, H. N., Kinghorn, E. W., Fleming, D. E., & Lephart, E. D. (1999). Relationship between sexual behavior and sexually dimorphic structures in the anterior hypothalamus in control and prenatally stressed male rats. *Brain Research Bulletin*, 50, 193–199.
- Rivera, O. E., Varayoud, J., Rodriguez, H. A., Munoz-de-Toro, M., & Luque, E. H. (2011). Neonatal exposure to bisphenol a or diethylstilbestrol alters the ovarian follicular dynamics in the lamb. *Reproductive Toxicology*, 32, 304–312.
- Roa, J., & Tena-Sempere, M. (2010). Energy balance and puberty onset: Emerging role of central mTOR signaling. *Trends in Endocrinology and Metabolism*, 21, 519–528.
- Robinson, J. E., Forsdike, R. A., & Taylor, J. A. (1999). In utero exposure of female lambs to testosterone reduces the sensitivity of the gonadotropin-releasing hormone neuronal network to inhibition by progesterone. *Endocrinology*, 140, 5797–5805.
- Ronnekleiv, O. K., & Kelly, M. J. (1986). Luteinizing hormone-releasing hormone neuronal system during the estrous cycle of the female rat: Effects of surgically induced persistent estrus. *Neuroendocrinology*, 43, 564–576.
- Roselli, C. E., Liu, M., & Hum, P. D. (2009). Brain aromatization: Classic roles and new perspectives. *Seminars in Reproductive Medicine*, 27(3), 207–217.
- Rubin, B. S., Lenkowski, J. R., & Schaeberle, C. M. (2006). Evidence of altered brain sexual differentiation in mice exposed perinatally to low, environmentally relevant levels of bisphenol A. *Endocrinology*, 147, 3681–3691.
- Saillenfait, A. M., Sabaté, J. P., & Gallissot, F. (2008). Diisobutyl phthalate impairs the androgen-dependent reproductive development of the male rat. *Reproductive Toxicology*, 26(2), 107–115.
- Salazar, V., Castillo, C., Ariznavarreta, C., Campon, R., & Tresguerres, J. A. (2004). Effect of oral intake of dibutyl phthalate on reproductive parameters of long Evans rats and pre-pubertal development of their offspring. *Toxicology*, 205, 131–137.

- Savabieasfahani, M., Kannan, K., Astapova, O., Evans, N., & Padmanabhan, V. (2006). Developmental programming: Differential effects of prenatal exposure to bisphenol-A or methoxychlor on reproductive function. *Endocrinology*, 147, 5956–5966.
- Scallet, A. C., Schmued, L. C., & Slikker, W. (2004). Developmental neurotoxicity of ketamine: Morphometric confirmation, exposure parameters, and multiple fluorescent labeling of apoptotic neurons. *Toxicological Sciences*, 81, 364–370.
- Schwarz, J. M., & McCarthy, M. M. (2008). Steroid-induced sexual differentiation of the brain: Multiple pathways, one goal. *Journal of Neurochemistry*, 105, 1561–1572.
- Seminara, S. B., Messenger, S., Chatzidakis, E. E., Thresher, R. R., Acierno, J. S., Jr., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwino, K. M., Hendrick, A. G., Zahn, D., Dixon, J., Kaiser, U. B., Slangenaupt, S. A., Gusella, J. F., O'Rahilly, S., Carlton, M. B., Crowley, W. F., Jr., Aparicio, S. A., & Colledge, W. H. (2003). The GPR54 gene as a regulator of puberty. *The New England Journal of Medicine*, 349, 1614–1627.
- Sena, G. C., Freitas-Lima, L. C., Merlo, E., Podratz, P. L., de Araújo, J. F., Brandão, P. A., Carneiro, M. T., Zicker, M. C., Ferreira, A. V., Takiya, C. M., de Lemos Barbosa, C. M., Morales, M. M., Santos-Silva, A. P., Miranda-Alves, L., Silva, I. V., & Gracell, J. B. (2017). Environmental obesogen tributyltin chloride leads to abnormal hypothalamic-pituitary-gonadal axis function by disruption in kisspeptin/leptin signaling in female rats. *Toxicology and Applied Pharmacology*, 319, 22–38.
- Shanle, E. K., & Xu, W. (2011). Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. *Chemical Research in Toxicology*, 24, 6–19.
- Sharma, T. P., Herkimer, C., West, C., Ye, W., Birch, R., Robinson, J. E., Foster, D. L., & Padmanabhan, V. (2002). Fetal programming: Prenatal androgen excess disrupts positive feedback actions of estradiol but has no effect on timing of onset of puberty in female sheep. *Biology of Reproduction*, 66, 924–933.
- Sharpe, R. M., Fisher, J. S., Millar, M., Jobling, S., & Sumpter, J. P. (1995). Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. *Environmental Health Perspectives*, 103, 1136–1143.
- Simerly, R. B., Chang, C., Muramatsu, M., & Swanson, L. W. (1990). Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: An in situ hybridization study. *The Journal of Comparative Neurology*, 294, 76–95.
- Skakkebaek, N. E., Rajpert-De Meyts, E., & Main, K. M. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. *Human Reproduction*, 16, 972–978.
- Stoker, T. E., Laws, S. C., Crofton, K. M., Hedge, J. M., Ferrell, J. M., & Cooper, R. L. (2004). Assessment of DE-71, a commercial polybrominated diphenyl ether (PBDE) mixture, in the EDSP male and female pubertal protocols. *Toxicological Sciences*, 78, 144–155.
- Strom, B. L., Schinnar, R., Ziegler, E. E., Barnhart, K. T., Sammel, M. D., Macones, G. A., Stallings, V. A., Drulis, J. M., Nelson, S. E., & Hanson, S. A. (2001). Exposure to soy-based formula in infancy and endocrinological and reproductive outcomes in young adulthood. *Journal of the American Medical Association*, 286, 807–814.
- Tahirović, H. F. (1998). Menarchal age and the stress of war: An example from Bosnia. *European Journal of Pediatrics*, 157, 978–980.
- Takeda, T., Fujii, M., Hattori, Y., Yamamoto, M., Shimazoe, T., Ishii, Y., Himeno, M., & Yamada, H. (2014). Maternal exposure to dioxin imprints sexual immaturity of the pups through fixing the status of the reduced expression of hypothalamic gonadotropin-releasing hormone. *Molecular Pharmacology*, 85, 74–82.
- Tan, B. L., Kassim, N. M., & Mohd, M. A. (2003). Assessment of pubertal development in juvenile male rats after sub-acute exposure to bisphenol A and nonylphenol. *Toxicology Letters*, 143, 261–270.
- Tanner, J. M. (1962). *Growth at adolescence; with a general consideration of the effects of hereditary and environmental factors upon growth and maturation from birth to maturity* (2nd edn., p. 325). Oxford: Blackwell Scientific Publications.
- Thomas, P., & Dong, J. (2006). Binding and activation of the seven-transmembrane estrogen receptor GPR30 by environmental estrogens: A potential novel mechanism of endocrine disruption. *The Journal of Steroid Biochemistry and Molecular Biology*, 102, 175–179.
- Tinwell, H., Haseman, J., Lefevre, P. A., Wallis, N., & Ashby, J. (2002). Normal sexual development of two strains of rat exposed in utero to low doses of bisphenol a. *Toxicological Sciences*, 68, 339–348.
- van Noord, P. A., & Kaaks, R. (1991). The effect of wartime conditions and the 1944–45 'Dutch famine' on recalled menarcheal age in participants of the DOM breast cancer screening project. *Annals of Human Biology*, 18, 57–70.
- Veiga-Lopez, A., Luense, L. J., Christenson, L. K., & Padmanabhan, V. (2013). Developmental programming: Gestational bisphenol-a treatment alters trajectory of fetal ovarian gene expression. *Endocrinology*, 154, 1873–1884.
- Wang, T., Zhao, L., Liu, M., Xie, F., Ma, X., Zhao, P., Liu, Y., Li, J., Wang, M., Yang, Z., & Zhang, Y. (2014). Oral intake of hydrogen-rich water ameliorated chlorpyrifos-induced neurotoxicity in rats. *Toxicology and Applied Pharmacology*, 280, 169–176.
- Warner, M., Samuels, S., Mocarrelli, P., Gerthoux, P. M., Needham, L., Patterson, D. G. J., & Eskenazi, B. (2004). Serum dioxin concentrations and age at menarche. *Environmental Health Perspectives*, 112, 1289–1292.
- Wiegand, S. J., Terasawa, E., & Bridson, W. E. (1982). Discrete lesions reveal functional heterogeneity of suprachiasmatic structures in regulation of gonadotropin secretion in the female rat. *Neuroendocrinology*, 34, 395–404.
- Wiersma, M., Long, P. J., & Forehand, R. L. (1993). Toward a new understanding of early menarche: The role of environmental stress in pubertal timing. *Adolescence*, 28, 913–924.
- Wood, R. I., & Foster, D. L. (1998). Sexual differentiation of reproductive neuroendocrine function in sheep. *Reviews of Reproduction*, 3, 130–140.
- Wright, C. L., Schwarz, J. S., Dean, S. L., & McCarthy, M. M. (2010). Cellular mechanisms of estradiol-mediated sexual differentiation of the brain. *Trends in Endocrinology and Metabolism*, 21, 553–561.
- Xi, W., Lee, C. K., Yeung, W. S., Giesy, J. P., Wong, M. H., Zhang, X., Hecker, M., & Wong, C. K. (2011). Effect of perinatal and postnatal bisphenol A exposure to the regulatory circuits at the hypothalamus-pituitary-gonadal axis of CD-1 mice. *Reproductive Toxicology*, 31, 409–417.
- Yu, B., Chen, Q. F., Liu, Z. P., Xu, H. F., Zhang, X. P., Xiang, Q., Zhang, W. Z., Cui, W. M., Zhang, X., & Li, N. (2010). Estrogen receptor alpha and beta expressions in hypothalamus-pituitary-ovary axis in rats exposed lactationally to soy isoflavones and bisphenol A. *Biomedical and Environmental Sciences*, 23, 357–362.

Further Reading

- Bourguignon, J. P., & Parent, A. S. (2016). *Puberty from bench to clinic. Lessons for clinical management of pubertal disorders*. Endocr dev. Basel: Karger.
- De Vries, G., Rissman, E. F., Simerly, R. B., Yang, L. Y., Scordalakes, E. M., Auger, C. J., Swain, A., Lovell-Badge, R., Burgoyne, P. S., & Arnold, A. P. (2002). A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral trait. In *Hormones, Brain and Behavior* (vol. 4, pp. 137–192). New York: Academic Press.
- Padmanabhan, V., Salvetti, N. R., Matiller, V., & Ortega, H. H. (2014). Developmental programming: Prenatal steroid excess disrupts key members of intraovarian steroidogenic pathway in sheep. *Endocrinology*, 155, 3649–3660.

Male Fertility Overview

Eduardo RS Roldan, National Museum of Natural Sciences (CSIC), Madrid, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

Fertility varies considerably between males. Thus, assessments of male fertility are necessary for different reasons, and in different situations, and this is generally not an easy task. Evaluation of male reproductive condition may rely on assessments of fertility or fecundity rates, lifetime reproductive success, or the examination of behavioral or physiological traits, including semen quality. These different approaches involve each potential difficulties. Firstly, fertility or reproductive success requires assignment of paternity in natural populations, or the separation of male and female factors in domestic species or humans. Secondly, in the absence of efficient or reliable methods to quantify reproductive success, it is necessary to rely on physiological traits, such as semen parameters; this, however, has limitations because of our incomplete knowledge of how different sperm or seminal plasma traits determine fertility. Despite these limitations, diagnosis and prognosis of male fertility is now of major concern for humans and animals worldwide. This is emphasized by recent studies in human males pointing towards a decline in sperm quality and an increase in several male reproductive problems, such as testicular cancer, cryptorchidism, low testosterone levels, and an accompanying rise in the demands for assisted reproductive techniques.

Differences in male fertility in natural populations of wildlife are vast. It was once considered that, because males are under strong selection pressure, they would have consistently high fertility (Gomendio et al., 2007). Furthermore, it was assumed that males had an almost unlimited supply of gametes, and that producing spermatozoa was “cheap.” Hence, males would not suffer constraints in their fertility. This view can no longer be held because it is now known that males vary widely in their fertilization success, even if they have access to females and copulate. In addition, in species under different forms of threat, such as excessive hunting or reduction of habitats, which results in reductions in population size, there will be a higher prevalence of matings among related individuals. This situation would lead to an increase in inbreeding and loss of genetic diversity, followed by a decrease in fertility, which would negatively feedback on the population by further reducing its size, increased matings among relatives and, thus, give rise to an extinction vortex. There is ample evidence now that inbreeding results in a decrease in some male reproductive parameters, including a deterioration in semen traits, with increases in sperm abnormalities and sperm DNA damage (Roldan and Gomendio, 2009).

Among livestock, there has been a long-standing interest in fertility because of its direct link to production in domestic species. It is estimated that about 50% of breeding failure is associated to male infertility. Considering that approximately 70% of dairy cattle and about 90% of pig livestock are currently produced by artificial insemination, reduction in male fertility accounts for huge economic losses in the animal industry. Furthermore, there is sometimes a tendency to overuse specific males, because of their desirable traits (i.e., for genetic improvement), and this can potentially lead to decrease in genetic variability with negative consequences on reproduction and fertility. In companion animals, or animals used in sports, such as dogs or horses, there is a more individual approach to fertility, with conditions affecting fertility being examined and treated with a strategy similar to that used in human males. For some species, or breeds, there is also interest in artificial insemination and, therefore, the need to evaluate the reproductive potential of males, or semen samples, is also of interest.

In humans, there is also considerable variation in fertility. Human male fertility is of interest to demographers and epidemiologists although the approaches they use, and the emphasis on fertility and fecundity, seem to differ among them. Thus, demographers are interested in circumstances in which live births occur, with the aim of predicting population changes, whereas epidemiologists place emphasis on what may be regarded as fecundity (“fecundability”), which is based on determination of the time to pregnancy. There is now considerable overlap, and some interchange in terminology, between the two approaches taken to understand variation in human fertility. Thus, there is both interest in understanding variation in timing to pregnancy and the factors that may affect it and how this, in turn, affects changes in population.

In the European Union and Japan there is currently a persistently low total fertility rate, below the replacement level of 2.1 child per woman; in the United States, the fertility rate has also declined but there are differences between ethnic groups, with Caucasians having rates that are below replacement values (Skakkebaek et al., 2016). This has led to the prediction that there will be appreciable decreases in their populations in the immediate future. Part of this decline may be due to social issues and part could be related to fertility problems. What is, then, the prevalence of human infertility and what proportion of such infertility is due to male factors? This is a simple question but, unfortunately, not an easy one to answer. It is important because defining the extent of a disease is crucial to estimating its impact, assess health economic arguments, know prevalence in different populations, and decide what resources should be applied to study and to alleviate it (Barratt et al., 2017). Nevertheless, despite a long-standing interest in these issues, most of the aspects mentioned here are still little known. Infertility is generally defined as the failure of a couple to establish a pregnancy despite 12 or more months of unprotected intercourse. General estimates indicate that about 10%–15% of couples exhibit difficulties for conception, with half of them experiencing some form of male factor; in other words, about 5%–8% of all males of reproductive age exhibit subfertility or infertility. Recent surveys suggest that about 1 in 8 women and 1 in 10 men have experienced infertility. Figures could be higher because there is currently no method to identify men, as individuals, who may be infertile (Barratt et al., 2017). Much variation has been identified between studies estimating infertility due to methods, geographic variation, or populations. One approach generally used to assess male infertility is to examine semen parameters in

men in the general population. Many studies have done this and the information has also been used to try to identify changes in semen quality over time. The information may be valuable if the populations are well characterized and standardized methods are employed across studies. There are also limitations due to the fact that it is not possible to equate the results of semen analyses with male fertility, particularly if only few parameters are assessed.

It seems that male fertility is declining. Evidence from studies reporting semen quality over the years appears to support this claim. A recent major meta-analysis (Levine et al., 2017) has examined sperm concentration and total sperm count from a total of 185 studies, including 42,935 men, and covering the period 1973–2011. The slopes of sperm concentration and total sperm count were estimated as functions of sample collection year. Results of the study showed that sperm concentration declined significantly in the period 1973–2011 with an overall slope in adjusted meta-regression models of -0.64 million/mL/year, which was influenced by fertility and geographic group. Among unselected Western studies, the mean sperm concentration declined, on average, 1.4% per year with an overall decline of 52% during this period. Tendencies for total sperm counts were similar to those of sperm concentration, with a marked decline among unselected Western studies (-5.33 million/year), an average decline in mean total sperm count of 1.6% per year, and an overall decline of about 60%. This study therefore revealed a significant decrease in sperm counts during a period of about 40 years, driven by a 50%–60% decline among men unselected by fertility from North America, Europe, Australia and New Zealand. Research on the causes of this decline is required because of the major public health implications of these findings.

Variation in male fertility is evaluated taking into account the many factors that can affect it, including genetic abnormalities, diseases, environmental factors and, among human males, occupational hazards or differences in lifestyle (Skakkebaek et al., 2016). Among the genetic component that may affect human male fertility, there are chromosomal abnormalities (e.g., aneuploidies or translocations), microdeletions of the Y chromosome, or mutations (e.g., cystic fibrosis or mutations associated to sperm formation or function). A variety of diseases have been identified to affect male fertility, including a series of ill-defined entities such as infections, varicocele or iatrogenic causes. Several environmental chemicals, as well as substances present in the workplace, may also have an impact on fertility. A series of endocrine disruptors may be responsible for effects on testicular development, hormone levels and sperm quality, whereas a variety of heavy metals such as lead or pesticides may impact on semen. Finally, an array of lifestyle conditions, including obesity, smoking, alcohol or drug use, clothing or stress are also known to influence male fertility. Another factor which also influences male fertility is age and with the current protracted life expectancy in many countries, and opportunities for fatherhood later in life, it is also relevant to understand the impact of male age on sperm quality and sperm DNA integrity particularly with regards to potential mutations that can lead to genetic disease in offspring.

There are thresholds in semen quality below which fertility is compromised, and there is considerable interest in understanding reasons behind this fall in semen quality and male fertility. The purpose is twofold. Firstly, by understanding reasons underlying subfertility or infertility, to try and develop methods to diagnose causes that lower fertility. Secondly, by manipulation of gametes or semen, try to enhance methods for treatment, including the development of assisted reproductive techniques. In this second category, there is also considerable interest in developing methods to evaluate and, hopefully, predict fertility potential in species of commercial interest for which a variety of methods of assisted reproduction are being used. In this regards, species in which semen cryopreservation, artificial insemination, sex preselection or embryo production, are widely employed technologies, increases in male fertility (including the possibility of predicting male fertility or, perhaps, sperm fertility) is a goal that commands much interest.

Overall, there are several factors that can influence fertility but one that seems to be on the rise (and that generates increasing concern) in humans and animals relates to the influence of environmental chemicals (Lewis and Ford, 2012; Vested et al., 2014). There are many studies now that have called our attention to this issue and, although the evidence still seems somewhat controversial, there is sufficient information to suspect an adverse environmental effect on male reproduction. In humans, there are now clear data showing a decrease in sperm counts, and rises in the prevalence of cryptorchidism and testicular cancer. Since these problems cluster in certain geographical areas they are thought to have common origins and have thus been associated to endocrine perturbations in early life. These problems are not restricted to humans. Many studies on wildlife suggest an important effect of environmental chemicals on male reproduction and, similarly, environmental contaminants may affect livestock and companion animals. In a study carried out in dogs it was found that they also may be experiencing a decline in fertility (Lea et al., 2016). Dogs of different breeds that were systematically examined for sperm quality for over 25 years were found to have a decline in sperm motility during this period of time, together with an increase in cryptorchidism and a bias in sex ratio, with less males being born over this period. Environmental chemicals are suspected to be responsible for these changes since they were found in dog food and testes. These results suggest that there may be some direct effect of chemicals on sperm, similar to what has been suggested for humans. Therefore, it is important to devote efforts to clarify how these contaminants may negatively affect male fertility in a variety of species including humans.

Determinants of Male Fertility

Male fertility is not determined by semen or sperm traits alone. There are other factors (i.e., hormonal, immunological, genetic/chromosomal, behavioral) that are relevant besides semen quality, but these are somewhat more difficult to quantify or relate to male fertility. Thus, much effort has been placed on trying to understand the relation between semen traits and male fertility (De Jonge and Barratt, 2017). Semen evaluation has moved towards more objective ways of examining the different constituents

of the ejaculate, including sperm numbers, cell morphology, sperm dimensions, various sperm functions and the underlying mechanisms that lead to successful fertilization (Barratt et al., 2017). Nevertheless, under routine conditions, semen analysis relies on just a few traits so the objective of uncovering links between sperm traits and fertility has remained elusive. In addition, there are substantial differences in the focus of attention when one considers studies in domestic species and those in human males. The former have been the subject of intense selection, including fertility traits, whereas among the latter studies are mainly of infertile patients; therefore, there may be little variation at each end of the range, with limited possibilities for the identification of critical sperm traits that could explain diversity in fertility. Studies on wildlife or populations of domestic species not yet subjected to strong selection has allowed for the identification of semen traits that are important determinants of fertility. In natural populations in which male fertility varies between 20% and 80% it was found that some traits seem to be directly associated to fertilization success, such as the proportion of morphologically normal sperm, and sperm swimming velocity, which are thus key determinants of male fertility (Gomendio et al., 2007).

In studies in human donors and infertile patients, and also in males from domestic species, it has been shown that one single sperm parameter cannot generally be associated to the fertility of the male. Even when a series of basic traits such as sperm concentration, morphology or motility are taken into account, a multi-parametric analysis is more informative than just a single one. With this in mind, research has focused on the identification of relevant sperm parameters that, being important for sperm transport, survival and fertilization, could be used in combination in the design of better assays. One aspect that should be borne in mind is that an ejaculate exhibits considerable heterogeneity in its sperm traits. As a consequence, it has been realized that it may not be very informative to rely solely on average values for each trait but, rather, that subpopulations of spermatozoa should be identified and, subsequently, that the structure of these subpopulations in different males, or ejaculates, may render more useful information (Ramón et al., 2014). One additional point to bear in mind is that spermatozoa need to survive for a long time, either in the female tract, between ejaculation and the time of fertilization, with many changes taking place during this period of time, or during storage either by means of refrigeration or cryopreservation. Survival in the female tract and storage bring about modifications that may hamper the chances of fertilization. Tests of sperm function may take these aspects into account so, rather than making a single assessment of a battery of traits, assays should probably incorporate assessments at different time points and report kinetics rather than static values (Petrunkina and Harrison, 2013). The sections below will briefly summarize information on sperm parameters, and tests that may be used to assess them, taking some of these issues into consideration.

Sperm Traits and Use in Diagnostic Tests

We tend to distinguish semen traits that relate to quantity from those that are linked to quality of spermatozoa. Quantity refers to sperm numbers in the ejaculate, expressed either as sperm concentration or total sperm count (that is, number of sperm in the total volume of the ejaculate). Quality is used to refer to traits linked to morphology, integrity, cell viability or motility and functional ability. This so-called qualitative traits are quantified expressing the proportion of sperm cells in different categories, but giving numbers to certain attributes is difficult. There has been enormous efforts, particularly for human semen analysis, to develop standardized methods and quality assurance. Recommendations are available in the form of laboratory manuals for clinical andrology, as endorsed by the World Health Organization (Barratt et al., 2017).

There has been a long-standing interest in identifying criteria for what may be regarded as normal sperm morphology. In humans, which have very pleiomorphic sperm, a set of “strict” criteria has been presented and promoted to identify “normal” spermatozoa (Barratt et al., 2017). Morphology can be assessed under the microscope using a variety of staining techniques or under phase contrast, or with more recent technologies to immobilize spermatozoa to avoid artifacts due to sample processing. By using a series of fluorochromes, shapes of the sperm nucleus and acrosomal granule can be distinguished and characterized separately. Computer-assisted sperm morphometry analysis (CASMA or ASMA) has been developed to reduce the subjectivity of assessments; several systems are available, either commercially or as free software (Yániz et al., 2015). In order to obtain more detailed information, some simple equations for elongation, rugosity or regularity have been used to estimate sperm head shape from dimensions and areas, with a view to assessing head morphology in more detail. These equations have been incorporated into computer-aided sperm morphology analyses. More accurate approaches to sperm morphology have been proposed, including the use of Fourier shape analyses or geometric morphometrics, which may prove to be more informative. These may also be amenable to automation and incorporation into computer-aided systems in the future.

Motility evaluation has developed from a subjective assessment under the microscope to computer-assisted sperm analysis (CASA) with many systems now available either commercially or as free software (Amann and Waberski, 2014; Mortimer et al., 2015). These systems have improved due to developments in image capture from a microscope, increases in computational power and concomitant reduction in computer size and new software, although the basic concepts seem to have remained essentially unchanged. It is important to distinguish the ways in which “sperm motility” is used. On the one hand, motility may refer to the quantification of the proportion of cells moving, even discriminating different types of motility (e.g., proportion of progressive or non-progressive motility), based on either subjective or CASA-derived estimates. On the other hand, motility is assessed as “kinematics” with a series of parameters, or descriptors, used to characterize sperm movement, that is, sperm swimming, with further discrimination of parameters that describe swimming velocity and others that refer to swimming trajectory. These parameters can be quantified with CASA systems. Sperm kinematic parameters can be quantified and analyzed either as averages or as subpopulations identified via other algorithms available now for mouse and human spermatozoa. The latter serve to quantify

subpopulations of hyperactivated sperm cells. CASA systems have now expanded beyond their use in motility evaluation. Being a combination of image capture and computer image assessment, the concept of CASA now refers to a suite of parameters that can be evaluated with the same set up. Thus, by varying sample preparations, sample examination (e.g., under phase contrast, bright field or fluorescence) CASA systems now provide options to estimating sperm concentration, viability, morphometry/morphology, acrosome integrity, or sperm DNA integrity. These systems have therefore evolved into an instrument of image analyses that can serve to quantify in an objective manner a variety of traits in spermatozoa subjected to a number of tests.

Sperm DNA Damage and Role of Protamines

Spermatozoa have the very important function of carrying the paternal haploid nuclear genome to the oocyte. Tests of sperm DNA integrity and sperm nuclear protein have shown potential to discriminate infertile from fertile men or animals or to identify changes in sperm DNA taking place during sperm storage by cryopreservation. Thus, in addition to testing the ability of sperm to fertilize, it is also crucial to examine whether there are damages in the sperm genetic load because such damages, in addition to affecting the sperm's fertilizing ability, may negatively affect embryo or fetal survival, postnatal development, or because damages to sperm DNA may give rise to genetic diseases in the offspring. Thus, assessments of sperm DNA integrity could provide additional information of sperm quality not identified by evaluation of other sperm parameters. Damage to sperm DNA can be examined by a variety of methods such as the sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase nick end labeling (TUNEL), the sperm chromatin dispersion (SCD) assay, or the Comet assay. Each assay measures different aspects of sperm DNA damage.

Sperm DNA damage is prevalent among infertile men and is known to influence natural reproduction. On the other hand, the impact of sperm DNA damage on the outcome of assisted reproduction is still controversial and this may be due to the variety of methods used. A recent meta-analysis ([Simon et al., 2017](#)) examined possible relations between human sperm DNA damage (assessed by SCSA, TUNEL, SCD or Comet assay) and clinical pregnancy after in vitro fertilization (IVF) and/or intracytoplasmic sperm injection (ICSI) treatments. From 56 studies including a total of 8068 treatment cycles of IVF, ICSI, and mixed IVF plus ICSI, the results indicated that sperm DNA damage affects clinical pregnancy following assisted conception. Interestingly, there were differences regarding the different methods used to assess sperm DNA damage, with methods that measure DNA integrity directly (TUNEL and Comet) revealing more consistent associations with pregnancy outcomes than the methods that indirectly measure DNA integrity (SCSA and SCD). Nonetheless, there seems to be sufficient evidence to suggest that sperm DNA damage has a negative effect on pregnancy.

In endangered species a relationship between sperm DNA damage and sperm abnormalities, which were related to inbreeding levels, was observed and, more importantly, sperm DNA damage was found to be associated to neonatal offspring survival ([Ruiz-López et al., 2010](#)). These results have important implications for conservation efforts and underscore again the negative impact of inbreeding in endangered species.

Storage of sperm by cryopreservation may have detrimental effects on sperm DNA integrity. Several studies in man, domestic species and wildlife have shown that sperm experience DNA damage when exposed to subzero conditions and that additions to semen extenders have different ability to protect sperm from such damage. Cooling and freezing rates also influence the integrity of sperm DNA and differences between individuals in their ability to survive cryopreservation are reflected also in the integrity of sperm DNA.

The realization that sperm may carry damaged DNA, and that this associates with pregnancy loss, or disease in the offspring, has led to an interest in the etiology of DNA damage. Several studies suggest that protamine deficiency is related to DNA damage and male fertility. In primates (including man), most rodents and a few other species there are two protamines (protamine 1 and protamine 2) and the ratio between them is important for sperm head (chromatin) compaction and the protection of DNA. There seems to be a constant protamine ratio for each species, although ratios vary between species. A recent review and analysis examined data from the literature on the relationship between the ratio of protamine 1 and protamine 2 with male fertility and the association of protamine deficiency with sperm DNA damage measured using different methods ([Ni et al., 2016](#)). A significantly higher protamine ratio was identified in subfertile men when compared with controls; both protamine mRNA ratio and protein ratio were found to be significantly increased in subfertile patients. The association between protamine deficiency and DNA damage exhibited a significant combined overall correlation coefficient. Interestingly, the level of "protamination" (i.e., protamine deficiency) measured by chromomycin A3 staining was significantly associated with sperm DNA damage whereas the protamine mRNA or protein ratio was not. These results should be interpreted with caution because of the diversity of methods used to assess DNA damage but, nevertheless, from this meta-analysis it seems that protamine ratio may be a suitable biomarker for the assessment of sperm quality whereas protamine deficiency (protamination) is closely related with sperm DNA damage.

Functional Sperm Tests

Taking into account the prevalence of human male infertility, the need to ensure high quality semen samples for domestic species of economic importance, and the interest in preserving gametes from endangered animals, it is important to have reliable methods for laboratory evaluation of semen traits. The predictive value of semen analysis is thus paramount. Here, it is important to distinguish the value of semen analysis as a method to assess the fertility of a male, from the capacity to predict the fertilizing potential of a given semen sample to be used in assisted reproductive techniques. When considering the predictive value of semen analysis for

reproductive outcome, the question is which is the best approach to analyzing sperm traits. Thus, is the evaluation of one ejaculate better than two or more ejaculates for referral to infertility investigation, is a combination of several parameters better than just a single parameter, or is a set of parameters representing a wide range of basic and functional traits better than a limited set of basic parameters?

For cases of human male infertility, it seems that assessment of one ejaculate may be adequate for referral to infertility investigation and treatment, although there is controversy regarding this conclusion because there may be a subgroup of men in the intermediate range requiring repeated semen analysis. On the other hand, a combination of basic parameters seems more predictive than the assessment of just one trait. In any case, it is now clear that the use of a simple set of parameters is insufficient, and calls to expand the array of traits to be examined argue for the inclusion of several tests that would examine sperm function (Barratt et al., 2017). In domestic species, narrow differences in sperm fertility may have enormous economic impact, so there is considerable interest in identifying and implementing a battery of tests that better allow for comprehensive assessment of the effect of storage conditions and fertilizing ability. Thus, in this framework, many studies have proposed and tested a variety of assays that examine the ability of sperm to survive under a variety of conditions, and assays that test for the capacity of sperm to respond to signals that prepare them for fertilization during the processes of capacitation, acrosomal exocytosis and interaction with the oocyte. Currently, a suite of parameters and possible tests can be used for these purposes, mainly in the research laboratory, but, nonetheless, more research is required to better understand processes of formation, maturation and preparation for fertilization, whose knowledge may result in more reliable tests that could be used in the future on a routine basis.

Semen analyses (for human males but also in general for other species) usually do not include functional tests that could identify cases of infertility (or predict fertilizing potential). Semen analysis is generally restricted to a set of basic semen traits including sperm concentration, morphology, or motility. Sperm cells experience a series of changes before fertilization. They need to undergo capacitation, acrosomal exocytosis (the so-called acrosome reaction), develop hyperactivated motility, traverse the oocyte coats and fuse with oocyte's membrane. If sperm are used in assisted reproduction, either in animals of economic interest or infertile patients, there is a need to ensure that spermatozoa will experience these changes with an appropriate timing because, otherwise, the success of fertilization would be compromised. These changes take place in the female tract but can be mimicked in the laboratory and efforts have thus been directed to understand the underlying mechanisms of these changes and, furthermore, design tests that can reliably and affordably assess these processes and the underlying mechanisms. Many efforts to design appropriate tests deal mainly with capacitation and acrosomal exocytosis, so a brief summary will be presented below.

Functional Tests for Capacitation

Capacitation is an essential process that involves a series of cellular and molecular changes and is required for acrosomal exocytosis and interaction with the oocyte. The process is still ill-defined and the understanding of what it entails and when it is completed varies between research laboratories. The main difficulties lie in the definition (and identification) of what represents a capacitated sperm. Capacitation has been examined using a series of tools and criteria, and some assays have been devised to examine the ability of spermatozoa to undergo this process.

The fluorescent probe chlortetracycline (CTC) has been used to monitor the acquisition of the capacitated state of spermatozoa, first in the mouse, and then in other species, including man. Although it is not clear what is the basis of the staining, use of CTC together with fluorescent microscopy allows for the distinction of three functional states which have been correlated to the ability of sperm to fertilize oocytes: fresh uncapacitated sperm, capacitated acrosome-intact spermatozoa, and acrosome-reacted sperm. Thus, three sperm subpopulations can be distinguished with CTC and one important feature is that it can identify one that has apparently completed capacitation but has not yet undergone acrosomal exocytosis. Incubating spermatozoa under capacitating conditions, and using CTC together with a vital stain (e.g., Hoechst 33258), it is possible to quantify the proportion of live, capacitated-acrosome intact sperm. If sperm are incubated further, or are stimulated with ligands thought to be natural inducers of acrosome reaction, such as progesterone or the zona pellucida, then exocytosis in response to stimulation can also be quantified.

Occurrence of capacitation can also be monitored by changes in sperm lipids. Capacitation involves changes such as loss of cholesterol, destabilization of the sperm membrane, exposure of specific phospholipids from the inner membrane leaflet, activation of phospholipases and changes in composition of membrane phospholipids. Some of these changes occur very rapidly and they have been identified using specific fluorochromes and flow cytometry or with a series of methods for lipid biochemistry and enzyme (phospholipase) activation. Some of these methods, especially those involving flow cytometry are used to assess capacitation in the research laboratory. Changes in monosialotetrahexosylganglioside (G_{M1}) localization patterns that take place upon completion of capacitation in mouse and human sperm have been identified and recently proposed as the basis for a microscopy-based test in human spermatozoa. The test has been validated and was found to be indicative of male fertility; importantly, it renders information that is independent of traditional semen measures (Moody et al., 2017).

During capacitation there are also changes in the pattern of phosphorylation of sperm proteins. Ever since the discovery that during capacitation there is an activation of the cAMP/protein kinase A (PKA) pathway that results in phosphorylation of substrates and also tyrosine phosphorylation in an additional series of substrates, efforts have been made to take these proteins as markers of capacitation. Although the identity of the phosphorylated proteins is largely unknown, the PKA- and tyrosine kinase-mediated phosphorylation serves as a tool to understand changes supporting (or inhibiting) capacitation. This is a useful approach that can be used as a complement (or as a replacement) of other approaches used to assess capacitation, such as the time-consuming CTC staining. Nonetheless, electrophoresis and Western blotting is also time-demanding and requires a specialized setting.

Thus, other means to assess the phosphorylation status of proteins has been explored, including the use of direct microscopic examination of spermatozoa labeled with antiphosphotyrosine antibodies conjugated to fluorescent probes. A similar approach, but using flow cytometry to quantify changes in proteins phosphorylated in tyrosine, is an alternative that deserves standardization for use in future functional tests.

Proteomics techniques allow for the identification of protein content of spermatozoa at different stages and may provide information on dynamics of sperm cell function and identification of relevant marker proteins. This is particularly important in spermatozoa because these modifications take place in the absence of gene transcription and translation and, thus, are the result of post-translational processes such as phosphorylation. Studies have examined proteomes of sperm from different species (mouse, rat, human, bull, ram, stallion, boar) and comparisons using quantitative methods have addressed differences between mature and immature spermatozoa, epididymal versus ejaculated sperm, fresh and frozen spermatozoa, and high- and low-fertility spermatozoa. A few studies have attempted to identify variation due to capacitation, mainly in boar spermatozoa, and many proteins were found to vary in their abundance between capacitated and non-capacitated spermatozoa, although the function of many are not known. Proteins identified as being more abundant in capacitated spermatozoa have various roles connected to metabolism, protection from oxidative stress, motility regulation and sperm-egg interaction. Thus, these proteins may perhaps serve as biomarkers for functional tests that can assess and predict fertility (Rahman et al., 2017). Little has been done yet to compare the abundance of these markers with male fertility or litter size, although, interestingly, some initial attempts have revealed two important points: There may be an association between abundance of certain protein markers in high fertility animals when comparing expression profiles between males rendering high or low fertility results. However, not all the protein markers may be informative when running subsequent field trials trying to discriminate (predict) fertility in males. In bulls, only a subfraction of all the proteins identified to change during capacitation were significantly correlated to fertility. On the other hand, in boars, it was possible to identify proteins in capacitated sperm that correlated with litter size whereas others correlated with male fertility. Overall, there are differences in proteins between males with low or high fertility or litter size, and the predictive value increases when the proteins of capacitated sperm (as opposed to non-capacitated sperm) are taken into consideration in the analyses.

Functional Tests for the Acrosome Reaction

The status of the acrosome (i.e., presence, damage, or absence) has been examined using a variety of methods ever since it was realized that this exocytotic granule is required at some point in the train of events leading to fertilization. The acrosome contains enzymes that, upon release during acrosomal exocytosis, help the sperm traverse the cellular and cellular coats of the oocytes. So, it is important that the integrity of the acrosome is preserved until the sperm reaches the vicinity of the oocyte. The status of the acrosome can be examined using phase contrast optics in species with prominent acrosomes (ungulates, many rodents) but requires staining in other species. In general terms, staining of the acrosome could be done with or without fixation and employing a variety of dyes, including fluorescent ones, and examination under the microscope. More recently, methods to stain acrosomes and examine them by using flow cytometry have been developed; they are generally combined with a dye that can distinguish live-dead cells thus making it possible to follow changes in the four sperm populations.

Although exocytosis of the acrosome occurs subsequent to capacitation, and the latter is required for the former, exocytosis can be triggered using molecular probes, in the presence of extracellular Ca^{2+} , even in uncapacitated spermatozoa. The ability of sperm to respond to probes such as the Ca^{2+} ionophores A23187 or ionomycin, and the assessment of acrosomal status by a variety of methods, has been used as the basis of tests to identify the sperm's ability to undergo the acrosome reaction in efforts to distinguish potential fertilizing capacity (or at least, functional competence). Other probes, or metabolites involved in signaling processes underlying the acrosomal exocytosis, have been examined for their ability to trigger or enhance exocytosis in capacitated sperm. Thus, analogs of cAMP, inhibitors of phosphodiesterases, diacylglycerol kinase, diacylglycerol lipase, protein kinase A or C, fusigenic lipids, or modulators of phospholipase C or A_2 , among others, have been used (alone or in conjunction with ionophores/ Ca^{2+}) to probe for various pathways leading to exocytosis. However, most of them have not been incorporated, on a regular basis, in the design of tests to examine functional capacities of spermatozoa. Induction of acrosomal exocytosis by natural ligands (progesterone, zona pellucida) has been tested in spermatozoa capacitated under appropriate conditions.

Other Functional Tests

There are a series of methods for flow cytometric evaluation of plasma membrane and acrosome integrity, mitochondrial membrane potential, lipid stability or peroxidation and intracellular Ca^{2+} content, which serve to assess effects of storage conditions or in preparation for fertilization. These traits are assessed mainly in a research set-up and have not yet being incorporated as diagnostic tools of male fertility.

There has also been interest in quantifying oxidative stress because it is a known cause of impairment of sperm function. Reactive oxygen species (ROS) appear to take part in sperm capacitation but an excess may impair membrane integrity, sperm motility and it can damage sperm DNA. Little use is made of the quantification of ROS or antioxidants in a clinical set-up but these parameters have receive attention in the investigation of conditions that affect sperm storage by cryopreservation. The production of ROS can be examined using various methods, including some based on chemiluminescent detection using luminol, or flow cytometry with various fluorochromes that serve to quantify generic intracellular ROS or mitochondrial superoxide. There is also a simple inexpensive method, based on a nitro blue tetrazolium test, which can be assessed microscopically and that could be incorporated to the

clinical andrology laboratory. Nevertheless, there is still debate as to the merits of measuring ROS because of the limited evidence available regarding standardization and links with pregnancy outcomes.

Combination of flow cytometry and image analyses will be very useful in the future for the analysis of cell subpopulations with the possibility of adding localization of changes taking place in sperm cells.

Functional Tests for Sperm–Oocyte Interactions

The best estimate of fertilizing ability is perhaps that obtained through the use of artificial insemination, in which most sperm functions come into play, or in vitro fertilization, which still assesses many sperm functions with the exception of factors that influence sperm transport. However, artificial insemination can be used as a test method in domestic species, particularly bulls, rams or boars, but it is not available for other species for a variety of reasons. Alternatives to test fertilizing capacity in the laboratory, through tests of gamete interaction, have been examined.

Sperm–zona pellucida binding assays were developed for human sperm in an attempt to identify cases in which patients could be directed to artificial insemination or ICSI (Oehninger et al., 2014). Although the results of some of these assays correlate significantly with fertilization in vitro, they have some drawbacks, because they require human material, such as intact eggs or zonae pellucidae, and they are technically and time demanding. Two of these assays, the sperm–zona binding assay, and the hemizona assay, provide clinically useful and prognostic information regarding sperm competence for fertilization in IVF, according to a meta-analysis. The hemi-zona assay also resulted predictive of intra-uterine insemination in couples with male factor infertility (Oehninger et al., 2014). The hemizona assay includes a comparison of two populations of fertile and infertile spermatozoa in the same assay using matching halves of human zonae from an oocyte with no developmental potential (e.g., salt-stored).

Tests based on sperm–zona pellucida binding cannot be applied to certain species for a variety of reasons, including limited access to females or oocytes. Alternative approaches such as in vitro fertilization assays using heterologous oocytes have been explored. In some cases, the oocyte's zona pellucida, important for species-specific gamete recognition, has to be removed to allow for fertilization, as in the so-called “sperm penetration assay” (SPA) for human sperm in which zona-free golden hamster oocytes are employed (Oehninger et al., 2014). The SPA measures the sperm ability to undergo capacitation, spontaneous acrosome reaction, fusion and penetration through the oolemma, and decondensation within the cytoplasm of hamster oocytes. This was one of the first functional tests to be introduced to evaluate male fertility potential but results have been difficult to interpret among other reasons because of rather high rate of false-negative results (men whose spermatozoa fail the SPA but successfully fertilize human oocytes in vitro or in vivo). A pair or meta-analysis revealed poor clinical value of the SPA as predictor of fertilization (Oehninger et al., 2014).

Heterologous sperm–oocyte interaction assays have been used in other species. A test resembling the human SPA has been used with domestic ungulates but it has not gained general acceptance. In felines, an heterologous test to assess fertilization is used but in this case, removal of the zona pellucida is not required. Domestic cat oocytes matured in vitro can be fertilized by a variety of feline species, thus serving as a useful test of sperm fertilization potential, particularly for endangered species (Roldan and Gomendio, 2009).

Towards New (and More Inclusive) Assays of Male Fertility

For many years now, the use of ICSI has become widespread for cases of male factor infertility and for couples who have failed conventional IVF. The high levels of fertilization achieved with ICSI, even in cases of multiple morphological and dysfunctional sperm defects, as well as the possibility of using testicular or epididymal spermatozoa, or cryopreserved spermatozoa, may have diverted attention from the need to identify causes of male infertility. There are thus many unanswered questions regarding sperm pathologies and the underlying molecular and cellular causes, or the role of sperm components in early development or postnatal development, with important implications for future reproductive performance, or wellbeing in general, in the offspring. Thus, the need to understand the many bases of male infertility is a priority. Furthermore, in order to have reliable evaluations of male fertility in general, or sperm function, better tests should be designed and implemented.

One overall priority in the area of male fertility is a better understanding of processes of production, formation, maturation and structure-function of spermatozoa. There is a crucial requirement to investigate the different genetic and phenotypic aspects of sperm biology, and obtain a much better grasp of molecular, biochemical, cellular and physiological mechanisms, with a view to design more reliable diagnostic and prognostic assays, consider more appropriate therapies and evaluate the impacts of lifestyles, age and environmental factors on these processes (Barratt et al., 2017). Despite much progress in recent years, there is still the need to make significant progress in certain areas, including taking into consideration new technological breakthroughs in other areas, and, perhaps, even rethink some of the strategies for diagnosis and treatment.

There seem to be two divergent trends nowadays that may determine the future of semen analyses and male fertility assessments. On the one hand, options for in-home evaluation of semen quality are becoming commercially available, and thus accessible and affordable kits may become the first line of action for a quick evaluation of one or more basic parameters, such as sperm numbers or sperm motility. Currently, there is much interest in the development of devices and software to be used in conjunction with smart-phones. Although this may appear at first as a drawback for the andrology laboratory it may in fact represent a considerable gain allowing more men to approach testing in a less stressful way. Beyond the use of kits in human fertility testing, these devices may

also become useful in other situations such as the quick assessment of semen quality in farms in which frozen or refrigerated semen is used in artificial insemination.

On the other hand, there is room for much improvement in research and clinical laboratory tests that would allow more exhaustive assessments of sperm function. There is an urgent need for the development and use of assays that go beyond basic semen parameters (such as sperm concentration, morphology and motility) and that can yield multiparametric dynamic evaluations of changes taking place in spermatozoa under conditions that mimic preparation for fertilization, or that would ensure long-term survival of the genetic resource. In this regard, development of assays that evaluate sperm metabolism, capacitation, acrosome reaction, swimming velocity and trajectory, sperm DNA integrity, chromatin compaction, or various protein changes including protamine ratios deserve attention. Consideration of the arena in which spermatozoa perform would also be important and, therefore, the development of chips for assessments through microfluidic devices should be considered. These devices will be most useful for the evaluation of spermatozoa, for sperm cryopreservation or in the selection of competent sperm for fertilization.

References

- Amann, R. P., & Waberski, D. (2014). Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology*, 81, 5–17.
- Barratt, C. L. R., Björndahl, L., De Jonge, C. J., et al. (2017). The diagnosis of male infertility: An analysis of the evidence to support the development of global WHO guidance—Challenges and future research opportunities. *Human Reproduction Update*, 23, 660–680.
- De Jonge, C. J., & Barratt, C. L. R. (Eds.). (2017). *The sperm cell. Production, maturation, fertilization, regeneration* (2nd edn.). Cambridge: Cambridge University Press.
- Gomendio, M., Malo, A. F., Garde, J., & Roldan, E. R. S. (2007). Sperm traits and male fertility in natural populations. *Reproduction*, 134, 19–29.
- Lea, R. G., Byers, A. S., Sumner, R. N., et al. (2016). Environmental chemicals impact dog semen quality in vitro and may be associated with a temporal decline in sperm motility and increased cryptorchidism. *Science Reports*, 6, 31281.
- Levine, H., Jørgensen, N., Martino-Andrade, A., et al. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*, 23, 646–659.
- Lewis, C., & Ford, A. T. (2012). Infertility in male aquatic invertebrates: A review. *Aquatic Toxicology*, 120–121, 79–89.
- Moody, M. A., Cardona, C., Simpson, A. J., et al. (2017). Validation of a laboratory-developed test of human sperm capacitation. *Molecular Reproduction and Development*, 84, 408–422.
- Mortimer, S. T., van der Horst, G., & Mortimer, D. (2015). The future of computer-aided sperm analysis. *Asian Journal of Andrology*, 17, 545–553.
- Ni, K., Spiess, A. N., Schuppe, H. C., & Steger, K. (2016). The impact of sperm protamine deficiency and sperm DNA damage on human male fertility: A systematic review and meta-analysis. *Andrology*, 4, 189–199.
- Oehninger, S., Franken, D. R., & Ombelet, W. (2014). Sperm functional tests. *Fertility and Sterility*, 102, 1528–1533.
- Petrunkina, A. M., & Harrison, R. A. P. (2013). Fluorescence technologies for evaluating male gamete (dys)function. *Reproduction in Domestic Animals*, 48(Suppl. 1), 11–24.
- Rahman, M. D., Kwon, W. S., & Pang, M. G. (2017). Prediction of male fertility using capacitation-associated proteins in spermatozoa. *Molecular Reproduction and Fertility*, 84, 749–759.
- Ramón, M., Jiménez-Rabadán, P., García-Álvarez, O., et al. (2014). Understanding sperm heterogeneity: Biological and practical implications. *Reproduction in Domestic Animals*, 49(Suppl. 4), 30–36.
- Roldan, E. R. S., & Gomendio, M. (2009). Sperm and conservation. In T. R. Birkhead, D. J. Hosken, & S. Pitnick (Eds.), *Sperm biology: An evolutionary perspective* (pp. 539–564). London: Academic Press.
- Ruiz-López, M. J., Espeso, G., Evenson, D. P., Roldan, E. R. S., & Gomendio, M. (2010). Paternal levels of DNA damage in spermatozoa and maternal parity influence offspring mortality in an endangered ungulate. *Proceedings of the Royal Society of London, Series B*, 277, 2541–2546.
- Simon, L., Zini, A., Dyachenko, A., Ciampi, A., & Carrell, D. T. (2017). A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. *Asian Journal of Andrology*, 19, 80–90.
- Skakkebaek, N. E., Rajpert-De Meyts, E., Buck Louis, G. M., et al. (2016). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*, 96, 55–97.
- Vested, A., Giwercman, A., Bonde, J. P., & Toft, G. (2014). Persistent organic pollutants and male reproductive health. *Asian Journal of Andrology*, 16, 71–80.
- Yáñez, J. L., Soler, C., & Santolaria, P. (2015). Computer assisted sperm morphometry in mammals: A review. *Animal Reproduction Science*, 156, 1–12.

Fertility, Sperm Number and Motility

Florence Eustache, University hospital of Paris Seine-Saint-Denis, Bondy, France; and Université Paris Descartes, Paris, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

Semen has two major quantifiable components: sperm cells and seminal fluid. The nature of the spermatozoa, motility, vitality and morphology, and the composition of seminal fluid are important for sperm function. Thus, evaluation of male fertility has traditionally relied on the analysis of spermatozoa, notably sperm count and motility.

This analysis has been carried out since the beginning of the 20th century and more particularly since the 1970s. In response to a growing need for the standardization of procedures for the examination of human semen, the first WHO laboratory manual for the Examination and processing of human semen has been published in 1980.

Numerous publications have been produced in an attempt to find a link between sperm parameters and the patient's fertility status. At this time, semen analysis is used as a marker of male fecundity in clinical andrology and is examined routinely in ART (Assisted Reproductive Technology) laboratory. It is considered an important component of the evaluation of couples presenting for fertility evaluation. Semen analysis is also performed in reproductive toxicology, epidemiology, and risk assessment in order to better understand the causes of variation in sperm quality.

Sperm Number and Motility: Methods and Limits

The routine evaluation of human semen including sperm count and motility is subjective by nature. All aspects of semen collection and analysis must be done by properly standardized procedures if the results are to provide valid, useful information. The WHO manual for the examination of human semen proposes a standard methodology for laboratories for several decades. In each given subject the characteristics of semen can be influenced by the conditions of sample collection, the length of abstinence, and any disease that may have occurred in the 3 months preceding the examination.

The semen samples are obtained by masturbation and ejaculated into a clean collection tube. WHO recommends that semen samples should be collected after a minimum of 48 h but not more than 7 days of ejaculation abstinence to standardize the influence this factor ([World Health Organization, 2010](#)). Ejaculate volume is estimated by weighing the collection tube with the semen sample and subsequently subtracting the predetermined weight of the empty tube, and assuming that 1 mL of ejaculate weighs 1 g. Phase-contrast microscopy is used for the examination of semen.

The number of spermatozoa in the ejaculate is calculated from the concentration of spermatozoa. The sperm concentration is assessed using the improved Neubauer hemocytometer. Briefly, each semen sample is thoroughly homogenized. An aliquot of the sample is put into the diluent (NaHCO₃, formaldehyde and distilled water) and mixed. One drop of the diluted specimen is transferred to each chamber of the hemocytometer, which are allowed to stand in a humid chamber before the cells were counted at a total microscope magnification of $\times 400$ ([WHO, 2010](#)). Only spermatozoa with tails are counted, and at least 200. A comparing replicate count is required to see if they are acceptably close. If so, calculations could be proceeded; if not, a new dilution should be prepared. Sperm concentration could be calculated according to the number of spermatozoa counted in n rows, and the factor of dilution, and the total number of spermatozoa per ejaculate could be obtained by multiplying the sperm concentration by the volume of the whole ejaculate. If no spermatozoa are observed in the ejaculate, azoospermia can be suspected. A centrifugation of the whole ejaculate is required to determine if any spermatozoa are present, and the term azoospermia can only be used if no spermatozoa are found in the sediment of a centrifuged sample. If there are spermatozoa, we will use the term cryptozoospermia. Azoospermia needs to be confirmed through another analysis 3 months after.

For the assessment of sperm motility, 10 μ L of well-mixed semen is placed on a clean glass slide (which had been kept at 37°C), and covered with a 22 mm $\times$ 22 mm coverslip. The preparation is placed on the heating stage of a microscope (37°C), and immediately examined at a total magnification of $\times 200$ or $\times 400$. In the previous edition ([WHO, 1999](#)), the progressively motile spermatozoa should be categorized as rapid (a) or slow (b), and after (c) for the nonprogressive motility and (d) for immotile spermatozoa. However, it is clear that it is difficult for technicians to define the forward progression so accurately without bias ([WHO, 2010](#)). Therefore, the motility of each spermatozoon is now classified as follows: either motile, with progressive motility (PR) and nonprogressive motility (NP) or immotile (IM) ([WHO, 2010](#)) and reported as a percentage ([Fig. 1](#)). A replicate is systematically performed. Two hundred spermatozoa should be assessed per replicate. If the replicate values are acceptably close, calculations could be done; if not, it is necessary to prepare new samples.

The computer-aided sperm analysis (CASA) system was developed in the late 1980s for analyzing sperm movement characteristics. It has been successful in the field of research and for measuring semen characteristics such as sperm concentration and motility in many animal species. The use of CASA for human semen analysis has met with poor success probably due to some difficulties presented by many human semen samples, aggregation/agglutination of spermatozoa, and others cells and debris ([Mortimer et al., 2015](#)). However, the use of fluorescent DNA stains and tail-detection algorithms, may allow sperm concentration and progressive motile spermatozoa to be determined more accurately ([Mortimer et al., 2015](#)). In the same way as subjective analysis, quality control procedures will be necessary.

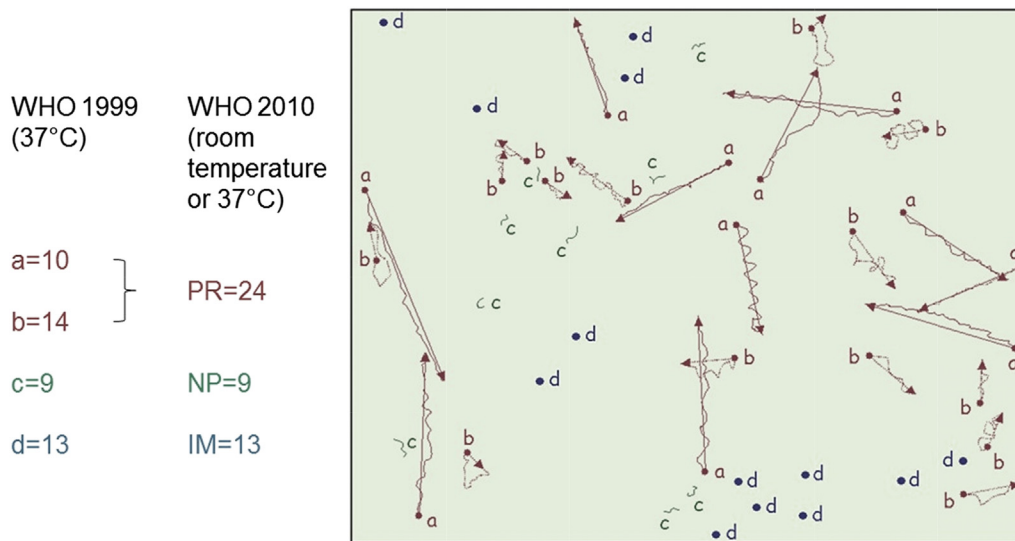


Fig. 1 Sperm trajectories in a microscopic field in the same time: PR, progressive motility, NP, nonprogressive motility and IM, immotile (WHO, 2010) and (a) rapid progressive, (b) slow progressive, and (c) nonprogressive and (d) for immotile (WHO, 1999).

Based on the subjective sperm count and motility assessment, quality control procedures are necessary to establish and maintain a high standard. Large discrepancies in sperm count and motility have been observed in different laboratories. Indeed, the coefficients of variation for sperm concentration can vary from 23% to 60%, and for motility from 20% to 30% (Auger et al., 2000). Many authors indicate interestingly that training and subsequent follow-up workshops can guarantee minimum variability in assessments of sperm concentration and motility among centers. Thus it is both necessary to standardize the analysis of these parameters, and to implement quality control (QC) to detect and correct systematic errors and high variability of results. Internal quality control (IQC) is essential to maintain accuracy, precision and competence in a laboratory. External quality control (EQC) is the evaluation of results for the same samples in several laboratories and is required to ensure that different laboratories produce comparable results. IQC and EQC should be performed in all laboratories and during all studies on semen quality.

The characteristics of semen can be influenced by several factors as the length of sexual abstinence and a disease and/or a fever that may have occurred in the 3 months preceding the analysis. There is general agreement that semen volume, sperm concentration and therefore total sperm count increase with prolonged sexual abstinence but the issue of sperm motility remains contradictory (WHO, 2010). The decrease of sperm motility with an increase of sexual abstinence might be due to the variation in epididymal and accessory sex gland secretions as well as the morphology of spermatozoa with an increased percentage of tail defects (WHO, 2010). Other factors such as a recent acute febrile episode may temporarily alter sperm parameters. Thus, a decrease in total sperm count but also in motility can be observed (WHO, 2010). These variable, and largely uncontrollable, factors explain the well-known intra-individual variation in semen composition (WHO, 2010).

It is therefore necessary to have information on possible exposure factors in the 3 months prior to the sperm analysis but also on the medical record and lifestyle for a better interpretation.

Thus, for a given man, spermatoc parameters can vary with time (Fig. 2). It would be misleading to draw hasty conclusions from one single analysis and a second examination is advised.

Reference Values

Until 1999, the WHO manual proposed reference values for semen parameters that had limitations. Indeed, the used data were derived from imprecisely defined reference populations, obtained from laboratories with unknown comparability with respect to analytical methodologies, and were limited by the lack of available data on semen variables in recent fathers. Moreover, there had been no consensus around the relevance of these values. A sperm concentration of $20 \times 10^6 \text{ mL}^{-1}$, the "normal" or "reference" value cited by WHO (1999), has been considered too low for a lower reference limit because the probability of pregnancy is essentially linear with sperm concentrations up to $40\text{--}50 \times 10^6 \text{ mL}^{-1}$ (Bonde et al., 1998; Slama et al., 2002). Conversely, sperm concentrations above this value are repeatedly observed in infertile patients.

In 2010, Cooper and collaborators provided reference values from a population of men, partners of pregnant women with a time to pregnancy (TTP) ≤ 12 months. Data from 1953 semen samples from five studies in eight countries on three continents were combined and analyzed. The samples were obtained and processed for analysis using adequately standardized methods under quality control.

A reference distribution for semen parameters was generated and the 95% confidence intervals for a range of semen variables and the lower (2.5th centile and 5th centile) reference limits, have been obtained, in line with clinical chemistry standards (Table 1).

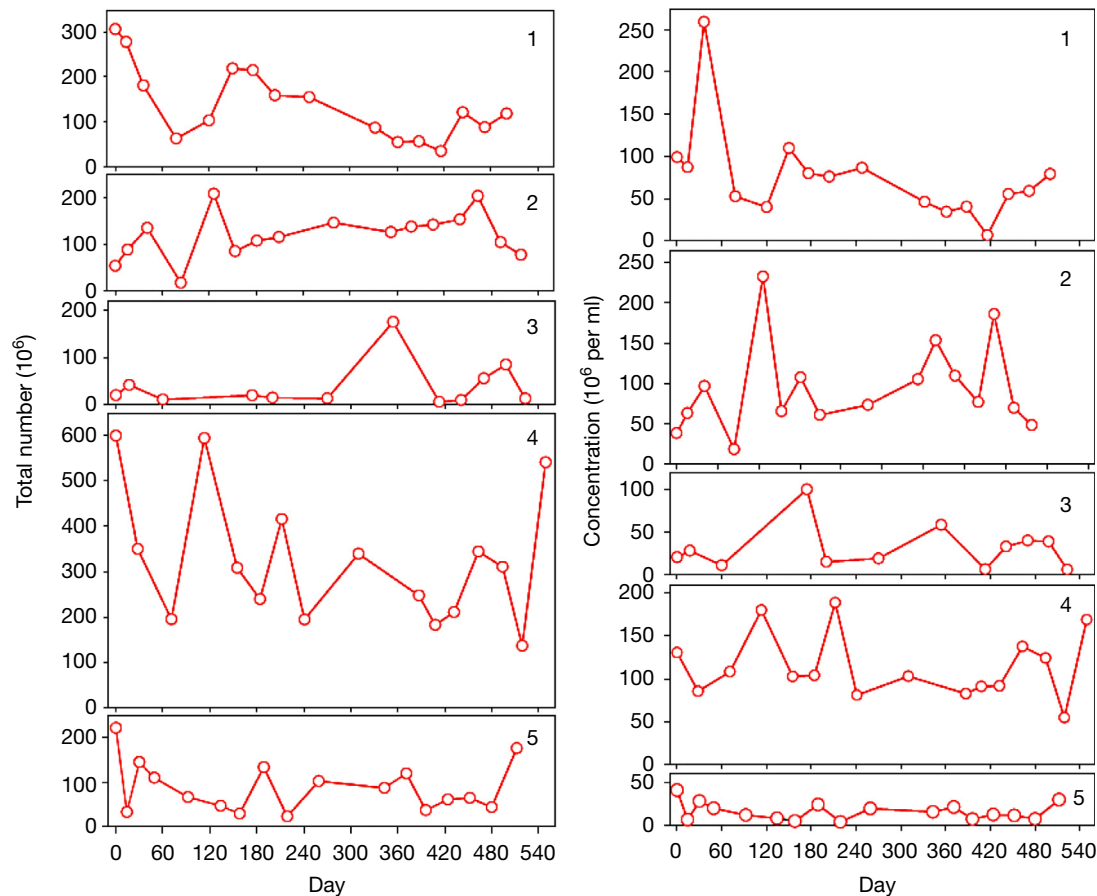


Fig. 2 Variation in total number of spermatozoa and sperm concentration over a one-and-a-half-year period From World Health Organization (2010). WHO laboratory manual for the examination and processing of human semen, 5th edn. Geneva, Switzerland: WHO Press.

Table 1 Distribution of values, lower reference limits and their 95% CI* for sperm number and motility from fertile men whose partners had a time-to- pregnancy of 12 months or less

	<i>N</i>	<i>Centiles</i>								
		2.5	5	10	25	50	75	90	95	97.5
Semen volume (mL)	1941	1.2	1.5	2	2.7	3.7	4.8	6	6.8	7.6
			(1.4–1.7)*							
Sperm concentration (10^6 mL^{-1})	1859	9	15	22	41	73	116	169	213	259
			(12–16)*							
Total number ($10^6/\text{ejaculate}$)	1859	23	39	69	142	255	422	647	802	928
			(33–46)*							
Total motility (PR + NP, %)	1781	34	40	45	53	61	69	75	78	81
			(38–42)*							
Progressive motility (PR, %)	1780	28	32	39	47	55	62	69	72	75
			(31–34)*							

From Cooper, T.G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H.W., *et al.* (2010). World Health Organization reference values for human semen characteristics. *Human Reproduction Update* **16**, 231–245.

WHO (2010) proposed a nomenclature with the aim to classify the quality of the semen without suggesting any biological cause. These terms are used to describe samples with values lying outside the reference range (Table 2).

Are Sperm Count and Motility Good Indicators for the Evaluation of Fertility?

For many years, the influence of semen parameters on the probability of conception or pregnancy has been studied in various situations and populations: in a fertile population, in an insemination program or couples attending an IVF/ICSI.

Table 2 Nomenclature related to semen quality (WHO, 2010)

<i>Aspermia</i>	<i>No semen (no or retrograde ejaculation)</i>
Normozoospermia	Total number (or concentration) of spermatozoa, and percentages of PR and morphologically normal spermatozoa, equal to or above the lower reference limits
Oligozoospermia	Total number (or concentration) of spermatozoa below the lower reference limit
Azoospermia	No spermatozoa in the ejaculate
Cryptozoospermia	Spermatozoa absent from fresh preparations but observed in a centrifuged pellet
Asthenozoospermia	Percentage of PR spermatozoa below the lower reference limit
Oligoasthenozoospermia	Total number (or concentration) of spermatozoa, and percentage of PR spermatozoa, below the lower reference limits
Necrozoospermia	Low percentage of alive, and high percentage of immotile, spermatozoa in the ejaculate

PR: progressively motile.

In 430 couples with men aged 20–35 who had no children (first pregnancy planners), [Bonde et al. \(1998\)](#) found that the probability of conception increased with increasing sperm concentration up to $40 \times 10^6 \text{ mL}^{-1}$. A prospective study with 210 reproductive-age healthy couples has shown an association between total sperm count, sperm concentration on the one hand, and time to pregnancy on the other ([Zinaman et al., 2000](#)). Thus, a decline in pregnancy rate was observed below approximately $30 \times 10^6 \text{ sperm mL}^{-1}$ and $80 \times 10^6 \text{ sperm}$, for sperm concentration and total sperm count, respectively. Another study also revealed for 942 partners of pregnant women that an increasing sperm concentration may influence the time to pregnancy (TTP) up to $55 \times 10^6 \text{ mL}^{-1}$ and a total sperm count up to 145×10^6 ([Slama et al., 2002](#)).

In these different studies no convincing relationships were observed between motility and pregnancy or TTP. This is probably due to the subjective analysis of this parameter and therefore to the existence of too large inter-individual variability.

Numerous studies have been carried out to investigate the value of sperm parameters to predict a pregnancy outcome in couples attending an insemination program. [Lemmens et al. \(2016\)](#) did not find a correlation between sperm count or motility and pregnancy in a fresh sample. In the same way, a recent review did not reveal a clear evident relationship between sperm quality and IUI success ([Ombelet et al., 2014](#)). However, the total motile spermatozoa (TMS) count obtained and calculated after centrifugation of a semen sample in a discontinuous density gradient column could be a good prognostic factor for pregnancy. In a recent meta-analysis, the literature did not reveal evidence on the relationship between sperm quality and IUI success probably due to a lack of prospective studies, a lack of standardization in semen analysis, an heterogeneity of patient groups and IUI treatment strategies ([Ombelet et al., 2014](#)) and authors recommended that more standardized prospective studies are needed.

In the present time, no evident correlation has been found between sperm parameters and success rates in IVF/ICSI program.

In conclusion, apart from the fertile population, sperm count and motility do not seem to be the best indicators for IUI/IVF/ICSI program. Further standardized studies should be carried out.

Factors Influencing Sperm Count and Motility

The etiology of sperm defects remains idiopathic in 30%–50% of cases. Male infertility is probably a multifactorial disorder. Indeed, different causes may be involved such as genetic, environmental, drugs, lifestyle ...factors.

Genetic factors

Azoospermia/severe oligozoospermia

Microdeletions of the Y chromosome, Klinefelter syndrome (47,XXY), and CFTR mutations are well known genetic causes of severe male infertility as azoospermia or severe oligozoospermia. Testicular causes include also germ cell aplasia (Sertoli cell-only syndrome, SCOS) or spermatogenesis arrest. The recent literature on the genetics of azoospermia and oligozoospermia has been shown that other genetic defects (X-chromosome or autosomal mutations) may have a direct quantitative effect on sperm production ([Table 3](#)).

Asthenozoospermia

Recent data allow to establish that some genes encoding for proteins involved in the assembly and/or structure of the flagellum (DNAH1, SEPTIN12) and affecting the flagella morphology or for ion channels located at the sperm plasma membrane and required for sperm motility (CATSPER, SLC26A8) are implicated in the physiopathology of isolated asthenozoospermia ([Table 3](#)).

Others factors

Western lifestyle (sedentary work/lifestyle) as well as other lifestyle factors (stress, smoking, maternal smoking, alcohol, heat...) could potentially be damaging to sperm production and quality. Endocrine disruptors including pesticide and plasticizers may also be important damaging factors. Thus, numerous epidemiological studies have been carried out from different populations to better understand the effects of lifestyle factors, endocrine disruptors on sperm production and quality. In most cases, humans studies have considered separately perinatal exposures, exposures during infancy and postpubertal to adulthood exposures.

Table 3 Genes involved in azoospermia /oligozoospermia/asthenozoospermia (Mitchell et al., 2017; Ray et al., 2017)

<i>Gene</i>	<i>Chromosome</i>	<i>Human phenotype</i>
TEX 11	X	Azoospermia
TAF4B	18	Azoospermia/oligozoospermia
ZMYND15	17	Azoospermia
SYCE1	10	Azoospermia
TEX15	8	Azoospermia/oligozoospermia
NPAS2	2	Azoospermia
SYCP3	12	Azoospermia
KLHL10	17	Oligozoospermia
SEPT 12	16	Asthenozoospermia
DNAH1	3	Asthenozoospermia
CATSPER1	11	Asthenozoospermia
CATSPER2	15	Asthenozoospermia
SLC26A8	6	Asthenozoospermia

Environmental factors

During the past decades, a temporal decline and geographical variations in semen quality has been observed. Rapidly, environmental factors and particularly, endocrine disruptors have been suspected of interfering with the sperm production and quality. Actually, the available data on the effects of environmental chemicals, such as pesticides, DDT, PCBs, or plasticizers, both in the perinatal period and in adult, on sperm quality do not permit any definitive conclusions (Skakkebaek et al., 2016). However, reports on reduced sperm counts and azoospermia in men exposed to dibromochloropropane (DBCP) and dioxin provide the proof of principle that exogenous chemicals during adult life can disturb human spermatogenesis as it has been shown in numerous rodent studies (Skakkebaek et al., 2016).

Heat

Exposure to hot occupational environment (bakers, welders, foundry workers), sedentary work habits, traveling by car for long time during commute to and from work, tight clothing, sauna, all these can disrupt regulation of intra-scrotal temperature and can lead to an increase in testicular temperature. Male heat exposure could have a deleterious effect on sperm characteristics (such as motility and morphology) as has been reported in animal models (Mieusset and Bujan, 1995) while others studies do not reach the same conclusion. Recently, it has been shown that the deleterious effects on semen quality may be reversible sometime after a cessation of heat exposure.

Alcohol/tobacco

Alcohol does not appear to have any obvious effects on sperm parameters whatever exposure period: in utero or during adulthood (Skakkebaek et al., 2016). The results of studies concerning the possible effects of adult smoking on semen quality are contrasted.

Therapeutic treatment

It is now clearly documented that treatment of cancer with chemo- or radiotherapy causes reduction of sperm counts often to azoospermic levels that may persist for several years or be permanent. Concerning the others drugs likely to affect semen parameters, a recent review showed that the levels of scientific evidence are still insufficient (at the exception of Sirolimus, Sulfasalazine, exogenous testosterone, Finasteride and Cyproterone acetate, for which the levels of evidence are higher) (Semet et al., 2017).

Psychological stress

Several studies have investigated associations between semen quality and stress due to different types of stressors: environmental disasters, occupational stress, stressful life events, stress due to infertility, etc.... Overall, these studies may reveal that semen quality including sperm count and sperm motility is impaired by different stresses. But study populations were mostly small and selected (infertile, students, healthy men, donors...). Moreover, numerous confounding factors (age, abstinence time, smoking...) were not taken into account. Lastly, there are different tools to assess stress, making difficult a comparison between studies. Psychological stress might be a modifiable or reversible factor of spermatogenesis. But, it remains controversial.

Thus, many environmental factors alone or associated can probably have effects on sperm parameters. At present, data remain controversial. However, it has been shown that in a special context such as the Seveso accident, dioxin may have a detrimental effect on sperm production confirming the adverse effects of these molecules on spermatogenesis. Futures studies are therefore still needed.

In conclusion, sperm concentration and motility are two parameters of semen analysis, and have an important role in male fertility. These analyzes are still subjective and require standardized methods but also quality control. Semen analysis is performed in the ART laboratory but also in the context of epidemiological studies. Recently, Cooper and collaborators provided reference

values from a population of fertile men and a reference distribution for semen parameters was generated. Sperm count and motility can be altered with consequences for the fertility. These causes are probably due to different factors such as genetic, environmental... but the cause can also be multifactorial.

References

- Auger, J., Eustache, F., Ducot, B., Blandin, T., Daudin, M., et al. (2000). Intra- and inter-individual variability in human sperm concentration, motility and vitality assessment during a workshop involving ten laboratories. *Human Reproduction*, 15, 2360–2368.
- Bonde, J. P., Ernst, E., Jensen, T. K., Hjøllund, N. H., Kolstad, H., et al. (1998). Relation between semen quality and fertility: A population-based study of 430 first-pregnancy planners. *Lancet*, 351, 1172–1177.
- Lemmens, L., Kos, S., Beijer, C., Brinkman, J. W., van der Horst, F. A., et al. (2016). Predictive value of sperm morphology and progressively motile sperm count for pregnancy outcomes in intrauterine insemination. *Fertility and Sterility*, 105, 1462–1468.
- Mieusset, R., & Bujan, L. (1995). Testicular heating and its possible contributions to male infertility: A review. *International Journal of Andrology*, 18, 169–184.
- Mitchell, M. J., Metzler-Guillemain, C., Toure, A., Coutton, C., Arnoult, C., & Ray, P. F. (2017). Single gene defects leading to sperm quantitative anomalies. *Clinical Genetics*, 91, 208–216.
- Mortimer, S. T., van der Horst, G., & Mortimer, D. (2015). The future of computer-aided sperm analysis. *Asian Journal of Andrology*, 17, 545–553.
- Ombelet, W., Dhont, N., Thijssen, A., Bosmans, E., & Kruger, T. (2014). Semen quality and prediction of IUI success in male subfertility: A systematic review. *Reproductive Biomedicine Online*, 28, 300–309.
- Ray, P. F., Toure, A., Metzler-Guillemain, C., Mitchell, M. J., Arnoult, C., & Coutton, C. (2017). Genetic abnormalities leading to qualitative defects of sperm morphology or function. *Clinical Genetics*, 91, 217–232.
- Semet, M., Paci, M., Saïas-Magnan, J., Metzler-Guillemain, C., Boissier, R., et al. (2017). The impact of drugs on male fertility: A review. *Andrology*, 5, 640–663.
- Skakkebaek, N. E., Rajpert-De Meyts, E., Buck Louis, G. M., Toppari, J., Andersson, A. M., et al. (2016). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*, 96, 55–97.
- Slama, R., Eustache, F., Ducot, B., Jensen, T. K., Jørgensen, N., et al. (2002). Time to pregnancy and semen parameters: A cross-sectional study among fertile couples from four European cities. *Human Reproduction*, 17, 503–515.
- World Health Organization. (1999). *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction* (4th edn.). United Kingdom: Cambridge University Press.
- World Health Organization. (2010). *WHO Laboratory Manual for the Examination and Processing of Human Semen* (5th edn.). Geneva, Switzerland: WHO Press.
- Zinaman, M. J., Brown, C. C., Selevan, S. G., & Clegg, E. D. (2000). Semen quality and human fertility: A prospective study with healthy couples. *Journal of Andrology*, 21, 145–153.

Further Reading

- Cooper, T. G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H. W., et al. (2010). World Health Organization reference values for human semen characteristics. *Human Reproduction Update*, 16, 231–245.

Autonomic Nervous System and Male Reproduction

Janet R Keast and Peregrine B Osborne, University of Melbourne, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Overview

The autonomic nervous system is essential for regulating the activities of male reproductive organs. It is required to orchestrate basic functions such as erection, emission and ejaculation that depend upon the precise coordinated control of smooth muscle activity, blood control and exocrine gland secretion. In addition, recent advances suggest the autonomic nervous system also regulates the immune system, tissue growth and other important functions of the reproductive organs.

The patterns of autonomic nerve activity determine the onset, intensity, duration and coordination of many reproductive organ functions. For example, propulsion of sperm and seminal fluid requires appropriately timed activation of sympathetic nerves to contract smooth muscle of the ejaculatory duct. By contrast, erection of the penis in response to mechano-sensory stimulation depends on activation of a different population of nerves—parasympathetic nerves—whereas the following period of detumescence requires the central nervous system (brain and spinal cord) to coordinate cessation of parasympathetic activity with initiation of sympathetic activity. To make these types of decisions, the central nervous system integrates sensory information from within the organs themselves (interoception) with sensory information relating to the external environment (exteroception, e.g. visual and olfactory cues). Brain and spinal circuits determine what type of response, if any, is necessary and then initiate a motor response to match the needs of the body or complete the behavior.

This chapter will first introduce general principles of autonomic regulation followed by more detailed discussion of how neural activity affects different reproductive organs, the impact of sex hormones on this neural communication and relevance to several clinical conditions.

Autonomic Regulation of Organs and Tissues: General Principles

What Is an Autonomic Axon?

Autonomic axons are present in almost every tissue of the body. They are sometimes referred to as *visceral motor axons* because their activity directly affects the organs rather than the brain. This term serves to distinguish them from *somatic motor axons* that supply skeletal muscle, bones, joints and skin. However, an important limitation of this definition of ‘visceral motor’ should be noted as some targets of autonomic nerves are found integrated within somatic tissues—blood vessels and sweat glands, for example. An alternate definition of the autonomic nervous system is the ‘involuntary motor system’ that focuses on the context and types of actions that occur when autonomic nerves are excited or inhibited. Irrespective of which definition is used, it is important to realize that “motor” means much more than causing movement—as it can refer to any change in the activity of a tissue or organ caused by the autonomic nerve. On this basis, motor events include cellular activities such as contraction or relaxation of smooth muscle, altered ion fluxes across epithelia (to stimulate absorption or secretion), glandular secretions (mucous or hormones), metabolic changes (e.g., lipolysis), cell proliferation, and modulation of immune cell function.

Divisions of the Autonomic Nervous System: Anatomical Criteria

Conventionally, the autonomic nervous system is classified into two divisions that have quite distinct anatomical and physiological features—the sympathetic and parasympathetic nervous systems (Jänig, 2008). These are the focus of this chapter. A third division is the enteric nervous system that comprises only the neuronal circuits embedded within the digestive tract (Furness, 2012). The enteric circuits have several features that are quite different from the sympathetic and parasympathetic systems, most notably having their own sensory neurons and the capacity to operate independently of the central nervous system.

A typical autonomic pathway (Fig. 1) from the central nervous system to a peripheral organ has two linked nodes. The first component is called a preganglionic neuron. This has a cell body (soma) located in the central nervous system (brainstem or spinal cord) and an axon that projects outside of the central nervous system via a ventral spinal root or cranial nerve. This preganglionic axon projects via peripheral nerves and terminates at synapses on one or more neurons that are aggregated into autonomic ganglia. Some autonomic ganglia consist of thousands of neurons but others can be very small (only tens of neurons). Autonomic ganglion neurons are the second component of the motor pathway and their axons are called postganglionic axons. Postganglionic axons project out of the autonomic ganglia and synapse with specific tissues within the organs, at neuroeffector junctions.

The sympathetic and parasympathetic nervous systems are most clearly distinguished by their anatomical features. Preganglionic neurons of the sympathetic nervous system lie in the thoracic and lumbar regions of the spinal cord. Autonomic ganglia innervated by these neurons are called sympathetic ganglia. Conversely, parasympathetic preganglionic neurons lie in the brainstem (cranial nuclei) and sacral spinal cord, so autonomic ganglia innervated by these neurons are called parasympathetic ganglia. Most sympathetic ganglia are quite distant from the organs (i.e., their postganglionic axons are quite long), whereas most parasympathetic ganglia lie very close to or even within their target organs (i.e., very short postganglionic axons). In most parts of the body,

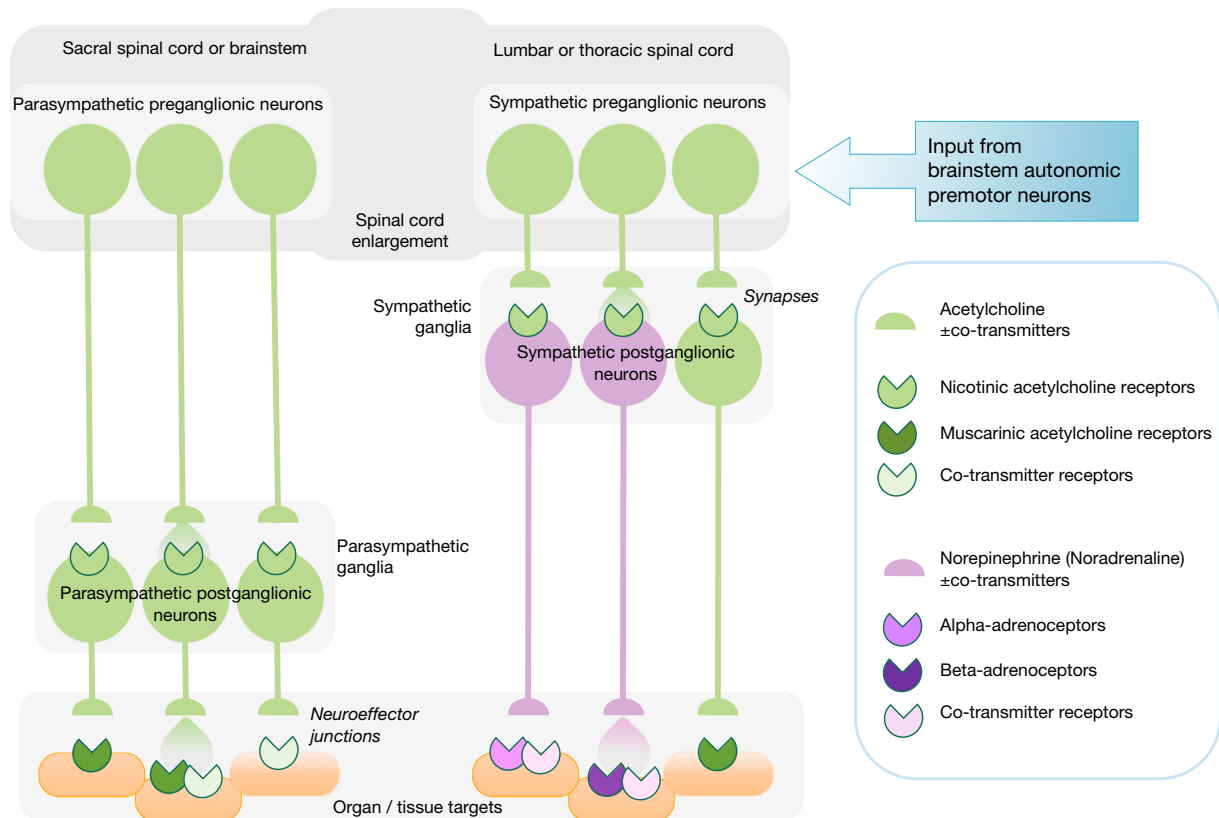


Fig. 1 Features of the sympathetic and parasympathetic nervous systems. These two systems are defined by their location of preganglionic neurons. Preganglionic neurons of both types receive inputs from specific areas of the brainstem. In most cases, parasympathetic ganglia lie closer to the organs, whereas sympathetic ganglia lie more distant. Communication between postganglionic axons and their target tissues (neuroeffector junctions) is mediated by acetylcholine or norepinephrine (synonym: noradrenaline), with or without various co-transmitters.

autonomic ganglion cells are clustered into separate sympathetic and parasympathetic ganglia, but as we will discuss later in this chapter, the ganglia innervating reproductive organs are quite different.

Divisions of the Autonomic Nervous System: Functional Differences

The sympathetic and parasympathetic nervous systems differ not only in their anatomy but also in their physiology and behavioral context of activation. These are summarized for the male reproductive organs later in this chapter. Specific brain circuits and mechanisms determine the type and duration of sympathetic and parasympathetic activity to ensure that relevant types of nerves are activated or inhibited appropriately. Many areas of the brain are involved in sexual and reproductive function, especially the hypothalamus and limbic system; specific regions of relevance include the paraventricular nucleus and medial preoptic area of the hypothalamus, ventral tegmentum, medial amygdala, and periaqueductal gray (Clement and Giuliano, 2016; Jänig, 2008).

Sympathetic and parasympathetic nerves generally have different, and often opposing, effects on shared or related organ targets (Jänig, 2008). This is most often caused by differential tissue-selective targeting of effectors in the organ. For example, in the vas deferens, sympathetic nerves innervate the smooth muscle, whereas parasympathetic nerves innervate the epithelium (Fig. 2A). When considered across the spectrum of actions across the body, the sympathetic nervous system is often described as enabling a 'fight-or-flight' response, with a range of activities that can be synchronized to optimize the response to a physical challenge (e.g., increasing heart rate and diverting blood flow from the gut to the limb muscles). Conversely, the function of the parasympathetic nervous system function is often generalized as 'rest-and-digest', because it has effects such as decreasing heart rate and redirecting blood from the limb muscles to the gut. These two terms are very relevant to some situations, but do not capture the full scope of autonomic nerve functions that are essential for numerous readjustments of tissue and organ function throughout every hour and day of our lives. More relevant to this chapter, these two terms cannot be readily applied to functions of the urogenital system, where the autonomic nervous system is instead critical for mediating reproductive behaviors, voiding and continence.

Sympathetic and parasympathetic ganglion neurons generally use different neurotransmitters to communicate at synapses with their target tissues (neuroeffector junctions) (Fig. 1). Neurotransmitters have transient actions close to their site of release from the axon, as they are released in small amounts and either metabolized or resorbed by nearby tissues. The primary neurotransmitter released by parasympathetic axons in the organs is acetylcholine (ACh), so events activated by this transmitter are referred to as

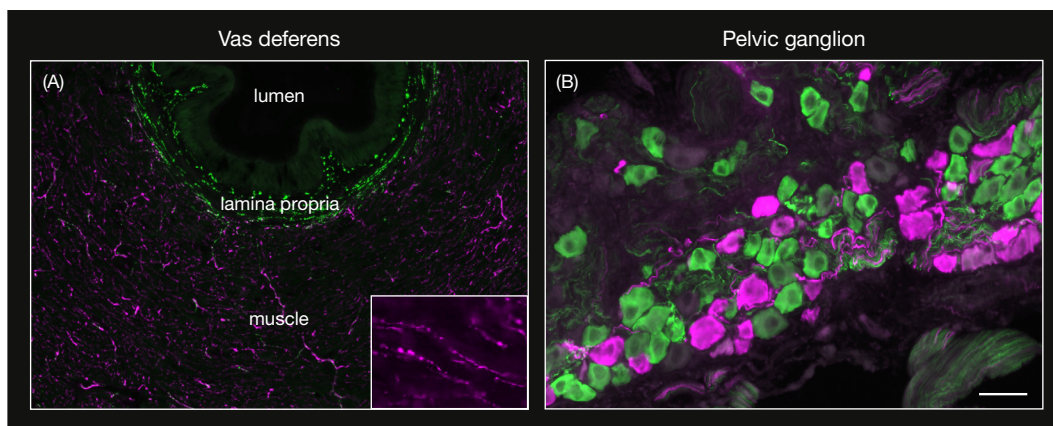


Fig. 2 Sympathetic and parasympathetic axons in the vas deferens and their cell bodies in pelvic ganglia. Panel **A** shows part of a transverse section through the rat vas deferens, using fluorescence immunohistochemistry to reveal the location of cholinergic parasympathetic axons (green) in the lamina propria, labeled with an antibody to vesicular acetylcholine transporter, and noradrenergic sympathetic axons (magenta) in the muscle, labeled with an antibody to tyrosine hydroxylase. The inset is a higher magnification view of axons in the muscle to illustrate the swellings (varicosities) that aggregate and release neurotransmitter. Here the noradrenergic axons were labeled with an antibody to a co-expressed peptide (neuropeptide Y) instead of tyrosine hydroxylase. Panel **B** shows part of a pelvic ganglion from a male mouse, using fluorescence immunohistochemistry to show the intermingling of cholinergic (green) and noradrenergic (magenta) neurons, using an antibody to nitric oxide synthase (that synthesizes nitric oxide, a co-transmitter with acetylcholine) and tyrosine hydroxylase. Calibration bar represents 50 μm (A), 15 μm (inset) and 30 μm (B).

cholinergic. ACh acts by binding to one or more subtypes of muscarinic cholinergic receptors expressed in tissues innervated by parasympathetic axons. It is common, however, for additional transmitters (co-transmitters) to be released at the same time from the same axons. These include nitric oxide (NO), adenosine triphosphate (ATP) and various neuropeptides (e.g., vasoactive intestinal peptide). In some tissues, the actions of co-transmitters can dominate over the actions of ACh. The primary sympathetic transmitter released in most tissues is norepinephrine (synonym: noradrenaline). Norepinephrine acts by binding to one or more subtypes of adrenoceptors; this is referred to as noradrenergic transmission. As discussed for the parasympathetic nerves, additional transmitters (such as ATP and neuropeptide Y) can also be released from many sympathetic axons. Some classes of sympathetic ganglion neurons—such as sympathetic nerves that activate secretion from sweat glands—release ACh but are not defined as parasympathetic as they receive input from thoracolumbar (i.e., sympathetic) preganglionic neurons.

There are several other ways in which neurotransmitter receptors can be activated in the organs. The first is relevant to the adrenoceptors. When the adrenal medulla is activated, high levels of norepinephrine and epinephrine (synonym: adrenaline) are released into the bloodstream. Therefore, any tissues that express adrenoceptors will be potentially affected. The second relates to cholinergic receptors (synonym: cholinceptors) and adrenoceptors expressed by autonomic neurons that function as autoreceptors on autonomic axons within the organs. Effects on these receptors by transmitters or circulating ligands have a range of complex positive- and negative-feedback effects on transmitter release. Therefore, to develop pharmaceutical therapies targeted to specific tissues or organs, a detailed understanding of these complex signaling scenarios is essential. These are confounded by species differences. Although many of the broad principles of autonomic regulation are shared between laboratory animals and people, there is diversity in the range of substances released by autonomic nerves (e.g., the types of neuropeptides), the dominant receptor subtypes expressed by the organs and their signaling mechanisms.

Autonomic Regulation of Male Reproductive Organs

The Pelvic Ganglia (Inferior Hypogastric Plexus)

The pelvic ganglia (synonym: inferior hypogastric plexus) contain all the parasympathetic and most of the sympathetic neurons that regulate the urogenital organs (Keast, 1999b; Keast, 2006). A small proportion of sympathetic innervation originates from other sympathetic ganglia, i.e., the lumbosacral sympathetic chain ganglia and the inferior mesenteric ganglion. An example is the sympathetic innervation of the corpus cavernosum that originates from the sympathetic chain. The pelvic ganglia also contain sympathetic and parasympathetic neurons that innervate the large intestine, supplementing the enteric nervous system.

The pelvic ganglia are very unusual autonomic ganglia because they contain both sympathetic and parasympathetic neurons (Figs. 2B and 3). In humans, these ganglia are aggregated into a complex network of microganglia called the inferior hypogastric plexus (Baader and Herrmann, 2003). Although they are intermingled within these ganglia, the sympathetic and parasympathetic ganglion neurons receive their commands from preganglionic neurons with different locations (sympathetic from the thoracolumbar level and parasympathetic from sacral level). The two main structures that carry these commands from the relevant spinal levels are the hypogastric nerves (sympathetic) and the pelvic nerves (parasympathetic; synonym, pelvic splanchnic nerves). These two

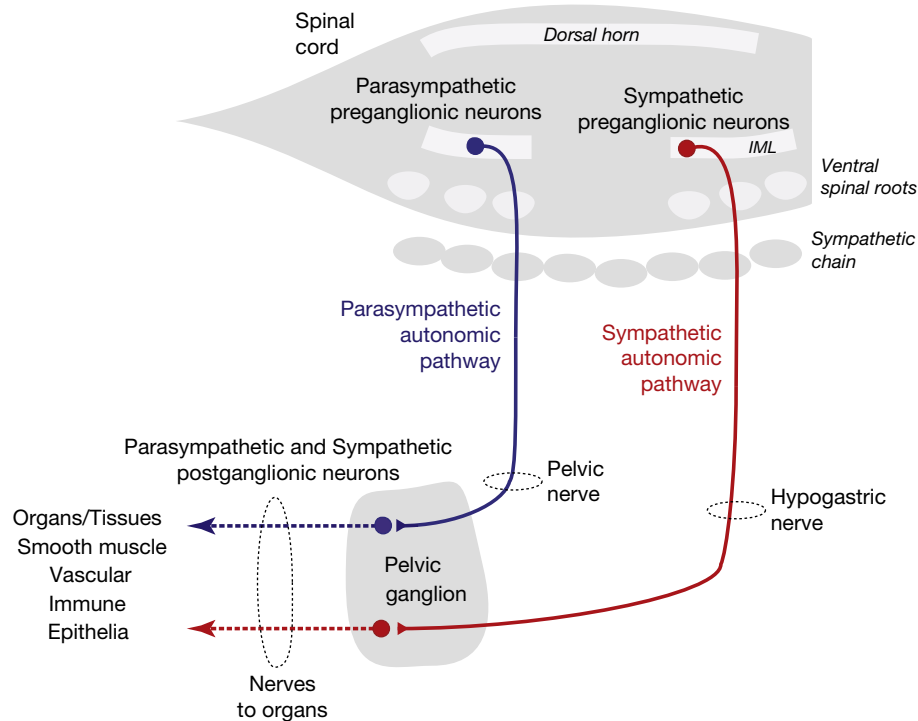


Fig. 3 Sympathetic and parasympathetic components of pelvic ganglia. Diagram shows the two types of spinal input to the pelvic ganglia, from sympathetic and parasympathetic preganglionic neurons that lie in the intermediolateral nucleus in two distinct spinal levels. The lumbar segments that form the lumbar enlargement separate these two sources of preganglionic neurons. The particular spinal levels have not been specified here as there are species differences in the number of lumbar and sacral spinal segments and, within these regions, which particular segments contribute to sympathetic and parasympathetic outflow. Sympathetic and parasympathetic postganglionic axons travel together in nerves that connect to the relevant organs. *IML*, intermediolateral nucleus.

nerves converge upon the pelvic ganglia, where their individual axons then make a synaptic connection with the appropriate ganglion neuron (or group of neurons). A minority of sympathetic ganglion cells in the pelvic ganglia use ACh as their primary transmitter, rather than norepinephrine (note: these neurons are defined as sympathetic because they are controlled by the thoracolumbar preganglionic neurons rather than sacral preganglionic neurons).

The unusual intermingling of sympathetic and parasympathetic neurons provides a challenge to understanding their development, connectivity, function and impact of disease or injury. In many species, including humans, this is made even more difficult because their pelvic autonomic neurons are not in discrete, large ganglia but embedded within a dispersed plexus that extends between and even within the outer layers of the pelvic organs. The breadth and complexity of this structure poses a considerable challenge to surgical procedures in these organs (Baader and Herrmann, 2003). Experimental studies on pelvic ganglia have instead focused on rats and mice, where the equivalent neurons are aggregated into a large, single ganglion on each side of the animal (Keast, 1999b). This simplicity enables precise experimental interventions and quantitative studies to be performed. Pelvic ganglia contain many more neurons in males than females, correlating with the higher density of autonomic axons in the reproductive organs in males. It is not known when this sexual dimorphism develops or how it is initiated.

Autonomic Reflexes and Male Sexual Function

The major role of sympathetic nerves in the male reproductive organs is to contract smooth muscle, whereas parasympathetic nerves enhance secretions and dilate the specialized vascular bed of the corpus cavernosum to cause penile erection. Therefore, to enable the full sequence of events—erection, emission and ejaculation—it is essential to coordinate activation of sympathetic and parasympathetic pathways (Andersson, 2011; Clement and Giuliano, 2015; Clement and Giuliano, 2016; Young et al., 2009). Moreover, ejaculation is not mediated purely by the autonomic nervous system. Complete expulsion requires carefully timed activation of striated perineal muscles that are innervated by sacral spinal motor neurons, not autonomic ganglia. Therefore, involuntary and voluntary components of the nervous system collaborate to complete this entire behavior. An additional autonomic function in each of the reproductive organs is control of tissue perfusion by regulation of vascular tone, specifically via activation of sympathetic vasoconstrictor axons.

Specific brain and/or spinal neural circuits contribute to each component of sexual function. In the case of erectile function, optimal strength and duration of erection occur if two components are activated. Direct, mechanical stimulation of the penis or

genital area activates sensory pathways that initiate activity in the sacral spinal preganglionic pathways to cause erection. This 'reflexogenic erection' is usually accompanied by 'psychogenic erection' that involves the brain, driven by awareness of erotic context, visual, auditory and olfactory stimuli. Psychogenic erection also involves more rostral levels of the spinal cord (lower thoracic and upper lumbar).

Emission, namely the release and propulsion of seminal fluid through the ejaculatory ducts to the urethra, involves spinal rather than brain circuits, especially the sympathetic pathways. Activation of these pathways causes contraction of smooth muscle of the ducts, prostate gland and seminal vesicles. The role of the parasympathetic nervous system here is unclear. The production of seminal fluid is most likely regulated by parasympathetic axons innervating various glandular epithelia, but it has yet to be determined how the activity of these nerves changes during sexual behavior. Expulsion involves a mixture of sympathetic spinal pathways to control smooth muscle of the ejaculatory ducts, bladder neck and proximal urethra. Both brain and spinal mechanisms integrate this with sensory stimuli from the genitals to coordinate the smooth muscle activity with rhythmic contractions of pelvic striated muscles. The spinal neurons involved in this coordination lie in the 'spinal ejaculation generator', a cluster of neurons in the mid-lumbar spinal cord. These neurons have a strong connection to the thalamus, a major sensory input center of the brain.

In addition to this integrated sequence of events in the reproductive system, there is further coordination with the neural circuits of the lower urinary tract. By contracting the smooth muscle of the bladder neck and pelvic (proximal) urethra, retrograde flow of seminal fluid is prevented during emission and ejaculation. The logistics of this coordination have not been defined, but likely involve a mixture of spinal and brain mechanisms. Central nervous system mechanisms also inhibit voiding reflexes of the lower urinary and digestive tracts during sexual activity.

Neural Communication with Tissues in Male Reproductive Organs

Across the tissues and organs of the male reproductive organs, there is diversity in the distribution and density of sympathetic and parasympathetic axons, and in the associated neurotransmitter receptor combinations that act as neuroeffectors. Nevertheless, these axons all share a common structure. Each autonomic axon branches near its point of termination, enabling simultaneous control of many cells at once by release of transmitter at multiple sites (Brock and Cunnane, 1993). Each of these terminal branches has numerous small swellings (varicosities) (Fig. 2A). Varicosities are functionally equivalent to synaptic boutons found on axons of the central nervous system. They serve as neuroeffector junctions where neurotransmitters are stored until being released, when an electrical signal (action potential) has reached the final length of axon. Each autonomic axon may have hundreds of varicosities, therefore transmitter can be released from many sites at one time.

Although the sites of transmitter release can be deduced from the location of varicosities, there is no easily visible structural specialization of the synapse on the cell that is innervated by an autonomic axon. This contrasts with the classical neuromuscular junction in skeletal muscle that has a single large transmitter release site adjacent to a highly folded region of membrane near the centre of the muscle cell. Also, sympathetic and parasympathetic axons look identical. Therefore, the precise sites and properties of autonomic communication in an organ can only be identified by electrophysiological, pharmacological or microscopic anatomy experiments.

The best characterized actions of autonomic nerves will be summarized here. Many other possible roles continue to be proposed and explored. This list has not included sympathetic vasoconstrictor axons that are present in each organ.

Testicular tissue does not have significant autonomic innervation beyond its vasculature. In the smooth muscle wall of the epididymis there is a pro-contractile sympathetic nerve supply. These axons increase in density towards the vas deferens. Parasympathetic axons are sparse or absent in these tissues. Of all regions in the male reproductive system, the testis and epididymis are the least dependent on autonomic nerves for their primary function.

The vas deferens has a thick muscular wall and high density of sympathetic noradrenergic axons that cause a strong contraction after electrical or pharmacological activation. Parasympathetic cholinergic axons are sparsely distributed in the muscle but form a dense network in the lamina and near the epithelium. The function of these parasympathetic axons is not known, but elsewhere in the body, parasympathetic activity stimulates epithelial secretion. These two contrasting patterns of innervation are shown in Fig. 2A. The seminal vesicles and bulbourethral glands show a comparable pattern, i.e., sympathetic noradrenergic pro-contractile innervation of smooth muscle and blood vessels, with a sparser parasympathetic cholinergic pro-secretory innervation of the epithelium.

In the prostate gland, sympathetic noradrenergic axons dominate and form a dense network within the smooth muscle trabeculae and stroma, as well as regulating vascular tone. Parasympathetic cholinergic axons primarily innervate the epithelial cells but their physiological role is less well understood. Studying prostate innervation is complicated by differences in structure and function across prostatic regions, and corresponding differences in axon density between these regions.

Penile erection and detumescence are complex physiological processes that require coordinated changes in blood flow across several vascular beds (Andersson, 2011). Erectile tissues of the corpora cavernosa and corpus spongiosum of the penis comprise networks of thin-walled vessels, filling of which causes erection. In total, this process involves increasing the blood flow to the penis, relaxing the smooth muscle of these thin-walled vessels and blocking the outflow of blood from the penis to sustain an increased intracavernosal pressure.

The primary driver for the active relaxation of intracavernous vessels is the parasympathetic nervous system, although there may also be some sympathetic component. Signals from sacral preganglionic neurons activate parasympathetic pelvic ganglion neurons that innervate the erectile tissue, releasing transmitters ACh and nitric oxide. Nitric oxide directly relaxes cavernosal smooth muscle

by activating the enzyme, guanylate cyclase, to synthesize cyclic GMP (cyclic guanosine monophosphate) from GTP (guanosine-5'-triphosphate). This cyclic nucleotide second messenger causes cavernosal smooth muscle relaxation. In contrast, ACh causes cavernosal muscle relaxation indirectly, by activating muscarinic ACh receptors on the endothelium (vessel lining) that in turn release nitric oxide; this causes further dilation of the cavernosal spaces. Many commonly used drugs that enhance erection have their primary action by increasing cyclic GMP levels within the cavernosum. Detumescence is triggered by activity of sympathetic nerves that release noradrenaline to contract the smooth muscle of cavernosal vessels and enhance the exit of blood from the penile vasculature. In the flaccid state, these sympathetic nerves provide a tonic contraction of the cavernosal smooth muscle. Striated muscles involved in ejaculation are controlled by the somatic nervous system but also contain some autonomic nerves, the physiological role of which during sexual behaviors has not been examined in depth.

Until this point, the focus of this chapter has been how nerves communicate with organs. There are also powerful ways that the organs communicate with nerves. For example, many tissues synthesize trophic factor proteins that during development are critical for establishing the appropriate axon connections and, by triggering signals back to the neuronal cell body in the autonomic ganglion, allowing the neuron to survive (Glebova and Ginty, 2005). Even in adulthood, any reduction in access to trophic factor has an impact on neuronal health. Therefore, if axon connections to an organ are damaged, these trophic signals can no longer reach the neuronal cell bodies in the ganglia. This is very relevant to developing strategies for restoring function.

Sensory Innervation of Male Reproductive Organs

Sensory nerves have a much lower density in the male reproductive organs than in the lower digestive or urinary tracts. The contrast is even greater for the genital skin—as the glans penis has one of the highest densities of sensory axons found anywhere in the body. Within the male reproductive organs, the sensory axons are greatly outnumbered by autonomic axons.

Sensory nerves supplying the reproductive organs originate from neuronal cell bodies in lumbar and sacral dorsal root ganglia. These particular sensory nerves have not undergone extensive study, but from studies of sensory nerves in the urinary and digestive system, it is predicted they would have the capacity to detect mechanical (e.g., distension) and chemical changes (e.g., inflammatory mediators or pH changes with tissue damage).

It is not known if sensory axons have a role in coordinating the components of normal sexual reflexes, but it is possible that they activate local reflex loops, functioning to detect the passage of seminal fluid (distension or flow detectors) or its contents (chemoreceptors). They also likely have roles in inflammatory conditions and several types of pelvic pain (Schwartz et al., 2015).

Sensory axons connecting with the reproductive organs project in the same nerve bundles as autonomic axons—the hypogastric and pelvic nerves, and the nerves that connect pelvic ganglia with the organs. Therefore, they are similarly vulnerable to injury or disease. In addition, several studies have proposed that some sensory neurons have branched axons that innervate both the bladder and the prostate. This raises the possibility that disease or damage in the bladder could impact on prostate function, or vice versa.

Effects of Gonadal Hormones on Autonomic Neural Circuits

The field of reproductive neuroendocrinology is strongly focused on regions of the brain, especially the hypothalamus and pituitary. However, many peripheral neurons that regulate autonomic function of the reproductive organs express proteins relevant to steroid signaling, such as androgen receptors (ARs), estrogen receptors (ERs) and aromatase (Purves-Tyson et al., 2007). These are expressed in subpopulations of neurons in the pelvic ganglia, lumbosacral dorsal root ganglia and spinal cord (Keast, 2006). Because of the many possible sites of action for circulating or exogenously administered steroids, it can be challenging to define exactly how a steroid affects each aspect of sexual function. Another layer of complexity is that steroids can stimulate synthesis of neurotrophic factors by the organs. Therefore, an effect of steroid exposure or deprivation on a peripheral neuron could be occurring directly on that neuron or indirectly via modulation of organ-derived trophic factors.

The impact of steroid exposure on pelvic ganglion neurons has been examined experimentally for only a limited number of functions. In rodent pelvic ganglion neurons that express ARs and ERs, there is a critical period for androgen exposure in the perinatal period, when various adult features of these circuits are established, and further changes at puberty (Brock et al., 2007; Keast, 1999a; Keast, 2006). Post-pubertal reduction in androgen exposure — whether by gonadectomy or pharmacological/genetic intervention — has a noticeable impact on neural features as diverse as cell body size, axon branching, neuropeptide and transmitter expression, capacity for regrowth after injury, and electrophysiological properties. Typically, these are reversible by subsequent exposure to androgens, or in some cases, estrogens.

The Relevance of Autonomic Nerves to Clinical Conditions in the Male Reproductive Organs

Many clinical conditions are caused by changes in the structure or activity of autonomic nerves, leading to organ dysregulation. The conventional therapeutic approach for autonomic systems has been to develop drugs that selectively activate or block particular classes of axons or transmitter signaling processes. There is now increasing interest in bioengineering approaches to modulate peripheral autonomic activity, by using implantable devices to electrically stimulate or block activity in specific types of axons (Famm et al., 2013).

A contributor to disorders of male reproductive organs is the reduction of activity of autonomic nerves, which may be caused by diverse neurodegenerative conditions such as diabetes and various small-fiber neuropathies (Dineen and Freeman, 2015). The

underlying pathophysiology is best characterized in the context of erectile dysfunction, but can also affect other urogenital organs leading to ejaculatory problems, lower urinary tract symptoms, and other clinical conditions. It is popular to focus therapies on enhancing the capacity of existing nerves, e.g., Viagra (sildenafil) for enhancing nitric oxide signaling in erectile tissue of the penis, but there is also interest in developing ways to protect autonomic nerves from degeneration or to enhance tissue perfusion by neural regulation of the microvasculature. The impact of hormonal environment on sexual function and clinical dysfunction also includes a component due to the effects of hormones on relevant autonomic nerves (Podlasek et al., 2016).

Autonomic regulation and sensory innervation of the reproductive organs is physically at risk during pelvic surgery (e.g., prostatectomy), commonly performed to remove malignant tissue. The complex anatomy of the inferior hypogastric plexus and its connections makes it extremely difficult to perform such surgery without injuring these nerves (Baader and Herrmann, 2003). Erectile dysfunction and impaired bladder control are the most common negative outcomes. Surgical procedures and methods continue to be improved to visualize this plexus in situ, but in some cases the location and extent of tissue removal required make it impossible to avoid some neural damage. Improved understanding of endogenous trophic factors that originally enabled these nerve-organ connections to be established during development has provided some strategies for enhancing their recovery after damage or degenerative triggers.

Clinical conditions that potentially benefit from reduction of autonomic or sensory activity in the reproductive organs have largely focused on the prostate gland and lower urinary tract but may have complex interactions with sexual dysfunction (Mazur et al., 2012). Lower urinary tract symptoms, comprising a spectrum of voiding disorders often age-related and coupled with benign prostatic hyperplasia, can be attenuated by blocking sympathetic activation of prostatic (and potentially urethral) smooth muscle. While this approach still has limitations of efficacy and specificity, additional ways of regulating smooth muscle tone in the prostate are still being explored. The actions of inflammatory mediators on these autonomic nerves and their neighboring sensory nerves are strongly implicated in neuronal overactivity and increased sensitivity of these urogenital sensory nerves to normal physiological stimuli. This is relevant not only to lower urinary tract symptoms but also pelvic pain driven by prostatitis.

There is growing interest in the concept that autonomic nerve effects on organ structure could be manipulated to reduce the growth of cancer. This is being explored in several types of cancer, including the prostate (Saloman et al., 2016). The original rationale for this approach was the observation that removing the nerve supply to a healthy prostate gland, or pharmacologically blocking the activity of these nerves, impacts on the structure of prostate glandular or stromal tissue. The nature of the effect depends on the type of nerve manipulation and the duration of intervention. It is logical to now investigate the impact of selectively blocking or stimulating the activity of various types of autonomic nerves in a situation of unregulated tissue growth.

References

- Andersson, K. E. (2011). Mechanisms of penile erection and basis for pharmacological treatment of erectile dysfunction. *Pharmacological Reviews*, 63, 811–859.
- Baader, B., & Herrmann, M. (2003). Topography of the pelvic autonomic nervous system and its potential impact on surgical intervention in the pelvis. *Clinical Anatomy*, 16, 119–130.
- Brock, J. A., & Cunnane, T. C. (1993). Neurotransmitter release mechanisms at the sympathetic neuroeffector junction. *Experimental Physiology*, 78, 591–614.
- Brock, J. A., Handelsman, D. J., & Keast, J. R. (2007). Postnatal androgen deprivation dissociates the development of smooth muscle innervation from functional neurotransmission in mouse vas deferens. *The Journal of Physiology*, 581, 665–678.
- Clement, P., & Giuliano, F. (2015). Anatomy and physiology of genital organs—men. *Handbook of Clinical Neurology*, 130, 19–37.
- Clement, P., & Giuliano, F. (2016). Physiology and pharmacology of ejaculation. *Basic & Clinical Pharmacology & Toxicology*, 119(Suppl 3), 18–25.
- Dineen, J., & Freeman, R. (2015). Autonomic neuropathy. *Seminars in Neurology*, 35, 458–468.
- Famm, K., Litt, B., Tracey, K. J., Boyden, E. S., & Slaoui, M. (2013). Drug discovery: A jump-start for electroceuticals. *Nature*, 496, 159–161.
- Furness, J. B. (2012). The enteric nervous system and neurogastroenterology. *Nature Reviews. Gastroenterology & Hepatology*, 9, 286–294.
- Glebova, N. O., & Ginty, D. D. (2005). Growth and survival signals controlling sympathetic nervous system development. *Annual Review of Neuroscience*, 28, 191–222.
- Jänig, W. (2008). *Integrative action of the autonomic nervous system: Neurobiology of homeostasis*. Cambridge: Cambridge University Press.
- Keast, J. R. (1999a). The autonomic nerve supply of male sex organs—An important target of circulating androgens. *Behavioural Brain Research*, 105, 81–92.
- Keast, J. R. (1999b). Unusual autonomic ganglia: Connections, chemistry, and plasticity of pelvic ganglia. *International Review of Cytology*, 193, 1–69.
- Keast, J. R. (2006). Plasticity of pelvic autonomic ganglia and urogenital innervation. *International Review of Cytology*, 248, 141–208.
- Mazur, D. J., Helfand, B. T., & Mcvary, K. T. (2012). Influences of neuroregulatory factors on the development of lower urinary tract symptoms/benign prostatic hyperplasia and erectile dysfunction in aging men. *The Urologic Clinics of North America*, 39, 77–88.
- Podlasek, C. A., Mulhall, J., Davies, K., Wingard, C. J., Hannan, J. L., Bivalacqua, T. J., Musicki, B., Khera, M., Gonzalez-Cadavid, N. F., & Burnett, A. L. (2016). Translational perspective on the role of testosterone in sexual function and dysfunction. *The Journal of Sexual Medicine*, 13, 1183–1198.
- Purves-Tyson, T. D., Arshi, M. S., Handelsman, D. J., Cheng, Y., & Keast, J. R. (2007). Androgen and estrogen receptor-mediated mechanisms of testosterone action in male rat pelvic autonomic ganglia. *Neuroscience*, 148, 92–104.
- Saloman, J. L., Albers, K. M., Rhim, A. D., & Davis, B. M. (2016). Can stopping nerves, stop cancer? *Trends in Neurosciences*, 39, 880–889.
- Schwartz, E. S., Xie, A., La, J. H., & Gebhart, G. F. (2015). Nociceptive and inflammatory mediator upregulation in a mouse model of chronic prostatitis. *Pain*, 156, 1537–1544.
- Young, B., Coolen, L., & McKenna, K. (2009). Neural regulation of ejaculation. *The Journal of Sexual Medicine*, 6(Suppl 3), 229–233.

Circadian Rhythms—Male

Katherine M Hatcher and Megan M Mahoney, University of Illinois at Urbana Champaign, Champaign, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The circadian (circa = about, dia = day) clock controls 24-h rhythms of physiological processes, including sleep/wake behavior, hormone levels, and body temperature. In the presence of rhythmic external stimuli, the circadian clock functions to synchronize the physiology and behavior of the organism with that of the environment. An essential attribute of circadian rhythms is not only that they synchronize, or entrain, to timing cues, but they also persist in the absence of these timing cues in constant conditions, such as constant darkness or constant light. Animals will continue to exhibit a rhythmic “period” around 24 h in these constant conditions.

For most organisms, light is the primary time cue for the circadian clock. In mammals, the master clock is located in the suprachiasmatic nucleus (SCN), a nucleus in the ventral hypothalamus of the brain, just dorsal to the optic chiasm. As light enters the eye and activates specialized retinal ganglion cells, this light information is sent to the optic chiasm via neuronal projections known as the retinohypothalamic tract. Then, the light information is conducted to the SCN and is interpreted by the animal’s internal circadian clock via complex molecular mechanisms (see Fig. 1).

The circadian clock responds to external timing cues, primarily light, because of the molecular components that exist in positive and negative molecular feedback loops, which oscillate in a 24-h rhythm. The proteins, translated from core clock genes, are essential for generating and regulating other circadian rhythms both within and outside of the SCN. The positive element of the feedback loop begins with the proteins CLOCK and BMAL1, which form the CLOCK:BMAL1 pair (i.e. heterodimer). Once the CLOCK:BMAL1 complex is formed, it associates with *E*-box promoter regions, located upstream of the gene, and promotes transcription of the clock genes *Period* (*Per*) and *Cryptochrome* (*Cry*). The negative feedback loop is achieved by the heterodimerization of PER and CRY proteins. Specifically, *Per* and *Cry* are translated in the cytosol, where their protein counterparts form a PER:CRY heterodimer. Then, the PER:CRY pair translocates back into the nucleus, where it inhibits the binding of CLOCK:BMAL1 to promoter regions, ultimately decreasing transcription of *Per* and *Cry*. This process takes approximately 24 h to complete one cycle, resulting in the rhythmic expression of the circadian genes and proteins. CLOCK:BMAL1 also binds to the *E*-box elements of other genes to regulate their transcription, generally known as clock controlled genes, which can influence downstream effects of the circadian timekeeping system, as well as fine-tune the expression of core clock genes. Furthermore, while the core clock gene loop located in the SCN is known as the master clock, this same molecular loop has been shown to occur in tissues throughout the body. (See Fig. 2.)

Circadian rhythms are an important research area for understanding reproductive physiology of both males and females, with circadian variations in sex steroid hormones, critical enzymes involved in steroidogenesis, mating behavior and receptivity, and other important factors involved in regulation of the reproductive system. Importantly, the core circadian molecular clock can be measured in almost all peripheral systems. However, the exact role that the circadian timekeeping system plays in regulating male reproduction remains poorly understood. This chapter will specifically examine the evidence for the presence of clock systems in different male reproductive tissues and functions, with emphasis on mice and rat studies. Specifically, we will focus on rhythms found in the testes, where spermatogenesis occurs, the epididymis, where sperm maturation occurs, and accessory glands and ducts, which aid in male reproductive function and sperm health. Additionally, we will discuss evidence for the male reproductive system acting on the circadian timekeeping system to fine tune behavior.

Rhythms of Circadian Clock Genes and Proteins in Testis

A handful of studies have identified rhythmic clock gene expression in the rodent testis. One early mouse study investigating mRNA expression found that the clock genes *mPer1* and *mPer2* exhibited daily rhythmic expression, peaking around 6 (*mPer1*) and 9 (*mPer2*) hours after lights on (Zylka et al., 1998). This rhythmic expression of clock genes in the testes was confirmed in a second

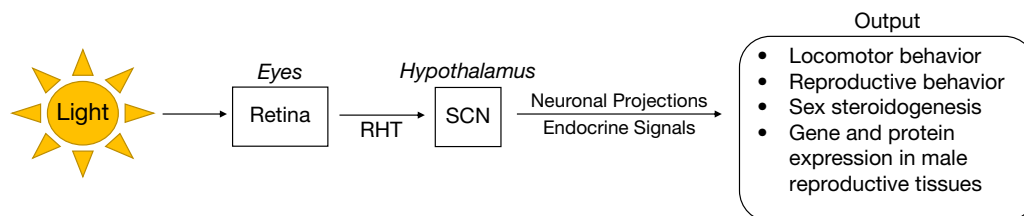


Fig. 1 Simplified schematic of the circadian timekeeping system. Light enters through the eye and stimulates the retina. Then, timing information from the light stimulus is communicated through the retinohypothalamic tract (RHT) to the hypothalamus. Within the hypothalamus, a specific subset of neurons located in the suprachiasmatic nucleus (SCN) are stimulated. Using either endocrine outputs or neuronal projections, the SCN communicates the light information to the rest of the brain and body to initiate multiple output rhythms. Specifically, this light information relayed by the SCN can regulate locomotor and reproductive behaviors, sex steroidogenesis, and gene and protein expression within the male reproductive system.

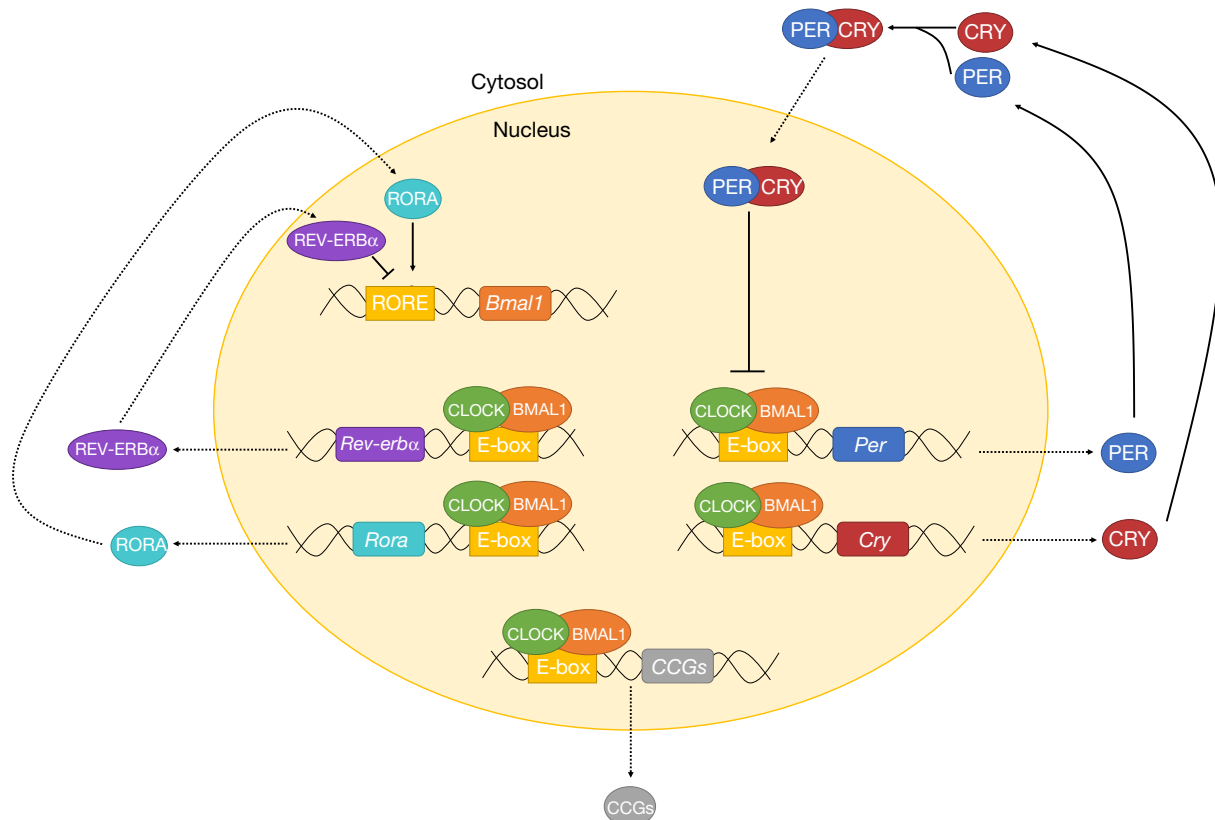


Fig. 2 Core circadian clock feedback loops. In the nucleus, the CLOCK:BMAL1 protein pair binds to E-box elements located upstream of the *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. This binding initiates the transcription of the clock genes *Per1* and *Cry1*, where they then are translated into their respective proteins, PER and CRY. PER and CRY bind in the cytosol to form the PER:CRY pair, and then translocate back into the nucleus. In the nucleus, PER:CRY can bind to and inhibit the action of CLOCK:BMAL1, therefore inhibiting their own expression. The expression of BMAL1 can be further finetuned by additional clock genes and proteins. Specifically, CLOCK:BMAL1 binds to the E-box elements upstream of the genes for *Rev-erbα* and *Rora*, initiating transcription of these two genes. Then, the RORA and REV-ERBα proteins are translated in the cytosol, where they can re-enter the nucleus and affect *Bmal1* gene transcription. Specifically, RORA and REV-ERBα bind to ROR elements (RORE) located upstream of the *Bmal1* gene where they initiate or inhibit transcription of the *Bmal1* gene, respectively. Overall, this precise timing of gene transcription and protein translation results in the daily rhythmic expression of the core molecular clock. Furthermore, CLOCK:BMAL1 can bind to additional E-box elements upstream of other circadian target genes known as clock-controlled genes (CCGs), thereby initiating transcription of genes outside of the core circadian clock loop. Importantly, this core circadian molecular loop is found within almost every tissue of the body and is thought to be important for regulating precisely timed events within those tissues.

study in mice (Bebas et al., 2009). In this study, rhythmic expression of the clock genes *mPer1*, *mPer2*, and *Bmal1* was found, with levels peaking in the middle-to-late night. Interestingly, this study did not observe rhythmic expression of mPER1 and BMAL1 protein expression. While both of these studies showed rhythmic expression of core circadian clock genes, the peak in expression of *mPer2* was opposite in timing (phase) between the two studies. Additionally, these studies documented clock gene rhythmicity in the male reproductive tract; however, they did not examine the functional role of rhythmic clock gene expression in the testis, which remains to be investigated.

Interestingly, additional studies examining the presence of rhythmic circadian clock gene expression have produced conflicting results. In one study, expression of the clock genes *mCry1*, *mCry2*, and *mPer1* was observed in the mouse testis; however, this expression was not rhythmic for any of these genes (Miyamoto and Sancar, 1999). An additional study investigated rhythmicity of *mPer1* in several endocrine tissues, including the mouse testis, using in situ hybridization, a technique that allows for the visualization of labeled nucleic acids in a section of tissue (Bittman et al., 2003). In this study, they did not observe rhythmic expression of the clock gene *mPer1*. However, they did observe daily rhythmic expression of mPER1 protein in specific sections of the seminiferous tubules, the portion the testes where spermatozoa are found. This finding indicates differential expression based on location. In another study, the expression of *mPer1* and *Clock* genes and proteins was observed in mouse testes; however, the expression was also not rhythmic (Morse et al., 2003). Moreover, this study found that *mPer1* and *Clock* were expressed in different portions of the seminiferous tubules. Overall, these studies observed that the traditional clock genes and proteins are expressed in the testes, albeit not rhythmically, potentially indicating a potential non-circadian function of these genes.

The conflicting results of whether or not there are circadian rhythms in gene and protein expression in the testes have yet to be resolved. However, it is important to note that most of these studies examined expression using whole testes. The testis is a very

diverse organ, with many cell types present, including seminiferous tubules, developing spermatozoa, interstitial cells, and Sertoli cells. It is possible that clock gene and/or protein expression is rhythmic in one or all of these different cell types. Alternatively, it is possible that circadian expression of these clock genes and proteins only occurs in some of these cell types, or at specific developmental time points. Indeed, one study using mice observed that *mPer1* was expressed in specific stages of spermatogenesis, specifically in the spermatids and not in the spermatogonia and spermatocytes (Morse et al., 2003). This study, among others, reiterates the importance of investigating rhythms of gene and protein expression at specific developmental time points may be a critical next step to understanding the circadian clock system in the testis.

Rhythms of Circadian Clock Genes and Proteins in the Epididymis, Accessory Glands, and Vas Deferens of the Male Reproductive Tract

Until recently, it has been unclear if there was a role for circadian genes in other tissues of the male reproductive tract beyond the testes, including the epididymis, accessory glands (i.e. seminal vesicles and prostate gland), and vas deferens. These tissues are essential for the further maturation and health of spermatozoa, production of semen, and the release of sperm during ejaculation, respectively. In addition to investigating the rhythmic expression of genes in the testes, one study also measured gene and protein expression of *mPer1*, *mPer2*, and *Bmal1* in the epididymis, vas deferens, seminal vesicles, and prostate gland (Bebas et al., 2009).

Interestingly, rhythmic gene and protein expression varied in the epididymis for all genes, depending on where the expression was measured. In the caput (head) of the epididymis, *mPer1* gene expression was rhythmic, peaking at 8 h after lights off, while mPER1 protein expression peaked 2 h after lights on. While *Bmal1* gene expression was not rhythmic in the caput epididymis, BMAL1 protein expression did have a daily expression pattern, with a peak 2 h after lights off. In the corpus (body) of the epididymis, they observed rhythmic expression of both *mPer1* and *mPer2* clock genes and mPER1 clock protein, all peaking 2 h after lights off. They also observed rhythmic *Bmal1* gene expression in the corpus, with an opposite timing (phase) to that of *mPer1*/*mPer2*, peaking in the late night. BMAL1 protein expression was rhythmic in the corpus, with a peak in expression at 2 h after lights on. In the cauda (tail) of the epididymis, *Bmal1* and *mPer1* were rhythmic and in phase with each other, peaking 8 h after lights off, while *mPer2* peaked 2 h after lights off. Overall, these data indicate that there are rhythms in the expression of clock genes and clock proteins throughout the epididymis, a tissue where further maturation of spermatozoa occurs. Furthermore, within each location within the epididymis, they observed that peak BMAL1 protein expression followed peak mPER1 expression, consistent with what is expected if BMAL1 is acting as the positive regulator in the circadian feedback loop.

Like the epididymis, this study also found rhythmicity in clock gene and protein expression throughout the accessory glands and vas deferens, with tissue-specific expression of genes. In the vas deferens, they found *mPer1* and *mPer2* gene expression peaked at 4 h before lights off and *Bmal1* gene expression peaked 2 h after lights on, with respective protein levels peaking at the same time. In the prostate, *mPer1* and *mPer2* both peaked 8 h after lights on, while *Bmal1* peaked 8 h after lights off, with mPER1 protein peaking at 8 h after lights on and BMAL1 protein peaking around 2 h after lights off. Overall, they found no significant rhythms in the seminal vesicles, however, both *mPer1* and *mPer2* genes and mPER1 and BMAL1 proteins were highly expressed. Together, these data show rhythmic expression of core circadian clock genes in a tissue-specific manner. It is plausible that the rhythmic expression of the core circadian genes and proteins is involved in regulating timed events in the male reproductive tract, including sperm maturation and hormone production. However, the functional role of these genes in the male reproductive tract remains to be examined (Table 1).

Traditional Clock Genes Have Non-Circadian Functions for Spermatogenesis

The continuous production of sperm throughout the reproductive lifetime of the male is essential for fertility. Spermatozoa develop with specific spatiotemporal patterning along the length of the seminiferous tubule. Briefly, the most undifferentiated cells, the spermatogonia, are located basally, while the more mature spermatids are located toward the lumen of the testis. While circadian rhythmicity in developing spermatozoa has been studied, more precise tools are needed to further evaluate individual components and specific locations that exhibit rhythmicity. In one study, location-specific expression of *Per1* was visualized with in situ hybridization (Morse et al., 2003). Using this technique on stage-specific samples of dissected tubules showed that *mPer1* expression was highest in the middle of the seminiferous tubules, where elongated spermatids and spermatozoa reside. To determine if *mPer1* expression is driven by the traditional CLOCK/BMAL1 dimer mechanism, the authors used in situ hybridization to measure *Clock* expression. They found that *Clock* was expressed at different locations along the tubule compared to expression of *mPer1*. Specifically, *Clock* was expressed in the periphery of the tubule, where spermatogonia and primary spermatocytes are located. In *Clock* mutant mice (without a functioning *Clock* gene), *Per1* expression persisted, indicating that *Per1* is expressed independently of *Clock* expression. Overall, it appears that *Per1* is differentially expressed relative to sperm development stages and suggesting it may not be related to circadian functions.

Interestingly, another group released similar results around the same time as Morse et al., except observing protein expression of CLOCK and mPER1 using immunohistochemistry, as opposed to mRNA expression (Alvarez et al., 2003). Immunohistochemistry allows for visualization of proteins in specific sections of tissues using labeled antibodies. Using this technique, they observed noncyclic expression of mPER1 at different stages of spermatogenesis depending on the age of the animal. In early postnatal testes (postnatal day 7), they observed that expression of mPER1 was restricted to spermatogonia in early stages (Type A spermatogonia)

Table 1 Summary of the rhythmic expression of clock genes and proteins observed in the male reproductive tract of mice

<i>Tissue</i>	<i>Gene or protein</i>	<i>Rhythms present?</i>	<i>Description of rhythms observed</i>	<i>References</i>
Testis	<i>Per1</i>	Yes	Peak 6 h after lights on Peak in late night	Zylka et al. (1998) Bebas et al. (2009)
		No rhythmic expression		Bittman et al. (2003) and Morse et al. (2003)
	<i>Per2</i>	Yes	Peak 9 h after lights on Peak in late night	Zylka et al. (1998) Bebas et al. (2009)
	<i>Bmal1</i>	Yes	Peak 8 h after lights off	
	<i>Cry1</i>	No rhythmic expression		Miyamoto and Sancar (1999)
	<i>Cry2</i>			
	<i>Clock</i> PER1 BMAL1			Morse et al. (2003) Bebas et al. (2009) and Bittman et al. (2003)
Epididymis	<i>Per1</i>	Yes	Caput (head): Peak at 8 h after lights off; Corpus (body): Peak in late morning; Cauda (tail): Peak at 8 h after lights off	Bebas et al. (2009)
	<i>Per2</i>		Corpus (body): Peak in the late morning/early night; Cauda (tail): Peak 2 h after lights off	
	<i>Bmal1</i>		Caput (head): No rhythmic expression; Corpus (body): Peak in the late night, in opposite phase of <i>mPer1</i> and <i>mPer2</i> in the corpus; Cauda (tail): in phase with <i>mPer1</i> , peak 8 h after lights off	
	PER1		Caput (head): Peak 2 h after lights on; Corpus (body): Peak 2 h after lights off	
	BMAL1		Caput (head): Peak 2 h after lights off; Corpus (body): Peak 2 h after lights on	
Vas deferens	<i>Per1</i>	Yes	Peak 4 h before lights off	
	<i>Per2</i>			
	<i>Bmal1</i>		Peak 2 h after lights on	
	PER1		Peak 4 h before lights off	
	PER2 BMAL1		Peak 2 h after lights on	
Prostate	<i>Per1</i>	Yes	Peak 8 h after lights on	
	<i>Per2</i>			
	<i>Bmal1</i>			
	PER1 BMAL1		Peak 2 h after lights off	
Seminal vesicles	<i>Per1</i>	No rhythmic expression		
	<i>Per2</i>			
	PER1			
	BMAL1			

and then this expression transitions and mPER1 is found in spermatocytes at postnatal day 14. In adult samples, mPER1 expression was only observed in spermatogonia in later stages of development (Type B spermatogonia) and condensing spermatids in adult seminiferous tubule. To evaluate if CLOCK is involved in regulating mPER1 expression in the seminiferous tubules of adults, CLOCK expression was localized and compared with mPER1 expression. Interestingly, CLOCK was expressed in the acrosome (the cap of the sperm head) of round spermatids and did not colocalize with mPER1, and was also found in different sections of the seminiferous tubules. These data indicate a differential function of these clock proteins compared with the SCN and other peripheral tissues that exhibit the canonical core circadian clock loops. While the function for the developmental stage-specific expression of mPer1 mRNA and protein remains unknown, recent studies suggest non-circadian functions for Clock expression in sperm. Indeed, using molecular techniques to reduce or knock down CLOCK expression in the adult mouse testis results in reduced fertility, potentially by indirectly reducing acrosin activity in sperm (Cheng et al., 2016).

Rhythms in Steroidogenesis

The production and secretion of gonadal hormones are tightly regulated by the hypothalamic-pituitary-gonadal axis. Small-bodied neurons in the hypothalamus release gonadotropin releasing hormone (GnRH), which acts upon receptors in the anterior pituitary

to stimulate the synthesis and release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) into circulation. These gonadotropins act differentially within the testis to stimulate synthesis and release of sex steroid hormones, including testosterone. The primary action of LH is to stimulate production of the androgen hormones, including testosterone, in Leydig cells. FSH primarily acts on Sertoli cells to synthesize growth factors that are important for spermatogenesis. In addition, FSH also stimulates Sertoli cells to synthesize aromatase, an enzyme that converts the testosterone produced by Leydig cells to estradiol. The testosterone produced by LH stimulation not only acts locally in the testis, but also negatively feeds back on the hypothalamus and pituitary to reduce the release of GnRH and gonadotropins, thereby indirectly regulating its own synthesis.

There are circadian variations in several hormones relevant to male reproduction. Testosterone exhibits a circadian rhythm in serum concentrations, peaking toward the middle-to-late portion of the light phase in rats (Wilson et al., 1976) and mice (Lucas and Eleftheriou, 1980), likely due to the circadian pattern of release of LH from the anterior pituitary in both rats (Taya and Igarashi, 1974) and mice (Jean-Faucher et al., 1986). There are many enzymes and proteins involved in the synthesis of testosterone by Leydig cells, including: StAR, which transports cholesterol into mitochondria; Cyp11a1, which catalyzes the cleavage of cholesterol to pregnenolone; and Cyp17a1, which is involved in several steps in biosynthesis of testosterone. Interestingly, expression of genes coding for these proteins and enzymes in rat Leydig cells exhibit a circadian rhythm in expression preceding that of the peak in testosterone, all peaking in the middle of the light phase of the light: dark cycle (Baburski et al., 2016).

In a study investigating the role of BMAL1 in male fertility, mice that had BMAL1 knocked out had reduced testosterone levels (Alvarez et al., 2008). Additionally, serum LH concentrations were 3-fold higher in knockout mice compared to control animals, suggesting an impairment of the Leydig cells to produce testosterone in response to LH stimulation. Additionally, after examining several key proteins and enzymes involved in steroidogenesis in the testis of BMAL1 knockout mice, they found that StAR gene and protein expression were significantly reduced, potentially affecting steroidogenesis by decreasing cholesterol uptake into the Leydig cells. Upon further investigation in vitro using a Leydig cell line, they found that BMAL1 induces StAR expression by acting on an upstream promoter.

Androgen Regulation of Behavioral Rhythms

Not only is there circadian regulation of testosterone synthesis in the testis, but testosterone also feeds back on the SCN to regulate gene expression and morphology and thereby modulating circadian rhythms in behavior. Studies that have removal of the gonads (gonadectomy) have revealed that testosterone is an important regulator of circadian rhythms in locomotor behavior. Circulating testosterone mediates behavior by acting locally on androgen receptors (AR) within the SCN. Specifically, AR was colocalized with neurons expressing gastrin-releasing peptide (GRP) within the core region of the SCN (Karatsoreos et al., 2007). GRP-expressing neurons in the SCN are important for the SCN's ability to respond to light, indicating that AR may be involved in regulating light responsiveness in the SCN. Indeed, when light pulses are administered, this causes an increase in the expression of immediate early genes within the SCN. Castration results in reduced immediate early gene expression in response to light pulses given in the subjective night. Replacement with dihydrotestosterone, an AR-specific androgen, restores immediate early gene expression in response to a light pulse given in the dark. Castration of male mice also modulated SCN astroglial abundance in concurrence with irregular expression of the clock genes *Per2* and *Per1* in response to light pulses in the early night and late night, respectively (Karatsoreos et al., 2011). The altered *Per2* expression also resulted in a larger shift in the timing of behavioral rhythms in response to a light pulse, whereas the hormone dihydrotestosterone restored the normal behavioral shift in response to light cues. While the role of astrocytes in the SCN is not well understood, it appears that they may be important for normal SCN function, especially for the regulating response to light cues in the SCN (Karatsoreos et al., 2011).

Gonadectomy has been shown to alter many aspects of locomotor rhythms in constant environmental conditions (i.e. constant darkness), with testosterone replacement rescuing most of these components (Butler et al., 2012). In an early study, castration (thereby removing circulating testosterone) reduces the total amount of wheel running activity, lengthens the free-running period, and shifts the distribution of activity throughout the day, while testosterone replacement restores normal activity patterns (Daan et al., 1975). In addition, a later study showed that gonadectomy induces a more variable onset of activity rhythms, reduces activity levels, and increases the duration of the activity bout (Butler et al., 2012). Interestingly, dihydrotestosterone replacement rescues these measures, indicating an AR-dependent mechanism of androgen regulation of these aspects of locomotor behavior. Model et al. (2015) sought to identify the SCN as the primary site of action in androgen regulation of circadian locomotor rhythms. To directly target the SCN, they gonadectomized mice and stereotactically implanted testosterone propionate (TP) in the SCN. They found that gonadectomy increased free-running period of wheel running activity to an average of 24.48 h, and the SCN TP implant restored the original average free-running period of 24.03 h. Interestingly, they also found that there was a positive correlation with the amount of decrease in free-running period to the amount of AR expression in the SCN. Additionally, they did not observe this same correlation between free-running period and AR expression in other related brain regions, including the preoptic area, bed nucleus of the stria terminalis, and premammillary nucleus. Overall, these studies implicate that androgen action in the SCN, primarily through the hormone receptor AR, is responsible for regulating aspects of circadian locomotor behavior.

Male reproductive behavior has also been shown to exhibit circadian rhythms. Male rats have shorter time (latencies) to first intromission of the penis into the vagina, and ejaculations, and shorter intervals between these events, during the dark than the light phase of the light: dark cycle (Beach and Levinson, 1949; Stefanick, 1983). Interestingly, research using mice that are lacking the clock gene *Bmal1* has connected the core circadian clock to reproductive behavior (Schoeller et al., 2016). Male *Bmal1* knockout

mice are infertile, and when mating behavior was evaluated, researchers found that the males did not mount receptive females. These males also presented with very little anogenital investigation of receptive females, an important behavior for copulation. This behavior is regulated by a complex neural circuit, and it was found that two brain regions involved in this circuit, the bed nucleus of the stria terminalis and medial preoptic area, were both significantly less active in the knockout males compared to wild-type males. While it is clear that the circadian timekeeping system is involved in regulating male reproductive behavior, there are still many details that remain to be elucidated.

Conclusion

Rhythmic expression of core clock genes and proteins has now been observed in rat, mouse, and hamster testes. Additionally, there is also rhythmic expression of these genes and proteins in additional male reproductive tissues, including the epididymis and prostate gland. Furthermore, there is also rhythmic regulation of testosterone production, as well as enzymes and proteins essential for the production of testosterone. Finally, there are rhythms in sexual behavior that are controlled, in part, by clock genes and testosterone. However, the role of the rhythmic expression of many of these genes and proteins remains to be elucidated. Future work in this field will examine the differential expression of clock genes and proteins across different ages of animals, as well as different tissues. Moreover, future work will identify functions for the expression of clock genes and proteins in the male reproductive tract.

References

- Alvarez, J. D., Chen, D., Storer, E., & Sehgal, A. (2003). Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. *Biology of Reproduction*, 69, 81–91.
- Alvarez, J. D., Hansen, A., Ord, T., Bebas, P., Chappell, P. E., Giebultowicz, J. M., Williams, C., Moss, S., & Sehgal, A. (2008). The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *Journal of Biological Rhythms*, 2, 26–36.
- Baburski, A. Z., Sokanovic, S. J., Bjelic, M. M., Radovic, S. M., Andric, S. A., & Kostic, T. S. (2016). Circadian rhythm of the Leydig cells endocrine function is attenuated during aging. *Experimental Gerontology*, 73, 5–13.
- Beach, F. A., & Levinson, G. (1949). Diurnal variations in the mating behavior of male rats. *Proceedings of the Society for Experimental Biology and Medicine*, 71, 78–80.
- Bebas, P., Goodall, C. P., Majewska, M., Neumann, A., Giebultowicz, J. M., & Chappell, P. E. (2009). Circadian clock and output genes are rhythmically expressed in extratesticular ducts and accessory organs of mice. *The FASEB Journal*, 23, 523–533.
- Bittman, E. L., Doherty, L., Huang, L., & Paroskie, A. (2003). Period gene expression in mouse endocrine tissues. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 285, R561–R569.
- Butler, M. P., Karatsoreos, I. N., LeSauter, J., & Silver, R. (2012). Dose-dependent effects of androgens on the circadian timing system and its response to light. *Endocrinology*, 153, 2344–2352.
- Cheng, S., Liang, X., Wang, Y., Jiang, Z., Liu, Y., Hou, W., Li, S., Zhang, J., & Wang, Z. (2016). The circadian Clock gene regulates acrosin activity of sperm through serine protease inhibitor A3K. *Experimental Biology and Medicine*, 241, 205–215.
- Daan, S., Damassa, D., Pittendrigh, C. S., & Smith, E. R. (1975). An effect of castration and testosterone replacement on a circadian pacemaker in mice (*Mus musculus*). *Proceedings of the National Academy of Sciences of the United States of America*, 72, 3744–3747.
- Jean-Faucher, C., Berger, M., de Turckheim, M., Veyssiere, G., & Jean, C. (1986). Circadian variations in plasma LH and FSH in juvenile and adult male mice. *Hormone Research*, 23, 185–192.
- Karatsoreos, I. N., Butler, M. P., LeSauter, J., & Silver, R. (2011). Androgens modulate structure and function of the suprachiasmatic nucleus brain clock. *Endocrinology*, 152, 1970–1978.
- Karatsoreos, I. N., Wang, A., Sasanian, J., & Silver, R. (2007). A role for androgens in regulating circadian behavior and the suprachiasmatic nucleus. *Endocrinology*, 148, 5487–5495.
- Lucas, L. A., & Eleftheriou, B. E. (1980). Circadian variation in concentrations of testosterone in the plasma of male mice: A difference between BALB/cBy and C57BL/6By inbred strains. *Journal of Endocrinology*, 87, 37–46.
- Miyamoto, Y., & Sancar, A. (1999). Circadian regulation of cryptochrome genes in the mouse. *Molecular Brain Research*, 71, 238–243.
- Model, Z., Butler, M. P., LeSauter, J., & Silver, R. (2015). Suprachiasmatic nucleus as the site of androgen action on circadian rhythms. *Hormones and Behavior*, 73, 1–7.
- Morse, D., Cermakian, N., Brancorsini, S., Parvinen, M., & Sassone-Corsi, P. (2003). No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. *Molecular Endocrinology*, 17, 141–151.
- Schoeller, E. L., Clark, D. D., Dey, S., Cao, N. V., Semaan, S. J., Chao, L. W., Kauffman, A. S., Stowers, L., & Mellon, P. L. (2016). Bmal1 Is Required for Normal Reproductive Behaviors in Male Mice. *Endocrinology*, 157, 4914–4929.
- Stefanick, M. L. (1983). The circadian patterns of spontaneous seminal emission, sexual activity and penile reflexes in the rat. *Physiology and Behavior*, 31, 737–743.
- Taya, K., & Igarashi, M. (1974). Circadian rhythms in serum LH, FSH and prolactin levels in adult male rats. *Endocrinology Japan*, 21, 211–215.
- Wilson, M. J., McMillin, J. M., Seal, U. S., & Ahmed, K. (1976). Circadian variation of serum testosterone in the adult male rat with a late morning acrophase. *Experientia*, 32, 944–945.
- Zylka, M. J., Shearman, L. P., Weaver, D. R., & Reppert, S. M. (June, 1998). Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. *Neuron*, 20, 1103–1110.

Further Reading

- Iwahana, E., Karatsoreos, I., Shibata, S., & Silver, R. (2008). Gonadectomy reveals sex differences in circadian rhythms and suprachiasmatic nucleus androgen receptors in mice. *Hormones and Behavior*, 53, 422–430.

Circannual Rhythms

Vinod Kumar and Ila Mishra, University of Delhi, Delhi, India

© 2018 Elsevier Inc. All rights reserved.

Glossary

Amplitude Difference of maximum or minimum from the mean value of a biological oscillation.

Biological rhythm Repeated cycles of a biological function at relatively stable interval.

Circadian rhythm Endogenous biological rhythm with period length of about 24 h.

Circannual rhythm Endogenous biological rhythm with period length of about 1 year.

Clock gene Gene involved in the transcription-translation feedback loop producing near 24 h time.

Free-running rhythm Endogenous rhythm repeats with its natural period under constant conditions.

Free-running period The time interval between consecutive recurrences of the phase marker (e.g., the time of peak or trough) of a free-running rhythm.

Phase An instantaneous point (particular reference point) within the biological rhythm (e.g., onset, mid point or end of activity).

Period The time interval after which a particular phase recurs during the complete biological rhythm.

Entrainment The process which enables endogenous rhythm to assume the period length of the external (environmental) periodicity, e.g., 24 h light-dark cycle or annual changes in the photoperiod cycle.

Synchronization The state in which endogenous and exogenous (environmental) periodicities assume same period length; i.e., both run with same frequency.

Introduction

Circannual rhythms (Latin: *circa* = about; *annum* = year) are endogenously generated oscillations in biological processes with period length of approximately 1 year (12 months) and time changes in the physiology and behavior within each year in long-lived animals. Corresponding to this are circadian rhythms (Latin: *circa* = about; *diem* = day), which are endogenously generated biological functions with period length of approximately 24 h time changes within each day. Among animals, there are many different examples of circannual rhythms; the most notable ones include the insect pupation rhythms, animal migration patterns, pelage growth, hibernation, reproduction and molt cycles. Hence, circannual rhythms are adaptive, and have been evolved to ensure the success of the long-term biological functions, since mistiming of these with the seasonal world will have serious fitness consequences. For example, organisms reproduce during the period of optimal food availability, which is directly responsible for the offspring survival. The life-span of Siberian chipmunks (*Eutamias sibirium*) is affected by their ability and inability to undergo hibernation: those who undergo hibernation cycles live about 11 years, and those failing to do so, live a reduced life-span of about 3 years (Kondo et al., 2006). Overall, circannual timing mechanisms appear to be universal. As we know now that humans themselves are no exception; we are far more a seasonal species than has previously been assumed (Stevenson et al., 2015). Studies have also revealed that basic mechanisms of the circannual rhythmicity are present in the primate lineage; the ancestral circannual clocks continue to tick in us in still unexplored ways, and significantly affect contemporary human life (Stevenson et al., 2015; Helm and Lincoln, 2017).

Naturalists have long speculated endogenous timing mechanism on yearly time scale in the control of biological events. At the beginning of eighteenth century, Ferdinand J. A. von Pernau, a pioneer student of bird behavior, suggested that migratory birds were "driven at the proper time by a hidden drive". Early in nineteenth century, Arnold A. Berthold suggested that the emergence from hibernation in spring was due to the action of endogenous timing factors, and not a simple response to the prevailing environmental stimulus. About a century later, William Rowan argued for the involvement of an internal factor, i.e., a physiological rhythm, in migratory birds that breed in the northern hemisphere, and winter around equator or in the southern hemisphere. In 1960, A. B. Misra suggested, "... the internal rhythm, guided and controlled by genetical factors and fixed by natural selection is the supreme governor of recurrent reproductive physiology of the birds". Both, naturalists and farmers have noted timely return of migratory great knots (*Calidris tenuirostris*) year-after-year, and in few instances this record worked as an agricultural calendar (Foster and Kreitzman, 2009).

Most evidence for the existence of endogenous circannual rhythms, as consolidated by experimental demonstrations in several species, are consistent with the hypothesis that annual timing is based on self-sustained circannual clock(s) which interacts with the environmental photoperiodic zeitgeber (German: *zeit* = time, *geber* = giver; Gwinner, 1986; Numata and Helm, 2014). Few taxonomic groups like insects, birds and hibernating mammals, in particular, captured human interest for detailed scientific investigations over the last about six decades, with seminal and extensive works of Eberhard Gwinner and Eric T. Pongelley on birds and mammals, respectively. An early evidence for circannual rhythm in mammals came from golden-mantled ground squirrel

(*Callospermophilus lateralis*), which displayed circannual cycles in hibernation, food consumption and body mass under constant light and ambient temperature conditions (Pengelley and Fisher, 1963). Strong evidence for avian circannual rhythms came from Ebo Gwinner's studies; the rhythms in migratory restlessness (*Zugunruhe*, demonstrated by intense night activity and wing whirring in night migrants) and molt persisted in captivity for 3 years, suggesting an endogenous regulation of annual migration in willow warblers (*Phylloscopus trochilus*; Gwinner, 1986).

Formal Properties, Stability, and Entrainment

Formal Properties

Circannual rhythms are self-sustained endogenously driven biological oscillations that persist with a regular periodicity of about 12 months in the absence of environmental cycles. Hence, a rhythmic process cannot be designated as 'circannual rhythm' unless it has been experimentally observed to persist in the organism under condition that provides no information about its period. Hence, the constant darkness (DD) or constant dim light (dimLL) can be considered as constant environment condition for circannual rhythm demonstration. Also, 24 h light-dark (LD) cycle (e.g., 12 L:12D) with an unchanging photorefraction contains no information about the year, and so it can be considered as a constant condition for circannual rhythms. Circannual rhythms are temperature compensated: cycle lengths do not vary significantly with change in the constant temperature environment. Overall, circannual rhythms share all fundamental properties with those of the circadian rhythms (Gwinner, 1986; Helm and Lincoln, 2017).

Irving Zucker (2001) reviewed annual rhythms in mammals and distinguished them in three categories, types I, II, and III (Fig. 1). Common among short-lived temperate and boreal species, type I rhythms, although with an endogenous component, do not persist beyond 1 cycle under constant conditions; hence, they are contingent on periodic environmental input. Type II circannual rhythms are found in long-lived species like rodents, carnivores, ungulates, bats and primates. These are endogenous and persist with cycle length of ~12 months under constant conditions; hence, they are not contingent on periodic environmental input. Type III rhythms occur almost with regular periodicity in response to the environmental periodicities; hence, these rhythms do not have an endogenous component, e.g., seasonal allergies in reaction to production of pollens. By-and-large, our present discussion includes types I and II rhythms, which are displayed by different groups, particularly in controlling seasonal reproduction.

Stability and Lability

The period length (Greek: tau, τ) of the free-running circannual rhythm deviates from a year, usually shorter than 12 months. Measured as intervals between two successive peaks in testis growth, circannual testicular rhythm in spotted munia (*Lonchura punctulata*) was found to have $\tau = \sim 11$ months under both constant photoperiod, LL or alternate 24 h of LL/DD at $\sim 20^\circ\text{C}$ temperature (Budki et al., 2012). Circannual reproductive rhythm shows period stability in, as evidenced by similar periods in the circannual testicular cycle in spotted munia under LL at 22- and 90-lx light intensities (cf. Budki et al., 2012, 2014; Fig. 2). Similarly, the average body mass in hibernating ground squirrels (*Citellus lateralis*) cycle with $\tau = 11.3$ and 10.9 months under high ($34 \pm 1^\circ\text{C}$) and low ($5 \pm 1^\circ\text{C}$) temperatures, respectively (Gwinner, 1986). Also, Siberian chipmunks (*Eutamias sibiricus*) maintain precise circannual rhythms of core body temperature for up to a decade both in continuous cold and darkness, although with τ ranging from 5 to 13 months, and hibernation period ranging from 2 to 8 months (Kondo et al., 2006). However, circannual cycles can differ between sexes. Whereas male spotted munia cycle with $\tau = \sim 11$ months (see above), females under same light conditions exhibit circannual cycles with relatively large period variations, $\tau = 10\text{--}13$ months (cf. Budki et al., 2012, 2014). Also, male Siberian chipmunks end their hibernation quite early in the spring when there is still sufficient snow cover, but females emerge from hibernaculum much later in the season when food resources become readily available (Florant and Healy, 2012). Furthermore, individuals can exhibit independent circannual phenotypes, as suggested by differences in the period length, hence frequency, between various circannual functions. For example, molt cycles are more frequent and widely scattered in the year than the gonadal cycles in spotted munia under LL (Budki et al., 2012, 2014; Fig. 3).

Entrainment

Circannual rhythms are synchronized (or "entrained") to local time by appropriate phase shifts, and may involve transients. ($\tau = T$; T is cycle length of an environmental *zeitgeber*, e.g., annual LD cycle). The range of entrainment is broader, and so an entraining LD cycle may vary in T from c. 6–15 months, i.e., $\sim 50\%\text{--}30\%$ of τ of the endogenous circannual rhythm (Gwinner, 1986). Apart from T , the light intensity can influence the synchronization, particularly the inter-individual synchrony. LL at 90 lx reduced variation and increased synchrony between circannual cycles of gonadal maturation and molt among individuals of a group of spotted munia, as compared to those in LL at 22 lx (cf. Budki et al., 2012, 2014).

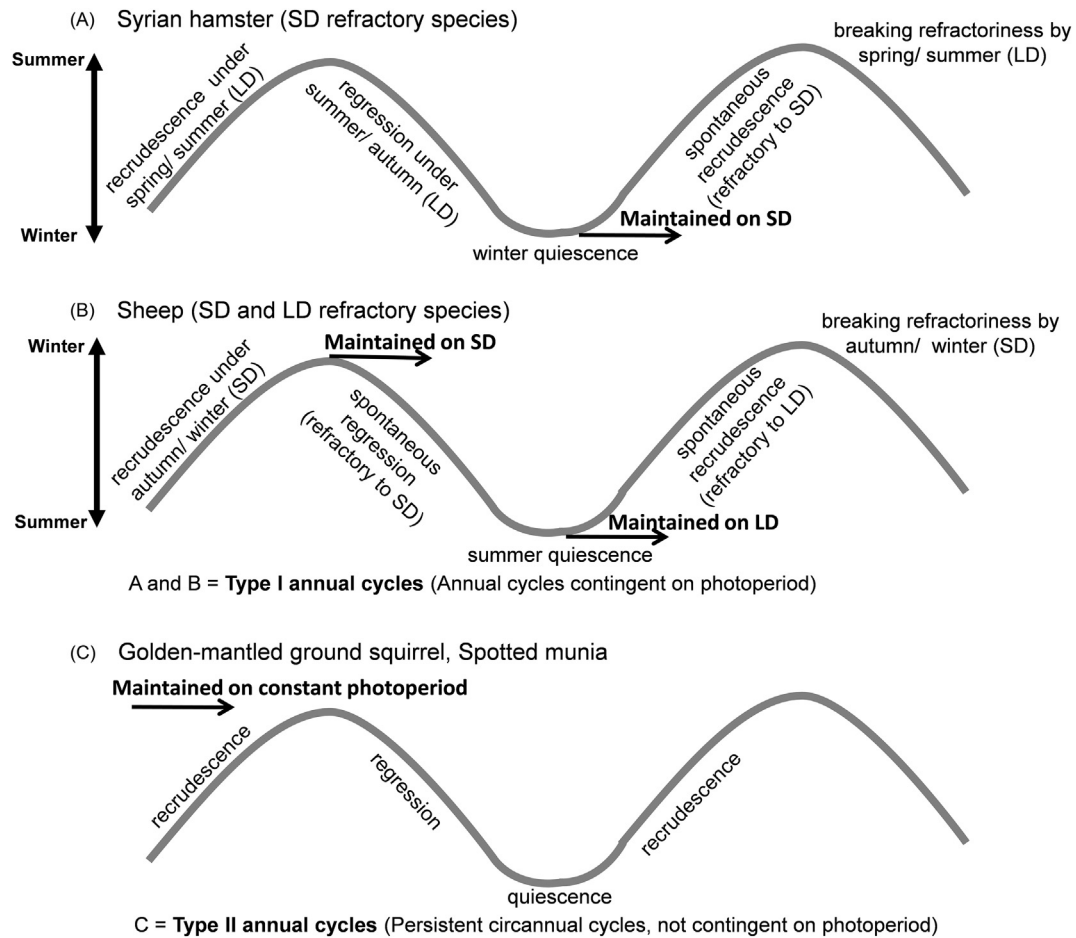


Fig. 1 Schematic representation of the annual reproductive cycle types. Upper 2 panels: Type I rhythms in which environmental photoperiod is the primary zeitgeber in the regulation and synchronization of annual gonadal cycles. (A): Syrian hamsters (*Mesocricetus auratus*) show gonadal recrudescence and regression under long and short days, and spontaneously regrow their testes under prolonged short day exposure; hence they exhibit refractoriness to the inhibitory effects of the short day, SD. (B): Sheep (*Ovis* sp.) shows refractoriness to both, the stimulatory effects of SD as well as inhibitory effects of LD. Hence, there is the recrudescence and spontaneous regression of gonads under SD; converse is true under LD. A photoperiod change is therefore required to break the refractoriness. (C): Type II rhythms are endogenous cycles and persist under constant photoperiod and constant light/darkness. There seems no particular day length requirement to induce the gonadal recrudescence—regression cycle. Drawn based on Zucker (2001).

Circannual Rhythms and Reproduction

Circannual rhythms temporally space out seasonal phenologies in the year such that a species can profitably utilize resources available in its surroundings and survive through the unfavorable conditions, if any. Reproduction, in particular, is timed to occur during the year when feeding resources are optimal and ensure the survival of newborns. This necessitates that physiological processes underlying reproduction (e.g., pair formation, as required; gonadal maturation; mating; incubation/gestation) are initiated, terminated and reinitiated within a specified temporal window in the year, with a great time precision. Hence, species have evolved and are genetically programmed with an endogenous circannual timekeeping system, which in interaction with prevailing environment helps individuals (or population) anticipate the most favorable time in the year. Experimental studies support this. The black caps (*Sylvia atricapilla*) from Cape Verde breed twice and from central Europe breed once a year, and they continue to show two and single circannual cycles in testis maturation, respectively, even when placed under constant artificial 13 h light per day (Helm and Lincoln, 2017). Similar trans-generational persistence of circannual gonadal and molt cycles has been demonstrated in equatorial African stonechats (*Saxicola torquatus axillaris*): both hand-raised and their offspring displayed similar circannual cycles under constant photoperiod conditions (Gwinner, 1996). Thus, circannual rhythm generation and photoperiodism (environmental photoperiod times component events of a seasonal process) may not be mutually exclusive and, in fact, might interact closely, albeit in species-specific manner, as per the adaptive needs.

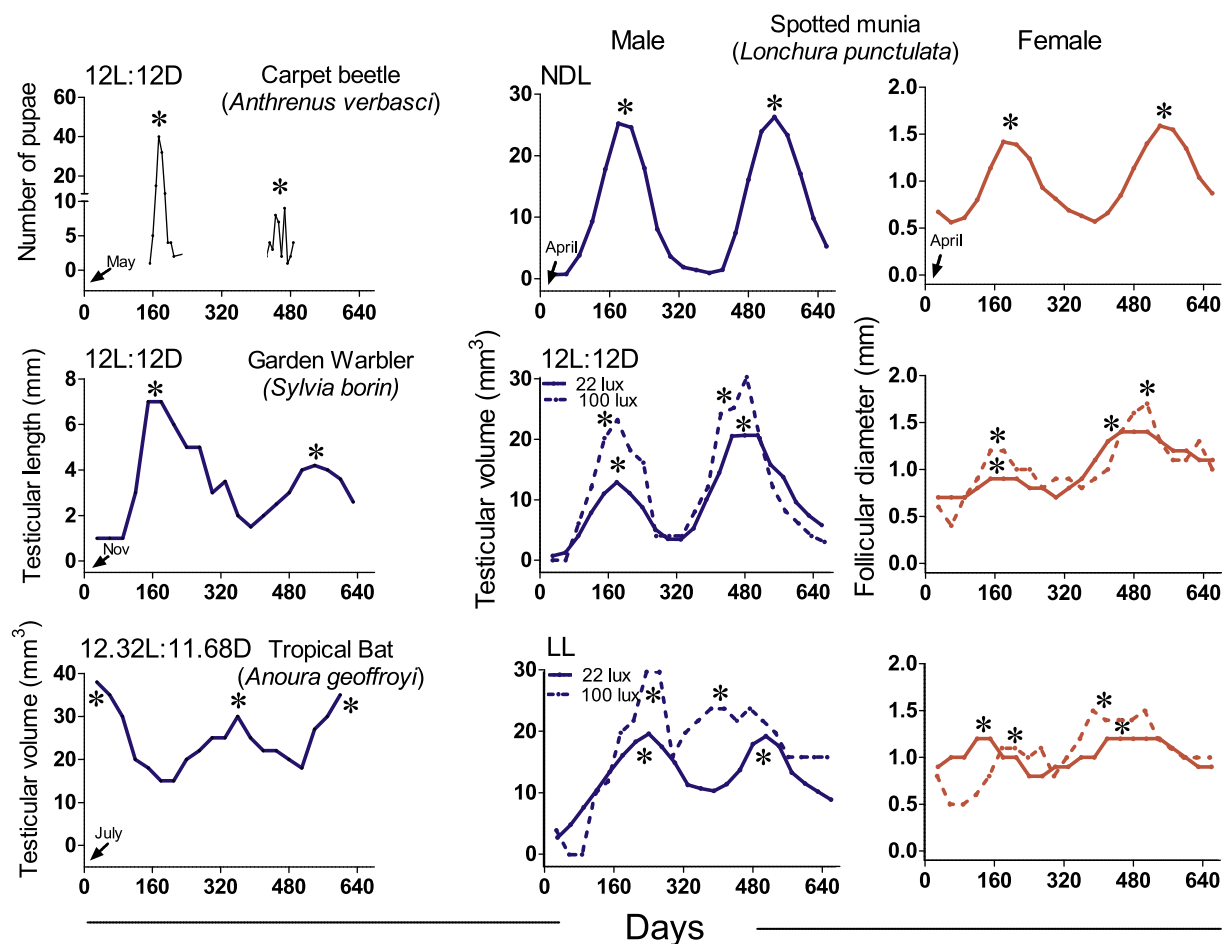


Fig. 2 Circannual reproductive cycles under constant photoperiods. Left panel: circannual cycles in the pupation in Carpet beetles (*Anthrenus verbasci*; 4-stage life cycle holometabolous insect), and in the testicular growth-regression in garden warblers (*Sylvia borin*; an avian migrant) and tropical bat (*Anoura geoffroyi*) under equinox, or near equinox, photoperiod (12L:12D, 12.32L:11.68D). Right panels: circannual gonadal growth-regression cycles in male and female spotted munia (*Lonchura punctulata*) under natural day length (NDL), and under 12L:12D or LL at 22- and 90-lx light intensities. Asterisks indicate the time of peak gonadal growth. Note light intensity dependent modulation of the circannual gonadal cycles in the spotted munia. Drawn based on Gwinner (1986), Heidemann and Bronson (1994), Budki et al. (2012, 2014), and Miyazaki et al. (2014).

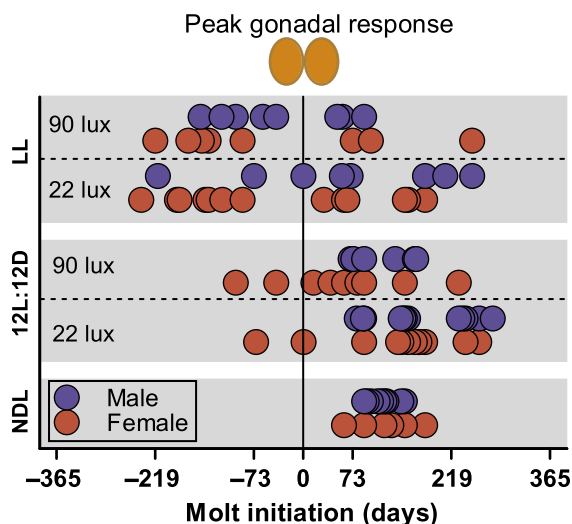


Fig. 3 Relationship of molt with reproductive cycle in spotted munia (*Lonchura punctulata*). The time of initiation of wing primaries molt of in the first circannual cycle plotted in relation to peak gonadal size (indicated by time '0' value on x-axis) under natural day length, and equinox photoperiod (12L:12D) or constant light (LL) at 22- and 90-lx intensities. The time scale on x-axis expands for 365 days on each side of '0', with minus (–) and plus (+) signs indicating the onset of molt prior to, and after the peak gonadal response (time 0), respectively. Blue and red circles represent data for individual male and female birds, respectively. Note light intensity dependent effects under 12L:12D and LL. Drawn based on Budki et al. (2012, 2014)

Insects

Most insect species have life cycle shorter than a year, and thus do not seem to be in need of the circannual timing. But, holometabolous insect like carpet beetles (*Anthrenus verbasci*) with 4-stage life cycle of ≥ 2 years utilize circannual timing for seasonal events. Adult beetles emerge, mate and lay eggs from spring to early summers, depending on the climate and nutritional conditions. Under constant temperature and darkness, the pupation and development of *A. verbasci* exhibits a circannual rhythm with $\tau = \sim 40$ weeks in both British and Japanese populations (Miyazaki et al., 2014). The rhythms were temperature compensated, although larvae pupated earlier at high (25°C) and later at low (15°C) temperatures. Light period also affected the circannual pupation rhythm, as shown by a clear rhythm under 12L:12D and complete arrhythmicity under 15L: 9D (Miyazaki et al., 2014). Similarly, circannual cycles persist in oviposition and larvae pupation over 5 years in ants (*Formica aquilina*, *F. polyctena*), although with huge individual differences in the cycle length, to some extent affected by the prevailing photoperiod and temperature (Miyazaki et al., 2014).

Birds

Circannual rhythms have been demonstrated in gonad development and “postnuptial” molt in several birds, with pioneering investigations of Ebo Gwinner (see Gwinner, 1986). The longest record of circannual cycles of gonad development and molt over 15 years is from African stonechats under 12.25L:11.75D (see Gwinner, 1996). In the most constant year-round environment of equatorial rainforest, chestnut-winged babblers (*Stachyris erythroptera*) and little spider hunter (*Arachnothera longirostris*) show circannual cycles in breeding and molt with a period length of about 9 months (see Gwinner, 1986). Similarly, subtropical spotted munia (*Lonchura punctulata*) show recurrent circannual cycles in gonadal maturation and molt under different light conditions, including LD cycles and LL (Figs. 2 and 3; Budki et al., 2014). A circannual cycle in reproduction is not the feature restricted to equatorial or tropical species. Temperate species also show circannual cycles in gonadal maturation. European starlings (*Sturnus vulgaris*) show recurrent testicular cycles under 12L:12D (Gwinner, 1986). Similarly, migratory dark-eyed juncos (*Junco hyemalis*) show circannual cycles in both testes and migratory phenotypes (e.g. spring pre-migratory body fattening, Zugunruhe) under dim LL of 1–3 lx (Rani and Kumar, 2013).

Mammals

Circannual rhythms have been shown in short and long day breeding mammals, such as sheep (*Ovis* sp.), sika deer (*Cervus nippon*), bats (*Antrozous pallidus*, *Anoura geoffroyi*, *Phyllostomus hastatus*) and rodents (Syrian hamster, *Mesocricetus auratus*; Siberian hamster, *Phodopus sungorus*; European hamster, *Cricetus cricetus*) (Heidemann and Bronson, 1994; Lincoln and Hazlerigg, 2014; Numata and Helm, 2014; Helm and Lincoln, 2017; Figs. 1 and 2), although with subtle differences in features, as compared to those found in birds (see above). Sheep, a short day breeder, undergoes circannual cycles of reproduction (gonadal maturation, luteinizing hormone and prolactin secretions) and molt under constant conditions. Sika deers display circannual cycles in the testicular growth, antler replacement, and coat color and molt under constant photoperiods. Circannual rhythms persist in gonad development cycle and pelage color in rodents, including squirrels, hamsters and mice. Circannual cycles of body mass and estrous freerun golden-mantled ground squirrels under $T = 23, 24$ or 25 h LD cycles, in which they synchronized circadian activity rhythms (Zucker, 2001). Similarly, small vesper mice (*Calomys laucha*), a south American rodent, show circannual cycles in pelage color (orange and light summer pelage, and gray and dark winter pelage) under 12L:12D at 22°C (Camargo et al., 2006).

Circannual rhythms have also been suggested operating in biological functions of humans including the reproduction-associated measures, albeit with inconsistencies between findings. Human birth pulses can fluctuate seasonally with an amplitude of about 10%, which is substantial for a species that effectively performs physiological functions under conditions of an eternal summer maintained within the body 37°C (the core body temperature). The amplitude of seasonal rhythms indicated by the timing of maxima can vary with the latitude, suggesting an underlying physiological regulatory mechanism. The pattern of birth pulses appears to have a longer-term relevance because birth-month correlates with life-long impacts on health, including the likelihood of general, psychiatric or neurological illnesses (Stevenson et al., 2015). An 8-year study of the semen analysis from human volunteers found a statistically significant high-amplitude seasonal variation in the sperm count (not ejaculate volume or percent motile sperms), with highest values in late winter and early spring, and lowest values in the late summer (Saint Pol et al., 1989). Furthermore, both the presence and absence of circannual cycles in circulating testosterone levels have been reported in humans (Smith et al., 2013).

Inter- and Intra-Specific Differences

Among species that do show circannual rhythms, all individuals may not show them, and the cycle length can also vary considerably from 12 months even among individuals that show them. Circannual cycles of gonadal maturation and pelage growth or molt can also differ between sexes. Unlike males, female European starlings lack a robust circannual response, and exhibit less clear rhythms with larger variations in the period and frequency of cycles in plasma LH and prolactin levels (Dawson, 2007). Also, circannual ovarian cycles, as shown by follicular maturation, were less pronounced and had larger variations than the testes in spotted munia (Budki et al., 2012; Fig. 2). These suggest sex-dependent timing strategies, with females appearing to share a greater role in defining

the reproductive season in relation with the environment. Different circannual rhythms can vary within an individual: cycles of “postnuptial” molt get out of phase with the gonadal cycle in spotted munia under LL (Fig. 3; Rani and Kumar, 2013).

Circannual Rhythms and Photoperiodism

In nature, various environmental factors, most reliably the changes in photoperiod, synchronize the timing of circannual clock-mediated physiological processes to occur at a particular time during the year, which is best-suited for survival and adaptability. Hence, organism-specific experimental strategies are manifested in circannual cycles. Circannual cycles freerun in several birds (e.g., spotted munia, dark-eyed juncos) under constant photoperiod or LL, but these require a photoperiod change at a particular phase of the cycle to express in many mammals (e.g., sheep, sika deer, hamsters; Fig. 1). European starlings held under constant photoperiods exceeding 12.5L:11.5D or < 11.5L:12.5D fail to exhibit circannual cycles. Furthermore, artificial simulations of annual photoperiod variation can entrain circannual cycles to periods as short as 4 months and as long as 2 years. Photoperiodic entrainment can also vary between populations. For example, post juvenile molt in the long distance migrant population of Siberian stonechats (*Saxicola torquata maura*) is earlier and faster than in the short distance migrating European stonechats (*S.t. rubicola*); molt is further slower and late in the nonmigrating east-African stonechats (*S.t. axillaris*).

Circannual Timing: Putative Mechanisms

Va. Endogenous clocks: Several line of evidence show that circadian pacemaker system (CPS) is not involved in the circannual rhythm generation. The ablation of the suprachiasmatic nuclei (SCN), which contains CPS in mammals, eliminates circadian rhythms in activity, but not the circannual cycles of body mass and reproductive phenotypes in golden-mantled ground squirrels (Zucker, 2001). Sika deers show circannual cycles under LD cycles (4.94 L:4.94D, 6L:6D, 8L:8D and 21L:21D) which fail to induce the circadian entrainment (Helm and Lincoln, 2017). Similarly, spotted munia show free-running circannual rhythms in gonad development and molt under LL which induces circadian rhythm disruption both at the behavioral and circadian gene transcriptional levels (Budki et al., 2014; our unpublished data). Thus, a biological year is not defined by 360-odd circadian days. CPS could still have modulatory role in the annual timing, as shown by alteration in the long-term timing and structure of circannual hibernation cycle in SCN ablated squirrels (see Zucker, 2001). Daily oscillations of core clock genes (*PERIODS*, *PERs*; *CRYPTOCHROMES*, *CRYs*; *BRAIN AND MUSCLE ARNT LIKE PROTEIN 1*, *BMAL1*; *CIRCADIAN LOCOMOTOR OUTPUT CYCLES KAPUT*, *CLOCK*) also show alterations between short and long days, and between seasonal states in photoperiodic birds (Cassone and Yoshimura, 2015; Singh et al., 2015).

Thyroid Hormone (TH) Responsive Hypothalamic Pathways

Highly conserved TH-responsive molecular mechanism mediates the photostimulation of gonadal growth and development in both short- and long-day species (Cassone and Yoshimura, 2015; Fig. 4). It begins with concurrent activation of the *EYA3* (eye absent 3) and *TSH-beta* (thyroid stimulating hormone-beta) genes in pars tuberalis (PT) thyrotrophs in response to stimulatory photoperiod, as perceived by brain photopigments (e.g., neuropsin) in birds and by CPS-controlled duration of night melatonin secretion in mammals (Fig. 4). TSH (product of *TSH-alpha* and *TSH-beta*) from PT thyrotrophs activates and suppresses transcription of genes coding for type 2 and 3 deiodinases (*DIO2* and *DIO3*), respectively, in tanycytes (ependymal cells lining the third ventricle). *DIO2* and *DIO3* mediate conversion of thyroxine (T4) into biologically active T3 (tri-iodothyronine) and inactive rT3 (reverse T3) forms, respectively. T3 regulates GnRH (gonadotropin releasing hormone) synthesis and/or release from the preoptic area into median eminence, and consequently the pituitary gonadotropins secretion and gonadal response (Fig. 4). TH signaling also plays a role in the seasonal timing of hibernation in golden-mantled ground squirrels. Recently, chromogranin (CHGA, a hormone-packaging molecule) has been identified as the robust marker of short photoperiod in sheep. Wood et al. (2015) report binary switching of PT thyrotrophs: they exist in CHGA+ form under short-day breeding state, TSH-beta/*EYA3*+ form under long-day nonbreeding state, and spontaneously switch from TSH-beta/*EYA3*+ to CHGA+ state under the long-day refractory state (Fig. 4). Thus, apart from TH signaling, PT seems to drive long-term circannual cycles with individual cells undergoing recapitulation of the developmental state. It remains to be tested however if TH-responsive mechanism is key to the circannual rhythm generation in a nonphotoperiodic species; our initial studies on spotted munia fail to this, however.

The Other Hypothalamic Nuclei/Candidate Molecules

Recent researches have implicated various other hypothalamic structures and candidate molecules in the circannual timekeeping. For example, ventromedial and lateral hypothalamus (VMH, LH), arcuate nucleus (ARC), paraventricular nucleus (PVN) and dorsomedial hypothalamus (DMH) play roles in the seasonal regulation of feeding and energy balance. In hibernators, ARC regulates body mass cycles, and mediates the torpor; T3 acts in ARC and PVN as a low energy signal during fasting (Florant and Healy, 2012). In chipmunks, hibernation-specific proteins (HP) complex concentration in blood closely correlates with the circannual hibernation rhythm, with down regulation of HP prior to the onset of hibernation (Kondo et al., 2006). VGF (nonacronymic) is

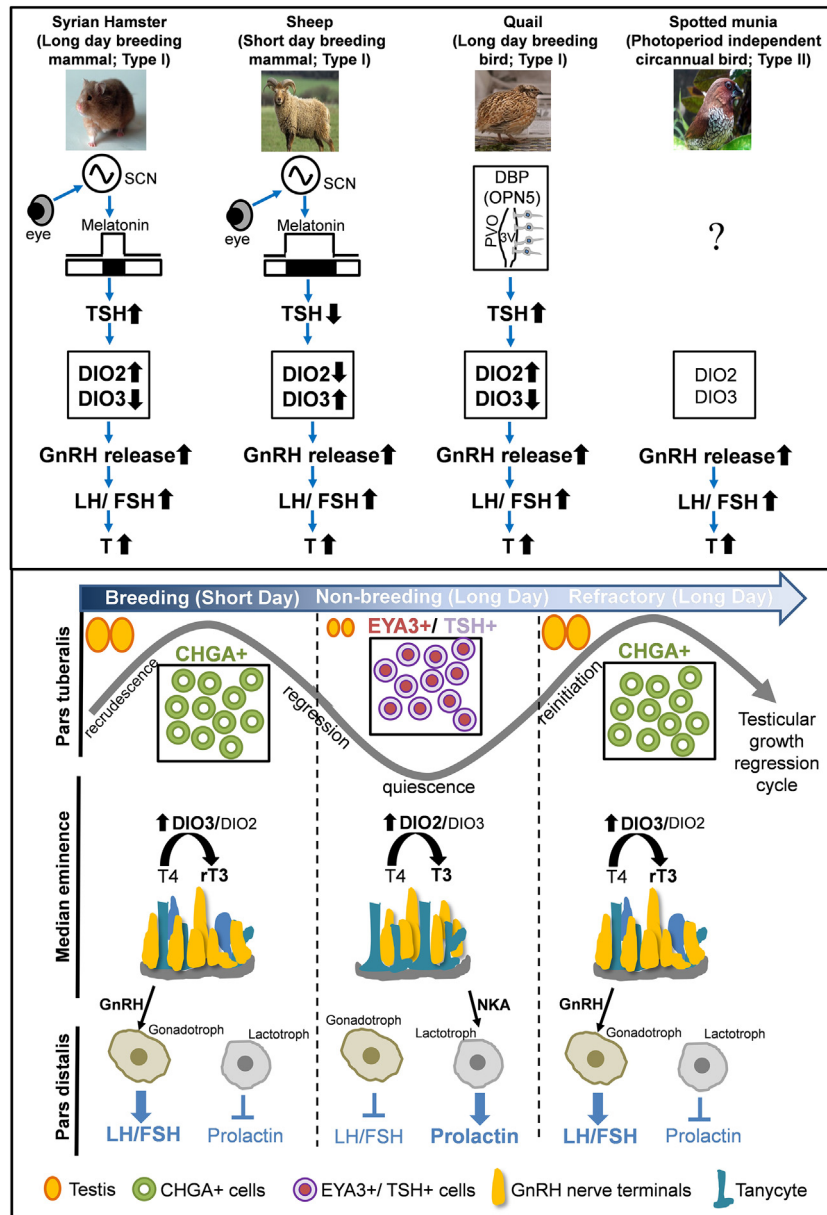


Fig. 4 Mechanisms for seasonal timing of gonad development. Upper half: Changes in the HPG (hypothalamic-pituitary-gonadal) axis involved in the regulation of type I and type II annual reproductive cycles. In type I annual reproductive cycles, the relay of light information in mammals is from the retina via SCN activity-dependent melatonin secretion, while in birds light is perceived through the deep brain photoreceptor photopigments, e.g., neuropsin, OPN5. PT-thyrotroph derived TSH activates DIO2 expression in response to long days in both summer and winter breeders. Although increased synthesis/ release of GnRH (gonadotropins releasing hormone) and pituitary gonadotropins cause gonad development in species that exemplify type II annual reproductive cycles, how is light information decoded and transduced upstream is not well understood, as yet. However, we found no difference in *DIO2* and *DIO3* mRNA expressions in mediobasal hypothalamus of a strong circannual species, the spotted munia (*Lonchura punctulata*; unpubl. Obs.). Up and down arrows indicate high and low expression levels, respectively. Lower half: A proposed model for circannual timekeeping in sheep. Individual pituitary pars tuberalis (PT) thyrotrophs exist in the binary state. The relative proportion of these binary state cells determines the phase of the circannual cycle (breeding: CHGA+, Chromogranin A positive; non-breeding: EYA3+/TSH+, eye absent 3/thyroid stimulating hormone positive). In SD breeding and LD refractory states (left and right panels), there is an increase in the deiodinase type 3 (DIO3) expression in the ependymal cells, the tanycytes lining the 3rd ventricle. DIO3 mediates the conversion of thyroxine (T4) into its inactive form (triiodothyronine, rT3), and hence reduces local concentration of its active, T3. As a consequence, the tanycyte end feet retract, and eliminate the physical barrier for GnRH secretion. In the nonbreeding LD state (middle panel), PT-thyrotroph derived TSH activates DIO2 expression and hence enhances T3. As a consequence occur the neuronal remodeling and the encasement of neuronal synapses by tanycyte end feet. These suggest a physical mechanism for the control of GnRH secretion, and downstream gonadotropin release and gonadal response. PT thyrotrophs thus operate as a calendar cell, and generate long-term neuroendocrine rhythms in both the hypothalamus and pituitary gland. Schematically drawn based on Cassone and Yoshimura (2015) and Wood et al. (2015).

another most abundantly expressed molecule in the hypothalamus, and involved in the energy balance and reproduction. VGF mRNA is induced in the ARC, in both Syrian and Siberian hamsters transferred from long to short days (Barrett et al., 2005). VGF also plays a role in the synaptic plasticity, and seem important for the reduction of synaptic connectivity in the song control system, as required post-breeding in songbirds and during torpor bouts in hibernating mammals (Schwartz and Andrews, 2013). Hypothalamic VGF might play a role in the seasonal switch from hyperphagic behavior in summer and early fall to the hypophagic behavior during the hibernation (Schwartz and Andrews, 2013). In hibernators like thirteen-lined ground squirrels (*Spermophilus tridecemlineatus*), brain VGF expression is increased in October when there is decreased food consumption and increased white adipose stores, and is the onset of shallow torpor bouts (Schwartz and Andrews, 2013).

Emerging Concepts in Circannual Rhythm Research

At the present time, three concepts seem emerging to explain the annual timing. Recent studies on seasonally breeding sheep suggest pituitary PT as the putative site of the circannual rhythm generation (see above). It remains inconclusive if PT acts as the circannual pacemaker, as does the SCN as the circadian pacemaker; the autonomous switching in PT genes may be governed by still an undefined structure/ mechanism. A second concept revolves around the involvement of circadian timing in seasonal processes, at least in photoperiodic species with a robust self-sustained circadian system. This is supported by recent demonstrations of alteration in the expression level, 24 h expression patterns, and 24 h rhythm waveforms (phase, amplitude) in the hypothalamic mRNA oscillations of core clock genes, and of genes involved in the photoperiodic transduction and neurosteroid dependent processes, in parallel with seasonal changes in the physiology and behavior (e.g., reproduction, migration) in migratory blackheaded buntings (*Emberiza melanocephala*; Singh et al., 2015; Mishra et al., 2017). The third concept underlies that circannual cycles are generated by tissue-autonomous histogenesis, which includes the formation of a complex tissue system from undifferentiated cells, as a result of complex developmental processes such as the cell differentiation, tissue remodeling and feedback signaling between and within tissues (Lincoln and Hazlerigg, 2014). Exclusive demonstration for this is still lacking, however.

Perhaps an answer to the question whether annual timing of temporally spaced seasonal behaviors has a common regulatory site, as is the SCN for the regulation of many daily responses, is key to finding the circannual pacemaker. Else, circannual timing might include independent phase-related seasonal processes? Perhaps, uncovering genes and protein pathways underlying the induction and maintenance, and the termination and reinitiation of seasonal response (e.g., molecular genetics of migratory or seasonally breeding birds) might enable us to understand the mechanism of circannual rhythm generation. Current advances in molecular tools, and its usage in studying the non-model organisms have greatly improved and should enhance our ability to understand where and how are the circannual rhythms generated.

Acknowledgements

Our research has been generously supported by grants to VK from the Science and Engineering Research Board, New Delhi (through IRHPA center for excellence grant) and Department of Biotechnology (DBT), New Delhi, India.

References

- Barrett, P., Ross, A. W., Balik, A., et al. (2005). Photoperiodic regulation of histamine H3 receptor and VGF messenger ribonucleic acid in the arcuate nucleus of the Siberian hamster. *Endocrinology*, 146, 1930–1939.
- Budki, P., Malik, S., Rani, S., & Kumar, V. (2014). Circadian rhythms are not involved in the regulation of circannual reproductive cycles in a sub-tropical bird, the spotted munia. *Journal of Experimental Biology*, 217, 2569–2579.
- Budki, P., Rani, S., & Kumar, V. (2012). Persistence of circannual rhythms under constant periodic and aperiodic light conditions: sex differences and relationship with the external environment. *Journal of Experimental Biology*, 215, 3774–3785.
- Camargo, C. R., Colares, E., & Castrucci, A. M. L. (2006). Seasonal pelage color change: News based on a South American rodent. *Anais da Academia Brasileira de Ciências*, 78, 77–86.
- Cassone, V. M., & Yoshimura, T. (2015). Circannual cycles and photoperiodism. In C. G. Scanes (Ed.), *Sturkie's avian Physiology* (6th edn., pp. 829–844). Amsterdam: Elsevier (Academic Press).
- Dawson, A. (2007). Seasonality in a temperate zone bird can be entrained by near equatorial photoperiods. *Proceedings of Royal Society Series B Biological Sciences*, 274, 721–725.
- Florant, G. L., & Healy, J. E. (2012). The regulation of food intake in mammalian hibernators: A review. *Journal of Comparative Physiology B*, 182, 451–467.
- Foster, R. G., & Kreitzman, L. (2009). *Seasons of life: The biological rhythms that enable living things to thrive and survive*. New Haven, CT: Yale University Press.
- Gwinner, E. (1986). *Circannual rhythms*. Heidelberg, Berlin: Springer.
- Gwinner, E. (1996). Circannual clocks in avian reproduction and migration. *Ibis*, 138, 47–63.
- Heidemann, P., & Bronson, F. H. (1994). An endogenous circannual rhythm of reproduction in a tropical bat, *Anoura geoffroyi*, is not entrained by photoperiod. *Biology of Reproduction*, 50, 607–614.
- Helm, B., & Lincoln, G. A. (2017). Circannual rhythms anticipate the Earth's annual periodicity. In V. Kumar (Ed.), *Biological timekeeping: Clocks, rhythms and behavior* (pp. 545–570). New Delhi: Springer.
- Kondo, N., Sekijima, T., Kondo, J., et al. (2006). Circannual control of hibernation by HP complex in the brain. *Cell*, 125, 161–172.
- Lincoln, G. A., & Hazlerigg, D. (2014). Stem cell regulation of circannual rhythms. In H. Numata, & B. Helm (Eds.), *Annual, lunar, and tidal clocks* (pp. 227–246). Tokyo, Heidelberg, New York, Dordrecht, London: Springer.

- Mishra, I., Singh, D., & Kumar, V. (2017). Seasonal alterations in the daily rhythms in hypothalamic expression of genes involved in the photoperiodic transduction and neurosteroid-dependent processes in migratory blackheaded buntings. *Journal of Neuroendocrinology*. <https://doi.org/10.1111/jne.12469>.
- Miyazaki, Y., Nisimura, T., & Numata, H. (2014). Circannual rhythms in insects. In H. Numata, & B. Helm (Eds.), *Annual, lunar, and tidal clocks* (pp. 333–350). Tokyo, Heidelberg, New York, Dordrecht, London: Springer.
- Numata, H., & Helm, B. (Eds.). (2014). *Annual, lunar, and tidal clocks*. Tokyo, Heidelberg, New York, Dordrecht, London: Springer.
- Pengelly, E. T., & Fisher, K. C. (1963). The effect of temperature and photoperiod on the yearly hibernating behavior of captive golden-mantled ground squirrels (*Citellus lateralis tesseorum*). *Canadian Journal of Zoology*, 41, 1103–1120.
- Rani, S., & Kumar, V. (2013). Avian circannual systems: Persistence and sex differences. *General and Comparative Endocrinology*, 190, 61–67.
- Saint Pol, P., Beuscart, R., Leroy-Martin, B., Hermand, E., & Jablonski, W. (1989). Circannual rhythms of sperm parameters of fertile men. *Fertility and Sterility*, 51, 1030–1033.
- Schwartz, C., & Andrews, M. T. (2013). Circannual transitions in gene expression: Lessons from seasonal adaptations. *Current Topics in Developmental Biology*, 105, 247–273.
- Singh, D., Trivedi, A. K., Rani, S., Panda, S., & Kumar, V. (2015). Circadian timing in central and peripheral tissues in a migratory songbird: Dependence on annual life-history states. *The FASEB Journal*, 29, 4248–4255.
- Smith, R. P., Coward, R. M., Kovac, J. R., & Lipshultz, L. I. (2013). The evidence for seasonal variations of testosterone in men. *Maturitas*, 74, 208–212.
- Stevenson, T. J., Visser, M. E., Arnold, W., et al. (2015). Disrupted seasonal biology impacts health, food security and ecosystems. *Proceedings of Royal Society B*. <https://doi.org/10.1098/rspb.2015.1453>.
- Wood, S. H., Christian, H. C., Miedzinska, K., et al. (2015). Binary switching of calendar cells in the pituitary defines the phase of the circannual cycle in mammals. *Current Biology*, 25, 2651–2662.
- Zucker, I. (2001). Circannual rhythms: Mammals. In J. S. Takahashi, F. W. Turek, & R. Y. Moore (Eds.), *Circadian Clocks: Vol. 12. Handbook of behavioral neurobiology* (pp. 509–528). New York: Kluwer Academic, Plenum Publishers.

Energetics of the Male Reproduction

Marco G Alves, University of Porto, Porto, Portugal

Branca M Silva, University of Beira Interior, Covilhã, Portugal

Pedro F Oliveira, University of Porto, Porto, Portugal

Luís Rato, University of Beira Interior, Covilhã, Portugal

© 2018 Elsevier Inc. All rights reserved.

Introduction

The formation of spermatozoa is an intricate process maintained through a complex network of both peripheral and local interactions. Several testicular cells are involved in this process that occurs in the seminiferous tubules and depends of a close interaction between those cells, particularly germ cells and Sertoli cells (SCs). Germ cells develop in a particular environment, isolated and protected by the blood-testis barrier (BTB) that is formed by adjacent SCs. The BTB divides the seminiferous epithelium into two compartments, the basolateral and the adluminal, which allow the developing germ cells to uptake different nutrients and energetic substrates. Germ cells at the basolateral compartment have access to substrates present in peripheral circulation, whilst secluded germ cells at adluminal compartment are completely dependent on metabolites resulting from SCs secretion (Rato et al., 2012). This partly explains why the metabolic phenotype of germ cells varies according to their differentiation state.

Spermatogenesis is a high-energy dependent process in which germ cells are constantly nourished to meet the energy requirements for several processes, including biosynthetic activities, otherwise, they degenerate. This topic was overlooked for a long time, but the relevance of testicular cells metabolism has gained interest from basic to clinical professionals due to its relevance to male fertility. The link between metabolism and reproductive function is extremely susceptible to insults, such as those promoted by external factors or diseases. It is generally accepted that both, our lifestyle and the surrounding environment, play a key role on that link. So there is an urgent need to find new possibilities that may reduce or even counteract the deleterious effects of those threats to the reproductive health of males. In this context, testicular metabolism has emerged as a possible site for therapeutic intervention. Indeed, cell metabolism has become a subject of renewal interest in the last years, possibly aided by the novel biochemical and molecular tools that allow an in-depth study of the metabolic pathways in cells and organs. It is expected that in the next years the emerging field of “omics” provide new opportunities and advances in our understanding of the testicular metabolic pathways and how they can be manipulated at the clinical level. In this chapter, we will present a brief overview of the main metabolic pathways occurring in the testis, with special emphasis on the metabolic cooperation established between SCs and germ cells, as well as the main pathways fueling sperm metabolism. Finally, it will be also discussed how the metabolic cues induced by metabolic diseases change the energetics of testicular tissue and may compromise male fertility.

Metabolic Cooperation in Testis Is a Key Event for a Normal Spermatogenesis

In the seminiferous tubules it occurs a cellular metabolic cooperation between SCs and germ cells. The latter are highly dependent on the physical and nutritional support of the former. SCs, also known as “nurse” cells, do not follow the same metabolic pattern of most normal cells and only use a minor part of glucose to face their own energy requirements. The majority of this sugar is converted to lactate and delivered to the developing germ cells. This metabolic cooperation is vital for male fertility and depends on the proper functioning of several metabolic pathways controlled by a complex network of hormones and endogenous factors that are mostly secreted by germ cells. Adjacent SCs are responsible for the formation of the blood-testis barrier (BTB), dividing the seminiferous epithelium into the basal and adluminal compartments, and allowing the development of germ cells in an adequate environment. The pH and ionic composition of the tubular fluid, as well the selective passage of substrates, is under the control of SCs, mainly through specific solute carriers located at the surface of plasma membrane. For instance, solute carrier family 2, also known as glucose transporters (GLUTs) is a family consisting of 14 members that mediate the facilitated diffusion of glucose. Of these members, SCs express glucose transporter 1 (GLUT1), glucose transporter 2 (GLUT2), glucose transporter 3 (GLUT3) and glucose transporter 4 (GLUT4), which are mainly involved in the uptake of extracellular glucose. Glucose transporter 8 (GLUT8) is also present in SCs but is only involved in the transport of glucose between cellular compartments.

Once inside the cells, glucose undergoes a series of multistep reactions catalyzed by several enzymes where phosphofructokinase (PFK) catalyzes a rate-limiting reaction converting fructose-6-phosphate to fructose-1,6-bisphosphate in an irreversible manner. The final product of glycolysis is pyruvate, which may follow three different pathways: (1) being converted into alanine, by alanine aminotransferase (ALT); (2) it can be converted into acetyl-CoA in mitochondria and enter the Krebs cycle; or (3) it can be converted to lactate by lactate dehydrogenase (LDH), with a concomitant oxidation of nicotinamide adenine dinucleotide (NADH) into NAD^+ . The resulting lactate is then preferentially exported through monocarboxylate transporters (MCTs) to the existing space between SCs and germ cells. From all the 14 members of the MCTs family, monocarboxylate transporter 1 (MCT1) and monocarboxylate transporter 4 (MCT4) are expressed by SCs, where MCT4 mainly acts as lactate exporter due to its low affinity for this metabolite (Fig. 1) (Oliveira et al., 2015). Not all germ cells are dependent on lactate supply by SCs. In fact, spermatogenic cells express several glycolytic enzymes, but only those located closest to the basement membrane of the seminiferous tubules are

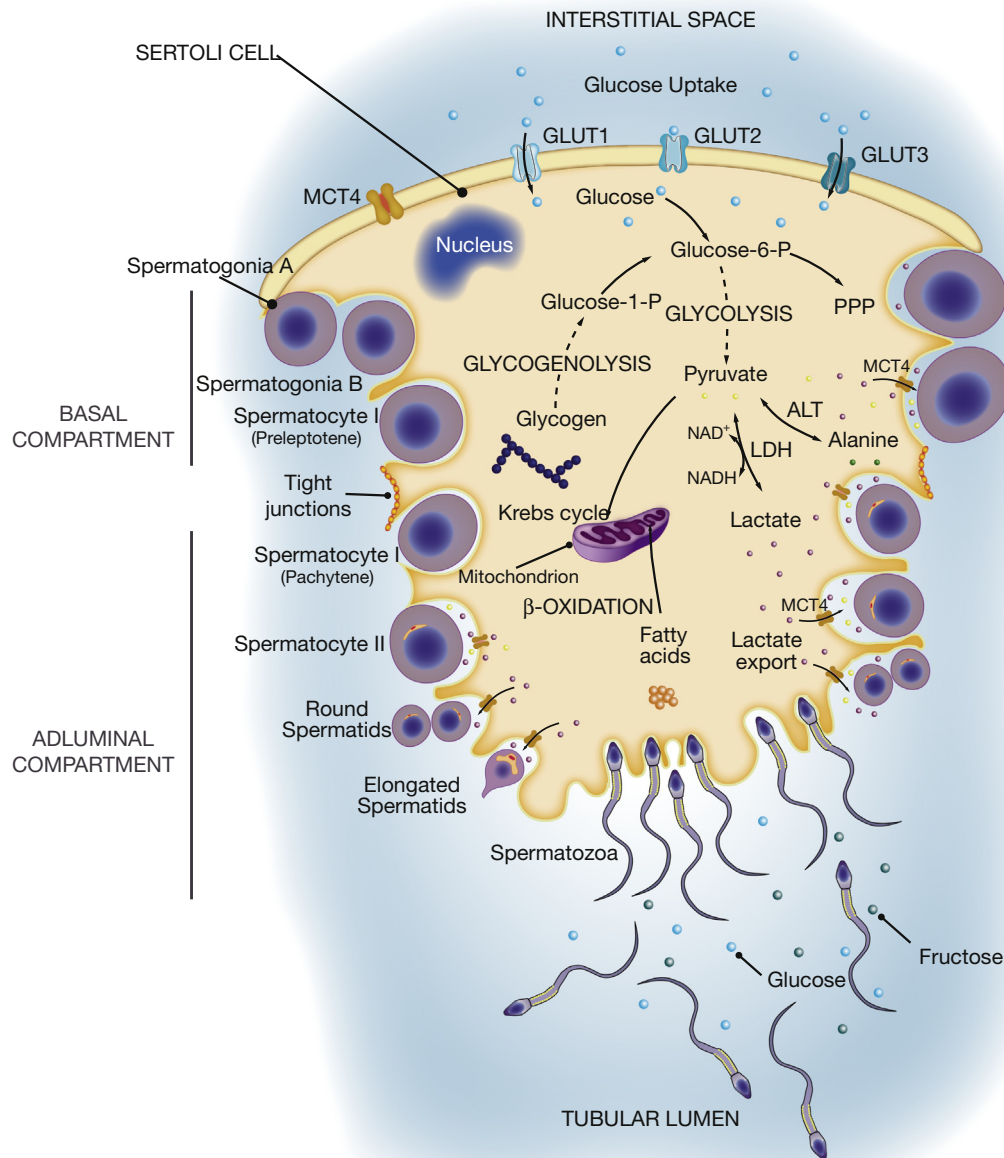


Fig. 1 Schematic illustration of the metabolic cooperation between the Sertoli cells (SCs) and developing germ cells. The SCs consume several substrates, such as glucose, lactate and fatty acids. Though glycolytic pathway, glucose is metabolized into pyruvate which can (i) be converted into Acetyl-CoA, which can enter the Krebs cycle; (ii) be converted into alanine via alanine transaminase or (iii) be converted into lactate via lactate dehydrogenase (LDH). The extracellular lactate and pyruvate are transported through the family of proton-linked plasma membrane transporters known as the monocarboxylate transporters (MCTs), while glucose is imported via the family of membrane proteins called glucose transporters (GLUTs). SCs can maintain their viability in the complete absence of glucose, still producing energy and lactate via β -oxidation of fatty acids. SCs also accumulate glycogen that can be readily hydrolyzed when glucose is not available and/or when its metabolism is deficient. In the lumen of the seminiferous tubules spermatozoa rely majorly in the glucose and fructose available in the fluid. *GLUT1*, glucose transporter 1; *GLUT2*, glucose transporter 2; *GLUT3*, glucose transporter 3; *ALT*, alanine aminotransferase; *LDH*, Lactate dehydrogenase; *MCT4*, monocarboxylate transporter 4; *PPP*, pentose phosphate pathway.

able to use glucose, whereas germ cells located beyond BTB cannot metabolize this sugar. For instance, pachytene spermatocytes rely on the supply of lactate from SCs. Lactate is also the preferred substrate of early spermatids and the production of energy in those cells is more efficient in the presence of high concentrations of this metabolite, illustrating their lower glycolytic potential (Bajpai et al., 1998). Despite the presence of all molecular machinery necessary for the metabolism of glucose, the high dependency of the secluded germ cells in lactate may be caused by inefficient oxygen diffusion through the epithelium but this hypothesis needs further investigation.

Lactate is more than an energy substrate to germ cells. It stimulates the synthesis of ribonucleic acid and protein in developing germ cells, but also helps to promote the activation of alternative metabolic pathways, such as gluconeogenesis and the pentose-phosphate pathway (PPP) since may be “recycled” to glucose-6-phosphate. In addition, lactate is a relevant factor for the survival of germ cells since the administration of exogenous lactate in adult cryptorchid rat testis was shown to suppress the loss of spermatocytes and spermatids (Courtens and Ploen, 1999). This was later observed in humans, since the treatment of seminiferous tubules with lactate prevented the apoptosis of germ cells, especially spermatocytes and spermatids (Erkkila et al., 2002).

Besides lactate, acetate is also produced at high rates by SCs. Acetate is a crossroad metabolite and is converted to acetyl-CoA either in the cytoplasm or in mitochondrial matrix by acetyl-CoA synthase. It is a central metabolite for the synthesis of cholesterol and other precursors of lipids. Thus, it has been proposed that acetate is essential for the maintenance of the high rate of lipids synthesis and the intense remodeling of the germ cells’ membranes during their development (Alves et al., 2012). However, lipids are also used by mature SCs as an energy substrate. These cells degrade residual bodies, as well as phagocytized apoptotic spermatogenic cells, which enables the recycling of lipids to produce ATP (Fig. 1). β -oxidation of fatty acids is the preferred pathway to guarantee their own energy requirements, which may explain why SCs maintain the synthesis of ATP and their viability even when glucose is absent (Xiong et al., 2009). However, the metabolism of aminoacids must not be excluded, since the enzyme branched-chain aminoacid aminotransferase, responsible for the conversion of the branched-chain aminoacids (such as valine, leucine, and isoleucine) to the corresponding branched-chain acids was identified in SCs (Kaiser et al., 2005). Apart from the role of these alternative substrates in the metabolism of SCs, endogenous substrates such as glycogen may also have huge relevance in the maintenance of SCs high glycolytic activity.

Metabolic Requirements of Spermatozoa

After being released in the lumen of the seminiferous tubules, spermatozoa become independent from the nutritional support of SCs. They will begin their journey towards the epididymis and pass through maturation in a series of events occurring in a synchronized manner. As they pass through epididymis, spermatozoa must fuel all molecular events taking place either in the cytoplasm as in the surface of the membrane. Even within the female reproductive tract, spermatozoa must ensure efficient energy production to successfully achieve acrosome reaction and capacitation, otherwise, fertilization will not occur. Unlike secluded germ cells, spermatozoa are able to maintain their energy levels through glycolysis, but also through oxidative phosphorylation (OXPHOS) (Fig. 2). In fact, both processes may occur independently or in combination depending on the surrounding environment. This is possible due to the highly specialized structure of spermatozoa, which allows the compartmentalization of energy production. The ATP generated through OXPHOS occurs in mitochondria, while the synthesis of ATP through glycolysis takes place in the head and the fibrous sheath, due to the absence of respiratory enzymes. Several glycolytic enzymes, such as hexokinase, PFK, LDH and glyceraldehyde-3-phosphate dehydrogenase (GAPD) are present in these segments of spermatozoa. The preferred substrates of spermatozoa are glucose and fructose. These hexoses are taken up through GLUTs present along the plasma membrane. GLUT1 and GLUT2 are expressed in the acrosomal region and in both main and end pieces of the flagellum, while GLUT3 has been described in the mid-piece of human spermatozoa (Angulo et al., 1998). Other members, such as GLUT8 can be found in the acrosomal region, post-acrosomal region, and flagellum. The presence of glucose transporter 9 (GLUT9) was reported in the acrosome, midpiece and main piece of the mouse sperm. It is interesting to observe that all GLUTs isoforms are mainly distributed in the head, particularly in the acrosomal region and in several parts of the tail (Kim and Moley, 2007), showing that those events preceded by oocyte fertilization may depend on the glucose-derived ATP. Indeed, glucose alone is sufficient to support the protein tyrosine phosphorylation events associated with spermatozoa capacitation. From all GLUTs isoforms expressed by spermatozoa, glucose transporter 5, is the one with higher affinity for fructose and is confined to the subequatorial region of the spermatozoa, midpiece and main piece. Glucose taken up from the seminal fluid is metabolized originating two molecules of ATP, pyruvate, and nicotinamide adenine nucleotide (NADH). Pyruvate is then further metabolized to produce lactate, which can also be used as energetic substrate. In the same way, fructose is internalized and phosphorylated by fructokinase to fructose-1-phosphate, which undergoes hydrolysis by fructose-1-phosphate aldolase yielding dihydroxyacetone phosphate (DHAP) and glyceraldehyde. DHAP is converted to glyceraldehyde-3-phosphate by triosephosphate isomerase or glyceraldehyde kinase. From this point, intermediates of the glycolytic pathway are obtained and can finally be converted to pyruvate and lactate for energy production. It is interesting that spermatozoa possess all molecular machinery involved in energy metabolism to support the sliding flagellar movements under oxygen deprivation. However, the use of metabolic substrates for ATP production may vary between species and consequently may dictate the main pathway required for ATP synthesis. When the availability of glycolytic substrates is scarce, the energy production is ensured by mitochondria. It is known that the most efficient energy process occurs under aerobic conditions and in these conditions pyruvate is irreversibly converted to acetyl-CoA by pyruvate dehydrogenase, which then enters the Krebs cycle to combine with oxaloacetate (OAA). OAA is then further oxidized to reduce NAD^+ and the complex II prosthetic group flavin adenine dinucleotide generating carbon dioxide. The oxidation of these substrates is coupled with the phosphorylation of adenosine diphosphate via the mitochondrial electron transport chain, with the subsequent production of ATP. These are the two main metabolic pathways in sperm metabolism, however, an exacerbated or deficient mitochondrial function is often associated with the production of reactive oxygen species (ROS). ROS are extremely dangerous for the viability of spermatozoa and their overproduction have been recognized as a major cause of male infertility. Spermatozoa are poor on antioxidant defenses, so these cells are dependent on the surrounding antioxidant systems.

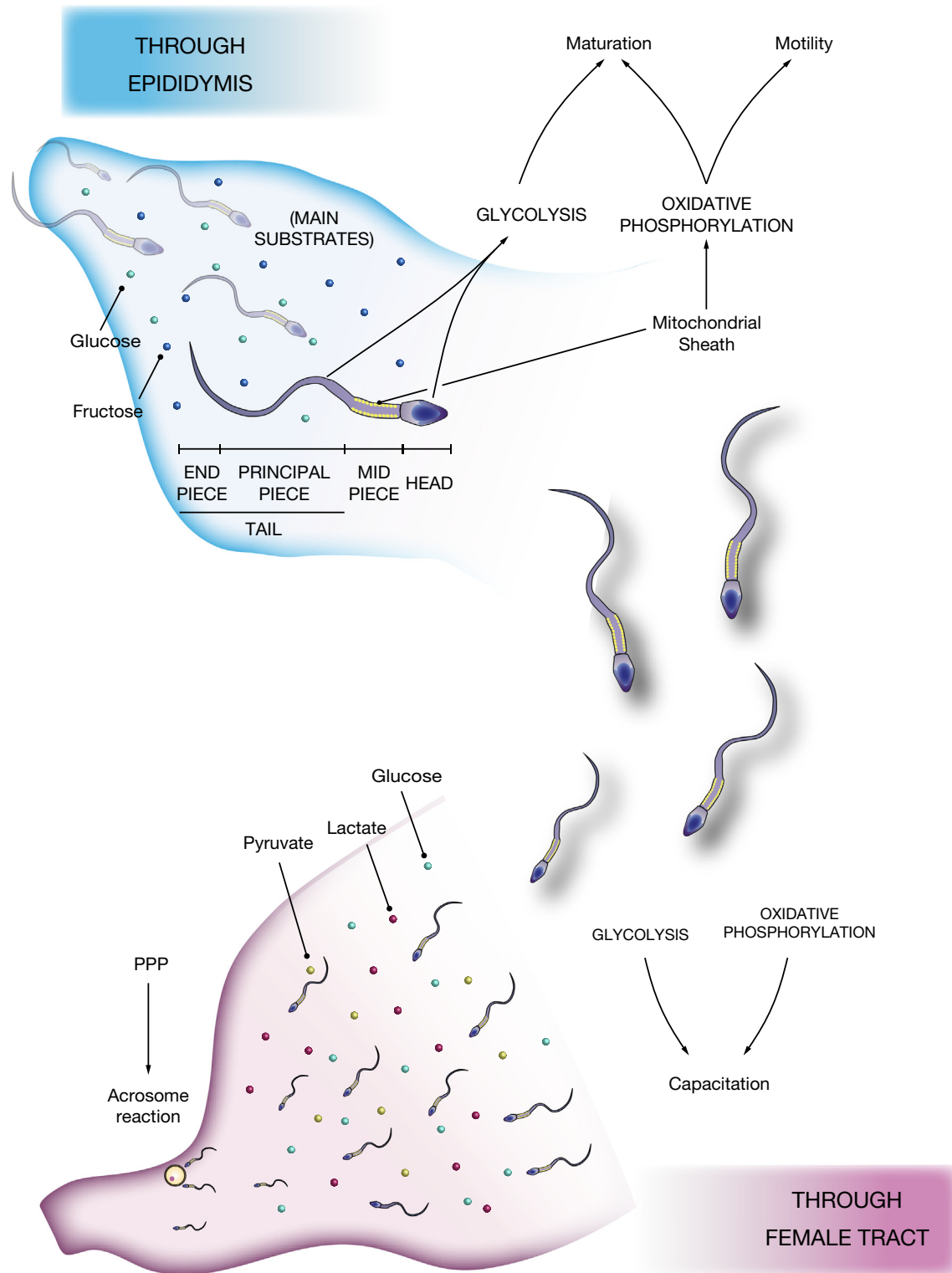


Fig. 2 Schematic illustration of the main metabolic pathways involved in the sperm functions. These cells rely on glucose and fructose available in the fluid of reproductive male tract, however they can also to obtain energy by using lactate, pyruvate and other substrates (e.g. citrate, fatty acids and aminoacids). The major pathways of sperm metabolism are glycolysis and oxidative phosphorylation which are essential to fuel all energy requirements of the maturation-associated events. In the reproductive female tract sperm rely in high concentration of glucose, lactate and pyruvate. Glycolysis is the predominant pathway for fertilization, since is used for sperm capacitation and during acrosome reaction, but this may vary between species. Glucose is also important to the pentose-phosphate pathway that is involved in the sperm entry into the oocyte. When substrates for glycolysis are absent, sperm use other substrates to obtain energy through oxidative phosphorylation. *PPP*, pentose phosphate pathway.

When other energy sources are absent, spermatozoa may also use intracellular lipid reserves. Acyl ester bonds of plasmalogen are broken during endogenous respiration resulting “in the release” of fatty acids, which can then be used as a substrate for endogenous respiration (Misro and Ramya, 2012). Glycerol can also be used by spermatozoa and may be converted to fructose and then metabolized via fructolysis. Sperm is also able to oxidize aminoacids. Another pathway that is often used by sperm during fertilization is PPP. Indeed, this is consistent with the increased activity of the glucose-6-phosphate dehydrogenase (G-6-PD) observed in human spermatozoa. G-6-PD is a rate-limiting enzyme essential for the production of reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH), which is involved in the activation of NADPH-oxidase. NADPH-oxidase produces radical species, which at low levels promote sperm capacitation, a process that occurs in the female reproductive tract (Travis et al., 2001). Interestingly, the oviductal fluid is highly rich in pyruvate and lactate, which are commonly used as energy substrates by mammalian spermatozoa. In fact, these metabolites may serve as a link between glycolysis and OXPHOS. As aforementioned, lactate derives from the enzymatic reduction of pyruvate by LDH with a concomitant production of NAD^+ . Under anaerobic conditions, this reaction regenerates cytosolic NAD^+ necessary for the progress of the glycolytic pathway. Cytosolic lactate can also be transported into mitochondria of spermatozoa by the mitochondrial lactate carrier, where the presence of the specific isoform of lactate dehydrogenase C reoxidizes lactate to pyruvate in the mitochondrial matrix.

Finally, it is interesting to note that sperm accumulates glycogen and has measurable activities of glycogen synthase and glycogen phosphorylase. However, the real impact of glycogen in the metabolism of spermatozoa needs to be fully explored (Ballester et al., 2000). Mammalian spermatozoa are able to use a variety of substrates allowing energy production independently from mitochondrial activity, thus allowing fertilization possible under divergent conditions.

Metabolic Cues and Male (in)Fertility: From Basic Research to Clinical Considerations

There has never been a greater need to understand the real relevance of testicular metabolism to male reproductive function. This process is complex and any dysregulation in metabolic homeostasis may negatively impact reproductive performance (Rato et al., 2012). Such effects are in part mediated by the endocrine system, which compromises testicular signaling pathways, especially those related to the metabolic cooperation occurring in the seminiferous epithelium. Even a subtle alteration in the consumption of substrates and/or in the production of metabolites may result in a deleterious effect for male fertility. This is alarming considering the epidemic numbers of men suffering from metabolic diseases and it may be expected a decline in male fertility in the upcoming years. Male fertility is extremely susceptible to external insults or diseases and policymakers and health authorities have recognized it as one of the hidden effects of metabolic diseases, particularly diabetes mellitus (DM) and obesity (Rato et al., 2014).

DM induces large hormonal fluctuations, as well as metabolic imbalance directly affecting the function of mature SCs. Glucose metabolism is altered in insulin-deprived SCs, mainly due to the modulation of GLUT1 and GLUT3 expression. Changes in the uptake of glucose may be reflected in the glycolytic potential of SCs. Indeed, insulin-deprived SCs present decreased lactate production due to downregulation of LDH and MCT4 expression (Oliveira et al., 2012). This was the first report showing how absence of insulin directly modulates SCs metabolism. Apart from insulin dysfunction, DM also induces severe alterations in the synthesis of sex steroids. SCs exposed to doses of testosterone (T) and 17β -estradiol occurring in prediabetic males did not uptake glucose as efficiently as compared to SCs exposed to sex steroids levels observed in type 2 diabetes mellitus (T2DM) (Rato et al., 2015). These findings suggest that alterations of T levels induced by progressive stages of DM alters the metabolism of SCs. Importantly, it was clearly evidenced that the most remarkable metabolic alterations of the SCs were concurrent with the severity degree of the disease. Although the testicular dysfunctions associated with DM go beyond the inhibition of sex steroid synthesis, this may be a key mechanism by which DM affects male fertility. There has been an increasing interest in what regards to the development of a possible strategy to counteract the deleterious effects of DM in these metabolic pathways. Indeed, anti-diabetic drugs, such as metformin (Alves et al., 2014) and pioglitazone (Meneses et al., 2016), are able to increase the glycolytic efficiency of human SCs, providing new evidence of how those drugs can be suitable for male diabetics in order to sustain the nutritional control of spermatogenesis.

Apart from the in vitro approaches, in vivo evidence explored how progressive stages of DM impact testicular metabolism. Prediabetes induced by overconsumption of a high-energy diet increased testicular glycolytic activity and the expression of all key intermediates of this pathway was upregulated, even those involved in the production of lactate such as LDH and MCT4. This evidence came from a prediabetic animal model and shows that testicular metabolism is vulnerable to the effects of the prodromal stage of DM (Rato et al., 2013). Nonetheless, the high glycolytic flux seems to be accompanied by a pro-oxidant environment, where the function of the axis composed by peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC1- α) and sirtuin 3 (SIRT3) is compromised (Rato et al., 2014). PGC1- α and SIRT3 are nuclear-encoded proteins with a key role in the maintenance of mitochondrial function, as well in the activation of antioxidant defenses. Together with the disrupted PGC1- α /SIRT3 axis, a dysregulation of mitochondrial respiration was noted being responsible for a decrease in testicular ATP content. So the hormonal and metabolic fluctuations occurred in the initial stages of DM development compromises normal testicular bioenergetics with the development of a deleterious pro-oxidative environment (Rato et al., 2014). However, as DM progresses to T2DM some of these alterations, especially in the testicular metabolism, tend to become more pronounced. In a T2DM animal model, testicular glycolytic flux is severely compromised as observed by the decreased lactate content and these alterations were associated with a remarkable decrease of sperm quality (Rato et al., 2015).

Similar results have also been observed in diabetic men. Recently the metabolic profile of testicular biopsies from type 1 diabetes mellitus (T1DM) men was established (Alves et al., 2015). The testicular tissue of those individuals showed a decreased content of

both lactate and alanine, which may be caused by impaired uptake of glucose, when compared with normoglycemic men. The expression of LDH and ALT was found to be decreased in the testes of these individuals, contributing to the lower testicular lactate content (Alves et al., 2015). Importantly, the decreased protein levels of ALT together with the decreased alanine content suggest that glycolysis-derived pyruvate is probably being redirected into mitochondria. These findings are consistent with the low intratesticular levels of citrate, an intermediate of the Krebs cycle (Alves et al., 2015). Until now there has never been a detailed study exploring the testicular metabolic profile of T1DM men and thus, that study opened some new insights into the molecular mechanism by which DM promotes subfertility/infertility in humans.

Concluding Remarks and Future Perspectives

The biochemical link established between metabolism and male fertility has been a matter of debate. Male fertility requires an adequate nutritional support of spermatogenesis. In this context, the metabolic cooperation established between SCs and germ cells is pivotal and any dysregulation in this crosstalk may end-up in anomalies in sperm production and thus, male infertility. This process is permanently targeted by hormones and external factors and is vulnerable to stress conditions. Malnutrition behaviors resulting in extreme deprivation of nutrients or excessive energy accumulation must be avoided otherwise the normal flow of testicular metabolic pathways is hampered. Metabolic diseases have also become a matter of great concern since they may be key players in the increased rates of male infertility. The whole hormonal and metabolic imbalance often associated with these diseases disrupts male reproduction at multiple levels, but especially the metabolic cooperation SCs:germ cells. There is no doubt that testicular metabolism is a point of control for spermatogenesis, so exploring these pathways provide new opportunities to allow clarifying how testicular metabolic disruption associated with pathological states end up in infertility. Potential threats for male reproductive health are encountered not only by the modern lifestyle (and consequent development of metabolic diseases such as prediabetes, T2DM, and obesity) but also through permanent exposure to environmental factors, which certainly hamper the metabolism of testicular cells and therefore compromise male fertility. These issues will deserve special attention from researchers and clinicians in the following decades.

Acknowledgment

This work was supported by the “Fundação para a Ciência e a Tecnologia”—FCT co-funded by Fundo Europeu de Desenvolvimento Regional—FEDER via Programa Operacional Factores de Competitividade COMPETE/QREN to UMIB (Pest OE/SAU/UI0215/2014); POCI—COMPETE 2020—Operational Programme Competitiveness and Internationalization in Axis I—Strengthening research, technological development and innovation (Project No. 007491) and National Funds by FCT—Foundation for Science and Technology (Project UID/Multi/00709); PF Oliveira (PTDC/BBB-BQB/1368/2014 and IFCT2015), MG Alves (PTDC/BIM-MET/4712/2014 and IFCT2015), L Rato was funded by Santander-Totta/University of Beira Interior (BIPD/ICI-CICS-BST-UBI).

References

- Alves, M. G., Martins, A. D., Moreira, P. I., et al. (2015). Metabolic fingerprints in testicular biopsies from type 1 diabetic patients. *Cell and Tissue Research*, 362, 431–440.
- Alves, M. G., Martins, A. D., Vaz, C. V., et al. (2014). Metformin and male reproduction: Effects on Sertoli cell metabolism. *British Journal of Pharmacology*, 171, 1033–1042.
- Alves, M. G., Socorro, S., Silva, J., et al. (2012). In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17beta-estradiol and suppressed by insulin deprivation. *Biochimica et Biophysica Acta-Molecular Cell Research*, 1823, 1389–1394.
- Angulo, C., Rauch, M. C., Droppelmann, A., et al. (1998). Hexose transporter expression and function in mammalian spermatozoa: Cellular localization and transport of hexoses and vitamin C. *Journal of Cellular Biochemistry*, 71, 189–203.
- Bajpai, M., Gupta, G., & Setty, B. (1998). Changes in carbohydrate metabolism of testicular germ cells during meiosis in the rat. *European Journal of Endocrinology*, 138, 322–327.
- Ballester, J., Fernandez-Novell, J. M., Rutilant, J., et al. (2000). Evidence for a functional glycogen metabolism in mature mammalian spermatozoa. *Molecular Reproduction and Development*, 56, 207–219.
- Courtens, J. L., & Ploen, L. (1999). Improvement of spermatogenesis in adult cryptorchid rat testis by intratesticular infusion of lactate. *Biology of Reproduction*, 61, 154–161.
- Erkkila, K., Aito, H., Aalto, K., et al. (2002). Lactate inhibits germ cell apoptosis in the human testis. *Molecular Human Reproduction*, 8, 109–117.
- Kaiser, G. R., Monteiro, S. C., Gelain, D. P., et al. (2005). Metabolism of amino acids by cultured rat Sertoli cells. *Metabolism, Clinical and Experimental*, 54, 515–521.
- Kim, S. T., & Moley, K. H. (2007). The expression of GLUT8, GLUT9a, and GLUT9b in the mouse testis and sperm. *Reproductive Sciences*, 14, 445–455.
- Meneses, M. J., Bernardino, R. L., Sa, R., et al. (2016). Pioglitazone increases the glycolytic efficiency of human Sertoli cells with possible implications for spermatogenesis. *International Journal of Biochemistry and Cell Biology*, 79, 52–60.
- Misro, M. M., & Ramya, T. (2012). Fuel/energy sources of spermatozoa. In S. J. Parekattil, & A. Agarwal (Eds.), *Male infertility: Contemporary clinical approaches, andrology, ART & Antioxidants*. New York: Springer.
- Oliveira, P. F., Alves, M. G., Rato, L., et al. (2012). Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. *Biochimica et Biophysica Acta - General Subjects*, 1820, 84–89.
- Oliveira, P. F., Martins, A. D., Moreira, A. C., et al. (2015). The Warburg effect revisited—Lesson from the Sertoli cell. *Medicinal Research Reviews*, 35, 126–151.
- Rato, L., Alves, M. G., Cavaco, J. E., & Oliveira, P. F. (2014). High-energy diets: A threat for male fertility? *Obesity Reviews*, 15, 996–1007.
- Rato, L., Alves, M. G., Dias, T. R., et al. (2015). Testicular metabolic reprogramming in neonatal Streptozotocin-induced type 2 diabetic rats impairs glycolytic flux and promotes glycogen synthesis. *Journal of Diabetes Research*, 2015, 13.
- Rato, L., Alves, M. G., Dias, T. R., et al. (2013). High-energy diets may induce a pre-diabetic state altering testicular glycolytic metabolic profile and male reproductive parameters. *Andrology*, 1, 495–504.

- Rato, L., Alves, M. G., Duarte, A. I., et al. (2015). Testosterone deficiency induced by progressive stages of diabetes mellitus impairs glucose metabolism and favors glycogenesis in mature rat Sertoli cells. *International Journal of Biochemistry and Cell Biology*, 66, 1–10.
- Rato, L., Alves, M. G., Socorro, S., et al. (2012). Metabolic regulation is important for spermatogenesis. *Nature Reviews. Urology*, 9, 330–338.
- Rato, L., Duarte, A. I., Tomas, G. D., et al. (2014). Pre-diabetes alters testicular PGC-1alpha/SIRT3 axis modulating mitochondrial bioenergetics and oxidative stress. *Biochimica et Biophysica Acta, Bioenergetics*, 1837, 335–344.
- Travis, A. J., Jorgez, C. J., Merdiushev, T., et al. (2001). Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. *Journal of Biological Chemistry*, 276, 7630–7636.
- Xiong, W. P., Wang, H. K., Wu, H., et al. (2009). Apoptotic spermatogenic cells can be energy sources for Sertoli cells. *Reproduction*, 137, 469–479.

Further Reading

- Cardoso, A. M., Alves, M. G., Mathur, P. P., et al. (2017). Obesogens and male fertility. *Obesity Reviews*, 18, 109–125.
- Oliveira, P. F., Cheng, C. Y., & Alves, M. G. (2017a). Emerging role for mammalian target of rapamycin in male fertility. *Trends in Endocrinology and Metabolism*, 28, 165–167.
- Oliveira, P. F., Sousa, M., Silva, B. M., et al. (2017b). Obesity, energy balance and spermatogenesis. *Reproduction*, 153, R173–R185.
- Rato, L., Alves, M. G., Silva, B. M., et al. (2016). Sirtuins: Novel players in male reproductive health. *Current Medicinal Chemistry*, 23, 1084–1099.

Nutritional Factors and Male Reproduction

Tânia R Dias, Universidade da Beira Interior, Covilhã, Portugal; and University of Porto, Porto, Portugal

Marco G Alves, University of Porto, Porto, Portugal

Branca M Silva, Universidade da Beira Interior, Covilhã, Portugal

Pedro F Oliveira, University of Porto, Porto, Portugal

© 2018 Elsevier Inc. All rights reserved.

Introduction

Nutrition is a primitive need essential for human survival and reproduction. However, the dietary patterns have changed along the years, especially with industrialization and technology that directly or indirectly controlled the food market and promoted a sedentary lifestyle. Over-processed products containing artificial sweeteners, preservatives and refined sugars gained ground over the biological ones. The fact is that over-processed foods are usually produced in a larger scale, making them cheaper and more accessible to everyone. Additionally, multi-million-dollar marketing strategies make those products very attractive, particularly to young people. But not all the alterations of modernization were unfavorable. It also brought increased knowledge and education about nutrition, a higher support by health care systems and the promotion of a healthy lifestyle. But in general, people do not just eat what the body requires to function, they eat bigger portions than they should because eating also gives a sense of relaxation, comfort and pleasure. So, individuals are consuming more and wasting fewer calories. This overeating scenario, together with the lack of physical activity, contributed to the alarming proportions of several diseases worldwide, including diabetes and obesity. On the other hand, there are many countries where food is not so abundant and people are undernourished, which also triggers the development of severe diseases. Even so, according to the World Health Organization (WHO), in many countries overnutrition is now killing more people than undernutrition. However, both malnutrition types contribute to the increasing incidence of diet-related diseases year by year.

In the last decade, there was accumulating evidence for the decline in male fertility potential. In fact, the number of couples seeking for medical assistance to conceive children has increased and in half of those cases the male is the sole cause or contributes to the fertility problem. Nevertheless, there are many undiagnosed cases and we should consider that in some cultures this is still a taboo subject. So, the real numbers of male subfertility and infertility can be even more disturbing. The impact of diet and other daily life activities in male reproductive potential is currently a matter of debate among researchers. In fact, the nutritional status of an individual is of great importance to maintain the well-functioning of the whole body, which may have an impact in the reproductive function. Many minerals, vitamins and other food components obtained through the diet have demonstrated an essential role in processes involved in sperm production and survival. Particularly, there are many dietary compounds with antioxidant properties that are essential for sperm protection from oxidative damages. This chapter will elucidate the influence of diet and lifestyle on male reproduction, unveiling several nutritional factors with a beneficial or negative impact on male fertility.

Nutrition, Lifestyle and Male Reproduction

Modern societies are currently aware of the benefits of having an adequate nutrition and practicing regular physical activity and how these factors can influence important aspects of people's life. Although this so called "healthy lifestyle" may vary among cultures, it has been associated with the prevention of many diseases, which led many people to change their daily habits. However, great part of the population still devalues the importance of having a well-balanced diet. On the other hand, it is difficult to promote a healthier lifestyle when there are so many attractive fast food restaurants to try out. For instance, in the USA there are over 200,000 fast food restaurants with a huge diversity of concepts and meals, leading countless Americans to eat fast food daily. Moreover, millions of vending machines containing soft drinks, sweets and refined foods are available pretty much everywhere, thus reflecting the actual tastes and choices of consumers. The scenario is even most worrying because children and adolescents are the most attracted ones. Generally, people are on a rush, thus making bad food choices, eating too fast, alone and less frequently or even resort to food supplements to save time. This situation usually leads to nutritional deficiencies, whether of one or more nutrients, having negative outcomes for human health. Additionally, there are many jobs with a sedentary nature or that require exposure to high temperatures or toxic compounds during long periods. Furthermore, couples are so focused on their professional careers that are postponing the age to have children and this could be a major obstacle to achieve conception. These current dietary and lifestyle routines make people more susceptible to develop pathological conditions, including male subfertility/infertility. Among the most prevalent diet-related diseases in the world, we can highlight diabetes mellitus, obesity, cardiovascular diseases, cancer and infections, since all these conditions have been linked with a decreased male fertility potential (Dias et al., 2016b). Oxidative stress has been reported as a major contributing factor for the development of those diseases, but it can also be a result of the unhealthy status created by the disease itself. This kind of stress occurs when the production of free radicals overwhelms the ability of inner antioxidant defenses to control them. High levels of oxidative stress are usually found in subfertile and infertile men, thus having a key role on the pathophysiology of male subfertility/infertility. In fact, high levels of free radicals were detected in the semen of 25–40% of infertile men, as well as less amounts of inner antioxidant enzymes than in fertile men (Agarwal et al., 2003). Antioxidant compounds obtained through the diet may be of extreme relevance to restore the reproductive tract antioxidant defense system and avoid fertility dysfunctions induced by oxidative stress.

Oxidative Stress, Antioxidants and Male Fertility

A free radical is a chemical compound with one or more unpaired electron(s) in an outer membrane orbital, which makes it unstable and highly reactive. The term reactive oxygen species (ROS) usually refer to oxygen-containing free radicals, such as superoxide anion ($O_2^{\bullet-}$), hydroxyl ($\bullet OH$) and nitric oxide ($NO^{\bullet}$) radicals, but also to some non-radical oxygen derivatives, including singlet oxygen (1O_2), ozone (O_3), hydrogen peroxide (H_2O_2), peroxyxynitrite anion ($ONOO^-$) and hydrogen hypochlorite ($HClO$). These latter do not contain unpaired electrons but are potentially reactive and can be converted to radical ROS. Cellular endogenous sources of ROS, mostly generated by oxidative phosphorylation in mitochondria or by the immune system, have an essential role in normal cellular processes. In physiological conditions, ROS are involved in the regulation of cell signaling pathways, having the ability to modulate the activity of enzymes, mediate inflammation and control pathogens intrusion. For instance, Sertoli cells, the somatic cells of the testis that are vital for a successful spermatogenesis, require ROS for normal metabolic processes, namely to obtain energy and produce important substrates for germ cells (Dias et al., 2013). Moreover, spermatozoa also need a controlled amount of ROS for essential fertilizing processes such as sperm capacitation, hyperactivation, acrosome reaction and sperm-egg fusion. However, when something disrupts the prooxidant-antioxidant homeostasis in those cells, it promotes an oxidative environment, impairing spermatogenesis and sperm function. Defective spermatozoa (immature or abnormal), high levels of leucocytes present in the ejaculate (leucocytospermia), pathological conditions or other exogenous factors (e.g. smoke, radiation or drugs), are major stimuli for ROS overproduction (Fig. 1). The most common ROS produced in sperm and that can harm their quality are superoxide anion, hydrogen peroxide, nitric oxide and peroxyxynitrite. Proteins, lipids, and DNA are the core targets of ROS since they can capture electrons from them to seek stability. However, this process triggers a cascade of reactions due to the generation of new unstable species that will subsequently try to capture electrons from a nearby molecule as well. These events lead to protein oxidation, lipid peroxidation and/or oxidation of DNA bases, which culminate in severe cellular damages and even cell death. Spermatozoa are quite susceptible to oxidative damages due to their distinctive lipidic membrane and limited intracellular defense system. When ROS attack the polyunsaturated fatty acids (PUFAs)-rich membrane of spermatozoa, lipid radicals are formed, which in the presence of oxygen generate lipid peroxy radicals. Those products contribute to the formation of some dangerous adducts, such as the 4-hydroxynonenal (4-HNE) or thiobarbituric acid reactive substances (TBARS), that stimulate a chain reaction of free radicals' generation usually described as lipid peroxidation. Consequently, spermatozoa membrane loses fluidity, which compromises motility and sperm-egg fusion. ROS can also inactivate key enzymes involved in sperm function by altering their structural conformations. Moreover, ROS-induced damages to mitochondrial and nuclear sperm DNA may end-up in low pregnancy rates, increased birth defects and high offspring morbidity.

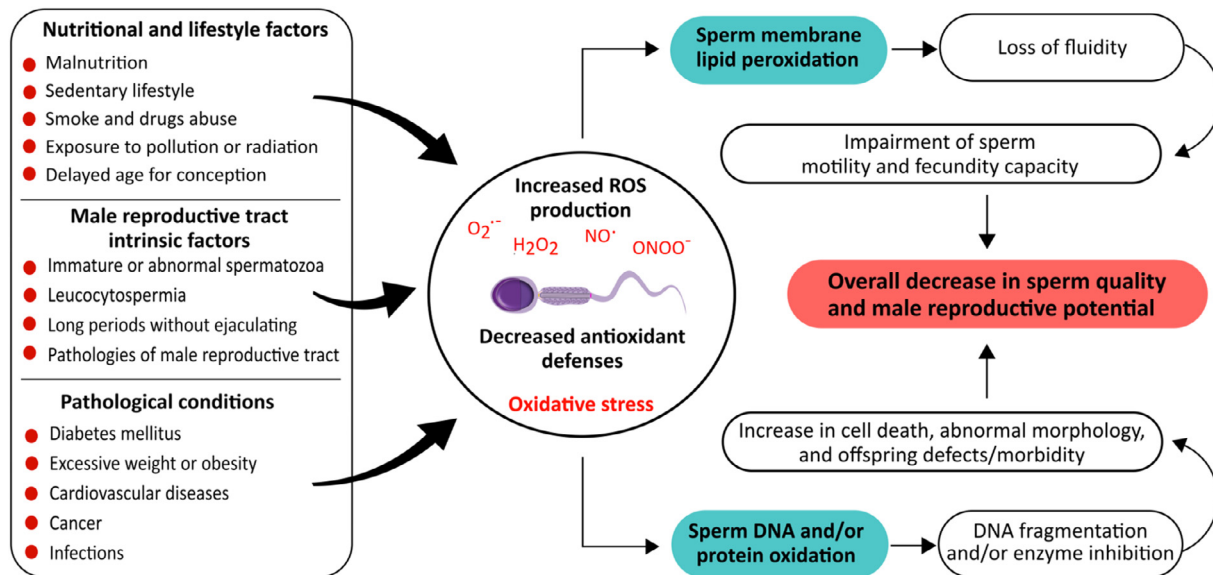


Fig. 1 Schematic representation of the oxidative stress-promoting factors in spermatozoa and the respective outcomes for male reproductive function. Nutrition, lifestyle and pathological conditions are among the extrinsic factors promoting oxidative stress in the male reproductive tract. There are also some intrinsic factors such as immature/abnormal spermatozoa and leucocytospermia that may increase ROS production within the male reproductive tract, including in spermatozoa. Superoxide anion radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), nitric oxide radical ($NO^{\bullet}$) and peroxyxynitrite anion ($ONOO^-$) are the main types of ROS produced by spermatozoa. ROS overproduction together with the decrease in antioxidant defenses may lead to sperm membrane lipid peroxidation and and/or oxidation of DNA bases. While lipid peroxidation alters the fluidity of spermatozoa membrane, impairing motility and fecundity, DNA oxidation lead to its fragmentation, thus increasing cell death, the number of abnormal spermatozoa and the occurrence of defects/morbidity in the offspring. This culminates in the overall decrease in sperm quality and impairment of male reproductive potential.

Generally, antioxidants are compounds with the ability to scavenge ROS or act as cofactors for the cellular enzymatic antioxidant defenses, thus preventing oxidative-induced damages. To counteract these severe damages, spermatozoa are endowed by an intrinsic antioxidant system that contains a myriad of enzymatic and non-enzymatic components, including superoxide dismutase, catalase, glutathione peroxidase, glutathione transferases, peroxiredoxins and thioredoxins. Still, the amount of those scavenging compounds in spermatozoa cytoplasm is very low. During spermatogenesis, germ cells differentiation is physically protected by the blood-testis barrier formed by Sertoli cells. Chromatin condensation is one of the vital processes occurring during spermatogenesis, in which most of sperm DNA histones are replaced by protamines to obtain a higher compaction and protection. If these events are disrupted and spermatozoa retain more histones than they should, sperm DNA becomes more susceptible to oxidative damages, which is critical since sperm DNA has a low ability for repair. On the other hand, during spermiation, most of spermatozoa cytoplasm and organelles are removed, thus diminishing sperm endogenous defenses. Sometimes, this process is not fully completed and some spermatozoa retain a residual cytoplasmic droplet, which contains enzymes that promote ROS production. Nevertheless, epididymal fluid and seminal plasma possesses a powerful antioxidant system that protect spermatozoa from oxidative stress after being released from the testis. These fluids contain many enzymatic antioxidants, namely superoxide dismutase, catalase and glutathione peroxidase. However, they also contain many non-enzymatic antioxidants that can be obtained through the diet, supporting the key role of nutrition in the control of reproductive fluids composition and antioxidant capacity. Thus, the nutritional state of a man really influences his fertility potential, as well as the frequency of ejaculation and hydration. Some nutritional factors that may influence the antioxidant-pro-oxidant balance in male reproductive tract will be discussed in the following section.

Nutritional Factors Affecting Male Reproductive Function

A well-balanced nutrition includes adequate amounts of vitamins, minerals, protides, carbohydrates and lipids, as well as a limited number of calories to maintain a productive and energetic lifestyle. Depending on the variability of individual tastes and the country-specific dietary habits, variable amounts of antioxidant compounds can be obtained through a healthy diet. But current lifestyle patterns are making this a nearly impossible task, as evidenced by the increasing prevalence of oxidative-related dysfunctions, including in male reproduction. This led to a huge launch of antioxidant-based compounds on the market, which now people incessantly seek as they are claimed as healthy. One of the major concerns is around the market of food supplements that is saturating grocery stores. The consumption of these food supplements to compensate for the lack of dietary antioxidant compounds is becoming a trend. However, they do not require medical prescription and are usually referred to as devoid of side effects. This is a critical issue since the use of non-validated dosages and combinations of several antioxidant compounds, may lead to the depletion of the physiologically required ROS amounts for a normal cellular function, thus doing more harm than good. But for many men that do not want to bring “public” their fertility problems, this self-medicating approach constitutes an appealing alternative. Nevertheless, the consciousness about antioxidant products and their effects in the overall health condition of an individual is increasing. In fact, alterations in nutrition and lifestyle become one of the primary medical recommendations to any treatment, which are commonly called nutritional therapies. For instance, the higher intake of fruits and vegetables, which are natural sources of antioxidants, was associated with the improvement of sperm quality (Gaskins et al., 2012). However, other studies reported that many edible antioxidant-rich plants have the ability to disrupt spermatogenesis and decrease sperm quality, being good candidates for contraceptive purposes but not for subfertility treatments (Dias et al., 2014). There is still a lack of knowledge in this area and many controversial data, due to the high variability of subjects, techniques, compounds, doses and evaluated parameters. Nevertheless, several nutrients and other food components (Table 1) demonstrated a protective effect in spermatozoa by decreasing lipid peroxidation, DNA fragmentation and improving sperm quality. So, the excessive or deficient ingestion of one or more nutrients/food components can affect several processes, such as Sertoli and Leydig cells function, spermatogenesis and sperm quality. The accurate knowledge concerning the recommended dosages of some nutrients to maintain a normal reproductive function is of extreme importance.

Vitamins

Vitamin A comprises a group of related compounds called retinoids, including retinol and beta-carotene, which can be uptake from eggs, sweet potatoes, carrots and pumpkins. Generally, it is essential for vision, immune system and skin health. Due to its characteristic lipid solubility, it is better absorbed when consumed together with fats. The body can store vitamin A in the liver to be used when needed. When it reaches the target sites, it is converted to retinoic acid, so it can bind to its specific receptors. It has been reported that vitamin A participates in certain stages of spermatogenesis by stimulating the transcription of several genes that are involved in meiosis. Moreover, the long-term deprivation of this vitamin in male rats led to the disruption of blood-testis barrier and germ cell aplasia, thus compromising fertility (Morales and Cavicchia, 2002). Higher serum concentrations of retinol were found in men presenting a normal spermogram relative to subfertile ones (oligozoospermia and asthenozoospermia) (Al-Azemi et al., 2009).

Other significant vitamin for male reproduction is vitamin B9 (or folate) that is mainly present in beans, lentils and spinach. This water-soluble vitamin plays an essential role in DNA biosynthesis cycle and repair, cell division and cell growth, which are crucial steps occurring during spermatogenesis. Due to the importance of this micronutrient, a synthetic version of vitamin B9, folic acid,

Table 1 Some nutritional factors obtained from diet that influence male reproductive potential

Nutritional factor	Dietary sources	Major outcomes	References
Vitamin A	Eggs, sweet potatoes, carrots, pumpkins	• Maintains blood-testis-barrier • Avoids germ cell aplasia • Higher concentration in seminal plasma of fertile men	(Morales and Cavicchia, 2002, Al-Azemi et al., 2009)
Vitamin B	Beans, lentils, spinach	• Important for DNA biosynthesis cycle and repair, cell division and cell growth • Decreases the occurrence of aneuploidy in spermatozoa • Improves total sperm count	(Wong et al., 2002, Young et al., 2008)
Vitamin C	Oranges, papaya, strawberries, pineapple, peppers, broccoli, cauliflower	• Sertoli cells express functionally active vitamin C transporters • Lower levels in seminal plasma of infertile men • Improves sperm quality (counts, motility and normal morphology)	(Akmal et al., 2006, Colagar and Marzony, 2009)
Vitamin D	Egg yolks, dairy products, fish	• Immature Sertoli cells and germ cells express vitamin D receptors • Influences intracellular calcium levels • Improves sperm motility	(Jensen et al., 2011)
Vitamin E	Nuts, seeds, green leafy vegetables	• Decreases lipid peroxidation in spermatozoa and seminal plasma • Improves sperm motility • Higher levels in fertile men	(Keskes-Ammar et al., 2003, Al-Azemi et al., 2009)
Vitamin K	Broccoli, cabbage, brussels sprouts	• Promotes testosterone production by Leydig cells	(Oury et al., 2013)
Selenium	Nuts, fish, beef	• Protects spermatozoa from ROS attack • Lower levels in oligospermic and azospermic men • Influences sperm motility and abnormal morphology	(Ahsan et al., 2014)
Zinc	Seafood, beef/lamb meat, wheat	• Cofactor of metalloenzymes involved in DNA transcription, expression of steroid hormone receptors, and protein synthesis • Higher levels in seminal plasma of fertile men • Reduces DNA fragmentation levels	(Colagar et al. 2009; Schmid et al., 2012)
Carnitines	Meat, milk	• Lower levels in azospermic men	(Ahmed et al., 2011)
Coenzyme Q10	Salmon, tuna, beef, nuts, seeds	• Involved in ROS control in spermatozoa • Improves sperm density and motility	(Safarinejad, 2009)
Polyunsaturated fatty acids (PUFAs)	Nuts, fish	• Increase the antioxidant potential of seminal plasma • Improve sperm quality	(Safarinejad et al., 2010)
Catechins	Tea, chocolate, berries	• Modulate Sertoli cells function • Improve sperm viability and motility • Increase male reproductive tract antioxidant potential	(De Amicis et al., 2012; Oliveira et al., 2015, Dias et al., 2016a; Dias et al., 2016c)
Caffeine	Coffee, tea, chocolate	• Alters Sertoli cells metabolism and oxidative profile	(Dias et al., 2015)

has been included in many fortified foods and supplements to maintain a sufficient daily supply of this vitamin. However, it is known that many men during the reproductive years do not reach the recommended daily dose of vitamin B9. The high intake of folates by healthy non-smoking men has been linked with lower occurrence of aneuploidy in spermatozoa (Young et al., 2008). Moreover, a study performed with subfertile men demonstrated that the consumption of folic acid together with zinc sulfate, led to a 74% improvement in total sperm count (Wong et al., 2002), thus supporting the vital role of vitamin B9 in male fertility potential.

Vitamin C, mostly known as L-ascorbic acid, can be consumed through fruits (e.g. oranges, papaya, strawberries, pineapple) and vegetables (e.g. peppers, broccoli, cauliflower). This vitamin is water-soluble and is part of the antioxidant machinery of seminal plasma, in which it is present at a 10-fold higher concentration than in blood plasma. Sertoli cells seem to be responsible for the availability of vitamin C levels for developing germ cells since they express functionally active vitamin C transporters. Lower levels of vitamin C in the seminal plasma of infertile men (relative to fertile ones) were associated with an increased spermatozoa abnormal morphology (Colagar and Marzony, 2009). Oligospermic men consuming vitamin C showed an overall improvement on sperm quality, including increased sperm counts, motility and normal morphology (Akmal et al., 2006).

Vitamin D is a group of vitamins that human body can generate. In humans, the most important compounds of this group are vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol). Vitamin D₃ is mainly produced by the epidermis after exposure to sunlight, thus being particularly important for skin health. It is also vital for calcium absorption and hence for bone health. However, it can also be obtained through diet, namely from egg yolks, dairy products and fish (e.g. salmon and sardines). In humans, vitamin D₃ receptor (VDR) is expressed by germ cells and immature Sertoli cells, evidencing the role of this vitamin in

those cells physiology. In fact, the serum levels of vitamin D have been positively correlated with human sperm motility by influencing the intracellular levels of calcium (Jensen et al., 2011). Calcium has a vital role not only in sperm maturation during epididymal transit, but also for acrosome reaction and fertilization.

Vitamin E can be obtained essentially through nuts, seeds and green leafy vegetables, and is one of the primary antioxidant compounds of spermatozoa. The most biologically active form of vitamin E, is α -tocopherol. Vitamin E is a fat-soluble antioxidant and for that reason it can be incorporated into cells membranes, protecting them from lipid peroxidation. It can scavenge lipid peroxyl radicals by generating other radicals, which can be further reduced by other electron donors (usually vitamin C) to return to a stable state, thus blocking the cascade of events that lead to lipids damage. For instance, infertile patients taking a combination of vitamin E and selenium demonstrated an improvement in sperm motility and reduced levels of lipid peroxidation in spermatozoa and seminal plasma (Keskes-Ammar et al., 2003). Additionally, lower levels of α -tocopherol were found in men with oligozoospermia and asthenozoospermia relative to men with normal sperm parameters (Al-Azemi et al., 2009).

Vitamin K is present in many vegetables such as broccoli, cabbage and brussels sprouts and it can be found in two different forms, vitamin K1 (phylloquinone) or vitamin K2 (menaquinone). Only a few studies have evaluated the role of vitamin K in male reproduction. For instance, it has been described that a vitamin K2-dependent protein called osteocalcin, which is secreted by osteoblasts, could promote testosterone production by murine Leydig cells through the binding to a specific receptor - GPRC6A. Although there is a controversy concerning the role of this protein in human testosterone levels and sperm count, one study demonstrated the lack of one of the transmembrane domains of GPRC6A in infertile men, which could prevent the receptor from localizing to the cell membrane (Oury et al., 2013).

Trace Elements

Several trace elements make part of the composition of seminal plasma, including calcium, magnesium, selenium, sodium, potassium and zinc, which are important for sperm physiology. All these elements can be obtained through diet, so variations in the nutritional status of a man can influence the levels of those components in the seminal plasma. These minerals are important for the maintenance of seminal plasma pH and antioxidant potential, thus being important for male fertility. For instance, selenium has a preponderant role in normal testicular development and spermatogenesis. Nuts, fish and beef are some of the selenium-rich food products. Selenium is a constituent of selenoproteins, which are present within spermatozoa midpiece. One major class of functional selenoproteins includes glutathione peroxidase enzymes that are crucial for spermatozoa protection against ROS attack. Lower serum levels of selenium have been described in men with oligospermia and azoospermia compared to fertile ones. Both selenium deficiency or excessive supplementation may lead to decreased sperm motility and increased abnormal morphology (Ahsan et al., 2014).

Zinc is very important for spermatogenesis since it acts as a cofactor of metalloenzymes involved in DNA transcription, expression of steroid hormone receptors, and protein synthesis. Among the zinc-rich foods are seafood, beef/lamb meat and wheat. Fertile men showed higher levels of zinc in the seminal plasma than infertile ones and these levels were positively correlated with sperm counts and normal morphology (Colagar et al., 2009). Moreover, the combined ingestion of zinc with vitamin C and E by older men (>44 years) was associated with lower levels of DNA fragmentation (Schmid et al., 2012).

Other Food Components

Carnitines are lipid-soluble compounds that can be synthesized in the body (25%) from the essential amino acids lysine and methionine, or it can be obtained through diet (75%). They are abundantly present in meat and milk. There are two major forms of carnitines, L-carnitine and acetyl-L-carnitine, which are found in the seminal plasma. The levels of seminal L-carnitine were reported to be lower in infertile individuals, especially when azoospermic, relative to fertile ones (Ahmed et al., 2011), thus evidencing its role in male fertility.

Coenzyme Q10 is a polar ubiquinone involved in the cellular energy metabolism that can also have an antioxidant protective role in cell membranes and lipoproteins. The human body can synthesize coenzyme Q10 through the cholesterol metabolic pathway, but it can also be obtained through diet by consuming salmon, tuna, beef, nuts and seeds. Coenzyme Q10 has a specific role in oxidative phosphorylation by mitochondria, thus being located in sperm midpiece and having a role in ROS control. Significant improvement in sperm density and motility was demonstrated by coenzyme Q10 ingestion (Safarinejad, 2009).

PUFAs are abundant constituents of sperm membranes, being essential to their integrity, fluidity and resistance. There are two main types of PUFAs, omega-3 and omega-6, which are considered essential fatty acids and are mostly obtained through the diet (e.g. fish and nuts). Alterations in dietary PUFAs have been linked with semen quality. In fact, the higher intake of omega-3 and omega-6 fatty acids may improve the antioxidant potential of seminal plasma, thus improving sperm count, motility and normal morphology (Safarinejad et al., 2010).

Catechins are a type of phenolic compounds very abundant in tea, cocoa and berries to which are ascribed a potent antioxidant activity, especially to epigallocatechin-3-gallate (EGCG). This catechin showed the ability to modulate Sertoli cells proliferation and function, having toxic effects at high doses (Dias et al., 2017). EGCG was shown to increase human sperm motility and viability, but at higher doses the opposite effects were observed (De Amicis et al., 2012). Interestingly, the effect of this catechin in sperm quality

seem to be better when acting synergistically with other tea components, including caffeine and L-theanine (Dias et al., 2016a). Caffeine itself has also been reported as a modulator of human Sertoli cells action, thus affecting spermatogenesis and male fertility (Dias et al., 2015). Additionally, the ingestion of white tea by prediabetic rats improved antioxidant defenses (Oliveira et al., 2015) and sperm quality (Dias et al., 2016a). These studies evidence the importance of using adequate doses and combinations between several compounds to reach beneficial outcomes.

Future Perspectives

A balanced diet and lifestyle are crucial to avoid the establishment of an oxidative environment within the male reproductive tract, which has been implicated in the high prevalence of subfertility/infertility. However, further investigation is needed to clarify the link between nutrition and male fertilizing ability, as well as the doses of certain nutrients and other important food components that influence male reproductive potential. Nutritional therapies using antioxidant compounds are frequently used and may be a good approach to restore the capacity of the inner antioxidant system to control the detrimental effects of oxidative stress and enhance natural conception rates. A wide range of antioxidants can be daily consumed through the diet, influencing the overall nutritional status of an individual. This status should be part of the couple evaluation when they seek for medical help to conceive, since it could improve the outcomes of assisted reproductive technologies (ART). Alterations in nutrition and lifestyle are the primary medical recommendations to any treatment since they are easily manipulated factors. However, according to the high prevalence of diet-related diseases, most people are not complying. So, global measures should be taken to make people follow a healthy lifestyle and this should be made at early age to avoid late complications. Further studies and controlled clinical trials are needed to elucidate the possible use of nutritional therapies in the improvement of an individual's reproductive health state, especially in the presence of oxidative stress-related pathological conditions.

Acknowledgements

The authors want to thank the support of “Fundação para a Ciência e a Tecnologia” – FCT to Tânia R. Dias (SFRH/BD/109284/2015); Marco G. Alves (SFRH/BPD/80451/2011); Pedro F. Oliveira (SFRH/BPD/108837/2015); CICS (UID/Multi/00709/2013) and UMIB (PEst-OE/SAU/UI0215/2014). The work was co-funded by FEDER through the COMPETE/QREN, FSE/POPH (PTDC/BIM-MET/4712/2014 and PTDC/BBB-BQB/1368/2014), and POCI - COMPETE 2020 (POCI-01-0145-FEDER-007491) funds.

References

- Agarwal, A., Saleh, R. A., & Bedaiwy, M. A. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertility and Sterility*, 79, 829–843.
- Ahmed, S., Karira, K. A., & Ahsan, S. (2011). Role of L-carnitine in male infertility. *The Journal of the Pakistan Medical Association*, 61, 732–736.
- Ahsan, U., Kamran, Z., Raza, I., et al. (2014). Role of selenium in male reproduction—A review. *Animal Reproduction Science*, 146, 55–62.
- Akmal, M., Qadri, J., Al-Waili, N. S., et al. (2006). Improvement in human semen quality after oral supplementation of vitamin C. *Journal of Medicinal Food*, 9, 440–442.
- Al-Azemi, M., Omu, A., Fatinikun, T., Mannazhath, N., & Abraham, S. (2009). Factors contributing to gender differences in serum retinol and α -tocopherol in infertile couples. *Reproductive Biomedicine Online*, 19, 583–590.
- Colagar, A. H., & Marzony, E. T. (2009). Ascorbic acid in human seminal plasma: Determination and its relationship to sperm quality. *Journal of Clinical Biochemistry and Nutrition*, 45, 144–149.
- Colagar, A. H., Marzony, E. T., & Chaichi, M. J. (2009). Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. *Nutrition Research*, 29, 82–88.
- De Amicis, F., Santoro, M., Guido, C., Russo, A., & Aquila, S. (2012). Epigallocatechin gallate affects survival and metabolism of human sperm. *Molecular Nutrition & Food Research*, 56, 1655–1664.
- Dias, T. R., Alves, M. G., Bernardino, R. L., et al. (2015). Dose-dependent effects of caffeine in human Sertoli cells metabolism and oxidative profile: Relevance for male fertility. *Toxicology*, 328, 12–20.
- Dias, T. R., Alves, M. G., Casal, S., Silva, B. M., & Oliveira, P. F. (2016a). The single and synergistic effects of the major tea components caffeine, epigallocatechin-3-gallate and L-theanine on rat sperm viability. *Food & Function*, 7, 1301–1305.
- Dias, T. R., Alves, M. G., Oliveira, P. F., & Silva, B. M. (2014). Natural products as modulators of spermatogenesis: The search for a male contraceptive. *Current Molecular Pharmacology*, 7, 154–166.
- Dias, T. R., Alves, M. G., Rato, L., et al. (2016c). White tea intake prevents prediabetes-induced metabolic dysfunctions in testis and epididymis preserving sperm quality. *The Journal of Nutritional Biochemistry*, 37, 83–93.
- Dias, T. R., Alves, M. G., Silva, J., et al. (2017). Implications of epigallocatechin-3-gallate in cultured human Sertoli cells glycolytic and oxidative profile. *Toxicology In Vitro*, 41, 214–222.
- Dias, T. R., Bernardino, R. L., Meneses, M. J., et al. (2016b). Emerging potential of natural products as an alternative strategy to pharmacological agents used against metabolic disorders. *Current Drug Metabolism*, 17, 582–597.
- Dias, T. R., Martins, A. D., Reis, V. P., et al. (2013). Glucose transport and metabolism in Sertoli cell: relevance for male fertility. *Current Chemical Biology*, 7, 282–293.
- Gaskins, A. J., Colaci, D. S., Mendiola, J., Swan, S. H., & Chavarro, J. E. (2012). Dietary patterns and semen quality in young men. *Human Reproduction*, 27, 2899–2907.
- Jensen, M. B., Bjerrum, P. J., Jessen, T. E., et al. (2011). Vitamin D is positively associated with sperm motility and increases intracellular calcium in human spermatozoa. *Human Reproduction*, 26, 1307–1317.
- Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., et al. (2003). Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Archives of Andrology*, 49, 83–94.
- Morales, A., & Cavicchia, J. C. (2002). Spermatogenesis and blood–testis barrier in rats after long-term vitamin A deprivation. *Tissue and Cell*, 34, 349–355.
- Oliveira, P. F., Tomás, G. D., Dias, T. R., et al. (2015). White tea consumption restores sperm quality in prediabetic rats preventing testicular oxidative damage. *Reproductive Biomedicine Online*, 31, 544–556.

- Oury, F., Ferron, M., Huizhen, W., et al. (2013). Osteocalcin regulates murine and human fertility through a pancreas-bone-testis axis. *The Journal of Clinical Investigation*, 123, 2421–2433.
- Safarinejad, M. R. (2009). Efficacy of coenzyme Q10 on semen parameters, sperm function and reproductive hormones in infertile men. *The Journal of Urology*, 182, 237–248.
- Safarinejad, M. R., Hosseini, S. Y., Dadkhah, F., & Asgari, M. A. (2010). Relationship of omega-3 and omega-6 fatty acids with semen characteristics, and anti-oxidant status of seminal plasma: A comparison between fertile and infertile men. *Clinical Nutrition*, 29, 100–105.
- Schmid, T. E., Eskenazi, B., Marchetti, F., et al. (2012). Micronutrients intake is associated with improved sperm DNA quality in older men. *Fertility and Sterility*, 98, 1130–1137.
- Wong, W. Y., Merkus, H. M., Thomas, C. M., et al. (2002). Effects of folic acid and zinc sulfate on male factor subfertility: A double-blind, randomized, placebo-controlled trial. *Fertility and Sterility*, 77, 491–498.
- Young, S., Eskenazi, B., Marchetti, F., Block, G., & Wyrobek, A. (2008). The association of folate, zinc and antioxidant intake with sperm aneuploidy in healthy non-smoking men. *Human Reproduction*, 23, 1014–1022.

Pineal Gland and Melatonin Biosynthesis

Pedro F Oliveira, Mário Sousa, and Mariana P Monteiro, University of Porto, Porto, Portugal
Branca Silva, University of Beira Interior, Covilhã, Portugal
Marco G Alves, University of Porto, Porto, Portugal

© 2018 Elsevier Inc. All rights reserved.

The Pineal Gland

The historical identification of the pineal gland has traces in the III and IV centuries BC, when Galen of Pergamon (AD 130–200) appeared to have described the location for this gland in the brain. Later, Andreas Vesalius Bruxellensis (1515–64) reported that this was an unpaired organ with extensive vascularization. The name of this gland itself underwent several alterations throughout time, mostly due to the increasing knowledge of its structure and functioning. The first consistent concept for pineal gland functioning, dated from 1965, was supported on the incomplete definition that the pineal organ was a photoneuroendocrine transducer that converted the coded photic information received into a hormonal signal of light and darkness (Fig. 1). Indeed, the pineal body is the only endocrine gland that is under the influence of the external stimuli received by the retina, thus being responsible for the conversion of those signals into neuroendocrine-mediated effects.

The remarkable anatomical features of the pineal gland, which is a spherical organ located in the midline of the brain, highlight a potential central role for this gland in the individual's physiological functions. Interestingly, most sudden death in infants occur during night and were associated with small pineal glands and with low biosynthetic rates, illustrating that a normal functioning and physiology of this gland is absolutely necessary for a good lifespan. In vertebrates, the pineal gland develops from the neural crest cells of the roof of the diencephalon. It is situated within the brain located adjacent to the superior *colliculi* and behind the *stria medullaris*. Based on this, there were several attempts to make a definitive anatomical classification of the pineal gland for which there is no consensus, though the most used and cited was proposed by Vollrath (1981), which takes in consideration the relative location, shape, and size of the gland. Essentially, he has proposed three anatomical subtypes of pineal glands, mostly according to their relationship with the third ventricle of the brain. The type A pineal rests at a proximal position to the posterior section of the diencephalon, while type AB pineal adopts a proximal intermediate position, and the ABC type is an elongated pineal spanning from the diencephalon till near the cerebellum and adopts a proximal–intermediate–distal position. Still, there is a great variation in the anatomic morphology of the pineal gland across and between species that reflects the adaptive needs for each and the adaptation to environmental factors. In addition, the pineal gland can have a wide range of weights and pineal glands weighing around 1 g were even reported in individuals with genetic disorders associated with insulin resistance. Nevertheless, in human adults without any known disordered clinical condition, the average pineal dimensions are the same as a grain of rice; with no variations according to age or gender. The pineal gland has sympathetic enervation, which consists of noradrenergic fibers, which have a central role for the regulation of the pineal gland functioning. It has also excellent blood irrigation and is usually presented as

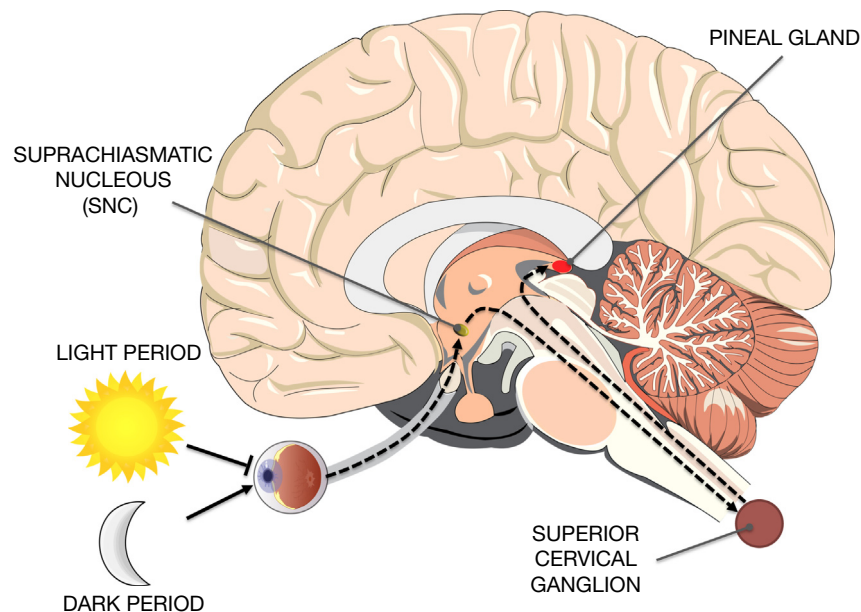


Fig. 1 Overview of the mechanism by which light availability modulates melatonin secretion by the pineal gland. This mechanism involves the neural pathway originating in the retina and passing through the suprachiasmatic nucleus, reaching pinealocytes in the pineal gland via adrenergic nerves and adrenergic receptors, and subsequently to the periphery.

the second organ with the highest rate of blood flow per tissue mass, only surpassed by the liver. Thus, there are numerous capillaries within the pineal parenchyma, which allow a greater metabolic activity. Indeed, during the night, when the pineal gland activity is more intense due to increased indole metabolism, the blood supply also increases.

Histologically, the pineal gland presents a parenchyma composed of two types of cells: the pinealocytes and the neuroglia. The former are organized into strings and rest on the basal membrane where they relate with the interstitial space. In fact, the most abundant cells in the pineal body are pinealocytes that produce the pineal hormones, from which melatonin arises as the most relevant for several physiological processes, being that the pineal gland also secretes indoles and polypeptides. In addition, other astrocyte-like interstitial cells were also identified in the pineal gland. Photoreceptors have been found in pineal glands, but mammalian pinealocytes are not capable of directly produce hormones in response to light treatment.

The human pineal is classified as type A. It is positioned within 1–2 mm of the brain midline, which makes it an ideal reference point for the midsagittal plane. The development of the pineal gland starts during the second month of gestation, as an invagination of the ependymal lining of the diencephalic aspect of the third ventricle, between the *habenula* and the posterior commissure, to which it retains connections composed of a rostral and a caudal lamina. The human pineal gland is usually described as consisting of a central core composed of lobules and a cortex with a more diffuse distribution of neurons, being the pinealocyte *sensu stricto* the main cell type. Human pinealocytes are histologically described as having a prominent nucleus and a granular appearance with a cytoplasm and capillaries with characteristic configuration of endocrine glands. Surrounding the pinealocytes, there is neuroglia composed of fibrous astrocytes and microglia. The pineal gland receives blood supply from the branches of the posterior choroidal arteries. In addition, veins and arteries are present in the pineal tissue and there is an extensive internal capillary system, which, together with the fact that it lacks an endothelial blood–brain barrier, makes it very reactive to peripheral drugs. Innervation is also a key feature of the human pineal gland and results from the presence of peripheral sympathetic and parasympathetic fibers, together with fibers originating in the central nervous system (CNS). However, although it is closely located to the midbrain, the pineal gland scarcely receives afferent innervation from the brain itself.

Melatonin

Melatonin (or *N*-acetyl-5-methoxytryptamine) was firstly isolated in 1958 as a pineal gland product and is an evolutionarily conserved molecule, which is often referred as a master regulator of the biological (or circadian) clock. It is a small and very lipophilic indoleamine, with a molecular weight of 232 Da. This hormone is not stored at significant levels in the pinealocytes, but is rapidly released into the bloodstream. Apart from the role of pineal gland in melatonin production, it is also produced in small quantities by other extrapineal organs including the retina, skin, bone marrow cells and ovaries, among others. Nevertheless, in humans, pinealectomy virtually removes all plasma melatonin availability. Melatonin is mostly secreted during the night in humans, as it occurs in most other diurnal species, and the maximum levels detected in the plasma in adults average 60–70 pg/mL, while in saliva are usually one-third lower. Nevertheless, in physiological conditions, melatonin is present in both fluids in concentrations that are usually below 5 pg/mL. In human adults, the peak of melatonin concentration usually occurs between 02.00 and 04.00 h, while the onset of secretion occurs around 21–22.00 h and the offset at 07.00–09.00 h. Moreover, plasma melatonin concentrations reflect the synthesizing activity of the pineal gland, while its concentration in retina and other organs may reflect a local action.

Melatonin Biosynthesis

The precursor for melatonin synthesis is tryptophan, an essential aminoacid uptaken, which is hydroxylated to 5-hydroxytryptophan and then converted into serotonin (Fig. 2). Several food products, like banana, soya, milk, meat, fish or egg, are rich in tryptophan. The rate of melatonin production depends mostly on the activity of the key enzyme aralkylamine *N*-acetyltransferase (AANAT), and to a lesser extent on that of the enzyme tryptophan hydroxylase (TPH). Compelling evidence suggests that AANAT catalyzes the most fundamental step in this pathway since it seems that the key mechanisms that control melatonin production are related with the regulation of its activity (Klein et al., 1997). Nevertheless, the mechanisms that regulate melatonin biosynthesis and AANAT activity vary between species, and there are several other factors that can control the production of melatonin, including the availability of the different intervenients and nutritional factors.

Tryptophan hydroxylase (TPH) catalyzes the conversion of tryptophan to 5-hydroxytryptophan, using tetrahydrobiopterin (BH4) as a pteridine cofactor. Interestingly, the localization of TPH is restricted to serotonin-synthesizing tissues, which highlights the relevance of this enzyme for the process. This enzyme has two main isoforms: TPH1 and TPH2. The former is localized in pineal gland and gut, while the latter is exclusively found in the brain. The regulation of the expression levels of these isoforms follows the clock-driven circadian rhythm, being that the highest values are detected at night, when melatonin levels are known to rise. Notably, when individuals are exposed to light during the night, the levels and activity of TPH drop.

5-Hydroxytryptophan is then readily and freely converted to serotonin by the action of the enzyme DOPA decarboxylase (Fig. 2), with the intervention of vitamin B6. Serotonin is then metabolized into melatonin in two steps. In the first step, *N*-acetyltransferase (AANAT) acetylates serotonin to form *N*-acetylserotonin. The second step occurs by the action of hydroxyindole-*O*-methyltransferase (HIOMT) that converts *N*-acetylserotonin into melatonin (Fig. 2). As referred, AANAT is usually described as the most relevant regulator of melatonin biosynthesis. In vertebrates, AANAT belongs to a superfamily of GCN5-related *N*-acetyltransferases (GNAT), requiring acetyl coenzyme A (AcCoA) as an acetyl group donor and presenting

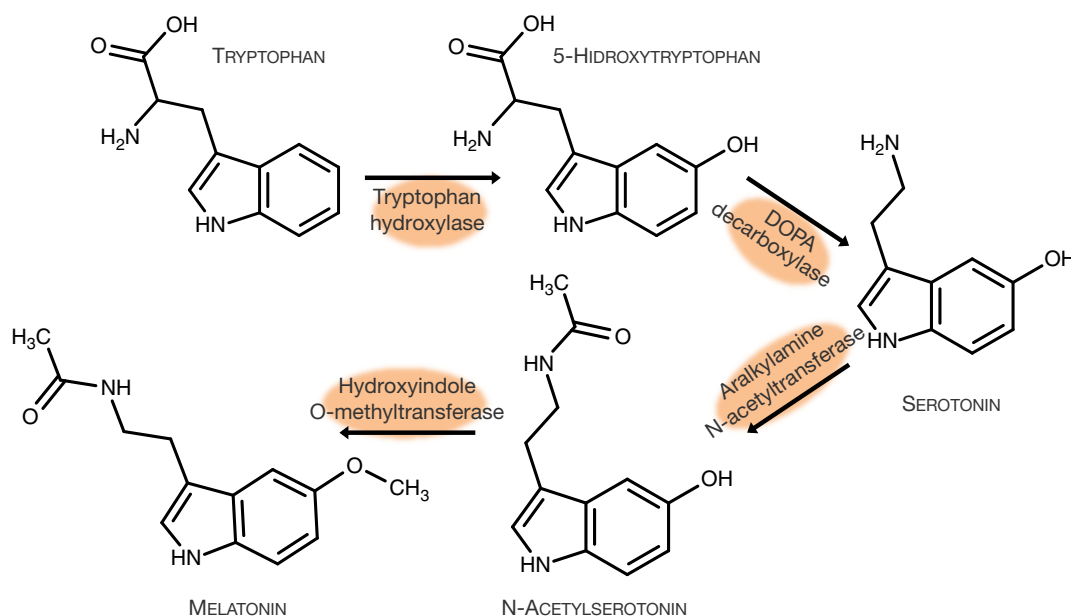


Fig. 2 Simplified overview of the biosynthetic pathway of melatonin in the pineal gland. Tryptophan is converted to 5-hydroxytryptophan by the action of tryptophan hydroxylase. This latter is converted to serotonin by DOPA decarboxylase, which is later converted to *N*-acetylserotonin by aralkylamine *N*-acetyltransferase. Melatonin is then originated from *N*-acetylserotonin by the action of hydroxyindole *O*-methyltransferase. The action of these enzymes is assisted by several co-factors (not shown).

a catalytic core and regulatory regions. The catalytic core binds to arylalkylamines and acetyl-CoA to facilitate the transfer of the acetyl group. The regulatory regions contain phosphorylation sites that function as regulatory domains for activation and stabilization of the catalytic core. The phosphorylation of those domains promotes the binding to 14-3-3 proteins, which mediate a reduction of the K_m for arylalkylamine substrates and also protects the enzyme from the proteosomal degradation, illustrating a complex functioning. The activity of AANAT can be regulated at three main levels: (1) transcription; (2) posttranscriptional, such as phosphorylation; and (3) binding to chaperones or by processes related with the regulation of protein degradation velocity through proteosomal degradation (for review Karolczak et al., 2005). Nevertheless, the relevance of those mechanisms is species-dependent. For instance, *de novo* transcription is required in rat since during the light phase the transcripts of *Aanat* gene are not detectable, while the increase of norepinephrine (NE) release during the night is followed by a strong increase of approximately 100-fold in the levels of *Aanat* gene, which promotes a rise in the levels of the protein and its activity. However, that is not the case in other species, such as the sheep or the rhesus monkey in which AANAT mRNA levels do not change much during the 24-h of the day and thus, changes in this protein activity are mainly modulated by the levels of the protein itself (Coon et al., 2002).

In addition to melatonin, there are other 5-hydroxyindoles that may also be synthesized in the pineal gland, such as 5-methoxytryptophan, 5-methoxytryptamine, 5-methoxyindole 3-acetic acid, and 5-methoxytryptophol. Nevertheless, the relevant biologically active methoxyindoles are melatonin, 5-methoxytryptophol, and 5-methoxytryptamine.

Regulation of Melatonin Production

Melatonin production is driven by the internal clock mediated by the suprachiasmatic nucleus (SCN) that is connected with the pineal gland. This multisynaptic pathway ends-up in a circuit that consists of sympathetic nerve fibers. Those fibers release NE exclusively at night, complying with the circadian rhythm. Information concerning the light availability is received in the retina and primarily passes through the retinohypothalamic pathway, until it reaches the SCN, where the internal circadian sensitive clock is located. This synchronizes the circadian clock phases with light–dark cycle. It is a complex process in which the SCN fibers pass through the paraventricular hypothalamic nucleus, medial forebrain bundle, and reticular formation. This sends information to the intermediolateral horn cells of the spinal cord, which contain preganglionic sympathetic neurons. The latter terminate on the pinealocytes that are responsible for the release of NE. NE acts as the master neurotransmitter that regulates AANAT action and the synthesis of melatonin in mammals. It stimulates the pinealocytes and initiates a sequence of reactions after activation of two subtypes of receptors, α_1 - and β_1 -adrenergic receptors.

The activation of the α_1 -adrenergic receptors results in a significant increase in the intracellular concentration of calcium ($[Ca^{2+}]_i$) (Schomerus et al., 1995; Sugden et al., 1987). It is known that $[Ca^{2+}]_i$ modulates melatonin biosynthesis in a manner that may be species-specific, though the exact mechanisms remain unknown. Nevertheless, it is known that increased $[Ca^{2+}]_i$ triggers L-glutamate exocytosis from pineal microvesicles, which inhibits NE-mediated melatonin synthesis through paracrine and/or autocrine mechanisms (Yamada et al., 1998a,b). From here, the downstream mechanisms that control AANAT expression

and activity appear to be species-specific. These NE-dependent mechanisms are on the basis of the control of melatonin biosynthesis, which usually is suspended before the end of the night due to several processes, such as attenuation of NE release from the sympathetic nerve fibers as the night progresses, or CREB dephosphorylation.

The activation of the β 1-adrenergic receptors increases the intracellular concentration of cAMP, which is followed by activation of the cAMP-dependent protein kinase A (PKA). This mechanism is common to all mammalian species and results in stimulation of AANAT and melatonin production. The cAMP/PKA pathway controls transcriptional mechanisms that regulate the synthesis of melatonin through the transcription factor cyclic AMP response element (CRE)-binding protein (CREB), which is constitutively expressed in the pineal gland (Klein et al., 1997; Korf et al., 1998). Phosphorylation of CREB enhances the transcription of genes bearing a CRE in their promoter region. The transcription of those genes ends up in the upsurge of AANAT protein levels and activity, which results in increased production of melatonin. Phosphorylated CREB results not only from stimulation of β 1-adrenergic receptors, but also from other cAMP-elevating agents, highlighting the relevance of CREB as a central master regulator of melatonin biosynthesis and production.

When discussing melatonin biosynthesis, one must not overlook the retinal biosynthesis of melatonin and its regulation by dopamine. Dopamine is the major catecholamine of the vertebrate retina and is localized to a subpopulation of amacrine and/or interplexiform cells. Nevertheless, that organization is dependent of the species, though the regulatory functions of dopamine as a biochemical signal for the presence of light is conserved. Indeed, the suppressive effect of light on retinal biosynthesis of melatonin seems to be mediated in part by D_4/D_2 -dopamine receptors that are localized in the photoreceptor cells. Those receptors seem to be involved in the phase-shifting effect controlled by light in the circadian melatonin/AANAT rhythm in some species, such as *xenopus*, but not in the chicken for instance. Interestingly, those D_4/D_2 -dopamine receptors, known regulators of melatonin biosynthesis in the retina, are also known to be indirectly linked in a negative way to the cAMP generating system.

Melatonin Receptors

Melatonin is mainly synthesized and secreted by the pineal gland during the dark phase at night, whose main function is related with the regulation of the circadian rhythm. The major physiological actions of this hormone are primarily mediated by interaction with G protein-coupled transmembrane receptors. Initially, melatonin receptors were divided into two classes, named ML1 and ML2, in accordance with their different affinity and binding kinetics for an agonist radioligand 2-[125 I]iodomelatonin ([125 I] Mel) and differential pharmacological profiles for the synthetic ligands. The ML1 receptors showed a range of affinity considered high (in picomolar concentration) to [125 I]Mel, while ML2 receptors have reportedly a much lower affinity to the radioligand, in a nanomolar range. But further studies revealed that this would not be a definitive classification, exposing new findings concerning the action of melatonin and indeed, up till now seven receptors have been identified. The first melatonin receptor to be discovered was cloned from *Xenopus laevis* and seems to be expressed only in nonmammalian species (Ebisawa et al., 1994).

Subsequently, two different forms of human melatonin receptors were cloned and named as melatonin receptors types 1 and 2 (MT1 and MT2, respectively) (Reppert et al., 1994, 1995). Both receptors have similar binding properties and have subnanomolar affinities for the hormone. Interestingly, a G protein-coupled receptor named GPR50 (or Mel1c), shares 45% amino acid sequence identity with MT1 (or Mel1a) and MT2 (or Mel1b) and, though melatonin does not directly binds to this receptor, it dimerizes with MT1 and MT2 but only inhibits the agonist binding to MT1, preventing the recruitment of signaling partners (Levoe et al., 2006).

Finally, melatonin has been suggested to bind to the orphan nuclear receptor of the retinoic acid receptor family, named as retinoid Z receptor (RZR) and retinoid acid receptor-related orphan receptor (ROR) (Becker-Andre et al., 1994). The RZR/ROR subfamily consists of three subtypes (α , β , γ) and four splice variants of the α -subtype. The mode of action of those receptors regarding melatonin binding and subsequent activity remains unknown.

Melatonin receptors act through a myriad of signaling pathways, including adenylyl cyclase inhibition, phospholipase C stimulation, and other relevant pathways. It is interesting to notice that in birds and lower vertebrates, melatonin receptors are widely distributed throughout the CNS, while in mammals its distribution is by far more restricted (Table 1), being highly weaker than in nonmammal species. Melatonin can induce the activation of different second messenger cascades, depending of the tissue and also of the species, which makes very difficult to understand the specificity of the process since distinct cascades can act on the same receptor subtype. Nevertheless, some specific characteristics are usually attributed to each receptor. For instance, MT1, MT2, and GPR50 receptors are primarily coupled in an inhibitory manner to adenylyl cyclase, cAMP and PKA signaling pathway, respectively. Notably, the hypothalamic neurons in the SCN and the neurons responsible for the secretion of GnRH are the main targets of melatonin in the hypothalamus. Nevertheless, melatonin receptors are disseminated and expressed in the *pars tuberalis* and *pars distalis* of the anterior pituitary (Table 1). The melatonin receptors in the anterior pituitary are time-dependent and it has been described a postnatal decline of those receptors in the *pars distalis* (Johnston et al., 2006). It is also interesting to highlight that melatonin receptors are not only expressed throughout human fetus (Thomas et al., 1998), but the pinealocytes of the fetus are capable to synthesize melatonin as early as the 26th week of gestation which highlights a possible role for this hormone during development.

Bioactivity and Bioavailability of Melatonin

In humans, melatonin secretion varies during the different life periods. It peaks at 3–5 years of age and starts to decrease from puberty onwards, though it is reported that melatonin values are relatively constant until late in life, when a significant decrease

Table 1 System, organ, tissue, and cell distribution of melatonin receptor 1 (MT1) and melatonin receptor 2 (MT2)

<i>Systems—organs—tissues—cells</i>	<i>Melatonin receptors</i>	<i>References</i>
Nervous system		
<i>Brain</i>	MT1, MT2	Gupta et al. (2017), Hirsch-Rodriguez et al. (2007), and Mazzucchelli et al. (1996)
Cerebellum	MT1, MT2	Al-Ghoul et al. (1998)
Hypothalamus	MT1, MT2	Gupta et al. (2017) and Mazzucchelli et al. (1996)
Hippocampus	MT1, MT2	Mazzucchelli et al. (1996)
Suprachiasmatic nucleus	MT1	Gupta et al. (2017)
<i>Retina</i>	MT1, MT2	Gupta et al. (2017), Savaskan et al. (2007), and Scher et al. (2002)
Cardiovascular system		
<i>Vasculature</i>	MT1, MT2	Ekmekcioglu et al. (2003) and Sallinen et al. (2005)
<i>Aorta</i>	MT1	Ekmekcioglu et al. (2003) and Sallinen et al. (2005)
<i>Heart</i>	MT1, MT2	Sallinen et al. (2005)
Immune system		
<i>Thymus</i>	MT1, MT2	Ahmad and Haldar (2010)
<i>Spleen</i>	MT1	Ahmad and Haldar (2010)
Endocrine system		
<i>Endocrine pancreas</i>	MT1, MT2	Mühlbauer and Peschke (2007)
<i>Pituitary gland</i>	MT1, MT2	Gupta et al. (2017)
<i>Adrenal glands</i>	MT1	Richter et al. (2008) and Torres-Farfan et al. (2003)
Female reproductive system		
<i>Uterus</i>	MT1, MT2	Steffens et al. (2003)
<i>Placenta</i>	MT1, MT2	Lanoix et al. (2012)
<i>Endometrium</i>	MT1, MT2	Steffens et al. (2003)
<i>Ovary</i>	MT1, MT2	Soares et al. (2003) and Niles et al. (1999)
<i>Granulosa cells</i>	MT1, MT2	Niles et al. (1999)
<i>Breast</i>	MT1	Ekmekcioglu (2006)
<i>Mammary glands</i>	MT1	Hill et al. (2011)
Male reproductive system		
<i>Testis</i>	MT1, MT2	Izzo et al. (2010)
<i>Sertoli cells</i>	MT1, MT2	Rocha et al. (2014)
<i>Leydig cells</i>	MT1, MT2	Valenti et al. (1997) and Roy et al. (2001)
<i>Spermatozoa</i>	MT1	Espino et al. (2011) and Irez et al. (1992)
<i>Epididymis</i>	MT1, MT2	Li et al. (1998) and Shiu et al. (2000)
Other systems		
<i>Skin</i>	MT1, MT2	Slominski et al. (2005)
<i>Kidney</i>	MT1, MT2	Sallinen et al. (2005) and Sánchez-Hidalgo et al. (2009)
<i>Liver</i>	MT1	Sallinen et al. (2005) and Venegas et al. (2013)
<i>Gastrointestinal tract</i>	MT1, MT2	Sallinen et al. (2005)
<i>Adipose tissue</i>	MT2	Brydon et al. (2001)

MT1, melatonin receptor 1; MT2, melatonin receptor 2.

is observed. However, there is a great range of values between individuals, which has captured the attention of the clinicians and researchers to understand if these differences do have an impact in human health. Melatonin intervenes in several important physiological and pathological conditions, including seasonal reproductive cycles in animals, mitochondrial function, cancer, cardiovascular function, cellular metabolism, reproductive system, and immunomodulation, among others. Thus, several clinical applications for melatonin have been highlighted. Due to the presence of the electron-rich aromatic indole ring and/or the absence of phenolic hydrogens in its structure, melatonin has been suggested to be involved in electron exchange, which allows it to neutralize oxygen derived radicals and other cellular oxidants, including peroxy (ROO•), singlet oxygen ($^1\text{O}_2$), hydroperoxide (H_2O_2), superoxide anion radical ($\text{O}_2^{\cdot-}$), hydroxyl radical ($\bullet\text{OH}$), among several others. In addition, the metabolites that result from the reaction between melatonin and free radicals also present interesting antioxidant properties, which makes possible to eliminate several molecules of reactive species with only one molecule of melatonin. Furthermore, melatonin has an indirect antioxidant action by activating endogenous antioxidant enzymes, such as superoxide dismutase or the glutathione peroxidase.

Overall, the biosynthesized melatonin is released into bloodstream and gains access to tissues and organs, interacting with virtually all the major biological processes in the body, through the direct action of melatonin itself or its biological metabolites. Since it is not stored at the pineal gland, though it can be locally produced, the bioavailability of this lipophilic hormone in plasma mostly reflects the activity of the pineal gland. Still, the plasma levels of melatonin are also dependent on its deactivation by the liver. More than 90% of the circulating melatonin is deactivated in this organ. It is firstly hydroxylated at the 6-position by hepatic P450, forming the 6-hydromelatonin, which then conjugates with sulfate and also glucuronic acid at less extent, to be then excreted

in the urine. This catabolism of melatonin is different between species but it is rapid and its half-life in humans after short-term exogenous administration ranges from 10 to 60 min. Also remarkable is the fact that the metabolic breakdown of melatonin produced in retina is distinct of that produced by the pineal gland.

Concluding Remarks

The pineal gland is an essential organ where most of melatonin biosynthesis occurs. It is a complex process that has implications in several physiological functions. Indeed, melatonin is predominantly synthesized in the pineal gland, although it can also be locally synthesized in several tissues. In addition, its production was also reported in edible plants (including fruits, cereals, and aromatic plants), bacteria and even unicellular protists, illustrating the conversed evolutionary relevance of melatonin. The most recognized function of melatonin is to carry information about the photoperiod during each day. That information is then differently used by the cells, tissues and even by the different species. There are several relevant biological functions dependent on melatonin biosynthesis, including seasoning-related processes; reproduction; appetite, body weight and most strikingly, in sleep. In addition, emerging evidence has shown that puberty is also under the influence of melatonin biosynthesis. Notably, the discovery that melatonin has a direct free radical scavenger and indirect antioxidant activity, attributed novel functions to this pineal hormone that go far beyond the classic regulation of seasonal reproductive events, photoperiod-dependent processes and breeding animals. In addition, this pineal hormone is a very safe molecule without known toxicity, even when administered in high doses, such as up to 200 mg/kg daily. Thus, there is a wide range of studies aiming to fill the gap of knowledge on the pharmacological potential of this hormone, particularly the best time and route for administration; the lowest effective dose to achieve the desired effects; the specific duration of treatment for each targeted disorder; frequency of administration; type of formulation; controlled released strategies, among several other crucial mechanisms that remain a matter of intense debate and can only arise after melatonin biosynthesis and action is completely known.

References

- Ahmad, R., & Haldar, C. (2010). Photoperiodic regulation of MT1 and MT2 melatonin receptor expression in spleen and thymus of a tropical rodent *Funambulus pennanti* during reproductively active and inactive phases. *Chronobiology International*, 27, 446–462.
- Al-Ghoul, W. M., Herman, M. D., & Dubocovich, M. L. (1998). Melatonin receptor subtype expression in human cerebellum. *Neuroreport*, 9, 4063–4068.
- Becker-Andre, M., Wiesenberger, I., Schaeren-Wiemers, N., et al. (1994). Pineal gland hormone melatonin binds and activates an orphan of the nuclear receptor superfamily. *The Journal of Biological Chemistry*, 269, 28531–28534.
- Brydon, L., Petit, L., Delagrange, P., et al. (2001). Functional expression of MT2 (Mel1b) melatonin receptors in human PAZ6 adipocytes. *Endocrinology*, 142, 4264–4271.
- Coon, S. L., Del Olmo, E., Young, W. S., & Klein, D. C. (2002). Melatonin synthesis enzymes in *Macaca mulatta*: Focus on arylalkylamine N-acetyltransferase (EC 2.3.1.87). *Journal of Clinical Endocrinology and Metabolism*, 87, 4699–4706.
- Ebisawa, T., Karne, S., Lerner, M. R., & Reppert, S. M. (1994). Expression cloning of a high-affinity melatonin receptor from *Xenopus* dermal melanophores. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 6133–6137.
- Ekmekcioglu, C. (2006). Melatonin receptors in humans: Biological role and clinical relevance. *Biomedicine & Pharmacotherapy*, 60, 97–108.
- Ekmekcioglu, C., Thalhammer, T., Humpeler, S., et al. (2003). The melatonin receptor subtype MT2 is present in the human cardiovascular system. *Journal of Pineal Research*, 35, 40–44.
- Espino, J., Ortiz, A., Bejarano, I., et al. (2011). Melatonin protects human spermatozoa from apoptosis via melatonin receptor – and extracellular signal – regulated kinase-mediated pathways. *Fertility and Sterility*, 95, 2290–2296.
- Gupta, T., Sahni, D., Gupta, R., & Gupta, S. K. (2017). Expanding the horizons of melatonin use: An immunohistochemical neuroanatomic distribution of MT1 and MT2 receptors in human brain and retina. *Journal of the Anatomical Society of India*, 66, 58–66.
- Hill, S. M., Cheng, C., Yuan, L., et al. (2011). Declining melatonin levels and MT1 receptor expression in aging rats is associated with enhanced mammary tumor growth and decreased sensitivity to melatonin. *Breast Cancer Research and Treatment*, 127, 91–98.
- Hirsch-Rodriguez, E., Imbesi, M., Manev, R., et al. (2007). The pattern of melatonin receptor expression in the brain may influence antidepressant treatment. *Medical Hypotheses*, 69, 120–124.
- Irez, T.Ö., Senol, H., Alagöz, M., et al. (1992). Effects of indoleamines on sperm motility in vitro. *Human Reproduction*, 7, 987–990.
- Izzo, G., Francesco, A., Ferrara, D., et al. (2010). Expression of melatonin (MT1, MT2) and melatonin-related receptors in the adult rat testes and during development. *Zygote*, 18, 257–264.
- Johnston, J. D., Klosin, P., Barrett, P., & Hazlerigg, D. G. (2006). Regulation of MT melatonin receptor expression in the foetal rat pituitary. *Journal of Neuroendocrinology*, 18, 50–56.
- Karolczak, M., Korf, H. W., & Stehle, J. H. (2005). The rhythm and blues of gene expression in the rodent pineal gland. *Endocrine*, 27, 89–100.
- Klein, D. C., Coon, S. L., Roseboom, P. H., et al. (1997). The melatonin rhythm-generating enzyme: Molecular regulation of serotonin N-acetyltransferase in the pineal gland. *Recent Progress in Hormone Research*, 52, 307–357. discussion 357–308.
- Korf, H. W., Schomerus, C., & Stehle, J. H. (1998). The pineal organ, its hormone melatonin, and the photoneuroendocrine system. *Advances in Anatomy, Embryology, and Cell Biology*, 146, 1–100.
- Lanoix, D., Guérin, P., & Vaillancourt, C. (2012). Placental melatonin production and melatonin receptor expression are altered in preeclampsia: New insights into the role of this hormone in pregnancy. *Journal of Pineal Research*, 53, 417–425.
- Levoye, A., Dam, J., Ayoub, M. A., et al. (2006). The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *EMBO Journal*, 25, 3012–3023.
- Li, L., Xu, J. N., Wong, Y. H., et al. (1998). Molecular and cellular analyses of melatonin receptor-mediated cAMP signaling in rat corpus epididymis. *Journal of Pineal Research*, 25, 219–228.
- Mazzucchelli, C., Pannacci, M., Nonno, R., et al. (1996). The melatonin receptor in the human brain: Cloning experiments and distribution studies. *Molecular Brain Research*, 39, 117–126.
- Mühlbauer, E., & Peschke, E. (2007). Evidence for the expression of both the MT1-and in addition, the MT2-melatonin receptor, in the rat pancreas, islet and β -cell. *Journal of Pineal Research*, 42, 105–106.
- Niles, L., Wang, J., Shen, L., et al. (1999). Melatonin receptor mRNA expression in human granulosa cells. *Molecular and Cellular Endocrinology*, 156, 107–110.

- Reppert, S. M., Godson, C., Mahle, C. D., et al. (1995). Molecular characterization of a second melatonin receptor expressed in human retina and brain: The Mel1b melatonin receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 8734–8738.
- Reppert, S. M., Weaver, D. R., & Ebisawa, T. (1994). Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. *Neuron*, 13, 1177–1185.
- Richter, H. G., Torres-Farfan, C., Garcia-Sesnich, J., et al. (2008). Rhythmic expression of functional MT1 melatonin receptors in the rat adrenal gland. *Endocrinology*, 149, 995–1003.
- Rocha, C. S., Martins, A. D., Rato, L., et al. (2014). Melatonin alters the glycolytic profile of Sertoli cells: Implications for male fertility. *Molecular Human Reproduction*, 20, 1067–1076.
- Roy, D., Angelini, N. L., Fujieda, H., et al. (2001). Cyclical regulation of GnRH gene expression in GT1–7 GnRH-secreting neurons by melatonin. *Endocrinology*, 142, 4711–4720.
- Sallinen, P., Saarela, S., Ilves, M., et al. (2005). The expression of MT 1 and MT 2 melatonin receptor mRNA in several rat tissues. *Life Sciences*, 76, 1123–1134.
- Sánchez-Hidalgo, M., Guerrero Montávez, J. M., Carrascosa-Salmoral, M. d. P., et al. (2009). Decreased MT1 and MT2 melatonin receptor expression in extrapineal tissues of the rat during physiological aging. *Journal of Pineal Research*, 46, 29–35.
- Savaskan, E., Jockers, R., Ayoub, M., et al. (2007). The MT2 melatonin receptor subtype is present in human retina and decreases in Alzheimer's disease. *Current Alzheimer Research*, 4, 47–51.
- Scher, J., Wankiewicz, E., Brown, G. M., & Fujieda, H. (2002). MT1 melatonin receptor in the human retina: Expression and localization. *Investigative Ophthalmology & Visual Science*, 43, 889–897.
- Schomerus, C., Laedtke, E., & Korf, H. W. (1995). Calcium responses of isolated, immunocytochemically identified rat pinealocytes to noradrenergic, cholinergic and vasopressinergic stimulations. *Neurochemistry International*, 27, 163–175.
- Shiu, S. Y., Li, L., Siu, S. W., et al. (2000). Biological basis and possible physiological implications of melatonin receptor-mediated signaling in the rat epididymis. *Neurosignals*, 9, 172–187.
- Slominski, A., Fischer, T. W., Zmijewski, M. A., et al. (2005). On the role of melatonin in skin physiology and pathology. *Endocrine*, 27, 137.
- Soares, J. M., Masana, M. I., Erşahin, Ç., & Dubocovich, M. L. (2003). Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. *Journal of Pharmacology and Experimental Therapeutics*, 306, 694–702.
- Steffens, F., Zhou, X.-B., Sausbier, U., et al. (2003). Melatonin receptor signaling in pregnant and nonpregnant rat uterine myocytes as probed by large conductance Ca^{2+} -activated K^{+} channel activity. *Molecular Endocrinology*, 17, 2103–2115.
- Sugden, L. A., Sugden, D., & Klein, D. C. (1987). Alpha 1-adrenoceptor activation elevates cytosolic calcium in rat pinealocytes by increasing net influx. *The Journal of Biological Chemistry*, 262, 741–745.
- Thomas, L., Drew, J. E., Abramovich, D. R., & Williams, L. M. (1998). The role of melatonin in the human fetus (review). *International Journal of Molecular Medicine*, 1, 539–543.
- Torres-Farfan, C., Richter, H. G., Rojas-García, P., et al. (2003). mt1 Melatonin receptor in the primate adrenal gland: Inhibition of adrenocorticotropin-stimulated cortisol production by melatonin. *The Journal of Clinical Endocrinology & Metabolism*, 88, 450–458.
- Valenti, S., Giusti, M., Guido, R., & Giordano, G. (1997). Melatonin receptors are present in adult rat Leydig cells and are coupled through a pertussis toxin-sensitive G-protein. *European Journal of Endocrinology*, 136, 633–639.
- Venegas, C., García, J. A., Doerrier, C., et al. (2013). Analysis of the daily changes of melatonin receptors in the rat liver. *Journal of Pineal Research*, 54, 313–321.
- Vollrath, L. (1981). *The pineal organ*. Berlin: Springer.
- Yamada, H., Ogura, A., Koizumi, S., et al. (1998a). Acetylcholine triggers L-glutamate exocytosis via nicotinic receptors and inhibits melatonin synthesis in rat pinealocytes. *The Journal of Neuroscience*, 18, 4946–4952.
- Yamada, H., Yatsushiro, S., Ishio, S., et al. (1998b). Metabotropic glutamate receptors negatively regulate melatonin synthesis in rat pinealocytes. *The Journal of Neuroscience*, 18, 2056–2062.

Pineal Gland and Regulatory Function

Pedro F Oliveira, University of Porto, Porto, Portugal

Mario Sousa and Mariana P Monteiro, University of Porto, Porto, Portugal

Branca Silva, CICS-UBI – Health Sciences Research Center, University of Beira Interior, Covilhã, Portugal

Marco G Alves, University of Porto, Porto, Portugal

© 2018 Elsevier Inc. All rights reserved.

The Pineal Gland and Melatonin

The major regulatory function of the pineal gland is to control several biological rhythms under the influence of the light/dark cycle of the surrounding environment. Pineal gland controls the vertebrate circadian biology in a very strict way. This is mainly achieved by the fluctuation of circulating melatonin levels, as consequence of the regulation of melatonin release into the blood by the dark–light cycle. Levels of *N*-acetylserotonin (NAS) and melatonin secreted by pineal gland are low during the day but increase during the night, and the levels of serotonin (5-HT) are up to 10 times higher during the day than at night. During darkness, the pineal gland is responsible for the increase of the circulating melatonin. Since the relative duration of day and night changes along with the seasons, the light cycle also changes and thus, melatonin concentration in circulation follows those alterations. It is interesting to note that whether the individuals are active during the day (diurnal species), during night (nocturnal species) or at crepuscular period, the levels of melatonin are higher during the night and follow a dark–light cycle. Up until now, only the retina of some salmonoid fish was found to break that rule, with melatonin levels being the same or even higher during the day light, when compared with the dark period. In fact, in lower vertebrates, the role of pineal gland goes far beyond that and functions as a third eye, with pinealocytes themselves containing biologically well-defined photoreceptors that in the presence of light respond just like the retina.

Highly specialized photoreceptors mediate the pineal gland regulatory mechanisms responsive for the precisely timed reproductive events. Those photoreceptors are specialized retinal ganglion cells that contain melanopsin, a unique photopigment. Those cells receive the information concerning the period of the day (light versus dark) via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. The SCN is then responsible for the transmission of information via central and peripheral sympathetic neurons to the pineal gland. Norepinephrine (NE) is released to the pinealocytes by the neurons and thus, the latter are responsible for the regulation of melatonin production during the night. The regulatory processes and the molecular mechanisms by which pineal gland produces melatonin and modulates the neuroendocrine axis and intervenes in the secretion of gonadotropins and prolactin are under intense research, particularly the effects mediated by the cellular receptors.

In addition to all these regulatory functions, it should also be highlighted that melatonin peak is closely related with central body temperature and some particular characteristics including the maximum fatigue, lowest alert and performance. For example, though the observations are not that consistent, several authors still associate the ovulatory rise in temperature during the menstrual cycle with a consistent decline in melatonin, while the concentration of melatonin during the luteal phase is suggested to be higher than during the follicular phase. This is still a matter of debate and remains one of the main unveiled mechanisms for melatonin function.

Melatonin and Reproductive Function

The pineal gland functioning, primarily through melatonin biosynthesis and action, has been extensively characterized and associated with several aspects of mammalian physiology, as for instance the reproductive function (Fig. 1). Some of those are quite remarkable, with melatonin being proposed to have a role in the selection of the sexual partner in zebra finches (*Taeniopygia guttata*) by at least two possible mechanisms: one related with its antioxidant activity that affects the color and pigmentation; the other related with melatonin receptors localization in the neurons of the sensorimotor areas which could intervene with the song pattern, known to be essential for mating in male zebra finches (for review (Reiter et al., 2009)).

There is also compelling evidence for a role of melatonin in human reproduction, particularly due to its correlation with gonadotrophins and steroids. Those findings suggest that melatonin may play a role in sexual maturation, control of puberty onset, folliculogenesis, spermatogenesis, oocyte maturation, ovulation, sperm activation, sperm-oocyte fusion, pregnancy and menopause (Rocha et al., 2015). Melatonin is expected to indirectly alter gonadal function through gonadotropin-releasing hormone (GnRH) and gonadotropin secretion, but direct effects may also occur since melatonin can be synthesized in the gonads. The direct action of melatonin is expected to influence steroids production and action as well as major cellular signals in those tissues. In mammals it has been described that melatonin influences the reproductive function by activating melatonin receptors sites that are distributed throughout the HPG axis, particularly in the hypothalamic neurons, which are responsible for the release of GnRH. The activation of those receptors decreases the production of cyclic AMP, as well as the activity of the protein kinase A and induces a decrease on GnRH-induced gonadotropin secretion. This is mainly caused by suppression of GnRH-stimulated calcium signaling due to activation of melatonin-mediated signaling (Ishii et al., 2009). The processes related with calcium mobilization from intracellular stores and the influx through voltage-dependent channels are inhibited by melatonin, mostly likely due

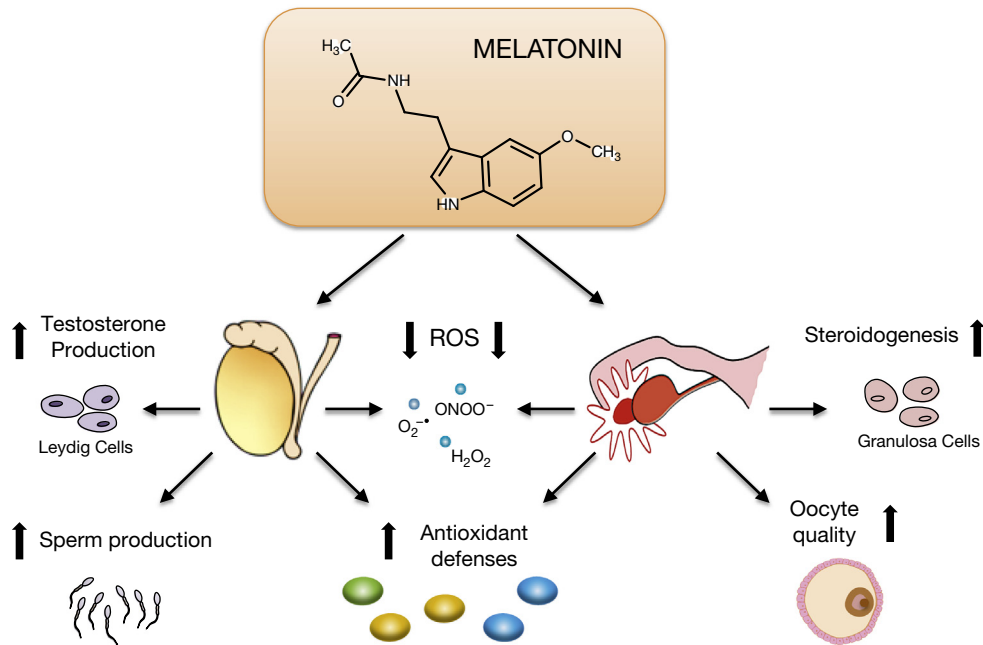


Fig. 1 Overview of the main melatonin functions in male and female reproductive gonads. In males, melatonin is capable of acting directly in the hypothalamic–pituitary axis, modulating the production and release of gonadotropins, which regulate testicular function (particularly spermatogenesis and testosterone synthesis). Melatonin is also able to directly influence sperm quality through its anti-apoptotic and radical scavenger properties. The antioxidant properties of melatonin allow the lessening of testicular damage in pathogenic conditions characterized by an overproduction of reactive oxygen species (such as in episodes of ischemia/reperfusion, in diabetes mellitus and obesity, among others). The ability to scavenge many types of radicals extends to the female reproductive tract, also by enhancing the antioxidant defenses, by regulating antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase. In the female gonads, melatonin has the ability to enhance the follicular steroidogenesis, and to be directly involved in oocytes physiology, particularly its growth, maturity and in other factors that control oocyte quality. Up arrow, increase; down arrow, decrease; ROS, reactive oxygen species.

to AMP/protein kinase C-mediated signaling (Zemkova and Vanecek, 1997). Those events seem to decline with age, promoting some disorders mostly due to a reduction in the expression of functional melatonin receptors.

More recently, it has also been suggested that melatonin can act directly on the cells that produce the gonadotropin-inhibitory hormone (GnIH), a multifunctional neuropeptide whose expression and synthesis increase on short days that abundantly express melatonin receptors (Bentley et al., 2009). Moreover, melatonin was reported not only to increase the number of GnRH-secreting cells, which has a relevant impact in the neuroendocrine activity, but it does not affect the size or morphology of those cells (Glass and Knotts, 1987). Thus, it has been suggested that the effect of melatonin in the hypothalamus is mediated by suppression of GnRH release, rather than its synthesis. Melatonin is also known to inhibit the expression of several hypothalamic genes, especially kisspeptin, which has been shown to act as modulator of GnRH release in breeders. All these evidence shows that melatonin interferes with GnRH release but it is unclear whether it can directly act on GnRH neurons or whether up- or downstream regulators of GnRH neurons mediate the actions.

Melatonin and Puberty Onset

The first evidence that melatonin could be involved in the control of the reproductive function was reported in 1963, in a study showing that the exogenous administration of melatonin reduced the weight of female rats ovaries (Wurtman et al., 1963). Since then, a growing number of reports have emerged exposing several regulatory functions of pineal gland, particularly through melatonin, in the reproductive function. For instance, melatonin has been suggested to play a pivotal role during puberty onset. In rats, the process of reproductive maturity was shown to be finely controlled by melatonin, as injections of this hormone during different light periods or dark phase of the pubertal development result in delay of reproductive development in both males and females without compromising the full process (Horton et al., 1989). Ovaries and testes maturation is triggered by secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), under pulsatile GnRH secretion. Melatonin was suggested to interact in that cascade by triggering the hypothalamus through molecular mechanisms that remain unknown. It seems that before puberty, the concentrations of melatonin are too high for hypothalamic activation to occur. However, by the onset of puberty, there is a decline in the serum levels of melatonin, triggering GnRH, which is pivotal for it to start. This reactivation of the hypothalamic–pituitary axis by the amplitude and frequency of GnRH pulses follows the pulsatile secretion of FSH and LH that triggers puberty. The role of melatonin in the onset of puberty is also highlighted by studies showing that children with delayed puberty

have high levels of nocturnal melatonin, whereas children with precocious puberty present low nocturnal melatonin levels (Waldhauser et al., 1991). In addition, there is clear evidence that melatonin is involved in the control of the pulsatile secretion of LH (Brzezinski et al., 1987) and there is a negative correlation between nocturnal melatonin and LH levels (Waldhauser et al., 1984). Interestingly, it was reported that melatonin serum levels in humans increase during the dark periods in winter and that this is related with reduced activity of the anterior pituitary–ovarian axis (Kauppila et al., 1987). Contrastingly, plasma levels of LH are higher in summer than in winter when the dark period is longer (Kivela et al., 1988). It was also shown that melatonin passes to the fetus through the placenta and by the umbilical cord illustrating that even during pregnancy, this hormone has a preponderant role.

Melatonin and Female Reproductive Function

Melatonin is also well known for its direct effects in the ovaries, which has implications in female reproduction (Fig. 1). Ovarian granulosa and theca cells, which are known to promote steroid hormone production and are present in mature follicles and in the corpus luteum (CL), possess melatonin receptors. In addition, melatonin seems to be directly involved in oocytes physiology, particularly its growth, maturity and in other factors that control oocyte quality. Interestingly, measurements of melatonin levels showed that these are higher in human ovarian follicular fluid (FF) than in plasma and depend on follicular growth. That is suggested to occur due to a high transport of melatonin from the bloodstream, but also due to local synthesis mostly by granulosa cells (Tamura et al., 2009). Among the several functions of melatonin in the ovary, it appears that this hormone is involved in follicular steroidogenesis via modulation of cAMP and also the expression of the steroidogenic acute regulatory protein (StAR). Melatonin influences the production of sex steroids at different stages of follicle maturation by inhibiting the expression of the steroidogenic genes *CYP11A* and *CYP17*. In humans it was shown that melatonin can change the follicular response to LH by elevating the mRNA expression of LH receptors in granulosa cells. It was also shown that this hormone promotes follicular development since it may increase the production of insulin-like growth factor I, which is known to stimulate the mitogenic growth of granulosa cells.

Also interesting is the fact that human births occur more frequently at night when the levels of melatonin are higher, which illustrates a possible role for melatonin on that process. That is also supported by the fact that myometrial cell membranes possess melatonin receptors. Indeed, MTNR1B levels were found to be upregulated in myometrium cells of women during labor and melatonin synergizes with oxytocin to promote and coordinate muscle contraction (Sharkey et al., 2009). Treatment of several diseases that occur during pregnancy may also benefit from melatonin and pineal gland function. For instance, preeclampsia, a condition that affects a high number of pregnant women, whose major signs include elevated systolic and diastolic blood pressures and proteinuria that can progress into both mother and fetal complications, was ameliorated by melatonin treatment. This is a condition that might be associated with increased oxidative stress and low antioxidant defenses thus, melatonin has great therapeutic potential.

Melatonin seems to have a role also during menopause. Women face a series of alterations during and after menopause that have an impact in their health, including hot flushes, mood disturbances, vulvovaginal atrophy, muscle weakness, bone mass loss and sexual dysfunction, among other health problems. In addition, there is a higher risk for the development of cardiovascular diseases, breast cancer and even Alzheimer's disease, among other conditions. The treatment of women in post-menopause with melatonin can elicit positive effects in most of those health conditions. For instance, it can revert some hormonal disturbances and menopause-related depression. In addition, it also has the ability to restore menstrual cycles and fertility in peri- or premenopausal women. Nevertheless, the mechanisms that mediate those effects are still under intense research and remain unknown.

Melatonin and Male Reproductive Function

Melatonin is also essential for male reproduction and testicular function. It has direct and indirect effects on testicular cells and sperm quality (Fig. 1). In males, it is known that melatonin alters the secretion of two key neurohormones, GnRH and LH; it also regulates the synthesis of testosterone and testicular maturation. The regulation of testosterone secretion by melatonin is still unclear, since it is a very complex process that depends not only on the indirect pathways involving the hypothalamic–pituitary–gonadal axis, but also on direct effects on Leydig cells. Indeed, melatonin receptors have been identified in several testicular cells (for review (Rocha et al., 2015)). By acting in Leydig cells, melatonin is reported to regulate testicular growth. In addition, melatonin receptors were identified in Sertoli cells, and in vitro exposure to melatonin modulated the nutritional support of spermatogenesis by these cells (Rocha et al., 2014) by enhancing glucose consumption though producing less lactate. Interestingly, treatment of Sertoli cells with melatonin plus insulin increased the efficiency of lactate production. This metabolite has a crucial role for spermatogenesis since it is an anti-apoptotic factor and a main energy source for developing germ cells.

Melatonin also interferes with the morphology of the male reproductive system, since its administration can cause atrophy and reduction of the seminiferous tubules diameter, which can arrest spermatogenesis. In addition, it can change the morphology of testicular cells. For instance, exposure to melatonin led to alterations in Leydig cells morphology, particularly by decreasing the size and reducing the cytoplasm. Melatonin can also act locally as an inhibitor of androgen production by promoting a downregulation of the expression of steroidogenic acute regulatory protein and key enzymes related with that process (Rossi et al., 2012). These studies illustrate that the regulatory function of pineal gland on male reproductive health, through melatonin, is finely tuned and depends of several aspects, including duration, intensity, and complexity of exposure, among others.

The effects of melatonin on sperm quality has also been reported (Luboshitzky et al., 2002) and widely inferred since its receptors are widely distributed in epididymis, prostate cells and even in spermatozoa. Indeed, several parameters associated with sperm

quality, including concentration, count and motility, are significantly lower during the summer when the days are longer (Levine et al., 1990), suggesting that melatonin has a regulatory role of sperm production and quality. In addition, melatonin was detected in human seminal fluid.

Melatonin Antioxidant Properties and Reproductive Function

The antioxidant properties of melatonin also play a pivotal role in melatonin action on reproductive function. This hormone is known to neutralize the highly reactive hydroxyl radicals resulting from oxygen- and nitrogen-based reactants in an antioxidant cascade, in which melatonin interacts with oxidants species generating further molecular species with important antioxidant properties (Fig. 2). In addition, melatonin boosts the expression and activity of antioxidant enzymes, such as superoxide dismutase (SOD) or the glutathione peroxidase (GPX), while can also inhibit the activity of pro-oxidative enzymes such as the nitric oxide synthase (NOS). Thus, the regulatory function of melatonin in several reproductive tissues, including the FF and seminal fluid, is strictly associated with its antioxidant activity, including a decrease in DNA damage resulting from lipid oxidation (Acuna-Castroviejo et al., 2007), which is correlated with ovum and sperm quality.

Melatonin stimulates maturation-inducing hormone (MIH (17α , 20β -dihydroxy-4-pregnen-3-one)), which heightens oocyte maturation (Reiter et al., 2014; Tamura et al., 2009). During ovulation, reactive oxygen species (ROS) and nitrogen species (RNS) are generated at high quantities, which can promote a high level of oxidative damage. The oxidative balance is very important at this step since ROS may act as second messengers controlling the expression of relevant genes for a regular maturation of the oocytes. On the other hand, a significant increase in ROS may result in damages in oocyte and granulosa cell membrane and structure. Thus, this strict control of oxidative environment is mandatory. In addition, melatonin can have a regulatory function in embryo development. For instance, inseminated mice embryos cultured in a medium supplemented with melatonin have higher rates of fertilization and blastocyst (Ishizuka et al., 2000). This beneficial effect was attributed to the high antioxidant activity of melatonin. In humans, these properties of melatonin are difficult to confirm. However, a recent study showed that infertile women seeking for assisted reproductive technology, in vitro fertilization and embryo transfer taking 3 mg of melatonin a day from day 5 of the previous menstrual cycle to the day of oocyte retrieval had a significant higher percentage of good embryos in the 2nd day after insemination when compared with the control cycle where there was no melatonin treatment (Tamura et al., 2012). Thus, data provide compelling evidence that melatonin is involved in fertilization and pregnancy due to direct and indirect effects.

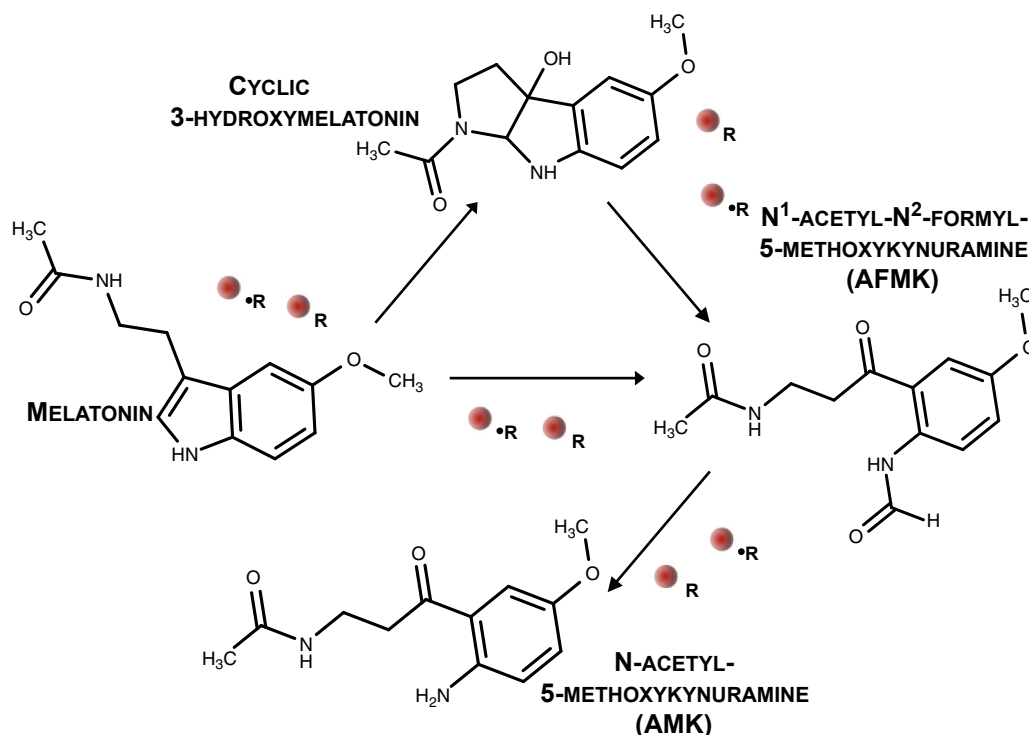


Fig. 2 Simplified overview of the antioxidant cascade of melatonin. When melatonin interacts with oxidants species it can generate cyclic 3-hydroxymelatonin and *N*¹-acetyl-*N*²-formyl-5-methoxykynuramine (AFMK). Both these compounds are also radical scavengers leading to the formation of *N*¹-acetyl-5-methoxykynuramine (AMK). This latter molecule is a radical scavenger as well. This cascade of reactions increases the effective concentration and scavenging capacity of melatonin.

Melatonin's antioxidant properties are also very important to prevent testicular damage caused by environmental aggressions or inflammation. Testicular torsion, which is a common and frequent pathology, induces several damages that can be prevented by treatment with melatonin which can decrease germ cell apoptosis, increase cell proliferation and testosterone production (Kanter, 2010). Most of those effects seem to be mediated by the antioxidant effect of melatonin that prevents the boost of free radicals that are formed during testicular torsion. That process is also suggested to mediate several other regulatory functions of melatonin in male reproductive system, particularly in sperm, since the increase of free radicals is directly correlated with poor semen quality and function. Spermatozoa are very susceptible to oxidative stress due to the presence of a large quantity of polyunsaturated fatty acids in their membranes along with a low quantity of antioxidant scavenging enzymes. There are several studies showing a positive effect of melatonin supplementation in sperm quality but the mechanisms by which it acts remain a matter of debate. Further studies are still needed to elucidate the molecular regulatory function of melatonin in sperm production and function. Moreover, melatonin has also been reported to prevent testicular damages and dysfunction promoted by toxic agents, such as bisphenol A, cadmium, diazinon, among others. The treatment of mice with melatonin after exposure to a chemotherapeutic agent, cyclophosphamide, reduced testicular lipid peroxidation and other oxidative damages illustrating the potential of this hormone to protect the male reproductive system during chemotherapy.

The aging-associated regulatory functions of the pineal gland are also under constant debate and might contribute to the reproductive decline that is frequently linked with aging. It has been shown that endogenous levels of melatonin consistently decrease with age and thus, the regulatory function of pineal gland becomes very relevant as the lack of the antioxidant properties of melatonin may result in oxidative damage in cells. Thus, it is still under debate the contribution of decreased pineal gland functioning during aging and its reproductive outcomes. Obviously, all circadian rhythm disorders such as jet lag, sleep-related problems and syndromes, including those in the elderly, are under the regulatory function of the pineal gland. Thus, taking in consideration the enormous range of pathologies and the high prevalence of those disorders, the biological regulatory function of pineal gland have emerged as a major clinical field of research.

Concluding Remarks

The regulatory functions of the pineal gland occur through several mechanisms, mostly orchestrated by melatonin, which exerts an action at multiple levels. However, melatonin is not just a pineal hormone since it is also peripherally produced by other organs. It regulates several relevant biological processes and not only reproduction, including in humans. In addition, some important regulatory functions of pineal gland are manipulated by melatonin administration to simulate a different photoperiod. That has important commercial relevance since it can, for instance, allow the control of reproduction in ruminants or the winter coat in most species including cashmere of goats and arctic foxes.

Melatonin is a molecule with multitasking properties and currently, clinical evidence supports a key role for this hormone in reproduction and reproductive processes both, in males and females. In addition, it appears as pivotal for puberty onset but it is difficult to study the specific signals coordinated by pineal gland as during this period there is a complex interplay among neuropeptides, neurotransmitters, steroids and other molecules that interact with the hypothalamic–pituitary–ovarian and the hypothalamic–pituitary–testis axes. Thus, contrarily to hormones synthesized in other glands, melatonin's optimal concentrations for each effect remains obscure and much research is still needed. In addition, most studies are performed in rodents making it very difficult to extrapolate to humans. Currently, melatonin-based therapies are still under debate and studies are needed to consubstantiate the health benefits of this hormone for each particular condition. The pineal gland emerged as an important regulatory organ of several pivotal functions, including reproduction since melatonin has a very low toxicity and can readily cross biological barriers such as the blood testis barrier.

References

- Acuna-Castroviejo, D., Escames, G., Rodríguez, M. I., & Lopez, L. C. (2007). Melatonin role in the mitochondrial function. *Frontiers in Bioscience*, 12, 947–963.
- Bentley, G. E., Ubuka, T., McGuire, N. L., et al. (2009). Gonadotrophin-inhibitory hormone: a multifunctional neuropeptide. *Journal of Neuroendocrinology*, 21, 276–281.
- Brzezinski, A., Lynch, H. J., Wurtman, R. J., & Seibel, M. M. (1987). Possible contribution of melatonin to the timing of the luteinizing hormone surge. *New England Journal of Medicine*, 316, 1550–1551.
- Glass, J. D., & Knotts, L. K. (1987). A brain site for the antigonadal action of melatonin in the white-footed mouse (*Peromyscus leucopus*): involvement of the immunoreactive GnRH neuronal system. *Neuroendocrinology*, 46, 48–55.
- Horton, T. H., Ray, S. L., & Stetson, M. H. (1989). Maternal transfer of photoperiodic information in Siberian hamsters. III. Melatonin injections program postnatal reproductive development expressed in constant light. *Biology of Reproduction*, 41, 34–39.
- Ishii, H., Tanaka, N., Kobayashi, M., et al. (2009). Gene structures, biochemical characterization and distribution of rat melatonin receptors. *Journal of Physiological Sciences*, 59, 37–47.
- Ishizuka, B., Kuribayashi, Y., Murai, K., et al. (2000). The effect of melatonin on in vitro fertilization and embryo development in mice. *Journal of Pineal Research*, 28, 48–51.
- Kanter, M. (2010). Protective effects of melatonin on testicular torsion/detorsion-induced ischemia–reperfusion injury in rats. *Experimental and Molecular Pathology*, 89, 314–320.
- Kaupilla, A., Kivela, A., Pakarinen, A., & Vakkuri, O. (1987). Inverse seasonal relationship between melatonin and ovarian activity in humans in a region with a strong seasonal contrast in luminosity. *Journal of Clinical Endocrinology and Metabolism*, 65, 823–828.
- Kivela, A., Kaupilla, A., Ylostalo, P., et al. (1988). Seasonal, menstrual and circadian secretions of melatonin, gonadotropins and prolactin in women. *Acta Physiologica Scandinavica*, 132, 321–327.

- Levine, R. J., Mathew, R. M., Chenault, C. B., et al. (1990). Differences in the quality of semen in outdoor workers during summer and winter. *New England Journal of Medicine*, 323, 12–16.
- Luboshitzky, R., Shen-Orr, Z., Nave, R., et al. (2002). Melatonin administration alters semen quality in healthy men. *Journal of Andrology*, 23, 572–578.
- Reiter, R. J., Tan, D. X., Manchester, L. C., et al. (2009). Melatonin and reproduction revisited. *Biology of Reproduction*, 81, 445–456.
- Reiter, R. J., Tan, D. X., Tamura, H., et al. (2014). Clinical relevance of melatonin in ovarian and placental physiology: a review. *Gynecological Endocrinology*, 30, 83–89.
- Rocha, C. S., Martins, A. D., Rato, L., et al. (2014). Melatonin alters the glycolytic profile of Sertoli cells: implications for male fertility. *Molecular Human Reproduction*, 20, 1067–1076.
- Rocha, C. S., Rato, L., Martins, A. D., et al. (2015). Melatonin and male reproductive health: relevance of darkness and antioxidant properties. *Current Molecular Medicine*, 15, 299–311.
- Rossi, S. P., Matzkin, M. E., Terradas, C., et al. (2012). New insights into melatonin/CRH signaling in hamster Leydig cells. *General and Comparative Endocrinology*, 178, 153–163.
- Sharkey, J. T., Puttaramu, R., Word, R. A., & Olcese, J. (2009). Melatonin synergizes with oxytocin to enhance contractility of human myometrial smooth muscle cells. *Journal of Clinical Endocrinology and Metabolism*, 94, 421–427.
- Tamura, H., Nakamura, Y., Korkmaz, A., et al. (2009). Melatonin and the ovary: physiological and pathophysiological implications. *Fertility and Sterility*, 92, 328–343.
- Tamura, H., Takasaki, A., Taketani, T., et al. (2012). The role of melatonin as an antioxidant in the follicle. *Journal of Ovarian Research*, 5, 5.
- Waldhauser, F., Weiszenbacher, G., Frisch, H., et al. (1984). Fall in nocturnal serum melatonin during prepuberty and pubescence. *Lancet*, 1, 362–365.
- Waldhauser, F., Boepple, P. A., Schemper, M., et al. (1991). Serum melatonin in central precocious puberty is lower than in age-matched prepubertal children. *Journal of Clinical Endocrinology and Metabolism*, 73, 793–796.
- Wurtman, R. J., Axelrod, J., & Chu, E. W. (1963). Melatonin, a pineal substance: effect on the rat ovary. *Science*, 141, 277–278.
- Zemkova, H., & Vanecek, J. (1997). Inhibitory effect of melatonin on gonadotropin-releasing hormone-induced Ca^{2+} oscillations in pituitary cells of newborn rats. *Neuroendocrinology*, 65, 276–283.

Further Reading

- Davis, F. C., & Mannion, J. (1988). Entrainment of hamster pup circadian rhythms by prenatal melatonin injections to the mother. *American Journal of Physiology*, 255, R439–448.

Male contraception

Peter Y Liu, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States; and Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute (LA BioMed), Torrance, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The Need for Contraception

Unintended pregnancies are unwanted, mistimed, or both unwanted and mistimed. Almost half of all pregnancies that occur in industrialized nations are unintended. Unintended pregnancies increase the risk for lower birth weights, poorer health outcomes, retarded development, and compromised education in the offspring, as well as postpartum depression and ongoing mental health problems in the mother. These adverse health and social consequences impact the entire family, and result in substantial societal costs that have been estimated to exceed \$15 billion in a single year in the United States alone. Broadening contraceptive choice by allowing both men and women to fully share family planning responsibilities would promote important, but largely unmet individual, couple, and societal needs; reduce the number of elective terminations; and decrease overpopulation.

In the United States, 30% of couples rely solely on a male-directed method (male sterilization, condom, withdrawal). However, the proportion of all couples that are using male-directed methods varies greatly from 0% to 50% throughout the world. Given this variability, the median (rather than the average) proportion of couples using male-directed methods across over 60 nations surveyed is approximately 10% ([United Nations, 2015](#)). After excluding couples that are not using any male or female-directed contraceptive method, about 20%, and up to 80% in some countries, rely on a male-directed method. These data confirm that many couples already depend upon male-directed methods despite such methods being far from ideal. Usage would presumably be even greater if male-directed methods could be developed that were more effective, convenient, reversible, and safe.

Features of an Ideal Contraceptive

An ideal male-directed contraceptive is safe, rapidly and consistently effective (i.e., usable by all men) and rapidly and consistently reversible (i.e., reversible in all men). In contrast, the two currently available male-directed methods are not widely acceptable because vasectomies are not easily reversible and condoms are not very effective. During the last few decades, many new female-directed reversible hormonal contraceptive options including pills, patches, injections, implants, and intrauterine devices with various drug and dose combinations have been marketed. In contrast, there has not been a single reversible male-directed contraceptive developed over this same period. Currently, the only reversible male-directed method available is the condom, which was invented 400 years ago and has failure rates as high as 20% in the first year. New and effective options are needed to broaden choice for couples.

Male Hormonal Contraception

Summary

More than 2000 eugonadal men with unimpaired fertility at baseline have been exposed to over 2000 person-years of male hormonal contraception (MHC, consisting of androgens with or without progestins) to assess contraceptive efficacy (i.e., the ability to prevent pregnancies) ([Table 1](#)). These studies show the effectiveness, reversibility and short-term safety of MHC. MHC satisfies many of the requirements for an ideal contraceptive method that is male-directed. The hope is that a range of MHC treatment options will become available to fulfill differing needs and preferences of couples.

Androgen-progestin MHC treatment regimens reduce sperm output and induce a predictable degree of infertility by exploiting the negative feedback suppression of pituitary gonadotropin secretion by sex hormones. The goal of male hormonal contraceptive methods, according to expert consensus opinion, is to achieve and maintain severe oligozoospermia, defined as a sperm concentration in the ejaculate that is below 1 million/mL, in all users. If this can be consistently achieved and maintained, then contraceptive failure (i.e., pregnancy) rates of 0.6 (95% confidence interval CI 0.09–2.7) % per year can be expected ([WHO Task Force on Methods for the Regulation of Male Fertility, 1996](#)). These failure rates are comparable to those achieved with perfect use of female hormonal contraceptive methods. Even higher failure rates of 1.4 (0.4–3.7) % occur with a higher threshold of 3 million/mL. These data indicate that the degree of infertility induced can be predicted from the semen analysis, and is consistent with semen analysis being the most widely used clinical measure of spermatogenesis and male fecundity.

Mechanism of Action

Spermatogenesis is tightly regulated by the hypothalamo-pituitary-gonadal network through stimulatory feedforward and inhibitory feedback signaling ([Liu and Veldhuis, 2014](#)). The decapeptide, gonadotropin-releasing hormone (GnRH), is secreted in a pulsatile and synchronized fashion by approximately 1200 specialized neurons in the arcuate-nucleus of the mediobasal hypothalamus of the human. GnRH is secreted in minute quantities directly into a portal microvasculature system, where anatomical proximity

Table 1 Contraceptive failure rates of MHC

References	Regimen	Enrolled (n)	Target sperm concentration (M/mL)	Documented failure to suppress by 6 months n (%)	Median time to enter efficacy (months)	Maximum treatment duration (months)	Entered efficacy (n)	Sperm rebound during efficacy n (%)	Pregnancies during efficacy n (%)	Failure rate/100 couple years
Gu et al. (2009)	^a TU	1045	1	43 (4)	3.6	30	855	10 (1.2)	9 (1.1)	1.1 (0.4–1.8)
WHO (1996)	^b TE	399	3 ^c	< 8 (2) ^c	2.2	18	349	4 (1.1)	4 (1.1)	1.4 (0.4–3.7)
Behre et al. (2016)	^d TU + NET-EN	320	1	9 (3)	3	18	266	6 (2.3)	4 (1.5)	2.2 (0.8–5.8)
Gu et al. (2003)	^a TU	308	3	9 (3)	2–3	12	296	6 (2.0)	1 (0.3)	2.3 (0.5–4.2)
WHO (1990)	^b TE	271	0	68 (25)	< 6	18	157	11 (7.0)	1 (0.6)	0.8 (0.02–4.5)
Turner et al. (2003)	^e Ti + DMPA	55	1	2 (4)	1.8	18	51	0 ^f	0	0 (0.0–8.0)
McLachlan et al. (2000)	^g Ti + various	36	1	8 (22)	Not reported	18	21	4 (19.0)	0	Not reported

^aTestosterone undecanoate 500 mg/month (with 1000 mg loading dose).^bTestosterone enanthate 200 mg/week.^cOriginal threshold was 5 M/mL, but data shown here is for those who suppressed to no more than 3 M/mL.^dTestosterone undecanoate 1000 mg and norethisterone enanthate 200 mg every 8 weeks.^eTestosterone implant 800 mg every 4 or 6 months with depot medroxyprogesterone acetate 300 mg every 3 months.^fFour had sperm rebound and symptoms of androgen deficiency in the group receiving testosterone implants 800 mg every 6 months.^gTestosterone implant 800 mg or 1200 mg every 3 months with or without finasteride.

allows entrainment of pituitary gonadotropes to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Pulsatile LH and pulsatile FSH circulate and act on distant testicular Leydig and Sertoli cells, to stimulate testosterone production and spermatogenesis, respectively. Circulating testosterone inhibits both hypothalamic GnRH, and pituitary LH and FSH secretion. The feedback inhibition of pituitary gonadotropins is through local aromatization to estradiol. Both FSH and high intratesticular levels of testosterone are required for spermatogenesis.

Male hormonal contraception exploits this negative feedback inhibition to impair spermatogenesis, in a manner that is analogous to female hormonal methods that prevent ovulation. The administration of exogenous androgens, with or without the addition of progestins, suppresses GnRH, LH, and FSH, reduces testicular testosterone concentrations and impairs spermatogenesis. In this context, exogenous progestins act to augment inhibition of the secretion of pituitary gonadotropins, so that unequivocally supraphysiological exogenous testosterone administration is no longer required. Other compounds could also be used alone or in combination to suppress pituitary gonadotropins more completely. Some of these, such as GnRH antagonists or non-pulsatile GnRH analogues that downregulate the GnRH receptor, have already been shown to reduce sperm output in humans, whereas other putative compounds that act upstream of the GnRH neuron through the kisspeptin-neurokinin B-dynorphin pathway remain to be directly tested for contraceptive efficacy. In all male hormonal contraceptive methods, testosterone or another androgen must be administered to maintain systemic testosterone exposure, in order to prevent the symptoms and consequences of unwanted hypogonadism.

Suppression of LH and FSH acutely decreases type B spermatogonia and blocks spermatogonial differentiation. A reduction in round and elongated spermatid production, and ultimately spermatozoa concentrations in the ejaculate, will therefore occur, but requires several weeks due to the duration of the spermatogenic cycle. Whereas azoospermia, the complete absence of spermatozoa in the ejaculate, would render fertilization impossible, this has not been consistently achievable with MHC in all users, nor has it proved necessary. Mechanistically, the physiological bases to explain why 5%–15% of men do not ever suppress sperm output to the desired goal of < 1 million/mL, are unknown. It could be that some men have low basal levels of gonadotropin independent spermatogenesis. Alternatively, the administration of exogenous testosterone itself could raise intratesticular testosterone by advection or diffusion sufficiently to maintain a low level of spermatogenesis in some men. In fact, these very low concentrations of intratesticular testosterone are able to maintain spermatogenesis in rodents and non-human primates; and there is no definable dose of testosterone that would both maintain sexual function and suppress gonadotropins without also activating spermatogenesis in rodents. Indirect evidence in humans supports this contention since the use of higher effective testosterone doses as part of MHC results in a greater proportion of men maintaining a low level of spermatogenesis (i.e., less complete suppression of sperm output) (Liu et al., 2008). However, intratesticular testosterone concentrations have not been consistently different between men who respond with lower or higher sperm output while taking MHC (Roth et al., 2016). Nevertheless, using the lowest effective dose of testosterone is advisable to reduce the theoretical risk of breakthrough spermatogenesis, and decrease androgenic side effects such as acne and male pattern balding.

Acceptability of MHC by Couples

Surveys of actual self-reported behaviors show that the male partner is solely responsible for the couple's contraceptive needs in a large proportion, indicating that women can and do trust their partners to share the responsibility to space and time pregnancies. Consistent with this finding, women surveyed concerning their attitudes to a theoretical MHC also indicate that this would be acceptable to them, provided it was a decision shared with their male partner, not with any man in general (Wang et al., 2016). At least a quarter of men, and in some cases many more, would be willing to use male hormonal contraception. These data together suggest that MHC would be widely used by men in cooperation with their female partners, if and when such methods become available. Furthermore, these surveys of a theoretical MHC were conducted in many large cities in Asia, North and South America, Europe and Africa, and indicate that MHC is acceptable by many couples from very diverse backgrounds throughout the world. However, marked geographical variability in theoretical acceptability was apparent, and is consistent with data of actual usage of existing male-directed methods (male sterilization, condom, withdrawal) discussed earlier. Understanding the cultural, religious or other attitudes that underpin this variability may inform strategies to improve usage of male-directed methods in general. Societal changes in attitudes as well as drug development may therefore both be needed to increase usage.

Men participating in MHC studies report that intramuscular injections every 1–3 months, subcutaneous implants every 3 months, or transdermal gels applied every day are acceptable contraceptive methods; however the reports may be biased by selection for, and active participation in, a clinical trial. Reliably understanding the factors that influence acceptability of MHC may therefore require such methods to first become available. Surveys of men contemplating the use of a theoretical MHC product indicate greater acceptability with oral mode of delivery, and higher income and education. However, female-directed methods that require daily dosing are less reliable due to irregular compliance, compared with long-term injectable and implantable contraceptive options. Male-directed methods that also require daily dosing (such as oral or transdermal delivery) are therefore also likely to be less reliable with typical, rather than perfect use. Nevertheless, data from potential users, and actual participants in clinical trials of specific MHC, both indicate that MHC is acceptable to many men and their partners. Multiple MHC treatment options, as exists for female-directed methods, need to be developed to broaden choice.

Effectiveness of MHC

The contraceptive effectiveness of MHC is highly predictable and varies depending on the threshold sperm output achieved, and how consistently suppression to this threshold can be maintained. Mechanistically, this contrasts greatly with hormonal methods in women which either prevent, or do not prevent, ovulation during each menstrual cycle. Men have been exposed to over 3000 person years of androgens with or without progestins, administered to determine the effect on sperm output, or on contraceptive failure rates. Although androgen-progestin drug combinations account for less than one-fifth of the total exposure, many different drug combinations have already been evaluated. This is because the specific delivery, dose and drug combination influences the rate and extent of spermatogenic suppression, which must be individually quantified in order to plan for a large scale contraceptive efficacy study (Liu et al., 2008). To date, only testosterone, or combinations of testosterone with a progestin, have been evaluated to determine contraceptive failure rates (Table 1). However, two novel synthetic androgens, 7- α -methyl-19-nortestosterone and dimethandrolone (7 α , 11 β -methyl-19-nortestosterone), have undergone preliminary testing of formulation and dose in humans, with suppression of sperm output as the surrogate endpoint. Both androgens are considerably more potent than testosterone, dimethandrolone also exhibits progestational activity, but cannot be aromatized, whereas 7- α -methyl-19-nortestosterone lacks progestational activity, is aromatized, but is resistant to 5- α reduction. These biochemical characteristics may allow for the development of a single oral agent with both androgenic and progestational activity (dimethandrolone), or a more favorable risk profile that spares the prostate from overandrogenization by preventing 5- α reduction (7- α -methyl-19-nortestosterone). Other synthetic androgens could also be developed with specific properties designed to increase effectiveness or limit adverse effects.

Weekly supraphysiological testosterone therapy is a highly effective contraceptive (i.e., prevents pregnancies) and profoundly suppresses spermatogenesis through negative feedback inhibition of gonadotropins (WHO Task Force on Methods for the Regulation of Male Fertility, 1990, 1996; Table 1). Longer-acting testosterone formulations administered alone (McLachlan et al., 2000; Gu et al., 2003, 2009) or in combination with either depot medroxyprogesterone acetate (DMPA) (Turner et al., 2003) or norethisterone enanthate (NET-EN) (Behre et al., 2016) are also highly effective (Table 1). Individual large scale studies show contraceptive failure rates of 0.8%–2.3%, with upper 95% confidence limits of 1.8%–5.8% (Table 1). However, some of these studies did not target sperm concentration to <1 million/mL, and hence may not be applicable for future MHC development.

An integrated analysis of individual participant data of all then-available studies report that 50% and 85% of men suppress sperm output to concentrations compatible with reliable contraception (<1 million/mL), by 3 and 6 months, respectively (Liu et al., 2008). However, this analysis included many exploratory MHC regimens where drug dose and frequency had not yet been optimized. More realistic estimates would be obtained by only examining contraceptive efficacy studies, which are usually extensively tested and optimized. These show that 80%–95% of men suppress sperm output to a threshold of 1 million/mL by 3 months (Table 1). The timeframe for this reduction in sperm output compares favorably with the disappearance of sperm after vasectomy.

Progestin co-administration enhances both the rate and extent of sperm output suppression by up to two-fold (Liu et al., 2008). Certain progestins are likely to be more effective in suppressing sperm output than others, due to differences in binding and

activation of progesterone and other steroid receptors. Pharmacokinetic differences are known to explain variation in anovulatory potency and the ability to support pregnancy in women. The hope is that a fully optimized androgen-progestin combination could be utilized by all men within a practical timeframe. To date however, at least 5% of men enrolled in MHC trials never suppress spermatogenesis to levels required for reliable contraception, for mechanistic reasons that are not entirely clear. Another limitation of the method is that sperm rebound, which occurs when sperm suppression below the target threshold is not maintained, is observed in approximately 2% of men. Specifically, 20 (1.7%) of 1193 men who initially suppressed sperm output to below the target of 1 million/mL experienced sperm rebound (Table 1). There have not been systematic attempts to determine the characteristics of this group of men, and the mechanisms underlying sperm rebound are not known. Sperm rebound is analogous to breakthrough ovulation that can sometimes occur in women, particularly when using lower dose hormonal contraceptive methods. In men, it cannot be solely explained by poor compliance since study personnel administered MHC in many of these studies.

It is also possible that MHC may not universally suppress sperm output adequately in all men, even after extensive drug and dose optimization. Being able to predict non-suppression for any given drug and dose combination used for MHC would then be important. Systematic analyses show that Caucasian ethnicity and higher BMI are important predictors of non-suppression, although the effect of BMI is small and may not be clinically relevant (Liu et al., 2008). Slower spermatogenic suppression occurs in Asian men, and also with older age, higher baseline testosterone and higher initial sperm concentration, but the independent effect sizes of these latter parameters are small. The large and differing effects of ethnicity on the rate and extent of spermatogenic suppression observed between Caucasian and Asian men may eventually be explained by pharmacogenetic differences, which may ultimately unveil more specific methods to identify the small number of men who do not suppress sperm output adequately.

Reversibility of MHC

An integrated analysis of individual participant data of all then-available studies demonstrated that it is realistic to expect full recovery of spermatogenesis to levels consistent with normal male fertility for all men ceasing male hormonal contraceptives (Liu et al., 2006). At the time, normal male fertility was defined by a sperm concentration of at least 20 million/mL, although sperm concentrations of only 13–15 million/mL are now known to be sufficient, based on an analysis published in 2010 (Cooper et al., 2010). Recovery of sperm output to concentrations consistent with normal male fertility occurs in more than 50% of all men after 6 months, and over 90% of all men by 12 months. The remaining men, if not all men who should recover, do so within 24 months. Older age, Asian ethnicity, lower baseline LH concentration, higher initial sperm concentration and faster initial suppression of sperm output predicted faster recovery. Amongst treatment-related factors, shorter treatment duration and the use of shorter-acting testosterone formulations were associated with faster recovery. Treatment duration had the largest clinically important effect.

Since the publication of the integrated analysis, three large studies, including two contraceptive efficacy studies (Gu et al., 2009; Behre et al., 2016), have been completed (Gu et al., 2009; Mommers et al., 2008; Behre et al., 2016). The first study was conducted in China (Gu et al., 2009), and reported a median time of 7.6 months for sperm output to recover to 20 million/mL (Gu et al., 2009). This is considerably slower than the median recovery time of 3.4 (95%CI 3.2–3.5) months calculated from all previous studies, particularly since faster recovery would have been anticipated for this Asian population (Liu et al., 2006). However, the slower recovery was likely explained by the longer treatment duration of up to 30 months, compared with 12–18 months utilized in all other studies (Table 2). The timeframe for recovery of sperm output following cessation of MHC in this study was also consistent with the previous analyses: all except 17 men had recovered by 12 months, and by 15 months one man had a sperm concentration of 13 M/mL (and was then not followed up) and the other presumably never recovered due to intercurrent epididymitis (Gu et al., 2009). The second study was conducted in Europe (Mommers et al., 2008), and 354 men were randomized to receive placebo (53 men) or various regimens of testosterone and the progestin, etonogestrel, to assess suppression of sperm output. The use of a placebo was possible because sperm output, not contraceptive efficacy (i.e., pregnancy rates) was being assessed. Men were treated for 42 or 44 weeks, and amongst those who received active therapy, the median time to recover to a sperm concentration of 20 million/mL was 3.5 months, and by 16 months all men completing follow up had recovered. These data are also compatible with the earlier integrated analysis. Remarkably, two or three men treated with placebo had sperm concentrations <20 million/mL at every assessment during the 24 week follow-up, and up to 14 men (28% of all men treated with placebo) did not consistently maintain sperm concentrations above 20 million/mL during the recovery period. These data from men treated with placebo indicates that supposed non-recovery of sperm output documented in a small number of men treated with MCH could represent regression to the mean, the occurrence of an intercurrent partially sterilizing illness, or both.

The final study was sponsored by the World Health Organization and was conducted in Europe, Asia, South America, and Australia (Behre et al., 2016). Men were treated with a testosterone-progestin combination for up to 18 months, 266 men suppressed sperm output to <1 million/mL, and 96 of these men were discontinued because the study was prematurely terminated (see next section on adverse events). Unlike any of the earlier contraceptive efficacy studies, normal male fertility was defined by a sperm concentration of 15 million/mL or a total sperm count of 39 million per ejaculate, since contemporaneous analyses had established this lower threshold to be compatible with normal male fertility (Cooper et al., 2010). The men treated in this study therefore had a lower initial sperm concentration compared with all previous studies. Interestingly, recovery was slightly delayed, with the median time to recovery to this lower threshold being approximately 6.5 months. This delay is consistent with the earlier integrated analysis which showed slower recovery to either a sperm concentration of either 10 or 20 million/mL, with lower initial sperm concentration. The extent of recovery was also compatible with the earlier integrated analyses since all participants, except 8, recovered by 12 months, and another 5 by 18 months. One participant did not recover even after 4 years, but sperm concentrations were not

Table 2 Pregnancy outcomes of MHC

References	Pregnancies												Recovery of sperm output		
	Pregnancy outcome						Pregnancy outcome						Known pregnancies during and after treatment (n)	Maximum treatment duration (months)	Median time to recovery 20 M/mL (months)
	Pregnancies during treatment (n)	LB	SA	IA	UK	CM	Pregnancies after treatment (n)	LB	SA	IA	UK	CM			
^a Gu et al. (2009)	28	0	0	0	28	0	Not reported	–	–	–	–	–	28	30	7.6
^b WHO (1996)	19	10	4	5	0	0	33	25	1	4	3	0	52	18	2.3
^c Behre et al. (2016)	4	3	0	1	0	0	Not reported	–	–	–	–	–	4	18	6.5 ^d
^a Gu et al. (2003)	4	0	0	0	4	0	3	0	0	0	3	0	7	12	2–3
^b WHO (1990)	10	3	0	6	1	0	10 ^e	4	1	2	3	0	20	18	3.7
^f Turner et al. (2003)	0	0	0	0	0	0	5	3	1	0		1 ^g	5	18	5.0
^h McLachlan et al. (2000)	2	2	0	0	0	0	Not reported	–	–	–	–	–	2	18	Approx. 7

LB, live birth; SA, spontaneous abortion; IA, induced abortions; UK, unknown; CM, congenital malformation.

^aTestosterone undecanoate 500 mg/month (with 1000 mg loading dose).

^bTestosterone enanthate 200 mg/week.

^cTestosterone undecanoate 1000 mg and norethisterone enanthate 200 mg every 8 weeks.

^dRecovery to 15 M/mL, not 20 M/mL.

^eDoes not include one pregnancy by a man other than the partner.

^fTestosterone implant 800 mg every 4 or 6 months with depot medroxyprogesterone acetate 300 mg every 3 months.

^gOne of the twins was born with Vater Anomalad, which was thought to be unrelated to the study drug.

^hTestosterone implant 800 mg or 1200 mg every 3 months with or without finasteride.

reported, so it is not known whether he remained severely oligozoospermic which could suggest an intercurrent sterilizing disorder, or had values that were closer to 13–15 million/mL which could suggest regression to the mean.

Together these new data from three large trials verify the earlier analyses that concluded that it is reasonable to expect full recovery of spermatogenesis to levels consistent with normal male fertility, and that the rate of recovery is dependent upon treatment duration and initial sperm concentration. Documented non-recovery of sperm output has been reported in only three men from over 2000 men enrolled in MHC trials (Turner et al., 2003; Gu et al., 2009; Behre et al., 2016). Of these three men, an intercurrent and unrelated sterilizing process, rather than any lasting adverse effect of MHC, was more likely to be the true cause: myotonic dystrophy in one man (Turner et al., 2003) and bilateral epididymitis in another (Gu et al., 2009). In the third man, important details regarding his final sperm concentration were not reported (Behre et al., 2016). Important limitations are that few data of MHC are available for men of African or Hispanic origin, or in subfertile men. Another limitation is that systematic studies of sperm recovery after long-term treatment of eugonadal men with androgens or androgen-progestins for contraceptive or non-contraceptive purposes are not available. Case reports do however suggest that sperm quality tends to recover spontaneously within 4–12 months of cessation of high dose androgens in long-term anabolic steroid abusers.

Actual pregnancies and live births have also been observed in couples after cessation of MHC. Specifically, all couples known to be actively seeking parenthood have reportedly been able to do so, and 51 pregnancies resulting in 32 live births have been identified (Table 2). A limitation of this analysis is that many couples were likely using other forms of contraception after cessation of MHC if parenthood was still not desired, and long term follow up of subsequent fertility was not systematically obtained.

Safety of MHC

Pregnancy outcomes occurring during or after MHC treatment suggest no increase in fetal miscarriage rates or congenital malformations (Table 2). Altogether, 118 pregnancies were reported which resulted in 50 live births, 7 spontaneous abortions, 18 induced abortions, and 1 congenital malformation. Fetal outcomes from the remaining 42 pregnancies were not reported. This corresponds with spontaneous miscarriage rates of 6% (95%CI 2%–12%) assuming no miscarriages amongst pregnancies with unknown outcomes. These miscarriage rates overlap with spontaneous abortion rates of 8%–20% in the general population. The aggregate congenital malformation rate was 0.9% (95%CI 0.0%–4.6%) assuming no malformations amongst pregnancies with unknown outcomes. Accordingly, these data are also consistent with, but are insufficiently powered to exclude the possibility of an increase over the 4% congenital malformation rate in the general population.

A recent methodological advance has been to conduct a randomized placebo controlled androgen-progestin MHC trial from which short-term adverse events due to active treatment could be defined for the first time (Mommers et al., 2008). Almost all men on either active (93%) or placebo (81%) therapy self-reported at least one adverse event, which was mild in severity in almost all cases. However, men receiving active treatment complained twice as frequently than those receiving placebo for certain adverse events: 20% versus 8%, on average for mood and libidinal changes, weight gain, acne and night sweats. These complaints were statistically more prevalent in those treated with active, were generally mild but occasionally led to subject discontinuation (Mommers et al., 2008). Many factors, particularly biochemical factors, were considered clinically irrelevant. Total and high density lipoprotein cholesterol both fell by about 10%, and none of the lipid changes in any treatment or placebo group were statistically significant.

Despite or possibly because of these findings, a recent androgen-progestin MHC efficacy study was prematurely stopped due to concerns regarding mood changes, depression, pain at injection site and increased libido, even though the independent data and safety monitoring board charged with directly overseeing this study found that all criteria for continuation had been met after reviewing the same adverse event data (Behre et al., 2016). Most of these adverse events were mild in severity, only 20 men actually discontinued due to these complaints, and to the contrary, participants themselves reported high levels of satisfaction with the method. Self-reported mood changes and depression in particular, were highly variable across study sites, suggesting interaction with non-drug related factors. Furthermore, depression and mood were only self-reported, and not systematically verified using validated instruments.

In women, the mood changes that occur with estrogen-progestin contraceptives have been attributed to the progestin, and this could also be true for the mood changes reported in both of these androgen-progestin MHC studies. Activation of the gamma-aminobutyric acid type A receptor, which is believed to be important for these mood changes, is also different depending on specific progestins and how they are metabolized. Accordingly, certain progestins could have minimal or no mood problems, but this requires verification. Ultimately, postmarketing surveillance of specific androgen-progestin regimens will be required to properly assess infrequently occurring adverse events. Determining long-term adverse cardiovascular or prostate effects of MHC will also likely require phase four studies since MHC will be most used by younger men in whom the background incidence of either cardiovascular or prostate disease is very low (Piotrowska et al., 2016). It may be that some couples will be willing to accept the theoretical risk of MHC, if the benefits of such therapy in the context of all available options are well-defined. In older men with age-related partial androgen deficiency, for example, lingering concerns regarding the cardiovascular or prostate safety of testosterone therapy remain, yet many men choose to be treated.

Non-hormonal Male Contraception

In contrast to hormonal methods where large-scale multicenter pivotal trials necessary for drug registration by the Food and Drug Administration in the United States are due to start by 2018, the majority of potential non-hormonal male-directed methods are yet

to be tested in humans (Page et al., 2008). Nevertheless, promising targets in the testis, epididymis, sperm, and sperm-egg interaction that appear to be specific and necessary for spermatogenesis, and/or fertilization have been identified. Drugs that can act reversibly and solely on these targets should have few or no systemic adverse effects if these targets are truly specific to spermatogenesis. Despite promising nonclinical preliminary work, all of the few compounds tested in humans thus far have not met these criteria, thereby illustrating the challenges inherent in developing a non-hormonal contraceptive. Gossypol and triptolide, for example, resulted in irreversible infertility. The bis-(dichloroacetyl)-diamines that have been developed thus far to inhibit aldehyde dehydrogenase 1A2, have also inhibited liver aldehyde dehydrogenase thereby causing unacceptable disulfiram-like reactions when co-administered with alcohol. Aldehyde dehydrogenase 1A2 is specific to the testis, and crucial for the conversion of retinaldehyde to retinoic acid, and blocking retinoic acid production in the testis prevents spermatogonial differentiation through suppression of STRA8. The challenge has been developing compounds that specifically inhibit the Aldehyde dehydrogenase 1A2 isoform present only in the testis. A fourth compound, miglustat, induced reversible infertility by inhibiting glycosphingolipid biosynthesis and sperm motility in mice, but miglustat did not alter spermatogenesis in humans. This illustrates that species differences in spermatogenesis exist, and further complicates the development of specific, reversible nonhormonal methods in humans.

Promising testicular targets include bromodomain testis-specific protein-1, which is critical for chromatin remodeling during spermatogenesis, and retinoic acid receptors, activation of which is required for spermatogonial differentiation. Epididymal targets include the epididymal G-protein coupled receptor HE6, which is responsible for reabsorption of tubular fluid, and therefore the sperm microenvironment. Other sperm targets include: epididymal protease inhibitor (EPPIN) which prevents binding of semenogelin 1, a semen coagulation protein, and therefore prevents impaired sperm motility, and; voltage-gated calcium-permeable ion channels (CatSper), which are important for sperm hyperactivation and motility.

Developing drugs that act on these or other targets would eventually require scalable drug synthesis, stability, and microbial testing, and certification of good manufacturing practice standards. Academic researchers are ill-equipped to meet the regulatory hurdles required for such an undertaking, and hence the National Institute of Child Health and Development has established the male Contraceptive Clinical Trials Network to implement late phase 2 and phase 3 studies for drug registration purposes, and assist in partnering with pharmaceutical industry. Currently this network has focused on hormonal methods. However, the extensive clinical trial experience developed for male hormonal contraceptive methods will be applicable for the testing of non-hormonal approaches, once suitable phase 1 and 2 studies of compounds directed to these targets have been performed.

Summary and Conclusions

Male-directed contraceptive methods are estimated to be worth 40–200 billion dollars worldwide, assuming a market size of 10 million in United States and 50 million worldwide. Many couples already depend upon less than ideal male-directed methods, and there is large unmet need to develop better methods. Despite this, many hurdles remain. Despite this, MHC is the closest to regulatory approval from a drug registration perspective. As discussed, much progress has been made in delineating the effectiveness, reversibility and short-term safety of MHC. Remaining uncertainties include whether such methods can be universally used by all men, how to prevent, predict or monitor sperm rebound, and whether clinically relevant and diagnosable mood changes actually occur, and if it does occur, whether these can be minimized with progestins with optimal characteristics. Fully characterizing the long-term prostate and cardiovascular safety will require post-marketing surveillance, but the low incidence of these disorders in young men provides some reassurance that the absolute risk with MHC is likely to be small. No single female or male directed method will be ideal for all couples at all times, and hence the priority should be to broaden choice to reduce unintended pregnancies, and the individual, family, and societal sequelae that are associated with unintended pregnancies. Male-directed hormonal and non-hormonal methods promise to allow both partners to share equally in family planning responsibilities.

Acknowledgments

This review was supported by the National Center for Advancing Translational Sciences through UCLA CTSI Grant UL1TR000124, Contraceptive Clinical Trials Network HHSN275201300241 and R01 HL124211, and research funds from Los Angeles Biomedical Research Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The funding sources had no role in the writing of the manuscript or the decision to submit it for publication.

References

- Behre, H. M., Zitzmann, M., Anderson, R. A., Handelsman, D. J., Lestari, S. W., McLachlan, R. I., Meriggiola, M. C., Misro, M. M., Noe, G., Wu, F. C., Festin, M. P., Habib, N. A., Vogelsong, K. M., Callahan, M. M., Linton, K. A., & Colvard, D. S. (2016). Efficacy and safety of an injectable combination hormonal contraceptive for men. *Journal of Clinical Endocrinology and Metabolism*, 101, 4779–4788.
- Cooper, T. G., Noonan, E., Von Eckardstein, S., Auger, J., Baker, H. W., Behre, H. M., Haugen, T. B., Kruger, T., Wang, C., Mbizvo, M. T., & Vogelsong, K. M. (2010). World Health Organization reference values for human semen characteristics. *Human Reproduction Update*, 16, 231–245.
- Gu, Y. Q., Wang, X. H., Xu, D., Peng, L., Cheng, L. F., Huang, M. K., Huang, Z. J., & Zhang, G. Y. (2003). A multicenter contraceptive efficacy study of injectable testosterone undecanoate in healthy Chinese men. *Journal of Clinical Endocrinology and Metabolism*, 88, 562–568.
- Gu, Y., Liang, X., Wu, W., Liu, M., Song, S., Cheng, L., Bo, L., Xiong, C., Wang, X., Liu, X., Peng, L., & Yao, K. (2009). Multicenter contraceptive efficacy trial of injectable testosterone undecanoate in Chinese men. *Journal of Clinical Endocrinology and Metabolism*, 94, 1910–1915.

- Liu, P. Y., & Veldhuis, J. D. (2014). The hypothalamo-pituitary unit, testis and male accessory organs. In J. F. Strauss, & R. L. Barbieri (Eds.), *Yen and Jaffe's reproductive endocrinology: physiology, pathophysiology and clinical management* (7th ed.,). Philadelphia: W. B. Saunders.
- Liu, P. Y., Swerdloff, R. S., Christenson, P. D., Handelsman, D. J., Wang, C., & Hormonal Male Contraception Summit Group. (2006). Rate, extent and modifiers of spermatogenic recovery after hormonal male contraception: an integrated analysis. *Lancet*, 367, 1412–1420.
- Liu, P. Y., Swerdloff, R. S., Anawalt, B. D., Anderson, R. A., Bremner, W. J., Elliesen, J., Gu, Y. Q., Kersemaekers, W. M., McLachlan, R. I., Meriggiola, M. C., Nieschlag, E., Sitruk-Ware, R., Vogelsong, K., Wang, X. H., Wu, F. C., Zitzmann, M., Handelsman, D. J., & Wang, C. (2008). Determinants of the rate and extent of spermatogenic suppression during hormonal male contraception: an integrated analysis. *Journal of Clinical Endocrinology and Metabolism*, 93, 1774–1783.
- McLachlan, R. I., McDonald, J., Rushford, D., Robertson, D. M., Garrett, C., & Baker, H. W. (2000). Efficacy and acceptability of testosterone implants, alone or in combination with a 5alpha-reductase inhibitor, for male hormonal contraception. *Contraception*, 62, 73–78.
- Mommers, E., Kersemaekers, W. M., Elliesen, J., Kepers, M., Apter, D., Behre, H. M., Beynon, J., Bouloux, P. M., Costantino, A., Gerbershagen, H. P., Gronlund, L., Heger-Mahn, D., Huhtaniemi, I., Koldewijn, E. L., Lange, C., Lindenberg, S., Meriggiola, M. C., Meuleman, E., Mulders, P. F., Nieschlag, E., Perheentupa, A., Solomon, A., Vaisala, L., Wu, F. C., & Zitzmann, M. (2008). Male hormonal contraception: a double-blind placebo-controlled study. *Journal of Clinical Endocrinology and Metabolism*, 93, 2572–2580.
- Page, S. T., Amory, J. K., & Bremner, W. J. (2008). Advances in male contraception. *Endocrine Reviews*, 29, 465–493.
- Piotrowska, K., Wang, C., Swerdloff, R. S., & Liu, P. Y. (2016). Male hormonal contraception: hope and promise. *The Lancet Diabetes and Endocrinology*, 5, 214–223.
- Roth, M. Y., Page, S. T., & Bremner, W. J. (2016). Male hormonal contraception: looking back and moving forward. *Andrology*, 4, 4–12.
- Turner, L., Conway, A. J., Jimenez, M., Liu, P. Y., Forbes, E., McLachlan, R. I., & Handelsman, D. J. (2003). Contraceptive efficacy of a depot progestin and androgen combination in men. *Journal of Clinical Endocrinology and Metabolism*, 88, 4659–4667.
- United Nations, D. O. E. A. S. A., Population Division 2015. World Contraceptive Use 2015 (POP/DB/CP/Rev2015).
- Wang, C., Festin, M. P., & Swerdloff, R. S. (2016). Male hormonal contraception: where are we now? *Current Obstetrics and Gynecology Reports*, 5, 38–47.
- WHO Task Force on Methods for the Regulation of Male Fertility. (1990). Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet*, 336, 955–959.
- WHO Task Force on Methods for the Regulation of Male Fertility. (1996). Contraceptive efficacy of testosterone-induced azoospermia and oligozoospermia in normal men. *Fertility and Sterility*, 65, 821–829.

COMPARATIVE MAMMALIAN MALE REPRODUCTION

Mammalian Reproduction Overview

William Vincent Holt, University of Sheffield, Sheffield, United Kingdom

Stephen Johnston, The University of Queensland, Gatton, QLD, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Altricial Offspring that are hatched or born in a very immature and helpless condition so as to require parental care for some time.

Delayed implantation Delayed implantation or embryonic diapause is a reproductive strategy used by approximately 100 different mammals in seven or eight different orders. In embryonic diapause, the embryo (blastocyst) does not immediately implant in the uterus after sexual reproduction has created the zygote, but is maintained in a state of dormancy.

Eutheria Is the taxonomic name for the main group of living mammals. This taxon contains the placental mammals, of which humans are one species.

Marsupials Mammals of an order whose members are born incompletely developed (i.e., altricial) and are typically carried and suckled in a pouch on the mother's belly. Marsupials are found chiefly in Australia and New Guinea, and also in America.

Metatheria Is a mammalian clade that includes all mammals more closely related to marsupials than to placentals. It is a slightly more inclusive group than the marsupials; it contains all of the living mammals with abdominal pouches (most female marsupials) as well as their more primitive ancestors and relatives.

Monotreme Any member of the Monotremata, the most primitive order of mammals, characterized by certain birdlike and reptilian features, such as hatching young from eggs, and having a single opening for the digestive, urinary, and genital organs. Platypus and echidnas are the only extant monotremes.

Phylogeny The evolutionary history of a group of organisms, especially as depicted in a family tree.

Prototherian Primitive oviparous mammals belonging to the order Monotremata.

Introduction

The diversity of reproductive strategies and mechanisms exhibited by mammals is bewildering and complex. This is not surprising as there are estimated to be more than 5400 species of mammals worldwide (Wilson and Reeder, 2005), subdivided into 1229 genera (groups of species that are highly related and share common phylogeny). These species have evolved to occupy multiple ecological niches, and therefore exhibit a huge amount of life history variation. Detailed evolutionary studies of mammalian origins have produced some contentious outcomes, partly because phylogenetic trees can be derived using both DNA and protein sequences, as well as paleobiological data interpreted by a diverse array of statistical approaches. However, a relatively recent publication in Science (Meredith *et al.*, 2011) presented a credible attempt to reconcile these divergent views and these authors published a helpful mammalian phylogeny based on data from 164 species.

As a group, mammals are distinguished from other species by their ability to support their newborn offspring with milk, which is produced by the female's mammary glands during lactation. Milk is a rich source of important nutrients, including fatty acids, proteins, vitamins and growth factors, but it also provides newborns with a range of important immunologically active compounds that protect against infections during early postnatal development (Blum and Baumrucker, 2008; Oguchi *et al.*, 1997). Lactation is thought to be an ancient process that evolved in a clade of amniotes (synapsids, or mammal-like reptiles (Kemp, 2007)) which may have existed before the first appearance of mammals on earth (Ofstedal, 2012). Owing to its important role in neonatal development, lactation has persisted in all forms of mammals, but the interactions between mothers and their offspring are highly variable between species. Monotremes (prototheria), i.e., platypus and echidnas, are the only egg laying mammals and yet their neonates still receive milk from their mothers. Likewise, the marsupials (metatheria), whose neonates are still in a fetal and poorly developed condition (altricial) when born are provided with milk for prolonged periods after birth. The placental mammals (eutheria), which constitute the overwhelming majority of mammalian species, despite having evolved placental function to support their offspring during embryonic and fetal growth, still lactate and thus provide their neonates with an important additional source of nutrition in early life. These differences, based on the various ways in which neonates are supported during early postnatal growth, represent a useful criterion for dividing the mammals into three distinct classes with different evolutionary histories. It is, therefore, worth beginning this review by making some general observations about the reproductive mechanisms that characterise these three mammalian groups.

Monotremes

The four extant species of echidna within the order *Tachyglossidae*, together with the duck-billed platypus (*Ornithorhynchus anatinus*) are the egg laying mammals, and show many reproductive characteristics reminiscent of birds and reptiles. These species are geographically restricted and occupy different habitats; echidnas are terrestrial mammals and are found only in Australia and New Guinea while platypus are aquatic, living in the river systems of eastern Australia and Tasmania. Genome research has suggested that monotremes diverged from the other mammalian lineage (therian mammals; which later further diverged, forming the marsupials and the placental mammals) about 166 million years ago. Detailed studies of platypus and echidna reproductive biology have only been conducted over the last few decades (see, e.g., [Grant and Temple-Smith, 1998](#); [Morrow and Nicol, 2009](#)) and given that these species are secretive and until recently ([Wallage et al., 2015](#)) have not been easy to breed in captivity, knowledge about their general biology is still rather rudimentary ([Johnston and Keeley, 2015](#)). In fact, some aspects of their biology seemed highly improbable to earlier generations of scientists, and indeed, when the first description of a platypus was sent to the Royal Society in London in the early 19th century ([Home, 1802](#)), it was widely regarded as a hoax!

Echidnas and the platypus are both seasonal breeders, and it is worth noting here that seasonality is an important reproductive adaptation for many, if not most, mammals. For detailed information on comparative mammalian reproductive biology the reader should consult the encyclopaedic book by Asdell entitled "Patterns in mammalian reproduction" ([Asdell, 1965](#)) and a subsequent revision of the same book, which presents further detailed information ([Hayssen, 1993](#)). The monotremes are testicond mammals, which means that the males' testes are situated inside the body cavity, rather than in a scrotum. The spermatozoa are filiform and bear more resemblance to avian and reptilian spermatozoa than to spermatozoa of other mammals; during sperm maturation in the epididymis, the spermatozoa become organized into bundles of approximately 100 cells, which swim together in synchrony. The microanatomy of the epididymis has been likened to that of birds and reptiles ([Bedford and Rifkin, 1979](#); [Carrick and Hughes, 1982](#)). Monotreme yolky oocytes are fertilized within the female's oviduct where they meet the spermatozoa ([Grutzner et al., 2008](#)). It is also worth noting that, as with marsupials (with the exception of some planigales), and, unlike eutherian mammals, the amino acid sequence of protamine 1 of monotreme spermatozoa does not include cysteine residues ([Retief et al., 1993](#)). The sperm heads are thus considered rather fragile as there is no capacity for the formation of stable crosslinked nuclear protein-DNA complexes. Presumably platypus and echidna spermatozoa typically ascend the female tract as motile bundles ([Johnston et al., 2007](#); [Nixon et al., 2016](#)), and although relatively little is known about fertilization mechanisms, it is interesting to see that the platypus genome encodes each of the four proteins of the human zona pellucida. Field and captive observations of echidna reproduction ([Nicol and Andersen, 2007](#); [Rismiller and McKelvey, 1996](#)) have shown that the egg is incubated in the pouch for approximately 10 days after a pregnancy of about 3 weeks, whereupon the female eventually deposits the young in a nursery burrow. As neither the platypus nor the echidna possess nipples, the offspring suckle directly from mammary lobules that empty into an invagination of skin surrounding a hair follicle ([Grant and Temple-Smith, 1998](#)).

Remarkably, it appears that only the left side of the female platypus reproductive tract is functional, while that of the echidna has two functional ovaries. The male echidna does not urinate through the penis, but via urogenital papillae. The echidna penis itself is most bizarre, being about a quarter of the animal's body length when erect and terminating in two sides, each side possessing a glans penis taking the form of 2 rosettes. The urethrae terminate on the surfaces of the rosette in a series of openings through which the semen emerges ([Johnston et al., 2007](#)); the platypus penis is equally bizarre and appears adapted for securing this organ into the urogenital sinus of the female during mating, which occurs in the water column.

Marsupials

Recent genomic data ([Nilsson et al., 2010](#); [Warren et al., 2008](#)) suggest that marsupials diverged from eutherian mammals 130–150 million years ago, and since then they have colonised both central and south America as well as Australia and New Guinea. The marsupials, which include about 330 extant species, show huge variation in terms of their ecology and reproductive characteristics. This remarkable diversity is, however, often underestimated by those not familiar with the group as a whole ([Johnston and Keeley, 2015](#); [Johnston and Holt, 2001](#); [Tyndale-Biscoe and Renfree, 1987](#)). In contrast to the popular belief that all marsupials possess a pouch and lack a placenta, some of the carnivorous dasyurid species (e.g., phascogales and antechinus) possess only a marginal pouch that does not cover the mammary area, while the Peramelidae family (e.g., bandicoots) even develop an invasive chorioallantoic placenta during pregnancy. The majority of marsupials are also seasonal breeders, and some species, including macropods (kangaroos and wallabies) and the honey possum ([Oates et al., 2007](#); [Renfree, 1981](#)), exhibit embryonic diapause, where the embryo remains in the uterus for up to several months without showing any development; development can be rapidly initiated by changes in day length or the loss of pouch young. The occurrence of embryonic diapause is not restricted to marsupials as it has also been recognized in a range of eutherian mammals ([Mead, 1993](#)) as a strategy for the temporal separation of mating events and subsequent embryonic development. While it is impossible to describe in this short review how reproductive systems vary between marsupials, it is worthwhile attempting to summarise a few key differences, as well as emphasising how some of their characteristics differ from eutherian mammals.

Reproductive Features of Marsupials

Species diversity among marsupials is as multifaceted as diversity among eutherian mammals. Some species, such as the Tasmanian devil (*Sarcophilus harrisii*), which is the largest of the dasyurids, are carnivorous, while others, such as the macropodidae are herbivores. Marsupials have also evolved multiple and diverse reproductive strategies and as a consequence the details of their reproductive biology are also highly varied. In an effort to classify and make sense of the different reproductive strategies found among marsupials, Tyndale-Biscoe and Renfree (1987) proposed that four distinct categories could be identified. However, because more knowledge of marsupial reproduction and its complexity has become available over the last 30 years, this simple classification needed further refinement (Johnston and Keeley, 2015).

Understanding reproductive anatomy in female marsupials is also challenging in itself, being different from that of the eutherian mammals (Fig. 1(D)). The caudal region of the female reproductive tract possesses two lateral vaginae, each of which opens into a urogenital sinus that also receives the urethra (Tyndale-Biscoe and Renfree, 1987). The differences arise during organogenesis owing to a difference in the migration of the embryonic urinary and genital ducts. The ureters pass medially to the Wolffian and Mullerian ducts, rather than laterally to them, as in eutherians. As a result, the female Mullerian ducts are prevented from fusing medially so that the uteri and vaginae on either side remain separate, except where the ducts fuse medially at the junction of the developing uterine and vaginal sections (Mackay *et al.*, 2004; Renfree *et al.*, 1996).

Prior to parturition, it is thought that most marsupials develop a new birth canal; this joins the posterior margins of the vaginal cul-de-sac to the anterior end of the urogenital sinus (Johnston and Keeley, 2015). In most species that have been examined, the connective tissue closes and repairs quite rapidly while in others (e.g., Honey possum, kangaroos and wallabies) it remains patent.

At the time of copulation, semen is deposited into the cranial portion of the urogenital sinus, from where it moves through the lateral vaginae, a vaginal complex or cul-de-sac, cervix and into separate uteri and oviducts. Although this is the general scheme, there is considerable anatomical variation between and within species (Tyndale-Biscoe and Renfree, 1987), which sets considerable challenges to the development of artificial insemination and embryo transfer technologies, where both semen and embryo deposition require both anatomical and temporal precision.

Inter-male competition is prevalent in many species, for example, wallabies (Jarman, 1991) and some researchers have used testis weight as a predictor of this reproductive strategy; in principle, it is likely that species engaging in male-male competition would benefit from high sperm production capacity and hence a high testis/body weight ratio (Rose *et al.*, 1997). Some species, especially the dasyuridae, combine such competitiveness with a more complex phenomenon known as semelparity (Naylor *et al.*, 2008). This strategy involves the almost complete death and ecological disappearance of males after mating combined with a heavy reliance on sperm storage in oviductal crypts within the female reproductive tract (Taggart *et al.*, 1999; Taggart and Temple-Smith, 1991).

The morphology and physiology of marsupial spermatozoa are highly variable between species (Breed, 1994), but it is notable that sperm morphology within a single species can either be highly pleiomorphic, as in the koala (*Phascolarctos cinereus*), or show very little variability (e.g., macropodidae). The new world marsupials have evolved an unusual reproductive specialization whereby their spermatozoa become “paired” during maturation in the epididymis (Krause and Cutts, 1979; Temple-Smith and Bedford, 1980); the pairing involves very specific and stable interactions between the flattened surfaces of the two sperm heads, and the end result appears to provide both of the spermatozoa with synchronized and rapid motility (Moore and Taggart, 1995; Taggart *et al.*, 1993). Experiments (Rodger and Bedford, 1982) have shown that the two spermatozoa become separated shortly before meeting the egg, but detailed studies of marsupial sperm capacitation and acrosome reaction have yet to be conducted.

The variations in spermiogenesis and epididymal sperm maturation result in significant differences in sperm morphology between marsupials (Rodger and Mate, 1993; Temple-Smith, 1994). These represent wonderful sources of information for researchers interested in the comparative evolution of reproductive biology; however, the relevant literature is far too detailed and rich to describe here. The reader is advised to consult J. M. Bedford’s thought provoking reviews (Bedford, 1996, 2004, 2008, 2014) on gamete form and function.

Fertilization and embryonic development in marsupials show many differences from the same processes in the eutherian mammals; the main points of these differences are well summarized in two excellent reviews (Baggott, 1992; Selwood, 2007). Marsupial oocytes are much larger (150–200 µm diameter) than those of the eutherians (typically about 100 µm diameter), and after fertilization the zygotes exhibit visible polarity as an early indication of embryonic cell commitment. The polarity predicts the eventual separation into two cell types, pluriblast (equivalent to the inner cell mass of the mouse), the trophoblast, and ultimately the alignment of the body axis.

Assisted Reproductive Technologies and Marsupials

Like animal populations everywhere, the unique fauna of Australia is under threat from many directions, including land management, industrial development and environmental pollution, habitat fragmentation, disease and invasive species. Many of the marsupials are therefore endangered and under imminent threat of extinction. However, there are also many conservation schemes that attempt to counter these problems. Almost all of the conservation measures are based around habitat protection and the mitigation of direct threats, but some researchers have argued that these policies should be backed up by the use of assisted reproductive technologies (ART), such as artificial insemination and the cryobanking of genetic resources (Czarny *et al.*, 2009; Johnston and Holt, 2001) that could be used for genetic management. Unfortunately, these technologies are still very underdeveloped with respect to

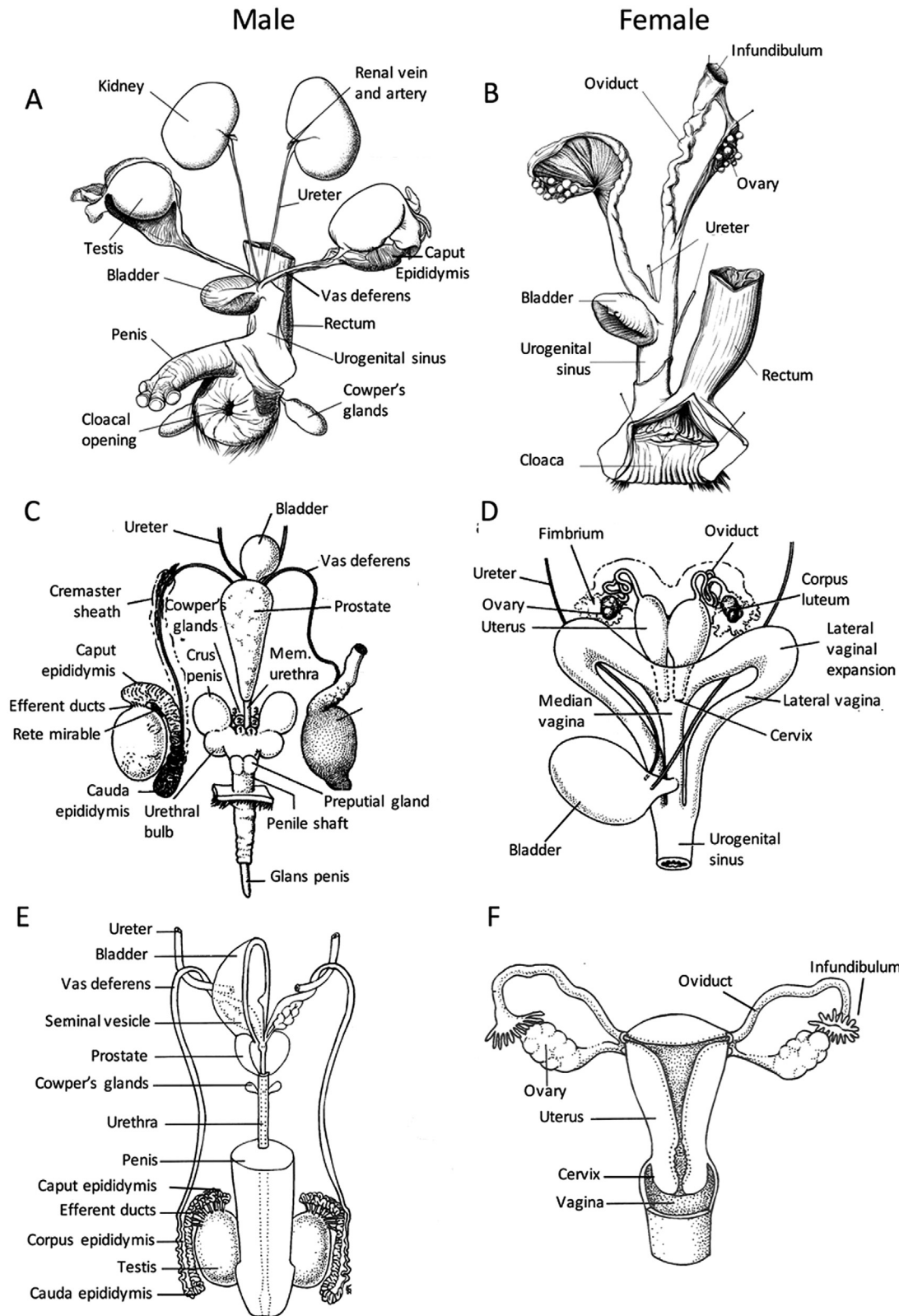


Fig. 1 Comparative mammalian reproductive anatomy. (A) Male prototheria – Echidna; (B) female prototheria – Echidna; (C) male metatheria – Tammar Wallaby; (D) female metatheria – Tammar Wallaby; (E) male eutheria – Human; (F) female eutheria – Human. A and B modified from Augee, M.L., Gooden, B., Musser, A., 2006. *Echidna: Extraordinary Egg-Laying Mammal*. Collingwood, VIC: CSIRO Publishing. C and D modified from Renfree, M., Shaw, G., 1999. *Marsupials*. In: Knobil, E., Neill, J.D. (Eds.), *Encyclopedia of Reproduction*. London: Academic Press, pp. 104–114. E and F modified from Lombardi, J., 1998. *Comparative Vertebrate Reproduction*. Boston; Dordrecht; London: Kluwer Academic Publishers.

the marsupials, mainly because there is such a paucity of knowledge about the reproductive physiology of marsupials (Johnston and Holt, 2001). Using ART requires detailed knowledge of both male and female physiology, coupled with practical methods for semen collection, the control of ovarian cycles, semen cryopreservation and reproductive anatomy. All of these factors have proven extremely challenging in marsupials. Having pointed out the difficulties, it is worth mentioning that appropriate investment of effort does indeed produce results, and that artificial insemination has been successful in the koala (Allen *et al.*, 2008; Johnston *et al.*, 2003) and tammar wallaby (Paris *et al.*, 2005; Rodger *et al.*, 2009).

Reproduction in Eutherian Mammals

The eutherians account for the vast majority of extant mammals. Their shared reproductive characteristic is that they are all viviparous; that is, they all produce live offspring that have been nurtured via an invasive chorioallantoic placenta throughout pregnancy. Unlike the marsupials, the newborn offspring reach an advanced stage of development before birth and continue to receive maternal support after birth by suckling milk from the mammary glands. Placental function during pregnancy is supported by the secretion of progesterone, produced initially from the corpus luteum, formed within the ovary when the post-ovulatory Graafian follicle becomes luteinized, and later in many species by the placenta itself. Circulatory progesterone and its metabolites (known collectively as progestagens) can readily be measured after excretion into fecal samples and/or urine. In practical terms this is the basis for the non-invasive detection of oestrous cycles and pregnancy, a technique that can be used with many wild mammalian species (Brown *et al.*, 1994; Burgess *et al.*, 2012; Wasser *et al.*, 1996). Fig. 2 illustrates this principle, using data from a study of the Mohor gazelle (*Gazella dama mhorr*) (Pickard *et al.*, 2001).

One important role of progesterone in eutherian mammals involves transforming the lining of the uterus in order to allow implantation of the early embryo and the subsequent formation of the placenta. Prior to ovulation the ovary has been more concerned with oocyte development within the follicle, a process supported by the production of estradiol from thecal and

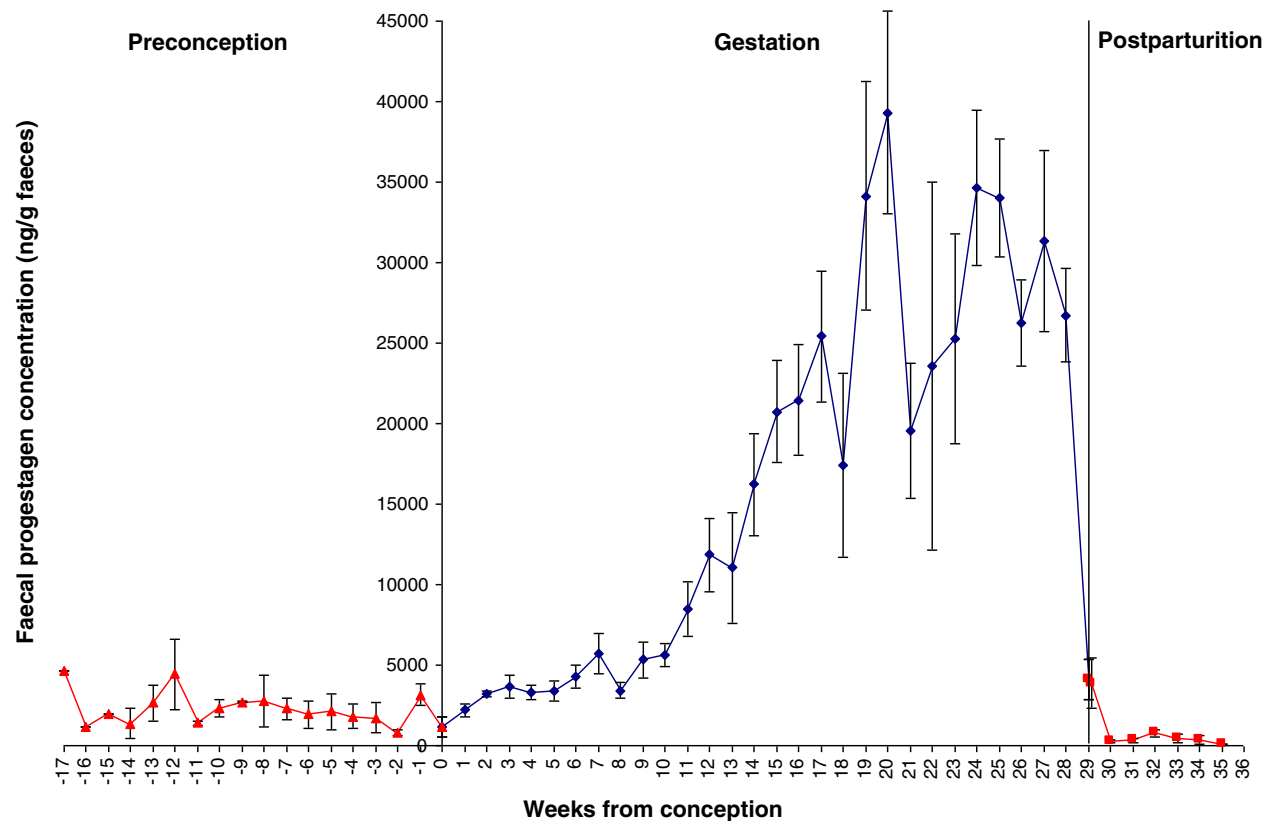


Fig. 2 Faecal progestagen concentrations (mean $\pm$ SEM; $n=7$ females) before conception (red triangles), during gestation (blue diamonds) and after parturition (red squares) in the Mohor gazelle (*Gazella dama mhorr*). The first vertical line indicates the date of insemination and the second vertical line indicates the date of parturition. The progestagen concentrations do not increase significantly until about 9 weeks after insemination, whereupon they rise steeply and remain high until shortly before parturition. Reproduced with permission from Pickard, A.R., Abaigar, T., Green, D.I., *et al.*, 2001. Hormonal characterization of the reproductive cycle and pregnancy in the female Mohor gazelle (*Gazella dama mhorr*). Reproduction 122, 571–580.

granulosa cells that line the follicle itself. These two sequential processes, which together result in the basic features of the estrous cycle (follicular and luteal phases), have been moulded by evolutionary processes in ways that are adapted to assure optimal reproductive success in different habitats and climates.

Control of the estrous cycle and its relationships to environmental factors such as photoperiod is exerted via the neuroendocrine system. These physiological processes are governed by the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from nerve endings in the hypothalamus which are then released into portal vessels connected to the anterior pituitary. The pituitary responds by a highly synchronized release of two gonadotropins (luteinizing hormone (LH)) and follicle stimulating hormone (FSH), which together and in association with inhibin and activin from the ovary, control gonadal function (i.e., both in the ovaries and the testes). In some mammals (e.g., human, cattle, pigs) the estrous cycle occurs regularly and spontaneously until it is interrupted by pregnancy, but it is important to note that in many species (e.g., rabbits, ferrets, cats, camelids, often grouped together and known as “induced ovulators”) there are sophisticated control mechanisms that help to ensure either that births occur at a favourable time of year, or that ovulation only takes place when a female is likely to encounter a male. It would, for example, be a waste of energy for females with solitary lifestyles to ovulate regardless of the likelihood of being mated. In many species, ovulation can be induced, often by physical stimulation of the female genitalia during mating, but also by the presence of ovulation-inducing hormones in seminal plasma (e.g., Adams and Ratto, 2013, Camelidae).

Reproduction in mammals is modulated by subtle and sophisticated mechanisms, details of which are beyond the scope of this article. However, it is worth mentioning the relatively recent discovery that the production of leptin, a peptide hormone produced by adipose tissues and also by the placenta, is now seen as an important modulator of diet, body condition and the neural control of reproduction (Barb *et al.*, 2005; Daniel *et al.* 2013; Evans and Anderson, 2017). Extremes of body condition such as obesity or malnutrition are known to impact the success of reproduction in humans and other species (Hausman *et al.*, 2012; Perez-Perez *et al.*, 2015; Walzem and Chen, 2014).

Reproductive Seasonality

Seasonality has a major impact on reproductive processes and many eutherian mammals have adjusted their entire reproductive repertoire accordingly. For example, the breeding season of the harbour porpoise (*Phocaena phocaena*) and common dolphin (*Delphinus delphis*) is restricted to a short period in spring or summer (Neimanis *et al.*, 2000; Westgate and Read, 2006). Testicular weight during the summer months is approximately five times greater than in winter because, while spermatogenesis and sperm production are maximally active during the summer, they cease during winter and the testes undergo regression. Cycles of seasonally activated testicular activity followed by regression also occur in many other mammalian taxonomic groups, including various deer species (Lincoln, 1971), rodents and bats.

Many species align their reproductive physiology with environmental factors so that their offspring are born when environmental conditions are most favourable. Two common tactics for achieving this objective involve introducing a delay between mating and either fertilization itself, or the development and implantation of embryos (for review, see Orr and Zuk, 2014). One approach involves the prolonged storage of spermatozoa in the female reproductive tract (Holt, 2011; Holt and Fazeli, 2016); this is usually limited to several days in mammals, although sperm storage in some bat species can last several months (Krishna and Dominic, 1978; Racey *et al.*, 1987; Roy and Krishna, 2011). An alternative approach to the temporal uncoupling of mating and fetal development, known as delayed implantation (Fenelon *et al.*, 2014; Lopes *et al.*, 2004) has been adopted by at least 130 eutherian mammals. In species such as the seal (Pomeroy, 2011), badger (Yamaguchi *et al.*, 2006), Giant panda (Zhang *et al.*, 2009), armadillo and stoat (reviewed by Mead, 1993), the embryos do not develop for some weeks or even months, before the diapause ends and the pregnancy resumes. Sixteen species of mustelids exhibit long gestations ranging from 172 to 365 days, with embryonic development being arrested for most of this period (Mead, 1993). In these species, the fertilized embryo remains within the uterus until an external signal such as shortening day length stimulates the embryo to resume its development and undergo implantation. Mead (1993) pointed out that the distribution of this trait within a genus is not constant, and cited long-tailed weasels and stoats (*Mustela frenata* and *Mustela erminea*) as examples; these species exhibit delayed implantation, whereas the least weasel (*Mustela rixosa*) does not. Eastern and western spotted skunks (*Spilogale putorius*) may be even more closely related, yet only those from west of the continental divide exhibit embryonic diapause.

Mechanisms for the control of embryonic diapause in mammals are usually classified as either “facultative” or “obligate” (Fenelon *et al.*, 2014). Facultative diapause is induced by the metabolic stress of lactation, and implantation occurs when the stress is relieved. Examples include some of the marsupials, where the diapause is terminated immediately if lactation ceases (Renfree, 1981). Obligate diapause is widespread among the mustelidae as described above (Mead, 1993), is rigidly controlled by factors such as photoperiod and occurs in every gestation. One of the mustelid species (the stoat, *Mustela erminea*) employs obligate embryonic diapause in a very unusual way. Reproductive activity in this species, which is highly seasonal, involves the young female stoat reaching sexual maturity at about 6–8 weeks of age; she is mated while still in the nest and before she is weaned (Gulamhusein and Tam, 1974). Mating takes place in June or July, but the female does not give birth until the following April or May; in this instance, the delay of implantation is almost certainly necessary to ensure that the female is sufficiently mature to bear the stress of pregnancy.

Intriguingly, such observations of reproductive heterogeneity among closely related species tend to suggest that the mechanisms governing embryonic diapause and delayed implantation evolved independently. Using similar arguments, evidence that sperm storage mechanisms also show considerable dissimilarity between species leads towards the same conclusion (Holt and Lloyd, 2010). It seems reasonable to suggest that neither long-term sperm storage nor embryonic diapause required drastic genomic

and metabolic reorganization during evolution in order to introduce these reproductive modifications. Recent research on the expression and function of microRNA, short RNA molecules that are increasingly recognized as being significant regulators of development, suggests that they have an important role in controlling delayed implantation and embryonic diapause (Su *et al.*, 2010). This significant example of epigenetic regulation might explain how functionally important and highly targeted physiological differences could evolve so rapidly in closely related species.

Reproductive Adaptations in Eutherian Mammals

Evolution has led to a few unusual reproductive outcomes for eutherian mammals, and rather than try to provide here a detailed description of basic mammalian reproductive biology, which can be found in textbooks and reviews (Bronson, 1989; Doty, 1976), it is more interesting to point out how groups of species have adapted their reproductive patterns and physiologies to suit their specific needs. Eutherian mammals have colonized multiple habitats all over the world, and have evolved a dazzling variety of reproductive strategies and physiological mechanisms. Some species live in social groups while others are mainly solitary; some are tropical and others live in high latitudes where they have to cope with highly seasonal environments with differing day lengths, ambient temperatures, varying amounts of rainfall and availability of food.

African mole rats belong to a family of about 18 species of rodents whose lifestyle is, as their name suggests, mainly subterranean, and have evolved a very unusual social system that bears more similarity to that of bees and other social insects, than those of other mammals (Faulkes and Bennett, 2001). About 14 of these species live in socially well-organized colonies, where individuals within the colony have to fulfil specific functional roles such as foraging, digging tunnels or defending the colony. The ability to breed is restricted to a very small group of individuals, which includes a single female or “queen” and two or three breeding males. All other members of the colony are reproductively suppressed; consequently there is a high level of inbreeding within colonies (Reeve *et al.*, 1990). When a queen dies, one of the other females can take her place, thus showing that reproductive suppression is not irreversible. The non-reproductive males possess testes and produce spermatozoa, but they do not attempt to mate with the non-breeding females. In addition, species such as the Damaraland High Veldt mole rat *Cryptomys hottentotus pretoriae* (Rensburg *et al.*, 2002), are seasonal breeders, thus showing a rich repertoire of reproductive adaptations that provide a wealth of material for researchers interested in reproductive control mechanisms. This is not the only example of cooperative breeding in mammals; it also happens in some primates such as marmosets (*Callithrix jacchus*) (Mattle *et al.*, 2008; Yamamoto *et al.*, 2014; Ziegler, 2013), where a dominant female can suppress the reproductive capacity of other females in the group, as well as in canids (Moehlman and Hofer, 1996), beavers, mongooses and porcupines (for review, see Lukas and Clutton-Brock, 2012).

As pointed out more than four decades ago by Weir and Rowlands (1973), there are few, if any, reproductive mechanisms whose evolution can be directly attributed to either habitat or taxonomy. These authors based their argument on the premise that if two species of a genus are found to be different in any respect, then that particular factor is not a generic characteristic. This complicates our understanding of mammalian reproductive evolution and implies that some traits, such as delayed implantation and sperm storage in the female tract, have evolved independently several times. This possibility holds some promise for the survival of mammals in the face of current changes in climate and other environmental challenges as it indicates that they can adapt quickly when conditions change. In a review that was directed at considering the likely impacts of climate change, Bronson (2009) predicted that small rodents will probably adapt to new conditions with relative ease because of their short generation time; however, he also cautioned that large and long-lived species whose reproduction is rigidly controlled by photoperiod will find it more difficult to adapt, especially as they might shift their geographical ranges.

Conclusions

This short review emphasises that mammalian reproduction owes its widespread success largely to the way that a few physiological processes such as sperm production, ovulation and embryonic development have developed within a framework that can be modified by the demands of habitats. While the detailed neural mechanisms controlling the endocrine system, the cell biology of sperm formation or early embryonic development can be studied in isolation using experimental animals, a comparative overview between species demonstrates that nature has treated these fundamental processes as reproductive building blocks that can be engineered together in an endless number of permutations and combinations. That these permutations characterize the whole animal is a feature of biology that is frequently overlooked in an era when research tends to focus mainly on gene and protein sequences, without necessarily remembering that a mouse or rat is not the same as an elephant. This principle has been succinctly stated as “a cow is not the same as a cheetah” in numerous lectures and reviews by Wildt *et al.* (2001) of the Smithsonian Conservation Biology Institute, Centre for Species Survival in Washington, DC, United States.

References

- Adams, G. P., & Ratto, M. H. (2013). Ovulation-inducing factor in seminal plasma: A review. *Animal Reproduction Science*, 136, 148–156.
- Allen, C. D., Burridge, M., Mulhall, S., *et al.* (2008). Successful artificial insemination in the koala (*Phascolarctos cinereus*) using extended and extended-chilled semen collected by electroejaculation. *Biology of Reproduction*, 78, 661–666.

- Asdell, S. A. (1965). *Patterns of Mammalian Reproduction* (second enlarged ed.). London: Constable & Co. Ltd. (Published in the U.S.A. in 1964).
- Baggott, L. M. (1992). A marsupial for a new approach to studies in reproductive biology. *Journal of Biological Education*, 26, 171–177.
- Barb, C. R., Hausman, G. J., & Czaja, K. (2005). Leptin: A metabolic signal affecting central regulation of reproduction in the pig. *Domestic Animal Endocrinology*, 29, 186–192.
- Bedford, J. M. (1996). What marsupial gametes disclose about gamete function in eutherian mammals. *Reproduction Fertility and Development*, 8, 569–580.
- Bedford, J. M. (2004). Enigmas of mammalian gamete form and function. *Biological Reviews*, 79, 429–460.
- Bedford, J. M. (2008). Puzzles of mammalian fertilization – And beyond. *International Journal of Developmental Biology*, 52, 415–426.
- Bedford, J. M. (2014). Singular features of fertilization and their impact on the male reproductive system in eutherian mammals. *Reproduction*, 147, R43–R52.
- Bedford, J. M., & Rifkin, J. M. (1979). An evolutionary view of the male reproductive tract and sperm maturation in a monotreme mammal – The echidna, *Tachyglossus aculeatus*. *American Journal of Anatomy*, 156, 207–230.
- Blum, J. W., & Baumrucker, C. R. (2008). Insulin-like growth factors (IGFs), IGF binding proteins, and other endocrine factors in milk: Role in the newborn. *Advances in Experimental Medicine and Biology*, 606, 397–422.
- Breed, W. G. (1994). How does sperm meet egg? In a marsupial. *Reproduction Fertility and Development*, 6, 485–506.
- Bronson, F. H. (1989). *Mammalian Reproductive Biology*. Chicago; London: University of Chicago Press.
- Bronson, F. H. (2009). Climate change and seasonal reproduction in mammals. *Philosophical Transactions of the Royal Society of London B*, 364, 3331–3340.
- Brown, J. L., Wasser, S. K., Wildt, D. E., et al. (1994). Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biology of Reproduction*, 51, 776–786.
- Burgess, E. A., Lanyon, J. M., Brown, J. L., et al. (2012). Diagnosing pregnancy in free-ranging dugongs using fecal progesterone metabolite concentrations and body morphometrics: A population application. *General and Comparative Endocrinology*, 177, 82–92.
- Carrick, F. N., & Hughes, R. L. (1982). Aspects of the structure and development of monotreme spermatozoa. *Cell and Tissue Research*, 222, 127–141.
- Czarny, N. A., Harris, M. S., Iulius, G. N. D., et al. (2009). Acrosomal integrity, viability, and DNA damage of sperm from dasyurid marsupials after freezing or freeze drying. *Theriogenology*, 72, 817–825.
- Daniel, J. A., Foradori, C. D., Whitlock, B. K., et al. (2013). Hypothalamic integration of nutrient status and reproduction in the sheep. *Reproduction in Domestic Animals*, 48(Suppl 1), 44–52.
- Doty, R. L. (Ed.). (1976). *Mammalian Olfaction, Reproductive Processes, and Behavior*. London: Academic Press.
- Evans, M. C., & Anderson, G. M. (2017). Neuroendocrine integration of nutritional signals on reproduction. *Journal of Molecular Endocrinology*, 58, R107–R128.
- Faulkes, C. G., & Bennett, N. C. (2001). Family values: Group dynamics and social control of reproduction in African mole-rats. *Trends in Ecology and Evolution*, 16, 184–190.
- Fenelon, J. C., Banerjee, A., & Murphy, B. D. (2014). Embryonic diapause: Development on hold. *International Journal of Developmental Biology*, 58, 163–174.
- Grant, T. R., & Temple-Smith, P. D. (1998). Field biology of the platypus (*Ornithorhynchus anatinus*): Historical and current perspectives. *Philosophical Transactions: Biological Sciences*, 353, 1081–1091.
- Gruzner, F., Nixon, B., & Jones, R. C. (2008). Reproductive biology in egg-laying mammals. *Sexual Development*, 2, 115–127.
- Gulamhusein, A. P., & Tam, W. H. (1974). Reproduction in the male stoat, *Mustela erminea*. *Journal of Reproduction and Fertility*, 41, 303–312.
- Hausman, G. J., Barb, C. R., & Lents, C. A. (2012). Leptin and reproductive function. *Biochimie*, 94, 2075–2081.
- Hayssen, V. (1993). Empirical and theoretical constraints on the evolution of lactation. *Journal of Dairy Science*, 76, 3213–3233.
- Holt, W. V. (2011). Mechanisms of sperm storage in the female reproductive tract: An interspecies comparison. *Reproduction in Domestic Animals*, 46(Suppl. 2), 68–74.
- Holt, W. V., & Fazeli, A. (2016). Sperm storage in the female reproductive tract. *Annual Reviews of Animal Biosciences*, 4, 291–310.
- Holt, W. V., & Lloyd, R. E. (2010). Sperm storage in the vertebrate female reproductive tract: How does it work so well? *Theriogenology*, 73, 713–722.
- Home, E. (1802). A description of the anatomy of the *Ornithorhynchus paradoxus*. *Philosophical Transactions of the Royal Society of London*, 92, 67–84.
- Jarman, P. J. (1991). Social behavior and organization in the macropodidae. *Advances in the Study of Behavior*, 20, 1–50.
- Johnston, S. D., & Holt, W. V. (2001). Germplasm Conservation in Marsupials. In P. F. Watson, & W. V. Holt (Eds.), *Cryobanking the Genetic Resource. Wildlife Conservation for the Future?* (pp. 203–225). London: Taylor and Francis.
- Johnston, S. D., Smith, B., Pyne, M., et al. (2007). One-sided ejaculation of echidna sperm bundles. *American Naturalist*, 170, E162–E164.
- Johnston, S., & Keeley, T. (2015). Enigmas in reproductive biology. In A. Klieve, L. Hogan, S. Johnston, & P. Murray (Eds.), *Marsupials and Monotremes: Nature's Enigmatic Mammals*. New York, NY: Nova Science Publishers.
- Johnston, S., McGowan, M., O'Callaghan, P., et al. (2003). Birth of koalas *Phascolarctos cinereus* at Lone Pine koala sanctuary following artificial insemination. *International Zoo Yearbook*, 38, 160–172.
- Kemp, T. S. (2007). The origin of higher taxa: Macroevolutionary processes, and the case of the mammals. *Acta Zoologica*, 88, 3–22.
- Krause, W. J., & Cutts, J. H. (1979). Pairing of spermatozoa in the epididymis of the opossum (*Didelphis virginiana*): A scanning electron microscopic study. *Archivum Histologicum Japonicum*, 42, 181–190.
- Krishna, A., & Dominic, C. J. (1978). Storage of spermatozoa in the female genital tract of the vespertilionid bat, *Scotophilus heathi*. *Journal of Reproduction and Fertility*, 54, 319–321.
- Lincoln, G. A. (1971). The seasonal reproductive changes in the red deer stag (*Cervus elaphus*). *Journal of Zoology (London)*, 163, 105–123.
- Lopes, F. L., Desmarais, J. A., & Murphy, B. D. (2004). Embryonic diapause and its regulation. *Reproduction*, 128, 669–678.
- Lukas, D., & Clutton-Brock, T. (2012). Cooperative breeding and monogamy in mammalian societies. *Proceedings of the Royal Society of London B: Biological Sciences*, 279(1736), 2151–2156.
- Mackay, S., Xie, Q., Ullmann, S. L., et al. (2004). Postnatal development of the reproductive system in the grey short-tailed opossum, *Monodelphis domestica*. *Anatomy and Embryology*, 208, 121–133.
- Mattie, F. M., Pryce, C. R., & Anzenberger, G. (2008). Different ovarian responses to potential mates underlie species-specific breeding strategies in common marmoset and Goeldi's monkey. *Hormones and Behavior*, 54, 302–311.
- Mead, R. A. (1993). Embryonic diapause in vertebrates. *Journal of Experimental Zoology*, 266, 629–641.
- Meredith, R. W., Janečka, J. E., Gatesy, J., et al. (2011). Impacts of the Cretaceous terrestrial revolution and KPG extinction on mammal diversification. *Science*, 334, 521–524.
- Moehman, P. D., & Hofer, H. (1996). Cooperative breeding, reproductive suppression, and body mass in Canids. In N. Solomon, & J. French (Eds.), *Cooperative Breeding in Mammals* (pp. 76–128). Cambridge, MA: Cambridge University Press.
- Moore, H. D., & Taggart, D. A. (1995). Sperm pairing in the opossum increases the efficiency of sperm movement in a viscous environment. *Biology of Reproduction*, 52, 947–953.
- Morrow, G., & Nicol, S. C. (2009). Cool sex? Hibernation and reproduction overlap in the echidna. *PLOS ONE*, 4, e6070.
- Naylor, R., Richardson, S. J., & McAllan, B. M. (2008). Boom and bust: A review of the physiology of the marsupial genus antechinus. *Journal of Comparative Physiology B, Biochemical, Systemic, and Environmental Physiology*, 178, 545–562.
- Neimanis, A. S., Read, A. J., Foster, R. A., et al. (2000). Seasonal regression in testicular size and histology in harbour porpoises (*Phocoena phocoena*, L.) from the Bay of Fundy and Gulf of Maine. *Journal of Zoology*, 250, 221–229.
- Nicol, S., & Andersen, N. A. (2007). The life history of an egg-laying mammal, the echidna (*Tachyglossus aculeatus*). *Écoscience*, 14, 275–285.
- Nilsson, M. A., Churakov, G., Sommer, M., et al. (2010). Tracking marsupial evolution using archaic genomic retroposon insertions. *PLOS Biology*, 8, e1000436.
- Nixon, B., Ecroyd, H., Dacheux, J. L., et al. (2016). Formation and dissociation of sperm bundles in monotremes. *Biology of Reproduction*, 95, 91.
- Oates, J. E., Bradshaw, F. J., Bradshaw, S. D., et al. (2007). Reproduction and embryonic diapause in a marsupial: Insights from captive female honey possums, *Tarsipes rostratus* (Tarsipedidae). *General and Comparative Endocrinology*, 150, 445–461.

- Ofstedal, O. T. (2012). The evolution of milk secretion and its ancient origins. *Animal*, 6, 355–368.
- Oguchi, S., Shinohara, K., Yamashiro, Y., et al. (1997). Growth factors in breast milk and their effect on gastrointestinal development. *Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi*, 38, 332–337.
- Orr, T. J., & Zuk, M. (2014). Reproductive delays in mammals: An unexplored avenue for post-copulatory sexual selection. *Biological Reviews*, 89, 889–912.
- Paris, D. B., Taggart, D. A., Shaw, G., et al. (2005). Birth of pouch young after artificial insemination in the tammar wallaby (*Macropus eugenii*). *Biology of Reproduction*, 72, 451–459.
- Perez-Perez, A., Sanchez-Jimenez, F., Maymo, J., et al. (2015). Role of leptin in female reproduction. *Clinical Chemistry and Laboratory Medicine*, 53, 15–28.
- Pickard, A. R., Abaigar, T., Green, D. I., Holt, W. V., & Cano, M. (2001). Hormonal characterization of the reproductive cycle and pregnancy in the female mohor gazelle (*Gazella dama mhori*). *Reproduction*, 122, 571–580.
- Pomeroy, P. (2011). Reproductive cycles of marine mammals. *Animal Reproduction Science*, 124, 184–193.
- Racey, P. A., Uchida, T. A., Mori, T., Avery, M. I., & Fenton, M. B. (1987). Sperm-epithelium relationships in relation to the time of insemination in little brown bats (*Myotis lucifugus*). *Journal of Reproduction and Fertility*, 80, 445–454.
- Reeve, H. K., Westneat, D. F., Noon, W. A., Sherman, P. W., & Aquadro, C. F. (1990). DNA "fingerprinting" reveals high levels of inbreeding in colonies of the eusocial naked mole-rat. *Proceedings of the National Academy of Sciences USA*, 87, 2496–2500.
- Renfree, M. B. (1981). Embryonic diapause in marsupials. *Journal of Reproduction and Fertility*, (Suppl 29), 67–78.
- Renfree, M. B., W.-S, O., Short, R. V., & Shaw, G. (1996). Sexual differentiation of the urogenital system of the fetal and neonatal tammar wallaby, *Macropus eugenii*. *Anatomy and Embryology*, 194, 111–134.
- Janse van Rensburg, L., Bennett, N. C., van der Merwe, M., & Schoeman, A. S. (2002). Seasonal reproduction in the highveld mole-rat, *Cryptomys hottentotus pretoriae* (rodentia: Bathyergidae). *Canadian Journal of Zoology*, 80, 810–820.
- Retief, J. D., Winkfein, R. J., & Dixon, G. H. (1993). Evolution of the monotremes. The sequences of the protamine p1 genes of platypus and echidna. *European Journal of Biochemistry*, 218, 457–461.
- Rismiller, P., McKelvey, M. (1996). Sex, torpor and activity in temperate climate echidnas. In: Adaptations to the Cold: Proceedings of the Tenth International Hibernation Symposium, Tasmania, Australia, pp. 23–30.
- Rodger, J. C., & Bedford, J. M. (1982). Separation of sperm pairs, and sperm-egg interaction in the opossum. *Journal of Reproduction and Fertility*, 64, 171–179.
- Rodger, J. C., & Mate, K. E. (1993). Marsupial gametes and fertilisation. *Today's Life Science*, 28–33.
- Rodger, J. C., Paris, D. B., Czarny, N. A., et al. (2009). Artificial insemination in marsupials. *Theriogenology*, 71, 176–189.
- Rose, R., Nevison, C., & Dixon, A. (1997). Testes weight, body weight and mating systems in marsupials and monotremes. *Journal of Zoology*, 243, 523–531.
- Roy, V. K., & Krishna, A. (2011). Sperm storage in the female reproductive tract of *Scotophilus heathii*: Role of androgen. *Molecular Reproduction and Development*, 78, 477–487.
- Selwood, L. (2007). Marsupial oocytes, fertilization and embryonic development can provide useful tools to study developmental mechanisms. *IUBMB Life*, 59, 617–621.
- Su, R. W., Lei, W., Liu, J. L., et al. (2010). The integrative analysis of microRNA and mRNA expression in mouse uterus under delayed implantation and activation. *PLOS ONE*, 5, e15513.
- Taggart, D. A., Johnson, J. L., O'Brien, H. P., et al. (1993). Why do spermatozoa of American marsupials form pairs? A clue from the analysis of sperm-pairing in the epididymis of the grey short-tailed opossum, *Monodelphis domestica*. *Anatomical Record*, 236, 465–478.
- Taggart, D. A., Shimmmin, G. A., McCloud, P., et al. (1999). Timing of mating, sperm dynamics, and ovulation in a wild population of agile antechinus (marsupialia: Dasyuridae). *Biology of Reproduction*, 60, 283–289.
- Taggart, D. A., & Temple-Smith, P. D. (1991). Transport and storage of spermatozoa in the female reproductive-tract of the brown marsupial mouse, *Antechinus stuartii* (Dasyuridae). *Journal of Reproduction and Fertility*, 93, 97–110.
- Temple-Smith, P. D. (1994). Comparative structure and function of marsupial spermatozoa. *Reproduction Fertility and Development*, 6, 421–435.
- Temple-Smith, P. D., & Bedford, J. M. (1980). Sperm maturation and the formation of sperm pairs in the epididymis of the opossum, *Didelphis virginiana*. *Journal of Experimental Zoology*, 214, 161–171.
- Tyndale-Biscoe, C. H., & Renfree, M. (1987). *Reproductive Physiology of Marsupials*. Cambridge: Cambridge University Press.
- Wallage, A., Clarke, L., Thomas, L., et al. (2015). Advances in the captive breeding and reproductive biology of the short-beaked echidna (*Tachyglossus aculeatus*). *Australian Journal of Zoology*, 63, 181–191.
- Walzem, R. L., & Chen, S. E. (2014). Obesity-induced dysfunctions in female reproduction: Lessons from birds and mammals. *Advances in Nutrition*, 5, 199–206.
- Warren, W. C., Hillier, L. W., Marshall Graves, J. A., et al. (2008). Genome analysis of the platypus reveals unique signatures of evolution. *Nature*, 453, 175–183.
- Wasser, S. K., Papageorge, S., Foley, C., & Brown, J. L. (1996). Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and patterns of fecal steroid concentrations throughout the estrous cycle. *General and Comparative Endocrinology*, 102, 255–262.
- Weir, B. J., & Rowlands, I. (1973). Reproductive strategies of mammals. *Annual Review of Ecology and Systematics*, 4, 139–163.
- Westgate, A. J., & Read, A. J. (2006). Reproduction in short-beaked common dolphins (*Delphinus delphis*) from the western north Atlantic. *Marine Biology*, 150, 1011.
- Wildt, D. E., Ellis, S., & Howard, J. G. (2001). Linkage of reproductive sciences: From 'quick fix' to 'integrated' conservation. *Journal of Reproduction and Fertility*, (Suppl. 57), 295–307.
- Wilson, D. E., & Reeder, D. M. (2005). *Mammal Species of the World: A Taxonomic and Geographic Reference*. JHU Press.
- Yamaguchi, N., Dugdale, H., & Macdonald, D. (2006). Female receptivity, embryonic diapause, and superfetation in the European badger (*Meles meles*: Implications for the reproductive tactics of males and females). *The Quarterly Review of Biology*, 81, 33–48.
- Yamamoto, M. E., Araujo, A., Arruda Mde, F., et al. (2014). Male and female breeding strategies in a cooperative primate. *Behavioural Processes*, 109(Pt. A), 27–33.
- Zhang, H., Li, D., Wang, C., & Hull, V. (2009). Delayed implantation in giant pandas: The first comprehensive empirical evidence. *Reproduction*, 138, 979–986.
- Ziegler, T. E. (2013). Social effects via olfactory sensory stimuli on reproductive function and dysfunction in cooperative breeding marmosets and tamarins. *American Journal of Primatology*, 75, 202–211.

Relevant Website

<https://www.britannica.com/science/animal-reproductive-system/>. – Encyclopedia Britannica.

Cattle Overview

John J Parrish, University of Wisconsin-Madison, Madison, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blood Testis Barrier (BTB) This is a junctional complex between adjacent Sertoli cells in the seminiferous tubule.

Bull A male bovine.

Genomic selection This is genetic selection based on analysis of a bull's DNA.

Leydig cells These cells are just outside of the seminiferous tubule and produce the male hormone testosterone.

Prepuce The tissue that covers the penis when the penis is not erect.

Primary spermatocyte This is a cell produced by mitosis of a spermatogonia but will enter into meiosis.

Round spermatid This is the final cell of meiosis in the male and comes from the secondary spermatocyte.

Secondary spermatocyte This is the second cell in meiosis and is produced by the primary spermatocyte.

Seminiferous tubule This is where sperm production occurs within the testis.

Sertoli cells A nurse cell for the developing male germ cells that become sperm.

Spermatogonia These are the germ cells in the male that undergo mitosis.

Testicular parenchyma The tissue in the testis in which the seminiferous tubules are located.

Testis The organ of the male that produces the male germ cells.

Introduction

Cattle belong to the family Bovidae and genus *Bos*. There are two subspecies of cattle, *Bos taurus* and *Bos indicus* however many breeds exist within each of the subspecies and include both meat, milking and dual purpose. In addition, some cattle are also used as beast's burden. This article will focus on reproduction of male cattle. Comparisons of *B. taurus* and *B. indicus* will be made as appropriate.

Anatomy of the Bull Testis

The development of germ cells occurs in the testis which is suspended vertically in the bull and located within the scrotum. The testicular parenchyma is composed of seminiferous tubules that connect to the rete testis in mediastinum and the interstitial space composed of Leydig cells, blood vessels, lymphatic vessels and nerves (Senger, 2012). The germ cells undergo mitosis and meiosis in the seminiferous tubules (Amann and Schanbacher, 1983; Amann, 1983) which both begin and end at the rete testis. The average bull testis contains 5.2 km (3.2 mi) of seminiferous tubules (Lennox and Logue, 1979). In the basic anatomy and physiology there are only minor differences between domestic cattle of the *B. taurus* and *B. indicus* subspecies. While *B. taurus* is adapted to temperate climates, *B. indicus* is adapted to warm climates and this reflects on how the subspecies differ in temperature regulation of the testes (Brito *et al.*, 2004).

Spermatogenesis

Spermatogenesis in the bull consists of proliferative (mitosis, spermatocytogenesis), meiotic (meiosis) and differentiation (spermiogenesis) phases (Courot *et al.*, 1970; Amann, 1983). The proliferative phase consists of mitotic division in which germ cells numbers increase but also differentiate through Type A1-A3, Intermediate and B1-B2 spermatogonia, ending in the formation of primary spermatocytes. The next phase is meiosis in which the DNA complement undergoes reduction and division from the diploid (2N) to the haploid (N) state. The cell types go from the primary spermatocyte (2N) to the secondary spermatocyte to the round spermatid (N). The round spermatid then undergoes a differentiation into the spermatozoa by elongation of the nucleus, growth of the axoneme and tail, formation of the mitochondria and the acrosome among other changes needed for a sperm to be motile and deliver its haploid DNA to the oocyte at fertilization. In theory, spermatogenesis in the bull can produce up to 256 spermatozoa from each type A1 spermatogonium that enters into the proliferating pool of germ cells. Some authors (Amann and Schanbacher, 1983) have suggested there may be fewer mitotic division and only 64 spermatozoa result but the higher number is now accepted for almost all mammals. However, as will be described below this theoretical maximum number is never reached.

While the developing germ cells undergo the process of spermatogenesis they are surrounded by Sertoli cells that serve to support and direct the division of the germ cells. The Sertoli cells fill the seminiferous tubules extending from the basement membrane to the lumen and interacting with adjacent Sertoli cells via junctional complexes including tight junctions known collectively as the blood



Fig. 1 A bull seminiferous tubule. The seminiferous tubule is the large circular structure in the image. Outside the seminiferous tubule are the Leydig cells that produce testosterone. From the outside towards the center of the seminiferous tubule the following cell types are visible: spermatogonia, primary spermatocytes, and round spermatids. Importantly, multiple types of germ cells can be seen within the tubule at any point in time.

testis barrier (BTB). The BTB allows the formation of the basal, adluminal, and luminal compartments (Amann, 1983; Cheng and Mruk, 2012). Cells in the basal compartment communicate with the interstitial compartment. Cells in this compartment are the Type A, I, B spermatogonia and the primary spermatocytes all of which undergo mitosis during the proliferative phase. The adluminal compartment is toward the lumen of the seminiferous tubule and beyond the BTB. In this compartment, substances reaching the developing germ cells must first pass through the Sertoli cells and thus Sertoli cells manipulate the environment so that meiosis and differentiation can occur to primary, secondary spermatocytes, round spermatids and elongating spermatids developing into spermatozoa. The spermatozoa are finally released into the lumen at the end of spermatogenesis in a process known as spermiation. An actual seminiferous tubule from a bull is shown in Fig. 1 and demonstrates how multiple germ cell types are present at the same time in a seminiferous tubule.

Unique to germ cell division in the male is the retention of cytoplasmic bridges between a cohort of germ cells developing from a single Type A spermatogonium and likely sharing plasma membrane components. The plasma membranes of X and Y bearing sperm are thus unlikely to have any differences and have never been shown to have such differences in the bull. Many germ cells also undergo the process of programmed cell death, apoptosis, although the frequency can increase due to disease, environmental heat exposure, or stress. We routinely find lower levels of germ cells than theoretically possible. For example, in Holstein bulls we found a mitotic yield of 8.9 ± 1.3 primary spermatocytes per A spermatogonia (theoretical max=64); a meiotic yield of 1.7 ± 0.2 round spermatids per primary spermatocyte (theoretical max=4); a spermatogenic yield of 17.1 ± 0.9 round spermatids per A spermatogonia (theoretical max=256). Loss of germ cells thus occur both in mitosis as well as meiosis although the largest number are lost in mitosis. Why so many germ cells die is unclear but may be a reserve ability of the male to recover from testicular environmental or physical damage. A single cohort of germ cells is also interacting with only 1 Sertoli cell. However due to the staggered nature of stages and cycles (Senger, 2012), a single Sertoli cell needs to coordinate germ cells going through mitosis, meiosis and differentiation at the same time.

Endocrinology of the Bull and Puberty

The endocrinology of the adult testis has been reviewed by Amann (1983) and is shown in Fig. 2. In the adult bull, production of GnRH comes from the hypothalamus which causes the production of LH and FSH from the anterior pituitary gland. The LH causes Leydig cells to produce testosterone which supports function of Sertoli cells, germ cells and binds to androgen binding protein in the seminiferous tubules to be carried down to the epididymis via seminiferous tubule fluid. Testosterone also enters the blood stream and acts via negative feedback to regulate GnRH and LH secretion. The FSH released from the anterior pituitary causes the Sertoli cells to convert testosterone to estradiol, produce inhibin, androgen binding protein and formation of the blood testis barrier. The estradiol and inhibin produced from Sertoli cells feedback on the anterior pituitary and hypothalamus in a negative feedback loop to regulate FSH.

Prior to birth the structures of the testis and endocrinology of the male is very different. The primordial germ cells migrate into the progenitors of the seminiferous tubules, the primary sex chords and differentiate as gonocytes. The Pre-Sertoli cells also migrate into the primary sex chords leaving fetal Leydig and stromal cells in the remaining tissue of the developing testis. Endocrinology in

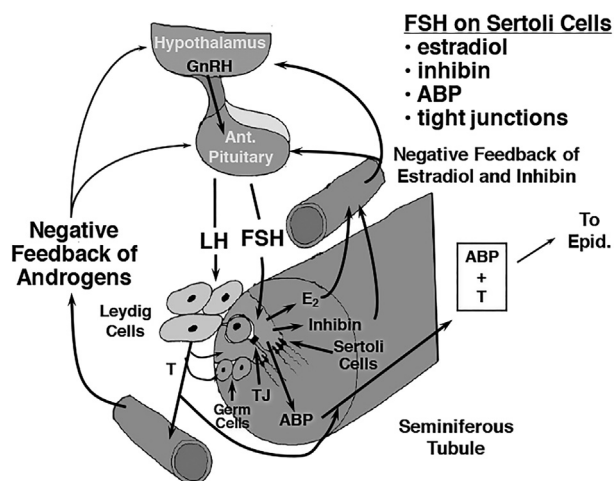


Fig. 2 Endocrinology of the adult bull. The hormones depicted are gonadotropin releasing hormone (GnRH), luteinizing hormone (LH), follicular stimulating hormone (FSH), estradiol (E₂), testosterone (T), and inhibin. Peripheral circulation is depicted as blood vessels into which T, E₂ or inhibin may diffuse. The impact of LH is on Leydig Cells and FSH on Sertoli cells. The tight junctions are indicated as TJ in the diagram. Androgen binding protein (ABP) is produced by the Sertoli cells and remains in the seminiferous tubules and binds T, which is then carried down in the seminiferous tubule fluid to the epididymis (Epid.). Based on work from Amann (1983).

the fetus is very different than the adult. At this point the Fetal Leydig cells secrete testosterone equal to amounts secreted in the adult however this secretion is independent of LH. The testosterone has a major function in the development of the primitive male reproductive tract, the Wolfian ducts. The Pre-Sertoli cells are secreting anti-mullerian hormone which leads to regression of the primitive female reproductive tract known as the Mullerian duct. The testosterone also is converted to estradiol and is secreted into general vascular circulation. Events in the bull from birth to puberty have been reviewed by Rawlings *et al.* (2008). Prior to birth the production of testosterone from fetal leydig cells decreases and fetal leydig cells degenerate by 8 weeks of age. At approximately 4 weeks of age the adult Leydig cells begin developing and respond to LH and produce testosterone that drives mitosis and maturation of Sertoli cells from 4–20 weeks of age with complete maturation of adult Sertoli cells by 30–40 weeks of age. Primary spermatocytes form by 20 weeks of age, secondary spermatocytes from 20–30 weeks of age, round spermatids from 25–30 weeks of age and elongated spermatids from 25–35 weeks of age, with mature spermatozoa forming from 32–40 weeks of age.

There is a gradual increase in production of sperm at the time of puberty so there is a need to define puberty in order to make comparisons of treatments or procedures that might alter the timing of puberty. Puberty has therefore been defined in the Holstein bull as when scrotal circumference is >28 cm, or there are more than 50×10^6 sperm/mL in the ejaculate with >10% progressively motile sperm (Amann, 1983). These are intended to be a measure when puberty occurs and not when an ejaculate would be suitable for breeding or cryopreservation. While scrotal circumference values might need differ for other breeds, the number of sperm is suggested to be useful for all breeds.

Age of Puberty and Impact of Nutrition

Using the criteria described above for puberty, puberty in Holstein bulls has not changed much since 1959 and ranges from 39–47 weeks. In the work of Bratton *et al.* (1959) there was a decrease in age of puberty by 4–7 weeks when the total digestible nutrients (TDN) in a ration were increased from 100% of the suggested daily requirements to 140–160% but bulls maintained on this high level of nutrition eventually decreased in libido. However, in multiple other studies since, there has been no ability to decrease puberty when increasing nutrition relative to a control or medium diet (Flipse and Almquist, 1961; Harstine *et al.*, 2015; Dance *et al.*, 2015). The mean age of puberty in Holstein bulls (Killan and Amann, 1972) was thus identified as 41.1 ± 4.1 weeks of age. The total sperm per ejaculate ($\times 10^9$; mean $\pm$ sem) at puberty was 0.3 ± 0.1 , at 10-weeks post puberty was 3.1 ± 0.4 , at 20-weeks post puberty was 6.2 ± 0.9 and at 30-weeks post puberty at 6.0 ± 1.9 . Holstein bulls should be monitored for puberty at approximately 10 months of age (43 weeks) with the expectation that semen will not be sufficient for normal processing, >70% normal sperm, >60% progressively motile sperm until 1 year of age. If the bull should not yet be producing sufficient normal sperm, then it is likely he will be by an additional 10–12 weeks or by 14 months of age. The reason for the delay in increasing sperm production is that there is a complex interplay between germ cells and Sertoli cells as development proceeds past puberty and it takes time to stabilize with normal cellular signaling. Puberty in well fed Holstein bulls, previously mentioned to be from 39–47 weeks of age and has not changed in the last 50+ years of reports on the subject (Bratton *et al.*, 1959; Killan and Amann, 1972; Amann, 1983).

Puberty in bulls of Jersey and Brown Swiss dairy breeds are similar to Holstein bull while the beef breeds such as Angus and Herford appear to be 1 month later (Senger, 2012). The *B. indicus* breeds are slower maturing with puberty listed as greater than

17 months age (Senger, 2012). We have found that there is quite a range in when *B. indicus* bulls reach puberty, with some bulls not even reaching puberty until after 2 years of age (Siddiqui *et al.*, 2008). This is despite being housed and fed under the same conditions. Selection of bulls which reach puberty earlier is possible and demonstrated that you could select *B. indicus* bulls that reach puberty at an average of 17 ± 2 months of age. The *B. taurus* breeds have already undergone selection and so do not show the same ability for selection and reduction of age of puberty.

Other Effects of Sexual Maturation

To determine if a bull is pubertal, you must collect a semen sample. For artificial insemination (AI), we would generally train a bull to jump a mount animal, usually a steer or bull of appropriate size. The semen would then be collected via an artificial vagina. The young bull should not be asked to mount a bull that is aggressive to that young bull or a negative interaction may impact the ability of that bull to mount and ejaculate in the future. If we simply want a semen sample and the bull would be used for natural breeding of females we would likely use an electroejaculator (Senger, 2012). In either case, the development of the penis is important. As puberty approaches the prepuce of the penis begins to retract from the free end of the penis with the scrotal tissue or the Raphe forming. Many young bulls are housed in groups and the normal activity of young bulls mounting other young bulls resulted in the prepuce retracting as the bull extended his penis and was masturbating. Changes occurring in the artificial insemination industry with the advent of genomic testing to predict genetic value (VanRaden *et al.*, 2009) has resulted in pre-pubertal animals being housed individually and so it may take some time and false mounts for the prepuce to retract from the free end of the penis.

Selection Changes of Bulls Used for Artificial Insemination

The young sire is becoming increasingly important in the AI industry of cattle with the extensive genomic selection of young bulls prior to puberty using whole-genome SNP markers (VanRaden *et al.*, 2009; Aguilar *et al.*, 2010). Where once Holstein bulls in AI centers were 5+ years of age, due to the need for progeny testing, as of 2017 it is rare to see bulls older than 3 and much more common to have bulls <2.5 years of age. As this type of selection becomes more common in beef breeds we are likely to see similar trends and this is already happening with Angus bulls within the US.

At What Age Can Semen be Collected From a Bull?

The theoretical production of sperm from Holstein bulls is depicted vs. age in Fig. 3 as an example for bulls in general. It can be observed that soon after puberty, approx. 10 months of age, sperm production increases yet does not peak until 60 months or 5 years of age. Precise sperm numbers for Holstein bulls can be found in Salisbury *et al.* (1978).

Semen production by the young bull, increases rapidly within 10 to 20 weeks of puberty (Killan and Amann, 1972). The percent motile sperm increased sooner however than the total number of sperm. Although the quality of semen may increase after 1 year of age, it is likely adequate to produce semen for cryopreservation. While the number of AI doses possible will increase with the age of the bull, at 1 year of age it is only 23% of the doses possible at 6–7 years of age. For artificial insemination organizations with bull

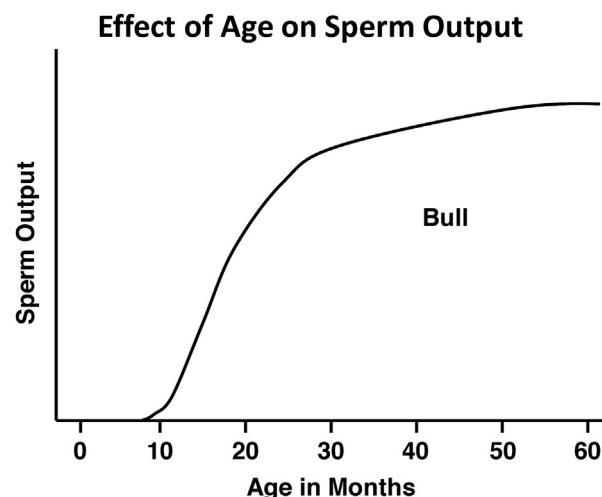


Fig. 3 Theoretical sperm output from Holstein bulls. Location now indicated in text following heading “At what age can semen be collected from a bull”; use the minimum of 166 mm width. Adapted from Amann R.P., 1970. Sperm production rates. In: Johnson, A.D., Gomes W.R., VanDemark, N.R. (Eds.), *The Testis* vol. 1. New York, NY: Academic Press, pp. 433–482.

studs, it is clear that the impact of younger bulls being collected is that more bulls are now required to produce the required number of semen doses being sold by each company.

When to Process Semen

The desire to collect semen on younger and younger bulls requires some standards on when to start processing semen. The ejaculates collected on young sires must pass routine quality control standards such as percentage of motile sperm ($>60\%$), percentage of normal sperm ($>70\%$) and the total number of sperm in an ejaculate (>1 billion). The number of sperm in the ejaculate may need to be reduced for sires younger than 14 months of age.

It is suggested that the first ejaculates collected from a bull that is just pubertal be used for disease testing. Semen may then be collected, cryopreserved and used for AI when at least 50 units can be produced at the company standard. Cost of semen processing may be too high per unit when fewer than 50 units will be produced from an ejaculate. With high genomic value bulls, semen should be collected within 5–10 weeks of puberty and may be used for in vitro fertilization if sperm numbers are low. This is particularly important to reduce the generation interval and the selection of bull calves again by genomics. Other bulls may be collected later when the required units of semen can be produced. Within the first 12 weeks following puberty, only 35% of Holstein bull-bulls produce ejaculates suitable for cryopreservation but between 13–24 weeks the acceptable ejaculates increased to 85% and then after to 96%.

There are other potential issues with collecting semen from very young bulls. The presence of a persistent frenulum can be a significant problem. If need be this can be corrected by surgery. Another problem may be genital warts which are a particular problem with group housed bulls. Genital warts are often seen in beef bulls which are commonly group housed prior to or even shortly after puberty. Genital warts can be surgically removed but the bull cannot be used for semen collection until healed. Lastly abrasions or lacerations to the penis may occur in the young or older bullbulls but must be allowed time to recover until routinely ejaculated.

The First Collections on a Bull

The first collections on a bull are critical to get right. There is a substantial difference in size of young bulls and they are also rapidly increasing in size at the time of puberty ([Dance et al., 2015](#)). The changing sizes of the bulls require the proper selection of mounts for semen collection. It is critical the young bull does not have a bad experience and falls off the mount, slips upon mounting or the mount animal is too large. The AI company needs to have mount animals, bulls, steers or a dummy of different sizes to accommodate the changing young bull. It should also be remembered to not false mount the young sire too many times at first. You need to get the young bull familiar with the process and not be intimidated. Turning a young bull out with females also has similar issues. A bull must also learn when a female is in estrus or he could be potentially injured by the female.

Heat Stress Impacts on Spermatogenesis

There are clearly effects of summer heat stress on semen quality and fertility in bulls. In 4 bulls identified as not passing semen quality control during the summer and then collected again in the fall during a cooler season of the year, post thaw motility increased (30 ± 3 vs. 45 ± 6 , $p=0.07$), viability decreased (40 ± 2 vs. 56 ± 3 , $p=0.07$), morphologically normal sperm increased (55 ± 6 vs. 76 ± 1 , $p<0.05$), and ability to fertilize oocytes in vitro increased (65 ± 4 vs. 76 ± 2 , $p<0.05$) ([Parrish et al., 2014](#)). Using a 48 h scrotal insulation model, spermatogenesis in bulls was impacted by this mild heat stress. The heat of scrotal insulation damaged zygotene to pachytene primary spermatocytes, secondary spermatocytes and round spermatids as evaluated by sperm morphology, nuclear shape and ability of sperm to fertilize oocytes in vitro and development of resulting embryos in vitro ([Parrish et al., 2014](#)). The studies suggest that damage to spermatogenesis is likely initiated via damage to the BTB as demonstrated by [Cheng and Mruk \(2012\)](#) to be essential for meiosis. As global climates warm, impacts of heat on bull spermatogenesis will become more pronounced.

Conclusion

Reproduction in the bull involves not only the production of sperm in the testes, but adequate nutrition to assure puberty at a young age, semen collection is maximized and attention to minimizing heat stress.

References

- Aguilar, I., Misztal, I., Johnson, D. L., et al. (2010). Hot topic: A unified approach to utilize phenotypic, full pedigree, and genomic information for genetic evaluation of Holstein final score. *J. Dairy Sci.*, *93*, 743–752.
- Amann, R. P. (1983). Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *J. Dairy Sci.*, *66*, 2606–2622.
- Amann, R. P., & Schanbacher, B. D. (1983). Physiology of male reproduction. *J. Anim. Sci.*, *57*, 380–403.
- Bratton, R., Musgrave, S., Dunn, H., & Foote, R. (1959). Causes and prevention of reproductive failures in dairy cattle. II. Influence of underfeeding and overfeeding from birth to 80 weeks of age on growth, sexual development, and semen production of Holstein bulls. *Bull. Cornell Agric. Exp. Sta.*, *940*, 1–45.
- Brito, L., Silva, A. E. D. F., Barbosa, R. T., & Kastelic, J. P. (2004). Testicular thermoregulation in *Bos indicus*, crossbred and *Bos taurus* bulls: Relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality and sperm production. *Theriogenology*, *61*, 511–528.
- Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implications for male contraception. *Pharmacol. Rev.*, *64*(1), 16–64.
- Courot, M., Reviers, M. H., & Ortavant, R. (1970). Spermatogenesis. In A. D. Johnson, W. R. Gomes, & N. L. Vandemark (Eds.), *The Testis, Volume I Development, Anatomy and Physiology* (pp. 339–432). New York: Academic Press.
- Dance, A., Thundathil, J., Wilde, R., Blondin, P., & Kastelic, J. (2015). Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls. *J. Dairy Sci.*, *98*, 987–998.
- Flipse, R. J., & Almquist, J. O. (1961). Effect of total digestible nutrient intake from birth to four years of age on growth and reproductive development and performance of dairy bulls. *J. Dairy Sci.*, *44*, 905–914.
- Harstine, B. R., Maquivar, M., Helser, L. A., et al. (2015). Effects of dietary energy on sexual maturation and sperm production in Holstein bulls. *J. Anim. Sci.*, *93*, 2759–2766.
- Killan, G. J., & Amann, R. P. (1972). Reproductive capacity of dairy bulls. IX. Changes in reproductive organ weights and semen characteristics of Holstein bulls during the first thirty weeks after puberty. *J. Dairy Sci.*, *55*, 1631–1635.
- Lennox, B., & Logue, D. N. (1979). Tubule length and Leydig cell volume in the normal bull testis. *Vet. Rec.*, *104*, 431–433.
- Parrish, J. J., Ostermeier, C., Schindler, J., et al., 2014. Quantifying sperm nuclear shape with Fourier harmonic analysis and relationship to spermatogenesis and fertility. In: Association for Applied Animal Andrology 2014 meeting. p. 30–49. International Veterinary Information Service, Available at: <http://www.ivis.org/proceedings/aaaa/2014/3.pdf>.
- Rawlings, N., Evans, A. C. O., Chandolia, R. K., & Bagu, E. T. (2008). Sexual maturation in the bull. *Reprod. Dom. Anim.*, *43*(Suppl. 2), 295–301.
- Salisbury, G. W., VanDemark, N. L., & Lodge, J. R. (1978). Management factors that affect the reproductive efficiency of the bull. In G. W. Salisbury (Ed.), *Physiology of Reproduction and Artificial Insemination of Cattle* (pp. 733–787). San Francisco, Ca: WH Freeman and Co.
- Senger, P. L. (2012). Spermatogenesis. *Pathways to Pregnancy & Parturition* (third ed., pp. 202–227). Redmond, OR: Current Conceptions, Inc.
- Siddiqui, M. A., Bhattacharjee, J., Das, Z. C., et al. (2008). Crossbred bull selection for bigger scrotum and shorter age at puberty with potentials for better quality semen. *Reprod. Domest. Anim.*, *43*(1), 74–79.
- VanRaden, P. M., Van Tassell, C. P., Wiggans, G. R., et al. (2009). Invited review: Reliability of genomic predictions for North American Holstein bulls. *J. Dairy Sci.*, *92*, 16–24.

Pig Overview

Peter Sutovsky, University of Missouri, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Artificial insemination Assisted reproductive technology used to reproduce livestock animals by using semen from male animal that is collected by artificial stimulation, then diluted and distributed as fresh or frozen insemination doses that are injected in the female reproductive tract by using special catheters, at the time during female reproductive cycle that favors conception.

Boar Adult male pig, domestic or wild, with intact sex organs.

Epididymus Male accessory sex gland that collects and stores sperm cells released from the testis, to convey to them the potential to fertilize an oocyte.

Farrowing Giving of birth by a female pig. Farrowing rate in sows is an indicator of reproductive efficiency and success of artificial insemination or natural mating.

Gilt A young adult or pubertal, virgin female pig.

Intracytoplasmic sperm injection (ICSI) Assisted reproductive technology in animals and infertility treatment in humans that bypasses the natural fertilization process by preselecting and injecting a single sperm cell in a fertilization and development-competent female oocyte, typically followed by in vitro embryo culture and embryo transfer.

Oviductal sperm reservoir Transient sperm storage site within the female Fallopian tube, the oviduct, to which sperm cells bind to await ovulation, the release of female ovum from the ovary in the oviduct.

Sperm capacitation Process by which spermatozoa within the female reproductive tract become competent to seek and fertilize an oocyte, encompassing extensive remodeling of various sperm structures, leading to increased sperm motility and ability of sperm plasma membranes to fuse with oocyte plasma membrane.

Semen sexing Automated process of separation of sperm cells bearing the male Y chromosome from the female X-chromosome-bearing ones, based on lesser DNA content of the former. The goal is to produce offspring of desired sex by artificial insemination.

Sow Adult female pig, domestic or wild, with intact sex organs.

Spermatogenesis Generation of spermatozoa in the testis that starts with multiplication of precursor germ cells by mitosis, followed by two rounds of meiotic division halving the number of chromosomes carried by each resulting daughter cell, and the differentiation of the resultant haploid germ cells, spermatids into spermatozoa with a compacted head and a tail capable of acquiring motility and fertilizing potential.

Transgenic animal An animal carrying a foreign gene (i.e. genetic material from another species) or set of genes inserted in its genome, typically during early embryo development.

Reproductive Organs

The boar has large elliptical testes with thick testicular tunics and adjacent large epididymides, connected to the testis by efferent ducts and to the urethra by the ductus deferens. The tail of the epididymis is dorsally oriented within the scrotum. The boar epididymis is composed of 9 distinct regions, parted into the initial segment, caput, corpus, and cauda epididymis. The scrotum is located in the sub-anal position and divided into two halves by a scrotal raphe. The testis descent occurs shortly before birth. Seminiferous tubules in the boar are very tightly packed, resulting in dense parenchyma from which individual tubules are impossible to extract unless enzymatic digestion is applied. The vesicular gland and bulbourethral glands are very large in the boar, and provide the bulk of seminal plasma volume. Inversely, the prostate is very small. Boars have a long, fibroelastic penis with a sigmoid flexure and a spiraling apex resting within a two-compartment prepuce. Such anatomy, (reviewed in detail by Constantinescu (2017)), facilitates cervical penetration and intracervical semen deposition during mating with a sow.

Spermatogenesis and Epididymal Sperm Maturation

Depending on collection schedule, the daily sperm output of a boar is approx. 15 billion and can bypass 20 billion (up to 120 billion per ejaculate). Sperm concentration can be as high as 200 million/mL, with the total ejaculate volume (both gel-rich and gel-free fraction) approaching 500 mL. As in other species, boar spermatogenesis is divided into proliferative/mitotic and meiotic phase, and a haploid, post-meiotic phase. During the proliferative phase, spermatogonia differentiate into spermatocytes entering meiosis, while a subpopulation of spermatogonial stem cells constantly renews itself to maintain a stable sperm output. The post meiotic phase, during which the haploid spermatids differentiate into spermatozoa capable of acquiring progressive motility and

fertilizing ability, can be divided into distinct steps by which the spermatid nucleus is remodeled and the sperm accessory structures such as the flagellum and acrosome arise. Parameters of boar spermatogenesis may vary between breeds. The duration of spermatogenesis from an undifferentiated A-type spermatogonium to spermatozoon is estimated at 34 days. The staging of seminiferous epithelium, i.e. the identification of the associations of specific germ cell types along the length of seminiferous tubule, points to 8 distinct stages. Spermiogenesis can be divided into 12 steps (Frankenhuis *et al.*, 1982). The length/duration of the cycle of seminiferous epithelium, i.e. the interval between two waves of sperm release from the same segment of a seminiferous tubule, is 8.6 days (Swierstra, 1968). The average duration of each of the 8 stages is 9 days (Franca and Cardoso, 1998). As in other species, boar spermatozoa complete their morphogenesis and undergo final maturation during epididymal transit when they acquire the potential for fertilization and progressive motility. This maturation process is mediated by the modification of existing sperm proteins and adsorption of new ones from epididymal fluid. Clusterin, glutathione peroxidase and lactoferrin are among the major secretory proteins in the boar epididymis (Syntin *et al.*, 1996). The sperm cytoplasmic droplet (CD) slides down the boar sperm tail during sperm transport from the caput to cauda epididymis. Compared to other mammals, a greater proportion of boar spermatozoa retain their distal CD until after ejaculation, at which time factors from seminal plasma are thought to contribute to its removal. Epididymal passage takes approximately 9–10 days and 120–165 billion spermatozoa are stored in a single cauda epididymis. The epididymal reserve could thus be replenished in 3–5 days after depletion.

Sperm Morphology and Abnormalities

The spatula shaped boar sperm head (Fig. 1) is very similar in appearance to bull. The head is approximately 9 μm long and 5 μm wide, and features a prominent acrosomal ridge and a clearly delineated, crescent shaped equatorial segment. The sperm tail (flagellum) measures approximately 30–35 μm , of which ~7–8 μm accounts for the midpiece with mitochondrial sheath. Similar to other mammals, the axonemal 9+2 microtubule arrangement is paralleled by nine outer dense fibers (ODF) along the length of the midpiece, principal piece and end piece, with the fibrous sheath wrapped around the principal piece. The connecting piece linking the sperm tail with the implantation fossa of the sperm head has a typical-for-mammals set of striated columns, directly connected to one ODF each, caging a functional proximal centriole composed of nine microtubule triples. The proximal centriole serves as the organizer of the zygotic centrosome at fertilization, a pattern typical for all mammals except rodents. The distal centriole is dismantled during spermiogenesis, leaving an empty distal centriolar vault (Manandhar *et al.*, 2006).

The most common ejaculated sperm abnormality in boars is cytoplasmic droplet retention (Fig. 2), which is negatively associated with sperm ability to bind to oviductal epithelia and with fertility in AI service (Lovercamp *et al.*, 2007). Other common abnormalities in semen of fertile boars include knobbed or otherwise abnormal acrosomes, and deformed sperm heads, as well as distal cytoplasmic reflex and abaxial attachment of the sperm tail. Nuclear vacuoles and diadems (craters) are less common than in bull spermatozoa. Presumed heritable sperm defects include immotile short tails (not to be confused with stump tail syndrome) and knobbed acrosomes. Examples of genomic influences on boar sperm phenotype and fertility include the of copy number variation in sperm function associated genes (e.g. *WBP2NL* and *ZAN*; (Wang *et al.*, 2015)), and sperm-phenotype affecting polymorphisms in genes such as ubiquitin-ligase *HECW2* (syn. *NEDL2*) associated with the knobbed acrosome defect and an intronic insertion in the *KLP2* gene implicated in flagellar/ciliary biogenesis and function in boars with short immotile tails (Sironen *et al.*, 2010). Conventional analysis of sperm morphology and motility provides useful baseline information on semen quality, but assays of membrane and DNA integrity, mitochondrial membrane potential, viability and sperm molecular makeup are increasingly sought to connect the sperm phenotypes to boar fertility in AI service. Biomarker based sperm/semen analysis and preselection/purification by flow cytometry and other techniques is increasingly used to evaluate boar sperm quality and improve the fertility of AI dose (Sutovsky, 2015).

Seminal Plasma

Seminal plasma (SP) supports ejaculated boar spermatozoa up until their passage through the uterotubal junction. Proposed roles include warding off the immune attack by uterus-infiltrating leucocytes, maintenance of a non-capacitated sperm state and metabolic support of spermatozoa (Rodríguez-Martínez *et al.*, 2009). Seminal plasma components such as micro RNAs and cytokines may stimulate gene expression in the uterus, as a way of priming the uterine lining for conception and implantation (Robertson *et al.*, 2017). Specific to boars, seminal plasma is thought to contain factors facilitating the rejection of CDs by ejaculated spermatozoa. Given the enormous size of vascular and bulbourethral glands, boars can produce as much as 500 mL of semen in one ejaculation, the majority of the volume contributed by SP. Boar SP is rich in inositol, fructose, bicarbonate, magnesium and calcium (Bearden and Fuquay, 2000; Rodríguez-Martínez *et al.*, 2009), and has the highest zinc content among all studied bodily fluids. Spermadhesins, PSP and DQH glycoproteins are the major SP proteins in boars (Jonakova *et al.*, 2007). Adsorbed mainly onto acrosomal surface during ejaculation, spermadhesins have been implicated in sperm-oviduct binding during the formation of the oviductal sperm reservoir. Conversely, spermadhesins such as AQN1 are proteolytically cleaved and shed from the sperm surface during sperm capacitation (Ekhlasi-Hundrieser *et al.*, 2005), to facilitate sperm detachment from the oviductal reservoir in response to ovulation. Such processing of sperm surface proteins may be mediated by intrinsic sperm proteases and possibly by proteolytic and glycolytic enzymes of the oviductal fluid. A high concentration of certain SP proteins is associated with high fertility in AI boars, while other SP proteins may be more abundant in low fertility ones (Flowers *et al.*, 2016).

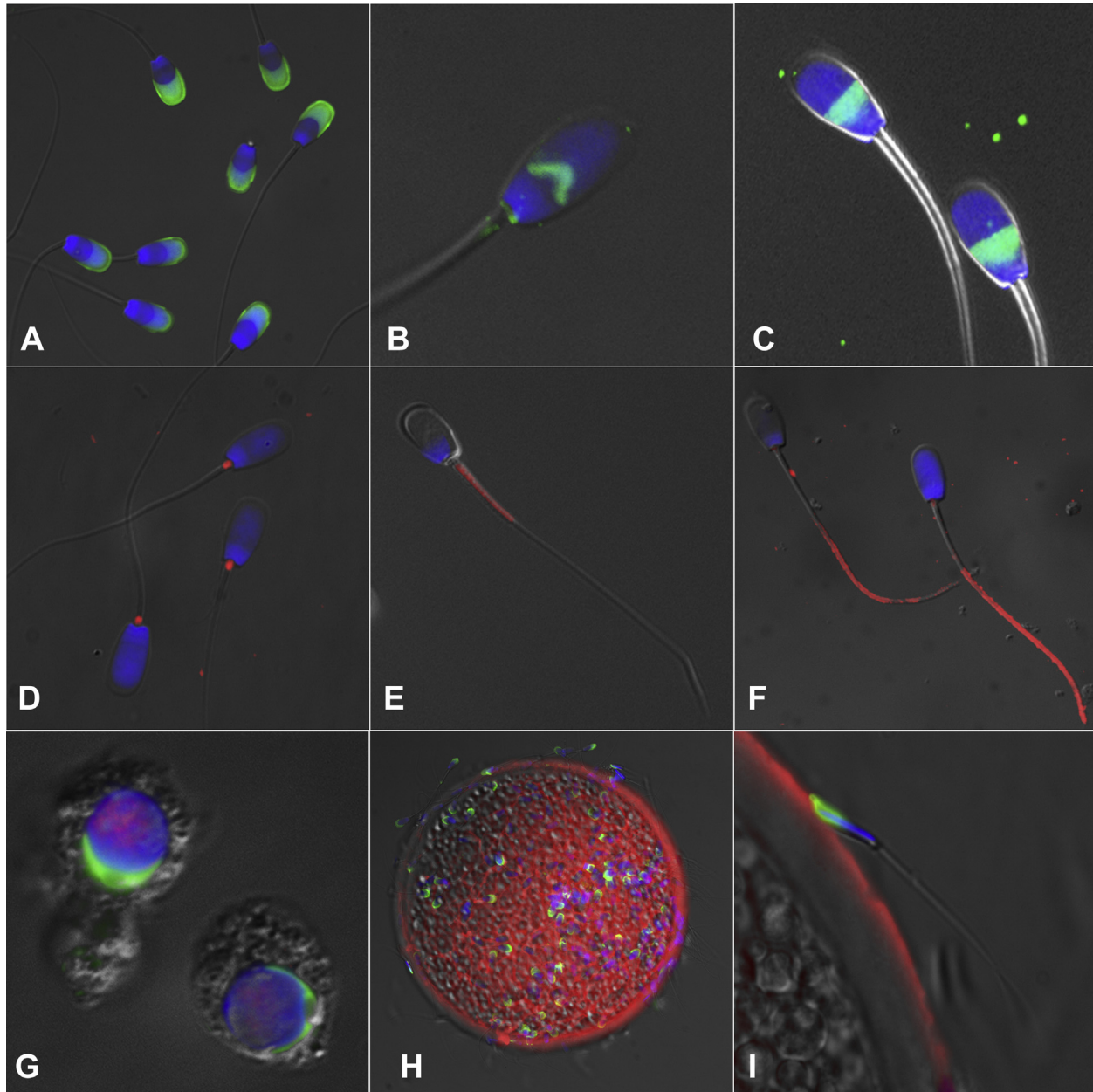


Fig. 1 Accessory structures of the boar spermatozoon. (A) Acrosome labeled with lectin PNA (green). (B) Equatorial segment, labeled with lectin LCA (green). (C) Postacrosomal sheath labeled with antibody against the WW-domain containing protein WBP2NL (alias PAWP; green). (D) Sperm tail connecting piece, housing the sperm centriole, labeled with antibody against thioredoxin TXL1 (red). (E) Sperm tail midpiece, covered with the mitochondrial sheath, labeled with ProteoStat aggresome detection kit for the labeling of ubiquitinated protein aggregates (red). (F) Sperm tail principal piece covered with fibrous sheath, labeled with antibody against male germline-specific thioredoxing TXND3 (alias SPTRX2; red). (G) Boar round spermatids, with acrosomal caps labeled with lectin PNA (green) and nuclei labeled with antibody against ubiquitin ligase MKRN1 (alias makorin 1, red). (H, I) Porcine oocytes with zona pellucida (red; antibody against ZP3 protein) bound spermatozoa (green acrosomes labeled with lectin PNA). Sperm nuclei were stained with DAPI. Epifluorescence images were superimposed on parfocal transmitted light images acquired using differential interference contrast (DIC) optics.

Mating, Seasonal Effects and Hormonal Control of Gonadal Function

Domestic pigs are not seasonal breeders as the females are cyclic all year. However, boar libido and sperm quality may be influenced by seasonal changes in photoperiod and ambient temperature, particularly summer heat stress (Ross *et al.*, 2017). Given the duration of spermatogenic cycle and epididymal passage, some such adverse effects can last for weeks/months after the exposure, and can be mimicked by scrotal insulation. In addition to reduced sperm quality, sperm content of proteins associated with aberrant sperm phenotypes, such as ubiquitin, increases after heat stress (Lovercamp *et al.*, 2007). Compared to other mammals,

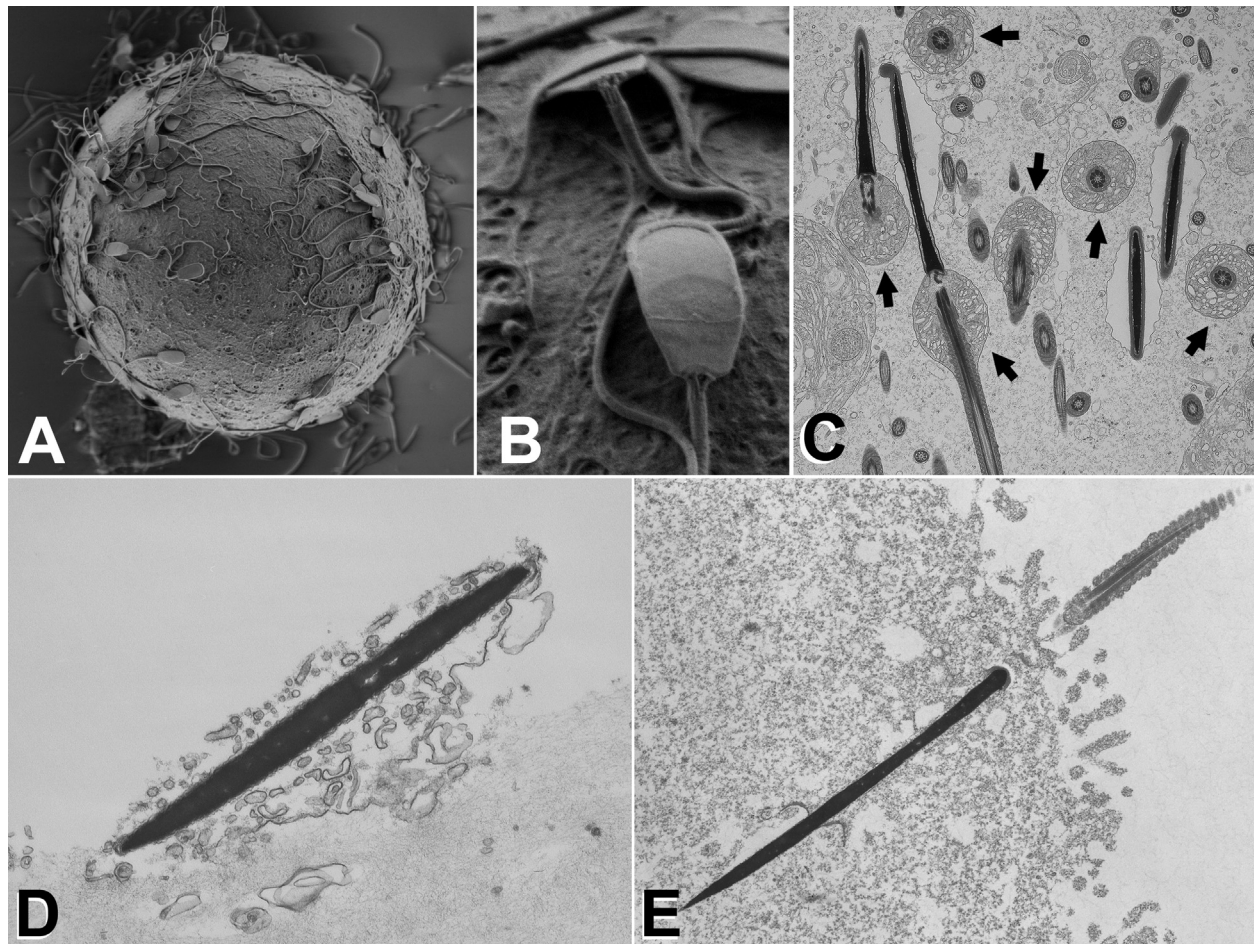


Fig. 2 Scanning (A, B) and transmission (C–E) electron micrographs of boar spermatozoa on the surface of oocyte zona pellucida (A, B, D; note the vesiculation of the acrosome in panel D, caused by zona-induced acrosomal exocytosis), in the epididymis (C; note prominent cytoplasmic droplets – arrows), and at the time of sperm incorporation in the oocyte cytoplasm (E; sperm tail is still outside of the oocyte, which was fertilized zona-free *in vitro*, in this case). Photo credit, panels (A) and (B), Shawn Zimmerman, PhD.

boars produce as much as 500 mL of ejaculate, mainly due to the large volume contribution by accessory sex gland secretions (Constantinescu, 2017). Boars are uterine semen depositors, meaning that their glans penis reaches and partially penetrates the cervix during mating. Ejaculation occurs in fractions (segmented ejaculation) over a period of time as long as 10 min (Bearden and Fuquay, 2000). The initial portion of the sperm-rich fraction appears to have the highest content of epididymal fluid proteins, while seminal plasma/fluid proteins produced by accessory sex glands prevail in the main volume of sperm-rich fraction (Rodríguez-Martínez *et al.*, 2009).

Boar libido and testicular function is guided by the hypothalamic-pituitary-gonadal axis. Hypothalamic gonadotropin releasing hormone (GNRH) stimulates pulsatile secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary, which in turn stimulates steroidogenesis and testosterone production by testicular interstitial Leydig cells (Lents *et al.*, 2017). Epididymal sperm maturation and epididymis sustenance is stimulated by lumicrine factors from rete testis fluid- accompanying spermatozoa as they travel through the efferent ducts to the caput epididymis. Puberty starts generally at around 120–180 days of age but as early as 56–84 days in Meishan boars. Between the two mammalian isoforms of gonadotropin releasing hormone, GNRH2 and its receptor seems to control testosterone production in boar, with a feedback loop regulated by RF-amide-related peptide 3, which appears to control both the testis and hypophysis, and suppresses GNRH-I secretion from the hypothalamus (Lents *et al.*, 2017). Within the seminiferous tubule, spermatogonial renewal, proliferation and differentiation are regulated by an intricate signaling network, involving growth factors, cell cycle regulators and transcription factors.

Sperm Transport and Capacitation

After uterine deposition, boar spermatozoa migrate to the oviduct within 1–2 h after mating as they reach the site of oviductal sperm reservoir in the distal part of the oviductal isthmus, where they await ovulation (Suarez and Pacey, 2006). Detachment from the reservoir upon capacitation and hyperactivation is mediated by proteolysis causing the shedding of spermadhesins from the sperm

head acrosomal surface (Ekhlesi-Hundrieser *et al.*, 2005), mirroring the cleavage and release of the BSP proteins from capacitating bull spermatozoa. Capacitation causes a series of signaling, biochemical and structural remodeling events that release spermatozoa from oviductal reservoir site, remodel the sperm surface, endow spermatozoa with hyperactivated motility and prepare them for interactions with the oocyte at fertilization. Hallmarks of boar sperm capacitation common with sperm capacitation in other mammals include tyrosine phosphorylation, Ca-influx and cholesterol removal (Gadella, 2017), as well as recently postulated regulation by the 26S proteasome (Kerns *et al.*, 2016). Capacitation is not fully reversible, and spermatozoa that fail to reach the oocyte after detaching from oviductal sperm reservoir die within 4–6 h (Suarez and Pacey, 2006). Consequently, premature sperm capacitation in the semen processed for AI reduces AI dose fertility.

Fertilization and Zygotic Development

The nature of oocyte produced sperm attractant, if any exists in pigs, is not known, though progesterone is currently favored for a role in mammalian sperm-oocyte chemotaxis. Of the three porcine zona pellucida (ZP) composing proteins (ZPA/ PZP1 - homologue of mouse ZP2; ZPB/PZP3 α , homologue of human ZP4; ZPC/PZP3 β , homologue of mouse ZP3), the ZPB-ZPC complex rather than a single ZP protein species is thought to act as receptor for sperm-ZP adhesion (Yonezawa *et al.*, 1997). Both conventional proteases and acrosomal proteasomes (Zimmerman *et al.*, 2011) have been implicated in ZP digestion and penetration by boar spermatozoa. After sperm passage through the ZP, oocyte JUNO and sperm IZUMO1 proteins are the likely surface ligands involved in sperm adhesion to the oocyte plasma membrane, the oolemma (Tanihara *et al.*, 2014). The nature of fusogenic molecules involved in the gamete plasma membrane fusion after initial adhesion is not known. An alternative model proposing a phagocytosis-like mechanism of sperm incorporation in the oocyte has been proposed in mammalian fertilization. Porcine gametes initially fuse at the sperm head equatorial segment and the postacrosomal perinuclear theca solubilizes quickly in the ooplasm, releasing sperm-borne oocyte activating factors (SOAF). The currently favorite SOAF molecule, phospholipase PLCZ1 is present in boar spermatozoa but its localization and solubility during fertilization do not support a role in oocyte activation, prompting research into alternative SOAF candidates such as PAWP/WBP2NL (Oko *et al.*, 2017). Following sperm incorporation in the ooplasm, which involves cortical microfilament cytoskeleton of the oocyte (sperm tail beating stops at gamete fusion), the sperm nucleus decondenses quickly, and both maternal and paternal pronuclei (PN) form in synchrony, with the paternal PN being larger than the maternal one. The sperm mitochondrial sheath, which remains associated with paternal PN early after fertilization, is degraded very quickly, i.e. before first embryo cleavage, to promote post-fertilization sperm mitophagy as way of enforcing maternal inheritance of mitochondria and mtDNA, and thus preventing heteroplasmy (coexistence of paternal and maternal mitochondrial genomes in the same individual; (Song *et al.*, 2016)). With such a mechanism being specific to gametes, somatic cell mitochondria are not recognized as foreign, causing some piglets born from embryos reconstructed by somatic cell nuclear transfer (SCNT) to be heteroplasmic.

In Vitro Fertilization (IVF) and Intracytoplasmic Sperm Injection (ICSI)

While the first live birth after surgical transfer of flushed porcine embryos reportedly occurred in 1950, IVF embryos by surgical (with frozen spermatozoa) or non-surgical transfer were produced more recently. Porcine oocytes mature in vitro in the presence of gonadotropins for 44 h, typically with a medium change at 24 h; thus, IVF is a three day cycle. Cumulus cells are removed prior to IVF. Typical sperm concentration is 0.5–1 million/mL of IVF medium based on a modified tris-buffer (Abeydeera and Day, 1997). In vitro capacitation prior to sperm mixing with oocytes is not required as the spermatozoa capacitate in IVF medium. Spermatozoa incorporated into oocyte cytoplasm are first observed at 5–8 h after gamete mixing. Polyspermy is common in vitro (but not prevalent in vivo) due to reduced quality/developmental potential of in vitro matured oocytes and lack of oocyte exposure to oviductal secretions. Polyspermy can be mitigated by IVF culture medium supplementation with isolated oviductal fluid or its components such as osteopontin, or with recombinant deubiquitinating enzymes. Common protocols call for transfer from fertilization medium to a development promoting embryo culture medium at 6 h after sperm-oocyte mixing. First embryo cleavage typically occurs within 24 h after gamete mixing. Compacting morulae appear on day 5 and hatching blastocysts on Day 7. Zona free oocytes can also be fertilized in vitro and the resultant embryos used to produce piglets by embryo transfer.

Apart from sperm vectored transgenic ICSI, porcine ICSI is performed for research rather than for clinical or production purposes. Contrary to humans and non-human primates, and similar to murid and bovine ICSI, boar spermatozoa disassemble poorly when injected in porcine oocytes, warranting the use of a piezo-driven injector. Sperm disassembly and pronuclear development can be improved by supplementation of culture media with cysteine, with which live births have been achieved (Katayama *et al.*, 2007).

Artificial Insemination (AI)

Semen is most commonly collected from phantom-trained boars by the gloved hand technique or automated collection devices (pneumatic or physically applied pressure), in fractions (Bearden and Fuquay, 2000). Only the sperm-rich second fraction is typically processed for AI out of concern for low sperm concentration and high bacterial count in the initial sperm fraction (first 10 mL); which, however, appears to be most resistant to cryodamage and most capable of reaching the oviductal sperm reservoir

after AI (Rodriguez-Martinez *et al.*, 2009). Commonly desired minimal sperm progressive motility is 80%, with total sperm count of at least 60 billion, concentration of 100–200 million/mL (sperm rich fraction) and 80–90% normal morphology by conventional light microscopic assessment; although the rejection limits for an ejaculate may vary by boar stud. Depending on deposition site, a single AI dose contains 1–3 billion spermatozoa suspended in 80 mL of extended semen (Bearden and Fuquay, 2000). Boar semen freezes poorly, and spermatozoa may capacitate prematurely (Yeste *et al.*, 2017). Semen pooling is a common practice in the US because of a high degree of variation in semen quality between boars and within a boar between collections. Single sire AI is desired by the swine industry, which may need high precision management of boar reproductive function. Increasing use of post-cervical and deep intrauterine insemination, semen freezing and lower sperm doses (Yeste *et al.*, 2017) drive the development of novel AI catheters. Sperm quality and fertility in boars is influenced by genetics, nutrition, housing, seasonal weather and photoperiod effects, and disease, and further impacted by semen handling and processing. Semen sexing is possible in boars and live births have been achieved, but sexing is not (yet) practical and thus in limited use due to large semen volume, high sperm number needed per AI dose and poor freezability. Progress is being made as the AI technology improves and tubal insemination becomes more common. Improvements of AI dose have been achieved by various purification techniques and by supplementation of boar semen extenders with antioxidants [reviewed by Sutovsky (2015)].

Transgenic Boar as a Biomedical and Biotechnological Animal Model

As a biomedical model, domestic pig is a valuable alternative to rodents because of a manageable generational interval (114 day gestation) and large litter size, and particularly because the size and anatomy of pig organs is similar to humans, favoring the pig as a model for testing surgical procedures, as well as for xenotransplantation. Furthermore, in some cases transgenic pigs bearing mutations mimicking human genetic disease are more likely to have human-like disease phenotypes and symptoms than transgenic rodents (Prather *et al.*, 2013). Importantly, domestic pigs are now routinely genetically modified. Boar-relevant transgenic pig models include green-fluorescent proteasome pig for the study of sperm function, cystic fibrosis transmembrane receptor (CFTR) mutant pig with congenital absence of vas deferens, the NANOS2 male germline ablated pig and the porcine reproductive and respiratory (PRRS) virus resistant pig which is relevant to boar fertility because the PRRS virus is transferred by semen during AI. Because of a lack of chimera-suitable porcine embryonic stem cells, the most common method for the production of transgenic pigs is by somatic cell nuclear transfer (SCNT) of genetically modified fibroblasts, and more recently by zygotic CRISPR/CAS microinjection. Earlier successes have been achieved, among other approaches, by direct zygotic-pronuclear injection of DNA constructs, and by sperm-vectored IVF and ICSI transgenesis.

Acknowledgements

Author wishes to thanks his colleagues, Professors Timothy Safranski and Randall Prather, and his graduate student Mr. Karl Kerns for valuable comments on this manuscript. Work in author's laboratory is currently funded by Agriculture and Food Research Initiative Competitive Grant no. 2015–67015-23231 from the USDA National Institute of Food and Agriculture, grant number 5 R01 HD084353-02 from NIH National Institute of Child and Human Development, and by seed funding from the Food for The 21st Century Program of the University of Missouri.

References

- Abeydeera, L. R., & Day, B. N. (1997). Fertilization and subsequent development in vitro of pig oocytes inseminated in a modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. *Biol. Reprod.*, 57, 729–734.
- Bearden, H. J., & Fuquay, J. W. (2000). *Applied Animal Reproduction* (5th ed). Upper Saddle River, NJ: Prentice-Hall.
- Constantinescu, G. M. (2017). Anatomy of the reproductive system. In H. Schatten, & G. M. Constantinescu (Eds.), *Animal Models Adn Human Reproduction* (pp. 1–58). Hoboken, NJ: John Wiley & sons.
- Ekhlas-Hundrieser, M., et al. (2005). Spermadhesin aqn1 is a candidate receptor molecule involved in the formation of the oviductal sperm reservoir in the pig. *Biol. Reprod.*, 73, 536–545.
- Flowers, W. L., Deller, F., & Stewart, K. R. (2016). Use of heterospermic inseminations and paternity testing to evaluate the relative contributions of common sperm traits and seminal plasma proteins in boar fertility. *Anim. Reprod. Sci.*, 174, 123–131.
- Franca, L. R., & Cardoso, F. M. (1998). Duration of spermatogenesis and sperm transit time through the epididymis in the piau boar. *Tissue Cell*, 30, 573–582.
- Frankenhuis, M. T., Kramer, M. F., & de Rooij, D. G. (1982). Spermatogenesis in the boar. *Vet. Q*, 4, 57–61.
- Gadella, B. M. (2017). Reproductive tract modifications of the boar sperm surface. *Mol. Reprod. Dev.*
- Jonakova, V., Manaskova, P., & Ticha, M. (2007). Separation, characterization and identification of boar seminal plasma proteins. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 849, 307–314.
- Katayama, M., et al. (2007). Improved fertilization and embryo development resulting in birth of live piglets after intracytoplasmic sperm injection and in vitro culture in a cysteine-supplemented medium. *Theriogenology*, 67, 835–847.
- Kerns, K., Morales, P., & Sutovsky, P. (2016). Regulation of sperm capacitation by the 26s proteasome: An emerging new paradigm in spermatology. *Biol Reprod*, 94, 117.
- Lents, C. A., Thorson, J. F., Desaulniers, A. T., & White, B. R. (2017). Rfamide-related peptide 3 and gonadotropin-releasing hormone-ii are autocrine-paracrine regulators of testicular function in the boar. *Mol. Reprod. Dev.*
- Lovercamp, K. W., et al. (2007). Arachidonate 15-lipoxygenase and ubiquitin as fertility markers in boars. *Theriogenology*, 67, 704–718.
- Manandhar, G., et al. (2006). Centrosomal protein centrin is not detectable during early pre-implantation development but reappears during late blastocyst stage in porcine embryos. *Reproduction*, 132, 423–434.
- Oko, R., Aarabi, M., Mao, J., Balakier, H., & Sutovsky, P. (2017). Sperm specific ww-domain binding proteins. In C. DeJonge, & C. Barratt (Eds.), *The Sperm Cell: Production, Maturation, Fertilization, Regeneration*. Cambridge, UK: Cambridge University Press.

- Prather, R. S., Lorson, M., Ross, J. W., Whyte, J. J., & Walters, E. (2013). Genetically engineered pig models for human diseases. *Annu. Rev. Anim. Biosci.*, 1, 203–219.
- Robertson, S. A., et al. (2017). MicroRNA regulation of immune events at conception. *Mol. Reprod. Dev.*
- Rodriguez-Martinez, H., et al. (2009). The physiological roles of the boar ejaculate. *Soc. Reprod. Fertil. Suppl.*, 66, 1–21.
- Ross, J. W., et al. (2017). Physiological mechanisms through which heat stress compromises reproduction in pigs. *Mol. Reprod. Dev.*
- Sironen, A., et al. (2010). Knobbed acrosome defect is associated with a region containing the genes *stk17b* and *hecw2* on porcine chromosome 15. *BMC Genomics*, 11, 699.
- Song, W. H., Yi, Y. J., Sutovsky, M., Meyers, S., & Sutovsky, P. (2016). Autophagy and ubiquitin-proteasome system contribute to sperm mitophagy after mammalian fertilization. *Proc. Natl Acad. Sci. USA*, 113, E5261–E5270.
- Suarez, S. S., & Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Hum. Reprod. Update*, 12, 23–37.
- Sutovsky, P. (2015). New approaches to boar semen evaluation, processing and improvement. *Reprod. Domest. Anim.*, 50(Suppl 2), 11–19.
- Swierstra, E. E. (1968). Cytology and duration of the cycle of the seminiferous epithelium of the boar; duration of spermatozoan transit through the epididymis. *Anat. Rec.*, 161, 171–185.
- Syntin, P., et al. (1996). Characterization and identification of proteins secreted in the various regions of the adult boar epididymis. *Biol. Reprod.*, 55, 956–974.
- Tanihara, F., et al. (2014). Roles of the zona pellucida and functional exposure of the sperm-egg fusion factor ‘izumo’ during in vitro fertilization in pigs. *Anim. Sci. J.*, 85, 395–404.
- Wang, H., et al. (2015). Genome wide distributions and functional characterization of copy number variations between chinese and western pigs. *PLOS One*, 10, e0131522.
- Yeste, M., Rodríguez-Gil, J. E., & Bonet, S. (2017). Artificial insemination with frozen-thawed boar sperm. *Mol. Reprod. Dev.*, 84(9), 802–813.
- Yonezawa, N., Mitsui, S., Kudo, K., & Nakano, M. (1997). Identification of an n-glycosylated region of pig zona pellucida glycoprotein zpb that is involved in sperm binding. *Eur. J. Biochem.*, 248, 86–92.
- Zimmerman, S. W., et al. (2011). Sperm proteasomes degrade sperm receptor on the egg zona pellucida during mammalian fertilization. *PLOS One*, 6, e17256.

Relevant Websites

- <http://porkgateway.org/resource/evaluating-boar-semen-for-quality/>. – Evaluating Boar Semen Quality.
- [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1098-2795/homepage/virtual_issue_control_of_pig_reproduction_x.htm](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1098-2795/homepage/virtual_issue_control_of_pig_reproduction_x.htm). – Molecular Reproduction & Development.
- <http://onlinelibrary.wiley.com/doi/10.1111/rda.2015.50.issue-S2/issuetoc>. – Reproduction in Domestic Animals 2015.

Horse Overview

Christine Aurich, Artificial Insemination and Embryo Transfer, Vienna, Austria

© 2018 Elsevier Inc. All rights reserved.

Anatomy of the Equine Male Reproductive Tract

In the stallion, the paired testicles are located in the inguinal region and covered by the scrotum. The scrotal skin is hairless or finely haired, often pigmented and contains numerous sweat glands. The testicles are in a horizontal position and have the shape of plump eggs with their size varying between a hen's egg and a man's fist depending on size and age of the stallion. The epididymal body (*corpus epididymidis*) is lying dorsally on the respective testis, its head (*caput epididymidis*) is covering the cranial testicular pole, and its tail (*cauda epididymidis*) is located at the caudal testicular pole. While the head is closely associated with the testis, the epididymal tail is clearly protruding and therefore easily accessible by manual palpation (Fig. 1). A fibrous structure, the proper ligament of the testis, attaches the epididymal tail to the testis. The efferent ducts of the testis terminate into the epididymal head and merge to form the epididymal duct. The deferent duct is a direct continuation of the epididymal duct. It ascends from the epididymis into the spermatic cord and enters the abdominal cavity through the inguinal canal. It finally opens into the proximal part of the urethra at the *colliculus seminalis* (seminal hillock). The terminal part of the duct is enlarged and forms the ampulla of the deferent duct, one of the accessory sex glands. The deferent duct is joined by the excretory duct of the respective vesicular gland; the shared termination of them is also called the ejaculatory duct. In horses, only the compact part of the prostate gland exists and consists of two lateral lobes and an isthmus (prostatic body) traversing the urethra. Multiple excretory ducts of the prostate terminate into the urethra in close proximity to the ejaculatory ducts. The paired bulbourethral glands are located near the pelvic outlet and are covered by the respective bulboglandular muscle. Several secretory ducts terminate dorsally into the urethra. The penis of the horse is of the musculocavernous type. The opening of the prepuce is located directly behind the umbilicus. The shape of the equine glans penis is similar to that of a mushroom, the *corona glandis* being its widest part. The urethra protrudes into a distinctive central fossa (*fossa glandis*) at the free end of the corona (Fig. 2). The pronounced increase in size of the penile glans at maximal erection allows for dilatation of the mare's cervical canal at copulation resulting in deposition of the ejaculate directly into the uterine lumen in this species.

Reproductive Activity and Behaviour in Stallions

Horses are seasonal breeders with sexual activity occurring in spring and summer. The increase in sexual activity is stimulated by increasing day length after the winter solstice. However, in domesticated horses, breeding selection has diminished seasonal reproductive activity because conception early in the year is considered advantageous to horse breeds participating in races or equestrian competitions early in life. While in less domesticated horse breeds, the ovulatory season is limited to the time period between

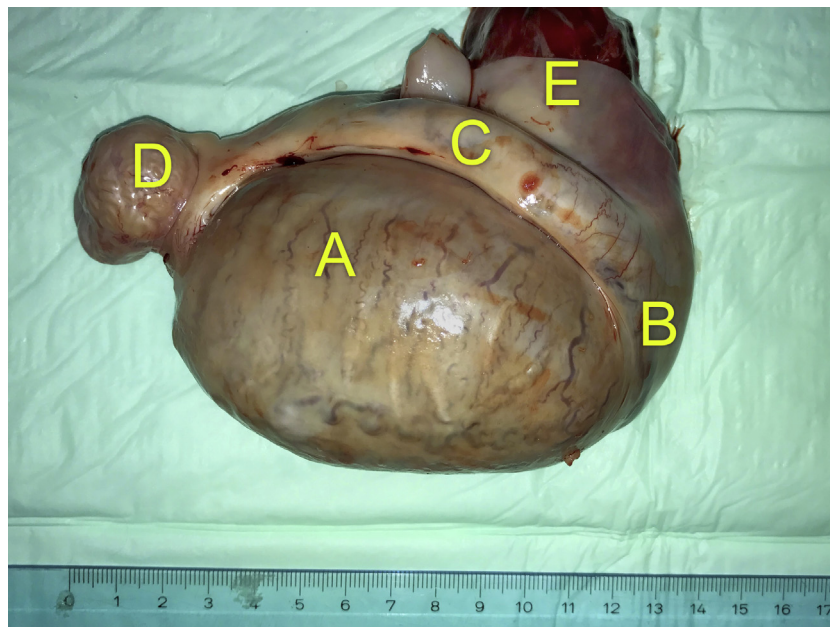


Fig. 1 Photograph depicting right testis and epididymis collected at surgery of a mature healthy stallion. (A) testis, (B) caput epididymidis, (C) corpus epididymidis, (D) cauda epididymidis, and (E) spermatic cord. Reproduced from Aurich, C., Vetmeduni Vienna, Austria.



Fig. 2 Erected penis of a warmblood stallion shortly before mounting. Reproduced from Aurich, C., Vetmeduni Vienna, Austria.

May and October, in more domesticated breeds like Thoroughbreds or riding-type horses a certain percentage of females continues cyclic ovarian activity throughout winter. In contrast, stallions are fertile throughout the year irrespective of breed, although testicular size and function as well as sexual behaviour are reduced outside the breeding season (**Fig. 3**). Testicular weight, parenchymal weight and seminiferous tubular volume increase by 30% from the beginning to the height of the breeding season. Due to increased activity of the accessory sex glands during the breeding season, also changes in ejaculate characteristics such as increased volume but decreased sperm concentration occur. In addition to photoperiod, exogenous factors such as age, reproductive state, nutrition, body condition and environmental temperature contribute to changes in reproductive activity of the horse (reviewed in [Aurich \(2016\)](#)).

In the normal male horse, testicular descend into the scrotum occurs between 30 days before and 10 days after birth. Puberty occurs at an age of 56–97 weeks with an average of 83 weeks ([Naden et al., 1990](#)). In stallions below three years of age, semen quality may be poor and male horses do not achieve maximal efficiency in spermatogenesis before an age of 5–6 years when semen production and fertility reach a plateau phase. From an age of 14 years onwards, semen quality may decline due to age-related testicular degeneration and impaired epididymal function. This may finally result in azoospermia and infertility in old stallions while characteristic male sexual behaviour is often maintained ([Amann, 2011](#)).

Under feral conditions, horses live in stable family groups that typically consist of adult mares, their young offspring and one to five adult harem stallions. Strong social bonds among stallions and mares exist resulting in continuous interaction among members of the harem. Under today's breeding conditions, the situation is completely different: the contact time of an individual stallion to mares is often minimal. If mating by natural cover takes place, reproductive receptivity of the mares is mostly not tested by the sire selected for breeding but instead the mare's sexual behaviour will be determined by exposing her to a less valuable stallion ("teasing"). The contact of stallions to oestrous mares is therefore often limited to the mating procedure itself. If they are used for artificial insemination, the time of contact to mares may be even more limited. The stallion is often only exposed to an oestrous mare for stimulation of sexual arousal when brought to the semen collection room. In well-trained stallions, semen collection may even take place on a breeding dummy in complete absence of a mare. It has been suggested that this man-made breeding routine contributes to dysfunctions of sexual behaviour and subfertility among hand-bred domestic horses when compared to equines breeding at liberty ([McDonnell, 2000](#)). The social environment of breeding stallions may impair ejaculate characteristics and contribute to increased cortisol concentration. Although fertility of horses kept under the described management may be lower than in their feral conspecifics, it is still considered acceptable and results in end of season pregnancy rates of 80% and foaling rates of 70%. However, unlike other domestic animal species, stallions are mainly selected for breeding based on their phenotypic

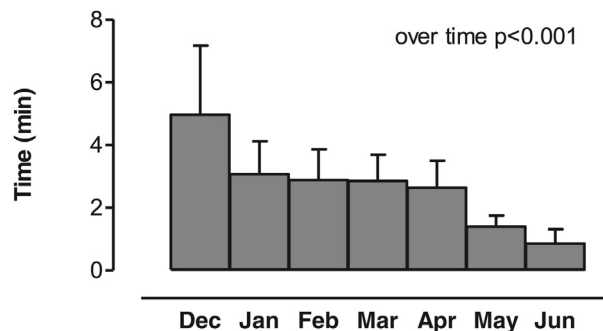


Fig. 3 Time (min; mean+SEM) from entering the breeding barn until ejaculation in Shetland stallions ($n=7$) kept under ambient photoperiod in different months of the year. Reproduced from Aurich, C., Vetmeduni Vienna, Austria.

characteristics and athletic performance, but not on reproductive performance. The current knowledge on genetic traits involved in fertility in the horse is still limited, but some candidate genes contributing to infertility in stallions have already been identified (Giesecke *et al.*, 2010).

If a stallion has the opportunity to breed mares at liberty, i.e., under conditions similar to the feral, an oestrous mare will usually start showing increased interest in the male in response to his sexual attractiveness. Presentation of characteristic oestrous behaviour of the mare (posture with lowered pelvis and straddled hind limbs accompanied by deviation of the tail, exposition of the perineal region and “clitoral winking”, i.e., rhythmic eversion of the clitoris, as well as frequent voidance of small quantities of urine) will stimulate sexual arousal in the stallion. This is mostly accompanied by an olfactory response of the male including frequent flehmen (curling of the upper lip for presentation of the vomeronasal organ for better perception of the mare’s odour, Fig. 4). It has been assumed that frequent urination of the oestrous mare induces chemosensory priming of the stallion for reproduction (Crowell-Davis, 2007). Erection is followed by mounting and insertion of the penis into the mare’s vagina. Some vigorous pelvic thrusts of the stallion will finally lead to ejaculation. In the horse this is characterized by deposition of five to eight jets of seminal fluid. Ejaculation starts with deposition of spermatozoa-free presperm fluid that originates from the bulbourethral glands. This is followed by the sperm-rich fraction and eventually a sperm-poor gelatinous fraction with its major component coming from the seminal vesicles (Kosniak, 1975). Presence and volume of the gelatinous fraction depends on season and sexual arousal with higher volumes during the breeding season and after prolonged stimulation of sexual behaviour.

Endocrine Regulation of Stallion Reproduction

In the horse, both endogenous and environmental factors affect the synthesis and secretion of GnRH from the hypothalamus via higher brain areas. These include factors like the day-night ratio, temperature, food range and composition, energy intake, social status of the animal and others. Hypothalamic activity is also modulated by endogenous steroid feedback mechanisms. In the stallion, testosterone activates endogenous opioidergic systems inhibiting GnRH release and thus limiting synthesis and release of testicular steroid hormones. Opioidergic activity is further modulated by season with highest opioidergic tone being present outside the breeding season (Aurich *et al.*, 1994). Reduced GnRH release is assumed to be the key to inhibition of reproductive function in stallions outside the breeding season. This depends on the prolonged presence of melatonin associated with long nights and short days during the non-breeding season in winter. During this time, LH and testosterone release are reduced. Besides seasonal changes in reproductive activity, not only in wild horses such as the Przewalski horse but also in domesticated horses, metabolism is reduced. This has been interpreted as the capacity of equines for seasonal adaptation to environmental conditions with the aim to limit energy expenditure. Such mechanisms have been suggested to further influence reproductive functions in horses.

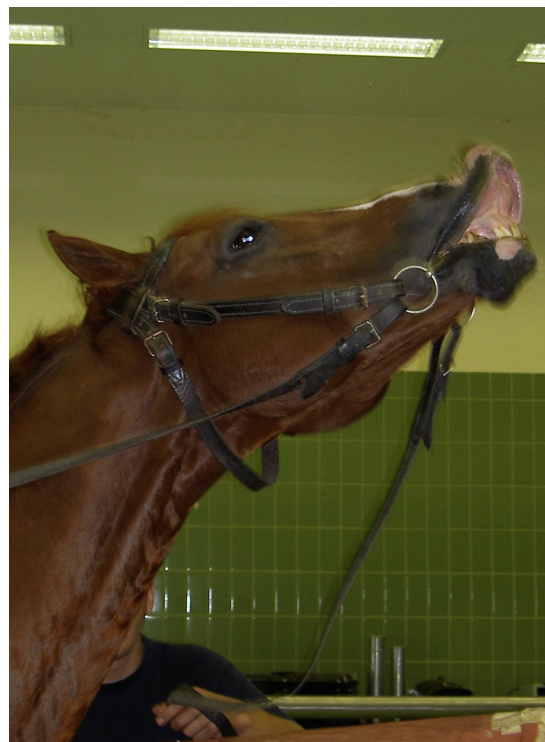


Fig. 4 Flehmen of a warmblood stallion when exposed to an oestrous mare for stimulation before semen collection. Reproduced from Aurich, C., Vetmeduni Vienna, Austria.

In the stallion, the mean GnRH pulse interval is approximately 30 min. A high synchrony between GnRH, LH and FSH pulses in pituitary effluent blood has been demonstrated (Irvine and Alexander, 1988). A differential release of LH and FSH at least in part may be caused by inhibin which is produced by the Sertoli cells and exerts a negative feedback on FSH secretion also in the stallion.

In prepubertal male horses, FSH and LH concentrations in plasma peak at about 40 weeks of age, decline thereafter and rise again at puberty. The rise at 40 weeks is not associated with increased testosterone concentration which will not increase before the onset of puberty. Before and during puberty, FSH is assumed to be critical for Sertoli cell development and proliferation, while after puberty it induces the release of paracrine Sertoli cell factors involved in regulation of spermatogenesis. LH regulates the synthesis and secretion of testicular steroid hormones from Leydig cells (reviewed by Roser (2008)). However, in the prepubertal stallion, LH receptors are also present in seminiferous tubules suggesting an involvement of LH in their development. In the equine testis, besides testosterone also oestrogens are produced and secreted in considerable amounts. While in prepubertal stallions, immunoreaction for aromatase, a key enzyme for oestradiol-17 β synthesis, was observed within both Leydig cells and seminiferous tubules, in adult stallions, aromatase was restricted to Leydig cells (Herrera-Luna *et al.*, 2016). In immature stallions, sexual steroid concentrations remain at baseline until puberty when the ratio of testicular testosterone to oestrogen changes due to a variation of Leydig cell responsiveness to LH (Roser, 2008). In adult stallions, oestrogen concentrations in the peripheral blood are considerably higher than in mares irrespective of reproductive state. The expression of aromatase (CYP19A1), in testicular tissue increases with stallion age (Herrera-Luna *et al.*, 2016). During the breeding season, oestrogen concentration in the blood circulation is higher than outside the breeding season. These findings highlight the importance of oestrogens for the maintenance of testicular function in stallions. High concentrations of steroid hormones around seminiferous tubules have been suggested to be essential for maintenance of spermatogenesis also in the horse. However, concentrations of steroid hormones in peripheral blood of stallions vary considerably and are not stable, but episodic elevations occur. Concentrations of steroid hormones determined in the testicular vein are up to 100fold higher than in jugular venous blood. Stimulation of testosterone release by injection of the LH analogue hCG is suggested a better tool for assessment of Leydig cell function than determination of testosterone in a single blood sample. In stallions with physiologic testicular function, administration of hCG results in an immediate rise of peripheral testosterone concentration with peak values being reached within 120–180 min. A second increase in testosterone concentration occurs 72–96 h after hCG as a result of increased steroid hormone synthesis. In contrast to other species, male behaviour in stallions is not directly related to peripheral testosterone and/or oestrogen concentration.

Recently, Anti-Müllerian Hormone (AMH) has gained some interest as a potential biomarker for the presence and function of testicular tissue in horses. AMH is produced by Sertoli cells from the initiation of testicular differentiation, i.e., already in the fetus. The production continues after birth and AMH concentrations are high in the plasma of prepubertal male horses but decrease after puberty. In mature stallions with physiologic reproductive functions, AMH concentration is increased during the breeding season as a cause of stimulated Sertoli cell function. AMH concentrations are elevated in cryptorchid stallions in comparison with intact stallions or geldings (Claes *et al.*, 2013).

Among paracrine factors suggested to be involved in the regulation of testicular function, prolactin and growth hormone (GH) have been addressed in more detail in the stallion. In the horse, prolactin concentration in blood significantly changes with season and is maximal during the breeding season. An involvement of prolactin in testicular recrudescence in spring has been suggested. In male golden hamsters, prolactin stimulates LH receptor expression on Leydig cells but such interactions could not be confirmed in the male horse. In humans, GH deficiency is associated with fertility problems. No seasonal changes in GH secretion in stallions were demonstrated. Treatment of healthy fertile stallions with the somatostatin analogue octreotide transiently decreased their semen motility and also reduced an hCG-induced testosterone release. An involvement of GH in testicular and/or epididymal function in stallions was therefore suggested (reviewed by Roser (2008)).

Spermatogenesis and Semen Characteristics in the Stallion

Complete spermatogenesis in the male horse requires approximately 57 days. It can be further divided into spermatocytogenesis (19.4 days), meiosis (19.4 days) and spermiogenesis (18.6 days). Transport through the epididymis takes another 9–14 days. Sperm morphology therefore reflects events that occurred in the past two months. In general, the structure of the equine spermatozoon is similar to that of other species. It has a length of approximately 60 μ m. The head is oval elongated, the anterior third being its widest part. From the side, the equine sperm head is relatively flat. Altogether, the sperm head resembles the shape of a spatula. The anterior two-thirds are overlaid by the acrosome. Significant stallion effects on sperm head dimensions have been reported, ranging from somewhat thinner and elongated to shorter and broader. The impact of these variations on fertility is unclear but a tendency of larger heads in subfertile stallions has been suggested (Casey *et al.*, 1995; Brito, 2007).

The efficiency of spermatozoal production is defined as the number of spermatozoa produced per day and gram of testicular parenchyma. In the stallion, this characteristic is affected by season, is maximal during the breeding season and assumed to be similar among individuals with physiologic fertility. In stallions, efficiency of spermatozoal production has been determined to average 19 million spermatozoa per day and gram testicular parenchyma. In consequence, the total daily spermatozoal production (DSP) of a given stallion depends on the size of his testes and varies considerably among stallions. Testicular size and DSP gradually increase from puberty onwards until approximately an age of 6 years. DSP has therefore not reached its maximum when most stallions start their breeding career at an age of 2 or 3 years (reviewed by Amann (2011)). In the stallion, spermatozoa are produced continuously from puberty onwards. Considerable degeneration of germinal cells occurs already during the breeding season but is increased during the non-breeding season. This is associated with a reduced number of germinal cells per Sertoli cell and reduced

daily sperm production outside the breeding season. Modulations in daily sperm output with regard to season also depend on breed and may be pronounced in less domesticated breeds like Shetland ponies (Fig. 5). While in seasonal anovulatory mares, the onset of ovulatory cycles in spring can be advanced approximately 60 days by exposure to a long-day light program (16 h light – 8 h darkness) from the beginning of December (northern hemisphere), such a light program was not effective in increasing sperm output in stallions early in the breeding season. Only a more complex light program (6 months of short days starting in July followed by 4 months of long days) was able to influence testicular function and daily sperm output in stallions (reviewed by Aurich (2016)).

Thermoregulation within the scrotum is of importance for maintenance of spermatogenesis which will be completely arrested in abdominal testes such as in cryptorchids due to the temperature within the abdomen. Increased temperature may also impair epididymal function and result in declining semen quality (increased number of detached sperm heads) within a few days. Within the scrotum, temperature is kept approximately 3–5°C below body temperature because of evaporation of moisture from the scrotal skin, counter current heat exchange within the *plexus pampiniformis* and changes in the position of the testes relative to the abdominal wall due to relaxation respective contraction of the *musculus cremaster* and *tunica dartos* muscle.

After having left the testes via the ductuli efferentes, spermatozoa are still infertile and achieve progressive motility and fertility only during passage transport through several sections of the epididymal tract. These have not yet been characterized in detail in the stallion. During sperm transport through the epididymal tract, fluids and other material of testicular origin are replaced by epididymal secretions. Function of the epididymal epithelium depends on the presence of testosterone and the detection of aromatase in the equine epididymis suggests that it is further converted into oestradiol-17 β in this location (Herrera-Luna *et al.*, 2016). In the stallion, the process of spermatozoal maturation takes place in the caput and corpus epididymidis and spermatozoa recovered from the cauda epididymidis after surgical castration have been successfully used for the insemination of mares. However, these spermatozoa do not start moving before being mixed with accessory sex gland fluids or appropriate buffer solutions. The epididymal tail together with the deferent duct and its ampulla is therefore assumed to be a major storage area for spermatozoa in the stallion. Movement of spermatozoa through the epididymal tract of the caput and corpus epididymidis depends on continuous peristaltic contractions of its smooth muscular wall and is not hastened by ejaculation. It requires approximately 4.1 days in a stallion. In contrast, the smooth muscular wall of the cauda epididymidis is usually inactive and the time spermatozoa spend in this part of the epididymal tract is influenced by ejaculation and ranges from 2 to 3 days in a sexually active stallion to 10 days in a sexually rested stallion. Depletion of the epididymal sperm reservoir can be achieved if semen collection is performed daily on at least 5 consecutive days, i.e. the total sperm number of the ejaculate collected on the last day is normally reflecting the daily sperm output of the testes in an individual stallion. However, in a sexually rested healthy stallion, accumulation of overaged spermatozoa in the cauda epididymidis does not occur. It has been assumed from other species that spermatozoa will pass from the deferent duct into the pelvic urethra and will be voided at urination resulting in a continuous discharge of spermatozoa from the epididymal reservoir (Amann, 2011). Masturbation in stallions occurs on a regular basis independent of sexual activity but will only rarely result in complete ejaculation and therefore not contribute to considerable spermatozoal loss (McDonnell, 2000).

The use of cooled-shipped semen has become a routine method in the equine breeding industry since the 1980s (Aurich, 2012). Equine semen stored at 5°C for about 24 h maintains fertility similar to that of fresh semen. When stored for longer time periods, motility, velocity and fertilization rates of equine sperm decline markedly but with considerable variation among stallions. Loss of motility and fertilizing ability has been mainly attributed to lipid peroxidation processes within the sperm plasma membrane. The two main metabolic pathways of energy production by spermatozoa are glycolysis and oxidative phosphorylation. Spermatozoa from most species appear to depend mainly on glycolysis for ATP production; however, the significance of glycolysis for equine spermatozoa is not yet completely clear. It has been recently shown that equine spermatozoa depend at least in part on oxidative phosphorylation to meet their energy requirements. It has therefore been suggested that rapidly metabolising spermatozoa and not

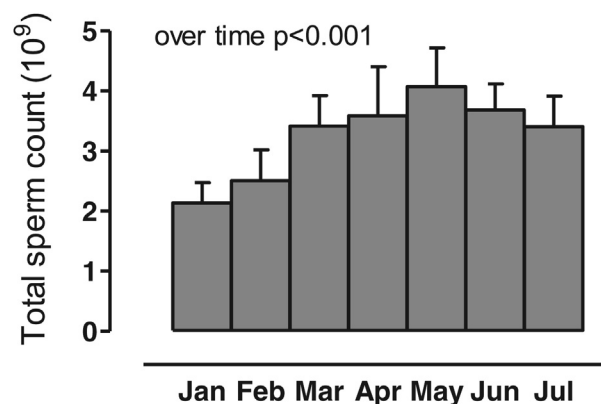


Fig. 5 Total sperm count (10⁹; mean+SEM) of ejaculates collected from Shetland pony stallions (n=8) kept under ambient photoperiod in different months of the breeding season. Reproduced from Aurich, C., Vetmeduni Vienna, Austria.

nonviable or dying sperm are responsible for the generation of reactive oxygen species in equine semen (Varner *et al.*, 2015). In addition to cooled-stored semen, also cryopreserved semen is gaining increasing interest in the horse industry despite the fact that post-thaw semen quality and fertilization rates are still lower than with fresh and cooled-stored semen. The variability in semen viability and pregnancy rates among stallions, breeds and even among ejaculates of a given stallion is large. The phospholipid profile of the equine sperm plasma membrane which is similar to that of the boar contains high levels of docosapentaenoic acid (DPA), an omega-6 polyunsaturated fatty acid (PUFA), and docosahexaenoic acid (DHA) an omega-3-PUFA. DPA content varies among stallions, is influenced by season and has been suggested the main reason for differences in sperm cryosurvival.

Semen analysis provides important information and allows to assess fertility problems or to develop strategies for optimizing fertility in an individual stallion. However, correlations between semen characteristics and fertility may be low. This is also due to the fact that the repeatability of semen characteristics among ejaculates within an individual stallion is only low to moderate. Factors such as season, time of sexual rest, state of sexual arousal, semen collection technique and other contribute to this variation. Fertility is further influenced by semen handling and processing procedures during and following semen collection, management of stallion and mares as well as the number of mares bred per stallion. Semen is routinely collected with an artificial vagina on either an oestrous mare or on a dummy (Fig. 6) in the presence of a mare. The time and number of mounts until successful semen collection is influenced by libido of the stallion as well as semen collection practice. Stallion and personnel have to be well trained and the equipment has to be adjusted to the individual stallion to provide optimal conditions. Repeated mounts until successful ejaculation do not only affect semen characteristics but also increase the probability that semen is contaminated with bacteria from the stallion's genital surface which may impair semen quality especially if semen is further processed for use in artificial insemination. Parameters that are included into routine semen analysis in stallions are semen volume (total volume, gel-free semen volume, gel-volume), sperm concentration ($10^6/\text{mL}$), total sperm count (10^9 ; gel-free volume $\times$ sperm concentration), sperm motility as well as the percentage of morphologically normal sperm. Mean values for these semen characteristics are given in Table 1. Semen volume is highly influenced by breed, size, age, season, state of sexual stimulation as well as collection technique. It may thus vary from a few to several hundred milliliters. Sexual arousal or repeated unsuccessful attempts of semen collection will increase the volume considerably due to increased contributions of the accessory sex glands. Semen volume is therefore negatively correlated with sperm concentration which in healthy normal stallions varies between approximately 100 and 300×10^6 spermatozoa mL^{-1} or even more. For semen processing with the aim to produce cooled-stored or cryopreserved semen, higher sperm concentration is advantageous. Total sperm count is highly correlated to testicular size and varies according to breed, size and age of the stallion. Sperm motility is historically an important characteristic in stallion semen analysis even if the impact on fertility is often questioned. Sperm motility in a stallion with good fertility will in most cases exceed 70% with the majority of sperm being progressively motile. Due to abaxial attachment of the sperm mid-piece to its head, most equine spermatozoa produce a circular motion of varying radius. If performing motility analysis, well-controlled conditions are inevitable. With the increasing impact of artificial insemination in the equine industry, computer assisted semen analysis is more and more used also with stallion semen. Sperm morphology is a parameter which reflects either disturbances in spermatogenesis, epididymal sperm maturation and storage or during semen collection and processing. In raw equine semen of acceptable quality, the percentage of spermatozoa with pathological morphology should not exceed 30% and increased percentages may reflect impaired fertility (Brito, 2007; Amann, 2011; Varner *et al.*, 2015).



Fig. 6 Semen collection from an Icelandic stallion on a dummy with an artificial vagina. Reproduced from Aurich, C., Vetmeduni Vienna, Austria.

Table 1 Mean values and standard deviations of semen characteristics from 47 stallions (age 2–26 years) belonging to 7 different breeds

	Mean	Standard deviation
Total volume (mL)	63.6	45.9
Gel-free volume (mL)	45.3	30.9
Gel volume (mL)	16.8	24.7
Sperm concentration ($\times 10^6/\text{mL}$)	178.0	168.0
Total sperm count ($\times 10^9$)	7.3	6.9
Total motility (%)	72	16
Morphologic aberrations of spermatozoa		
Abnormal sperm heads (%)	7.1	5.9
Proximal plasma droplets (%)	13.1	10.3
Distal plasma droplets (%)	8.1	11.7
Abnormal tails (%)	10.9	9.2
Detached heads (%)	2.6	4.8

Source: Modified from Dowsett, K.F., Pattie, W.A., 1982. Characteristics and fertility of stallionsemen. *Journal of Reproduction and Fertility Supplement* 32, 1–8; Aurich, C., Vetmeduni Vienna, Austria.

References

- Amann, R. P. (2011). Physiology and endocrinology. In A. O. McKinnon, E. L. Squires, W. E. Vaala, & D. D. Varner (Eds.), *Equine Reproduction* (second ed., pp. 881–908). Chichester: Wiley-Blackwell.
- Aurich, C. (2016). Seasonal influences on cooled-shipped and frozen-thawed stallion semen. *Journal of Equine Veterinary Sciences*, 43, 1–5.
- Aurich, C., Sieme, H., Hoppe, H., et al. (1994). Involvement of endogenous opioids in the regulation of LH and testosterone release in the male horse. *Journal of Reproduction and Fertility*, 102, 327–336.
- Aurich, J. (2012). Artificial insemination in horses – More than a century of practice and research. *Journal of Equine Veterinary Sciences*, 32, 458–463.
- Brito, L. F. C. (2007). Evaluation of stallion sperm morphology. *Clinical Technology in Equine Practice*, 6, 249–264.
- Casey, P. J., Gravance, C. G., Davis, R. O., et al. (1995). Morphometric differences in sperm head dimensions of fertile and subfertile stallions. *Theriogenology*, 47, 575–582.
- Claes, A., Ball, B. A., Almeida, J., et al. (2013). Serum anti-Müllerian hormone concentrations in stallions: Developmental changes, seasonal variation, and differences between intact stallions, cryptorchid stallions, and geldings. *Theriogenology*, 79, 1229–1235.
- Crowell-Davis, S. L. (2007). Sexual behaviour of mares. *Hormones and Behavior*, 52, 12–17.
- Giesecke, K., Sieme, H., & Distl, O. (2010). Infertility and candidate gene markers for fertility in stallions: A review. *Veterinary Journal*, 186, 265–271.
- Herrera-Luna, C. V., Scarlet, D., Walter, I., et al. (2016). Effect of stallion age on the expression of LH and FSH receptors and aromatase P450 in equine male reproductive tissue. *Reproduction Fertility and Development*, 28, 2016–2026.
- Irvine, C. H. G., & Alexander, S. L. (1988). Secretion rates and short-term patterns of gonadotrophin-releasing hormone, FSH and LH in the normal stallion in the breeding season. *Journal of Endocrinology*, 117, 197–206.
- Kosniak, K. (1975). Characteristics of the successive jets of ejaculated semen in stallions. *Journal of Reproduction and Fertility Supplement*, 23, 59–61.
- McDonnell, S. M. (2000). Reproductive behavior of stallions and mares: Comparison of free-running and domestic in-hand breeding. *Animal Reproduction Science*, 60–61, 211–219.
- Naden, J., Amann, P., & Squires, E. L. (1990). Testicular growth, hormone concentrations, seminal characteristics and sexual behaviour in stallions. *Journal of Reproduction and Fertility*, 88, 167–176.
- Roser, J. F. (2008). Regulation of testicular function in the stallion: An intricate network of endocrine, paracrine and autocrine systems. *Animal Reproduction Science*, 107, 179–196.
- Varner, D. D., Gibb, Z., & Aitken, R. J. (2015). Stallion fertility: A focus on the spermatozoon. *Equine Veterinary Journal*, 47, 16–24.

Further Reading

- Amann, R. P. (2011). Physiology and endocrinology. In A. O. McKinnon, E. L. Squires, W. E. Vaala, & D. D. Varner (Eds.), *Equine Reproduction* (second ed., pp. 881–908). Chichester: Wiley-Blackwell.
- Aurich, C. (2008). Recent advances in cooled-semen technology. *Animal Reproduction Science*, 107, 268–275.
- Aurich, C. (2016). Seasonal influences on cooled-shipped and frozen-thawed stallion semen. *Journal of Equine Veterinary Sciences*, 43, 1–5.
- Aurich, J. (2012). Artificial insemination in horses – More than a century of practice and research. *Journal of Equine Veterinary Sciences*, 32, 458–463.
- Brito, L. F. C. (2007). Evaluation of stallion sperm morphology. *Clinical Technology in Equine Practice*, 6, 249–264.
- Crowell-Davis, S. L. (2007). Sexual behaviour of mares. *Hormones and Behavior*, 52, 12–17.
- Giesecke, K., Sieme, H., & Distl, O. (2010). Infertility and candidate gene markers for fertility in stallions: A review. *Veterinary Journal*, 186, 265–271.
- McDonnell, S. M. (2000). Reproductive behavior of stallions and mares: Comparison of free-running and domestic in-hand breeding. *Animal Reproduction Science*, 60–61, 211–219.
- Roser, J. F. (2008). Regulation of testicular function in the stallion: An intricate network of endocrine, paracrine and autocrine systems. *Animal Reproduction Science*, 107, 179–196.
- Varner, D. D., Gibb, Z., & Aitken, R. J. (2015). Stallion fertility: A focus on the spermatozoon. *Equine Veterinary Journal*, 47, 16–24.

Sheep Overview

Wansheng Liu and Troy Ott, Pennsylvania State University, University Park, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Reproductive Organs of the Ram

In comparison with males of most species the testes of rams (**Fig. 1**) are large (each testis is approximately 0.5% of body weight) and highly productive. For example, they are 25 times as large relative to body weight as human testes. The paired testis are situated outside of the body in the scrotum and contain networks of tubules, termed seminiferous tubules, in which spermatozoa are formed from progenitor stem cells called spermatogonia. Testicle size, as measured by the scrotal circumference (width of the paired testicles at the widest point), is positively correlated with sperm production (Ley *et al.*, 1990; Soderquist and Hulten, 2006). Interestingly, rams with larger testicles sire more prolific ewes suggesting a genetic component to fertility spanning both genders (Goelz, 1999). Sperm exit the seminiferous tubules through the rete testes and the efferent ducts before entering the epididymis. The epididymis is single highly coiled tube on the outer surface of each testicle where sperm finish maturing and then are stored for later ejaculation. The epididymis is composed of a caput (head), corpus (body) and cauda (tail), and is attached to the testicle on the cranial pole and wraps around it to the caudal pole of the testis. It can be visualized and easily felt by palpation as a large lump under each testis. A large firm tail to the epididymis indicates that the ram has good reserves of sperm (Schoenian, 2016). Sperm then move from the cauda epididymis to the ductus deferens (vas deferens). During sexual excitement, contractions of the cauda epididymis and ductus deferens move sperm to the point where both ducts converge at the pelvic urethra in an area termed the colliculus seminalis. The final section of the ductus deferens is enlarged and glandular and is termed the ampulla. As the sperm enter the colliculus seminalis they are combined with seminal plasma contributed by the ampullae of each ductus, a set of paired seminal vesicles that are large and distinct, the prostate gland and the bulbourethral gland (cowpers gland). The sperm and the seminal plasma together are termed semen. At ejaculation, strong contractions of the urethralis muscle surrounding the pelvic urethra and contractions of the ischiocavernosus and bulbospongiosus muscles force the semen through the penile urethra and out through the urethral process (filiform appendage), which spins rapidly and sprays the ejaculate on the external os (opening) of the cervix. The bulk of the ejaculate, which is approximately 3–5 mL, is deposited at the external cervical os in the anterior vagina. The ram has a fibroelastic penis which is retracted into the body cavity by the retractor penis muscle creating an “S” shaped sigmoid flexure in the penile shaft. Upon sexual excitement the retractor penis muscle relaxes and extends the penis in preparation for intromission.

Spermatogenesis

The Rate of Spermatogenesis in the Ram

Rams achieve puberty between 5 and 12 months of age depending on breed and season of birth. Spermatogenesis occurs in the seminiferous tubules of testes – about 7000 m (m) of them in each testis of the ram (Setchell, 1984). As in other species, ram spermatogenesis is divided into proliferative/mitotic and meiotic phases, and a haploid, post-meiotic phase. Development of a single sperm from the first mitotic division of a spermatogonium until it reaches the center of the seminiferous tubules takes 31 days. It then takes 3–4 days to pass through the tubules, out of testis, and into the epididymis. Sperm are matured in the epididymis and held in the cauda epididymis awaiting ejaculation. Each epididymis is approximately 50 m long. Transit of sperm through the epididymal duct takes 14 days with most of this interval spent within the cauda epididymis. Thus the total passage time from the original division of the spermatogonium to a mature sperm ready for ejaculation is 49 days.

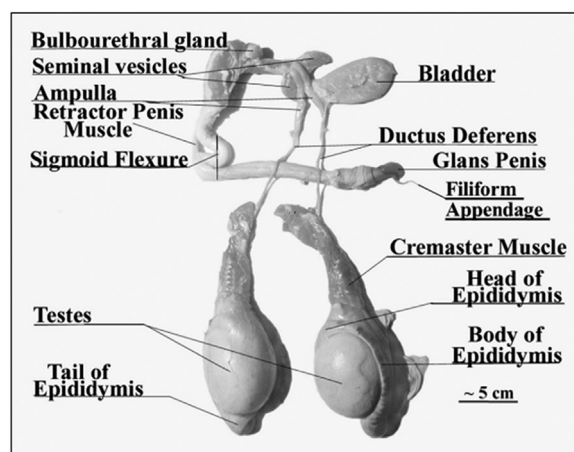


Fig. 1 The dissected reproductive tract of a ram. Adapted from American Sheep Industry Association, 2015. Sheep Production Handbook, vol. 8. Centennial, CO: American Sheep Industry Association.

The rate of sperm production can be determined in various ways. One is to determine the number of sperm produced per gram testis per day as this estimation is reasonably constant regardless of the size of the testis. In comparison with bulls, dogs and stallions that have a relatively low sperm production ($10\text{--}19 \text{ million g}^{-1} \text{ day}^{-1}$), rams have a high sperm production of $20 \text{ million g}^{-1} \text{ day}^{-1}$, similar to that of boars (Amann, 2010). As testicle size and weight vary among sheep breeds, the daily sperm output of a ram is estimated between 5 and 9 billion (Amann, 2010).

Major Factors That Affect Sperm Production in Rams

The two most important factors that can affect the amount and quality of sperm produced are nutritional status (body condition) and the ability of the ram to regulate testicular temperature. While sperm production continues even in very thin rams, the amount and quality of sperm produced is sensitive to nutritional status, with testicle size (volume) being reduced during times of poor forage quality and availability. Rams are seasonal breeders and mating activities contribute to the loss of both the testicle volume and body weight (Fig. 2) (Masters and Fels, 1984). It was reported that feeding supplements to rams on poor pasture for a month before breeding can increase testicle size and subsequent sperm production by up to 100% (Lindsay, 1988). At the same time, overfeeding can have a detrimental effect on sperm production. Overfeeding also reduces the numbers of years a ram lives and the number of ewes he mates per year. It can cause welfare issues due to joint and kidney problems.

For normal sperm production to occur, the testes are maintained at a temperature $4\text{--}6^\circ\text{C}$ below normal body temperature (38.5°C). The ram has numerous sweat glands in the skin of the scrotum and a system of muscles that raise or lower the testes for temperature regulation. If this temperature difference is reduced for extended periods resulting in excessive heating, then spermatogenesis is severely disrupted (Lindsay, 1988).

Puberty and Sexuality

Age at Sexual Maturity and Initial Breeding

Puberty is the age at which the ram's reproductive organs become functional, secondary sex characteristics develop, and he is ready to successfully mate ewes. Most ram lambs reach puberty between 5 and 8 months of age at 50%–60% of their mature weight. The onset of puberty is affected by breed, genetics, season of birth and nutrition. Ram lambs on a low plane of nutrition may not reach puberty until they are 12 months of age or older. Some breeds reach puberty earlier than other breeds, such as prolific breeds and hair sheep. Meat breeds tend to reach puberty earlier than wool breeds.

Age and growth of rams is an important consideration in maximizing flock fertility. Rams at puberty will have limited serving capacity because they are still growing. It is known that ejaculates collected shortly after puberty in rams contain a high proportion of abnormal sperm which exhibit poor motility.

Sexual Behavior

Sexual behavior or sex drive (libido) is controlled by androgens produced in the testis. It is also influenced by developmental events between conception and puberty, and is exhibited on a continuum. The hypothalamus in the brain coordinates information about the external and internal environment of the ram and produces gonadotropin-releasing hormone (GnRH), which causes the pituitary to produce luteinizing hormone (LH) and follicle stimulate hormone (FSH). Both LH and FSH are released in pulses into blood circulation to regulate testes function. The frequency of pulses changes seasonally, and is more important than the total amount released (Lindsay, 1988). LH and FSH stimulate steroidogenesis and testosterone production by testicular Leydig cells.

Testosterone regulates the ram's libido or desire to mate. Some breeds of rams show high libido continuously once they reach puberty. In other breeds, there is a marked decline in libido during the non-breeding season. Underfed and overfat rams may show reduced libido. A ram's desire to mate also decreases with age and disease conditions.

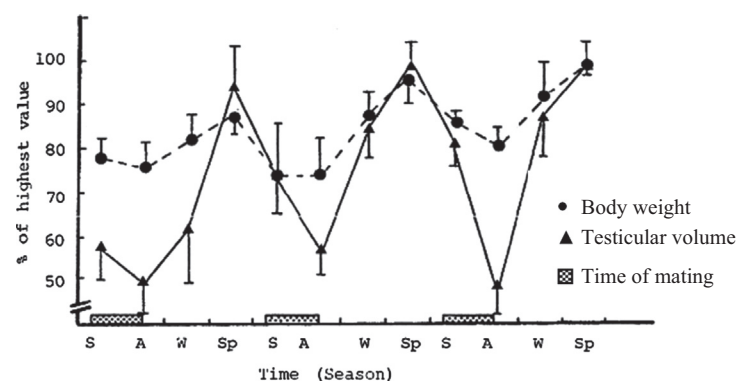


Fig. 2 The testicular volume of Merino rams is sensitive to nutrition. Adapted from Masters, D., Fels, H., 1984. Seasonal changes in the testicular size of grazing rams. *Anim. Prod. Aust.* 15, 444–447.

A sexual performance score of the ram (also known as ram libido score) is an attempt to quantify libido. It is generated based upon numbers of mounts and ejaculations in the presence of estrual ewes. Scores range from 1 to 6 with scores increasing from sexually inactive to highly sexually active. The sexual performance score is a highly repeatable and reliable predictor of the ram's libido under field conditions (Snowder *et al.*, 2004). Rams with high scores for sexual behavior can improve flock fertility during breeding. Like other traits, ram sexual performance is genetically influenced and serum testosterone level exhibit a high correlation ($r=0.90$) with sexual performance of rams during mating.

The Ram Effect

The "ram effect" is when non-cycling ewes are stimulated to ovulate by the sudden introduction of a novel ram. Rams produce chemical substances called pheromones, which are perceived by the ewe and can stimulate the onset of estrus in peripubertal ewes or ewes approaching the onset of the breeding season. For the ram effect to work, ewes should be isolated from rams for at least 6 weeks. Ewes must have no contact with rams by either sight, sound, or smell, which means that they must be separated by considerable distance. The great value of the ram effect is synchronization of estrus activity. In addition, when using a vasectomized or teaser ram, this can allow ewes to achieve several estrous cycles before the desired date of breeding, which will increase fertility (American Sheep Industry Association, 2015; Schoenian, 2016).

Seasonal Breeding

Like some goats and horses, many sheep breeders are seasonal breeders. Seasonal breeders optimize survival of young by synchronizing birth of offspring with favorable conditions including ambient temperature, weather conditions, and food and water availability. The breeding season of sheep in the northern hemisphere is fall and winter during shorter photoperiods (the period of time during the day when there is daylight), so sheep are known as short-day breeders (Gomez-Brunet *et al.*, 2008). Sheep breeds that originated in the northern hemisphere exhibit stronger seasonality than those that originated closer to the equator. The effects of photoperiod on estrous cyclicity are mediated by stimulation of the retina by light. During the fall, with reduced photoperiodic stimulation, melatonin release from the pineal gland increases. Increased melatonin results in onset of pulsatile release of GnRH and onset of reproductive cyclicity.

Unlike other seasonal breeders, the influence of day length on puberty onset in sheep is not equal in ram-lambs and ewe-lambs. In other words, the responsiveness to photoperiod is sexually differentiated in sheep. LH secretion in ewe-lambs is highly influenced by day length, whereas it is not in ram-lambs (Herbosa *et al.*, 1995). For mature sheep, ram breeding behavior is less seasonal compared with ewes even though testicle size, sperm production, and mating capacity of rams varies according to the season of the year, being highest during the normal fall breeding season. These changes are guided by the hypothalamic-pituitary-gonadal axis responding to photoperiod.

Ram Breeding Soundness Evaluation

A breeding soundness evaluation prior to ram introduction to ewes is essential to assess a ram's breeding capacity. However, careful consideration should also be given to nutritional management of rams so they do not enter the breeding season too fat or too thin. The purpose of the breeding soundness exam is to evaluate a ram's potential breeding ability. Approximately 10%–15% of rams have low fertility and low libido. The breeding soundness evaluation, which should be conducted by a veterinarian or trained technician, consists of three parts: physical examination of the ram, semen evaluation and breeding capacity test.

Physical examination of the ram should include palpation of the testicles, epididymis and examination of the penis in addition to visual appraisal of feet, legs, eyes, jaws and body condition. We focus here only on the importance of testicle size. A detailed guideline for ovine breeding soundness examination can be found in the American Sheep Industry Association (2015).

Testicle Size

Testicle size is a good indication of a ram's fertility and varies with season and body condition. Sperm production is positively correlated to testicle size. Testicle size is usually estimated by measuring scrotal circumference (SC) in centimeters (cm) (Foster *et al.*, 1989). The recommended SC minimums for ram lambs and mature meat-type rams are listed in Table 1 but will differ somewhat by breed.

Scrotal circumference and testicle volume decrease dramatically during the breeding season when rams are sexually active. This is often associated with a substantial decline in the body weight of the ram because of its mating activity (Gordon, 1997). It has been reported that rams with small testes are less fertile than those with larger testes in field mating due to production of fewer sperm (Gherardi *et al.*, 1980). However, there was no difference in the semen quality between rams with large and those with small testes (Gordon, 1997).

Semen Evaluation

Semen should be evaluated for sperm concentration, motility, morphology, and white blood cells. Motility is the percentage of spermatozoa showing forward progressive movement. The minimum acceptable standards are $\geq 30\%$ motile and $\geq 50\%$ normal

Table 1 Scrotal circumference guidelines in rams

Age of rams	Questionable	Satisfactory	Exceptional
Ram lambs, 8–14 months	<30	30–36	>36
Mature ram, >14 months	<32	32–40	>40

Source: Reproduced from American Sheep Industry Association, 2015. Sheep Production Handbook, vol. 8. Centennial, CO: American Sheep Industry Association.

morphology. Semen of an exceptional ram should contain >70% motile sperm with >80% normal morphology. The presence of white blood cells in the semen is an indication of infection (e.g., epididymitis). To reduce the occurrence of epididymitis, a serological test is recommended for *Brucella ovis*, which causes epididymitis. A positive test usually renders a ram unsatisfactory as a breeder.

Breeding Capacity

Breeding capacity refers to the number of ewes that a ram can mate and still achieve high fertility within a short breeding interval. It is not only a function of testicle size, semen quality and sperm production, but also depends upon sex drive, mating behavior and serving capacity of the ram. Serving capacity refers to the total number of times a ram can successfully breed the same or different ewes during a heat cycle. Serving capacity affects conception rate in the ewe in several ways. First, under continuous mating systems and at lower ram: ewe ratios (1:100), rams ejaculate fewer sperm than are optimally needed for high conception rates. Second, rams exhibit preference for mating with certain females, inseminating preferred ewes several times at the expenses of other ewes in estrus on any given day. Third, a dominate ewe can prevent a submissive ram from mating with other ewes in estrus. Finally, when ewes are synchronized in estrus by hormonal treatment a greater number of rams will be needed to achieve optimal fertility in a short breeding interval (American Sheep Industry Association, 2015). The working ram shows definite patterns of sexual behavior towards the ewe including genital sniffing, foreleg kick, vocalization, chin resting and mounting. More intensive screening can be done by pen mating test of rams exposed to several ewes that have been synchronized in estrus (American Sheep Industry Association, 2015).

The recommended ram: ewe ratios are 1:15–30 for well-matured ram lambs, 1:25–50 for yearling to five year old rams. However, in some western sheep production systems, the ram: ewe ratio can be as low as 1:100. The ability of rams to successfully inseminate ewes depends on the physical condition of the ram and the ambient temperature, sex drive, topography and area of pasture. For 6 year old and older rams, there is no recommendation of ram: ewe ratios as these rams are mainly suited for pasture or hand breeding only depending on the physical condition of the ram.

Failure to identify low-performance rams can result in a longer lambing season and possibly fewer lambs per ewe. However, failure to identify high-performance rams can result in underutilization of ram power. So, it is important to evaluate the ram breeding capacity before the main breeding season. The simplest way is by observing breeding performance and calculating sexual performance score (see above) as rams are exposed to ewes.

Artificial Insemination

Artificial insemination (AI) is a management tool that requires collection of semen from the male and depositing it into (inseminate) the female. It is estimated that more than 60 million ewes are artificially inseminated each year, although this represents a very small portion of the total ewes worldwide. A major advantage for using an AI program is the potential for genetic gains from more extensive use of superior sires. As discussed earlier, in a seasonal breeding system, a ram can breed approximately 50 to 100 ewes per year, whereas in the AI program, one ram can be used to inseminate thousands of ewes. In controlled-breeding and large-scale estrus-synchronization programs, AI is an essential component. There are some limitations to more widespread adoption of AI in the sheep industry. Primary among them has been the difficulty in passing an insemination pipette through the sheep cervix (especially with primiparous ewes), the limited options for synchronizing estrus, and the lower conception using frozen-thawed semen.

Collection and Evaluation of Semen

An artificial vagina is used most commonly for collection of ram semen. The semen can also be collected by electroejaculation, although this method is less reliable and the collected specimens vary in quality and quantity, and can be contaminated with urine. Normally 2–3 ejaculates are collected from a ram in a day. The volume of semen collected with the artificial vagina is 0.5–2.0 mL, and the concentration of the spermatozoa is $2.5\text{--}6 \times 10^9 \text{ mL}^{-1}$. Semen obtained by electroejaculation generally is of larger volume but lower concentration. Immediately after collection, the semen is assessed for color, volume, concentration of sperm, sperm motility and morphology, percent live sperm, and contamination.

Semen handling is crucial for the sperm cell viability and quality. High temperatures of 39°C or greater induce the acrosome reaction, resulting in greater proportion of dead or damaged sperm. Sudden decreases in temperature, particularly in the 5 to

18°C range, can cause irreversible loss of cell viability. Unnecessary delays from time of collection to AI of undiluted semen can be detrimental to the quality of the sample. However, controlled, slow cooling of diluted semen for extended term use does not seriously reduce sperm viability or fertilization capacity (American Sheep Industry Association, 2015).

Extension of Semen

Once semen evaluation is completed, the sample can be processed by extending or diluting, packaging, and storing. Semen may be diluted up to 10-fold, depending on the initial concentration, the processing and storage method, and whether the semen will be used fresh, chilled, or frozen-thawed. Most semen extenders have a buffered egg yolk base and cryoprotectants such as glycerol. These extenders can be used for semen destined to be used fresh or frozen. The number of motile sperm and the volume of an insemination dose for the ewe depends on the site of insemination and the method of processing. For vaginal insemination, 0.2 mL with a minimum of 1.5×10^8 motile spermatozoa is used; for cervical insemination, 0.05–0.2 mL is used with 1×10^8 , 1.5×10^8 , and 1.8×10^8 sperm of fresh, liquid-stored, and frozen-thawed semen, respectively. Intrauterine insemination by laparoscopy requires 0.08–0.25 mL (with a total of 2×10^7 motile sperm) into each uterine horn (Menzies, 2016).

Storage of Semen

Following the dilution of the semen, it may be prepared for short- and long-term storage. For short-term storage up to 24 h, the extended semen should be slowly cooled to 2–5°C over a 1.5–2 h interval and held at this temperature. Fertility decreases rapidly after 24 h and is low by 48 h. Long-term storage of semen requires that the samples be stored frozen in liquid nitrogen (–196°C). The extended semen is usually packaged in 0.25 or 0.5 mL straws. Sperm motility after thawing varies among rams and processing batches. Use of frozen-thawed semen may result in lambing rates of 50% with cervical insemination and 50%–80% with intrauterine insemination.

Insemination

Ewes can be inseminated either into the vagina, the cervix (cervical insemination) or into the uterus (intrauterine insemination). Extended fresh or chilled semen can be placed into the vagina or cervix, and extended fresh, chilled, or frozen-thawed semen can be placed into the uterus. The optimal time for insemination with fresh or chilled semen is 12–18 h after the onset of estrus. Ewes exhibit estrus for about 30 h and ovulate near the end of estrus.

Conclusions

Rams are seasonal breeders that have large testes in comparison with other mammalian species. Testicle size is estimated by measuring scrotal circumference, which is a good indication of a ram's sperm producing ability and varies with season, body condition and mating activity. A breeding soundness evaluation prior to ram introduction to ewes is critical to assess a ram's breeding capacity. In a seasonal breeding system, a ram can breed up to 100 ewes per year. In an AI program, one ram can be used to inseminate thousands of ewes. However, artificial insemination is not popular in the sheep industry because of the difficulty in passing an insemination pipette through the sheep cervix, the limited options for synchronizing estrus, and the lower conception rate using frozen-thawed semen.

Acknowledgements

The authors would like to thank Dr. Stephen White at the United States Department of Agriculture, Agricultural Research Service (USDA-ARS) and Dr. Lixin Du at the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS) for their help in materials collection and critical comments on the manuscript.

References

- Amann, R. (2010). How is male-factor subfertility minimized in companion and food-producing animals?. *Handbook of Andrology* (second ed.). The American Society of Andrology.
- American Sheep Industry Association. (2015). *American Sheep. Sheep Production Handbook* (vol. 8). Centennial, CO: American Sheep Industry Association.
- Foster, R. A., Ladds, P. W., Hoffmann, D., & Briggs, G. D. (1989). The relationship of scrotal circumference to testicular weight in rams. *Aust. Vet. J.*, 66, 20–22.
- Gherardi, P. B., Lindsay, D. R., & Oldham, C. M. (1980). Testicle size in rams and flock fertility. *Proc. Aust. Soc. Anim. Prod.*, 13, 48–50.
- Goetz, J. (1999). Breeding soundness exam rams. *Int. Sheep Lett.*, 19, 1–2.
- Gomez-Brunet, A., Santiago-Moreno, J., del Campo, A., et al. (2008). Endogenous circannual cycles of ovarian activity and changes in prolactin and melatonin secretion in wild and domestic female sheep maintained under a long-day photoperiod. *Biol. Reprod.*, 78, 552–562.
- Gordon, I. (1997). Controlled reproduction in goats and sheep. In *Controlled Reproduction in Farm Animals Series* (vol. 2). New York, NY: CAB International.
- Herbosa, C. G., Wood, R. I., & Foster, D. L. (1995). Prenatal androgens modify the reproductive response to photoperiod in the developing sheep. *Biol. Reprod.*, 52, 163–169.
- Ley, W. B., Sprecher, D. J., Lessard, P., et al. (1990). Scrotal circumference measurements in purebred Dorset, Hampshire and Suffolk lamb and yearling rams. *Theriogenology*, 34, 913–925.
- Lindsay, D. (1988). *Breeding the Flock: Modern Research and Reproduction in Sheep*. Melbourne: Inkata Press.

Masters, D., & Fels, H. (1984). Seasonal changes in the testicular size of grazing rams. *Anim. Prod. Aust.*, 15, 444–447.

Menzies, P.I., 2016. Management of reproduction: Sheep. Available at: <http://www.merckvetmanual.com/management-and-nutrition/management-of-reproduction-sheep/ram-management>.

Schoenian, S., 2016. Sheep 201: A beginner's guide to raising sheep.

Setchell, B. (1984). The functions of testes and epididymis in rams. In D. R. Lindsay, & D. T. Pearce (Eds.), *Reproduction in Sheep* (pp. 53–58).

Snowder, G. D., Stellflug, N. J., & Van Vleck, L. D. (2004). Genetic correlation of ram sexual performance with ewe reproductive traits of four sheep breeds. *Appl. Anim. Behav. Sci.*, 88, 253–261.

Soderquist, L., & Hulten, F. (2006). Normal values for the scrotal circumference in rams of gotlandic breed. *Reprod. Domest. Anim.*, 41, 61–62.

Rodent Overview

Nina Zupanič, Nutrition Institute, Ljubljana, Slovenia; and Wageningen University, Wageningen, The Netherlands
Jaap Keijer and Katja Teerds, Wageningen University, Wageningen, The Netherlands

© 2018 Elsevier Inc. All rights reserved.

Introduction

Male fertility depends on the continuous production of sperm by the testes, comprising a process of cellular events, called spermatogenesis. Spermatogenesis is a complex process consisting of stem-cell proliferation and self-renewal, meiotic divisions, and several steps of cell differentiation that ultimately result in the production of millions of motile gametes daily. This process is highly dependent of the presence of sufficient amounts of testosterone (T) produced by the interstitial Leydig cells (LC) as well as on the pituitary synthesis and release of the gonadotropic hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH specifically targets the Sertoli cells (SC) within the seminiferous tubules. SC are nursing cells that are indispensable for the normal progression of spermatogenesis. LH is responsible for the development of the adult LC population as well as stimulation of LC T production. Insufficient T production will hamper the progression of meiosis and can thus severely affect spermatogenesis.

Defects at any stage of spermatogenesis, as well as reproductive tract obstruction, inflammation and other sexual disorders may all contribute to male infertility, causing a significant distress to couples trying to conceive. Fertility problems affect up to 20% of all couples in Western societies, whereby nearly half of these involve a male factor. Environmental factors, lifestyle, and genetics all seem to be involved in the pathogenesis of semen anomalies, though in most cases the exact cause and underlying mechanisms of infertility remain unknown. It is speculated that in the coming decades infertility rates will further increase, bringing an additional burden on fertility clinics and raising costs of health care systems (Agarwal *et al.*, 2015).

Assisted reproductive technologies, such as *in vitro* fertilization (IVF) are most commonly used as treatments of male infertility. The development of the so-called intracytoplasmic sperm injection technique, or ICSI, in which a single sperm is injected directly into an egg, has enabled biological offspring even in cases of severe male infertility. This and other treatments are, although relatively efficient, only a means to circumvent rather than treat male infertility. Moreover, assisted reproductive techniques rely on the presence of sperm cells in the ejaculate or within the testis. With the increasing survival rates of male cancer patients that have undergone chemotherapy and/or irradiation, a new category of infertile patients becomes in search of treatment; patients in whom the testis is depleted of sperm. To develop novel treatments to help patients with the wide variety of infertility causes that is observed, a profound basal knowledge on spermatogenesis and testis function is required, coupled with the understanding of the triggers and processes leading to infertility (Cooke and Saunders, 2002).

The purpose of this article is to provide an overview of selected rodent models that have significantly contributed to our understanding of male fertility problems and consequently in their treatment.

Purpose of Animal Models

Since the beginning of 20th century, an enormous progress has been made in the field of male reproduction. Attempts to fill the existing knowledge gaps have resulted in an array of different *in vivo* and *in vitro* model systems, developed as tools to study spermatogenesis and testis function, each of them with specific advantages and disadvantages. From an ethical point of view, cell culture, or *in vitro* models are the preferred choice of research method and to date two models have been developed that are able to provide complete spermatogenesis, namely tissue culture with or without germ cell transplantation and 3D germ cell culture. In the three-dimensional culture systems, comprising of LC, SC, peritubular and spermatogonial stem cells (SSCs), SSC can differentiate into post-meiotic spermatids, albeit with a very low efficiency. Despite the huge progress made in mimicking the intricate spatial organization of the testis, the optimal endocrine and paracrine stimulations in these cultures remain largely unknown and thus present limitations for *in vitro* studies (Dores *et al.*, 2012). Nevertheless, such culture systems hold a great potential for studies trying to unravel the regulation of sperm differentiation as well as to study gene expression of each individual testicular cell type at different stages of spermatogenesis. Once developed for clinical use, they can contribute to much needed options for fertility treatment of infertile male patients. As an example, germ cells in many patients with non-obstructive azoospermia are malformed or underdeveloped, which makes them unsuitable for current IVF treatment. Culturing SSC of these patients under a controlled *in vitro* environment may lead to appropriate germ cell maturation and development of viable spermatids, suitable for IVF. Even though extremely desirable, the path to achieving complete and functional human spermatogenesis under *in vitro* conditions might still be a long and difficult one. Even if complete functional spermatogenesis is achieved, insight needs to be obtained in potential imprinting effects of the *in vitro* conditions, which may have undesirable consequences in the long term. Because of the lack of effective *in vitro* spermatogenesis models, rodent models can provide us a much-needed way forward to better mechanistic understanding of spermatogenesis.

Animal models have contributed much to our understanding of physiological processes not only in reproductive biology but also in biomedical sciences in general. Due to their physiological and genetic similarities with humans, rodents are one of the most commonly used model organisms and most of our knowledge on male reproduction indeed arises from studies performed on mice and rats. There are several reasons for the usefulness of rodent models in male reproductive biology. Firstly,

spermatogenesis in rodents and humans is highly comparable. Secondly, rodents have a relatively short reproductive cycle with large litters; but most importantly, with the introduction of genomic technologies in mice, the mouse genome became extremely easy to manipulate. Next to the more classical rodent models, the advancement of techniques such as gene targeting and homologous recombination lead to unique models for elucidating phenotypic effects caused by specific genes.

Up to date, at least 400 different mutant mouse models with mutations in fertility-related genes have been described, the majority of these being whole body or cell type specific gene silencing or overexpression models. Also, mutated alleles can be introduced. However, the use of genetically modified models does not come without limitations. Whole body gene silencing or overexpression can result in phenotypic defects in other organs, which may have a confounding influence on the effects observed in the tissue of interest, the testis. Cell-type-specific gene silencing or overexpression is usually a preferable option, as it eliminates many of the side effects from whole body gene silencing or overexpression. Furthermore, inducible promoters can be used, which are especially relevant to circumvent developmental effects. However, such models may not always be available due to the absence of cell-type unique promoters, and besides the generation of new models requires a considerable amount of time and costs.

Due to phenotypic and genetic robustness of organisms, gene silencing models often display so-called compensatory mechanisms. This means that a system can withstand a certain amount of change, caused by intrinsic or extrinsic factors while still function normally. There are two ways in which an organism can compensate for a missing protein: either through genetic buffering, with an alternative pathway taking over, or through genetic redundancy, where a given function of the deleted gene is redundantly encoded by other gene(s). Both mechanisms can efficiently mask the phenotypic effects of the mutation (Barbaric *et al.*, 2007).

In addition to the silencing of a specific gene, it is also possible to introduce specific mutations into the mouse genome. This makes it possible to create mouse models that carry specific, known mutations related to, for instance, human male infertility, allowing an in depth study of the pathway along which such a mutation influences fertility, thus contributing to a possible treatment. Models with gene mutations have, however, a serious limitation due to the large percentage of mice suffering from unexpected embryonic/neonatal lethality, limiting the analysis of gene function in reproductively mature animals (Jamsai and O'Bryan, 2011). The use of inducible promoters may circumvent this, since the mutated genes can be activated in later developmental stages.

In overexpression mouse models, phenotypic defects as a consequence of tissue specific or ectopic (over)expression of a gene can be studied *in vivo*. In the newer models, transgenesis is targeted at specific loci (e.g., Rosa26) for ubiquitous expression and the expression level of the transgene can, among others be influenced by the transgene copy number (Jamsai and O'Bryan, 2011).

An emerging possible cause of fertility problems in males that has received increasing attention, is the increasing exposure to endocrine disrupting chemicals (EDCs) that may through their androgenic or estrogenic action interfere with normal gonadal development during pregnancy (Wang *et al.*, 2017). Rodent models have significantly contributed to our knowledge on especially the consequences of EDC exposure during pregnancy and its consequences for fetal and postnatal gonadal development and fertility in adulthood.

In the following section a selection of rodent models and their contribution to our understanding and possible treatment of male fertility problems will be discussed.

Application of Rodent Models

Spermatogonial Stem Cell Transplantation

SSCs are unipotent stem cells, responsible for the maintenance of the continuous production of sperm throughout man's life. From the pool of SSC located at the basement membrane of the seminiferous tubules interconnected pairs of daughter cells can initiate differentiation to become mature spermatozoa. A fine balance between self-renewal and differentiation of SSCs is dependent on their close association with the surrounding somatic SC and needs to be carefully orchestrated by underlying signaling pathways. Knowledge of these regulatory cues is crucial to understand the initiation of spermatogenesis, SSCs renewal after cytotoxic insult, and possible treatments of infertility.

In the early 1990s, the understanding of SSC characteristics was based largely on morphological studies. There was a clear consent that both, endocrine and paracrine signaling molecules were involved in the regulation of SSC proliferation and spermatogonial differentiation, but very little was known about the biochemical and molecular properties of the process. One of the common techniques to study the behavior of SSCs at that time was a depletion of the seminiferous epithelium in rodent testes by cytotoxic agents. Radiation was one of such agents commonly used to differentially kill rapidly dividing cells such as differentiating spermatogonia, while leaving the SSC population largely intact. Subsequent repopulation of the seminiferous epithelium was due to proliferation and differentiation of the remaining SSCs, which made it possible to trace the entire progeny of an individual stem cell (Meistrich and Van Beek, 1993).

In 1994, an enormous progression in our understanding of SSCs occurred when Brinster and colleagues reported the first successful mouse-to-mouse SSCs transplantation, leading to a viable progeny from a previously sterile recipient mouse. The procedure comprised of isolation of SSC from the testes of donor mice, successful *in vitro* maintenance of the cells, followed by their transplantation into the seminiferous tubules of the previously established infertile mice. Infertility of recipient mice was ensured experimentally by cytotoxic treatment, using busulfan, which completely destroyed SSC's. Following microinjection, donor SSCs were able to attach to SC and colonize the basal membrane from the luminal side, where they clonally expanded and subsequently formed patchy repopulated areas. These authors showed, for the first time, that SSC transplantation can establish normal

spermatogenesis within donor's testis and, moreover, produce fully functional spermatozoa that restore the fertility of the donor (Brinster and Zimmermann, 1994).

The most critical phase during homing of newly transplanted spermatogonia is the migration towards the seminiferous tubule basement membrane, especially the crossing of the SC tight junction barrier. The formation of this blood-testis barrier in mouse testis occurs early in the postnatal period, approximately 10 to 12 days pp, when proliferation of SC ceases. SSC transplantation performed in immature mouse pups shows that colonization of the recipient testis is much more efficient when SC tight junctions are not yet formed. The number of colonies generated in recipient pup's testis was 5–10 times greater as when compared with adult recipients. These observations suggest that seminiferous tubules of an immature testis may possess a much more favorable microenvironment for transplanted SSC colonization and expansion, regardless of the donor's age (Shinohara *et al.*, 2001; Kanatsu-Shinohara and Shinohara, 2013).

Successful development of the mouse SSC transplantation assay together with the development of a functional *in vitro* system for SSC culturing initiated active research in the field of fundamental aspects of spermatogenesis, as well as in stem cell biology. Several xenogeneic SSC transplantation studies that followed the initial mouse-to-mouse transplantation revealed not only the extent of evolutionary conservation in regulation of spermatogenesis between species, but also the extent of SSC autonomy in proliferation and differentiation. Transplantation of rat SSCs into testes of immunodeficient mice resulted in the establishment of complete rat spermatogenesis within mouse seminiferous tubules. Mouse SC were entirely capable of supporting rat stem cells, while the timing and organizational pattern of spermatogenesis appeared to be intrinsic to the transplanted germ cells. Thus, the entire process of spermatogenesis initiated from rat SSCs still required 52 days and not 35 days, as in mouse spermatogenesis, despite being controlled by mouse somatic cells (Brinster, 2002).

The stem cell transplantation assay further provided an extremely useful approach for studies aiming at unraveling the regulatory pathways within the SSC niche. The SSC niche is a specialized microenvironment consisting of supporting somatic SC, LC, and peritubular-myoid cells, extracellular matrix components, and several signaling molecules produced by the niche cells, which jointly regulate the SSC identity and capacity of the niche. Polarized SC provide physical support for SSCs and differentiating germ cells, supply nutrients and emit external signals for initiation and coordination of the complex process of spermatogenesis. They are also responsible for sustaining the blood-testis barrier and the segregation of the seminiferous epithelium into a basal and an adluminal part. With the development of functional SSC transplantation technique, the testicular SSC niche became an ideal model to study.

During the last decade, our understanding of the regulation and coordination within the mammalian SSC niche on a molecular level has progressed immensely. The discovery of glial cell line-derived neurotrophic factor (GDNF) secreted by SC has unexpectedly identified one of the most important paracrine molecules involved in SSC self-renewal in the mouse. The GDNF signaling pathway is triggered through the binding to a RET receptor tyrosine kinase and GDNF family receptor- α 1 (GFR α 1) complex, which in turn activates downstream intracellular responses. Using mouse models, *Gdnf* under- or overexpression studies have helped us understand its role in the lineage determination and maintenance of undifferentiated spermatogonia (Hofmann, 2008). For example, *Gdnf* targeted overexpression has been shown to result in rapidly proliferating spermatogonia, leading to testicular germ-cell tumor formation.

Production of GDNF in SC is influenced by FHS, growth factors and cytokines, stimulating *Gdnf* expression throughout life. Its constant levels are required for the maintenance of undifferentiated spermatogonia in the germ cell niche. A drop in GDNF levels, together with a surge of retinoic acid represents a signal that is sufficient for initiation of differentiation in adjacent spermatogonia. While GDNF is indisputably required for self-renewal of mouse SSCs, several other factors are involved in defining a SSC niche.

Recent advances in SSC cryopreservation, *in vitro* propagation and transplantation hold a potentially valuable clinical application; the techniques may offer a successful treatment option for infertility of young male cancer patients who have previously undergone an aggressive treatment, such as chemotherapy or irradiation, often resulting in a complete and permanent depletion of pre-meiotic germ cells. As the spontaneous recovery of spermatogenesis in oncological patients is generally not observed regardless of the patient's age, assisted reproductive techniques are the only treatment option available to father biological children. Semen cryopreservation before therapy is an established clinical procedure and a solution to preserve the fertility of adult cancer patients. Still, the chances of achieving a successful fertilization and live delivery through assisted *in vitro* fertilization in male cancer survivors are as low as 22%. Semen cryopreservation is not an option in pre-pubertal boys, in which the onset of sperm production has not yet begun. The isolation and cryopreservation of SSCs before the beginning of cancer treatment with the subsequent re-injection of SSC after the therapy is thus a promising technique that may allow natural conception even in immature oncological patients (Ehmcke *et al.*, 2006; Mulder *et al.*, 2016).

The EDS Model in Understanding Leydig Cell Development

In the early 1980s, largely by chance, rats treated with the glutathione-dependent alkylating agent ethane-1,2-dimethyl sulphate (EDS) became a prime model for studies aiming at understanding the formation of the LC population in the adult rat testis and the underlying developmental cues. Initial studies showed that a single dose of EDS was able to destroy the entire population of LC while leaving the remaining testicular cells intact. Within 3 days after the administration of the toxicant, the complete LC population was degenerated through apoptosis. For approximately 2 weeks after treatment, the testicular interstitium remained depleted of LC. Concomitantly with LC apoptosis, the testosterone production ceased and was completely inhibited for 2–3 weeks (reviewed in Teerds and Rijntjes, 2007).

The mechanism by which EDS exerts its cytotoxic effect upon LC was elucidated many years later when it became clear that EDS compromises the steroid production pathway by disrupting the mitochondrial electrochemical potential, affecting among others steroidogenesis acute regulatory protein (StAR) import across the mitochondrial membrane. Since StAR is responsible for the transfer of cholesterol from the mitochondrial outer membrane to the mitochondrial inner membrane, where it then is cleaved into pregnenolone, its absence severely compromises steroidogenesis (reviewed in [Teerds and Rijntjes, 2007](#), chapters 36, 37).

One of the important findings of the EDS model was the observation, that stem LC (SLC) persist throughout adulthood and as such hold the potential to regenerate the entire LC population. The origin of adult LC was a matter of dispute for a long time, but more recent studies have shown that SLC are mainly located in close vicinity of the seminiferous tubules, and to a lesser degree around the interstitial blood vessels. The regeneration of the adult LC population after EDS in many aspects closely mirrors the initial establishment of the adult population in the developing testis, thus providing a good model to study this process. LC ablation has also proven to be extremely informative when it comes to understanding androgen function in the testis, especially with regards to SC functioning and maintenance of spermatogenesis. Interestingly, several studies have shown that spermatogenesis remains largely undisrupted if EDS exposed animals are simultaneously treated with sufficient doses of androgen. This suggests that, when it comes to spermatogenesis, androgen production is by far the most important role of adult LC.

The EDS-treated rat model is one of several cell ablation animal models used in male reproductive research. Various testis-specific cell types, for example, germ cells, macrophages, and SC, have all been successfully ablated using different approaches. The major criticism of this method relates to its unpredictability, as the elimination of an entire cell population disrupts an innumerable amount of signaling pathways within the testis tissue and beyond. While this is indeed the case with such a severe and radical approach, the method is however not meant to elucidate specific mechanisms or signaling pathways within the testis. Rather it is a powerful tool to reveal the role of a specific cellular type in the development and functioning of the tissue ([Smith et al., 2015](#)).

Rodents as Model for Testicular Dysgenesis Syndrome

Several adverse trends in male reproductive health such as the increasing incidence of undescended testis and hypospadias, subnormal sperm count, and testicular germ cancer, all seem to be related to one underlying entity called testicular dysgenesis syndrome (TDS). Even though some of these disorders usually manifest later in life, abnormalities are thought to start to accumulate already during fetal development and share several pregnancy-associated risk factors. Based on the prevalence of these disorders in Western countries, environmental and/or lifestyle factors are speculated to be involved in its pathogenesis, albeit with an important contribution of genetic predisposition.

It has been hypothesized that TDS may arise from several different prenatal primary causes, which lead to LC and/or SC malfunctioning during sexual maturation, abnormal germ cell differentiation, and androgen insufficiency. All these defects may result in immediate symptoms, such as the above mentioned hypospadias, undescended testes or increase disease susceptibility later in life. Testing this hypothesis has proven difficult due to relative inaccessibility of the fetal testis at early stages of gonadal sex differentiation as well as long periods required for development of potential adverse health outcomes later in life. With the exception of epidemiological studies and testicular biopsies of TDS patients, clinical studies on the development of TDS are very difficult to perform. In such cases, rodent models can contribute greatly to our understanding of the pathogenesis of the disease.

A model of TDS was first proposed when male offspring of rats exposed in utero to di-n-butyl phthalate (DBP), were shown to display symptoms very similar to those of TDS. In essence they exhibited all symptoms of TDS with the exception of testicular germ cell cancer, for which no suitable animal model has been reported so far. DBP is a commercially important phthalate ester with anti-androgenic properties, used for production of soft plastics for a variety of commercial products. Prenatally affected male rats exhibit focal areas of dysgenic tubules, malformed seminiferous tubules, impaired LC functioning, and abnormal SC proliferation, consistent with the proposed hypothesis of TDS development in men. Studies performed in the DBP-induced TDS rat model as well as animals treated with the nonsteroidal antiandrogen flutamide, are helping to dissect the underlying mechanisms that lead to male reproductive disorders and provide some important information about the critical timing for initiation of dysgenesis during fetal development ([Skakkebaek et al., 2001](#); [Sharpe and Skakkebaek, 2008](#)). An important finding is the observation that prenatal blockade of androgen action during a specific fetal programming window can induce hypospadias, cryptorchidism, and altered penile length. Several studies have confirmed that androgen-driven masculinization of the gonads is indeed mediated by a common early programming window, which occurs before maximal androgen secretion. The existence of such programming window in humans has not been confirmed yet, but based on this rat model it is speculated to occur between 8 and 14 wk of gestation ([Wohlfahrt-Veje et al., 2009](#)).

The inhibition of testosterone production during the prenatal and early postnatal period results in a substantial reduction in SC numbers, indicating the essential role of androgens in SC proliferation during this period. This finding was rather unexpected, as SC in the fetal human and rat testis do not express nuclear androgen receptors (AR). It seems that the effects of androgens on SC during this phase of testicular development are mediated by another testicular cell type, presumably AR-positive peritubular-myoid cells. Low SC numbers are one of the main determinants of low sperm count in adult men, therefore reduced androgen levels and/or impaired androgen signaling in the fetal testis might be involved in the etiology of TDS-related disorders ([Skakkebaek et al., 2001](#)).

Despite all the knowledge we have gained from animal models and clinical research, it is still not clear to what extent environmental and lifestyle exposures participate in the development of testicular dysgenesis. Maternal lifestyle during pregnancy, including obesity, alcohol consumption and smoking, might all influence fetal sexual development. Interestingly, smoking during pregnancy has been shown to have more pronounced adverse effects on male fertility than man's own smoking in adulthood.

Moreover, people living in modern societies are exposed to numerous man-made chemicals on a daily basis, which have been implicated to influence normal fetal development. There is an accumulating body of evidence suggesting that prenatal exposure to endocrine-disrupting chemicals, such as phthalates, pesticides, and dioxins, is likely to affect normal male fetal reproductive development. If ever confirmed that TDS-related abnormalities can be attributed to modern lifestyle and environmental factors, most cases of TDS will in principle be preventable. However, proving the hypothesis of causal relationship between genetic predisposition, environmental chemicals and fetal testis development will certainly not be an easy task (Juul *et al.*, 2014).

Conclusion

- Rodent models have contributed much to our understanding of physiological processes in reproductive biology.
- The mouse model of SSC transplantation has not only increased our understanding of the complexity of spermatogonial proliferation and differentiation, but also offers future opportunities for fertility preservation in prepubertal and adult oncological patients.
- The ethane-1,2-dimethylsulphonate-treated (EDS) rat model is one of several cell specific ablation animal models that has contributed significantly to our understanding of the ontogeny of the LC lineage, and androgen function in the testis especially with regard to SC functioning and maintenance of spermatogenesis.
- Rodent models have made clear that prenatal blockade of androgen action during a specific programming window can induce cryptorchidism, hypospadias and altered penile length, underlying TDS. Based on clinical evidence, it is speculated that such a programming window will also exist in humans.

References

- Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. *Reproductive Biology & Endocrinology*, 13, 37.
- Barbaric, I., Miller, G., & Dear, T. N. (2007). Appearances can be deceiving: Phenotypes of knockout mice. *Briefings in Functional Genomics and Proteomics*, 6, 91–103.
- Brinster, R. L. (2002). Germline stem cell transplantation and transgenesis. *Science*, 296, 2174–2176.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences USA*, 91, 11298–11302.
- Cooke, H. J., & Saunders, P. T. (2002). Mouse models of male infertility. *Nature Reviews Genetics*, 3, 790–801.
- Dores, C., Alpaugh, W., & Dobrinski, I. (2012). From in vitro culture to in vivo models to study testis development and spermatogenesis. *Cell and Tissue Research*, 349, 691–702.
- Ehmcke, J., Wistuba, J., & Schlatt, S. (2006). Spermatogonial stem cells: Questions, models and perspectives. *Human Reproduction Update*, 12, 275–282.
- Hofmann, M. C. (2008). Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Molecular and Cellular Endocrinology*, 288, 95–103.
- Jamsai, D., & O'Bryan, M. K. (2011). Mouse models in male fertility research. *Asian Journal of Andrology*, 13, 139–151.
- Juul, A., Almstrup, K., Andersson, et al. (2014). Possible fetal determinants of male infertility. *Nature Reviews Endocrinology*, 10, 553–562.
- Kanatsu-Shinohara, M., & Shinohara, T. (2013). Spermatogonial stem cell renewal and development. *Annual Review of Cellular and Developmental Biology*, 29, 163–187.
- Meistrich, M. L., & Van Beek, M. E. A. B. (1993). Spermatogonial stem cells. In C. Desjardins, & L. L. Ewing (Eds.), *Cell and Molecular Biology of the Testis* (pp. 266–295). Oxford University Press.
- Mulder, C. L., Zheng, Y., Jan, S., Z., et al. (2016). Spermatogonial stem cell autotransplantation and germline genomic editing: A future cure for spermatogenic failure and prevention of transmission of genomic diseases. *Human Reproduction Update*, 22, 561–573.
- Sharpe, R. M., & Skakkebaek, N. E. (2008). Testicular dysgenesis syndrome: Mechanistic insights and potential new downstream effects. *Fertility and Sterility*, 89, e33–e38.
- Shinohara, T., Orwig, K. E., Avarbock, M. R., & Brinster, M. R. (2001). Remodeling of the postnatal mouse testis is accompanied by dynamic changes in stem cell number and niche availability. *Proceedings of the national Academy of Science USA*, 98, 6189–6191.
- Skakkebaek, N. E., Rajpert-De Meyts, E., & Main, K. M. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. *Human Reproduction*, 16, 972–978.
- Smith, L. B., O'Shaughnessy, P. J., & Rebourcet, D. (2015). Cell-specific ablation in the testis: What have we learned? *Andrology*, 3, 1035–1049.
- Teerds, K., & Rijntjes, E. (2007). Dynamics of Leydig cell regeneration after EDS. In A. H. Payne, & M. P. Hardy (Eds.), *The Leydig Cell in Health and Disease* (pp. 91–116). Totowa, New Jersey: Humana Press.
- Wang, Y., Chen, F., Ye, L., Zirkin, B., & Chen, H. (2017). Steroidogenesis in Leydig cells: Effects of aging and environmental factors. *Reproduction*, 154, R111–R122.
- Wohlfahrt-Veje, C., Main, K. M., & Skakkebaek, N. E. (2009). Testicular dysgenesis syndrome: Foetal origin of adult reproductive problems. *Clinical Endocrinology*, 71, 459–465.

Male Dog Overview

Mushtaq A Memon, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Dog is considered man's best friend, whether it is for companionship, helping herding cattle, guarding home or helping a blind to find his way. Due to some similarities between human and dog physiology, dog has provided beneficial information as a model for human disease investigation and finding cure for many diseases (Harkey *et al.*, 2013). Reproductive biologists and scientists have been equally fascinated with the unique reproductive physiology and anatomy of the dog.

Puberty or Sexual Maturity

Puberty in the dog refers to first appearance of sperm in the ejaculate, and the sexual desire and the ability to copulate (Johnston *et al.*, 2001). Most of the male dogs of various breeds reach puberty at about 80% of their adult body weight. Large breed dogs, e.g., Great Dane may reach sexual maturity about 2-year age, whereas small breed such as Dachshund may reach puberty at about 6-month age. To confirm if the dog has reached puberty, breeding with a female or collecting semen for presence of sperm is recommended.

Gender Determination

Gender differentiation occurs at embryonic level, and it is dependent on the type of chromosomes. All dogs have 78 chromosomes, of which 2 are sex chromosomes. In the female, the sex chromosomes are XX, whereas in the male have XY. At embryonic stage, the mullerian and wolffian ducts form the female and male reproductive tract, respectively. With the help of Sry gene on the Y chromosome influences the indifferent gonad to form into testis. The testis produces mullerian-inhibiting factor which inhibits development of female reproductive system (Moore and Lambert, 1963; Meyers-Wallen and Patterson, 1986; Johnston *et al.*, 2001).

Reproductive Anatomy and Physiology

The reproductive system of the male dog consists two testes and epididymides enclosed in scrotum, the prostate gland, and penis. Palpation of the testes is performed to confirm that both testes are present in the scrotum, and they are normal size and consistency (Gradil *et al.*, 2006; Memon, 2007). The scrotum in the dog is sparsely covered with hair, feel relatively smooth and soft, having skin that is freely movable over the testes, non-painful on touch and uniform thickness (Feldman and Nelson, 2004). Testicular size varies with body weight in the dog. Body weight is positively correlated testicular weight, volume, and testicular width (Woodall and Johnstone, 1988; Eilts *et al.*, 1993). Even though there is limited information available regarding testicular size in different breeds (Table 1), but most experts agree that there is positive correlation between testicular size and sperm production.

The testes perform two functions, spermatogenesis and production of hormones. The endocrine control of spermatogenesis is governed by the neuroendocrine activity along the hypothalamic-pituitary-testicular axis (Ramaswamy and Weinbauer, 2014). The Leydig or interstitial cells secrete testosterone (T) hormone in response to luteinizing hormone (LH) stimulation. Inhibin and activin hormones are secreted by Sertoli cells, and T exert feedback on the pituitary, controlling secretion of the gonadotropins LH and follicle-stimulating hormone (FSH). Formation of androgen-binding protein in Sertoli cells is stimulated by FSH. Binding of T to androgen-binding protein maintains intratesticular T concentration necessary for spermatogenesis (Johnston *et al.*, 2001). Spermatogenesis take place in seminiferous tubules in the testes. Spermatogonia or immature sperm go through series of developmental cycles, which may take about 45 days to become mature spermatozoa. The sperm are transported from seminiferous tubules to rete testis, located in the middle part of the testes. The sperm then move from rete testes to head, body and finally reach tail of the

Table 1 Mean ($\pm$ sem) total scrotal width, sperm output and sperm counts for normal dogs of selected weight ranges

Body weight range lbs (Kgs)	Total scrotal width (mm)	Daily sperm output per dog $\times 10^6$	Sperm count in ejaculate ^a 10^6
10–34 (4.5–15.5)	33–40	287 $\pm$ 33	400 $\pm$ 110
35–59 (15.9–26.8)	49–52	472 $\pm$ 32	1120 $\pm$ 130
60–84 (27.3–38.2)	54–58	750 $\pm$ 111	1430 $\pm$ 460

^aafter sexual rest.

Source: Adapted from Amann, R.P., 1986. Reproductive physiology and endocrinology of the dog. In: Morrow, D.A., (Eds.), Current Therapy in Theriogenology. second ed. Philadelphia: W.B. Saunders Co, pp. 532–538 and Gradil, C.M., Yeager, A., Concannon, P.W., 2016. Assessment of reproductive problems in the male dog. In: Concannon, P.W., England, G., Verstegen, III J., Linde Forsberg, C., (Eds.), Recent Advances in Small Animal Reproduction. Ithaca, NY: International Veterinary Information Service. Available at: <http://www.ivis.org/advances/Concannon/gradil/chapter.asp?LA=1>. (assessed 15.11.17).

epididymis. As sperm is transported through the epididymis, it goes thru various chemical and morphological changes necessary for sperm to gain capability of fertilization. The sperm transport thru epididymis may take about 15 days to complete. The sperm is stored in tail of the epididymis until ejaculated. Therefore, it takes about 60–62 days (Amann, 1989) from development of spermatogonia to spermatozoa (spermatogenesis - 45 days), and sperm transport and maturation in the epididymis (15 days).

Some unique features of dog reproductive anatomy include the presence of a bone in the penis and presence of only one accessory sex gland, the prostate.

The prostate gland is comprised of two lobes, enclosed by an outer layer of tissue and surrounds the urethra (Evans and Christensen, 1993). It produces seminal plasma; during ejaculation, the seminal plasma is secreted into the urethra to dilute the sperm. In prostatic epithelial cells, testosterone, produced by the Leydig cells of the testes, is converted by an enzyme, 5-alpha reductase to dihydrotestosterone (DHT), a much more active form of this hormone that regulates prostatic development and function.

Semen Collection

The most common semen collection technique is by digital manipulation of the penis. Semen may be collected with artificial vagina (AV) comprised of a latex rubber cone attached to a 15 mL calibrated plastic centrifuge tube (Reproduction Resources, Hebson, IL). Other technique is gloved hand and semen collection in color-coded Funnels (Minitube of America, Verona, WI).

The semen collection environment, collector's experience and dog's temperament influence the outcome of the collection. A 'door mate' or a carpet for 'good footing' for the male is helpful.

Most experienced dogs will ejaculate with simple hand stimulation, whereas in-experienced or younger dogs may require a teaser bitch in-heat. If a teaser bitch not familiar with the male, may require some time to 'get-to-know' each other. The semen collector, if right handed, kneels at the dog's left side and holds the AV with top folded in the left hand, and uses the right to move the prepuce up and down the shaft of the penis. Left-handed collectors may reverse the sides described here. At this time, many dogs will begin to thrust the penis in to the rubber cone, and the collector should then move the AV above the engorging bulbous glandis (Fig. 1) and keep firm, steady pressure around as much of the penis as possible to maintain the erection. If the male does not begin to thrust, the AV should be worked up on the penis in an up and down massaging motion to stimulate erection and ejaculation. If using the gloved hand/funnel technique (Fig. 2), the gloved hand is used instead of the AV, and all other steps are the same as discussed above.



Fig. 1 Normal erect penis of the dog. Note the engorged proximal portion, the bulbous glandis. Os penis (bone in the penis, not shown) provides rigidity to penis to penetrate in the female before erection. The bulbous glandis form rigid bulb during erection creating the copulatory lock between male and the female during breeding.



Fig. 2 Funnel technique for semen collection in the dog. The operator is holding the erected penis proximal to bulbus glandis, tip of the erected penis is directed in the funnel, and the ejaculate is collected in the attached tube. This techniques provides ease in changing the tubes to collect 3 semen fraction separately by different collecting tubes.

Consideration is given to when the dog was last collected as well as possible recent breedings and ejaculations, as these may affect results of semen collection and evaluation. Most dogs ejaculate the pre-sperm (clear) and sperm rich fraction (cloudy) of semen during the time of most rapid pelvic thrusting and then dismount and lift one hind leg as though trying to step over the bitch and achieve the tie. At this time, the penis is rotated at about 180 degrees and is directed caudally while still maintaining tight pressure around the bulbus glandis until the third prostatic fraction of semen is ejaculated. The prostatic fluid is ejaculated after a short pause. At the same time, anal contractions can be seen and rhythmic pulsations of the penile urethra can be felt by the collector. After a few mL of the prostatic fractions are collected, the AV may be removed. Let the dog ejaculate the rest of the prostatic fluid, which may take between 5 to 15 min. The teaser bitch (if used) may be removed from the collection area. Allow some time for the dog to lose his erection and retract the penis into the prepuce before leaving the collection area.

Prostate Examination

The prostate is the only accessory sex gland present in the dog and it contributes its secretion as part of the ejaculate. A lubricated gloved index finger is inserted in the rectum to feel the walnut-shaped, bi-lobed prostate on the floor of the pelvis. In medium to large size dogs, the examiner may have to push the ventral abdomen upward to facilitate feeling of the whole prostate gland.

Semen Evaluation

The semen sample or sperm-rich fraction is evaluated based upon color, sperm motility, sperm concentration, and percentage of morphological normal sperm. Additional semen tests (pH, cytology, presence of WBCs, alkaline phosphatase) are done in cases of infertility diagnosis of the dog. The color of normal semen should be cloudy to milky white.

Semen volume can be read from the calibrated collection tube. Semen volume varies with the breed, size of the dog and the amount of prostatic fluid collected

Sperm motility should be evaluated within a few minutes of semen collection. Place a drop of semen on a warm (37 degrees C) microscope slide, place a glass cover glass over the drop and examine under 100–200X magnification. The percentage of motile sperm are estimated, those moving in progressive forward manner. Sperm moving in circles or wiggling in one spot can indicate

Table 2 Characteristics of normal canine semen

Parameter	Normal values
First fraction	0.5–2.0 mL, clear
Second or sperm-rich fraction	1.0–4.0 mL, opalescent
Third fraction	2.0–50 mL, clear
Total ejaculate volume	2.5–60 mL
Sperm concentration	4–400×10 ⁶ /mL
Total sperm per ejaculate	300–2000×10 ⁶ /mL
Sperm motility	>70%
Normal morphology	>80%

Source: Adapted from Gradil, C.M., Yeager, A., Concannon, P.W., 2016. Assessment of reproductive problems in the male dog. In: Concannon, P.W., England, G., Verstegen, III J., Linde Forsberg, C., (Eds.), Recent Advances in Small Animal Reproduction. Ithaca NY: International Veterinary Information Service. Available at: <http://www.ivis.org/advances/Concannon/gradil/chapter.asp?LA=1>. (assessed 15.11.17).

chemical or cold shock to the semen. These sperm are considered mobile but are not motile (sperm moving in forward, progressive manner). It is desirable to examine 4 to 5 different microscopic fields, make an average of the fields to arrive the final percentage of sperm motility. Even though sperm motility is a subjective test, but it is a quick functional test, commonly used to assess semen quality. Other objective methods of semen evaluation include computer-assisted sperm motility and flow cytometry are available (Martinez, 2004).

Sperm concentration or sperm numbers are more important to count in whole ejaculate (total volume) than per milliliter of the semen, because total semen volume is going to depend upon how much prostate fluid is collected. Again, expensive automated semen analyzers are also available for this purpose (Martinez, 2004), but sperm count by hemocytometer is the most common technique used. A white blood cell unopette system (Becton Dickson, Ruthford, NJ) is used to process the semen sample for sperm count with hemocytometer (Johnston *et al.*, 2001). It utilizes a premeasured volume of diluent in a chamber into which a specified amount of semen is drawn. The semen sample is mixed gently and drawn into the capillary pipette of the Unopette, which holds 20 µL. Place the contents of the capillary pipette into the container of diluent according to the kit instructions. The diluted semen is mixed and is loaded into the 2 counting chambers of the hemocytometer and the sperm are counted under the microscope. The number of sperm counted×10⁶ is the concentration per mL. For the total number of sperm multiply concentration×volume. The total number of sperm for a small dog is reported as 400×10⁶; whereas the large dogs yield an ejaculate with around 1.4×10⁹ sperm (Amann, 1986).

Sperm morphology (normal and abnormal sperm) is assessed by examination of the stained slide of the semen. Smear of sperm-rich fraction of the semen is made, air-dried and stained with eosin-nigrosin (Rouge, 2004). One drop of semen and a similar sized drop of stain are placed on one end of a glass slide and the drops are mixed gently with a wooden applicator stick. A second slide is used as a spreader slide to draw out a thin film. The smear is air dried, and checked under the microscope. Other option is using Diff-Quick stain, the air-dried slide is immersed in each (total 3) solution for 5 min. Some prefer examining the semen wet mount slide. This technique requires placing 1–2 drops of semen in 1 mL buffered formol saline in a tube, the contents are mixed, and taken drop from the mixture, and placed on the microscope slide, cover glass is applied and examined under phase contrast microscope.

One to two hundred (100–200) sperm are examined using the oil-immersion (1000X) microscope objective. The smear must be thin enough to visualize individual sperm. Each sperm is classified as either: normal sperm; sperm showing a primary abnormality, which includes all sperm head abnormalities (e.g., micro or macro head), proximally coiled sperm tails, and proximal cytoplasmic droplets. The secondary sperm abnormalities are considered for sperm with detached heads, bent tails, and sperm with distal cytoplasmic droplets.

Primary abnormalities represent abnormalities that originated during spermatogenesis in the testes. Secondary abnormalities are believed to occur during epididymal transport and/or a result of improper semen sample handling.

Primary abnormalities should not exceed 20%; however, pregnancies have been achieved when the abnormalities exceeded this number. Most normal dogs, which ejaculate regularly, would have >200 million sperm in an ejaculate, of which over 80% are normal (Table 2).

References

- Amann, R. P. (1986). Reproductive physiology and endocrinology of the dog. In D. A. Morrow (Ed.), *Current Therapy in Theriogenology* (second ed., pp. 532–538). Philadelphia: W.B. Saunders Co.
- Amann, R. P. (1989). Structure and function of the normal testis and epididymis. *J. Am. Coll. Toxicol.*, 8, 457–471.
- Eilts, B. E., Williams, D. B., & Moser, E. B. (1993). Ultrasonic measurement of canine testes. *Theriogenology*, 40, 819–828.
- Evans, H. E., & Christensen, G. C. (1993). The urogenital system. In H. E. Evan (Ed.), *Miller's Anatomy of the Dog: Diagnosis, Treatment and Prevention of Reproductive Disorders in Small and Large Animals* (third ed., pp. 494–558). Philadelphia: WB Saunders.

- Feldman, E. C., & Nelson, R. W. (2004). Clinical and diagnostic evaluation of the male reproductive tract. *Canine and Feline Endocrinology and Reproduction* (third ed.). Saunders.
- Gradil, C. M., Yeager, A., & Concannon, P. W. (2006). Assessment of reproductive problems in the male dog. In P. W. Concannon, G. England, J. Verstegen, III, & C. Linde Forsberg (Eds.), *Recent Advances in Small Animal Reproduction*. Ithaca NY: International Veterinary Information Service. Available at: <http://www.ivis.org/advances/Concannon/gradil/chapter.asp?LA=1>. (accessed 15.11.17).
- Harkey, M. A., Asano, A., Zoulas, M. E., et al. (2013). Isolation, genetic manipulation, and transplantation of canine spermatogonial stem cells: Progress toward transgenesis through the male germ line. *Reproduction*, 146, 75–90.
- Johnston, S. D., Root Kustritz, M. V., & Olson, P. N. S. (2001). Semen collection, evaluation and preservation. *Canine and Feline Theriogenology*. Philadelphia: WB Saunders.
- Martinez, A. I. P. (2004). Canine fresh and cryopreserved semen evaluation. *Anim. Reprod. Sci.*, 82, 209–224.
- Memon, M. A. (2007). Common causes of male dog infertility. *Theriogenology*, 68, 322–328.
- Meyers-Wallen, V. N., & Patterson, D. F. (1986). Disorders of sexual development in the dog. In D. A. Morrow (Ed.), *Current Therapy in Theriogenology* (2nd ed, pp. 567–574). Philadelphia: WB Saunders.
- Moore, W., Jr., & Lambert, P. D. (1963). The chromosomes of the beagle dog. *J. Hered.*, 54, 273–276.
- Ramaswamy, S., & Weinbauer, G. F. (2014). Endocrine control of spermatogenesis: Role of FSH and LH/testosterone. *Spermatogenesis*. Available at: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4581062/>. (accessed 15.11.17).
- Rouge M., 2004. Sperm morphology. In: Collection and Evaluation of Semen: Introduction and Index. Available at: <http://www.vivo.colostate.edu/hbooks/pathphys/reprod/semeneval/morph.html> (accessed 15.11.17).
- Woodall, P. F., & Johnstone, I. P. (1988). Scrotal width as an index of testicular size in dogs and its relationship to body size. *J. Small Anim. Pract.*, 29, 543–547.

The Male Cat

Emmanuel Fontaine, Royal Canin Canada, ON, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Just in the United States, recent surveys indicate that there are nearly 74 million of cats kept as pets. Their independent nature indeed seems like a great fit to our sedentary lifestyle. There is definitely no doubt: cats are on the rise.

Interestingly enough though, cats are very often mistaken as “smaller versions” of dogs. It is an easy parallel indeed. People – even veterinarians sometimes – easily assume both species “work” the same... This is obviously not true; they are both unique in their own way, and cats have specific features that set them apart; especially when it comes to the reproductive function.

This article focuses on the male cat. Having a good understanding of its reproductive physiology and specificities will come handy in many situations – and not only to veterinarians and veterinary staff. For instance, a pet owner might want to know when its male kitten will reach puberty. Somebody working or volunteering at a cat rescue or a humane society will be interested in knowing the consequences of neutering. A cat breeder will obviously want to know more about breeding behavior and breeding activity.

We have more and more answers – and solutions – to provide to all those questions we get from the field. There is indeed more and more research published on the reproductive function in male cats, driven by two things:

1. Cats are prolific animals and cat overpopulation is definitely a concern in many areas of the world, even in developed countries. More and more research is done on how to efficiently control their population via controlling their reproductive function;
2. There are 37 different felid species on Earth and the only one that is not endangered is the domestic cat. This makes it the perfect model in conservation biology. Several groups are therefore studying its reproductive function in order to build better strategies to help out wild felids in the field.

This article will give us an opportunity to review the specific reproductive characteristics of the male cat. We will describe its anatomy, its reproductive physiology as well as discuss neutering and most common reproductive disorders encountered in this species.

General Anatomy of the Male Cat Genital Tract

The male cat genital tract (see Fig. 1) is composed of:

1. The gonads:

The two testes of the adult cat are spherical to ovoid and are located in the scrotal sac ventral to the anus. This is where spermatogenesis occurs. The testes are also endocrine organs producing androgens and are the main source of testosterone in the cat. Testosterone is necessary for the development of secondary sex characteristics, such as the heavy jowls of male cats, and normal sex behavior.

The extra-abdominal localization of the testes is of great importance: in this position, the testes are submitted to a cooler temperature than the body's, which is a mandatory requirement for spermatogenesis to occur.

2. The epididymes:

These tubular anatomical structures are attached to the testis. This is where the spermatozoa will undergo maturation (spermiogenesis). Only after maturation do the spermatozoa have the ability to be mobile and fertilize and egg.

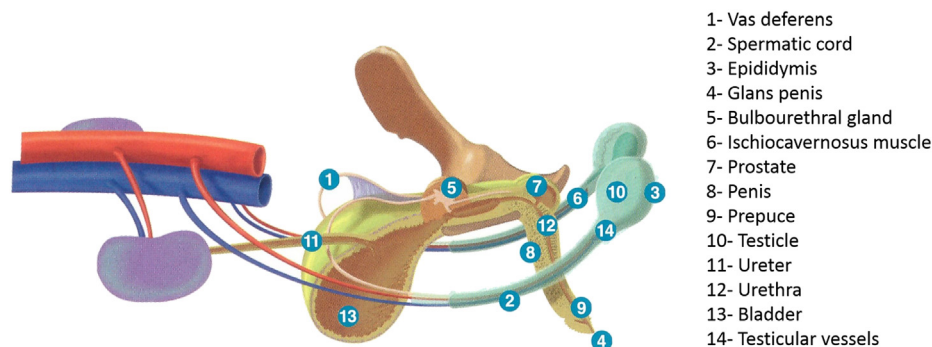


Fig. 1 Organization of the male cat genital tract.

In cats, this is also the only storage structure of the male gametes. Spermatozoa present in the ejaculate originate in fact from the caudal part of the epididymis (Parkinson, 2009).

1. Accessory glands:

Only two glands are found in the genital tract of a male cat: the prostate and the bulbo-urethral glands. The secretions of those glands play a role in the formation of the male's ejaculate.

2. A copulatory organ:

The penis is located inside the prepuce.

Some of the unique anatomical characteristics of the male cat genital tract are definitely worth noticing, and concern the testes and the penis.

The Testes

Testicular development starts at an early age, in fact even before birth during the embryonic and fetal phases. The testes will emerge caudally to the kidney and will then progressively migrate to reach the scrotum.

In cats, on the contrary to dogs, the testes are present in the scrotum right after the kittens are born (Johnston *et al.*, 2001).

However, because of their small size at this age, it is usually difficult – even not possible – to properly palpate and identify them in newborns. This will become easier as kittens get older and usually, both testes can be properly identified around the time of nutritional weaning, when the kittens are 4–4.5 weeks old.

Determining the sex of a newborn kitten might therefore be tricky. This is usually achieved by looking at the ano-genital distance (see Fig. 2). This distance is shorter in the male than in the female and will help differentiate both genders (Malandain, 2006).

The Penis

During its early formation, the penis adheres to the prepuce via a structure called the balanopreputial fold. Dissolution of this fold is an androgen-dependent event that occurs after birth in most mammals.

In male cats however, breakdown of this fold and resulting ability to completely protrude the penis from the prepuce occurs at 7–12 months of age, unless the cat is castrated prior to that time, in which case the adhesion persists.

The other particularity of the cat penis is that it carries penile spines. At about 9 weeks of age, elongated pits become evident on the surface of the glans penis, from which androgen-dependent penile spines (see Fig. 3) erupt between 9 and 13 weeks of age.

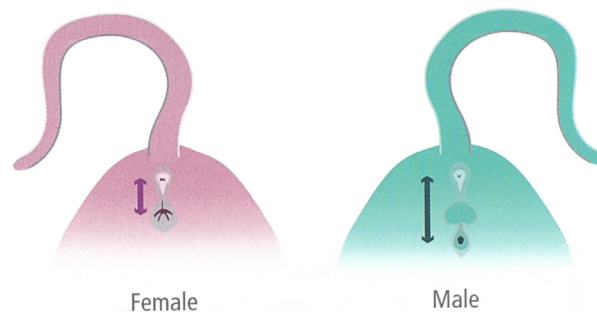


Fig. 2 Gender determination in newborn kittens based on the ano-genital distance.



Fig. 3 Penile spines.

In the adult intact male cat, the penis free end – the glans – is a 5–10 mm long conical structure directed caudally that contains a band of 120–150 of those androgen dependent penile spines in 6 to 8 circular rows. Following castration of the adult cat, spines regress rapidly, reaching hair-like structures in 5–6 weeks.

The presence of the penile spines is an easy clinical way to confirm testosterone production – and therefore presence of a testis -. This is an interesting clinical feature used often when the presence of an ectopic testis – which is the most common genital disorder seen in males of this species- is suspected in a male cat that is supposedly castrated (Fontbonne *et al.*, 2007).

The physiologic role of the spines in copulation is unknown; they have been proposed to provide sexual stimulation for the male or female, to impede withdrawal of the penis from the vagina (as they are directed towards the base of the penis) or to increase vaginal stimulation therefore favoring successful induction of ovulation.

Of interest also is the presence of an os penis, which presence seems to vary depending on the size of the cat. The os penis is 3–5 mm long and may be present inside the tip of the glans. In one study, the os penis was present in 3 of 9 medium size cats and 10 of 12 large size cats; no os penis was however found in kittens and small adult cats (Jackson, 1902).

Physiology of Reproduction in the Male Cat

Puberty

Earliest evidence of spermatogenesis in the cat occurs at approximately 20 weeks of age and spermatozoa are present when the testes exceed 1 g in combined weight.

Sperm may be ejaculated in semen of male kittens as young as 7 weeks of age, but the age at which mating begins varies with physical condition and body size, with average onset of puberty occurring at 8–10 months or at body weight of 2.5 kg, whichever occurs first.

It is interesting to note here that there is definitely a breed effect. While Persian cats and related breeds usually have a tendency to reach puberty later in their life (a male Persian might not properly breed before 2 years of age), Oriental breeds on the other hand are known to be rather precocious and might start breeding as early as 6 months of age (Malandain, 2006).

Seasonality

In the female cat, reproduction has a seasonal pattern that is influenced by the hormonal secretion of melatonin. While this has been well established in females, seasonality is however still a controversial topic in male cats.

Some studies have described a fall of libido during the winter months, associated with lower quantities of sperm cells in the ejaculate as well as an increased percentage of abnormal forms in the sperm (Tsutsui *et al.*, 2009).

One thing however remains: sperm cells are always found in the seminiferous tubules of the male cat's testes. Ejaculates containing fertile spermatozoa can be found all year long. Therefore, male cats definitely have the ability to breed throughout the year.

The limiting factor does not appear to be the tomcat's seasonality, but rather the presence of females in estrus.

Normal Breeding Behavior of Male Cats

The calling of estrous females when they are in season is definitely a well-known feature of their reproduction. This is how the males will locate them. When an adult male cat is in presence of an estrous female, the courtship phase will begin.

The male cat may exhibit a flehmen reaction by opening his mouth, slightly closing his eyes while sniffing, an action associated with reception of pheromones of the female by the male's vomeronasal organ. The flehmen reaction in the male is influenced by presence of testosterone in the male, as well as by fluid-borne chemical stimuli in urine and vaginal secretions of the female.

Neck grasp of the female

When the female accepts the male, this one will typically grasp her skin over the dorsal part of her neck with his teeth. This leads to immobilization of the female – a reflex that comes from kittenhood – which allows the male to perform coitus. This neck grasp is of great importance: if the male cannot perform it, breeding usually cannot occur.

It is therefore interesting to note that males that are suffering from dental disorders (like periodontal disease), joint issues or any other painful disorder will not be able to grasp the female's neck. That will impair breeding performances and even decrease libido (Malandain, 2006).

Breeding per se is very quick in cats. Pelvic thrusting by the male is followed by intromission and ejaculation which occurs in less than 20 s. The male then pulls away quickly to avoid being struck by the female in her post-coital reaction, which is an aggressive reaction towards the male that happens right after coitus.

Post-coital reaction & number of breedings

Visualizing this post-coital reaction is of great important from a clinical standpoint: it is indeed the sign that confirms penetration did occur. That is therefore an important behavioral sequence to visualize in terms of breeding management in catteries (Fontbonne *et al.*, 2007).

The number one cause of infertility in the feline species is indeed absence of breeding. Observing the breedings and confirming the post-coital reaction is therefore a mandatory prerequisite when dealing with infertility in this species.

As that reaction subsides, the male usually will mount and breed the female again. The number of breedings that can be observed varies a lot and basically depends on the individual male.

Since ovulation is generally induced by coitus in the feline species, it is interesting to note as well that one breeding only induces ovulation in 50% of the females (Fontbonne *et al.*, 2007). Not only should the post-coital reaction need to be observed to confirm that breeding indeed occurred, but it is also important to observe how many breedings occurred. That will be an important data to consider when dealing with infertility issues in a breeding cattery for instance.

In one study, sperm extra-gonadal reserves were exhausted after 4 days of daily ejaculation and the sperm numbers obtained thereafter were equivalent to the daily sperm output. Those results suggest that male cats can be used at stud three times weekly without showing a drop in the numbers of sperm per ejaculate (Sojka *et al.*, 1970).

Since the queen typically ovulates several oocytes, superfecundation can happen in cats and inside the same litter, kittens from different fathers can be found. While this might happen often in free-roaming cats, this is something cat breeders try to avoid for obvious reason: in a breeding cattery, an estrous female usually should only have access to a single male (Malandain, 2006).

Environmental conditions

We often hear that cats thrive on familiarity and routine. This is definitely true in feline reproduction as well. Copulation may not be completed if the male is not yet acclimated to his environment (a process that may take 1–2 months). That is the reason why in breeding catteries, the females are usually brought to the male, and not the contrary.

Also, with breeding cats, it is very often recommended to use an experienced and quiet female with a young male. If the female's post coital reaction is aggressive, a young male might associate breeding with potential danger and source of stress. It might then simply refuse to further breed (Malandain, 2006).

Mating behavior usually ceases following castration of the male cat, but it may persist for as long as several years following castration of the experienced male cat.

Semen Analysis and Collection in Male Cats

The Male Cat's Ejaculate

Production of a fully functional spermatozoa typically takes around 60 days in cats.

Volume

The volume of sperm produced per ejaculation in cats is small ranging typically from 0.01 to 0.7 mL.

This is definitely a limitation when it comes to semen evaluation in this species, and one of the reason why this type of fertility test is usually not performed in routine in veterinary clinics, but only in veterinary reference centers or research facilities.

Sperm motility

Mean progressive motilities of 60–90% have been reported as normal in this species.

Sperm count

A cat ejaculate contains in average 8–30 million sperm cells, different reports mentioning that there can be between 3 and 153 million sperm cells per ejaculate (Johnston *et al.*, 2001). This number is of great importance, especially when it comes to assisted reproductive techniques.

Performing an intravaginal artificial insemination is the easiest assisted reproductive technique in cats. However, to obtain 80% fertility rate with this technique, studies have shown that at least 80 million sperm cells should be deposited inside the vagina, which represents the equivalent of nearly 10 ejaculates (Tanaka *et al.*, 2000).

On the other hand, when performing intra-uterine insemination, the same 80% fertility rate could be obtained with only 8 million sperm cell (which represents a single ejaculate) (Tsutsui *et al.*, 2000).

Based on those results, it appears that intra-uterine insemination is the best option when considering assisted reproductive techniques in cats (Fontaine *et al.*, 2010).

Abnormal forms

Cats – especially pure-bred cats – are known to be prone to teratozoospermia, which means that there are lots of abnormal forms in the ejaculate. One study reported that the median percentage of normal spermatozoa was 44% (ranging from 1% to 91%) (Axner and Linde Forsberg, 2007).

This is something that is also well described in wild felids, especially in those species with low genetic diversity.

Retrograde ejaculation

The tom cat is reported to ejaculate from 15% to 90% (average 46.8%) of the ejaculate retrograde into the urinary bladder during ejaculation (Dooley *et al.*, 1984).

From a clinical standpoint, this is something definitely interesting. Collection of urine by cystocentesis from the tom following ejaculation with examination of the urine sediment for sperm is a useful procedure in small animal practice to determine whether the cat is producing sperm or not.

From a clinical standpoint again, this is a simple technique that most veterinarians can have access to. It is certainly not as accurate as a performing a full spermogram – during which motility, numeration and percentage of abnormal forms of the sperm are analyzed – , but as we are going to see in the next paragraph, there are definitely limitations in this regard in the feline species for sure.

Semen Collection

The least we can say is that it is not easy to perform semen collection in cats. Different techniques are however described.

Artificial vagina

Male cats can be trained to be collected with an artificial vagina. Training is accomplished by repeated gentle handling of the male cat in the presence of an estrous female. When cats are used to the procedure, an estrous female might not be needed anymore.

This technique is however difficult to reproduce outside of a controlled environment. As mentioned before, cats thrive on familiarity and routine... and that is definitely not what would happen if male cats were to be brought to a veterinary clinic for semen collection.

As interesting as it sounds, this technique is unfortunately not suitable to a busy veterinary clinic environment and it is a clear limitation to the development of fertility assessment in male cats – while in dogs, this is something that can be performed in routine in most veterinary clinics (Fontbonne *et al.*, 2007).

Electro-ejaculation

Collection via electro-ejaculation is often considered as the norm in felines.

The nervous pathways of ejaculation are stimulated via electric shots delivered through a rectal probe, leading to ejaculation. This technique is obviously much more invasive and in domestic cats require general anesthesia of the male (Platz and Seager, 1978).

It is to date the technique that allows for the collection of the best semen sample in the male cat.

While there is no doubt the technique allows for quick and efficient semen collection in male cats – a full electroejaculation protocol does not require more than 15 min – in recent years, more and more ethical concerns were raised regarding this type of collection.

Moreover, the anesthesia that is required is also a concern for many owners, and definitely limits the use of the technique in veterinary clinics.

Urethral catheterization (see Fig. 4)

This technique is based on the use of a special anesthetic drug, called alpha2-agonists.

Ejaculation is indeed an alpha2 agonist process and in other species like in stallions for instance the use of those drugs like xylazine can even lead to ejaculation (Parkinson, 2009).

In cats, the use of alpha2- agonists like medetomidine or dexmedetomidine will not lead to ejaculation per se, but will induce a passive flow of semen from the cauda of the epididymes to the urethra. By then placing a urinary catheter inside the male's urethra, it then becomes possible to collect the semen of the male cat (Zambelli and Cunto, 2006).

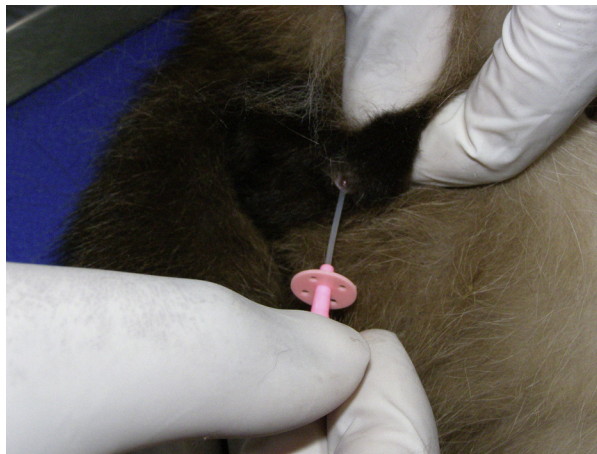


Fig. 4 Semen collection via urethral catheterization.

The technique is very simple to use but there are two main limitations:

1. It requires to use high doses of alpha2-agonists, which might increase the anesthesia risk;
2. Moreover the volumes of semen collected are nearly 10 times lower than what is collected when performing electroejaculation, which makes it very hard to manipulate then. Indeed with electroejaculation, secretions from the prostate and the bulbourethral glands are collected as well while when using this technique, we only get the spermatic fraction of the sperm which makes handling of the semen even more difficult.

Assisted Reproductive Techniques

As described above, semen collection in cats is definitely not something simple to perform.

Only certain specialized veterinary centers (universities or research centers) will typically perform these kind of procedures. Moreover, the small volumes of ejaculate that can be collected by the various techniques previously described is definitely a limitation to the development and use of assisted reproductive techniques like described in other mammalian species (Fontaine *et al.*, 2010).

Semen freezing and pregnancy following the use of it has been successfully reported in cats, however, because of the aforementioned difficulties, there is no commercial semen extender available to date.

Prevention of Fertility in Male Cats

It is not common to discuss how to evaluate or enhance semen quality in cats because of the limitations we just discussed. On the other hand however, neutering and prevention of fertility is a heavily discussed topic that is definitely worth mentioning in this article.

As we mentioned in the introduction, cat overpopulation is a problem in many parts of the world, including developed countries. While cat population control programs obviously need to focus on both the male and female aspects, we will focus here only on what is important to know about the male.

This is obviously something of interest for people involved in cat rescues and humane societies, as well as for any pet owner who considers neutering their pet.

Why Consider Neutering in Male Cats

Not all cats are intended to be bred. In order to better control the cat population, it is recommended that those animals that are not part of a breeding program should be neutered.

On top of population control measures, neutering also brings some strong health benefits to consider for pet owners.

Castration of the male cat will indeed lead to the decrease of many disturbing behaviors: aggressive behavior, mounting, roaming, and especially urine marking and urine odor.

Castration will eliminate sexual behavior immediately in most tom cats (except maybe the experienced ones as discussed earlier in this article) and will decrease the associated roaming.

Prepuberal castration is most effective in eliminating or preventing undesirable sexual behavior.

Inter-male aggression, which is normal in cats, subsides in 80%–90% of male cats castrated for this reason.

Prepuberal castration usually prevents urine spraying while male cats castrated in adulthood usually show postoperative decline in urine marking. A rapid decline in urine spraying occurred in 78% of 42 male cats castrated as adults, and another 9% showed gradual decline in spraying.

Side Effects of Castration

If there are obviously positive aspects to consider, castration – like all medical procedure however, also has its cons. Those are important to know as well.

Undesirable effects of feline castration include adhesion of the prepuce to the penis and delayed long bone physeal closure in cats castrated prior to 7 months of age, decreased nutritional caloric needs that may predispose to obesity.

When it comes to adhesion of the prepuce, in theory, these adhesions may be associated with buildup of debris and urine and predispose to local irritation, inflammation or ascending urinary tract infection. That being said, observations of such adverse sequelae has not been reported.

Physeal closure is delayed to 5–7 months in cats neutered at 7 weeks or 7 months of age; this results in long bone length that is about 10% longer than that of sexually intact littermate. Originally, this was thought to predispose cats to long bone fractures but studies have shown that in fact, it is not the case (Spain *et al.*, 2004).

Heat production, a measure of metabolic rate, was decreased an average of 28% in cats castrated at 7 weeks or 7 months when compared to intact toms (Root and Johnston, 1995). Therefore, neutered male cats require intake of 28% fewer calories than do sexually intact males in order to avoid signs of obesity.

This is definitely the biggest issue encountered after castration in male cats. Overweight/obesity in pets is indeed considered as a huge threat today in veterinary medicine, recent reports even indicate that 59% of cats seen in veterinary clinics are overweight or obese, and this condition is obviously associated with deleterious health effects.

That is why an important recommendation given to pet owners after castration in a male cat is to focus on their nutrition. Neutered cats should see their daily caloric intake monitored and decreased by around 30%.

Techniques for Neutering Cats

Today, the norm for neutering cats remains orchiectomy. Both testes are removed surgically. This is a fast procedure that is performed every day in most veterinary clinics (Fontbonne *et al.*, 2007).

Vasectomy can also be performed, but is usually only requested by cat breeders. A vasectomized tomcat can indeed be used as a teaser tom, which role is to induce ovulation in females and therefore increase their inter-estrus interval between litters.

Other noninvasive techniques exist and have been well documented recently.

While melatonin implants that are used more and more in female cats do not appear to fully prevent sperm production in males, there has been several publications done on the use of GnRH-agonists implants containing deslorelin in cats.

Those studies have shown that it is possible to induce reversible chemical sterilization using those implants in male cats for up to 2 years using a single implant. In this study, they indeed reported a mean duration of efficacy of 78.8 ± 12.9 weeks, ranging from 61.1 to 100.7 weeks (Goericke-Pesch *et al.*, 2014).

This can offer interesting alternative for breeders who do not want to neuter their cat but want to find a way to prevent the problems that comes with an entire male. This can also be an interesting alternative for cats that cannot undergo surgeries because of other health concerns (like cardiac or kidney disease for instance).

Pathology of Reproduction in Male Cats

Pathology of the Male Cat Genital Tract

Genital disorders in male cats are rather uncommon.

As mentioned earlier, cryptorchidism is the most common congenital defect of the urogenital system (Priester *et al.*, 1970). Feline cryptorchidism is more commonly unilateral than bilateral.

With the exception of a small number of prostatic adenocarcinomas, prostatic disease is almost unknown in cats, while it is extremely frequent in dogs for instance (Fontbonne *et al.*, 2007).

The Calico or Tortoise-Shell Males

Cats have 19 pairs of chromosomes and a normal male's chromosome formula will therefore be 38,XY (Parkinson, 2009).

Like in other mammals, abnormalities of sexual differentiation are described in cats such as abnormalities of chromosomal sex (such as 39, XXY trisomy or 38 XX/38 XY chimeras and mosaics); abnormalities of the gonadal sex (such as presence of ovotestes in true hermaphrodites) and abnormalities of phenotypic sex (such as male pseudohermaphrodite) (Johnston *et al.*, 2001).

Of particular interest in this species is what is observed in tortoise shell and calico males.

The male tortoise shell (black and orange – see Fig. 5) or calico (black orange and white – see Fig. 6) has been known since the early 1900s to be exceedingly uncommon and when found, to have a high incidence of sterility (Johnston *et al.*, 2001).

Orange and black coat colors are sex-limited traits in the cat. The orange coat color in this species is determined by a single gene located on the X chromosome. Therefore, black and orange may occur together in the female, but not in the male since they are carrying only one X chromosome.

Calico or tortoise-shell male cats are therefore three sex chromosomes: XXY.

While this is frequently associated with infertility, it is however interesting to note that some of those males have a normal fertility: one male tortoiseshell cat was reported to have sired 65 kittens in 2 years (Malandain, 2006).

Nutrition-Induced Testicular Degeneration

Testicular degeneration is typically associated with aging and lead to decrease sperm output and sperm quality, that will obviously ultimately impact fertility results (Parkinson, 2009).

In cats, testicular degeneration has also been associated with nutritional deficiencies.

Cats fed diets deficient in riboflavin, vitamin A or essential fatty acids (specifically linoleate, which the cat testis can convert to arachidonate) have been shown to undergo testicular degeneration and atrophy.

Male cats fed a riboflavin-deficient diet for 6 months showed complete aspermia, with decreased number of spermatids and an occasional increase in number of Sertoli cells (Gershoff *et al.*, 1959).

Kittens fed raw meat diet devoid of vitamin A from 12 weeks of age failed to show pubertal testicular development (Scott and Scott, 1964).

This is worth noticing because raw feeding is a growing trend in the cat breeder world.



Fig. 5 A tortoise-shell cat.



Fig. 6 A calico cat.

While the infectious diseases risks associated with this practice are now well described, it is worth adding that unbalanced diet – like what can be achieved when raw feeding the cats – can also impact fertility parameters as well.

Conclusion

Feline reproduction is definitely an interesting field of veterinary medicine.

Because of the current research projects in both animal population control and conservation biology, we now have a better understanding of the male cat reproductive function and a better idea of how to approach the different aspects of fertility in this field.

That provides us with clear elements to answer questions people might have on the male cat's reproductive function.

There are obviously still some limitations, especially in terms of assisted reproductive techniques. With the emphasis on conservation biology, there might however be more answers and alternatives in a near future.

References

- Axner, E., & Linde Forsberg, C. (2007). Sperm morphology in the domestic cat, and its relation with fertility: A retrospective study. *Reprod Domest Anim*, 42(3), 282–291.
- Dooley, M., Pineda, M., Hopper, J., Hsu, W., 1984. Retrograde flow of semen caused by electroejaculation in the domestic cat. Urbana, IL, s.n., p. 363.
- Fontaine, E., Vannier, F., & Fontbonne, A. (2010). Insémination artificielle chez la chatte: du rêve à la réalité. *Le Nouveau Praticien Vétérinaire*, 219, 55–58.
- Fontbonne, A., Levy, X., Fontaine, E., & Gilson, C. (2007). *Guide Pratique de Reproduction Clinique Canine Et Féline*. Paris: Med'com.
- Gershoff, S., Andrus, S., & Hegsted, D. (1959). The effect of the carbohydrate and fat content of the diet upon the riboflavin requirement of the cat. *J Nutr*, 68, 75–88.
- Goericke-Pesch, S., et al. (2014). Reversibility of germinative and endocrine testicular function after long-term contraception with a GnRH-agonist implant in the tom: A follow-up study. *Theriogenology*, 81(7), 941–946.
- Jackson, C. (1902). On the structure of the corpora cavernosa in the domestic cat. *Am J Anat*, 2, 73–80.
- Johnston, S., Root Kustritz, M., & Olson, P. (2001). Sexual differentiation and normal anatomy of the Tom Cat. *Canine and Feline Theriogenology* (p. 592). Philadelphia: WB Saunders.
- Malandain, E. (2006). Reproduction: From oestrus to kitting. *Practical Guide: Cat Breeding* (pp. 63–131). Paris: Aniwa.
- Parkinson, T. (2009). Normal reproduction in male animals. *Veterinary Reproduction and Obstetrics* (Ninth edition., pp. 681–704). London: Saunders.
- Platz, C., & Seager, S. (1978). Semen collection by electroejaculation in the domestic cat. *J Am Vet Med Assoc*, 173, 1353–1355.
- Priester, W., Glass, A., & Waggoner, N. (1970). Congenital defects in domesticated animals: General considerations. *Am J Vet Res*, 31, 1871–1879.
- Root, M., & Johnston, S. (1995). The effect of early spay-neuter in the development of feline obesity. *Vet Forum*, 12, 38–43.
- Scott, P., & Scott, M. (1964). Vitamin A and reproduction in the cat. *J Reprod Fertil*, 8, 270–271.
- Sojka, N., Jennings, L., & Hamner, C. (1970). Artificial insemination in the cats (*Felis catus* L.). *Lab Anim Care*, 20, 198–204.
- Spain, C., Scarlett, J., & Houpt, K. (2004). Long-term risks and benefits of early-age gonadectomy in cats. *J Am Med Assoc*, 224, 372.
- Tanaka, A., Takagi, Y., & Nakagawa, K. (2000). Artificial intravaginal insemination in domestic cats (*Felis catus*). *J Vet Med Sci*, 62(11), 1163–1167.
- Tsutsui, T., et al. (2009). Plasma hormone levels and semen quality in cats during non-breeding and breeding seasons. *Reprod Domest Anim*, 44(Suppl. 2), 291–293.
- Tsutsui, T., Tanaka, A., & Takagi, Y. (2000). Unilateral intrauterine horn insemination of fresh semen in cats. *J Vet Med Sci*, 62(12), 1241–1245.
- Zambelli, D., & Cunto, M. (2006). Semen collection in cats: Techniques and analysis. *Theriogenology*, 66, 159–165.

Human and Primate Overview

Gerhard F Weinbauer, Covance Preclinical Services GmbH, Muenster, Germany

C Marc Luetjens, Covance Preclinical Services GmbH, Muenster, Germany

© 2018 Elsevier Inc. All rights reserved.

Background

Phylogenetically, primates are a mammalian Order and that Order is still growing considerably with 234 species reported in 1996 and 505 species reported in 2016 including human (Rowe and Myers, 2016). Male reproductive functions can generally be described across four main categories A–D: (A) production and secretion of male reproductive hormones (e.g., testosterone) that are indispensable for achieving B–D but also influence a large variety of non-reproductive parameters in the male organism; (B) the production and maturation of male germ cells culminating in the production of spermatozoa (sperm) in the testis; (C) production of seminal fluid in the accessory male reproductive organs (e.g., epididymis/seminal vesicles/prostate/bulbourethral glands) and (D) transport of spermatozoa and seminal fluid into the female reproductive tract via a penis and ejaculation process. This article provides a comparative overview on these four categories across the Order Primates (men and male nonhuman primates (NHPs)).

The overall morphology of the male genital system of NHPs is similar to humans and other mammals. In adult primates, the testes are located in a scrotum outside the body. In all primates, the testes descend into the scrotum around birth (Martin, 2007). The scrotal location is necessary since the process of primate spermatogenesis – unlike in other mammals such as whales or elephants – requires temperatures below body temperature. However, testes can be retracted back into the external inguinal ring for protecting the organ or in species with distinct seasonality of reproduction. Anus, scrotum and penis are also denoted as external perineum. In some primates, this perineum can be intensely coloured and fulfils social and reproductive signalling function. For example, in the adult Patas monkey (*Erythrocebus patas*) the anal triangle is bright red and the scrotum is bright blue; and in the rhesus monkey (*Macaca mulatta*) the perineum is coloured intensely red during the active reproductive season.

Production and Secretion of Male Reproductive Hormones

Testicular functions, i.e., production of gametes and testosterone are controlled by endocrine factors derived from the brain across many species such as rodents, dogs and (nonhuman) primates (Ramaswamy and Weinbauer, 2015). In primates, the key hormones are testosterone, inhibin B and the gonadotropins Luteinizing hormone (LH), and Follicle-stimulating hormone (FSH). Hypothalamic gonadotropin releasing hormone (GnRH) controls pituitary LH and FSH synthesis, release and GnRH secretion is regulated by the neurohormone kisspeptin. The regulation of pituitary gonadotropin release is regulated by an ultradian cycle generated by a neuronal pulsatile network (Herbison, 2016). In the testis, Leydig cells produce testosterone under the influence of LH; Sertoli cells produce inhibin B under the influence of FSH, and both testosterone and inhibin B exert brain feedback regulation and a controlled interaction between hypothalamus, pituitary and testis (hypothalamus-pituitary-testis axis) is essential for intact male primate reproductive physiology. In peripheral blood testosterone is converted into estradiol which also exerts feedback effects on the secretion of GnRH, LH and FSH.

In primates, this axis is already active during embryonic development, but apart from short periods of activation after birth, remains suppressed during a prolonged period of reproductive quiescence until start of puberty (Plant, 2015), ultimately resulting in sexual maturity and fertility. In this context, primates are different from rodents lacking such prolonged period of reproductive hiatus. During pubertal onset, the hypothalamic pulse generator reactivates via kisspeptin, GnRH and gonadotropic hormones. The age at onset of puberty differs between males and females with females generally experiencing an earlier start, and the duration of the pubertal phase can be highly variable either between individuals of the same species (Luetjens and Weinbauer, 2012) or among closely related species such as chimpanzees (*Pan troglodytes*) and bonobos (*Pan paniscus*) (Behringer et al., 2014). In male primates, the reawakening of the GnRH pulse generator at the termination of the juvenile phase of development leads to re-initiation of gonadotropin release, reactivation of testicular testosterone secretion and the initiation of spermatogenesis relatively readily identified (Plant, 2015). Albeit under separate control, adrenal hormone secretion (adrenarche) starts in primates with chimpanzees and gorillas having similar adrenarche development to humans. During adrenarche the adrenal cortex secretes increased levels of androgens such as dehydroepiandrosterone and dehydroepiandrosterone sulfate, but without increased cortisol levels. Adrenarche means the development of a new zone in the adrenal cortex, the zona reticularis.

LH (indirectly via testosterone) and FSH directly stimulate Sertoli cells and spermatogenesis. In the immature primate testis, FSH potentiates the effect of LH on Leydig cell testosterone production. Beyond that, testosterone also exerts a variety of peripheral non-gametogenic effects such a sexual differentiation, testicular descent, perineum coloration, puberty-associated voice maturation, sexual behaviour and libido and on bone, muscle and lipid metabolism (Weinbauer et al., 2010). It is also assumed that testosterone may influence “male priming” during brain development and the maturation of the immune system. Recent investigations support direct production and activity of kisspeptin in testis and its involvement in the control of Leydig cells, germ cells progression and sperm function. The combined action of testosterone and FSH results in quantitatively normal production of germ cell numbers and sperm. In primates, either hormone alone can support the complete spermatogenic process but not the production of normal

germ cell numbers. Testicular concentrations of androgen levels can exceed peripheral blood concentrations up to 100-fold or beyond leading to the view that high local androgen concentrations are required for normal spermatogenesis. In primates, however, there is no direct correlation between intratesticular testosterone levels and the level of germ cell/sperm production. It is believed, that high local testicular androgen are rather required in order to provide sufficient peripheral testosterone levels and a pulsatile pattern of hormone concentrations.

Reproductive and developmental physiology has been described for number of primate species regularly used a research model and/or as preclinical model for human, comprising Old World and New World monkeys (Ramaswamy and Weinbauer, 2015; Tardif et al., 2012). Similar to other mammals, NHPs display significant annual differences in their reproductive activities depending on the habitat latitude. Species living in the northern hemisphere and exposed to distinct season with cold winters, exhibit seasonal reproductive patterns with sexual activity during autumn/winter and reproductive quiescence during spring/summer in order to ensure delivery of infants during appropriate climate conditions. Species with distinct reproductive seasonality are for example rhesus monkey (*Macaca mulatta*), Japanese macaque (*Macaca fuscata*) and bonnet monkey (*Macaca radiata*). Primates are short-day breeders with the reproductive activity being triggered by a shortened day length in autumn as opposed to long-day breeders such as sheep or rodents becoming sexually active during spring. During the out-of-season periods, reproductive hormone secretion and gonadal activity are at a complete halt triggered by the specific and day-length-related pattern of pineal melatonin secretion. Species living in the equatorial regions lacking significant seasons, typically are fertile throughout the entire year – e.g., long-tailed macaque (cynomolgus monkey, *Macaca fascicularis*), African NHP species and some New World monkey species such as marmoset (*Callithrix jacchus*). Long-tailed macaques may exhibit some adaption of reproductive activity based upon the onset of the raining season. Human are considered a non-seasonal species in term of reproductive activities.

In general, endocrine regulation of male reproductive functions is similar in anthropoid Old World monkeys (NHPs with African and Southeast-Asian habitats) and human with LH/androgen and FSH as the main factors. The LH/androgen setup, however, is fundamentally different in neotropical (New World) monkeys. This difference has initially been unravelled in the tuffed ear marmoset (*Callithrix jacchus*) but has been confirmed to other neotropical NHP species (Grzesik et al., 2015). These species lack LH but use chorionic gonadotropin (CG) instead of LH. Thus, CG is produced in the pituitary, stimulates testosterone production in the testicular Leydig cells and constitutes another primate gonadotropic hormone. LH and CG are heterodimeric glycoprotein hormones, consisting of a common alpha subunit and a specific beta subunit. It is believed that CG evolved by gene duplication of an ancient LH beta subunit gene approximately 40 million years ago. Specific to CG is a C-terminal peptide of 24 amino acids which was generated during evolution by the introduction of a point mutation shifting the open reading frame. The number of the CG beta gene amplifies with progressing evolution towards 7 CG beta gene copies (Troppmann et al., 2013). Along with this gene duplication, a mutation occurred in the sixth transmembrane domain of the LH receptor (LHR). While it was assumed that the function of CG in adult primates is confined to maintenance of pregnancy and early sexual differentiation, studies on the LH receptor revealed a dual action of LH and CG in primates. Thus, in primates two native LH receptor types exist. The wild-type LHR type 1 containing exon 10, which is present in Old World monkeys, great apes and the human lineage and the LHR type 2, lacking exon 10 in the New World monkey lineage. The LH/CG system independently evolved by geographical isolation and the ancestral functions of the LH/CG system remained in the neotropical taxa - double role of CG for pregnancy and male reproduction. Leydig cells with LHR type 2 produce testosterone in response to CG but not to LH. Interestingly patients lacking LHR exon 10 have been identified and are deficient in testosterone production. Based upon the knowledge about CG and LHR type 2, therapy with CG has been successfully introduced for some of these patients (Troppmann et al., 2013).

The key factors driving spermatogenesis are LH/androgen and FSH in Old World monkeys and human. Compared to rodents, spermatogenesis is particularly dependent on FSH vs androgens in Old World monkeys and human. Also, rodent and primate spermatogenesis respond quite differently to reproductive hormone withdrawal in terms of germ cell type-specific effects (Ramaswamy and Weinbauer, 2015). The primary spermatogenic lesion initially affects different germ cell types with spermatogonia in primates and spermatocytes/spermatids in rodents and the maximal spermatogenic suppression is more pronounced in NHPs compared to rat. It is less clear to what extent spermatogenesis in New World monkeys is dependent on established reproductive hormones. Administration of androgenic and gestagenic steroids – a well-established regimen for suppression of testicular functions and male contraception in macaques and men – produced comparable endocrine suppression (reduction of pituitary CG and testicular testosterone) in the marmoset but interestingly without effects on testicular function and fertility (Wistuba et al., 2013). Potentially, marmoset spermatogenesis is particularly dependent on FSH and/or rather independent of CG/testosterone. Alternatively, other unknown regulatory factors for male reproductive functions are active in the marmoset and possibly also in other neotropical primate species.

Production and Maturation of Male Germ Cells

Primate spermatogenesis encompasses germ cell development features common to other mammals but also primate-specific aspects. Mitotically active stem cells and spermatogonia provide the base of testicular gametogenesis followed by formation of meiotic spermatocytes and subsequently haploid spermatids which develop into testicular sperm via morphological and functional differentiation. Testicular sperm production depends on the number of spermatogonial multiplications, successful meiosis yielding four spermatids, differentiation of spermatids into testicular sperm and germ cell loss during the various development and division phases. In general primate spermatogenesis is comparatively efficient (Fig. 1). Primates differ from rodents with respect to the

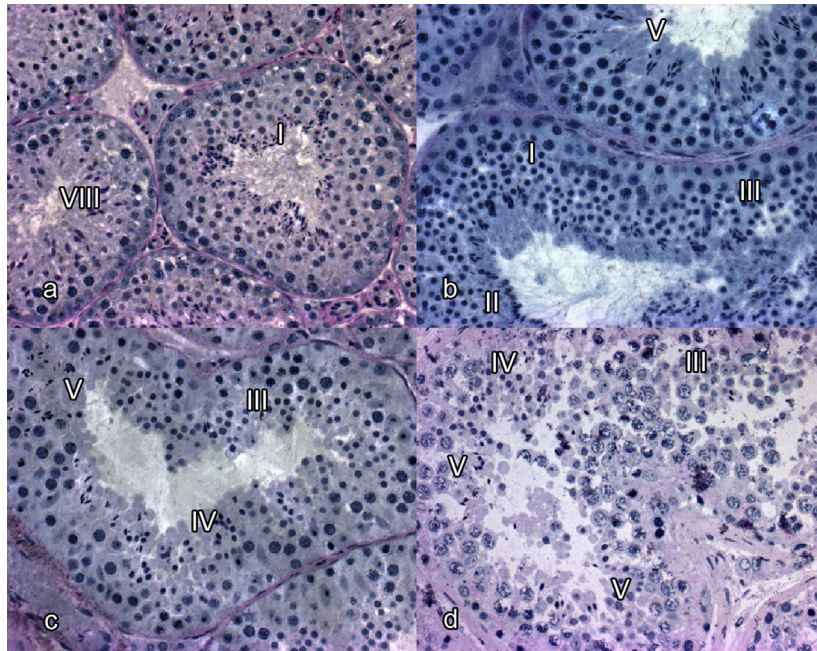


Fig. 1 Micrographs of testicular histology from four different species showing typical organisation of the germinal epithelium. Roman numerals indicate stage of spermatogenesis. (a) Macaques (*Macaca fascicularis*): Predominantly single-stage tubules; (b) Baboon (*Mandrillus sphinx*): Mixture between single-stage and multi-stage tubules; (c) Marmoset (*Callithrix jacchus*): Multi-stage tubules; (d) Chimpanzee (*Pan troglodytes*): Multi-stage tubules.

testicular stem cell systems such that in primates fewer number/generations of stem cells are present. There are two separate cell types of undifferentiated spermatogonia: the A dark spermatogonia represent the mitotically inactive reserve stem cells (which only start to proliferate upon severe testicular damage), whereas the Apale spermatogonia represent a type of mitotically active progenitor cell. For example, the differentiating germ cells derived from the Apale population produce 128 sperm in the rhesus monkey and 16 sperm in man from each cell. By contrast, in rodents several generations of A-type spermatogonia exist and over 4000 sperm are being produced from a differentiating spermatogonium (Ramm *et al.*, 2014; Fig. 2). Other factors that determine testicular germ cell production are the conversion of spermatogonia into spermatocytes and the maturation spermatids. In this context, primate spermatogenesis is generally quite efficient (Fig. 3).

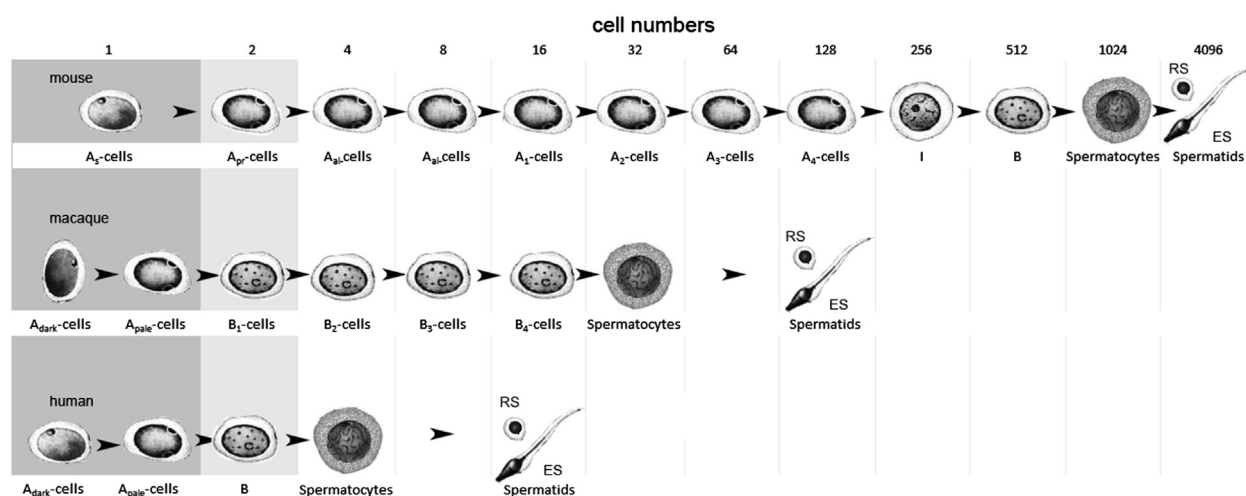


Fig. 2 Schematic depiction of male germ cell differentiation in the mouse, the macaque and man. Spermatogonial subtypes: As: Asingle; Apr: Apair; Aal: Aaligned; I: intermediate spermatogonium; B: B-spermatogonium. RS: round spermatid; ES: elongated spermatid. Note the very different number of spermatid produced per spermatogonial stem cell division in these three species. For this figure the spermatogonial renewal model proposed by Ramm, S.A., Schärer, L., Ehmecke, J., Wistuba, J., 2014. Sperm competition and the evolution of spermatogenesis. Molecular Human Reproduction 20, 1169–1179 was adopted.

The complex pattern of germ cell differentiation results in specific germ cell associations known as stages of spermatogenesis with each stage comprising spermatogonia, spermatocytes and spermatids during a specific development stage. The topography and number of stages is species-specific. For a stage-associated group of germ cells to pass through all stages requires approx. 10 days for most NHP species that have been studied, 14 days for the chimpanzee and 16 days for human (Amann, 2008; Luetjens *et al.*, 2005). For a differentiating spermatogonium to develop into testicular sperm, takes four spermatogenic cycles, i.e., 40 days for most NHP species, 56 days for chimpanzee and 64 days for human. A seminiferous tubule cross-section can either contain one spermatogenic stage (single-stage tubule) or several stages (multi-stage tubule) (Luetjens *et al.*, 2005). In men, great apes and neotropical monkeys multi-stages tubules prevail although New World monkeys separated from human and great ape line around 35 million years ago. In Old World monkeys (Afrika and Asia; Catarrhini) an intermediate type of organisation has been reported for baboon and mandrill. Single-stage tubules are predominantly found in macaques and vervet monkeys. The prosimians (Strepsirrhini) exhibit the lowest proportion of tubules with multi-stage. It appears that the single-stage organisation seems to represent a more ancestral stage which developed during evolution into an increasing number of stages per tubular cross section (Fig. 4). All germ cells within a given spermatogenic stage represent a single cell-derived clone. It is assumed that the origin of single-stage versus multi-stage tubules relates to the size of these clones: Small clones are associated with the multi-stage arrangement whereas large clones are observed in single-stage organisation. The comparison of efficiency rates among primates revealed similar values for all species indicating that the structural organisation is related to the yield of spermatozoa (Luetjens *et al.*, 2005) because they obviously need more space. The clonal size is determined by the number of Sertoli cells. A single Sertoli cell supports only a limited number of germ cells and the number of Sertoli cells determines the final testis size in mammals. The ratio between Sertoli cell and germ cells defines the Sertoli cell workload which obviously does not influence the spermatogenic efficiency, but is very likely to be a species-specific feature of the seminiferous epithelium (Luetjens *et al.*, 2005). The total number of spermatozoa produced is generally correlated only with the testis size, which is determined by the Sertoli cell number (Fig. 3).

Intrasexual selection occurs between males competing for access to females and vice versa. This access selection may not only mean to have physical intercourse. It could also be at a post-copulatory level – sperm from different males having to compete within the individual female tract for the oocytes leading to sperm competition from different males. Primates have many differing mating systems comprising single-male, multi-male, single-female and multi-female strategies.

As a result of different male mating systems the requirements for daily sperm production may have to differ reflected by the testis size. As an example, chimpanzees have bigger testes relative to their body weight than gorillas, orang-utans or humans (Ramm *et al.*, 2014). In some primates, testis size can be 1–5% of body weight (e.g., *Loris tardigradus*, *Saguinus oedipus*, *Microcebus murinus*) – for comparison relative testis size in men as around 0.06%. The testis/body weight correlates strongly with the mating system indicating that primates with lifelong partnership do not invest into testicular size. This finding supports the concept that species living in multi-male systems have often larger testes in relation to body weight than those with single-male systems (e.g., macaques and chimpanzees vs. marmosets, gorillas, orang utans and human). Sperm in the latter breeding systems supposedly do not have to compete for the oocytes against sperm from other males (Dixon and Anderson, 2004).

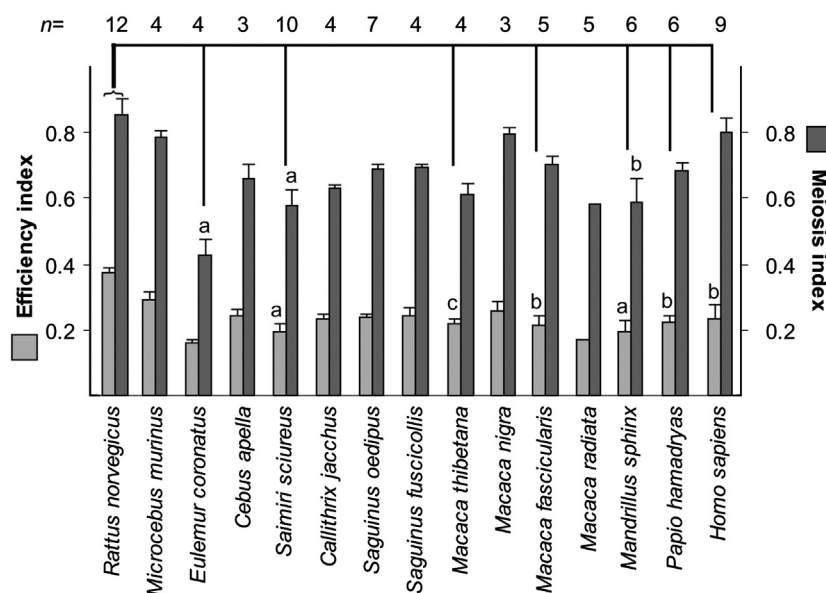


Fig. 3 Spermatogenic efficiency and meiosis indices for the rat, 13 primate species and man based upon flow cytometric data. The efficiency index is defined as the number of elongated cells divided by the total cell number. The meiosis index is defined as the number of haploid cells divided by the total cell number. n denotes the number of testes analysed. Data are means±SEM. The rat differs significantly from seven primate species (one-way ANOVA; P values encoded as af0.001, bf0.01, and cf0.05). Based upon Luetjens, C.M., Weinbauer, G.F., Wistuba, J., 2005. Primate spermatogenesis: New insights into comparative testicular organisation, spermatogenic efficiency and endocrine control. *Biological Reviews of the Cambridge Philosophical Society* 80, 475-488.

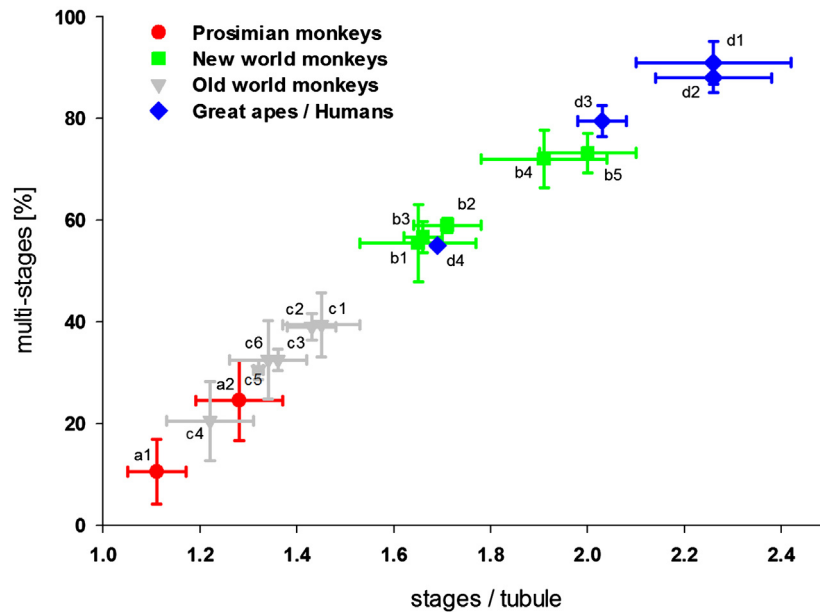


Fig. 4 Correlation between the percentage of multistage tubules and the average number of stages per tubular cross-section. The linear correlation elucidates that the species belonging to a certain systematic entity are grouped together. Overlapping is rare – the error bars are SD. a1 *Otolemur spec.*, a2 *Microcebus murinus*, b1 *Cebus apella*, b2 *Saimiri sciureus*, b3 *Saguinus oedipus*, b4 *Saguinus fuscicollis*, b5 *Callithrix jacchus*, c1 *Papio hamadryas*, c2 *Mandrillus sphinx*, c3 *Macaca nigra*, c4 *Macaca fascicularis*, c5 *Macaca tibetana*, c6 *Cercopithecus aethiops*, d1 *Pan paniscus*, d2 *Pan troglodytes*, d3 *Homo sapiens*, d4 *Pongo pygmaeus*. Modified from Wistuba, J., Schrod, A., Greve, B., et al., 2003. Organisation of the seminiferous epithelium in primates: Relationship to spermatogenic efficiency, phylogeny and mating system. *Biology of Reproduction* 69, 582–591.

Production of Seminal Fluid

Testicular spermatozoa are released from the testis into the epididymis via longitudinal contractions of the seminiferous tubules. There upon spermatozoa enter the vas (ductus) deferens that connects the epididymis to the urethra. The epididymis and a variety of glands along the vas deferens contribute to the composition of seminal fluid. The purpose of these contributions is to provide a suitable environment for spermatozoa for preserving their functional integrity (progressive motility and intact morphology) and to render seminal fluid as a transport medium for spermatozoa to enter the female reproductive tract.

Epididymis and Vas Deferens

This gland is composed of a singly and highly coiled duct and separated structurally and functionally into an initial segment that connects the organ to the rete testis followed by a caput, corpus and cauda segment. The vas deferens is a long, muscular tube carrying mature sperm from the epididymis to the urethra prior to ejaculation. The vas deferens joins the urethra just behind the bladder.

In some primate Genus the ejaculatory duct does not enter the prostate (e.g., Lorisidae, Tarsiidae). The primary function of the epididymis is maturation of sperm and storage of sperm. During transport along the epididymal duct, sperm mature functionally and gain forward progressive motility with sperm being only slightly motile in the caput region but having complete motility characteristics when leaving the cauda region. This is achieved by interaction of sperm with proteins and other factors provided by epididymal cells in a region-specific manner. The cauda region also serves for sperm storage and as sperm reservoir. Typically, the caudal fluid has low inorganic ions concentration but high concentrations of organic constituents, e.g., phosphatases, glycosidases, sialic acid, lactic acid, ascorbic acid, dehydrogenases, carnitine and glycerophosphocholine (Tardif et al., 2012). L-carnitine, glycerophosphocholine and neutral alpha-glucosidase are secreted from the epididymis and are also present in semen. Sperm maturation is defined by the ability to undergo capacitation and acrosome reaction. Macaques and marmosets display an acrosome maturation profile that is comparable to that in human and this functionality increases as sperm move from the caput to the cauda of the epididymis. Functional sperm maturation also comprises remodelling of sperm chromatin into a highly condensed form. It appears that this remodelling can occur independent of reproductive hormones.

Prostate/Seminal Vesicles/Bulbourethral Glands

The prostate surrounds the urethra, the tube that carries the ejaculate that is expelled from the body during orgasm. In macaques, prostate anatomy and zonation are roughly comparable to the human prostate whilst in other primate species the anterior part

found in humans maybe lacking. Single-lobed prostate has been described for the marmoset (*Callithrix jacchus*) and pigmy chimpanzee (*Pongo pygmaeus*). The prostate gland provides zinc, citric acid, prostate-specific antigen, acid phosphatase and acid alpha-glucosidase to the seminal fluid.

The paired seminal vesicles and the vas deferens join together to form the ejaculatory ducts, which empty into the urethra. Seminal vesicles produce fructose, prostaglandins, carnitine, glycerophosphocholine and seminogelin. The latter is a key factor for liquefaction of seminal fluid outside the male body. Bulbourethral glands are also known as Cowper glands, provide mucus proteins that lubricate the urethra and counteract the acidity of any urine leftover in the urethra.

Semen

Semen is composed of seminal fluid and sperm. In some primate species, e.g., rhesus monkey and cynomolgus monkey, the semen solidifies into a coagulum upon expulsion whilst in men semen is viscous initially but liquefies over time after ejaculation. Basic sperm morphology is similar across the Primate Order and sperm are composed of four regions: Head containing chromatin and the acrosome, neck with a centriole, midpiece containing mitochondria and tail. Species-specific differences relate to size of sperm, shape/size of sperm head and kinematic properties of sperm. Overall, sperm morphology and shape is extremely variable and sperm are considered among the most variable cell when comparing different species (quoted after [Ramm et al. \(2014\)](#)).

Transport of Spermatozoa

Transport of semen into the female reproductive tract is achieved via penile erection and ejaculation. Mammals typically use a penis to deposit the semen within the vagina/uterus (internal fertilization). The penis is typically cylinder-shaped and has several chambers inside made of erectile tissue (Corpus cavernosum penis). During sexual arousal these spaces fill with blood, which causes the penis to become rigid and erect, and enables the penis to penetrate the female reproductive tract during sexual intercourse. In general, penile size and penile morphology are species-specific and/or related to the mating system with corresponding vaginal/uterine morphological adaptations. The penis is responsible for expelling (ejaculating) sperm inside the female reproductive tract. In men, the urethra besides urine also carries semen, which is ejaculated during orgasm. Urine is prevented from entering the urethra while the penis is erect, which allows only semen to be ejaculated. In all Primates, the penis is pendulous but varies considerably with respect to size and details of surface structure e.g. penile spines and size/shape of the glans penis. In men, the penis is described as cavernous type as opposed to the fibro-elastic type (e.g., in cattle) in which the erectile tissue contains much connective tissue and during blood inflow the bent penis is being prolonged but grows little in diameter. In many NHPs an os penis (baculum) is present but such structure is lacking in men except for disease conditions and in some New World monkey species. The baculum is an ossification of part of the erectile tissue structures. The penis type in species with a baculum is therefore intermediate between cavernous and fibro-elastic type. Overall, there is great variation in baculum size and the presence of a baculum appears related to mating systems. The length of the baculum correlates with the duration of intromission over the course of primate evolution in extant primates and carnivores, and both polygamous and seasonal breeding systems predict significantly longer bacula in primates ([Brindle and Opie, 2016](#)). In men, a specific deletion in the androgen receptor gene eliminated a penile spine/vibrissae enhancer sequence ([McLean et al., 2011](#); [Reno et al., 2013](#)). Hence men maybe the only primate without baculum and sensory vibrissae and this appears to be the case also for extinct Homo species, e.g., Neandertal and Denisova. The presence of a baculum and vibrissae is regulated by androgenic hormones and this has been demonstrated experimentally in prosimian primates (*Galago* sp., [Reno et al. \(2013\)](#)).

References

- Amann, R. P. (2008). The cycle of the seminiferous epithelium in humans: A need to revisit? *Journal of Andrology*, 29, 469–487.
- Behringer, V., Deschner, T., Deimel, C., Stevens, J. M., & Hohmann, G. (2014). Age-related changes in urinary testosterone levels suggests differences in puberty onset and divergent life history strategies in bonobos and chimpanzees. *Hormone Behaviour*, 66, 525–533.
- Brindle, M., & Opie, C. (2016). Postcopulatory sexual selection influences baculum evolution in primates and carnivores. *Proceedings of the Royal Society Biological Sciences B*, 283(1844), 20161736.
- Dixon, A. F., & Anderson, M. J. (2004). Sexual behavior, reproductive physiology and sperm competition in male mammals. *Physiological Behavior*, 83, 361–371.
- Grzesik, P., Kreuchwig, A., Rutz, C., et al. (2015). Differences in signal activation by LH and hCG are mediated by the LH/CG receptor's extracellular hinge region. *Frontiers in Endocrinology ((Lausanne))*, 140, 1–14.
- Herbison, A. E. (2016). Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nature Reviews Endocrinology*, 12, 452–466.
- Luetjens, C. M., & Weinbauer, G. F. (2012). Functional assessment of sexual maturity in male macaques (*Macaca fascicularis*). *Regulatory Toxicology and Pharmacology*, 63, 391–400.
- Luetjens, C. M., Weinbauer, G. F., & Wistuba, J. (2005). Primate spermatogenesis: New insights into comparative testicular organisation, spermatogenic efficiency and endocrine control. *Biological Reviews of the Cambridge Philosophical Society*, 80, 475–488.
- Martin, R. D. (2007). The evolution of human reproduction: A primatological perspective. *American Journal of Physical Anthropology*, (Suppl. 45), 59–84.
- McLean, C. Y., Reno, P. L., Pollen, A. A., et al. (2011). Human-specific loss of regulatory DNA and the evolution of human-specific traits. *Nature*, 471, 216–219.
- Plant, T. M. (2015). Neuroendocrine control of the onset of puberty. *Frontiers in Neuroendocrinology*, 38, 73–88.
- Ramaswamy, S., & Weinbauer, G. F. (2015). Endocrine control of spermatogenesis: Role of FSH and LH/ testosterone. *Spermatogenesis*, 26, e996025.
- Ramm, S. A., Scharer, L., Ehmkke, J., & Wistuba, J. (2014). Sperm competition and the evolution of spermatogenesis. *Molecular Human Reproduction*, 20, 1169–1179.
- Reno, P. L., McLean, C. Y., & Hines, J. E. (2013). A penile spine/vibrissae enhancer sequence is missing in modern and extinct humans but is retained in multiple primates with penile spines and sensory vibrissae. *PLOS ONE*, 8(12), e84258.

- Rowe, R., & Myers, M. (2016). *All the Worlds Primates* (second ed.). Charlestown, Rhode Island: Pogonias Press.
- Tardif, S., Carville, A., Elmore, D., Williams, L. E., & Rice, K. (2012). Reproduction and breeding of nonhuman primates. In C. R. Abee, K. Mansfield, S. D. Tardif, & T. Morris (Eds.), *Nonhuman Primates in Biomedical Research: Biology and Management* (second ed., pp. 197–249). Amsterdam: Elsevier (Chapter 8).
- Troppmann, B., Kleinau, G., Krause, G., & Gromoll, J. (2013). Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. *Human Reproduction Update*, 19, 583–602.
- Weinbauer, G. F., Luetjens, C. M., Simoni, M., & Nieschlag, E. (2010). Physiology of testicular function. In E. Nieschlag, H. M. Behre, & S. Nieschlag (Eds.), *Andrology: Male Reproductive Health and Dysfunction* (third ed., pp. 11–59). Berlin: Springer Verlag.
- Wistuba, J., Luetjens, C. M., Ehmecke, J., et al. (2015). Experimental endocrine manipulation by contraceptive regimen in the male marmoset (*Callithrix jacchus*). *Reproduction*, 154(4), 439–451.

Further Reading

- Aslam, H., Schneiders, A., Perret, M., Weinbauer, G. F., & Hodges, J. K. (2002). Quantitative assessment of testicular germ cell production and kinematic and morphometric parameters of ejaculated spermatozoa in the grey mouse lemur, *Microcebus murinus*. *Reproduction*, 123, 323–332.
- Behringer, V., Hohmann, G., Stevens, J. M., Weltring, A., & Deschner, T. (2012). Adrenarche in bonobos (*Pan paniscus*): Evidence from ontogenetic changes in urinary dehydroepiandrosterone-sulfate levels. *Journal of Endocrinology*, 214, 55–65.
- Blumel, J., Korte, S., Schenck, E., & Weinbauer, G. F. (2015). *The Nonhuman Primate in Nonclinical Drug Development and Safety Assessment* (first ed.). Amsterdam: Academic Press.
- Chianese, R., Cobellis, G., Chioccarelli, T., et al. (2016). Kisspeptins, estrogens and male fertility. *Current Medicinal Chemistry*, 23, 1–23.
- Chowdhury, A. K., & Marshall, G. R. (1980). Irregular pattern of spermatogenesis in the Baboon (*Papio anubis*) and its possible mechanism. In A. Steinberger, & E. Steinberger (Eds.), *Testicular Development, Structure and Function* (pp. 129–137). New York, NY: Raven Press.
- Gromoll, J., Eiholzer, U., Nieschlag, E., & Simoni, M. (2000). Male hypogonadism caused by homozygous deletion of exon 10 of the luteinizing hormone (LH) receptor: Differential action of human chorionic gonadotropin and LH. *Journal of Clinical Endocrinology & Metabolism*, 85, 2281–2286.
- Huhtaniemi, I. (2015). A short evolutionary history of FSH-stimulated spermatogenesis. *Hormones (Athens)*, 14, 468–478.
- Martin, P. L., & Weinbauer, G. F. (2010). Developmental toxicity testing of biopharmaceuticals in nonhuman primates: Previous experience and future directions. *International Journal of Toxicology*, 29, 552–568.
- Maston, G. A., & Ruvolo, M. (2002). Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. *Molecular Biology and Evolution*, 19, 320–335.
- Mineshima, H., Endo, Y., Ogasawara, H., et al. (2004). Impact of globalization under the ICH guidelines on the conduct of reproductive toxicity studies – Report on current status in Japan, Europe and the U.S. by questionnaire survey. *The Journal of Toxicological Sciences*, 29, 201–215.
- Onyango, P. O., Gesquiere, L. R., Altmann, J., & Alberts, S. C. (2013). Puberty and dispersal in a wild primate population. *Hormones and Behavior*, 64, 240–249.
- Rode-Margono, E. J., Nekaris, K. A., Kappeler, P. M., & Schwitzer, C. (2015). The largest relative testis size among primates and aseasonal reproduction in a nocturnal lemur, *Mirza zaza*. *American Journal of Physical Anthropology*, 158, 165–169.
- Short, R. V. (1977). The testis: The witness of the mating system, the site of mutation and the engine of desire. *Acta Paediatrica*, (Suppl. 422), 3–7 (Oslo, Norway: 1992).
- Uenoyama, Y., Pheng, V., Tsukamura, H., & Maeda, K. I. (2016). The roles of kisspeptin revisited: Inside and outside the hypothalamus. *Journal of Reproductive Development*, 62, 537–545.

Relevant Websites

- www.phallus.is. — Phallus.
- www.pin.primat.wisc.edu. — Primate Info Net.
- <http://www.tutorvista.com>. — Tutorvista.com.

Male ART in Animal Species

Robert V Knox and David J Miller, University of Illinois, Champaign-Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Artificial insemination (AI) Deposition of semen into the female reproductive tract by a method other than natural mating.

Artificial vagina (AV) A device that mimics the vagina and used to collect semen.

Assisted reproductive technology (ART) A group of technologies that promote reproduction.

Embryo transfer (ET) Transferring embryos from one female to another.

Intra cytoplasmic sperm injection (ICSI) Microinjection of a single sperm into an oocyte to achieve fertilization.

In vitro fertilization (IVF) Fertilization of oocytes in a laboratory culture dish.

Semen extender A medium into which semen is diluted in order to increase inseminate volume and sperm lifespan.

Super Ovulation (SOV) Hormone induced development of many ovarian follicles.

Introduction

Assisted reproductive technologies (ART) that manipulate the use of mammalian sperm in order to control fertility have been developed to the greatest extent for practical use in livestock species. The same technologies developed for use in livestock, were adapted for aiding human fertility control, and to help conserve wild and endangered animal species. Artificial insemination (AI) is perhaps one of the most widely used male ART that has impacted nearly all levels of animal production for controlled gene transfer to specific breeds of animals, herds, and even specific regions or countries around the world (Foote, 2002). The outcome from sire selection and distribution of the semen from these genetically superior sires has increased the rate and extent of genetic change with the most notable effects in milk, meat and fiber production. The ability to collect and preserve semen from superior breeding males has become the gateway technology for all other male ART. This article will describe several related technologies, such as in vitro fertilization (IVF), super ovulation (SOV), embryo transfer (ET), intra-cytoplasmic sperm injection (ICSI) and sperm sexing, as to their stage of development and use towards their potential to impact fertility in the future.

Artificial Insemination

Methods of Semen Collection

The earliest documented reports for semen collection for use in AI with domesticated mammalian species can be traced back to Russia in the 18th century using the dog. Subsequent efforts in the late 19th and early 20th century continued with dogs, rabbits, foxes, horses, cattle and sheep. Many of the significant advances in the development of AI occurred throughout the remainder of the 20th century in livestock species around the globe. Today, the majority of AI is performed to enhance the efficiency of food production in livestock. However, AI is also used in companion animal and exotic animal breeding, although to a much lesser extent.

For use with AI, semen collection is the initial step, and can be accomplished using an artificial vagina (AV), digital manipulation (the gloved hand technique), or with an electroejaculator. In the majority of species, semen collection is quick, with ejaculation lasting seconds and up to a minute. However, with pigs and dogs, ejaculation can take several minutes. The use of the AV and gloved hand technique rely on the male becoming sexually excited to achieve penile erection. This can be accomplished using an estrual female and allowing the male to naturally mate so as to help him learn to associate the collection area with sexual excitement and ejaculation. The male can be trained to mount a collection dummy or phantom that mimics the height and width of a female. Estrual females can also be used to collect semen, but may need to be restrained so the male can mount and semen can be collected using the AV or gloved hand. However, a male trained to a collection dummy is much more practical and efficient. Training the male to become excited in the collection pen, to mount the dummy and achieve an erection is accomplished by association. One male can be stimulated by watching another trained male mount the dummy while in an observation pen. The male can also be sexually stimulated by the smells and secretions of males previously in the pen and on the collection dummy.

Stallions, bulls, bucks, rams, boars, as well as the males of many other species such as cats and dogs, respond well to the use of the AV, while the digital or gloved hand technique is most commonly used for the boar and dog (Hopkins and Evans, 2003). The dimensions of the AV need to be suited to species length and width of the penis for collection. The AV must function to mimic the temperature, pressure and tactile stimulation of the vagina to induce ejaculation. One end of the AV is open for insertion of the penis, and the other end tapers into a collection tube. The AV is filled with water to the required temperature and pressure. Funnel shaped rubber liners and disposable plastic liners are used to attach the AV to the semen collection tube. The innermost disposable liner helps prevent disease transfer among males, and limits bacterial contamination of the ejaculate. For commercial swine semen

production, semi-automated semen collection systems have been developed and placed into large commercial boar studs. These machines are based on the dummy and AV collection system, but with automated pressure and collection that do not require the gloved hand digital pressure for the 7–10 min of ejaculation. Electroejaculation of semen is also effective in many animal species, with different sized devices appropriate for animals ranging in size from cats to cattle. Other species such as the horse and pig do not tolerate the electroejaculation procedure well and therefore it is seldom used. For performing the procedure, an electrical probe is inserted into the rectum and a technician applies a series of low level electrical pulses to stimulate erection and then ejaculation. This method does not use a collection dummy, but is often used in combination with an AV to collect semen under proper temperature and hygienic conditions.

Methods of Sperm Preservation

Efficient distribution and utilization of sperm from genetically superior sires is the key to improved animal breeding for performance and production efficiency. To accomplish this, dilution and preservation of sperm following ejaculation is essential for use with ART. Hundreds of millions to billions of sperm are ejaculated in small or large volumes of seminal plasma, specific to each species. Sperm are highly sensitive to changes in temperature and the chemical conditions while in liquid suspension. Since the viability of sperm declines over time in the undiluted state, efficient processing of the ejaculate is required. To preserve sperm fertility for storage, avoiding temperature fluctuation is critical at all stages. Rather than transient increases and decreases in temperature after collection, a gradual decline in temperature is desired until dilution in a thermally matched extender. The ejaculate contains highly concentrated sperm in seminal plasma and both must be diluted to avoid extensive sperm damage during processing and storage.

Evaluation of the ejaculate immediately following collection is an important step in successful sperm preservation. Ejaculates are evaluated for concentration and volume to determine total sperm numbers, while sub-samples are evaluated by qualitative or quantitative methods for determining sperm motility and extent of abnormalities. These and other assessments are used to quickly determine whether the ejaculate meets the minimum quality criteria for preservation. With quantitative ejaculate measures, the dilution rate can be adjusted for numbers of motile and normal sperm for efficient sire utilization. When the ejaculate meets the quality criteria, the semen is diluted to the proper concentration and volume for packaging.

Preservation of semen in frozen or liquid form involves packaging sperm into single doses for storage and for later use with AI or in vitro procedures. As a liquid, sperm are packaged into bottles, tubes or bags and refrigerated. When frozen, sperm are most often packaged into straws and stored in liquid nitrogen at -196°C . Preservation of sperm for storage as a liquid is most often used for boars and stallions using a 1 step dilution process and slow cooling to storage temperature of $5\text{--}18^{\circ}\text{C}$. Stallion ejaculates result in ~ 14 AI doses with 1 billion sperm/50 mL of diluent and boar ejaculates provide up to 30 AI doses with $\sim 2\text{--}3$ billion sperm/in 75 mL. Extended liquid semen can retain much of its fertility for 2–4 days before use. In cattle, semen is stored frozen with the ejaculate extended and allowed to slow cool to 5°C before dilution in the freezing solution. A bull ejaculate containing ~ 10 billion sperm can be extended to create 500 AI doses with 20 million sperm/0.5 mL straw. The semen that has been frozen can be stored in liquid nitrogen to retain its fertility for many years.

The semen diluents used for refrigerated sperm in liquid form vary considerably but generally are formulated to provide an energy source (glucose), a pH buffering agent, electrolytes (potassium and sodium chloride so that the diluent is iso-osmotic), and selected additional ingredients to enhance membrane stabilization, reduce oxidative damage, and prevent bacterial growth for different periods of time. Milk based extenders have been popular and widely used for sperm membrane stabilization. For storage in the frozen state, the diluents are similar but contain egg yolk in the cooling extender, and glycerol in the freezing solution. The egg yolk components provide protection for the sperm membrane while cooling, and the cryoprotectant serves to prevent ice crystal formation inside and outside the cell during freezing.

One other form of sperm processing, encapsulation, has been under investigation in dogs, cattle and swine for many years (Perteghella *et al.*, 2015). Suspensions of sperm are mixed to create alginate droplets, and mixed with ions to form a gel. The sperm remain viable in the droplet for days and when placed in-vivo or in-vitro in a physiological salt solution, the droplets slowly dissolve to release the sperm. There is limited information on the commercial use of this technology, but it holds great potential for application with advanced ART using sexed sperm and IVF.

Technologies for Artificial Insemination

The use of ART with intensive livestock production predominates in developed countries in Europe and North America where $\sim 80\%$ of the dairy cattle and 90% of the swine are bred using AI (Riesenbeck, 2011; Stevenson, 2014). While AI can be successful in other livestock species, it is used to a much lesser extent in horses, sheep, goats, deer, buffalo, dogs and cats (Morrell, 2011). In these species, limits to wider application arise due to uncertainty in genetic value and economic return, as well as reduced sperm fertility and difficulty in the technical aspects of AI. In cattle, frozen sperm is imported and exported from *bos taurus* and *bos indicus* dairy and beef bulls maintained in numerous semen collection centers around the world (Thibier and Wagner, 2002). However, AI in beef cattle is uncommon since much of the commercial production is extensive on open grazing lands and estrus detection or ovulation synchronization are not widespread practices (Foote, 2002).

For successful AI in many of the species described, deposition of semen into the uterine body by transcervical passage of a catheter is desired to achieve good fertility with low numbers of sperm in small volumes. Locating and passing the catheter

Table 1 Ejaculate parameters of males and most common methods used in artificial insemination programs

Male (species)	Ejaculate				Artificial Insemination				
	Collection	Aids	Volume (mL)	Sperm ($\times 10^9$)	Sperm/AI dose	Storage form	Insemination	Species use	Inseminations/Female
Bull (<i>Bos taurus</i>)	AV/EEJ	Phantom/ Female	6	7	$20 \times 10^6/0.5$ mL	Frozen	Uterus	80%	AI once in mid estrus
Boar (pig)	DM	Phantom/ Female	250	60	$2.5 \times 10^9/75$ mL	Chilled	Cervix/ Uterus	90%	AI on Days 1 and 2 of estrus
Stallion (horse)	AV/EEJ	Phantom/ Female	70	9	$500 \times 10^8/75$ mL	Chilled	Uterus	Low	1 AI every 48 h during estrus
Ram (sheep)	AV/EEJ	Female	1	3	$200 \times 10^6/0.5$ mL	Chilled/ Frozen	Uterus	Low	1 AI near end of estrus
Buck (goat)	AV/EEJ	Female	1	2	$200 \times 10^6/0.5$ mL	Chilled/ Frozen	Uterus	Low	1 AI near end of estrus
Buck (rabbit)	AV	Female	0.8	0.4	$5 \times 10^7/0.5$ mL	Chilled	Vagina	Low	1 AI after induced ovulation
Dog	DM/AV	Female	10	2	$200 \times 10^6/5$ mL	Fresh/Chilled	Vagina	Low	AI on Days 2 and 4 of estrus
Tom (cat)	AV/EEJ	Female	0.06	0.1	$20 \times 10^6/0.2$ mL	Fresh/Chilled	Vagina	Low	1 AI after induced ovulation

Abbreviations: AV, artificial vagina; EEJ, electroejaculator; DM, digital method (gloved hand).

through the cervix may require manipulation through the rectal wall in cattle and horses, or by locating the opening using a speculum and laparoscope such as with small ruminants. Other species such as the pig are most often inseminated intracervically with high fertility while the cat is most often inseminated vaginally due to the structure of the cervix. The AI conditions used in various species are shown in **Table 1**.

Superovulation, Embryo Collection and Transfer

Being able to collect embryos from valuable donors and transfer them to surrogates that carry them through gestation has allowed females to produce many more embryos and offspring than they would if they gestated. Some cows have produced over 200 embryos in their lifetime. Superovulation (SOV) and embryo transfer (ET) are most commonly used in cattle. Although ET was performed successfully as a surgical procedure in rabbits over 100 years ago, the current non-surgical techniques used for cattle were not developed until the 1970s and 1980s (Kuzan and Seidel, 1986). Today, ET is commonly used in cattle reproduction. The first step in embryo production is to inject donor females in diestrus with FSH. These injections rescue ovarian follicles that would otherwise undergo atresia, resulting in super-stimulation of follicle development. Following an injection of prostaglandin $F_{2\alpha}$ to induce luteolysis, multiple follicles develop and ovulate following estrus. Cows are usually inseminated once based on signs of estrus. About one week after AI, each uterine horn is flushed with medium to collect embryos. The donor embryos can be transferred to the uterine horn ipsilateral to the corpus luteum in a recipient female that is in the same stage of the estrous cycle as the embryo donor. Alternately, the embryos may be cryopreserved and transferred later after thawing. The ability to cryopreserve embryos allows owners of animals with high genetic merit to ship embryos around the world at a significant cost savings compared with shipping live animals. In cattle, the economic value of producing more offspring with high genetic merit makes the ET procedure profitable. However, in other species, ET is seldom used due to the uncertainty in the economic value and absence of practical methodology.

In addition to increasing the number of offspring produced by a single female, another advantage of ET is that the generation interval can be reduced to increase the rate of genetic selection by flushing embryos from females soon after they have reached puberty. In this scheme, the surrogates could deliver offspring from the transferred embryos before the embryo donor carries an embryo to term. Indeed, some very high genetic merit cows spend more of their lifetimes producing embryos than carrying them.

In-Vitro Fertilization

In vitro fertilization (IVF) in most mammals, with the exception of humans, is primarily a research procedure. It has been used to develop a clearer understanding of the principles of fertilization and what factors influence its outcome. In cattle, IVF is becoming a more common commercial practice to produce embryos from cows that do not respond well to SOV. In addition, it can be used to produce embryos of a specific gender using sex chromosome-sorted sperm. Despite these opportunities, IVF is still not commonly performed. In other species, such as the horse, IVF yields poor fertilization rates for reasons that remain unclear.

Successful IVF requires that both gametes undergo final maturation. Oocytes can be collected from animals in an abattoir or from live animals by aspiration of oocytes via laparotomy guided by ultrasound. Oocytes can even be collected from young females prior to puberty. Oocyte maturation is usually accomplished by in vitro culture in medium supplemented with hormones such

as FSH and LH or the growth factor EGF. Fully mature oocytes have an expanded cumulus oophorus, have resumed meiotic maturation and progressed to metaphase II, and have developed the ability to decondense the sperm nucleus (Fig. 1).

Sperm must also complete a final maturation, known as capacitation. The requirements for capacitation vary to some degree between species. For example, mouse and porcine sperm are usually capacitated by incubation in a simple salt solution including calcium with bicarbonate and albumin. In contrast, bovine sperm capacitate most effectively when the glycosaminoglycan heparin is added to the medium and glucose is absent (Parrish, 2014). The reasons for the differences between species are not clear. Regardless of medium composition, a certain minimum incubation time is necessary for capacitation. The duration of capacitation also varies between species. Mouse sperm capacitate in an hour whereas it takes 4–5 h for bovine sperm to complete capacitation. The next step in IVF is to add capacitated sperm to mature oocytes to allow fertilization. Zygotes are then transferred to fresh medium and allowed to develop to the morula or blastocyst stage, a stage appropriate for transfer to the uterus of recipient females or for cryopreservation (Fig. 1).

Separating Sperm by Sex Chromosomes

The sex chromosome of the fertilizing sperm determines the sex of the offspring. The ability to separate live sperm based on the presence of an X or Y chromosome to produce nearly all males or females can be accomplished based on the difference in total DNA. The X chromosome is larger than the Y chromosome, yielding a difference in total DNA of about 3–4% in several species whose sperm have been sorted, including cattle, swine and sheep (Gamer, 2006). Following staining with a fluorescent DNA-binding dye, this difference can be used to identify and separate X and Y sperm using a flow cytometer. The process is limited by the speed at which sperm can be sorted, currently about 20 million per hour. But despite this obstacle, bovine sex-sorted semen has been available commercially in the cattle industry for about 10 years. It is used mostly by dairy farmers because of the higher value of female offspring compared to male offspring. The same process can be applied to sperm from other agricultural species but there are a couple of limitations. In swine, conventional artificial insemination requires billions of sperm, compared to the 2–4 million sex-sorted cattle sperm that are typically inseminated. And the difference in the value of females and males in most other agricultural animals is much lower, providing less profit incentive at the production level, to sex-sort sperm.

Intracytoplasmic Sperm Injection

Typically, successful IVF requires thousands of sperm in the droplet volume with oocytes to obtain satisfactory fertilization results whereas AI requires millions to billions more. In contrast, using a micromanipulator, one can select a single sperm and inject it into the oocyte cytoplasm to obtain fertilized eggs using ICSI (Galli *et al.*, 2014). Oocytes may be aspirated from ovaries at slaughter or by ultrasound guided ovum pickup via standing laparotomy. If oocytes were from small follicles, they must be matured in vitro. If they were aspirated from large follicles, they will require less time for maturation after collection. After maturation, the cumulus cells will be removed using hyaluronidase. Maturation can be confirmed by assessing extrusion of the first polar body. Fresh or cryopreserved sperm are usually used for ICSI but even freeze-dried sperm can be used. Using a micromanipulator, a large diameter holding pipette (120–140 μm outer diameter) is used to hold the oocyte while another small diameter pipette (7–8 μm outer diameter) is used to aspirate a sperm following immobilization. Usually a hole is made in the zona pellucida using a Piezo drill so that the sperm injection pipette can be passed through the zona to reach the oocyte plasma membrane. The injection pipette is then advanced through the oocyte plasma membrane and the sperm is ejected into the oocyte cytoplasm without aspirating any

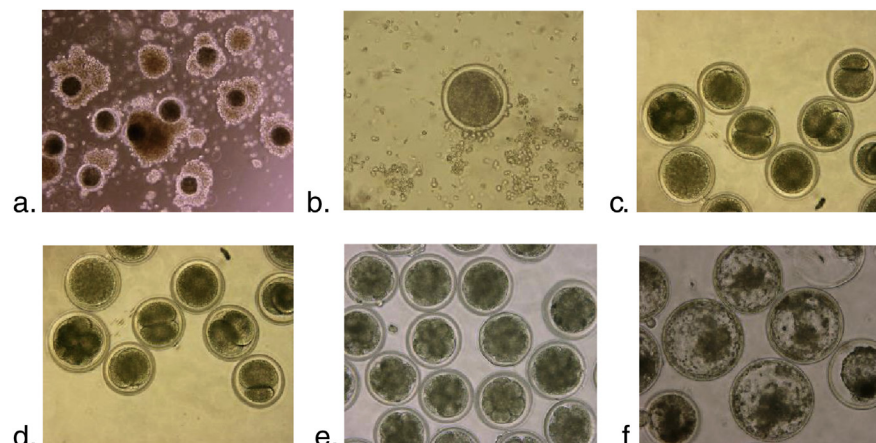


Fig. 1 Microscopic images of (a) unfertilized eggs; (b) cumulus-free egg and sperm for in-vitro fertilization; (c) fertilized egg with male and female pronuclei; (d) unfertilized eggs and 2–4 cell embryos; (e) 8 cell embryos; (f) blastocyst stage embryos ready for transfer into recipients. Photos (a), (b) and (d)–(f) are courtesy of Wheeler, M.B., Rubessa, M., University of Illinois.

cytoplasm. The resulting zygotes are cultured in droplets to assess cleavage and development and then either transferred or cryopreserved. A major advantage of ICSI is that few sperm are needed. Abnormal sperm and even epididymal or testicular sperm can be used for ICSI or sperm that have been freeze-dried and are dead. Although this technique is used routinely in humans, success rates in some species such as cattle are much lower. Yet in horses, ICSI is used more commonly because of the very poor fertilization and development rates with IVF (Rader *et al.*, 2016). One interesting application of ICSI is to inject sperm coated with vector DNA to produce transgenic animals.

Sperm Selection Techniques

Although millions to billions of sperm are deposited in the female reproductive tract with natural mating or when using AI, only a very small fraction of the sperm are found in the oviduct, the site of fertilization. The genital tract removes the majority of sperm by retrograde flow, phagocytosis or retention in the lower tract. The mechanisms by which sperm are selected to advance to the oviduct are unclear. In the laboratory, there are several techniques employed to select elite sperm and improve fertility with ART but it is not clear how well any mimic the natural selection that occurs in the genital tract or even if this natural selection actually selects the very elite sperm (Sakkas *et al.*, 2015). For most ART procedures, one needs only a small number of cells, so the goal is to select the sperm that will give the greatest success. Only a couple of techniques are used routinely to select sperm for ART procedures, such as IVF. The sperm swim-up technique is a sperm motility challenge. To be selected, sperm must swim from the semen layer into a layer of medium overlaid above the semen. Motile sperm are selected but the yield is low. Another common technique is to separate sperm based on density by centrifugation through Percoll or other coated silica. The density gradients that are used may have continuous or discontinuous densities. The yield of sperm after density gradient centrifugation is higher than swim-up but correspondingly there may be less stringent selection. The procedures for swim-up and Percoll density gradient centrifugation for sperm selection are very similar across species.

There are several other sperm selection techniques that are not used frequently for animals, such as hyaluronic acid binding, DNA/chromatin integrity separation, and removal of apoptotic cells by Annexin V binding (Rappa *et al.*, 2016; Sakkas *et al.*, 2015). Annexin V binds to sperm that have phosphatidylserine exposed on the sperm exterior, indicating that membrane integrity is compromised. In normal non-capacitated sperm, phosphatidylserine is located on the inner leaflet of the sperm plasma membrane and inaccessible to Annexin V. Coupling these selection approaches with flow cytometry to separate high and low fertility sperm can be done on a small scale appropriate for most ART procedures.

In addition to flow cytometry, physical separation of sperm that bind Annexin V has also been accomplished using magnetic activated cell sorting (MACS). Annexin V coupled to colloidal supramagnetic beads binds dead and apoptotic sperm and when placed into a magnetic field, bound sperm can be removed. MACS separation can even be combined with density gradient centrifugation for even greater selection enhancement. Microfluidic devices have been developed to sort sperm in small channels based upon motility and morphology. The flow of medium through these channels is used to flush out immotile sperm. These procedures have been limited mostly to animals in a research setting and the procedures have not been standardized for routine use in clinics or in sperm selection for genetics company sires. Furthermore, these selection procedures only yield relatively small numbers of sperm; too few for routine use with AI.

The fertility of live normal sperm in a semen sample with a large number of dead cells could be compensated by simply inseminating more sperm, which is why the number of dead cells is considered a compensable trait. But it is possible that the reactive oxygen species produced by a large number of dead or abnormal sperm may compromise the fertility of the normal sperm. Timely removal of the damaged sperm prior to AI could improve fertility. There is research interest in the use of nanoparticles for removing defective sperm in scaled-up systems that would yield an adequate number of sperm for AI doses. Further, nanoparticles conjugated with lectins or Annexin V may remove acrosome-reacted or apoptotic sperm from large semen volumes. Using nanoparticle conjugates to remove poor sperm might improve the fertility of males that produce sperm that do not survive cryopreservation or are affected significantly by thermal stress.

References

- Foot, R. H. (2002). The history of artificial insemination: Selected notes and notables. *Journal of Animal Science*, 80, 1–10.
- Galli, C., Duchi, R., Colleoni, S., Lagutina, I., & Lazzari, G. (2014). Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: From the research laboratory to clinical practice. *Theriogenology*, 81, 138–151.
- Garner, D. L. (2006). Flow cytometric sexing of mammalian sperm. *Theriogenology*, 65, 943–957.
- Hopkins, S. M., & Evans, L. E. (2003). Artificial insemination. In M. H. Pineda, & M. P. Dooley (Eds.), *McDonald's Veterinary Endocrinology and Reproduction* (pp. 341–376). Ames, IA: Blackwell Publishing.
- Kuzan, F. B., & Seidel, G. E., Jr. (1986). Embryo transfer in animals. *Developmental Biology (New York 1985)*, 4, 249–278.
- Morrell, J. M. (2011). Artificial insemination: Current and future trends. In M. Manafi (Ed.), *Artificial Insemination in Farm Animals*. Rijeka: InTech (Ch. 01).
- Parrish, J. J. (2014). Bovine in vitro fertilization: In vitro oocyte maturation and sperm capacitation with heparin. *Theriogenology*, 81, 67–73.
- Perteghella, S., Viganì, B., Crivelli, B., et al. (2015). Sperm encapsulation from 1985 to date: Technology evolution and new challenges in swine reproduction. *Reproduction in Domestic Animals*, 50, 98–102.
- Rader, K., Choi, Y. H., & Hinrichs, K. (2016). Intracytoplasmic sperm injection, embryo culture, and transfer of in vitro-produced blastocysts. *Veterinary Clinics of North America: Equine Practice*, 32, 401–413.
- Rappa, K. L., Rodríguez, H. F., Hakkarainen, G. C., et al. (2016). Sperm processing for advanced reproductive technologies: Where are we today? *Biotechnology Advances*, 34, 578–587.

- Riesenbeck, A. (2011). Review on international trade with boar semen. *Reproduction in Domestic Animals*, 46, 1–3.
- Sakkas, D., Ramalingam, M., Garrido, N., & Barratt, C. L. (2015). Sperm selection in natural conception: What can we learn from mother nature to improve assisted reproduction outcomes? *Human Reproduction Update*, 21, 711–726.
- Stevenson, J. S. (2014). Impact of reproductive technologies on dairy food production in the dairy industry. In G. C. Lamb, & N. DiLorenzo (Eds.), *Current and Future Reproductive Technologies and World Food Production* (pp. 115–129). New York, NY: Springer.
- Thibier, M., & Wagner, H. G. (2002). World statistics for artificial insemination in cattle. *Livestock Production Science*, 74, 203–212.

Male Contraception in Animal Species

Marc A Driancourt, Astek Consult, Chateaufort/Sarthe, France

Linda Rhodes, Alliance for Contraception in Cats and dogs (ACC&D), Portland, OR, United States

© 2018 Elsevier Inc. All rights reserved.

Strategies to Interfere With the Function of the Hypothalamo-Pituitary-Testicular Axis in Males

The purpose of contraception in males is to prevent endocrine (production of testosterone that supports sperm production and generates sexual behavior) and exocrine (sperm production) production by the testis.

As sperm production is under the control of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), as well as testosterone (itself LH dependent), efficient contraceptive strategies have attempted to act at the top of the hypothalamo-pituitary axis to prevent synthesis and release of LH and FSH. When FSH and LH concentrations reach low levels, testosterone production in the testes is significantly reduced. The low gonadotropin and testosterone concentrations do not support sperm production, and when testosterone concentrations are low, male sexual behavior is inhibited. Therefore three main approaches to suppress gonadotropins have been tested: (1) sustained administration of GnRH agonists (e.g., deslorelin or azagly nafarelin) that trigger down regulation of the GnRH receptors and desensitization of pituitary gonadotropes, (2) active immunization against GnRH resulting in antibody binding of GnRH in portal blood before it reaches the pituitary and (3) the use of GnRH antagonists that bind to the GnRH receptors (therefore preventing binding of native GnRH) blocking their activation and therefore suppressing LH, FSH and testosterone.

The first two options, for which extensive data are available, will be discussed throughout this review, with emphasis being placed on products that have been achieved regulatory approval, and therefore have extensive safety and efficacy data available (Fig. 1).

In addition, in recent years, a strategy whereby sterilization is achieved by intra-testicular injections of compounds (Kutzler, 2015) that destroy the seminiferous tubules has been proposed and shown to work in pets and cattle. This option will be briefly discussed in the last section of this review.

Why Suppress Male Reproductive Activity: Which Needs in Specific Species?

There are several reasons (summarized in Table 1) for asking for a contraceptive strategy for males. The most obvious indication for requesting contraception (if the animals are not simply surgically castrated) is stopping the males from mating and producing unwanted progeny. This indication applies to all companion animal species (particularly dogs and cats) and is usually life-long. For this sub-population, the main goal is high efficacy and the global lack of treatment associated safety issues. In animals where surgical castration is contra-indicated because the animal may be used for breeding some time in the future, or because anesthesia may be risky, a non-surgical alternative may be preferred.

A similar need for lifelong contraceptive/sterilant strategies is triggered by the large population of stray dogs and cats living in some highly populated environments where their interactions with the human population may not be safe, where they may

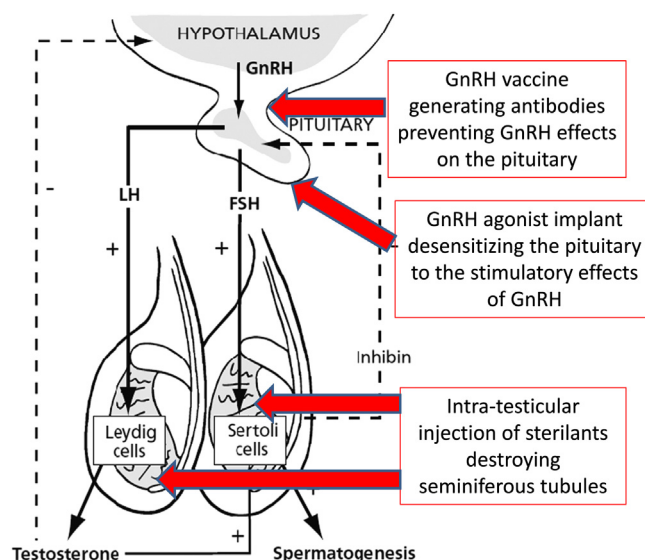


Fig. 1 The 3 main strategies used for achieving contraception in males.

Table 1 Possible reasons for requesting a contraceptive treatment in males for a range of species and associated technical solutions

<i>Indication</i>	<i>Species</i>	<i>Main needs</i>	<i>Treatment regime</i>	<i>Preferred treatment</i>
Fertility control	Pets (cats & dogs)	High and long efficacy, very good safety	Yearly implant injection by vet	GnRH implant
Behavioural control	Pets (cats, dogs and stallions)	Obvious long lasting efficacy & very good safety	Implant placement by vet & owner's monitoring	GnRH implant
Testosterone dependent pathologies	Pets (cats, dogs and stallions)	Very high efficacy	Repeated treatments and monitoring of effects by vet	GnRH implant or GnRH vaccine
Population control	Stray dogs and cats & wild deer, boars	Single, low cost treatment, easy to administer	Handling and monitoring by field workers	One shot GnRH vaccine or testicular sterilant
Zootechnical use	Boars & bulls	Failure proof unexpensive treatment, OK for consumers	Administration of vaccine (primary and booster) by farmers	GnRH vaccine

interfere with the local biodiversity and where they may carry diseases such as rabies. This need also applies to wildlife species living in zoos or reserves where the amount of land available only allows carrying a specific population size. For this sub-population, the main constraints are a long lasting efficacy, easy implementation and cost effective treatments.

Short/medium term contraception may also be useful in some farm animal species (swine, cattle) if surgical castration is perceived as unethical owing to the pain and discomfort generated by some surgical procedures.

In farm animals, testosterone production improves feed efficiency (i.e., the conversion ratio of food into muscle), but may have negative effects on meat quality in some species. Meat of boars (at least a fraction of them, ranging between 5% and 30% according to their genetics) slaughtered after puberty, displays “boar taint” an off flavor where the meat has a urine and faeces odor when cooked. This feature is obviously not wanted by consumers, and can be prevented by surgical castration. However, the same consumers may consider unethical the pain induced by piglet castration. Pharmaceutical suppression of testicular androgen production is therefore important in post-pubertal boars presented for slaughter. Furthermore, in all intact animals used for meat production, meat is less tender and leaner (i.e., less tasty and juicy). This is why, in many beef production systems worldwide, steers are treated with steroid implants during the fattening phase. A short term contraceptive treatment if available to the producers, could offer a combination of high feed efficiency during most of the growth phase of the animal, followed by a “no testosterone” phase caused by the contraceptive treatment (during the 2–4 weeks before slaughter) to ensure good meat quality. For this sub-population, the main constraint is consumer acceptance as well as a cost effective treatment.

Finally, treatments whereby production of testicular steroids is prevented may be significant treatment aids to stop testosterone dependent diseases (benign prostatic hyperplasia in dogs, equine arteritis virus infection in stallions). For this (small) sub-population, the main goal of such treatments is high efficacy and an acceptable risk/benefit ratio owing to acceptable adverse effects of treatment.

The Available Solution for Contraception in Male Dogs: GnRH Implants

There are several reasons why owners may seek contraception for their male dog. They include reluctance to get the dog surgically castrated for ethical (animal welfare) or anesthetic risk reasons, or the desire to breed this dog in the future. In addition, some owners may want to take time before making the decision to surgically castrate their pet and therefore need a temporary contraception before making a final decision. The GnRH agonist releasing implants act by causing desensitization of the pituitary, therefore reducing gonadotropin support to testicular function (endocrine and exocrine) during the time frame during which the GnRH agonist is released. Towards the end of the release period, testicular function and fertility may resume or a new treatment may be applied. Two GnRH agonist implants have been developed for this purpose. The first one (Suprelorin, commercially available in Europe and Oceania) uses deslorelin as GnRH agonist (with 4.7 and 9.4 mg of deslorelin to achieve 6 or 12 months of fertility suppression, respectively) in a biodegradable controlled release matrix. The second one (Gonazon, approved by EU regulatory authorities but not commercially available) uses azagly nafarelin, a different GnRH agonist, in a solid silicone based matrix.

Mode of Action

Following a brief (1–2 weeks) flare up period during which gonadotropin concentrations are increased, irrespective of the GnRH agonist used, gonadotropin concentrations drop to basal levels owing to the combined effects of down regulation of pituitary GnRH

receptors together with a drop in LH/FSH gene expression (review: Goericke Pesch (2017)). Consequently, testosterone concentrations also drop to undetectable values, 6–43 days following insertion of a deslorelin implant (Trigg *et al.*, 2006) and 17 ± 8 days following insertion of an azagly nafarelin implant (Ludwig *et al.*, 2009). The underlying mechanism causing azoospermia or aspermia is an arrest of spermatogenesis at the spermatogonia/spermatocyte level (Goericke Pesch, 2017).

Safety and Efficacy

The duration of fertility suppression of the deslorelin implants is variable, and although the minimum duration of efficacy has been shown to reach 180 days (4.7 mg implant) and 400 days (9.4 mg implant) (Trigg *et al.*, 2006), the median duration of efficacy reach 300–400 days (large and small dogs respectively), when using the 4.7 mg implant (Trigg *et al.*, 2006). The lack of endocrine support to the testis (basal LH and testosterone concentrations) triggers a drop in sperm quality (reduced semen volume, massive increase in tail abnormalities of the spermatozoa produced), together with a strong decrease in libido. By 30–35 days post implantation, semen production has ceased and libido is absent (Trigg *et al.*, 2006). Such a treatment appears to be very safe, as there was no inflammation or pain detected at the implantation site. In addition, repeated treatment of the same male dogs proved to be well tolerated, therefore offering an option to generate permanent efficacy (Trigg *et al.*, 2006). Duration of efficacy of the azagly nafarelin containing implant (Gonazon) ranged between 223 and over 365 days (Ludwig *et al.*, 2009).

Reversibility

All endocrine and exocrine effects of treatment were shown to be fully reversible following implant removal (Gonazon) or exhaustion of the deslorelin load (Suprelorin). Recrudescence of testicular steroidogenesis is rapid, as testosterone concentrations are back to pre-treatment values at 7–8 weeks post implant removal (Ludwig *et al.*, 2009). Testicular size and normal semen quality were back to normal values around 9 weeks after a 4.7 mg deslorelin implant has emptied (Trigg *et al.*, 2006) and 16–30 weeks after Gonazon removal (Ludwig *et al.*, 2009). As the dynamics of the post removal recovery process are very variable, no specific claim of reversibility time is made on the approved product.

The Available Solutions for Contraception in Tom Cats (GnRH Implants)

If a male cat is an indoor pet, an owner may request contraception to control behavioural conditions such as vocalization and urine marking. Having the option to breed once from their pet cat may sometimes be a wish. In such cases, treatment of the cat with a GnRH agonist containing implant (such as Suprelorin or Gonazon), may provide a suitable solution to such requests. For male cats living outdoors, the reasons for requesting contraception are different and relate to the owner's perception of the responsibility to keep feral cat populations under control and avoid fights and wounds generated by fights between breeding tom cats. Suprelorin in cats is not approved and therefore any use would be 'off label'.

Mode of Action

The mode of action of GnRH agonist releasing implants is essentially similar in dogs and cats. Hence the reader is referred to the dog GnRH implant section for information.

Safety and Efficacy

As in dogs, there is a brief flare up period following GnRH agonist implant insertion. Following application of a 4.7 mg deslorelin implant, the time to suppression of fertility ranged between 3 and 11 weeks (Goericke Pesch *et al.*, 2011). The reasons underlying this high variability are not fully understood. Lowest semen quality was usually reached around two months after treatment. However, a similar large variation in sperm features was also detected with some tom cats having no motile sperm and very low total sperm counts, while others display larger numbers of total and motile spermatozoa in their ejaculates. When testicles of treated tom cats were examined by histology, a large between male variability was again detected with spermatogenesis being arrested at variable levels including elongated spermatids. Overall, the mean duration of efficacy of a 4.7 mg deslorelin implant was 551 ± 90 days, again displaying a large individual variability 432–705 days) (Goericke Pesch *et al.*, 2011).

Reversibility

Testosterone concentrations start to recover within one month after removal of a deslorelin implant, as does testicular size. Total sperm counts are back to normal values 3 months post removal (Novotny *et al.*, 2012). After removal of an azagly-nafarelin implant, normal fertility was regained and following mating, litter size was not different from that of untreated males (Goericke Pesch *et al.*, 2011).

The Available Non-Surgical Solutions for Interfering With Gonadal Function in Male Farm Animals (GnRH Vaccines)

Castration of young male farm animals (swine, cattle) is common as (1) it helps management of male animals, particularly when they are co-mingled with females, (2) it improves meat quality (by promoting marbling) and (3) it prevents meat from male pigs to display boar taint, a off-flavor of meat. However these benefits are at the expense of the improved feed efficiency (efficiency of conversion of feedstuffs into meat proteins) that is better in intact males. This is why several groups have attempted to develop GnRH vaccines that would allow high feed efficiency, while also promoting the benefits listed above.

The purpose of this strategy is production based (alternative to castration to optimize animal welfare and to optimize protein and fat accretion in meat).

Mode of Action and Purpose

The vaccination schedule includes a primary injection and a booster injection, usually several weeks later. GnRH antibody titers rise following the booster injection, therefore preventing LH release hence blocking testicular function. More specifically, Leydig cells when deprived from LH support stop producing the steroids responsible of lean meat (testosterone) and boar taint (androstenone).

Safety and Efficacy

Scientists at CSL in Australia developed a GnRH vaccination strategy that proved very efficient in swine (Improvac vaccine: [Dunshea et al. \(2001\)](#)) as well as in cattle (Bopriva vaccine: [Amatayakul-Chantler et al., 2012](#)). In swine ([Dunshea et al., 2001](#)), this vaccination has multiple benefits: first, male piglets do not need to be castrated in the days following birth, a procedure raising ethical issues because it is commonly done without anesthesia. Second, in the time interval between the primary injection and the booster, boars display the high feed efficiency typical of intact males, as the primary immunization does not cause high levels of GnRH antibodies and testosterone is not suppressed. Third, following a booster injection, testicular function of GnRH immunized boars is significantly reduced, stopping the production of the testicular steroids (androstenone) responsible of boar taint. Finally, GnRH immunized boars, when achieving high GnRH titers, have an increased ability to accumulate fat in meat (marbling) generating more tender and more tasty meat. Boars need to be slaughtered no earlier than 4 weeks and no later than 8 weeks after the booster injection.

Similar results have been reported in bulls, although meat of intact bulls does not display any detrimental taste (as in boars) and is only less tender and less tasty ([Amatayakul-Chantler et al., 2012](#)). In both species, no safety issues secondary to vaccination have been observed. Local reactions at the injection site are minimal and risks for the consumers are minimized by the long time lag between the booster injection and slaughter.

The Available Contraceptive Solutions for Controlling Populations of Wildlife (Single Injection GnRH Vaccine)

There is a need for population control in wild animals where keeping a suitable balance between animal numbers, such as deer or wild horses, and the feeding capacity of their environment and minimizing issues with agricultural damage, negative conservation impacts and risks for public safety are needed. The target strategy for this purpose is a single injection treatment given when the animals are captured in traps, or given by darting that induces very long term infertility. Another important constraint for this use is that treatments must be cost effective.

Mode of Action and Purpose

The joint need for a cost effective single injection treatment ensuring long term efficacy suggested that a potent GnRH vaccine might be the best candidate. Gonacon, a GnRH vaccine developed by the National Wildlife Research Center (NWRC) of the United States Department of Agriculture (USDA) which combines GnRH conjugated to a carrier protein (keyhole limpet hemocyanin or “blue protein”, another marine mollusk hemocyanin) in a strong adjuvant (AdjuVac) containing *Mycobacterium avium* proved to deliver consistent results in several populations of wild animals and was approved by the US Environmental Protection Agency (EPA) as an option to control populations of deer and wild horses.

Safety and Efficacy

While most of the data documenting success of this approach have been generated in females, which are the obvious sex to be targeted for population control, there are some lines of evidence showing efficacy in males as well. For example, [Gionfriddo et al. \(2012\)](#) demonstrated that Gonacon vaccinated male deer displayed a titer dependent suppression of gonadal function with reduced testicular sizes and antler development.

The Available Non Hormonal Solutions for Controlling Populations of Stray Tom Cats and Male Dogs (Intra-Testicular Injection of Chemical Solutions)

The aim of this approach is population control. In some cities and environmental niches, population of stray dogs and cats may proliferate if food supply is available and if there are no predators. The idea of inducing long term sterility by intra-testicular compounds triggering prolonged azoospermia (or teratospermia) has been around for several decades.

As a cost effective and not technically difficult to achieve and potentially generating permanent contraception, such an approach may be attractive for controlling the populations of stray dogs and tom cats.

There are two solutions that have been evaluated for intra-testicular sterilants in male animals, calcium chloride, and zinc gluconate (also known as Neutersol, Esterisol and Zeuterin).

Mode of Action

The intratesticular injection of these solutions targets the seminiferous tubules which are selectively destroyed. The Leydig cell population may be affected to some extent, but testosterone concentrations, although somewhat reduced, are not consistently reduced to castrate levels with this strategy.

Safety and Efficacy

The physiological effects of two sterilants (Calcium chloride dehydrate and zinc gluconate buffered with arginine) have been described. In a pioneering study, [Jana and Samanta \(2011\)](#) tested the effects of intra-testicular injection of 0.25 mL of 5%, 10% and 20% calcium chloride solution (with 1% lidocaine) and showed that the 10% solution induced necrosis of the seminiferous tubules as well as of the interstitial tissue. In a second study using zinc gluconate (13.1 mg zinc/mL), [Oliveira et al. \(2013\)](#) have established that intratesticular injection (0.3 mL) to male cats (using a similar injection strategy as above) induced testicular atrophy and a drop in the presence of testosterone-dependent penile spines. Histological analysis of testes of treated cats showed a lack or very few germ cells within the seminiferous tubules, with clusters of Leydig cells remaining at some places.

Intra-testicular calcium chloride injection is usually done by inserting a 27 gauge needle into one pole of the testis and pushing it towards the other pole to ensure a homogeneous spread of the sterilant through testicular tissue. Such a treatment does not appear to be painful and mild discomfort is only observed during the minutes following injection. Testicular size usually increases following injection with the peak size reached within 2–4 days later. This early rise in testicular size is followed by a decrease over the next 2–4 weeks ([Kutzler, 2015](#)). Based on the damage done to the intra-testicular structures, it is assumed that the treated animals are sterile after a 4–6 weeks delay (linked to the exhaustion of the sperm reserves present in the epididymis). However, there is no solid clinical information documenting the ability to mate and fertility of treated tom cats in the long term.

The zinc solution approved by US FDA for use in male dogs 3–10 months of age (Zeuterin, Ark Sciences, previously known as Neutersol) has been extensively tested in these young dogs to document its safety and efficacy. A very low occurrence of injection site reactions has been reported in male dogs and cats (review: [Kutzler \(2015\)](#)). Apart from a transient testicular swelling on the day post injection, no testicular pain or tenderness were detected. On day 60 post injection, 91% of the tom cats were azoospermic and most of them displayed a reduction in testosterone dependent penile spines, suggesting a reduction in circulating testosterone concentrations. On day 120 post treatment, only 73% of the tom cats were azoospermic while the others displayed asthenospermia or teratospermia. Whether testicular function may remain fully suppressed over extended durations of time is yet to be demonstrated.

The Available Solutions for Treating Testosterone Dependent Diseases (GnRH Vaccine or GnRH Implants)

In pets (dogs and cats as well as horses) the expression of sexual behavior, a feature partly related to testicular testosterone production, may make animals unsuitable pets. In addition, in horses it may strongly interfere with performance during training and competition. In addition, in aging intact male dogs, benign prostatic hyperplasia, a testosterone dependent pathology, is common. Finally, in breeding stallions, the presence of the equine arteritis virus (AEV) in the accessory sex glands and in the ejaculate carries the risk of spreading the disease at each mating. In this case, viral persistence is known to be testosterone dependent and reduction of testosterone concentrations for a long enough period appears to be a suitable approach to “clean” AEV shedder stallions.

Purpose and Mode of Action

For these indications (behavior, benign prostatic hyperplasia and EAV treatment), a contraceptive treatment depressing testosterone concentrations may be useful. This may involve using GnRH vaccination, or a GnRH agonist implant. In EAV shedder stallions, the

effects of treatment should be reversible to allow them recovering a normal endocrine and exocrine testicular function, and breeding, if desired.

Efficacy and Safety

There are no studies that clearly demonstrate the link between the suppression of testosterone to the change in behavior in dogs, and more data are needed. Reduction of testosterone in male dogs, either by castration or one of the methods listed above, causes reduction in the size of the prostate gland. In AEV shedder stallions, [Burger et al. \(2006\)](#) demonstrated the efficacy of GnRH immunization to reduce testosterone concentrations and remove all EAV viral contamination from the male genital tract. This study did not report any issues with treatment's safety.

Conclusions

Three main strategies (GnRH vaccination, GnRH agonist implantation and injection of intra-testicular sterilants) have been shown to be useful for contraception in males. These options are the end result of years of active research aiming to provide solutions to the diversity of issues associated with raising/keeping intact males. In spite of significant research, only a few of these options have achieved regulatory approval in some markets, although they may be used off label for specific clinical needs. Many other treatments options (such as immunization against the LH receptor, or selective destruction of the pituitary gonadotropin secreting cells by GnRH-toxin conjugates) have also been studied without demonstrating that they reach the level of efficacy or duration to be practical for clinical use. Novel scientific breakthroughs may help to revive interest in these strategies. The area where active research remains mostly needed is the development of a life-long contraceptive treatment (perhaps by adeno associated virus mediated gene therapy) that could become an alternative to surgical castration of pets and, if accepted by the consumers, also in farm animals.

References

- Amatayakul-Chantler, S., King, V., Rubio, L. M. S., et al. (2012). Immunocastration of Bos indicus X Brown Swiss bulls in feedlot with gonadotropin-releasing vaccine Bopriva provides improved performance and meat quality. *Journal of Animal Science*, 90, 3718–3728.
- Burger, D., Janett, F., Vidament, M., et al. (2006). Immunization against GnRH in stallions: Effects on semen characteristics, behavior and shedding of equine arteritis virus. *Animal Reproduction Science*, 94, 107–111.
- Dunshea, F. R., Colantoni, C., Howard, K., et al. (2001). Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increases growth performance. *Journal of Animal Science*, 79, 2524–2535.
- Gionfriddo, J. P., Denicola, A. J., Miller, L. A., & Fagerstone, K. A. (2012). Efficacy of GnRH immunocontraception of white tailed deer in New Jersey. *Wildlife Society Bulletin*, 35, 142–148.
- Goericke Pesch, S. (2017). Long term effects of GnRH agonists on fertility and behavior. *Reproduction in Domestic Animals*, 52, 336–347.
- Goericke Pesch, S., Georgiev, P., Antonov, P., Albouy, M., & Wehrend, A. (2011). Clinical efficacy of a GnRH agonist implant containing 4.7 mg deslorelin, Suprelorin, regarding suppression of reproductive function in tomcats. *Theriogenology*, 75, 803–810.
- Jana, K., & Samanta, P. K. (2011). Clinical evaluation of non surgical sterilization of male cats with single intra-testicular injection of calcium chloride. *BMC Veterinary Research*, 7, 39.
- Kutzler, M. A. (2015). Intratesticular and intra-epididymal injections to sterilize male cats. *Journal of Feline Medicine and Surgery*, 17, 772–776.
- Ludwig, C., Desmoulins, P. O., Driancourt, M. A., Goericke Pesch, S., & Hoffmann, B. (2009). Reversible down regulation of endocrine and germinative testicular function in the dog with the GnRH agonist azagly-nafarelin as a removable implant "Gonazon". *Theriogenology*, 71, 1037–1045.
- Novotny, R., Cizek, P., Vitasek, R., et al. (2012). Reversible suppression of sexual activity in tomcats with deslorelin implant. *Theriogenology*, 78, 848–857.
- Oliveira, E. C., Fagundes, A. K., Melo, C. C., et al. (2013). Intratesticular injection of a zinc based solution for contraception of domestic cats: A randomized clinical trial of efficacy and safety. *Veterinary Journal*, 197, 307–310.
- Trigg, T. E., Doyle, A. G., Wash, J. D., & Swangchan-uthai, T. (2006). A review of advances in the use of the GnRH agonist deslorelin in control of reproduction. *Theriogenology*, 66, 1507–1512.

Testis Toxicants

Ming Yan, Baiping Mao, Linxi Li, and Stephen YT Li, The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, NY, United States

Chris KC Wong, Hong Kong Baptist University, Hong Kong, China

Bruno Silvestrini, SBM Pharmaceuticals S.r.l., Rome, Italy

C Yan Cheng, The Mary M. Wohlford Laboratory for Male Contraceptive Research, Center for Biomedical Research, Population Council, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Spermatogenesis is composed of a series of complex cellular events that take place in the seminiferous tubule – the functional unit of the testis that produces spermatozoa. These cellular events include: (i) self-renewal of spermatogonial stem cells (SSCs) and undifferentiated spermatogonia, (ii) meiosis I/II to generate haploid spermatids, (iii) postmeiotic spermatid development through spermiogenesis, and the release of mature spermatids (i.e., spermatozoa) at spermiation (Ehmcke and Schlatt, 2006; Schlatt and Ehmcke, 2014; Sharpe, 1994; O'Donnell, 2014; O'Donnell et al., 2006; de Kretser and Kerr, 1988). Furthermore, many of these cellular events including (ii)–(iv) take place in a specialized microenvironment known as the adluminal (apical) compartment of the seminiferous epithelium (Fig. 1). In short, cellular events pertinent to self-renewal of SSCs and undifferentiated spermatogonia, and the subsequent differentiation of type B spermatogonia to preleptotene spermatogonia take place in the basal compartment of the epithelium. But all cellular events pertinent to (ii)–(iv) that take place in the mammalian testis are behind the blood-testis barrier (BTB) (Fig. 1). Thus, cellular events pertinent to meiosis, spermiogenesis and spermiation are protected by the BTB – one of the tightest blood-tissue barriers, which also divides the epithelium of the seminiferous tubule into the basal and the adluminal (apical) compartments (Cheng and Mruk, 2012; Franca et al., 2012; Stanton, 2016; Pelletier, 2011). It is of interest to note that seminiferous tubules in the testis also maintain a daily production of ~70 million of sperm in an adult male rat versus ~300 million of sperm in an adult man (Johnson et al., 1980, 1983, 1984; Amann and Howards, 1980) through spermatogenesis. Thus, there is an enormous cellular output that takes place in the testis throughout the entire adulthood of any male. In order to protect spermatogenesis, besides the BTB, the testis is equipped with an array of drug transporters, either expressed by Sertoli or germ cells (or both) to avoid the passage of harmful substances and xenobiotics into the adluminal compartment to perturb spermatogenesis (Qian et al., 2013; Mruk and Cheng, 2012; Mruk et al., 2011; Setchell, 2008; Su et al., 2011). Despite the unique morphological settings in the testis to protect spermatogenesis, the testis, however, is highly sensitive to toxicants as noted in earlier studies, such as when rodents were exposed to cadmium or other toxicants more than six decades ago (Parizek and Zahor, 1956; Parizek, 1960; Kanakis and Goullis, 2015; Boekelheide et al., 1989, 2003, 2005). Since then, studies reporting the disruptive effects of toxicants, including heavy metals, plasticizers, and surfactants on male reproductive health including humans, such as reducing sperm counts, perturbing human sperm chromatin integrity, and causing idiopathic infertility in men in particular the epigenetic effects of male infertility have grown exponentially (Nordkap et al., 2012; Delbes et al., 2010; Cheng et al., 2011; Guerrero-Bosagna and Skinner, 2014). Herein, we focus our discussion on toxicants which induce disruption of endocrine systems in both humans and rodents known as EDCs (endocrine disrupting chemicals), by focusing on their effects in the testis in vivo or Sertoli cells cultured in vitro (Table 1). In brief, EDCs are agents that can interfere with the synthesis, secretion, metabolism, binding, and action of naturally occurring hormones in the body that are used for the maintenance of reproduction, development and/or behavior. As such, numerous environmental toxicants which are common EDCs that are known to cause testis injuries can be broadly categorized into three types, namely heavy metals (e.g., cadmium, mercury, lead), plasticizers (e.g., bisphenol A (BPA), phthalates), and surfactants (e.g., perfluorooctanesulfonate (PFOS), PFOA (perfluorooctanoic acid)) (Table 1). Since the subject of testis toxicants has been widely reviewed (O'Donnell, 2014; Boekelheide et al., 2000, 2003, 2012; Wan et al., 2013; Siu et al., 2009a; Wong et al., 2010; Johnson, 2014; Boekelheide, 2005), instead of providing a redundant review of the subject, we elect to provide a timely review based on recent studies regarding the likely common molecular targets of several EDCs. This information, in our opinion, is of interest not just to research scientists, but possibly clinicians since these recent studies have illustrated some potential candidates through which the toxicant-induced testis injury can possibly be therapeutically managed or treated, thereby providing a means to interfere toxicant-induced Sertoli cell injury. While we may not be able to reverse the toxicant-induced permanent damage to the testis, such as the BTB disrupted by an acute dose of cadmium, but proper preventive measures can be developed to help industrial workers to prevent testis injuries or manage toxicant-induced reproductive dysfunction. This is the goal of this review.

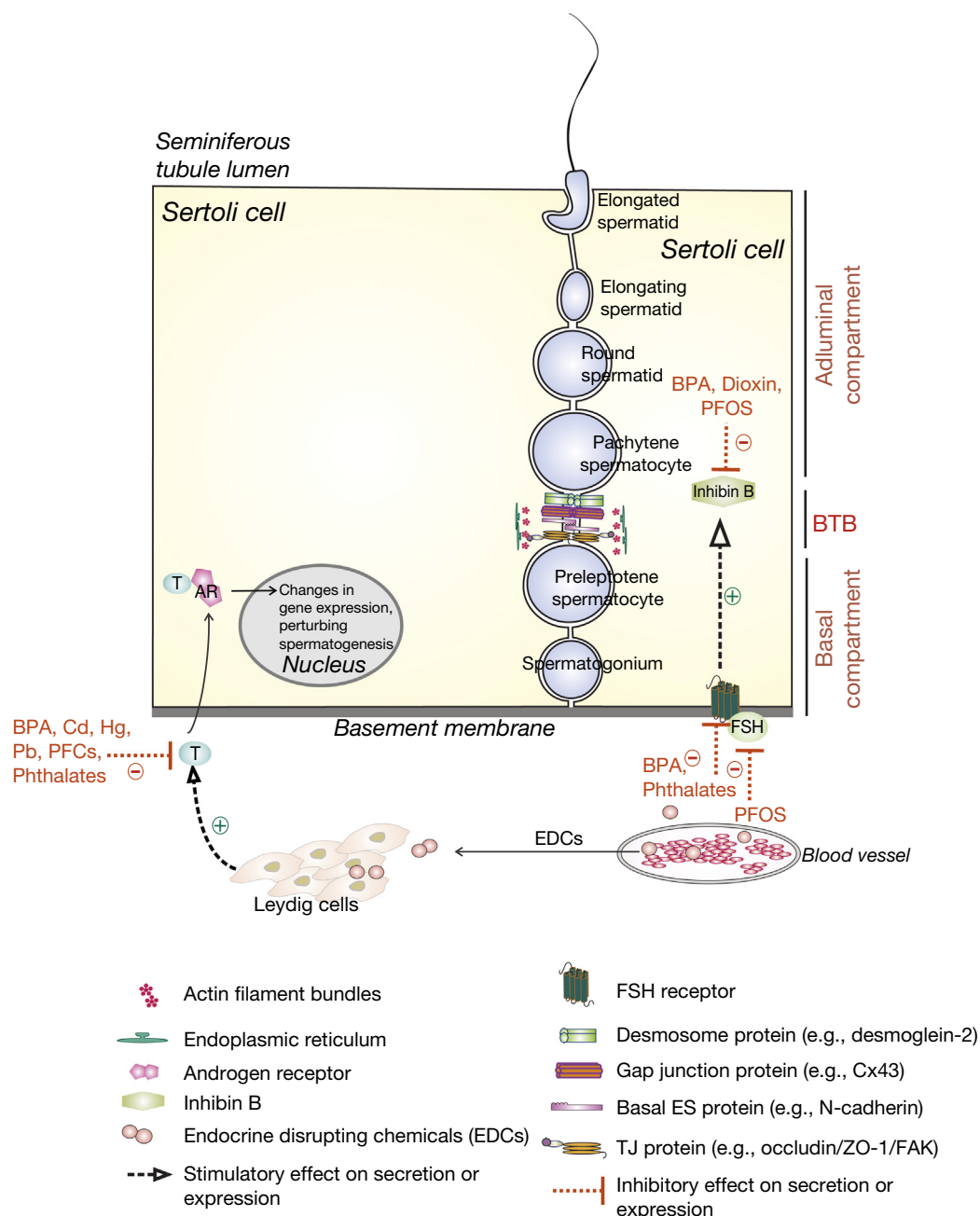


Fig. 1 A schematic drawing that illustrates the morphological features of the seminiferous epithelium from an adult rat testis. The blood-testis barrier (BTB) comprised of actin-based tight junction, basal ES and gap junction, as well as intermediate filament-based desmosome, which divides the seminiferous epithelium into the basal and the adluminal (apical). Endocrine disrupting chemicals (EDCs) enter the testis through the microvessels, some of which perturb Leydig cell steroidogenesis, perturbing gene expression through a disruption of androgen receptor (AR) function. Other EDCs perturb Sertoli cell function by modulating FSH receptor activity, perturbing spermatogenesis.

Cytoskeletons Are the Target of Environmental Toxicants

Studies using various toxicants, such as 2,5-hexanedione, carbendazim, colchicine, cadmium (e.g., CdCl_2), PFOS, BPA, and adjuvant (which is being investigated as a reversible male contraceptive and it has no considerable effects on either serum FSH, LH, testosterone or inhibin B in the treated animals Cheng et al., 2005; Mruk and Cheng, 2008; Mruk et al., 2008) have shown that Sertoli cell cytoskeletons including the actin- and/or microtubule (MT)-based cytoskeletons are the primary target of multiple toxicants (Boekelheide et al., 1989, 2003; Johnson, 2014; Boekelheide, 1993; Cheng, 2014; Siu et al., 2009b; Li et al., 2016a; Wan et al., 2014a; Gao et al., 2017) (Table 1). For instance, it has been reported that colchicine and carbendazim that induce germ cell sloughing is mediated primarily by Sertoli cell microtubule disruption (Boekelheide et al., 1989; Allard et al., 1993; Nakai and Hess, 1994;

Table 1 Disruptive effects of environmental toxicants or drugs on adult rodent testes in vivo or Sertoli cells cultured in vitro

Toxicant or drug	Phenotypes	Treatment dose(s)	Species	References
PFOS	Sertoli cell injury due to (i) mislocalization of Arp3 and palladin, (ii) disruptive organization of F-actin- and MT-based cytoskeletons; (iii) an up-regulation of p-rpS6 coupled with a down regulation of p-Akt1-S473 and p-Akt2-S474 that led to Sertoli cell TJ-permeability disruption	In vitro: 20–50 μ M	Rat/primary SC cultures	Gao et al. (2017)
	Loss of BTB integrity due to (i) p38/ATF2 activation, (ii) down-regulation of MMP9, occludin, and Cx43	In vivo: 0.5–10 mg/kg/b.w.	Mouse	Qiu et al. (2016)
	Sertoli cell injury due to down-regulation of the GJ protein Cx43	In vitro: 20 μ M	Rat/primary SC cultures	Li et al. (2016a)
	Sertoli cell TJ-barrier disruption due to: (i) disruptive F-actin organization as a result of truncation of actin filaments; (ii) disruptive inter-Sertoli cell gap junction (GJ) communication as a result of down-regulating of C43	In vitro: 10–20 μ M	Rat/primary SC cultures	Wan et al. (2014b)
BPA	An increase in Sertoli cell autophagy activity and cell apoptosis; an increase in Bax:Bcl-2 ratio	In vitro: 100–600 μ M	Goat/primary SC cultures	Zhang et al. (2017)
	BTB injury due to a down-regulation of occludin, ZO-1, β -catenin, AR and TGF- β 2	In vitro: 20 μ M	Human/ primary SC cultures	de Freitas et al. (2016)
	Sertoli cell apoptosis; down-regulation of Bcl-2; up-regulation of CaM and caspase-3 expression; increase in CaMKII phosphorylation	In vitro: 20 μ M	TM4 cell line	Qian et al. (2014)
Cadmium	Sertoli cell injury, BEX4 up-regulation	In vivo: 3.5 mg/kg	TM4 cell line	Yu et al. (2017)
	Reduced Sertoli cell migration with impaired cell viability, a loss in actin and tubulin expression and a reduced phosphorylation status of the MTs and actin microfilaments; down-regulation of F-actin cleavage protein cofilin and MT polymerization inhibitory protein stathmin	In vitro: 1 and 12 μ M	TM4 cell line	Egbowon et al. (2016)
	BTB injury, likely mediated by: (i) an up-regulation in extracellular matrix regulatory protein dermatopontin expression, and (ii) a down-regulation in claudin-11 expression	In vivo: 3.5 mg/kg b.w.	Mouse	Yang et al. (2014)
Adjudin	Premature release of elongating/elongated spermatids, followed by round spermatids and spermatocytes mediated by a down-regulation of Eps8, formin 1, EB1, and MARK4; and an up-regulation of Arp3	In vivo: 50 mg/kg/ b.w.	Rat	Li et al. (2017b)
	BTB injury due to a down-regulation of Cx43, which impeded meiosis and spermiogenesis; overexpression of Cx43 rescued the adjudin-induced BTB injury, resealing the BTB and re-initiating spermatogenesis	In vivo: 125 mg/kg/b.w.	Rat	Li et al. (2016b)
Triptolide	Sertoli cell injury, likely due to a down-regulation of β -actin, Rho GTPase (e.g., RhoA, RhoB, Cdc42 and Rac1) expression	In vivo: 100 μ g/kg/ b.w.	Rat	Wang et al. (2016)
	Testis injury due to a failure in intracellular transport of fatty acids and a down-regulation of PPARs	In vivo: 60 μ g/kg/ b.w.	Mouse	Ma et al. (2015)

Abbreviations used: *Arp3*, actin-related protein 3; *ATF2*, activating transcription factor 2; *Bcl-2*, B-cell lymphoma 2; *BEX4*, brain expressed X-linked 4; *BTB*, blood-testis barrier; *CaM*, calmodulin; *CaMKII*, calcium/calmodulin dependent kinase II; *Cdc42*, Cell division cycle 42.

Lim and Miller, 1997; Nakai et al., 1992). Carbendazim exerts its disruptive effects by blocking tubulin polymerization (Johnson, 2014; Winder et al., 2001). As such, MTs no longer are capable of undergoing the dynamic restructuring to confer germ cell adhesion and other crucial endocytic vesicle-mediated protein trafficking events (e.g., protein endocytosis and recycling), as well as the transport of spermatids and organelles (e.g., residual bodies, phagosomes) across the epithelium to support spermatogenesis, thereby causing defects in spermatogenesis and infertility in rodents. In this context, it is of interest to note that this disruptive effect is stage-specific, and spermatids that are embedded deep inside the seminiferous epithelium, such as in stages I–V, are less susceptible to carbendazim-induced loss (Nakai et al., 2002). Subsequent studies using the adjudin model have shown that this is likely due to the gross disruption of the track-like structures conferred by either MTs and/or F-actin across the seminiferous epithelium, thereby depleting the ability of the Sertoli cells to support spermatid transport across the epithelium so that spermatids can be released to the tubule lumen similar to those spermatids residing near the tubule lumen (Tang et al., 2016). This possibility is supported by the observation that the anchoring device between Sertoli cells and elongated spermatids known as the apical ES has been grossly disrupted by adjudin for spermatids resided near the tubule lumen, but also for spermatids embedded deep inside the epithelium (Tang et al., 2016). Furthermore, treatment of rats with glycerol following intratesticular injection (Wiebe and Barr, 1984; Wiebe et al., 1989) was also found to cause gross disruption of microtubules and actin microfilaments (Wiebe et al., 2000). Similarly, treatment of rats with CdCl₂ was also found to induce defragmentation of the actin microfilaments and actin-based TJ fibrils (Hew et al., 1993), illustrating the cytoskeleton is a target of many toxicants. In order to better define the molecular mechanism(s) underlying toxicant-induced cytoskeletal disruption, we had used Sertoli cells cultured in vitro as a study model to examine the biology of toxicant-induced cytoskeleton dysfunction including studies in both rat (Li et al., 2016a; Wan et al., 2014a; Gao et al., 2017) and human Sertoli cells (Xiao et al., 2014). Based on these studies, it is increasingly clear that the toxicant-induced cytoskeletal dysfunction in Sertoli cells utilizes similar mechanistic pathways and signaling molecules both in vitro and in vivo. These findings, if adequately expanded to cover additional toxicants, should be helpful for future studies designed to interfere with the toxicant mediated Sertoli cell injury by blocking or activating specific signaling pathways and/or signaling molecule(s).

Toxicants-Induced Sertoli Cell Injury Through Cytoskeletal Disruption Is Mediated by Changes in the Spatial Expression of Actin- and MT-Binding and Regulatory Proteins

When Sertoli cells are cultured in vitro for ~2–3 days, distinctive actin filaments and also microtubules (MTs) are clearly visible which stretch across the entire cell cytosol (Gao et al., 2017) (Table 1). These track-like ultrastructures are essential to support Sertoli cell shape and provide structural support for cellular transport of endocytic vesicles, phagosomes, and other organelles to maintain cellular homeostasis. However, following exposure of these cells to PFOS for 24 h, F-actin was truncated, failing to stretch across the entire cell cytosol (Gao et al., 2017). Interestingly, it was noted that these changes were the result of disruptive spatial expression of the actin bundling protein palladin and also branched actin nucleation protein Arp3 (Gao et al., 2017). In this context, it is of interest to note that palladin confers actin microfilaments to a bundled configuration to support Sertoli cell adhesion, such as at the Sertoli cell blood-testis barrier (BTB). On the other hand, actin-related protein 3, which together with the Arp2, create the Arp2/3 complex known to induce branched actin polymerization when activated by N-WASP (neuronal Wiskott-Aldrich syndrome protein), effectively converting a linear actin filament to a branched configuration, thereby making actin filament bundles to become a branched network. Thus, the combined action of palladin and the Arp2/3 complex would provide the plasticity to the actin filaments, necessary to changes Sertoli cell shape in response to the stage of the epithelial cycle to support spermatogenesis. However, treatment of Sertoli cells to PFOS for 24 h led to disruptive changes in the spatial expression of both Arp3 and palladin but also p-FAK-Tyr407 which is known to promote Sertoli cell BTB function through its effects to maintain actin-cytoskeletal function (Lie et al., 2012). As the actin-based cytoskeleton was disrupted by PFOS due to unwanted changes in the spatial expression of these actin regulatory proteins, the Sertoli cell TJ-permeability barrier function was also disrupted (Wan et al., 2014a). Since virtually all the adhesion protein complexes at the Sertoli cell BTB, such as the TJ (e.g., CAR (coxsackievirus and adenovirus receptor)/ZO-1 (zonula occludens-1)) and basal ES (N-cadherin/ β -catenin) adhesion protein complexes utilize actin for their attachment, the disorganization of actin filaments in Sertoli cells thus led to gross mis-localization of CAR, ZO-1, N-cadherin and β -catenin at the Sertoli cell cortical zone (Gao et al., 2017). These observations are also consistent with results of an earlier report in which treatment of PFOS for 24 h at 20 μ M would induce mis-localization of TJ and basal ES proteins at the Sertoli cell-cell interface (Wan et al., 2014a). While these findings are of interest, it is not known if similar changes could be observed in humans. Interestingly, studies using human Sertoli cells cultured in vitro (Chui et al., 2011) indeed have shown that treatment of human Sertoli cells with either CdCl₂ (5 or 20 μ M) or BPA (0.4, 40 or 200 μ M) also impedes the organization of F-actin, causing truncation of actin microfilaments (Xiao et al., 2014). This, in turn, perturbs the localization ZO-1 (a TJ adaptor protein), and N-cadherin and β -catenin (a basal ES adhesion protein complex) at the Sertoli cell cortical zone, making them diffusively localized at the BTB site (Xiao et al., 2014). More important, these changes are likely contributed by the disruptive spatial expression of Arp3 and Eps8 (epidermal growth factor receptor pathway substrate 8, an actin barbed end capping and bundling protein, conferring actin filaments to a bundled configuration (Ahmed et al., 2010; Cheng and Mruk, 2011)) and also c-Src (Xiao et al., 2014) (note: c-Src and its closely related member c-Yes within the Src family kinases (SFK) were earlier shown to be involved in modulating F-actin organization in Sertoli cells (Xiao et al., 2011, 2013)). For instance, following exposure of human Sertoli cells cultured in vitro to BPA (200 μ M) or CdCl₂ (20 μ M) for 24 h, Arp3, Eps8 and c-Src no longer evenly distributed across the human Sertoli cell cytosol and the cell cortical zone, instead, they were found to be retracted and moved closer to the cell nuclei, thereby failing to support the F-actin network across the cell to maintain adhesion protein complexes at the cell-cell interface (Xiao et al., 2014).

Role of p-FAK-Tyr407 in Toxicant-Induced Sertoli Cell Injury

FAK and its phosphorylated forms (such as p-FAK-Tyr397 and p-FAK-Tyr576) were initially shown to be involved to spermatid adhesion in early 2000s when these proteins were found to be intensively localized at the apical ES in stage VII-late VIII tubules (Siu et al., 2003, 2005; Beardsley et al., 2006). These findings were later expanded, developing into a novel concept that p-FAK-Tyr397 and/or p-FAK-Tyr576 may be working in concert with the Sertoli cell-specific adhesion protein $\alpha 6 \beta 1$ -integrin to support apical ES function, serving as a novel signal protein that contributes to the release of sperm at spermiation (O'Donnell et al., 2011; O'Donnell, 2014; Cheng and Mruk, 2015). Subsequent studies have shown that another FAK isoform, p-FAK-Tyr407, is playing a crucial role to support Sertoli cell adhesion function at the BTB as well (Lie et al., 2012). Using different phosphomimetic mutants versus nonphosphorylatable mutants of p-FAK-Tyr397 and p-FAK-Tyr407, it was shown that p-FAK-Tyr397 and p-FAK-Tyr407 supports apical ES and basal ES/BTB function, respectively (Lie et al., 2012). For instance, overexpression of the phosphomimetic mutant of p-FAK-Tyr407, namely p-FAK-Y407E mutant (in which Tyr-407 was mutated to Glu-407, making it a constitutively active mutant) or a constitutively inactive mutant of p-FAK-Tyr397, namely p-FAK-Y397F mutant (in which Tyr-397 was mutated to Phe-397, making it a constitutively inactive mutant) in Sertoli cells, they would both promote the Sertoli cell TJ-permeability barrier function (Lie et al., 2012). Furthermore, the promoting effect of p-FAK-Y407E mutant to the Sertoli cell TJ-barrier function is mediated by increasing the Sertoli cell actin nucleation function (Lie et al., 2012). These findings are important since they illustrate p-FAK-Tyr407 exerts its effects by making the Sertoli TJ-barrier "tighter" is mediated through changes in the organization of F-actin across the Sertoli cells. Subsequent studies by using an endogenous produced miRNA, called miR-135, which is known to knockdown FAK specifically in rats (Linsen et al., 2010; Landgraf et al., 2007), was capable of perturbing F-actin organization in Sertoli cells, potentiating the disruptive effects of PFOS (20 μ M) (Wan et al., 2014a). More important, it was also shown that treatment of PFOS also down-regulated the expression of connexin 43 (Cx43, a Sertoli cell integral membrane protein that constitutes the gap junction (GJ) in the rat testis) considerably (Wan et al., 2014a). Additionally, this considerable down-regulation of Sertoli cell Cx43 expression also perturbed the Sertoli intercellular GJ communications based on a fluorescence recovery after photobleaching (FRAP) functional assay (Wan et al., 2014a). Subsequent studies have shown that overexpression of Cx43 in Sertoli cells can rescue the PFOS-induced Sertoli cell GJ dysfunction by re-establishing the GJ communication between Sertoli cells (Li et al., 2016a). Furthermore, overexpression of Cx43 in Sertoli cells also prevent the PFOS-induced F-actin dis-organization (Li et al., 2016a). Taking collectively, these data thus illustrate the PFOS-induced Sertoli cell injury is mediated through a disruption of Sertoli cell GJ-based communication function, involving p-FAK-Tyr407, which in turn perturbs the F-actin organization in Sertoli cells.

Role of mTORC1-p-rpS6-p-Akt1/2 Signaling Pathway in Toxicant-Induced Sertoli Cell Injury

mTOR (mammalian target of rapamycin), a well conserved Ser/Thr protein kinase across different species including flies, worms and mammals, is an ubiquitous signaling molecule found in virtually all mammalian cells including Sertoli and germ cells in the testis (Shimobayashi and Hall, 2014; Laplante and Sabatini, 2012). It supports a wide range of cellular functions and pathological conditions including energy metabolism, protein synthesis, cell growth, cell survival, differentiation, cytoskeletal dynamics, maintenance of pluripotency of stem cells, cell signaling, tumorigenesis, and others (Bockaert and Marin, 2015; Stransky et al., 2016; Goodman, 2014; Ito and Suda, 2014; Mok et al., 2013). When mTOR binds to adaptor protein Raptor (regulatory-associated protein of mTOR) or Rictor (rapamycin-insensitive companion of mTOR), it creates the mTORC1 (mammalian target of rapamycin complex 1, i.e., mTOR+Raptor) or mTORC2 (mammalian target of rapamycin complex 2, i.e., mTOR+Rictor), respectively. Studies have shown that mTORC1 exerts its regulatory effects downstream through rpS6 (ribosomal protein S6) and Akt1/2 (Borrie et al., 2017) by affecting Sertoli cell TJ-barrier function, making the immunological barrier "leaky" (Mok et al., 2012, 2014, 2015) (Fig. 2). For instance, treatment of Sertoli cells cultured in vitro with rapamycin or a knockdown of rpS6 by RNAi was found to promote the Sertoli cell barrier, making its "tighter" (Mok et al., 2012), illustrating its physiological function during the epithelial cycle promotes BTB disruption, making it "leaky". Indeed, overexpression of rpS6 or a quadruple phosphomimetic mutant, by mutating S235, S236, S240 and S244 to E235, E236, S240 and S244 by site directed mutagenesis to create a constitutively active form of rpS6, in Sertoli cells was found to induce disruption of the Sertoli cell TJ-barrier (Mok et al., 2014, 2015). More important, these changes were also associated with an extensive disruption on the organization of the Sertoli F-actin network in which actin microfilaments across the Sertoli cells were grossly truncated (Mok et al., 2015). Since these studies were based on the use of Sertoli cells in vitro, the physiological relevancy of this information to the testis in vivo was not established. Interestingly, a recent study by overexpressing rpS6 wild type (WT, i.e., full-length rpS6 cDNA) versus p-rpS6-mutant (i.e., the quadruple constitutively active mutant of rpS6) and empty vector (i.e., the mammalian expression vector pCI-neo alone) in the testis in vivo was found to induce damage to the BTB in vivo (Li et al., 2017a). Additionally, considerably more severe damaged was detected following overexpression of p-rpS6 mutant versus rpS6-WT based on the use of a functional assay to assess the BTB integrity and when defects of spermatogenesis in the testis were assessed by histological analysis (Li et al., 2017a). In this context, it is of interest to note that earlier studies using Sertoli cells cultured in vitro have shown that mTORC1 exerts its disruptive effects on the Sertoli cell TJ-barrier function is mediated by an up-regulation rpS6, which is associated with a considerable down-regulation of Akt1/2 expression downstream (Mok et al., 2014, 2015), illustrating Akt1/2 is part of the mTORC1/rpS6 signaling pathway (Fig. 2). Interestingly, exposure of Sertoli cells cultured in vitro to PFOS at 50 μ M indeed caused a considerable down-regulation of Akt1/2, associated with consideration dis-organization of F-actin and also MT across the Sertoli cell cytosol (Gao et al., 2017), implicating

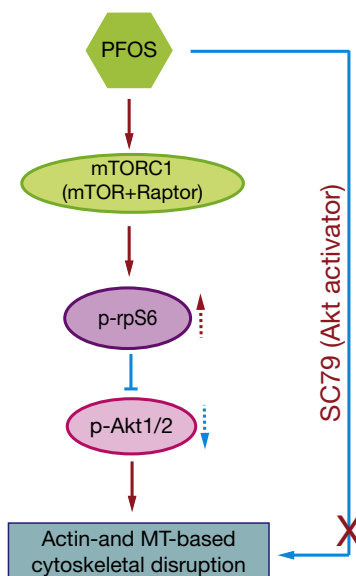


Fig. 2 A possible mechanism by which PFOS induces Sertoli cell injury through disruptive changes in Sertoli cell actin- and MT-based cytoskeletons. (see text for details).

Akt1/2 may be the signaling molecule involving in the PFOS-mediated Sertoli cell injury. When a Akt1/2 activator SC79 was included in the Sertoli cell cultures together with PFOS treatment, it was shown that SC79 was capable of blocking the PFOS-induced actin- and MT-based disruption (Gao et al., 2017) (Fig. 2). SC79 was also found to block the PFOS-induced changes in the disruptive spatial expression of p-FAK-Tyr407 (Gao et al., 2017). These findings are significant since they illustrate that the mTORC1-rpS6-pAkt1/2 and p-FAK-Tyr407 signaling proteins may be working in concert to modulate the organization of Sertoli cell actin- and MT-based cytoskeletons, but they are also one of the targets of toxicants. In short, these findings, if adequately expanded in future studies, may provide a novel approach to therapeutically manage toxicant-induced Sertoli cell and testis injury.

Concluding Remarks and Future Perspectives

Based on some recently published findings, we provide herein insightful information regarding the underlying molecular mechanism(s) in particular the cellular targets and signaling proteins that are involved in toxicant-induced Sertoli cell and testis injury. The use of human Sertoli cells cultured *in vitro* as a study model is of considerable help to relate findings based on the use rodent testicular cells to humans. It is increasingly clear that the Sertoli cell actin- and MT-based cytoskeletons are the target of toxicants based on studies using PFOS, BPA and cadmium. We have also identified p-FAK-Tyr407 and mTOR-rpS6-Akt1/2 signaling proteins are possibly working in concert to modulate PFOS-induced testis and Sertoli cell injury, involving both actin- and MT-binding regulatory proteins. Many questions remain unanswered. For instance, what is the upstream molecule(s) that trigger or regulate p-FAK-Tyr407 and mTORC1/rpS6/Akt1/2 signaling proteins? How does an activation of p-FAK-Tyr407, mTORC1, rpS6 or Akt1/2 relate to changes in the organization of F-actin and/or MTs? Does this involve any small GTPases, such as Rho and Rac GTPases? It is expected that some of these questions will be answered in the upcoming years when more data are available in the field. It is also expected that some of the unexplained infertility caused by environmental toxicants could be therapeutically managed when additional information is available based on studies using the human Sertoli cell model.

Acknowledgments

This work was supported by grants from the National Institutes of Health (NICHD R01 HD056034 to C.Y.C.; U54 HD029990 Project 5 to C.Y.C.).

References

- Ahmed, S., Goh, W. I., & Bu, W. (2010). I-BAR domains, IRSp53 and filopodium formation. *Seminars in Cell & Developmental Biology*, 21, 350–356.
- Allard, E. K., Johnson, K. J., & Boekelheide, K. (1993). Colchicine disrupts the cytoskeleton of rat testis seminiferous epithelium in a stage-dependent manner. *Biology of Reproduction*, 48, 143–153.
- Amann, R. P., & Howards, S. S. (1980). Daily spermatozoal production and epididymal spermatozoal reserves of the human male. *The Journal of Urology*, 124, 211–215.
- Beardsley, A., Robertson, D. M., & O'Donnell, L. (2006). A complex containing $\alpha 6 \beta 1$ -integrin and phosphorylated focal adhesion kinase between Sertoli cells and elongated spermatids during spermatid release from the seminiferous epithelium. *The Journal of Endocrinology*, 190, 759–770.
- Bockaert, J., & Marin, P. (2015). mTOR in brain physiology and pathologies. *Physiological Reviews*, 95, 1157–1187.
- Boekelheide, K. (1993). Sertoli cell toxicants. In L. Russell, & M. Griswold (Eds.), *The sertoli cell* (pp. 551–575). Clearwater: Cache River Press.
- Boekelheide, K. (2005). Mechanisms of toxic damage to spermatogenesis. *Journal of the National Cancer Institute. Monographs*, 34, 6–8.
- Boekelheide, K., Neely, M. D., & Sioussat, T. M. (1989). The Sertoli cell cytoskeleton: A target for toxicant-induced germ cell loss. *Toxicology and Applied Pharmacology*, 101, 373–389.

- Boekelheide, K., Fleming, S., Johnson, K., Patel, S., & Schoenfeld, H. (2000). Role of Sertoli cells in injury-associated testicular germ cell apoptosis. *Proceedings of the Society for Experimental Biology and Medicine*, 225, 105–115.
- Boekelheide, K., Fleming, S. L., Allio, T., Embree-Ku, M. E., Hall, S. J., Johnson, K. J., et al. (2003). 2,5-Hexanedione-induced testicular injury. *Annual Review of Pharmacology and Toxicology*, 43, 125–147.
- Boekelheide, K., Johnson, K. J., & Richburg, J. H. (2005). Sertoli cell toxicants. In M. K. Skinner, & M. D. Griswold (Eds.), *Sertoli cell biology* (pp. 345–382). New York: Elsevier Science.
- Boekelheide, K., Blumberg, B., Chapin, R. E., Cote, I., Graziano, J. H., Janesick, A., et al. (2012). Predicting later-life outcomes of early-life exposures. *Environmental Health Perspectives*, 120, 1353–1361.
- Borrie, S. C., Brems, H., Legius, E., & Bagni, C. (2017). Cognitive dysfunctions in intellectual disabilities: The contributions of the Ras-MARK and PI3K-Akt-mTOR pathways. *Annual Review of Genomics and Human Genetics*, 18, 115–142.
- Cheng, C. Y. (2014). Toxicants target cell junctions in the testis—insights from the indazole-carboxylic acid model. *Spermatogenesis*, 4, e981485. <https://doi.org/10.4161/21565562.2014.981485>.
- Cheng, C. Y., & Mruk, D. D. (2011). Regulation of spermiogenesis, spermiation and blood-testis barrier dynamics: Novel insights from studies on Eps8 and Arp3. *The Biochemical Journal*, 435, 553–562.
- Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implication in male contraception. *Pharmacological Reviews*, 64, 16–64.
- Cheng, C. Y., & Mruk, D. D. (2015). Biochemistry of Sertoli cell/germ cell junctions, germ cell transport, and spermiation in the seminiferous epithelium. In M. D. Griswold (Ed.), *Sertoli cell biology* (2nd edn., pp. 333–383). Amsterdam: Elsevier. <https://doi.org/10.1016/B978-0-12-417047-6.00012.0>.
- Cheng, C. Y., Mruk, D. D., Silvestrini, B., Bonanomi, M., Wong, C. H., Siu, M. K. Y., et al. (2005). AF-2364 [1-(2,4-dichlorobenzyl)-1H-indazole-3-carboxylate] is a potential male contraceptive: a review of recent data. *Contraception*, 72, 251–261.
- Cheng, C. Y., Wong, E. W. P., Lie, P. P. Y., Li, M. W. M., Su, L., Siu, E. R., et al. (2011). Environmental toxicants and male reproductive function. *Spermatogenesis*, 1, 2–13.
- Chui, K., Trivedi, A., Cheng, C. Y., Cherubavaz, D. B., Dazin, P. F., Huynh, A. L. T., et al. (2011). Characterization and functionality of proliferative human Sertoli cells. *Cell Transplantation*, 20, 619–635.
- Delbes, G., Hales, B. F., & Robairer, B. (2010). Toxicants and human sperm chromatin integrity. *Molecular Human Reproduction*, 16, 14–22.
- Egbowon, B. F., Harris, W., Arnott, G., Mills, C. L., & Hargreaves, A. J. (2016). Sub-lethal concentrations of CdCl₂ disrupt cell migration and cytoskeletal proteins in cultured mouse TM4 Sertoli cells. *Toxicology In Vitro*, 32, 154–165. An international journal published in association with BIBRA.
- Ehmcke, J., & Schlatt, S. (2006). A revised model for spermatogonial expansion in man: lessons from non-human primates. *Reproduction*, 132, 673–680.
- Franca, L. R., Auharek, S. A., Hess, R. A., Dufour, J. M., & Hinton, B. T. (2012). Blood-tissue barriers: morphofunctional and immunological aspects of the blood-testis and blood-epididymal barriers. *Advances in Experimental Medicine and Biology*, 763, 237–259.
- de Freitas, A. T., Ribeiro, M. A., Pinho, C. F., Peixoto, A. R., Domeniconi, R. F., & Scarano, W. R. (2016). Regulatory and junctional proteins of the blood-testis barrier in human Sertoli cells are modified by monobutyl phthalate (MBP) and bisphenol A (BPA) exposure. *Toxicology In Vitro*, 34, 1–7. An international journal published in association with BIBRA.
- Gao, Y., Chen, H., Xiao, X., Lui, W. Y., Lee, W. M., Mruk, D. D., et al. (2017). Perfluorooctanesulfonate (PFOS)-induced Sertoli cell injury through a disruption of F-actin and microtubule organization is mediated by Akt1/2. *Scientific Reports*, 7, 1110.
- Goodman, C. A. (2014). The role of mTORC1 in regulating protein synthesis and skeletal muscle mass in response to various mechanical stimuli. *Reviews of Physiology, Biochemistry and Pharmacology*, 166, 43–95.
- Guerrero-Bosagna, C., & Skinner, M. K. (2014). Environmentally induced-epigenetic transgenerational inheritance of male infertility. *Current Opinion in Genetics & Development*, 26, 79–88.
- Hew, K. W., Heath, G. L., Jiwa, A. H., & Welsh, M. J. (1993). Cadmium *in vivo* causes disruption of tight junction-associated microfilaments in rat Sertoli cells. *Biology of Reproduction*, 49, 840–849.
- Ito, K., & Suda, T. (2014). Metabolic requirements for the maintenance of self-renewing. *Nature Reviews in Molecular and Cellular Biology*, 15, 243–256.
- Johnson, K. J. (2014). Testicular histopathology associated with disruption of the Sertoli cell cytoskeleton. *Spermatogenesis*, 4, e979106. <https://doi.org/10.4161/21565562.2014.979106>.
- Johnson, L., Petty, C. S., & Neaves, W. B. (1980). A comparative study of daily sperm production and testicular composition in humans and rats. *Biology of Reproduction*, 22, 1233–1243.
- Johnson, L., Petty, C. S., & Neaves, W. B. (1983). Further quantification of human spermatogenesis: germ cell loss during postprophase of meiosis and its relationship to daily sperm production. *Biology of Reproduction*, 29, 207–215.
- Johnson, L., Zane, R. S., Petty, C. S., & Neaves, W. B. (1984). Quantification of the human Sertoli cell population: its distribution, relation to germ cell numbers, and age-related decline. *Biology of Reproduction*, 31, 785–795.
- Kanakakis, G. A., & Goulis, D. G. (2015). Male contraception: a clinically-oriented review. *Hormones (Athens, Greece)*, 14, 598–614.
- de Kretser, D. M., & Kerr, J. B. (1988). The cytology of the testis. In E. Knobil, J. B. Neill, L. L. Ewing, G. S. Greenwald, C. L. Markert, & D. W. Pfaff (Eds.), *The physiology of reproduction* (vol. 1, pp. 837–932). New York: Raven Press.
- Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., et al. (2007). A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*, 29, 1401–1414.
- Laplante, M., & Sabatini, D. M. (2012). mTOR signaling in growth control and disease. *Cell*, 149, 274–293.
- Li, N., Mruk, D. D., Chen, H., Wong, C. K., Lee, W. M., & Cheng, C. Y. (2016a). Rescue of perfluorooctanesulfonate (PFOS)-mediated Sertoli cell injury by overexpression of gap junction protein connexin 43. *Scientific Reports*, 6, 29667.
- Li, N., Mruk, D. D., Mok, K. W., Li, M. W., Wong, C. K., Lee, W. M., et al. (2016b). Connexin 43 reboots meiosis and reseals blood-testis barrier following toxicant-mediated aspermatogenesis and barrier disruption. *The FASEB Journal*, 30, 1436–1452.
- Li, S. Y. T., Yan, M., Chen, H., Jesus, T. T., Lee, W. M., Xiao, X., et al. (2017a). mTORC1/rpS6 regulates blood-testis barrier (BTB) dynamics and spermatogenic function in the testis *in vivo*. *American Journal of Physiology. Endocrinology and Metabolism*. <https://doi.org/10.1152/ajpendo.00263.2017>.
- Li, L., Tang, E. I., Chen, H., Lian, Q., Ge, R., Silvestrini, B., et al. (2017b). Sperm release at spermiation is regulated by changes in the organization of actin- and microtubule-based cytoskeletons at the apical ectoplasmic specialization—A study using the adjuvant model. *Endocrinology*, 158, 4300–4316.
- Lie, P. P. Y., Mruk, D. D., Mok, K. W., Su, L., Lee, W. M., & Cheng, C. Y. (2012). Focal adhesion kinase-Tyr⁴⁰⁷ and -Tyr³⁹⁷ exhibit antagonistic effects on blood-testis barrier dynamics in the rat. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 12562–12567.
- Lim, J. P., & Miller, M. G. (1997). The role of the benomyl metabolite carbendazim in benomyl-induced testicular toxicity. *Toxicology and Applied Pharmacology*, 142, 401–410.
- Linsen, S. E., de Wit, E., de Bruijn, E., & Cuppen, E. (2010). Small RNA expression and strain specificity in the rat. *BMC Genomics*, 11, 249. <https://doi.org/10.1186/1471-2164-11-249>.
- Ma, B., Qi, H., Li, J., Xu, H., Chi, B., Zhu, J., et al. (2015). Triptolide disrupts fatty acids and peroxisome proliferator-activated receptor (PPAR) levels in male mice testes followed by testicular injury: a GC-MS based metabolomics study. *Toxicology*, 336, 84–95.
- Mok, K. W., Mruk, D. D., Silvestrini, B., & Cheng, C. Y. (2012). rpS6 regulates blood-testis barrier dynamics by affecting F-actin organization and protein recruitment. *Endocrinology*, 153, 5036–5048.
- Mok, K. W., Mruk, D. D., & Cheng, C. Y. (2013). Regulation of blood-testis barrier (BTB) dynamics during spermatogenesis via the “Yin” and “Yang” effects of mammalian target of rapamycin complex 1 (mTORC1) and mTORC2. *International Review of Cell and Molecular Biology*, 301, 291–358.
- Mok, K. W., Mruk, D. D., & Cheng, C. Y. (2014). rpS6 regulates blood-testis barrier dynamics through Akt-mediated effects on MMP-9. *Journal of Cell Science*, 127, 4870–4882.

- Mok, K. W., Chen, H., Lee, W. M., & Cheng, C. Y. (2015). rpS6 regulates blood-testis barrier dynamics through Arp3-mediated actin microfilament organization in rat Sertoli cells. An *in vitro* study. *Endocrinology*, 156, 1900–1913.
- Mruk, D. D., & Cheng, C. Y. (2008). Delivering non-hormonal contraceptives to men: Advances and obstacles. *Trends in Biotechnology*, 26, 90–99.
- Mruk, D. D., & Cheng, C. Y. (2012). Drug transporters in spermatogenesis: A re-evaluation of recent data on P-glycoprotein. *Spermatogenesis*, 2, 70–72.
- Mruk, D. D., Silvestrini, B., & Cheng, C. Y. (2008). Anchoring junctions as drug targets: Role in contraceptive development. *Pharmacological Reviews*, 60, 146–180.
- Mruk, D. D., Su, L., & Cheng, C. Y. (2011). Emerging role for drug transporters at the blood-testis barrier. *Trends in Pharmacological Sciences*, 32, 99–106.
- Nakai, M., & Hess, R. A. (1994). Morphological changes in the rat Sertoli cell induced by the microtubule poison carbendazim. *Tissue & Cell*, 26, 917–927.
- Nakai, M., Hess, R. A., Moore, B. J., Gutoff, R. F., Strader, L. F., & Linder, R. E. (1992). Acute and long-term effects of a single dose of the fungicide carbendazim (methyl 2-benzimidazole carbamate) on the male reproductive system in the rat. *Journal of Andrology*, 13, 507–518.
- Nakai, M., Miller, M. G., Carnes, K., & Hess, R. A. (2002). Stage-specific effects of the fungicide carbendazim on Sertoli cell microtubules in rat testis. *Tissue & Cell*, 34, 73–80.
- Nordkap, L., Joensen, U. N., Blomberg, J. M., & Jorgensen, N. (2012). Regional differences and temporal trends in male reproductive health disorders: Semen quality may be a sensitive marker of environmental exposures. *Molecular and Cellular Endocrinology*, 355, 221–230.
- O'Donnell, L. (2014). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis*, 4, e979623.
- O'Donnell, L., Meachem, S. J., Stanton, P. G., & McLachlan, R. I. (2006). Endocrine regulation of spermatogenesis. In J. D. Neill (Ed.), *Physiology of reproduction* (3rd edn., pp. 1017–1069). Amsterdam: Elsevier.
- O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation: The process of sperm release. *Spermatogenesis*, 1, 14–35.
- Parizek, J. (1960). Sterilization of the male by cadmium salts. *Journal of Reproduction and Fertility*, 1, 294–309.
- Parizek, J., & Zahor, Z. (1956). Effect of cadmium salts on testicular tissue. *Nature*, 177, 1036–1037.
- Pelletier, R. M. (2011). The blood-testis barrier: the junctional permeability, the proteins and the lipids. *Progress in Histochemistry and Cytochemistry*, 46, 49–127.
- Qian, X., Cheng, Y. H., Mruk, D. D., & Cheng, C. Y. (2013). Breast cancer resistance protein (Bcrp) and the testis—An unexpected turn of events. *Asian Journal of Andrology*, 15, 455–460.
- Qian, W., Zhu, J., Mao, C., Liu, J., Wang, Y., Wang, Q., et al. (2014). Involvement of CaM-CaMKII-ERK in bisphenol A-induced Sertoli cell apoptosis. *Toxicology*, 324, 27–34.
- Qiu, L., Qian, Y., Liu, Z., Wang, C., Qu, J., Wang, X., et al. (2016). Perfluorooctane sulfonate (PFOS) disrupts blood-testis barrier by down-regulating junction proteins via p38 MAPK/ATF2/MMP9 signaling pathway. *Toxicology*, 373, 1–12.
- Schlatt, S., & Ehmecke, J. (2014). Regulation of spermatogenesis: an evolutionary biologist's perspective. *Seminars in Cell & Developmental Biology*, 29, 2–16.
- Setchell, B. P. (2008). Blood-testis barrier, functional and transport proteins and spermatogenesis. *Advances in Experimental Medicine and Biology*, 636, 212–233.
- Sharpe, R. M. (1994). Regulation of spermatogenesis. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction* (pp. 1363–1434). New York: Raven Press.
- Shimobayashi, M., & Hall, M. N. (2014). Making new contacts: the mTOR network in metabolism and signalling crosstalk. *Nature Reviews. Molecular Cell Biology*, 15, 155–162.
- Siu, M. K. Y., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2003). Adhering junction dynamics in the testis are regulated by an interplay of β 1-integrin and focal adhesion complex (FAC)-associated proteins. *Endocrinology*, 144, 2141–2163.
- Siu, M. K. Y., Wong, C. H., Lee, W. M., & Cheng, C. Y. (2005). Sertoli-germ cell anchoring junction dynamics in the testis are regulated by an interplay of lipid and protein kinases. *The Journal of Biological Chemistry*, 280, 25029–25047.
- Siu, E. R., Mruk, D. D., Porto, C. S., & Cheng, C. Y. (2009a). Cadmium-induced testicular injury. *Toxicology and Applied Pharmacology*, 238, 240–249.
- Siu, E. R., Wong, E. W. P., Mruk, D. D., Sze, K. L., Porto, C. S., & Cheng, C. Y. (2009b). An occludin-focal adhesion kinase protein complex at the blood-testis barrier: A study using the cadmium model. *Endocrinology*, 150, 3336–3344.
- Stanton, P. G. (2016). Regulation of the blood-testis barrier. *Seminars in Cell & Developmental Biology*, 59, 166–173.
- Stransky, L., Cotter, K., & Forgacs, M. (2016). The function of V-ATPases in cancer. *Physiological Reviews*, 96, 1071–1091.
- Su, L., Mruk, D. D., & Cheng, C. Y. (2011). Drug transporters, the blood-testis barrier and spermatogenesis. *The Journal of Endocrinology*, 208, 207–223.
- Tang, E. I., Lee, W. M., & Cheng, C. Y. (2016). Coordination of actin- and microtubule-based cytoskeletons supports transport of spermatids and residual bodies/phagosomes during spermatogenesis in the rat testis. *Endocrinology*, 157, 1644–1659.
- Wan, H. T., Mruk, D. D., Wong, C. K. C., & Cheng, C. Y. (2013). Targeting testis-specific proteins to inhibit spermatogenesis—Lesson from endocrine disrupting chemicals. *Expert Opinion on Therapeutic Targets*, 17, 839–855.
- Wan, H. T., Mruk, D. D., Wong, C. K. C., & Cheng, C. Y. (2014a). Perfluorooctanesulfonate (PFOS) perturbs male rat Sertoli cell blood-testis barrier function by affecting F-actin organization via p-FAK-Tyr⁴⁰⁷—an *in vitro* study. *Endocrinology*, 155, 249–262.
- Wan, H. T., Mruk, D. D., Wong, C. K. C., & Cheng, C. Y. (2014b). Perfluorooctanesulfonate (PFOS) perturbs male rat Sertoli cell blood-testis barrier function by affecting F-actin organization via p-FAK-Tyr(407): An *in vitro* study. *Endocrinology*, 155, 249–262.
- Wang, X., Zhao, F., Lv, Z. M., Shi, W. Q., Zhang, L. Y., & Yan, M. (2016). Triptolide disrupts the actin-based Sertoli-germ cells adherens junctions by inhibiting Rho GTPases expression. *Toxicology and Applied Pharmacology*, 310, 32–40.
- Wiebe, J., & Barr, K. (1984). The control of male fertility by 1,2,3-trihydroxypropane (THP; glycerol): Rapid arrest of spermatogenesis without altering libido, accessory organs, gonadal steroidogenesis, and serum testosterone, LH, and FSH. *Contraception*, 29, 291–302.
- Wiebe, J., Barr, K., & Buckingham, K. (1989). Sustained azoospermia in squirrel monkey, *Saimiri sciureus*, resulting from a single intratesticular glycerol injection. *Contraception*, 39, 447–457.
- Wiebe, J., Kowalik, A., Gallardi, R., Egeler, O., & Clubb, B. (2000). Glycerol disrupts tight junction-associated actin microfilaments, occludin, and microtubules in Sertoli cells. *Journal of Andrology*, 21, 625–635.
- Winder, B. S., Strandgaard, C. S., & Miller, M. G. (2001). The role of GTP binding and microtubule-associated proteins in the inhibition of microtubule assembly by carbendazim. *Toxicological Sciences*, 59, 138–146.
- Wong, E. W. P., Yan, H. H. N., Li, M. W. M., Lie, P. P. Y., Mruk, D. D., & Cheng, C. Y. (2010). Cell junctions in the testis as targets for toxicants. In P. B. Hoyer, J. H. Richburg (Series Eds.) & C. A. McQueen (Vol. Ed.), *Comprehensive toxicology: 2nd edn., vol. 11. Reproductive and endocrine toxicology* (pp. 167–188). Oxford: Academic Press, Elsevier.
- Xiao, X., Mruk, D. D., Lee, W. M., & Cheng, C. Y. (2011). c-Yes regulates cell adhesion at the blood-testis barrier and the apical ectoplasmic specialization in the seminiferous epithelium of rat testes. *The International Journal of Biochemistry & Cell Biology*, 43, 651–665.
- Xiao, X., Mruk, D. D., & Cheng, C. Y. (2013). c-Yes regulates cell adhesion at the apical ectoplasmic specialization-blood-testis barrier axis via its effects on protein recruitment and distribution. *American Journal of Physiology. Endocrinology and Metabolism*, 304, E145–E159.
- Xiao, X., Mruk, D. D., Tang, E. I., Wong, C. K. C., Lee, W. M., John, C. M., et al. (2014). Environmental toxicants perturb human Sertoli cell adhesive function via changes in F-actin organization mediated by actin regulatory proteins. *Human Reproduction*, 29, 1279–1291.
- Yang, Q., Hao, J., Chen, M., & Li, G. (2014). Dermatopontin is a novel regulator of the CdCl₂-induced decrease in claudin-11 expression. *Toxicology In Vitro*, 28, 1158–1164. An international journal published in association with BIBRA.
- Yu, W., Yaping, L., Mingjun, W., Jie, H., Xiaogang, L., & Gang, L. (2017). BEX4 upregulation alters Sertoli cell growth properties and protein expression profiles: An explanation for cadmium-induced testicular Sertoli cell injury. *Journal of Biochemical and Molecular Toxicology*, 31, e21908. <https://doi.org/10.1002/jbt.21908>.
- Zhang, Y., Han, L., Yang, H., Pang, J., Li, P., Zhang, G., et al. (2017). Bisphenol A affects cell viability involved in autophagy and apoptosis in goat testis Sertoli cell. *Environmental Toxicology and Pharmacology*, 55, 137–147.

Toxicant Actions: Mode of Action of Endocrine Disruptors

Patrick Balaguer and William Bourguet, Université de Montpellier, Montpellier, France

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

α -ZA α -Zearalanol
BPA Bisphenol-A
CAR Constitutive activated receptor
9-*cis*RA 9-*cis* Retinoic acid
CYP Cytochrome P450
DDT Dichlorodiphenyltrichloroethane
 K_d Dissociation constant
EDCs Endocrine-disrupting chemicals
E2 Estradiol
ER Estrogen receptor
GPR30 G protein-coupled receptor 30
GPER G protein-coupled estrogen receptor
hsp Heat shock protein
LBD Ligand binding domain
LBP Ligand binding pocket
MEHP Mono(2-ethylhexyl) *phthalate*
NRs Nuclear receptors
PPAR Peroxisome proliferator-activated receptor
PXR Pregnane X receptor
RXR Retinoid X receptor
TTR Transthyretin
TBT Tributyltin

Nuclear Receptors Are Primary Targets of EDCs

Most of the reported harmful effects of EDCs are currently attributed to their interference with hormonal signaling mediated by nuclear receptors (NRs) (Guilivo et al., 2016). Human NRs are a family of 48 ligand-regulated transcription factors that control a plethora of biological processes such as development, organ homeostasis, metabolism, immune function, or reproduction. As a consequence, inappropriate exposure to EDCs, can cause proliferative reproductive and metabolic disorders, including hormonal cancers, infertility, and obesity. NRs harbor transcription regulation as well as DNA- and ligand-binding properties embedded in three distinct structural domains, and respond to a large variety of small endogenous agonist ligands such as hormones, vitamins, fatty acids or metabolites. Upon binding of agonist ligands, conformational changes in the C-terminal region of the ligand-binding domain (LBD) allow NRs to recruit transcriptional activators. Reciprocally, interactions with antagonists enable repressors recruitment. Most of the studies on EDCs have originally focused on NRs involved in reproductive processes, in particular the estrogen (ER α and ER β) and the androgen (AR) receptors. More recently, studies have shown that the activity of the retinoid X receptors (RXRs), the peroxisome proliferator-activated receptors γ (PPAR γ) or the pregnane X receptor (PXR) is also modulated by EDCs.

The group of molecules acting as environmental ligands of NRs is highly heterogeneous and comprises compounds that are often distantly related to endogenous ligands in terms of size or chemical structure. This group contains substances as chemically different as bisphenols, phthalates, parabens, dioxins, alkylphenols, organotins, polychlorinated biphenyls, perfluoroalkyls, or benzophenones, as well as natural compounds. This large structural diversity renders the interaction of EDCs with their biological targets barely predictable. Most of the studies have revealed that chemicals bind to NRs with medium affinities ranging from sub to high micromolar (le Maire et al., 2010). However, recent studies have revealed some binding modes by which EDCs achieve high affinity binding to NRs. Since humans and wildlife are simultaneously and chronically exposed to low doses of multiple contaminants, understanding the molecular mechanisms underlying the physiological consequences of exposure to environmentally relevant concentrations of EDCs is of prime importance.

The Estrogen Receptors

ER α and ER β (NR3A1-2) are receptors for the sex hormone 17 β -estradiol (E2), that plays important roles in the growth and maintenance of a diverse range of tissues such as the mammary gland, uterus, bone or the cardiovascular system. Furthermore, estrogens are key regulators of primary breast, endometrial, and ovarian cancer growth. Both ERs are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues (Couse and Korach, 1999). Although ER α and ER β share similar mechanisms of action, several differences in the transcriptional abilities of each receptor as well as distinct phenotypes between gene null animals have been identified, suggesting that these receptors may regulate distinct cellular pathways (Couse and Korach, 1999).

Endogenous estrogens (estradiol, estriol, estrone) are high affinity ligands of ERs with dissociation constants (K_d s) in the subnanomolar range. On the contrary, xenoestrogens such as the phytoestrogens genistein and ferutinin, the metabolites of pesticides DDE, HPTE, and M2 compound (respectively DDT, methoxychlore, and vinclozolin metabolites), the plasticizers bisphenols and phthalates, and the benzophenones and parabens used as UV filters and preservatives, respectively, bind to ERs with affinities in the sub- to micromolar range which is far above the environmental concentrations (Table 1). All of them bind to the hormone-binding site of ERs and engage in different sets of ligand/protein interactions according to their size and chemical structures. The smallest compounds making fewer contacts with the receptor cavity are generally associated with lower binding affinities whereas bigger EDCs adopting a binding mode reminiscent of that used by the endogenous ligands are characterized by higher interaction capacities. This is for example the case of the mycoestrogens α -zearalanol (α -ZA) and its derivatives which bind and activate ERs with high affinity (Table 1). Comparison of the crystal structures of ER α in complex with E2 and α -ZA (Delfosse et al., 2014a), reveals that, even though the mycoestrogen is not a steroidal compounds, it recapitulates most of the interaction network observed with E2.

The Androgen Receptor

Androgen receptor (AR) (NR3C4) plays a crucial role in the regulation of target genes expression in physiological processes like development and differentiation of the male embryo, spermatogenesis initiation and maintenance, as well as neuroendocrine

Table 1 EDCs and their NR targets

EDCs	NR targets	Affinity	Environmental compounds	Nature
Natural estrogens	ER α , ER β	High	E2	Agonist
	GPR30	Controversial	E2	Agonist
	PXR	Low	E2	Agonist
Pharmaceuticalestrogens	ER α , ER β	High	EE2	Agonist
	PXR	Low	EE2	Agonist
Alkylphenols	ER α , ER β	Medium	4-tert-Octylphenol	Agonist
	AR	Low	4-tert-Octylphenol	Antagonist
	PXR	Low	4-tert-Octylphenol	Agonist
Benzophenones	ER α , ER β	Medium	BP2, THB	Agonist
	AR	Low	BP2, THB	Antagonist
Bisphenols	ER α , ER β	Medium	BPA	Agonist
	GPR30	Controversial	BPA	Agonist
	AR	Medium	BPC	Antagonist
	PXR	Low	BPA	Agonist
Halogenated bisphenols	ER α , ER β	Low	TetrachloroBPA	Agonist
	PPAR γ	Low	TetrachloroBPA	Agonist
Mycoestrogens	ER α , ER β	High	α -zearalanol	Agonist
	AR	Low	α -zearalanol	Antagonist
	PXR	Low	α -zearalanol	Agonist
Organotins	PPAR γ	High	TPropT	Agonist
		High	TBT	Agonist
Parabens	ER α , ER β	Low	Butyl paraben	Agonist
Perfluorinated compounds	PPAR γ	Low	PFOA	Agonist
Pesticides	ER α , ER β	Low	DDE, HPTE, M2	Agonist
	AR	Medium	M2 vinclozolin	Agonist
	PXR	Low	Pretilachlor, T NC	Agonist
Phytoestrogens	ER α , ER β	Medium	Genistein, ferutinin	Agonist
	PPAR γ	Low	Genistein	Agonist
Phthalates	ER α , ER β	Low	BBP	Agonist
	PPAR γ	Low	MEHP	Agonist

BP2, benzophenone 2; BPA, bisphenol A; BBP, benzyl butyl phthalate; DDE, dichlorodiphenyldichloroethylene; E2, estradiol; EE2, ethinil estradiol; MEHP, mono ethyl hexyl phthalate; PFOA, perfluorinated octanoic acid; TBT, tributyltin; THB, trihydroxy-benzophenone; TNC trans-nonachlore; TpropT, tripropyltin. K_d s of high affinity are lower than 1 nM. K_d s of medium affinity range from 1 nM to 1 μ M. K_d s of low affinity are higher than 1 μ M.

system functioning (Matsumoto et al., 2013). Furthermore, androgens are key regulators of primary prostatic growth. In the absence of ligand, AR is essentially localized in the cytoplasm. Binding to androgens enables heat shock proteins (hsps) dissociation and AR translocation to the nucleus. The presence of androgens is essential for the regulation of male embryo development and differentiation processes and spermatogenesis initiation and maintenance. Environmental compounds have been extensively studied for their action on AR and most of the compounds described as being estrogenic display also antiandrogenic activities. The most potent of them are (α -ZA) and its derivatives, the pesticides metabolites M2 compound and DDE, cosmetics like benzophenone 2 and some bisphenols such as BPA and BPC. The affinities of these diverse environmental antiandrogens are in the sub- to micromolar range (Table 1).

The Retinoid X Receptors

RXR α , β , and γ (NR2B1-3) occupy a central position in the NR superfamily as they are the common heterodimerization partners for one-third of the 48 family members including PPAR γ and PXR. As such, RXRs play key roles in the control of many NR-dependent signaling pathways. RXR ligands and ligands of the partner receptors can act synergistically to activate heterodimers (Germain et al., 2002). This regulatory control of nuclear signaling pathways by multiple RXR heterodimers allows environmental RXR ligands to potentially trigger a multitude of adverse effects on human health.

RXRs are activated by endogenous retinoids like 9-*cis* retinoic acid (9-*cis*RA) (Kd in the nanomolar range) and some other vitamin A metabolites and unsaturated fatty acids (Kds in the micromolar range). On the other hand, RXRs can be activated by some organotin compounds at nanomolar concentrations (Table 1). Organotins are ubiquitously present throughout the environment due to their widespread use since the 1960s in many industrial and agricultural processes. Since the 1980s, they were found to be responsible for a wide variety of deleterious effects in the endocrine systems of humans and wildlife at nanomolar concentrations. They do not, neither structurally nor chemically, resemble known NR ligands so the mechanism by which organotins act as endocrine disruptors has remained enigmatic until the crystal structure of RXR α in complex with tributyltin (TBT) was solved (le Maire et al., 2009). The structure shows that as compared with 9-*cis*RA, the organotin occupies only a small part of the ligand binding pocket (LBP). However, it also reveals that the high affinity of TBT for RXRs derives from the formation of a covalent bond between the tin atom of the organotin and the sulfur atom of the conserved cysteine in the LBP. Although TBT interacts with only a subset of LBP residues, it is engaged in enough essential contacts to efficiently stabilize RXR α in an active conformation which is crucial for the recruitment of transcriptional activators. Hence, in addition to bind to RXR at very low concentrations, TBT acts as a full agonist activating the receptor as efficiently as 9-*cis*RA.

The Peroxisome Proliferator Activated Receptor γ

The NR subfamily of peroxisome proliferator-activated receptors (PPARs) includes three members, PPAR α (NR1C1), PPAR β / δ (NR1C2), and PPAR γ (NR1C3). These receptors bind to PPAR-responsive DNA regulatory elements in the form of heterodimers with RXR. PPARs have distinct tissue distributions and physiological roles (Berger and Moller, 2002). PPAR α is preferentially expressed in the heart, liver, and brown adipose tissue, whereas PPAR β / δ is ubiquitously expressed. They both play an important role as activators of fatty acid oxidation pathways and thus in the regulation of energy homeostasis. PPAR γ , for its part, is highly expressed in adipose tissues and plays a key role in regulating adipogenesis, lipid metabolism, and glucose homeostasis by improving insulin sensitivity.

PPARs bind and respond to dietary fatty acids and various lipid metabolites, including eicosanoids, prostaglandins, and oxidized phospholipids. In accordance with their tissue distributions and roles as sensors of lipids/fatty acids levels, in regulating fatty acid catabolism, and in lipid storage, all three PPARs are thought to be strongly involved in the metabolic syndrome. However, in light of the particular role of PPAR γ in adipose tissue development and maintenance, it has been suggested that the disruption of regulatory pathways controlled by PPAR γ may be specifically implicated in the onset of diabetes and obesity. It was thus proposed that activation of PPAR γ by environmental compounds could contribute to the "obesogen hypothesis" stating that the growing obesity epidemic due to the imbalance between caloric intake and expenditure could also implicate chemicals, so-called "obesogens," which directly or indirectly increase fat accumulation and obesity.

As a matter of fact, activation of PPAR γ by some xenobiotic compounds like BPA and its halogenated derivatives, perfluorooctanoic acid, mono(2-ethylhexyl) phthalate (MEHP), phytoestrogens and organotins has been shown to stimulate adipogenesis *in vitro* and *in vivo* by promoting the differentiation of preadipocytes of the fibroblastic lineage into mature adipocytes (Delfosse et al., 2014b). With the exception of organotins that can bind to PPAR γ with nanomolar affinities, all of these compounds activate PPAR γ with EC50s in the micromolar range (Table 1). The crystal structure of PPAR γ in complex with the tripropyltin (Delfosse et al., 2014b) shows that the interacting cysteine anchors the organotin in a region of the LBP that does not allow the efficient stabilization of the active receptor conformation. This is in line with the weak PPAR γ agonistic activity of the compound. Interestingly, some of these environmental (BPA, MEHP) and pharmaceuticals (ibuprofene, indomethacine) PPAR γ ligands have also been shown to cause direct endocrine disorders in the human fetal testis and alteration of the germ cell biology (Albert and Jégou, 2014; Mazaud-Guittot et al., 2013).

The Pregnane X Receptor

Pregnane X receptor (NR1I2) is a broad-specificity sensor playing a critical role in the regulation of phase I oxidative enzymes, phase II conjugating enzymes, and phase III transport uptake and efflux transporters, co-ordinately regulating steroid, drug, and xenobiotic

clearance in the liver and intestine (Orans et al., 2005). Activated PXR binds to gene promoters as a heterodimer with RXR and triggers the expression of target genes such as the cytochrome P450 3A4 (CYP3A4), UDP-glycosyltransferase (UGT1A1), and multidrug resistance protein 1 (MDR1). PXR plays an important role in protecting the endocrine system from EDCs by sensing concentration increases of these chemicals and stimulating detoxification pathways, resulting in a decreased interaction of EDCs with other NRs. This PXR-driven elimination of xenobiotics confers a positive role to the activation of this NR. On the contrary, PXR activation can also prevent effects of endogenous hormones or drugs by stimulating prematurely their metabolism, which could lead to adverse interactions or harmful effects. In addition, the activation of PXR has been linked to chemoresistance, growth, and aggressiveness of colon and hepatic cancers (Banerjee et al., 2014).

Unlike many NRs that tend to be specialized to bind few ligands with structural homologies, PXR is able to interact with a large number of structurally diverse compounds with medium affinities (K_{ds} between 0.1 and 100 μM). Known ligands of PXR include pesticides, phenols, cosmetics, phytoestrogens, pharmaceuticals, etc. (Table 1) (Banerjee et al., 2014). Crystallographic studies have revealed the unique characteristics of PXR that account for its promiscuous ligand-binding properties. Firstly, PXR possesses a large LBP that can accommodate compounds with larger volumes than that of classical NR ligands, and secondly, several loops of the LBD confer a high plasticity allowing the receptor to adopt different shapes according to the bound ligands. A recent study demonstrated that a pharmaceutical estrogen (the contraceptive 17 α -ethinylestradiol, EE2) and a persistent organochlorine pesticide (*trans*-nonachlor, TNC), both exhibiting low efficacy when studied separately, cooperatively bind to PXR, leading to synergistic activation (Delfosse et al., 2015). High-resolution crystal structures showed that individually, EE2 and TNC are too small to make all the necessary interactions ensuring high binding affinity and effective stabilization of the active conformation of the receptor. In contrast, when associated in a binary mixture, EE2 and TNC fill a larger fraction of the PXR LBP. Strong interligand contacts between EE2 and TNC generate a mutual stabilization of the compounds in the LBP and account for the enhanced binding affinity of the binary mixture. This study provided the first detailed mechanistic explanation and a proof of concept for the synergistic action of a mixture of compounds (cocktail effect) via their simultaneous interaction with a NR, as well as a new insight on how low doses of EDCs or drugs may affect physiology and homeostasis.

Other Disruption Pathways of EDCs

Beside their interference with the NR signaling, EDCs could also interact with membrane-associated receptors (Fig. 1). It is now well accepted that a small amount of NRs, including ER α and β (mbER α and mbER β), are located at the membrane and could mediate the effects of xenoestrogens such as BPA or alkylphenols outside the nucleus (Nadal et al., 2017). It has been suggested that such

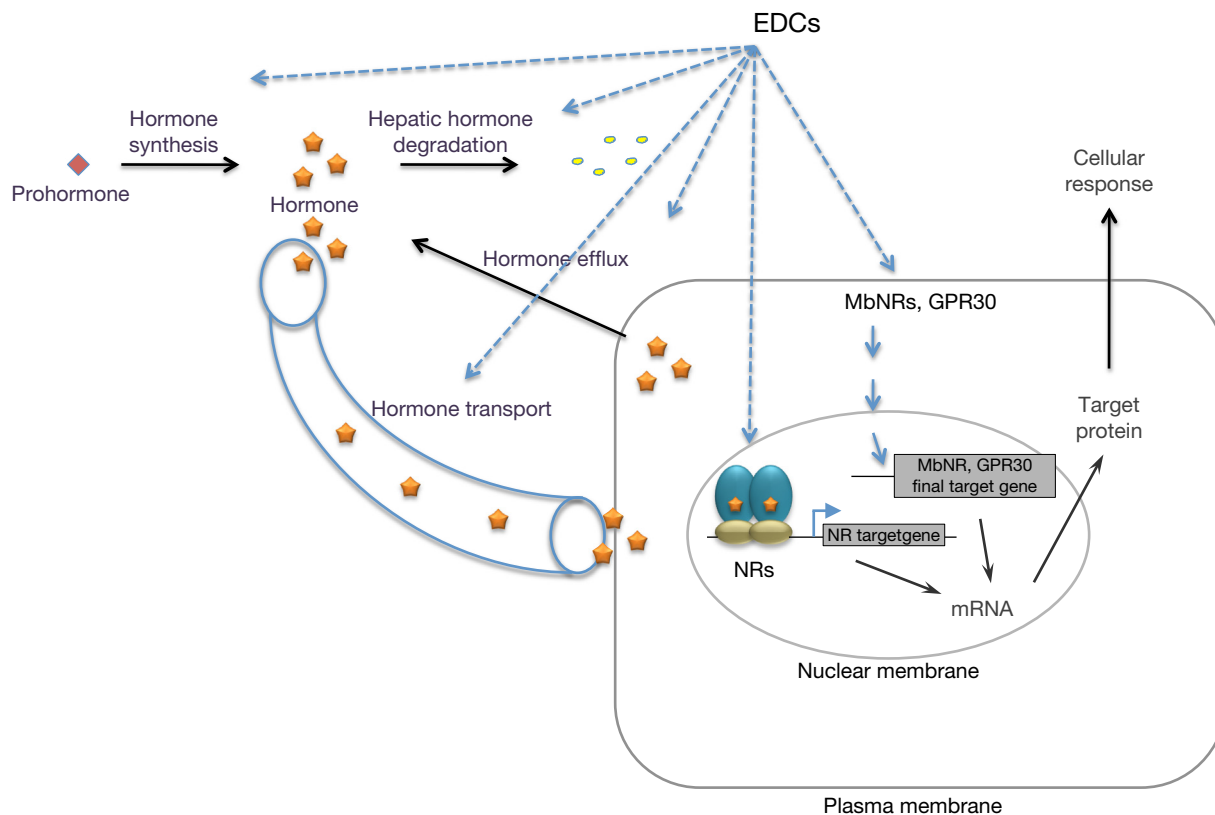


Fig. 1 Possible sites of action of EDCs on hormonal signaling.

rapid nongenomic activation of signal transduction pathways could explain the low-dose effects of EDCs, but this hypothesis is still controversial and needs further investigations (Arnal et al., 2017).

The G protein-coupled receptor 30 (GPR30) also referred to as G protein-coupled estrogen receptor (GPER) was also proposed to act as a nonclassical estrogen receptor localized at the plasma membrane (Revankar et al., 2005). However some recent data contradict this hypothesis suggesting that GPR30 would rather be a collaborator of mbERs (Levin, 2009).

Additionally, EDCs could also alter hormone actions by interfering with their synthesis, transport or metabolism (Fig. 1), and in turn, modify their physiologic concentrations. For instance, several pesticides have been reported to inhibit aromatase activity (CYP19) (Whitehead and Rice, 2006). This enzyme plays a crucial role in steroidogenesis because it allows the synthesis of estrogens (estradiol and estrone) from androgens (testosterone and androsterone). Thus, pesticide-induced inhibition of aromatase can lead to masculinization of an individual (human or animal) by increasing endogenous androgen concentrations and decreasing estrogen levels. The masculinising effect of organotins used in antifouling paints on female gastropods, which is termed “imposex,” was initially attributed to the inhibition of aromatase activity. However, the effective concentrations for aromatase inhibition by organotins are in the high micromolar range that largely exceeds their environmental doses. It is now assumed that the effects of tin compounds on CYP19 are by RXRs whose affinity for organotin compounds is in the nanomolar range (Nakanishi, 2008).

Many EDCs including flame retardants have also been suspected to disrupt thyroid function by binding to thyroid hormone transport proteins such as transthyretin (TTR) (Weiss et al., 2009). By inhibiting the binding of triiodothyronine precursor thyroxine to TTR, these compounds can lead to insufficient intracellular levels of thyroid hormones. However, these effects are rarely observed at concentrations below the micromolar range which is far above the concentrations of EDCs encountered in the environment (Hamers et al., 2006).

Another mechanism of action of EDCs is on hormonal clearance. Exposure to some EDCs may lead to an increase in the expression of metabolizing enzymes and transporting proteins that in turn increase hormonal clearance. For instance, the insecticide fipronil has been described as a thyroid hormone signaling disruptor by increasing thyroid hormones clearance. However, this effect is most likely indirect as fipronil has been shown to be an activator of PXR and the constitutive activated receptor (CAR) which are master regulators of the expression of many genes involved in xeno- and endo-biotics metabolism (Roques et al., 2013).

Concluding Remarks

EDCs represent a broad class of substances that are suspected to interfere with hormone synthesis, transport, degradation, or action, and thus alter the proper function of the endocrine system. Several research groups have proposed that the nonclassical GPER/GPR30 or mbERs could mediate the effects of xenoestrogens through rapid nongenomic activation of signal transduction pathways. However his hypothesis is still controversial and there is a urgent need to develop assays to detect low-doses extranuclear initiated actions of EDCs to further explore this hypothesis. As of today, the deregulation of NR-mediated transcription appears to account for most of the deleterious effects EDCs. Thus characterization of the interaction between NRs and environmental compounds is important for the assessment of the global hormonal activity of a large number of chemicals and the understanding of their mechanisms of action at environmentally relevant concentrations.

High affinity interaction between some EDCs and NRs could explain some of these effects. For instance, in spite of its structural differences with E2, the interaction of α -ZA (a mycoestrogen with nonestrogenic chemical structure) with ER α resembles that of E2. As a consequence, α -ZA is one of the most active xenoestrogen that can modulate ER activity at concentrations as low as 0.1 nM. In contrast, it has been shown that organotins such as TBT do not recapitulate any of the specific interactions made by the classical ligands of RXRs and PPAR γ with their corresponding receptors. Instead, tin compounds use a Sn-S covalent interaction to bind to and modulate the transcriptional activity of the RXR–PPAR heterodimer at nanomolar concentrations. It is thus not excluded that the low dose effects of some environmental compounds could also be explained by their covalent interaction with the dozen NRs containing a cysteine residue in their LBP. Accordingly, it has been reported that dibutyltin acts as a potent antagonist of the glucocorticoid receptor (GR) which contains two cysteine residues in its hormone-binding site. Finally, experimental evidences have been recently provided for the concomitant interaction of two EDCs with PXR. It was shown that the two compounds interact with each other in the PXR LBP, forming a more potent activator than either of the two chemicals alone. The two compounds not only bind concomitantly to the LBP of the receptor, but they do so cooperatively, that is, binding of one molecule promotes high affinity binding of the second. Structural studies revealed that the ligands mutually stabilize each other via strong interligand contacts and that a large number of interactions with LBP amino acids allow the so-called “supramolecular ligand” to tightly hold the receptor in the transcriptionally-competent conformation. A synergistic/additive effect resulting from the simultaneous activation or inhibition of different NRs could also account for the low dose action of certain chemicals. BPA, which is a moderate environmental agonist of ERs, PXR, and AR antagonist, is a good example of an EDC whose adverse effects could result from its combined estrogenic and antiandrogenic properties.

References

- Albert, O., & Jégou, B. (2014). A critical assessment of the endocrine susceptibility of the human testis to phthalates from fetal life to adulthood. *Human Reproduction Update*, 20, 231–249.
- Arnal, J. F., Lenfant, F., Metivier, R., et al. (2017). Membrane and nuclear estrogen receptor alpha actions: From tissue specificity to medical implications. *Physiological Reviews*, 97, 1045–1087.

- Banerjee, M., Robbins, D., & Chen, T. (2014). Targeting xenobiotic receptors PXR and CAR in human diseases. *Drug Discovery Today*, 20, 618–628.
- Berger, J., & Moller, D. E. (2002). The mechanisms of action of PPARs. *Annual Review of Medicine*, 53, 409–435.
- Couse, J. F., & Korach, K. S. (1999). Estrogen receptor null mice: What have we learned and where will they lead us? *Endocrine Reviews*, 20, 358–417.
- Delfosse, V., Grimaldi, M., Cavaillès, V., Balaguer, P., & Bourguet, W. (2014a). Structural and functional profiling of environmental ligands for estrogen receptors. *Environmental Health Perspectives*, 122, 1306–1313.
- Delfosse, V., le Maire, A. L., Balaguer, P., & Bourguet, W. (2014b). A structural perspective on nuclear receptors as targets of environmental compounds. *Acta Pharmacologica Sinica*, 133.
- Delfosse, V., Dendele, B., Huet, T., et al. (2015). Synergistic activation of human pregnane X receptor by binary cocktails of pharmaceutical and environmental compounds. *Nature Communications*, 6, 8089.
- Germain, P., Iyer, J., Zechel, C., & Gronemeyer, H. (2002). Co-regulator recruitment and the mechanism of retinoic acid receptor synergy. *Nature*, 415, 187–192.
- Guilivo, M., Lopez de Alda, M., Capri, E., & Barceló, D. (2016). Human exposure to endocrine disrupting compounds: Their role in reproductive systems, metabolic syndrome and breast cancer. A review. *Environmental Research*, 151, 251–264.
- Hamers, T., Kamstra, J. H., Sonneveld, E., Murk, A. J., & Kester, M. H. (2006). In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicological Sciences*, 92, 157–173.
- Levin, E. R. (2009). G protein-coupled receptor 30: Estrogen receptor or collaborator? *Endocrinology*, 19, 1951–1959.
- le Maire, A., Grimaldi, M., Roecklin, D., et al. (2009). Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. *EMBO Reports*, 10, 367–373.
- le Maire, A., Bourguet, W., & Balaguer, P. (2010). A structural view of nuclear hormone receptor: Endocrine disruptor interactions. *Cellular and Molecular Life Sciences*, 67, 1219–1237.
- Matsumoto, T., Sakari, M., Okada, M., et al. (2013). The androgen receptor in health and disease. *Annual Review of Physiology*, 75, 201–224.
- Mazaud-Guittot, S., Nicolas Nicolaz, C., Desdoits-Lethimonier, C., et al. (2013). Paracetamol, aspirin, and indomethacin induce endocrine disturbances in the human fetal testis capable of interfering with testicular descent. *The Journal of Clinical Endocrinology and Metabolism*, 98, 1757–1767.
- Nadal, A., Fuentes, E., Ripoll, C., et al. (2017). Extranuclear-initiated estrogenic actions of endocrine disrupting chemicals: Is there toxicology beyond paracelsus? *The Journal of Steroid Biochemistry and Molecular Biology* (Epub ahead of print).
- Nakanishi, T. (2008). Endocrine disruption induced by organotin compounds: Organotins function as a powerful agonist for nuclear receptors rather than an aromatase inhibitor. *The Journal of Toxicological Sciences*, 33, 269–276.
- Orans, J., Teotico, D. G., & Redinbo, M. R. (2005). The nuclear xenobiotic receptor pregnane X receptor: Recent insights and new challenges. *Molecular Endocrinology*, 19, 2891–2900.
- Revankar, C. M., Cimino, D. F., Sklar, L. A., et al. (2005). A transmembrane intracellular estrogen receptor mediates rapid cell signalling. *Science*, 307, 1625–1630.
- Roques, B. B., Leghait, J., Lacroix, M. Z., et al. (2013). The nuclear receptors pregnane X receptor and constitutive androstane receptor contribute to the impact of fipronil on hepatic gene expression linked to thyroid hormone metabolism. *Biochemical Pharmacology*, 86, 997–1039.
- Weiss, J. M., Andersson, P. L., Lamoree, M. H., et al. (2009). Competitive binding of poly- and perfluorinated compounds to the thyroid hormone transport protein transthyretin. *Toxicological Sciences*, 109, 206–216.
- Whitehead, S. A., & Rice, S. (2006). Endocrine-disrupting chemicals as modulators of sex steroid synthesis. *Best Practice & Research: Clinical Endocrinology & Metabolism*, 20, 45–61.

Temperature and Testis

Mieusset Roger, Paule de Viguier Hospital, Toulouse, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

Mammals function at high core body temperatures (from approximately 35°C–39°C) obtained from combustion of fuel material to achieve heat production. Body temperature is closely regulated by matching heat production with heat loss to the environment via conduction, convection, radiation, and evaporation. Regulation of core body temperature is a priority over several other physiological functions, as endotherms can better tolerate low body temperatures than high body temperatures (Hansen, 2009). Most mammals have testes suspended in a scrotum outside the body cavity, and there is abundant evidence that in a thermoneutral environment the scrotal testes are appreciably cooler than the body, but this does not mean that they are kept at a constant lower temperature.

Mechanism of Testicular Cooling

In eutherian mammals with scrotal testes, the testicular venous blood in vessels running on the surface of the testis loses heat through the scrotal skin, and testis temperature falls to the same as the subcutaneous tissue. In the spermatic cord, the coiled spermatic artery—warm blood—runs among the multiple veins of the anterior pampiniform plexus—cool blood at the temperature of subcutaneous tissue. This arrangement acts as a counter-current heat exchanger (Waites, 1970) and results in a precooling of the arterial blood arriving to the testis. In marsupials, dolphins and seals there are analogous arrangements acting as heat exchanger (Setchell, 1998).

The key to the reduced temperature of the testis is the scrotal skin which in animals (rams, bulls, boars) is well supplied with apocrine sweat glands. In men, there are no eccrine sweat glands in the scrotal skin; but the water loss is much greater from this area than from sole or areola. The other important mechanism for controlling testis temperature is the tunica dartos muscle which is a layer of smooth muscle inserted into the skin in such a way that contraction of the muscle causes the scrotal skin to be thrown into folds. The dartos contracts in response to cold; under hot conditions, it relaxes, allowing the testis to move away from the body wall, and extending the scrotal surface area (Waites, 1970; Mieusset and Bujan, 1995). As a result, testicular temperature is lower than core body temperature and slightly higher than scrotal temperature in most mammals (Table 1) including men (Table 2).

Ways Used to Induce an Increase in Testicular Temperature

The effects of elevated temperatures on the testes have been studied in different ways: (1) translocation of the testis into the abdominal cavity: done in immature and adult animals, generally used for periods of days or weeks; (2) local heating of the testes by immersion in a water bath at various temperatures, on a single occasion or repeated; (3) whole body exposure to hot environments; (4) scrotal insulation. The two last-mentioned can either be continuous or intermittent, in order better to simulate the temperature fluctuations to which an animal would normally be exposed.

Among these different ways, the value of the achieved testicular temperature differentiates two types heating. In supraphysiological heating, the achieved testicular temperature is higher than the normal core body temperature. This heating is mainly used in rodents, with short exposures (30–90 min) requiring anesthesia—that suppresses scrotal thermo-regulative functions—and induces general

Table 1 Testis and rectal temperatures (°C) in conscious animals with scrotal testes

Species	Number of animals	Rectum	Testis	Rectum-testis difference	Authors
Rat	9	37.4	34.3	3.1	Kormano (1967)
	20	37.7	32.7	5.0	Collins and Lacy (1969)
	8	37–38	33.2–34.7	3.3–3.8	Brooks (1973)
Rabbit	6	38.9	36.7	2.2	Waites (1970)
Boar	6	38.6	35.7	2.9	McNitt et al. (1972)
	4	38.2	35–36.5	1.7–3.2	Stone (1981)
Bull	3	38.6	34.6	4.0	Riemerschmid and Duilian (1941)
Ram	5	39.2	35.3	3.9	Moule and Knapp (1950)
	10	39.1	35.3	3.8	Foote et al. (1957)
	8	39.8	34.2	5.6	Waites and Moule (1961)

Values are means or ranges.

Adapted from Waites, G. M. H. (1970) Temperature regulation and the testis. In Johnson, A.D., Gomes, W.R. and Vandermark, N.L. (eds.)

The testis. pp 241–279. London: Academic Press

Table 2 Scrotal temperatures (°C) in conscious men

Authors	Number of men	Technique	Scrotal temperatures right left	
Zorgniotti and McLeod (1973)	35 (f)	Mercury thermometer	33.5 (32.1–34.8)	33.6 (32.2–34.8)
Zorgniotti et al. (1979)	11 (f)	Infrared thermometry	31.8 (30.7–33.1)	32.2 (31.0–33.6)
Zorgniotti et al. (1982)	7 (f)	Cutaneous thermocouple	32.0 (30.0–33.4)	32.5 (31.3–33.6)
Mieusset et al. (1989)	37 (F)	Mercury thermometer	34.6 (32.0–35.6)	34.6 (32.1–35.7)
Ali et al. (1989)	10 (F)	Mercury thermometer	34.9 (34.6–35.1)	34.8 (34.6–35.1)
Dewasmes et al. (1991)	5 (f)	Cutaneous thermocouple	33.3 (± 0.55 SD)	NR
Jockenhövel et al. (1990)	8 (F)	Cutaneous thermocouple	NR	34.9 (± 1.2 SEM)
Mieusset et al. (1995)	85 (F)	Mercury thermometer	34.5 (32.6–35.6)	34.6 (32.2–35.7)
Hjollund et al. (2002a)	101 (f)	Thermistor ATU 24 h	34.5 a (0.7)	
Hjollund et al. (2002b)	99 (f)	Thermistor ATU daytime	34.2 b (33.7, 34.8)	
Bengoudifa and Mieusset (2007)	11 (PM)	Cutaneous thermocouple	34.38 (± 0.37 SD)	34.56 (± 0.25 SD)
	11 (BD)	Cutaneous thermocouple	35.43 (± 0.25 SD)	35.92 (± 0.19 SD)

Values are means and ranges, or standard deviation (SD) or standard error of the mean (SEM); a = percentiles of individual readings: values (interquartile range); b = median value. (25th, 75th percentile); f = fecund; F = fertile; NR = not recorded. TATU = thermistor attached to the underwear (between the caudal poles of the testes). PM = postmen working 90 consecutive minutes in a standing position; BD = bus drivers driving (sitting) consecutively for 90 min. Measurements were done with men in supine position (except where indicated), but clothing, previous activities as well as room temperature and acclimatization were various.

changes—for example, the vascular flow—that are source of direct non-thermal testicular effects. In physiological heating, the achieved testicular values are never higher than body core temperature. Such a heating includes involve increased ambient temperature as well as induced cryptorchidism, or scrotal insulation; exposures are daily or repeated.

Effects of Exposure to Elevated Temperatures on the Testicles

Spermatogenesis

Pachytene and diplotene spermatocytes have been reported to be damaged in the testes exposed either to high increased temperatures in rats (43°C, 15 min), rams (40°C, 150 min) and mice (43°C, 15 min), or for continuous period of time in pigs (scrotal insulation, 100 h); the same germ cells were affected with experimental cryptorchidism in rats, rabbits, and mice, associated with a negative effect on round spermatids. Most of these affected germ cells undergo apoptosis; (Liu, 2010) but some of them escape to apoptosis, and this damage can extend to an effect on the development of preimplantation embryos. When the degree of heat exposure is more increased (by increasing the temperature or the time of exposure, e.g., cryptorchidism) other testis components are affected such as Sertoli cells and spermatogonia (Jégou et al., 1983; Setchell, 1998; Liu, 2010).

In humans submitted to different conditions increasing testicular temperatures, indirect data about germ cells were obtained based on both the three first constitutive phases of spermatogenesis: proliferation phase (Pp), meiosis phase (Mp) and spermiogenesis (Sg), and the epididymal transit (Et), which take place about 58–86 (Pp), 34–58 (Mp), 12–34 (Sg) and 0–12 days (Et) before ejaculation (Ahmad et al., 2012).

Whole body (fever)

Over a 16 months follow-up period in 27 men 15 reported at least one episode of fever (from 1 to 11 days; median 5; up to 40°C for 6 days). From the monthly delivered semen samples, the occurrence of fever had significant effect: sperm morphology and motility are adversely affected by a febrile episode during spermiogenesis. Sperm concentration was adversely affected by fever during the meiosis phase. These results are in line with previous studies dealing with fever either induced (40.5°C–41°C) or associated with diseases (Carlsen et al., 2003).

Scrotal exposure to high elevated temperature

Increasing scrotal temperature to 42°C by an electric lamp (30 min/day; 14–28 days) induced a drop in total sperm count (TSC) to 40%–70% of the initial TSC 3–11 weeks after heating, with a recovery to the initial TSC within 11–13 weeks after heating (Robinson et al., 1968). Using the timing of the different phases of spermatogenesis and Et, these data indicate a negative effect on sperm output during the first two phases, proliferation and meiosis.

Testes exposure to slight elevated temperature

A 2°C increase in testicular temperature was induced in 5 men by using underwear designed to push up and maintain the testes at the upper part of the root of the penis for 15 h/day (h/day) for 120 consecutive days. According to the chronology of spermatogenesis and Et, TSC was not affected by this heating when spermatozoa were stored in the epididymis when heating was induced. The major decrease in TSC at 34 days of heating suggests an active impact mainly on the meiosis phase from such a slight increase in testicular temperature. Furthermore, during heating, the first stage of spermatogenesis (mitosis and differentiation of spermatogonia) seems not to be affected, as has been reported in similar conditions of induced cryptorchidism in rats and rabbits (Ahmad et al., 2012).

Testicular volume

Following a single exposure of testicles in rats (41°C; 90 min; or 43°C; 30 min) or in mouse (43°C; 30 min), testes weight falls to almost half, reaching a minimum between 14 and 21 days afterwards, and then slowly returning to baseline values (Setchell, 1998). Results from these supraphysiological heatings differ from those of physiological heatings with no modifications in testes weight in boars submitted to whole body heating or in rats and mice under chronic ambient exposure (35°C and 33°C, respectively). In humans, a slight increase in testicular temperature (+2°C) every day (15 or 24 h/day; 12 months) induced a reversible reduction in testes weight of almost 50% for 24 h/day (Shafik 1991) of about 25% for 15 h/day.

Reproductive Hormones

Concerning the changes observed in circulating pituitary hormones, they are separated in time from the actual thermal stress; indicating these changes are unlikely to be caused directly by the heat exposure but may result from specific germ cell changes, either directly, or secondary to disruption of Sertoli or Leydig cells function (Jégou et al., 1984). Besides these delayed changes in circulating pituitary hormones, only few studies have experimented on the direct effects of temperature.

Elevated ambient temperature

When boars were exposed to 35°C (8 h/day; 90 min), there were slightly reduced testosterone (T) levels in blood on the 1st day of heating and after 7 and 14 days, with no significant differences thereafter. In rams exposed to an environmental temperature of 28°C–32°C (2 weeks), T concentrations were reduced in the testes and in blood from the spermatic internal vein. In bulls, exposure to an environment of 35°C (7 weeks) is associated with a fall in plasma T concentrations during the first 2 weeks followed by a return to control values during the rest of the exposure.

Scrotal heating

In boars subjected to scrotal insulation, plasma T levels decrease during the insulation period and return to normal afterwards. In rats, T concentration was not affected when measured in arterial blood at the end of a 30 min exposure of the scrota to temperatures of 33°C, 37°C, 41°C, and 43°C; but the response to LH and the amount of T in the testes after LH were severely repressed at the two high temperatures of 41°C and 43°C (Setchell, 1998).

Cryptorchidism and wet local heating

When spermatogenesis is arrested either by making the testes cryptorchid or by local heating of the scrotum using water bath, the T peripheral levels are not affected, but the concentrations of LH are increased.

Humans

A daily slight elevation in testes temperature (+2°C; 24 h/day) for a period as long as 12 months is without any effect on peripheral levels of FSH and LH; an observed reduction in peripheral T levels, to values still within normal range, was reversible within 3 months after the end of heating (Shafik, 1991). No significant modifications in reproductive hormones (FSH, LH, T and free T) were observed during the 30 follow-up weeks in 18 men whom scrota were submerged into a warm water bath (43°C; 30 min/day; six consecutive days; Wang et al., 2007); or during the 16 follow-up weeks in 20 men in whom the lower half body was soaked in a bathtub (43°C; 30 min/day; 10 consecutive days or once every 3 days, 10 times; Rao et al., 2015).

Effects of Exposure to Elevated Temperatures on the Epididymides

The epididymides, similar to the testes, are also maintained at a lower temperature than that of core body temperature: compared to the last one in conscious animals, temperature of cauda epididymis is 4°C lower in rats (Brooks, 1973), and those of caput and cauda epididymis 1.5°C and 1.1°C respectively lower in pigs (Stone, 1981). Epididymis subjection to body temperature was obtained by reflecting the epididymis to the abdomen (called cryptepididymis), while testes were kept in scrotal position. In rabbits, hamster and rats, cryptepididymis is associated with the reversible following consequences: (1) a rapid reduction in the cauda size, that is, a reduced capacity of sperm storage; (2) a major increase in the speed of transit of sperm, that is, a shorter epididymal transit time; (3) an inability of the cauda epididymis to maintain sperm viability; (4) changes in oxygen levels, water and ion transport mechanisms, proteins synthesis in cauda fluid (Bedford, 1994).

Moreover, epididymal sperm are susceptible to heat-induced DNA damage. In mice (42°C and 40°C; 30 min), a significant increase in the number of sperm with DNA damage was observed at the 24-h time point (Paul et al., 2008).

Effects of Exposure to Elevated Temperatures on Semen Parameters

It is usually reported that elevations in testis temperature (using whole body heating or scrotal heating) lead to reduced total sperm count (TSC) and decreased sperm motility while increasing the number of abnormal spermatozoa in bulls, rams, boars, pigs, stallions, and men.

While such effects are generally true, they results from both the value of the achieved temperature and the length of exposure to such an achieved value; for example, in animals: three groups of 4 rams were submitted to an identical regimen of scrotal insulation ($+2^{\circ}\text{C}$) for 8, 16, or 24 h/day. With scrotal insulation for 8 h/day, no significant effect on TSC appeared during the 162 consecutive days of insulation; however a slight depression of TSC was observed from day 100 for 16 h/day; and a fall in TSC started as soon as day 12 for a 24 h/day exposure (Mieusset et al., 1992); for example, in humans: a 1°C increase in scrotal temperature (20 h/day; 12 months) is without effects on sperm parameters while a 2°C daily increase in testicular temperature (15 h/day) induces in 3 months of exposure a 90% drop in TSC and a drastic reduction in the percentage of motile sperm (Soufir, 2017). In all experiments of a physiological induced increase in testis temperature in animals and humans, semen parameters recover their initial values within 1–2 spermatogenesis cycles after cessation of exposure depending of the length of exposure.

Effects of Exposure to Elevated Temperatures on Fertility

Male mice were exposed to elevated ambient temperature (32°C ; 24 h) then mated with females; fertilization rate, calculated from the ratio of cleaved ova to total ova recovered, fell from 79% between days 1–5 to 11% for days 16–20, and then recovered to 79% for days 26–30 after heat exposure. In male animals, infertility following exposure to heat has been known for a long time. The infertile period after heating the testes (rats: 43°C ; 30 min; mice: 42°C ; 30 min) lasted from between 7 and 14 days after heating to between 49 and 56 days (rats) and between 10 days and 38 days (mice). Such an infertile period has been reported in rams submitted to elevated ambient temperature or scrotal heating, the value of the achieved testicular temperature and the length of exposure defining the percentage of infertile male.

However, as exposure to heat induces a decrease in TSC and sperm motility, this infertility period could be explained by the reduced number of sperm being available to fertilize the oocytes. But when an equal number of motile swum-up sperm from scrotal-insulated and control rams were used for in vitro fertilization (IVF), the percentage of oocytes fertilized decreased from 73% to 7% with 16 h/day insulation for 18 days, and there were significant falls with 8 h/day insulation for 5 weeks, both during the insulation period and during the following 2 weeks (Setchell, 1998). These last results indicate that in such a situation of $a + 2^{\circ}\text{C}$ increase in testis temperature in rams, the reduced oocytes fertilization rate (1) is not only due to the number of sperm being available to fertilize the oocytes; (2) is linked to the daily length of exposure for the same achieved testicular temperature ($+2^{\circ}\text{C}$); (3) is reversible after cessation of exposure.

Effects of Exposure to Elevated Temperatures on Embryo Development

Embryonic death rate fell by 27% when rams were kept in air-conditioned chambers during the summer months. Ewes inseminated with frozen-thawed semen collected from rams on the 4th day of scrotal insulation ($+2^{\circ}\text{C}$; 16 h/day) showed a greater loss of embryos between day 17 and day 65 of pregnancy; as sperm used for insemination involve only sperm that were in the epididymis in the heating period (4th day), these data suggests that even epididymal sperm may be affected in such a way as to affect the developmental capacity of the embryos they produce (Mieusset et al., 1992; Mieusset, 1995). Such an epididymal effect may be observed in a previous study in which male mice were exposed to elevated ambient temperature (32°C ; 24 h); the ratio of implantation sites to fertilized ova was already less for the heated versus non-heated males in the first 5 days (71% vs. 90%; Setchell, 1998).

When normal female rats were mated to males whose testes had been heated (43°C ; 30 min), there were periods when litter size and the fetus to corpus luteum ratios were reduced, suggesting that there was significant mortality of the embryos. The sheep embryos produced by IVF, using semen from scrotal-insulated rams ($+2^{\circ}\text{C}$; 16 h/day) developed slightly less well than those obtained with control semen, and there was increased degeneration at the blastocyst stage. When the testes of male mice were heated (42°C ; 20 min) the animals remained fertile and litter size was not reduced, but the embryos they sired were about 25% smaller than controls at 10.5 days of gestation (Setchell, 1998). These findings of smaller fetuses with normal litter size from heat exposed sires have been confirmed for embryos at 14.5 days (Jannes et al., 1998).

Different studies looking at the effect of a single scrotal heating (36°C ; 24 h or 12 h) in mice have reported abnormal development in embryos recovered from females mated to heat-stressed males (36°C ; 24 h) including reduced number of embryos and blastomeres or impaired embryo development in IVF (Yaeram et al., 2006). The lower third of the body of mice was submerged in a water bath (38°C , 40°C or 42°C ; 30 min); paternal heat stress at 42°C resulted in reduced pregnancy rate, placental weight and litter size; abnormalities in embryonic development were detected at 3.5 days and IVF with sperm recovered 16 h or 23 days after scrotal stress at 42°C revealed a block in development between the 4-cell and blastocyst stages. By reporting DNA strand breaks present in spermatocytes recovered from testes subjected to 40°C or 42°C this study has provided evidence of temperature-dependent effects on germ cell DNA integrity and highlighted the importance of an intact paternal genome for normal embryo development (Paul et al., 2008).

Routes of Potential Exposure in Humans

Posture, Clothing and Activity

Scrotal temperatures (St) have been measured by different techniques in naked normal men in at least two of the following postures: supine, sitting with legs either together or apart, and standing. The lowest St observed was whilst standing, with

a mean lowering of from 0.3°C to 0.6°C when changing from supine to a standing posture. When sitting with legs together, St was higher than with legs apart, but in both sitting situations, St was higher than when standing. Being clothed elevates scrotal temperatures by 1.5°C–2°C when standing or supine, compared with an unclothed state (Jung et al., 2005; Mieusset et al., 2007).

Activity is associated with a lower St: when walking, scrotal temperature is 0.3°C–1°C lower than when sitting, independent of the type of clothing worn. Sleep is associated with the highest St, though values are lower when sleeping naked than when wearing pajamas or underwear (Jung et al., 2003).

Lifestyle Factors

Hot bath and sauna bathing are suspected sources of potential testicular heating. When St was monitored continuously with a miniaturized portable digital data recorder, it rose considerably in response to exogenous heat from the sauna, and reached core body temperature within 10 min of exposure (Jockenhövel et al., 1990). These data were confirmed in men submitted to two sauna sessions per week (15 min each; 80°C–90°C; 3 months); moreover sperm count and motility were reversibly decreased (Garolla et al., 2013).

Occupational Heat Exposure

Valid information was obtained from three case-control studies in which fertility status and/or semen analyses were compared between exposed and non-exposed workers to occupational heat stress: they reported (1) an increased odds ratio for occupational exposure to heat among men with sperm abnormalities; (2) a higher incidence of childlessness and of self-reported difficulty in conceiving; (3) a decline in the proportion of spermatozoa with normal forms (Mieusset and Bujan, 1995).

Driving or being seated is associated with an increase in St, alterations in semen parameters and longer time to pregnancy (Thonneau et al., 1997).

Clinical Factors

There are mainly two types of clinical factors (endogenous factors) that can be congenital or acquired.

Testicular mal descent (TMD)

Formerly called cryptorchidism, TMD is any testicle not within the scrotum at birth (congenital) or between 4 and 10 years old (acquired). When located in the inguinal canal (main TMD location), testis temperature is 1.5°C higher than the contralateral normally descended testis in impuberal boys. TMD treatment is a surgical positioning into the scrotum; in adult life, half these men will have still an elevated St associated with lower testicular volume, lower TSC and motile sperm than the other half in whom St has recovered normal value (Mieusset and Bujan, 1995).

Varicocele

Varicocele is a venous dilation of the spermatic cord, a factor associated with male infertility as its incidence ranges from 8% to 23% in the general or fertile versus 21%–39% in the infertile population. One of the suggested explanations for the pathophysiology of varicocele in male infertility is an increase in testicular temperature due to impaired heat transfer in the spermatic cord and/or decreased testicular blood flow. In men with varicocele, mean scrotal or testicular temperatures are significantly increased in uni- or bilateral varicocele compared with values in control men; and recovered after surgical treatment of the varicocele. However, the only way to prove the association of varicocele with increased St is to measure St in every infertile man with varicocele (Mieusset and Bujan, 1995).

References

- Ahmad, G., Moinard, N., Esquerré-Lamare, C., Mieusset, R., & Bujan, L. (2012). Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men. *Fertility and Sterility*, 97, 546–553.
- Ali, J. I., Weaver, D. J., Weinstein, S. H., & Grimes, E. M. (1989). Scrotal temperature and semen quality in men with or without varicocele. *Archives of Andrology*, 24, 215–219.
- Bedford, J. M. (1994). The status and the state of the human epididymis. *Human Reproduction*, 9, 2187–2199.
- Bengoudifa, B., & Mieusset, R. (2007). Thermal asymmetry of the human scrotum. *Human Reproduction*, 22, 2178–2182.
- Brooks, D. E. (1973). Epididymal and testicular temperature in the unrestrained conscious rat. *Journal of Reproduction and Fertility*, 35, 157–160.
- Carlsen, E., Andersson, A. M., Petersen, J. H., & Skakkebaek, N. E. (2003). History of febrile illness and variation in semen quality. *Human Reproduction*, 18, 2089–2092.
- Collins, P., & Lacy, D. (1969). Studies on the structure and function of the mammalian testis. II. Cytological and biochemical observations on the testis of the rat after a single exposure to heat applied for different lengths of time. *Proceedings of the Royal Society B*, 172, 17–38.
- Dewasmes, G., Bothorel, B., Hsiung, R., Clavert, A., & Candas, V. (1991). Human scrotal temperature during heat exposure associated with passive leg heating. In A. W. Zorngiotti (Ed.), *Temperature and environmental effects on the testis* (pp. 187–191). New York: Plenum Press.
- Foote, W. C., Pope, A. L., Nichols, R. E., & Casida, L. E. (1957). The effect of variation in ambient temperature and humidity on rectal and testis temperature on sheared and unsheared rams. *Journal of Animal Science*, 16, 144–150.
- Garolla, A., Torino, M., Sartini, B., et al. (2013). Seminal and molecular evidence that sauna exposure affects human spermatogenesis. *Human Reproduction*, 28, 877–885.
- Hansen, P. J. (2009). Effects of heat stress on mammalian reproduction. *Philosophical Transactions of the Royal Society B*, 364, 3341–3350.
- Hjollund, N. H. I., Storgaard, L., Ernst, E., Bonde, J. P., & Olsen, J. (2002a). The relation between daily activities and scrotal temperature. *Reproductive Toxicology*, 16, 209–214.
- Hjollund, N. H. I., Storgaard, L., Ernst, E., Bonde, J. P., & Olsen, J. (2002b). Impact of diurnal scrotal temperature on semen quality. *Reproductive Toxicology*, 16, 215–221.

- Jannes, P., Spiessens, C., Van der Auwera, I., et al. (1998). Male subfertility induced by acute scrotal heating affects embryo quality in normal female mice. *Human Reproduction*, 13, 372–375.
- Jégou, B., Lawns, A. O., & de Kretser, D. M. (1983). The effect of cryptorchidism and subsequent orchidopexy on testicular function in adult rats. *Journal of Reproduction and Fertility*, 69, 137–145.
- Jégou, B., Lawns, A. O., & de Kretser, D. M. (1984). Changes in testicular function induced by short-term exposure of the rat testis to heat: Further evidence for interaction of germ cells, Sertoli cells and Leydig cell. *International Journal of Andrology*, 7, 244–257.
- Jockenhövel, F., Gräwe, A., & Nieschlag, E. (1990). A portable digital data recorder for long-term monitoring of scrotal temperature. *Fertility and Sterility*, 54, 694–700.
- Jung, A., Hofstötter, J. P., Schuppe, H. C., & Schill, W. B. (2003). Relationship between sleeping posture and fluctuations in nocturnal scrotal temperature. *Reproductive Toxicology*, 17, 433–438.
- Jung, A., Leonhardt, F., Schill, W. B., & Schuppe, H. C. (2005). Influence of the type of undertrousers and physical activity on scrotal temperature. *Human Reproduction*, 20, 1022–1027.
- Kormano, M. (1967). Development of the rectum-testis temperature difference in the post-natal rat. *Journal of Reproduction and Fertility*, 14, 427–437.
- Liu, Y.-X. (2010). Temperature control of spermatogenesis and prospect of male contraception. *Frontiers in Bioscience*, S2, 730–755.
- McNitt, J. I., Tanner, C. B., & First, N. I. (1972). Thermoregulation in the scrotal system of the boar. I. Temperature distribution. *Journal of Animal Science*, 34, 112–116.
- Mieusset, R. (1995). Spermatozoa and embryonic development. In S. Hamamah, R. Mieusset, & J. L. Dacheux (Eds.), *Epididymis: Role and importance in male infertility treatment. Frontiers in Endocrinology*, 11 pp. 105–128. Roma: Ares-Serono Symposia Publications.
- Mieusset, R., & Bujan, L. (1995). Testicular heating and its possible contributions to male infertility: A review. *International Journal of Andrology*, 18, 169–184.
- Mieusset, R., Bujan, L., Plantavid, M., & Grandjean, H. (1989). Association of scrotal hyperthermia with impaired spermatogenesis in infertile men. *Fertility and Sterility*, 48, 1006–1011.
- Mieusset, R., Quintana Casares, P., Sanchez Partida, L. G., et al. (1992). Effects of heating the testes and epididymides of rams by scrotal insulation on fertility and embryonic mortality in ewes inseminated with frozen semen. *Journal of Reproduction and Fertility*, 94, 337–343.
- Mieusset, R., Bujan, L., Massat, G., Mansat, A., & Pontonnier, F. (1995). Clinical biological characteristics of infertile men with a history of cryptorchidism. *Human Reproduction*, 10, 613–619.
- Mieusset, R., Bengoudifa, B., & Bujan, L. (2007). Effect of posture and clothing on scrotal temperature in fertile men. *Journal of Andrology*, 28, 170–175.
- Moule, G. R., & Knapp, B. (1950). Observations on intra-testicular temperature of Merino rams. *Australian Journal of Agricultural Research*, 1, 456–464.
- Paul, C., Murray, A. A., Spears, N., & Saunders, P. T. (2008). A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. *Reproduction*, 136, 73–84.
- Rao, M., Zhao, X. L., Yang, J., et al. (2015). Effect of transient scrotal hyperthermia on sperm parameters, seminal plasma biochemical markers, and oxidative stress in men. *Asian Journal of Andrology*, 17, 668–675.
- Riemerschmid, G., & Duilian, J. (1941). Further observations on the scrotal skin temperature of the bull, with some remarks on the intra-testicular temperature. *Journal of Veterinary Science and Animal Husbandry*, 17, 123–140.
- Robinson, D., Rock, J., & Menkin, M. F. (1968). Control of human spermatogenesis by induced changes of intrascrotal temperature. *Journal of the American Medical Association*, 204, 80–87.
- Setchell, B. P. (1998). The Parkes lecture. Heat and the testis. *Journal of Reproduction and Fertility*, 114, 179–194.
- Soufir, J.-C. (2017). Hormonal, chemical and thermal inhibition of spermatogenesis: Contribution of French teams to international data with the aim of developing male contraception in France. *Basic and Clinical Andrology*, 27, 3.
- Stone, B. A. (1981). Thermal characteristics of the testis and epididymis of the boar. *Journal of Reproduction and Fertility*, 63, 551–557.
- Thonneau, P., Ducot, B., Bujan, L., Mieusset, R., & Spira, A. (1997). Effect of male occupational heat exposure on time to pregnancy. *International Journal of Andrology*, 20, 274–278.
- Waites, G. M. H. (1970). Temperature regulation and the testis. In A. D. Johnson, W. R. Gomes, & N. L. Vandermark (Eds.), *The testis* (pp. 241–279). London: Academic Press.
- Waites, G. M. H., & Moule, G. R. (1961). Relation of vascular heat exchange to temperature regulation in the testis of the ram. *Journal of Reproduction and Fertility*, 2, 213–224.
- Wang, C., Cui, Y. G., Wang, X. H., et al. (2007). Transient scrotal hyperthermia and levonorgestrel enhance testosterone-induced spermatogenesis suppression in men through increased germ cell apoptosis. *Journal of Clinical Endocrinology and Metabolism*, 92, 3292–3304.
- Yaeram, J., Setchell, B. P., & Maddocks, S. (2006). Effect of heat stress on the fertility of male mice in vivo and in vitro. *Reproduction, Fertility and Development*, 18, 647–653.
- Zorgniotti, A. W., & McLeod, J. (1973). Studies in temperature, human semen quality and varicocele. *Fertility and Sterility*, 24, 854–863.
- Zorgniotti, A. W., Toth, A., & McLeod, J. (1979). Infrared thermometry for testicular temperature determinations. *Fertility and Sterility*, 32, 343–347.
- Zorgniotti, A. W., Reiss, H., Toth, A., & Sealfon, A. (1982). Effect of clothing on scrotal temperature in normal men and patients with poor semen quality. *Urology*, 19, 176–178.

Metals

Pallav Sengupta and Sulagna Dutta, MAHSA University, Kuala Lumpur, Malaysia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Endocrine disruptor “Is an exogenous substance or mixture that alters functions(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or in progeny, or (sub)populations” (WHO/IPCS, 2002).

Reproductive disruptor Is an exogenous substance or mixture that causes subfertility or infertility by interfering with normal functions of endocrine axes concerned with reproductive functions and their cross-talks, gonadal activities including gametogenesis, as well as postgonadal events associated with maturation, release, and fertilization of germ cells.

Abbreviations

ABP Androgen binding protein
AP-1 Activator protein 1
AR Androgen receptor
DMT1 Divalent metal transporter 1
EDCs Endocrine disrupting chemicals
EGF Epidermal growth factor
EGFR Epidermal growth factor receptor
FSH Follicle stimulating hormone
HIF1 Hypoxia-inducible factor 1
I κ B Inhibitor of kappa B
IUDs Intrauterine devices
LDH Lactate dehydrogenase
LH Luteinizing hormone
LPO Lipid peroxidation
MAPKs Mitogen activated protein kinases
NF- κ B Nuclear factor- κ B
NFAT Nuclear factor of activated T-cells
OS Oxidative stress
PI3K Phosphoinositide 3-kinase
PSA Prostate specific antigen
ROS Reactive oxygen species
ZIP, ZRT Iron regulated transporter (IRT)-like Protein

Introduction

The man-made changes to the environment vex the natural regulations altering the level of exposure of human to various essential and nonessential metals. Since last few decades, concern has been increasing about the possible deterioration of male reproductive health owing to various exogenous and endogenous flaws (Sengupta et al., 2017). Among the exogenous factors, the toxic consequences following pervasive exposure to metals stand at the top crosspiece (Sengupta, 2013a). The vulnerability of mammalian reproductive system to the adverse effects of metals is a threat to the power of procreation of the planet. Some metals are known to be beneficial to reproductive functions in men, involved in several reproductive processes, whereas environmental or occupational exposure to others are proven to disrupt male fertility. The male reproductive function usually be affected through the direct and indirect impacts of the toxic metals on the reproductive axis causing hormonal imbalance (pretesticular effect) leading to impaired sperm production in the testis, genomic constituents' alterations of the germ cells (testicular effect) as well as alterations of secretion of the accessory sex glands and disruption in sperm maturation at different levels (posttesticular effect) (Sengupta, 2013b). These propagations of metal toxicity are evoked by cellular macromolecules mediating intracellular catalytic, structural, and functional modulations. They may overcome the O₂ spin restriction donating a single electron, thus propelling free radical species which undergo uncontrolled destructive chain reactions causing oxidative damage to the cells. Furthermore, the suppressed activity of antioxidant enzymes, owing to metal toxicity, collapse body's own capability to combat and nullify such oxidative

damage. Other essential toxic effects of metals include DNA damage, impairment of cellular respiration, inhibition of several cell signaling pathways, etc. The mentioned impairments, individually or in combination by the toxic metals acting as “*reproductive disruptors*,” upset male fertility. As male reproductive system lack definite measurable cycle, these changes can be gauged by assessing testis size, semen quality, functioning of prostate and seminal vesicles, hormonal profile, tenacity of libido, and other basic parameters.

Major Sites of Action of Metals in Male Reproductive System

Exposure of men to metals is increasing with global advent, owing to their ubiquity in nature, wide use in industry, and long-term persistence in the environment. Metals may have direct effect on male reproductive system, targeting specific reproductive organs, or indirect effect, acting on the neuroendocrine system (Fig. 1).

These adverse effects can be irreversible if Sertoli cells are disrupted at the time of fetal development. The number of sperm generated in adulthood is determined by the number of Sertoli cells present, because each Sertoli cell can make provision for only a finite number of germ cells that mature into sperm. Proliferation of the Sertoli cells occurs during the fetal, neonatal as well as prepubertal period which predominantly sensitive to the hostile effects of metals. Disruption of spermatogenesis at any phase of cellular differentiation can reduce the total sperm count, heighten abnormal sperm numbers, and damage the stability of sperm chromatin. Metals may get accumulated in the prostate, epididymis, vesicular seminalis or seminal fluid, and thereby impair sperm motility. Metals can affect the male reproductive system indirectly leading to hormonal imbalance by disturbing the neuroendocrine system, and thus secretion of androgens from Leydig cells or Inhibin B, activins, and androgen binding protein (ABP) from Sertoli cells also get adversely altered. Oxidative stress (OS) is associated with the pathogenesis of male infertility and as human spermatozoa are particularly susceptible to OS, metals lead to propelling of an excessive reactive oxygen species (ROS) in the spermatozoa. This results in the lipid peroxidation (LPO) of the cell membrane. The decrease glutathione and other antioxidant levels, by exposure to metals, further contribute to the oxidative damage to spermatozoa even damaging its DNA. Destruction to the sperm membrane diminishes sperm's motility and capacity to fuse with the oocyte, while mutilation to the sperm DNA impede healthy paternal genomic influence to the embryo and escalates the risk of infertility, miscarriage, or any serious disease in the offspring. Exposure to “endocrine disrupting chemicals (EDCs)” may give rise to certain male reproductive malformations such as cryptorchidism, prostate cancer, hypospadias, and testicular are suspected to affect the endocrine system (Table 1).

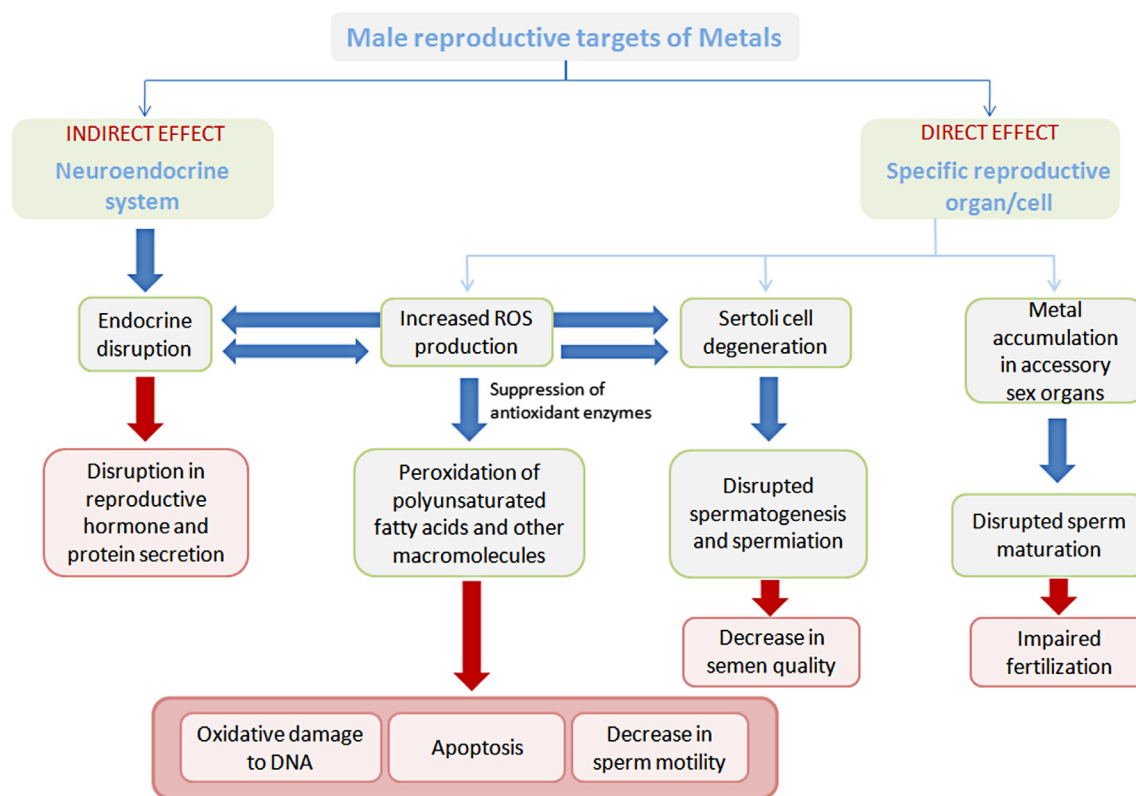


Fig. 1 Major sites of metal action on male reproductive tract with their mode of actions. Through indirect effect metals disrupt endocrine regulations and by direct mechanisms they cause cellular toxicity and impairment of reproductive functions.

Table 1 Effects of metals on male reproductive system and their evaluation

Potential sites	Functions	Effects	Evaluative tests
Sertoli cells	Sertoli cells create the blood-testis barrier by the help of tight junctions. The luminal environment is regulated by FSH and inhibins These Sertoli cells: (1) nourish the developing germ cells; (2) remove the defective sperms; (3) secrete fluid aiding transport of sperm in the epididymis; and (4) release inhibins to control sperm production	↓ HPG-axis, ↓ spermatogenesis, abnormal spermiation, x CNS effects, testicular damage	Receptor analysis, hormone assay
Leydig cells	Secrete testosterone under the influence of LH. They are situated between the seminiferous tubules in the connective tissue		Receptor analysis, Radio-immuno assay (RIA); In vitro tests (co-culture), morphological analysis through staining procedures
Spermatogenesis	A chronological process, spans approximately 72 days in men. During this period, the immature undifferentiated spermatogonia grow into specialized spermatozoa. Spermatogonia go through several mitotic divisions to produce a large population of primary spermatocytes, which give rise to haploid spermatids by two meiotic cell divisions. The transformation of spermatids into elongated flagellar matured germ cells capable of motility is referred to as spermiogenesis. The release of finally formed mature germ cells is known as spermiation		Germ cell count and calculating the percentage of tubules without germ cells, spermatid counts and percentage of tubules with luminal sperm, germ cell culture, morphological analysis
Epididymis	Maturation of sperms (capacitation)		Histopathology, biochemical analysis
Seminal fluid	Regular sperm production		Spermatid counts, semen evaluation
Neuroendocrine system	Hypothalamo-pituitary axis, through LH and FSH, regulate the secretions of Leydig cells and Sertoli cells and thus impinging effects on spermatogenesis and every reproductive functioning of male		In vitro pituitary cell culture, hypothalamus perfusion, histopathology, hormone assay, RIA

Adversities and Benefits of Metals on Male Reproductive System

Metals With Adverse Effects

Arsenic

Arsenic is intensely found in the environment owing to anthropogenic and natural occurrence, such as contaminated drinking water. Its intrusion in male reproductive organ affects spermatogenesis adversely, reduces the organ weights among which testis and accessory sex glands are the most mentionable. Arsenic also causes reduction of serum luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone. Its accumulation in testis, epididymis, and accessory sex glands attributes to decreased sperm count, motility, elevated abnormalities in sperm morphology, undesirable alterations in testicular enzyme activities, etc. It impinges the nocuous OS in the testicular tissues paving the way to testicular toxicity and lay deleterious impacts upon the hypothalamo-pituitary-gonadal (HPG) axis. It also causes DNA damage, germ cell deficiency, testosterone reduction, decreased sperm capacitation, and abnormal fertilization (Huang et al., 2016).

Bismuth

Men working in close proximity or in direct contact to bismuth and its compounds (such as bismuth oxychloride, bismuth subgallate, and bismuth subsalicylate), easily get inflicted by this metal. Bismuth tends to get accumulated in the lysosomes of the Leydig cells, in the testis and in the pituitary gland resulting in reduced activities of sperm creatine kinase owing to the displacement of its magnesium ions. The impairment of the Leydig cells also attributes to diminished testosterone production and thereby adversely afflicting the male reproduction.

Boron

Boron is absent in nature in elemental state but found in combination in borates, boric acid, borax, kernite, ulexite, colemanite, and borates. Boric acid is abundant in volcanic spring water. The global production of borates is as high as 2 million tons per year and mined mainly in the United States, Turkey, and Chile where the miners are the ones mostly affected by boron exposure. Boron, at low and high levels of exposure, inhibits spermiation, and epididymal sperm count respectively, thereby inducing testicular toxicity. It triggers the increase in weight, size and volume of the testis, and seminal vesicles. The testicular epithelium becomes disrupted

with very few spermatids owing to boron exposure. Moreover, spermatogonia is prompted to undergo apoptosis as can be perceived by the presence of their picnotic nuclei. Thus, boron has severe cytotoxic effects including distortion of seminiferous tubule epithelium, reduced spermiation, and hypertrophy of testis.

Cadmium

Cadmium may get access to human body via drinking water, ingested food, cigarette smoking or through inhaled air as well as dust or soil. Food crops harvested in polluted soil are the main contributors for inseminating the general population with cadmium. People are more susceptible to cadmium toxicity if they lack enough protein, calcium or iron in their body. Cadmium may interfere and impede sex hormone synthesis by acting as an EDC. High level of cadmium in blood mutilates the functioning of male reproductive system via distortion of sperm morphology, shrinkage of the testis, impairment of sperm motility, diminutions of semen volume, and decline in sperm count (Cheng et al., 2011). It is also worthy to know that this metal lays its detrimental effects on male prostate gland in the process of deregulating the level of PSA (prostate specific antigen) and morphological distortion of the gland. Its noxious effects on testicular vasculature may lead to testicular toxicity, necrosis and atrophy, damage to the germinal cells, decreasing the level of testosterone and also ravaging the accessory genital organs (Telisman et al., 2000). Owing to such far reaching damage to the reproductive organs, continuous exposure to cadmium can inculcate loss of libido and infertility in male.

Chromium

Drinking water, the ambient air, and food may expose human body to chromium or its compounds. Through any of the sources if chromium comes in contact with skin, it gets access to the body's internal environment. It is present in very low concentration in air (about $0.02 \mu\text{g m}^{-3}$) and water (< 2 ppb) in CrIII and CrVI forms. One may get exposed to chromium through wood preservative, household utensils, products used for cleaning, cement, tanned leather, and also several textiles. If we consider high level of exposure to chromium then we must focus on the workers in the industries using or processing chromium or its compounds. Both chromium III and VI are able to inflict adversities in spermatogenic process while the latter is reportedly more toxic. Hexavalent chromium (CrVI) is the widely used form of chromium in industries, and its exposure on male reproductive system may cause disturbed spermatogenesis, deteriorated semen quality, and reduced level of zinc and lactate dehydrogenase levels, all of which cumulate to impart male infertility. This may trigger OS and the following detrimental complications such as increased LPO in the testes leading to reduced sperm count and elevated sperm morphology distortions. Chromium is also capable of obstructing epididymal lumen thereby impeding the release of sperm.

Cobalt

Cobalt is an important oligoelement which is found in composition of vitamin B₁₂. Mainly beverages and foods convey cobalt to the body, several household products and cement being the other sources. The industries concerned with the use or production of various forms of cobalt such as those producing cobalt powder, using it to polish diamonds, processing cobalt alloy or preparing enamels and pigments, expose their workers to this metal. Though cobalt affects male reproductive system in a sluggish manner, in case of chronic exposure it intrudes into the normal functioning of male reproductive system thereby depressing sperm motility, testicular weight, and epididymal sperm concentration. Cobalt imposes indirect or direct repugnant effects on spermatogenesis and testosterone synthesis. Chronic exposure to cobalt may damage epididymal functioning to severe extent as the epididymis is the most susceptible organ to cobalt toxicity.

Fluoride

Human is vulnerable to fluoride exposure via toothpaste, soda, juices, fluoridated salts, and drinking water. Chronic to this metal develops structural and functional disorientation of the spermatozoa, debacle of spermatogenesis and disturbances in neuroendocrine control over reproductive functioning. Zinc deficiency, oxidative damage, and interference in signaling pathways prompted by fluorides lead to the mentioned damages to male reproductive components. Excess fluoride interferes with spermatogenesis by depressing levels of epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR), modifying G-protein signaling, diminishing levels of testosterone, and its androgen receptor (AR). It also lays its impact upon thyroid hormone directly or indirectly hampering normal spermatogenesis.

Indium

High level of exposure to indium is most prominent among the workers in semiconductor or welding industries. It foists undesirable changes to male reproductive systems such as testicular epithelium vacuolization, distortions in sperm shape and reduction in sperm count, impaired libido and sexual behavior, decreased testicular and epididymal weights. It also reduces the activities of the sperm creatine kinase. Both indium arsenide and indium phosphide are able to inflict testicular toxicity, the former being more potent. Indium containing compounds also first adversely target the Sertoli cells instead of spermatogonia.

Lead

Lead is one of the leading metals triggering occupational health hazards as a wide proportion of industry workers and researchers deal with lead and their organs are exposed to its contamination. It is noxious to male reproductive system on account of reduced sperm parameters both qualitatively and quantitatively even at blood lead levels $< 150 \mu\text{g L}^{-1}$ (Jurasovic et al., 2004). Lead leads

to decline in semen volume, sperm count as well as sperm motility, while increased abnormalities in sperm head morphology, and also afflicts prostate secretions (Telisman et al., 2000). Treatment with lead chelators may reverse the complications inflicted by lead or it may also be reversed with time after cessation of regular exposure to lead. If paternal blood contains lead of approximately $300\text{--}400\text{ }\mu\text{g L}^{-1}$ then probability of spontaneous abortions is elevated, and also of the rate of live births (Lindbohm et al., 1991).

Mercury

At the level of its being toxic, mercury adversely affects the male fertility in both organic, and inorganic forms. The globulin levels in the serum which binds sex hormones and transports them to specific tissue locations show reduction in the workers exposed to mercury vapor. With increased mercury content in the paternal urine, the rate of spontaneous abortions increases. Testes are susceptible to both inorganic and methylmercury, the uptake and clearance of the latter being faster. This restrains normal spermatogenesis resulting in reduced sperm count and motility. It deforms testicular morphology and also depress DNA synthesis in spermatogonial stem cells. Cellular distortion of the Leydig cells and the seminiferous as well as reduced testosterone level in serum are the other established complications inflicted by mercury to pave the way to male infertility.

Molybdenum

Ingestion of industrial contaminant foods or even naturally occurring food and water may expose human to this metal. Elevated usage of molybdenum rich multiminerals/multivitamin supplements and occupational liabilities for workers of certain building materials, also lead to molybdenum exposure. The most distressing consequences owing to molybdenum exposure of male reproductive system are lack of libido, pronounced reduction in weight of testis and other vital reproductive organs such as epididymis, seminal vesicles and prostate glands, almost complete inhibition of spermatogenesis owing to severe histological damage to the testes especially to the germinal epithelium and interstitial cells. Accumulation of this metal to testis and other male reproductive organs may impose alterations in the activities of essential enzymes, such as it decreases sorbitol dehydrogenase (essential for sperm motility) activity and increases gamma-glutamyl transpeptidase (essential for spermatid maturation) and LDH (essential for energy metabolism of sperm) activities. Sperms, even if produced in reduced quantity, lack proper functional morphology following high molybdenum exposure.

Nickel

Nickel salts are regarded as industrial hazards to which human gets exposed through natural components. They perpetrate toxic progression in the peripheral tissues and also in the male reproductive tract. Nickel accumulation in testis may impose toxicity by various factors which are not yet specifically established. These effects are accentuated if its exposure gets coupled to protein deficiency in the male body. Increased nickel exposure is associated with decline in weight of essential male reproductive organs and activities of steroidogenic enzymes, impaired sperm morphology with sperm tail defects rendering them immotile or poorly motile. Nickel propels its toxicity via generation of free radicals leading to OS in the male reproductive tissues, evidenced through DNA damage, reduced total protein in testis, sperm count decrements, increase in the level of OS markers like LPO and induction of apoptosis in the testicular cells. The OS and the associated reproductive damage cumulate to impair male fertility. Besides being rigorously toxic, this metal also possesses some beneficial prospects to the male reproductive system since at low concentration it actually stimulates sperm motility, which is otherwise inhibited at high nickel concentration.

Silver

Silver is a precious metal which occurs naturally in the environment normally in the form of metal ore. In small quantity, it is regarded as beneficial to general health as it possesses certain antimicrobial capabilities. But in case of high level of exposure, silver is potent to reduce the activity of creatine kinase by displacing the Mg^{2+} from the enzyme thus impairing sperm energy homeostasis. Silver nanoparticles are able to percolate through the blood-testes barrier and get deposited in the testis where they can bind to the testicular tissues and induce generation of free radicals thereby initiating OS, toxicity and eventually cell death effects on sperm cells (McAuliffe and Perry, 2007).

Vanadium

Vanadium is an industrial hazard which inculcates its adverse effects on male reproductive system via ROS induced pathway which inflict toxicity in the germ cells bringing about reduced levels of gonadotropin and testosterone which in turn decline the sperm count. Gamma tubules are integral components of the microtubules which are vital for cell division thereby for spermatogenesis. Vanadium alters the structure of these gamma tubules poisoning inhibition to sperm production. Other indirect effects of vanadium on male reproductive system consists of hypertrophy of the adrenal gland causing increased secretion of glucocorticoids, elevated activities of the adrenal steroidogenic enzymes and consequently increased testicular LPO initiating damage to the cells.

Metals With Beneficial Effects

Gold

Gold is considered as one of the immensely precious and auspicious metals with enormous goodwill of having innumerable health benefits since ancient treatment regime. It is being utilized in "*Ayurveda*" for ailing several malaises, infertility of men being one. The

quantity of gold in human body is petite, and in semen, being < 1 mg mL may be detected (Jain et al., 2010). There is controversy regarding the source of gold in human body which may be the exposure to sea water or in vivo conversion of digested entities into gold. Although gold is hypothesized to lay ameliorating effects on male reproductive health, the beneficial effects of gold upon male reproductive is yet to be revealed with proper mechanisms.

Magnesium

Magnesium is vital for essential physiological processes and acts as cofactors for several enzymes in crucial energy metabolic pathways and biosynthesis of nucleic acid and proteins. Consumption of magnesium rich food and through hard water in various parts of the world are the sources through which magnesium get access inside the body. The observed consequences of intake of excess magnesium in the diet are enhancement of male fertility by improving the functions of testicular steroidogenic enzymes (Δ^5 3 β -HSD and 17 β -HSD) and testosterone level. It also improves other reproductive hormone levels (FSH and LH) in blood, and reduces OS in male gonadal system (Chandra et al., 2013). Magnesium is reported to improve sperm count in men and germ cell viability in male gonads.

Zinc

Zinc is essential for metabolic, morphologic as well as physiologic aspects of the male reproductive system. Zinc is omnipresent in serum and its constituents in considerable concentration. Its deficiency retards testicular development, degenerates tubular epithelium and decreases DNA, RNA, and protein contents of the vital reproductive organs. Zinc reportedly contributes to sperm count, motility, fertility capacity, and to improve the physical features of the sperm and the physical characteristics of sperm in infertile men (Hidioglou and Knipfel, 1984). Zinc can regulate male reproductive functioning via imposing stimulatory effects to pituitary gonadotropin and in turn testosterone by the activation of steroidogenic enzymes, while it is also linked with the—SH groups and disulfide linkages within the spermatozoa at the tail region. Zinc apparently regulates the contraction and the motility of sperm by controlling the energy utilization via adenosine triphosphate system and phospholipid energy reserves. Zinc is, thus, an imperative metal for customary agility of male reproductive organs and its deficiency is noxious to male fertility.

Metals With Dual Effects

Calcium

The trend of immense use of calcium supplementation through commercial food products, drugs, etc. are the heinous causatives for excessive exposure to calcium. Hard water contains calcium in abundance and drinking the same aids calcium access to the internal environment of body. Though calcium owns pivotal role in male reproductive functioning including different levels of sperm maturation, its detrimental effects on the same are also not under veils. Besides being a chemocastrative culprit, it is also considered to be a generator of free radicals promoting oxidative damage to the testicular germ cells, Leydig cells and interfere in the interactions of hypothalamus-pituitary-adrenal (HPA) and HPG axes. These, through a sequel of events, disrupt the normal spermatogenesis. Excess calcium exposure decreases weight of testis and accessory sex organs; reduces epididymal sperm count; declines activities of vital testicular enzymes such as Δ^5 3 β -hydroxy steroid dehydrogenase (HSD) and 17 β -hydroxy steroid dehydrogenase; diminishes serum levels of LH, FSH, and testosterone; and degenerative changes in the testicular histoarchitecture (Chandra et al., 2012).

Copper

Copper reaches the general population in abundance through innumerable sources of food, air, and water. This trace element is necessary for proper physiological functioning of the human body including fertility, but above a physiological limit it paves the way to eminent adverse implications including severe problems in the male reproductive system. As copper is an essential part of antioxidant enzymes, and involved in several redox reactions with iron, it improves male reproductive functions. But, people at close proximity to the smelters developing copper ore into metal are the ones mostly exposed to high range. Copper plumbing at homes also lead to significant exposure of this metal, owing to pipe corrosions emancipating copper into drinking water. Male reproductive system, when exposed to copper over prohibitive extent, is subjected to undergo distortion, and deregulations. It is severely detrimental to the spermatozoa mobility. It is able to bind to pituitary receptors ravaging LH regulations and to the FSH receptors to impede spermatogenesis. It interferes in oxidative process and reduces glucose consumption leading to immobility of spermatozoa and so, it is used for intrauterine devices (IUDs) in female whereas inflicts toxicity when used for male contraceptive purposes.

Iron

Iron is an essential element playing important role in male fertility. However, it can cause toxic effects when accumulating in large quantities. Anthropogenic sources of iron include steel industry, sewage, and mine-dust. Within the male reproductive system, Sertoli cell and Leydig cells are chief sources of ferritin. This protein acts as a readily accessible source of iron for developing sperm, while providing an extra layer to the testicular tissue. As an integral part of antioxidant enzymes, it protects germ cells from oxidative damage. It is also involved in cellular differentiation, proliferation, and metabolism. It is involved in several redox reactions in germ

cell, and thus energy metabolism and sperm motility. Iron deficiency anemia and thalassemia is known to be associated with male infertility, whereas iron overload causes testicular atrophy, morphological changes, impaired spermatogenesis, epididymal lesions, and impaired reproductive functions.

Manganese

Human body gets exposed to manganese through the ambient air where they occur naturally and also gets accumulated through mines and gasoline additives. It shows biphasic action on male reproductive system: being an integral part of antioxidant enzymes, it reduces OS in male reproductive system, while exposed in excess concentration, it disrupts reproductive functions. Manganese is considered essential to restore the oxidative balance in the sperms and neurons by impeding LPO generated by free radicals. It is an integral component of various enzymes involved in redox reactions while manganese itself acts as an oxidative chain breaker being able to quench peroxy radicals.

The clinical complications in the male reproductive system owing to the excess exposure to this metal may be reduced libido even impotency and the origin of manganese impacts are partly neurological. In case, manganese level exceeds the permitted level in blood, men can experience testicular tissue degenerations, reduction in testicular weight, sperm motility and sperm count, and also decline in serum levels of FSH and testosterone.

Selenium

Selenium is a vital trace element which is essential to maintain proper physiological functioning which also aid male reproductive operations such as antioxidant defense systems, immune function, and thyroid metabolism. Spermatogenesis and maintenance of semen quality are aided by two vital selenoproteins, phospholipid hydroperoxide glutathione peroxidase (PHGPx/GPx4), and selenoprotein-P, the former being the major one expressed by the testicular germ cells. Selenium deficiency leads to decline in sperm motility and sperm morphological distortions, including splintering at the mid-piece and abnormal shape of the sperm head. The antioxidant enzyme, glutathione peroxidase, contains selenium, and is inevitable for maintaining oxidative stability in the male reproductive cells.

The adverse effects of selenium dioxide on the testis and the observations such as oligospermia, intertubular edema, and degeneration of spermatids owing to chronic exposure to selenium, are the contrary effects of this otherwise beneficial metal. Moreover, in case of sustained high level exposure to selenium may be more detrimental as it can inhibit the testicular steroidogenic enzyme activities and markedly reduce the tubular areas, diameters and their perimeters obstructing sperm movement and ejaculation, thereby fostering male infertility (Boitani and Puglisi, 2008).

Mechanism of Action

Direct Effect

Metals interfere with the normal male reproductive functioning by thwarting the neuroendocrine regulation (pretesticular) or by acting directly upon the vital reproductive cells (testicular and posttesticular). In order to achieve access to the internal components, they must scout out the ways to enter the cells. In most of the cases, metals mimic essential endogenous ligands in order to bind to their receptors and in the process, they enter the respective cell to disorient the concerned signaling pathways resulting in reproductive disruption. For example, transferrin that transports iron may turn responsible for cellular toxicity by allowing excess iron as well as other metals which may mimic iron, like nickel, manganese, cobalt, copper, and zinc. Similarly, ZIP transporters may transfer some toxic metals, along with its patent ligand, zinc in excess. The phosphate/sulfate transporters aid the ingress of toxic metals which resemble phosphates and sulfates such as metal oxyanions, selenite, vanadate, and arsenate. Metals pave their way into the reproductive cells also by forming organic complexes, for example methylmercury may combine with cysteine to mimic methionine and with glutathione to mimic glutathione in order to use L-type neural amino acid carrier and glutathione carrier respectively. Lead mimics calcium to probe into the cells through the voltage-gated calcium channels.

While internalized, metals impede cellular signaling cascades within the male reproductive cells. Metals, such as arsenic, cadmium, chromium. Cobalt and nickel, targets the activation of ROS inducing oxidative damage to cytoplasmic components, lipid membrane as well as DNA and other cellular micromolecules. They also interfere MAPKs activities to bring about uncontrolled cell proliferation by inhibiting apoptosis. Arsenic, nickel, and copper are able to stimulate PI3K pathway to accelerate cellular growth induce HIF1 and thus establish a pseudohypoxia stimulating tumorigenesis, angiogenesis etc. They also lead to dislodging of I κ B from the NF- κ B resulting in unregulated activation of NF- κ B which in turn initiates carcinogenesis. The NFAT and AP-1 mediated signaling pathways may also get unnecessarily activated by the interfering metals leading to undesirable cellular differentiation, inflammatory responses and impaired repair process.

These cellular massacres by toxic metals increases cellular toxicity of reproductive cells. Leydig cells necrosis diminishes testosterone level which causes germ cell death and atrophy of secondary organs. In the Sertoli cells, the implications of metals distort cellular morphology and thus inhibit sperm development. Metal toxicity causes spermatogonial damage, perturbed growth, and death while damage to the spermatids' morphology give rise to abnormal sperms. The toxic progressions intervene

into the blood vessels supplying the testicles where endothelial necrosis lead to reduced blood flow thereby initiating oncotic necrosis and cellular apoptosis. In the epididymis and vas deferens, effects of metal insemination results in blockage, rupture of epithelial cells lining the lumen and formation of immotile sperm granulomes. Metals, if perpetuate inside the prostate and seminal vesicles, they adversely alter the intracellular mechanisms and curtail their secretions. Hence, the metals induct into the male reproductive cells, take up varied routes to ultimately establish a grievous toxic scenario and curb every vital male reproductive function (Fig. 2).

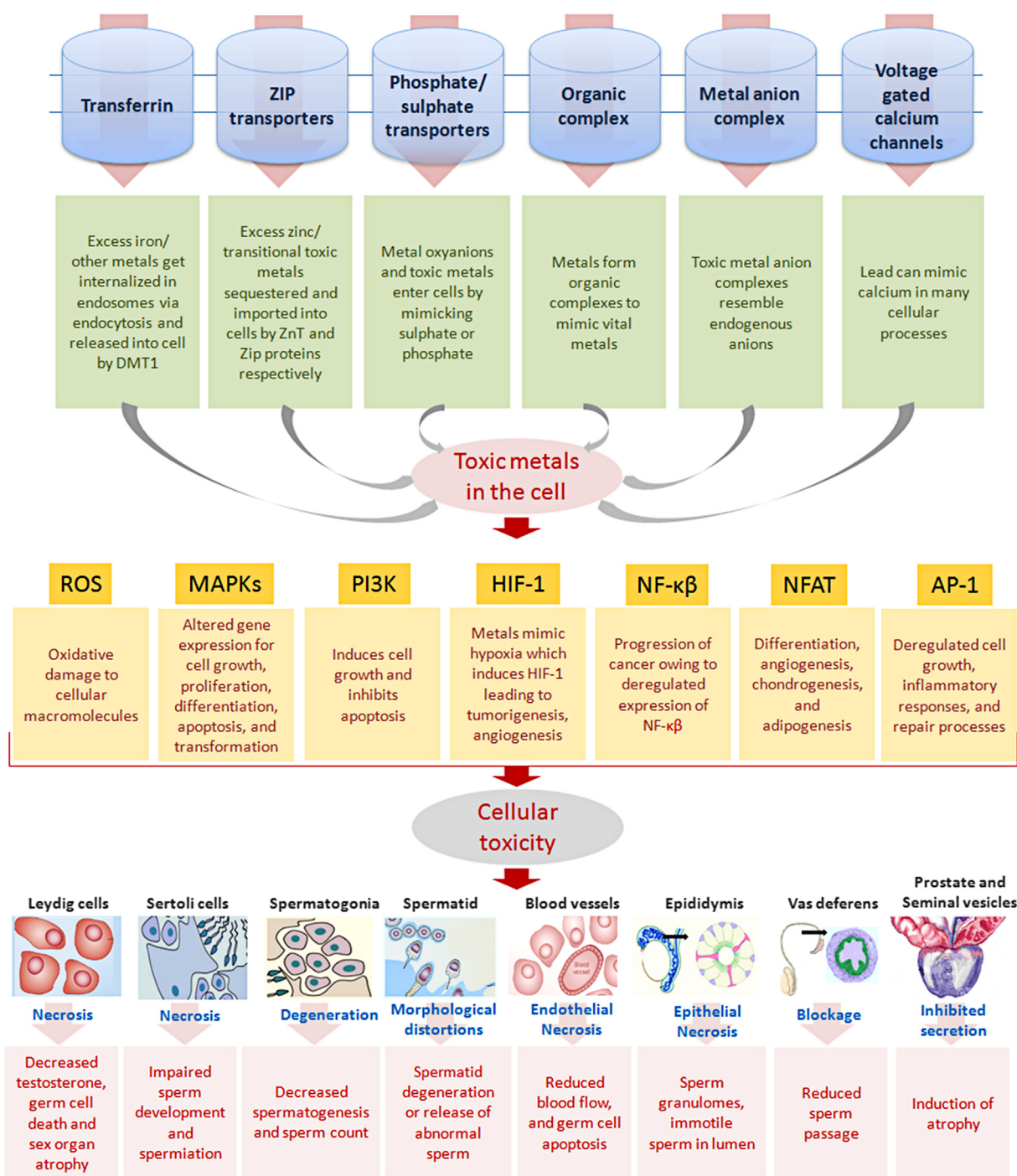


Fig. 2 The key routes of entry of toxic metals inside the reproductive cells and their mechanisms of action. Within these cells they disrupt various subcellular signaling pathways resulting in cellular toxicity that leads to various reproductive functional dysregulations.

Indirect Effect

The indirect effect of metals is mediated through the cross-talk among different endocrine axes. Most of the metals, following exposure, generate physiological stress and thus activate HPA axis to release the *stress hormone*, cortisol. Cortisol, through the cross-talk with HPG axis, restrains the secretion of LH and thus testosterone from Leydig cells (IPCS, 2002). Chronic exposure of toxic metals affects FSH release and impaired spermatogenesis. Activation of HPA axis is also concerned with generation of ROS and oxidative damage of gonadal cells. Some metals also impact on thyroid gland and disrupts hypothalamo-pituitary-thyroid (HPT)-HPG cross-talk to interfere the thyroid hormone mediated endocrine control of spermatogenesis (Rajender et al., 2011). By affecting the interplays among the different neuroendocrine axes with male gonadal system, toxic metals hinder normal endocrine control and spermatogenesis as well as sperm maturation to induce male infertility.

References

- Boitani, C., & Puglisi, R. (2008). Selenium, a key element in spermatogenesis and male fertility. *Advances in Experimental Medicine and Biology*, 636, 65–73.
- Chandra, A. K., Sengupta, P., Goswami, H., & Sarkar, M. (2013). Effects of dietary magnesium on testicular histology, steroidogenesis, spermatogenesis and oxidative stress markers in adult rats. *Indian Journal of Experimental Biology*, 51, 37–47.
- Chandra, A. K., Sengupta, P., Goswami, H., & Sarkar, M. (2012). Excessive dietary calcium in the disruption of structural and functional status of adult male reproductive system in rat with possible mechanism. *Molecular and Cellular Biochemistry*, 364, 181–191.
- Cheng, C. Y., Wong, E. W., Lie, P. P., et al. (2011). Environmental toxicants and male reproductive function. *Spermatogenesis*, 1, 2–13.
- Hidiroglou, M., & Knipfel, J. E. (1984). Zinc in mammalian sperm: A review. *Journal of Dairy Science*, 67, 1147–1156.
- Huang, Q., Luo, L., Alamdar, A., et al. (2016). Integrated proteomics and metabolomics analysis of rat testis: Mechanism of arsenic-induced male reproductive toxicity. *Scientific Reports*, 6, 32518.
- International Programme on Chemical Safety (IPCS). (2002) International Programme on Chemical Safety (IPCS). *Global assessment of the state-of-the-science of endocrine disruptors*. Geneva: World Health Organization.
- Jain, V., Rai, A., Misra, S., & Singh, K. M. (2010). Seminal gold content in healthy fertile men in India. *International Journal of Ayurveda Research*, 1, 172–174.
- Jurasovic, J., Cvitkovic, P., Pizent, A., Colak, B., & Telisman, S. (2004). Semen quality and reproductive endocrine function with regard to blood cadmium in Croatian male subjects. *Biometals*, 17, 735–743.
- Lindbohm, M. L., Sallmen, M., Anttila, A., Taskinen, H., & Hemminki, K. (1991). Paternal occupational lead exposure and spontaneous abortion. *Scandinavian Journal of Work Environment Health*, 17, 95–103.
- McAuliffe, M. E., & Perry, M. J. (2007). Are nanoparticles potential male reproductive toxicants? A literature review. *Nanotoxicology*, 1, 204–210.
- Rajender, S., Monica, M. G., Walter, L., & Agarwal, A. (2011). Thyroid, spermatogenesis, and male infertility. *Frontiers in Bioscience*, (3), 843–855.
- Sengupta, P., Dutta, S., & Krajewska-Kulak, E. (2017). The disappearing sperms : Analysing the reports published between 1980 and 2015. *American Journal of Men's Health*, 11, 1279–1304.
- Sengupta, P. (2013a). Potential health impacts of hard water. *International Journal of Preventive Medicine*, 4, 866–875.
- Sengupta, P. (2013b). Environmental and occupational exposure of metals and their role in male reproductive functions. *Drug and Chemical Toxicology*, 36, 353–368.
- Telisman, S., Cvitkovic, P., Jurasovic, J., et al. (2000). Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men. *Environmental Health Perspectives*, 108, 45–53.

Further Reading

- Sengupta, P., Banerjee, R., Nath, S., Das, S., & Banerjee, S. (2015). Metals and female reproductive toxicity. *Human and Experimental Toxicology*, 34, 679–697.

Anogenital Distance: A Marker of Steroidal Endocrine Disruption

Shanna H Swan, Icahn School of Medicine at Mount Sinai, New York, NY, United States
David M Kristensen, University of Copenhagen, Copenhagen, Denmark

© 2018 Elsevier Inc. All rights reserved.

Anogenital Distance: Definition and Brief History

Anogenital distance (AGD) is defined as the distance from the anus to the genitals. In rodents, the distance is defined by a single measurement, as the genital tubercle is a single point at birth. In human infants and adults, several genital landmarks have been used to measure AGD, with two measures used most frequently. In males, the shorter of the two most frequently used measures is the anoscrotal distance (anus to posterior insertion of scrotum) or AGD_{AS}, while the longer of the two measures, the anopenile distance (anus to anterior insertion of penis) or AGD_{AP} is, on average, twice as long as the anoscrotal distance in newborn male infants (see Fig. 1A). The corresponding measures in females are the anoclitoral distance (AGD_{AC}) and the anofourchette distance (AGD_{AF}) (Fig. 1B). This article will only discuss male AGD.

AGD has been used to identify offspring sex since 1912 (Jackson, 1912). It has been used as a marker of reproductive toxicity since 1996 when the United States Environmental Protection Agency (USEPA) identified AGD as an endpoint for animal-based reproductive toxicity assay (USEPA, 1996). The first studies of AGD in human populations suggested that AGD is under hormonal influence during fetal development (Salazar-Martinez et al., 2004), but it was not used as a measure of human reproductive toxicity until 2005 (Swan et al., 2005a).

Several measurement methods have been used in both infants and adults. One study compared the feasibility and reproducibility of adult male AGD measurements by two of the most commonly used methods ("lithotomy" or "frog-legged" position) (Mendiola et al., 2016). In this study men attending an outpatient clinic at a university hospital underwent an andrological examination at which AGD_{AS} and AGD_{AP} were measured in 70 men by these two methods. The overall agreement ($ICC \geq 0.80$) was similar and acceptable for both measures, and both methods were deemed adequate for epidemiological studies although the two methods differed systematically in their AGD measurements. Similarly, two methods have been used in measuring infant AGD. While no published study has systematically compared these methods in infants, unpublished data demonstrate that the coefficients of variation for the two methods are similar.

AGD and Body Size

Like all anatomical structures, AGD increases with age and body size. Because of the dependence of AGD's on body size, rodent studies frequently use an anogenital Index (AGI) (AGD divided by a function of body weight). Some human studies have also used AGI (Swan et al., 2005b; Loreto-Gomez et al., 2017) though most human studies have used the untransformed AGD measurement and included a term for body size (a function of weight or height) in multivariable models (Swan, 2008; Swan et al., 2015a).

Stability of AGD in Rodents and Humans

The stability of (body-size adjusted) AGD (referred to here as Adj-AGD) across the lifespan is an important issue. Data from multiple rodent studies indicate that (body-size adjusted) AGD is permanent. Several studies have examined the stability of

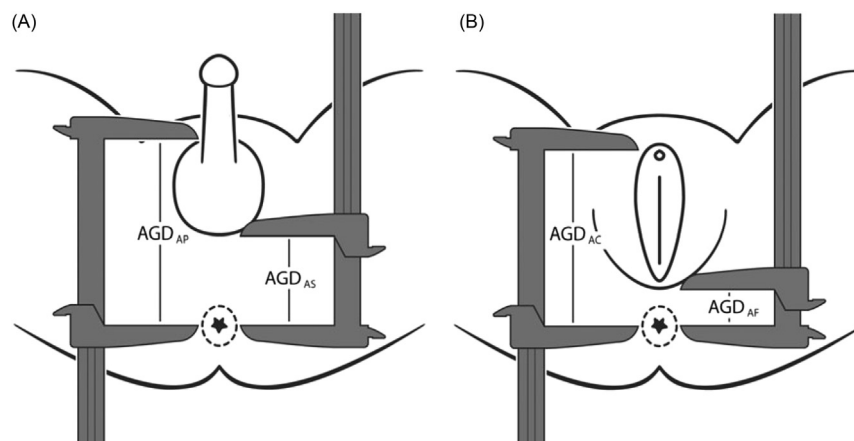


Fig. 1 Alternative measures of anogenital distance in males and female infants. From: Swan, S.H., Sathyanarayana, S., Barrett, E.S., Janssen, S., Liu, F., Nguyen, R.H.N., Redmon, J.B. and the TIDES Study Team. (2015). First trimester phthalate exposure and anogenital distance in newborns. *Human Reproduction*. **30**(4), 963–972.

Adj-AGD as rodents age and have found little change in Adj-AGD over time, although recent data (Kita et al., 2016) suggest that post-natal exposure to exogenous androgens and anti-androgens may alter this measurement. Body-size-adjusted AGD appears stable in humans, but to date there are no human populations in which AGD has been measured both in infancy and again in adulthood to confirm this. A recent unpublished analysis of The Infant Development and the Environment Study (TIDES) compared AGD in 281 boys at birth and 1-year of age and found that within-child measurements correlated significantly ($r = 0.56$ for AGD_{AS} and 0.51 for AGD_{AP}, $P < 0.0001$ for both). Further longitudinal follow-up is needed before concluding that Adj-AGD is stable over the lifespan.

Mechanisms of Action of Anti-Androgens on Male AGD

The term anti-androgen and AGD are closely entwined. The term “anti-androgen” is used to describe a class of compounds that oppose the action of steroidal androgenic hormones. The pharmacologic uses of anti-androgens began in the 1960s when these compounds were first prescribed to treat androgen-dependent conditions. It was not until the 1990s that the term was used to characterize an anthropogenic compound in the environment with endocrine action. In the same decade, studies performed in rats showed that the AGD is sensitive to anti-androgens, reflecting androgen action during fetal life (Gray et al., 1999). This research was followed by the discovery that AGD reflects fetal androgen exposure only within a specific “masculinization programming window” (Welsh et al., 2008).

Critical Developmental Windows for AGD in Rodents and Humans

The spectrum and severity of effects induced by EDCs are largely dependent on timing of exposure. Masculinization, a process driven by gonadal hormones at critical time windows in utero, is particularly sensitive to endocrine disruption. Androgen action during the MPW is essential for the differentiation of internal and external male genitalia and to ensure that reproductive tissues are responsive to subsequent hormonal stimuli (Welsh et al., 2008; Macleod et al., 2010). In rats, the MPW occurs during gestation days 15–18, whereas in humans it is believed to occur between weeks 8–14 (Welsh et al., 2008). Suboptimal androgen action during the MPW may result in short (feminized) anogenital distance (AGD) and several reproductive tract abnormalities, including abnormal development of sex accessory organs, micropenis, and hypospadias (Macleod et al., 2010; van den Driesche et al., 2011). Experimental manipulation of pre- and postnatal androgen environments in rodents suggests that adequate androgen exposure during the male programming window (MPW) is critical to achieving male-typical AGD length, although subsequent growth and development outside the MPW may require additional androgen exposure (Macleod et al., 2010; van den Driesche et al., 2011).

It is now evident that exposure to compounds that interfere with androgen action, including disruption of androgen production (e.g., dibutyl phthalate, ketoconazole, and acetaminophen) or androgen receptor signaling (flutamide) may result in abnormal male reproductive development if the exposure occurs during the MPW (embryonic days 15.5–19.5) in the rat. This is a time window that precedes morphological differentiation of the male tissues, where the androgens are not pivotal. Hence, it is only when blocking androgen action in the MPW that hypospadias and cryptorchidism and altered penile length in male rats can be induced, all of which are correlated with AGD.

In humans, the sexual dimorphism in AGD is evident by weeks 11–13 gestation increasing till week 17 and remaining approximately 50%–100% longer in males than in females (Fowler et al., 2011). To date, only one study in humans has examined the effect of varying exposure timing to identify the most sensitive period for AGD development. That study compared associations between maternal anti-androgen exposure (prenatal exposure to the phthalate diethylhexyl phthalate, DEHP, in three trimesters) and genital morphology (Martino-Andrade et al., 2016). Concentrations of DEHP metabolites in first trimester urine samples were inversely related to male AGD, while no appreciable associations were seen between AGD measures and any DEHP metabolite in the second and third trimesters. These data are consistent with the MPW for AGD development in rodent studies.

Clinical Correlates of Male AGD

Numerous rodent studies have reported associations between anti-androgen exposure and the joint occurrence of short AGD and impaired genital tract development (Foster, 2006; Ema et al., 2003). Congenital abnormalities of the genitalia (hypospadias, cryptorchidism) are, together with shortened male AGD, visible at birth, supporting the conclusion that these conditions reflect disruption of reproductive tract development during the fetal period (Dean and Sharpe, 2013). Several recent studies suggest that a short male AGD has implications for reproductive health in humans as well as rodents. Boys with genital defects (hypospadias and cryptorchidism) have significantly shorter AGD_{AS} than controls (Hsieh et al., 2012; Thankamony et al., 2013). In adult males, shorter AGD by either measure is associated with poorer semen quality and reduced testosterone and testicular volume (Eisenberg et al., 2012). In fact, short male AGD may be considered as one of several clinical markers of the Testicular Dysgenesis Syndrome (TDS) (Skakkebaek et al., 2001). While females are not the focus of this article, it is important to note that altered AGD may have implications for female. As well as male, reproductive development. Longer female AGD has been associated with conditions linked to increased androgenization in females such as congenital adrenal hyperplasia (Callegari et al., 1987) and multifollicular ovaries (Mendiola et al., 2012). In rodents, a shorter female AGD has been associated with reduced follicle counts in adulthood and

decreased fertility (Holm et al., 2016). AGD may therefore be an important biomarker of reproductive health in both males and females.

AGD as a Measure of Reproductive Toxicity

Brief Review of the Literature

At the turn of the 21st century, prenatal exposure to several phthalates, a class of environmentally pervasive industrial chemicals, was shown to cause a cluster of reproductive tract changes in male rodents. In 2000, Gray et al. showed that prenatal exposure to diethylhexyl phthalate (DEHP) and butyl benzyl phthalate (BBP) reduced male AGD by 30% (Gray et al., 2000). In 2006 Foster et al. reported that DEHP, BBP as well as dibutyl phthalate (DBP) produced a syndrome of male reproductive abnormalities that included demasculinization of the growth of the perineum resulting in a reduced anogenital distance (AGD) (Foster, 2006). Other changes included: malformations of the epididymis, vas deferens, seminal vesicles, prostate, external genitalia (hypospadias), cryptorchidism and testicular injury together with retention of nipples/areolae. The authors termed this cluster of endpoints the phthalate syndrome and noted that this syndrome had parallels with the human testicular dysgenesis syndrome (TDS). TDS was the term given to the cluster of interrelated male reproductive abnormalities in 2001 (Skakkebaek et al., 2001). While the phthalate syndrome and TDS are distinct, the former being defined in rodents, while the latter is defined in humans, short male AGD is now considered a component of both syndromes (Skakkebaek et al., 2016).

Because of the strong rodent data on the reproductive toxicity of phthalates and the ubiquitous exposure to phthalates, most human studies (10 studies) that have examined prenatal exposure to environmental chemicals have focused on phthalates (5 studies). However, studies showing associations between pre- and perinatal exposure to bisphenol A (BPA) (2 studies), DDE (2 studies) and dioxin (1 study) are available. In contrast, one study found that levels of the antiandrogen 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (DDE) did not correlate with male infant AGD (Longnecker et al., 2007). These studies are summarized in Table 1. Here we provide a summary of results from the Study for Future Families (SFF), the study that initially reported associations between male AGD and prenatal exposure to an anti-androgen (phthalates) and the follow-up study that confirmed these associations; The Infant Development and the Environment Study (TIDES).

The Study for Future Families (SFF)

The Study for Future Families (SFF) examined AGD and other genital measurements in relation to prenatal phthalate exposure in humans. Standard measures of AGD (anopenile and anoscrotal) were obtained in 134 boys at 2–36 months of age as well as penile volume and degree of testicular descent. AGD was significantly correlated with penile volume ($R = 0.27$, $P = 0.001$) and the proportion of boys with incomplete testicular descent ($R = 0.20$, $P = 0.02$). This initial publication from SFF used anogenital index (AGI), defined as AGD divided by weight at examination [$AGI = AGD/\text{weight (mm/kg)}$] to examine age-adjusted AGI in relation to phthalate metabolite concentration in prenatal urine samples (median week 27 gestation in regression and categorical analyses that included all data available in 2005 ($n = 85$) (Swan et al., 2005b). The study reported that urinary concentrations of four phthalate metabolites [monoethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), monobenzyl phthalate (MBzP), and monoisobutyl phthalate (MiBP)] were inversely related to AGI. This study also examined a phthalate mixture using a summary phthalate score and the age-adjusted AGI decreased significantly with increasing phthalate score (p -value for slope = 0.009). This initial publication was followed by a second publication which included a larger data set and control of AGD for infant body size in regression analyses, rather than by using AGI (Swan, 2008). This second publication looking at AGD and prenatal phthalate exposure in SFF included 106 boys, 68 with two AGD measurements. Mixed models were used to include multiple measurements per child and body size was controlled by including a term for weight percentile (weight-for-age based on was determined by sex-specific estimates of weight percentiles in the US population (<http://www.cdc.gov/growthcharts/>). All metabolites of diethylhexyl phthalate (DEHP), diethyl phthalate (DEP) and dibutyl phthalate (DBP) were significantly associated with shorter AGD, as was the summary phthalate score. For all metabolites, an interquartile increase in concentration was associated with a 3%–5% shorter AGD.

The Infant Development and the Environment Study (TIDES)

The Infant Development and the Environment Study (TIDES), which recruited women in their first trimester of pregnancy in four US cities in 2010–2012, was designed as a follow-up to SFF and included larger sample size ($N = 366$ boys), phthalate measurement in urine from all three trimesters and AGD measurements in boys at birth and 1 year of age. In this population, concentration of DEHP metabolites were significantly reduced relative to those in SFF women, in whom phthalates were measured 10 years earlier. Despite these lower exposure levels, three metabolites of DEHP were significantly and inversely associated with both measures of boys' AGD. Associations (b, 95% confidence interval (CI)) between AGD_{AS} and (log₁₀) SpG-adjusted phthalate concentrations were: -21.12 (-22.16 , -20.07) for mono-2-ethylhexyl phthalate (MEHP), -21.43 , (-22.49 , -20.38) for mono-2-ethyl-5-oxohexyl phthalate (MEOHP) and -21.28 (-22.29 , -20.27) for mono-2-ethyl-5-hydroxyhexyl (MEHHP). Associations were of similar magnitude for AGD_{AP} (Swan et al., 2015a). No other phthalate metabolites were associated with any genital measurement in boys nor were phthalate metabolites associated with either AGD measure in girls. These phthalate-AGD associations were significant only for exposure in the first trimester, consistent with rodent data on the male programming window (Martino-Andrade et al., 2016).

Table 1 Male AGD and perinatal exposure to EDCs

<i>First author (year) country [citation]</i>	<i>Exposure(s)</i>	<i>Age at AGD assessment</i>	<i>Results (N for AGD/EDC associations)</i>	<i>Time of sample collection time (sample medium)</i>
Swan et al. (2005b)	DEHP, DBP, DEP, other and phthalates summary of phthalate exposure	9–27 months	MBP, MiBP, MBzP, MEP and summary phthalate score significantly and inversely associated with AGI (ano-penile AGD/weight) ($N = 85$)	Median 27 weeks (urine)
Longnecker et al. (2007)	DDE	Birth	DDE not associated with any AGD measure ($N = 781$)	Postpartum (serum)
Swan (2008)	DEHP, DBP, DEP, other phthalates and summary of phthalate exposure	9–27 months	DEHP, DBP, and DEP metabolites and summary phthalate score associated with shorter (ano-penile) AGD; ($N = 106$, 68 with two measurements)	Median 27 weeks (urine)
Torres-Sanchez et al. (2008)	DDT and DDE	3,6, 12, or 18 months	DDE (not DDT) in first trimester samples associated with shorter API (ano-scrotal AGD/coccyx-scrotal distance) ($N = 37$)	Three trimesters (serum)
Suzuki et al. (2012)	DEHP (MEHP), other phthalates	Birth	MEHP sig. associated with shorter (ano-penile) AGD/weight; other phthalates not associated ($N = 111$)	Mean 29 weeks (urine)
Miao et al. (2011)	BPA	0–17 years	Maternal occupational BPA exposure associated with shorter (ano-penile) AGD ($N = 153$)	Prenatal (air)
Bustamante-Montes et al. (2013)	Summary of phthalate exposure	Birth	Phthalate index in first trimester associated with shorter AGD (ano-penile); individual phthalates not associated ($N = 73$)	Each trimester (urine)
Vafeiadi et al. (2013)	Dioxins (dioxin-like activity)	Birth or mean 16 months	Dioxin associated with shorter AGD (ano-penile) ($N = 119$)	Postpartum (serum)
Swan et al. (2015b)	DEHP, other phthalates	Birth and 1 year	DEHP metabolites in first trimester associated with shorter AGD (both anopenile and anoscrotal); other phthalates not associated ($N = 366$)	Each trimester (urine)
Bornehag et al. (2015)	DINP, DEHP, other phthalates	3 years	DINP metabolites associated with shorted AGD (anoscrotal); other phthalates not associated ($N = 196$)	First trimester (urine)
Liu et al. (2014)	BPA	Birth	BPA not associated with AGD ($N = 137$)	Late third trimester (urine)

Most studies prior to TIDES examined AGD and prenatal EDC exposure collected samples in late pregnancy, except one other study that collected samples in the first trimester (Bornehag et al., 2015) and one which collected samples in the second trimester (Huang et al., 2008). In TIDES, all birth exam measurements (including weight and length) were measured with standardized equipment and protocols and timed to minimize variability.

Conclusions

Anogenital distance (AGD), an easily measured sexually dimorphic measurement reflecting the androgenicity of the early fetal environment has been used for over 100 years to sex newborn pups and as a measure of reproductive toxicity in rodent studies. While no epidemiological study had used this measure prior to 2004 (Salazar-Martinez et al., 2004), since that time 42 studies on human AGD have been listed in PubMed, most of these (59.5%) since 2011. These studies document the feasibility of obtaining reliable AGD measurements in infants, children and adults of both sexes. Several factors have been reported to alter infant AGD and it has been shown to have important clinical correlates. The available literature on human AGD and prenatal exposure to endocrine disrupting chemicals demonstrates the value of this measurement in human, as well as non-human toxicology. As additional studies undertake these measurements it will be possible to generate populations norms, which may be useful in clinical settings as well as epidemiological studies.

References

- Bornehag, C. G., et al. (2015). Prenatal phthalate exposures and anogenital distance in Swedish boys. *Environmental Health Perspectives*, 123(1), 101–107.
- Bustamante-Montes, L. P., et al. (2013). Prenatal exposure to phthalates is associated with decreased anogenital distance and penile size in male newborns. *Journal of Developmental Origins of Health and Disease*, 4(4), 300–306.
- Callegari, C., et al. (1987). Anogenital ratio: Measure of fetal virilization in premature and full-term newborn infants. *The Journal of Pediatrics*, 111(2), 240–243.
- Dean, A., & Sharpe, R. M. (2013). Clinical review: Anogenital distance or digit length ratio as measures of fetal androgen exposure: Relationship to male reproductive development and its disorders. *The Journal of Clinical Endocrinology and Metabolism*, 98(6), 2230–2238.
- Eisenberg, M. L., et al. (2012). The relationship between anogenital distance and reproductive hormone levels in adult men. *The Journal of Urology*, 187(2), 594–598.
- Ema, M., et al. (2003). Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate. *Reproductive Toxicology*, 17(4), 407–412.
- Foster, P. M. (2006). Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters. *International Journal of Andrology*, 29(1), 140–147. discussion 181–5.
- Fowler, P. A., et al. (2011). Maternal cigarette smoking and effects on androgen action in male offspring: Unexpected effects on second-trimester anogenital distance. *The Journal of Clinical Endocrinology and Metabolism*, 96(9), E1502–6.
- Gray, L. E., Jr., et al. (1999). Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, *p,p'*-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. *Toxicology and Industrial Health*, 15(1–2), 94–118.
- Gray, L. E. J., et al. (2000). Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. *Toxicological Sciences*, 58, 350–365.
- Holm, J. B., et al. (2016). Intrauterine exposure to paracetamol and aniline impairs female reproductive development by reducing follicle reserves and fertility. *Toxicological Sciences*, 150(1), 178–189.
- Hsieh, M. H., et al. (2012). Caucasian male infants and boys with hypospadias exhibit reduced anogenital distance. *Human Reproduction*, 27(6), 1577–1580.
- Huang, P. C., et al. (2008). Association between prenatal exposure to phthalates and the health of newborns. *Environment International*, 35, 14–20.
- Jackson, C. M. (1912). On the recognition of sex through external characters in the young rat. *Biological Bulletin*, 23(3), 171–173.
- Kita, D. H., et al. (2016). Manipulation of pre and postnatal androgen environments and anogenital distance in rats. *Toxicology*, 368–369, 152–161.
- Liu, C., Xu, X., & Huo, X. (2014). Anogenital distance and its application in environmental health research. *Environmental Science and Pollution Research International*, 21(8), 5457–5464.
- Longnecker, M. P., et al. (2007). In utero exposure to the antiandrogen 1,1-dichloro-2, 2-bis(*p*-chlorophenyl)ethylene (DDE) in relation to anogenital distance in male newborns from Chiapas, Mexico. *American Journal of Epidemiology*, 165(9), 1015–1022.
- Loreto-Gomez, C., et al. (2017). Anogenital distance: A longitudinal evaluation of its variants and indices in boys and girls of Sonora, Mexico. *Reproductive Toxicology*, 73, 167–174.
- Macleod, D. J., et al. (2010). Androgen action in the masculinization programming window and development of male reproductive organs. *International Journal of Andrology*, 33(2), 279–287.
- Martino-Andrade, A. J., et al. (2016). Timing of prenatal phthalate exposure in relation to genital endpoints in male newborns. *Andrology*, 4(4), 585–593.
- Mendiola, J., et al. (2012). Anogenital distance is related to ovarian follicular number in young Spanish women: A cross-sectional study. *Environmental Health*, 11, 90.
- Mendiola, J., et al. (2016). Comparability and reproducibility of adult male anogenital distance measurements for two different methods. *Andrology*, 4(4), 626–631.
- Miao, M., et al. (2011). In utero exposure to bisphenol-A and anogenital distance of male offspring. *Birth Defects Research. Part A, Clinical and Molecular Teratology*, 91(10), 867–872.
- Salazar-Martinez, E., et al. (2004). Anogenital distance in human male and female newborns: A descriptive, cross-sectional study. *Environmental Health*, 3(1), 8.
- Skakkebaek, N. E., Rajpert-De Meyts, E., & Main, K. M. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. *Human Reproduction*, 16(5), 972–978.
- Skakkebaek, N. E., et al. (2016). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*, 96(1), 55–97.
- Suzuki, Y., et al. (2012). Foetal exposure to phthalate esters and anogenital distance in male newborns. *International Journal of Andrology*, 35(3), 236–244.
- Swan, S. H. (2008). Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans. *Environmental Research*, 108(2), 177–184.
- Swan, S. H., et al. (2005). Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environmental Health Perspectives*, 113(8), 1056–1061.
- Swan, S. H., et al. (2015). First trimester phthalate exposure and anogenital distance in newborns. *Human Reproduction*, 30(4), 963–972.
- Thankamony, A., et al. (2013). Anogenital distance and penile length in infants with hypospadias or cryptorchidism: Comparison with normative data. *Environmental Health Perspectives*, 122(2), 207–211.
- Torres-Sanchez, L., et al. (2008). Dichlorodiphenyldichloroethylene exposure during the first trimester of pregnancy alters the anal position in male infants. *Annals of the New York Academy of Sciences*, 1140, 155–162.
- USEPA. (1996). Guidelines for reproductive toxicity risk assessment. *Federal Register*, 61(212), 56274–56322.

- Vafeiadi, M., et al. (2013). In utero exposure to dioxins and dioxin-like compounds and anogenital distance in newborns and infants. *Environmental Health Perspectives*, 121(1), 125–130.
- van den Driesche, S., et al. (2011van). Relative importance of prenatal and postnatal androgen action in determining growth of the penis and anogenital distance in the rat before, during and after puberty. *International Journal of Andrology*, 34(6 Pt 2), e578–e586.
- Welsh, M., et al. (2008). Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *The Journal of Clinical Investigation*, 118(4), 1479–1490.

Antiandrogens

Océane Albert, McGill University, Montreal, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Glossary

Androgen Steroid molecule responsible for male characteristics.

Androgen receptor Intranuclear protein that, when activated by androgens, will initiate the transcription of selected genes.

Anogenital distance The distance from the anus to the genitalia, which is a marker of exposure to androgens during fetal life. The anogenital distance is longer in males than in females.

Cryptorchidism Undescended or incompletely descended testis.

Endocrine disruptive compound An exogenous substance or mixture that alters function(s) of the endocrine system.

FSH A hormone synthesized in the pituitary gland that regulates the development, growth, pubertal maturation, and reproductive processes of the body.

Gonadotropins Hormones secreted by the anterior pituitary of vertebrates and regulating normal growth, sexual development, and reproductive function.

Hypospadias Congenital anomaly characterized by failure of the urethra to form completely.

LH A hormone synthesized by the pituitary gland that stimulates Leydig cell production of testosterone.

Testosterone An androgen produced by the interstitial Leydig cell in the testis.

Xenobiotic A chemical compound that is foreign to a living organism.

Abbreviations

5 alpha-DHT 5 alpha-dihydrotestosterone

AR Androgen receptor

ARE Androgen responsive element

BPA Bisphenol A

DBP Di-*n*-butyl phthalate

DDT Dichlorodiphenyltrichloroethane

DEHP Di(2-ethylhexyl) phthalate

DINCH 1,2-Cyclohexane dicarboxylic acid diisononyl ester

EDC Endocrine disruptive compound

FSH Follicle stimulating hormone

Gamma-HCH Gamma-hexachlorocyclohexane

HPDE 2,2-Bis-(*p*-hydroxyphenyl)-1,1,1-dichloroethane

HPTE 2,2-Bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane

LH Luteinizing hormone

***p,p'*-DDE** *p,p'*-Dichlorodiphenyldichloroethylene

PBDE Polybrominated diphenyl ether

PFAS Perfluoroalkylated substance

PPAR Peroxisome proliferator-activated receptor

StAR Steroidogenic acute regulatory protein

TDS Testicular dysgenesis syndrome

Definition

In the early 2000s, the documented increase in male reproductive disorders, should they be developmental (cryptorchidism, hypospadias), pubertal (delayed puberty, gynecomastia), or related to the adult testicular function (declining sperm quality, subfertility, testicular cancer), was theorized to be part of a common disorder named testicular dysgenesis syndrome (TDS). Conceptualized by Skakkebaek et al. (2001), the TDS hypothesis identifies adverse environmental factors acting upon a susceptible genetic background to disrupt embryonal programming and gonadal development during fetal life. Environmental factors acting as endocrine disrupting compounds (EDCs) have since then been identified as part of the TDS etiology.

When the field of endocrine disruption first emerged in the late 1960s, investigation was largely focused on environmental chemicals displaying estrogenic properties. The term antiandrogenic was used for the first time in 1994 to describe the properties of the pesticide vinclozolin. Since then, numerous compounds and/or their metabolites have been identified as antiandrogens (Table 1), herein defined as any class of xenobiotic that opposes the action of androgens by either altering androgen production, acting as an androgen receptor (AR) antagonist, or a combination of these different modes of action. An extensive body of literature now associates exposure to such compounds to antigonadotropic effects at large, including adverse effects on masculinization, morphological development of the urogenital system, secondary sex characteristics establishment, and adult testicular function.

Steroidogenesis Inhibitors

Androgens derive from cholesterol, either synthesized endogenously from acetyl-CoA or imported from exogenous sources by receptor-mediated uptake of low and high-density lipoproteins. In steroidogenic cells, cholesterol is first transported from the cytoplasm to the mitochondria under the regulation of the steroidogenic acute regulatory protein (StAR). The biosynthesis of androgens then requires a battery of oxidative enzymes to convert cholesterol, first to pregnenolone in the mitochondria, and then to various precursors leading to testosterone in the smooth endoplasmic reticulum near here. Two major families of enzymes are at play: the cytochrome P450 superfamily (i.e., CYP11A1, CYP17A1, CYP21), and the steroid dehydrogenase family (i.e., HSDB3, HSD17B3). Several environmental pollutants, among which industrial chemicals and pharmaceuticals, have been shown to exert antiandrogenic effects by acting as steroidogenic enzymes inhibitors.

Industrial Chemicals

Plasticizers

Phthalates are used as plastic emollients, matrices, solvents, and excipients in industrial applications. They are used in the production of a wide range of consumer products, including construction materials, toys, packaging films and sheets, medical tubing and blood storage bags. Because they are not covalently bound to their supports, phthalates leach out to the environment and have become ubiquitous environmental contaminants, with numerous studies reporting widespread human exposure through oral, inhalational, dermal, and parenteral routes. The most commonly studied antiandrogenic phthalates include di(2-ethylhexyl) phthalate (DEHP) and di-*n*-butyl phthalate (DBP).

Gestational exposure to phthalic acid esters in murine models produces a unique combination of phenotypes in the male offspring sometimes labeled “phthalate syndrome” that includes—depending on the window and dose of exposure—hypospadias, cryptorchidism, decreased anogenital distance, retained nipples, and epididymal and accessory glands agenesis. Histologically, in utero exposure to phthalates can translate postnatally into the presence of multinucleated gonocytes or testicular hemorrhage. At adult age, these disorders have been shown to result in impaired testosterone and sperm production. Such effects are corroborated by epidemiological studies showing associations between levels of phthalate metabolites in body fluids and reproductive

Table 1 Main environmental antiandrogens and their known mechanisms of action^a

Type	Class	Antiandrogen	AR antagonist	Steroidogenesis inhibitor
Agrochemicals	Dicarboximide fungicides	Vinclozolin	x	
		Procymidone	x	
	Imidazole fungicide	Prochloraz	x	x
	Organochloride insecticides	DDT	x	
		Methoxychlor	x	x
		Aldrin	x	
		Dieldrin	x	
		Lindane	x	x
	Phenylurea herbicide	Linuron	x	
Industrial chemicals	Brominated flame retardants	PBDEs	x	
	Plasticizers	Phthalates		x
		Bisphenol A	x	x
	Surfactants	PFASs		x
Pharmaceuticals	Mild analgesics	Acetaminophen		x
		Acetylsalicylic acid		x
		Indomethacin		x
		Ibuprofen		x
	Antifungal	Ketoconazole		x

^aClassification was established based on the available literature.

development and function disorders, such as decreased anogenital distance and higher incidence of gynecomastia in boys, or decreased testosterone and semen quality in men (Albert and Jégou, 2014). Proposed mechanisms of action for the antiandrogenic effects of phthalate include an impairment of steroidogenic enzyme gene expression (among which StAR, CYP11A1, CYP17A1, HSD3B) or a dysregulation of the peroxisome proliferator-activated receptor (PPAR) pathway.

Bisphenol A (A2,2-bis (4-hydroxyphenol) propane or BPA) is another synthetic compound used primarily in the manufacture of polycarbonate plastic and epoxy resins, and as an antioxidant or inhibitor of polymerization additive to other plastics. Sources of human exposure to BPA include food contamination, dermal exposure, and inhalation of indoor dust.

While BPA was originally known for its estrogenic activity, it is also considered to be a weak antiandrogen because of its affinity for AR and its ability to inhibit steroidogenic enzymes, such as HSD3B, CYP17A1, or HSD17B3. In rodent models, the effects of in utero exposure to BPA are still controversial, with some studies only showing decreased anogenital distance and plasma testosterone levels in male pups. However, in vitro organotypic cultures using mouse, rat, and human fetal testes showed decreased testosterone production after exposure to BPA. Intrauterine exposure to BPA also possibly induces a range of effects in the adult testis, including decreased sperm counts, DNA damage, and reduced sperm motility (Peretz et al., 2014). These effects are maintained in subsequent generations, suggesting an epigenetic reprogramming of germ cells by mechanisms that persists well beyond the initial exposure. These antiandrogenic reproductive effects are corroborated by epidemiological studies reporting associations between BPA exposure during fetal life and masculinization defects, such as shorter anogenital distance or hypospadias. In men, exposure to BPA is associated with reduced sperm quality, decreased sex hormone levels, and sexual function impairment. In applying the precautionary principle, several countries have restricted or banned the use of BPA in food contact products, especially for small children.

With increasing concern from the scientific community and emerging governmental regulations, the chemical industry substituted some of these plasticizers on the market. In some products, BPA was thus replaced by compounds such as bisphenol F or bisphenol S, while DEHP was replaced by 1,2-cyclohexane dicarboxylic acid diisononyl ester (DINCH). Recent studies however suggest that these replacements are bioactive in different settings, and may exert hazardous effects as well, although deemed safe by classical toxicology regulatory standards (Sartain and Hunt, 2016).

Surfactants

Perfluoroalkylated substances (PFASs) are synthetic organofluorine compounds with high stability and low surface tension. Insoluble in water and organic solvents, they repel water, dirt, and oils, and are used as surfactants in a wide range of products such as food packaging, paints, textiles, footwear, furniture, carpets, lubricants, waxes, and fire-fighting foam for oil fires. Human exposure occurs through food, water, and inhalation of domestic dusts contaminated with PFASs used as surfactants for impregnation of consumer goods; children crawling and playing on floors are therefore particularly exposed. Due to their hydrophobic and lipophobic properties, PFASs are readily absorbed and bind to proteins in blood serum and accumulate mainly in organs such as liver, kidney, and spleen, but also in testes and brain.

Carcinogenic in animal models, PFASs also display endocrine disruptive properties. Exposure of adult male rats to certain PFASs results in a decrease in testosterone production and steroidogenic gene expression, as well as Leydig cell hyperplasia and adenomas (Jensen and Leffers, 2008). In men, the overall evidence concerning PFAS exposure and sperm quality or androgen levels remains inconsistent, and necessitates further studies.

Pharmaceuticals

Mild Analgesics

Acetaminophen, aspirin, ibuprofen, and indomethacin are among the most commonly used over-the-counter painkillers. Due to their wide use for self-medication and prophylactic purposes, some mild analgesics are also found in wastewaters. Several independent epidemiological studies found significant associations between the intake of acetaminophen alone or in combination with nonsteroidal anti-inflammatory drugs during pregnancy and an increased risk of cryptorchidism in newborn boys. Ibuprofen consumption during pregnancy was also associated with an increased prevalence of hypospadias in male infants.

These observations lead to investigations in murine models, in which intrauterine exposure to indomethacin, acetaminophen, and aspirin reduced anogenital distance in the male offspring; the latter two also significantly reduced intratesticular testosterone levels. Direct evidence that acetaminophen, aspirin, indomethacin, and ibuprofen display antiandrogenic properties was also provided using ex vivo organotypic culture of rat fetal, human fetal, and human adult testes (Kristensen et al., 2016), although extremely sensitive to dosage and windows of exposure. The precise mechanisms underlying the antiandrogenic action of mild analgesics are still unknown; one proposed mechanism involves the inhibition of steroidogenic enzyme gene expression, such as CYP17A1.

Androgen Receptor Antagonists

During fetal and early life, androgens are responsible for the differentiation, growth, and development of the male urogenital system, accessory sex glands, and external genitalia. After this initial action, the testis remains quiescent until puberty, when gonadal

activity increases under the control of gonadotropins. Acting in multiple primary loci, the elevated circulating levels of androgens, both testosterone and 5 alpha-dihydrotestosterone (5 alpha-DHT), are then responsible for the establishment of secondary sex characteristics and the initiation of spermatogenesis.

Androgen action is mediated through the androgen receptor, a member of the nuclear receptor superfamily with specified structural domains that define their molecular actions as ligand-inducible transcription factors (Fig. 1). In target cells, androgens diffuse passively into the cytoplasm and bind to the AR forming dimers in response. This binding is hypothesized to result in a conformational change exposing the receptor's DNA-binding domain and enabling it to bind to androgen response elements (AREs) on the nuclear chromatin, thus initiating gene transcription.

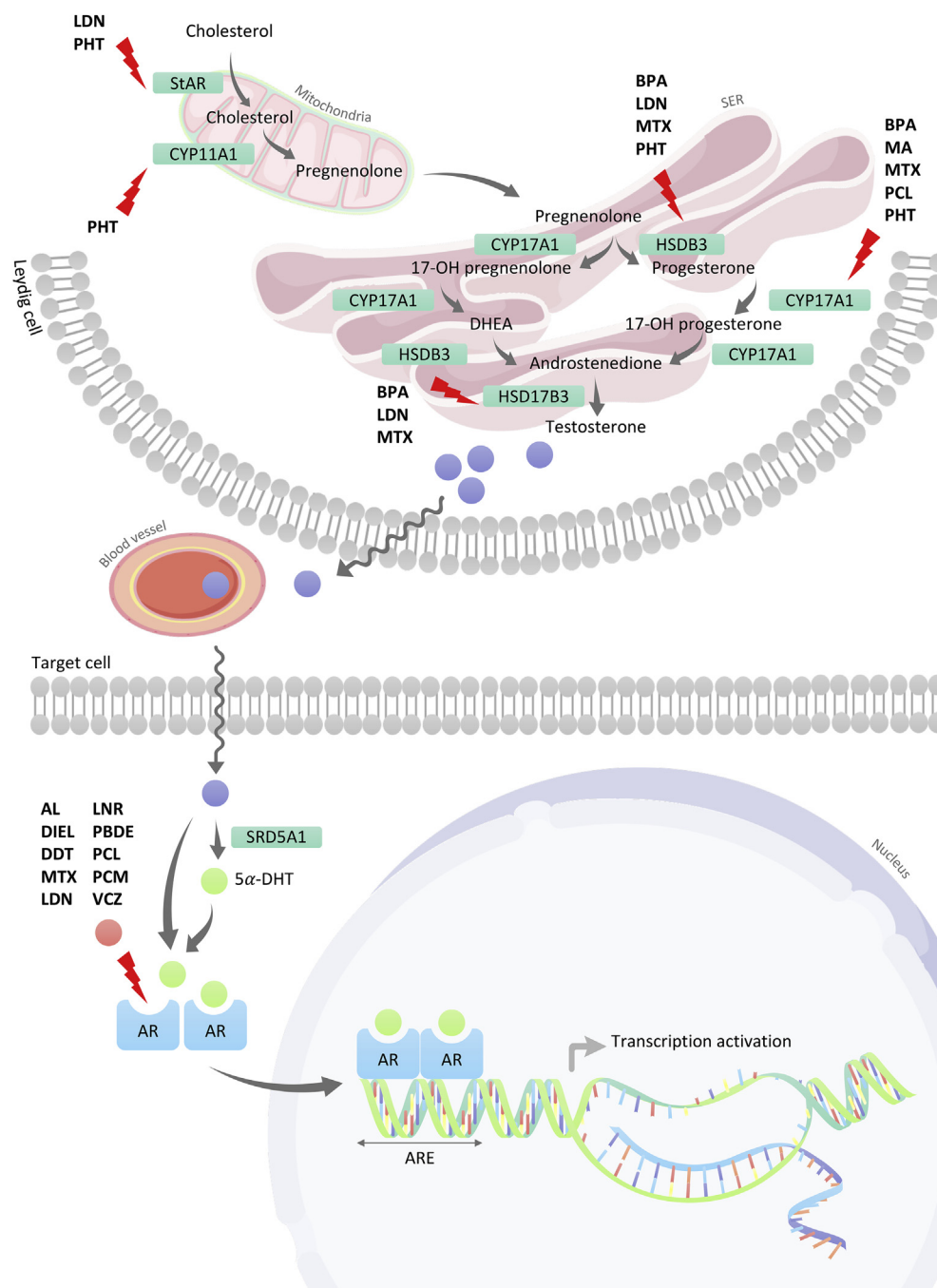


Fig. 1 Targeted effects of environmental antiandrogens. *AL*, aldrin; *AR*, androgen receptor; *ARE*, androgen responsive element; *BPA*, bisphenol A; *DDT*, dichlorodiphenyltrichloroethane; *DIEL*, dieldrin; *LDN*, lindane; *LNR*, linuron; *MA*, mild analgesics; *MTX*, methoxychlor; *PBDE*, polybrominated diphenyl ether; *PCL*, prochloraz; *PCM*, procymidone; *PHT*, phthalates; *SER*, smooth endoplasmic reticulum; *VCZ*, vinclozolin.

Several environmental antiandrogens adversely affect the male reproductive development and function by acting as AR antagonists. These compounds bind to the AR with moderate affinity and induce a conformation that either fails to protect the AR against degradation, is incompatible with AR dimerization, or prevents binding to the AREs, resulting in impaired transcriptional activation. Among them are agrochemicals and industrial additives.

Agrochemicals

Fungicides

Vinclozolin and procymidone are dicarboximide fungicides used to control diseases, such as blights, rots and molds in vineyards and on certain fruits and vegetables. Workers can be exposed to them during handler activities such as mixing, loading, applying and flagging, or by re-entering treated sites. Exposure to vinclozolin and procymidone can also occur non occupationally to golfers playing on treated golf courses and homeowners coming into contact with sod which has been previously treated on a sod farm, or through residues in food and water.

Vinclozolin and its two primary metabolites, 2-[[[(3,5-dichlorophenyl)-carbamoyl] oxy]-2-methyl-3-butenic acid (M1) and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide (M2), competitively bind to AR (Fang et al., 2003) and inhibit DHT-induced transcriptional activation by blocking AR binding to AREs. Procymidone, on the contrary, exhibits almost identical affinity for the AR than the antiandrogenic drug flutamide, while its metabolites exhibit varying but lower levels of affinity. When administered during sexual development in a murine model, vinclozolin and procymidone produce an undervirilization and feminization of male pups, attested by a reduction in anogenital distance, retained nipples, hypospadias, a blind vaginal pouch, reduced to absent accessory glands, and a delay in pubertal maturation; such malformations lead to a greater rate of infertility at adult age (Gray et al., 2006). Vinclozolin has also been shown to promote transgenerational inheritance of reduced male fertility: after a transient embryonic exposure at a critical time during gonadal sex determination, an adult testis phenotype of decreased spermatogenic capacity and male infertility was found to be transgenerational, and appeared to be associated with altered primordial germ cell epigenetic programming via noncoding RNAs and alterations in gene expression. In spite of these concordant data in laboratory models, no epidemiological study has specifically looked into the antiandrogenic effects of vinclozolin or procymidone in men. Both vinclozolin and procymidone have now been phased-out for most domestic food uses.

The imidazole fungicide prochloraz was introduced in the late 1970s for horticulture and agriculture purposes in Europe, Asia, Australia, and South America. It is still currently in use on wheat, barley, mushrooms, cherries, turf on golf courses, and in flower production. The action of imidazoles used as fungicides or antimycotic drugs (e.g., ketoconazole) is based on the inhibition of the cytochrome P450-dependent 14 α -demethylase activity required in the conversion of lanosterol to ergosterol, an essential component of fungal cell membranes. The molecular basis of this inhibition is the presence of an imidazole moiety that interacts strongly with the iron atom of cytochrome P450. However, this binding is fairly unspecific, and prochloraz also inhibits the activities of a broad spectrum of other cytochrome P450-dependent enzymes, including key enzymes involved in steroidogenesis such as CYP17A1. Prochloraz is also an AR antagonist, and even though in vitro studies suggest that its dominant mechanism of action is the inhibition of testosterone production, its AR antagonistic properties are likely to be magnified by decreased circulating testosterone levels.

In murine models, gestational exposure to prochloraz produces a significant decrease in testosterone levels in male fetuses and smaller anogenital distance, nipple retention, and hypospadias in the male offspring (Vinggaard et al., 2006). Peripubertal exposure to prochloraz also delays pubertal development. A brief exposure at adult age does not significantly affect the adult male reproductive function. As of today, there are no epidemiological studies available on the effects of prochloraz in men reproductive function.

Insecticides

Dichlorodiphenyltrichloroethane (DDT) is an organochlorine. Originally used to control malaria and typhus among civilians and troops, DDT was increasingly used as an agricultural insecticide after World War II. This widespread use was famously condemned by Rachel Carson in her 1962 book *Silent Spring*, where she cataloged environmental impacts that coincided with the use of DDT in the United States, revealing its threat to wildlife and human health. The publication triggered a large public outcry, followed by an environmental movement, that eventually lead to a global ban of the use of DDT for disease control in more than 170 countries. In 1995, DDT was listed as a persistent organic pollutant by the Stockholm Convention. Since 2004, the use of DDT is legally limited to vector control, but agricultural use is possibly sustained in countries such as India or North Korea.

The main DDT isomer, *p,p'*-DDT, as well as its two primary metabolites *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE) and *p,p'*-dichlorodiphenyldichloroethane (*p,p'*-DDD), are persistent organic pollutants, bioaccumulate in fat tissues and biomagnify up the food chain, causing them to be detected in human tissues despite the ban. *p,p'*-DDE is a potent AR antagonist and inhibits the response to FSH (follicle stimulating hormone) and androgen-dependent gene expression in vitro. When administered during gestation in the rat, *p,p'*-DDE reduces anogenital distance, induces nipple retention, and permanently reduces androgen-dependent organ weights, but does not increase the incidence of hypospadias. Exposure during puberty produces a delay in preputial-separation. In adult male rats, exposure to DDT alters testosterone production, produces a dose-dependent reduction of testicular and seminal vesicle weights, and reduces the number and proportion of motile spermatozoa in cauda epididymides (Gray et al., 2006; Wilson et al., 2008).

Epidemiological studies investigating the effects of in utero exposure to DDT and *p,p'*-DDE remain inconclusive as to whether environmental exposure levels significantly decrease anogenital distance or increase the risk of hypospadias and cryptorchidism in newborn boys. The effects of DDT in adult men are also very much debated, with studies showing negative associations between levels of DDT and *p,p'*-DDE in body fluids and reproductive hormone levels, semen volume, sperm count, motility, morphology, and chromatin quality, while others show no such association. A few studies also identified positive associations between *p,p'*-DDE exposure and sperm concentration, reinforcing the need for further investigation.

Methoxychlor, a synthetic organochlorine, was initially intended to be a replacement for DDT. More rapidly metabolized and excreted in mammals, it is less acutely toxic than DDT and more readily biodegraded. Methoxychlor is used against a wide range of pests encountered in agriculture, households, and ornamental plantings. Despite a ban in the United States and Europe, methoxychlor is still used in several countries, and still detected in human urines, blood, cord blood, and follicular fluid.

Methoxychlor can be metabolized by the liver into two demethylated compounds, (2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-dichloroethane (HPDE), and two 0-ring methylated compounds. Each of these metabolites have been demonstrated to act differentially on the estrogen and androgen receptors. Both methoxychlor and HPTE are AR antagonists (Fang et al., 2003) and inhibit steroidogenic enzymes such as CYP11A1, HSD3B, and HSD17B3. Methoxychlor alters testosterone production both in vitro and in vivo. In male murine models, exposure to methoxychlor results in a pubertal delay, as well as reduced growth, seminal vesicle weight, cauda epididymis weight, and caudal sperm count. However, epidemiological studies in humans remain scarce.

Other DDT replacements from the 1940s include aldrin and dieldrin. Aldrin is a chlorinated insecticide used against soil-dwelling pests, for wood protection and, in the case of dieldrin, against insects of public health importance. The two compounds are closely related with respect to their toxicology and mode of action. Aldrin is rapidly converted to dieldrin under most environmental conditions and in the body. Dieldrin is a highly persistent organochlorine compound that has low mobility in soil, can be lost to the atmosphere and bioaccumulates. Since the early 1970s, a number of countries have either severely restricted or banned the use of both compounds, particularly in agriculture, and since 1995, they are both listed as persistent organic pollutants by the Stockholm Convention.

Both aldrin and dieldrin are AR antagonists. Reproductive health failures following exposure to aldrin and dieldrin have been reported in numerous wildlife species such as fish, birds, alligators, harbor seals, and minks (Jorgenson, 2001). In vivo exposure in laboratory male murine models produced significant reductions in testosterone and LH (luteinizing hormone) serum levels, as well as a reduction in sperm count and degeneration of germ cells. The effects of aldrin and dieldrin in men reproductive function have not been extensively studied.

Lindane, also known as gamma-hexachlorocyclohexane (gamma-HCH), is another organochlorine that was used as both an insecticide and pharmaceutical treatment in the 1940s, resulting in wildlife and human exposure through inhalation, ingestion, and dermal absorption. In 2001, lindane was officially listed as a persistent organic pollutant by the Stockholm Convention. Since then, its use has been largely banned or restricted worldwide, with the exception of second-line treatment of lice and scabies. However, due to its extreme persistence in the environment and bioaccumulation properties, lindane is detected in air, surface water, groundwater, sediment, soil, ice, snowpack, fish, wildlife, and humans.

Lindane acts as both an AR antagonist (Fang et al., 2003) and an inhibitor of steroidogenic enzymes, such as StAR, HSDB3, and HSD17. Male gonads have been found to be particularly sensitive to lindane, with several studies in adult murine models showing degenerative changes such as tubular atrophy, spermatogenic cells necrosis, enlargement of the interstitial space, or decreased sperm production and quality. Following in utero or lactational exposure, lindane produces testicular alterations, a reduction in spermatogenesis, serum testosterone levels, testicular weight, spermatid and sperm counts, and increased chromatin abnormalities in epididymal sperm in male pups (Pagès et al., 2002). In humans, a sparse literature reveals possible associations between exposure to lindane and increased risk of cryptorchidism, sex hormone disruption, and a decline in sperm quality.

Herbicides

Linuron (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a phenylurea herbicide used to control the growth of grass and weeds in crops such as soybeans. Linuron inhibits electron transport in chloroplast photosystem II complex, thereby preventing plant growth and promoting cell membrane destruction. Human exposure to linuron may occur through diet (food and water), when handling and applying the product, or by entering treated sites.

Linuron competitively inhibits the binding of androgens to AR and acts as a weak AR antagonist in transcriptional activation assays in vitro. In utero exposure to linuron drastically affects sexual differentiation and development by causing reduced anogenital distance and retained nipples as well as a high degree of epididymal malformation and testicular atrophy in male offspring. When administered to the dam during the critical period of sex differentiation, high doses of in utero linuron treatment also reduce ex vivo fetal testosterone synthesis in male offspring. In castrate-immature testosterone propionate-treated adult male rats, a 7-days treatment with linuron reduced testosterone and DHT-dependent tissue weights. Contrary to other pesticides such as vinclozolin or procymidone, malformed external genitalia or undescended testes are rarely displayed by linuron-exposed males (Gray et al., 2006). There are no studies available on associations between exposure to linuron and TDS-related phenotypes in boys or men.

Industrial Chemicals

Brominated Flame Retardants

Polybrominated diphenyl ethers (PBDEs) are organobromine compounds used as flame retardants. The family of PBDEs consists of 209 congeners classified by the average number of bromine atoms in the molecule. The available commercial PBDE products are not single congeners but rather mixtures of congeners. Due to their widespread use as nonchemically bound additives to a wide array of consumer products (e.g., building materials, electronics, furnishing, and textiles), PBDEs have become ubiquitous environmental pollutants. Since 2009, PBDEs are listed as persistent organic pollutants by the Stockholm Convention. Despite bans and voluntary withdrawals, lipophilic PBDE congeners persist in the environment, biomagnify up the food chain, and bioaccumulate in breast milk, serum, and adipose tissue.

The AR antagonistic activities of several PBDE congeners, metabolites and mixtures have been shown in vitro, in particular those of commercial mixture DE-71 and congeners BDE-17, -19, -28, -47, and -100. However, there is only limited evidence for the anti-androgenic properties of PBDEs to result in reproductive disorders. Peripubertal exposure to PBDEs in murine models results in delayed puberty and suppression of androgen-dependent tissue growth (Linares et al., 2015). In a single study, maternal exposure to PBDEs resulted in decreased sperm production in the offspring at adult age; such effects are corroborated by epidemiological studies in small cohorts on men recruited in fertility clinics showing associations between exposure to PBDEs and reproductive hormone levels or sperm motility. However, a study on partners of pregnant women showed conflicting results with the latter, reinforcing the need for large cohort studies that are more representative of the general population with background exposure. This past decade, PBDEs have been gradually replaced by novel mixtures of organophosphate flame retardants, but recent investigations suggest that these alternatives also constitute a toxic hazard.

Summary and Perspectives

If the field of environmental antiandrogens was born with agrochemicals, it has since expanded to a wide range of compounds, from industrial additives in everyday products to pharmaceutical drugs sold over the counter. Mechanistically, these chemicals can disrupt the physiological androgen pathway by antagonizing or modulating the androgen receptor, altering androgen production, or both. As more and more molecular based studies on antiandrogens are conducted, it has been suggested that this definition should be broadened to chemicals exerting antigonadotropic effects at large (for example, drugs altering the expression of hormones involved in gonadal differentiation, like the anti-Müllerian hormone, or in testicular descent, like insulin-like factor 3).

The evidence for implications of exposure to environmental antiandrogens on male reproductive defects from in vitro and in vivo laboratory models is ever-growing. However, the clinical and epidemiological evidence for effects in humans is still controversial: if some studies have found direct associations between exposure to some of these compounds and the incidence of cryptorchidism, hypospadias, pubertal delay, gynecomastia, or decreased semen quality, others have not. Such discrepancy is attributable to the complexity and variety of clinical protocols used, the degree of exposure, the subpopulation studied, the variables measured, the sample size, or, with regard to semen quality, the contribution of seasonal and geographical variations.

The growing body of evidence in support of the deleterious effects of environmental antiandrogens has prompted legislation to protect consumers, stimulating the chemical industry to develop alternative compounds. However, from DDT to phthalates and bisphenols, there is an inexorable history of regrettable substitutions entering the market, with some chemicals phased out at great cost in scientific, advocacy and legal efforts being replaced by hazardous substitutes. The scientific community is now calling for the use of proactive approaches to replace harmful chemicals, including a thorough investigation comparing the endocrine-disruptive effects of the candidate compounds to those of the original chemical.

References

- Albert, O., & Jégou, B. (2014). A critical assessment of the endocrine susceptibility of the human testis to phthalates from fetal life to adulthood. *Human Reproduction Update*, 20(2), 231–249.
- Fang, H., Tong, W., Branham, W. S., Moland, C. L., et al. (2003). Study of 202 natural, synthetic, and environmental chemicals for binding to the androgen receptor. *Chemical Research in Toxicology*, 16(10), 1338–1358.
- Gray, L. E., Wilson, V. S., Stoker, T., Lambright, C., et al. (2006). Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals. *International Journal of Andrology*, 29(1), 96–104. discussion 105–8.
- Jensen, A. A., & Leffers, H. (2008). Emerging endocrine disruptors: Perfluoroalkylated substances. *International Journal of Andrology*, 31(2), 161–169.
- Jorgenson, J. L. (2001). Aldrin and dieldrin: A review of research on their production, environmental deposition and fate, bioaccumulation, toxicology, and epidemiology in the United States. *Environmental Health Perspectives*, 109(Suppl 1), 113–139.
- Kristensen, D. M., Mazaud-Guittot, S., Gaudriault, P., Lesné, L., et al. (2016). Analgesic use—Prevalence, biomonitoring and endocrine and reproductive effects. *Nature Reviews. Endocrinology*, 12(7), 381–393.
- Linares, V., Bellés, M., & Domingo, J. L. (2015). Human exposure to PBDE and critical evaluation of health hazards. *Archives of Toxicology*, 89(3), 335–356.
- Pagès, N., Sauviat, M.-P., Bouvet, S., & Goudey-Perrière, F. (2002). Toxicité du lindane sur la reproduction. *Journal de la Société de Biologie*, 196(4), 325–338.
- Peretz, J., Vrooman, L., Rieke, W. A., Hunt, P. A., et al. (2014). Bisphenol a and reproductive health: Update of experimental and human evidence, 2007–2013. *Environmental Health Perspectives*, 122(8), 775–786.
- Sartain, C. V., & Hunt, P. A. (2016). An old culprit but a new story: Bisphenol A and 'NextGen' bisphenols. *Fertility and Sterility*, 106(4), 820–826.

- Skakkebaek, N. E., Rajpert-De Meyts, E., & Main, K. M. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. *Human Reproduction*, 16(5), 972–978.
- Vinggaard, A. M., Hass, U., Dalgaard, M., Andersen, H. R., et al. (2006). Prochloraz: An imidazole fungicide with multiple mechanisms of action. *International Journal of Andrology*, 29(1), 186–192.
- Wilson, V. S., Blystone, C. R., Hotchkiss, A. K., Rider, C. V., et al. (2008). Diverse mechanisms of anti-androgen action: Impact on male rat reproductive tract development. *International Journal of Andrology*, 31(2), 178–187.

Antiestrogens

Timothy J Doyle and Kwanhee Kim, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Androgens A type of steroidal hormones produced in the testis and adrenal glands of the males and primarily in the ovary and adrenal glands of the females. They have roles in the development of male secondary sex characteristics and the brain, reproduction, and regulation of lipid metabolism.

Antiestrogens A type of estrogen blockers or estrogen antagonists that inhibit or block the estrogen receptor activity or interfere with the production of estrogens in cells.

Aromatases A group of enzymes that convert androgens to estrogens.

Endocrine disruptors A group of artificially produced chemicals that show hormone-like properties and can interfere with the synthesis, metabolism, secretion, and transport, and the function of endogenous hormones of the endocrine system in the body.

Estrogens A type of steroidal hormones produced primarily by the ovary, adipose tissue, and the testis that has a role in the development of female secondary sex characteristics and the brain, reproduction, regulation of body fat, and the growth of long bones.

Introduction

Endocrine hormones and their signaling pathways play a vital role in the ability of organisms to develop from a fetus into a healthy adult. Steroid hormones such as estrogen and testosterone are known to play a critical role in sexual differentiation and reproduction. Estrone, a precursor to estrogen, and androsterone, a metabolite of testosterone, were isolated in the 1920s and 1930s. Then, it took another few decades before it was shown that radiolabeled estradiol was explicitly retained in estrogen-sensitive tissues compared to nonestrogen target tissues and that estrogen-sensitive tissues indeed contained extractable proteins known as estrogen receptors (Tata, 2005). Furthermore, only in the 1980s with the cloning of cDNAs were there enough protein materials to decipher the molecular mechanism of estrogen receptor action.

Today, we have a better understanding of how estrogen interacts with estrogen receptors and which tissues are sensitive to estrogens. Estrogen can transduce cellular signaling by two pathways, the classical, genomic pathway and nongenomic pathway (Fig. 1). In the genomic pathway (Fig. 1A), estrogen binds the ligand-binding domain of the estrogen receptor in the cytoplasm. With estrogen binding, the receptor is activated to move into the nucleus, where the receptors bind specific estrogen response elements (EREs) on target DNA as a dimer of receptors and regulate the expression of the target genes. Using this genomic pathway are two primary estrogen receptors, estrogen receptor alpha (ESR1), and estrogen receptor beta (ESR2), each responsible for modulation of its physiological functions in estrogen-sensitive cells (Kiyama and Wada-Kiyama, 2015). In the nongenomic pathway, estrogen can either activate a membrane-associated G protein-coupled estrogen receptor 1 (GPER1) or the classical ESR1 or ESR2 that then translocates to the membrane (Fig. 1B) (Arnal et al., 2017). Either of these nongenomic activation processes initiates a cascade of cellular signaling and cross talks of proteins, eventually leading to the recruitment of transcription factors and gene regulation in the nucleus.

In recent decades, research has been shifted to focus on how industrial and pharmacological chemicals and phytoestrogens in the environment may alter the availability of endogenous hormones and disrupt the action of estrogen-activated receptors, either in a genomic pathway or a nongenomic signaling cascade pathway (Fig. 1). The terms, environmental endocrine disruptors (EEDs) or endocrine disrupting chemicals (EDCs) have been coined to label any environmental chemicals that can mimic receptor ligand and disrupt normal endocrine function, causing abnormal cellular changes in the hormone-sensitive tissues and leading to the onset of diseases.

Classes of Ligand Mimics

Many endocrine disruptors (EEDs or EDCs) acting as receptor ligand mimics are industrial, synthetic chemicals or drugs with a similar structure to the natural hormone receptor ligands produced in the body (Fig. 2A and B). Binding of ligand mimics (Fig. 2C–G) to the receptor usually blocks the binding of the endogenous hormone ligand (Fig. 2A and B), and as a consequence, ligand mimics usually alter hormone-sensitive physiological reactions in the cell negatively. Although some ligand mimics show a clear preference for a specific receptor (distinguish between ESR1 and ESR2 receptors), most ligand mimics are promiscuous and inhibit the function of a variety of receptors including receptors for estrogens, androgens, progestins, and sometimes, adrenal

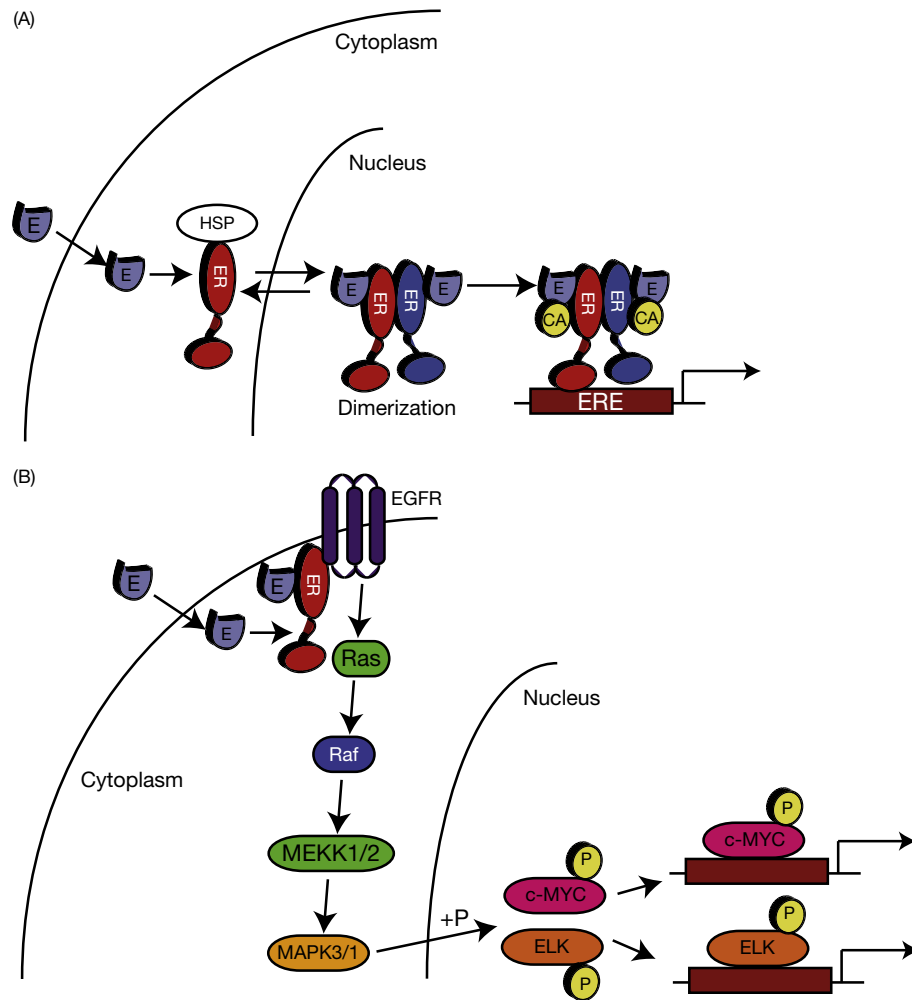


Fig. 1 Steroid hormone receptors regulate target genes by two distinct pathways. (A) In the classical, genomic pathway, estrogen (E) binds estrogen receptor (ER) and activates the receptor for the nuclear entry and dimerization. The dimer of receptors then binds estrogen response element (ERE) on the target gene to regulate its gene expression in the presence of co-activator (CA). (B) In a nongenomic pathway, E interacts with ER, which is modified to interact with a membrane receptor protein (such as epidermal growth factor or EGFR), which in turn activates the membrane receptor signaling cascade leading to the phosphorylation of transcription factors and the regulation of gene expression.

hormones in different tissues. Similarly, a ligand mimic can have opposite effects on cells, either estrogenic or antiestrogenic. For estrogen receptors, the ligand mimics can be classified as being estrogenic, stimulating the estrogen receptor activity in the presence of co-activator (Fig. 3A), or antiestrogenic, inhibiting estrogenic receptor activity in the presence of co-repressor (Fig. 3B). The ligand mimics in the first case are working as agonists, and in the second case, they are working as antagonists. In both cases, the ligand mimics interfere with the normal signaling mechanisms, either prolonging estrogen stimulus at inappropriate times or merely reducing or blocking any estrogen stimulus in the cell.

Whether the ligand mimics act as an agonist, or an antagonist is dependent on a complex set of variable contexts, among which are: what hormone receptors are expressed in the cell or tissue; what genes ligand mimics are acting on; what concentration of ligand mimics is used; and what other proteins, such as co-activators, co-repressors, kinases, or phosphatases, are permitted to interact with the hormone-receptor complex. One such example is a breast cancer drug tamoxifen that acts antiestrogenic in estrogen receptor-positive breast cancer cells but acts estrogenic in the endometrium by promoting Src kinase phosphorylation of the estrogen receptor. Phosphorylation stabilizes the interaction of estrogen receptor with co-activator and ERE on the target genes (Shah and Rowan, 2005). Prolonged exposure of tamoxifen in the endometrium can lead to endometrial cancer, which remains a primary concern for tamoxifen therapy on breast cancers (Shah and Rowan, 2005). Usually, antihormone ligand mimics are structurally different enough that they simply cannot bind the ligand-binding domain in the same way as the endogenous hormones, which blocks co-repressor release, thus restricting a room for co-activator binding to stimulate estrogenic activity (Fig. 3B).

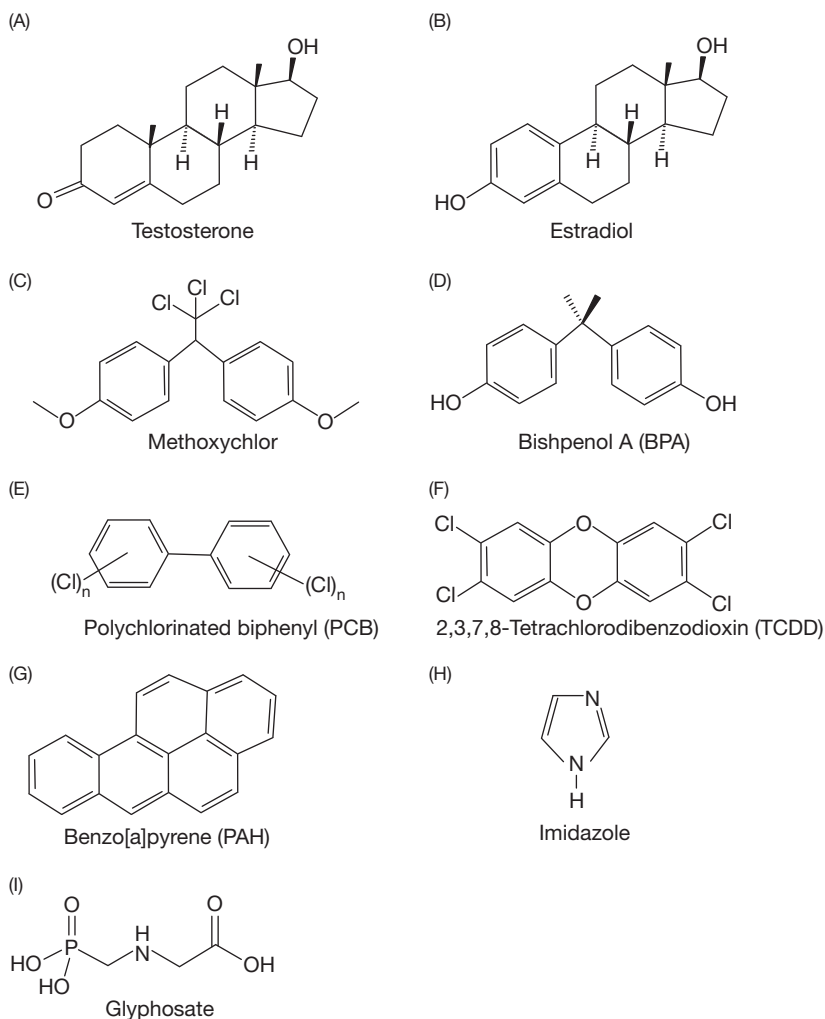


Fig. 2 Chemical structures of natural ligands and endocrine disrupting ligand mimics. The ligands made in the body: (A) testosterone and (B) estrogen. Examples of antiestrogen endocrine disruptors: (C) bisphenol A (BPA), (D) methoxychlor, (E) 2,3,7,8-tetrachlorodibenzodioxin (TCDD), (F) polychlorinated biphenyl (PCB), and (G) benzo[a]pyrene, a type of polycyclic aryl hydrocarbon. The Structures of aromatase inhibitors: (H) imidazole and (I) glyphosate.

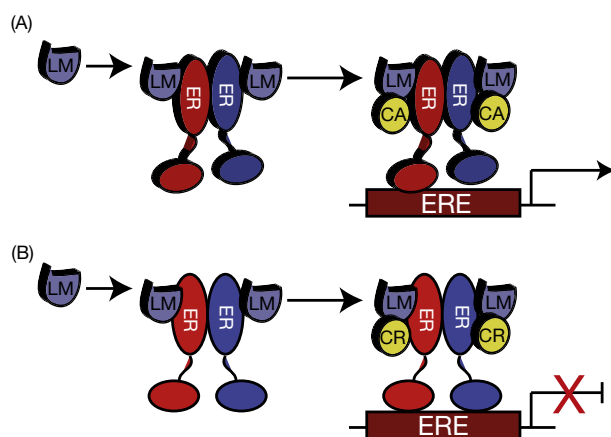


Fig. 3 Ligand mimics can be estrogenic or antiestrogenic. When a ligand mimic binds to a steroid hormone receptor, it may activate or repress the function of the receptor. (A) When the ligand mimics are estrogenic (agonist), the complex of ligand mimic (LM) and the receptor (ER) interacts with co-activators (CA) and induces the transcription of ERE-containing target genes. (B) When LMs are antiestrogenic (antagonist), the LM and ER complex interacts with co-repressors (CR) that keeps the LM and ER complex turned off and decreased the transcription of ERE-containing target genes.

Antiestrogens

There are several classes of ligand mimics that have been identified as antiestrogens or estrogen antagonists. They are classified by their mode of action: compounds that selectively bind the estrogen receptors and modulate their activity; compounds that act by binding and activating another nuclear receptor, which in turn inhibits estrogen receptor function; or compounds that reduce estrogen levels by inhibiting the cytochrome P450 enzyme aromatase (CYP19A1) needed in the production of estrogen. It should be noted that we have taken a relaxed definition of antiestrogens by including aromatase inhibitors, which may not act directly through estrogen receptors, but nonetheless, promote antiestrogenic activities.

Selective Estrogen Receptor Modulators (SERMs)

Many of the endocrine disruptors with antiestrogenic activities behave as selective estrogen receptor modulators (SERMs). SERMs are typically nonsteroidal in structure and act as competitive inhibitors for the binding of estrogen to the estrogen receptors (Kiyama and Wada-Kiyama, 2015). SERMs usually target specific estrogen receptors in estrogen-sensitive tissues, causing either estrogenic (agonist) or antiestrogenic (antagonist) activities. Examples of such SERMs are drugs such as tamoxifen, clomifene, and raloxifene, which are used to decrease estrogenic activities in estrogen-sensitive cancer cells. Similar chemicals, termed SERMs-like, are environmental endocrine disruptors such as an organochlorine pesticide methoxychlor (Fig. 2C), a component of plastics, bisphenol A (BPA) (Fig. 2D), and a methoxychlor derivative, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) (Fig. 4D). As with many of the metabolic derivatives of ligand mimics (Fig. 4), HPTE is at least 100 times more potent in biological activity (e.g., antiestrogenic or antiandrogenic activities) compared to the parental methoxychlor.

BPA is a small, synthetic, phenolic chemical (Fig. 2D) used in the manufacture of consumer products, such as the resin linings for canned foods and polyvinylchloride (PVC) plastics in medical tubings, toys and bottles, and water pipes. There are numerous variants of BPA including bisphenol C (BPC), a fluorinated derivative bisphenol AF (BPAF), used as a polycarbonate copolymer in high-temperature composites, electronic materials, and gas-permeable membranes, and a chlorinated tetrachlorobisphenol A (TCBPA), used as a fire retardant (Delfosse et al., 2014).

For many years, BPA was described as weakly estrogenic, having the ability to directly bind both ESR1 and ESR2, or as antagonists to both androgen and thyroid hormones (Welshons et al., 2006). However, more recent evidence indicates that BPA are SERMs, having the ability to serve as both estrogenic and antiestrogenic compounds, dependent on what tissues are examined, which receptors are present in the cell, what concentrations of BPA are used, and which BPA is examined (Welshons et al., 2006). BPA acts biphasically for ESR1 and ESR2 as a cell type-specific agonist at ≥ 10 nM or as an antagonist at ≤ 10 nM (Li et al., 2012). Both BPC and TCBPA inhibited ESR2 signaling, while only TCBPA, and not BPC, activated ESR1 signaling (Delfosse et al., 2014). Additionally, BPA can mediate its action through a nongenomic mechanism, where it activates a membrane-associated G protein-coupled estrogen receptor 1 (GPER1), which in turn associates with and activates the Egfr/Mapk3/1 pathway, influencing a cascade of cellular events (Fitzgerald et al., 2015) (Fig. 1B).

Methoxychlor is an organochlorine pesticide that replaced banned DDT and was used to control insects on many crops around the world (Fig. 2C). Although the production of methoxychlor was halted in the United States in 2003, methoxychlor is present in food and water samples and persists in the environment due to the evaporation/rain cycle redistributing waters on earth. Methoxychlor and HPTE are regarded as SERMs (Delfosse et al., 2014). In the mouse uterus, where ESR1 is the primary estrogen receptor expressed, methoxychlor operates as an ESR1 agonist and induces tissue growth (Tiemann, 2008). In contrast, methoxychlor operates as an antiestrogen in the ovary, where ESR2 is the dominant subtype and inhibits FSH/cAMP-induced estradiol synthesis mediated by ESR2 (Harvey et al., 2015). The results of methoxychlor exposure are the inhibition of follicle growth and increased atresia of antral follicles in the ovary.

Antiestrogen Activity Through Induction of Aryl Hydrocarbon Receptor

Not all antiestrogens inhibit estrogen signaling by directly binding to the classical estrogen receptors or the nongenomic membrane estrogen receptor. Several known ligand mimics work through binding and activation of other nuclear receptors. Well-studied, such compounds are polychlorinated biphenyls (PCBs), chlorinated dibenzo-*p*-dioxins (CDDs), and polycyclic aromatic hydrocarbons (PAHs) (Fig. 2E–G). Typically, CDDs are formed as industrial byproducts during the chlorination processes or incineration of garbage and fuel combustion. 2,3,7,8-TCDD (Fig. 2F) is produced during the chlorine bleaching process used by pulp and paper mills and during the manufacture of 2,4,5-trichlorophenol (2,4,5-TCP). 2,4,5-TCP is a chemical used in the manufacturing of bactericide hexachlorophene and the herbicide, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Manzetti et al., 2014). TCDD is persistent in the environment and bioaccumulates in the fatty tissue of the body. It is well known for being a compound found in Agent Orange, which contained herbicide 2,4,5-T to remove foliage during the Vietnam War (Manzetti et al., 2014). PAHs are also industrial byproducts of incomplete incineration and combustion, primarily from car emissions, burning coals, and in the manufacture of petroleum-derived substances.

On the other hand, PCBs are commonly used as coolants and lubricants in transformers, as flame retardants, and used in microelectronic circuits, capacitors, and other electrical equipment, since they do not burn easily and make good insulator material (Manzetti et al., 2014). It should be noted that the PCB being referred to here is 3,3',4,4',5-pentachlorobiphenyl (PCB126), which is known to act similarly to TCDD (Matthews et al., 2007). There is a variety of PCBs, and other PCBs may act differently, with most of

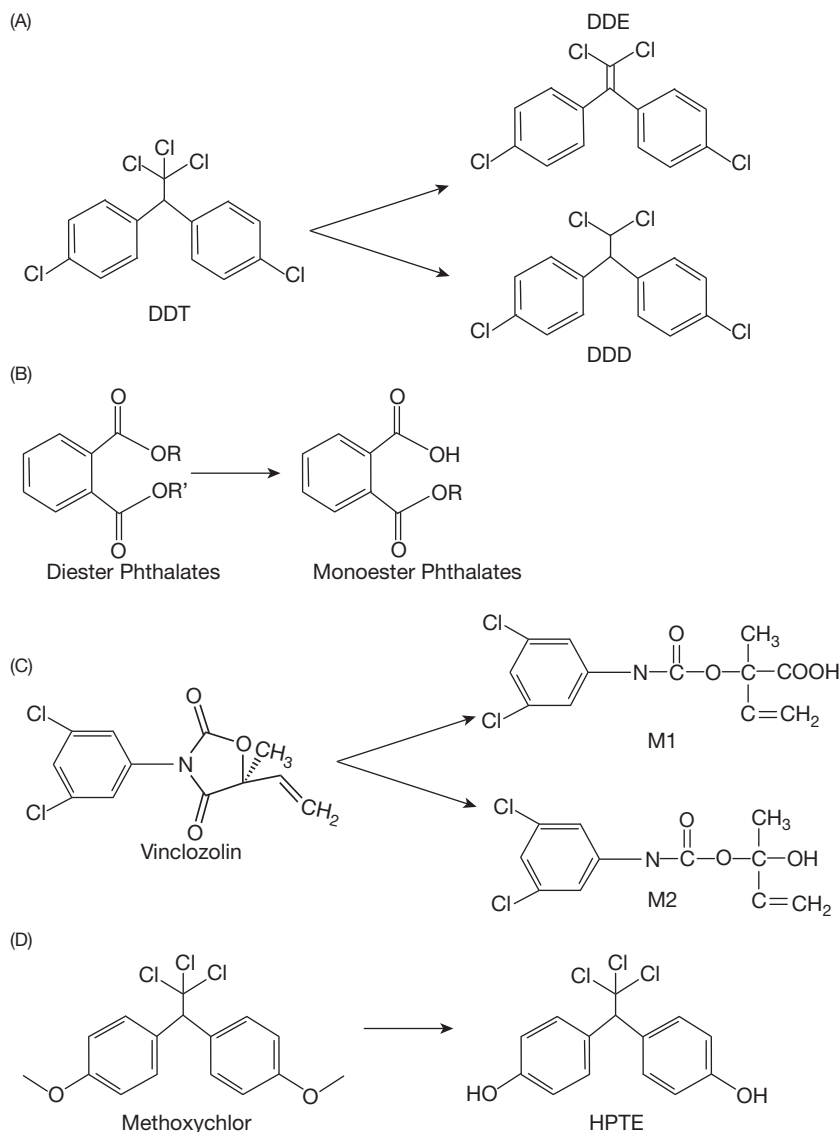


Fig. 4 Chemicals are metabolized to their more active metabolites in the body. (A) Chlorinated aromatic hydrocarbon insecticide DDT is metabolized to DDE and DDD. (B) Plasticizer diester phthalates are metabolized to monoester phthalates. (C) Dicarboximide fungicide vinclozolin is metabolized to M1 and M2. (D) Organochlorine pesticide methoxychlor, which was a replacement for DDT, is metabolized into its active metabolite HPTE.

them being estrogenic. TCDD, PAHs, and PCBs are suspected carcinogens and are implicated in cancer, reproductive disorders, and neurological and developmental disorders.

Upon entering the body, the dioxin and related compounds are taken up into tissues, where they activate the aryl hydrocarbon receptor (AHR) (Fig. 5). Upon activation, AHR receptor interacts with aromatic hydrocarbon receptor nuclear translocator (ARNT, or HIF1 β) in the nucleus, which together associates with dioxin-response elements (XRE) on DNA and regulates AHR-regulated genes. The antiestrogen action of the compounds like TCDD and PCB126 comes from their activation of the AHR signaling pathway, which in turn recruits ESR1 as a co-activator (Fig. 5) to AHR/ARNT bound dioxin-response elements (Matthews et al., 2007). This recruitment of ESR1 removes the estrogen receptors from the normal transcription activity on estrogen-responsive genes, leading to antiestrogenic activity.

Antiestrogen Activity Through Inhibition of Aromatase

The third class of antiestrogenic chemicals does not interact with the estrogen receptors directly or indirectly but modulates the production of estrogen and the homeostasis of estrogenic activities in the cell. The principal target for this type of endocrine disruptors is the cytochrome P450 enzyme aromatase (CYP19A1), the enzyme responsible for converting testosterone to estradiol, and androstenedione to estrone (Blakemore and Naftolin, 2016) (Fig. 6). CYP19 is expressed in many tissues including the ovary,

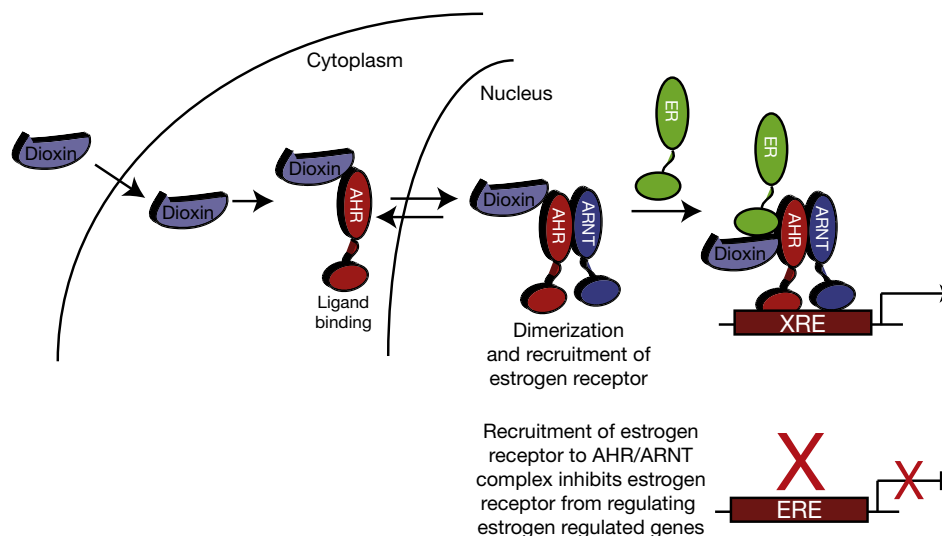


Fig. 5 Dioxins and dioxin-like chemicals act as antiestrogens through the activation of the aryl hydrocarbon receptor pathway. Dioxin and dioxin-like compounds bind and activate the aryl hydrocarbon receptor (AHR), which then moves into the nucleus and dimerizes with ARNT. Upon binding of the aryl hydrocarbon receptor response element (XRE), the estrogen receptor (ER) is recruited to the AHR/ARNT complex as a co-activator. The recruitment of ER to the AHR/ARNT complex removes ER from being able to bind and regulate ERE containing target genes, thus decreasing estrogenic activity.

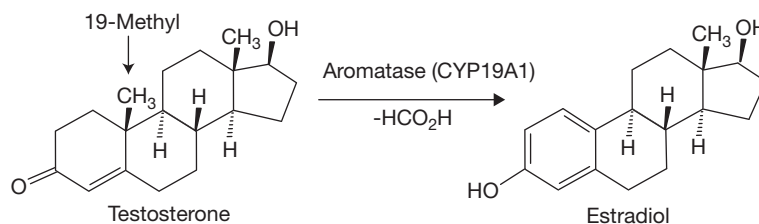


Fig. 6 Aromatase converts androgens into estrogens through the removal of the 19-methyl group of androgen in three successive oxidation steps.

testis, brain, adrenal, placenta, adipose, bone, and skin, but regulation of CYP19 expression is highly tissue-specific with alternative promoter usage and cross-talks with other second messengers, such as cAMP. Changes in aromatase expression (loss or gain of expression) have been associated with numerous male and female reproductive and immunological disorders, as well as in the induction of cancer.

The aromatase inhibitors used in agriculture are nonsteroidal chemicals called azoles (epoxiconazoles, imidazoles, and triazoles) that have antifungal activity, and glyphosate and glyphosate-containing compounds, like Roundup, that are herbicides (Richard et al., 2005) (Fig. 2H and I). Many aromatase inhibitors were developed for the treatment of breast cancer, endometriosis, and prevention of menopausal symptoms as well (Blakemore and Naftolin, 2016). Successfully used clinical aromatase inhibitors are letrozole and anastrozole that inhibit the aromatase activity by binding the heme group of aromatase reversibly (Blakemore and Naftolin, 2016). Additionally, estrogen receptor and aryl hydrocarbon receptor could be involved in the expression of aromatase. The gene for aromatase has both EREs and XREs in the promoter (Sanderson, 2006). It means that the expression of aromatase can be downregulated by antiestrogens through classical estrogen receptors or by dioxin-like chemicals through aryl hydrogen receptors. Another chemical that reduces aromatase activity by aryl hydrogen receptor and estrogen receptor is benzopyrene, a dioxin-like PAH from incomplete combustion of organic materials (Alharthy et al., 2017).

Reproductive Disorders and Cancer

As many environmental disruptor chemicals are components of industrial/consumer products in large quantities, they have a great potential to contaminate the environment. Humans are in direct contact with these chemicals, setting up the possibility of disease onset. Routes of entry into the body occur in any number of different ways, from ingestion through food and water, inhalation of the dust particles in the air, to absorption through the skin. Antiestrogen endocrine disruptor chemicals have been linked to many different diseases, be it the initiation and promotion of different types of cancer, or the induction of various types of reproductive disorders. As estrogen plays a role in maintaining the hormone balance in both sexes, it is not surprising that

these compounds can induce diseases in both males and females. In this section, we will focus on the diseases more commonly observed in males over those found in females and focus on chemicals known to have antiestrogenic activities (BPA, methoxychlor, and dioxins).

As endocrine disruptors with antiestrogenic activities are targeting estrogen signaling, it is no surprise that BPA, dioxin, and methoxychlor affect the reproductive system, where estrogen signaling function is essential. The reproductive disorder in males induced by endocrine disruptors with antiestrogenic activity range from mild (a small decrease in sperm count) to major as in hypogonadism, infertility and testicular cancer. In humans, fetal BPA exposure has been linked with feminization, shorter anogenital distances, increased incidences of hypospadias, and undescended testes (Fernandez et al., 2016; Komarowska et al., 2015; Miao et al., 2011). In rodents exposed perinatally to BPA, observations have been made for defective spermatogenesis, increase in meiotic cell arrest, increased apoptosis, decreased sperm quality, and more recently, transgenerational impairment of male fertility (Williams et al., 2014).

Additionally, BPA exposure has been associated with high risks of developing prostate cancer, testicular interstitial cell tumors, and mammary cancer in males (Sweeney et al., 2015; Seachrist et al., 2016; Wang et al., 2017). Supporting this, young men with histopathologically verified prostate cancer had higher serum levels of BPA than healthy controls.

For methoxychlor, different dosing and concentration schemes have identified multiple disruptions in male reproductive function. Perinatal exposure to mice showed a reduction in serum testosterone levels, an increase in estradiol concentrations, a decrease in pachytene and spermatid cell numbers, and increases in Leydig cell numbers (Du et al., 2014). Some of these changes were attributed to decreased androgen levels and altered estrogen metabolism enzymes, notably a decrease in CYP11 and an increase CYP19 expressions. Similar to BPA, the embryonic exposure of pregnant rats decreased sperm number and motility and increased germ cell apoptosis (Anway et al., 2005). These changes were transmitted to male progeny in multiple generations (first to fourth generations) by epigenetic mechanisms (Anway et al., 2005). Further investigation is required to elucidate the exact mechanism by which methoxychlor interferes with epigenetics.

TCDD, PAHs, and PCBs are suspected carcinogens and are implicated in cancer, reproductive disorders, and neurological and developmental disorders. These chemicals working through the AHR pathway have been observed in rodent males to cause decreases in androgen levels and sperm counts, reductions in gonadal, seminal vesicle, and prostate weights, hypospadias, and delays in male puberty. The exact magnitude of these decreases was found to vary, depending on the concentration and timing of exposure. A low dose of TCDD has been found to decrease sperm quality, reduce androgen levels and cause transgenerational changes in fertility over multiple generations (Sanabria et al., 2016). Moreover, dioxin-like compounds such as PCBs have been positively correlated with increased incidence of prostate cancer and male breast cancer (Prins, 2008; Sweeney et al., 2015).

A transparent common theme emerging from these studies is that the type of diseases initiated by ligand mimics or the severity of disease is dependent on the concentration and the time of exposure (embryonic versus postnatal) of the ligand mimics. Exposure to ligand mimics during embryonic and perinatal times, which span the critical periods of sexual differentiation, masculinization, and development, seems to have more dramatic effects on reproductive disorders or cancers than postnatal times. Accordingly, many of the adult diseases are thought to originate from fetal and perinatal exposures to endocrine disruptors. At the same time, the concentration of ligand mimics has a high influence on whether a ligand mimic operates as an estrogen or an antiestrogen mimic. At a low dose, the ligand mimics may be estrogenic, whereas, at a high dosage, the ligand mimics may be antiestrogenic, or vice versa. Low concentration of ligand mimics could have totally opposite physiological effects from the high concentration.

However, not much information is available in the literature, directly linking the mechanisms of action to pathological effects on male reproduction and cancers for each ligand mimic following in vivo exposures in animals or real-life exposures to humans. As endocrine disruptors with antiestrogenic properties discussed here are ubiquitously contaminating the environment and humans are continually exposed and at risk to develop pathological conditions, there is urgent need to fully decipher the mechanisms of each ligand mimic underlying specific male reproductive disorder or cancer. Understanding the mechanisms of estrogenic and antiestrogenic ligand mimics may provide guidance for the development of potential counteractive therapy for different reproductive disorders and cancer.

References

- Alharthy, K. M., Albaqami, F. F., Thornton, C., et al. (2017). Mechanistic evaluation of benzo[a]pyrene's developmental toxicities mediated by reduced Cyp19a1b activity. *Toxicological Sciences*, 155, 135–147.
- Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308, 1466–1469.
- Arnal, J. F., Lenfant, F., Metivier, R., et al. (2017). Membrane and nuclear estrogen receptor alpha actions: From tissue specificity to medical implications. *Physiological Reviews*, 97, 1045–1087.
- Blakemore, J., & Naftolin, F. (2016). Aromatase: Contributions to physiology and disease in women and men. *Physiology (Bethesda)*, 31, 258–269.
- Delfosse, V., Grimaldi, M., Cavailles, V., et al. (2014). Structural and functional profiling of environmental ligands for estrogen receptors. *Environmental Health Perspectives*, 122, 1306–1313.
- Du, X., Zhang, H., Liu, Y., et al. (2014). Perinatal exposure to low-dose methoxychlor impairs testicular development in C57BL/6 mice. *PLoS One*, 9, e103016.
- Fernandez, M. F., Arrebola, J. P., Jimenez-Diaz, I., et al. (2016). Bisphenol A and other phenols in human placenta from children with cryptorchidism or hypospadias. *Reproductive Toxicology*, 59, 89–95.

- Fitzgerald, A. C., Peyton, C., Dong, J., & Thomas, P. (2015). Bisphenol A and related alkylphenols exert nongenomic estrogenic actions through a G protein-coupled estrogen receptor 1 (Gper)/epidermal growth factor receptor (Egfr) pathway to inhibit meiotic maturation of zebrafish oocytes. *Biology of Reproduction*, 93, 135.
- Harvey, C. N., Chen, J. C., Bagnell, C. A., & Uzumcu, M. (2015). Methoxychlor and its metabolite HPTE inhibit cAMP production and expression of estrogen receptors alpha and beta in the rat granulosa cell in vitro. *Reproductive Toxicology*, 51, 72–78.
- Kiyama, R., & Wada-Kiyama, Y. (2015). Estrogenic endocrine disruptors: Molecular mechanisms of action. *Environment International*, 83, 11–40.
- Komarowska, M. D., Hermanowicz, A., Czyzewska, U., et al. (2015). Serum bisphenol A level in boys with cryptorchidism: A step to male infertility? *International Journal of Endocrinology*, 2015, 973154.
- Li, Y., Burns, K. A., Arao, Y., et al. (2012). Differential estrogenic actions of endocrine-disrupting chemicals bisphenol A, bisphenol AF, and zearalenone through estrogen receptor alpha and beta in vitro. *Environmental Health Perspectives*, 120, 1029–1035.
- Manzetti, S., Van Der Spoel, E. R., & Van Der Spoel, D. (2014). Chemical properties, environmental fate, and degradation of seven classes of pollutants. *Chemical Research in Toxicology*, 27, 713–737.
- Matthews, J., Wihlen, B., Heldring, N., et al. (2007). Co-planar 3,3',4,4',5-pentachlorinated biphenyl and non-co-planar 2,2',4,6,6'-pentachlorinated biphenyl differentially induce recruitment of oestrogen receptor alpha to aryl hydrocarbon receptor target genes. *The Biochemical Journal*, 406, 343–353.
- Miao, M., Yuan, W., He, Y., et al. (2011). In utero exposure to bisphenol-A and anogenital distance of male offspring. *Birth Defects Research Part A: Clinical and Molecular Teratology*, 91, 867–872.
- Prins, G. S. (2008). Endocrine disruptors and prostate cancer risk. *Endocrine-Related Cancer*, 15, 649–656.
- Richard, S., Moslemi, S., Sipahutar, H., et al. (2005). Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environmental Health Perspectives*, 113, 716–720.
- Sanabria, M., Cuciolo, M. S., Guerra, M. T., et al. (2016). Sperm quality and fertility in rats after prenatal exposure to low doses of TCDD: A three-generation study. *Reproductive Toxicology*, 65, 29–38.
- Sanderson, J. T. (2006). The steroid hormone biosynthesis pathway as a target for endocrine-disrupting chemicals. *Toxicological Sciences*, 94, 3–21.
- Seachrist, D. D., Bonk, K. W., Ho, S. M., et al. (2016). A review of the carcinogenic potential of bisphenol A. *Reproductive Toxicology*, 59, 167–182.
- Shah, Y. M., & Rowan, B. G. (2005). The Src kinase pathway promotes tamoxifen agonist action in Ishikawa endometrial cells through phosphorylation-dependent stabilization of estrogen receptor (alpha) promoter interaction and elevated steroid receptor coactivator 1 activity. *Molecular Endocrinology*, 19, 732–748.
- Sweeney, M. F., Hasan, N., Soto, A. M., & Sonnenschein, C. (2015). Environmental endocrine disruptors: Effects on the human male reproductive system. *Reviews in Endocrine & Metabolic Disorders*, 16, 341–357.
- Tata, J. R. (2005). One hundred years of hormones. *EMBO Reports*, 6, 490–496.
- Tiemann, U. (2008). In vivo and in vitro effects of the organochlorine pesticides DDT, TCPM, methoxychlor, and lindane on the female reproductive tract of mammals: A review. *Reproductive Toxicology*, 25, 316–326.
- Wang, Z., Liu, H., & Liu, S. (2017). Low-dose bisphenol A exposure: A seemingly instigating carcinogenic effect on breast cancer. *Advanced Science*, 4, 1600248.
- Welshons, W. V., Nagel, S. C., & Vom Saal, F. S. (2006). Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure. *Endocrinology*, 147, S56–69.
- Williams, C., Bondesson, M., Kremontsov, D. N., & Teuscher, C. (2014). Gestational bisphenol A exposure and testis development. *Endocrine Disruptor*, 2, e29088–1.

Estrogen Agonists

Frederick S vom Saal and Wade V Welshons, University of Missouri-Columbia, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

AR	Androgen receptor
CDC	Centers for disease control and prevention
DES	Diethylstilbestrol
DOHaD	Developmental origins of adult health and disease
EC50	Excitatory concentration 50%
EDC	Endocrine disrupting chemical
EDSP	Endocrine Disruptor Screening Program
EPA	Environmental Protection Agency
ER α	Estrogen receptor alpha
ER β	Estrogen receptor beta
ERK1/2	Extracellular signal-regulated kinase
FQPA	Food Quality Protection Act of 1996
K _{ATP}	ATP-sensitive potassium (K ⁺) channels
K _d	Dissociation constant
NIEHS	National Institute of Environmental Health Sciences
NOEC	No observable effect concentration
NOAEL	No adverse effect level
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
SERM	Selective estrogen receptor modulator
TR	Thyroid hormone receptor
YES	Yeast estrogen screen

Background

There are many chemicals present in the environment that have been reported to disrupt normal endocrine function (TEDX, 2017). A number of these chemicals, used as insecticides, herbicides and fungicides, components of plastic and resins, and in a wide variety of other products, bind to estrogen receptors and stimulate or inhibit estrogenic responses (they act as receptor agonists or antagonists). Other chemicals, such as atrazine, glyphosate, lindane and bisphenol A (BPA) impact estrogen responses by altering the activity of the estrogen synthesizing enzyme, aromatase, in addition to impacting receptor function (Table 1).

Estrogens as a hormonal class were initially recognized in animal science and agriculture with respect to their effects in female reproduction and their responses generated in the female reproductive tract. However, after the estrogen receptor proteins and the mechanisms of estrogen responses were recognized beginning in the 1960s, estrogens have been defined specifically, as for all other hormones in endocrinology, by interactions with the estrogen receptor signaling systems. Specifically, this occurs by actions which start with specific, high affinity, saturable binding to and activation of estrogen receptors, and not by the final response(s), which are usually regulated by many additional factors and mechanisms in the animal's physiology (Fig. 1).

Therefore, unlike classical toxicology, where a chemical is characterized by its measurable effects on generic responses through unknown mechanisms, hormones and chemicals with hormonal activity, such as estrogens and xenoestrogens, are by definition known first by the mechanism of action through the linked hormone receptor signaling systems. This links the chemical immediately to the existing literature spanning over a century on the sensitive, specific estrogenic effects and responses, including normal reproduction, normal nonreproductive physiology, and estrogen-stimulated cancers. This crucial difference in the starting points and consequent approaches has been responsible for the failure of classical toxicology to adequately identify and characterize estrogenic endocrine disrupting chemicals before incontrovertible and massive human damage has already occurred, as for the DES tragedy.

Table 1 Common xenoestrogens (A) and disruptors of estrogen signaling (B)

A. Xenoestrogens
Insecticides
o,p'DDT
Flame retardants
PBDE's
PCB's
Herbicides
Glyphosate
Plasticizers
Phthalates
Plastic or resin monomers
Bisphenol A (BPA)
Bisphenol S (BPS)
Bisphenol F (BPF)
Detergent Alkylphenols
Octylphenol
Nonylphenol
Pharmaceuticals
Diethylstilbestrol (DES)
Ethinylestradiol (EE)
Phytoestrogens
Genistein
Daidzein
Biochanin A
Coumestans
Resveratrol
Mycotoxins
Zearalenone
Zearalenol
Metals
Cadmium
Nanoparticles
Gold Nanoparticles (GNP)
B. Aromatase modulators, estrogen antagonists, antagonists related to estrogen signaling, and other mechanisms
Aromatase Modulators
Glyphosate
Atrazine
BPA
Lindane
Estrogen Antagonists
Lindane
Antagonists for Estrogen-Related Receptor α (ESRRA)
Toxaphene
Chlordane
Other Mechanisms
Insecticide
Toxaphene
Fungicide
Hexachlorobenzene
Endosulfan
Nicotine
Pyrethroids

Diethylstilbestrol (DES): An Latrogenic Tragedy Predicted Long-Latency Effects of Xenoestrogens

Compared to other components of the endocrine system, there is a considerable amount known regarding the mechanisms of estrogen action. One reason is that there has been interest in developing estrogenic drugs to impact female fertility since the early 1900s (Dodds and Lawson, 1936). The publication of the estrogenic activity of the potent estrogenic drug diethylstilbestrol (DES) in 1938 (Dodds et al., 1938) resulted in millions of women being administered DES during pregnancy between the late 1940s through 1972 in the misguided belief that supplemental estrogen would reduce the incidence of miscarriage. Instead, fetal exposure to DES

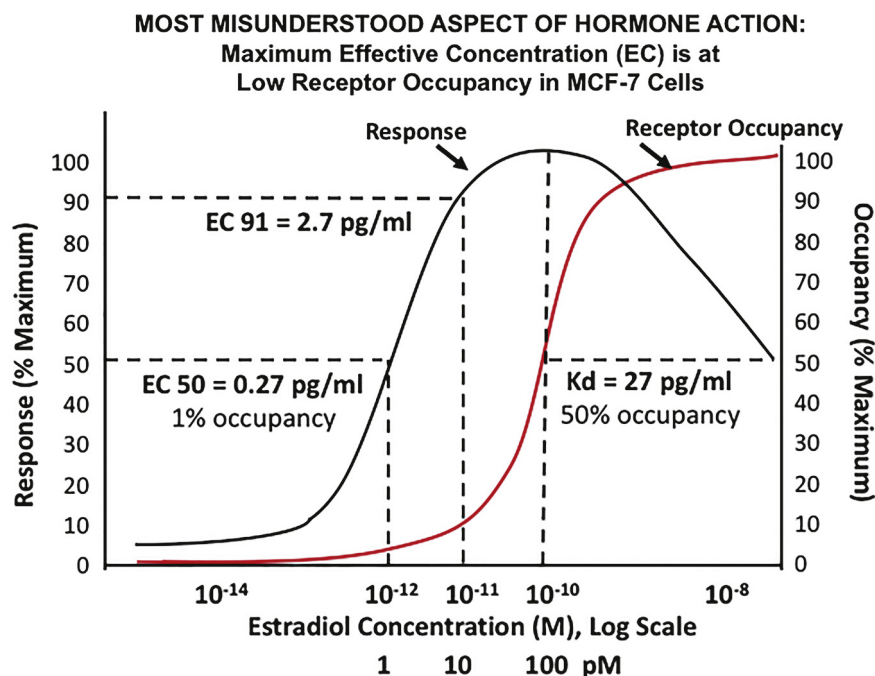


Fig. 1 The effect of very low (femtomolar) to high (nanomolar) concentrations of estradiol on cell proliferative response (percent of maximum response) in relation to percent receptor occupancy of MCF-7 human breast cancer cells. The data show that the physiological range for proliferative response to estradiol is within the sub picomolar to very low picomolar range. Above the nanomolar range, nuclear estrogen receptors are saturated, and no additional estradiol can cause an increase in receptor occupancy and thus receptor-mediated response (as proliferation here). In this cell culture system, the peak proliferative response occurs at the K_d for estradiol (50% receptor occupancy), while 50% of the maximum response (the EC_{50}) occurs at 100-fold lower estradiol concentration and approximately 1% receptor occupancy. Thus, estradiol (and other hormones) acts via receptors at very low percent receptor occupancy, where the increase in estradiol concentration is approximately proportional to an increase in receptor occupancy that mediates an increase in the proliferative response. Derived from data published in Welshons, W. V., Thayer, K. A., Judy, B. M. et al. (2003). Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environmental Health Perspectives* **111**, 994–1006.

during the first and second trimesters disrupted development of the reproductive system in both males and females (Reed and Fenton, 2013), and also impacted neurobehavioral development (Hines, 2011). Unfortunately, since fetal DES exposure did not result in an easily identified external malformation, the use of DES continued for decades before it was identified as a human carcinogen. Fetal DES exposure was related to clear cell adenocarcinoma of the vagina and cervix in female offspring in their teens and 20s, and an increase in the risk of breast cancer in their 40s (Palmer et al., 2006). The DES iatrogenic tragedy alerted the medical community to the hazards of exposure to drugs that could cross the placenta, but sadly, the implications of this tragedy for exposure to environmental chemicals with estrogenic activity has remained largely unnoticed by clinicians (Cohen and vom Saal, 2017).

In response to the DES tragedy, the US National Institute of Environmental Health Sciences (NIEHS) began a research program aimed at elucidating life-history effects of DES exposure during development using mice as the model organism. In a number of cases, effects were observed in DES-exposed mice that led clinicians to look for the same effects in DES exposed human offspring, and only then did clinicians observe in humans the effects that had been observed in mice (Haney et al., 1986). Of great concern is the finding in mice that fetal DES exposure not only harmed the offspring (F1 generation) of the exposed mothers, but the offspring (F2) of the F1 exposed females; that is, the grandchildren of the DES exposed mother (Newbold et al., 2006), tentatively confirmed in a human epidemiological study (Kalfa et al., 2011). The multigenerational impact of environmental chemicals, including those with estrogenic activity, as well as effects on subsequent generations (transgenerational—beyond F2) is now a major research focus, based on findings that hormones and hormonally active chemicals can alter the epigenetic mechanisms that control gene function, particularly during critical periods in tissue differentiation. These findings are predicted to be due to epigenetic changes in the germ line that somehow escape the deprogramming and/or reprogramming of the genome that would otherwise occur without chemical exposure (Nilsson and Skinner, 2015).

The NIEHS research on DES effects identified that there was a very close relationship between effects observed in mice and effects found in human DES exposed male and female offspring (Newbold, 1995; Newbold et al., 2006). The experimental demonstration that the laboratory mouse accurately predicted virtually all developmental effects of an estrogenic drug in humans has had a profound impact on the study of environmental chemicals with estrogenic activity. In particular, the DES literature led to a focus on the long-term effects of exposure to environmental chemicals during “critical periods” in organogenesis, during which epigenetic changes impact the differentiation and life-long functioning of organ systems. There is now a medical/scientific society whose focus is on the “Developmental Origins of Adult Health and Disease” (the DOHaD Society and journal).

Estrogenic Endocrine Disrupting Chemicals

The potential impact of chemicals in the environment, both naturally occurring and manmade, that could disrupt the functioning of the endocrine system was first raised at a meeting held at Wingspread in Racine Wisconsin in 1991, and a review of endocrine disrupting chemicals (EDCs) was published in 1993 with a focus on the long-term consequences of exposure during development (Colborn et al., 1993). At that time there was already evidence that a number of EDCs had estrogenic activity, and in 1996 the US Congress passed the Food Quality Protection Act (FQPA) that mandated that the EPA screen pesticide chemicals for estrogenic activity. The EPA expanded their screening program, The Endocrine Disruptor Screening Program (EDSP), to include disruption of androgen and thyroid hormones as well as estrogens. A combination of industry interference, EPA mismanagement, and then a lack of funding, resulted in the failure of the EDSP (Zoeller et al., 2012). However, over the past 25 years thousands of studies have been published documenting the effects of EDCs using *in vitro* assays, experiments with laboratory animals, observational and experimental studies with wildlife, and human epidemiological studies. Environmental estrogens (xenoestrogens) have been the focus of a large number of these EDC studies.

Ligand-Receptor Interaction: Affinity vs. Potency

One of the crucial issues regarding xenoestrogens that has created considerable confusion is that, in general, xenoestrogens exhibit a lower binding affinity relative to estradiol for the classical nuclear estrogen receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β). This has led those who believe that dose–response relationships for EDCs, and all other environmental chemicals, are monotonic and exhibit a threshold below which there is no effect to not be concerned with the potential for these chemicals to pose a hazard to human health. However, it is now well documented that neither of these assumptions is valid for EDCs, which do not exhibit thresholds and show nonmonotonic dose–response relationships (Sheehan, 2006; Vandenberg et al., 2012); despite the extensive data, these remain the core assumptions (monotonic dose responses with thresholds) used by regulatory agencies in chemical risk assessments (vom Saal and Sheehan, 1998; Zoeller et al., 2012). In addition, it is well recognized that affinity (K_d) of a chemical for a receptor is not equivalent to its potency (EC₅₀) (Welshons and Jordan, 1987), which is impacted by coregulatory proteins (coactivators and corepressors) that together with the receptor form the transcriptional complex. For example, using HeLa cells transiently transfected with ER β , the increase in response to estradiol was significantly greater with the co-activator TIF2 relative to SRC-1, demonstrating that estimations of potency based only on affinity of a ligand for a receptor was not valid (Routledge et al., 2000).

The issue of potency is also greatly complicated by the fact that while in chemical risk assessments only one chemical is tested, the typical person is exposed to hundreds or thousands of chemicals that can potentially interact with each other. The Centers for Disease Control and Prevention (CDC) has managed to obtain funding from the US Congress to examine 308 chemicals out of a much larger number that there is a need to examine in the US population; one glaring example of the need to expand the number of chemicals being tested is that the CDC does not determine the levels of glyphosate in the US population, although glyphosate is the highest volume herbicide used in the US and worldwide, and there is evidence that glyphosate is a carcinogen and an EDC (Myers et al., 2016).

Concentration Additive Effects of Xenoestrogens

Kortenkamp and colleagues have published studies concerning the consequences of examining mixtures of estrogenic chemicals, with each xenoestrogen being added to the mixture at a concentration that individually produced no observable effect (the NOEC). They tested a mixture of eight xenoestrogens using the yeast estrogen screen (YES) and showed that the xenoestrogen mixture stimulated a response consistent with “concentration addition” (Silva et al., 2002). They also showed the same result when a mixture of 11 xenoestrogens (each at concentration that individually produced no effect) was tested in combination with a dose of estradiol that alone stimulated a significant response (Rajapakse et al., 2002). Thus, very low doses of estrogenic chemicals that act through the same receptor can have additive effects that can alter the response to the potent steroid estradiol, even though when tested individually, the xenoestrogen dose tested would have been declared “safe.” In addition, a take-home message from these studies is that testing chemicals individually with the objective of establishing a NOEC or no adverse effect level (NOAEL) results in an invalid estimation of a safe exposure level, because we are not exposed to only one chemical at a time.

Synergistic Effects of Xenoestrogens

Synergy is a naturally occurring feature of estrogens. Estradiol stimulates mammary gland progesterone receptors, and after a brief exposure to estradiol, there is a substantial synergistic effect of estradiol with progesterone responsiveness by stimulation of progesterone receptors. In addition, estradiol stimulates prostate androgen receptors (vom Saal et al., 1997; Taylor et al., 2012) and uterine oxytocin receptors (Richter et al., 2004). The interaction of estrogens with receptors for other hormones thus leads to synergistic as opposed to additive effects, which are observed when different estrogens produce similar effects through the same receptor mechanism.

Rapid Signaling via Nonnuclear Estrogen Receptors

A major change in understanding estrogen action occurred with the discovery that estrogens acted through receptors associated with rapid signaling systems. While for some time there had been evidence for rapid responses to estrogen that were not consistent with responses mediated by changes in gene activity mediated by nuclear transcription factors, there is now an extensive literature on the cellular response pathways that mediate the rapid responses to endogenous and xeno-estrogens. Of great importance, the potency of xenoestrogens, such as BPA, are equivalent to estradiol for effects mediated by these cell membrane associated receptors.

For example, in rat cerebellar cortex neurons in primary culture, estradiol activated rapid estrogen-mediated extracellular signal-regulated kinase (ERK1/2) signaling with a peak response occurring at 10^{-10} M and another peak occurring at 10^{-7} and 10^{-6} M, while at 10^{-9} and 10^{-8} M the response was at baseline. A nonmonotonic dose-response profile is common for membrane-receptor mediated responses to hormones, and the multiple peaks at 1000–10,000 fold different doses suggest that different mechanisms are involved in the peak at 10^{-10} M as opposed to 10^{-6} M. In addition, in this study the response to the same doses of BPA administered alone tracked identically with estradiol, in sharp contrast to the often-stated conclusion that BPA is a “weak” estrogen. However, when the low-dose peak response dose of estradiol (10^{-10} M) was coadministered with increasing doses of BPA, there was a linear inhibition of estradiol phosphorylation of ERK1/2 by BPA beginning at 10^{-12} M BPA (Zsarnovszky et al., 2005).

This finding of antagonism by BPA of estradiol-mediated activation of responses in the brain is consistent with in vivo findings in African green monkeys that BPA completely abolished the stimulatory effect of 10^{-10} estradiol on spine synapse formation in the cerebral cortex and hippocampus, and thus acted in these areas of the primate brain as an estrogen antagonist. The ability of BPA to interfere with spine synapse formation has clear implications for cognitive functioning (Leranth et al., 2008). With regard to BPA acting in some tissues as an agonist or antagonist, this is not unique and is why BPA is referred to as a selective estrogen receptor modulator or SERM (Jordan and Murphy, 1990). Virtually all xenoestrogens have been identified as SERMs as well.

Nonadditive Effects of Mixtures of Xenoestrogens via Membrane-Associated Estrogen Receptors and Nonmonotonic Dose-Response Relationships

Watson and colleagues identified modulation of ERK1/2 signaling in rat pituitary (GH3/B6/F10) cells in response to endogenous estrogens as well as numerous xenoestrogens, including phytoestrogens, alkylphenols, and BPA. ERK1/2 activation by estradiol and these xenoestrogens occurred in the femtomolar range, but the response profiles were unique for the different xenoestrogens relative to estradiol. Similar to findings by Zsarnovszky, the xenoestrogens showed nonmonotonic dose-response curves. However, when estradiol and each xenoestrogen were examined as a mixture, entirely different, typically monotonic, response profiles were observed. Thus, simple concentration additive effects were not observed, since each chemical had a unique response profile. This is expected for xenoestrogens that act as SERMs and multiple response mechanisms are involved. In addition, using selective agonists of ER α and ER β , the low-dose ERK1/2 activation was shown to be via ER α (Jeng et al., 2009; Jeng and Watson, 2011).

Experiments with mouse pancreatic beta cells identified unique effects of nonnuclear ER α and ER β , and also shed light on the intracellular pathways that can mediate nonmonotonic dose-responses. Specifically, glucose entry into pancreatic beta cells results in K_{ATP} channel closure, membrane depolarization, opening of voltage-gated Ca²⁺ channels and Ca²⁺ influx, which results in exocytosis of insulin granules (first phase insulin release). However, including either a 0.1 or 1 nM concentration of BPA resulted in a diminished Ca²⁺ influx, while 10 or 100 nM BPA had no effect relative to negative control levels. Use of selective agonists and antagonists for ER α and ER β and ER β knockout mouse cells revealed that the low-dose BPA effects on Ca²⁺ channels and insulin release were mediated via activation of ER β pathways. In contrast, the 100 nM BPA concentration inhibited the low-dose BPA effect via activation of ER α , which opposed ER β actions on Ca²⁺ currents by acting through P13K kinase, resulting in a nonmonotonic dose response. ERK1/2 pathways did not contribute to these findings. In pancreatic beta cells there is thus an activation of ER β at very low doses of BPA and at higher doses (≥ 10 nM), the low-dose BPA effect is blocked via pathways mediated by ER α (Villar-Pazos et al., 2017).

Importantly, nonmonotonic dose-responses are observed due to the interaction of xenoestrogens with nuclear ERs due to differences in the profile of gene expression as the dose increases. For example, high doses of estradiol result in gene expression for growth factors that inhibit the proliferation stimulated by estradiol at low doses in MCF-7 breast cancer cells (Cosér et al., 2003). One possibility for the marked difference in gene expression at subpicomolar and picomolar concentrations of estradiol, as opposed to nonphysiological nanomolar concentrations is that high doses of estradiol bind to receptors for other hormones, such as androgen receptors (AR), which is referred to as receptor cross-talk. The cross talk with receptors for androgen and thyroid hormone at increasing doses of BPA could be a mechanism that accounts for some nonmonotonic dose responses identified for BPA (Vandenberg et al., 2012). Binding of BPA to AR or TR antagonized the action of androgens and thyroid hormone (Fig. 2). This is a reason that if one is interested in the estrogenic actions of BPA, they cannot be determined by testing at doses that saturate estrogen receptors, because estrogen-receptor saturating doses lead to interactions with receptors for other hormones as well as oppose the saturated estrogenic actions.

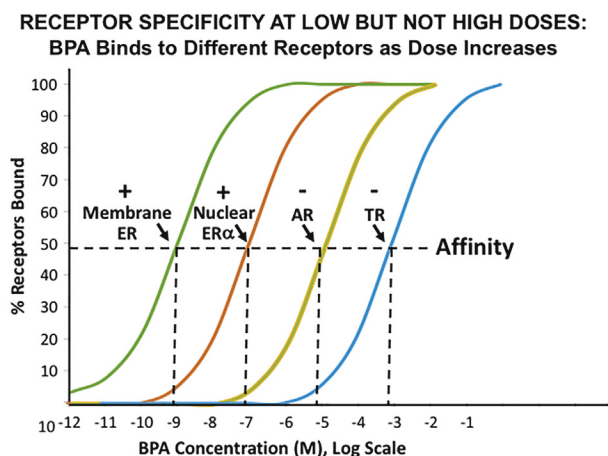


Fig. 2 BPA acts as an estrogen receptor agonist (+) for membrane and nuclear estrogen receptors at femtomolar and nanomolar concentrations, respectively. The binding of BPA to androgen receptors (AR) and thyroid hormone receptors (TR) at much higher concentrations (at which both membrane and nuclear estrogen receptors are saturated) is antagonistic (–) to androgen and thyroid hormone action. As shown in studies of pancreatic beta cells (Villar-Pazos et al., 2017), the high-dose binding with different receptors (a common feature of hormones) can account for non-monotonic dose–response curves.

All Environmental Chemical Interactions With the Normal Receptor-Mediated Estrogenic Signaling Are Disruptive

Estrogens in the organism are active and required throughout life to induce normal fetal development and to regulate normal physiology in the adult (males as well as females). For these reasons, the interactions of external environmental estrogenic EDCs, which are not controlled by the animal, will be disruptive and adverse to the normal hormone action and all of the physiology controlled by estrogens, known now or discovered in the future. The reasons for this are as follows. (1) A xenoestrogen of identical action to the endogenous estrogens (that is, a full agonist), will add to the normal signaling to estrogenize the animal abnormally, including effects on estrogen-stimulated cancers. The additivity of xenoestrogens with each other was described above. (2) A xenoestrogen of identical action to endogenous estrogens, but which can stimulate only a partial response (that is, a partial agonist), will blunt the normal signaling in the animal, inhibiting the normal effects of endogenous estrogens. (3) A xenoestrogen which binds to estrogen receptors but activates responses different from the endogenous estrogens (that is, a SERM), will change normal signaling in the animal and disrupt normal estrogen actions. (4) A chemical which binds to estrogen receptors but does not activate the receptor (that is, an estrogen antagonist or pure antiestrogen), will by competitive inhibition prevent the binding of endogenous receptors, and inhibit normal signaling by the endogenous estrogens, disrupting all of the physiology normally controlled by endogenous estrogens. And (5), environmental chemicals that change endogenous estrogen synthesis (aromatase modulators), or normal estrogen-controlled receptor activation (ligand-independent activators of steroid receptors; see below), or any other aspect of estrogenic hormone action and control, will change normal estrogen-regulated physiology in one direction or another. As indicated previously, for all of the above cases the presence of a threshold has been shown to not exist for ligand–receptor interactions (Sheehan, 2006).

Disruption of Estrogen Homeostasis by Altering Estrogen Synthesis and Ligand-Independent Effects

Another mechanism by which environmental chemicals can act as endocrine disruptors is by altering the activity of the enzyme, aromatase, which is the estrogen synthesizing enzyme that is sometimes referred to “estrogen synthetase.” A number of chemicals alter aromatase activity: The herbicide atrazine increases aromatase activity in the ovary (Holloway et al., 2008). BPA increases aromatase activity in the prostate, and the increase in intracellular estradiol may contribute the effects of very low doses of BPA on the prostate through additive effects (Arase et al., 2011; Taylor et al., 2012). In addition, BPA and the insecticide lindane increase aromatase activity in the placenta (Nativelle-Serpentini et al., 2003, while glyphosate, as well as other chemicals in commercial formulations of this herbicide, decrease aromatase activity in placenta cells (Defarge et al., 2016).

There is evidence that steroid receptors can be activated without binding of a ligand to the receptor, which is referred to as a “ligand-independent” mechanism. This can result from stimulation of growth factors, such as EGF and IGF-1, that bind to their receptors, which then leads to activation of the growth factor receptor and subsequent phosphorylation and activation of the estrogen receptor. There are a number of examples of ligand-independent effects: for example, BPA and DES have been reported in experiments involving cell and organ culture to produce ligand-independent effects (Gupta, 2000; Mesnage et al., 2017).

Regrettable Substitutions

In the United States, the EPA has only banned the use of a few chemicals over more than 40 years. The FDA has an even more dismal record. The US congress passed the Food Additive Amendment in 1958, which was intended to protect the public from harmful chemicals by requiring an affirmation of safety by the FDA and toxicity testing before they were used in or on foods. Specifically, the FDA supposedly considers chemicals to be safe when there is “a reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use” [21 CFR §170.3(i)]. In other words, a chemical is considered unsafe until proven safe, but sadly, exactly the opposite is the case for the “food” division at the FDA, in sharp contrast to the approach used in the “drug” division of the FDA. The 1958 law included chemical ingredients intentionally added to foods or “direct additives” such as preservatives, flavors, sweeteners, and emulsifiers, as well as chemicals unintentionally added to food, such as chemicals used in production, packaging, preparation and transporting that through migration or leaching may enter the food supply; one of the major examples is BPA that is used in food packaging and the resin lining of food and beverage cans. The law required the FDA to consider chemicals that were structurally similar as being potentially similar to each other in terms of toxicity unless proven otherwise. The FDA has simply chosen to ignore this law (Maffini and Vogel, 2017). The consequence of the EPA ignoring the requirements of the Food Quality Protection Act, and of the FDA ignoring the Food Additive Amendment is that chemicals that are found to pose a hazard to animal and human health, such as BPA and its analogues, remain unregulated by federal agencies in the United States. This also leads to chemicals that pose a hazard to human health being used to replace (regrettably) other chemicals that pose a hazard to human health.

Similar to smoking, it has been cities and states, and other countries, that have regulated BPA, and there are ongoing efforts to regulate BPA analogues. Due to public pressure on corporations, there has been a gradual voluntary transition to the use of other chemicals as substitutes for BPA. Many of these are now being referred to as “regrettable substitutions,” because they are BPA analogues that are structurally similar to BPA and show estrogenic activity in a variety of assay systems with ER α and ER β (examples are: BPB, BPF, BPS, BPZ, BPAF, BPAP, TM-BPA, MH-BPF; there are dozens of other BPA analogues). In some cases, the replacement analogues are worse than BPA, but they are being used in “BPA-free” products, which the public thus assumes are safe (Stossi et al., 2014; Rochester and Bolden, 2015; Mesnage et al., 2017). There are other examples of “regrettable substitutions,” such as the use of brominated flame retardants (polybrominated diphenyl ethers or PBDEs) as replacement chemicals of polychlorinated biphenyls (PCBs), and over the last 40 years PBDEs have been shown to be as bad or worse than PCBs. Attempts to increase federal oversight of chemicals prior to their use in products, with the potential for widespread exposure, have not been successful in the United States, while the EU has taken a more precautionary approach.

Politics of Xenoestrogen Evaluation, and Failure by Regulatory Agencies to Protect the Public

A consequence of political effects on evaluation of endocrine disruptors that are in commerce is that reading just the abstract of a paper is dangerous in this field. This is because, while falsification (changing existing data) or fabrication (creation of data that did not exist) reported as experimental findings are defined as prosecutable scientific misconduct, conclusions drawn in a publication which are contraindicated by the data, that is statements characterizing results in the text of a paper which are blatantly opposite to the data, are not administratively/legally defined as scientific misconduct. There are a number of examples of this for studies involving BPA, one of the most glaring being a study published by FDA scientists in which they concluded in the abstract that “No age-related changes were seen in internal exposure metrics for aglycone BPA in monkeys,” which was contradicted by their findings (Doerge et al., 2010).

Therefore, unlike in most of the scientific literature and in the independent academic literature, for studies involving chemicals in commerce it is essential to the evaluation of experimental findings to read the paper's results and examine the data, to determine independently whether the data in fact support the conclusions as stated in the Abstract or the Discussion. Unfortunately, in many cases in regulatory and industry papers claiming some version of “no harm,” the data may clearly show the opposite of the written conclusions: an example was the inappropriate analysis of data to justify the conclusion of “no harm” in an industry-funded BPA study (Elswick et al., 2000) that was harshly criticized by NIH statisticians who had reviewed the study for the National Toxicology Program (Haseman et al., 2001).

In summary, the current approach by federal regulatory agencies of considering environmental chemicals safe until “proven” not to be safe (the opposite of the law in the United States) has led to thousands of chemicals, an unknown number of which may have estrogenic endocrine disrupting activity, being used in food and food packaging, as well as in a wide range of consumer products. This approach is leading to wide-spread exposure of the general population to chemicals about which, in many cases, nothing is known about the potential hazards posed to human and animal health. Even when there is extensive documentation of harm, the chemical industry and their lobbyists have managed to block attempts to regulate the chemicals; some examples are dioxin, BPA, perfluorinated compounds, flame retardants, and additives such as phthalates, although there are many others. When there is public pressure to stop using a specific chemical, there is a history of “regrettable substitution” with chemicals that turn out to be as harmful as the chemical that was replaced.

The fact that the interaction of the thousands of chemicals to which the general population is exposed is not considered, for the relative few (out of an estimated 100,000) chemicals that are tested for health effects, is a major problem that has proven to be very difficult to address in terms of developing experimental protocols that regulatory agencies would be willing to adopt. We thus are

living in the “chemical age” but do not have appropriate safeguards in place to address the findings that environmental chemicals are implicated in many of the chronic diseases, such as obesity and diabetes, and hormone-dependent cancers, that have been increasing since the post World War II dramatic increase in the use of manmade chemicals (Soto et al., 2013; Cohen and vom Saal, 2017; Heindel et al., 2017).

References

- Arase, S., Ishii, K., Igarashi, K., et al. (2011). Endocrine disrupter bisphenol A increases in situ estrogen production in the mouse urogenital sinus. *Biology of Reproduction*, 84, 734–742.
- Cohen, A., & vom Saal, F. S. (Eds.). (2017). *Weil integrative medicine library/Integrative environmental medicine*. New York: Oxford University Press.
- Colborn, T., vom Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives*, 101, 378–384.
- Coser, K. R., Chesnes, J., Hur, J., et al. (2003). Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/bus breast cancer cells by DNA microarray. *Proceedings of the National Academy of Sciences*, 100, 13994–13999.
- Defarge, N., Takacs, E., Lozano, V. L., et al. (2016). Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *International Journal of Environmental Research and Public Health*, 13.
- Dodds, E. C., & Lawson, W. (1936). Synthetic oestrogenic agents without the phenanthrene nucleus. *Nature*, 137, 996.
- Dodds, E. C., Lawson, W., & Noble, R. L. (1938). Biological effects of the synthetic oestrogenic substance 4: 4'-dihydroxy- a: B-dimethylstilbene. *Lancet*, 234, 1389–1391.
- Doerge, D. R., Twaddle, N. C., Woodling, K. A., & Fisher, J. W. (2010). Pharmacokinetics of bisphenol A in neonatal and adult rhesus monkeys. *Toxicology and Applied Pharmacology*, 248, 1–11.
- Elswick, B. A., Welsch, F., & Janszen, D. B. (2000). Effect of different sampling designs on outcome of endocrine disruptor studies. *Reproductive Toxicology*, 14, 359–367.
- Gupta, C. (2000). The role of estrogen receptor, androgen receptor and growth factors in diethylstilbestrol-induced programming of prostate differentiation. *Urological Research*, 28, 223–229.
- Haney, A. F., Newbold, R. R., Fetter, B. F., & McLachlan, J. A. (1986). Paraovarian cysts associated with prenatal diethylstilbestrol exposure. Comparison of the human with a mouse model. *American Journal of Pathology*, 124, 405–411.
- Haseman, J. K., Bailer, A. J., Kodell, R. L., et al. (2001). Statistical issues in the analysis of low-dose endocrine disruptor data. *Toxicological Sciences*, 61, 201–210.
- Heindel, J. J., Blumberg, B., Cave, M., et al. (2017). Metabolism disrupting chemicals and metabolic disorders. *Reproductive Toxicology*, 68, 3–33.
- Hines, M. (2011). Prenatal endocrine influences on sexual orientation and on sexually differentiated childhood behavior. *Frontiers in Neuroendocrinology*, 32, 170–182.
- Holloway, A. C., Anger, D. A., Crankshaw, D. J., et al. (2008). Atrazine-induced changes in aromatase activity in estrogen sensitive target tissues. *Journal of Applied Toxicology*, 28, 260–270.
- Jeng, Y. J., & Watson, C. S. (2011). Combinations of physiologic estrogens with xenoestrogens alter ERK phosphorylation profiles in rat pituitary cells. *Environmental Health Perspectives*, 119, 104–112.
- Jeng, Y. J., Kochukov, M. Y., & Watson, C. S. (2009). Membrane estrogen receptor- α -mediated nongenomic actions of phytoestrogens in GH3/B6/F10 pituitary tumor cells. *Journal of Molecular Signaling*, 4, 2.
- Jordan, V. C., & Murphy, C. S. (1990). Endocrine pharmacology of antiestrogens as antitumor agents. *Endocrine Reviews*, 11, 578–610.
- Kalfa, N., Paris, F., Soyer-Gobillard, M. O., et al. (2011). Prevalence of hypospadias in grandsons of women exposed to diethylstilbestrol during pregnancy: A multigenerational national cohort study. *Fertility and Sterility*, 95, 2574–2577.
- Leranth, C., Hajszan, T., Szigeti-Buck, K., et al. (2008). Bisphenol A prevents the synaptogenic response to estradiol in hippocampus and prefrontal cortex of ovariectomized nonhuman primates. *Proceedings of the National Academy of Sciences*, 105, 14187–14191.
- Maffini, M. V., & Vogel, S. (2017). Food additives: Health consequences of regulatory oversight failure. In A. Cohen, & F. S. vom Saal (Eds.), *Integrative environmental medicine* (pp. 255–277). New York: Oxford University Press.
- Mesnage, R., Phedonos, A., Bisemi, M., et al. (2017). Evaluation of estrogen receptor α activation by glyphosate-based herbicide constituents. *Food and Chemical Toxicology*, 108, 30–42.
- Myers, J. P., Antoniou, M. N., Blumberg, B., et al. (2016). Concerns over use of glyphosate-based herbicides and risks associated with exposures: A consensus statement. *Environmental Health*, 15(19).
- Nativelle-Serpentini, C., Richard, S., Seralini, G. E., & Sourdaire, P. (2003). Aromatase activity modulation by lindane and bisphenol-a in human placental JEG-3 and transfected kidney E293 cells. *Toxicology In Vitro*, 17, 413–422.
- Newbold, R. R. (1995). Cellular and molecular effects of developmental exposure to diethylstilbestrol: Implications for other environmental estrogens. *Environmental Health Perspectives*, 103, 83–87.
- Newbold, R. R., Padilla-Banks, E., & Jefferson, W. N. (2006). Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology*, 147, S11–17.
- Nilsson, E. E., & Skinner, M. K. (2015). Environmentally induced epigenetic transgenerational inheritance of reproductive disease. *Biology of Reproduction*, 93, 145.
- Palmer, J. R., Wise, L. A., Hatch, E. E., et al. (2006). Prenatal diethylstilbestrol exposure and risk of breast cancer. *Cancer Epidemiology, Biomarkers & Prevention*, 15, 1509–1514.
- Rajapakse, N., Silva, E., & Kortenkamp, A. (2002). Combining xenoestrogens at levels below individual no-observed-effect-concentrations dramatically enhances steroid hormone action. *Environmental Health Perspectives*, 110, 917–921.
- Reed, C. E., & Fenton, S. E. (2013). Exposure to diethylstilbestrol during sensitive life stages: A legacy of heritable health effects. *Birth Defects Research. Part C, Embryo Today*, 99, 134–146.
- Richter, O. N., Kubler, K., Schmolling, J., et al. (2004). Oxytocin receptor gene expression of estrogen-stimulated human myometrium in extracorporeally perfused non-pregnant uteri. *Molecular Human Reproduction*, 10, 339–346.
- Rochester, J. R., & Bolden, A. L. (2015). Bisphenol S and F: A systematic review and comparison of the hormonal activity of bisphenol A substitutes. *Environmental Health Perspectives*, 123, 643–650.
- Routledge, E. J., White, R., Parker, M. G., & Sumpter, J. P. (2000). Differential effects of xenoestrogens on coactivator recruitment by estrogen receptor (ER) α and β . *The Journal of Biological Chemistry*, 275, 35986–35993.
- Sheehan, D. M. (2006). No-threshold dose-response curves for nongenotoxic chemicals: Findings and applications for risk assessment. *Environmental Research*, 100, 93–99.
- Silva, E., Rajapakse, N., & Kortenkamp, A. (2002). Something from “nothing”—eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environmental Science & Technology*, 36, 1751–1756.
- Soto, A. M., Briskin, C., Schaeberle, C., & Sonnenschein, C. (2013). Does cancer start in the womb? Altered mammary gland development and predisposition to breast cancer due to in utero exposure to endocrine disruptors. *Journal of Mammary Gland Biology and Neoplasia*, 18, 199–208.
- Stossi, F., Bolt, M. J., Ashcroft, F. J., et al. (2014). Defining estrogenic mechanisms of bisphenol A analogs through high throughput microscopy-based contextual assays. *Chemistry & Biology*, 21, 743–753.

- Taylor, J. A., Richter, C. A., Suzuki, A., et al. (2012). Dose-related estrogen effects on gene expression in fetal mouse prostate mesenchymal cells. *PLoS One*, 7(10), e48311.
- TEDX (2017). List of endocrine disrupting chemicals. The endocrine disruption exchange. <https://endocrinedisruption.org/interactive-tools/tedx-list-of-potential-endocrine-disruptors/search-the-tedx-list>. Accessed: November 26, 2017.
- Vandenberg, L. N., Colborn, T., Hayes, T. B., et al. (2012). Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*, 33, 378–455.
- Villar-Pazos, S., Martinez-Pinna, J., Castellano-Munoz, M., et al. (2017). Molecular mechanisms involved in the non-monotonic effect of bisphenol-A on Ca^{2+} entry in mouse pancreatic beta-cells. *Scientific Reports*, 7(11770).
- vom Saal, F. S., & Sheehan, D. M. (1998). Challenging risk assessment. *Forum for Applied Research and Public Policy*, 13, 11–18.
- vom Saal, F. S., Timms, B. G., Montano, M. M., et al. (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proceedings of the National Academy of Sciences*, 94, 2056–2061.
- Welshons, W. V., & Jordan, V. C. (1987). Adaptation of estrogen-dependent MCF-7 cells to low estrogen (phenol red-free) culture. *European Journal of Cancer & Clinical Oncology*, 23, 1935–1939.
- Zoeller, R. T., Brown, T. R., Doan, L. L., et al. (2012). Endocrine-disrupting chemicals and public health protection: A statement of principles from the Endocrine Society. *Endocrinology*, 153, 4097–4110.
- Zsamovszky, A., Le, H. H., Wang, H. S., & Belcher, S. M. (2005). Ontogeny of rapid estrogen-mediated extracellular signal-regulated kinase signaling in the rat cerebellar cortex: Potent nongenomic agonist and endocrine disrupting activity of the xenoestrogen bisphenol A. *Endocrinology*, 146, 5388–5396.

Further Reading

- Welshons, W. V., Thayer, K. A., Judy, B. M., et al. (2003). Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environmental Health Perspectives*, 111, 994–1006.

Plastics

Julie Boberg, Kit Granby, Terje Svingen, and Anne Marie Vinggaard, Technical University of Denmark, Lyngby, Denmark

© 2018 Elsevier Inc. All rights reserved.

Introduction to Plastics

Plastics comprise a diverse group of polymeric materials that can easily be molded and shaped into a variety of different products. Because of their malleable properties, the use of plastics has increased dramatically since it was first introduced on the market a century ago. Especially since the 1950s the annual growth rate has been high, reaching an annual world-wide production of 322 million tons in 2015, corresponding to 43 kg per person (Plastic Europe, 2017). Packaging is the main application for plastics, followed by building and construction materials, automotive parts, electronics, to mention just a few.

Within the food sector, different types of plastic polymers are used. Polyethylenes (PE) and polypropylenes (PP) are among the most common due to their attractive physical and chemical properties, as well as their resistance to moisture. Polyethylene terephthalate (PET) and polycarbonate (PC) are examples of polyesters that are used for disposable bottles suitable for recycling or for nondisposable food and drinking containers, respectively. Polyvinylchloride (PVC) is used to make rigid or cling film, although PE is now the preferred material for cling film since it requires less softener.

Because of the very large world-wide production of plastics, an extremely slow breakdown process, combined with disposable consumer behaviors and a limited fossil fuel reserve, there is a great need for a circular plastic economy. For the most part, the waste management plans are limited or insufficient, which has resulted in a planet literally flooded with plastics. Recycling will make plastic production more sustainable, and reprocessed waste plastic such as multilayer plastic food packaging is expected to be used for many more applications in the future. However, contamination of the recycled products may become very complex and difficult to track and handle. Therefore, alternative bio-based polymers made from nonfossil biomass have been introduced to the markets and are expected to become much more common (Plastic Europe, 2017).

Endocrine Disrupting Chemicals in Plastic

Plastics represent a major source of human exposure to endocrine disrupting chemicals (EDCs). Some of the best known EDCs to which most—if not all—humans are exposed, such as the nonpersistent phthalates and bisphenols, can originate from plastics. Persistent organic pollutants (POPs), such as polychlorinated compounds or brominated flame retardants (BFR), are used in certain nonfood plastic products or are present as nonintentional contaminants and thereby also contribute to the overall exposure burden from plastics.

Plastic polymers have covalent bonds and are therefore not considered likely to migrate from packaging materials to foods or the environment. However, monomers may be present if left unreactive in the polymerization process, as is the case for bisphenols. Furthermore, additives that are not covalently bound to the polymeric plastic material can be added to plastics to improve the physical or chemical properties during production and storage, as well as appearance of the final products. These additives are used as pigments, preservatives, flame retardants or plasticizers.

Substances not covalently bound to plastic polymers can migrate into foods or drinks depending on the physico-chemical properties and concentrations of the chemicals, type of food, contact time and temperature. If the chemicals migrate to the food or are released into the environment, human exposure can occur by the diet, dust, air or dermal contact.

The contents of additives or monomers in food contact materials or migration from these materials are regulated in Europe by the Plastic Regulation 10/2011 (EU, 2011).

Among additives that are allowed in food contact materials, certain preservatives may be of concern for reproductive health. For instance, developmental exposure to the preservatives butylparaben or propylparaben has been shown to affect sperm production in rats, but the general human exposure to parabens originating from plastic is considered very low when compared to other sources, not least cosmetics.

Fact box

<i>Examples of chemicals allowed to be added to plastics, which are either suspected or known to exert endocrine activity</i>	
Bisphenols (monomers)	2,2-Bis(4-hydroxyphenyl)-propan (BPA)
	4,4'-Dihydroxydiphenylsulfon (BPS)
Phthalates (plasticizers)	4-Cumylphenol
	Dibutylphthalate (DBP)
	Benzylbutylphthalate (BBP)
	Bis(2-ethylhexyl)phthalate (DEHP)
	Dimethylterephthalate
	Diallylphthalate
	Dimethylisophthalate
	Diphthalate with primary, saturated C8-C10-alcohols
<i>(Continued)</i>	

Examples of chemicals allowed to be added to plastics, which are either suspected or known to exert endocrine activity

Parabens (preservatives)	Methyl-4-hydroxybenzoate Ethyl-4-hydroxybenzoate Propyl-4-hydroxybenzoate Isopropyl-4-hydroxybenzoate 2,4-di- <i>tert</i> -butylphenyl-3,5-di- <i>tert</i> -butyl-4-hydroxybenzoate
Benzophenons (UV filters)	2,4-dihydroxybenzophenon (benzophenone-1) 4,4'-dihydroxybenzophenon 2-hydroxy-4-methoxybenzophenon 2,2'-dihydroxy-4-methoxybenzophenon 2-hydroxy-4- <i>n</i> -hexyloxybenzophenon 2-hydroxy-4- <i>n</i> -octyloxybenzophenon (benzophenone-12) 4,4'-difluorbenzophenon
Adhesives	Abietic acid

Plastics can enter the aquatic environment as litter or through wastewater. In the aquatic environment the weathering processes can transform plastic materials into microplastics; i.e., plastic particles typically smaller than 5 mm. These may act as vectors for transfer of added or adsorbed chemicals such as chlorinated or brominated POPs to aquatic organisms. These POPs can be highly concentrated on plastic particles due to their high affinity for certain plastics such as PE. In turn, the occurrence of contaminated plastic may affect aquatic organisms, but it is presently unclear if human consumption of fish and seafood leads to exposure levels that may be harmful to humans (Ziccardi et al., 2016).

Phthalates

A wide range of phthalates are used worldwide as additives to plastic materials to soften and make the material more stretchable. Phthalates are used in PVC and PE polymers, where diethylhexyl phthalate (DEHP) has been the most widely used for PVC production so far, but di-*n*-butyl phthalate (DBP), benzylbutylphthalate (BBP), di-isononylphthalate (DiNP), and di-isodecylphthalate (DiDP) are also used. Phthalates are also found in inks, lacquers, and glues for food contact materials (Fierens et al., 2012), in tubing for milk transfer and in numerous applications for nonfood plastics. DEHP, DBP, and DiBP have been shown to be the most abundant phthalate in plastics from household waste and also recycled waste, originating both from plastics and other sources such as adhesives, sealants or inks. However, the use of phthalates is changing over time and the low to intermediate molecular weight ortho-phthalates such as DEHP and DBP are substituted by a range of high molecular weight phthalates, including DiNP and DiDP, as they are currently considered less toxic.

Although the FCM legislation within the EU has set maximum quantities and specific migration limits of different phthalates in plastic for food contact materials, these limits have been exceeded in some cases (Petersen and Jensen, 2016).

Bisphenols

Bisphenol A (BPA) is used in the manufacture of plastics, including PC, polyacrylate, and coatings such as epoxy resins. PC is mainly used to make beverage bottles, infant feeding bottles, tableware (plates and mugs) and storage containers such as water coolers and chocolate molds. BPA-based epoxy resin is used as inner coatings of food cans and lids of glass jars to ensure the preservation of the packaged food. BPA may also be present to some extent in printing inks and adhesives and can migrate in small amounts into food and beverages stored in food contact materials containing the substance. BPA is also used in other consumer products such as thermal paper receipts, toys and medical devices. Thermal paper has been identified as the major source of BPA and bisphenol S in the paper recycling process (Pivnenko et al., 2015).

Due to its adverse effects on human health, BPA is increasingly being substituted by structural analogues such as bisphenol S, E or F. However, several of these have been shown to qualitatively cause the same adverse effects as BPA, making such substitutions problematic (Rosenmai et al., 2014).

Legislation on the presence and migration of BPA from food contact materials varies between countries and regions. United States, European Union, and Canada have prohibited the use of polycarbonate in infant feeding bottles, and specific migration limits have been established for use in other food contact materials.

Persistent Organic Compounds (POPs)

General unintentional contamination of plastics with persistent halogenated compounds is another area of concern for male reproductive health. PVC plastic waste contains chloride which, when incinerated, can release the toxic chlorinated dioxins to the environment unless appropriate filters are applied. Consequently, dioxins can be inhaled by humans, deposited in terrestrial or aquatic environments or biomagnified in the food chain through crops and edible animals. Dioxins, together with chlorinated

biphenyls previously produced in massive amounts for various industrial applications, persist in the environment and accumulate in the food chain.

Brominated flame retardants are added to a wide variety of products such as plastics, textiles and electronic equipment to make them less flammable. But recycling of plastics can potentially reintroduce brominated flame retardants to other food contact materials unintentionally. Among the many types of brominated flame retardants, it is mainly the polybrominated diphenylethers (PBDEs) that have been associated with adverse male reproductive health effects (EFSA, 2011) (Fig. 1).

Chemical Exposure and Effects on Male Reproductive Health

Phthalates

Phthalates are among the most common synthetic chemicals to which humans are exposed. As phthalates are not covalently bound to the plastic, they can leak out of products and enter the environment this way. Oral exposure by ingestion of contaminated foods or dust is considered to be the main route of exposure, but consumer and building products made of plastics may also release phthalates in to the indoor environment, thereby contributing to the overall exposure. Finally human exposure can occur through direct dermal contact with clothes, bags, or toys, but also by using cosmetics. Children are among those highest exposed to phthalates, and infants in neonatal intensive care units that are treated with PVC plastic medical devices containing DEHP, can be significantly exposed.

Certain phthalates have been found to interfere with early male reproductive development by altering fetal germ cell development or inhibiting synthesis of sex steroid hormones. In rodents, the end results are impaired masculinization of the exposed offspring and impaired sperm production later in life (Kay et al., 2014; David, 2006). This is supported by some human epidemiological studies showing associations between maternal exposure to these phthalates and reproductive malformations in their sons (Kay et al., 2014). At high doses, testicular toxicity is also induced with adult exposure. Therefore, DEHP, DBP, BBP, and DIBP have been classified as being reproductive toxicants (ECHA, 2017).

It is generally understood that phthalates with backbones of three to six carbons share similar modes of action when it comes to adversely affecting the reproductive system in experimental animals. These phthalates seem to act via multiple modes of action. Their ability to reduce testicular testosterone production during late gestation in rats is associated with impaired development of androgen-dependent tissues. The exact initiating event is currently unclear, but marked reductions in gene or protein expression

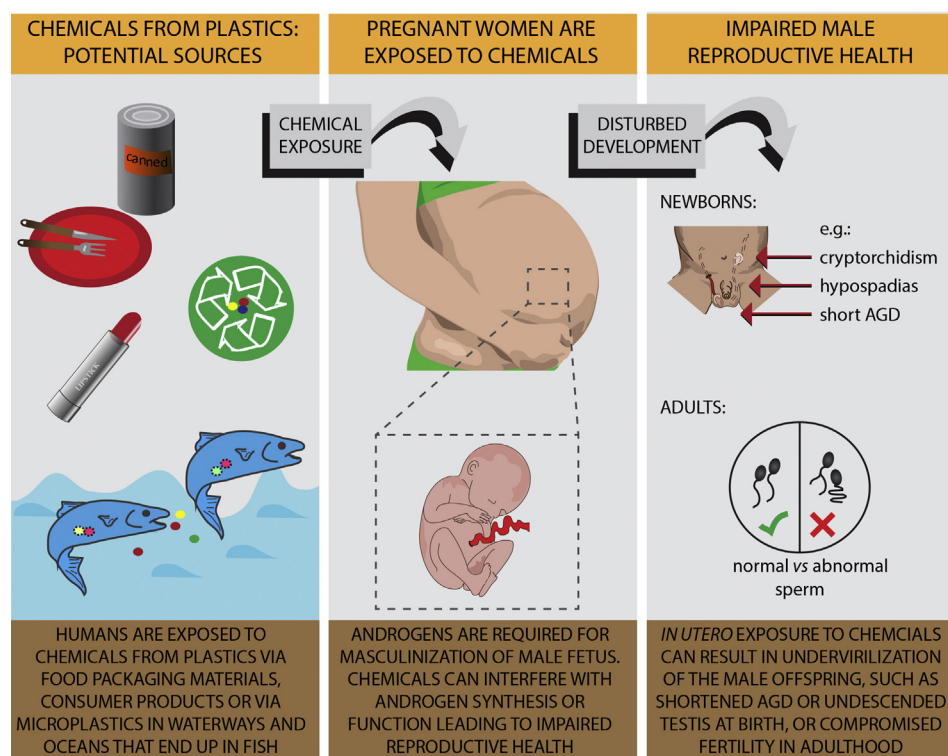


Fig. 1 Various sources of chemical exposure of pregnant women to EDCs such as BPA, phthalates, and POPs are illustrated. If exposure to these male reproductive toxicants occurs during a specific programming window during fetal life, normal development of the male fetus can be disturbed, resulting in male reproductive health disorders such as hypospadias, cryptorchidism or poor sperm quality. The antiandrogenic mode of action is the best described mechanism causing these effects, but other mechanisms may also play a role.

are seen for several steroidogenic factors, including StAR, SR-B1, CYP11A1, and CYP17A1 (David, 2006). In mice, only minor changes in fetal steroid production are seen, whereas changes in early germ cell development are seen in both rats and mice (Albert and Jégou, 2014). Possible species differences have been studied using xenografted human testicular tissues. Xenotransplants of testis tissue from different species appear to respond differently to phthalate exposure with respect to testosterone production, but similarly with respect to germ cell changes. The windows of sensitivity appear to differ between rodents and primates (Albert and Jégou, 2014).

A high number of other phthalates are used as alternatives to the abovementioned phthalates. It is not currently clear whether other phthalates than those with intermediate chain lengths can also adversely affect the male reproductive system. For phthalates with shorter or longer chain lengths, a few human epidemiological studies have found associations between prenatal exposures and anogenital distance, a marker of prenatal androgen insufficiency. Long-chain phthalates such as diisododecyl phthalate (DIDP) and diisononyl phthalate (DINP) are abundantly used, and while DIDP does not appear to influence reproductive function or markers of androgen insufficiency in early life, DINP does seem to affect male reproduction via alterations of testosterone production, though with less potency than e.g., DEHP and DBP (ECHA, 2013). This means that even though the health risk associated with exposure to phthalates from plastics is likely reduced due to current regulations of the most toxic phthalates, the possible impact of other phthalates and alternative plasticizers on male reproductive function is not fully understood.

Bisphenols

The main source of exposure to BPA and other bisphenols is considered to be through foods, but also through the skin by direct contact with thermal paper or by ingestion of dust. Dust seems to be a particular important source for toddlers and young children, who are often playing on floors or chewing on toys that have gathered dust particles.

The potential adverse effects of BPA on human health have been extensively studied over the past decades (Peretz et al., 2014). In the 1940s BPA was identified as being an estrogenic compound, as it was found capable of increasing the size of the uterus. In more recent years, reproductive effects caused by BPA exposure have been a significant focus, culminating in a large body of studies dealing with particularly its endocrine disrupting activity. BPA may act by multiple modes of action including altered steroid production, nuclear receptor interaction and causing epigenetic changes in various tissues.

For regulatory purposes, a large number of studies have been evaluated by different regulatory bodies, including the European Food Safety Agency (EFSA). Based on their extensive review, EFSA concluded that developmental exposure to BPA affects the male and female reproductive system at high doses (EFSA, 2015). The possible influence of developmental exposure at lower doses, however, remains controversial, and EFSA has considered the evidence for male reproductive effects less strong at lower doses. Male reproductive toxicity of BPA at high doses has been seen in several studies in mice and rats evidencing increased incidence of undescended testes at weaning, delayed puberty and reduced sperm quality (Peretz et al., 2014; EFSA, 2015). Other animal studies indicate a role of perinatal BPA exposure in later prostate cancer pathogenesis (Peretz et al., 2014). Collectively, BPA does appear to affect male reproduction, although it is unclear which developmental period is most sensitive, and whether this is only observed at higher doses.

The potential adverse effects of other bisphenols on male reproductive function is less well studied, but several alternatives such as BPB, BPE, BPF, and BPS have shown a similar toxicological profile in vitro (Rosenmai et al., 2014).

Persistent Organic Pollutants

The dioxin 2,3,7,8-TCDD and some PCBs are known to adversely affect sperm parameters in experimental animals exposed prenatally at very low doses. In humans, PCB/dioxin exposure also seems to influence semen quality, leading to increased abnormal morphology and reduced motility. The timing of the exposure is important with prenatal or prepubertal exposure periods being most sensitive (Leijts et al., 2014).

For brominated flame retardants, particularly PBDEs, some evidence exists that they have the potential to disrupt endocrine systems at multiple target sites, and thereby cause adverse effects on male reproductive health. While the thyroid hormone system appears to be the main target of these compounds, recent in vivo studies demonstrated effects on both the estrogen- and androgen-mediated processes (EFSA, 2011). Reproductive and developmental toxicity studies have shown changes in the development of androgen-sensitive male reproductive organs and impaired spermatogenesis following exposure to certain PBDEs (EFSA, 2011), but for many brominated flame retardants there is a general lack of data and knowledge on the possible consequences for human male reproductive function.

Conclusion and Perspectives

Plastics represent an important source of human exposure to chemicals, and of the many potential adverse effects this may cause, male reproductive health is one of them. The most studied and debated endocrine disrupting chemicals, bisphenols and phthalates, originate primarily from plastic materials. From both animal experiments and human epidemiological studies, these chemicals are known to affect male reproductive health. Importantly, most of the health effects that are observed throughout life are believed to arise from exposure during fetal life, the period when the male reproductive system is developing. Based on these findings, the use of

four phthalates, as well as BPA, has been restricted for certain uses within the EU and some other countries. In consequence, these chemicals are now being substituted by structural analogues with differing chain length or small variations in chemical structure. These remain to be investigated in-depth; therefore these substitutions should be used with caution, as evidence exists that chemicals belonging to the same class can often share the same modes of action.

Certain persistent endocrine disrupting chemicals such as brominated flame retardants, PCBs, or dioxins may also be present in plastics, either because they are ubiquitous contaminants in the environment or because they accumulate during recycling, or adhere to microplastics present in the environment.

Although there are specific regulations on plastics used for food contact, humans cannot avoid being exposed to chemicals from plastics. Finally, even though human exposure to each single chemical may be relatively low, the fact that many of them are able to affect male reproductive health makes it conceivable that the combined effects of several chemicals may result in mixture effects among the highest exposed individuals with severe consequences for development of the male fetus.

References

- Albert, O. and Jégou, B. (2014). A critical assessment of the endocrine susceptibility of the human testis to phthalates from fetal life to adulthood Human Reproduction Update 20(2), 231–49.
- David, R.M. (2006). Proposed mode of action for in utero effects of some phthalate esters on the developing male reproductive tract Toxicologic Pathology 34(3), 209–19.
- ECHA. (2013). *Evaluation of new scientific evidence concerning DINP and DIDP in relation to entry 52 of Annex XVII to regulation (EC) No 1907/2006 (REACH)*.
- ECHA. (2017). *Authorization list, List of substances included in Annex XIV of REACH*. <https://echa.europa.eu/addressing-chemicals-of-concern/authorisation/recommendation-for-inclusion-in-the-authorisation-list/authorisation-list>.
- EFSA. (2011). Scientific opinion of polybrominated diphenyl ethers (PBDEs) in food (EFSA CONTAM panel). *EFSA Journal*, 9, 2156.
- EFSA. (2015). Panel on food contact materials, enzymes, flavourings and processing aids: Scientific opinion on the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs: PART II—Toxicological assessment and risk characterisation. *EFSA Journal*, 13(1), 3978.
- EU. (2011). *Commission regulation (EU) No.10/2011 of 14th Jan 2011 on plastic materials and articles intended to come into contact with food*.
- Fierens, T., Servaes, K., Van Holderbeke, M., Geerts, L., De Henauw, S., & Sioen, V. (2012). Analysis of phthalates in food products and packaging materials sold on the Belgian market. *Food and Chemical Toxicology*, 50, 2575–2583.
- Kay, V.R., Bloom, M.S. and Foster, W.G. (2014). Reproductive and developmental effects of phthalate diesters in males. *Critical Reviews in Toxicology* 44(6), 467–98.
- Leijts, M. M., van der Linden, L. M., Koppe, J. G., Olie, K., van Aalderen, W. M. C., & ten Tusscher, G. W. (2014). The influence of perinatal and current dioxin and PCB exposure on reproductive parameters (sex-ratio, menstrual cycle characteristics, endometriosis, semen quality, and prematurity): A review. *Biomonitoring*, 1, 1–15.
- Plastic Europe. (2017). available from: <http://www.plasticseurope.org/what-is-plastic/types-of-plastics-11148/bio-based-plastics/qas.aspx>.
- Petersen, J. H., & Jensen, L. K. (2016). Phthalates in soft PVC products used in food production equipment and in other food contact materials on the Danish and the Nordic Market 2013–2014. *International Journal of Food Contamination*, 3(1), 1–7.
- Peretz, J., Vrooman, L., Ricke, W. A., Hunt, P. A., Ehrlich, S., Hauser, R., Padmanabhan, V., Taylor, H. S., Swan, S. H., VandeVoort, C. A., & Flaws, J. A. (2014). Bisphenol A and reproductive health: Update of experimental and human evidence, 2007–2013. *Environmental Health Perspectives*, 122(8), 775–786.
- Pivnenko, K., Pedersen, G. A., Eriksson, E., & Astrup, T. F. (2015). Bisphenol A and its structural analogues in household waste paper. *Waste Management*, 44, 39–47.
- Rosenmai, A. K., Dybdahl, M., Pedersen, M., Van Vugt-Lussenburg, B., Wedebye, E. B., Taxvig, C., & Vinggaard, A. M. (2014). Are structural analogues to bisphenol A safe alternatives? *Toxicological Sciences*, 139(1), 35–47.
- Ziccardi, L. M., Edgington, A., Hentz, K., Kulacki, K. J., & Driscoll, S. K. (2016). Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science. *Environmental Toxicology and Chemistry*, 35, 1667–1676.

Pesticides

Terje Svingen, Sofie Christiansen, Camilla Taxvig, and Anne M Vinggaard, Technical University of Denmark, Kongens Lyngby, Denmark

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acceptable daily intake The amount of a substance that can be orally ingested—by foods and drinks—on a daily basis over a lifetime without an appreciative health risk. ADI is expressed in milligrams of substance per kilogram of body weight per day ($\text{mg kg}^{-1} \text{ day}^{-1}$).

Androgens The androgens are any natural or synthetic compound, usually a steroid hormone that can stimulates or controls the development and maintenance of male characteristics in vertebrates by binding to androgen receptors.

Anogenital distance The distance between anus and genitalia. Male AGD is approximately twice that of female in laboratory rodents, as well as humans. The anatomical region is called the perineum and develops differently between the sexes by way of androgen stimulation during fetal life in males, but not in females.

Cryptorchidism The absence of one or both testes from the scrotum. The condition can be caused by undescended testis, but also ectopic, atrophic or absent testis. It is the most common reproductive birth defect in males.

Endocrine disruptors An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations. A potential endocrine disruptor is an exogenous substance or mixture that possesses properties that might be expected to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations.

Hypospadias A condition where the opening of the urethra is not located at the tip of the penis, but somewhere along the underside of the penile shaft between the penis head and the scrotum.

Pesticides A pesticide is any substance intended to repel, mitigate, or kill pests. Depending on their target organism, pesticides are typically grouped into different classes: insecticides are used against insects; herbicides (or weed-killers) are used against unwanted plants; fungicides are used against molds or fungi; rodenticides are used against mammals, typically mice and rats; and antimicrobials are used against microorganisms such as bacteria and viruses.

Abbreviations

ADI Acceptable daily intake
AGD Anogenital distance
AMH Anti-Müllerian hormone
AR Androgen receptor
DHT Dihydrotestosterone
E Embryonic day
EDC Endocrine disrupting chemical
FSH Follicle stimulating hormone
GW Gestational week
LH Luteinizing hormone
PCB Polychlorinated biphenyl
SHBG Sex-hormone binding globulin

Introduction

Pesticides are substances used to control or kill pests. A pest, in turn, can broadly be defined as any living organism that is either a nuisance or detrimental to human health, or that can have negative impact on human interests such as agriculture and livestock production. Pesticides thus comprise a broad range of substances with varied biological effects, but have in common that they often interfere with essential biological functions of the target organism. Because living organisms share many biomolecular processes, pesticides are rarely specific to just one species. While a pesticide may effectively harm or kill their intended target, the same pesticide may inadvertently also harm other organisms, including humans.

Humans are exposed to many different pesticides from various sources. This includes occupational exposure, consumption of foods containing pesticide residues, by use of domestic herbicides and insecticides, or by contact with contaminated water or air. If

the biological effects for which the pesticides were selected for can also affect humans, exposure can have detrimental consequences. The resulting adverse effects will in turn depend on different parameters such as level and timing of exposure, or mechanisms of action. In this article we will focus on potential effects on the male reproductive system, many of which are believed to arise from exposure during fetal life.

Androgen Signaling and Male Sexual Development

Sexual development is a complex process that involves several key steps that takes place sequentially from early fetal life to puberty. This ensures the development of two sexually mature phenotypes, male and female, which is necessary for procreation. This is a very complex subject and beyond the scope of this article, but additional information can be found in other articles. However, some key points need to be addressed and understood in order to grasp how pesticides potentially can affect male reproductive health.

Firstly, male and female embryos initially develop similarly and are morphologically indistinguishable from each other until testicles or ovaries have formed. This event, referred to as gonadal sex determination, is initiated at around gestational week (GW) 6 in humans, but midway through gestation in mice and rats, starting at around embryonic day (E) 10 and 12, respectively. The differentiation of the gonads into testicles or ovaries is independent of sex hormones, and instead relies on the timely expression of certain sex-determining genes (Svingen and Koopman, 2013). It is after the gonads have differentiated that the development of the rest of the body also starts to diverge between the sexes. In simple terms, the fetal testicles will start to produce various hormones that will influence the differentiation of other cells and tissues throughout the body so that they develop into male tissues and organs. For instance, the production of anti-Müllerian hormone (AMH) will cause the regression of the Müllerian ducts, hence preventing the female reproductive tracts to develop, but promote the development of the male reproductive tracts from the Wolffian ducts. Production of the main male sex hormone testosterone together with other androgens will stimulate peripheral tissues—primarily through the Androgen receptor (AR)—to differentiate into male phenotypes. In contrast, it is the absence of androgens (in other words; ovaries, rather than testicles) that ensures female development.

Since proper masculinization of the fetus depends on androgens produced by the testicles, male sexual development is particularly sensitive to endocrine disruption during these critical periods. Endocrine disrupting chemicals (EDCs) with antiandrogenic properties are believed to be of particular concern, since they can block normal androgen function either by reducing the amount of available testosterone or by interfering with the AR. Exposure to antiandrogenic compounds during pregnancy can therefore result in undervirilized, or feminized, offspring. The question then is, do pesticides have endocrine disrupting properties and if yes, can they be detrimental to male reproductive health? Fig. 1

Pesticides as Endocrine Disruptors

Pesticide exposure has been linked to endocrine disruption and consequently, with reproductive health issues (Mnif et al., 2011). The type of adverse effects is dependent on several factors, especially the type of pesticide, the dose level of the pesticide(s), and the

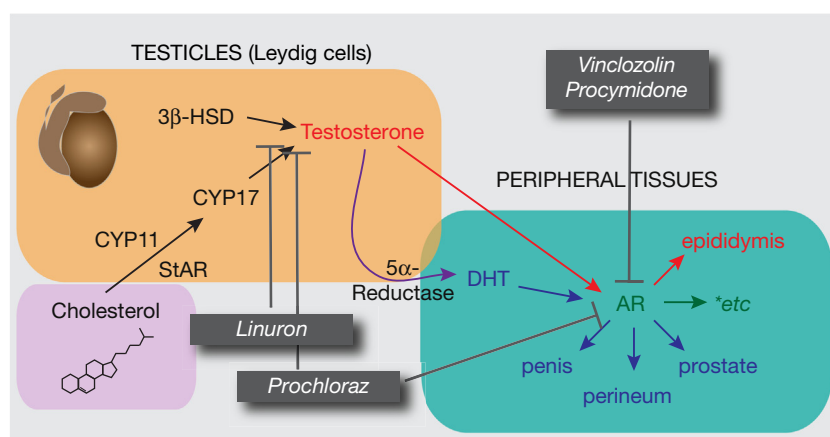


Fig. 1 Examples of pesticides that can interfere with male reproductive development. The testicular Leydig cells are responsible for the production of testosterone. The process by which cholesterol is converted into sex steroid hormones is referred to as steroidogenesis; a multistep pathway involving several key enzymes. Testosterone is secreted from the testicular Leydig cells. In many peripheral tissues, 5 α -reductase converts testosterone into DHT, a powerful androgen that can bind to and activate the AR. In turn, activated AR translocates to the nucleus of the cells and activates gene expression by directly binding to DNA. Some pesticides can interfere with activation of AR itself, or with various steps involved in androgen synthesis or metabolism, which can ultimately disrupt masculinization of the fetus.

Table 1 Examples of pesticides reported to cause effects on male reproductive health

<i>Pesticide</i>	<i>Pesticide class</i>	<i>Possible adverse effects</i>
Carbaryl	Insecticide	Reduce sperm counts and motility
Chlordecone	Fungicide and insecticide	Reduce sperm counts and motility
Linuron	Herbicide	Disrupts Leydig cell steroidogenesis. Shorter AGD in rats.
Mancozeb	Fungicide	Various effects in rodents including: reduced sperm counts, toxic effects on testis, suppressed testosterone levels
Prochloraz	Fungicide	Interacts with nuclear receptors such as AR and ER. Nipple retention, reproductive malformations, reduced testosterone levels in rats
Procymidone	Fungicide	Inhibits AR activation. Multiple reproductive malformations, including nipple retention, shorter AGD, hypospadias, and cryptorchidism
Vinclozolin	Fungicide	Inhibits AR activation. Multiple reproductive malformations, including nipple retention, shorter AGD, hypospadias, and cryptorchidism

time of exposure. With regards to male reproductive health, it is the fetal period that is of particular concern. The causative molecular mechanisms can be quite diverse, and many pesticides are also known to affect more than one molecular pathway (e.g., AR antagonism, induction or inhibition of aromatase activity, affecting steroid hormone production). The most common modes of action with regards to endocrine disruption include antiandrogenic effects, estrogenic and antiestrogenic effects, disruption of aromatase activity, or estradiol or androgen production. Disruption of thyroid hormone action and interference with steroid hormone metabolism have also been reported (Mnif et al., 2011).

Organochlorine pesticides and conazole fungicides have been linked to adverse male reproductive health effects. From studies involving laboratory animals or wildlife, as well as human epidemiological studies, these pesticide classes are believed to affect the steroid hormone balance in organisms by various mechanisms, ranging from binding to various hormone receptors, disruption of hormone synthesis, or effects on metabolism. In the following section, we will highlight a handful of pesticides and discuss what effects they may have on the male reproductive system in laboratory animals. This information is also summarized in Table 1.

Animal Studies

Pesticides can affect the endocrine system of different animal species, ranging from invertebrates to reptiles, fish, birds, and mammals. Since sexual development varies significantly between distant animal taxa, we will focus on mammals, more specifically mice and rats, in this article. Furthermore, intrauterine exposure is the main focus, since it is during fetal life that male fetuses are particularly sensitive to endocrine disruption. In other words, in utero exposure experiments can best forewarn us about what effects maternal exposure can have for the future reproductive health of the male offspring.

It is now clear that exposure to endocrine disrupting pesticides during fetal and prenatal life can adversely affect male reproductive development. The most common effects readily observed at birth include shorter anogenital distance (AGD), retained nipples, cryptorchidism and penile malformations, typically hypospadias or cleft phallus. There are also proposed late-life effects such as reduced fertility. Below, we will describe four pesticides for which there is clear evidence that they can adversely affect male reproductive development in rodents.

Linuron is a selective organochlorine herbicide used to control broadleaf and grassy weeds for crops such as carrots, beans, and peas. When administered to pregnant rats, the male offspring show a significantly reduced testosterone production (Wilson et al., 2009). Linuron appears to directly affect Leydig cell steroidogenesis by suppressing the expression of key enzymes, leading to low testosterone levels in the male fetus, and ultimately significantly shorter AGD than normal (McIntyre et al., 2002). In other words, exposure to linuron during the male masculinization window prevents the fetus from masculinizing properly, resulting in feminization phenotypes at birth and potential reproductive health issues later in life.

Vinclozolin is a fungicide commonly used in vineyards, but also for the protection of other fruit and vegetable crops, albeit now banned in some countries. It acts by inhibiting spore germination and mycelial growth in fungi. It is the metabolites of vinclozolin, M1 and M2, that can bind to the AR and prevent binding to DNA, ultimately inhibiting androgen-dependent gene expression. Following fetal exposure to high doses of vinclozolin, male rat offspring are born with a significantly shorter AGD, resembling the distance in female rats. They also often present with retained nipples, cleft phallus with hypospadias, cryptorchidism, vaginal pouch, epididymal granulomas, and small to absent sex accessory glands (Gray et al., 1994). So again, the end result of exposure is undervirilization of the fetus, but by different mechanisms of action than what was the case with linuron.

Procymidone, like vinclozolin, is a dicarboximide fungicide widely used as a seed dressing and for the general control of fungal diseases pre- and postharvest. Procymidone looks to display a nearly identical toxicity profile as vinclozolin, both in vitro and in vivo, albeit with lower potency. It is also an AR antagonist. In rats, perinatal exposure to procymidone can lead to shorter AGD, increased nipple retention, hypospadias, cryptorchidism, as well as reduced prostate, testis and epididymal weights (Ostby et al., 1999).

Prochloraz is an imidazole fungicide used to protect a wide range of crops such as cereals and fruits. In rats, high-dose exposure during fetal life can adversely affect several organ systems by various mechanisms of action (Vinggaard et al., 2005). A common feature of azole fungicides is their propensity to affect steroid hormone levels in pregnant rats and their fetuses, as well as prolonging the gestational period. Male offspring that have been exposed in utero to prochloraz often display nipple retention (Christiansen et al., 2009; Vinggaard et al., 2005), whereas effects on AGD are conflicting. The effects are believed to be the result of reduced intra-testicular and plasma testosterone levels caused by prochloraz interfering with steroidogenesis (Laier et al., 2006).

From these four examples, it is clear that that some pesticides can disrupts male sexual development by interfering with androgen signaling, at least in rodent models. Notably, the adverse effects are typically seen at relatively high dose levels. Humans are rarely, if ever, exposed to such high doses, so the question remains if humans are at real risk from exposure to pesticides. Although a topic of debate, one possible way in which humans can be put at risk is through a combined exposure to a large number of different pesticides and other environmental chemicals with similar modes of action. There are literally hundreds of pesticides of various chemical classes currently in use, but tens of thousands of environmental chemicals more broadly. This scenario is commonly referred to as the “mixture,” or “cocktail effect,” whereby single chemicals that are present at doses that alone does not elicit an effect, can cause adverse effects in combination when many chemicals with similar mechanisms of action are present at the same time. A clear example of this has been shown in rat studies where in utero exposure to five pesticides combined can cause significant effects at doses where the pesticides give little or no effect alone (Hass et al., 2012). There are also indications that cumulative exposure to EDCs can affect human development, with epidemiological studies reporting associations between organochlorine pesticides and congenital cryptorchidism (Damgaard et al., 2006). But to get an understanding of any potential risk, human exposure data must also be obtained and analyzed.

Human Epidemiological Studies

Pesticides with endocrine disrupting properties have been associated with disrupted reproductive development in humans. Also, as is the case for animals, fetuses, and infants are more vulnerable to exposure than adults, since much of the damage caused by EDCs take place during critical stages of fetal life (Mnif et al., 2011). A higher prevalence of cryptorchidism and hypospadias in newborn boys has been observed in areas with extensive farming and pesticide use, as well as in sons of women that work in greenhouses (Kristensen et al., 1997; Weidner et al., 1998). A relationship has also been reported between cryptorchidism and persistent pesticide concentration in maternal breast milk (Damgaard et al., 2006).

A meta-analysis of human epidemiological studies found that pesticide exposure may affect spermatogenesis, leading to poor semen quality and reduced male fertility, as well as a possible link to prostate cancer (Mnif et al., 2011). Notably, the former conditions are often linked to antiandrogenic effects, whereas for instance prostate cancer has been associated with exposure to pesticides with estrogenic properties, as for instance chlordecon (Kepone), an insecticide previously used heavily in banana crop protection (Multigner et al., 2010). In many ways, chlordecon stands out as a clear example where heavy and prolonged pesticide use can have deleterious effects on male reproductive health, shown by a string of studies carried out on the populations in the French West Indies where chlordecon was intensively used on banana crops from around 1973 to 1993 (Multigner et al., 2016).

Despite some incidences, individual pesticides are by themselves generally not believed to cause adverse reproductive health effects in humans at exposure levels relevant for the general population. However, some studies suggest an increased prevalence of cryptorchidism, decreased penile length, and testicular size in sons born from women working as gardeners or living on farms where pesticides have been used (Andersen et al., 2008). In particular, female greenhouse workers had an increased risk of delivering boys with classic symptoms of disrupted fetal masculinization programming, including cryptorchidism, smaller penis or testicles, lower serum concentrations of testosterone and inhibin B, higher serum concentrations of SHBG and FSH, or a generally higher LH:testosterone ratio (Andersen et al., 2008). Taken together, these data all point in a direction where certain pesticides can disrupt normal androgen signaling during fetal life, whereupon the male fetus fail to masculinize properly.

Beyond disrupted fetal development, there are also examples where pesticide exposure can adversely affect the reproductive health of adult men. For instance, 1,2-dibromo-3-chloropropane (DBCP) is a classic example of a man-made chemical (pesticide) that affects spermatogenesis. Historically, DBCP was used as a soil fumigant to control nematode worms. Several animal studies have shown effects on the testicles, particularly on sperm quality, but also that DBCP is a water-soluble and persistent chemical that lingers on in the environment. Several years after it was discovered that men had been occupationally exposed to DBCP, their sperm counts were either zero, or so low that it rendered the men sterile (Pryor et al., 2008).

Pesticide Exposure and Consequences for Male Reproduction

The average person is generally exposed to very low levels of pesticides, much lower than what is the case with many other chemicals. However, some people may be exposed to greater levels, especially those who regularly work with pesticides, for instance in greenhouses or farmland. This is evident in that most epidemiological studies that show associations between male reproductive disorders and pesticide exposure have been conducted on gardeners or farmers. For the general population, the risk of developing

reproductive health issues from exposure to single pesticides would be considered low, if not negligible. In fact, pragmatic acceptable daily intake (ADI) calculations—typically derived from rodent experiments—suggest that humans are at low risk from exposure to either single pesticides, or combinations of pesticides. It should be noted, however, that the ADIs are derived from animal data and that there are indications that humans may be more sensitive toward pesticide exposure than rodents. Especially, epidemiological data from the general human population have shown associations between neurological disorders and some pesticides, indicating a discrepancy between animal-derived ADIs and actual risk to humans.

As a final note; when evaluating the potential risk of chemical exposure, it is not sufficient to simply evaluate exposure to single chemicals. The combined exposure of all chemicals, being it pesticides or any other type of environmental chemical, must be considered together as a mixture. Humans are exposed to a large number and variety of chemicals at any given time, and will typically include chemicals such as phthalates, bisphenols, PCBs, dioxins, and so forth, all of which have the potential to affect male reproductive health parameters. Therefore, although pesticides alone may be of less concern, they could become a concern if contributing toward the total load of endocrine disrupting compounds, ultimately putting the male reproductive system at risk.

References

- Andersen, H. R., Schmidt, I. M., Grandjean, P., Jensen, T. K., Budtz-Jørgensen, E., Kjaerstad, M. B., Baelum, J., Nielsen, J. B., Skakkebaek, N. E., & Main, K. M. (2008). Impaired reproductive development in sons of women occupationally exposed to pesticides during pregnancy. *Environmental Health Perspectives*, 116, 566–572.
- Christiansen, S., Scholze, M., Dalgaard, M., Vinggaard, A. M., Axelstad, M., Kortenkamp, A., & Hass, U. (2009). Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. *Environmental Health Perspectives*, 117, 1839–1846.
- Damgaard, I. N., Skakkebaek, N. E., Toppari, J., Virtanen, H. E., Shen, H., Schramm, K. W., Petersen, J. H., Jensen, T. K., Main, K. M., & Nordic Cryptorchidism Study, G. (2006). Persistent pesticides in human breast milk and cryptorchidism. *Environmental Health Perspectives*, 114, 1133–1138.
- Gray, L. E. J., Ostby, J. S., & Kelce, W. R. (1994). Developmental effects of an environmental antiandrogen: The fungicide vinclozolin alters sex differentiation of the male rat. *Toxicology and Applied Pharmacology*, 129, 46–52.
- Hass, U., Boberg, J., Christiansen, S., Jacobsen, P. R., Vinggaard, A. M., Taxvig, C., Poulsen, M. E., Herrmann, S. S., Jensen, B. H., Petersen, A., Clemmensen, L. H., & Axelstad, M. (2012). Adverse effects on sexual development in rat offspring after low dose exposure to a mixture of endocrine disrupting pesticides. *Reproductive Toxicology*, 34, 261–274.
- Kristensen, P., Irgens, L. M., Andersen, A., Bye, A. S., & Sundheim, L. (1997). Birth defects among offspring of Norwegian farmers, 1967–1991. *Epidemiology*, 8, 537–544.
- Laier, P., Metzdorff, S. B., Borch, J., Hagen, M. L., Hass, U., Christiansen, S., Axelstad, M., Kledal, T., Dalgaard, M., McKinnell, C., Brokken, L. J., & Vinggaard, A. M. (2006). Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz. *Toxicology and Applied Pharmacology*, 213(2), 160–171.
- McIntyre, B. S., Barlow, N. J., & Foster, P. M. (2002). Male rats exposed to linuron in utero exhibit permanent changes in anogenital distance, nipple retention, and epididymal malformations that result in subsequent testicular atrophy. *Toxicological Sciences*, 65, 62–70.
- Mnif, W., Hassine, A. I. H., Bouaziz, A., Bartegi, A., Thomas, O., & Roig, B. (2011). Effect of endocrine disruptor pesticides: A review. *International Journal of Environmental Research and Public Health*, 8, 2265–2303.
- Multigner, L., Ndong, J. R., Giusti, A., Romana, M., Delacroix-Maillard, H., Cordier, S., Jégou, B., Thome, J. P., & Blanchet, P. (2010). Chlordecone exposure and risk of prostate cancer. *Journal of Clinical Oncology*, 28, 3457–3462.
- Multigner, L., Kadhel, P., Rouget, F., Blanchet, P., & Cordier, S. (2016). Chlordecone exposure and adverse effects in French West Indies populations. *Environmental Science and Pollution Research International*, 23, 3–8.
- Ostby, J., Kelce, W. R., Lambright, C., Wolf, C. J., Mann, P., & Gray, L. E. J. (1999). The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. *Toxicology and Industrial Health*, 15, 80–93.
- Pryor, J. L., Hughes, C., Foster, W., Hales, B. F., & Robaire, B. (2008). Critical windows of exposure for children's health: The reproductive system in animals and humans. *Environmental Health Perspectives*, 108(Suppl 3), 491–503.
- Svingen, T., & Koopman, P. (2013). Building the mammalian testis: Origins, differentiation, and assembly of the component cell populations. *Genes and Development*, 27, 2409–2426.
- Vinggaard, A. M., Christiansen, S., Laier, P., Poulsen, M. E., Breinholt, V., Jarfelt, K., Jacobsen, H., Dalgaard, M., Nellemann, C., & Hass, U. (2005). Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring. *Toxicological Sciences*, 85, 886–897.
- Weidner, I. S., Møller, H., Jensen, T. K., & Skakkebaek, N. E. (1998). Cryptorchidism and hypospadias in sons of gardeners and farmers. *Environmental Health Perspectives*, 106, 793–796.
- Wilson, V. S., Lambright, C. R., Furr, J. R., Howdeshell, K. L., & Earl Gray, L., Jr. (2009). The herbicide linuron reduces testosterone production from the fetal rat testis during both in utero and in vitro exposures. *Toxicology Letters*, 186, 73–77.

Endocrine Disruptors and Male Reproductive Function

João Ramalho-Santos and Renata S Tavares, University of Coimbra, Coimbra, Portugal

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome reaction An exocytosis event in which the sperm outer acrosomal membrane and the sperm plasma membrane fuse at multiple sites, leading to the release of hydrolytic enzymes and to the exposure of new membrane domains, both of which are essential for fertilization to occur.

Capacitation Fertilizing competence acquisition where spermatozoa undergo a series of poorly understood maturation steps that include increased plasma membrane permeability and fluidity due to cholesterol efflux, influx of ions specially Ca^{2+} and bicarbonate (HCO_3^-), internal pH rise and protein phosphorylation, typically on tyrosine residues.

Cryptorchidism Genital abnormality characterized by the absence of one or both testes in the scrotum. Commonly referred as undescended testis.

Endocrine-disrupting chemical or endocrine disruptor (EDC) Man-made or naturally occurring substance that can mimic natural hormone activity at different levels.

Hypospadias Congenital disorder in which the opening of the urethra is located on the underside of the penis, instead of on the tip.

Spermatozoon/sperm Male haploid cell that has resulted from the process of spermatogenesis. Male sexual cell responsible for the delivery of the paternal DNA to the oocyte.

Testicular dysgenesis syndrome A proposed hypothesis in which poor semen quality, testicular cancer, cryptorchidism, and hypospadias result from errors during the development of the fetal testes. Therefore, as they share a common origin, these different aspects should be recognized as a single syndrome.

What Are Endocrine Disruptors (EDCs)? Context and Sources of Exposure

Published in 1992 a pioneer study involving a group of men with no history of infertility highlighted a decrease in both sperm concentration and mean semen volume, and a concomitant reduction in total sperm count over a 50-year period. Since then, a considerable amount of papers, including an extensive study published in 2017, has reported diminished (and still declining) human sperm quality and increasing trends of testicular cancer and anomalies in male reproductive organs, such as cryptorchidism and hypospadias, over the last decades in several areas of the world, thus jeopardizing male reproductive health. Indeed, this type of evidence led Skakkebaek and colleagues to propose that all these conditions are part of one single underlying issue—the testicular dysgenesis syndrome (TDS). The authors hypothesized that these abnormalities originate from specific errors that take place during fetal testes development. Given that this developmental origin is common, they should thus be recognized as a single entity, the so-called TDS, although it is obviously acknowledged that different individuals might display distinct severities. This proposal was based on the understanding that variations in fetal hormone levels may affect the development of the male reproductive tract, and that hormone-mimicking compounds in the environment could possibly play a role in disturbing this intricately regulated process.

Although it has not been widely accepted, the worrying increase in male reproductive tract anomalies and poor sperm quality does suggest that exposure to environmental factors, such as endocrine-disrupting chemicals (EDCs) might be, at least in part, responsible for these outcomes. EDCs are an extremely heterogeneous group of compounds that include man-made substances such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides, phthalates, alkylphenols, bisphenol A (BPA), diethylstilbestrol (DES); and naturally occurring compounds, such as phytoestrogens and mycotoxins. By definition, these compounds can mimic endogenous hormones, and promote endocrine imbalance via different mechanisms of action. Thus, by interfering with hormone synthesis, binding and/or action, EDCs can potentially disturb the normal development and function of tissues and organs, and the male reproductive system does not seem to be an exception. Indeed, it has been broadly argued that by presenting (anti)estrogenic- or antiandrogenic-like activities EDCs are likely to affect male reproductive health and fertility, in both human, animal and wildlife populations.

Populations are continuously exposed to EDCs due to their widespread use and high persistency in the environment. Even the restriction or ban of several persistent organic pollutants—including PCDDs, PCBs, PAHs, and several pesticides—in many countries (as agreed in the Stockholm Convention), did not necessarily spare these populations, given the strong resistance of many of these compounds to degradation. EDCs are present in our daily lives, and we are exposed mainly through diet, although dermal contact and inhalation may also contribute. Moreover, given their lipophilic nature, persistent organic pollutants may bioaccumulate in the food chain, since they can be stored in body fat for extended periods. This implies that species on the top of food chains are especially susceptible, given that they can be exposed both directly, and via ingestion of contaminated food sources

that have previously accumulated EDCs. Other EDCs, such as phthalates and BPA, do not accumulate in the body, but chronic exposure to low levels via dietary intake and commonly used products can provide continuous steady state doses in living organisms that may produce undesirable effects.

Additionally, as an obvious consequence of exposure, EDCs and/or their metabolites can be found not only in body fluids such as urine, blood serum or breast milk, but many can also be detected in reproductive fluids, including seminal fluid, cervical mucus, and female reproductive tract secretions. This is an important route of exposure that should not be overlooked, as spermatozoa steep in these fluids/secretions on their journey toward the oocyte, thus coming into direct contact with these compounds with possible deleterious consequences. Indeed, it is known that EDCs may act both through genomic and nongenomic hormone-independent pathways (e.g., by affecting ion channels and signaling pathways) causing a wide spectrum of effects on male reproductive function, ultimately compromising sperm fertilizing ability, and thus jeopardizing male fertility.

In general, whether they persist in tissues and body fluids or are rapidly excreted by the organism, continuous exposure to EDCs may threaten the reproductive health status of both human and animal/wildlife populations. Furthermore, in accordance with the United Nations Environment Program "Endocrine Disrupting Chemicals" and the 2013 report of the World Health Organization (WHO), nearly 800 substances are recognized as supposedly able of disturbing the endocrine system, and only a limited number of these substances have been studied. With more and more compounds being produced daily, for example, to replace certain pesticides or other dangerous chemicals, the numbers are increasing. A nonexhaustive list of some of the major compounds described as EDCs relevant for male fertility is shown in Table 1, and their prevalence constitutes a serious public health issue that should not be neglected.

This article intends to briefly summarize the effects of EDCs on male reproductive function, integrating both epidemiological and experimental evidence. Special emphasis was placed on effects related to sperm quality and function.

Epidemiological and Experimental Data: An Overview

An increasing number of reports have documented the effects of several EDCs, in many cases using laboratory animals as a strategy to (cautiously) infer about putative human exposure effects. Others have been studying the impact of EDCs in commercially valuable species and in wildlife. Understandably, studies with humans can only involve: (1) epidemiological data; or, (2) in vitro experimental settings in which human cells (including spermatozoa) are exposed to different concentrations of distinct compounds for precise amounts of time. In addition, the epidemiological strategy may include an analysis of both populations with high exposure episodes (industrial accidents, areas with a heavy use of pesticides) or high dietary intakes (the consumption of foodstuffs with high levels of EDCs); and populations with no obvious exposure to EDCs, that are often enrolled when undergoing routine semen analysis and/or seeking assisted reproduction treatments (which can constitute a sampling bias).

Reproductive abnormalities such as altered anogenital distance, nipple retention, modifications in the structure and function of the testis and other reproductive organs have been attributed to EDC exposure in animal models and, in wildlife, the most notorious cases are possibly the egg shell thinning in birds, and the poorly organized testis and abnormally small phalli of male juvenile alligators in contaminated areas.

Although with some exceptions, possibly due to the different concentrations found in urine, seminal or blood serum samples, exposure to EDCs in men has been associated with a reduction of the sperm parameters monitored using standard WHO criteria, namely sperm concentration, motility, and morphology. Sperm motility is of obvious importance for fertilization in vivo, and it has been shown to be affected by a vast range of EDCs, not only in humans but also in rodents, rabbits, boar/pig and horse, as shown by many in vivo and in vitro studies using different experimental approaches, doses and times of exposure. Similarly, the functional status of sperm mitochondria, which is correlated with sperm fertilizing ability, seems to be a target of EDCs. A reduction in sperm antioxidant scavenging defenses and/or a rise in the production of reactive oxygen species (ROS) have also been observed following EDCs exposure, not only in human male gametes but also in sperm from other species. This may potentially lead to downstream complications, such as DNA fragmentation, lipid peroxidation and apoptosis, all of which have been reported to occur at least in an in vitro scenario of exposure. Furthermore, although not extensively addressed, sperm capacitation in vitro is affected after treatment with a putative environmentally relevant EDC mixture containing mainly PCBs and pesticides. Because prematurely acrosome-reacted sperm cannot fertilize the oocyte, several EDCs could also provoke undesirable effects on male fertility given their ability to interfere with the acrosome reaction. Other studies have also shown reduced sperm energy status and inhibition of fertilization rates due to EDC exposure. Moreover, the discovery that many (but not all) EDCs are able to interfere with sperm plasma membrane channels such as CatSper (a calcium ion channel), and disturb normal cytoplasmic ion concentrations that are relevant to both capacitation and the acrosome reaction, add further evidence to the potential adverse of these compounds on male reproductive health. Importantly, recent data clearly shows that concentrations of EDCs that can cause sperm dysfunction are physiologically and environmentally relevant, thus preemptively answering possible criticism in terms of doses used in vitro being considered unrealistic for in vivo situations.

Reports using sperm samples collected from human populations frequently lack the evaluation of functional parameters that more accurately reflect male fertility, in comparison with the standard WHO parameters. However, to some extent they give similar results to what was obtained in vitro. For instance, studies on *p,p'*-dichlorodiphenyl dichloroethylene (*p,p'*-DDE), the major and most stable metabolite of the pesticide dichlorodiphenyl trichloroethane (DDT) sprayed in dwellings as a way to control malaria-bearing mosquitoes in some countries, have been shown to adversely affect human reproductive outcomes. In these

Table 1 Some compounds known for their endocrine-disrupting properties in male fertility

<i>EDCs</i>	<i>Examples</i>
Polychlorinated dibenzo- <i>p</i> -dioxins (PCDDs)	TCDD
Polychlorinated biphenyls (PCBs)	PCB 54
	PCB 77
	PCB 153
	PCB 118
	Aroclor 1254
Polycyclic aromatic hydrocarbons (PAHs)	B[a]P, BPDE
Pesticides	Alachlor
	β -HCH
	Lindane
	DDT and related metabolites (e.g., <i>p,p'</i> -DDD, <i>p,p'</i> -DDE)
	Methoxychlor
	Vinclozolin
	Chlorpyrifos
	Deltamethrin
	Dimethoate
	Permethrin
	Cypermethrin
	Atrazine
	Fenoxapropethyl
	Diazinon
	Malathion
	Bendiocarb
	Endosulfan
	Parathion
	Paraoxon
	Flutamide
	DBCP
Phthalates	2,4-D
	DBP
	DEP
	DMP
	DOP
	DEHP
	DBP
	DEHP
	DnBP
	BBzP
	DiBP
	DPP
Alkylphenols	Nonylphenol, octylphenol
Bisphenol A (BPA)	
Diethylstilbestrol (DES)	
Phytoestrogens	Genistein, daidzein
Mycotoxins	Aflatoxin B1
	ZEA
	α -ZOL
	α -ZAL
	β -ZOL

Dioxins: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; *micotoxins*: ZEA, zearalenone; α -ZOL, α -zearalenol; β -ZOL, β -zearalenol; α -ZAL, α -zearalanol; *pesticides*: β -HCH, β -hexachlorocyclohexane; PCB54, 2,2',6,6'-tetrachlorobiphenyl; PCB77, 3,3',4,4'-tetrachlorobiphenyl; PCB153, 2,2',4,4',5,5'-hexachlorobiphenyl; PCB118, 2,3',4,4',5-pentachlorobiphenyl; 2,4-D-, 2,4-dichlorophenoxyacetic acid; DBCP, 1,2-dibromo-3-chloropropane; *phthalates*: BBzP, benzyl butyl phthalate; DiBP, di-*isobutyl* phthalate; DnBP, dibutyl phthalate; DPP, dipentyl phthalate; DBP, dibutyl phthalate; DEP, diethyl phthalate; DMP, dimethyl phthalate; DOP, dioctyl phthalate; DEHP, di(2-ethylhexyl)phthalate.

Please note that this list is not exhaustive, it merely indicates the major classes that comprise the wide and heterogeneous group of EDCs and associated compounds. See text for discussion.

populations, semen volume, sperm count, concentration, motility, morphology, and chromatin integrity were inversely correlated with *p,p'*-DDE levels in serum of men exposed to extremely high DDT and *p,p'*-DDE concentrations. However, opposing reports have shown no correlations between *p,p'*-DDE serum concentrations and reproductive hormone levels or standard sperm parameters and chromatin/DNA damage in different populations. But, although *p,p'*-DDE was considered elevated in these populations,

due to a high intake of contaminated food, it was at least 1000-fold lower than the levels reported in individuals from malaria-endemic regions, possibly justifying the different outcomes. Furthermore, increasing levels of the EDC 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153), frequently used as a proxy for the total PCB body burden, have been correlated with decreased chromatin/DNA integrity in European men.

In addition, many other studies have raised important concerns. For instance, the exposure to what is believed to be the most powerful toxic agent ever made by mankind—2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)—was found to exert a broad spectrum of effects, including decreased sperm count, reduced reproductive organ weights, altered hormone levels, and altered urogenital features (e.g., anogenital distance) in many studies, suggesting that the male reproductive system is a TCDD-sensitive target *in vivo*. However, data in humans is scarce, and most of the understanding regarding TCDD effects comes from studies carried out with the population of Seveso (Italy) following an explosion that released high amounts of TCDD into the environment. Of note, breast-fed sons whose Seveso mothers presented a low median serum dioxin concentration at conception have lower sperm counts, concentration and motility and altered serum hormone levels, showing that *in utero* and lactation exposure of children to reasonably low TCDD concentrations can permanently decrease sperm quality. Strikingly, even 22 years after exposure men exposed to TCDD during childhood presented abnormal sperm concentration, motility and hormone levels, while men exposed only during puberty or adulthood showed an increase in these parameters, or no differences relative to controls. However, exposure during either infancy or puberty led to a permanent alteration of hormone levels, such as those of FSH and estradiol. These long-lasting endocrine changes were observed at serum TCDD doses within one order of magnitude of what was determined in the 1970–80s in modern countries, leading to the premise that the reported decrease in sperm quality, principally in younger men, could be partially attributed to TCDD exposure.

Finally, recent reports have suggested a completely new mechanism of EDC action that involves the potential transgenerational inheritance of effects. So far, studies performed in rodents have suggested that exposure to EDCs during gonadal sex determination may lead to a reprogramming of the male germline, not only leading to detrimental effects in the F1 generation but also inducing a transgenerational effect up to the third or fourth generation (F3 or F4) without additional exposure besides the initial F0 pregnant dam, in essence signifying that great-grandparents may transmit unwanted characteristics to their male descendants. Although some high doses were employed in these studies, and strain-specific effects have been reported, the hypothesis is that EDCs may alter the programming of the male germline. This will possibly transmit an altered epigenome in an imprinted-like manner across generations, thus inducing adult onset diseases, such as testis pathologies and a reduced fertilizing capability phenotype, emphasizing that male subfertility might be maintained throughout many generations. However, it is still to be determined whether this effect might also take place in humans.

Conclusions

The data currently available shows that several EDCs may induce impairments in male reproductive function, ultimately contributing towards an increase in male infertility/subfertility. Whether acting via genomic or nongenomic signaling pathways, many EDCs may compromise sperm quality and function during (1) testis and male reproductive tract development; (2) spermatogenesis; (3) storage/transit through the male reproductive tract; and/or (4) in the female reproductive tract. In general, EDCs may not only affect sperm production, motility, mitochondrial function, oxidative status, and/or DNA integrity but also capacitation and sperm ability to undergo the acrosome reaction and fuse with the oocyte, among other possible effects, and this will necessarily be reflected on fertilization success.

However, it is also important to note that some studies have used EDC concentrations that are far higher than what populations are normally exposed to, and higher than what is detected in body tissues and fluids. Although this may be crucial in order to unveil the molecular mechanisms of action underlying EDC toxicity, the relevance in an environmental context may be questioned. The only exception might be the cases in involving heavily contaminated scenarios (such as in the Seveso disaster, the Yucheng contamination, or in malaria-endemic areas, among others). These populations were accidentally exposed to extremely high levels of certain EDCs, but little is known about the resulting levels of these compounds in reproductive fluids and secretions, although it is expected to be higher than in other populations.

Importantly, although recently physiologically and environmentally relevant EDC concentrations have already been shown to have potential deleterious effects, currently available studies tend to focus on the effects of an individual EDC and not many pay attention to mixtures, which represent the truly relevant situation. In fact, human and animal populations are simultaneously exposed to numerous EDCs, and there is limited information on the putative synergistic effects of such compounds on male reproductive function, an issue that should be taken seriously into consideration in forthcoming years. Indeed, when acting together lower concentrations of EDCs may be needed to promote undesirable effects, making the environmentally relevant EDC concentrations more threatening than what might have been anticipated solely from *in vitro* data using single compounds.

Finally, it is also important to keep in mind that a key aspect that may lead EDCs to affect human and animal/wildlife populations in some contexts, but not in others, may be related to when the exposure takes place. Indeed, besides the concentrations used, duration and routes of exposure, or individual EDCs versus mixtures, many studies show that future male reproductive capacity may be more affected if the organism is exposed during critical windows of development, such as during fetal development and the early postnatal period. The data available also suggests that deleterious changes in male reproductive function may be more severe, even permanent, during these time periods, when compared to instances of exposure in adulthood.

Further Reading

- Aneck-Hahn, N. H., Schulenburg, G. W., Bornman, M. S., Farias, P., & De Jager, C. (2007). Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa. *Journal of Andrology*, 28(3), 423–434.
- Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308(5727), 1466–1469.
- Anway, M. D., Memon, M. A., Uzumcu, M., & Skinner, M. K. (2006). Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *Journal of Andrology*, 27(6), 868–879.
- Carlsen, E., Giwercman, A., Keiding, N., & Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ*, 305(6854), 609–613.
- Guerrero-Bosagna, C., Covert, T. R., Haque, M. M., Settles, M., Nilsson, E. E., Anway, M. D., & Skinner, M. K. (2012). Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reproductive Toxicology*, 34(4), 694–707.
- Levine, H., Jørgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. H. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*, 25(7), 1–14. <https://doi.org/10.1093/humupd/dmx022>.
- Mocarelli, P., Gerthoux, P. M., Patterson, D. G., Jr., Milani, S., Limonta, G., Bertona, M., Signorini, S., Tramacere, P., Colombo, L., Crespi, C., Brambilla, P., Sarto, C., Carreri, V., Sampson, E. J., Turner, W. E., & Needham, L. L. (2008). Dioxin exposure, from infancy through puberty, produces endocrine disruption and affects human semen quality. *Environmental Health Perspectives*, 116(1), 70–77.
- Mocarelli, P., Gerthoux, P. M., Needham, L. L., Patterson, D. G., Jr., Limonta, G., Falbo, R., Signorini, S., Bertona, M., Crespi, C., Sarto, C., Scott, P. K., Turner, W. E., & Brambilla, P. (2011). Perinatal exposure to low doses of dioxin can permanently impair human semen quality. *Environmental Health Perspectives*, 119(5), 713–718.
- Schiffer, C., Muller, A., Egeberg, D. L., Alvarez, L., Brenker, C., Rehfeld, A., Frederiksen, H., Waschle, B., Kaupp, U. B., Balbach, M., Wachten, D., Skakkebaek, N. E., Almstrup, K., & Strünker, T. (2014). Direct action of endocrine disrupting chemicals on human sperm. *EMBO Reports*, 15(7), 758–765.
- Skakkebaek, N. E., Rajpert-De Meyts, E., & Main, K. M. (2001). Testicular dysgenesis syndrome: An increasingly common developmental disorder with environmental aspects. *Human Reproduction*, 16(5), 972–978.
- Stronati, A., Manicardi, G. C., Cecati, M., Bordinchia, M., Ferrante, L., Spano, M., Toft, G., Bonde, J. P., Jönsson, B. A., Rignell-Hydbom, A., Rylander, L., Giwercman, A., Pedersen, H. S., Bonefeld-Jørgensen, E. C., Ludwicki, J. K., Lesovoy, V., Sakkas, D., & Bizzaro, D. (2006). Relationships between sperm DNA fragmentation, sperm apoptotic markers and serum levels of CB-153 and p,p'-DDE in European and Inuit populations. *Reproduction*, 132(6), 949–958.
- Tavares, R. S., Mansell, S., Barratt, C. L., Wilson, S. M., Publicover, S. L., & Ramalho-Santos, J. (2013). p'-DDE activates CatSper and compromises human sperm function at environmentally relevant concentrations. *Human Reproduction*, 28(12), 3167–3177.
- Tavares, R. S., Escada-Rebelo, S., Correia, M., Mota, P. C., & Ramalho-Santos, J. (2016). The non-genomic effects of endocrine-disrupting chemicals on mammalian sperm. *Reproduction*, 151(1), R1–R13.
- Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillette, L. J., Jr., Jégou, B., Jensen, T. K., Jouannet, P., Keiding, N., Leffers, H., McLachlan, J. A., Meyer, O., Müller, J., Rajpert-De Meyts, E., Scheike, T., Sharpe, R., Sumpter, J., & Skakkebaek, N. E. (1996). Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives*, 104(4), 741–803.

Transgenerational and Epigenetic Impacts of Environmental Exposures in Male Reproduction

Manuel Álvarez-Rodríguez, Heriberto Rodríguez-Martínez, and Carlos Guerrero-Bosagna, Linköping University, Linköping, Sweden

© 2018 Elsevier Inc. All rights reserved.

Glossary

Epigenetic modifications Accessory chemical modifications of the DNA that are mitotically stable and able to regulate gene expression and post-translational processes.

Epigenome Set of all epigenetic modifications found in a given genome.

Endocrine disruptor An exogenous substance that mimic the action of endogenous hormones on organisms.

Transgenerational epigenetic inheritance Process of inheritance in which environmental exposures modify the epigenome of the gametes, which will consequently influence the phenotype formation in the descendants. Heritable changes are observed in an unexposed offspring as consequence of parental exposure to environmental insults.

DNA methylation An enzymatic chemical reaction in which CH₃ (methyl) groups are covalently added to nucleotides, preferentially cytosines neighboring guanines (direction 5' to 3'); these dinucleotides are known as CpG sites.

Histone Protein directly involved in the structural compaction of DNA, which regulate the access of transcription factors to the genome, and consequently gene expression and post-translational processes.

ncRNA Small RNA molecules;(e.g., miRNAs, tRNAs, piRNAs) that are not translated into proteins, but that are able to regulate gene expression and post-translational processes.

Reprogramming Depletion and re-establishment of epigenetic marks, which occurs at different developmental windows.

Introduction

When we think of reproduction, what generally comes to mind is sexual intercourse with the union of gametes and the consequent generation of a new individual. However, even though these are important aspects, reproduction is much more than the action of hormones, sexual coupling or the generational transfer of genes. Reproduction, which depends on both intrinsic and extrinsic factors, can be defined as the parents passing to the progeny both structural (e.g., molecules, cellular compartments) and environmental features involved in the emergence of a living organization (Maturana-Romesin and Mpodozis, 2000). Simply put, reproduction involves parents perpetuating genes (among other chemical structures), and the maintenance of the parental environment involved in reproductive events. Therefore, the environmental context plays a fundamental role in the process of reproduction.

The question of how an adult organism unfolds after the merging of two gametes have been around since Aristotelian time. Adherents of “preformation” answered this question by stating that all the components needed for the embryo to become an adult individual were already inside the gametes. On the other side of the spectrum, those supporting “epigenesis” thought that gametes and embryonic cells needed to interact with external signals in order to generate an adult individual.

The concept of epigenesis had vital importance in forming the field of “epigenetics.” The term “epigenetics” was firstly introduced by Conrad H. Waddington, a British Geneticist, in 1942 in his paper “The Epigenotype,” before DNA and the molecular machinery of gene expression were described. Waddington asked “how genes interact with their products and the environment to bring phenotypes into being?” By combining the term “epigenesis” and “genetics” he came up with a term that is now becoming highly important in all fields of biological sciences: “Epigenetics.”

One of the main aspects of epigenetics is to see the genome as a reactive chemical entity rather than as a “code” directing the development of organisms towards an adult phenotype. This is because behind the A, C, G, and T letters of this so-called “genetic code,” there is an enormous complexity: the one inherent to every chemical structure.

Just as lipids or proteins, the DNA has a molecular structure, and as such, it interacts with an array of other molecular structures. These can be proteins such as histones, small fragments of RNA or simply methyl groups that attach to the DNA structure and block or modify the translation of parts of the genome. When such interactive modifications with the DNA are maintained even after cell divisions, they are defined as “epigenetic” modifications, with a remarkable feature: not only can they regulate gene expression, but can be influenced by environmental cues.

A major breakthrough in epigenetic research is the ability to understand the etiology of diseases for which genetic associations have never been found, or where these only explain a limited number of cases. By focusing on environmental exposures during early development, and the epigenetic changes they produce, we can start to understand the causes and mechanisms of many diseases of common and increasing occurrence. Early developmental exposures and epigenetic changes are nowadays correlated with an array of pathologies, which includes metabolic syndrome, cardiovascular diseases, allergies or different types of cancer.

Recently, evidence has even shown that environmental exposure during pregnancy can generate epigenetic disturbances and increase the rate of diseases in the offspring and their descendants to be. This phenomenon is called transgenerational epigenetic inheritance (TEI). The initial description of cases of TEI was done through experiments studying reproductive dysfunction (particularly in males) after exposure to environmental toxicants, categorized as endocrine disrupting chemicals (EDCs); these are exogenous chemical compounds that can interact with endogenous hormone receptors and cause hormone-like responses in the affected organism.

Initial Evidence for Transgenerational Effects Induced by the Environment

The first evidence in animal models indicating that gestational exposure to EDCs could generate disease phenotypes capable of being transmitted to future generations was obtained in a study testing for the reproductive effects of Diethylstilbestrol (DES). DES is an estrogenic compound originally prescribed to women to prevent miscarriages and other reproductive complications, in use from the 1930 to the 1970s. Later observations revealed that DES administration was associated with reproductive defects in the offspring of the treated women. While studying reproductive effects of a gestational exposure to DES in rodents, researchers reported in the year 2000 that administration of DES to pregnant rats during early post-implantation development generated reproductive abnormalities in the following generations (Newbold et al., 2000). These abnormalities in the male offspring of the F1 and F2 generation included increased susceptibility for proliferative lesions in the *rete testis*, which are usually of rare incidence, as well as increased tumors formed in other areas of the reproductive tract (Newbold et al., 2000). Based on these results, the authors suggested that epigenetic alterations could be involved in the generation of these reproductive abnormalities, and their transmission to future generations. In the year 2005, while reproductive effects of the fungicide vinclozolin were being studied, came the first description of an environmentally-induced transgenerational inheritance process confirmed to be related to epigenetic changes in the germ line (Anway et al., 2005). Vinclozolin is a fungicide with anti-androgenic effects (widely used in crops around the world) that was previously shown to generate increased apoptosis in spermatogenic cells of male rodents after their mothers were treated with the compound during gestation (Uzumcu et al., 2004). Follow-up experiments demonstrated that this vinclozolin-induced increased apoptosis in spermatogenic cells was also observed in the next three generations, in absence of any further exposure of the affected offspring (Anway et al., 2005, 2006a,b).

Current Evidence for Reproductive Transgenerational Effects Induced by the Environment in Males

In addition to increased events of programmed death (apoptosis) during spermatogenesis, other male reproductive abnormalities are shown to be transmitted through TEI (Fig. 1). Moreover, a variety of environmental toxicants can trigger this phenomenon. These transgenerationally transmitted altered phenotypes in males include: kidney abnormalities (after ancestral exposures to

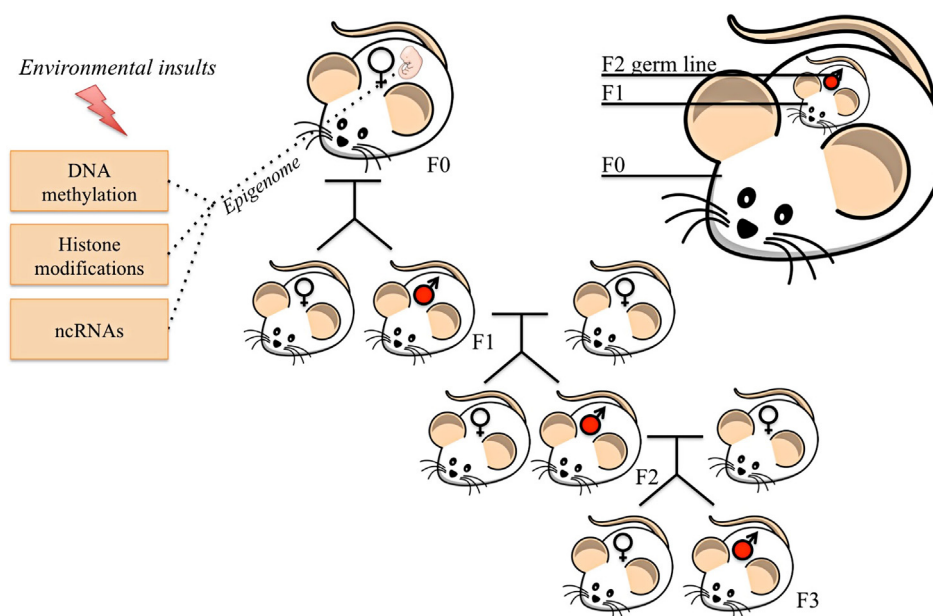


Fig. 1 Transgenerational epigenetic effect up to the male germinal line of the third generation of environmentally-exposed mice. Such effects “travel” throughout generations, displaying changes on the offspring directly related to the original parental exposure (F0), but also affecting the germline of the F1, which –via its semen– would pass the transgenerational epigenetic inheritance phenomenon to an F3-generation individual. Figure modified from Nilsson, E., Skinner, M.K. (2015). Environmentally Induced Epigenetic Transgenerational Inheritance of Disease Susceptibility. *Translational Research* 165(1), 12–17, <https://doi.org/10.1016/j.trsl.2014.02.003>.

vinclozolin (Anway et al., 2006a), dioxin (Manikkam et al., 2012b), DDT (Skinner et al., 2013b), and methoxychlor (Manikkam et al., 2014)), prostate abnormalities (after ancestral exposure to vinclozolin (Anway et al., 2006a)), pubertal abnormalities (after ancestral exposures to dioxin (Manikkam et al., 2012b)), and testis abnormalities (after ancestral exposures to a mixture of the pesticides Permethrin and DEET (Manikkam et al., 2012c), and to DDT (Skinner et al., 2013b)). Ancestral exposure to pharmacological levels of all of these compounds induced sperm epimutations (i.e., DNA methylation changes) in the unexposed F3 generation, therefore opening the conceptual possibility that sperm epimutations can be indicators of both ancestral exposure and susceptibility to disease (Manikkam et al., 2012a; Skinner et al., 2013b; Guerrero-Bosagna et al., 2010). Independent studies have reported TEI in response to a variety of EDCs. For example, in males, early developmental exposure to di-(2-ethylhexyl) phthalate induces transgenerationally transmitted disruption of testicular germ cell associations, reduced sperm count and decreased sperm motility in mice (Doyle et al., 2013). Also, early developmental exposure to DDE is reported to increase testicular germ line apoptosis from the F1 to the F3 generations in rats (Song et al., 2014). Interestingly, increased rate of infertility and incidence of small testes were observed in this study in the F3 but not in the F1 or F2 generations (Song et al., 2014). A summary of the current evidence reporting EDC-related TEI is shown in Table 1.

Reprogramming and Epigenetics

The transgenerational transmission of disease phenotypes is attributed to disturbances in DNA methylation that occur during the migration of primordial germ cells (at initial stages of sex determination) (Skinner et al., 2013a). This ultimately affects the epigenome of the mature sperm, which is then maintained altered in the following generations, thereby affecting the adult phenotype of the descendants. A particular aspect of this developmental period is that it is one of the developmental windows during which a so-called “epigenetic reprogramming” can occur.

Reproduction in vertebrates depends on two critical participants: the gametes (the maternal egg and the paternal sperm) and the zygote resulting from fertilization. In the latter, a most relevant event occurs under the generic name of *Reprogramming* (Fig. 2) of both the maternal and paternal genome (Hill et al., 2014). This allows the zygote to develop full potency (totipotency) to generate all the cell types present in the new embryo, as well as the extraembryonic tissues (including placenta). The primary epigenetic factors (also called marks) that determine potency are DNA methylation, histone modifications and the action of non-coding RNA (ncRNA) (Piferrer, 2013). During this reprogramming, epigenetic “marks” are removed from the DNA of the egg and the sperm, thereby restoring their cell potency immediately before the zygote is formed. This demethylation process occurs either by active or passive mechanisms (Hackett et al., 2012). Later in development, another reprogramming will occur during the formation of the primordial germ cells (PGCs) which will develop into the gametes of the offspring, depending on its chromosomal sex. The phenomenon of PGC reprogramming is basically the same as previously described for the zygote: the genome is to be stripped of essentially all heterochromatic factors/marks that could restrict developmental capacity. Imprinted genes, transposable elements (TE's) as retrotransposons (RTs), and centromeric heterochromatin, which together compose almost 50% of the genome (Lander et al., 2001) are, however, excepted from this stripping during the reprogramming that occurs during the early embryonic cleavage stage (Morgan et al., 2005). The second wave of reprogramming that occurs in the embryonic PGCs is absolutely necessary to re-establish fetal-specific imprinting, to regain the epigenetic potential required for fertilization, and for the development of the next generation individuals. In this way, some loci do not undergo demethylation, and thus become the carriers of transgenerational epigenetic memory (Lane et al., 2003).

Transposable Elements and Embryo Vulnerability

As mentioned above, demethylation of DNA in either gametes or embryos is a critical component of reprogramming. Yet it opens for the most vulnerable period owing to the risk of the expression or even the expansion of integrated ancestral viral elements within the genome (retroviruses, transposons) and are normally kept “silenced” by DNA methylation (Guo et al., 2014). Demethylation during reprogramming can cause mobilization of TEs (either retrotransposons or DNA-transposons, Wicker et al., 2007) with deleterious effects ranging from genetic instability (causing genome mutations) to cell death, genetic diseases, cancer and even infertility (Maksakova et al., 2006). However, considering that TEs are a significant part of the genome, their role might also be determinant for evolution driving, and TE's should not necessarily be related to negative effects (Chuong et al., 2017). In any case, there seems to be a liaison between environmental pressure and TEs-mediated genetic changes, particularly in relation to stress and immune responses (Van De Lagemaat et al., 2003).

Environmental Insults and Non-Coding Small RNAs

Emerging evidence suggests that miRNAs play an important role in male fertility. MicroRNAs (miRNA) are single-strand RNAs of 21–23 nucleotides, transcribed from DNA but not translated into proteins (thus generically named non-coding RNAs, ncRNAs). Important roles for these ncRNAs have been ascribed in different molecular pathways, both at transcriptional and post-transcriptional level. miRNAs undergo a maturation process from primary miRNA to a precursor of miRNA, in which the enzyme

Table 1 Transgenerational and epigenetic effects derived from exposure to EDCs

<i>Exposure</i>	<i>Species</i>	<i>Route of administration</i>	<i>Period of exposure</i>	<i>Generation and cell type analyzed</i>	<i>Type of epigenetic analysis</i>	<i>Epigenetic change(s)</i>	<i>Correlation with gene expression</i>	<i>Abnormalities observed (F: females; M: males)</i>	<i>Reference</i>
Vinclozolin	Rat	Intraperitoneal	Gestational from E8-E15	F2 and F3 generations, sperm	Methylation-sensitive restriction enzyme PCR; bisulfite sequencing	Within the LPLase gene and around the start site of the cytokine-inducible SH2 protein	No	M: spermatogenic cell apoptosis, reduced sperm numbers and motility	Anway et al. (2005)
Vinclozolin	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array; mass spectrometry with bisulfite sequencing; bisulfite-pyrosequencing	52 regions in 48 genes in initial screening; 16 regions confirmed	No	—	Guerrero-Bosagna et al. (2010)
Vinclozolin	Mouse	Intraperitoneal	Gestational from E7-E13	F3 generation, sperm	MeDIP with comparative promoter chip array; MeDIP with RT qPCR	68 regions in 66 genes in initial screening; 40 regions confirmed	No	F: PCOS M: spermatogenic cell apoptosis, testis, prostate and kidney abnormalities	Guerrero-Bosagna et al. (2012)
BPA + Phtalates	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array; MeDIP with RT qPCR	197 regions	No	M&F: increased adiposity F: PCOS; primordial follicle loss, pubertal abnormalities	Manikkam et al. (2012a,d)
Permethrin + DEET	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array; MeDIP with RT qPCR	363 regions	No	M: testis abnormalities	Manikkam et al. (2012a,c)
Jet fuel 8	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array; MeDIP with RT qPCR	33 regions	No	F: PCOS, primordial follicle loss	Manikkam et al. (2012a) and Tracey et al. (2012)
TCDD	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array; MeDIP with RT qPCR	50 regions	No	M: kidney, pubertal abnormalities	Manikkam et al. (2012a,b)
Vinclozolin	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, Sertoli cells	MeDIP with comparative promoter chip array	101 regions	Yes	M: spermatogenic cell apoptosis	Guerrero-Bosagna et al. (2013)
Vinclozolin	Mouse	Intraperitoneal	Gestational from E10-E18	F1, F2, and F3 generations, sperm, tail, liver, skeletal muscle	Bisulfite-pyrosequencing	Imprinted genes H19 and Peg3 showed a transgenerational effect in the sperm; Peg3 showed a transgenerational effect in tail and liver	No	M: reduced sperm motility (only F1)	Stouder and Paoloni-Giacobino, (2010)
Vinclozolin	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, primordial germ cells	MeDIP with comparative promoter chip array	24 regions in E13 PGCs; 13 regions in E16 PGCs	Yes	—	Skinner et al. (2013a)
DDT	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array	39 regions	No	M&F: kidney, increased adiposity F: PCOS; M: testis	Skinner et al. (2013b)
Methoxychlor	Rat	Intraperitoneal	Gestational from E8-E14	F3 generation, sperm	MeDIP with comparative promoter chip array	37 regions	No	M&F: kidney, increased adiposity F: PCOS	Manikkam et al. (2014)
TCDD	Rat	Oral gavage	Gestational from E8-E14	F1 and F3 generation, liver	Bisulfite sequencing	Imprinted gene Igf2, and altered DNMTs expression	Yes	M: liver abnormalities	Ma et al. (2015)
DDE	Rat	Oral gavage	Gestational from E8-E15	F1, F2, and F3 generations, sperm	Bisulfite sequencing	Imprinted DMR at the Igf2/H19 cluster	Yes	M: testis abnormalities, spermatogenic cell apoptosis	Song et al. (2014)

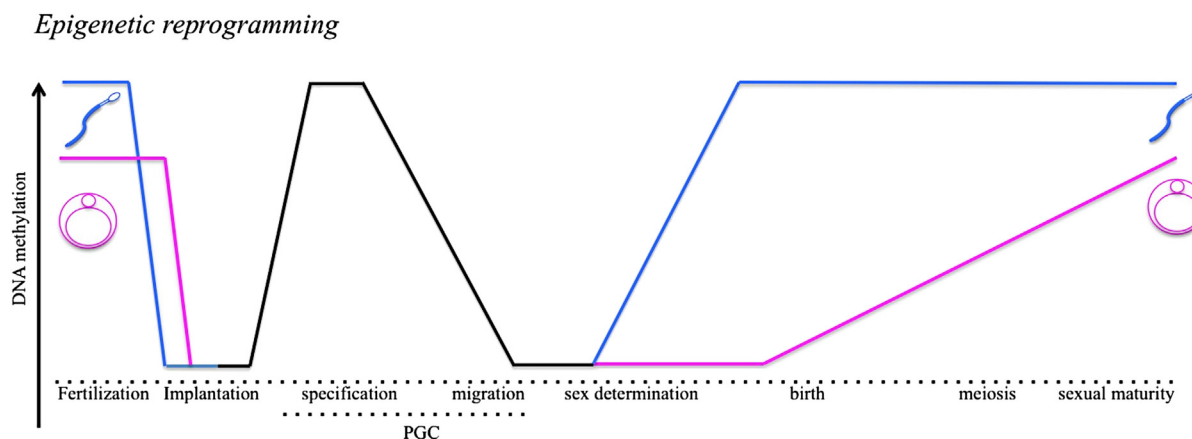


Fig. 2 Epigenetic reprogramming. Levels of DNA methylation from fertilization to sexual maturity. Reprogramming waves mainly occur during fertilization and gametogenesis of the new conceptus, and are necessary for the correct transmission of the epigenetics patterns through generations. Subtle changes, for example, produced by environmental toxicants, could induce epigenetically heritable changes in the offspring which, albeit not coded genetically, are made permanent in future generations. Male gametes are represented in blue line and female in pink. PGC: primordial germinal cell. Figure modified from <http://www.hannonlab.org/epigenetics>.

Drosha plays a fundamental role. The final step in the development of these molecules is carried out in the cytoplasm by Dicer and loaded onto the Argonaute protein to result in a RNA-silencing complex (RISC). This final complex is able either to restrain, or increase translation.

It is also becoming increasingly evident that non-coding RNAs in testicular somatic or germ cells play a fundamental role in spermatogenesis. Small non-coding RNAs involved in sperm production include micro RNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and PIWI-interacting RNAs (piRNAs). These small RNAs are categorized depending on the involvement of the RNase III endonuclease DICER in their biogenesis mechanisms. DICER is essential for microRNA processing during the haploid differentiation of germ cells. Endo-siRNAs and miRNAs are DICER-dependent, while piRNAs are DICER-independent but predominantly expressed in tissues involved in male germ line development (Yadav and Kotaja, 2014). The miRNA knockout of *Dicer* in Sertoli cells leads to testicular dysgenesis and infertility due to the disruption of Sertoli cell architecture (Papaioannou et al., 2009). The identification of the roles of particular miRNAs during spermatogenesis should lead to the use of miRNAs as predictive or diagnostic indicators.

In addition to DNA methylation, miRNAs are starting to emerge as important players in the process of TEI. Particularly, a specific type of small RNAs named sperm transfer RNA-derived smallRNAs (tsRNAs), could be implicated as paternal epigenetic factor that may mediate the inheritance of diet-induced metabolic disorders. For example, injection of sperm tsRNA fractions from males consuming a high fat diet into normal zygotes has generated metabolic disorders in the F1 offspring, together with altered gene expression of metabolic pathways in early embryos and pancreatic islets of F1 offspring (Chen et al., 2016). Interestingly, this sperm tsRNA-mediated inheritance is unrelated to DNA methylation at CpG-rich regions. Moreover, while these tsRNAs are found at minimal levels in testicular spermatozoa, their amount increases substantially through their maturation within the epididymis. Therefore, the presence of tsRNAs in mature spermatozoa does not merely result from degradation of tsRNAs present during spermatogenesis (Sharma et al., 2016). In this sense epididymosomes, exosomal vesicles from the epididymis epithelium, are able to fuse with the luminal spermatozoa and most likely load these RNAs along the sperm maturation process. Overall, recent evidence points towards (i) the paternal diet being able to influence the phenotype of the offspring via the inclusion of varying amounts of specific small RNAs in spermatozoa (including tRNA fragments) throughout the male reproductive tract, and (ii) that these tRNA fragments in mature sperm can regulate expression of transcripts, driven by endogenous retro-transposable elements (Sharma et al., 2016).

TE-expression during reprogramming seems to act via the so-called PIWI (P-element Induced Wimpy testes) pathway. This is a small RNA-based pathway of a post-transcriptionally regulatory system of RNA interference, built by PIWI proteins (belonging to the Argonaute family) and associated PIWI-interacting RNAs (piRNAs), all functionally acting in a similar fashion as the miRNAs described above, being key controllers of retrotransposon expression (Vagin et al., 2006).

Germline Development: Is it Here Where Transgenerational Somatic Epigenetic Effects Are Caused?

The development of the germ line plays a crucial role in the phenomenon of TEI. DNA methylation and expression patterns are disrupted in PGCs when the maternal exposure to vinclozolin occurs between the onset of gonadal sex determination and after testis cord formation (Skinner et al., 2013a,b). These alterations are maintained in subsequent generations (Skinner et al., 2013a,b; Anway et al., 2008) and will, in theory, influence the epigenome of the mature spermatozoa.

However, despite what is known today and of an increasing number of studies focusing on the mechanisms behind TEI, there are still gaps in knowledge regarding the exact biological pathways by which environmental insults interfere with germ line epigenetic

THE 'EPIGENETIC DOMINO' EFFECT

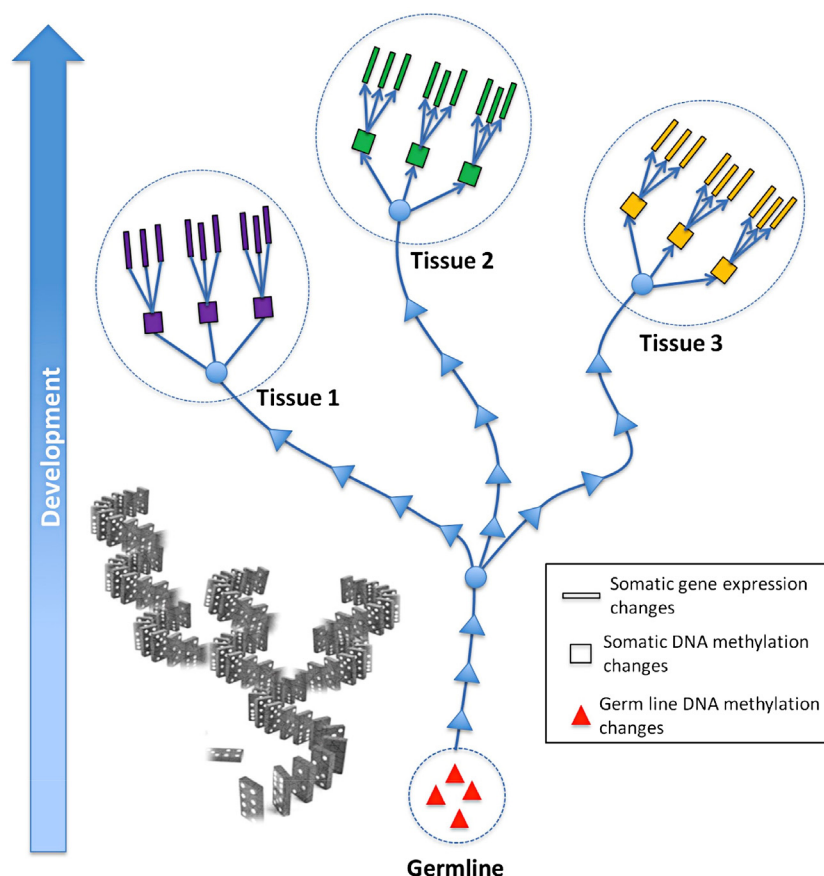


Fig. 3 Schematic representation of an “Epigenetic Domino” effect in which environmentally-induced germ line epigenome modifications will generate subsequent amplified epigenomic and transcriptomic effects in somatic cells. Figure modified from Guerrero-Bosagna, C., Skinner, M.K. (2014). Environmentally induced epigenetic transgenerational inheritance of male infertility. *Current Opinion in Genetics & Development* **26**, 79–88, <https://doi.org/10.1016/j.gde.2014.06.005>.

reprogramming and subsequently, with the phenotype of individuals in future generations. Experiments have been performed to determine whether somatic gene expression and epigenetic patterns are transgenerationally altered in somatic tissues in close contact with gonadal development (Guerrero-Bosagna et al., 2013; Nilsson et al., 2012). Studies in Sertoli (Guerrero-Bosagna et al., 2013) and granulosa (Nilsson et al., 2012) cells have shown altered epigenomes and transcriptomes in the F3-generation after an ancestral exposure to vinclozolin. In Sertoli cells, vinclozolin-induced transgenerational DNA methylation changes were observed with distinct degree of connections to gene expression changes in the same cells (Guerrero-Bosagna et al., 2013). These connections included genomic proximity as well as functional correlations. Importantly, changes in gene expression do not necessarily correlate to changes in DNA methylation. This is due, in part, to the fact that changes in DNA methylation in somatic cell types developmentally precede and influence later changes in gene expression in the same tissues. Similarly, DNA methylation changes in the germ line will influence somatic DNA methylation patterns later on in the individuals that will be originated out of these germ cells; however, the genes affected in each case will not necessarily be the same. This corresponds to an “epigenetic domino” process in which the germ line epigenome will influence the epigenome somatic cells in the originating individual, hence, ultimately affecting gene expression. During this process, an amplification effect is observed, in which few altered genes in the germ line can produce an increased number of somatic epigenetic alterations, which will in turn generate an even larger number of genes altered in terms of expression (Fig. 3).

Concluding Remarks

There is increasing evidence that parental exposures to environmental insults affect male reproduction, both in exposed individuals and in coming generations. These alterations are mostly due to changes in the epigenome of somatic and germ cells. In the scenario when the germ line epigenome is affected by environmental exposures, then epigenetic alterations can be transmitted to future

generations, in a process known as TEI. Although this phenomenon have only started to be described in detail at the first decade of this century, evidence is rapidly accumulating not only in reproduction but also in many other biological aspects. Considering the relevance of the fact that certain environmental exposures can trigger diseases that last for generations, more is needed to be know about which specific compounds are able to trigger a process of TEI, at which amounts, in which developmental stages, and what are the effects of combined exposures (of multiple compounds). These gaps in knowledge, together with the relevance of this biological process to human and animal health, warrant an expanding interest by the scientific community in performing research in the many aspects related to TEI.

References

- Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308, 1466–1469.
- Anway, M. D., Leathers, C., & Skinner, M. K. (2006a). Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. *Endocrinology*, 147, 5515–5523.
- Anway, M. D., Memon, M. A., Uzumcu, M., & Skinner, M. K. (2006b). Transgenerational effect of the endocrine disruptor vinclozolin on male spermatogenesis. *Journal of Andrology*, 27, 868–879.
- Anway, M. D., Rekow, S. S., & Skinner, M. K. (2008). Transgenerational epigenetic programming of the embryonic testis transcriptome. *Genomics*, 91, 30–40.
- Chen, Q., Yan, M., Cao, Z., et al. (2016). Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science*, 351, 397–400.
- Chuong, E. B., Elde, N. C., & Feschotte, C. (2017). Regulatory activities of transposable elements: From conflicts to benefits. *Nat. Rev. Genet.*, 18, 71–86.
- Doyle, T. J., Bowman, J. L., Windell, V. L., McLean, D. J., & Kim, K. H. (2013). Transgenerational effects of di-(2-ethylhexyl) phthalate on testicular germ cell associations and spermatogonial stem cells in mice. *Biology of Reproduction*, 88, 1–15.
- Guerrero-Bosagna, C., Settles, M., Lucker, B., & Skinner, M. K. (2010). Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. *PLoS One*, 5(9), e13100.
- Guerrero-Bosagna, C., Covert, T. R., Haque, M. M., et al. (2012). Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reproductive Toxicology*, 34, 694–707.
- Guerrero-Bosagna, C., Savenkova, M., Haque, M. M., Nilsson, E., & Skinner, M. K. (2013). Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: Molecular etiology of male infertility. *PLoS One*, 8, e59922.
- Guo, H., Zhu, P., Yan, L., et al. (2014). The DNA methylation landscape of human early embryos. *Nature*, 511, 606–610.
- Hackett, J. A., Zyllicz, J. J., & Azim Surani, M. (2012). Parallel mechanisms of epigenetic reprogramming in the germline. *Trends in Genetics*, 28, 164–174.
- Hill, P. W. S., Amoroux, R., & Hajkova, P. (2014). DNA demethylation, Tet proteins and 5-hydroxymethylcytosine in epigenetic reprogramming: An emerging complex story. *Genomics*, 194, 324–333.
- Lander, E. S., Consortium, I. H. G. S., Linton, L. M., et al. (2001). Initial sequencing and analysis of the human genome. *Nature*, 409, 860–921.
- Lane, N., Dean, W., Erhardt, S., et al. (2003). Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. *Genesis*, 35, 88–93.
- Ma, J., Chen, X., Liu, Y., et al. (2015). Ancestral TCDD exposure promotes epigenetic transgenerational inheritance of imprinted gene Igf2: Methylation status and DNMTs. *Toxicology and Applied Pharmacology*, 289, 193–202.
- Maksakova, I. A., Romanish, M. T., Gagnier, L., Dunn, C. A., van de Lagemaat, L. N., & Mager, D. L. (2006). Retroviral elements and their hosts: Insertional mutagenesis in the mouse germ line. *PLOS Genetics*, 2(1), e2.
- Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, M. M., & Skinner, M. K. (2012a). Transgenerational actions of environmental compounds on reproductive disease and epigenetic biomarkers of ancestral exposures. *PLoS One*, 7, e31901.
- Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. K. (2012b). Plastic derived endocrine disruptors (BPA, DEHP, and DBP) induce epigenetic transgenerational inheritance of adult-onset disease and sperm epimutations. *PLoS One*, 8, e55387.
- Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. K. (2012c). Pesticide and insect repellent mixture (Permethrin and DEET) induces epigenetic transgenerational inheritance of disease and sperm epimutations. *Reproductive Toxicology*, 34, 708–719.
- Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. K. (2012d). Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. *PLoS One*, 7, e46249.
- Manikkam, M., Haque, M. M., Guerrero-Bosagna, C., Nilsson, E. E., & Skinner, M. K. (2014). Pesticide methoxychlor promotes the epigenetic transgenerational inheritance of adult-onset disease through the female germline. *PLoS One*, 9, e102091.
- Maturana-Romesin, H., & Mpodzis, J. (2000). The origin of species by means of natural drift. *Revista Chilena de Historia Natural*, 73, 261–310.
- Morgan, H. D., Santos, F., Green, K., Dean, W., & Reik, W. (2005). Epigenetic reprogramming in mammals. *Human Molecular Genetics*, 14, R47–R58.
- Newbold, R. R., Hanson, R. B., Jefferson, W. N., et al. (2000). Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol. *Carcinogenesis*, 21, 1355–1363.
- Nilsson, E., Larsen, G., Manikkam, M., et al. (2012). Environmentally induced epigenetic transgenerational inheritance of ovarian disease. *PLoS One*, 7, e36129.
- Papaioannou, M. D., Pitetti, J. L., Ro, S., et al. (2009). Sertoli cell Dicer is essential for spermatogenesis in mice. *Developmental Biology*, 326, 250–259.
- Piferrer, F. (2013). Epigenetics of sex determination and gonadogenesis. *Developmental Dynamics*, 242, 360–370.
- Sharma, U., Conine, C. C., Shea, J. M., et al. (2016). Biogenesis and function of tRNA fragments during sperm maturation and fertilization in mammals. *Science*, 351, 391–396.
- Skinner, M. K., Guerrero-Bosagna, C., Haque, M. M., et al. (2013a). Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. *PLoS One*, 8, e66318.
- Skinner, M. K., Manikkam, M., Tracey, R., et al. (2013b). Ancestral dichlorodiphenyltrichloroethane (DDT) exposure promotes epigenetic transgenerational inheritance of obesity. *BMC Medicine*, 11(228).
- Song, Y., Wu, N., Wang, S., et al. (2014). Transgenerational impaired male fertility with an Igf2 epigenetic defect in the rat are induced by the endocrine disruptor p,p'-DDE. *Human Reproduction*, 29, 2512–2521.
- Stouder, C., & Paoloni-Giacobino, A. (2010). Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. *Reproduction*, 139, 373–379.
- Tracey, R., Manikkam, M., Guerrero-Bosagna, C., & Skinner, M. K. (2012). Hydrocarbon (jet fuel JP-8) induces epigenetic transgenerational inheritance of adult-onset disease and sperm epimutations. *Reproductive Toxicology*, 36, 104–116.
- Uzumcu, M., Suzuki, H., & Skinner, M. K. (2004). Effect of the anti-androgenic endocrine disruptor vinclozolin on embryonic testis cord formation and postnatal testis development and function. *Reproductive Toxicology*, 18, 765–774.
- Vagin, V. V., Sigova, A., Li, C., et al. (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science*, 313, 320–324.
- Van De Lagemaat, L. N., Landry, J. R., Mager, D. L., & Medstrand, P. (2003). Transposable elements in mammals promote regulatory variation and diversification of genes with specialized functions. *Trends in Genetics*, 19, 530–536.

- Wicker, T., Sabot, F., Hua-Van, A., et al. (2007). A unified classification system for eukaryotic transposable elements. *Nat. Rev. Genet.*, 8, 973–982.
- Yadav, R. P., & Kotaja, N. (2014). Small RNAs in spermatogenesis. *Molecular and Cellular Endocrinology*, 382, 498–508.

Further Reading

- Groenen, M. A. M., Archibald, A. L., Uenishi, H., et al. (2013). Analyses of pig genomes provide insight into porcine demography and evolution. *Nature*, 491, 393–398.
- Skinner, M. K., & Skinner, M. (2008). What is an epigenetic transgenerational phenotype? F3 or F2. *Reproductive Toxicology*, 25, 2–6.
- Waddington, C. H. (1942). The epigenotype. *Endeavour*, 1, 18–20.

Preconception Generational Impacts Male

Aurore Gely-Pernot, EHESP/Inserm, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Glossary

Aneuploidy Aneuploidy is presence of abnormal number of chromosome in cell.

Azoospermic men Men whose sperm contains no spermatozoa.

Cryptorchidism It is a congenital anomaly of male sexual development where undescended testis are absent of the scrotum and stay in abdominal cavity.

DNA methyltransferase DNA methyltransferase is an enzyme involve in the transfer of methyl group on DNA sequence.

Epigenetic Epigenetic is a study of changes in gene expression without affecting DNA sequence.

Hypospadia Hypospadia is a congenital anomaly where urethra opening is not at the head of the penis but on its ventral side resulting to an incomplete closure of the urethral folds.

Methylation It is an addition of a methyl group on a substrate.

miRNAs Small RNA segments able to bind messenger RNA and to suppress gene expression.

Oligozoospermic men Men with a quantity of spermatozoa in sperm lower than normal (< 20 million of spermatozoa per milliliter).

Paternally imprinted loci Position on the chromosome marked with information about its parental origin, here the father.

Secondary sex ratio It is defined as the ratio of male-to-female live births. The first sex ratio is the ratio of male-to-female conceptions.

Telomere Telomere is tandemly repeated hexameric sequence of nucleotides whose role is to preserve the stability of the genome.

X-chromosome inactivation Randomly inactivation of one X chromosome in mammal's females (XX) during development to have the same content of X-located genes than in males (XY).

Introduction

Environmental factors can disrupt early developmental process during pregnancy and early childhood, which constitute critical windows of vulnerability and lead to diseases or dysfunctions development after birth until adulthood. This concept corresponds to the Developmental Origins of Health and Disease (DOHaD) which was first given by David Barker in the 1980s. Now, other evidences have shown that environmental stressors can affect female and male gametes before conception and may transfer effects to the future individual through different mechanisms.

In this article, we will see first how preconception exposure of male to physical, chemical and socio-cultural agents will be able to affect offspring (Fig. 1) and then, we will do an overview of the main epigenetic mechanisms possibly involved in paternal inheritance.

Impact of Male Preconception Period on Human Reproduction

Physical Agents

We will focus here on the preconceptional effect of two physical agents, the temperature and the radiations.

Temperature

In Human, spermatogenesis occurs between 34°C and 35°C that mean 2–3°C below the temperature of the body. Many studies have shown that an increase of 1°C to 1.5°C of the scrotal temperature can induce a decrease of testis size, a disruption of sperm production and can affect sperm morphology. In the case of cryptorchidism, a congenital malformation where testis fails to descend from the peritoneal cavity to the scrotum, spermatogenesis does not occur due to a high temperature (Mieusset et al., 1995). The potential use of testicular heating for contraceptive purposes has been investigate the first time in 1921 by Martha Voegli, a Swiss physician. The method was for men to take a bath at 46–47°C during 45 min every day during 3 weeks. She reported this technique was efficient and induces no malformation in babies after recovery of fertility. One study performed in sauna-exposed Finnish men demonstrated that two sauna sessions per week for 3 months during 15 min induces a significant, but reversible, impairment of spermatogenesis (Garolla et al., 2013). Use of laptop on the lap induce also an increase of genital area temperature in men witch can represent a risk for normal spermatogenesis process. A reduction of fertility relative to occupational heat stress has also been

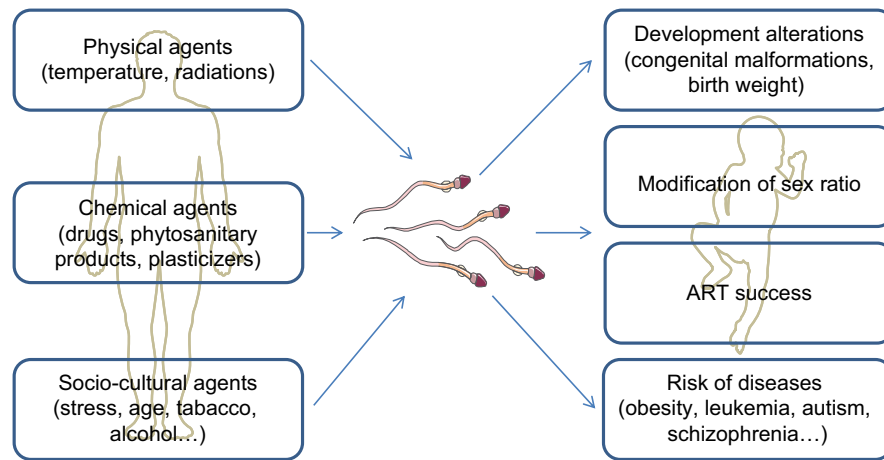


Fig. 1 Male preconception exposition affects development and health of the offspring. Environmental factors as physical, chemical and socio-cultural agents affect male gametes. Spermatozoa are responsible to the transmission of information to the next generation leading to an alteration of development, a modification of the sex ratio, a decrease of the success of assisted reproductive technologies (ART) and an increase of the risk of diseases.

observed. It is the case for welders or men working in ceramic industry exposed to high temperature whose have an alteration of spermatogenesis process leading to an increase of infertility incidence.

Radiations

Radiations are another physical agents affecting reproduction in male. It corresponds to energy coming from a source and traveling through space and some material as Human body. Human can be exposed to two types of radiation: non-ionizing radiations via phone, computer, radio and so on or ionizing radiations via medical X-rays or radioactive source.

A meta-analysis performed in 2016 by Ryan C. Lewis and collaborators report that exposure to power-frequency magnetic fields (50 or 60 Hz), some non-ionizing radiations, may be link to infertility and adverse pregnancy outcomes etiology (Lewis et al., 2016). For example, exposition to magnetic field ≥ 1.6 mG increases the risk of poor semen quality and the risk increases with exposure duration in a dose-dependent manner. In vitro experiment show that radiofrequency electromagnetic waves (870 MHz) from cellular phone may lead to oxidative stress in semen samples and a decrease of sperm mobility and viability. Electromagnetic waves coming from laptop computers decrease spermatozoa motility and increase DNA fragmentation.

Ionizing radiation is a classical mutagen witch can increase chromosomal aberration, the frequencies of chromatin exchange and DNA double-strand breaks. Claudius Regaud shown in 1977 that radon, a chemical element witch produce ionizing radiation, induces spermatogonia cell death and ram sterilization (Regaud, 1977). Populations occupationally exposed to ionizing radiation have less good sperm parameters with an increase of sperm DNA fragmentation. A strong association was done between preconception radiation exposure of father and development of leukemia in offspring living near Sellafield nuclear plant in united Kingdom (Gardner et al., 1990). Other studies about workers exposed to ionizing radiation failed to confirm these finding. In fact, any correlation was done between exposition and an increase of adult-onset multifactorial diseases among offspring of A-bomb or an increase of cancer in offspring of surviving cancer patients receiving an high dose of mutagen by radio- and chemotherapy.

Chemical Agents

Human are exposed every day to chemical agents produce by human activity. More than 100,000 substances are present around us and can impact our health and health of our children. In this section, we will focus only on some categories of substances, the drugs, the phytosanitary products and some plasticizers for which scientific community accumulate significant data.

Drugs

Chemotherapy induces gonadal dysfunction due to a toxic effect on spermatogonia stem cell. The differentiated germ cell and the Leydig cell seem to be more resistant to treatment. Patients who received chemotherapy treatment may become oligospermic or azoospermic but our secondary sexual characteristics are conserved as testosterone production by Leydig cell is in principle not affected. The effect on testicular function is really specific of the drug and doses used during treatment. For example, children who received chemotherapy treatment for Hodgkin's lymphoma presented an important alteration on seminiferous epithelium up to 10 years after treatment. Abdomino-pelvic or testicular radiotherapy can affect reproductive function. At the median testicular radiation dose (0.1 Gy), any reduction of sperm count is observed 1 year after treatment. In some cases, irradiation of testis at a high dose (20 Gy) leads to permanent azoospermia. Chemotherapy has the potential to induce germ cell mutation which can lead to

genetic disease in the next generation. For the moment, there is not clear evidence in the literature of a relationship between chemotherapy treatment in male survivors of cancer and genetic disease in children. As the risk of sperm aneuploidy decreases over the time, contraception is recommended for 1 year after the end of the treatment.

Other drugs can impact male spermatogenesis. If the treatment cannot be stop or if the effect is potentially irreversible, the cryopreservation of sperm patient must be proposed. It is the case for some immunosuppressant as Azathioprine and Mycophenolate mofetil. These compounds are mutagenic and teratogen when taken by pregnant women but no significant increase of preterm birth and malformation have been observed in children born of treated-father. Some drugs affect sperm parameters in a reversible manner. It is the case for hormonal treatment using anabolic steroids or testosterone, for antiandrogenic drugs as cyproterone acetate, for opiates, and for some nonsteroidal anti-inflammatory drug (NSAID) and salicylates. Tramadol, an analgesic, and some antidepressant therapy as sartans can also affect sexual function (Semet et al., 2017).

Phytosanitary products

Human is exposed every day to a large scale of chemical compounds affecting male fertility alone or in association. The population of greenhouse workers or farmer is importantly exposed to phytosanitary products. Some studies showed that male exposure to pesticides induces a decrease of fecundability and a prolonged time-to-pregnancy while others did not. It is very difficult to establish the causal link between pesticide exposure and endocrine disruptors found in environment and male congenital malformations. Despite this, some epidemiological studies correlated parental occupations in agriculture work and a higher prevalence of cryptorchidism, and a moderate association with hypospadias in son (Fernandez et al., 2007). Some studies associate an increase of the risk with mother exposure during gestation, other one with father exposure during preconception period. Preconceptional maternal and paternal presence of several persistent organic pollutants (POPs) in serum was associated with variation of birth weight offspring (Robledo et al., 2015). These compounds can be found in industrial chemicals or pesticides.

Another indicator of reproductive problem can be a modification of the sex ratio. Generally 105 males are born for 100 females. Men exposition to dioxin, a POP found among other in few pesticides, after explosion at a chemical plant in Seveso (Italy, 1976) or to the gonadotoxic nematocide DBCP (1,2-dibromo-3-chloropropane), induce a reduction of sex ratio compared with children born before paternal exposure.

During the last decades, several studies highlighted the relationship between paternal occupational exposure and the risk factors for some childhood cancers. There are now strong evidences between childhood leukemia, childhood nervous system tumor and paternal exposure to pesticides. With these case-report studies, it can be hypothesized than exposition to chemical product can cause alteration in sperm DNA of father before conception and then affect cancer risk in their children. We cannot exclude here the possibility that father can bring some mutagen pollutants into home where embryo and/or fetus is exposed via the mother.

Plasticizers: The cases of Bisphenol A and phthalates

Plastics are widely used and present everywhere around us. This induces the production of a very high quantity of plasticizers as Bisphenol A (BPA) and Phthalates found in a variety of commercial products as plastics bottles and epoxy resins for BPA and food packaging and cosmetics for phthalates. BPA and phthalates are known to be endocrine disrupting chemicals. Some epidemiological studies have shown that exposure to phthalates or BPA during pregnancy are associated with a decrease of anogenital distance in newborn boys, an indicator of an antiandrogenic exposure. Recently, Smarr and collaborators designed a population-based prospective cohort in United States to explore the effects of environmental factors on critical windows of reproduction and development. They examined the association between parental urinary presence of BPA and 14 phthalates metabolites and birth size in a cohort of 233 to 237 children from 501 enrolled couples. Authors suggest that "preconception maternal and paternal urinary concentration of BPA and specific phthalates metabolites may be associated with smaller birth size and increased gestational age", but this correlation is parent and chemical specific and that paternal preconception exposure to BPA and phthalates were associated with female excess, that mean a decrease of the secondary sex ratio (Smarr et al., 2015). Contrarily, maternal preconception exposure to these compounds were significantly associated with a male excess. In the same cohort the authors shown that father preconception urinary concentrations of phthalates metabolites were associated with an increase of the time-to-pregnancy (Buck Louis et al., 2014).

In couples undergoing an assisted technology treatment (ART), the presence of phthalates metabolites in the urine of father can be associated with a decrease of blastocyst quality (Wu et al., 2017) implantation odds and live birth (Dodge et al., 2015). Urinary concentrations in mother of a known metabolite of phthalates, the di(2-ethylhexyl)phthalate (DEHP), were associated with a reduction of the probability of life birth and of oocyte yield (Hauser et al., 2016) in women undergoing ART or with pregnancy loss in women without known problem of reproduction (Toft et al., 2012).

Preconceptional and gestational exposition to plasticizers can affects reproduction and birth outcome but can also have long term effects which are easier to highlight in animal models. Studies performed in rodent demonstrate that preconception exposure to BPA in adult male rats can transmit spatial memory deficits to male and female offspring (Fan et al., 2013) and that gestational exposure to BPA alters social behavior in juvenile female's mice (Wolstenholme et al., 2011). In this last one, authors shown that BPA induced change in the expression of two methyltransferases genes (Dnmt3a and Dnmt1) coding for enzyme involved for DNA methylation. We can suppose here than change in DNA sequence methylation status can be responsible of the behavior effect of BPA.

Sociocultural Agents

We saw above that physical and chemical agents have a preconceptional impact. We will see here that reproductive function can also be affected by sociocultural agents as stress, age, the consumption of tobacco and alcohol and the nutrition.

Stress

A rich literature focuses on the effect on maternal stress on offspring development but very little things are known about the preconception paternal stress effect. Harker et al. demonstrate in rat that preconception paternal stress can have an effect on the neurodevelopment and behavior of male and female offspring (Harker et al., 2015). This change in behavior can be linked to change in methylation in prefrontal cortex (Mychasiuk et al., 2013). In mice, maternal separation of the offspring during postnatal development induces altered anxiety and depressive-like behaviors that persist across generation. In these maternally-separated males, there is a modification of the status of DNA methylation in some genes in sperm (Franklin et al., 2010).

Natural disaster or war can constitute a stress for population. For example, some authors reported a reduction in sperm motility just after the Kobe earthquake and then, a decline in the secondary sex ratio (Fukuda et al., 1996, 1998). A decrease of sex ratio also been observed after September 11 attack in New York, economic downturns and war which can be due to an alteration in semen quality or spontaneous abortion. A population-based prospective cohort study confirmed these data and associates the presence of preconception salivary cortisol, a physiological indicator of stress, with a decrease of odds of male birth (Chason et al., 2012).

Age

Over the last decade, the average of paternal age significantly increases due to an increase of life expectancy, the use of contraception methods or change in various socioeconomic factors. Studies have demonstrated that the increase of paternal age affects testicular function, reproductive hormone production and sperm parameters (for review, Sharma et al., 2015). The increase of paternal age can also be associated with sperm DNA fragmentation (Johnson et al., 2015) affecting the success rate of ART, the level of miscarriages, as well as the early embryonic development (for review, Sharma et al., 2015). However it has also been reported that telomere length in sperm increases with paternal age and that there is a positive correlation between paternal age and sperm telomere length in offspring. For now, the role of telomere length in spermatogenesis and fertility is unclear but length of telomere is significantly shorter in oligozoospermic men compared to normozoospermic one (Ferlin et al., 2013). Increase of paternal age can also be responsible for the increase of error in DNA sequence of germline leading to the accumulation of de novo mutations in spermatozoa. A study performed on the entire genome of Icelandic parent-offspring estimate an increase of two mutations per year of father's age (Kong et al., 2012). The increase in de novo mutation rate is the major source of paternal age affect disorders. In fact, advanced paternal age has been associated to neuro-cognitive disorders as schizophrenia and autism, autosomal dominant disorders as retinoblastoma, congenital abnormalities as cleft lips and so on in offspring (for review, Sharma et al., 2015). The epigenetics pattern of sperm is also modified with age as the global level of 5-methyl-cytosine increase by 1.76% per year (Jenkins et al., 2013). Age can also induce chromosomal aneuploidy. Only 1% of aneuploidy pregnancy leads to live birth with high number of congenital malformation and/or mental retardation.

Tobacco

A meta-analysis of 20 studies with 5865 males participants over 13 years-old conducted by Sharma and colleagues concluded that cigarette smoking has a negative effect on sperm parameters (Sharma et al., 2016). It has been shown that fathers smoking affect the time-to-pregnancy and then couples fertility. This risk increase with the number of cigarette smoked per day and the smoking duration. Paternal smoking can also affect health of the offspring through damage to the genome and epigenome of sperm. For example, studies performed in China, Colombia or US highlight that the risk of childhood acute leukemia significantly increases with paternal preconception smoking.

Alcohol

Alcohol is known to impair fertility and to be teratogen. Alcohol consumption by women during pregnancy lead to abnormal fetal development resulting in fetal alcohol spectrum disorders (FASDs). FASDs were first attributable to maternal alcohol consumption but some studies suggest that preconception paternal alcohol consumption may contribute to FASDs development. Some epidemiological data associated preconceptional paternal alcohol consumption and a lowered birth weight, hyperactivity and lowered cognitive abilities in offspring. Alcohol has been shown to lower the level of DNA methyltransferase transcription in some organs such as testis. It has been shown that chronic alcohol consumption can be correlated with demethylation of paternally imprinted loci in sperm DNA. This effect could result in epigenetic modification on sperm DNA and an alteration of gene expression required for normal embryonic development leading to offspring with FASDs symptoms (Ouko et al., 2009).

Nutrition

In human, the impact of maternal nutrition on offspring development and risk disease was fully described in the literature. More recently, research has shown that paternal diet can affect future generations. In men, an increase of the body mass index is associated to a decrease of sperm mobility, an increase of sperm abnormality and sperm DNA fragmentation and a reduction of pregnancy rates. Paternal obesity is also associated with a decrease of blastocyst development and live birth rate following ART. It has been shown that low amount of dietary recourses during preadolescent period of fathers or grandfathers was correlated with a decrease

of cardiovascular or diabetes mortality of children. As this phenotype appears very quickly, it could not be the consequences of DNA mutation but probably due to modification to epigenetics status of some genes. Heijmans and collaborators shown than men and women prenatally exposed to famine during the Dutch Hunger during the winter 1944–45 of the II world war have, 6 decades later, a decrease of the level of DNA methylation of the imprinted insulin-like growth factor II (IGF2 gene) (Heijmans et al., 2008). This gene is a key factor in human growth and development and his hypomethylated status can be associated with increased likelihood of obesity (Soubry et al., 2013). The effect of father diet on the next generation was verified in rodent models. In rat, an high-fat diet in adulthood induce appearance of impaired pancreatic function in female offspring which is correlated with an alteration of DNA methylation of genes involved in insulin or glucose metabolism or expressed in pancreatic tissue. In male mice, a low-protein diet affects the status of DNA methylation of genes or the expression of some microRNAs in hepatic tissue.

Some studies have shown that preconceptional mother diet affects sex ratio. In Human, male fetus appears to need a higher caloric demand. For example, malnourished African woman produced more daughters than sons. In Ethiopia, a positive correlation between the percentage of male birth and a better nutritional state was observed. A study performed on a cohort of 740 nulliparous British women who were unaware of their fetus's gender, demonstrated than the sex of the fetus is associated with diet of the mother at conception. Fifty six per cent of women with the highest energy intake give boys, against 45% with a lowest energy diet (Mathews et al., 2008). These data support the hypothesis that mother had a better chance to give male if the nutriment intake was higher prior conception.

The preconceptional diet of the father affects, as for the mother, the disease risk in descendants. This paternal transmission can be inherited via the modification of epigenetic status of genes involved in development or in the regulation of physiological process.

Overview of Epigenetic Mechanisms Possibly Involved in Paternal Inheritance

Epigenetics changes in sperm have been proposed as one of the possible mechanism through which paternal gamete can impact offspring. Epigenetic changes affect gene expression without modification of DNA sequence. This epigenetic inheritance is mediated by epigenetic modifications as DNA methylation, posttranslational modifications of histone or small noncoding RNAs in male germline and is transmitted to the next generations where metabolism, behavior, neurodevelopment or reproduction capacity can be affected (Fig. 2).

DNA Methylation

DNA methylation corresponds to an addition of a methyl group (CH_3) on DNA sequence on cytosine on cytosine/guanine (C/G) dinucleotides. The most widely characterized type of methylation is the addition of a methyl group on the carbon number 5 of the cytosine giving rise to 5-methylcytosine (5-mC). DNA methyltransferases (DNMTs) are enzymes required for the establishment but also the maintenances of DNA methylation patterns. Other enzymes are required to remove methylation group as enzymes of TETs family. This process is essential for normal development, cell differentiation and involved in key process as parental imprinting and X-chromosome inactivation. As CpG islands are present in promoter regions, DNA methylation acts in this case as repressor of transcription. DNA methylation patterns are largely erased, soon after fertilization, to remove paternal mark and then reestablished in Human when fetal gonocyte give rise to prespermatogonia. This remethylation process continues in male germ cell until spermatocyte stage just before the beginning of meiosis.

An alteration of DNA methylation can be an explanation of the preconceptional impact of environmental factors on offspring. It has been shown in literature than some compounds as BPA and phthalates can affect DNA methylation. As mention before, other factors as stress, nutrition or alcohol consumption can affect this epigenetic process and then lead to an alteration of gene expression involved in development and disease.

Histone Modifications

Histones are proteins linked to DNA which participate to special organization of chromatin. Posttranslational modifications of histones by acetylation, methylation, ubiquitination or phosphorylation induce change of chromatin organization affecting inactivation/activation of gene transcription.

In sperm, histones are exchange by protamines in spermatocyte to allow a better compaction of DNA. In Human, approximately 15% of histones are retained in chromatin of spermatozoa predominantly in some regions of genome important for early development of embryo. Anomalies in histone–protamine transition or histone retention have been detected in patient with problem of fertility.

It can be possible that some environmental factors can altered the level of histone in sperm or modify histone marks and in this way affect the next generation. In China, a case-control study performed on semen samples from 147 heavy smokers and 175 nonsmokers receiving infertility treatment highlight that smoking and abnormal spermatogenesis are associated with anomalies in histone-to-protamine transition (Yu et al., 2014). Atrazine, a widely-used pesticide in United States banned in Europe in 2003, have been shown to interfere with the level of histone methylation and to induce a transgenerational decrease of protamine level in mouse sperm (Hao et al., 2016). Other compound as diethylstilbestrol (DES), an antiabortive medication used by woman

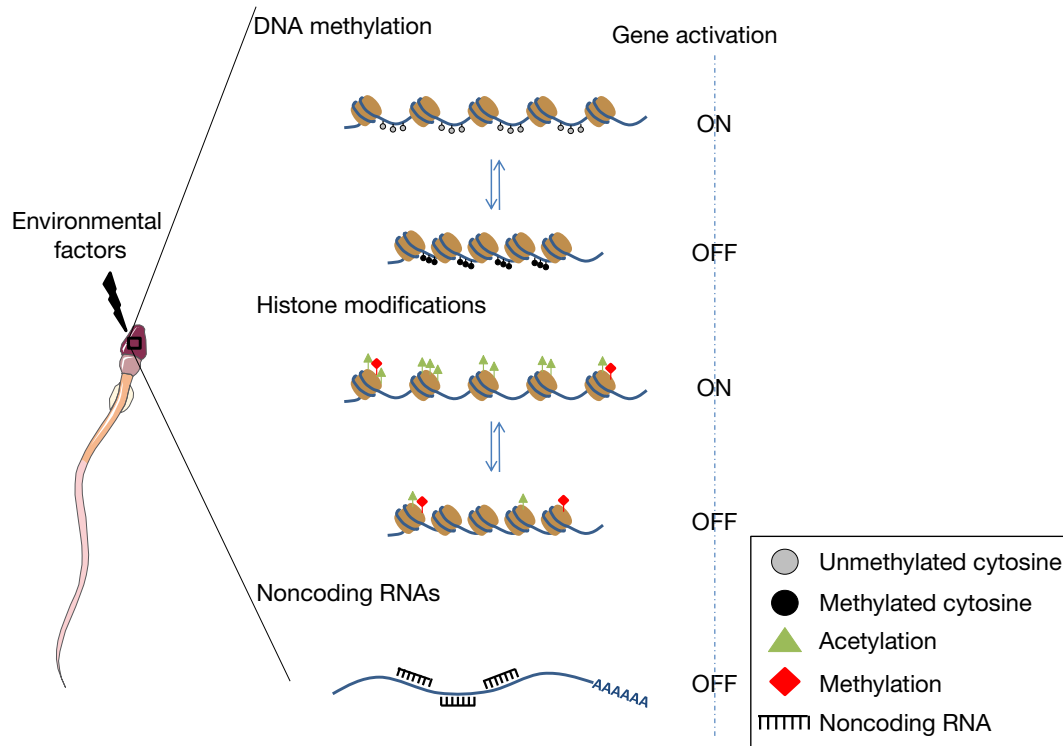


Fig. 2 Overview of epigenetic modification possibly involved in transmission of paternal preconceptional information. The modification of spermatozoa DNA content by epigenetic process induced by some factors can activate (ON) or silence (OFF) genes involved in development and disease. These include (1) the DNA methylation: the methylation of DNA leads to repression of gene transcription (2) the modification of histone where acetyl or methyl groups are added. Histone acetylation leads to opening of DNA molecule and gene activation. The effect of methylation on gene activation/repression is more complex and depends of the localization of methylation site and (3) the noncoding RNAs can bind messenger RNAs (mRNA) and induce their degradation.

until 1971, interferes with histone methylation. DES was associated to an increased risk of hypospadias, a congenital anomaly, in males exposed during development which can persist until the third generation (for review, [Bouty et al., 2015](#)).

Noncoding RNAs

Noncoding RNAs (ncRNAs) are functional RNA molecules coming from DNA molecules but not translated into proteins. ncRNAs are divided into two groups, the long ncRNAs (≥ 200 nucleotides) and the small ncRNAs (≤ 30 nucleotides). The small ncRNAs are composed of three major classes: the microRNAs (miRNAs), the short interfering RNAs (siRNAs) and the piwi-interacting RNAs (piRNAs). ncRNAs can act transcriptionally and posttranscriptionally on the control of gene expression.

Experiments performed in knockout mouse have revealed that small RNAs are key actors of spermatogenesis. In Human, down-regulation of miRNAs was observed in spermatozoa of subfertility patients compared to control men ([Abu-Halima et al., 2014](#)) and miRNAs clusters are over-expressed in malignant germ cell testis cancer (TGCC) ([Novotny et al., 2012](#)). The proof was done in literature that modification of the pattern of ncRNAs in sperm can be the mirror of paternal exposition to environmental factors and can modify behavior and offspring physiology.

A Swiss team published in 2014 a paper in *Nature Neuroscience* on the impact of sperm small ncRNAs transmission of early life traumatic sperm across generation ([Gapp et al., 2014](#)). They first demonstrated that early life exposure of mice to traumatic stress affects microRNAs expression in sperm and behavior and metabolic responses in offspring. More importantly, they injected sperm RNAs of traumatized animals into wild-type fertilized oocytes and showed a comparable effect than previously described in the next generation. These results showed a causal link between an alteration of sperm RNA in father due to an early life stress and an effect on behavior in offspring.

References

- Abu-Halima, M., Hammadeh, M., Backes, C., Fischer, U., Leidinger, P., Lubbad, A. M., Keller, A., & Meese, E. (2014). Panel of five microRNAs as potential biomarkers for the diagnosis and assessment of male infertility. *Fertility and Sterility*, 102, 989–997.e1.
- Bouty, A., Ayers, K. L., Pask, A., Heloury, Y., & Sinclair, A. H. (2015). The genetic and environmental factors underlying hypospadias. *Sexual Development*, 9, 239–259.

- Buck Louis, G. M., Sundaram, R., Sweeney, A. M., Schisterman, E. F., Maisog, J., & Kannan, K. (2014). Urinary Bisphenol A, phthalates, and couple fecundity: The longitudinal investigation of fertility and the environment (LIFE) study. *Fertility and Sterility*, 101, 1359–1366.
- Chason, R. J., McLain, A. C., Sundaram, R., Chen, Z., Segars, J. H., Pyper, C., & Louis, G. M. B. (2012). Preconception stress and the secondary sex ratio: A prospective cohort study. *Fertility and Sterility*, 98, 937–941.
- Dodge, L., Williams, P. L., Williams, M. A., Missmer, S. A., Souter, I., Calafat, A. M., Hauser, R., & EARTH Study Team. (2015). Associations between paternal urinary phthalate metabolite concentrations and reproductive outcomes among couples seeking fertility treatment. *Reproductive Toxicology*, 58, 184–193.
- Fan, Y., Ding, S., Ye, X., Manyande, A., He, D., Zhao, N., Yang, H., Jin, X., Liu, J., Tian, C., et al. (2013). Does preconception paternal exposure to a physiologically relevant level of Bisphenol A alter spatial memory in an adult rat? *Hormones and Behavior*, 64, 598–604.
- Ferlin, A., Rampazzo, E., Rocca, M. S., Keppel, S., Frigo, A. C., De Rossi, A., & Foresta, C. (2013). In young men sperm telomere length is related to sperm number and parental age. *Human Reproduction*, 28, 3370–3376.
- Fernandez, M. F., Olmos, B., Granada, A., Lopez-Espinosa, M. J., Molina-Molina, J. M., Fernandez, J. M., Cruz, M., Olea-Serrano, F., & Olea, N. (2007). Human exposure to endocrine-disrupting chemicals and prenatal risk factors for cryptorchidism and hypospadias: A nested case-control study. *Environmental Health Perspectives*, 115(Suppl. 1), 8–14.
- Franklin, T. B., Russig, H., Weiss, I. C., Graff, J., Linder, N., Michalon, A., Vizi, S., & Mansuy, I. M. (2010). Epigenetic transmission of the impact of early stress across generations. *Biological Psychiatry*, 68, 408–415.
- Fukuda, M., Fukuda, K., Shimizu, T., & Moller, H. (1998). Decline in sex ratio at birth after Kobe earthquake. *Human Reproduction*, 13, 2321–2322.
- Fukuda, M., Fukuda, K., Shimizu, T., Yomura, W., & Shimizu, S. (1996). Kobe earthquake and reduced sperm motility. *Human Reproduction*, 11, 1244–1246.
- Gapp, K., Jawaid, A., Sarkies, P., Bohacek, J., Pelczar, P., Prados, J., Farinelli, L., Miska, E., & Mansuy, I. M. (2014). Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nature Neuroscience*, 17, 667–669.
- Gardner, M. J., Snee, M. P., Hall, A. J., Powell, C. A., Downes, S., & Terrell, J. D. (1990). Results of case-control study of leukaemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. *BMJ*, 300, 423–429.
- Garolla, A., Torino, M., Sartini, B., Cosci, I., Patassini, C., Carraro, U., & Foresta, C. (2013). Seminal and molecular evidence that sauna exposure affects human spermatogenesis. *Human Reproduction*, 28, 877–885.
- Hao, C., Gely-Pernot, A., Kervarrec, E., Boudjema, M., Becker, E., Khil, P., Tevosian, S., Jegou, B., & Smagulova, F. (2016). Exposure to the widely used herbicide atrazine results in deregulation of global tissue-specific RNA transcription in the third generation and is associated with a global decrease of histone trimethylation in mice. *Nucleic Acids Research*, 44, 9784–9802.
- Harker, A., Raza, S., Williamson, K., Kolb, B., & Gibb, R. (2015). Preconception paternal stress in rats alters dendritic morphology and connectivity in the brain of developing male and female offspring. *Neuroscience*, 303, 200–210.
- Hauser, R., Gaskins, A. J., Souter, I., Smith, K. W., Dodge, L., Ehrlich, S., Meeker, J. D., Calafat, A. M., Williams, P. L., & EARTH Study Team. (2016). Urinary phthalate metabolite concentrations and reproductive outcomes among women undergoing in vitro fertilization: Results from the EARTH Study. *Environmental Health Perspectives*, 124, 831–839.
- Heijmans, B. T., Tobi, E. W., Stein, A. D., Putter, H., Blauw, G. J., Susser, E. S., Slagboom, P. E., & Lumey, L. H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 17046–17049.
- Jenkins, T. G., Aston, K. I., Cairns, B. R., & Carrell, D. T. (2013). Paternal aging and associated intraindividual alterations of global sperm 5-methylcytosine and 5-hydroxymethylcytosine levels. *Fertility and Sterility*, 100, 945–951.e2.
- Johnson, S. L., Dunleavy, J., Gemmell, N. J., & Nakagawa, S. (2015). Consistent age-dependent declines in human semen quality: A systematic review and meta-analysis. *Ageing Research Reviews*, 19, 22–33.
- Kong, A., Frigge, M. L., Masson, G., Besenbacher, S., Sulem, P., Magnusson, G., Gudjonsson, S. A., Sigurdsson, A., Jonasdottir, A., Jonasdottir, A., et al. (2012). Rate of de novo mutations and the importance of father's age to disease risk. *Nature*, 488, 471–475.
- Lewis, R. C., Hauser, R., Maynard, A. D., Neitzel, R. L., Wang, L., Kavet, R., & Meeker, J. D. (2016). Exposure to power-frequency magnetic fields and the risk of infertility and adverse pregnancy outcomes: Update on the human evidence and recommendations for future study designs. *Journal of toxicology and environmental health Part B, Critical Reviews*, 19, 29–45.
- Mathews, F., Johnson, P. J., & Neil, A. (2008). You are what your mother eats: Evidence for maternal preconception diet influencing foetal sex in humans. *Proceedings of the Biological Sciences*, 275, 1661–1668.
- Mieusset, R., Bujan, L., Massat, G., Mansat, A., & Pontonnier, F. (1995). Clinical and biological characteristics of infertile men with a history of cryptorchidism. *Human Reproduction*, 10(3), 613–619.
- Mychasiuk, R., Harker, A., Illytsky, S., & Gibb, R. (2013). Paternal stress prior to conception alters DNA methylation and behaviour of developing rat offspring. *Neuroscience*, 241, 100–105.
- Novotny, G. W., Bellington, K. C., Bramsen, J. B., Nielsen, J. E., Bork-Jensen, J., Almstrup, K., Sonne, S. B., Kjems, J., Rajpert-De Meyts, E., & Leffers, H. (2012). MicroRNA expression profiling of carcinoma in situ cells of the testis. *Endocrine-Related Cancer*, 19, 365–379.
- Ouko, L. A., Shantikumar, K., Knezovich, J., Haycock, P., Schnugh, D. J., & Ramsay, M. (2009). Effect of alcohol consumption on CpG methylation in the differentially methylated regions of H19 and IG-DMR in male gametes: Implications for fetal alcohol spectrum disorders. *Alcoholism, Clinical and Experimental Research*, 33, 1615–1627.
- Regaud, C. (1977). The influence of the duration of irradiation on the changes produced in the testicle by radium. *International Journal of Radiation Oncology, Biology, Physics*, 2, 565–567.
- Robledo, C. A., Yeung, E., Mendola, P., Sundaram, R., Maisog, J., Sweeney, A. M., Barr, D. B., & Louis, G. M. (2015). Preconception maternal and paternal exposure to persistent organic pollutants and birth size: The LIFE study. *Environmental Health Perspectives*, 123, 88–94.
- Semet, M., Paci, M., Saias-Magnan, J., Metzler-Guillemain, C., Boissier, R., Lejeune, H., & Perrin, J. (2017). The impact of drugs on male fertility: A review. *Andrology*, 5, 640–663.
- Sharma, R., Agarwal, A., Rohra, V. K., Assidi, M., Abu-Elmagd, M., & Turki, R. F. (2015). Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reproductive Biology and Endocrinology*, 13, 35.
- Sharma, R., Harlev, A., Agarwal, A., & Esteves, S. C. (2016). Cigarette smoking and semen quality: A new meta-analysis examining the effect of the 2010 World Health Organization Laboratory methods for the examination of human semen. *European Urology*, 70, 635–645.
- Smarr, M. M., Grantz, K. L., Sundaram, R., Maisog, J. M., Kannan, K., & Louis, G. M. B. (2015). Parental urinary biomarkers of preconception exposure to Bisphenol A and phthalates in relation to birth outcomes. *Environmental Health*, 14, 73.
- Soubry, A., Schildkraut, J. M., Murtha, A., Wang, F., Huang, Z., Bernal, A., Kurtzberg, J., Jirtle, R. L., Murphy, S. K., & Hoyo, C. (2013). Paternal obesity is associated with IGF2 hypomethylation in newborns: Results from a newborn epigenetics study (NEST) cohort. *BMC Medicine*, 11, 29.
- Toft, G., Jonsson, B. A., Lindh, C. H., Jensen, T. K., Hjelund, N. H., Vested, A., & Bonde, J. P. (2012). Association between pregnancy loss and urinary phthalate levels around the time of conception. *Environmental Health Perspectives*, 120, 458–463.
- Wolstenholme, J. T., Taylor, J. A., Shetty, S. R. J., Edwards, M., Connelly, J. J., & Rissman, E. F. (2011). Gestational exposure to low dose Bisphenol A alters social behavior in juvenile mice. *PLoS ONE*, 6, e25448.
- Wu, H., Ashcraft, L., Whitcomb, B. W., Rahil, T., Tougas, E., Sites, C. K., & Pilsner, J. R. (2017). Parental contributions to early embryo development: Influences of urinary phthalate and phthalate alternatives among couples undergoing IVF treatment. *Human Reproduction*, 32, 65–75.
- Yu, B., Qi, Y., Liu, D., Gao, X., Chen, H., Bai, C., & Huang, Z. (2014). Cigarette smoking is associated with abnormal histone-to-protamine transition in human sperm. *Fertility and Sterility*, 101, 51–57.e1.

Pharmaceutical Drugs

David M Kristensen, Danish Headache Center, Glostrup, Denmark

© 2018 Elsevier Inc. All rights reserved.

Introduction

During the last century, pharmaceutical drugs have become ever more intertwined in everyday life. At the same time, infertility affects approximately 10%–15% of the population in developed countries, with about half of the cases recognized as being caused by male infertility. Genetic differences, fetal exposures, recreational drug or alcohol abuse, dietary issues, environmental or occupational exposure to reprotoxicological agents, and pharmaceutical drugs are all among known causes that may result in decreased fertility (Skakkebaek et al., 2016). Due to these multiple factors underlying male infertility, it is hard to estimate the number of men whose fertility is clinically obstructed by drugs. It remain, however, that drug use is high. During the period from 2009 to 2012, approximately half of the U.S. population (48.7%) had taken a prescription drug in the last 30 days, with more than 20% of the population (21.8%) having taken three or more prescription drugs during the last month (Center for Disease Control and Prevention). At the same time, an increasing number of patients are placed on long-term medication regimens at a young age (including potential mothers) due to for example, chronic migraine, epilepsy or clinical depression, resulting in a substantial number of males being at risk of experiencing a decrease in fertility due to side effects of drug treatment. The last 50 years have shown that this risk is not negligible, as drugs have been shown to disrupt male reproduction through both prenatal and adult exposures by both hormonal and non-hormonal mechanisms.

Fetal Exposure to Pharmaceutical Drugs and Reproduction

Thalidomide

The maternal use of thalidomide (alpha-phthalimido-glutarimide) and the resulting prenatal exposure resulted in a medical disaster that still resonate today more than a half a century later. The exposure included more than 10,000 children being born with a range of severe and debilitating malformations (Smithells and Newman, 1992). Thalidomide was developed and introduced in an era where formal testing for harmful effects was not undertaken. It was prescribed as a nonbarbiturate hypnotic sedative able to produce deep sleep without hangover or risk of dependency and with the important property that overdoses caused merely prolonged sleep. Importantly, it became very popular for its secondary anti-emetic effects among pregnant women suffering of morning sickness and it was widely available without prescription. In Germany, it became a top-selling sedative and an estimated 14.6 tons was sold in 1960. Among the many birth defects caused by thalidomide, the prenatal exposure resulted in large range of external and internal malformations including small or complete absent testes, mal-descended testes (cryptorchidism), and midline fusion defect of the male ventral urethra resulting in misplaced urethra opening (hypospadias; see Table 1). Evidence show that there is likely no safe time for taking the drug during pregnancy. However, the majority of damages to the forming infant, including the reproductive defects, was in a short time sensitive window extending no more the 16 days between day 20 and 36 after conception during first trimester. Reports indicate that as little as a single 50 mg tablet of thalidomide during this sensitive window is sufficient to cause birth defects in up to 50% of pregnancies. The mechanisms behind the malformations are still debated, and it is now likely that thalidomide may act in multiple tissues through different and likely tissue specific mechanisms. These mechanisms include anti-angiogenesis, induction of reactive agents, and by binding specific molecular targets, including tubulin and the androgen receptor (AR) (Smithells and Newman, 1992). The fact that thalidomide can bind as antagonist to AR and hence block pivotal androgen like testosterone during development may account for some of the milder malformations like cryptorchidism and hypospadias, while the complete absent of testis are more likely associated with the anti-angiogenesis effects. Hence, the defects observed after prenatal thalidomide exposure are likely results of both reduced or blocked hormonal actions and non-hormonal effects.

Table 1

Prenatal exposure	Timing window	Reproductive effects	Mechanisms
Thalidomide	Throughout gestation but increased sensitivity in the first trimester	Absent or malformed testes (infertility), cryptorchidism, hypospadias	Multiple mechanisms incl. anti-angiogenesis and blocking the androgen receptor
Diethylstilbestrol (DES)	First trimester	Cryptorchidism, hypospadias, transgenerational effects	Estrogenic
Mild analgesics	First and second trimester	Cryptorchidism, hypospadias, reduction in anogenital distance	Anti-androgenic

Diethylstilbestrol

A decade after the initial reports showing effects of thalidomide, prenatal exposure to a hormonal active compound, the synthetic, nonsteroidal estrogen diethylstilbestrol (DES), was shown to be associated with reproductive developmental disruption and subsequent congenital malformations (Swan, 2000; see Table 1). DES was prescribed for pregnant women from the late 1930s to the 1970s in the belief that it would prevent spontaneous abortions and premature births, resulting in >2 million pregnant women in the US using the compound between 1940s and 1960s. In the 1970s, studies published on prenatally exposed males showed increased risk of the congenital reproductive malformations cryptorchidism and hypospadias. In 2009, a study with >1000 exposed sons supported these earlier investigations, showing an increased risk of urogenital abnormalities with associations strongest for exposure occurring in early gestation. Hence, cryptorchidism was for example, significantly associated with prenatal DES exposure that began before the 11th week of gestation (Palmer et al., 2009). Moreover, DES may increase the risk of congenital malformations even in the second generation, as the sons of in prenatally exposed women have a higher prevalence of hypospadias than other males suggesting an trans-generational epigenetic effect by DES (Skakkebaek et al., 2016). Effects on sperm quality remain disputed, as some studies have reported lower sperm counts, decreased sperm motility, and abnormal sperm morphology in men exposed prenatally to DES, while other studies have reported no differences. As DES is associated with the occurrence of vaginal/cervical clear cell adenocarcinoma in DES exposed daughters, an increased risk of testicular cancer in DES sons has also for a long time been suspected without clear associations been made (Swan, 2000). Moreover, reports have suggested a possible link between behavioral and psychosexual effects of prenatal DES exposure. However, a study from 2003 of 2600 men taking part of the world's largest cohort of individuals with documented prenatal exposure to DES researchers concluded that not enough evidence was available to prove such associations. Although the effects on sperm quality, testicular cancer and sexual behavior remain to be completely elucidated, the prenatal exposure to DES, and the subsequent investigations conducted in order to understand the effects of an estrogenic compound on male development, contributed to laying the foundation for the development of the field of endocrine disruption. Through the estrogenic effects of DES it became clear that hormonal active compounds can disturb the delicate reproductive development. This realization in turn was instrumental for the development of the estrogen hypothesis in the early 1990s, proposing that prenatal exposure to multiple xenobiotic estrogens in for example, plastic and the diet from plants and milk could disrupt development, resulting in reproductive decline among men (Sharpe and Skakkebaek, 1993).

Mild Analgesics

In the recent decade, it has been suggested that xenobiotic estrogens could function in connection with anti-androgens during gestation in a cocktail effect (Skakkebaek et al., 2016). In this context, mild analgesics (hereafter called analgesics) have received increasing attention as they show anti-androgen properties (see Table 1). Comprising of NSAIDs and acetaminophen (paracetamol), analgesics are typically used to relieve pain, fever and malaise. NSAIDs are also used in connection with pre-term labor and in inflammatory diseases and chronic musculoskeletal pain and through the inhibition of auto- and paracrine active prostaglandins. The drugs have been on the market for many decades (>100 years for acetylsalicylic acid (aspirin), ~60 years for acetaminophen and ~50 years for ibuprofen) and they are now in most households in the Western world. Moreover, sales of analgesics have been steadily increasing over the past 20 years and are now the most commonly used over-the-counter drugs worldwide. Of concern is that analgesic use during pregnancy is generally high especially for acetaminophen as it is used for common symptoms without the need to consult a physician or pharmacist. This concern have increased after analgesics in two studies in 2010 suggested an association between prenatal analgesic exposure and congenital cryptorchidism (Kristensen et al., 2016). A purely epidemiological study, using hospital registry data for cryptorchidism, reported that exposure to acetaminophen for 4 weeks resulted in an increased risk (Jensen et al., 2010). The second study using a standardized assessment of cryptorchidism combined with experimental rodent approaches showed that use of analgesics during especially the second trimester increased the risk of cryptorchidism through anti-androgenic mechanisms. The study also showed that this risk remained significant when ibuprofen and acetylsalicylic acid were examined separately, and a similar tendency was seen for acetaminophen. The risk was increased if the exposure was >2 weeks with similar results obtained in separate analyses for acetaminophen and acetylsalicylic acid. The risk was highest if the exposure included more than one compound simultaneously for >2 weeks, suggesting an additive cocktail effect. The study also included a cohort from Finland in which the associations were not significant (Kristensen et al., 2011). However, subsequent studies have found similar associations to both cryptorchidism and hypospadias together with reductions in anogenital distance among infants, all supporting the notion that analgesics are anti-androgenic compounds. As the same time, it has become clear that from urinary analysis for acetaminophen that people in Western world are ubiquitously exposed to this drug (Kristensen et al., 2016). In addition to intentional/therapeutic use of acetaminophen, the ubiquitous detection of urinary concentrations of acetaminophen indicates the existence of possible unintentional (background) sources of exposure. The unintentional exposure may occur by direct ingestion of acetaminophen residues in food and water or through exposure to aniline, an important source material in the chemical industry that is converted in vivo to the analgesic. Data show that among different groups investigated for acetaminophen in the urine, pregnant women are those with the highest proportion of individuals with recent intentional use of acetaminophen. This may perhaps reflect the fact that acetaminophen is often recommended by doctors during all stages of pregnancy and, therefore, be perceived by the pregnant woman as a medication without risks (Kristensen et al., 2016). Research on the mechanism of analgesics is still too premature to

enable a precise explanation of their anti-androgenic action. There is for example, no clear association between analgesic suppression of testosterone and the analgesic-induced blocking of prostaglandin synthesis. This may suggest that alternative mechanisms could be involved and experiments using animal models and xenograft models of human testicular development have found that analgesics can have the ability to inhibit the expression of genes encoding steroidogenic enzymes and thereby inhibit androgen synthesis (Kristensen et al., 2016). It is obvious that the effects of prenatal analgesics exposure are much less severe than both thalidomide and DES. It is therefore likely that analgesics, unlike thalidomide and DES, will continue to play a vital role in the clinic for pregnant women in many years to come. It is, however, also clear that thalidomide, DES and analgesics all share the fact that they are drugs developed during the 18th and the first half of the 19th century prior to the thorough testing seen today.

Adult Exposure to Pharmaceutical Drugs and Reproduction

Multiple pharmaceutical drugs have been shown to affect fertility including exogenous hormones, antineoplastic drugs, psychotropic medications, antihypertensive medications and anti-infection medications (Samplaski and Nangia, 2015). These diverse drug effects can be classed in three groups based on their mechanisms of action: (i) disruption of the hypothalamic–pituitary–gonadal (HPG) hormonal axis; (ii) impairment of sperm production and function; (iii) or changes in sexual function including erections, ejaculation, and libido (Samplaski and Nangia, 2015). Among these groups, especially the investigation of impairment of sperm production and function is difficult due to the high variability in spermatogenesis. Healthy men not taking any medications exhibit daily, monthly and seasonal variations in sperm counts and motility and at the same time all the sperm parameters are highly dependent on the duration of abstinence prior to ejaculation. Moreover, the effects on fertility by many drugs is not well investigated or conflicting due to the above-mentioned limitations and the fact that the men are using the drugs for a reason. Hence, in human epidemiological studies focusing on side effects on reproduction, it is difficult to control for whether the underlying disease for which the drug is used also is changing for example the hormonal balance or spermatogenesis. The following will therefore summarize and highlight examples that are particularly adverse documenting the three above-mentioned groups.

Changes of the Hypothalamic–Pituitary–Gonadal Hormonal Axis

Exogenous male hormones, hormone substitutes and hormone antagonists all have potential negative impact on male fertility. Gonadotropin-releasing hormone, follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone in the HPG axis are essential for the successful completion of spermatogenesis (see Fig. 1). All drugs containing male hormones or their derivatives have therefore potential to impair sperm proliferation and/or maturation, suppress spermatogenesis, and inhibit the testicular function through interfering with the balance of these essential hormones. A classic case of such disruption is exogenous testosterone used by a high number of men in their reproductive years to obtain a particular body figure or in the faulty belief that it enhances fertility (Samplaski and Nangia, 2015). Exogenous testosterone impairs spermatogenesis through suppression of the HPG axis through decreasing levels of FSH, LH and the endogenous levels of testosterone. In most cases, the spermatogenic arrest induced by exogenous testosterone is resolved by 4–12 months after cessation. Other common drugs with known effects on the HPG axis are opioids, shown to disrupt gonadotropin secretion from the hypothalamus, and mild analgesics that can decrease testosterone secretion from Leydig cells and hence reduce sensitivity to LH (De Maddalena et al., 2012; Albert et al., 2013; Kristensen et al., 2016).

Impairment of Sperm Production and Function

As mentioned above, exogenous hormones can induce spermatogenesis arrest through obstruction of the reproductive hormones under control of the HPG axis, as these hormones control the continuous cell proliferation and differentiation associated with spermatogenesis. However, drugs can also affect directly the spermatogenesis. Such effects have been reported for inhibitors of cyclic nucleotide phosphodiesterases (PDEs) that selectively catalyze hydrolysis of cAMP and/or cGMP. These inhibitors are used to promote an increase in cAMP and cGMP levels to increase endovascular smooth muscle relaxation for example the treatment of erectile dysfunction. Spermatogenesis is regulated through adenylate cyclase and other enzymes involved in the creation of cAMP, which play a vital role during early meiosis. It is therefore likely that PDEs interfere with sperm production by disrupting the balance of cAMP during spermatogenesis (Samplaski and Nangia, 2015). Antineoplastic drugs used in cancer treatment are another group of compounds with effects on male fertility. These drugs have the potential to damage both germ cells and the supporting Sertoli cells, resulting in severe oligozoospermia or azoospermia. The rapidly proliferating and differentiating spermatogonia are particularly sensitive to the actions of these compounds that are used in the clinic to target proliferating cancer cells. For example, busulfan, used for leukemia, is considered a direct sterilizing agent, as it kills the spermatogonial stem cells (Howell and Shalet, 2001). Other antineoplastic drugs do not cause azoospermia, or cause only temporary and/or modest reductions in the sperm counts because they kill only the differentiating spermatogonia. Another group of drugs known to affect fertility through effects on sperm are the anti-depressive drugs selective 5-hydroxytryptamine/serotonin re-uptake inhibitors (SSRIs). These drugs have been found to have a direct effect on sperm function due to oxidative phosphorylation of mitochondria or an effect on the sperm cell membrane.

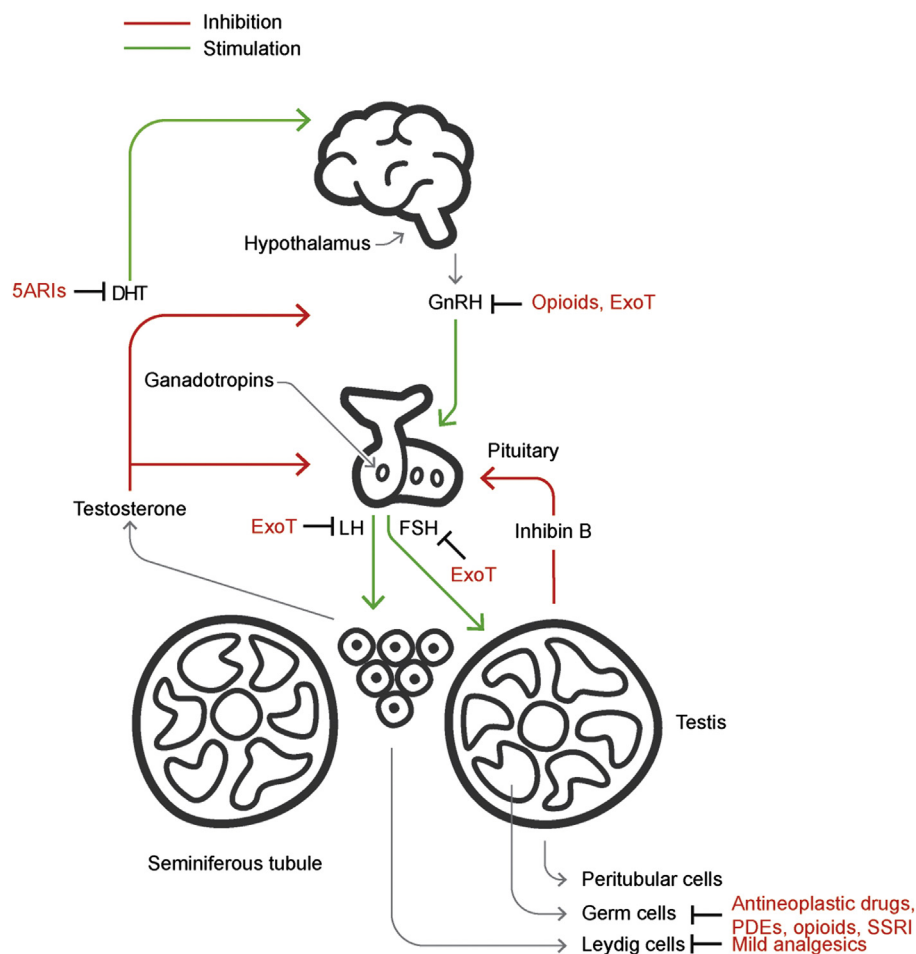


Fig. 1 A major mechanism of pharmaceutical drugs resulting endocrine disruption in the adult male is the hypothalamic–pituitary–gonadal (HPG) axis that control sex hormones and spermatogenesis. The schematic representation show how drugs can interfere with this hormonal axis and directly spermatogenesis. *GnRH*, Gonadotropin-releasing hormone; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *ExoT*, exogenous testosterone; *DHT*, dihydrotestosterone; *PDEs*, phosphodiesterases; *SSRIs*, selective 5-hydroxytryptamine/serotonin re-uptake inhibitors; *5ARIs*, 5 α -reductase inhibitors.

A controlled trial have showed that SSRI use decreased motility and increased DNA damage in sperm cells as a function of duration of use. Hence, a study with 35 men taking SSRI for 5 weeks have shown that while using the SSRI mean sperm DNA fragmentation was higher (Tanrikut et al., 2010). Other compounds used in psychiatric care are opioids and these compounds have been shown to have similar effects as SSRIs including increased sperm DNA fragmentation.

Sexual Function Including Erections, Ejaculation, and Libido

Sexual function is obviously important for male fertility and important for these actions are testosterone and its derivatives. Of particular interest in this regard is the 5 α -reductase inhibitors (5ARIs) that block the conversion of testosterone to dihydrotestosterone (DHT). This conversion happens in certain tissues including the prostate gland, seminal vesicles, epididymis, skin, hair follicles, liver, and brain. Central for understanding the effects of DHT is that it is a more potent as an agonist of the androgen receptor (AR) than testosterone. 5ARIs are used for the treatment of benign prostate hyperplasia and male baldness through inhibition of two primary isozymes of 5 α -reductase, the enzyme converting testosterone to DHT (Samplaski and Nangia, 2015). The drug finasteride inhibits type II 5 α -reductase, while dutasteride inhibits both type I and type II 5 α -reductase. While conflicting data exist for the effects on spermatogenesis, effects on men after using 5ARIs and increased risk of sexual dysfunction are robust (see Fig. 1). This include erectile dysfunction, diminished sexual desire and ejaculatory dysfunction (Samplaski and Nangia, 2015). The sexual adverse effects of 5ARIs are thought to be caused by alterations in steroid levels in the cerebrospinal fluid. Hence, the fluid have been shown to be altered in men with persistent sexual adverse effects following finasteride use. In some men, the sexual dysfunction continues after cessation of 5ARI use and one study found persistent sexual dysfunction among 50% of affected patients following discontinuation of finasteride use (Wessells et al., 2003). Another class of common drugs with

known effects on libido, erectile dysfunction and ejaculatory dysfunction are psychotropic medications. Tricyclic antidepressants and selective SSRIs have shown to have these effects together with monoamine oxidase inhibitors and medications for bipolar conditions, such as lithium. These drugs have multiple action on the reproductive apparatus likely through anticholinergic, α -blocker and/or dopaminergic properties. Hence, where the 5ARI drugs interfere with HPG axis, the psychotropic drugs are believed to act primarily through changes of neurotransmitters in the CNS in such a way that the SSRIs are now used off-label by urologists for the treatment of premature ejaculation (Samplaski and Nangia, 2015).

Conclusions

As use of drugs become more and more common, it is likely that effects on male reproduction will become more pronounced. Although it is unlikely that the disastrous experiences after maternal use of thalidomide and DES will be repeated, recent years work on analgesics suggest that prenatal exposure to all drugs should be considered with concern. Obviously, human physiology is much more robust in adult life compared to the prenatal era. Nonetheless, the effects of drugs on the adult can be nothing but subtle as seen with young men using exogenous testosterone. The years to come will therefore undoubtedly show more cases of drugs obstructing male fertility.

References

- Albert, O., Desdoits-Lethimonier, C., Lesné, L., et al. (2013). Paracetamol, aspirin and indomethacin display endocrine disrupting properties in the adult human testis in vitro. *Human Reproduction*, 28, 1890–1898.
- De Maddalena, C., Bellini, M., Berra, M., et al. (2012). Opioid-induced hypogonadism: Why and how to treat it. *Pain Physician*, 15, 111–118.
- Howell, S. J., & Shalet, S. M. (2001). Testicular function following chemotherapy. *Human Reproduction Update*, 7, 363–369.
- Jensen, M. S., Rebordosa, C., Thulstrup, A. M., et al. (2010). Maternal use of acetaminophen, ibuprofen, and acetylsalicylic acid during pregnancy and risk of cryptorchidism. *Epidemiology*, 21, 779–785.
- Kristensen, D. M., Hass, U., Lesne, L., Lottrup, G., et al. (2011). Intrauterine exposure to mild analgesics is a risk factor for development of male reproductive disorders in human and rat. *Human Reproduction*, 26, 235–244.
- Kristensen, D. M., Mazaud-Guittot, S., Gaudriault, P., et al. (2016). Analgesic use-prevalence, biomonitoring and endocrine and reproductive effects. *Nature Reviews. Endocrinology*, 12, 381–393.
- Palmer, J. R., Herbst, A. L., Noller, K. L., et al. (2009). Urogenital abnormalities in men exposed to diethylstilbestrol in utero: A cohort study. *Environmental Health*, 8, 8–37.
- Samplaski, M. K., & Nangia, A. K. (2015). Adverse effects of common medications on male fertility. *Nature Reviews. Urology*, 12, 401–413.
- Sharpe, R. M., & Skakkebaek, N. E. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet*, 29, 1392–1395.
- Skakkebaek, N. E., Rajpert-De Meyts, E., Buck Louis, G. M., et al. (2016). Male reproductive disorders and fertility trends: Influences of environment and genetic susceptibility. *Physiological Reviews*, 96(1), 55–97.
- Smithells, R. W., & Newman, C. G. (1992). Recognition of thalidomide defects. *Journal of Medical Genetics*, 29, 716–723.
- Swan, S. H. (2000). Intrauterine exposure to diethylstilbestrol: Long-term effects in humans. *APMIS*, 108, 793–804.
- Tanrikut, C., Feldman, A. S., Altemus, M., et al. (2010). Adverse effect of paroxetine on sperm. *Fertility and Sterility*, 94, 1021–1026.
- Wessells, H., Roy, J., Bannow, J., et al. (2003). Incidence and severity of sexual adverse experiences in finasteride and placebo-treated men with benign prostatic hyperplasia. *Urology*, 61, 579–584.

Further Reading

- Kristensen, D. M., Mazaud-Guittot, S., Gaudriault, P., et al. (2016). Analgesic use—Prevalence, biomonitoring and endocrine and reproductive effects. *Nature Reviews. Endocrinology*, 12, 381–393. This is a recent review that gives an oversight of mild analgesic use during pregnancy and the potential reproductive consequences. It stresses the urgent need to reconsider the safety and dosage of especially acetaminophen/paracetamol during pregnancy and advocates the development of interdisciplinary research and clinical studies in this domain together with more counseling for pregnant women.
- Samplaski, M. K., & Nangia, A. K. (2015). Adverse effects of common medications on male fertility. *Nature Reviews. Urology*, 12, 401–413. This is a review that describes how many drugs commonly used in urology can have effects on male fertility through a multitude of mechanisms. It stresses the need for men to be counseled about potential drug-related adverse effects on their fertility.
- Anon, n.d. Centers for Disease Control and Prevention. National Center for Health Statistics. Therapeutic Drug Use. <http://www.cdc.gov/nchs/fastats/drug-use-therapeutic.htm> Accessed 4.20.16.
- Farshi, A., Ghaedi, G., Kolahi, A. A., et al. (2013). The effects of opiate consumption on serum reproductive hormone levels, sperm parameters, seminal plasma antioxidant capacity and sperm DNA integrity. *Reproductive Toxicology*, 36, 18–23.

Content and Volume Overview

Jodi A Flaws, Department of Comparative Biosciences, University of Illinois, Urbana-Champaign, IL, USA
Thomas E Spencer, Division of Animal Sciences, University of Missouri, Columbia, MO, USA

© 2018 Elsevier Inc. All rights reserved.

Female reproduction is required for the continuity of life and preservation of the species. It also plays a major role in evolution by transmitting advantageous changes/variations from one generation to the next. Due to the vital roles of female reproduction, it is considered to be one of the most important systems in the body.

The female reproductive system includes the ovaries, oviducts, uterus, vagina, and mammary glands, as well as the placenta and fetus in pregnant animals. This system is primarily regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Levine & Hadley, 2007). The hypothalamus is part of the brain and receives stimulatory and inhibitory signals from the brain and rest of the body as necessary. The pituitary consists of two lobes: the posterior pituitary and the anterior pituitary. The posterior pituitary is an extension of the hypothalamus and receives direct chemical signals from the hypothalamus. The anterior pituitary is not directly connected to the hypothalamus and receives hormonal signals from the hypothalamus via the hypophyseal portal vein to function properly. The ovaries have a dual function in the HPG axis: an endocrine function, producing and secreting hormones (steroidogenesis), and a nonendocrine function, developing and releasing germ cells for reproduction (gametogenesis). Through positive and negative feedback loops, the hypothalamus, pituitary, and ovaries work together to orchestrate the function of the female reproductive system.

Within the HPG axis, the hypothalamus produces gonadotropin-releasing hormone (GnRH), the anterior pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the ovaries produce sex steroid hormones, including estrogens, progesterone, and androgens (Hirshfield, 1991; Levine & Hadley, 2007). GnRH from the hypothalamus directly stimulates secretion of FSH and LH from the anterior pituitary. FSH then stimulates folliculogenesis and steroidogenesis, and LH induces steroidogenesis, ovulation, and the development of the corpus luteum (Levine & Hadley, 2007).

The HPG axis is regulated and maintained by a balance of positive and negative hormonal feedback loops. This provides a delicate balance of stimulatory and inhibitory signals between the components of the axis. Thus, hormones secreted by the ovaries, specifically progesterone and estradiol, feed back on the hypothalamus and anterior pituitary and inhibit either GnRH or FSH and LH release, respectively. Without GnRH, FSH, or LH stimulation, hormones from the ovaries will not be secreted, releasing the negative feedback hold on the hypothalamus and pituitary and maintaining homeostasis.

The ovary is a complex tissue and is responsible for oocyte development and sex steroid hormone production (Hirshfield, 1991). The follicle is the functional unit of the ovary. Follicles are present within the ovary in various stages of development. Primordial follicles consist of a single germ cell, termed an oocyte, surrounded by a single layer of flattened somatic cells, known as granulosa cells. The primordial follicle develops into the primary follicle, which consists of an oocyte surrounded by a single layer of cuboidal granulosa cells. The primary follicle then develops into the preantral follicle, which consists of a single oocyte surrounded by at least two layers of granulosa cells and a newly formed theca cell layer. The final follicular stage is the antral follicle; this follicle has a single oocyte with multiple layers of granulosa cells, two theca layers (theca interna and theca externa), and a fluid-filled space called the antrum. The antral follicle is the only follicle in the ovary that can release an oocyte during ovulation, and it produces and secretes the majority of sex steroid hormones within the ovary. Once the oocyte has been ovulated, the follicle develops into a corpus luteum, a temporary endocrine gland responsible for making hormones that act on the brain and uterus to establish and maintain pregnancy.

Pregnancy is a complex event and involves fertilization, implantation, placentation, and development of the conceptus into a fetus (Spencer, Dunlap, & Filant, 2012; Wang, Wu, & DeMayo, 2017). During fertilization, the sperm cell penetrates the zona pellucida, a glycoprotein membrane that surrounds the oocyte. Fusion of the plasma membranes of the sperm and oocyte triggers the cortical reaction, which prevents other sperm from entering the oocyte. The union of oocyte and sperm occurs in the ampulla of the oviduct and marks the initiation of prenatal development. Implantation is the process by which the blastocyst attaches to the uterine lining. Implantation is divided into several stages: orientation, apposition and attachment (establishes contact with the uterus), adhesion (attaches to the luminal epithelium of the uterus), and invasion (penetrates the uterine stroma). This process is followed by placentation, during which time the placenta is formed to facilitate the transfer of maternal nutrients to the developing embryo. In several species such as the human and mouse, the stromal cells of the uterus undergo a process termed decidualization, which regulates development of the placenta, remodeling of the vasculature, and immune cells.

The end of pregnancy is marked by parturition and the birth of the offspring. In humans and several other mammalian species, labor is characterized by the sustained development of weak and irregular contractions of the uterus, which progressively become stronger and more coordinated. Finally, labor results in the expulsion of the fetus, placenta, and membranes. Prostaglandins and oxytocin are involved in the stimulation of uterine contractions, which are necessary for the progression of parturition (Vannuccini, Bocchi, Severi, Challis, & Petraglia, 2016). Prostaglandins synthesized by uterine decidual cells and the placenta act on the smooth muscle cells of the uterus. Prostaglandins, mainly prostaglandin $F_{2\alpha}$ (PGF_{2 α}) and prostaglandin E₂ (PGE₂), stimulate contraction of smooth muscle cells of the myometrium and promote the softening, dilation, and effacement of the cervix.

Prostaglandin synthesis by uterine myometrium is stimulated by oxytocin. Oxytocin is synthesized by neurons in the supraoptic and paraventricular nuclei of the hypothalamus, but it is stored in and released from the posterior pituitary. Oxytocin signals through its receptors in uterine smooth muscle cells to stimulate contractions and increase intrauterine pressure. Finally, contraction

of the myometrium is triggered by increases in intracellular calcium concentrations. Contraction is coordinated by formation of membrane channels known as gap junctions that allow rapid transfer of small molecules from cell-to-cell.

After birth, the mammary gland supplies a balanced mixture of nutrients, specifically tailored for neonatal health, growth, and development of the offspring (Neville, 1999). In humans, full development of the mammary glands during pregnancy is mainly governed by the actions of prolactin (PRL), estrogen, and progesterone. Prolactin is made by lactotrophs within the anterior pituitary, and its release is constantly inhibited by dopamine (DA). The main functions of PRL are to promote mammary growth, initiate milk secretion, and maintain milk secretion once it is established. PRL binds to PRL receptors (tyrosine kinase receptor type) present in the breast and stimulates the expression of milk protein genes, such as casein and lactalbumin. To nourish the offspring, milk must be successfully ejected from the breast, and this process is governed by oxytocin, which stimulates contraction of the myoepithelial cells surrounding the alveoli and ducts of the breast. Suckling stimulates both PRL and oxytocin release via neural pathways. Specifically, these pathways inhibit dopaminergic neurons in the median eminence of the hypothalamus to stimulate the release of PRL, whereas they stimulate neurons to release oxytocin.

Offspring progress through puberty and become capable of reproduction themselves. Eventually, the reproductive life span will end and the organism will undergo reproductive senescence. The timing of puberty and the onset of reproductive senescence can vary considerably between species and in response to environmental exposures (Gore et al., 2015).

In conclusion, female reproduction is complex and involves many organs and hormones (Gore et al., 2015; Hirshfield, 1991; Levine & Hadley, 2007; Neville, 1999; Spencer et al., 2012; Vannuccini et al., 2016; Wang et al., 2017). This volume highlights the major organs, hormones, and processes that control female reproduction. It contains several chapters on various aspects of the ovary, including ovarian development, folliculogenesis, steroidogenesis, ovulation, and corpus luteum formation. Further, this volume contains several chapters on the hypothalamus and pituitary, as well as hormones that are produced by or regulate the hypothalamus and pituitary. This volume also contains several chapters on other important female reproductive organs such as the oviducts, uterus, cervix, vagina, placenta, and mammary glands. Further, this volume contains several chapters on pregnancy, including the endocrinology of pregnancy, pregnancy complications, parturition, labor, and delivery. It also contains several chapters on important physiological events in female reproduction such as puberty, menopause, and reproductive senescence. Finally, this volume includes chapters on female reproduction in multiple species, and it provides several chapters on the impacts of environmental chemicals on female reproduction.

References

- Gore, A. C., Chappell, V. A., Fenton, S. E., Ja, F., Nadal, A., Prins, G. S., et al. (2015). EDC-2: the Endocrine Society's second scientific statement on endocrine-disrupting chemicals. *Endocrine Reviews*, 36, E1–E150.
- Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. *International Review of Cytology*, 124, 43–101.
- Levine, J. E., & Hadley, M. E. (2007). Pituitary hormones. In M. E. Hadley, & J. E. Levine (Eds.), *Endocrinology* (p. 93).
- Neville, M. C. (1999). Physiology of lactation. *Clinics in Perinatology*, 26, 251–279.
- Spencer, T. E., Dunlap, K. A., & Filant, J. (2012). Comparative developmental biology of the uterus: insights into mechanisms and developmental disruption. *Molecular and Cellular Endocrinology*, 354, 34–53.
- Vannuccini, S., Bocchi, C., Severi, F. M., Challis, J. R., & Petraglia, F. (2016). Endocrinology of human parturition. *Annales d'Endocrinologie*, 77, 105–113.
- Wang, X., Wu, S. P., & DeMayo, F. J. (2017). Hormone dependent uterine epithelia-stromal communication for pregnancy support. *Placenta*. <https://doi.org/10.1016/placenta2017.07.003> [Epub ahead of print].

OVARY

Ovary, Overview

Janice M Bahr, University of Illinois, Urbana-Champaign, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Corpus luteum An endocrine gland formed from the granulosa and theca layers of an ovulated follicle.

Follicle A structure in the ovary consisting of the oocyte and surrounding granulosa and theca cell layers.

Granulosa cells Somatic cells directly surrounding the oocyte.

Meiosis A type of cell division which the oocyte undergoes reducing the number of chromosomes so that the oocyte has one copy of each chromosome.

Oocyte The female gamete.

Ovary The female gonad.

Steroids Molecules with a basic structure similar to that of cholesterol.

Theca cells Layer of steroidogenic cells and connective tissue surrounding the granulosa cells and forming the outer layer of the follicle.

Introduction

Ovaries are female gonads responsible for the generation of female gametes (oocytes) and synthesis of hormones necessary for the regulation of reproductive functions. Since the first description of the ovary reported by Aristotle more than 2000 years ago, information about the ovary has expanded significantly. Knowledge of the formation of the ovary and its endocrine function is essential to understand the mystery of the regeneration of life.

Anatomy of the Ovary

Most vertebrates develop a pair of ovaries with the exception of some birds, reptiles and a few mammals that only have one ovary. Ovaries lie on either side of the upper pelvic cavity and against the pelvic wall. They are held in place by a mesentery (mesovarium) connected to a broad ligament. Ovaries are one of the most vascular organs in the body. The ovarian artery (or utero-ovarian artery) which arises from the abdominal aorta reaches the ovary along with the mesovarium. Branches of the ovarian artery enter the ovary through the hilus, the same site at which the venous blood exits. Adrenergic and cholinergic nerves also enter the ovary through the hilus.

Even though the size of the ovary varies, the structure of the ovary is similar among mammalian species (**Fig. 1**). The ovary consists of an inner medulla, containing a rich vascular bed within loose connective tissue and an outer cortex, where the ovarian follicles are located. The outermost layer of the cortex is a single squamous or cuboidal surface epithelium derived from the peritoneum. Under the surface epithelium lies the tunica albuginea, a poorly delineated layer of dense connective tissue that gives the ovary a whitish color. The cortex of the ovary is made up of numerous follicles of various sizes and stages of development embedded in the stroma. The stroma is composed of at least three different cell types: connective tissue cells (fibroblasts) performing support functions, smooth muscle cells regulating the contraction of blood vessels and interstitial cells including undifferentiated theca cells and degenerated cells from atretic follicles and regressed corpora lutea. The follicles (follicle is Latin for "little bag") are structurally very conspicuous because of their variation in size. The microscopic appearance of follicles is different depending on the stage of follicular development whereas the basic cellular organization of follicles is the same. A follicle consists of an oocyte and surrounding follicular wall. Between the oocyte and surrounding follicular wall is a thin transparent membrane, the zona pellucida. The follicular wall contains an inner granulosa layer and an outer theca layer. The granulosa layer surrounds the oocyte and is separated from the theca layer by the basement membrane. The number and function of the granulosa cells changes during follicular growth. In mature follicles, the theca layer can be divided into the theca externa and interna. The theca externa consists of concentrically arranged smooth muscle cells innervated with autonomic nerves. The theca interna has epithelioid cells called interstitial cells, which are steroid producing cells. These cells contain LH and insulin receptors

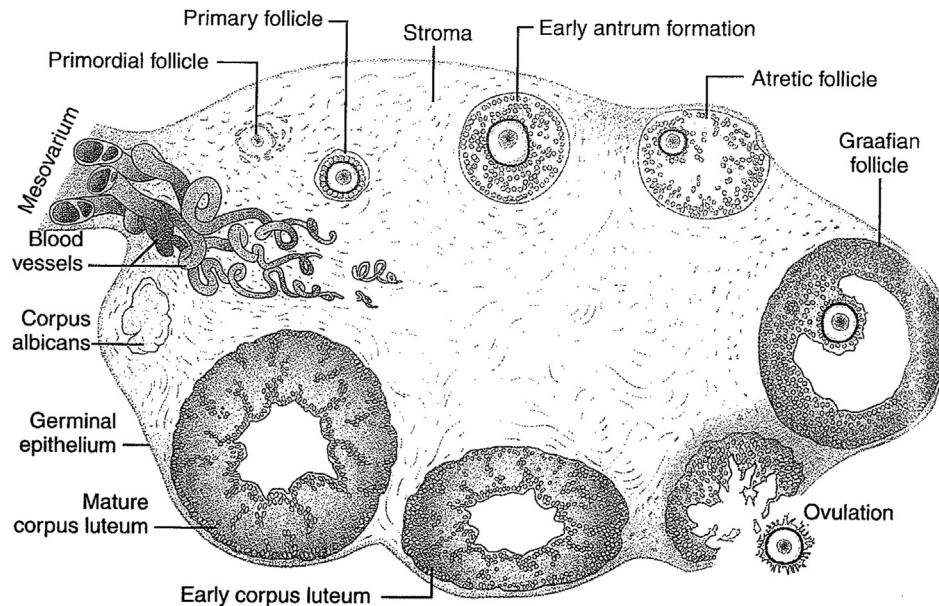


Fig. 1 A cross-section of the ovary illustrating follicles at different stages of development (from primordial to Graafian follicles), corpus hemorrhagicum, corpus luteum, and corpus albicans. The microscopic structures of follicles are also shown. Adapted from Jones, R.E. (1991) *The ovaries in human reproductive biology*, pp. 39–53. Academic Press, San Diego. p. 42.

and synthesize primarily androgens, of which the predominant steroid is androstenedione. The theca interna has both blood vessels and nerves. The granulosa layer is devoid of blood vessels and nerves at all times.

Once ovulation of the Graafian follicle (tertiary) has occurred, blood derived from torn blood vessels of the theca layer infiltrates the collapsed follicle and results in the formation of the corpus hemorrhagicum, a developing corpus luteum with a bloody core. Luteinizing granulosa and thecal cells begin to divide and invade the antral cavity, which remains after ovulation of the oocyte, forming the corpus luteum (Latin for “yellow body”). Blood vessels from the theca layer grow and penetrate the developing luteal cell mass. If pregnancy does not occur, the corpus luteum degenerates after a certain length of time depending upon the species. The connective tissues replaces the luteal cells and forms the corpus albicans (Latin for “white body”). The ovarian medulla devoid of follicles, contains large, spirally arranged blood vessels, lymphatic vessels and nerves.

Functions of the Ovary

Generation of the Female Gametes

Oogenesis

Female gametes, or oocytes, provide the maternal genetic material for the formation of an embryo. The ovary nurtures thousands of oocytes and functions as an incubator for their development. The development of oocytes (oogenesis) starts with primordial germ cells, residing in sex cords which divide mitotically producing oogonia. Oogonia then become primary oocytes and undergo the first meiosis. The primary oocytes are arrested at the diplotene stage of the first meiosis until they experience the preovulatory LH surge. Then the first meiosis is reinitiated and the membrane of the oocyte nucleus (germinal vesicle) disintegrates, which is called germinal vesicle breakdown. Meiosis of the oocyte is unequal producing a large haploid secondary oocyte and a tiny haploid first polar body. This polar body can divide again or remain single; in either case, it degenerates. Then the secondary oocyte begins the second meiotic division but this division is arrested at metaphase until after sperm penetration of the oocyte, which occurs in the oviduct. Completion of the second meiosis results in a haploid ovum and the second polar body.

Folliculogenesis

Folliculogenesis is a developmental sequence regulated by a number of genes, transcription factors and hormones. During fetal development of humans and postnatal development of mice, oocytes are present in clusters or nests. Majority of these oocytes enter meiosis during embryonic life. As the oocytes separate into individual oocytes, they form primordial follicles and undergo further development called oogenesis. Maturation of oocytes (oogenesis) is closely associated with the development of follicles because factors produced by the oocytes have a major impact on the development of the granulosa and theca layers. Folliculogenesis always begins in the innermost part of the ovarian cortex in mammals. Primordial follicles consist of primary oocytes surrounded by flat squamous pre-granulosa cells. Primordial follicles are the only available source of oocytes during the entire reproductive period of the female. As primordial follicles develop into primary follicles, there are changes in the oocyte. It

increases in diameter and develops an extracellular matrix, the zona pellucida. Reactivation of the oocyte genome causes the oocyte to secrete growth factors which play a crucial role in the growth of the follicle. As primary follicles grow, the granulosa cells divide mitotically so that secondary follicles have two to six layers of cuboidal-shaped granulosa cells. Secondary follicles also acquire an additional somatic cell layer, the theca. There are at least two sources of the theca progenitor cells: somatic precursors of the fetal ovary and mesenchymal cells in the neighboring mesonephros. The formation of this theca layer is dependent upon the presence of growth differentiation factor-9 (GDF-9) produced by the oocyte. The theca layer forms around the basement membrane in secondary follicles and ultimately forms the theca interna and theca externa. Follicular growth from primordial to secondary follicles is gonadotrophin-independent. During the formation of tertiary follicles or preantral follicles, follicles continue to grow in size. As follicles progress from secondary follicles to antral follicles, granulosa cells secrete a fluid that accumulates between cells. Large amounts of additional fluid diffuse out of the thecal blood vessels and are added to the fluid which is called follicular fluid. Follicular fluid contains steroid and protein hormones, anticoagulants, enzymes, and electrolytes and is similar to blood serum in appearance and contents. The follicle filled with follicular fluid is the tertiary or preovulatory follicle. These follicles have a mural granulosa layer of four to six layers and the theca layer is differentiated into an inner theca interna and an outer theca externa. Oocytes in preovulatory follicles are suspended in follicular fluid by a stalk of granulosa cells, the cumulus oophorus. Immediately surrounding the oocyte is a thin ring of granulosa cells, the corona radiata. At this state the follicle is called the Graafian follicle and appears as a transparent vesicle that bulges from the surface of the ovary.

Even though one of the function of the ovary is to produce oocytes, the majority of oocytes never ovulate. The number of oocytes reaches its maximum soon after the ovaries are formed. After that time oocyte number decreases dramatically. At birth, a female has all the oocytes she will have in her life; no new oocytes are formed after birth. The vast majority of oocytes, enclosed in follicles, around 99.9%, are eliminated before ovulation through a process called atresia which is due to the activation of apoptosis in the oocyte and granulosa cells. Follicles can become atretic at any stage of development.

Production of Hormones

Another function of the ovary is to secrete hormones which act on the hypothalamus and pituitary to regulate the secretion of hormones by these two tissues, thus establishing the hypothalamic-pituitary-ovarian axis. The ovarian hormones also regulate the function of the reproductive tract and ultimately reproduction.

Protein and peptide hormones

- (i) *Inhibin and activin*: Inhibin and activin were first isolated from gonadal fluids because of their effects on production of follicle stimulating hormone (FSH) by the pituitary in mammals. Inhibins consist of two disulfide-bridged subunits, the α and β subunits, whereas activins consist of two β subunits. The primary source of inhibin and activin in the ovary is the maturing follicles and the corpus luteum. The function of inhibins is to modulate FSH secretion at the level of the pituitary, whereas the function of activins is to increase FSH secretion at the level of the pituitary. Inhibins and activins have antagonistic actions. Inhibins and activins also function as intraovarian hormones.
- (ii) *Follistatin*: Follistatin is a FSH-modulating polypeptide not related to TGF- β . Follistatin acts as a binding protein and a functional antagonist of activin. Granulosa cells in antral follicles and luteal cells secrete follistatin.
- (iii) *Relaxin*: Relaxin is produced by the corpus luteum. The structure of relaxin is very similar to that of insulin but has <20% amino acid homology. In the human, relaxin is the highest during the first trimester of pregnancy after which the concentrations are relative stable. In the rat and the pig, relaxin reaches the highest concentration prior to parturition. Relaxin in these species functions to soften the cervix and vagina for the passage of the fetus during parturition and to promote the growth of nipples. Relaxin also acts on nonreproductive tissues, such as skin and the gastrointestinal tract.
- (iv) *Growth factors*: The ovary not only secretes endocrine hormones to regulate functions of other reproductive organs but also produces growth factors to coordinate the activities of different ovarian compartments. Many growth factors, such as insulin-like growth factors, transforming growth factors and epidermal growth factor are produced by the oocyte and somatic cells in the ovary. This complex intraovarian regulation system is no less important than the extraovarian regulation by the pituitary hormones. These growth factors form a delicate interactive communication web inside the ovary. Without them, the ovarian cells cannot interact with each other and the growth of the ovary is halted.

Steroid hormones

The ovary uses cholesterol as the precursor for steroid synthesis. Cholesterol is metabolized into progestins, androgens, and estrogens by different compartments of the follicles (Fig. 2).

- (i) *Progestins pregnenolone*. Is the most important progestins (C21 pregnane family) produced by follicles because of its key position as the precursor of all steroid hormones. The most abundant progestin is progesterone, produced as a biosynthetic intermediate by follicles at all growing stages of development and as a secretory end product of the corpus luteum. In the developing follicles, the theca layer is the primary site of progestin productions. Immediately prior to ovulation, the granulosa cells stimulated by LH also synthesize progesterone. After ovulation the corpus luteum

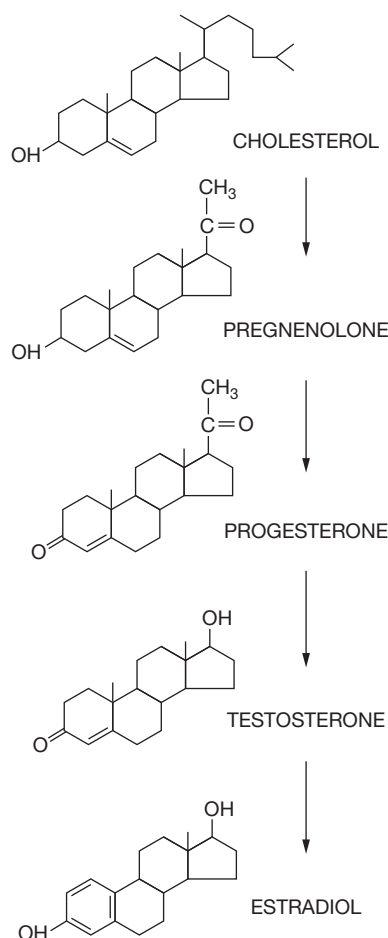


Fig. 2 Biosynthesis of steroid hormones from cholesterol. This scheme provides a simplistic view of a highly organized and complicated process that requires multiple enzymes. Adapted from Hafez, E.S.E (1993) Folliculogenesis, egg maturation, and ovulation. In *Reproduction in farm animals*, 6th ed., pp. 114–143. Lea and Febiger, Philadelphia, p. 79.

synthesizes copious amounts of progesterone needed to prepare the uterus for implantation and later for the maintenance of pregnancy.

- (ii) **Androgens.** The follicle is a significant source of ovarian androgens (C19 androstane family). Pregnenolone and progesterone are converted into androgen metabolites, dehydroepiandrosterone and androstenedione, respectively. These two metabolites are then transformed into testosterone. The theca layer of the follicle is the primary source of ovarian androgens.
- (iii) **Estrogens.** Physiologically, the estrogens (C18 estrane family) especially estrone and estradiol-17- β , are the most important of the ovarian steroids. Androstenedione and testosterone are the immediate biosynthetic precursors of estrone and estradiol-17- β , respectively. Their names reflect their roles in the induction of sexual receptivity (estrus) in female mammals. Estrone was the first sex steroid isolated and identified. The granulosa layer is the major site of estrogen synthesis in the mammalian ovary.

Regulation of Ovarian Functions

Regulation of Folliculogenesis

Growth of primordial follicles to the preantral stage is independent of gonadotropins and is controlled by intraovarian growth factors. Growth of follicles after the preantral stage depends on appropriate patterns of secretion, sufficient concentrations and adequate ratios of FSH and LH in the blood. FSH plays a major role in early follicular development. FSH stimulates granulosa cell mitosis and accumulation of follicular fluid. Granulosa cells synthesize estrogens in response to FSH which further enhance the mitotic effect of FSH. Moreover, FSH induces granulosa cell sensitivity to LH by increasing LH receptor expression. Abundant LH receptors in granulosa cells prepare the luteinization of granulosa cells in response to the ovulatory LH surge in mammals. In contrast, theca cells are stimulated only by LH and LH receptors are present from the beginning of the formation of the theca layer.

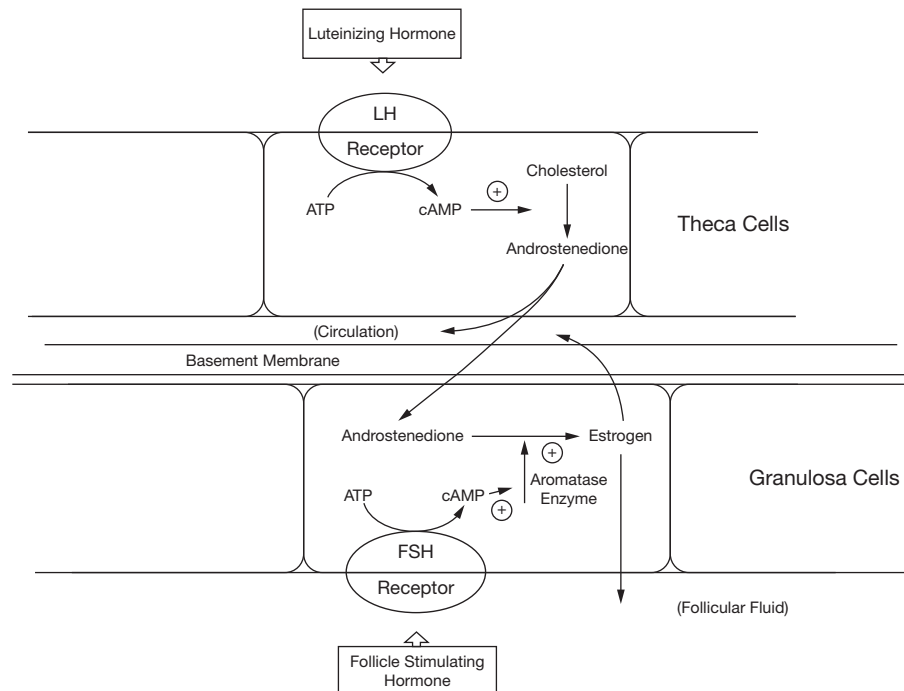


Fig. 3 “Two-cell, two-hormone” theory of follicular steroidogenesis. LH binds to specific membrane receptors on theca cells and stimulates cyclic AMP production and the conversion of cholesterol to androgens, primarily androstenedione and testosterone. These androgens diffuse into the circulation and across the basement membrane into granulosa cells. FSH binds to specific membrane receptors on granulosa cells and stimulates cyclic AMP production, which leads to increased aromatase enzyme activity and the conversion of theca androgens to estrogens. Adapted from Yen, S. S. C. and Jaffe, R. B. (1986). *Reproductive Endocrinology* (2nd ed.), Philadelphia: Saunders.

Regulation of Steroidogenesis

The steroidogenic output of the ovary is a function of coordinated actions of theca and granulosa cells. Differences in gonadotropin receptors on the membrane, in the activity of steroidogenic enzymes and in compartmentalization in the follicle result in a unique partnership in steroid synthesis between theca and granulosa cells. The principal site of estrogen synthesis in the mammalian ovary is granulosa cells under the control of FSH. Androgen production appears to be the primary steroidogenic function of theca cells in response to LH. Androgens from theca cells provide substrates for granulosa cells to synthesize estrogens. The action of LH on theca androgen production, together with the action of FSH on granulosa estrogen synthesis, forms the basis of the “two-cell, two-hormone” theory for the control of steroidogenesis in the ovary (Fig. 3).

Further Reading

- Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N., & Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, 383, 531–535.
- Hafez, E. S. E. (1993). Folliculogenesis, egg maturation, and ovulation. In *Reproduction in farm animals* (6th ed., pp. 114–143). Philadelphia: Lea and Febiger.
- Jones, R. E. (1991). *The ovaries in human reproductive biology*. San Diego: Academic Press. pp. 39–53.
- Liu, C., Peng, J., Matzuk, M.M. and Yao, H.H. (2015). *Nature Communications*. <https://doi.org/10.1038/ncomms7934>.
- Rajkovic, A., Panagas, S. A., & Matzuk, M. M. (2006). Follicular development: Mouse, sheep and human models. In J. D. Neill (Ed.), *Knobil and Neill's physiology of reproduction* (3rd edn). Amsterdam: Elsevier.
- Strauss, J.F. III and Williams, C.J. (2009). The ovarian life cycle, Strauss, J.F. III, Barberi, R.L. eds., Yen and Jaffe's reproductive endocrinology, physiology, pathophysiology, and management, 6th edn, Saunders, Philadelphia, PA.
- Williams, C. J., & Erickson, G. F. (2012). In L. J. De Groot, G. Chrousos, & K. Dungan (Eds.), *Morphology and physiology of the ovary*. South Dartmouth, MA: Endotext.

Granulosa Cells

Sarah C Baumgarten and Carlos Stocco, University of Illinois College of Medicine, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Granulosa cells, their precursors, or their final differentiation stages are critical components of the ovary that nurture germ cells, sustain oocyte maturation, provide the hormonal milieu required to synchronize uterus receptivity with the release of the mature eggs, and support early pregnancy. Granulosa cells are the only somatic cells that closely interact with the oocyte from the moment the follicle forms until the release of the oocyte at ovulation. Throughout this long relationship, both the oocyte and the granulosa cells go through significant functional and morphological changes.

Embryonic Progenitors

Pregranulosa cell differentiation is vital for fertility because it ensures the differentiation of the oogonia, the formation of the definitive follicle reserve, and, ultimately, the formation of the ovary. Pregranulosa cells originate from coelomic proliferating epithelial cells that proliferate and invade the developing gonads. Pregranulosa cells can be classified into medullary and cortical cells depending on their location in the developing gonad. Some precursor cells migrate to the medulla of the fetal gonads at approximately embryonic day 11.5 (E11.5) in the mouse and are known as medullary cells. These cells stop proliferating and get incorporated into medullary follicles. A second population of precursors enters the ovary from the epithelium between E15.5 and postnatal day 4 and remains closer to the ovarian surface and is, therefore, called cortical cells. Some of these cortical cells also stop proliferating, differentiate into pregranulosa cells, and get incorporated into cortical primordial follicles a few days after birth in the mouse. The follicles in the medulla of the ovary are activated immediately after birth and reach antral stages before puberty, whereas cortical follicles are activated throughout the reproductive years of the female (Zheng et al., 2014).

Pregranulosa cells can be recognized by the expression of forkhead box L2 (FOXL2) and LGR5. FOXL2 is a forkhead transcription factor expressed not only in medullary pregranulosa cells in the developing XX gonad but also in mature granulosa cells. Recently, the adult stem cell marker LGR5 was found to be expressed in the cortical region of the fetal ovary in cells that give rise to adult cortical granulosa cells. LGR5 binds R-spondin proteins with high affinity and thereby enhances WNT signaling. *Lgr5* deletion blocks germ cell differentiation (Fig. 1, pregranulosa cell differentiation).

The commitment of the coelomic proliferating epithelial cells that invade the developing gonads to the granulosa fate defines sex determination. Thus, if these cells have a Y chromosome and express SRY and SOX9, the gonad develops into a testis. In the absence of SRY and SOX9, the expression of female-specific genes encoding secreted factors such as R-spondin1 (RSPO1), WNT4, and follistatin and transcription factors such as GATA4, β -catenin, and FOXL2 triggers ovarian development and actively suppresses testis differentiation. Of these genes, *Rspo1* and *Wnt4* are already expressed in the bipotential gonad, but they become ovary-specific at E11.5. WNT4 and RSPO1 are parts of a common signaling pathway that acts through β -catenin, which is essential for pregranulosa cell differentiation.

The transcription factors GATA4 and GATA6 are also present in the pregenital ridge region as early as E10. Both factors are required for the proliferation of the coelomic epithelium leading to the formation of the genital ridge. GATA4 remains expressed at E11.5 in the primitive gonads of both XX and XY mouse embryos. By E13.5, GATA4 is found in most ovarian somatic cells where it is expressed at high levels over the entire fetal period. In animals that lack GATA4 and GATA6 during embryogenesis, the expression of key markers of pregranulosa cells, including FOXL2, is reduced leading to a decrease in proliferation and an increase in apoptosis. In mice lacking GATA, the majority of the oocytes are lost after birth due to the lack of granulosa cell support.

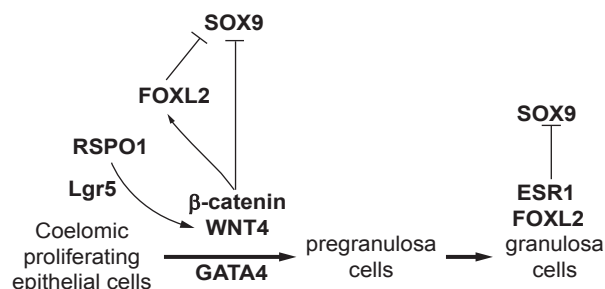


Fig. 1 Genes involved in pregranulosa cell differentiation. During embryonic gonad development, the ovary-specific genes *Foxl2*, *Rspo1*, *Wnt4*, and β -catenin suppress the male pathway through inhibition of *Sox9*. In the adult ovary, FOXL2 and ESR1 are required for tissue maintenance and follicle maturation.

Follicle Assembly

Once primordial germ cells arrive at the developing gonad, they stop migrating but continue dividing. At this point, these cells are known as oogonia. Due to incomplete cytokinesis, oogonia remain attached in clusters forming cysts or nests that become enclosed by somatic pregranulosa cells. The oogonia continue dividing until they receive signals from surrounding somatic and pregranulosa cells to shift from mitosis to meiosis. It is clear that the signal received by the oogonia to enter meiosis is dependent on the genomic sex of the surrounding cells. In fetal testis, primordial germ cells stop mitotic division and enter a period of mitotic quiescence in G₀. In the ovary, retinoic acid (RA) produced, in part, by the pregranulosa cells is responsible for inducing meiosis.

Under the influence of RA, clusters of oogonia enter meiosis synchronously, becoming oocytes. Later, the clusters break down, and individual oocytes become surrounded by squamous pregranulosa cells forming primordial follicles, which are the basic functional units of the ovary. This represents the first stage of folliculogenesis taking place during the latter half of fetal development in humans or during the days following birth in mice. In animals lacking factor in the germ line (FIGL α), pregranulosa cells fail to form a monolayer around individual primordial oocytes resulting in rapid oocyte loss. Similarly, mice lacking Notch2 signaling in the pregranulosa cells fail to break down the clusters of oogonia and form primordial follicles containing multiple oocytes.

Importantly, all granulosa cells in the postnatal ovary, in both the medullary and cortical follicles, are marked by the expression of FOXL2. Moreover, postnatal deletion of FOXL2 or estrogen receptor alpha (ESR1) induces a transdifferentiation of granulosa cells to sertoli-like cells, indicating that FOXL2 acts as an antitestis factor and is required to maintain the granulosa cell phenotype.

Gonadotropin-Independent Activation and Growth

Activation

The pregranulosa cells and the oocytes of cortical primordial follicles remain dormant for months in rodents or for decades in humans. Primordial follicles then transition to primary follicles. During this transition, the single layer of flattened granulosa cells changes morphology to become cuboidal granulosa cells. Primordial follicle activation occurs independently of pituitary gonadotropins. Instead, it is regulated by factors produced within the ovary and requires the interaction between pregranulosa cells and oocyte.

In FOXL2-null mice, granulosa cells remain flattened resulting in the absence of follicle growth suggesting that the activation of these cells in adult ovaries requires FOXL2. Similarly, inhibition of mTORC1 in granulosa cells blocks dormant follicle activation, while its overactivation increases this process. These findings indicate that granulosa cells play a major role in the activation of primordial follicles. An interaction between the flattened granulosa cells and the oocyte is also critical for follicle activation and is carried out by the KIT and KIT ligand (KITL) system. The oocyte expresses KIT, a tyrosine kinase receptor, on its surface, whereas flattened granulosa cells and later the granulosa cells express KITL, which is able to bind to KIT to promote follicle activation. Preventing KIT/KITL interaction in mice pauses follicle growth at the primordial stage, and few, if any, follicles transition to the primary stage. On the other hand, *in vitro* experiments demonstrated that if ovaries are treated with KITL, a significant number of follicles transition from the primordial to the primary stage (Saatchioglu et al., 2016; Zhang and Liu, 2015). However, the molecular changes involved in the transition from flattened to cuboidal granulosa cells remain to be determined.

Proliferation to Preantral Stage

As the ovarian follicle progresses from the primary stage to the preantral stage, the layer of cuboidal granulosa cells proliferates to form multiple layers. Granulosa cell proliferation occurs independently of gonadotropins. However, it is possible that the oocyte influences proliferation. Thus, mouse studies demonstrated that oocytes from follicles with two layers of granulosa cells could stimulate granulosa cell proliferation and differentiation in primordial follicles, suggesting that oocyte-secreted factors are crucial for follicle growth. One such factor is growth differentiation factor 9 (GDF9). For instance, folliculogenesis is arrested at the primary stage in animals lacking GDF9. Direct communication between the granulosa cells and the oocyte is also essential for follicle maturation beyond the primary stage. For instance, knockout of connexins 43 and 37 (CX43 and CX37), which both are components of gap junctions between the oocyte and surrounding granulosa cells, halts folliculogenesis at the primary (unilaminar) stage, and granulosa cell differentiation is impaired. The full spectrum of molecules and signals that travel through gap junctions to promote follicle growth remains to be determined.

Differentiation to the Preovulatory Stage

During follicle progression from the preantral to the preovulatory stage, a fluid-filled cavity called the antrum forms within the follicle. This final period of follicle growth is mainly driven by the expansion of the fluid inside the antrum than by the proliferation of the granulosa cells. Antrum formation depends on gonadotropins, which stimulate the production and secretion of polysaccharides and proteins by the granulosa cells. Granulosa cells produce and secrete hyaluronan and versican, a chondroitin sulfate proteoglycan, toward the extracellular space. The accumulation and the cross-links of these molecules generate an osmotic gradient, which in turn draws in fluid from the thecal vasculature. This idea is supported by genetic models. For instance, in animals lacking

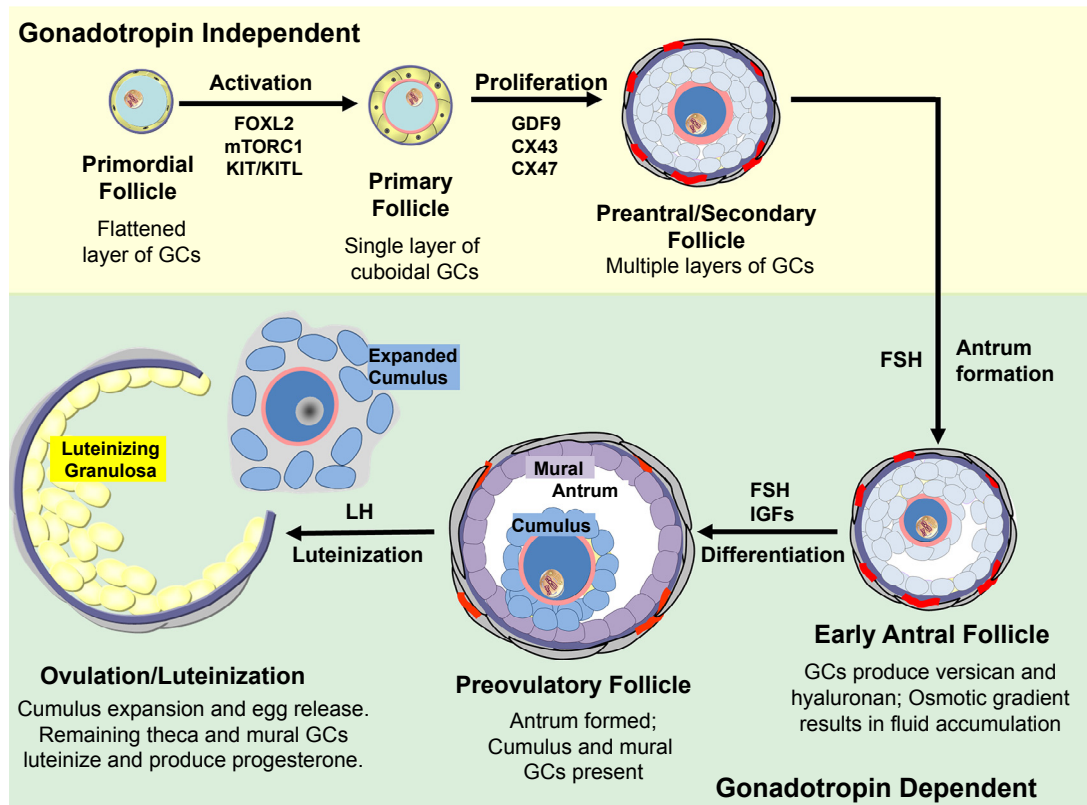


Fig. 2 Changes taking place in granulosa cells (GCs) during folliculogenesis. The progression of follicles from the primordial to the preantral stage is dependent on intraovarian factors and does not require gonadotropins. The processes of antrum formation and granulosa cell differentiation to the preovulatory follicle stage are regulated by FSH. Ovulation and luteinization are controlled by LH.

GATA4 and GATA6 in the granulosa cells, versican expression is abolished in the granulosa cells, an effect that correlates with the lack of antrum formation.

The antrum physically separates the granulosa cells into two functionally distinct cell populations, cumulus and mural. The cumulus granulosa cells surround the oocyte and actively participate in oocyte maturation. The mural granulosa cells are close to the follicle wall and are the primary site of sex steroid hormone synthesis and produce estradiol as the follicle matures to the preovulatory stage (Fig. 2).

While intraovarian factors are the primary regulators of preantral granulosa cell growth, extraovarian factors, in particular follicle-stimulating hormone (FSH), are crucial for the differentiation of mural granulosa cells. This is illustrated in female FSH knockout mice, where folliculogenesis is halted at the preantral stage. Although FSH is essential for the survival, proliferation, and differentiation of preovulatory mural granulosa cells, local factors such as the insulin-like growth factor (IGF) system are also crucial for mural granulosa cell differentiation. Thus, granulosa-cell-specific IGF1R-depleted mice are infertile and lack mural cells.

Cumulus Cells

In antral follicles, cumulus granulosa cells remain intimately associated with the oocyte. The first layer around the oocyte is called the corona radiata. Both corona cells and cumulus cells in outer layers communicate with the oocyte via extensions that reach around the corona cells to contact the oocyte. Oocyte-secreted factors (OSFs) play crucial roles in the development and function of the cumulus cells.

Two well-recognized OSFs involved in cumulus cell regulation are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15). GDF9 and BMP15 protect cumulus cells from FSH-induced differentiation, and an opposing gradient of FSH and OSF signaling throughout granulosa cell layers establishes the cumulus or the mural phenotypes in vivo (Diaz et al., 2007). In mice, GDF9 and BMP15 activate SMAD2/3 signaling within the granulosa cells, which prevents FSH from stimulating genes expressed in mural granulosa cells, including luteinizing hormone (LH) receptor. Importantly, FSH cannot stimulate the expression of steroidogenic genes in the presence of SMAD2/3 activation, so cumulus cells are functionally distinct from mural granulosa cells and do not serve as a source of steroid hormone production during follicle maturation.

In humans, cumulus cells isolated from in vitro fertilization patients also express lower levels of differentiation markers than mural cells including CYP19a1, CYP11a1, StAR, and LH receptor indicating that cumulus cells are also protected from

differentiation in humans. Moreover, once separated from the oocyte, human cumulus cells respond to FSH with the activation of a genomic program that resembles the transformation of preantral to mural cells observed in laboratory animals. Thus, human cumulus granulosa cell cultures can serve as an experimental approach to study the differentiation of these cells. This has allowed for the identification of key genes and mechanisms of differentiation that were previously unable to be studied in humans (Stocco et al., 2017).

Cumulus granulosa cells nurture the oocyte by providing specific amino acids, glycolysis products, and cholesterol biosynthesis substrates via gap junctions. Additionally, cumulus granulosa cells prevent premature oocyte maturation and resumption of meiosis in the oocyte. The oocyte remains paused at the prophase stage of meiosis I throughout follicle maturation until shortly before ovulation. The pause in meiosis is sustained by cAMP within the oocyte. In a normal follicular cycle, the gonadotropin surge that triggers ovulation also stimulates the activation of phosphodiesterases within the oocyte that reduces the levels of cAMP and allows for the resumption of meiosis. Before the LH surge, cAMP levels in the oocyte are maintained through an intimate interaction between the oocyte and surrounding cumulus granulosa cells via gap junctions. In this system, the cumulus granulosa cells express NPR2, a receptor with guanylyl cyclase activity. Mural granulosa cells produce the ligand NPPC, which is released and can activate NPR2 on cumulus cells. When activated, NPR2 increases intracellular levels of cGMP, which travels into the oocyte through gap junctions. In the oocyte, cGMP inhibits the activity of phosphodiesterases to maintain cAMP levels. Interestingly, oocyte-secreted factors, such as GDF9 and BMP15, can enhance NPR2 expression in cumulus cells, thus enhancing the system necessary to maintain its pause in meiosis I (Egbert et al., 2016).

Cumulus expansion

A distinctive characteristic of cumulus cells is their capacity to change from a compact cell mass into a dispersed structure of cells during the preovulatory period. This process is referred to as cumulus expansion and involves the synthesis and deposition of a mucoid intercellular matrix. Cumulus expansion influences a variety of developmental changes during oocyte maturation.

Cumulus granulosa cells express very low levels of LH receptor compared with mural granulosa cells; however, the LH surge drives the process of cumulus cell expansion. In vitro studies have shown that the effect of LH on cumulus cells is mediated by mural granulosa cells. Upon LH stimulation, the mural cells produce epidermal growth factor (EGF)-like family members, which are released and activate the EGF receptor on cumulus cells to enhance expression of genes necessary for the formation and stabilization of ECM and cumulus expansion. Interestingly, cumulus expansion in response to EGF receptor activation also requires the presence of factors secreted by the oocyte. The expression of several genes including *Has2*, *Ptgs2*, *Ptx3*, and *Tnfaip6* are needed for cumulus expansion, and their deletion impairs this process.

Mural Cells

During the transition from the preantral to the preovulatory follicle, the granulosa cells that line the wall of the follicle and are distant from the oocyte differentiate into mural granulosa cells under the influence of FSH and locally produced factors such as IGFs. FSH stimulates the expression of hundreds of genes, including those necessary for the production of estradiol and expression of the LH receptor. FSH activates the FSH receptor, a G-protein-coupled receptor, on the surface of mural granulosa cells. This stimulates an increase in intracellular cAMP and PKA activation, finally resulting in the activation of CREB transcription factor. Many key genes for steroidogenesis are regulated by CREB, including aromatase. Additionally, FSH activates AKT signaling, which is essential, although not enough, to promote the expression of differentiation genes in granulosa cells (Fig. 3, cumulus and mural granulosa cell differentiation and function).

As mentioned earlier, the IGF family is also essential for the differentiation of mural granulosa cells. IGF1 and IGF2 and their target receptor, the IGF1R, are needed for the stimulation of AKT by FSH. In vitro studies in rodents and humans demonstrated that granulosa-cell-produced IGFs are essential for differentiation. Activation of the IGF1R by IGFs and subsequent AKT activation is needed for FSH to maximally stimulate the expression and activity of aromatase to enhance estradiol production. Interestingly, IGF production is species-specific. Thus, rodent granulosa cells produce IGF1, while human granulosa cells produce only IGF2. However, their role in granulosa cell differentiation is comparable.

Steroidogenesis

Antral follicles begin producing increasing levels of progestins, estrogens, and androgens. The mural granulosa cells and the adjacent theca cells surrounding the ovarian follicle are the sites of steroidogenesis. According to the “two cell theory” of steroidogenesis, the cells work cooperatively to produce estradiol. The theca cells express enzymes necessary for androgen synthesis, while the mural cells express aromatase, which converts androgens to estradiol. Theca cells produce androgens by expressing 17 α -hydroxylase/17,20-lyase (CYP17A1). This enzyme is not expressed in granulosa cells. However, the granulosa expresses StAR, CYP11A1, and 3 β -HSD. Therefore, they are able to produce progesterone.

Aromatase (CYP19A1) is the main steroidogenic enzyme expressed in mural granulosa cells. The timely expression of aromatase in the ovarian follicle is responsible for the cyclic changes in serum estradiol levels. These changes modulate the structure and function of the female reproductive tract, including the oviducts, the uterus, and the vagina. These endocrine actions are essential for oocyte survival, fertilization, and implantation. More importantly, the strong expression of aromatase and subsequent high level of estradiol production in preovulatory follicles lead to the surge of LH required to trigger ovulation. Locally produced estrogens also stimulate granulosa cell proliferation and facilitate FSH and LH actions in a paracrine/autocrine manner. Therefore, the

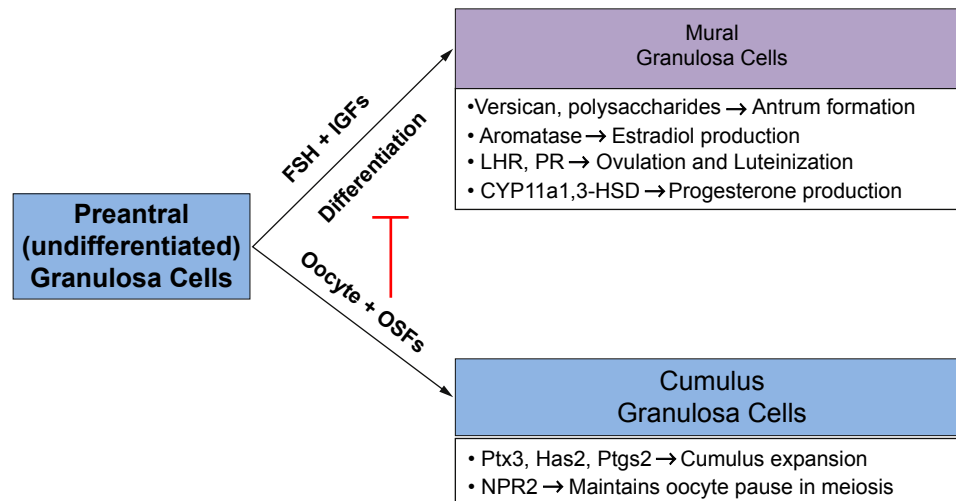


Fig. 3 Granulosa cell differentiation. As antral follicle development proceeds, mural granulosa cells and cumulus granulosa cells develop distinguishing characteristics. FSH drives the differentiation of mural granulosa cells, whereas the oocyte and oocyte-secreted factors (OSFs) actively prevent this in cumulus granulosa cells. Mural cells express genes involved in estradiol production and on the preparation of the cells for ovulation and luteinization. Oocyte-secreted factors stimulate the expression of cumulus cell-specific genes required to cumulus expansion and oocyte maturation.

well-timed and cell-specific expression of aromatase in mural granulosa cells is crucial for the autocrine regulation of folliculogenesis, the endocrine control of the female reproductive tract, and the coordination of gonadotropin secretion.

Ovulation

The high level of estradiol produced by the developing dominant follicle exerts a positive feedback on the anterior pituitary resulting in a surge of FSH and LH production and release into the circulation. This surge in LH acts on mural granulosa cells, which express high levels of the LH receptor, to initiate a series of events leading to cumulus cell expansion and follicle rupture. During this time, LH also stimulates the resumption of meiosis in the oocyte. Although the mechanisms are not entirely clear, a closure in gap junctions between the cumulus cells and oocyte or an increase in phosphodiesterase activity within the oocyte could contribute to a fall in cAMP and resumption of meiosis.

LH receptor activation in the preovulatory granulosa cells results in the induction of a broad range of genes that are crucial for ovulation and differentiation of the granulosa cells. Some of these genes are transiently induced, whereas others remain constitutively expressed in luteal cells. The transiently induced genes include progesterone receptor (*Pr*), cyclooxygenase (*Cox2*), CCAAT/enhancer-binding protein β (*C/EBP β*), and early growth response protein-1 (*Egr-1*). Mice deficient in *PR*, *C/EBP β* , or *COX-2* are infertile because preovulatory follicles fail to ovulate. LH also enhances the transcription of proteases, including matrix metalloproteinases (MMPs) and *Adamst1*, which degrade components of the surrounding ECM during the process of ovulation. This remodeling is essential for follicle rupture and release of the cumulus-oocyte complex from the ovary.

Final Differentiation: Luteinization

Mural and cumulus cells have different destinies after the preovulatory surge of LH. The mural cells remain in the ovary, luteinize, and participate in the formation of the corpus luteum, whereas the expanded cumulus cells accompany the metaphase II (secondary) oocyte to the oviduct. During luteinization, the granulosa cells express high levels of *StAR*, *CYP11a1*, and 3β -HSD, which are needed for the production of progesterone. After luteinization, luteinized granulosa cells are called luteal cells and produce high amounts of progesterone. Progesterone stimulates blood vessel growth and nutrient secretion in the endometrium of the uterus to support the implantation of the embryo.

FSH stimulates proliferation of granulosa cells; however, after the LH surge, granulosa cells stop dividing and exit the cell cycle. Luteal cells are found arrested predominantly at the G_0/G_1 phase of the cell cycle. Cessation of cell proliferation during luteinization is associated with a progressive loss of positive cell-cycle regulators, including cyclins and *Cdk2*, and with increased expression of the *Cdk* inhibitors *p21^{cip1}* and *p27^{kip}*. In addition, luteinizing granulosa cells express factors that stimulate the development of an extensive web of blood vessels, which provide not only the substrates for progesterone synthesis but also a mechanism to transfer of the high amounts of progesterone produced by luteal cells to the systemic circulation. One such factor is VEGF, which is expressed in the granulosa compartment only at the preovulatory stage. After ovulation, luteal cells continue expressing VEGF. The block of VEGF before ovulation inhibits luteal angiogenesis (Stocco et al., 2007).

Aging

Aging has a significant effect on ovarian function and, in particular, on granulosa cells. With age, granulosa cells show an increase in oxidative stress, leading to the formation of reactive oxygen species (ROS), which can damage lipids, proteins, and DNA. The formation of ROS could be triggered by a decline in superoxide dismutase, which neutralizes ROS, observed in aging granulosa cells. Additionally, the expression of glutathione S-transferase theta 1 (GSTT1) is increased in the granulosa cells of older women, an indication of increased oxidative stress. In studies of in vitro fertilization patients, women with granulosa cells that expressed high levels of GSTT1 have a significantly lower oocyte developmental capacity and lower cumulus-oocyte complex maturity compared to patients with low GSTT1. The increase in ROS correlates with FSH receptor downregulation and dysregulation of FSH action including disrupted steroidogenesis. This effect may explain the poor response to FSH observed in older women.

In addition to oxidative stress, reactive carbonyl species (RCS) are also thought to contribute to the functional decline of granulosa cells with aging. RCS are derivatives of metabolic processes, such as glycolysis, and they promote the formation of advanced glycation end products (AGEs). AGE-modified proteins and receptor for advanced glycation end products (RAGE) have been detected in granulosa cells. Activation of RAGE by AGE-modified proteins results in the generation of ROS and the expression of proinflammatory genes. Studies of granulosa cells retrieved from in vitro fertilization patients revealed that older women expressed significantly more RAGE than younger women. Thus, RCS accumulation during aging could lead to granulosa cell dysfunction by increasing ROS and inflammatory pathways (Avila et al., 2016).

Tumors

Granulosa cell tumors (GCT) are the most common type of ovarian sex cord tumors, which represent approximately 8% of ovarian cancers. GCTs are classified into juvenile GCT or adult GCT, each having unique histology and genetic mutations. Juvenile GCTs are rare and make up only 5% of all GCTs. Approximately 30% of these tumors have activating mutations in the *Gsp* oncogene, which encodes the α subunit of Gs. Also, roughly 60% of GCT tumors contain activating mutations of AKT. In contrast, more than 97% of adult GCTs contain a single missense mutation in the *FOXL2* gene, *FOXL2* C134W. Interestingly, although the biological action of *FOXL2* in many aspects of ovarian physiology is well understood, the mechanisms by which *FOXL2* C134W results in tumorigenesis have not been established.

Both juvenile and adult GCTs produce high levels of estrogens that can become symptomatic. Hyperestrogenism can cause precocious puberty in children and irregular bleeding in reproductive-age or postmenopausal women. These symptoms often lead to the GCT diagnosis. The high levels of estrogen can also result in endometrial hyperplasia or carcinoma. Granulosa cell markers, such as AMH and inhibin, are used in diagnosis and monitoring of GCTs. Overall, the majority of GCTs are diagnosed at an early stage and have a good prognosis after surgical intervention.

Conclusion

The granulosa cells are central for female reproduction. These cells establish a very close relationship with the female gamete even before oögonia differentiation during embryogenesis. This relationship continues for months or years and during the maturation of primary and secondary oocytes. After ovulation, cumulus granulosa cells escort the oocyte to the oviduct, whereas the mural granulosa cells that remain in the ovary are transformed into luteal cells. Luteal cells are necessary for implantation and early development of the zygote. The understanding of the intricate mechanisms, pathway activation, and gene expression in the granulosa cells at each of these steps is still evolving.

References

- Avila, J., Gonzalez-Fernandez, R., Rotoli, D., Hernandez, J., & Palumbo, A. (2016). Oxidative stress in granulosa-lutein cells from in vitro fertilization patients. *Reproductive Sciences*, 23, 1656–1661.
- Diaz, F. J., Wigglesworth, K., & Eppig, J. J. (2007). Oocytes determine cumulus cell lineage in mouse ovarian follicles. *Journal of Cell Science*, 120, 1330–1340.
- Egbert, J. R., Ullasz, T. F., Shuhaibar, L. C., Geerts, A., Wunder, F., Kleiman, R. J., Humphrey, J. M., Lampe, P. D., Artemyev, N. O., Rybalkin, S. D., Beavo, J. A., Movsesian, M. A., & Jaffe, L. A. (2016). Luteinizing hormone causes phosphorylation and activation of the cGMP phosphodiesterase PDE5 in rat ovarian follicles, contributing, together with PDE1 activity, to the resumption of meiosis. *Biology of Reproduction*, 94, 110.
- Saatcioglu, H. D., Cuevas, I., & Castrillon, D. H. (2016). Control of oocyte reawakening by kit. *PLoS Genetics*, 12, e1006215.
- Stocco, C., Telleria, C., & Ghibori, G. (2007). The molecular control of corpus luteum formation, function, and regression. *Endocrine Reviews*, 28, 117–149.
- Stocco, C., Baumgarten, S. C., Armouti, M., Fierro, M. A., Winston, N., Scoccia, B., & Zama, A. M. (2017). Genome-wide interactions between follicle-stimulating hormone and insulin-like growth factors in the regulation of human granulosa cell differentiation. *Human Reproduction*, 32, 905–914.
- Zhang, H., & Liu, K. (2015). Cellular and molecular regulation of the activation of mammalian primordial follicles: somatic cells initiate follicle activation in adulthood. *Human Reproduction Update*, 21, 779–786.
- Zheng, W., Zhang, H., & Liu, K. (2014). The two classes of primordial follicles in the mouse ovary: their development, physiological functions and implications for future research. *Molecular Human Reproduction*, 20, 286–292.

Theca Cells

JoAnne S Richards, Baylor College of Medicine, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Definition of the Theca Cell Layer

Theca is a Latin word for a casing or sheath. The theca cell layer of the ovarian follicle is an envelope of connective tissue surrounding the granulosa cells. The inner-most layer that is closest to the granulosa cells is called the theca interna; the outer-most layer is called the theca externa. The theca interna contains the endocrine cells that produce the steroid hormones. The theca externa is a connective tissue layer derived from fibroblast-like cells that produce extracellular matrix factors, such as collagen. The theca interna and externa also contain a rich supply of blood vessels called the vascular tissue that brings nutrients, oxygen, and immune cells to the growing follicles. Thus, the theca layer of ovarian follicles is critical not only for maintaining the structure of the follicle but also for delivering nutrients to the granulosa cells and oocyte that reside in a non-vascular environment within the follicle (Young and McNeilly, 2010) (Fig. 1).

Endocrine Functions of Theca Cells

The cells within the theca that produce and secrete steroid hormones are called theca endocrine cells. The secreted steroid hormones act as messengers that travel through the blood stream to alter the function of distant tissues and thereby coordinate the development and metabolic homeostasis of the body. Studies in the 1970s documented that theca cells in growing follicles produce steroid hormones called androgens, primarily androstenedione, testosterone, and dihydrotestosterone. Moreover, it was discovered that androgens produced by the theca were then converted to another steroid hormone, estradiol, by the aromatase enzyme in granulosa cells (Young and McNeilly, 2010).

Theca endocrine cells produce androgens in response to the pituitary hormone called luteinizing hormone (LH). Studies in the 1970s documented that LH stimulated theca cell steroidogenesis by binding to a molecule on the surface of the theca cells called the LH receptor. By binding to this receptor, LH triggers a cascade of intracellular signaling pathways leading to the production of androgens. Furthermore, LH receptors were shown to be present in theca endocrine cells of small preantral, antral, and preovulatory

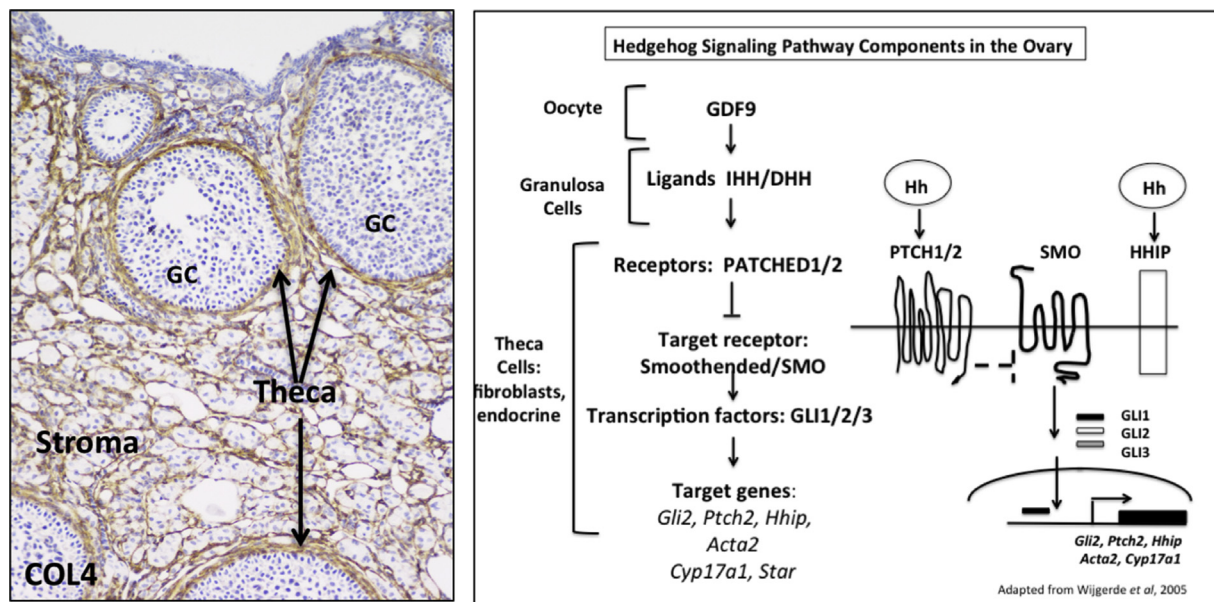


Fig. 1 Theca cells in growing follicles express collagen IV (COLIV) an extracellular matrix protein that provides structural integrity. COLIV is also present in the ovarian stroma but is not present in granulosa cells. Theca cells are recruited to the growing follicles and are functionally regulated by the Hedgehog (HH) signaling pathway. Specifically, GDF9 secreted from the oocyte induces the expression of IHH and DHH in granulosa cells. These ligands then bind to and activate their receptors (PATCHED1 and PATCHED2) in theca cells. This activation prevents PATCHED1/2 inhibition of another theca cell membrane signaling molecule SMOOTHENED. Activated Smoothened then increases the transcription of specific genes via the nuclear transcription factors, Gli1 and Gli2. Two genes of particular interest are alpha smooth muscle actin (*Acta2*) that is a marker of stromal fibroblast and perivascular cells and the steroidogenic gene, cytochrome P450 17a1 (*Cyp17a1*) that is critical for androgen biosynthesis. Adapted from Richards, J.S., Ren, Y.A., Canderlaria, N., Adams, J.E., Rajkovic, A. (2017). Ovarian follicular theca cell recruitment, differentiation and impact on fertility. *Endocrine Reviews* (advanced publication). <https://doi.org/10.1210/er.2017-00164>.

follicles but not to primordial follicles. The LH receptor was only detected in granulosa cells of the preovulatory follicles whereas receptors for follicle stimulating hormone (FSH) were only expressed in granulosa cells. Collectively, these seminal studies led to the two-cell, two gonadotropin theory of steroidogenesis; namely that theca endocrine cells bind LH and produce androgens whereas the granulosa cells bind FSH and produce estradiol (Richards, 1994). Thus, the theca endocrine cells are homologous to the LH responsive, androgen-producing Leydig cells of the testis.

In the ovary, the theca and granulosa endocrine cells control the levels of steroids not only within the follicle but also in the peripheral blood to act on cells in the hypothalamus and pituitary to control the production and release of LH and FSH that control follicle growth and ovulation. Estradiol, in particular, is essential for stimulating the mid-cycle surge of LH that stimulates ovulation (Young and McNeilly, 2010).

Theca Cell Derivation

The Role of GDF9

Recent studies have shown that theca cells within the theca layer of growing follicles are derived from two different sources in the embryo (Liu et al., 2015). One source is comprised of cells migrating into the embryonic ovary from a region called the mesonephros that eventually gives rise to the kidney and adrenal as well as contributing precursor cells to the gonads. The other source is cells present in the medullary region of the embryonic ovary itself. These fetal progenitor cells differentiate into what are known as adult theca cells. Those from the mesonephros become the steroidogenic androgen-producing cells; those from the ovary become theca fibroblasts, perivascular smooth muscle cells, and interstitial ovarian tissue.

A major discovery about what factors regulate the formation of the theca layer came in 1996 when Dong et al. (1996) reported that ovarian function was disrupted in transgenic mutant mice in which a factor, growth differentiation factor 9 (GDF9) was genetically deleted. Because GDF9 is produced by the oocyte of growing follicles, these observations provided the first evidence that the oocyte was controlling the recruitment of theca cells to the developing follicle. Hence, theca cells in the *Gdf9* null mice fail to organize around the granulosa cells to form a follicle, and as a consequence, ovarian follicular growth is arrested at the primary, small pre-antral stage (Dong et al., 1996). Arrested follicular development was also observed in sheep and humans harboring mutations in the *GDF9* gene (Young and McNeilly, 2010). These observations provided the first evidence that a factor from the oocyte was regulating the function and formation of the theca cell layer (Dong et al., 1996). Recent studies have shown that the effects of GDF9 on theca cell organization and function are mediated, at least in part, by the induction and activation of hedgehog (HH) signaling pathways (Liu et al., 2015) (Fig. 1).

The Role of Hedgehog Signaling

The HH signaling pathway has many components and acts to control local cell–cell interactions and patterning. The extracellular ligands called Desert hedgehog (DHH), Indian hedgehog (IHH) are expressed selectively in granulosa cells of growing follicles. These ligands bind the HH cell surface transmembrane receptors PATCHED 1 (PTCH1) and PTCH2 present in theca cells as well as in interstitial/fibroblast and/or perivascular cells within the theca layer. This paracrine activation of PTCH1/2 releases PTCH1/2 inhibition of Smoothened (SMO), a G protein coupled receptor, leading to down-stream induction and/or activation of the transcription factors GLI1, 2, or 3. GLI1 originally isolated from glioblastoma cells and is known to regulate many genes and in some contexts can be an oncogene. Based on elegant cell lineage analyses, GLI1 is now a known marker of theca precursor cells that differentiate into the theca steroidogenic cells in the adult ovary (Liu et al., 2015). Activation of SMO also leads to the induction of inhibitory factors, *Ptch2* and hedgehog interacting protein, *Hhip*, providing autocrine negative regulatory loops within the follicle. HH signaling is thus tightly controlled at a local level within the follicle.

Interestingly, GLI1, 2, and 3 are also markers of Leydig precursor cells in the testis (DeFalco et al., 2011) indicating that there are parallels between the neonatal and embryonic development of the ovary and testis, respectively, with the main differences being related to when these factors and pathways are activated in the developing gonads. For example, Morohashi and colleagues identified a fetal Leydig cell specific enhancer that drives expression of the transcription factor SF-1 in the fetal testis. When female mice expressing the same EGFP-tagged-fetal Leydig enhancer were analyzed, EGFP positive cells were identified not in the fetal ovary but rather in the postnatal ovary that represented theca cells and interstitial cells, some of which were positive for 3-beta hydroxysteroid dehydrogenase/isomerase (3βHSD) and were steroidogenic whereas other cells were not (Shima et al., 2011). These results support observations that there are steroidogenic and non-steroidogenic cells within the theca layer that likely derive from two different progenitor cell types in the embryonic and neonatal ovaries (Liu et al., 2015). Furthermore, overexpression of the HH pathway in the fetal ovary of *Sf-1/Cre; Smo^{YFP}* mice leads to the appearance of fetal Leydig cells, premature increases in androgen production and female pseudohermaphroditism in the female mutant mice (Barsoum et al., 2009). A similar phenotype was not induced in the *Amhr2-Cre; Smo* mutant mice (Ren et al., 2012) indicating that *Amhr2* is not as strongly expressed in steroidogenic precursor theca cells but rather is a more potent driver theca precursor cells that give rise to components of the stroma, including fibroblasts and perivascular smooth muscle cells. Because *Amhr2* is expressed in the embryonic (E17.5) gonad and in some cells of the theca and stroma of the adult ovary, it is also possible that the effects of over-expressing SMO are developmentally stage dependent and precursor cell specific.

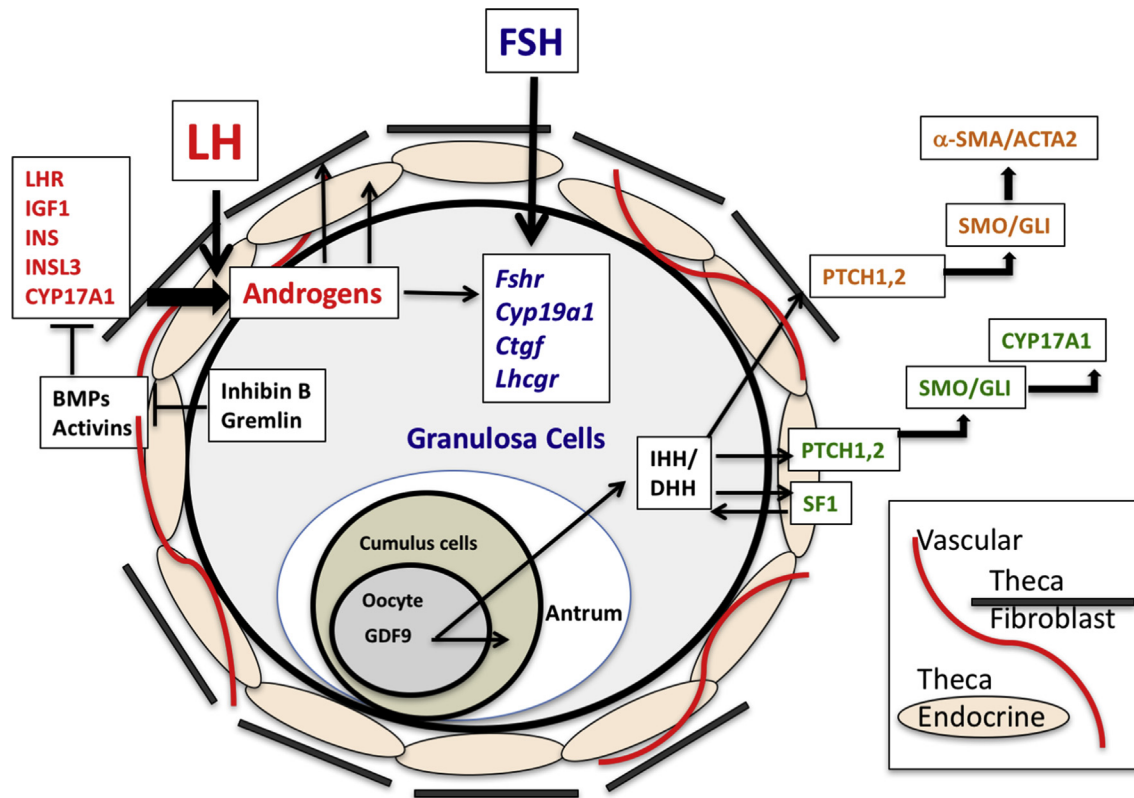


Fig. 2 Theca cell production of androgens, primarily testosterone, is critical of normal follicular development. Androgen biosynthesis is increased not only by the HH signaling pathway but also by luteinizing hormone (LH), insulin (INS), insulin-like growth factor 1 (IGF1), and insulin like 3 signaling pathways. Conversely, members of the BMP/TGF β family can antagonize theca cell androgen biosynthesis. Androgens impact follicular development by binding to androgen receptors (AR) present in granulosa cells, theca cells, and stromal cells. In granulosa cells androgens regulate expression of the FSH receptor (*Fshr*) and connective tissue growth factor (*Ctgf*) and in the presence of FSH regulate the expression of aromatase (*Cyp19a1*) and the LH receptor (*Lhcgr*), markers of preovulatory follicles. The genes regulated by androgens and AR in theca cells and stromal cells remain to be identified. HH signaling also controls expression of *Acta2* in theca fibroblasts and endothelial cells. Adapted from Richards, J.S., Ren, Y.A., Canderlaria, N., Adams, J.E., Rajkovic, A. (2017). Ovarian follicular theca cell recruitment, differentiation and impact on fertility. *Endocrine Reviews* (advanced publication). <https://doi.org/10.1210/er.2017-00164>.

Mouse models in which SMO is over-expressed have revealed key functions of the HH pathway, particularly in regulating genes within the steroidogenic pathway including *Cyp11a1* and *Cyp17a1*. CYP11A1 controls the first step in steroidogenesis, namely the conversion of cholesterol to pregnenolone that is eventually, by a series of enzymes including CYP17A1, converted to testosterone. Over-activation of SMO also leads to the absence of perivascular smooth muscle cells in the theca of growing follicles. The lack of α SMA is evident as early as postnatal day 8, continues throughout follicle development and is associated with defective ovulation (Ren et al., 2009). Thus, over-activation of SMO in the embryonic ovary appears to enhance the functions of theca endocrine precursor cells but suppresses functions of fibroblasts and vascular smooth muscle cells (Fig. 2).

Recent studies also show that the oocyte-derived factor GDF9 regulates the expression of DHH and IHH in granulosa cells, thereby providing a potential explanation for why loss of GDF9 disrupts theca cell recruitment and proliferation, steroidogenesis, and hence follicle growth (Liu et al., 2015). As proof of principle, genetic disruption of the *Dhh* and *Ihh* in mice in ovarian cells markedly reduces expression of steroidogenic enzymes, including CYP17A1, and levels of testosterone and progesterone. Loss of DHH and IHH also reduces α -SMA/ACTA2 expression and impairs follicular growth (Liu et al., 2015). Thus, the HH signaling pathways impacts many aspects of the cell function and follicular development.

Theca Endocrine Cell Differentiation and Function

The nuclear receptor SF-1 or NR5A1 regulates many steroidogenic genes in the gonads and adrenal. Mice null for *Sf-1* revealed that SF1 has far reaching functions in endocrine cells: the mice are infertile and lack gonads, pituitary gonadotrophs, as well as adrenal glands (Young and McNeilly, 2010). Based on deletion studies, DHH and IHH are implicated in regulating the differentiation and steroidogenic capacity of endocrine cells including adrenal, theca, and Leydig cells, in part, by regulating the expression of *Sf-1*/*Nr5a1* and steroidogenic enzymes *Cyp11a1* (Liu et al., 2015). Over-expression of HH signaling by *Amhr2-Cre* driven expression

of SMO leads to abnormal expression of *Shh*, *Star*, and *Cyp21a1* in what appear to be adrenal-like cells in the day 2 postnatal ovary but does not alter steroidogenesis in ovaries of adult mice (Ren et al., 2012). By contrast, activation of HH signaling with *Sf-1/Cre*; *Smo^{YFP}* leads to the induction of Leydig cells in the fetal ovary and female mice that exhibit pseudohermaphroditism (Barsoum et al., 2009). These results indicate that activation of HH signaling in theca cells impacts steroidogenesis in a stage, cell, and context specific manner.

Recent studies have further shown that theca “stem” cells can be isolated from neonatal mouse ovaries and stimulated to differentiate in culture or when transplanted to a recipient host ovary in vivo (Honda et al., 2007). Specifically, theca stem cells cultured in serum free media expressed theca cell markers, *Ptch1* and *Gli3* but were morphologically undifferentiated, and non-steroidogenic. When serum was added to the culture media, small lipid droplets accumulated and LH receptor (*Lhcgr*) mRNA was noted. However, the most dramatic changes were observed when theca stem cells were cultured in granulosa cell conditioned media or were co-culture with granulosa cells. Under these conditions, the theca stem cells acquired a steroidogenic morphology (lipid droplets and smooth endoplasmic reticulum), increased expression of *Lhcgr* and *Gli2*, and elevated androstenedione production. When EGFP-tagged theca stem cells were transplanted to an ovary of a recipient mouse in vivo, EGFP+ cells were found in the theca layer of growing follicles indicating that they had been recruited to growing follicles. It is likely that these theca stem cells differentiate not only in response to IHH/DHH but also to LH and other granulosa cell-derived factors, including insulin like growth factor, stem cell factor (kit-ligand), epidermal growth factors, as well as by intrafollicular regulatory factors including inhibins, activins, and BMPs (Glister et al., 2013; Young and McNeilly, 2010) (Fig. 2).

Other Regulators of Theca Cell Function

Insulin-like 3 (INSL3) is expressed at high levels in testicular Leydig cells and is critical for testicular descent in males. INSL3 is also expressed in and impacts theca cell functions in the ovary (Glister et al., 2013). Specifically, INSL3 and its receptor (relaxin/insulin-like family peptide receptor 2; RXFP2) appear to modulate LH-mediated androgen biosynthesis. INSL3 null female mice exhibit increased apoptosis and luteal regression whereas over-expression of INSL3 in fetal and neonatal INSL3 null mice leads to ovarian descent within the peritoneum but does not impair ovarian function. The effects of LH and INSL3 are antagonized dramatically by activin and BMPs (BMP2, 4, 6, 7) that are produced by granulosa cells and/or oocytes (Glister et al., 2013; Young and McNeilly, 2010). Inhibin B blocks the inhibitory effects of activin and BMPs, thereby creating a local regulatory network within growing follicles (Young and McNeilly, 2010). BMPs are also blocked by the BMP antagonist gremlin that is induced by oocyte-derived GDF9 but markedly reduced by loss of *Smad1/5* or *Bmpr1* in granulosa cells (Edson et al., 2010), providing another local regulatory network, especially in small follicles and within the cumulus cell–oocyte complex of larger follicles.

Circadian rhythms impact reproduction as evidenced by more than 4000 publications listed in PubMed. At the cellular level circadian rhythms are controlled by the molecular clock, a feedback loop of transcription factors that impacts the cyclic expression of target genes in peripheral tissues. Two of these factors, CLOCK (circadian locomotor output cycle kaput) and BMAL1 (brain and muscle ARNT-like protein 1), impact reproductive function not only at the hypothalamic-pituitary level but also in the ovary (Mereness et al., 2015). Theca cell specific disruption of *Bmal1* gene in mice expressing *Cyp17-Cre* leads to changes in the circadian expression of the LH receptor (*Lhcgr*), reduced sensitivity of the ovaries to LH and subfertility, suggesting that BMAL1 acts as a pacemaker of LH sensitivity (Mereness et al., 2015). Disruption of *Bmal1* in mice expressing *Sf1-Cre* blocks expression of *Star*, progesterone production in corpora lutea and implantation, indicating that BMAL1 also impacts steroidogenesis.

Androgens produced by theca cells exert paracrine functions within the ovary by binding and activating the androgen nuclear receptor (AR/NR3C4) that is present in multiple cell types within the ovary (Lenie and Smitz, 2009) including granulosa cells, theca cells, and stromal cells. Targeted disruption of the androgen receptor (*AR*; *Nr3c4*) in granulosa cells impairs follicular development (Lebbe and Woodruff, 2013). Androgens are made in small follicles and regulate the expression of FSH receptor (*Fshr*) leading to early follicle growth and eventually, with FSH, they promote expression of *Cyp19a1* (aromatase) and *Lhcgr* in granulosa cells of antral follicles (Young and McNeilly, 2010). Moreover, androgens act in granulosa cells via AR canonical and non-canonical pathways to promote cell survival (Sen et al., 2014). However, the role of androgens in other cell types (theca, stromal, fibroblastic, immune) within the ovary is less well characterized but appears to impact not only normal processes in the ovary but also the ovarian phenotype of women with PCOS (see below).

Structural and Matrix-Related Functions of Theca Cells

The theca layer of growing follicles provides a structural framework that is largely composed extracellular matrix factors, including different types of collagen, vimentin, vascular cell adhesion molecule 1, laminin, fibronectin, proteoglycans, tenascin C, and fibrillins. Although the matrix appears highly structured, it is not static. Rather the extracellular matrix within the theca of growing follicles undergoes dynamic changes during follicular development (Irving-Rodgers et al., 2010) and contributes to follicular function. Pioneering studies of Woodruff and Shea have shown that the rigidity of the extracellular matrix impacts not only follicle growth but also its steroidogenic potential (Woodruff and Shea, 2011). Using a tissue-engineered artificial matrix made of alginate, they have also documented that follicles grown in this extracellular milieu produce live, fertile offspring. Moreover small follicles cultured in

the presence of stroma, containing theca cells and macrophages, grow better and survive longer than follicles cultured in the absence of stroma indicating that the dependence of follicles on the ovarian stroma is stage dependent (Tingen et al., 2011).

Based on these studies, it is not surprising that the composition of the theca matrix undergoes constant remodeling and that expression of factors within the theca and that impact the matrix change during follicle growth and apoptosis (Hatzirodos et al., 2014a,b). However, microarray analyses have revealed that the gene expression patterns in bovine theca cells exhibit fewer changes during follicular growth and atresia than the gene expression patterns in bovine granulosa cells (Hatzirodos et al., 2014a,b). In particular, the microarray analyses reveal that theca derived from small and large bovine follicles express not only collagen 1 (COL1A1) but also many other types of collagen. Also of note, theca of small follicles express neuronal cell adhesion molecule (NCAM1) and intercellular adhesion molecule (ICAM1) that likely impact vascular cells; theca of large follicles also express vascular cell adhesion molecule (VCAM1) that helps recruit immune cells, is a marker of theca cell lineage in the embryonic mouse gonad (DeFalco et al., 2011), and is a potential androgen target gene (Death et al., 2004; Solano et al., 2011). Connective tissue growth factor (CTGF) that is an androgen target gene in murine granulosa cells is also expressed in bovine theca (Hatzirodos et al., 2014a). Although marked changes in gene expression patterns were not noted in bovine theca cells from small and large follicles, the tight junction protein Claudin11 was the most highly up-regulated gene in theca of large follicles (Hatzirodos et al., 2014a). The function of CLDN11 in theca cells is not known but may impact vascular permeability; it may also be an androgen target gene.

Bovine theca cells of small follicles undergoing atresia do not exhibit increased expression of genes or factors mediating apoptosis (Hatzirodos et al., 2014b), such as cleaved-caspase 3, BMP2, and FOXO1 as is observed in granulosa cells of mouse and presumably other species. Rather, the theca exhibited changes in genes related to cell cycle arrest and DNA replication (Hatzirodos et al., 2014b). In addition, the expression of cytokines, including interleukin IL6 and a macrophage related protein, glycoprotein non-metastatic melanoma protein B/osteostatin (GPNMB/OA) were increased (Hatzirodos et al., 2014b).

Vascular and Immune Cells in the Theca

The vascular network in the theca layer is essential for providing nutrients to the theca cells as well as to the avascular granulosa cells, cumulus cells, and oocyte. Although critical, little is known about what regulates the vascular tissue in the theca during follicular growth and what factors exclude vascular invasion of the granulosa cell layer until after ovulation. However, vascular endothelial growth factor (VEGF) is expressed in granulosa cells and is one important factor (Young and McNeilly, 2010). Targeted disruption of VEGF in granulosa cells or over-expression of an anti-angiogenic splice variant of VEGF leads to profound changes in follicular development and reduced fertility (Sargent et al., 2015). Based on bovine theca microarrays there appear to be only minor changes in vascular markers in theca of small and large follicles suggesting that the maintenance of vascular tissue is relatively stable and critical during follicle growth. However, markers of vascular cells are clearly evident in the theca (Hatzirodos et al., 2014a). As noted above, VCAM1 is higher in theca of large follicles (Hatzirodos et al., 2014a), perhaps due to increased androgen levels. Immune cell markers are also present, including members of the complement pathway (Hatzirodos et al., 2014a). Furthermore, the number of immune cells, namely macrophages, associated with growing follicles increases during the pre-pubertal period in mice and these macrophages are associated not only with apoptotic follicles but also with healthy secondary follicles (Tingen et al., 2011). Clearly, additional studies are needed to fully characterize what controls the vascular tissue and immune cell functions in the theca of growing follicles. It is highly likely that there are specific molecular markers that characterize the vascular tissue and immune cells associated with follicles at defined stages of growth (Nolan Daniel et al., 2013).

By contrast to events in small growing follicles, marked changes occur in gene expression profiles in the theca following LH induction of ovulation (Christenson et al., 2013). In addition, there are major changes in the follicular vasculature following ovulation when vessels rapidly invade the granulosa cell layer and contribute to the formation of the corpus luteum (Richards et al., 2015). Targeted disruption of the MAP kinases (MAPK1/3; ERK1/2) in granulosa cells prevents not only ovulation but also the invasion of vascular cells into the granulosa cell layer. These observations provide evidence that the MAPK pathway is essential for LH induction of ovulation and that vascular invasion does not occur in the absence of ovulation. Additionally, targeted disruption of genes encoding the transcription factors, CCAAT enhancer binding proteins alpha and beta (*Cebpa* and *Cebpb*) in granulosa cells also blocks ovulation and vascular invasion (Richards et al., 2015). Gene expression profiles in granulosa cells of these mice document that specific genes other than, or in addition to VEGF, are markedly increased during ovulation and likely contribute to the vascular invasion of the granulosa cell compartment. Immune cells are essential for this process, based on evidence that depletion of immune cells either by clodronate treatment (Turner et al., 2011) or diphtheria toxin mediated immune cell disruption (Care et al., 2013), prior to ovulation blocks corpus luteum formation. The recruitment of immune cells is dependent on follicular expression of cytokines and chemokines, including IL6 and CCL20 (Adams et al., 2016; Al-Alem et al., 2015), and expression of these factors appears to depend on LH, androgens, and IL6 (Adams et al., 1991).

Theca Cell Functions in Disease

Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is the leading cause of anovulatory infertility in women in their reproductive years (Diamanti-Kandarakis and Dunaif, 2012; Rosenfield and Ehrmann, 2016). Patients with PCOS exhibit excess androgens (hyperandrogenemia),

the defining characteristic of this endocrine disorder (Diamanti-Kandarakis and Dunaif, 2012; Rosenfield and Ehrmann, 2016). The underlying causes of excess androgen appear to be multi-faceted but elevated theca production of androgens is considered a key event in determining the PCOS phenotype and theca cells derived from follicles of PCOS patients exhibit elevated, intrinsic androgen production and secrete higher levels of DHEA and 17OH progesterone in response to LH compared to theca cells of normal patients. Many other genes are also differentially expressed in theca of PCOS compared to non-PCOS patients. A genome-wide screen identified an DENND1A isoform variant V2 that, when over-expressed in normal theca cells in culture led to elevated androgen production and responses to LH (McAllister et al., 2014). Moreover, when this isoform was expressed in mice, it recapitulated a PCOS ovarian phenotype in vivo (McAllister et al., 2014).

Elevated androgens in PCOS follicles alter not only the functions of theca and granulosa cell obtained from patients undergoing IVF but also may impact premature arrest follicle growth that leads to the anovulatory phenotype of PCOS (Rosenfield and Ehrmann, 2016). The mechanisms by which elevated androgens lead to follicle arrest in PCOS are not entirely clear but may be related to an increase in AR mediated expression of FSHR and increased responsiveness of small growing follicles to FSH leading to premature differentiation and luteinization. Alternatively, elevated AMH in the arrested follicles appears to impact and reduce early follicle growth by antagonizing FSH action.

Importantly, the expression of AR is not restricted to granulosa cells. Rather, AR is present in multiple cells types within the ovary (theca, fibroblast, stroma, immune, and endothelial cells), can act on these cells, and contribute to the multifaceted effects of androgens on the PCOS ovarian phenotype. For example, the ovarian stroma of PCOS patients is characterized by increased fibroblast proliferation, collagen deposition, and thus inherent rigidity (Lebbe and Woodruff, 2013). Increased rigidity provides another explanation for follicle growth arrest in PCOS (Lebbe and Woodruff, 2013).

Androgens can also stimulate the expression of VEGFA in PCOS ovaries that impacts vascular cell growth (Young and McNeilly, 2010). As mentioned above, VEGFA and its proangiogenic and anti-angiogenic splice variants have been identified in the ovary. Targeted disruption of VEGF in granulosa cells in mice leads to impaired follicular development and expression of specific genes. An anti-angiogenic splice variant of VEGF is expressed in theca cells of marmosets. Over-expression of this anti-angiogenic splice variant in the ovary of mice impaired follicular development leading to increased follicular atresia (Qiu et al., 2012).

In addition, there appears to be a functional link between elevated androgens in PCOS patients and inflammatory conditions both at the systemic level, within the stroma and granulosa cells of ovulatory follicles of PCOS patients undergoing IVF (Schmidt et al., 2014), especially those that are obese (Adams et al., 2016). Specifically, the expression of many cytokines and chemokines, such as IL6, IL7, CCL20, are elevated in granulosa cells isolated from PCOS patients compared to non-PCOS patients undergoing IVF (Adams et al., 2016; Al-Alem et al., 2015). These observations document that there is an enhanced local, intra-ovarian inflammatory response associated with a PCOS patients, many of whom are obese (Adams et al., 2016). The elevated levels of cytokines appear to be associated, in part, with the enhanced local recruitment of CD45⁺ immune cells because the cytokine profile in follicular fluid differs from that observed in peripheral serum (Adams et al., 2016). Whether theca-derived androgens directly alter immune cells is not yet clear. However, that androgen receptors are present in immune cells and can exert specific functions may explain, in part, the proinflammatory condition of PCOS.

Animal models of PCOS have been established in mice, rats, primates, and sheep. These models are based mainly on elevating androgens prenatally or post-natally and mimic many but not all aspects of the human condition. Importantly, these studies indicate that androgens may exert epigenetic effects leading to metabolic syndrome and anovulation associated with PCOS (Rosenfield and Ehrmann, 2016; Walters et al., 2012).

Premature Ovarian Failure

Although most causes of POF remain idiopathic and unknown, it is clear that disruption of theca cell derivation or function during embryonic and post-natal periods can lead to abnormal follicle formation and growth. As mentioned above, depletion of the oocyte derived factor GDF9 in mice prevents the organization of the theca cell layer leading to follicle growth arrest, POF, and infertility (Dong et al., 1996). Mutations in the human *GDF9* gene have been reported and are associated with POF (Laissue et al., 2008), providing evidence that GDF9 is critical for ovarian function in women. One would predict that disruption or mutation of factors regulating GDF9 expression, levels, or activity would also lead to POF. Of relevance, newborn ovary homeobox (NOBOX) protein that is expressed in the oocyte, and if deleted, leads to impaired expression of *GDF9* and follicle growth arrest (Rajkovic et al., 2004). Moreover, the *GDF9* gene is a direct target of NOBOX. Recent studies have further shown that some women (6.2% of a cohort analyzed) have mutations in the *NOBOX* gene and these women present POF (Bouilly et al., 2014). Significantly, although disruption of *Ihh* and *Dhh* or over-expression of SMO in ovarian cells in mice is associated with abnormal theca cell development and function, mutations in the HH signaling pathway genes have not yet been linked to POF in women.

References

- Adams, S. R., Harootunian, A. T., Buechler, Y. J., Taylor, S., & Tsien, R. (1991). Fluorescence ratio imaging of cyclic AMP in single cells. *Nature*, 349, 694–697.
- Adams, J., Liu, Z., Ren, Y. A., Wun, W.-S., Zhou, W., et al. (2016). Enhanced inflammatory transcriptome in granulosa cells of women with polycystic ovarian syndrome. *The Journal of Clinical Endocrinology and Metabolism*, 101, 3459–3468. PMID: 27228368.
- Al-Alem, L., Puttabyatappa, M., Rosewell, K., Brännström, M., Akin, J., et al. (2015). Chemokine ligand 20: A signal for leukocyte recruitment during human ovulation? *Endocrinology*, 156, 3358–3369. PMID: 26125463.

- Barsoum, I. B., Bingham, N. C., Parker, K. L., Jorgensen, J. S., & Yao, H. H. C. (2009). Activation of the hedgehog pathway in the mouse fetal ovary leads to ectopic appearance of fetal leydig cells and female pseudohermaphroditism. *Developmental Biology*, 329, 96–103. PMID: 19268447.
- Bouilly, J., Roucher-Boulez, F., Gompel, A., Bry-Gaillard, H., Azibi, K., et al. (2014). New NOBOX mutations identified in a large cohort of women with primary ovarian insufficiency decrease KIT-L expression. *The Journal of Clinical Endocrinology & Metabolism*, 100, 994–1001. PMID: 25514101.
- Care, A. S., Diener, K. R., Jasper, M. J., Brown, H. M., Ingman, W. V., et al. (2013). Macrophages regulate corpus luteum development during embryo implantation in mice. *The Journal of Clinical Investigation*, 123, 3472–3487. PMID: 23867505.
- Christenson, L. K., Gunewardena, S., Hong, X., Spitschak, M., Baufeld, A., et al. (2013). Research resource: Preovulatory LH surge effects on follicular theca and granulosa transcriptomes. *Molecular Endocrinology*, 27, 1153–1171. PMID: 23716604.
- Death, A. K., McGrath, K. C. Y., Sader, M. A., Nakhla, S., Jessup, W., et al. (2004). Dihydrotestosterone promotes vascular cell adhesion molecule-1 expression in male human endothelial cells via a nuclear factor- κ B-dependent pathway. *Endocrinology*, 145, 1889–1897. PMID: 14684616.
- DeFalco, T., Takahashi, S., & Capel, B. (2011). Two distinct origins for leydig cell progenitors in the fetal testis. *Developmental Biology*, 352, 14–26. PMID: 21255566.
- Diamanti-Kandarakis, E., & Dunaif, A. (2012). Insulin resistance and the polycystic ovary syndrome revisited: An update on mechanisms and implications. *Endocrine Reviews*, 33, 981–1030. PMID: 23065822.
- Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N., et al. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, 383, 531–535. PMID: 8849725.
- Edson, M. A., Nalam, R. L., Clementi, C., Franco, H. L., Demayo, F. J., et al. (2010). Granulosa cell-expressed BMP1A and BMP1B have unique functions in regulating fertility but act redundantly to suppress ovarian tumor development. *Molecular Endocrinology*, 24, 1251–1266. PMID: 20363875.
- Glistler, C., Satchell, L., Bathgate, R. A. D., Wade, J. D., Dai, Y., et al. (2013). Functional link between bone morphogenetic proteins and insulin-like peptide 3 signaling in modulating ovarian androgen production. *Proceedings of the National Academy of Sciences of the United States of America*, 110, E1426–E1435. PMID: PMC3625357.
- Hatzirados, N., Hummitzsch, K., Irving-Rodgers, H. F., & Rodgers, R. J. (2014a). Transcriptome profiling of the theca interna in transition from small to large antral ovarian follicles. *PLoS One*, 9, e97489. PMID: 24830430.
- Hatzirados, N., Irving-Rodgers, H. F., Hummitzsch, K., & Rodgers, R. J. (2014b). Transcriptome profiling of the theca interna from bovine ovarian follicles during atresia. *PLoS One*, 9, e99706. PMID: 2495638.
- Honda, A., Hirose, M., Hara, K., Matoba, S., Inoue, K., et al. (2007). Isolation, characterization, and in vitro and in vivo differentiation of putative thecal stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 12389–12394. PMID: PMC1941479.
- Irving-Rodgers, H. F., Hummitzsch, K., Murdiyaro, L. S., Bonner, W. M., Sado, Y., et al. (2010). Dynamics of extracellular matrix in ovarian follicles and corpora lutea of mice. *Cell and Tissue Research*, 339, 613–624. PMID: PMC2831189.
- Laissue, P., Vinci, G., Veitia, R. A., & Fellous, M. (2008). Recent advances in the study of genes involved in non-syndromic premature ovarian failure. *Molecular and Cellular Endocrinology*, 282, 101–111. PMID: 18164539.
- Lebbe, M., & Woodruff, T. K. (2013). Involvement of androgens in ovarian health and disease. *Molecular Human Reproduction*, 19, 828–837. PMID: 24026057.
- Lenie, S., & Smits, J. (2009). Functional AR signaling is evident in an in vitro mouse follicle culture bioassay that encompasses most stages of folliculogenesis. *Biology of Reproduction*, 80, 685–695. PMID: 19152831.
- Liu, C., Peng, J., Matzuk, M. M., & Yao, H. H. C. (2015). Lineage specification of ovarian theca cells requires multi-cellular interactions via oocyte and granulosa cells. *Nature Communications*, 6, 6934. PMID: PMC4413950.
- McAllister, J. M., Modi, B., Miller, B. A., Biegler, J., Bruggeman, R., et al. (2014). Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype. *Proceedings of the National Academy of Sciences of the United States of America*, 111, E1519–E1527. PMID: 24706793.
- Mereness, A. L., Murphy, Z. C., Forrestel, A. C., Butler, S., Ko, C., et al. (2015). Conditional deletion of Bmal1 in ovarian theca cells disrupts ovulation in female mice. *Endocrinology*, 157, 913–927. PMID: 26671182.
- Nolan Daniel, J., Ginsberg, M., Israely, E., Palikuqi, B., Poulos, M. G., et al. (2013). Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. *Developmental Cell*, 26, 204–219. PMID: 23871589.
- Qiu, Y., Seager, M., Osman, A., Castle-Miller, J., Bevan, H., et al. (2012). Ovarian VEGF(165)b expression regulates follicular development, corpus luteum function and fertility. *Reproduction (Cambridge, England)*, 143, 501–511. PMID: PMC3325318.
- Rajkovic, A., Pangas, S. A., Ballow, D., Suzumori, N., & Matzuk, M. M. (2004). Nobox deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science*, 305, 1157. PMID: 15326356.
- Ren, Y., Cowan, R. G., Harman, R. M., & Quirk, S. M. (2009). Dominant activation of the hedgehog signaling pathway in the ovary alters theca development and prevents ovulation. *Molecular Endocrinology*, 23, 711–723. PMID: 19196835.
- Ren, Y., Cowan, R. G., Migone, F. F., & Quirk, S. M. (2012). Overactivation of hedgehog signaling alters development of the ovarian vasculature in mice. *Biology of Reproduction*, 86, 174. PMID: 22402963.
- Richards, J. S. (1994). Hormonal control of gene expression in the ovary. *Endocrine Reviews*, 15, 725–751. PMID: 7705279.
- Richards, J. S., Liu, Z., & Shimada, M. (2015). Ovulation. In T. M. Plant, & A. J. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn, pp. 997–1021). Waltham, MA: Academic Press.
- Rosenfield, R. L., & Ehrmann, D. A. (2016). The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited. *Endocrine Reviews*, 37, 467–520. PMID: 27459230.
- Sargent, K. M., Lu, N., Clopton, D. T., Pohlmeier, W. E., Brauer, V. M., et al. (2015). Loss of vascular endothelial growth factor A (VEGFA) isoforms in granulosa cells using pDmrt-1-Cre or Amhr2-Cre reduces fertility by arresting follicular development and by reducing litter size in female mice. *PLoS One*, 10, e0116332. PMID: PMC4320103.
- Schmidt, J., Weijdegård, B., Mikkelsen, A. L., Lindenberg, S., Nilsson, L., et al. (2014). Differential expression of inflammation-related genes in the ovarian stroma and granulosa cells of PCOS women. *Molecular Human Reproduction*, 20, 49–58. PMID: 23900753.
- Sen, A., Prizant, H., Light, A., Biswas, A., Hayes, E., et al. (2014). Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microrna-125b expression. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 3008–3013. PMID: 24516121.
- Shima, Y., Miyabayashi, K., Baba, T., Otake, H., Oka, S., et al. (2011). Identification of an enhancer in the Ad4BP/SF-1 gene specific for fetal Leydig cells. *Endocrinology*, 153, 417–425. PMID: 22128023.
- Solano, M. E., Sander, V. A., Ho, H., Motta, A. B., & Arck, P. C. (2011). Systemic inflammation, cellular influx and up-regulation of ovarian VCAM-1 expression in a mouse model of polycystic ovary syndrome (PCOS). *Journal of Reproductive Immunology*, 92, 33–44. PMID: 22018827.
- Tingen, C. M., Kiesewetter, S. E., Jozefik, J., Thomas, C., Tagler, D., et al. (2011). A macrophage and theca cell-enriched stromal cell population influences growth and survival of immature murine follicles in vitro. *Reproduction (Cambridge, England)*, 141, 809–820. PMID: 21389078.
- Turner, E. C., Hughes, J., Wilson, H., Clay, M., Mylonas, K. J., et al. (2011). Conditional ablation of macrophages disrupts ovarian vasculature. *Reproduction*, 141, 821–831. PMID: 21393340.
- Walters, K. A., Allan, C. M., & Handelsman, D. J. (2012). Rodent models for human polycystic ovary syndrome. *Biology of Reproduction*, 86, 149. PMID: 22337333.
- Woodruff, T. K., & Shea, L. D. (2011). A new hypothesis regarding ovarian follicle development: ovarian rigidity as a regulator of selection and health. *Journal of Assisted Reproduction and Genetics*, 28, 3–6. PMID: 20872066 PMID: PMC23045494.
- Young, J. M., & McNeilly, A. S. (2010). Theca: the forgotten cell of the ovarian follicle. *Reproduction*, 140, 489–504. PMID: 20628033.

The Oocyte

Allison R Grover, Northwestern University, Evanston, IL, United States

Barbara Fegley, University of Kansas Medical Center, Kansas City, KS, United States

Timothy V Duncan, US Food and Drug Administration, Bedford Park, IL, United States

Francesca E Duncan, Northwestern University, Evanston, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

In his book, “*Exercitationes de generatione animalium*,” first published in 1651, the renowned physician scientist William Harvey stated that “*ex ovo omnia*,” or all comes from the oocyte. This statement aptly encompasses the concept that the foundation for embryo development (embryogenesis) lies in oocyte development (oogenesis). The oocyte is one of the largest, rarest, and most vital cells in the human body—capable of giving rise to an entirely new generation. During its growth phase within the ovarian follicle, the oocyte generates maternal products (i.e., organelles, RNAs, and proteins), which are essential for supporting subsequent fertilization and early embryo development (Figs. 1 and 2A). There is a corresponding volumetric expansion due to these accumulated products, and human and mouse oocytes increase in volume > 100-fold and 200-fold, respectively, during their growth phase. In this article, we consider the mammalian oocyte—from its structure to critical biological processes in its development and to factors that influence its quality.

Cellular Architecture

The bulk cytoplasm or volume of the embryo originates from the oocyte rather than from the sperm at the time of fertilization. Therefore, the maternal products that accumulate with the oocyte during its growth phase endow the embryo with materials essential for early development. Here, we describe the key cellular structures within the mammalian oocyte and highlight unique features that make it distinct from other cells.

Germinal Vesicle (Oocyte Nucleus)

The germinal vesicle (GV) is the nucleus of the oocyte (Figs. 1B and 2B). The GV is large and spherical and contains chromatin (DNA) and the nucleolus (Figs. 1B and 2B). The oocyte chromatin is located throughout the GV, and its exact configuration

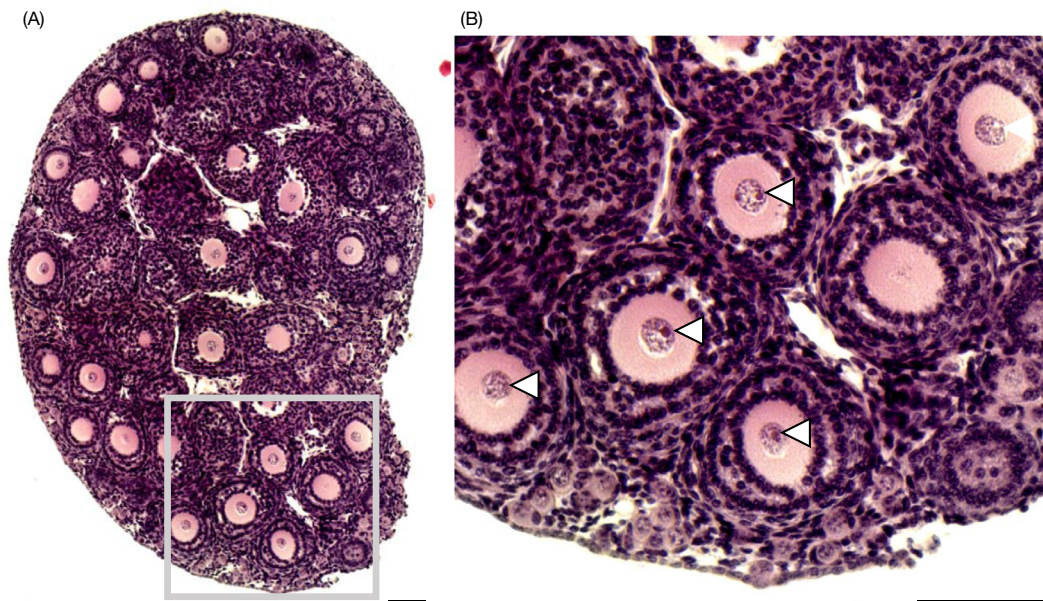


Fig. 1 Histology of a prepubertal mouse ovary showing oocytes within the context of the follicle. (A) A scan of a histological section of a prepubertal mouse ovary (postnatal day 10) stained with hematoxylin and eosin that stains cell nuclei and cytoplasm, respectively. Oocytes within follicles in early stages of development are visible throughout the ovary. The region outlined by the gray box is further magnified in (B). All oocytes within the ovary, regardless of follicle stage, are arrested in prophase of meiosis I and have an intact, visible nucleus that contains DNA. The oocyte nucleus is called the germinal vesicle (GV) and is highlighted by the arrowhead. Scale bars are 100 μ m.

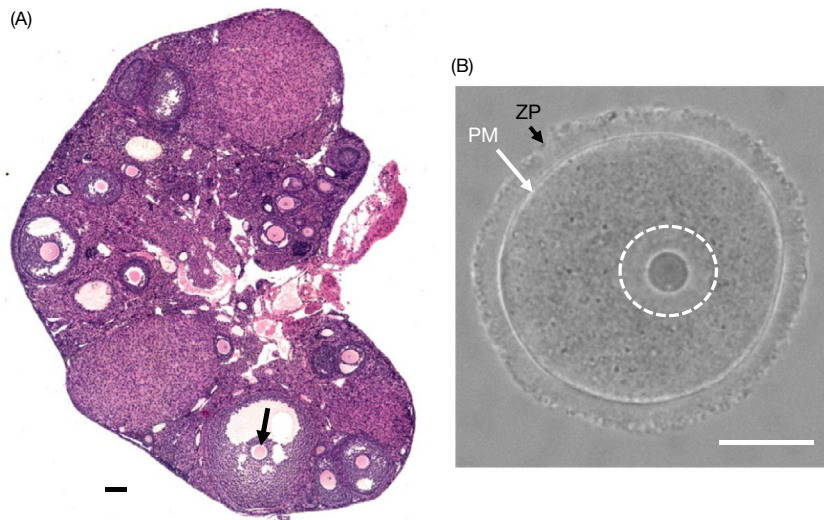


Fig. 2 Histology of an adult mouse ovary and a transmitted light image of a fully grown mouse oocyte. (A) A scan of a histological section of an adult mouse ovary stained with hematoxylin and eosin. Oocytes within follicles at all stages of development are visible, including one in a large antral follicle (*black arrow*). A transmitted light image of a fully grown oocyte that was isolated from a large antral follicle is shown in (B). The oocyte nucleus or germinal vesicle (GV) is outlined with a *dotted white line*, and the nucleolus is visible within it. The oocyte plasma membrane (PM) is highlighted by the *white arrow*, and the zona pellucida (ZP)—or glycoprotein matrix—that surrounds the oocyte is highlighted by the *black arrow*. The scale bar is 100 μm (A) and 25 μm in (B).

depends on the transcriptional activity of the oocyte as described further in the section “**Meiotic Competence**” (Fig. 1B). All oocytes within the ovary are arrested in the dictyate stage of prophase of meiosis I (MI), and this meiotic stage is characterized by the presence of an intact GV, which can be identified morphologically by histology and light microscopy (Figs. 1B and 2B). Oocytes maintain this meiotic arrest throughout oogenesis as they grow in size and accumulate the necessary maternal products to support the completion of meiosis, fertilization, and early preimplantation development. The prophase I arrest is maintained by high intra-oocyte cyclic adenosine monophosphate (cAMP) levels (Jaffe and Egbert, 2017). In the oocyte, cAMP is regulated by the opposing activities of adenylyl cyclase 3 (AC3) and the oocyte-specific phosphodiesterase (PDE3A). AC3 produces cAMP, whereas PDE3A destroys it. In the granulosa cells (mural and cumulus), the NPR2 guanylyl cyclase produces cyclic guanosine monophosphate (cGMP) that diffuses into the oocyte and inhibits PDE3A, thus keeping oocyte cAMP levels high and maintaining meiotic arrest (Jaffe and Egbert, 2017).

Within the GV is the nucleolus, an electron-dense organelle whose prime function is ribosome biogenesis (Figs. 2B, 3, and 4A). Ribosomes are small organelles responsible for protein synthesis. The size of the nucleolus is related to the ribosomal requirements of the cells in which they are found. Because the oocyte has high metabolic demands, it has a very prominent nucleolus that is visible by light microscopy, and some oocytes have multiple nucleoli (Fig. 2B).

The GV is lined by a nuclear envelope, which is supported by the nuclear lamina—a proteinaceous layer composed of lamin proteins situated between the chromatin and inner nuclear membrane (Figs. 2B, 3, and 4B). The nuclear envelope is punctuated by pores that provide the means for the regulated exchange of material between the nucleus and cytoplasm (Figs. 3 and 4B). For example, ribosomal components and newly transcribed RNAs must be transported to the cytoplasm for protein production to occur.

Cytoplasm and Organelles

The cytoplasm of the oocyte contains the many organelles vital to its growth. Early in development, when oocytes are within germ-line cysts or primordial follicles, they contain a large distinctive organelle aggregate called the Balbiani body that consists of mitochondria, endoplasmic reticulum (ER), and Golgi elements (Pepling et al., 2007). However, the Balbiani body disperses later during oocyte growth, and organelles have a broader cytoplasmic distribution. Mitochondria are the energy-producing organelles of the oocyte. Mitochondria increase dramatically in number from a small founder population by fusion or fission during oogenesis. This accumulation is necessary to endow the early embryo with mitochondria, since their biogenesis ceases in the mature oocyte and does not resume until after fertilization (Babayev and Seli, 2015). Mitochondria in the oocyte have three distinct morphologies: those with typical cristae (or folds) seen in somatic cells, those with vacuoles between cristae, and those without cristae (Figs. 3 and 4C; Ruby et al., 1969). Mitochondria are usually in close proximity to the ER.

There are two types of ER in the oocyte. In general, the smooth ER aids in lipid synthesis and transport. In the maturing oocyte, the smooth ER near the oocyte cortex is also a main storage site for intracellular calcium stores, which are ultimately responsible for the rise in cytosolic calcium that occurs during fertilization (Figs. 3 and 4D; Machaty, 2016). The rough ER is distinct from the

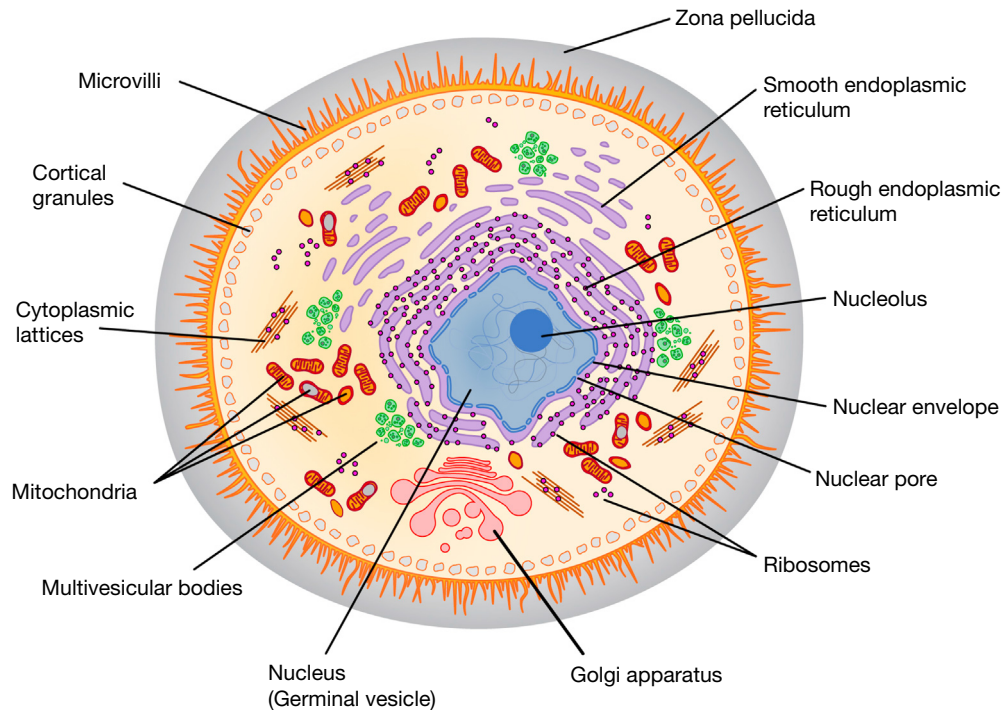


Fig. 3 Schematic of the subcellular architecture of the mammalian oocyte. This schematic highlights the cell biology of the mammalian oocyte, and corresponding structures and organelles are shown at the ultrastructural level in Fig. 4.

smooth ER because it is punctuated with ribosomes and aids in the production and transport of proteins and other biomolecules (Figs. 3 and 4E). In oocytes, the ER is typically poorly developed or connected to neighboring mitochondria, perhaps to aid in protein transport (Ruby et al., 1969). In addition to being associated with the ER, ribosomes are also found on unique structures known as cytoplasmic lattices (CPLs) (Figs. 3 and 4F). CPLs are made of a fibrillar matrix composed of proteins and RNA and could possibly contain intermediate filaments, like keratin (Yurttas et al., 2008). A major protein component of CPLs is peptidylarginine deiminase 6 (Yurttas et al., 2008). As the oocyte grows, CPLs increase, while cytoplasmic ribosomes decrease, suggesting that CPLs function as storage structures for the maternal contribution of ribosomes and RNA to the early embryo.

The Golgi apparatus is composed of a stack of membranous compartments or cisternae and is the organelle responsible for the secretion and intracellular transport of proteins from the ER (Figs. 3 and 4G). The main clusters of Golgi complexes are near the nucleus and on the periphery of the cytoplasm. In the oocyte, the Golgi apparatus is also involved in the generation of other critical structures and organelles. For example, the Golgi apparatus modifies several secreted glycoproteins that form a thick glycoprotein matrix or extracellular coat called the zona pellucida (ZP) (Figs. 2B and 3, discussed further later). Vesicles of hypertrophied Golgi complexes also fuse and become multivesicular bodies (MVBs) or cortical granules (CGs) (Liu, 2011). MVBs are late-stage endosomes that have intraluminal vesicles contained within larger vesicles. MVBs can target their contents to lysosomes for degradation. In addition, when MVBs fuse with the plasma membrane, they release their internal vesicles, which are called exosomes. Exosomes are now recognized as a new form of intercellular communication as they can contain several types of molecules, including miRNAs, proteins, and lipids. MVBs are clearly prominent within the oocyte cytoplasm, and their exact biological function is an active area of research (Figs. 3 and 4H). Hypertrophied Golgi clusters also form CGs, which are specialized organelles that are essential for proper fertilization. CGs form a monolayer-to-multilayer sheath beneath the plasma membrane (Figs. 3 and 4D). In the mature egg, upon fertilization and under the influence of a rise in intracellular calcium levels, CGs exocytose their contents in what is known as the cortical reaction (Liu, 2011). The CGs contain a protease, ovastacin, which cleaves ZP proteins in such a manner that modifies the glycoprotein matrix and prevents multiple sperm from entering the egg (i.e., polyspermy). Zinc-containing granules, which may be CGs or a subset of CGs, release zinc at the time of fertilization, and this zinc release also modifies the ZP to prevent polyspermy (Que et al., 2017).

Microvilli, Transzonal Projections, and the Zona Pellucida

The oocyte plasma membrane is covered by microvilli or small actin-rich membrane protrusions (Figs. 3 and 4D). In the mature egg, the microvilli become asymmetrically distributed, and sperm-egg interactions take place predominantly or exclusively in the region of the egg surface that contains microvilli (Calarco, 1991). The oocyte plasma membrane is surrounded by the ZP (Figs. 2B and 3). At the peak of protein synthesis in the oocyte, 5% of all proteins are destined for ZP production. More than 95% of the ZP is

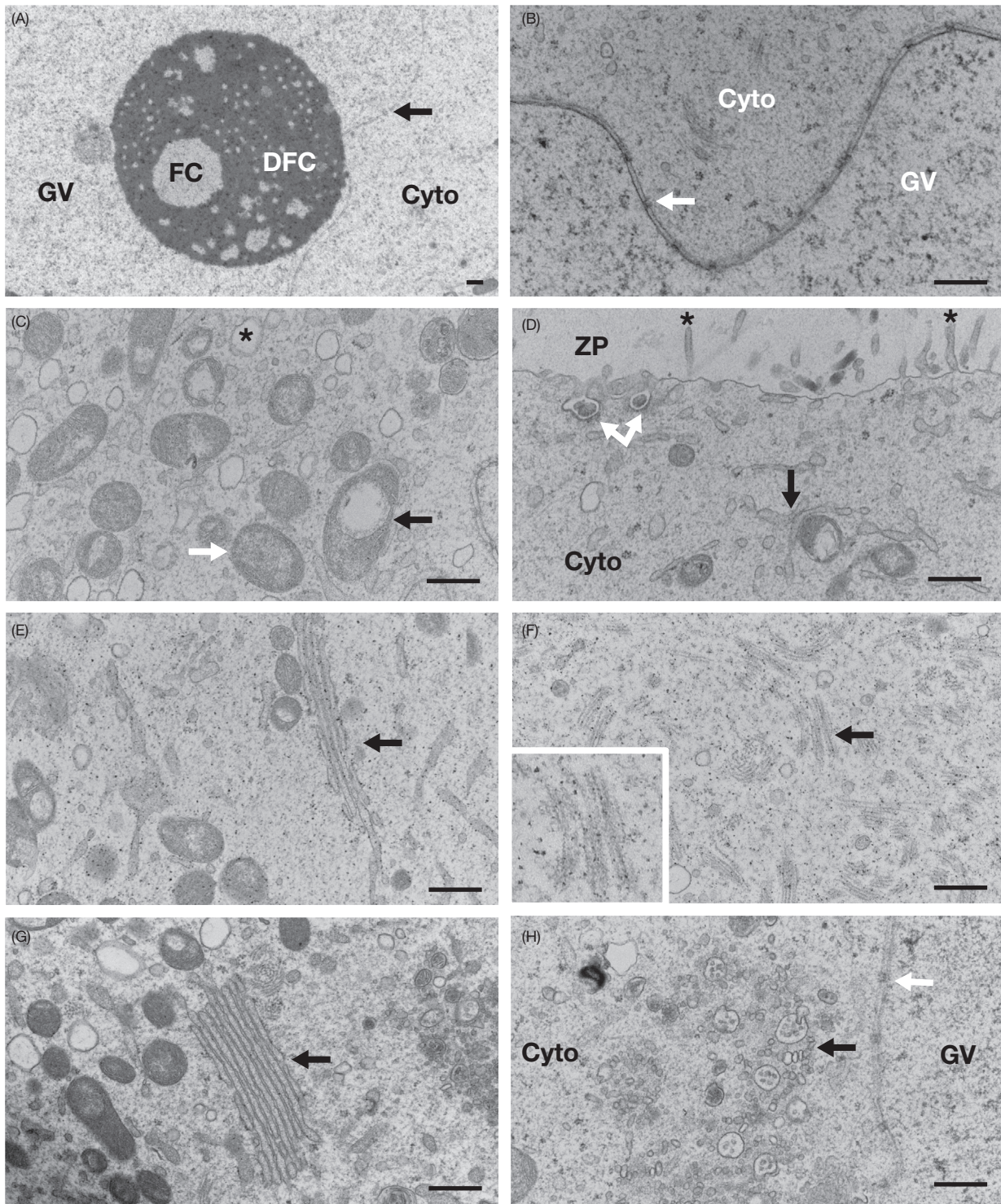


Fig. 4 Ultrastructure of key organelles in the growing mouse oocyte. Late primary and secondary follicles were isolated from mouse ovaries and fixed and processed for electron microscopy. Regions within the oocyte were imaged using a JEOL JEM-1400 transmission electron microscope. (A) The nucleolus is the circular electron-dense structure. The nucleolus is a tripartite structure composed of dense fibrillar centers (DFCs), granular center, and fibrillar centers (FCs). The DFCs and FCs are clear at this magnification. The *black arrow* highlights the nuclear envelope. (B) The nuclear envelope forms a boundary between the GV and the cytoplasm. The *white arrow* highlights one of the nuclear pores that punctuates the nuclear envelope. (C) Oocyte mitochondria have unique morphologies including those with typical cristae (*white arrow*), those with vacuoles in between cristae (*black arrow*), and those without cristae (*asterisks*). (D) A view of the oocyte cortex or outer edge shows the plasma membrane, which abuts the ZP and is punctuated by microvilli (*asterisks*). The smooth ER is highlighted by the *black arrow*, and vesicles containing electron-dense material (possibly cortical granules) are highlighted by the *white arrows*. (E) The rough ER is typically found near mitochondria and has a rough-appearing surface due to the presence of ribosomes (*black arrow*). (F) Cytoplasmic lattices are unique oocyte structures that are prevalent in the cytoplasm and involved in ribosome storage. The *black arrow* highlights this structure, which is further magnified in the inset. The black dot-like structures are individual ribosomes. (G) The Golgi apparatus is visible as a stack of several membrane-covered sacs called cisternae (*black arrow*). (H) Large aggregates of multivesicular bodies are found in the oocyte cytoplasm (*black arrow*). The nuclear envelope is highlighted by the *white arrow*. In all images, GV is the germinal vesicle, cyto is the cytoplasm, and the scale bars are 500 nm.

composed of three glycoproteins: ZP1, ZP2, and ZP3 (Wassarman and Litscher, 2013). The ZP physically separates the oocyte from adjacent granulosa cells. Transzonal projections (TZPs) are actin- and microtubule-rich structures that extend from granulosa cells, transverse the ZP at varying angles, and make contact with the oocyte (Plancha et al., 2005). TZPs allow bidirectional communication and passage of molecules to occur between the oocyte and its surrounding granulosa cells via gap junctions. Gap junctions are composed of a family of proteins called connexins. Connexin-37, for example, is expressed by the oocyte and is essential for proper follicle development as it bridges the oocyte to the surrounding somatic cells (Winterhager and Kidder, 2015). Of note, diverse factors and molecules have been observed within TZPs, including nutrients, small molecules (i.e., cGMP), and organelles. In addition to playing a vital role in cell growth and communication, the ZP also plays a vital role in fertilization and the block of polyspermy. The glycoprotein ZP3 induces the sperm acrosome reaction, which is necessary for fertilization. Moreover, as described earlier, CG exocytosis releases proteases that modify or “harden” the ZP and prevent sperm entry following fertilization.

Developmental Determinants of Oocyte Quality

Only a high-quality oocyte will have sufficient developmental potential to complete meiosis, undergo fertilization, and initiate the events of early embryo development prior to implantation in the uterus. Oocyte quality is largely determined by the acquisition of both cytoplasmic and meiotic competence during oogenesis, and these two quality determinants are explained further later.

Cytoplasmic Competence

Cytoplasmic competence refers to the accumulation of key maternal factors during oogenesis. This maternal legacy is critical, since developmental events prior to the activation of the zygotic genome (i.e., completion of meiosis, fertilization, egg activation, and preimplantation embryo development) occur largely in the absence of transcription. Moreover, the stored cellular components of the sperm do not contribute significantly to cleavage-stage embryogenesis. To accumulate these maternal products, the oocyte is one of the most transcriptionally and translationally active cells in the human body. Transcription and translation levels, however, decrease significantly when the oocyte is fully grown.

Transcription is the process by which a cell's DNA is read and turned into RNA, and this is a necessary first step in the production of proteins. Transcription occurs in the cell's nucleus (oocyte GV), and the messenger RNAs (mRNAs) that are to be translated into proteins are transported through nuclear pores into the cell's cytoplasm. In the mouse oocyte, RNA content increases by 300% during its active growth phase, and the fully grown oocyte contains ~200 times as much RNA found in a typical somatic cell (~0.6 ng total RNA) (Eichenlaub-Ritter and Peschke, 2002). Some of the mRNAs are recruited and translated into proteins to meet the metabolic demands of the oocyte, but some are selectively degraded or stored for use in the fertilized egg and early embryo. Whether an mRNA is translated or repressed is regulated in large part by the length of its cytoplasmic polyadenylation (polyA) tail within its 3' untranslated region (Eichenlaub-Ritter and Peschke, 2002). In general, translating mRNAs have long polyA tails, whereas ones that are dormant have short ones.

Translation is the process by which mRNA is converted into proteins, and this occurs in the cell cytoplasm mediated by ribosomes. Active ribosome biogenesis and protein synthesis during oocyte growth are key aspects of cytoplasmic competence. To accommodate high levels of protein production, oocytes synthesize and accumulate large amounts of ribosomal RNA (rRNA) that are key components of ribosome biogenesis. rRNA synthesis in the growing oocyte is the highest among all cell types, and rRNA accounts for approximately 60%–70% of the total amount of RNA in oocytes. Mouse oocytes rapidly accumulate ribosomes during the initial growth phase, with 95% of the ribosomal pool established when the oocyte is only two-thirds its terminal size (Eichenlaub-Ritter and Peschke, 2002). Interestingly, several *Drosophila* mutants in ribosomal protein genes have low fertility and reduced fecundity due to defects in oocyte development (Marygold et al., 2007). Thus, ribosome production and protein synthesis are major cellular events during oogenesis that are essential for proper oocyte development.

Meiotic Competence

Meiotic competence refers to the ability of the fully grown oocyte, which is arrested at prophase I, to resume the cell cycle in response to hormonal cues and complete meiosis to ultimately produce a haploid gamete, or a cell that contains half its DNA content. This reduction in DNA content ensures that at fertilization, the unification of a haploid egg with a haploid sperm will produce a diploid zygote with the correct chromosome complement. Central to the oocyte's ability to resume meiosis is global silencing of transcription that is tightly correlated to changes in the oocyte's DNA or chromatin structure. In the prophase-I-arrested oocyte, the configuration of chromatin is classified in one of the three ways: nonsurrounded nucleolus (NSN), intermediate, and surrounded nucleolus (SN) (De La Fuente, 2006). The NSN chromatin configuration is characterized by uncondensed chromatin and is found in smaller, growing oocytes that are actively transcribing their DNA. In contrast, the SN configuration is characterized by condensed chromatin that forms a tight ring around the nucleolus and is associated with fully grown oocytes that are transcriptionally silent. The intermediate configuration is somewhere between the NSN and SN states. In general, oocytes that are in the SN configuration have an increased ability to resume and complete meiosis.

Meiosis resumes in the prophase-I-arrested oocyte at the time of ovulation, which can happen anytime between puberty and menopause. The ovulatory luteinizing hormone (LH) surge from the anterior pituitary triggers the resumption of meiosis. LH binds

to its receptors in the mural granulosa cells of the ovulatory antral follicle and trigger signaling pathways that decrease NPR2 activity and the production of cGMP (Jaffe and Egbert, 2017). As a result, cGMP in the oocyte diffuses down its concentration gradient, which results in a decrease in cGMP levels in the oocyte. This decrease in oocyte cGMP relieves the inhibition of PDE3A and causes a decrease in cAMP levels in the oocyte, triggering cell cycle resumption. Importantly, simply removing the oocyte from the context of the follicle will lead to a drop in cAMP levels, which will result in spontaneous resumption of meiosis.

Once the oocyte resumes meiosis, it will undergo events that are collectively referred to as meiotic maturation. One of the first manifestations of meiotic maturation is the breakdown of the nuclear envelope or GV breakdown (GVBD). During GVBD, the chromosomes will condense completely, and the meiotic spindle will begin to form. The meiotic spindle is the cellular machinery that segregates chromosomes and mediates cell division. At metaphase of meiosis I (MI), the chromosomes will align in the center of the meiotic spindle. Once chromosomes are stably attached to the meiotic spindle, the first meiotic division will occur with the segregation of chromosomes and extrusion of the first polar body. The polar body is the by-product of a meiotic cell division that contains excess DNA. Immediately following MI and without an intervening round of DNA replication, the oocyte will proceed into meiosis II (MII), rapidly reform a spindle, and arrest at metaphase of MII. Once at MII, the cell is termed an “egg” and is capable of fertilization if sperm are present. It is not until fertilization occurs that the second meiotic division will be completed with an additional round of chromosome segregation and extrusion of the second polar body. Both cell divisions at MI and MII are asymmetrical, such that both polar bodies are small to ensure that the bulk cytoplasm remains in the egg cell that will ultimately contribute its contents to the embryo.

Factors That Influence Oocyte Quality

According to dogma, the number of oocytes in the ovary is fixed and nonreplenishable. Thus, the approximate 400 oocytes that are ovulated over a human female’s reproductive life span have existed within the ovary for up to decades. These cells, therefore, are particularly susceptible to accumulated damage due to a variety of factors including aging, obesity, clinical conditions, medical interventions, and environmental exposures, which are discussed further later.

Age

Advanced reproductive age, which typically refers to females > 35 years of age, is associated with a significant decline in oocyte quantity. Females are born with ~1 million oocytes (enclosed in primordial follicles) in the ovary, and this number declines steadily with age, such that the ovary contains ~400,000 oocytes at puberty and only ~1000 at menopause (Broekmans et al., 2009). Paralleling the decline in oocyte quantity is a deterioration in oocyte quality with age (Broekmans et al., 2009). Advanced reproductive age, for example, is unequivocally associated with an increase in oocytes with the incorrect chromosome number—or aneuploidy. Aneuploidy in the female gamete results from errors in meiosis and is a leading cause of infertility, miscarriages, and birth defects. The most common example of age-related aneuploidy is trisomy 21 or Down syndrome. The mechanisms underlying the age-associated increase in aneuploidy are complex and multifactorial, including alterations in chromosome structure, chromosome-associated proteins, cell cycle regulators, and the cell division machinery (Chiang et al., 2012). In addition, oocyte aging is also associated with significant changes in the mitochondria, including decreased function (i.e., energy production) and increased frequency of mitochondrial DNA mutations (May-Panloup et al., 2016). The ovarian microenvironment in which oocytes develops becomes fibrotic and highly inflammatory with age, and this likely indirectly affects oocyte quality (Briley et al., 2016). The overall impact of reproductive aging on fertility is becoming a tangible concern as more women worldwide are delaying childbearing.

Obesity

In addition to aging, obesity impacts oocyte quality. Obesity, defined as having a body mass index of $> 30 \text{ kg/m}^2$, has reached epidemic proportions—affecting > 10% of the world’s adult population. Maternal obesity has been linked to difficulty in conception and adverse fetal health outcomes. Studies in various genetic and diet-induced obesity mouse models have shown that oocytes from obese mice, relative to controls, have reduced size, altered gene expression patterns and epigenetic modifications, defects in meiosis and meiotic spindle formation, aberrant mitochondria, and increased levels of apoptosis (Broughton and Moley, 2017). Interestingly, when examining the effect of obesity on offspring health, pups from obese mothers were initially smaller than those from control mice (Jungheim et al., 2010). However, the pups from the obese mothers ultimately showed signs of metabolic syndrome (glucose intolerance and increased cholesterol and body fat). Maternal programming of metabolic disease can be passed through the female germ line across multiple generations through the transfer of aberrant oocyte mitochondria (Jaeger et al., 2017).

Clinical Conditions and Medical Interventions

There are several clinical conditions or medical interventions that can also impact oocyte quality. For example, maternal diabetes can result in miscarriages and poor pregnancy outcomes due in part to poor oocyte quality (Wang and Moley, 2010). In mouse models of maternal acute and chronic insulin-dependent diabetes, oocytes were smaller and had a decreased ability to progress through meiosis relative to controls. These oocytes were also surrounded by somatic cells with higher levels of apoptosis. Diabetic

mouse ovaries had reduced connexin-43 expression, which may be a potential mechanism underlying the observed defects in oocyte quality. Connexin-43 is a gap junction protein that allows for granulosa cells that surround the oocyte to communicate with each other. Eliminating this essential connexin likely negatively impacts granulosa cell function and alters bidirectional communication with the oocyte, which is required for gamete quality.

Another condition in which oocyte quality is compromised is polycystic ovarian syndrome (PCOS). PCOS is the most common heterogeneous endocrine disorder in reproductive age women, with a prevalence of ~5%–10%. Criteria for diagnosis include elevated androgen levels; ovulation that is infrequent, irregular, or lacking; and the presence of multiple cysts on the ovaries. Women with PCOS suffer from infertility even when using assisted reproductive technologies, and this is in part due to poor oocyte quality. In fact, oocytes from women with PCOS have reduced expression of critical oocyte-specific paracrine factors (i.e., GDF9) and have altered growth dynamics within the follicle (Dumesic and Abbott, 2008). Ovaries from women with PCOS have excess extracellular matrix components, and the ovarian microenvironment in which oocytes develop is more rigid (Wood et al., 2015). Data in the mouse show that follicles grown in rigid biomaterials that mimic the PCOS ovary produce higher levels of androgens relative to estrogen and contain poor-quality oocytes that have compromised developmental competence (Woodruff and Shea, 2011). In addition, there is a higher frequency of insulin resistance and obesity in women with PCOS, and as described earlier, these factors on their own can contribute to reduced oocyte quality and difficulty with conception.

Medical interventions for various clinical conditions can also have the unintended consequence of damaging oocytes. For example, life-preserving cancer treatments—such as chemotherapy and radiation—can trigger DNA damage and cell death pathways in the oocyte. In fact, the oocyte is highly sensitive to such exposures. The dose of radiation sufficient to destroy half the population of human oocytes is <2 Gy (Wallace et al., 2003). Because the number of oocytes in the ovary is nonreplenishable, oocyte loss due to chemotherapy- or radiation-induced damage can accelerate reproductive aging and lead to early onset menopause. The effect of cancer therapies on reproduction is significant because a greater number of women, men, and children are surviving their cancer diagnoses, and long-term quality-of-life factors such as fertility and normal reproductive function post cancer are of paramount importance. This need has resulted in the generation of a new field of oncofertility that bridges oncology and reproduction to explore and expand the fertility preservation options and the reproductive potential of those with cancer (Woodruff, 2015).

Environmental Exposures

Exposures to several chemicals in the environment are also harmful to oocytes. One primary example is bisphenol A (BPA), a compound that is widely used in the production of plastics, resins, and epoxies (Sartain and Hunt, 2016). The chemical structure of BPA is similar to estrogen and is weakly estrogenic. Thus, it acts as an endocrine disruptor. After prolonged exposure, BPA may be found in many biological fluids, including blood, urine, amniotic fluid, cord blood, breast milk, and follicular fluid. In the mouse model, BPA exposure significantly disrupted various aspects of meiosis in oocytes and resulted in spindle abnormalities and increased aneuploidy. A similar phenotype has also been observed in human oocytes. Chronic low exposure to BPA during oocyte growth can also disrupt the epigenome of the oocyte by altering methylation of imprinted genes. The exact molecular mechanisms by which BPA leads to these outcomes are an active area of investigation. Importantly, transgenerational effects have been observed in the offspring of BPA-exposed rodents, so perturbations that occur in the oocyte may have impacts not only on fertility but also on the health of future generations.

Outlook

The oocyte has the great potential of transforming into another living organism. Understanding the biological mechanisms regulating how this cell develops and what factors impacts its quality has significant impact for the health and well-being of subsequent generations. In addition, recent scientific and technological advances are changing the way we think about reproduction. In the not-so-distant future, oocytes not only will develop within ovaries but also will be derived from induced pluripotent stem cells and gonadal bioprotheses. For these ex vivo approaches to be successful, we must recapitulate key aspects of in vivo development and understand how these in vitro manipulations affect the resulting oocyte.

Acknowledgments

The authors thank Dr. Karen Schindler and Dr. Teresa Woodruff for the critical review of the manuscript and Dr. Atsuko Kato and Shawn Briley for providing images. This work was supported by the Master of Science in Reproductive Science and Medicine Program and the Center for Reproductive Health After Disease (P50 HD076188) from the National Centers for Translational Research in Reproduction and Infertility (NCTRI).

References

- Babayev, E., & Seli, E. (2015). Oocyte mitochondrial function and reproduction. *Current Opinion in Obstetrics and Gynecology*, 27, 175–181.
- Briley, S. M., Jasti, S., McCracken, J. M., Hornick, J. E., Fegley, B., Pritchard, M. T., & Duncan, F. E. (2016). Reproductive age-associated fibrosis in the stroma of the mammalian ovary. *Reproduction*, 152, 245–260.

- Broekmans, F. J., Soules, M. R., & Fauser, B. C. (2009). Ovarian aging: mechanisms and clinical consequences. *Endocrine Reviews*, 30, 465–493.
- Broughton, D. E., & Moley, K. H. (2017). Obesity and female infertility: potential mediators of obesity's impact. *Fertility and Sterility*, 107(4), 840–847.
- Calarco, P. G. (1991). Fertilization of the mouse oocyte. *Journal of Electron Microscopy Technique*, 17, 401–411.
- Chiang, T., Schultz, R. M., & Lampson, M. A. (2012). Meiotic origins of maternal age-related aneuploidy. *Biology of Reproduction*, 86, 1–7.
- De la Fuente, R. (2006De). Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. *Developmental Biology*, 292, 1–12.
- Dumesic, D. A., & Abbott, D. H. (2008). Implications of polycystic ovary syndrome on oocyte development. *Seminars in Reproductive Medicine*, 26, 53–61.
- Eichenlaub-Ritter, U., & Peschke, M. (2002). Expression in in-vivo and in-vitro growing and maturing oocytes: focus on regulation of expression at the translational level. *Human Reproduction Update*, 8, 21–41.
- Jaeger, K., Saben, J. L., & Moley, K. H. (2017). Transmission of metabolic dysfunction across generations. *Physiology (Bethesda)*, 32, 51–59.
- Jaffe, L. A., & Egbert, J. R. (2017). Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. *Annual Review of Physiology*, 79, 237–260.
- Jungheim, E. S., Schoeller, E. L., Marquard, K. L., Loudon, E. D., Schaffer, J. E., & Moley, K. H. (2010). Diet-induced obesity model: abnormal oocytes and persistent growth abnormalities in the offspring. *Endocrinology*, 151, 4039–4046.
- Liu, M. (2011). The biology and dynamics of mammalian cortical granules. *Reproductive Biology and Endocrinology*, 9, 149.
- Machaty, Z. (2016). Signal transduction in mammalian oocytes during fertilization. *Cell and Tissue Research*, 363, 169–183.
- Marygold, S. J., Roote, J., Reuter, G., Lambertsson, A., Ashburner, M., Millburn, G. H., Harrison, P. M., Yu, Z., Kenmochi, N., Kaufman, T. C., Leever, S. J., & Cook, K. R. (2007). The ribosomal protein genes and minute loci of *Drosophila melanogaster*. *Genome Biology*, 8, R216.
- May-Panloup, P., Boucret, L., Chao de la Barca, J. M., Desquiere-Dumas, V., Ferre-Hotellier, V., Moriniere, C., Descamps, P., Procaccio, V., & Reynier, P. (2016). Ovarian ageing: the role of mitochondria in oocytes and follicles. *Human Reproduction Update*, 22, 725–743.
- Pepling, M. E., Wilhelm, J. E., O'Hara, A. L., Gephardt, G. W., & Spradling, A. C. (2007). Mouse oocytes within germ cell cysts and primordial follicles contain a Balbiani body. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 187–192.
- Plancha, C. E., Sanfins, A., Rodrigues, P., & Albertini, D. (2005). Cell polarity during folliculogenesis and oogenesis. *Reproductive Biomedicine Online*, 10, 478–484.
- Que, E. L., Duncan, F. E., Bayer, A. R., Phillips, S. J., Roth, E. W., Bleher, R., Gleber, S. C., Vogt, S., Woodruff, T. K., & O'Halloran, T. V. (2017). Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy. *Integrative Biology (Cambridge)*, 9, 135–144.
- Ruby, J. R., Dyer, R. F., & Skalko, R. G. (1969). Continuities between mitochondria and endoplasmic reticulum in the mammalian ovary. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 97, 30–37.
- Sartain, C. V., & Hunt, P. A. (2016). An old culprit but a new story: bisphenol A and "NextGen" bisphenols. *Fertility and Sterility*, 106, 820–826.
- Wallace, W. H., Thomson, A. B., & Kelsey, T. W. (2003). The radiosensitivity of the human oocyte. *Human Reproduction*, 18, 117–121.
- Wang, Q., & Moley, K. H. (2010). Maternal diabetes and oocyte quality. *Mitochondrion*, 10, 403–410.
- Wassarman, P. M., & Litscher, E. S. (2013). Biogenesis of the mouse egg's extracellular coat, the zona pellucida. *Current Topics in Developmental Biology*, 102, 243–266.
- Winterhager, E., & Kidder, G. M. (2015). Gap junction connexins in female reproductive organs: implications for women's reproductive health. *Human Reproduction Update*, 21, 340–352.
- Wood, C. D., Vijayvergia, M., Miller, F. H., Carroll, T., Fasanati, C., Shea, L. D., Brinson, L. C., & Woodruff, T. K. (2015). Multi-modal magnetic resonance elastography for noninvasive assessment of ovarian tissue rigidity in vivo. *Acta Biomaterialia*, 13, 295–300.
- Woodruff, T. K. (2015). Oncofertility: a grand collaboration between reproductive medicine and oncology. *Reproduction*, 150, S1–10.
- Woodruff, T. K., & Shea, L. D. (2011). A new hypothesis regarding ovarian follicle development: ovarian rigidity as a regulator of selection and health. *Journal of Assisted Reproduction and Genetics*, 28, 3–6.
- Yurtas, P., Vitale, A. M., Fitzhenry, R. J., Cohen-Gould, L., Wu, W., Gossen, J. A., & Coonrod, S. A. (2008). Role for PADI6 and the cytoplasmic lattices in ribosomal storage in oocytes and translational control in the early mouse embryo. *Development*, 135, 2627–2636.

The Ovarian Interstitium

Patricia B Hoyer, The University of Arizona, Tucson, AZ, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Ovarian stroma Soft tissue of the ovary that contains an abundant supply of blood vessels and surrounds the functional units, follicles and corpora lutea.

Atresia Designation for programmed cell death of ovarian follicles by apoptosis.

CYP Cytochrome P450 family of enzymes that actively participate in phase 1 metabolism of many small chemicals. Enzyme activity can include bioactivation or detoxification of a chemical as well as steroidogenesis.

Sex cord stromal tumors Malignancies arising in the ovarian stroma.

PCOS Polycystic ovarian syndrome, associated with menstrual disorders.

Introduction

The stroma of the ovary is a vascularized soft tissue that consists of interstitial cells (Wikipedia). This comprises the compartment that forms the body of the ovary. Interstitial cells pack the areas between the functional units within the ovary, oocyte-containing follicles, and corpora lutea (Fig. 1). Interstitial cells are designated as primary or secondary. Primary interstitial cells originate from fibroblastlike stromal cells. Generally, in species with long gestational periods, they differentiate during fetal development. This is the case in human ovaries where their development is maximal at 18 weeks of gestation (Konishi et al., 1986). Whereas, in species with short gestational periods, they differentiate during the postnatal period. They were even found to develop on different

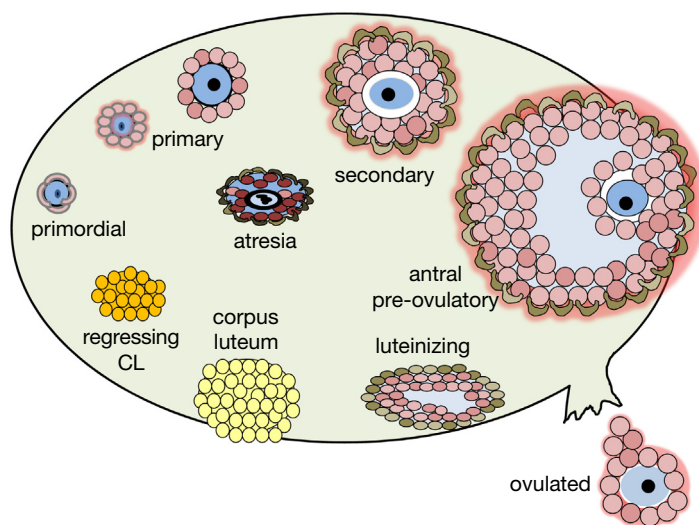


Fig. 1 The reproductively active mammalian ovary. The major functional unit within the cycling ovary consists of the oocyte-containing follicle. The primordial follicle is formed during fetal development and contains an oocyte arrested in prophase of the first meiotic division. Therefore, after birth, the ovary contains a set number of follicles that must serve the female throughout her lifespan. The oocyte is surrounded by a single layer of squamous granulosa like cells. Primordial follicles may remain dormant for up to a number of years in women, or may become activated toward development to ovulation at any time after birth. Following activation of a primordial follicle, it goes through a number of maturation stages. The first stage is primary in which the surrounding granulosa cells become cuboidal, a sign they are activated and are beginning to function. In the secondary stage, granulosa cells have begun to proliferate and form a multi-layer around the oocyte. At this stage, theca interna cells form an outer layer around the proliferating granulosa cells. As the follicle keeps growing, a fluid filled space within the granulosa-oocyte space called an antrum begins to form. The antrum enlarges and the follicle grows to the pre-ovulatory stage. Following ovulation, the oocyte enters the oviduct for potential ovulation, whereas, residual granulosa and theca cells infiltrate to form a solid gland and differentiate by luteinization to become the corpus luteum (CL). If fertilization of the oocyte has not occurred, the CL undergoes regression and disappears. The vast majority of primordial follicles in the ovaries at birth do not become ovulated, but undergo a form of cell death by apoptosis called atresia. The remainder of the ovary constitutes the stromal compartment comprised of blood vessels and interstitial cells. Reproduced from Hoyer, P. B., Devine, P. J. (2001). Endocrinology and toxicology: The female reproductive system. In: Derelanko, M. J., Hollinger, M. A. (eds.) *Handbook of toxicology* (2nd edn.). Boca Raton FL: CRC Press, with permission from CRC Press.

postnatal days (pnd) between different strains of rats and mice (Guraya, 1991). Postnatal day 12 was the time of their appearance in C57Bl/6J mice, whereas it was pnd 10 in C57Bl/6Pa mice. They were first observed on pnd 7 in Swiss albino mice. Primary interstitial cells that have developed in the fetal guinea pig ovary, begin to regress during postnatal life (Guraya, 1977). However, factors that regulate this regression are not known. Primary interstitial cells in a number of species possess cytological and ultrastructural characteristics of steroidogenic cells. These characteristics include sudanophilic lipid droplets (staining positive with Sudan black) and orangeles related to steroidogenesis.

Secondary interstitial cells form from residual theca interna cells in atretic follicles (Guraya, 1991). Thus, whereas, primary interstitial cells form the ovarian stroma as the ovary undergoes fetal development, secondary are derived from theca interna remaining after follicular atresia. Theca cells are acquired at the secondary stage during development of a follicle as it is recruited for development toward possible ovulation. However, the vast majority of developing follicles do not ovulate, but undergo cell death by apoptosis (atresia). In the ovary the oocyte is enclosed in a spherical-shaped follicle along with granulosa cells. Theca interna cells surround the membrane a propria that forms the follicle. During follicular development theca interna cells undergo steroidogenesis to produce androgens (androstenedione and testosterone). The androgens passively diffuse across the membrane a propria and are converted to 17 β -estradiol in the granulosa cells. During atresia, the oocyte and its surrounding granulosa cells degenerate, yet, the layer of theca interna cells that surround the follicle remain. These cells form the secondary interstitial compartment. A major function of the theca interna cells is to undergo steroidogenesis, providing substrate for follicular steroid production. As a result, as with primary, secondary interstitial cells display an ultrastructure typical of steroid-producing cells (Konishi et al., 1986).

Genes Expressed

As suggested by morphological evidence, the interstitial compartment appears to be steroidogenic. Investigation of gene expression in the stromal compartment can provide evidence to support that theory. Histochemical analysis of pnd 3 mouse ovaries demonstrated staining for the steroidogenic enzyme 3- β hydroxysteroid dehydrogenase (3 β HSD, Hoyer and Byskov, 1981). Expression of the cytochrome P450 enzyme CYP2B which is involved in phase 1 metabolism of xenobiotics was also demonstrated in interstitial cells in mouse ovaries (Fig. 2). Other studies have also provided evidence of 3 β HSD activity in rat ovaries (Presl et al., 1964; Schlegel et al., 1967). Tsai-Morris and Johnson (1982) demonstrated 17 α -hydroxylase activity by conversion of 17 α [³H]-pregnenolone to 17 α [³H] progesterone in primary interstitial membranes of immature ovaries.

According to reported high throughput analysis of human ovarian stromal cells, 879 proteins were determined to be expressed (LifeMap). Expression of these gene products is also supported by morphological observations of rough endoplasmic reticulum and ribosomes in intermediate stages of primary interstitial cell differentiation (Guraya, 1991).

Rat mature interstitial cells have also been shown to express receptors for androgen (AR), estrogen (ER), and luteinizing hormone (LHR). It was reported that injection of adult female rats with testosterone propionate induced the appearance of aged interstitial cells with enhanced expression of AR, but reduced ER and LHR expression, compared with normal mature interstitium (Bukovsky et al., 2002). This resulted in premature aging of adult rat ovaries.

Functional Significance

The functional significance of ovarian stroma is not completely understood. Much morphological information has been described, and a large number of genes have been demonstrated to be expressed in interstitial cells. However, what functional capacity these genes have has been hard to determine. This is partly due to the difficulty of separating interstitial cells from the dynamic compartments within the ovary, follicles and corpora lutea. There is ample evidence that the tissue is steroidogenic as has been discussed above, but whether steroid produced in those cells supplements that produced in follicles (androgens and 17 β -estradiol) and corpora lutea (progesterone) is not clear. A recent immunohistochemical study of stromal cells adjacent to epithelial ovarian tumors in women found that the stroma in those ovaries express markers of sex-steroid differentiation and steroidogenic enzymes (calretinin, inhibin, steroidogenic factor 1; Blanco et al., 2017). Further, this tissue expressed genes involved in steroid hormone biosynthesis (CYP17, CYP 19, HSD17B1, AKR, C3). The growth of ovarian neoplasms is known to be stimulated by steroid hormones. This led the authors to propose that the stroma surrounding ovarian epithelial tumors elaborates steroid hormones to further stimulate neoplastic growth.

Isolation of Interstitial Cells

Many studies of the stromal compartment have focused on cytological and ultrastructural imaging of ovarian sections. However, in vitro incubation of neonatal whole ovaries from laboratory animals cannot distinguish steroid production from follicular versus interstitial cells. Thus, performing in vitro biochemical or molecular studies of interstitial cells might involve separating them from within the ovary. This could be done on isolated ovaries that were gently dissociated with collagenase (Flaws et al., 1994). By this method, follicles remain intact and the dissociate can be filtered through a 250 μ pore membrane which will retain the intact

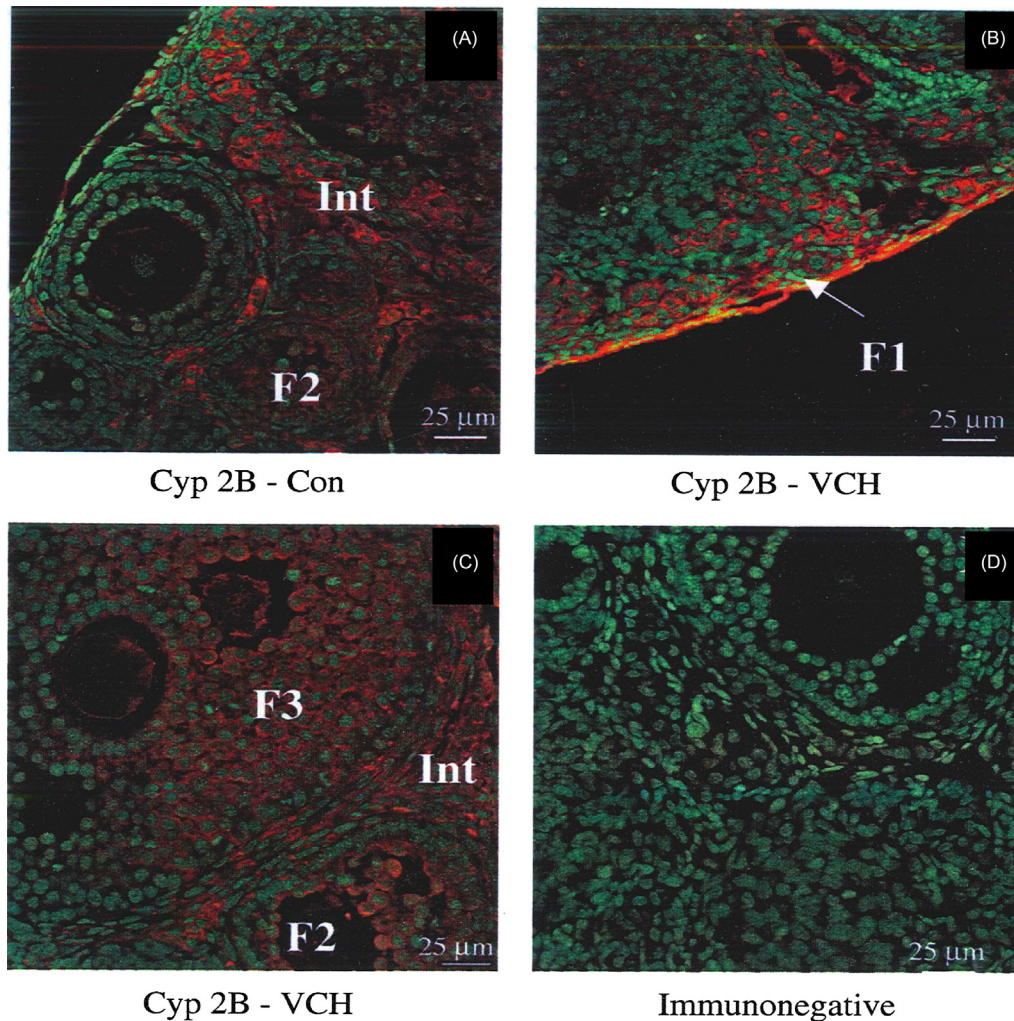


Fig. 2 Ovarian distribution of CYP2B protein in ovarian follicles and interstitial tissue. B6C3F1 mice (day 28) were given a daily injection (15d, i.p.) of sesame oil (vehicle control) or 4-vinylcyclohexene (VCH; 7.4 mmol/kg/d). Ovaries were removed 4 h. after the final injection and processed for immunostaining by confocal microscopy. CYP2B protein was visualized in small preantral (F1), early antral (F2), and antral (F3) follicles with high intensity staining in the interstitial compartment (Int). Ovaries were from vehicle-treated (A), or VCH-treated (B, C) mice. Ovarian section stained with YOYO-1 and CY5, with no primary antibody added (D). Green stain = YOYO (DNA stain). Red stain = CY-5 labeled antibody for CYP2B. Modified from Cannady, E. A., Dyer, C. A., Christian, P. J., Sipes, I. G., Hoyer, P. B. (2003). Expression and activity of cytochromes P450 2E1, 2A, and 2B in the mouse ovary: The effect of 4-vinylcyclohexene and its diepoxide metabolite. *Toxicological Sciences* **73**, 423–430, with permission from Toxicological Sciences.

follicles and the filtrate will be highly enriched in interstitial cells. NOTE: It is important to use ovaries collected during the follicular development stage to avoid collecting luteal cells, which would also be dissociated by collagenase, and thereby contaminate the interstitial fraction.

Pathological Involvement

Ovarian stromal tissue can develop into malignancies. This is often termed sex cord-stromal tumor. This classification of ovarian tumor, however, is rare comprising about 7%, when compared with other categories of malignant ovarian neoplasms (Colombo et al., 2007). Sex cord stromal tumor is usually composed of various combinations of granulosa, theca and luteal cells. These can be classified as granulosa cell tumors or thecomas. The ultimate classification of these tumors as sex cord-stromal can only be confirmed by histological evaluation which reveals predominately solid cellular tumor with components of variable mixtures of granulosa, theca and fibroblast cells. Immunohistochemical analysis of stromal cell ovarian tumors observed expression of the steroidogenic enzymes 17 α hydroxylase and 3 β HSD (Ishikura and Sasano, 1998).

CYP450 aromatase is usually expressed in follicular granulosa cells, where high levels of estrogen are produced by large antral pre-ovulatory follicles. Aromatase expression in granulosa cells is regulated by follicle stimulating hormone (FSH) produced in the anterior pituitary. Under normal conditions, pituitary hormone secretion is kept relatively low due to negative feedback from ovarian hormones. However, CYP450 aromatase, was exclusively expressed in stromal cells of ovarian tumors (Kato et al., 2013).

Sex cord-stromal tumors are most prevalent in post-menopausal women. One hypothesis to explain this age group distribution of the disease is that, after oocyte depletion (menopause) negative feedback from the ovary is lost, resulting in increases in the pituitary hormones FSH and luteinizing hormone (LH) which in turn stimulate tumor growth (Colombo et al., 2007).

Another disorder that involves ovarian stroma is polycystic ovarian syndrome (PCOS). PCOS is an ovarian syndrome that affects 5%–10% of reproductive age women worldwide (Chun, 2014). Two of three criteria must be met to support a diagnosis of PCOS (Guraya, 2013). The three criteria are oligo or anovulation (dysmenorrhea), hyperandrogenism, and ultrasonographic evidence of the presence of > 12 follicles in either ovary measuring 2–9 mm in diameter. The presence of multiple large follicles can contribute to increased ovarian volume. One cardinal feature of PCOS as visualized by ultrasonography has been the presence of a bright, highly echogenic stroma (Buckett et al., 1999). In this study, a stromal index was calculated (ratio of mean stromal echogenicity to mean echogenicity of the entire ovary). Total stromal echogenicity was also calculated. Ovarian volume, stromal volume and stromal peak blood flow velocity were significantly higher in ovaries from women with PCOS compared with those from normal women. The stromal index was increased in PCOS ovaries likely due to increased volume of the ovarian stroma. However, the mean stroma echogenicity was not different between PCOS and normal patients, likely due to overall lower mean echogenicity of the entire ovary in PCOS women.

In summary, the stroma of the ovary has not been as widely studied as its functional units, follicles and corpora lutea, however, it serves an important role in providing the architecture to support follicular development, ovulation and luteinization. Additionally, steroid production by interstitial cells is likely to contribute to the regulation of ovarian function under normal and pathological conditions.

References

- Blanco, L. Z., Kuhn, E., Morrison, J. C., Bahadirli-Talbot, A., et al. (2017). Steroid hormone synthesis by the ovarian stroma surrounding epithelial ovarian tumors: A potential mechanism in ovarian tumorigenesis. *Modern Pathology*, 30, 563–576.
- Buckett, W. M., Bouzayen, R., Watkin, K. L., Tulandi, T., & Tan, S. L. (1999). Ovarian stromal echogenicity in women with normal and polycystic ovaries. *Human Reproduction*, 14, 618–621.
- Bukovsky, A., Ayala, M. E., Dominguez, R., et al. (2002). Changes of ovarian interstitial cell hormone receptors and behavior of resident mesenchymal cells in developing and adult rats with steroid-induced sterility. *Steroids*, 67, 277–289.
- Chun, S. (2014). Serum luteinizing hormone level and luteinizing hormone/follicle-stimulating hormone ratio but not serum anti-müllerian hormone level is related to ovarian volume in Korean women with polycystic ovary syndrome. *Clinical and Experimental Reproductive Medicine*, 41, 86–91.
- Colombo, N., Parma, G., Zanagnolo, V., & Insinga, A. (2007). Management of ovarian stromal tumors. *Journal of Clinical Oncology*, 25, 2944–2951.
- Flaws, J. A., Salyers, K. L., Sipes, I. G., & Hoyer, P. B. (1994). Reduced ability of rat preantral ovarian follicles to metabolize 4-vinyl-1-cyclohexene diepoxide *in vitro*. *Toxicology and Applied Pharmacology*, 126, 286–294.
- Guraya, S. S. (1977). Recent advances in the morphology, histology, biochemistry and physiology of the developing mammalian ovary. *International Review of Cytology*, 51, 49–131.
- Guraya, S. (1991). Interstitial cells. In G. Familiari, S. Makabe, & P. M. Motta (Eds.), *Ultrastructure of the ovary* (pp. 199–223). Kluwer Acad. Press (Chapter 13).
- Guraya, S. S. (2013). Prevalence and ultrasound features of polycystic ovaries in young unmarried Saudi females. *Journal of Microscopy and Ultrastructure*. <https://doi.org/10.1016/j.jmau.2013.06.002>.
- Hoyer, P. E., & Byskov, A. G. (1981). Quantitative cytochemical study of hydroxysteroid dehydrogenase activity in the rete system of the immature mouse ovary. In A. G. Byskov, & H. Peters (Eds.), *Development and function of reproductive organs* (pp. 216–224). Amsterdam Excerpta Medica.
- Ishikura, H., & Sasano, H. (1998). Histopathologic and immunohistochemical study of steroidogenic cells in the stroma of ovarian tumors. *International Journal of Gynecological Pathology*, 17, 261–265.
- Kato, N., Hayasaka, T., Takeda, J., Osakabe, M., & Kurachi, H. (2013). Ovarian tumors with functioning stroma: A clinicopathologic study with special reference to serum estrogen level, stromal morphology, and aromatase expression. *International Journal of Gynecological Pathology*, 32, 556–561.
- Konishi, I., Fujii, S., Okamura, H., Parmley, T., & Mori, T. (1986). Development of interstitial cells and ovigerous cords in the human fetal ovary: An ultrastructural study. *Journal of Anatomy*, 148, 121–135.
- LifeMap Discovery. <http://discovery.lifemapsc.com/gene-expression-signals/highthroughputexperiments-ovary;ovarianstromacells>.
- Presl, J., Jirasek, J., Horsky, J., & Henzl, H. (1964). Observations on steroid-3 β -ol-dehydrogenase activity in the ovary during early postnatal development of the rat. *Journal of Endocrinology*, 31, 293–294.
- Schlegel, R. J., Farias, E., Russo, N. C., Moore, J. R., & Gardner, L. I. (1967). Structural changes in the fetal gonads and gonads during maturation of an enzyme, steroid 3 β -ol-dehydrogenase, in the gonads, adrenal cortex and placenta in fetal rats. *Endocrinology*, 81, 565–572.
- Tsai-Morris, C. H., & Johnson, D. C. (1982). The steroid 17 α -hydroxylase activity of the immature ovary. *Journal of Steroid Biochemistry*, 17, 404–414.

Further Reading

- Cannady, E. A., Dyer, C. A., Christian, P. J., Sipes, I. G., & Hoyer, P. B. (2003). Expression and activity of cytochromes P450 2E1, 2A, and 2B in the mouse ovary: The effect of 4-vinylcyclohexene and its diepoxide metabolite. *Toxicological Sciences*, 73, 423–430.
- Hoyer, P. B., & Devine, P. J. (2001). Endocrinology and toxicology: The female reproductive system. In M. J. Derelanko, & M. A. Hollinger (Eds.), *Handbook of toxicology* (2nd edn.). Boca Raton, FL: CRC Press.

Cell-to-Cell Communication in the Ovarian Follicle

Claude Robert and Isabelle Gilbert, Université Laval, Québec, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Establishment of the Ovarian Reserves

In order to grasp the significance of the main signaling pathways occurring within the ovary, it is helpful to understand how the ovarian reserves are established. During fetal development, primordial germ cells migrate to the gonadal ridge, which will form the gonads (Wear et al., 2016). In females, these cells proliferate and interconnect with surrounding somatic cells to form cysts (Greenbaum et al., 2011). The ovarian reserves are established through a selection process in which apoptosis eliminates most of these cysts. Those that are selected will become primordial follicles (Wear et al., 2016). In cattle and humans, the number of oocytes apparently peaks at about 5 million in the developing fetus and then plummets to an average of about 300,000 per ovary at birth, with a range of 35,000–2.5 million (Erickson, 1966; Wallace and Kelsey, 2010). In most species, primordial follicles are composed of a single oocyte surrounded by a few flattened somatic cells. Canine follicles have been reported to harbor more than one oocyte (Payan-Carreira and Pires, 2008).

These systemic signaling pathways are active to various degrees throughout the lifetime of the animal. Non-ovulatory waves of follicular cell proliferation occur already during fetal growth and then become dormant around the time of birth. Ovulatory follicular waves begin at puberty and then occur more or less regularly, peaking in reproductive potency and then declining until the end of reproductive life, at which point the ovarian reserves are depleted. The gradual faltering of signaling that occurs as menopause is reached is well documented (Prior and Hitchcock, 2011). In contrast, little is known about what switches the signaling on at puberty to initiate reproductive life. Discovered in 1997, neuropeptides known as kisspeptins appear to initiate GnRH secretion by the hypothalamus (Messenger et al., 2005).

The growth and differentiation of the cells surrounding the oocyte in the sexually mature animal is called folliculogenesis, whereas the growth and differentiation of the cell destined to yield an ovule is called oogenesis. Throughout folliculogenesis, the somatic cells forming the follicles proliferate and differentiate to become a highly structured unit (Fig. 1). Once recruited

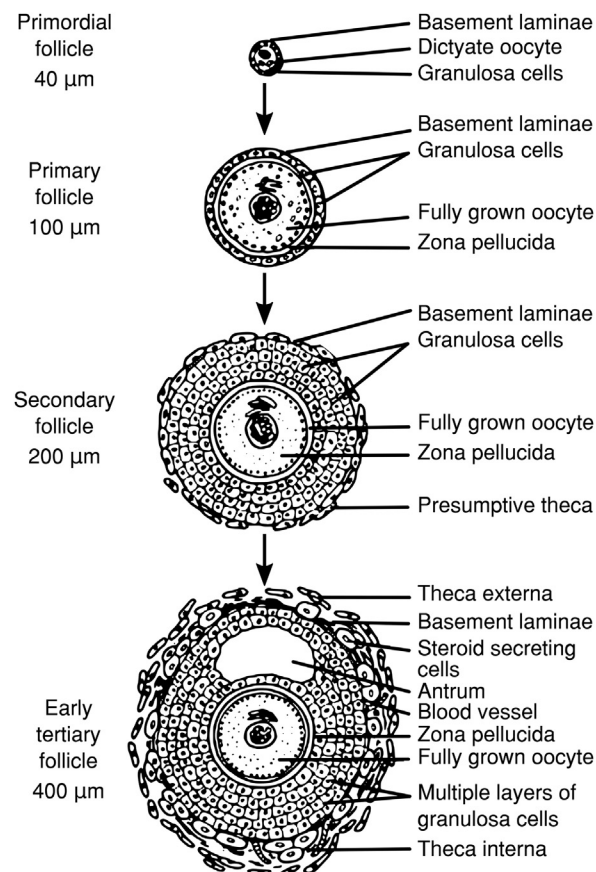


Fig. 1 Diagram illustrating the development of early follicles and their architectures. Erickson, G. F. (1995). The ovary: Basic principles and concepts. In Felig, P., Baxter, J.D., Frohman, L.A. (eds.) *Endocrinology and metabolism*, pp. 973–1015. New York: McGraw Hill.

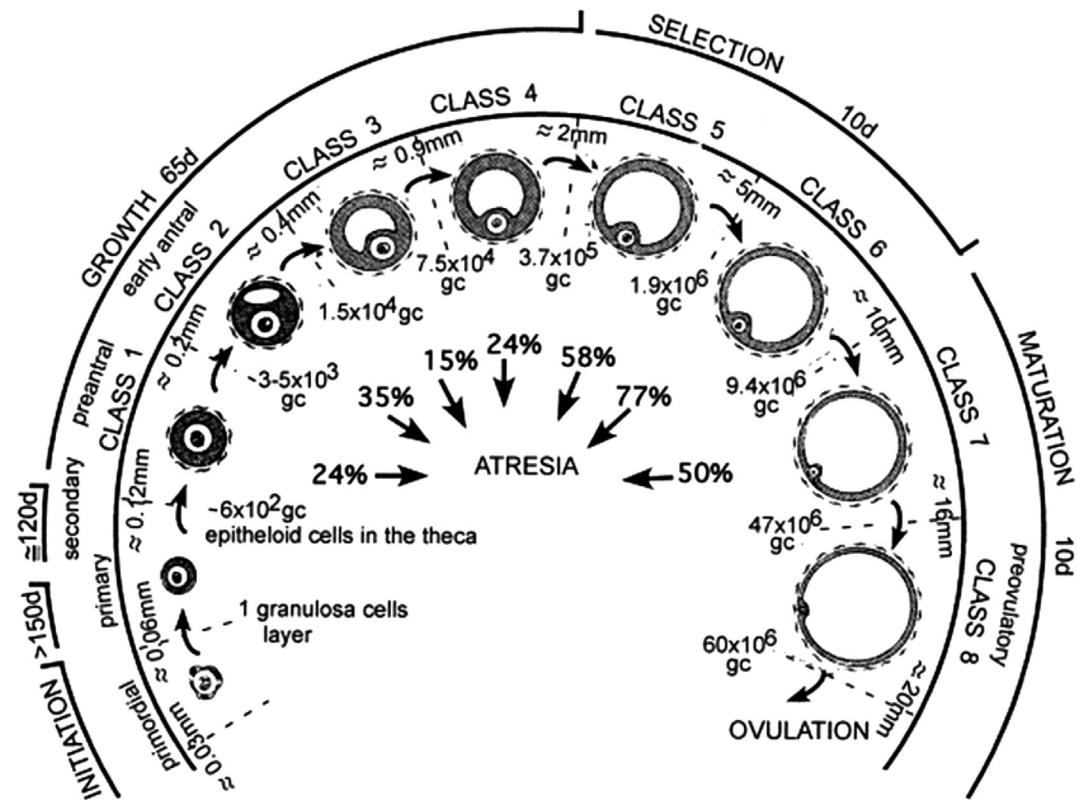


Fig. 2 Chronology of folliculogenesis in the human ovary. gc, granulosa cell. From Gougeon, A. (1986). Dynamics of follicular growth in the human: A model from preliminary results. *Human Reproduction* 1, 81–87.

from the stored pools, primordial follicles will develop into primary follicles, which are characterized by the presence of cuboid cells surrounding the oocyte. As somatic cell proliferation progresses, the oocyte grows in volume. The secondary follicle stage is reached when the oocyte is enclosed in a few layers of somatic cells called granulosa cells, which are further enclosed within an extracellular collagen matrix called the basal lamina. Somatic cells attached to the outside surface of the basal lamina are called theca cells. As the follicle develops, an antrum is formed among the granulosa cells and becomes filled with follicular fluid. The presence of this fluid-filled cavity marks the tertiary follicle stage. Although our general model of endocrine signaling implies a menstrual/estrus cycle lasting 23–35 days in humans (Munster et al., 1992), 18–24 days in cattle (Adams et al., 2008) and only 4 or 5 days in mice and rats (Felicio et al., 1986), the entire process of primordial follicle recruitment up to ovulation spans a much longer period of time. Bringing a human primordial follicle to the early tertiary follicle stage may take up to 300 days, followed by another 50 days to bring a follicle with a small antrum to the pre-ovulatory stage (Gougeon, 1986). It takes about a year (or 13 menstrual cycles) for a primordial follicle to reach ovulation size. In pre-pubertal gilts (the porcine equivalent of a heifer), the time from follicular activation to ovulation has been estimated at 100 days (Morbeck et al., 1992). In cattle, antral growth alone takes 40 days to complete (Lussier et al., 1987) whereas in mice, folliculogenesis overall appears to require only 17–19 days (Pedersen, 1970). In order for each menstrual/estrus cycle to result in ovulation, follicles are recruited in waves. When a wave of antral follicles is being monitored, subsequent waves are already in preparation. Most of our understanding of endocrine control of folliculogenesis is based on monitoring the final interval of gonadotropin-responsive antral growth leading to ovulation (the last 20 days in Fig. 2). It is known that in cattle at least, this interval does not correspond to the entire estrus cycle (18–24 days) and that two or three cohorts of follicles may grow and die during this period (Evans, 2003). The human menstrual cycle might also coincide with two or three follicular waves (Baerwald et al., 2012). When considering endocrine support for ovulatory waves in cattle, and possibly in humans, the monitoring period should therefore span 8–10 days. Our understanding of the intra-follicular communications occurring during this final phase (i.e., antral growth) is much more detailed than our understanding of the signaling occurring during the follicular recruitment and basal growth phases or throughout folliculogenesis overall.

Intra-Follicular Signaling

Signaling inside the follicle is complicated. It must first ensure a proper response of follicular cells to the systemic support provided by the HPX axis gonadotropins. This occurs mainly through transduction of external signals to intercellular communications. Since

the follicle and the gamete behave like a syncytium of inter-dependent cells, signals from the follicle center (i.e., the oocyte) reach peripheral follicular cells and vice versa. This bi-directional communication allows maintenance of synchrony between follicular development and oogenesis. Asynchrony between the follicular compartment and the gamete will lead to the demise of the follicle and hence to reduced fertility or even infertility.

Recruitment

Intercellular communications during the recruitment phase of folliculogenesis is only partially understood. Gene inactivation studies of reproductive phenotypes characterized by high turnover of primordial follicles have allowed identification of the currently known regulators of recruitment. Some genes appear to enhance recruitment rate, thus leading to faster depletion of the ovarian reserves, while others lead to infertility due to inability of the activated primordial follicles to progress through folliculogenesis, caused by blockage of proliferation and differentiation of the somatic cells surrounding the oocyte.

Follicle recruitment is gonadotropin-independent (Glasier et al., 1989), and current understanding based on gene expression studies suggest that inhibitory signals play a major role by keeping primordial follicles in latency. Among the suppressors of recruitment is the anti-Müllerian hormone (AMH), of which the inactivation (by suppressing gene expression) appears to stimulate recruitment, whereas supplementing in vitro cultures of ovarian cortex with AMH suppresses follicular recruitment and growth (Durlinger et al., 1999). AMH is expressed in the somatic cells surrounding the oocyte (Visser et al., 2006). Other suppressors originating from the oocyte itself have appeared to be part of the PTEN–PI3K–AKT–mTOR canonical pathway (Kim, 2012). Inactivating genes such as *phosphatase and tensin homolog (PTEN)* or *mechanistic target of rapamycin (mTOR)* and the corresponding driver molecules tuberous sclerosis 1 and 2 (Tsc1 and Tsc2) lead to over-active recruitment of primordial follicles and subsequently to rapid depletion of reserves (Reddy et al., 2008; Adhikari et al., 2009). A similar phenotype has been obtained by depleting downstream effector molecules such as forkhead box O3 (Castrillon et al., 2003). In many cell types, growth-factor-mediated stimulation of the PTEN–PI3K–AKT–mTOR pathway leads to increased gene translation as result of phosphorylation of ribosomal proteins (Kim, 2012). Although depletion of these growth factors is associated with follicle recruitment, it is still unclear what triggers the shift from the inhibited state. Nerve growth factor and brain-derived neurotrophic factor might be involved (Spears et al., 2003), as might positioning of the primordial follicle within the ovarian cortex, which could act through physical pressure, since infertile patients with normal ovarian reserves but very low antral follicle counts have responded well to incisions made on the ovary surface to the release intra-ovarian pressure (Eftekhari et al., 2016). Since the Hippo pathway is involved in limiting organ size, and in the case of ovaries its activity would result in pressure on resting follicles, it has been proposed as a long-term negative regulator of follicle recruitment (Kawamura et al., 2013).

Basal Growth

A variety of other factors could also impede the lengthy process by which primordial follicles progress towards ovulation. Bidirectional communication between the somatic cells and the gamete is important even at the earliest stage of folliculogenesis. Since the immature oocyte inside a primordial follicle is embedded amidst flattened somatic cells in direct contact with each other (Fig. 1), inter-cellular signaling likely occurs through autocrine and paracrine mechanisms with passage of molecules through gap junctions between plasma membranes (Gittens et al., 2005; Luciano et al., 2011).

One of the first communication routes found to function throughout folliculogenesis is the tandem composed of the tyrosine protein kinase receptor (expressed in the oocyte) and its ligand, secreted by the somatic cells (Driancourt et al., 2000). This receptor–ligand interaction is important for antrum formation and oocyte survival.

Many members of the transforming growth factor beta (TGFB) superfamily also appear to mediate communication between the gamete and the somatic cells. One of the key members is growth differentiation factor 9, which is produced by the oocyte and promotes expression of the tyrosine protein kinase ligand and the LH receptor and stimulates the somatic cells to proliferate and differentiate, showing the central role of the gamete in follicle development (Su et al., 2004). In the absence of GDF9, the primordial follicle fails to complete the transition to the primary follicle stage. Other members of the TGFB superfamily shown to play a functional role early in folliculogenesis are bone-morphogenic proteins (BMP) 2, 4, 6, 7 and 15, as illustrated in Figs. 3 and 4 (Chang et al., 2016).

Several additional members of the TGFB superfamily appear to be involved in folliculogenesis during the long basal growth period. These include fibroblast growth factor 2, platelet-derived growth factor, leukemia inhibitory factor and other neurotrophins, in particular NGF and BDNF (Pangas and Matzuk, 2004). It is still unclear how these factors are regulated and how essential they are, since in vitro culture of ovarian cortex leading to follicular development has been achieved successfully in serum-free media, thus in the absence of a complex mixture of growth factors (Eppig and O'Brien, 1996; Xiao et al., 2015).

Although the complexities of the interplay between these molecules are not yet understood, it is known that the transduction of their signals is mediated in large part by the SMAD pathway (Pangas, 2012). SMAD factors can translocate to the nucleus to act within transcriptional complexes and target transcription factors such as forkhead box L2, spermatogenesis and oogenesis-specific basic helix-loop-helix 1 and 2 (SOHLH1, SOHLH2), LIM homeobox 8 (LHX8), newborn ovary homeobox (NOBOX) and folliculogenesis-specific basic helix-loop-helix (FIGLA), as illustrated in Fig. 5 (Lim and Choi, 2012). Inhibition of SMAD

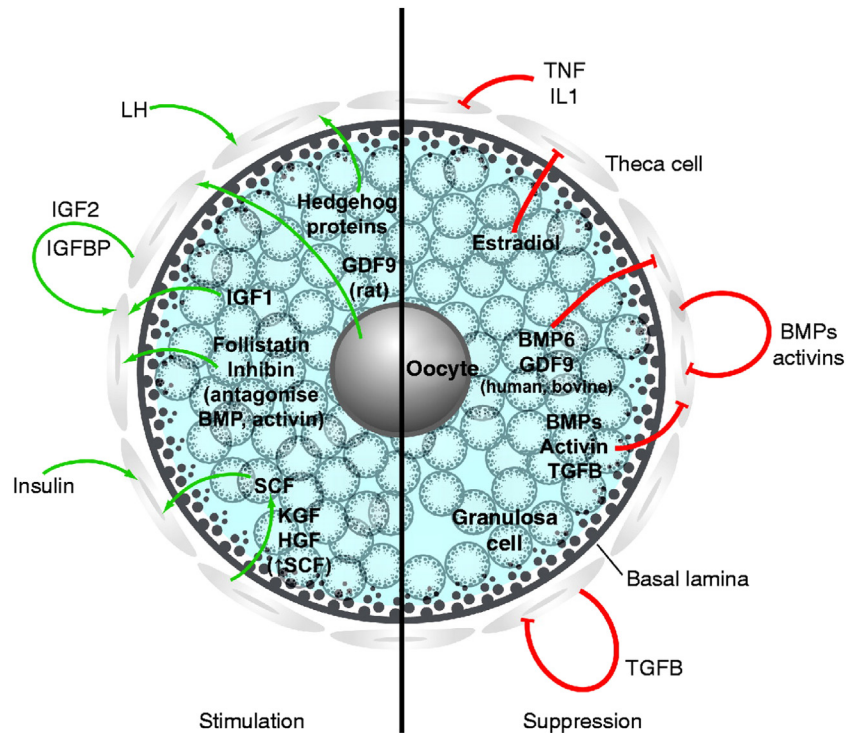


Fig. 3 Various factors, such as TGFB superfamily, are implicated in the bidirectional communication between the gamete and the somatic cells. From Young, J. M. And Mcneilly, A. S. (2010). Theca: The forgotten cell of the ovarian follicle. *Reproduction* **140**, 489–504.

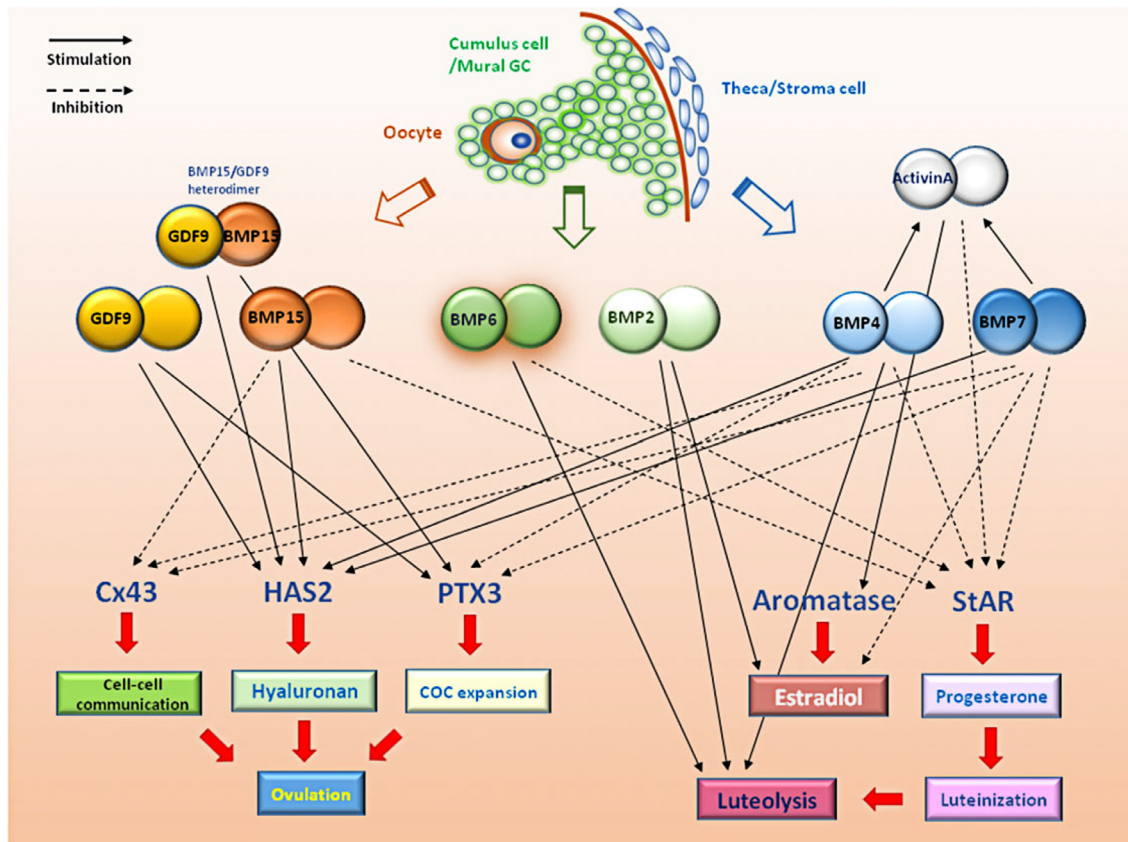


Fig. 4 Schematic diagram summarizing functional roles of BMPs and GDF9 in ovary. From Chang, H. M., Qiao, J. and Leung, P. C. (2016). Oocyte-somatic cell interactions in the human ovary-novel role of bone morphogenetic proteins and growth differentiation factors. *Human Reproduction Update* **23**, 1–18.

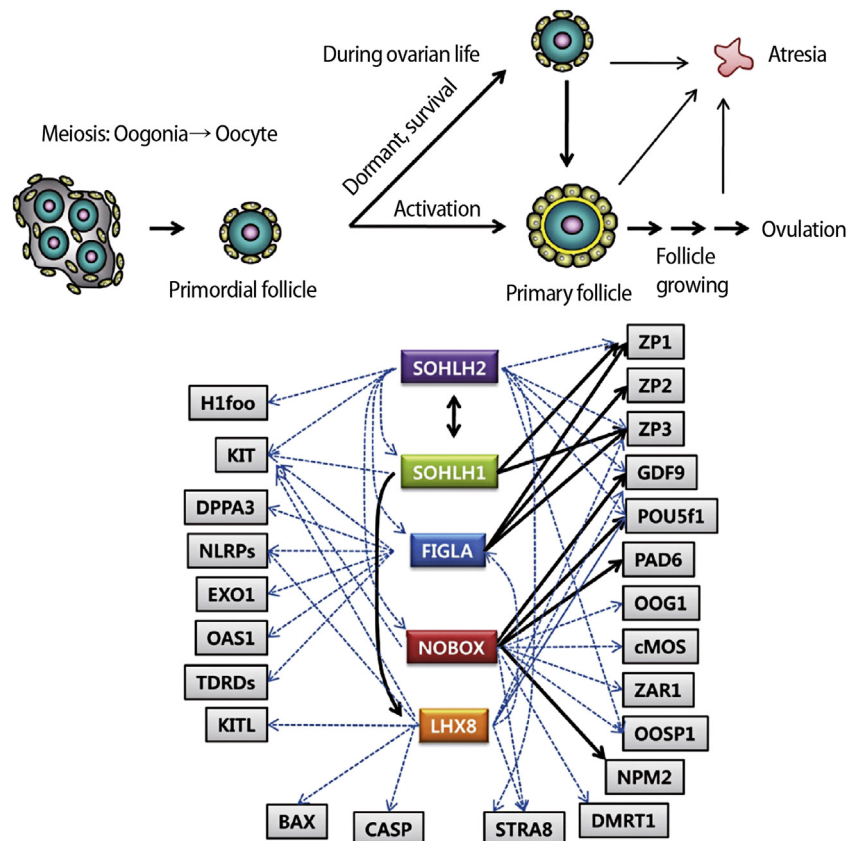


Fig. 5 Regulatory networks of transcriptional factors. From (Lim, E. J. and Choi, Y. (2012). Transcription factors in the maintenance and survival of primordial follicles. *Clinical and Experimental Reproductive Medicine* **39**, 127–131.

signaling also inhibits folliculogenesis very early on (Kaivo-Oja et al., 2006). The role of these factors appears to be to activate genes involved in the growth and morphogenesis of both the somatic cells and the oocyte (Pangas and Matzuk, 2004).

In mice, zona pellucida glycoproteins are secreted solely by the oocyte, whereas in pigs and horses, the somatic cells also participate in this secretion (Flechon et al., 2003; Kolle et al., 2007). Connectivity through the physical boundary formed by the zona pellucida between the oocyte and the somatic cells is maintained through the formation of filipodia-like cellular projections by the somatic cells, as illustrated in Fig. 6 (Allworth and Albertini, 1993; Macaulay et al., 2014). These span the zona pellucida and attach to the oocyte plasma membrane. Such transzonal projections are conserved across species, since they play a major role in nourishing the developing oocyte (Allworth and Albertini, 1993; Macaulay et al., 2016). Various types of physical bridges between somatic cells have been described, such as tunneling nanotubes and epithelial bridges, which have been shown to allow exchanges of material and even mitochondria between cells (Zani and Edelman,

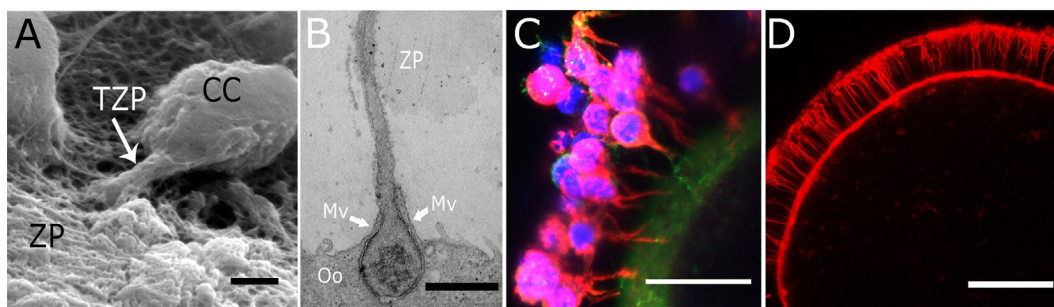


Fig. 6 Transzonal projection structure. (A) Scanning electron micrograph of a cumulus cell (CC) and its TZP interaction with the zona pellucida (ZP). (B) A TZP that penetrates the ZP and interacts with the microvilli (Mv) extensions of the oocyte's (Oo) oolemma. (C) and (D) Numerous actin microfilaments in projections interact with the oocyte membrane. From (Macaulay, A. D., Gilbert, I., Caballero, J., Barreto, R., Fournier, E., Tossou, P., Sirard, M. A., Clarke, H. J., Khandjian, E. W., Richard, F. J., Hyttel, P. and Robert, C. (2014). The gametic synapse: RNA transfer to the bovine oocyte. *Biology of Reproduction* **91**, 90.

2010; Torralba et al., 2016). It has been shown that in transzonal projections, the plasma membranes of the different cells do not fuse (Macaulay et al., 2014). The points of contact are thus gap junctions (Luciano et al., 2011). These channels allow the passage of small molecules (<1 kDa) including cyclic nucleotides (cAMP and cGMP) from the somatic cells in order to maintain the oocyte in meiotic arrest (Gilchrist et al., 2016). Little is known about the nature and the extent of transfers mediated via transzonal junctions from the basal growth phase up to the secondary follicle phase, at which point several layers of granulosa cells and presumptive theca cells are differentiating into a fully formed antral follicle.

Antral Development

The redistribution of granulosa cells to form an antrum marks the passage of the follicle to the tertiary phase. Such a follicle is composed of various cell types, the outermost being more or less flattened cells making up the theca *externa*, in contact with rounder cells making up the theca *interna*, both separated from the granulosa cells by the basal membrane, and finally the innermost and largest cell, namely the oocyte (Fig. 1). Granulosa cells are further delineated according to their position relative to the oocyte. Mural granulosa cells line the entire inner surface of the follicle, while cumulus cells lie closer to the gamete. Cells in direct contact with the oocyte through transzonal projections (via the zona pellucida) make up what is called the *corona radiata*. From primordial follicle activation through basal growth up to the development of the antrum, folliculogenesis seems to depend mostly on local signaling supported by endocrine stimulation. In antral follicles, an important change occurs, making them responsive to gonadotropins (Hillier, 2001) and activating a new set of intra-follicular communications. The two-cell, two-gonadotropin model proposes that pulses of LH stimulate receptors on the surface of theca cells, inducing cholesterol uptake and production of androstenedione by CYP17 activity (Hillier, 2001; Hillier et al., 1994). The neighboring granulosa cells take up the androgen, while stimulation of their FSH receptors activates CYP19 to convert the steroid to estradiol (Fig. 7). In some species, receptor expression is not restricted to one follicular compartment (Peng et al., 1991; Yung et al., 2014) and both receptors are coupled to intracellular signaling mediated by the cAMP-protein kinase A pathway (Gloaguen et al., 2011). In mono-ovulating species, follicles are selected, and this is believed to occur when granulosa cells differentiate to acquire LH receptors, thereby becoming responsive to the basal LH pulses and the pre-ovulatory LH surge and therefore insensitive to the negative feedback of estradiol on FSH secretion (Fortune et al., 2001). FSH stimulation activates epidermal growth factor signaling inside the follicle (El-Hayek et al., 2014).

Most models incorporate the notion of highly compartmentalized follicles in which the different types of granulosa cells (i.e., mural, cumulus and the *corona radiata*) have different functions, as exemplified by differences in the sets of expressed genes. In the late stages of folliculogenesis, the follicular cells and the gamete are still in bilateral communication. Oocyte-secreted factors (e.g., growth differentiation factor 9 and bone-morphogenic protein 15) promote granulosa cell proliferation, and it is believed that gonadotropin signaling is amplified, since it is transmitted over a decreasing LH receptor expression gradient from the outside of the follicle towards the gamete (Gilchrist et al., 2004). This may be important given that once the oocyte reaches its full size, its chromatin becomes compacted and transcription ceases (Lodde et al., 2008; Tan et al., 2009). Although this compaction is species-specific, transcriptional silence means in any case that the oocyte is less capable of responding to environmental cues and must rely on its stored reserve of transcripts to produce its proteins (De La Fuente and Eppig, 2001).

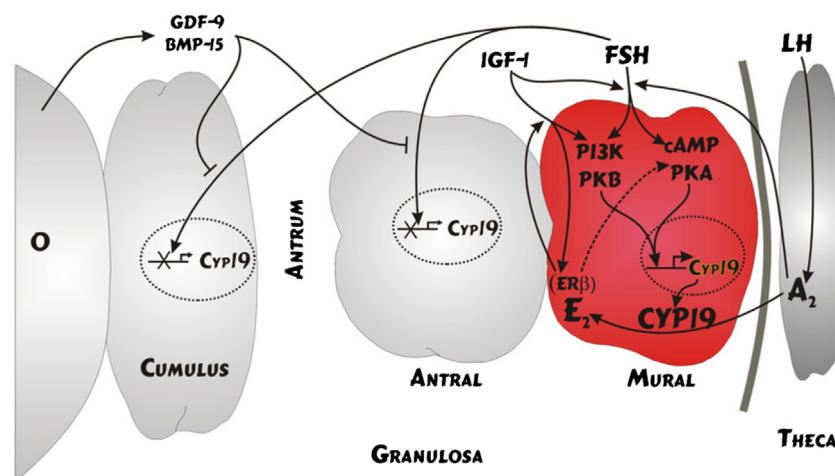


Fig. 7 Illustration of the two-cell, two-gonadotropin model. From Stocco, C. (2008). Aromatase expression in the ovary: Hormonal and molecular regulation. *Steroids* 73, 473–487.

Corona radiata cells bearing transzonal projections have a nourishing role that includes continued delivery of cyclic nucleotides to the gamete to maintain meiotic arrest as well as providing energy substrates such as pyruvate and lactate (Scantland et al., 2014). In many species, the oocyte contains immature mitochondria and an inactive glycolytic apparatus and needs a supply of 3' carbon substrates in order to maintain at least some energy production by oxidative phosphorylation (Rieger and Loskutoff, 1994; Trimarchi et al., 2000).

In addition to proximal cell-to-cell communications, paracrine signaling through the follicular fluid occurs. The composition of antral fluid is quite similar to that of serum and fluctuates during the menstrual or estrous cycle. It contains ions, proteins, hormones, lipids and energy substrates and even accumulates toxins such as organochlorines (Leroy et al., 2004; Meeker et al., 2009). The differences in composition between antral fluid and serum suggest selective transport from one to the other as well as accumulation of substances secreted by follicular cells. Among these, certain proteins are believed to sequester insulin-like growth factors, thereby controlling ovarian stimulation (Amato et al., 1999). In follicles at the early antral stage, this sequestering lasts until pregnancy-associated plasma protein A intervenes to potentiate a pre-ovulatory surge of stimulation (Nyegaard et al., 2010). Communication in pre-ovulatory follicles also involves paracrine signaling among the different types of granulosa cells. Following stimulation by LH, epidermal-growth-factor-like substances (epiregulin, amphiregulin, and betacellulin) are expressed as precursor hormones at the surface of mural granulosa cells. Matrix metalloproteinases cleave the precursor protein to release the hormone subunit into the follicular fluid, which binds to its target receptors on cumulus cells, as illustrated in Fig. 8 (Conti et al., 2006; Hsieh et al., 2009; Light and Hammes, 2015). Signal transduction occurs through the MAPK/ERK pathway to stimulate cumulus cells in preparation for ovulation (Fan et al., 2009). Another signaling molecule requiring proteolysis is the C-type natriuretic peptide (NPPC). The precursor protein and its receptor are expressed in granulosa and cumulus cells (Zhang et al., 2010; Franciosi et al., 2014). Cleavage of the precursor releases a peptide 22 amino acid residues in length, which signals the follicle to grow and maintain the oocyte in meiotic arrest (Franciosi et al., 2014; Zhang et al., 2010).

More recently, follicular cells have been shown to secrete extracellular vesicles into the antral fluid for absorption by cumulus cells (da Silveira et al., 2012; Di Pietro, 2016). The protein and RNA contents of these microvesicles and exosomes could fluctuate during the menstrual (or estrous) cycle and reflect the metabolic status of the mother. These vesicles might constitute another signaling route, and their composition could indicate both follicle viability and oocyte quality. Although the gamete itself appears to have a very limited capacity for exosome uptake, the surrounding cumulus cells could provide an entry point and deliver the contents to the oocyte. Although it was believed that transzonal projections are closed-ended and that only small molecules (<1 kDa) such as cAMP, cGMP, energy substrates and miRNAs are transferred through gap junctions, the presence of microvesicles at junctions and transfer of mRNA and lipids from cumulus cells to the oocyte through transzonal projections have been demonstrated recently (Macauley et al., 2014, 2016; Del Collado et al., 2017). The transfer of large cargo to the oocyte is well described in invertebrates such as *Drosophila* and hydra, where nurse cells “dump” nearly all their contents (including organelles other than the nucleus) into the oocyte. In *Xenopus*, nurse cells transfer germ-cell granules containing mRNA and ribosomes to the oocyte (Guild et al., 1997; Alexandrova et al., 2005).

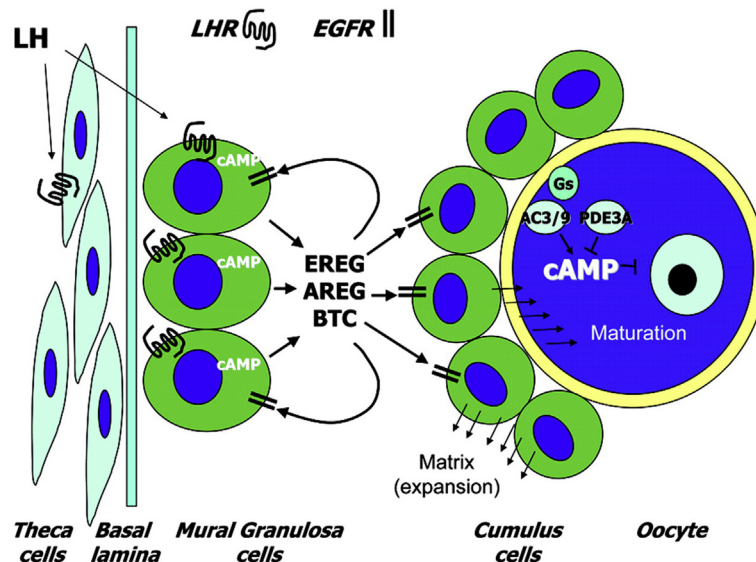


Fig. 8 A model for LH regulation of the EGF network in different cells of the ovarian follicle. From Conti, M., Hsieh, M., Park, J. Y. & Su, Y. Q. (2006). Role of the epidermal growth factor network in ovarian follicles. *Molecular Endocrinology* 20, 715–723.

Folliculogenesis and Developmental Competence of the Oocyte

Folliculogenesis and oogenesis progresses concomitantly until the oocyte reaches its full size. In mice, fully grown oocytes are found in antral follicles up to 2 mm in diameter and ovulatory follicles can reach 4 mm (Griffin et al., 2006). In humans, full-sized oocytes are found in antral follicles 5–7 mm in diameter, and the diameter of a follicle at ovulation is about 20 mm (Gougeon, 1986). In cattle, full-sized oocytes are found in 3 mm follicles and ovulation occurs 7–8 days later in a follicle 20–25 mm in diameter (Fair et al., 1995). Folliculogenesis therefore continues sometimes for several days after the oocyte has reached its full size and passed the chromatin compaction stage (Lodde et al., 2008; Tan et al., 2009). At this point, corona radiata cells could act as surrogate nuclei, much like nurse cells do, and support the transcriptional needs of the gamete during the final stage of folliculogenesis.

Regarding the capacity of the oocyte to undertake the post LH surge steps, it should be considered that full-sized oocytes collected from follicles that have not reached ovulation size are sometimes already developmentally competent, in terms of zygote formation and subsequent embryogenesis at least up to the blastocyst stage. In cattle for example, oocytes from follicles smaller than 2 mm are undersized and incapable of resuming meiosis, whereas a large proportion of oocytes from follicles from 3 to 6 mm are able to reach metaphase II in vitro (Fair et al., 1995). About 30%–40% of these are able to form a zygote and develop into a blastocyst in vitro, compared to about 60% of oocytes collected from healthy follicles larger than 8 mm (Rizos et al., 2002). The developmental competence of immature oocytes is highly sensitive to manipulation of the hormonal regimen (Blondin et al., 2002; Nivet et al., 2012). These observations suggest strongly that a considerable proportion of oocytes have already acquired the capability of resuming meiosis, forming a zygote and sustaining early development but that this proportion increases significantly when the oocyte is kept inside the follicle, even if it is full-sized and transcriptionally inactive. The nature of the essential events that occur during the final step of folliculogenesis to ensure the acquisition of developmental competence is still largely unknown, as are the characteristics that define oocyte quality.

It has been observed recently in cattle that the RNA contents of transzonal projections of cumulus oocyte complexes in antral follicles 3–6 mm in diameter increase significantly during the first 4 h following removal of the ovaries from the animal (Macauley et al., 2016). At this point, the oocyte is still in meiotic arrest and resumes meiosis spontaneously once the cumulus oocyte complex is extracted from the follicle (Tsafiri and Pomerantz, 1986). The ability of cumulus cells to react to some unknown cue following removal of the ovary and to keep the oocyte in meiotic arrest (which requires the transfer of cAMP) suggests a parallel signaling within the compartments of a follicle. Furthermore, the timing of post-mortem RNA accumulation matches post-mortem acquisition of developmental competence observed in cattle (Blondin et al., 1997). It is unclear what this means in terms of the natural post-LH surge events that lead to a complete change in communications and transformation of theca and mural granulosa cells into the corpus luteum while cumulus cells begin to express a new set of genes for cloud expansion and disconnection from the oocyte as it resumes meiosis.

Various interesting observations have clarified the role of somatic cells and endocrine signaling in producing developmentally competent oocytes. First it was shown that in vitro culture of mouse follicles can provide oocytes that occasionally yield live offspring (Eppig and O'Brien, 1996) and that culture conditions can be improved in order to increase the percentage of oocytes that do so (Morohaku et al., 2016). It was noted also that stripped oocytes are not competent, but their developmental capacity improves when they are co-cultured with cumulus cells and even more when they are in physical contact with them (Luciano et al., 2005). Meanwhile, as scientists pondered the complex interplay of signaling pathways and systemic as well as local feedback loops, their assumption that these are essential was challenged when mouse oocytes were obtained in vitro from undifferentiated cells originating outside the follicular environment. This was achieved first using primordial germ-cell-like cells (Hayashi et al., 2012) and later repeated using embryonic stem cells and pluripotent cells (Hayashi and Saitou, 2013; Hikabe et al., 2016). Some of these oocytes were competent for fertilization and even underwent embryonic development to produce live offspring (Hikabe et al., 2016). However, the vast majority of them were incompetent, and this was attributed to inadequate endocrine support and physical interaction between somatic cells and the oocyte (Morohaku et al., 2016; Hikabe et al., 2016). This in vitro production of competent mouse oocytes nevertheless did provide a new workbench for studying the adequacy of culture conditions and the timing of specific signaling pathways and for distinguishing essential requirements from helpful but not critical factors. It remains to be seen whether or not a similar investigational tool will be developed for species in which folliculogenesis is a much longer process and the gamete is more dependent on support from neighboring cells. Improving our understanding of intra-follicular communications throughout folliculogenesis will not only bring advances in the science of reproductive biology but should also reveal new physiological targets for the treatment infertility.

References

- Adams, G. P., Jaiswal, R., Singh, J., & Malhi, P. (2008). Progress in understanding ovarian follicular dynamics in cattle. *Theriogenology*, 69, 72–80.
- Adhikari, D., Flohr, G., Gorre, N., Shen, Y., Yang, H., Lundin, E., Lan, Z., Gambello, M. J., & Liu, K. (2009). Disruption of Tsc2 in oocytes leads to overactivation of the entire pool of primordial follicles. *Molecular Human Reproduction*, 15, 765–770.
- Alexandrova, O., Schade, M., Bottger, A., & David, C. N. (2005). Oogenesis in Hydra: Nurse cells transfer cytoplasm directly to the growing oocyte. *Developmental Biology*, 281, 91–101.
- Allworth, A. E., & Albertini, D. F. (1993). Meiotic maturation in cultured bovine oocytes is accompanied by remodeling of the cumulus cell cytoskeleton. *Developmental Biology*, 158, 101–112.

- Amato, G., Izzo, A., Tucker, A. T., & Bellastella, A. (1999). Lack of insulin-like growth factor binding protein-3 variation after follicle-stimulating hormone stimulation in women with polycystic ovary syndrome undergoing in vitro fertilization. *Fertility and Sterility*, 72, 454–457.
- Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Human Reproduction Update*, 18, 73–91.
- Blondin, P., Bousquet, D., Twagiramungu, H., Barnes, F., & Sirard, M. A. (2002). Manipulation of follicular development to produce developmentally competent bovine oocytes. *Biology of Reproduction*, 66, 38–43.
- Blondin, P., Guilbault, L. A., & Sirard, M. A. (1997). The time interval between FSH-P administration and slaughter can influence the developmental competence of beef heifer oocytes. *Theriogenology*, 48, 803–813.
- Castrillon, D. H., Miao, L., Kollipara, R., Horner, J. W., & Depinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*, 301, 215–218.
- Chang, H. M., Qiao, J., & Leung, P. C. (2016). Oocyte-somatic cell interactions in the human ovary-novel role of bone morphogenetic proteins and growth differentiation factors. *Human Reproduction Update*, 23, 1–18.
- Conti, M., Hsieh, M., Park, J. Y., & Su, Y. Q. (2006). Role of the epidermal growth factor network in ovarian follicles. *Molecular Endocrinology*, 20, 715–723.
- da Silva, J. C., Veeramachaneni, D. N., Winger, Q. A., Carnevale, E. M., & Bouma, G. J. (2012). Cell-secreted vesicles in equine ovarian follicular fluid contain miRNAs and proteins: A possible new form of cell communication within the ovarian follicle. *Biology of Reproduction*, 86, 71.
- De La Fuente, R., & Eppig, J. J. (2001). Transcriptional activity of the mouse oocyte genome: companion granulosa cells modulate transcription and chromatin remodeling. *Developmental Biology*, 229, 224–236.
- Del Collado, M., da Silva, J. C., Sangalli, J. R., Andrade, G. M., Sousa, L., Silva, L. A., Meirelles, F. V., & Perecin, F. (2017). Fatty acid binding Protein 3 and transzonal projections are involved in lipid accumulation during in vitro maturation of bovine oocytes. *Scientific Reports*, 7, 2645.
- Di Pietro, C. (2016). Exosome-mediated communication in the ovarian follicle. *Journal of Assisted Reproduction and Genetics*, 33, 303–311.
- Driancourt, M. A., Reynaud, K., Cortvrindt, R., & Smits, J. (2000). Roles of KIT and KIT ligand in ovarian function. *Reviews of Reproduction*, 5, 143–152.
- Durlinger, A. L., Kramer, P., Karels, B., de Jong, F. H., Uilenbroek, J. T., Grootegoed, J. A., & Themmen, A. P. (1999). Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. *Endocrinology*, 140, 5789–5796.
- Eftekhari, M., Deghani Firoozabadi, R., Khani, P., Ziaei Bideh, E., & Forghani, H. (2016). Effect of laparoscopic ovarian drilling on outcomes of in vitro fertilization in clomiphene-resistant women with polycystic ovary syndrome. *International Journal of Fertility & Sterility*, 10, 42–47.
- El-Hayek, S., Demeestere, I., & Clarke, H. J. (2014). Follicle-stimulating hormone regulates expression and activity of epidermal growth factor receptor in the murine ovarian follicle. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 16778–16783.
- Eppig, J. J., & O'Brien, M. J. (1996). Development in vitro of mouse oocytes from primordial follicles. *Biology of Reproduction*, 54, 197–207.
- Erickson, B. H. (1966). Development and senescence of the postnatal bovine ovary. *Journal of Animal Science*, 25, 800–805.
- Evans, A. C. (2003). Characteristics of ovarian follicle development in domestic animals. *Reproduction in Domestic Animals*, 38, 240–246.
- Fair, T., Hyttel, P., & Greve, T. (1995). Bovine oocyte diameter in relation to maturational competence and transcriptional activity. *Molecular Reproduction and Development*, 42, 437–442.
- Fan, H. Y., Liu, Z., Shimada, M., Sterneck, E., Johnson, P. F., Hedrick, S. M., & Richards, J. S. (2009). MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science*, 324, 938–941.
- Felicio, L. S., Nelson, J. F., & Finch, C. E. (1986). Prolongation and cessation of estrous cycles in aging C57BL/6J mice are differentially regulated events. *Biology of Reproduction*, 34, 849–858.
- Flechon, J. E., Degrouard, J., Kopečný, V., Pivko, J., Pavlok, A., & Motlik, J. (2003). The extracellular matrix of porcine mature oocytes: Origin, composition and presumptive roles. *Reproductive Biology and Endocrinology*, 1, 124.
- Fortune, J. E., Rivera, G. M., Evans, A. C., & Turzillo, A. M. (2001). Differentiation of dominant versus subordinate follicles in cattle. *Biology of Reproduction*, 65, 648–654.
- Franciosi, F., Coticchio, G., Lodde, V., Tessaro, I., Modina, S. C., Fadini, R., Dal Canto, M., Renzini, M. M., Albertini, D. F., & Luciano, A. M. (2014). Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. *Biology of Reproduction*, 91, 61.
- Gilchrist, R. B., Luciano, A. M., Richani, D., Zeng, H. T., Wang, X., Vos, M. D., Sugimura, S., Smits, J., Richard, F. J., & Thompson, J. G. (2016). Oocyte maturation and quality: Role of cyclic nucleotides. *Reproduction*, 152, R143–57.
- Gilchrist, R. B., Ritter, L. J., & Armstrong, D. T. (2004). Oocyte-somatic cell interactions during follicle development in mammals. *Animal Reproduction Science*, 82–83, 431–446.
- Gittens, J. E., Barr, K. J., Vanderhyden, B. C., & Kidder, G. M. (2005). Interplay between paracrine signaling and gap junctional communication in ovarian follicles. *Journal of Cell Science*, 118, 113–122.
- Glasier, A. F., Baird, D. T., & Hillier, S. G. (1989). FSH and the control of follicular growth. *Journal of Steroid Biochemistry*, 32, 167–170.
- Gloaguen, P., Crepeux, P., Heitzler, D., Poupon, A., & Reiter, E. (2011). Mapping the follicle-stimulating hormone-induced signaling networks. *Frontiers in Endocrinology (Lausanne)*, 2, 45.
- Gougeon, A. (1986). Dynamics of follicular growth in the human: A model from preliminary results. *Human Reproduction*, 1, 81–87.
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3, a005850.
- Griffin, J., Emery, B. R., Huang, I., Peterson, C. M., & Carrell, D. T. (2006). Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *Journal of Experimental & Clinical Assisted Reproduction*, 3, 2.
- Guild, G. M., Connelly, P. S., Shaw, M. K., & Tilney, L. G. (1997). Actin filament cables in Drosophila nurse cells are composed of modules that slide passively past one another during dumping. *The Journal of Cell Biology*, 138, 783–797.
- Hayashi, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H., & Saitou, M. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science*, 338, 971–975.
- Hayashi, K., & Saitou, M. (2013). Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. *Nature Protocols*, 8, 1513–1524.
- Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., Shimamoto, S., Imamura, T., Nakashima, K., Saitou, M., & Hayashi, K. (2016). Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature*, 539, 299–303.
- Hillier, S. G. (2001). Gonadotropic control of ovarian follicular growth and development. *Molecular and Cellular Endocrinology*, 179, 39–46.
- Hillier, S. G., Whitelaw, P. F., & Smyth, C. D. (1994). Follicular oestrogen synthesis: The "two-cell, two-gonadotrophin" model revisited. *Molecular and Cellular Endocrinology*, 100, 51–54.
- Hsieh, M., Zamah, A. M., & Conti, M. (2009). Epidermal growth factor-like growth factors in the follicular fluid: Role in oocyte development and maturation. *Seminars in Reproductive Medicine*, 27, 52–61.
- Kaivo-Oja, N., Jeffery, L. A., Ritvos, O., & Mottershead, D. G. (2006). Smad signalling in the ovary. *Reproductive Biology and Endocrinology*, 4, 21.
- Kawamura, K., Cheng, Y., Suzuki, N., Deguchi, M., Sato, Y., Takae, S., Ho, C. H., Kawamura, N., Tamura, M., Hashimoto, S., Sugishita, Y., Morimoto, Y., Hosoi, Y., Yoshioka, N., Ishizuka, B., & Hsueh, A. J. (2013). Hippo signaling disruption and Akt stimulation of ovarian follicles for infertility treatment. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 17474–17479.
- Kim, J. Y. (2012). Control of ovarian primordial follicle activation. *Clinical and Experimental Reproductive Medicine*, 39, 10–14.
- Kolle, S., Dubois, C. S., Caillaud, M., Lahuec, C., Sinowatz, F., & Goudet, G. (2007). Equine zona protein synthesis and ZP structure during folliculogenesis, oocyte maturation, and embryogenesis. *Molecular Reproduction and Development*, 74, 851–859.
- Leroy, J. L., Vanholder, T., Delanghe, J. R., Opsomer, G., Van Soom, A., Bols, P. E., & de Kruij, A. (2004). Metabolite and ionic composition of follicular fluid from different-sized follicles and their relationship to serum concentrations in dairy cows. *Animal Reproduction Science*, 80, 201–211.
- Light, A., & Hammes, S. R. (2015). LH-induced steroidogenesis in the mouse ovary, but not testis, requires matrix metalloproteinase 2- and 9-mediated cleavage of upregulated egf receptor ligands. *Biology of Reproduction*, 93, 65.

- Lim, E. J., & Choi, Y. (2012). Transcription factors in the maintenance and survival of primordial follicles. *Clinical and Experimental Reproductive Medicine*, 39, 127–131.
- Lodde, V., Modina, S., Maddox-Hyttel, P., Franciosi, F., Lauria, A., & Luciano, A. M. (2008). Oocyte morphology and transcriptional silencing in relation to chromatin remodeling during the final phases of bovine oocyte growth. *Molecular Reproduction and Development*, 75, 915–924.
- Luciano, A. M., Franciosi, F., Modina, S. C., & Lodde, V. (2011). Gap junction-mediated communications regulate chromatin remodeling during bovine oocyte growth and differentiation through cAMP-dependent mechanism(s). *Biology of Reproduction*, 85, 1252–1259.
- Luciano, A. M., Lodde, V., Beretta, M. S., Colleoni, S., Lauria, A., & Modina, S. (2005). Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: Role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. *Molecular Reproduction and Development*, 71, 389–397.
- Lussier, J. G., Matton, P., & Dufour, J. J. (1987). Growth rates of follicles in the ovary of the cow. *Journal of Reproduction and Fertility*, 81, 301–307.
- Macaulay, A. D., Gilbert, I., Caballero, J., Barreto, R., Fournier, E., Tossou, P., Sirard, M. A., Clarke, H. J., Khandjian, E. W., Richard, F. J., Hyttel, P., & Robert, C. (2014). The gametic synapse: RNA transfer to the bovine oocyte. *Biology of Reproduction*, 91, 90.
- Macaulay, A. D., Gilbert, I., Scantland, S., Fournier, E., Ashkar, F., Bastien, A., Saadi, H. A., Gagne, D., Sirard, M. A., Khandjian, E. W., Richard, F. J., Hyttel, P., & Robert, C. (2016). Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. *Biology of Reproduction*, 94, 16.
- Meeker, J. D., Missmer, S. A., Alttshul, L., Vitonis, A. F., Ryan, L., Cramer, D. W., & Hauser, R. (2009). Serum and follicular fluid organochlorine concentrations among women undergoing assisted reproduction technologies. *Environmental Health*, 8, 32.
- Messenger, S., Chatzidaki, E. E., Ma, D., Hendrick, A. G., Zahn, D., Dixon, J., Thresher, R. R., Malinge, I., Lomet, D., Carlton, M. B., Colledge, W. H., Caraty, A., & Aparicio, S. A. (2005). Kisspeptin directly stimulates gonadotropin-releasing hormone release via G protein-coupled receptor 54. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 1761–1766.
- Morbeck, D. E., Esbenshade, K. L., Flowers, W. L., & Britt, J. H. (1992). Kinetics of follicle growth in the prepubertal gilt. *Biology of Reproduction*, 47, 485–491.
- Morohaku, K., Tanimoto, R., Sasaki, K., Kawahara-Miki, R., Kono, T., Hayashi, K., Hirao, Y., & Obata, Y. (2016). Complete in vitro generation of fertile oocytes from mouse primordial germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 9021–9026.
- Munster, K., Schmidt, L., & Helm, P. (1992). Length and variation in the menstrual cycle—A cross-sectional study from a Danish county. *British Journal of Obstetrics and Gynaecology*, 99, 422–429.
- Nivet, A. L., Bunel, A., Labrecque, R., Belanger, J., Vigneault, C., Blondin, P., & Sirard, M. A. (2012). FSH withdrawal improves developmental competence of oocytes in the bovine model. *Reproduction*, 143, 165–171.
- Nyegaard, M., Overgaard, M. T., Su, Y. Q., Hamilton, A. E., Kwintkiewicz, J., Hsieh, M., Nayak, N. R., Conti, M., Conover, C. A., & Giudice, L. C. (2010). Lack of functional pregnancy-associated plasma protein-A (PAPPA) compromises mouse ovarian steroidogenesis and female fertility. *Biology of Reproduction*, 82, 1129–1138.
- Pangas, S. A. (2012). Bone morphogenetic protein signaling transcription factor (SMAD) function in granulosa cells. *Molecular and Cellular Endocrinology*, 356, 40–47.
- Pangas, S. A., & Matzuk, M. M. (2004). Genetic models for transforming growth factor beta superfamily signaling in ovarian follicle development. *Molecular and Cellular Endocrinology*, 225, 83–91.
- Payan-Carreira, R., & Pires, M. A. (2008). Multioocyte follicles in domestic dogs: A survey of frequency of occurrence. *Theriogenology*, 69, 977–982.
- Pedersen, T. (1970). Follicle kinetics in the ovary of the cyclic mouse. *Acta Endocrinologica*, 64, 304–323.
- Peng, X. R., Hsueh, A. J., Lapolt, P. S., Bjersing, L., & Ny, T. (1991). Localization of luteinizing hormone receptor messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. *Endocrinology*, 129, 3200–3207.
- Prior, J. C., & Hitchcock, C. L. (2011). The endocrinology of perimenopause: Need for a paradigm shift. *Frontiers in Bioscience (Scholar Edition)*, 3, 474–486.
- Reddy, P., Liu, L., Adhikari, D., Jagarlamudi, K., Rajareddy, S., Shen, Y., Du, C., Tang, W., Hamalainen, T., Peng, S. L., Lan, Z. J., Cooney, A. J., Huhtaniemi, I., & Liu, K. (2008). Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science*, 319, 611–613.
- Rieger, D., & Loskutoff, N. M. (1994). Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. *Journal of Reproduction and Fertility*, 100, 257–262.
- Rizos, D., Ward, F., Duffy, P., Boland, M. P., & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Molecular Reproduction Development*, 61, 234–248.
- Scantland, S., Tessaro, I., Macabelli, C. H., Macaulay, A. D., Cagnone, G., Fournier, E., Luciano, A. M., & Robert, C. (2014). The adenosine salvage pathway as an alternative to mitochondrial production of ATP in maturing mammalian oocytes. *Biology of Reproduction*, 91, 75.
- Spears, N., Molinek, M. D., Robinson, L. L., Fulton, N., Cameron, H., Shimoda, K., Telfer, E. E., Anderson, R. A., & Price, D. J. (2003). The role of neurotrophin receptors in female germ-cell survival in mouse and human. *Development*, 130, 5481–5491.
- Su, Y. Q., Wu, X., O'Brien, M. J., Pendola, F. L., Denegre, J. N., Matzuk, M. M., & Eppig, J. J. (2004). Synergistic roles of BMP15 and GDF9 in the development and function of the oocyte-cumulus cell complex in mice: Genetic evidence for an oocyte-granulosa cell regulatory loop. *Developmental Biology*, 276, 64–73.
- Tan, J. H., Wang, H. L., Sun, X. S., Liu, Y., Sui, H. S., & Zhang, J. (2009). Chromatin configurations in the germinal vesicle of mammalian oocytes. *Molecular Human Reproduction*, 15, 1–9.
- Torralba, D., Baixauli, F., & Sanchez-Madrid, F. (2016). Mitochondria Know No Boundaries: Mechanisms and Functions of Inter cellular Mitochondrial Transfer. *Frontiers in Cell and Development Biology*, 4, 107.
- Trimarchi, J. R., Liu, L., Porterfield, D. M., Smith, P. J., & Keefe, D. L. (2000). Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. *Biology of Reproduction*, 62, 1866–1874.
- Tsafiri, A., & Pomerantz, S. H. (1986). Oocyte maturation inhibitor. *Clinics in Endocrinology and Metabolism*, 15, 157–170.
- Visser, J. A., de Jong, F. H., Laven, J. S., & Themmen, A. P. (2006). Anti-Müllerian hormone: A new marker for ovarian function. *Reproduction*, 131, 1–9.
- Wallace, W. H., & Kelsey, T. W. (2010). Human ovarian reserve from conception to the menopause. *PLoS One*, 5, e8772.
- Wear, H. M., Mcpike, M. J., & Watanabe, K. H. (2016). From primordial germ cells to primordial follicles: A review and visual representation of early ovarian development in mice. *Journal of Ovarian Research*, 9, 36.
- Xiao, S., Zhang, J., Romero, M. M., Smith, K. N., Shea, L. D., & Woodruff, T. K. (2015). In vitro follicle growth supports human oocyte meiotic maturation. *Scientific Reports*, 5, 17323.
- Yung, Y., Aviel-Ronen, S., Maman, E., Rubinstein, N., Avivi, C., Orvieto, R., & Hourvitz, A. (2014). Localization of luteinizing hormone receptor protein in the human ovary. *Molecular Human Reproduction*, 20(9), 844.
- Zani, B. G., & Edelman, E. R. (2010). Cellular bridges: Routes for intercellular communication and cell migration. *Communicative & Integrative Biology*, 3, 215–220.
- Zhang, M., Su, Y. Q., Sugiura, K., Xia, G., & Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*, 330, 366–369.

Further Reading

- Erickson, G. F. (1995). The ovary: basic principles and concepts. In P. Felig, J. D. Baxter, & L. A. Frohman (Eds.), *Endocrinology and metabolism* (pp. 973–1015). New York: McGraw Hill.
- Stocco, C. (2008). Aromatase expression in the ovary: Hormonal and molecular regulation. *Steroids*, 73, 473–487.
- Young, J. M., & Mcneilly, A. S. (2010). Theca: The forgotten cell of the ovarian follicle. *Reproduction*, 140, 489–504.

Cumulus Cells

Darryl L Russell and Rebecca L Robker, The University of Adelaide, Adelaide, South Australia, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

The somatic cells that directly surround the oocyte are a unique sub-lineage of the granulosa cells, known as “cumulus cells”, that play a unique and critical role providing a niche environment for development of the pluripotent oocyte. The name is derived from a description of their physical appearance in the final stage of folliculogenesis; the periovulatory period, when a burst of mucinous extracellular matrix production (discussed below) gives the cell complex a puffy cumulus cloud-like appearance. The cumulus cells and the oocyte together form a visibly distinguishable complex known collectively as the cumulus oocyte complex or COC; comprising 3 to 4 layers of cumulus cells (totalling around 2000 cells) tightly packed together in concentric layers enveloping the oocyte. The COC becomes apparent in antral follicles where it protrudes into the antrum from the inner granulosa cell layers and is surrounded by follicular fluid. Strong specific interactions among cumulus cells and between cumulus cells and the oocyte mean the COC detaches readily as a discrete intact complex from the granulosa layers and can be isolated by simply aspirating the antral fluid, as is the standard method of collecting oocytes for in vitro fertilization procedures.

Origin of Cumulus Cells

Cumulus and granulosa cells have a shared origin, but are separate sub-lineages. The cumulus are clearly a distinct cell type since they have different functions, hormone responsiveness and fate to the granulosa cells. In fact granulosa cells can be divided into three sub-lineages based on distinctions in hormone responsiveness and gene expression: 1. Mural granulosa cells, which line the follicle wall and usually have some contact with the follicular basement membrane. 2. Antral granulosa cells, which line the antral cavity and contact the follicular fluid; and 3. cumulus cells, which have direct contact with the oocyte through cellular projections that traverse the zona pellucida and form gap junction connections with the oocyte plasma membrane (Winterhager and Kidder, 2015). The cumulus lineage is specified by the actions of oocyte-secreted morphogens Growth and Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15). These two oocyte-specific growth factors induce the cumulus gene expression profile, which includes suppression of luteinizing hormone receptor and progesterone receptor expression preventing the cumulus cells responding to key endocrine stimuli of granulosa cells (Elvin et al., 1999; Eppig et al., 1997). Meanwhile GDF9 and BMP15 signals activate the expression of key cumulus-specific genes; such as glycolysis enzymes (Sugiura et al., 2007) and cholesterol synthetic pathway enzymes (Su et al., 2008) that are essential mediators of healthy oocyte function. The importance of oocyte secreted growth factors in specifying cumulus phenotype is illustrated by the fact that when mural granulosa cells are treated in vitro with these factors, they respond by adopting the cumulus cell gene expression profile (Peng et al., 2013). Furthermore, the cumulus cells adopt a more granulosa like gene expression profile, including increased LH-receptor and steroidogenic enzyme expression, after ovulation when they have become separated from the oocyte (Chaffin et al., 2012). Thus cumulus specification is principally a function of exposure to these oocyte secreted factors through close proximity to the oocyte. The first divergence between cumulus and granulosa cell fates arises due to the formation of the antrum (Eppig et al., 1997), when mural granulosa cells differentiate because they become separated from oocyte derived signals by the antral space and follicular fluid. It is often assumed that the granulosa cell phenotype is the default prior to antrum formation. However, the divergence of the two cell types at antrum formation is synchronous with an increase in LH-receptor expression and increased aromatase expression (Brown et al., 2010b) as the granulosa compartment first emerges as an endocrine organ. The somatic cells of follicles prior to antrum formation have more in common with cumulus cells than granulosa cells, considering that oocytes produce GDF9 and BMP15 from the earliest stages of follicle growth, that close proximity to the oocyte promotes cumulus phenotype and that oocytes require the supportive functions of cumulus cells for their growth and survival. The role of the fluid-filled follicular antrum is an enduring unknown, but this important divergence of somatic cell phenotypes into separate endocrine and oocyte support functions is one clearly important outcome of antrum development.

Cumulus Cell Functions

Metabolic and Nutritional Support of the Oocyte

An intimate association with cumulus cells is essential for oocyte survival and healthy development. Even in the final hours of maturation, dissociation of the cumulus vestment from the oocyte severely impacts oocyte competence (Zhang et al., 2012). The cumulus cells directly contact the oocyte through long transzonal projections with terminal gap junctions connecting cumulus cells to the oocyte (Winterhager and Kidder, 2015). These gap junctions allow transit of small (~1 kDa) molecules between the oocyte and cumulus cell cytoplasm (Edry et al., 2006; Sela-Abramovich et al., 2006). That small cytoplasmic contents are shared between cumulus cells and the oocyte is demonstrated when fluorescent dyes are injected into one cell type but then become rapidly visible in both (Li et al., 2007; Veitch et al., 2004). Endogenous molecules that are transported from the cumulus cells into oocytes include

metabolites of glucose; lactate and pyruvate, ATP, cAMP and cGMP. Transit of glucose metabolites is important as oocytes cannot independently metabolize glucose and they depend on cumulus cells to perform glycolysis and transfer the products intercellularly for energy production in the oocyte mitochondria (Johnson et al., 2007). The cyclic nucleotides cAMP and cGMP are important regulators of oocyte meiotic arrest and their supply from the somatic cells (cumulus as well as granulosa) into the oocyte is a critical means by which the status of the somatic compartments control germ cell maturation (Russell et al., 2016). During its growth the oocyte must accumulate large RNA stores that mediate the early stages of embryogenesis post fertilization, and some evidence exists to suggest that RNA is passed to the oocyte from cumulus cells (Macaulay et al., 2016), but this role is not definitively proven. On the other hand, it is less certain what, if any, endogenous molecules are directly transported from oocyte to cumulus cytoplasm through the gap junctions, although small molecule dyes do flux readily in that direction. The cumulus cells also provide protection for the oocyte from damage caused by elevated reactive oxygen species through transfer of antioxidant reduced glutathione through Gap junctions (Ozawa et al., 2010). In humans the capacity of cumulus cells to produce glutathione is positively associated with oocyte developmental potential (Ceko et al., 2015).

Control of Oocyte Meiosis

The cumulus cells and granulosa cells play a critical role in controlling oocyte progression through meiosis. The oocyte will exit meiotic arrest upon activation of meiosis promoting factor (MPF) a complex of cell cycle proteins that are kept switched off through phosphorylation by protein kinase-A, activated by high cAMP. Cumulus and granulosa cells maintain high oocyte cAMP levels through the transport of cAMP through the gap junctions and cGMP which inhibits cAMP degrading phosphodiesterases in the oocyte, thus keeping oocytes in meiotic arrest. Separation of the oocyte from its cumulus vestment thus results in spontaneous meiotic resumption due to the loss of cAMP and cGMP transport. Once ovulation is triggered, oocytes re-enter meiosis due to closure of the gap junctions and separation of cumulus cells from the oocyte due to cumulus matrix expansion (see below) which lead to reduced cAMP in the oocyte releasing the block on MPF action.

Ovulation

In addition to provision of metabolism and nutrients to the oocyte, the cumulus cells also play a unique and critical role mediating ovulation of the oocyte. Ovulation is triggered by a surge of Luteinizing Hormone from the pituitary gland. This high level of LH acts on granulosa and theca cells of the follicle, but not cumulus cells because the action of GDF9 and BMP15 from the oocyte represses expression of the *Lhcgr* gene, and hence cumulus cells lack LH receptors and remain unresponsive (Eppig et al., 1997). Cumulus cells, like granulosa cells, do have FSH receptors and thus likely sense the smaller surge in FSH from the pituitary at the beginning of ovulation. But granulosa cells principally transmit the ovulatory signal to cumulus cells by activating the production of EGF-like Ligand (EGF-L) genes; *Areg*, *Ereg* and *Btc* and these factors strongly influence cumulus cells in the periovulatory period (Park et al., 2004). FSH and EGF-L promote very large, rapid production of a unique ECM in the COC. The glycosaminoglycan hyaluronan (HA) is synthesized via strong induction of hyaluronan synthase 2 gene (*Has2*) as well as glycolytic genes necessary to synthesize the HA components from glucose (Sugimura et al., 2015). The very long HA molecules are cross-linked by an HA-binding protein complex of TSG6 and PTX3 also rapidly and simultaneously made by the cumulus cells (Russell and Robker, 2007). Expression of *Has2*, *Tnfrsf10b* and *Ptx3* genes requires the presence of oocyte-secreted signals GDF9 and BMP15 which specify the cumulus phenotype but cannot activate the ovulatory gene induction. Expression of each of these cumulus cell genes is essential for ovulation and additionally some proteins synthesized by the granulosa cells that are incorporated into the COC matrix are also necessary for normal ovulation. Specifically, versican a large HA-binding proteoglycan and ADAMTS1 a proteoglycanase enzyme that processes versican are incorporated in the HA matrix of the COC (Brown et al., 2010a), and are important for normal ovulation, but are both synthesized in the GC in response to the LH surge. Some blood borne proteins are also incorporated and play key roles in the COC matrix, including liver derived inter-alpha trypsin inhibitor (*IαI*) and fetuin B (Dietzel et al., 2013; Zhu et al., 2008). Combined, the findings that each individual component of the COC matrix is essential for ovulation to succeed demonstrates that the COC matrix is a key mediator of ovulation. The fact that oocyte factors promote the synthesis of this matrix provides a mechanism whereby oocytes can 'guide' the ovulation process and hence ovulation may be considered a "checkpoint" event that ensures the release of only high quality oocytes, i.e. ones that can sustain the appropriate responsiveness in the cumulus cells.

Oocyte Developmental Competence

The role of cumulus cells in establishing oocyte developmental competence is supported by growing cumulative evidence. As the cumulus complex provides metabolic and nutritional support for the developing oocyte, removal of the cumulus vestment results in slow starvation and death of the oocyte. Even when cumulus cells are removed acutely in the final stages of maturation, the competence of oocytes to fertilize and develop normally into embryos is severely compromised. The importance of cumulus gene expression for health of the oocyte is demonstrated by a range of studies showing that the expression level of key cumulus genes is directly associated with the developmental outcomes for that oocyte. Genes whose expression is positively correlated with high quality oocyte outcomes include *Has2*, *Grem1*, *Tnfrsf10b*, *Ptx3*, *Glut4*, *Vcan*, and several others (Mckenzie et al., 2004; Anderson et al., 2009; Assou et al., 2008; Gebhardt et al., 2011; Wathlet et al., 2012). Thus acquisition of oocyte developmental competence is a bi-directional mechanism whereby oocyte-derived signals act on cumulus cells to promote expression of genes and pathways

required to support oocyte growth; and the cumulus cells in turn feedback the reciprocal trophic support. Cumulus cells and the COC matrix also play important roles after ovulation in the interaction of the COC with the fallopian tube (or oviduct) epithelial cells. Adhesive interactions between the cumulus and fallopian epithelial cells mediates the collection of the oocyte and its transport through this long tube to meet the sperm and be fertilized (Lam et al., 2000).

Fertilization

Contact with the COC matrix signals to the sperm that they are near their final target and sperm are intricately influenced by the cumulus cells and matrix which induce the final maturation phases in the sperm; capacitation and the acrosome reaction, making them competent for fertilization. The acrosome reaction in sperm is promoted upon contact with the COC which maximizes sperm's potential to fertilize the oocyte upon reaching the zona pellucida (Hong et al., 2009; Jin et al., 2011).

As well as being important for ovulation, the composition of the COC matrix is also critical for fertilization. Mutant mouse models with ablation of each of the cumulus matrix proteins uniformly show deficiencies in ability to become fertilized, due to changes in matrix stiffness, specific protein interactions between the COC and sperm as well as possible reduction in oocyte competence (Russell and Robker, 2007). Protease inhibitors I α I and Fetuin B in the COC matrix and prevent premature hardening of the zona pellucida which must occur only after fertilization as it prevents sperm binding (Dietzel et al., 2017; Dietzel et al., 2013). The matrix is also capable of excluding certain molecules including glucose, while sequestering and concentrating cumulus secreted factors such as prostaglandins –and most likely oocyte secreted factors also (Dunning et al., 2007). This process may intensify the signaling actions of these factors prolonging their action and possibly leading to concentration dependent changes in the response both of cumulus cells and sperm. Post-ovulation the cumulus cells steroidogenic activity (Vanderhyden and Tonary, 1995) increases and high concentrations of progesterone are known to promote the sperm acrosome reaction, hyperactivation of sperm motility and guidance of hyperactivated sperm towards the oocyte (Unates et al., 2014). The abundant hyaluronan making up the bulk of the COC matrix is digested by a specific hyaluronidase enzyme expressed on the sperm head to facilitate sperm penetration. The matrix also presents a barrier which selects sperm by preventing the penetration of any individual sperm with defective hyaluronidase or motile activity. This has been illustrated by showing that using COC matrix penetration as a laboratory method to select sperm used in IVF is an effective way to enrich high quality sperm (Hong et al., 2009). The digested hyaluronan fragments interact with Toll-like receptors (TLR) on the cumulus cells leading to production of cytokines, chemokines and prostaglandins which accumulate after fertilization and trigger further degradation of the COC matrix mediated by the cumulus cells (Tamba et al., 2010). Importantly chemokines secreted by the cumulus cells, activate receptors on the sperm mid-piece to enhance successful fertilization (Caballero-Campo et al., 2014).

References

- Anderson, R. A., et al. (2009). Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. *Reproduction*, 138, 629–637.
- Assou, S., et al. (2008). A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: A proof of concept study. *Molecular Human Reproduction*, 14, 711–719.
- Brown, H. M., et al. (2010a). Adamts1 cleavage of versican mediates essential structural remodeling of the ovarian follicle and cumulus-oocyte matrix during ovulation in mice. *Biology of Reproduction*, 83, 549–557.
- Brown, H. M., et al. (2010b). Development and hormonal regulation of the ovarian lymphatic vasculature. *Endocrinology*, 151, 5446–5455.
- Caballero-Campo, P., et al. (2014). A role for the chemokine receptor ccr6 in mammalian sperm motility and chemotaxis. *Journal of Cellular Physiology*, 229, 68–78.
- Ceko, M. J., et al. (2015). X-ray fluorescence imaging and other analyses identify selenium and gp1 as important in female reproductive function. *Metallomics: Integrated Biometal Science*, 7, 71–82.
- Chaffin, C. L., et al. (2012). Rhesus monkey cumulus cells revert to a mural granulosa cell state after an ovulatory stimulus. *Endocrinology*, 153, 5535–5545.
- Dietzel, E., et al. (2017). Recombinant fetuin-b protein maintains high fertilization rate in cumulus cell-free mouse oocytes. *Molecular Human Reproduction*, 23, 25–33.
- Dietzel, E., et al. (2013). Fetuin-b, a liver-derived plasma protein is essential for fertilization. *Developmental Cell*, 25, 106–112.
- Dunning, K. R., et al. (2007). Altered composition of the cumulus-oocyte complex matrix during in vitro maturation of oocytes. *Human Reproduction*, 22, 2842–2850.
- Edry, I., et al. (2006). Meiotic arrest of oocytes depends on cell-to-cell communication in the ovarian follicle. *Molecular and Cellular Endocrinology*, 252, 102–106.
- Elvin, J. A., et al. (1999). Paracrine actions of growth differentiation factor-9 in the mammalian ovary. *Molecular Endocrinology*, 13, 1035–1048.
- Eppig, J. J., et al. (1997). Murine oocytes suppress expression of luteinizing hormone receptor messenger ribonucleic acid by granulosa cells. *Biology of Reproduction*, 56, 976–984.
- Gebhardt, K. M., et al. (2011). Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertility and Sterility*, 96, 47–52.
- Hong, S. J., et al. (2009). Cumulus cells and their extracellular matrix affect the quality of the spermatozoa penetrating the cumulus mass. *Fertility and Sterility*, 92, 971–978.
- Jin, M., et al. (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 4892–4896.
- Johnson, M. T., et al. (2007). Oxidative metabolism of pyruvate is required for meiotic maturation of murine oocytes in vivo. *Biology of Reproduction*, 77, 2–8.
- Lam, X., et al. (2000). Assay and importance of adhesive interaction between hamster (mesocricetus auratus) oocyte-cumulus complexes and the oviductal epithelium. *Biology of Reproduction*, 62, 579–588.
- Li, T. Y., et al. (2007). Rescue of oogenesis in cx37-null mutant mice by oocyte-specific replacement with cx43. *Journal of Cell Science*, 120, 4117–4125.
- Macaulay, A. D., et al. (2016). Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation. *Biology of Reproduction*, 94, 16.
- Mckenzie, L. J., et al. (2004). Human cumulus granulosa cell gene expression: A predictor of fertilization and embryo selection in women undergoing IVF. *Human Reproduction*, 19, 2869–2874.
- Ozawa, M., et al. (2010). Cumulus cell-enclosed oocytes acquire a capacity to synthesize gsh by fsh stimulation during in vitro maturation in pigs. *Journal of Cellular Physiology*, 222, 294–301.

- Park, J. Y., et al. (2004). Egf-like growth factors as mediators of lh action in the ovulatory follicle. *Science*, 303, 682–684.
- Peng, J., et al. (2013). Growth differentiation factor 9: Bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proceedings of the National Academy of Sciences of the United States of America*, 110, E776–85.
- Russell, D. L., et al. (2016). Bidirectional communication between cumulus cells and the oocyte: Old hands and new players? *Theriogenology*, 86, 62–68.
- Russell, D. L., & Robker, R. L. (2007). Molecular mechanisms of ovulation: Co-ordination through the cumulus complex. *Human Reproduction Update*, 13, 289–312.
- Sela-Abramovich, S., et al. (2006). Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology*, 147, 2280–2286.
- Su, Y. Q., et al. (2008). Oocyte regulation of metabolic cooperativity between mouse cumulus cells and oocytes: Bmp15 and gdf9 control cholesterol biosynthesis in cumulus cells. *Development*, 135, 111–121.
- Sugimura, S., et al. (2015). Promotion of egf receptor signaling improves the quality of low developmental competence oocytes. *Developmental Biology*, 403, 139–149.
- Sugiura, K., et al. (2007). Oocyte-derived bmp15 and fgfs cooperate to promote glycolysis in cumulus cells. *Development*, 134, 2593–2603.
- Tamba, S., et al. (2010). Expression profiling of cumulus cells reveals functional changes during ovulation and central roles of prostaglandin ep2 receptor in camp signaling. *Biochimie*, 92, 665–675.
- Unates, D. R., et al. (2014). Versatile action of picomolar gradients of progesterone on different sperm subpopulations. *PLoS ONE*, 9, e91181.
- Vanderhyden, B. C., & Tonary, A. M. (1995). Differential regulation of progesterone and estradiol production by mouse cumulus and mural granulosa cells by a factor(s) secreted by the oocyte. *Biology of Reproduction*, 53, 1243–1250.
- Veitch, G. I., et al. (2004). Selective assembly of connexin37 into heterocellular gap junctions at the oocyte/granulosa cell interface. *Journal of Cell Science*, 117, 2699–2707.
- Wathlet, S., et al. (2012). New candidate genes to predict pregnancy outcome in single embryo transfer cycles when using cumulus cell gene expression. *Fertility and Sterility*, 98, 432–439.
- Winterhager, E., & Kidder, G. M. (2015). Gap junction connexins in female reproductive organs: Implications for women's reproductive health. *Human Reproduction Update*, 21, 340–352.
- Zhang, A., et al. (2012). The effect of human cumulus cells on the maturation and developmental potential of immature oocytes in icsi cycles. *Journal of Assisted Reproduction and Genetics*, 29, 313–319.
- Zhu, L., et al. (2008). Equivalent involvement of inter-alpha-trypsin inhibitor heavy chain isoforms in forming covalent complexes with hyaluronan. *Connective Tissue Research*, 49, 48–55.

OVARY DEVELOPMENT

Fetal/Gonadogenesis

Melissa E Pepling and Joshua JN Burton, Syracuse University, Syracuse, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Anti-Müllerian hormone (AMH) A growth factor secreted by Sertoli cells to induce the degeneration of the Müllerian ducts in male embryos.

Coelomic epithelium Mesothelium derived from the splanchnopleura of lateral plate mesoderm that forms the lining of the organs in the coelomic cavity.

Folliculogenesis The growth, development, and maturation of an ovarian follicle.

Genital/gonadal ridge The anlagen of the testis in males and the ovary in females.

Germ cells Cells whose descendants become either sperm or egg.

Granulosa cells Ovary-specific cells that produce estrogen and progesterone, and support female germ cells throughout oogenesis.

Leydig cells The steroidogenic cells of the testis that synthesize testosterone.

Mesonephroi A pair of kidney-like organs derived from the intermediate mesoderm that function temporarily in the embryo, and from which the male and female reproductive tract are derived.

Oogonia Ovarian germ cells derived from PGCs that give rise to primary oocytes.

Primordial germ cell (PGC) Germ cell stage preceding sex differentiation.

Sertoli cells Testis-specific cells that secrete AMH in the developing embryo, and function to support male germ cells throughout spermatogenesis.

Sex-determining region Y (Sry) Gene encoding a transcription factor that initiates male sex determination; also known as testis determining factor (TDF).

Theca cells Endocrine cells associated with ovarian follicles that produce the androgen substrate for estrogen biosynthesis.

Introduction

Mammalian embryonic gonads initially form as a bipotential primordium that is morphologically indistinguishable in male and female embryos. A chromosomally determined sexual fate decision elicits the activation of the testis-specific or the ovary-specific pathway, and initiates the transformation of the common primordial gonads into either the male or female phenotype. The differentiated ovary produces and maintains a pool of oocytes, of which a subset is ovulated for fertilization. Additionally, the ovary functions as an endocrine organ, synthesizing and secreting the steroid hormones estrogen and progesterone that stimulate the development of secondary sex characteristics and prepare the accessory reproductive organs to facilitate pregnancy, birth, and lactation. Consequently, organogenesis of the ovaries is a highly important process for female fertility and reproductive success. Defects during gonad formation and development are pertinent to disorders of sex development (DSDs), and may also contribute to infertility or ovarian cancer. Knowledge of the process of gonadogenesis is therefore crucial to our understanding of reproductive pathologies, and may inform new treatment approaches for associated abnormalities. This article provides an overview of the key events that occur during the formation of the bipotential primordial gonad and the differentiation of the ovary, with particular focus on organogenesis of the ovary in mice, the most extensively studied mammalian model, and in humans.

The Urogenital System

Early in mammalian gestation, the embryo becomes trilaminar as it undergoes gastrulation to form three germ layers: the ectoderm, mesoderm, and endoderm. All embryonic tissues and organs are derived from these germ layers as the cells proliferate, migrate, and differentiate. The mesodermal layer is regionalized into the paraxial, intermediate, and lateral plate mesoderm. The intermediate mesoderm forms the urogenital system which includes the gonads and kidneys, as well as their associated duct systems. The urogenital ridges form bilaterally as longitudinal mesodermal elevations in the dorsal body wall of the embryo. Three sets of kidneys—the pronephroi (transitory and non-functional), mesonephroi (interim kidneys), and the metanephroi (becomes the permanent

kidneys)—develop from the urogenital ridges. Formation of the gonads is concurrent and closely associated with the differentiation of the mesonephroi. Invaginations of the surface epithelium of the mesonephroi give rise to the mesonephric (Wolffian) ducts and the paramesonephric (Müllerian) ducts. In male embryos, the testes produce testosterone which influences the differentiation of the Wolffian ducts to form the internal male genitalia, such as the vas deferens, epididymis, and the seminal vesicle. Additionally, the male gonad produces Müllerian-inhibiting substance (MIS), also known as anti-Müllerian hormone (AMH), which induces the degeneration of the Müllerian ducts. In contrast, the developing female gonad lacks these factors and the Müllerian ducts give rise to the fallopian tubes (oviducts), the uterus, the cervix, and the cranial portion of the vagina while the Wolffian ducts degenerate. Recent evidence suggests that the transcription factor, COUP-TFII, suppresses signaling that is required to maintain the Wolffian ducts and therefore actively regulates differentiation of the female reproductive tract (Zhao et al., 2017).

Formation of the Bipotential Gonad: The Indifferent Stage of Sexual Development

Gonadogenesis begins with the formation of the genital (gonadal) ridges which are the somatic precursor structures of both the testis and ovaries. The genital ridges form as a bulge or outgrowth of the coelomic epithelium on the ventromedial side of the mesonephroi by the fifth week of gestation in humans, and by embryonic day 10 (E10) in mice (Satoh, 1991; Waldeyer, 1870; Kaufman, 1992; Byskov, 1986). This proliferation of the coelomic epithelium and the underlying mesenchyme produces the somatic constituents of the gonads. There are at least two distinct bipotential somatic precursor lineages: (1) supporting cell precursors that differentiate into Sertoli cells in the testis or granulosa cells in the ovary, and (2) steroidogenic progenitors that produce Leydig cells in the testis or theca cells in the ovary (Karl and Capel, 1998; Schmahl et al., 2000). Stromal cells which are not specialized between sexes are also present at the genital ridge, and are responsible for structural patterning and angiogenic vascularization of the gonad. Genital ridge formation is therefore an indispensable prerequisite for development of both testis and ovaries, which remain bipotential at this stage until an intricate network of cellular signals begins to drive sexual differentiation.

Genetic Regulation of Gonad Formation

Several transcription factors and signaling proteins perform crucial regulatory roles in the development of the bipotential gonad. These were recently reviewed (Eggers et al., 2014; Tanaka and Nishinakamura, 2014), and are summarized here (also see Table 1). Steroidogenic factor 1 (*Sf1*), also known as *Nr5a1*, encodes an orphan nuclear hormone receptor that is expressed in both the gonad and steroidogenic tissues such as the adrenal glands (Lu and Yamashita, 2017). *Sf1* null mice exhibit agenesis of the gonads and the adrenal glands. *Sf1* is also an important regulator of *Sox9*, an essential gene for mediating testis differentiation. The Wilms tumor gene (*Wt1*) encodes a zinc finger transcription factor that is involved in the regulation of early gonad development, and differentiation of the testis (Kreidberg et al., 1993). *Wt1* null mice fail to undergo gonadogenesis, and their kidneys do not develop. Additionally, the *Wt1* protein has two isoforms, one lacking and one containing a KTS (lysine, threonine, and serine) amino acid motif. Mice lacking the *Wt1*(+KTS) isoform lose the ability to upregulate the testis-determining gene *Sry*, and exhibit male-to-female sex reversal. The *Wt1*(−KTS) isoform, in conjunction with *Lhx9*, directly activates the *Sf1* gene promoter and regulates the expression of testis-inducing factors such as *Amh*. This isoform is also important for the maintenance of the gonadal primordium. In humans, *WT1* mutations are also implicated in a form of kidney cancer that primarily affects children. *Lhx9* encodes the Lim/homeobox 9 protein which is another regulator of *Sf1* (Birk et al., 2000). *Lhx9* null mice also fail to undergo gonadogenesis. A deficiency in *Lhx9* contributes to the loss of *Amh* and testosterone, which causes XY mice to develop as females.

Table 1 Genes involved in mouse gonad formation

Gene	Protein/Function	Mutant phenotype	References
<i>Cbx2</i>	Chromobox, chromatin modification and remodeling factor	Impaired ovary development, male to female sex reversal.	Katoh-Fukui et al. (1998)
<i>Emx2</i>	Homeobox transcription factor	Lack kidney, uterine, gonad, reproductive tract.	Miyamoto et al. (1997)
<i>Gata4</i>	Zinc finger transcription factor	Inhibition of genital ridge formation.	Hu et al. (2013)
<i>Igfr1</i>	Insulin like growth factor receptor	Decreased proliferation of somatic progenitor cells.	Nef et al. (2003)
<i>Insr</i>	Insulin receptor	Decreased proliferation of somatic progenitor cells.	Nef et al. (2003)
<i>Lhx9</i>	Lim homeobox	Impaired gonadogenesis	Birk et al. (2000)
<i>Sf1</i>	Steroidogenic factor, orphan nuclear hormone receptor	No gonads or adrenal glands	Lu and Yamashita (2017)
<i>Six1/4</i>	Six family homeobox	Double knockouts have smaller gonads.	Kawakami et al. (2000)
<i>Wt1</i>	Zn finger transcription factor	No gonad development.	Kreidberg et al. (1993)

Empty spiracles homeobox 2 (Emx2) encodes a homeobox transcription factor involved in urogenital development (Miyamoto et al., 1997). *Emx2* null mice fail to develop kidneys, ureters, gonads, and genital tracts. Cellular polarity is also lost in the forming gonadal ridges of *Emx2* null mice leading to the abnormal assembly of tight junctions. Two other homeobox containing genes, *Six1* and *Six4*, are mammalian homologs of the *sine oculis homeobox (Six)* family in *Drosophila* (Kawakami et al., 2000). Both function redundantly in mouse embryogenesis. *Six1* and *Six4* double-mutant mouse embryos exhibit decreased gonad size in both sexes and abnormal differentiation of the testis in XY gonads.

The chromobox homolog 2 protein encoded by the *Cbx2* gene is a chromatin modification and remodeling factor that is involved in early gonadal development (Katoh-Fukui et al., 1998). *Cbx2* has been shown to upregulate *Sf1*, *Wt1*, and *Sry*. Deletion of *Cbx2* leads to impaired development of the ovary in XX mouse models, and male-to-female sex reversal in XY mice. *Cbx2* null mice appear to have normal development of the coelomic epithelium, but the gonadal cells later exhibit defective proliferation.

The GATA-binding protein 4 (*Gata4*) is a zinc finger transcription factor whose deletion is lethal to mouse embryos prior to genital ridge formation (Hu et al., 2013). Conditional knockdown of *Gata4* expression in mouse embryos after E8.75 causes the coelomic epithelium to remain as a morphologically undifferentiated monolayer, thereby preventing genital ridge formation. The insulin/insulin-like growth factor (IGF) signaling pathway is another crucial regulator of gonadogenesis (Nef et al., 2003). Mouse embryos without the insulin receptor (*Insr*) and the IGF receptor (*Igfr1*) have decreased proliferation of somatic progenitor cells in the gonads of both genotypic sexes prior to gonadal sex determination, and the testis fail to develop. Loss of insulin/IGF signaling can induce male-to-female sex reversal, and is also associated with reduced *Sry* expression levels. Proper spatiotemporal expression of each of these genes is imperative for establishing the somatic gonad.

Sex Determination

In mammals, dimorphic sex determination and differentiation progresses in three distinct phases. Firstly, the genetic sex of the embryo is determined at fertilization by the chromosomal complement of the zygote. Females are normally endowed with two X chromosomes, while males have an X and a Y sex chromosome. This genetic information facilitates gonadal sex determination during the second phase, by triggering growth of the bipotential gonad towards a testicular or ovarian fate. The short arm of the Y chromosome contains the gene encoding the mammalian testis-determining factor (TDF) known as *Sry* (sex-determining region Y). The spatiotemporal expression pattern of *Sry* is strictly controlled, and is first detected in the mouse gonad around E11, peaking at E11.5, before disappearing after E12.5 (Tanaka and Nishinakamura, 2014). *Sry* upregulates *Sox9*, and is both necessary and sufficient to direct the indifferent gonad towards the male phenotype in mice and humans (Koopman et al., 1991; Sinclair et al., 1990). *Sry* induces testicular morphogenesis by coordinating the differentiation of Sertoli cells and other testis-specific lineages. The presumptive ovarian equivalent of *Sry* has not yet been identified, and there is no evidence of a specific ovary determining factor. However, the ovary differentiation pathway is activated in the absence of functional *Sry* protein, and is primarily driven by the WNT/ β -catenin signaling pathway. Accordingly, although initial morphogenesis of the gonad is macroscopically similar between sexes, the gonadal transcriptome is notably different. The third stage, phenotypic sex determination, begins perinatally and continues throughout sexual maturation as the endocrine products of the gonads stimulate the development of secondary sex characteristics. Mutations or defects that compromise development during any of these three stages could lead to urogenital abnormalities or sex reversal.

The Ovary Differentiation Pathway

Differentiation of the bipotential gonads to the ovaries is not simply a passive process that occurs if there is no *Sry*. There are several ovary specific genes and signaling pathways that elicit female gonadal determination and development and this gonadal fate requires active repression of the testis pathway (Eggers et al., 2014; Chassot et al., 2014) (see Table 2). *Foxl2* encodes the forkhead box L2 transcription factor and is upregulated in the developing ovary (Uhlenhaut et al., 2009). Mutations of this gene in humans lead to blepharophimosis ptosis epicanthus inversus syndrome (BPES), which is characterized by eyelid malformation as well as, in some cases, primary ovarian insufficiency. Conditional knockdown of the *Foxl2* gene causes granulosa cells to become reprogrammed into Sertoli cells.

Table 2 Genes involved in mouse ovary differentiation

Gene	Protein/function	Mutant phenotype	References
<i>Ctnnb1</i>	β -catenin, Wnt signaling pathway	Ectopic expression causes male to female sex reversal.	Liu et al. (2009)
<i>Foxl2</i>	forkhead box transcription factor	Female to male sex reversal of somatic cells	Uhlenhaut et al. (2009)
<i>Fst</i>	Follistatin, activin antagonist	Mutants have female to male sex reversal.	Yao et al. (2004)
<i>Rspo1</i>	R-spondin homolog 1	Mutants have female to male sex reversal	Chassot et al. (2008)
<i>Wnt4</i>	Wnt signaling	Decreased proliferation of somatic progenitor cells.	Tomizuka et al. (2008)

β -Catenin, also known as Catenin beta-1 (Ctnnb1), transcriptionally regulates a variety of genes including *Wnt4* and *Fst* which are important for ovarian development (Liu et al., 2009). Ectopic expression of β -catenin in the developing XY mouse gonad causes male-to-female sex reversal, but the mechanism driving this trans-differentiation is unclear. WNT family members are conserved secreted proteins with roles in various biological processes. WNT3A participates in the stabilization of β -catenin and allows it to transcribe its target genes. WNT4 is known to activate canonical WNT signaling during gonadal development (Tomizuka et al., 2008). Females with heterozygous missense *WNT4* mutations exhibit agenesis of the reproductive tract, while *Wnt4* in the mouse is also important for gonadal morphogenesis via its role in sex determination and female development.

RSPO proteins activate the WNT/ β -catenin signaling pathway by binding to the LGR4, LGR5, and LGR6 G protein-coupled receptors, or by binding to ZNRF3 and RNF43 which are negative-feedback regulators of WNT signaling (Chassot et al., 2008). Disruption of human *RSPO1* can lead to female-to-male sex reversal, suggesting that it plays a crucial role in sex determination and female differentiation. At E12.5 in the mouse *Rspo1* and *Wnt4* become expressed in an ovary specific manner, and their gene products are secreted by the somatic cells of the ovary. *Rspo1* null mice exhibit sex reversal, bearing ovotestes. The *Rspo1*^{-/-} embryo demonstrates abnormalities in the XX gonad such as the presence of testis-like vasculature, and reduced germ cell proliferation. Similarly, a deficiency in *Wnt4* is associated with inducing male-like vascularization of the gonad in XX embryos. There is also evidence suggesting that *Wnt4* functions as a survival factor in female germ cells, as there are nearly three times as many apoptotic germ cells in the *Wnt4* deficient gonad, when compared to wild type gonads.

Follistatin (*Fst*) expression is stimulated by *Wnt4* in the XX gonad from E11.5 onwards, but is not expressed in the XY gonad (Yao et al., 2004). *Fst* encodes an activin-binding protein that antagonizes Activin B. This inhibitory action prevents male-like vascularization of the gonad. Subsequent development of the differentiated ovary will involve the production and development of the ovarian follicles.

Primordial Germ Cells: The Embryonic Precursors of Gametes

Primordial germ cells (PGCs) colonize the bipotential gonad in association with the somatic cells, and differentiate to produce haploid eggs or sperm. During gastrulation in mice, at around E7, inductive cell-cell interactions establish the germ line by specifying the allocation of PGCs. The genetic basis for germ cell specification is thought to involve the expression of the *fragilis* and *stella* genes in PGC founder cell lineages, allowing them to retain pluripotency while neighboring cells acquire a mesodermal fate (Saitou et al., 2002). Intercellular signaling involving Bone Morphogenetic Protein 4 (*Bmp4*) is also critical for initiating the formation of the germ line. *Bmp4* null mouse embryos lack PGCs and an allantois which are both derived from the proximal epiblast (Lawson et al., 1999). The *Bmp* signaling pathway regulates the expression of two key genes that are essential for germ cell fate: PR domain containing protein 1 (*Prdm1*) which acts to repress the somatic cell fate, and PR domain containing protein 14 (*Prdm14*) which transcriptionally regulates germ cell pluripotency and epigenetic reprogramming (Windley and Wilhelm, 2015).

PGCs migrate via the hindgut and the dorsal mesentery to the developing gonadal ridges from approximately E9.5 to E11.5 in the mouse and from the fifth to seventh week of gestation in humans (Clark and Eddy, 1975; Fujimoto et al., 1977). During this time, the number of PGCs increases from approximately 40 to 250 in mice, and from approximately 100 to 5000 in humans (Cummings and Kavlock, 2004; Saitou and Yamaji, 2012). The PGCs rapidly proliferate by mitosis and increase in number to colonize the developing gonads as oogonia or spermatogonia. This proliferation produces a maximum of almost 7 million germ cells by the 5th month of gestation in humans, and around 25,000 germ cells by E13.5 in mice. At E11.5 in the mouse embryo and in the seventh to ninth week of gestation in humans, PGCs display similar expression patterns of germline and pluripotency genes. These germ cell specific genes include *BLIMP1*, *AP2 γ* , *UTF1*, *DAZL*, *Kit*, and *DDX4*, while the pluripotency genes include *OCT4*, *NANOG*, *PRDM14*, and *LIN28* (Dolci et al., 2015). The KIT receptor and its ligand KITL, are well known for promoting cell survival and proliferation during gametogenesis. Loss of KIT function impairs PGC proliferation and migration, results in ectopic PGCs, and affects the growth of early ovarian follicles (Buehr et al., 1993; Huang et al., 1993; McCoshen and McCallion, 1975; Mintz and Russell, 1957). By E13.5 in mice and by the tenth week of gestation in humans, oogonia begin to enter meiosis and are called oocytes. Developing oocytes arrest in the diplotene stage of the first meiotic prophase until ovulation begins during puberty, while spermatogonia enter a state of quiescence and reinitiate development at the onset of puberty.

Development of the Ovaries

The differentiated ovary displays notable complexity in structure and function, and is histologically identifiable around the twelfth week of gestation in humans and E13.5 in mice, by the presence of loose cordlike structures termed ovigerous cords (Loffler and Koopman, 2002; Odor and Blandau, 1969). Ovigerous cords consist of clusters of primordial germ cells that are surrounded by somatic cells. Ovarian PGCs in these clusters are physically interconnected by cytoplasmic bridges, and develop synchronously as germ cell cysts (Pepling and Spradling, 1998). Cysts subsequently undergo programmed breakdown to form primordial follicles, consisting of an individual germ cell or oocyte surrounded by a monolayer of somatic granulosa cells (Pepling and Spradling,

2001). Somatic cells extend thin cytoplasmic prolongations between germ cells in ovigerous cords, thereby facilitating cyst breakdown and follicle assembly (Odor and Blandau, 1969; Pepling and Spradling, 2001). The features of early oocyte development are very similar in mice and humans, with the main difference being that cyst breakdown and primordial follicle formation occur perinatally in the mouse, but before birth in humans (Sarraj and Drummond, 2012).

References

- Birk, O. S., Casiano, D. E., Wassif, C. A., Cogliati, T., Zhao, L., Zhao, Y., Grinberg, A., Huang, S., Kreidberg, J. A., Parker, K. L., Porter, F. D., & Westphal, H. (2000). The LIM homeobox gene *Lhx9* is essential for mouse gonad formation. *Nature*, 403, 909–913.
- Buehr, M., McLaren, A., Bartley, A., & Darling, S. (1993). Proliferation and migration of primordial germ cells in We/We mouse embryos. *Developmental Dynamics*, 198, 182–189.
- Byskov, A. G. (1986). Differentiation of mammalian embryonic gonad. *Physiological Reviews*, 66, 71–117.
- Chassot, A. A., Ranc, F., Gregoire, E. P., Roepers-Gajadien, H. L., Taketo, M. M., Camerino, G., De Rooij, D. G., Schedl, A., & Chaboissier, M. C. (2008). Activation of beta-catenin signaling by *Rspo1* controls differentiation of the mammalian ovary. *Human Molecular Genetics*, 17, 1264–1277.
- Chassot, A. A., Gillot, I., & Chaboissier, M. C. (2014). R-spondin1, WNT4, and the CTNNB1 signaling pathway: Strict control over ovarian differentiation. *Reproduction*, 148, R97–110.
- Clark, J. M., & Eddy, E. M. (1975). Fine structural observations on the origin and associations of primordial germ cells of the mouse. *Developmental Biology*, 47, 136–155.
- Cummings, A. M., & Kavlock, R. J. (2004). Function of sexual glands and mechanism of sex differentiation. *The Journal of Toxicological Sciences*, 29, 167–178.
- Dolci, S., Campolo, F., & De Felici, M. (2015). Gonadal development and germ cell tumors in mouse and humans. *Seminars in Cell & Developmental Biology*, 45, 114–123.
- Eggers, S., Ohnesorg, T., & Sinclair, A. (2014). Genetic regulation of mammalian gonad development. *Nature Reviews: Endocrinology*, 10, 673–683.
- Fujimoto, T., Miyayama, Y., & Fuyuta, M. (1977). The origin, migration and fine morphology of human primordial germ cells. *The Anatomical Record*, 188, 315–330.
- Hu, Y. C., Okumura, L. M., & Page, D. C. (2013). *Gata4* is required for formation of the genital ridge in mice. *PLoS Genetics*, 9, e1003629.
- Huang, E. J., Manova, K., Packer, A. I., Sanchez, S., Bachvarova, R. F., & Besmer, P. (1993). The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Developmental Biology*, 157, 100–109.
- Karl, J., & Capel, B. (1998). Sertoli cells of the mouse testis originate from the coelomic epithelium. *Developmental Biology*, 203, 323–333.
- Kato-Fukui, Y., Tsuchiya, R., Shiroishi, T., Nakahara, Y., Hashimoto, N., Noguchi, K., & Higashinakagawa, T. (1998). Male-to-female sex reversal in M33 mutant mice. *Nature*, 393, 688–692.
- Kaufman, M. H. (1992). *The atlas of mouse development*. London: Academic Press.
- Kawakami, K., Sato, S., Ozaki, H., & Ikeda, K. (2000). Six family genes—structure and function as transcription factors and their roles in development. *BioEssays*, 22, 616–626.
- Koopman, P., Gubbay, P., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature*, 351, 117–121.
- Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D., & Jaenisch, R. (1993). *WT-1* is required for early kidney development. *Cell*, 74, 679–691.
- Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P., & Hogan, B. L. (1999). *Bmp4* is required for the generation of primordial germ cells in the mouse embryo. *Genes & Development*, 13, 424–436.
- Liu, C. F., Bingham, N., Parker, K., & Yao, H. H. (2009). Sex-specific roles of beta-catenin in mouse gonadal development. *Human Molecular Genetics*, 18, 405–417.
- Loffler, K. A., & Koopman, P. (2002). Charting the course of ovarian development in vertebrates. *The International Journal of Developmental Biology*, 46, 503–510.
- Lu, K. L., & Yamashita, Y. M. (2017). Germ cell connectivity enhances cell death in response to DNA damage in the *Drosophila* testis. *eLife*, 6, e27960.
- McCoshen, J. A., & McCallion, D. J. (1975). A study of the primordial germ cells during their migratory phase in steel mutant mice. *Experientia*, 31, 589–590.
- Mintz, B., & Russell, E. S. (1957). Gene-induced embryological modifications of primordial germ cells in the mouse. *The Journal of Experimental Zoology*, 134, 207–237.
- Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I., & Aizawa, S. (1997). Defects of urogenital development in mice lacking *Emx2*. *Development*, 124, 1653–1664.
- Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J. D., Efstratiadis, A., Accili, D., & Parada, L. F. (2003). Testis determination requires insulin receptor family function in mice. *Nature*, 426, 291–295.
- Odor, D. L., & Blandau, R. J. (1969). Ultrastructural studies on fetal and early postnatal mouse ovaries. I. Histogenesis and organogenesis. *The American Journal of Anatomy*, 124, 163–186.
- Pepling, M. E., & Spradling, A. C. (1998). Female mouse germ cells form synchronously dividing cysts. *Development*, 125, 3323–3328.
- Pepling, M. E., & Spradling, A. C. (2001). Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Developmental Biology*, 234, 339–351.
- Saitou, M., & Yamaji, M. (2012). Primordial germ cells in mice. *Cold Spring Harbor Perspectives in Biology*, 4, a008375.
- Saitou, M., Barton, S. C., & Surani, M. A. (2002). A molecular programme for the specification of germ cell fate in mice. *Nature*, 418, 293–300.
- Sarraj, M. A., & Drummond, A. E. (2012). Mammalian foetal ovarian development: Consequences for health and disease. *Reproduction*, 143, 151–163.
- Satoh, M. (1991). Histogenesis and organogenesis of the gonad in human embryos. *Journal of Anatomy*, 177, 85–107.
- Schmah, J., Eicher, E. M., Washburn, L. L., & Capel, B. (2000). *Sry* induces cell proliferation in the mouse gonad. *Development*, 127, 65–73.
- Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A. M., Lovell-Badge, R., & Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*, 346, 240–244.
- Tanaka, S. S., & Nishinakamura, R. (2014). Regulation of male sex determination: Genital ridge formation and *Sry* activation in mice. *Cellular and Molecular Life Sciences*, 71, 4781–4802.
- Tomizuka, K., Horikoshi, K., Kitada, R., Sugawara, Y., Iba, Y., Kojima, A., Yoshitome, A., Yamawaki, K., Amagai, M., Inoue, A., Oshima, T., & Kakitani, M. (2008). R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Human Molecular Genetics*, 17, 1278–1291.
- Uhlenhaut, N. H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A. C., Klugmann, C., Klasen, C., Holter, N. I., Riethmacher, D., Schutz, G., Cooney, A. J., Lovell-Badge, R., & Treier, M. (2009). Somatic sex reprogramming of adult ovaries to testes by *FOXL2* ablation. *Cell*, 139, 1130–1142.
- Waldeyer, W. (1870). *Eierstock und Ei*. Leipzig: Engelmann.
- Winkley, S. P., & Wilhelm, D. (2015). Signaling pathways involved in mammalian sex determination and gonad development. *Sexual Development*, 9, 297–315.
- Yao, H. H., Matzuk, M. M., Jorgez, C. J., Menke, D. B., Page, D. C., Swain, A., & Capel, B. (2004). *Follistatin* operates downstream of *Wnt4* in mammalian ovary organogenesis. *Developmental Dynamics*, 230, 210–215.
- Zhao, F., Franco, H. L., Rodriguez, K. F., Brown, P. R., Tsai, M. J., Tsai, S. Y., & Yao, H. H. (2017). Elimination of the male reproductive tract in the female embryo is promoted by *COUP-TFII* in mice. *Science*, 357, 717–720.

Cellular and Structural Aspects of Fetal Ovarian Differentiation

Barbara Nicol and Humphrey H-C Yao, National Institute of Environmental Health Sciences (NIEHS/NIH), Research Triangle Park, NC, United States

© 2018 Elsevier Inc. All rights reserved.

Contrary to other organs in the embryo, embryonic gonads are unique for their capacity to become two morphologically distinct organs: testes or ovaries. In mammals, the decision of embryonic gonads to develop into testes or ovaries relies on the sex chromosomes and the presence or absence of the sex-determining gene of the Y chromosome or SRY. As a consequence, embryonic gonads undergo dimorphic cellular and morphological changes that are specific for either the testis or the ovary. While sex determination of the testis results in radical changes in the morphology of the embryonic gonads, sex determination of the ovary yields subtle changes. This article focuses on the cellular and morphological changes that transform undifferentiated embryonic gonad into an ovary.

Sex Determination of the Ovary: Cellular Changes

Long before the undifferentiated gonads acquire the characteristic structures of an ovary, they already contain precursor cell populations that serve as the foundation of the ovary. Through a process called cell differentiation, these cell populations change their cellular characteristics to become specialized cell types specific for the testis or the ovary. When the undifferentiated gonads transform into ovaries, major cellular events occur in three cell populations: (1) the supporting cells, a subtype of somatic cells that become granulosa cells, (2) the steroidogenic cell precursors, another subtype of somatic cells that later become theca cells, and (3) the primordial germ cells, which become oocytes through the process called oogenesis.

Origins and Differentiation of the Somatic Cells in the Ovary

Supporting Cells or Granulosa Cells

Among the somatic cells in the undifferentiated gonads, supporting cells play the most critical role in sex determination. This is the first cell population that chooses its fate, and differentiates into cell types that are specific to the sex of the embryo: Sertoli cells in the testis or granulosa cells in the ovary. Sertoli and granulosa cells derive from a common precursor population that arises from the coelomic epithelium on the surface of the undifferentiated gonads (Karl and Capel, 1998; Albrecht and Eicher, 2001). The precursor cells contribute to the thickening of the gonads by expanding their population and then entering the gonads. Sex determination of these cells relies on their sex chromosome constitution, particularly the Y-chromosome-linked SRY gene. In the presence of SRY, the supporting cell progenitors become Sertoli cells whereas in the absence of SRY, supporting cell progenitors develop into granulosa cells, marking the first event of ovary sex determination.

Studies in rodents and larger mammals suggest that granulosa cell population does not solely arise from the common supporting cell progenitors at the time of sex determination (Mork et al., 2012; Rastetter et al., 2014; Smith et al., 2014). In fact, granulosa cell populations could derive from at least two distinct sources at different stages of development (Fig. 1):

- The first wave of development corresponds to the appearance of the above-mentioned population of supporting cell precursors, which give rise to the first cohort of granulosa cells at the time of sex determination. In female embryos, this recruitment of

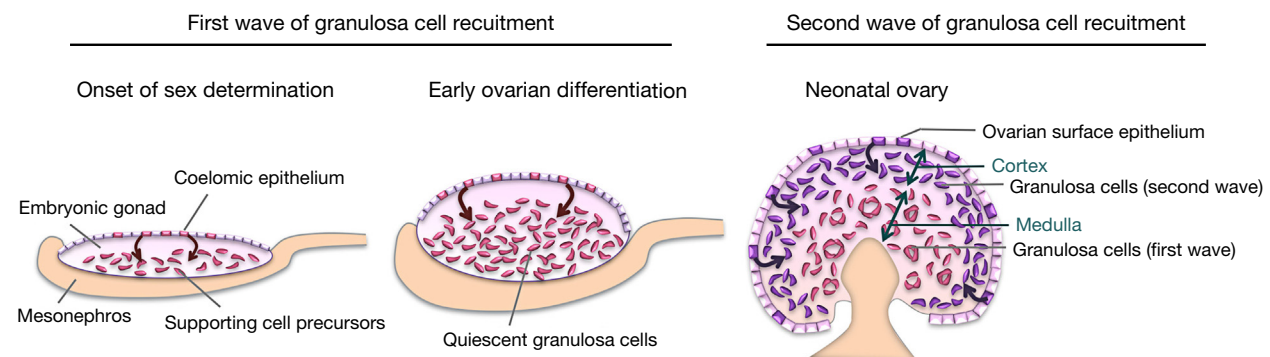


Fig. 1 *Origins of the granulosa cells.* Two waves of cell differentiation give rise to the granulosa cells. The first population of granulosa cells originates from the coelomic epithelium at the onset of ovary sex determination, and gives rise to the granulosa cells located in the medulla of the ovary. The second population of granulosa cells originates from the ovarian surface epithelium of the neonatal ovary, and gives rise to the granulosa cells of the cortex. This model is based on the findings by Mork et al. (2012).

supporting cell precursors from the coelomic epithelium continues for a short time after sex determination. These granulosa cells remain mostly located at the innermost part of the ovary, called medulla. This first cohort of granulosa cells later participates in the formation of the first wave of follicles in the medulla of the ovary.

- The second wave of granulosa cell differentiation appears late in gestation. Similar to the first wave, granulosa cells from the second wave of development arise from the ovarian surface epithelium. The cell recruitment from the surface epithelium is continuous from fetal to early postnatal stage, concomitant with the initiation of follicle assembly. The second cohorts of granulosa cells remain close to the surface epithelium, contributing to the growth of this region called cortex. These granulosa cells participate in the formation of the second wave of follicles in the cortex of the ovary and give rise to the majority of adult granulosa cells (Zheng et al., 2014).

Contrary to Sertoli cells in the fetal testis that proliferate during the course of sex determination, fetal granulosa cells cease proliferation once their fate is determined. As the fetal ovary develops, granulosa cell population expands through the cell recruitment from the ovarian epithelium surface. Proliferation of granulosa cells only resumes after folliculogenesis is initiated. Establishment of the supporting granulosa cell populations is the first step of ovary sex determination, and it influences the appearance of other somatic cell populations in the embryonic ovary.

Steroidogenic Cells or Theca Cells

In addition to the supporting cells, steroidogenic cells are another somatic cell population that plays a critical role in ovarian physiology. Both testes and ovaries contain a population of steroidogenic cells that contributes to the production of sex steroids. In the ovary, these steroidogenic cells are called theca cells. Leydig cells, the testis counterpart of theca cells, differentiate and become steroidogenically active soon after testis sex determination. The timing of theca cell differentiation in the ovary, on the other hand, varies among species. For instance, theca cells in the mouse become distinguishable after birth, when the follicles are assembling (Mannan and O'Shaughnessy, 1991). In larger mammals, theca cells differentiate during embryonic development of the ovary. Similar to the testicular Leydig cells, the ovarian theca cells arise from non-steroidogenic precursor cells of two distinctive fetal origins (Liu et al., 2015):

- The majority of the theca cells originate from the coelomic epithelium of the embryonic ovary. After the coelomic epithelium has given rise to the supporting cell precursors, some cells from the coelomic epithelium ingress into the embryonic ovary to become mesenchymal cells of the ovarian stroma (Karl and Capel, 1998). Then, after ovarian differentiation is established, some of these mesenchymal cells differentiate into theca cells in response to signals coming from neighboring granulosa cells and oocytes.
- In addition to the mesenchymal cells in the fetal ovary, a subset of theca cells has an extragonadal origin: they come from the mesonephros connected to the ovary. In the mouse, the theca cell precursors in the mesonephros migrate into the ovary around birth, right before steroidogenic theca cells are detected. As the theca cells differentiate, their populations expand and begin to surround primary follicles, which are composed of a single oocyte enclosed in a layer of granulosa cells.

Establishment of the Germ Cells in the Ovary

Oocytes in the ovary and sperm in the testis both arise from the same embryonic cell population: the primordial germ cells. Primordial germ cells are formed early during embryogenesis, before the primitive gonads are even established. As the undifferentiated gonads start to form in the embryo, primordial germ cells migrate and colonize the gonads. Contrary to the supporting cells, the sexual fate of germ cells does not directly rely on their sex chromosome content. Instead, sex determination of the germ cells is controlled by signals that they receive from the surrounding gonadal environment, in particular from the supporting cells. If the supporting cells differentiate into ovary-specific granulosa cells, the germ cells commit to a developmental pathway that ultimately lead them to become oocytes. Once sex determination of the ovary is initiated, primordial germ cells (or oogonia) undergo several rounds of synchronous cell division or mitosis. At the end of each cell division, the oogonia are not completely separated: they remain connected between each other through intercellular bridges and form clusters of oogonia known as ovarian germ cell cysts (Pepling and Spradling, 1998) (Fig. 2). Within the cysts, the intercellular bridges allow oogonia to exchange cytoplasmic material. From insects to vertebrates, the formation of ovarian germ cell cysts is highly conserved throughout evolution. Each cyst is initially composed of oogonia that originate from a single founder primordial germ cell. Depending of the species, germ cell cysts can partially fragment and aggregate with other germ cell cysts that arose from another primordial germ cell (Lei and Spradling, 2013).

At the time of ovarian cyst formation, the ovary reaches the highest number of germ cells it will ever contain. Soon after, the oogonia progressively stop proliferating, and begin to condense their chromosomes. This chromosome condensation indicates that female germ cells are initiating meiosis, a unique cellular division process that occurs only in germ cells. Meiosis initiation in the fetal ovary is a key morphological indicator of sex differentiation of the germ cells. In the testis, germ cell meiosis is only initiated at puberty. In the fetal ovary, meiosis initiation occurs in an asynchronous way both spatially and temporally. In mice, meiosis progresses in an anterior-to-posterior wave (Menke et al., 2003; Yao et al., 2003). On the other hand, in human and large mammals, meiosis is established in an inner-to-outer fashion, from the ovarian cortex extending to the region close to the ovarian

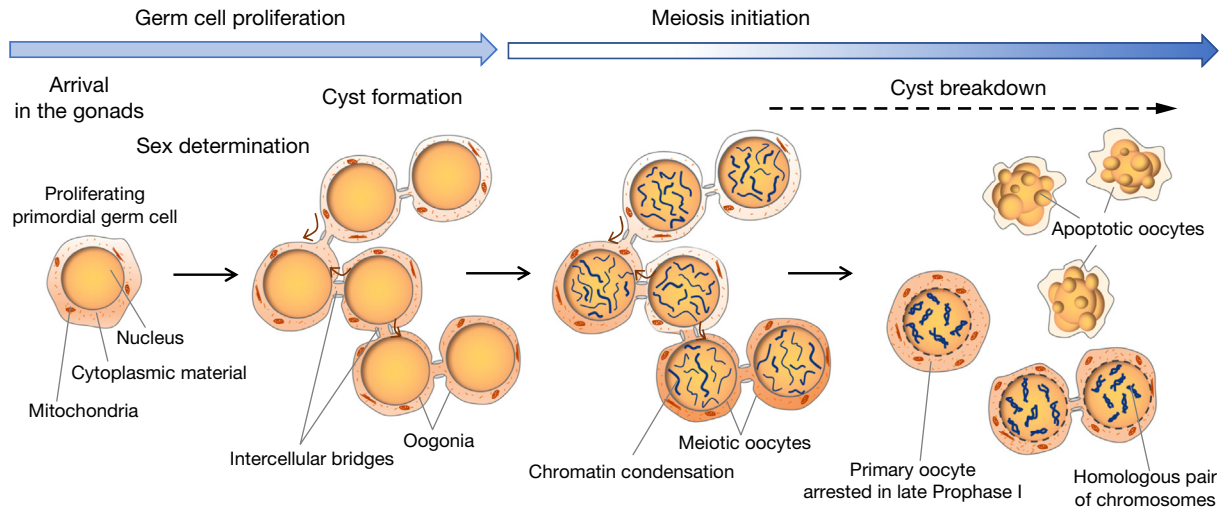


Fig. 2 Sex differentiation of the female germ cells. Primordial germ cells migrate into the embryonic ovary before sex determination. Once they colonize the gonad, primordial germ cells (or oogonia) undergo incomplete cell divisions, resulting in the formation of germ cell cysts in which the oogonia remain connected through intercellular bridges. These intercellular bridges allow the oogonia to exchange cytoplasmic materials. The oogonia then initiate meiosis, and their chromosomes start to condense. The female germ cells are now referred as oocytes. In parallel to meiosis initiation, germ cell cysts start to break down. Some oocytes undergo apoptotic cell death whereas the remaining oocytes arrest in late prophase I of meiosis.

surface epithelium. Initiation of meiosis is thought to be controlled by retinoid acids (Bowles et al., 2006). The sources of retinoid acids vary among species from the mesonephros close to the anterior part of the ovary in rodents to the ovary itself in humans and large mammals. As oogonia enter meiosis, they develop into oocytes. While the chromosomes progressively condense, homologous chromosomes pair together and exchange genetic material through a process called crossing-over. During fetal ovary development, oocytes progress up to the late prophase of meiosis I. Once oocytes reach the diplotene stage of prophase I, meiosis is arrested, only to resume after puberty.

In parallel to the event of meiosis initiation, oocytes undergo a progressive loss called attrition, another hallmark of germ cell commitment to female differentiation. In the beginning, soon after meiosis is initiated, the pace of oocyte loss is slow; then, around the time of follicle formation, the loss becomes massive. Oocyte attrition occurs through programmed cell death of two types: apoptosis that is triggered by signals from the oocytes themselves and the neighboring granulosa cells; and autophagy. It remains unclear why the fetal ovary undergoes such a big loss of oocytes. Three hypotheses have been proposed to explain the massive loss of oocytes in the fetal/neonatal ovary:

- First, loss of oocytes may result from an intrinsic quality control checkpoint: oocytes that initiate meiosis improperly, or have an abnormal chromosomal composition/DNA damage, are eliminated whereas healthy oocytes survive.
- Second, within the ovarian germ cell cysts, some oocytes only serve as nursing cells, providing organelles and cytoplasmic material through the intercellular bridges to other oocytes. Loss of organelles such as mitochondria, “the powerhouse” of the cell, results in the death of these nursing cells (Lei and Spradling, 2016).
- Third, extrinsic stimuli could affect germ cell survival/loss: the contact between oocytes and granulosa cells could be necessary for oocyte survival. As a consequence, the oocytes that fail to be surrounded by granulosa cells degenerate. Loss of oocytes balances the ratio between oocytes and granulosa cells in order to allow proper formation of follicles.

Survival of oocytes is based on multiple criteria of selection. Therefore, these three hypotheses are not mutually exclusive, and oocyte death probably results from a combination of these intrinsic and extrinsic mechanisms. Consequently, depending upon the species, up to 90% of the total oocyte population is lost in the fetal or neonatal ovary.

Sex Determination of the Ovary: Morphological Changes

The morphological changes leading to ovarian development depends on the recruitment, structural organization, and communication between the cell populations mentioned in the first part of this article. The undifferentiated gonad is an oblong, streak-like structure undistinguishable between male and female embryos. After the onset of gonadal sex determination, the embryonic ovary progressively becomes thicker in diameter and shorter in length, and ultimately acquires a round shape. The morphological change of fetal ovaries is slow and unremarkable compared to the fast-paced growth of the embryonic testis. As a result, soon after sex determination is initiated, embryonic ovary and testis can be distinguished by their size, with the ovary being significantly smaller than the testis. In addition, while the embryonic testis undergoes dramatic structural reorganization, the structure of the fetal ovary

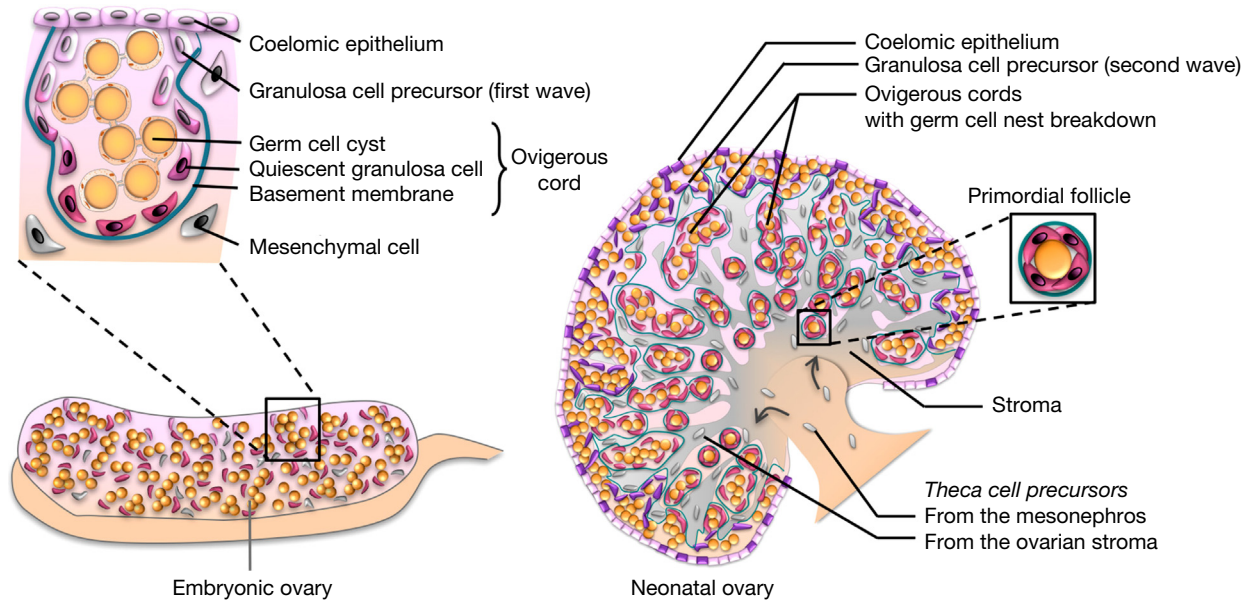


Fig. 3 Morphological changes in the mouse fetal ovary: from ovigerous cord formation to primordial follicles. In the embryonic ovary, the ovigerous cords are formed by the loose surrounding of germ cell cysts by granulosa cells. As the ovigerous cords form, they are in contact with the surface epithelium, source of granulosa cell precursors, and become delimited from the stroma by a basement membrane. In the neonatal ovary, the germ cell cysts progressively breakdown in a medulla-to-cortex fashion, resulting in smaller ovigerous cords and ultimately to the formation of primordial follicles. Theca cells differentiate from mesenchymal cells originating from both the ovarian stroma and the mesonephros (arrows).

initially remains similar to that of the undifferentiated gonads, with somatic and germ cells mixed throughout the gonad. From the time of ovary sex determination in fetal life to folliculogenesis in adulthood, granulosa cells and female germ cells form a tight cellular and structural connection. Their capacity to interact is critical for their cellular development, as well as for their ability to form particular structures, from the ovigerous cords in the embryonic ovary to follicles later on (Fig. 3).

During sex determination of the ovary, oogonia and granulosa cells assemble to form structures called “ovigerous cords”. Each cord is composed of a germ cell cyst with proliferating oogonia, loosely surrounded by a layer of quiescent granulosa cells. These ovigerous cords are separated from the ovarian stroma by a basement membrane (or basal lamina), a thin layer of extra-cellular matrix. The ovigerous cords remain connected to the surface of the ovary, in contact with the coelomic epithelium, the source of newly differentiating granulosa cells. Depending upon the species, the timing of ovigerous cords formation varies, and their morphology is not always well-defined.

The formation of the ovigerous cords demarcates the two different regions in the embryonic ovary: the cortex and the medulla. The cortex consists of the coelomic epithelium and the region underneath it. Proliferation of the surface epithelium causes the expansion of the cortex. The cells resulting from this proliferation then ingress into the ovary and become a source of granulosa cells. This recruitment of granulosa cells contributes to the development of the ovigerous cords in the fetal ovary and the follicles later in life. The cortex is the region of the ovary where most oocytes accumulate. As the fetal ovary grows, the cortex becomes the predominant region of the organ. The medulla, on the other hand, is the region at the center of the ovary that is closely associated with the adjacent mesonephros. As the ovigerous cords form, the ovarian stroma, composed of non-granulosa somatic cells outside of the cords, migrate from the medulla toward the cortex between ovigerous cords. The ovarian stroma contains precursor cells that give rise to most of the steroidogenic theca cells. Medulla of the ovary is where stroma cells, connective tissue and vasculature concentrate. Ultimately, in the adult ovary, the medulla becomes devoid of follicle, and contains only the vasculature and innervation.

Once ovigerous cords are formed, they gradually become isolated from the ovarian surface epithelium by a basement membrane. During the ovarian morphogenesis, fetal ovaries changes from a long structure full of multiple ovigerous cords to a round structure composed of primordial follicles. This morphological change begins when oocytes enter meiosis, and germ cell cysts within the ovigerous cords begin to break down (Pepling and Spradling, 2001). The germ cell cyst breakdown is the combined result of oocyte death and invasion of granulosa cells between the oocytes. As a consequence, intercellular bridges between the oocytes are disrupted, and germ cell cysts gradually disintegrate and become smaller. The decline in oocyte numbers due to cell death results in an increased ratio of granulosa cells to oocytes within the ovigerous cords. More granulosa cells surround the remaining oocytes and their cytoplasmic extensions enclose each oocyte, isolating the oocyte from the cyst. Eventually, the ovigerous cords disappear to give rise to primordial follicles that are isolated from the stroma by a basement membrane. The process of germ cell cyst breakdown and primordial follicle formation starts in the medulla and expands toward the cortex. The first cohorts of primordial follicles are formed in the medulla while the cortex still contains ovigerous cords that undergo germ cell cyst breakdown. Following the formation of the first cohort of primordial follicle, the majority of the primordial follicles are established in the cortex

region soon after birth. The outcome of the ovary morphogenesis during fetal life ultimately leads to the neonatal ovary that contains primordial follicles, the functional unit of the ovary. These follicles will be responsible for the development of healthy and fertilizable oocytes as well as the production of female hormones in the adulthood.

References

- Albrecht, K. H., & Eicher, E. M. (2001). Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. *Developmental Biology*, 240(1), 92–107.
- Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., et al. (2006). Retinoid signaling determines germ cell fate in mice. *Science*, 312(5773), 596–600.
- Karl, J., & Capel, B. (1998). Sertoli cells of the mouse testis originate from the coelomic epithelium. *Developmental Biology*, 203(2), 323–333.
- Lei, L., & Spradling, A. C. (2013). Mouse primordial germ cells produce cysts that partially fragment prior to meiosis. *Development*, 140(10), 2075–2081.
- Lei, L., & Spradling, A. C. (2016). Mouse oocytes differentiate through organelle enrichment from sister cyst germ cells. *Science*, 352(6281), 95–99.
- Liu, C., Peng, J., Matzuk, M. M., & Yao, H. H. (2015). Lineage specification of ovarian theca cells requires multicellular interactions via oocyte and granulosa cells. *Nature Communications*, 6, 6934.
- Mannan, M. A., & O'Shaughnessy, P. J. (1991). Steroidogenesis during postnatal development in the mouse ovary. *Journal of Endocrinology*, 130, 101–106.
- Menke, D. B., Koubova, J., & Page, D. C. (2003). Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. *Developmental Biology*, 262, 303–312.
- Mork, L., Maatouk, D. M., McMahon, J. A., et al. (2012). Temporal differences in granulosa cell specification in the ovary reflect distinct follicle fates in mice. *Biology of Reproduction*, 86(2), 37.
- Pepling, M. E., & Spradling, A. C. (1998). Female mouse germ cells form synchronously dividing cysts. *Development*, 125(17), 3323–3328.
- Pepling, M. E., & Spradling, A. C. (2001). Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Developmental Biology*, 234, 339–351.
- Rastetter, R. H., Bernard, P., Palmer, J. S., et al. (2014). Marker genes identify three somatic cell types in the fetal mouse ovary. *Developmental Biology*, 394(2), 242–252.
- Smith, P., Wilhelm, D., & Rodgers, R. J. (2014). Development of mammalian ovary. *Journal of Endocrinology*, 221(3), R145–61.
- Yao, H. H., DiNapoli, L., & Capel, B. (2003). Meiotic germ cells antagonize mesonephric cell migration and testis cord formation in mouse gonads. *Development*, 130(24), 5895–5902.
- Zheng, W., Zhang, H., Gorre, N., et al. (2014). Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions. *Human Molecular Genetics*, 23, 920–928.

Further Reading

- Piprek, R. P. (2016). *Molecular mechanisms of cell differentiation in gonad development*. Place: Springer international publishing.
- Suzuki, H., Kanai-Azuma, M., & Kanai, Y. (2015). From sex determination to initial Folliculogenesis in mammalian ovaries: Morphogenetic waves along the anteroposterior and Dorsoventral axes. *Sexual Development*, 9(4), 190–204.

Female Sex Determination: Molecular

Joan S Jorgensen, Anqi Fu, and Megan Hornung, University of Wisconsin, Madison, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Sex, or male versus female determination is a critical developmental event in all sexually reproducing organisms. While male versus female sex is typically considered a binary differentiation event, and common sense would dictate a process that is evolutionarily conserved, the diversity and plasticity of sex determination is both fascinating and astounding. The variations across animal kingdoms are vast, but even among vertebrates, diversity in sex determination between species include those that rely on temperature gradients, sex-specific chromosome genes, or other modes of genetic control, many still unknown. Still others, including certain species of fish, mole, or alligators, have the ability to transdifferentiate from one sex to another or harbor remnants of both sexes so that they may expand male or female tissue depending on external cues (Guillette et al., 1997; Jimenez et al., 2013; Wu and Chang, 2013). For mammalian species, it has long been established that sex is determined by XX versus XY chromosome presence with *SRY* (sex determining region of the Y chromosome) identified as the single inciting gene from the Y chromosome that is required to stimulate male development (Hacker et al., 1995). Although this genetic pathway appears straightforward, the diversity in biological presentations suggests otherwise. For example, in humans, there are a wide variety of disorders of sex development (DSDs), many of which remain unexplained. Even if birth defects are not obvious, there is also increasing recognition of people suffering from gender dysphoria, of which there is no known cause. Together, these truths, along with others not mentioned here, suggest that female versus male sex determination might better be represented by a gray scale instead of a black or white outcome.

Unlike any other developing organ, cells within the early gonad have the potential to differentiate into one of two very different organs: a testis or an ovary. While they share the same functional outcome of passing along sex-specific gametes for the next generation, their modes of operation are necessarily quite distinct. In typical XY individuals, the adult testes produce millions of gametes each day that are released in large quantities along with nutritive fluids contributed by accessory sex organs through specially designed ductwork and external genitalia. Meanwhile, in the typical XX individual, the entire population of gametes is present at birth, and in the adult, a single gamete is released once every cycle into its specially designed ductwork that will receive male gametes and provide the protective environment for a new embryo to develop. In both cases, a lifetime of intricately timed sex-specific hormone production is required to successfully achieve each outcome. And in both cases, this coordinated program stems from events that occur in early gonad development.

The Bipotential State

Early on, the paired gonads differentiate and acquire four distinct cell types: (1) support and (2) interstitial/stroma cells that arise from a common mesodermal progenitor, and then (3) endothelial and (4) germ cells that differentiate elsewhere and migrate into the gonad. The initial focus of the nascent developing gonad, like any other newly forming organ, is cell proliferation and survival. Early genes that are known to contribute to this phase include *Lhx9* (Lim homeobox 9), *Wt1* (Wilms tumor 1), *Odd1* (Odd-skipped 1), *Emx2* (empty spiracles homeobox 2), and *Sf1* (steroidogenic factor 1, also known as NR5A1, ADBP4) (Birk et al., 2000; Kreidberg et al., 1993; Luo et al., 1994; Miyamoto et al., 1997; Sadovsky et al., 1995; Wang et al., 2005). Previous studies using knockout models for each of these genes resulted in impaired cell proliferation and gonad agenesis or degeneration. While knockout models of other genes including *Wnt4/Rspo1* (wingless-type MMTV integration site family, member 4, R-spondin homolog 1), *Pbx1* (pre-B-cell homeobox 1) and *Insr/Igfr2* signaling factors (insulin receptor/insulin-like growth factor receptor 1) also disrupted cell proliferation, they were still able to initiate sex determination albeit in rudimentary form (Chassot et al., 2012; Nef et al., 2003; Pitetti et al., 2013; Schnabel et al., 2003) (Table 1).

Ultimately, the support cell population initiates sex determination. Certain somatic cells will differentiate into the support cell precursors, Sertoli cells in XY and granulosa cells in XX, and henceforth, trigger the sex-specific commitment of all other cell lineages (interstitial/stromal, germ, and endothelial cells) within the nascent developing gonad. The onset of *SRY* expression as the initiator of Sertoli cell fate and testis determination was discovered over a decade ago (Gubbay et al., 1990; Koopman et al., 1991; Sekido et al., 2004), but the mechanisms by which granulosa cell fate and ovarian determination is just beginning to be understood. Landmark studies by Alfred Jost suggested that female differentiation was a default pathway because female structures prevailed in XY individuals that lacked male factors (Jost, 1947). In addition, the search for a single XX-female initiating factor has thus far not been fruitful. Instead, studies suggest an active balancing act that imposes mutual antagonism between a cadre of female and male-promoting factors that stabilize the sex-specific fate. Maintenance of either fate depends on an intricate network of molecular controls that include signal cascade pathways along with transcription factor and epigenetic mechanisms for genetic control.

Table 1 Genes associated with cell proliferation in the bipotential gonad

Knockout description	Gene	Gene name	Reference(s)
Impaired proliferation, gonad agenesis or degeneration	<i>Lhx9</i>	Lim homeobox 9	Birk et al. (2000)
	<i>Wt1</i>	Wilms tumor 1	Kreidberg et al. (1993)
	<i>Odd1</i>	Odd-skipped 1	Wang et al. (2005)
	<i>Emx2</i>	Empty spiracles homeobox 2	Miyamoto et al. (1997)
	<i>Sf1 (Nr5a1, AdBP4)</i>	Steroidogenic factor 1 (nuclear receptor subfamily 5 group A member 1, adrenal 4 binding protein)	Luo et al. (1994), Sadovsky et al. (1995)
Impaired proliferation, rudimentary sex determination	<i>Wnt4/Rspo1</i>	Wingless-type MMTV integration site family, member 4; R-spondin homolog 1	Chassot et al. (2012), Nef et al. (2003)
	<i>Pbx1</i>	Pre-B-cell homeobox 1	Schnabel et al. (2003)
	<i>Insr/Igfr2</i>	Insulin receptor/insulin-like growth factor receptor 1	Pitetti et al. (2013)

Sex Determination

Careful analysis of gene expression profiles from embryonic mouse gonads taken at multiple time-points within a 24-h window highlighted two fundamental concepts: (1) both XX and XY gonads harbor all genes necessary for generating either a testis or an ovary, and (2) more female than male genes are expressed at the late bipotential stage, suggesting that a female bias exists (Jameson et al., 2012; Munger et al., 2013). In the early bipotential stage, XX and XY gonad transcriptomes are nearly identical and include typical testis genes *Sox9* (Sry box 9) and *Fgf9* (fibroblast growth factor 9) along with typical ovary genes *Wnt4* and *Rspo1* (Munger et al., 2013; Nef et al., 2005). This stage has been described as a transient balanced transcriptional state with genes associated with either sex poised for activation or repression depending on molecular cues (Lin and Capel, 2015). The presence of a female bias suggests that testis differentiation must provide the means to block the ovary pathway. This is where *Sry* comes in.

Male Sex Determination

It is impossible to discuss female gonad sex determination without some understanding of what's happening in the male. By definition, only XY gonads can express *Sry*, which encodes the transcription factor that tilts the balance of genes toward the testis fate. The timing for SRY activity, however, is critical and exposes a weakness in the male pathway. *Sry*/SRY expression initiates starting at 10.5dpc (days post coitum) in mouse (out of ~19.5 days gestation) (Bullejos and Koopman, 2001; Hacker et al., 1995; Jeske et al., 1995) and between 41 and 44 days post ovulation in humans (out of ~240 days, relatively much sooner) (Hanley et al., 2000). Expression in the mouse is fleeting, only 36 h; whereas SRY expression is maintained in human cells throughout testis development and into adulthood (Hacker et al., 1995; Salas-Cortes et al., 2001). Studies in mouse have shown that *Sry* expression must increase to a certain level within a short timeline (6 h!) to successfully promote the testis fate. Any delay or failure in reaching the defined threshold of expression results in a range of gonad abnormalities from formation of an ovotestis (containing both ovarian and testicular tissue in the same gonad) to formation of an ovary and complete XY sex reversal (Carre and Greenfield, 2014; Hiramatsu et al., 2009). Although the path to increased *Sry* expression is not entirely clear, key regulatory proteins include insulin signaling factors (*Insr/Igfr1*), GADD45G (growth arrest and DNA damage response), MAP3K4 (mitogen-activated protein kinase), and the GATA4/FOG2 (GATA binding protein 4, Friend of GATA 2) transcription complex (Bogani et al., 2009; Gierl et al., 2012; Manuylov et al., 2008; Nef et al., 2003; Tevosian et al., 2002; Warr et al., 2012).

To tilt the balance of gonad genes, SRY stimulates transcription of another member of the same family of transcription factors, *Sox9*, the real powerhouse behind testis determination. The increase in SOX9 presence feeds forward to increase other testis determining genes including its own expression to solidify the Sertoli cell identity and a testis fate. One of these, *Fgf9*, is translated into a factor that is released from Sertoli cells, only to interact with its receptor (FGFR2) on the same or neighboring Sertoli cells (paracrine signaling) to amplify *Sox9* expression (Bagheri-Fam et al., 2008; Colvin et al., 2001; Kim et al., 2007; Kim et al., 2006; Siggers et al., 2014). *Fgf9* and *Fgfr2* will come up again later in the discussion regarding mutual antagonism of male versus female genes. Finally, SOX9 stimulates transcription of *Ptgds* (prostaglandin D2 synthase), which encodes another signaling molecule, PGD2 that provides paracrine signaling to promote a positive feedback loop on *Sox9* transcription (Adams and McLaren, 2002; Moniot et al., 2009; Wilhelm et al., 2005). Unlike *Sry* in the mouse, *Sox9* expression is maintained throughout life where it plays a central role in reinforcing the Sertoli cell identity.

Female Sex Determination

RSP01/WNT4/ β -catenin

The strength of the female bias in genetic expression eventually tips toward ovary development if SRY is not present to block it. The first female genes that increase are *Wnt4* and *Rspo1* (Jameson et al., 2012; Munger et al., 2013). Both of these genes encode signaling factors that stimulate activity within support cells to facilitate accumulation of β -catenin in the cytoplasm. β -catenin can then be transported to one of two different locations in the cell: the cell membrane or the nucleus (Gottardi and Gumbiner,

2004). At the cell membrane, β -catenin interacts with other adhesion proteins to promote cell–cell adhesion and communication. Alternatively, in the nucleus, β -catenin interacts with particular protein partners, for example TCF/LEF (T-cell factor/lymphoid enhancer-binding factor), that bind to DNA sequences to stimulate transcription of specific WNT4/RSPO1-responsive genes. The identity of these downstream genes is still unclear, but some notable factors include *Fst* (Follistatin), and *Irx3/Irx5* (Iroquois homeobox family members 3 and 5), each of which contributes to WNT4/RSPO1 activity (Jorgensen, 2013; Yao et al., 2004) (Fig. 1).

Each aspect of the WNT4/RSPO1/ β -catenin pathway has been knocked out in mice; notably, their loss does not cause female to male gonad sex reversal, but they are all similarly masculinized (Liu et al., 2009; Tomizuka et al., 2008; Vainio et al., 1999). Common features include abnormal formation of the large coelomic vessel, differentiation of functional steroid producing cells, and massive oocyte death before birth. Activation of β -catenin and *Wnt4* requires RSPO1 signaling within early ovaries, but once activated, WNT4 can synergize with RSPO1 to further increase β -catenin activity (Chassot et al., 2008). In general, mutations of WNT4 in humans can cause a variety of phenotypes that may range from a masculinized outcome that is remarkably similar to the mouse models, to those that are much more severe, including female to male sex reversal. Altogether, the complexities behind the range of outcomes in individuals with WNT4 mutations support the idea that the relative amount or dosage of WNT4 activity is key to female versus male differentiation (Biason-Lauber, 2012). Meanwhile, human mutations of RSPO1 also exhibited more severe abnormalities in sex differentiation than in the mouse. Indeed, at least two reports identified RSPO1 mutations that caused complete female to male sex reversal in patients (Parma et al., 2006; Tomaselli et al., 2008). There is currently no explanation for the difference seen in outcomes for humans versus mice. It is noteworthy, however, that these reports marked the first occasions that complete female to male sex reversal had been attributed to something other than translocation of SRY, which still only explains approximately 10% of these cases, the remainder are still unsolved.

FOXL2

While *Wnt4* and *Rspo1* are present in the bipotential gonad of both sexes, *Foxl2* is never detected in the XY gonad. Similar to *Wnt4*/*Rspo1*/ β -catenin mutant mice, knockout of *Foxl2* does not cause female to male sex reversal and ovarian defects are not apparent until after birth (Schmidt et al., 2004; Uda et al., 2004). Likewise, human patients with FOXL2 mutations exhibit ovarian defects associated with premature ovarian insufficiency in adult female and no sex reversal (Crisponi et al., 2001). In the goat, however, loss of FOXL2 causes female to male sex reversal and is considered a female determining gene (Boulanger et al., 2014). The diversity of outcomes among species caused by a lack of FOXL2 function is intriguing and in this case, has been explained by the timing of its expression in relationship to the onset of meiotic division, and the ovary's ability to make and respond to estrogen (Pannetier et al., 2016).

In the mouse, *Foxl2* expression initiates after the gonad has already tilted toward the ovarian path at approximately 12.5dpc (Schmidt et al., 2004). The onset of FOXL2 expression coincides with support cell transition to mitotic arrest and marks

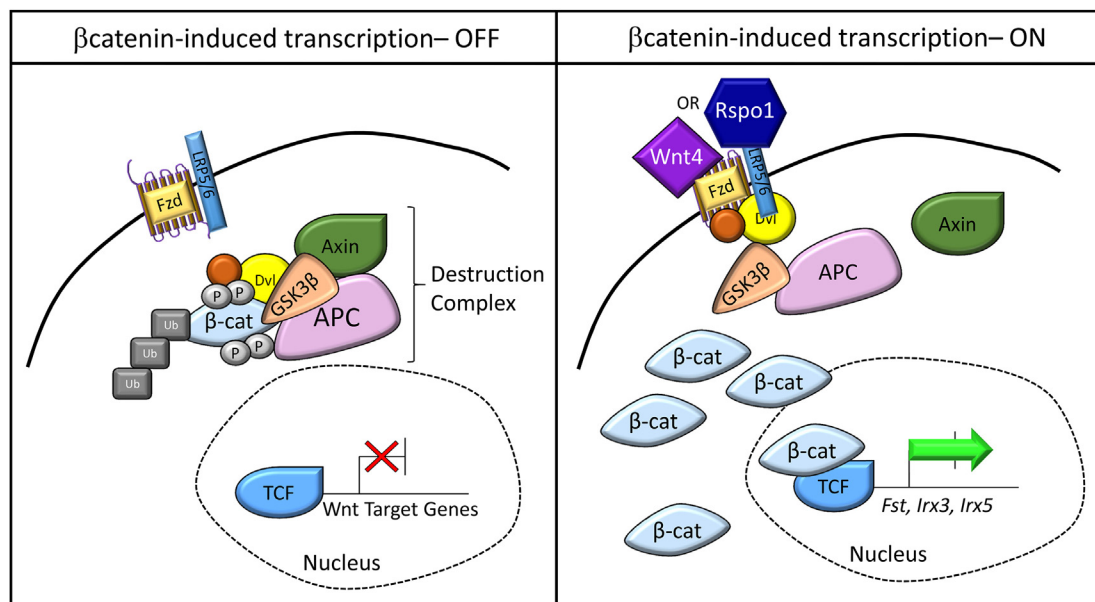


Fig. 1 Canonical Wnt/Rspo1 signaling leads to β -catenin induced transcription. Left: Without a Wnt or Rspo1 ligand present at the membrane receptors Frizzled (Fzd) or Lrp5/6, a destruction complex forms, targeting β -catenin for degradation by the proteasome. Right: If Wnt or Rspo1 ligands bind to the membrane receptors, the destruction complex cannot form, allowing β -catenin to stabilize and translocate to the nucleus to bind to its DNA binding partners TCF/LEF family members. This will turn on ovary target genes such as *Fst*, *Irx3*, or *Irx5*.

differentiation to the pre-granulosa cell fate (Maatouk et al., 2013). This is also just about the time that germ cells begin to transition from mitotic to meiotic division. Although loss of *Foxl2* doesn't appear to affect the fetal ovary, within a week after birth, ovarian cells that were meant to carry on a granulosa cell fate transdifferentiated into SOX9-positive, male support cells (Ottolenghi et al., 2005; Schmidt et al., 2004; Uhlenhaut et al., 2009). In the mouse, the ovary does not synthesize estrogen until after birth. Thus, the delay in support cell transdifferentiation has been attributed to the timing of estrogen synthesis, which will then allow FOXL2 to interact with the estrogen receptor (ESR1) to suppress *Sox9* and maintain the female support cell program (Uhlenhaut et al., 2009). Further, FOXL2 can then interact with ESR1 and ESR2 (estrogen receptor beta) along with other nuclear receptor transcription factors to impact estrogen production itself (Georges et al., 2014). Meanwhile, in the goat, FOXL2 enhances the ovary's ability to make estrogen at a time that corresponds to sex determination, far earlier than in the mouse (Boulanger et al., 2014) (Fig. 2). These interactions have yet to be described in human perinatal ovaries, but they are known to impact sex determination in several other non-mammalian vertebrate species including reptiles, birds, and fish, making an intriguing case for *Foxl2*, *Esr1*, and *Esr2* as female-determining genes (Nakamura, 2010).

RSP01/WNT4/ and FOXL2 in pre-granulosa cell proliferation and differentiation

Proliferation of support cells is a fundamental difference between female and male gonad differentiation just after the onset of SRY expression (Schmahl and Capel, 2003; Schmahl et al., 2000). Careful studies in mice showed that just after *Sry* turns on and then stimulates *Fgf9*, there is a discrete 8-h window of time in which the testis increases in size. In fact, if FGF9 activity or cell proliferation is blocked during this time frame, male-specific genes and cell types fail to differentiate and the testis fate is thwarted (Schmahl and Capel, 2003; Schmahl et al., 2004). In contrast, support cells of the female gonad transition to mitotic arrest once they differentiate into pre-granulosa cells. There are, however, two different transition events and the timing of each marks the future follicles into one of two populations. All support cells appear to originate from a common precursor lineage that is positive for transcription factors GATA4, IRX3, and IRX5 (Manuylov et al., 2008) (Fu et al., 2018). The first population of precursor cells enters mitotic arrest and express cell cycle inhibitor factors including p27, p21, and p57 along with FOXL2 (Bouma et al., 2007; Jameson et al., 2012; Mork et al., 2012). These cells remain in mitotic arrest until after birth, when they are activated and recruited as granulosa cells that are destined for the first wave of follicles that mature within the medulla of the ovary. They facilitate the onset of puberty and early fertility (Mork et al., 2012; Rastetter et al., 2014; Zheng et al., 2014). The surface epithelium of the developing ovary, however, express WNT4/RSP01 and RSP01 receptor, LGR5, and remain proliferative until they exit the surface and then transition to mitotic arrest as they begin to contribute to the support cell pool (Gustin et al., 2016; Hummitzsch et al., 2013). Once they leave the ovarian surface, they begin to express FOXL2 and differentiate into granulosa cells that remain quiescent within primordial follicles in the ovary cortex until recruited later after puberty (Mork et al., 2012; Zheng et al., 2014). What helps to maintain this quiescent state? Studies indicate that oocyte status in regard to their transition into meiosis plays an important role in communicating granulosa cell activity during fetal development (Maatouk et al., 2013). These studies highlighted the first meaningful outcome in ovary differentiation that results from interactions between support cells and oocytes.

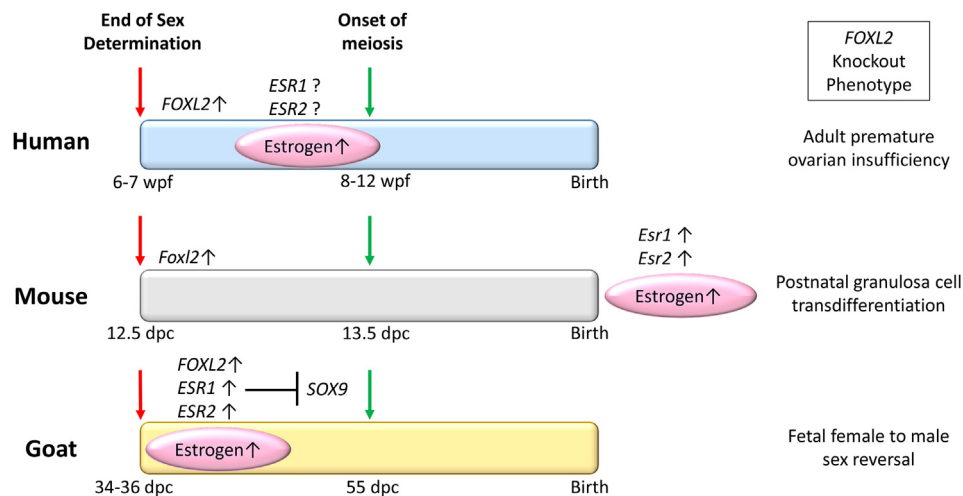


Fig. 2 Comparison of FOXL2–estrogen/ESR1/ESR2 interactions in the ovary across different mammalian species: The onset of FOXL2 and estrogen signaling via estrogen receptors, ESR1 and ESR2 differ between human, mouse, and goat. In the goat, FOXL2 can interact with estrogen-bound ESR1 and ESR2 to block *Sox9* at the time of sex determination and before the onset of meiosis. In the mouse, FOXL2 is present just after sex determination and before the onset of meiosis; however, estrogen is not active until after birth. In the human, FOXL2 and estrogen are present before the onset of meiosis; however, the timing for estrogen receptor-mediated activity is unknown. wpf, week post fertilization; dpc, days post coitum; timeline not drawn to scale.

Mutual Antagonism

The initial status of the developing gonad is bipotential as it harbors genetic signatures that are typically associated with both male and female pathways. Once the molecular cues within gonad support cells tilt toward one particular sex, those cells maintain their sex-specific fate and steer other resident cells, including germ cells, toward the same sex-specific outcome. Historically, this decision was considered irreversible. Recent studies, however, suggest otherwise. Instead, key factors have been shown to play dual roles during gonad development: they promote sex determination while they actively antagonize the molecular pathways of the opposite sex. This should not be a big surprise as it has long been established that other vertebrate species can readily transdifferentiate from one sex to another in response to external cues. Ultimately though, the mammalian species are less susceptible to external cues and transdifferentiation from one committed fate to another is unusual and no human case has been reported.

Antagonistic relationships exist among the major sex determining factors and are maintained from developmental stages through adulthood (for review, see [Wilhelm et al., 2013](#)). First, consider the developmental state and sex-determining factors present in the bipotential gonad, SOX9/FGF9 and the RSPO1/WNT4/ β -catenin pathways. In male gonads, knockout of *Sox9*, *Fgf9* or its receptor, *Fgfr2*, causes partial to complete male to female sex reversal ([Bagheri-Fam et al., 2008](#); [Chaboissier et al., 2004](#); [Colvin et al., 2001](#); [Kim et al., 2006](#); [Siggers et al., 2014](#)). Notably, in *Fgf9* or *Fgfr2* null testes, expression of *Wnt4* increased dramatically suggesting the FGF9 pathway plays an active role in suppressing the female pathway ([Kim et al., 2006](#)). These findings were supported by double knockout studies in which elimination of any member of the *Rspo1/Wnt4/Ctnnb1* (β -catenin) pathway along with *Fgf9* or *Fgfr2* allowed for rescue of the male to female sex reversal ([Jameson et al., 2012](#)). Given the relationship that SOX9 and FGF9 mutually support each other, it was not surprising to find that, double knockout of *Sox9* along with any member of the *Rspo1/Wnt4/Ctnnb1* pathway also blocked the male to female sex reversal ([Lavery et al., 2012](#); [Nicol and Yao, 2015](#)). The power of the β -catenin pathway to block male differentiation is especially illustrated in XY gonads that exhibit male to female sex reversal when male support cells over-express the stabilized form of β -catenin ([Maatouk et al., 2008](#)). In the case of the female gonad, loss of any member of the *Rspo1/Wnt4/Ctnnb1* pathway caused increased expression of *Sox9* and *Fgf9* and incomplete female–male sex reversal; however, this phenotype was not rescued after either of these factors was also eliminated ([Kim et al., 2006](#); [Nicol and Yao, 2015](#)). These studies suggest that the β -catenin pathway blocks male-driving factors in addition to *Sox9* and *Fgf9*, such as other Sox family members ([Nicol and Yao, 2015](#)).

Other antagonistic relationships that maintain sex fate are not manifested until after birth. Identity of Sertoli and granulosa cells in adult testis and ovary, respectively, is mediated by transcription factor activity. In the adult testis, the highly conserved male promoting factor, DMRT1 (doublesex and mab-3 related transcription factor 1) promotes testis-specific factors while repressing ovary-driving genes ([Minkina et al., 2014](#)). Indeed, elimination of *Dmrt1* in the adult testis causes significant reductions in *Sox9* and activation of *Foxl2* to result in Sertoli–granulosa cell transdifferentiation ([Matson et al., 2011](#)). On the flip side, the presence of DMRT1 alone was sufficient to silence *Foxl2* and reprogram granulosa cells into Sertoli cells in the adult ovary ([Lindeman et al., 2015](#)). Also in the adult mouse ovary, the aforementioned transcription factors FOXL2 and activated estrogen receptors (ESR1 and ESR2) have been shown to interact with critical enhancer sequences to repress *Sox9* transcription ([Matson et al., 2011](#); [Minkina et al., 2014](#); [Uhlenhaut et al., 2009](#)). Knockout of *Foxl2* in adult granulosa cells was sufficient to cause rapid upregulation of *Sox9* and *Dmrt1* transcription followed by granulosa and theca cell transdifferentiation into Sertoli and Leydig cells ([Uhlenhaut et al., 2009](#)) ([Fig. 3](#)). As might be expected, these transdifferentiation events destined all germ cell populations to death.

Epigenetics

The developmental program of any organ depends on exquisite timing of gene expression as cell fates are decided. Appropriate timing is defined by the activity of relevant transcription factors along with the nature of the chromatin environment or epigenetic marks of the collection of sex determination genes. Epigenetics is defined as the molecular approach for genetic activity that does not depend on the DNA sequence itself. Instead, epigenetic control is typically mediated by reading biochemical marks that decorate DNA. These marks are inherited and reversible, so biochemical reactions along the DNA can switch genes on or off depending on other cellular signals. The major marks include methyl (–CH₃) and acetyl groups that affix themselves to the DNA itself or to histone proteins that surround the DNA sequences. Their addition will cause chromatin to either coil up tightly (high levels of DNA methylation, some forms of histone methylation) to silence genes, or relax (low levels of DNA methylation, some forms of histone methylation and acetylation) to allow access and stimulate gene transcription. Recent studies used DNase1-Seq technology to uncover thousands of open chromatin sites that were unique to Sertoli cells just after sex determination, a subpopulation of these were also positive for histone acetylation binding. Together, these new data illustrate the possibility that there are collections of sites that are available for either repressor or activator regulation ([Maatouk et al., 2017](#)).

The first solid piece of evidence for epigenetic control for sex determination was the discovery that *Sry* gene activity was controlled by DNA methylation status ([Nishino et al., 2004](#)). Since then, studies have also shown that *Sry* transcription is also under the control of chromatin binding protein CBX2 and histone demethylase enzyme activity ([Biaison-Laubier et al., 2009](#); [Katoh-Fukui et al., 2012](#); [Kuroki et al., 2013](#)). The most exciting discoveries cementing the role for DNA methylation activity and sex determination have been in non-mammalian vertebrate species. The link between temperature and sex determination has long been a mystery. Recently, several groups have shown that temperature gradients exert tight control of DNA methylation of critical

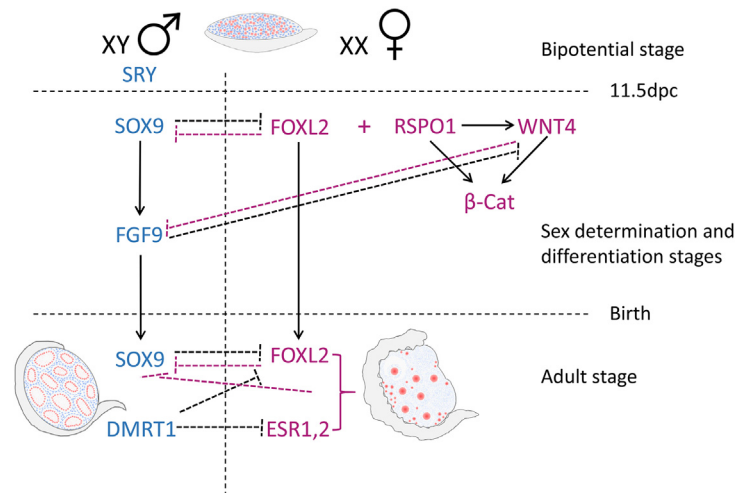


Fig. 3 *Mutual antagonism during gonad development.* Summary of mutual antagonism between male promoting factors (blue) and female promoting factors (pink) during gonad development through adulthood. Adapted from Wilhelm, D., Yang, J. X. and Thomas, P. (2013). Mammalian sex determination and gonad development. *Current Topics in Developmental Biology* **106**, 89–121.

sex determination genes including *Sox9* and *Cyp19a1*, the enzyme responsible for the final step in estrogen synthesis (Navarro-Martin et al., 2011; Parrott et al., 2014). At this time, however, epigenetic control remains a mystery. For example, in the absence of SRY-SOX9 action, what controls upregulation of the *Rspo1/Wnt4/Ctmb1* pathway? How are dosages for critical genes such as *Sry* ramped up or down? What senses the intricate timing schedule? Clearly, there is much to discover and new discoveries are awaited with anticipation.

Conclusions

Sex determination is a fascinating process that illuminates the beautiful diversity of living beings. When the process is disrupted, variations of disorders of sex differentiation can occur. While not life threatening, at least in humans, successful management requires an entire team of medical and social support networks for both patient and family. As increasingly sophisticated tools become available, researchers have set increasingly realistic goals to identify causes that will lead to ways to prevent and reverse potential disorders.

References

- Adams, I. R., & McLaren, A. (2002). Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. *Development*, *129*, 1155–1164.
- Bagheri-Fam, S., Sim, H., Bernard, P., Jayakody, I., Taketo, M. M., Scherer, G., & Harley, V. R. (2008). Loss of *Fgfr2* leads to partial XY sex reversal. *Developmental Biology*, *314*, 71–83.
- Blason-Lauber, A. (2012). WNT4, RSP01, and FOXL2 in sex development. *Seminars in Reproductive Medicine*, *30*, 387–395.
- Blason-Lauber, A., Konrad, D., Meyer, M., DeBeaufort, C., & Schoenle, E. J. (2009). Ovaries and female phenotype in a girl with 46,XY karyotype and mutations in the *CBX2* gene. *American Journal of Human Genetics*, *84*, 658–663.
- Birk, O. S., Casiano, D. E., Wassif, C. A., Cogliati, T., Zhao, L., Zhao, Y., Grinberg, A., Huang, S., Kreidberg, J. A., Parker, K. L., Porter, F. D., & Westphal, H. (2000). The LIM homeobox gene *Lhx9* is essential for mouse gonad formation. *Nature*, *403*, 909–913.
- Bogani, D., Siggers, P., Brixey, R., Warr, N., Beddow, S., Edwards, J., Williams, D., Wilhelm, D., Koopman, P., Flavell, R. A., Chi, H., Ostrer, H., Wells, S., Cheeseman, M., & Greenfield, A. (2009). Loss of mitogen-activated protein kinase kinase 4 (MAP3K4) reveals a requirement for MAPK signalling in mouse sex determination. *PLoS Biology*, *7*, e1000196.
- Boulanger, L., Pannetier, M., Gall, L., Allais-Bonnet, A., Elzaiat, M., Le Bourhis, D., Daniel, N., Richard, C., Cotinot, C., Ghyselinck, N. B., & Pailhoux, E. (2014). FOXL2 is a female sex-determining gene in the goat. *Current Biology*, *24*, 404–408.
- Bourma, G. J., Affourtit, J. P., Bult, C. J., & Eicher, E. M. (2007). Transcriptional profile of mouse pre-granulosa and Sertoli cells isolated from early-differentiated fetal gonads. *Gene Expression Patterns*, *7*, 113–123.
- Bullejos, M., & Koopman, P. (2001). Spatially dynamic expression of *Sry* in mouse genital ridges. *Developmental Dynamics*, *221*, 201–205.
- Carre, G. A., & Greenfield, A. (2014). Characterising novel pathways in testis determination using mouse genetics. *Sexual Development*, *8*, 199–207.
- Chaboissier, M. C., Kobayashi, A., Vidal, V. I., Lutzendorf, S., van de Kant, H. J., Wegner, M., de Rooij, D. G., Behringer, R. R., & Schedl, A. (2004). Functional analysis of *Sox8* and *Sox9* during sex determination in the mouse. *Development*, *131*, 1891–1901.
- Chassot, A. A., Ranc, F., Gregoire, E. P., Roepers-Gajadien, H. L., Taketo, M. M., Camerino, G., de Rooij, D. G., Schedl, A., & Chaboissier, M. C. (2008). Activation of beta-catenin signaling by *Rspo1* controls differentiation of the mammalian ovary. *Human Molecular Genetics*, *17*, 1264–1277.
- Chassot, A. A., Bradford, S. T., Auguste, A., Gregoire, E. P., Pailhoux, E., de Rooij, D. G., Schedl, A., & Chaboissier, M. C. (2012). WNT4 and RSP01 together are required for cell proliferation in the early mouse gonad. *Development*, *139*, 4461–4472.

- Colvin, J. S., Green, R. P., Schmah, J., Capel, B., & Ornitz, D. M. (2001). Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell*, 104, 875–889.
- Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., Amati, P., Biscaglia, L., Zelante, L., Nagaraja, R., Porcu, S., Ristaldi, M. S., Marzella, R., Rocchi, M., Nicolini, M., Lienhardt-Roussie, A., Nivelon, A., Verloes, A., Schlessinger, D., Gasparini, P., Bonneau, D., Cao, A., & Pilia, G. (2001). The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nature Genetics*, 27, 159–166.
- Fu A, Oberholzer SM, Bagheri-Fam S, Rastetter RH, Caceres V, John S, Shaw S, Zhang X, Hui C-C, Wilhelm D, and Jorgensen JS. (January 15, 2018). Dynamic Expression Patterns of *Irx3* and *Irx5* During Germline Cyst Breakdown and Primordial Follicle Formation Promote Follicle Survival in Mouse Ovaries.
- Georges, A., L'Hôte, D., Todeschini, A. L., Auguste, A., Legois, B., Zider, A., & Veitia, R. A. (2014). The transcription factor FOXL2 mobilizes estrogen signaling to maintain the identity of ovarian granulosa cells. *eLife*, 3.
- Gierl, M. S., Gruhn, W. H., von Seggern, A., Maltry, N., & Niehrs, C. (2012). GADD45G functions in male sex determination by promoting p38 signaling and Sry expression. *Developmental Cell*, 23, 1032–1042.
- Gottardi, C. J., & Gumbiner, B. M. (2004). Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. *Journal of Cell Biology*, 167, 339–349.
- Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1990). A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature*, 346, 245–250.
- Guillette, L. J., Jr., Woodward, A. R., Crain, D. A., Masson, G. R., Palmer, B. D., Cox, M. C., You-Xiang, Q., & Orlando, E. F. (1997). The reproductive cycle of the female American alligator (*Alligator mississippiensis*). *General and Comparative Endocrinology*, 108, 87–101.
- Gustin, S. E., Hogg, K., Stringer, J. M., Rastetter, R. H., Pelosi, E., Miles, D. C., Sinclair, A. H., Wilhelm, D., & Western, P. S. (2016). WNT/beta-catenin and p27/FOXL2 differentially regulate supporting cell proliferation in the developing ovary. *Developmental Biology*, 412, 250–260.
- Hacker, A., Capel, B., Goodfellow, P., & Lovell-Badge, R. (1995). Expression of Sry, the mouse sex determining gene. *Development*, 121, 1603–1614.
- Hanley, N. A., Hagan, D. M., Clement-Jones, M., Ball, S. G., Strachan, T., Salas-Cortes, L., McElreavey, K., Lindsay, S., Robson, S., Bullen, P., Ostrer, H., & Wilson, D. I. (2000). SRY, SOX9, and DAX1 expression patterns during human sex determination and gonadal development. *Mechanisms of Development*, 91, 403–407.
- Hiramatsu, R., Matoba, S., Kanai-Azuma, M., Tsunekawa, N., Katoh-Fukui, Y., Kurohmaru, M., Morohashi, K., Wilhelm, D., Koopman, P., & Kanai, Y. (2009). A critical time window of Sry action in gonadal sex determination in mice. *Development*, 136, 129–138.
- Hummitzsch, K., Irving-Rodgers, H. F., Hatzirodos, N., Bonner, W., Sabatier, L., Reinhardt, D. P., Sado, Y., Ninomiya, Y., Wilhelm, D., & Rodgers, R. J. (2013). A new model of development of the mammalian ovary and follicles. *PLoS One*, 8, e55578.
- Jameson, S. A., Natarajan, A., Cool, J., DeFalco, T., Maatouk, D. M., Mork, L., Munger, S. C., & Capel, B. (2012). Temporal transcriptional profiling of somatic and germ cells reveals biased lineage priming of sexual fate in the fetal mouse gonad. *PLoS Genetics*, 8, e1002575.
- Jeske, Y. W., Bowles, J., Greenfield, A., & Koopman, P. (1995). Expression of a linear Sry transcript in the mouse genital ridge. *Nature Genetics*, 10, 480–482.
- Jimenez, R., Barrionuevo, F. J., & Burgos, M. (2013). Natural exceptions to normal gonadal development in mammals. *Sexual Development*, 7, 147–162.
- Jorgensen, J. S. (2013). Defining the neighborhoods that escort the oocyte through its early life events and into a functional follicle. *Molecular Reproduction and Development*, 80, 960–976.
- Jost, A. (1947). Recherches sur la différenciation sexuelle de l'embryon de lapin. *Archives d'Anatomie Microscopique et de Morphologie Experimentale*, 36, 271–315.
- Katoh-Fukui, Y., Miyabayashi, K., Komatsu, T., Owaki, A., Baba, T., Shima, Y., Kidokoro, T., Kanai, Y., Schedl, A., Wilhelm, D., Koopman, P., Okuno, Y., & Morohashi, K. (2012). Cbx2, a polycomb group gene, is required for Sry gene expression in mice. *Endocrinology*, 153, 913–924.
- Kim, Y., Kobayashi, A., Sekido, R., DiNapoli, L., Brennan, J., Chaboissier, M. C., Poulat, F., Behringer, R. R., Lovell-Badge, R., & Capel, B. (2006). Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. *PLoS Biology*, 4, e187.
- Kim, Y., Bingham, N., Sekido, R., Parker, K. L., Lovell-Badge, R., & Capel, B. (2007). Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 16558–16563.
- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for Sry. *Nature*, 351, 117–121.
- Kreidberg, J. A., Sariola, H., Loring, J. M., Maeda, M., Pelletier, J., Housman, D., & Jaenisch, R. (1993). WT-1 is required for early kidney development. *Cell*, 74, 679–691.
- Kuroki, S., Matoba, S., Akiyoshi, M., Matsumura, Y., Miyachi, H., Mise, N., Abe, K., Ogura, A., Wilhelm, D., Koopman, P., Nozaki, M., Kanai, Y., Shinkai, Y., & Tachibana, M. (2013). Epigenetic regulation of mouse sex determination by the histone demethylase Jmjd1a. *Science*, 341, 1106–1109.
- Lavery, R., Chassot, A. A., Pauper, E., Gregoire, E. P., Klopstein, M., de Rooij, D. G., Mark, M., Schedl, A., Ghyselinck, N. B., & Chaboissier, M. C. (2012). Testicular differentiation occurs in absence of R-spondin1 and Sox9 in mouse sex reversals. *PLoS Genetics*, 8, e1003170.
- Lin, Y. T., & Capel, B. (2015). Cell fate commitment during mammalian sex determination. *Current Opinion in Genetics & Development*, 32, 144–152.
- Lindeman, R. E., Gearhart, M. D., Minkina, A., Krentz, A. D., Bardwell, V. J., & Zarkower, D. (2015). Sexual cell-fate reprogramming in the ovary by DMRT1. *Current Biology*, 25, 764–771.
- Liu, C. F., Bingham, N., Parker, K., & Yao, H. H. (2009). Sex-specific roles of beta-catenin in mouse gonadal development. *Human Molecular Genetics*, 18, 405–417.
- Luo, X., Ikeda, Y., & Parker, K. L. (1994). A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. *Cell*, 77, 481–490.
- Maatouk, D. M., DiNapoli, L., Alvers, A., Parker, K. L., Taketo, M. M., & Capel, B. (2008). Stabilization of beta-catenin in XY gonads causes male-to-female sex-reversal. *Human Molecular Genetics*, 17, 2949–2955.
- Maatouk, D. M., Mork, L., Chassot, A. A., Chaboissier, M. C., & Capel, B. (2013). Disruption of mitotic arrest precedes precocious differentiation and transdifferentiation of pregranulosa cells in the perinatal Wnt4 mutant ovary. *Developmental Biology*, 383, 295–306.
- Maatouk, D. M., Natarajan, A., Shibata, Y., Song, L., Crawford, G. E., Ohler, U., & Capel, B. (2017). Genome-wide identification of regulatory elements in Sertoli cells. *Development*, 144, 720–730.
- Manuylov, N. L., Smagulova, F. O., Leach, L., & Tevosian, S. G. (2008). Ovarian development in mice requires the GATA4-FOG2 transcription complex. *Development*, 135, 3731–3743.
- Matson, C. K., Murphy, M. W., Sarver, A. L., Griswold, M. D., Bardwell, V. J., & Zarkower, D. (2011). DMRT1 prevents female reprogramming in the postnatal mammalian testis. *Nature*, 476, 101–104.
- Minkina, A., Matson, C. K., Lindeman, R. E., Ghyselinck, N. B., Bardwell, V. J., & Zarkower, D. (2014). DMRT1 protects male gonadal cells from retinoid-dependent sexual transdifferentiation. *Developmental Cell*, 29, 511–520.
- Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I., & Aizawa, S. (1997). Defects of urogenital development in mice lacking Emx2. *Development*, 124, 1653–1664.
- Moniot, B., Declosmenil, F., Barrionuevo, F., Scherer, G., Aritake, K., Malki, S., Marzi, L., Cohen-Solal, A., Georg, I., Klattig, J., Englert, C., Kim, Y., Capel, B., Eguchi, N., Urade, Y., Boizet-Bonhoure, B., & Poulat, F. (2009). The PGD2 pathway, independently of FGF9, amplifies SOX9 activity in Sertoli cells during male sexual differentiation. *Development*, 136, 1813–1821.
- Mork, L., Maatouk, D. M., McMahon, J. A., Guo, J. J., Zhang, P., McMahon, A. P., & Capel, B. (2012). Temporal differences in granulosa cell specification in the ovary reflect distinct follicle fates in mice. *Biology of Reproduction*, 86, 37.
- Munger, S. C., Natarajan, A., Looger, L. L., Ohler, U., & Capel, B. (2013). Fine time course expression analysis identifies cascades of activation and repression and maps a putative regulator of mammalian sex determination. *PLoS Genetics*, 9, e1003630.
- Nakamura, M. (2010). The mechanism of sex determination in vertebrates—are sex steroids the key-factor? *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology*, 313, 381–398.
- Navarro-Martin, L., Vinas, J., Ribas, L., Diaz, N., Gutierrez, A., Di Croce, L., & Piferrer, F. (2011). DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLoS Genetics*, 7, e1002447.

- Nef, S., Verma-Kurvari, S., Merenmies, J., Vassalli, J. D., Efstratiadis, A., Accili, D., & Parada, L. F. (2003). Testis determination requires insulin receptor family function in mice. *Nature*, 426, 291–295.
- Nef, S., Schaad, O., Stallings, N. R., Cederroth, C. R., Pitetti, J. L., Schaer, G., Malki, S., Dubois-Dauphin, M., Boizet-Bonhoure, B., Descombes, P., Parker, K. L., & Vassalli, J. D. (2005). Gene expression during sex determination reveals a robust female genetic program at the onset of ovarian development. *Developmental Biology*, 287, 361–377.
- Nicol, B., & Yao, H. H. (2015). Gonadal identity in the absence of pro-testis factor SOX9 and pro-ovary factor beta-catenin in mice. *Biology of Reproduction*, 93, 35.
- Nishino, K., Hattori, N., Tanaka, S., & Shiota, K. (2004). DNA methylation-mediated control of Sry gene expression in mouse gonadal development. *Journal of Biological Chemistry*, 279, 22306–22313.
- Ottolenghi, C., Omari, S., Garcia-Ortiz, J. E., Uda, M., Crisponi, L., Forabosco, A., Pilia, G., & Schlessinger, D. (2005). Foxl2 is required for commitment to ovary differentiation. *Human Molecular Genetics*, 14, 2053–2062.
- Pannetier, M., Chassot, A. A., Chaboissier, M. C., & Pailhoux, E. (2016). Involvement of FOXL2 and RSP01 in ovarian determination, development, and maintenance in mammals. *Sexual Development*, 10, 167–184.
- Parma, P., Radi, O., Vidal, V., Chaboissier, M. C., Dellambra, E., Valentini, S., Guerra, L., Schedl, A., & Camerino, G. (2006). R-spondin1 is essential in sex determination, skin differentiation and malignancy. *Nature Genetics*, 38, 1304–1309.
- Parrott, B. B., Kohno, S., Cloy-McCoy, J. A., & Guillet, L. J., Jr. (2014). Differential incubation temperatures result in dimorphic DNA methylation patterning of the SOX9 and aromatase promoters in gonads of alligator (*Alligator mississippiensis*) embryos. *Biology of Reproduction*, 90, 2.
- Pitetti, J. L., Calvel, P., Romero, Y., Conne, B., Truong, V., Papaioannou, M. D., Schaad, O., Docquier, M., Herrera, P. L., Wilhelm, D., & Nef, S. (2013). Insulin and IGF1 receptors are essential for XX and XY gonadal differentiation and adrenal development in mice. *PLoS Genetics*, 9, e1003160.
- Rastetter, R. H., Bernard, P., Palmer, J. S., Chassot, A. A., Chen, H., Western, P. S., Ramsay, R. G., Chaboissier, M. C., & Wilhelm, D. (2014). Marker genes identify three somatic cell types in the fetal mouse ovary. *Developmental Biology*, 394, 242–252.
- Sadovsky, Y., Crawford, P. A., Woodson, K. G., Polish, J. A., Clements, M. A., Tourtellotte, L. M., Simburger, K., & Milbrandt, J. (1995). Mice deficient in the orphan receptor steroidogenic factor 1 lack adrenal glands and gonads but express P450 side-chain-cleavage enzyme in the placenta and have normal embryonic serum levels of corticosteroids. *Proceedings of the National Academy of Sciences of the United States of America*, 92, 10939–10943.
- Salas-Cortes, L., Jaubert, F., Bono, M. R., Fellous, M., & Roseblatt, M. (2001). Expression of the human SRY protein during development in normal male gonadal and sex-reversed tissues. *Journal of Experimental Zoology*, 290, 607–615.
- Schmahl, J., & Capel, B. (2003). Cell proliferation is necessary for the determination of male fate in the gonad. *Developmental Biology*, 258, 264–276.
- Schmahl, J., Eicher, E. M., Washburn, L. L., & Capel, B. (2000). Sry induces cell proliferation in the mouse gonad. *Development*, 127, 65–73.
- Schmahl, J., Kim, Y., Colvin, J. S., Ornitz, D. M., & Capel, B. (2004). Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. *Development*, 131, 3627–3636.
- Schmidt, D., Ovitt, C. E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A. C., & Treier, M. (2004). The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development*, 131, 933–942.
- Schnabel, C. A., Selleri, L., & Cleary, M. L. (2003). Pbx1 is essential for adrenal development and urogenital differentiation. *Genesis*, 37, 123–130.
- Sekido, R., Bar, I., Narvaez, V., Penny, G., & Lovell-Badge, R. (2004). SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. *Developmental Biology*, 274, 271–279.
- Siggers, P., Carre, G. A., Bogani, D., Warr, N., Wells, S., Hilton, H., Esapa, C., Hajhosseini, M. K., & Greenfield, A. (2014). A novel mouse Fgfr2 mutant, hobbyhorse (hob), exhibits complete XY gonadal sex reversal. *PLoS One*, 9, e100447.
- Tevosian, S. G., Albrecht, K. H., Crispino, J. D., Fujiwara, Y., Eicher, E. M., & Orkin, S. H. (2002). Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. *Development*, 129, 4627–4634.
- Tomaselli, S., Megiorni, F., De Bernardo, C., Felici, A., Marrocco, G., Maggiulli, G., Grammatico, B., Remotti, D., Saccucci, P., Valentini, F., Mazzilli, M. C., Majore, S., & Grammatico, P. (2008). Syndromic true hermaphroditism due to an R-spondin1 (RSP01) homozygous mutation. *Human Mutation*, 29, 220–226.
- Tomizuka, K., Horikoshi, K., Kitada, R., Sugawara, Y., Iba, Y., Kojima, A., Yoshitome, A., Yamawaki, K., Amagai, M., Inoue, A., Oshima, T., & Kakitani, M. (2008). R-spondin1 plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Human Molecular Genetics*, 17, 1278–1291.
- Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J. E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., & Pilia, G. (2004). Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Human Molecular Genetics*, 13, 1171–1181.
- Uhlenhaut, N. H., Jakob, S., Anlag, K., Eisenberger, T., Sekido, R., Kress, J., Treier, A. C., Klugmann, C., Klasen, C., Holter, N. I., Riethmacher, D., Schutz, G., Cooney, A. J., Lovell-Badge, R., & Treier, M. (2009). Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell*, 139, 1130–1142.
- Vainio, S., Heikkila, M., Kispert, A., Chin, N., & McMahon, A. P. (1999). Female development in mammals is regulated by Wnt-4 signalling. *Nature*, 397, 405–409.
- Wang, Q., Lan, Y., Cho, E. S., Maltby, K. M., & Jiang, R. (2005). Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. *Developmental Biology*, 288, 582–594.
- Warr, N., Carre, G. A., Siggers, P., Faleato, J. V., Brixey, R., Pope, M., Bogani, D., Childers, M., Wells, S., Scudamore, C. L., Tedesco, M., del Barco Barrantes, I., Nebreda, A. R., Trainor, P. A., & Greenfield, A. (2012). Gadd45gamma and Map3k4 interactions regulate mouse testis determination via p38 MAPK-mediated control of Sry expression. *Developmental Cell*, 23, 1020–1031.
- Wilhelm, D., Martinson, F., Bradford, S., Wilson, M. J., Combes, A. N., Beverdam, A., Bowles, J., Mizusaki, H., & Koopman, P. (2005). Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. *Developmental Biology*, 287, 111–124.
- Wilhelm, D., Yang, J. X., & Thomas, P. (2013). Mammalian sex determination and gonad development. *Current Topics in Developmental Biology*, 106, 89–121.
- Wu, G. C., & Chang, C. F. (2013). The switch of secondary sex determination in protandrous black porgy, *Acanthopagrus schlegelii*. *Fish Physiology and Biochemistry*, 39, 33–38.
- Yao, H. H., Matzuk, M. M., Jorgez, C. J., Menke, D. B., Page, D. C., Swain, A., & Capel, B. (2004). Follistatin operates downstream of Wnt4 in mammalian ovary organogenesis. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 230, 210–215.
- Zheng, W., Zhang, H., Gorre, N., Risal, S., Shen, Y., & Liu, K. (2014). Two classes of ovarian primordial follicles exhibit distinct developmental dynamics and physiological functions. *Human Molecular Genetics*, 23, 920–928.

Primordial Follicle

Megan A Gura and Richard N Freiman, Brown University, Providence, RI, United States

© 2018 Elsevier Inc. All rights reserved.

Development of the Primordial Follicle

What is a Primordial Follicle?

A human primordial follicle is approximately 40 μm in diameter, and comprised of a 30 μm oocyte in the center of approximately 10 squamous pregranulosa cells (Fig. 1; Townson and Combelles, 2012). Although not exclusive to any part of the ovary, primordial follicles are typically located in the peripheral cortex of the ovary rather than the inner medulla (Sforza et al., 2003). Primordial follicles contain a Balbiani body which is a unique assembly of cytoplasmic organelles including mitochondria and Golgi bodies associated with the nucleus of germ cells. In mice, the Balbiani body develops during germ cell cyst breakdown and primordial follicle assembly and is no longer present in the oocyte after primary follicle formation (Pepling et al., 2007). The total number of primordial follicles within the ovary at the time of birth is considered a finite pool from which the reproductive system will draw from over a lifetime. Although some research posits that there are cells within the adult human ovary that may be capable of forming new germ cells, through a process called neooogenesis (Virant-Klun, 2015), such studies remain to be confirmed independently and contrast with the evidence provided by a wealth of controlled mouse genetic studies (Zarate-Garcia et al., 2016). In humans, primordial follicles that make up the adult ovarian reserve are arrested in meiotic prophase I and remain quiescent until being activated for ovulation, which can be decades later (Pelosi et al., 2015). However, most reawakened follicles end up undergoing atresia, a specialized form of apoptosis of oocytes and their mature granulosa cells. For clinical purposes, there is not a test available that will directly assess the number of quiescent primordial follicles in the ovarian reserve (Gleicher et al., 2011). However, there is a strong and positive correlation between ovarian volume and the number of primordial follicles within the human ovary, with approximately 1000 primordial follicles corresponding to 3.01 cm^3 of ovarian volume (Kelsey and Wallace, 2012). Indirectly, the human ovarian reserve can be measured via serum AMH levels secreted by granulosa cells of the primordial follicle pool (van Rooij, 2002). In the following sections, we will discuss the developmental steps and dynamics that take place to establish the proper primordial follicle pool.

Germ Cell Numbers Over Time

In 1963, T.G. Baker published a seminal paper that estimated the population dynamics of the human ovarian reserve over time. This study posited that approximately one million oocytes were present in newborn females, which is often repeated amongst lay and scientific communities alike (Baker, 1963). A more precise estimation of how many oocytes within primordial follicles are present at the time of birth is less certain and seemingly more variable between individuals than the estimate Baker proposed. A more recent and comprehensive estimation of the primordial follicles within the human ovarian reserve suggests the peak number of follicles to be present at 18–22 weeks post fertilization (wpf) of approximately 300,000 non-growing follicles per ovary, with a 95% prediction interval of 35,000–2,534,000 follicles. Furthermore, the population at the time of birth is estimated to be similar at 295,000 follicles with a 95% prediction interval of 34,000–2,508,000 cells (Wallace and Kelsey, 2010). While in similar ranges as Baker first proposed, these large prediction intervals illustrate the general uncertainty surrounding the size and dynamics of the human ovarian reserve. In female mice, where genetic background, tissue procurement and environment can be controlled, these numbers are much clearer and help inform us how the mammalian ovary regulates oocyte numbers through development. At embryonic day 7.25 (E7.25), bone morphogenetic protein (BMP) signaling instructs approximately 40 primordial germ cells (PGCs) to form in the embryonic mesoderm. These initial PGCs migrate to the genital ridge from E8.5 to E10.5 and during this journey undergo mitosis with incomplete cytokinesis until E14.5. Incomplete cytokinesis of these PGCs creates “germ cell cysts” or “germline cysts” (discussed further in the next section). The peak number of PGCs at E14.5 is approximately 20,000 germ cells per ovary and this number decreases over time as many germ cells undergo apoptosis

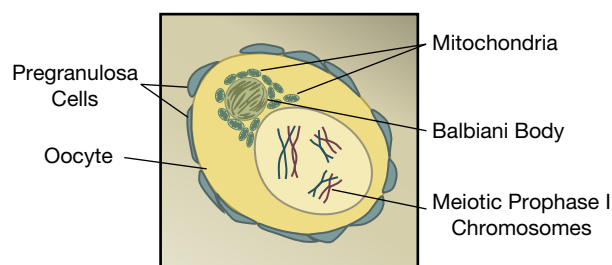


Fig. 1 Anatomy of a primordial follicle. Schematic diagram of a mammalian primordial follicle which is composed of a layer of squamous pregranulosa cells surrounding a single oocyte. The oocyte contains meiotic chromosomes arrested in the diplotene stage of prophase I, and a specialized perinuclear Golgi and mitochondrial cloud called a Balbiani body (green circle). The oocyte cytoplasm (dark yellow) and nucleus (light yellow) are highlighted.

during cyst breakdown. By postnatal day 4 (PND4) approximately 4000 meiotically arrested oocytes per ovary survive thus establishing the initial primordial follicle pool and subsequent ovarian reserve (Ikami et al., 2017).

Germ Cell Cyst Formation and Breakdown

The formation of germ cell cysts in the human fetal ovary occurs 5–16 wpf (Wang et al., 2017) and is evolutionarily conserved across vertebrate species, including fish, frogs, and mammals (Pepling and Spradling, 1998; Filosa and Taddei, 1976). Much of our current understanding of this process in mammals is based upon extensive studies of oogenesis in the invertebrate model organism *Drosophila melanogaster*. The germline cyst in *Drosophila* is comprised of 16 cells in which their cytoplasm is connected through ring canals (Jenkins et al., 2013). Of these 16 cells within the cyst, there are two cells that have four ring canal connections. One of these two cells is designated to become the oocyte and the rest become nurse cells for the oocyte (Hong et al., 2003). These intercellular bridges allow for the movement of RNA, proteins, and organelles from the nurse cells into the developing oocyte within the cyst (Marlow, 2010).

In mammals, there are similar strategies implemented for determining and nourishing developing oocytes. Mouse female germ cells also form germline cysts through mitotic divisions with incomplete cytokinesis and are connected through cytoplasmic bridges (Pepling and Spradling, 1998) and these cysts begin to breakdown at the time of birth (Pepling and Spradling, 2001). At their greatest number, there are approximately 30 germ cells in a cyst. However, the functionality of these cytoplasmic bridges as ring canals in mammals has been disputed. Intercellular bridges using ring canals require the protein TEX14 to connect male germ cells but female *Tex14*-knockout mice are fertile. This suggests that in mammals, the ring canal connections between germ cells in a cyst are an evolutionary vestige rather than an essential development structure (Greenbaum et al., 2009). Alternatively, TEX14 function might be redundant with other proteins expressed in the cytoplasmic bridges in the developing mammalian ovary. Most importantly, the surviving oocytes still gain many useful organelles from other germ cells in the cyst that are destined to undergo apoptosis. It has been demonstrated that centrioles, Golgi, and mitochondria are moved into the surviving oocyte through cell membrane connections rather than ring canals. This would explain why TEX14 is not necessary for female fertility. This transfer of cellular equipment into the surviving oocyte leads to the Balbiani body forming within the oocyte (Lei and Spradling, 2016). After germ cell cyst breakdown and survival of specific oocytes, only 20% of the original germ cells in the cyst remain (Lei and Spradling, 2016). Thus, comparing how invertebrates and vertebrates initiate oogenesis has been an invaluable asset to our understanding of human oogenesis.

Primordial Follicle Assembly

Primordial follicle assembly is characterized by a minority of individual oocytes within the developing ovary being enveloped by a layer of squamous pregranulosa cells (Fig. 2). The majority of early oocytes that do not assemble into primordial follicles are lost by apoptosis. Formation of the primordial follicle occurs in mice after birth and in humans as early as 14 wpf, but is more common at 18 wpf (Pepling, 2006; Coward and Wells, 2013). The productive interaction of pregranulosa cells and their corresponding oocyte is critical for establishing the proper ovarian reserve during embryogenesis (Nicol and Yao, 2014). While the bidirectional communication between granulosa cells and oocytes during folliculogenesis in the adult is well characterized, how these cells first initiate their intimate and critical relationship remains to be further illuminated.

Pioneering genetic studies of the forkhead transcription factor FOXL2 indicated that proper differentiation of embryonic pregranulosa cells was required for development of the primordial follicle pool in the postnatal mouse (Schmidt, 2004). Strikingly, mutation of the human *FOXL2* gene causes blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) type I, which is a rare eye malformation and associated with primary ovarian insufficiency (Crisponi et al., 2001; De Baere et al., 2001). Together these studies suggest that proper differentiation of pregranulosa cells is highly regulated during embryonic development and critical for proper human fertility.

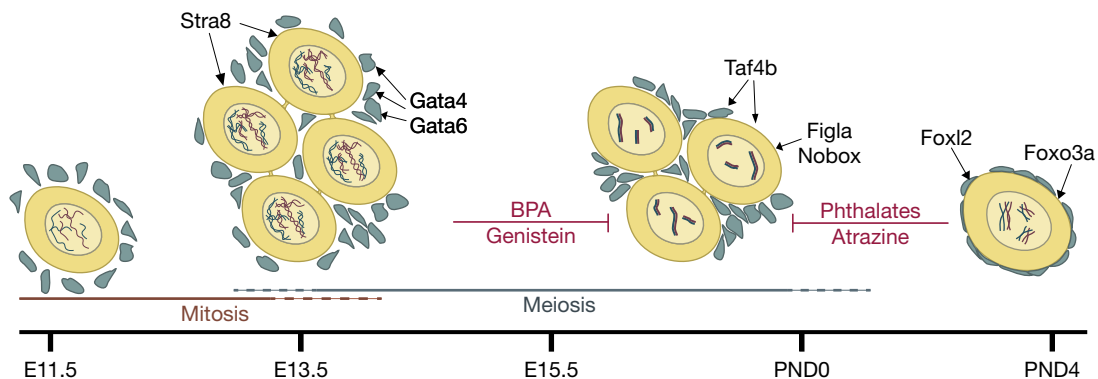


Fig. 2 Temporal regulation of mouse primordial follicle assembly. Based on extensive mouse genetic and developmental studies, formation of primordial follicles covers embryonic (E) and postnatal day (PND) timepoints. At E13.5, mitotically active oogonia begin meiotic differentiation. By PND4, oocytes are arrested in prophase I of meiosis and cyst breakdown is complete forming a finite pool of individual primordial follicles. Genes regulating entry into meiosis and the early development of primordial follicle are indicated in *black font*. Environmental toxicants known to disrupt these early steps in primordial follicle development are indicated in *red font*.

One of the major challenges to understanding the function of specific genes in pregranulosa cell-dependent regulation of primordial follicle assembly is that potential regulators are often expressed both in oocytes and somatic pregranulosa cells. The transcriptional regulator of primordial follicle development TAF4b is expressed in both oocytes and pregranulosa cells and may direct unique transcriptional programs in each cell type (Grive and Freiman, 2015). In such cases, assigning precise cell type-specific function to individual genes and signaling pathways is difficult. However, recent studies in mice have begun to identify pregranulosa-specific factors, functions and mechanisms that drive establishment of the initial primordial follicle pool. For example, deletion of the transcription factors *Gata4* and *Gata6* in embryonic pregranulosa cells prevents formation of the initial primordial follicle pool (Padua et al., 2014). These studies further support the notion that highly regulated pregranulosa cell-specific functions are essential for proliferation and/or differentiation of pregranulosa cells to support oocyte nest breakdown and embryonic formation of the adult ovarian reserve. Adding to this complexity, recent evidence supports the existence of heterogeneity amongst pregranulosa cell and primordial follicle populations that arise in the embryonic ovary (Harikae et al., 2013; Zheng et al., 2014). Thus, future studies are required to identify the molecular nature of these sub-populations of pregranulosa cells and primordial follicles and how they may uniquely contribute to mammalian fertility.

Further progress has been made in understanding oocyte-specific genes that contribute to primordial follicle assembly. Two genes that have come into focus as important for primordial follicle assembly expressed in the oocyte are *Figla* and *Nobox*, both transcription factors that regulate the expression of other genes. *Figla*-null mice suffer from infertility due to a lack of primordial follicles and its encoded protein Figla has been shown to regulate multiple germ cell-specific genes in the embryonic ovary while repressing male germline genes (Soyal et al., 2000; Joshi et al., 2007). The human *Figla* homolog is expressed in primordial follicles and is correlated with the development of these follicles (Huntriss et al., 2002; Bayne et al., 2004). Similar to Figla, Nobox expression is thought to repress male germline gene expression and is needed for proper cyst breakdown, oocyte survival, and primordial follicle assembly in mice (Choi et al., 2007; Rajkovic et al., 2004). When Nobox is absent, there is impaired invasion of pregranulosa cells into the germ cell cysts for primordial follicle formation (Lechowska et al., 2011). The common theme that transcription factors play a critical role in primordial follicle development suggests that particular sets of gene networks are exquisitely regulated in a stage and cell type-specific manner to initiate this process for successfully for subsequent fertilization and development.

Primordial Follicle Quiescence and Maintenance

In humans, quiescent follicles must have the potential to survive a woman's reproductive lifespan of about 50 years (McGee and Hsueh, 2000). The dormant primordial follicles are mainly localized to the outer cortical areas of the adult human ovary (Leung et al., 2004). While this quiescence phase is the longest state of a female gamete's life, how exactly this phase is maintained is not well understood. It has been shown that inhibitory factors repress the activation of the primordial follicle from entering folliculogenesis and survival factors prevent primordial follicle loss via apoptosis (Reddy et al., 2010). The major genes and pathways that support these concepts have been identified using mutant mouse genetic models. For example, the PTEN signaling pathway, which includes genes like *Foxo3a*, is believed to be critical for germ cell quiescence due to premature primordial follicle activation in mice (Fig. 2; Castrillon et al., 2003; Reddy et al., 2008). Furthermore, factors that promote primordial follicle survival are also connected to this pathway such as 3-phosphoinositide-dependent protein kinase-1 (PDK1), whose loss in germ cells causes early primordial follicle death (Reddy et al., 2009). The further understanding of what genes and pathways contribute to primordial follicle quiescence and survival will need to be improved by future research in rodents and other large animal models in addition to further examination of these signaling pathways in the context of the human ovary.

Meiosis Initiates Within the Developing Oocyte

Meiosis During Oogenesis

Concurrent with germ cell cyst breakdown and primordial follicle development, prophase I of meiosis I has begun. Meiosis is the process by which a single germ cell duplicates its genome and divides twice into haploid daughter cells. The gametes that are produced by this process provide half the genetic information to the next generation. In female mammals, meiosis begins during embryonic development and is critical for establishing a functional ovarian reserve. In this section, we will briefly cover the stages of meiosis that occur and are critical for proper primordial follicle development.

Meiotic Initiation

The reductive divisions of meiosis begin with DNA replication. In mice, meiotic initiation begins at embryonic day 13.5 (E13.5, Fig. 2) and in humans at 11 wpf (Childs et al., 2011; Coward and Wells, 2013). In the developing mouse ovary, meiosis initiates and progresses through a synchronous anterior to posterior wave, whereas human meiosis progresses less synchronously but generally more developed oocytes are in the medulla of the ovary in comparison to the peripheral cortex (Menke et al., 2003; Le Bouffant et al., 2010; Childs et al., 2011). Retinoic acid (RA) and stimulated by retinoic acid 8 (Stra8) are critical for meiotic initiation. In mice RA signaling originates from the adjacent mesonephros, an organ that acts as a transient embryonic kidney, and induces Stra8 expression in the oocytes via retinoic acid receptors (Feng et al., 2014; Gray and Cohen, 2016). Stra8 expression is the first indication that meiosis has begun in the oocyte and is critical for both pre-meiotic DNA replication and proper execution of prophase I (Baltus

et al., 2006). Despite being discovered in 1995 and studied extensively, the function of the STRA8 protein remains unknown (Bouillet et al., 1995; Oulad-Abdelghani et al., 1996; Feng et al., 2014). In humans, it is believed that similar mechanisms occur to promote meiotic initiation, however RA signaling, and thus the cue for meiotic initiation, instead originates from the ovary, rather than the mesonephros (Le Bouffant et al., 2010; Childs et al., 2011). Many aspects of meiotic initiation are not well understood, especially in regards to the regulation of gene expression associated with promoting the timely initiation of meiosis that directly leads to the establishment of the proper adult ovarian reserve.

Primordial Follicle Stages of Meiosis—Prophase I

Female mammalian oocytes progress through prophase of meiosis I (prophase I) before arresting for the duration of primordial follicle quiescence. During this process, chromosomes condense, synapse, recombine, repair and then arrest until primordial follicle activation. These actions within prophase I are often broken down into five distinct steps of development:

Leptotene

Leptotene, also known as leptonema, is based in the Greek meaning “thin thread” due to the thin strands the oocyte’s chromosomes are at this stage (Gilbert, 2016). Leptotene is predominantly observed in oocytes at E14.5 in mice and approximately 13 wpf in humans (Borum, 1961; Coward and Wells, 2013). Chromosomes are loosely condensed and connections between sister chromatids are formed during leptotene through axial elements (AEs), which connect the sister chromatids from end to end. At this point, the homologous chromosomes, which each have a sister chromatid due to pre-meiotic DNA replication, are not rigidly attached to one another. Double-stranded DNA breaks also occur in preparation for genetic recombination and repair by non-sister homologous chromosomes. While the process of DNA recombination requires many steps and proteins, PRDM9 is a methyltransferase that works in both mice and humans as a marker of meiotic recombination hotspots and SPO11 is a topoisomerase that is responsible for breaking the DNA strands (McNicoll et al., 2013; Gray and Cohen, 2016).

Zygotene

Zygotene, also known as zygonema, comes from the Greek meaning “yoked threads” due to the chromosomes becoming slightly more condensed and homologous chromosomes beginning to attach using the synaptonemal complex (SC), a set of proteins appropriately named synaptonemal complex protein 1–3 (SYCP 1–3) that zip up the homologous chromosomes from end to end. The zygotene substage of prophase I predominantly occurs at E14.5 but also can be found at E15.5 and E16.5 in mouse development (Borum, 1961). Similarly, zygotene in human development can first be observed at approximately 14 wpf (Coward and Wells, 2013). DNA repair proteins such as MSH4/MSH5 localize to sites of DNA damage induced for meiotic recombination by SPO11. Most DNA double-stranded breaks will not result in genetic crossover between homologous chromosomes, but instead be simply repaired (Bolcun-Filas and Schimenti, 2012; Gray and Cohen, 2016).

Pachytene

Pachytene, also known as pachynema, comes from the Greek meaning of “thick thread” because by this stage the homologous chromosomes are synapsed, or have been fully zipped up and are connected from end to end by the synaptonemal complex. This fully synapsed unit of chromosomes is referred to as a tetrad. Pachytene can begin to be observed as early as E15.5 in mice but can be found in oocytes up until the time of birth and in humans can begin to be observed at approximately 16 wpf (Borum, 1961; Coward and Wells, 2013). During this time, strand invasion and meiotic recombination occurs and is repaired by the end of pachytene. The DNA repair mechanisms tend to favor the homologous chromosome as the template for DNA repair over using the sister chromatid. However, the basis for the concept of homolog preference comes from studies in yeast and the facilitating proteins do not have clear homologues in mice and humans but the proteins HORMAD1 and HORMAD2 are suspected to be such homologues (McNicoll et al., 2013; Bolcun-Filas and Schimenti, 2012; Rinaldi et al., 2017).

Diplotene

Diplotene, or diplonema, meaning “double threads” is the final substage of prophase I and retains the compact appearance of pachytene chromosomes that appear more Y-shaped due to the loss of the synaptonemal complex between the homologous chromosomes except in a few areas where meiotic recombination occurred. These points of contact that tether the chromosomes together are called chiasmata. In mice, diplotene is observable during late gestation time points such as E17.5, with nearly all oocytes reaching this stage by postnatal day 2 (PND2), and in humans can begin to be observed at approximately 18 wpf (Dutta et al., 2016; Borum, 1961; Coward and Wells, 2013).

Meiotic arrest

Oocytes remain arrested in diplotene until meiotic resumption weeks to months later in mice and decades later in humans. Cell cycle regulators such as nitrous oxide (NO) and cyclic guanine monophosphate (cGMP) promote meiotic arrest. Meiotic oocytes that are removed from their somatic surroundings are capable of spontaneous resumption of meiosis, indicating that factors from the somatic cells are important for inhibiting this resumption (Tiware et al., 2017). One major remaining question is how does the ovary maintain these dormant cells over such long periods of time and how time affects the quality of these oocytes. Human trisomies are more frequent when compared to other species and these defects can originate early in meiosis. Furthermore,

the incidence of genetic defects increases with maternal age, although the molecular mechanisms for why these defects arise are largely unclear. One major model for age-related aneuploidy is that cohesin proteins that bind sister chromatids together within a tetrad degenerate with age due to many possible factors including premature loss of protection or oxidative damage (Cheng and Liu, 2017). Other factors underlying meiosis I aneuploidies remain to be understood but likely are critical in the development of human birth defects like fragile X syndrome one of the most common causes of childhood intellectual disability.

Disruption of the Primordial Follicle Reserve and Infertility

Environmental Susceptibility of Primordial Follicle Development

The ovarian reserve, and thus a woman's reproductive potential, is dependent upon the population of properly developed primordial follicles during fetal ovarian development. After primordial follicle assembly, follicles enter a long phase of dormancy. Both primordial follicle assembly and the ability to remain quiescent are vulnerable to errors in development and environmental insult. For example, multi-oocyte follicles (MOFs) have been observed in many species including mice and humans (Iguchi and Takasugi, 1986; Telfer and Gosden, 1987). There is no single cause underlying the formation of MOFs, but both environmental contaminants such as exposure to estrogenic substances and genetic defects have been demonstrated to increase the frequency of multi-oocyte follicles (Yan et al., 2001; Iguchi and Takasugi, 1986).

The potential mechanisms leading to the early demise of primordial follicle assembly and MOF development has been intensively studied in rodents where precise doses of environmental toxicants can be assessed in the context of isogenic strains. A major result of this research has led to the importance of the estrogen signaling pathway in the formation of the normal primordial follicle pool (Chen et al., 2007) and how sensitive this developmental process is to estrogen disrupting chemicals (EDCs). The well-studied and ubiquitous EDC bisphenol A (BPA) has been shown to disrupt multiple aspects of early primordial follicle development in both mice and non-human primates (Zhang et al., 2012; Hunt et al., 2012). In addition, embryonic exposure to the phytoestrogen genistein has been shown to disrupt primordial follicle assembly leading to MOFs (Jefferson et al., 2006; Cimafranca et al., 2010; Patel et al., 2017). Strikingly, prenatal exposure to a mixture of phthalates, a ubiquitous plasticizer, can lead to multi- and transgenerational effects on female reproduction (Zhou et al., 2017). A recent study implicates embryonic exposure of the herbicide atrazine in the early demise of the primordial follicle pool (Gely-Pernot et al., 2017). Taken together with the genetic factors affecting this process, establishing the primordial follicle pool is a critical window in development that is highly regulated and sensitive to diverse perturbations.

Early Aspects of Later Infertility

The average woman's ovarian reserve lasts for approximately 55 years before being depleted during menopause. While the timing of reproductive senescence is highly variable between individuals, many cases of reduced female fertility are caused by primary ovarian insufficiency (POI). Affecting 1% of women worldwide, POI is diagnosed by primary or secondary amenorrhea in women under 40 years of age, elevated follicle stimulating hormone (FSH) serum levels that are akin to levels found in menopausal women with reduced estradiol levels (Nelson, 2009). In addition to a reduced reproductive capacity at an early age, POI has other health consequences including increasing the risk of cardiovascular disease, osteoporosis, anxiety, and depression (Hewlett and Mahalingaiah, 2015). POI is one of the leading causes of female infertility, it is mainly spontaneous in nature, but there is a positive family history in 10%–15% of POI patients. The probability of POI diagnosis increases from 1 in 10,000 before 20 years of age, to 1 in 1000 before 30, to 1 in 100 women before age 40 (Fortuño and Labarta, 2014). Although POI is often sporadic in nature, there are likely complex genetic factors underlying the disorder. Many POI cases can be attributed to X chromosome abnormalities, including X chromosome monosomy called Turner Syndrome. Genes on the X chromosome disproportionately contribute to POI risk. The *Fragile X Mental Retardation 1* (*FMR1*) gene contains CGG triplet repeats that can cause fragile X syndrome if repeat numbers are above 200. However, unaffected mothers of these fragile X individuals, whose CGG repeats range from 55 to 200 have a 13%–26% chance of experiencing POI (Wittenberger et al., 2007; Fortuño and Labarta, 2014). Despite the common trend of the X chromosome and particularly *FMR1* contributing to POI susceptibility, many other factors can also be responsible for POI including many of the genes discussed in this article. Mutations in the oogenesis genes *Nobox* and *Figla* and the meiosis gene *MSH5* have been found in different populations to be associated with POI in women (Fortuño and Labarta, 2014). Future research into POI will likely focus on the additive effects of genetic, environmental and epigenetic factors that together contribute to the demise of the ovarian reserve in POI and other related fertility disorders in women.

References

- Baker, T. G. (1963). A quantitative and cytological study of germ cells in human ovaries. *Proceedings of the Royal Society of London. Series B, Biological sciences*, 158, 417–433.
- Baltus, A. E., Menke, D. B., Hu, Y. C., Goodheart, M. L., Carpenter, A. E., de Rooij, D. G., & Page, D. C. (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nature Genetics*, 38(12), 1430–1434.
- Bayne, R. A. L., da Silva, S. J. M., & Anderson, R. A. (2004). Increased expression of the FIGLA transcription factor is associated with primordial follicle formation in the human fetal ovary. *Molecular Human Reproduction*, 10(6), 373–381.
- Bolcun-Filas, E., & Schimenti, J. C. (2012). Genetics of meiosis and recombination in mice. *International Review of Cell and Molecular Biology*, 298, 179–227.
- Borum, K. (1961). Oogenesis in the mouse. A study of meiotic prophase. *Experimental Cell Research*, 24(3), 495–507.

- Bouillet, P., Oulad-Abdelghani, M., Vicaire, S., Garnier, J.M., Schuhbaur, B., Dolle, P., Chambon, P. (1995). Efficient cloning of cDNAs of retinoic acid-responsive genes in P19 embryonal carcinoma cells and characterization of a novel mouse gene, *Stra1* (mouse LERK-2/Eplg2). *Developmental Biology*, 170(2), 420–433.
- Castro-Alfonso, D. H., Miao, L., Kollipara, R., Horner, J. W., & DePinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*, 301(5630), 215–218.
- Chen, Y., Jefferson, W. N., Newbold, R. R., Padilla-Banks, E., & Pepling, M. E. (2007). Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology*, 148(8), 3580–3590.
- Cheng, J. M., & Liu, Y. X. (2017). Age-related loss of cohesion: Causes and effects. *International Journal of Molecular Sciences*, 18(7), 1–14.
- Childs, A. J., Cowan, G., Kinnell, H. L., & Anderson, R. A. (2011). Retinoic acid signalling and the control of meiotic entry in the human fetal gonad. *PLoS One*, 6(6), e20249.
- Choi, Y., Qin, Y., Berger, M. F., Ballow, D. J., Bulik, M. L., & Rajkovic, A. (2007). Microarray analyses of newborn mouse ovaries lacking Nobox. *Biology of Reproduction*, 77(2), 312–319.
- Cimafranca, M., Davila, J., Ekman, G. C., Andrews, R. N., et al. (2010). Acute and chronic effects of oral genistein administration in neonatal mice. *Biology of Reproduction*, 83, 114–121.
- Coward, K., & Wells, D. (2013). *Textbook of clinical embryology illustrate*. New York: Cambridge University Press.
- Crisponi, L., Deiana, M., Loi, A., Chiappe, F., Uda, M., et al. (2001). The putative forkhead transcription factor FOXL2 is mutated in blepharophimosis/ptosis/epicanthus inversus syndrome. *Nature Genetics*, 27, 159–166.
- De Baere, E., et al. (2001). Spectrum of FOXL2 gene mutations in blepharophimosis-ptosis-epicanthus inversus (BPES) families demonstrates a genotype–phenotype correlation. *Human Molecular Genetics*, 10(15), 1591–1600.
- Dutta, S., Burks, D. M., & Pepling, M. E. (2016). Arrest at the diplotene stage of meiotic prophase I is delayed by progesterone but is not required for primordial follicle formation in mice. *Reproductive Biology and Endocrinology*, 14(1), 82.
- Feng, C. W., Bowles, J., & Koopman, P. (2014). Control of mammalian germ cell entry into meiosis. *Molecular and Cellular Endocrinology*, 382(1), 488–497.
- Filosa, S., & Taddei, C. (1976). Intercellular bridges in lizard oogenesis. *Cell Differentiation*, 5(3), 199–206.
- Fortuño, C., & Labarta, E. (2014). Genetics of primary ovarian insufficiency: A review. *Journal of Assisted Reproduction and Genetics*, 31(12), 1573–1585.
- Gely-Pernot, A., Saci, S., Kernanec, P. Y., Hao, C., & Giton, F. (2017). Embryonic exposure to the widely-used herbicide atrazine disrupts meiosis and normal follicle formation in female mice. *Scientific Reports*, 7(1), 3526.
- Gilbert, S. F. (2016). *Developmental biology*. New York: Oxford University Press.
- Gleicher, N., Weghofer, A., & Barad, D. H. (2011). Defining ovarian reserve to better understand ovarian aging. *Reproductive Biology and Endocrinology*, 9(1), 23.
- Gray, S., & Cohen, P. E. (2016). Control of meiotic crossovers: From double-strand break formation to designation. *Annual Review of Genetics*, 50(1), 175–210.
- Greenbaum, M. P., Iwamori, N., Agno, J. E., & Matzuk, M. M. (2009). Mouse TEX14 is required for embryonic germ cell intercellular bridges but not female fertility. *Biology of Reproduction*, 80(3), 449–457.
- Grive, K. J., & Freiman, R. N. (2015). The developmental origins of the mammalian ovarian reserve. *Development*, 142(15), 2554–2563.
- Harikae, K., Miura, K., & Kanai, Y. (2013). Early gonadogenesis in mammals: Significance of long and narrow gonadal structure. *Developmental Dynamics*, 242(4), 330–338.
- Hewlett, M., & Mahalingaiah, S. (2015). Update on primary ovarian insufficiency. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 22(6), 483–489.
- Hong, A., Lee-Kong, S., Iida, T., Sugimura, I., & Lilly, M. A. (2003). The p27cip/kip ortholog dacapo maintains the Drosophila oocyte in prophase of meiosis I. *Development (Cambridge, England)*, 130(7), 1235–1242.
- Hunt, P. A., Lawson, C., Gleske, M., Murdoch, B., Smith, H., Marre, A., Hassold, T., & VandeVoort, C. A. (2012). Bisphenol A alters early oogenesis and follicle formation in the fetal ovary of the rhesus monkey. *Proceedings of the National Academy of Sciences*, 109(43), 17525–17530.
- Huntriss, J., Gosden, R., Hinkins, M., Oliver, B., Miller, D., Rutherford, A. J., & Picton, H. M. (2002). Isolation, characterization and expression of the human factor in the Germline alpha (FIGLA) gene in ovarian follicles and oocytes. *Molecular Human Reproduction*, 8(12), 1087–1095.
- Iguchi, T., & Takasugi, N. (1986). Polyovular follicles in the ovary of immature mice exposed prenatally to diethylstilbestrol. *Anatomy and Embryology*, 175(1), 53–55.
- Ikami, K., Nuzhat, N., & Lei, L. (2017). Organelle transport during mouse oocyte differentiation in germline cysts. *Current Opinion in Cell Biology*, 44, 14–19.
- Jefferson, W., Newbold, R., Padilla-Banks, E., & Pepling, M. (2006). Neonatal genistein treatment alters ovarian differentiation in the mouse: Inhibition of oocyte nest breakdown and increased oocyte survival. *Biology of Reproduction*, 74(1), 161–168.
- Jenkins, V. K., Timmons, A. K., & McCall, K. (2013). Diversity of cell death pathways: Insight from the fly ovary. *Trends in Cell Biology*, 23(11), 567–574.
- Joshi, S., Davies, H., Sims, L. P., Levy, S. E., & Dean, J. (2007). Ovarian gene expression in the absence of FIGLA, an oocyte-specific transcription factor. *BMC Developmental Biology*, 7, 67.
- Kelsey, T. W., & Wallace, W. H. B. (2012). Ovarian volume correlates strongly with the number of nongrowing follicles in the human ovary. *Obstetrics and Gynecology International*, 2012, 305025.
- Le Bouffant, R., Guerguin, M. J., Duguenne, C., Frydman, N., Coffigny, H., Rouiller-Fabre, V., Frydman, R., Habert, R., & Livera, G. (2010). Meiosis initiation in the human ovary requires intrinsic retinoic acid synthesis. *Human Reproduction*, 25(10), 2579–2590.
- Lechowska, A., Bilinski, S., Choi, Y., Shin, Y., Kloc, M., & Rajkovic, A. (2011). Premature ovarian failure in nobox-deficient mice is caused by defects in somatic cell invasion and germ cell cyst breakdown. *Journal of Assisted Reproduction and Genetics*, 28(7), 583–589.
- Lei, L., & Spradling, A. C. (2016). Mouse oocytes differentiate through organelle enrichment from sister cyst germ cells. *Science*, 352(6281), 95–99.
- Leung, P. C. K., Adashi, E. Y., & Gougeon, A. (2004). Dynamics of Human Follicular Growth: Morphologic, Dynamic, and Functional Aspects. In *The Ovary* (pp. 25–43). San Diego: Elsevier Academic Press. Chapter 2.
- Marlow, F. L. (2010). *Maternal control of development in vertebrates*. San Rafael, CA: Morgan & Claypool Life Sciences.
- McGee, E. A., & Hsueh, A. J. W. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews*, 21(2), 200–214.
- McNicoll, F., Stevenson, M., & Jessberger, R. (2013). Cohesin in gametogenesis. *Current Topics in Developmental Biology*, 102, 1–34.
- Menke, D. B., Koubova, J., & Page, D. C. (2003). Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. *Developmental Biology*, 262(2), 303–312.
- Nelson, L. M. (2009). Clinical practice. Primary ovarian insufficiency. *The New England Journal of Medicine*, 360(6), 606–614.
- Nicol, B., & Yao, H. H. C. (2014). Building an ovary: Insights into establishment of somatic cell lineages in the mouse. *Sexual Development*, 8(5), 243–251.
- Oulad-Abdelghani, M., Bouillet, P., Decimo, D., Gansmuller, A., Heyberger, S., et al. (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by *Stra8*, a novel retinoic acid-responsive gene. *Journal of Cell Biology*, 135(2), 469–477.
- Padua, M. B., Fox, S. C., Jiang, T., Morse, D. A., & Tevosian, S. G. (2014). Simultaneous gene deletion of Gata4 and Gata6 leads to early disruption of follicular development and germ cell loss in the murine Ovary1. *Biology of Reproduction*, 91(1), 24.
- Patel, S., Hartman, J. A., Helferich, W. G., & Flaws, J. A. (2017). Preconception exposure to dietary levels of genistein affects female reproductive outcomes. *Reproductive Toxicology*, 74, 174–180.
- Pelosi, E., Simonsick, E., Forabosco, A., Garcia-Ortiz, J. E., & Schlessinger, D. (2015). Dynamics of the ovarian reserve and impact of genetic and epidemiological factors on age of menopause. *Biology of Reproduction*, 92(5), 130, 1–9.
- Pepling, M. E. (2006). From primordial germ cell to primordial follicle: Mammalian female germ cell development. *Genesis*, 44(12), 622–632.
- Pepling, M. E., & Spradling, A. C. (1998). Female mouse germ cells form synchronously dividing cysts. *Development*, 125(17), 3323–3328.
- Pepling, M. E., & Spradling, A. C. (2001). Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. *Developmental Biology*, 234(2), 339–351.

- Pepling, M. E., Wilhelm, J. E., O'Hara, A. L., Gephardt, G. W., & Spradling, A. C. (2007). Mouse oocytes within germ cell cysts and primordial follicles contain a Balbiani body. *Proceedings of the National Academy of Sciences of the United States of America*, 104(1), 187–192.
- Rajkovic, A., Pangas, S. A., Ballow, D., Suzmori, N., & Matzuk, M. M. (2004). NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science*, 305(5687), 1157–1159.
- Reddy, P., Liu, L., Adhikari, D., Jajarlamudi, K., Rajareddy, S., Shen, Y., et al. (2008). Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. *Science*, 319(5863), 611–613.
- Reddy, P., Adhikari, D., Zheng, W., Liang, S., Hamalainen, T., et al. (2009). PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Human Molecular Genetics*, 18(15), 2813–2824.
- Reddy, P., Zheng, W., & Liu, K. (2010). Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends in Endocrinology and Metabolism*, 21(2), 96–103.
- Rinaldi, V. D., Bolcun-Filas, E., Kogo, H., Kurahashi, H., & Schimenti, J. C. (2017). The DNA damage checkpoint eliminates mouse oocytes with chromosome synapsis failure. *Molecular Cell*, 67(6), 1026–1036.
- van Rooij, I. A. J. (2002). Serum anti-Müllerian hormone levels: A novel measure of ovarian reserve. *Human Reproduction*, 17(12), 3065–3071.
- Schmidt, D. (2004). The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development*, 131(4), 933–942.
- Sforza, C., Vizzotto, L., Ferrario, V. F., & Forabosco, A. (2003). Position of follicles in normal human ovary during definitive histogenesis. *Early Human Development*, 74(1), 27–35.
- Soyal, S. M., Amleh, A., & Dean, J. (2000). FlGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development*, 127, 4645–4654.
- Telfer, E., & Gosden, R. G. (1987). A quantitative cytological study of polyovular follicles in mammalian ovaries with particular reference to the domestic bitch (*Canis familiaris*). *Journal of Reproduction and Fertility*, 81(1), 137–147.
- Tiwari, M., Prasad, S., Pandey, A. N., Premkumar, K. V., Tripathi, A., Gupta, A., et al. (2017). Nitric oxide signaling during meiotic cell cycle regulation in mammalian oocytes. *Frontiers in Bioscience – Scholar*, 9(3).
- Townson, D. H., & Combelles, C. M. H. (2012). Ovarian follicular atresia. In *Basic Gynecology—Some Related Issues* (pp. 43–76). Rijeka, Croatia: InTech.
- Virant-Klun, I. (2015). Postnatal oogenesis in humans: A review of recent findings. *Stem Cells Cloning*, 8, 49–60.
- Wallace, W. H. B., & Kelsey, T. W. (2010). Human ovarian reserve from conception to the menopause. *PLoS One*, 5(1), e8772.
- Wang, C., Zhou, B., & Xia, G. (2017). Mechanisms controlling germline cyst breakdown and primordial follicle formation. *Cellular and Molecular Life Sciences*, 74(14), 2567.
- Wittenberger, M. D., Hagerman, R. J., Sherman, S. L., McConkie-Rosell, A., Welt, C. K., et al. (2007). The FMR1 premutation and reproduction. *Fertility and Sterility*, 87(3), 456–465.
- Yan, C., Wang, P., DeMayo, J., Elvin, J. A., Carlino, C., Prasad, S. V., et al. (2001). Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology*, 15(6), 854–866.
- Zarate-Garcia, L., Lane, S. I., Merriman, J. A., & Jones, K. T. (2016). FACS-sorted putative oogonial stem cells from the ovary are neither DDX4-positive nor germ cells. *Scientific Reports*, 6(1), 27991.
- Zhang, H. Q., Zhang, X. F., Zhang, L. J., Chao, H. H., Pan, B., et al. (2012). Fetal exposure to bisphenol A affects the primordial follicle formation by inhibiting the meiotic progression of oocytes. *Molecular Biology Reports*, 39(5), 5651–5657.
- Zheng, W., Zhang, H., & Liu, K. (2014). The two classes of primordial follicles in the mouse ovary: Their development, physiological functions and implications for future research. *Molecular Human Reproduction*, 20(4), 286–292.
- Zhou, C., Gao, L., & Flaws, J. A. (2017). Exposure to an environmentally relevant phthalate mixture causes transgenerational effects on female reproduction in mice. *Endocrinology*, 158(6), 1739–1754.

Folliculogenesis

Patrick R Hannon and Thomas E Curry, University of Kentucky, Lexington, KY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

There are two main functions of the ovary, (1) the development of the female gamete (oocyte) for fertilization and the propagation of the species, and (2) the production and secretion of sex steroid hormones that regulate reproductive and nonreproductive health. Both of these critical ovarian functions are controlled by the ovarian follicle, which is the functional unit of the ovary.

The follicle consists of the oocyte, or egg, surrounded by granulosa cells and, at later stages of development, theca cells; both of which are somatic cells that support the oocyte. Follicles undergo several irreversible developmental transitions, and this process of follicular development is known as ovarian folliculogenesis. Follicles form by the breakdown of germ cell nests during fetal development to establish the most immature follicle type, known as the primordial follicle. Females are born with a finite supply of primordial follicles, which constitutes the ovarian reserve and represents a female's reproductive potential. There are, however, controversial reports of egg producing stem cells in women suggesting that these rare cells may have the ability to form oocytes and follicles. By default, primordial follicles are quiescent, but once they are selected to develop via a process called initial recruitment, they become primary follicles. These primary follicles continue to develop to become secondary and tertiary follicles (also known as preantral follicles), as discussed in detail below. Preantral follicles then develop into antral follicles. Most early antral follicles undergo atresia, which is a controlled apoptosis-mediated process of follicle death. However, during the follicular phase of a female's reproductive cycle, a small cohort of antral follicles are rescued from atresia and selected to develop further into preovulatory follicles (also known as Graafian follicles) via a process called cyclic recruitment. These preovulatory follicles are the major producers of estrogens in the female and are the only follicle type that is capable of ovulation. The culmination of folliculogenesis results in rupture of the preovulatory follicle and release of the oocyte during the process of ovulation. The ruptured follicle undergoes dynamic transformation into the corpus luteum, which is a progesterone producing structure needed for pregnancy. Fig. 1 outlines follicles at the different stages of development. Important for human health, defects in folliculogenesis can lead to infertility and primary ovarian insufficiency (POI; also known as premature ovarian failure or premature menopause), which underscores the importance of understanding how folliculogenesis is controlled.

Multidirectional interactions between the oocyte, granulosa cells, theca cells, and the hypothalamic-pituitary axis of the endocrine system are paramount for all of the complex developmental transitions of folliculogenesis. Specifically, the cells within the follicle produce growth and signaling factors that act in an autocrine and paracrine manner to regulate the early stages of folliculogenesis (i.e., initial recruitment). Further, hormones secreted from the hypothalamic-pituitary-ovarian axis act in an endocrine manner to coordinate the later stages of folliculogenesis (i.e., cyclic recruitment). The steps involved in each stage of folliculogenesis are outlined in the detailed sections below.

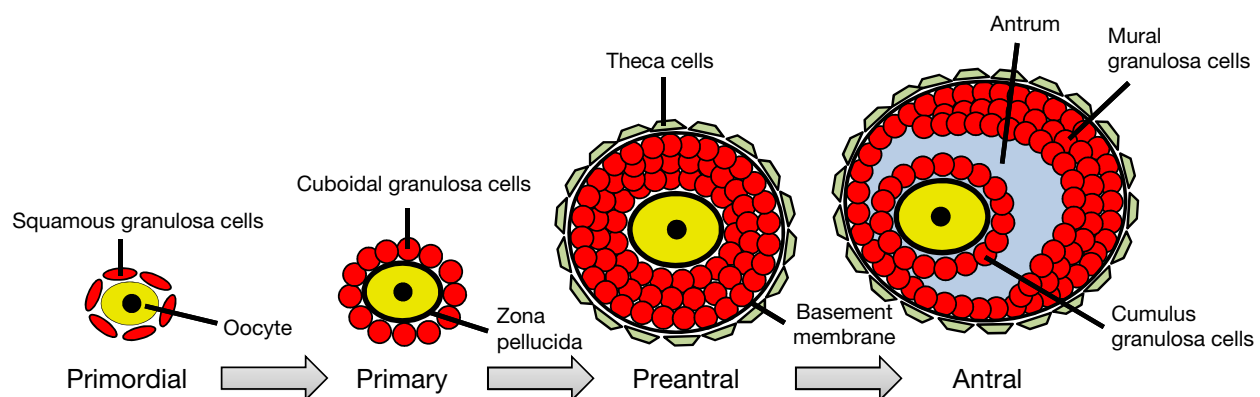


Fig. 1 Stages of folliculogenesis from the immature primordial follicle to the mature antral follicle. Follicles contain the female germ cell (oocyte) surrounded by somatic cells (granulosa cells and theca cells), and, at later stages of development, a fluid filled space called the antrum. Primordial follicles contain the oocyte surrounded by a single layer of squamous granulosa cells. These follicles develop into primary follicles, which contain the oocyte surrounded by a single layer of cuboidal granulosa cells. Primary follicles develop into preantral follicles (secondary and tertiary follicles), which contain the oocyte surrounded by several layers of granulosa cells and a basement membrane separating these cells from theca cells. Preantral follicles develop into antral follicles, which contain the oocyte surrounded by multiple layers of granulosa cells, the antrum, and a basement membrane separating the granulosa cells from the theca cells. Antral follicles develop further into preovulatory follicles, which are capable of ovulation. Figure modified from (Hannon, P. R., Flaws, J. A. (2015). The effects of phthalates on the ovary. *Frontiers in Endocrinology* 6, 8).

Primordial Follicle Formation

The establishment of the ovarian reserve, or primordial follicle formation, occurs during the later stages of fetal life in the human and during the early postnatal life in the rodent. Prior to this, primordial germ cells, which will give rise to oocytes, migrate during embryonic development from the yolk sac to the genital ridge where the undifferentiated gonad resides. These germ cells, now termed oogonia, massively proliferate via mitosis to a peak of 6–7 million oogonia by the 20th week of gestation in the human, and develop into germ cell nests that contain multiple oogonia surrounded by squamous pregranulosa cells. Once established in the germ cell nests, mitosis of oogonia ceases and meiosis begins. It is here that the oogonia become oocytes, and the oocytes progress through meiosis until they are arrested in the diplotene stage of meiotic prophase I.

Primordial follicle assembly then occurs around the 6–9th month of gestation in the human and around the first few days after birth in the mouse. Primordial follicle assembly occurs via a process called germ cell nest breakdown. This process involves the atretic demise of several of the oocytes contained within the germ cell nests. The interaction of several molecular events that leads to oocyte death causes the remaining oocytes to become associated with a single layer of flattened, squamous granulosa cells, resulting in the formation of the primordial follicle.

Fate of the Follicle

Once the primordial follicle population is established, the follicle is destined to four fates: (1) to remain quiescent for varying lengths of time to constitute the ovarian reserve, (2) to directly undergo atresia, (3) to undergo initial recruitment and eventually ovulate, or (4) to experience the most common fate, which is to undergo initial recruitment but later die via atresia. In fact, greater than 99% of all follicles in the ovary are lost via atresia, meaning < 1% of follicles ovulate. As previously discussed, oogonia numbers peak at approximately 6 million during midgestation. Due to germ cell nest breakdown and primordial follicle formation, the newborn ovary contains about 1 million follicles. Of these, only 200,000 remain at puberty, and only 300 will ovulate. This loss of follicles is because atresia is a dynamic process that occurs throughout the entire life of the female, even during embryonic development. Thus, understanding the mechanisms that control folliculogenesis is imperative because the female's finite supply of follicles is constantly dwindling to the eventual point of exhaustion, which occurs at about 51 years of age in women and is known as menopause.

Initial Recruitment—The Primordial to Primary Transition

Primordial follicle recruitment to the primary follicle stage is the first developmental transition that the follicle can undergo following follicle formation. Although the default fate of the primordial follicle is to remain quiescent and immature, the rate-limiting transition of initial recruitment can occur at any stage of life. In humans, it can occur in utero, prior to puberty, during the female's reproductive lifespan, and through the late stages of the menopausal transition. The first change in the primordial follicle in response to initial recruitment is the transformation of the morphology of the granulosa cells from a squamous to cuboidal shape. The diameter of the oocyte also increases in size, and the oocyte now acquires the zona pellucida, which is a thick membrane surrounding the oocyte that is required for fertilization. Initial recruitment to the primary stage of development is complete when the follicle contains a larger oocyte surrounded by a single layer of cuboidal granulosa cells. This process is the longest in duration for folliculogenesis, in which the completion of initial recruitment is estimated to take over 150 days in the human.

Since the primordial follicle is devoid of gonadotropin receptors and theca cells, initial recruitment is gonadotropin-independent and relies on a balance of ovarian-derived factors that either inhibit or promote primordial follicle recruitment. In fact, in vitro and in vivo studies have shown that primordial follicles develop to the early antral stage in the absence of gonadotropins. Under basal conditions, inhibitory factors keep the follicle in a quiescent state and favor follicle survival. A decrease in the levels/activity of these inhibitory factors in addition to the increase/action of stimulatory factors results in development to the primary follicle stage. These factors originate from the oocyte, the squamous granulosa cells, stromal cells that are precursor theca cells that will later be recruited to the follicle during development, and even cells from more mature follicles. [Table 1](#) contains a list of several of the well-documented intraovarian factors that act to promote or inhibit initial recruitment, some of which will be discussed below.

Phosphatidylinositol 3-Kinase Signaling

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is a regulatory pathway that controls processes such as cell proliferation, survival, migration, metabolism, and even the initial recruitment stage of ovarian folliculogenesis. This signaling pathway contains proteins that drive PI3K signaling (i.e., 3-phosphoinositide dependent protein kinase-1 (PDPK1), mammalian target of rapamycin complex 1 (mTORC1), protein kinase B (AKT), and ribosomal protein s6 (rpS6)), and proteins that inhibit PI3K signaling (i.e., forkhead box O3 (FOXO3), phosphatase and tensin homolog (PTEN), and tuberous sclerosis 1 and 2 (TSC1 and TSC2)). Conditional deletion of *Foxo3a*, *Pten*, *Tsc1*, or *Tsc2* from mouse oocytes results in global activation of all primordial follicles,

Table 1 Intrinsic ovarian factors involved in initial recruitment

Factor	Source	Target of action	Action on initial recruitment
AKT	Oocyte	Oocyte	Promote
bFGF	Oocyte	Granulosa cells, precursor theca cells, and stroma	Promote
BMP4/7	Precursor theca cells	Granulosa cells	Promote
KGF	Precursor theca cells	Granulosa cells, precursor theca cells, and stroma	Promote
Kit	Oocyte (also low levels in precursor theca cells)	Oocyte and precursor theca cells	Promote
Kit ligand	Granulosa cells	Oocyte and precursor theca cells	Promote
LIF	Granulosa cells	Oocyte and granulosa cells	Promote
mTORC1	Oocyte	Oocyte	Promote
PDGF	Oocyte and granulosa cells	Granulosa cells	Promote
AMH	Antral granulosa cells	Primordial follicle	Inhibit
FOXL2	Granulosa cells	Granulosa cells	Inhibit
FOXO3	Oocyte	Oocyte	Inhibit
PTEN	Oocyte	Oocyte	Inhibit
TSC1/2	Oocyte	Oocyte	Inhibit

leading to primary ovarian insufficiency (POI); thus, these inhibitory regulators of PI3K signaling maintain primordial follicle quiescence. In addition, studies indicate that acceleration of primordial follicle recruitment occurs because of over-activation of the PI3K signaling pathway brought about by the unrestricted regulation of stimulatory PI3K factors, such as AKT, mTORC1, PDPK1, and rpS6. Therefore, a basal level of PI3K signaling is needed for oocyte survival, a shift in the balance of signaling favoring the PI3K inhibitory components maintains quiescence, while signaling favoring an increase in stimulatory PI3K proteins promotes primordial follicle recruitment. Of importance, primordial follicles from mouse and human ovarian cortical tissue can be stimulated to undergo initial recruitment with the treatment of a PTEN inhibitor and a PI3K stimulating phosphopeptide. This treatment regimen is currently being investigated as a potential therapeutic option for in vitro activation protocols that are used on cryopreserved ovarian tissue from women that have experienced chemotherapy-induced infertility/POI. The PI3K signaling pathway is perhaps the most well-studied signaling pathway related to early ovarian folliculogenesis, and Fig. 2 is a simplified outline of the signaling cascades that bring about initial recruitment.

Kit and Kit Ligand

One of the receptor tyrosine kinases thought to stimulate PI3K signaling in the ovary is the mast/stem cell growth factor receptor, also known as kit. The kit receptor can be activated by binding to kit ligand (also known as stem cell factor, steel factor, and multipotent growth factor) in its soluble and membrane bound forms. Unlike intracellular PI3K signaling that utilizes factors that are restricted to the oocyte, kit–kit ligand signaling involves the multidirectional coordination between the oocyte and precursor theca cells, that express kit, and the granulosa cells, that produce kit ligand. Mutations of the soluble form of kit ligand in

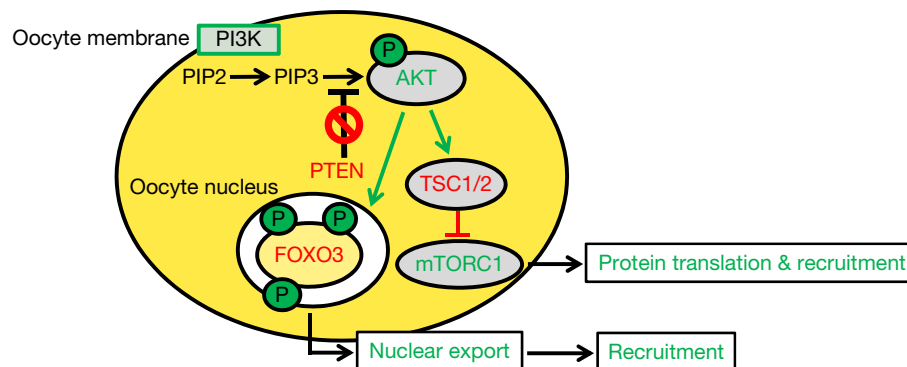


Fig. 2 Regulation of initial recruitment by the phosphatidylinositol 3-kinase (PI3K) signaling pathway. Growth factor-mediated binding to receptor tyrosine kinases recruits PI3K to the inner oocyte membrane, which stimulates the conversion of PIP2–PIP3. PIP3 phosphorylates AKT, while PTEN inhibits PIP3-mediated AKT phosphorylation by converting PIP3 back to PIP2. Phosphorylated AKT can phosphorylate FOXO3, which exports FOXO3 from the oocyte nucleus leading to primordial follicle recruitment. Phosphorylated AKT can also phosphorylate TSC1/2, which destabilizes the protein leading to activation of the mTORC1 cascade and ultimately protein translation and primordial follicle recruitment. Proteins in green are stimulators of initial recruitment, while proteins in red are inhibitors of initial recruitment.

mice arrests follicle development at the primary follicle stage, and neonatal mice treated with a neutralizing antibody for kit disturbs initial follicle recruitment. Previous studies have suggested that granulosa cell derived kit ligand can bind to kit on stromal precursor theca cells to induce their transition into theca cells, promote their migration to become closely associated to the follicle, and initiate proliferation of these soon to be theca cells. Simultaneously, kit ligand has been shown to bind to kit on the primordial follicle oocyte to initiate oocyte growth and development. Of importance, treatment with kit ligand to neonatal rodent ovaries increased the percentage of growing follicles, further suggesting that kit–kit ligand signaling may be a potential mediator of initial recruitment.

Other Stimulatory Factors

Similar to treatment with kit ligand, rat ovary cultures treated with basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), keratinocyte growth factor (KGF), bone morphogenetic proteins 4 and 7 (BMP4 and BMP7), and platelet-derived growth factor (PDGF) exhibited a dramatic increase in initial recruitment, as evident by a decrease in primordial follicle numbers and an increase in growing follicle numbers. Further, blocking bFGF and LIF action with neutralizing antibodies caused a reduction in primordial follicle activation. Following binding to its receptors located on granulosa cells, precursor theca cells, and stroma cells, bFGF appears to regulate granulosa, theca, and stromal cell growth. LIF produced by the granulosa cells is thought to act upon both the granulosa cells and the oocyte to initiate oocyte growth and the recruitment of precursor theca cells to the follicle. KGF is found in newly recruited precursor theca cells that are in contact with the squamous granulosa cells surrounding the primordial follicle and is thought to act upon the granulosa cells to initiate proliferation, the change to cuboidal morphology, or the upregulation of kit ligand. BMP4, localized to precursor theca cells, and BMP7, localized to theca cells of more developed follicles, also stimulate initial recruitment. Further, BMP4 has been shown to aid in the survival of primordial follicle oocytes, as treatment with a neutralizing antibody to BMP4 resulted in atresia of all the oocytes in a rat ovary culture system. PDGF expressed in oocytes, and granulosa cells to a lesser degree, acts on granulosa cells to potentially increase kit ligand levels for primordial follicle activation.

Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) belongs to the transforming growth factor beta (TGF- β) superfamily and is involved in the inhibition of initial recruitment. Although the mechanism by which AMH suppresses initial recruitment is largely unknown, AMH is recognized as a paracrine factor secreted from maturing follicles that maintains primordial follicle quiescence. Primordial follicles do not express AMH, and controversy exists whether they express receptors for AMH; however, follicular AMH levels that are produced by granulosa cells begin to rise after the follicle is initiated to develop. The highest levels of follicular AMH are present in the preantral and early antral follicle stages before declining again with preovulatory follicle development. *Amh* knockout mice exhibited depletion of the primordial follicle reserve as these mice aged, albeit much slower than that of *Pten* knockout mice. Further, treatment with AMH in vitro in human and rodent ovarian samples resulted in an increase in the number of primordial follicles with a subsequent decline in the number of growing follicles. Serum AMH levels are also used clinically to assess ovarian function, primarily because serum levels do not fluctuate during a female's menstrual cycle. In accordance with follicle development and the dwindling supply of follicles with age, serum AMH is highest at puberty when the number of maturing follicles is high and decreases with age until it is negligible at menopause when the ovary is devoid of follicles. Since the production of AMH is highest in the early antral follicle, serum AMH levels are predictive of the number of antral follicles that can be stimulated by gonadotropins and, consequently, of in vitro fertilization outcomes. Important for initial recruitment, there is also a positive correlation between serum AMH levels and the number of primordial follicles present in the ovary. This is because AMH is thought to be a regulator of the status of the female's ovarian reserve. Thus, AMH is increasingly being used to assess a woman's fertility and potential reproductive lifespan.

Forkhead Box L2

Forkhead box L2 (FOXL2) is a wing-helix/forkhead transcription factor that has been shown to be a negative regulator of initial primordial follicle recruitment. FOXL2 is expressed in granulosa cells from the primordial follicle and throughout several stages of folliculogenesis. Results obtained from *Foxl2* knockout mice suggest that FOXL2 plays a critical role in the development of granulosa cells within primordial follicles. Specifically, these mice exhibited premature growth/development of oocytes that resemble oocytes of more mature follicles. However, these changes in the oocyte occur in the absence of the squamous to cuboidal granulosa cell transition. The entire follicle pool then undergoes atresia because the oocyte and somatic cells must develop simultaneously. Due to this POI, these mice are infertile. Thus, FOXL2 appears to mediate the squamous to cuboidal transition and proliferation of granulosa cells, which are required steps in initial recruitment. Importantly, sequences of the *FOXL2* gene are highly conserved across species, and mutations of the *FOXL2* gene in humans cause Blepharophimosis, ptosis, and epicanthus inversus syndrome (BPES). Females with BPES type I are at a high risk of POI-induced infertility, which underscores the potential importance of this inhibitory factor of folliculogenesis in humans.

Initial Recruitment—Summary

Contrary to cyclic recruitment that occurs during the follicular phase of a female's reproductive cycle, the molecular mechanisms that control initial recruitment are poorly understood. Although the list of factors involved in initial recruitment within this article is not exhaustive, it should be emphasized that primordial follicle recruitment is a complex, multidirectional process involving several cell types and signaling molecules that converge on multiple signaling pathways. It is likely that the intricate coordination between these stimulatory and inhibitory substances determines a primordial follicle's fate of survival, quiescence, or recruitment.

Transitions From the Primary Stage to the Early Antral Stage

Once established, primary follicles are devoid of theca cells, but precursor theca cells derived from ovarian stroma have already begun migrating to the follicle as early as the final stages of initial recruitment. The granulosa cells contained in a single layer in the primary follicle undergo mitosis and multiple layers of granulosa cells that are capable of producing steroids form, establishing the preantral follicle. It takes roughly 120 days for this process to occur in the human.

Once the follicle has reached the preantral stage, a basement membrane and two layers of theca cells form. The theca interna is the inner layer of cells that are adjacent to the basement membrane separating the theca cells from the granulosa cells and are steroid producing cells. The theca externa is the outer layer of cells comprised predominantly of nonsteroidogenic fibroblast-like cells that become part of a collagenous connective tissue. Vascularization of the theca cells provides the follicle with direct access to the bloodstream, and thus access to nutrients and hormones, such as gonadotropins.

As the follicle develops further, granulosa cells continue to proliferate forming additional layers and theca cell vascularization increases. The substances brought in through the bloodstream and the secretory products produced by the proliferating granulosa cells result in the formation of fluid-filled spaces within the granulosa cell layer. These spaces eventually coalesce to form a singular fluid cavity within the granulosa cell layer, which is known as the antrum, or antral cavity. Following the initial appearance of the antral spaces, the follicle is called an early antral follicle. This process occurs over a span of roughly 70 days in the human.

Similar to initial recruitment, the transitions from the primary stage to the early antral stage are largely driven by intraovarian factors and signaling pathways. This is confirmed by the evidence that early antral follicles are formed in the complete absence of gonadotropins. Some of the intraovarian factors that control these transitions are presented in **Table 2**. However, some studies suggest that gonadotropins may help mediate follicle development during these stages. Specifically, theca cells contain luteinizing hormone (LH) receptors, and granulosa cells contain follicle-stimulating hormone (FSH) receptors at early stages of development. Thus, the middle transitions of folliculogenesis are not entirely gonadotropin-independent like initial recruitment, but rather, these transitions appear to be gonadotropin-responsive.

Cyclic Recruitment—The Early Antral to Preovulatory Transition

To this point in folliculogenesis, the ovary now contains a cohort of early antral follicles that are gonadotropin responsive and able to be further stimulated by FSH for final maturation to the preovulatory stage. Hormonal feedback loops that exist within the

Table 2 Intrinsic ovarian factors involved in preantral/early antral follicle development

<i>Factor</i>	<i>Source</i>	<i>Action</i>
Activins	Granulosa cells	Stimulate granulosa cell proliferation
AMH	Granulosa cells	Inhibitory factor on small follicle development
BMP15	Oocyte	Stimulate granulosa cell proliferation
BMP4/7	Theca cells	Modulate FSH signaling to increase estradiol levels, prevent premature luteinization
Connexins 37 and 43	Granulosa cells and granulosa-oocyte junction	Communication between granulosa cells to granulosa cells and oocyte to granulosa cells. Knockouts have inhibited development beyond primary stage
Cyclin D2	Granulosa cells	FSH stimulated factor that controls granulosa cell proliferation
GDF9	Oocyte	Theca cell recruitment. Knockouts have inhibited development beyond primary stage
Hedgehog signaling members	Granulosa cells and theca cells	Proper theca cell function
IGF1/IGFR	Granulosa cells	Enhance granulosa cell responsiveness to FSH
KGF	Theca cells	Modulating communication between theca cells and granulosa cells
Kit/kit ligand	Granulosa cells and oocyte	Continued follicle development, oocyte growth, and theca cell organization
NTF5/BDNF/NTRK2	Granulosa cells and oocyte	Knockouts have impaired development beyond primary stage
WT1	Granulosa cells	Inhibitory factor on small follicle development

IGF1, insulin-like growth factor 1; IGFR, insulin-like growth factor receptor; NTF5, neurotrophin 5; BDNF, brain-derived neurotrophic factor; NTRK2, neurotrophic tyrosine kinase receptor type 2; WT1, Wilms tumor 1.

hypothalamic-pituitary-ovarian axis lead to the fluctuation of FSH levels during the menstrual and estrous cycles. The rising level of FSH during the late luteal/early follicular stages of the menstrual/estrous cycle is undeniably the most important catalyst for preovulatory follicle development. Without FSH stimulation, the early antral follicle undergoes atresia, and in fact, early antral follicles are the most susceptible to atretic demise. Thus, further development at this stage is now gonadotropin-dependent. Since the pattern of FSH secretion is cyclical, and FSH is the predominant factor in antral follicle development and survival, this period of folliculogenesis is referred to as cyclic recruitment. This development is the most rapid and occurs during the follicular phase of the menstrual cycle, or about 14–20 days.

Physical characteristics of cyclic recruitment include the coalescence of antral spaces of the early antral follicle to form a singular antral cavity. As theca cell vascularization continues to increase, so does the size of the antrum. This is accompanied by FSH-mediated mitosis of granulosa cells, all of which leads to a massive increase in the size of the maturing antral follicle over a very short period of time. As the antrum increases in size, different subpopulations of granulosa cells emerge. This is largely driven by a concentration gradient of oocyte-derived growth differentiating factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) that diminishes with distance from the oocyte. The mural granulosa cells are the outermost cells surrounding the basement membrane and are the most steroidogenically active, estradiol-producing cells. The cumulus granulosa cells surround the oocyte to promote its growth and developmental competence. These two subpopulations of granulosa cells respond differently to gonadotropins but both are needed for successful ovulation.

Maturing antral follicles are major producers of sex steroid hormones in the female, and these hormones (namely estradiol) are required for further follicle development. Steroidogenesis requires both follicular somatic cell types and both gonadotropins from the anterior pituitary; thus, this process is called the two-cell, two-gonadotropin hypothesis (Fig. 3). Theca cells produce androgens under the control of LH signaling, but they lack the ability to synthesize estrogens, such as estradiol. Granulosa cells produce estradiol under the regulation of FSH signaling, but they lack the ability to synthesize androgens. Both of these cell types and gonadotropins are needed for ovarian steroidogenesis as theca cells provide precursor androgens, which are converted to estradiol in the granulosa cells by an enzyme called aromatase. Estradiol production is largely driven by FSH signaling, as FSH increases the levels of aromatase in granulosa cells. Although granulosa cells are initially devoid of LH receptors, both FSH and estradiol signaling lead to the acquisition of LH receptors on the granulosa cells, which is required for ovulation.

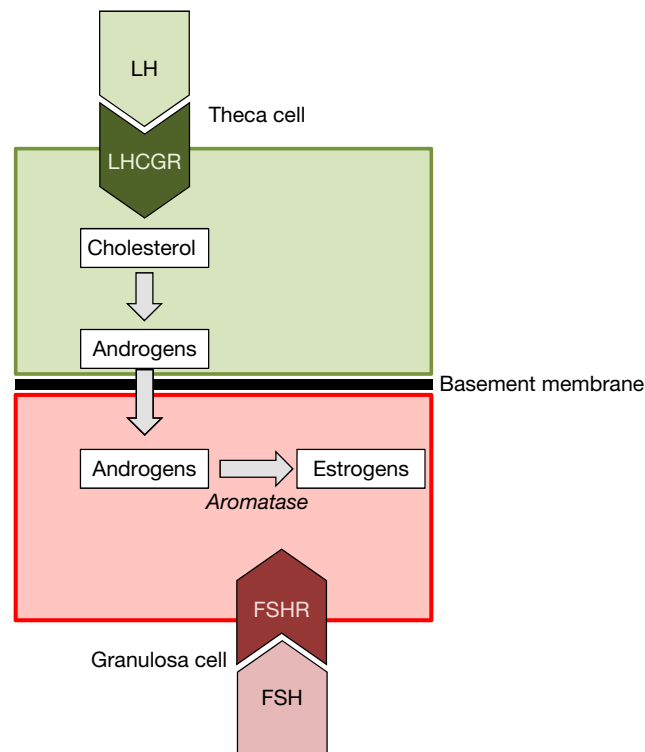


Fig. 3 The two-cell, two-gonadotropin hypothesis of ovarian steroidogenesis. Theca cells contain luteinizing hormone (LH) receptors (LHCGR). Under the action of LH signaling, cholesterol is converted to androgens via several enzymatic reactions in the theca cells. These androgens can diffuse through the basement membrane to the granulosa cells. Granulosa cells contain follicle-stimulating hormone (FSH) receptors (FSHR). Under the action of FSH signaling, aromatase is dramatically increased, which is the enzyme that converts androgens to estrogens, such as estradiol. Estradiol can then act within the follicle or can be released in circulation to act throughout the body. The theca cells lack the enzymes required to synthesize estradiol, and granulosa cells lack the enzymes to synthesize androgens. Thus, both cell types and gonadotropins are required for ovarian steroidogenesis.

As stated, cyclic recruitment corresponds to the cyclic hormonal patterns of the menstrual/estrous cycle and leads to the development of a dominant follicle(s), depending on the species, that will be selected to ovulate. Increasing FSH levels give rise to maturing antral follicles, and thus increasing estradiol levels. Insulin-like growth factors and activin A have also been shown to facilitate the increase in estradiol levels during this period. Estradiol, in turn, negatively feeds back to the hypothalamic-pituitary axis causing a decrease in FSH levels. This is also accomplished by the action of inhibin B, which is a granulosa cell-produced peptide hormone that inhibits the anterior pituitary from producing and secreting FSH. As this is occurring, a dominant preovulatory follicle(s) continues to grow and diverges from subordinate antral follicles within the cohort. Since FSH is the predominant survival factor for the antral follicle, a decrease in FSH leads to atresia of the subordinate follicles. The dominant follicle escapes this demise because it is more sensitive to and more efficiently utilizes gonadotropins, possibly due to increased granulosa cell numbers, FSH and LH receptors, and vasculature supply, thereby providing mechanisms to thrive in the dwindling FSH environment. As such, the dominant antral follicle selected to ovulate further enhances its dominance by creating a hormonal milieu that inhibits, both directly and indirectly, the development of subordinate antral follicles. This, accompanied by the increase in granulosa cell-derived LH receptors, ensures a fail-safe mechanism for ovulation.

The dominant follicle is now producing threshold levels of estradiol that positively feedback to the hypothalamic-pituitary axis initiating the ovulatory LH surge. The rapid rise in LH induces a cascade of events in the follicle, including meiotic resumption in the oocyte, cumulus granulosa cell expansion, breakdown of the follicular wall, and differentiation of follicular cells to luteal cells. These events lead to ovulation and the transformation of the follicle into the corpus luteum. The periovulatory period, or the time between the LH surge and oocyte release, varies across species from 12 to 18 h in rodents to 36 h in humans and nonhuman primates.

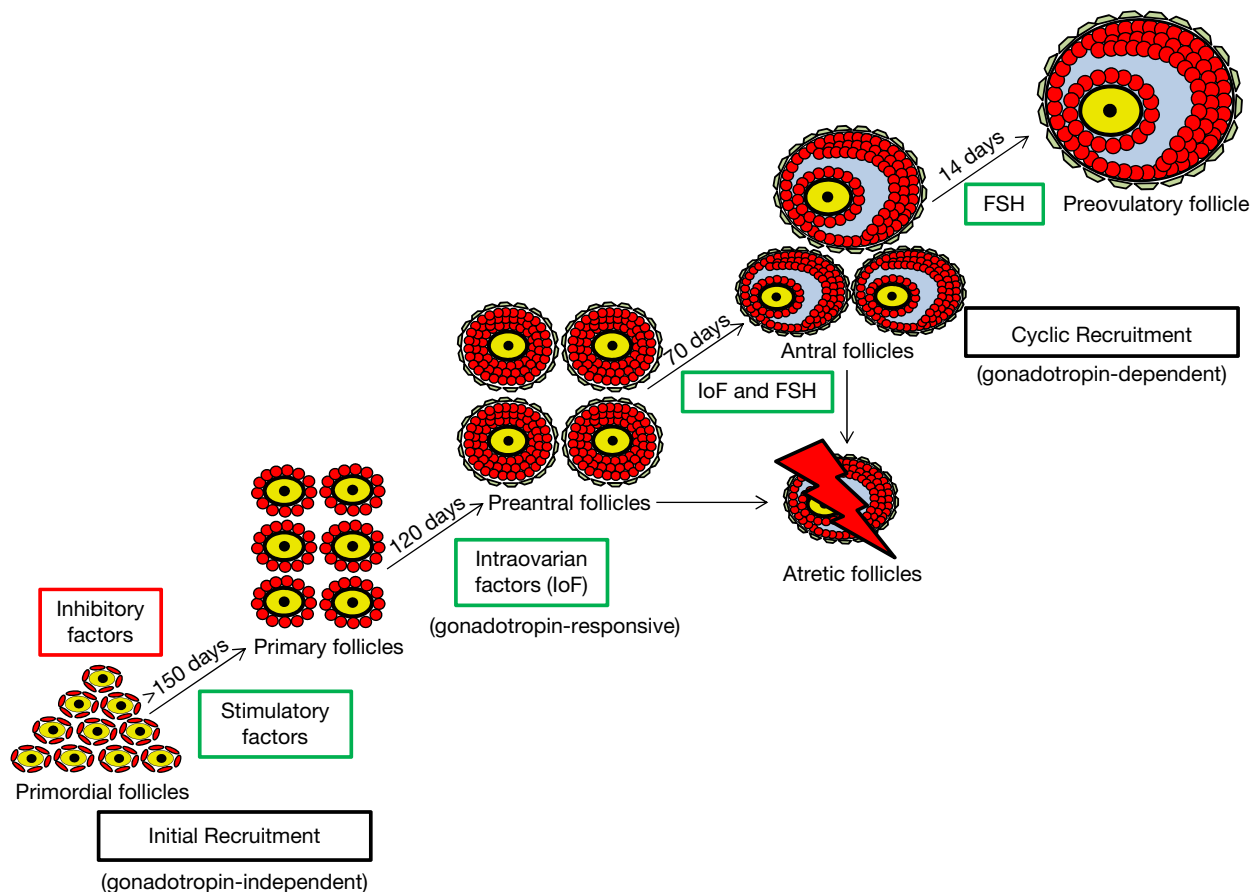


Fig. 4 Progression and regulation of ovarian folliculogenesis. The finite supply of primordial follicles remains quiescent under the action of intraovarian inhibitory factors. A decrease in these factors and/or an increase in intraovarian stimulatory factors leads to primordial follicle activation to the primary stage of development. This process is gonadotropin-independent and is known as initial recruitment. Primary follicles develop into preantral and early antral follicles via the action of intraovarian factors and gonadotropins. These follicles are the most susceptible to atresia, or follicle death. Select antral follicles are rescued from atresia by responding to the cyclic changes in FSH secretion, and they become preovulatory follicles that are capable of oocyte release and corpora lutea formation. This final stage of development is gonadotropin-dependent and is known as cyclic recruitment. Figure modified from (Hsueh, A. J. W., Kawamura, K., Cheng, Y., Fauser, B. C. J. M. (2015). Intraovarian control of early folliculogenesis. *Endocrine Reviews* 36(1), 1–24; (McGee, E. A. and Hsueh, A. J. W. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews* 21(2), 200–214.).

Conclusion

Folliculogenesis, from primordial follicle activation to the culmination of ovulation, is a complex and dynamic ovarian process involving multiple ovarian and endocrine cells and numerous signals that takes about 1 year in the human (Fig. 4). Folliculogenesis is essential for ovarian function and the propagation of the species because it allows for ovulation and the synthesis of the vital sex steroid hormones estradiol and progesterone. Thus, understanding how folliculogenesis is controlled and regulated is crucial for women's reproductive health. Primarily, this is because defects in folliculogenesis have been shown to cause infertility and POI. Infertility and POI negatively impact a woman's physical and mental state and has potential dire economic impacts. Elucidating the mechanisms that control all stages of folliculogenesis will greatly benefit patients that are diagnosed with ovarian-derived fertility defects. These include infertile women, women of advanced maternal age, women that are poor responders to conventional infertility treatments, women with a low ovarian reserve, prepubertal cancer patients undergoing chemotherapeutic treatments that inadvertently target and deplete the finite supply of primordial follicles, and women that have other ovarian disorders such as polycystic ovary syndrome. Therapeutic manipulations of folliculogenesis are being used as novel assisted reproductive technologies (ART) in these female patients. These include in vitro fertilization protocols, activation protocols for immature oocytes for in vitro fertilization, activation of immature follicles from cryopreserved ovarian tissues either in vitro or following transplantation, and bioengineering synthetic ovarian tissue.

Further Reading

- Adhikari, D., & Liu, K. (2009). Molecular mechanisms underlying the activation of mammalian primordial follicles. *Endocrine Reviews*, 30(5), 438–464.
- Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Human Reproduction Update*, 18(1), 73–91.
- Edson, M. A., Nagaraja, A. K., & Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine Reviews*, 30(6), 624–712.
- Fortune, J. E. (2003). The early stages of follicular development: Activation of primordial follicles and growth of preantral follicles. *Animal Reproduction Science*, 78, 135–163.
- Hannon, P. R., & Flaws, J. A. (2015). The effects of phthalates on the ovary. *Frontiers in Endocrinology*, 6, 8.
- Hsueh, A. J. W., Kawamura, K., Cheng, Y., & Fauser, B. C. J. M. (2015). Intraovarian control of early folliculogenesis. *Endocrine Reviews*, 36(1), 1–24.
- McGee, E. A., & Hsueh, A. J. W. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews*, 21(2), 200–214.
- Oktem, O., & Urman, B. (2010). Understanding follicle growth in vivo. *Human Reproduction*, 25(12), 2944–2954.
- Skinner, M. K. (2005). Regulation of primordial follicle assembly and development. *Human Reproduction Update*, 11(5), 461–471.

Antral Follicles: Recruitment and Selection of Ovulatory Follicles

Joanne E Fortune, Cornell University, Ithaca, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The pace of ovarian follicular development is extremely slow. For example, development from the primordial to the preovulatory stage is estimated to take about 6 weeks in rats, about 6 months in cattle, and up to a year in humans (reviewed in Fortune, 1994). Follicles spend most of that time traversing the preantral stages (primordial, primary, and secondary). When a certain number of granulosa cells layers have formed, fluid-filled spaces begin to appear within the granulosa cell/oocyte compartment of the follicle. The spaces gradually enlarge and coalesce into the follicular antrum. The granulosa cells differentiate into mural granulosa cells, lining the basement membrane and cumulus granulosa cells, which surround the oocyte and develop special structural and functional relationships with it (Fig. 1). Mammalian follicles become antral at a diameter of 0.2–0.4 mm.

The mechanisms of antrum formation and the role of the antrum and its follicular fluid are far from completely understood (see reviews by Rodgers and Irving-Rodgers, 2010; Hirshfield, 1991). No capillaries penetrate the basement membrane and it has been hypothesized that formation and progressive enlargement of the antrum serves to keep all the mural granulosa cells close to the vascularized thecal compartment of the follicle as the number of granulosa cells increases and thereby, facilitates their access to components of blood such as oxygen, nutrients, and hormones. In species like mice and rats, which ovulate when follicles are about 0.5 or 1 mm in diameter, respectively, follicular growth is almost complete when the antrum forms and the oocyte has completed its development and can resume meiosis if it is released from the follicle. In other species, considerable increases in size occur during the antral phase of follicular development to produce much larger ovulatory follicles (e.g., ~15 mm in humans and cattle, ~7–8 mm in sheep and pigs, 40–45 mm in horses) and oocyte development may continue during the early antral phase. Fig. 1 represents a small antral follicle; in species with large ovulatory follicles, the follicular fluid comprises a much larger percentage of the follicular volume than in small follicles. The follicular fluid provides the microenvironment of the granulosa cells and oocyte and it comprises an increasingly greater proportion of the follicular volume as follicular diameter increases. In some ways its composition is similar to blood plasma, but some components of the follicular fluid are present in dramatically different concentrations vs. their concentrations in the general circulation, especially in preovulatory follicles. Thus, the granulosa cells and oocyte can be regulated both by compounds in the general circulation that diffuse across the basement membrane from the thecal layer and also by components of the follicular fluid, i.e., by their microenvironment.

The “job” of antral follicles is (1) to nurture the oocyte and maintain or facilitate its meiotic and developmental capacities and (2) to grow to a size with enough theca and granulosa cells to produce sufficient androgen precursor (theca) to enable granulosa cells to secrete enough estradiol to trigger the surge release of gonadotropins from the anterior pituitary gland and thereby stimulate ovulation. Many follicles enter the antral stage of development, but only a very few complete it by ovulating. Although atresia (programmed death) of follicles occurs at every stage of follicular development, atresia does not occur evenly across the follicular stages. Analysis of patterns of atresia in several species has revealed that the rate of atresia is highest late in antral follicular development. A careful analysis by Hirshfield (1991) of the course of follicular development in rats revealed that atresia is most prevalent during the “penultimate” stage of follicular development, i.e., the stage just before they might enter the pool of potentially ovulatory follicles (Fig. 2). A study by Gougeon of the course of human follicular development and data for follicular development in cattle showed that in both species the highest percentages of atretic follicles are observed late in follicular development (~4–10 mm diameter) (reviewed in Fortune, 1994).

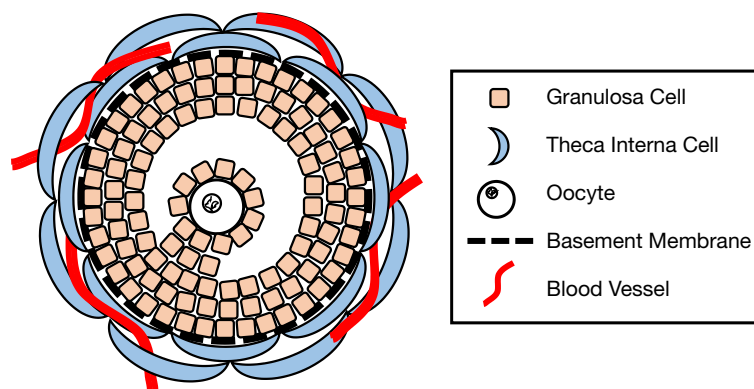


Fig. 1 Schematic diagram of a cross section through a mammalian antral follicle, showing the oocyte and surrounding cumulus cells, follicular antrum, granulosa cells, theca cells, and thecal blood vessels. The size of preovulatory follicles varies greatly in different mammalian species (from ~0.5 to ~45 mm in diameter). The antrum comprises an increasingly greater proportion of the follicle as follicular size increases.

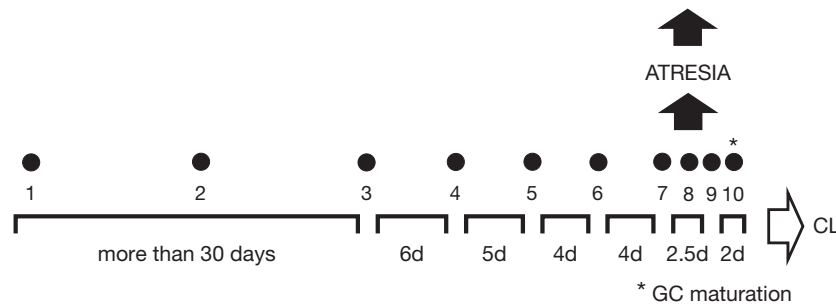


Fig. 2 Schematic representation of the temporal progression of follicular growth in rats, showing that the initial stages of follicular development occur much more slowly than the final stages and that most atresia occurs during a few granulosa cell generations. The numbers and filled circles above the line indicate granulosa cell generations (time required for doubling of granulosa cells in the largest cross section through the follicle). GC, granulosa cell; CL, corpus luteum. Reprinted, with permission, from Fortune, J.E. (1994). Ovarian follicular growth and development in mammals. *Biology of Reproduction* 50, 225–232; figure provided by Dr. A.N. Hirshfield.

Taken together, these observations suggest that many antral follicles reach a particular, species-specific size at which atresia is their normal fate. It is as though they run into a brick wall or reach a closed door and can develop no further. In more scientific terms, it seems likely that basal hormonal conditions (gonadotropin concentrations) are sufficient to sustain follicular growth to a certain, species-specific size and in the absence of some additional endocrine signal, regression ensues. This article is focused on the patterns of development of follicles that survive beyond this point of natural follicular death and the mechanisms that allow some follicles to develop beyond the usual end of follicular life and even, in some cases, to ovulate. During reproductive cycles one or more groups (depending on the species) of antral follicles are recruited to grow larger than most antral follicles and then, from the follicles of the recruited cohorts, a species-specific number of follicles is selected for further development and potential for ovulation. The patterns of follicular development in various species and the mechanisms that subserve follicular recruitment and selection for dominance are reviewed in this article. This topic is of practical interest because understanding these mechanisms has been (and will be) important for the development of interventions to subvert the natural regulatory mechanisms, such as superovulation of domestic animals and hormonal stimulation of women prior to in vitro fertilization.

Patterns of Follicular Development and Regression

Follicular development is by its nature a dynamic process, but careful histological analysis can reveal temporal patterns and in some species serial rectal or vaginal palpation of the ovaries can detect changes in large structures over time (large follicles or corpora lutea). A more direct tool for assessing changes in ovarian structures over time, ovarian ultrasonography, became available in the 1980's. In species with fairly large ovulatory follicles, the final stages of follicular development have been visualized by trans-rectal, trans-vaginal, and/or trans-abdominal ultrasonography. The fluid-filled follicular antrum absorbs ultrasound waves and appears as a black spherical space which can be measured to estimate follicular diameter. In contrast to histology, ultrasonography allows repeated visualization of individual follicles in the same animal (or person) over time and their growth and/or demise can thus be followed.

Studies of the dynamics of antral follicular development by histology or ultrasonography revealed that follicles above a certain species-specific size (the size when follicles naturally die) do not appear at random during reproductive cycles. Rather there are specific times when a group of follicles contemporaneously grows larger than normal. This is referred to as "follicular recruitment" or sometimes as "cyclic recruitment" by authors who consider the activation of primordial follicles to begin growth as "initial recruitment". Recruitment of follicular cohorts or "waves" is most obvious in species with large preovulatory follicles, where individual follicles can be followed over time with ultrasonography (cattle and other large ruminants, horses, humans), but it can be deduced by careful histological analyses of the ovaries of species with smaller preovulatory follicles.

These studies have revealed several different patterns. Cattle have been studied extensively because the large size of their ovulatory follicles, the position of their ovaries within the abdominal cavity, and their availability make them excellent candidates for serial transrectal ultrasonography. Ultrasonography revealed that large, ovulatory-size follicles do not appear continuously or at random during the bovine estrous cycle (as some researchers had hypothesized), but rather cohorts of follicles are recruited to grow larger than normal two or three times during the cycle, about every 7–10 days (reviewed in Fortune, 1994; Ginther et al., 2001). The follicles in each wave increase in size for several days and then one follicle is "selected" for further growth (the dominant follicle), whereas the other (subordinate) follicles stop growing and begin to regress. The dominant follicle of the final wave of the cycle (second or third) ovulates (Fig. 3A). It is interesting that similar, but always nonovulatory, follicular waves occur in heifers during the prepubertal period and during pregnancy (reviewed in Fortune, 1994). The wave-like pattern seems to be inherent to the bovine ovary after a certain age. Sheep display a similar pattern of multiple waves throughout the cycle, but they are less regular than in cattle; there can be 2–6 follicular waves per cycle and ovulation of the dominant follicles of both the final and penultimate waves has been observed (reviewed in Goodman and Inskeep, 2015; Scaramuzzi et al., 2011). It is more difficult to study follicular

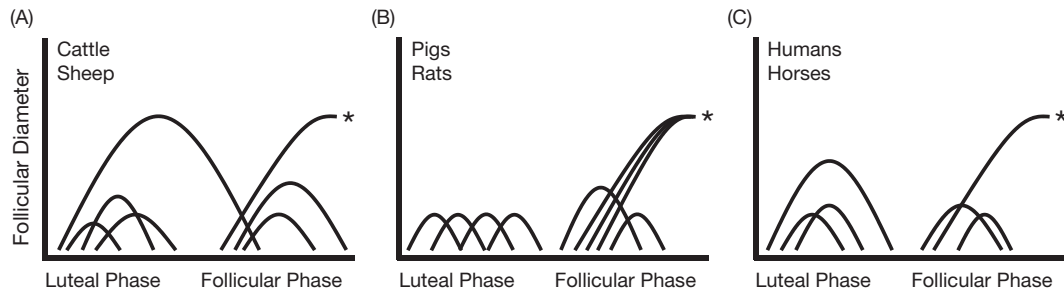


Fig. 3 Schematic diagrams of three patterns of follicular development during mammalian reproductive cycles. The *lines* represent changes in follicular diameter as follicles grow or regress; the asterisks denote ovulation. Panel A depicts a pattern observed in cattle and sheep, in which dominant, ovulatory-size follicles develop throughout the estrous cycle. Panel B shows a pattern typical of species such as rats and pigs, with dominant follicles developing only during the follicular phase. Panel C shows a pattern observed in horses and humans, with a minor wave of follicular development (dominant follicle smaller than ovulatory size) during the luteal phase.

dynamics in litter-bearing species like rodents and pigs, but it appears that follicles are recruited for potential ovulation during the follicular phase and then a subset of the recruited follicles is selected for dominance and ovulation (**Fig. 3B**) (reviewed in [Fortune, 1994](#); [Guthrie, 2005](#); [Soede et al., 2011](#)).

Initially, it was reported that ovulatory follicles of humans and nonhuman primates such as rhesus monkeys develop during a single wave of follicular recruitment and selection during the follicular phase of the cycle (reviewed in [Fortune, 1994](#)). More recent and very careful ultrasonographic analyses of follicular development during human menstrual cycles revealed the presence of one or two “minor” waves of follicular development during the luteal phase in most women studied and an occasional minor wave during the follicular phase (in women with long follicular phases) (**Fig. 3C**) (reviewed in [Baerwald et al., 2012](#)). The dominant follicle of the minor waves is generally smaller than the preovulatory follicle that develops during the ovulatory wave of the follicular phase (**Fig. 3C**). Mares have a similar pattern of follicular development, with some, but not all, mares exhibiting a wave of follicular development during the luteal phase with a dominant follicle smaller than ovulatory size (a minor wave) (reviewed in [Ginther et al., 2001](#); [Baerwald et al., 2012](#)). It is not clear whether nonhuman primates also have minor waves; their follicles are smaller than human follicles and thus much harder to visualize by ultrasonography. The mechanisms that regulate these follicular patterns are discussed in the sections below.

Recruitment of Follicles Into Follicular Waves (Cohorts)

A common pattern of recruitment of a wave or cohort of follicles at a species-specific diameter has been documented, as discussed above. But what causes groups of follicles to grow synchronously beyond the stage when most follicles become atretic and why do some species have several waves of follicular development per reproductive cycle, whereas only one cohort is recruited in others? In species in which this question has been examined carefully, a small rise in circulating FSH accompanies the initiation of each follicular wave (reviewed in [Baerwald et al., 2012](#); [Fortune et al., 2001](#); [Fortune, 1994](#); [Ginther et al., 2001](#); [Mihm and Evans, 2008](#)). In primates and pigs, circulating FSH is slightly higher at the beginning of the follicular phase than during the mid-luteal or mid to late follicular phases. Rats have a secondary surge of FSH on the day of estrus, preceding recruitment of the next cohort of ovulatory follicles. Cattle also exhibit a secondary surge of FSH around the time that the first follicular wave of the cycle begins and small rises in FSH were detected just prior to the later follicular waves of the estrous cycle. It is not clear if the minor waves during the luteal phase in women are preceded by an increase in FSH. The differing patterns of follicular wave dynamics during the luteal phase in different species may be due to the extent to which their corpora lutea secrete feedback inhibitors of FSH (estradiol, inhibin) and/or to differing sensitivities to such feedback.

The temporal relationships between increases in circulating FSH and the initiation of follicular waves that have been documented in a number of species suggest that the two events are causally related (see **Fig. 4**). Indeed, the hypothesis of a cause and effect relationship is supported by the results of experiments in which the elevation in FSH was suppressed, mimicked, or enhanced. For example, in cattle a secondary surge of FSH occurs about 1 day after the LH/FSH surge and injection of bovine follicular fluid (containing inhibin which suppresses FSH) delayed the first wave of follicular development for the duration of the suppression of FSH. Similar experiments with rats provided evidence that the secondary surge of FSH in that species prevents the recruitment of the next cohort of ovulatory follicles. When LH was held constant in cynomolgus monkeys and plasma FSH was gradually increased in a manner designed to mimic the early follicular phase, increases in circulating estradiol provided evidence that the small increase in FSH had allowed follicles to develop beyond the stage when they would normally become atretic ([Zelevnik and Kubik, 1986](#)). In addition, treating animals or women with exogenous FSH increases the number of follicles recruited and has been used to superovulate domestic animals and promote the development of multiple ovulatory follicles in women. The critical role of FSH in recruitment of follicles into waves appears to be well established and has been reviewed in more detail by [Baerwald et al. \(2012\)](#), [Fortune et al. \(2001\)](#), [Fortune \(1994\)](#), and [Ginther et al. \(2001\)](#).

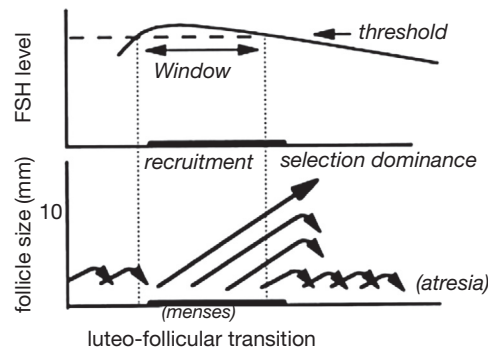


Fig. 4 FSH threshold/window concept. Schematic depiction of the intercycle rise in FSH during the human menstrual cycle that recruits a cohort of follicles for growth during the early follicular phase. The number of follicles recruited and selected for dominance depends on width of the “window,” the time when FSH is elevated above the threshold level. Figure modified from Macklon, N.S. and Fauser, B.C. (1998). Follicle development during the normal menstrual cycle. *Maturitas* 30(2), 181–188 with permission from Elsevier.

Selection of Dominant (Ovulatory) Follicles

Introduction

The number of follicles recruited into waves is typically greater than the number of follicles that ovulate. During waves of follicular development only some of the recruited follicles (a species-specific number) continue growth towards ovulatory status (Fig. 3). How the dominant follicle(s) is selected for dominance, exerts and maintains dominance, and loses dominance (in species with multiple follicular waves) has been the focus of numerous studies. The key characteristic of dominant follicles is their greater capacity to secrete estradiol, compared with their sister subordinate follicles. Secretion of increasingly greater quantities of estradiol is the hallmark of preovulatory follicles. Dominant follicles already have a higher capacity for estradiol production at the time when their growth trajectory diverges from that of the subordinate follicles (reviewed in Fortune et al., 2001; Ginther et al., 2001) and estradiol is a negative feedback regulator of FSH. This suggests that they may “beat back” the subordinate follicles by reducing FSH to levels insufficient to sustain continued growth of the subordinate follicles. However, this hypothesis assumes that dominant follicles can continue their development with levels of circulating FSH insufficient for growth of subordinate follicles. The experiments with cynomolgus monkeys mentioned above showed that estradiol continued to increase after the level of circulating FSH was progressively reduced (Zelevnik and Kubik, 1986), which implies that once dominant follicles have been selected they can continue to develop and secrete estradiol in an environment of lower FSH.

Cattle have been a useful model in efforts to understand how the dominant follicle is selected and they provide an excellent animal model for human follicular development (Campbell et al., 2003). They are more available for experimental manipulation *in vivo* than nonhuman primates and their dominant and subordinate follicles are large enough to be visualized by ultrasonography and to be removed for analysis of follicular characteristics (follicular fluid hormones, levels of mRNA in follicular cells, etc.) and/or for culture of follicular cells. The dominant follicle of bovine nonovulatory waves can ovulate if the corpus luteum is regressed by injection of prostaglandin F_{2α} (PGF_{2α}) during the follicle’s tenure as dominant follicle (Fortune et al., 1991), indicating that all dominant follicles of the cycle have full developmental potential if the right endocrine environment is present. The first wave of follicular development of the bovine estrous cycle is a particularly useful model because it begins at a predictable time of the cycle, i.e., following ovulation of the previous dominant follicle. Hence much of the literature reviewed below is based on studies with cattle.

Characteristics of Dominant Follicles

At first, attempts to understand how follicles are selected in cattle were focused on describing the characteristics of dominant vs. subordinate follicles at various stages of the first follicular wave (reviewed in Fortune et al., 2001; Ginther et al., 2001). Estradiol concentrations in the follicular fluid and estradiol secretion by follicular wall tissue were the only characteristics examined that were different (higher) in the dominant follicle than in the largest subordinate follicle at a time when the dominant follicle had just reached a significantly larger diameter. It makes sense that estradiol production is a key characteristic of emerging dominant follicles because estradiol can exert negative feedback on FSH secretion by the anterior pituitary, depriving the subordinate follicles of sufficient FSH for their further growth and development.

Ovarian follicles produce estradiol via interactions between their two endocrine cell types, the inner granulosa cells, which line the antrum of the follicle, and the outer theca cells, which lie outside the basement membrane between the theca and granulosa cells (Fig. 1). From very early stages of follicular development, theca cells have LH receptors and granulosa cells have FSH receptors. Theca cells are stimulated by LH to convert cholesterol to progesterins and hence to androgens, but they lack the aromatase enzyme necessary to convert androgens to estrogens (estradiol and estrone). The granulosa cells cannot synthesize androgens, but they can convert androgens that diffuse from the theca across the basement membrane to estrogens, primarily in the form of estradiol.

Granulosa cells also acquire LH receptors as follicles develop towards ovulation. Comparisons of levels of mRNA for LH receptor, FSH receptor, aromatase, and P450 17 α -hydroxylase in follicular cells of the dominant vs. the largest subordinate follicle revealed that they were all higher in the dominant follicle, but not until a day after the difference in estradiol was detected. Therefore, a number of differences were detected, but all of them followed selection for dominance (Fortune et al., 2001). However, it seems logical that biochemical/molecular changes in the future dominant follicle, before it can be detected as significantly larger in diameter, allow it to grow a little faster and to begin to make more estradiol than its sister subordinate follicles and thus achieve morphological (size) dominance.

Role of the Insulin-Like Growth Factor (IGF) System in Selection of Follicles for Dominance

Studies of the characteristics of dominant follicles summarized above revealed that they have a greater capacity to produce estradiol when they are first detected as significantly larger in diameter than other follicles of the wave. Mechanisms that enable this capacity must precede, and facilitate, their selection for dominance. There is evidence for cattle that changes in the insulin-like growth factor (IGF) system in the future dominant follicle (i.e., before divergence in follicular size) enable increased production of estradiol (reviewed in Ginther et al., 2001; Fortune et al., 2001; Mihm and Evans, 2008; Spicer, 2004). The IGF system comprises two ligands (IGF-I and -II), two receptors (types 1 and 2), and six IGF binding proteins (IGFBP-1 through -6). In mice, IGF is essential for follicular development beyond the late preantral/early antral stage and numerous effects of IGF on follicular function have been documented. Effects of IGF on follicle cells are inhibited by low molecular weight IGFBPs (-2, -4, and -5) because they bind IGF and prevent it from binding to its receptor. Specific IGFBP proteases can cleave IGFBPs, thus freeing IGF to bind to its receptor.

In bovine follicles, FSH induces the IGFBP-4/-5 protease PAPP-A (pregnancy-associated plasma protein A), suggesting that changes in PAPP-A might be important in selection of follicles for dominance. To test that hypothesis, follicles of the first follicular wave of the bovine estrous cycle were isolated before and after the dominant follicle could be detected by a larger diameter; the follicular fluid was collected and measured for concentrations of estradiol and free IGF-1 and abundance of IGFBP-2, -4, and -5 and PAPP-A (reviewed in Fortune et al., 2004). The results supported the conclusion that, of the follicular components examined, an increase in PAPP-A was the earliest change, followed by a decrease in IGFBP-4 and -5 and an increase in free IGF-1, and then an increase in estradiol (Fig. 5). Thus, biochemical changes were detected in one follicle of the cohorts before size deviation, suggesting that the dominant follicle is selected biochemically (functionally) prior to size deviation. Since IGF synergizes with FSH to increase estradiol production, the increase in free IGF (due to the increase in PAPP-A) in one follicle of the wave would allow that follicle to ramp up its production of estradiol very quickly, rapidly suppressing basal FSH to levels insufficient to support similar developmental changes in other follicles of the cohort. This postulated involvement of the IGF system in selection for dominance is further supported by experiments in vivo showing that increasing circulating FSH slightly promotes the development of co-dominant follicles, both of them with higher PAPP-A than in subordinates. In addition, a low level of IGFBP-4 in the follicular fluid of a follicle in a recruited wave, prior to morphological selection of the dominant follicle, was more predictive of future dominance than follicular diameter or concentration of estradiol in the follicular fluid. Further details about these studies in vivo can be found in Fortune et al. (2001, 2004).

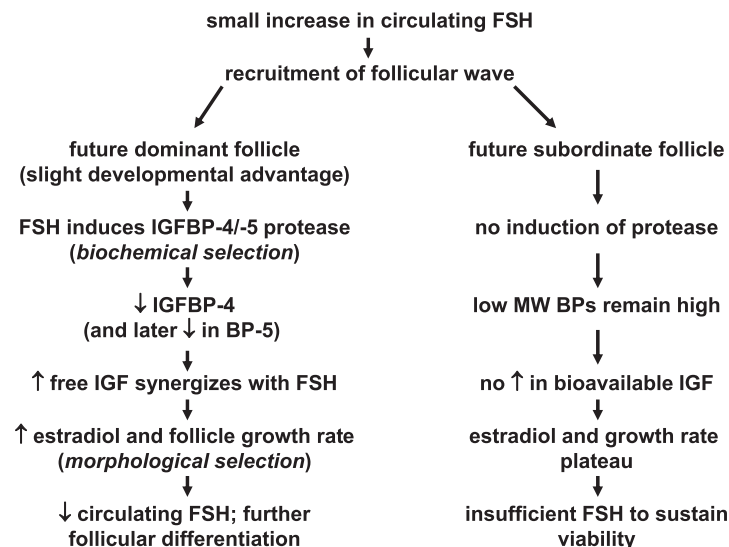


Fig. 5 Hypothesized sequence of events during selection of the dominant follicle in cattle. This model suggests that a critical event of “biochemical selection” is the induction by FSH of a protease for IGFBP-4/–5 in one follicle of a cohort and that biochemical selection quickly leads to “morphological selection” and further differentiation of the dominant follicle. Taken from Fortune, J.E., Rivera, G.M., Evans, A.C.O., and Turzillo, A.M. (2001). Differentiation of dominant vs. subordinate follicles in cattle. *Biology of Reproduction* 65, 648–654, with permission.

These results provide an example of how a biochemical change (increase in PAPP-A) can precede and lead to morphological (size) dominance. There is also extensive evidence for changes in components of the IGF system, including PAPP-A, during development of dominant follicles in a number of mammalian species besides cattle, including pigs, sheep, horses, and humans (see review by Spicer, 2004). There may well be other changes/mechanisms besides changes in the IGF system that serve to promote selection of follicles for dominance before size deviation by promoting the same end result of increased capacity for estradiol synthesis (Mihm and Evans, 2008). Different species may have somewhat different mechanisms and it would not be surprising if there were redundant mechanisms because selection of a species-specific number of dominant follicles is so important for reproductive success of females.

Development of Preovulatory Follicles

In species with multiple waves of follicular development, it is useful to distinguish morphological dominance from one aspect of functional dominance, the ability to suppress new follicular waves. Nonovulatory dominant follicles lose functional dominance before they lose morphological dominance. In nonovulatory waves in cattle, the growth of the dominant follicle plateaus for several days before a decrease in diameter (regression) is observed. Early in the plateau phase, the dominant follicle can resume growth if the corpus luteum is regressed with PGF2 α , but a day or two later it cannot and the dominant follicle of the new wave that has just emerged will ovulate in response to luteolysis (reviewed in Fortune et al., 1991).

In species with multiple waves of follicular development, the follicle that is dominant at the time of luteal regression transitions to a new phase of development; it continues to grow and to increase its capacity to produce estradiol. This is stimulated by the decrease in circulating progesterone during luteal regression, which causes an increase in basal levels of LH. In sheep and cattle the increase is manifested as an increase in LH pulse frequency once the negative feedback effects of luteal progesterone are removed. This change, coupled with the lower levels of circulating FSH, is sometimes described as a shift in the follicle from FSH-dependence to LH-dependence. Although the estradiol made by the dominant follicle decreases basal FSH, there is evidence that dominant follicles are still dependent on those normal basal levels and undergo regression if FSH is depressed further (reviewed in Fortune, 1994). However, the increase in basal LH during the latter part of the follicular phase seems critically important to convert the dominant follicle into a preovulatory follicle, capable of secreting quantities of estradiol sufficient to elicit the gonadotropin surge. Although the granulosa cells acquire LH receptors as the ovulatory follicle develops, it is not clear that LH directly increases the follicle's ability to make estradiol via effects on granulosa cell aromatization of androgen to estradiol (and some evidence against this). However, it may do so indirectly by increasing production of progestins by granulosa cells for conversion to thecal androgens. It seems possible that the major effect of the increased circulating LH is on the theca cells, to increase their production of androgen precursor for conversion to estradiol by granulosa cells in which aromatase has been induced and is maintained by FSH (Hampton et al., 2004).

Periovulatory Follicles

The preovulatory surge of LH and FSH is induced when the feedback of estradiol on the hypothalamus/pituitary changes from negative to positive, as circulating estradiol increases beyond a species-specific threshold. The gonadotropin surge converts preovulatory follicles into periovulatory follicles; a complex cascade of changes is triggered by the surge, causing profound morphological and functional changes in the follicles. The changes induced by the surge lead to meiotic maturation of the oocyte, cumulus expansion, and the structural and functional changes that produce rupture at the apex of the follicle. Androgen and estradiol secretion by the follicle cells decrease rapidly and progesterone levels increase, as the follicle enters the initial stages of luteinization. Detailed consideration of the events of the periovulatory phase of antral follicle development is beyond the scope of this article.

Summary

Mammalian follicles begin to form an antral cavity when they are 0.2–0.4 mm in diameter. Once they reach a particular, species-specific size, they either become atretic or are recruited by a small increase in circulating FSH to grow larger. From each recruited wave of follicles, a species-specific number is selected for dominance (i.e., further growth and potential ovulation), whereas the remaining, subordinate follicles gradually regress. Waves of follicular development occur once or more than once during estrous and menstrual cycles, depending on the species. Dominant follicles are characterized by a greater capacity to produce estradiol and by the appearance of LH receptors on their granulosa cells. There is evidence that changes in the IGF system within the follicle precede and facilitate the increase in estradiol production by dominant follicles. The increase in estradiol terminates the small rise in FSH that initiated the waves by negative feedback, thus depriving the subordinate follicles of the FSH they need to develop further. As dominant follicles develop towards ovulation, the increasing levels of estradiol in the circulation induce the gonadotropin surge that causes meiotic maturation of the oocyte and rupture of the follicle (ovulation).

Acknowledgments

The author is grateful to Dr. Jeremy J. Allen for preparing the figures and providing feedback on the manuscript.

References

- Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Human Reproduction Update*, 18(1), 73–91.
- Campbell, B. K., Souza, C., Gong, J., et al. (2003). Domestic ruminants as models for the elucidation of the mechanisms controlling ovarian follicle development in humans. *Reproduction Supplement*, 61, 429–443.
- Fortune, J. E. (1994). Ovarian follicular growth and development in mammals. *Biology of Reproduction*, 50, 225–232.
- Fortune, J. E., Sirois, J., Turzillo, A. M., & Lavoie, M. (1991). Follicle selection in domestic ruminants. *Journal of Reproduction and Fertility. Supplement*, 43, 187–198.
- Fortune, J. E., Rivera, G. M., Evans, A. C. O., & Turzillo, A. M. (2001). Differentiation of dominant vs. subordinate follicles in cattle. *Biology of Reproduction*, 65, 648–654.
- Fortune, J. E., Rivera, G. M., & Yang, M. Y. (2004). Follicular development: The role of the follicular microenvironment in selection of the dominant follicle. *Animal Reproduction Science*, 82–83, 109–126.
- Ginther, O. J., Beg, M. A., Bergfelt, D. R., Donadeu, F. X., & Knot, K. (2001). Follicle selection in monovular species. *Biology of Reproduction*, 65, 638–647.
- Goodman, R. L., & Inskeep, E. K. (2015). Control of the ovarian cycle of the sheep. In T. Plant, & A. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn., pp. 1259–1305). San Diego, CA: Elsevier Inc.
- Guthrie, H. D. (2005). The follicular phase in pigs: Follicle populations, circulating hormones, follicle factors and oocytes. *Journal of Animal Science*, 83(E. Supplement), E79–89.
- Hampton, J. H., Bader, J. F., Lamberson, W. R., et al. (2004). Gonadotropin requirements for dominant follicle selection in GnRH agonist-treated cows. *Reproduction*, 127(6), 695–703.
- Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. *International Review of Cytology*, 124, 43–101.
- Mihm, M., & Evans, A. C. O. (2008). Mechanisms for dominant follicle selection in monovulatory species: A comparison of morphological, endocrine, and intraovarian events in cows, mares, and women. *Reproduction in Domestic Animals*, 43(Suppl. 2), 48–56.
- Rodgers, R. J., & Irving-Rodgers, H. F. (2010). Formation of the ovarian follicular antrum and follicular fluid. *Biology of Reproduction*, 82, 1021–1029.
- Scaramuzzi, R. J., Baird, D. T., Campbell, B. K., et al. (2011). Regulation of folliculogenesis and the determination of ovulation rate in ruminants. *Reproduction, Fertility, and Development*, 23, 444–467.
- Soede, N. M., Langendijk, P., & Kemp, B. (2011). Reproductive cycles in pigs. *Animal Reproduction Science*, 124, 251–258.
- Spicer, L. J. (2004). Proteolytic degradation of insulin-like growth factor binding proteins by ovarian follicles: A control mechanism for selection of dominant follicles. *Biology of Reproduction*, 70(5), 1223–1230.
- Zeleznik, A. J., & Kubik, C. J. (1986). Ovarian responses in macaques to pulsatile infusion of follicle-stimulating hormone (FSH) and luteinizing hormone: Increased sensitivity of the maturing follicle to FSH. *Endocrinology*, 119, 2025–2032.

Follicle Atresia

Elizabeth A McGee and Jessica Horne, University of Vermont Larner College of Medicine, Burlington, VT, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Atresia 1. Absence or closure of a natural passage of the body. 2. Absence or disappearance of an anatomical part (such as an ovarian follicle).

Bleb A vesicular outpocketing.

Caspase Any of several intracellular proteases that have a cysteine residue at their active site, that cleave substrate proteins at specific aspartic acid residues, and that are involved in the initiation and mediation of apoptosis.

Dictyate The dictyate or dictyotene is a prolonged resting phase in oogenesis. It occurs in the stage of meiotic prophase I in oogenesis. It starts late in fetal life and is terminated shortly before ovulation by the LH surge.

Graafian follicle The follicular stage after the first meiotic division but before ovulation containing a 2N haploid oocyte. It is characterized by a large follicular antrum that makes up most of the follicle.

Hypophysectomy The surgical removal of the pituitary gland.

Oogonia An immature female reproductive cell that gives rise to primary oocytes by mitosis.

Rete ovarii A transient network of cells in the developing ovary formed from the primary sex cords in females; homologous to the rete testis. It is a narrow hilus, at which nerves and vessels enter the ovary.

Introduction

Atresia in biology generally means the closing or failure to open of a tubular structure such as with vaginal atresia or esophageal atresia. However, follicle atresia has come to mean the failure of a follicle to develop to ovulate and release an egg. There is some disagreement on what stages of follicle loss are included when describing follicle atresia. Some scholars consider follicle atresia to have occurred only after follicles have grown to a certain size, but other scholars also include follicle or oocyte loss at any time after oogonia have migrated to the genital ridge as putative oocytes. In this article, we will discuss follicle loss over the span of follicle development.

There has been a great deal of research and advancement in the study of follicle loss over the preceding decades and much is known about the regulation of the growth and death of ovarian follicles in mammals, however little is known about why some follicles survive many years, even decades, while others are lost during development (McGee and Hsueh, 2000). A key advancement in understanding follicle atresia was the discovery that it is the result of the highly regulated process of apoptosis or programmed cell death. Apoptosis is a very important process in mammalian ovarian physiology. It is differentially regulated in different follicle stages and different ovarian cell types (Markstrom et al., 2002).

Apoptosis is a highly regulated process, important throughout development of virtually every system. It eliminates cells by enzymatic deconstruction and generally does not engage the inflammatory process. Apoptotic cells shrink, form blebs, and undergo patterned DNA fragmentation and membrane fragmentation into apoptotic bodies. Many of the events of apoptosis are measurable. Pro- and antiapoptotic genes are expressed and cell and follicle stage specific patterns and can be regulated by growth factors and cytokines and even small RNA. Apoptosis can be detected and quantified by numerous techniques such as those that measure DNA fragmentation, detect particular lipid patterns, gene expression analysis, and even morphological analysis (Elmore, 2007). These techniques and others have been used to characterize the role of apoptosis in ovarian function. The following sections will discuss what we know about apoptosis pathways and their regulation in ovarian tissues and follicles.

Pathways of Apoptosis

Intrinsic Pathway

There are at least two pathways that when activated lead to apoptotic death. They are often described as the intrinsic and extrinsic pathways in the classic canonical terminology (Fig. 1). The intrinsic pathway can be activated by cell stress events such as radiation, oxidative stress, or hormone withdrawal and may involve the activation of P53. The common feature is that small death molecules, either Bax or Bak, are activated resulting in the release of cytochrome *c* from the mitochondria. When cytochrome *c* is released into the cytoplasm it results in the activation of caspase 9 which then cleaves caspases 3, 6, 7 and initiating the caspase cascade.

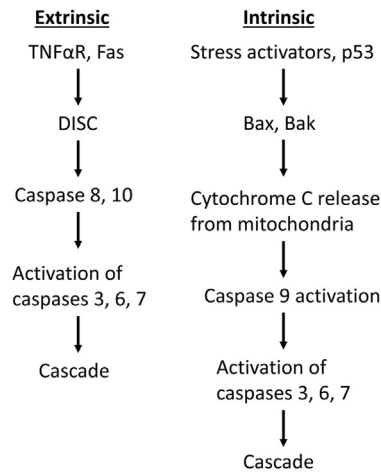


Fig. 1 A basic schematic comparison of the extrinsic and intrinsic pathways of apoptosis. *TNF α* , tissue necrosis factor alpha; *BAX*, Bcl-2-associated X protein; *BAK*, Bcl-2 homologous antagonist killer.

Extrinsic Pathway

In the extrinsic pathway of apoptosis, cell surface receptors are activated by cytokines or factors such as Fas ligand or *TNF α* . In this pathway, the death receptors (Fas or *TNF α* R) are activated and then activate a multi-protein death inducing signal complex (DISC). The assembled DISC apparatus can activate caspase 8 or caspase 10, which can in turn activate caspases 3, 6, and 7 and initiate the caspase cascade similarly to the intrinsic pathway.

Caspase Cascade

Caspases are the enzymatic engine of apoptosis. These protein cleaving enzymes affect their cut at locations adjacent to aspartic acid residues. Caspases can cut a large variety of proteins that are essential to maintaining cellular function. Caspases cleave and activate the endonuclease that causes the fragmentation of DNA into the characteristic sizes as well as cleaving other proteins with additional downstream effects. The collective action of the mass cleavage of cellular and structural proteins defines the features of apoptosis and results in dissolution and ultimately phagocytosis of the cell.

The process of apoptosis can be regulated by factors that either promote (proapoptotic) or prevent (antiapoptotic) the activation of the pathways of apoptosis.

Bcl-2 is a molecule that acts to block the actions of the death initiating proteins (Bad, Bak, and Bok). Factors that up regulate Bcl-2 are generally survival factors. Factors that decrease Bcl-2 or increase the expression of death proteins increase apoptosis. Individual cell life or death is often based on the balance of pro- or anti-factors that either push the cell into the caspase cascade or block progression towards cascade activation. The extrinsic pathway can be regulated by proteins such as c-FLIP that bind or block the death domain in the receptor or associated adaptor proteins that can then prevent the formation of the DISC (Hussein, 2005).

Ovarian Formation and Folliculogenesis

Germ cells migrate from the yolk sac to the genital ridge as oogonia. They become organized into cords within the embryonic ovary, and the putative pregranulosa cells migrate into the cords around the oogonia, thus forming the primordial follicles. As the somatic granulosa cells separate an oogonia from others in the cord, it becomes an oocyte within a primordial follicle. Primordial follicles can remain relatively inert in mammals for many years (up to 5 decades in humans), though some will begin to grow almost immediately after formation. Germ cells that do not become invested with a ring of primordial granulosa cells undergo apoptosis.

The regulation of follicular growth is complex and stage specific. Follicles progress from primordial to primary, multilaminar preantral, early antral, antral, and ultimately a few survivors become a Graafian follicle. Graafian follicles may ovulate, releasing an egg and then transforming into a corpus luteum (Fig. 2). If pregnancy does not occur, the corpus luteum itself regresses into a corpus atretica. Less than 0.01% of human primordial follicles will ever ovulate. The fate of the vast majority of follicles is atresia.

The entire population of follicles is generally thought to be created early in mammalian life; in utero in humans and just after birth in rodents. The first wave of follicles begins to grow just after formation, though these early follicles do not make it to ovulation. Only after puberty does the pituitary secrete gonadotropins in the patterns necessary to support the growth of a dominant follicle and its ovulation (McGee and Hsueh, 2000).

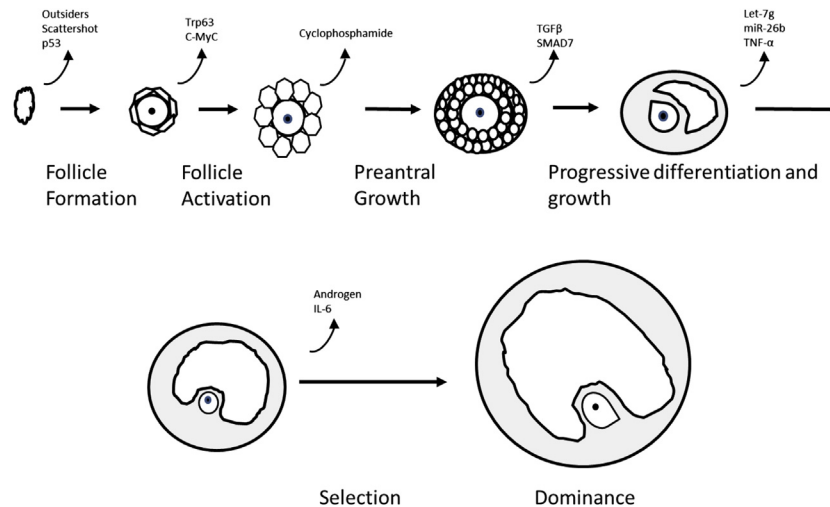


Fig. 2 A schematic of the progression of follicle development. The *curved arrows* represent the loss of follicles to apoptosis at the various stages labeled with factors or miRNAs that promote atresia/apoptosis at that stage.

Atresia Across Early Folliculogenesis

Migration and Loss or Survival

Migration of germ cells from the yolk sac endoderm to the genital ridge is a critical event of gonad formation. Interaction of the germ cells with coelomic epithelium and the mesonephric tissue are required for formation of the ovary. However the germ cells that do not complete the journey to the gonadal ridge generally do not survive and are lost to apoptosis.

Though the process has not yet been clearly characterized in humans, there are genes expressed in both drosophila and mice that are involved in apoptosis of errant germ cells. *Scattershot* and *outsiders* both can cause apoptosis of germ cells that do not make it to the gonadal ridge. The regulation of the removal of ectopic germ cells is not yet fully understood. It is thought that germ cells that find a niche in which prosurvival pathway activation can occur may elude the usual fate of apoptosis and survive to become germ cell tumors.

After Arrival at the Gonad

Once the oogonia reach the genital ridge they must continue to proliferate but also avoid apoptosis. Oogonia that become invested with a layer of pregranulosa cells from the cords derived from the rete ovarii become primordial follicles, whereas oogonia that are not incorporated into follicles, ultimately undergo apoptosis (McGee et al., 1998). In mice and rats, the primordial follicle units begin assembly in the first day after birth. Most investigators think that the oogonia that do not enter meiosis and become oocytes in primordial follicles are lost to apoptosis. During this time there is an increased rate of apoptosis as shown in the DNA fragmentation patterns in Fig. 3.

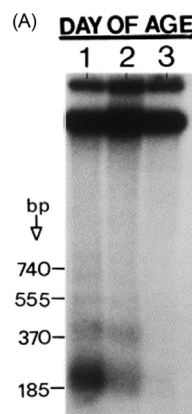


Fig. 3 A ladder gel of DNA fragmentation patterns from whole ovary lysates from rats on day 1, 2, or 3 of life. There are prominent ladder bands in the lane for Day 1 but almost no DNA fragmentation is detected by Day 3. Modified from McGee, E. A., Hsu, S. Y., Kaipia, A. and Hsueh, A. J. W. (1998). Cell death and survival during ovarian follicle development. *Molecular and Cellular Endocrinology* **140**(1–2), 15–18 with permission.

Primordial Follicle Resting Pool

Once the oocytes are in primordial follicles, they arrest at the dictyate stage of meiosis one and are seemingly suspended in development. The regulation of the initiation of growth of the primordial follicle is quite complex and covered in another chapter. Follicles can be lost from the resting or nongrowing pool of follicles by death or damage but also by entering the growth phase. The difficulty in studying small follicles and lack of material available for investigation has limited progress in understanding which process is more responsible for follicle loss related to various biological and iatrogenic processes. It has been shown that chemotherapeutic agents can differentially affect oocytes versus granulosa cells in primordial follicles and some investigators have demonstrated follicle loss prior to entering the growing pool ([Morgan et al., 2012](#)).

Growth or Death; Apoptosis Over the Course of Follicle Maturation

Once follicles are activated and enter the growing pool, only a small fraction will continue to develop to ovulation. Though growing follicles are lost at all follicle stages there are some developmental points where apoptosis is more likely to occur.

In small follicles the establishment of oocyte/granulosa cell communication is important to further development. GDF-9 an oocyte derived growth factor in the TGF β family is important to developing a theca layer and in granulosa cell proliferation. Without GDF9, follicles are lost prior to the development of the full second layer of granulosa cells. Stem cell growth factor receptor, more commonly known as c-Kit, is also an important factor in early follicle cell–cell communication and is a survival factor for preantral follicles ([Huang et al., 1993](#)).

Prior to development of an antrum, follicles can grow in response to FSH, but they are not dependent upon it for survival. Survival of preantral follicles is dependent on the presence of cGMP, though the exact mechanisms controlling cGMP production and maintenance in small follicles have not yet been completely described.

There are other factors that have been identified as growth factors for preantral follicles such as KGF, GDF9, IGF1, and activin. However, TGF β can promote apoptosis of granulosa cells in preantral and small antral follicles. One mechanism by which TGF β promotes apoptosis is by increasing the expression of the inhibitory signaling molecule Smad7 ([McGee and Raj, 2015](#)). There is also a microRNA that can block Smad7 and acts to promote survival ([Worku et al., 2017](#)). With the development of in vitro folliculogenesis and other fertility preservation strategies there will likely be further definition of the regulators and regulatory pathways involved in both growth and survivorship of preantral follicles.

In small antral follicles, FSH is the primary factor that rescues follicles from atresia. Follicles at this developmental stage are dependent upon FSH for survival and further development. Withdrawal of FSH by hypophysectomy or pituitary suppression results in widespread apoptosis of all follicles beyond the antral stage in rodents. This can be prevented by adding back or treating with FSH. In culture, substances that elevate cAMP (the second messenger of the FSH receptor) in the cytoplasm can rescue antral stage granulosa cells from apoptosis. Thus the FSH stimulated production of cAMP and activation of the protein kinase A (PKA) pathway is necessary to prevent apoptosis of antral follicles.

Interestingly, there are far more factors that have been identified as survival factors for preovulatory follicles than at the earlier stages of folliculogenesis. Though both FSH and LH are primary survival factors for preovulatory follicles, there are many other paracrine and autocrine factors with a role in promoting survival such as GH, IGF1, EGF, FGF-2, IL1 β , and nitric oxide. These factors can augment the gonadotropin effect and can also reduce the threshold of gonadotropin needed to promote survival ([Table 1](#)).

Clinical Relevance of Follicle Atresia

Atresia of follicles during development permanently removes an egg from the pool. As such the process of atresia has a profound effect on the selection of mature germ cells that have the possibility to become embryos. Regulation of atresia can have an effect on fertility and over time, evolution of a species. The default pathway of a follicle seems to be apoptosis after the antral stage is reached and is regulated by phosphorylation state of the BCL-2 interacting proapoptotic proteins present in granulosa cells. Apoptosis is a differentiated energy dependent process. Under appropriate conditions a few follicles are rescued from atresia and continue on in development. FSH is the primary survival factor in antral follicles and is not readily available until after puberty. In the post pubertal ovary, FSH is the primary survival factor from the antral stage up to the preovulatory follicle. We know from animal studies and human IVF that increasing FSH levels can increase the number of follicles in a cohort that survive and grow to produce a mature egg.

The dynamics of follicle selection and dominance in humans remains an area of many unknowns. We know that there is variation in egg capacity for fertilization and competence for development as an embryo after fertilization. We also know that more maturing follicles containing healthy eggs are available in a penultimate cohort than the ovulatory quota for a species. However we do not fully know why one follicle achieves dominance and ovulates while the others are relegated to atresia. It could be that the process of cyclic production of a cohort of follicles competing to become one of the ovulatory complement could be key underlying feature regulating the reproductive process and assuring a fit oocyte becomes available for fertilization. We also do not know what role the egg plays in the earlier stages of folliculogenesis in assisting in the escape from atresia. However we do know that the follicle unit comprised of theca, extracellular matrix, granulosa, and oocyte is a complex structure with inter- and intracellular

Table 1 Survival and death factors by follicle stage

Stage	Proapoptotic	Prosurvival
Oocyte and primordial	Bad C-Myc Trp63	Bcl-2 GDF-9 cKit cAMP
Preantral	Bad C-Myc TGF β	Bcl-2 cGMP cKit GDF-9 Connexin 37 Androgens miR-92a
Antral	Bax Bad TRAIL Fas/FasL Caspases p53 Let-7g miR-26b TNF- α ceramide	Bcl-2 FSH Lh/hCG IGF-I IL1- β EGF FGF2 Activin Estrogen NGF TGF- β
Preovulatory	Bad p53 TGF- β SMAD7 Androgens IL-6	Bcl-2 FSH LH/hCG GH IGF-I EGF FGF2 IL1- β NO cGMP

communication and is responsive in stage specific ways to endocrine, paracrine, and autocrine signals that determine follicular fate.

References

- Elmore, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology*, 35, 495–516.
- Huang, E. J., Manova, K., Packer, A. I., Sanchez, S., Bachvarova, R. F., & Bes, P. (1993). The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Developmental Biology*, 157, 100–109.
- Hussein, M. R. (2005). Apoptosis in the ovary: Molecular mechanisms. *Human Reproduction Update*, 11(2), 162–178.
- Markstrom, E., Svensson, E. C., Shao, R., Svanberg, B., & Billig, H. (2002). Survival factors regulating ovarian apoptosis—Dependence on follicle differentiation. *Reproduction*, 123, 23–30.
- McGee, E. A., & Hsueh, A. J. W. (2000). Initial and cycle recruitment of ovarian follicles. *Endocrine Reviews*, 21(2), 200–214.
- McGee, E. A., & Raj, R. S. (2015). Regulators of ovarian preantral follicle development. *Seminars in Reproductive Medicine*, 33, 177–182.
- McGee, E. A., Hsu, S. Y., Kaipia, A., & Hsueh, A. J. W. (1998). Cell death and survival during ovarian follicle development. *Molecular and Cellular Endocrinology*, 140(1–2), 15–18.
- Morgan, S., Anderson, R. A., Gourley, C., Wallace, W. H., & Spears, N. (2012). How do chemotherapeutic agents damage the ovary? *Human Reproduction Update*, 11(5), 525–535.
- Worku, T., Rehman, Z. U., Talpur, H. S., Bhattarai, D., Ullah, F., Malobi, N., Kebede, T., & Yang, L. (2017). MicroRNAs: New insight in modulating follicular atresia: A review. *International Journal of Molecular Sciences*, 18(333), 1–13.

Further Reading

- Quezada, M., Wang, J., Hoang, V., & McGee, E. A. (2012). Smad7 is a transforming growth factor-beta-inducible mediator of apoptosis in granulosa cells. *Fertility and Sterility*, 97, 1452–1459.

Ovulation

JoAnne S Richards, Baylor College of Medicine, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

The text has been adapted from Richards JS, Liu Z, Shimada M (2015). Ovulation. In: Plant TM, Zeleznik AJ (eds). *Knobil and Neill's physiology of reproduction*, 4th edn. Academic Press: Waltham, MA. pp. 997–1021.

Introduction to Ovulation

Ovulation of an oocyte capable of being fertilized is a critical function of the female gonad, the ovary and is obligatory for reproductive success. Ovulation is a *unique biological process* that is so precisely regulated that in most mammals less than 1% of all oocytes present at birth are ever released from the surface of the ovary. Most ovarian follicles die by the process of atresia; granulosa cells undergo programmed cell death (apoptosis), oocytes degenerate, and theca cells become part of the ovarian stroma. Thus, the follicle that ovulates is the one that is in the right place at the right time. More specifically, the follicle that ovulates has acquired specific functions that allow it to respond to the ovulatory surge of luteinizing hormone, LH. A specific characteristic of preovulatory follicles is the induction of LH receptors in granulosa cells; LH receptors are not present in granulosa cells of small growing follicles. The LH responsive granulosa cells in preovulatory follicles control the molecular and cellular events that culminate in ovulation.

Although ovulation is often referred to as “rupture” of the ovulatory follicle, the process is not explosive. Rather, the release of the oocyte surrounded by cumulus cells is an extrusion process and occurs, in part, by degradation of the follicle wall (Adams et al., 2016).

Introduction to the Preovulatory Follicle

Successful ovulation in response to the LH surge involves multiple cell types (Richards et al., 2015; Adams et al., 2016). These include the theca cells, granulosa cells and cumulus cells within the preovulatory follicle itself. In essence, the follicle is a multilayered sphere, each layer being separated from the other by a specialized extracellular matrix. The theca cell layer is the outmost layer of cells in the preovulatory follicle and is composed of the theca externa and theca interna. The theca externa contains fibroblasts that generate extracellular collagen to provide a semirigid, supportive structure around the follicle. The theca externa is also endowed with an elaborate vascular network that supplies nutrients and oxygen to the follicle. The granulosa cell layer is separated from the theca layer by a thin collagen/laminin-rich basal lamina. This matrix provides an attachment surface for the outer-most layer of granulosa cells, the mural granulosa cells. The remaining granulosa cells form a syncytium in which they are interconnected by specialized gap-junctions, composed primarily of connexin 43 (GJA1). The cumulus cells, the inner layer of somatic cells, surround the oocyte, forming the cumulus cell–oocyte complex (COC). The cumulus cells are separated from the oocyte by the zona pellucida, a matrix of specific glycosylated zona proteins that serve to protect the oocyte and to provide structure and function during fertilization. The zona also allows for the attachment of cumulus cells and their connections with the oocyte via gap junctions, comprised exclusively of connexin 37 (GJA4), in the COCs that are critical for oocyte development.

Ovulation also depends on other cell types associated with the preovulatory follicle, including surrounding stromal and vascular tissues, immune cells, and the ovarian surface epithelium (OSE). These tissues undergo structural and functional changes during ovulation. For example, at the ovarian surface, the morphological events associated with ovulation involve the dissolution of collagen fibers in the theca layer and the consequent thinning of the surrounding stromal tissue. The vascular network in the theca cells of ovulating follicles becomes highly hyperemic and dilated, indicative of the inflammatory-like activities that are occurring in the somatic cells. The prostaglandins (PGs), cytokines, and chemokines that are being released alter vascular functions and recruit immune cells that are essential for ovulation and luteinization. Ultimately, the basal lamina of the OSE, that separates the apex of the follicle from the peritoneal cavity (human and other species) or bursa (mouse, rat), loosens and permits the release of the expanded COCs. The COCs are then picked up by the ciliated cells of the fimbria and transported to the oviduct where fertilization occurs. Successful release of the oocyte must occur before luteinization, otherwise the oocytes are trapped with the corpora lutea.

The Role of LH in Ovulation

The LH Receptor

It is now axiomatic that only preovulatory follicles are capable of ovulating in response to the ovulatory surge of pituitary LH. This has been experimentally established in most (all?) mammals studied by the evidence showing that granulosa cells in preovulatory follicles selectively express elevated levels of LH receptors that bind LH or exogenously administered hCG (Richards et al., 2002). Moreover, only granulosa cells and not cumulus cells express the LH receptor. Biochemically, the LH receptor belongs to the G-protein coupled receptor superfamily. When the ligand LH binds to the LH receptor it activates adenylyl cyclase leading to the production of cAMP and stimulation of cAMP-dependent protein kinase (protein kinase A). Protein kinase A in turn phosphorylates proteins that regulate the transcription of specific genes. These genes then lead to other events that impact the ovulation process (Fig. 1).

A major milestone in our understanding of the molecular events controlling ovulation came with the discovery that the LH surge via the cAMP-protein kinase A pathway induced the expression of specific epidermal growth factor (EGF)-like ligands (amphiregulin,

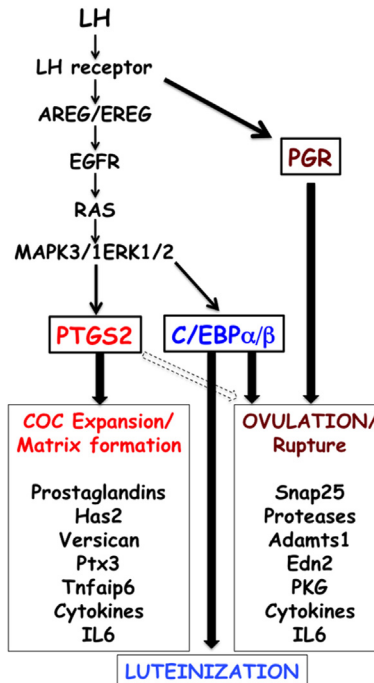


Fig. 1 The ovulatory LH surge initiates a cascade of cellular and molecular events that culminate in expansion of the cumulus oocyte complex (COC) and ovulation. COC expansion is induced by the EGF-like factor-EGF receptor (EGFR)-RAS-MAPK pathway that leads to the production of prostaglandins. Prostaglandins regulate the expression of genes involved in the formation and stabilization of the COC matrix. The EGF-like factor-EGFR pathway also leads to the activation of the transcription factors C/EBP α/β that impact ovulation and formation of the corpus luteum (luteinization). LH also induces the progesterone receptor (PGR) that impacts events leading to successful rupture of the preovulatory follicle and ovulation. Adapted from Richards JS, Pangas SA (2010). The ovary: Basic biology and clinical implications. *Journal of Clinical Investigation* 120, 963–972 PMID: 20364094; PMCID:PMC22846061 and Richards JS, Liu Z, Shimada M (2015). Ovulation. In: Plant TM, Zeleznik AJ (eds). *Knobil and Neill's physiology of reproduction*, 4th edn. Academic Press: Waltham, MA. pp. 997–1021.

AREG; epiregulin, EREG, and betacellulin, BTC) in granulosa cells (Conti et al., 2005). These EGF-like ligands bind and activate the EGF receptors present on granulosa cells and cumulus cells. Activation of EGF receptors leads to the activation of down-stream targets including the mitogen activated protein kinases (MAPK3/1) (also known as ERK1/2). Based on gene knock out studies in mice, this cascade is essential for ovulation. Mice null for the EGF receptor in granulosa cells exhibit impaired ovulation, cumulus cell–oocyte expansion, oocyte maturation, and luteinization. Conditional disruption of the mitogen activated protein kinases (MAPK3/1; ERK1/2) in mice blocks these same events (Richards and Pangas, 2010). Collectively, these observations document that the EGF-like factors and their receptors are key intracellular mediators of LH action and impact many critical events controlling ovulation. These include the production of prostaglandins, the induction of the progesterone receptor, and the expansion of the cumulus cell oocyte complex (COC).

A Central Role of ERK1/2 (MAPK3/1)

The expression of the *Lhcgr* gene is not altered in granulosa cells of *Mapk3/1* mutant mice indicating that the LH receptor is up-stream of these genes. Many genes that are expressed at high levels in granulosa cells of preovulatory follicles are normally down-regulated as a consequence of the LH surge. These genes include *Fshr*, *Cyp19a1*, *Foxo1*, *Ctgf*, *Ccnd2*, *Inhba* as well as *Lhcgr* itself (Richards and Pangas, 2010). However, in the *Mapk3/1* mutant mice these genes are not down-regulated. Thus, activation of the MAPK3/1 pathway by LH and the EGF-like factors is essential not only for turning on genes that regulate ovulation and luteinization but also for turning off genes that establish and maintain the preovulatory granulosa cell phenotype. It is impressive to observe that healthy preovulatory follicles remain present in the *Mapk3/1* mutant mice following administration of an ovulatory dose of LH whereas corpora lutea are fully formed in the wild type control mice similarly treated. Thus, activation of MAPK3/1 is critical for both ovulation and the terminal differentiation of granulosa cells to luteal cells. It is also important to note that disruption of *Mapk3/1* in the pituitary causes infertility in female (but not male) mice by blocking the LH. Thus, the MAPK3/1 pathways in the pituitary and ovary are both critical and tightly integrated to control ovulation.

C/EBP α and C/EBP β

Among the many transcription factors that are essential for ovulation are the CCAAT enhancer binding proteins alpha and beta (C/EBP α/β) (Richards et al., 2005). These are phosphorylated and activated by MAPK3/1. Conditional depletion of *Cebpb* and *Cebpa*

genes in granulosa and cumulus cells using *Cyp19-Cre* mice disrupts ovulation and luteinization and the mice are infertile. Gene profiling of granulosa cells obtained from the *Cebpa/b* mutant mice revealed a novel set of genes that are mostly down-stream of MAPK3/1 and appear to control the neovascularization of the ruptured, ovulated follicle. Major target genes are *Runx2*, specific transporters, the prolactin receptor, and the acute phase marker serum amyloid A.

The Role of Prostaglandins in Ovulation

Studies in the 1980–90s documented that an ovulating follicle exhibits physical characteristics of inflammation, including hyperemia and edema as well as biochemical features of inflammation including the production of prostaglandins (PGs) and the presence of immune cells. That ovulation was blocked by an inhibitor of prostaglandin biosynthesis, indomethacin supported the hypothesis that ovulation is an inflammatory-like response (Espey, 1980). Since then many studies have supported and expanded this insightful hypothesis (Richards et al., 2002).

From 1991 to 1993, two distinct forms and molecular weight variants (69 and 72 kDa) of the prostaglandin synthase (PGS), the rate-limiting step in prostaglandin biosynthesis, were identified in rat ovarian tissues (Richards, 2006; Richards et al., 2015). These observations provided the first evidence that there was not just one enzyme but two enzymes controlling prostaglandin biosynthesis. Furthermore, LH induced the 72 kDa form specifically in granulosa cells of ovulating follicles, whereas the 69 kDa form was constitutively expressed in theca cells and other tissues. Purification of the 72 kDa inducible form of PGS and amino-terminal sequence results documented that the inducible form was distinct from the constitutive form. Ultimately, the genes encoding each distinct protein were cloned and are now known as prostaglandin synthase1, PTGS1 (also known as cyclooxygenase 1; COX1) and PTGS2 (cyclooxygenase 2; COX2).

Prostaglandins and COC Expansion

Prostaglandins are essential for stimulating the expansion of the cumulus cell–oocyte complex (COC) and COC expansion is essential for ovulation (Richards and Pangas, 2010). Specifically, the cumulus cells within the COC of a preovulatory follicle are tightly packed together around the oocyte. In response to the LH surge, the cumulus cells make an extracellular hyaluronan-rich matrix, dissociate and migrate from the oocyte to fill the follicular antral cavity. Because neither the cumulus cells nor the oocyte express LH receptors, LH induction of this process occurs by an indirect mechanism. Specifically, LH induces the EGF-like factors in granulosa cells which then activate the EGF receptors in the cumulus cells. Activation of the cumulus EGF receptors leads to the induction of PTGS2 and production of prostaglandins, mainly PGE2. Prostaglandins made by cumulus cells then bind their G-protein coupled receptor (EP2) present in cumulus cells leading to activation of AC, cAMP, and protein kinase A and additional induction of EGF-like factors. Thus, cumulus cell-derived prostaglandins assume the role of LH. EGF-like factors alone or prostaglandins alone can induce COC expansion in the absence of LH. These observations established a unique intrafollicular regulatory loop (Richards et al., 2015).

Although the precise mechanisms by which COCs are released from the ovary remain to be determined, successful expansion of COCs by EGF-like factors and prostaglandins depends on their ability to induce factors that bind to and stabilize the hyaluronan polymers that form the backbone of the expanded matrix. Specifically, the LH surge rapidly induces expression of hyaluronan synthase 2 (*Has2*) that generates hyaluronan. The factors that stabilize hyaluronan include tumor necrosis factor alpha induced protein 6 (*Tnfaip6*), pentraxin 3 (*Ptx3*), and the proteoglycan versican (*Vcan*). TNFAIP6 delivers a serum factor IxI to the hyaluronan backbone. PTX3 binds exclusively to TNFAIP6. Versican binds hyaluronan and is the preferred substrate for the protease ADAMTS1. Collectively, these components form a scaffold on the matrix and each is essential for COC expansion. Targeted disruption of matrix-related genes *Ptgs2*, *Ep2*, *Has2*, *Adams1*, *Tnfaip6*, *Ptx3*, and *Ial* in mice (or in culture) impairs COC expansion; the mutant mice are infertile or subfertile (Richards et al., 2015). If the COC matrix is not made appropriately and lacks the hyaluronan backbone (made by HAS-2) or is not stabilized by the scaffolding of proteins that bind the hyaluronan polymers directly (IxI heavy chain and versican) or each other (IxI-TNAIP6-PTX3), COCs are not released and, as a consequence, the oocytes are trapped inside the non-ovulated, luteinized follicles.

Cumulus Cells Are Unique

Cumulus cells are distinct from granulosa cells in many ways (Richards et al., 2002). As indicated above, they are in close physical contact with the oocyte and thereby are exposed to high levels of oocyte-derived regulatory factors that control their function. The oocyte-derived factors, such as GDF9, BMP15 and FGF8, act singly or in combination to suppress cumulus cell expression of the LH receptor (*Lhcgr*) and steroidogenic genes and promote the expression of other genes in cumulus cells (Hernandez-Gonzalez et al., 2006). Cumulus cells also have a distinct fate. During COC expansion, they are embedded within the expanded extracellular matrix, produce many cytokines and inflammatory factors, and are released from the ovary along with the oocyte at the time of ovulation (Espey and Richards, 2006). Importantly, factors within the matrix also control cumulus cell functions. Thus, each COC is a highly specialized microenvironment with inflammatory-like responses that control the formation and stabilization of the matrix (Richards et al., 2015). Hence, during ovulation, the oocyte is the center of a pronounced inflammatory reaction and is essentially treated as a foreign body and kicked out of the nest. The expanded COC matrix is also sticky and this feature is essential for the

ovulated COCs to adhere to the surface of the ovary. The fimbria of the Fallopian tube that is lined with cilia then picks up the COCs for transport to the uterus for fertilization.

The Role of Progesterone in Ovulation

Studies in the 1970–90s revealed that the ovulation process was also dependent on the increased production of the steroid hormone progesterone that occurs rapidly following the LH surge (Espey and Lipner, 1994). The progesterone synthesis inhibitor epostane blocked ovulation; this effect was reversed by exogenous progesterone. LH also rapidly induces the expression of a nuclear receptor that bind progesterone, progesterone receptor (PGR) in granulosa cells (Park and Mayo, 1991). The induction of PGR preferentially impacts the process of follicle “rupture” and extrusion of the oocyte more than it impacts COC expansion. Activation of PGR facilitates the induction of specific genes encoding ADAMTS1 that is a novel protease, synaptosome associated factor 25 (SNAP25) that is involved in protein secretion, cGMP dependent protein kinase 2 (PRKG2) and endothelin 2 (END2) that regulate vascular endothelial cells. Genes that are induced at later times after the LH surge and closer to the time of actual release of the oocyte from the surface of the ovary include, cytokines and chemokines as well as other genes whose functions remain to be defined. The essential roles of progesterone and its nuclear receptor were unequivocally documented by targeted disruption of the progesterone receptor in mice. The progesterone receptor knockout (PRKO) mice fail to ovulate, contain corpora lutea with entrapped oocytes and are completely infertile (Lydon et al., 1995). Molecular analyses comparing control and mutant follicles have documented that progesterone regulates the expression of several genes that are distinct from those regulated by prostaglandins but are also critical for ovulation (Russell and Robker, 2007). Thus, the roles of progesterone and PGRs in the ovulation process are distinct from those of PGs (Richards et al., 2015).

The Role of Cytokines and Chemokines in Ovulation

In cumulus cells, AREG induction of *Ptgs2* and the production of prostaglandins represent key mediators of inflammation. AREG also leads to the induction of other genes (*Alcam*, *Il6*, *Cd34*, *Cd52*, *Pdcd1*) associated with inflammation (Hernandez-Gonzalez et al., 2006). In addition to IL6 and other cytokines (*Ccl2*, *Ccl4*, *Ccl5*) are expressed in cumulus cells raising the possibility that cytokines and their receptors play distinct roles in the functions and fate of COCs prior to and after ovulation. IL6 alone can stimulate COC expansion and induce genes required for this process by activating the MAPK3/1 pathway and JAK/STAT pathways. IL6 also enhances embryo development (Richards et al., 2015). However, *Il6* null mice are fertile indicating that other cytokines being released from COCs may act in a redundant manner during ovulation to facilitate COC expansion and release. Many cytokines and chemokines recruit immune cells to the ovulating follicles. Recent studies have also proposed that there is a pituitary–ovary–spleen axis associated with ovulation that promotes the trafficking of immune cells to the ovulating follicle. That immune cells are recruited to ovulating follicles is highly relevant because recent studies also show that macrophages and dendritic cells are essential for ovulation, luteinization and vascularization of newly formed corpora lutea (Care et al., 2013; Cohen-Fredarow et al., 2014).

The Role of Immune-Related Factors

Components of the innate immune system are an evolutionarily conserved receptor recognition system that includes the pattern recognition receptors (*Cq1a*, *Cd14*, *Trl4*, *Thr2*). In immune cells these receptors are used to discriminate self from non-self and bind such factors as bacterial lipopolysaccharide, LPS/Pam3Cys. These receptors are also expressed in nonimmune cells including cumulus cells. In cumulus cells they are functional and bind LPS as well as fragments of hyaluronan (Richards et al., 2015). Specifically, LPS itself induces marked induction of *Il6* and *Ptgs2* in cumulus cells. Furthermore, fragments of hyaluronan that are generated by hyaluronidase that is released from cumulus cells or sperm also bind to these receptors and increase *Il6* and *Ptgs2*. Thus, cumulus cells have the potential to monitor their environment within the follicle and the oviduct.

The cGMP Pathway in Cumulus Cells

The resumption of meiosis is initiated rapidly by the LH surge and involves an intrafollicular paracrine network that controls production of cyclic GMP (Zhang et al., 2010). This network is comprised of the natriuretic peptide precursor C (NPPC) that is produced and secreted by granulosa cells. The NPPC ligand binds the natriuretic peptide receptor 2/guanylyl cyclase B (NPR2) that is present primarily in cumulus cells. Cyclic cGMP that is produced by activation of NPR2 in cumulus cells is then transferred to oocytes through gap junctions. Elevated levels of cGMP block PDE3A activity leading to elevated intra-oocyte levels of cAMP that block meiosis. The LH surge not only disrupts gap junctions and prevents cGMP from entering the oocytes but also reduces the expression of *Nppc* and *Npr2*. Activation of the EGF-MAPK pathway also disrupts this pathway leading to decreased cGMP in cumulus cells and the oocyte. Reduced levels of cGMP in oocytes leads to increased activity of PDE3A and lower levels of cAMP. Decreased levels of cAMP in oocytes permit the resumption of meiosis of ovulatory follicles.

In granulosa cells, another pathway can generate cGMP (Richards et al., 2015). This pathway is comprised of nitric oxide synthase 3 (NOS-3) that is induced by the LH surge and generates nitric oxides that activate soluble guanylyl cyclases and produce cGMP. cGMP-dependent protein kinase II (cGKII or PRKG2) is also induced by LH in a progesterone receptor (PGR)-dependent manner. Thus, PRKG2 may play a critical role in granulosa cells of ovulatory follicles by mediating the effects of cGMP, however, the precise functions of PRKG2 in ovulatory granulosa cells remain to be resolved.

The Role of Proteases in Ovulation

Ovulation involves and requires the breakdown of the basal lamina separating mural granulosa cells from theca cells as well as the dissolution of the basement membrane underlying the ovarian surface epithelium (Curry and Osteen, 2003). This “thinning” of the ovulatory follicle wall and the surface epithelium ultimately permits the extrusion of the COCs through the ovulation pore. This latter event appears to depend, in part, on EDN2 and smooth muscle contractions within the theca layer. The former events appear to involve various proteases and tissue protease inhibitors, the list of which is long and the functions of which are complex (Curry and Osteen, 2003). Despite much research, data from knockout mice lacking various proteases have shed little insight into which ones might be the key players close to or at the time ovulation. However, protease inhibitors can block ovulation as shown recently in primates (Peluffo et al., 2011). An outstanding and comprehensive reviews have covered this topic recently therefore, only one protease will be discussed herein (Curry and Osteen, 2003; Rosewell et al., 2015).

One novel protease called A disintegrin and metalloproteinase with thrombospondin-like repeats 1, ADAMTS1 is rapidly and markedly induced in granulosa cells of preovulatory follicles by the LH surge and in a progesterone-PGR-dependent manner. Not only does the temporal expression of ADAMTS1 (Richards et al., 2002) and its control by PGR (Robker et al., 2000; Russell and Robker, 2007) suggest its key role in ovulation, but also the functional activities of ADAMTS1 indicate that it is a key protease related to ovulation. Mice null for *Adams1*, as well as mice in which *Adams1* has been conditionally disrupted in granulosa cells, are sub-fertile. The proteoglycan versican is one of the preferred substrates for ADAMTS1 and is a key component of the extracellular matrix in expanded COCs and in the follicle wall. In the absence of ADAMTS1 both the invasion of the theca cell layer into the granulosa cell layer as well as the structure of the COC matrix are abnormal. How these defects ultimately cause subfertility and reduce release of COCs remains to be more clearly defined. ADAMTS4 and ADAMTS5 are also expressed and regulated in the mouse ovary (Richards et al., 2005) whereas ADAMTS1 and ADAMTS9 are regulated in human granulosa cells during ovulation (Wissing et al., 2014).

Translational-Clinical Implications

Polycystic Ovarian Syndrome at the Ovarian Level

Polycystic ovarian syndrome (PCOS) is the most common endocrine defect and cause of anovulatory infertility in women in their reproductive years. PCOS afflicts more than 5%–10% of women in the United States, costs more than \$4 million/year to manage and is becoming increasingly prevalent. A PubMed search of “ovulation” lists scores of papers that are predominantly related to current clinical studies describing ways to improve treatment of infertile women, especially those with anovulatory PCOS. The number of basic research papers on ovulation pales in comparison. Although some molecular aspects of theca cell androgen production have been determined in PCOS, our understanding of the molecular consequences of PCOS in granulosa cells and cumulus cells is less well defined.

Although elevated androgens (hyperandrogenemia) are one defining characteristic of PCOS, PCOS is also frequently associated with metabolic defects related to insulin resistance and a proinflammatory state that may contribute to insulin resistance. Thus, understanding the consequences of endocrine, cytokine and metabolic defects at the ovarian level are critical for improving ovulation, IVF procedures and oocyte quality in PCOS patients (Rosenfield and Ehrmann, 2016). PCOS is often, but not always, associated with obesity suggesting that cytokines or other factors coming from adipose tissue likely impact the hypothalamic-pituitary-ovarian axis. Two major cytokines derived from adipose tissue are leptin and adiponectin. Whereas leptin regulates appetite, adiponectin has been shown to alter steroidogenesis, genes involved in ovulation and those involved in apoptosis. That adiponectin enhances oocyte and embryo development in mice indicates that this may be a “good” cytokine. Furthermore, expression of adiponectin receptors is correlated positively with oocyte maturation in samples obtained from IVF patients. That levels of adiponectin are reduced in PCOS patients may indicate that it plays some role in optimal ovulation and oocyte quality.

In addition, there is a functional link between elevated androgens in PCOS patients and proinflammatory conditions at the systemic level. Cytokines such as CRP and TNF alpha released from adipocytes can cause proinflammatory reactions in cells. Moreover, inflammatory conditions within the ovarian stroma and granulosa cells of ovulatory follicles of PCOS patients undergoing IVF release elevated levels of cytokines and chemokines including IL6, IL7, CCL20 that recruit immune cells (Adams et al., 2016; Al-Alem et al., 2015). These observations document that there is an enhanced local, intraovarian inflammatory response associated with a PCOS patients, many of whom are obese.

Ovaries in women with anovulatory PCOS contain numerous small antral follicles that are characterized by high levels of androgens. However, these follicles also produce estradiol. Successful ovulation can be achieved frequently in PCOS patients by

decreasing estradiol or its actions and by judiciously increasing endogenous or exogenous FSH. These results indicate that anovulation is related not only to ovarian problems but also to hypothalamus–pituitary events. Ovarian hyperstimulation syndrome (OHSS) in which the ovary produces excessive amounts of estradiol and progesterone and ovarian blood vessels become highly leaky, is frequently associated with hormonal stimulation of IVF patients and complicates IVF outcomes. Therefore it is important to understand what factors contribute to PCOS and to OHSS, including what functions are altered in PCOS ovaries, including the ovarian vasculature.

Restoring Fertility With IVF, ICSI, and IVM

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the most frequent methods used for treating infertile couples. However, the effectiveness of IVF/ICSI procedures is compromised by the transfer of multiple embryos and multiple births. Now that we know many of the genes regulating COC expansion, these genes might be used as markers to identify healthy versus compromised oocytes during IVF. In vitro maturation (IVM) of oocytes provides promise as an alternative to IVF but oocyte quality remains an issue and better techniques need to be developed in the years ahead.

Summary

Ovulation is a complex process essential for the reproductive success of every species. Although we have learned a great deal about how LH initiates this process in mice and other mammals, there are still many blank spaces on the canvas and in the script. We know some of the transcription factors that are essential for ovulation but the regulatory networks that they control remain incompletely defined.

World population continues to soar and therefore, the need to regulate population growth remains critical in many areas of the globe. Conversely, rescuing fertility has a major impact on allowing infertile couples to have the children they desire. Therefore, understanding what regulates ovulation and oocyte quality are of prime importance for the development of new contraceptive approaches as well as for designing improved procedures for in vitro fertilization (IVF) of an oocyte and in vitro maturation (IVM) of follicles and oocytes. Although the transfer of some of our current knowledge to clinical issues is occurring, much still remains to be applied to IVF and especially IVM where conditions are less than optimal.

References

- Adams, J., Liu, Z., Ren, Y. A., Wun, W.-S., Zhou, W., et al. (2016). Enhanced inflammatory transcriptome in granulosa cells of women with polycystic ovarian syndrome. *The Journal of Clinical Endocrinology and Metabolism*, 101, 3459–3468. PMID: 27228368.
- Al-Alem, L., Puttabyatappa, M., Rosewell, K., Brännström, M., Akin, J., et al. (2015). Chemokine ligand 20: A signal for leukocyte recruitment during human ovulation? *Endocrinology*, 156, 3358–3369. PMID: 26125463.
- Care, A. S., Diener, K. R., Jasper, M. J., Brown, H. M., Ingman, W. V., et al. (2013). Macrophages regulate corpus luteum development during embryo implantation in mice. *The Journal of Clinical Investigation*, 123, 3472–3487. PMID: 23867505.
- Cohen-Fredarow, A., Tadmor, A., Raz, T., Meterani, N., Addadi, Y., et al. (2014). Ovarian dendritic cells act as a double-edged pro-ovulatory and anti-inflammatory sword. *Molecular Endocrinology*, 28, 1039–1054. PMID: 24825398.
- Conti, M., Hsieh, M., Park, J.-Y., & Y-Q, S. (2005). Role of the egf network in ovarian follicles. *Molecular Endocrinology*, 20, 715–723.
- Curry, T. E. J., & Osteen, K. G. (2003). The matrix metalloproteinase system: Changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. *Endocrine Reviews*, 24, 428–465.
- Espey, L. L. (1980). Ovulation as an inflammatory reaction—A hypothesis. *Biology of Reproduction*, 22, 73–106.
- Espey, L. L., & Lipner, H. (1994). *Ovulation*. New York, NY: Raven Press.
- Espey, L. L., & Richards, J. S. (2006). *Ovulation* (3rd edn.). San Diego, CA: Academic Press, Elsevier.
- Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Shimada, M., Wayne, C. M., Ochsner, S. A., et al. (2006). Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: Does this expand their role in the ovulation process? *Molecular Endocrinology*, 20, 1300–1321. PMID: 16455817.
- Lydon, J. P., DeMayo, F., Funk, C. R., Mani, S. K., Hughes, A. R., et al. (1995). Mice lacking progesterone receptor exhibit reproductive abnormalities. *Genes & Development*, 9, 2266–2278.
- Park, O.-K., & Mayo, K. (1991). Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. *Molecular Endocrinology*, 5, 967–978. PMID: 1840636.
- Peluffo, M. C., Murphy, M. J., Talcott Baughman, S., Stouffer, R. L., & Hennebold, J. D. (2011). Systematic analysis of protease gene expression in the rhesus macaque ovulatory follicle: Metalloproteinase involvement in follicle rupture. *Endocrinology*, 152, 3963–3974.
- Richards, J. S. (2006). Genetics of ovulation. *Seminars in Reproductive Medicine*, 25, 235–242.
- Richards, J. S., & Pangas, S. A. (2010). The ovary: Basic biology and clinical implications. *Journal of Clinical Investigation*, 120, 963–972. PMID: 20364094. PMCID:PMC22846061.
- Richards, J. S., Russell, D. L., Ochsner, S., & Espey, L. L. (2002). Ovulation: New dimensions and new regulators of the inflammatory-like response. *Annual Review of Physiology*, 64, 69–92.
- Richards, J. S., Hernandez-Gonzalez, I., Gonzalez-Robayna, I., Teuling, E., Lo, Y., et al. (2005). Regulated expression of adamts family members in follicles and cumulus oocyte complexes: Evidence for specific and redundant functions. *Biology of Reproduction*, 72, 1241–1255. PMID: 15659705.
- Richards, J. S., Liu, Z., & Shimada, M. (2015). Ovulation. In T. M. Plant, & A. J. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn, pp. 997–1021). Waltham, MA: Academic Press.
- Robker, R. L., Russell, D. L., Espey, L. L., Lydon, J. P., O'Malley, B. W., et al. (2000). Progesterone-regulated genes in the ovulation process: Adamts-1 and cathepsin I proteases. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 4689–4694. PMID: 10781075.

- Rosenfield, R. L., & Ehrmann, D. A. (2016). The pathogenesis of polycystic ovary syndrome (pcos): The hypothesis of pcos as functional ovarian hyperandrogenism revisited. *Endocrine Reviews*, 37, 467–520. PMID: 27459230.
- Rosewell, K. L., Al-Alem, L., Zakerkish, F., McCord, L., Akin, J. W., et al. (2015). Induction of proteinases in the human preovulatory follicle of the menstrual cycle by human chorionic gonadotropin. *Fertility and Sterility*, 103, 826–833. PMID: 25516084.
- Russell, D. L., & Robker, R. L. (2007). Molecular mechanisms of ovulation: Co-ordination through the cumulus complex. *Human Reproduction Update*, 13, 289–312.
- Wissing, M. L., Kristensen, S. G., Andersen, C. Y., Mikkelsen, A. L., Høst, T., et al. (2014). Identification of new ovulation-related genes in humans by comparing the transcriptome of granulosa cells before and after ovulation triggering in the same controlled ovarian stimulation cycle. *Human Reproduction*, 29, 997–1010.
- Zhang, M., Su, Y.-Q., Sugiura, K., Xia, G., & Eppig, J. J. (2010). Granulosa cell ligand nppc and its receptor npr2 maintain meiotic arrest in mouse oocytes. *Science*, 330, 366–369. PMID: 20947764. PMCID:PMC: 23056542.

Corpus Luteum

Jon D Hennebold, Oregon Health & Science University, Beaverton, OR, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Angiogenesis The formation of new blood vessels.

Luteinization The differentiation of follicular granulosa and theca cells into the cells that comprise the corpus luteum.

Luteinizing hormone A protein hormone secreted by the anterior pituitary that is responsible for initiating events necessary for ovulation and initiating corpus luteum development.

Luteolysis Loss of luteal progesterone producing potential and luteal cell numbers at the end of a reproductive cycle.

Maternal recognition of pregnancy Substances generated by the conceptus that signal the corpus luteum of the mother to maintain a level of progesterone synthesis that will support pregnancy.

Progesterone A steroid hormone produced by the corpus luteum that is necessary for preparing the uterus for embryo implantation and subsequently sustaining pregnancy.

Prostaglandins Lipid-derived molecules that are key mediators of luteal development (prostaglandin E₂; PGE₂) or luteolysis (prostaglandin F_{2α}; PGF_{2α}).

Introduction

The corpus luteum is remarkable in that it is critically important for reproduction in numerous species (mammals and some reptiles), but exists only as a transient structure that arises from the ovarian follicle after ovulation. Marcello Malpighi first coined the term corpus luteum (yellow body) in 1681. Regnier de Graaf (1641–73) was the first to describe and provide detailed illustrations of the corpus luteum present in the ovaries of pregnant rabbits, where it was noted that they possessed “globular bodies.” The essential role of the corpus luteum was not defined until 1901, when Ludwig Frankel demonstrated that rabbits could not maintain a pregnancy when all of the corpora lutea were removed. It was subsequently discovered in 1929 that extracts obtained from corpora lutea were able to replace the function of the ovaries once removed. Around this same time, several groups purified and defined this luteal derived steroid hormone and named it progesterone by joining the terms “progestin” and “luteo-sterone.” In the intervening decades, studies focused primarily on understanding the cellular processes that allow for the development of the corpus luteum following ovulation, its ability to produce large quantities of the steroid hormone progesterone, its regression in non-fecund cycles, as well as luteal regulation of pregnancy.

Since the corpus luteum forms from the antecedent ovulatory follicle, the number of corpora lutea are proportional to the number of ovulatory follicles that form and ovulate through each reproductive cycle (Fig. 1). Thus, in polyovulatory species, numerous corpora lutea are generated per cycle; whereas most ovine, bovine, and primate species develop a single follicle that yields one corpus luteum per reproductive cycle. Species have also evolved several different mechanisms that control the lifespan of the corpus luteum in the nonfertile cycle as well and how pregnancy affects luteal survival. Mammals can be divided into three major categories based on how long the corpus luteum functions after ovulation in the non-fecund ovarian cycle and how luteal lifespan is affected by pregnancy onset and gestation, including the following: (1) ultrashort-lived corpora lutea, (2) short-lived corpora lutea, and (3) long-lived corpora lutea.

Species that have ultrashort-lived corpora lutea do not form a functional corpus luteum (e.g., rodents) or any corpus luteum during the ovarian cycle unless ovulation is induced following copulation (reflex ovulators, e.g., rabbit) and/or pseudopregnancy or pregnancy (e.g., rat, mouse). In rodents, the ruptured follicle takes on certain structural and cellular characteristics of a corpus luteum, but does not secrete sufficient progesterone to allow for implantation. A requirement for the development of significant progesterone producing potential by the murine corpus luteum includes either mating that does not result in pregnancy (pseudopregnancy) or fertilization and pregnancy. Pseudopregnancy results in the development of functional corpora lutea that secrete progesterone for 12–14 days, whereas fertilization and implantation leads to continued progesterone secretion by the corpus luteum throughout gestation that is critical for maintaining pregnancy (~21 days).

Species that possess short-lived corpora lutea include many of the ungulates, such as domesticated farm animals (e.g., sheep and cattle), and primate species (e.g., monkeys, great apes, and humans). The short-lived corpus luteum develops and functions for a finite interval during the ovarian cycle in the absence of pregnancy (typically between 2 and 3 weeks), but its function is extended markedly if pregnancy ensues. This period of luteal progesterone synthesis allows for the timely movement of the early embryo through the oviduct and preparation of the uterus for implantation. If implantation and the establishment of pregnancy occur, progesterone production by the corpus luteum continues until the placenta assumes this activity (referred to as the luteal-placental shift). In primates, the luteal-placental transition takes place between 3 and 7 weeks of gestation. In domesticated

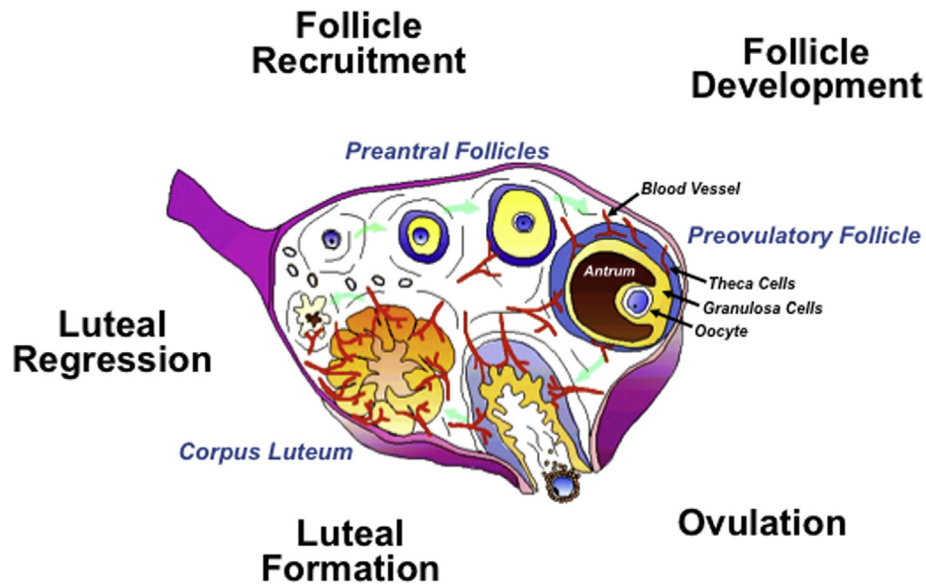


Fig. 1 The ovarian cycle and the formation of the corpus luteum. The luteal phase of the cycle encompasses the period of corpus luteum formation and its subsequent regression. The corpus luteum forms at the end of the ovarian cycle from the remnants of the ruptured ovulatory follicle. This cartoon depicts the ovarian cycle in a monovulatory species that results in the growth and development of a single follicle that ruptures and transforms into a single corpus luteum.

animal species, progesterone synthesis by the corpus luteum is sustained by pregnancy for several months, or even until the end of pregnancy, as occurs in the pig.

The absence of pregnancy leads to rapid luteolysis in species that have short-lived corpora lutea. However, there are important distinctions between primates and ungulates in terms of the processes that determine the length of the luteal phase as well as how maternal recognition of pregnancy is mediated. In the ungulate corpus luteum, antral follicle growth occurs during the luteal phase, which results in a very short (2–3 days) and well-defined interval between luteolysis and the development of the next ovulatory follicle. In most primates, however, small antral follicles do not develop until after luteolysis, resulting in a relatively long follicular phase (~1–2 weeks). Another distinguishing feature between ungulates and primates is that luteolysis in the former is dependent on a uterine-derived luteolytic signal that orchestrates corpus luteum regression; whereas in primates, hysterectomy does not alter the functional lifespan of the corpus luteum, indicating the absence of a uterine luteolytic signal. Lastly, while early pregnancy in ungulates and some New World monkeys prevents the synthesis of a uterine luteolytic signal, an embryonic hormone (chorionic gonadotropin) is produced by the conceptuses of Old World monkeys, apes, and women that sustains corpus luteum progesterone synthesis.

In some species that have estrous cycles of very long duration (i.e., every 6–12 months), the lifespan of a fully functioning corpus luteum does not differ between fertile or nonfertile cycles. Such species, including carnivores (e.g., dogs, wolves, foxes, cats, ferrets, skunks) and other animals such as the roe deer, armadillo and marsupials, possess corpora lutea that survive for as long as 6 months. In dogs for example, the length of the luteal phase in nonpregnant and pregnant animals is ~65 days, with rapid luteolysis occurring after delivery in pregnant animals. In the absence of pregnancy, luteal regression in these species generally encompasses a relatively prolonged period of time (i.e., weeks).

Corpus Luteum Development

After ovulation, the adjacent vasculature and the cellular remnants of the ruptured follicle undergo structural and cellular remodeling that ultimately results in the formation of the functional corpus luteum. The key events necessary for this transformation (e.g., luteinization) are coordinated by a midcycle surge of the pituitary-derived luteinizing hormone (LH). LH acts by directly binding to and signaling through LH receptors present on granulosa and theca cells. Alternatively, LH induces the synthesis of paracrine acting factors, such as progesterone or the fatty acid metabolite prostaglandin E₂ (PGE₂), both of which have been shown to promote luteal development and survival. Luteinization is a remarkable event involving initial rounds of cell proliferation, cell differentiation, and tissue remodeling that are unparalleled in terms of normal physiological processes. Luteal cell differentiation primarily refers to the transformation from predominantly estradiol synthesis via the steroidogenic activities present in both follicular granulosa and theca cells to progesterone production by the cells that comprise the corpus luteum.

Based on differences in size, morphology and cellular markers, the general belief is that the corpus luteum is comprised of at least two types of luteal cells, large luteal cells and small luteal cells. This distinction is somewhat species dependent in that rodents, for example, possess luteal cells that are relatively homogenous in terms of their size and distribution throughout the corpus luteum. In

contrast, the primate and ungulate corpus luteum possesses distinct small and large luteal cell populations. In ruminants, the larger luteal cells are endowed with organelles associated with a higher rate of cholesterol uptake and steroid/protein synthesis, which includes greater levels of smooth and rough endoplasmic reticulum, mitochondria, Golgi bodies, and secretory granules relative to small luteal cells. The most widely held view is that large luteal cells are derived from the granulosa cells of the follicle, whereas the theca cells of the luteinizing follicle give rise to the small luteal cell population. This hypothesis is supported by several lines of evidence, including that isolated granulosa and theca cells can be induced to undergo differentiation *in vitro* and develop many of the characteristics of large and small luteal cells, respectively. Moreover, monoclonal antibodies that uniquely recognize granulosa and theca cell surface antigens revealed that small luteal cells bound theca-specific antibodies, whereas large luteal cells bound either granulosa- or theca-specific antibodies. In primates, there is a spatial organization of small luteal cells and large luteal cells that mirrors the orientation of theca and granulosa cells in the follicle, wherein the inner layer of large luteal cells is surrounded by an infolded layer of small luteal cells (Fig. 2). Furthermore, removal of granulosa cells from the preovulatory follicle leads to reductions in luteal mass and serum progesterone levels in both domesticated animal species and primates. These studies establish granulosa-derived luteal cells as being a key contributor to the progesterone-secreting cells in the corpus luteum.

The overall steroidogenic potential increases as the corpus luteum develops, especially through the early-to-mid luteal phase in species with a short-lived corpus luteum. Increases in luteal cell number/size, along with vascular formation, are tightly coupled to overall corpus luteum function after ovulation as these events occur in parallel with the rise in circulating progesterone levels. Angiogenesis in the corpus luteum is unique from the standpoint of its rapidity and that it occurs as part of a recurring physiological event. Even in comparison to what is observed in tumors or the placenta, the level of luteal vessel and lymphatic development is remarkable. The initial cellular proliferation that is observed in the developing corpus luteum can be attributed mostly to microvascular endothelial cell expansion. Prior to the midcycle LH surge, the vasculature of the preovulatory follicle is restricted to a capillary network that does not extend beyond the theca layer (Fig. 1). After the LH surge, cellular and extracellular matrix remodeling results in the breakdown of the basement membrane that separates the theca cell layer from the granulosa cell layer, which also allows for the growth and invasion of new capillary beds and lymphatics into the forming corpus luteum. After the network of luteal vessels is formed, the number of endothelial cells is estimated to equal the number of luteal cells. This is particularly true in the primate corpus luteum, wherein there is a microvessel in direct contact with every luteal cell. It is thought that this close contact between steroidogenic luteal cells and the vasculature allows for rapid transfer of progesterone into the circulation for its distribution to responsive tissues.

The induction of endothelial cell proliferation and vessel growth is controlled by angiogenic factors that are secreted primarily by luteal cells. The pro-angiogenic substances studied to date in the greatest detail include the proteins that comprise the vascular endothelial growth factors (VEGFs). There are several different VEGF family members, but the various isoforms that constitute the VEGFA subgroup have received the most attention with regard to promoting luteal angiogenesis. VEGFA is produced by luteal cells and acts in a paracrine manner through specific cell surface VEGFA receptors that are present on microvascular endothelial cells. Whereas VEGFA production is strongly induced by hypoxia in many tissues or cells, evidence suggests that its production in the developing corpus luteum may also be directly regulated by transcriptional or post-transcriptional mechanisms that are induced by the midcycle LH surge.

Other factors acting locally often promote or counter-act the effects of the VEGFs, thereby controlling the overall density of the vasculature in the corpus luteum by directing vessel growth, stability, and regression. Thus, the level of angiogenesis that occurs

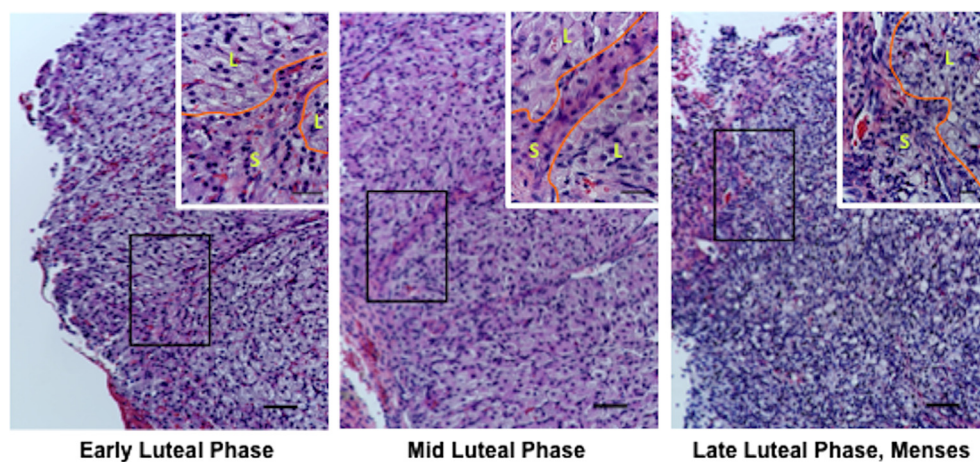


Fig. 2 Distinct small and large luteal cell populations in the primate corpus luteum. Histological hematoxylin and eosin stained sections of corpora lutea isolated from rhesus macaques 2–3 days after ovulation (early luteal phase; period of corpus luteum development), 4–5 days after ovulation (mid luteal phase; period of peak corpus luteum progesterone synthesis), or at the time of menses (late luteal phase; 2–3 days after luteal progesterone production ceases, time of menses). In contrast to rodent species, the corpus luteum from primates possesses distinct small and large populations of luteal cells. Insets are a higher magnification (scale bar = 25 μ m) of the region outlined by the black box present in each larger panel (scale bar = 100 μ m). The lines in the insets highlight the boundaries between regions containing primarily small (S) and large (L) cells.

during luteal development is tightly controlled by balancing the production of pro- and anti-angiogenic factors. As an example, the proteins angiopoietin-1 and -2 (ANGPTs) are essential for balancing vessel growth, stabilization, and regression. ANGPT1 recruits pericytes that surround and stabilize small vessels, whereas ANGPT2 promotes endothelial cell proliferation and capillary tube formation in the presence of VEGFs. A local ratio favoring ANGPT1 over ANGPT2 promotes capillary growth and maturation. When the levels of ANGPT2 exceed VEGF or ANGPT1, blood vessels are destabilized and capillary regression ensues. While the VEGF/ANGPT systems have received the most attention with regard to controlling angiogenesis in the corpus luteum, advances in genomic and molecular techniques revealed numerous other factors exist that also play an important role in this process.

Corpus Luteum Function in the Absence of Pregnancy

Producing sufficient quantities of progesterone to prepare the uterus for implantation and sustain early pregnancy is arguably the most important function of the corpus luteum. In women, it is estimated that at mid luteal phase, when steroid synthesis is at its peak, the corpus luteum produces 40 mg of progesterone every day, making it one of the most highly steroidogenic structures on a per weight basis. In contrast to nonprimate species, the primate corpus luteum also synthesizes significant amounts of estrogens. In primate species, pituitary-derived LH is critical for luteal function since blocking its secretion at mid luteal phase through the administration of gonadotropin releasing hormone (GnRH) antagonist stops progesterone synthesis and causes premature regression of the developed corpus luteum. By mid luteal phase, pulses of LH are actually entrained to corpus luteum progesterone secretion. In ruminants, the majority of circulating progesterone is derived from the large luteal cells that are thought to be independent of LH action. GnRH antagonist treatment of cows and sheep only modestly reduce circulating progesterone levels in a stage-specific manner. Large luteal cells of ungulates appear, therefore, to undergo a differentiation process that results in constitutive, LH-independent production of progesterone. In rodents, progesterone production by corpora lutea that develop following pseudopregnancy or pregnancy is not dependent on LH. Instead, prolactin production by the anterior pituitary or prolactin-like hormones from the uterine decidua and placenta are the essential luteotropins.

To accommodate such a high rate of steroid production, a significant supply of the steroid precursor cholesterol is required by luteal cells (Fig. 3). Data from studies utilizing inhibitors of *de novo* cholesterol synthesis revealed that the corpus luteum possesses only a very limited capacity to synthesize its own cholesterol. Instead, the corpus luteum is reliant on the uptake of circulating cholesterol from either high-density lipoprotein (HDL) or low-density lipoprotein (LDL) particles. Primate species preferentially utilize LDL, whereas in rodents HDL is the predominant source of cholesterol for steroid synthesis. Ruminants utilize either HDL or LDL as the source of cholesterol for steroid synthesis. Following cholesterol uptake from HDL or LDL, cholesterol can be stored in lipid droplets as cholesterol esters. Free cholesterol is then liberated by the actions of one or more lipase enzyme(s) and can then be transported to the mitochondria for entry into the progesterone synthesis pathway. Thus, another mechanism whereby luteal cells can acutely increase the availability of substrate for steroidogenesis is by hydrolysis of intracellular stores of cholesterol esters in lipid droplets.

The expression of key enzymes responsible for progesterone production, including CYP11A1 (side chain cleavage enzyme, converts cholesterol to pregnenolone) and HSD3B (3 β -hydroxysteroid dehydrogenase, converts pregnenolone to progesterone), increase in the follicle shortly after ovulation and through the initial stages of corpus luteum development (Fig. 3). In parallel, reduced expression of enzymes involved in androgen (e.g., CYP17A1) and estradiol (e.g., aromatase or CYP19A1) synthesis occurs, being more prevalent in species that do not secrete significant amounts of estradiol (i.e., ruminants). While elevated steroidogenic enzyme expression is important for luteal function, the key rate-limiting step in luteal progesterone production appears to be movement of cholesterol from the outer to the inner mitochondrial membrane, where the first step in steroid synthesis occurs. The protein responsible for mitochondrial cholesterol transport is termed steroidogenic acute regulatory protein (STAR), which has a high level of activity as each STAR molecule shuttles hundreds of molecules of cholesterol before undergoing a cleavage/inactivation event. In rodents, prolactin maintains steroidogenesis by upregulating enzymes involved in progesterone and estradiol synthesis, including STAR, as well as by inhibiting the expression of a gene (AKR1C3) that encodes an enzyme responsible for degrading progesterone. In ruminants, the differential control of STAR expression in large and small luteal cell types matches their ability to produce progesterone. Large luteal cells of ruminants constitutively express high levels of STAR in an LH-independent manner and are the source for the majority of the progesterone (~80%) that comes from the corpus luteum, whereas STAR expression and the limited amount of progesterone produced in small luteal cells is LH dependent. The primate corpus luteum, in contrast, is clearly dependent upon acute LH action since numerous studies demonstrated that blocking LH secretion from the pituitary at any point of the luteal phase inhibits the expression of key steroidogenic enzymes (e.g., STAR, HSD3B) and progesterone production.

Corpus Luteum Luteolysis

The key regulatory mechanisms that determine when and how the corpus luteum ceases to produce progesterone and undergo structural regression in non-fertile cycles is highly species dependent. A common underlying initiator of luteolysis in several species is prostaglandin PGF $_{2\alpha}$. PGF $_{2\alpha}$ is produced by the uterine endometrium of numerous species (e.g., sheep, cow, horse, pig, rodent, and rabbit) and is then transported through the circulation to the corpus luteum to initiate events that ultimately result in luteolysis. In mice, both luteolysis and parturition are intertwined and dependent on the action of PGF $_{2\alpha}$ since it is required to halt

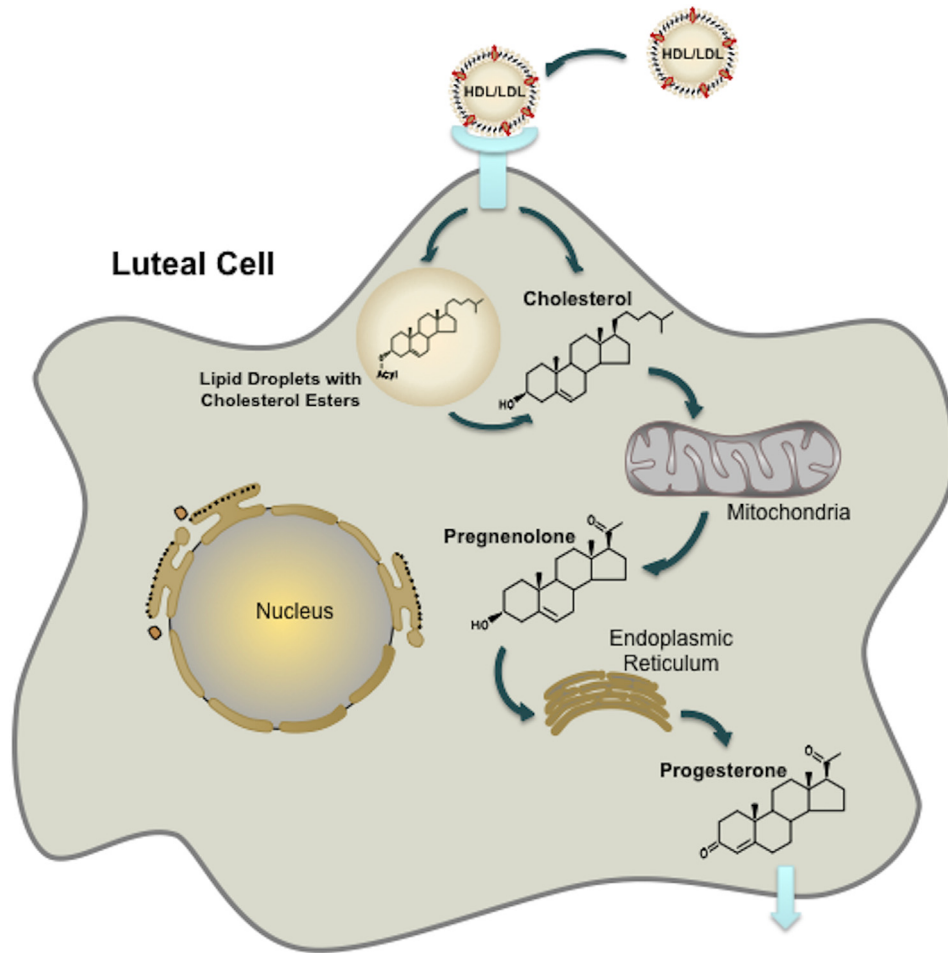


Fig. 3 Luteal cell cholesterol uptake and utilization. Steroidogenic luteal cells obtain cholesterol from circulating sources, including HDL and/or LDL particles or from internal stores found within lipid droplets, which collectively provides the necessary substrate for progesterone synthesis. Cholesterol is transported into the mitochondria, where it is converted to pregnenolone. Pregnenolone leaves the mitochondria and is subsequently converted to progesterone in the endoplasmic reticulum, where it moves out of the cell and into the extensive vasculature that exists throughout the corpus luteum. Figure generated using ScienceSlides from VisiScience.

progesterone synthesis that in turn allows for the initiation of labor. $\text{PGF2}\alpha$ is well documented as the key luteolytic agent in domesticated animal species where it is produced by the uterine endometrium in non-fecund cycles. Hysterectomy in these species results in an extension of luteal lifespan by removing the source of $\text{PGF2}\alpha$ production. In primate species, however, regression is independent of a uterine-derived luteolytic factor as illustrated by the fact that luteal lifespan is not changed by hysterectomy. The finding that a uterine derived signal is not responsible for luteal regression suggests that an internal “self-destruct” mechanism is responsible for primate luteolysis at the end of the non-fecund menstrual cycle. One mechanism that has emerged as a regulator of luteal function in primates is the loss of sensitivity to the luteotropic hormone LH. It is well established that in the early luteal phase, LH pulses from the pituitary are frequent and luteal cells exhibit the greatest degree of LH sensitivity in terms of its ability to stimulate progesterone production. By mid-late to late luteal phase, LH pulse frequency declines and luteal cells are less responsive to LH due to the desensitization of the LH receptor. Loss of sensitivity to LH is an early indicator of functional regression in the primate corpus luteum and appears to be critical for its demise, since sustaining LH levels either by promoting endogenous LH pulses or administering exogenous LH does not prolong primate luteal lifespan. It is presently unclear if the loss of LH sensitivity in the primate corpus luteum is the primary initiator of luteolysis or if it is secondary to other luteolytic processes. With regard to the latter, intraluteal $\text{PGF2}\alpha$ synthesis and action has been proposed to serve as a primate luteolysin because the administration of exogenous $\text{PGF2}\alpha$ will directly induce luteolysis. Recent characterization of the cellular and molecular processes known to control prostaglandin synthesis, metabolism, and signaling suggest that changes in the balance from luteotropic prostaglandins (e.g., PGE2) to luteolytic prostaglandins (e.g., $\text{PGF2}\alpha$) may serve as intraluteal determinants of primate luteal regression. Another proposed pro-luteolytic substance is estradiol, which is based on the ability of the primate corpus luteum to produce and respond to estradiol through the estrogen receptor β ($\text{ER}\beta$, or ESR2). As progesterone production becomes entrained to pituitary-derived LH pulses just prior to the onset of functional regression, there are intervals where estradiol production continues that could promote luteal

demise during the nadir of luteal progesterone secretion. However, such a role for estradiol in serving as a key primate luteolysin has yet to be established. A more detailed overview of luteal regression, primarily as related to the well-characterized luteolytic mechanisms that occur in non-primate species, is provided in the next article.

Extended Corpus Luteum Function in Pregnancy

As is the case for luteolysis, the processes that are responsible for ensuring luteal survival and progesterone production through pregnancy are highly species dependent, contingent on whether they possess ultra-short, short, or long-lived corpora lutea. In rodents, continued luteal structure/function during pregnancy is dependent on a combination of prolactin and prolactin-related (e.g., placental lactogens) factors derived from maternal (pituitary, uterine) and fetal (trophoblast) sources that bind to the prolactin receptor expressed by luteal cells. Twice-daily pulses of prolactin production by the pituitary gland, induced by mating and continue through the first 8–9 days of pregnancy, serve to sustain rodent corpus luteum function. By approximately day 10–11 of pregnancy, placental lactogens assume the role as the predominant luteotropic hormone. In ruminants, the anti-luteolytic substance is interferon-tau (IFN- τ) produced by the embryo around the time of implantation. There is a high sequence homology of IFN- τ across ruminant species, where it apparently has a unique role in pregnancy initiation. One mechanism through which IFN- τ prolongs the function of the corpus luteum is by preventing high amplitude pulses of luteolytic PGF 2α . In many primates, from Old World monkeys to humans, embryonic trophoblast production of chorionic gonadotropin (CG) “rescues” the corpus luteum and extends luteal function in early pregnancy. CG binds to and signals through the same receptor that binds to LH. Similar to the gonadotropic hormones follicle stimulating hormone (FSH) and LH, CG is composed of a common alpha subunit as well as a unique beta subunit that is highly glycosylated and endows an increased half-life in the maternal bloodstream. After circulating CG is first detected around the time of implantation, its levels increase exponentially and peak in the first trimester. Administration of increasing amounts of recombinant human LH, as well as human CG, around the expected time of implantation increased progesterone levels and prevented timely luteolysis during the menstrual cycle in macaque monkeys. These data are consistent with the concept that CG’s ability to rescue the primate corpus luteum is due to differences in the circulating gonadotropin support; with LH being secreted in non-fecund cycles in intermittent pulses that result in periods (i.e., troughs) of LH deprivation, whereas CG levels increase continuously and at increasing levels in fertile cycles. Other studies have also provided evidence that CG signaling through the LH/CG receptor preferentially activates pathways responsible for promoting steroidogenesis, whereas LH is more potent in regulating the pathways that are important for cell survival during luteal development. By the end of the first trimester, the progesterone production needed to maintain pregnancy shifts from the corpus luteum to the placental trophoblast.

Summary and Future Research

Recent advances in methods that allow for genome-wide analyses are providing insight into the dynamic changes in corpus luteum gene expression that occur as a consequence of its development, function, regression during non-fecund cycles, as well as in response to fertilization and pregnancy. From these studies, there is now an unprecedented opportunity to carefully and systematically understand the coordinated events that dictate luteal structure and function. Potential areas of research that would benefit from recent high-throughput “omic” approaches include defining the underlying initiators and effectors of luteal regression in primate species as well as whether luteal regression after the luteal-placental shift is comparable to that occurring at the end of the non-fecund cycle. Due to the dynamic and ephemeral nature of the corpus luteum, there are also opportunities to understand basic biological processes relevant to various pathologies and diseases. For example, the corpus luteum offers a unique model for studying microvessel development, maintenance, and regression in a normal tissue. Based on the considerable amount of cholesterol required to support luteal progesterone production, the corpus luteum may also provide insight into the cellular processes that are important for cholesterol ester utilization and disposition. Studying the regression of the corpus luteum would also yield information regarding the processes involved in initiating cellular death and senescence.

It is obvious recent advances in reproductive and cell biology that are made from studying the corpus luteum are possible only because of the research conducted by the pioneers in the field that came before us. In this regard, acknowledgement of Drs. Gordon Niswender and William Hansel, both of which passed in 2017 is warranted. We owe them a great deal of gratitude for setting the foundation for our current understanding of corpus luteum biology.

Further Reading

- Davis, J. S., & Rueda, B. R. (2002). The corpus luteum: An ovarian structure with maternal instincts and suicidal tendencies. *Frontiers in Bioscience*, 7, 1949–1978.
- Devoto, L., Fuentes, A., Kohen, P., Cespedes, P., Palomino, A., Pommer, R., Munoz, A., Strauss, J. F., 3RD (2009). The human corpus luteum: Life cycle and function in natural cycles. *Fertility and Sterility* 92, 1067–1079.
- Murphy, B. D. (2000). Models of luteinization. *Biology of Reproduction*, 63, 2–11.
- Niswender, G. D., Davis, T. L., Griffith, R. J., Bogan, R. L., Monser, K., Bott, R. C., Bruemmer, J. E., & Nett, T. M. (2007). Judge, jury and executioner: The auto-regulation of luteal function. *Society of Reproduction and Fertility Supplement*, 64, 191–206.

- Niswender, G. D., Juengel, J. L., Silva, P. J., Rollyson, M. K., & McIntush, E. W. (2000). Mechanisms controlling the function and life span of the corpus luteum. *Physiological Reviews*, 80, 1–29.
- Pate, J. L., Johnson-Larson, C. J., & Ottobre, J. S. (2012). Life or death decisions in the corpus luteum. *Reproduction in Domestic Animals*, 47(Suppl. 4), 297–303.
- Pate, J. L., & Landis Keyes, P. (2001). Immune cells in the corpus luteum: Friends or foes? *Reproduction*, 122, 665–676.
- Smith, G. W., & Meidan, R. (2014). Ever-changing cell interactions during the life span of the corpus luteum: Relevance to luteal regression. *Reproductive Biology*, 14, 75–82.
- Stouffer, R. L., Bishop, C. V., Bogan, R. L., Xu, F., & Hennebold, J. D. (2013). Endocrine and local control of the primate corpus luteum. *Reproductive Biology*, 13, 259–271.
- Stouffer, R. L., & Hennebold, J. D. (2015). Structure, function, and regulation of the corpus luteum. In T. M. Plant, & A. J. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn, pp. 1023–1076). London, UK: Academic Press.

Luteolysis

Joy L Pate, The Pennsylvania State University, State College, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

AKR1C3	Aldo-keto reductase family 1 member C3 (also known as PGFS and PTGFS)
ANGPT	Angiopoietin
ATF3	Activating transcription factor 3
cAMP	Cyclic adenosine monophosphate
CASP3	Caspase 3
CBR1	Carbonyl reductase 1
CCL	C-C motif chemokine ligand
CRE	cAMP response elements
CREB	cAMP responsive element binding protein
CYP11A1	Cytochrome P450 family 11 subfamily A member 1
CXCL	C-X-C motif chemokine ligand
EGR1	Early growth response 1
FOS	Fos proto-oncogene, AP-1 transcription factor subunit
LGALS	Galectin
ICAM1	Intercellular adhesion molecule 1
IFNG	Interferon gamma
IL	Interleukin
JUN	Fun proto-oncogene, AP-1 transcription factor subunit
KDR	Kinase insert domain receptor (also known as VEGFR2)
MAPK	Mitogen-activated protein kinases
NR4A1	Nuclear receptor subfamily four group A member 1
PG	Prostaglandin
PGF2A	Prostaglandin F2alpha
PTGFR	Prostaglandin F receptor
PROK1	Prokineticin 1
PTGS2	Prostaglandin-endoperoxide synthase 2
RIPK	Receptor interacting serine/threonine kinases
SELP	Selectin P
SERPINE1	Serpin family E member 1
SPP1	Secreted phosphoprotein 1
StAR	Steroidogenic acute regulatory protein
TGFB1	Transforming growth factor beta 1
THBS1	Thrombospondin 1
TNF	Tumor necrosis factor
TNFRSF1A	TNF receptor superfamily member 1A
VEGFA	Vascular endothelial growth factor A

Introduction

The corpus luteum (CL) is a transitory endocrine gland that forms on the ovary from the granulosa and thecal cells that remain in the postovulatory follicle. Its function is to secrete progesterone, preparing the uterus for implantation, as well as maintaining pregnancy by promoting uterine quiescence. If fertilization does not occur, or if embryonic signaling is insufficient, the CL will regress. Luteal regression (luteolysis) results in pregnancy failure, but also removes the negative feedback of progesterone on gonadotropin release, allowing for maturation and ovulation of a new follicle and another opportunity to establish a pregnancy. The ephemeral nature of the CL has intrigued scientists for decades and understanding the mechanisms responsible for luteolysis could shed light on the nature of cellular life or death in other tissues, including tumors. Although a viable CL is essential for reproduction in all mammals, the hormonal events that initiate luteolysis and the cellular mechanisms to sustain or overcome it remain at least

partially elusive. This article will highlight the established pathways leading to luteal regression or rescue. For more information and specific citations of work to elucidate luteolytic mechanisms, the reader is referred to previous reviews listed in the Bibliography for this article.

Initiation of Luteolysis

Luteolysis is the process by which functional and structural changes culminate in the rapid degradation and eventually, complete disappearance of the CL. The hallmark of luteolysis is the functional change that occurs, which is cessation of steroid production. This is followed by the somewhat more gradual structural changes, such as breakdown of extracellular matrix, cellular death, and removal of cellular debris. Hysterectomy prolongs the lifespan of the CL in sheep, cows, and guinea pigs, demonstrating that the uterus is necessary for normal timing of luteal regression. This is true for most species studied, albeit it is not the case in primates or dogs, in which hysterectomy does not alter ovarian cyclicity. In nonpregnant bitches, luteolysis appears to be a gradual degenerative process, whereas in pregnant, periparturient bitches, luteolysis is initiated by uteroplacental PGF₂A and progesterone declines more precipitously. The signal that initiates luteolysis in the primate is unknown. There is evidence that luteolysis results from decreased sensitivity of the CL to LH, but may also be initiated by a change in the balance of luteotropic (PGE₂) to luteolytic (PGF₂A) prostaglandins.

One of the major discoveries in reproductive biology during the middle of the 20th century was that the uterine luteolytic signal is prostaglandin F₂ α (PGF₂A). Estrogen from the developing preovulatory follicle stimulates PGF₂A release from the endometrium, with the effectiveness of estrogen being greater after a period of progesterone priming. Progesterone supports accumulation of prostaglandin precursor molecules in the endometrium and ultimately downregulates its own receptor, allowing for estrogen-stimulated expression of oxytocin receptors. A role for oxytocin in timing of luteolysis was first apparent when it was discovered that daily injections of oxytocin during the first week of the bovine estrous cycle resulted in premature regression of the CL and a shortened estrous cycle. Oxytocin infused into the uterus of cyclic ewes stimulates production of PGF₂A, with the greatest effect of oxytocin occurring near the end of the estrous cycle, and the least effect occurring in midcycle ewes. The pattern of endometrial expression of oxytocin receptors parallels uterine responsiveness to oxytocin, which explains why the uterus becomes more sensitive to the effects of oxytocin around the time of luteolysis. Further, after stimulation by oxytocin, the oxytocin receptors are internalized and recycled to the plasma membrane, a process that takes about 6 h, coinciding with the frequency of PGF₂A pulses from the endometrium. Oxytocin therefore serves as a pulse generator for uterine PGF₂A release.

The ruminant CL contains large concentrations of oxytocin. Oxytocin is synthesized and stored in the CL, and is released into the ovarian vein in response to PGF₂A. It was subsequently proposed that the oxytocin pulse generator that is operational during luteolysis involved a positive feedback loop between uterine PGF₂A and luteal oxytocin. In sheep, many of the oxytocin and PGF₂A (measured as the metabolite, 13,14-dihydro-15-keto-PGF₂A, or PGFM) pulses appear to be coincident, whereas in cows, only 50% of PGF₂A and oxytocin pulses are coincident. Continuous infusion or repeated injections of oxytocin in sheep late in the estrous cycle, which should downregulate oxytocin receptors, yielded contradictory results, with an increase in cycle length in one study but no effect in another study. Depletion of luteal oxytocin from the CL of cows also had no effect on estrous cycle length. Furthermore, it has been argued that concentration of luteal oxytocin at the end of the estrous cycle may be insufficient to promote uterine PGF₂A release, because the concentration of luteal oxytocin is greatest on days 5–10 of the estrous cycle and declines thereafter, decreasing before the decline in progesterone. The initial release of PGF₂A from the uterus is likely triggered by pituitary release of oxytocin and the contribution of luteal oxytocin to maintenance of PGF₂A pulses may differ somewhat between sheep and cows, and certainly between ruminants and nonruminants. Some of the controversial results may be due to subtle differences in timing or background concentrations of steroid hormones.

Treatment with progesterone on days 3–5 of the ewe estrous cycle causes premature upregulation of endometrial oxytocin receptors, PGF₂A release, and premature luteolysis, whereas a progesterone receptor antagonist administered on the same days delays luteolysis. Administration of the antagonist on days 12–14 similarly advances oxytocin receptor expression and uterine responsiveness to oxytocin, but the timing of luteolysis is unchanged. Progesterone administered to ewes on estrous cycle days 1–6 also decreases the content of luteinizing hormone (LH) receptors in the CL, and this action of progesterone can be prevented by administration of PGE₁ or PGE₂, resulting in normal luteal weights and estrous cycle lengths, despite progesterone-induced premature release of uterine PGF₂A. This implies that advancing the timing of luteolysis by progesterone involves not only changes in the oxytocin-PGF₂A pulse generator, but also changes within the CL that render it less sensitive to gonadotropins.

Acquisition of Luteolytic Capacity

The developing CL does not regress in response to PGF₂A, although it is not completely refractory to PGF₂A effects. The developing ruminant CL has a sufficient number of PGF₂A receptors and responds to PGF₂A with changes in gene expression, but these CL do not undergo luteolysis. On about day 6 in cows and sheep and day 13 in pigs, CL will regress in response to PGF₂A. This ability to undergo luteolysis in response to PGF₂A has been termed “acquisition of luteolytic capacity.” Unlike in ruminants, in pigs there is a marked increase in the number of PGF₂A receptors in the CL around the time of acquisition of luteolytic capacity. From a practical standpoint, the refractoriness of the early CL to the luteolytic effects of PGF₂A limits use of PGF₂A in estrous synchronization

protocols to the period of time after acquisition, but from a scientific perspective, the physiological differences in CL before and after acquisition have provided a useful model for understanding the changes that must occur for luteolysis to progress normally.

Differences in luteal responses to PGF_{2A} before and after acquisition of luteolytic capacity indicate that luteal prostaglandin metabolism, calcium signaling, and endothelial and immune cell responses to PGF_{2A} are likely critical mediators of the ability of the CL to undergo luteolysis in response to PGF_{2A}. Even with a double dose of PGF_{2A} given on Day 5, progesterone decreases but the CL fully recovers and is functional, indicating that the CL responds to PGF_{2A}, but is protected from its luteolytic effects. Prior to acquisition of luteolytic capacity, luteal endothelial cells do not respond to PGF_{2A} with changes in endothelin 1 or its receptors, as they do in CL with luteolytic capacity, nor do early CL produce cytokines in response to PGF_{2A}. Comparisons of Day 4 and Day 10 CL also indicate differences in the ability of PGF_{2A} to initiate signaling through calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2) and 5' adenosine monophosphate-activated protein kinase (AMPK), which may be necessary for luteolytic capacity. Genes related to immune responses, angiogenesis, and apoptosis were activated in mature CL by PGF_{2A}, but not in early CL. Thus, the ability of PGF_{2A} to cause luteolysis is dependent upon the development and maturation of endothelial and immune cells that, in addition to the steroidogenic cells, characterize the cellular makeup of the mature CL.

Once the CL has acquired luteolytic capacity, the progression of luteolysis depends on the PGF_{2A} concentration and pattern of pulsatility to which the CL is exposed. Administration of subluteolytic doses of PGF_{2A} to ruminants results in a transient decline in progesterone and reduction in luteal volume, but with no effect on the timing of luteal regression. The concentration of PGF_{2A} required to ensure luteolysis depends on the metabolic or reproductive state of the animal, because multiparous cows are less sensitive to PGF_{2A} than primiparous cows and pregnant sheep are less sensitive to PGF_{2A} than nonpregnant sheep. A luteolytic dose of PGF_{2A} will induce expression of immediate early genes that are a part of the luteolytic changes that occur in luteal cells, but without repeated exposure to pulses of PGF_{2A}, these transcripts decline in concentration and the CL is not committed to cell death pathways. The pulsatile pattern of PGF_{2A} release can be disrupted by selective inhibition of the prostaglandin transporter protein (PGT) in the endometrium, and this disruption extends the lifespan of the CL.

Experiments with unilaterally hysterectomized ewes and heifers during normal and oxytocin-induced luteolysis revealed that the retained uterine horn must be ipsilateral to the ovary bearing the CL in order for regression to occur, indicating that the effect of the uterus to initiate luteolysis required a local mechanism of transport of PGF_{2A}. Prostaglandin travels from the uterus to the ovary via counter-current transfer across the anastomoses of the uterine vein and ovarian artery. In sheep, approximately 97% of the PGF_{2A} released from the uterus into the general circulation is metabolized after just one passage through the lungs, making counter-current transport critical for luteolysis. In cows, less, but still greater than 50%, of the PGF_{2A} is quickly metabolized, so delivery of the large concentrations of PGF_{2A} necessary for luteolysis also relies on counter-current transfer in this species. The counter-current mechanism is accomplished by expression of prostaglandin transporter (PGT) in the tunica intima, tunica media, and tunica adventitia of the uteroovarian vein and the ovarian artery. PGT is a 12-membrane-spanning protein that mediates the movement of prostaglandin through cellular membranes, and in this case, across the endothelium from the uterine vein to the ovarian artery.

Although uterine PGF_{2A} does not initiate luteolysis in the primate, withdrawal of gonadotropin support can cause luteolysis, but restoration of gonadotropin results in recovery of the CL, with normal concentrations of progesterone and luteal lifespan. This, coupled with the response of the ruminant CL to subluteolytic doses of PGF_{2A}, indicates that the early stages of luteolysis, as manifested by the initial decline in progesterone, are reversible.

PGF_{2A} Signaling in the Corpus Luteum

The ability of PGF_{2A} to cause luteolysis is dependent on activation of multiple intracellular events within the steroidogenic cells to reduce progesterone secretion, as well as activation of nonsteroidogenic cells to facilitate cellular death and structural degradation of the tissue. In the CL, PGF_{2A} binds to a G protein coupled receptor in the plasma membrane (PTGFR) and initiates luteolysis by activation of phospholipase C, which cleaves inositol triphosphate and diacylglycerol from membrane phospholipids, leading to an increase in intracellular free calcium and activation of protein kinase C. The functional effects of PGF_{2A} (inhibition of steroidogenesis) are mediated by activation of the protein kinase C pathway. The actions of PGF_{2A} are thought to be primarily on the large luteal cells, which contain many PTGFR, although the small steroidogenic cells, as well as endothelial and immune cells, can express PTGFR. PGF_{2A} directly inhibits LH-stimulated progesterone production in cultured steroidogenic luteal cells from rats, ewes, and cows. The PGF_{2A}-induced decrease in progesterone is likely due to an uncoupling of the mechanism by which cholesterol moves to, or associates with, the cholesterol side-chain cleavage enzyme, CYP11A1. This enzyme is located in the mitochondria and converts cholesterol to pregnenolone, the immediate precursor of progesterone. Association of cholesterol with CYP11A1 is the rate-limiting step in steroidogenesis, which makes it a perfect target to achieve a rapid loss of progesterone production. When cultured bovine luteal cells are treated with PGF_{2A}, cholesterol transport to the mitochondria is not disrupted, but the cholesterol is not converted to progesterone. However, side-chain cleavage of 25-OH-cholesterol, which is readily diffusible across membranes, is not inhibited by PGF_{2A}. Therefore, the acute effect of PGF_{2A} may be to prevent movement of cholesterol from the outer to the inner mitochondrial membrane. The StAR protein mediates cholesterol transfer in the mitochondria. Progesterone declines prior to the decrease in StAR mRNA or protein content, but this does not rule out an effect of PGF_{2A} on the process of StAR-mediated cholesterol transfer. The exact mechanism by which PGF_{2A} disrupts cholesterol transfer or metabolism in the mitochondria is unknown.

The activation of protein kinase C induced by PGF2A leads to activation of mitogen-activated protein kinase (MAPK) signaling pathways, including MAPK3 (ERK1/2), MapK8 (JNK), MAPK11 (p38) and ATF3, and upregulation of the transcription factors, Fos, Jun, NR4A1, and EGR1. ATF3 inhibits LH-induced progesterone synthesis by binding to CRE, the transcription factor that is activated by the cAMP/protein kinase A signaling pathway. Activation of MAPK11 and MAPK8 have been linked to regulation of apoptosis and autophagy, and to stimulation of interleukin (IL)8, which serves as a chemoattractant to neutrophils during luteolysis. Gene expression studies have indicated that early changes in luteolysis likely involve immune response, cytokine signaling and cell death pathways, whereas later in luteolysis, genes associated with regulation of cholesterol and steroid biosynthesis predominate.

Upon initiation of luteolysis by PGF2A, there is autoamplification of the PGF2A signal in luteal cells. PGF2A stimulates the enzymes that metabolize arachidonic acid and PGH2–PGF2A (PTGS2 and AKR1C3, respectively), or convert PGE2–PGF2A (CBR1), but downregulate the enzymes that catabolize PGF2A to inactive metabolites. PGE2 activates the cAMP pathway in luteal cells, stimulating progesterone production. Thus, conversion of PGE2–PGF2A may be important to maintain the decline in steroidogenesis. This may be especially critical for luteolysis in species such as primates, in which uterine PGF2A is not required for luteolysis. The high concentrations of progesterone that are present in the CL prior to luteolysis reduce luteal prostaglandin synthesis, but the initial decline in progesterone allows for the autoamplification of PGF2A. In pigs, inhibition of progesterone production prior to the onset of luteolytic capacity allows for exogenous PGF2A to elicit some luteolytic responses. Thus, the life or death of the CL depends, in part, on the balance of progesterone and prostaglandin synthesis that occurs within the CL itself.

Increased concentrations of Ca^{2+} in response to PGF2A lead to upregulation of CAMKK2, which phosphorylates AMPK. Downstream effects of this pathway are reduced expression of the low density lipoprotein receptor and an increase in acyl coenzymeA: cholesterol acyltransferase (ACAT1), which collectively make free cholesterol less available for conversion to pregnenolone. By 12 h after the initiation of luteolysis, genes responsible for cholesterol uptake are downregulated, whereas genes responsible for cholesterol efflux are upregulated. Thus, as cholesterol balance is a critically important feature of the maintenance of steroidogenesis, disruption of cholesterol supply to the cells is also part of the mechanism to reduce steroidogenesis.

The conversion of cholesterol to pregnenolone and the high metabolic activity of luteal cells results in mitochondrial production of free radicals, which have negative effects on both progesterone production and cell viability. Prior to luteolysis, the reactive oxygen species may be neutralized by high concentrations of antioxidant enzymes and lipids, such as glutathione peroxidase and beta-carotene. Recently, it has been demonstrated that lysophosphatidic acid (LPA) completely prevents reactive oxygen species-induced decreases in progesterone production and stimulation of apoptosis. Reactive oxygen species are also produced by luteal endothelial cells and mediate some of the luteolytic effects of PGF2A. Superoxide dismutase concentration and activity is increased by a direct action of PGF2A on endothelial cells, and hydrogen peroxide further enhances PGF2A production by endothelial cells. Therefore, this provides another positive feedback loop to advance luteolysis. Conversely, progesterone suppresses nitric oxide production by luteal endothelial cells, perhaps protecting the CL from premature luteolysis while progesterone is elevated. The intracellular pathways and known downstream mediators of PGF2A signaling in luteal steroidogenic cells are depicted in Fig. 1.

During PGF2A-induced luteolysis, concentrations of VEGFA and ANPT1 in the CL decrease, whereas concentrations of TGFB1 and ANPT2 increase. This leads to destabilization of blood vessels. In fact, capillary endothelial cells are the first cells in the CL to undergo degenerative changes, although the decline in progesterone production by steroidogenic cells precedes the decline in blood flow to the CL, reinforcing the concept that luteolysis is achieved by a multifactorial insult from PGF2A. Another effect of TGFB1 during luteolysis is to promote conversion of fibroblasts to myofibroblasts and the formation of arteriovenous anastomoses, limiting nutrient supply to the extravascular cells. Lymphoangiogenic factors are downregulated by PGF2A, indicating that there is a simultaneous breakdown of the draining lymphatic vessels in addition to the capillary vasculature in the CL.

Recent evidence indicates that viability of luteal cells may be regulated by galectins (LGALS) and by thrombospondin 1 (THBS1). Galectin 1, which binds to glycans on KDR and supports viability of steroidogenic cells, is decreased in regressing corpora lutea. Conversely, LGALS 3 expression is increased by PGF2A during luteal regression. LGALS binds to beta 1 integrin, with a downstream effect of caspase 3 (CASP3) cleavage, resulting in decreased cell viability, but with no direct effect on the steroidogenic pathway. Further, alpha 2,6-sialylation of glycoproteins is increased in response to PGF2A during luteolysis, and this prevents binding of LGALS. For this reason, LGALS1 cannot support viability of late stage luteal steroidogenic cells. Thus, the balance of LGALS1 and LGALS3, and the degree of alpha 2,6-sialylation of glycoproteins may be important regulators of life or death of the steroidogenic cells. Apoptosis of steroidogenic cells is also directed by THBS1, which is increased during luteolysis and activates CASP3. THBS1 also increases expression of TGFB1, which stimulates expression of SERPINE1, facilitating breakdown of extracellular matrix.

In addition to apoptosis, luteolysis may involve both autophagy and programmed necrosis. Within 4 h of PGF2A treatment, the sensors of cellular stress, RIPK1 and RIPK3, are increased in concentration. These proteins are stimulated by TNF and IFNG and lead to necroptotic cell death. Progesterone can prevent apoptosis in luteal steroidogenic cells, but may not be a regulator of programmed necrosis because inhibition of RIPK1 by necrostatin 1 increases cell viability, with no effect on progesterone or caspases. The different pathways by which cell death occurs during luteolysis may be related to the heterogeneous cell types in the CL.

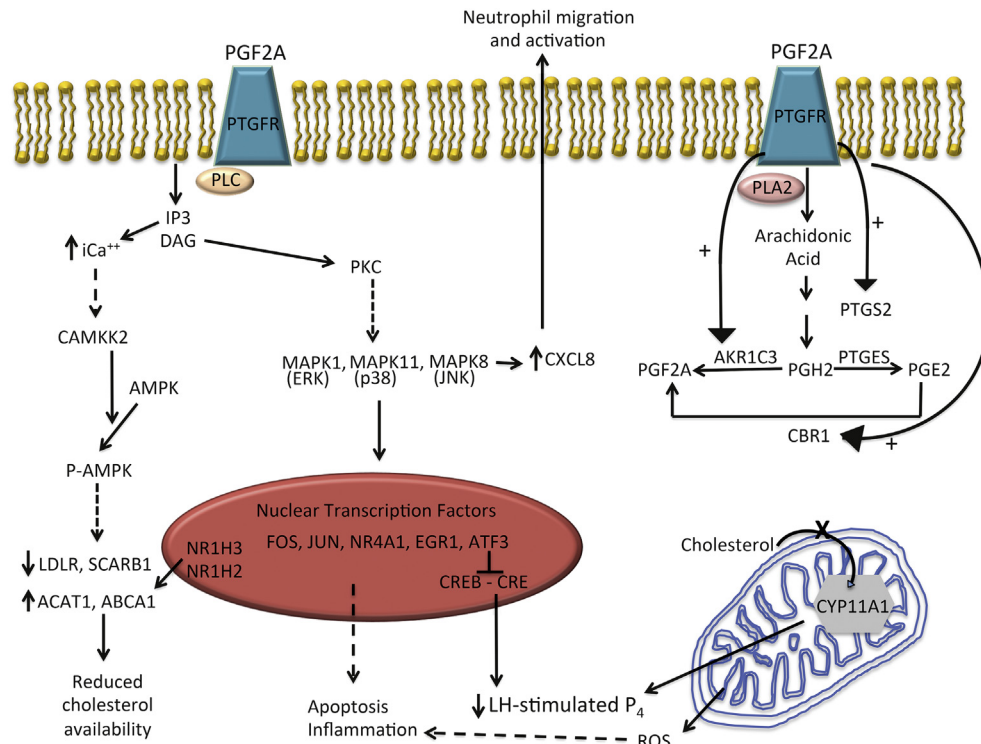


Fig. 1 Mechanisms of prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) action in steroidogenic cells of the CL. PGF $_{2\alpha}$ binds to a receptor (PTGFR) located in the plasma membrane of cells. Activation of phospholipase C (PLC) leads to generation of inositol triphosphate (IP3) and diacylglycerol (DAG), leading to an increase in intracellular calcium and activation of protein kinase C (PKC). Increased concentration of CAMKK2 (calcium/calmodulin-dependent protein kinase kinase 2) results in phosphorylation of adenosine monophosphate kinase (AMPK), leading to decreased expression of low density lipoprotein receptor (LDLR) and scavenger receptor class B member 1 (SCARB1) and increased concentration of acyl coenzymeA: cholesterol acyltransferase (ACAT1) and ATP binding cassette subfamily A member 1 (ABCA1). Collectively, these changes lead to reduced cholesterol availability in luteal cells. Activation of map kinases (MAPK1, MAPK8, and MAPK11) causes upregulation of a number of transcription factors that regulate genes responsible for apoptosis, inflammation and cholesterol homeostasis. One such factor, ATF3 prevents CREB (cAMP responsive element binding protein) binding to CRE (cAMP responsive element), which inhibits LH-stimulated progesterone production. MAPK8 leads to increased synthesis of interleukin (IL)8, which activates neutrophils and promotes migration into the CL. An important effect of PGF $_{2\alpha}$ in luteal cells is to prevent association of cholesterol with the cytochrome P450 side-chain cleavage enzyme (CYP11A1), although the mechanism for this action of PGF $_{2\alpha}$ is unknown. Excessive production of reactive oxygen species (ROS) during luteolysis likely contributes to apoptosis and promotion of an inflammatory response. Finally, activation of phospholipase A2 (PLA2) causes membrane phospholipids to be converted to arachidonic acid, which is the precursor for prostaglandin synthesis. PGF $_{2\alpha}$ stimulates prostaglandin synthase 2 (PTGS2) and prostaglandin F synthase (AKR1C3), as well as carbonyl reductase 1 (CRB1), which results in increased synthesis of PGF $_{2\alpha}$ and a decrease in the ratio of PGE2 (a luteotropic prostaglandin) to PGF $_{2\alpha}$ (a luteolytic prostaglandin). Together, the cascades of signaling events that are initiated by PGF $_{2\alpha}$ in the CL lead to acute inhibition of progesterone production, activation of immune cells, chronic reduction in cholesterol availability for steroidogenesis and apoptosis of endothelial and steroidogenic cells. (Dashed lines indicate multiple steps).

Participation of Nonsteroidogenic Cells in Luteolysis

The direct action of PGF $_{2\alpha}$ to initiate luteolysis is the inhibition of steroidogenesis. Although some of the biochemical changes in luteal cells may promote cell death, a direct effect of physiological concentrations of PGF to initiate cell death in isolated steroidogenic cells has not been demonstrated. The CL is comprised of a heterogeneous population of cells, with capillary endothelial cells being particularly abundant, a network of fibroblasts that develop the supporting extracellular matrix and immune cells scattered throughout the luteal tissue. These cells participate in the luteolytic process. PGF $_{2\alpha}$ stimulates production of endothelin-1 by capillary endothelial cells, which inhibits progesterone production by steroidogenic cells. Cooperative interaction among steroidogenic cells, immune cells and endothelial cells occurs to ensure the progression of luteolysis by recruiting additional immune cells and orchestrating the destruction of the tissue itself.

Immune cells are present in the CL when it is fully functional. Macrophages are necessary for proper angiogenesis during luteal development, and immune cells may facilitate extracellular matrix remodeling and homeostasis of the metabolically active tissue. However, during luteolysis, chemokines (CCL2, CCL8, CXCL2, SPP1, and IL8), and adhesion molecules (SELP, ICAM1, and PROK1) are increased and recruit additional macrophages, T lymphocytes, and neutrophils into the CL. There is evidence of spatial regulation of macrophage recruitment into the human CL, with more abundant macrophages located in areas that lack the enzymes for production of progesterone or PGE, suggesting that these molecules may suppress local immune cell trafficking. Adhesion of

immune cells to bovine luteal endothelial cells is reduced when progesterone concentration is high, but the decline in progesterone early in luteolysis may allow for extravasation of immune cells.

The immune cells produce cytokines, such as TNF, IFNG, and IL1B, which inhibit progesterone production and stimulate prostaglandin production by luteal steroidogenic cells. The latter effect of the cytokines further contributes to the amplification of PGF2A production in luteal cells. TNF and IFNG also modify synthesis of prostaglandins, leukotrienes and endothelins, and promote nitric oxide production in luteal endothelial cells. Expression of both TNF and TNFRSF1A becomes widespread on many types of cells in the CL during luteolysis. TNF and IFNG promote apoptosis of luteal steroidogenic and endothelial cells. IFNG also contributes to structural degradation of the CL by activation of matrix metalloproteinases, resulting in breakdown of the extracellular matrix. TNF is an obligatory component of the luteolytic cascade, because PGF2A fails to induce luteolysis in either TNFRSF1A null mice or in mice treated with TNF antibody.

The immune cells in the regressing CL are distinct from those in the fully functional CL. The immune cells that reside in the functional CL may exit and be replaced with alternative cell types during luteolysis, or more likely, these immune cells are induced to change their function by the luteal microenvironment. Although it was once thought that immune cell phenotype was fixed, it is now widely recognized that many immune cells can exhibit phenotypic plasticity. In coculture experiments, luteal steroidogenic and endothelial cells altered the expression of immune cell membrane and secreted proteins, indicating that the parenchymal cells could program immune cells toward a particular functional phenotype. In the primate CL, the luteal-resident macrophages and neutrophils produce more cytokines/chemokines after progesterone has declined compared to when the CL is fully functional. In the bovine CL, there is a decline in the proportion of regulatory T cells during luteolysis, and a robust upregulation of the CD8 $\alpha\alpha$ homodimer on CD8⁺ T cells in the CL by 8 h after exposure to PGF2A. This represents a shift from suppression of an inflammatory response in the functional CL, to resolution of an inflammatory response in the regressing CL. Studies of acute changes in mRNA in the CL indicate that proinflammatory cytokines may be early mediators of luteolysis, but it would be detrimental to ovarian function if such a response was maintained. Thus, functional changes in immune cells are necessary to resolve any inflammatory process that was initiated during luteolysis. Cellular interactions and mediators of luteal regression are highlighted in Fig. 2.

Luteal Rescue

The transitory nature of the CL is necessary to allow for repeated opportunities for fertilization, because follicular maturation and ovulation are usually suppressed when progesterone concentrations are high. However, if fertilization has occurred, it is essential that the CL be maintained. Therefore, most species that rely on the CL for pregnancy maintenance have developed mechanisms by which signals from the developing embryo result in rescue of the CL from luteolysis. Prevention of luteolysis by embryonic signals in early pregnancy has been termed “maternal recognition of pregnancy.”

In species in which uterine PGF2A initiates luteolysis, rescue of the CL involves changes in uterine secretion of PGF2A, and the ratio of PGE2–PGF2A released from the uterus is greater than that observed during luteolysis. In ruminants, these changes are in response to embryonic secretion of a Type I interferon, IFN τ (IFNT), which acts on endometrial cells to inhibit expression of estrogen receptors. The lack of estrogen action in the endometrium results in less expression of oxytocin receptors, which disrupts the normal pulsatile release of PGF2A that is necessary to cause luteolysis. In pigs, the pregnancy recognition signal is estrogen, which redirects endometrial PGF2A from endocrine (into the uterine vein) to exocrine (into the uterine lumen) secretion. Equine embryos migrate between the two uterine horns and presumably signal to the endometrium, because there is lesser expression of oxytocin receptors and prostaglandin synthase 2 than observed in cyclic mares, and PGF release is not pulsatile. However, the identity of the embryonic signal in the mare is unknown. In primates, the embryonic trophoblast produces chorionic gonadotropin, which directly provides gonadotropic support and stimulation of steroidogenesis in the CL. In the domestic dog, no embryonic signal is required to maintain the CL, because the CL of a nonfertile cycle is maintained beyond that of a CL of pregnancy.

Rescue of the CL also involves mechanisms within the tissue to maintain steroidogenesis and capillary integrity. During maternal recognition of pregnancy, the CL becomes less sensitive to the luteolytic effects of PGF2A, and the autoamplification of PGF2A that occurs during luteolysis is prevented by inhibition of AKR1C3 and stimulation of the enzymes that convert PGF2A to inactive metabolites. Luteal cells *in vitro* are responsive to IFNT, and during the period of maternal recognition of pregnancy, interferon-stimulated genes (ISG) are expressed in the CL. Some IFNT does exit the uterus to enter the general circulation and may reach the CL, although the amount and role of embryonic IFNT within the CL is controversial. Circulating white blood cells also express ISG in response to embryonic IFNT. During maternal recognition of pregnancy there is a transient influx of neutrophils into the CL, so it has been proposed that IFNT-activated neutrophils may induce the changes in ISG that are observed in the CL.

The CL of pregnancy is protected from effects of oxygen radicals by maintenance of antioxidant enzymes and synthesis of lysophosphatidic acid (LPA). LPA also stimulates synthesis of PGE2, which protects the CL from oxytocin-induced luteolysis, possibly by preventing loss of LH receptors to maintain steroidogenesis. Small noncoding RNA (microRNA) that regulate protein synthesis are differentially expressed in the CL of pregnancy compared to the CL of the cycle, indicating these molecules may support intracellular pathways that result in cell survival. Based on *in silico* analyses of luteal microRNA, they likely regulate immune cell functions within the CL of early pregnancy. This is supported by the observation that immune cell phenotype is altered in the CL of pregnancy compared to the CL of the cycle. Although the exact cellular interactions and intracellular biochemical pathways have

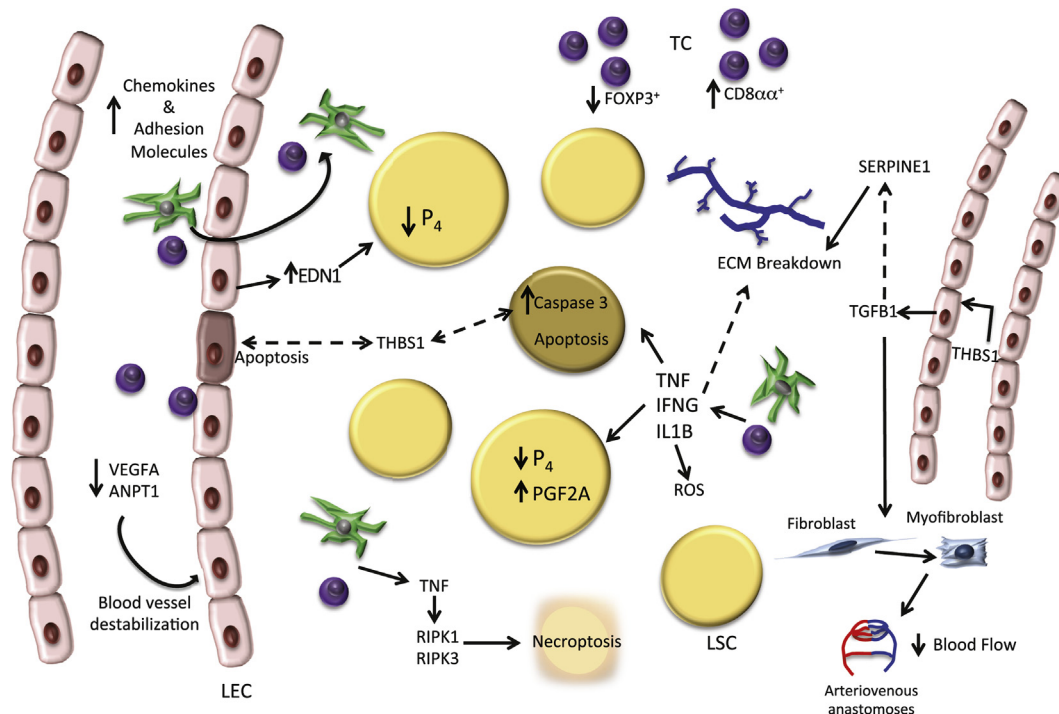


Fig. 2 Changes that occur in the CL during luteolysis. Functions of luteal endothelial cells (LEC) change early in luteolysis. LEC produce endothelin 1 (EDN1), which acts on luteal small and large steroidogenic cells (LSC) to decrease progesterone production. Endothelial cell expression of chemokines (CCL2, CCL8, CXCL2) and adhesion molecules (SELP, ICAM1, PROK1; see nomenclature), results in recruitment of immune cells and extravasation into the luteal parenchyma, where they can be in intimate contact with LSC. There is decreased expression of growth factors (VEGFA, ANPT1) in the CL, which results in destabilization of blood vessels and disruption of blood flow. Thrombospondin 1 (THBS1) is produced by both LEC and LSC and has multiple effects in the CL during luteolysis, leading to activation of caspase 3, which is part of the apoptotic cascade, and to increased expression of transforming growth factor beta 1 (TGFβ1). TGFβ1 increases synthesis of SERPINE1, facilitating breakdown of the extracellular matrix (ECM). TGFβ1 also causes transformation of fibroblasts into myofibroblasts, which leads to formation of arteriovenous anastomoses in the CL during luteolysis, decreasing oxygen availability to the luteal cells. Activated immune cells in the CL produce cytokines (TNF, IFNG, IL1B) that inhibit LH-stimulated progesterone and stimulate PGF2A production in LSC, promote production of reactive oxygen species (ROS) and ECM breakdown, and cause apoptosis of LSC. TNF also stimulates receptor interacting serine/threonine kinases (RIPK1, RIPK3), which promote another cellular death pathway, necroptosis. There are also changes in the phenotype of T cells (TC) in the CL. Loss of FoxP3⁺ cells and increased surface expression of the CD8αα/CD8αβ heterodimer indicates a shift from suppression of a proinflammatory response to resolution of the transient proinflammatory response that occurs early in luteolysis. Although the localization of some events in a particular cell type is as yet undefined, it is clear that each of these types of cells plays a role in orchestrating the changes associated with regression of the CL.

yet to be elucidated, ultimately the signal from the embryo results in maintenance of survival genes and prevents upregulation of death pathway-related genes, protecting the integrity of the luteal vasculature and maintaining steroidogenesis. This event is critical for successful reproduction, so future studies to understand the mechanisms associated with luteal rescue are of the utmost importance.

Further Reading

- Arosh, J. A., Banu, S. K., & McCracken, J. A. (2016). Novel concepts on the role of prostaglandins on luteal maintenance and maternal recognition and establishment of pregnancy in ruminants. *Journal of Dairy Science*, 99, 5926–5940.
- Bazer, F. W. (2013). Pregnancy recognition signaling mechanisms in ruminants and pigs. *Journal of Animal Science and Biotechnology*, 4, 23.
- Kato, H., Sugino, N., Takiguchi, S., Kashida, S., & Nakamura, Y. (1997). Roles of reactive oxygen species in the regulation of luteal function. *Reviews of Reproduction*, 2, 81–83.
- Kowalewski, M. P. (2012). Endocrine and molecular control of luteal and placental function in dogs: A review. *Reproduction in Domestic Animals*, 47, 19–24.
- Kowalewski, M. P., Gram, A., Kautz, E., & Graubner, F. R. (2015). The dog: Nonconformist, not only in maternal recognition signaling. In R. D. Geisert, & F. W. Bazer (Eds.), *Advances in anatomy, embryology and cell biology: vol. 216. Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"* (pp. 215–237). Basel: Springer International Publishing.
- Maalouf, S. W., Liu, W.-S., Albert, I., & Pate, J. L. (2014). Regulating life or death: Potential role of microRNA in rescue of the corpus luteum. *Molecular and Cellular Endocrinology*, 398, 78–88.
- McCracken, J. A. (1980). Hormone receptor control of PGF2a secretion by the ovine uterus. *Advances in Prostaglandin and Thromboxane Research*, 8, 1329–1344.
- McCracken, J. A. (1999). Luteolysis. *Encyclopedia of Reproduction*, 2, 1083–1094.
- Meidan, R., Girsh, E., Mamluk, R., Levy, N., & Farberov, S. (2017). Luteolysis in ruminants: Past concepts, new insights, and persisting challenges. In R. Meidan (Ed.), *The life cycle of the corpus luteum* (pp. 159–182). Basel: Springer International Publishing.

- Pate, J. L., Johnson-Larson, C. J., & Ottobre, J. S. (2012). Life or death decisions in the corpus luteum. *Reproduction in Domestic Animals*, 47(Suppl. 4), 297–303.
- Smith, G. W., & Meidan, R. (2014). Ever-changing cell interactions during the lifespan of the corpus luteum: Relevance to luteal regression. *Reproductive Biology*, 14, 75–82.
- Townson, D. H. (2006). Immune cell-endothelial cells interactions in the bovine corpus luteum. *Integrative and Comparative Biology*, 46, 1055–1059.
- Walusimbi, S. S., & Pate, J. L. (2013). The role of immune cells in the corpus luteum. *Journal of Animal Science*, 91, 1650–1659.
- Wiltbank, M. C., Meidan, R., Ochoa, J., et al. (2016). Maintenance or regression of the corpus luteum during multiple decisive periods of bovine pregnancy. *Animal Reproduction*, 13, 217–233.

FEMALE ENDOCRINOLOGY

Estrous and Menstrual Cycles

Romana A Nowak, University of Illinois, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Corpus luteum A yellow ovarian endocrine structure formed after the oocyte is released and composed of transformed granulosa cells and theca interna cells that secretes progesterone and oxytocin.

Decidualization Process that results in significant morphological and functional changes in endometrial stromal cells under the influence of progesterone during the luteal phase of the cycle.

Endometrium The mucosal lining of the uterus consisting of glandular epithelium and stroma.

Estradiol The predominant estrogen produced by the granulosa cells of the ovarian follicle from androgen precursors. Estradiol is an 18'carbon steroid.

Follicle A spherical structure in the ovary that contains an oocyte surrounded by granulosa cells, theca interna and theca externa. Follicles will progress from primordial to primary, secondary, and finally antral follicles.

Follicle stimulating hormone (FSH) A pituitary glycoprotein hormone that initiates and sustains follicle development and steroidogenesis.

Follicular phase The phase of the estrous cycle characterized by the presence of a dominant follicle that produces estradiol. It is the period before ovulation.

GnRH pulse generator A group of endogenously pulsatile interconnected neurons located in the mediobasal hypothalamus that synthesize and release GnRH into the portal vasculature to thereby stimulate pituitary gonadotropins synthesis and secretion.

Gonadotropins The hormones FSH and LH are the two gonadotropins produced by the gonadotropes in the anterior pituitary. These are both glycoprotein hormones consisting of a common alpha subunit and a unique beta subunit. Both hormones are regulated by GnRH from the hypothalamus.

Hypothalamus Part of the brain located around the inferior portion of the third ventricle. It receives ascending neural signals from the brain stem and descending neuronal input from the limbic lobe and frontal cortex and projects primarily to the median eminence and posterior pituitary.

Kisspeptin A protein hormone that plays an important role in initiating the secretion of GnRH from the hypothalamus. Kisspeptin producing neurons are located in the anteroventral periventricular nucleus and in the arcuate nucleus.

Luteal phase The second half of the cycle following the LH surge and ovulation, which is characterized by progesterone dominance and the presence of a functional corpus luteum. The luteal phase begins after ovulation and ends when the corpus luteum regresses.

Luteinizing hormone (LH) A pituitary glycoprotein hormone that is produced by the gonadotropes. LH has two main functions in the ovary; (1) to stimulate theca cells of the ovary to secrete androgens including androstenedione; and (2) to cause ovulation of ovarian follicles and formation of the corpus luteum.

Ovary The female gonad that is the source of oocytes needed for reproduction in the female.

Pituitary Endocrine gland that consists of the anterior and intermediate glandular lobes and the neurohypophysis or neural lobe. It is connected to the hypothalamus by the hypothalamo-hypophyseal-portal blood system.

Progesterone A 21'carbon steroid made and secreted by the corpus luteum in high concentrations after ovulation for approximately 14 days. Progesterone is required for maintenance of pregnancy.

Proliferative phase Endometrial events that occur during the first half of the menstrual cycle, namely the proliferation and growth of the endometrium in response to sustained exposure to estradiol.

Secretory phase Endometrial events that occur during the second half of the menstrual cycle, when the endometrial glands gain full secretory activity in response to progesterone and estrogen.

Estrous Cycles

Unlike mature male mammals, where gametogenesis and steroidogenesis occur continuously throughout life, females exhibit repetitive cyclic changes that occur either as estrous cycles or menstrual cycles. This reproductive cyclicity is initiated after puberty and allows female mammals with repeated opportunities to copulate, conceive, and bear offspring. The cyclic nature of reproduction in female mammals depends on carefully coordinated cyclic changes in the hypothalamic-pituitary-ovarian axis which result in the production of both mature oocytes and a uterine environment that is prepared to support a conceptus should fertilization occur. Estrous or menstrual cycles will be interrupted by pregnancy and lactation and occur in seasonal breeders only during a specific time of the year that is dependent on daylength.

The beginning of the estrous cycle is the period when the female is sexually receptive (known as estrus or heat) and this stage is soon followed by ovulation of one or more follicles. The overall length of an estrous cycle is measured from one period of estrus to the next succeeding period of estrus. Each estrous cycle can be divided into two main phases, the follicular phase and the luteal phase. The follicular phase of the cycle is dominated by the actions of the ovarian steroid hormone estradiol, whereas the luteal phase is dominated by the steroid hormone progesterone produced by the corpus luteum. Estrous cycles can be very short, lasting only 4–5 days in rodents, or last for much longer periods ranging from 17 to 21 days for most domestic production animals.

Estrous cycles in rodents can be divided into four stages called proestrus, estrus, metestrus, and diestrus each of which last approximately 1–2 days. Proestrus is the stage right before estrus. It is the time during which the corpus luteum undergoes luteolysis resulting in a sharp decline in progesterone, allowing for increases in follicle stimulating hormone (FSH) and luteinizing hormone (LH) which lead to increased estradiol production by ovarian follicles. Estrus then occurs in response to the increasing estradiol levels causing the female to become sexually receptive to the male and this is soon followed by ovulation. Metestrus is the period of transition between ovulation and the formation of the functional corpus luteum. The formation of the corpus luteum from the follicle wall involves significant tissue and cell remodeling and is called luteinization. The final stage of the estrous cycle in rodents is called diestrus and is the period when the completely functional corpus luteum is secreting high amounts of progesterone. If no conceptus is present, the corpus luteum will undergo regression and the female will enter proestrus, the beginning of the next cycle.

The duration of the estrous cycle in domestic animals such as the cow, sow, and mare average 21 days in length whereas the cycle is 17 days in the ewe (see [Table 1](#)). Estrus, the time during which the female is receptive to mating with the male, is variable in length within species. Ruminants such as the cow and ewe experience short estrus periods of 29 h or less while species such as the sow and mare show estrus periods of several days. The cow and sow are polyestrous, with a number of estrous cycles occurring each year, whereas the mare and ewe are seasonally polyestrous. The mare is a long day breeder exhibiting estrous cycles in spring and summer while the ewe is a short day breeder with cycles occurring in the late summer and fall. Some species such as the dog, wolf, and bear experience only one estrous cycle each year and are called monoestrous.

The estrous cycle in domestic animals is divided into two unequal phases ([Fig. 1A and B](#)). The first is the relatively short follicular phase which is the period of rapid follicular development beginning with regression of the corpus luteum of the previous cycle and ending with estrus and ovulation of one or more follicles. The second is the much longer luteal phase which extends from the formation of the corpora lutea after ovulation until their regression at the end of the cycle. The estrous cycle of domestic animals differs from that in rodents due to the fact that rodents lack a true luteal phase. The corpus luteum that forms after ovulation in the absence of mating is very short-lived, lasting only 1–2 days. If mating does occur, the corpus luteum will secrete progesterone for a length of time comparable to that of the corpus luteum of domestic animals. This extended lifespan of the corpus luteum is called pseudopregnancy and also occurs in other species such as rabbits and dogs.

Reproductive Hormones Throughout the Estrous Cycle

The reproductive hormones that regulate ovarian cyclicity show a characteristic ebb and flow which is schematically illustrated in [Fig. 1A](#). The two pituitary gonadotropin hormones luteinizing hormone (LH) and follicle stimulating hormone (FSH) are important regulators of follicular development, ovarian steroidogenesis, and ovulation. The levels of LH are relatively low during the luteal phase and FSH levels follow a similar pattern to LH with a modest rise during the time of luteal regression. These basal levels of gonadotropins are needed to initiate the next round of follicular development. As the ovary enters the follicular phase, and the follicle(s) begin to grow rapidly, estradiol synthesis will increase leading to a marked rise in circulating estradiol levels. Estradiol levels will peak prior to or at the onset of estrus. It is this rise in estradiol levels that leads to the LH surge and ovulation. FSH levels

Table 1 Average reproductive cycles

Species	Length of estrous cycle	Length of estrus	Ovulation	Length of pregnancy
Cow	21 days polyestrous	18 h	11 h after end estrus	282 days
Ewe	17 days seasonal (fall)	29 h	Near end estrus	148 days
Sow	21 days polyestrous	48–72 h	35–45 h after start estrus	115 days
Mare	21 days seasonal (spring) polyestrous	4–8 days	3–6 day of estrus (1–2 days before end of estrus)	335 days

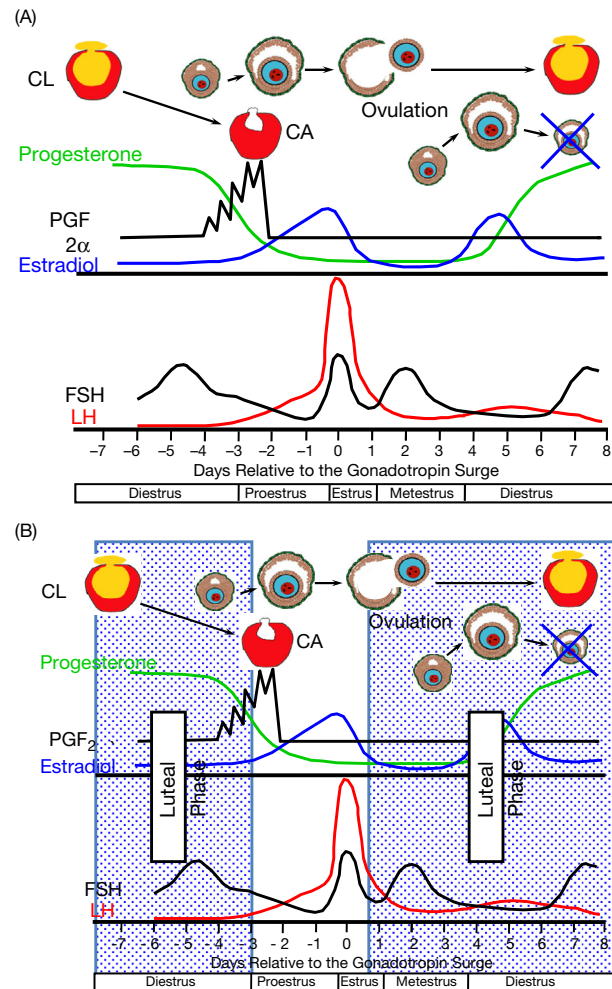


Fig. 1 Schematic outline of ovarian events and changes in reproductive hormone levels throughout the estrous cycle. (A) corpus luteum (CL) regresses to form corpus albicans (CA). New follicles develop, ovulate and form new corpora lutea. Changes in the hormones progesterone, estradiol, prostaglandin F₂ α (PGF₂ α), follicle stimulating hormone (FSH) and luteinizing hormone (LH) are depicted. Day 0 is the day of the LH surge/ovulation. (B) The *shaded areas* represent the luteal phase of the estrous cycle, the *unshaded area* is the follicular phase of the estrous cycle.

also show a peak during this peri-ovulatory period. Once the follicle(s) has ovulated, it will form a corpus luteum which will begin to produce progesterone to prepare the uterus for implantation of a conceptus.

The corpus luteum is formed from the granulosa cells remaining in the follicle wall and by theca interna cells that penetrate into the granulosa layer. These two cell types of the corpus luteum become large (from granulosa) and small (from theca interna) luteal cells and synthesize progesterone. Progesterone levels begin to rise soon after ovulation with a noticeable increase in concentrations beginning days 3–4 after ovulation and they remain elevated for the duration of the lifespan of the corpus luteum. The rapidity with which progesterone secretion rises after ovulation and the maximum levels reached are proportional to the number of corpora lutea in litter bearing animals such as the pig. If no pregnancy occurs, luteolysis will occur in response to increases in circulating levels of prostaglandin F₂ α coming primarily from the uterus in the domestic animal species. The increase in prostaglandin production by uterine epithelial cells occurs in response to oxytocin. In the absence of a conceptus, oxytocin receptors are induced in the uterine epithelial cells and episodic pulses of oxytocin secretion from the ovary then become coupled with the release of prostaglandin F₂ α (PGF₂ α) from the uterus. If a conceptus is present, luteal regression will not occur due to production of an anti-luteolytic factor by the conceptus. This anti-luteolytic factor will prevent oxytocin receptor expression in uterine epithelial cells and consequently the pulsatile pattern of PGF₂ α secretion needed to achieve luteolysis. These anti-luteolytic factors have been identified in some domestic animal species and include interferon-Tau in cattle and sheep, estrogens in pigs, and a mix of small proteins in the mare.

Hypothalamic-Pituitary–Ovarian Axis

The pattern of regular ovulatory cycles is dependent on a complex and tightly regulated interplay between the hypothalamus, pituitary, and ovary, which together make up the reproductive axis. The cyclic changes in the pituitary gonadotropins and in the ovarian

steroid hormones are the result of negative and positive feedback effects of the ovarian hormones estradiol and progesterone on the hypothalamus and pituitary gland. The reproductive system functions in a classic endocrine mode initiated by pulsatile secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus into the pituitary portal venous system. GnRH regulates the synthesis and release of both FSH and LH from the gonadotropes of the anterior pituitary. FSH and LH secretion differ somewhat throughout the cycle and this differential regulation of the two hormones produced by the same cells occurs in response to changing pulse frequencies of GnRH. A rapid pulse frequency favors LH synthesis and secretion while a slower pulse frequency favors the production of FSH.

The key unique feature of ovarian steroid hormone regulation of the ovulatory cycle is that estradiol is able to exert both inhibitory and stimulatory effects on LH secretion. The direction of estradiol feedback is dependent on both the levels of estradiol and duration of exposure with low levels of estradiol inhibiting LH secretion, while positive feedback of estradiol occurs in response to higher concentrations over a more prolonged duration. The negative feedback effects of estradiol occur at the level of the hypothalamus resulting in a decreased amplitude of GnRH pulses. Estradiol exerts positive feedback effects at the level of the hypothalamus and also directly on the pituitary. As the follicular phase progresses and ovarian follicles produce more and more estradiol, the sustained rise in estradiol leads to the positive feedback effect on LH secretion. This positive feedback effect of estradiol is manifested at the hypothalamus as increased frequency and amplitude of GnRH pulses. In addition, there is a positive feedback effect directly at the pituitary with a 20-fold increase in sensitivity of the pituitary gonadotropes to GnRH resulting in a marked increase in LH synthesis and secretion. The positive feedback effect of rising estradiol in the hypothalamus occurs through the kisspeptin neurons. GnRH neurons do not express estrogen receptors but the kisspeptin neurons do. In the female there are two kisspeptin neuron nuclei as compared to only one in the male. The second kisspeptin neuron nucleus is called the surge center and it is this cluster of kisspeptin neurons that responds to the positive feedback effect of estradiol leading to stimulation of pulsatile GnRH secretion. The result of the positive feedback effect of estradiol on the hypothalamus and pituitary is initiation of the ovulatory LH surge. A small rise in FSH occurs simultaneously with the marked rise in LH at ovulation. Following ovulation, the corpus luteum forms and begins to produce significant quantities of progesterone and also low levels of estradiol. Progesterone exerts a very strong negative feedback effect on the hypothalamic GnRH pulse generator causing GnRH pulse frequency to slow dramatically. The inhibitory effect of progesterone on GnRH neurons is mediated through the production of the endogenous opioid β -endorphin in the brain. The sustained levels of progesterone will inhibit LH and FSH secretion during the luteal phase. Once luteal regression is initiated and progesterone levels begin to fall, FSH levels will begin to rise and initiate a new round of follicular development.

Menstrual Cycle

The menstrual cycle in women and non-human primates differs from the estrous cycles of domestic mammals in that the follicular phase is much longer, lasting about 2 weeks. The length of the luteal phase (about 2 weeks) in women is similar to that of domestic animals. In addition, only women and non-human primates undergo the cyclic shedding of the endometrium during menstruation. The human menstrual cycle can be divided into three phases based on functional and morphological changes in the ovary and uterus (Fig. 2A and B). These three phases are (1) the menstrual phase also called menses; (2) the follicular phase also called the proliferative phase; and (3) the luteal or secretory phase. The terms proliferative and secretory refer to the fact that the uterine epithelial cells in the endometrium are undergoing marked proliferation during the follicular phase while during the luteal phase they become highly differentiated and secretory. These changes in the endometrium during the menstrual cycle are dependent on the ovarian hormones estradiol and progesterone and are exquisitely timed so that they can be used to document the day of ovulation. Endometrial biopsies are typically taken during the secretory phase where the histological changes can be used to accurately date the cycle to within a day. It is customary to designate the first day of the cycle (day 1) as the first day of the menstrual phase (i.e., when menstruation begins). The menstrual cycle averages 28 days in length. However, only about 10%–20% of menstrual cycles are exactly 28 days. Cycle length can vary greatly among different women and may also vary significantly in a single woman. Most cycle are between 25 and 30 days long, but some women experience cycles that last less than 25 or greater than 30 days. Cycle length is more variable in teenage and older women than in women in their peak reproductive years.

The Menstrual Phase

The first day of menstruation marks the beginning of the menstrual cycle. Menstruation is triggered by the loss of progesterone support for the endometrium and results in the shedding of the upper layer of the endometrial lining. The endometrium consists of two layers called the stratum functionalis and the stratum basalis. It is the upper functionalis layer that is shed with each menstrual cycle and then regenerated anew in the next succeeding cycle. The shedding of the functionalis layer is regulated by ovarian steroid hormones. Fig. 2A and B show the pattern of reproductive hormone levels during the phases of the menstrual cycle in women. Regression of the corpus luteum during the late secretory phase leads to a sharp decline in progesterone and estradiol levels, initiating the process of tissue breakdown and menstruation in the endometrium. The onset of menstruation is an inflammatory response leading to the influx and activation of leukocytes and various immune cells including macrophages, natural killer cells, dendritic cells, and mast cells. These cells increase substantially in the endometrium during the late secretory phase of the cycle and make up as much as 40% of the cell population of the endometrium prior to menstruation. As progesterone levels decline sharply these cells are activated which triggers a cascade of matrix metalloproteinases (MMPs) that degrade the extracellular matrix

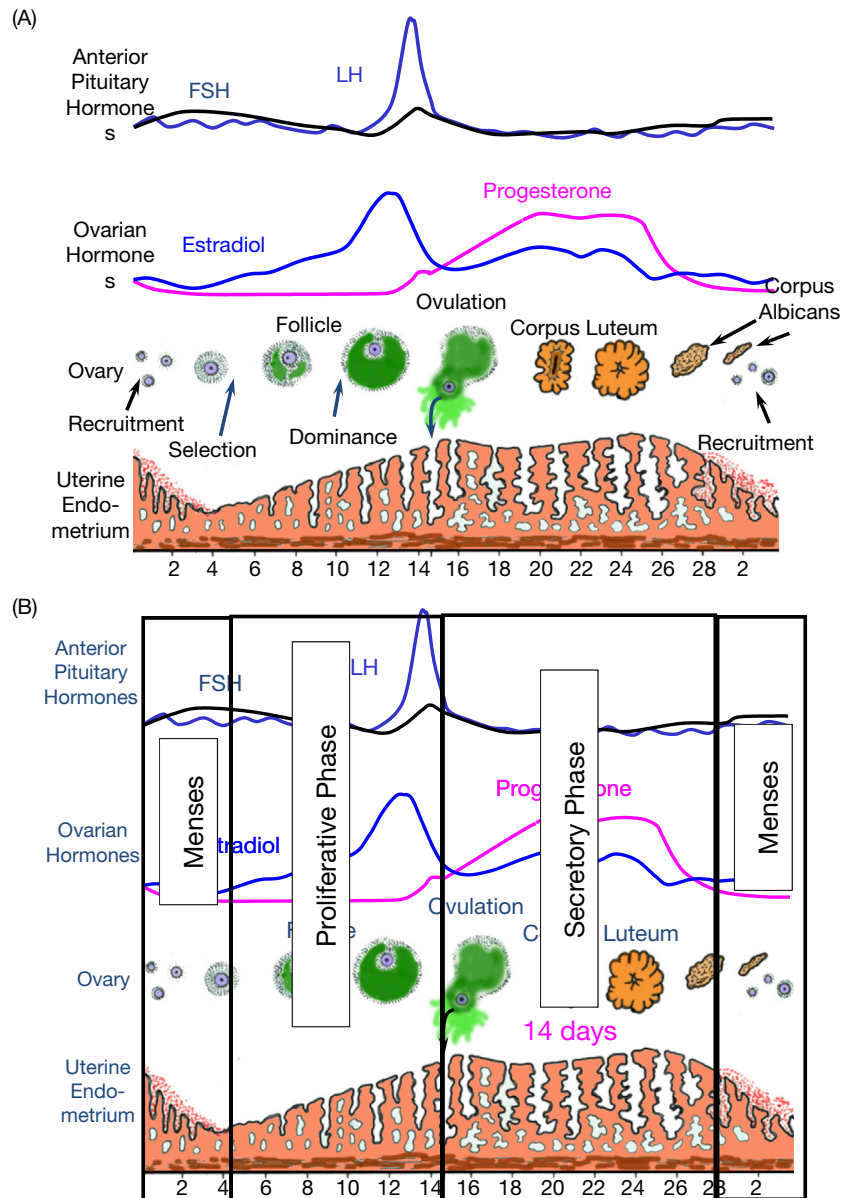


Fig. 2 Schematic outline of the ovarian events, endometrial and reproductive hormone level changes throughout the menstrual cycle. (A) New primary follicles are recruited, selected, and one becomes the dominant follicle destined to ovulate. Following ovulation the corpus luteum forms and produces progesterone during the secretory phase. Changes in the hormones estradiol, progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) are depicted. (B) The three phases of the menstrual cycle are indicated. Day 1 of the menstrual cycle is the first day of menses. Ovulation occurs at the end of the proliferative phase of the cycle.

and destabilize blood vessels in the endometrium. MMPs show increased expression and activity at menstruation when ovarian hormones are low. Of the MMP family, seven members are expressed in cell- and menstrual cycle-specific patterns in the uterus and their substrates include collagens, laminin, fibronectin, and other matrix proteins. Expression of inflammatory mediators in the endometrium such as interleukins and chemoattractant proteins is also increased. The sharp drop in circulating progesterone levels also causes vasoconstriction of the spiral arteries in the basal endometrium leading to local hypoxia and further tissue breakdown. This vasoconstriction occurs in response to the high levels of endothelins, potent vasoconstrictors, that show maximal activity at the end of the secretory phase. As these vessels constrict and then dilate they bleed and contribute further to the sloughing of the endometrium. Finally, the premenstrual fall in progesterone also leads to a decline in 15-hydroxyprostaglandin dehydrogenase activity, which increases the availability of prostaglandin $\text{PGF2}\alpha$, a potent stimulator of myometrial contractility, further facilitating the sloughing of the functionalis layer of the endometrium. Amazingly, the repeated cycles of inflammation and tissue breakdown during menstruation completely resolves without any residual scarring or loss of function. During this period of endometrial breakdown the levels of LH, FSH, estradiol, and progesterone are low due to low frequency and low amplitude pulses of

GnRH. However, by day 3, both FSH and LH levels begin to rise slowly and these increases in gonadotropins will stimulate growth of some of the ovarian follicles recruited during the late secretory phase. As the follicles grow in size they also achieve steroidogenic capacity and begin to secrete estradiol, so that estradiol levels also begin to rise by day 3 (Fig. 2).

The Proliferative Phase

The phase of the menstrual cycle leading up to ovulation is called the proliferative phase. Although the proliferative phase typically occupies the first half of the cycle, its length can vary. In ovulatory cycles of more or less than 28 days' duration, the deviation from the average is largely due to differences in the length of the follicular phase.

During the first 4–5 days of the proliferative phase, development of the recruited ovarian follicles continues. Due to the low levels of estradiol and progesterone both FSH and LH secretion increase. FSH induces granulosa cell proliferation and the recruited follicles build up several layers of granulosa cells. FSH also induces the enzyme aromatase in granulosa cells, increasing the amount of estradiol produced from androgens that diffuse from the theca cells to the granulosa cells. The proliferative phase of the cycle is characterized by a progressive increase in the synthesis and secretion of estradiol from the developing early antral follicles. FSH also induces expression of LH receptors on the granulosa cells making them LH responsive. By days 5–7 of the cycle, a single follicle predominates which will mature and ovulate between days 13 and 15. The remaining recruited follicles will undergo atresia. The process by which one follicle from a group of recruited follicles is selected to become the dominant follicle destined to ovulate is not well understood, but is thought to involve androgens. During the mid- to late proliferative phase, the rising estradiol levels in the circulation suppress FSH secretion, thereby preventing new follicular recruitment. The rising estradiol also leads to more LH secretion by the pituitary as well as increased ovarian responsiveness to LH. The increasing estrogen levels also cause growth of the endometrial tissue lining the uterus. These visible changes in the endometrium are defined as the "proliferative phase."

The uterine endometrium is a primary target organ of estradiol. During the proliferative phase the uterine endometrium begins to thicken and the uterine glands enlarge. The increase in thickness of the endometrium can be measured by ultrasound. The uterine epithelial cells on the surface of the lumen and lining the glands proliferate in response to rising estrogen. The glands proliferate and elongate deep into the stroma of the endometrial basalis. Immediately prior to ovulation, the glands are maximally elongated and markedly coiled. The supply of blood vessels in the endometrium is also markedly increased due to proliferation of the spiral arteries that grow upwards from the basal layer into the functionalis layer of the endometrium.

By days 10–13 of the proliferative phase the antral follicle(s) reaches peak size and estradiol levels also continue to rise to reach their maximum levels by day 12 or 13. This sustained rise in estradiol activates positive feedback at the level of the hypothalamus (through kisspeptin) and also directly at the pituitary to increase LH secretion. Approximately 24–48 h after the peak in estradiol, an LH surge occurs characterized by a marked, rapid increase in frequency and amplitude of LH pulses. Ovulation occurs about 9–12 h after the peak of the LH surge accompanied by a smaller FSH surge. The LH surge initiates the resumption of meiosis in the oocyte within the largest antral follicle and extrusion of the mature ovum through the follicle wall.

The Luteal Phase

The length of the luteal phase in women is normally approximately 14 ± 2 days. The corpus luteum forms from the follicle after ovulation and its production of progesterone becomes the dominant feature of the luteal phase. The corpus luteum in humans is also made up of large and small luteal cells that produce estrogen and inhibin, as well as progesterone and 17-hydroxyprogesterone. These hormones are all produced in quantities that are measureable in the peripheral circulation.

The high concentrations of progesterone present during the luteal phase exert a marked negative feedback effect on both FSH and LH secretion in spite of the moderate levels of estradiol being produced by the corpus luteum at this time. These negative feedback effects occur at the level of the hypothalamus with progesterone causing a significant inhibition in the frequency of GnRH pulses, resulting in only baseline FSH and LH secretion. The corpus luteum is dependent on these basal levels of LH for its ability to produce progesterone. If pregnancy does not ensue, the corpus luteum will spontaneously regress due to apoptosis of the luteal cells. When progesterone, estradiol and inhibin levels begin to decline at the end of the luteal phase, secretion of GnRH is no longer inhibited and a new cycle of follicular development is initiated. FSH secretion will begin to rise in response to the drop in hormone production by the corpus luteum and a new crop of follicles will be recruited for the next cycle. If a conceptus is present, the corpus luteum will not undergo regression but will be maintained due to the production of human chorionic gonadotropin (hCG) by the trophoblast cells of the early embryo. hCG is a glycoprotein hormone that is homologous to LH and can bind to LH receptors in the corpus luteum with an affinity similar to LH. hCG is critical for maintenance of the corpus luteum and continued progesterone secretion. The corpus luteum of pregnancy will continue to be maintained and secrete progesterone until the fetal placenta assumes this role near the end of the first trimester of pregnancy.

Progesterone is the dominant hormone during the secretory phase and promotes endometrial maturation so that implantation can occur. During the luteal phase the endometrial glands become fully differentiated and secrete the nutrients that will be needed by an implanting embryo. The endometrium is thick and spongy, due to increased vascularity and maturation of the spiral arterioles. There is also a marked influx of leukocytes and the endometrial stromal cells undergo a unique process known as decidualization. The stromal cells enlarge and acquire a foamy appearance indicative of increased metabolism, glycogen and lipid accumulation, and synthetic capacity. The cells change in appearance from fibroblast-like cells to large round cells with an abundant

cytoplasm and become terminally differentiated. Decidualization of the endometrium is first initiated in the stromal cells located around the elongated and coiled spiral arteries and then spreads under and around the glands by the mid- to late luteal phase (around 10 days after ovulation). The presence of decidual cells and the influx of leukocytes leads to an increase in cytokines and chemokines within the endometrium as well as hormones such as prolactin and insulin-like growth factor binding protein 1. If implantation does not occur, the corpus luteum will regress, leading to a drop in progesterone production and the endometrium will undergo ischemic necrosis leading to menstruation. If pregnancy does occur, the lifespan of the corpus luteum will be extended by hCG prolonging progesterone secretion and decidualization of the stroma will continue.

In spite of some fundamental differences between the estrous cycle of domestic mammals and the menstrual cycle of primates and women, many of the physiologic mechanisms are comparable between all these species.

Further Reading

- Aurich, C. (2011). Reproductive cycles in horses. *Animal Reproduction Science*, 124, 220–228.
- Downey, B. R. (1980). Regulation of the estrous cycle in domestic animals. *The Canadian Veterinary Journal*, 21, 301–306.
- Erickson, G. F. (1986). An analysis of follicle development and ovum maturation. *Seminars in Reproductive Endocrinology*, 4, 233–254.
- Evans, A. C. O. (2003). Characteristics of ovarian follicle development in domestic animals. *Reproduction in Domestic Animals*, 38, 240–246.
- Fierro, S., Gil, J., Vinales, C., & Olivera-Muzante, J. (2013). The use of prostaglandins in controlling estrous cycle of the ewe: A review. *Theriogenology*, 79, 399–408.
- Hall, J. E. (2014). Neuroendocrine control of the menstrual cycle. In J. F. Strauss, & R. L. Barbieri (Eds.), *Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management* (pp. 141–156). Philadelphia, PA: Elsevier Press.
- Maybin, J. A., & Critchley, H. O. D. (2012). Steroid regulation of menstrual bleeding and endometrial repair. *Reviews in Endocrine & Metabolic Disorders*, 13, 253–263.
- Maybin, J. A., & Critchley, H. O. D. (2015). Menstrual physiology: Implications for endometrial pathology and beyond. *Human Reproduction Update*, 21, 748–761.
- Okuda, K., Miyamoto, Y., & Skarzynski, D. J. (2002). Regulation of endometrial prostaglandin F_{2α} synthesis during luteolysis and early pregnancy in cattle. *Domestic Animal Endocrinology*, 23, 255–264.
- Sakamoto, R. (2016). Pregnancy-associated changes in uterine-luteal relationships in cows: A mini-review. *Reproductive Biology*, 16, 182–188.
- Sato, J., Nasu, M., & Tsuchitani, M. (2016). Comparative histopathology of the estrous or menstrual cycle in laboratory animals. *Journal of Toxicologic Pathology*, 29, 155–162.
- Senger, P. L. (2004). Reproductive cyclicity. In P. L. Senger (Ed.), *Pathways to pregnancy and parturition* (pp. 144–213). Pullman, WA: Current Conceptions.
- Thackray, V. G., Mellon, P. L., & Coss, D. (2010). Hormones in synergy: Regulation of the pituitary gonadotropin genes. *Molecular and Cellular Endocrinology*, 314, 192–203.

Hypothalamus–Pituitary–Ovary Axis

Vasantha Padmanabhan and Muraly Puttabyatappa, University of Michigan, Ann Arbor, MI, United States
Rodolfo C Cardoso, Texas A&M University, College Station, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

In female mammals, reproductive processes such as puberty, ovarian cyclicity and pregnancy require precise coordination of the hypothalamic–pituitary–ovarian (HPO) axis. The major components of the HPO axis include the hypothalamic gonadotropin-releasing hormone (GnRH) neurons, the pituitary gonadotropes and ovarian structures, such as follicles and the corpus luteum (Fig. 1). The gonadotropes respond to GnRH by releasing the gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn stimulate folliculogenesis and steroid and peptidergic hormone secretion by the ovaries. The hypothalamic, pituitary, and ovarian hormones integrate to orchestrate the different events within the reproductive cycle so they occur in a timely manner to ensure successful reproduction (see earlier review (Beshay and Carr, 2013)). In this chapter, we present an overview of the key components and hormones of the HPO axis, discuss the complex endocrine interactions that exist between the different levels of this axis, and summarize recent findings on the main factors and mechanisms that regulate the HPO axis in the female mammals.

The Hypothalamus

The hypothalamus encompasses the lower part of the lateral wall and the floor of the third ventricle, containing several localized clusters of neurons, called nucleus, that control multiple processes such as metabolism, reproduction, and behavior. The median eminence located at the base of the hypothalamus extends to the pituitary and contains neurosecretory neurons that control hormone synthesis and secretion from the pituitary.

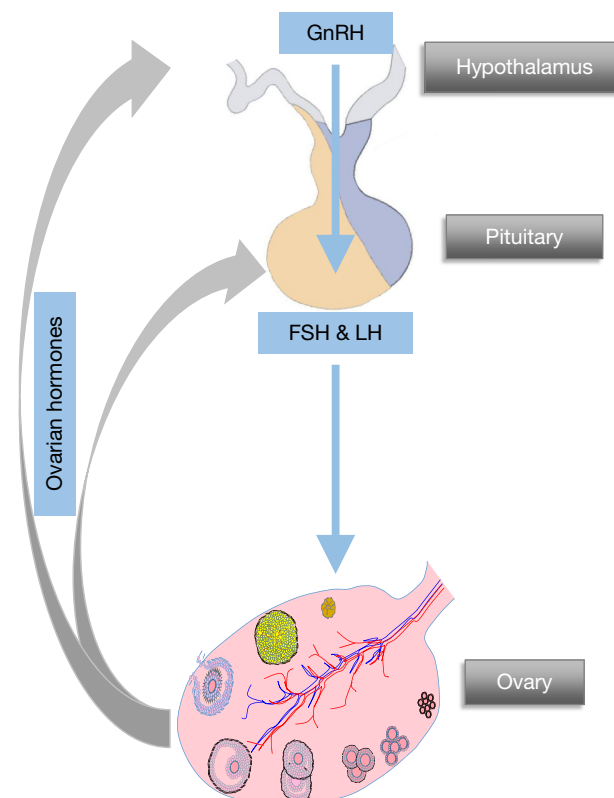


Fig. 1 Schematic showing the components of the hypothalamic–pituitary–ovarian axis.

GnRH and Its Mode of Release

The GnRH neuron

The decapeptide, GnRH—the primary hypothalamic regulator of reproductive function, is synthesized in specialized neurons as part of a larger precursor molecule (prohormone) containing 92 amino acids. GnRH neurons originate in the olfactory placode and migrate to the hypothalamus where several hundreds of these cells can be found diffusely distributed in the anterior portion of the hypothalamus, the preoptic area (POA), and more caudally in the medial basal hypothalamus (MBH). Most GnRH neurons have two dendritic projections, which may extend remarkable distances (2–3 mm) from the cell body. The main target of hypothalamic GnRH projections is the primary plexus of the pituitary portal system in the median eminence.

The hypothalamic–pituitary portal vasculature

Upon reaching the median eminence, GnRH axons end on capillary loops that give rise to long portal veins that descend along the pituitary stalk to terminate in the pituitary sinusoids. The portal system facilitates the rapid exchange between the hypothalamus and the pituitary, requiring only minute amounts of neurohormones to stimulate release of hormones by the pituitary. The superior hypophyseal arteries form the primary capillary plexus that supplies blood to the median eminence. From this capillary system, the blood is drained in hypophyseal portal veins to the secondary plexus, a network of fenestrated sinusoid capillaries that provide blood to the anterior portion of the pituitary. This anatomical arrangement allows rapid and undiluted transport of GnRH to the pituitary gland.

GnRH biosynthesis

To date, three isoforms of GnRH (GnRH-I, GnRH-II, and GnRH-III) have been identified in humans and many others have been detected in fish, amphibians, and protochordates. GnRH-I is the classic hypothalamic peptide responsible for regulating the synthesis and secretion of gonadotropins. GnRH-II, first reported in brain tissue and since been found in other peripheral tissues, such as the uterus and ovaries is also capable of stimulating LH release. The role of GnRH-III, first identified in the lamprey, in humans remains unclear (Schneider et al., 2006).

Upon synthesis, GnRH-I gets transported to the median eminence of the hypothalamus and then released in the portal circulation in a pulsatile manner. The lifespan of GnRH-I is very short with a half-life of 2–4 min. Because of its short half-life and rapid dilution in the peripheral circulation, GnRH-I concentrations are very difficult to measure in the peripheral circulation.

GnRH pulsatility

The cellular network that elicits the pulsatile increases in GnRH secretion is referred to as the “GnRH pulse generator” and includes the synchronously firing GnRH neurons. While there is supporting evidence that GnRH pulsatility is intrinsic to GnRH neurons themselves and that extensive intercellular mechanisms orchestrate synchrony within the network, more recent observations strongly indicate that other neurons in the MBH target the GnRH neuronal network directly to drive the pulsatile release of GnRH (Fig. 2). Kisspeptin, the most potent stimulator of GnRH secretion yet discovered, appears to be an integral component of the GnRH pulse generator. Of particular interest are the neurons in the arcuate nucleus (ARC) that co-express the neuropeptides kisspeptin, neurokinin B, and dynorphin, termed KNDy neurons. In contrast to the endogenous opioid dynorphin that has inhibitory actions on GnRH release, neurokinin B is generally stimulatory to GnRH secretion. Based on the action of these neuropeptides on GnRH release and the neuroanatomical evidences that KNDy neurons communicate with GnRH neurons (particularly at the level of the median eminence), a model for GnRH pulse generation has been established. This

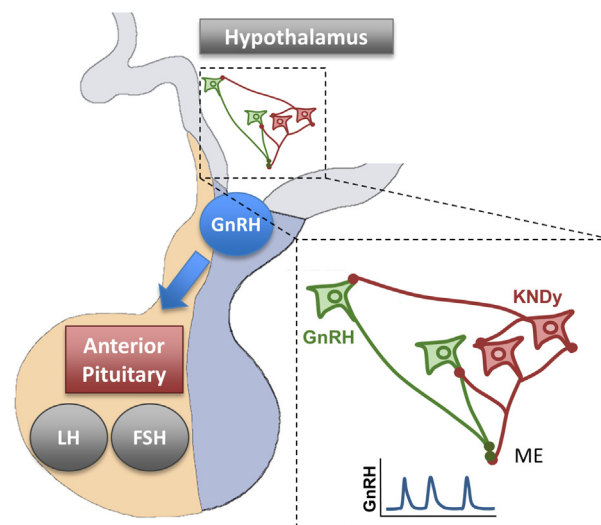


Fig. 2 Schematic depicting the control of the GnRH neurons in the hypothalamus. KNDy neurons in the arcuate nucleus project to GnRH cell bodies and axonal projections to modulate GnRH release into the median eminence (ME).

model proposes that GnRH pulse generation is initiated in the KNDy neuronal network in the ARC by a reciprocating interplay of stimulatory neurokinin B signals and inhibitory dynorphin inputs and the output of the pulse generator is relayed to GnRH neurons via the release of kisspeptin from axonal terminals originating from KNDy neurons (Fig. 2; Lehman et al., 2010).

The Pituitary Gland

The pituitary, also known as the master endocrine gland, is a small gland located beneath the third ventricle of the brain and above the sphenoidal sinus in a bone cavity called sella turcica. The pituitary gland contains two major lobes: anterior and posterior.

The Anterior Pituitary

Development

The anterior pituitary, also known as adenohypophysis, is a classic endocrine gland in that it is composed of secretory cells of epithelial origin supported by connective tissue rich in blood and lymphatic capillaries. The anterior pituitary is derived from the ectoderm, more specifically from the Rathke's pouch, part of the developing hard palate in the fetus. The Rathke's pouch eventually loses its connection with the pharynx, giving rise to the anterior pituitary. The pouch becomes committed to organ development several days prior to the expression of markers of individual cell types within the mature anterior pituitary gland. Between the time of organ commitment and maturation, a series of cell type-specific differentiation and proliferative events occur.

Cell types

The five cell types present in the mature anterior pituitary gland are defined based on the trophic factors that they synthesize and secrete. Corticotropes secrete adrenocorticotrophin, thyrotropes produce thyroid-stimulating hormone, somatotropes secrete growth hormone, lactotropes produce prolactin and gonadotropes secrete the gonadotropins, LH and FSH.

The Posterior Pituitary

The posterior pituitary, also known as neurohypophysis, is a grouping of axonal projections that secretes oxytocin responsible for increasing uterine contractions and vasopressin, an antidiuretic hormone.

Gonadotropes

Gonadotropes are specialized basophilic cells in the anterior pituitary that express cell surface receptors for GnRH and produce LH and FSH.

The GnRH receptor

The GnRH-I receptor is a G-protein coupled receptor that utilizes inositol triphosphate and diacylglycerol as second messenger molecules to stimulate protein kinase, release calcium ions, and cyclic adenosine monophosphate activity. While the pituitary is the classic target tissue for GnRH actions, the GnRH-I receptor is expressed in many other tissues, including ovarian follicles, pancreas, liver, heart, and the placenta. Importantly, the continuous activation of the GnRH receptor results in its desensitization thus enabling the use of GnRH agonists for treatment of reproductive disorders such as precocious puberty and endometriosis, and to prevent GnRH actions prior to ovarian stimulation cycles for in vitro fertilization. Therefore, only pulsatile GnRH administration can restore normal gonadotropin secretion in individuals lacking endogenous GnRH secretion (e.g., Kallmann syndrome).

Luteinizing hormone

LH is a glycoprotein heterodimer consisting of two subunits: α - and β -subunits. The α -subunit is common between LH, FSH, and TSH and consists of 92 amino acids. The β -subunit is distinct and hormone-specific, which allows the different biological actions of each hormone. The LH β -subunit has 120 amino acids and one to two sialic acid residues, giving it its shorter half-life of approximately 20 min. LH is secreted in a pulsatile manner except preceding ovulation when a massive release of LH (LH surge) occurs to trigger ovulation. Since LH secretion is primarily regulated by GnRH, peripheral levels of LH serve as good surrogate markers for assessing pattern of GnRH release.

Follicle-stimulating hormone

FSH is also a glycoprotein heterodimer consisting of two subunits: α - and β -subunits. The FSH β -subunit contains 118 amino acids and 5 sialic acid residues. The sialic acid content of FSH and LH is responsible for the half-life of these hormones, with greater number of sialic acid residues yielding longer half-life. FSH has a half-life of approximately 3–4 h, thus providing sustained support to facilitate growth of ovarian follicles.

Ovaries

The ovaries are the female gonads and are primarily responsible for the generation of gametes and production of hormones that regulate reproductive function. In most mammals, a pair of ovaries is present on either side of the body and is structurally

made of inner medulla and the outer cortex that is encapsulated by a connective tissue rich membrane called tunica albuginea. While the medulla contains vascular structures such as blood and lymphatic vessels, the cortex contains the follicles, which are the functional units of the ovary.

Follicular Development

An ovarian follicle contains the female gamete—oocyte, and the somatic cells, granulosa and theca cells. The number and type of somatic cells varies depending on the stage of follicular development. During the embryonic development, the primordial germ cells (PGCs) originate from the endoderm of the yolk sac and migrate into the genital ridge. These PGCs mitotically divide to form the oogonia, which later undergoes meiosis to form the oocyte. The primordial follicles are formed when the naked oocytes are surrounded by the pregranulosa cells (Fig. 3). These primordial follicles form the ovarian follicular pool or reserve and majority of these follicles remain in a quiescent state. A few follicles emerge from the reserve at specific intervals, a process termed as activation, and either undergo atresia (degeneration) or develop into primary follicles with the transformation of granulosa cells from spindle to cuboidal shaped. Follicular growth continues with granulosa cells dividing to form several layers around the oocyte leading to the development of the preantral follicle. During this developmental period, the follicle also acquires the theca cells that surround the granulosa cells. It has been suggested that follicular development up until the preantral stage occurs in a gonadotropin-independent manner, however, the role of gonadotropins cannot be ruled out because FSH receptors are present in early follicular stages.

Subsequent follicular differentiation occurs under the influence of gonadotropins with preantral follicles progressing to the antral stage with development of a fluid-filled cavity, termed the antrum. Selection of a dominant follicle from the antral follicular pool that is destined for ovulation (Fig. 3) occurs under the influence of FSH. Further maturation into Graafian or preovulatory follicle occurs predominantly under LH stimulation. Upon ovulation, the release of egg from the follicle, subsequent transformation of the follicular remnant into the endocrine structure corpus luteum is driven by LH.

Ovarian Hormones

The ovarian hormones produced under the influence of LH and FSH include steroidal and non-steroidal polypeptide hormones. Androgens and estrogens are produced by theca and granulosa cells, respectively. Under the influence of LH, theca cells take up cholesterol and convert it into 17-hydroxy-pregnenolone through the Δ^5 pathway, which is then acted on by 17-hydroxylase to synthesize the androgen androstenedione. Majority of androstenedione is aromatized into the major estrogen, estradiol, by the enzyme aromatase (CYP19) in granulosa cells, a process that occurs under the influence of FSH. In the corpus luteum, synthesis of progesterone is stimulated by LH through the Δ^4 pathway (Miller, 2008).

The non-steroidal hormones, among others, include those belonging to the transforming growth factor beta (TGF β) family namely inhibin, activin, anti-Mullerian hormone (AMH), TGF α , bone morphogenetic proteins, and growth differentiation factors. Other factors produced by the ovary include insulin-like growth factors (IGF), kit ligand, fibroblast growth factors, epidermal growth factors, and prostaglandins.

Inhibins

Inhibins are protein hormones consisting of two subunits α and β and are secreted mainly by the granulosa cells under the influence of FSH but are also produced by the pituitary gonadotropes. Two forms of inhibin have been identified, Inhibin-A and -B. The α subunits are identical, while the β -subunit differs between both forms of inhibin (denoted as β_A and β_B , respectively). Inhibin-B is predominantly secreted during the follicular phase, while the A form is secreted during the luteal phase. Inhibins function to suppress FSH secretion by the anterior pituitary.

Activins

Activins are secreted by granulosa cells and are homo- or hetero-dimers of the inhibin β subunits, with activin A and B being the homodimer of two β_A and β_B subunits, respectively and AB being the heterodimer. Activin is also produced in the pituitary and

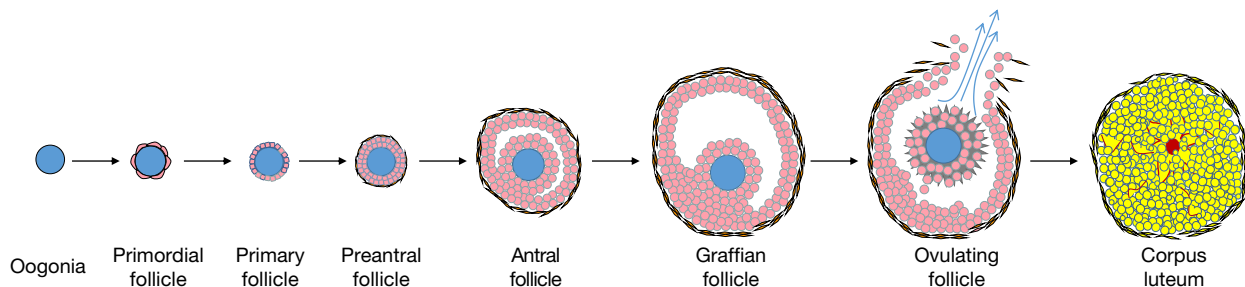


Fig. 3 Schematic representation of the different stages in the ovarian follicular development.

stimulates FSH release. Other β forms have been identified, raising the possibility of additional homo- and hetero-dimeric forms although their role in regulation of gonadotropin secretion is still being ascertained (Wijayarathna and De Kretser, 2016).

Follistatin

Follistatin is a monomeric glycoprotein produced by the ovary as well as anterior pituitary folliculostellate cells. It is a high affinity activin binding protein therefore its main role in female reproduction is to sequester activin and inhibit FSH secretion.

Anti-Müllerian hormone

AMH is produced by granulosa cells. AMH production increases from primordial to preantral follicle stage, being highest in preantral and small antral follicles but declining thereafter. AMH is implicated in inhibition of follicular recruitment from the resting pool. It acts on antral follicles to decrease FSH sensitivity, reduce aromatase activity and allow the selection of the dominant follicle (Visser and Themmen, 2014). AMH is increasingly being used as a biomarker of the relative size of the ovarian reserve (Pankhurst, 2017). Recently, receptors for AMH have also been identified in the mouse and human hypothalamic neurons suggesting a potential direct role in the neuroendocrine system (Cimino et al., 2016).

All intra-ovarian factors act in a paracrine manner to influence follicular growth and maturation (Gougeon, 2010).

Regulation of the HPO Axis

The cyclic functioning of the HPO axis involves a complex interaction between the hypothalamus, pituitary and ovary that include feed-forward, feedback and paracrine loops (Fig. 4).

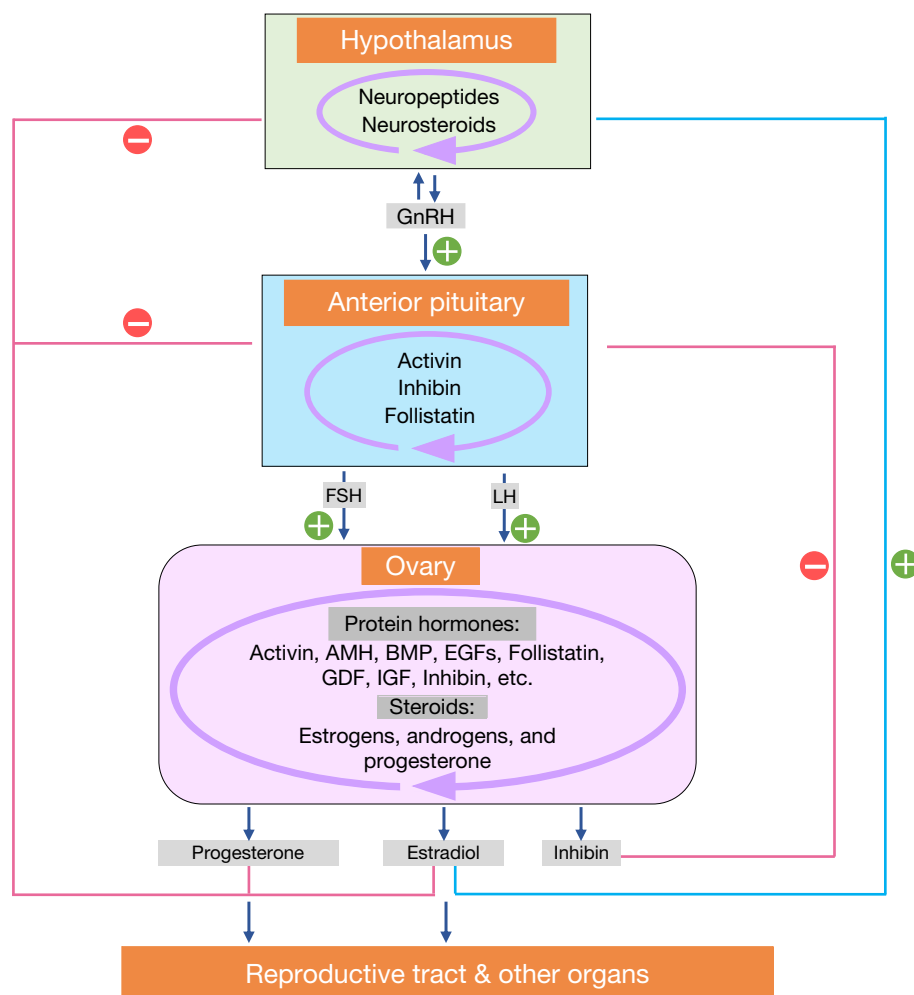


Fig. 4 Hypothalamic–pituitary–ovarian axis depicting feed-forward, feedback, and paracrine loops.

The Feed-Forward Regulation in HPO Axis

A pulsatile release of GnRH by the hypothalamic GnRH pulse generator leads to feed-forward regulation of the anterior pituitary gonadotropes that promotes synthesis, storage and secretion of gonadotropins (Fig. 4). Gonadotropin secretion depends on the frequency of pulsatile GnRH secretion with faster frequency favoring LH- β subunit gene transcription and LH release, and slower frequency favoring FSH- β gene transcription and FSH release.

The gonadotropins released under the influence of GnRH have feed-forward actions at the ovarian level (Fig. 4) where they bring about morphological and hormonal changes. These include regulation of follicular differentiation by FSH, induction of ovulation and formation of corpus luteum by the surge release of LH, stimulation of theca cell androgen production by LH, conversion of androgen to estrogen as well as synthesis of inhibin and activin by FSH, and the stimulation of progesterone production in the corpus luteum by LH.

The Feedback Regulation of the HPO Axis

The feedback regulation of HPO axis, which may be negative or positive, involves control of hormonal release from the hypothalamus, pituitary or ovary by the end products of their stimulation (Fig. 4). Such feedback ensures that follicular growth, maturation, ovulation, corpus luteum formation and preparation of the uterine lining occur in a controlled and timely manner so as to facilitate successful conception or renewal of the reproductive cycle in the event of unsuccessful conception.

Negative feedback regulation

Estradiol, progesterone, and inhibin are the main ovarian hormones that participate in the negative feedback regulation of the neuroendocrine axis to decrease gonadotropin release (Fig. 4). At the level of the hypothalamus, the exact afferent neuronal pathways and mechanisms mediating the gonadal steroid-dependent negative feedback process remain unresolved as the majority of GnRH neurons do not express receptors for gonadal steroids. Current evidence suggests that kisspeptin neurons located in the POA and ARC play a key role conveying the feedback regulatory effects of gonadal steroids on GnRH neurosecretory activity. Kisspeptin neurons located in the ARC (KNDy neurons) express receptors for gonadal steroids and have been postulated to mediate the negative feedback actions of estradiol on GnRH secretion (Lehman et al., 2010). There is considerable evidence that the inhibitory effects of progesterone on GnRH pulsatile secretion is mediated through dynorphin from KNDy neurons. This premise is supported by the observations that KNDy neurons express the progesterone receptor, local administration of an antagonist to the dynorphin receptor in the MBH increases LH pulse frequency during the luteal phase in sheep, ovariectomy decreases the expression of dynorphin in the ARC and local administration of a progesterone receptor antagonist to the ARC disrupts the negative feedback actions of progesterone on GnRH/LH secretion (Lehman et al., 2010).

The inhibitory effects of estradiol on gonadotropin secretion, apart from its action on GnRH release, also appear to be partially mediated via direct pituitary effects. This involves repression of FSH- β subunit expression by estradiol. Inhibin from the ovary also exerts negative feedback on the pituitary FSH secretion and one mechanism proposed involves competing with activin in binding to its receptors, thereby preventing activin's role in stimulating FSH secretion.

Positive feedback regulation

The ovarian hormone estradiol is the main player that exerts positive feedback action in the HPO axis (Fig. 4) and research findings suggest that there are important differences in the neuronal systems mediating its positive actions on GnRH surge release between different animal models. While in rodents it appears that the anteroventral periventricular area is the main hypothalamic area involved, in sheep and primates the estradiol positive feedback is largely mediated in the MBH. Despite these differences, in most female mammals increased expression of kisspeptin during the late follicular phase promotes a robust release of GnRH that in turn stimulates the surge release of gonadotropins. In humans, the anterior pituitary appears to be an important site of estradiol positive feedback regulation of LH secretion.

Paracrine Regulation

In addition to the positive and negative feedback regulation discussed earlier, there are several paracrine/autocrine regulatory loops at all levels of the HPO axis. For instance, GnRH has been shown to regulate its own secretion (Padmanabhan et al., 1995). Several neuropeptides produced by hypothalamic neurons, such as kisspeptin, also participate in paracrine regulation of GnRH release. More recent studies suggest neurosteroids produced within the hypothalamus may also participate in feedback regulation of GnRH release (Kenealy et al., 2013). At the pituitary level, activin, inhibin and follistatin produced by the pituitary modulate FSH synthesis through paracrine actions. Activins promote FSH secretion by increasing the number of GnRH receptors on gonadotropes (Fortin et al., 2015) while inhibin and follistatin oppose activin actions.

At the level of the ovary, there are several paracrine systems operational. Some examples of such paracrine regulation include: (1) modulation of activin action by its binding protein, follistatin, (2) promotion of theca androgen production by inhibin, (3) down-regulation of FSH receptors in antral follicles by AMH, which helps reduce FSH sensitivity thus allowing emergence of dominant follicles, (4) induction of inhibin and gonadotropin receptors by estradiol, and (5) promotion of FSH-induced estrogen production in granulosa cells and LH-induced androgen production in theca cells by inhibins. Another important example of a paracrine

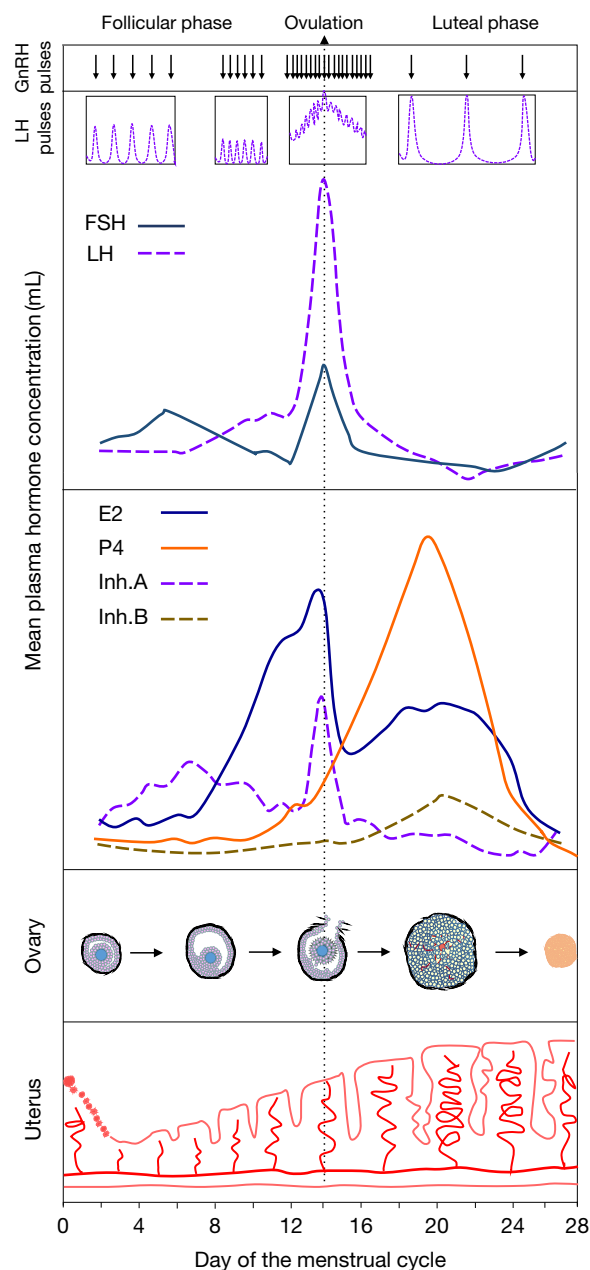


Fig. 5 Hypothalamic, pituitary, and ovarian hormonal, ovarian follicular and corpus luteum and uterine epithelial changes during the menstrual cycle. The mean concentrations of each of the hormonal changes are shown with LH pulsatile secretion shown as insets during the early and later follicular, periovulatory and luteal phases. *E2*, estradiol; *P4*, progesterone; *inh.A*, inhibin A; *inh.B*, inhibin B. Adapted with permission from Mahutte, N. G. and Ouhilal, S. (2007). In: Hurd, W. W. and Falcone, T. (eds.) *Clinical reproductive medicine and surgery*. St. Louis, MO: Elsevier.

feedback loop within the ovary involves androgen and inhibin with inhibin acting on theca cells to regulate androgen production and androgen acting back on granulosa cells to regulate inhibin production.

Other Regulators of HPO Axis

In addition to regulation of the HPO axis by its components and their secretions, nutrition, metabolic status and seasonality, among other factors, also regulate the HPO axis. Reproductive processes in female mammals can be energetically costly, thus requiring a coordinated regulation by the nutritional and metabolic systems. Therefore, the HPO axis must receive and integrate information about peripheral metabolic cues in order to regulate reproductive function. Neuropeptides and leptin are the major cues of the body's metabolic status.

Although leptin, a metabolic hormone produced primarily by adipocytes, does not affect GnRH secretion directly as its receptor is not present on GnRH neurons, the effects of leptin are mediated through intermediate neuronal pathways that encompass hypothalamic neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and proopiomelanocortin (POMC) neurons. While NPY has inhibitory action, melanocyte-stimulating hormone alpha (α MSH), one of the products of the POMC gene, has stimulatory effect on GnRH neurons (Evans and Anderson, 2017). Undernutrition upregulates NPY expression in the ARC reducing the GnRH pulsatility while increased leptin concentrations in circulation not only downregulates NPY but also increases α MSH increasing the GnRH/LH pulsatility (Evans and Anderson, 2017).

Photoperiod is a major factor determining annual changes in reproductive function in mammalian species that breed seasonally such as sheep (short day breeders) and mares (long day breeders). While the neuronal mechanisms involved in photoperiod control of GnRH/LH pulses remain uncertain it may involve hypothalamic dopaminergic neurons, KNDy neurons in the ARC, and neurons expressing RF-amide related peptide 3, which is also referred to gonadotropin inhibitory hormone 3 (Clarke, 2011). Another important factor modulating GnRH release in female mammals is stress. Stress influences the GnRH pulse generator reducing the frequency of GnRH/LH pulses through increased cortisol secretion (Whirledge and Cidlowski, 2010).

Apart from actions at the GnRH neurons, metabolic hormones such as insulin, leptin and adiponectin enhance gonadotropin synthesis and release via direct action on the gonadotropes. Stress in addition to reducing GnRH pulsatility also reduces pituitary responsiveness to GnRH, and amplifies the negative feedback while inhibiting the positive feedback effects of estradiol (Whirledge and Cidlowski, 2010).

At the level of the ovary, the major metabolic hormones modulating ovarian function are insulin and adiponectin. Insulin is referred to as a co-gonadotropin in the ovary as in addition to acting alone can potentiate gonadotropin action. Insulin can also activate IGF receptors in the ovary. The ovary also expresses adiponectin receptors and adiponectin promotes ovulatory and luteinization processes induced by the LH surge by increasing steroidogenesis, prostaglandin synthesis and angiogenesis (Palin et al., 2012). While glucocorticoids have an anti-inflammatory role in the ovary and aid in the formation of the corpus luteum, stress-induced increase in glucocorticoids has negative effects by suppressing the LH-induced steroidogenesis (Whirledge and Cidlowski, 2010).

Conclusions

Precise coordination of components of the HPO axis via endocrine and paracrine feedback mechanisms is essential to maintain adequate reproductive cyclic function. At the beginning of reproductive cycle, increasing levels of LH and FSH facilitate growth of multiple follicles to early antral stage (Fig. 5). The estradiol and inhibin B produced by the growing follicles exert negative feedback effects at the hypothalamic–pituitary level leading to a reduction in FSH secretion. The increased estradiol also promotes uterine epithelial growth to prepare for receiving the conceptus. The follicle that develops increased sensitivity to FSH in the face of reduced FSH support differentiates into the dominant follicle. The dominant follicle contributes to a further increase in estradiol secretion leading to positive feedback response at the hypothalamic–pituitary level and surge release of gonadotropins triggering ovulation. Upon release of the oocyte, the follicle remnant develops into a progesterone and inhibin A producing organ called corpus luteum. The increase in progesterone and inhibin secreted by the corpus luteum exerts negative feedback at the hypothalamic–pituitary level to reduce LH and FSH secretion. In the event of conception, luteal function is maintained by factors produced by the developing conceptus. If conception fails, the corpus luteum undergoes regression leading to a decline in progesterone and inhibin A secretion that result in removal of negative feedback effects at the hypothalamic–pituitary level leading to an increase in LH and FSH secretion and reinitiation of the cycle. All in all, the HPO axis integrates peripheral signals and via feed-forward, feed-back and paracrine mechanisms it enables reproductive success to ensure the survival of the species.

References

- Beshay, V. E., & Carr, B. R. (2013). Hypothalamic-pituitary-ovarian axis and control of the menstrual cycle. In *Clinical reproductive medicine and surgery*. New York: Springer.
- Cimino, I., Casoni, F., Liu, X., Messina, A., Parkash, J., Jamin, S. P., Catteau-Jonard, S., Collier, F., Baroncini, M., DeWailly, D., Pigny, P., Prescott, M., Campbell, R., Herbison, A. E., Prevot, V., & Giacobini, P. (2016). Novel role for anti-Müllerian hormone in the regulation of GnRH neuron excitability and hormone secretion. *Nature Communications*, 7, 10055.
- Clarke, I. J. (2011). Control of GnRH secretion: one step back. *Frontiers in Neuroendocrinology*, 32, 367–375.
- Evans, M. C., & Anderson, G. M. (2017). Neuroendocrine integration of nutritional signals on reproduction. *Journal of Molecular Endocrinology*, 58, R107–R128.
- Fortin, J., Ongaro, L., Li, Y., Tran, S., Lamba, P., Wang, Y., Zhou, X., & Bernard, D. J. (2015). Minireview: activin signaling in gonadotropes: what does the FOX say ... to the SMAD? *Molecular Endocrinology*, 29, 963–977.
- Gougeon, A. (2010). Human ovarian follicular development: from activation of resting follicles to preovulatory maturation. *Annales D'Endocrinologie*, 71, 132–143.
- Kenealy, B. P., Kapoor, A., Guerriero, K. A., Keen, K. L., Garcia, J. P., Kurian, J. R., Ziegler, T. E., & Terasawa, E. (2013). Neuroestradiol in the hypothalamus contributes to the regulation of gonadotropin releasing hormone release. *The Journal of Neuroscience*, 33, 19051–19059.
- Lehman, M. N., Coolen, L. M., & Goodman, R. L. (2010). Minireview: kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: a central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology*, 151, 3479–3489.
- Miller, W. L. (2008). Steroidogenic enzymes. *Endocrine Development*, 13, 1–18.
- Padmanabhan, V., Evans, N. P., Dahl, G. E., McFadden, K. L., Mauder, D. T., & Karsch, F. J. (1995). Evidence for short or ultrashort loop negative feedback of gonadotropin-releasing hormone secretion. *Neuroendocrinology*, 62, 248–258.

- Palin, M. F., Bordignon, V. V., & Murphy, B. D. (2012). Adiponectin and the control of female reproductive functions. *Vitamins and Hormones*, 90, 239–287.
- Pankhurst, M. W. (2017). A putative role for anti-Müllerian hormone (AMH) in optimising ovarian reserve expenditure. *The Journal of Endocrinology*, 233, R1–R13.
- Schneider, F., Tomek, W., & Gründker, C. (2006). Gonadotropin-releasing hormone (GnRH) and its natural analogues: a review. *Theriogenology*, 66, 691–709.
- Visser, J. A., & Themmen, A. P. (2014). Role of anti-Müllerian hormone and bone morphogenetic proteins in the regulation of FSH sensitivity. *Molecular and Cellular Endocrinology*, 382, 460–465.
- Whirledge, S., & Cidlowski, J. A. (2010). Glucocorticoids, stress, and fertility. *Minerva Endocrinologica*, 35, 109–125.
- Wijayarathna, R., & De Kretser, D. M. (2016). Activins in reproductive biology and beyond. *Human Reproduction Update*, 22, 342–357.

Gonadotropins

T Rajendra Kumar, University of Colorado Anschutz Medical Campus, Aurora, CO, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Activating mutation A modification in DNA sequence that confers gain of function phenotype.

Activin A gonadal peptide that promotes FSH production from pituitary.

Adipocyte The major cell type in the fat or adipose tissue.

Anovulatory A failure to ovulate or produce eggs.

Antral follicle A mature follicle in the ovary that consists of an antrum.

Atrophy A tissue/cell that shows degeneration.

Autocrine A hormone or a chemical messenger produced from and acting on the same cell.

Blocking antibody An antibody that prevents the binding of a ligand to its binding partner and blocks its actions.

Chorionic gonadotropin A glycoprotein hormone produced by trophoblast cells in placenta.

Corpus luteum A steroidogenic structure formed after the egg is released from a fully grown ovarian follicle.

Counter current chromatography A classical separation technique that allows distribution of different proteins into different solvent phases.

Ectopic expression Expression from tissues other than the original tissue of expression.

Endothelial cell A cell that lines the inner surface of blood and lymphatic vessels.

Enzyme linked immunosorbent assay A technique that measures the concentration of antigens by using enzyme linked antibodies as detection reagents. Commonly abbreviated as ELISA.

Equine Horse or its family member.

Estrogen A female steroid mostly produced from ovarian granulosa cells.

Expression vector A synthetically designed plasmid or virus-based carrier to express genes in cells.

Follicular atresia Breakdown of follicles within an ovary.

Folliculogenesis Formation of new follicles.

Follistatin A gonadal peptide that antagonizes activin actions in many cells.

Gonadectomy Removal of gonads, usually by surgical methods.

Gonadotrope A pituitary cell that produces gonadotropins LH and FSH.

Gonadotropin-releasing hormone A 10-amino acid peptide hormone released from hypothalamus in the brain and binds to gonadotropes to regulate gonadotropin secretion from pituitary.

Granulosa cell A cell that is the functional unit of a ovarian follicle.

Homologous hormones Hormones from the same species.

Hyperandrogenemia A condition in which androgen is in excess.

Hyperplasia A condition in which there is an increase in cell proliferation leading to tissue enlargement.

Hypogonadism A condition that manifests in small gonads with reduced function.

Hypophysectomy Surgical removal of the pituitary.

Hypoplastic Incomplete development of a tissue that consists of less number of cells.

Hypothalamus A tiny structure at the base of the brain that controls the pituitary and other organs in the body.

Immunoneutralization Blocking the hormone action by specific antibodies.

In vitro Outside the biological context, usually culturing and treating cells in a petri dish.

In vivo Inside a living organism or whole animal.

Inactivating mutation A modification in DNA sequence that confers loss of function phenotype.

Inhibin A gonadal peptide that inhibits FSH production from pituitary.

Lactotrope A pituitary cell type that produces prolactin hormone.

Leydig cell The predominant cell type within the testis interstitium, produces testosterone.

Macro-heterogeneity Glycoforms that have differences in the number of N-linked glycans.

Micro-heterogeneity Refers to glycoforms that have differences in terminal sugars of N-glycan chains.

Mutagenesis Method to introduce mutations into DNA.

Osteoclast A cell type in the bone that destroys bone.

Ovarian senescence A condition in which ovary ceases to produce eggs.

Ovariectomy Surgical removal of the ovary.

Paracrine A hormone secreted by one cell acting on a neighboring cell within a tissue.

Perfusion Culturing cells in a solution phase and testing the effects of drugs on them.

Pituitary An endocrine gland closely connected with the hypothalamus and consists of multiple hormone-producing cell types.

Placenta An organ that connects fetus to the uterus and facilitates exchange of nutrients and other components via maternal blood circulation. Placenta also produces hCG during pregnancy.

Polarized cell A cell that has distinct compartments called apical and basolateral and also exhibits electrical differences along the cell membrane.

Pre-ovulatory follicle A fully grown follicle from which oocyte is released.

Progesterone A female steroid hormone produced from the corpus luteum.

Radioimmunoassay A radioactive technique that is used to measure hormones.

Radioreceptor binding assay A radioactive technique that is used to measure the binding characteristics of a hormone to its receptor.

Sedimentation The settling of particles from a liquid upon centrifugation.

Seminal vesicle An accessory male sex gland whose secreted fluids combine with sperm.

Sertoli cell A somatic nurse cell that maintains germ cells in the seminiferous tubules of testis.

Spermatid An advanced stage male germ cell that ultimately gives rise to sperm.

Spermatogenesis A process that occurs in the testis and produces sperm.

Targeted expression Expression of a foreign gene in the same cell type as the endogenous cell using cell-specific promoters.

Testosterone A male steroid produced from Leydig cells in the testis.

Theca cell A cell located outside the follicle that produces testosterone in response to LH.

Thyrotrope A pituitary cell type that produces thyroid stimulating hormone.

Transgene A natural or genetically modified gene transferred from one species to another.

3'-Untranslated region A region at the 3'-end of a gene that is transcribed but not translated, usually plays a role in stability of mRNA, commonly designated as 3'-UTR.

Abbreviations

cDNA	Complementary DNA
CGA	Common gonadotropin α -subunit
CTP	Carboxy terminal peptide
DNA	Deoxy ribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle-stimulating hormone
FSHR	FSH receptor
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
hpg	Hypogonadal
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MDCK	Madin-Darby canine kidney
miRNA	Micro-RNA
mRNA	Messenger RNA
RIA	Radio immunoassay
RNA	Ribonucleic acid
TSH	Thyroid-stimulating hormone

Introduction

Gonadotropins are glycoprotein hormones produced by gonadotropes in the anterior pituitary, an endocrine gland located beneath the hypothalamus of the brain. Their existence was identified nearly 100 years ago by hypophysectomy, a surgical procedure in which the pituitary gland is excised, that resulted in gonadal atrophy. Subsequently, they were biochemically purified from pituitary glands of a number of vertebrate species including post-mortem humans. Gonadotropes produce two

Table 1 A summary of different gonadotropins

<i>Hormone</i>	<i>Subunits</i>	<i>Tissue</i>	<i>Source</i>
Luteinizing hormone (LH)	$\alpha + \text{LH}\beta$	Pituitary	Gonadotrope
Follicle-stimulating hormone (FSH)	$\alpha + \text{FSH}\beta$	Pituitary	Gonadotrope
Chorionic gonadotropin (CG)	$\alpha + \text{CG}\beta$	Placenta	Trophoblast
Pregnant mare serum gonadotropin (PMSG) ^a	$\alpha + \text{eCG}\beta$	Endometrial cups	Serum
Equine chorionic gonadotropin (eCG) ^a	$\alpha + \text{eCG}\beta$	Placenta	Chorion

^aPMSG and eCG are the same.

gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH), named for their actions on ovaries, although they both act on testes as well (Table 1). During pregnancy, another gonadotropin is secreted in high levels from placental syncytio-trophoblast cells into urine. This is called chorionic gonadotropin (CG), also known as the pregnancy hormone. CG is also produced by pregnant mare, donkey and zebra placentas.

Gonadotropin Structure and Biosynthesis

Early biochemical studies using classical techniques such as counter current chromatography and sedimentation under denaturing conditions indicated that gonadotropins were heterodimers consisting of an α -subunit and a β -subunit. The subunits are non-covalently associated with each other and are easily separable. Meticulous manual protein sequencing techniques were used to discern amino acid sequences of individual gonadotropin subunits. These studies revealed that within a given species, the α -subunit was common between the gonadotropins. The α -subunit is also present in thyrotropin (thyroid-stimulating hormone or TSH) produced by thyrotropes, a distinct cell population in the pituitary. Significant protein sequence homology also exists between the gonadotropin subunits and across species. The non-covalent nature of the subunits allowed cross-species subunit reconstitution experiments such that inter-species hybrid heterodimers were assembled in test tubes. Depending on the species, the molecular weight of pituitary gonadotropin heterodimers range from 28 to 35 kDa. The critical amino acids that confer inter-subunit interaction, subunit assembly and antigenicity in gonadotropins were identified by various protein modification reagents. However, these studies were limited by unstable nature of the modified subunits and problems with subunit assembly often leading to biological inactivity of the resultant heterodimeric hormones.

One key feature identified by various protein modification and sequencing techniques was the presence of carbohydrate moieties covalently attached to Asn residues in both subunits of LH and FSH. N-glycosylation, the process by which sugars are assembled onto Asn residues, is a co-translational event that occurs early in the gonadotropin subunit synthesis in the rough endoplasmic reticulum. In the case of CG beta subunits, sugars are also attached onto Ser/Thr amino acid residues in the carboxy terminus ends. These O-linked sugars contribute to the longer half-life of hCG in circulation. Variations in terminal sugars are known to contribute to micro-heterogeneity observed in general in all glycoproteins, including gonadotropins. Recent studies also indicate that macro-heterogeneity due to lack of an entire sugar chain attached to one or two of the Asn residues is found in human FSH β -subunit. Using a variety of techniques to bulk produce pure hormones and biophysical and computational approaches, crystal structures of FSH and hCG have been solved and found to contain a characteristic cystine knot. These data gave clues about the 3-dimensional architecture of the hormone subunits, molecular basis of their physical interaction between the subunits and how carbohydrates are positioned on the dimer.

Gonadotropin Secretion and Action

Gonadotropin subunits must assemble within the gonadotropes and only FSH and LH heterodimers are secreted and detected in blood. In the case of human CG, free α - or β -subunits are ectopically expressed under some pathological conditions. Gonadotropin secretion is under tight physiological control involving feed-forward and -feedback endocrine regulatory loops. The hypothalamus-derived gonadotropin releasing hormone (GnRH) is the main activator of gonadotropin secretion. Sex steroids and gonad-derived peptides known as inhibins and follistatin act as negative regulators of FSH secretion. Another related peptide, activin, also identified originally in gonadal extracts, positively regulates FSH secretion. Activins, inhibins and follistatin are also locally expressed within the pituitary and regulate FSH secretion by autocrine and paracrine mechanisms. In the female, gonadotropin secretion varies according to the estrus or menstrual cycle stage and involves complex modes of regulation by numerous factors including the nutritional and metabolic status, age of the species and other pathological conditions. During ovarian senescence, when the ovarian feedback regulation no longer exists, FSH synthesis in the pituitary and the circulating FSH levels steadily increase. Gonadotropin biosynthesis and secretion are mostly aberrant in pituitary tumors of gonadotrope origin, often termed as non-functional or null cell adenomas. Rarely, these tumors produce high levels of functional gonadotropins. Circulating gonadotropins in blood and tissues were originally measured by radioimmunoassay (RIA) methods using

^{125}I -radioisotopes. During recent years, several non-radioactive automated methods namely ELISA and chemiluminiscence are routinely used in many basic research and clinical laboratories.

The availability of ^{125}I -labeled hormones provided a means to identify specific binding sites of gonadotropins in gonads of various species. These membrane-bound receptors were identified in low abundance on granulosa (LH and FSH receptors) and theca cells (LH receptors) within ovarian follicles. In the male, FSH receptors were localized to Sertoli cells and LH receptors to Leydig cells. Biochemical methods were used to purify these extremely labile receptors from target tissue membranes, although crude membrane receptor preparations were earlier used in radioreceptor binding assays.

LH and FSH act in a coordinated fashion in normal physiology. In the female, LH acts on ovarian theca cells resulting in production of testosterone which is then converted to estrogen by aromatase, an enzyme expressed in granulosa cells and regulated by FSH. LH also acts on granulosa cells and controls critical events leading to ovulation. After ovulation, LH acts on the corpus luteum, a structure derived from granulosa cells, theca, and endothelial cells to regulate progesterone production in many species. FSH regulates pre-antral follicle growth and granulosa cell proliferation. In the male, FSH acts during pre-pubertal period in a narrow window of time and regulates Sertoli cell proliferation and differentiation. LH binds to Leydig cells in the interstitium and regulates testosterone production, which in turn acts on Leydig cells, Sertoli cells and peritubular myoid cells to maintain spermatogenesis. A summary of gonadotropin actions is listed in **Table 2**. Despite several unique species-specific characteristics, in the majority of instances, gonadotropin signaling is mediated by the protein kinase-A and cAMP pathway. Several in vitro studies indicate that other pathways involving MAP kinase and AKT pathways are also regulated by FSH. Because, gonadotropins regulate several events leading to optimal gametogenesis and steroidogenesis in both males and females, immunoneutralization approaches using specific antibodies against full-length or small peptide regions of LH or FSH hormones or their corresponding receptors were used as potential contraceptive reagents in several species.

Gonadotropin Subunit-Encoding Genes and Their Regulation

With the emergence of gene cloning and advanced molecular biology techniques, research on gonadotropins, like many other areas of reproductive physiology, also witnessed major advances. Gonadotropin subunit-encoding genes are distinct and their corresponding cDNAs were cloned, their chromosomal positions mapped and their regulation studied in vitro in immortalized gonadotrope cell lines as well as in pituitaries of different species. Similarly, the gene/cDNA sequences for LH and FSH receptors were cloned from gonads from multiple species, their chromosome positions mapped and regulation of their gene expression studied.

Both the β -subunit encoding genes typically contain three exons and two introns. Human *FSHB* gene consists of a characteristic long 3'-untranslated region. The gonadotropin subunit gene regulation is well coordinated in response to GnRH, activins and inhibins and gonadal steroids. Transcriptional regulation of each of the subunits is studied using gonadotrope cell lines encased in a dynamic perfusion cell culture system. A combinatorial cascade of several transcriptional repressors and activators regulated by various physiological factors was identified. Their specific binding sites on gonadotropin subunit gene regulatory regions were further mapped in mutagenesis experiments where the putative regions or single nucleotide base pairs were modified. Moreover, gonadotropin subunit gene expression analyses across estrus cycles were also performed in pituitaries of various species under normal physiological conditions or under conditions where steroid negative hormone feedback is removed by gonadectomy (removal of gonads, testes or ovaries) or GnRH pulses were administered or suppressed with changing characteristics such as pulse frequency, and magnitude. The common α -subunit mRNA/protein is always present in excess whereas the β -subunits are rate-limiting. High frequency pulses of GnRH favor *Lhb* and low frequency pulses favor *Fshb* gene transcription. Recent studies identified microRNAs (miRNAs), small RNAs, 21–24 nucleotides in length, bind to 3' regions of LH β and FSH β encoding mRNAs and post-transcriptionally regulate their expression.

Table 2 A summary of important biological actions of gonadotropins

Hormone	Major sites of action	Physiological role
LH	Male: Leydig cells in testis	Testosterone production
	Female: Theca cells in ovary	Androgen production
	Granulosa cells in ovary	Ovulation
	Luteal cells in ovary	Progesterone production
FSH	Male: Sertoli cells in testis	Sertoli cell proliferation
		Germ cell maintenance
	Female: Granulosa cells in ovary	Estrogen production
CG ^a	Male: Leydig cells in testis	Testosterone production
	Female: Theca cells in ovary	Androgen production
	Granulosa cells in ovary	Ovulation
	Luteal cells in ovary	Progesterone production
		Maintenance of pregnancy

^aBinds to LH receptors and acts like LH; PMSG/eCG acts like both LH and FSH.

Gene manipulation techniques have permitted cloning of the human gonadotropin subunit genes into expression vectors and subsequent expression in Chinese hamster ovary (CHO) cell lines. The secreted recombinant gonadotropins designated rec-hLH and rec-hFSH have been highly purified and are currently used for all physiological studies. Other expression systems such as insect cell (using baculovirus vectors) and yeast were also used to produce recombinant gonadotropins for structure-function studies. Cleverly designed single chain gonadotropin analogs were expressed in CHO expression systems. This strategy bypassed the need for subunit assembly step, thereby allowing design of desired mutations on these gonadotropin single chain templates. Furthermore, novel double domain (LH and FSH) and triple domain (LH, FSH and TSH) analogs were produced that contain sub-populations of dually active gonadotropin molecules. The single, dually active and triple domain analogs were efficiently secreted into the medium, bound to receptors in vitro and exhibited biological activities when injected into animal models.

The unique O-glycosylated carboxy terminal peptide (CTP) of hCG β subunit-encoding gene sequences were genetically fused to that in the LH β subunit, expressed along with the α -subunit in polarized MDCK (Madin-Darby canine kidney) epithelial cells and the secretion behavior of LH containing this chimeric LH β -CG β CTP subunit was studied. In these MDCK cells, LH normally exited from the basolateral compartment and hCG from the apical compartment. Mutant LH containing the LH β -CG β CTP subunit exited from the apical compartment similar to hCG. Similarly, hCG containing a mutant CG β subunit lacking the CTP sequence exited from the basolateral compartment like LH. These studies implicate that while hCG and LH are highly similar in structure and function, they have different secretion patterns encoded by the presence or absence, respectively, of the CTP sequence in their β -subunits.

Mutations in Human Gonadotropins

Because gonadotropins are essential for reproduction, inactivating mutations in genes encoding the hormone-specific β -subunits are rare. But, when they do occur, they result in various gonadal defects and infertility. Two types of classical inactivating mutations in *LHB* gene were identified in men. In one type, the α /LH β dimer assembly was impaired and as a result no bioactive LH was produced in the patient. In the second type, the mutant LH β -containing LH dimer did assemble and was secreted, but did not recognize the gonadal receptors. In both cases, early testis development was not impaired, the patients exhibited normal testicular descent, however, delayed puberty, reduced spermatogenesis, Leydig cell hypoplasia and suppressed testosterone were noted. Therapy with hCG alleviated the gonadal and fertility problems in these patients. A third type affected the splicing event and resulted in an abnormal inclusion of second intronic sequences in *LHB* mRNA. Chronic testosterone treatment in conjunction with high endogenous FSH levels perhaps restored the normal testicular volume in these men. Their sister harboring the same inactivating mutation in the *LHB* gene had normal female reproductive tract growth. She displayed cystic ovaries and oligomenorrhea (infrequent menstrual cycles) with low levels of circulating estrogen. Estrogen did stimulate her ovarian follicle growth but hCG supplementation was absolutely required for ovulation induction.

Inactivating mutations in the human *FSHB* gene were also reported. Most of these are located in exon 3 and result in production of a truncated FSH β protein that fails to assemble with the α -subunit and hence FSH dimer is absent in these patients. In some patients, FSH heterodimer is formed but cannot bind to the receptor and transduce the signals in gonads. Men with mutations in *FSHB* gene present variable testis volume but are azoospermic and infertile. Bio and immunoactive FSH levels are undetectable and LH and testosterone values vary in these patients and some exhibit Leydig cell hyperplasia. Women with mutations in *FSHB* gene present common phenotypic features such as primary amenorrhea, low levels of serum FSH and estradiol and elevated LH. These women fail to respond to GnRH stimulation because bioactive and immunoactive FSH is unmeasurable indicating defective FSH dimer assembly as a result of formation of a mutant FSH β subunit. Exogenous FSH treatment rescues the ovarian folliculogenesis defects by promoting antral follicle formation. In addition to inactivating mutations in gonadotropin β -subunit encoding genes, activating and inactivating mutations in their cognate gonadotropin receptors (*LHR* and *FSHR*) were also reported. These mutations manifest in defects in reproductive function as a result of failure of normal gonad development.

Genetically Modified Mouse Models for Gonadotropins

The gonadotropin gene and protein sequences are relatively highly conserved between mice and humans. Hence, mouse genome manipulation techniques provide a convenient in vivo model system to study gonadotropin synthesis, secretion and action in a whole animal context. In one approach, gain-of-function effects of gonadotropins were tested by overexpressing individual gonadotropin subunits or both subunits separately but simultaneously in the same transgenic mouse and the consequences studied. Either a homologous or a heterologous tissue/cell specific promoter was used for directing the transgene (cDNA or gene) expression directly into gonadotropes or ectopically in tissues other than pituitary. In a second approach, loss-of-function mutations, were first created in mouse embryonic stem cells in a heterozygous state and the mutations propagated via germline transmission. Subsequently, homozygous mutant (knockout or null) were obtained by crossbreeding. A functional rescue was feasible by introducing the gain of function transgene onto the null genetic background.

During mouse pituitary development, the common α -subunit encoding gene (*Cga*) is activated first followed by LH β and finally FSH β in gonadotropes. Later, it is also activated in thyrotropes within the pituitary where it heterodimerizes with the TSH β subunit to produce TSH. Targeted overexpression of a human *CGA* transgene in pituitaries or ectopically in multiple tissues of transgenic mice did not result in any overt phenotypes and the mice were normal without any reproductive deficits. The in vivo physiological

role of the α -subunit was demonstrated by a loss of function mutation by which the α -subunit encoding gene was deleted and the α -subunit was absent from birth in these null mice. *Cga* null mice lacked both gonadotropins (LH, FSH) and thyrotropin because α -subunit is required for production of all three heterodimeric hormones. These mice exhibit profound hypothyroidism and develop thyrotrope tumors at old age. Both ovaries and testes were formed normally in these infertile mutant mice indicating early gonad formation is not dependent on circulating gonadotropins.

Gain-of-function effects of LH were studied by pituitary targeted expression of an LH analog, called LH β -CTP using a transgene encoding the carboxy terminal sequence from hCG β fused in frame with the LH β -sequence. In this way, LH remained for longer periods of time in serum of these transgenic mice. These mice initially developed hemorrhagic ovarian cysts and depending on the genetic background, also eventually developed ovarian granulosa cell tumors. Two other LH gain of function transgenic mouse models were also developed. In these models, hCG α and β subunit encoding transgenes were ubiquitously expressed in multiple tissues either by a ubiquitin C or a mouse metallothionein-I promoter. This ectopic expression of hCG in multiple tissues caused various phenotypes in both the sexes including hyperandrogenemia, Leydig cell hyperplasia and Leydig cell tumors, impaired spermatogenesis in transgenic males and ovarian cysts, thecal cell expansion and development of mammary tumors and estrogen-dependent lactotrope hyperplasia/tumors in female mice.

Because both LH and FSH are absent in *Cga* null mice, to independently study the loss of function effects of LH, *Lhb* null mice were developed. In the absence of LH β subunit and hence LH heterodimer from birth, male mice were infertile, exhibited hypogonadism and demonstrated severely suppressed intra testicular and circulating testosterone. Leydig cells were developmentally arrested and no mature adult type Leydig cells that normally produce testosterone were present in the testes. Spermatogenesis was arrested at the round spermatid stage and no elongated spermatids and mature sperm were present in the mutant testes. *Lhb* null female mice were also infertile and demonstrated suppressed levels of serum estrogen and progesterone. Ovarian histology showed most of the follicles developed up to the antral stage but pre-ovulatory follicles were absent. A prominent thecal layer was evident but all the LH-regulated genes in thecal cells were suppressed in ovaries of mutant mice. Administration of exogenous hCG to immature males and superovulation of immature females (by a combination of equine CG and hCG) resulted in rescue of *Lhb* mutants.

Targeted overexpression of a human *FSHB* transgene in gonadotropes of transgenic mice was also achieved. The human *FSHB* transgene was expressed two- to threefold higher than the endogenous mouse *Fshb* gene and was regulated similarly in mouse pituitaries. An interspecies hybrid FSH dimer, consisting of mouse α - and human FSH β , was assembled and efficiently secreted because the serum FSH levels were elevated in these mice. Male transgenic mice showed a marginal increase in testis size and normal testosterone levels. Both male and female transgenic mice were normal and did not exhibit any reproductive deficits. Human FSH was also ectopically expressed from multiple tissues in another transgenic line by using a ubiquitously active mouse metallothionein-1 promoter. This ectopic expression resulted in production of very high levels of serum FSH in these transgenic mice. Male transgenic mice exhibited enlarged seminal vesicles, elevated testosterone levels in serum and reduced testis size with apparently normal spermatogenesis but these males were infertile. Female transgenic mice were infertile and exhibited massively hemorrhagic ovarian cysts with totally disrupted folliculogenesis. These mice exhibited elevated serum testosterone and estrogen and died early around 12–14 weeks due to kidney defects.

Ectopic expression of hFSH was achieved by targeting human *CGA* and *FSHB* cDNA transgenes using a rat insulin II promoter and introducing them onto hypogonadal (*hpg*) null genetic background by crossbreeding. The *hpg* mice harbor a mutation in the GnRH-encoding gene resulting in loss of functional GnRH and hence both LH and FSH were severely suppressed in these mutant mice. This genetic strategy permitted direct assessment of physiological effects of only ectopically expressed FSH in the absence of endogenous LH. Biologically active human FSH partially rescued phenotypes in *hpg* male mice, but testosterone supplementation fully rescued them. Female transgenic mice expressing human FSH on a *hpg* genetic background demonstrated increased ovarian follicle reserve and increased antral follicle development. Those transgenic mice not on the *hpg* background also demonstrated premature ovarian failure as a result of accelerated ovarian aging.

FSH loss of function condition was created by selectively deleting the FSH β subunit-encoding gene in mice. The resultant *Fshb* null male mice showed reduced testis size, Sertoli cell number and a correspondingly reduced number of sperm as well as reduced sperm motility. Qualitatively, spermatogenesis was normal with all stages apparent in the mutant testes. Leydig cell number per testis remained the same and these mice demonstrated normal circulating testosterone levels. Interestingly, loss of FSH in mice did not affect male fertility unlike in men with inactivating mutations in the human *FSHB* gene. Unlike males, female *Fshb* null mice were infertile. They were anovulatory and demonstrated hypoplastic ovaries with a block in ovarian folliculogenesis at the pre-antral stage. There were no corpora lutea present in the mutant ovaries. In a pharmacological rescue approach, superovulation rescued *Fshb* female null mice and resulted in normal one-cell number of embryos confirming that FSH responsiveness was not lost in *Fshb* null mutant female mice.

Fshb null mice were also genetically rescued. This was achieved in two independent ways. In one approach, the 10 kb human *FSHB* transgene was introduced onto *Fshb* null genetic background. This gonadotrope-targeted human *FSHB* transgene fully rescued *Fshb* null mice. Testis size was restored, sperm number and motility reverted back to normal levels. Female mice showed normal ovarian folliculogenesis, ovulated normally and became fertile. In a second approach, low levels of human FSH were ectopically expressed and this resulted in full rescue of male mice but the rescue was somewhat incomplete in female mice. This suggested pituitary-derived but not ectopically expressed FSH was more efficient for a full functional rescue of *Fshb* null mice. *Fshb* null mice proved very useful in a number of other physiological and genetic studies in male and female reproductive physiology.

More recently, genetic rescue of *Fshb* null mice was achieved using constitutively secreted and re-routed FSH released via the LH secretory pathway. In these studies, a human LH β carboxy terminal heptapeptide encoding DNA sequence was genetically fused to that of human FSH β encoding gene sequence and the resultant *FSHB* transgene was introduced onto *Fshb* null genetic background. For comparison, the conventional wild-type human *FSHB* transgene was similarly introduced onto the *Fshb* null background. The mutant FSH β containing FSH dimer entered the LH secretory pathway and was released in response to acute GnRH treatment, similar to endogenous LH and unlike that of constitutively released FSH dimer (containing the FSH β subunit encoded by the wild-type human *FSHB* transgene). The mutant FSH expressing female mice demonstrated vastly improved and prolonged reproductive performance, producing large number of ovulations as a result of suppressed ovarian follicular atresia. Thus, the target cells (ovarian granulosa cells) can effectively decode the pattern of FSH signal inputs and respond distinctly.

Extra-Gonadal Actions of Gonadotropins

The central dogma that gonadotropins regulate gonadal function by binding to gonadal cell receptors has been challenged. In a series of in vitro studies, extra-gonadal actions of hCG were uncovered in many different tissues that express LH/CG receptors. Recently, extra-gonadal expression of FSHRs and FSH actions via these receptors were also identified. Significant effects of FSH signaling in bone osteoclasts, and white and brown adipocytes were noted particularly with implications to age-related bone loss and visceral adiposity in post-menopausal women. In ovariectomized animal models, a blocking polyclonal antibody against FSH reduced both bone loss and enhanced conversion of white to brown adipose tissue.

Clinical Applications of Gonadotropins

Human gonadotropin preparations purified from pituitary and urine sources were used for clinical research in the past. However, recombinant gonadotropins expressed in mammalian cell expression systems replaced the earlier preparations and have now been in use for various human in vitro fertilization protocols at the clinic. These recombinant gonadotropins are routinely used for ovarian follicle induction in vivo and follicle maturation in vitro. These preparations are pathogen-free and thus safe for human use. Elegant molecular cloning techniques have also allowed expressing improved versions of gonadotropins such as single chain gonadotropin analogs in which the two gonadotropins subunits are genetically fused in tandem. A long-acting FSH analog with enhanced serum half-life of FSH was also generated by genetically fusing the DNA sequence encoding the carboxy terminus of hCG β to 3'-end of DNA sequence encoding the human FSH β subunit. This FSH analog is currently approved for use in human IVF protocols in some countries. Although the primary goal of recombinant human gonadotropins is to benefit human reproductive healthcare, recombinant homologous hormones are equally efficacious in many veterinary/agricultural livestock species.

Acknowledgments

Work done in the Author's laboratory is supported in part by National Institutes of Health Grants AG029531, CA166557, HD081162, AG056046, and The Makowski Foundation. The author expresses his sincere thanks to Dr. George Bousfield for critically reading and his comments on the manuscript. This chapter is dedicated to the memory of Dr. P. Michael Conn who deceased in November 2016.

Further Reading

- Bousfield, G. R., Jia, L., & Ward, D. N. (2006). Gonadotropins: Chemistry and biosynthesis. In J. D. Neill (Ed.) (3rd edn), *vol. I. Knobil's physiology of reproduction* (pp. 1581–1634). Amsterdam: Academic Press.
- Huhtaniemi, I. T., & Themmen, A. P. (2005). Mutations in human gonadotropin and gonadotropin-receptor genes. *Endocrine*, 26, 207–217.
- Kumar, T. R. (2016). Mouse models for the study of synthesis, secretion, and action of pituitary gonadotropins. *Progress in Molecular Biology and Translational Science*, 143, 49–84.
- Narayan, P., Ulloa-Aguirre, A., & Dias, J. A. (2018). Gonadotropin hormones and their receptors. In J. Strauss, A. Barbieri, & A. Gargiulo (Eds.), *Yen & Jaffe's reproductive endocrinology* (pp. 25–57). Philadelphia: Elsevier.
- Pierce, J. G., & Parsons, T. F. (1981). Glycoprotein hormones: Structure and function. *Annual Review of Biochemistry*, 50, 465–495.
- Richards, J. S., & Pangas, S. A. (2010). The ovary: Basic biology and clinical implications. *The Journal of Clinical Investigation*, 120, 963–972.
- Skinner, M. K. (1991). Cell–cell interactions in the testis. *Endocrine Reviews*, 12, 45–77.
- Stamatiades, G. A., & Kaiser, U. B. (2017). Gonadotropin regulation by pulsatile GnRH: Signaling and gene expression. *Molecular and Cellular Endocrinology*.
- Ulloa-Aguirre, A., Dias, J. A., & Bousfield, G. R. (2017). Gonadotropins. In M. Simoni, & I. Huhtaniemi (Eds.), *Endocrinology of the testis and male reproduction* (pp. 1–52). New York: Springer International.
- Yuen, T., Sun, L., Liu, P., Blair, H. C., New, M., Zallone, A., & Zaidi, M. (2016). Beyond reproduction: Pituitary hormone actions on bone. *Progress in Molecular Biology and Translational Science*, 143, 175–185.

Gonadotropin Receptors

Kirsten S Eckstrum and Lori T Raetzman, University of Illinois at Urbana-Champaign, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The gonadotropin receptors (luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR)) play critical roles in male and female reproduction. LHR and FSHR belong to the G protein coupled receptor (GPCR) family and have a large extracellular domain, a transmembrane segment consisting of seven membrane spanning α helices, and a short intracellular domain. These receptors, found in male testes and female ovaries, bind to the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), secreted from the gonadotroph cells of the anterior pituitary. Activation of these receptors leads to development of the gonads and production of sex steroids. In both males and females the production of hormones through the gonadotropin receptors is important for proper development and functioning of the hypothalamic-pituitary-gonadal (HPG) axis. The LHR is also called LH/hCG receptor due to its ability to bind human chorionic gonadotropin, a hormone produced by the placenta during pregnancy. Here we will focus on the structure and function of the gonadotropin receptors in the female ovaries. The ovary has two fundamental functions: one is to develop oocytes and the second is to produce steroid hormones. The gonadotropin receptors have roles in each of these processes.

Gene to Protein

Both the gonadotropin receptor genes (*LHR* and *FSHR*) in humans are located on chromosome 2 and are separated by about 200 kb. In the mouse, they are located on chromosome 17, and in the rat on chromosome 6. Interestingly, for both *Lhr* and *Fshr*, the extracellular domain is encoded by the first 10 and 9 exons respectively, while the transmembrane domain and the C-terminal intracellular domain are encoded by the last exon. Between these receptors, the N-terminal domain is highly conserved, whereas the C-terminal is variable. Thus, the intracellular C-terminal domain is important in specific functions of each of the receptors. *LHR* and *FSHR* transcript size varies from species to species and with different splice variants. In general, the transcript size for *Lhr* in rats ranges from 6.7 to 1.8 kb and for *Fshr* there are two predominant transcripts of 7.0 and 2.5 kb (reviewed in Menon and Menon, 2012). Once the mRNA is transcribed, it is translated into protein. LHR consists of 699 amino acids and the mature protein has a molecular mass of 75 kDa. FSHR has 678 amino acids and a molecular weight of 76.5 kDa. Precursor forms of LHR and FSHR are also detectable as well. The precursors tend to accumulate in the endoplasmic reticulum and do not bind the hormone with high affinity. The LHR precursor is a mannose-rich 68 kDa protein and the FSHR precursor is an 81 kDa mannose-rich protein. LHR and FSHR are folded in the endoplasmic reticulum by a number of chaperone proteins including calnexin and calreticulin.

Once LHR and FSHR are translated into protein, they go through protein modifications. The rat LHR has been shown to contain six N-glycans on the predicted glycosylation sites. However, if protein glycosylation is inhibited, binding of hormone is unaffected. On the other hand, FSHR was shown to be glycosylated on two of three possible sites. Interestingly, glycosylation of FSHR is necessary for proper functioning of the receptor, as deglycosylated FSHR does not bind FSH. This is because FSHR does not fold into the right conformation and move to the cell surface unless at least one of the two sites is properly glycosylated (Davis et al., 1995). The fully glycosylated form of LHR is 85 kDa and the fully glycosylated form of FSHR is 87 kDa. Soluble forms of LHR, formed from alternative splicing, also occur in the ovary. Soluble forms of LHR contain either the extracellular domain only or the extracellular and intracellular domain but no transmembrane domain. The soluble form has a similar affinity for LH as the membrane bound receptor. Therefore, the precursor, soluble, and full LHR can bind LH but only the full FSHR can bind FSH.

Interestingly, when gel electrophoresis was first done to determine the size of LHR and FSHR, only two bands were visualized for these receptors, the immature and the mature forms. However, more recently, molecular weight bands have been discovered consistent with dimeric or oligomeric forms of the gonadotropin receptors. The dimerization/oligomerization of the gonadotropin receptors is a process where the receptor self-associates in the endoplasmic reticulum and is eventually put in the plasma membrane in its self-associated form. This association of the receptors is not altered by the activation state of the receptor, meaning activation of one receptor does not cause the receptors to dimerize or cause them to break apart, however, data suggest that the transmembrane and extracellular domains of the receptors can mediate the homodimerization of the receptors (reviewed in Segaloff, 2012). Therefore, many mechanism and associations take place to get a fully functional LHR and FSHR in the cell membrane and ready to be activated.

Receptor Structure and Activation

The gonadotropin receptors are both transmembrane G-protein coupled receptors containing a large N-terminal extracellular domain. LHR and FSHR need to have such large extracellular domains because their ligands, the glycoproteins LH and FSH, are dimeric proteins consisting of an α and a β subunit, making these rather large proteins. Like other G-protein coupled receptors, LHR and FSHR have seven transmembrane α helices with three extracellular loops and three intracellular loops, and a short intracellular or cytoplasmic domain. Normally the receptors remain in an inactive state. Gonadotropin receptor activation occurs in two

steps. First, there is high affinity hormone binding to the extracellular domain. Second, there is hormone or extracellular domain interaction with the extracellular loops of the transmembrane domain to cause receptor activation via a conformational change, leading to activation of intracellular signaling cascades.

The ligands for the receptors share a common α subunit, but each have distinct β subunits. The extracellular domain has irregular leucine rich repeats that create successive β -strands and α -helices which makes a cusped structure with a β -sheet at the concave surface of the cusp. The hormone ligands of these receptors bind in the cusp region. The interaction with the extracellular loops of the transmembrane domain is important for receptor activation and the large extracellular domain helps to increase the binding affinity. Mutations of the third extracellular loop of LHR impairs receptor activation without altering hormone binding. In FSHR, mutations in the first extracellular loop impair hormone binding and receptor activation. Therefore, all three of the extracellular domains play a role in hormone binding and mutations in any of the three extracellular loops can affect receptor activation and/or hormone binding (reviewed in [Banerjee and Mahale, 2015](#)). The precise mechanism by which the interaction between the ligand and the receptor activates the receptor is unknown, however, there are three proposed models. First, the portion of the ligand bound to the extracellular domain interacts with the hinge region of transmembrane helices, leading to receptor activation. Second, the interaction of the ligand on the extracellular domain causes the extracellular domain to interact with the transmembrane helices to cause receptor activation. Third, the extracellular domain normally causes an inhibitory effect on the transmembrane region, keeping it in the inactivated state, and binding of the ligand relieves the inhibitory effect (reviewed in [Menon and Menon, 2012](#)). Despite the lack of understanding of precisely how the receptor is activated, it is known that the extracellular domain, extracellular loops, and the ligand are all important, and that specific binding leads to a conformational change that conducts the signal into the cell.

The transmembrane domain is the site of signal transduction. Once the hormone binds the receptor, it is phosphorylated by G-protein related kinases. The receptor is then functionally uncoupled from the G-protein (Gs), meaning the G protein dissociates to bind with other factors in the signal transduction pathway. Once Gs becomes uncoupled, it activates adenylate cyclase, resulting in increased intracellular cyclic AMP (cAMP). This then leads to activation of protein kinase A (PKA) ([Fig. 1](#)). LHR and FSHR are also able to activate signaling pathways leading to increased inositol triphosphate (IP3) production, increased calcium, and activation of mitogen-activated protein kinases (MAPK) (reviewed in [Themmen and Huhtaniemi, 2000](#)). Additionally, these receptors have been shown to activate Akt pathways. Signaling is terminated in part by either desensitization or down regulation of LHR and FSHR expression, mechanisms to prevent excessive stimulation. Desensitization involves receptor phosphorylation and uncoupling to signaling pathways. For down regulation, the receptor brings in beta arrestins, which assist in internalization of hormone-receptor complex. The three intracellular loops play roles in either the internalization or phosphorylation process (reviewed in [Banerjee and Mahale, 2015](#)). In addition to receptor internalization, receptor mRNA levels are also reduced upon prolonged stimulation, which will further lead to receptor downregulation. Overall, once the gonadotropin receptors are activated, there are series of signaling cascades initiated that lead to many effects within the cells, including additional signaling through protein modification by phosphorylation and activation of gene transcription. Importantly, changes in the cell will be important in folliculogenesis and steroid hormone production.

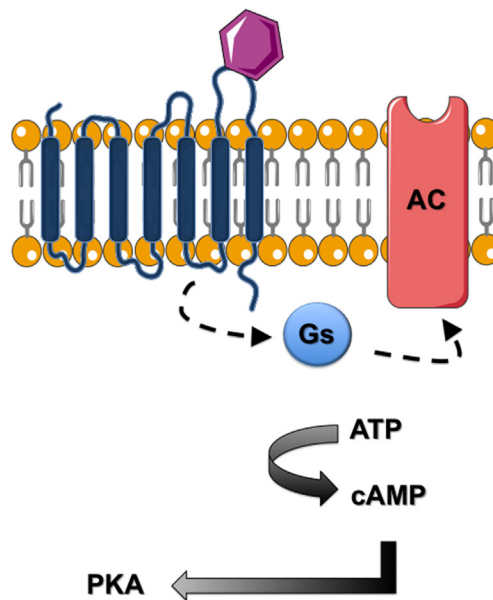


Fig. 1 Schematic of gonadotropin receptor signaling. When the gonadotropin (LH or FSH, purple) binds to its receptor (blue structure) in the cell membrane, a signaling pathway is activated through adenylate cyclase (AC) leading to protein kinase A (PKA) activation.

Gonadotropin Receptors in Follicular Development

The oocytes develop inside a follicle that consists of granulosa and theca cells. The gonadotropins, LH and FSH, are important in folliculogenesis or development of the follicle, induction of ovulation, and overall reproductive capability of the female. Primordial follicles remain quiescent until selected to become a primary follicle. Once primordial follicles are recruited, they progress to primary follicles containing a single layer of cuboidal granulosa cells and a basement membrane. The primary follicle is considered the first stage of the growing pool of follicles. Next, the follicle progresses to a secondary follicle containing several layers of granulosa cells surrounding the oocyte and a layer of theca cells. Eventually the granulosa cells secrete fluid to the center of the follicle, which collects to create a fluid filled spaces. This marks the tertiary follicle or early antral follicle. Tertiary follicles also have multiple layers of granulosa and theca cells. With increasing secretions from the granulosa cells, the fluid filled spaces fuse into what is termed the antrum. The antrum eventually becomes large enough so that it nearly encompasses the oocyte. At this point the follicle is called the preovulatory or Graafian follicle. It is from this follicle that the fertilized oocyte will be released from (reviewed in [Boron and Boulpaep, 2009](#); [Sánchez and Smitz, 2012](#)). This process of follicular growth, once initiated, continues until the oocytes is ovulated or degraded. At any given point in the reproductive cycle, an ovary will possess follicles of all the different stages.

To understand how the gonadotropin receptors function in follicular development and hormone production, it is important to know when and where they are expressed during the cycle described above. In humans and rodents, LHR protein and mRNA expression are not detected in primordial or primary follicles. LHR begins to be detected in theca cells of small antral follicles and is present in granulosa cells of the late antral follicles. The LH surge results in downregulation of LHR mRNA in rodents, but then expression reappears in the corpus luteum. On the other hand, primordial follicles do not express full length *FSHR* mRNA. In humans, *FSHR* protein is detectible in follicles beginning in the secondary stage and FSH binding is detectible in pre-granulosa cells that have matured to cuboidal forms. Although granulosa cells express *FSHR* and can respond to exogenous gonadotropins before the antral phase, follicular growth prior to the antral phase is not dependent on gonadotropins (reviewed in [Misrahi et al., 1998](#); [Yamamoto et al., 1992](#)). *FSHR* mRNA is present in the antral through pre-ovulatory follicle, but levels decrease in the corpus luteum.

There are three key phases of the gonadotropin receptor activity in follicular development: initial follicle development, FSH-dependent progression, and LH-responsive maturation. Initial follicle development from primordial to secondary follicle is believed to be *independent* of gonadotropin involvement. This has been determined based on evidence that mice that lack gonadotropin releasing hormone (GnRH), and thus have low FSH and LH levels, still show follicular growth up to the pre-antral stage. Similarly, women with hypogonadotropic hypogonadism, who have low levels of gonadotropins, have follicular development up to the primordial follicle. If exogenous gonadotropins are given to patients with this condition, ovulation and pregnancy can be obtained ([Sungurtekin et al., 1995](#)). This suggests that the ovaries already had a population of follicles that achieve their basal growth independent of the actions of gonadotropin receptors and administration of gonadotropins allowed them to progress to antral follicles.

It is clear that FSH is required for development of large antral follicles, bringing us to the FSH-dependent progression phase. With rising levels of FSH secreted from the anterior pituitary gland, a cohort of follicles will progress to further stages of development during the follicular phase of the ovarian cycle. Gonadotropins, in particular FSH, are important for sustained growth. FSH acts through its receptor on the granulosa cell to stimulate proliferation of granulosa cells leading to increased layers of granulosa cells. It also causes locally produced growth factors and formation of glycosaminoglycans that are then secreted to create the antral fluid (reviewed in [Hillier, 1994](#)). Dependence on FSH during folliculogenesis is low during basal growth and becomes more and more dependent as the follicle progresses. Granulosa cell growth is impaired when gonadotropins are low or absent, so there are less follicles in the growing phase without gonadotropins. Additionally, giving more gonadotropins accelerates follicular growth in experimental systems. However, gonadotropin receptor signaling is not the only determinant of follicular growth as many factors have also been shown to be important, including estradiol, insulin-like growth factor (IGF) and IL-6 (reviewed in [Matsuda et al., 2012](#)). If any of these factors are depleted or absent, the follicle will undergo atresia. The granulosa cells are programmed to be the first to undergo a form of cell death known as apoptosis if any of these pro-growth factors, including FSH, are missing.

As the follicles continue to grow, they become selectable, meaning they can be chosen to ovulate. During the early follicular phase, one follicle is selected to ovulate in humans, the most developed and healthy of all the follicles. The dominant follicle has been hypothesized to release follicular regulatory proteins to prevent the development of less developed follicles. In terms of hormonal regulation, the selected dominant follicle begins expressing FSH-induced aromatase activity, induces expression of LHR, and starts producing estrogens instead of androgen. It is also the one with the lowest FSH threshold. As the follicle develops, it becomes more and more sensitive to FSH. In the latter half of the ovarian cycle, FSH levels decrease due to increased estradiol and inhibin. Despite the decrease in FSH, the preovulatory follicle remains responsive to circulating FSH. The reason for increased responsiveness of pre-ovulatory follicles compared to small antral follicles is not entirely understood as smaller antral follicles and pre-ovulatory follicles express a similar number of FSH binding sites (reviewed in [Misrahi et al., 1998](#)). However, it may be because the dominant follicle is more vascularized compared to the less developed follicles thus increasing the amount of FSH the dominant follicle receives.

Follicular development is not only sensitive to FSH, but it is also sensitive to LH during the third stage, LH-responsive maturation. By the mid-follicular phase LH receptors are present on the theca cells and on granulosa cells, whose expression was stimulated by FSH signaling through *FSHR* (reviewed in [Richards et al., 1987](#)). The increased presence of LHR in the dominant follicle protects the follicle from the fall in plasma FSH concentrations and the follicle becomes more dependent and responsive to LH. There is a threshold level of LH that is required for follicular development and maturation during this stage. Too little LH and there is no androgen or estrogen synthesis, too much LH and granulosa cell proliferation is inhibited and follicles become atretic or go

through premature luteinization. As the pre-ovulatory follicle continues to grow there are increasing amounts of estrogen being made by this follicle. This estrogen then causes a positive feedback event leading to a LH surge mid-cycle. This surge causes the egg to finish meiotic maturation, it inhibits granulosa cell division and begins luteinization of the follicle. LH is primarily responsible for the shift in steroid production after the LH surge from estrogen to progesterone. Furthermore, in the corpus luteum the breakdown of the basal lamina allows cholesterol to get to the granulosa cells for progesterone production (reviewed in [Amsterdam et al., 1992](#)). Thus, both FSHR and LHR are of critical importance in the ovarian cycle. FSHR is more critical in the follicular phase while LHR is more important in the luteal phase. However, both receptors are necessary at the same time for estrogen production in the two-cell two-gonadotropin model.

Steroid Biosynthesis: The Two-Cell, Two-Gonadotropin Hypothesis

LHR and FSHR play important roles in steroid biosynthesis of estrogens via a two-cell concerted system. The two cells are theca and granulosa cells and the two gonadotropins are LH and FSH. Theca cells are the more superficial cells of the follicle making them close to blood vessels so they can easily pick up cholesterol. Cholesterol is the first essential component in steroid biosynthesis. Theca cells express LHR only, not FSHR. When LH binds its receptor, it increases the synthesis of steroidogenic acute regulatory protein (STAR), leading to an increase of cholesterol in the mitochondria. LHR signaling also increases the expression of enzymes necessary to produce androstenedione and testosterone in the theca cell including p450 side-chain cleavage (CYP11A1), 17 α -hydroxylase (CYP17A), and 3 β -hydroxysteroid dehydrogenase (HSD3B). Here, steroid biosynthesis in the theca cell ends.

The next step is the androstenedione created in the theca cell freely diffuses through the lamina basalis, or basement membrane, into the granulosa cells. FSH binding to FSHR initiates a signal cascade in the granulosa cell to stimulate expression of the enzyme aromatase, allowing the granulosa cell to convert the testosterone from the theca cell into estradiol, one of the main estrogens. Estradiol is then released into the blood stream. Interestingly, granulosa cells also express LHR however, they only create their own testosterone during the luteal phase of the menstrual cycle. This is because granulosa cells are located more centrally in the follicle where there are not many blood vessels to provide cholesterol to the cells. However, in the luteal phase, the theca and granulosa cells transform into the theca-lutein and granulosa-lutein cells respectively, which make up the corpus luteum. The corpus luteum is more highly vascularized allowing for granulosa-lutein cells to pick up cholesterol from the blood stream. However, another problem exists for the granulosa cells in making testosterone in that they lack the necessary enzyme, 17 α -hydroxylase, which converts progesterone into 17 α -hydroxyprogesterone. Therefore, progesterone from the granulosa cell must diffuse to the theca cell to be converted to androstenedione (reviewed in [Boron and Boulpaep, 2009](#)). In sum, LHR stimulates the production of testosterone in the theca cells, which is sent to the granulosa cells that express aromatase, and whose activity is increased by FSHR, in order to make estrogen. Therefore, both LH and FSH are necessary and both theca and granulosa cells are necessary to make estradiol.

Relevant Mutations to Human Health

Genetic mutations in *LHR* or *FSHR* can impact reproductive function in the female. These mutations can either lead to inactivation or overactivation of the receptors. Inactivating *LHR* mutations have few effects on the early development of female sex characteristics. However, patients have a small uterus due to decreased estrogen, reduced bone mass, increased plasma LH concentrations, amenorrhoea, and anovulation. Loss of function mutations in *FSHR* show ovarian histology resembling that of the pre-pubertal period with mainly primordial and primary follicles ([Aittomäki et al., 1996](#)). Women with loss of function mutations in *FSHR* have an ovarian dysgenesis syndrome associated with delayed puberty, primary amenorrhea, and infertility. Most mutations in *LHR* and *FSHR* result in decreased expression of the mutant receptor due to misfolding of the receptor. Misfolded proteins are usually prevented from leaving the ER and become targeted for degradation. Interestingly, a mutation in the *LHR* that prevents interaction between Asp578 and Asn615 in the transmembrane helix 7 mimics the ligand occupied state of LHR and leads to a constitutively active form of LHR. Although this can cause precocious puberty (reviewed in [Shenker, 2002](#)), other studies have suggested that overexpression of LHR alone is insufficient to induce puberty in girls because both LHR and FSHR co-operate to allow steroid production in the ovary. Further, mutations in extracellular binding domain of *FSHR* that permit hCG to bind to the receptor have been associated with ovarian hyperstimulation syndrome, including fluid filled ovarian cysts and pregnancy complications. These examples illustrate the importance of normal gonadotropin receptor function in human health.

Conclusion

Taken together, the gonadotropin receptors are a unique class of GPCRs that play a vital role in ovarian development and function. Not only are the actions of LHR and FSHR important for the ovary and reproduction, as we have focused on here, but they are important for the proper functioning of the entire body because they are critical in the creation of estrogens and estrogens can affect many tissues including the brain, pituitary, mammary glands, bone, heart, and many more. Therefore, it is important to continue to study the components of these GPCR structure, activation, and function to fully understand the ovary, reproduction, and the overall human body.

References

- Aittomäki, K., Herva, R., Stenman, U. H., Juntunen, K., Ylöstalo, P., Hovatta, O., & de la Chapelle, A. (1996). Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. *The Journal of Clinical Endocrinology and Metabolism*, 81(10), 3722–3726. <https://doi.org/10.1210/jcem.81.10.8855829>.
- Amsterdam, A., Plehn-Dujowich, D., & Suh, B. S. (1992). Structure-function relationships during differentiation of normal and oncogene-transformed granulosa cells. *Biology of Reproduction*, 46(4), 513–522.
- Banerjee, A. A., & Mahale, S. D. (2015). Role of the extracellular and intracellular loops of follicle-stimulating hormone receptor in its function. *Frontiers in Endocrinology*, 6(110). <https://doi.org/10.3389/fendo.2015.00110>.
- Boron, W. F., & Boulpaep, E. L. (2009). *Medical physiology* (2nd edn). Philadelphia, PA: Saunders Elsevier.
- Davis, D., Liu, X., & Segaloff, D. L. (1995). Identification of the sites of N-linked glycosylation on the follicle-stimulating hormone (FSH) receptor and assessment of their role in FSH receptor function. *Molecular Endocrinology*, 9(2), 159–170. <https://doi.org/10.1210/mend.9.2.7776966>.
- Hillier, S. G. (1994). Current concepts of the roles of follicle stimulating hormone and luteinizing hormone in folliculogenesis. *Human Reproduction (Oxford, England)*, 9(2), 188–191.
- Matsuda, F., Inoue, N., Manabe, N., & Ohkura, S. (2012). Follicular growth and atresia in mammalian ovaries: Regulation by survival and death of granulosa cells. *The Journal of Reproduction and Development*, 58(1), 44–50. <https://doi.org/10.1262/jrd.2011-012>.
- Menon, K. M. J., & Menon, B. (2012). Structure, function and regulation of gonadotropin receptors—A perspective. *Molecular and Cellular Endocrinology*, 356(1), 88–97. <https://doi.org/10.1016/j.mce.2012.01.021>.
- Misrahi, M., Beau, I., Meduri, G., Bouvattier, C., Atger, M., Loosfelt, H., et al. (1998). Gonadotropin receptors and the control of gonadal steroidogenesis: Physiology and pathology. *Baillière's Clinical Endocrinology and Metabolism*, 12(1), 35–66. [https://doi.org/10.1016/S0950-351X\(98\)80444-8](https://doi.org/10.1016/S0950-351X(98)80444-8).
- Richards, J. S., Jahnsen, T., Hedin, L., Lifka, J., Ratoosh, S., Durica, J. M., & Goldring, N. B. (1987). Ovarian follicular development: From physiology to molecular biology. *Recent Progress in Hormone Research*, 43, 231–276.
- Sánchez, F., & Smits, J. (2012). Molecular control of oogenesis. *Biochimica et Biophysica Acta*, 1822(12), 1896–1912. <https://doi.org/10.1016/j.bbadis.2012.05.013>.
- Segaloff, D. L. (2012). Regulatory processes governing the cell surface expression of LH and FSH receptors. *Subcellular Biochemistry*, 63, 113–129. https://doi.org/10.1007/978-94-007-4765-4_7.
- Shenker, A. (2002). Activating mutations of the lutropin choriogonadotropin receptor in precocious puberty. *Receptors & Channels*, 8(1), 3–18.
- Sungurtekin, U., Fraser, I. S., & Shearman, R. P. (1995). Pregnancy in women with Kallmann's syndrome. *Fertility and Sterility*, 63(3), 494–499. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/7851576>.
- Themmen, A. P. N., & Huhtaniemi, I. T. (2000). Mutations of gonadotropins and gonadotropin receptors: Elucidating the physiology and pathophysiology of pituitary-gonadal function. *Endocrine Reviews*, 21(5), 551–583. <https://doi.org/10.1210/edrv.21.5.0409>.
- Yamamoto, M., Shima, K., & Nakano, R. (1992). Gonadotropin receptors in human ovarian follicles and corpora lutea throughout the menstrual cycle. *Hormone Research*, 37(Suppl. 1), 5–11.

Steroid Receptors Classical

Rong Li and Francesco J DeMayo, National Institute of Environmental Health Sciences, Research Triangle Park, NC, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Steroid receptors (SRs) are a subfamily of the nuclear receptor superfamily, containing five classical members: estrogen receptors (ESRs), progesterone receptors (PGRs), androgen receptors (ARs), glucocorticoid receptors (GRs), and mineralocorticoid receptor (MR) (Williams-Ashman and Reddi, 1971; Toft and Gorski, 1966; Kuiper et al., 1996; Bruchovsky and Wilson, 1968; Munck and Brinck-Johnsen, 1968; Porter and Edelman, 1964). They are likely to stem from a common ancestor gene, and share highly conserved structures and functions (Thornton, 2001). SRs regulate a multitude of genes and proteins via both genomic and non-genomic pathways, leading to alterations in a wide range of biological processes (Levin, 2015; Beato and Klug, 2000; Levin and Hammes, 2016). The critical regulation of SRs in female reproduction has already been recognized (Geraghty and Kaufer, 2015; Findlay et al., 2010; Conneely et al., 2000; Zhou, 2010). This chapter summarizes the molecular structures and actions of SRs, and focuses on their functions in female reproduction.

Molecular Biology

Gene

In humans, SR genes are located at different chromosomes. ESR has two major isoforms ESR1 and 2, also called NR3A1 and 2 which is encoded by two different genes located on chromosome 6q and chromosome 14q, respectively (Gosden et al., 1986; Enmark et al., 1997). PGR, also called NR3C3, is encoded by one gene that is located at Chromosome 11q which transcribes two major isoforms PGR-A and -B (Law et al., 1987; Kastner et al., 1990). AR, also called NR3C4, is encoded by one gene on chromosome X q11-q12 (Lubahn et al., 1988). GR is encoded by gene NR3C1, located on chromosome 5q31-32 with two major isoforms GR α and GR β (Theriault et al., 1989; Hollenberg et al., 1985). MR is encoded by gene NR3C2 located on Chromosome 4 q31.1 (Morrison et al., 1990).

Protein

As shown in Fig. 1, all SRs display a modular structure comprised of six regions (A-F) (Misrahi et al., 1993): a hypervariable amino-terminal domain region A/B, a highly conserved DNA-binding domain (DBD) region C, a linker region D, a less conserved ligand binding domain (LBD) region E, and a hypervariable carboxyl-terminal domain region F (Beato and Klug, 2000). There are two transcription activation domains in SRs, one is ligand independent and located at the A/B region referred to as activation function (AF)-1, the other is ligand dependent and resides at the E region, called AF-2 (Wammark et al., 2003). Cofactors and basal transcription machinery factors can interact with AF-1 and -2 (Kumar and Thompson, 2005). Little is known about F region, but it may play a role in ESR1 transcription (Pawlak et al., 2012).

Isoforms

Protein isoforms are generated from the same gene but with distinct amino acid sequences and biological roles (Gunning, 2006). Alternative promoter, splicing and translation initiation sites are common mechanisms of producing protein isoforms (Chen and Manley, 2009; Pal et al., 2011; Kochetov, 2008), and are identified in SRs.

Multiple promoters reside at SR genes. At a minimum, the number of promoters is eleven for ESR1 (Kos et al., 2001; Zou et al., 2009; Okuda et al., 2003), four for ESR2 (Hirata et al., 2001; Shoda et al., 2002; Smith et al., 2010; Lee et al., 2013), five for PGR (Yamanaka et al., 2002; Hirata et al., 2000, 2002; Saner et al., 2003; Kastner et al., 1990), nine for GR (Palma-Gudiel et al., 2015; Breslin et al., 2001; Turner and Muller, 2005; Presul et al., 2007) and two for MR (Zennaro et al., 1995), but only one identified for AR so far (Ahrens-Fath et al., 2005; Dehm et al., 2008; Tilley et al., 1990).

Coupling with the multiple promoters, alternative splicing produces dozens of transcription isoforms by intron/exon skipping, inserting, or replicating. Many SR transcription isoforms have been demonstrated with physiological and/or pathological functions, such as ER β cx (Herynk and Fuqua, 2004), PGR-C, -M, -S (Wei et al., 1990; Cork et al., 2008), AR-V7, -V9 (Lu et al., 2015; Dehm and Tindall, 2011), GR γ , GR-A and GR-P (Moalli et al., 1993; Ray et al., 1996), and MR248, MR260, MR+4 (Bloem et al., 1995; Zennaro et al., 2001) etc.



Fig. 1 Schematic diagram of the protein structure of steroid receptors (SRs). SRs display a modular structure comprised of six regions (A–F). AF1, activation function 1 is located at A/B region; DBD, DNA binding domain is located at C region; LBD, ligand binding domain; AF2, activation function 2 are located at E region.

Alternative translation further increases the number of protein isoforms. For example, 8 alternative translation initiation sites (TIS) have been identified in GR, thus 8 translation isoforms could be generated from a single GR transcription isoform, such as GR α and β (Lu and Cidlowski, 2005). In the same paper, the authors predict more species conserved TIS at the N-terminal of ESR1, PGR, AR, and MR (Lu and Cidlowski, 2005). Several of these internal TIS have already been identified as truly functional alternative translational start sites (Pascual-Le Tallec et al., 2004; Barraille et al., 1999; Wilson and McPhaul, 1994; Chantalat et al., 2016).

Coregulators

Transcription coregulators are molecules that interact with nuclear receptors (NRs) to regulate the gene transcription (Lonard and O'Malley, 2012; Millard et al., 2013). They are recruited by the NRs in a dynamic arrangement to modify the transcription factors including NRs, chromatin, and other coregulators (Millard et al., 2013). Most coregulators can either activate or repress the transcription (Lonard and O'Malley, 2007). To date, more than 300 coregulators can interact with SRs (Obeid et al., 2016).

These coregulators not only act as a physical adaptor, but also exert some enzymatic activities, including the acetyltransferase activity associated with steroid receptor coactivator-1 (SRC-1) family and CREB binding protein (CBP)/p300 coactivator family (Dasgupta and O'Malley, 2014), the methyl transferase activity of protein arginine methyltransferase (PRMT) 1 and 5 (Wysocka et al., 2006), the ATPase dependent chromatin remodeling by the switch/sucrose non-fermentable (SWI/SNF) complex (Roberts and Orkin, 2004), ubiquitin-ligase activity of E6-associated protein (E6-AP) and receptor potentiating factor 1 (RPF1) (Ramamoorthy and Nawaz, 2008; Imhof and McDonnell, 1996).

Actions

Nuclear SRs directly regulate gene transcription through genomic pathways, while membrane or mitochondria SRs might regulate the gene or protein expression through non-genomic pathways which will not be discussed in this chapter (see Fig. 2; Losel and Wehling, 2003; Levin, 2015; Schwartz et al., 2016).

For the genomic actions, SRs bind to a specific DNA sequence in the chromatin, called hormone response element (HRE) (Klinge, 2001; Glass et al., 1988; Umesono and Evans, 1989; Klein-Hitpass et al., 1986; Aranda and Pascual, 2001). After binding to the HRE, SRs recruit multiple enzymes to modify the chromatin state in preparation of the gene transcription (Beato et al., 1995; Metivier et al., 2006). The recent development of high throughput sequencing techniques has unveiled the DNA binding patterns of SRs throughout the genome.

Genomic analysis of SR binding sites has demonstrated that only a small fraction of SR binding sites are within the proximal promoter of target genes, while the majority are at the enhancers, the distal regulatory regions (Ballare et al., 2013; Carroll et al., 2006; Wang et al., 2011; Le Billan et al., 2015; Yu et al., 2010; Hewitt et al., 2012). The distribution patterns of these binding sites are markedly changed with different species, cell lines, and experimental designs (O'Hara et al., 2014; Le Billan et al., 2015; Aranda and Pascual, 2001; Rubel et al., 2012; Handel et al., 2013).

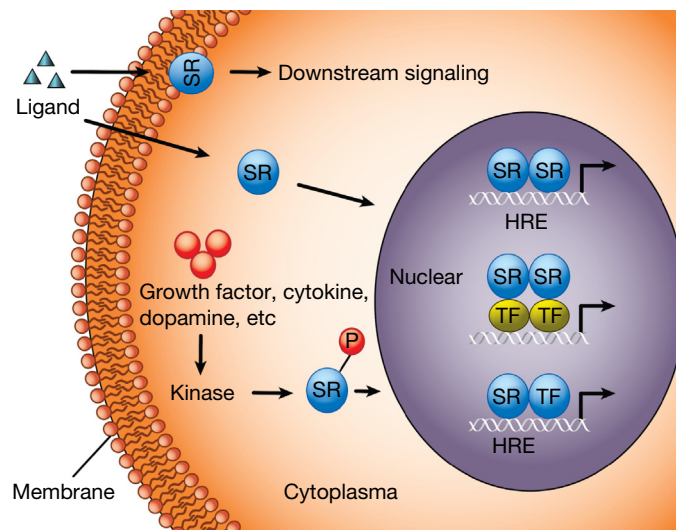


Fig. 2 Molecular mechanism of steroid receptor (SR) actions. Non-genomic actions of SR could be mediated by membrane SR and its downstream signaling pathways. SR genomic actions could be induced by ligand in a ligand dependent pathway, or growth factors, cytokines, etc. and its downstream kinases in ligand independent pathways. In the nucleus, SR directly binds to the chromatin at HRE, or tethers or co-binds with TF to the chromatin to regulate the gene transcription. *SR*, Steroid receptor; *HRE*, Hormone response element; *TF*, Transcription factor.

In addition to the direct binding, more often, SRs bind to the chromatin via tethering, or co-binding with other transcription factors (Owen et al., 1998; Gao et al., 2001; Gizard et al., 2005; Stender et al., 2010; Kassel and Herrlich, 2007; Reddy et al., 2009). The effects of the crosstalk between SRs and other transcription factors on transcription can be either positive or negative (Ratman et al., 2013).

Based on the presence of ligand, the genomic actions of SRs could be divided into ligand dependent or independent actions.

Ligand Dependent Actions

The classical model of SR actions presumes that SRs reside in the cytoplasm in association with heat shock proteins, once activated by its ligand, SRs transiently dissociate from the complex, dimerize, translocate to the nucleus and bind to the chromatin to regulate the transcription (Beato, 1989; Jensen et al., 1968; Pratt et al., 1989). Despite of its popularity, several problems arise from the classical model. One is that unliganded SRs do not necessarily reside in the cytoplasm. It is well known that ESR, PGR, and MR are mainly in the nucleus even in the absence of ligand (Greene et al., 1984; Perrot-Applanat et al., 1985; Wikstrom et al., 1987; Lombes et al., 1990). To make it more complicated, SR isoforms, such as PGR-A, PGR B, GR α , and GR β , may reside in different cellular compartments (Nadeem et al., 2016; Leslie et al., 2005; Oakley et al., 1997; Wan et al., 2001). More importantly, ligand recruitment is not essential for SR genomic actions as will be discussed below.

Ligand Independent Actions

In the absence of cognate ligand, ESR, PGR, AR and GR can be transcriptionally activated by a large variety of signals, such as Epidermal growth factor (EGF), insulin, dopamine, cytokine, etc. (Bennesch and Picard, 2015). As ligand binding can recruit kinase to activate SRs through phosphorylation (Lannigan, 2003; Trevino and Weigel, 2013; Koryakina et al., 2014; Bagchi et al., 1992), ligand independent kinase recruitment could also activate SRs. For example, Mitogen activated protein kinase (MAPK), Protein kinase A (PKA), and Protein kinase B (AKT) are recruited to activate ESR (Maggi, 2011; Becker et al., 2011); MAPK, Cyclin dependent kinase 2 (CDK2) and casein kinase 2 (CK2) are recruited to activate PGR (Hagan et al., 2012); Protein kinase C (PKC) and Rho GTPase are recruited to activate AR (Ponguta et al., 2008; Chan and Dehm, 2014; Lyons et al., 2008); and MAPK, Tumor necrosis factor (TNF) α are recruited to activate GR (Verhoog et al., 2011; Kotitschke et al., 2009) etc.

After activation, unliganded SRs bind to the chromatin to regulate gene transcription. Chromatin immunoprecipitation-sequencing (ChIP-Seq) analysis identifies the binding sites of ESR, PGR and GR with chromatin at ligand free conditions (Ahmad and Kumar, 2011; John et al., 2011; Rubel et al., 2012; Ballare et al., 2013). And the ligand independent GR bindings at the promoter regions are found to up-regulate several gene expressions (Ritter et al., 2012; Ritter and Mueller, 2014).

Specific Topics

SRs are expressed throughout the female reproductive system and act as critical regulators. In this chapter, we will focus on their functions in hypothalamus, pituitary, ovary, uterus, mammary gland and placenta. The hypothalamus and pituitary are the central part of the reproductive system. They coordinate with the female gonad, the ovary, to establish the hypothalamus–pituitary–gonad (HPG) axis to regulate the peripheral reproductive organs including the uterus, mammary gland and the placenta.

ESR

ESRs play a crucial role in the female reproductive system (Hamilton et al., 2014).

Hypothalamus

ESR1 and ESR2 are expressed in specific nuclei in the hypothalamus (Kuiper et al., 1998). Results from genetically modified mouse models indicate that ESR1 is the predominate regulator of female reproduction at the level of the central nervous system (Couse and Korach, 1999; Herbison, 1998; Wintermantel et al., 2006; Jayes et al., 2014). However, the importance of ESR2 in the central regulation of female reproduction has also been suggested (Hu, 2009; Weiser et al., 2008; Acevedo-Rodriguez et al., 2015).

In the hypothalamus, Gonadotropin-Releasing Hormone (GnRH) neurons, the central regulator of the reproductive HPG axis, is under the control of estrogen. Although the direct estrogen actions on GnRH neurons are still elusive (Butler et al., 1999; Roy et al., 1999; Martinez-Morales et al., 2001; Hu, 2009; Ng et al., 2009), it has been well established that GnRH neurons are innervated by ESR1 expressing neurons in other regions, such as the anteroventral periventricular nucleus (AVPV), the arcuate nucleus (ARC) and preoptic and anterior hypothalamic divisions of the periventricular nucleus (PVPOA) (Smith et al., 2005; Clarkson et al., 2008). ESR1 signaling in Kisspeptin (Kiss) 1 neurons at AVPV and ARC is essential for GnRH neuron activities (Skorupskaitė et al., 2014).

In addition to regulating the HPG axis, ESR1 is also directly involved in the neural circuits of some reproductive behaviors, such as female sexual receptivity which is initiated by ESR1 in the ventromedial hypothalamus (VMH) (Musatov et al., 2006; Kudwa and Rissman, 2003; Dominguez-Ordóñez et al., 2016). Maternal behaviors are enhanced by ESR1 actions in the medial preoptic area (Pena and Champagne, 2015).

Pituitary

ESR1 is the major isoform of ESRs in the pituitary. ESR1 primes the pituitary for the GnRH-induced luteinizing hormone (LH) surge (Kim et al., 2011). Pituitary-specific ESR1 null mice show dysregulation of the HPG axis (Singh et al., 2009), which could be partially rescued by the induction of ESR2. This demonstrates both ESR1 and 2 functions in the pituitary (Sanchez-Criado et al., 2012). Furthermore, ESR1 is important for the proliferation, apoptosis, prolactin expression and secretion of lactotrope in the anterior pituitary during estrous cycle and lactation (Ben-Jonathan et al., 2008; Yin et al., 2002; Zarate and Seilicovich, 2010; Zarate et al., 2010).

Ovary

In the ovary, ESR2 is abundant in the follicular granulosa cells, while ESR1 is mainly in the thecal and interstitial cells (Heldring et al., 2007; Sar and Welsch, 1999; Drummond and Fuller, 2012). Accordingly, ESR2 is found to be essential for the development of the ovarian follicles (Drummond and Fuller, 2012). ESR2 deletion from ovarian follicles impairs follicle development and functions (Emmen et al., 2005; Couse et al., 2005; Inzunza et al., 2007) leading to subfertility of mice (Jayes et al., 2014). In contrast, ESR1 deletion has been shown to have minimal effects on follicle development (Emmen et al., 2005). However, the ovulation and folliculogenesis resulted in thecal-specific ESR1 null mice emphasizing the importance of ESR1 in the ovary (Lee et al., 2009). More interestingly, ESR1 and ESR1/2 global knockout mice have testicular structures in the ovary, which suggests the roles of ESR1 in embryonic development of the ovary (Couse et al., 1999, 2006; Dupont et al., 2000).

Uterus

Results from genetically modified mice indicate that ESR1 is the major isoform in the uterus, which is dispensable for uterine development but essential for uterine functions (Hewitt et al., 2016). In response to the cyclic levels of estrogen during reproductive cycles and pregnancy, uterine ESR1 signaling drives epithelial proliferation (Koos, 2011), increases vasculature permeability (Rockwell et al., 2002; Koos, 2011), and establishes endometrial receptivity and decidualization (Robertshaw et al., 2016). Interestingly, the action of ESR1 is not cell autonomous in the uterus. ESR1 mediates a network of paracrine signaling between uterine stroma and epithelium to regulate epithelial cell proliferation (Winuthayanon et al., 2010, 2014).

Mammary gland

Both ESR1 and ESR2 are expressed in the mammary gland (Saji et al., 2000). ESR1 is expressed at all stages of mammary gland development including embryonic, pubertal, adult virgin and pregnant stages (Mehta et al., 2014). During the embryonic stage, ESR1 is detected (Keeling et al., 2000; Lemmen et al., 1999), but not required for mammary gland development (Briskin and O'Malley, 2010). At puberty, epithelial not stromal ESR1 drives the epithelium proliferation via paracrine signaling to promote the rapid growth of mammary ducts, such as elongation and side-branching (Zeps et al., 1998; Russo et al., 1999; Clarke et al., 1997; Mueller et al., 2002; Mallepell et al., 2006). During pregnancy, ESR1 regulates ductal and alveoli development (Feng et al., 2007).

Placenta

Virtually all cellular compartments of the human term placenta express either ESR1, ESR2, or both (Bukovsky et al., 2003a, b; Kim et al., 2016). Estrogen stimulates the differentiation of trophoblast, remodeling of uterine arteries, hormone production in trophoblast cells, placenta villous angiogenesis, and proliferation of decidualized endometrial stromal cells in the metrial gland in both human and rodents (Gambino et al., 2012; Albrecht and Pepe, 2010; Furukawa et al., 2012, 2013).

PGR

Hypothalamus

In rodents, PGR is highly expressed in several hypothalamic regions, such as anteroventral periventricular nucleus (AVPV), arcuate nucleus (ARC), and ventromedial nucleus of the hypothalamus (VMH) etc. (Quadros et al., 2007; Schwarz et al., 2010; Mahfouz et al., 2016a; Sa et al., 2013), including GnRH and Kiss1 neurons located at these regions (Ng et al., 2009; Clarkson et al., 2008; Dubois et al., 2016). Estradiol induction of PGR is a critical event for GnRH surge (Brom and Schwartz, 1968; Labhsetwar, 1970; Rao and Mahesh, 1986). Especially, PGR signaling is essential for the activities of Kiss1 neuron (Gal et al., 2016; Stephens et al., 2015; Leite et al., 2016). In addition, progesterone facilitates and inhibits female sexual behavior via PGR, mainly PGR-A, expressing neurons in VMH (Gomez-Camarillo et al., 2011; Portillo et al., 2016; Flanagan-Cato et al., 2006; Sanathara et al., 2014; White et al., 2007; Acharya et al., 2015).

Pituitary

PGR is expressed in the pituitary gonadotropes (Sprangers et al., 1990; Turgeon et al., 1999; Turgeon and Waring, 2001). Gonadotrope PGR is essential for the GnRH stimulated and estrogen induced LH surge *in vivo* (Turgeon and Waring, 2001; Chappell et al., 1999; Attardi et al., 2007). *In vitro*, PGR mediates GnRH stimulation on the expression of the common α subunit (Chen et al., 2010), progesterone inhibition of the LH β subunit (Thackray et al., 2009) and activation of follicle stimulating hormone (FSH) β subunit expression (An et al., 2009; Thackray et al., 2006).

Ovary

In rodents, PGR expression is initiated in the pre-ovulatory granulosa cells and then rapidly induced by a LH surge, and dramatically decreases in luteinized granulosa cells (Sriraman et al., 2003; Park and Mayo, 1991; Shao et al., 2003; Kim et al., 2008). PGR is also detected in the peri-ovulatory follicles of humans, but persists in the active corpus luteum until regression (Chang et al., 2005; Robker et al., 2009). PGR deletion does not affect follicle development and luteinization in the mouse ovary (Robker et al., 2000). However, PGR is a key regulator of follicle rupture in both rodents and humans (Russell and Robker, 2007; Robker et al., 2009). Since PGR-A is more abundant in granulosa cells than PGR-B (Natraj and Richards, 1993; Toms et al., 2015), and mice lacking only PGR-A show severely reduced ovulation (Mulac-Jericevic et al., 2000), PGR-A is the predominant isoform in the murine ovary. PGR deletion does not impair luteinization in mouse ovary (Robker et al., 2000) but may play a role in the function of the corpus luteum of primate ovary (Stouffer, 2003).

Uterus

Similar to ESR, PGR is dynamically expressed in the uterus and regulates multiple events via paracrine factors [Reviewed in (Wetendorf and DeMayo, 2012; Bhurke et al., 2016)]. Interestingly, one of the major functions of PGR is to inhibit estrogen induced epithelial proliferation (Martin et al., 1973; Lydon et al., 1995; Tong and Pollard, 1999; Li et al., 2011). However, unlike ESR1 in which stromal ESR1 regulates epithelial cell proliferation, the inhibition of epithelial cell proliferation is initiated by PGR expressed in the epithelium (Nanjappa et al., 2015; Franco et al., 2012).

PGR is crucial for uterine functions during pregnancy. PGR ablation in the germline or specifically in the uterine epithelium of mouse shows disrupted embryo implantation and endometrial decidualization (Franco et al., 2012). A similar phenotype is observed in PGR-A knockout mice indicating that PGR-A is the major functional isoform in the mouse uterus (Mulac-Jericevic et al., 2000, 2003). In contrast, PGR-B has been found as functionally more potent for human endometrium decidualization than PGR-A (Bagchi et al., 2015). During pregnancy, PGR modulates physiological vascular permeability (Goddard et al., 2014), uterine fluid pH (Karim et al., 2016), decidua angiogenesis (Kim et al., 2013), and immune responses (Bhurke et al., 2016) of the uterus.

Not only is the expression of PGR critical for uterine function, but also the timing of PGR action and the ratio of PGR isoforms is critical for uterine function. Uterine epithelial PGR is high in the neonatal uterus even though its ligand progesterone is low. Uterine gland formation, adenogenesis, is disrupted by neonatal progesterone injections in the mouse through PGR signaling (Stewart et al., 2011; Cooke et al., 2012; Filant et al., 2012; Dhakal et al., 2015; Franco et al., 2012). During the receptive period of embryo attachment, epithelial PGR expression is decreased, extending PGR action in the uterine epithelium through this receptive phase disrupts pregnancy (Wetendorf et al., 2017). In humans, the ability of the uterus to support pregnancy and parturition is dependent upon the ratio of PGR-A and PGR-B. During pregnancy PGR-B is the dominant isoform maintaining pregnancy, in part, through its anti-inflammatory action. At the end of pregnancy, there is a shift in the ratio of PR-B to PGR-A. In this case PGR-A promotes parturition in part through its pro-inflammatory action (Nadeem et al., 2016; Ke et al., 2016; Tan et al., 2012; Patel et al., 2015).

Mammary gland

PGR is co-expressed with ESR1 in the mammary gland epithelium (Obr and Edwards, 2012). PGR is not essential for mammary gland development during embryonic and pubertal stages (Lydon et al., 1995). Despite this, a role of epithelial PGR in mammary duct growth during puberty has been suggested using pharmacological treatment and mammary epithelial transplant models (Shi et al., 2004; Briskin et al., 1998; Aupperlee et al., 2013; Atwood et al., 2000). During pregnancy, PGR is required for the extensive side-branching and alveologenesis via a paracrine mechanism (Satoh et al., 2007; Briskin et al., 1998; Lain et al., 2013). The fact that PGR-B not PGR-A knockout mice resemble the mammary defects of PGR knockout mice (Mulac-Jericevic et al., 2000, 2003) suggests the PGR-B is the major isoform that functions in the mammary gland.

Placenta

The presence of PGR in human fetal membranes is still controversial (Mesiano et al., 2011). PGR is detected in human decidua and declines after the onset of labor to maintain decidua before labor (Blanks and Brosens, 2012; Goldman et al., 2005). Meanwhile, functional withdrawal of PGR by shifting from PGR-B dominance to PGR-A dominance is also observed in the decidua (Zhuang et al., 2015; Goldman and Shalev, 2007). In human term placenta, PGR-A is found as the only form in the cytotrophoblast (Wang et al., 2014) while PGR-C is the major isoform in syncytiotrophoblast (Taylor et al., 2009). Both PGR-A and -B are expressed in several trophoblast cell lines, such as JEG3, BeWo cells, etc. (Yasuda et al., 2009; Zachariades et al., 2011; Lee et al., 2016). And progesterone can inhibit the invasion of BeWo cells (Jo et al., 2015; Chen et al., 2011).

AR

AR functions in male reproduction have been well defined, but the importance of AR in female reproduction has gradually drawn more attention. Actually, androgen deficiency and excess in females contribute to several pregnancy- and fertility-related disorders, such as polycystic ovarian syndrome (Apparao et al., 2002; Wang et al., 2015), endometriosis (Huhtinen et al., 2014), and recurrent pregnancy loss (Okon et al., 1998).

Hypothalamus, pituitary, ovary

AR is expressed in a variety of nuclei that are known to project to or regulate the hypothalamus in rodents and humans (Williamson and Viau, 2007; Mahfouz et al., 2016b; Dart et al., 2013; Fernandez-Guasti et al., 2000).

Deletion of AR in pituitary gonadotrope reduces the basal FSH levels and pre-ovulatory FSH and LH surge (Wu et al., 2014). Androgen, the cognate ligand of AR, stimulates FSH β expression in castrated rats (Wierman and Wang, 1990) and suppresses LH β expression in immortalized gonadotrope cells (Curtin et al., 2001; Jorgensen and Nilson, 2001). AR knockout mice exhibit hypothalamus–pituitary defects (Walters et al., 2009).

In the ovary, AR is expressed in granulosa cells, theca cells and the oocytes at almost all the estrous stages (Tetsuka et al., 1995; Horie et al., 1992). AR is involved in the follicle development and ovulation (Prizant et al., 2014; Walters et al., 2008) while global knockout of AR impairs follicle development and ovulation leading to subfertility (Walters et al., 2010).

Uterus

AR is found in both the stromal and epithelial compartments of the human endometrium with a cyclic expression pattern during the menstrual cycle (Ito et al., 2002; Marshall et al., 2011) and is still detected in the first trimester decidua (Milne et al., 2005). Similar expression patterns are found in the mouse uterus (Pelletier et al., 2004; Rothman et al., 2011; Xu et al., 2015). Previous studies suggest androgen function in uterine epithelium proliferation and gland formation (Simitsidellis et al., 2016), uterine receptivity during embryo implantation (Mokhtar et al., 2014b; Gibson et al., 2016), endometrial decidualization (Kajihara et al., 2012, 2014; Gibson et al., 2016), uterine ion secretion and water transport (Salleh et al., 2015; Mokhtar et al., 2014a), proliferation and apoptosis of uterine myometrium (Liu et al., 2013; Li et al., 2014). More interestingly, uterine gland epithelial-specific AR null mice have defective endometrial and myometrial proliferation suggesting that AR mediates the crosstalk between epithelium and other uterine compartments (Choi et al., 2015).

Mammary gland, placenta

AR is expressed in the luminal layer of mammary ducts and acini and in a subset of basal myoepithelial cells in pre-menopausal women and mammary epithelium in mice (Li et al., 2010; Tarulli et al., 2014). During puberty, androgens inhibit while AR antagonists stimulate the proliferation of mammary epithelium in humans and rodents (Tarulli et al., 2014; Peters et al., 2011).

In human placenta, AR is primarily present in the syncytiotrophoblasts and stromal cells, and dysregulation of AR may contribute to the placenta abnormality in preeclampsia pregnancy and gestational diabetes (Hsu et al., 2009; Sathishkumar et al., 2012; Uzelac et al., 2010).

GR

Due to the wide spread distribution of GR, glucocorticoid, the cognate ligand of GR, signaling has been found throughout the female reproductive system (Whirledge and Cidlowski, 2010, 2013b; Geraghty and Kaufer, 2015). On one hand, stress responses, mainly mediated through glucocorticoids, adversely affects female reproduction (Sapolsky et al., 2000). On the other hand, glucocorticoids are used to treat a variety of medical disorders in pregnant women (Roberts and Dalziel, 2006; Lunghi et al., 2010). Therefore, identifying the potential GR signaling pathways in female reproduction is urgent (Emin Turkyay Korgun et al., 2012).

Hypothalamus, pituitary, ovary

At central level, GR is expressed in the hippocampus, hypothalamus and amygdala etc., thus, GR may regulate GnRH neuron actions both directly and indirectly (Son et al., 2014; Gojska and Belsham, 2014; Takumi et al., 2012; Dondi et al., 2005; Nahar et al., 2016; Johnson et al., 2005; McEwen et al., 1986; Gore et al., 2006).

In the pituitary cells, glucocorticoids enhance FSH, but inhibit LH production (Suter and Schwartz, 1985; McGillivray et al., 2007; Breen et al., 2012), and synergize with GnRH to regulate the transcription of GnRH receptor (GnRHR) (Kotitschke et al., 2009).

In the ovaries, GR is present in the granulosa cells, theca cells, and surface epithelium cells (Tetsuka et al., 1999; Rae et al., 2004; Yuan et al., 2016; Myers et al., 2007). Glucocorticoids modulate the responsiveness of the ovary to FSH and LH (Michael et al., 1993; Hsueh and Erickson, 1978; Schoonmaker and Erickson, 1983), maintain the corpus luteum (Myers et al., 2007), and promote oocyte development (Jimena et al., 1992; Andersen, 2003; Yuan et al., 2016).

Uterus, mammary gland, placenta

GR is abundantly expressed in the rodent uterus and human endometrial cancer cell lines (Korgun et al., 2003; Thompson et al., 2002; Whirledge and Cidlowski, 2013a). Specific deletion of GR from the reproductive tract impairs embryo implantation and decidualization in mice (Whirledge et al., 2015). Glucocorticoids antagonize estrogen actions in the uterus probably via GR (Gunin et al., 2000, 2001, 2003; Rabin et al., 1990; Whirledge et al., 2013).

GR is ubiquitously expressed in human mammary glands (Lien et al., 2006; Buxant et al., 2010). GR null mammary glands when transplanted into wild-type mice show abnormal pubertal development (Kingsley-Kallesen et al., 2002). Although GR is essential for milk protein production *in vitro* (Stocklin et al., 1996), mammary epithelial deletion of GR during pregnancy and lactation disrupts the alveoli development, but not milk production in mice (Wintermantel et al., 2005). *In vitro* culture of mouse mammary epithelium also demonstrates the importance of glucocorticoids in acini formation (Murtagh et al., 2004).

GR is highly expressed in the placenta (Thompson et al., 2002; Clifton et al., 2016; Lee et al., 2005). Glucocorticoids reduce placenta weight (Ozmen et al., 2011; Mark et al., 2013; Baisden et al., 2007), probably by inhibiting proliferation (Zhang et al., 2016; Gennari-Moser et al., 2011) and inducing apoptosis (Chan et al., 2007; Baisden et al., 2007), and prevent angiogenesis in the placenta (Hewitt et al., 2006; Nugent et al., 2013).

MR

Studies concerning MR functions in female reproductive systems are limited. But MR has been found to be expressed throughout the female reproductive system.

At central level, MR is found in several distinct brain regions including the hypothalamus (de Kloet et al., 1993; Mahfouz et al., 2016b; Varga et al., 2013; Haque et al., 2015) and the pituitary (Moguilewsky and Raynaud, 1980; Krozowski and Funder, 1981) of rodents. In the rat ovary, MR immunostaining is most intense in the corpus luteum, light to moderate in oocytes and granulosa cells, and least intense in theca cells (Gomez-Sanchez et al., 2009). MR is also found in the ductal epithelial cells of human breast (Sasano et al., 1997). In the uterus, MR is found at the different stages of the menstrual cycle and the first-trimester of human pregnancy (McDonald et al., 2006). In the placenta, MR is moderately detected in human placenta, such as syncytiotrophoblasts, some cytotrophoblasts, and interstitial cells of the villous core (Hirasawa et al., 2000).

Some MR functions have been suggested in the uterus and placenta. MR deletion or repression alters lipid droplet biogenesis and retinoid metabolism in the decidualized primary human endometrial stromal cells (HESC) (Kuroda et al., 2013a, b). The activation of MR appears to be required for the proliferation of the trophoblasts, sodium transport of cytotrophoblast cells, and intracellular pH value of syncytiotrophoblast in the placenta (Gennari-Moser et al., 2011; Driver et al., 2003; Speake et al., 2010).

Future Directions

Previous studies on genetically modified mouse models and human cell lines demonstrate the critical roles and broad actions of SRs in the female reproductive system. However, with these studies come more questions that need answering. Further studies on the underlying mechanisms of the tissue-specific functions of multiple isoforms of EGR, PGR, and GR, etc., the reproductive roles of MR, AR, and GR, and the integrated analysis of SR activities with the recently developed high throughput bioinformatics are all needed. Furthermore, SRs, as well known nuclear receptors are good models to study the molecular mechanism of general transcription factors.

References

- Acevedo-Rodriguez, A., Mani, S. K., & Handa, R. J. (2015). Oxytocin and estrogen receptor beta in the brain: an overview. *Frontiers in Endocrinology (Lausanne)*, 6, 160.
- Acharya, K. D., Finkelstein, S. D., Bless, E. P., Nettles, S. A., Mulac-Jericevic, B., Conneely, O. M., Mani, S. K., & Tetel, M. J. (2015). Estradiol preferentially induces progesterin receptor-A (PR-A) over PR-B in cells expressing nuclear receptor coactivators in the female mouse hypothalamus(1,2,3). *eNeuro*, 2.
- Ahmad, N., & Kumar, R. (2011). Steroid hormone receptors in cancer development: a target for cancer therapeutics. *Cancer Letters*, 300, 1–9.
- Ahrens-Fath, I., Politz, O., Geserick, C., & Haendler, B. (2005). Androgen receptor function is modulated by the tissue-specific AR45 variant. *The FEBS Journal*, 272, 74–84.
- Albrecht, E. D., & Pepe, G. J. (2010). Estrogen regulation of placental angiogenesis and fetal ovarian development during primate pregnancy. *The International Journal of Developmental Biology*, 54, 397–408.
- An, B. S., Poon, S. L., So, W. K., Hammond, G. L., & Leung, P. C. (2009). Rapid effect of GNRH1 on follicle-stimulating hormone beta gene expression in LbetaT2 mouse pituitary cells requires the progesterone receptor. *Biology of Reproduction*, 81, 243–249.
- Andersen, C. Y. (2003). Effect of glucocorticoids on spontaneous and follicle-stimulating hormone induced oocyte maturation in mouse oocytes during culture. *The Journal of Steroid Biochemistry and Molecular Biology*, 85, 423–427.
- Apparao, K. B., Lovely, L. P., Gui, Y., Lininger, R. A., & Lessey, B. A. (2002). Elevated endometrial androgen receptor expression in women with polycystic ovarian syndrome. *Biology of Reproduction*, 66, 297–304.
- Aranda, A., & Pascual, A. (2001). Nuclear hormone receptors and gene expression. *Physiological Reviews*, 81, 1269–1304.
- Attardi, B., Scott, R., Pfaff, D., & Fink, G. (2007). Facilitation or inhibition of the oestradiol-induced gonadotrophin surge in the immature female rat by progesterone: effects on pituitary responsiveness to gonadotrophin-releasing hormone (GnRH), GnRH self-priming and pituitary mRNAs for the progesterone receptor A and B isoforms. *Journal of Neuroendocrinology*, 19, 988–1000.
- Atwood, C. S., Hovey, R. C., Glover, J. P., Chepko, G., Ginsburg, E., Robison, W. G., & Vonderhaar, B. K. (2000). Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. *Journal of Endocrinology*, 167, 39–52.
- Aupperlee, M. D., Leipprandt, J. R., Bennett, J. M., Schwartz, R. C., & Haslam, S. Z. (2013). Amphiregulin mediates progesterone-induced mammary ductal development during puberty. *Breast Cancer Research*, 15.
- Bagchi, M. K., Kaya, H. S., Hantak, A. M., Taylor, R. N., & Bagchi, I. C. (2015). Roles of progesterone receptor A and B isoforms during human endometrial decidualization. *Reproductive Sciences*, 22, 129a.
- Bagchi, M. K., Tsai, S. Y., Tsai, M. J., & O'Malley, B. W. (1992). Ligand and DNA-dependent phosphorylation of human progesterone receptor in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 89, 2664–2668.
- Baisden, B., Sonne, S., Joshi, R. M., Ganapathy, V., & Shekhawat, P. S. (2007). Antenatal dexamethasone treatment leads to changes in gene expression in a murine late placenta. *Placenta*, 28, 1082–1090.
- Ballare, C., Castellano, G., Gaveglia, L., Althammer, S., Gonzalez-Vallinas, J., Eyraes, E., Le Dily, F., Zaurin, R., Soronellas, D., Vicent, G. P., & Beato, M. (2013). Nucleosome-driven transcription factor binding and gene regulation. *Molecular Cell*, 49, 67–79.
- Barraille, P., Chineastra, P., Bayard, F., & Faye, J. C. (1999). Alternative initiation of translation accounts for a 67/45 kDa dimorphism of the human estrogen receptor ERalpha. *Biochemical and Biophysical Research Communications*, 257, 84–88.

- Beato, M. (1989). Gene-regulation by steroid-hormones. *Cell*, 56, 335–344.
- Beato, M., Herrlich, P., & Schutz, G. (1995). Steroid-Hormone Receptors - Many Actors in Search of a Plot. *Cell*, 83, 851–857.
- Beato, M., & Klug, J. (2000). Steroid hormone receptors: an update. *Human Reproduction Update*, 6, 225–236.
- Becker, M. A., Ibrahim, Y. H., Cui, X., Lee, A. V., & Yee, D. (2011). The IGF pathway regulates ERalpha through a S6K1-dependent mechanism in breast cancer cells. *Molecular Endocrinology*, 25, 516–528.
- Ben-Jonathan, N., Lapensee, C. R., & Lapensee, E. W. (2008). What can we learn from rodents about prolactin in humans? *Endocrine Reviews*, 29, 1–41.
- Bennesch, M. A., & Picard, D. (2015). Minireview: Tipping the balance: ligand-independent activation of steroid receptors. *Molecular Endocrinology*, 29, 349–363.
- Bhurke, A. S., Bagchi, I. C., & Bagchi, M. K. (2016). Progesterone-regulated endometrial factors controlling implantation. *American Journal of Reproductive Immunology*, 75, 237–245.
- Blanks, A. M., & Brosens, J. J. (2012). Progesterone action in the myometrium and decidua in preterm birth. *Facts, Views & Vision in ObGyn*, 4, 33–43.
- Bloom, L. J., Guo, C., & Pratt, J. H. (1995). Identification of a splice variant of the rat and human mineralocorticoid receptor genes. *The Journal of Steroid Biochemistry and Molecular Biology*, 55, 159–162.
- Breen, K. M., Thackray, V. G., Hsu, T., Mak-McCully, R. A., Coss, D., & Mellon, P. L. (2012). Stress levels of glucocorticoids inhibit LHbeta-subunit gene expression in gonadotrope cells. *Molecular Endocrinology*, 26, 1716–1731.
- Breslin, M. B., Geng, C. D., & Vedeckis, W. V. (2001). Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. *Molecular Endocrinology*, 15, 1381–1395.
- Briskin, C., & O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harbor Perspectives in Biology*, 2.
- Briskin, C., Park, S., Vass, T., Lydon, J. P., O'Malley, B. W., & Weinberg, R. A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 5076–5081.
- Brom, G. M., & Schwartz, N. B. (1968). Acute changes in the estrous cycle following ovariectomy in the golden hamster. *Neuroendocrinology*, 3, 366–377.
- Bruchovsky, N., & Wilson, J. D. (1968). The intranuclear binding of testosterone and 5-alpha-androstan-17-beta-ol-3-one by rat prostate. *The Journal of Biological Chemistry*, 243, 5953–5960.
- Bukovsky, A., Caudle, M. R., Cekanova, M., Fernando, R. I., Wimalasena, J., Foster, J. S., Henley, D. C., & Elder, R. F. (2003a). Placental expression of estrogen receptor beta and its hormone binding variant—comparison with estrogen receptor alpha and a role for estrogen receptors in asymmetric division and differentiation of estrogen-dependent cells. *Reproductive Biology and Endocrinology*, 1, 36.
- Bukovsky, A., Cekanova, M., Caudle, M. R., Wimalasena, J., Foster, J. S., Henley, D. C., & Elder, R. F. (2003b). Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. *Reproductive Biology and Endocrinology*, 1, 13.
- Butler, J. A., Sjoberg, M., & Coen, C. W. (1999). Evidence for oestrogen receptor alpha-immunoreactivity in gonadotrophin-releasing hormone-expressing neurones. *Journal of Neuroendocrinology*, 11, 331–335.
- Buxant, F., Engohan-Aloghe, C., & Noel, J. C. (2010). Estrogen receptor, progesterone receptor, and glucocorticoid receptor expression in normal breast tissue, breast in situ carcinoma, and invasive breast cancer. *Applied Immunohistochemistry & Molecular Morphology*, 18, 254–257.
- Carroll, J. S., Meyer, C. A., Song, J., Li, W., Geistlinger, T. R., Eeckhoutte, J., Brodsky, A. S., Keeton, E. K., Fertuck, K. C., Hall, G. F., Wang, Q., Bekiranov, S., Sementchenko, V., Fox, E. A., Silver, P. A., Gingeras, T. R., Liu, X. S., & Brown, M. (2006). Genome-wide analysis of estrogen receptor binding sites. *Nature Genetics*, 38, 1289–1297.
- Chan, J., Rabbitt, E. H., Innes, B. A., Bulmer, J. N., Stewart, P. M., Kilby, M. D., & Hewison, M. (2007). Glucocorticoid-induced apoptosis in human decidua: a novel role for 11 beta-hydroxysteroid dehydrogenase in late gestation. *Journal of Endocrinology*, 195, 7–15.
- Chan, S. C., & Dehm, S. M. (2014). Constitutive activity of the androgen receptor. *Advances in Pharmacology*, 70, 327–366.
- Chang, S. Y., Kang, H. Y., Lan, K. C., Chang, C. Y., Huang, F. J., Tsai, M. Y., & Huang, K. E. (2005). Expression of steroid receptors, their cofactors, and aromatase in human luteinized granulosa cells after controlled ovarian hyperstimulation. *Fertility and Sterility*, 83, 1241–1247.
- Chantalat, E., Boudou, F., Laurell, H., Paliere, G., Houtman, R., Melchers, D., Rochaix, P., Filleron, T., Stella, A., Burlet-Schiltz, O., Brouchet, A., Flouriot, G., Metivier, R., Arnal, J. F., Fontaine, C., & Lenfant, F. (2016). The AF-1-deficient estrogen receptor ER alpha 46 isoform is frequently expressed in human breast tumors. *Breast Cancer Research*, 18.
- Chappell, P. E., Schneider, J. S., Kim, P., Xu, M., Lydon, J. P., O'Malley, B. W., & Levine, J. E. (1999). Absence of gonadotropin surges and gonadotropin-releasing hormone self-priming in ovariectomized (OVX), estrogen (E2)-treated, progesterone receptor knockout (PRKO) mice. *Endocrinology*, 140, 3653–3658.
- Chen, J., An, B. S., So, W. K., Cheng, L., Hammond, G. L., & Leung, P. C. (2010). Gonadotropin-releasing hormone-I-mediated activation of progesterone receptor contributes to gonadotropin alpha-subunit expression in mouse gonadotrophs. *Endocrinology*, 151, 1204–1211.
- Chen, J. Z. J., Wong, M. H., Brennecke, S. P., & Keogh, R. J. (2011). The effects of human chorionic gonadotrophin, progesterone and oestradiol on trophoblast function. *Molecular and Cellular Endocrinology*, 342, 73–80.
- Chen, M., & Manley, J. L. (2009). Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. *Nature Reviews Molecular Cell Biology*, 10, 741–754.
- Choi, J. P., Zheng, Y., Skulte, K. A., Handelsman, D. J., & Simanainen, U. (2015). Development and Characterization of Uterine Glandular Epithelium Specific Androgen Receptor Knockout Mouse Model. *Biology of Reproduction*, 93, 120.
- Clarke, R. B., Howell, A., Potten, C. S., & Anderson, E. (1997). Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Research*, 57, 4987–4991.
- Clarkson, J., D'anglemont, X., Moreno, A. S., Colledge, W. H., & Herbison, A. E. (2008). Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge. *Journal of Neuroscience*, 28, 8691–8697.
- Clifton, V. L., Cuffe, J., Moritz, K. M., Cole, T. J., Fuller, P. J., Lu, N. Z., Kumar, S., Chong, S., & Saif, Z. (2016). Review: The role of multiple placental glucocorticoid receptor isoforms in adapting to the maternal environment and regulating fetal growth. *Placenta*.
- Conneely, O. M., Lydon, J. P., De Mayo, F., & O'Malley, B. W. (2000). Reproductive functions of the progesterone receptor. *Journal of the Society for Gynecologic Investigation*, 7, S25–32.
- Cooke, P. S., Ekman, G. C., Kaur, J., Davila, J., Bagchi, I. C., Clark, S. G., Dziuk, P. J., Hayashi, K., & Bartol, F. F. (2012). Brief exposure to progesterone during a critical neonatal window prevents uterine gland formation in mice. *Biology of Reproduction*, 86.
- Cork, D. M., Lennard, T. W., & Tyson-Capper, A. J. (2008). Alternative splicing and the progesterone receptor in breast cancer. *Breast Cancer Research*, 10, 207.
- Couse, J. F., Hewitt, S. C., Bunch, D. O., Sar, M., Walker, V. R., Davis, B. J., & Korach, K. S. (1999). Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science*, 286, 2328–2331.
- Couse, J. F., & Korach, K. S. (1999). Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews*, 20, 358–417.
- Couse, J. F., Yates, M. M., Deroo, B. J., & Korach, K. S. (2005). Estrogen receptor-beta is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. *Endocrinology*, 146, 3247–3262.
- Couse, J. F., Yates, M. M., Rodriguez, K. F., Johnson, J. A., Poirier, D., & Korach, K. S. (2006). The intraovarian actions of estrogen receptor-alpha are necessary to repress the formation of morphological and functional Leydig-like cells in the female gonad. *Endocrinology*, 147, 3666–3678.
- Curtin, D., Jenkins, S., Farmer, N., Anderson, A. C., Haisenleder, D. J., Rissman, E., Wilson, E. M., & Shupnik, M. A. (2001). Androgen suppression of GnRH-stimulated rat LHbeta gene transcription occurs through Sp1 sites in the distal GnRH-responsive promoter region. *Molecular Endocrinology*, 15, 1906–1917.
- Dart, D. A., Waxman, J., Aboagye, E. O., & Bevan, C. L. (2013). Visualising androgen receptor activity in male and female mice. *Plos One*, 8.

- Dasgupta, S., & O'Malley, B. W. (2014). Transcriptional coregulators: emerging roles of SRC family of coactivators in disease pathology. *Journal of Molecular Endocrinology*, 53, R47–R59.
- de Kloet, E. R., Sutanto, W., Van Den Berg, D. T., Carey, M. P., Van Haarst, A. D., Hornsby, C. D., Meijer, O. C., Rots, N. Y., & Oitzl, M. S. (1993). Brain mineralocorticoid receptor diversity: functional implications. *The Journal of Steroid Biochemistry and Molecular Biology*, 47, 183–190.
- Dehm, S. M., Schmidt, L. J., Heemers, H. V., Vessella, R. L., & Tindall, D. J. (2008). Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer Research*, 68, 5469–5477.
- Dehm, S. M., & Tindall, D. J. (2011). Alternatively spliced androgen receptor variants. *Endocrine-Related Cancer*, 18, R183–R196.
- Dhakal, P., Rumi, M. A. K., Kubota, K., Chakraborty, D., Chien, J., Roby, K. F., & Soares, M. J. (2015). Neonatal progesterone programs adult uterine responses to progesterone and susceptibility to uterine dysfunction. *Endocrinology*, 156, 3791–3803.
- Dominguez-Ordóñez, R., García-Juarez, M., Lima-Hernandez, F. J., Gomora-Aratti, P., Blaustein, J. D., Etgen, A. M., & Gonzalez-Flores, O. (2016). Estrogen receptor alpha and beta are involved in the activation of lordosis behavior in estradiol-primed rats. *Hormones and Behavior*, 86, 1–7.
- Dondi, D., Piccolella, M., Messi, E., Demissie, M., Cariboni, A., Selleri, S., Piva, F., Samara, A., Consalez, G. G., & Maggi, R. (2005). Expression and differential effects of the activation of glucocorticoid receptors in mouse gonadotropin-releasing hormone neurons. *Neuroendocrinology*, 82, 151–163.
- Driver, P. M., Rauz, S., Walker, E. A., Hewison, M., Kilby, M. D., & Stewart, P. M. (2003). Characterization of human trophoblast as a mineralocorticoid target tissue. *Molecular Human Reproduction*, 9, 793–798.
- Drummond, A. E., & Fuller, P. J. (2012). Ovarian actions of estrogen receptor-beta: an update. *Seminars in Reproductive Medicine*, 30, 32–38.
- Dubois, S. L., Wolfe, A., Radovick, S., Boehm, U., & Levine, J. E. (2016). Estradiol restrains prepubertal gonadotropin secretion in female mice via activation of ER alpha in kisspeptin neurons. *Endocrinology*, 157, 1546–1554.
- Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., & Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ER alpha) and beta (ER beta) on mouse reproductive phenotypes. *Development*, 127, 4277–4291.
- Emin Turkyay Korgun, A. O., Unek, G., & Mendilcioglu, I. (2012). The effects of glucocorticoids on fetal and placental development. In *Glucocorticoids—New Recognition of Our Familiar Friend*. InTech.
- Emmen, J. M., Couse, J. F., Elmore, S. A., Yates, M. M., Kissling, G. E., & Korach, K. S. (2005). *in vitro* growth and ovulation of follicles from ovaries of estrogen receptor (ER){alpha} and ER{beta} null mice indicate a role for ER{beta} in follicular maturation. *Endocrinology*, 146, 2817–2826.
- Enmark, E., Peltö-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjöld, M., & Gustafsson, J. A. (1997). Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology & Metabolism*, 82, 4258–4265.
- Feng, Y., Manka, D., Wagner, K. U., & Khan, S. A. (2007). Estrogen receptor-alpha expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 14718–14723.
- Fernandez-Guasti, A., Kruijver, F. P., Fodor, M., & Swaab, D. F. (2000). Sex differences in the distribution of androgen receptors in the human hypothalamus. *Journal of Comparative Neurology*, 425, 422–435.
- Filant, J., Zhou, H. J., & Spencer, T. E. (2012). Progesterone Inhibits Uterine Gland Development in the Neonatal Mouse Uterus. *Biology of Reproduction*, 86.
- Findlay, J. K., Liew, S. H., Simpson, E. R., & Korach, K. S. (2010). Estrogen signaling in the regulation of female reproductive functions. *Handbook of Experimental Pharmacology*, 29–35.
- Flanagan-Cato, L. M., Lee, B. J., & Calizo, L. H. (2006). Co-localization of midbrain projections, progesterin receptors, and mating-induced fos in the hypothalamic ventromedial nucleus of the female rat. *Hormones and Behavior*, 50, 52–60.
- Franco, H. L., Rubel, C. A., Large, M. J., Wetendorf, M., Fernandez-Valdivia, R., Jeong, J. W., Spencer, T. E., Behringer, R. R., Lydon, J. P., & DeMayo, F. J. (2012). Epithelial progesterone receptor exhibits pleiotropic roles in uterine development and function. *The FASEB Journal*, 26, 1218–1227.
- Furukawa, S., Hayashi, S., Usuda, K., Abe, M., Hagio, S., Kuroda, Y., & Ogawa, I. (2013). Effect of estrogen on rat placental development depending on gestation stage. *Experimental and Toxicologic Pathology*, 65, 695–702.
- Furukawa, S., Hayashi, S., Usuda, K., Abe, M., & Ogawa, I. (2012). The impairment of metrial gland development in tamoxifen exposed rats. *Experimental and Toxicologic Pathology*, 64, 121–126.
- Gal, A., Lin, P. C., Cacioppo, J. A., Hannon, P. R., Mahoney, M. M., Wolfe, A., Fernandez-Valdivia, R., Lydon, J. P., Elias, C. F., & Ko, C. (2016). Loss of fertility in the absence of progesterone receptor expression in kisspeptin neurons of female mice. *PLoS One*, 11, e0159534.
- Gambino, Y. P., Maymo, J. L., Perez Perez, A., Calvo, J. C., Sanchez-Margalet, V., & Varone, C. L. (2012). Elsevier Trophoblast Research Award lecture: Molecular mechanisms underlying estrogen functions in trophoblastic cells—focus on leptin expression. *Placenta*, 33(Suppl), S63–70.
- Gao, J. G., Mazella, J., Seppala, M., & Tseng, L. (2001). Ligand activated hPR modulates the glycodelin promoter activity through the Sp1 sites in human endometrial adenocarcinoma cells. *Molecular and Cellular Endocrinology*, 176, 97–102.
- Gennari-Moser, C., Khankin, E. V., Schuller, S., Escher, G., Frey, B. M., Portmann, C. B., Baumann, M. U., Lehmann, A. D., Surbek, D., Karumanchi, S. A., Frey, F. J., & Mohaupt, M. G. (2011). Regulation of placental growth by aldosterone and cortisol. *Endocrinology*, 152, 263–271.
- Geraghty, A. C., & Kaufer, D. (2015). Glucocorticoid regulation of reproduction. *Advances in Experimental Medicine and Biology*, 872, 253–278.
- Gibson, D. A., Simitsidellis, I., Cousins, F. L., Critchley, H. O., & Saunders, P. T. (2016). Intracrine androgens enhance decidualization and modulate expression of human endometrial receptivity genes. *Scientific Reports*, 6, 19970.
- Gizard, F., Robillard, R., Gervois, P., Faucompre, A., Revillon, F. O., Peyrat, J. P., Hum, W. D., & Staels, B. (2005). Progesterone inhibits human breast cancer cell growth through transcriptional upregulation of the cyclin-dependent kinase inhibitor p27(Kip1) gene. *FEBS Letters*, 579, 5535–5541.
- Glass, C. K., Holloway, J. M., Devary, O. V., & Rosenfeld, M. G. (1988). The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in thyroid hormone and estrogen response elements. *Cell*, 54, 313–323.
- Goddard, L. M., Murphy, T. J., Org, T., Enciso, J. M., Hashimoto-Partyka, M. K., Warren, C. M., Domigan, C. K., McDonald, A. I., He, H. H., Sanchez, L. A., Allen, N. C., Orsenigo, F., Chao, L. C., Dejana, E., Tontonoz, P., Mikkola, H. K. A., & Iruela-Arispe, M. L. (2014). Progesterone receptor in the vascular endothelium triggers physiological uterine permeability preimplantation. *Cell*, 156, 549–562.
- Gojska, N. M., & Belsham, D. D. (2014). Glucocorticoid receptor-mediated regulation of Rfrp (GnIH) and Gpr147 (GnIH-R) synthesis in immortalized hypothalamic neurons. *Molecular and Cellular Endocrinology*, 384, 23–31.
- Goldman, S., & Shalev, E. (2007). Progesterone receptor profile in the decidua and fetal membrane. *Frontiers in Bioscience*, 12, 634–648.
- Goldman, S., Weiss, A., Almalah, I., & Shalev, E. (2005). Progesterone receptor expression in human decidua and fetal membranes before and after contractions: possible mechanism for functional progesterone withdrawal. *Molecular Human Reproduction*, 11, 269–277.
- Gomez-Camarillo, M. A., Beyer, C., Lucio, R. A., Garcia-Juarez, M., Gonzalez-Arenas, A., Camacho-Arroyo, I., Komisaruk, B. R., & Gonzalez-Flores, O. (2011). Differential effects of progesterone and genital stimulation on sequential inhibition of estrous behavior and progesterone receptor expression in the rat brain. *Brain Research Bulletin*, 85, 201–206.
- Gomez-Sanchez, E. P., Gomez-Sanchez, M. T., De Rodriguez, A. F., Romero, D. G., Warden, M. P., Plonczynski, M. W., & Gomez-Sanchez, C. E. (2009). Immunohistochemical demonstration of the mineralocorticoid receptor, 11beta-hydroxysteroid dehydrogenase-1 and -2, and hexose-6-phosphate dehydrogenase in rat ovary. *J Histochem Cytochem*, 57, 633–641.
- Gore, A. C., Attardi, B., & Defranco, D. B. (2006). Glucocorticoid repression of the reproductive axis: effects on GnRH and gonadotropin subunit mRNA levels. *Molecular and Cellular Endocrinology*, 256, 40–48.
- Gosden, J. R., Middleton, P. G., & Rout, D. (1986). Localization of the human oestrogen receptor gene to chromosome 6q24–q27 by in situ hybridization. *Cytogenetics and Cell Genetics*, 43, 218–220.

- Greene, G. L., Sobel, N. B., King, W. J., & Jensen, E. V. (1984). Immunochemical studies of estrogen receptors. *Journal of Steroid Biochemistry*, 20, 51–56.
- Gunin, A. G., Emelianov, V. U., & Tolmachev, A. S. (2003). Expression of estrogen receptor- α , glucocorticoid receptor, β -catenin and glycogen synthase kinase-3 β in the uterus of mice following long-term treatment with estrogen and glucocorticoid hormones. *European Journal of Obstetrics Gynecology and Reproductive Biology*, 107, 62–67.
- Gunin, A. G., Mashin, I. N., & Zakharov, D. A. (2001). Proliferation, mitosis orientation and morphogenetic changes in the uterus of mice following chronic treatment with both estrogen and glucocorticoid hormones. *Journal of Endocrinology*, 169, 23–31.
- Gunin, A. G., Sharov, A. A., & Nikolaev, D. V. (2000). Two month glucocorticoid treatment increases estradiol-induced stromal and myometrial cell proliferation in the uterus of ovariectomized rats. *European Journal of Obstetrics Gynecology and Reproductive Biology*, 88, 171–179.
- Gunning, P. W. (2006). Protein isoforms and isozymes. *eLS*. <https://doi.org/10.1038/npg.els.0005717>.
- Hagan, C. R., Daniel, A. R., Dressing, G. E., & Lange, C. A. (2012). Role of phosphorylation in progesterone receptor signaling and specificity. *Molecular and Cellular Endocrinology*, 357, 43–49.
- Hamilton, K. J., Arao, Y., & Korach, K. S. (2014). Estrogen hormone physiology: reproductive findings from estrogen receptor mutant mice. *Reproductive Biology*, 14, 3–8.
- Handel, A. E., Sandve, G. K., Disanto, G., Handunnetthi, L., Giovannoni, G., & Ramagopalan, S. V. (2013). Integrating multiple oestrogen receptor α ChIP studies: overlap with disease susceptibility regions, DNase I hypersensitivity peaks and gene expression. *BMC Medical Genomics*, 6.
- Haque, M., Wilson, R., Sharma, K., Mills, N. J., & Teruyama, R. (2015). Localisation of 11-hydroxysteroid dehydrogenase type 2 in mineralocorticoid receptor expressing magnocellular neurosecretory neurones of the rat supraoptic and paraventricular nuclei. *Journal of Neuroendocrinology*, 27, 835–849.
- Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., Tujague, M., Strom, A., Treuter, E., Warner, M., & Gustafsson, J. A. (2007). Estrogen receptors: how do they signal and what are their targets. *Physiological Reviews*, 87, 905–931.
- Herbison, A. E. (1998). Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons. *Endocrine Reviews*, 19, 302–330.
- Herynk, M. H., & Fuqua, S. A. W. (2004). Estrogen receptor mutations in human disease. *Endocrine Reviews*, 25, 869–898.
- Hewitt, D. P., Mark, P., & Waddell, B. J. (2006). Glucocorticoids prevent the normal increase in placental vascular endothelial growth factor expression and placental vascularity during late pregnancy in the rat. *Endocrinology*, 147, 5568–5574.
- Hewitt, S. C., Li, L., Grimm, S. A., Chen, Y., Liu, L., Li, Y., Bushel, P. R., Fargo, D., & Korach, K. S. (2012). Research resource: whole-genome estrogen receptor α binding in mouse uterine tissue revealed by ChIP-seq. *Molecular Endocrinology*, 26, 887–898.
- Hewitt, S. C., Winuthayanon, W., & Korach, K. S. (2016). What's new in estrogen receptor action in the female reproductive tract. *Journal of Molecular Endocrinology*, 56, R55–R71.
- Hirasawa, G., Takeyama, J., Sasano, H., Fukushima, K., Suzuki, T., Muramatsu, Y., Darnel, A. D., Kaneko, C., Hiwatashi, N., Toyota, T., Nagura, H., & Krozowski, Z. S. (2000). 11 β -hydroxysteroid dehydrogenase type II and mineralocorticoid receptor in human placenta. *The Journal of Clinical Endocrinology & Metabolism*, 85, 1306–1309.
- Hirata, S., Shoda, T., Kato, J., & Hoshi, K. (2000). The novel isoform of the progesterone receptor cDNA in the human testis and detection of its mRNA in the human uterine endometrium. *Oncology*, 59, 39–44.
- Hirata, S., Shoda, T., Kato, J., & Hoshi, K. (2001). The multiple untranslated first exons system of the human estrogen receptor β (ER β) gene. *Journal of Steroid Biochemistry and Molecular Biology*, 78, 33–40.
- Hirata, S., Shoda, T., Kato, J., & Hoshi, K. (2002). The novel exon, exon T, of the human progesterone receptor gene and the genomic organization of the gene. *Journal of Steroid Biochemistry and Molecular Biology*, 80, 365–367.
- Hollenberg, S. M., Weinberger, C., Ong, E. S., Cerelli, G., Oro, A., Lebo, R., Thompson, E. B., Rosenfeld, M. G., & Evans, R. M. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. *Nature*, 318, 635–641.
- Horie, K., Takakura, K., Fujiwara, H., Suginami, H., Liao, S., & Mori, T. (1992). Immunohistochemical localization of androgen receptor in the human ovary throughout the menstrual cycle in relation to estrogen and progesterone-receptor expression. *Human Reproduction*, 7, 184–190.
- Hsu, T. Y., Lan, K. C., Tsai, C. C., Ou, C. Y., Cheng, B. H., Tsai, M. Y., Kang, H. Y., Tung, Y. H., Wong, Y. H., & Huang, K. E. (2009). Expression of androgen receptor in human placentas from normal and preclimptic pregnancies. *Taiwanese Journal of Obstetrics and Gynecology*, 48, 262–267.
- Hsueh, A. J. W., & Erickson, G. F. (1978). Glucocorticoid inhibition of FSH-induced estrogen production in cultured rat granulosa-cells. *Steroids*, 32, 639–648.
- Hu, L. (2009). Converse regulatory functions of estrogen receptor- α and - β subtypes expressed in hypothalamic gonadotropin-releasing hormone neurons (vol 22, pg 2250, 2008). *Molecular Endocrinology*, 23, 423.
- Huhtinen, K., Saloniemi-Heinonen, T., Keski-Rahkonen, P., Desai, R., Laajala, D., Stahle, M., Hakkinen, M. R., Awosanya, M., Suvitie, P., Kujari, H., Aittokallio, T., Handelsman, D. J., Auriola, S., Perheentupa, A., & Poutanen, M. (2014). Intra-tissue steroid profiling indicates differential progesterone and testosterone metabolism in the endometrium and endometriosis lesions. *Journal of Clinical Endocrinology and Metabolism*, 99, E2188–97.
- Imhof, M. O., & McDonnell, D. P. (1996). Yeast RSP5 and its human homolog hRPF1 potentiate hormone-dependent activation of transcription by human progesterone and glucocorticoid receptors. *Molecular and Cellular Biology*, 16, 2594–2605.
- Inzunza, J., Morani, A., Cheng, G., Warner, M., Hreinsson, J., Gustafsson, J. A., & Hovatta, O. (2007). Ovarian wedge resection restores fertility in estrogen receptor β knockout (ER β -/-) mice. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 600–605.
- Ito, K., Suzuki, T., Akahira, J., Moriya, T., Kaneko, C., Utsunomiya, H., Yaegashi, N., Okamura, K., & Sasano, H. (2002). Expression of androgen receptor and 5 α -reductases in the human normal endometrium and its disorders. *International Journal of Cancer*, 99, 652–657.
- Jayes, F. L., Burns, K. A., Rodriguez, K. F., Kissling, G. E., & Korach, K. S. (2014). The naturally occurring luteinizing hormone surge is diminished in mice lacking estrogen receptor β in the ovary. *Biology of Reproduction*, 90, 24.
- Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W., & Desombre, E. R. (1968). A two-step mechanism for the interaction of estradiol with rat uterus. *Proceedings of the National Academy of Sciences of the United States of America*, 59, 632–638.
- Jimena, P., Castilla, J. A., Peran, F., Ramirez, J. P., Vergara, F., Molina, R., Vergara, F., & Herruzo, A. (1992). Adrenal hormones in human follicular-fluid. *Acta Endocrinologica*, 127, 403–406.
- Jo, Y. S., Lee, G. S. R., Nam, S. Y., & Kim, S. J. (2015). Progesterone inhibits leptin-induced invasiveness of BeWo cells. *International Journal of Medical Sciences*, 12, 773–779.
- John, S., Sabo, P. J., Thurman, R. E., Sung, M. H., Biddie, S. C., Johnson, T. A., Hager, G. L., & Stamatoyannopoulos, J. A. (2011). Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. *Nature Genetics*, 43, 264–268.
- Johnson, L. R., Farb, C., Morrison, J. H., McEwen, B. S., & Ledoux, J. E. (2005). Localization of glucocorticoid receptors at postsynaptic membranes in the lateral amygdala. *Neuroscience*, 136, 289–299.
- Jorgensen, J. S., & Nilson, J. H. (2001). AR suppresses transcription of the LH β subunit by interacting with steroidogenic factor-1. *Molecular Endocrinology*, 15, 1505–1516.
- Kajihara, T., Tanaka, K., Oguro, T., Tochigi, H., Prechapanich, J., Uchino, S., Itakura, A., Sucurovic, S., Murakami, K., Brosens, J. J., & Ishihara, O. (2014). Androgens modulate the morphological characteristics of human endometrial stromal cells decidualized in vitro. *Reproductive Sciences*, 21, 372–380.
- Kajihara, T., Tochigi, H., Prechapanich, J., Uchino, S., Itakura, A., Brosens, J. J., & Ishihara, O. (2012). Androgen signaling in decidualizing human endometrial stromal cells enhances resistance to oxidative stress. *Fertility and Sterility*, 97, 185–191.
- Karim, K., Giribabu, N., Muniandy, S., & Salleh, N. (2016). Vacuolar-ATPase (V-ATPase) mediates progesterone-induced uterine fluid acidification in rats. *Journal of Membrane Biology*, 249, 65–76.
- Kassel, O., & Herrlich, P. (2007). Crosstalk between the glucocorticoid receptor and other transcription factors: molecular aspects. *Molecular and Cellular Endocrinology*, 275, 13–29.
- Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., & Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO Journal*, 9, 1603–1614.

- Ke, W., Chen, C., Luo, H., Tang, J., Zhang, Y., Gao, W., Yang, X., Tian, Z., Chang, Q., & Liang, Z. (2016). Histone deacetylase 1 regulates the expression of progesterone receptor A during human parturition by occupying the progesterone receptor A promoter. *Reproductive Sciences*, 23, 955–964.
- Keeling, J. W., Ozer, E., King, G., & Walker, F. (2000). Oestrogen receptor alpha in female fetal, infant, and child mammary tissue. *The Journal of Pathology*, 191, 449–451.
- Kim, H. J., Gieske, M. C., Trudgen, K. L., Hudgins-Spivey, S., Kim, B. G., Krust, A., Chambon, P., Jeong, J. W., Blalock, E., & Ko, C. (2011). Identification of estradiol/ERalpha-regulated genes in the mouse pituitary. *Journal of Endocrinology*, 210, 309–321.
- Kim, J., Sato, M., Li, Q., Lydon, J. P., DeMayo, F. J., Bagchi, I. C., & Bagchi, M. K. (2008). Peroxisome proliferator-activated receptor gamma is a target of progesterone regulation in the preovulatory follicles and controls ovulation in mice. *Molecular and Cellular Biology*, 28, 1770–1782.
- Kim, M., Park, H. J., Seol, J. W., Jang, J. Y., Cho, Y. S., Kim, K. R., Choi, Y., Lydon, J. P., DeMayo, F. J., Shibuya, M., Ferrara, N., Sung, H. K., Nagy, A., Alitalo, K., & Koh, G. Y. (2013). VEGF-A regulated by progesterone governs uterine angiogenesis and vascular remodelling during pregnancy. *EMBO Molecular Medicine*, 5, 1415–1430.
- Kim, S. C., Park, M. N., Lee, Y. J., Joo, J. K., & An, B. S. (2016). Interaction of steroid receptor coactivators and estrogen receptors in the human placenta. *Journal of Molecular Endocrinology*, 56, 239–247.
- Kingsley-Kallesen, M., Mukhopadhyay, S. S., Wyszomierski, S. L., Schanler, S., Schutz, G., & Rosen, J. M. (2002). The mineralocorticoid receptor may compensate for the loss of the glucocorticoid receptor at specific stages of mammary gland development. *Molecular Endocrinology*, 16, 2008–2018.
- Klein-Hitpass, L., Schorpp, M., Wagner, U., & Ryffel, G. U. (1986). An estrogen-responsive element derived from the 5' flanking region of the *Xenopus vitellogenin A2* gene functions in transfected human cells. *Cell*, 46, 1053–1061.
- Klinge, C. M. (2001). Estrogen receptor interaction with estrogen response elements. *Nucleic Acids Research*, 29, 2905–2919.
- Kochetov, A. V. (2008). Alternative translation start sites and hidden coding potential of eukaryotic mRNAs. *Bioessays*, 30, 683–691.
- Koos, R. D. (2011). Minireview: Putting physiology back into estrogens' mechanism of action. *Endocrinology*, 152, 4481–4488.
- Korgun, E. T., Dohr, G., Desoye, G., Demir, R., Kayisli, U. A., & Hahn, T. (2003). Expression of insulin, insulin-like growth factor I and glucocorticoid receptor in rat uterus and embryo during decidualization, implantation and organogenesis. *Reproduction*, 125, 75–84.
- Koryakina, Y., Ta, H. Q., & Gioeli, D. (2014). Androgen receptor phosphorylation: biological context and functional consequences. *Endocrine-Related Cancer*, 21, T131–45.
- Kos, M., Reid, G., Denger, S., & Gannon, F. (2001). Minireview: genomic organization of the human ER alpha gene promoter region. *Molecular Endocrinology*, 15, 2057–2063.
- Kotischke, A., Sadie-Van Gijzen, H., Avenant, C., Fernandes, S., & Hapgood, J. P. (2009). Genomic and nongenomic cross talk between the gonadotropin-releasing hormone receptor and glucocorticoid receptor signaling pathways. *Molecular Endocrinology*, 23, 1726–1745.
- Krozowski, Z., & Funder, J. W. (1981). Mineralocorticoid receptors in rat anterior pituitary: toward a redefinition of "mineralocorticoid hormone". *Endocrinology*, 109, 1221–1224.
- Kudwa, A. E., & Rissman, E. F. (2003). Double oestrogen receptor alpha and beta knockout mice reveal differences in neural oestrogen-mediated progesterone receptor induction and female sexual behaviour. *Journal of Neuroendocrinology*, 15, 978–983.
- Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 5925–5930.
- Kuiper, G. G., Shughrue, P. J., Merchenthaler, I., & Gustafsson, J. A. (1998). The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems. *Frontiers in Neuroendocrinology*, 19, 253–286.
- Kumar, R., & Thompson, E. B. (2005). Gene regulation by the glucocorticoid receptor: structure: function relationship. *The Journal of Steroid Biochemistry and Molecular Biology*, 94, 383–394.
- Kuroda, K., Venkatakrishnan, R., James, S., Sucurovic, S., Mulac-Jericevic, B., Lucas, E. S., Takeda, S., Shmygol, A., Brosens, J. J., & Quenby, S. (2013a). Elevated periimplantation uterine natural killer cell density in human endometrium is associated with impaired corticosteroid signaling in decidualizing stromal cells. *Journal of Clinical Endocrinology and Metabolism*, 98, 4429–4437.
- Kuroda, K., Venkatakrishnan, R., Salker, M. S., Lucas, E. S., Shaheen, F., Kuroda, M., Blanks, A., Christian, M., Quenby, S., & Brosens, J. J. (2013b). Induction of 11beta-HSD 1 and activation of distinct mineralocorticoid receptor- and glucocorticoid receptor-dependent gene networks in decidualizing human endometrial stromal cells. *Molecular Endocrinology*, 27, 192–202.
- Labhsetwar, A. P. (1970). Role of estrogens in ovulation: A study using the estrogen-antagonist, I.C.I. 46,474. *Endocrinology*, 87, 542–551.
- Lain, A. R., Creighton, C. J., & Conneely, O. M. (2013). Research resource: progesterone receptor targetome underlying mammary gland branching morphogenesis. *Molecular Endocrinology*, 27, 1743–1761.
- Lannigan, D. A. (2003). Estrogen receptor phosphorylation. *Steroids*, 68, 1–9.
- Law, M. L., Kao, F. T., Wei, Q., Hartz, J. A., Greene, G. L., Zarucki-Schulz, T., Conneely, O. M., Jones, C., Puck, T. T., O'Malley, B. W., et al. (1987). The progesterone receptor gene maps to human chromosome band 11q13, the site of the mammary oncogene int-2. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 2877–2881.
- Le Billan, F., Khan, J. A., Lamrabet, K., Viengchareun, S., Bouligand, J., Fagart, J., & Lombres, M. (2015). Cistrome of the aldosterone-activated mineralocorticoid receptor in human renal cells. *FASEB Journal*, 29, 3977–3989.
- Lee, B. H., Kim, J. H., Park, T. C., & Lee, H. J. (2016). Relationship between gonadotropin-releasing hormone/gonadotropin-releasing hormone receptor signaling and progesterone receptors in human trophoblasts. *Journal of Reproductive Medicine*, 61, 275–281.
- Lee, M. J., Wang, Z., Yee, H., Ma, Y., Swenson, N., Yang, L., Kadner, S. S., Baergen, R. N., Logan, S. K., Garabedian, M. J., & Guller, S. (2005). Expression and regulation of glucocorticoid receptor in human placental villous fibroblasts. *Endocrinology*, 146, 4619–4626.
- Lee, M. T., Ouyang, B., Ho, S. M., & Leung, Y. K. (2013). Differential expression of estrogen receptor beta isoforms in prostate cancer through interplay between transcriptional and translational regulation. *Molecular and Cellular Endocrinology*, 376, 125–135.
- Lee, S., Kang, D. W., Hudgins-Spivey, S., Krust, A., Lee, E. Y., Koo, Y., Cheon, Y., Gye, M. C., Chambon, P., & Ko, C. (2009). Theca-specific estrogen receptor-alpha knockout mice lose fertility prematurely. *Endocrinology*, 150, 3855–3862.
- Leite, C. M., Kalil, B., Uchoa, E. T., Antunes-Rodrigues, J., Elias, L. K. L., Levine, J. E., & Anselmo-Franci, J. A. (2016). Progesterone-induced amplification and advancement of GnRH/LH surges are associated with changes in kisspeptin system in preoptic area of estradiol-primed female rats. *Brain Research*, 1650, 21–30.
- Lemmen, J. G., Broekhof, J. L. M., Kuiper, G. G. J. M., Gustafsson, J. A., Van Der Saag, P. T., & Van Der Burg, B. (1999). Expression of estrogen receptor alpha and beta during mouse embryogenesis. *Mechanisms of Development*, 81, 163–167.
- Leslie, K. K., Stein, M. P., Kumar, N. S., Dai, D., Stephens, J., Wandinger-Ness, A., & Glueck, D. H. (2005). Progesterone receptor isoform identification and subcellular localization in endometrial cancer. *Gynecologic Oncology*, 96, 32–41.
- Levin, E. R. (2015). Extranuclear steroid receptors are essential for steroid hormone actions. *Annual Review of Medicine*, 66(66), 271–280.
- Levin, E. R., & Hammes, S. R. (2016). Nuclear receptors outside the nucleus: extranuclear signalling by steroid receptors. *Nature Reviews Molecular Cell Biology*, 17, 783–797.
- Li, H., Li, Y., Morin, D., Plymate, S., Lye, S., & Dong, X. (2014). The androgen receptor mediates antiapoptotic function in myometrial cells. *Cell Death & Disease*, 5, e1338.
- Li, Q. X., Kannan, A., DeMayo, F. J., Lydon, J. P., Cooke, P. S., Yamagishi, H., Srivastava, D., Bagchi, M. K., & Bagchi, I. C. (2011). The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science*, 331, 912–916.
- Li, S. J., Han, B., Liu, G. J., Li, S. Y., Ouellet, J., Labrie, F., & Pelletier, G. (2010). Immunocytochemical localization of sex steroid hormone receptors in normal human mammary gland. *Journal of Histochemistry & Cytochemistry*, 58, 509–515.
- Lien, H. C., Lu, Y. S., Cheng, A. L., Chang, W. C., Jeng, Y. M., Kuo, Y. H., Huang, C. S., Chang, K. J., & Yao, Y. T. (2006). Differential expression of glucocorticoid receptor in human breast tissues and related neoplasms. *The Journal of Pathology*, 209, 317–327.
- Liu, L., Li, Y., Xie, N., Shynlova, O., Challis, J. R., Slater, D., Lye, S., & Dong, X. (2013). Proliferative action of the androgen receptor in human uterine myometrial cells—a key regulator for myometrium phenotype programming. *Journal of Clinical Endocrinology and Metabolism*, 98, 218–227.

- Lombes, M., Farman, N., Oblin, M. E., Baulieu, E. E., Bonvalet, J. P., Erlanger, B. F., & Gasc, J. M. (1990). Immunohistochemical localization of renal mineralocorticoid receptor by using an anti-idiotypic antibody that is an internal image of aldosterone. *Proceedings of the National Academy of Sciences of the United States of America*, 87, 1086–1088.
- Lonard, D. M., & O'Malley, B. W. (2007). Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. *Molecular Cell*, 27, 691–700.
- Lonard, D. M., & O'Malley, B. W. (2012). Nuclear receptor coregulators: modulators of pathology and therapeutic targets. *Nature Reviews Endocrinology*, 8, 598–604.
- Losel, R., & Wehling, M. (2003). Nongenomic actions of steroid hormones. *Nature Reviews Molecular Cell Biology*, 4, 46–56.
- Lu, J., van der Steen, T., & Tindall, D. J. (2015). Are androgen receptor variants a substitute for the full-length receptor? *Nature Reviews Urology*, 12, 137–144.
- Lu, N. Z., & Cidlowski, J. A. (2005). Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. *Molecular Cell*, 18, 331–342.
- Lubahn, D. B., Joseph, D. R., Sullivan, P. M., Willard, H. F., French, F. S., & Wilson, E. M. (1988). Cloning of human androgen receptor complementary-DNA and localization to the X-chromosome. *Science*, 240, 327–330.
- Lunghi, L., Pavan, B., Biondi, C., Paolillo, R., Valerio, A., Vesce, F., & Patella, A. (2010). Use of glucocorticoids in pregnancy. *Current Pharmaceutical Design*, 16, 3616–3637.
- Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Shyamala, G., Conneely, O. M., & O'Malley, B. W. (1995). Mice lacking progesterone-receptor exhibit pleiotropic reproductive abnormalities. *Genes & Development*, 9, 2266–2278.
- Lyons, L. S., Rao, S., Balkan, W., Faysal, J., Maiorino, C. A., & Burnstein, K. L. (2008). Ligand-independent activation of androgen receptors by Rho GTPase signaling in prostate cancer. *Molecular Endocrinology*, 22, 597–608.
- Maggi, A. (2011). Liganded and unliganded activation of estrogen receptor and hormone replacement therapies. *Biochimica et Biophysica Acta*, 1812, 1054–1060.
- Mahfouz, A., Lelieveldt, B. P., Grefhorst, A., Van Weert, L. T., Mol, I. M., Sips, H. C., Van Den Heuvel, J. K., Datson, N. A., Visser, J. A., Reinders, M. J., & Meijer, O. C. (2016a). Genome-wide coexpression of steroid receptors in the mouse brain: identifying signaling pathways and functionally coordinated regions. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 2738–2743.
- Mahfouz, A., Lelieveldt, B. P., Grefhorst, A., Van Weert, L. T., Mol, I. M., Sips, H. C., Van Den Heuvel, J. K., Datson, N. A., Visser, J. A., Reinders, M. J., & Meijer, O. C. (2016b). Genome-wide coexpression of steroid receptors in the mouse brain: identifying signaling pathways and functionally coordinated regions. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 2738–2743.
- Mallepell, S., Krust, A., Chambon, P., & Briskin, C. (2006). Paracrine signaling through the epithelial estrogen receptor alpha is required for proliferation and morphogenesis in the mammary gland. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 2196–2201.
- Mark, P. J., Jones, M. L., Lewis, J. L., Waddell, B. J., & Smith, J. T. (2013). Kiss1 and Kiss1r mRNA expression in the rat placenta: changes with gestational age and regulation by glucocorticoids. *Placenta*, 34, 657–662.
- Marshall, E., Lowrey, J., Macpherson, S., Maybin, J. A., Collins, F., Critchley, H. O., & Saunders, P. T. (2011). In silico analysis identifies a novel role for androgens in the regulation of human endometrial apoptosis. *Journal of Clinical Endocrinology and Metabolism*, 96, E1746–55.
- Martin, L., Finn, C. A., & Trinder, G. (1973). DNA synthesis in the endometrium of progesterone-treated mice. *Journal of Endocrinology*, 56, 303–307.
- Martinez-Morales, J. R., Morales, A., Marin, R., Hernandez-Jimenez, J. G., Acevedo, A., Guerra, B., Hernandez, G., Lopez-Coviella, I., Prieto, L., & Alonso, R. (2001). Estrogen modulates norepinephrine-induced accumulation of adenosine cyclic monophosphate in a subpopulation of immortalized luteinizing hormone-releasing hormone secreting neurons from the mouse hypothalamus. *Neuroscience Letters*, 298, 61–64.
- McDonald, S. E., Henderson, T. A., Gomez-Sanchez, C. E., Critchley, H. O., & Mason, J. I. (2006). 11Beta-hydroxysteroid dehydrogenases in human endometrium. *Molecular and Cellular Endocrinology*, 248, 72–78.
- McEwen, B. S., de Kloet, E. R., & Rostene, W. (1986). Adrenal steroid receptors and actions in the nervous system. *Physiological Reviews*, 66, 1121–1188.
- McGillivray, S. M., Thackray, V. G., Coss, D., & Mellon, P. L. (2007). Activin and glucocorticoids synergistically activate follicle-stimulating hormone beta-subunit gene expression in the immortalized LbetaT2 gonadotrope cell line. *Endocrinology*, 148, 762–773.
- Mehta, R. G., Hawthorne, M., Mehta, R. R., Torres, K. E., Peng, X., McCormick, D. L., & Kopelovich, L. (2014). Differential roles of ERalpha and ERbeta in normal and neoplastic development in the mouse mammary gland. *PLoS One*, 9, e113175.
- Mesiano, S., Wang, Y. G., & Norwitz, E. R. (2011). Progesterone receptors in the human pregnancy uterus: do they hold the key to birth timing? *Reproductive Sciences*, 18, 6–19.
- Metivier, R., Reid, G., & Gannon, F. (2006). Transcription in four dimensions: nuclear receptor-directed initiation of gene expression. *EMBO Reports*, 7, 161–167.
- Michael, A. E., Pester, L. A., Curtis, P., Shaw, R. W., Edwards, C. R. W., & Cooke, B. A. (1993). Direct inhibition of ovarian steroidogenesis by cortisol and the modulatory role of 11-beta-hydroxysteroid dehydrogenase. *Clinical Endocrinology*, 38, 641–644.
- Millard, C. J., Watson, P. J., Fairall, L., & Schwabe, J. W. (2013). An evolving understanding of nuclear receptor coregulator proteins. *Journal of Molecular Endocrinology*, 51, T23–36.
- Milne, S. A., Henderson, T. A., Kelly, R. W., Saunders, P. T., Baird, D. T., & Critchley, H. O. (2005). Leukocyte populations and steroid receptor expression in human first-trimester decidua; regulation by antiprogesterin and prostaglandin E analog. *Journal of Clinical Endocrinology and Metabolism*, 90, 4315–4321.
- Misrahi, M., Venencie, P. Y., Saugier-Verber, P., Sar, S., Dessen, P., & Milgrom, E. (1993). Structure of the human progesterone receptor gene. *Biochimica et Biophysica Acta*, 1216, 289–292.
- Moalli, P. A., Pillay, S., Krett, N. L., & Rosen, S. T. (1993). Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells. *Cancer Research*, 53, 3877–3879.
- Moguilewsky, M., & Raynaud, J. P. (1980). Evidence for a specific mineralocorticoid receptor in rat pituitary and brain. *Journal of Steroid Biochemistry*, 12, 309–314.
- Mokhtar, H. M., Giribabu, N., Kassim, N., Muniandy, S., & Salleh, N. (2014a). Testosterone decreases fluid and chloride secretions in the uterus of adult female rats via down-regulating cystic fibrosis transmembrane regulator (CFTR) expression and functional activity. *The Journal of Steroid Biochemistry and Molecular Biology*, 144B, 361–372.
- Mokhtar, H. M., Giribabu, N., Muniandy, S., & Salleh, N. (2014b). Testosterone decreases the expression of endometrial pinopode and L-selectin ligand (MECA-79) in adult female rats during uterine receptivity period. *International Journal of Clinical and Experimental Pathology*, 7, 1967–1976.
- Morrison, N., Harrap, S. B., Arriza, J. L., Boyd, E., & Connor, J. M. (1990). Regional chromosomal assignment of the human mineralocorticoid receptor gene to 4q31.1. *Human Genetics*, 85, 130–132.
- Mueller, S. O., Clark, J. A., Myers, P. H., & Korach, K. S. (2002). Mammary gland development in adult mice requires epithelial and stromal estrogen receptor alpha. *Endocrinology*, 143, 2357–2365.
- Mulac-Jericevic, B., Lydon, J. P., DeMayo, F. J., & Conneely, O. M. (2003). Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 9744–9749.
- Mulac-Jericevic, B., Mullinax, R. A., DeMayo, F. J., Lydon, L. P., & Conneely, O. M. (2000). Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science*, 289, 1751–1754.
- Munck, A., & Brinck-Johnsen, T. (1968). Specific and nonspecific physicochemical interactions of glucocorticoids and related steroids with rat thymus cells in vitro. *The Journal of Biological Chemistry*, 243, 5556–5565.
- Murtagh, J., McArdle, E., Gilligan, E., Thornton, L., Furlong, F., & Martin, F. (2004). Organization of mammary epithelial cells into 3D acinar structures requires glucocorticoid and JNK signaling. *Journal of Cell Biology*, 166, 133–143.
- Musatov, S., Chen, W., Pfaff, D. W., Kaplitt, M. G., & Ogawa, S. (2006). RNAi-mediated silencing of estrogen receptor [alpha] in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 10456–10460.
- Myers, M., Lamont, M. C., Van Den Driesche, S., Mary, N., Thong, K. J., Hillier, S. G., & Duncan, W. C. (2007). Role of luteal glucocorticoid metabolism during maternal recognition of pregnancy in women. *Endocrinology*, 148, 5769–5779.

- Nadeem, L., Shynlova, O., Matysiak-Zablocki, E., Mesiano, S., Dong, X., & Lye, S. (2016). Molecular evidence of functional progesterone withdrawal in human myometrium. *Nature Communications*, 7, 11565.
- Nahar, J., Rainville, J. R., Dohanich, G. P., & Tasker, J. G. (2016). Further evidence for a membrane receptor that binds glucocorticoids in the rodent hypothalamus. *Steroids*, 114, 33–40.
- Nanjappa, M. K., Medrano, T. I., Lydon, J. P., Bigsby, R. M., & Cooke, P. S. (2015). Maximal dexamethasone inhibition of luminal epithelial proliferation involves progesterone receptor (PR)- and non-PR-mediated mechanisms in neonatal mouse uterus. *Biology of Reproduction*, 92.
- Natraj, U., & Richards, J. S. (1993). Hormonal regulation, localization, and functional activity of the progesterone receptor in granulosa cells of rat preovulatory follicles. *Endocrinology*, 133, 761–769.
- Ng, Y., Wolfe, A., Novaira, H. J., & Radovick, S. (2009). Estrogen regulation of gene expression in GnRH neurons. *Molecular and Cellular Endocrinology*, 303, 25–33.
- Nugent, J. L., Wareing, M., Palin, V., Sibley, C. P., Baker, P. N., Ray, D. W., Farrow, S. N., & Jones, R. L. (2013). Chronic glucocorticoid exposure potentiates placental chorionic plate artery constriction: implications for aberrant fetoplacental vascular resistance in fetal growth restriction. *Endocrinology*, 154, 876–887.
- O'Hara, B., Alvarez de la Rosa, D., & Rajendran, V. M. (2014). Multiple mineralocorticoid response elements localized in different introns regulate intermediate conductance K⁺ (Kcnk4) channel expression in the rat distal colon. *PLoS One*, e98695, 9.
- Oakley, R. H., Webster, J. C., Sar, M., Parker, C. R., Jr., & Cidlowski, J. A. (1997). Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. *Endocrinology*, 138, 5028–5038.
- Obeid, J. P., Zafar, N., & El Hokayem, J. (2016). Steroid Hormone Receptor Coregulators in Endocrine Cancers. *IUBMB Life*, 68, 504–515.
- Obr, A. E., & Edwards, D. P. (2012). The biology of progesterone receptor in the normal mammary gland and in breast cancer. *Molecular and Cellular Endocrinology*, 357, 4–17.
- Okon, M. A., Laird, S. M., Tuckerman, E. M., & Li, T. C. (1998). Serum androgen levels in women who have recurrent miscarriages and their correlation with markers of endometrial function. *Fertility and Sterility*, 69, 682–690.
- Okuda, Y., Hirata, S., Watanabe, N., Shoda, T., Kato, J., & Hoshi, K. (2003). Novel splicing events of untranslated first exons in human estrogen receptor alpha (ER alpha) gene. *Endocrine Journal*, 50, 97–104.
- Owen, G. I., Richer, J. K., Tung, L., Takimoto, G., & Horwitz, K. B. (1998). Progesterone regulates transcription of the p21(WAF1) cyclin- dependent kinase inhibitor gene through Sp1 and CBP/p300. *The Journal of Biological Chemistry*, 273, 10696–10701.
- Ozmen, A., Unek, G., Kipmen-Korgun, D., & Korgun, E. T. (2011). The expression of Akt and Erk1/2 proteins decreased in intrauterine growth restricted rat placental development. *Placenta*, 32, A99.
- Pal, S., Gupta, R., Kim, H., Wickramasinghe, P., Baubet, V., Showe, L. C., Dahmane, N., & Davuluri, R. V. (2011). Alternative transcription exceeds alternative splicing in generating the transcriptome diversity of cerebellar development. *Genome Research*, 21, 1260–1272.
- Palma-Gudiel, H., Cordova-Palamera, A., Leza, J. C., & Fananas, L. (2015). Glucocorticoid receptor gene (NR3C1) methylation processes as mediators of early adversity in stress-related disorders causality: a critical review. *Neuroscience & Biobehavioral Reviews*, 55, 520–535.
- Park, O. K., & Mayo, K. E. (1991). Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge. *Molecular Endocrinology*, 5, 967–978.
- Pascual-Le Tallec, L., Demange, C., & Lombes, M. (2004). Human mineralocorticoid receptor A and B protein forms produced by alternative translation sites display different transcriptional activities. *European Journal of Endocrinology*, 150, 585–590.
- Patel, B., Elguero, S., Thakore, S., Dahoud, W., Bedaiwy, M., & Mesiano, S. (2015). Role of nuclear progesterone receptor isoforms in uterine pathophysiology. *Human Reproduction Update*, 21, 155–173.
- Pawlak, M., Lefebvre, P., & Staels, B. (2012). General molecular biology and architecture of nuclear receptors. *Current Topics in Medicinal Chemistry*, 12, 486–504.
- Pelletier, G., Luu-The, V., Li, S., & Labrie, F. (2004). Localization and estrogenic regulation of androgen receptor mRNA expression in the mouse uterus and vagina. *Journal of Endocrinology*, 180, 77–85.
- Pena, C. J., & Champagne, F. A. (2015). Neonatal overexpression of estrogen receptor-alpha alters midbrain dopamine neuron development and reverses the effects of low maternal care in female offspring. *Developmental Neurobiology*, 75, 1114–1124.
- Perrot-Appianat, M., Logeat, F., Groyer-Picard, M. T., & Milgrom, E. (1985). Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. *Endocrinology*, 116, 1473–1484.
- Peters, A. A., Ingman, W. V., Tilley, W. D., & Butler, L. M. (2011). Differential effects of exogenous androgen and an androgen receptor antagonist in the peri- and postpubertal murine mammary gland. *Endocrinology*, 152, 3728–3737.
- Ponguta, L. A., Gregory, C. W., French, F. S., & Wilson, E. M. (2008). Site-specific androgen receptor serine phosphorylation linked to epidermal growth factor-dependent growth of castration-recurrent prostate cancer. *The Journal of Biological Chemistry*, 283, 20989–21001.
- Porter, G. A., & Edelman, I. S. (1964). The action of aldosterone and related corticosteroids on sodium transport across the toad bladder. *The Journal of Clinical Investigation*, 43, 611–620.
- Portillo, W., Gonzalez-Flores, O., Camacho, F. J., Mani, S. K., & Paredes, R. G. (2016). Participation of progesterone receptors in facilitation and sequential inhibition of lordosis response induced by ring A-reduced progesterone metabolites in female mice. *Behavioral Neuroscience*, 130, 624–634.
- Pratt, W. B., Sanchez, E. R., Bresnick, E. H., Meshinchi, S., Scherrer, L. C., Dalman, F. C., & Welsh, M. J. (1989). Interaction of the glucocorticoid receptor with the Mr 90,000 heat shock protein: an evolving model of ligand-mediated receptor transformation and translocation. *Cancer Research*, 49, 2222s–2229s.
- Presul, E., Schmidt, S., Kofler, R., & Helmborg, A. (2007). Identification, tissue expression, and glucocorticoid responsiveness of alternative first exons of the human glucocorticoid receptor. *Journal of Molecular Endocrinology*, 38, 79–90.
- Prizant, H., Gleicher, N., & Sen, A. (2014). Androgen actions in the ovary: balance is key. *Journal of Endocrinology*, 222, R141–51.
- Quadros, P. S., Pfau, J. L., & Wagner, C. K. (2007). Distribution of progesterone receptor immunoreactivity in the fetal and neonatal rat forebrain. *Journal of Comparative Neurology*, 504, 42–56.
- Rabin, D. S., Johnson, E. O., Brandon, D. D., Liapi, C., & Chrousos, G. P. (1990). Glucocorticoids inhibit estradiol-mediated uterine growth: possible role of the uterine estradiol receptor. *Biology of Reproduction*, 42, 74–80.
- Rae, M. T., Niven, D., Critchley, H. O., Harlow, C. R., & Hillier, S. G. (2004). Antiinflammatory steroid action in human ovarian surface epithelial cells. *Journal of Clinical Endocrinology and Metabolism*, 89, 4538–4544.
- Ramamoorthy, S., & Nawaz, Z. (2008). E6-associated protein (E6-AP) is a dual function coactivator of steroid hormone receptors. *Nuclear Receptor Signaling*, 6, e006.
- Rao, I. M., & Mahesh, V. B. (1986). Role of progesterone in the modulation of the preovulatory surge of gonadotropins and ovulation in the pregnant mare's serum gonadotropin-primed immature rat and the adult rat. *Biology of Reproduction*, 35, 1154–1161.
- Ratman, D., Vanden Bergh, W., Dejager, L., Libert, C., Tavernier, J., Beck, I. M., & De Bosscher, K. (2013). How glucocorticoid receptors modulate the activity of other transcription factors: a scope beyond tethering. *Molecular and Cellular Endocrinology*, 380, 41–54.
- Ray, D. W., Davis, J. R., White, A., & Clark, A. J. (1996). Glucocorticoid receptor structure and function in glucocorticoid-resistant small cell lung carcinoma cells. *Cancer Research*, 56, 3276–3280.
- Reddy, T. E., Pauli, F., Sprouse, R. O., Neff, N. F., Newberry, K. M., Garabedian, M. J., & Myers, R. M. (2009). Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res*, 19, 2163–2171.
- Ritter, H. D., Antonova, L., & Mueller, C. R. (2012). The unliganded glucocorticoid receptor positively regulates the tumor suppressor gene BRCA1 through GABP beta. *Molecular Cancer Research*, 10, 558–569.

- Ritter, H. D., & Mueller, C. R. (2014). Expression microarray identifies the unliganded glucocorticoid receptor as a regulator of gene expression in mammary epithelial cells. *BMC Cancer*, 14.
- Roberts, C. W., & Orkin, S. H. (2004). The SWI/SNF complex—chromatin and cancer. *Nature Reviews. Cancer*, 4, 133–142.
- Roberts, D., & Dalziel, S. (2006). Antenatal corticosteroids for accelerating fetal lung maturation for women at risk of preterm birth. *The Cochrane Database of Systematic Reviews*, CD004454.
- Robertshaw, I., Bian, F., & Das, S. K. (2016). Mechanisms of uterine estrogen signaling during early pregnancy in mice: an update. *Journal of Molecular Endocrinology*, 56, R127–R138.
- Robker, R. L., Akison, L. K., & Russell, D. L. (2009). Control of oocyte release by progesterone receptor-regulated gene expression. *Nuclear Receptor Signaling*, 7, e012.
- Robker, R. L., Russell, D. L., Espey, L. L., Lydon, J. P., O'Malley, B. W., & Richards, J. S. (2000). Progesterone-regulated genes in the ovulation process: ADAMTS-1 and cathepsin L proteases. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 4689–4694.
- Rockwell, L. C., Pillai, S., Olson, C. E., & Koos, R. D. (2002). Inhibition of vascular endothelial growth factor/vascular permeability factor action blocks estrogen-induced uterine edema and implantation in rodents. *Biology of Reproduction*, 67, 1804–1810.
- Rothman, M. S., Carlson, N. E., Xu, M., Wang, C., Swerdloff, R., Lee, P., Goh, V. H., Ridgway, E. C., & Wierman, M. E. (2011). Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids*, 76, 177–182.
- Roy, D., Angelini, N. L., & Belsham, D. D. (1999). Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor-alpha (ER alpha)- and ER beta-expressing GT1-7 GnRH neurons. *Endocrinology*, 140, 5045–5053.
- Rubel, C. A., Lanz, R. B., Kommagani, R., Franco, H. L., Lydon, J. P., & DeMayo, F. J. (2012). Research resource: genome-wide profiling of progesterone receptor binding in the mouse uterus. *Molecular Endocrinology*, 26, 1428–1442.
- Russell, D. L., & Robker, R. L. (2007). Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Human Reproduction Update*, 13, 289–312.
- Russo, J., Ao, X., Grill, C., & Russo, I. H. (1999). Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Research and Treatment*, 53, 217–227.
- Sa, S. I., Pereira, P. A., Malikov, V., & Madeira, M. D. (2013). Role of estrogen receptor alpha and beta in the induction of progesterone receptors in hypothalamic ventromedial neurons. *Neuroscience*, 238, 159–167.
- Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M., & Gustafsson, J. A. (2000). Estrogen receptors alpha and beta in the rodent mammary gland. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 337–342.
- Salleh, N., Mokhtar, H. M., Kassim, N. M., & Giribabu, N. (2015). Testosterone induces increase in aquaporin (AQP)-1, 5, and 7 expressions in the uteri of ovariectomized rats. *The Journal of Membrane Biology*, 248, 1097–1105.
- Sanathara, N. M., Moreas, J., Mahavongtrakul, M., & Sinchak, K. (2014). Estradiol upregulates progesterone receptor and orphanin FQ colocalization in arcuate nucleus neurons and opioid receptor-like receptor-1 expression in proopiomelanocortin neurons that project to the medial preoptic nucleus in the female rat. *Neuroendocrinology*, 100, 103–118.
- Sanchez-Criado, J. E., Trudgen, K., Millan, Y., Blanco, A., Monterde, J., Garrido-Gracia, J. C., Gordon, A., Aguilar, R., de las Mulas, J. M., & Ko, C. (2012). Estrogen receptor (ESR) 2 partially offsets the absence of ESR1 in gonadotropes of pituitary-specific ESR1 knockout female mice. *Reproduction*, 143, 549–558.
- Saner, K. J., Welter, B. H., Zhang, F., Hansen, E., Dupont, B., Wei, Y. Z., & Price, T. M. (2003). Cloning and expression of a novel, truncated, progesterone receptor. *Molecular and Cellular Endocrinology*, 200, 155–163.
- Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, 21, 55–89.
- Sar, M., & Welsch, F. (1999). Differential expression of estrogen receptor-beta and estrogen receptor-alpha in the rat ovary. *Endocrinology*, 140, 963–971.
- Sasano, H., Frost, A. R., Saitoh, R., Matsunaga, G., Nagura, H., Krozowski, Z. S., & Silverberg, S. G. (1997). Localization of mineralocorticoid receptor and 11 beta-hydroxysteroid dehydrogenase type II in human breast and its disorders. *Anticancer Research*, 17, 2001–2007.
- Sathishkumar, K., Balakrishnan, M., Chinnathambi, V., Chauhan, M., Hankins, G. D. V., & Yallampalli, C. (2012). Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia. *Journal of Perinatology*, 32, 328–335.
- Satoh, K., Hovey, R. C., Malewski, T., Warri, A., Goldhar, A. S., Ginsburg, E., Saito, K., Lydon, J. P., & Vonderhaar, B. K. (2007). Progesterone enhances branching morphogenesis in the mouse mammary gland by increased expression of Msx2. *Oncogene*, 26, 7526–7534.
- Schoonmaker, J. N., & Erickson, G. F. (1983). Glucocorticoid modulation of follicle-stimulating hormone-mediated granulosa-cell differentiation. *Endocrinology*, 113, 1356–1363.
- Schwartz, N., Verma, A., Bivens, C. B., Schwartz, Z., & Boyan, B. D. (2016). Rapid steroid hormone actions via membrane receptors. *Biochimica et Biophysica Acta*, 1863, 2289–2298.
- Schwarz, J. M., Nugent, B. M., & McCarthy, M. M. (2010). Developmental and hormone-induced epigenetic changes to estrogen and progesterone receptor genes in brain are dynamic across the life span. *Endocrinology*, 151, 4871–4881.
- Shao, R., Markstrom, E., Friberg, P. A., Johansson, M., & Billig, H. (2003). Expression of progesterone receptor (PR) A and B isoforms in mouse granulosa cells: stage-dependent PR-mediated regulation of apoptosis and cell proliferation. *Biology of Reproduction*, 68, 914–921.
- Shi, H. Y., Lydon, J. P., & Zhang, M. (2004). Hormonal defect in maspin heterozygous mice reveals a role of progesterone in pubertal ductal development. *Molecular Endocrinology*, 18, 2196–2207.
- Shoda, T., Hirata, S., Kato, J., & Hoshi, K. (2002). Cloning of the novel isoform of the estrogen receptor beta cDNA (ERbeta isoform M cDNA) from the human testicular cDNA library. *The Journal of Steroid Biochemistry and Molecular Biology*, 82, 201–208.
- Simitsidellis, I., Gibson, D. A., Cousins, F. L., Esnal-Zufiaurre, A., & Saunders, P. T. (2016). A role for androgens in epithelial proliferation and formation of glands in the mouse uterus. *Endocrinology*, 157, 2116–2128.
- Singh, S. P., Wolfe, A., Ng, Y., Divall, S. A., Buggs, C., Levine, J. E., Wondisford, F. E., & Radvick, S. (2009). Impaired estrogen feedback and infertility in female mice with pituitary-specific deletion of estrogen receptor alpha (ESR1). *Biology of Reproduction*, 81, 488–496.
- Skorupskaitė, K., George, J. T., & Anderson, R. A. (2014). The kisspeptin-GnRH pathway in human reproductive health and disease. *Human Reproduction Update*, 20, 485–500.
- Smith, J. T., Cunningham, M. J., Rissman, E. F., Clifton, D. K., & Steiner, R. A. (2005). Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology*, 146, 3686–3692.
- Smith, L., Coleman, L. J., Cummings, M., Satheesha, S., Shaw, S. O., Speirs, V., & Hughes, T. A. (2010). Expression of oestrogen receptor beta isoforms is regulated by transcriptional and post-transcriptional mechanisms. *Biochemical Journal*, 429, 283–290.
- Son, Y. L., Ubuka, T., Narihiro, M., Fukuda, Y., Hasunuma, I., Yamamoto, K., Belsham, D. D., & Tsutsui, K. (2014). Molecular basis for the activation of gonadotropin-inhibitory hormone gene transcription by corticosterone. *Endocrinology*, 155, 1817–1826.
- Speake, P. F., Glazier, J. D., Greenwood, S. L., & Sibley, C. P. (2010). Aldosterone and cortisol acutely stimulate Na⁺/H⁺ exchanger activity in the syncytiotrophoblast of the human placenta: effect of fetal sex. *Placenta*, 31, 289–294.
- Sprangers, S. A., West, N. B., Brenner, R. M., & Bethea, C. L. (1990). Regulation and localization of estrogen and progestin receptors in the pituitary of steroid-treated monkeys. *Endocrinology*, 126, 1133–1142.
- Sriraman, V., Sharma, S. C., & Richards, J. S. (2003). Transactivation of the progesterone receptor gene in granulosa cells: evidence that Sp1/Sp3 binding sites in the proximal promoter play a key role in luteinizing hormone inducibility. *Molecular Endocrinology*, 17, 436–449.
- Stender, J. D., Kim, K., Charn, T. H., Komm, B., Chang, K. C. N., Kraus, W. L., Benner, C., Glass, C. K., & Katzenellenbogen, B. S. (2010). Genome-wide analysis of estrogen receptor alpha DNA binding and tethering mechanisms identifies Runx1 as a novel tethering factor in receptor-mediated transcriptional activation. *Molecular and Cellular Biology*, 30, 3943–3955.

- Stephens, S. B., Tolson, K. P., Rouse, M. L., Jr., Poling, M. C., Hashimoto-Partyka, M. K., Mellon, P. L., & Kauffman, A. S. (2015). Absent progesterone signaling in kisspeptin neurons disrupts the LH surge and impairs fertility in female mice. *Endocrinology*, 156, 3091–3097.
- Stewart, C. A., Fisher, S. J., Wang, Y., Stewart, M. D., Hewitt, S. C., Rodriguez, K. F., Korach, K. S., & Behringer, R. R. (2011). Uterine gland formation in mice is a continuous process, requiring the ovary after puberty, but not after parturition. *Biology of Reproduction*, 85, 954–964.
- Stocklin, E., Wissler, M., Gouilleux, F., & Groner, B. (1996). Functional interactions between Stat5 and the glucocorticoid receptor. *Nature*, 383, 726–728.
- Stouffer, R. L. (2003). Progesterone as a mediator of gonadotrophin action in the corpus luteum: beyond steroidogenesis. *Human Reproduction Update*, 9, 99–117.
- Suter, D. E., & Schwartz, N. B. (1985). Effects of glucocorticoids on secretion of luteinizing hormone and follicle-stimulating hormone by female rat pituitary cells in vitro. *Endocrinology*, 117, 849–854.
- Takumi, K., Iijima, N., Higo, S., & Ozawa, H. (2012). Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. *Neuroscience Letters*, 531, 40–45.
- Tan, H. Q., Yi, L. J., Rote, N. S., Hurd, W. W., & Mesiano, S. (2012). Progesterone receptor-A and -B have opposite effects on proinflammatory gene expression in human myometrial cells: implications for progesterone actions in human pregnancy and parturition. *Journal of Clinical Endocrinology & Metabolism*, 97, E719–E730.
- Tarulli, G. A., Butler, L. M., Tilley, W. D., & Hickey, T. E. (2014). Bringing androgens up a NOTCH in breast cancer. *Endocrine-Related Cancer*, 21, T183–T202.
- Taylor, A. H., McParland, P. C., Taylor, D. J., & Bell, S. C. (2009). The cytoplasmic 60 kDa progesterone receptor isoform predominates in the human amnionchorion and placenta at term. *Reproductive Biology and Endocrinology*, 7.
- Tetsuka, M., Milne, M., Simpson, G. E., & Hillier, S. G. (1999). Expression of 11beta-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. *Biology of Reproduction*, 60, 330–335.
- Tetsuka, M., Whitelaw, P. F., Bremner, W. J., Millar, M. R., Smyth, C. D., & Hillier, S. G. (1995). Developmental regulation of androgen receptor in rat ovary. *Journal of Endocrinology*, 145, 535–543.
- Thackray, V. G., Hunnicutt, J. L., Memon, A. K., Ghochani, Y., & Mellon, P. L. (2009). Progesterone Inhibits basal and gonadotropin-releasing hormone induction of luteinizing hormone beta-subunit gene expression. *Endocrinology*, 150, 2395–2403.
- Thackray, V. G., McGilivray, S. M., & Mellon, P. L. (2006). Androgens, progestins, and glucocorticoids induce follicle-stimulating hormone beta-subunit gene expression at the level of the gonadotrope. *Molecular Endocrinology*, 20, 2062–2079.
- Therlaux, A., Boyd, E., Harrop, S. B., Hollenberg, S. M., & Connor, J. M. (1989). Regional chromosomal assignment of the human glucocorticoid receptor gene to 5q31. *Human Genetics*, 83, 289–291.
- Thompson, A., Han, V. K., & Yang, K. (2002). Spatial and temporal patterns of expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 messenger RNA and glucocorticoid receptor protein in the murine placenta and uterus during late pregnancy. *Biology of Reproduction*, 67, 1708–1718.
- Thornton, J. W. (2001). Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 5671–5676.
- Tilley, W. D., Marcelli, M., & McPhaul, M. J. (1990). Expression of the human androgen receptor gene utilizes a common promoter in diverse human tissues and cell-lines. *Journal of Biological Chemistry*, 265, 13776–13781.
- Toft, D., & Gorski, J. (1966). A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proceedings of the National Academy of Sciences of the United States of America*, 55, 1574–1581.
- Toms, D., Xu, S. Y., Pan, B., De, W., & Li, J. L. (2015). Progesterone receptor expression in granulosa cells is suppressed by microRNA-378-3p. *Molecular and Cellular Endocrinology*, 399, 95–102.
- Tong, W., & Pollard, J. W. (1999). Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E- and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice. *Molecular and Cellular Biology*, 19, 2251–2264.
- Trevino, L. S., & Weigel, N. L. (2013). Phosphorylation: a fundamental regulator of steroid receptor action. *Trends in Endocrinology and Metabolism*, 24, 515–524.
- Turgeon, J. L., Van Patten, S. M., Shyamala, G., & Waring, D. W. (1999). Steroid regulation of progesterone receptor expression in cultured rat gonadotropes. *Endocrinology*, 140, 2318–2325.
- Turgeon, J. L., & Waring, D. W. (2001). Luteinizing hormone secretion from wild-type and progesterone receptor knockout mouse anterior pituitary cells. *Endocrinology*, 142, 3108–3115.
- Turner, J. D., & Muller, C. P. (2005). Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. *Journal of Molecular Endocrinology*, 35, 283–292.
- Umesono, K., & Evans, R. M. (1989). Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell*, 57, 1139–1146.
- Uzelac, P. S., Li, X., Lin, J., Neese, L. D., Lin, L., Nakajima, S. T., Bohler, H., & Lei, Z. (2010). Dysregulation of leptin and testosterone production and their receptor expression in the human placenta with gestational diabetes mellitus. *Placenta*, 31, 581–588.
- Varga, J., Ferenczi, S., Kovacs, K. J., Garafova, A., Jezova, D., & Zelena, D. (2013). Comparison of stress-induced changes in adults and pups: is aldosterone the main adrenocortical stress hormone during the perinatal period in rats? *Plos One*, 8.
- Verhoog, N. J., Du Toit, A., Avenant, C., & Hapgood, J. P. (2011). Glucocorticoid-independent repression of tumor necrosis factor (TNF) alpha-stimulated interleukin (IL)-6 expression by the glucocorticoid receptor: a potential mechanism for protection against an excessive inflammatory response. *The Journal of Biological Chemistry*, 286, 19297–19310.
- Walters, K. A., Allan, C. M., & Handelsman, D. J. (2008). Androgen actions and the ovary. *Biology of Reproduction*, 78, 380–389.
- Walters, K. A., McTavish, K. J., Seneviratne, M. G., Jimenez, M., McMahon, A. C., Allan, C. M., Salamonsen, L. A., & Handelsman, D. J. (2009). Subfertile female androgen receptor knockout mice exhibit defects in neuroendocrine signaling, intraovarian function, and uterine development but not uterine function. *Endocrinology*, 150, 3274–3282.
- Walters, K. A., Simanainen, U., & Handelsman, D. J. (2010). Molecular insights into androgen actions in male and female reproductive function from androgen receptor knockout models. *Human Reproduction Update*, 16, 543–558.
- Wan, Y., Coxe, K. K., Thackray, V. G., Housley, P. R., & Nordeen, S. K. (2001). Separable features of the ligand-binding domain determine the differential subcellular localization and ligand-binding specificity of glucocorticoid receptor and progesterone receptor. *Molecular Endocrinology*, 15, 17–31.
- Wang, B. B., Parobchak, N., Rosen, M., Roche, N., & Rosen, T. (2014). Negative effects of progesterone receptor isoform-A on human placental activity of the noncanonical NF-kappa B signaling. *Journal of Clinical Endocrinology & Metabolism*, 99, E320–E328.
- Wang, D., Garcia-Bassets, I., Benner, C., Li, W., Su, X., Zhou, Y., Qiu, J., Liu, W., Kaikkonen, M. U., Ohgi, K. A., Glass, C. K., Rosenfeld, M. G., & Fu, X. D. (2011). Reprogramming transcription by distinct classes of enhancers functionally defined by eRNA. *Nature*, 474, 390–394.
- Wang, F., Pan, J., Liu, Y., Meng, Q., Lv, P., Qu, F., Ding, G. L., Klausen, C., Leung, P. C., Chan, H. C., Yao, W., Zhou, C. Y., Shi, B., Zhang, J., Sheng, J., & Huang, H. (2015). Alternative splicing of the androgen receptor in polycystic ovary syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 4743–4748.
- Warmark, A., Treuter, E., Wright, A. P., & Gustafsson, J. A. (2003). Activation functions 1 and 2 of nuclear receptors: molecular strategies for transcriptional activation. *Molecular Endocrinology*, 17, 1901–1909.
- Wei, L. L., Gonzalez-Aller, C., Wood, W. M., Miller, L. A., & Horwitz, K. B. (1990). 5'-Heterogeneity in human progesterone receptor transcripts predicts a new amino-terminal truncated "C"-receptor and unique A-receptor messages. *Molecular Endocrinology*, 4, 1833–1840.
- Weiser, M. J., Foradori, C. D., & Handa, R. J. (2008). Estrogen receptor beta in the brain: from form to function. *Brain Research Reviews*, 57, 309–320.
- Wetendorf, M., & DeMayo, F. J. (2012). The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. *Molecular and Cellular Endocrinology*, 357, 108–118.
- Wetendorf, M., Wu, S. P., Wang, X. Q., Creighton, C. J., Wang, T. Y., Lanz, R. B., Blok, L., Tsai, S. Y., Tsai, M. J., Lydon, J. P., & DeMayo, F. J. (2017). Decreased epithelial progesterone receptor A at the window of receptivity is required for preparation of the endometrium for embryo attachment. *Biology of Reproduction*, 96, 313–326.

- Whirledge, S., & Cidlowski, J. A. (2010). Glucocorticoids, stress, and fertility. *Minerva Endocrinologica*, 35, 109–125.
- Whirledge, S., & Cidlowski, J. A. (2013a). Estradiol antagonism of glucocorticoid-induced GILZ expression in human uterine epithelial cells and murine uterus. *Endocrinology*, 154, 499–510.
- Whirledge, S., & Cidlowski, J. A. (2013b). A role for glucocorticoids in stress-impaired reproduction: beyond the hypothalamus and pituitary. *Endocrinology*, 154, 4450–4468.
- Whirledge, S., Xu, X., & Cidlowski, J. A. (2013). Global gene expression analysis in human uterine epithelial cells defines new targets of glucocorticoid and estradiol antagonism. *Biology of Reproduction*, 89, 66.
- Whirledge, S. D., Oakley, R. H., Myers, P. H., Lydon, J. P., DeMayo, F., & Cidlowski, J. A. (2015). Uterine glucocorticoid receptors are critical for fertility in mice through control of embryo implantation and decidualization. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 15166–15171.
- White, M. M., Sheffer, I., Teeter, J., & Apostolakis, E. M. (2007). Hypothalamic progesterone receptor-A mediates gonadotropin surges, self priming and receptivity in estrogen-primed female mice. *Journal of Molecular Endocrinology*, 38, 35–50.
- Wierman, M. E., & Wang, C. (1990). Androgen selectively stimulates follicle-stimulating hormone-beta mRNA levels after gonadotropin-releasing hormone antagonist administration. *Biology of Reproduction*, 42, 563–571.
- Wikstrom, A. C., Bakke, O., Okret, S., Bronnegard, M., & Gustafsson, J. A. (1987). Intracellular localization of the glucocorticoid receptor: evidence for cytoplasmic and nuclear localization. *Endocrinology*, 120, 1232–1242.
- Williams-Ashman, H. G., & Reddi, A. H. (1971). Actions of vertebrate sex hormones. *Annu Rev Physiol*, 33, 31–82.
- Williamson, M., & Viau, V. (2007). Androgen receptor expressing neurons that project to the paraventricular nucleus of the hypothalamus in the male rat. *Journal of Comparative Neurology*, 503, 717–740.
- Wilson, C. M., & McPhaul, M. J. (1994). A and B forms of the androgen receptor are present in human genital skin fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 1234–1238.
- Wintermantel, T. M., Bock, D., Fleig, V., Greiner, E. F., & Schutz, G. (2005). The epithelial glucocorticoid receptor is required for the normal timing of cell proliferation during mammary lobuloalveolar development but is dispensable for milk production. *Molecular Endocrinology*, 19, 340–349.
- Wintermantel, T. M., Campbell, R. E., Porteous, R., Bock, D., Grone, H. J., Todman, M. G., Korach, K. S., Greiner, E., Perez, C. A., Schutz, G., & Herbison, A. E. (2006). Definition of estrogen receptor pathway critical for estrogen positive feedback to gonadotropin-releasing hormone neurons and fertility. *Neuron*, 52, 271–280.
- Winuthayanon, W., Hewitt, S. C., & Korach, K. S. (2014). Uterine epithelial cell estrogen receptor alpha-dependent and -independent genomic profiles that underlie estrogen responses in mice. *Biology of Reproduction*, 91.
- Winuthayanon, W., Hewitt, S. C., Orvis, G. D., Behringer, R. R., & Korach, K. S. (2010). Uterine epithelial estrogen receptor alpha is dispensable for proliferation but essential for complete biological and biochemical responses. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 19272–19277.
- Wu, S., Chen, Y., Fajobi, T., Divall, S. A., Chang, C., Yeh, S., & Wolfe, A. (2014). Conditional knockout of the androgen receptor in gonadotropes reveals crucial roles for androgen in gonadotropin synthesis and surge in female mice. *Molecular Endocrinology*, 28, 1670–1681.
- Wysocka, J., Allis, C. D., & Coonrod, S. (2006). Histone arginine methylation and its dynamic regulation. *Front Biosci*, 11, 344–355.
- Xu, J., Li, M., Zhang, L., Xiong, H., Lai, L., Guo, M., Zong, T., Zhang, D., Yang, B., Wu, L., Tang, M., & Kuang, H. (2015). Expression and regulation of androgen receptor in the mouse uterus during early pregnancy and decidualization. *Mol Reprod Dev*, 82, 898–906.
- Yamanaka, T., Hirata, S., Shoda, T., & Hoshi, K. (2002). Progesterone receptor mRNA variant containing novel exon insertions between exon 4 and Exon 5 in human uterine endometrium. *Endocrine Journal*, 49, 473–482.
- Yasuda, S., Kobayashi, M., Itagaki, S., Hirano, T., & Iseki, K. (2009). Response of the ABCG2 promoter in T47D cells and BeWo cells to sex hormone treatment. *Molecular Biology Reports*, 36, 1889–1896.
- Yin, P., Kawashima, K., & Arita, J. (2002). Direct actions of estradiol on the anterior pituitary gland are required for hypothalamus-dependent lactotrope proliferation and secretory surges of luteinizing hormone but not of prolactin in female rats. *Neuroendocrinology*, 75, 392–401.
- Yu, C. Y., Mayba, O., Lee, J. V., Tran, J., Harris, C., Speed, T. P., & Wang, J. C. (2010). Genome-wide analysis of glucocorticoid receptor binding regions in adipocytes reveal gene network involved in triglyceride homeostasis. *Plos One*, 5.
- Yuan, H. J., Han, X., He, N., Wang, G. L., Gong, S., Lin, J., Gao, M., & Tan, J. H. (2016). Glucocorticoids impair oocyte developmental potential by triggering apoptosis of ovarian cells via activating the Fas system. *Scientific Reports*, 6, 24036.
- Zachariades, E., Foster, H., Goumenou, A., Thomas, P., Rand-Weaver, M., & Karteris, E. (2011). Expression of membrane and nuclear progesterone receptors in two human placental choriocarcinoma cell lines (JEG-3 and BeWo): Effects of syncytialization. *Int J Mol Med*, 27, 767–774.
- Zarate, S., & Seilicovich, A. (2010). Estrogen receptors and signaling pathways in lactotropes and somatotropes. *Neuroendocrinology*, 92, 215–223.
- Zarate, S., Zaldivar, V., Jaita, G., Magri, L., Radl, D., Pisera, D., & Seilicovich, A. (2010). Role of estrogens in anterior pituitary gland remodeling during the estrous cycle. *Front Horm Res*, 38, 25–31.
- Zennaro, M. C., Keightley, M. C., Kotelevtsev, Y., Conway, G. S., Soubrier, F., & Fuller, P. J. (1995). Human mineralocorticoid receptor genomic structure and identification of expressed isoforms. *The Journal of Biological Chemistry*, 270, 21016–21020.
- Zennaro, M. C., Souque, A., Viengchareun, S., Poisson, E., & Lombes, M. (2001). A new human MR splice variant is a ligand-independent transactivator modulating corticosteroid action. *Molecular Endocrinology*, 15, 1586–1598.
- Zeps, N., Bentel, J. M., Papadimitriou, J. M., D'Antuono, M. F., & Dawkins, H. J. (1998). Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. *Differentiation*, 62, 221–226.
- Zhang, D., Liu, H., Zeng, J., Miao, X., Huang, W., Chen, H., Huang, Y., Li, Y., & Ye, D. (2016). Glucocorticoid exposure in early placentation induces preeclampsia in rats via interfering trophoblast development. *General and Comparative Endocrinology*, 225, 61–70.
- Zhou, X. (2010). Roles of androgen receptor in male and female reproduction: lessons from global and cell-specific androgen receptor knockout (ARKO) mice. *Journal of Andrology*, 31, 235–243.
- Zhuang, Y., Cui, H., Liu, S., Zheng, D., & Liu, C. (2015). Progesterone receptor B promoter hypermethylation in human placenta after labor onset. *Reproductive Sciences*, 22, 335–342.
- Zou, Y., Ding, L., Coleman, M., & Wang, Z. Y. (2009). Estrogen receptor-alpha (ER-alpha) suppresses expression of its variant ER-alpha 36. *FEBS Letters*, 583, 1368–1374.

Nonclassical Steroid Receptors and Their Role in Regulating Female Reproduction

John J Peluso, University of Connecticut School of Medicine, Farmington, CT, United States

© 2018 Elsevier Inc. All rights reserved.

A Brief History of Nonclassical Steroid Receptors

The first edition of the *Encyclopedia of Reproduction* was published in 1998. In this massive text of 1297 pages, the idea that steroids could have rapid and/or membrane-initiated actions that were not dependent on the genomic action of classical nuclear steroid receptors was not even mentioned. Coincidentally in that same year, a group of about 100 scientists gathered in Mannheim, Germany to attend the First International Symposium on Rapid Responses to Steroids. Although rapid actions of steroids had been described, the cloning of classical nuclear steroid receptors in the late 1980s focused scientific research on the genomic mechanism through which steroids act. So much so that it prompted Norman and Wehling to state in their forward to the proceedings of this symposium that “nuclear receptors are believed by some researchers to account for the mechanism of action of all biological responses initiated by steroid hormones... and any unorthodox observations of apparent nongenomically mediated steroid effect [i.e., an action to rapid to be the result of differential gene expression] was either not believed by the researchers themselves or was in some instances, judged to be almost unpublishable due to the objections of the editors and/or the referees” (Norman and Wehling, 1999). It has taken nearly 20 years and 2489 publications since this symposium for the concept of nonclassical actions for steroids to be accepted (PubMed search terms: nonclassical or noncanonical and steroid; conducted June 20, 2017). However, the details as to the precise mechanisms that mediate these actions still remain to be elucidated. Consistent with the topics of Volume 2 of *Encyclopedia of Reproduction*, the focus of this article will be restricted to nonclassical actions of estrogen and progesterone as these steroids are the major steroids that regulate female reproduction. Finally, it is important to appreciate that this article is not intended to be a comprehensive review. Rather this article is intended to provide a general overview of how estrogen and progesterone mediate their actions through mechanisms that do not involve steroid receptor: DNA interaction and thereby regulate gene transcription. Unfortunately, this approach necessitates limiting the number of citations and omitting citations to many primary references.

Structural Features of Classical and Nonclassical Steroid Receptors

Estrogen Receptors

The first estrogen receptor cloned was the classical estrogen receptor α (ER α), which is encoded by the gene *Esr1*. ER α is a 66 kDa protein composed of six domains: A and B domains that contain transactivation site AF-1, a C domain which has the DNA binding domain (DBD), a D domain which includes the hinge region and the nuclear localization site (NLS), an E domain containing a ligand binding domain (LBD) and second transactivation site, AF-2 and the F domain comprising the c-terminal sequence. Exons 1–8 of *Esr1* encode these domains. There also exist splice variants of *Esr1*. Exons 2–8 encode ER α -46, while ER α -36 is encoded by Exons 2–6 and 9 of *Esr1*. *Esr2* encodes estrogen receptor β (ER β), which is a 56 kDa protein that possesses the same functional domains and shares significant sequence homology with ER α , especially in the C and E domains (Fig. 1A). Each of these estrogen receptor family members has a palmitoylation site that accounts in part for their ability to localize to cellular membranes (Soltysik and Czekaj, 2015).

In addition to estrogen receptors derived from either *Esr1* or *Esr2*, a G-protein coupled receptor was cloned in 2005. This G-protein coupled receptor was initially referred to a GPR30 but now is referred to as G-protein coupled estrogen receptor 1 (GPER1). GPER1 is a member of the rhodopsin-like receptor family and possesses seven transmembrane domains (Fig. 1B). GPER1 associates various G proteins that ultimately lead to either the stimulation or inhibition of different signal transduction pathways depending on its association with specific G proteins.

There are also other potential nonclassical mediators of estrogen's actions that have been proposed such as the putative Gq-mER (Micevych et al., 2015). These potential mediators of estrogen's actions are mainly associated with the regulatory functions of the hypothalamus. However, their molecular identities and functions have not been clearly demonstrated and therefore will not be discussed further.

Progesterone Receptors

The receptors that activate the nonclassical signaling for progesterone vary considerably. Basically, these receptors include members of (1) the classical nuclear progesterone receptor family (2) the progestin and adipoQ receptor family and (3) the membrane-associated progesterone receptor family. These receptor families are structurally unique as outlined below.

Classical nuclear progesterone receptors

The nuclear progesterone receptors have the five functional domains. There are two basic isoforms of the nuclear progesterone receptor referred to as progesterone receptor A (PR-A) and progesterone receptor B (PR-B). The PRs are encoded by the gene *Pgr*,

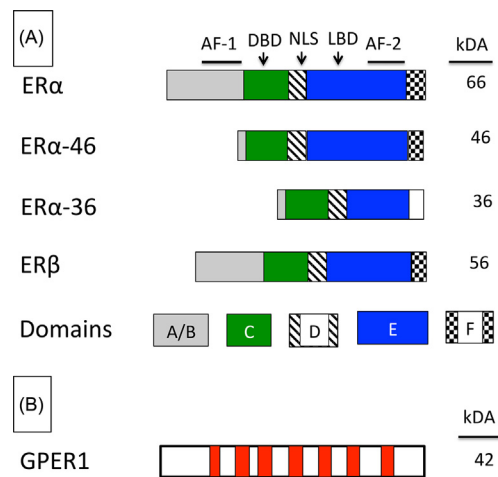


Fig. 1 The structural domains of estrogen receptor α (ER α), its splice variants (ER α -46 and ER α -36), estrogen receptor β , and G-protein coupled estrogen receptor 1 (GPER1). In panel (A), the localization of the transactivation site (AF-1), the DNA binding domain (DBD), the nuclear localization site (NLS), the ligand binding domain (LBD) and second transactivation site (AF-2) is shown in relationship to the color-coded domain. For these receptors the A/B domains are shown in *gray*, the C domain in *green*, the D domain in *white and black stripes*, E domain in *blue* and the F domain in *white and black checks*. In panel (B), the transmembrane domains of GPER1 are shown in *red*.

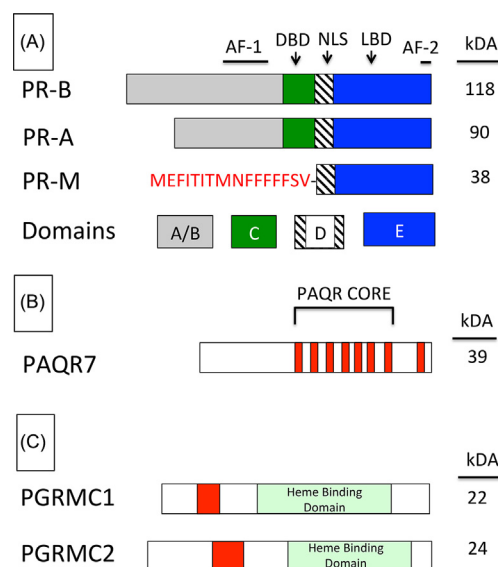


Fig. 2 The structural domains of progesterone receptors, PR-B, PR-A, and PR-M. In panel (A), the A/B domains are shown in *gray*, the C domain in *green*, the D domain in *white and black stripes*, E/F domains in *blue*. Note that in PR-M, the A, B, and C domains are replaced by a 16 amino acid sequence that allows for its mitochondrial localization. The transmembrane domains of PAQR7 are shown in *red* in panel (B). The area demarked by brackets and labeled as PAQR core also contains sequences similar to those of alkaline ceramidase. The transmembrane domain and heme binding domains of PGRMC1 and 2 are illustrated by the *red and green* areas, respectively in panel (C).

but because two different promoters are used PR-A lacks the first 164 N-terminal amino acids. Other than this, the two isoforms are identical (Fig. 2A). Interestingly, a small percentage of PR-B localizes to the plasma membrane and has the capacity of influence cellular function through membrane-initiated mechanisms (Kawprasertsri et al., 2016). The membrane-initiated action is largely mediated through a polyproline motif in the amino-terminal domain of PR-B. This polyproline motif allows PR-B to bind the SRC Homology 3 domain of Src kinase and leads to a rapid activation MAPK (mitogen-activated protein kinase) which is often referred to as ERK (extracellular signal regulated kinase) pathway (Kawprasertsri et al., 2016).

In addition there is another PR that has some structural similarities to the classic nuclear PRs. This PR is referred to as mitochondrial progesterone receptor, PR-M. PR-M is a truncated isoform of PR in which the initial 16 amino acids are encoded by a sequence located distal to the third intron followed by the sequence identical to exons 4–8 of *Pgr*. It is this N-terminal 16 amino acid sequence that is required for PR-M to localize to the outer mitochondrial membrane (Fig. 2A) (Price and Dai, 2015).

Progestin and adipoQ receptors

There are at least 11 members of the progestin and adipoQ receptor (PAQR) family. These receptors are similar to G-protein coupled receptors in that they have seven transmembrane domains. They also have similarities to the alkaline ceramidase family and may possess ceramidase activity (Moussatche and Lyons, 2012). These receptors localize to the plasma and cellular membrane where they regulate various signal transduction pathways. Although there are many members in this family, PAQR7, which is often referred to as membrane progesterone receptor α (mPR α), is more abundantly expressed in the ovary, uterus, and placenta thereby potentially contributing to progesterone-mediated responses that influence female reproduction. Its structure is illustrated in Fig. 2B. PAQR5 (a.k.a. mPR γ) is also expressed with its highest expression occurring mostly in the hypothalamus (Moussatche and Lyons, 2012).

Membrane-associated progesterone receptors

There are four members of the membrane-associated progesterone receptor (MAPR) family. These members include progesterone receptor membrane component 1, progesterone receptor membrane component 2, neudesin, and neuferricin. Each family member possesses a single transmembrane domain and the cytochrome *b5*-like heme/steroid binding domain. Moreover, these family members have numerous phosphorylation and sumoylation sites (Cahill, 2017). The first two family members cloned were progesterone receptor membrane component 1 (PGRMC1) and PGRMC2, respectively. Their structural features are illustrated in Fig. 2C. These two MAPR family members are expressed throughout the female reproductive axis and are the most extensively studied MAPR family members. As such, their mode of action will be discussed in subsequent sections of this article.

Nonclassical Steroid Receptors and Their Mechanisms of Action

Estrogen Receptors

Classical estrogen receptors and their splice variants

As previously indicated there are two classical nuclear estrogen receptors, ER α and ER β . In addition, there are several splice variations of *Esr1*. The two most frequently detected splice variants are ER α -46 and ER α -36. All four of these ERs have been detected at the plasma membrane and can potentially activate various signal transduction pathways. Precise details as to how these ER receptors mediate their membrane-initiated actions are still being investigated. However, each of these receptors contains palmitoylation sites and when palmitoylated bind to caveolin-1, which facilitates their localization to the plasma membrane. Once at the plasma membrane the ERs can interact with various growth factor receptors, G-proteins, and kinases (Fig. 3). Through these interactions, exposure to estrogen can result in the activation of different signal transduction pathway (e.g., PI3 Kinase, ERK, AKT, and Protein Kinase A) (Arnal et al., 2017). These actions have most predominately been shown for ER α and its splice variant ER α -36. Also Levin's group has shown membrane-initiated actions of ERs promote the production of nitrous oxide. It is important then to appreciate that each of these ER-regulated downstream signal transduction cascades could play essential roles in regulating the function of specific organs and tissues that comprise the female reproductive system. Whether nonclassical actions of estrogen are mediated via membrane-initiated actions of ERs is dependent on the expression profile of the various ERs, their palmitoylation status, and the level of estrogen associated with specific physiological conditions.

G-protein coupled estrogen receptor 1

Most studies indicate that ligand activation of GPER1 stimulates the ERK signaling pathway. An interesting model to explain how ligand activation of GPER1 increases ERK activity has been proposed by Gonzalez de Valdivia et al. (2017). In this model GPER1 is

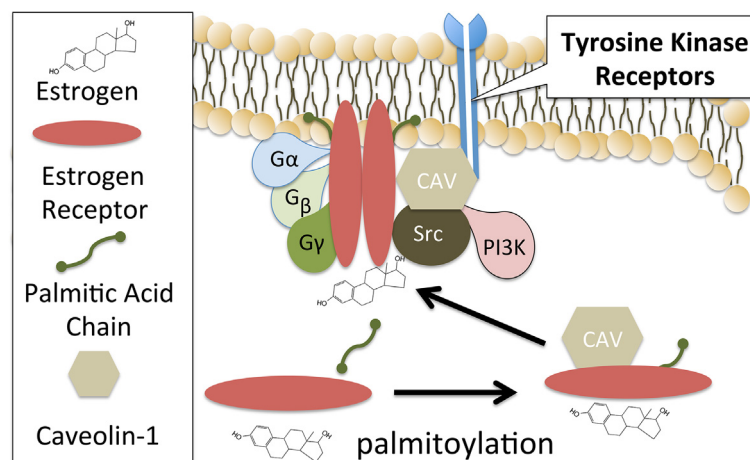


Fig. 3 Membrane-initiated mechanism regulated by classical estrogen receptors. The various components are identified within the figure.

bound to a complex composed of membrane-associated guanylate kinase, A-kinase anchor protein 5 and the regulatory II component of protein kinase A. This protein complex ultimately constitutively inhibits cAMP production. Exposure to the GPER1 agonist, G-1, disrupts this complex and thereby removes the inhibition on cAMP production and increases the coupling of Gi/o proteins to GPER1, which results in greater stimulation of the ERK signaling pathway. GPER1 is also thought to modulate intracellular free calcium levels. While this is an interesting model of GPER1's actions, it is mainly based on *in vitro* studies, often after forced expression of GPER1. Therefore, caution must be exercised regarding the GPER1's capacity to regulate these signaling pathways in such a way as to have a major influence on female reproductive function.

Progesterone Receptors

Classical progesterone receptors

The N-terminal region of PR-B possesses a polyproline domain, which binds to the Src homology domain 3 (SH3) of Src kinase. Moreover, this domain is required for ligand activation of PR-B to stimulate Src and subsequently ERK activity (Fig. 4). This is evidenced by the fact that mutating the polyproline domain eliminates the capacity of PR-B to stimulate these kinases. Interestingly, elevated levels of PR-B attenuate the function of the epidermal growth factor receptor, indicating that PR-B competes with this growth factor receptor for the binding to the SH3 domain of Src (Kawprasertsri et al., 2016). The ability of PR-B to localize to the plasma membrane may be facilitated by its undergoing palmitoylation, but the functional evidence for this is not as strong as that involving ER α .

PR-M shares structural similarities to PR-A and PR-B. PR-M appears to be a truncated form of PR with its major characteristic being that it localizes to the outer mitochondria membrane (Fig. 4). In addition genetic enhancement and ablation experiments reveal that PR-M mediates progesterone's ability to increase cellular metabolism *in vitro*. It has been suggested that PR-M may account in part for progesterone's well-known ability to increase basal body temperature. Since progesterone is cell permeable, it is likely that PR-M influences cellular energy production to meet the energy demands of women, especially during pregnancy (Price and Dai, 2015).

Progesterone and adipoQ receptor family

A detailed discussion of the potential signal transduction pathways that could be activated by each of the 11 members of this family is not within the scope of this article. Since PAQR7 expressed throughout the female reproductive axis, the effect of progesterone that are mediated via PAQR7 will be presented. One of the more defined pathways involves that activation of both the PI3 kinase/AKT and the ERK pathways, while inhibiting cAMP production (Fig. 4). Other G-protein coupled modes of PAQR7's action have been proposed and are reviewed in more detail by Valadez-Cosmes et al. (2016).

In addition to the G-protein coupled mechanisms, another signaling mechanism has been proposed based on the observation that PAQR family members share sequences consistent with alkaline ceramidases. It has been suggested that progesterone could stimulate PAQR-based alkaline ceramidase activity, which would convert ceramides into sphingolipids. Further metabolism of sphingolipid results in sphingosine-1-phosphate, which in turn could activate one of five different G-protein coupled sphingosine-1-phosphate receptors. This mode of action could lead to modulation of various G-protein dependent signaling pathway (Moussatche and Lyons, 2012).

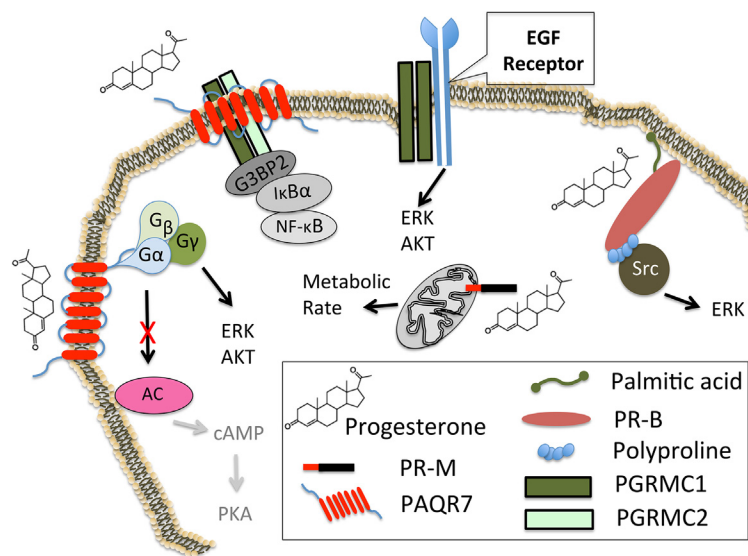


Fig. 4 Membrane-initiated mechanism regulated by the various progesterone receptors. The different components of each pathway are identified within the figure. Components connected by *black arrows* are stimulated while components in *gray* and connected by *gray arrows* are inhibited.

Membrane-associated progesterone receptor component family

Most of the studies related to mechanism through which PGRMC1 and PGRMC2 regulate female reproductive function have been largely restricted to cell lines derived from the ovary. These studies have implicated (1) the activation of protein kinase G, (2) the inhibition of ERK, and (3) the suppression T-cell-specific transcription factor/lymphoid enhancer factor activity as components of the progesterone-PGRMC1 signaling pathway that preserves cell viability (Peluso and Pru, 2014). Progesterone's ability to suppress the entry of granulosa cells into the cell cycle is mediated by PGRMC1 and PGRMC2 as treatment with siRNA directed against either MAPR family member results in an inappropriate entry into the cell cycle. This frequently results in the cells undergoing apoptosis. How progesterone acts through PGRMC1 and PGRMC2 to restrict entry into the cell cycle is not known. What is known is that PGRMC1 interacts with PGRMC2 and both MAPR family members bind GTPase-activating protein-binding protein 2 (G3BP2). G3BP2 binds nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor, which is commonly referred to as I κ B α . I κ B α binds nuclear factor κ B (NF- κ B), thereby retaining the I κ B α :NF- κ B complex within the cytoplasm (Fig. 4). Based on this, it is proposed that (1) PGRMC1 and PGRMC2 restrict entry into the cell cycle by anchoring NF- κ B to the cytoplasm and (2) progesterone intensifies these interactions making it more difficult for NF- κ B to translocate to the nucleus and up regulate genes that initiate entry into the cell cycle (Peluso et al., 2014). These observations provide the rationale for further investigation of this potential signal transduction cascade.

PGRMC1 could also affect epidermal growth factor signaling. It could do so by facilitating the plasma membrane localization of the epidermal growth factor receptor to the plasma membrane (Fig. 4) (Kabe et al., 2016). Similarly, PGRMC1 directs the ER β to the membrane (Thomas et al., 2014). In this manner PGRMC1 has the capacity to influence numerous signal transduction pathways.

Function of the Nonclassical Steroid Receptors in the Female Reproductive System

Estrogen Receptors

Classical estrogen receptors and their splice variants

The classic estrogen receptors, ER α and ER β , are expressed throughout the female reproductive axis and play essential roles in female reproduction. Functional evidence of their roles in reproduction is demonstrated by the observations that various knockout and knockin mice are infertile (Hewitt et al., 2016). Females from the different ER α mouse lines are all infertile, due to alterations ovarian and uterine function, while impaired fertility in female ER β knockout mice is largely due to the failure of the ovary to respond to gonadotropins (Hewitt et al., 2016).

Interestingly, both ER α and ER β are involved in regulating gonadotropin releasing hormone (GnRH) and in turn gonadotropin secretion (Radovick et al., 2012). The ER α is expressed by the gonadotropes within the pituitary and the kisspeptin expressing neurons of the anteroventral periventricular nucleus and arcuate nucleus within the hypothalamus. These kisspeptin expressing neurons activate GnRH neurons, which express ER β thereby providing multiple sites for estrogen's action. Moreover, the mechanism through which estrogen mediates its effects most likely includes both classical and nonclassical actions. For example, Yan et al. have shown that estrogen-dependent gene expression in the arcuate nucleus of the hypothalamus is not dependent on ER α interactions with the estrogen response element (ERE) within the promoters of specific genes. Rather it appears that ERE-independent genomic actions are due to ER α "tethering" to other DNA-associated transcription factors. This nonclassical mode of action could be important in regulating gonadotropin secretion and thereby female reproduction (Yang et al., 2017). As previously discussed, ERs can localize to the plasma membrane and in turn activate ERK pathway through an interaction with growth factors receptors. Thus, ER α and ER β likely have both classical and nonclassical modes of action, which mediate estrogen's effects at the level of both the hypothalamus and pituitary.

The potential roles for ERs derived from *Esr1* (i.e., ER α -46 and ER α -36) in regulating female fertility are difficult to discern. For example, ER α -46 localizes to cytoplasmic membrane and is expressed in numerous cell types including vascular endothelial cells and many cancers. It is also expressed in the ovary and uterus but at levels much lower than in nonreproductive tissue such as liver (Irsik et al., 2013). While its role in regulating reproduction has not been characterized, the most plausible site of action is the vascular endothelial cells within organs of the reproductive axis (Arnal et al., 2017). Unfortunately genetically modified mice are not available to examine the specific actions of ER α -36, therefore definitive conclusion regarding ER α -36's role in female reproductive function cannot be drawn at this time.

G-protein coupled estrogen receptor 1

In most mammalian species that have been examined, GPER1 is expressed in the hypothalamus specifically in the neurons expressing gonadotropin-releasing hormone (Zimmerman et al., 2016). It is also present in ovary and uterus but its level of expression is considerably less than that of ER α (Zimmerman et al., 2016). This expression pattern is suggestive of a role for GPER1 in female reproduction, but this is controversial as several different knockout mouse lines are fertile (Otto et al., 2009; Langer et al., 2010; Zimmerman et al., 2016). Based on these findings, it does not appear that GPER1 has a significant role in the regulation mammalian female reproductive function. However, some caution should be exercised in drawing this conclusion, because most fertility trials were conducted over a short period of time and GPER1 function may only be revealed as the female mice age. Support for age-related effects of GPER1 is provided by the findings of Wang et al. (2008) who showed that GPER1 is expressed in the somatic cells of the neonatal hamster ovary and GPER1 siRNA treatment suppressed estrogen-induced formation of primordial follicles. It is possible then that the GPER1 knockout mice will have fewer primordial follicles and therefore have a shorter reproductive lifespan, which would not have been detected in the previous 2-month fertility trials. This possibility merits further investigation.

It is also possible that GPER1 plays a subtle role in female reproduction. For example, female GPER1 knockout mice show estrous cycle dependent alterations in serum corticosterone levels and stress coping abilities (Kastenberger and Schwarzer, 2014). Thus, GPER1 could be involved in the regulation of age-related changes in blood pressure, vascular remodeling, heart failure (Zimmerman et al., 2016), and metabolic disorders including estrogen-regulated anorectic affects (Kwon et al., 2014).

Progesterone Receptors

Classical progesterone receptors and their splice variant

It is indisputable that the PR-A and PR-B are important regulators of female fertility given that their ablation results infertile mice. This is not surprising since these receptors are present through the reproductive axis. The ablation of PRs adversely alters the ability of progesterone to regulate uterine function, as well as ovulation and the regulation of GnRH-induced luteinizing hormone secretion (Radovick et al., 2012). Although these PR-dependent functions have a profound effect on female reproductive function, it is not entirely clear whether the classical PRs mediate their affects solely through a direct interaction with the promoters of specific genes or whether any of their actions are initiated at the plasma membrane.

Progesterone and adiponectin receptor family

As with the PRs, the PAQR family members are expressed in the hypothalamic nuclei (Petersen et al., 2013), the mammalian ovary, most notably within the corpora lutea (Peluso and Pru, 2014) and in the uterus (Peluso and Pru, 2014). Unfortunately, PAQR knockout mice have not been generated so the role that PAQR family members play in regulating female fertility remains to be conclusively demonstrated.

One potential mechanism by which PAQR7 could influence progesterone's actions in female reproductive system was revealed by in vitro studies involving PGR negative breast cancer cells lines and PGR negative spontaneously immortalized granulosa cells. In this study, forced expression of *Pgrmc1* increased the amount of PAQR7 that localized to the plasma membrane with a corresponding increase in the capacity of the cells to bind progesterone and activate G proteins. Conversely, forced expression of *Paqr7* increased (1) the amount of PAQR7 and PGRMC1 that localized to the plasma membrane, (2) the interaction between PAQR7 and PGRMC1, and (3) progesterone's ability to inhibit apoptosis. Importantly, progesterone's antiapoptotic actions were attenuated by treatment with *Paqr7* siRNA (Thomas et al., 2014). Therefore, this study suggests that a functional interaction between these two nonclassical progesterone receptors exists, but this remains to be demonstrated in vivo.

Membrane-associated progesterone receptor component family

To investigate the role that PGRMC1 and PGRMC2 play in regulating female fertility, various conditional knockout mouse lines have been generated (McCallum et al., 2016; Clark et al., 2017). The effect of depleting these MAPRs was a decline in fertility as judged by a decrease in litter size, with the decline in litter size generally increasing maternal age. The most dramatic decline in litter size was observed in mice in which the *Pgr-cre* driver was used to ablate *Pgrmc1* and *Pgrmc2* (Clark et al., 2017). In this mouse line, the conditional *Pgrmc1/2* knockout mice ovulated and became pregnant but the birth of pups was rarely observed after 6 months of age compare to controls whose fertility was not affected at 6 months of age. The major cause of the reproductive wastage was associated with implantation site reabsorption due to faulty decidualization. A much more limited decline in female fertility was observed when an antimüllerian hormone receptor type 2-*cre* driver was used to ablate *Pgrmc1* with reproductive loss associated with uterine defects, although some impairment of follicle growth was also observed (Peluso and Pru, 2014). Finally, a role for progesterone-PGRMC1 signaling in regulating the formation of primordial ovarian follicles has been proposed (Guo et al., 2016). It is likely that disrupting PGRMC1 expression during primordial follicle formation shortens the reproductive lifespan, since lower levels of PGRMC1 are observed in some women who have undergone the premature loss of the primordial follicle population and premature menopause (i.e., premature ovarian insufficiency) (Mansouri et al., 2008).

Summary and Future Directions

In this article each of the nonclassical estrogen and progesterone receptors was presented as a separate entity. However, the responses to estrogen and/or progesterone are dictated by both the level of expression of each classical and nonclassical steroid receptor as well as the steroid levels to which the cell is exposed. One thing that is striking about the membrane-initiated actions of both estrogen and progesterone is that they often regulate the same downstream signal transduction pathways, most notably the PI3K/AKT and the ERK pathways. The physiological rationale for this commonality between estrogen and progesterone actions is unknown. It may actually reflect our tendency to monitor a few well-established markers of biological function and our failure to examine responses in a more global sense. In this light the use of more discovery approaches such as kinase activity screening might provide a more complete picture of the components of the membrane-initiated mode of action.

Another perplexing issue that limits our understanding of how estrogen and progesterone modulate biological responses is that the nonclassical steroid actions, which localize to the plasma membrane, often lead to the transcription of specific genes. Thus, differentiating these genomic responses from those triggered by direct nuclear steroid receptor: DNA interaction is difficult. Resolving this issue will be necessary in order to truly understand the complete and complex mechanism through which each steroid acts.

What is essential then is that these variables be considered in both designing and, more importantly, interpreting the outcomes of experiments. This is especially true for studies involving genetically modified mice as depleting one gene may either enhance the

expression of genes and/or induce redundant signal pathways to compensate for the loss of the targeted gene. These realities should be incorporated into our thinking in order to deepen our understanding of how a specific cell will respond to estrogen and/or progesterone under different physiological conditions.

In conclusion over the last 20 years, the concept of nonclassical membrane-initiated actions of both estrogen and progesterone has been established and is no longer thought of as an experimental artifact. Importantly, the receptors that mediate these steroid actions have been identified and with their identification genetic approaches and in vitro systems used to demonstrate that these receptors account for specific aspects of estrogen and progesterone signaling. More recently the generation of genetically modified mice has been used to conclusively illustrate the physiological relevance of some of these nonclassical steroid receptors. The major void now is in defining components of the signal transduction pathways that are activated in response to the membrane-initiated actions of each steroid and to different these components from those associated the direct nuclear steroid receptor: DNA interactions. This is not a trivial task and will likely require the application of discovery analytical tools such as RNAseq and proteomic analysis coupled with intensive bioinformatic analyses. This approach alone however will not be sufficient in that genetic approaches will be required to dissect each newly proposed component of these putative pathways. Hopefully over the course of the next 20 years, these details of steroid signaling will be elucidated.

References

- Arnal, J. F., Lenfant, F., et al. (2017). Membrane and nuclear estrogen receptor alpha actions: From tissue specificity to medical implications. *Physiological Reviews*, 97(3), 1045–1087.
- Cahill, M. A. (2017). The evolutionary appearance of signaling motifs in PGRMC1. *Bioscience Trends*, 11(2), 179–192.
- Clark, N. C., Pru, C. A., et al. (2017). Conditional ablation of progesterone receptor membrane component 2 causes female premature reproductive senescence. *Endocrinology*, 158(3), 640–651.
- Gonzalez de Valdivia, E., Broselid, S., et al. (2017). G protein-coupled Estrogen Receptor 1 (GPER1)/GPR30 Increases ERK1/2 Activity Through PDZ-dependent and -independent Mechanisms. *The Journal of Biological Chemistry*, 292, 9932–9943.
- Guo, M., Zhang, C., et al. (2016). Progesterone receptor membrane component 1 mediates progesterone-induced suppression of oocyte meiotic prophase I and primordial folliculogenesis. *Scientific Reports*, 6, 36869.
- Hewitt, S. C., Winuthayanon, W., et al. (2016). What's new in estrogen receptor action in the female reproductive tract. *Journal of Molecular Endocrinology*, 56(2), R55–71.
- Irsik, D. L., Carmines, P. K., et al. (2013). Classical estrogen receptors and ERalpha splice variants in the mouse. *PLoS One*, 8(8), e70926.
- Kabe, Y., Nakane, T., et al. (2016). Haem-dependent dimerization of PGRMC1/Sigma-2 receptor facilitates cancer proliferation and chemoresistance. *Nature Communications*, 7, 11030.
- Kastenberger, I., & Schwarzer, C. (2014). GPER1 (GPR30) knockout mice display reduced anxiety and altered stress response in a sex and paradigm dependent manner. *Hormones and Behavior*, 66(4), 628–636.
- Kawprasertsri, S., Pietras, R. J., et al. (2016). Progesterone receptor (PR) polyproline domain (PPD) mediates inhibition of epidermal growth factor receptor (EGFR) signaling in non-small cell lung cancer cells. *Cancer Letters*, 374(2), 279–291.
- Kwon, O., Kang, E. S., et al. (2014). GPR30 mediates anorectic estrogen-induced STAT3 signaling in the hypothalamus. *Metabolism*, 63, 1455.
- Langer, G., Bader, B., et al. (2010). A critical review of fundamental controversies in the field of GPR30 research. *Steroids*, 75(8–9), 603–610.
- Mansouri, M. R., Schuster, J., et al. (2008). Alterations in the expression, structure and function of progesterone receptor membrane component-1 (PGRMC1) in premature ovarian failure. *Human Molecular Genetics*, 17(23), 3776–3783.
- McCallum, M. L., Pru, C. A., et al. (2016). Conditional ablation of progesterone receptor membrane component 1 results in subfertility in the female and development of endometrial cysts. *Endocrinology*, 157(9), 3309–3319.
- Micevych, P. E., Wong, A. M., et al. (2015). Estradiol membrane-initiated signaling and female reproduction. *Comprehensive Physiology*, 5(3), 1211–1222.
- Moussatche, P., & Lyons, T. J. (2012). Non-genomic progesterone signalling and its non-canonical receptor. *Biochemical Society Transactions*, 40(1), 200–204.
- Norman, A. W., & Wehling, M. (1999). Overview of the first international meeting on rapid responses to steroid hormones. *Steroids*, 64(1–2), 3–4.
- Otto, C., Fuchs, I., et al. (2009). GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biology of Reproduction*, 80(1), 34–41.
- Peluso, J. J., & Pru, J. K. (2014). Non-canonical progesterone signaling in granulosa cell function. *Reproduction*, 147(5), R169–178.
- Peluso, J. J., Griffin, D., et al. (2014). Progesterone receptor membrane component-1 (PGRMC1) and PGRMC-2 interact to suppress entry into the cell cycle in spontaneously immortalized rat granulosa cells. *Biology of Reproduction*, 91(5), 104.
- Petersen, S. L., Intlekofer, K. A., et al. (2013). Nonclassical progesterone signalling molecules in the nervous system. *Journal of Neuroendocrinology*, 25(11), 991–1001.
- Price, T. M., & Dai, Q. (2015). The role of a mitochondrial progesterone receptor (PR-M) in progesterone action. *Seminars in Reproductive Medicine*, 33(3), 185–194.
- Radovick, S., Levine, J. E., et al. (2012). Estrogenic regulation of the GnRH neuron. *Frontiers in Endocrinology*, 3, 52.
- Soltysik, K., & Czekaj, P. (2015). ERalpha36—Another piece of the estrogen puzzle. *European Journal of Cell Biology*, 94(12), 611–625.
- Thomas, P., Pang, Y., et al. (2014). Enhancement of cell surface expression and receptor functions of membrane progesterin receptor alpha (mPRalpha) by progesterone receptor membrane component 1 (PGRMC1): Evidence for a role of PGRMC1 as an adaptor protein for steroid receptors. *Endocrinology*, 155(3), 1107–1119.
- Valadez-Cosmes, P., Vazquez-Martinez, E. R., et al. (2016). Membrane progesterone receptors in reproduction and cancer. *Molecular and Cellular Endocrinology*, 434, 166–175.
- Wang, C., Prossnitz, E. R., et al. (2008). G protein-coupled receptor 30 expression is required for estrogen stimulation of primordial follicle formation in the hamster ovary. *Endocrinology*, 149(9), 4452–4461.
- Yang, J. A., Stires, H., et al. (2017). The arcuate estrogen-regulated transcriptome: Estrogen response element-dependent and -independent signaling of eralpha in female mice. *Endocrinology*, 158(3), 612–626.
- Zimmerman, M. A., Budish, R. A., et al. (2016). GPER-novel membrane oestrogen receptor. *Clinical Science*, 130(12), 1005–1016.

Ovarian Production of Estradiol: The Two-Cell, Two-Gonadotropin Model

Joanne E Fortune, Cornell University, Ithaca, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Female reproductive cycles are regulated by endocrine signals between the hypothalamus-pituitary and the ovary. Mammalian ovaries synthesize all three classes of sex steroid hormones: progestins, androgens, and estrogens. The estrogenic steroid estradiol is the most important endocrine signal produced by the ovary during the follicular phase of female reproductive cycles. A consideration of circulating levels of reproductive hormones during estrous and menstrual cycles of mammalian species reveals a consistent dramatic increase in circulating estradiol. The rise in estradiol peaks just prior to and induces the preovulatory surge of gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), that triggers ovulation. The developing preovulatory follicle(s) is responsible for the large and rapid increase in circulating estradiol prior to the LH/FSH surge. During the follicular phase, circulating estradiol also has a number of other targets in addition to the hypothalamus-anterior pituitary. It promotes changes in the reproductive tract (vagina, cervix, uterus, and oviducts) and the mammary glands that prepare the female for gamete transport and fertilization and for potential pregnancy and lactation. In addition, estradiol exerts paracrine and autocrine effects within developing preovulatory follicles that promote their development.

Despite the central importance of follicular estradiol production to female reproduction, the cellular site and regulation of estradiol synthesis within developing preovulatory follicles were difficult to determine. One reason was the lack of sufficiently purified gonadotropins before the 1970's. The gonadotropic hormones, LH and FSH, synthesized and secreted by the anterior pituitary gland, are glycoproteins with unique beta subunits, but identical alpha subunits. This structural similarity made it difficult to obtain completely (or even mostly) pure preparations of either hormone by isolating them from pituitary glands. The availability of purer preparations of LH and FSH, together with the development of methods for separating and culturing the two follicular endocrine cell types, theca and granulosa cells, were advances important for studying follicular steroid production. In addition, the development of radioimmunoassays (RIAs) provided much more sensitive methods for measuring hormones and was critical for accurate measurement of the small amounts of steroids secreted by follicular cells *in vitro*. These advances allowed the two-cell, two-gonadotropin hypothesis of follicular estradiol production to be tested more directly than in previous experiments. There is now experimental evidence that the two-cell, two-gonadotropin model describes the regulation of estradiol biosynthesis by preovulatory follicles in a number of mammalian species.

Pathways of Steroidogenesis

As mentioned above, sex steroids are members of one of three general classes: progestins, androgens, and estrogens. The steroids within each class share structural and functional similarities. Cholesterol is the precursor of all steroid hormones (Fig. 1). Within the biosynthetic pathway of sex steroids, cholesterol is converted to the progestin pregnenolone via the P450 side-chain cleavage enzyme (CYP11A1). Progestins serve as precursors for androgenic steroids and androgens in turn are precursors for estrogen synthesis (Fig. 1). Thus, steroid hormones play roles both as precursors for the synthesis of other steroids and as endocrine factors that exert actions by binding to receptors. Both ovaries and testes synthesize all three classes of sex steroids, but the relative amounts produced and the steroids that are used for endocrine signaling to other parts of the body differ in males and females. In mammalian females, estradiol is the dominant circulating sex steroid during the follicular phase of the reproductive cycle, but it is obvious from Fig. 1 that synthesis of progestins and androgens is necessary for the production of estradiol. In contrast, progesterone made by

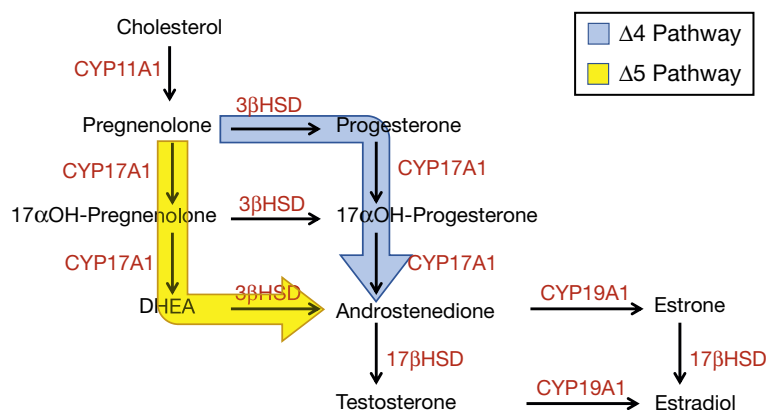


Fig. 1 The pathway of ovarian sex steroid biosynthesis. Enzymes that catalyze each conversion are indicated in red. Androgens can be synthesized via the delta 4 ($\Delta 4$, blue arrow) and/or delta 5 ($\Delta 5$, yellow arrow) pathways.

the corpus luteum is the dominant circulating sex steroid during the luteal phase of the cycle, although estradiol is also elevated in some species (e.g., rodents, primates).

Estradiol can be synthesized via two different pathways (Fig. 1). In the delta 5 pathway, the progestin pregnenolone is converted to 17 α -hydroxypregnenolone, which in turn is converted to the androgen dehydroepiandrosterone (DHEA) via 17 α -hydroxylase (CYP17A1). In the delta 4 pathway, pregnenolone is converted by 3 β -hydroxysteroid dehydrogenase (3 β HSD) to progesterone, which is then converted to androgens. Androgens are converted to the estrogens estrone and estradiol by the enzyme P450 aromatase (CYP19A1). In mammalian ovaries, estradiol is made in much greater quantities than estrone and it is also a much more potent estrogen (i.e., has greater activity on a molar basis).

Ovarian Follicular Synthesis of Estradiol: The Two-Cell, Two-Gonadotropin Model

By the 1970's there was evidence that both LH and FSH and both follicular cell types play essential roles in the synthesis of estradiol, but their relative roles were not clear (the early literature is reviewed in [Gore-Langton and Armstrong, 1994](#)). It was believed that both LH and FSH are important for follicular estradiol synthesis because both were required to restore ovarian development and estradiol secretion in hypophysectomized animals, but their relative roles were not understood. Ovarian follicles contain an oocyte and two types of endocrine-active follicular cells, granulosa and theca cells (Fig. 2). As antral follicles develop, the granulosa cells differentiate as either mural granulosa cells, surrounding the follicular antrum or cumulus granulosa cells, which surround the oocyte and interact with it in specific ways. Because no capillaries penetrate the basement membrane that separates the theca and granulosa cell compartments of the follicle, the granulosa cells and the cumulus-oocyte complex are in an avascular environment, with the follicular fluid providing their "microenvironment." Several layers of theca interna cells lie just outside the basement membrane and the thecal compartment is vascularized (Fig. 2). The two follicular cell types are thus physically separated, but they can communicate biochemically since molecules, even large ones such as LH and FSH, can pass through the basement membrane.

Experiments by Falck in the late 1950's provided compelling indirect evidence that both theca and granulosa cells are required for estradiol production ([Falck, 1959](#)). The relative roles of the two follicular endocrine cell types began to be understood in the 1970's. Before that, many reproductive biologists, including Falck, hypothesized that the theca interna cells of follicles were the major site of estradiol synthesis. They reasoned that, since the theca cell layer is vascularized whereas the inner granulosa compartment is not, it would be logical for the theca cells to be the site of estradiol synthesis. However, there was also some evidence for granulosa cells as the site of estradiol synthesis (reviewed by [Gore-Langton and Armstrong, 1994](#)).

Elucidation of the roles of the follicular endocrine cells and of the two gonadotropins was made possible in the 1970's by the availability of highly purified preparations of LH and FSH and much more sensitive methods for measuring hormones (radioimmunoassays) and by the development of methods for isolating and culturing theca and granulosa cells. One hypothesis, based partly on studies of steroidogenesis by testicular Sertoli and Leydig cells of immature rats, was that the theca cells synthesize androgens under the influence of LH and that granulosa cells cannot synthesize androgens, but can convert androgens to estradiol under the influence of FSH. This two-cell, two-gonadotropin hypothesis was first comprehensively tested with theca and granulosa cells from rat preovulatory follicles. Because of the controversy in this area, it was not clear for some time whether the results for rats applied to other mammalian species or not. The following sections will summarize findings for several mammalian species.

Rat Preovulatory Follicles

The first complete test of the two-cell, two-gonadotropin hypothesis in one species and with a single experimental model was with rats. Prepubertal rats were injected with equine chorionic gonadotropin (eCG, also known as PMSG) to initiate an estrous cycle. The

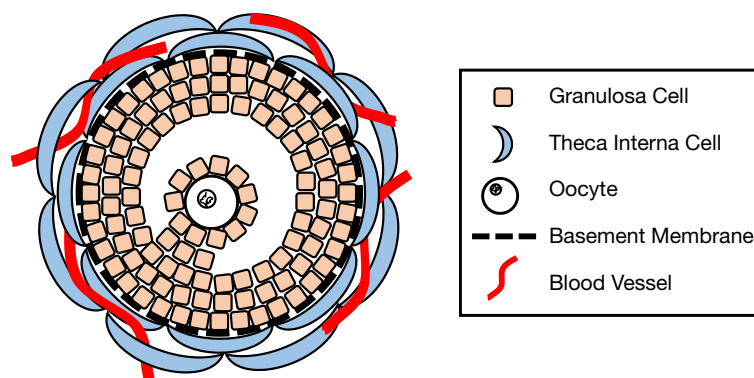


Fig. 2 Schematic diagram of a cross section through a mammalian preovulatory follicle, showing the oocyte and surrounding cumulus cells, follicular antrum, granulosa cells, theca cells, and thecal blood vessels. The size of preovulatory follicles varies greatly in different mammalian species (from ~0.5 to ~45 mm in diameter).

dose of eCG used was not superovulatory; in this model, a normal number of preovulatory follicles develops and an endogenous LH/FSH surge occurs and triggers ovulation. The developing preovulatory follicles were dissected from the ovaries on the morning of proestrus, well before the expected time of the LH/FSH surge (late on the afternoon of proestrus), and theca and granulosa cells were separated by scraping the inner granulosa cells from the basement membrane (Fig. 2). The granulosa cells are loosely connected to each other by gap junctions and were dispersed by gentle pipetting and then cultured as monolayers. The thecal layers, with cells that adhere tightly to each other, were maintained in vitro as organ cultures. Theca and granulosa cultures were maintained for 3 days, without or with highly purified preparations of LH and/or FSH.

Androgen accumulated in the medium of thecal cultures and LH increased androgen production by theca cells (Fortune and Armstrong, 1977). FSH had no effect on thecal androgen production and the effects of LH + FSH were not different from LH alone. In contrast, there were only negligible amounts of androgen in cultures of granulosa cells in the presence or absence of gonadotropins. These results confirmed earlier reports of the theca as the source of follicular androgens and established that the theca cells are a target of LH, but not FSH, action. Treatment of thecal cultures with exogenous progesterone alone did not increase androgen production and this suggested that LH increases thecal androgen synthesis by promoting the conversion of progestins to androgens.

In control medium, both theca and granulosa cells produced only very low amounts of estradiol and neither gonadotropin affected estradiol accumulation; production of estradiol by thecal cultures was greater than by granulosa cells (Fortune and Armstrong, 1978). However, the results were completely different when testosterone was added to the culture medium as a substrate for estradiol synthesis. In the presence of the androgen precursor, secretion of estradiol by granulosa cells was many-fold higher than in its absence. Estradiol was highest during the first 12 h of culture and progressively lower amounts were produced with time in culture. However, addition of FSH or FSH + LH to the medium maintained estradiol production throughout the culture period; LH alone had no effect.

Addition of testosterone to thecal cultures increased estradiol production, but gonadotropins had no further effect. In the presence of testosterone + FSH, granulosa cells produced about 8- to 9-fold more estradiol per follicle than preparations of theca from the same follicles. These results might be interpreted to mean that granulosa cells, using androgens provided by the theca cells, are the primary, but not the sole source of the estradiol produced by rat proestrous follicles. However, it is extremely difficult to remove all granulosa cells from the basement membrane by dissection and histological analysis of the thecal preparations revealed the presence of some granulosa cells attached to the theca (i.e., close to a source of androgen precursor). When theca prepared with special care to try to remove all granulosa cells ("clean" theca) was compared with preparations where the theca was scraped lightly to remove most granulosa cells ("crude" theca), estradiol production by "clean" theca was 7- to 30-fold lower than "crude" theca. These experiments provided evidence that the granulosa cells are likely the sole source of follicular estradiol in rat preovulatory follicles. Taken together, the results support the two-cell, two-gonadotropin model of follicular estradiol synthesis (Fig. 3).

Bovine Preovulatory Follicles

The experiments described above provided strong evidence for the two-cell, two-gonadotropin model in rats, but there were previous reports in the literature of thecal production of estradiol in other species and it was not clear to what extent the model might apply to other mammalian species. Hence, the two-cell, two-gonadotropin model was not uniformly embraced by reproductive biologists. Cattle were the next species to be examined extensively via experiments that allowed direct comparison to the studies in rodents. Cattle have much larger preovulatory follicles than rats (~15 mm vs. ~1 mm diameter) and a much longer estrous cycle (average about 21 days vs. 4 or 5 days for rats) and they usually ovulate only one follicle versus 10–12. The bovine follicular phase, initiated by the regression of the corpus luteum and a decline in circulating progesterone, is 2–4 days long and estrus occurs at around the time of the peak of the gonadotropin surge; ovulation follows about 29 h later.

Theca and granulosa cells interact to promote estradiol production

Because the length of an individual bovine estrous cycle is not readily predictable, preovulatory follicles at a stage similar to those in the experiments with rats were obtained by daily measurements of progesterone and removal of the preovulatory follicle about 24 h after progesterone first decreased to <1 ng/mL, which is indicative of luteolysis. The preovulatory follicle was dissected from the ovary and the granulosa cells were separated from the theca interna by scraping them from the basement membrane. As with

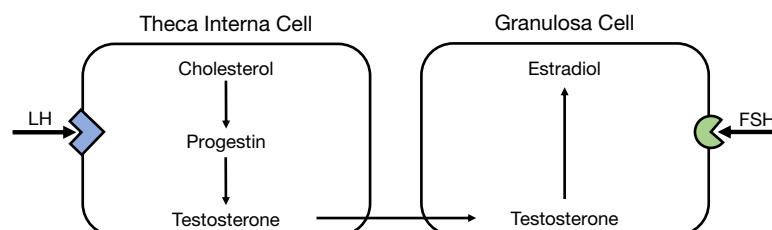


Fig. 3 The two-cell, two-gonadotropin model of estradiol synthesis in rat proestrous follicles. LH binds to receptors on theca cells to stimulate them to synthesize androgens. Granulosa cells cannot convert progestins to androgens, but convert exogenous androgen to estradiol via the enzyme aromatase, which is induced and maintained by FSH.

the studies on rat proestrous follicles, pieces of theca interna were maintained in organ culture and granulosa cells were dispersed and grown in monolayer cultures. The large size of bovine preovulatory follicles allowed all treatments to be applied to thecal and granulosa cultures from each follicle. Theca cells secreted androgen into the medium, primarily in the form of androstenedione, and LH, but not FSH, stimulated its production. Accumulation of estradiol in the medium of thecal cultures was negligible. Granulosa cells did not secrete androgens, even in the presence of exogenous progesterin precursor, but readily converted exogenous testosterone to estradiol (reviewed in [Fortune and Quirk, 1988](#)).

Importance of gonadotropin concentration

In initial experiments with bovine follicular cells in vitro, neither FSH nor LH stimulated the aromatization of testosterone to estradiol by granulosa cells and the effects of LH on thecal androgen production were exerted primarily during the first day of culture. Later dose-response experiments revealed that low doses of LH (2–4 ng/mL) or FSH (1–2 ng/mL) stimulated androstenedione or estradiol (in medium with testosterone) production by theca or granulosa cells, respectively, over the course of 3-day cultures ([Berndtson et al., 1995](#)). In contrast, higher doses of the gonadotropins did not stimulate (androstenedione) or inhibited (estradiol) accumulation of those steroids, but higher doses stimulated progesterone secretion by both cell types and initiated oxytocin secretion by granulosa cells ([Berndtson et al., 1995](#)). These experiments both confirmed the two-cell, two-gonadotropin model for bovine preovulatory follicles and underscored the importance of the concentration of gonadotropin used in vitro. A narrow range of low doses of LH and FSH promoted a follicular-phase profile of steroid production (androgen and estradiol), whereas higher doses luteinized the cells in culture. This makes physiological sense, because follicular cells are exposed to low circulating concentrations of LH and FSH during the follicular phase of the cycle in vivo and they increase secretion of estradiol into the blood under those conditions. In contrast, the LH/FSH surge rapidly decreases follicular androgen and estradiol secretion in vivo and promotes production of the luteal phase steroid progesterone (and in cattle and sheep, the luteal peptide hormone oxytocin).

Theca and granulosa cells interact to promote thecal androgen production

In the course of experiments with both rat proestrous follicles and bovine preovulatory follicles, preparations of follicle wall (theca with granulosa cells attached) secreted more androgen than isolated theca. These results suggested the hypothesis that granulosa cells increase the capacity of theca cells to synthesize androgens by providing them with progesterin precursor. When bovine thecal cultures were treated with graded doses of pregnenolone or progesterone, pregnenolone was much more effective than progesterone at increasing androstenedione production (reviewed in [Fortune and Quirk, 1988](#)). This suggests that bovine theca cells use the delta 5 pathway to synthesize androgens ([Fig. 1](#)). Although secretion of pregnenolone by bovine granulosa or theca cells, cultured without or with gonadotropins, is low, adding estradiol to the culture medium increased pregnenolone in the culture medium 11-fold (granulosa) or 3-fold (theca) and decreased progesterone secretion by both cell types (reviewed in [Fortune and Quirk, 1988](#)). The concentrations of estradiol that produced maximal effects were similar to concentrations that granulosa and theca cells of preovulatory follicles are exposed to in vivo (i.e., concentrations in the follicular fluid; [Fortune and Hansel, 1985](#)).

These results, summarized in [Fig. 4](#), indicate that interactions between the two follicular cell types to promote estradiol production go beyond the theca providing the granulosa cells with androgen precursor. The results suggest that the granulosa cells provide progesterin precursor in the form of pregnenolone to the theca cells to enhance their production of androstenedione. As estradiol production increases during follicular development, its concentration in the follicular fluid increases to levels that inhibit the delta 4 pathway in both cell types, thus further driving androgen production via the delta 5 pathway ([Fig. 4](#)). This is a positive feedback loop in which follicular estradiol increases its own production. The LH/FSH surge breaks this loop by decreasing androgen and

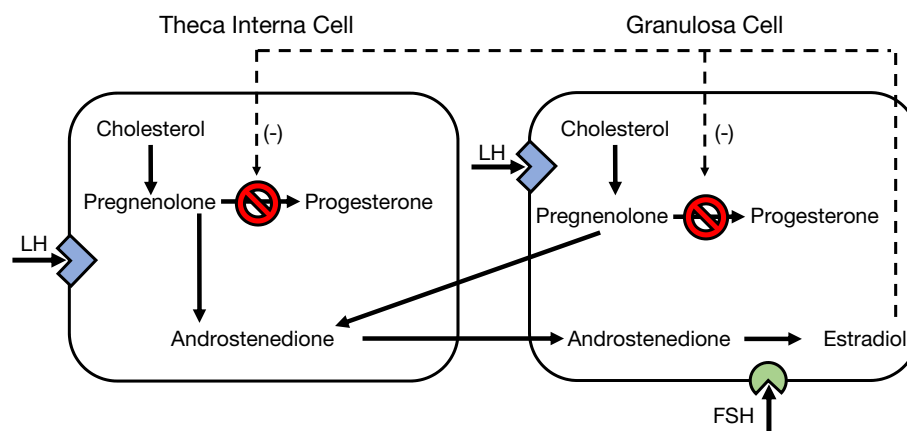


Fig. 4 Interactions between theca and granulosa cells of bovine preovulatory follicles that promote estradiol production. In addition to the interactions shown in [Fig. 3](#), experimental evidence suggests that granulosa cells interact with theca cells to increase their synthesis of androstenedione by providing them with progesterin precursor in the form of pregnenolone and that high intrafollicular concentrations of estradiol inhibit the $\Delta 4$ pathway, promoting androgen synthesis by conversion of pregnenolone via the $\Delta 5$ pathway.

estradiol synthesis and increasing progesterone production by the periovulatory follicle and the surge induces other changes that culminate in ovulation of the oocyte and luteinization of the remaining follicular cells.

Porcine Preovulatory Follicles

In porcine follicles, the FSH receptor (FSHR) and its mRNA are localized exclusively to the granulosa cell layer. During the later stages of growth of preovulatory follicles, but shortly before the gonadotropin surge, FSHR and its mRNA decrease and granulosa cells begin to acquire LH receptors. In contrast, theca cells express LH receptors throughout follicular development, but never have FSH receptors. In granulosa cells, FSH signaling increases aromatase activity, allowing the cells to synthesize estrogens from androgen precursors. *CYP19A1* mRNA, aromatase immunoreactivity, and/or estradiol secretion were detected in theca cells in some studies, albeit at lower levels than in granulosa cells, but were not detected in other studies. Theca cells express mRNAs for enzymes necessary for progestin and androgen synthesis, whereas those mRNAs have little or no expression in porcine granulosa cells. Taken together, these findings suggest that developing porcine preovulatory follicles produce large quantities of estradiol via interactions between the theca and granulosa cells in which the theca cells are stimulated by LH to produce androgens that are converted to estradiol by granulosa cells under the influence of FSH. It is not clear whether porcine theca cells also contribute to follicular estradiol production to a limited extent or not. This area of research was reviewed recently by [LaVoie \(2017\)](#).

Equine Preovulatory Follicles

Horses differ from other domestic species and from rodents and primates in their long period of estrus (4–6 days) at the end of the follicular phase. In addition, the preovulatory follicle is much larger than in other species (40–45 mm in diameter) and ovulation occurs only at a specific region of the ovary, the ovulation fossa. Initial studies on the relative roles of equine theca and granulosa cells led to a model which held that granulosa cells produce progesterone, whereas theca cells produce estradiol, perhaps using progesterone from the granulosa cells as precursor. In vitro studies in the early 1990's, similar to those that supported the two-cell, two-gonadotropin model in rats and cattle, led to a different conclusion about the source of follicular estradiol ([Sirois et al., 1991](#)). Theca and granulosa cells were isolated from preovulatory follicles in early estrus (day 1–2 of estrus) and cultured for 3 days. Theca cells secreted primarily androstenedione and its production was increased by LH, but not by FSH. In contrast, theca cells secreted only negligible quantities of progesterone and estradiol. Granulosa cells had very limited to no ability to produce androstenedione and this is consistent with their secretion of only very negligible amounts of estradiol in control medium. However, when testosterone was added to the medium, granulosa cells secreted about 170-fold more estradiol over 3 days of culture, but neither gonadotropin affected estradiol production. Granulosa cells secreted progesterone and its production was stimulated by both gonadotropins, especially during the second and third days of culture.

The results of the studies in vitro support the model of cooperation between theca and granulosa cells in the production of estradiol, with the theca cells producing androgen under the influence of LH and granulosa cells aromatizing androgen to estradiol. These conclusions are supported by later molecular studies that localized *CYP17A1* to the theca cells and *CYP19A1* to the granulosa cells of equine preovulatory follicles ([Watson et al., 2004](#)). The role of the gonadotropins in aromatization by equine granulosa cells is less clear. Dose-response studies might clarify this issue, based on the results for cattle where only low doses of FSH increased the conversion of testosterone to estradiol ([Berndtson et al., 1995](#)).

Primate Preovulatory Follicles

Investigations of the relative roles of theca and granulosa cells and of LH and FSH in the production of estradiol by preovulatory human and nonhuman primate follicles have yielded results that support a two-cell, two-gonadotropin model (reviewed by [Zeleznik and Benyo, 1994](#); [Strauss and Williams, 2014](#)). Primate theca cells synthesize and secrete aromatizable androgens, primarily androstenedione and LH, but not FSH, stimulates androgen synthesis and secretion. Granulosa cells do not make androgens, but can aromatize exogenous androgens to estradiol under the influence of FSH.

Regulation of Steroidogenesis at Earlier Stages of Follicular Development

Preovulatory follicles are by far the most active in producing estradiol, but it is interesting that what is known about steroidogenesis by earlier follicular stages is also consistent with the two-cell, two-gonadotropin model. LH and FSH receptors have been detected on theca and granulosa cells, respectively, from early preantral stages onwards. Mouse ovaries began to secrete sex steroids and to respond to LH and FSH in vitro as early as 7 days of age, when only primordial, primary, and early secondary follicles are present ([Fortune and Eppig, 1979](#)). In those studies, LH stimulated progesterone and androgen production, whereas FSH stimulated the conversion of exogenous androgen to estradiol.

Fetal ovaries produce steroids, even before follicles have formed and the level of ovarian steroid production varies over the course of gestation. In cattle, fetal ovarian production of estradiol and progesterone is higher before follicles start to form than during follicle formation. The results of in vitro studies on the regulation of fetal ovarian steroid production in ovaries obtained just before the initiation of follicle formation suggest that two different steroidogenic cell types are involved, with LH promoting

androgen production and FSH responsible for increasing aromatization (Allen et al., 2016). Exactly which fetal ovarian cells are involved remains to be determined. Taking all these results together suggests that the two-cell, two-gonadotropin model is an inherent mechanism that allows mammalian ovaries to produce estradiol at various stages of ovarian and follicular development.

Summary and Additional Considerations

The literature reviewed above for various mammalian species supports a two-cell, two-gonadotropin model that describes an interaction between the two follicular endocrine cell types, responding to different gonadotropins, that enables the dramatic increase in estradiol to levels high enough to elicit the LH/FSH surge that triggers ovulation. All the evidence thus far points to this model as the mechanism for preovulatory estradiol production in mammals and probably for follicular synthesis of estradiol at all stages. However, there may be modulations of the basic model that are species-specific.

It seems clear that only theca cells can synthesize androgens from progestins. Some species (cattle, sheep, primates) use predominantly the delta-5 pathway to produce thecal androgen, whereas others use the delta 4 pathway (guinea pigs) or are able to use both pathways (rats, pigs) (reviewed by Conley and Bird, 1997). That LH stimulates androgen synthesis is also clear; with the use of reasonably pure preparations of gonadotropins, there have been no suggestions that theca cells are a target for FSH. There have been reports for some species that theca cells can synthesize estradiol. However, theca cells can appear to produce estradiol if the preparations contain contaminating granulosa cells, which can readily convert the androgens produced by the adjacent theca to estradiol. In addition, theca cells may contain estradiol that has diffused in from the granulosa cell compartment in vivo and this may diffuse out of the cells in the early hours of culture. When immunohistochemistry is used, the antibodies may cross-react with similar steroidogenic enzymes and so results are not definitive unless such cross-reactivity is ruled out. These considerations can make it difficult to evaluate reports of thecal estradiol production. It is possible that the theca produces a low level of estradiol in some species (e.g., pigs), but that level would be many-fold less than the levels produced by granulosa cells.

It seems clear that granulosa cells do not produce androgens (although small amounts may diffuse from the cells during the early hours of culture), but do convert androgens to estradiol and that FSH induces the aromatase enzyme (CYP19A1) that converts androgens supplied by the theca cells to estradiol. As follicular development proceeds and follicles approach the time of the LH/FSH surge, granulosa cells also acquire LH receptors. It is not clear if these receptors are acquired solely in preparation for granulosa cells to respond to the LH surge or if they enable granulosa cells to convert androgens to estradiol in response to both LH and FSH, thereby increasing follicular capacity to produce estradiol. The latter possibility is frequently assumed, but in at least one species (cattle) granulosa cells with LH receptors did not aromatize androgen in response to LH (Berndtson et al., 1995). Furthermore, in species where the corpus luteum produces estradiol, an increase in aromatization in response to LH can be due to luteinization of the granulosa cells in vitro. As discussed above, the dose of LH or FSH used in vitro is critically important. Thus, whether the LH receptors acquired late in follicular development by granulosa cells directly increase aromatization is still uncertain and there may be as yet unappreciated species differences.

The production of a high, and continuously increasing, level of estradiol is, arguably, the most important endocrine function of developing preovulatory follicles. This article has reviewed the basis for the current model of estradiol production via interactions between the two endocrine cell types, responding to different gonadotropins. However, there is extensive evidence that a number of other hormones and growth factors, particularly intra-follicular factors, impinge on this basic model to modulate steroid biosynthesis (e.g., steroids, activin, inhibin, and various growth factors). Consideration of these factors is beyond the scope of this article and readers are referred to reviews that consider this topic (Hillier, 2001; Macklon et al., 2006; Strauss and Williams, 2014).

Acknowledgments

The author is grateful to Dr. Jeremy J. Allen for preparing the figures and providing feedback on the manuscript.

References

- Allen, J. J., Herrick, S. L., & Fortune, J. E. (2016). Regulation of steroidogenesis in fetal bovine ovaries: Differential effects of LH and FSH. *Journal of Molecular Endocrinology*, 57(4), 275–286.
- Berndtson, A. K., Vincent, S. E., & Fortune, J. E. (1995). Low and high concentrations of gonadotropins differentially regulate hormone production by theca interna and granulosa cells from bovine preovulatory follicles. *Biology of Reproduction*, 52(6), 1334–1342.
- Conley, A. J., & Bird, I. M. (1997). The role of cytochrome P450 17 α -hydroxylase and 3 β -hydroxysteroid dehydrogenase in the integration of gonadal and adrenal steroidogenesis via the Δ 5 and Δ 4 pathways of steroidogenesis in mammals. *Biology of Reproduction*, 56, 789–799.
- Falck, B. (1959). Site of production of oestrogen in rat ovary as studied in micro-transplants. *Acta Physiologica Scandinavica*, 47(Suppl. 163), 1–101.
- Fortune, J. E., & Armstrong, D. T. (1977). Androgen production by theca and granulosa isolated from proestrous rat follicles. *Endocrinology*, 100, 1341–1347.
- Fortune, J. E., & Armstrong, D. T. (1978). Hormonal control of estradiol-17 β biosynthesis in proestrous rat follicles: Estradiol production by isolated theca versus granulosa. *Endocrinology*, 102, 227–235.
- Fortune, J. E., & Eppig, J. J. (1979). Effects of gonadotropins on steroid secretion by infantile and juvenile mouse ovaries in vitro. *Endocrinology*, 105, 760–768.
- Fortune, J. E., & Hansel, W. (1985). Concentrations of steroids and gonadotropins in follicular fluid from normal heifers and heifers primed for superovulation. *Biology of Reproduction*, 32, 1069–1079.
- Fortune, J. E., & Quirk, S. M. (1988). Regulation of steroidogenesis in bovine preovulatory follicles. *Journal of Animal Science*, 66(Suppl. 2), 1–8.

- Gore-Langton, R. E., & Armstrong, D. T. (1994). Follicular steroidogenesis and its control. In E. Knobil, & J. D. Neill (Eds.) *The physiology of reproduction* (2nd edn., vol. 1, pp. 571–627). New York: Raven Press.
- Hillier, S. G. (2001). Gonadotropic control of ovarian follicular growth and development. *Molecular and Cellular Endocrinology*, 179, 39–46.
- LaVoie, H. A. (2017). Transcriptional control of genes mediating ovarian follicular growth, differentiation, and steroidogenesis in pigs. *Molecular Reproduction and Development*, 84(9), 788–801.
- Macklon, N. S., Stouffer, R. L., Giudice, L. C., & Fauser, B. C. (2006). The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocrine Reviews*, 27, 170–207.
- Sirois, J., Kimmich, T. L., & Fortune, J. E. (1991). Steroidogenesis by equine preovulatory follicles: Relative roles of theca interna and granulosa cells. *Endocrinology*, 128, 1159–1166.
- Strauss, J. F., III, & Williams, C. J. (2014). The ovarian life cycle. In J. F. Strauss, III, & R. L. Barbieri (Eds.), *Yen and Jaffe's reproductive endocrinology* (7th edn., pp. 157–191). Philadelphia, PA: Elsevier Inc.
- Watson, E. D., Bae, S.-E., Steele, M., et al. (2004). Expression of messenger ribonucleic acid encoding for steroidogenic acute regulatory protein and enzymes, and luteinizing hormone receptor during the spring transitional season in equine follicles. *Domestic Animal Endocrinology*, 26(3), 215–230.
- Zelevnik, A. J., & Benyo, D. F. (1994). Control of follicular development, corpus luteum function, and the recognition of pregnancy in higher primates. In E. Knobil, & J. D. Neill (Eds.) *The physiology of reproduction* (2nd edn., vol. 2, pp. 751–782). New York: Raven Press.

Hormone Effects on Follicular Growth and Differentiation

So-Youn Kim, Yi Luan, and Teresa K Woodruff, Northwestern University, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

The Hypothalamic–Pituitary–Gonadal Axis

When the hypothalamic neurons produce the neuropeptide *gonadotropin-releasing hormone* (GnRH), GnRH is released into the portal vessels in pulses (Maffucci and Gore, 2009). The biological regulation of GnRH secretion is controlled by the pulsatile mode and keeps continuing throughout the reproductive lifespan in female. The pulse of GnRH occurs every 1–2 h in primate, while it does at intervals of about 30 min in rat (Maffucci et al., 2008). The GnRH from GnRH neurons is synthesized as a prehormone in the anterior hypothalamus and secreted as a 10-amino acid peptide hormone. The pulsatile pattern of decapeptide GnRH induces secretion of the gonadotropin hormones—*follicle-stimulating hormone* (FSH) and *luteinizing hormone* (LH)—from gonadotropes in the anterior pituitary, also in a pulsatile fashion. FSH and LH are comprised of a unique β -subunit and a common α -subunit (Thompson and Kaiser, 2014). In the ovary, FSH stimulates early follicle growth while LH causes ovulation of the dominant or Graafian follicles. They also stimulate the ovarian follicles to secrete the sex steroid hormones—*androgens*, *estrogens*, and *progesterones*—as well as the peptide hormones *activin*, *inhibin*, and *folliculin*. In addition to acting locally on the ovary, these hormones feed back to modulate the secretion of FSH and LH through a positive/negative feedback system, forming the hypothalamic–pituitary–gonadal (HPG) axis. This homeostatic feedback system is critical for maintaining the regular fluctuations in serum levels of FSH and LH and ovarian production of steroid and peptide hormones during the follicular and luteal phases of the menstrual cycle.

Hormones at Puberty

In the fetus and newborn babies, the levels of FSH and LH are high, but then drop quickly and remain low until puberty (Kuri-Hanninen et al., 2014). The amplitude of LH pulses, stimulated by the pulsatile release of GnRH from the hypothalamus, changes from hour to hour prior to puberty; however, FSH and LH do not reach sufficient levels to initiate gonadal function. At early puberty, blood levels of FSH and LH begin to rise at night and pulses increase in amplitude and frequency. Primordial follicles are selected from the ovarian reserve to enter folliculogenesis, and the estrogens from these growing follicles acts as an agonist to the increasing gonadotropin secretion before menarche. As puberty progresses, GnRH induces sufficient release of FSH and LH from the pituitary to trigger the first ovulatory surge. When the uterus synchronizes to the hypothalamic–pituitary–ovarian axis hormonal cycles, the endometrium builds (follicular phase) and is shed (luteal phase) with each cycle (Fig. 1).

Hormone Production and Action Through the Stages of Folliculogenesis

The female fetal ovary contains 6–7 million germ cells contained within primordial follicles (Findlay et al., 2015). Germ cells enter meiosis at 7–15 weeks of gestation and maintain meiotic arrest until puberty. Most of the primordial follicles in the fetal ovary are lost before birth, leaving only 1 million distributed into each ovary at birth. In humans, the total number of primordial follicles at birth represents the ovarian reserve—a measure of reproductive capacity and endocrine health. Here, we introduce the critical bioactive steroid and peptide hormones involved in the selection of follicles from the reserve to resume meiosis and grow, as well as the processes of folliculogenesis and ovulation. Primordial follicles contain a single immature oocyte covered in a layer of squamous-shaped pregranulosa cells. Primordial follicles exist in a dormant status and do not produce any steroid hormones. The phosphoinositide 3-kinase (PI3K) pathway plays a central role in the activation of quiescent primordial follicles and initiation of folliculogenesis (Zheng et al., 2012). PTEN and FOXO3 inhibit PI3K, which ensures dormancy of primordial follicles. Internal and external signals inhibit PTEN and FOXO3, releasing the block and allowing primordial follicles to develop into primary follicles. The squamous pregranulosa cells develop into cuboidal-shaped granulosa cells during this transition.

The activated follicle progresses through the primary and secondary (preantral) stages, which are characterized by oocyte growth, granulosa cell proliferation, and acquisition of theca cells (Zhang and Liu, 2015). Early growth of follicles relies on various autocrine and paracrine factors between the oocyte and the somatic cell compartments of the follicle and is not dependent on pituitary gonadotropin signals. The granulosa cells of primary follicles start to make *activins* and *inhibins*, express proliferation markers, and form multiple cell layers around the oocyte. Stromal cells are recruited to form the outer theca cell layer and produce secondary follicles. In each human menstrual cycle, one secondary follicle is selected in response to FSH and continues to grow and mature, both in terms of hormone production and egg maturation. As the granulosa cells proliferate, greater amounts of estrogens are produced and released. The granulosa cells also further differentiate into cumulus cells that surround the oocyte and mural cells that surround a fluid-filled antrum. Mural granulosa cells are important for steroidogenesis and ovulation, whereas cumulus granulosa cells

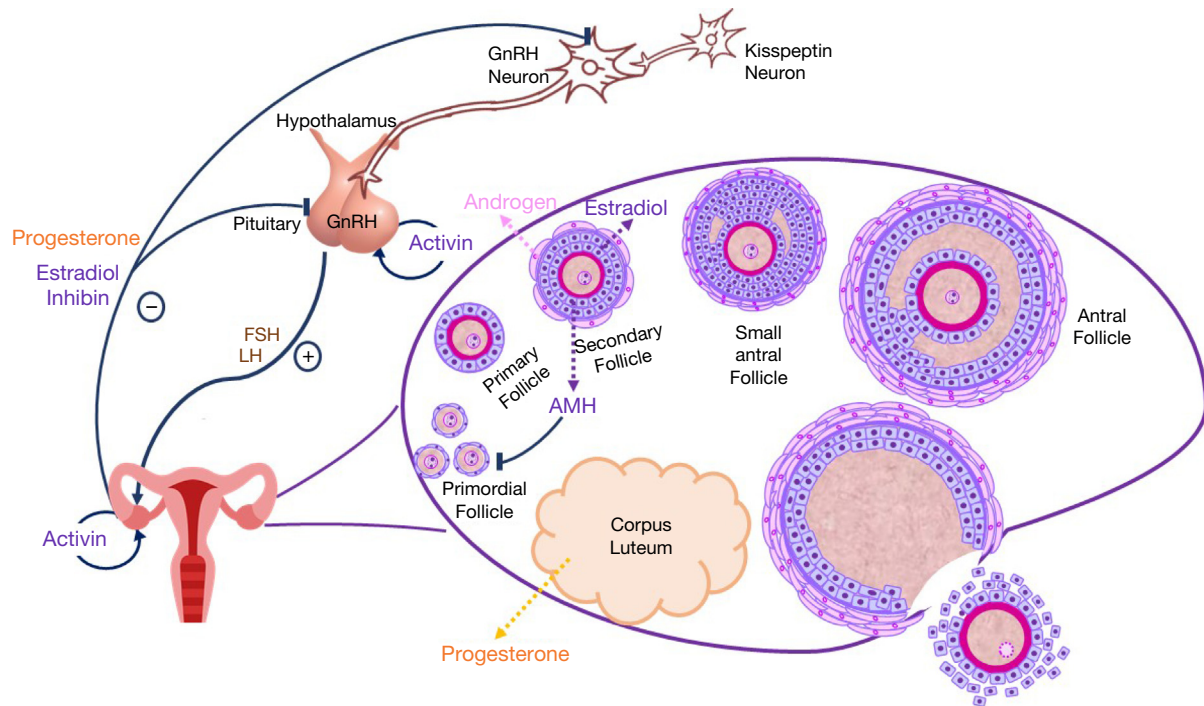


Fig. 1 Secretion of hormones during folliculogenesis and their effects on HPG axis. GnRH neurons excited by Kisspeptin neurons secrete GnRH. The GnRH stimulates FSH and LH secretion to activate folliculogenesis in ovaries. While primordial follicles are covered with a layer of pregranulosa cells before being stimulated, those are surrounded by a layer or multilayers of granulosa cells after being waken up. In the ovaries, immature primordial follicles are awakened by activation factors and grow until becoming antral follicles. As follicles grow, estradiol, AMH, activins, and inhibins are secreted. The AMH from growing follicles inhibits the activation of primordial follicles. Estradiol and inhibins from growing follicles and progesterone from corpus lutea have an effect of negative feedback on pituitary and GnRH neurons. The negative feedback signaling suppresses the excitatory signals from hypothalamus–pituitary to ovaries and shifts follicular to luteal phase, resulting in successful menstrual cycle.

support the growth and maturation of the oocyte. During antral follicle growth, FSH is critical for granulosa cell proliferation, estradiol production, and LH receptor expression (Richards, 1994).

Sex Steroids in Folliculogenesis

Estradiol is produced by granulosa cells of growing follicles in the ovary, starting at the late preantral stage; up to this point, production of estradiol is limited by insufficient androgen substrate for aromatization. Theca cells express key steroidogenic enzymes such as CYP11A1, HSD3B1, and CYP17A1, while granulosa cells contain CYP19A1. Once the theca cell layer forms, aromatizable androgen and its estradiol precursor, androstenedione, are produced in theca cells and transferred to granulosa cells for conversion to estradiol. As late preantral follicles continue to grow through the antral stages, the level of estradiol increases with proliferation of granulosa cells (Edson et al., 2009). Estradiol from granulosa cells plays a positive feedback role in the HPG axis, stimulating production of FSH in the pituitary that promotes further follicle growth and production of steroid hormones in the ovary. Estradiol may also have intrafollicular actions: administration of estradiol to hypophysectomized rats stimulated the proliferation of granulosa cells in small preantral follicles and reduced atresia. Studies indicate that a combination of FSH and estradiol or androgen aromatized to estradiol in vitro can stimulate granulosa cell proliferation. The estrogen receptor, ER β , is expressed in granulosa cells of growing follicles, while ER α is expressed in thecal and interstitial cells (Britt and Findlay, 2003). Based on the expression patterns of these receptors, ER β in granulosa cells is likely involved in ER-stimulated granulosa cell proliferation and follicle growth, decreasing follicle atresia, and increasing the number of oocytes released following ovulation induction.

During puberty, androgen blood levels increase in females and induce body changes during sexual maturation, mainly acting on the adrenal glands and ovaries. Androstenedione that is converted from dehydroepiandrosterone converts to testosterone, and testosterone converts to 5 α -dihydrotestosterone (DHT) (Lebbe and Woodruff, 2013). Among these androgens, testosterone and DHT are the only two hormones that bind and activate the androgen receptor (AR), which is localized in granulosa cells; stromal cells; theca cells in humans; and oocytes in rats, mice, and pigs. It has been shown that androgens induce follicle activation through AR in the granulosa cells in primates, suggesting that androgens, like estradiol through ER β , also boost the effect of FSH on growing

follicles (Weil et al., 1998). Testosterone stimulates the transition of primary to secondary follicles in a dose-dependent manner in the fetal bovine ovary, which is inhibited by the AR antagonist flutamide. A similar effect of androgens has also been reported in ovaries from testosterone-treated transsexual women.

Peptide Hormones in Folliculogenesis

In addition to sex steroid hormones, the ovary produces many peptides that work as autocrine, paracrine, or endocrine factors. Among these peptides, *activins*, *inhibins*, and *follicle-stimulating hormone* are produced by the granulosa cells. *Activins* are glycoprotein dimers composed of two subunits, βA and βB , which form activin A ($\beta A\beta A$), B ($\beta B\beta B$), or AB ($\beta A\beta B$) (Thompson et al., 2004). As autocrine and paracrine factors produced by granulosa cells, the activins are important in the development of early-stage follicles (Pangas and Woodruff, 2000). Activin A produced by granulosa cells of primary and secondary follicles and cumulus cells of antral follicles locally enhances FSH action on granulosa cells. In vitro assays have shown that activin and FSH signaling synergize to stimulate follicle growth and secretion of estradiol and inhibin. Activins from granulosa cells also inhibit androstenedione production by suppressing excess androgen biosynthesis in preantral and small antral follicles through modulation of LH signals (Makanji et al., 2014; Woodruff et al., 1988). Activin is also synthesized in the pituitary where it plays a role in regulating FSH production.

Inhibins are also glycoprotein dimers composed of a unique α -subunit and either the activin βA -subunit (inhibin A) or βB -subunit (inhibin B). During the human menstrual cycle, inhibin B is dominantly expressed in the early follicular phase, decreases in the midfollicular phase, and is undetectable after the LH surge in the luteal phase. Inhibin A is very low during the early follicular phase, increases gradually in the midfollicular phase, and peaks during the luteal phase (Woodruff and Mayo, 1990). As endocrine factors, ovarian inhibins play a very important feedback role of selectively inhibiting expression of the FSH β -subunit in the pituitary, thereby limiting FSH production.

Follicle-stimulating hormone is a peptide hormone that is mainly produced in the pituitary and the ovary and binds and neutralizes activin. In the pituitary, follicle-stimulating hormone plays an inhibitory role by reducing activin-stimulated FSH production (Michel et al., 1993).

Folliculogenesis is also negatively regulated by *anti-Müllerian hormone* (AMH), also known as Müllerian-inhibiting substance (Teixeira et al., 2001). AMH is a peptide growth factor belonging to the TGF- β family. It was discovered as the hormone produced by fetal testis at the time of Müllerian duct regression during development of male reproductive tissues. The ovaries of homozygous AMH-null female mice were found to contain no primordial follicles and a greater number of growing preantral follicles compared to wild-type mice (Durlinger et al., 1999). It was subsequently found that AMH is mainly secreted by granulosa cells of preantral follicles in mice and antral follicles in humans. AMHR2, an AMH type II receptor, is expressed in granulosa cells of primary and growing follicles. In both mice and humans, AMH negatively regulates initial follicular recruitment from the quiescent pool by exerting an inhibitory effect on the transition from primordial to primary follicles. AMH can be detected in serum, and because of its expression pattern in growing preantral follicles, it has become a measurement of the ovarian reserve and ovarian function. Because the levels of AMH in serum positively correlate with the size of the primordial follicle pool and AMH levels decline with age and ovarian dysfunction, AMH has been suggested as a clinical marker for predicting the age of menopause, assessing the ovarian reserve before assisted reproduction procedures, and predicting gonadal function after cancer treatment. AMH is a more sensitive marker for ovarian reserve compared to FSH, which has been used in clinical care as a standard biomarker of reproductive capacity.

Hormones at and After Ovulation

As the dominant antral follicle reaches its final stage, serum estradiol reaches its peak and secretion of pituitary FSH is suppressed. Pituitary LH production increases, eventually resulting in an LH surge that induces ovulation of the oocyte from the dominant antral follicle. As oocytes do not have LH receptors, cyclic AMP in granulosa cells is transported into oocytes through gap junctions when the LH surge occurs. The ovulated oocyte resumes meiosis while the cumulus granulosa cells left behind expand and terminally differentiate to form the corpus luteum (CL). The CL becomes a very important endocrine organ, as CL cells (10–20 mm) secrete high levels of progesterone and moderate amounts of estradiol. Progesterone from the CL acts on the lining of the uterus, increasing its thickness in preparation for implantation of the fertilized egg, and thus is critical to sustain early pregnancy. The size of the CL correlates with the production of serum progesterone during early pregnancy. The CL produces progesterone until the placenta begins to produce progesterone around 10 weeks gestation in human. If implantation does not occur, the CL undergoes atresia and progesterone production stops; in response, the lining of the uterus gradually stops thickening and menstruation begins.

Progesterone receptor (PR) is localized in theca cells of small antral follicles and granulosa cells of preovulatory follicles that are exposed to LH/human chorionic gonadotropin, or granulosa cells undergoing luteinization, in primate and rabbit ovaries (Hild-Petito et al., 1988). In the porcine and rat ovary, LH may induce PR mRNA expression in granulosa cells, and short-form PR-A is more abundant than the long-form PR-B. It has been shown that progesterone can enhance the activity of steroidogenic enzymes in granulosa cells and progesterone production by these cells (Fanjul et al., 1983). In rat granulosa cells, progesterone may enhance the response to FSH by increasing cAMP and inhibiting FSH-stimulated estradiol production.

References

- Britt, K. L., & Findlay, J. K. (2003). Regulation of the phenotype of ovarian somatic cells by estrogen. *Molecular and Cellular Endocrinology*, 202, 11–17.
- Durlinger, A. L., Kramer, P., Karels, B., de Jong, F. H., Uilenbroek, J. T., Grootegoed, J. A., & Themmen, A. P. (1999). Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. *Endocrinology*, 140(12), 5789–5796.
- Edson, M. A., Nagaraja, A. K., & Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine Reviews*, 30, 624–712.
- Fanjul, L. F., Ruiz de Galarreta, C. M., & Hsueh, A. J. (1983). Progesterin augmentation of gonadotropin-stimulated progesterone production by cultured rat granulosa cells. *Endocrinology*, 112(1), 405–407.
- Findlay, J. K., Hutt, K. J., Hickey, M., & Anderson, R. A. (2015). How is the number of primordial follicles in the ovarian reserve established? *Biology of Reproduction*, 93, 111.
- Hild-Petito, S., Stouffer, R. L., & Brenner, R. M. (1988). Immunocytochemical localisation of oestradiol and progesterone receptors in the monkey ovary throughout the menstrual cycle. *Endocrinology*, 123, 2896–2905.
- Kuiri-Hanninen, T., Sankilampi, U., & Dunkel, L. (2014). Activation of the hypothalamic-pituitary-gonadal axis in infancy: Minipuberty. *Hormone Research in Paediatrics*, 82, 73–80.
- Lebbe, M., & Woodruff, T. K. (2013). Involvement of androgens in ovarian health and disease. *Molecular Human Reproduction*, 19, 828–837.
- Maffucci, J. A., & Gore, A. C. (2009). Chapter 2: Hypothalamic neural systems controlling the female reproductive life cycle gonadotropin-releasing hormone, glutamate, and GABA. *International Review of Cell and Molecular Biology*, 274, 69–127.
- Maffucci, J. A., Walker, D. M., Ikegami, A., Woller, M. J., & Gore, A. C. (2008). NMDA receptor subunit NR2b: Effects on LH release and GnRH gene expression in young and middle-aged female rats, with modulation by estradiol. *Neuroendocrinology*, 87, 129–141.
- Makanji, Y., Zhu, J., Mishra, R., et al. (2014). Inhibin at 90: From discovery to clinical application, a historical review. *Endocrine Reviews*, 35, 747–794.
- Michel, U., Farnworth, P., & Findlay, J. K. (1993). Follistatins: More than follicle-stimulating hormone suppressing proteins. *Molecular and Cellular Endocrinology*, 91, 1–11.
- Teixeira, J., Maheswaran, S., & Donahoe, P. K. (2001). Müllerian inhibiting substance: An instructive developmental hormone with diagnostic and possible therapeutic applications. *Endocrine Reviews*, 22, 657–674.
- Pangas, S. A., & Woodruff, T. K. (2000). Activin signal transduction pathways. *Trends in Endocrinology and Metabolism*, 11, 309–314.
- Richards, J. S. (1994). Hormonal control of gene expression in the ovary. *Endocrine Reviews*, 15, 725–751.
- Thompson, T. B., Cook, R. W., Chapman, S. C., Jardtzy, T. S., & Woodruff, T. K. (2004). Beta A versus beta B: Is it merely a matter of expression? *Molecular and Cellular Endocrinology*, 225, 9–17.
- Thompson, L. R., & Kaiser, U. B. (2014). GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression. *Molecular and Cellular Endocrinology*, 385, 28–35.
- Weil, S. J., Vendola, K., Zhou, J., et al. (1998). Androgen Receptor Gene Expression in the primate ovary: Cellular localization, regulation, and functional correlations. *The Journal of Clinical Endocrinology and Metabolism*, 83, 2479–2485.
- Woodruff, T. K., D'Agostino, J., Schwartz, N. B., & Mayo, K. E. (1988). Dynamic changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle. *Science*, 239, 1296–1299.
- Woodruff, T. K., & Mayo, K. E. (1990). Regulation of Inhibin synthesis in the rat ovary. *Annual Review of Physiology*, 52, 807–821.
- Zhang, H., & Liu, K. (2015). Cellular and Molecular Regulation of the activation of mammalian primordial follicles: Somatic cells initiate follicle activation in adulthood. *Human Reproduction Update*, 21, 779–786.
- Zheng, W., Nagaraju, G., Liu, Z., & Liu, K. (2012). Functional roles of the phosphatidylinositol 3-kinases (PI3Ks) signaling in the mammalian ovary. *Molecular and Cellular Endocrinology*, 356, 24–30.

Estrogens

Istvan Merchenthaler, University of Maryland, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cytochrome P450 A superfamily of > 220 genes that are grouped into 36 gene families that encode heme-based enzymes. Three of the enzymes that are required for estrogen biosynthesis (cholesterol side chain cleavage enzyme, 17-hydroxylase, and aromatase) are members of this superfamily.

Estrogen Female sex hormone (although also present in males) with steroid hormone structure.

Estrogen receptor-alpha and beta; GPR30, and ER-X *ER α* and *ER β* are about 66-kDa nuclear proteins that bind to estrogens with high affinities. The gene structures of *ER α* and *ER β* are similar, including N- and C-terminal activational domains, a hinge region, a nuclear binding domain, and a ligand binding domain. The nuclear ERs are ligand-regulated transcription factors. GPR30 is a membrane ER activated by 17 β -estradiol and it is involved in mediating fast estrogen responses via an interaction of second messenger systems. ER-X is also a membrane-associated ER activated primarily by 17 α -estradiol but it also binds 17 β -estradiol.

Estrogen response element A palindromic nucleotide sequence that is specifically recognized and bound by the DNA-binding domain of estrogen receptors. The consensus estrogen response element is GGTCAnnnTGACC.

Sex hormone binding globulin A glycoprotein found in the blood that binds some androgens and estrogens with relatively high affinities. This protein contributes to the regulation of the amount of free estrogen present in plasma.

Historical Background

The name “estrogen” comes from the observations more than 100 years ago when it was found that ovariectomy (castration of females) resulted in a number of changes in the reproductive tract and the ovaries transplanted back into ovariectomized animals ameliorated these effects. These observations indicated that the ovary produced a substance that acted in an endocrine manner, that is, it secreted the substance into the systemic circulation by which it reached the responsive organs/tissues. This conclusion was supported by the observations that reproductive organs, such as the vagina and uterus, underwent cyclical changes in a fashion similar to the ovaries. Because the substance produced by the ovaries induced estrus, it was referred to as estrogen (estrogen). The laboratories of Doisy and Butenandt (Doisy, 1972) crystallized a low-molecular-weight lipophilic steroidal substance from the urine of pregnant women which they originally termed “theelin” and later renamed it estrone. Many years of effort were dedicated to identify the mechanism by which estrogens stimulated the growth in reproductive tract tissues. A major discovery occurred in 1957 when Jensen reported that tritium-labeled estradiol was concentrated in uterine cells and induced growth without itself undergoing chemical changes. The subsequent demonstration that increasing amounts of the antiestrogen nafoxidine could inhibit uterine uptake of labeled estradiol as well as estrogen-stimulated cell growth corroborated that steroid binding to a bona fide receptor is required for estrogen action. The classic estrogen receptor (ER) (now called *ER α*) was discovered in 1985/1986 (Walter et al., 1985; Green et al., 1986) and the second ER (now called *ER β*) was discovered in 1996 (Kuiper et al., 1996).

Like other members of the superfamily, *ER α* and *ER β* are nuclear, ligand-activated transcription factors, although *ER α* is also associated with the cell membrane (*mER α*) (Levin, 2005; Zivadinovic and Watson, 2005).

In addition to the nuclear *ER α* and *ER β* , 17 β -estradiol also binds to membrane-associated ERs that are different from the nuclear *ER α* and *ER β* . Among these, the G-protein associated ER (GP-ER or GPR30) localizes to the endoplasmic reticulum and induces adenylate cyclase (AC), extracellular signal-related kinase (ERK), protein kinase A (PKA), protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), and epidermal growth factor receptor (EGFR) signaling. GP-ER or GPR30 plays a role in the rapid non-genomic signaling events widely observed following stimulation of cells and tissues with estradiol (Prossnitz et al., 2007).

Another membrane-associated ER is called ER-X that is bound and activated by 17 α -estradiol and 17 β -estradiol. It shows sequence homology with *ER α* and *ER β* and activates the MAPK/ERK pathways (Toran-Allerand et al., 2002). The receptor is insensitive to the antiestrogen ICI-182,780 (fulvestrant).

Structural Features

Estrogens are steroid hormones. The estrane steroid nucleus, which is common to all estrogens and distinct from other classes of steroids such as androgens and progestins, contains 18-carbon atoms arranged into four rings as shown in Fig. 1A. The aromatic A ring with the phenolic hydroxyl group located at the C3 position and an oxygen atom in the form of a hydroxyl or ketone group at the C17 position are critical for the hormonal activity of all estrogens (see below).

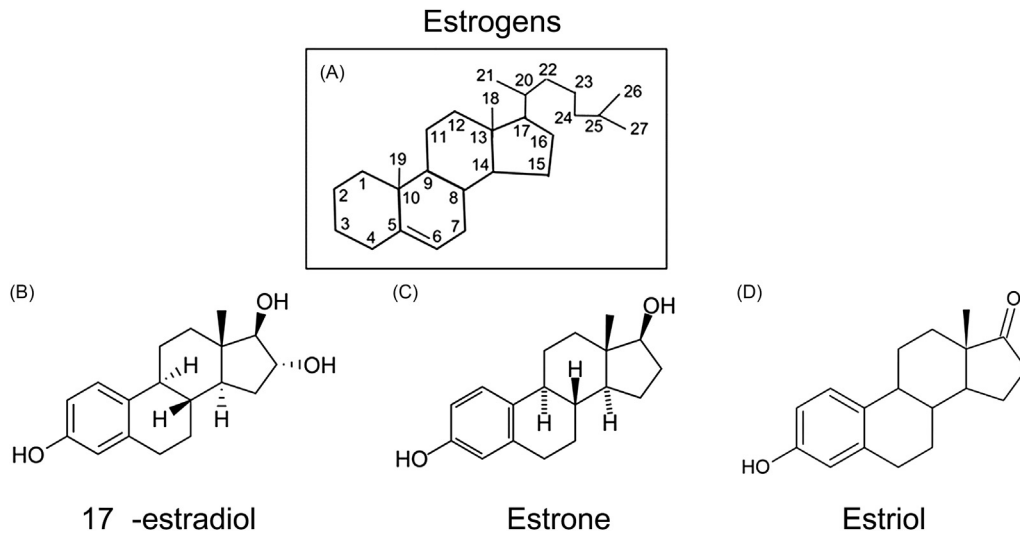


Fig. 1 The steroid structure and estrogens. The estrane steroid nucleus, which is common to all estrogens and distinct from other classes of steroids such as androgens and progestins, contains 18-carbon atoms arranged into four rings. The aromatic A ring with the phenolic hydroxyl group located at the C3 position and an oxygen atom in the form of a hydroxyl or ketone group at the C17 position are critical for the hormonal activity of all estrogens (see below). (B) 17β-estradiol; note the aromatic (aliphatic) A ring, a –OH group at C3 and C17 positions. (C) estrone: note the lack of –OH at C17 position. (D) estriol: note the keto group at C20 position.

The most potent, naturally occurring estrogen is 17β-estradiol, which has 3-hydroxyl and 17β-hydroxyl groups (**Fig. 1B**). In humans, there are two other major forms of estrogen in the circulation, estrone (**Fig. 1C**) and estriol (**Fig. 1D**). In contrast to 17β-estradiol, estrone has a ketone group at the C17 position instead of a hydroxyl, and estriol differs from 17β-estradiol by the addition of a 16α-hydroxyl group. The biological activity of estrogens is determined in part by their ability to bind to and activate the estrogen receptors. 17β-Estradiol is the most active as an estrogen; estrone is about one-third as active as 17β-estradiol and estriol is about one-sixteenth as active as 17β-estradiol (Estradiol > Estrone > Estriol).

Biosynthesis of Estrogens

The vast majority of estrogens are synthesized by the ovarian follicles (**Fig. 2A,B**). In the cortex, just below the tunica albuginea of the ovary, the primordial follicles first appear in the third month of fetal development. Then, during each menstrual cycle, a dozen or two of these cells grow but only one or two completes fill maturation and becomes Graafian follicle. The growth and maturation

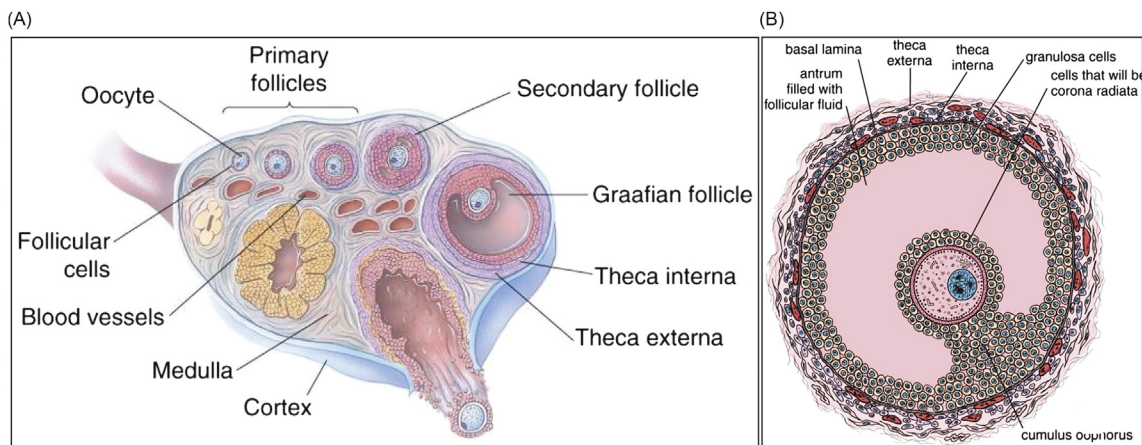


Fig. 2 The ovary. (A) The ovary and folliculogenesis. (B) The mature Graafian follicle. The granulosa cells around the egg (corona radiata) and around the antrum secrete estrogen and the theca cells around the granulosa cells secrete testosterone which diffuses to the granulosa cells. The granulosa cells convert testosterone to 17β-estradiol via the P450arom and release it into the general circulation. From Ross MH, Pawlina W (2006) Histology: A Text and Atlas (5th edn.). Lipincott Williams & Wilkins.

of these follicles is stimulated by gonadotropins from the anterior pituitary. During this process, the single layer of follicle cells gives rise to a stratified epithelium, the stratum granulosum. The granulosa cells are surrounded by theca cells (interna and externa) of stromal origin. In humans, luteinizing hormone (LH) stimulates the cells of the theca interna to secrete androgens, which serve as estrogen precursors. In response to follicle-stimulating hormone (FSH) from the anterior pituitary, the granulosa cells catalyze the conversion of androgens to estrogens, which in turn stimulate the granulosa cells to proliferate resulting in increased estrogen levels until the mid-cycle ovulation. After ovulation, the Graafian follicle becomes corpus luteum and secretes primarily progesterone but estrogens as well.

All steroid hormones originate from C27 cholesterol (Fig. 3). The main source of cholesterol required for the synthesis of steroid hormones (steroidogenesis) is LDL-cholesterol. Cholesterol is metabolized down to a number of enzymatic pathways and is converted to the 21-, 19-, and 18-carbon steroid hormones, respectively (Samavat and Kurzer, 2015).

Humans produce several different estrogens whose biosynthesis occurs in a variety of tissues and they are gender and age-dependent. In premenopausal women, 17 β -estradiol is the most abundant estrogen and the majority of this steroid is produced by the granulosa cells of the ovary (see above). Plasma 17 β -estradiol levels vary during menstrual cycle and the highest levels are found in the late follicular stage. Relatively high levels of estrone also are present in cycling women, but little originates in the ovary. Most estrone is generated by the aromatization of androstenedione in adipose tissue. Aromatase is present in skeletal muscle, skin, hair follicles, bone tissue, several sites in the brain, and the Leydig cells of the adult testes. During pregnancy, estriol, which is produced by the placental aromatization of fetal-derived androgens, is the most abundant estrogen and its levels are

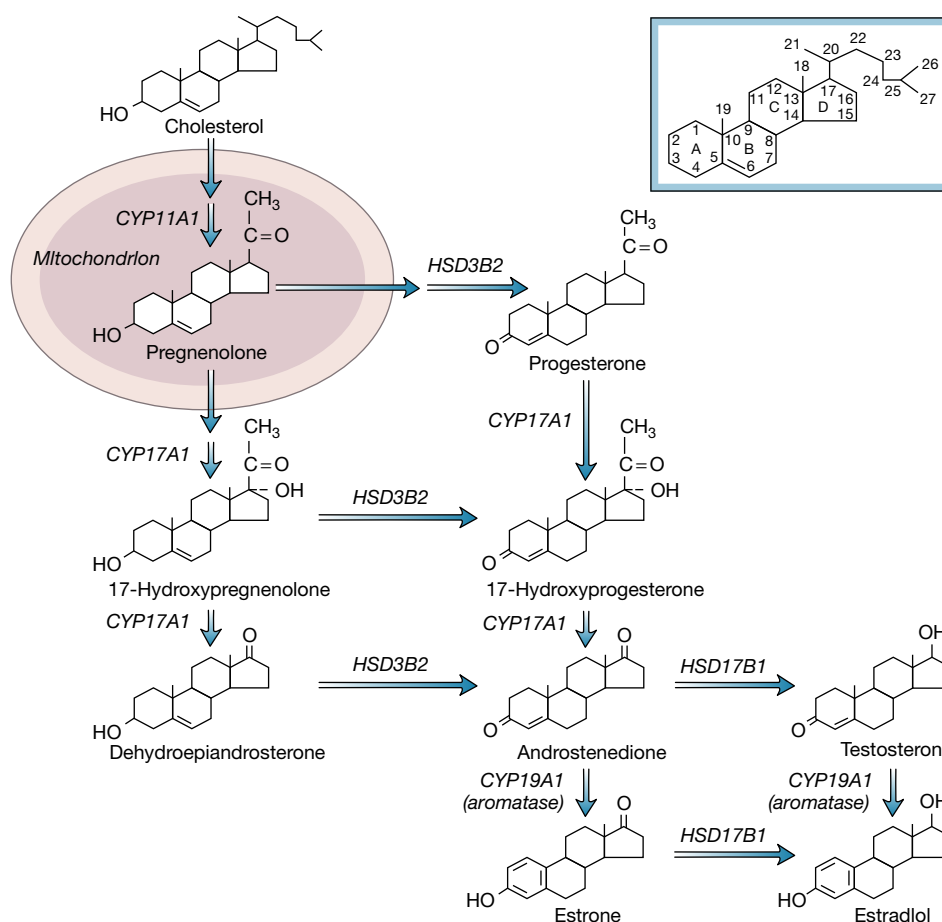


Fig. 3 Biosynthesis of estrogens. All steroid hormones originate from C27 cholesterol. Estrogen biosynthesis requires three hem-based cytochrome P450 enzyme complexes and two different dehydrogenases. The rate-limiting step in all steroid synthesis is the removal of the cholesterol side chain by the cytochrome P450 side chain cleavage enzyme which results in the generation of the C21 steroid, pregnenolone. Subsequent processing by 17 α -hydroxylase cytochrome P450, which converts the steroid from a C21 to a C19 structure, and 3/3-hydroxysteroid dehydrogenase, which oxidizes the hydroxyl group at the C3 position to a ketone group, results in the synthesis of the C19 estrogen precursor, androstenedione. The cytochrome P450 aromatase enzyme (P450arom) then catalyzes the unsaturation and aromatization of the A ring to produce estrone. Then, 16 α -hydroxylase converts estrone to estriol. Androstenedione can also be converted to testosterone and then, testosterone is converted to 17 β -estradiol by the P450arom. Abbreviations: StAR: steroidogenic acute regulatory protein; CYP11A1: side-chain-cleavage of P450; CYP17A1: 17-hydroxylase/17,20-lyase; HSD3B2:3 β -hydroxysteroid dehydrogenase-delta5,4 isomerase type 2; CYP19A1: aromatase; HSD17B1: 17 β -hydroxysteroid dehydrogenase type 1. From Samavat H, Kurzer MS (2015) Estrogen metabolism and breast cancer. *Cancer Letters* 356, 231-243.

a marker of fetal/placental functions. In postmenopausal women and in men, relatively low levels of 17β -estradiol (< 20 pg/mL) are generated by the peripheral conversion of androgens to estrogens. The total amount of estrogens synthesized by extragonadal sites may be small, but the local tissue concentrations achieved are probably high and exert biological influence locally.

Estrogen biosynthesis requires three hem-based cytochrome P450 enzyme complexes and two different dehydrogenases. The rate-limiting step in all steroid synthesis is the removal of the cholesterol side chain by the cytochrome P450 side chain cleavage enzyme, which results in the generation of the C21 steroid, pregnenolone. Subsequent processing by 17α -hydroxylase cytochrome P450, which converts the steroid from a C21 to a C19 structure, and 3β -hydroxysteroid dehydrogenase, which oxidizes the hydroxyl group at the C3 position to a ketone group, results in the synthesis of the C19 estrogen precursor, androstenedione. The cytochrome P450 aromatase enzyme (P450arom) then catalyzes the unsaturation and aromatization of the A ring to produce estrone.

Both androstenedione and estrone are substrates for 17β -hydroxysteroid dehydrogenase (17β -HSD), which can reversibly convert these oxosteroids to their 17β -hydroxysteroid counterparts, testosterone and 17β -estradiol, respectively.

Most cells do not express the complete complement of steroid biosynthetic enzymes required to synthesize estrogens. Thus, the predominant C18 estrogen produced by a given tissue depends on the C19 steroid presented to the aromatase enzyme complex. For example, granulosa cells of the ovarian follicles lack 17α -hydroxylase activity and are, therefore, dependent on an external source for the C19 steroid necessary for estradiol biosynthesis. These precursors (androstendione and testosterone) are synthesized in the thecal cell layer of the ovarian follicle (Fig. 2) and then CYP450 aromatase convert them to estrogen and 17β -estradiol in the granulosa cells. The formation of estrone by adipose tissue utilizes androstenedione made in the adrenal cortex as its C19 precursor. Estriol is produced by the syncytiotrophoblasts within the placenta from 16α -hydroxydehydroisoandrosterone-sulfate produced by the combined activities of the fetal adrenal and liver.

Transport of Estrogens

After secretion into the circulation, only 2% or 3% of biosynthetic 17β -estradiol remains free in plasma. Of the remaining amount, approximately 60% is weakly bound to albumin and 38% is bound to a 95 kDa glycoprotein, sex hormone-binding globulin (SHBG). SHBG binds both estrogens and androgens. Its binding affinity to androgens (testosterone and dihydrotestosterone) is approximately five- and three-fold greater, respectively, than to 17β -estradiol. The protein-bound hormone is inactive, and that only the free fraction is able to enter target cells and exert biological effects. The metabolic clearance rate of estrogens is inversely related to their affinity for SHBG and other plasma-binding proteins. The concentration of SHBG, therefore, influences estrogen metabolism and the amount of hormone available for entry to the target cell. Plasma SHBG levels are affected by a number of clinical conditions including pregnancy, diabetes, obesity, and sex. SHBG levels are two-fold higher in women than men.

Metabolism of Estrogens

Circulating estrogens are quickly metabolized in the liver to inactive, water-soluble compounds which are then excreted in the urine and feces. The vast majority of estrogen end up in the urine as glucuronosides. A second major class of catabolic products is the catechol estrogens (they have catecholaminergic and estrogenic activities) and are primarily 2-hydroxyestrogens (Samavat and Kurzer, 2015) (Fig. 4). Some catechol estrogens (e.g., 3-hydroxy estrone) by interacting with ER and the noradrenergic, dopaminergic and serotonergic pathways in the CNS may modulate behavior (cognition, memory) and may play a role in CNS diseases such as Alzheimer's disease.

Mechanism of Estrogen Action

Estrogens are lipophilic substances and it is believed that they passively diffuse across the plasma membrane and then bind to high-affinity receptor proteins, known as ERs. The ERs ($ER\alpha$ and $ER\beta$) are members of the nuclear receptor superfamily (Walter et al., 1985; Green et al., 1986; Kuiper et al., 1996), which includes receptors for other steroids (e.g., progesterone and androgens), vitamins (e.g., vitamin D and vitamin A (retinoids)), and thyroid hormone. Like other members of the superfamily, the ERs are nuclear, ligand-activated transcription factors, although $ER\alpha$ also associated with the membrane. After entering the cell through the plasma membrane, estrogen binds to ERs in the nucleus. In the nucleus, ERs are present as inactive monomers bound to heat-shock proteins. Upon binding to estrogen ERs dissociate from the heat-shock protein. This causes receptor dimerization which increases the affinity and the rate of receptor binding to estrogen response element (ERE) in the promoter of estrogen-regulated genes. In addition, the activated ERs bind to a number of nuclear proteins that act as bridging factors between the ERs and the general transcriptional machinery. By this complex mechanism, estrogens regulate the expression of target genes. The regulation can be stimulation or repression. The changes in the expression of these genes and the proteins that they encode result in an estrogenic response within a given target cell.

Estrogen may also bind to G-protein associated ER (GP-ER or GPR30) and induces AC, ERK, PKA, PKC, PI3K, and EGFR signaling (Zivadinovic et al., 2005; Prossnitz et al., 2007). This protein is a member of the rhodopsin-like family of G-protein-coupled receptors and is a multi-pass membrane protein that unlike the other members of the GPCR family localizes to the

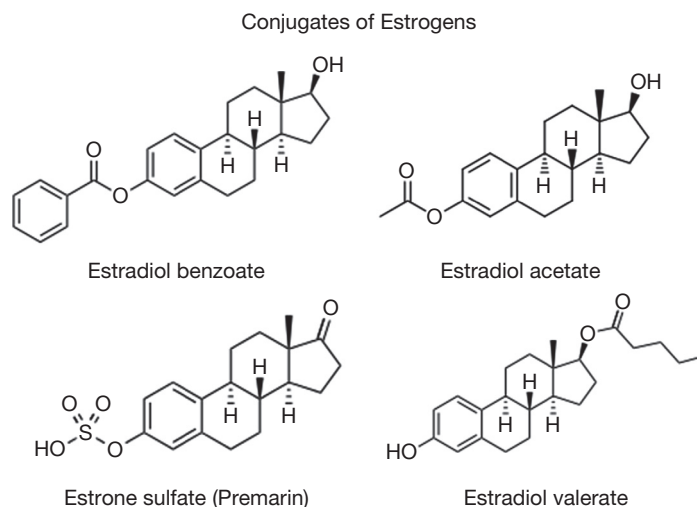


Fig. 5 Conjugates of estrogens: estradiol benzoate, estradiol valerate, estrone sulfate and estradiol valerate.

cyclopentylpropionate (**Fig. 5**). De-esterification of these compounds via esterases in the liver produces biologically active 17β -estradiol in vivo which then reaches all ERs in the body.

Brain-selective bioprecursor approach

As estrogens beside their many beneficial effects (see below) also have unwanted effects in the periphery (overstimulation of the uterus leading to spotting or bleeding or even cancer; stimulation of breast and potentially causing cancer; elevated coagulation; etc.) efforts have been devoted to develop brain-selective estrogens which would be beneficial in preventing/treating menopausal hot flushes, cognitive and memory declines, depression, sleep fragmentation, reduced sexual desire, etc.)

Recently published studies ([Prokai et al., 2015](#)) have identified $10\beta,17\beta$ -dihydroxyestra-1,4-dien-3-one (DHED) as a brain-selective bioprecursor prodrug for the potential treatment of estrogen-responsive diseases of the central nervous system. While typical prodrugs rely on the attachment of hydrolysis-sensitive “pro-moieties” to overcome caveats associated with the use of the parent agents (see above), DHED is actually a bioprecursor prodrug produced by a bioreversible chemical modification (i.e., a stereo-selective oxidation and without the use of an auxiliary pro-moiety) of 17β -estradiol (**Fig. 6**). As prodrugs, DHED completely lacks the biological activities of estrogens and other steroid hormones, until it is metabolized to 17β -estradiol (as shown in **Fig. 1**). Since DHED is orally bioavailable and is converted to 17β -estradiol only in the brain and not in the periphery, once it is developed for human use, it could be a safe estrogen therapy to treat menopausal symptoms (see above) without stimulating the uterus and the breast or increasing the chance of coagulation in the blood. Moreover, DHED could be used for men as well. Men going through the natural andropause or castrated because of hormone-sensitive prostate cancer have similar symptoms as menopausal women. The similarity of the symptoms in both sexes indicates that most of these symptoms are due to a common cause; that is, the lack of estrogen (primarily 17β -estradiol) action in the brain. The lack of 17β -estradiol is obvious in menopausal women; however, it does not seem to be obvious in men because 17β -estradiol is considered primarily a feminine steroid hormone. On the other hand, 17β -estradiol is well distributed in males’ brain due to its conversion from testosterone through the actions of aromatase ([MacLusky et al., 1987](#)). Indeed, estrogens (e.g., ethinyl estradiol and diethylstilbestrol (see below) (**Fig. 7**) have been successfully used in men receiving androgen depletion therapy for the alleviation of hot flushes, cognitive deficits and depression/anxiety associated with the hypoandrogenic conditions (for reviews, see ([Gillies and McArthur, 2010](#))). Select studies have suggested that 17β -estradiol has antidepressant-like and anxiolytic-like effects in males as well. Furthermore, although testosterone itself has clear effects on behavior, its mood- and anxiety-related actions might also be mediated through aromatase action and ERs ([Frye et al., 2008](#)). Except for hot flushes, the above neurological and psychiatric symptoms may be treated or prevented with medications other

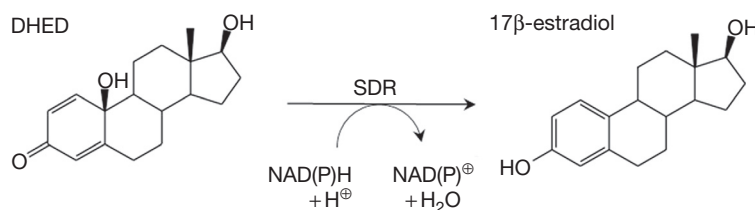


Fig. 6 Bioprecursors: The bioprecursor DHED is converted to 17β -estradiol via a reductase (SDR) utilizing NAD(P)H.

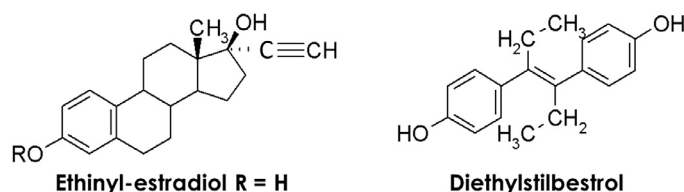


Fig. 7 Synthetic estrogens: ethinyl estradiol and diethylstilbestrol.

than estrogens. However, only estrogens can provide full relieve from hot flushes based on current clinical practices. Therefore, current preclinical studies support the notion that DHED could be a safe therapy not only in women but in men as well.

Synthetic, non-steroidal estrogens: 17 α -ethinyl estradiol (EE) and diethylstilbestrol (DES)

17 α -ethinyl estradiol (EE) has an ethinyl group linked to C17 of the estradiol molecule (Fig. 7). This compound is the most widely used synthetic oral estrogen and is the estrogen component found in most oral contraceptives.

Another synthetic, nonsteroidal compound which mimics estrogen-like effects is diethylstilbestrol (DES; Fig. 7), which was generated for clinical use. Diethylstilbestrol binds to the ER with high affinity yet does not bind to the plasma transport protein, sex hormone-binding globulin (see Section **Transport of Estrogens**), and is therefore a more potent estrogen than 17 β -estradiol.

In addition, there are a variety of chemicals found in the environment that possess weak estrogenic activity; they are referred to as “xenoestrogens.” This group of compounds includes a variety of industrial products, including pesticides such as DDT and other organochlorine compounds (e.g., polychlorinated bisphenols) as well as the monomer used to produce polycarbonate plastics, bisphenol A. Many of these molecules have a phenolic ring structure and hydroxyl groups that contribute to their ability to bind to the ER with moderate affinity. Although the impact of these molecules on human health is controversial, these chemicals are able to mimic estrogen effects in laboratory tests and several have been shown to disrupt reproductive processes in wildlife, suggesting that they may impinge on estrogen signaling pathways in nature.

It is worth noting that antiestrogens also have been developed for clinical uses, such as the treatment of breast cancer. However, today the most promising therapy for ER-positive breast cancer is the use of aromatase inhibitors which block the formation of estrogens from androgens.

Conjugated equine estrogens

A unique approach of estrogen therapy is the use of conjugated equine alone for estrogen replacement therapy or in combination with progestins as hormone replacement therapy. PREMARIN (pregnant mare urine; Fig. 5) contains primarily estrone, equilin, equilinenin as water soluble sodium sulfate conjugates. At least 10 other active estrogenic products are in the mixture including 17 α -dihydroequilin sulfate, 17 α -dihydro-equilin sulfate and 17 α -estradiol sulfate.

Selective estrogen receptor modulators (SERMS)

SERMS are a class of drugs that have features that can act estrogen receptor agonist and an antagonist, depending on the target tissue and include tamoxifene, raloxifene, nafoxidine, toremifene, and bazedoxifene (Fig. 8). SERMS are now being used as a treatment of breast cancer, osteoporosis, and postmenopausal symptoms.

Natural estrogens

In addition to men-made xenoestrogens discussed above, there are a number of naturally occurring substances that exhibit estrogenic activity. These chemicals are produced by plants and are more specifically referred to as phytoestrogens. The most abundant phytoestrogen is genistein, which is found in high concentration in soybeans. Diadzein (Fig. 9), S-equol (Fig. 9), coumestrol, resveratrol, quercetin, etc. also belong to this group. Phytoestrogens have weaker estrogenic activity compared to 17 β -estradiol or estrone. As other estrogens, they can bind sex hormone binding proteins. Dietary phytoestrogens are metabolized by intestinal bacteria,

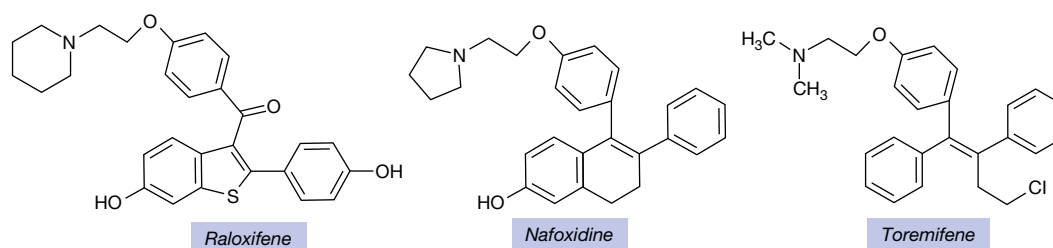


Fig. 8 Selective estrogen receptor modulators (SERMS): Raloxifene, Nafoxidine, and Toremifene.

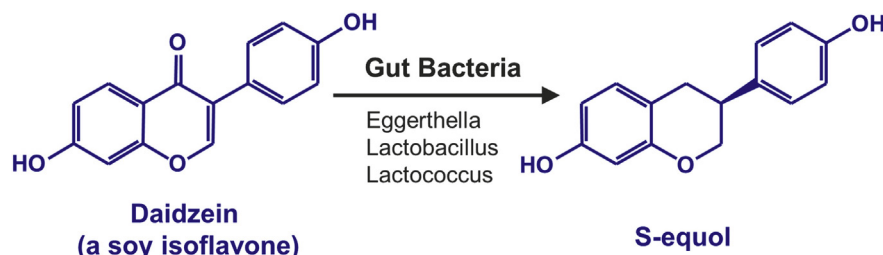


Fig. 9 Phytoestrogens: the phytoestrogens daidzein and S-equol. Daidzein is converted to S-equol by gut bacteria.

absorbed, conjugated in the liver by sulfotransferases and UDP-glucuronyl transferases, circulated in plasma, and excreted in urine. Phytoestrogens can bind to the ERs with varying degrees of effectiveness. However, the mechanism by which they exert their estrogenic effects in vivo is a matter of ongoing investigation.

References

- Doisy, E. A. (1972). Isolation of a crystalline estrogen from urine and the follicular hormone from ovaries. *American Journal of Obstetrics and Gynecology*, 114, 701–702.
- Ettmayer, P., Chloupek, S., & Weigand, K. (2003). Solid-phase synthesis of 7-acylamino-1,4-benzodiazepine-2,5-diones. *Journal of Combinative Chemistry*, 5, 253–259.
- Frye, C. A., Koonce, C. J., Edinger, K. L., Osborne, D. M., & Walf, A. A. (2008). Androgens with activity at estrogen receptor beta have anxiolytic and cognitive-enhancing effects in male rats and mice. *Hormones & Behavior*, 54, 726–734.
- Gillies, G. E., & McArthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: A case for sex-specific medicines. *Pharmacological Reviews*, 62, 155–198.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornert, J. M., Argos, P., & Chambon, P. (1986). Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb-A. *Nature*, 320, 134–139.
- Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S., & Gustafsson, J. A. (1996). Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 5925–5930.
- Levin, E. R. (2005). Integration of the extranuclear and nuclear actions of estrogen. *Molecular Endocrinology*, 19, 1951–1959.
- MacLusky, N. J., Clark, A. S., Naftolin, F., & Goldman-Rakic, P. S. (1987). Estrogen formation in the mammalian brain: Possible role of aromatase in sexual differentiation of the hippocampus and neocortex. *Steroids*, 50, 459–474.
- Prokai, L., Nguyen, V., Szarka, S., Garg, P., Sabnis, G., Bimonte-Nelson, H. A., McLaughlin, K. J., Talboom, J. S., Conrad, C. D., Shughrue, P. J., Gould, T. D., Brodie, A., Merchenthaler, I., Koulen, P., & Prokai-Tatrai, K. (2015). The prodrug DHED selectively delivers 17β-estradiol to the brain for treating estrogen-responsive disorders. *Science Translational Medicine*, 7, 297ra113.
- Prossnitz, E. R., Arterburn, J. B., & Sklar, L. A. (2007). GPR30: A G protein-coupled receptor for estrogen. *Molecular Cellular Endocrinology*, 265–266, 138–142.
- Samavat, H., & Kurzer, M. S. (2015). Estrogen metabolism and breast cancer. *Cancer Letters*, 356, 231–243.
- Toran-Allerand, C. D., Guan, X., MacLusky, N. J., Horvath, T. L., Diano, S., Singh, M., Connolly, E. S., Jr., Nethrapalli, I. S., & Tinnikov, A. A. (2002). ER-X: A novel, plasma membrane-associated, putative estrogen receptor that is regulated during development and after ischemic brain injury. *Journal of Neuroscience*, 22, 8391–8401.
- Walter, P., Green, S., Greene, G., Krust, A., Bornert, J. M., Jeltsch, J. M., Staub, A., Jensen, E., Scrace, G., Waterfield, M., et al. (1985). Cloning of the human estrogen receptor cDNA. *Proceedings of the National Academy of Sciences of the United States of America*, 82, 7889–7893.
- Zivadinovic, D., & Watson, C. S. (2005). Membrane estrogen receptor-α levels predict estrogen-induced ERK1/2 activation in MCF-7 cells. *Breast Cancer Research*, 7, R130–144.
- Zivadinovic, D., Gametchu, B., & Watson, C. S. (2005). Membrane estrogen receptor-α levels in MCF-7 breast cancer cells predict cAMP and proliferation responses. *Breast Cancer Research*, 7, R101–112.

Progesterone Signaling in the Endometrium

Juanmahel Dávila, Indrani C Bagchi, and Milan K Bagchi, University of Illinois at Urbana-Champaign, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

For more than a century, it is known that corpus luteum (CL) and its secretions are critical for endometrial function and pregnancy. In 1903, it was demonstrated that the removal of the corpora lutea of rabbits during early pregnancy resulted in pregnancy loss (Vaisbuch et al., 2015). Decades later, it was shown that injection of CL extracts into ovariectomized pregnant rabbits could sustain normal implantation as well as embryo survival and growth (Lessey, 2011; Vaisbuch et al., 2015). It was proposed that the biological effects of the CL extracts are due to the hormone “progestin” (Vaisbuch et al., 2015). By the mid-1930s, four different research groups reported the purification and characterization of progesterone (P), a steroid hormone derived from cholesterol (Lessey, 2011; Vaisbuch et al., 2015).

A large body of work has established that P is a critical regulator of normal female reproductive functions. It elicits a broad spectrum of responses in tissues, such as the ovary, uterus and mammary gland (Graham and Clarke, 1997). These include ovulation, proliferation and differentiation of the uterine tissue during pregnancy, lobuloalveolar development in the mammary gland, and orchestration of the menstrual cycle (Conneely et al., 2001; Kim et al., 2009; Vaisbuch et al., 2015). Besides its role in the reproductive tissues, P also influences the functions of the anterior pituitary, ventromedial hypothalamus, and preoptic areas. In the mouse brain, it elicits signals that are required for the regulation of the female sexual receptivity response (Conneely et al., 2001; Graham and Clarke, 1997; Vaisbuch et al., 2015). Lastly, there is emerging evidence of P action in diverse other tissues, such as the bone, lung, adipose tissue, kidney, and immune system (Al-Sabbagh et al., 2012; Graham and Clarke, 1997; Vaisbuch et al., 2015).

Progesterone produced by the ovary plays a critical role in regulating many biologically distinct processes that are associated with the establishment and maintenance of pregnancy. In the ovary, P signaling is implicated in ovulation and luteinization. In rodents, prior to ovulation, a surge of the luteinizing hormone stimulates a transient expression of the progesterone receptor (PGR) in granulosa cells of the preovulatory follicles (Conneely et al., 2001). The ovarian P then signals via the PGR to promote the expression of downstream molecular pathways that mediate the ovulatory process, culminating in the rupture of the follicle to yield the mature oocyte (Conneely et al., 2001; Kim et al., 2009). In humans, P secreted by the CL, which is formed following ovulation, dominates the secretory phase of the menstrual cycle. P signaling also exerts profound effects on mammary gland development during pregnancy in preparation for lactation (Conneely et al., 2001; Graham and Clarke, 1997). Although P has many roles in diverse tissues and cell types, this chapter will focus on the effects of this hormone on endometrial functions.

Structure and Function of the Progesterone Receptors

In target tissues, the cellular actions of P are mediated by its intracellular progesterone receptors (PGR). The PGR protein, which is a ligand-inducible transcription factor, was purified from the chick oviduct and human uterus (Graham and Clarke, 1997; Kaya et al., 2015; O'Malley, 2005; Vaisbuch et al., 2015). The cloning of the *PGR* gene, which is conserved among the vertebrate species, including humans and rodents, revealed that it belongs to the nuclear receptor superfamily (Conneely et al., 2001; O'Malley, 2005). A common feature of PGR and other members of this superfamily is a modular structure harboring distinct functional domains that allow them to function as ligand-inducible transcription factors (Al-Sabbagh et al., 2012; Binder et al., 2015; Vaisbuch et al., 2015). Accordingly, PGR has multiple distinct functional domains: an amino-terminal domain (A/B) harboring a transactivation function AF-1, a centrally located DNA binding domain (C), a hinge region (D), and a ligand binding domain (E) containing a second transactivation function AF-2 (Fig. 1). Moreover, like all other steroid receptors, PGR harbors signature amino acid motifs that serve as nuclear import and export signals, enabling the receptor to shuttle actively between the nuclear and cytoplasmic compartments (Al-Sabbagh et al., 2012; Binder et al., 2015; Vaisbuch et al., 2015).

The gene encoding human PGR is located on chromosome 11q22.1 and has eight exons (Al-Sabbagh et al., 2012; Binder et al., 2015; Conneely et al., 2001; Vaisbuch et al., 2015). There are two isoforms of human PGR, PGR-A (94 kDa) and PGR-B (114 kDa). These proteins are generated from alternate transcripts arising from a single *PGR* gene via different promoter usage. Both isoforms have the same DNA binding and ligand binding domains, but PGR-B possesses an additional transactivation domain AF-3 in the amino-terminal region (Fig. 1) (Binder et al., 2015; Graham and Clarke, 1997; Kaya et al., 2015; Li and O'Malley, 2003; Vaisbuch et al., 2015; Young, 2013). The presence of this additional transactivation domain in PGR-B allows it to interact with adjacent PGR-B dimers, different transcription factors, and coregulatory factors, which helps explain why PGR-B is a stronger transcriptional activator than PGR-A (Binder et al., 2015; Li and O'Malley, 2003; Patel et al., 2015; Vaisbuch et al., 2015).

The PGR isoforms are expressed at different levels in progesterone-responsive tissues, depending on developmental stage, hormonal status, or disease (Conneely et al., 2001; Graham and Clarke, 1997). The biological significance of the ratios of PGR expression is still unclear. However, the functional differences between the two PGR isoforms suggest that their differential expression may be critical for the appropriate cellular responses to P (Conneely et al., 2001). Notably, several additional alternatively

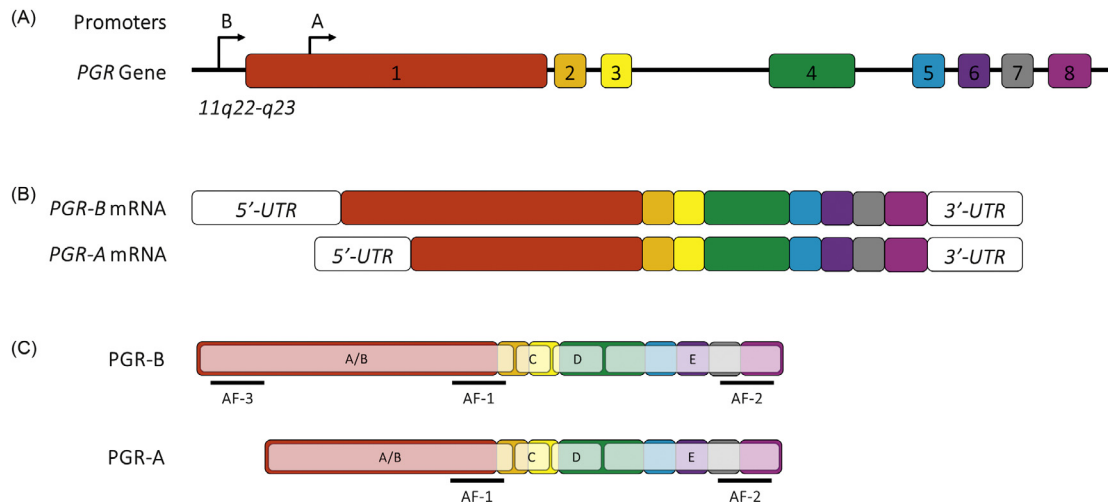


Fig. 1 Progesterone receptor (PGR) gene and its protein isoforms. (A) Exon organization map of the human PGR gene located on chromosome 11, regions q22-q23, showing the PGR-A and PGR-B promoters (arrows). (B) The primary mRNA transcripts derived from different transcription start sites. (C) Structural organization of the PGR-B and PGR-A proteins. Both PGR-B and PGR-A contain a transcriptional activation function AF-1 in the N-terminal domain (A/B) and a transcriptional activation function AF-2 in the C-terminal domain (E). PGR-B isoform contains an additional transcriptional activation function AF-3. The DNA binding domain (C), the hinge (D), and the ligand-binding domain (E) are shown.

transcribed, translated or spliced PGR variants have also been described in the literature, but their relevance and potential biological role remains controversial and will not be discussed in this chapter.

Genomic Actions of Progesterone Receptors

In PGR-expressing cells, the unliganded receptor exists in a large, multi-protein complex containing various heat shock proteins (e.g., HSP90, HSP70, and p23) and immunophilins (e.g., FKBP51 and FKBP52), which maintain the receptor in a transcriptionally inactive state (Fig. 2) (Al-Sabbagh et al., 2012; Vaisbuch et al., 2015). P, which is lipophilic, passively diffuses through the plasma membrane and binds to the intracellular PGR. Binding of P induces a change in the conformation of PGR that results in its dissociation from the chaperone proteins, dimerization, and translocation into the nucleus. The hormone-occupied PGR then binds to target genes via direct interaction with discrete DNA response elements, known as progesterone response elements (PREs) or via tethering interactions with other transcription factors (Conneely et al., 2001; Vaisbuch et al., 2015). The target gene-bound PGR recruits specific coactivator proteins and general transcription factors to form a productive transcription initiation complex to activate or repress RNA synthesis (Al-Sabbagh et al., 2012; Conneely et al., 2001; Patel et al., 2015; Vaisbuch et al., 2015).

Both PGR isoforms are capable of binding P and interacting with PREs and the transcriptional machinery to regulate gene expression (Conneely et al., 2001; Graham and Clarke, 1997; Patel et al., 2015). In target cells, these isoforms can potentially form three types of dimers: A:A homodimer, B:B homodimer, and A:B heterodimer. The specific contribution of each of these dimer species in mediating the regulatory effects of P depends on their differential transactivation properties. Several lines of evidence have suggested that PGR-A and PGR-B proteins are functionally distinct. When expressed individually in cultured cells, they display different transactivation properties that are specific to both cell type and target gene promoter used (Conneely et al., 2001). Studies have shown that PGR-A acts as a trans-repressor of PGR-B and other steroid receptors in certain cell and promoter contexts, presumably through competition for common limiting coactivators (Binder et al., 2015; Conneely et al., 2001; Li and O'Malley, 2003; O'Malley, 2005; Vaisbuch et al., 2015). Therefore, the ratios of the isoforms within a target cell under specific physiological conditions would likely alter the relative complement of dimeric complexes and exert a significant impact on the overall cellular responses to P (Conneely et al., 2001).

Modulation of Progesterone Receptor Activity by Co-regulatory Factors

It is well documented that the activity of nuclear PGR, as well as other nuclear steroid receptors, is regulated not only by the cognate hormone itself, but also by its interaction with co-regulatory factors, such as coactivators, corepressors, and chromatin modifiers (Al-Sabbagh et al., 2012; Binder et al., 2015; Vaisbuch et al., 2015; Young, 2013). Prominent among these factors are the three members of the steroid receptor coactivator (p160/SRC) family: SRC1, SRC2, and SRC3 (Binder et al., 2015; Vaisbuch et al., 2015). The SRCs, which share a high degree of sequence homology, harbor several LXXLL signature motifs that interact directly with the ligand-binding domain of the steroid receptors (Binder et al., 2015; Vaisbuch et al., 2015; Young, 2013). Binding of

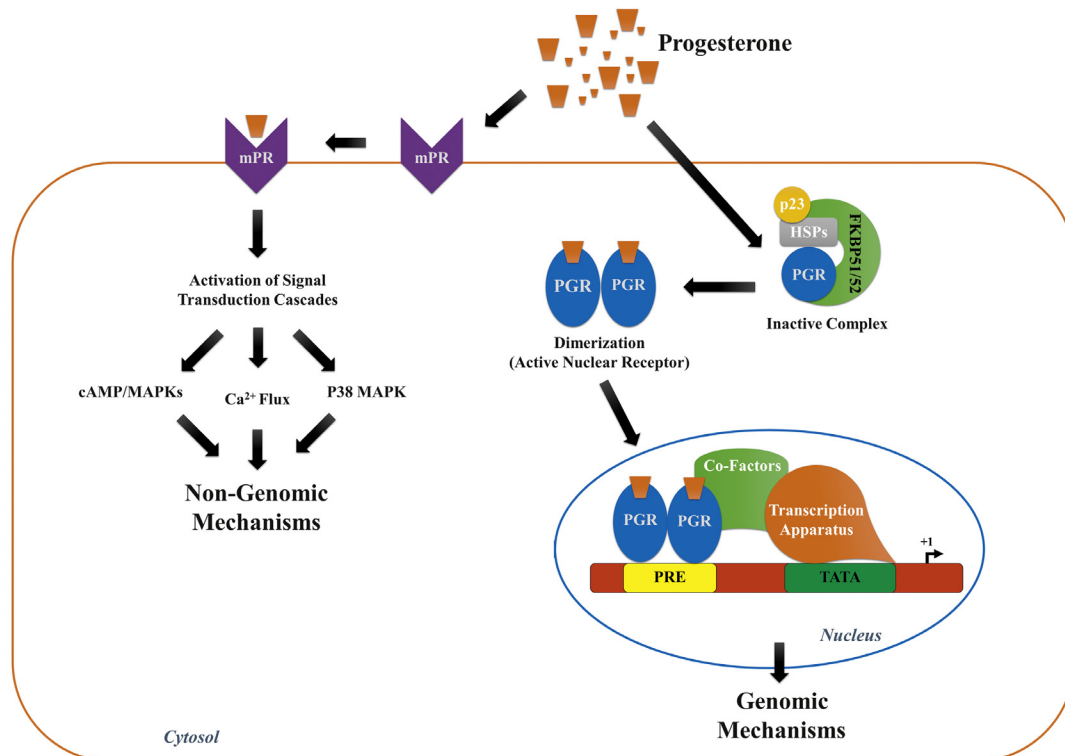


Fig. 2 Progesterone enters the target cell via passive diffusion, binds to the intracellular progesterone receptor (PGR), which then translocates to the nucleus and initiates a series of genomic events that result in altered gene expression and cellular function. Binding of the hormone to the membrane-bound progesterone receptor (mPR) mediates non-genomic mechanisms.

SRCs to PGR creates a surface that recruits additional coactivator molecules, such as the coactivator-associated arginine methyltransferase-1 (CARM1), cAMP responsive element binding protein (CREB)-binding protein (CBP), and the related protein p300 (Al-Sabbagh et al., 2012; Binder et al., 2015; Li and O'Malley, 2003). The resulting multimeric coactivator protein complex then controls transcription by modulating local chromatin structure via histone acetylation/methylation. The SRCs also harbor a bHLH-PAS motif within the amino-terminal region, which can interact with other transcription factors binding to nearby genomic sites. Besides interacting with the SRCs, PGR is also able to recruit other multiprotein complexes, such as the SWI/SNF chromatin remodeler, mediator complex, or proteasomes to dynamically mediate and coordinate the processes necessary to accomplish transcription of PGR target genes (Al-Sabbagh et al., 2012; Binder et al., 2015; Li and O'Malley, 2003).

In cell-based assays, all three members of the SRC family enhance PGR-mediated gene activation. Knockout mouse studies, however, have revealed that these coactivators play tissue-specific roles *in vivo*. Whereas SRC-1 regulates steroid hormone sensitivity in the uterus, SRC-3 controls mammary gland development. SRC-2 plays a critical role in both organs (Vaisbuch et al., 2015). The SRC-2 knockout mice manifested a deficient uterine decidual response to P stimulation and an early block in embryo implantation (Vaisbuch et al., 2015; Young, 2013). Ablation of SRC-2 also suppresses P-induced ductal side branching and alveologenesis in the mammary gland. Collectively, these data substantiate the importance of SRCs in regulating the biological actions of P.

Non-Genomic Actions of Progesterone

During the past several years, a growing body of literature supports the concept that P activates a variety of rapid signaling events, independent of its role as a nuclear receptor (Moussatche and Lyons, 2012; Pru and Clark, 2013). This mode of signaling involves the activation of signal transduction cascades that generate intracellular second messengers and changes in Ca^{2+} ion flux. Additionally, this mode of PGR signaling has been shown to activate intracellular kinases, including the cAMP-dependent protein kinase A, the cyclin-dependent kinase-2, the mitogen activating protein kinase (MAPK), and the stress-activated p38 MAPK (Fig. 2) (Moussatche and Lyons, 2012; Pru and Clark, 2013; Vaisbuch et al., 2015). Non-genomic signaling is thought to be mediated by membrane-bound PGRs (mPRs), which have been identified in reproductive tissues, immune cells, the nervous system, and cancer cells (Moussatche and Lyons, 2012). Two families of non-classical membrane receptors have been identified, including the progestin and adipoQ receptor (PAQR) and progesterone receptor membrane component (PGRMC) (Pru and Clark, 2013). Members of the PAQR family with P binding activity belong to the G protein-coupled receptor superfamily and include mPR α , mPR β , and mPR γ (Pru and Clark, 2013; Valadez-Cosmes et al., 2016). Recent studies have reported that, in the myometrial cells

of pregnant humans, mPR α and mPR β are directly coupled to G-inhibitory proteins and may play a role in facilitating myometrial contractions (Vaisbuch et al., 2015). However, whereas the genomic actions are relatively well defined, in-depth mechanistic insights into non-genomic P actions are still needed, and therefore this mode of P-signaling will not be the focus of this chapter.

Progesterone Receptor-Mediated Signaling in the Endometrium

Lessons from the PGR Knockout Mouse Models

The development of a genetically engineered mouse model lacking PGR established that this receptor is essential for female fertility (Conneely et al., 2001; Lydon et al., 1995). Female mice lacking both PGR-A and PGR-B exhibit pleiotropic reproductive abnormalities including an inability to ovulate, uterine hyperplasia and inflammation, and defects in uterine receptivity and implantation. Analysis of their uteri revealed impaired decidualization and a hypertrophied epithelium that is non-functional for embryo implantation. Creation of knockout mice in which PGR-A expression, but not that of PGR-B, was selectively ablated resulted in a uterine phenotype similar to that exhibited by the PGR-null mice. In contrast, mice that lacked uterine PGR-B expression, but retained PGR-A, displayed no obvious uterine phenotypic defects. Collectively, these data indicated that PGR-A is the major isoform that mediates P-dependent uterine functions in the mouse. However, as described below, in the human, PGR-B seems to play a predominant role in controlling endometrial functions, particularly decidualization.

Role of PGR in Regulating Stromal-Epithelial Crosstalk

Successful implantation requires a receptive endometrium, which arises from a synchronized dialogue between epithelial and stromal cells in this tissue (Cakmak and Taylor, 2011; Conneely et al., 2001; Lessey, 2011). P plays a central role in directing this crosstalk that dictates endometrial cell proliferation and differentiation during early pregnancy. P-dependent pathways modulate the functions of endometrial epithelial and stromal cells creating an environment necessary for the establishment of pregnancy. Various mechanisms have been proposed to explain how P acts via PGR to (1) arrest epithelial proliferation, a necessity for the acquisition of uterine receptivity, and (2) stimulate stromal cell proliferation and differentiation, a requirement for the establishment of pregnancy.

17 β -estradiol (E2) stimulates uterine growth by inducing epithelial cell proliferation in the pro-estrous and estrous stages of the reproductive cycle in rodents or the proliferative phase of the menstrual cycle in humans (Binder et al., 2015; Conneely et al., 2001; Patel et al., 2015; Young, 2013). The postovulatory surge of P triggers the arrest of E2-dependent epithelial cell proliferation, followed by the transformation of the stromal cells into decidual cells, which secrete many factors that promote endometrial neoangiogenesis and guide recruitment of various immune cells to the pregnant uterus (Al-Sabbagh et al., 2012; Binder et al., 2015). The expression of the transcription factor Heart and Neural Crest Derivatives Expressed 2 (HAND2) in uterine stroma plays a central role in mediating the suppressive effects of P on epithelial proliferation (Li et al., 2011). During the receptive phase, P induces HAND2 expression in uterine stromal cells by directly interacting with the PREs in the *Hand2* promoter (Li et al., 2011; Kaya et al., 2015). HAND2 then suppresses the production of several fibroblast growth factors (FGFs), which act as paracrine mediators of mitogenic effects of E2 on uterine epithelium (Li et al., 2011). When the *Hand2* gene was deleted in the uterine stroma in a conditional knockout mouse model, persistent activation of the FGF receptor and the downstream ERK1/2 pathway was evident in the luminal epithelium, creating a non-receptive endometrium and leading to implantation failure. These findings unveiled a critical role for P-induced HAND2 in controlling epithelial function and uterine receptivity.

Another plausible mechanism by which P controls E2-induced epithelial proliferation and uterine receptivity is by inducing the epithelial expression of Kruppel-like transcription factor 15 (KLF15), which opposes the action of the Kruppel-like transcription factor 4 (KLF4) in the preimplantation uterus (Bhurke et al., 2016). E2 promotes epithelial expression of KLF4, which stimulates synthesis of the minichromosome maintenance (MCM)-2 protein, an essential component of the DNA synthesis machinery (Bhurke et al., 2016). P-induced KLF15 disrupts the transcriptional function of KLF4, suppressing the level of MCM-2 and thereby causing the cessation of E2-dependent epithelial proliferation.

The PGR-mediated expression of the morphogen Indian hedgehog (IHH) in uterine luminal epithelium has also been implicated in the promotion of uterine receptivity at the time of implantation. Transient epithelial production of IHH is a paracrine signal that induces the expression of its receptor, Patched (PTCH), in uterine stromal cells. The IHH then acts via PTCH to elevate the expression of the transcription factor COUP-TFII, which controls stromal cell proliferation and differentiation (Bhurke et al., 2016). Uteri of mice harboring a conditional deletion of *Ihh* fail to attain the receptive state, indicating that PGR-induced IHH production plays an important role during implantation by controlling the epithelial-stromal crosstalk.

Role of Progesterone Receptor in Decidualization

The most well-known reproductive role of P is to trigger the differentiation process in the stromal compartment, known as “decidualization,” which is characterized by the transformation of endometrial stromal cells into specialized secretory decidual cells (Al-Sabbagh et al., 2012).

One of the key P-regulated factors that control uterine decidualization is the bone morphogenetic protein 2 (BMP2), a member of the TGF- β superfamily. Decidual stage-specific expression of BMP2, which acts via its receptor on the uterine stromal cells during

early pregnancy, is critical for implantation and decidualization (Bhurke et al., 2016). Conditional deletion of the BMP2 gene in the uterus led to female infertility, primarily due to a failure of decidualization. Consistent with this finding, in vitro experiments showed that addition of BMP2 to undifferentiated mouse or human endometrial stromal cells markedly advanced their differentiation into decidual cells (Bhurke et al., 2016). Conversely, siRNA-mediated knockdown of BMP2 expression in these cells efficiently blocked the differentiation process (Bhurke et al., 2016).

The mechanism of action of BMP2 in the uterus is linked to the induction of the signaling molecule WNT4. BMP2 and WNT4 proteins exhibit overlapping spatiotemporal expression in mouse decidua in vivo. Addition of BMP2 robustly induces *Wnt4* transcripts in the primary mouse endometrial stromal cultures (Bhurke et al., 2016), giving rise to the view that BMP2 induces WNT4 expression to regulate stromal decidualization (Bhurke et al., 2016). Consistent with this view, conditional loss of *Wnt4* in mouse uterus impaired decidualization and led to implantation failure. BMP2 and WNT4 are also strongly induced in human endometrial stromal cells during decidualization. siRNA-mediated attenuation of their expression in these cells showed that both BMP2 and WNT4 are critical for this differentiation process. Chromatin immunoprecipitation (ChIP)-sequencing analysis revealed the existence of PREs directly targeted by both PGR-A and PGR-B in the *BMP2* gene (Kaya et al., 2015). Collectively, these results established that the BMP2-WNT4 pathway, operating downstream of PGR signaling, critically controls decidualization (Bhurke et al., 2016).

It was recently reported that BMP2 signaling through the BMP type 1 receptor on endometrial stromal cells promotes the expression of the transcription factor C/EBP β during decidualization (Bhurke et al., 2016). A global knockout of C/EBP β in mice displayed marked impairment in stromal cell proliferation and differentiation and female infertility, indicating a critical role of this factor in the regulation of decidualization (Bhurke et al., 2016). Further analysis revealed that, in C/EBP β -null uteri, stromal proliferation is blocked at G2 to M transition (Bhurke et al., 2016). Interestingly, ChIP analysis in human endometrial stromal cells showed that PGR interacts with C/EBP β at the P-regulated region of *AURKA*, which encodes the aurora kinase A, which is involved in microtubule formation and/or stabilization at the spindle pole during cell division (Kaya et al., 2015). These results are consistent with the view that C/EBP β and PGR act in concert to regulate human endometrial proliferation and differentiation.

PGR-dependent pathways also regulate the expression of the homeobox gene *Hoxa10* in mouse uterine stromal cells during decidualization. The *Hoxa10*-null mice exhibit a severe defect in decidualization and female infertility (Bhurke et al., 2016). The steroid receptor chaperon protein FKBP52 is a downstream target of HOXA10. Mice lacking FKBP52 also displays implantation and decidualization defects, phenocopying *Hoxa10*-deficiency (Bhurke et al., 2016). HOXA10 is also induced during human endometrial stromal cell differentiation in response to P. Genomic analysis identified PREs in the *HOXA10* gene promoter, indicating that direct regulation of *HOXA10* by PGR controls decidualization (Kaya et al., 2015).

Role of Progesterone Receptor in Human Endometrium

In the human, cyclic patterns of the ovarian E2 and P secretion produce changes in the endometrium that are responsible for the menstrual rhythm. The endometrium undergoes cyclic proliferative and secretory phases and exhibits only a short period of receptivity to embryo implantation in the mid-secretory phase of the cycle (Cakmak and Taylor, 2011). Within this time window, which lasts for a few days following ovulation, the endometrium becomes competent to implant the embryo to initiate a potential pregnancy (Cakmak and Taylor, 2011). P is critical to endometrial receptivity and functions to turn the E2-primed endometrium into a secretory tissue that promotes embryo survival and implantation (Lessey, 2011). The expression of PGR-A and PGR-B increases in all cell types during the proliferative phase (Binder et al., 2015; Patel et al., 2015). In the secretory phase, as serum P levels increase, PGR levels are maintained in the stroma and myometrium but decrease sharply in the glandular epithelium (Binder et al., 2015).

In the presence of high levels of P in the secretory phase, the stromal cells in human endometrium undergo a differentiation process known as pre-decidualization. If pregnancy is established, it triggers a more robust differentiation process that results in the formation of the decidual tissue. Both PGR isoforms, PGR-A and PGR-B, are expressed during decidualization (Kaya et al., 2015; Vaisbuch et al., 2015). Recent work demonstrated that PGR-B controls a substantially larger cistrome and transcriptome than PGR-A during human endometrial decidualization (Kaya et al., 2015). There is also evidence that PGR-B directly regulates the expression of PGR-A. Furthermore, the study found that PGR-A and PGR-B regulate overlapping as well as distinct sets of genes, many of which are known to play critical roles in decidualization and establishment of pregnancy in the mouse (Kaya et al., 2015). When PGR-A and PGR-B were co-expressed during human endometrial stromal cell differentiation, PGR-B played a predominant role, although both isoforms influence each other's transcriptional activity (Kaya et al., 2015; Vaisbuch et al., 2015). PGR-A and PGR-B also interact with transcription factors, such as FOS, JUN, CEBP β , and STAT3, which bind to neighboring genomic sites and modulate their transcriptional activities (Bhurke et al., 2016). Collectively, these interactions regulate the expression of many P-target genes that function in concert to properly control epithelial proliferation, stromal differentiation, angiogenesis, and immune response in the endometrium to render it receptive for implantation.

Perspectives and Future Directions

In normal endometrial cells, the hormone-occupied PGR acts in concert with cell-specific transcription factors and co-regulators to modulate chromatin structure and gene expression, which in turn control cell proliferation and promote differentiation. Analysis of the profiles of PGR occupancy at genomic sites and that of downstream gene expression during endometrial differentiation have offered a better understanding of the role of this receptor in uterine biology. These studies have revealed that several

PGR-regulated pathways profoundly influence endometrial functions, such as decidualization and angiogenesis, during pregnancy and these are often conserved in species ranging from the rodents to humans. Reduced P sensitivity and consequent disruptions in PGR signaling contribute not only to recurrent miscarriage and infertility but also to other reproductive pathologies, such as endometriosis (Bhurke et al., 2016). Endometriosis, a prevalent gynecological disease, is associated with dysregulation of a subset of P-dependent genes in the secretory endometrium, leading to the concept that it is a disease with underlying P resistance, perhaps arising from epigenetic alterations at PGR target loci in the genome. Further refinement of cellular and mouse models using CRISPR/Cas9 gene editing and continued analysis of a wide range of endometrial specimens obtained from human subjects suffering from infertility or endometriosis will provide a comprehensive molecular understanding of the genetic and epigenetic alterations that lead to dysregulation of PGR-regulated pathways in these disease conditions.

References

- Al-Sabbagh, M., Lam, E. W., & Brosens, J. J. (2012). Mechanisms of endometrial progesterone resistance. *Molecular and Cellular Endocrinology*, 358(2), 208–215. <https://doi.org/10.1016/j.mce.2011.10.035>.
- Bhurke, A. S., Bagchi, I. C., & Bagchi, M. K. (2016). Progesterone-regulated endometrial factors controlling implantation. *American Journal of Reproductive Immunology*, 75(3), 237–245.
- Binder, A. K., Winuthayanon, W., Hewitt, S. C., Couse, J. F., & Korach, K. S. (2015). Steroid receptors in the uterus and ovary. In T. M. Plant, & A. J. Zeleznik (Eds.) (4th edn, *Knobil and Neill's physiology of reproduction* (4th edn, vol. 1, pp. 1099–1193). London: Elsevier.
- Cakmak, H., & Taylor, H. S. (2011). Implantation failure: Molecular mechanisms and clinical treatment. *Human Reproduction Update*, 17(2), 242–253. <https://doi.org/10.1093/humupd/dmq037>.
- Conneely, O. M., Mulac-Jericevic, B., Lydon, J. P., & De Mayo, F. J. (2001). Reproductive functions of the progesterone receptor isoforms: Lessons from knock-out mice. *Molecular and Cellular Endocrinology*, 179(1–2), 97–103.
- Graham, J. D., & Clarke, C. L. (1997). Physiological action of progesterone in target tissues. *Endocrine Reviews*, 18(4), 502–519. <https://doi.org/10.1210/edrv.18.4.0308>.
- Kaya, H. S., Hantak, A. M., Stubbs, L. J., Taylor, R. N., Bagchi, I. C., & Bagchi, M. K. (2015). Roles of progesterone receptor A and B isoforms during human endometrial decidualization. *Molecular Endocrinology*, 29(6), 882–895. <https://doi.org/10.1210/me.2014-1363>.
- Kim, J., Bagchi, I. C., & Bagchi, M. K. (2009). Control of ovulation in mice by progesterone receptor-regulated gene networks. *Molecular Human Reproduction*, 15(12), 821–828. <https://doi.org/10.1093/molehr/gap082>.
- Lessey, B. A. (2011). Assessment of endometrial receptivity. *Fertility and Sterility*, 96(3), 522–529. <https://doi.org/10.1016/j.fertnstert.2011.07.1095>.
- Li, X., & O'Malley, B. W. (2003). Unfolding the action of progesterone receptors. *The Journal of Biological Chemistry*, 278(41), 39261–39264. <https://doi.org/10.1074/jbc.R300024200>.
- Li, Q., Kannan, A., DeMayo, F. J., Lydon, J. P., Cooke, P. S., Yamagishi, H., et al. (2011). The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science*, 331(6019), 912–916. <https://doi.org/10.1126/science.1197454>.
- Lydon, J. P., DeMayo, F. J., Funk, C. R., Mani, S. K., Hughes, A. R., Montgomery, C. A., Jr, et al. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes & Development*, 9(18), 2266–2278.
- Moussatche, P., & Lyons, T. J. (2012). Non-genomic progesterone signalling and its non-canonical receptor. *Biochemical Society Transactions*, 40(1), 200–204. <https://doi.org/10.1042/BST20110638>.
- O'Malley, B. W. (2005). A life-long search for the molecular pathways of steroid hormone action. *Molecular Endocrinology*, 19(6), 1402–1411. <https://doi.org/10.1210/me.2004-0480>.
- Patel, B., Elguero, S., Thakore, S., Dahoud, W., Bedaiwy, M., & Mesiano, S. (2015). Role of nuclear progesterone receptor isoforms in uterine pathophysiology. *Human Reproduction Update*, 21(2), 155–173. <https://doi.org/10.1093/humupd/dmu056>.
- Pru, J. K., & Clark, N. C. (2013). PGRMC1 and PGRMC2 in uterine physiology and disease. *Frontiers in Neuroscience*, 7, 168. <https://doi.org/10.3389/fnins.2013.00168>.
- Vaisbuch, E., Erez, O., & Romero, R. (2015). Physiology of progesterone. In H. J. A. Carp (Ed.), *Progestogens in obstetrics and gynecology* (pp. 1–32). Cham: Springer International Publishing.
- Valadez-Cosmes, P., Vazquez-Martinez, E. R., Cerbon, M., & Camacho-Arroyo, I. (2016). Membrane progesterone receptors in reproduction and cancer. *Molecular and Cellular Endocrinology*, 434, 166–175. <https://doi.org/10.1016/j.mce.2016.06.027>.
- Young, S. L. (2013). Oestrogen and progesterone action on endometrium: A translational approach to understanding endometrial receptivity. *Reproductive Biomedicine Online*, 27(5), 497–505. <https://doi.org/10.1016/j.rbmo.2013.06.010>.

Further Reading

- Mazur, E. C., Vasquez, Y. M., Li, X., Kommagani, R., Jiang, L., Chen, R., et al. (2015). Progesterone receptor transcriptome and cistrome in decidualized human endometrial stromal cells. *Endocrinology*, 156(6), 2239–2253. <https://doi.org/10.1210/en.2014-1566>.

Androgens

Carlos Stocco, University of Illinois College of Medicine, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

For decades, *in vitro* studies have consistently demonstrated that androgens participate in the regulation of follicle growth and modulate gonadotropin action in the ovary. Conversely, clinical or biochemical evidence of androgen excess is one of the main criteria for the diagnosis of polycystic ovarian syndrome (PCOS), a disease that significantly decreases fertility. Androgens actions are mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily. The development of global and conditional AR knockout mouse models showed that androgenic activity is required for normal follicle development and function. In the ovary, AR activation is mainly carried out by testosterone, which is produced by the theca cells of the follicle. Depending on the stage of follicle maturation, androgens exert direct autocrine and/or paracrine effects to regulate follicular function, or they can also act as a substrate for aromatase of the granulosa cells, which convert the androgens to estrogens. In females, circulating androgens including dehydroepiandrosterone, androstenedione, and dihydrotestosterone (DHT) may also participate in the regulation of ovarian function. However, only testosterone and DHT (a nonaromatizable androgen) activate the AR, while the other androgens require conversion to testosterone or DHT.

Androgen Receptor Expression in the Ovary

In the ovary, the AR is expressed in the theca-interstitial cells, in the oocytes, and in the granulosa cells. This suggests that all compartments of the follicle are putative targets of androgen action.

Theca Cells

In situ hybridization studies showed extensive AR mRNA labeling in the theca cells of rat ovaries (Hirai et al., 1994). Recently, strong AR protein labeling in the nuclei of the theca cells was also observed in mouse follicles especially after treatment with DHT. However, a decrease in AR signaling in the theca cells has no effect on steroid secretion, development, or reproductive function of females under physiological androgen levels (Ma et al., 2017).

In rhesus monkeys, the AR mRNA is detected in theca-interstitial cells of developing follicles but at distinctly lower levels than granulosa cells. Moreover, in contrast to granulosa cells, there are no significant differences between AR mRNA levels in the theca of preantral and large antral follicles. In this species, the AR mRNA is also present in the ovarian stroma (Weil et al., 1998).

The Oocyte

The AR has been detected in the oocyte of several species. Particularly in rats, the AR is clearly detectable in the oocyte of primary and secondary follicles. Interestingly, the oocyte nucleolus becomes strongly positive for the AR as the follicles grow. In mouse, expression of the AR in the oocyte is detectable by immunohistochemistry mainly in the cytoplasm, and it is virtually absent in the nucleus. The function of the AR in the oocyte in mice is still unclear as the knockout of the AR in the oocyte has no effects on female reproduction (Sen and Hammes, 2010).

Granulosa Cells

In rodents, the AR is expressed in granulosa cells of smaller follicles and steadily increases toward the early antral stage, whereas a progressive decrease has been observed from the early antral to the preovulatory stage. In rats, the abundance of AR mRNA decreases ~50% during the maturation from small to large antral follicles, whereas the abundance of aromatase mRNA increased > 250%. Immunofluorescence studies demonstrated AR protein expression in primordial, transitional, and primary follicles in mice (Laird et al., 2017). In rats, the AR protein is located predominantly in the nuclei of the granulosa layer of preantral and very early antral rat follicles. Interestingly, as follicles grow to the preovulatory stage, a decline in AR expression occurs in the mural granulosa cells closer to the basement membrane of the follicles. As follicle maturation advances, the decline in AR expression proceeds toward the antrum. However, the antral regions connected with cumulus cells and the cumulus cells themselves remain strongly positive for the AR. Thus, in preovulatory follicles, the AR is confined to the cumulus cells. High nuclear AR expression can also be observed in the expanded cumulus oophorous cells (Galas et al., 2012). These findings indicate that during the final stages of follicle maturation, the AR decline starts in the mural granulosa cells and progresses toward the antrum, but before and after ovulation, the AR remains highly expressed in the cumulus cells (Fig. 1).

In humans, the mRNA for the AR is not present in primordial follicles but appears in transitional and primary follicles. Moreover, AR immunostaining is most abundant in healthy preantral/early antral follicles and low in preovulatory follicles (Saunders et al., 2000). In premenopausal women, AR nuclear staining has been shown in granulosa cells and in a small proportion of stromal

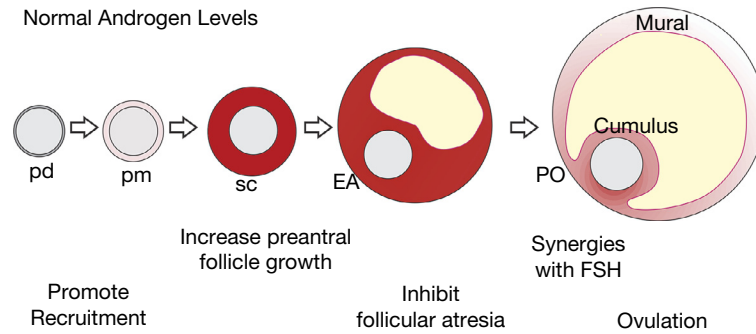


Fig. 1 Androgen receptor (AR) expression in granulosa cells throughout folliculogenesis and primary follicular mechanisms controlled by androgens under normal conditions. Increasing intensity of red color indicates higher AR expression. *pd*, primordial; *pm*, primary; *sc*, secondary; *EA*, early antral; *PO*, preovulatory. Granulosa cells, mural and cumulus.

cells. At the protein levels, human cumulus cells express high levels of AR, but it is almost undetectable in mural cells. By ICC, it was shown that AR is present in the nucleus of cumulus cells, and it is not affected by DHT treatment. More recent findings using PCR confirmed that the AR is not detectable in primordial human follicles, but as folliculogenesis progresses, the percentage of AR positive follicles at each stage increased linearly. This report also demonstrated that early follicles acquire the AR before they express the receptor for follicle-stimulating hormone (FSHR). In rhesus monkeys, there is noticeable heterogeneity in the follicular pattern of AR gene expression, with only a subpopulation of follicles demonstrating intense AR mRNA hybridization. AR mRNA appears more abundant in small- and medium-sized follicles compared with the large preovulatory follicles. In common marmoset, there are clear differences in AR immunostaining in the granulosa cells of immature and preovulatory follicles. Thus, in this species, the average AR level in the granulosa cells of immature follicles is 4.2-fold higher than that in the granulosa cells of preovulatory follicles. These findings strongly support the importance of androgens in early folliculogenesis in higher primates including humans.

In all species studied thus far, there is an evident decline in AR expression in mural granulosa cells, which are close to the wall of the follicle. In contrast, the receptor remains highly expressed in cumulus cells, which are in close contact with the oocyte (Fig. 1). These findings suggest that androgens can play a paracrine role in oocyte maturation by controlling cumulus cells function, whereas androgens actions on mural cells wane as the follicles reach the preovulatory state. Thus, in preovulatory follicles, androgens are mainly used to produce estradiol, a role that is potentiated by the high levels of aromatase expressed by the mural granulosa cells. However, despite the progressive decline toward the preovulatory stage, the AR remains clearly detectable at the mRNA and protein levels in preovulatory mural granulosa cells of rodents and primates. The AR mRNA not only remains detectable in the granulosa cells of preovulatory follicles but also increased in response to the ovulatory gonadotropin stimulus, suggesting it may play a role during ovulation.

Genes Targeted by Androgens in the Ovary

Only a few genes have been confirmed to be direct targets of the AR in ovarian granulosa cells.

FSHR

Androgens seem to sustain FSHR expression in mouse granulosa cells, and a positive association between AR and FSHR mRNA levels has been described in small antral follicles in humans. In mice, androgens appear to increase FSHR protein levels without affecting the transcription of the *Fshr* gene (Sen et al., 2014). However, how the AR may only enhance the translation of FSHR is not understood, although paxillin, a cytoskeletal protein involved in cell adhesion to the extracellular matrix, seems to be involved. The effect of androgens on the FSHR protein synthesis does not appear to be universal as androgens were shown to increase FSHR mRNA levels by several authors. For instance, testosterone increases FSHR mRNA expression in primate primary follicles, and DHT increases FSHR in porcine preovulatory follicles. In female rhesus monkeys, testosterone also augments follicular FSHR expression.

Furthermore, global AR knockout mice showed no decrease in FSHR mRNA levels (Shiina et al., 2006), whereas other global AR knockout mice showed a decline in FSHR expression in ovaries of 10-day-old mice (Hu et al., 2004). Similarly, conditional knockout of the AR in the granulosa cells has no effect on FSHR mRNA levels when compared with controls. In addition, testosterone stimulation of mouse and rat granulosa cells does not affect FSHR mRNA or protein levels. Therefore, the role of androgen on FSHR expression is not entirely understood yet. In AR knockout females (see succeeding text), follicles develop until the antral and preovulatory stages, which are dependent on FSH actions. Therefore, even in the absence of androgen effects, FSH appears to promote follicle development suggesting that androgens are not essential for FSHR expression, at least in rodents.

Experiments using luteinized human granulosa cells showed that testosterone could increase the expression of the FSHR. However, this report used an extremely high concentration of testosterone ($> 30 \mu\text{M}$). As androgen levels in the follicular fluid are in the nanomolar range, the effects observed at these higher concentrations do not seem to have physiological importance.

IGF1 and IGF1R

In rhesus monkeys, androgen treatment results in a threefold increase in insulin-like growth factor 1 (IGF1) and a fivefold increase in IGF1R in primordial follicle oocytes (Vendola et al., 1999). Similarly, testosterone and DHT increase IGF1 mRNA levels in granulosa, thecal, and interstitial cells of small antral follicles in primates. In the same system, androgens have no effect on the expression of the insulin receptor.

Kit Ligand

Microarray analysis in ovaries of AR knockout mice identified kit ligand (kit-L) as a possible target of androgens (Shiina et al., 2006). In fact, in primary rat granulosa cells, androgens stimulate kit-L expression. In addition, androgens increase the activity of the kit-L promoter in a human granulosa cell line. Moreover, the AR binds to the kit-L promoter in living cells. These findings suggest that kit-L is a direct target of the AR in the granulosa cells.

COX2 and AREG

Exogenous gonadotropin-induced superovulation increases serum androgen levels robustly in female mice suggesting that androgens may be involved in ovulation. In fact, in rodents, DHT treatment induces the expression of prostaglandin-endoperoxidase synthase 2 (COX2) and of epidermal growth factor-like factor, amphiregulin (AREG), which both are involved in ovulation. These genes are also induced by DHT in granulosa tumor-derived cells. Further studies demonstrated the presence of androgen-response sequence(s) upstream of COX2 and AREG, and that these regions are occupied by the AR in periovulatory granulosa cells.

Cyclin D2

In rat granulosa cells, DHT inhibits FSH-mediated granulosa cell proliferation by reducing cyclin D2 mRNA expression causing cell cycle arrest at the G1/S interphase. DHT exerts this inhibitory effect on cyclin D2 mRNA expression by reducing FSH-mediated phosphorylation of ERK via inhibition of protein kinase A. In these cells, DHT also reduces insulin-mediated cyclin D2 mRNA expression (Kayampilly and Menon, 2006).

miR-125b

In mouse granulosa cells, the AR targets miR-125b. The authors demonstrated the presence of AR response elements in the miR-125b promoter and revealed that the AR binds to the miR-125b promoter only in the presence of androgens.

Role of Androgens in Folliculogenesis

Currently, it is widely accepted that androgen actions change from stimulation to inhibition as follicles mature. Moreover, it has been suggested that inappropriately high levels of androgens promote follicular atresia. Here, we will briefly describe known effects of androgens throughout folliculogenesis.

Primordial Follicles

Androgens promote the initiation of primordial follicle growth in mice, sheep, and primates. However, it has been shown that primordial human follicles do not express the AR. Therefore, it is expected that this effect of androgen is indirect via androgen activation of primary and secondary follicles, which in turn produce factors, such as IGF1, that stimulate primordial follicle activation.

Preantral and Early Antral Follicle

In primates, bovines, and rodents, androgens promote early follicle growth. In vitro findings demonstrated that testosterone and DHT improve the survival of preantral follicles in primates. In rhesus monkeys, DHT rescues follicle growth after treatment with drugs that inhibit steroid synthesis. In rhesus monkeys, high levels of AR mRNA correlate with elevated levels of granulosa cell proliferation and the absence of apoptotic cell death in these same follicles. In contrast, low or undetectable levels of AR mRNA are associated with barely detectable proliferation but abundant apoptotic cell death (Weil et al., 1998). Testosterone stimulates the primary to secondary follicle transition in fetal bovine ovaries, an effect that is inhibited by antagonists of the AR. Also in bovines, testosterone promotes the growth of follicles, whereas estradiol has no effects, suggesting that the effect of testosterone

is not due to its conversion to estradiol. Recent findings in rats demonstrated that DHT promotes follicle growth in a stage-dependent manner via changes in granulosa cell proliferation and apoptosis. Thus, androgen stimulation of preantral follicles increases rat granulosa cell proliferation and decreases apoptosis. The combination of these effects augments follicular growth. However, androgens effects on follicle growth and antrum formation seem to follow a biphasic pattern. Thus, low levels of androgens induce follicle maturation, while high levels inhibit oocyte maturation and follicle growth (Lebbe et al., 2017).

Large Antral to Preovulatory Follicles

At the antral stage, androgens may have positive or adverse effects on follicular growth (Fig. 2). In rat granulosa cells of antral follicles, androgens augment the stimulatory effect of FSH on estradiol, aromatase, and plasminogen expression. On the other hand, androgens increase follicular atresia and inhibit FSH-induced granulosa cell proliferation in antral follicles. Moreover, in primates, it has been shown that DHT inhibits the production of estradiol, which is exclusively produced by antral follicles. Strikingly, AR antagonists can amplify the stimulatory effects of FSH and LH, suggesting that endogenous androgens block gonadotropin-stimulated ovulation. In fact, in primates, high concentrations of DHT are antagonistic to gonadotropin-stimulated ovarian function. The mechanisms involved in these differential effects of androgens during folliculogenesis remain to be determined.

Finally, in rhesus monkeys, AR expression increases during the induction of ovulation along with a temporary rise in androstenedione levels in the follicular fluid. This suggests that androgens may also play a role in the periovulatory follicles. In mice lacking the AR, treatment with gonadotropins causes an increase in apoptosis in antral follicles (see succeeding text). These results suggest that the AR plays a significant role in granulosa cell survival during the periovulatory stage, and the absence of AR may cause granulosa cells to be more susceptible to apoptosis.

Effect of the AR Knockdown on Female Fertility

Female global AR knockout mice are distinctively subfertile (Shiina et al., 2006; Hu et al., 2004). All global AR knockout female mice produce fewer pups/litter and exhibit abnormal estrous cycles, which in the mutant animals are longer and irregular. Interestingly, the ovaries of AR knockout mice contain a typical population of follicles at least up to 16 weeks of age. However, older AR knockout females exhibit an accelerated depletion of the follicle pool with a complete loss of follicles by 40 weeks of age. Atresia is also significantly increased in the ovaries of AR knockout mice. In general, evidence from the AR knockout mice suggests that the observed reduction in the numbers of pups produced by AR knockout females is due to dysfunctional follicular growth and the development of fewer preovulatory follicles (Fig. 2).

Mice that express a mutant AR lacking the DNA binding domain show a similar phenotype to the global knockouts, which include subfertility, dysfunctional follicle development, ovulation disruption, and age-dependent subfertility (Walters et al., 2007). Therefore, classical genomic AR-mediated effects are critical for optimal follicle growth and successful ovulation.

The knockdown of the AR specifically in the granulosa cells also leads to impaired fertility, with reduced numbers of litters and smaller litter sizes and irregular cyclicity (Sen and Hammes, 2010). However, the knockdown of the AR in the theca cells or in the oocyte has no major effects on female reproduction (Ma et al., 2017; Sen and Hammes, 2010). This suggests that the role of androgens in female fertility is mainly mediated by their regulation of granulosa cell function.

The deletion of the AR in the pituitary does not alter the estrous cycles, but these animals are also subfertile and produce fewer pups per litter (Wu et al., 2014). In these animals, all follicle populations are normal except for a decrease in antral follicles and

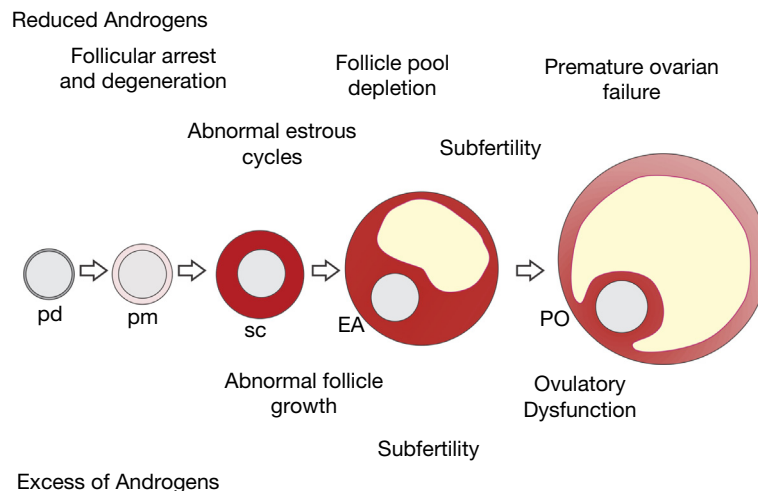


Fig. 2 A decrease or an excess of androgens significantly alter the normal progress of folliculogenesis. See Fig. 1 for more details.

corpora lutea count. Pituitary-specific AR knockout females also have reduced FSH levels at proestrus and diminished ovulatory LH levels. This suggests that AR signaling in the pituitary plays a significant role in maintaining optimal gonadotropin levels.

In humans, androgen depletion also reduces fertility. Indeed, patients with diminished ovarian reserve appear to have an androgen deficiency and benefit from androgen supplementation. However, this clinical practice remains controversial because the most basic ovarian mechanisms controlled by androgens remain unknown. This controversy is also fueled by the fact that elevated androgen levels are associated with poor reproductive health. For instance, hyperandrogenemia is present in up to 70% of women with PCOS, a disease that significantly reduces fertility and has an incidence of 6%–10% in reproductive-aged women. Thus, the evidence available clearly indicates a delicate equilibrium between beneficial and harmful effects of androgens in female reproduction.

Conclusions

The evidence supports an important role of androgens in follicle growth and ovarian function. Androgens appear to participate in follicle development from the initiation of primordial follicle growth all the way to ovulation. During this process, the granulosa cells are the primary target of the androgens, although a neuroendocrine effect of androgens may also participate in the regulation of normal ovarian function. Most strikingly, it is clear that optimal androgen levels are required for normal ovarian function. Thus, diminished androgenic activity leads to subfertility, while androgen excess negatively affects ovulation. The mechanisms that control the balance between harmful and beneficial effects of androgens in ovarian function are starting to be elucidated.

References

- Galas, J., Slomczynska, M., Knapczyk-Stwora, K., Durlaj, M., Starowicz, A., Tabarowski, Z., Rutka, K., & Szoltys, M. (2012). Steroid levels and the spatiotemporal expression of steroidogenic enzymes and androgen receptor in developing ovaries of immature rats. *Acta Histochemica*, 114, 207–216.
- Hirai, M., Hirata, S., Osada, T., Hagihara, K., & Kato, J. (1994). Androgen receptor mRNA in the rat ovary and uterus. *The Journal of Steroid Biochemistry and Molecular Biology*, 49, 1–7.
- Hu, Y. C., Wang, P. H., Yeh, S., Wang, R. S., Xie, C., Xu, Q., Zhou, X., Chao, H. T., Tsai, M. Y., & Chang, C. (2004). Subfertility and defective folliculogenesis in female mice lacking androgen receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 11209–11214.
- Kayampilly, P. P., & Menon, K. M. (2006). Dihydrotestosterone inhibits insulin-stimulated cyclin D2 messenger ribonucleic acid expression in rat ovarian granulosa cells by reducing the phosphorylation of insulin receptor substrate-1. *Endocrinology*, 147, 464–471.
- Laird, M., Thomson, K., Fenwick, M., Mora, J., Franks, S., & Hardy, K. (2017). Androgen stimulates growth of mouse Preantral follicles in vitro: Interaction with follicle-stimulating hormone and with growth factors of the TGFbeta superfamily. *Endocrinology*, 158, 920–935.
- Lebbe, M., Taylor, A. E., Visser, J. A., Kirkman-Brown, J., Woodruff, T. K., & Arit, W. (2017). The steroid metabolome in the isolated ovarian follicle and its response to androgen exposure and antagonism. *Endocrinology*, 158, 1474–1485.
- Ma, Y., Andrisse, S., Chen, Y., Childress, S., Xue, P., Wang, Z., Jones, D., Ko, C., Divall, S., & Wu, S. (2017). Androgen receptor in the ovary theca cells plays a critical role in androgen-induced reproductive dysfunction. *Endocrinology*, 158, 98–108.
- Saunders, P. T., Millar, M. R., Williams, K., Macpherson, S., Harkiss, D., Anderson, R. A., Orr, B., Groome, N. P., Scobie, G., & Fraser, H. M. (2000). Differential expression of estrogen receptor-alpha and -beta and androgen receptor in the ovaries of marmosets and humans. *Biology of Reproduction*, 63, 1098–1105.
- Sen, A., & Hammes, S. R. (2010). Granulosa cell-specific androgen receptors are critical regulators of ovarian development and function. *Molecular Endocrinology*, 24, 1393–1403.
- Sen, A., Prizant, H., Light, A., Biswas, A., Hayes, E., Lee, H. J., Barad, D., Gleicher, N., & Hammes, S. R. (2014). Androgens regulate ovarian follicular development by increasing follicle stimulating hormone receptor and microRNA-125b expression. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 3008–3013.
- Shiina, H., Matsumoto, T., Sato, T., Igarashi, K., Miyamoto, J., Takemasa, S., Sakari, M., Takada, I., Nakamura, T., Metzger, D., Chambon, P., Kanno, J., Yoshikawa, H., & Kato, S. (2006). Premature ovarian failure in androgen receptor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 224–229.
- Vendola, K., Zhou, J., Wang, J., & Bondy, C. A. (1999). Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. *Human Reproduction*, 14, 2328–2332.
- Walters, K. A., Allan, C. M., Jimenez, M., Lim, P. R., Davey, R. A., Zajac, J. D., Illingworth, P., & Handelsman, D. J. (2007). Female mice haploinsufficient for an inactivated androgen receptor (AR) exhibit age-dependent defects that resemble the AR null phenotype of dysfunctional late follicle development, ovulation, and fertility. *Endocrinology*, 148, 3674–3684.
- Weil, S. J., Vendola, K., Zhou, J., Adesanya, O. O., Wang, J., Okafor, J., & Bondy, C. A. (1998). Androgen receptor gene expression in the primate ovary: Cellular localization, regulation, and functional correlations. *The Journal of Clinical Endocrinology and Metabolism*, 83, 2479–2485.
- Wu, S., Chen, Y., Fajobi, T., Divall, S. A., Chang, C., Yeh, S., & Wolfe, A. (2014). Conditional knockout of the androgen receptor in gonadotropes reveals crucial roles for androgen in gonadotropin synthesis and surge in female mice. *Molecular Endocrinology*, 28, 1670–1681.

Aromatization

Jennifer R Wood and Andrea S Cupp, UNL Animal Science, Lincoln, NE, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Dehydroepiandrosterone (DHEA) 19-Carbon androgen synthesized in the adrenal glands, gonads, and brain; it can also have a sulfate group attached (DHEA-S).

DNA methylation Process by which a methyl group is attached to DNA; increases or decreases in this process determines if a DNA sequence can be transcribed into RNA.

Endogenous A protein or hormone that is normally synthesized by a cell or tissue in the body.

Endoplasmic reticulum A structure in the cytoplasm of a cell; it usually has ribosomes attached and is involved in protein and lipid synthesis by a cell.

Epiphyseal fusion The epiphysis is located at the end of long bones and is the site of bone growth; at the end of adolescence the epiphysis fuses with the rest of the bone effectively terminating future bone growth.

Exogenous A factor (organic or synthetic) that is produced outside of the body.

Exon Segment of RNA sequence that contains the coding information for a protein or peptide.

Formic acid A simple carboxylic acid with the chemical formula HCOOH; an intermediate in chemical synthesis reactions.

Genitalia Organs associated with reproduction; commonly used to describe the external reproductive organs.

Genome Complete set of genes or genetic material present in a cell.

Gonad Organ that produces germ cells or gametes; in mammals, it refers to the ovary which produces oocytes or testis which produces sperm.

Histone modifications Histones are proteins that DNA wraps around within the nucleus of a cell; each histone protein has a tail which can have acetyl, phosphate, or methyl groups attached; these modifications to histone tails regulate DNA transcription rates.

Homozygous Having identical pairs of genes (one on each chromosome pair) for any given hereditary characteristic.

Kinases Enzyme that catalyzes the transfer of a phosphate group from ATP to a specific protein or molecule; important regulator of signal transduction.

Missense mutation A change in a single nucleotide of DNA; when copied to mRNA the mutation causes a switch in the amino acid added to the protein.

Nicotinamide adenine dinucleotide phosphate (NADPH) Cofactor used in anabolic chemical reactions associated with the synthesis of lipids and nucleic acids.

Osteopenia Condition of lower than normal bone mineral density; precursor to osteoporosis.

P450 oxidoreductase An enzyme inserted in the endoplasmic reticulum membrane which transfers electrons from NADPH to other cytochrome P450 enzymes (e.g., aromatase).

Phenyl ring Cyclic group of atoms with the formula C₆H₅.

Senescence Biological aging; in the context of the ovary it refers to depletion of oocytes in the ovary and marks the end of reproductive function in the female.

Sexually dimorphic nucleus (SDN) of the preoptic area (POA) Cluster of cells within the brain; located in the anterior hypothalamus and thought to be an important determinant of sexual behavior.

Transcription Biological process of synthesizing an RNA message (mRNA); copy of the DNA gene sequence.

Translation Biological process of synthesizing protein from mRNA; mRNA code is recognized and determines how amino acids are added to the growing protein.

Virilization Development of male physical characteristics in a female or a boy prior to puberty; usually occurs due to exposure to excess androgens.

X-ray crystallography A technique that uses X-ray technology to examine the structure of DNA or a protein which is captured in a crystal.

Introduction

Estrogens are a class of 18-carbon steroid hormones that, like all steroid hormones, are synthesized from cholesterol. In mammalian species, a series of enzymatic reactions result in conversion of cholesterol into one of three endogenously produced estrogens; estrone, estradiol, and estriol (**Fig. 1A**). The common characteristic of all three estrogens is the presence of a phenyl ring (**Fig. 1A**) which is the result of a final aromatization step. The substrates for this irreversible aromatization reaction are androgens,

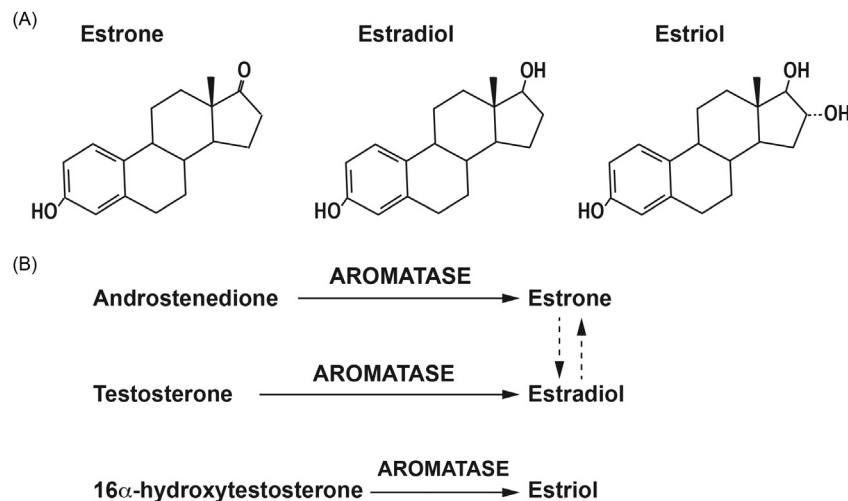


Fig. 1 (A) Chemical structure of estrone, estradiol, and estriol. (B) Schematic showing androgen substrates that are converted to specific estrogens by the enzyme aromatase. Based on DiNardo, G. and Gianfranco, G. (2013). Human aromatase: perspectives in biochemistry and biotechnology. *International Union of Biochemistry and Molecular Biology* **60**, 92–101.

a class of 19-carbon steroids. Specifically, the immediate steroid precursor for estrone is androstenedione while testosterone is the precursor to estradiol and 16 α -hydroxytestosterone is the precursor to estriol (Fig. 1B). The focus of this article is on the enzyme aromatase, which catalyzes the aromatization of each 19-carbon androgen into an 18-carbon estrogen. The regulation of aromatase expression and activity, which represents an important regulatory aspect of the physiological actions of estrogens, will also be discussed.

Conversion of Androgens to Estrogens Is Regulated by the P450 Enzyme, Aromatase

Aromatase is a single enzyme that belongs to the cytochrome P450 family. This family includes enzymes involved in drug metabolism and hormone biosynthesis and degradation. Like many of its family members aromatase is located in the endoplasmic reticulum of the cell. Purification and sequencing of the aromatase protein was accomplished in the 1980s using tissues from several species including human placenta. These studies showed that the aromatase protein sequence is highly conserved between mammalian species especially in the predicted androgen binding site. Recently, the three-dimensional structure of the protein was also defined. Using X-ray crystallography, investigators identified not only how the protein is folded into its final structure but also the site where androstenedione binds (Ghosh et al., 2010). In addition to the binding site for androstenedione, and presumably other androgens, a binding site for NADPH and cytochrome P450 oxidoreductase (CPR) which are essential for the activity of aromatase, was also identified. Interestingly, CPR is a common partner of all cytochromes P450 and therefore is expressed throughout the body.

The consensus is that the aromatase enzyme catalyzes three independent steps that convert androgens to estrogens (Yoshimoto and Guengerich, 2014; Di Nardo and Gilardi, 2013). The first two steps are well established hydroxylation reactions (Fig. 2). During the third step, the 19th carbon is released as formic acid due to a demethylation reaction which concomitantly causes the formation of the characteristic phenolic ring of estrogen (Fig. 2). However, the exact sequence of biochemical events associated with this third step is still under investigation. The energetic cost of this three-step enzymatic reaction is the consumption of oxygen (Fig. 2). Likewise, the reactions require the donation of electrons from NADPH to aromatase which is mediated by CPR. The product of the reaction is of course estrone, estradiol, or estriol dependent on the substrate used in the reaction (e.g., Fig. 2 shows conversion of androstenedione to estrone).

Aromatase Is Expressed in a Variety of Tissues

Ovary and Placenta

The aromatase protein is normally expressed in a wide variety of cells and tissues in both males and females (Table 1). The classic and most abundant site of estrogen synthesis is the female ovary. Specifically, aromatase is expressed in granulosa cells and to a lesser extent theca cells (Corbin et al., 2003), which are the somatic cells within the ovary that directly surround oocytes. Aromatase is also expressed in the ovarian corpus luteum (Tripathy et al., 2016), a transient ovarian cell type that arises from theca and granulosa cells after oocyte ovulation. Due to the high levels of aromatase activity in the ovary, the primary estrogen produced in pre-menopausal women is estradiol, which is secreted into the circulation and therefore acts as an endocrine hormone. As described above, the substrate for this aromatase is testosterone which in humans is predominately produced by the theca cells. The placenta, a transient organ essential for pregnancy, is also a significant source of aromatase activity in women. Indeed, as indicated above, the placenta was an important tissue source for aromatase protein purification. Within the placenta, aromatase synthesizes all three estrogens;

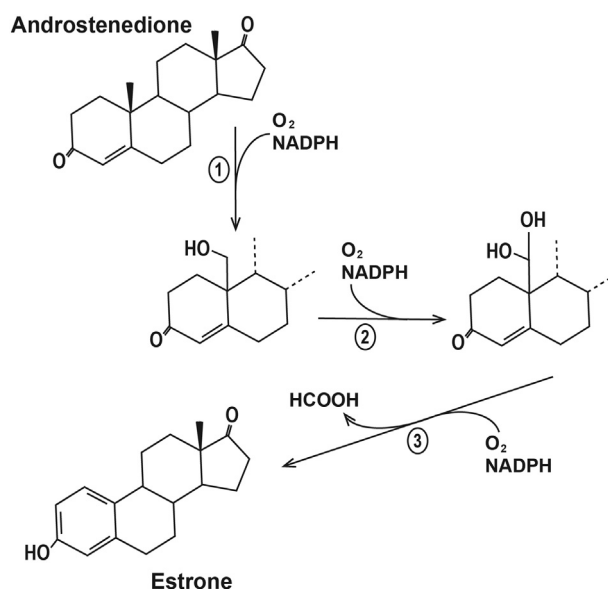


Fig. 2 Chemical reaction whereby androstenedione is converted to estrone. The consumption of oxygen (O_2) and use of NADPH at each step (1–3) are indicated. The production of formic acid in the third and final step of the reaction is indicated as well. Based on Yoshimoto, F. K. and Guengerich, F. P. (2014). Mechanism of the third oxidative step in the conversion of androgens to oestrogen by cytochrome P450 19A1 steroid aromatase. *Journal of the American Chemical Society* **136**, 15016–15025.

Table 1 Normal tissue expression of aromatase

Ovary—follicular and luteal cells
Testis—somatic and germ cells
Placenta
Brain/neurons
Adipose tissue
Bone
Vascular endothelium

that is, estrone, estradiol, and estriol. The result of placental aromatase is a 100-fold increase in circulating estrone and estradiol in women at the end of pregnancy. Furthermore, there is a 1000-fold increase in circulating estriol in pregnant compared to non-pregnant women. Interestingly, as pregnancy progresses, the predominate source of androgen substrates is the fetus (Kaludjerovic and Ward, 2012). Specifically, within the fetal adrenal gland, dehydroepiandrosterone sulfate (DHEA-S) is produced from maternal cholesterol. The DHEA-S is subsequently converted to androstenedione, testosterone, or 16 hydroxytestosterone by placental enzymes which are then converted to estrone, estradiol, or estriol by aromatase.

Testis

In the last few decades, the importance of aromatase expression and activity in the male testis has become fully realized. In adult animals and humans, the primary site of aromatase expression is in the Leydig cells which are a steroidogenically active somatic cell of the testis. Because the major androgen synthesized by Leydig cells is testosterone, the major estrogen produced by testicular aromatase is estradiol. Like the ovary, testis-derived estradiol enters the circulation; however, the concentration is significantly less than the amount of estradiol secreted by the ovary. Interestingly, studies in numerous species also show aromatase expression in developing and mature sperm (Haverfield et al., 2011). It is hypothesized that aromatase produces local estrogens which may regulate sperm motility; although, additional studies are required to confirm this finding. Finally, aromatase expression has been detected in the epididymis which is an important site of sperm storage. The estradiol produced within the epididymis is important for regulating the environment within this compartment where the final steps in sperm maturation occur that result in its ability to effectively fertilize eggs (Haverfield et al., 2011).

Brain

In addition to the gonads, distinct regions of the brain express aromatase which results in localized synthesis and action but not significant secretion of estrogens. Within the hypothalamus, conversion of testosterone to estradiol by aromatase is important

for endocrine regulation of gonad function particularly in males. Likewise, aromatase activity is positively correlated to the expression of male sexual behaviors. These reproductive effects are best described in the male brain due to increased availability of circulating substrate (i.e., testosterone) and increased responsiveness of aromatase within the male brain to the circulating androgens (Roselli et al., 2009). Aromatization of testosterone to estradiol in the fetal brain is also an important determinant of sexual behavior in the adult male. Based on studies in sheep, rats, mice and pigs, it appears that differential development of the sexually dimorphic nucleus (SDN) of the preoptic area (POA) of the brain, which is an important site that determines sexual behavior including partner preference, is correlated to aromatase activity. Specifically, in male fetuses, testosterone from the fetal testis is converted to estradiol in neural tissue of the POA which results in development of the SDN. Furthermore, the production of estradiol increases the expression and activity of the neural aromatase. Alternatively, in female fetuses, there is low circulating testosterone and therefore local estrogen synthesis and SDN development is significantly reduced. Interestingly, the female brain can be masculinized if the fetal brain is exposed to exogenous testosterone. Likewise, the male brain can be feminized if aromatase expression or activity is impaired in the developing POA. While these hypothalamic sites are important for reproductive function and behaviors, local aromatase activity within the hippocampus, cerebral cortex, and spinal cord produces estrogen which contributes to neural health particularly at sites of neural injury. Interestingly, it appears that aromatase is also expressed by neurons including at the site of neuron-to-neuron communication and therefore may influence important functions including cognition, memory, and sensory perception (e.g., pain) (Roselli, 2007).

Adipose Tissue and Bone

Aromatase is also expressed in several other extragonadal tissues in men and women (Stocco, 2012). For example, its expression and activity in bone cells is correlated with increased bone density. Likewise, adipose tissue is a significant source of aromatase activity. Interestingly, expression and activity of aromatase in adipose tissue increases with age making it an important source of estrogens, particularly in post-menopausal women. It should be noted that like aromatase activity in the brain, the majority of estrogen synthesized by aromatase within these non-reproductive tissues are not released into the circulation but rather stay and act locally in the surrounding tissue. Reviews on additional functions of locally-produced estrogens in extragonadal tissues can be found in Simpson et al. (2000).

Regulation of Aromatase Expression Is Tissue Specific

Expression of the aromatase protein is positively correlated with transcriptional activity at the gene encoding its messenger RNA (mRNA). The aromatase gene is called *CYP19* and it spans approximately 130 kilobases on human chromosome 15. Human *CYP19*, a single copy gene, is arranged into 10 exons with exons II-X containing the coding sequence for the aromatase protein. Exon I encodes for untranslated sequence of the mRNA and together with the DNA proximal to exon I (i.e., the promoter) contributes to the regulation of *CYP19* mRNA synthesis. Ten alternative exon I sequences have been identified in human tissues that express *CYP19* (Fig. 3). For example, exon I.1 is transcribed in placenta whereas exon I.6 is transcribed in bone. Alternatively, in both male and female gonads, transcription starts in exon IIa which is located adjacent to the common exon II (Fig. 3). Interestingly, the

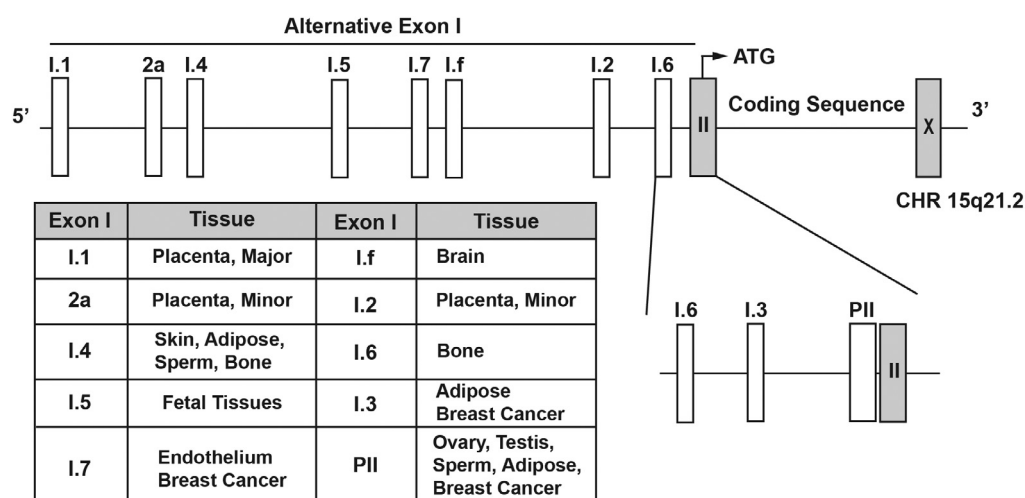


Fig. 3 Schematic organization of the *CYP19* gene which is located on human chromosome 15. The translational start site (ATG) and alternative exon I sequences are indicated. Tissue specific expression of alternative exon I sequence is also indicated in the table. Based on Bulun, S. E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H. and Shozu, M. (2003). The human *CYP19* (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. *Journal of Steroid Biochemistry and Molecular Biology* **86**, 219–224.

promoter and alternative exon I used in a particular cell type is often altered if/when it transforms into a cancer cell. For example, adipocytes associated with breast cancer cells express *CYP19* mRNA from exon IIa rather than exon I.4 which is used in normal adipocytes (Fig. 3). In contrast to the human genome, the genomes of some other species contain more than one *CYP19* gene. For example, in pigs, there are three different *CYP19* genes which produce three similar but unique aromatase proteins in gonads, placenta, and embryo (Kamat et al., 2002). Likewise, goldfish and zebrafish genomes contain two copies of the *CYP19* gene one of which is expressed in the gonads and the other in the brain.

The genomic arrangement (i.e., the use of alternative exon I and promoter sequences) of human *CYP19* provides regulatory diversity of its expression in the absence of gene duplication. Indeed, an important area of research is understanding tissue-specific regulation of *CYP19* transcription by different growth factors and signaling pathways. These collective studies have, to date, been performed using predominately in vitro cell culture models. Future studies will be required to fully understand the complexities by which transcription and signaling factors regulate the expression of *CYP19* within diverse cells and tissues. In addition to these traditional mechanisms of transcriptional regulation, interesting novel regulators of *CYP19* mRNA abundance have been identified. For example, in the turtle fetus, temperature changes alter DNA methylation and histone modifications resulting in increased or decreased aromatase expression within the developing gonad (Matsumoto et al., 2016). In this way, temperature determines development of the female ovary versus the male testis in developing turtles. Recent studies also show an important role for micro RNAs in the regulation of *CYP19* mRNA abundance. MicroRNAs (miRs) are small non-coding pieces of single stranded RNA that bind to messenger RNAs and promote their degradation and/or inhibit mRNA translation. In developing placental cells, miR-17~92 and miR-106a~363 bind to and reduce the amount of *CYP19* transcripts (Kumar et al., 2013).

Despite the differential expression of the unique exon I (or IIa) sequences in unique tissue and cell types, each first exon joins with the common exon II at the same nucleotide position. Furthermore, the translation of aromatase protein initiates within the common exon II; and therefore, the same aromatase protein is produced in each cell and tissue. It is important to note growing evidence that regulation of aromatase enzyme activity is also an important regulatory mechanism particularly in cells where local estrogen production is rapidly increased or decreased. An example of this type of regulation has been described in quail brain and cell lines expressing human aromatase. Specifically, these studies show that the rate of aromatase enzymatic activity is rapidly decreased when the protein has a phosphate group added to specific amino acids by calcium-dependent and calcium-independent kinases (Charlier et al., 2011).

Aromatase Deficiency Causes Abnormalities in Reproductive and Non-Reproductive Systems

Genetic mutations in the human aromatase gene are rare. In fact, prior to the 1990s it was believed that the loss of aromatase expression would result in embryonic or fetal loss. However, missense mutations in the *CYP19* gene have since been identified in a small number of individuals (Bulun, 2014). Homozygous mutations in *CYP19* render the aromatase enzyme essentially inactive resulting in dramatic reductions in circulating estrogen. In females, the loss of aromatase is immediately apparent. Due to the loss of placental derived estrogens, the female fetus exhibits masculinized external genitalia. Furthermore, disrupted communication between the ovary and pituitary results in pubertal failure and multi-cystic ovaries. In the male, the loss of aromatase activity associated with genetic mutation in the *CYP19* gene is not apparent until after puberty. In the adult male, sperm are immotile resulting in infertility. There is also reduced male libido although the severity of this phenotype is variable. Interestingly, women who are pregnant with a fetus carrying a *CYP19* mutation also exhibit phenotypes associated with virilization including increased acne and hirsutism. These phenotypes are a result of reduced placental estrogen production combined with increased fetal adrenal androgen synthesis; however, it is a transient condition which resolves after parturition.

In addition to reproductive phenotypes, both males and females with a *CYP19* mutation have abnormalities in bone growth. Specifically, there is increased linear growth due to a lack of epiphyseal fusion and at the same time osteopenia resulting in reduced bone density. Individuals carrying a *CYP19* mutation are also at increased risk for metabolic dysfunction due to insulin resistance and abnormal lipid metabolism. Interestingly, post-menopausal women, who experience a natural drop in aromatase expression due to ovarian senescence, experience similar bone and metabolic phenotypes. Clinically, these phenotypes can be reversed by estrogen replacement. It should be noted that an aromatase knock-out (ArKO) mouse model was generated in 1998 by two independent groups (Simpson et al., 2005). Phenotypes of the ArKO mouse include reproductive abnormalities, bone density loss, insulin resistance, obesity, and lipid excess in the liver and are consistent with the phenotypes identified in humans with a naturally occurring *CYP19* mutation.

Aromatase Inhibitors Represent Important Therapeutics Against Estrogen Excess

While estrogen deficiencies associated with *CYP19* mutations are rare, estrogen excess associated with benign (endometriosis, stunted growth) and malignant (cancers of the breast, uterus, and prostate) disease is quite prevalent in the human population. Estrogen excess is generally due to increases in local rather than circulating estrogen production. For example, altered exon I and promoter usage leads to increased estrogen production by adipose tissue within the mammary gland (Fig. 3). In some cases, aromatase is abnormally expressed in a tissue. Indeed, it is the abnormal expression and activity of aromatase in uterine stroma which increases local estrogen levels and results in development of endometriosis. Alternatively, increases in estrogen may be simply due

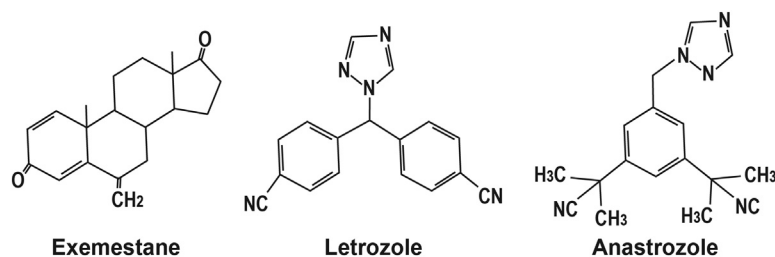


Fig. 4 Chemical structure of aromatase inhibitors including a steroidal analog (exemestane) and non-steroidal molecules (letrozole, anastrozole). Based on Dunkel, L. (2006). Use of aromatase inhibitors to increase final height. *Molecular and Cellular Endocrinology* **254–255**, 207–216.

to increases in the amount of adipose tissue which some individuals carry (i.e., obesity). To combat estrogen excess, aromatase inhibitors have been designed which bind to the enzyme and prevent the binding of endogenous C19-androgens. Unlike androgens, these synthetic molecules cannot be aromatized and this causes dramatic decreases in the amount of locally produced estrogen. Inhibitors that are currently used clinically include exemestane, anastrozole, and letrozole (Fig. 4, note their structural similarity to androgens). Given the potential ability of aromatase inhibitors to combat breast cancer and endometriosis, new inhibitors that are more potent, more stable, and have greater specificity (i.e., they do not inhibit any other P450 enzymes) are desired. Therefore, this is an active area of current research (Ghosh et al., 2016).

References

- Bulun, S. E. (2014). Aromatase deficiency. *Fertility and Sterility*, *101*, 323–329.
- Charlier, T. D., Harada, N., Balthazart, J., & Cornil, C. A. (2011). Human and quail aromatase activity is rapidly and reversibly inhibited by phosphorylating conditions. *Endocrinology*, *152*, 4199–4210.
- Corbin, C. J., Moran, F. M., Vidal, J. D., Ford, J. J., Wise, T., Mapes, S. M., Njar, V. C., Brodie, A. M., & Conley, A. J. (2003). Biochemical assessment of limits to estrogen synthesis in porcine follicles. *Biology of Reproduction*, *69*, 390–397.
- Di Nardo, G., & Gilardi, G. (2013). Human aromatase: perspectives in biochemistry and biotechnology. *International Union of Biochemistry and Molecular Biology*, *60*, 92–101.
- Ghosh, D., Griswold, J., Erman, M., & Pangborn, W. (2010). X-ray structure of human aromatase reveals an androgen-specific active site. *Journal of Steroid Biochemistry and Molecular Biology*, *118*, 197–202.
- Ghosh, D., Lo, J., & Egbuta, C. (2016). Recent progress in the discovery of next generation inhibitors of aromatase from the structure-function perspective. *Journal of Medicinal Chemistry*, *59*, 5131–5148.
- Haverfield, J. T., Ham, S., Brown, K. A., Simpson, E. R., & Meachem, S. J. (2011). Teasing out the role of aromatase in the healthy and diseased testis. *Spermatogenesis*, *1*, 240–249.
- Kaludjerovic, J., & Ward, W. E. (2012). The interplay between estrogen and fetal adrenal cortex. *Journal of Nutrition and Metabolism*, *2012*, 837901.
- Kamat, A., Hinshelwood, M. M., Murry, B. A., & Mendelson, C. R. (2002). Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans. *Trends in Endocrinology and Metabolism*, *13*, 122–128.
- Kumar, P., Luo, Y., Tudela, C., Alexander, J. M., & Mendelson, C. R. (2013). The c-Myc-regulated microRNA-17~92 (miR-17~92) and miR-106a~363 clusters target hCYP19A1 and hGCM1 to inhibit human trophoblast differentiation. *Molecular and Cellular Biology*, *33*, 1782–1796.
- Matsumoto, Y., Hannigan, B., & Crews, D. (2016). Temperature shift alters DNA methylation and histone modification patterns in gonadal aromatase (cyp19a1) gene in species with temperature-dependent sex determination. *PLoS One*, *11*, e0167362.
- Roselli, C. F. (2007). Brain aromatase: roles in reproduction and neuroprotection. *Journal of Steroid Biochemistry and Molecular Biology*, *106*, 143–150.
- Roselli, C. E., Liu, M., & Hum, P. D. (2009). Brain aromatization: classic roles and new perspectives. *Seminars in Reproductive Medicine*, *27*, 207–217.
- Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Jones, M., & Davis, S. (2000). The role of local estrogen biosynthesis in males and females. *Trends in Endocrinology and Metabolism*, *11*, 184–188.
- Simpson, E. R., Misso, M., Hewitt, K. N., Hill, R. A., Boon, W. C., Jones, M. E., Kovacic, A., Zhou, J., & Clyne, C. D. (2005). Estrogen-the good, the bad, and the unexpected. *Endocrine Reviews*, *26*, 322–330.
- Stocco, C. (2012). Tissue physiology and pathology of aromatase. *Steroids*, *77*, 27–35.
- Tripathy, S., Asaithambi, K., Jayaram, P., & Medhamurthy, R. (2016). Analysis of 17beta-estradiol (E2) role in the regulation of corpus luteum function in pregnant rats: involvement of IGFBP5 in the E2-mediated actions. *Reproductive Biology and Endocrinology*, *14*, 19.
- Yoshimoto, F. K., & Guengerich, F. P. (2014). Mechanism of the third oxidative step in the conversion of androgens to estrogens by cytochrome P450 19A1 steroid aromatase. *Journal of the American Chemical Society*, *136*, 15016–15025.

Further Reading

- Balthazart, J. (2017). Steroid metabolism in the brain: from bird watching to molecular biology, a personal journey. *Hormones and Behavior*, *93*, 137–150.
- Blakemore, J., & Naftolin, F. (2016). Aromatase: contributions to physiology and disease in women and men. *Physiology*, *31*, 258–269.
- Boon, W. C., Chow, J. D. Y., & Simpson, E. R. (2010). The multiple roles of estrogens and the enzyme aromatase. *Progress in Brain Research*, *81*, 209–232.
- Bulun, S. E., Sebastian, S., Takayama, K., Suzuki, T., Sasano, H., & Shozu, M. (2003). The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. *Journal of Steroid Biochemistry and Molecular Biology*, *86*, 219–224.
- Carreau, S., Bourguiba, S., Lambard, S., Galeraud-Denis, I., Genissel, C., & Levallet, J. (2002). Reproductive system: aromatase and estrogens. *Molecular and Cellular Endocrinology*, *193*, 137–143.
- Dunkel, L. (2006). Use of aromatase inhibitors to increase final height. *Molecular and Cellular Endocrinology*, *254–255*, 207–216.
- Gruber, C. J., Tschugguel, W., Schneeberger, C., & Huber, J. C. (2002). Production and action of estrogens. *New England Journal of Medicine*, *346*, 340–352.
- Hong, Y., Li, H., Yate-Ching, Y., & Chen, S. (2009). Molecular characterization of aromatase. *Annals of the New York Academy of Sciences*, *1155*, 112–120.

- Karaer, O., Oruc, S., & Koyuncu, F. M. (2004). Aromatase inhibitors: possible future applications. *Acta Obstetrica et Gynecologica Scandinavica*, 83, 699–706.
- Pasqualini, J. R. (2005). Enzymes involved in the formation and transformation of steroid hormones in the fetal and placental compartments. *Journal of Steroid Biochemistry and Molecular Biology*, 97, 401–415.
- Santen, R. J., Brodie, H., Simpson, E. R., Siiteri, P. K., & Brodie, A. (2009). History of aromatase: saga of an important biological mediator and therapeutic target. *Endocrine Reviews*, 30, 343–375.
- Simpson, E., & Santen, R. J. (2015). Celebrating 75 years of oestradiol. *Journal of Molecular Endocrinology*, 55, T1–T20.

Activins and Inhibins in Female Reproduction

Daniel J Bernard, Gauthier Schang, Yining Li, and Luisina Ongaro, McGill University, Montreal, QC, Canada
Thomas B Thompson, University of Cincinnati, Cincinnati, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Autocrine When a cell secretes a chemical messenger that acts back on that same cell.

Affinity The strength of the interaction between a receptor and its ligand.

β -strand A polypeptide chain of 3–10 amino acids with backbone in an extended conformation.

Competitive receptor antagonist An agent that displaces an endogenous ligand by binding to the same site on the receptor, without activating downstream intracellular signaling.

Corpus luteum The remnant of the ovarian follicle following ovulation; and the principal source of progesterone during the luteal phase.

Cystine-knot Protein structure composed of three disulfide bridges (between pairs of cysteine residues). Two of the bridges form a loop through which the third bridge passes.

Dimeric A protein that is composed of two subunits (can be different or identical).

Estrus The stage of the female rodent reproductive cycle during which the female is sexually receptive following ovulation.

Follicle The functional unit of the ovary, which is composed on the oocyte surrounded by somatic cells.

Furin A protein belonging to the proprotein convertase family. A calcium-dependent serine protease that cleaves precursor proteins.

Gonadotrope cell/gonadotropes A cell type of the anterior pituitary gland that produces the gonadotropins: LH and FSH.

Gonadotropins Dimeric glycoprotein hormones produced by gonadotrope cells. LH and FSH are the canonical members of this protein hormone family; however, human chorionic gonadotropin (hCG) and equine chorionic gonadotropin (eCG), secreted from the placenta, are also considered gonadotropins.

Granulosa cell Somatic cells within the ovarian follicle that surround the oocyte. They produce estrogens and inhibins.

Kinase Enzymes that catalyze phosphorylation reactions.

Luteal cell The cells that comprise the corpus luteum. They produce progesterone and, in humans, inhibin A.

Paracrine When a cell secretes a chemical messenger that acts on neighboring cells.

Proestrus The stage of the female rodent reproductive cycle just prior to ovulation. High levels of estrogens trigger the LH surge on the afternoon of proestrus.

Prohormone A precursor form of a hormone that must be processed (usually by proteolysis) into the biologically active form.

Proteoglycan Proteins with glycosaminoglycan chains covalently attached.

Radioimmunoassay A test used to quantitatively measure concentrations of hormones in circulation or in tissues.

Theca cell Somatic cells of the ovarian follicle that produce androgens in response to LH. Theca cells are the outermost layer of the follicle, encapsulating the granulosa cells.

Introduction

Reproduction is a complex physiological process controlled by hormones from the brain (hypothalamus), pituitary gland, and gonads (ovaries, in the case of females) (Fig. 1). The classical hormones in this hypothalamic-pituitary-gonadal (HPG) axis are gonadotropin-releasing hormone (GnRH) from the brain, the pituitary gonadotropins (FSH and LH), and the gonadal steroid hormones (e.g., estrogens and progesterone). GnRH is released from the brain into the pituitary portal vasculature and stimulates the synthesis and secretion of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by gonadotrope cells in the anterior pituitary. FSH and LH then travel via the bloodstream to the ovary, where they stimulate follicle growth (Fig. 2), estrogen production, and ovulation. Through a process called negative feedback, estrogens travel back to the brain to slow the rate of GnRH release and, consequently, FSH and LH secretion. A lesser known, but equally important, hormonal feedback system exists between the gonads and the pituitary in which protein hormones called inhibins (Fig. 3) travel from the ovary (and testes) to suppress FSH, but not LH, synthesis directly at the pituitary level. Inhibins suppress FSH by blocking the actions of activins, which are produced in the pituitary and promote FSH synthesis. Inhibins and activins also play local roles in the ovary, regulating follicle development. In this article, we describe the history of inhibin and activin research, the structure of the proteins, and how they regulate both pituitary and ovarian function.

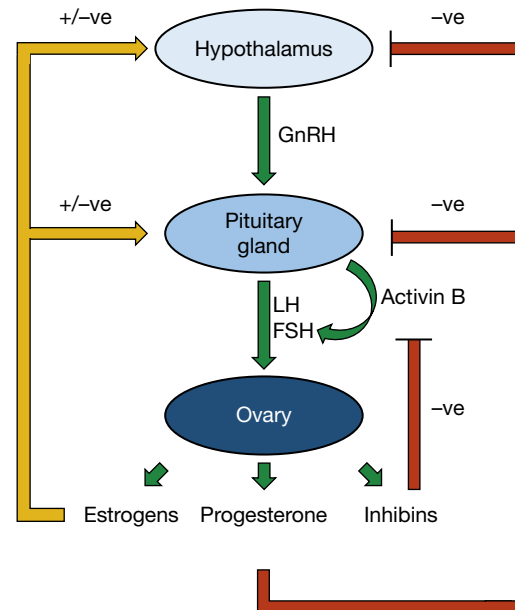


Fig. 1 Schematic representation of the hypothalamic-pituitary-gonadal axis. The hypothalamus secretes pulses of GnRH that enter the pituitary portal vessels at the level of the median eminence and thereby reach gonadotrope cells of the anterior pituitary gland. There, GnRH stimulates the production and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH enter the general circulation to reach the ovary where LH stimulates the production of androgens by theca cells; FSH stimulates granulosa cell proliferation and estrogen production. LH also stimulates ovulation. The remnants of the ovulated follicles form corpora lutea, which produce progesterone. Progesterone and estrogens enter the general circulation and feedback on the hypothalamus and the pituitary gland to negatively regulate the release of GnRH, and LH and FSH, respectively. Estrogens can also have positive feedback effects, promoting GnRH and LH release prior to ovulation. Gonadotropes also secrete activins, which act in an autocrine/paracrine fashion to specifically stimulate FSH. Activin signaling, and therefore FSH synthesis and secretion, is antagonized by inhibins from the ovaries. -ve, negative feedback. +ve, positive feedback. Green and red arrows/lines denote positive and negative arms of the axis. Estrogen feedback can be both negative and positive (in females) and is depicted with yellow arrows.

Discovery of Inhibins and Activins

Inhibin was discovered, or at least hypothesized, in the late 1920s to early 1930s. Scientists observed that removing the testes from rats (i.e., castration) caused structural changes (specifically hypertrophy) in a subset of cells of the anterior pituitary gland. These “castration” cells, as they were then called, are now known to be gonadotropes, which produce FSH and LH. As these large cells emerged only following castration, researchers reasoned that the testes must produce factors that regulate pituitary cell function,

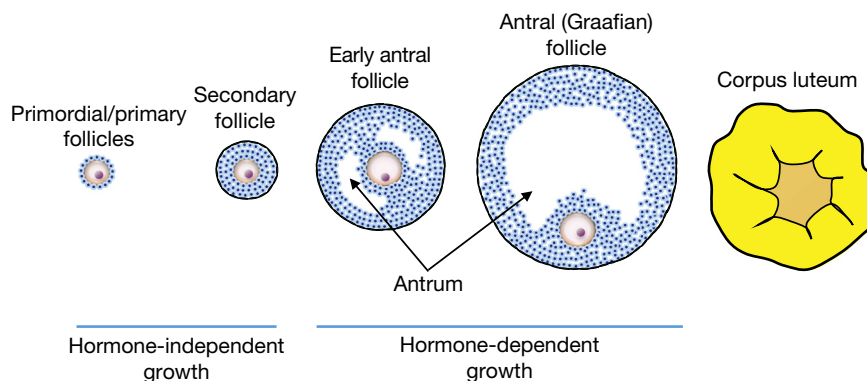


Fig. 2 Stages of ovarian follicle development. Follicles are formed in embryonic (human) or early postnatal (rodent) development. Primordial and primary follicles contain an oocyte surrounded by a single layer of immature or mature granulosa cells (blue). As follicles begin to grow to the secondary stage, granulosa cells proliferate, the oocyte grows, and the follicle becomes encapsulated by theca cells (black line encircling follicles). These early stages of follicle development occur in the absence of gonadotropins. Once follicles reach the early antral stage, their growth into preovulatory (antral/Graafian) follicles is driven by FSH. Following the mid-cycle surge of LH, the large follicle(s) ovulate (not pictured). The remnants of the follicle then transdifferentiate into the corpus luteum, which secretes progesterone and, in the case of primates, inhibin A. The fluid filled antra are labeled. Inhibins and activins were purified from this follicular fluid.

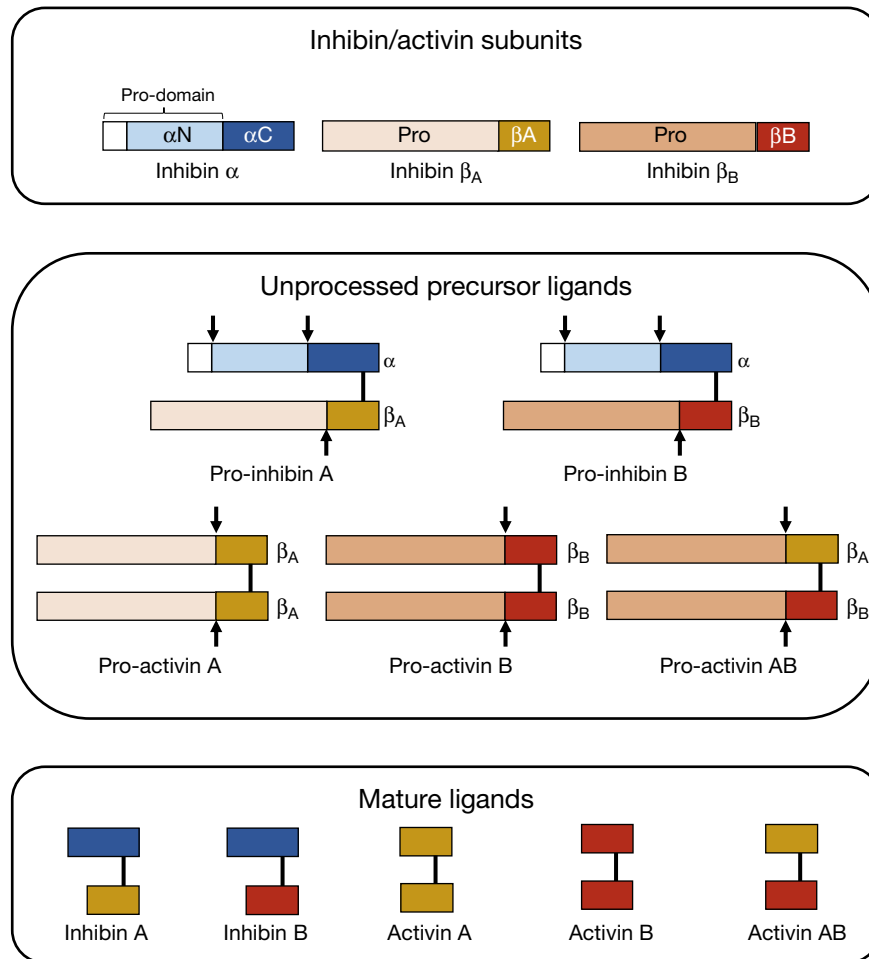


Fig. 3 Schematic representation of activins and inhibins. Top: The three inhibin subunits (inhibin α , β_A , and β_B) are composed of a pro-domain and a C-terminal mature region. The prodomain of inhibin α is cleaved in two; the large product is referred to as αN . The mature domain of inhibin α is referred to as αC . Middle: The different subunits can hetero- or homodimerize to form precursor forms of the inhibin and activin molecules. The mature regions are linked by intermolecular disulfide bonds (*thick vertical lines*). The prodomains are essential for dimerization and protein folding. The *arrows* indicate where furin and related proteases cleave the precursor molecules. Bottom: The mature forms of the inhibin (A or B) and activin (A, B, or AB) ligands.

either directly or indirectly. In an effort to determine the nature of these factors, investigators fractionated fat- and water-soluble components of the testes and injected them back into castrated rats. The water-soluble fraction prevented the formation of castration cells. As testosterone (then called androinitin) is fat-soluble, these results indicated that it was not a steroid hormone that regulated castration cells. Instead, researchers proposed that there must be a new hormone linking the testes and the pituitary. They dubbed this hormone “inhibin.”

Attempts to purify inhibin from testicular tissue were uniformly unsuccessful from the 1930s–1960s. With the benefit of hindsight, we now know that much of this failure derived from the fact that the adult testis produces relatively low amounts of inhibins, making it a poor source for protein purification. Research in the 1970s, however, represented a rebirth of inhibin research. Development of radioimmunoassays for FSH and LH in the mid-1960s enabled quantitative analyses of both hormones from the blood and tissues. Using these assays, researchers discovered that testicular extracts and ovarian follicular fluid (from the antrum of large follicles; Fig. 2) contained water-soluble proteins that could selectively suppress FSH secretion without affecting LH in castrated mice. They reasoned that these proteins were likely the long-sought inhibin and realized that its activity was that of a selective FSH inhibitor. As a result, the hypertrophy of gonadotrope (castration) cells reflected the increase in FSH synthesis in the absence of inhibin negative feedback.

Armed with the information that follicular fluid (which could be acquired in large volumes from cows, pigs, and sheep in slaughterhouses) was a rich source of inhibin, researchers now only needed an assay (test) in which to purify the protein(s). They found such a system in the form of cultured rat pituitary glands. When placed in culture, pituitary cells continue to produce FSH and LH, and secrete the hormones into the culture medium where they can be measured. When pituitary cultures were treated with unfractioated follicular fluid, they showed selective decreases in FSH secretion. These results demonstrated that inhibin could affect the

pituitary directly, and not indirectly via the hypothalamus and GnRH. With this powerful in vitro culture assay in hand, researchers biochemically processed large volumes of follicular fluid and honed in on the components (fractions) that retained selective FSH-suppressing activity. This purification procedure led to the isolation of two forms of inhibin (inhibin A and inhibin B) as well as a structurally-unrelated protein (follistatin), which selectively suppressed FSH (i.e., did not affect LH secretion). Serendipitously, and unexpectedly, two other proteins with structural similarity to the inhibins were purified, but were found to selectively stimulate FSH secretion. These proteins were dubbed activins (in this case, activin A and activin AB; Fig. 3). It was subsequently shown that follistatins bind and bionutralize the activins. Follistatins are the subject of another chapter in this volume and are therefore not considered in detail here.

Structure of Inhibins and Activins

The purification of inhibins from follicular fluid lead quickly to their molecular characterization as dimeric proteins of an α subunit (inhibin α) disulfide-linked to one of two β subunits (inhibin β A and inhibin β B; Fig. 3). The three proteins are derived from different genes on chromosomes 2 (α and β B) and 7 (β A), and are expressed as preprohormones. The amino-terminal prodomains are essential for proper folding and dimerization of the biologically active carboxy-terminal mature domains. Prodomains are cleaved from the mature domains by furin or related enzymes. Dimers of mature inhibin α with inhibin β A form inhibin A, whereas the inhibin α -inhibin β B heterodimer constitutes inhibin B. Remarkably, despite their opposing actions, activins are formed through homodimeric or heterodimeric bonding of inhibin β subunits. In the context of reproduction, three activins have been described: Activin A (β A- β A), B (β B- β B), and AB (β A- β B) (Fig. 3). Despite their high sequence identity, the β subunits (and hence the activin and inhibin isoforms) have both common and distinct biological functions, the latter of which are dictated by differences in their patterns of expression and their structures.

Inhibins and activins are members of the transforming growth factor β (TGF β) superfamily of secreted ligands, which includes TGF β s, growth differentiation factors, bone morphogenetic proteins, anti-Müllerian hormone, and nodal. Like other proteins in the family, inhibins and activins are secreted from cells noncovalently linked to their prodomains. Prodomain association can restrict the actions of other TGF β family members, but not the inhibins and activins. Indeed, recent data suggest that the inhibin α prodomain may actually facilitate receptor binding of the mature hormone, though the underlying mechanism is presently unresolved. Nonetheless, the mature dimer must eventually dissociate from the prodomains to bind to cell surface receptors or other binding proteins. Both inhibins and activins can be detected in the bloodstream, but only inhibins appear to act as bona fide hormones. Inhibins have short half-lives in circulation (less than 10 min). As a result, changes in circulating inhibins (e.g., Fig. 7) are directly linked to changes in subunit expression in the ovary. That is, when inhibin subunits are down-regulated, circulating levels of the hormone quickly decrease. Most activins in the bloodstream are bound to follistatins, which appear to irreversibly block activin action. Follistatins bind activins in a 2:1 stoichiometry (two molecules of follistatin for every activin dimer), blocking receptor binding sites on the ligand. Rather than functioning as hormones, activins instead act in autocrine or paracrine fashion, like most members of the TGF β family.

Inhibins and activins adopt a growth factor-like fold, classically described as a hand with a pair of reciprocating anti-parallel β -strands (representing four fingers), a central helix (representing the wrist) and an amino-terminal segment representing the thumb (Fig. 4A). A cystine-knot motif is found within the fingers near the wrist helix where two disulfide bonds from finger 2 to finger 4 form a ring-like structure. The knot-like structure comes from a third disulfide bond that loops through the center of the ring, joining fingers 1 and 3. Near the cystine-knot is an additional unpaired cysteine that forms the intermolecular disulfide bond linking the two monomers (Fig. 4B). This brings the two monomers together at the central wrist helix with the fingers from each monomer extending in different directions, giving the dimer a propeller or butterfly appearance (Fig. 4B, top).

Activin and Inhibin Signaling Mechanisms

In general, TGF β family members signal in cells through a conserved mechanism in which the dimeric proteins bind to complexes of transmembrane serine/threonine kinase receptors. Though there are 33 genes encoding TGF β ligands (subunits), there are only 12 receptors for the whole family. Thus, ligands share receptors. These receptors fall into two general classes: type I and type II. There are seven type I and five type II receptors in mammals. Both receptor types have extracellular amino-terminal domains, a single transmembrane domain, and an intracellular carboxy-terminus that contains the kinase domain. In general, the dimeric ligand binds to heterotetrameric complexes of two type II and two type I receptors (e.g., Figs. 4C and 5). Different ligands associate with receptors in different ways. In the case of activins, the ligand binds first to the type II receptors. The lower affinity type I receptors are then recruited into the complex. Other family members bind first to the type I receptor, others may bind to preassembled receptor complexes, and others require co-receptors (accessory membrane proteins) to engage type I or II receptors.

Regardless of the order of ligand binding, upon complex formation, type II receptors phosphorylate the associated type I receptors in a juxtamembrane domain referred to as the GS box. This activates the type I receptor kinases, enabling them to phosphorylate their intracellular substrates, the best known of which are the SMAD proteins. SMAD is a contraction of SMA and MAD (mothers against decapentaplegic), the founding members of the family in *Caenorhabditis elegans* (worms) and *Drosophila melanogaster* (flies), respectively. There are eight SMADs in mammals, which come in three forms: receptor-regulated (R-SMAD), common (SMAD4),

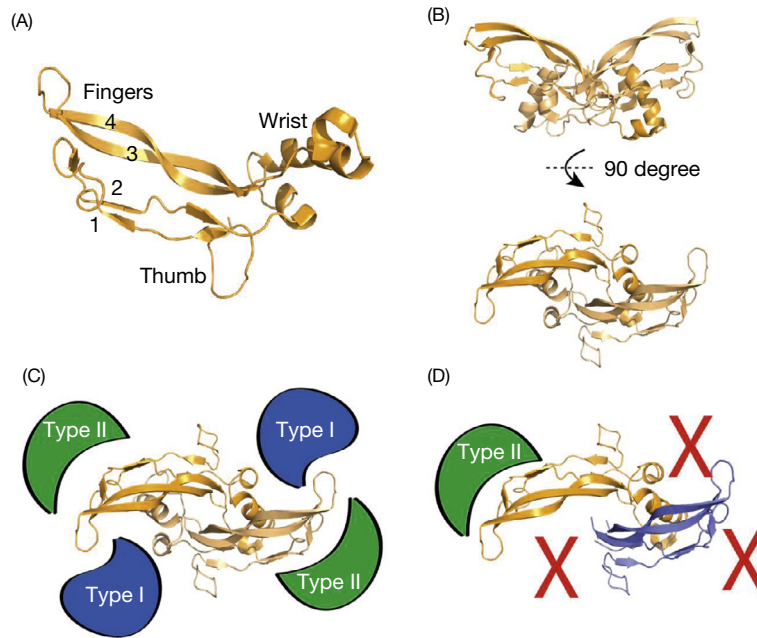


Fig. 4 Structure of activins and inhibins. Structural representation of the (A) activin A (individual βA subunit) monomer and (B) two views of the activin A dimer. In panel A, the four fingers are numbered. (C) The relative location of receptor binding sites for activin A. Note how the knuckles of the fingers of each monomer contact distinct type II receptors (green), which are positioned at opposite ends of the dimeric ligand. The structure of activin A bound to its type I receptor has not yet been solved. The positioning of the type I receptors here (blue) is modeled based on structures of other ligands and receptors in the family. Note how each type I receptor is predicted to bind at the dimer interface. (D) The structure of inhibin alone or in complex with receptors has not been solved. Here, we show a homology model of inhibin A and the predicted positioning of the activin type II receptor. The Xs denote how the presence of the α subunit (in blue) prevents binding of a second type II receptor and both type I receptors.

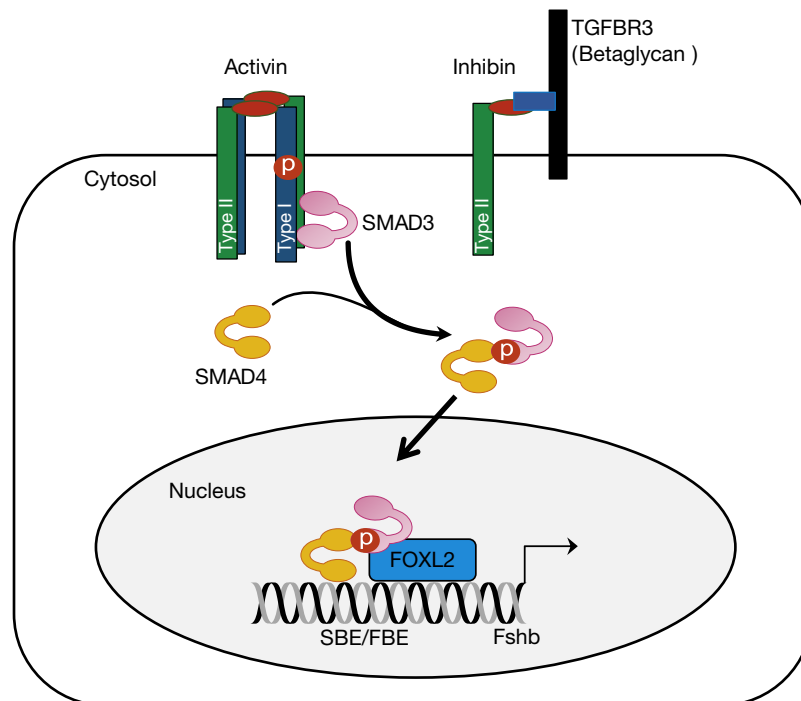


Fig. 5 Mechanisms of activin and inhibin regulation of FSH synthesis. Activins bind to complexes of type I (blue rectangles) and type II (green rectangles) receptor serine-threonine kinases. The type II receptors phosphorylate (P in red circles) the type I receptors in their juxtamembrane GS-domains. The type I receptors then phosphorylate SMAD3 on C-terminal serine residues. Phosphorylated SMAD3 then forms complexes with SMAD4, which accumulate in the cell nucleus. SMAD3/4 complexes partner with the transcription factor forkhead box L2 (FOXL2) and bind to the proximal promoter of the follicle-stimulating hormone β subunit gene (*Fshb*). SBE, SMAD binding element. FBE, FOXL2 binding element. Inhibins bind to complexes of the activin type II receptor and the TGF β type III receptor, betaglycan. Type I receptors are not recruited into these complexes. As a result, inhibins do not signal in cells and prevent activins from doing so.

and inhibitory (SMADs 6 and 7) (**Fig. 6**). R-SMADs and SMAD4 have a common domain structure with an amino-terminal MAD homology domain (MH1) and carboxy-terminal MH2 domain, joined by a variable linker region. The R-SMADs (SMADs 1, 2, 3, 5, and 8) are phosphorylated by type I receptors on serine residues at their extreme carboxy-termini. Phosphorylated R-SMADs then dissociate from the receptors and form heteromeric complexes, via their MH2 domains, with themselves and/or the common SMAD (SMAD4). These SMAD complexes then translocate to and accumulate in the nucleus. The MH1 domains of R-SMADs (except full-length SMAD2) and SMAD4 have a DNA binding domain, enabling them to interact directly with target genes to regulate transcription. SMAD3 and SMAD4 can bind a simple DNA sequence, GTCT (or its reverse-complement, AGAC). However, this binding is relatively weak; therefore, SMADs usually partner, again via their MH2 domains, with other DNA-binding proteins for higher affinity and specificity interactions.

Activins can bind to at least three type II receptors: the two activin type II receptors (ACVR2A and ACVR2B) and the bone morphogenetic protein receptor type II (BMPRII). They can similarly bind to at least three type I receptors (also known as activin receptor-like kinases or ALKs): ACVR1A (ALK2), ACVR1B (ALK4), and ACVR1C (ALK7); however, activin binding to wild-type ALK2 does not activate intracellular (SMAD) signaling. Interestingly, activin B and activin AB, but not activin A, can signal via ALK7. ALK4 is the primary signaling receptor for activin A. This demonstrates functional consequences for the structural (sequence) differences between the β subunits. ALK4 and ALK7 preferentially phosphorylate SMAD2 and SMAD3. As such, these SMADs are classically considered the principal signaling molecules activated downstream of activin receptor complexes. Phosphorylated SMAD2 and SMAD3 form complexes with SMAD4 before accumulating in the nucleus. The stoichiometry of these complexes is not entirely clear and may be context-specific. According to X-ray crystallographic studies, the MH2 domains can form trimers of two R-SMADs and one SMAD4.

Mechanisms of inhibin action are not fully described, particularly in vivo, but extant data converge to suggest that inhibins function as endogenous competitive receptor antagonists. By virtue of sharing β subunits with activins, inhibins can bind to activin type II receptors, which occurs exclusively through the β subunit (**Fig. 4D**). However, inhibins do not functionally bind type I receptors and therefore cannot propagate intracellular signals via classical TGF β pathways (i.e., via SMAD proteins). This is due to the α -subunit, which is expected to disrupt the type I binding surfaces that are formed at the interface of the two chains (**Fig. 4D**). Since inhibins only have one type II binding epitope they bind the type II receptors with 10-fold lower affinity than activins and are therefore weak antagonists on their own. However, in the presence of the TGF β type III receptor (TGFR3, also known as betaglycan), inhibins can form high affinity complexes with type II receptors that potently block activin binding and signaling (**Fig. 5**). Inhibins bind betaglycan via their α subunits, explaining why activins do not share this co-receptor. Betaglycan is a transmembrane proteoglycan. Inhibins bind the juxtamembrane portion of the extracellular domain. The intracellular carboxy-terminus of betaglycan lacks known functional motifs and is therefore not considered a signaling receptor. Instead, it acts to facilitate binding of inhibins (and TGF β isoforms) to type II receptors.

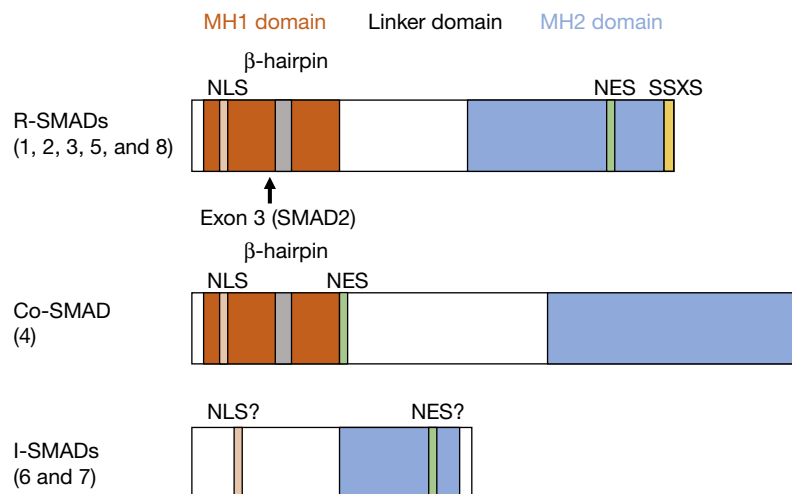


Fig. 6 Schematic representation of SMAD protein domain structures. SMAD proteins can be classified into three categories: (top) receptor-regulated (R-SMADs); (middle) common (co-SMAD); and (bottom) inhibitory (I-SMADs). Both R-SMADs and SMAD4 possess an N-terminal MAD homology 1 domain (MH1), which confers DNA binding activity via a β -hairpin structure that inserts into the major groove of DNA. Full-length SMAD2 cannot bind DNA because an additional exon (exon 3) introduces a sequence near the β -hairpin that sterically hinders DNA binding. The MH1 domain also encodes the nuclear localization signal (NLS), which directs the protein to the nuclear compartment. This activity is counteracted by the nuclear export signal (NES) in the MH1 (SMAD4) or C-terminal MH2 domain (R-SMADs). The linker domain can be phosphorylated by various kinases (such as ERK, AKT, JNK, and PI3K) and can regulate transactivation and binding to different partners. The SMAD activation domain (SAD) in the SMAD4 linker region is required for transcriptional activity. The MH2 domain, present in all SMADs, is essential for protein-protein interactions. The R-SMADs are phosphorylated by the type I receptors in their SSXS motif at the extreme C-terminus of the MH2 domain.

Inhibin and Activin Regulation of Follicle-Stimulating Hormone Synthesis

The main source of circulating inhibins is the ovary (and testes in males). Indeed, ovariectomy reduces inhibins in the blood to undetectable levels. Inhibin A and B are dynamically regulated across female reproductive cycles and are produced by different cell types, in species-specific fashion. In humans, inhibin B is principally produced by granulosa cells of growing follicles during the follicular phase of the menstrual cycle, whereas inhibin A is mainly derived from the corpus luteum following ovulation (Fig. 7A). In rats, inhibin B is produced by granulosa cells of small follicles, whereas inhibin A is the product of large, preovulatory follicles. As such inhibin B is elevated through most of the estrous cycle, where inhibin A increases steadily to a peak on the afternoon of proestrus (Fig. 7B). The preovulatory surges of LH (and FSH) down-regulate inhibin α production, leading to a rapid decline in circulating inhibins. In response to this loss of negative feedback, the pituitary gland rapidly upregulates FSH synthesis and secretion, leading to a 'secondary surge' of FSH on the morning of estrus, which drives the next wave of follicle development (Fig. 7B). Indeed, inhibins and FSH are strongly negatively correlated throughout the rat estrous cycle. Whether inhibins similarly restrain FSH during the human menstrual cycle is a matter of some debate; however, it is clear that inhibin B is the major negative feedback modulator of FSH in male primates. Also, perimenopausal increases in circulating FSH correlate more with loss of inhibin B than estrogen negative feedback. There is ample evidence to support the notion that inhibin B contributes to the pattern of FSH release during the follicular phase of the menstrual cycle. Whether inhibin A restrains FSH secretion during the luteal phase is less clear.

As indicated above, inhibins function as receptor antagonists. They therefore suppress FSH by blocking the actions of other proteins, most likely the activins, rather than by generating intracellular signals of their own. According to current dogma, pituitary gonadotrope cells produce activin B (the homodimer of $\beta\beta$ subunits). Activin B then acts on the gonadotrope cell that made it (autocrine) or on neighboring gonadotropes (paracrine) to stimulate FSH synthesis. The mechanisms through which activins regulate FSH synthesis have been most thoroughly characterized in mice. Some, though perhaps not all, of the mechanisms are likely to be conserved across mammalian species, including humans. FSH is a dimeric protein composed of the glycoprotein α subunit (not to be confused with the inhibin α subunit) noncovalently bound to the FSH β subunit. FSH β is rate-limiting in the production of FSH and activins stimulate transcription of the FSH β gene (*Fshb*). In the current model, activin B binds to complexes of ACVR2A and ALK4 or ALK7. The latter then phosphorylate SMAD3, which partners with SMAD4 and translocates into the nucleus. SMAD3/4 complexes then partner with the forkhead transcription factor FOXL2 and bind to the promoter (regulatory sequence) of the *Fshb* gene (Fig. 5). Some aspects of this model await in vivo validation. In particular, it is unclear whether ACVR2A is the only

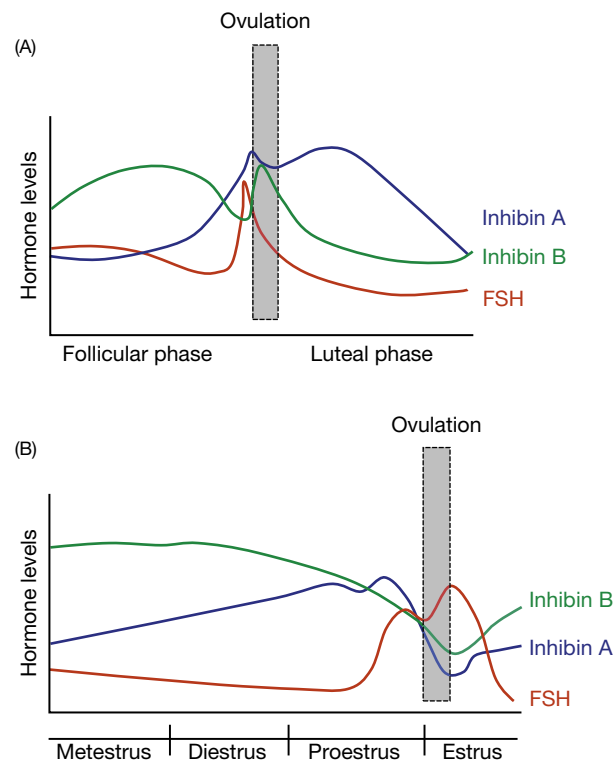


Fig. 7 Schematic representation of inhibin and FSH secretion across the human menstrual (top) and rodent estrous cycles (bottom). The figures show relative, not absolute amounts of inhibin A (blue), inhibin B (green), and FSH (red) in circulation. The different stages of the cycles are labeled. The approximate timing of ovulation (gray box) is shown. Ovulation is triggered by a surge of LH (not pictured, but the timing coincides with that of the primary FSH surge just before ovulation).

type II required or which type I receptors play a role. Genetic studies in mice also indicate that activin B is unlikely to be the only TGF β family member that stimulates the *Fshb* gene. Finally, there is some suggestion that activins not only stimulate *Fshb* transcription but also increase the half-life of the *Fshb* mRNA. This latter mechanism has not been investigated in detail.

Roles for Inhibins and Activins in the Ovary

Although best known for their roles in the selective regulation of FSH synthesis and secretion, inhibins and activins were first purified from follicular fluid and, indeed, have important local actions in the ovary. In fact, many intra-ovarian TGF β family members play essential roles in follicle development. Ovarian follicles consist of germ cells (oocytes) surrounded by somatic cells (granulosa and theca) (Fig. 2). The number and nature of these cells depends on the stage of follicle development. Immature (primordial or primary) follicles are surrounded by a single layer of granulosa or pregranulosa cells. As follicles mature, the granulosa cells proliferate and, themselves, become surrounded by theca cells. TGF β ligands are made by and act in oocytes, granulosa, and theca cells. Inhibin and activin subunits, however, are principally expressed in granulosa cells and, in some species, in luteal cells.

Our understanding of the intra-ovarian actions of inhibins and activins is incomplete; however, both appear to regulate steroid hormone production and granulosa cell proliferation, at least in certain contexts. Androgens (like androstenedione and testosterone) are produced by theca cells in response to pituitary LH. Inhibins stimulate and activins inhibit thecal androgen biosynthesis. As with FSH synthesis, activins appear to be the main actors in the system, with inhibins antagonizing their actions. Granulosa cells produce estrogens, principally in response to FSH. Here, FSH stimulates production of the aromatase enzyme, which converts theca-derived androgens into estrogens. Activin actions in granulosa cells are complex, and are both age- and developmental-stage dependent. For example, in follicles from immature mice, activins can potentiate the effects of FSH on granulosa cell proliferation and estrogen biosynthesis, in part by upregulating FSH receptor levels. However, in older mice, activins actually block FSH-stimulated follicle growth. It is presently unclear how activins produce these opposing activities.

Genetic studies in mice demonstrate important roles for inhibins and activins in follicle development and ovarian tissue homeostasis *in vivo*. Mice lacking the inhibin α subunit (*Inha* $-/-$) develop sex-cord stromal tumors beginning around 4 weeks of age. This observation led to the concept that inhibins act as tumor suppressors in the gonads and that excess activin signaling may promote tumor development. In the absence of the inhibin α subunit, the ovaries fail to make inhibins and instead produce excess activins, as β subunits can freely homo- or hetero-dimerize. These activins appear to contribute to tumor formation, as mice lacking both inhibin α and the activin type IIA receptor (*Acr2a*) show slower tumor progression than mice lacking inhibin α alone. However, *Acr2a* $-/-$ mice also have reduced FSH levels (because of impaired activin signaling in the pituitary). Therefore, it is possible that the loss of both activin and FSH action in the ovaries explains the slowing of tumor growth in these mice. Consistent with this idea, deletion of the *Fshb* gene (and therefore FSH) similarly slows tumor growth in *Inha* $-/-$ mice. Tumor development is entirely blocked in *Inha* $-/-$ mice lacking both FSH and LH. These data indicate a fundamental role for gonadotropins in gonadal tumor formation in *Inha* $-/-$ mice. More recent data also indicate that the loss of inhibin or gain of activin action may alter how the gonads respond to gonadotropins. Prior to tumor development, juvenile *Inha* $-/-$ mice exhibit aberrant ovarian physiology, with precocious granulosa cell development. When treated with exogenous gonadotropins these animals fail to produce antral follicles. Thus, both gonadotropin-independent and -dependent aspects of ovarian follicle development are dysregulated in the absence of inhibin (and/or in the presence of enhanced activin) action in the ovary.

Mice lacking inhibin β subunits display distinct phenotypes to those of *Inha* $-/-$ mice. Inhibin β A subunit knockout mice (*Inhba* $-/-$), which cannot make activin A, activin AB, or inhibin A, die shortly after birth. Inhibin β B subunit knockout (*Inhbb* $-/-$) develop relatively normally and are fertile; though the females exhibit impaired maternal behavior. These data suggest that activin B, activin AB, and inhibin B do not play essential roles in female fertility in mice or that activin A and inhibin A can compensate in their absence. Deletion of the inhibin β A subunit specifically in granulosa cells leads to subfertility, whereas mice lacking both β subunits in the ovary are sterile. These mice have complex, but intriguing ovarian phenotypes. There are no apparent alterations in the numbers of primordial, primary, or secondary follicles, suggesting that activins (and inhibins) may not be required for follicle formation or early (gonadotropin-independent) stages of follicle development. However, these animals, especially those lacking both β subunits, have increased numbers of antral (gonadotropin-dependent) follicles and an unusually large number of corpora lutea. Enhanced antral follicle development likely derives from elevated FSH levels (again due to loss of inhibin negative feedback). The increased numbers of corpora lutea may indicate a role for activins in regression of corpora lutea (luteolysis). That is, corpora lutea may persist in the absence of activin signaling.

Summary and Conclusions

Inhibins were first postulated in the 1930s, but were only purified over 50 years later. The following year, the activins were unexpectedly discovered as the yang to the yin of inhibins. Over the past 30 years, we have learned a great deal about how inhibins and activins are synthesized and how they act in cells. In the last 15 years, we have come to understand how activins (or related proteins) regulate FSH synthesis, the context in which they were discovered and, in fact, named. Though inhibins appear to act as receptor antagonists, this has not yet been confirmed *in vivo*. Biochemical analyses have revealed the three-dimensional structure of activins, how they bind to their type II receptors, and how they are antagonized by follistatins. Comparable structures for inhibins are needed

but are currently lacking. Though inhibins and activins were discovered in the context of reproduction, it is now abundantly clear that they play diverse roles during development and adulthood, in health and disease. For example, inhibins have been proposed to regulate bone physiology, whereas activins regulate muscle mass and stem cell fate. As we approach the 100 year anniversary of the naming of inhibin, it is exciting to ponder what the next century of inhibin and activin research will reveal.

Further Reading

- Bernard, D. J., & Tran, S. (2013). Mechanisms of activin-stimulated FSH synthesis: The story of a pig and a FOX. *Biology of Reproduction*, 88, 78.
- Burger, H. G. (1993). Evidence for a negative feedback role of inhibin in follicle stimulating hormone regulation in women. *Human Reproduction*, 8(Suppl 2), 129–132.
- Carroll, R. S., Corrigan, A. Z., Gharib, S. D., et al. (1989). Inhibin, activin, and follistatin: Regulation of follicle-stimulating hormone messenger ribonucleic acid levels. *Molecular Endocrinology*, 3, 1969–1976.
- Corrigan, A. Z., Bilezikjian, L. M., Carroll, R. S., et al. (1991). Evidence for an autocrine role of activin B within rat anterior pituitary cultures. *Endocrinology*, 128, 1682–1684.
- De Jong, F. H., & Sharpe, R. M. (1976). Evidence for inhibin-like activity in bovine follicular fluid. *Nature*, 263, 71–72.
- Fortin, J., Boehm, U., Deng, C. X., et al. (2014). Follicle-stimulating hormone synthesis and fertility depend on SMAD4 and FOXL2. *The FASEB Journal*, 28, 3396–3410.
- Lewis, K. A., Gray, P. C., Blount, A. L., et al. (2000). Betaglycan binds inhibin and can mediate functional antagonism of activin signaling. *Nature*, 404, 411–414.
- Li, Y., Schang, G., Boehm, U., et al. (2017). SMAD3 regulates follicle-stimulating hormone synthesis by pituitary gonadotrope cells in vivo. *The Journal of Biological Chemistry*, 292, 2301–2314.
- Makanji, Y., Zhu, J., Mishra, R., et al. (2014). Inhibin at 90: From discovery to clinical application, a historical review. *Endocrine Reviews*, 35, 747–794.
- Mason, A. J., Hayflick, J. S., Ling, N., et al. (1985). Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-beta. *Nature*, 318, 659–663.
- Matzuk, M. M., Finegold, M. J., JG, S., et al. (1992). Alpha-inhibin is a tumor-suppressor gene with gonadal specificity in mice. *Nature*, 360, 313–319.
- McCullagh, D. R. (1932). Dual endocrine activity of the testes. *Science*, 76, 19–20.
- Myers, M., Middlebrook, B. S., Matzuk, M. M., et al. (2009). Loss of inhibin alpha uncouples oocyte-granulosa cell dynamics and disrupts postnatal folliculogenesis. *Developmental Biology*, 334, 458–467.
- Pangas, S. A., Jorgez, C. J., Tran, M., et al. (2007). Intraovarian activins are required for female fertility. *Molecular Endocrinology*, 21, 2458–2471.
- Pangas, S. A., & Matzuk, M. M. (2004). Genetic models for transforming growth factor beta superfamily signaling in ovarian follicle development. *Molecular and Cellular Endocrinology*, 225, 83–91.
- Robertson, D. M., Foulds, L. M., Leversha, L., et al. (1985). Isolation of inhibin from bovine follicular fluid. *Biochemical and Biophysical Research Communications*, 126, 220–226.
- Schwartz, N. B., & Channing, C. P. (1977). Evidence for ovarian “inhibin”: Suppression of the secondary rise in serum follicle stimulating hormone levels in proestrous rats by injection of porcine follicular fluid. *Proceedings of the National Academy of Sciences of the United States of America*, 74, 5721–5724.
- Vale, W., Rivier, J., Vaughan, J., et al. (1986). Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature*, 321, 776–779.
- Vassalli, A., Matzuk, M. M., Gardner, H. A., et al. (1994). Activin/inhibin beta B subunit gene disruption leads to defects in eyelid development and female reproduction. *Genes & Development*, 8, 414–427.
- Woodruff, T. K., Besecke, L. M., Groome, N., et al. (1996). Inhibin A and inhibin B are inversely correlated to follicle-stimulating hormone, yet are discordant during the follicular phase of the rat estrous cycle, and inhibin A is expressed in a sexually dimorphic manner. *Endocrinology*, 137, 5463–5467.
- Woodruff, T. K., D’Agostino, J., Schwartz, N. B., et al. (1988). Dynamic changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle. *Science*, 239, 1296–1299.

Follistatin and Transforming Growth Factor β (TGF β) Family

Paul T Fullerton, Jr., Diana Monsivais, and Martin M Matzuk, Baylor College of Medicine, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

TGF β Family

The human transforming growth factor β (TGF β) superfamily, which includes > 35 members, is the largest family of growth factors in vertebrates. TGF β signaling regulates development and adult tissue homeostasis through context-dependent and cell-type-specific actions. The superfamily is named for the first member discovered, TGF β 1, which was first detected as one of two fractions, the first dubbed TGF α and the second TGF β , purified from acid-ethanol extracts of murine sarcoma cells transformed by Moloney sarcoma virus that enabled large colonies of normal rat kidney epithelial cells to grow in soft agar. Purification of the TGF β 1 peptide from human tissues confirmed that this transforming factor had a role in normal biology. Although first identified for its ability to promote anchorage-independent growth in vitro, it soon became clear that TGF β has cell-type- and context-dependent effects. Subsequent studies demonstrated that TGF β 1 is one member of a family of structurally-related ligands.

The TGF β family operates through a shared mechanism. The signaling cascade is initiated by a ligand homodimer or heterodimer binding to and bringing together pairs of type 1 receptor kinases with pairs of type 2 receptor kinases. This newly formed transmembrane receptor complex allows the type 2 receptor kinase to phosphorylate the type 1 receptor kinase, removing a conformational block on the kinase domain of the type 1 receptor and activating it. The activated type 1 receptor kinase can then phosphorylate receptor SMADs, allowing the receptor SMADs to form heterotrimeric complexes of two phosphorylated receptor SMADs and one common SMAD (co-SMAD, SMAD4). This receptor SMAD/SMAD4 complex concentrates in the nucleus, where it regulates gene expression directly and through interactions with numerous cofactors. This process is outlined in Fig. 1.

There are three major groups of related ligands within the TGF β family: the bone morphogenetic protein (BMP)/growth differentiation factor (GDF) group, the activin/nodal group, and the TGF β group. These groups all have partially overlapping preferences for type 1 receptor kinases, dubbed activin receptor-like kinases (ALK), each of which acts through one of two distinct sets of receptor SMADs. The BMP/GDF group binds ALK1, 2, 3, and 6, which phosphorylate SMAD1, SMAD5, and SMAD8. The activin/nodal group binds ALK4 and 7, which phosphorylate SMAD2 and SMAD3. The TGF β group binds ALK1, 2, 3, and 5; however,

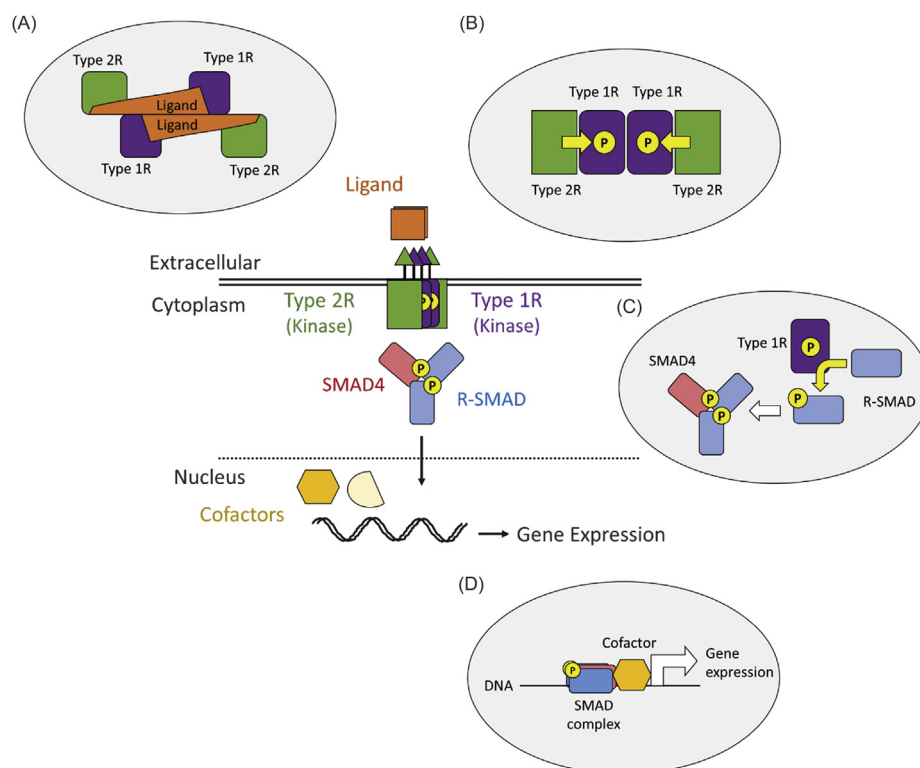


Fig. 1 The basic mechanism of TGF β family signaling. (A) Binding of the ligand dimer to two type 1 receptor kinases and two type 2 receptor kinases. (B) Upon ligand binding, the type 2 receptor kinase phosphorylates the type 1 receptor kinase, activating it. (C) The activated type 1 receptor kinase phosphorylates the receptor SMADs, which, once phosphorylated, form a heterotrimeric complex with SMAD4, which remains unphosphorylated. (D) The SMAD complex concentrates in the nucleus, where it regulates gene expression as a transcription factor by binding DNA with the cooperation of a wide array of cofactors.

Table 1 Type 1 receptor kinase interactions

Type 1 receptor	Alternate symbol	Ligands	R-SMAD
ALK1	ACVRL1	BMPs, TGF β s	SMAD1/5/8
ALK2	ACVR1A	BMPs, GDF5/6/7/9b/10/11/15, AMH, TGF β s	SMAD1/5/8
ALK3	BMPR1A	BMPs, GDF5/6/7/9b/10/11/15, AMH, TGF β s	SMAD1/5/8
ALK4	ACVR1B	myostatin, GDF1/3/9, activins, nodal	SMAD2/3
ALK5	TGFR1	myostatin, GDF1/3/9, TGF β s	SMAD2/3
ALK6	BMPR1B	BMPs, GDF5/6/7/9b/10/11/15, AMH	SMAD1/5/8
ALK7	ACVR1C	myostatin, GDF1/3/9, activins, nodal	SMAD2/3

R-SMAD is an abbreviation for receptor SMAD.

TGF β typically signals through ALK5, which phosphorylates SMAD2 and SMAD3. Type 1 receptor kinase interactions with ligands and receptor SMADs are detailed in [Table 1](#).

The type 2 receptor kinase binding follows a different pattern: activin receptor type 2A (ACVR2A) and activin receptor type 2B (ACVR2B) can bind BMPs, GDFs, and activin/nodal; BMP receptor type 2 (BMPR2) can bind BMPs; TGF β receptor type 2 (TGFR2) exclusively binds the TGF β s; and anti-Müllerian hormone receptor type 2 (AMHR2) exclusively binds AMH, a BMP-group ligand. Type 2 receptor kinase interactions with ligands are detailed in [Table 2](#).

Because of its critical roles in development and adult tissue homeostasis, the TGF β family includes regulators that can promote or inhibit signaling. Regulation occurs primarily at two levels, the TGF β family ligands by factors that sequester and bind ligands and the receptor SMADs by factors that regulate receptor SMAD phosphorylation.

Ligand binding to the type 1 and 2 receptor kinases is regulated by secreted ligand-binding proteins and by membrane-anchored co-receptors. Secreted ligand-binding proteins bind TGF β family ligands, preventing them from interacting with the type 1 and 2 receptor kinases. Follistatin, chordin, noggin, cerberus, gremlin, DAN, and follistatin-like proteins 1 and 3 are examples of ligand-binding proteins that regulate signaling through preferential binding to specific sets of ligands ([Table 3](#)). Similarly, membrane-anchored co-receptors bind TGF β family ligands to regulate ligand signaling; however, the effects of co-receptors on ligand signaling are more variable than those of secreted ligand-binding proteins. Co-receptor binding can promote ligand signaling, as in the case of betaglycan, which is required for TGF β 2 binding to TGFR2 or cripto, which facilitates nodal interactions with ALK4/7. Co-receptor binding can also inhibit signaling, as in the inhibition of activin signaling by cripto, which complexes with activin but prevents activin from binding its type 2 receptors. Additional examples of co-receptor regulation include DRAGON, a BMP co-receptor; repulsive guidance molecule (RGM), a BMP co-receptor; and endoglin, a co-receptor that interacts with ALK1 and 5 to regulate TGF β and BMP signaling ([Table 4](#)).

Receptor SMADs are regulated before and after phosphorylation. Receptor SMAD phosphorylation is promoted by recruitment of receptor SMADs to type 1 receptor kinases by anchor proteins; SMAD anchor for receptor activation (known as SARA) recruits SMAD2/3 and endoglin recruits SMAD1/5/8. Phosphorylation of receptor SMADs is also negatively regulated. The inhibitory

Table 2 Type 2 receptor kinase interactions

Type 2 receptor	Ligands
BMPR2	BMPs, GDFs
ACVR2A	BMPs, GDFs, myostatin, activins, nodal
ACVR2B	BMPs, GDFs, myostatin, activins, nodal
TGFR2	TGF β s
AMHR2	AMH

Table 3 Secreted ligand binding proteins

Secreted proteins	Target ligands
Follistatin	Activin; myostatin; BMP2, 4, 5, 6, 7, 15; GDF11; TGF β 3
Chordin	BMP2, 4, 7
Noggin	BMP2, 4, 5, 6, 7; GDF5, 6
Cerberus	BMP2, 4; nodal
Gremlin	BMP2, 4, 7
DAN	BMP2, 4; GDF5
Follistatin-like protein 1	BMP4
Follistatin-like protein 3	Activin; myostatin; BMP2

Table 4 Membrane-anchored co-receptors

Coreceptors	Targets	Action
betaglycan	TGF β 2	Promotes ligand binding to TGFBR2
cripto	nodal, activin	Promotes nodal binding to ALK4/7 Inhibits activin binding to type 2 receptors
DRAGON	BMP2, 4	Promotes binding to type 1 and type 2 receptors
RGM	BMP2, 4	Promotes binding to type 1 and type 2 receptors
endoglin	ALK1, ALK5	Promotes signaling through ALK1 and ALK5

Table 5 SMAD proteins

Name	Type	Summary
SMAD1	receptor SMAD	Phosphorylated by ALK1/2/3/6
SMAD2	receptor SMAD	Phosphorylated by ALK4/5/7
SMAD3	receptor SMAD	Phosphorylated by ALK4/5/7
SMAD4	common SMAD	Binds phosphorylated receptor SMADs
SMAD5	receptor SMAD	Phosphorylated by ALK1/2/3/6
SMAD6	inhibitor SMAD	Inhibits receptor SMADs
SMAD7	inhibitor SMAD	Inhibits receptor SMADs
SMAD8	receptor SMAD	Phosphorylated by ALK1/2/3/6

SMADs, SMAD6 and SMAD7, compete with receptor SMADs for access to activated type 1 receptor kinase. SMAD6 also continues to regulate receptor SMADs after phosphorylation, binding them and preventing complex formation with SMAD4. Mammalian SMAD family members are described in [Table 5](#).

In addition to signaling through phosphorylation of receptor SMADs, activated TGF β family type 1 receptors can phosphorylate non-SMAD cytoplasmic proteins to regulate ERK MAPK, JNK/p38 MAPK, Rho-like GTPase, and PI3K/AKT signaling pathways. Termed non-canonical TGF β signaling, these alternative signaling pathways play important roles in cancer and may have underappreciated roles in normal biology. The effects of non-canonical TGF β signaling include promoting epithelial to mesenchymal transition, migration, and invasion—cell behaviors critical in cancer, embryonic development, and placentation.

Follistatin

Discovery of Follistatin

The discovery of follistatin is intimately connected to the discovery of its signaling partners inhibin and activin. The concept of an inhibiting factor was originally articulated by Dr. Douglas Roy McCullagh in 1932 who proposed that a water-soluble hormone capable of abrogating gonadotrophin secretion by the pituitary is produced by the testis. The term inhibin was subsequently refined to refer to the factor produced by the gonads that regulates the secretion of follicle-stimulating hormone (FSH) by the pituitary and numerous studies were conducted to attempt to isolate the responsible protein. The development of a reliable and specific bioassay for regulation of basal FSH production, the rat anterior pituitary monolayer culture system, allowed protein extracts to be tested for inhibin activity. In 1985, three groups purified two highly similar 32 kDa proteins that inhibited FSH production in the bioassay from porcine ovarian follicular fluid. Their inhibins, termed inhibin A and B, were composed of a 20 kDa amino-acid chain and a 14 kDa amino-acid chain bound together by disulfide bonds. Subsequent studies would reveal that inhibin A and B are actually heterodimers formed of inhibin α with one of two inhibin β subunits, A or B, and that homo- and heterodimers formed by two inhibin β subunits have an opposite effect on pituitary FSH secretion, leading inhibin β dimers to be termed activins. Follistatin entered this mix as an incidental finding reported in the original papers describing the discovery of inhibin. A side fraction of the protein purification that isolated inhibin also had the ability to inhibit FSH secretion in the bioassay, but it was less potent, leading the original studies to focus on inhibin. Purification of the FSH secretion-inhibiting protein from the side fraction revealed a 35 kDa protein termed follistatin.

Biological Activity of Follistatin

The mechanism behind the FSH secretion inhibition by follistatin was uncovered when characterization of an activin-binding protein in the rat ovary showed the activin-binding protein was follistatin. Although initially described as a regulator of the pituitary, it quickly became clear that activin has diverse biological roles and belongs to a family of structurally-related secreted factors, the transforming growth factor β (TGF β) family. Follistatin binds not only activin, but other TGF β family ligands, including

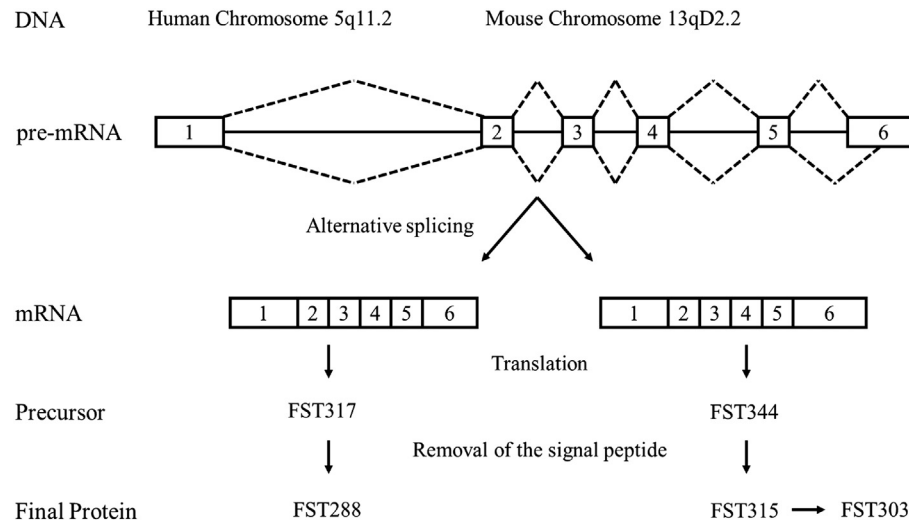


Fig. 2 The structure of follistatin and the generation of its three protein isoforms. FST288 and FST315 are generated through alternative splicing in exon 6 and FST305 is generated through proteolytic cleavage of FST315.

myostatin, BMP2, 4, 6, 7, 15, GDF11, and TGF β 3. However, follistatin strongly prefers binding to activin rather than to BMPs. Follistatin inhibits ligand activity by preventing binding of the ligand to its receptor complex. Follistatin also promotes clearance of its ligands through uptake of cell surface heparin-bound follistatin-ligand complexes, which are subsequently targeted for lysosomal degradation.

Structure of Follistatin

Characterization of the porcine and human cDNAs revealed the primary structure of follistatin. Follistatin has six exons expressed as two isoforms, FST288 and FST315, with alternate splicing of exon six in the smaller FST288 isoform. As a secreted protein, follistatin is expressed as a precursor of 317 or 344 amino acids with a 29-amino acid signal peptide that is proteolytically cleaved to generate a final product of 288 or 315 amino acids (Fig. 2). The FST288 and FST315 isoforms differ in their localization; FST288 is found tethered to cell surface-bound heparins, allowing it to act locally on paracrine and autocrine activin signaling whereas FST315 is the primary isoform in serum and can inhibit endocrine activin signaling. A third isoform, FST303, is produced by proteolytic cleavage of FST315 and shows similar localization to FST288 (Fig. 2). Structural studies show that follistatin is composed of an N-terminal unique domain, three consecutive repeated follistatin-like domains (Fs1, 2, 3), and, in the FST315 isoform, a C-terminal acidic tail. Each follistatin-like domain of 70–74 amino acids is composed of two subdomains—an epidermal growth factor (EGF)-like subdomain of ~30 amino acids and a Kazal protein inhibitor-like subdomain of ~40 amino acids. The three Fs domains bind target ligands, with Fs1 and Fs2 required for ligand binding, and Fs1 contains the heparin-binding domain. The C-terminal acidic tail of the FST315 isoform can interact with the heparin-binding domain, modulating its affinity for heparin from negligible to strong, based on local salt concentrations and whether FST315 is bound to a target ligand. The crystal structures of follistatin bound to two target ligands, activin A and myostatin, have been described. One follistatin molecule binds each monomer of the activin or myostatin dimer. In the case of activin, the convex surface of activin, which interacts with the type 2 receptor binding site, is blocked by the Fs1 and Fs2 domains of follistatin; however, the concave surface of activin, which interacts with the type 1 receptor binding site of myostatin, is not occluded. In contrast, both the type 2 and type 1 receptor binding sites of myostatin are blocked by follistatin, with the type 1 receptor binding site bound by the N-terminal domain of follistatin. Although crystal structures of the interaction between follistatin and BMPs have not been reported, sequence similarity/identity between BMPs and activin suggests a similar mode of binding that does not cover the type 1 receptor binding site of BMPs.

Mouse Models of Follistatin Dysfunction

Multiple transgenic models have been produced to study follistatin function (see Table 6).

Follistatin Knockout

Follistatin knockout mice are born at expected Mendelian ratios, but have multiple developmental defects and die within hours of birth because they fail to breathe. Development of muscles, bone, and skin are disrupted. Muscle mass is reduced, particularly in the diaphragm and intercostal muscles, likely leading to the respiratory failure. Skeletal defects include absent or delayed incisor

Table 6 Reproductive phenotypes of follistatin mouse models

Manipulation	Reproductive phenotype
knockout	Perinatal lethal; none reported
overexpression	Infertile; thin uteri, block in folliculogenesis, FSH deficient
hFST315 on <i>Fst</i> KO	Infertile; postnatal uterine developmental defects, uterine inflammation, premature ovarian failure
hFST288 on <i>Fst</i> KO	Perinatal lethal; none reported
FST315 KO	Subfertile; premature ovarian failure
<i>Fst</i> cKO by <i>Amhr2-cre</i>	Subfertile; premature ovarian failure
<i>Fst</i> cKO by <i>Pgr-cre</i>	Subfertile; embryo attachment failure and defective decidualization

cKO, conditional knockout.

development, cleft or soft palates, abnormalities in the thirteenth pair of ribs, and missing lumbar vertebrae. Skin is shiny and taut. Whiskers are unusually thin and improperly oriented. No defects in the development of the female reproductive tract were reported. Because follistatin null pups die shortly after birth, the effects on reproduction cannot be studied using a constitutive knockout.

Follistatin Overexpression

To study the roles of follistatin in reproduction and development, a gain-of-function mutant mouse model with overexpression of follistatin was developed. Adult females have reproductive defects, including small thin uteri, variable ovarian defects ranging from an early block in folliculogenesis before antral follicle formation to a block in the early primary follicle stage, and reduced concentrations of FSH. This model cannot distinguish between the effects of lower FSH concentrations on reproduction and possible roles of follistatin in the ovary and uterus.

Single-Isoform Follistatin Knockout

Because follistatin knockout is perinatal lethal, several studies have sought to use knockouts of only one of the two follistatin isoforms to study both general and isoform-specific roles of follistatin.

To study the isoform-specific roles of follistatin, mice that expressed FST315 or FST288 isoforms with native human promoter sequences on a *Fst* KO background were generated. hFST288 expressing mice are similar to *Fst* KO mice, exhibiting similar phenotypes and surviving only slightly longer. hFST315-expressing mice survive past birth and grow to adulthood, allowing the effects of FST288 knockout to be assessed. Females are infertile with ovarian and uterine abnormalities. Folliculogenesis is disrupted with fewer follicles of all stages, including no antral follicles or corpus luteum observed at 7 weeks of age. The phenotype appears to worsen over time with young adult mice displaying estrous behavior, but losing the behavior as they age, suggesting that hFST315 mice suffer from premature ovarian failure. Uterine development is abnormal, with dramatically shorter uterine horns and vagina. Uteri of hFST315 females respond abnormally to mating, with leukocyte infiltration in the lumen and disruption of the luminal epithelium that is consistent with an inflammatory response. Three key limitations of the study make interpreting the reproductive phenotypes difficult. First, expression of hFST315 is under the control of human, not mouse, promoters. Expression of hFST315 is reduced relative to wild-type FST315, making this a FST-deficient model in addition to a FST315-only model. It is also possible that hFST315 expression does not follow the expression pattern of wild-type FST315. Second, it is difficult to distinguish between the effects of FST288 absence during development and in the adult, particularly on uterine and ovarian function. Third, the severe ovarian defects make any analysis of the uterine phenotype difficult. The absence of corpus luteum indicates that the uteri would have been exposed to estrogen, but not progesterone. This could account for the inflammation phenotype independent of follistatin insufficiency. It could also account for uterine developmental abnormalities as later stages of uterine development are ovarian dependent. To attempt to address these limitations, the reproductive phenotypes of this mouse model were further characterized. The follow-up study showed that the abnormal reproductive tract observed in hFST315-only females arises from defects in postnatal uterine development, not embryonic Müllerian duct development. It also examined whether removing the ovaries would improve the inflammatory phenotype seen in adult uteri, but found that whereas estrogen treatment of ovariectomized hFST315 mice does increase leukocyte abundance in the uterus, removing the ovaries does not eliminate the abnormally high baseline number of leukocytes. These data are difficult to interpret given the developmental and hormonal abnormalities of hFST315-only mice prior to ovariectomy.

In an alternative approach, mice expressing only the FST288 isoform were generated by deleting the splice acceptor site within intron 5. Unlike mice with hFST288 expression on a FST KO background, which could not rescue perinatal lethality, FST288-only mice survive to adulthood allowing reproductive phenotypes to be studied. FST288-only females are subfertile with 25% smaller litters and fewer litters per month. In FST288-only females, the primordial follicle pool is significantly larger; however, this pool is depleted to wild-type amounts by 40 days of age and decreases to below wild-type levels by 100 days of age. The higher initial number of primordial follicles is matched by greater numbers of primary and secondary, but not antral follicles, suggesting that

there is a defect in folliculogenesis. Supporting this, FST288-only mice have a significantly reduced response to superovulation. No uterine phenotypes were reported.

***Amhr2-cre* cKO of Follistatin in the Ovary**

To study the roles of follistatin in the ovary, mice with a conditional knockout (cKO) of follistatin in the granulosa cells of the postnatal ovary were generated. This was achieved using an allele of follistatin with LoxP sites flanking exons 2 through 6 and *Amhr2-cre*, which expresses Cre in the granulosa cells of all secondary and small antral follicles and in the myometrium of the uterus, but not in primordial and primary follicles nor in the uterine endometrium. *Amhr2-cre Fst* cKO mice are subfertile, producing less than half as many pups per litter and litters per month. An ovarian phenotype is not apparent at 3 months of age when follicles of all stages are present in *Amhr2-cre Fst* cKO mice. However, at 6 months of age, fewer antral follicles and corpora lutea are observed. At 8 months of age, the phenotype is even more pronounced with few follicles of any stage present. In line with the ovarian defects observed, *Amhr2-cre Fst* cKO mice have elevated levels of FSH and LH. *Amhr2-cre Fst* cKO mice also have reduced testosterone levels starting at 6 months of age, a symptom of premature ovarian failure in women. Altogether, the data suggest follistatin regulates folliculogenesis and supports oocyte survival.

***Pgr-cre* cKO of Follistatin in the Uterus**

The roles of follistatin in the uterus have been addressed in a recent study, which describes a cKO of follistatin in the uterus using Progesterone Receptor Cre (*Pgr-cre*). *Pgr-cre* acts on the epithelium and stroma of the uterus, allowing roles in the uterus to be studied. *Pgr-cre Fst* cKO mice are infertile or severely subfertile, producing only 2% of the pups generated by control mice over a 4-month breeding trial. Uteri of *Pgr-cre Fst* cKO mice fail to become receptive to embryo attachment despite normal ovarian function. The failure of uterine receptivity in *Pgr-cre Fst* cKO mice is characterized by luminal epithelial proliferation and aberrant expression of mucin-1 and e-cadherin at 3.5dpc when the epithelium must stop proliferating and differentiate to become receptive to embryo attachment. In the absence of follistatin, activin signaling is increased while BMP signaling, critical for uterine receptivity, is decreased in the uterus. The expression of E2-regulated genes is also increased, possibly because of cooperation between activin and E2 signals. Although the roles of follistatin during mid- or late-gestation periods cannot be assessed using this model, *Pgr-cre Fst* cKO mice are unable to properly decidualize in response to an artificial decidual stimulus, supporting a role for uterine follistatin in regulating decidualization.

TGF β Family in the Uterus

Introduction

Because of the important roles TGF β family members play in development, knockout mice yield few clues into the importance of TGF β family signaling in the uterus. When a female infertility phenotype is observed, as in the inhibin β B or the ACVR2A knockout, the involvement of both ovarian and uterine defects confounds analysis of uterine phenotypes. For the interested reader, the phenotypes of mice with constitutive knockout of TGF β family signaling proteins are thoroughly reviewed in [Chang et al. \(2002\)](#).

Pgr-Cre

The advent of tissue-specific cKOs has allowed the roles of TGF β family members to be studied in the uterus. These cKO mice are created using two different genetic modifications. The first is replacing the coding region of one copy of a tissue-specific gene with a sequence encoding the Cre recombinase; this ensures that the Cre recombinase is expressed only in the tissues where the replaced gene would have been expressed. The second is modifying both alleles of the target gene to include sites, termed loxP sites, recognized by the Cre recombinase bracketing an essential region of the target gene; in any cell where the Cre recombinase is expressed, Cre-mediated recombination will excise the bracketed region, resulting in a non-functional allele. For studying the uterus, *Pgr-cre* has been invaluable. According to the original paper describing it, *Pgr-cre* is expressed postnatally in the anterior lobe of pituitary glands, granulosa cells of the postovulatory follicles, oviducts, and epithelium, and stroma of the uterus; however, cKO mice generated using *Pgr-cre* have shown a specific knockout in the uterus with excellent knockout in the epithelium and stroma (see studies described below).

BMP Signaling

The first TGF β family member studied using *Pgr-cre* was BMP2. In the mouse, BMP2 is expressed in the uterine stroma starting after embryo implantation and is strongly expressed in decidualizing stroma. Uterine cKO of *Bmp2* produces infertile females. *Bmp2* cKO females cannot form implantation sites because of an impaired decidual response to embryo attachment, demonstrating *Bmp2* is essential for decidualization.

Seeking to follow up on the role of BMP2 in decidualization, a uterine cKO of one of the type 2 receptors for BMP2, BMPR2, was generated. Intriguingly, although *Bmpr2* cKO mice are infertile, their phenotype differs from *Bmp2* cKO mice. *Bmpr2* cKO mice establish pregnancies with normal implantation sites until embryonic day 8, well past the beginning of decidualization at day 5 when pregnancies in *Bmp2* cKO mice fail. *Bmpr2* cKO mice can decidualize; however, their decidualization is defective and cannot support placentation because of poor vascularization and deficient recruitment of uterine natural killer cells. This suggests that whereas BMP2 may signal through BMPR2, the other type 2 receptors, ACVR2A or ACVR2B, may compensate for its absence during the initial days of decidualization.

The divergence between the uterine phenotypes of *Bmp2* and *Bmpr2* cKO mice suggests that ACVR2A and ACVR2B play an important role as ligands for BMP2 during decidualization; however, neither gene has yet been ablated using *Pgr-cre*. Knockout of *Acrv2b* is perinatal lethal, precluding any study of its role in female reproduction. Most *Acrv2a* knockout mice do survive to reproductive age and females are infertile; however, their infertility phenotype is caused by a severe reduction in pituitary production of follicle stimulating hormone (FSH) that causes dramatic defects in ovarian development and function, thus shedding little light on the roles of uterine ACVR2A during pregnancy.

In addition to BMPR2, two of the type 1 receptors used by BMP2—ALK2 and ALK3 have been conditionally ablated using *Pgr-cre*. *Alk2* cKO mice are sterile because of several defects during pregnancy. First, invasion of the embryo into the uterine epithelium and stroma is delayed after attachment, occurring a full day later in *Alk2* cKO mice. Second, decidualization is compromised with reduced proliferation and differentiation of stromal cells; however, the phenotype is less severe than that of the *Bmp2* cKO phenotype. Together, the delay in invasion and poor decidual reaction leave the uterus unable to support the implanted embryo and pregnancy terminates at 7.5dpc before placentation. Because *Alk2* cKO mice do not phenocopy *Bmp2* cKO mice, it is clear that BMP2 has an additional type 1 receptor partner during early pregnancy. *Alk3* cKO mice were generated to determine whether ALK3 is an essential partner for BMP2 in the uterus during pregnancy. *Alk3* cKO females are sterile; however, the cause of their infertility is distinct from the other uterine cKOs in the BMP signaling pathway. At 3.5dpc in a normal pregnancy, the uterine luminal epithelium is directed by progesterone signaling to stop proliferating and differentiate to facilitate embryo attachment and invasion. In *Alk3* cKO mice, this process fails and embryos are unable to attach to the apical surface of the luminal epithelium. Analysis of transcription factor binding in the uteri of wild-type females at 3.5dpc showed that SMAD4 and progesterone receptor (PR) bind the same region in the promoter of *Klf15*, a critical negative regulator of luminal epithelial proliferation, suggesting that BMP signaling cooperates with P4 signaling to promote uterine receptivity to embryo attachment. Because *Bmp2* cKO mice have normal embryo attachment, another BMP must be responsible for regulating uterine receptivity. The defect in uterine receptivity precluded observation of a natural decidual response, but artificial decidualization experiments show that ALK3 is required for proper decidualization, in line with its role as a receptor for BMP2.

Uterine BMP7 is also crucial for female reproduction. Indeed, conditional deletion of BMP7 with *Pgr-cre* (*Bmp7* cKO) resulted in female subfertility, with endometrial defects displayed during the window of implantation, such as epithelial proliferation, unopposed estrogen response and decreased progesterone signaling. Gene expression analysis of other BMPs during the window of implantation showed strong *Bmp5* expression in the luminal epithelium and stroma, suggesting that BMP5 compensates for the absence of BMP7 during implantation. To test this, *Bmp7* cKO were crossed to *Bmp5* KO mice to generate *Bmp7* cKO *Bmp5* KO mice. Fertility trials showed that the absence of BMP5 did not have additive effects on the fertility of *Bmp7* cKO mice, indicating that other BMP ligands may be involved in conferring endometrial receptivity. *Bmp7* cKO mice also demonstrated placental defects during mid-gestation, such as expansion of the trophoblast giant cells and absent spongiotrophoblast development. These defects indicate that BMP7 is crucial at two stages of pregnancy, during embryo implantation and later during placental development. An alternate explanation is that the absence of BMP7 during the window implantation perturbs normal embryonic and placental development that are manifested at later stages of gestation. Further studies are needed to establish the complete mechanism by which BMPs sensitize the endometrium for embryo receptivity as well as their roles in placental development.

SMADs

To date, no *Pgr-cre*-driven cKOs of the receptor SMADs or SMAD4 have been published. A recent paper, however, described the uterine phenotype of *Amhr2-cre* cKO of *Smad1/5/4*. *Amhr2-cre* acts in ovarian granulosa cells, the smooth muscle of the oviduct and uterus, and the uterine stroma. The *Smad1/5/4* triple cKO made using *Amhr2-cre* has defects in oviduct and uterine smooth muscle development, implantation failure, and defective decidualization. The phenotypes described differ notably from *Pgr-cre* models of BMP signaling. *Pgr-cre* *Alk3* cKO mice are not receptive to embryo attachment, whereas embryos can attach to the uteri of *Amhr2-cre* *Smad1/5/4* cKO mice. Additionally, whereas decidualization is defective in *Amhr2-cre* *Smad1/5/4* cKO mice, it is entirely absent in *Pgr-cre* *Bmp2* cKO mice. These differences, particularly with the *Pgr-cre* *Alk3* cKO mice, might be attributable to the lack of epithelial knockout in *Amhr2-cre* cKO; however, it could also be that *Amhr2-cre* is less effective than *Pgr-cre* in the uterine stroma. For example, *Amhr2-cre* cKO of COUP-TFII produces less severe phenotypes in the uterus than *Pgr-cre* cKO, despite the lack of COUP-TFII expression in the uterine epithelium. Alternatively, there could be underappreciated roles for non-SMAD TGF β family signaling in the uterus during early pregnancy.

TGF β Signaling

BMP-mediated signaling has been studied thoroughly in vivo; however, a few studies have examined the roles of TGF β - or activin/nodal-mediated signaling during pregnancy. Ablation of ALK5, the primary type 1 receptor for TGF β s, in the uterus causes nearly

complete infertility. ALK5 is expressed in response to embryo implantation at 4.5dpc and continues to be expressed during decidualization, declining after 6.5dpc. *Alk5* cKO mice show subtle abnormalities during implantation including delayed luminal closure, likely owing to the dysregulation of E2 and P4 signals observed at this time point. Although proliferation and differentiation occur normally during decidualization, uterine natural killer cells are not recruited and placentation is impaired with excessive trophoblast giant cells and a reduced placental labyrinth. The defects in placentation are likely responsible for spontaneous abortion later in pregnancy as mice with reduced numbers of uterine natural killer cells do not share this phenotype. Given the subtle phenotypes described in *Alk5* cKO mice, further in vivo studies are needed to determine the contribution of TGF β s to a successful pregnancy.

Nodal Signaling

Uterine cKO of *Nodal*, a member of the TGF β /activin group that signals through ACVR2A/B and ALK4/7, results in infertility because of reduced pregnancy rate, reduced survival to birth, and an ~80% rate of pre-term birth. The cause of the reduced pregnancy rate, measured as the percentage of mice with implantation sites at 5.5dpc, was not detailed, frustrating an assessment of the apparent function of nodal during early pregnancy. Pregnancy proceeds normally in *Nodal* cKO mice through decidualization until defects become apparent at 12.5dpc. Through a combination of reduced proliferation and apoptosis, the maternal decidua adjacent to the placenta regresses dramatically. Because the maternal decidua provides the blood supply and regulatory signals important for placental and embryonic development, it is likely that the excessive regression of the decidua is responsible for the reduced survival of pups to birth.

Knockout of ALK7, one of the type 1 receptors of activins and nodal, has no female reproductive phenotype, suggesting that it is dispensable during pregnancy. To investigate the role of the other activin/nodal type 1 receptor, ALK4, during pregnancy, a uterine cKO was generated using *Pgr-cre*. Intriguingly, *Alk4* cKO mice have a phenotype similar to *Nodal* cKO mice but it is not a phenocopy. *Alk4* cKO mice are subfertile, but do not experience preterm birth. Implantation and decidualization appear normal, but placental development is impaired. At 10.5dpc, there are excessive trophoblast giant cells and at 12.5dpc placentation is defective with reduced spongiotrophoblast and labyrinth. Because the labyrinth forms the site of nutrient exchange between mother and fetus, this defect in placentation likely accounts for the reduced survival rate of *Alk4* cKO pregnancies. The disparities between the *Nodal* and *Alk4* cKO phenotypes suggest that ALK7 likely plays a redundant role in transmitting nodal signals and that ALK4 may be transmitting activin signals to regulate placentation.

TGF β Family in the Ovary and Oviduct

Introduction

In addition to its important roles in the uterus, TGF β family signaling regulates ovarian function. Animal models have shown that TGF β family ligands including BMPs, GDFs, TGF β s, and activins regulate primordial germ cell development, folliculogenesis, and ovulation. Please note that our discussion excludes inhibins and activins.

Amhr2-Cre

Although global KO mice have provided important clues about the roles of the TGF β family in the ovary, critical developmental roles for many TGF β family members makes the engineering of tissue-specific cKOs essential. *Amhr2-cre* replaces one copy of *Amhr2* with the coding sequence for Cre recombinase. This allele expresses Cre in the granulosa cells of secondary and small antral follicles in the ovary and in the myometrium and stroma of the uterus. *Amhr2-cre* is not active in primordial or primary follicles, preventing analysis of the roles of TGF β family members in these early follicles using *Amhr2-cre* cKO.

BMP and GDF Signaling

Early studies of BMPs and GDFs using knockout mice quickly revealed important roles for BMP signaling in germ-cell development. *Bmp2* KO mice have fewer primordial germ cells (PGC) at 7.5 days of gestation and die between days 7 to 10.5 of gestation, preventing analysis beyond this early stage. *Bmp4* KO mice lack PGCs. A more severe PGC deficit in *Bmp2 Bmp4* heterozygous KO mice than in *Bmp4* heterozygous KO mice suggests that embryonic endoderm-expressed BMP2 and extraembryonic ectoderm-expressed BMP4 cooperate to regulate PGC generation. BMP8B is also critical for PGC generation as *Bmp8b* KO mice have reduced or absent PGCs; however, its role appears to be distinct from those of BMP2 and BMP4 because *Bmp8b Bmp4* heterozygous KO mice do not show a more severe phenotype than that of *Bmp4* heterozygous KO mice. In line with the important roles of BMP2/4/8B in PGC generation, *Alk2* KO mice have no primordial germ cells. Despite its critical roles, the mechanisms by which BMP signaling regulate PGC generation remain unclear.

Limited data suggests that BMP4 and BMP7 play a role in regulating the transition from primordial to primary follicles, which is marked by the shift of oocyte-supporting granulosa cells from flat to cuboidal structure. In rats, BMP4 is expressed by thecal cells starting in primary follicles. Treatment of rat ovaries in whole-organ in vitro cultures with BMP4 promotes the development of primary follicles and treatment with a BMP4-neutralizing antibody reduces the number of oocyte and primordial follicles. Treatment of adult rat ovaries with BMP7 by injection into the ovarian bursa increases the fraction of primary follicles and more

advanced follicles, while reducing primordial follicles; however, rat BMP7 is not expressed in the follicle until the secondary follicle stage in vivo, suggesting it may only support later stages of folliculogenesis.

Oocyte-derived BMP15 and GDF9 also play critical roles in folliculogenesis. *Bmp15* KO female mice are subfertile with incompletely penetrant defects in folliculogenesis and oocyte quality. *Gdf9* KO female mice are sterile because of a block in follicular development at the primary to secondary follicle transition. Sheep without functional BMP15 are infertile with a block at the primary stage in folliculogenesis, but heterozygotes for the inactivating mutation have an increased ovulation rate. Sheep without functional GDF9 show a similar phenotype, with infertility in homozygotes and excesses ovulation in heterozygotes. In humans, a dominant negative heterozygous nonsynonymous mutation in *BMP15* has been reported in women with hypergonadotrophic ovarian failure. Rare mutations in *GDF9* have been identified in families with a history of dizygotic twinning, supporting a role for GDF9 in regulating folliculogenesis or ovulation in women. Female mice with knockout of *Bmp15* and heterozygous knockout of *Gdf9* are infertile with defects in cumulus-cell development and expansion, suggesting that oocyte-produced BMP15 and GDF9 cooperate to promote folliculogenesis. In fact, BMP15 and GDF9, which are closely related paralogs, cooperate by forming heterodimers that regulate cumulus cell expansion. Mouse GDF9:BMP15 heterodimers are 10- to 30-fold more potent than mouse GDF9 homodimers in an in vitro granulosa cells cell assay, and human GDF9:BMP15 heterodimers are 1000- to 3000-fold more potent than human BMP15 homodimers in a human granulosa cell assay. BMP family heterodimers can achieve higher potency than homodimers because of the different receptor binding activities of each ligand allow heterodimers to interact more strongly with available type 1 and 2 receptors than homodimers. GDF9 binds the type 1 receptors ALK4/5/7, which signal through SMAD2/3, and BMP15 binds ALK1/2/3/6, which signal through SMAD1/5/8. Treatment with type 1 receptor kinase inhibitors demonstrates that GDF9:BMP15 heterodimers signal through ALK4, but not ALK6; however, ALK6-null mouse granulosa cells do not respond to GDF9:BMP15 heterodimer. Therefore, ALK4 and ALK6 are both required for heterodimer binding, but the type 2 receptor in the GDF9:BMP15 heterodimer-binding receptor complex, BMPR2, only phosphorylates and activates ALK4. In line with this, *Alk6* KO mice are infertile with defects in cumulus-cell expansion, similar to the phenotype of GDF9 KO mice, despite the lack of direct interaction between GDF9 and ALK6. Data from humans, sheep, and rodents diverge on the relative importance of BMP15 and GDF9 homodimers for cumulus granulosa cell regulation. Human and sheep granulosa cells do not respond to GDF9 homodimers in vitro and respond only mildly to BMP15 homodimers. In contrast, mouse and rat granulosa cells respond to GDF9 homodimers, but do not respond to BMP15 homodimers.

SMADs

Global and conditional *Amhr2-cre* knockouts of SMAD proteins have informed our understanding of the roles of TGF β family signaling in PGC development, follicle development, and cancer prevention. In line with the defects in PGC development seen in *Bmp2*, *Bmp4*, and *Bmp8b* KO mice, knockouts of their downstream receptor SMADs, SMAD1 and SMAD5, have fewer PGCs and SMAD4 cKO in the early mouse epiblast precludes PGC development. PGC development is not SMAD8-dependent, as no PGC deficits are seen in *Smad8* KO mice.

Amhr2-cre cKO of *Smad1* or *Smad5* are fertile without ovarian phenotypes; however, double *Smad1/5* cKO mice are infertile and develop metastatic granulosa cell tumors. Intriguingly, knockout of inhibin α , an inhibitor of activin signaling through SMAD2/3, also enables development of granulosa cell tumors. Gene and protein expression analysis reveals that granulosa-cell tumors in both *Smad1/5* cKO mice and inhibin α KO mice are driven by excessive activin signaling, supporting a model in which development of granulosa-cell tumors is caused by disruption of an antagonistic equilibrium between BMP-SMAD1/5 and activin-SMAD2/3 signaling. In this instance, BMP-SMAD1/5 signaling suppresses tumorigenesis while activin-SMAD2/3 signaling promotes it. Signaling through SMAD8 does not appear to play a significant role in suppressing tumorigenesis, as *Smad8* cKO, *Smad1/8* cKO, and *Smad5/8* cKO mice have no reproductive phenotypes and *Smad1/5/8* cKO phenocopies *Smad1/5* cKO.

Smad3 KO mice are subfertile with impaired follicle growth, increased atresia, and defects in differentiation; however, the contribution of ovarian knockout is unclear as hormonal imbalances caused by pituitary loss of SMAD3 confound analysis. Neither *Smad2* nor *Smad3* *Amhr2-cre* cKO mice have female fertility defects, whereas double *Smad2/3* cKO mice are subfertile with disrupted follicular development, ovulation, and cumulus-cell expansion. At 3 and 8 months of age, *Smad2/3* cKO mice show significantly fewer antral follicles and an accumulation of zone pellucida remnants, indicating follicular atresia owing to oocyte loss.

Smad4 *Amhr2-cre* cKO mice have a phenotype distinct from either *Smad1/5* or *Smad2/3* cKO mice. *Smad4* cKO causes subfertility with decreasing fertility over time characterized by multiple defects in folliculogenesis. In response to superovulation with equine chorionic gonadotrophin, antral follicles in *Smad4* cKO mice undergo premature luteinization. Cumulus-cell expansion is also abnormal or absent in COCs isolated from *Smad4* cKO mice after superovulation. Supporting the hypothesis that granulosa cell tumors in *Smad1/5* cKO mice arise from excess activin signaling through *Smad2/3*, *Smad4* cKO mice do not develop granulosa cells tumors.

ALK3 and ALK6

Knockout of *Alk6* (*Bmpr1b*) in mice causes female infertility because of a failure of cumulus cell expansion. Notably, *Amhr2-cre* cKO of *Alk3* reveals a distinct function from ALK6, despite their shared ligand partners and downstream receptor SMADs; *Alk3* cKO females are subfertile with reduced spontaneous ovulation. Females with *Amhr2-cre* conditional double knockout of *Alk3/6* develop

granulosa cell tumors, phenocopying *Smad1/5* cKO and *inhibin α* KO mice. In sheep, an ALK6 kinase domain missense mutation increases ovulation rate and litter size.

TGF β Signaling

The roles of TGF β s in the ovary remain unclear. Mouse knockouts for TGF β 1, TGF β 2, and TGF β 3 have been produced and are perinatal lethal, permitting only prenatal and perinatal phenotypes to be assessed. TGF β 1 and TGF β 3 KO mice show no ovarian defects at birth. TGF β 2 KO mice have an increased number of germ cells in the ovary, suggesting that TGF β 2 may play a role in regulating PGC development. However, data in various adult models is muddled. For example, rat thecal cells in preantral and antral follicles express TGF β 1 and TGF β 2, preantral expression has been reported in sheep, and in vitro evidence suggests a role for TGF β s in regulating granulosa cells in cattle and humans. However, the absence of ovarian phenotypes in *Amhr2-cre* cKO of ALK5, the primary type 1 receptor for TGF β s, indicates that TGF β signaling through ALK5 is not essential for normal mouse ovarian function. *Alk5* cKO mice do suffer from developmental defects of the oviduct and uterus, particularly in smooth muscle development, but which TGF β family ligands signal through ALK5 to regulate oviduct and uterine development remains unclear.

Summary

The expression of ligands, receptors, and downstream effectors of the TGF β family are carefully coordinated throughout life, participating critically in development and adult tissue function. Data from genetic mouse models have demonstrated that TGF β family signaling in the uterus and ovaries is essential in female reproduction. Although much progress has been made towards characterizing the roles of TGF β family signaling in female reproduction, the complexity of ligand/receptor combinations and the context-dependent nature of responses to TGF β family signals leave open numerous avenues for future study.

Acknowledgements

We thank Dr. John Nelson for editing and critical comments. Support was provided by the Eunice Kennedy Shriver National Institute of Child Health and Human Development through grant R01-HD033438 and R01-HD032067, by National Institute of General Medical Sciences grant T32GM008307, the Institutional Research and Career Development Award K12-GM084897, and by a Postdoctoral Enrichment Program Award from the Burroughs Wellcome Fund.

References

- Chang, H., Brown, C. W., & Matzuk, M. M. (2002). Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocrine Reviews*, 23, 787–823. <https://doi.org/10.1210/er.2002-0003>.
- Di Pasquale, E., Beck-Peccoz, P., & Persani, L. (2004). Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *American Journal of Human Genetics*, 75, 106–111. <https://doi.org/10.1086/422103>.
- Dong, J., Albertini, D., Nishimori, K., Kumar, T., Lu, N., & Matzuk, M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature*, 383, 531–535. <https://doi.org/10.1038/383531a0>.
- Galloway, S. M., McNatty, K. P., Cambridge, L. M., Laitinen, M. P., Juengel, J. L., Jokiranta, T. S., McLaren, R. J., Luiro, K., Dodds, K. G., Montgomery, G. W., Beattie, A. E., Davis, G. H., & Ritvos, O. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature Genetics*, 25, 279–283. <https://doi.org/10.1038/77033>.
- Hashimoto, O., Nakamura, T., Shoji, H., Shimasaki, S., Hayashi, Y., & Sugino, H. (1997). A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells: Endocytotic degradation of activin and its acceleration by Follistatin associated with cell-surface heparan sulfate. *The Journal of Biological Chemistry*, 272, 13835–13842. <https://doi.org/10.1074/jbc.272.21.13835>.
- Lawson, K. A., Dunn, N. R., Roelen, B. A., Zeinstra, L. M., Davis, A. M., Wright, C. V., Korving, J. P., & Hogan, B. L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev*, 13, 424–436.
- Lin, S.-Y., Craythorn, R. G., O'Connor, A. E., Matzuk, M. M., Girling, J. E., Morrison, J. R., & de Kretser, D. M. (2008). Female infertility and disrupted angiogenesis are actions of specific follistatin isoforms. *Molecular Endocrinology*, 22, 415–429. <https://doi.org/10.1210/me.2006-0529>.
- Massagué, J. (2008). TGF-beta in cancer. *Cell*, 134, 215–230. <https://doi.org/10.1016/j.cell.2008.07.001>.
- Matzuk, M. M., & Burns, K. H. (2012). Genetics of mammalian reproduction: modeling the end of the germline. *Annual Review of Physiology*, 74, 503–528. <https://doi.org/10.1146/annurev-physiol-020911-153248>.
- Matzuk, M. M., Kumar, T. R., & Bradley, A. (1995). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature*, 374, 356–360. <https://doi.org/10.1038/374356a0>.
- Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R., & Bradley, A. (1995). Multiple defects and perinatal death in mice deficient in follistatin. *Nature*, 374, 360–363. <https://doi.org/10.1038/374360a0>.
- Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., & Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. *Science*, 247, 836–838. <https://doi.org/10.1126/science.2106159>.

- Pangas, S. A. (2012). Bone morphogenetic protein signaling transcription factor (SMAD) function in granulosa cells. *Molecular and Cellular Endocrinology*. <https://doi.org/10.1016/j.mce.2011.06.021>.
- Peng, J., Li, Q., Wigglesworth, K., Rangarajan, A., Kattamuri, C., Peterson, R. T., Eppig, J. J., Thompson, T. B., & Matzuk, M. M. (2013). Growth differentiation factor 9:bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proceedings of the National Academy of Sciences of the United States of America*, 110, E776–85. <https://doi.org/10.1073/pnas.1218020110>.
- Robertson, D. M., Klein, R., de Vos, F. L., McLachlan, R. I., Wettenhall, R. E., Hearn, M. T., Burger, H. G., & de Kretser, D. M. (1987). The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. *Biochemical and Biophysical Research Communications*, 149, 744–749.
- Shi, Y., & Massagué, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell*, 113, 685–700. [https://doi.org/10.1016/S0092-8674\(03\)00432-X](https://doi.org/10.1016/S0092-8674(03)00432-X).
- Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S. Y., Ling, N., & Guillemin, R. (1988). Primary structure of the human follistatin precursor and its genomic organization. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 4218–4222. <https://doi.org/10.1073/pnas.85.12.4218>.
- Ueno, N., Ling, N., Ying, S. Y., Esch, F., Shimasaki, S., & Guillemin, R. (1987). Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 8282–8286. <https://doi.org/10.1073/pnas.84.23.8282>.
- Wijayarathna, R., & de Kretser, D. M. (2016). Activins in reproductive biology and beyond. *Human Reproduction Update*, 22, 342–357. <https://doi.org/10.1093/humupd/dmv058>.
- Wrana, J. L., Ozdamar, B., Roy, C. L., & Benchabane, H. (2008). 6 Signaling receptors of the TGF- β family. *Cold Spring Harbor Monograph Archive of the TGF- β Family*, 50.
- Zhang, Y. E. (2009). Non-Smad pathways in TGF- β signaling. *Cell Research*, 19, 128–139. <https://doi.org/10.1038/cr.2008.328>.

Anti-Müllerian Hormone (AMH)

Francesca Mossa, University of Sassari, Sassari, Italy

James J Ireland, Michigan State University, East Lansing, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

What is AMH and Its Source in Females

AMH is a dimeric glycoprotein and a member of the transforming growth factor β (TGF β) superfamily of growth and differentiation factors. It is produced by Sertoli cells of the testes in the male and by granulosa cells of healthy, growing ovarian follicles in females (Visser et al., 2006).

AMH Mechanism of Action

Like other TGF β family members, AMH signals through a receptor complex consisting of two type I and two type II receptors. AMH binding causes type II receptor mediated phosphorylation and activation of the type I receptor, which in turn activates the SMAD pathway. Receptor-activated SMADs then interact with SMAD₄ and translocate to the nucleus to modify target gene expression.

The type II AMH receptor (AMHRII) is specific for AMH, whereas there are three type I AMH receptors (ACVR₁, BMPR_{1A}, and BMPR_{1B}) that are shared with bone morphogenetic proteins (BMPs), which are also members of the TGF β family. AMHRII mRNA is expressed in granulosa cells of small growing follicles. Type I AMH receptors mRNA is expressed in oocytes, granulosa, and theca cells at different stages of follicular development (reviewed in Visser et al., 2006).

AMH Functions

Mammals, such as human beings, cattle, swine, and sheep are born with a finite number of morphologically healthy follicles and oocytes in the ovaries (the ovarian reserve) that dwindle rapidly during aging and are never replenished. Follicular recruitment starts soon after birth and is continuous throughout life. In female AMH null mice, follicles are recruited at a faster rate, resulting in an exhausted pool of primordial follicles at a younger age compared to wild-type controls. Also, AMH inhibits primordial follicle assembly and decreases the initial primordial follicular pool size in a rat ovarian organ culture. Similarly, AMH inhibited the initiation of follicle growth in cultured pieces of human ovaries (reviewed in Dewailly et al., 2014) and recently, AMH was shown to inhibit follicular activation and attenuate the growth of primary follicles in bovine fetal ovarian cortical pieces in vitro (Yang et al., 2017). These findings indicate that AMH acts as a brake on follicular development in humans, rodents and in cattle, inhibiting primordial follicular growth from the ovarian reserve and avoiding its premature exhaustion.

AMH is also believed to reduce the responsiveness of preantral and small antral follicles to FSH, modulating follicular development. In cultured human granulosa cells, AMH inhibited FSH-induced aromatase mRNA and protein expression and estradiol production and AMH inhibited FSH effects on follicular growth in women and mice (reviewed in Dewailly et al., 2014).

Regulation of AMH Secretion

AMH secretion changes as the ovarian follicle develops; AMH expression starts in primary follicles, reaches its highest level in preantral and small antral follicles, whereas it then decreases as the selected, FSH-dependent follicle progresses toward the preovulatory stage and is absent in atretic follicles. This pattern of expression was first assessed in rodents and subsequently in women, cattle and sheep. Nevertheless cumulus cells of preovulatory follicles continue to express AMH in women and mice (reviewed by Monniaux et al., 2012).

AMH production is stimulated by the oocyte: evidence indicates that BMPs, such as BMP4, BMP6, and BMP15 originating from oocyte and theca cells, stimulate AMH mRNA expression by granulosa cells. On the contrary, FSH appears to have an inhibitory effect on AMH secretion, albeit still poorly understood. FSH decreased AMH mRNA expression in rat and cattle, but not in sheep (reviewed by Monniaux et al., 2012); however, FSH enhanced AMH gene expression and protein secretion in bovine granulosa cells in vitro at low concentrations, but inhibited them at higher concentrations (Scheetz et al., 2012). In summary, the regulation of AMH secretion by FSH is complex and the mechanism that regulates AMH secretion is unclear.

Alterations in Circulating AMH Concentrations From Birth to Puberty

In healthy women, peripheral AMH concentrations change throughout life. A marked postnatal rise in AMH concentrations was reported in girls, with higher AMH at 3 months of age compared to those at birth, followed by a decrease at 12 months. This peak in early infancy was interpreted as an ovarian response to prevent FSH-induced follicle growth at a time when further differentiation of follicles would be inappropriate (Hagen et al., 2010).

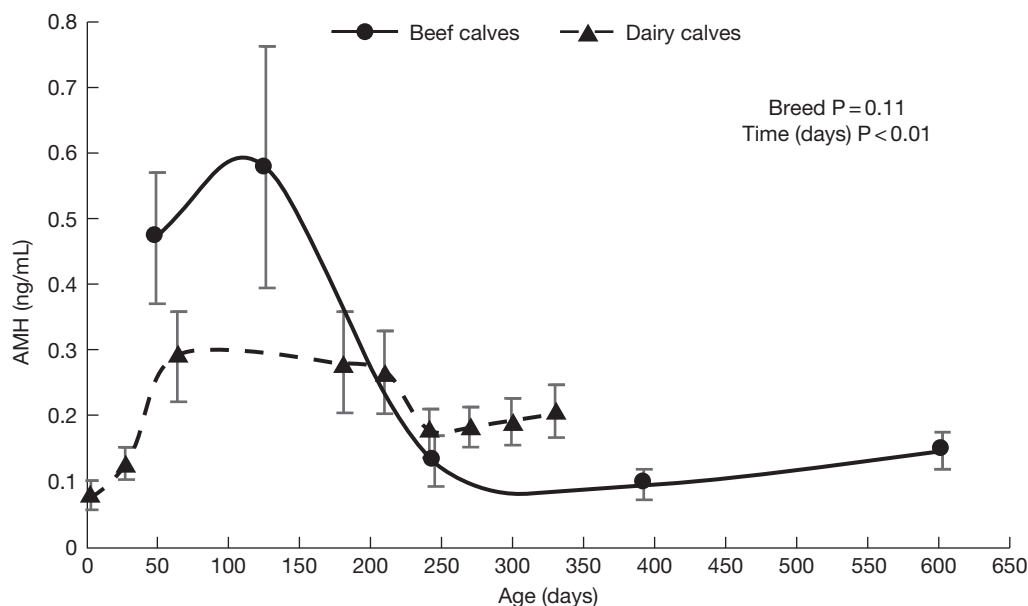


Fig. 1 Circulating AMH concentrations (ng/mL) in dairy ($n = 9$) and cross bred beef ($n = 13$) female calves. Results of ANOVA indicated that mean AMH concentrations were similar between dairy and beef calves ($P = 0.11$), but varied with time ($P < 0.01$) (Mossa et al., 2017).

Several studies report that serum AMH levels rise steadily during childhood with greatest levels at around 15–18 years, but are stable during early adulthood (18–25 years). This pattern has been assessed in Caucasian and Chinese women, and may suggest that ethnicity does not have an impact on age-related AMH variations in women (Hagen et al., 2010). Nevertheless, the mechanisms responsible for the increase in AMH concentrations during childhood in girls remain to be elucidated.

We conducted a study to illustrate the variations of AMH from birth to puberty in Holstein female calves (Mossa et al., 2017). Results depicted in Fig. 1 show that AMH concentrations increase during the first 2 months of age, decrease at 5 and are stable at 8–9 months of age, around the time of first ovulation. We also described a similar pattern in beef calves (Mossa et al., 2017). In Maine-Anjou beef heifers, plasma AMH concentrations increased rapidly between 1 and 3 months of age, remained high at 6 months of age then declined slowly until 12 months of age, which corresponds to the age of ovulation for this breed (Monniaux et al., 2012). These findings are supported by results from others indicating that 3- to 4-month old calves have greater AMH levels compared to young adult heifers in both Holstein (14–16 months) and *Bos indicus* Nelore (18–24 months) cattle (reviewed in Mossa et al., 2017). In summary, AMH concentrations increase in the first months of life and decrease before puberty in cattle, like women, but the timing of such fluctuations may vary among breeds and genetic groups. Prepubertal heifers experience waves of antral follicular growth like adult cattle and number of follicles increases from 2 to 14 weeks of age (Evans et al., 1994). Thus, it is plausible that the variations in AMH concentrations observed before puberty are reflective of changes in growth patterns of small antral follicles. Another possible explanation is that prepubertal variations in circulating AMH concentrations are due to changes in the ability of granulosa cells to secrete AMH.

Alterations in Circulating AMH Concentrations during Estrous and Menstrual Cycles

In women, AMH peripheral concentrations remain relatively constant during the menstrual cycle (La Marca et al., 2009). For example, in a study conducted on young (aged 19–35 years) normo-ovulatory women, the fluctuations of serum AMH levels were not significant either when aligned to day of menstrual cycle or to day of ovulation. Also, another study reported that AMH concentrations were not different within individual young women (aged 24–40) across a number of up to four consecutive menstrual cycles, ranging between 5.7 and 6.0 ng/mL (van Disseldorp et al., 2010). The limited variation in AMH peripheral concentration during the menstrual cycle indicates that AMH serum levels are not affected by dominant follicle growth during the late follicular phase of the normal menstrual cycle despite the finding that AMH expression declines in large follicles. These findings taken together imply that a single measurement of AMH is predictive of circulating AMH concentration during the menstrual cycle. The static nature of AMH concentrations enhances its clinical usefulness for example to assess response to ovarian stimulation protocols during ART compared with other currently available clinical markers of reproductive function, such as inhibin B, estradiol (E_2), and FSH, which are all menstrual cycle dependent.

Evidence is also accumulating that AMH concentration varies minimally during estrous cycles in cattle. For example, we reported that a single AMH measurement in young adult beef heifers was highly correlated ($r = 0.97$) with the average for multiple AMH measurements during different days of the same or multiple estrous cycles (Ireland et al., 2011). In Holstein cows, AMH varied

minimally during the same estrous cycle and on different days of two estrous cycles (reviewed in [Mossa et al., 2017](#)). Taken together these findings illustrate the static nature of AMH during the estrous cycle and its repeatability across multiple estrous cycles in cattle.

Conversely, in mice serum AMH levels were increased at estrus and gradually declined to basal levels during the following days of the cycle ([Visser et al., 2006](#)). Such differences between mice compared with women and cattle may be explained by species differences in the dynamics of follicular growth and associated AMH secretion during reproductive cycles.

Correlation of Circulating AMH Concentrations With Antral Follicle Count, Size of the Ovarian Reserve (All Healthy Follicles), and Aging

In women, antral follicle count (AFC) is defined as the number of follicles measuring 2–10 mm in diameter and is assessed by transvaginal ultrasound. AFC is proportionally related to the size of the ovarian reserve ([La Marca et al., 2009](#)). Similar to AFC, circulating AMH is a marker of the size of the ovarian follicular reserve in humans and in mice. Several studies report that AFC and serum AMH are strongly ($r = 0.74$) to moderately ($r = 0.57$ – 0.47) positively associated. For example, when AFC and AMH were repeatedly measured during a menstrual cycle, the moderate fluctuations in serum AMH concentration paralleled the variation in number of follicles 2–8 mm in diameter ([Depmann et al., 2016b](#)). This positive correlation between AFC and AMH can be explained by the AMH production by the small antral follicles that are also detected by ultrasonography. Both AFC and AMH are often used independently or jointly to estimate the size of the ovarian reserve and to predict the ovarian response to hormonal stimulation during ART ([La Marca et al., 2009](#)).

A high positive correlation was observed between the variation in AMH, AFC and histological determination of total number of morphologically healthy follicles and oocytes in ovaries of young adult cattle ([Ireland et al., 2008](#)). In beef heifers, circulating AMH concentrations were approximately six- and twofold greater in animals with high (≥ 25 follicles, ≥ 3 mm in diameter) or intermediate (16–24 follicles) compared with a low (≤ 15 follicles) AFC during follicular waves ([Fig. 2](#), left panel). Also, the overall average AMH concentration during ovulatory follicular waves per animal was highly correlated ($r = 0.88$; [Fig. 2](#), right panel) with average peak AFC during the two or three waves of an estrous cycle ([Ireland et al., 2008](#)). In another study, AMH plasma concentrations were highly positively correlated with the numbers of 3–7 mm antral follicles detected by ovarian ultrasonography in primiparous dairy cattle ([Rico et al., 2009](#)). Also, a positive association was detected between the antral follicle population and circulating AMH concentrations in Murrah (*Bubalus bubalus*), Holstein (*Bos taurus*), and Gyr (*Bos indicus*) heifers ([Mossa et al., 2017](#)). It can be

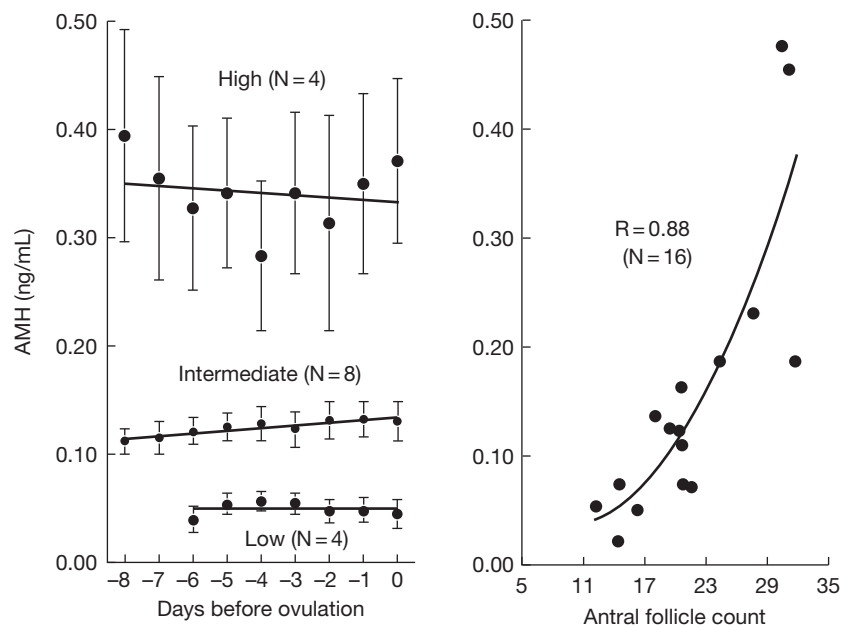


Fig. 2 Alterations in circulating AMH concentrations during ovulatory follicular waves in cattle. Serial ovarian ultrasonography was used to identify animals with a consistently low (≤ 15 follicles ≥ 3 mm in diameter per wave, $n = 4$ animals), intermediate (16–24 follicles, $n = 8$), or high (≥ 25 follicles, $n = 4$ animals) peak AFC during ovarian follicular waves. Prostaglandin $F_{2\alpha}$ then was used to synchronize estrus, and ovarian ultrasonography was used to reconfirm that animals were correctly identified and to determine day of ovulation. Blood samples were taken daily at 1100 h beginning on Day 6 of the estrous cycle and ending 1 day after ovulation. Serum samples obtained 6–8 days preceding ovulation, which corresponded with ovulatory follicular waves, and on the day of ovulation were analyzed for AMH concentrations. In the left graph, data for each animal were aligned relative to day of ovulation, and data are plotted based on results of linear regression analysis. Each point represents the mean $\pm$ SEM for four or eight animals. In the right graph, average AMH concentration per animal was plotted relative to the average peak number of antral follicles per wave during the estrous cycle for each animal; r = correlation coefficient and n = total number of animals ([Ireland et al., 2008](#)).

concluded, therefore, that AMH and AFC are positively correlated in cattle and both AFC and AMH can be used interchangeably and reliably to estimate total number of morphologically healthy follicles and oocytes in the ovaries of an individual.

Diagnostic Use of AMH to Predict Fertility, Age at Menopause and Response to ART

Predict Fertility

In cattle, in our recent work, AMH concentrations were determined in young adult Holstein heifers at 11–12 months of age and a variety of fertility measurements made before and after calving in the same individuals. Results showed that conception rates to first artificial insemination (AI), services per conception and days open after calving until pregnant were similar among individuals in the different AMH quartiles before calving and during the first, second and third lactations. However, the quartile (Q) of cows with the lowest AMH concentrations (Q1) as heifers tended ($P < 0.10$) to be lower at each lactation and had the lowest overall average for total percentage pregnant compared with cows in Q2 or Q3 but not Q4 AMH quartiles (Jimenez-Krassel et al., 2015). These findings contrast somewhat with our other study that examined fertility in dairy cows (up to eight parities) with low, intermediate or high AFC and presumably corresponding differences in AMH concentrations. In this study, dairy cows with a low AFC had a lower conception rate to first AI, greater number of AI to conceive and higher calving interval compared with cows with an intermediate or high AFC (Mossa et al., 2012). Like our finding for dairy cows with low AMH as a heifer (Jimenez-Krassel et al., 2015) overall pregnancy rate was lower for the dairy cows with a low compared with a high AFC (Mossa et al., 2012). Another study shows that dairy cows with high AMH had greater pregnancy rates and lower incidence of pregnancy loss between days 30 and 65 of gestation. Lower fertility for cattle with a relatively low AFC is further supported by evidence of lower pregnancy rates in beef heifers with a low vs. a higher AFC and by a study showing that lactating cows with a low AFC had a longer interval from calving to conception and lower pregnancy rates than cows with a high AFC (reviewed in Mossa et al., 2017).

The reasons for the differences between some fertility measurements in the studies that classified cattle based on AMH or AFC are unknown but likely caused by relatively small numbers of cattle in AMH quartiles and some AFC groups coupled with mixed ages of cows in AFC groups. In addition, cross-sectional studies using older cows may be biased by previous removal (culling) of individuals from the herd with a relatively low AFC or AMH. Nevertheless, combined results (Mossa et al., 2017) imply that fertility is suboptimal in cows with low AMH concentrations as heifers and in cows with low AMH or a low AFC compared with herd mates with higher AMH concentrations or a higher AFC.

Age at Menopause

In women, the size of the ovarian reserve declines with age until there are too few follicles to sustain menstrual activity and menopause (final menstrual period) is reached. Menopause occurs at a mean age of 51 years when the ovarian reserve drops to approximately 1000 follicles, but the age at which natural menopause occurs varies considerably between 40 and 60 years (te Velde & Pearson, 2002). Similarly, the rate of decline of the ovarian reserve varies significantly among individuals and the high variability of age at menopause is believed to be caused by the high variation in the number of ovarian follicles and oocytes at birth. Since AMH is a marker of the ovarian reserve, it is considered a promising marker to predict age at natural menopause. So far, six studies showed that AMH concentration is predictive of age at natural menopause (hazard ratio, 5.6–9.2) (Depmann et al., 2016), but long-term studies on large numbers of women are lacking.

Response to ART

Assisted reproductive technology (ART), which includes hormonal stimulation, AI, in vitro fertilization and surrogacy, is used in both women and cattle. Ovarian stimulation consists of the artificial increase of FSH concentration that leads to the simultaneous growth of several follicles. The oocytes can be recovered from the stimulated follicles and fertilized in vitro or they can be allowed to ovulate (superovulation) and fertilized in vivo. In women oocytes are usually collected before ovulation, whereas in cattle superovulation is commonly followed by AI and the nonsurgical recovery of the embryos. The ovarian response to hormonal stimulation varies greatly among individuals in both women and cattle; such responsiveness is negatively associated with aging and growing evidence indicates that it can be predicted by measuring serum AMH concentrations (La Marca et al., 2009).

In women AMH correlates strongly with the number of oocytes retrieved following ovarian stimulation for IVF (La Marca et al., 2009), hence it is now widely used to assess the size of the ovarian reserve before assisted conception. Women with high AMH are likely to respond excessively to exogenous gonadotrophins (Dewailly et al., 2014), leading to a serious condition named ovarian hyperstimulation syndrome (OHSS), thus their treatment strategy can be modified by using a less intense ovarian stimulation regime. Conversely women with a low AMH are likely to respond poorly to stimulation with consequently a low chance of pregnancy (Dewailly et al., 2014) and their expectations can be managed appropriately, reducing the financial and emotional burden on couples seeking ART treatment.

Evidence is now growing to support the use of AMH as a predictor of the responsiveness to ovarian stimulatory treatment in agricultural species. In primiparous dairy cows, AMH concentrations before the superovulatory treatment were positively correlated with the number of follicles before treatment and with the numbers of large follicles and corpora lutea (CL) after treatment (Rico et al., 2009). In nonlactating Holstein cows, plasma AMH concentrations before gonadotropin treatments were highly correlated

with the numbers of large follicles and oocytes recovered at ovum pick up (OPU), as well as with the number of large follicles at estrus and the number of embryos collected from multiple ovulation and embryo transfer protocols. Further, in Japanese Black beef cattle, AMH concentrations were positively correlated with the number of follicles, number of oocytes/embryos recovered, fertilized embryos and transferable embryos. In lactating Holstein cows, AMH concentrations for each individual cow were correlated with superovulation response (number of CL on the day of the flush), total oocytes collected and total transferable embryos. Also, when cows were classified into quartiles of circulating AMH, Q4 cows had a > 2-fold greater response to superovulation including embryo production compared with cows in other quartiles (Mossa et al., 2017). In addition, a positive correlation existed between AMH with OPU and in vitro embryo production in Holstein, beef (Korean Hanwoo) and *Bos indicus* (Zebu) cattle (Mossa et al., 2017).

Summary and Conclusion

Evidence from studies conducted in women, cattle and rodents indicates that AMH circulating concentration (1) increases in the first months of life and then decreases until puberty; (2) is repeatable in the same or multiple estrous/menstrual cycles within individuals; (3) is positively correlated with AFC; (4) is a reliable indicator of the total number of healthy follicles and oocytes; (5) is positively associated with measures of fertility (women and cattle: response to ovarian stimulation; cattle: pregnancy rate, herd longevity); (6) is a promising candidate to predict age at menopause. Taken together, these findings support the potential utility of AMH measurement in human ART and in agricultural species as a predictor of fertility. However, uncertainty still exists concerning AMH mechanism of action and regulation of secretion. Cattle may be used as a valid animal model to further corroborate the association between AMH and fertility in mammals.

References

- Depmann, M., Broer, S. L., van der Schouw, Y. T., Tehrani, F. R., Eijkemans, M. J., Mol, B. W., & Broekmans, F. J. (2016a). Can we predict age at natural menopause using ovarian reserve tests or mother's age at menopause? A systematic literature review. *Menopause*, 23(2), 224–232.
- Depmann, M., van Disseldorp, J., Broer, S. L., Eijkemans, M. J., Laven, J. S., Visser, J. A., de Rijke, Y. B., Mol, B. W., & Broekmans, F. J. (2016b). Fluctuations in anti-Müllerian hormone levels throughout the menstrual cycle parallel fluctuations in the antral follicle count: A cohort study. *Acta Obstetrica et Gynecologica Scandinavica*, 95(7), 820–828.
- Dewailly, D., Andersen, C. Y., Balen, A., Broekmans, F., Dilaver, N., Fanchin, R., Griesinger, G., Kelsey, T. W., La Marca, A., Lambalk, C., Mason, H., Nelson, S. M., Visser, J. A., Wallace, W. H., & Anderson, R. A. (2014). The physiology and clinical utility of anti-Müllerian hormone in women. *Human Reproduction Update*, 20(3), 370–385.
- Evans, A. C., Adams, G. P., & Rawlings, N. C. (1994). Follicular and hormonal development in prepubertal heifers from 2 to 36 weeks of age. *Journal of Reproduction and Fertility*, 102(2), 463–470.
- Hagen, C. P., Aksglaede, L., Sørensen, K., Main, K. M., Boas, M., Cleemann, L., Holm, K., Gravholt, C. H., Andersson, A. M., Pedersen, A. T., Petersen, J. H., Linneberg, A., Kjaergaard, S., & Juul, A. (2010). Serum levels of anti-Müllerian hormone as a marker of ovarian function in 926 healthy females from birth to adulthood and in 172 Turner syndrome patients. *The Journal of Clinical Endocrinology and Metabolism*, 95(11), 5003–5010.
- Ireland, J. J., Smith, G. W., Scheetz, D., Jimenez-Krassel, F., Folger, J. K., Ireland, J. L. H., Mossa, F., Lonergan, P., & Evans, A. C. O. (2011). Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-Müllerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. *Reproduction, Fertility and Development*, 23, 1–14.
- Ireland, J. L., Scheetz, D., Jimenez-Krassel, F., Themmen, A. P., Ward, F., Lonergan, P., Smith, G. W., Perez, G. I., Evans, A. C., & Ireland, J. J. (2008). Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biology of Reproduction*, 79(6), 1219–1225.
- Jimenez-Krassel, F., Scheetz, D. M., Neuder, L. M., Ireland, J. L., Pursley, J. R., Smith, G. W., Tempelman, R. J., Ferris, T., Roudebush, W. E., Mossa, F., Lonergan, P., Evans, A. C., & Ireland, J. J. (2015). Concentration of anti-Müllerian hormone in dairy heifers is positively associated with productive herd life. *Journal of Dairy Science*, 98(5), 3036–3045.
- La Marca, A., Sighinolfi, G., Radi, D., Argento, C., Baraldi, E., Artenisio, A. C., Stabile, G., & Volpe, A. (2009). Anti-Müllerian hormone (AMH) as a predictive marker in assisted reproductive technology (ART). *Human Reproduction Update*, 16(2), 113–130.
- Monniaux, D., Drouilhet, L., Rico, C., Estienne, A., Jarrier, P., Touzé, J. L., Sapa, J., Phocas, F., Dupont, J., Dalbiès-Tran, R., & Fabre, S. (2012). Regulation of anti-Müllerian hormone production in domestic animals. *Reproduction, Fertility, and Development*, 25(1), 1–16.
- Mossa, F., Jimenez-Krassel, F., Scheetz, D., Weber-Nielsen, M., Evans, A. C. O., & Ireland, J. J. (2017). Anti-Müllerian hormone (AMH) and fertility management in agricultural species. *Reproduction*, 154(1), R1–R11.
- Mossa, F., Walsh, S. W., Butler, S. T., Berry, D. P., Carter, F., Lonergan, P., Smith, G. W., Ireland, J. J., & Evans, A. C. (2012). Low numbers of ovarian follicles ≥ 3 mm in diameter are associated with low fertility in dairy cows. *Journal of Dairy Science*, 95(5), 2355–2361.
- Rico, C., Fabre, S., Médigue, C., di Clemente, N., Clément, F., Bontoux, M., Touzé, J. L., Dupont, M., Briant, E., Rémy, B., Beckers, J. F., & Monniaux, D. (2009). Anti-müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. *Biology of Reproduction*, 80(1), 50–59.
- Scheetz, D., Folger, J. K., Smith, G. W., & Ireland, J. J. (2012). Granulosa cells are refractory to FSH action in individuals with a low antral follicle count. *Reproduction, Fertility, and Development*, 24(2), 327–336.
- te Velde, E. R., & Pearson, P. L. (2002). The variability of female reproductive ageing. *Human Reproduction Update*, 8(2), 141–154.
- van Disseldorp, J., Lambalk, C. B., Kwee, J., Looman, C. W., Eijkemans, M. J., Fauser, B. C., & Broekmans, F. J. (2010). Comparison of inter- and intra-cycle variability of anti-Müllerian hormone and antral follicle counts. *Human Reproduction*, 25(1), 221–227.
- Visser, J. A., de Jong, F. H., Laven, J. S., & Themmen, A. P. (2006). Anti-Müllerian hormone: A new marker for ovarian function. *Reproduction*, 131(1), 1–9.
- Yang, M. Y., Cushman, R. A., & Fortune, J. E. (2017). Anti-Müllerian hormone inhibits activation and growth of bovine ovarian follicles in vitro and is localized to growing follicles. *Molecular Human Reproduction*, 23(5), 282–291.

FEMALE REPRODUCTIVE PHYSIOLOGY

Female Puberty Overview

Juan M Castellano, Violeta Heras, Francisco Ruiz-Pino, and Manuel Tena-Sempere, Instituto Maimónides de Investigación Biomédica de Córdoba (IMIBIC), Córdoba, Spain; University of Córdoba, Córdoba, Spain; Hospital Universitario Reina Sofía, Córdoba, Spain; and Instituto de Salud Carlos III, Córdoba, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Central-drive hypothesis of puberty It postulates that the initiation of puberty emerges from changes in the activity of the central pathways controlling GnRH neurons. According to this concept, in addition to sex steroid-mediated negative feedback, a central inhibitory system restrains GnRH secretion at early stages of pubertal development. At the onset of puberty, this inhibitory tone is vanished or is dominated by a central stimulatory system. For further details, see Fig. 1.

Epigenetics Inheritable information that is not encoded by the nucleotide sequence of a given gene. Epigenetic regulatory mechanisms include DNA methylation, histone modifications, and microRNAs (miRNA).

Gonadostat hypothesis of puberty It proposes that the onset of puberty results from a developmental change in the sensitivity of the hypothalamic-pituitary unit to the inhibitory feedback action of sex steroids. This sensitivity gradually decreases when puberty approaches, resulting in an increased gonadotropin-releasing hormone (GnRH) secretion that leads to the initiation of puberty.

Kisspeptins Family of structurally related RF-amide peptides, encoded by the KISS1/Kiss1 gene, that act through binding and subsequent activation of the G protein-coupled receptor, Gpr54, which is also termed kisspeptin receptor or Kiss1R.

Kisspeptins have emerged as important gatekeepers of key aspects of reproductive maturation and function, including sexual differentiation of the brain, puberty onset, adult regulation of gonadotropin secretion, and metabolic control of fertility.

KNDy neurons Subset of Kisspeptin neurons located in the hypothalamic arcuate (ARC) nucleus that co-express Neurokinin B (NKB) and Dynorphin A (Dyn) (acronym made using the underlined words). The KNDy hypothesis sets the notion that the dynamic release of kisspeptin from ARC kisspeptin neurons onto GnRH neurons is controlled by the opposite actions of NKB, which are mostly stimulatory, and Dyn, which are mainly of inhibitory nature.

Leptin Adipose hormone that is encoded by the Lep gene. Leptin is released to circulation in proportion to the size of fat stores. In terms of metabolic control, leptin acts as an anorexigenic, and thermogenic factor at the hypothalamus to adjust energy requirements, fat reserves, and food intake. In the context of reproductive function, leptin operates as an essential gatekeeper of puberty and fertility in mammals.

Makorin 3 (MKRN3) Intronless, maternally imprinted gene located on human chromosome 15q11–13. This gene encodes a protein with a conserved RING finger domain and several ZNF motifs with predicted ubiquitin-protein isopeptide ligase E3 activity. MKRN3 has been recently proposed as a potential repressor of puberty onset.

MicroRNAs (miRNAs) Small, noncoding RNAs that can modulate (mainly repress) gene/protein expression, mainly by interaction with seed regions at the 3' untranslated region (3'-UTR) of target genes, thereby promoting mRNA degradation or preventing protein translation.

Polycomb group (PcG) Family of epigenetic regulators of transcription that modify histones (and other proteins) and silence target genes. They function within multiprotein complexes, called Polycomb repressive complexes (PRCs), which include PRC1, PRC2, and PhoRC. Cbx7 and Eed, members of PRC1 and PRC2, respectively, have been shown to be essential components of a novel epigenetic mechanism of transcriptional repression involved in the central control of female puberty.

Abbreviations

ARC Arcuate nucleus

Dyn Dynorphin

FE First estrus

FSH Follicle-stimulating hormone

GABA γ -Aminobutyric acid

GnRH Gonadotropin-releasing hormone

LH Luteinizing hormone

miRNA microRNA

NKB Neurokinin B
 RFRP RFamide-related peptides
 RP3V Rostral periventricular area of the 3rd ventricle
 VO Vaginal opening
 VP Vaginal plug

Introduction

Puberty is a fascinating developmental period that culminates with the attainment of reproductive capacity (Parent et al., 2003). This maturational phenomenon starts to be shaped at early stages of development and involves a complex series of morphological, physiological, behavioral, and psychological changes that allows the acquisition of a complete adult phenotype (Parent et al., 2003). The timing of puberty critically depends on the interplay between genetic and environmental factors (Parent et al., 2003). Indeed, when the dynamic interaction between these two elements is inappropriate, maturation of the reproductive axis is affected, and the timing of puberty is altered. Hence, in addition to its physiological dimension as an essential developmental process, puberty may serve as a biological sensor (and eventual sentinel) for perturbations of gene–environment interactions during pre- and postnatal development.

Assessment of Pubertal Development: External Markers

The assessment of pubertal maturation is particularly relevant in clinical practice and relies on specific changes related to body growth and composition, as well as the development of secondary sexual characteristics. Marshall and Tanner were the first to provide a standard frame of reference for such assessment in both girls and boys. This reference method, known as Tanner scale, allows classifying pubertal development into five continuum stages (from infantile to adult) on the basis of somatic changes in breast, pubic hair, and genital development (Marshall and Tanner, 1969, 1970). In girls, the first sign of puberty initiation is often thelarche (that typically occurs at Tanner stage II), which consists of the onset of breast bud development (Snyder, 2016). This phenomenon is usually followed by pubarche; a process that involves the development of axillary and pubic hair and depends on an increase in adrenal androgen production—termed adrenarche—(Bordini and Rosenfield, 2011). Approximately 2 or 2.5 years after thelarche (at Tanner stage III or, more often, at IV) typically occurs menarche, which represents the first menstrual cycle and, eventually, the final marker of puberty completion in girls (Snyder, 2016).

The study of the mechanistic basis of puberty and its eventual alterations also requires the precise assessment of pubertal maturation in laboratory rodents (i.e., rats and mice), which are widely used in biomedical research. External signs of puberty onset conventionally assessed in female rodents are: (i) the complete canalization of the vagina (i.e., vaginal opening; VO); (ii) the first appearance of cornified epithelial cells in the vagina (i.e., first vaginal estrus; FE); (iii) and the presence of a vaginal plug (VP) after mating (Gaytan et al., 2017; Ojeda and Skinner, 2006). However, it is worth to note that those pubertal markers show relevant differences between rats and mice, especially in terms of reliability. For instance, VO and FE are tightly coupled with the first ovulation in female rats, and consequently, the age of VO is a reliable marker of puberty onset for this species (Ojeda and Skinner, 2006). In contrast, VO and FE are not always immediately followed by first ovulation in mice, which the value of these external pubertal markers for accurate detection of completion of puberty in female mice. In this species, postmating VP seems to be the most accurate external sign of first ovulation (Safrański et al., 1993).

Trends in Pubertal Timing

Recent epidemiological studies conducted in different countries have documented a downward trend in the age of puberty in girls. In the United States, a higher prevalence of thelarche among girls at ages 7 and 8 has been detected when compared with epidemiological data obtained a decade earlier (Biro et al., 2010). In the same vein, a European study conducted over 15 years (1990–2006) in Copenhagen has reported an advancement of approximately 1 year (from 10.88 to 9.86 years) in the onset of breast development in two cohorts of girls (Akslaede et al., 2009b). This phenomenon has also been documented in China, where a recent cross-sectional study of over 20,000 girls has detected an earlier onset of thelarche (Ma et al., 2009).

Although the above epidemiological data requires further validation and demonstration in other populations, this trend toward an earlier puberty onset in girls has caused considerable concerns among scientists and lay public. This is mainly due to the potential health issues associated with this pubertal phenotype, which includes increased risks for up to 48 adverse outcomes across a range of cancer, cardiovascular, gynecological, metabolic, musculoskeletal, and neurocognitive categories (Day et al., 2015; Elks et al., 2013; Velie et al., 2005). The higher prevalence of childhood obesity and the increased exposure to endocrine disruptors have been suggested as relevant factors for this trend in pubertal timing (Akslaede et al., 2009a,b).

Neuroendocrinology of Female Puberty

Female puberty involves the full activation of the so-called hypothalamic-pituitary-ovarian (HPO) axis, which is responsible for the completion of ovarian development and the attainment of sexual phenotypic maturity (Tena-Sempere, 2012; Tena-Sempere and Huhtaniemi, 2003). The function of this intricate neurohormonal system depends primarily on the coordinated interaction of three major groups of signals: (i) the decapeptide gonadotropin-releasing hormone (GnRH), which is synthesized and released in a pulsatile manner by a scarce group of hypothalamic neurons; (ii) the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are produced and secreted by gonadotropic cells of the anterior pituitary; and (iii) the sex steroids, which are generated along with fertilizable gametes and specific regulatory peptides by the ovaries. These components are connected each other via feed-forward and feedback loops, thus facilitating its homeostatic regulation. Furthermore, these regulatory circuits are also sensitive to other endogenous factors and exogenous cues, which contribute to its dynamic modulation (Tena-Sempere and Huhtaniemi, 2003).

Mechanisms for the Onset of Puberty: The Essential Role of GnRH Neurons

In female mammals, it is well known that the initiation of puberty requires a sustained increase in the pulsatile release of GnRH from the hypothalamus (Ojeda and Skinner, 2006). However, it is still a great mystery how such increase occurs. For several decades, it was thought that the increase in the neurosecretory activity of GnRH neurons resulted from a decreased sensitivity of the hypothalamic-pituitary unit to the inhibitory feedback action of ovarian estrogen (Gore, 2002; Rapisarda et al., 1983). Following this assumption, named “gonadostat” hypothesis, the removal of the immature ovary and (eventually) estrogen should result in an increased GnRH/LH response, while estrogen replacement after ovariectomy should cause a suppression of GnRH/LH secretion at prepubertal developmental stages but not at the onset of puberty. In good agreement with this hypothesis, initial studies in female rats demonstrated that ovariectomy (OVX) followed by estrogen replacement suppressed LH secretion at prepubertal stages of development, but not at the time when VO occurs (Steele and Weisz, 1974). However, further evidence brought the above assumption into question. Thus, studies conducted in female rats demonstrated that (i) the loss of responsiveness to the estrogen-negative feedback takes place after, but not before, the first ovulation (Andrews et al., 1981) and (ii) the effectiveness of estrogen in suppressing gonadotropin secretion only changes slightly before puberty (Kawagoe and Hiroi, 1983). In the same vein, results obtained in female rhesus monkeys and humans revealed that GnRH/LH secretion is highly sensitive to the inhibitory actions of estrogen at the time of puberty, that is, when gonadotropin levels are already increasing (Chongthammakun et al., 1993; Maruca et al., 1983). Overall, the above data suggest that the pubertal increase in GnRH/LH secretion may primarily be an ovarian-independent phenomenon in females, especially in humans.

Compelling evidence supports that the increase in the pulsatile GnRH secretion responsible for the onset of female puberty may emerge from changes in the activity of the central pathways controlling GnRH neurons (Ojeda and Skinner, 2006). According to this assumption, known as “central drive” hypothesis (see Fig. 1), the intrinsic oscillatory nature of GnRH neurons along with the wide array of afferents that integrate the so-called GnRH pulse generator, are the key components for the pubertal activation of GnRH neurosecretory activity (Ojeda and Skinner, 2006). The latter is based on the following pieces of evidence: (i) GnRH neurons, which migrate from the olfactory placode to the basal forebrain during embryonic development, reach their final destination (hypothalamus) and project their axons to the median eminence before birth (Jennes, 1989; Prevot, 2015); (ii) the secretory activity of GnRH neurons is prematurely enhanced by the electrical stimulation of the hypothalamus (Claypool et al., 1990; Meijs-Roelofs, 1972); (iii) the dendritic morphology of GnRH neurons changes substantially during the juvenile–pubertal transition, with increased numbers of spines and dendritic rearrangements as puberty approaches (Herbison, 2016); and (iv) GnRH neurons are sensitive to inhibiting and stimulating signals of GnRH release before the onset of puberty (Ojeda et al., 2006b). Considering the above data, it has been proposed that the pubertal activation of GnRH neurons is brought about by coordinated changes in trans-synaptic and glial inputs to the GnRH neuronal network, consisting of a reduction of inhibitory influences and an increase in stimulatory signals (Ojeda et al., 2006b).

The inhibitory trans-synaptic circuitry controlling GnRH release is mostly composed of neurons that produce γ -aminobutyric acid (GABA), endogenous opioids, and eventually RFamide-related peptides (RFRP) (Lomniczi et al., 2013b). GABAergic neurons can modulate GnRH release through indirect actions on neurons connected to the GnRH neuronal network (Ojeda et al., 2006a) or direct actions mediated by the activation of GABA-A receptors on GnRH neurons (Moenter and DeFazio, 2005); it must be noted, however, that direct depolarizing actions of GABA on GnRH neurons have repeatedly been reported. Opiatergic neurons repress GnRH neuronal activity through different peptides acting on different receptors (Fabbri et al., 1989). These actions may be exerted either on GnRH neurons (Dudas and Merchenthaler, 2006) or on neurons involved in the stimulatory control of the GnRH neuronal network (Navarro et al., 2009). RFRP is the mammalian ortholog of the gonadotropin-inhibitory hormone (GnIH), originally described in birds (Leon and Tena-Sempere, 2015). RFRP neurons produce two different peptides, RFRP1 and RFRP3, which can be recognized by a high-affinity receptor, named NPFF1R or GPR147, and a low-affinity receptor, termed NPFFR2 or GPR74 (Leon and Tena-Sempere, 2015). The fact that NPFF1R is expressed in GnRH neurons suggests that RFRP neurons may directly suppress GnRH secretion through this receptor (Ducret et al., 2009; Poling et al., 2012).

A significant fraction of the stimulatory inputs that modulate GnRH secretion is provided by neurons that synthesize glutamate, kisspeptins, and neurokinin B (NKB) as neuronal transmitters (Lomniczi et al., 2013b) (for further details, see sections “Kisspeptins as major regulators of GnRH neurons and puberty onset” and “NKB and Dynorphin as key partners of kisspeptins in the control of GnRH neurons and puberty onset”).

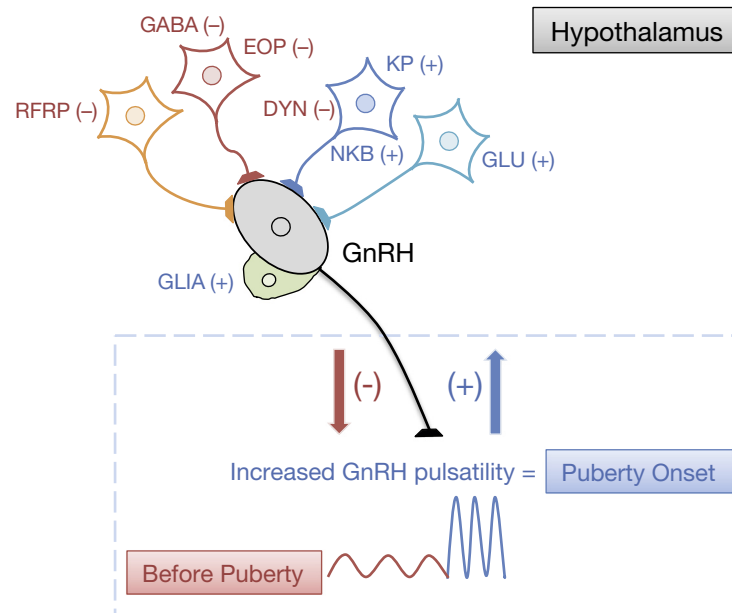


Fig. 1 Schematic representation of the “central drive” hypothesis of puberty. The increase in the pulsatile GnRH release that leads to the onset of puberty is brought about by coordinated changes in trans-synaptic and glial inputs to GnRH neuronal network, consisting of a reduction of inhibitory influences (–) and an increase in stimulatory signals (+). The inhibitory trans-synaptic circuitry is mostly composed of neurons that synthesize RFamide-related peptides (RFRP), γ -aminobutyric acid (GABA), endogenous opioids (EOP), and dynorphin (DYN), whereas the stimulatory counterpart is provided by neurons that produce kisspeptins (KP), neurokinin B (NKB), and glutamate (GLU). Glial cells facilitate GnRH secretion by the release of growth factors and other bioactive molecules. According to this hypothesis, GnRH secretion is restrained by inhibitory influences at early stages of development. At the time of puberty onset, this inhibitory tone is lifted and/or replaced by stimulatory inputs. From Pinilla, L., Aguilar, E., Dieguez, C., Millar, R.P., and Tena-Sempere, M. (2012). Kisspeptins and reproduction: Physiological roles and regulatory mechanisms. *Physiological Reviews* **92**, 1235–1316, with substantial modifications.

Glial cells, such as astrocytes and tanycytes, facilitate GnRH secretion by the release of growth factors and other bioactive molecules, as well as the plastic rearrangement of glial-to-GnRH neuron contacts and adhesiveness (Ojeda et al., 2010).

Kisspeptins as Major Regulators of GnRH Neurons and Puberty Onset

Among the plethora of neuropeptides controlling pubertal GnRH release, kisspeptins hold a prominent place. Indeed, they are currently recognized as one of the most powerful activators, if not the most, of the GnRH/gonadotropin system during the pubertal transition (Pinilla et al., 2012). Kisspeptins are a family of structurally related peptides, encoded by *KISS1/Kiss1* gene, that act through binding and subsequent activation of the G protein-coupled receptor, Gpr54, which is also termed kisspeptin receptor or Kiss1R (Oakley et al., 2009; Pinilla et al., 2012). The pubertal dimension of kisspeptins was unveiled in 2003, when inactivating mutations of *GPR54* were associated with lack of pubertal progression and hypogonadotropic hypogonadism in humans (de Roux et al., 2003; Seminara et al., 2003). These findings were later supported by similar phenotypes of mice with genetic inactivation of *Gpr54* or *Kiss1*, and humans with inactivating mutations of *KISS1* (Pinilla et al., 2012). Those observations paved the way for the analysis of the physiological roles of kisspeptin neurons in the control of GnRH pulsatile secretion and (eventually) the onset of puberty. These studies have documented a sophisticated developmental program responsible for the activation of the Kiss1 neuronal system during the pubertal transition (Pinilla et al., 2012; Tena-Sempere, 2013). Such program involves the following major components: (i) a hypothalamic rise of Kiss1 mRNA/peptide content during the juvenile–pubertal transition, which seems to be sufficient to induce a state of full activation of the GnRH/LH system (Navarro et al., 2004a,b; Plant et al., 2006; Shahab et al., 2005); (ii) an increase in the sensitivity to the excitatory actions of kisspeptins on GnRH/LH release (Castellano et al., 2006a; Han et al., 2005); (iii) an augmentation of Gpr54 signaling efficiency at GnRH neurons, coupled to a state of resistance to desensitization to kisspeptin stimulation (Han et al., 2005; Roa et al., 2008); and (iv) an increase in the number of kisspeptin-positive neurons and their projections to GnRH neurons (Bentsen et al., 2010; Clarkson and Herbison, 2006).

Additional pharmacological and genetic studies have further attested the relevance of proper kisspeptin signaling for the precise timing of puberty in females. Thus, daily injections of a synthetic kisspeptin analog, Kp-10, in prepubertal female mice resulted in precocious puberty onset, as estimated by earlier VO and FE. Furthermore, this pubertal phenotype was associated with a direct stimulation of GnRH neurons (Decourt et al., 2016). In contrast, central infusion of a kisspeptin antagonist, p234, in prepubertal female rats led to delayed VO and reduced uterus and ovarian weights (Pineda et al., 2010); a phenomenon that is seemingly linked to a suppression of GnRH secretion, as subsequently demonstrated in prepubertal and pubertal female rhesus monkeys (Guerriero

et al., 2012). In the same vein, experiments of genetic manipulation showed that the timed ablation of Kiss1-expressing neurons in juvenile female mice prevented the attainment of reproductive competence (Mayer and Boehm, 2011).

Detailed neuroanatomical studies in mammals have identified two major hypothalamic populations of kisspeptin neurons in the ventral forebrain. One of these populations is located in the rostral periventricular area of the 3rd ventricle (RP3V), and the other is found in the arcuate nucleus (ARC; infundibular region in humans) (Garcia-Galiano et al., 2012; Smith et al., 2005a). These two populations seem to be functionally different. While RP3V kisspeptin neurons respond to estrogen with increased production of kisspeptin, the synthesis of kisspeptin is inhibited by estrogen in the ARC (Smith et al., 2005a,b). These and other observations have led to the concept that RP3V kisspeptin neurons are involved in positive feedback and the genesis of the preovulatory LH surge in females, whereas ARC kisspeptin neurons mediate the inhibitory effect of estrogen on GnRH/gonadotropin release and are therefore crucial for the tonic pulsatile secretion of gonadotropins (Pinilla et al., 2012).

The relative contribution of the different kisspeptin neuronal populations to the precise timing of puberty has been partially addressed in recent years. Expression analyses revealed an increase of kisspeptin content in the RP3V of female mice from the infantile to the pubertal period (Clarkson and Herbison, 2006). Furthermore, virogenetic experiments involving the partial knocking down of kisspeptin expression either into the RP3V or ARC of female rats documented a partial delay in the onset of puberty only after targeting RP3V kisspeptin neurons (Hu et al., 2015). Although the latter may suggest a relevant role of RP3V kisspeptin neurons in the timing of female puberty, these findings must be taken with caution due to the moderate kisspeptin suppression obtained with this virogenetic approach in both kisspeptin neuronal populations.

Anyhow, it is worth to note that experimental evidence arguing against an indispensable role of kisspeptin signaling in the onset of puberty has also been presented, as congenital ablation of kisspeptin neurons in female mice appeared to be compatible with the attainment of adult fertility (Mayer and Boehm, 2011). It can be argued, however, that this phenotype could be due to an incomplete neuronal ablation and/or developmental compensation. Indeed, as mentioned above, the same study showed that timed elimination of kisspeptin neurons during the early juvenile period prevented normal pubertal maturation and caused infertility (Mayer and Boehm, 2011).

NKB and Dynorphin as Key Partners of Kisspeptins in the Control of GnRH Neurons and Puberty Onset

The recent identification of NKB and dynorphin (Dyn), as products and putative co-regulators of kisspeptin neurons, has represented an important breakthrough in our understanding of the mechanisms controlling kisspeptin release and, eventually, the awakening of GnRH/gonadotropin system at puberty (Lehman et al., 2010; Navarro et al., 2009). These co-transmitters are only expressed in a percentage of ARC kisspeptin neurons, which can vary depending on the species, the sex and the functional state of the reproductive axis (Hrabovszky et al., 2012; Overgaard et al., 2014). Because this Kisspeptin neuronal population co-express NKB and Dyn, they have been termed KNDy neurons (acronym made using the underlined words).

Compelling evidence suggests that KNDy neurons are important modulators of GnRH pulsatile release, by dictating patterned kisspeptin secretion on GnRH neurons (Navarro, 2012). In particular, the KNDy hypothesis sets the notion that the dynamic release of kisspeptin from the ARC kisspeptin neurons onto GnRH neurons is tightly regulated by the reciprocal stimulatory and inhibitory actions of NKB and Dyn, respectively (Navarro, 2012). This assumption is primarily based on the presence of numerous collateral projections interconnecting ARC KNDy neurons and the proven expression of NKB and Dyn receptors (NK3R and Oprk1, respectively) in these neurons. The physiological relevance of such system is further strengthened by the clinical findings that patients with mutations of the NKB and NK3R gene display a state of hypogonadotropic hypogonadism similar to that shown in patients with genetic inactivation of KISS1 or GPR54 (Topaloglu et al., 2009).

Our current knowledge about the roles of NKB and Dyn signaling in the control of puberty onset is still limited; yet, mounting evidence suggests their contribution in pubertal timing. Thus, central administration of the NKB agonist, senktide, to prepubertal female rats stimulated LH secretion (Navarro et al., 2012). Furthermore, the developmental expression of the NKB gene in the hypothalamus increases before the rise of Kiss1 mRNA levels in female rodents (Gill et al., 2012), which is compatible with a role of NKB in the pubertal activation of kisspeptin/GnRH secretion (Fergani and Navarro, 2017). The latter is further supported by the moderate delay of puberty onset induced by the central blockade of NKB signaling in female rats (Navarro et al., 2012). Interestingly, an independent study later showed that either stimulation of NKB signaling or inhibition of Dyn induces early puberty onset in female rats (Nakahara et al., 2013). These observations have led to the speculation that the inhibitory action of Dyn on kisspeptin release declines when puberty approaches, whereas the stimulatory effect of NKB on kisspeptin secretion gradually augments during this transition. As a result of these sequential modulatory events, the pulsatile kisspeptin/GnRH/LH release would increase, thus leading to the onset of puberty (Uenoyama et al., 2014).

Finally, it is important to note that congenital NK3R null female mice seem to display normal puberty onset (Yang et al., 2012); yet, these animals do present various reproductive defects, including abnormal estrous cycles, reduced number of corpora lutea, decreased uterine weights, and subfertility (Yang et al., 2012).

Metabolic Influences Regulating the Timing of Female Puberty

The scientific basis for the connection between the magnitude of energy (fat) stores and the timing of puberty was formulated for the first time by Frisch and Revelle in 1970. Their “critical fat mass hypothesis” proposed the need to reach

a certain threshold of body fat mass for the attainment of reproductive capacity (Frisch and Revelle, 1970). This assumption is especially evident in females, in which the acquisition of reproductive capacity involves the potential metabolic drainage of pregnancy and lactation and, therefore requires reaching a minimal level of energy stores. Furthermore, the timing of puberty in females seems to be extremely sensitive to conditions of energy insufficiency (i.e., undernutrition) and energy excess (i.e., overnutrition), as evidenced by different experimental and epidemiological studies (Castellano and Tena-Sempere, 2016).

Leptin as an Essential Gatekeeper of Puberty Onset

The formulation of the critical fat mass hypothesis, and its supporting evidence, fuelled the search of the potential endogenous mediators responsible for transmitting the metabolic information to the brain reproductive centers (mainly, the hypothalamus) that control the onset of puberty. In this context, the identification of the adipose hormone, leptin, represented a significant breakthrough (Vazquez et al., 2015). Indeed, leptin is an ideal candidate for connecting metabolism and reproduction, due to its ability for conveying information regarding the magnitude of fat stores to the hypothalamus, where it acts as an anorexigenic and thermogenic factor to adjust energy requirements, fat reserves, and food intake (Casaneuva and Dieguez, 1999).

The pubertal dimension of leptin is clearly illustrated by the alterations in the timing of puberty associated with conditions of leptin deficiency (e.g., inactivating mutations affecting leptin signaling and malnutrition) and situations of leptin excess (e.g., genetic models of leptin overexpression and early overfeeding), which have been linked to delayed and advanced puberty, respectively (Castellano and Tena-Sempere, 2016; Manfredi-Lozano et al., 2017).

Despite some initial controversy, there is now a broad consensus that leptin plays a major permissive role in the metabolic gating of puberty; an assumption that is based on a large body of evidence. For instance, chronic leptin administration has shown to prevent the delay in the onset of puberty induced by severe undernutrition in female rodents (Cheung et al., 1997, 2001). In the same vein, it has been reported that the maintenance of adequate levels of leptin in conditions of energy insufficiency prevents pubertal delay in female prepubertal rats (Gruaz et al., 1998); a phenomenon that has also been documented in humans, where subcutaneous injections of leptin facilitate pubertal development in obese children congenitally deficient in leptin (Farooqi et al., 2002). As a whole, the above evidence suggests that leptin, rather than a trigger, enables puberty to proceed when its circulating levels achieve a certain threshold.

Interplay of Leptin and Kisspeptins in the Control of Puberty: Direct, Indirect or Independent Actions

How the permissive actions of leptin are transmitted to the GnRH neuronal network during the pubertal transition is still an open question. Studies in rodents and primates suggest that such actions are carried out in an indirect manner, as the expression levels of leptin receptor (LepR) in GnRH neurons seems to be very low or negligible (Finn et al., 1998; Hakansson et al., 1998). Furthermore, when LepR is detected, as it has been shown in female mice, its selective elimination in GnRH neurons does not cause any alteration in the timing of puberty (Quennell et al., 2009).

The above observations clearly suggest the potential involvement of central mediators for transmitting the pubertal actions of leptin onto GnRH neurons. Among them, kisspeptin neurons have received special attention in recent years (see Fig. 2). The first studies addressing the potential role of kisspeptins as putative mediators of leptin actions onto GnRH neurons supported a direct connection between leptin and kisspeptin. This assumption was based on the following pieces of evidence: (i) conditions of leptin deficiency (i.e., undernutrition) suppresses hypothalamic *Kiss1* expression in rodents and sheep (Castellano and Tena-Sempere, 2016; Manfredi-Lozano et al., 2017), (ii) leptin administration activates kisspeptin neurons or increase *Kiss1* expression in vivo (Backholer et al., 2010; Castellano et al., 2006b; Qiu et al., 2011; Smith et al., 2006), and in vitro (Luque et al., 2007; Morelli et al., 2008); and (iii) LepR mRNA is detected in a subset of ARC kisspeptin neurons in mice and ewes (Quennell et al., 2011; Smith et al., 2006).

Despite the above data suggested a direct mode of action of leptin on kisspeptin neurons, subsequent findings have (partially) questioned this assumption. Thus, studies in a *Kiss1-Cre* mouse model that allows the identification of kisspeptin neurons evidenced that only a small fraction of ARC kisspeptin neurons responds to leptin stimulation (Cravo et al., 2011). Moreover, the congenital ablation of LepR from kisspeptin neurons did not prevent the onset of puberty in female mice (Cravo et al., 2011). Additional studies have not been able to detect functional LepRs in kisspeptin neurons before puberty (Cravo et al., 2013; Qiu et al., 2015), while suggested that LepR might become expressed in this neuronal population in adulthood (Cravo et al., 2013; Qiu et al., 2011, 2015). One of those studies also demonstrated the expression of LepR in populations of ARC and RP3V neurons, of unknown nature, in close vicinity of kisspeptin neurons. Moreover, a relevant population of neurons expressing LepR has also been described in the hypothalamic premammillary nucleus (PMV). The physiological relevance of such population is demonstrated by the rescue of puberty onset induced by the selective re-expression of LepR in the PMV of female db/db mice, devoid of LepR (Donato et al., 2011). This study also showed that PMV neurons expressing LepR innervate kisspeptin neurons, suggesting their potential interplay with kisspeptin signaling in the metabolic control of puberty. Overall, these observations are compatible with an indirect (or even independent) mode of action of leptin on kisspeptin neurons in the control of female puberty.

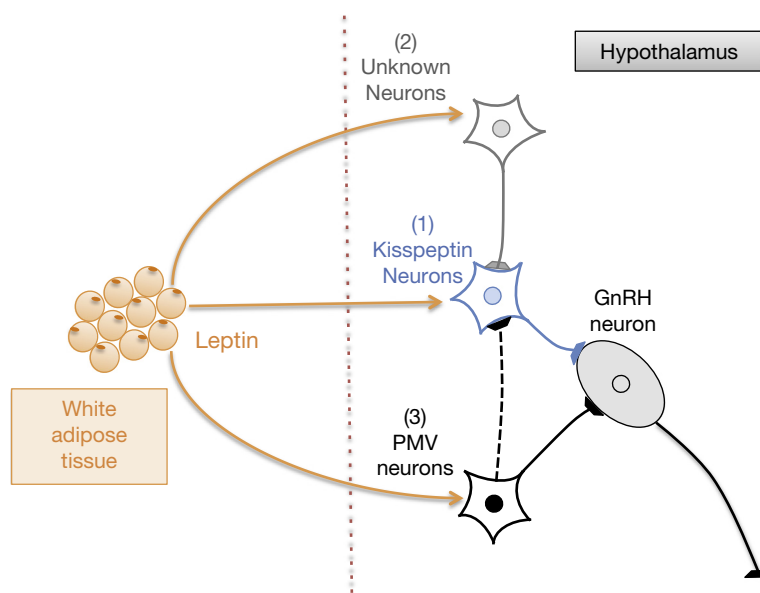


Fig. 2 Tentative scheme showing the direct (1), indirect (2) and independent (3) mode of action of leptin on kisspeptin pathways controlling GnRH neurons. While initial evidence supported a direct connection between leptin and kisspeptin (1), further studies suggest the potential involvement of an unknown population of ARC and R3PV neurons located in close vicinity of kisspeptin neurons (2) and the novel role of a neuronal population expressing LepR in the hypothalamic PMV (3). Of note, the major actions of leptin on PMV nucleus, which were initially considered as kisspeptin-independent, do not rule out the existence of some interaction with kisspeptin circuits (represented as a *dotted line* projection).

Novel Signals and Mechanisms for the Control of Female Puberty

The complex and sophisticated nature of puberty, and its sensitivity to different regulators, strongly suggest the involvement of diverse but complementary regulatory mechanisms, enabling such a degree of precise control. Emerging evidence is mounting that such intricate control likely involves epigenetic regulatory mechanisms. Those mechanisms mainly include: (i) DNA methylation, which consists of the incorporation of methyl groups to CpG islands in the DNA and is often associated with gene repression (Paulsen et al., 2008); (ii) histone modifications (e.g., acetylation, methylation, and phosphorylation), whose effects on gene expression, in terms of activation or repression, depends on the histone and residue subjected to modification (Sims et al., 2008); (iii) and miRNAs, which are small, noncoding RNAs that can modulate (mainly repress) gene/protein expression, predominantly by interaction with seed regions at the 3' untranslated region (3'-UTR) of target genes (Sayed and Abdellatif, 2011).

Roles of the Polycomb Group and Zinc Finger Proteins

Recent evidence suggests the involvement of an epigenetic mechanism of transcriptional repression in the central control of female puberty (Lomniczi et al., 2013a). This mechanism is driven by two members of the so-called Polycomb group, namely Eed and Cbx7. Lomniczi and colleagues showed that the hypothalamic expression of these two genes decreases during the juvenile–pubertal transition owing to the enhanced methylation pattern of their promoters (Lomniczi et al., 2013a). In line with this finding, the pharmacological inhibition of DNA methylation in juvenile female rats, a manipulation that may prevent the pubertal decrease of *Eed* and *Cbx7* expression, delayed the onset of puberty (Lomniczi et al., 2013a). This epigenetic repressive mechanism was shown to modulate the hypothalamic expression of *Kiss1* (Lomniczi et al., 2013a). Thus, the increase of *Kiss1* mRNA levels preceding the onset of puberty was accompanied by the removal of EED from *Kiss1* promoter and the enrichment of histone modifications linked to gene activation (Lomniczi et al., 2013a). In contrast, the prevention of the loss of EED from the *Kiss1* promoter disrupted GnRH pulsatile release and delayed the timing of puberty (Lomniczi et al., 2013a).

Further studies have reported a similar epigenetic mechanism of transcriptional repression in female primates and rats. This mechanism is conducted by GATAD1 and ZNF573, two members of the family of transcriptional repressors Zinc Finger (ZNF) genes (Lomniczi et al., 2015). The hypothalamic expression of these two genes has been shown to decrease in peripubertal female monkeys (Lomniczi et al., 2015). The physiological relevance of this phenomenon was confirmed by further functional analyses in female rats. Thus, *GATAD1* or *ZNF573* overexpression in the ARC of juvenile female rats delayed the onset of puberty; a phenomenon that was associated with changes in the epigenetic landscape of the hypothalamus (Lomniczi et al., 2015). Importantly, GATAD1 evoked the transcriptional repression of *KISS1* and *TAC3* genes through an epigenetic mechanism that implies the eviction of the activating histone mark H3K4me2 from *KISS1* and *TAC3* promoters via the recruitment of the histone demethylase KDM1A (Lomniczi et al., 2015).

An additional member of the superfamily of ZNF genes that may also contribute to the transcriptional silencing of female puberty is Makorin 3 (MKRN3). *MKRN3* is an intron-less, maternally imprinted gene that encodes a protein with a conserved RING finger domain and several ZNF motifs with predicted ubiquitin-protein isopeptide ligase E3 activity (Jong et al., 1999). The potential repressive role of MKRN3 in the control of puberty has been suggested by the presence of inactivating mutations of MKRN3 in girls and boys with precocious puberty (Abreu et al., 2013); a phenomenon that has been documented by several independent studies in different human populations (Abreu et al., 2015). In line with these findings, a decline in the hypothalamic expression of *Mkfn3* has been detected in both male and female mice during the prepubertal development (Abreu et al., 2013). Furthermore, recent studies have demonstrated a decrease in the serum levels of MKRN3 before the onset of puberty in healthy girls and boys (Abreu et al., 2015). Overall, these data support the potential repressive role of MKRN3 in the control of puberty; yet, further studies are required to demonstrate the precise mechanism of action of MKRN3 in such context.

Roles of miRNA Regulatory Pathways

The first indirect evidence for a role of miRNAs in the timing of puberty came from genome-wide association (GWAS) studies that revealed an association of the age at menarche with variability at 6q21 chromosome, in or near the *LIN28B* gene (Elks et al., 2010). *LIN28B* encodes a cytoplasmic protein that blocks the processing of mature miRNAs of the let-7 family (Viswanathan et al., 2008). Interestingly, reciprocal changes in the hypothalamic expression of *Lin28b* and let-7 (as well as related miRNAs, for example, miR-132, miR-145) have been documented in female rats during postnatal development (Sangiao-Alvarellos et al., 2013). Thus, while *lin28b* mRNA levels decrease along the prepubertal period, the mRNA expression of let7 increases during this transition (Sangiao-Alvarellos et al., 2013). Moreover, altered ratios of *Lin28b*/let7 levels have also been documented in different rodent models of perturbed puberty (Sangiao-Alvarellos et al., 2013). As a whole, the above findings strongly suggest the potential role of the *Lin28b*/let7 miRNA system in pubertal modulation; yet, functional data showing the impact of such alterations in the onset of puberty are still missing.

Interestingly, a very recent study has provided a more direct evidence for the pubertal role of miRNA regulatory pathways in the control of puberty. In particular, Messina and coworkers have demonstrated a switch in the miRNA expression profiles of GnRH neurons during the infantile period that precedes to, and seems mandatory for, the onset of puberty in rodents (Messina et al., 2016). This switch involves significant alterations in the expression of members of the miR-155 and miR-200 families that seem to be mandatory for appropriate activation of GnRH neurosecretion during the pubertal transition (Messina et al., 2016). In line with this finding, genetic ablation of *Dicer*, the enzyme responsible for the generation of mature miRNAs, in GnRH neurons prevented normal pubertal maturation in both male and female mice and resulted in central hypogonadism and infertility (Messina et al., 2016).

References

- Abreu, A. P., Dauber, A., Macedo, D. B., Noel, S. D., Brito, V. N., Gill, J. C., Cukier, P., Thompson, I. R., Navarro, V. M., Gagliardi, P. C., et al. (2013). Central precocious puberty caused by mutations in the imprinted gene MKRN3. *The New England Journal of Medicine*, 368, 2467–2475.
- Abreu, A. P., Macedo, D. B., Brito, V. N., Kaiser, U. B., & Latronico, A. C. (2015). A new pathway in the control of the initiation of puberty: The MKRN3 gene. *Journal of Molecular Endocrinology*, 54, R131–139.
- Aksglaede, L., Juul, A., Olsen, L. W., & Sorensen, T. I. (2009a). Age at puberty and the emerging obesity epidemic. *PLoS One*, 4, e8450.
- Aksglaede, L., Sorensen, K., Petersen, J. H., Skakkebaek, N. E., & Juul, A. (2009b). Recent decline in age at breast development: The Copenhagen puberty study. *Pediatrics*, 123, e932–939.
- Andrews, W. W., Advis, J. P., & Ojeda, S. R. (1981). The maturation of estradiol-negative feedback in female rats: Evidence that the resetting of the hypothalamic "gonadostat" does not precede the first preovulatory surge of gonadotropins. *Endocrinology*, 109, 2022–2031.
- Backholer, K., Smith, J. T., Rao, A., Pereira, A., Iqbal, J., Ogawa, S., Li, Q., & Clarke, I. J. (2010). Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. *Endocrinology*, 151, 2233–2243.
- Bentsen, A. H., Ansel, L., Simonneaux, V., Tena-Sempere, M., Juul, A., & Mikkelsen, J. D. (2010). Maturation of kisspeptinergic neurons coincides with puberty onset in male rats. *Peptides*, 31, 275–283.
- Biro, F. M., Galvez, M. P., Greenspan, L. C., Succop, P. A., Vangeepuram, N., Pinney, S. M., Teitelbaum, S., Windham, G. C., Kushi, L. H., & Wolff, M. S. (2010). Pubertal assessment method and baseline characteristics in a mixed longitudinal study of girls. *Pediatrics*, 126, e583–590.
- Bordini, B., & Rosenfield, R. L. (2011). Normal pubertal development: Part II: Clinical aspects of puberty. *Pediatrics in Review*, 32, 281–292.
- Casanueva, F. F., & Dieguez, C. (1999). Neuroendocrine regulation and actions of leptin. *Frontiers in Neuroendocrinology*, 20, 317–363.
- Castellano, J. M., & Tena-Sempere, M. (2016). Metabolic control of female puberty: Potential therapeutic targets. *Expert Opinion on Therapeutic Targets*, 20, 1181–1193.
- Castellano, J. M., Navarro, V. M., Fernandez-Fernandez, R., Castano, J. P., Malagon, M. M., Aguilar, E., Dieguez, C., Magni, P., Pinilla, L., & Tena-Sempere, M. (2006a). Ontogeny and mechanisms of action for the stimulatory effect of kisspeptin on gonadotropin-releasing hormone system of the rat. *Molecular and Cellular Endocrinology*, 257–258, 75–83.
- Castellano, J. M., Navarro, V. M., Fernandez-Fernandez, R., Roa, J., Vigo, E., Pineda, R., Dieguez, C., Aguilar, E., Pinilla, L., & Tena-Sempere, M. (2006b). Expression of hypothalamic KISS-1 system and rescue of defective gonadotropic responses by kisspeptin in streptozotocin-induced diabetic male rats. *Diabetes*, 55, 2602–2610.
- Cheung, C. C., Thornton, J. E., Kuijper, J. L., Weigle, D. S., Clifton, D. K., & Steiner, R. A. (1997). Leptin is a metabolic gate for the onset of puberty in the female rat. *Endocrinology*, 138, 855–858.
- Cheung, C. C., Thornton, J. E., Nurani, S. D., Clifton, D. K., & Steiner, R. A. (2001). A reassessment of leptin's role in triggering the onset of puberty in the rat and mouse. *Neuroendocrinology*, 74, 12–21.
- Chongthammakun, S., Claypool, L. E., & Terasawa, E. (1993). Ovariectomy increases in vivo luteinizing hormone-releasing hormone release in pubertal, but not prepubertal, female rhesus monkeys. *Journal of Neuroendocrinology*, 5, 41–50.
- Clarkson, J., & Herbison, A. E. (2006). Postnatal development of kisspeptin neurons in mouse hypothalamus; sexual dimorphism and projections to gonadotropin-releasing hormone neurons. *Endocrinology*, 147, 5817–5825.

- Claypool, L. E., Watanabe, G., & Terasawa, E. (1990). Effects of electrical stimulation of the medial basal hypothalamus on the in vivo release of luteinizing hormone-releasing hormone in the prepubertal and peripubertal female monkey. *Endocrinology*, 127, 3014–3022.
- Cravo, R. M., Margatho, L. O., Osborne-Lawrence, S., Donato, J., Jr., Atkin, S., Bookout, A. L., Rovinsky, S., Frazao, R., Lee, C. E., Gautron, L., et al. (2011). Characterization of Kiss1 neurons using transgenic mouse models. *Neuroscience*, 173, 37–56.
- Cravo, R. M., Frazao, R., Perello, M., Osborne-Lawrence, S., Williams, K. W., Zigman, J. M., Vianna, C., & Elias, C. F. (2013). Leptin signaling in Kiss1 neurons arises after pubertal development. *PLoS One*, 8, e58698.
- Day, F. R., Elks, C. E., Murray, A., Ong, K. K., & Perry, J. R. (2015). Puberty timing associated with diabetes, cardiovascular disease and also diverse health outcomes in men and women: The UK Biobank study. *Scientific Reports*, 5, 11208.
- Decourt, C., Robert, V., Anger, K., Galibert, M., Madinier, J. B., Liu, X., Dardente, H., Lomet, D., Delmas, A. F., Caraty, A., et al. (2016). A synthetic kisspeptin analog that triggers ovulation and advances puberty. *Scientific Reports*, 6, 26908.
- Donato, J., Jr., Cravo, R. M., Frazao, R., Gautron, L., Scott, M. M., Lachey, J., Castro, I. A., Margatho, L. O., Lee, S., Lee, C., et al. (2011). Leptin's effect on puberty in mice is relayed by the ventral premammillary nucleus and does not require signaling in Kiss1 neurons. *The Journal of Clinical Investigation*, 121, 355–368.
- Ducet, E., Anderson, G. M., & Herbison, A. E. (2009). RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. *Endocrinology*, 150, 2799–2804.
- Dudas, B., & Merchenthaler, I. (2006). Three-dimensional representation of the neurotransmitter systems of the human hypothalamus: Inputs of the gonadotrophin hormone-releasing hormone neuronal system. *Journal of Neuroendocrinology*, 18, 79–95.
- Elks, C. E., Perry, J. R., Sulem, P., Chasman, D. I., Franceschini, N., He, C., Lunetta, K. L., Visser, J. A., Byrne, E. M., Cousminer, D. L., et al. (2010). Thirty new loci for age at menarche identified by a meta-analysis of genome-wide association studies. *Nature Genetics*, 42, 1077–1085.
- Elks, C. E., Ong, K. K., Scott, R. A., van der Schouw, Y. T., Brand, J. S., Wark, P. A., Amiano, P., Balkau, B., Barricarte, A., Boeing, H., et al. (2013). Age at menarche and type 2 diabetes risk: The EPIC-InterAct study. *Diabetes Care*, 36, 3526–3534.
- Fabbri, A., Jannini, E. A., Gnassi, L., Ulisse, S., Moretti, C., & Isidori, A. (1989). Neuroendocrine control of male reproductive function. The opioid system as a model of control at multiple sites. *Journal of Steroid Biochemistry*, 32, 145–150.
- Farooqi, I. S., Matarese, G., Lord, G. M., Keogh, J. M., Lawrence, E., Agwu, C., Sanna, V., Jebb, S. A., Perna, F., Fontana, S., et al. (2002). Beneficial effects of leptin on obesity, T cell hyporesponsiveness, and neuroendocrine/metabolic dysfunction of human congenital leptin deficiency. *The Journal of Clinical Investigation*, 110, 1093–1103.
- Fergani, C., & Navarro, V. (2017). Expanding the role of Tachykinins in the neuroendocrine control of reproduction. *Reproduction*, 153, R1–R14.
- Finn, P. D., Cunningham, M. J., Pau, K. Y., Spies, H. G., Clifton, D. K., & Steiner, R. A. (1998). The stimulatory effect of leptin on the neuroendocrine reproductive axis of the monkey. *Endocrinology*, 139, 4652–4662.
- Frisch, R. E., & Revelle, R. (1970). Height and weight at menarche and a hypothesis of critical body weights and adolescent events. *Science*, 169, 397–399.
- Garcia-Galiano, D., Pinilla, L., & Tena-Sempere, M. (2012). Sex steroids and the control of the Kiss1 system: Developmental roles and major regulatory actions. *Journal of Neuroendocrinology*, 24, 22–33.
- Gaytan, F., Morales, C., Leon, S., Heras, V., Barroso, A., Avendano, M. S., Vazquez, M. J., Castellano, J. M., Roa, J., & Tena-Sempere, M. (2017). Development and validation of a method for precise dating of female puberty in laboratory rodents: The puberty ovarian maturation score (Pub-Score). *Scientific Reports*, 7, 46381.
- Gill, J. C., Navarro, V. M., Kwong, C., Noel, S. D., Martin, C., Xu, S., Clifton, D. K., Carroll, R. S., Steiner, R. A., & Kaiser, U. B. (2012). Increased neurokinin B (Tac2) expression in the mouse arcuate nucleus is an early marker of pubertal onset with differential sensitivity to sex steroid-negative feedback than Kiss1. *Endocrinology*, 153, 4883–4893.
- Gore, A. C. (2002). GnRH neurons changes across the life cycle. In *The master molecule of reproduction* (pp. 53–92). Norwell, MA: Kluwer Academic Publishers.
- Gruaz, N. M., Laloui, M., Pierroz, D. D., Englaro, P., Sizonenko, P. C., Blum, W. F., & Aubert, M. L. (1998). Chronic administration of leptin into the lateral ventricle induces sexual maturation in severely food-restricted female rats. *Journal of Neuroendocrinology*, 10, 627–633.
- Guerriero, K. A., Keen, K. L., Millar, R. P., & Terasawa, E. (2012). Developmental changes in GnRH release in response to kisspeptin agonist and antagonist in female rhesus monkeys (*Macaca mulatta*): Implication for the mechanism of puberty. *Endocrinology*, 153, 825–836.
- Hakansson, M. L., Brown, H., Ghilardi, N., Skoda, R. C., & Meister, B. (1998). Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. *The Journal of Neuroscience*, 18, 559–572.
- Han, S. K., Gottsch, M. L., Lee, K. J., Popa, S. M., Smith, J. T., Jakawich, S. K., Clifton, D. K., Steiner, R. A., & Herbison, A. E. (2005). Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. *The Journal of Neuroscience*, 25, 11349–11356.
- Herbison, A. E. (2016). Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. *Nature Reviews. Endocrinology*, 12, 452–466.
- Hrabovszky, E., Sipos, M. T., Molnar, C. S., Ciofi, P., Borsay, B. A., Gergely, P., Herczeg, L., Bloom, S. R., Ghatel, M. A., Dhillon, W. S., et al. (2012). Low degree of overlap between kisspeptin, neurokinin B, and dynorphin immunoreactivities in the infundibular nucleus of young male human subjects challenges the KNDy neuron concept. *Endocrinology*, 153, 4978–4989.
- Hu, M. H., Li, X. F., McCausland, B., Li, S. Y., Gresham, R., Kinsey-Jones, J. S., Gardiner, J. V., Sam, A. H., Bloom, S. R., Poston, L., et al. (2015). Relative importance of the arcuate and anteroventral periventricular kisspeptin neurons in control of puberty and reproductive function in female rats. *Endocrinology*, 156, 2619–2631.
- Jennes, L. (1989). Prenatal development of the gonadotropin-releasing hormone-containing systems in rat brain. *Brain Research*, 482, 97–108.
- Jong, M. T., Gray, T. A., Ji, Y., Glenn, C. C., Saitoh, S., Driscoll, D. J., & Nicholls, R. D. (1999). A novel imprinted gene, encoding a RING zinc-finger protein, and overlapping antisense transcript in the Prader-Willi syndrome critical region. *Human Molecular Genetics*, 8, 783–793.
- Kawagoe, S., & Hiroi, M. (1983). Maturation of negative and positive estrogen feedback in the prepubertal female rat. *Endocrinologia Japonica*, 30, 435–441.
- Lehman, M. N., Coolen, L. M., & Goodman, R. L. (2010). Minireview: Kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: A central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology*, 151, 3479–3489.
- Leon, S., & Tena-Sempere, M. (2015). Dissecting the roles of gonadotropin-inhibitory hormone in mammals: Studies using pharmacological tools and genetically modified mouse models. *Frontiers in Endocrinology*, 6, 189.
- Lomniczi, A., Loche, A., Castellano, J. M., Ronnekleiv, O. K., Bosch, M., Kaidar, G., Knoll, J. G., Wright, H., Pfeifer, G. P., & Ojeda, S. R. (2013a). Epigenetic control of female puberty. *Nature Neuroscience*, 16, 281–289.
- Lomniczi, A., Wright, H., Castellano, J. M., Sonmez, K., & Ojeda, S. R. (2013b). A system biology approach to identify regulatory pathways underlying the neuroendocrine control of female puberty in rats and nonhuman primates. *Hormones and Behavior*, 64, 175–186.
- Lomniczi, A., Wright, H., Castellano, J. M., Matagne, V., Toro, C. A., Ramaswamy, S., Plant, T. M., & Ojeda, S. R. (2015). Epigenetic regulation of puberty via Zinc finger protein-mediated transcriptional repression. *Nature Communications*, 6, 10195.
- Luque, R. M., Kineman, R. D., & Tena-Sempere, M. (2007). Regulation of hypothalamic expression of KISS-1 and GPR54 genes by metabolic factors: Analyses using mouse models and a cell line. *Endocrinology*, 148, 4601–4611.
- Ma, H. M., Du, M. L., Luo, X. P., Chen, S. K., Liu, L., Chen, R. M., Zhu, C., Xiong, F., Li, T., Wang, W., et al. (2009). Onset of breast and pubic hair development and menses in urban Chinese girls. *Pediatrics*, 124, e269–277.
- Manfredi-Lozano, M., Roa, J., & Tena-Sempere, M. (2017). Connecting metabolism and gonadal function: Novel central neuropeptide pathways involved in the metabolic control of puberty and fertility. *Frontiers in Neuroendocrinology*.
- Marshall, W. A., & Tanner, J. M. (1969). Variations in pattern of pubertal changes in girls. *Archives of Disease in Childhood*, 44, 291–303.
- Marshall, W. A., & Tanner, J. M. (1970). Variations in the pattern of pubertal changes in boys. *Archives of Disease in Childhood*, 45, 13–23.
- Maruca, J., Kulin, H. E., & Santner, S. J. (1983). Perturbations of negative feedback sensitivity in agonadal patients undergoing estrogen replacement therapy. *The Journal of Clinical Endocrinology and Metabolism*, 56, 53–59.

- Mayer, C., & Boehm, U. (2011). Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nature Neuroscience*, 14, 704–710.
- Meijs-Roelofs, H. M. (1972). Effect of electrical stimulation of the hypothalamus on gonadotrophin release and the onset of puberty. *The Journal of Endocrinology*, 54, 277–284.
- Messina, A., Langlet, F., Chachlaki, K., Roa, J., Rasika, S., Jouy, N., Gallet, S., Gaytan, F., Parkash, J., Tena-Sempere, M., et al. (2016). A microRNA switch regulates the rise in hypothalamic GnRH production before puberty. *Nature Neuroscience*, 19, 835–844.
- Moenter, S. M., & DeFazio, R. A. (2005). Endogenous gamma-aminobutyric acid can excite gonadotropin-releasing hormone neurons. *Endocrinology*, 146, 5374–5379.
- Morelli, A., Marini, M., Mancina, R., Luconi, M., Vignozzi, L., Fibbi, B., Filippi, S., Pezzatini, A., Forti, G., Vannelli, G. B., et al. (2008). Sex steroids and leptin regulate the “first Kiss” (KISS 1/G-protein-coupled receptor 54 system) in human gonadotropin-releasing-hormone-secreting neuroblasts. *The Journal of Sexual Medicine*, 5, 1097–1113.
- Nakahara, T., Uenoyama, Y., Iwase, A., Oishi, S., Nakamura, S., Minabe, S., Watanabe, Y., Deura, C., Noguchi, T., Fujii, N., et al. (2013). Chronic peripheral administration of kappa-opioid receptor antagonist advances puberty onset associated with acceleration of pulsatile luteinizing hormone secretion in female rats. *The Journal of Reproduction and Development*, 59, 479–484.
- Navarro, V. M. (2012). New insights into the control of pulsatile GnRH release: The role of Kiss1/neurokinin B neurons. *Frontiers in Endocrinology*, 3, 48.
- Navarro, V. M., Castellano, J. M., Fernandez-Fernandez, R., Barreiro, M. L., Roa, J., Sanchez-Criado, J. E., Aguilar, E., Dieguez, C., Pinilla, L., & Tena-Sempere, M. (2004a). Developmental and hormonally regulated messenger ribonucleic acid expression of KISS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KISS-1 peptide. *Endocrinology*, 145, 4565–4574.
- Navarro, V. M., Fernandez-Fernandez, R., Castellano, J. M., Roa, J., Mayen, A., Barreiro, M. L., Gaytan, F., Aguilar, E., Pinilla, L., Dieguez, C., et al. (2004b). Advanced vaginal opening and precocious activation of the reproductive axis by KISS-1 peptide, the endogenous ligand of GPR54. *The Journal of Physiology*, 561, 379–386.
- Navarro, V. M., Gottsch, M. L., Chavkin, C., Okamura, H., Clifton, D. K., & Steiner, R. A. (2009). Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *The Journal of Neuroscience*, 29, 11859–11866.
- Navarro, V. M., Ruiz-Pino, F., Sanchez-Garrido, M. A., Garcia-Galiano, D., Hobbs, S. J., Manfredi-Lozano, M., Leon, S., Sangiao-Alvarellos, S., Castellano, J. M., Clifton, D. K., et al. (2012). Role of neurokinin B in the control of female puberty and its modulation by metabolic status. *The Journal of Neuroscience*, 32, 2388–2397.
- Oakley, A. E., Clifton, D. K., & Steiner, R. A. (2009). Kisspeptin signaling in the brain. *Endocrine Reviews*, 30, 713–743.
- Ojeda, S. R., & Skinner, M. K. (2006). Puberty in the rat. In J. D. Neill (Ed.), *The physiology of reproduction* (pp. 2061–2126). San Diego: Academic Press/Elsevier.
- Ojeda, S. R., Lomniczi, A., Mastronardi, C., Heger, S., Roth, C., Parent, A. S., Matagne, V., & Mungenast, A. E. (2006a). Minireview: The neuroendocrine regulation of puberty: Is the time ripe for a systems biology approach? *Endocrinology*, 147, 1166–1174.
- Ojeda, S. R., Roth, C., Mungenast, A., Heger, S., Mastronardi, C., Parent, A. S., Lomniczi, A., & Jung, H. (2006b). Neuroendocrine mechanisms controlling female puberty: New approaches, new concepts. *International Journal of Andrology*, 29, 256–263. discussion 286–290.
- Ojeda, S. R., Lomniczi, A., & Sandau, U. (2010). Contribution of glial-neuronal interactions to the neuroendocrine control of female puberty. *The European Journal of Neuroscience*, 32, 2003–2010.
- Overgaard, A., Ruiz-Pino, F., Castellano, J. M., Tena-Sempere, M., & Mikkelsen, J. D. (2014). Disparate changes in kisspeptin and neurokinin B expression in the arcuate nucleus after sex steroid manipulation reveal differential regulation of the two KNDy peptides in rats. *Endocrinology*, 155, 3945–3955.
- Parent, A. S., Teilmann, G., Juul, A., Skakkebaek, N. E., Toppari, J., & Bourguignon, J. P. (2003). The timing of normal puberty and the age limits of sexual precocity: Variations around the world, secular trends, and changes after migration. *Endocrine Reviews*, 24, 668–693.
- Paulsen, M., Tierling, S., & Walter, J. (2008). DNA methylation and the mammalian genome. In J. Tost (Ed.), *Epigenetics* (pp. 1–22). Norfolk: Caister Academic Press.
- Pineda, R., Garcia-Galiano, D., Roseweir, A., Romero, M., Sanchez-Garrido, M. A., Ruiz-Pino, F., Morgan, K., Pinilla, L., Millar, R. P., & Tena-Sempere, M. (2010). Critical roles of kisspeptins in female puberty and preovulatory gonadotropin surges as revealed by a novel antagonist. *Endocrinology*, 151, 722–730.
- Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P., & Tena-Sempere, M. (2012). Kisspeptins and reproduction: Physiological roles and regulatory mechanisms. *Physiological Reviews*, 92, 1235–1316.
- Plant, T. M., Ramaswamy, S., & Dipietro, M. J. (2006). Repetitive activation of hypothalamic G protein-coupled receptor 54 with intravenous pulses of kisspeptin in the juvenile monkey (*Macaca mulatta*) elicits a sustained train of gonadotropin-releasing hormone discharges. *Endocrinology*, 147, 1007–1013.
- Poling, M. C., Kim, J., Dhamiya, S., & Kauffman, A. S. (2012). Development, sex steroid regulation, and phenotypic characterization of RFamide-related peptide (Rfrp) gene expression and RFamide receptors in the mouse hypothalamus. *Endocrinology*, 153, 1827–1840.
- Prevot, V. (2015). Puberty in mice and rats. In T. M. Plant, & J. Zelezniak (Eds.), *Knobil and Neill's physiology of reproduction* (pp. 1395–1439). Amsterdam: Elsevier.
- Qiu, J., Fang, Y., Bosch, M. A., Ronnekleiv, O. K., & Kelly, M. J. (2011). Guinea pig kisspeptin neurons are depolarized by leptin via activation of TRPC channels. *Endocrinology*, 152, 1503–1514.
- Qiu, X., Dao, H., Wang, M., Heston, A., Garcia, K. M., Sangal, A., Dowling, A. R., Faulkner, L. D., Molitor, S. C., Elias, C. F., et al. (2015). Insulin and Leptin signaling interact in the mouse Kiss1 neuron during the peripubertal period. *PLoS One*, 10, e0121974.
- Quennell, J. H., Mulligan, A. C., Tups, A., Liu, X., Phipps, S. J., Kemp, C. J., Herbison, A. E., Grattan, D. R., & Anderson, G. M. (2009). Leptin indirectly regulates gonadotropin-releasing hormone neuronal function. *Endocrinology*, 150, 2805–2812.
- Quennell, J. H., Howell, C. S., Roa, J., Augustine, R. A., Grattan, D. R., & Anderson, G. M. (2011). Leptin deficiency and diet-induced obesity reduce hypothalamic kisspeptin expression in mice. *Endocrinology*, 152, 1541–1550.
- Rapisarda, J. J., Bergman, K. S., Steiner, R. A., & Foster, D. L. (1983). Response to estradiol inhibition of tonic luteinizing hormone secretion decreases during the final stage of puberty in the rhesus monkey. *Endocrinology*, 112, 1172–1179.
- Roa, J., Vigo, E., Garcia-Galiano, D., Castellano, J. M., Navarro, V. M., Pineda, R., Dieguez, C., Aguilar, E., Pinilla, L., & Tena-Sempere, M. (2008). Desensitization of gonadotropin responses to kisspeptin in the female rat: Analyses of LH and FSH secretion at different developmental and metabolic states. *American Journal of Physiology. Endocrinology and Metabolism*, 294, E1088–E1096.
- de Roux, N., Genin, E., Carel, J. C., Matsuda, F., Chaussain, J. L., & Milgrom, E. (2003). Hypogonadotropic hypogonadism due to loss of function of the KISS1-derived peptide receptor GPR54. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 10972–10976.
- Safranski, T. J., Lamberson, W. R., & Keisler, D. H. (1993). Correlations among three measures of puberty in mice and relationships with estradiol concentration and ovulation. *Biology of Reproduction*, 48, 669–673.
- Sangiao-Alvarellos, S., Manfredi-Lozano, M., Ruiz-Pino, F., Navarro, V. M., Sanchez-Garrido, M. A., Leon, S., Dieguez, C., Cordido, F., Matagne, V., Dissen, G. A., et al. (2013). Changes in hypothalamic expression of the Lin28/let-7 system and related microRNAs during postnatal maturation and after experimental manipulations of puberty. *Endocrinology*, 154, 942–955.
- Sayed, D., & Abdellatif, M. (2011). MicroRNAs in development and disease. *Physiological Reviews*, 91, 827–887.
- Seminara, S. B., Messager, S., Chatzidakis, E. E., Thresher, R. R., Acierno, J. S., Jr., Shagoury, J. K., Bo-Abbas, Y., Kuohung, W., Schwino, K. M., Hendrick, A. G., et al. (2003). The GPR54 gene as a regulator of puberty. *The New England Journal of Medicine*, 349, 1614–1627.
- Shahab, M., Mastronardi, C., Seminara, S. B., Crowley, W. F., Ojeda, S. R., & Plant, T. M. (2005). Increased hypothalamic GPR54 signaling: A potential mechanism for initiation of puberty in primates. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 2129–2134.
- Sims, J. K., Magazinnik, T., Houston, S. I., Wu, S., & Rice, J. C. (2008). Histone modifications and epigenetics. In J. Tost (Ed.), *Epigenetics* (pp. 127–152). Norfolk: Caister Academic Press.
- Smith, J. T., Cunningham, M. J., Rissman, E. F., Clifton, D. K., & Steiner, R. A. (2005a). Regulation of Kiss1 gene expression in the brain of the female mouse. *Endocrinology*, 146, 3686–3692.
- Smith, J. T., Dungan, H. M., Stoll, E. A., Gottsch, M. L., Braun, R. E., Eacker, S. M., Clifton, D. K., & Steiner, R. A. (2005b). Differential regulation of KISS-1 mRNA expression by sex steroids in the brain of the male mouse. *Endocrinology*, 146, 2976–2984.

- Smith, J. T., Acohido, B. V., Clifton, D. K., & Steiner, R. A. (2006). KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *Journal of Neuroendocrinology*, 18, 298–303.
- Snyder, C. K. (2016). Puberty: An overview for pediatric nurses. *Journal of Pediatric Nursing*, 31, 757–759.
- Steele, R. E., & Weisz, J. (1974). Changes in sensitivity of the estradiol-LH feedback system with puberty in the female rat. *Endocrinology*, 95, 513–520.
- Tena-Sempere, M. (2012). Deciphering puberty: Novel partners, novel mechanisms. *European Journal of Endocrinology*, 167, 733–747.
- Tena-Sempere, M. (2013). Keeping puberty on time: Novel signals and mechanisms involved. *Current Topics in Developmental Biology*, 105, 299–329.
- Tena-Sempere, M., & Huhtaniemi, I. (2003). Gonadotropins and gonadotropin receptors. In B. C. J. M. Fauser (Ed.), *Reproductive medicine—molecular, cellular and genetic fundamentals* (pp. 225–244). New York: Parthenon Publishing.
- Topaloglu, A. K., Reimann, F., Guclu, M., Yalin, A. S., Kotan, L. D., Porter, K. M., Serin, A., Mungan, N. O., Cook, J. R., Imamoglu, S., et al. (2009). TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a key role for Neurokinin B in the central control of reproduction. *Nature Genetics*, 41, 354–358.
- Uenoyama, Y., Tsukamura, H., & Maeda, K. (2014). KNDy neuron as a gatekeeper of puberty onset. *The Journal of Obstetrics and Gynaecology Research*, 40, 1518–1526.
- Vazquez, M. J., Romero-Ruiz, A., & Tena-Sempere, M. (2015). Roles of leptin in reproduction, pregnancy and polycystic ovary syndrome: Consensus knowledge and recent developments. *Metabolism*, 64, 79–91.
- Velie, E. M., Nechuta, S., & Osuch, J. R. (2005). Lifetime reproductive and anthropometric risk factors for breast cancer in postmenopausal women. *Breast Disease*, 24, 17–35.
- Viswanathan, S. R., Daley, G. Q., & Gregory, R. I. (2008). Selective blockade of microRNA processing by Lin28. *Science*, 320, 97–100.
- Yang, J. J., Caligioni, C. S., Chan, Y. M., & Seminara, S. B. (2012). Uncovering novel reproductive defects in neurokinin B receptor null mice: Closing the gap between mice and men. *Endocrinology*, 153, 1498–1508.

Female Puberty: Nutrition and Endocrinology

Gary L Williams, Texas A&M AgriLife Research, Beeville, TX, United States; and Texas A&M University, College Station, TX, United States

Bruna RC Alves, University of Nevada, Reno, NV, United States

Rodolfo C Cardoso, Texas A&M University, College Station, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

α -MSH A neuropeptide hormone encoded by the *POMC* gene that suppresses appetite and stimulates GnRH release.

Anterior pituitary Glandular organ that secretes several hormones, including FSH and LH, to regulate several physiological processes, such as reproduction, stress, growth, and lactation.

Arcuate nucleus Hypothalamic region adjacent to the third ventricle that contains several neurons responsible for integrating nutritional and metabolic information.

Epigenetics The study of changes in phenotype caused by modification of gene expression rather than alteration of the genetic code itself.

Estradiol Steroid hormone produced chiefly by the ovarian follicle and the primary female sex hormone.

Follicle-stimulating hormone (FSH) Hormone produced by the pituitary gonadotropes responsible for controlling the initiation of follicular growth.

Gonadotropin-releasing hormone (GnRH) Hypothalamic neuropeptide responsible for the release of FSH and LH from the anterior pituitary.

Gonadotrope Specialized cell in the anterior pituitary that secretes LH and/or FSH.

Hypothalamus Portion in the ventral region of the brain that links the nervous system to the endocrine system to control reproduction and other biological processes.

Hypothalamic-hypophyseal portal system A system of blood vessels in the brain that connects the hypothalamus with the anterior pituitary. Its main function is the transport and delivery of hormones to the anterior pituitary (adenohypophysis).

Juvenile period Usually defined as the period beginning when the offspring no longer needs nourishment from the dam but before sexual maturation.

Kisspeptin Peptide encoded by the *KISS1* gene and a potent stimulator of GnRH neurons.

KNDy neurons Neurons located in the arcuate nucleus that co-express kisspeptin, neurokinin B, and dynorphin.

Leptin Hormone produced by adipose cells that acts at the brain level to regulate energy metabolism and reproduction.

Luteinizing hormone (LH) Hormone produced by the pituitary gonadotropes responsible for controlling follicular development and ovulation.

Median eminence Part of the hypothalamus from which regulatory hormones are released via the hypophyseal portal system; connects the neural and peripheral endocrine systems.

Menarche Beginning of menstrual cycles in humans and some nonhuman primates.

Neuropeptide Y Neuropeptide produced in several brain regions, including the hypothalamus, that stimulates food intake and suppresses GnRH release.

Ovulation Release of an oocyte (egg) from the ovaries.

Proopiomelanocortin (POMC) Precursor polypeptide that is cleaved in different peptide hormones that control energy balance and reproduction among other processes.

Puberty Process of biological and physical changes through which an individual matures to acquire reproductive competence.

Introduction

Puberty in females is a complex biological process involving both physical and behavioral changes, including those associated with the activation of specific neuronal pathways within the hypothalamus. The latter events lead to the initiation of high-frequency episodic release of gonadotropin-releasing hormone (GnRH) from GnRH neurons, a corresponding pattern of luteinizing hormone (LH) secretion from anterior pituitary gonadotropes, functional maturation of ovarian follicles, and subsequent establishment of ovulatory cycles (1). Pubertal development is controlled largely by genetic and environmental factors, among which nutrition plays a prominent role. The timing of pubertal onset has important relevance for both human health and livestock production. Both epidemiological data and animal studies have shown that increased postnatal nutrient intake, particularly during the juvenile period, has major impacts on the programming of puberty in females (2). For example, precocious puberty is associated with increased risk of unwanted pregnancies in all female mammals, and in girls a heightened risk of polycystic ovary syndrome,

reproductive cancers, and psychological distress (3). In contrast, delayed puberty negatively impacts reproductive performance and lifetime productivity in livestock species. The objectives of this article are to present an overview of the events within the brain and pituitary that control puberty in female mammals and to summarize recent research findings regarding the nutritional and metabolic control of the pubertal process.

Developmental Processes Underlying the Path to Fertility

Female puberty, which culminates ultimately in the functional potential to become pregnant, occurs as the result of a series of developmental events that begin as early as the prenatal period and conclude under normal circumstances after the juvenile period. At least three definitions of female puberty can be considered, all of which have relevance depending on one's focus: (1) *Neuroendocrine puberty*: a state of development reflecting maturity of hypothalamic centers responsible for both tonic (basal) and surge release of gonadotropins (LH and follicle-stimulating hormone (FSH)) in response to the negative and positive feedback of the most important biologically-functional estrogen, estradiol-17 β ; (2) *First estrus*: the exhibition of female sexual receptivity in nonhuman species in association with the development of a mature, estrogen-active ovarian follicle; (3) *First ovulation*: onset of ovarian cyclicity and menstrual cycles (girls and some nonhuman primates), thus the ability to become pregnant. Although the ability to become pregnant is the most frequently used definition of puberty, both first estrus and first ovulation are the most common markers used for assessing this potential in female (nonhuman) domestic species. However, both are subject to errors of interpretation. For example, the phenomenon of "nonpubertal estrus" is a documented phenomenon in some females (e.g., bovine females) and first ovulation is not always associated with estrus or fertility. Numerous genetic and environmental factors can influence all the foregoing events but, as noted earlier and discussed in detail later, both pre- and postnatal nutrition play a remarkable role in timing their onset.

Hypothalamic–Pituitary Axis

The endogenous secretion of gonadotropins (FSH and LH) during early (pre- and postnatal) development exhibits distinct patterns characteristic of the species. In general, these patterns tend to favor relatively high circulating concentrations of FSH during early development, followed by marked declines in advance of the juvenile period. Diminution of the early postnatal FSH rise in the laboratory rat begins at about 3 weeks of age, whereas in sheep, circulating FSH tends to remain elevated throughout the developmental period leading up to puberty. A mid-gestational rise in both LH and FSH is observed in human and nonhuman primate fetuses that declines at birth, followed by a postnatal rise and a second decline during the juvenile period.

During the postnatal period, the synthesis and secretion of gonadotropins appears to be largely gonadal steroid-independent. However, as development proceeds to the juvenile period, a gonad-dependent suppression of LH develops due to an increase in sensitivity to the negative feedback effects of estradiol. Although both FSH and LH are ultimately controlled by GnRH from the hypothalamus, secretion patterns of these two gonadotropins are often quite different. This differential pattern of release is controlled by contrasting responses of gonadotropes that secrete primarily either FSH or LH, by differences in estradiol ovarian steroid feedback effects on FSH- and LH-secreting gonadotropes, and by the release of regulatory peptides (e.g., activin and inhibin) from maturing ovarian follicles. The biological activity of FSH can also vary dramatically because of the production of heterogeneous forms of FSH diverging in their degree of glycosylation. However, in general, a limitation in availability of FSH is not a primary factor regulating the timing of puberty in the female, but rather the lack of a high-frequency pattern of pulsatile LH release. In many species, a decrease in estradiol negative feedback sensitivity during the late juvenile period times the onset of puberty. This modulatory effect was first described in the rat and is the basis for the transition to a high-frequency pattern of GnRH/LH release, a neuroendocrine event critical to the onset of puberty in the female. However, the escape from enhanced negative feedback sensitivity to estradiol apparently is not a critical event for pubertal progression in humans and nonhuman primates. Thus, a juvenile hiatus in both LH and FSH secretion occurs in these species that appears to be gonad independent, at least in the female. After the juvenile hiatus, the increase in circulating LH, and in some species FSH, results in heightened stimulation of ovarian follicles, an increase in circulating concentrations of estradiol, and initiation of an LH surge (Fig. 1). The latter occurs through a separate positive feedback effect of estradiol at the hypothalamic level which induces a prolonged surge of GnRH. In most species, estradiol positive feedback mechanisms, and thus the ability of estradiol to induce a surge release of GnRH/LH, is functional well before puberty.

Hierarchical Network Regulating Secretion of GnRH

For many years, hypothalamic secretion of GnRH has been defined as the master controller of reproduction. The inherent propensity for the GnRH neuronal network to produce a pulsatile pattern of GnRH release, which depends upon synchronous firing of GnRH neurons, has been referred to as the "GnRH pulse generator." However, insight into the exact mechanisms through which the pulse generator is regulated has until recently been lacking. In the mid-1990s, the discovery and characterization of the peptide, kisspeptin dramatically changed our understanding of how GnRH neuronal activity is synchronized. Kisspeptin, which is part of the RF-amide related peptide (RFRP) superfamily, was originally identified simply as a regulator of tumor metastasis and was called metastin. It was soon discovered that the gene for this peptide, named *KISS1* after the location (Hershey, PA) where it was first identified, as well as its G protein-coupled receptor (GPR54), were expressed in a variety of tissues, including the hypothalamus.

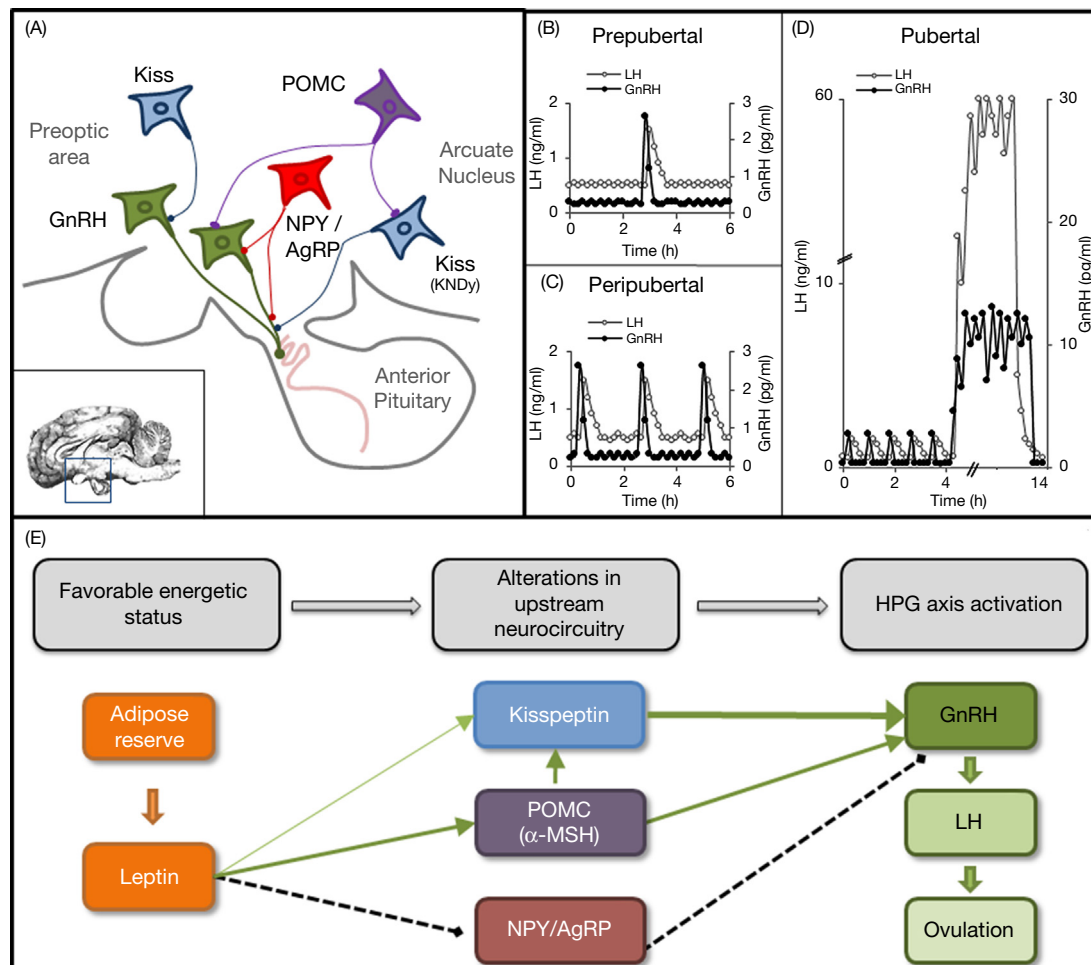


Fig. 1 Developmental changes and nutritional facilitation of puberty. (A) Representative scheme of major neuronal groups involved with the nutritional control of puberty that are located in the basal portion of the brain (*A-inset*) within the preoptic area and the hypothalamic arcuate nucleus (ARC). The population of kisspeptin (Kiss) neurons located in the ARC is also known as KNDy neurons. Kisspeptin, POMC, NPY/AgRP neurons communicate with gonadotropin-releasing hormone (GnRH) neurons (and with each other) by delivery of their respective neuropeptides through axonal projections directed to neuronal (GnRH) cell bodies. Kisspeptin neurons are important conveyors of the estradiol effects on GnRH neurons while POMC and NPY/AgRP neurons are the major ARC neurons that communicate metabolic status to GnRH neurons. GnRH neurons send projections to the hypophyseal portal circulation (represented in pink), allowing the access of this neurohormone to the anterior pituitary, where it stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). (B–D) Representative graphs depicting the pattern of GnRH and LH secretion in the prepubertal (B), peripubertal (C), and pubertal phases (D) of development in the female. Concentrations of LH refer to those in peripheral plasma, while GnRH is measured directly in hypophyseal portal blood or indirectly in third ventricle cerebrospinal fluid. The pulsatile pattern of GnRH and LH release occurs sporadically during the prepubertal period (B), increases in frequency as puberty approaches (C), and reaches a high frequency pattern (D, hours 0–4) before the first ovulation. The enhancement of GnRH/LH pulse frequency in many species is related to the reduction in the sensitivity to the negative feedback of estradiol. Ovulation occurs when a threshold level of estradiol is released that initiates a positive feedback effect at the hypothalamus that induces the release of GnRH and LH in a surge fashion (D, hours 4–14). (E) Schematic representation of events that culminate with the nutritional facilitation of puberty. Full *green arrows* represent stimulatory (thickness relates to the intensity of the stimulus) while *black dashed lines* represent inhibitory inputs. A favorable energetic status increases stimulatory and suppresses inhibitory pathways, permitting, or even accelerating, activation of the hypothalamic–pituitary–gonadal (HPG) axis, culminating with the first ovulation.

The peptide, now renamed kisspeptin, and mutations in the GPR54 receptor (now termed KISS1R), were found subsequently to play a major role in a condition in humans termed idiopathic hypogonadotropic hypogonadism. Through additional study with KISS1R knockout mice, it became apparent that kisspeptin was the natural ligand for KISS1R. This led to studies which localized both KISS1 and KISS1R within the hypothalamus and observations that KISS1R colocalized with GnRH neurons. Ultimately, it became obvious that kisspeptin-containing neurons within the hypothalamus were responsible for regulating the release of GnRH. A plethora of subsequent studies has now confirmed that the kisspeptin-KISS1R-connection in GnRH neurons is a critical element in the normal regulation of GnRH secretion (Fig. 1). Gene mutations resulting in a breakdown of this signaling pathway result in the failure to attain puberty and thus persistent infertility.

Recently, a further delineation of the chemical architecture of kisspeptin neurons has revealed additional variants that may explain the ontogeny of the GnRH pulse generator itself. Kisspeptin cells localized to the arcuate nucleus (ARC) of the hypothalamus are now referred to as KNDy neurons. They have been given this name because they co-express two other neuronal peptides, neurokinin B (NKB) and dynorphin. KNDy neurons express receptors for their own ligands, namely NKB and dynorphin, but do not contain kisspeptin receptors. These cells function as an interconnected network, secreting kisspeptin in response to their own release of NKB. Activation of the NKB receptor results in kisspeptin release at two locations: GnRH cell bodies and their terminals in the median eminence of the hypothalamus. This cascade initiates the transport of GnRH to the anterior pituitary via hypophyseal portal vessels and causes release of gonadotropins. It is believed that the activation of KNDy neurons results in the release of dynorphin which then terminates KNDy neuron activity. The repeating nature of this locally-controlled cycle provides one of the first plausible accounts of cellular activity that could represent the physical source of the GnRH pulse generator. It has also become obvious that the hypothalamus of at least some species contains two populations of kisspeptin neurons. Those located in the ARC are KNDy neurons because of their chemical constituents. A second population of kisspeptin neurons can be observed in the rostral or frontal part of the hypothalamus that do not contain NKB or dynorphin. However, both sets of neurons contain estrogen receptor alpha ($ER\alpha$). Estradiol suppresses *KISS1* expression in the ARC but has a positive feedback effect on kisspeptin neurons located in the rostral hypothalamus. Deletion of $ER\alpha$ in all kisspeptin-expressing neurons in mice advances the onset of puberty, supporting the contention that $ER\alpha$ has the inherent ability to restrain GnRH/LH secretion, ovarian function, and pubertal onset. The interrelationship between $ER\alpha$ and kisspeptin neurons provides a potential basis through which changes in negative feedback sensitivity to estradiol could regulate the timing of puberty, as described earlier in this article.

Energy Nutrition and the Central Control of Puberty

Reaching a threshold of body size and amount of stored energy before activation of reproductive processes that lead to pregnancy is an effective evolutionary strategy for female mammals to use for successful reproduction. The physiological mechanisms controlling energy metabolism, which include intake, expenditure and storage, are in part intermingled with the components that limit or time pubertal development. Not surprisingly, nutritional influences on puberty appear to be more robust in females, likely due to the energy-consuming activities of gestation and lactation, and in some species the final phases of growth. In mammals, it is broadly accepted that an adequate adipose reserve is necessary for puberty, an event often delayed under conditions of undernourishment and advanced in response to overnutrition.

Nutritional and Metabolic Signals that Time the Onset of Puberty

The central nervous system (CNS) perceives information regarding the energetic status of an individual by means of circulating nutritional and metabolic signals. Several of those metabolic signals influence the release of GnRH and LH, including their prepubertal rise. The hormones insulin, insulin-like growth factor-1 (IGF1), leptin, and ghrelin are circulating metabolically-controlled hormones that have been demonstrated to have the greatest influence on puberty. Both insulin, produced in the pancreas, and IGF1, a mediator of growth hormone (GH) action and produced in the liver, signal favorable energetic scenarios. Conversely, ghrelin, produced mainly in the stomach, is released under circumstances of fasting or low energy status. As an inhibitory signal to GnRH neurons, ghrelin appears to be dispensable for normal reproductive function but may play a role in postponing puberty onset. The structurally-homologous hormones insulin and IGF1 have stimulatory roles regarding GnRH release and facilitate growth and developmental events that support the pubertal process.

The adipokine leptin, which is synthesized primarily by adipocytes and whose synthesis and secretion increase in concert with increasing adiposity, plays a permissive role in the establishment of puberty. The complete knockout of leptin or its receptor results in infertility. Replacement of leptin in leptin-deficient females restores fertility. In this context, it has long been known that a critical amount of fat is necessary for adequate fertility. In female mammals, drastic reductions in adipose reserve are associated with adverse reproductive effects that persist until adiposity is restored to normal levels. Because reproduction is suspended well in advance of starvation, protective mechanisms that communicate negative energy balance to the central reproductive axis must be operable. The idea that a permissive signal produced by the adipose tissue informs the brain of energy status was first proposed in the 1950s and led to the characterization of the obese (*ob/ob*) mouse and the discovery of the leptin gene in 1994. Since its discovery as a peptide hormone synthesized primarily by adipose cells, and the finding that recombinant leptin not only normalized the obese and diabetic state in *ob/ob* mice but also restored fertility, leptin's role as a key player communicating energy reserves to the hypothalamus has become well-accepted. A concerted effort to characterize the physiological role of leptin and the mechanisms that govern its actions followed. In ruminant females, the stimulatory effects of leptin on secretion of LH are confined predominantly to periods of nutritional stress and occur through direct actions at both the hypothalamic and pituitary levels. Importantly, leptin appears to influence the secretion of GnRH at the hypothalamic level by modulating the sensitivity of the neuroendocrine system to steroid feedback as well as via steroid-independent mechanisms. The majority of GnRH neurons do not express the leptin receptor or intracellular sex steroid receptors, and leptin and other hormonal/metabolic factors regulate GnRH release via a complex neuronal network that has only recently begun to be characterized (Fig. 1).

Morphological Changes in Neuronal Pathways Controlling Puberty

Among the various functions of leptin is the ability to modulate axonal growth, neurogenesis and synaptogenesis. Leptin signaling during early life influences adult morphology and function of hypothalamic circuitries related to metabolism. Insulin and IGF1 also elicit neuronal remodeling, and IGF1 has been demonstrated to alter the morphology of GnRH neurons. Therefore, nutrition can influence reproductive function by promoting morphological alterations that result in remodeling of neurocircuitries capable of balancing inhibitory and stimulatory inputs to GnRH neurons. Interestingly, such modifications appear to be long-lasting. For example, neonatal nutritional manipulation in rodents alters the number or density of kisspeptin fibers in close apposition to GnRH neurons, and thus are increased by overnutrition and decreased by undernutrition. This is reinforced by our findings in the female bovine showing that a high nutritional plane during the juvenile period results in an acute increase in circulating concentrations of leptin, which promotes altered expression of several genes related to neuronal remodeling in the ARC, a reduction of direct NPY innervation of GnRH neurons, reductions in NPY tone as measured in third ventricle cerebrospinal fluid, and increased alpha melanocyte-stimulating hormone (α -MSH) innervation of kisspeptin neurons. Thus, nutritional plane during early life can create favorable metabolic conditions characterized primarily by increased circulating concentrations of leptin, lower hypothalamic NPY release, heightened pulsatile release of LH, and hastened onset of puberty (Fig. 1).

Epigenetic Modulation of Puberty

Recent advances in the emerging field of epigenetics have revealed that the fetal environment during mid- to late gestation may play a pivotal role in determining both postnatal and adult phenotype, including mechanisms involved in the restraint or acceleration of pubertal development. In this context, the neuroendocrine axis is responsible for integrating the operational control of a variety of nutritional and reproductive processes, including feed intake, satiety, metabolic homeostasis, reproductive cyclicity, and pregnancy. Mounting evidence has shown that nutritional imbalances during perinatal development can permanently alter the organization of this system, resulting in lifelong alterations of reproductive function in female offspring. Of significant importance are observations that both *excessive* and *deficient* maternal nutrition during gestation can cause phenotypically indistinguishable disorders of metabolism in the offspring. Restraining or stimulating pubertal development by altering the functions of neuronal populations related to the control of GnRH release is one potential mechanism through which metabolic state can create epigenetic effects. For example, it is plausible that KNDy neurons are targets for epigenetic modifications which would involve gene groups controlling *KISS1* transcription, *KISS1* or *Tac3* which encodes NKB. Epigenetic modifications in other ARC neuronal populations, such as those affecting the *POMC* gene, may also occur as a result of altered nutritional conditions that have been linked with advanced or delayed puberty. In female cattle, altered DNA methylation within the ARC has been shown to target many genes, including those encoding the growth hormone receptor (*GHR*) and a chromatin-associated protein (*HMG2*). Both genes have been implicated in the control of puberty onset in this species.

Genetic and Environmental Perturbations of Puberty

Precocious Puberty

Precocious puberty in girls is defined by the development of secondary sexual characteristics before the age of 8 years and is one of the most common conditions encountered in pediatric endocrinology practice. Central precocious puberty (CPP) is characterized by premature activation of the hypothalamic-pituitary-ovarian axis and increased secretion of GnRH/LH, accounting for approximately 90% of precocious puberty cases. In girls, precocious puberty is associated with greater risks of breast cancer, endometrial cancer, obesity, and cardiovascular disease. While most CPP cases are considered idiopathic, compelling evidence from population studies indicates that genetic factors influence timing of puberty in girls.

During the past few decades, several of the genetic determinants regulating pubertal development in females have been identified. In addition to mutations in the *KISS1* gene and its receptor *KISS1R* as discussed earlier in this chapter, loss-of-function mutations in the maternally-imprinted gene *MKRN3*, the gene encoding makorin RING-finger protein 3, has also been associated with CPP in humans. Notably, expression of *Mkrn3* mRNA in the ARC declines drastically immediately before puberty in female mice, providing further support for *MKRN3* as an important inhibitory factor controlling puberty in females. While these studies clearly implicate genetic factors in the control of precocious puberty, the rapid increase in frequency of accelerated timing of puberty in the last two decades point to an environmental etiology. In this regard, the obesity epidemic affecting both children and adults worldwide has received special attention.

Many studies have investigated the relationship between obesity, measured primarily by body mass index, and timing of puberty in girls. These studies have demonstrated that increased adiposity during childhood is associated with early reproductive maturation in girls. Obesity is approximately 1.5-fold more prevalent in girls who reach puberty at an earlier-than-average age and overweight girls are twice more likely to achieve puberty than average-weight girls of the same age. Similarly, animal studies have also identified a link between increased adiposity during juvenile development and precocious puberty in females. In female rats, postnatal overfeeding resulted in increased circulating concentrations of leptin and earlier age of vaginal opening, an external sign of puberty. Moreover, feeding female rhesus monkeys a high-calorie diet during juvenile development results in increased circulating concentrations of leptin and IGF1, and a greater incidence of precocious menarche. In cattle, a larger proportion of females fed to gain body weight at high rates during the juvenile period also exhibit precocious puberty, which is associated with an attenuation of

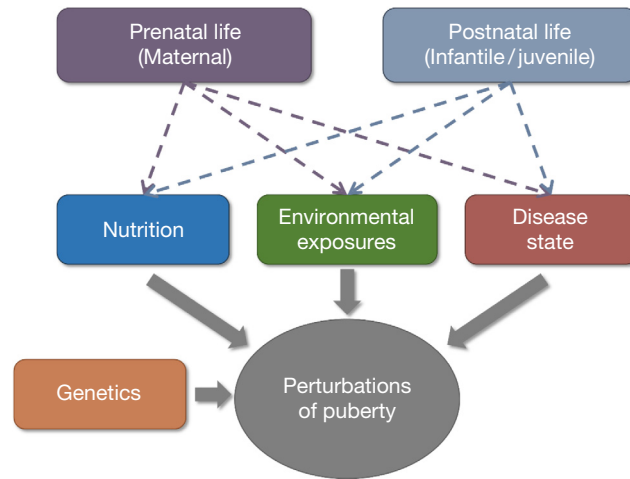


Fig. 2 Genetic and environmental perturbations of puberty. Genetic alterations (e.g., loss-of-function mutations) and environmental insults (e.g., malnutrition and exposure to endocrine disrupting chemicals (EDCs)) can disrupt the normal pubertal development in females resulting in perturbations of puberty, such as precocious or delayed puberty. Importantly, environmental insults occurring not only during postnatal life but also during in utero development can disrupt the progression of puberty in female mammals.

estradiol inhibitory effects on secretion of LH. Although there are several differences in the endocrine events that precede puberty among the foregoing species, the causal link between juvenile weight gain and adiposity and greater incidence of precocious puberty appears to be conserved in females across species, including women, rodents, nonhuman primates, and livestock species such as ruminants.

More recent evidence from epidemiological and animal studies indicates that not only does metabolic status during peripubertal development influence the timing of puberty in females but also metabolic changes occurring during prenatal/early postnatal life. For example, girls considered to be small for gestational age (SGA) at birth due to maternal malnutrition exhibit a greater incidence of precocious puberty. Daughters of obese mothers also present with advanced reproductive maturation; thus, the relationship between maternal nutrition and timing of puberty in female offspring is not a simple linear association. Instead, it appears to follow a U-shaped curve. Similar findings have also been observed in the laboratory rat, in which both maternal caloric restriction and maternal high fat nutrition result in early pubertal onset in the female offspring. Likewise, both maternal under- and overnutrition have been shown to induce juvenile obese phenotype and precocious puberty in female pigs. While a comprehensive review of the factors associated with the development of precocious puberty is beyond the scope of this chapter, it is noteworthy that a clear cause-and-effect relationship between environmental exposure to endocrine disrupting chemicals (EDCs) and perturbations of puberty has been observed. Taken together, these observations emphasize the idea that puberty is the endpoint of a maturational continuum that likely begins before birth and is modulated throughout development by genetic and environmental factors (Fig. 2).

Delayed Puberty

Delayed puberty in girls is defined as the absence of breast development at an age that is 2–2.5 standard deviations later than the population average (traditionally, 13 years in girls). Late pubertal onset can have negative effects on psychosocial well-being and can impact the normal pubertal growth spurt, thus affecting multiple organ systems. The cause of delayed puberty is unknown but, similar to precocious puberty, it has a strong genetic basis. It has been estimated that approximately 50%–75% of patients with constitutional delay of growth and puberty (CDGP) have a family history of delayed puberty. However, the specific genes involved in the development of CDGP remain largely unknown.

Decreased body fat, which is often associated with low circulating concentrations of leptin, is also a major cause of pubertal delay in females (Fig. 2). Studies examining pubertal onset across multiple populations have shown a negative correlation between socioeconomic status and the age of menarche in girls, suggesting that puberty occurs later in undernourished individuals. Excessive physical activity during childhood and adolescence has also been shown to negatively affect growth and pubertal development in girls. Sports that emphasize strict weight control and high energy output, such as gymnastics and dancing, are of particular concern for pubertal disorders. For example, the hard training schedule associated with low food intake typically observed in young female ballet dancers is often related to excessive thinness and a marked delay in pubertal maturation. Other conditions that result

in reduced energy reserves and low circulating levels of metabolic hormones during juvenile development, such as anorexia nervosa and inflammatory bowel disease, have also been shown to delay the establishment of menstrual cycles in girls.

A number of animal studies support the notion that undernutrition during juvenile life delays the onset of puberty. In rats, postnatal undernutrition associated with large litter size resulted in a persistent reduction in body weight, lower ovarian and uterine weights, and delayed vaginal opening; changes that were paralleled by a reduction in circulating concentrations of leptin and *Kiss1* mRNA expression in the hypothalamus. Similarly, restricted feed intake during juvenile development reduced the pulsatile secretion of LH during the peripubertal period and delayed first ovulation in female sheep. In female pigs, long-term moderate feed restriction during early postnatal life results in over a 3-week delay in the onset of puberty. Collectively, these studies emphasize the fact that a minimal amount of energy reserves and consequently circulating leptin are necessary for the normal progression of puberty in female mammals.

Further Reading

- Abreu, A. P., & Kaiser, U. B. (2016). Pubertal development and regulation. *The Lancet Diabetes & Endocrinology*, 4(3), 254–264.
- Amstalden, M., Alves, B. R., Liu, S., Cardoso, R. C., & Williams, G. L. (2011). Neuroendocrine pathways mediating nutritional acceleration of puberty: Insights from ruminant models. *Frontiers in Endocrinology*, 2(109.10), 3389.
- Elias, C. F. (2012). Leptin action in pubertal development: Recent advances and unanswered questions. *Trends in Endocrinology & Metabolism*, 23(1), 9–15.
- Lomniczi, A., Loche, A., Castellano, J. M., Ronnekleiv, O. K., Bosch, M., Kaidar, G., Knoll, J. G., Wright, H., Pfeifer, G. P., & Ojeda, S. R. (2013). Epigenetic control of female puberty. *Nature Neuroscience*, 16(3), 281–289.
- Sanchez-Garrido, M. A., & Tena-Sempere, M. (2013). Metabolic control of puberty: Roles of leptin and kisspeptins. *Hormones and Behavior*, 64(2), 187–194.
- Sisk, C. L., & Foster, D. L. (2004). The neural basis of puberty and adolescence. *Nature Neuroscience*, 7(10), 1040–1047.

Menarche/Menopause

Jacqueline Y Maher and Howard A Zacur, The Johns Hopkins University School of Medicine, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

- Adrenarche** Appearance of pubic and axillary hair resulting from secretion of androgens from the adrenal gland.
- Thelarche** Beginning of breast development.
- Menarche** First menstrual cycle resulting from activation of the hypothalamic-pituitary-ovarian-uterine axis.
- Menopause** The final menstrual cycle in a woman's life.
- Perimenopause** The transition between menstrual cycles occurring at regular intervals to the final menstrual period characterized by irregular menstrual cycles and intermittent menopausal symptoms such as hot flashes.

Introduction and Definitions

The human menstrual cycle discussed in detail elsewhere in this Encyclopedia represents a sequence of biologic events beginning with the growth and development of a follicle containing an oocyte within the ovary. During this time of development hormones are secreted from the ovary that produce proliferation of the lining of the uterus while priming the hypothalamus and pituitary gland that eventually results in a “spike” in the release of both luteinizing hormone (LH) and follicle stimulating hormone (FSH). This hormonal spike in turn causes the release of the oocyte from the follicle (ovulation) which is then followed by the luteinization or transformation of the cells in the follicle into a corpus luteum. Progesterone and estradiol are secreted by the corpus luteum which causes “decidualization” of the lining of the uterus making it receptive for the implantation of an embryo that defines the initiation of pregnancy. When this occurs the pregnancy’s placental tissues begins to secrete human chorionic gonadotropin (HCG) which maintains the survival of the cells in the corpus luteum allowing them to continue to secrete estrogen and progesterone, and menses does not occur. Should an embryo not implant into the decidualized uterine lining, HCG is not produced and the corpus luteum ceases to secrete estradiol and progesterone and the rapid decline in the level of these hormones triggers vascular events in the lining of the uterus resulting in the shedding of its outer two layers. As this occurs the smooth muscle walls of the uterus begin to contract expelling the endometrial lining which is mixed with blood into the vagina and this is eventually discharged from the vagina and is now defined as menstrual blood. This entire process is defined as a menstrual cycle and the first time that this occurs in a woman’s life it is defined as the first menstrual period or menarche. Menopause is defined as the age at which the menstrual cycle no longer occurs.

Puberty and Menarche

Menarche represents the final physical event in a series of steps known as puberty. A growth spurt is the first physical event which is closely followed by secondary sex characteristic development typified by breast budding (thelarche) then the arrival of pubic hair growth (pubarche) and then lastly the onset of menses (menarche). Marshall and Tanner were among the first to carefully classify pubertal changes in 192 white British girls in 1969 (see [Table 1](#)). Genetics, overall health, social and physical environment are all thought to play a role in determining the onset of puberty. Depending upon the reference cited, in girls, the average age of menarche is between 12 and 13 years of age, with racial and ethnic differences affecting this. Black girls experience menarche earlier than white girls while Hispanic or Mexican American girls will have their menarche at ages intermediate to that of black and white girls. Menarche will usually occur within 2–3 years after the onset of breast development. As previously mentioned genetics appears to play a large role in determining the onset of menarche, but better social conditions and increased weight are also involved ([Zacharias and Wurtman, 1969](#)).

Table 1 Average age and standard deviation of pubertal events in 192 British girls

	Average age (years)	Standard deviation
Breast budding (thelarche)	11.15	1.1
Pubic hair (adrenarche)	11.69	1.21
Menarche	13.47	1.02

Data from Marshall, W. A. and Tanner, J. M. (1969). Variations in pattern of pubertal changes in girls. *Archives of Disease in Childhood* 1969, **44**, 291–303.

Puberty and menarche result from the changes in the hormone levels of estrogens, androgens and progestogens which begin to be secreted in increasing amounts from the ovaries and adrenal glands. Release of LH and FSH from the pituitary gland stimulates ovarian hormone secretion of estrogens and progestogens coupled with an increased ability of the adrenal gland to respond to adrenal corticotrophic hormone (ACTH) by secreting androgens. Pituitary release of LH and FSH is regulated by gonadotropic releasing hormone or GnRH which is released into pituitary portal blood by the hypothalamus.

At birth the hypothalamus and the pituitary gland are fully capable of synthesizing and releasing GnRH and LH and FSH respectively, but this does not occur until the onset of puberty. At birth the ovaries contain a fixed number of oocytes surrounded by granulosa cells contained in structures known as follicles which are responsible for the secretion of estrogen. Follicles at birth are fully capable of responding to stimulation by LH and FSH resulting in follicle growth and even ovulation. It is therefore believed that the initiation of puberty begins with the ability of the hypothalamus to release GnRH which prior to puberty has been under inhibition by numerous inhibitory factors. How these inhibitory factors are prevented from keeping the hypothalamus from releasing GnRH from birth until the onset of puberty remains an unanswered question.

Occurring independently with the activation of the hypothalamic pituitary axis is the release of androgens from the zona reticularis of the adrenal gland causing adrenarche which is followed by pubarche. Adrenarche usually precedes activation of the hypothalamic pituitary axis but may occur contemporaneously. Circulating levels of ACTH do not appear to change during this time and the identification of an adrenal androgen stimulating hormone has been sought, but not yet found. The cause of adrenarche also remains an unanswered question.

Disorders of Puberty

It is generally stated that the onset of puberty prior to age eight is considered to be precocious and is deserving of evaluation. Results from a pediatric office practice study conducted in 1997 called this recommendation into question (Herman-Giddens et al., 1997). In this study involving over 18,000 girls ages 3–12, over 90% were white while 9.6% were African American. At age seven, 27.2% of African American girls and 6.7% of white girls had breast and/or pubic hair growth. Nevertheless given how disturbing precocious breast development is emotionally to young girls and their parents, a medical evaluation is often requested and agreed to when breast development begins before age eight.

Precocious puberty may result by premature activation of the hypothalamic pituitary ovarian axis which is called central, complete, isosexual or true precocious puberty. Over 90% of the time no identifiable cause may be found (Chemaitilly et al., 2001) and the diagnosis is given as idiopathic and made by exclusion. However premature activation of the hypothalamic pituitary axis may result from brain lesions that either release GnRH or disturb the inhibition of GnRH so brain imaging is indicated in these patients.

Precocious puberty may also result from the secretion of steroid hormones by the ovaries or the adrenal glands independently of hypothalamic-pituitary stimulation and this is known as gonadotropin independent precocious puberty. Transient secretion of estradiol from autonomously functioning ovarian cysts is one of the most common causes of this which may result in a temporary increase in breast size and even vaginal bleeding. In contrast tumors of the ovary such as granulosa cell tumors or Leydig cell tumors may secrete estrogens and or androgens causing breast development and/or pubic hair growth which is not temporary and their existence must be ruled out.

Delayed puberty is usually defined as the lack of any secondary sex characteristic development by age 12 to 13 and by absence of menses by age 16. Failure of the hypothalamic pituitary axis to become active as a result of a deficiency in GnRH may be causative resulting from genetic defects or tumors. This is known as hypogonadotropic hypogonadism and is typified by low levels of LH and FSH and estradiol. Alternatively the ovaries may fail to function as a result of genetic conditions such as gonadal dysgenesis due to Turner's syndrome and this is typified by elevated LH and FSH levels and low levels of estradiol.

Menopause

The final menstrual period in a woman's life defines menopause, but this is only determined retrospectively and occurs on average between 50 and 51 years of age with a range of 40–60 years (Treloar, 1981). Menopause results from a decline in the number follicles within the ovaries with increasing age. At birth there are approximately 300,000–500,000 follicles in each ovary (Wallace and Kelsey, 2010) and during reproductive life it is estimated that roughly only 400–600 oocytes will be ovulated depending upon the length of time between the age of menarche and age of menopause with the remaining oocytes lost through a process known as atresia.

Prediction of the age of menopause has proven elusive as the rate of follicle loss within the ovary may be influenced by factors such as smoking, obesity and family history, but all over the world the average age of menopause has remained similar as a recent meta-analysis involving 170,000 women in 24 countries on 6 continents has demonstrated (Schoenaker et al., 2014). Distinct hormonal changes are seen as menopause approaches and the follicle number in the ovary begins to decline. Inhibin B levels, made by granulosa cells, fall as the number of granulosa cells declines due to the decline in the number of ovarian follicles. Inhibin B normally acts to inhibit pituitary secretion of FSH so as inhibin B levels decline, FSH levels begin to rise. Antimüllerian hormone or AMH is produced by the granulosa cells of small follicles and AMH is believed to thwart the ability of FSH to recruit resting into growing follicles.

With the rise in FSH as follicle numbers decline, there is a fall in the level of AMH, and the elevation of FSH is now believed to act more unimpeded by stimulating the recruitment of more resting follicles into growing follicles. This is believed to be the explanation for the rise in the follicular phase estradiol concentration seen in older ovulatory women (Hansen et al., 2005). In addition to these ovarian changes with aging, an aging effect on the hypothalamus and pituitary appears to be occurring as well. For example there is a 30% decrease in serum LH and FSH concentrations between ages 50 and 75 (Hall et al., 2000), coupled with a 22% decrease in the frequency of GnRH pulses (Hall, 2004) and with a 30% decrease in the pituitary's release of LH and FSH in response to GnRH stimulation (Shaw et al., 2010).

The decline in the number of what are called resting ovarian follicles is rather constant between menarche and the age of 37.5, but after that age the rate of follicle loss increases more than two-fold (Faddy et al., 1992). After age 37.5 either a faster rate of recruitment of resting into growing follicles by FSH occurs and/or there is excess atresia of the resting follicles has been proposed as explanations for this more rapid rate of follicle loss (Faddy and Gosden, 1996). It is believed that this more rapid rate of follicle loss ultimately results in only about 1000 resting follicles remaining at the time of the final menstrual period.

Perimenopause

As the final menstrual period approaches menstrual cycles may occur irregularly with 60%–70% of the cycles being anovulatory and the menstrual cycle interval becoming either short or prolonged (Van Voorhis et al., 2008). Measurement of LH, FSH, estrone conjugates and pregnanediol conjugates in daily morning urine samples taken from perimenopausal women reveals marked variation with elevations in LH and FSH and estrone conjugates and declines in pregnanediol conjugates (Table 2) (Santoro et al., 1996). This time period in life has been termed the perimenopause.

The duration of the perimenopause has not been well studied. In a recent large prospective clinical study of hormonal changes in women approaching their final menstrual period known as the Study of Women's Health Across the Nation (SWAN) Daily Hormone Study (DHS) less than 2% of the menstrual cycles appeared to be anovulatory 10 years before the final menstrual period whereas almost 80% of menstrual cycles were anovulatory 1 year prior to the final menstrual period (Santoro et al., 2017).

Vasomotor Symptoms

Hot flashes or flushes are vasomotor symptoms that occur during the perimenopause and menopause. These events are characterized by a sensation of warmth beginning at the head which then radiates down the neck and chest which is followed by perspiration and often concludes with a chill. Hot flashes frequently occur at night awakening a woman from sleep who then has difficulty falling back to sleep.

In the SWAN study it was reported that 60%–80% of women experience hot flashes during the menopausal transition (Gold et al., 2006), with some having hot flashes even earlier in life and others continuing to have them years afterwards (Barnabei et al., 2002).

The cause of hot flashes remains unknown but is associated with the decline in estrogen levels, and the administration of estrogen can prevent them. There are other risk factors for having hot flashes which include race, alcohol consumption, body mass index, exercise and smoking (Smith et al., 2016).

Hormone Replacement

Conjugated equine estrogens were made available to Canadians for treatment of menopausal symptoms by the Wyeth-Ayerst Company in the late 1930s. In 1942 the Food and Drug Administration granted permission for the Wyeth-Ayerst Company to market Premarin 1.25 mg for the treatment of menopausal symptoms to women in the United States (Stefanick, 2005). Estrogen has been proven over time to be the most effective drug treatment for hot flashes and in addition to conjugated estrogens, may be

Table 2 Range in urinary hormone levels in perimenopausal women compared to menstruating women

	Menstruating women (n = 11)	Perimenopausal women (n = 11)
Mean estrone (ng/mg Cr)	23–60	13–135
Integrated pregnanediol (μg/mg Cr)	1.6–12.7	1.0–8.4
Mean LH (IU/gCr)	1.1–4.2	1.4–6.8
Mean FSH (IU/gCr)	3–7	4–32

Hormones measured by immunoassays in first morning daily urine samples with results normalized in terms of creatinine (Cr).

Data compiled from Santoro, N., Brown, J. R., Adel, T. and Skurnick, J. H. (1996). Characterization of reproductive hormonal dynamics in the perimenopause. *The Journal of Clinical Endocrinology and Metabolism* **81**, 1495–1501.

given orally as micronized estradiol, or transdermally or transvaginally as estradiol. Estrogens may be safely given alone to women without a uterus, but if given alone to women with a uterus, endometrial hyperplasia and cancer may occur. If progestins are given with estrogen to women with a uterus there is almost no increased risk of uterine cancer.

In the 1980s retrospective studies reported that menopausal women who had taken unopposed estrogen hormone replacement had a reduced risk of dying from cardiovascular disease or from any other cause (Bush et al., 1983, 1987). These observations were confirmed by others and ultimately led to a clinical study funded by the Wyeth-Ayerst pharmaceutical company known as the HERS study or Heart and Estrogen/progestin Replacement Study (Hulley et al., 1998). Postmenopausal women with known heart disease were enrolled in this clinical trial to determine if estrogen plus progestin therapy alters the risk of cardiovascular events. Over 2700 women with coronary disease participated whose average age was 66.7 years and who were randomized to receive either conjugated equine estrogens plus 2.5 mg of medroxyprogesterone acetate daily or placebo. Results from this study were published in 1998 and surprisingly showed that postmenopausal women with heart disease who took hormone replacement had the same rate of cardiovascular events as the women who took placebo. In addition, the women who took hormone replacement (1380 volunteers) had more thromboembolic events, (34 vs. 12) than the women who took placebo (1383 volunteers).

Prior to the publication of the results from the HERS study, the National Institutes of Health began recruiting volunteers for a large prospective clinical study in 1993 that became known as the Women's Health Initiative or WHI study. In this study over 27,000 menopausal women volunteers with (16,608) and without (10,739) a uterus were recruited. Women with a uterus were given either conjugated equine estrogens (0.625 mg) combined with 2.5 mg of medroxyprogesterone acetate (Prempro) or placebo. For women volunteers without a uterus, they were given either 0.625 mg conjugated equine estrogens alone (Premarin) or placebo. The primary objective of this study was to determine if hormone replacement given to menopausal women could reduce their risk of death from heart disease. By design the study deliberately enrolled older women (2/3 of the participants were older than 59 years of age at the beginning of the study) with the mean age of volunteers being 63 (Prentice et al., 1998).

The estrogen and progestin arm of the study was stopped after 5.2 years because no benefit from a reduction in cardiovascular events was demonstrated but an increased risk in breast cancer was observed (Writing Group for the Women, 2002). The estrogen only arm of the study was stopped after 6.8 years of treatment because a reduction in cardiovascular events was not seen while an increase in risk of ischemic stroke was observed (The Women's Health Initiative Steering Committee, 2004). Risk of breast cancer was not increased in the estrogen arm of this study, but was almost significantly decreased, a fact that did not receive much public attention.

Following the publications of these findings from the WHI many women stopped taking hormone replacement for treatment of their menopausal symptoms. Results from human and subhuman primate studies then led to the development of what is now known as the "timing hypothesis" to reconcile the results of the WHI with previous observational data (Clarkson, 2002). In brief this hypothesis states that if hormone replacement is begun at the time of menopause it may protect coronary vessels from atherosclerotic vascular disease. But if hormone replacement is begun many years after menopause it may not only not protect coronary vessels from atherosclerotic vascular disease, but may worsen it. Over time there has been a gradual re-evaluation of the risks and benefits of hormone therapy with a consensus now beginning to form that for younger women there is a favorable risk-benefit profile to the use of hormone replacement to treat menopausal symptoms (Lobo, 2017). Overall, it is recommended that patients with menopausal symptoms discuss the risks and benefits of hormone replacement therapy with their physician and that treatment should be individualized.

References

- Barnabei, V. M., Grady, D., Stovall, D. W., Cauley, J. A., Lin, F., Stuenkel, C. A., Stefanick, M. L., & Pickar, J. H. (2002). Menopausal symptoms in older women and the effects of treatment with hormone therapy. *Obstetrics and Gynecology*, 100, 1209–1218.
- Bush, T. L., Cowan, L. D., Barrett-Conner, E., Criqui, M. H., Karon, J. M., Wallace, R. B., Tyroler, H. A., & Rifkind, B. M. (1983). Estrogen use and all-cause mortality. Preliminary results from the lipid research clinics program follow-up study. *JAMA*, 249, 903–906.
- Bush, T. L., Barrett-Conner, E., Cowan, L. D., Criqui, M. H., Wallach, R. B., Suchindran, C. M., Tyroler, H. A., & Rifind, B. M. (1987). Cardiovascular mortality and noncontraceptive use of estrogen in women: Results from the lipid research clinics program follow-up study. *Circulation*, 75, 1102–1109.
- Chemaitilly, W., Trivin, C., Adan, L., Gall, V., Sainte-Rose, C., & Brauner, R. (2001). Central precocious puberty; clinical and laboratory features. *Clinical Endocrinology*, 54, 289–294.
- Clarkson, T. B. (2002). The new conundrum: Do estrogens have any cardiovascular benefits? *International Journal of Fertility and Women's Medicine*, 47, 61–68.
- Faddy, M. J., & Gosden, R. G. A. (1996). Model conforming the decline in follicle numbers to the age of menopause in women. *Human Reproduction*, 11, 1484–1486.
- Faddy, M. J., Gosden, R. G., Gougeon, A., Richardson, S. J., & Nelson, J. F. (1992). Accelerated disappearance of ovarian follicles in mid-life: Implications for forecasting menopause. *Human Reproduction*, 7, 1342–1346.
- Gold, E. B., Colvin, A., Avis, N., Bromberger, J., Greendale, G. A., Powell, L., Sternfeld, B., & Matthews, K. (2006). Longitudinal analysis of the association between vasomotor symptoms and race/ethnicity across the menopausal transition: Study of women's health across the nation. *American Journal of Public Health*, 96, 1226–1235.
- Hall, J. E. (2004). Neuroendocrine physiology of the early and late menopause. *Endocrinology and Metabolism Clinics of North American*, 33, 637–659.
- Hall, J. E., Lavoie, H. B., Marsh, E. E., & Martin, K. A. (2000). Decrease in gonadotropin-releasing hormone (GnRH) pulse frequency with aging in postmenopausal women. *The Journal of Clinical Endocrinology and Metabolism*, 85, 1794–1800.
- Hansen, K. R., Thyer, A. C., Sluss, P. M., Bremner, W. J., Soules, M. R., & Klein, N. A. (2005). Reproductive ageing and ovarian function: Is the early follicular phase FSH rise necessary to maintain adequate secretory function in older ovulatory women? *Human Reproduction*, 20, 89–95.
- Herman-Giddens, M. E., Siora, E. J., Wasserman, R. C., Bourdony, C. J., Bhapkar, M. V., Koch, G. G., & Hassemer, C. M. (1997). Secondary sexual characteristics and menses in young girls seen in office practice: A study from the pediatric research in office settings network. *Pediatrics*, 99, 505–512.
- Hulley, S., Grady, D., Bush, T., Furberg, C., Herrington, D., Riggs, B., & Vittinghoff, E. (1998). Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA*, 280, 605–613.

- Lobo, R. (2017). Hormone-replacement therapy: Current thinking. *Nature Reviews Endocrinology*, 217(13), 220–231.
- Prentice, R., Rossouw, J., Furburg, C., Johnson, S., Henderson, M., Cummings, S., Manson, J., Freedman, L., Oberman, A., Kuller, L., & Anderson, G. (1998). Design of the WHI clinical trial and observational study. *Controlled Clinical Trials*, 19, 61–109.
- Santoro, N., Brown, J. R., Adel, T., & Skurnick, J. H. (1996). Characterization of reproductive hormonal dynamics in the perimenopause. *The Journal of Clinical Endocrinology and Metabolism*, 81, 1495–1501.
- Santoro, N., Crawford, S. L., El Khoudary, S. R., Allshouse, A. A., Burnett-Bowie, S.-A., Finkelstein, J., Derby, C., Matthews, K., Kravitz, H. M., Harlow, S. D., Greendale, G. A., Gold, E. B., Kazlauskaitė, R., McConnell, D., Neal-Perry, G., Pavlovic, J., Randolph, J., Weiss, G., Chen, H.-Y., & Lasley, B. (2017). Menstrual cycle hormone changes in women traversing menopause: Study of Women's health across the nation. *Journal of Clinical Endocrinology and Metabolism*, 102, 2218–2229.
- Schoenaker, D. A., Jackson, C. A., Rowlands, J. V., & Mishra, G. D. (2014). Socioeconomic position, lifestyle factors and age at natural menopause: A systematic review and meta-analysis of studies across six continents. *International Journal of Epidemiology*, 5, 1542–1562.
- Shaw, N. D., Histed, S. N., Srouji, S. S., Yang, J., Lee, H., & Hall, J. E. (2010). Estrogen negative feedback on gonadotropin secretion: Evidence for a direct pituitary effect in women. *The Journal of Clinical Endocrinology and Metabolism*, 95, 1955–1961.
- Smith, R. L., Gallicchio, L., Miller, S. R., Zacur, H. A., & Flaws, J. A. (2016). Risk factors for extended duration and timing of peak severity of hot flashes. *PLoS One*, 11(5), e0155079.
- Stefanick, M. L. (2005). Estrogens and progestins: Background and history, trends in use, and guidelines and regimens approved by the US Food and Drug Administration. *American Journal of Medicine*, 118(Suppl), 64S–73S.
- The Women's Health Initiative Steering Committee. (2004). Effects of conjugated equine estrogen in postmenopausal women with hysterectomy; the women's health initiative randomized controlled trial. *JAMA*, 291, 1701–1712.
- Treloar, A. E. (1981). Menstrual cyclicity and the pre-menopause. *Maturitas*, 3-4, 249–264.
- Van Voorhis, B. J., Santoro, N., Harlow, S., Crawford, S. L., & Randolph, J. (2008). The relationship of bleeding patterns to daily reproductive hormones in women approaching menopause. *Obstetrics and Gynecology*, 112, 101–108.
- Wallace, W. H., & Kelsey, T. W. (2010). Human ovarian reserve from conception to the menopause. *PLoS One*, 5, e8772.
- Writing Group for the Women. (2002) Writing Group for. S health initiative investigators. Risks and benefits of estrogen plus progestin in healthy postmenopausal women; principal results from the women's health initiative randomized controlled trial. *JAMA*, 288, 321–333.
- Zacharias, L., & Wurtman, R. J. (1969). Age at menarche. Genetic and environmental influences. *New England Journal of Medicine*, 280, 868–875.

Further Reading

- Gracia, C. R., & Driscoll, D. A. (2003). Molecular basis of pubertal abnormalities. *Infertility and Reproductive Medicine Clinics of North America*, 14, 11–27.
- Hall, J. E. (2015). Endocrinology of the menopause. *Endocrinology and Metabolism Clinics of North America*, 44, 485–496.
- Lalwani, S., Reindollar, R. H., & Davis, A. J. (2003). Normal onset of puberty: Have definitions of onset changed? *Infertility and Reproductive Medicine Clinics of North America*, 14, 29–36.

Reproductive Senescence in the Female

Katherine L Rosewell and Thomas E Curry, Jr, University of Kentucky, Lexington, KY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

With advancing age there is a decline in a woman's overall fertility and fecundity. Historical reports examining fecundity from the 16th to 19th century have documented that women who married late in life, after the age of 35, were more likely to die childless. Recent studies have shown that the decline in fertility begins at approximately the age of 30. Women then undergo a period of menstrual cycle irregularity known as the menopausal transition starting around age 46. With a permanent ending of menstruation at approximately 51, women enter menopause. Classically defined, natural menopause is the permanent cessation of menstruation recognized to have occurred after 12 consecutive months of amenorrhea, for which no other obvious pathological or physiological cause could be determined. These age related changes are due to alterations in ovarian function, dominated by a gradual decrease in both the quantity and the quality of the egg or oocytes, which play an essential role in the transition from a normal fertile menstrual cycle to reproductive senescence.

Ovarian Aging

To fully comprehend the changes in the ovarian follicular pool that occur throughout a woman's lifespan, one has to understand the dynamics of follicular growth and the accompanying hormonal fluctuations. As the ovary develops during the fetal period, it has about 6–7 million eggs or oogonia. During gestation these oogonia either form primordial follicles that are composed of the oocyte surrounded by a flattened layer of granulosa cells or are removed from the ovary through a process of apoptotic programmed cell death known as atresia. Primordial follicles are the precursors for follicles which will go on to develop and ovulate a mature egg which can be fertilized and lead to pregnancy. At birth the number of primordial follicles has declined to about 1–2 million, mainly through atresia. This atretic process continues after birth, and at puberty a woman has approximately 300,000 primordial follicles (Fig. 1). This pool of primordial follicles is known as the ovarian reserve and reflects a woman's reproductive potential and overall fertility in the coming years.

Primordial follicles will either continue to undergo atresia or will be activated from the quiescent primordial follicle pool to undergo follicular growth and development through a process known as folliculogenesis. Folliculogenesis can be broken down into three broad and successive phases. The first phase is initiation or activation of the quiescent primordial follicle, which occurs from birth to the age of menopause and is independent of support from the gonadotropins, follicle stimulating hormone (FSH) or luteinizing hormone (LH). Upon initiation, the granulosa cells of the primordial follicle change from a squamous shape to become

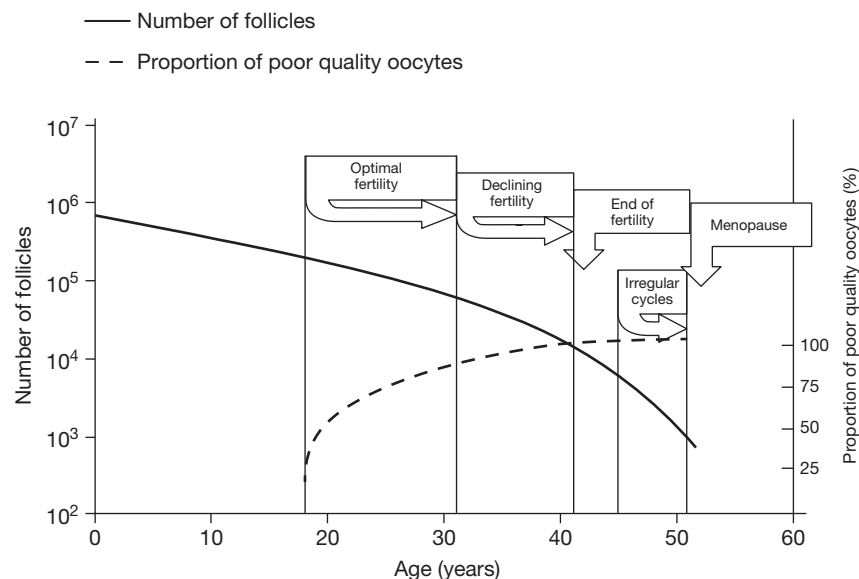


Fig. 1 Schematic representation of the age related changes and corresponding reproductive events in women. The number of primordial follicles present in the ovaries from birth until menopause is illustrated along with the quality of oocytes across the reproductive lifespan. Reproduced with permission from Broekmans, F. J., Soules, M. R. and Fauser, B. C. (2009). Ovarian aging: mechanisms and clinical consequences. *Endocrine Reviews* 30(5), 465–493.

cuboidal and the oocyte enlarges as the follicle becomes a primary follicle. With continued granulosa cell proliferation and differentiation, there is the development of the theca cell layer and a basement membrane as these primary follicles develop to become secondary and tertiary preantral follicles. The second stage known as recruitment requires tonic stimulation by FSH. At this time, FSH drives continued granulosa cell proliferation as the preantral follicles develop a fluid filled cavity forming the antral follicle. There is an increase in vascularization of the theca supplying the follicle with various growth factors which further drives follicular growth and the production of estradiol. The third and final stage of follicular growth requires luteinizing hormone (LH), which sets in motion a cascade of intracellular signaling pathways in the large preovulatory follicle leading to oocyte release through the process of ovulation.

By the time a woman reaches her late teens to early 20s she has entered into a fertile period characterized by regular monthly menstrual cycles (Fig. 1). During the early follicular phase of a woman's 28 day menstrual cycle, a number of preantral follicles are recruited through the actions of FSH. FSH stimulates granulosa cell proliferation, growth of the follicles and the production of estradiol and the inhibins A & B. Increasing levels of inhibin B during the early follicular phase feedback on the hypothalamic pituitary axis to inhibit FSH. In the face of declining levels of FSH, one follicle becomes dominant while the remaining cohort of growing follicles undergo atresia. The remaining dominant follicle is responsible for a rapid increase in estradiol during the latter stages of the follicular phase of the menstrual cycle which in turn decrease inhibin B from the ovary. At the midpoint of the cycle, elevated levels of estradiol act to stimulate the production of both FSH and LH from the hypothalamic pituitary axis along with an increase in inhibin A & B. The peak of LH sets in motion a cascade of events that result in ovulation and the transformation of the ruptured dominant follicle into a corpus luteum. The midcycle increase in FSH and inhibin A are thought to contribute to oocyte maturation and/or ovulation. The corpus luteum is responsible for estradiol, progesterone and inhibin A production during the midluteal phase of the cycle. Without the appropriate signals of pregnancy, the CL undergoes regression and there is a subsequent fall in progesterone, estradiol, and inhibin A from the CL which allows the levels of FSH to begin to rise again at the onset of the subsequent menstrual cycle. These cellular and hormonal changes associated with normal follicular growth, such as FSH and inhibins, have been used as important indicators of reproductive aging as described below.

From the pool of about 200,000 primordial follicles at puberty only approximately 60,000 remain at the age of 30. In addition to the loss of follicles that occurs, there is a decline in the quality of the oocyte as reflected by an increased incidence of miscarriages and chromosomal alterations that occur after 35 (Fig. 1). This is attributed to an increase in meiotic nondisjunction of the chromosomes during meiosis resulting in an increasing rate of aneuploidy in older women. Also around this time, there is an increase in FSH as the follicular pool begins to shrink. This increase in FSH occurs because factors produced by follicles which inhibit FSH secretion from the hypothalamic–pituitary axis, such as inhibin B and anti-Müllerian hormone (AMH), begin to decline. This decline in negative FSH regulators results in an increase in circulating levels of FSH in an attempt to increase the growing follicular pool. The elevated FSH promotes inappropriate maturation of the follicular granulosa cells resulting in the demise of these follicles by atresia. This vicious cycle results in a loss of additional follicles which continues to drive increasing levels of FSH to attempt to compensate for the diminished growing follicular pool.

There is evidence of internal self regulation of the size of the ovarian pool by local ovarian factors. As the pool of primordial follicles is depleted and the number of developing follicles declines, there is compensation by the ovary to increase the number of primordial follicles activated. Previously it was thought that at around the age of 35–38, the number of primordial follicles falls below 25,000 and the rate of follicle loss is accelerated. However, using stereologic techniques to determine ovarian follicle numbers there appears to be no sudden change in primordial follicle number but rather a consistently increasing rate of follicle loss as women age. This model for primordial follicle loss may be more biologically plausible as the ovary tries to compensate for the diminishing follicular pool.

With continued follicle loss a woman enters the menopausal transition of the perimenopausal period characterized by the onset of menstrual cycle irregularity. This transition period has been divided into an early and late stage. The early stage occurs when there are two or more cycles with a difference in length of greater than 7 days. This cycle irregularity leads to increasing periods of amenorrhea. The onset of the late stage of the perimenopausal period occurs when there are 60 or more days of amenorrhea. This menopausal transition, from the onset of menstrual cycle irregularity to menopause, is reported to be occur around the age of 46 (range 34–54 years) and lasts approximately 6 years. As the primordial follicle pool continues to be depleted and approaches 1000 follicles, a woman reaches menopause which occurs around the age of 51 (range 40–60 years). The time it takes to reach a pool of 1000 follicles from the critical threshold of about 25,000 follicles seems to be fairly constant, at approximately 13 years leading to a “fixed interval” hypothesis between the age at onset of cycle irregularity and the age at menopause.

Premature Ovarian Failure

Premature ovarian failure (POF) in women, also known as primary ovarian insufficiency (POI), is characterized by the absence of normal ovarian function due to depletion of the primordial follicle pool before the age of 40. POF is classified as an absence of menses for at least 4 months in combination with elevated FSH levels exceeding 40 IU/L before age of 40. POF affects approximately 1% of women below the age of 40% and 0.1% before the age of 30 (approximately 700,000 and 70,000 women respectively).

In addition to the decline in fecundity in women with POF, the loss of follicles results in a loss of ovarian hormones that has far reaching health consequences. Estradiol produced by the follicle during the late stage of the follicular phase and during the midluteal phase of the menstrual cycle provides numerous health benefits. Estradiol is responsible for maintenance of bone mineral density throughout life, preventing atherosclerosis, and improving mental health by attenuating mood and decreasing depression.

Thus, the premature loss of follicles results in an increased incidence of stroke and cardiovascular mortality, osteoporosis and bone fractures, and certain cancer risks such as colorectal cancer. To circumvent these health risks, hormonal replacement therapy using combined estrogen and progesterone medications are currently used to circumvent the loss of ovarian steroids and clinically manage these women.

Factors Influencing Ovarian Aging

Multiple factors appear to influence ovarian aging. These include genetic, iatrogenic, immunologic, metabolic, and infectious elements as well as exposure to environmental factors. The strong link to a genetic component has been demonstrated in studies examining the age when a woman undergoes natural menopause. Women experiencing relatively early menopause have mothers or will have daughters who also were likely to have become menopausal early in life as well. Likewise, it has been proposed that the size of the pool of primordial follicles, the proportion of these follicles that undergo atresia, as well as the rate of follicle activation is genetically determined.

Genetic Factors

Women with POF have provided a unique clinical model to begin to explore the genetic changes associated with alterations in ovarian aging. Early studies examined candidate genes associated with fetal formation of the primordial follicle, initial recruitment from the primordial follicle pool or follicular growth and development in women with POF. POF has been linked with mutations in genes associated with hormone and hormone actions (FSHR, LHR, PGRMC1, AMHR2, CYP17, CYP19), genes that regulate the early stages of oocyte development and ovarian formation (NOBOX, FIGLA, LHX8) as well as genes that impact initial folliculogenesis (BMP15, GDF9, GPR3). For example, polymorphisms in the AMH receptor 2 gene, which would decrease AMH signaling, have been associated with an earlier age at menopause in two large cohorts of Dutch postmenopausal women. Altering AMH signaling could potentially weaken the inhibition of initial follicle recruitment, resulting in a possible increased rate of follicle loss thereby advancing the age at menopause.

Abnormalities of the X chromosome have long been recognized as a frequent cause of many forms of familial POF. The more common of these include Turner's syndrome, triple X syndrome, Fragile X, and BMP15 variants. Women with Turner's syndrome, which is the loss of one X chromosome, have a loss of oocytes during the early stages of meiosis and ovarian development. Mutations of a trinucleotide repeat sequence in the fragile X mental retardation 1 (FMR1) gene is one of the most common genetic abnormalities associated with POF (incidence of 4%–15%). Although the mechanism is unclear, the FMR protein may play a role in early ovarian development by regulating oogonia proliferation which would determine the set point of the initial size of the ovarian reserve.

There are other autosomal mutations that have been linked with POF. Blepharophimosis, ptosis, epicanthus inversus syndrome (BPES) is an autosomal dominant eyelid malformation associated with POF. Mutations in the forkhead transcription factor L2 (FOXL2) are linked with BPES and may influence the follicular pool through the actions of FOXL2. FOXL2 regulates AMH expression as well as estradiol signaling through the estrogen receptor B (ESR2) which could potentially impact the ovarian follicular pool. Other alterations such as the ataxia telangiectasia mutated gene (ATM) have been associated with primordial germ cell development. Mutations in Factor V Leiden and apolipoprotein E-2 and their association with age at natural menopause has led to the hypothesis that genetically altered vascular support has long-term effects on ovarian follicle depletion. Thus, there are a large number of genetic alterations that have been reported to be associated with POF and appear to act through a variety of different mechanisms to affect the ovarian follicular pool.

More recently, genome wide association studies (GWAS) have examined possible genetic determinants and POF. A GWAS approach compares the DNA of women with POF to the DNA of women who do not exhibit POF to identify single nucleotide polymorphisms (SNPs) associated with POF. An advantage to the GWAS approach is that a non-candidate driven approach provides a basis to examine genetic changes across the genome as opposed to single gene specific candidate driven studies. Early genome-wide association analysis has revealed a candidate region linked to the ADAMTS19 and PTHB1 genes. Ongoing studies are identifying single nucleotide polymorphisms that are possible candidate mutations for POF. These studies provide evidence that the age at which the menopause occurs may vary among individuals and may be determined by genetic factors.

Environmental Factors

Many environmental and lifestyle factors have been suggested to negatively influence the ovarian follicular pool thereby leading to POF, such as smoking and environmental chemical exposure. Meta-analysis studies of environmental pollutants and POF have revealed that tobacco, pesticides and endocrine disrupting chemicals were the most reported substances having a negative impact on ovarian function resulting in an increased follicular depletion leading to an earlier age of menopause onset.

Cigarette smoking has been shown to be one of the most common and important factors resulting in a reduction of the primordial follicle pool where compounds present in tobacco enhance follicle damage and premature ovarian failure. Cigarette smoke contains more than 4000 chemicals including hydrocarbons, alcohols, phenols, aldehydes, and heavy metals. Hydrocarbons can act on the aryl hydrocarbon receptor of the ovary and activation of this receptor decreases oogonia proliferation in human fetal ovaries.

Endocrine disrupting chemicals include the phthalates and bisphenol A. Phthalates are chemicals that are ubiquitous in the environment and are currently used in the manufacture of plastics and consumer products such as clothing, children's toys and

cosmetics. Phthalates act to decrease the number of primordial follicles in mice and decrease antral follicles in women. Bisphenol A (BPA) is found in plastics used for food packaging, the epoxy resins that coat the interior of metal containers and beverage cans as well as papers receipts. BPA acts at the estrogen receptor to mimic the actions of estrogen and like the phthalates, BPA can decrease the number of primordial follicles in rodent studies.

Women undergoing chemotherapy or radiation therapy for cancer treatment are often infertile or sterile. Alkylating agents such as cyclophosphamide act to deplete the pool of primordial follicles. The mechanism of action varies depending upon the specific agent but is thought to be directed to the oocyte or the surrounding supporting granulosa or theca cells.

Predictors of Ovarian Aging

The loss of follicles and diminution of the follicular pool is only recognized at the late stages by alterations in menstrual cycle regularity. Because of this inability to accurately predict the vitality of the follicular pool before this menopausal transition, there has been a keen interest in the development of diagnostic markers of the ovarian follicular reserve and numerous potential markers have been explored.

Follicle Stimulating Hormone

As noted above, levels of FSH begin to increase as the primordial pool is depleted, long before the onset of menstrual cycle irregularity is observed. This increase in FSH levels begins and continues to rise during the perimenopausal period making it a predictor of the size of the follicular pool. FSH has remained a keystone in the classification of the current phases of reproductive aging according to the Stages of Reproductive Aging Workshop. However, during the regular menstrual cycle FSH demonstrates a cyclic pattern of secretion with an increase during the luteal period which acts to recruit follicles for the next menstrual cycle and another midcycle surge of FSH. Thus, there are naturally occurring changes in FSH secretion throughout the menstrual cycle. With aging, this naturally occurring increase in FSH during the luteal period becomes elevated with a loss of follicles. Using this rise in basal levels of serum FSH during the luteal period when paired with a woman's chronological age provides a crude surrogate marker of biological ovarian age. However, the capacity of FSH to predict the timing of future changes in the reproductive state (i.e., occurrence of menopause or the menopausal transition) has been reported to be weak, stimulating interest in other biomarkers of ovarian aging.

Antral Follicle Count (AFC)

The pool of primordial follicles is too small to observe by conventional imaging techniques such as ultrasound or transvaginal sonography (TVS) imaging. However, this pool of primordial follicles gives rise to follicles that develop into fluid filled antral follicles which can be imaged and counted. Histological analysis has demonstrated that the number of primordial follicles in the ovary is correlated with the number of antral follicles at all ages of a woman's life. This correlation is not a 1:1 association but rather a percentage of the primordial follicle pool. Since the decline in primordial follicle numbers parallels the decrease in size of the antral follicle pool with female aging, antral follicle count represents a better marker than either chronological age or basal FSH for assessing the ovarian biological age and has become an acceptable standard for assessment of the ovarian reserve.

Inhibin B

Inhibin B is a peptide hormone that is produced primarily by growing antral follicles that are responsive to FSH. As the follicle continues to grow and become dominant, inhibin B secretion declines. As the growing pool of antral follicles decreases during aging, levels of inhibin B are diminished. Studies in women have demonstrated that decreased inhibin B secretion is associated with elevated FSH levels and decreased oocyte quality and fertility potential. Thus, inhibin B has been used as a predictor of ovarian reserve. However, the late appearance of inhibin B in the menopausal transition has led to the suggestion that it may be a better indicator of ovarian activity than of ovarian reserve, due to its direct link with growing follicles.

Anti-Mullerian Hormone (AMH)

Anti-Mullerian hormone or AMH is a peptide hormone that is produced predominately by preantral and early antral follicles. AMH secretion begins when follicles differentiate from the primordial to the primary stage and declines when the follicle has reached the midantral stage (diameter of 2–6 mm). Granulosa cells are the predominate source of AMH production. Studies have demonstrated that with the decrease in the number of antral follicles with age, AMH serum levels also decline and will invariably become undetectable near menopause. Because AMH does not exhibit cyclic changes across the menstrual cycle, AMH levels have gained favor as a serum marker for the ovarian follicular reserve and may provide an index of age at menopause.

Menopausal Consequences on Overall Health

The age of the onset of menopause has remained fairly constant at approximately 51 years of age. However, life expectancy for women born in the United States has continued to increase. In 1917 the average life expectancy was 56 years where a woman would

live only 3–5 years after menopause. Women now have an average life expectancy of approximately 87 years resulting in women spending about one third of their life in a postmenopausal hypoestrogenic state. The loss of follicles and the accompanying loss of hormonal steroids during the late stage of the follicular phase and during the midluteal phase of the menstrual cycle have far reaching health consequences.

Early changes associated with the onset of menopause include vasomotor symptoms comprising hot flushes and night sweats. The duration and severity of these vasomotor changes that women experience vary among individuals. Symptoms can develop during the menopausal transition, during menopause, or after menopause and can last for a few years or for extended periods in the postmenopausal period. Night sweats often lead to insomnia and overall fatigue. The mechanism behind the onset of hot flushes and night sweats appears to involve the central nervous system, possibly linked to a narrowing of the thermoregulatory set point in women.

Women are protected from cardiovascular problems (ischemic heart disease, stroke and other heart diseases) mainly because of the protective role of estrogens against atherosclerotic disease. Estrogens maintain the flexibility of the arterial wall by acting on the endothelial and smooth muscle cells comprising the blood vessel. In the postmenopausal period, arterial vessels become more rigid decreasing blood flow. With the loss of estrogen, the risk of cardiovascular problems in postmenopausal women grows and becomes similar to that of men by the age of 75.

Estrogens are responsible for maintenance of bone mineral density throughout life. Estrogens act to stimulate osteoblasts, the cells responsible for bone formation. In the years after menopause, the dynamics of bone density are shifted away from the balance of bone formation and resorption toward bone resorption. This results in osteoporosis leading to bone fragility and fractures.

With the onset of menopause there are changes in cognition, mood and an increase in depression. Observational studies suggest that the symptoms of depression are more frequent in the perimenopause, particularly in the late menopausal transition with a peak around the last menstrual period. Animal studies have convincingly demonstrated a protective role for estradiol in preventing these CNS changes. However, studies in women have provided conflicting evidence about the benefit of estrogen replacement in preventing many of these neurocognitive and mood changes.

Genitourinary syndrome of menopause describes the changes in the vagina, vulva and bladder associated with loss of estrogen and other hormones. Estrogen acts to lubricate and keep the vagina flexible. A loss of hormones results in vaginal dryness and dyspareunia or painful intercourse, which in turn results in lower libido and hypoactive sexual desire disorder. Other genitourinary changes associated with menopause include genital dryness, burning or irritation as well as an increased urgency to urinate, pain with urination and recurring urinary tract infections. Thus, the loss of follicles and the changing hormonal milieu results in an increased incidence of stroke and cardiovascular mortality, osteoporosis and bone fractures, and certain genitourinary changes. To circumvent these health risks, hormonal replacement therapy using combined estrogen and progesterone medications are currently used to circumvent the loss of ovarian steroids and clinically manage many of these symptoms.

Further Reading

- Alviggi, C., Humaidan, P., Howles, C. M., Tredway, D., & Hillier, S. G. (2009). Biological versus chronological ovarian age: implications for assisted reproductive technology. *Reproductive Biology and Endocrinology*, 7, 101.
- Bilgin, E. M., & Kovanci, E. (2015). Genetics of premature ovarian failure. *Current Opinion in Obstetrics & Gynecology*, 27(3), 167–174. Review.
- Broekmans, F. J., Soules, M. R., & Fauser, B. C. (2009). Ovarian aging: mechanisms and clinical consequences. *Endocrine Reviews*, 30(5), 465–493.
- Depmann, M., Broer, S. L., van der Schouw, Y. T., Tehrani, F. R., Eijkemans, M. J., Mol, B. W., & Broekmans, F. J. (2016). Can we predict age at natural menopause using ovarian reserve tests or mother's age at menopause? A systematic literature review. *Menopause*, 23(2), 224–232.
- Gleicher, N., Weghofer, A., & Barad, D. H. (2011). Defining ovarian reserve to better understand ovarian aging. *Reproductive Biology and Endocrinology*, 9(23), 1–11.
- Hansen, K. R., Knowlton, N. S., Thyer, A. C., Charleston, J. S., Soules, M. R., & Klein, N. A. (2008). A new model of reproductive aging: the decline in ovarian non-growing follicle number from birth to menopause. *Human Reproduction*, 23, 699–708.
- Harlow, S. D., Gass, M., Hall, J. E., Lobo, R., Maki, P., Rebar, R. W., Sherman, S., Sluss, P. M., & de Villiers, T. J. (2012). Executive summary of the Stages of Reproductive Aging Workshop +10: addressing the unfinished agenda of staging reproductive aging. *Journal of Clinical Endocrinology and Metabolism*, 97, 1159–1168.
- Monniaux, D., Clément, F., Dalbiès-Tran, R., Estienne, A., Fabre, S., Mansanet, C., & Monget, P. (2014). The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link? *Biology of Reproduction*, 90(4), 85, 1–11.
- Rossetti, R., Ferrari, I., Bonomi, M., & Persani, L. (2017). Genetics of primary ovarian insufficiency. *Clinical Genetics*, 91(2), 183–198.

Female Contraception

Diane M Duffy and David F Archer, Eastern Virginia Medical School, Norfolk, VA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Actual efficacy (also called practical efficacy or typical use efficacy) Contraceptive efficacy when a method is used by people not involved in clinical trials.

Breakthrough bleeding Unanticipated or unscheduled bleeding or spotting, originating in the uterus and similar to menstrual bleeding.

Efficacy Effectiveness of a contraceptive method, the expected number of women who do not get pregnant in a group of 100 women using the method for an entire year.

Estrogens A group of structurally related steroid hormones that act at estrogen receptors.

Endometrium Lining of the uterus, is lost with menstruation.

Intrauterine device (IUD) T-shaped device placed in the uterus for contraception.

Long-acting reversible contraception (LARC) Contraceptives that remain in place and provide long-lasting but reversible contraceptive action.

Luteinizing hormone (LH) A protein hormone produced by the anterior pituitary.

Progestins A group of structurally related steroid hormones that act at progesterone receptors.

Subcutaneous Under the skin.

Synthetic Human-made in the laboratory, not found in nature.

Theoretical efficacy Contraceptive efficacy during perfect use, usually determined in clinical trials.

Transdermal Across the skin.

Opportunities for Female Contraception

Fertility requires interaction between the oocyte and the sperm. During intercourse, ejaculation results in the release of hundreds of millions of sperm in the vagina, near the opening of the uterine cervix. Sperm penetrate the cervical mucous and move through the lumen of the uterus into the oviduct (also called the fallopian tube or uterine tube). The oocyte (also known as the egg or ovum) leaves the ovary at ovulation, enters the oviduct through the fimbria, and is propelled through the oviduct and toward the uterus by the movement of cilia and muscle contractions of the oviduct. Fertilization, the fusion of oocyte and sperm, occurs in the oviduct. After fertilization, the single cell embryo, or zygote, continues to move through the oviduct and toward the uterus as the early cell divisions of embryonic development get underway.

The oocyte develops within the ovarian follicle. The mature ovarian follicle (also called a dominant, ovulatory, or Graafian follicle) produces large amounts of the steroid hormone estrogen, which enters the circulation. High serum levels of estrogen stimulate the anterior pituitary to release of a large amount or “surge” of LH. This surge of LH acts at the ovarian follicle to initiate a series of events that culminate in ovulation, including rupture of the follicle and release of the oocyte. After ovulation, the ruptured follicle is transformed into a corpus luteum and produces large amounts of the steroid hormone progesterone.

Estrogen and progesterone stimulate changes in the woman's body that affect fertility. Estrogen stimulates the oviduct to produce fluid and increases muscle contractions, which assist the movement of the oocyte from the fimbria and toward the uterus. Estrogen results in the production of a thin, watery cervical mucous, which is easily penetrated by sperm moving from the vagina into the uterus and toward the oviduct. Estrogen causes the endometrial lining of the uterus to regrow after menstruation. In turn, progesterone reduces fluid secretion and muscle contractions in the oviduct, limiting oocyte entry into the oviduct and transport through the oviduct. Progesterone increases the viscosity of cervical mucous, reducing sperm transit from the vagina to the uterus and oviduct. Progesterone also acts at the endometrial lining of the uterus to prepare for implantation and pregnancy. Falling progesterone levels after a period of progesterone exposure causes shedding of the uterine endometrium or menstruation. However, continuous exposure to progesterone prevents growth and development of the uterine endometrium.

Structure and function of the female reproductive tract offers many opportunities to intervene and prevent fertility (Table 1). Barrier methods prevent movement of sperm from the vagina, thereby preventing fertilization. Occlusion of the oviducts also prevents sperm–oocyte interaction. Spermicides damage or destroy sperm cells, so sperm are unable to fertilize the oocyte. Chemical spermicides and the intrauterine device (IUD) utilize this approach to fertility prevention. Hormonal contraception disrupts many aspects of female reproductive function, ultimately reducing fertility. Behavioral methods to prevent pregnancy include abstinence, periodic abstinence, and withdrawal of the penis from the vagina prior to ejaculation.

Table 1 Most effective contraceptive method used in the past month by US women in 2012

<i>Method</i>	<i>Number of users</i>	<i>Percent of women aged 15–44</i>
The pill	9,720,000	16.0
Female permanent contraception	9,443,000	15.5
IUD	3,884,000	6.4
Withdrawal	1,817,000	3.0
Progestin injection	1,697,000	2.8
Vaginal ring	759,000	1.2
Periodic abstinence	509,000	0.8
Progestin implant	492,000	0.8
Hormone patch	217,000	0.4
Emergency contraception	91,000	0.2
Other female methods ^a	133,000	0.3
Male methods ^b	8,823,000	14.5
No method; at risk of unintended pregnancy	4,175,000	6.9
No method; not at risk of unintended pregnancy	19,126,000	31.4

^aIncludes diaphragm, female condom, spermicide, cervical cap, and sponge.

^bIncludes male condom and male sterilization (vasectomy).

Table adapted with permission from Guttmacher Institute, Contraceptive use in the United States, Fact Sheet, New York: Guttmacher Institute, 2016, <https://www.guttmacher.org/fact-sheet/contraceptive-use-united-states>.

Selection of a Contraceptive Method

There are many considerations for selection of a contraceptive method. Permanent contraception eliminates the possibility of all future pregnancies. Reversible contraceptives can prevent pregnancy in the short-term while preserving long-term fertility. Contraception can be used with the intention of preventing all pregnancies. However, reduction of overall family size by increasing time interval between pregnancies can also be achieved with contraception. When selecting a contraceptive method, a woman may also consider safety, effectiveness, cost of the method, how often the user needs to employ the contraceptive, whether the method requires action immediately before intercourse, side effects caused by use of the method, impact on the sexual experience, if her partner should be involved, and whether a clinician must be consulted to acquire or employ the method properly.

Steroid Hormones as Components of Contraceptives

Steroid hormones, including estrogen and progesterone, are important to the proper function of the female reproductive system. High levels of estrogen are characteristic of the first half of the menstrual cycle. During this period of follicle growth and maturation, estrogen supports the health of reproductive tract organs including the oviduct, uterus, and cervix. Estrogen also increases the number of progesterone receptors in these organs. After ovulation, the follicle transforms into the corpus luteum, which produces large amounts of progesterone. Reproductive tract organs respond to progesterone via progesterone receptors to decrease oviductal motility, thicken cervical mucous, and prepare the uterine endometrium for possible implantation. Progesterone also decreases the number of estrogen receptors in these tissues, decreasing the effects of estrogen and limiting the ability of estrogen to increase progesterone receptor numbers.

Progesterone or a progesterone-like molecule (progestins) is an essential component of all hormonal contraceptives. As described earlier, progesterone decreases many reproductive tract functions required for fertilization. Progesterone also decreases estrogen action at these tissues due to the decrease in estrogen receptors. Finally, progesterone prevents the release of surge levels of LH, so LH cannot initiate events within the ovary that lead to ovulation, including follicle rupture and release of the oocyte. Many hormonal contraceptives also include an estrogen to reduce unwanted and unpredictable uterine bleeding (often called breakthrough bleeding) and to help maintain the overall health of nonreproductive tissues, such as the bone, skin, and heart.

The development of synthetic, orally active progestins and estrogens was a major advance in female contraception. Progestins, including progesterone and 17 α -hydroxyprogesterone, and estrogens, including estradiol and estrone, are produced in the body primarily by the ovary. For a contraceptive, circulating levels of steroid hormones must be maintained at relatively constant levels. Natural progestins and estrogens circulate in the bloodstream for several hours after production and then are removed from circulation by the liver and kidneys. Natural steroid hormones are damaged by passage through the digestive system, so oral administration of progesterone and estrogen is impractical. For these reasons, use of naturally occurring steroid hormones would require frequent readministration.

These limitations were overcome with the development of synthetic progestin and estrogen molecules. Synthetic hormones maintain bioactivity after oral administration, passage through the digestive system, and uptake into the circulation. Synthetic hormones can also be administered via transdermal patches, subcutaneous implants, and injections. Synthetic hormones remain

in the circulation longer than natural steroids because their chemical structure prevents rapid modification, conjugation, and elimination in the liver and kidneys. Synthetic progestins and estrogens activate progesterone receptors and estrogen receptors, respectively, but often with higher potency than natural progesterone and estrogen. These are just some of the reasons that synthetic progestin and estrogen molecules are used as components of hormonal contraceptives.

Synthetic progestins that bind to progesterone receptors but block activity of progesterone receptors are called antiprogestins. These synthetic hormones can prevent fertility by disrupting the activity of progesterone in reproductive tissues. The most common contraceptive actions of antiprogestins are to (1) reduce or eliminate the ovulatory LH surge and (2) block necessary actions of progesterone within the ovulatory follicle prior to ovulation. Actions of antiprogestins in the uterus to disrupt implantation or early stages of placenta development are not contraceptive since these actions occur after fertilization has taken place.

Hormonal Contraception: Combination Hormonal Contraceptives

The first combination oral hormonal contraceptive, commonly known as The Pill, contained large amounts of a synthetic estrogen and a synthetic progestin. Since The Pill was introduced in 1960, there has been a steady decrease in the concentration of the progestin and the estrogen in oral contraceptive products. Products with lower steroid doses remain highly effective at preventing pregnancy with a substantial reduction in side effects, especially deep vein thrombosis and other cardiovascular events.

The regimen of oral hormonal contraceptives was initially designed to mimic the 28-day interval between menstruations. A common formulation involved 21 days of steroid-containing pills, with 7 days of placebos to allow circulating steroid hormone levels to fall and permit menstruation. Changes in duration of administration have resulted in a variety of regimens, including 24 days of steroids with 4 days of placebo, continuous steroid administration for 84 days, and continuous steroid administration for 365 days. Continuous pill use increases the incidence of no menstrual bleeding, which is highly acceptable to some women. However, unscheduled endometrial bleeding, known as breakthrough bleeding, can occur with any steroid regimen. Oral hormonal contraceptives containing estrogen and progestin have a pregnancy rate of 1%–4% in well-controlled clinical trials (Table 2).

Hormonal contraception is also available in nonpill forms. Estrogen plus progestin contraceptives can be administered via a transdermal delivery system, vaginal ring, or injection. Transdermal and vaginal delivery use a regimen of 21 days of active hormone and 7 days of no hormone to permit menstruation and approximate the length of the typical menstrual cycle. The monthly injection has a duration of efficacy of 28 days and must be administered every 4 weeks. The contraceptive efficacy of these methods is very high, with pregnancy rates of 1%–2% in well-controlled clinical trials.

The lower dose combination oral hormonal contraceptive products in current use are relatively safe. The principal significant adverse events resulting from use of combination oral hormonal contraception are increased incidence of venous thrombosis in the leg and pulmonary emboli. Use of a modern, low-dose combination oral hormonal contraceptive doubles a woman's risk for these cardiovascular events. However, the risk of cardiovascular events associated with these contraceptives is much lower than the risk of cardiovascular events associated with pregnancy. Women with certain inherited clotting disorders or a family history of certain cardiovascular disorders should consider other methods of contraception. Women over the age of 35 who have hypertension, have diabetes mellitus, or smoke cigarettes are at increased risk of stroke and myocardial infarction. In women without these risk factors, low-dose combination oral hormonal contraceptives can be used during a woman's reproductive years up to menopause without a significant age-related increased risk of cardiovascular disease.

Table 2 Pregnancy rates (number out of 100 women) during the first year of continuous use of the contraceptive method

<i>Method</i>	<i>Perfect use^a</i>	<i>Typical use</i>
Intrauterine device—levonorgestrel	0.1	0.1
Progestin implant	0.1	0.1
Progestin injection	0.3	0.3
Female permanent contraception ^b	0.5	0.5
Intrauterine device—copper	0.6	0.8
Oral combined hormonal	1	9
Oral progestin only	0.5	9
Withdrawal	4	19
Diaphragm with spermicide	6	20
Female condom	5	24
Periodic abstinence	5	25
Spermicide alone	6	26
No contraception	85	85

^aFrom product label or other information.

^bIncludes both surgical and nonsurgical methods.

Side effects include nausea, weight gain, mood disturbances, breast tenderness, headache, and unscheduled endometrial bleeding. These side effects may disappear or improve with continued use. However, for some women, side effects are significant and result in discontinuation of the method.

Typical failure rates for oral, transdermal, vaginal, or injectable combined hormonal contraceptives are approximately 9% and are likely the result of compliance errors by the user.

Hormonal Contraception: Progestin-Only Methods

Progestins inhibit the pituitary LH surge and, therefore, prevent ovulation as the principal mechanism of action. Additional actions of progestins to decrease the likelihood of fertilization contribute to the high effectiveness of progestin contraceptives. Clinical testing of synthetic progestin-only oral methods reports pregnancy rates of <1%. Typical user's failure rates for daily oral progestin-only methods are about 9%.

Contraceptive efficacy can be enhanced with delivery systems that provide extended duration of progestin activity and minimize or eliminate the need to remember to use the contraceptive. Progestins can be delivered by subcutaneous implants, vaginal ring, IUD, and injection. These methods are termed long-acting reversible contraceptives (LARCs). Subcutaneous implants, vaginal rings, and IUDs contain the progestin within a plastic membrane. The plastic membrane controls the amount of hormone released each day, resulting in a duration of action of 1 month to 8 years, depending on the individual device. The high efficacy associated with these methods is due, in large part, to the fact that the user does not need to remember to use the method on a daily or weekly basis. Implants, vaginal rings, and IUDs can be removed for relatively rapid return to fertility. However, injections form a subcutaneous depot of progestin that cannot be removed. As such, return to fertility after discontinuing injections can require up to a year. The delivery system has a significant effect on typical user pregnancy rates with progestin-only contraceptives, making LARCs highly effective. Theoretical and typical user pregnancy rates are both <1%.

The primary side effect of progestin-only contraceptives is unscheduled and irregular endometrial bleeding. This bleeding does not reflect a malignancy but is the principal reason for discontinuation of progestin-only methods. Progestins have none of the estrogen-related significant side effects, most importantly blood clots. For this reason, hormonal contraceptives that contain progestin with no estrogen may be appropriate for women at higher risk of cardiovascular issues, including older women and women who smoke. Progestin-only contraceptives are appropriate for breastfeeding women since the estrogen component of combined hormonal contraceptives interferes with lactation.

Intrauterine Devices

The IUD is a T-shaped plastic device that is placed within the lumen of the uterus, such that the arms of the "T" point toward the oviducts, with the lower end of the "T" pointing toward the cervix. The plastic of the IUD has contraceptive action, creating a sterile inflammatory response that has spermicidal activity. However, all currently available IUDs are designed to have enhanced activities. Some IUDs include copper wound on the arms and stem, which enhances the spermicidal activity. Other IUDs include a progestin within the stem of the device. With progestin-releasing IUDs, locally elevated progestin within the uterine lumen decreases endometrial development and reduces or eliminates menstruation; progestin also decreases uterine contractions to reduce the rate of spontaneous expulsion of the IUD.

An IUD should be placed in the uterus by an experienced clinician. For insertion, the arms of the T are folded parallel to the stem of the device and held in place with an inserter. The inserter is introduced through the cervix and into the uterine cavity. Once properly placed, the IUD is deployed and achieves its active T shape. Each IUD has a different length of time approved for use, with a range of 5–10 years. Fertility is typically restored within a few weeks of IUD removal. IUDs provide highly effective protection against pregnancy, with theoretical and typical user pregnancy rates <1%.

Barrier Methods

Perhaps the best known barrier method is the male condom. The female condom is also a barrier contraceptive method. Female condoms are made out of latex, nitrile, or polyurethane. The female condom is designed to fit into the vagina, with a plastic ring anchoring it in the upper vagina. The condom covers the vaginal walls and extends outside of the vagina where a second ring prevents it from being pressed inward at the time of intercourse. Similar to the male condom, sperm are retained within the female condom after ejaculation, preventing sperm transit from the vagina to the cervix. Clinical studies of consistent use are associated with pregnancy rates of 4%–5%, while typical use pregnancy rates of 21% for the female condom have been reported. Condoms, including both male and female condoms, are highly recommended for use by couples at risk for sexually transmitted infections.

The diaphragm is a round device with a pouch, and the outer edge contains a spring designed to hold the device in place in the upper vagina. The uterine cervix fits into the diaphragm pouch, which is filled with a spermicide by the user prior to insertion. The diaphragm should be inserted before penile penetration and left in place for 1 h after ejaculation. Diaphragms are reusable and come in a variety of sizes. A diaphragm should be fitted by a clinician; the fit should be checked regularly, especially after pregnancy

or significant weight gain/loss. Diaphragms used consistently and correctly have a failure rate of 6%. Typical use failure rates are 20% for the diaphragm.

The cervical cap is a reusable contraceptive device similar to the diaphragm, except that the cap is smaller and fits tightly around the opening to the cervix. Effectiveness is enhanced when used with a spermicide. A cervical cap should be fitted by a clinician and refitted after pregnancy. Failure rates with consistent and typical use are slightly higher than for the diaphragm.

Side effects of barrier methods are minimal. Individuals with allergies to latex should select products that do not contain latex.

Spermicides

Spermicides are detergents that damage the cell membrane of the sperm. Spermicides must be placed within the vagina before penile penetration to be effective. The spermicide most commonly available is nonoxynol-9.

Spermicidal products include foam, creams, gel, vaginal suppositories, and vaginal film. When a spermicide is used as the only method of contraception, the pregnancy rate is 6% in clinical studies but 26% with typical use. Spermicides are often combined with a barrier method, such as a condom or diaphragm.

Spermicides are also impregnated into other contraceptive products to enhance efficacy. The contraceptive sponge is a single-use contraceptive that is inserted into the vagina to cover the uterine cervix. The sponge contains spermicide, which disables or destroys sperm during transit from the vagina to the uterus. The sponge must be inserted prior to intercourse and should be left in place for at least 6 h after intercourse. In women who have never given birth, consistent and correct use of the contraceptive sponge has a failure rate of 1%–9%; typical use failure rates are 11%–24%. Typical failure rates are higher for women who have experienced pregnancy (24%–32%).

Side effects with spermicides are minimal.

Emergency Contraception

Emergency contraception is intended for use after unprotected intercourse, in cases where a contraceptive method was not used or when the primary contraceptive method may have failed.

Two forms of oral emergency contraception, also called morning-after pills, are currently available in the United States. A variety of products, including Plan B[®], contain the progestin levonorgestrel as the active ingredient. Another option is ulipristal acetate, a progesterone receptor modulator, currently marketed as Ella[®]. These progestins prevent pregnancy by preventing the LH surge and, therefore, ovulation. For this reason, emergency contraceptive pills are most effective when taken within 72 h of intercourse. Emergency contraception does not interfere with fertilization or implantation, so these medications are less effective when taken more than 3 days after intercourse. Emergency contraceptives are also less effective in obese women. Levonorgestrel-containing emergency contraceptives reduce the estimated pregnancy rate by 33%. Ulipristal acetate is more effective, reducing the estimated pregnancy rate by 66%. Side effects are similar to those reported for other hormonal contraceptives and include nausea, headache, uterine cramping, unscheduled uterine bleeding, fatigue, and breast tenderness.

The copper IUD is a highly effective emergency contraceptive method. The copper IUD can be inserted up to 5 days following intercourse as an emergency method. Following insertion, the copper IUD will provide continued contraceptive action for up to 10 years unless removed.

Permanent Contraception

Permanent female contraception (also called sterilization) involves obstruction of the oviducts. Surgical tubal ligation is typically performed as a laparoscopic outpatient procedure. During surgery, the oviducts are severed or banded to prevent transit of oocytes or sperm, and major side effects are those of surgery. Nonsurgical methods involve introduction of a device inserter through the cervix, a procedure called a hysteroscopy. During the procedure, the contraceptive device is placed into the opening between each of the oviducts and the uterus. The contraceptive device creates a local inflammatory response and facilitates fibrosis, which blocks the oviduct. Complete blockage of both oviducts can take up to 3 months to be complete, so the woman should use an alternative, effective form of contraception during this period. Reversals of tubal obstruction have been reported. However, tubal obstruction by any method should be considered to be permanent. Female permanent contraception is highly effective, with a pregnancy rate of < 1%.

Removal of the uterus (hysterectomy) for contraceptive purposes is not performed in the United States.

Behavioral Contraceptive Methods

The most utilized nonhormonal contraceptive method is withdrawal or removal of the penis from the vagina prior to ejaculation. While well-controlled clinical trials are lacking, this method is thought to have an overall contraceptive failure rate of approximately 19%.

Periodic abstinence, also known as natural family planning, the rhythm method, or the symptothermal method, is based on the concept that intercourse can result in pregnancy only during a few days in each menstrual cycle. Pregnancy can be avoided by (1) predicting when a woman is fertile and (2) abstaining from intercourse, using a barrier contraceptive or using withdrawal during the fertile period.

The fertile period of each menstrual cycle is defined as the days when intercourse can result in fertilization and formation of an embryo. Sperm travel from the vagina to the oviduct, where fertilization occurs. Sperm can survive in the oviduct for about 5 days, waiting for the oocyte to travel from the ovary after ovulation. The 5 days where intercourse can result in fertilization include the 4 days before ovulation and the day of ovulation.

Predicting the day of ovulation requires regular, consistent, and noninvasive monitoring of the woman's menstrual cycle. Purchased kits can be used to measure urinary LH and determine the day of the LH surge. Progesterone increases basal body temperature, so daily oral temperature measurements can be charted to determine the days of the luteal phase. Cervical mucous is watery during the follicular phase and viscous during the luteal phase. A sample of cervical mucous can be assessed for presence and consistency in order to determine if a woman is in the follicular phase or luteal phase of her menstrual cycle. Menstruation signals the first day of the follicular phase. Charting these changes on a calendar can document if a woman has consistent, regular cycles of similar length. If so, then a prediction of the fertile period can be made for future menstrual cycles, with the fertile period beginning at least 3 days before the expected LH surge and lasting for a total of 5 days. During the fertile period, a woman can choose to avoid intercourse or use an alternative form of contraception to avoid pregnancy.

Few women have such predictable menstrual cycles, contributing to a high pregnancy rate of 25% for this method for contraception. However, periodic abstinence can be used successfully by highly motivated couples to increase time between pregnancies and reduce overall family size.

Summary

There are a variety of contraceptive options currently available for sexually active women. Choices for reversible contraception include highly effective hormonal methods and less effective barrier methods. IUDs and other LARCs provide effective, long-lasting, and reversible pregnancy prevention. Methods that obstruct the oviducts are highly effective and should be considered permanent. When selecting a method, effectiveness and reversibility should be balanced with other considerations, including safety, cost, and side effects.

Further Reading

- Benagiano, G., Bastianelli, C., & Farris, M. (2006). Contraception today. *Annals of the New York Academy of Sciences*, 1092, 1–32.
- Cheng, L., Che, Y., & Gulmezoglu, A. M. (2012). Interventions for emergency contraception. *Cochrane Database of Systematic Reviews*, 8, CD001324.
- Cook, L., Nanda, K., & Grimes, D. (2003). Diaphragm versus diaphragm with spermicides for contraception. *Cochrane Database of Systematic Reviews*, 1, CD002031.
- Curtis, K. M., & Peipert, J. F. (2017). Long-acting reversible contraception. *The New England Journal of Medicine*, 376, 461–468.
- Edelman, A. B., Cherala, G., & Stanczyk, F. Z. (2010). Metabolism and pharmacokinetics of contraceptive steroids in obese women: a review. *Contraception*, 82, 314–323.
- Edelman, A., Micks, E., Gallo, M. F., Jensen, J. T., & Grimes, D. A. (2014). Continuous or extended cycle vs. cyclic use of combined hormonal contraceptives for contraception. *Cochrane Database of Systematic Reviews*, (7), CD004695.
- Gemzell-Danielsson, K., Berger, C., & Lalitkumar, P. G. (2013). Emergency contraception—mechanisms of action. *Contraception*, 87, 300–308.
- Grimes, D. A., Lopez, L. M., O'Brien, P. A., & Raymond, E. G. (2013a). Progestin-only pills for contraception. *Cochrane Database of Systematic Reviews*, (11), CD007541.
- Grimes, D. A., Lopez, L. M., Raymond, E. G., Halpern, V., Nanda, K., & Schulz, K. F. (2013b). Spermicide used alone for contraception. *Cochrane Database of Systematic Reviews*, (12), CD005218.
- Lopez, L. M., Grimes, D. A., Gallo, M. F., & Schulz, K. F. (2010). Skin patch and vaginal ring versus combined oral contraceptives for contraception. *Cochrane Database of Systematic Reviews*, (3), CD003552.
- Stanczyk, F. Z., Archer, D. F., & Bhavnani, B. R. (2013). Ethinyl estradiol and 17beta-estradiol in combined oral contraceptives: pharmacokinetics, pharmacodynamics and risk assessment. *Contraception*, 87, 706–727.

Relevant Websites

- <https://www.cdc.gov>—Center for Disease Control and Prevention.
- <https://www.guttmacher.org>—Guttmacher Institute.
- <https://www.nichd.nih.gov>—National Institutes of Health.
- <https://www.plannedparenthood.org>—Planned Parenthood.
- <http://www.who.int>—World Health Organization.

FEMALE REPRODUCTIVE TRACT

Fetal and Postnatal Female Tract Development

Zer Vue, Rachel D Mullen, Shuo-Ting Yen, Alejandra E Ontiveros, Allison C Stewart, and Richard R Behringer, University of Texas MD Anderson Cancer Center, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

AKT	Protein kinase B
AMH	Anti-Müllerian hormone
AR	Androgen receptor
BMP	Bone morphogenetic protein
DES	Diethylstilbestrol
FGF	Fibroblast growth factor
GE	Glandular epithelium
GW	Gestational week
LE	Luminal epithelium
MD	Müllerian duct
MODY5	Maturity-onset diabetes of the young type 5
MRKH	Mayer-Rokitansky-Küster-Hauser
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
WD	Wolffian duct

Introduction

The female reproductive tract organs form and differentiate during fetal and postnatal stages of development (Kobayashi and Behringer, 2003) (Fig. 1). The oviducts, uterus, cervix and upper portion of the vagina are derived from the paramesonephric ducts or

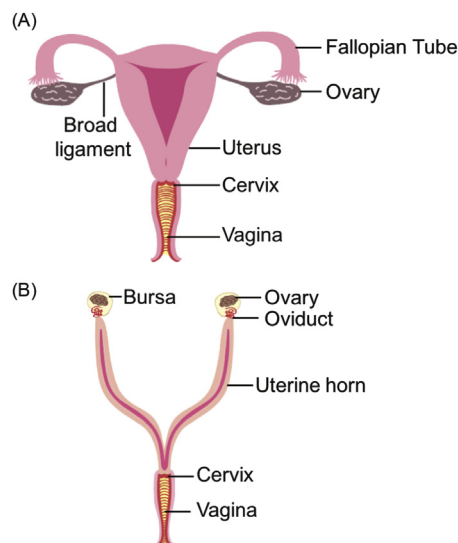


Fig. 1 Schematic illustration of the female reproductive tract in human and mouse. The female reproductive tracts of human (A) and mouse (B) consist of the ovary, Fallopian tube (oviduct in mouse), uterus, cervix and vagina. A bursal membrane surrounds the ovary in the mouse by not in human.

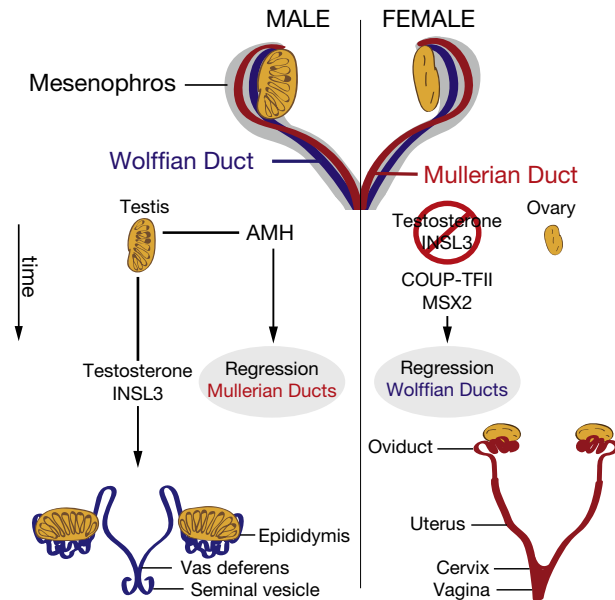


Fig. 2 Reproductive tract sexual differentiation. The reproductive tract progenitor tissues prior to sexual differentiation are equivalent and contain a fully formed Wolffian duct (blue) and Müllerian duct (red) within the mesenophros (gray). Hormones produced by the fetal testis, anti-Müllerian hormone (AMH), testosterone, and insulin-like 3 (INSL3), activate regression of the MD, differentiation of the WD into the male genital tract (vas deferens, epididymides, and seminal vesicles), and testicular descent, respectively. In females, at this developmental time point the ovary lacks AMH, testosterone, and INSL3. This permits differentiation of the MD into the female reproductive tract (oviducts, uterus and upper vagina), regression of the WD by allowing COUP-TFII signaling and MSX2 expression, and maintenance of the ovaries in an abdominal position, respectively. Modified from Mullen, R.D. and Behringer, R.R. (2014). Molecular Genetics of Müllerian Duct Formation, Regression and Differentiation. *Sex Dev.* 8, 281–296.

Müllerian ducts (MD) and adjacent mesenchyme that form within the fetal kidneys, the mesonephroi (Fig. 2). The MD is an epithelial tube with adjacent mesenchyme cells. The MDs are located adjacent and lateral to the mesonephric ducts or Wolffian ducts (WD) that also reside within the mesonephroi (Fig. 2). The WDs can give rise to male reproductive tract organs, including the seminal vesicles, vasa deferentia and epididymides. In male fetuses, the MDs are eliminated by the action of anti-Müllerian hormone (AMH), whereas the WDs differentiate in response to androgens. However, during female fetal development, the ovaries do not secrete AMH or androgens. Thus, in females the MDs differentiate, whereas the WDs regress (Fig. 2).

Once the MDs have formed, they will become regionalized into the oviduct, uterus, cervix and vagina. Depending on the species, the posterior region of the MDs will fuse to various extents, leading to different uterine morphologies (Kobayashi and Behringer, 2003). At birth, the uterus is composed of a lumen lined by a single layer of epithelial cells with a surrounding undifferentiated mesenchyme. Subsequently, the mesenchyme differentiates into an inner stromal compartment surrounded by inner circular and outer longitudinal smooth muscle layers, the myometrium. The adult uterus contains endometrial glands that produce factors required for uterine receptivity, embryo implantation, embryo survival and development (Gray et al., 2001). Endometrial glands from the luminal epithelium will invade into the uterine stroma in a process called adenogenesis (Spencer et al., 2005). Thus, the development of the fetal and postnatal female reproductive tract organs is complex and essential for the fertility of an individual female.

Formation of the Müllerian Duct

MDs form in amniotes, i.e., birds, reptiles and mammals. Current understandings of MD formation are mostly based on studies in the chicken and mammals. The corresponding developmental stages when MDs form are embryonic day (E) 11.5–13.5 in the mouse, E13.5–16.5 in the rat, Hamburger Hamilton stages 20–30 in the chicken, day 25 of gestation to 2–7 days postpartum in the wallaby and gestational week (GW) 6 to 9.5 (Carnegie Collection stages 16–18) in human (Renfree et al., 1996). Although the timing of MD formation varies between species, the process of how the MD forms is likely similar for each organism.

The formation of the MD can be separated into three phases: initiation, invagination, and elongation (Mullen and Behringer, 2014) (Fig. 3). The initiation phase occurs when a thickened placode-like structure forms on the anterior mesonephric epithelium near the WD. These cells are LHX1 positive and specified by an FGF/LHX1 axis, which, in turn, is regulated by a BMP/PAX2 axis (Atsuta and Takahashi, 2016) (Fig. 3).

The invagination phase occurs when the cells in the placode-like structure become elongated and form apical tight junctions, resulting in a depression of the mesonephric epithelium. Some cells appear to detach from the mesonephric epithelium and move into the space between the mesonephric epithelium and the WD. As the depression becomes deeper, it transforms into

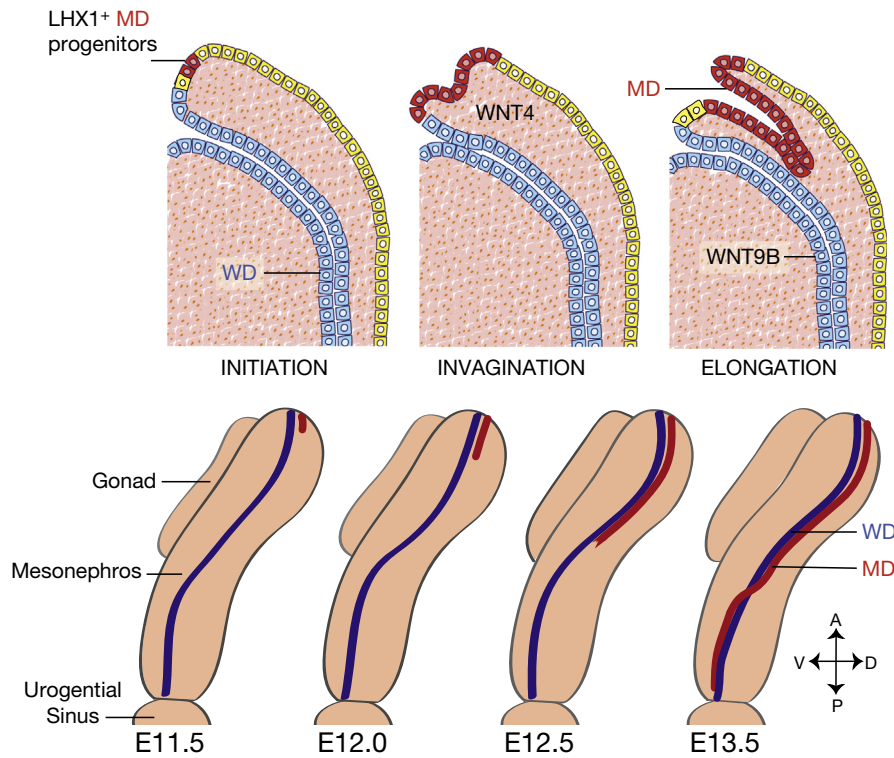


Fig. 3 Müllerian duct formation. (A) MD (red) formation occurs in three phases: initiation, invagination, and elongation. Initiation phase: MD progenitor cells in the mesonephric epithelium (yellow) are specified and begin to express LHX1. Invagination phase: in response to WNT4 signaling from the mesonephric mesenchyme, LHX1 positive (LHX1⁺) MD progenitor cells invaginate posteriorly into the mesonephros towards the WD (blue). Elongation phase: the tip of the MD contacts the WD and elongates caudally in close proximity to the WD requiring WNT9B signaling from the WD. The formation of the MD begins at around E11.5 in the mouse (B) The MD invaginates from the anterior mesonephric epithelium and extends posteriorly guided by the WD. During elongation, mesenchymal cells separate the WD and MD anterior to growing tip. However, at the MD tip, the MD and WD are in physical contact. At around E13.0 the MD crosses over the WD to become located medially. Elongation is complete by E13.5 with the MD reaching the urogenital sinus. E, embryonic day in mouse; D, dorsal; MD: Müllerian duct; P, posterior (caudal); V, ventral; WD, Wolffian duct. Adapted from Kobayashi, A., and Behringer, R.R. (2003). Developmental Genetics of the Female Reproductive Tract in Mammals. *Nature Reviews Genetics* 4, 969–980.

a funnel-like structure. The invagination process is possibly driven by *Wnt4* expressing cells in the mesonephric mesenchyme because *Wnt4* mutant mice have *Lhx1*-specified cells but do not form the MD (Fig. 3).

As the specified MD cells move posteriorly, MD formation enters the elongation phase. The posterior tip cells of the MD, which have shown to be *Wnt4* positive, will invade through the common basal lamina between the mesonephric epithelium and the lateral side of the WD (Prunskaitė-Hyryläinen et al., 2016). Following the tip cells, the rest of the MD cells will move along the WD in an anterior to posterior manner. When the MD elongates past the middle of the WD (posterior to the gonad), the MD will elongate dorsomedially across the WD, but will remain in close contact with it. After reaching the medial side of the WD, the MD resumes its anterior-posterior elongation along the medial side of the WD. At the end of the elongation phase, the MD tip reaches the urogenital sinus and fuses (Fig. 3).

Although the cellular mechanisms of MD formation are not fully understood, recent studies have shown that both cell proliferation and migration are involved in MD elongation. Studies in both chicken and mouse have shown that the MD cells are proliferative along the entire anterior to posterior length. In addition, cell migration may play an important role during the elongation process. The tip cells extend prominent processes, suggesting that the tips cells are actively investigating their environment for MD elongation (Huang et al., 2014). PI3K/AKT activity has been shown to be required for MD cell migration and elongation in rat embryos (Mullen and Behringer, 2014). It is also possible that cell shape changes may also contribute to MD elongation.

The relatively rapid elongation of the MD during mouse development has led to speculation that cells may be contributed from neighboring tissues, such as the adjacent WD, the mesonephric mesenchyme or the mesonephric epithelium. However, recent studies show that cell contributions from neighboring tissues are not found in both chicken and mouse (Mullen and Behringer, 2014). Therefore, cell recruitment is not a major cellular mechanism that contributes to the elongation of the MD.

The MD elongates in a unique manner, i.e., tube-dependent tubulogenesis. In 1937, Grünwald found that the MD elongation is dependent on the presence of the WD (Grünwald, 1937). It was found that the *Wnt9b* mutant mouse lacked MD formation. *Wnt9b*

is expressed in the WD. Thus, WNT9B secreted from the WD is required for MD elongation, providing a molecular explanation why MD elongation is dependent upon the WD (Mullen and Behringer, 2014).

Interestingly, MD cell differentiation switches between mesenchymal and epithelial states during MD formation. In the initiation phase, the specified MD cells are considered “mesoepithelial” and invade into the mesonephric mesenchyme. The MD cells are histologically epithelial but express mesenchymal molecular markers (Mullen and Behringer, 2014). After MD elongation is completed, the MD cells in female fetuses down-regulate mesenchyme markers and up-regulate epithelial molecular markers.

Wolffian Duct Regression

In amniotes, the initial formation of the reproductive tracts of genetic male and female embryos is identical with two pairs of simple epithelial tubes, the WD and MD, surrounded by mesenchymal cells. However for proper sexual differentiation, only one of these pairs of tubes will differentiate while the other is eliminated. As discussed above, this is regulated by the presence or absence of fetal gonadal hormones. The fetal male gonad secretes androgens, causing the WD to differentiate into the mature male reproductive tract organs. In females, it is necessary to eliminate or regress the WD. The absence of androgens in female fetuses leads to the elimination or regression of the WD. In female rodents, without androgens, degeneration of the WD is observed beginning midway between the gonads and point of contact with the urogenital sinus and proceeds cranial (head) to caudal (tail). Lower, caudal segments of the WD remain and fuse with the MD and urogenital sinus to form the lower portion of the vagina (Fig. 2). The ability of androgens (from the testis) to block WD regression in females has been shown in tammar wallaby. Grafting of a testis in female tammar pouch young resulted in a block of WD regression and differentiation of the WD. Similarly, mutations in the *androgen receptor* (AR) gene in humans and rodents result in intersex phenotypes and genetically male (XY) individuals lack WD-derived tissues. Further, observations in rodent models indicate androgen signaling in the WD mesenchyme may allow cell survival and differentiation of the adjacent WD epithelial tube and lack of androgen signaling in the mesenchyme results in cell death, thus facilitating WD regression (Shaw and Renfree, 2014).

Early studies of female reproductive tract differentiation during WD regression were limited to two-dimensional analyses in animal models. Recently, light-sheet microscopy has made it possible to quickly generate high-resolution three-dimensional images of fluorescently-labeled fetal organs. Light-sheet microscopy was used to visualize the developing human female reproductive tract at GW 10.5, 11.5 and 13 weeks. The human embryos were immuno-fluorescently stained with PAX2 antibody (which binds WD and MD epithelial cells) and imaged. At GW 10.5 fusion of the MD to form the uterovaginal canal was observed in female embryos. The WD was still intact however there was initial regression of the mesonephric tubules. At GW 11.5 WD regression was apparent and the MDs had grown in length. By GW 13, the WD was fragmented and completely regressed distally (Belle et al., 2017).

WD regression has long been considered a passive process, where lack of androgens in female fetuses fails to support the differentiation of the WD. However, several recent studies suggest that WD regression requires active signaling to promote cell death of the epithelium. MSX2, a transcription factor expressed in the WD epithelium, and orphan nuclear receptor chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) found in the WD mesenchyme have both been identified as potential mediators of WD regression in female reproductive tract differentiation. Down-regulation of *Msx2* expression in the WD epithelium either in response to diethylstilbestrol (DES) exposure or in a *Msx2* mutant mouse model in females results in persistent WD remnants dorsal to the vagina and reduced apoptosis (programmed cell death) in the WD epithelium (Yin et al., 2006). COUP-TFII, a mesenchyme specific transcriptional regulator, is required for WD regression during differentiation of the female reproductive tract in the mouse. Loss of the *Coup-tfII* gene in the WD mesenchyme results in retention of the WD independent of androgen signaling. In fetal males, androgens secreted from the testis presumably antagonize COUP-TFII function and prevent WD regression (Zhao et al., 2017).

Oviduct Development

The oviduct, or Fallopian tube in women, is a paired organ that is essential for fertility. In mature animals, the oviduct is the conduit for oocyte and embryo transfer to the uterus and is the site of fertilization. The ovulated oocyte enters the oviduct through the infundibulum, which is the most anterior region of the oviduct, and travels through the ampulla, which contains numerous longitudinal epithelial folds and abundant cilia to aid in oocyte transport. Upon fertilization, the zygote will travel through the isthmus region of the oviduct. The isthmus has fewer epithelial folds and cilia than the ampulla, but thicker smooth muscle layers. To leave the oviduct, the zygote must travel through the uterotubal junction to enter into the uterine horn/body. This junction is an ovarian hormone-controlled valve that controls the movement of spermatozoa/zygotes between the oviduct and uterus.

Defects in oviduct formation or the formation of occlusions can cause infertility. This may be overcome via superovulation, in vitro fertilization and embryo transfer into the uterus, but these are costly methods with demanding hormonal regimens and relatively low success rates. Tubal occlusions are caused most frequently by infections, but structural abnormalities arising during peri-natal development can have the same result. Very little is known of how and what regulates oviduct development.

The study of Fallopian tube development in women is limited, requiring the use of other animal models including both mammals and birds. However, there are some striking differences in the gross morphology and histology of various species. Oviduct coiling is observed in some species (e.g., mice), but not others (e.g., women, sheep, chickens). A bursa surrounds the oviduct and

ovary (e.g., mice) in certain species, which is absent in others (e.g., women). Oviduct epithelial folding, particularly in the ampulla region can be minimal (e.g., mice) or very extensive (e.g., women, sheep). Despite these differences, the oviducts function in a very similar manner.

Mammalian female reproductive organs, including the oviduct, uterus, cervix, and anterior vagina, are all derived along the anterior-posterior axis of the MD during embryonic development. The most anterior aspect of the MD gives rise to the oviduct. The developing MD forms a shepherd's crook shape around the ovary. The end of the curved portion of the "crook," posterior to the ovary, is referred to as the *flexura medialis* and is proposed to define the border between the region of the MD that will become the oviduct and that of the uterus (Agduhr, 1927).

The TGF β , WNT and mTOR signaling pathways have been identified as potential regulators of oviduct development. TGF β may play a key role in controlling cell proliferation, differentiation and apoptosis during oviduct development (Conery et al., 2004; Elliott and Blobe, 2005; Li et al., 2011; Rodriguez et al., 2016). Regulation of TGF β signaling during oviduct development likely involves extracellular matrix proteins, including matrix metalloproteinases and tissue inhibitors of metalloproteinases (ex. MMP-2, -9, TIMP-2) which act via enzymatic cleavage and activation or repression of signal transducers (Hu et al., 2004; Imai et al., 1997; Lesniak-Walentyń and Hrabia, 2016).

In addition to TGF β signaling, the WNT pathway appears to play a direct role in oviduct development. Oviduct development and formation is regulated tightly by correct expression of canonical WNT signaling pathway members in both the epithelia (*Wnt7a*) and mesenchyme (*Wnt4*, *Wnt5a*, *Ctnnb1*). WNT signaling during oviduct development is associated with the appearance of coiling and initial formation of the anterior region of the MD, suggesting that this pathway plays a key role in anterior-posterior oviduct extension and differentiation.

mTOR signaling appears to play a key role in smooth muscle differentiation and function in the oviduct. mTOR signaling is downstream of PI3K/AKT signaling and regulates cell growth and proliferation in response to growth factors and nutrients and is negatively regulated by a heterodimeric complex of TSC1 and TSC2. In the mouse, conditional deletion of *Tsc1* in both the MD mesenchyme and in all MD cell types results in infertility related to oviduct hyperplasia and formation of occlusions and hydrosalpinx in the ampulla (Daikoku et al., 2013; Tanaka et al., 2012). Conditional deletion of *Tsc2* in the MD mesenchyme resulted in infertility that may be related to the formation of oviductal blockages, but oviductal histology was not reported. The uterine phenotype was characterized by the presence of myometrial hyperplasia (Kaneko-Tarui et al., 2014). It is possible that this also occurred in the oviductal smooth muscle layers, which would adversely affect oocyte/zygote transport, resulting in a phenotype similar to oviduct blockage.

Uterine Development

In eutherian mammals, the majority of the development and differentiation of the female reproductive tract is completed by birth. However, the uterus is not fully developed or differentiated by birth and the histoarchitecture of this organ is established postnatally. Postnatal radial patterning morphogenesis establishes two functional compartments, the endometrium and the myometrium, surrounded by the perimetrium. The endometrium consists of two epithelial cell types, luminal epithelium (LE) and glandular epithelium (GE), and two stratified stromal compartments including a densely organized stromal zone, blood vessels and immune cells. The myometrium includes the smooth muscle layers of the uterine wall, an inner circular layer and an outer longitudinal layer (Gray et al., 2001). Morphogenic events common to morphogenesis of the uterus include: (1) organization and stratification of the endometrial stroma, (2) differentiation and growth of the myometrium and (3) coordinated development of the endometrial glands. The LE will invaginate into the stroma to generate the GE (endometrial or uterine glands), resulting in an extensive network of glands that extends towards the myometrium (Gray et al., 2001; Spencer et al., 2005).

Humans have a simplex uterus that consists of a single uterine body. The endometrium is lined by a LE that contains glands that radiate from the surface to the endometrial-myometrial interface. The endometrium is divided into two functional layers, the upper *stratum functionalis* (containing glands and is surrounded by loose stroma) and the lower *stratum basalis* (containing branched glands and dense stroma). During menses, the endometrial *stratum functionalis* is shed. The *stratum basalis* includes a zone that contains loose stroma and endometrial glands and another zone where endometrial glands terminate and endometrial progenitor and stem cells are thought to reside (Spencer et al., 2005).

During pregnancy, uterine glands secrete histotroph that is essential for endometrial receptivity of the embryo, conceptus survival, implantation, development and growth in sheep, cattle, pigs, horses and rodents (Gray et al., 2001). Histotroph is present in the uterine luminal fluid and is a complex, undefined mixture of ions, amino acids, carbohydrates, proteins, lipids, and other substances that are selectively transported into the uterine lumen by the epithelium, as well as specific secretory products encoded by genes and expressed in the LE and GE. Evidence shown in mouse and sheep suggests that uterine glands are required for female fertility, with defects resulting in abnormal implantation and early pregnancy loss (Filant and Spencer, 2014; Spencer et al., 2005).

Knowledge of prenatal uterine development is most complete in rodents. However, the basic biology of this process is assumed to be similar across mammalian species and the morphogenesis of the postnatal uterus is dependent on the maturity of the uterus at birth (e.g., gestational length) and perhaps the interval between birth and puberty (Gray et al., 2001). For example, in rodents, at birth, the uterus has not yet differentiated into endometrial stroma and myometrium, whereas in certain domestic animals and humans, the endometrial stroma and myometrium are present at birth (Spencer et al., 2005).

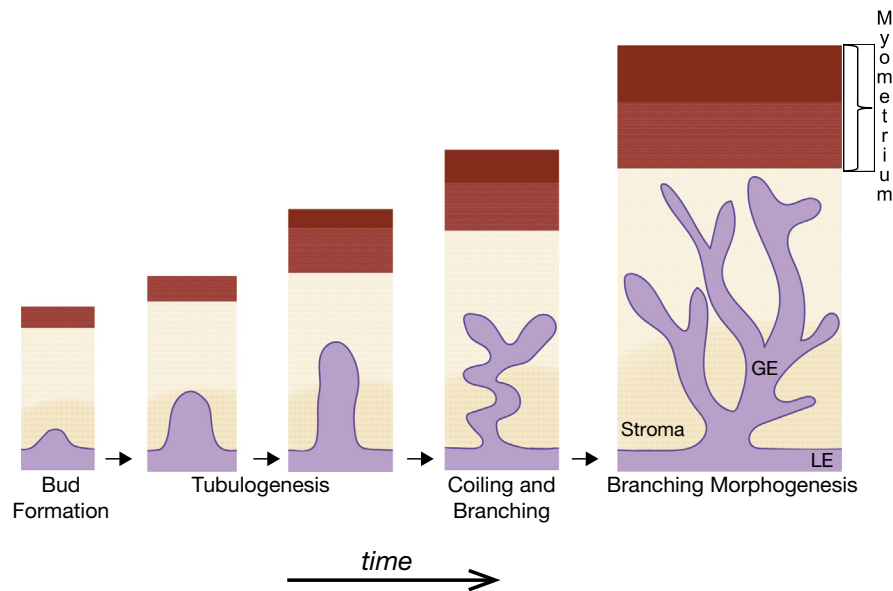


Fig. 4 Schematic illustration of endometrial adenogenesis in the mouse uterus. The uterus consists of two epithelial cell types (purple), the luminal epithelium (LE) and glandular epithelium (GE). The myometrium is composed of two smooth muscle layers: the inner circular layer (pink) and outer longitudinal layer (dark red). Stages of adenogenesis are indicated. GE, glandular epithelium; LE, luminal epithelium.

Uterine adenogenesis is the process of endometrial gland formation from the LE. It includes epithelial budding, extension and penetration into the stroma with coiling and branching. In humans, rodents and livestock, this process is completed postnatally (Gray et al., 2001). In mice, at birth, the uterus is comprised of a simple epithelium surrounded by undifferentiated mesenchyme with no endometrial glands. At Postnatal Day (P) 5, three mesenchymal layers are radially oriented and segregated into the endometrial stroma and inner circular and prospective outer longitudinal myometrial layers and the formation of epithelial buds by epithelial invaginations. Between P9 to P15, simple tubular glands develop that are not tightly coiled or branched (Fig. 4). By P10, the outer longitudinal layer of the myometrium becomes organized into bundles. At P15, the adult configuration of the mouse uterus is already established and as females mature, the glands lengthen as the uterus grows (Gray et al., 2001). P21 marks the end of the postnatal stage of gland formation. Many of these studies were performed using two-dimensional histological analyses. Recently, the three-dimensional morphology and organization of adult uterine glands has been examined (Arora et al., 2016).

Knowledge of prenatal and postnatal female reproductive tract development in humans is limited. By GW 12, the uterine corpus and cervix is has formed and the LE invaginates to give rise to epithelial buds. By GW 20–22, the myometrium is well defined but endometrial gland development has not progressed beyond epithelial buds. At birth, the uterine histoarchitecture is similar to that of an adult, but less developed. From birth to the onset of puberty, the glands develop slowly. A female at 6 years of age will have endometrial glands that will extend from one-third to one-half of the distance of the stroma to the myometrium. The mature uterine histoarchitecture is observed at puberty with glands extending to the inner circular layer of the myometrium. Endometrial gland formation in humans (fetus and neonate) involves differentiation of the GE from the LE, followed by radial development of the tubular glands through the endometrial stroma extending to the myometrium.

Multiple studies have established that prenatal urogenital tract development in female mammals is an ovary (hormonal) independent process (Gray et al., 2001). These studies have shown that uterine development and endometrial adenogenesis can proceed in the absence of the ovary for varying periods of time during early postnatal development. In rats, circulating estrogens increase between P9 and P11 in association with gland remodeling, but early postnatal uterine development and adenogenesis are both ovary- and adrenal-independent (Gray et al., 2001; Spencer et al., 2005). In mice, the introduction of hormones during a critical postnatal window causes a delay in gland formation or the loss of glands (Filant and Spencer, 2014).

Gland morphogenesis is highly complex and mediated by diverse mechanisms (hormonal, cellular and molecular). Despite being studied for decades, very little details are available, compared with other epitheliomesenchymal organs. The communication between the epithelium and stroma appears to be mediated by *Wnt* and *Hox* genes, intrinsic growth factors systems and the extracellular matrix (Spencer et al., 2005). In recent years, knockout (*Hoxa10*, *Hoxa11*, *Lef1*, *Wnt4*, *Wnt5a*) and conditional knockout (*Ctnnb1*, *Foxa2*, *Wnt7a*) mutants mouse models have been used to identify genes involved in uterine gland development (Filant and Spencer, 2014). Although some cellular events and molecular pathways have been identified through gene expression and mouse models, there is still a significant gap in knowledge of how glands develop and their morphogenesis.

Malformations of the Uterus

Uterine malformations can be classified into three main groups, (1) formation defects, (2) fusion defects, and (3) septal absorption defects (Jacquinet et al., 2016). The actual prevalence of uterine malformations has been difficult to evaluate because some defects may be considered normal variants of uterine anatomy, for example, arcuate uterus. Chan et al. (2011) reported a 5.5% prevalence of uterine malformations in an unselected population, 8.0% in infertile women, 13.3% in women with a history of miscarriage, and 24.5% in patients with a history of miscarriage and infertility. This lead to the conclusion that women who are infertile and/or have had spontaneous abortions are more likely to have a uterine malformation (Chan et al., 2011).

Mayer-Rokitansky-Küster-Hauser (MRKH) syndrome or Müllerian agenesis is characterized by the absence of the uterus, cervix and upper portion of the vagina in a 46,XX female; it is the second most common cause of primary amenorrhea (Fontana et al., 2017). It is divided into two main subtypes: (1) MRKH type 1 in which only the female reproductive tract is affected and (2) MRKH type 2 which can manifest with malformations of other organs systems such as: renal, skeletal (spine and limb) and less frequently auditory and heart defects. Even though MRKH is most severe in the spectrum of uterine defects, its incidence is relatively low, with only 1 in every 4500–5000 newborn females being affected. However, the association of MRKH type 2 with other organ system defects suggests that abnormal MD development involves the disruption of developmental pathways important for structures derived from the intermediate mesoderm of the embryo (Fontana et al., 2017).

The cause of uterine malformations is thought to be multifactorial and in the case of MRKH, the mode of transmission is thought to be autosomal dominant with incomplete penetrance and variable expressivity. First-degree relatives of patients presenting with a uterine anomaly are said to have a 1%–5% recurrence risk. There are reports of familial cases suggesting a predisposing genetic background. Conversely, there have been studies of monozygotic twins that show discordant phenotypes: MRKH vs. normal uterine anatomy, suggesting nongenetic mechanisms that point towards epigenetic and/or environmental factors (Jacquinet et al., 2016).

Relatively little is known about the genetic pathways that regulate the development of the female reproductive tract and lead to uterine malformations in humans. However mutation or deletion of certain genes have been found to be associated with reproductive tract defects in humans including: *EMX2*, *HNF1 β* , *LHX1*, *PBX1*, *WNT4*, *WNT7A*, and *WNT9B*. In patients with MRKH syndrome, a rare pathogenic deletion in region 17q12 containing *LHX1*, as well as *HNF1 β* , has been found to be statistically significant compared to a control population (Jacquinet et al., 2016). Mutations in *HNF1 β* are the cause of a form of maturity-onset diabetes of the young type 5 (MODY5). MODY5 clinically manifests with diabetes, renal disease and genital malformations (MRKH syndrome). Mutations in *HNF1 β* have only been found in patients with both renal and uterine malformations, and are rare in cases of isolated uterine defects (Fontana et al., 2017). Recently, in a case control study of 517 Chinese women with incomplete Müllerian fusion, a novel nonsense mutation in the *EMX2* gene (p.E142X) was detected in one patient (0.19%). The authors report functional studies in cultured cells, suggesting a dominant negative effect of the mutation (Jacquinet et al., 2016). Even though this mutation is uncommon in the studied population, *EMX2* is the first gene to be identified suggestive of a cause for an isolated uterine malformation (Jacquinet et al., 2016). An association study performed in a Chinese Han female population with MRKH found two susceptibility SNPs (single nucleotide polymorphism) in *WNT9B* and *PBX1* associated with MRKH syndrome risk (Ma et al., 2015). In humans, *WNT4* was the first gene to be associated with uterine defects accompanied by hyperandrogenism (Fontana et al., 2017). *WNT4* mutations are more commonly associated to an MRKH-like syndrome because of the concomitant virilization. *WNT7A* mutations have been linked to Al-Awadi/Raas-Rothschild and Fuhrmann syndromes which are characterized by skeletal dysplasia, hypoplastic pelvis and females may present with an absent uterus (Jacquinet et al., 2016).

Prenatal exposure of fetuses to endocrine disruptors can affect the development of the uterus in mice and humans. Diethylstilbestrol (DES) is a synthetic estrogen that was used from 1938 to 1971 to prevent miscarriages in millions of pregnant women. However, it was later discovered that prenatal and perinatal exposure to DES disturbs the development of the reproductive tract in both humans (males and females) and mice (Spencer et al., 2005). Prenatal exposure of human fetuses to DES alters the organizational program of the female reproductive tract tissues and disrupts the normal expression or function of genes in an epigenetic manner. These induced abnormalities have set the stage for infertility, cervicovaginal cancer and other complications in exposed females and their offspring in a transgenerational manner.

References

- Agduhr, E. (1927). Studies on the structure and development of the bursa ovarica and the tuba uterina in the mouse. *Acta Zoologica*, 8, 1–133.
- Arora, R., Fries, A., Oelerich, K., Marchuk, K., Sabeur, K., Giudice, L. C., & Laird, D. J. (2016). Insights from imaging the implanting embryo and the uterine environment in three dimensions. *Development*, 143, 4749–4754.
- Atsuta, Y., & Takahashi, Y. (2016). Early formation of the Mullerian duct is regulated by sequential actions of BMP/Pax2 and FGF/Lim1 signaling. *Development*, 143, 3549–3559.
- Belle, M., Godefroy, D., Couly, G., Malone, S. A., Collier, F., Giacobini, P., & Chedotal, A. (2017). Tridimensional visualization and analysis of early human development. *Cell*, 169, 161–173 e112.
- Chan, Y. Y., Jayaprakasan, K., Zamora, J., Thornton, J. G., Raine-Fenning, N., & Coomarasamy, A. (2011). The prevalence of congenital uterine anomalies in unselected and high-risk populations: a systematic review. *Human Reproduction Update*, 17, 761–771.
- Conery, A. R., Cao, Y., Thompson, E. A., Townsend, C. M., Jr., Ko, T. C., & Luo, K. (2004). Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nature Cell Biology*, 6, 366–372.
- Daikoku, T., Yoshie, M., Xie, H., Sun, X., Cha, J., Ellenson, L. H., & Dey, S. K. (2013). Conditional deletion of Tsc1 in the female reproductive tract impedes normal oviductal and uterine function by enhancing mTORC1 signaling in mice. *Molecular Human Reproduction*, 19, 463–472.
- Elliott, R. L., & Blobe, G. C. (2005). Role of transforming growth factor Beta in human cancer. *Journal of Clinical Oncology*, 23, 2078–2093.

- Filant, J., & Spencer, T. E. (2014). Uterine glands: Biological roles in conceptus implantation, uterine receptivity and decidualization. *The International Journal of Developmental Biology*, 58, 107–116.
- Fontana, L., Gentilin, B., Fedele, L., Gervasini, C., & Miozzo, M. (2017). Genetics of Mayer-Rokitansky-Kuster-Hauser (MRKH) syndrome. *Clinical Genetics*, 91, 233–246.
- Gray, C. A., Bartol, F. F., Tarleton, B. J., Wiley, A. A., Johnson, G. A., Bazer, F. W., & Spencer, T. E. (2001). Developmental biology of uterine glands. *Biology of Reproduction*, 65, 1311–1323.
- Grünwald, P. (1937). Zur Entwicklungsmechanik des Urogenitalsystems beim Huhn. *Wilhelm Roux Archiv für Entwicklungsmechanik der Organismen*, 136, 786–813.
- Hu, J., Zhang, X., Nothnick, W. B., & Spencer, T. E. (2004). Matrix metalloproteinases and their tissue inhibitors in the developing neonatal mouse uterus. *Biology of Reproduction*, 71, 1598–1604.
- Huang, C. C., Orvis, G. D., Kwan, K. M., & Behringer, R. R. (2014). Lhx1 is required in Müllerian duct epithelium for uterine development. *Developmental Biology*, 389, 124–136.
- Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D., & Okada, Y. (1997). Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. *The Biochemical Journal*, 322(Pt. 3), 809–814.
- Jacquinet, A., Millar, D., & Lehman, A. (2016). Etiologies of uterine malformations. *American Journal of Medical Genetics. Part A*, 170, 2141–2172.
- Kaneko-Tarui, T., Commandeur, A. E., Patterson, A. L., DeKuiper, J. L., Petillo, D., Styer, A. K., & Teixeira, J. M. (2014). Hyperplasia and fibrosis in mice with conditional loss of the TSC2 tumor suppressor in Mullerian duct mesenchyme-derived myometria. *Molecular Human Reproduction*, 20, 1126–1134.
- Kobayashi, A., & Behringer, R. R. (2003). Developmental genetics of the female reproductive tract in mammals. *Nature Reviews. Genetics*, 4, 969–980.
- Lesniak-Walentyn, A., & Hrabia, A. (2016). Expression and localization of matrix metalloproteinases (MMP-2, -7, -9) and their tissue inhibitors (TIMP-2, -3) in the chicken oviduct during maturation. *Cell and Tissue Research*, 364, 185–197.
- Li, Q., Agno, J. E., Edson, M. A., Nagaraja, A. K., Nagashima, T., & Matzuk, M. M. (2011). Transforming growth factor beta receptor type 1 is essential for female reproductive tract integrity and function. *PLoS Genetics*, 7, e1002320.
- Ma, W., Li, Y., Wang, M., Li, H., Su, T., Li, Y., & Wang, S. (2015). Associations of polymorphisms in WNT9B and PBX1 with Mayer-Rokitansky-Kuster-Hauser syndrome in Chinese Han. *PLoS One*, 10, e0130202.
- Mullen, R. D., & Behringer, R. R. (2014). Molecular genetics of Mullerian duct formation, regression and differentiation. *Sexual Development*, 8, 281–296.
- Prunskaitė-Hyyryläinen, R., Skovorodkin, I., Xu, Q., Miinalainen, I., Shan, J., & Vainio, S. J. (2016). Wnt4 coordinates directional cell migration and extension of the Mullerian duct essential for ontogenesis of the female reproductive tract. *Human Molecular Genetics*, 25, 1059–1073.
- Renfree, M. B., O, W. S., Short, R. V., & Shaw, G. (1996). Sexual differentiation of the urogenital system of the fetal and neonatal tammar wallaby, *Macropus eugenii*. *Anatomy and embryology*, 194, 111–134.
- Rodriguez, A., Tripurani, S. K., Burton, J. C., Clementi, C., Larina, I., & Pangas, S. A. (2016). SMAD signaling is required for structural integrity of the female reproductive tract and uterine function during early pregnancy in mice. *Biology of Reproduction*, 95, 44.
- Shaw, G., & Renfree, M. B. (2014). Wolffian duct development. *Sexual Development*, 8, 273–280.
- Spencer, T. E., Hayashi, K., Hu, J., & Carpenter, K. D. (2005). Comparative developmental biology of the mammalian uterus. *Current Topics in Developmental Biology*, 68, 85–122.
- Tanaka, Y., Park, J. H., Tanwar, P. S., Kaneko-Tarui, T., Mittal, S., Lee, H. J., & Teixeira, J. M. (2012). Deletion of tuberous sclerosis 1 in somatic cells of the murine reproductive tract causes female infertility. *Endocrinology*, 153, 404–416.
- Yin, Y., Lin, C., & Ma, L. (2006). MSX2 promotes vaginal epithelial differentiation and wolffian duct regression and dampens the vaginal response to diethylstilbestrol. *Molecular Endocrinology*, 20, 1535–1546.
- Zhao, F., Franco, H. L., Rodriguez, K. F., Brown, P. R., Tsai, M. J., Tsai, S. Y., & Yao, H. H. (2017). Elimination of the male reproductive tract in the female embryo is promoted by COUP-TFII in mice. *Science*, 357, 717–720.

Sperm Transport and Selection in Mammals

William V Holt, The University of Sheffield, Sheffield, United Kingdom

Alireza Fazeli, University of Tartu, Tartu, Estonia; and The University of Sheffield, Sheffield, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chemokines A family of small cytokines or signaling proteins secreted by cells. Their name is derived from their ability to induce directed chemotaxis in nearby responsive cells.

Cryptic female choice A form of mate choice which occurs both in pre- and postcopulatory circumstances when females in certain species use physical or chemical mechanisms to influence a male's reproductive success.

Glycome Strictly speaking this is the entire complement of sugars, whether free or present in more complex molecules, of an organism. In the context of this article it refers specifically to the sugars present on the outer surfaces of the cell.

microRNA A microRNA (abbreviated miRNA) is a small noncoding RNA molecule (containing about 22 nucleotides) found in plants, animals, and some viruses, that functions in RNA silencing and posttranscriptional regulation of gene expression.

Polyspermy Polyspermy occurs when two or more spermatozoa enter the cytoplasm of a single oocyte. In mammals this usually leads to abnormal fertilization and embryonic death, but in some other taxonomic groups (e.g., amphibians) it is perfectly normal.

Sperm competition This is the competitive process that results when spermatozoa from two or more different males strive to fertilize the same cohort of egg(s) during sexual reproduction.

Utero-tubal junction A narrow and tortuous region of the female reproductive tract that links the uterus to the oviduct.

Introduction

Why do most vertebrates produce and ejaculate far more spermatozoa than the numbers they actually need for fertilization? For species with external fertilization (many fishes and amphibians) a simple answer is that when spermatozoa are delivered into the watery environment near to the egg most will be lost and only a minority will find their way towards the egg. The situation for species with internal fertilization is different. With a few exceptions such as the Viscacha (*Lagostomus maximus*), a South American rodent that ovulates about 900 eggs at once (Weir, 1971), females of internally fertilizing species usually ovulate small numbers of eggs, and it seems logical that they should only need similarly small sperm numbers to assure fertilization success. However, it is clear that the logical scenario is wildly unrealistic because males produce billions of spermatozoa every day. In the early 1970s Parker (1970) proposed that because females frequently accept matings by more than one male, rivalry between males, and between their ejaculates would lead to "sperm competition." In this scenario, ability to produce and ejaculate larger numbers of spermatozoa than competing males represents an advantage in terms of breeding success. A detailed review of the massive body of research into sperm competition and "cryptic female choice," a complementary mechanism whereby the females exhibit a high degree of postcopulatory sperm selection, is beyond the scope of this article (for reviews, see Birkhead, 1998; Orr and Zuk, 2014). Nevertheless, cryptic female choice (between males) is a reproductive mechanism that somehow involves the assessment of sperm quality, including their genetic complement, thereby favoring some males at the expense of others. As such it represents a powerful influence in terms of sexual selection and evolution. However, it is increasingly clear that the female reproductive tract distinguishes between the individual spermatozoa within a single ejaculate. Recent studies have shown that there are numerous molecular differences between individual spermatozoa, which are able to bias the genetic outcomes of mating and fertilization. Sperm transport and selection are therefore now seen as highly complex and sophisticated mechanisms in which every anatomical region of the female reproductive tract has a distinct role. The subtlety underlying this statement is well illustrated in a conclusion reached by Rodriguez-Martinez et al. (2016) in a study of hyaluronan function, where its role within the deep folds and crevices of the oviduct is not necessarily the same as its role within the lumen:

Both hyaluronan and its CD44 receptor are particularly evident in the deep mucosal furrows of the sperm reservoir, in which most spermatozoa are embedded in; kept alive, uncapacitated but also undetected by the immune system of the female.

Sperm Transport in Mammals

After copulation, spermatozoa in the mammalian ejaculate make their way through the female reproductive tract and eventually reach the vicinity of the ovulated eggs within the oviduct. This journey, known generally as "sperm transport," involves several physiological processes acting in concert. Ejaculation itself forces spermatozoa to interact directly with the mucus produced by

the cervix. In many species, including humans (Yudin et al., 1989), cervical mucus consists of a fine network of microscopic fibers and the spermatozoa have to penetrate and pass through this mesh-like network powered by their own motility in order to reach the uterus. Thus the cervical mucus represents an initial selective barrier for spermatozoa, and many with inadequate motility cannot make any further progress. Transport of those spermatozoa that reach the uterus is assisted by muscle contractions, pushing the spermatozoa towards the next major barrier; the utero-tubal junction (UTJ). This is a narrow and tortuous, mucus-filled, tubular structure that controls access to the oviducts (also known as the Fallopian tubes) and, in fact, prevents the entry of most spermatozoa. It is now known that the UTJ is another important barrier, blocking the progress of many spermatozoa towards the oocyte and ensuring that only a small and privileged subpopulation of spermatozoa are ever allowed even to approach oocytes. Understanding sperm transport, and especially the key factors which distinguish the “privileged” sperm subpopulation from all the others, is extremely important in many different aspects of reproductive biology. From an evolutionary or genetic viewpoint we might ask whether the female reproductive tract is only permitting the progress of spermatozoa that fulfill specific criteria? It is known, for example, that mate choice in species from fishes to humans is driven, among other factors, by efforts to match male and female genetic criteria such as the major histocompatibility complex (MHC) (Burger et al., 2017; Wedekind and Furi, 1997; Wintemitz et al., 2017; Ziegler et al., 2002) and it is likely that similar processes are involved in postcopulatory sperm selection.

The development of artificial insemination (AI) technology between the 1950s and 1980s led to some extensive studies of sperm transport within the female reproductive tract being undertaken in cattle, pigs, and sheep. These studies typically involved the experimental insemination of oestrous females with known doses of semen; the females were slaughtered at intervals after insemination, the reproductive tracts flushed, and the different anatomical regions examined for the presence of spermatozoa. Data from studies in cattle and sheep revealed that only about 1%–3% of the total number of uterine spermatozoa reached the site of fertilization, while in pigs the reduction in sperm numbers was much greater (less than one thousandth of the inseminated population) (First et al., 1968). Interestingly First et al. (1968) estimated that 83,000 spermatozoa had reached the oviducts after about 4 h, and that this value remained constant over the ensuing 24 h period. This figure is surprisingly close to the mean of 79,055 spermatozoa detected by Mburu et al. (1996) when preovulatory inseminations were performed. In this study the majority of the oviductal spermatozoa (75,293; >96% of the total) were located at the UTJ, and the remaining 4% of spermatozoa (3132) were situated within the oviductal isthmus. The oviductal isthmus represents the initial anatomical region of the oviduct and is characterized by a narrow lumen lined by columnar epithelial cells.

The results obtained in agricultural species agreed with observations that hamster and rabbit spermatozoa accumulate in the oviductal isthmus 1 or 2 h after mating (Overstreet and Cooper, 1975; Yanagimachi and Chang, 1963), and remain there until the onset of ovulation. Direct video imaging of sperm behavior within the oviducts (Demott and Suarez, 1992; Kölle et al., 2010; Suarez, 1987) confirmed many of these observations and led to the recognition that the oviductal isthmus functions as a specialized storage reservoir, in which the spermatozoa are able to survive for varying durations prior to ovulation. Observations of spermatozoa taken from the isthmus reservoir of several species have consistently shown that their motility is suppressed (see, for example, Burkman et al., 1984; Overstreet and Cooper, 1975) and motility suppression has also been observed in spermatozoa exposed to oviductal fluid.

When a suspension of mammalian spermatozoa is placed on a slide and viewed under the microscope, the cells appear to move in random directions. However, it is important to recognize that this type of movement is not representative of the situation pertaining within the reproductive tract. There is convincing evidence that sperm behavior is markedly influenced by the physical and chemical characteristics of their local environment. Ovulation stimulates alterations to the way that fluids flow within the female reproductive tract, and it has recently been shown that spermatozoa sense these changes and use them to navigate their way towards the oocytes (Miki and Clapham, 2013). This is not, however, the only sperm guidance system in operation; it is now known that capacitated spermatozoa are sensitive to minute concentration gradients of chemoattractants within the oviduct and use these for chemotactic guidance in their progress towards the oocytes (Bian et al., 2012; Guidobaldi et al., 2012; Perez-Cerezales et al., 2015). Temperature gradients are also known to exist within the oviduct (Hunter, 2012) and it has been proposed that spermatozoa therefore can also employ thermotaxis for guidance towards the oocytes.

Investigations of chemotactic mechanisms that guide spermatozoa towards the oocyte(s) have identified progesterone as a significant molecular signal that modifies sperm flagellar activity (Armon and Eisenbach, 2011; Blengini et al., 2011; Guidobaldi et al., 2012; Oren-Benaroya et al., 2008; Teves et al., 2009). The action of progesterone is elicited via its receptor, Catsper (Arnoult et al., 2011; Lishko et al., 2011; Strunker et al., 2011), and the consequent modulation of intracellular calcium concentrations that change flagellar activity (Kaupp et al., 2008). Two recent studies (Caballero-Campo et al., 2014; Zuccarello et al., 2011) have demonstrated that sperm chemotaxis is not only modulated by progesterone but also via chemokine-receptor interactions involving factors produced by oocytes, granulosa cells, and endometrial cells. One of the studies (Zuccarello et al., 2011) focused on the interaction between CXCR4 (chemokine CXC motif receptor 4) present in human spermatozoa and SDF1 (chemokine stromal cell-derived factor-1), a member of the CXC chemokine family also known as CXCL12. It is of specific interest here that <30% of live human spermatozoa express CXCR4 (Kim et al., 1999; Zuccarello et al., 2011), meaning that approximately 70% of spermatozoa would be unresponsive to chemical signals emanating from granulosa cells and oocytes. Caballero-Campo et al. (2014), who identified CCR6, a chemokine receptor common to several chemoattractant peptides (Yang et al., 1999), in human and mouse spermatozoa, noted that CCR6 expression on the sperm surface was more intense after capacitation and also noted that the protein was not detected in every cell. That chemotaxis is stimulated by several different mechanisms is something of a puzzle: do the separate mechanisms operate synergistically or do they perhaps indicate the existence of an exceedingly sensitive and discriminatory sperm selection system?

What Are the Mechanisms Behind Sperm Storage in the Female Reproductive Tract?

Although the duration of sperm storage in mammals is usually measured in hours, in some mammals such as the dog (England et al., 2006) the storage period may last several days. This reflects the unusual situation in canids whereby the oocytes are ovulated as primary oocytes. These canine oocytes are not capable of being fertilized until about 60 h after ovulation; this explains why the presence of capacitated spermatozoa near oocytes is not required until some hours after ovulation. It is also worth noting that a number of bat species have the ability to store spermatozoa in the female reproductive tract for several months (for review, see Holt and Fazeli, 2016). Although not the focus of this article, it is worth noting that many species, especially the nonmammalian vertebrates such as birds and reptiles, store spermatozoa within the female tract for weeks, months, or even years. This adaptation enables them to mate and receive spermatozoa, but then to delay the processes involved in ovulation and embryo development until a more opportune time.

The mechanisms responsible for prolonging sperm survival in the female reproductive tract involve interactions between the sperm heads and the surface of the oviductal epithelium. Experimentally, coculture of spermatozoa with oviductal epithelial cells (OEC) has repeatedly been shown to prolong sperm viability across a variety of mammalian species (Dubuc and Sirard, 1995; Ellington et al., 1998; Sidhu et al., 1998; Thomas et al., 1995; Yeung et al., 1994). The formation of intimate associations between spermatozoa and the oviductal epithelial surface suggests that direct membrane–membrane contact is important. In fact, if direct contact is prevented, the beneficial effects of coculture are also prevented (Yeste et al., 2009). Intermembrane contact stimulates mutual cell signaling dialogues between the spermatozoa and oviductal epithelial cells, with resultant effects on the activity of both cell types. Murine and porcine oviductal epithelial cells are known to respond to the presence of spermatozoa in vivo by modulating gene transcription and protein synthesis (Fazeli et al., 2004; Yeste et al., 2014), while (boar) spermatozoa respond to the presence of oviductal proteins with reduced motility and an accelerated increase in intracellular pH (Satake et al., 2006).

The observation that exposure to oviductal proteins causes an increase in intracellular pH is of interest because it is not correlated with increases in capacitation and the acrosome reaction, both of which are processes that precede sperm–egg interactions and facilitate fertilization (Gadella and Luna, 2014). In fact, the oviductal proteins inhibit the completion of these processes and maintain the spermatozoa in a quiescent and stable state, while simultaneously preparing them for fertilization as soon as conditions are appropriate (Maillo et al., 2016; Rodriguez-Martinez et al., 2016) (see Fig. 1).

Several investigations of prolonged sperm survival have shown that membrane fractions and protein extracts isolated from OEC can support sperm viability in vitro. Efforts to identify a subset of OEC membrane proteins that bound specifically to the surface of boar spermatozoa demonstrated the presence, among others, of a group of heat shock proteins (Hsp70 kDa 1A, Hsp90, Hspa8 (formerly known as Hsc70) and glucose regulated protein 78 (GRP78)) (Lachance et al., 2007). Other studies that focused on bovine sperm–OEC interactions also identified GRP78 and Hsp60 for their sperm binding ability (Boilard et al., 2004). Subsequent experiments on in vitro sperm survival in the presence of recombinant Hspa8 showed convincingly that this protein could extend the lifespan of boar, bull, and ram spermatozoa under culture conditions and even when added to sperm extenders (Elliott et al., 2009; Lloyd et al., 2008, 2009). Hspa8 has been shown to exert its effects immediately upon contact with the sperm plasma membrane by increasing membrane fluidity as measured by fluorescent recovery and photobleaching (Moein-Vaziri et al., 2014). In this study the membrane fluidization and protective effects were shown to be (a) dependent on the presence of sperm plasma membrane cholesterol, (b) to be reduced by incubation in a capacitating environment, and (c) to be reversibly restored

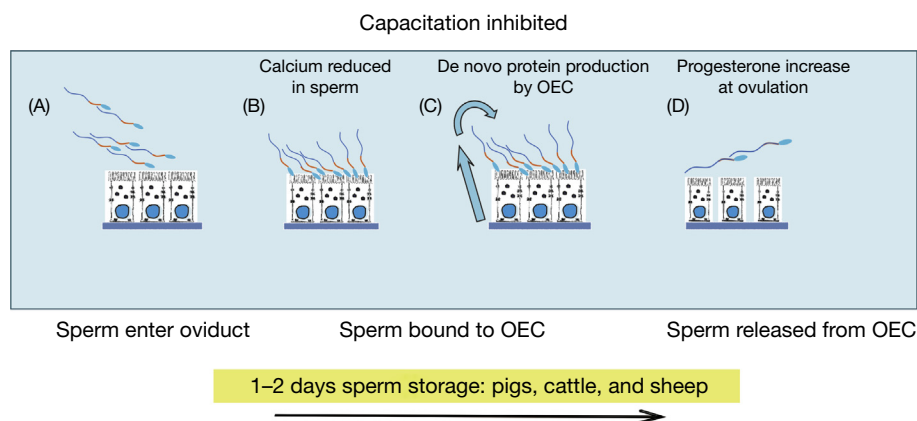


Fig. 1 Diagram summarizing the main events that occur during oviductal sperm storage in pigs, cattle, and sheep. (A) Spermatozoa enter the oviduct isthmus via the utero-tubal junction. (B) Some spermatozoa bind to oviductal epithelial cells (OEC) surfaces, whereupon their motility and intracellular calcium concentrations are reduced; capacitation is also inhibited. (C) The direct contact between spermatozoa and epithelial cells induces de novo gene expression and protein synthesis. Multiple proteins, including heat shock proteins, are secreted into the oviductal lumen where they protect sperm membranes and facilitate sperm storage. (D) Increased periovulatory progesterone production induces spermatozoa to escape and resume their progress towards the oocyte(s). Reproduced with permission from Holt, W. V. and Fazeli, A. (2016). *Annual Review of Animal Biosciences* 4, 291–310.

by depleting and then replenishing membrane cholesterol through incubating spermatozoa with cholesterol-loaded cyclodextrins. In addition, sperm exposure to hspA8 induced a decrease in mitochondrial activity. The heat shock proteins are multifunctional molecules that occur in multiple taxonomic groups and species, including bacteria, plants, and higher vertebrates (Liao and Tang, 2014; Liu et al., 2012). Moreover, HspA8 is an exceptionally well conserved member of the Hsp70 family.

What Governs Sperm Selection in the Female Reproductive Tract?

What cues are available to the female reproductive tract that enable sperm selection? These probably include responses to external signaling molecules, the presence of sperm surface markers such as ubiquitin (Sutovsky et al., 2001), and the complex arrays of carbohydrates that constitute the “glycome” (Kadirvel et al., 2012; Kuo et al., 2009; Pang et al., 2009; Silva et al., 2014). In addition, there is emerging evidence that each individual spermatozoon contains its own population of microRNAs (Curry et al., 2011; Das et al., 2013; Salas-Huetos et al., 2014). These are RNA sequences of about 20 bases (Hausser and Zavolan, 2014), which are known to be powerful modulators of gene expression. As the various microRNA species adopt unique three-dimensional shapes they are important candidates in any mechanism that involves selection. Little information is currently available about their role in sperm selection *in vivo*, but evidence from clinical studies supports the hypothesis that microRNAs can make the difference between successful and unsuccessful infertility treatment (Garcia-Herrero et al., 2011; Garrido et al., 2009, 2013). Correlations between the fertility of artificially inseminated bull semen and the nature of its microRNA content have also been demonstrated (Kasimanickam et al., 2012).

As mentioned above, the cervix and UTJ present spermatozoa with physical, chemical, and anatomical barriers that control their progress (Martyn et al., 2014; Yaniz et al., 2014). Blind-ended cervical crypts can block sperm transport, but may also allow live spermatozoa to be stored for several days before continuing. Because the physical nature of cervical mucus is under hormonal control during the reproductive cycle, it can both facilitate and inhibit sperm transport towards the uterus and oviduct. In human clinical medicine the “post-coital” test has been widely used to test for pathological incompatibility between spermatozoa and mucus (Barratt et al., 1992), which can be a cause of infertility.

Paradoxically, if the female reproductive tract allowed too many spermatozoa to reach the oviduct there would be a heightened risk of polyspermy (where individual oocytes are simultaneously penetrated by two or more spermatozoa) and embryonic death would ensue. The female reproductive tract has consequently developed at least three mechanisms for the prevention of polyspermy. These involve (1) selectively preventing sperm passage through the UTJ unless spermatozoa express an appropriate suite of proteins on their surface (calmegin, calreticulin, ADAM1a, 2, 3, and angiotensin converting enzyme, among others) (Cho et al., 1998; Ikawa et al., 2001, 2011; Shen et al., 2013); (2) hardening the zona pellucida through the action of oviductal proteins (e.g., oviduct-specific glycoprotein and heparin-like glycosaminoglycans), making it resistant to hydrolytic enzymes and turning it into a selective barrier that impedes the progress of spermatozoa towards the oocyte plasma membrane (Coy and Avilés, 2010; Coy et al., 2008); and (3) deploying intracytoplasmic hydrolytic enzymes sequestered in cortical granules situated beneath the oocyte plasma membrane to harden the zona pellucida still further after entry of the first, fertilizing, spermatozoon (Gadella and Evans, 2011; Puppo et al., 2008).

Ensuring that sufficient competent spermatozoa reach the vicinity of the egg, while simultaneously restricting the numbers and preventing polyspermy, requires delicate balancing mechanisms, but also provides scope for females to exercise a degree of choice. Compelling evidence for the existence of mechanisms that provide such choice has been provided by the use of heterospermic artificial insemination (HI). Highly controlled experiments conducted mainly on farm animals have demonstrated unequivocally that the female reproductive tract can significantly skew the outcome of AIs carried out by inseminating females with balanced sperm numbers taken from two or more males (for reviews, see Dziuk, 1996; Robl and Dziuk, 1988). This approach to relative fertility estimation eliminates female effects, such as the timing of insemination relative to ovulation and also eliminates the influence of relative sperm numbers. In some studies the skewed fertilization rates are as high as 97% in favor of one semen sample over another (Kasimanickam et al., 2006). In this study of bovine fertility, total progressive motility was positively correlated with the degree of skew but none of the other usual measures of sperm quality was significantly correlated. In similar vein, an HI study carried out with semen from two boars (Stahlberg et al., 2000) found a 70%–30% ratio of embryo paternity (95 embryos from 11 females were tested for paternity). When the same semen samples were used for homospermic inseminations there was no difference in the fertilization rates. Evidence of this nature implies that sperm selection *in vivo* is based on a complex molecular dialogue between the spermatozoa and the female reproductive tract.

In an effort to explain the mechanism responsible for the skewed fertility rates in cattle, Kasimanickam et al. (2006) found that the extent of skew was highly and negatively correlated with the DNA fragmentation rate in the semen samples (%DFI; $r = -0.87$; $P < 0.005$) while plasma membrane integrity (%PMI) was positively correlated ($r = 0.87$; $P < 0.005$) with the extent of skew. This study is of interest because it highlights the possibility that the female reproductive tract rejects membrane-damaged spermatozoa, but is then able to discriminate between the remaining intact cells on the basis of their DNA integrity. If this is true, it implies that membrane-intact spermatozoa somehow express membrane surface information about their DNA-status. Evidence that human cervical mucus acts as a filter capable of reducing the proportion of spermatozoa carrying fragmented DNA (Bianchi et al., 2004) supports the idea that the sperm surface mirrors sperm DNA status.

Hyaluronic acid has been also been found to assist with the formation of the oviductal sperm reservoir (Liberda et al., 2006; Rodriguez-Martinez et al., 2001). Once the spermatozoa gain access to the oviductal isthmus they exert some control over their

own environment. This was first noted by Ellington et al. (1993), who demonstrated that if cultured OEC are coincubated with spermatozoa, the epithelial cells respond by the de novo synthesis of proteins. Several follow-up studies in mice and pigs (Fazeli et al., 2004; Georgiou et al., 2007; Yeste et al., 2014) have lent support to these findings (for review, see Holt and Fazeli, 2010).

Conclusions

The term “sperm transport” understates the importance and complexity of the in vivo processes that ensue after ejaculation and culminate in fertilization. In fact, it could be argued that research conducted over the last two or three decades has revolutionized our view of sperm transport. Prior to this period, the female reproductive tract was largely regarded as a conduit, and the spermatozoa could simply make their way along it if their motility was good enough. Now it is understood that the sperm population in the ejaculate is essentially presented with a series of obstacles, and as the spermatozoa proceed via the cervix, uterus, and oviducts, perhaps becoming sequestered in epithelial glands or phagocytosed by polymorphonuclear leucocytes, their number is significantly depleted. Eventually a small remaining sperm population does, in fact, reach the vicinity of the oocyte and engage in fertilization. It has been estimated that the sperm:egg ratio within the oviduct at any given moment after ovulation may be as low as 1:1 (Hunter and Gadea, 2014), which presumably reflects the operation of stringent in vivo sperm selection mechanisms. The oviduct may be one of the most important sites for determining many aspects of sperm selection and competition as it possesses the potential for enhancing sperm survival, suppressing and activating sperm motility as required, and is now known to respond to the arrival of spermatozoa by producing novel proteins. Elegant proteomic experiments have even shown that the cohorts of novel proteins produced in response to the arrival of spermatozoa differ according to the sperm genotype (Almiñana et al., 2014). This implies that the spermatozoa are potentially responsible for establishing their own environment, and could certainly be involved in setting up the environment for optimal embryo development. Biochemical and biophysical studies of sperm motility have also revealed the existence of mechanisms that guide the spermatozoa towards the oocytes, probably using ancient mechanisms that may have initially evolved in marine invertebrates. Many puzzles remain to be solved, not least of which is how the female reproductive tract apparently has the ability to screen spermatozoa for their inherent genetic qualities, when sperm DNA is tightly and stably complexed with basic proteins (mainly protamines). Just as the last three decades have shown that sperm function is more complex than previously thought, it is likely that the next three decades will uncover yet more unsuspected but crucially important properties of spermatozoa.

References

- Almiñana, C., Caballero, I., Heath, P. R., et al. (2014). The battle of the sexes starts in the oviduct: Modulation of oviductal transcriptome by X and Y-bearing spermatozoa. *BMC Genomics*, 15, 293.
- Armon, L., & Eisenbach, M. (2011). Behavioral mechanism during human sperm chemotaxis: Involvement of hyperactivation. *PLoS One*, 6, e28359.
- Arnoult, C., Pierre, V., & Ray, P. F. (2011). Chemotaxis of spermatozoa is regulated by progesterone binding on calcium channel CatSper. *Medicine Sciences: M/S*, 27, 702–704.
- Barratt, C. L., McLeod, I. D., Dunphy, B. C., et al. (1992). Prognostic value of two putative sperm function tests: Hypo-osmotic swelling and bovine sperm mucus penetration test (Penetrak). *Human Reproduction*, 7, 1240–1244.
- Bian, F., Mao, G., Guo, M., et al. (2012). Gradients of natriuretic peptide precursor A (NPPA) in oviduct and of natriuretic peptide receptor 1 (NPR1) in spermatozoon are involved in mouse sperm chemotaxis and fertilization. *Journal of Cellular Physiology*, 227, 2230–2239.
- Bianchi, P. G., De Agostini, A., Fournier, J., et al. (2004). Human cervical mucus can act in vitro as a selective barrier against spermatozoa carrying fragmented DNA and chromatin structural abnormalities. *Journal of Assisted Reproduction and Genetics*, 21, 97–102.
- Birkhead, T. R. (1998). Cryptic female choice: Criteria for establishing female sperm choice. *Evolution*, 52, 1212–1218.
- Blengini, C. S., Teves, M. E., Unates, D. R., et al. (2011). Human sperm pattern of movement during chemotactic re-orientation towards a progesterone source. *Asian Journal of Andrology*, 13, 769–773.
- Boilard, M., Reyes-Moreno, C., Lachance, C., et al. (2004). Localization of the chaperone proteins GRP78 and hsp60 on the luminal surface of bovine oviduct epithelial cells and their association with spermatozoa. *Biology of Reproduction*, 71, 1879–1889.
- Burger, D., Meuwly, C., Marti, E., et al. (2017). MHC-correlated preferences in diestrous female horses (*Equus caballus*). *Theriogenology*, 89, 318–323.e1.
- Burkman, L. J., Overstreet, J. W., & Katz, D. F. (1984). A possible role for potassium and pyruvate in the modulation of sperm motility in the rabbit oviducal isthmus. *Journal of Reproduction and Fertility*, 71, 367–376.
- Caballero-Campo, P., Buffone, M. G., Benencia, F., et al. (2014). A role for the chemokine receptor ccr6 in mammalian sperm motility and chemotaxis. *Journal of Cellular Physiology*, 229, 68–78.
- Cho, C., Bunch, D. O., Faure, J. E., et al. (1998). Fertilization defects in sperm from mice lacking fertilin-β. *Science*, 281, 1857–1859.
- Coy, P., & Avilés, M. (2010). What controls polyspermy in mammals, the oviduct or the oocyte? *Biological Reviews*, 85, 593–605.
- Coy, P., Canovas, S., Mondejar, I., et al. (2008). Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during fertilization and contribute to the control of polyspermy. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 15809–15814.
- Curry, E., Szafranski, T. J., & Pratt, S. L. (2011). Differential expression of porcine sperm microRNAs and their association with sperm morphology and motility. *Theriogenology*, 76, 1532–1539.
- Das, P. J., McCarthy, F., Vishnoi, M., et al. (2013). Stallion sperm transcriptome comprises functionally coherent coding and regulatory RNAs as revealed by microarray analysis and RNA-seq. *PLoS One*, 8, e56535.
- Demott, R. P., & Suarez, S. S. (1992). Hyperactivated sperm progress in the mouse oviduct. *Biology of Reproduction*, 46, 779–785.
- Dubuc, A., & Sirard, M. A. (1995). Effect of coculturing spermatozoa with oviductal cells on the incidence of polyspermy in pig in vitro fertilization. *Molecular Reproduction and Development*, 41, 360–367.
- Dziuk, P. J. (1996). Factors that influence the proportion of offspring sired by a male following heterospermic insemination. *Animal Reproduction Science*, 43, 65–88.
- Ellington, J. E., Ignatz, G. G., Ball, B. A., et al. (1993). De novo protein-synthesis by bovine uterine tube (oviduct) epithelial-cells changes during coculture with bull spermatozoa. *Biology of Reproduction*, 48, 851–856.
- Ellington, J. E., Evenson, D. P., Fleming, J. E., et al. (1998). Coculture of human sperm with bovine oviduct epithelial cells decreases sperm chromatin structural changes seen during culture in media alone. *Fertility and Sterility*, 69, 643–649.

- Elliott, R. M., Lloyd, R. E., Fazeli, A., et al. (2009). Effects of Hspa8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. *Reproduction*, 137, 191–203.
- England, G. C., Burgess, C. M., Freeman, S. L., et al. (2006). Relationship between the fertile period and sperm transport in the bitch. *Theriogenology*, 66, 1410–1418.
- Fazeli, A., Affara, N. A., Hubank, M., et al. (2004). Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biology of Reproduction*, 71, 60–65.
- First, N. L., Short, R. E., Peters, J. B., et al. (1968). Transport and loss of boar spermatozoa in the reproductive tract of the sow. *Journal of Animal Science*, 27, 1037–1040.
- Gadella, B. M., & Evans, J. P. (2011). Membrane fusions during mammalian fertilization. *Advances in Experimental Medicine and Biology*, 713, 65–80.
- Gadella, B. M., & Luna, C. (2014). Cell biology and functional dynamics of the mammalian sperm surface. *Theriogenology*, 81, 74–84.
- Garcia-Herrero, S., Garrido, N., Martinez-Conejero, J. A., et al. (2011). Differential transcriptomic profile in spermatozoa achieving pregnancy or not via ICSI. *Reproductive Biomedicine Online*, 22, 25–36.
- Garrido, N., Martinez-Conejero, J. A., Jauregui, J., et al. (2009). Microarray analysis in sperm from fertile and infertile men without basic sperm analysis abnormalities reveals a significantly different transcriptome. *Fertility and Sterility*, 91, 1307–1310.
- Garrido, N., Garcia-Herrero, S., & Meseguer, M. (2013). Assessment of sperm using mRNA microarray technology. *Fertility and Sterility*, 99, 1008–1022.
- Georgiou, A. S., Snijders, A. P., Sostaric, E., et al. (2007). Modulation of the oviductal environment by gametes. *Journal of Proteome Research*, 6, 4656–4666.
- Guidobaldi, H. A., Teves, M. E., Unates, D. R., et al. (2012). Sperm transport and retention at the fertilization site is orchestrated by a chemical guidance and oviduct movement. *Reproduction*, 143, 587–596.
- Hausser, J., & Zavolan, M. (2014). Identification and consequences of miRNA-target interactions—Beyond repression of gene expression. *Nature Reviews Genetics*, 15, 599–612.
- Holt, W. V., & Fazeli, A. (2010). The oviduct as a complex mediator of mammalian sperm function and selection. *Molecular Reproduction and Development*, 77, 934–943.
- Holt, W. V., & Fazeli, A. (2016). Sperm storage in the female reproductive tract. *Annual Review of Animal Biosciences*, 4, 291–310.
- Hunter, R. H. (2012). Temperature gradients in female reproductive tissues. *Reproductive Biomedicine Online*, 24, 377–380.
- Hunter, R. H. F., & Gadea, J. (2014). Cross-talk between free and bound spermatozoa to modulate initial sperm:egg ratios at the site of fertilization in the mammalian oviduct. *Theriogenology*, 82, 367–372.
- Ikawa, M., Nakanishi, T., Yamada, S., et al. (2001). Calmegin is required for fertilin [α/β] heterodimerization and sperm fertility. *Developmental Biology*, 240, 254–261.
- Ikawa, M., Tokuihara, K., Yamaguchi, R., et al. (2011). Caldesmon is a testis-specific chaperone required for sperm fertility. *Journal of Biological Chemistry*, 286, 5639–5646.
- Kadivel, G., Machado, S. A., Korneli, C., et al. (2012). Porcine sperm bind to specific 6-sialylated biantennary glycans to form the oviduct reservoir. *Biology of Reproduction*, 87, 147.
- Kasimanickam, R., Nebel, R. L., Peeler, I. D., et al. (2006). Breed differences in competitive indices of Holstein and Jersey bulls and their association with sperm DNA fragmentation index and plasma membrane integrity. *Theriogenology*, 66, 1307–1315.
- Kasimanickam, V., Kasimanickam, R., Arangasamy, A., et al. (2012). Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. *Theriogenology*, 78(2007–2019), e2002.
- Kaupp, U. B., Kashikar, N. D., & Weyand, I. (2008). Mechanisms of sperm chemotaxis. *Annual Review of Physiology*, 70, 93–117.
- Kim, L. U., Johnson, M. R., Barton, S., et al. (1999). Evaluation of sperm washing as a potential method of reducing HIV transmission in HIV-discordant couples wishing to have children. *AIDS*, 13, 645–651.
- Kölle, S., Reese, S., & Kummer, W. (2010). New aspects of gamete transport, fertilization, and embryonic development in the oviduct gained by means of live cell imaging. *Theriogenology*, 73, 786–795.
- Kuo, C. W., Chen, C. M., Lee, Y. C., et al. (2009). Glycomics and proteomics analyses of mouse uterine luminal fluid revealed a predominance of Lewis Y and X epitopes on specific protein carriers. *Molecular and Cellular Proteomics*, 8, 325–342.
- Lachance, C., Bailey, J. L., & Leclerc, P. (2007). Expression of Hsp60 and GRP78 in the human endometrium and oviduct, and their effect on sperm functions. *Human Reproduction*, 22, 2606–2614.
- Liao, Y., & Tang, L. (2014). The critical roles of hsc70 in physiological and pathological processes. *Current Pharmaceutical Design*, 20, 101–107.
- Liberda, J., Manaskova, P., Prelowska, L., et al. (2006). Saccharide-mediated interactions of boar sperm surface proteins with components of the porcine oviduct. *Journal of Reproductive Immunology*, 71, 112–125.
- Lishko, P. V., Botchkina, I. L., & Kirichok, Y. (2011). Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature*, 471, 387–391.
- Liu, T., Daniels, C. K., & Cao, S. (2012). Comprehensive review on the Hsc70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential. *Pharmacology and Therapeutics*, 136, 354–374.
- Lloyd, R. E., Watson, P. F., & Holt, W. V. (2008). Heat shock cognate 70 protein improves the long-term survival of ram spermatozoa during storage at 17°C in a commercial extender. *Reproduction, Fertility, and Development*, 20, 88.
- Lloyd, R. E., Elliott, R. M., Fazeli, A., et al. (2009). Effects of oviductal proteins, including heat shock 70 kDa protein 8, on survival of ram spermatozoa over 48 h in vitro. *Reproduction, Fertility, and Development*, 21, 408–418.
- Maillo, V., Sanchez-Calabuig, M. J., Lopera-Vasquez, R., et al. (2016). Oviductal response to gametes and early embryos in mammals. *Reproduction*, 152, R127–141.
- Martyn, F., McAuliffe, F. M., & Wingfield, M. (2014). The role of the cervix in fertility: Is it time for a reappraisal? *Human Reproduction*, 29, 2092–2098.
- Mburu, J. N., Einarsson, S., Lundheim, N., et al. (1996). Distribution, number and membrane integrity of spermatozoa in the pig oviduct in relation to spontaneous ovulation. *Animal Reproduction Science*, 45, 109–121.
- Miki, K., & Clapham, D. E. (2013). Rheotaxis guides mammalian sperm. *Current Biology*, 23, 443–452.
- Moein-Vaziri, N., Phillips, I., Smith, S., et al. (2014). Heat-shock protein A8 restores sperm membrane integrity by increasing plasma membrane fluidity. *Reproduction*, 147, 719–732.
- Oren-Benaroya, R., Orvieto, R., Gakamsky, A., et al. (2008). The sperm chemoattractant secreted from human cumulus cells is progesterone. *Human Reproduction*, 23, 2339–2345.
- Orr, T. J., & Zuk, M. (2014). Reproductive delays in mammals: An unexplored avenue for post-copulatory sexual selection. *Biological Reviews*, 89, 889–912.
- Overstreet, J. W., & Cooper, G. W. (1975). Reduced sperm motility in the isthmus of the rabbit oviduct. *Nature, London*, 258, 718–719.
- Pang, P. C., Tissot, B., Drobnis, E. Z., et al. (2009). Analysis of the human seminal plasma glycome reveals the presence of immunomodulatory carbohydrate functional groups. *Journal of Proteome Research*, 8, 4906–4915.
- Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, 45, 525–567.
- Perez-Cerezales, S., Boryshpolets, S., & Eisenbach, M. (2015). Behavioral mechanisms of mammalian sperm guidance. *Asian Journal of Andrology*, 17, 628–632.
- Puppo, A., Chun, J. T., Gragnaniello, G., et al. (2008). Alteration of the cortical actin cytoskeleton deregulates Ca^{2+} signaling, monospermic fertilization, and sperm entry. *PLoS One*, 3, e3588.
- Robl, J. M., & Dziuk, P. J. (1988). Comparison of heterospermic and homospermic inseminations as measures of male fertility. *Journal of Experimental Zoology*, 245, 97–101.
- Rodriguez-Martinez, H., Tienthai, P., Suzuki, K., et al. (2001). Involvement of oviduct in sperm capacitation and oocyte development in pigs. Control of Pig Reproduction VI. *Reproduction, Supplement*, 58, 129–145.
- Rodriguez-Martinez, H., Tienthai, P., Atikuzzaman, M., et al. (2016). The ubiquitous hyaluronan: Functionally implicated in the oviduct? *Theriogenology*, 86, 182–186.
- Salas-Huetos, A., Blanco, J., Vidal, F., et al. (2014). New insights into the expression profile and function of micro-ribonucleic acid in human spermatozoa. *Fertility and Sterility*, 102(213–222), e214.
- Satake, N., Elliott, R. M. A., Watson, P. F., et al. (2006). Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. *The Journal of Experimental Biology*, 209, 1560–1572.

- Shen, C., Kuang, Y., Liu, J., et al. (2013). Prss37 is required for male fertility in the mouse. *Biology of Reproduction*, 88, 123.1–11.
- Sidhu, K. S., Mate, K. E., & Rodger, J. C. (1998). Sperm-oviduct epithelial cell monolayer co-culture: An in vitro model of sperm-female tract interactions in a marsupial, the tammar wallaby (*Macropus eugenii*). *Journal of Reproduction and Fertility*, 114, 55–61.
- Silva, E., Kadivel, G., Jiang, R., et al. (2014). Multiple proteins from ejaculated and epididymal porcine spermatozoa bind glycan motifs found in the oviduct. *Andrology*, 2, 763–771.
- Stahlberg, R., Harlizius, B., Weitze, K. F., et al. (2000). Identification of embryo paternity using polymorphic DNA markers to assess fertilizing capacity of spermatozoa after heterospermic insemination in boars. *Theriogenology*, 53, 1365–1373.
- Strunker, T., Goodwin, N., Brenker, C., et al. (2011). The catsper channel mediates progesterone-induced Ca^{2+} influx in human sperm. *Nature*, 471, 382–386.
- Suarez, S. S. (1987). Sperm transport and motility in the mouse oviduct: Observations in situ. *Biology of Reproduction*, 36, 203–210.
- Sutovsky, P., Terada, Y., & Schatten, G. (2001). Ubiquitin-based sperm assay for the diagnosis of male factor infertility. *Human Reproduction*, 16, 250–258.
- Teves, M. E., Guidobaldi, H. A., Unates, D. R., et al. (2009). Molecular mechanism for human sperm chemotaxis mediated by progesterone. *PLoS One*, 4, e8211.
- Thomas, P. G., Ignatz, G., Ball, B. A., et al. (1995). Effect of coculture with stallion spermatozoa on de novo protein synthesis and secretion by equine oviduct epithelial cells. *American Journal of Veterinary Research*, 56, 1657–1662.
- Wedekind, C., & Furi, S. (1997). Body odour preferences in men and women: Do they aim for specific MHC combinations or simply heterozygosity? *Proceedings of the Royal Society of London B: Biological Sciences*, 264, 1471–1479.
- Weir, B. J. (1971). The reproductive physiology of the plains viscacha, *Lagostomus maximus*. *Journal of Reproduction and Fertility*, 25, 355–363.
- Wintemitz, J., Abbate, J. L., Huchard, E., et al. (2017). Patterns of mhc-dependent mate selection in humans and nonhuman primates: A meta-analysis. *Molecular Ecology*, 26, 668–688.
- Yanagimachi, R., & Chang, M. C. (1963). Sperm ascent through the oviduct of the hamster and rabbit in relation to the time of ovulation. *Journal of Reproduction and Fertility*, 6, 413–420.
- Yang, D., Chertov, O., Bykovskaia, S. N., et al. (1999). β -Defensins: Linking innate and adaptive immunity through dendritic and t cell CCR6. *Science*, 286, 525–528.
- Yaniz, J. L., Carretero, T., Recreo, P., et al. (2014). Three-dimensional architecture of the ovine oviductal mucosa. *Anatomia Histologia Embryologia*, 43, 331–340.
- Yeste, M., Lloyd, R. E., Badia, E., et al. (2009). Direct contact between boar spermatozoa and porcine oviductal epithelial cell (OEC) cultures is needed for optimal sperm survival in vitro. *Animal Reproduction Science*, 113, 263–278.
- Yeste, M., Holt, W. V., Bonet, S., et al. (2014). Viable and morphologically normal boar spermatozoa alter the expression of heat-shock protein genes in oviductal epithelial cells during co-culture in vitro. *Molecular Reproduction and Development*, 81, 805–819.
- Yeung, W. S. B., Ng, V. K. H., Lau, E. Y. L., et al. (1994). Human oviductal cells and their conditioned medium maintain the motility and hyperactivation of human spermatozoa in vitro. *Human Reproduction*, 9, 656–660.
- Yudin, A., Hanson, F., & Katz, D. (1989). Human cervical mucus and its interaction with sperm: A fine-structural view. *Biology of Reproduction*, 40, 661–671.
- Ziegler, A., Dohr, G., & Uchanska-Ziegler, B. (2002). Possible roles for products of polymorphic mhc and linked olfactory receptor genes during selection processes in reproduction. *American Journal of Reproductive Immunology*, 48, 34–42.
- Zuccarello, D., Ferlin, A., Garolla, A., et al. (2011). How the human spermatozoa sense the oocyte: A new role of SDF1-CXCR4 signalling. *International Journal of Andrology*, 34, e554–565.

Further Reading

- Bedford, J. M. (2004). Enigmas of mammalian gamete form and function. *Biological Reviews*, 79, 429–460.
- Bedford, J. M. (2008). Puzzles of mammalian fertilization—And beyond. *International Journal of Developmental Biology*, 52, 415–426.
- Bedford, J. M. (2014). Singular features of fertilization and their impact on the male reproductive system in eutherian mammals. *Reproduction*, 147, R43–R52.
- Bedford, J. M., Mock, O. B., & Goodman, S. M. (2004). Novelities of conception in insectivorous mammals (Lipotyphla), particularly shrews. *Biological Reviews*, 79, 891–909.
- Edward, D. A., Stockley, P., & Hosken, D. J. (2014). Sexual conflict and sperm competition. *Cold Spring Harbor Perspectives in Biology*, 7, a017707.
- Fitzpatrick, J. L., & Lupold, S. (2014). Sexual selection and the evolution of sperm quality. *Molecular Human Reproduction*, 20, 1180–1189.
- Orr, T. J., & Zuk, M. (2014). Reproductive delays in mammals: An unexplored avenue for post-copulatory sexual selection. *Biological Reviews*, 89, 889–912.
- Palumbi, S. R. (2009). Speciation and the evolution of gamete recognition genes: Pattern and process. *Heredity* 102, 66–76.
- Ramm, S. A., & Scharer, L. (2014). The evolutionary ecology of testicular function: Size isn't everything. *Biological Reviews*, 89, 874–888.

Relevant Website

Encyclopedia Britannica—<https://www.britannica.com/science/animal-reproductive-system/>.

Fallopian Tube/Oviduct

Pilar Coy Fuster, Manuel Avilés, and Rafael Latorre Reviriego, University of Murcia, Murcia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Infundibulum Funnel shaped ovarian end of the uterine tube.

Ampulla Relatively wide part of the tubular portion of the uterine tube, from the abdominal opening to the isthmus part.

Estradiol The primary female sex hormone produced primarily in the ovary.

Isthmus Narrow portion of the uterine tube after the ampulla and connecting to the uterus by the uterotubal junction.

Polyspermy A pathological condition in mammals consisting of the entrance of more than one spermatozoon in the oocyte, resulting in an aneuploid product.

Progesterone The hormone produced in the ovary and placenta necessary to prepare and keep a suitable environment for pregnancy in the female reproductive tract.

Zona pellucida Extracellular coat formed by glycoproteins that surrounds the oocyte and first stages of embryo divisions before implantation in mammals.

Anatomy of the Oviduct

Topography and Morphological Characteristics

The uterine tube (salpinx, oviduct, fallopian tube in humans) is a tubular, narrow, and sinuous structure with a length that can vary between species (approximately 10 cm in human species, 20 cm in sheep and horses, and about 25–30 cm in pigs).

It is located between the ovary and the uterus and is attached to the pelvic walls by a part of the broad ligament called mesosalpinx, which together with the oviduct itself and the proper ovarian ligament configures the ovarian bursa in most mammals, which surrounds the ovary (Fig. 1).

In its course five main parts are differentiated (Fig. 2). The first one, called infundibulum (i), is the closest to the ovary. It has a funnel shape, thin walls and irregular conspicuous folds, called tubular fimbriae, which converge toward the abdominal orifice of the tube. The infundibulum continues with a tubular portion composed of two main segments, ampulla and isthmus. The ampulla (ii) runs laterally to the ovary describing a sinuous trajectory that ends in a narrow ampullary–isthmic junction (iii) connecting with the isthmus. The isthmus (iv) is the caudal part of the uterine tube which communicates with the uterus through a uterine orifice in the so-called uterotubal junction (v). The three-dimensional architecture of this junction acts as a barrier or sphincter between the uterine cavity and the isthmus. While smooth and progressive in ruminants and pigs, it is abrupt in horses and carnivores so that the uterine tube protrudes within the uterine lumen with a small uterine papilla. In the particular case of the human species the isthmus

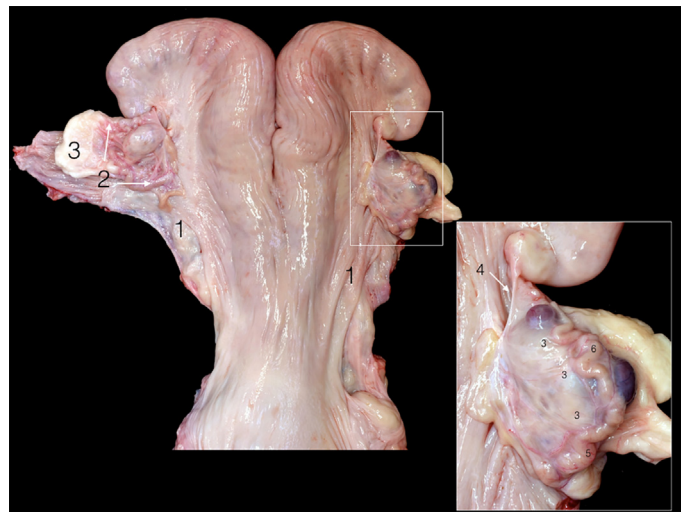


Fig. 1 Dorsal view of uterus and ovaries from adult cow. Detail of the right ovary covered by the infundibulum. 1: Broad ligament, 2: Oviduct, 3: Mesosalpinx, 4: Proper ovarian ligament, 5: Ampulla, 6: Isthmus.

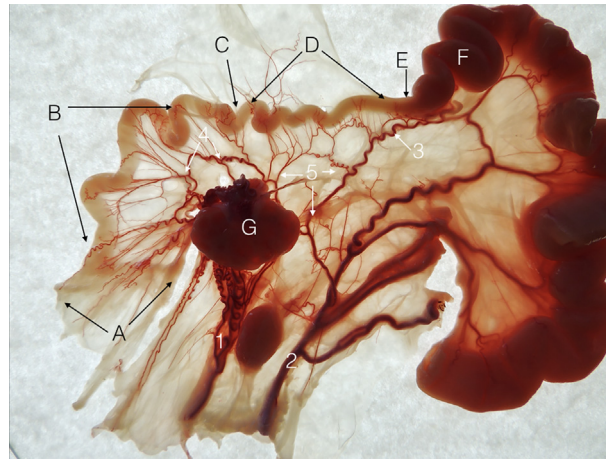


Fig. 2 Dorsal view of left horn and left ovary from a pig. (A) Infundibulum, (B) Ampulla, (C) ampullary-isthmic junction, (D) Isthmus, (E) Uterotubal junction, (F) Uterine horn, (G) Ovary, 1: Ovarian artery, 2: Uterine artery, 3: Tubal branch from the uterine artery, 4: Tubal branch from the ovarian artery, 5: arterial network in the mesosalpinx.

presents a transitional portion to join the uterus (uterine portion) which is a barrier not so functionally evident, as it will be seen later.

The inner morphology of the oviductal lumen is characterized by the presence of folds that are especially wide in the infundibulum and in the ampulla (**Fig. 3**). The largest ones or primary folds are arranged longitudinally along the whole length of the uterine tube. There are also smaller secondary folds with perpendicular disposition to the primary ones. Among the folds, there are deep grooves with sinuous pathways, and both structures together (folds and grooves) determine an irregular and highly complex lumen trajectory on the mucosa or endosalpinx (**Fig. 3A**). On the other hand, the number of folds is considerably reduced in the ampullary-isthmic junction, remaining only a few longitudinal folds in the isthmus, smaller and simpler than in the ampulla (**Fig. 3B**). In the ampulla and uterotubal junction, excavations are described in blind pouches and crypts, as well as elevations like polyps. These differences between the ampulla and the isthmus are related to functional aspects, as it will be seen later.

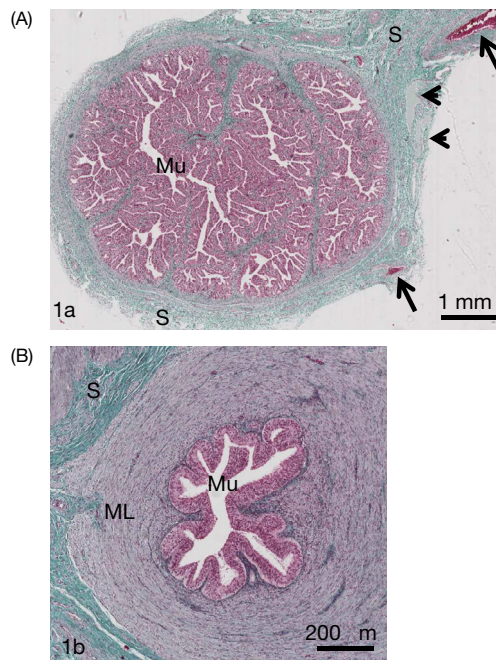


Fig. 3 Histological section of the mare oviduct stained with trichrome of Masson and viewed using light microscopy. (A) ampulla. The mucosa layer (Mu) presents numerous and big folds. Blood (arrows) and lymphatic vessels (arrowheads) are observed in the serosa layer (S). (B) isthmus. The mucosa layer (Mu) presents a important reduction of the number and size of the folds. The muscular layer (ML) is more developed in this region compared with the ampulla. Collagen fibers of the connective tissue present a green staining.

The reproductive status of the females as well as the phase of the reproductive cycle seem to influence the morphometric characteristics of the uterine tube. Thus, the length and its sinuous pathway increase in sexually adult females compared to prepubertal females, as well as among nulliparous and multiparous females. Similarly, the area of the oviductal lumen seems to be affected by these reproductive conditions. However, morphological aspects of the uterine tube, such as the length ratio between the ampulla and the isthmus, have been defined since puberty and are not affected by reproductive status or number of deliveries.

Vascularization and Innervation

In most of the domestic mammals the blood supply to the uterine tube comes mainly from the tubal branches of the ovarian and uterine arteries. These branches either form an extensive arterial network in the mesosalpinx before releasing terminal arterioles toward the mesotubarc border of the tube or directly terminate in the uterine tube with a minimal network of vascular anastomosis (Fig. 2).

The venous drainage of the uterine tube ends into the ovarian vein, as it does most of the blood from the uterus. The topography of the ovarian vein can be intimately related to the ovarian artery, which allows arteriovenous connections that are considered responsible for the local counter-current transfer of luteolytic substances between the uterus and ovary. In these large contact areas, the wall's thickness of the arteries and veins is diminished, specifically in adventitia where the fascicles of connective tissue form a single layer, which facilitates communication.

The lymphatic vessels of the uterine tube drain into the subserous and mucosal plexus and end up in the mesovary where they join the lymphatic vessels from the uterus and ovary. Together with the arteries and veins a periovaric vascular complex is described, which functionally interact creating a specific environment for physiological processes such as the mentioned hormone counter-current transfer.

Certain phases of the reproductive cycle may result in significant increases in vascular supply to the uterine tube as well as to the uterus and ovary. However, it has been found in the sow that the number of terminal arterioles reaching the ampulla or the isthmus does neither vary between prepubertal and adult females nor affect the phase of the reproductive cycle. Therefore, the increase of vascularization during the reproductive cycle is supposed to be mainly due to vasodilation phenomena.

Innervation is provided by sympathetic and parasympathetic nerves from the autonomic nervous system. Branches from the aortic plexus, that accompanies the ovarian artery, to innervate the ovary, and branches from the pelvic plexus supply the female genital tract.

Histology of the Oviduct

The oviduct is formed by three layers with a predominant or characteristic tissue. Thus, internally, a mucosa layer is present formed by an epithelium. The middle layer is the muscular layer formed mainly by smooth muscular cells and the external layer is the serosa formed mainly by connective tissue. The size and organization of these three layers is different depending on the region of the oviduct, the species and, to some extent, the phase of the estrus cycle.

Main Layers

The mucosa layer (Fig. 3), covering internally the oviduct, is formed by a simple epithelium of columnar or cuboidal cells that rest on a basal lamina. Below this, a connective tissue called lamina propria is present. Regarding the muscular layer (Fig. 3), two different types of structure can be observed in the ampulla and isthmus. It was observed in some species (rabbit, cow, sow, ewe, and woman) that the extrinsic and intrinsic components of the muscular layer are closed intermingled showing a plexiform architecture. However, in the rat, both musculatures are independent.

In the uterotubal junction, the myoarchitecture allows the classification in two main groups: a barrier-like and a sphincter-like structure. In the barrier-like structure, the smooth muscle fibers are densely packed (e.g., rat and sow). In the sphincter-like structure, the smooth muscle fibers can be geometrically organized (e.g., rabbit and ewe) with an independent relationship among the extrinsic and intrinsic musculature or with a plexiform organization (e.g., cow and woman) with a closely interwoven between both musculatures.

The serosa layer is formed by a simple squamous epithelia of mesothelial cells which cover externally the oviduct. A submesothelial connective tissue is observed which contains blood and lymphatic vessels. It can also be observed the presence of cholinergic and adrenergic nervous fibers which modulate the activity of the muscular layer.

Main Cells in the Oviductal Epithelium in the Mucosa

The epithelium covering the lumen of the oviduct is mainly formed by two types of cells: ciliated and secretory cells. The ciliated cells are characterized by the presence of a high number of motile cell prolongations called cilia that are formed by tubulin protein. The number of ciliated cells is different in the different regions of the oviduct with a lower number of cells in the isthmus (Fig. 4), and this number varies depending on the phase of the reproductive cycle, with the ciliated cells increasing around the ovulation time. In the human fallopian tube, the ciliated cells can be up to 80% in the fimbriae close to the ovary and 30% in the isthmus.

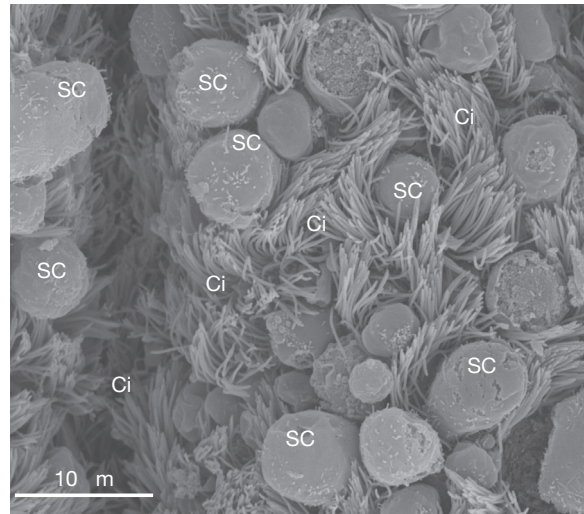


Fig. 4 Bovine oviduct. Scanning electron microscopy image showing the ciliated (Ci) and secretory (SC) cells of the oviductal epithelium.

The cilia move coordinately in the same direction and participate in the movement of the oocytes and embryos in direction to the uterus.

The secretory cells are dispersed among the ciliated cells, and the main characteristic is the presence of abundant secretory granules in their apical region that are released to the oviductal lumen mainly by exocytosis. At the surface of the cell, short immotile prolongations called microvilli are present. Recently, it was observed the presence of unique nonmotile cilia in these cells known as primary cilia. It was demonstrated that these primary cilia function as antennae for sensory reception and consequently provide information about the oviduct lumen. However, it was recently reported that the motile cilia, not only the primary cilia, are also involved in the sensory mechanism.

Oviductal Fluid's Composition

The oviductal fluid is the result of the specific secretions from the oviductal epithelial cells and the transudation from the blood. Although similar to the blood plasma, it shows some differences regarding electrolytes, amino acids, and carbohydrates proportions, among others. It contains glycosaminoglycans and a large number of proteins including some specific proteins only found at this organ, being the oviduct-specific glycoprotein (OVGP1) the best known and studied. The most abundant protein present in the oviductal fluid is the albumin which is originated in the liver and transported by the blood to the oviduct. Albumin plays an important role in the capacitation of the sperm by removal of the cholesterol from the sperm plasma membrane. Other important proteins contained in the oviductal fluid are growth factors that contribute to the efficient development of the early embryos by favoring the cell division. Another important group of proteins are proteases and their inhibitors whose key functions were revealed recently. Thus, in the oviductal milieu a well regulation of the proteolysis is produced which avoid the death of the oocyte and embryos. An additional more complex component present in the oviductal fluid consists of extracellular vesicles which are released by cells in the oviductal lumen and serve as vehicles for the transfer of lipids, proteins, and RNA. These extracellular vesicles can be differentiated according to their size in exosomes (40–100 nm) and microvesicles (50 nm–1 μm). Recently, these extracellular vesicles have been reported to modify the gametes and the embryo and even contribute to the maternal-embryo communication.

Oviductal Functions

According to the ovarian morphology, explained in previous chapters, the reproductive cycle of most of mammals, including primates, could be divided in two phases. The follicular phase is that in which the predominant structure observed in the ovary is the single (or are the multiple) follicle(s) growing (Fig. 5A), depending on the typical number of offspring delivered in each species. The luteal phase is that in which the corpora lutea, resulting from the transformation of the follicles after ovulation, can be observed. Ovulation, on the other side, is a process occurring between the follicular and the luteal phase of the cycle. While the ovarian follicles produce estradiol by means of the granulosa cells covering their internal wall and supporting the oocyte inside, the cells of the corpora lutea produce progesterone, the so-called hormone of pregnancy. All the events happening in the oviduct are regulated and orchestrated by these changing ratios of estradiol/progesterone.

The phase of the reproductive cycle in which the oviductal physiology acquires its maximum importance is that comprising the end of the follicular phase and the beginning of the luteal phase. At the end of the follicular phase the surge of LH is triggering the

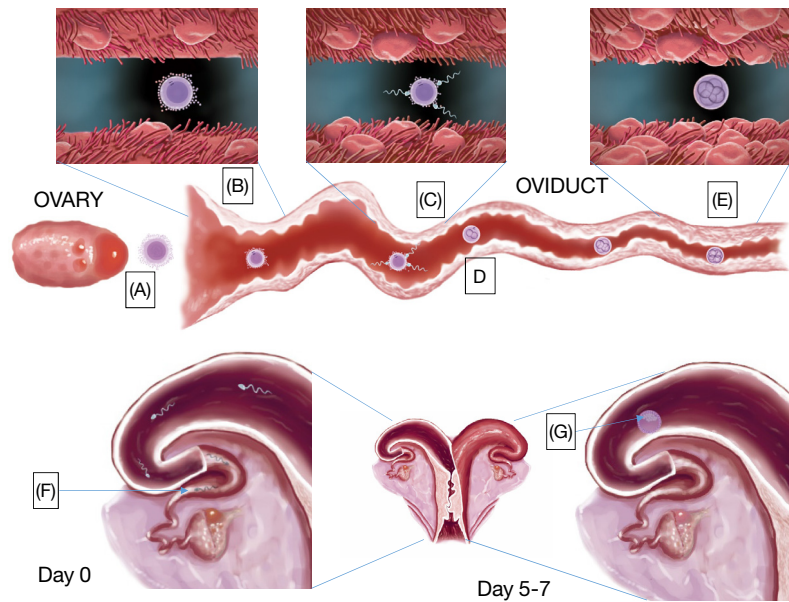


Fig. 5 Diagrammatic representation of the oviductal regions and their different functions. (A) The oocyte is released from the preovulatory follicle in the ovary. (B) The oocyte enters the oviduct through the infundibulum. (C) Spermatozoa and oocyte meet at the ampullary–isthmic junction and fertilization occurs. (D) After the first mitosis, the zygote divides into two cells. (E) The embryo proceeds through the isthmus while the divisions progress. (F) Hours or minutes before the arrival of the oocytes, the spermatozoa are retained in the isthmus by binding to the ciliated cells in the epithelium forming the sperm reservoir. (G) The embryo reaches the uterus and look for a place for implantation.

important hormonal changes (drop of the estradiol levels and increase of the progesterone) that modify the whole reproductive tract, for example, reshaping the mucosal epithelium from the oviduct, through the uterus until to the vagina or reverting the sense of the muscular layer contractions. In the oviduct in particular, as it has been explained in the histology section, the proportions between secretory and epithelial cells in the mucosa change depending on the estrogen/progesterone ratio and so does the composition of the fluid bathing the lumen of the oviduct. During these days, the crucial events taking place in this organ include the ovulation and oocyte transport, the end of the sperm capacitation, the fertilization, the first embryonic divisions (cleavage), and finally, the entrance in the uterus of the recently formed conceptus.

In the next three sections, the events occurring in the oviduct between the end of the follicular phase and the beginning of the luteal one are described.

Events Happening in the Oviduct Before Fertilization (End of Follicular Phase)

In Fig. 5, the different functions of the oviduct are represented. Just after ovulation (Fig. 5B), the cilia-covered fimbria of the infundibulum are responsible for the process of ovum-catchment, which, in most cases, is 100% effective. This is so because, around the ovulation time, the coordination between the nervous and hormonal systems makes this free end of the oviduct approach to the ovary. This movement is favored by the spontaneous contractions of the smooth muscular fibers in the oviduct wall that couple with phasic contractions of the myosalpinx. It seems that oviduct interstitial cells of Cajal display pacemaker activity by electrical slow waves coordinating these contractions. Once in the oviduct, the oocytes must be transported to the distal end of the ampulla. Together with the oviductal contractions, ciliary beating is crucial for this event. At this stage, the oocyte is still surrounded by the cumulus cells, and the level of adhesion of these cells to the infundibulum wall is depending on the extent of cumulus expansion. If this extent is very high, then the adhesion can be stronger and the transportation turns difficult. Oocytes surrounded by the cumulus cells (oocyte–cumulus complexes) reach the ampullary–isthmic junction (Fig. 5C) relatively soon (30–45 min), and their level of coupling is diminishing during the transport. The presence of spermatozoa in the oviduct accelerates cumulus cells dispersion. The contact of the oocytes with the oviductal fluid during this time is also important to induce modifications at the zona pellucida level that will influence the initial recognition and binding of the spermatozoa.

Hours or minutes before the arrival of the oocytes, the spermatozoa have usually reached the isthmus and are retained there by their binding to the ciliated cells in the epithelium forming the sperm reservoir (Fig. 5F). This retention is important for the sperm to finish their capacitation process, which enable them to fertilize the oocyte. It seems that the presence of cumulus–oocyte complexes in the ampulla contributes to the release of the spermatozoa from the sperm reservoir, thus reinitiating their journey to the fertilization site, at the ampullary–isthmic junction, where they finally meet the oocyte. Spermatozoa released from the reservoir show a specific motility pattern characterized by a very vigorous flagellar beating called “hyperactive motility.” This pattern is necessary for the fertilization.

Fertilization

Fertilization consists of the fusion of male and female gametes at the ampullary–isthmic junction of the oviduct to form the zygote (Fig. 5C). The oviduct favors the rendezvous of both gametes and provides a fine-tuned environment to avoid failures in this crucial event. As example of its perfect coordination, not only its tubular structure, the proportions between secretory and epithelial cells in the mucosa, or the spontaneous and phasic contractions of the smooth muscular fibers in the oviduct wall contribute to avoid the massive arrival of spermatozoa to the oocyte's proximity, but also the oviductal fluid, inducing modifications at the zona pellucida level, is responsible for regulating the pathological condition of polyspermy in mammals.

Events Happening in the Oviduct After Fertilization (Beginning of Luteal Phase)

Once the zygote is formed, it remains in the ampullary–isthmic junction for a few hours until the first cleavage takes place and it divides into two cells (Fig. 5D). Then, the two-cells embryo restarts its journey and slowly shifts along the isthmus. The thick muscular layer at this region, which was heavily contracted under the influence of estradiol, is now relaxed by the effect of progesterone, allowing the embryo proceeds while the divisions progress (Fig. 5E). The rich product of the secretory cells in the epithelium makes the fluid dense and keeps the perfect environment for the embryo survival, providing the necessary nutrients for the recently formed conceptus. Depending on the species, the entrance in the uterus through the uterotubal junction occurs approximately at the 4 cell stage on day 2–3 postfertilization (e.g., in the pig) or at more advanced stages on day 4–5 after fertilization (e.g., at the morula stage in the human) (Fig. 5G).

References

- Brüssow, K. P., Rátky, J., & Rodríguez-Martínez, H. (2008). Fertilization and early embryonic development in the porcine fallopian tube. *Reproduction in Domestic Animals*, 4, 245–251.
- Coy, P., Cánovas, S., Romar, R., et al. (2008). Oviduct-specific glycoprotein and heparin modulate sperm-zona pellucida interaction during mammalian fertilization. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 15809–15814.
- Coy, P., García-Vázquez, F. A., Visconti, P., & Avilés, M. (2012). Roles of the oviduct in mammalian fertilization. *Reproduction*, 144, 649–660.
- Dixon, R. E., Hwang, S. J., Hennig, G. W., et al. (2009). Chlamydia infection causes loss of pacemaker cells and inhibits oocyte transport in the mouse oviduct. *Biology of Reproduction*, 80, 665–673.
- Ginther, O. J. (1974). Internal regulation of physiological processes through local venoarterial pathways: A review. *Journal of Animal Science*, 39, 550–564.
- Gutiérrez, H. A., Latorre, R., Martínez Bagán, E., Sánchez Margallo, F. M., & López Albors, O. (2015). Quantitative evaluation of the vasculature supplying the oviduct in pre-pubertal and sexually mature sows. *Anatomical Record (Hoboken)*, 298, 1978–1983.
- Krzymowski, T., & Stefanczyk-Krzymowska, S. (2012). Local retrograde and destination transfer of physiological regulators as an important regulatory system and its role. Facts and hypothesis. *Journal of Physiology and Pharmacology*, 63, 3–16.
- Leese, H. J., Tay, J. I., Reischl, J., & Downing, S. J. (2001). Formation of fallopian tubal fluid: Role of a neglected epithelium. *Reproduction*, 121, 339–346.
- Mouguelar, H., Díaz, T., Borghi, D., et al. (2015). Morphometric study of the mare oviductal mucosa at different reproductive stages. *Anatomical Record (Hoboken)*, 298, 1950–1959.
- Muglia, U., & Motta, P. M. (2001). A new morpho-functional classification of the fallopian tube based on its three-dimensional myoarchitecture. *Histology and Histopathology*, 16, 227–237.
- Nomenclature ICoVA. (2012). *Nomina Anatomica Veterinaria* (5th (revised version) edn.). Columbia: World Association of Veterinary Anatomist, 5th Editorial Committee.
- Oxenreider, S. L., McClure, R. C., & Day, B. N. (1965). Arteries and veins of the internal genitalia of female swine. *Journal of Reproduction and Fertility*, 9, 19–27.
- Pazour, G. J., & Witman, G. B. (2003). The vertebrate primary cilium is a sensory organelle. *Current Opinion Cell Biology*, 15, 105–110.
- Wehrenberg, W. B., Chaichareon, D. P., Dierschke, D. J., Rankin, J. H., & Ginther, O. J. (1977). Vascular dynamics of the reproductive tract in the female rhesus monkey: Relative contributions of ovarian and uterine arteries. *Biology of Reproduction*, 17, 148–153.

Fallopian Tube/Oviduct: Structure and Cell Biology

Wipawee Winuthayanon and Shuai Li, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Axoneme The central strand of a cilium or flagellum. It is composed of an array of microtubules, typically in nine pairs around two single central ones.

B cells A type of lymphocyte developed from stem cells originated from bone marrow; responsible for producing antibodies and participating in the immune response.

Cell atrophy Decrease in size of the cells.

Ectopic pregnancy Embryo implants somewhere other than the main cavity of the uterus.

Fimbriae A series of fringe-like structure at the ovarian end of the Fallopian tube.

Polyspermy An egg fertilized by multiple sperm, this will lead to embryonic cell death.

T cells A type of lymphocyte produced or processed by the thymus gland and actively participating in the immune response.

Transudation Passage of the fluid through cell membrane as a result of osmotic pressure.

Zygote A fertilized egg or one-cell embryo.

Gross Anatomy

The human Fallopian tubes (known as oviducts in animals) are part of the female reproductive tract and are responsible for gamete (egg and sperm) transport. The Fallopian tube is the site for fertilization and preimplantation embryo development. Progressing from the ovary to the uterus, the three distinct segments of the Fallopian tube are infundibulum, ampulla, and isthmus (Fig. 1).

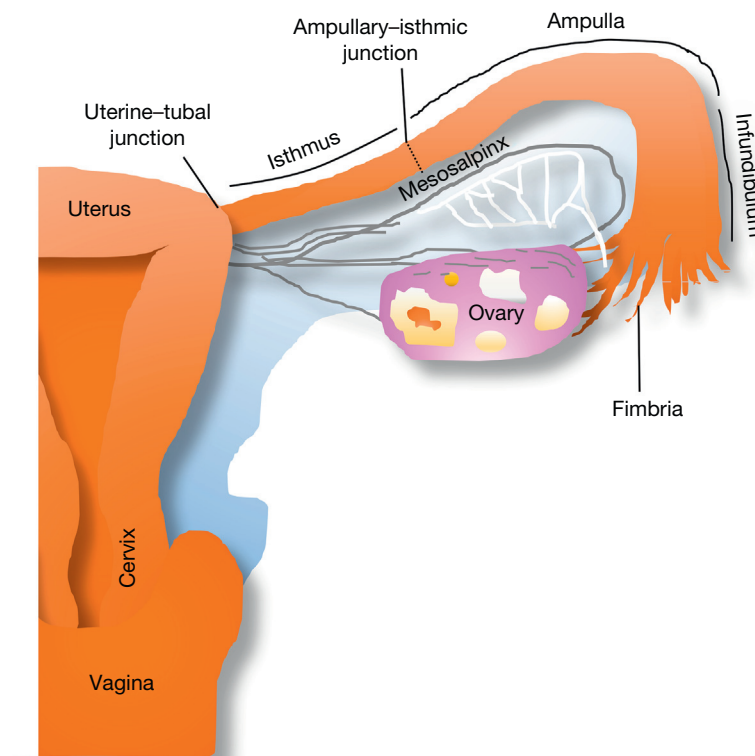


Fig. 1 Illustration of one side of the human female reproductive tract, including ovary, Fallopian tube, uterus, cervix, and vagina. The fimbria of infundibulum comes in contact with the ovary during ovulation to enable the Fallopian tube to pick up the eggs. The ampulla is the fertilization site and connects to the isthmus region of the Fallopian tubes. The isthmus is adjacent to the uterus and connected through the uterotubal junction. The Fallopian tube is supported by connective tissue embedded with vascular, nervous, and lymphatic system known as the mesosalpinx.

Each segment in the Fallopian tube has distinct cell morphology and function. The infundibulum is a trumpet-like segment of the Fallopian tube. The fimbriated end of the infundibulum opens up to the peritoneal cavity. Fimbriae are lined with multiciliated epithelial cells with a threadlike extension toward the ovary. These multiciliated epithelial cells of the infundibulum function to aid oocyte pickup and transport. The ampulla represents two-thirds of the length of Fallopian tube and is the site for fertilization of oocytes. The proportionality of epithelial cell lining in the infundibulum and ampulla gradually changes from ciliated cell population to secretory cell population in the isthmus, the final segment of the Fallopian tube. The wall of the isthmus is thicker and more resistant to palpation than the ampullary region.

Segments of the Fallopian tube are connected with three junctional areas: fimbria abdominal ostium, ampullary–isthmic junction, and uterotubal junction. The fimbria is a fingerlike structure surrounding the infundibulum and extending outward from the Fallopian tube. The fimbria abdominal ostium is the opening that connects the Fallopian tube with the peritoneal cavity. The largest fimbria, called the ovarian fimbria, is attached to the ovary. At ovulatory phase, the luteinizing hormone peaks, causing the follicle to rupture. The oocyte–cumulus complex is then released from the ovary and picked up by a fluid current generated from ciliated epithelial cells beating on the surface of the fimbria. Ciliary movement transports oocyte–cumulus complex further deep into the Fallopian tube toward the uterus. Aptly named, the ampullary–isthmic junction is a junction between the ampullary and isthmic region of the oviduct. This junction is identified by an abrupt change in muscle wall thickness between the ampulla and the isthmus. The ampullary–isthmic junction regulates the delay of ovum transport, allowing sperm to advance into the ampulla in a timely manner (Anand and Guha, 1982). Lastly, the isthmus joins the uterus via the uterotubal junction. This junction serves as a selective sperm barrier, allowing only viable sperm to advance to the site of fertilization.

There is a recognized anatomical distinction between species. Human Fallopian tubes, rabbit oviducts, and nonhuman primate Fallopian tubes have a slight curve in their structure. Other animals such as rodents, birds, and amphibians have a coiled structure. This difference is a developmental characteristic, but the mechanism for coiling or curvature is still not well understood (Stewart and Behringer, 2012). Despite the anatomical differences between species, the Fallopian tubes of mammals consist of the same three main structural layers (Fig. 2A): mesosalpinx (outer serous layer), myosalpinx (muscle layer), and endosalpinx (mucosa layer).

Physiological function of the Fallopian tubes is regulated mainly by estrogen (E_2) and progesterone (P_4), two major sex steroid hormones. E_2 and P_4 signals through estrogen receptors and progesterone receptors to control tubal muscle contraction, ciliary beat frequency of the ciliated epithelial cells, secretory factors from the nonciliated epithelial cells, and other cellular

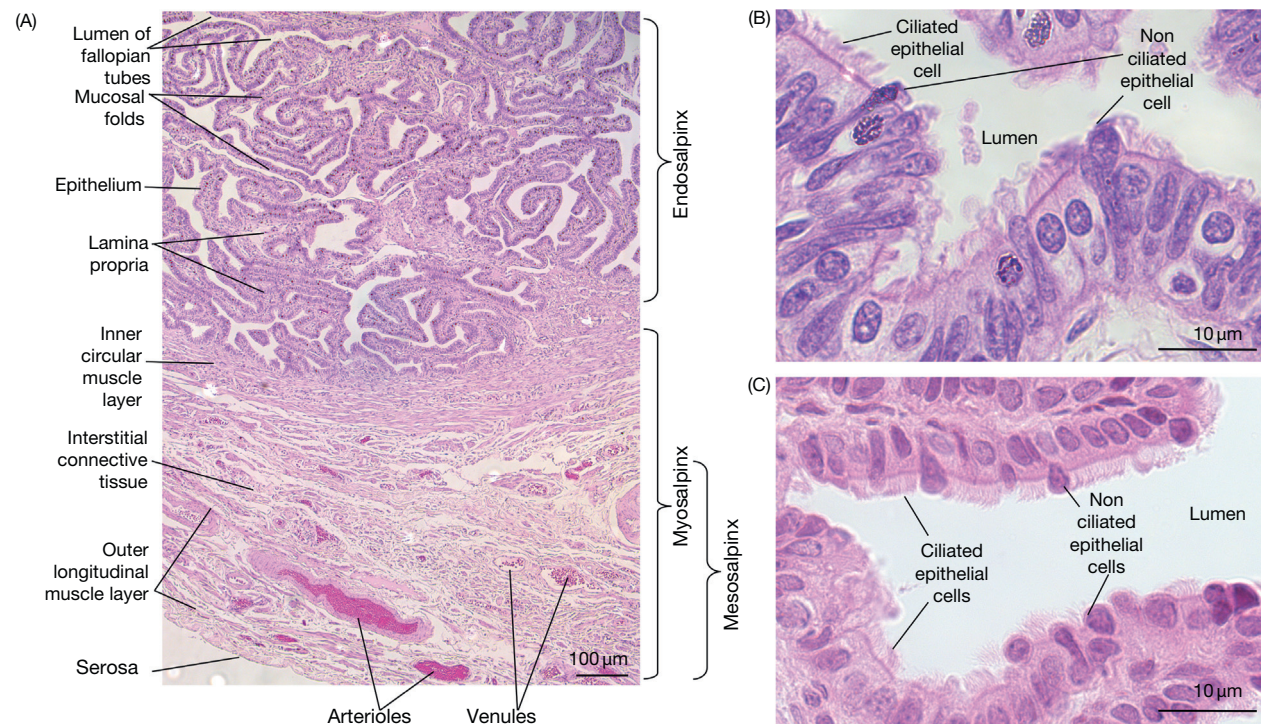


Fig. 2 Histological images of a human Fallopian tube in comparison with a mouse oviduct. (Human Fallopian tube obtained from Dr. Ken-Ichi Takemaru (Stony Brook University, School of Medicine) with institutional ethics approval.) (A) Histological structure of human Fallopian tube consists of mesosalpinx, myosalpinx, and endosalpinx. Epithelial cell layers of (B) human Fallopian tube and (C) mouse oviduct. Epithelial cells comprise two cell types: ciliated and nonciliated (or secretory) cells, facing the lumen.

functions to optimize the microenvironment for reproductive events in the Fallopian tube. This topic will be discussed in detail in the following sections.

Cell Biology and Physiology of the Fallopian Tube

Mesosalpinx

Serous layer of the Fallopian tube, also known as mesosalpinx (**Fig. 2A**), is the outer tissue layer that wraps around the Fallopian tube and is part of the peritoneal cavity lining. Mesosalpinx is composed of epithelial cells of mesothelium and a mesh of serous membrane lining (interstitial connective tissue). The mesothelium is the outermost boundary of the serous layer and is directly exposed to the pelvic cavity. Underneath the mesothelium is the serous membrane, surrounding the muscle layer. Blood vessels, nervous system, and lymphatic system are located within the serous membrane (**Pauerstein and Eddy, 1979**).

The vascular system of the Fallopian tube is supplied by the uterine and ovarian arteries. The capillaries innervate into the muscle and mucosal layers (**Fig. 2A**). This vascular system not only is critical for circulation but also is crucial for embryo development (**Garcia-Pascual et al., 1996**). Blood flow in the Fallopian tube is responsible for the generation and maintenance of the tubal fluid, which nourishes and protects gametes and embryos against external stress.

The nervous innervation of the Fallopian tube depends on the segment and the type of sensory nervous system. For example, the sympathetic nervous system from the celiac plexus innervates the infundibulum and the ampullary portion adjacent to the infundibulum, whereas the ampullary portion adjacent to the isthmic region and the isthmus receive sympathetic nervous supply from the inferior mesenteric plexus. For the parasympathetic nervous system, similar areas of the infundibulum and the ampulla are innervated by ovarian plexus, and similar areas of the isthmus and part of the ampulla are innervated by pelvic plexus of the pelvic nerve. Innervation of the nervous system runs parallel with the vascular system.

The lymphatic system of the Fallopian tube is supplied by the ovarian and uterine lymphatic systems, which originate from the iliac and lateral aortic nodes. Lymphatic system runs into the serous and mucosal layer enabling the lymphocyte to infiltrate and protect the Fallopian tube from pathogen infection. In the Fallopian tube, the lymphatic system acts as a drainage to reabsorb tubal fluid. The major immune cells found in the Fallopian tubes are the T cells. B cells are rare in this tissue (**Otsuki et al., 1989**).

Myosalpinx

Myosalpinx is the tubal muscle of the Fallopian tube, which consists of an outer longitudinal muscle layer and an inner circular muscle layer. These two muscle layers control the Fallopian tubal contraction responsible for various reproductive functions. First, the contraction retains the unfertilized oocyte in the ampullary region of the Fallopian tube. This is achieved through contraction of the muscle in the ampullary–isthmic junction. Ampullary–isthmic contraction also prevents polyspermy by restricting the rate of the sperm entering the ampulla (**Ezzati et al., 2014**). Both the ampullary–isthmic junction and uterotubal junction are pivotal for the timing of embryo implantation in the uterus. It takes the oocyte approximately 8 h to reach the ampullary–isthmic junction; then, after fertilization, the zygote is retained in the isthmus for approximately 72 h (**Croxatto et al., 1978; Lyons et al., 2006**). However, the time an embryo spends in the Fallopian tube is species-specific (**Croxatto, 2002**). Overaccelerated embryo transport results in premature arrival of the embryo to the uterus, in which the uterine environment is not conducive for embryo implantation (**Adams, 1979**).

Unlike intestinal smooth muscle, muscle contraction of the Fallopian tube not only is controlled by adrenergic neurons but also is regulated by P_4 and prostaglandins (**Wanggren et al., 2008**). Progesterone and prostaglandin receptors have been recently identified on the telocyte, a newly discovered cell type in human Fallopian tubes (**Cretoiu and Cretoiu, 2016**). Telocytes are interstitial cajal-like cells with long cytoplasmic processes (**Cretoiu et al., 2009**) that reside in the interstitial connective tissue layer of the Fallopian tube. The function of telocytes is not yet fully understood. However, evidence indicates that a reduction in telocytes results in defective peristaltic movement of the Fallopian tube (**Roatesi et al., 2015**).

Endosalpinx

Endosalpinx is the inner mucosal layer of the Fallopian tube responsible for transporting gametes and embryos and tubal fluid secretions. The endosalpinx is composed of epithelial cells and the lamina propria mucosa; the latter is a network of connective tissue with a mixed cell population. The epithelial tissue on the inner surface of the Fallopian tube is made up of two main cell types: ciliated epithelial cells and nonciliated secretory epithelial cells (**Fig. 2B and C**). Beneath the lining of epithelium is the lamina propria, which consists of fibroblast, immune, and progenitor cells. Mucosal layer forms dense internal folds, increasing the surface area of epithelial cell lining to achieve high fluid secretory rate (**Fig. 2A**).

Multiciliated epithelial cells in the Fallopian tube are featured with a classical 9 + 2 microtubule structure (axoneme) to create a beating movement (**Lodish et al., 2000**). The movement of axoneme requires adenosine triphosphate (ATP) as the energy source. Elevated E_2 concentration stimulates ATP production at the apical surface of the epithelial cells and results in an increase in ciliary beat frequency. In rabbits, the absence of calcium results in nonbeating cilium. This suggests the mechanism involves a calcium-dependent ciliary regulation (**Verdugo, 1980**), potentially through a voltage-gated calcium

channel (Doerner et al., 2015). Adrenomedullin-induced activation of calcitonin-gene-related peptide receptor is also involved in ciliary beating (Liao et al., 2013; Liao et al., 2011). In this adrenomedullin-related mechanism, E_2 stimulates the secretory epithelia to produce cyclic adenosine monophosphate, which subsequently promotes adrenomedullin activation in ciliated cells.

Nongenomic membrane-bound progesterone receptors (mPGRs) have been observed on the apical surface of epithelial cells in the Fallopian tube. mPGRs facilitate rapid cellular signaling pathways activated by P_4 and regulate ciliary beat frequency. Therefore, an alteration of these pathways could cause defective ciliary function and lead to embryo transport defects.

Ciliary beat activity of the epithelial cells results in oocyte pickup, embryo transport, and tubal fluid flow. Tubal fluid flow acts as a carrier, allowing embryo transport from the Fallopian tube to the uterus. Simultaneously, the fluid flow removes cell debris within the lumen (Miki and Clapham, 2013). The direction of the fluid flow is usually from the ovary toward the uterus in mammals. However, tubal flow in pigs progresses toward the ovary, which suggests that fluid flow orientation varies between species (Gaddum-Rosse and Blandau, 1976).

Approximately, 60% of epithelial cell lining is nonciliated epithelial (secretory) cells. The percentage differs between tube regions (Pedrero-Badillo et al., 2013). These secretory epithelial cells are morphologically distinct from ciliated epithelia. Not only the nonciliated epithelial cells lack cilia at the apical membrane, but also the cell bodies show apical protrusion into the lumen, also known as intercalary cells or peg cells (Fig. 2B and C). The cytosol of secretory cells contains an extensive Golgi network and apical granules. Secretory cells mainly secrete growth factors, nutrients, antimicrobial agents, and anticellular stress agents into the tubal fluid. Some of the secretory factors are enzymatically active including proteases and peptidases, which could potentially harm the embryos. Recent findings demonstrate that E_2 can have a protective effect on the embryo by reducing protease production within the mouse oviduct (Winuthayanon et al., 2015).

The lamina propria mucosa consists of connective tissue, secretory cells, microcapillary vessels, and a population of leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5)-positive progenitor cells. Lamina propria provides structural and nutritional support for the endosalpinx, while Lgr5-positive progenitor cells allow for tubal epithelial cell regeneration (Ng et al., 2014; Snegovskikh et al., 2014).

Tubal Fluid

Tubal fluid is composed of transudation from circulating plasma and secretions from the secretory epithelial cells. After ovulation, follicular fluid is combined with tubal fluid. Main functions of the tubal fluid are facilitating sperm transport and providing nutrients, growth hormone, protective agents for the embryo, and defense system against pathogens (Guerin and Menezo, 2011). In addition, the mucosal layer removes cell debris such as sperm, macrophages, bacteria, and dead epithelial cells (Miki and Clapham, 2013).

Fluid composition in the Fallopian tube is listed in Table 1. Tubal fluid contains glycoproteins and amino acids, with the most abundant nutritional compositions in the fluid being lactate (3–10 mM), glucose (0.2–1.9 mM), and pyruvate (0.02–0.2 mM). The most abundant amino acids are arginine (0.19 mM), alanine (0.11 mM), and glutamate (0.09 mM) (Dickens et al., 1995; Tay et al., 1997). The listed nutrients are used primarily by oocytes and embryos, while the excessive nutrients are reabsorbed and used by cells in the Fallopian tube. In general, protein concentration in the tubal fluid changes during the menstrual cycle and reaches the maximal concentrations at the time closest to menstruation. This dynamic change in protein concentration alters fluid viscosity and impacts the rate of tubal flow. The transient receptor vanilloid 4 (TRPV4) on epithelia cells detects fluid viscosity and regulates ciliary beating (Andrade et al., 2005). A viscous fluid in the Fallopian tube during fertilization stabilizes tubal microenvironment and protects the embryo against osmotic changes (Leese et al., 2001).

Growth factors in the tubal fluid include epidermal growth factor, embryotrophic factors, transforming growth factor, insulin-like growth factor, and fibroblast growth factor. Coculture of the embryos with these growth factors improves embryo development (Coy and Yanagimachi, 2015; Kane et al., 1992). Several embryo protective factors are present in the fluid, including heat shock protein, taurine and its derivatives, catalase, and antioxidative enzymes (Guerin and Menezo, 2011). Embryo protective factors prevent both gametes and embryos from environmental stress including, heat, free radicals, and internal and external reactive oxygen species.

Infection causes damage to Fallopian tube structure and function and can subsequently lead to complications in pregnancy. For example, gonococcal (gonorrhea) and chlamydia infections in the Fallopian tube cause ciliated cell damage, resulting in a loss of ciliary beat activities (McGee et al., 1981). Fortunately, the maternal immune system has macrophages patrolling within the tubal fluid to protect against microbial invasion. Fallopian tube secretory epithelial cells also produce cytokine/interleukin signal to induce inflammatory response (Maisey et al., 2003). Additionally, tubal fluid flow mechanistically eliminates unwanted bacteria and cell debris to the lower reproductive tract. In conclusion, the tubal fluid promotes fertilization, supports early embryo development, and maintains an optimal microenvironment in the Fallopian tube against pathogen invasion.

Cell Distribution in Different Regions of the Fallopian Tube

Each segment of the Fallopian tube serves a distinct function; therefore, structural and cellular compositions vary in each region. Two major structural differences are the muscle cell layer and the distinct epithelial cell distribution along the Fallopian tube. Less

Table 1 Known tubal fluid composition and their roles. Water and soluble gas are omitted from the table

<i>Roles</i>	<i>Components</i>
Embryo energy source	Pyruvate Lactate Lipids and fatty acid Amino acids Glucose and sugar glycogen
Fertilization-related	Oviductin (OVGP1) Plasmin Fetuin B
Sex steroid hormones	Estrogen Progesterone
Glycogen–sugar conversion	Amylase
RNA synthesis	HCO ₃ [−]
Growth factor	Embryotrophic factor 3 Epidermal growth factor (EGF) Transforming growth factor (TGF) Fibroblast growth factor (FGF) Insulin-like growth factor (IGF)
Antioxidants	Glutathione (GSH) Taurine Hypotaurine Cysteamine Catalase Superoxide dismutases Glutathione peroxidases
Antiheat stress	Heat-shock protein 70 Heat-shock factors
Antimicrobial	Serine proteases and inhibitors Defensins Cytokines
Muscle contractility	Endothelin, increase contractility Prostaglandins, depends on receptors Estrogen, increase contractility Progesterone, decrease contractility
Ciliary beat frequency (CBF)	Adrenomedullin, increase CBF Estrogen, increase CBF Progesterone, decrease CBF

muscle cells are present around the infundibulum and more abundant in the isthmic end of the tube (**Fig. 3**). A high percentage of ciliated epithelial cells are found at the infundibulum end, while the isthmic end is predominantly composed of secretory cells (**Pedrero-Badillo et al., 2013**). This is believed to be the evolutionary result of functional differences between the two ends of the tube. The infundibulum end is responsible for oocyte pickup and transport of the embryo; hence, it has more ciliated cells and less muscle cells than the isthmic end. A lack of muscle contraction in the infundibulum leads to a gentle motion, which allows smooth transition of the oocyte or embryos in the Fallopian tube (**Croxatto, 2002**). Strong muscle contraction at the isthmic end is due to the abundance of muscle fibers in this region. The mechanical control of muscle contraction at the isthmic region, together with the ampullary–isthmic junction and the uterotubal junction, allows embryo retention inside the Fallopian tube during the first few days of early development (**Halbert et al., 1988**).

Influences of E₂ and P₄ on Fallopian Tube Physiology and Function

In mammals, Fallopian tube physiology is significantly influenced by E₂ and P₄. Fallopian tube tissue displays a cyclical structural change in correlation with circulating levels of E₂ and P₄ during the menstrual cycle. Granulosa cells in the ovary produce E₂, in which the circulating level peaks during follicular phase of the menstrual cycle, right before ovulation, then decreases gradually during the luteal phase (**Fig. 4**). E₂ exerts its activity through estrogen receptor alpha (ESR1) and beta (ESR2). ESR1 is located in all layers of the Fallopian tube, and ESR2 presence is limited to some of the epithelial cells. E₂ increases epithelial cell hypertrophy, production of secretory proteins from the secretory cells, and ciliogenesis (**Verhage et al., 1979**) (**Fig. 4**). In humans, however, E₂ and P₄ work together to regulating ciliary beat frequency of the ciliated cells (**Mahmood et al., 1998**).

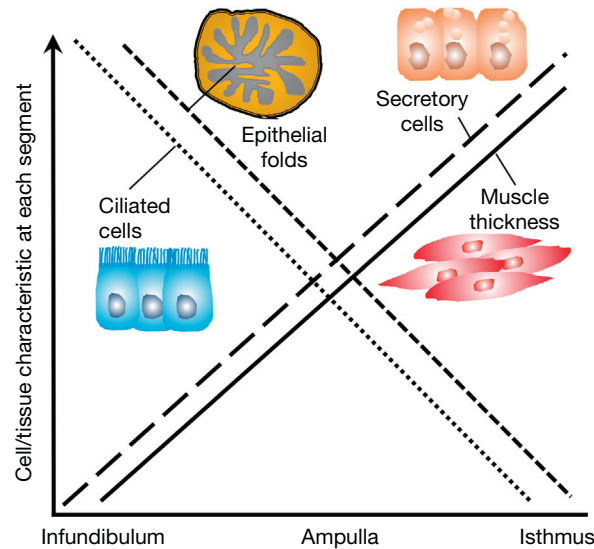


Fig. 3 Changes in cell population and tissue characteristics along the infundibulum, ampullary, and isthmic regions of the Fallopian tube. Ciliated epithelial cells and epithelial (mucosal) folds are highly abundant in the infundibulum and ampullary regions and gradually declined in the isthmic region. Isthmic region has high proportion of secretory epithelial cells and muscle thickness compared with the infundibulum and ampullary regions. Note that the figure does not reflect the actual percentile of the cell population proportion, but rather a general trend.

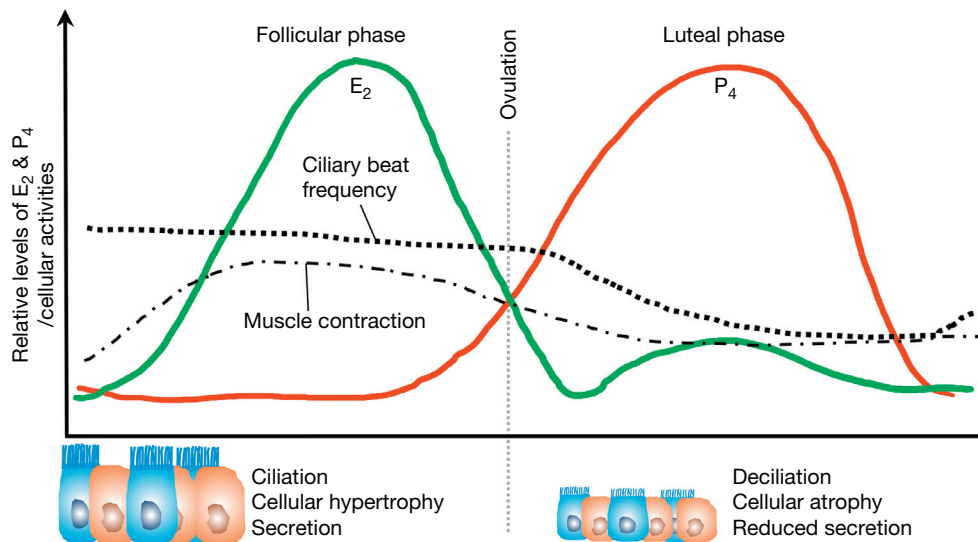


Fig. 4 The effects of circulating 17 β -estradiol (E_2) and progesterone (P_4) on cell morphology, ciliary beat frequency, and muscle contraction in Fallopian tubes during the follicular and luteal phases.

In mammals, P_4 is mainly secreted from the cumulus cells surrounding the oocyte. The circulating level of P_4 increases after ovulation and then declines at the end of the luteal phase. P_4 binds to progesterone receptors (PGRs), classical nuclear PGR-A and PGR-B, and membrane-bound PGRs. Classical PGRs respond to P_4 to decrease muscle contraction and ciliary beat frequency (Wanggren et al., 2008; Bylander et al., 2013). Membrane-bound PGRs (also known as membrane-bound progestin receptors, mPRs) are a family of PGR proteins recently discovered that consist of three members in humans and other vertebrates known as mPR α , mPR β , and mPR γ . The mPRs are localized mainly in the membrane of the cell and endoplasmic reticulum, and the expression of mPRs is tissue-specific (Kowalik et al., 2013). In humans and mice, mPR β is exclusively expressed in the cilia, whereas mPR γ primarily resides at the cilia basal area. Expression of mPRs in the Fallopian tube is regulated by P_4 and E_2 (Nutu et al., 2009). However, the function of these membrane-bound PGRs is still unclear.

In summary, E_2 promotes embryo transport by increasing epithelial cell hypertrophy, causing fluid secretion (Hamner and Fox, 1968), subsequently elevating ciliary beat frequency to induce a high rate of flow, and increasing the rate of muscle contraction

(via inhibition of P_4 activity) (Mahmood et al., 1998) (Fig. 4). P_4 induces deciliation (a loss of cilia on the ciliated epithelial cells), cell atrophy, and loss of secretory activity in secretory epithelial cells (Donnez et al., 1985; Pedrero-Badillo et al., 2013). Loss of secretory activity leads to a reduced fluid influx, resulting in viscous tubal fluid that slows the rate of embryo transport. When both E_2 and P_4 drop to basal levels, secretory epithelial cells restore their secretory activities, and ciliary cells regain ciliation for the next menstrual cycle (Verhage et al., 1979).

Health Condition Related to Defective Fallopian Tube

The most relevant health condition related to Fallopian tube defect is the ectopic pregnancy. Ectopic pregnancy is a form of abnormal gestation where a fertilized egg implants in tissues other than the endometrium of the uterus. During the early 2000s in the United States, 1%–2% of all pregnancies were ectopic, a dramatic increase from 0.4% in 1948. Many developed countries in Europe have also observed a doubling incident rate of ectopic pregnancies from the 1970s to 1990s.

The most common ectopic pregnancy site (>98%) is in the Fallopian tube. Among tubal ectopic pregnancies, one-third of the cases are associated with smoking, which damages ciliary function (Farquhar, 2005). Less than a third of tubal ectopic pregnancies are caused by tubal damage that can be sustained from infection or surgery. These physical damages disrupt muscle and ciliary function and lead to impaired embryo transport. Remaining cases are diagnosed with an unknown cause. Though deaths associated with ectopic pregnancy have declined due to advancement in medical improvement. However, it is still the most common maternal mortality with a morbidity rate of 9%–13% of all pregnancy related death during the first trimester. The mortality rate caused by ectopic pregnancy is much higher in developing countries (Shaw et al., 2010b).

Women with ectopic pregnancies have a reduced number of ciliated cells, suggesting that defective cell proliferation or differentiation could alter pregnancy outcome (Vasquez et al., 1983). Regarding molecular mechanisms, nitric oxide synthase (iNOS), prokineticins, cannabinoid receptors, mucin proteins, and immune system signaling pathways all are shown to be associated with the etiology of ectopic pregnancy (Shaw et al., 2010b). Women with ectopic pregnancy briefly have a higher level of iNOS in their Fallopian tube tissue. iNOS can alter tubal contractility and possibly ciliary beat frequency through the induction of nitric oxide (Vasquez et al., 1983; Al-Azemi et al., 2010). Prokineticins regulate smooth muscle contraction, and women with ectopic pregnancy show a reduced expression level of prokinetics (Maldonado-Perez et al., 2007; Shaw et al., 2010a). Interestingly, a defective cannabinoid receptor signaling pathway in mice causes embryo retention (Wang et al., 2004; Horne et al., 2008). A glycoprotein called mucin 1 (MUC1) is expressed in the epithelial cells of female reproductive tract. Epithelial mucin expression is normally suppressed to promote the embryo attachment in receptive uteri. In women with ectopic pregnancies, it is shown that expression of MUC1 is decreased in the tubal epithelial cells (Al-Azemi et al., 2009). Infection and inflammation can also induce high levels of interleukins and leukemia inhibitory factor (LIF), which promote tubal implantation of the embryo (Buchholz and Stephens, 2007; Guney et al., 2008). Several other signal pathways involved in the ectopic pregnancy are discussed in a comprehensive review (Shaw et al., 2010b).

Summary

This chapter summarizes what is known about the structure and function of the Fallopian tubes. The three segments of the Fallopian tube—mesosalpinx, myosalpinx, and endosalpinx—are described in details with basic cell biology and physiology. The article also summarizes the content and functionality of the tubal secretion and the cellular difference between each region. Sex steroids estrogen and progesterone influencing the physiology and the function of Fallopian tube are discussed. Lastly, we included the most common health condition related to pregnancy complication resulted from defective Fallopian tube—ectopic pregnancy. This condition impacts 1%–2% of pregnancies in the United States.

References

- Adams, C. E. (1979). Consequences of accelerated ovum transport, including a re-evaluation of Estes' operation. *Journal of Reproduction and Fertility*, 55, 239–246.
- Al-Azemi, M., Refaat, B., Aplin, J., & Ledger, W. (2009). The expression of MUC1 in human Fallopian tube during the menstrual cycle and in ectopic pregnancy. *Human Reproduction*, 24, 2582–2587.
- Al-Azemi, M., Refaat, B., Amer, S., et al. (2010). The expression of inducible nitric oxide synthase in the human fallopian tube during the menstrual cycle and in ectopic pregnancy. *Fertility and Sterility*, 94, 833–840.
- Anand, S., & Guha, S. K. (1982). Dynamics of the ampullary-isthmic junction in rabbit oviduct. *Gynecologic and Obstetric Investigation*, 14, 39–46.
- Andrade, Y. N., Fernandes, J., Vazquez, E., et al. (2005). TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *The Journal of Cell Biology*, 168, 869–874.
- Buchholz, K. R., & Stephens, R. S. (2007). The extracellular signal-regulated kinase/mitogen-activated protein kinase pathway induces the inflammatory factor interleukin-8 following Chlamydia trachomatis infection. *Infection and Immunity*, 75, 5924–5929.
- Bylander, A., Lind, K., Goksor, M., et al. (2013). The classical progesterone receptor mediates the rapid reduction of fallopian tube ciliary beat frequency by progesterone. *Reproductive Biology and Endocrinology*, 11, 33.

- Coy, P., & Yanagimachi, R. (2015). The common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. *Bioscience*, 65, 973–984.
- Cretoi, D., & Cretoi, S. M. (2016). Telocytes in the reproductive organs: current understanding and future challenges. *Seminars in Cell & Developmental Biology*, 55, 40–49.
- Cretoi, S. M., Cretoi, D., Suciu, L., & Popescu, L. M. (2009). Interstitial Cajal-like cells of human Fallopian tube express estrogen and progesterone receptors. *Journal of Molecular Histology*, 40, 387–394.
- Croxatto, H. B. (2002). Physiology of gamete and embryo transport through the fallopian tube. *Reproductive Biomedicine Online*, 4, 160–169.
- Croxatto, H. B., Ortiz, M. E., Diaz, S., et al. (1978). Studies on the duration of egg transport by the human oviduct. II. Ovum location at various intervals following luteinizing hormone peak. *American Journal of Obstetrics and Gynecology*, 132, 629–634.
- Dickens, C. J., Maguiness, S. D., Comer, M. T., et al. (1995). Human tubal fluid: formation and composition during vascular perfusion of the fallopian tube. *Human Reproduction*, 10, 505–508.
- Doerner, J. F., Delling, M., & Clapham, D. E. (2015). Ion channels and calcium signaling in motile cilia. *eLife*, 4.
- Donnez, J., Casanas-Roux, F., Caprasse, J., et al. (1985). Cyclic changes in ciliation, cell height, and mitotic activity in human tubal epithelium during reproductive life. *Fertility and Sterility*, 43, 554–559.
- Ezzati, M., Djahanbakhch, O., Arian, S., & Carr, B. R. (2014). Tubal transport of gametes and embryos: a review of physiology and pathophysiology. *Journal of Assisted Reproduction and Genetics*, 31, 1337–1347.
- Farquhar, C. M. (2005). Ectopic pregnancy. *Lancet*, 366, 583–591.
- Gaddum-Rosse, P., & Blandau, R. J. (1976). Comparative observations on ciliary currents in mammalian oviducts. *Biology of Reproduction*, 14, 605–609.
- Garcia-Pascual, A., Labadia, A., Triguero, D., & Costa, G. (1996). Local regulation of oviductal blood flow. *General Pharmacology*, 27, 1303–1310.
- Guerin, P., & Menezo, Y. (2011). Review: role of tubal environment in preimplantation embryogenesis: application to co-culture assays. *Zygote*, 19, 47–54.
- Guney, M., Erdemoglu, E., Oral, B., et al. (2008). Leukemia inhibitory factor (LIF) is immunohistochemically localized in tubal ectopic pregnancy. *Acta Histochemica*, 110, 319–323.
- Halbert, S. A., Szal, S. E., & Broderson, S. H. (1988). Anatomical basis of a passive mechanism for ovum retention at the ampulloisthmic junction. *The Anatomical Record*, 221, 841–845.
- Hamner, C. E., & Fox, S. B. (1968). Effect of oestrogen and progesterone on physical properties of rabbit oviduct fluid. *Journal of Reproduction and Fertility*, 16, 121–122.
- Horne, A. W., Phillips, J. A., Ill, Kane, N., et al. (2008). CB1 expression is attenuated in Fallopian tube and decidua of women with ectopic pregnancy. *PLoS One*, 3, e3969.
- Kane, M. T., Carney, E. W., & Ellington, J. E. (1992). The role of nutrients, peptide growth factors and co-culture cells in development of preimplantation embryos in vitro. *Theriogenology*, 38, 297–313.
- Kowalik, M. K., Rekawiecki, R., & Kotwica, J. (2013). The putative roles of nuclear and membrane-bound progesterone receptors in the female reproductive tract. *Reproductive Biology*, 13, 279–289.
- Leese, H. J., Tay, J. I., Reischl, J., & Downing, S. J. (2001). Formation of Fallopian tubal fluid: role of a neglected epithelium. *Reproduction*, 121, 339–346.
- Liao, S. B., Ho, J. C., Tang, F., & O, W. S. (2011). Adrenomedullin increases ciliary beat frequency and decreases muscular contraction in the rat oviduct. *Reproduction*, 141, 367–372.
- Liao, S. B., Cheung, K. H., Cheung, M. P., et al. (2013). Adrenomedullin increased the short-circuit current in the pig oviduct through chloride channels via the CGRP receptor: mediation by cAMP and calcium ions but not by nitric oxide. *Biology of Reproduction*, 89, 99.
- Lodish, H., Berk, A., Zipursky, S. L., et al. (2000). Cilia and flagella: structure and movement. In *Molecular cell biology* (4th edn.). New York: W. H. Freeman.
- Lyons, R. A., Saridogan, E., & Djahanbakhch, O. (2006). The reproductive significance of human Fallopian tube cilia. *Human Reproduction Update*, 12, 363–372.
- Mahmood, T., Saridogan, E., Smutna, S., et al. (1998). The effect of ovarian steroids on epithelial ciliary beat frequency in the human fallopian tube. *Human Reproduction*, 13, 2991–2994.
- Maisey, K., Nardocci, G., Imarai, M., et al. (2003). Expression of proinflammatory cytokines and receptors by human fallopian tubes in organ culture following challenge with *Neisseria gonorrhoeae*. *Infection and Immunity*, 71, 527–532.
- Maldonado-Perez, D., Evans, J., Denison, F., et al. (2007). Potential roles of the prokineticins in reproduction. *Trends in Endocrinology and Metabolism*, 18, 66–72.
- Mcgee, Z. A., Johnson, A. P., & Taylor-Robinson, D. (1981). Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or type 4. *The Journal of Infectious Diseases*, 143, 413–422.
- Miki, K., & Clapham, D. E. (2013). Rheotaxis guides mammalian sperm. *Current Biology*, 23, 443–452.
- Ng, A., Tan, S., Singh, G., et al. (2014). Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. *Nature Cell Biology*, 16, 745–757.
- Nutu, M., Weijdegard, B., Thomas, P., et al. (2009). Distribution and hormonal regulation of membrane progesterone receptors beta and gamma in ciliated epithelial cells of mouse and human fallopian tubes. *Reproductive Biology and Endocrinology*, 7, 89.
- Otsuki, Y., Maeda, Y., Magari, S., & Sugimoto, O. (1989). Lymphatics and lymphoid tissue of the fallopian tube: immunoelectronmicroscopic study. *The Anatomical Record*, 225, 288–296.
- Pauerstein, C. J., & Eddy, C. A. (1979). Morphology of the Fallopian tube. In F. K. Keller, & G. F. B. Schumacher (Eds.), *The biology of the fluids of the female genital tract*. Amsterdam: Elsevier/North-Holland.
- Pedrero-Badillo, F., Anaya-Hernandez, A., Corona-Quintanilla, D. L., et al. (2013). Morphohistological characteristics of rabbit oviduct: a proposal for a single regionalization. *Animal Reproduction Science*, 143, 102–111.
- Roatesi, I., Radu, B. M., Cretoi, D., & Cretoi, S. M. (2015). Uterine telocytes: a review of current knowledge. *Biology of Reproduction*, 93, 10.
- Shaw, J. L., Denison, F. C., Evans, J., et al. (2010a). Evidence of prokineticin dysregulation in fallopian tube from women with ectopic pregnancy. *Fertility and Sterility*, 94(1601–8 e1).
- Shaw, J. L., Dey, S. K., Critchley, H. O., & Horne, A. W. (2010b). Current knowledge of the aetiology of human tubal ectopic pregnancy. *Human Reproduction Update*, 16, 432–444.
- Snegovskikh, V., Mutlu, L., Massasa, E., & Taylor, H. S. (2014). Identification of putative fallopian tube stem cells. *Reproductive Sciences*, 21, 1460–1464.
- Stewart, C. A., & Behringer, R. R. (2012). Mouse oviduct development. *Results and Problems in Cell Differentiation*, 55, 247–262.
- Tay, J. I., Rutherford, A. J., Killick, S. R., et al. (1997). Human tubal fluid: production, nutrient composition and response to adrenergic agents. *Human Reproduction*, 12, 2451–2456.
- Vasquez, G., Winston, R. M., & Brosens, I. A. (1983). Tubal mucosa and ectopic pregnancy. *British Journal of Obstetrics and Gynaecology*, 90, 468–474.
- Verdugo, P. (1980). Ca^{2+} -dependent hormonal stimulation of ciliary activity. *Nature*, 283, 764–765.
- Verhage, H. G., Bareither, M. L., Jaffe, R. C., & Akbar, M. (1979). Cyclic changes in ciliation, secretion and cell height of the oviductal epithelium in women. *The American Journal of Anatomy*, 156, 505–521.
- Wang, H., Guo, Y., Wang, D., et al. (2004). Aberrant cannabinoid signaling impairs oviductal transport of embryos. *Nature Medicine*, 10, 1074–1080.
- Wanggren, K., Stavreus-Evers, A., Olsson, C., et al. (2008). Regulation of muscular contractions in the human Fallopian tube through prostaglandins and prostaglandins. *Human Reproduction*, 23, 2359–2368.
- Winuthayanon, W., Bernhardt, M. L., Padilla-Banks, E., et al. (2015). Oviductal estrogen receptor alpha signaling prevents protease-mediated embryo death. *eLife*, 4, e10453.

Further Reading

- Croxatto, H. B. (2002). Physiology of gamete and embryo transport through the fallopian tube. *Reproductive Biomedicine Online*, 4, 160–169.
- Coy, P., & Yanagimachi, R. (2015). The common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. *Bioscience*, 65, 973–984.
- Li, S., & Winuthayanon, W. (2017). Oviduct: roles in fertilization and early embryo development. *The Journal of Endocrinology*, 232, R1–R26.
- Lyons, R. A., Saridogan, E., & Djahanbakhch, O. (2006). The reproductive significance of human Fallopian tube cilia. *Human Reproduction Update*, 12, 363–372.
- Shaw, J. L., Dey, S. K., Critchley, H. O., & Horne, A. W. (2010b). Current knowledge of the aetiology of human tubal ectopic pregnancy. *Human Reproduction Update*, 16, 432–444.
- Suarez, S. S. (2016). Mammalian sperm interactions with the female reproductive tract. *Cell and Tissue Research*, 363, 185–194.

Aspects of Rodent Implantation

Jeeyeon M Cha, Vanderbilt University Medical Center, Nashville, TN, United States

Wenbo Deng, Jia Yuan, and Sudhansu K Dey, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Sexual reproduction and genetic diversification are foundations for evolutionary success to ensure perpetuation of the species by selecting for traits optimal for survival. Along this vein, mammalian pregnancy involves dynamic changes within the hormonally responsive pregnant uterus, nascent embryo, and transiently-lived mosaic tissues that promote their growth and reciprocal interactions. This involves the complex sequence of embryo development and implantation, decidualization, placentation and finally birth of offspring through the process of parturition (Dey et al., 2004; Lim and Wang, 2010; Cha et al., 2012). While the success of each event is critical for advancement to the next stage, the hierarchical directives that orchestrate embryo-uterine crosstalk are not fully characterized. These events are primarily directed by ovarian estrogen and progesterone (Dey et al., 2004); however, the molecular dialogue between the mother and embryo governing the orderly transitions of pregnancy events is yet to be fully understood.

The uterus is embryologically derived from the intermediate mesoderm and Mullerian ducts, which fuse at their caudal ends and differentiate to form the distinct cell types of the myometrium and the endometrium (Finn and Porter, 1975). The endometrium is hormonally responsive to ovarian hormones and comprised of the luminal epithelium, stroma and interspersed tubular glands which open and release contents into the uterine lumen. While the majority of endometrial cell types are defined during development, gland formation occurs postnatally from the uterine lumen as invaginations projecting toward the antimesometrial domain and is believed to be hormonally mediated (Filant and Spencer, 2014). A recent study shows that a differential Wnt gradient at the mesometrial (M) and anti-mesometrial (AM) domains directs the distribution of glands at the AM domain (Goad et al., 2017). Additional evidence shows that glands opening into the lumen secrete LIF under the regulation of FoxA2, which is essential for implantation in mice (Kelleher et al., 2017).

Molecular and cellular changes occur early in pregnancy to prepare the uterus for implantation (receptive phase). With the acquisition of blastocyst competency in the receptive uterus, implantation ensues (Dey et al., 2004). This “window of implantation” is a time-sensitive transient period when blastocyst competency coincides with the receptive state of the uterus on specific day(s) of pregnancy. When this coordination falls out of phase, implantation fails or becomes defective and generates adverse ripple effects throughout the course of pregnancy, leading to a poor pregnancy outcome (Song et al., 2002; Cha et al., 2012). The receptive window for implantation is also thought to be transient in humans; implantation past this window can lead to spontaneous miscarriages (Wilcox et al., 1999). In humans, the natural rate of conception per cycle (~30%), and up to 75% of failed pregnancies seem to be due to implantation failure (Norwitz et al., 2001). However, a significant number of miscarriages result from abnormal embryo development due to aneuploidy (reviewed in (Cha et al., 2012).

Implantation Stages and Types

Embryo implantation was historically dubbed “nidation” which originates from the word “nidus”, meaning a nest or breeding place. It is the first cooperative physical and physiological interaction between the epithelium (trophectoderm) of the blastocyst and the maternal endometrial lining (luminal epithelium). The prerequisites for mammalian embryo implantation include preimplantation embryo development and blastocyst competency for implantation, differentiation of the uterus to a receptive state, formation of uterine crypts (implantation chambers) and attachment of the blastocyst with the luminal epithelium. These events are the result of a reciprocal blastocyst-uterine dialogue (Lim and Wang, 2010; Cha et al., 2012). In mice and rats, initiation of implantation occurs on the evening of day 4 (day 1 = morning of finding vaginal plug) or day 5 of pregnancy (day 1 = sperm in vaginal smears), respectively. One of the early signs of implantation in many species is a local increase in vascular permeability at the site of blastocyst apposition, which can be monitored by an intravenous injection of a macromolecular blue dye that binds to serum proteins and leaks out at sites of increased vascular permeability; this process demarcates the implantation sites as distinct blue bands (Psychoyos, 1973). Following implantation, the underlying stromal cells proliferate and differentiate to decidual cells (decidualization) accompanied by marked matrix remodeling and angiogenesis to establish communications between the nascent vascular system of the conceptus and mother to form the presumptive placenta (Cha et al., 2012).

Enders and Schlafke characterized implantation as occurring in three discrete stages: apposition, adhesion, and penetration (Enders and Schlafke, 1969). During apposition, the blastocyst trophoctoderm becomes closely apposed to the uterine luminal epithelium (LE). When this intimate association is sufficient to resist dislocation of the blastocyst upon flushing the uterine lumen, implantation has progressed to the adhesion stage. Penetration is then associated with the breaching of LE by the trophoctoderm. By this stage, decidualization of underlying stromal cells progresses with extensive loss of the epithelial cells.

The entry of trophoblast cells into the stroma with the advancement of implantation was thought to be mediated by epithelial apoptosis. However, recent studies in mice have provided evidence that epithelial cells in contact with the blastocyst are engulfed by trophoctoderm cells by a nonapoptotic, cell-in-cell internalization process called entosis (Li et al., 2015). This process, which has

been previously characterized in cancer, was shown to have a physiologic role in normal pregnancy. However, the molecular mechanism behind this process is yet to be defined.

Implantation strategies vary between species and have been classified on the basis of divergent cell-cell interactions between the blastocyst and uterus. Bonnet classified implantation into three categories: central, eccentric and interstitial (Bonnet, 1884). Central implantation, observed in rabbits, ferrets, and some marsupials, occurs when blastocysts extensively expand prior to implantation to be closely apposed and maximally interact with the LE. In contrast, blastocysts in mice, rats, and hamsters show only modest expansion and undergo eccentric implantation, during which implantation chambers are formed within evaginations from the uterine lumen. Notably in rodents, implantation always occurs at the anti-mesometrial (AM) domain of the uterus, opposite to the mesometrial (M) domain, the entry site of blood vessels into the uterus (see below: **Uterine Changes in Implantation**). In contrast, implantation occurs at the mesometrial site in bats. Interestingly, during interstitial implantation observed in guinea pigs, chimpanzees and humans, blastocysts implant by entrenching into the subepithelial stroma (Dey et al., 2004).

Schlafke and Enders further classified implantation types as intrusive, displacement and fusion based on the results of ultra-structural electron microscopy studies (Schlafke and Enders, 1975). During intrusive types of implantation, which is seen in humans and guinea pigs, trophoblast cells penetrate through the LE to reach the basal lamina. In displacement type of implantation which occurs in rodents, the basal lamina disassociates from the overlying LE to facilitate trophoblast invasion into the subepithelial stromal bed. In contrast, rabbits exhibit fusion type of implantation in which trophoblast cells fuse with the LE by forming symplasma (trophoblastic knob, the syncytial aggregates that attach to and invade the endometrium) (Schlafke and Enders, 1975). In rabbits, attachment stimulates an angiogenic response, particularly at the trophoblastic knobs, the syncytial aggregates that attach to and invade the endometrium with increased expression of vascular endothelial growth factor (Das et al., 1997).

Noninvasive implantation has been observed in large animals and marsupials, such as the pig, sheep, cow, horse, and wallaby (Renfree and Shaw, 2000; Roberts et al., 2008; Bazer, 2015). For example, pig blastocysts remain in a “free-floating” state with noninvasive implantation until day 12 (termed pregnancy recognition) at which point it elongates up to 100 mm in length, primarily due to the rapid growth of the extraembryonic tissue. This strategy allows an efficient nutrient and metabolite exchanges between the uterus and conceptus until the attachment reaction.

Uterine Changes During Implantation

Corner once remarked: “the uterine chamber is actually a less favorable place for early embryos to implant than say, the anterior chamber of the eye, except when the hormones of the ovary act upon it and change it into a place of superior efficiency for its new functions” (Corner, 1947). The differentiation of the uterus to a receptive state during pregnancy that renders it favorable for the implantation and development of an embryo was first described by Alexandre Psychoyos. By reciprocal embryo transfer experiments using pseudopregnant and delayed-implanting rodent models, he established the concept of the transient window of receptivity by showing that blastocysts only implanted when transferred into a hormonally prepared, receptive uterus (Psychoyos, 1973). It was found that blastocysts implantation in uteri of mice or rats requires at least 24–48 h of P₄ priming superimposed with a small amount of estrogen (Psychoyos, 1973). Later, reciprocal blastocyst transfer experiments showed that the acquisition of blastocyst activation and uterine receptivity for implantation both require in vivo exposure to estrogen (Paria et al., 1993). Thus far, all mammals studied exhibit a transient window of uterine receptivity of varying duration for implantation (Cha et al., 2012; Yoshinaga, 2013).

Although both P₄ and estrogen are essential for implantation in mice and rats, ovarian estrogen is not a requirement for implantation in hamsters, rabbits and pigs. Blastocysts can implant in these species only in the presence of P₄; surprisingly, neither P₄ nor estrogen is required for implantation in guinea pigs (Deanesly, 1963). It was conjectured that embryos in these species can locally produce the steroid hormones required for implantation. Indeed, biochemical experiments provided evidence that the rabbit and pig blastocysts have the capacity to synthesize estrogens (Perry et al., 1973; Hoversland et al., 1982); this capacity was not found in mouse blastocysts (Stromstedt et al., 1996).

The acquisition of uterine receptivity in preparation for blastocyst attachment is reflected in both cellular and molecular changes involving the three major uterine compartments (epithelium, stroma, and myometrium) uniquely responding to changing ovarian P₄ and estrogen secretion. To create the window of uterine receptivity, the cooperative interactions between P₄ and estrogen regulate uterine cell proliferation and/or differentiation in a spatiotemporal manner in mice and rats. In rodents and humans, there is a gradual loss of apicobasal LE cell polarity and formation of microprotrusions called pinopodes or uterodomes on the apical surface of the LE impending blastocyst attachment (Thie et al., 1996; Nikas and Psychoyos, 1997).

Along the same vein, the epithelial apico-basal cell polarity is considered critical to implantation. In the search for the molecular mechanism underpinning this event, muscle-segment homeobox genes *Msx1* and *Msx2* were found to play a major role in modulating apico-basal polarity and implantation by altering uterine *Wnt5a* levels (Daikoku et al., 2011). MSX transcription factors are also important for regulating delayed implantation in the mouse, tammar wallaby and North American mink (Cha et al., 2013b). The molecular aspects of embryo competency and uterine receptivity are described in separate chapters.

Blood vessels travel to the uterus through the mesometrium and anatomically orient the uterus into mesometrial and anti-mesometrial domains (Cha et al., 2012). In rodents, embryo homing and implantation occur within a crypt (implantation

chamber) invariably at the AM domain along the uterus. On day four of pregnancy in mice (day 1 = vaginal plug), epithelial evaginations (folding) project from the uterine lumen toward the AM domain. A certain number of projections provokes blastocysts to form crypts for embryo implantation. The unique architectural organization of crypts for blastocyst homing and attachment was first recognized in rodents more than a century ago (Burckhard, 1901) and later by Enders' group (Enders et al., 1980). However, the mechanism by which epithelial evaginations are appropriately directed to form crypts at the AM domain for embryo homing and implantation remained unknown. It also remains undefined how the crypts homing the embryos are regularly spaced.

Recent studies show that crypt formation at the AM domain requires non-canonical Wnt5a-ROR signaling. Epithelial projections form along a *Wnt5a* expression gradient, and mice with disruption of uterine Wnt5a-ROR signaling with conditional uterine loss or gain of function of *Wnt5a* or loss of function of *Ror1/Ror2* (*Wnt5a* co-receptors) resulted in disorderly epithelial projections, crypt formation, embryo spacing, and impaired implantation (Cha et al., 2014). These early disturbances under abnormal Wnt5a-ROR signaling were reflected in adverse late pregnancy events (Cha et al., 2014). Further studies showed that Wnt5a-ROR signaling intersects planar cell polarity (PCP) signaling orchestrating the formation of directed epithelial evaginations to form crypts for implantation in mice (Yuan et al., 2016).

PCP is a developmental pathway essential for establishing spatial cues in multicellular tissues during organogenesis and branching morphogenesis during mammary gland development (Yang and Mlodzik, 2015). In animal models, disrupting PCP signaling genetically or pharmacologically results in abnormal hair cell orientation, giving rise to defects in neural tube closure and left-right asymmetry; in humans, polymorphisms of PCP components are associated with an array of developmental defects including spina bifida and cardiac outflow malformations (van Amerongen and Nusse, 2009). While PCP has been studied extensively during development, its role in adult tissues in physiological conditions has been limited. Recent studies show that uterine deletion of Vang-like protein 2 (*Vangl2*), but not *Vangl1*, confers aberrant PCP signaling, misdirected epithelial evaginations, defective crypt formation, and blastocyst attachment, leading to compromised pregnancy outcomes (Yuan et al., 2016). The study identified a novel role for PCP in executing spatial cues for crypt formation and implantation (Fig. 1).

PCP signaling is evolutionarily conserved, although it is not known whether uterine PCP activity is important for implantation across species including humans. Additional studies have shown that the correct orientation of the embryonic axis within the crypt is also critical for pregnancy success in mice (Zhang et al., 2014); Notch signaling was determined to be a key component.

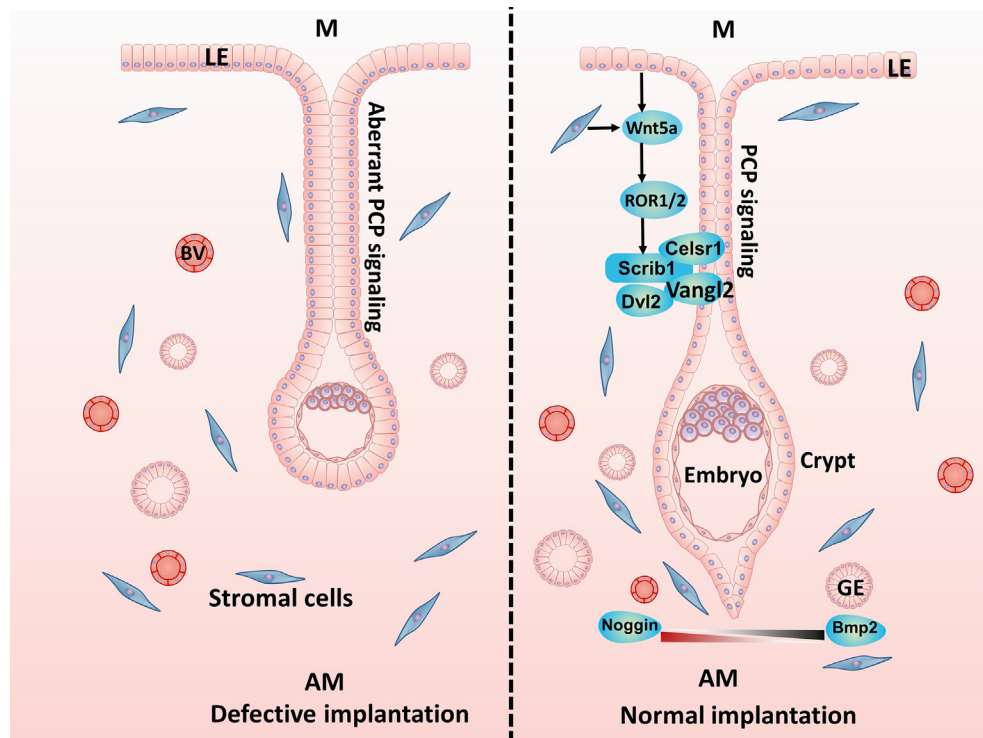


Fig. 1 A schematic diagram of PCP signaling pathway in designing epithelial evagination and crypt formation for embryo implantation at the AM domain of the uterus. As the downstream signaling of Wnt5a-ROR, *Vangl2* directly interacts with *Scrib*, *Celsr1*, and *Dvl2* to form a complex to maintain epithelium planar polarity. After blastocyst attachment within the crypt epithelium, an opposing balance between *Noggin* and *Bmp2* signaling in the subepithelial stroma facilitates the progression of implantation and decidualization. Right panel shows Wnt5a-ROR-PCP signaling regulates the luminal epithelium to form a “conch-shaped” crypt to home the embryo (normal implantation). Left panel shows defective implantation in a roundish crypt due to aberrant PCP signaling. M, mesometrium; AM, antimesometrium; BV, blood vessel; GE, glandular epithelium; LE, luminal epithelium.

Defective Implantation and Decidualization Causes Adverse Ripple Effects

Despite scientific advances in human fertility by assisted reproductive technologies, the implantation rate and number of ‘take-home’ babies still remain low (Norwitz et al., 2001). Poor embryo quality, transfer of embryos into uteri of unknown states of receptivity, and complications from inferior quality of implantation are all barriers to successful pregnancy. Indeed, defective implantation was shown to propagate adverse ripple effects throughout the remainder of pregnancy, resulting in compromised pregnancy outcomes, including preterm birth and preeclampsia which can have life-long health effects on the offspring.

Mouse models have helped to define propagation of early defects through the remaining course of pregnancy. The first study of adverse ripple effects from defective implantation was noted in mice deficient in *Pla2g4a* (encoding cPLA 2α), which generates arachidonic acid from membrane phospholipids for prostaglandin synthesis via COX enzymes. Implantation in *Pla2g4a* $-/-$ mice occurs beyond the normal window of implantation (deferred implantation), resulting in embryo crowding, conjoined placenta, arrested fetoplacental development, increased resorptions and reduced litter size (Song et al., 2002). The authors also performed proof-of-principle physiological experiments in which blastocysts were transferred to wild-type mouse uteri beyond the anticipated window of uterine receptivity and showed similar adverse phenotypes of reduced litter size and increased resorption. Finally, similar findings were observed for mice deficient in *Lpar3* (LPA3), a receptor for lysophosphatidic acid (Ye et al., 2005). The similar phenotypes were attributed to reduced production of prostaglandins generated by COX2, implicating a LPA3-cPLA 2 -Cox2 signaling axis as a critical determinant for on-time implantation.

Preeclampsia is believed to result from abnormal placentation. A recent study in a mouse model which spontaneously develops the cardinal features of preeclampsia showed periimplantation defects including upregulation of Cox2 and IL-15 at the maternal-fetal interface (Sones et al., 2016). This was associated with decreased decidual natural killer (dNK) cells, which have important roles in placental perfusion. A single administration of a Cox2 inhibitor (celecoxib) during decidualization restrained Cox2 activity and IL-15 expression, restored dNK cell numbers, improved fetal growth, and attenuated late gestational hypertension. This study provides evidence that decidual overexpression of Cox2 and IL-15 may trigger the adverse pregnancy outcome reflected in the preeclampsia syndrome.

Aberrant decidualization can also give rise to adverse pregnancy phenotypes including aberrations in parturition. Preterm birth is one example of an adverse pregnancy effect of defective decidualization. The Dey lab has shown that mice with uterine deletion of *Trp53* (encoding p53; *Trp53*^{d/d}) show normal implantation, however, 50–60% of *Trp53*^{d/d} mice exhibit preterm birth with dystocia and fetal death (Hirota et al., 2010). These mice have compromised decidualization with more terminally differentiated decidual cells than control littermates with increased polyploidy, senescence-associated growth restriction, and heightened expression of pAkt, p21 and Cox2. Many risk factors, such as genetic mutation, infection, inflammation and stress that lead to preterm birth are also reported to exacerbate cellular senescence via mammalian target of rapamycin complex 1 (mTORC1) signaling. Rapamycin attenuates senescence and increase life span in mice (Harrison et al., 2009). *Trp53*^{d/d} decidua have increased mTORC1 activity, which is inhibited by rapamycin or metformin with attenuation of premature decidual senescence and rescue of preterm birth (Hirota et al., 2010; Cha et al., 2013a). This finding is intriguing since women of advanced age exhibit higher rates of preterm birth (Cnattingius et al., 1992) and a cohort of Japanese women who delivered preterm showed increased decidual senescence compared to term counterparts (Cha et al., 2013a). These results are a paradigm shift in our understanding of the physiology of birth timing and pathogenesis of preterm birth by identifying a decidual origin of preterm birth which can be targeted using mTORC1 inhibitors (Fig. 2). Whether this intervention can be applied broadly remains to be studied; clinical research to study decidual senescence in women with higher risk factors for preterm birth will be useful to target this global problem.

In this regard, higher levels of endocannabinoid signaling have also been shown to enhance preterm birth in response to a mild inflammatory stimulus. Increased p38/MAPK independent of mTORC1 signaling accelerates decidual senescence (Sun et al., 2016), suggesting that decidual senescence may function as a common final pathway integrating multiple signaling pathways toward birth timing.

Future Considerations

With marked advances in technology, much remains to be revealed about the dynamic physiological and molecular interactions encompassing early pregnancy events. The precise timing of implantation and the molecular discourse between the embryo and uterus have yet to be determined in humans. Already, advances in high fidelity RNA-Seq and MALDI-MS proteomics have helped to identify low-abundance molecular and modified mediators not previously noted. Recent application of in situ mass spectrometry in periimplantation mouse uteri also provide opportunities to generate spatiotemporal maps of proteins and lipids as well as their modifications in other species including humans (Burnum et al., 2009). Furthermore, analysis of the single cell transcriptome of human preimplantation embryos and the epigenetic signature of primordial germ cells has opened new avenues to map the transcriptome of single epithelial and stromal cells in the uterus around the time of implantation (Yan et al., 2013; Guo et al., 2015; Petropoulos et al., 2016). These datasets coupled with those from MALDI-MS proteomics and lipidomics profiles can be used to

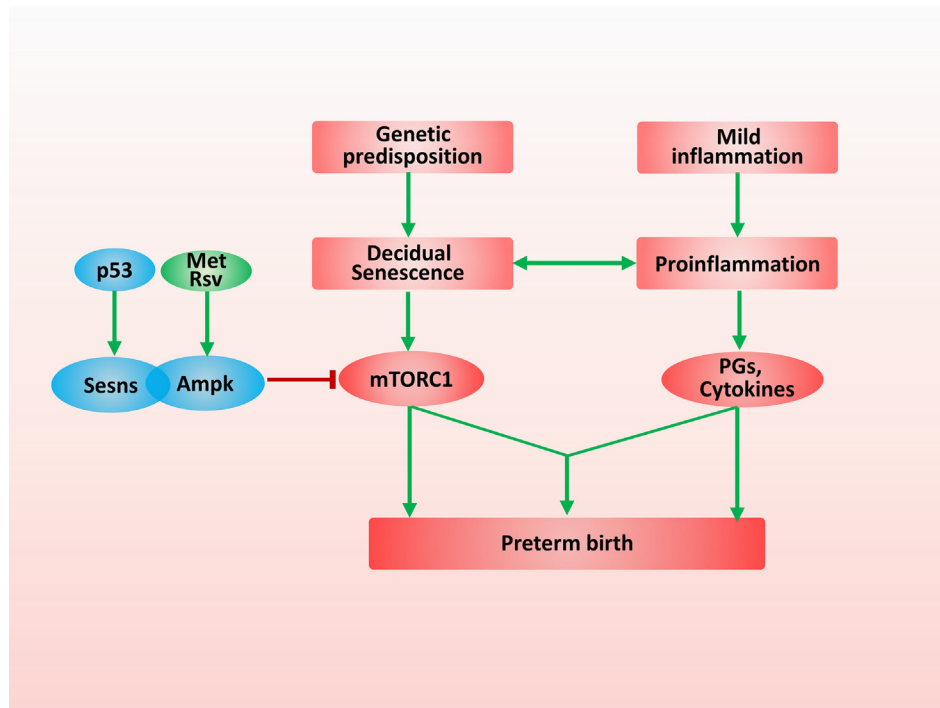


Fig. 2 A scheme showing that defective decidualization leads to preterm birth. Decidual senescence caused by genetic predisposition due to gene mutation superimposed by mild inflammation confers compromised decidual health and increased susceptibility to preterm birth. Heightened mTORC1 signaling is a signature of premature decidual. Signaling by p53-Sesn2 dampens mTORC1 signaling which is further helped by Met and Rsv through increased AMPK signaling in the context of inflammatory insult.

identify mediators secreted by embryos freely or in exosomes and their downstream effects that can promote their own growth or signal the endometrium for implantation.

Epigenetic regulation allows for versatile and reversible changes in gene expression without changing the underlying DNA sequence. New insights on epigenetic regulation of reproductive events continue to be gleaned, including the roles of histone modifications, chromatin remodeling, DNA methylation, microRNA and long noncoding RNAs (lncRNA), and novel transcription factor response elements such as the super enhancer identification (Pott and Lieb, 2015; Dekker and Mirny, 2016). The signature of estrogen and progesterone receptors binding sites and their downstream mediators have been assessed by genome-wide assays and are likely to have a substantial impact on implantation and pregnancy events (Pott and Lieb, 2015; Rubel et al., 2016; Vasquez et al., 2016). There is evidence regarding the roles of histone modifications in reproduction, especially the roles of the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) which are distinguishable by their core components. PRC1 components, including the E3 protein ligases responsible for ubiquitination of histone H2A, Ring1A and Ring1B, (Margueron and Reinberg, 2011) and chromobox (CBX) family (Schwartz and Pirrotta, 2013) are differentially and spatiotemporally expressed in the periimplantation uteri. Notably, inhibition of PRC1 activity by a Ring1A/B inhibitor compromises decidualization and polyploidy development during early pregnancy in vivo, while interference of Cbx4 expression in stromal cells also show defective stromal cell decidualization and polyploidy development in vitro (Bian et al., 2016). During decidualization and decidual polyploidy, DNA methylation was shown to be important in hormone-dependent gene expression in the pregnant uterus (Gao et al., 2015). H3K27 methyltransferase activity by PRC2 is relayed by its component proteins including EZH1/EZH2, SUZ12 polycomb repressive complex 2 subunit (SUZ12) and RB binding protein 7 (RBAP46) or RB binding protein 4 (RBAP48) (Blackledge et al., 2015). The increased H3K27me3 at the promoter of chemokine (C–C motif) ligand 8 (CCL8) and chemokine (C–C motif) ligand 9 (CCL9), critical chemokines for T cell migration from stroma to myometrium, confers a local immune-privileged region for embryo development, indicating the importance of this epigenetic mark in pregnancy (Nancy et al., 2012). Finally, EZH2 has dynamic expression during the menstrual cycle in humans and is suppressed in decidualized stromal cells, implicating a role for EZH2-PRC2 mediated chromatin remodeling in the human endometrium (Grimaldi et al., 2012). The role of these epigenetic marks in pregnancy events remains to be validated in a physiological setting. The novel Crispr-Cas9 system will provide an efficient way to systematically isolate the function of these targets in mammalian systems (Hsu et al., 2014; Gorski et al., 2017). Progress in precisely defining the molecular window of implantation and identifying regulators of dynamic embryo-uterine interactions will continue in stride with refined technological tools. These evolving areas of research warrant careful investigation in animal models which can then be expanded to human studies.

Acknowledgements

Our sincere appreciation to Katie Gerhardt for her help in editing this review article. The work described from Dey lab has been funded by NIH (R01HD068524 and DA006668) and March of Dimes (21-FY12-127 and 22-FY13-543). We regret that space limitations precluded us from citing many relevant references.

References

- Bazer, F. W. (2015). History of maternal recognition of pregnancy. *Advances in Anatomy, Embryology, and Cell Biology*, 216, 5–25.
- Bian, F., Gao, F., Kartashov, A. V., Jegga, A. G., Barski, A., & Das, S. K. (2016). Polycomb repressive complex 1 controls uterine decidualization. *Scientific Reports*, 6, 26061.
- Blackledge, N. P., Rose, N. R., & Klose, R. J. (2015). Targeting Polycomb systems to regulate gene expression: Modifications to a complex story. *Nature Reviews. Molecular Cell Biology*, 16(11), 643–649.
- Bonnet, R. (1884). Beitrage zur embryologie der wiederkauer, gewonnen am schafei. *Archiv für anatomie und physiologie*, 8, 170–230.
- Burnum, K. E., Cornett, D. S., Puolitaival, S. M., Milne, S. B., Myers, D. S., Tranguch, S., Brown, H. A., Dey, S. K., & Caprioli, R. M. (2009). Spatial and temporal alterations of phospholipids determined by mass spectrometry during mouse embryo implantation. *Journal of Lipid Research*, 50(11), 2290–2298.
- Cha, J., Bartos, A., Egashira, M., Haraguchi, H., Saito-Fujita, T., Leishman, E., Bradshaw, H., Dey, S. K., & Hirota, Y. (2013a). Combinatory approaches prevent preterm birth profoundly exacerbated by gene-environment interactions. *The Journal of Clinical Investigation*, 123(129), 4063–4075.
- Cha, J., Bartos, A., Park, C., Sun, X., Li, Y., Cha, S. W., Ajima, R., Ho, H. Y., Yamaguchi, T. P., & Dey, S. K. (2014). Appropriate crypt formation in the uterus for embryo homing and implantation requires Wnt5a-ROR signaling. *Cell Reports*, 8(2), 382–392.
- Cha, J., Sun, X., Bartos, A., Fenelon, J., Lefevre, P., Daikoku, T., Shaw, G., Maxson, R., Murphy, B. D., Renfree, M. B., & Dey, S. K. (2013b). A new role for muscle segment homeobox genes in mammalian embryonic diapause. *Open Biology*, 3(4).
- Cha, J., Sun, X., & Dey, S. K. (2012). Mechanisms of implantation: Strategies for successful pregnancy. *Nature Medicine*, 18(12), 1754–1767.
- Cnattingius, S., Forman, M. R., Berendes, H. W., & Isotalo, L. (1992). Delayed childbearing and risk of adverse perinatal outcome. A population-based study. *JAMA*, 268(7), 886–890.
- Comer, G. (1947). *The hormones in human reproduction*. Princeton, NJ: Princeton University Press.
- Daikoku, T., Cha, J., Sun, X., Tranguch, S., Xie, H., Fujita, T., Hirota, Y., Lydon, J., DeMayo, F., Maxson, R., & Dey, S. K. (2011). Conditional deletion of Msx homeobox genes in the uterus inhibits blastocyst implantation by altering uterine receptivity. *Developmental Cell*, 21(6), 1014–1025.
- Das, S. K., Chakraborty, I., Wang, J., Dey, S. K., & Hoffman, L. H. (1997). Expression of vascular endothelial growth factor (VEGF) and VEGF-receptor messenger ribonucleic acids in the peri-implantation rabbit uterus. *Biology of Reproduction*, 56(6), 1390–1399.
- Deanesly, R. (1963). Further observations on the effects of oestradiol on tubal eggs and implantation in the guinea-pig. *Journal of Reproduction and Fertility*, 5, 49–57.
- Dekker, J., & Mirny, L. (2016). The 3D genome as moderator of chromosomal communication. *Cell*, 164(6), 1110–1121.
- Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T., & Wang, H. (2004). Molecular cues to implantation. *Endocrine Reviews*, 25(3), 341–373.
- Enders, A. C., & Schlafke, S. (1969). Cytological aspects of trophoblast-uterine interaction in early implantation. *The American Journal of Anatomy*, 125(1), 1–29.
- Enders, A. C., Schlafke, S., & Welsh, A. O. (1980). Trophoblastic and uterine luminal epithelial surfaces at the time of blastocyst adhesion in the rat. *The American Journal of Anatomy*, 159(1), 59–72.
- Filant, J., & Spencer, T. E. (2014). Uterine glands: Biological roles in conceptus implantation, uterine receptivity and decidualization. *The International Journal of Developmental Biology*, 58(2–4), 107–116.
- Finn, C., & Porter, D. (1975). *The Uterus*. London: Paul Elek (Scientific Books) Ltd.
- Gao, L., Rabbitt, E. H., Condon, J. C., Renthal, N. E., Johnston, J. M., Mitsche, M. A., Chambon, P., Xu, J., O'Malley, B. W., & Mendelson, C. R. (2015). Steroid receptor coactivators 1 and 2 mediate fetal-to-maternal signaling that initiates parturition. *The Journal of Clinical Investigation*, 125(7), 2808–2824.
- Goad, J., Ko, Y. A., Kumar, M., Syed, S. M., & Tanwar, P. S. (2017). Differential Wnt signaling activity limits epithelial gland development to the anti-mesometrial side of the mouse uterus. *Developmental Biology*, 423(2), 138–151.
- Gorski, S. A., Vogel, J., & Doudna, J. A. (2017). RNA-based recognition and targeting: Sowing the seeds of specificity. *Nature Reviews. Molecular Cell Biology*.
- Grimaldi, G., Christian, M., Quenby, S., & Brosens, J. J. (2012). Expression of epigenetic effectors in decidualizing human endometrial stromal cells. *Molecular Human Reproduction*, 18(9), 451–458.
- Guo, F., Yan, L., Guo, H., Li, L., Hu, B., Zhao, Y., Yong, J., Hu, Y., Wang, X., Wei, Y., Wang, W., Li, R., Yan, J., Zhi, X., Zhang, Y., Jin, H., Zhang, W., Hou, Y., Zhu, P., Li, J., Zhang, L., Liu, S., Ren, Y., Zhu, X., Wen, L., Gao, Y. Q., Tang, F., & Qiao, J. (2015). The Transcriptome and DNA Methylation landscapes of human primordial germ cells. *Cell*, 161(6), 1437–1452.
- Harrison, D. E., Strong, R., Sharp, Z. D., Nelson, J. F., Astle, C. M., Flurkey, K., Nadon, N. L., Wilkinson, J. E., Frenkel, K., Carter, C. S., Pahor, M., Javors, M. A., Fernandez, E., & Miller, R. A. (2009). Rapamycin fed late in life extends lifespan in genetically heterogeneous mice. *Nature*, 460(7253), 392–395.
- Hirota, Y., Daikoku, T., Tranguch, S., Xie, H., Bradshaw, H., & Dey, S. K. (2010). Uterine-specific p53 deficiency confers premature uterine senescence and promotes preterm birth in mice. *Journal of Clinical Investigation*, 120(3), 803–815.
- Hoversland, R. C., Dey, S. K., & Johnson, D. C. (1982). Catechol estradiol induced implantation in the mouse. *Life Sciences*, 30(21), 1801–1804.
- Hsu, P. D., Lander, E. S., & Zhang, F. (2014). Development and applications of CRISPR-Cas9 for genome engineering. *Cell*, 157(6), 1262–1278.
- Kelleher, A. M., Peng, W., Pru, J. K., Pru, C. A., DeMayo, F. J., & Spencer, T. E. (2017). Forkhead box a2 (FOXA2) is essential for uterine function and fertility. *Proceedings of the National Academy of Sciences of the United States of America*, 114(6), E1018–E1026.
- Li, Y., Sun, X., & Dey, S. K. (2015). Entosis allows timely elimination of the luminal epithelial barrier for embryo implantation. *Cell Reports*, 11(3), 358–365.
- Lim, H. J., & Wang, H. (2010). Uterine disorders and pregnancy complications: Insights from mouse models. *The Journal of Clinical Investigation*, 120(4), 1004–1015.
- Margueron, R., & Reinberg, D. (2011). The Polycomb complex PRC2 and its mark in life. *Nature*, 469(7330), 343–349.
- Nancy, P., Tagliani, E., Tay, C. S., Asp, P., Levy, D. E., & Erlebacher, A. (2012). Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal-fetal interface. *Science*, 336(6086), 1317–1321.
- Nikas, G., & Psychoyos, A. (1997). Uterine pinopodes in peri-implantation human endometrium. Clinical relevance. *Annals of the New York Academy of Sciences*, 816, 129–142.
- Norwitz, E. R., Schust, D. J., & Fisher, S. J. (2001). Implantation and the survival of early pregnancy. *The New England Journal of Medicine*, 345(19), 1400–1408.
- Paria, B. C., Huet-Hudson, Y. M., & Dey, S. K. (1993). Blastocyst's State of activity determines the "window" of implantation in the receptive mouse uterus. *Proceedings of the National Academy of Sciences of the United States of America*, 90(21), 10159–10162.
- Perry, J. S., Heap, R. B., & Amoroso, E. C. (1973). Steroid hormone production by pig blastocysts. *Nature*, 245(5419), 45–47.
- Petropoulos, S., Edsgard, D., Reinius, B., Deng, Q., Panula, S. P., Codeluppi, S., Reyes, A. P., Linnarsson, S., Sandberg, R., & Lanner, F. (2016). Single-cell RNA-Seq reveals lineage and X chromosome dynamics in human Preimplantation embryos. *Cell*, 167(1), 285.
- Pott, S., & Lieb, J. D. (2015). What are super-enhancers? *Nature Genetics*, 47(1), 8–12.
- Psychoyos, A. (1973). Hormonal control of oviimplantation. *Vitamins and Hormones*, 31, 201–256.

- Renfree, M. B., & Shaw, G. (2000). Diapause. *Annual Review of Physiology*, 62, 353–375.
- Roberts, R. M., Chen, Y., Ezashi, T., & Walker, A. M. (2008). Interferons and the maternal-conceptus dialog in mammals. *Seminars in Cell & Developmental Biology*, 19(2), 170–177.
- Rubel, C. A., Wu, S. P., Lin, L., Wang, T., Lanz, R. B., Li, X., Kommagani, R., Franco, H. L., Camper, S. A., Tong, Q., Jeong, J. W., Lydon, J. P., & DeMayo, F. J. (2016). A Gata2-dependent transcription network regulates uterine progesterone responsiveness and endometrial function. *Cell Reports*, 17(5), 1414–1425.
- Schlafke, S., & Enders, A. C. (1975). Cellular basis of interaction between trophoblast and uterus at implantation. *Biology of Reproduction*, 12(1), 41–65.
- Schwartz, Y. B., & Pirrotta, V. (2013). A new world of Polycombs: Unexpected partnerships and emerging functions. *Nature Reviews. Genetics*, 14(12), 853–864.
- Sones, J. L., Cha, J., Woods, A. K., Bartos, A., Heyward, C. Y., Lob, H. E., Isroff, C. E., Butler, S. D., Shapiro, S. E., Dey, S. K., & Davisson, R. L. (2016). Decidual Cox2 inhibition improves fetal and maternal outcomes in a preeclampsia-like mouse model. *Journal of Clinical Investigation Insight*, 1(3).
- Song, H., Lim, H., Paria, B. C., Matsumoto, H., Swift, L. L., Morrow, J., Bonventre, J. V., & Dey, S. K. (2002). Cytosolic phospholipase A2alpha is crucial [correction of A2alpha deficiency is crucial] for 'on-time' embryo implantation that directs subsequent development. *Development*, 129(12), 2879–2889.
- Stromstedt, M., Keeney, D. S., Waterman, M. R., Paria, B. C., Conley, A. J., & Dey, S. K. (1996). Preimplantation mouse blastocysts fail to express CYP genes required for estrogen biosynthesis. *Molecular Reproduction and Development*, 43(4), 428–436.
- Sun, X., Deng, W., Li, Y., Tang, S., Leishman, E., Bradshaw, H. B., & Dey, S. K. (2016). Sustained Endocannabinoid signaling compromises Decidual function and promotes inflammation-induced preterm birth. *The Journal of Biological Chemistry*, 291(15), 8231–8240.
- Thie, M., Fuchs, P., & Denker, H. W. (1996). Epithelial cell polarity and embryo implantation in mammals. *The International Journal of Developmental Biology*, 40(1), 389–393.
- van Amerongen, R. and Nusse, R. (2009) Towards an integrated view of Wnt signaling in development. *Development*, 136(19), 3205–3214.
- Vasquez, Y. M., Wu, S. P., Anderson, M. L., Hawkins, S. M., Creighton, C. J., Ray, M., Tsai, S. Y., Tsai, M. J., Lydon, J. P., & DeMayo, F. J. (2016). Endometrial expression of Steroidogenic factor 1 promotes cystic glandular morphogenesis. *Molecular Endocrinology*, 30(5), 518–532.
- Wilcox, A. J., Baird, D. D., & Weinberg, C. R. (1999). Time of implantation of the conceptus and loss of pregnancy. *The New England Journal of Medicine*, 340(23), 1796–1799.
- Yan, L., Yang, M., Guo, H., Yang, L., Wu, J., Li, R., Liu, P., Lian, Y., Zheng, X., Yan, J., Huang, J., Li, M., Wu, X., Wen, L., Lao, K., Li, R., Qiao, J., & Tang, F. (2013). Single-cell RNA-Seq profiling of human preimplantation embryos and embryonic stem cells. *Nature Structural & Molecular Biology*, 20(9), 1131–1139.
- Yang, Y., & Mlodzik, M. (2015). Wnt-frizzled/planar cell polarity signaling: Cellular orientation by facing the wind (Wnt). *Annual Review of Cell and Developmental Biology*, 31, 623–646.
- Ye, X., Hama, K., Contos, J. J., Anliker, B., Inoue, A., Skinner, M. K., Suzuki, H., Amano, T., Kennedy, G., Arai, H., Aoki, J., & Chun, J. (2005). LPA3-Mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature*, 435(7038), 104–108.
- Yoshinaga, K. (2013). A sequence of events in the uterus prior to implantation in the mouse. *Journal of Assisted Reproduction and Genetics*, 30(8), 1017–1022.
- Yuan, J., Cha, J., Deng, W., Bartos, A., Sun, X., Ho, H. H., Borg, J. P., Yamaguchi, T. P., Yang, Y., & Dey, S. K. (2016). Planar cell polarity signaling in the uterus directs appropriate positioning of the crypt for embryo implantation. *Proceedings of the National Academy of Sciences of the United States of America*, 113(50), E8079–E8088.
- Zhang, S., Kong, S., Wang, B., Cheng, X., Chen, Y., Wu, W., Wang, Q., Shi, J., Zhang, Y., Wang, S., Lu, J., Lydon, J. P., DeMayo, F., Pear, W. S., Han, H., Lin, H., Li, L., Wang, H., Wang, Y. L., Li, B., Chen, Q., Duan, E., & Wang, H. (2014). Uterine Rbpj is required for embryonic-uterine orientation and decidual remodeling via notch pathway-independent and -dependent mechanisms. *Cell Research*, 24(8), 925–942.

Cell Biology of the Uterus

Paul S Cooke, Manjunatha K Nanjappa, and Ana M Mesa, University of Florida, Gainesville, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Functions of the Mammalian Uterus

The mammalian uterus plays a large number of critical roles in reproduction. The most familiar of these is that the uterus is the site of fetal growth and development, but the uterus has extensive other functions in reproduction as well. The uterine cervix has a key barrier function in inhibiting passage of bacteria or other infectious agents into the uterus, but under appropriate hormonal conditions allows and even facilitates sperm passage from the vagina into the uterus. Although semen is deposited in the vagina near the cervix in humans, the uterus is the site of semen deposition in a number of species such as the pig, horse and dog. The uterine environment is crucial for sperm capacitation, and uterine peristaltic contractions following semen deposition help move sperm toward the oocyte.

Following fertilization, the uterus nourishes and maintains the embryo until implantation and placentation, typically by the secretions of uterine glands. In some species, such as mice, embryos spend only a short period of time in the uterus before implantation, which occurs shortly after fertilization. In humans, implantation occurs about 9 days after fertilization, and since the embryo spends a few days in the oviduct before entering the uterus, the time that the embryo spends unattached in the uterus is fairly limited. In contrast, in species like cattle, embryo implantation does not occur until more than 3 weeks post-fertilization (Blomberg et al., 2008), and the bovine embryo is dependent on nutrition from the uterus for a significant amount of time before implantation.

A more extreme example of the uterus sustaining embryos for extended periods is the phenomenon known as delayed implantation or embryonic diapause, which has been reported in over 100 mammalian species. In some species that have delayed implantation, such as the tammar wallaby, nine-banded armadillo and western spotted skunk, embryos develop up to the blastocyst stage and then remain semi-dormant in the uterus for months. Furthermore, under experimental conditions, intrauterine embryos can remain unattached but viable in the uterus for up to 2 years before implantation, and the uterus sustains the embryo during this time. Entry into embryonic diapause can be induced as a result of lactation and/or seasonal factors, depending on the specific species, with some species such as the tammar wallaby showing both lactationally and seasonally controlled diapause.

The uterine wall is the site of embryo implantation, and this process is a complex and intricately regulated one that shows extensive species variation and must occur normally to allow embryonic development. The uterus is also where placentation occurs. Placentation differs markedly in various mammalian species. In some species (e.g., mice, humans), the blastocyst invades into the endometrium and disappears from the lumen, while in other species (e.g., cattle) the fetus remains on the luminal surface of the uterus and the fetal portion of the placenta develops in close apposition to the uterine epithelium but does not invade through it. Thus, the uterus plays a critical role in placentation and fetal development.

Lastly, the uterus plays an essential role in parturition. When considering parturition in elephants, where a newborn may weigh up to 120 kg, or in a giraffe where a newborn may be 1.9 m tall, the enormity of delivering a mature fetus from the intrauterine environment into the outside world while not injuring the fetus or otherwise compromising fetal/newborn respiration becomes clearer.

Uterine Anatomy in Mammals

In most mammals, the uterus has three distinct segments: cervix, body and horns. Although uterine morphology varies significantly amongst species, both histological structure and function are highly conserved. Humans possess a *simplex uterus* lacking uterine horns and composed of the body compartment and cervix. Rodents, rabbits and marsupials have a *duplex uterus* with separate uterine horns. Rodents such as rats and mice have cervixes that branch into each uterine horn compartment, while rabbits, hares, and marsupials possess two individual cervical canals. The *bicornuate uterus* is the most prevalent type of mammalian uterus and is characterized by two distinct horns, and a uterine body. Polytocous species such as the pig, dog and cat possess relatively large horns with a smaller body, while monotocous species (e.g., cattle and horse) have less developed horns.

Similar to other tubular organs, uterine histoarchitecture (Fig. 1B) consists of four circular tissue layers called tunics (mucosa, submucosa, muscularis and adventitia/serosa) surrounding a central uterine lumen. However, in general, the uterus is divided into three layers; *endometrium* (mucosa and submucosa), *myometrium* (muscularis) and *perimetrium* (adventitia/serosa).

The endometrial mucosa is lined by a cuboidal to columnar luminal epithelium (LE). The connective tissue layer immediately below the LE is the lamina propria. During uterine development (Fig. 1A), the uterus initially consists of an undifferentiated mesenchyme surrounding a central single-layered LE. The proliferating LE give rise to glandular epithelium (GE) that invaginates into the submucosa and forms simple or branched coiled tubular uterine glands, depending on the species (Cooke et al., 2013). In mice, uterine glands are spatially arranged in lateral to anti-mesometrial regions (opposite side of the broad ligament) of the uterus as a result of active Wnt signaling being limited to the anti-mesometrial region, and consequently implantation typically occurs anti-mesometrially (Goad et al., 2017). However, it is unclear whether this phenomenon occurs in other species. Below the mucosa,

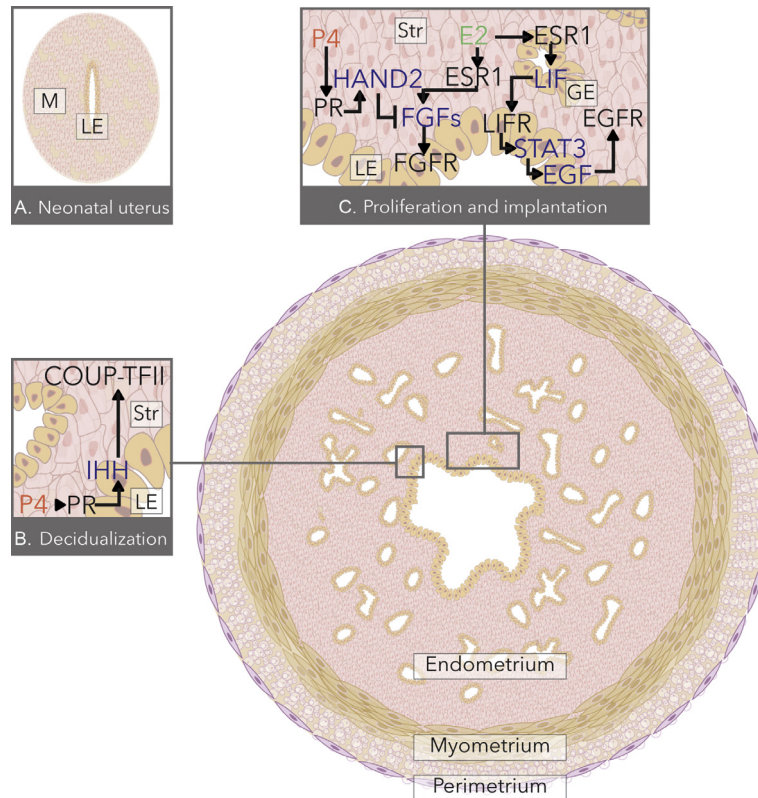


Fig. 1 Schematic representation of cross-sections of adult and developing uterus. Panel A represents the undifferentiated neonatal rodent uterus, which consists of a single layer of luminal epithelium (LE) surrounded by undifferentiated mesenchymal cells (M). The adult uterus consists of endometrial, myometrial, and perimetrial layers. Panel B shows a high-power view of cell–cell interactions that are critical for progesterone (P4) response and decidualization. P4 stimulates Indian Hedgehog (IHH) expression through progesterone receptors (PR). Production of IHH by LE promotes decidualization of neighboring stromal cells (Str) through COUP-TFII signaling. Panel C diagrams proliferation and implantation pathways regulated by steroid hormones. 17 β -Estradiol (E2) acts through estrogen receptor 1 (ESR1) in the stromal cells to promote secretion of various members of the fibroblast growth factor family (FGFs). This stromally-derived FGF acts on adjacent epithelial cells through FGF receptor, inducing cellular proliferation. High concentrations of P4 at the time of implantation act through PR in the stromal cells to induce the production of HAND2, which inhibits secretion of FGFs from the stromal compartment and thus blocks epithelial proliferation. In addition to effects on stromal FGFs, E2 also acts through ER in glandular epithelium (GE) to induce expression of leukemia inhibitory factor (LIF). The LIF produced in the GE then acts on LIF receptors (LIFR) in the LE to activate epithelial STAT3. This leads to the production of a number of EGF-family ligands (EGF), which act on stromal EGF receptors (EGFR) to promote implantation.

fibroblasts, blood vessels, lymphatics, nerves, white blood cells and type III collagen make up the stroma located between the LE and GE.

Although general endometrial structure is similar, there are species differences. The ruminant endometrium has specialized protuberances called caruncles, which are highly vascularized non-glandular structures that contribute to maternal side of the placenta. The endometrium of mares and sows is highly folded compared to other species. In primates, the endometrium is further divided into the basalis and functionalis regions (**Fig. 2**), the latter of which thickens during the cycle and then sloughs off during menstruation.

The adult uterine myometrium has two smooth muscle layers, an inner circular and outer longitudinal layer, separated by connective tissue. Myometrial contraction helps move the uterine contents either toward the oviduct during estrus/fertilization or the cervix during parturition. Estrogens increase myometrial tone or degree of contraction while progesterone decreases the tone. During pregnancy, the myometrium undergoes major remodeling and is responsible for the majority of the increase in uterine weight through myometrial hyperplasia and hypertrophy, and collagen deposition. After parturition, uterine involution is marked by myometrial involution resulting from cell death, hypotrophy and enzymatic degradation of collagen.

The perimetrium consists of a thin layer of squamous epithelium that is continuous with the serosal layer of the peritoneum and broad ligament of the uterus. The broad ligament houses blood vessels, lymphatics and nerves that supply the uterus.

The uterus is a dynamic organ that undergoes sequential remodeling during different phases of the estrous or menstrual cycle, and during pregnancy and after parturition. There is evidence that stem cells for each uterine cell type are a source of LE, GE, stroma and myometrial cells that are lost during remodeling (**Teixeira et al., 2008**). In addition, bone marrow-derived stem cells are capable of contributing to uterine repair after injury, and these stem cells may have clinical benefit in certain conditions such as Asherman's

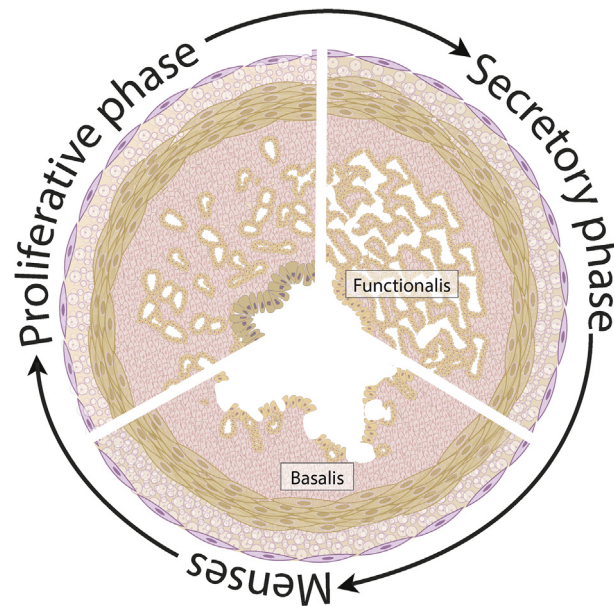


Fig. 2 Schematic diagram of cyclic changes in human uterine histology during the menstrual cycle. The menstrual phase begins with onset of bleeding and lasts for about 4 days. In the absence of pregnancy, the ovarian corpus luteum undergoes luteolysis, resulting in a rapid decline in P4 that results in local inflammation and tissue breakdown and shedding of the functional layer of the endometrium. By late menses, the endometrium consists of only the basal layer. The proliferative phase (follicular phase) lasts for an average of 10 days and coincides with a gradual increase in serum E2 produced by developing follicles. This phase is characterized by increased proliferation of glandular and luminal epithelium under the influence of E2, which reconstitutes the functional endometrium. Glands at this stage are simple tubular and have narrow lumens. The secretory phase (luteal phase) lasts about 14 days, and starts after the ovulation. This phase is marked by rising progesterone secretion from the corpus luteum and a sudden fall in E2 concentrations. Under the influence of progesterone, endometrium grows further and reaches maximal thickness. Glands become highly coiled and dilated with secretory products. During this phase, the endometrium is edematous and shows little cell proliferation. If pregnancy is not established during the secretory phase, progesterone concentrations fall and the shedding of the functional layer begins the next menstrual cycle.

syndrome (Sahin Ersoy et al., 2017), which is characterized by uterine fibrosis and adhesions. It is hypothesized that uterine stem cells also play a role in pathogenesis of uterine fibroids and cancer (Teixeira et al., 2008).

Uterine Development

In females, the Müllerian (paramesonephric) ducts present in both sexes during the ambisexual stage develop into the uterus, oviduct and cranial portion of the vagina. The area of fusion of the two bilateral Mullerian ducts produces the uterine body, while unfused regions give rise to uterine horns. A functional adult uterus, capable of sustaining successful pregnancy, is established through biphasic postnatal growth and differentiation during the pre-pubertal and post-pubertal periods. At birth, the uterus is a simple tube consisting of epithelium and mesenchyme in rodents (Fig. 1B). However, in other domestic animals, the uterus is already radially patterned into epithelium, stroma and myometrium at birth (Cooke et al., 2013). In contrast, human uterine architecture at birth is similar to adults due to exposure to maternal hormones (Gray et al., 2001). After birth, the uterus remains quiescent during early childhood years. Around puberty in humans, the uterus starts to grow and uterine endometrium begins to proliferate and assumes the adult uterine structure. In all species, including humans, continued postnatal uterine development and morphogenesis into functional endometrium with glands and myometrium from birth to puberty is crucial for adult function. The uterus attains its adult histo-architecture by 2 weeks of age in rodents, and by 2–3 months in sheep and pig. These observed temporal differences between species in postnatal uterine development and histo-architecture are attributed to the degree of maturity at the time of birth and the maternal hormonal milieu during gestation.

The exact mechanisms or signaling pathways that govern Müllerian duct development and regulate the differentiation of the various organs derived from this fetal structure are not completely understood. Recent seminal work by Nakajima et al. (2016) has demonstrated that retinoic acid signaling is critical for initiating the unique sequence of uterine development from the multipotent Mullerian duct. In addition, many genes are known to be necessary for normal uterine development, and mutations of genes such as PAX2, LIM1, Emx2, Wnt4, Wnt9b and other members of the WNT gene family of secreted signaling molecules results in Müllerian duct agenesis or impaired uterine development [reviewed in (Teixeira et al., 2008)].

As the uterus differentiates, mesenchyme surrounding the LE differentiates into the stroma and two myometrial layers. The mechanisms regulating this are unclear, but epithelium is required for normal development and differentiation of uterine myometrium, as well as stroma, as described in detail subsequently in this chapter. Thus, reciprocal communication between

epithelium and mesenchyme is essential for normal uterine differentiation and morphogenesis in rodents (Kurita et al., 2001).

In mice, higher rates of cell proliferation during the early neonatal (pre-pubertal) and post-pubertal period (Nanjappa et al., 2015) are major contributors to the size of the adult organ. Somewhat surprisingly, pre-pubertal uterine development in mice occurs normally in the absence of endogenous 17 β -estradiol (E2) and progesterone (P4), even though the neonatal uterus expresses steroid receptors [estrogen receptor 1 (ESR1), estrogen receptor (ESR2), G protein-coupled estrogen receptor 1 (GPER) and progesterone receptor (PR)] and responds to exogenous E2 or P4 (Hewitt et al., 2016). Signaling molecules that drive pre-pubertal uterine cell proliferation and development are not completely elucidated, although growth factors may play a role. However, for normal post-pubertal uterine growth and functions, E2 and P4 actions through their receptors are required (Hewitt et al., 2016).

Regulation of Endometrial Gland Development

Uterine glands are formed from the proliferating LE that differentiate into GE and form invaginations into the submucosa. In contrast to humans, uterine glands development (adenogenesis) occurs after birth in most domestic species and rodents. Uterine glands are formed around 20–22 weeks of gestation in humans. Adenogenesis begins in the first postnatal week of life and is completed by about day 15 postnatal in rodents, and day 56 and day 120 in sheep and pig, respectively (Gray et al., 2001). In mice, uterine glands are relatively simple tubes with limited branching, while in other species such as sheep and pig, uterine glands are tightly coiled and highly branched tubular glands (Goad et al., 2017).

Mechanisms that regulate adenogenesis are unclear, but cell–cell and cell–ECM interactions and stromally-derived factors influence adenogenesis (Gray et al., 2001). Several genes have been linked to uterine gland development in mice, such as the Wnt gene family (Wnt4, Wnt7a, Wnt5a, Wnt11, Wnt16) and their receptors (Fzd6 and Fzd10), Beta-catenin 1, Hoxa10, Hoxa11, Lef1 Vangl2 and Cdh1 [reviewed in (Cooke et al., 2013; Goad et al., 2017)].

Development of a functional adult uterus capable of sustaining and allowing early conceptus survival, implantation and decidualization requires initial adenogenesis during the pre-pubertal and post-pubertal period. Thus, impaired adenogenesis during the critical window of normal development has long-term consequences and cause infertility in adults. Exposure to hormones such as P4 during adenogenesis delays or completely prevents formation of glands in the adult uterus and causes infertility (Gray et al., 2001; Cooke et al., 2013). However, neonatal ovariectomy or inactivating mutations in major receptors of steroid hormones (e.g., ESR1, ESR2, PR) do not prevent neonatal or pre-pubertal adenogenesis. Once glands are established during the neonatal/pre-pubertal period, post-pubertal gland maintenance and further development are primarily dependent on the E2/ESR1 signaling (Nanjappa et al., 2015). Thus, uterine differentiation and adenogenesis during the pre-pubertal and post-pubertal periods are essential for normal fertility in adults.

Estrous Vs. Menstrual Cycle

Beginning at puberty, females begin to show reproductive cycles, although the length and features of these cycles varies amongst species. In the uterus, hormonal changes during the cycle induce functional and architectural changes in preparation for implantation of a fertilized egg and subsequent gestation (Fig. 2). All these events are intricately orchestrated, and if embryonic implantation does not occur the uterine endometrium either sheds in species that undergo a menstrual cycle or regresses in species with estrous cycles. Menstrual cycles are characteristic of primates (Fig. 2), but have also been observed in mammals such as elephant shrews and several families of bats [reviewed in (Emera et al., 2012)]. In the absence of pregnancy in primates, declining progesterone causes local inflammation and tissue breakdown and shedding of the upper two-thirds of the endometrium during the menstrual phase of the cycle (Maybin and Critchley, 2015). The non-primate mammals that have a menstrual cycle exhibit endometrial growth and differentiation during the cycle and also expel endometrial epithelial necrotic debris and erythrocytes if pregnancy does not occur, analogous to menses in primates.

The estrous cycles of placental mammals can be classified according to their frequency throughout the year. Monoestrous species such as wolves, foxes and bears have one cycle per year, while seasonally polyestrous animals such as horses, sheep and goats cycle multiple times during specific seasons of the year. Both monoestrous and seasonally polyestrous species have evolved to allow birth of offspring when environmental conditions are most favorable for their offspring. Lastly, rodents, cows and pigs are polyestrous and cycle regularly through the year.

Uterine remodeling and proliferation occurs in waves during different stages of the cycle, and these changes are mediated by E2 and P4. Ovaries are the major site of E2 and P4 synthesis, and concentrations of these hormones vary in concert with ovarian changes during the cycle. Systemic concentrations of E2 rise during the follicular phase of the cycle, as the ovary contains one or more dominant follicles developing for ovulation. The actions of E2 and other signaling molecules prepare reproductive organs for estrus and fertilization by inducing a wide array of processes, including uterine LE and GE proliferation. After ovulation, the corpus luteum is formed in the ovary, leading to rising systemic P4 concentrations. Progesterone induces uterine stromal differentiation and proliferation, and inhibits E2-induced LE and GE proliferation. Additionally, P4 plays a fundamental role in the decidualization process of estrogen-primed fibroblast cells in the uterine stroma necessary for pregnancy establishment [Reviewed in (Dunn et al., 2003)].

Uterine Hormonal Responses Involve Reciprocal Epithelial–Connective Tissue Communication

As described above, the uterus is a major target of estrogen and progesterone signaling, and ESR1, ESR2, GPER and PR are abundant in this organ. Expression of ESR1 and PR, for example, occurs in all epithelial cells and the vast majority of mouse uterine stromal cells. Estrogens are the major regulator of uterine epithelial development and function, and epithelial proliferation, secretory protein production and height are all estrogen-regulated. Progesterone blocks mitogenic estrogen effects and other estrogen-stimulated processes in uterine epithelium. Given the high uterine epithelial ESR1 expression and the critical estrogen roles in uterine epithelial function, it seems obvious that estrogens must induce processes such as epithelial proliferation directly through epithelial ESR1. Similarly, progesterone inhibition of mitogenic estrogen effects on uterine epithelial proliferation would logically occur directly through epithelial PR. However, over the past 4 decades, it has become clear that stimulatory and inhibitory effects of E2 and P4, respectively, on uterine epithelial proliferation are mediated indirectly through effects on connective tissue steroid hormone receptors.

Extensive research extending back to pioneering studies of the German embryologist Hans Spemann in the early 20th century established that connective tissue controls initial differentiation, proliferation, patterning and function of epithelial cells in developing organs. Work in the 1980s examining estrogen responses in neonatal mice suggested that connective tissue might also control epithelial response to estrogen in female reproductive organs, and estrogen effects on endpoints such as uterine epithelial proliferation might be mediated indirectly through the connective tissue. Neonatal mice express ESR1 in uterine mesenchyme, the undifferentiated connective tissue that is the precursor of the stroma, but not in the epithelium. Despite this, estrogen treatment of these mice induced robust increases in epithelial proliferation, and epithelial ESR1 was not detectable even after estrogen stimulation (Bigsby and Cunha, 1986). This suggested that mesenchymal ESR1 could mediate mitogenic estrogen effects on uterine epithelial proliferation, potentially analogous to the situation in males where androgen stimulation of epithelial proliferation in organs such as the prostate is indirect through connective tissue. However, concerns that neonatal uterine epithelium might express estrogen receptor in amounts sufficient to mediate estrogen-induced epithelial proliferation but insufficient to be detected by the techniques then in use for analyzing estrogen receptor expression made it impossible to definitively conclude that estrogen stimulation of uterine epithelial proliferation was mediated indirectly.

Estrogen Effects on Uterine Epithelial Proliferation Are Mediated Indirectly through Connective Tissue

Development of methodologies to produce mice in which a specific gene had been knocked out or disabled at the end of the 1980s provided a powerful new tool to establish the role of various proteins in reproduction in ways that had not been previously possible. This original technique and subsequent variations such as conditional knockouts that allowed a gene to be deleted in a specific cell type or at a particular time have been mainstays of reproductive biology research and have provided important insights into many aspects of reproductive biology and steroid endocrinology for over two decades, and continue to do so today. Knockouts of key enzymes that are involved in steroid hormone synthesis (e.g., aromatase), co-factors in steroid hormone signaling or downstream mediators of steroid hormone action have all provided key insights into steroid hormone actions, but knockouts of steroid hormone receptors have been the most critical in advancing our understanding of steroid hormone action.

The first of these was a knockout of ESR1 by Lubahn, Korach and Smithies (Lubahn et al., 1993). Using tissues from this *Esr1* knockout (*Esr1*KO) mouse, definitive proof was obtained that estrogen-induced uterine epithelial proliferation was mediated indirectly through ESR1 in the uterine mesenchyme/stroma (Cooke et al., 1997). In contrast, production of epithelial secretory proteins required both epithelial and stromal ESR1 (Cooke et al., 1997). Critical progesterone effects on uterine epithelium were mediated indirectly through the stroma as well, as progesterone inhibition of estrogen-induced uterine epithelial proliferation was also shown to be mediated through stromal PR.

Use of a Transgenic Mice Lacking ESR1 Expression in Uterine Epithelium

The development of conditional knockout mice where ESR1 is deleted only in the epithelium of organs such as the uterus and oviduct has recently provided an elegant model system for determining the role of loss of epithelial ESR1 in certain reproductive organs (Winuthayanon et al., 2010). In addition to confirming earlier results that estrogen-induced uterine epithelial proliferation is mediated through stromal ESR1, results from this mouse have shown that epithelial ESR1 in both the oviduct and uterus is necessary for fertility, and that uterine implantation requires epithelial ESR1 (Winuthayanon et al., 2010).

How Does Mesenchyme/Stroma Regulate Epithelial Proliferation and Other Processes in Response to Estrogen in Uterine Epithelial Cells?

The demonstration that estrogen-induced uterine epithelial proliferation is mediated indirectly through mesenchymal/stromal ESR1 raised the obvious question of how uterine mesenchymal/stromal cells stimulate proliferation in overlying epithelium.

Cell–cell communication between stromal and epithelial cells seemed unlikely, since the basement membrane on which the uterine epithelium rests should preclude direct cell–cell contact with the underlying connective tissue. This suggested that other means of communication, such as production of diffusible stromal factors that could affect neighboring epithelial cells, were most likely. It was also possible that stromal cells could have effects on the basement membrane, either by inducing compositional changes or by altering or activating growth factors present there, which could result in changes in epithelial proliferation.

Uterine Epithelial Proliferation Is Induced by Stromal Factors Produced in Response to Estrogen Stimulation, and Progesterone Inhibits the Production of these Stromal Factors

Despite work by many laboratories, it took almost 15 years from the initial demonstration that estrogen-induced uterine epithelial proliferation and progesterone inhibition of this process were mediated indirectly through uterine stromal ESR1 and PR until the stromal factors involved in these processes were identified. In 2011, Li et al. (Li et al., 2011) used a conditional knockout of HAND2, a basic helix–loop–helix transcription factor expressed in the uterine stroma, to show that this protein is induced by P4 in the uterus. Importantly, loss of HAND2 abolished P4 inhibition of estrogen-induced uterine epithelial proliferation, suggesting that HAND2 was essential for normal stromal P4 effects. HAND2 expression was shown to induce production and secretion of a group of stromal FGFs that then cross the basement membrane and act through FGF receptors on the LE to stimulate epithelial ERK1/2 signaling that normally occurs in response to estrogen treatment and drives proliferation. Thus, this landmark study determined the mechanisms both for estrogen-induced epithelial proliferation through stromal ESR1 as well as P4 inhibition of this effect through stromal PR.

Reciprocal Epithelial–Stromal Communication Is Critical for a Variety of Hormone Responses

Although uterine stromal–epithelial communication that mediates mitogenic estrogen effects on uterine epithelium and inhibitory P4 effects on this process are probably the best characterized, recent evidence suggests extensive cell–cell communication between different uterine cell types is essential for other aspects of normal uterine function. This involves reciprocal epithelial–stromal communication, as well as intraepithelial communication between LE and GE.

Leukemia inhibitory factor (LIF) in the uterus is produced primarily by GE under the influence of estrogen. It has been known for years that LIF is necessary for embryo attachment and implantation [reviewed in (Hantak et al., 2014)], but recent results examining LIF signaling have revealed complex crosstalk between various uterine cell types that are essential for normal function. In response to LIF signaling, STAT3 is activated in uterine epithelium (Fig. 1C). Use of a mouse with a conditional knockout of STAT3 only in uterine epithelium [reviewed in (Hantak et al., 2014)] revealed that STAT3 is necessary for normal LIF effects on epithelial junctional integrity that are involved in implantation. Recently, conditional deletion of LIF PR-expressing cells of the uterus was shown to cause implantation failure. Critically, loss of only epithelial STAT3 impaired stromal cell differentiation and proliferation. Additional work indicated that this effect resulted from loss of epithelial production of various members of the EGF family of ligands normally induced by epithelial STAT3 signaling that act through stromal receptors to regulate their differentiation (Fig. 1C). This unexpected signaling pathway from GE to LE to stromal cells to regulate key implantation events [Fig. 1C; reviewed in (Hantak et al., 2014)] illustrates both the complexity and importance of cell–cell signaling in uterine endometrium.

Other stromal–epithelial communication in the uterus has also been documented. A member of the hedgehog family of proteins, Indian Hedgehog (IHH) is stimulated by P4 and is a major mediator of P4 action in the uterus. Following stimulation by P4, IHH produced in the uterus induces production of the transcription factor COUP-TFII in the uterine stroma (Fig. 1B). Results from the last decade have finally established the molecular basis of critical intra-uterine signaling pathways, and undoubtedly other stromal–epithelial signaling pathways will be revealed and characterized in the decades to come.

It is likely that uterine myometrium also participates in uterine cell–cell communications, both as a source and target of signals from other uterine cell types, although this has not yet been established. Thus, all major uterine cell types may be involved in communication with other uterine cell types/compartments, and it is becoming increasingly clear that this intrauterine communication is a major factor in normal uterine function and hormonal response.

Communication between Uterine Tissues and the Uterine Microbiome

A rapidly expanding literature indicates that the gut microbiome present in humans and other animals has wide-ranging effects on metabolism, body weight and other aspects of the host animal's physiology. In recent years, a microbiome has been demonstrated in the uterus, and abnormalities in this uterine microbiome have been linked to implantation failure and pregnancy loss (Moreno and Franasiak, 2017). Based on emerging data regarding the critical role of the microbiome in the uterus, it is becoming increasingly clear that cell–cell interactions in the uterus are not confined exclusively to the constituent cells of the uterus, but extend to interactions between the uterine microbiome and the endometrium as well.

References

- Bigsby, R. M., & Cunha, G. R. (1986). Estrogen stimulation of deoxyribonucleic acid synthesis in uterine epithelial cells which lack estrogen receptors. *Endocrinology*, 119, 390–396.
- Blomberg, L., Hashizume, K., & Viebahn, C. (2008). Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. *Reproduction*, 135, 181–195.
- Cooke, P. S., Buchanan, D. L., Young, P., Setiawan, T., Brody, J., Korach, K. S., Taylor, J., Lubahn, D. B., & Cunha, G. R. (1997). Stromal estrogen receptors mediate Mitogenic effects of estradiol on uterine epithelium. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 6535–6540.
- Cooke, P. S., Spencer, T. E., Bartol, F. F., & Hayashi, K. (2013). Uterine glands: Development, function and experimental model systems. *Molecular Human Reproduction*, 19, 547–558.
- Dunn, C. L., Kelly, R. W., & Critchley, H. O. (2003). Decidualization of the human endometrial stromal cell: An enigmatic transformation. *Reproductive Biomedicine Online*, 7, 151–161.
- Emera, D., Romero, R., & Wagner, G. (2012). The evolution of menstruation: A new model for genetic assimilation: Explaining molecular origins of maternal responses to fetal invasiveness. *BioEssays*, 34, 26–35.
- Goad, J., Ko, Y. A., Kumar, M., Syed, S. M., & Tanwar, P. S. (2017). Differential Wnt signaling activity limits epithelial gland development to the anti-Mesometrial side of the mouse uterus. *Developmental Biology*, 423, 138–151.
- Gray, C. A., Bartol, F. F., Tarleton, B. J., Wiley, A. A., Johnson, G. A., Bazer, F. W., & Spencer, T. E. (2001). Developmental biology of uterine glands. *Biology of Reproduction*, 65, 1311–1323.
- Hantak, A. M., Bagchi, I. C., & Bagchi, M. K. (2014). Role of uterine stromal-epithelial crosstalk in embryo implantation. *The International Journal of Developmental Biology*, 58, 139–146.
- Hewitt, S. C., Winuthayanon, W., & Korach, K. S. (2016). What's new in estrogen receptor action in the female reproductive tract. *Journal of Molecular Endocrinology*, 56, R55–71.
- Kurita, T., Cooke, P. S., & Cunha, G. R. (2001). Epithelial-stromal tissue interaction in paramesonephric (Müllerian) epithelial differentiation. *Developmental Biology*, 240, 194–211.
- Li, Q., Kannan, A., Demayo, F. J., Lydon, J. P., Cooke, P. S., Yamagishi, H., Srivastava, D., Bagchi, M. K., & Bagchi, I. C. (2011). The Antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science*, 331, 912–916.
- Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S., & Smithies, O. (1993). Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 11162–11166.
- Maybin, J. A., & Critchley, H. O. (2015). Menstrual physiology: Implications for endometrial pathology and beyond. *Human Reproduction Update*, 21, 748–761.
- Moreno, I., & Franasiak, J. M. (2017). Endometrial microbiota-new player in town. *Fertility and Sterility*, 108, 32–39.
- Nakajima, T., Iguchi, T., & Sato, T. (2016). Retinoic acid signaling determines the fate of uterine stroma in the mouse Mullerian duct. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 14354–14359.
- Nanjappa, M. K., Medrano, T. I., March, A. G., & Cooke, P. S. (2015). Neonatal uterine and vaginal cell proliferation and Adenogenesis are independent of estrogen receptor 1 (Esr1) in the mouse. *Biology of Reproduction*, 92, 78.
- Sahin Ersoy, G., Zolbin, M. M., Cosar, E., Moridi, I., Mamillapalli, R., & Taylor, H. S. (2017). Cxcl12 promotes stem cell recruitment and uterine repair after injury in Asherman's syndrome. *Mol Ther Methods Clin Dev*, 4, 169–177.
- Teixeira, J., Rueda, B. R., & Pru, J. K. (2008). Uterine Stem Cells. In *StemBook*. Cambridge, MA: Harvard Stem Cell Institute (Internet).
- Winuthayanon, W., Hewitt, S. C., Orvis, G. D., Behringer, R. R., & Korach, K. S. (2010). Uterine epithelial estrogen receptor A is dispensable for proliferation but essential for complete biological and biochemical responses. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 19272–19277.

Uterus-Hormonal Regulation

Tae Hoon Kim and Jae-Wook Jeong, Michigan State University College of Human Medicine, Grand Rapids, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The uterus consists of heterogeneous cell types that undergo dynamic changes to support embryo development and implantation. The mature uterus consists of three tissue layers, the endometrium, myometrium, and serosa. The serosa is a single outer cell layer of tissue made of epithelial cells that envelop the uterus. The myometrium is the muscular layer, composed of inner circular and outer longitudinal smooth muscle layers. The endometrium is the most active layer and composed of stroma and epithelial cells (Brody and Cunha, 1989).

The ovarian steroid hormones estrogen (E2) and progesterone (P4) are essential regulators of uterine functions necessary for embryo implantation, development, and normal pregnancy, and the balance of these two steroid hormones working cooperatively is required for the maintenance of a healthy endometrium (Fig. 1) (Dharma et al., 2001; Wood et al., 2007). These steroids act primarily through their respective cognate nuclear receptors, the progesterone receptor (PGR) and the estrogen receptor (ESR). E2 stimulates proliferation of both the uterine epithelial and stromal cells in neonatal mice. However, this proliferative action of E2 is restricted to epithelial cells in the adult mouse uterus (Martin et al., 1973a; Huet-Hudson et al., 1989). In contrast, while P4 is inhibitory to E2-mediated proliferation of the luminal and glandular epithelial cells, P4, alone or in conjunction with E2, leads to uterine stromal cell proliferation (Huet-Hudson et al., 1989; Martin et al., 1973b; Paria et al., 1993).

The cross talk between epithelial and stroma cells changes due to P4 and E2 (Franco et al., 2012; Winuthayanon et al., 2010). These changes are critical for embryo implantation and development. E2 regulates uterine epithelial proliferation unlike P4 which inhibits the E2-induced proliferation of the epithelium (Martin et al., 1973a,b). P4 inhibits the proliferation via stromal-epithelial cross-talk. Successful embryo apposition, attachment, and implantation can be established under well-organized uterine receptivity (Wetendorf and DeMayo, 2012). A defect in maintaining the status of uterine receptivity is the major cause of most pregnancy losses. The ability of ovarian steroids to mediate endometrial proliferation occurs through the ability of hormonal stimulation to regulate growth factor communication networks between the uterine stromal and epithelial cells. P4 inhibits E2-stimulated epithelial cell proliferation in the uterus by the regulation of gene expression in endometrial stromal cells (Rubel et al., 2010).

Inappropriate activation of endometrial proliferation and differentiation by dysregulation of steroid hormones is one of the major features of uterine disease (Kao et al., 2003). Uterine diseases of the female reproductive tract represent a significant women's health problem. Excessive uterine proliferation caused by dysregulation of the steroid hormones results in several uterine diseases including infertility, endometriosis, and endometrial cancer (Bokhman, 1983; Sherman et al., 1997; Deligdisch and Holinka, 1987; Kurman et al., 1985). Endometrial cancer is a frequently occurring gynecological disorder (Siegel et al., 2016). Estrogen-dependent endometrioid carcinoma is the most common type of gynecological cancer. Endometriosis was found in 10% of reproductive-age women, and 35%–50% of infertile women had endometriosis (Jemal et al., 2006; Eskenazi and Warner, 1997; Bulun, 2009). Understanding the role of steroid hormone regulation of uterine function is critical in evaluating the pathology of these uterine diseases.

Progesterone

Progesterone (P4) is one of the major ovarian steroid hormones. P4 is produced by the corpus luteum in the ovaries (Graham and Clarke, 1997), and its synthesis and secretion are regulated by luteinizing hormone and chorionic gonadotropin during the menstrual cycle and pregnancy (Graham and Clarke, 1997). P4 is absolutely necessary for uterine implantation, decidualization, and maintenance of pregnancy (Wetendorf and DeMayo, 2012). Implantation is the most critical step to establish pregnancy in the uterus. Successful implantation requires the attachment of the embryo to a uterine luminal epithelium and its subsequent

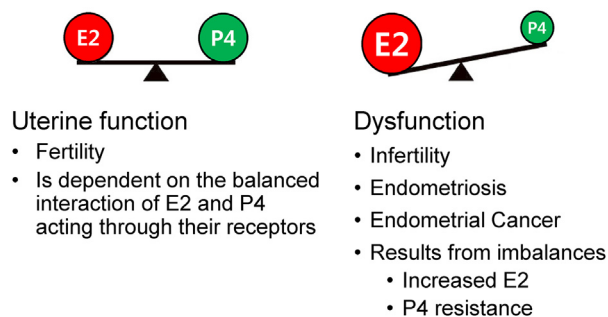


Fig. 1 Steroid hormone regulation of endometrium.

invasion into the stromal tissue (Young, 2013). P4 prepares the endometrium to implant the embryo into the uterine epithelium and maintain the pregnancy after fertilization and implantation (Mote et al., 1999). After implantation, the uterine stroma of the surrounding implantation site starts to proliferate and differentiate, resulting in decidual cells that support the growth of the embryo which is known as decidualization (Cha et al., 2012). Importantly, the high level of P4 is maintained to control the generation of other eggs throughout the pregnancy progression, and to inhibit lactation before birth (Capuco and Akers, 2009).

P4 has an important role in the regulation of the immune and endocrine system, which mediate the role of the trophoblasts at the implantation site (Drake et al., 2001). Trophoblasts are cells that form the outer layer of a blastocyst which plays a critical role in embryo implantation and interaction with the decidual cells (Red-Horse et al., 2004). It is reported that P4 increases the cytokines released by Th2 cells which are dominant within the decidua in early pregnancy, resulting in the maintenance of pregnancy. Th2-induced cytokines trigger the secretion of hCG from trophoblasts. The secreted hCG leads to P4 production from the corpus luteum in pregnancy (Drake et al., 2001). This supports the maintenance of pregnancy by P4.

Estrogen

Estrogen (E2) is an imperative hormone that stimulates uterine epithelium capacity and regulates the menstrual cycle. Its interplay with P4 is critical in the changes of the uterus during pregnancy (Hantak et al., 2014). E2 is highly secreted by ovaries, and secretion peaks at ovulation and its level is rapidly decreased after ovulation (Burns and Korach, 2012). Before ovulation, E2 plays an important in the regeneration and growth of the endometrium. E2 prepares the endometrial tissue to respond to progesterone after ovulation (Groothuis et al., 2007).

The proliferation of the uterine endometrium is triggered by E2. This phenomenon enhances the growth of the endometrium and increases the uterine weight. E2 also helps the uterine wall to slough off dead tissue during menstruation (Gargett et al., 2012; Vasquez and DeMayo, 2013). In addition, E2 is the most important hormone in preparation for parturition. E2 stimulates proliferation in the myometrium, accomplishing the considerable growth that is necessary for the forceful contractions of labor (Kumar and Magon, 2012; Kota et al., 2013).

Steroid Hormone Receptors

Nuclear receptors are a family of ligand-activated transcription factors that are activated by steroid hormones, such as estrogen and progesterone, and thyroid hormones, oxysterols and fat-soluble vitamins. After nuclear receptors are activated, their main function is to regulate gene expression for numerous biological processes including cell proliferation, development, and reproduction as transcription factors (Tsai and O'Malley, 1994). Nuclear receptors include several distinct modular domains for transactivation regions (AF-1), DNA-binding domain (DBD) that includes two Zn fingers, ligand-binding domain (LBD) that contributes to interactions of the subset of nuclear receptors on their mode of action, and hormone response elements (HRE) act as binding regions for steroid hormone receptors (Sever and Glass, 2013).

Steroid hormone receptors, in particular the progesterone and estrogen receptors (PGR and ESR), are important functional regulators of female reproductive biology. PGR and ESR are members of the nuclear receptor superfamily. PGR and ESR1 are expressed in all compartments of the uterus, and their tight regulation is critical for uterine function (Large and DeMayo, 2012). The steroid hormones, P4 and E2, exhibit their effects via the progesterone receptor (PGR) and estrogen receptors (ESR). PGR and ESR regulate the effect of P4 and E2 under physiological conditions, either by activation of P4 or E2 target genes transcription on the uterine tissues (Li and O'Malley, 2003; Acconcia and Kumar, 2006).

The ovarian steroid P4 mediates its effects through the PGR, which belongs to the nuclear receptor superfamily. As in all members of this family, the PGR contains modular functional domains. The PGR contains an N-terminal transactivating function domain (AF-1), a central DNA binding domain, and a ligand binding domain with a second activation domain (AF-2) (Sever and Glass, 2013). The PGR is found as two major isoforms, PRA and PRB, which are expressed from one gene with two alternative translation start sites. The PRB isoform contains an additional 164 amino acids at the N-terminus and a third activation domain (AF-3) (Wierman, 2007).

The importance of PGR in uterine function was demonstrated with the genetic ablation of PGR in mice (PRKO), which results in pleiotropic reproductive defects including those of sexual behavior, gonadotropin regulation, anovulation, lack of decidualization, and mammary branching morphogenesis. Ablation of PGR does not impact uterine development or morphology. However, ablation of PR renders the mouse uterus incapable of supporting embryo implantation (Lydon et al., 1996).

The total level of PGR and the ratio of PR-A/PR-B varies in reproductive tissues. The function of PR-A and PR-B were studied using an animal mouse model. Ablation of PRA alone (PRAKO) demonstrated that PRA is the major mediator of P4 signaling in the mouse reproductive tract, and these mice show similar ovarian and uterine abnormalities seen in the PRAKO mice. In addition, the PRAKO mice, in which PRB is solely expressed, show an abnormal P4-induced proliferative effect on the epithelium in opposition from its normal antiproliferative inhibition of E2. Ablation of the PRB isoform (PRBKO), although exhibiting mammary morphogenesis defects, did not display any uterine phenotype, and the mice were fertile (Vasquez and DeMayo, 2013). The mice with ablation of specific PR-A and PR-B demonstrated that PR-A plays a crucial role in the mouse uterus and PR-B is mediated in mammary gland maturation (Kariagina et al., 2008).

ESR1 and ESR2 are expressed in distinct tissues. ESR1 is mainly expressed in the mammary glands, pituitary, hypothalamus, ovarian theca cells and uterus, while ESR2 is detected in the ovarian granulosa cells, lung and prostate. ESR1 expression is regulated by very firm hormonal and temporal control, indicating spatial and temporal roles during implantation. Specific ablation of epithelial ESR1 in the murine uterus led to infertility due to dysregulated estrogen signaling. It was discovered that epithelial ESR1 has an important role in the regulation of epithelial apoptosis, while stromal ESR1 mediated E2-induced epithelial proliferation (Vasquez and DeMayo, 2013; Wierman, 2007; Deroo and Korach, 2006).

Interactions with many transcriptional coregulators are required to activate PGR and ESR1 signaling. These coactivators or corepressors make large complexes that engage in the remodeling of chromatin through histone post-translational modifications, and link transcription factors to the basal transcriptional RNA polymerase II machinery for all subsequent genomic actions (Szwarc et al., 2015). Steroid receptor coactivator (SRC) family members (SRC-1, SRC-2, SRC-3) interact with nuclear receptors and enhance target gene transcription. The function of steroid receptor coactivator is associated with regulation of steroid hormone signaling. Many studies have found that steroid receptor coactivator plays an important role in the regulation of reproductive biology including ovulation, implantation, and parturition. Furthermore, dysregulation of steroid receptor coactivator actions led to infertility, cancer, and endometriosis (Szwarc et al., 2014; Losel and Wehling, 2003).

Nongenomic Actions of Steroid Hormones

According to the classical model (genomic action) of steroid action, in the absence of P4 and E2 hormones, PGR and ESR1 are localized in the cytoplasm. To make an activated state of PGR and ESR, after hormone binding, it undergoes a conformational change and translocates to the nucleus where it regulates target gene expression. In contrast, a nongenomic action is distinguished from genomic steroid actions (Fig. 2). These responses are known as “non-classic,” “non-genomic” or “extra-nuclear” steroid effects.

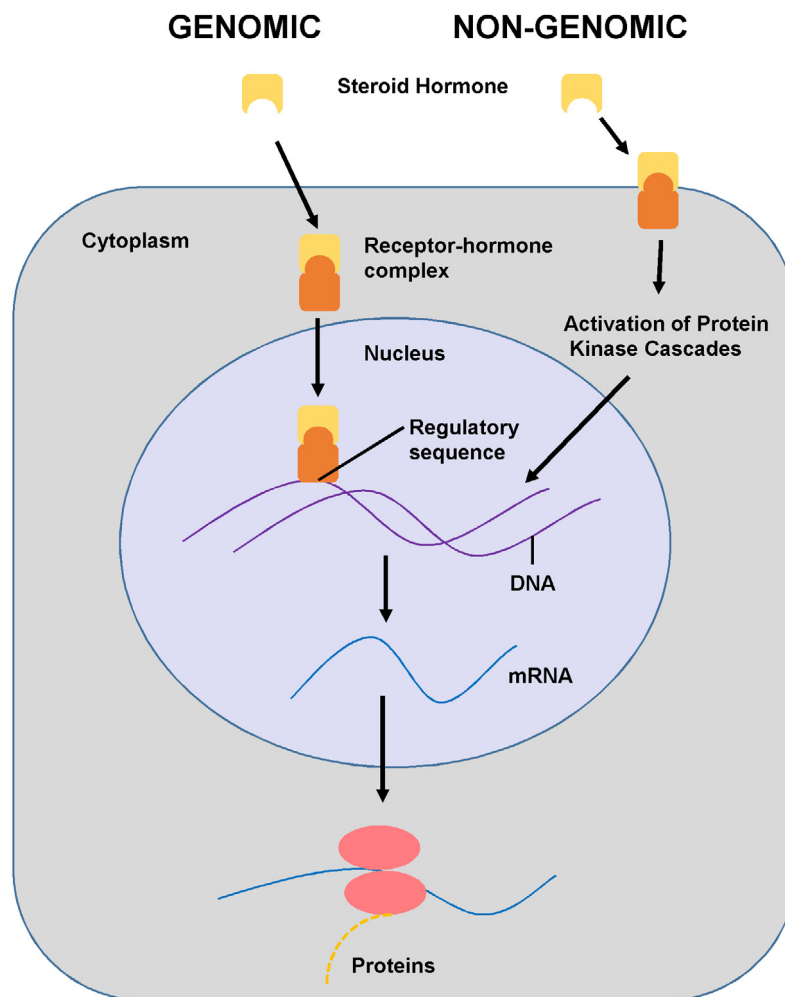


Fig. 2 Steroid hormone actions. Modified from Boonyaratankornkit, V. and Edwards, D. P. (2007). Receptor mechanisms mediating non-genomic actions of sex steroids. *Seminars in Reproductive Medicine* 25(3), 139–153.

Nongenomic actions are (1) observed in some cell types without a nucleus such as erythrocytes and platelets; (2) not blocked by inhibitors of transcription; (3) very rapid actions; (4) initiated by membrane-steroid signaling (Losel and Wehling, 2003).

In the uterus, this transcriptional activation can also occur in a ligand independent fashion as a result of phosphorylation of PGR and ESR1. In addition, PGR and ESR1 interact with src to activate the MAP kinase pathway to regulate target genes during the regulation of nuclear receptors target genes, and coregulator complexes are formed (Vasquez and DeMayo, 2013; Tsai and O'Malley, 1994; Li and O'Malley, 2003; Szwarc et al., 2014). Nongenomic E2 plays a crucial role in the regulation of endometrial inflammatory response during implantation, pregnancy, and menstruation by modulating gene expression and secretion that support fertilization and embryo viability. Furthermore, nongenomic E2 exhibits effects on the calcium-signaling pathway that are associated with muscle contraction in the reproductive tract (Solar and Velasquez, 2013).

Three membrane progesterone receptors are identified: mPR α , mPR β , and mPR γ in a variety of species. mPRs modulate the activity of various signal transduction cascades binding P4, including modulation of ERK1/2 or p38 MAPKs, inhibition of cAMP production. Furthermore, these membrane proteins are also associated with eliciting contraction in parturition (Losel and Wehling, 2003).

Dysregulation of Steroid Hormone Signaling

E2 induces proliferation of epithelial cells in the uterus, while P4 has an inhibitory effect on E2-induced proliferation (Martin et al., 1973a,b). An unbalancing of this steroid signaling causes infertility and several gynecological diseases such as endometriosis, endometrial cancer, and leiomyomas. The major pathologic phenomenon of uterine disease is the dysregulation of ovarian steroid hormones across epithelial proliferation and apoptosis in the uterus (Bokhman, 1983; Sherman et al., 1997; Deligdisch and Holinka, 1987; Kurman et al., 1985). These uterine diseases display characteristic dysregulation of PGR and ESR expression and of their downstream target genes in their pathologies. All of these disorders have been mainly associated with increased E2 sensitivity or dysregulation of P4. Loss of functioning P4 signaling leads to P4 resistance, either at the level of the PGR or its target genes, and it can lead to the disruption of the critical epithelial-stromal cross talk. The antagonistic effect of P4 on E2 forms the rationale for progestin-based therapeutics for endometrial cancers (Benshushan, 2004; Minaguchi et al., 2007; Hahn et al., 2009; Yamazawa et al., 2007). However, many studies indicate limiting the use of P4 therapy due to its low response rates (Ramirez et al., 2004; Park et al., 2012; Decruze and Green, 2007; Randall and Kurman, 1997; Kim et al., 1997). Resistance to P4 treatment, via loss of progesterone receptors (PGR) or its signaling pathways, is a major hurdle in the treatment of many of diseases in the endometrium of women including endometriosis and endometrial cancer (Al-Sabbagh et al., 2012; Burney et al., 2007; Soyal et al., 2005; Attia et al., 2000).

The endometrium undergoes well-defined and regulated gene expression in preparation for implantation. The timing of endometrial receptivity coincides with the down-regulation of epithelial ESR1 in normal mid-secretory endometrium. P4 and PGR are essential for successful embryo implantation, but there is a shift in PGR out of the epithelium to the stromal compartment that also occurs during the implantation. Persistence of ESR1 and PGR in the glandular epithelium is associated with infertility and suspected implantation defects. Infertility is a major reproductive health problem. Assisted reproductive technologies (ART) is the gold standard tool to help couples who had pregnancy failure due to an implantation defect (Levgur et al., 2000). P4 supplementation has been studied to improve the success of ART (Allahbadia, 2015; Practice Committee of American Society for Reproductive Medicine, 2008). P4 supplementation is associated with improvement of implantation and pregnancy rates, compared to treatment with placebo or no treatment. In addition, any type of P4 supplementation administration enhanced pregnancy outcomes in ART cycles (van der Linden et al., 2015).

The efficacy of P4 supplementation in women with unexplained recurrent miscarriages were investigated, considering its role in pregnancy (Hussain et al., 2012; El-Zibdeh and Yousef, 2009). Several clinical trials were studied to improve the pregnancy outcome that is associated with P4 supplementation in unexplained infertility or recurrent pregnancy loss patients (El-Zibdeh and Yousef, 2009; Clifford et al., 1997). A study on 203 women who had experienced at least three unexplained recurrent miscarriages was randomized to receive P4 supplementation for examining the miscarriage rate. The incidence of miscarriage rate was lower in the P4 treated group than in the untreated group when compared with historical data. The majority of cited clinical trials revealed that P4 supplementation can have beneficial effects in women with otherwise unexplained recurrent miscarriages (Hussain et al., 2012). P4 supplementation can be administrated via vaginal suppositories, intramuscular injection or oral intake (Walch and Huber, 2008).

Endometrial cancer is the most often diagnosed disease of the female uterus in the United States and the incidence of new cases has increased over the past several decades (Siegel et al., 2016). The major treatment for endometrial cancer is surgery to remove the uterus and cervix (called a hysterectomy) (ACOG Committee, 2009; Temkin et al., 2016). Although most cases of endometrial cancer are diagnosed in post-menopausal women, 5% of women have endometrial cancer before age 40 and 20%–25% are diagnosed with endometrial cancer before menopause (Pellerin and Finan, 2005). Furthermore, the number of endometrial cancer diagnoses in younger patients will continue to increase due to an increase of known endometrial cancer risk factors such as obesity, high blood pressure, and diabetes mellitus (Chassot et al., 2002; Charytan et al., 2012; Varon and Marik, 2008).

A steroid hormone imbalance could lead to aberrant endometrial proliferation and endometrial cancer. P4 therapy has been used in the conservative endocrine treatment of endometrial complex atypical hyperplasia and early endometrial cancer in young women with a desire to maintain their fertility (Hahn et al., 2009; Kim et al., 2013). P4 and its analogues can have an effect on

suppression of endometrial cancer proliferation (Yang et al., 2011). However, more than 30% of patients fail to respond to progestin due to de novo or acquired P4 resistance (Decruze and Green, 2007). Further, P4 resistance is seen in a wide variety of endometrial diseases such as infertility, endometriosis, as well as endometrial cancer. Therefore, the identification of P4-regulated signaling pathways in the uterus is crucial for understanding the impairments that underlie disruption of steroid hormone control of uterine cell proliferation and differentiation.

Endometriosis is defined as the presence of endometrial cells outside of the uterine cavity. Endometriosis is one of the most common causes of chronic pelvic pain, dysmenorrhea, and infertility, affecting 10% of all reproductive age women and 35%–50% of infertile women (Eskenazi and Warner, 1997; Bulun, 2009; Kim et al., 2013; Johnson and Hummelshoj, 2013; Burney and Giudice, 2012). Surgical excision of lesions and hormonal suppression are the current gold standards of therapy, but both approaches are associated with a significant risk of morbidity and a high incidence of relapse (Bulun, 2009; Sinaii et al., 2002). Therefore, identification of mechanisms involved in the pathogenesis of endometriosis and development of novel therapies are critical. Endometriosis is an estrogen (E2) dependent, inflammatory condition. Pro-inflammatory cytokines contribute to chronic pelvic pain and inflammation, the latter a key factor for pathogenesis and pathophysiology (Bulun, 2009).

Adenomyosis is a common benign gynecological disease caused by the presence of endometrial glands and stroma found within the myometrium (Tamai et al., 2005; Ferenczy, 1998). The prevalence of adenomyosis ranges from 10% to 66% in women diagnosed at the time of hysterectomy (Vercellini et al., 2006). Adenomyosis is one of the most common causes of menorrhagia, dyspareunia, dyschezia, dysmenorrhea, and chronic pelvic pain (Levgur et al., 2000). Most women are not diagnosed until later stages of the disease and invasive surgical intervention is required for severely symptomatic women due to less of an effect of pharmacological therapy (Vercellini et al., 2006).

References

- Acconcia, F., & Kumar, R. (2006). Signaling regulation of genomic and nongenomic functions of estrogen receptors. *Cancer Letters*, 238(1), 1–14.
- Al-Sabbagh, M., Lam, E. W., & Brosens, J. J. (2012). Mechanisms of endometrial progesterone resistance. *Molecular and Cellular Endocrinology*, 358(2), 208–215.
- Allahbadia, G. N. (2015). Has ART finally got a patient-friendly progesterone? *Journal of Obstetrics and Gynaecology of India*, 65(5), 289–292.
- Attia, G. R., Zeitoun, K., Edwards, D., Johns, A., Carr, B. R., & Bulun, S. E. (2000). Progesterone receptor isoform A but not B is expressed in endometriosis. *Journal of Clinical Endocrinology and Metabolism*, 85(8), 2897–2902.
- Benshushan, A. (2004). Endometrial adenocarcinoma in young patients: evaluation and fertility-preserving treatment. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 117(2), 132–137.
- Bokhman, J. V. (1983). Two pathogenetic types of endometrial carcinoma. *Gynecologic Oncology*, 15(1), 10–17.
- Brody, J. R., & Cunha, G. R. (1989). Histologic, morphometric, and immunocytochemical analysis of myometrial development in rats and mice: I. Normal development. *American Journal of Anatomy*, 186(1), 1–20.
- Bulun, S. E. (2009). Endometriosis. *New England Journal of Medicine*, 360(3), 268–279.
- Burney, R. O., & Giudice, L. C. (2012). Pathogenesis and pathophysiology of endometriosis. *Fertility and Sterility*, 98(3), 511–519.
- Burney, R. O., Talbi, S., Hamilton, A. E., Vo, K. C., Nyegaard, M., Nezhat, C. R., et al. (2007). Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. *Endocrinology*, 148(8), 3814–3826.
- Burns, K. A., & Korach, K. S. (2012). Estrogen receptors and human disease: an update. *Archives of Toxicology*, 86(10), 1491–1504.
- Capuco, A. V., & Akers, R. M. (2009). The origin and evolution of lactation. *Journal of Biology*, 8(4), 37.
- Cha, J., Sun, X., & Dey, S. K. (2012). Mechanisms of implantation: strategies for successful pregnancy. *Nature Medicine*, 18(12), 1754–1767.
- Charytan, D. M., Li, S., Liu, J., & Herzog, C. A. (2012). Risks of death and end-stage renal disease after surgical compared with percutaneous coronary revascularization in elderly patients with chronic kidney disease. *Circulation*, 126(11 Suppl. 1), S164–9.
- Chassot, P. G., Delabays, A., & Spahn, D. R. (2002). Preoperative evaluation of patients with, or at risk of, coronary artery disease undergoing non-cardiac surgery. *British Journal of Anaesthesia*, 89(5), 747–759.
- Clifford, K., Rai, R., & Regan, L. (1997). Future pregnancy outcome in unexplained recurrent first trimester miscarriage. *Human Reproduction*, 12(2), 387–389.
- Decruze, S. B., & Green, J. A. (2007). Hormone therapy in advanced and recurrent endometrial cancer: a systematic review. *International Journal of Gynecological Cancer*, 17(5), 964–978.
- Deligdisch, L., & Holinka, C. F. (1987). Endometrial carcinoma: two diseases? *Cancer Detection and Prevention*, 10(3–4), 237–246.
- Deroo, B. J., & Korach, K. S. (2006). Estrogen receptors and human disease. *Journal of Clinical Investigation*, 116(3), 561–570.
- Dharma, S. J., Kholkute, S. D., & Nandedkar, T. D. (2001). Apoptosis in endometrium of mouse during estrous cycle. *Indian Journal of Experimental Biology*, 39(3), 218–222.
- Drake, P. M., Gunn, M. D., Charo, I. F., Tsou, C. L., Zhou, Y., Huang, L., et al. (2001). Human placental cytotrophoblasts attract monocytes and CD56(bright) natural killer cells via the actions of monocyte inflammatory protein 1alpha. *Journal of Experimental Medicine*, 193(10), 1199–1212.
- El-Zibdeh, M. Y., & Yousef, L. T. (2009). Dydrogesterone support in threatened miscarriage. *Maturitas*, 65(Suppl. 1), S43–6.
- Eskenazi, B., & Warner, M. L. (1997). Epidemiology of endometriosis. *Obstetrics and Gynecology Clinics of North America*, 24(2), 235–258.
- Ferenczy, A. (1998). Pathophysiology of adenomyosis. *Human Reproduction Update*, 4(4), 312–322.
- Franco, H. L., Rubel, C. A., Large, M. J., Wetendorf, M., Fernandez-Valdivia, R., Jeong, J. W., et al. (2012). Epithelial progesterone receptor exhibits pleiotropic roles in uterine development and function. *FASEB Journal*, 26(3), 1218–1227.
- Gargett, C. E., Nguyen, H. P., & Ye, L. (2012). Endometrial regeneration and endometrial stem/progenitor cells. *Reviews in Endocrine & Metabolic Disorders*, 13(4), 235–251.
- Graham, J. D., & Clarke, C. L. (1997). Physiological action of progesterone in target tissues. *Endocrine Reviews*, 18(4), 502–519.
- Groothuis, P. G., Dassen, H. H., Romano, A., & Punyadeera, C. (2007). Estrogen and the endometrium: lessons learned from gene expression profiling in rodents and human. *Human Reproduction Update*, 13(4), 405–417.
- Hahn, H. S., Yoon, S. G., Hong, J. S., Hong, S. R., Park, S. J., Lim, J. Y., et al. (2009). Conservative treatment with progestin and pregnancy outcomes in endometrial cancer. *International Journal of Gynecological Cancer*, 19(6), 1068–1073.
- Hantak, A. M., Bagchi, I. C., & Bagchi, M. K. (2014). Role of uterine stromal-epithelial crosstalk in embryo implantation. *International Journal of Developmental Biology*, 58(2–4), 139–146.
- Huet-Hudson, Y. M., Andrews, G. K., & Dey, S. K. (1989). Cell type-specific localization of c-myc protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. *Endocrinology*, 125(3), 1683–1690.

- Hussain, M., El-Hakim, S., & Cahill, D. J. (2012). Progesterone supplementation in women with otherwise unexplained recurrent miscarriages. *Journal of Human Reproductive Sciences*, 5(3), 248–251.
- Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C., et al. (2006). Cancer statistics, 2006. *CA: a Cancer Journal for Clinicians*, 56(2), 106–130.
- Johnson, N. P., & Hummelshoj, L. (2013). World Endometriosis Society Montpellier C. Consensus on current management of endometriosis. *Human Reproduction*, 28(6), 1552–1568.
- Kao, L. C., Germeyer, A., Tulac, S., Lobo, S., Yang, J. P., Taylor, R. N., et al. (2003). Expression profiling of endometrium from women with endometriosis reveals candidate genes for disease-based implantation failure and infertility. *Endocrinology*, 144(7), 2870–2881.
- Kariagina, A., Aupperlee, M. D., & Haslam, S. Z. (2008). Progesterone receptor isoform functions in normal breast development and breast cancer. *Critical Reviews in Eukaryotic Gene Expression*, 18(1), 11–33.
- Kim, Y. B., Holschneider, C. H., Ghosh, K., Nieberg, R. K., & Montz, F. J. (1997). Progesterone alone as primary treatment of endometrial carcinoma in premenopausal women. Report of seven cases and review of the literature. *Cancer*, 79(2), 320–327.
- Kim, J. J., Kurita, T., & Bulun, S. E. (2013). Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocrine Reviews*, 34(1), 130–162.
- Kota, S. K., Gayatri, K., Jammula, S., Kota, S. K., Krishna, S. V., Meher, L. K., et al. (2013). Endocrinology of parturition. *Indian Journal of Endocrinology and Metabolism*, 17(1), 50–59.
- Kumar, P., & Magon, N. (2012). Hormones in pregnancy. *Nigerian Medical Journal: Journal of the Nigeria Medical Association*, 53(4), 179–183.
- Kurman, R. J., Kaminski, P. F., & Norris, H. J. (1985). The behavior of endometrial hyperplasia. A long-term study of “untreated” hyperplasia in 170 patients. *Cancer*, 56(2), 403–412.
- Large, M. J., & DeMayo, F. J. (2012). The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. *Molecular and Cellular Endocrinology*, 358(2), 155–165.
- Levgur, M., Abadi, M. A., & Tucker, A. (2000). Adenomyosis: symptoms, histology, and pregnancy terminations. *Obstetrics and Gynecology*, 95(5), 688–691.
- Li, X., & O'Malley, B. W. (2003). Unfolding the action of progesterone receptors. *Journal of Biological Chemistry*, 278(41), 39261–39264.
- Losel, R., & Wehling, M. (2003). Nongenomic actions of steroid hormones. *Nature Reviews Molecular Cell Biology*, 4(1), 46–56.
- Lydon, J. P., DeMayo, F. J., Conneely, O. M., & O'Malley, B. W. (1996). Reproductive phenotypes of the progesterone receptor null mutant mouse. *Journal of Steroid Biochemistry and Molecular Biology*, 56, 67–77 (1–6 Spec No).
- Martin, L., Finn, C. A., & Trinder, G. (1973a). Hypertrophy and hyperplasia in the mouse uterus after oestrogen treatment: an autoradiographic study. *Journal of Endocrinology*, 56(1), 133–144.
- Martin, L., Das, R. M., & Finn, C. A. (1973b). The inhibition by progesterone of uterine epithelial proliferation in the mouse. *Journal of Endocrinology*, 57(3), 549–554.
- Minaguchi, T., Nakagawa, S., Takazawa, Y., Nei, T., Horie, K., Fujiwara, T., et al. (2007). Combined phospho-Akt and PTEN expressions associated with post-treatment hysterectomy after conservative progestin therapy in complex atypical hyperplasia and stage Ia, G1 adenocarcinoma of the endometrium. *Cancer Letters*, 248(1), 112–122.
- Mote, P. A., Balleine, R. L., McGowan, E. M., & Clarke, C. L. (1999). Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*, 84(8), 2963–2971.
- ACOG Committee Opinion No. 444: choosing the route of hysterectomy for benign disease. *Obstetrics and Gynecology*, 114(5)(2009), 1156–1158.
- Paria, B. C., Huet-Hudson, Y. M., & Dey, S. K. (1993). Blastocyst's state of activity determines the “window” of implantation in the receptive mouse uterus. *Proceedings of the National Academy of Sciences of the United States of America*, 90(21), 10159–10162.
- Park, H., Seok, J. M., Yoon, B. S., Seong, S. J., Kim, J. Y., Shim, J. Y., et al. (2012). Effectiveness of high-dose progestin and long-term outcomes in young women with early-stage, well-differentiated endometrioid adenocarcinoma of uterine endometrium. *Archives of Gynecology and Obstetrics*, 285(2), 473–478.
- Pellerin, G. P., & Finan, M. A. (2005). Endometrial cancer in women 45 years of age or younger: a clinicopathological analysis. *American Journal of Obstetrics and Gynecology*, 193(5), 1640–1644.
- Practice Committee of American Society for Reproductive Medicine in collaboration with Society for Reproductive E, Infertility. (2008). Progesterone supplementation during the luteal phase and in early pregnancy in the treatment of infertility: an educational bulletin. *Fertility and sterility*, 90(5 Suppl.), S150–S153.
- Ramirez, P. T., Frumovitz, M., Bodurka, D. C., Sun, C. C., & Levenback, C. (2004). Hormonal therapy for the management of grade 1 endometrial adenocarcinoma: a literature review. *Gynecologic Oncology*, 95(1), 133–138.
- Randall, T. C., & Kurman, R. J. (1997). Progesterone treatment of atypical hyperplasia and well-differentiated carcinoma of the endometrium in women under age 40. *Obstetrics and Gynecology*, 90(3), 434–440.
- Red-Horse, K., Zhou, Y., Genbacev, O., Prakobphol, A., Foulk, R., McMaster, M., et al. (2004). Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *Journal of Clinical Investigation*, 114(6), 744–754.
- Rubel, C. A., Jeong, J. W., Tsai, S. Y., Lydon, J. P., & DeMayo, F. J. (2010). Epithelial-stromal interaction and progesterone receptors in the mouse uterus. *Seminars in Reproductive Medicine*, 28(1), 27–35.
- Sever, R., & Glass, C. K. (2013). Signaling by nuclear receptors. *Cold Spring Harbor Perspectives in Biology*, 5(3), a016709.
- Sherman, M. E., Sturgeon, S., Brinton, L. A., Potischman, N., Kurman, R. J., Berman, M. L., et al. (1997). Risk factors and hormone levels in patients with serous and endometrioid uterine carcinomas. *Modern Pathology*, 10(10), 963–968.
- Siegel, R. L., Miller, K. D., & Jemal, A. (2016). Cancer statistics, 2016. *CA: a Cancer Journal for Clinicians*, 66(1), 7–30.
- Sinaii, N., Cleary, S. D., Ballweg, M. L., Nieman, L. K., & Stratton, P. (2002). High rates of autoimmune and endocrine disorders, fibromyalgia, chronic fatigue syndrome and atopic diseases among women with endometriosis: a survey analysis. *Human Reproduction*, 17(10), 2715–2724.
- Solar, P., & Velasquez, L. (2013). Consequences of nongenomic actions of estradiol on pathogenic genital tract response. *Journal of Molecular Signaling*, 8(1), 1.
- Soyal, S. M., Mukherjee, A., Lee, K. Y., Li, J., Li, H., DeMayo, F. J., et al. (2005). Cre-mediated recombination in cell lineages that express the progesterone receptor. *Genesis*, 41(2), 58–66.
- Szwarc, M. M., Kommagani, R., Lessey, B. A., & Lydon, J. P. (2014). The p160/steroid receptor coactivator family: potent arbiters of uterine physiology and dysfunction. *Biology of Reproduction*, 91(5), 122.
- Szwarc, M. M., Lydon, J. P., & O'Malley, B. W. (2015). Reprint of “Steroid receptor coactivators as therapeutic targets in the female reproductive system”. *Journal of Steroid Biochemistry and Molecular Biology*, 153, 144–150.
- Tamai, K., Togashi, K., Ito, T., Morisawa, N., Fujiwara, T., & Koyama, T. (2005). MR imaging findings of adenomyosis: correlation with histopathologic features and diagnostic pitfalls. *Radiographics*, 25(1), 21–40.
- Temkin, S. M., Minasian, L., & Noone, A. M. (2016). The end of the hysterectomy epidemic and endometrial cancer incidence: what are the unintended consequences of declining hysterectomy rates? *Frontiers in Oncology*, 6, 89.
- Tsai, M. J., & O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Review of Biochemistry*, 63, 451–486.
- van der Linden, M., Buckingham, K., Farquhar, C., Kremer, J. A., & Metwally, M. (2015). Luteal phase support for assisted reproduction cycles. *The Cochrane database of systematic reviews*, 7(7), CD009154.
- Varon, J., & Marik, P. E. (2008). Perioperative hypertension management. *Vascular Health and Risk Management*, 4(3), 615–627.
- Vasquez, Y. M., & DeMayo, F. J. (2013). Role of nuclear receptors in blastocyst implantation. *Seminars in Cell & Developmental Biology*, 24(10–12), 724–735.
- Vercellini, P., Vigano, P., Somigliana, E., Daguati, R., Abbiati, A., & Fedele, L. (2006). Adenomyosis: epidemiological factors. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 20(4), 465–477.

- Walch, K. T., & Huber, J. C. (2008). Progesterone for recurrent miscarriage: truth and deceptions. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 22(2), 375–389.
- Wetendorf, M., & DeMayo, F. J. (2012). The progesterone receptor regulates implantation, decidualization, and glandular development via a complex paracrine signaling network. *Molecular and Cellular Endocrinology*, 357(1–2), 108–118.
- Wierman, M. E. (2007). Sex steroid effects at target tissues: mechanisms of action. *Advances in Physiology Education*, 31(1), 26–33.
- Winuthayanon, W., Hewitt, S. C., Orvis, G. D., Behringer, R. R., & Korach, K. S. (2010). Uterine epithelial estrogen receptor alpha is dispensable for proliferation but essential for complete biological and biochemical responses. *Proceedings of the National Academy of Sciences of the United States of America*, 107(45), 19272–19277.
- Wood, G. A., Fata, J. E., Watson, K. L., & Khokha, R. (2007). Circulating hormones and estrous stage predict cellular and stromal remodeling in murine uterus. *Reproduction*, 133(5), 1035–1044.
- Yamazawa, K., Hirai, M., Fujito, A., Nishi, H., Terauchi, F., Ishikura, H., et al. (2007). Fertility-preserving treatment with progestin, and pathological criteria to predict responses, in young women with endometrial cancer. *Human Reproduction*, 22(7), 1953–1958.
- Yang, S., Thiel, K. W., & Leslie, K. K. (2011). Progesterone: the ultimate endometrial tumor suppressor. *Trends in Endocrinology and Metabolism*, 22(4), 145–152.
- Young, S. L. (2013). Oestrogen and progesterone action on endometrium: a translational approach to understanding endometrial receptivity. *Reproductive Biomedicine Online*, 27(5), 497–505.

The Primate Uterus: Cyclic Changes

By Daniel Slayden and Andrea Calhoun, Oregon National Primate Research Center, Beaverton, OR, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Basalis zone The deepest zone of the endometrium adjacent to the myometrium.

Decidualization The postovulatory transformation of the stromal compartment of the endometrium in preparation for pregnancy.

Endometrium The inner mucosal lining of the uterus.

Functional Zone The upper zones of the endometrium that undergo cycling differentiation and shedding.

Menstruation Cyclic shedding of the upper zones of the endometrium at the beginning of each menstrual cycle.

Myometrium The smooth muscle layer of the uterine wall.

Spiral Arteries Coiled arteries that supply blood to endometrial functionalis zone during the luteal phase of the menstrual cycle.

Old World monkeys (Cercopithecidae) have reproductive tract anatomy and menstrual cycles similar to women. Herein, we describe the cyclic effects of steroid hormones on the primate uterus. In previous reviews we reference the historic literature extensively (see References). The focus here will be on nonhuman primates (NHPs) including rhesus (*Macaca mulatta*) and cynomolgus macaques (*M. fascicularis*), which our laboratory has studied extensively, with brief comparisons to other species including the vervet (*Chlorocebus aethiops*), the baboon (*Papio*), and women. At the beginning of each cycle, the luminal portion of the uterine lining, the endometrium, of these species is sloughed in menstruation. Macaques, baboons, and women display “frank” menstruation where bleeding is detectable externally. In contrast, the vervet displays minimal bleeding that can only be detected by vaginal swab. After menstruation, estradiol (E_2) secreted by the ovary during the follicular phase of the cycle stimulates endometrial cell proliferation and regrowth. Thus, the follicular phase is also referred to as the proliferative phase. In primates, the typical proliferative phase is 10–14 days but can be highly variable in length. After ovulation, progesterone (P_4) secreted from the corpus luteum stimulates endometrial secretory differentiation in preparation for embryo implantation. If embryo implantation does not occur, the luteal (secretory) phase of the cycle usually lasts for 12–14 days. The decline of P_4 at the end of the cycle (approximately 28 days) triggers menstruation. Estrogen and P_4 are the only ovarian factors needed to elicit cyclic responses in the uterus. In a proof of concept study, Hodgen (1983) demonstrated that pregnancy could occur in an ovariectomized macaque treated with E_2 and P_4 in this sequential fashion. In that study, an embryo was transferred into the fimbrial ostium, indicating that both fallopian tube and uterine function were preserved by E_2 and P in the absence of ovaries.

Cyclic Changes in Morphology

The primate (simplex) uterus can be divided into the fundus (dome-shaped top), the corpus (body), and the isthmus (neck) which leads into the cervix. Fig. 1 shows a picture of a macaque uterus. The endometrium that lines the uterine cavity is surrounded by a muscular wall, the myometrium. Fig. 2 shows full-thickness histological sections of the macaque endometrium. During menstruation (Fig. 2A) the upper portion of the endometrium is friable with evidence of breakdown and bleeding (Fig. 2D) of the luminal surface. In the proliferative and secretory phases of the cycle, the luminal surface displays a simple columnar epithelium, which is ciliated in women but predominately nonciliated in macaques and baboons. The primate endometrium contains simple tubular or branched glands that extend to the endometrial–myometrial border. The lamina propria or stroma is a highly cellular connective tissue with an amorphous extracellular matrix. The macaque and human endometrium were described by Bartelmez (1933, 1951) as having four morphological zones. Zone I is defined as the surface epithelium with an underlying band of stromal cells. Zone II the glands that run perpendicular to the surface, and deeper; Zone III contains branched glands. Zone IV is the basal layer where the glands terminate adjacent to the myometrium. Zones I–III are commonly termed the functionalis zones because these zones undergo cyclic secretory differentiation and menstrual sloughing. Zone IV, the basalis zone, is reported to be relatively unresponsive to hormonal changes in women, but in the macaque, the basalis zone undergoes cell proliferation in the early secretory phase of the cycle. Development of zonal anatomy is dependent on ovarian steroids and in premenarchial and postmenopausal primates the zonal anatomy is often indistinct. Hormone supplementation or replacement in postmenopausal animals results in reappearance and hypertrophy of the zones.

The myometrium surrounds the endometrium with four distinct layers of smooth muscle that include (1) the *stratum submucosum*, longitudinal muscle fibers underlying the endometrium; (2) the *stratum vasculare*, a blood vessel-rich layer with fibers running in multiple directions; (3) the *stratum supravascular*, a layer of circular and longitudinal bundles; and (4) the *stratum*

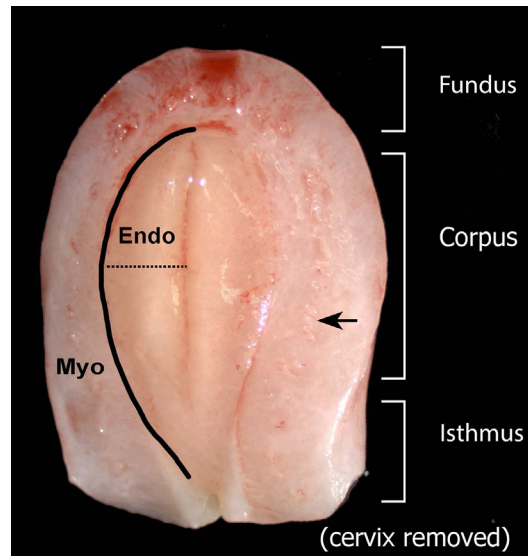


Fig. 1 Macrophotograph showing half of a proliferative phase rhesus macaque uterus that has been cut along the longitudinal axis. A solid black line has been drawn to delineate the endometrial–myometrial border, and a dashed line has been drawn to delineate the plane of sectioning for histological analysis.

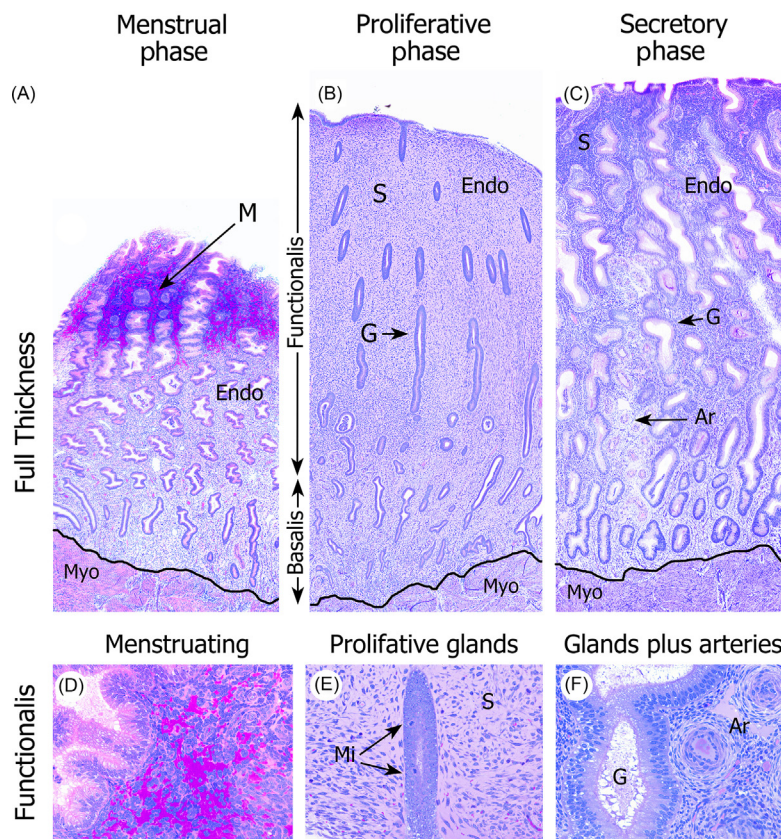


Fig. 2 Photomicrographs of glycol methacrylate embedded, Hematoxylin/Eosin stained sections of rhesus macaque endometrium showing cyclic changes in histology. Phase of the menstrual cycle is shown in columns. A black line has been drawn on the full thickness sections to show the myometrial border. *Endo*, endometrium; *Myo*, myometrium; *Mi*, mitosis; *G*, gland; *S*, stroma; *Ar*, spiral artery.

subserosum; a layer that is continuous with the myosalpinx (oviduct) and the connective tissues of the broad ligament. Blood vessels, individual nerve fiber, and nerve bundles intersect these layers. A unique junctional zone adjacent to the endometrial basalis has been extensively studied in women by magnetic resonance imaging and macaques by ultrasound.

The pattern of vascular circulation in the primate uterus has been described in a series of classic papers (Bartelmez, 1956, 1957). Briefly, the uterine artery on each side enters the uterus and runs as an arcuate artery to supply the myometrium. The arcuate sends numerous radial arteries into the endometrium. The functionalis zone derives its blood supply from specialized spiral arteries (Fig. 2C and F). Basal arteries supply the basalis zone. In an alternative classification the arteries that vascularize the functionalis zone are called Type I endometrial arteries, and the basal arteries that only vascularize the basalis and lower part of Zone III as Type II. The basal arteries produce a capillary bed that maintains the viability of the lower regions when the upper layers slough and degenerate during menstrual breakdown.

Noyes et al. (1950) created a scheme for morphologically dating the endometrium of women relative to an ideal 28-day cycle, and similar cyclic changes have been described in NHPs. In this scheme, the first day of menstruation is designated cycle day 1, and menses typically lasts 2–5 days. After menstruation in the early proliferative phase the endometrial glands are straight and short, and the stroma is compacted. By the midproliferative period (days 8–10), the glands are longer with abundant mitotic cells (see Fig. 2E), and a mitotically active stroma is somewhat edematous. In the late proliferative phase (days 11–14), the rapidly growing glands become more tortuous, and the epithelium appears pseudostratified due to the accelerated proliferation of the elongated cells. The stroma continues to be mitotically active and is less edematous than during the midproliferative stage. The effects of the P_4 are evident by the second day after ovulation (day 16 in an ideal cycle). At this time, glycogen accumulates in the basal portion of the glandular epithelium, displacing the nuclei and creating a pseudostratified appearance. Glycogen is often solubilized in histological preparations, leaving the appearance of vacuoles in the cells. By mid-secretory phase (days 18–20) the vacuoles become smaller as glycogen moves into the apex of the cells (see Fig. 2F), continuing into the gland lumen. On cycle days 21 and 22 the endometrial stroma becomes highly edematous. On day 23 stromal cells surrounding the spiral arterioles begin to enlarge and along with stromal mitoses constitutes the earliest visible predecidual change. By day 24, in the macaque, predecidual cells are observed cuffing the spiral arterioles and stromal mitoses are more numerous. By day 25, hypertrophied stromal cells are present under the surface epithelium, and by day 27, the upper portion of the endometrial stroma consists of a sheet of well-developed decidual-like cells. Lymphocytes and polymorphonuclear leukocytes invade the upper regions on days 25–27. The glands dilate during the late secretory phase giving glandular epithelium a saw-toothed appearance, with shrunken nuclei and jagged apical surfaces, an appearance that has been referred to as secretory exhaustion.

The Noyes criteria for endometrial dating have been challenged, primarily for its use in diagnosing the impact of low P_4 in the secretory phase (luteal-phase deficiency) and for determining the fertile window for embryo transfer. Advances in the genomics of endometrial receptivity have identified an array of molecular biomarkers that can better define the window of embryo implantation. More recently, the endometrial proteome has been probed by MALDI-MS to unveil an array of differentially expressed proteins that define the receptive endometrium of women.

Cell Proliferation

To circumvent the pitfalls of Noyes criteria and precisely time endocrine control of the monkey reproductive tract, we have sequentially treated macaques with implants that release E_2 alone for 14 days (an artificial proliferative phase) and then E_2 plus P_4 for 14 days (an artificial secretory phase). In this model E_2 priming results in tubular glands with abundant mitotic cells identical to the follicular phase of the natural cycle. In our studies and those of others, immunohistochemistry (IHC) for proliferation-associated markers including Ki-6, phosphor H3 (a marker of mitotic cells), and in vivo labeling with bromodeoxyuridine has been used extensively to detect and quantify cell proliferation. All three techniques show similar trends during the cycle. Fig. 3 shows IHC staining for Ki-67 in the macaque endometrial functional and basalis zones.

Use of artificial cycles has demonstrated that cell proliferation is minimal in both the functionalis and basalis zones during menstruation. The endometrium that remains after menstruation has a ragged and torn surface marked by multiple gland openings surrounded by denuded stroma that lacks a covering epithelium in both macaques and women. Healing of this denuded stromal surface begins with the transformation of the uppermost gland cells into a migratory phenotype. These cells proliferate and move out from the necks of the glands, spread out, and meet migrating cells from other glands to form a new luminal surface (Ludwig and Spornitz, 1991). In the macaque there are abundant proliferating cells in the Zone 1 glands, but epithelial cell proliferation in the basalis is quiescent (compare Fig. 3B and E). Healing is complete by 4–5 days after P_4 withdrawal at which time estrogen-dependent mitotic activity begins in the uppermost regions of the glands. Counts of proliferating cells reveal that almost all of the epithelial cell replacement during the late proliferative phase occurs in the mid- and upper-functional zones. Also during the proliferative phase, the upper zones contain abundant apoptotic cells, indicating a balance of cell death and cell birth, with the overall trend toward more growth. Apoptosis and phagocytosis of dead cells play major roles in endometrial remodeling. The spiral arteries are extensively remodeled or partially lost during menstruation. New growth in the proliferative phase features small vessels in the upper functionalis zone, appearing to peak on Day 8.

In the artificial cycle the effects of P_4 on the epithelium (Fig. 3G–I) become evident on cycle day 17, after day 3 of P_4 treatment. P_4 gradually suppresses cell proliferation in the glandular epithelium of the functionalis zone and induces hypertrophy and

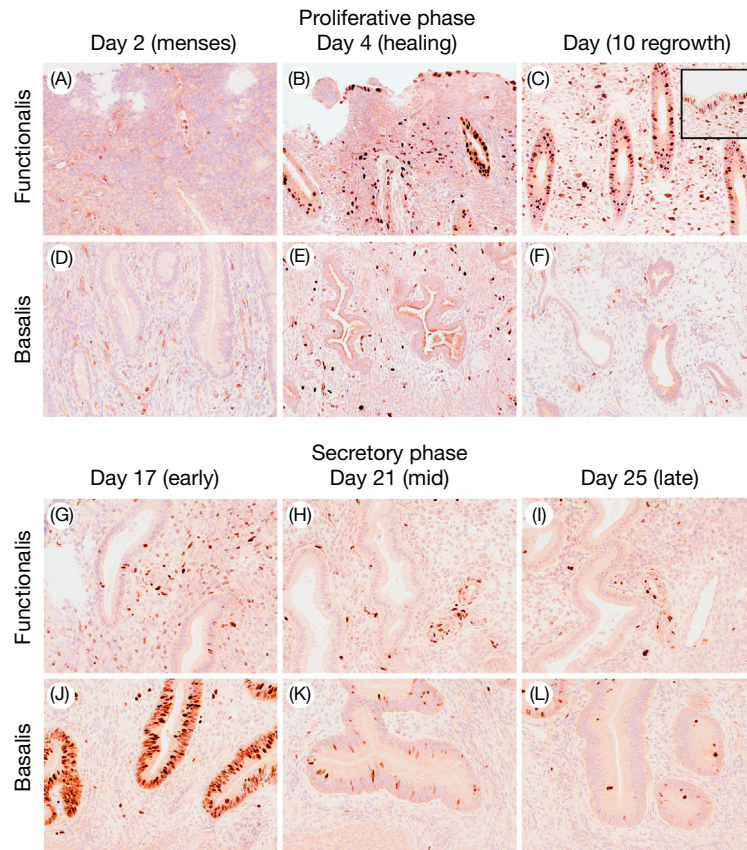


Fig. 3 Immunostaining for Ki-67 a marker of cell proliferation in the proliferative and secretory phases of the cycle. Slides were stained with the antihuman Ki-67 antibody (Dako Corp.; Carpinteria, CA); positive staining is dark brown.

accumulation of glycogen in epithelial cells. Conversely, the basalis zone, which is quiescent during the proliferative phase, displays an intense period of cell proliferation that gradually subsides by late secretory phase.

Menstruation

Old world monkeys, like women, exhibit true menstruation in which the functionalis zone of the endometrium is shed each cycle. In the macaque, exposure to P_4 (< 1 ng/ml in serum) for at least 3 days is required to prime the endometrium for menstruation. After that, withdrawal of P_4 triggers expression of matrix metalloproteinase (MMPs) enzymes in the upper endometrial zones that are the primary effectors resulting in dissociation of functionalis zone tissues. Macaque endometrial MMP-1, -2, -3, -10, -11, and -14 transcripts peak 2–4 days after P_4 withdrawal and then declines spontaneously during the proliferative phase of the cycle. Studies in the macaque have led to the view that a “critical period” consisting of 24–30 h of P_4 withdrawal exists during which P_4 replacement will prevent tissue breakdown. After this critical period, sloughing and bleeding become inevitable even if P_4 is replaced, indicating that P_4 does not simply block MMP synthesis.

Withdrawal of P_4 results in vasoconstriction of the spiral arteries. [Markee \(1940\)](#) proposed vasoconstriction of these arteries could lead to localized ischemic hypoxia in the superficial zones. While vasoconstriction may not play a regulatory role in menses, hypoxia is a strong regulator of vascular endothelial growth factor (VEGF), and both stromal and glandular VEGF are upregulated premenstrually in the macaque endometrium. In the macaque, hypoxia-inducible factor 1 alpha (HIF-1 α) is elevated in the small vessels of the endometrial functionalis, supporting a role for hypoxia during menses.

The focal nature of menstrual breakdown and MMP expression in the mid- and upper-functional zones supports the concept that local factors such as cytokines act to relay the effects of P_4 withdrawal preceding menstruation. Studies have promulgated the concept that the entire functionalis zone is lost during menstruation and that a new functionalis arises from proliferating stem cells located in the basalis. It has been demonstrated that an entire endometrium could regenerate from remnants left after dissecting out the endometrium with a scalpel and then wiping the uterine cavity clean with a cotton swab. Adult stem cells are present in other tissues that undergo regeneration; it has been proposed that endometrial stem cells could reside within the basalis zone and account for endometrial regrowth. However, during normal menstruation, the entire endometrium is not shed, and as menstruation ends, the surface epithelium undergoes a brief period of hormone-independent healing and reepithelialization from the remaining

glands. Bartelmez and Markee proposed that during natural menstruation little tissue is shed and the reduction in the thickness of the endometrium is primarily due to loss of fluid, resulting in shrinkage of the spongy layer. As indicated above found that mitotic activity is very low in the basal region until midcycle when it increased after the periovulatory period. In the early repair phase, synthesized DNA is primarily in the gland and surface epithelial cells, involved both cellular migration and replication, and is essentially a wound-healing reaction that was not dependent upon E_2 . Moreover, the highest rates of proliferation were evident on days 8–10 in the upper third of the functionalis, not the basalis. The sum of these studies indicate that P_4 stimulates cell proliferation in the basalis while it inhibits glandular proliferation in the upper zones.

Steroid Receptors

Two estrogen receptors ESR1 and ESR2 are expressed in the endometrium and undergo cyclic changes in expression and localization. ESR1 transcript and protein increase following E_2 treatment and decrease in response to P_4 treatment in the endometrium of women and NHPs. Fig. 4A–D shows immunostaining for ESR1 in the macaque endometrium. After E_2 treatment alone for 14 days, staining for ESR1 is strongly positive in the nucleus of glandular epithelium, and in the stroma of both functionalis and basalis zones. Cotreatment with P_4 during artificial secretory phase results in a P_4 -induced blockade of E_2 -stimulated ESR1, resulting in minimal ESR1 staining in glands and stroma of the functionalis zone. However, P_4 does not inhibit ESR1 expression in the glands and stroma of the basalis zone. Unlike ESR1, ESR2 staining in the glands and stroma of the macaque does not undergo cyclic changes. Also, though there is no detectable ESR1 staining in the endothelium of endometrial vessels of the macaque or women, there is strong staining for ESR2 throughout the proliferative and secretory phases. ESR1 is detectable by IHC and in situ hybridization muscle and pericytes of the spiral arteries of macaques, baboon, and women. In the macaque and vervet, the basal arteries are negative for ESR1. Also, many of the radial and arcuate arteries in the myometrium were negative for both ESR1 protein and ESR1 mRNA. Therefore E_2 could act on the vessels through endothelial ESR2 during the whole cycle, uninfluenced by P_4 .

Two functional progesterone receptors (PGRs) (PGR-A and PGR-B) have been identified. PGR-B is a larger molecule than PGR-A due to the presence of an additional 165 amino acids in the N-terminal domain; this distinction between the two isoforms appear to cause differential expression in the human endometrium. IHC with antibodies that recognize both PGR forms (Fig. 4E–H) shows that E_2 treatment increases PGR nuclear staining in all cell types except vascular smooth muscle and endothelium. Strong staining is

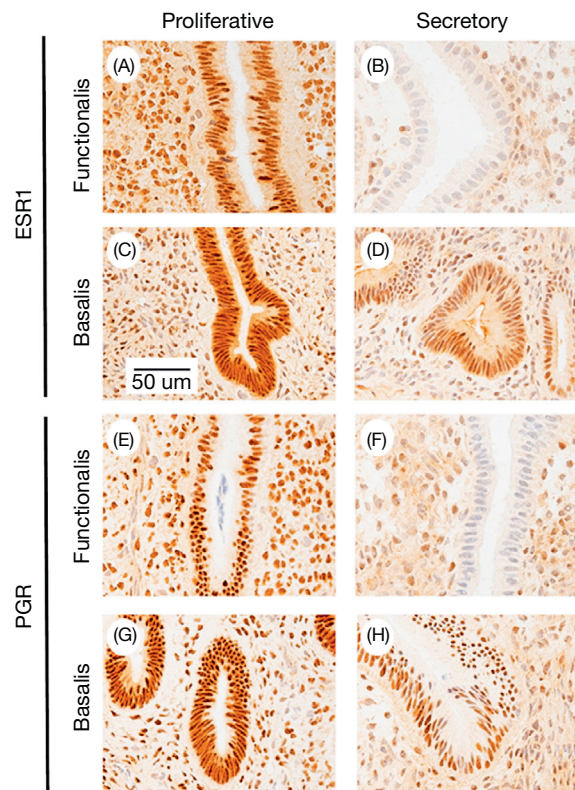


Fig. 4 Immunohistochemical staining for ESR1 and PGR in endometrial cryosections, ESR1 was detected with monoclonal antibody 1D-5 (Biogenex, San Ramon, CA). PGR staining was detected with monoclonal PR Ab-8 antibody (Neomarker Inc., Fremont, CA). Strong staining was detected for both ESR1 and PGR in the glands and stroma of the proliferative phase. In the secretory phase, P_4 resulted in reduction of glandular and stromal ESR1, whereas PGR staining was retained in the stroma of the functionalis but retained in the basalis zone.

observed in both the glands and stroma during the proliferative phase. In the luteal phase or after cotreatment with P_4 , functionalis glandular PGR staining is strikingly decreased, yet PGR is retained in the functionalis stroma, basalis glands, and stroma.

PGR staining is strong in perivascular stromal cells, but there is no specific PGR staining in either the endothelium or the smooth muscle cells of the endometrial vasculature. PGR of the vascular wall is present in the spiral arteries of the functionalis but not the basalis. Consequently, we suggest that the genomic actions of progestins on some uterine vascular elements are likely to be indirect.

Androgen receptor (AR) is also present in the macaque and human endometrium. E_2 increases and P_4 decreases expression of AR mRNA and protein within the endometrial stroma. No AR is detectable in the endothelium or vascular smooth muscle of the spiral arteries.

Paracrine Factors

It is well recognized that ovarian steroids drive the synthesis of paracrine factors, which have the potential to regulate cell proliferation in target tissues. While steroid stimulation of growth factors have been reported, none have been *unequivocally* identified as a universal mediator of steroid action in the primate uterus. Among the likely contenders are the insulin-like growth factor (IGF) and epidermal growth factor (EGF) and fibroblast growth factor (FGF) families. Estrogens upregulate EGF in nonprimates and EGF has been shown to have estrogen-like effects on uterine growth. EGF mRNA is present in human endometrium and the EGF peptide has been detected by IHC. Insulin-like growth factor I and II (IGF-I, IGF-II) can that stimulate mitosis in a wide variety of tissues. Regulation of IGFs in the uterus has been studied extensively in nonprimates in which the predominant stimulator of IGF-I secretion is E_2 . Cell membrane receptors for IGF-I and IGF-II have been identified in the rodent and human endometrium in both the proliferative and secretory phases. Receptors for IGF-I appear to be increased by E_2 . IGFs are typically found bound to specific insulin-like growth factor-binding proteins (IGFBPs) that modulate their action. IGFBP-1, -2, and -3 have been reported in the human and baboon endometrium. In women, IGFBP-1 has been localized by IHC in decidualized stromal cells during both the cyclic luteal phase and pregnancy. IGFBP-1 mRNA is reported to be expressed by the secretory but not proliferative human endometrium. IGFBP can be detected via IHC in the endometrium of baboons during the late cyclic luteal phase, pregnancy, and after E_2 plus P_4 treatment, but not during follicular phase or after E_2 treatment alone. However, the histological distribution of IGFBP-1 in baboon appears to be different from that reported in women. In the nonpregnant baboon, IGFBP-1 synthesis is confined to the epithelial cells of the deep basal glands during the late luteal phase and shifts to the decidualized stromal cells during pregnancy. This pattern of IGFBP localization closely parallels IHC staining of PGR during the luteal phase and pregnancy as reviewed above, which supports the hypothesis that P_4 induces both IGFBP-1 secretion and decidualization in humans and NHPs. Two other IGFBPs, IGFBP-2 and IGFBP-3, have been examined in the human and baboon endometrium. In women, IGFBP-2 and IGFBP-3 transcripts are present in the proliferative and secretory endometrium.

Keratinocyte growth factor (KGF; FGF-7) is strikingly increased in the macaque endometrium during the secretory phase. Recent studies have indicated that KGF can also be produced by endometrial epithelial cells in several species. KGF receptor expression is primarily localized to epithelial cells, and it was proposed that KGF could play a role in regulation of epithelial cell function during the secretory phase. Administering KGF directly to the uterus via an oviductal catheter system induced antiapoptotic effects on the glandular epithelium.

Decidualization

In women, decidualization begins during the latter half of the secretory phase around the spiral arterioles and then spreads throughout the upper 2/3 of the endometrium. In the macaque, the endometrium does not fully decidualize until either pregnancy occurs or when P_4 treatment is maintained for longer than 14 days. Compared to animals treated with E_2 plus P_4 for only 2 weeks, treatment for 5 months with E_2 plus P induces a highly decidualized endometrium with enlarged stromal cells, excessive amounts of extracellular matrix in the functionalis, enlarged spiral arteries, and a greatly reduced number of glands.

In macaques the presence of an implanting conceptus stimulates the formation of an epithelial plaque on the endometrial surface (Enders, 1991). The plaque arises from a proliferation of cells of the luminal surface epithelial cells and ESR1 and PGR are negative. A striking difference observed in human and macaque endometrium is the degree to which the periarteriole stromal cells differentiate during the final week of the menstrual cycle. In the macaque, these changes that occur beyond the arterioles in the stroma are minimal in the nonpregnant animals. In this case, the stromal cells enlarge, but only cells surrounding the spiral arterioles develop decidual characteristics. Extended treatment with P_4 results in a more dramatic hypertrophy of most of the stromal cells in macaque endometrium and has been called a decidual reaction. Expression of tissue inhibitor of metalloproteinase 3 (TIMP-3) is a clear marker of decidual changes and appears first around the spiral arteries. A mechanical stimulus can be used to induce decidualization in nonpregnant rhesus monkeys in the secretory phase. Approximately 1/3 of macaques treated with chronic P_4 will develop breakthrough bleeding in the presence of decidualization. Decidualization of the endometrial stroma and glandular atrophy has also been reported in macaques treated for 14 weeks with intrauterine devices that released the synthetic progestin levonorgestrel. It is noteworthy that the effects of P_4 do not appear to require continuous E_2 support during the secretory phase; animals treated with E_2 for 14 days followed by P_4 alone undergo morphological changes that appear nearly identical to animals cotreated.

In conclusion, our studies have revealed cyclic morphological and biochemical changes in the NHP endometrium during the menstrual cycle. We conclude that these changes are identical in naturally cycling and artificially cycled macaques. These

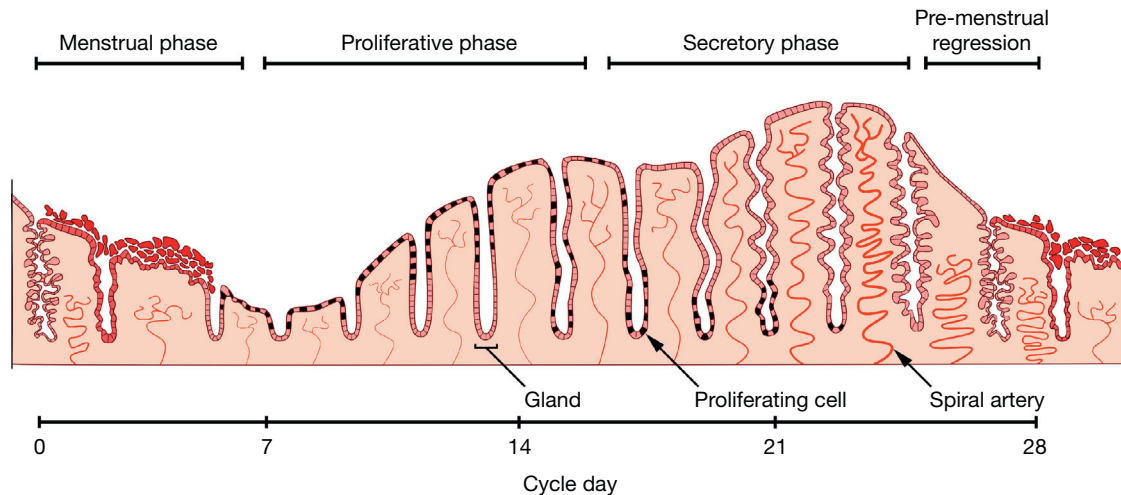


Fig. 5 Diagram depicting the cyclic changes in endometrial thickness, vascular growth, cell proliferation, and menstruation during the menstrual cycle.

endometrial changes are summarized in Fig. 4. At the end of the menstrual cycle, immediately preceding menstruation, the action of P_4 has suppressed ESR1 and PGR in endometrial functionalis glands, yet persist in the functionalis stroma and the basalis glands and stroma. When the functional corpus luteum regresses and circulating P_4 declines, the endometrium responds within a few days by menstruating. Menstruation must therefore be considered a stromal function, as the only cells in the functionalis with PGR that could “sense” P_4 withdrawal are in the stroma. As the next cycle begins after menstruation, the endometrial surface repairs itself independent of estrogen action. Then in the proliferative phase E_2 -dependent glandular and vascular growth begins. At mid-cycle, serum P_4 rises again and the endometrium steadily responds by stromal cell enlargement, increased glandular sacculization, spiral artery development, stromal edema, and increased mitotic activity in the basalis. P_4 acts to induce the expression of an array of steroid-regulated genes in both stromal and epithelial cells, but epithelial cells lack PGR during much of this time. The ability of P_4 to keep the epithelium suppressed may be a function of PGR-positive cells located within the stroma. This also generates other mediators that affect epithelial cell response during the late luteal phase until serum P_4 declines and the cycle begins anew (Fig. 5).

Acknowledgement

Dr. Slayden and Ms. Calhoun are supported by the Oregon National Primate Research Center (NIH OD011092 and U54 HD055744).

References

- Bartelmez, G. W. (1933). Histological studies on the menstruating mucous membrane of the human uterus. *Contributions to Embryology*, 142, 142–186.
- Bartelmez, G. W. (1951). Cyclic changes in the endometrium of the rhesus monkey (*Macaca mulatta*). *Contributions to Embryology*, 34, 99–144.
- Bartelmez, G. W. (1956). Premenstrual and menstrual ischemia and the myth of endometrial arteriovenous anastomoses. *Annals of the New York Academy of Sciences*, 98, 69–95.
- Bartelmez, G. W. (1957). The form and the functions of the uterine blood vessels in the rhesus monkey. *Contributions to Embryology*, 36, 153–182.
- Enders, A. C. (1991). Current topic: Structural responses of the primate endometrium to implantation. *Placenta*, 12, 309–325.
- Hodgen, G. D. (1983). Surrogate embryo transfer combined with estrogen-progesterone therapy in monkeys. *JAMA*, 250, 2167–2171.
- Ludwig, H., & Spornitz, U. M. (1991). Microarchitecture of the human endometrium by scanning electron microscopy: Menstrual desquamation and remodeling. *Annals of the New York Academy of Sciences*, 622, 28–46.
- Markee, J. E. (1940). Menstruation in intraocular endometrial transplants in the rhesus monkey. *Contributions to Embryology*, 177, 219–308.
- Noyes, R. W., Hertig, A. T., & Rock, J. (1950). Dating the endometrial biopsy. *Fertility and Sterility*, 1, 3–25.

Further Reading

- Brenner, R. M., & Slayden, O. D. (1994). Cyclic changes in the primate oviduct and endometrium. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction* (pp. 541–569). New York: Raven Press.
- Brenner, R. M., & Slayden, O. D. (2004). Steroid receptors in blood vessels of the rhesus macaque endometrium: A review. *Archives of Histology and Cytology*, 67, 411–416.
- Brenner, R. M., Slayden, O. D., Rodgers, W. H., Critchley, H. O., Carroll, R., Nie, X. J., & Mah, K. (2003). Immunocytochemical assessment of mitotic activity with an antibody to phosphorylated histone H3 in the macaque and human endometrium. *Human Reproduction*, 18, 1185–1193.
- Dominguez, F., Garrido-Gomez, T., Lopez, J. A., Camafeita, E., Quinonero, A., Pellicer, A., & Simon, C. (2009). Proteomic analysis of the human receptive versus non-receptive endometrium using differential in-gel electrophoresis and MALDI-MS unveils stathmin 1 and annexin A2 as differentially regulated. *Human Reproduction*, 24, 2607–2617.

- Horcajadas, J. A., Pellicer, A., & Simon, C. (2007). Wide genomic analysis of human endometrial receptivity: New times, new opportunities. *Human Reproduction Update*, 13, 77–86.
- Milne, S. A., Critchley, H. O. D., Drudy, T. A., Kelly, R. W., & Baird, D. T. (2001). Perivascular interleukin-8 messenger ribonucleic acid expression in human endometrium varies across the menstrual cycle and in early pregnancy decidua. *Journal of Clinical Endocrinology and Metabolism*, 84, 2563–2567.
- Noyes, R. W. (1959). The underdeveloped secretory endometrium. *American Journal of Obstetrics and Gynecology*, 77, 929–945.
- Slayden, O. D. (2014). Cyclic remodeling of the nonhuman primate endometrium: A model for understanding endometrial receptivity. *Seminars in Reproductive Medicine*, 32, 385–391.
- Slayden, O. D. (2016). Translational in vivo models for Women's health: The nonhuman primate endometrium—A predictive model for assessing steroid receptor modulators. *Handbook of Experimental Pharmacology*, 232, 191–202.
- Slayden, O. D., & Brenner, R. M. (2005). Role of progesterone in the structural and biochemical remodeling of the primate endometrium. *Ernst Schering Research Foundation Workshop*, 89–118.
- Slayden, O. D., & Keator, C. S. (2007). Role of progesterone in nonhuman primate implantation. *Seminars in Reproductive Medicine*, 25, 418–430.

Menstruation and Endometrial Repair

Lois A Salamonsen and Jemma Evans, Hudson Institute of Medical Research and Monash University, Clayton, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Decidualization Differentiation of the fibroblastic endometrial stromal cells into a more epithelial-like phenotype (epithelioid), known as (pre-) decidual cells. In humans, this occurs spontaneously under the influence of ovarian steroid hormones and local factors within the endometrial tissue. This differentiation process takes place progressively throughout the secretory phase of the menstrual cycle with cellular transformation becoming histologically evident during the mid-late secretory phase. In a conception cycle, these cells become the decidual component of the placenta.

Description of Menstruation

Menstruation is the process whereby most of the superficial or functionalis layer of the endometrium that lines the uterine cavity, disintegrates and is expelled from the uterine lumen at the end of the secretory phase of a nonconception cycle. In the absence of the luteotrophic signal from a developing embryo (human chorionic gonadotropin) the corpus luteum degrades leading to rapid fall in progesterone levels. This drop in progesterone is the major signal which triggers activation of the menstrual cascade of events. The first day that menstrual blood is visible defines day 1 of the menstrual cycle, with menstrual bleeding generally proceeding for 4–5 days. Our understanding of the specific mechanisms underlying menstruation is limited by the small number of species which experience menstruation as a normal physiological process; menstruation occurs in only a very few mammals—women, old-world primates, some bats, the elephant shrew and the spiny mouse. It is thought that menstruation is limited to these species as only they experience decidualization of the stroma in the absence of an embryo. Since decidualization is an irreversible differentiative process, the terminally differentiated tissue must be shed and subsequently restored for potential embryo implantation in the following cycle (Finn, 1986).

The concept of monthly menstruation is a modern world phenomenon arising from the widespread use of contraception, meaning less pregnancies and less breast feeding (lactational amenorrhoea). Thus modern women have between 350 and 400 episodes of menstruation. With a mean menstrual blood loss of 43 mL per cycle, the average woman loses more than 20 L of blood vaginally during her reproductive life. International standards now define that the 90th percentile of blood loss during each normal menstruation is >80 mL, and that anemia is significantly increased in women with a loss of >60 mL (Hallberg et al., 1966). Women with >80 mL blood loss are considered to have heavy or prolonged menstrual bleeding also known as menorrhagia. It is difficult to quantify menstrual blood loss based on the patient's history or even the use of pictorial blood loss assessment charts—actual measurement by analysis of tampons, pads or volumes harvested in menstrual cups provides the only truly accurate data. The duration of bleeding can be determined accurately with the use of menstrual diaries, adapted by the World Health Organization for use as a standard method for assessment of bleeding and spotting episodes, particularly in research studies on the breakthrough bleeding associated with the use of progestin-only contraception (reviewed in Salamonsen et al., 1999).

Importantly endometrial repair (re-epithelialization) occurs simultaneously with tissue breakdown in a piecemeal fashion. This ensures that the endometrial “wound” is not open for days at a time but rather is essentially rapidly repaired at a similar rate to which it is shed. Menstruation is generally complete within 4–5 days and remarkably among adult tissues, repair occurs without scarring.

Mechanisms of Endometrial Breakdown

Decidualization

Decidualization of endometrial stromal cells is an irreversible differentiation process that takes place gradually throughout the secretory phase of the menstrual cycle, reaching completion during the mid-late secretory phase of a woman's cycle. The parent stromal fibroblasts undergo a limited mesenchymal to epithelial transition (MET) to become decidualized cells; these are of quite a different phenotype to their parent cell. In menstrual cycles the extent of decidualization is limited, being initiated around the spiral arterioles, and then spreading, the extent of this spreading being very variable between women and between cycles. In a conception cycle, however, decidualization spreads throughout the entire endometrium and these specialized cells provide the decidual component of the placenta. Initially, decidualization is driven by rising progesterone levels in the secretory phase of the cycle, acting via cyclic AMP, but regulatory factors (such as interleukin (IL)-11, and activin A) produced by the already decidualized cells and acting via diverse intracellular pathways create a cascade of differentiation, enhancing the process in adjacent cells. Furthermore the decidualized cells are also a major endometrial source of chemokines which are leukocyte chemoattractants, and are proposed to provide the initial stimulus for the leukocyte influx associated with menstruation. Importantly, the decidualized

cells are the major sensor of progesterone withdrawal; they are the only endometrial cell type to retain expression of the progesterone receptor in the mid-late secretory phase. Because the decidualized cells are terminally differentiated and cannot revert to their fibroblastic phenotype, menstruation and regeneration of the endometrial functionalis layer is necessary.

Progesterone Withdrawal

The Inflammatory Response

In the mid 1980's, Finn (1986) proposed that menstruation could be viewed as an inflammatory process since it shared the features of tissue edema, the presence of decidual cells resembling granulation tissue fibroblasts, and an influx of a remarkable number of inflammatory cells. Increased knowledge of inflammatory events and tools to examine these in detail, have strongly supported and extended this proposal. For example, mice lacking the progesterone receptor have a highly inflamed uterus, with considerable infiltration of leukocytes, while in cell culture studies, progesterone inhibits production of matrix metalloproteinases (MMPs) but withdrawal of progesterone from decidualized endometrial stromal cells increases production of MMPs, and members of the plasminogen activator family (Henriet et al., 2012).

Cytokine and chemokine release

The first phase of menstrual events occurs in the decidualized stromal cells, which express progesterone receptors and hence act as sensors of progesterone withdrawal. This initiates a sequence of inter-dependent events of an inflammatory nature, with intracellular responses identified within the decidualized cells. With progesterone withdrawal there is a decrease in cytoplasmic I-kappaB and a progressive increase in NF-kappaB which accumulates in the nucleus. Concomitant with this nuclear translocation, production of a host of inflammatory mediators, including cytokines and chemokines increases [interferon (IFN) α ; IL-6; CCL11; granulocyte-monocyte colony stimulating hormone (GM-CSF); CCL2; IL-1ReceptorA; CXCL10; CXCL8; IL-12; IL-15; vascular endothelial growth factor (VEGF) and CCL5]; this increase is blocked by an NF-kappaB inhibitor. Together these mediators can recruit inflammatory leukocytes into the premenstrual endometrium (Fig. 1).

Prostaglandins and related mediators

In addition, withdrawal of progesterone from decidualized cells upregulates prostaglandin G/H synthase 2 (PTGS2, also known as COX-2) signaling, along with the enzymes prostaglandin (PG)E synthase, PGI synthase and others, resulting in production of the pro-inflammatory prostaglandins PGF2alpha, PGE2, PGI2 and TXA2. Their related receptors are also elevated at menses, with at least the PGF receptor being increased via NF-kappa B activation (Evans and Salamonsen, 2012).

Reactive oxygen species and superoxide dismutases

Reactive oxygen species (ROS) and their scavenging enzymes, the superoxide dismutases (SOD) participate in a dynamic relationship in the endometrium. Since the ROS (which include superoxide radicals, hydroxyl radicals and hydrogen peroxide), are produced as by-products of normal metabolism, they must be kept in check by SOD (in three forms, Cu-, Zn-SOD in cell cytosol and Mn-SOD in mitochondria) which provide the first cellular defense against the cytotoxic actions of ROS. SOD are present in both glands and stroma throughout the cycle, and in vitro decidualization of stromal cells increases Cu-, Zn-SOD and Mn-SOD. However, their activity is significantly decreased just prior to menstruation. In decidualized stromal cells in vitro, Cu- and Zn-SOD decrease with removal of hormonal control, providing a potential mechanism for their loss of regulation at menstruation (summarized Evans and Salamonsen, 2012).

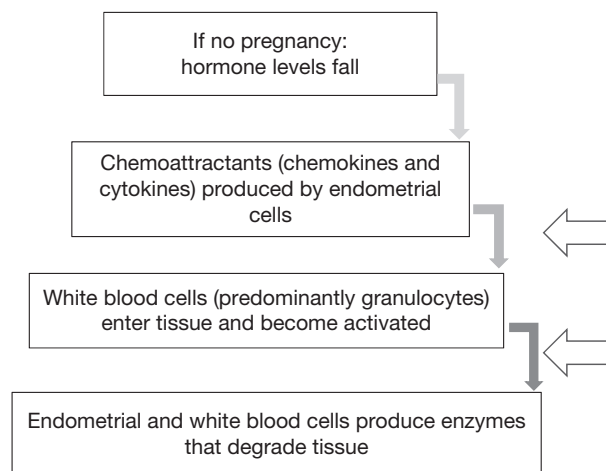


Fig. 1 The menstrual cascade: a process of controlled inflammation and tissue breakdown. Filled arrows represent the cascade, open arrows represent potential targets for restraint.

Leukocytes

The leukocyte population within the endometrium exhibits dynamic changes throughout the menstrual cycle. Leukocytes are present in the endometrium in only small numbers during the proliferative phase of the cycle, with an increase in natural killer cells and macrophages associated with the 'receptive phase'. During the secretory phase of the cycle, progesterone has antiinflammatory properties and its dramatic withdrawal during the late secretory phase leads to a massive influx and activation of inflammatory cells, particularly the granulocytes (mast cells, neutrophils, eosinophils, and basophils) which can release factors stored intracellularly in granules, but also monocytes/macrophages and uterine natural killer cells. These inflammatory cells can comprise up to 50% of the total cells in peri-menstrual endometrium. Leukocytes within tissues have constantly changing phenotypes and responses to paracrine stimuli which are difficult to define in tissue sections which represent only a snapshot in time (Evans and Salamonsen, 2012). The activation status of the granulocytes is critically important as they only become functional once activated, resulting in the release of their contents (rich in enzymes and cytokines) into the local tissue. Such activation has been demonstrated for both mast cells and neutrophils within menstrual tissue (Fig. 2). Due to the dynamic nature of the cascade of events involved in menstruation and the difficulties inherent in studying the processes involved, the roles of individual menstruation-associated leukocytes and their molecular mediators in women are not well defined. However, studies in mouse models indicate that functionally, neutrophils (Kaitu'u-Lino et al., 2007a) and macrophages (Cousins et al., 2016) are more important in repair than breakdown—whether this also applies in women, remains to be established.

Extracellular Matrix Breakdown

MMPs are the major class of enzymes responsible for breakdown of extracellular matrix (ECM): they can also activate or deactivate other enzymes and regulatory molecules such as chemokines and cytokines by proteolysis or cleavage of proteins from a latent to an active form. Most MMPs are released in latent forms and require extracellular activation by proteolytic removal of their pro-peptide. During the normal menstrual cycle, MMPs mRNA and protein are variable and at very low levels in the endometrium, but there is a dramatic increase immediately prior to and during menses. The natural tissue inhibitors of matrix metalloproteinases (TIMPs) that bind the active enzymes with 1:1 stoichiometry and hence inactivate them, are also locally present, with only minor changes during the cycle. It is only when the activated MMPs are in excess over the TIMPs that they can act to degrade ECM molecules: each MMP has an individual cohort of specific ECM that they can degrade. Importantly, cleavage of the native collagen fibrils which predominate within the endometrium, is restricted to specific proteases, the collagenases MMP1 and MMP8. Other MMPs can degrade the additional ECM molecules and the already cleaved fragments of native collagen: these include MMP2, MMP3, MMP7, and MMP9. All of these enzymes are present and active during menstruation, when they are induced both by progesterone withdrawal and also by locally produced cytokines such as IL-1 α . A pivotal role for MMPs in ECM breakdown at menstruation has been clearly

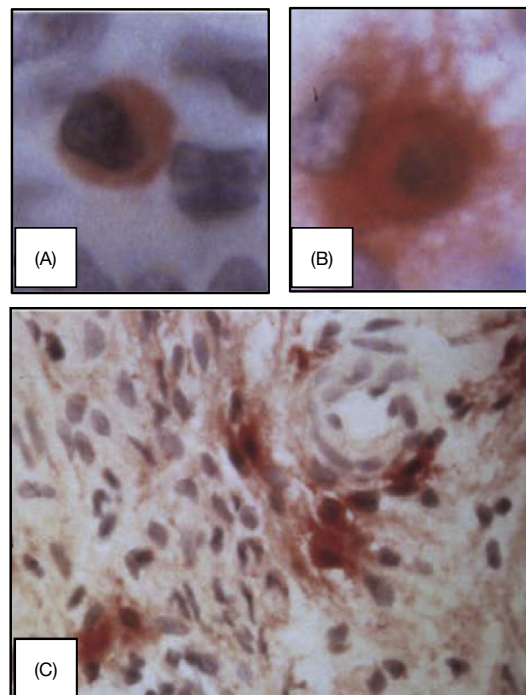


Fig. 2 Activation of granulocytes is required for their functionality. Shown here is mast cell tryptase staining (red) in day 1 menstrual tissue, (A) an inactive mast cell, (B) an activated mast cell which has released tryptase into the surrounding tissue, (C) lower power of B, showing the extent of tryptase release. Reprinted from M. Jeziorska et al. (1995). *Biology of Reproduction* 53, 312–320 with permission.

demonstrated both *ex vivo* and in endometrial explants. MMPs-1, -2, -3, -8, -9, -10, and -11 are predominantly produced by endometrial stromal cells while MMP1, -2, -3, and -9 are also expressed by endometrial vascular cells and MMP2, -7, -9, and -26 by epithelial cells. Importantly specific MMPs are also present in the infiltrating leukocytes at menstruation. It is now clear that MMP release and activation occurs in a cascade of events at menses: for example, pro-MMPs produced by stromal, epithelial and vascular cells can be activated by enzymes including neutrophil elastase, the mast cell enzymes chymase and tryptase, members of the kallikrein family, and by other MMPs such as MMP3 which is produced by stromal fibroblasts (review [Henriet et al., 2012](#)). The enzyme activities are very carefully controlled: fluorescent visualization of active MMP2 and MMP9 in menstrual tissue sections showed precise spots of activity throughout the tissue rather than the much wider activity observed using the same technique in cancers. This demonstration of specific foci of degradation enzymes supports the histological findings that menstrual breakdown is not a synchronized shedding of the whole tissue at a single time. Rather, tissue shedding initially occurs at very focal points, spreading across the tissue with time. Indeed a single histological section of menstrual tissue may contain intact decidualized tissue alongside areas of both breakdown and repair, highlighting the complexity of this tissue and the piecemeal nature of this tissue breakdown.

Vascular Events

While the very early studies on menstruation suggested that it results from a physiological ischemia-reperfusion injury, whether or not hypoxia is truly present in menstrual endometrium and involved in the menstruation process is still under debate. Work in a range of models indicates that hypoxia is not essential for either endometrial breakdown or repair although it may promote these processes ([Maybin and Critchley, 2015](#)). However, the vasculature clearly plays a role in the menstrual cascade and the balance of vasoconstriction versus vasodilation during menses will contribute to the extent of blood loss. At least two vasoconstrictors, PGF2 α and endothelin (ET) 1, are implicated in heavy menstrual bleeding (HMB). Highly potent ET1 is decreased in concert with an increase in its metabolizing enzyme neutral endopeptidase in women with HMB, with a similar decrease seen in the PGF2 α (vasoconstrictor)/PGE (vasodilator) ratio. Differences in spiral arteriole maturation may also contribute to the extent of vasoconstriction at menses, with vessel wall circumference, focal discontinuities, and reduced vascular smooth muscle myosin heavy chain (a marker of maturation) all altered in women with HMB. Menstrual disorders, including HMB are discussed in detail elsewhere in this series.

Endometrial coagulation

Cessation of menstruation requires an intact endometrial coagulation system. Indeed, HMB is reported by a majority of women with the inherited bleeding disorder, von Willebrand's disease, and this considerably impairs their quality of life. Von Willebrand factor interaction with platelets is one of two mechanisms resulting in a hemostatic plug; the other mechanism involves tissue factor generation of thrombin and the subsequent formation of fibrin via the coagulation cascade. Most of the components of this system are present in human endometrium (tissue factor, tissue plasminogen activator (tPA) its inhibitor (PAI-1), urokinase (u)PA, and the uPA receptor-1. PAI-1 and tissue factor are reduced by progesterone withdrawal, thus promoting menstrual bleeding. Evidence also exists for over-activation of the fibrinolytic system in women with HMB: indeed, the successful use of the hemostatic agent, tranexamic acid, as a treatment for HMB, provides strong evidence for such fibrinolytic over-activation as a driver of excessive bleeding (reviewed [Schatz et al., 2016](#)).

Mechanisms of Endometrial Repair

The shedding of endometrial tissue at menstruation is very carefully controlled: the lower third of the functionalis and the entire basalis layer generally remain, and it is from these maintained areas that endometrial repair occurs. However, it is clear that repair can occur from even tiny foci of residual tissue as the endometrium can become fully restored following extensive surgical curettage or ablation and indeed, it is almost impossible to create an endometrial-free uterus by surgical means. Electron microscopy and histology, have demonstrated clearly that re-epithelialization and repair of the endometrial surface are in progress as early as day 2 of menses in areas denuded of tissue, even while active proteolysis is occurring in adjacent areas of the endometrium ([Ludwig and Spornitz, 1991](#)). Indeed, in tissues obtained on day 2 of menses and even later, areas of active lysis are observed alongside both repairing tissue and decidualized tissue remaining from the previous cycle in histological sections ([Gaide Chevronnay et al., 2009](#)). However, to date, the mechanisms of endometrial repair are less well defined than those of menstrual breakdown at least in part because the two are proceeding concurrently.

As evidenced from the study of ovariectomised women and women in natural menopause, ovarian estrogen is not required for endometrial repair/re-epithelialization. Further, a mouse model in which all possible sources of estrogen were removed (even that from fat and food), demonstrated normal progress of repair progresses after induced menses ([Kaitu'u-Lino et al., 2007b](#)). However, estrogen is clearly needed for subsequent growth and regeneration of the endometrium during the proliferative phase of the cycle. The separate process of estrogen dependent tissue regeneration is reliant on initial re-epithelialization; studies in the mouse suggest that the stromal compartment does not become responsive to ovarian steroids until re-epithelialization is complete ([Bigsby, 2002](#)) and proliferation is not observed in the human endometrium until day 5–6 when a fully epithelialized surface is present (reviewed [Maybin and Critchley, 2015](#)).

Since endometrial repair occurs in a piecemeal manner, simultaneously with breakdown, the microenvironment in its early stages of re-epithelialization is highly inflammatory. However, once the inflammation is resolved, restoration of the full cellular

cohort and thickness of the functionalis, requires angiogenesis, glandular development, ECM formation and tissue remodeling and these are not complete until late in the proliferative phase of the endometrial cycle.

Importantly, repair of the endometrium is completely ‘scar-free,’ unusual among adult tissues. Thus while postmenstrual repair has commonalities with restoration of wounds in other tissues, there are also important differences which could, when fully understood, have significant translational benefits beyond the endometrium.

Re-epithelialization

Very rapid re-epithelialization is needed to cover the endometrial surface; it stops the bleeding and enables subsequent regeneration of the stromal compartment. It is observed as early as day 2, and occurs during active bleeding. Thus, repair occurs within a micro-environment of menstrual fluid which contains many proteins not found in peripheral blood. Menstrual fluid itself, and a number of these unique proteins within the fluid, stimulate re-epithelialization of wounded human endometrial epithelial cell monolayers in vitro primarily by promoting cellular migration (Evans and Salamonsen, unpublished). This strongly supports the contention that the very rapid re-epithelialization arises from retained epithelial luminal or glandular cells as initially indicated by scanning electron microscopy of menstrual endometrium (days 2 to 6) (Ludwig and Spornitz, 1991), in which re-epithelialization appeared to be initiated by cell migration from the mouths of open glands. However, more recent combined histologic and hysteroscopic studies, suggest that at least some of the epithelial cells may arise from MET of stromal cells (Garry et al., 2009). The exposed stromal surface following shedding is covered with a fibrinous matrix, upon which new epithelial cells were observed either as single cells or in small groups, not associated with the glandular stumps and it is these that are proposed to arise from the stromal compartment. However, these findings still require independent validation. The MET required for stromal fibroblasts to transition to the epithelial phenotype is commonly observed at least in part, during decidualization during which the fibroblasts become ‘epithelioid’. However, to form true epithelial cells the transformation would need to proceed further to include induction of junctional complexes and formation of tight baso-lateral connections, not observed in decidual cells. Nevertheless, since both proteins and genes known to be involved in MET are present during menstruation (reviewed Maybin and Critchley, 2015), it is possible that epithelial cell migration and MET occur simultaneously. Endometrial leukocytes are also present during repair. Indeed, in mouse models, both neutrophil and macrophage depletion reduces endometrial repair: emerging evidence indicates that at least the macrophages differentiate into repair-specific phenotypes within the tissue. How these cells promote or participate in endometrial repair is still to be determined.

Revascularization

Concomitant with re-epithelialization, restoration of the circulation is essential to maintain endometrial survival. As visualized by scanning electron microscopy, transverse endometrial arterioles in the subepithelial stroma and the ascending spiral arterioles are torn open as the tissue is shed (Ludwig and Spornitz, 1991; Garry et al., 2009). While such vessels will eventually fully regenerate, there is a need for very rapid repair of vascular integrity to minimize the risks of hemorrhage. A number of vascular repair agents including VEGF are present during menses (see above). Indeed, VEGF is produced by in vitro decidualized stromal cells after hormone withdrawal; this and other factors likely play key roles in rapid vascular repair. Additional factors, most likely reliant on estrogen action, are expected to drive subsequent slower vascular development during the regenerative proliferative phase.

Regeneration

Small populations of adult stem/progenitor cells with classic stem cell properties have been identified in human endometrium and proposed to contribute to the ability of the endometrium to regenerate during each menstrual cycle (Gargett et al., 2016). These include epithelial progenitors, mesenchymal stem cells and side-population cells. The epithelial progenitor cells which are located in vivo in the basalis layers of the endometrial glands, can differentiate into gland-like structures. Appropriately, the mesenchymal stem cells are detected peri-vascularly in both endometrial functionalis and basalis. It is not yet clear whether in vivo, one or more stem/progenitor cell type regenerates endometrial tissue. However, it is most likely that epithelial outgrowth is initially needed to rapidly cover the wound, with regeneration from stem cells contributing to the subsequent development of multicellular full thickness tissue.

Conclusions

Understanding how the menstrual endometrium limits inflammation, modulates immune cell activity, rapidly repairs and remains scar free, all within a 4 to 5 day time-frame, has implications for the development of treatments for a number of endometrial pathologies including abnormal uterine bleeding, Asherman’s syndrome and endometriosis. Furthermore, understanding processes within this dynamic reproductive tissue has widespread implication for understanding and treating pathologies in other organs. Skin wounds, for example, commonly exhibit deficient re-epithelialization, burn wounds often repair with massive scarring, while diabetic wounds can persist for months. Given that the inflammation associated with menstruation is highly controlled, translation of these regulatory mechanisms could also lead to novel therapies for resolution of chronic inflammation. Regrettably, since discussion of ‘menstruation’ is not well accepted socially and also carries cultural taboos, progress on its understanding has been slow, in spite of the many women who suffer menstrual abnormalities and the broader contributions that this could provide.

Acknowledgments

Work in the author's laboratory has been supported by the National Health and Medical Research Council of Australia (Fellowship (#1002028; to LAS), Program (#494802) and Project (#1047756) grants) and by the Victorian Government's Operational Infrastructure Funding Program.

References

- Bigsby, R. M. (2002). Control of growth and differentiation of the endometrium: The role of tissue interactions. *Annals of the New York Academy of Sciences*, 955, 110–117. discussion 8, 396–406.
- Cousins, F. L., Kirkwood, P. M., Saunders, P. T., & Gibson, D. A. (2016). Evidence for a dynamic role for mononuclear phagocytes during endometrial repair and remodelling. *Scientific Reports*, 6, 36748.
- Evans, J., & Salamonsen, L. A. (2012). Inflammation, leukocytes and menstruation. *Reviews in Endocrine & Metabolic Disorders*, 13(4), 277–288.
- Finn, C. A. (1986). Implantation, menstruation and inflammation. *Biological Reviews of the Cambridge Philosophical Society*, 61(4), 313–328.
- Gaide Chevonnay, H. P., Galant, C., Lemoine, P., Courtoy, P. J., Marbaix, E., & Henriot, P. (2009). Spatiotemporal coupling of focal extracellular matrix degradation and reconstruction in the menstrual human endometrium. *Endocrinology*, 150(11), 5094–5105.
- Gargett, C. E., Schwab, K. E., & Deane, J. A. (2016). Endometrial stem/progenitor cells: The first 10 years. *Human Reproduction Update*, 22(2), 137–163.
- Garry, R., Hart, R., Karthigasu, K. A., & Burke, C. (2009). A re-appraisal of the morphological changes within the endometrium during menstruation: A hysteroscopic, histological and scanning electron microscopic study. *Human Reproduction*, 24(6), 1393–1401.
- Hallberg, L., Hogdahl, A. M., Nilsson, L., & Rybo, G. (1966). Menstrual blood loss—A population study. Variation at different ages and attempts to define normality. *Acta Obstetrica et Gynecologica Scandinavica*, 45(3), 320–351.
- Henriot, P., Gaide Chevonnay, H. P., & Marbaix, E. (2012). The endocrine and paracrine control of menstruation. *Molecular and Cellular Endocrinology*, 358(2), 197–207.
- Kaitu'u-Lino, T. J., Morison, N. B., & Salamonsen, L. A. (2007a). Neutrophil depletion retards endometrial repair in a mouse model. *Cell and Tissue Research*, 328(1), 197–206.
- Kaitu'u-Lino, T. J., Morison, N. B., & Salamonsen, L. A. (2007b). Estrogen is not essential for full endometrial restoration after breakdown: Lessons from a mouse model. *Endocrinology*, 148(10), 5105–5111.
- Ludwig, H., & Spornitz, U. M. (1991). Microarchitecture of the human endometrium by scanning electron microscopy: Menstrual desquamation and remodeling. *Annals of the New York Academy of Sciences*, 622, 28–46.
- Maybin, J. A., & Critchley, H. O. (2015). Menstrual physiology: Implications for endometrial pathology and beyond. *Human Reproduction Update*, 21(6), 748–761.
- Salamonsen, L. A., Kovacs, G. T., & Findlay, J. K. (1999). Current concepts of the mechanisms of menstruation. *Baillière's Best Practice & Research. Clinical Obstetrics & Gynaecology*, 13(2), 161–179.
- Schatz, F., Guzeloglu-Kayisli, O., Arlier, S., Kayisli, U. A., & Lockwood, C. J. (2016). The role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding. *Human Reproduction Update*, 22(4), 497–515.

Uterus—Endometrium

John Aplin, University of Manchester, Manchester, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Bicornuate Having two horns; here the context is the mouse uterus, a v-shaped organ fused at the two cervixes.

Deciduochoorial placenta The boundary between maternal and conceptus-derived tissue is between placental trophoblast and maternal uterine decidua. Seen in the first trimester of human pregnancy.

Hemochorial placenta The interface is between syncytiotrophoblast at the outer surface of the placenta, and maternal blood. Occurs in the second and third trimesters of human pregnancy.

Proliferative phase Period of about 10 days after menstruation ends, during which the endometrium regrows from a thin basal layer.

Secretory phase Period of about 2 weeks during which the endometrium undergoes changes that allow it to accept and support an implanting embryo, if fertilization has occurred.

Anatomy

The uterus comprises a mucosal lining, the endometrium, and outer muscle sheath, the myometrium, enclosed in a serosal (perimetrial) layer. Both are targets of steroid hormone action. In human and many primates the uterus has a single corpus connected via two fallopian tubes to the right and left ovaries. During pregnancy, the organ expands with growth of the conceptus, and the endometrium and myometrium become thinner.

The vascular supply to the uterus is from the ovarian artery and the descending aorta. In human there are two uterine arteries running laterally in a cephalocaudal direction. In bicornuate species such as mouse and rat the vascular supply is from a central (mesometrial) plexus emanating from a single uterine artery. Arcuate (circumferential) arteries connect the uterine artery to a series of radial arteries, which in turn supply spiral arteries that become arterioles in the endometrium. They feed a substantial capillary plexus that surrounds glands and opens into wider channels near the tissue surface. Close to a doubling of flow through the uterine artery occurs during the second half of pregnancy in women, its diameter increasing apparently in response to estrogen. The spiral-shaped arteries in the inner myometrium and endometrium produce increased impedance in the circuit, possibly as a way of protecting the implanting embryo. During pregnancy, spiral arteries are remodeled to allow a low pressure, high volume flow through the placenta.

The Non-Pregnant Endometrium

The uterine lumen is lined by a simple cuboidal surface epithelium, opening into tubular glands that extend radially, ending at the junctional zone with the myometrium. Glands are also lined by a single-layered epithelium that varies from columnar to cuboidal in women. Beneath is stromal tissue containing mesenchymal and immune cells, blood vessels and nerves all set in a collagenous extracellular matrix. The junctional zone is a discrete layer, visible by ultrasound or magnetic resonance imaging.

Menstrual and Estrous Cycles

Between menarchy and menopause, women normally experience about 400 menstrual cycles in response to changing levels of ovarian hormones. There is a gradient of hormone responsiveness in endometrial tissue: the upper functionalis (about 80% of the tissue depth at mid cycle) undergoes marked histological changes, while the basalis exhibits more subtle alterations. Over three quarters of the endometrial lining is lost at menstruation, leaving a thin basalis from which scar-free regeneration occurs. The ensuing proliferative phase equates to the follicular phase in the ovary. Regeneration is stimulated by ovarian estradiol (E2), the endometrium growing from <2 mm (**Fig. 1**) to 7 mm by the end of the proliferative phase. Within 2 days of the end of menstruation, epithelial cells migrating from gland openings have recovered the tissue surface with a new luminal epithelium. The tissue thickens under the influence of estrogen, glands elongate, stromal cells proliferate and produce an abundant collagenous extracellular matrix, and angiogenesis restores the vascular network.

In the late proliferative phase, luteinizing hormone (LH) levels rise to a peak, and progesterone production is initiated in the follicle. Ovulation occurs, and progesterone (P4) and E2 from the corpus luteum act together on the endometrium, which now enters its secretory phase with onset of cell differentiation and a reduction in proliferative activity. Initially the effects are most prominent in the glandular epithelium, which starts to amass glycogen in subnuclear cytoplasmic masses (early secretory phase).

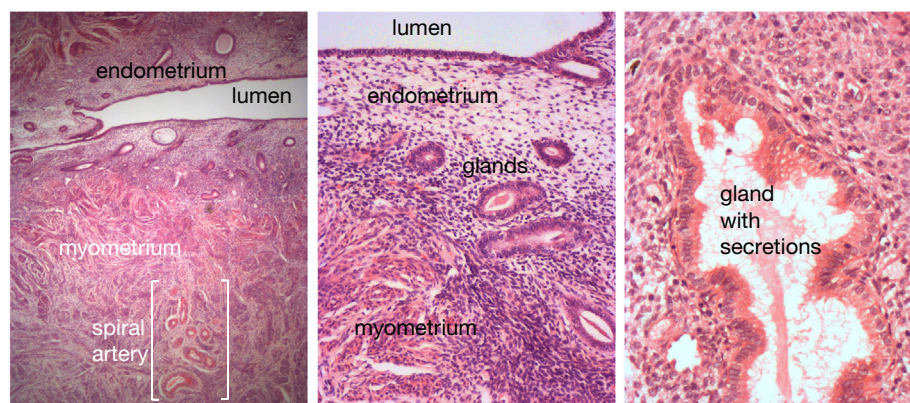


Fig. 1 Uterine histology in the menstrual cycle. *Left and centre*, proliferative phase showing endometrial luminal epithelium and tubular glands above myometrial tissue containing spiral arteries. *Right*, late secretory phase endometrium. H&E staining.

An unusual spherical stack of interdigitating tubules known as the nucleolar channel system (NCS), is present in 5% of epithelial cell nuclei in the mid-secretory phase. Enlargement of epithelial mitochondria occurs. In the mid secretory phase epithelial cells start to release secretory substances, filling the gland lumens. Ciliated epithelial cells, which are prominent at the necks of glands and are at a maximum at this time, waft the secretions into the uterine lumen. Secretions persist into the late secretory phase (**Fig. 1**).

In the mid secretory phase, areas of edema appear in the stroma, indicating ECM remodeling. Arterioles in the functionalis elongate and assume the characteristic spiral form. In proliferative phase, lymphoid aggregates form in the endometrial basal; these contain T and B cells which disperse later in the cycle. T_{reg} cells, macrophages, dendritic cells and mast cells are also present. Further immune cells, particularly uterine natural killer cells, are recruited later in the cycle.

The mid secretory phase represents a nodal point. If fertilization occurs, the CL is rescued by the conceptus as described below. Progesterone levels rise and the endometrial stroma begins to differentiate. If not, steroid levels reach a maximum about a week after ovulation, and then decrease, leading to menstruation.

Sex Hormones and Their Receptors

The actions of the sex steroids in the endometrium occur largely as a result of binding to their cognate nuclear receptors, estrogen receptors (ER α and ER β) and progesterone receptor (PGR, with splice variants A and B), which are transcription factors. Many, but not all, cells in the endometrium express these receptors. In addition there are “non-classical” receptors at the cell surface: for estrogen, a variant of the nuclear estrogen receptor and a G-protein-coupled receptor; for progesterone, members of the PGRMC and membrane progesterone receptor (mPR) families. Cell surface receptors mediate more rapid responses to steroidal stimulation that do not depend on changes in transcription.

Some steroid effects require paracrine actions between cell populations in the 3D tissue environment, and there is clear evidence for bidirectional communication between epithelial and stromal cells, both in the non-pregnant cyclic and at implantation. Some description is included below but the scope of this article does not permit a detailed account.

ER α and ER β are encoded by two distinct genes. ER α is the predominant isoform expressed in the endometrium, and ligand binding stimulates proliferation in gland and stromal cells during the proliferative phase, with a steep decline in the secretory phase. Signaling through the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway and hyperphosphorylation of retinoblastoma (Rb) and p107 proteins activate the DNA replication machinery for S-phase entry. In laboratory rodents, E2 stimulates epithelial proliferation by means of a paracrine pathway in which binding to stromal ER α leads to production of FGF, in turn driving epithelial proliferation by activating extracellular signal-regulator kinase (ERK) 1 and 2 or PI3K/AKT. Persistent ER α expression in epithelial cells may help them avoid apoptosis. The same may well be the case in human, as most endometrial epithelial cells rapidly cease proliferation in vitro, that is, in the absence of stroma.

A further important action of E2 is to induce PR in endometrial cells. During the secretory phase, P4 counteracts the mitogenic effects of E2 and stimulates stromal differentiation and the secretory function of the glands. Ablation of murine PR results in a hyperplastic response to E2 and P4 in epithelium. The anti-proliferative actions of P4 on the epithelium are mediated by down-regulation of ER and induction of 17 β -hydroxysteroid dehydrogenase Type 2, which catalyzes the conversion of E2 to estrone, which is less active. Other contributory mechanisms include a paracrine interaction in which the transcription factor HAND2 is induced in the stroma, and suppresses FGF expression.

PRA and PRB are splice variants of the receptor. PRA predominates in the endometrium. However, after ablation of murine endometrial PRA, epithelial proliferation is retained upon P4 treatment, and this response is dependent on PRB. Epithelial PR also affects stromal function: the selective knockdown of epithelial *Pgr* in mice results in infertility with failed decidualization.

Other cells in the endometrium that express steroid receptors include vascular endothelial cells (PRB, ER) and uNK cells (PRB).

Menstruation

In mid to late secretory phase, progesterone exerts an anti-inflammatory effect on the endometrium. Stromal cells that have been exposed for over a week to P4 begin the characteristic changes known as decidualization, which is discussed in “**Decidualization**” section. Decidualising endometrial stromal cells express tissue factor, which can activate the clotting cascade if interstitial leakage of plasma occurs. The steep decline in progesterone in late secretory phase leads to a reduction of Cu/Zn superoxide dismutase and loss of protection against oxidative damage. As a result, I κ B is degraded and its inhibition of NF- κ B lifts. NF- κ B moves into cell nuclei and binds to the progesterone receptor, adding to effective progesterone withdrawal. It activates production of the enzyme cyclooxygenase 2 (COX2), which catalyzes the synthesis of prostaglandins E2 and F2 α ; PGF2 α is vasoconstrictive, while PGE2 increases vessel leakage. Endothelin (ET-1) increases, adding further to vasoconstriction. Spiral arteries, a key locus of control of menstruation, are the main target for these actions. These vessels are unique to the human/primate endometrium and are highly sensitive to progesterone. They are controlled by perivascular cells (smooth muscle and pericytes).

Another action of nuclear NF- κ B is to upregulate transcription from genes encoding a large repertoire of pro-inflammatory cytokines and chemokines. Chemokines, produced initially by stromal cells, recruit inflammatory cells—neutrophils, monocytes and eosinophils—from circulation. Mast cells are activated. Inflammatory cells and resident epithelial and stromal cells produce a repertoire of matrix metalloproteinases (MMPs) which, following extracellular activation, proceed to break down ECM to destabilize the functionalis. Glands collapse, acquiring a serrated appearance. Plasminogen activator levels increase to digest fibrin, and tissue breakdown leads to shedding of approximately the upper two thirds of the endometrium, including most of the functionalis. Vessels constrict to limit bleeding, and locally available tissue factor triggers clotting.

Vascular constriction results in ischaemia and hypoxia in the tissue; in turn this switches on hypoxia-activated factor 1 (HIF1 α), a transcription factor that activates a set of downstream genes required for postmenstrual regeneration. One of these is vascular endothelial growth factor (VEGF), which is important for regeneration of blood vessels. Inflammatory cell phenotypes, especially in macrophages and neutrophils, shift from pro- to an anti-inflammatory, and these cells produce other growth factors that contribute to regeneration.

Menstruation occurs in rather few species: human, a few other primates and certain bats. The cascade of events that initiates and then controls menstruation in non-conception cycles depends on the presence of decidualised stromal cells. This is nicely demonstrated in the mouse, where decidualization occurs in steroid-sensitized stroma only when a stimulus is received from the implanting embryo. In this species, experimental withdrawal of steroid produces a much steeper decline in circulating levels than in the normal estrous cycle. This triggers menstruation, but only if the stroma has already initiated decidualization. In non-menstruating species, tissue remodeling occurs as the corpus luteum regresses at the end of the estrous cycle, and this is achieved without shedding of endometrium.

Progenitor and Stem Cells and Renewal of Endometrium

Based on growth in vitro of clones from single cells, there is a population of stromal progenitor cells (~1%) as well as a smaller (~0.2%) epithelial reserve population (CD44+/label-retaining and clonogenic in vitro). The stromal cells seem to reside in vivo in a peri-vascular location based on their marker expression profile CD146(+)/PDGFR β (+)/SUSD2(+), and they are probably drawn from the pericyte population. Though the endometrial basalis is normally expected to act as a reservoir of tissue from which post-menstrual regeneration can occur, both epithelial and stromal clonogenic cells can be recovered from functionalis tissue sampled using, for example, a pipelle suction device. They are of interest in the context of endometrial proliferative disorders such as adenomyosis and Asherman's syndrome, as well as in the establishment of endometriotic lesions from tissue shed at menstruation and refluxed through the fallopian tubes.

Though most of the cells of the functionalis at full thickness are probably derived by activation of cells resident in the basalis into cycles of proliferation, there is evidence that some can be recruited from cells in circulation. Women who have had blood or bone marrow transplants carry donor cells that can be recognized by virtue of their histocompatibility antigens at the cell surface. Such cells can be found in endometrium several years after transplantation, and they contribute to both stromal and epithelial cell compartments. In mice, similar observations have been made after transplantation of fluorescent-tagged bone marrow cells, indicating that this process occurs even in a non-menstruating species.

Pregnancy

There is remarkable inter-species diversity in placental form, with deep or superficial implantation and from one to many offspring. Comparative genomic analysis suggests that the placenta has evolved in response to constraints imposed by the maternal uterine environment.

Initially the hatched blastocyst attaches to the endometrial luminal epithelium. This may result in a long-lived adhesive interaction, as required in epitheliochorial placentation (e.g., in pig, sheep and cow). Here the conceptus is retained in the uterine lumen for the duration of pregnancy and nutrients are transferred from maternal capillaries across the luminal epithelium to apposed or adherent trophoblast. In interstitial implantation (human (**Fig. 2**) and laboratory rodents) attachment to the luminal epithelium is followed rapidly by invasion of the underlying stroma.

In mice and rats, implantation induces a transient increase in vascular permeability that is dependent on P4 binding to PR in venous endothelium, where it activates another transcription factor, NR4A1 to initiate a relaxation in the barrier function of the vessel wall.

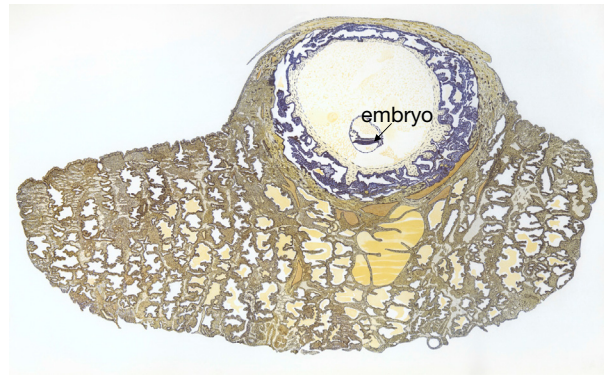


Fig. 2 The Falkiner embryo: secondary villous stage, about day 27 from the last menstrual period. Blue represents the embryo (bilaminar disc stage) and placenta (secondary villus stage; blue). Maternal tissue is colored mustard. Note the highly glandular, hypersecretory decidua beneath the embryo. Decidua has extended over the implant, encasing it. Trophoblast invasion has begun but the cells are not colored as this was not recognized at the time of publication. Reprinted with permission from Falkiner, N. (1932) *British Empire Journal of Obstetrics and Gynecology*, **39**(3), 471–506.

Rescue of the corpus luteum is effected in human by chorionic gonadotrophic released by trophoblast, which travels to the ovary and binds the LH receptor. Only primates and equids produce CG, and there are many alternative mechanisms of CL rescue in the animal world. For example, mice use prolactin, while in ruminants, trophoblast-derived interferon tau binds to the luminal epithelium, initiating a signaling cascade that blocks its production of luteolytic prostaglandin F2 α .

Decidualization

Decidualization is a process of stromal differentiation that is confined to species in which embryo implantation occurs interstitially, creating an interface between trophoblast and the maternal uterine stroma.

In women, decidual cells start to appear at the mid-secretory-late secretory transition, about day 23 of the cycle. It is notable therefore that implantation occurs in human *before* decidual changes have been initiated in the endometrial stroma; though there are few direct observations of these stages, decidualization is probably initiated 1–3 days after trophoblast starts to penetrate the stroma. Decidual cells arise by differentiation of resident stromal cells in response to a prolonged exposure to progesterone, with binding to PRA leading to receptor phosphorylation, dissociation from chaperone proteins, dimerisation, binding to response elements in specific genes, recruitment of coactivators and finally transcription. The resultant chromatin reorganization and genome-wide alteration of histone modification give rise to a profoundly distinct repertoire of gene expression.

Elevation of intracellular cAMP is also required for decidualization, and is achieved by stimulation of several G-protein coupled receptors leading to adenylyl cyclase activation and, in turn, the activation of cAMP-dependent protein kinase A which acts on targets in the cytoplasm or nucleus. Extracellular ligands involved in the initiation of this cascade include the prostaglandin PGE₂, hCG, LH, follicle stimulating hormone (FSH), and corticotropin-releasing hormone (CRH).

In addition, a discrete subfamily of genes is activated by androgen binding to its receptor in cells undergoing decidualization; this may be required for cytoskeletal reorganization leading to morphological change. The cells enlarge, accumulate fat and glycogen granules, acquire a distinct polygonal shape, and begin to produce a characteristic repertoire of secretory substances including prolactin and insulin-like growth factor binding protein 1 (IGF-BP1). They express a repertoire of antioxidant enzymes including glutathione peroxidase (GPX) 3, monoaminoxidase A, superoxide dismutase (SOD) 2, glutathione transferase, peroxiredoxin 4 and thioredoxin, and as a result are resistant to DNA damage and cell death induced by reactive oxygen free radical species.

Not all stromal cells decidualise; the characteristic morphological changes are initially seen most prominently in cells near spiral arterioles and beneath the luminal epithelium. There is evidence for a periarteriolar niche that contains a discrete subpopulation of cells (~5% of the stromal population) that may contribute to regeneration after pregnancy. They bear the surface protein Sushi domain containing 2 (SUSD2) and express vascular-related genes such as ELN (elastin), MYH11 (myosin 11) and α smooth muscle actin, as well as NOTCH3 and the niche cell marker MCAM (CD146). A few per cent are clonogenic *in vitro*, while the SUSD2-negative population contains at least 10 times fewer clonogenic cells. *In vitro* cells can shift from SUSD2-positive to negative and vice versa, and both can decidualise *in vitro* under a standard stimulation protocol. However, positive cells respond to decidualization by enhanced production of a group of immunomodulators and trophic factors. Stromal cells are apparently sensitive to local community signaling, cooperating to achieve a tissue-level biological end point by diversifying within a phenotypic envelope characteristic of the specific environment. The reappearance of SUSD2+ cells after menstruation or pregnancy probably relies on the re-creation of a permissive periarteriolar niche during regeneration.

Decidualization initiates reorganization of extracellular matrix in the functionalis. Basement membrane components including collagen IV and laminin are produced (Fig. 3), and in human are elaborated into a pericellular basement membrane in cells that have undergone the classical morphological transition into large polygonal shapes. These cells are probably sessile, while other cells may remain motile, allowing the space above the implanting embryo to be repaired and infilled by stroma with a covering

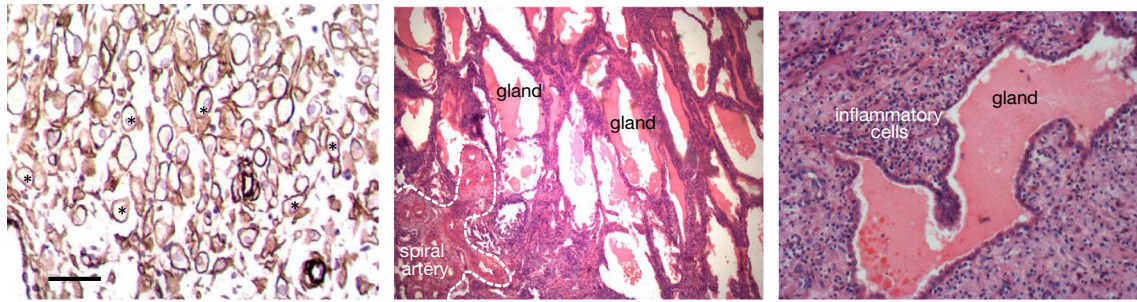


Fig. 3 Decidua in human first trimester pregnancy. *Left*, laminin (brown staining) in the pericellular basement membrane of decidualised stromal cells (a few examples are labelled (*)). *Centre*, glands and a spiral artery in hypersecretory decidua spongiosa beneath an implanted embryo, 5th week. *Right*, gland in compact decidua parietalis in the first trimester. Note the adjacent stromal inflammatory infiltrate. The scale bar (*left*) represents 25 μm at left, 650 μm in the center, and 50 μm at right.

epithelium. Active turnover of the major fibrillar collagens (types I, III and V) occurs; if the MMP inhibitor TIMP1 is overexpressed in stromal cells in the mouse, the extent of decidualization is reduced, and the embryo is displaced mesometrially with less stringent control of its orientation. Type VI collagen, which forms microfibrils that wrap around and stabilize the organization of the major fibrillar components of the ECM, is lost from most of the stroma, but is retained around blood vessels. The looser ECM probably has the capacity to expand to accommodate the implanting embryo. In addition ligands such as laminin in the decidual ECM (Fig. 3) engage integrins at the surface of trophoblast to promote invasion. In this context it is instructive that implantation fails in mouse embryos lacking the beta1 integrin subunit, which pairs with numerous different alpha subunits to form a family of surface receptors for ECM ligands; the embryos cannot progress beyond the initial breach of the epithelium.

As discussed in “Menstruation” section in the context of menstruation, decidual cells play an important role in initiating and controlling inflammation in the endometrium. At the same time, immunological management is required to ensure survival of the fetal allograft. This is not achieved by means of wholesale immune suppression, rather immune recognition of the placenta is required, with both local and systemic adjustment of the immunological environment. Trophoblast interaction with the innate immune system via uterine NK cells is one element in this process. Regulatory T cells are also important, indeed mice lacking T_{regs} are infertile. Stromal cell decidualization may therefore have evolved to manage the specific inflammatory and immune processes associated with invasive implantation. In some eutherians, decidual cells are lost after the implantation phase of pregnancy, indicating they are not required for the maintenance of these complex relationships.

In mouse and rat, decidualization of the uterine stroma requires steroid hormone priming, but is dependent on the presence of an embryo in the uterus. In these species the maternal tissue closes in to create an implantation chamber, a crypt-like branch off the main luminal axis. Decidualization is initiated by a signal from the embryo transduced by the epithelium to the underlying stroma, beginning in the superficial antimesometrial area and spreading deeper and towards the mesometrial pole. In these species (unlike in human) invading trophoblast encounters decidual cells as soon as the epithelium is breached. Steroid-sensitized uterus can also be stimulated to decidualise by means of a physical stimulus such as the instillation of oil or a superficial needle scratch to the luminal surface; this response is known as a deciduoma.

Embryo Selection and Correction

At least half of all fertilized human eggs are thought to fail to proceed to a clinically detectable pregnancy. One important reason is that embryos show a surprisingly high level of chromosomal abnormality, which arises in the first place from meiotic errors during gamete formation and secondly from errors in mitosis during pre-implantation development which, depending on the stage at which they occur, can give rise to different levels of genetic mosaicism. Abnormal embryos often develop the blastocyst stage, and may appear morphologically normal. Since some abnormal karyotypes (monosomies for example) are detected in preimplantation stage embryos but not in miscarriages, it has been hypothesized that the endometrium exercises a degree of quality control, to limit maternal investment in a compromised conceptus. In cows, where implantation is epitheliochorial (that is, the embryo remains positioned in the uterine lumen throughout pregnancy), gene expression in the endometrium differs depending in whether embryos were produced by in vitro fertilization, somatic cell nuclear transfer or artificial insemination.

Selection may occur at the stage of initial interaction with the epithelium: the apical glycocalyx, a protective mucin layer, has been suggested to act as a barrier, requiring an apposed embryo to send a signal to activate a process of clearance in order to advance to attachment and then breach the epithelium. In interstitially implanting species such as human, there is evidence for biosensing of embryo quality by decidual tissue, with suppression of supportive (e.g., growth-promoting) cytokines in the presence of an abnormal (e.g., developmentally arresting) embryo. This response, which requires the stromal cells to have decidualised, may arise after endoplasmic reticulum stress has been triggered. Decidualization also establishes an immune tolerant environment that might be overridden in the presence of an abnormal embryo. The incidence of mosaicism in human preimplantation embryos is so high that self-correction mechanisms are likely to exist; for example, karyotypically abnormal cells might apoptose or become segregated into the placenta.

Post-Implantation Development of the Placenta

Soon after implantation in human, rapidly growing cytotrophoblast occludes the superficial spiral arteries to that maternal blood cannot flow into the developing intervillous space. During this period (the first 11 weeks, coinciding with organogenesis) the placenta is very hypoxic, with an oxygen partial pressure of <20 mmHg at 8 weeks. By 12 weeks it has risen to >50 mmHg and protective antioxidant enzymes, such as catalases, GPXs and SODs have begun to be expressed in the placenta. Antioxidant enzymes in decidual cells offer protection for the developing placenta.

Meanwhile trophoblast invades more deeply, both into the decidual stroma and arteriolar walls. Changes to the arterioles are initiated by maternal uterine NK cells and macrophages, with disruption of the smooth muscle layers and endothelial hypertrophy, and the invasive trophoblast population collaborates in this process as it moves deeper through the decidua. Vascular smooth muscle is eventually replaced by trophoblast embedded in a fibrinoid extracellular matrix, so that the ability to respond to vasoconstrictors is reduced or lost.

At 11 weeks the trophoblast plugs are displaced and maternal blood can flow through the intervillous space. Thus the placental interface becomes hemochorial rather than deciduochorial. Trophoblast invasion progresses more deeply into the endometrium, crossing the junctional zone and continuing as far as the inner third of the myometrium. Spiral arterial segments are transformed to the corresponding level.

The idea that decidua may acts to restrain the invasion of trophoblast has gained traction from the genetics of imprinting. Imprinted genes are differentially expressed from their maternal and paternal alleles, a phenomenon found only in eutherian species. An example that has been explored in mouse is *igf2* (encoding insulin-like growth factor II), which is important in stimulating fetal growth. *Igf2* is expressed exclusively from the paternal allele, and imprinting theory interprets this as a paternal drive to produce larger offspring, which must be constrained by the mother so that her resources are not invested disproportionately in this offspring as compared with those arising in other pregnancies, and her continuing survival is secured. In turn, the gene encoding the recycling receptor *igf2* is imprinted with expression from the maternal allele, so the amount of available IGF is influenced by imprinting from the maternal side. Whether this applies to the uterine control of invasive trophoblast is unclear. However there is evidence in mouse that trophoblast does influence decidual gene expression: antimesometrial decidua differs in its gene expression signature from mesometrial decidua, arguably because the two are receiving paracrine signals from different populations of placentally-derived trophoblast.

Endometrial Glands in Pregnancy

In some species (though not human), endometrial glands form postnatally. In sheep and mouse this process has been inhibited by administering neonatal progestin. In both cases, the animals grow into infertile adults. Gland cell functions are therefore critical to fertility and pregnancy outcome. In the mouse, leukemia inhibitory factor (LIF) is released from glands in response to an estrogen pulse that occurs about 3 days after mating. It diffuses into the uterine lumen to activate receptors on luminal epithelium, producing a receptive state for embryo attachment.

In human, glands are important in the deciduochorial period, before maternal blood circulates in the intervillous space (Figs. 2 and 3). Glycogen stores in epithelial cells are broken down, with release of glucose oligomers into gland lumens. Glycoproteins and lipids are also present in gland secretions. These substances diffuse through narrow channels in the cytotrophoblast shell to reach the intervillous space, from where nutrient import can take place by villous syncytiotrophoblast. In this way they contribute

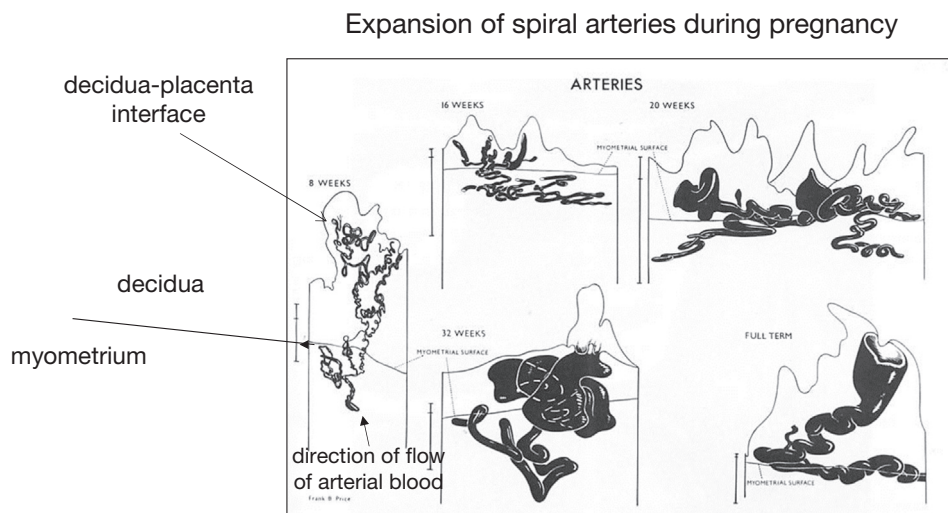


Fig. 4 Uteroplacental arteries at various stages of pregnancy, reconstructed from serial sections. Reproduced with permission from Ramsey, E.M. and Harris, J.W.S. (1966) *Carnegie Contributions to Embryology* 38, 59.

(with components of plasma and stromal secretions) to embryonic nutrition. From 11 weeks the placenta is predominantly hemochorial. In the second trimester, the gland cells regress to a flatter shape with reduced cytoplasmic contents and secretions.

Blood Flow in Pregnancy

From mid pregnancy, flow through the uterine artery increases steadily, as a result of NO-mediated relaxation resulting from stimulation of endothelial nitric oxide synthase by estrogen, and the effects of circulating relaxin. A larger volume of blood therefore enters the spiral arteries. However, remodeling has increased their diameter and abolished any contractile capacity, so following upstream relaxation, a high volume can traverse the intervillous space at low velocity, minimizing shear damage to placental villi (Fig. 4). Venous drainage channels are prominent at the periphery of the placental bed.

Further Reading

- Aplin, J. D., Fazleabas, A. T., Glasser, S. R., & Giudice, L. C. (Eds.). (2008). *The endometrium: Molecular, cellular and clinical perspectives*. London: Informa Healthcare.
- Aplin, J. D., & Ruane, P. T. (2017). Embryo-epithelium interactions during implantation at a glance. *Journal of Cell Science*, 130(1), 15–22. <https://doi.org/10.1242/jcs.175943>. 28043966.
- Du, H., & Taylor, H. S. (2007). Contribution of bone marrow-derived stem cells to endometrium and endometriosis. *Stem Cells*, 25(8), 2082–2086. 17464086.
- Evans, J., & Salamonsen, L. A. (2014). Decidualized human endometrial stromal cells are sensors of hormone withdrawal in the menstrual inflammatory cascade. *Biology of Reproduction*, 90(1), 14. <https://doi.org/10.1095/biolreprod.113.108175>. 24227758.
- Foidart, J. M., Hustin, J., Dubois, M., & Schaaps, J. P. (1992). The human placenta becomes haemochorial at the 13th week of pregnancy. *The International Journal of Developmental Biology*, 36(3), 451–453. 1445791.
- Jones, C. J., Choudhury, R. H., & Aplin, J. D. (2015). Tracking nutrient transfer at the human maternofetal interface from 4 weeks to term. *Placenta*, 36(4), 372–380. <https://doi.org/10.1016/j.placenta.2015.01.002>. 25618838.
- Lynch, V. J., Nnamani, M. C., Kapusta, A., Brayer, K., Plaza, S. L., Mazur, E. C., Emera, D., Sheikh, S. Z., Grützner, F., Bauersachs, S., Graf, A., Young, S. L., Lieb, J. D., DeMayo, F. J., Feschotte, C., & Wagner, G. P. (2015). Ancient transposable elements transformed the uterine regulatory landscape and transcriptome during the evolution of mammalian pregnancy. *Cell Reports*, 10(4), 551–561. <https://doi.org/10.1016/j.celrep.2014.12.052>. 25640180.
- Macklon, N. S., & Brosens, J. J. (2014 Oct). Human endometrium as a sensor of embryo quality. *Biology of Reproduction*, 91(4), 98. <https://doi.org/10.1095/biolreprod.114.122846>. 25187529.
- Schatz, F., Guzeloglu-Kayisli, O., Arlier, S., Kayisli, U. A., & Lockwood, C. J. (2016). The role of decidual cells in uterine hemostasis, menstruation, inflammation, adverse pregnancy outcomes and abnormal uterine bleeding. *Human Reproduction Update*, 22(4), 497–515. <https://doi.org/10.1093/humupd/dmw004>. 26912000.
- Smith, S. D., Dunk, C. E., Aplin, J. D., Harris, L. K., & Jones, R. L. (2009). Evidence for immune cell involvement in decidual spiral arteriole remodeling in early human pregnancy. *The American Journal of Pathology*, 174(5), 1959–1971. <https://doi.org/10.2353/ajpath.2009.080995>. 19349361.

Uterus: Growth Factors and Cytokines

Arpita Bhurke, Milan K Bagchi, and Indrani C Bagchi, University of Illinois at Urbana-Champaign, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Growth factors are a family of secreted signaling proteins that act in a paracrine and endocrine fashion to induce cellular proliferation and differentiation. Cytokines are polypeptide glycoproteins, which are produced in relatively small quantities and have a short half-life, restricting their sphere of influence to the local environment. Cytokines can promote or suppress multiple aspects of target cell behavior, impacting metabolism, stress response, survival, proliferation, and differentiation. Signaling via growth factors or cytokines is mediated by specific cell surface receptors. Across mammalian species, there is considerable similarity in the types of growth factors and cytokines that are expressed in the uterus, suggesting a high degree of conservation. In this article, we summarize the current understanding of the nature and role of growth factors and cytokines in the rodent and human uteri during the reproductive cycle and early pregnancy.

Uterine tissue is comprised of heterogeneous cell types. The cells lining the uterine lumen are epithelial cells. Subjacent to the luminal epithelium is the stroma, which harbors the fibroblastic stromal cells, endothelial cells, and a variety of immune cells. Dispersed within the stroma are glands, which are also lined by a single layer of epithelial cells. The cellular components of the uterus constitute the endometrium. The myometrium, defined as the outer layer of the uterus, houses the muscle cells. The focus of this article is the function of growth factors and cytokines produced by the endometrium. Secretion of cytokines and growth factors is not constitutive. In response to the ovarian steroid hormones, estrogen and progesterone, epithelial and stromal cells promote the synthesis of multiple growth factors and cytokines during the reproductive cycle and early pregnancy. Discussed below are some of these cytokines and growth factors that play critical roles in regulating uterine physiology. **Tables 1–3** show the list of growth factors and cytokines produced by the uterine cells during the reproductive cycle and early pregnancy, respectively.

Growth Factors

Insulin-Like Growth Factors (IGFs)

Among growth factors, insulin-like growth factors (IGFs) play a pivotal role in stimulating endometrial proliferation. Studies using mice lacking IGF1 revealed that in the absence of this growth factor, the uteri are hypotrophic. Further studies revealed a dramatic upregulation of IGF1 followed by phosphorylation of insulin-like growth factor 1 receptor (IGF1R) expressed in the uterine epithelium in response to treatment with estrogen. Activation of IGF1 signaling leads to nuclear accumulation of cyclin D1 and epithelial cell proliferation. Thus, IGFs mediate mitogenic action of estrogen in the uterus during the reproductive cycle (Nayak and Giudice, 2003).

During early pregnancy, *Igf1* mRNAs are observed in stromal cells of mouse uterus at days 4–6 of gestation. While the precise role of IGF1 during early pregnancy remains unclear, studies have shown that supplementation of the culture media with physiological concentration of IGF1 during IVF significantly increased blastocyst formation. Further, when extravillous trophoblast cells (EVT) were cultured in the presence of IGF1, it induced migration of EVT cells during the implantation process. Insulin-like growth factor binding proteins (IGFBPs) modulate the mitogenic and metabolic effects of IGFs, which play an important role in cell growth,

Table 1 Relative expression of growth factors and cytokines in the uterus during estrous cycle (mouse)

Growth factors and cytokines		Estrogen dominated phase Proestrus/estrus	Progesterone dominated phase Diestrus
1.	LIF	E ++	E +
2.	IL6	E ++	E +
3.	IL11	*	E ++
4.	GM-CSF	E ++	E +
5.	CSF1	E ++	E +
6.	EGFs	E +	E +
7.	HB-EGF	E ++	E +
8.	FGFs	S ++	S +
9.	IGF1	E +	E ++
10.	VEGF-A	E ++	E +

S, Stroma; E, epithelium; +, baseline levels; ++, increase in expression levels; +++, surge/maximal expression levels; *, not expressed or insufficient information on the precise expression levels.

Table 2 Relative expression of growth factors and cytokines in the uterus during menstrual cycle (human)

Growth factors and cytokines		Estrogen dominated phase Mid-late proliferative phase (LH – 7 to LH)	Estrogen + progesterone Early-mid secretory phase (LH to LH+ 10)	Progesterone dominated phase Mid-late secretory phase (LH+ 6 to LH+ 14)
1.	LIF	E +	E ++	E +++ (uNK)
2.	IL6	E ++	E ++	E +
3.	IL11	E +	E ++	E ++
4.	CSF1	E ++	E +	E +
5.	GM-CSF	E +	E +++	E +
6.	EGFs	Late proliferative: E +++	Mid secretory (LH+6–10): E ++	E ++
7.	HB-EGF	E +	Mid secretory (LH+6–10): E ++	E +
8.	FGFs	S +	S ++	S +
9.	IGF1	Late proliferative: E +++	E +	E +
10.	VEGF-A	E ++	E ++	E ++

LH, Luteinizing hormone surge; S, stroma; E, epithelium; +, baseline levels; ++, increase in expression levels; +++, surge/maximal expression levels.

Table 3 Growth factors and cytokines expressed in the uterus during pregnancy in mice and humans

Growth factors and cytokines		Pre-receptive (pre-implantation) Mice: day 1–3 Human: day LH – LH+ 6	Window of receptivity (implantation) Mice: day 4–5 Human: day LH+ 6–10	Refractory Mice: post day 6 Human: post day 10
1.	LIF	E ++	E +++	E + + (uNK)
2.	IL6	E ++	E ++	E +
3.	IL11	E +	E ++	E + +
4.	CSF1	E +	E +++	E + + (placenta)
5.	EGFs	E +	E ++	E + +
6.	HB-EGF	E +	Prior to embryo attachment: E +++ (mouse)	E +
7.	FGFs	S ++	S –	S –
8.	IGFs	E ++ (mouse)	S +	S +
9.	VEGF-A	E +	E, S ++	S +++

LH, Luteinizing hormone surge; S, stroma; E, epithelium; +, baseline levels; ++, increase in expression levels; +++, surge/maximal expression levels; –, expression inhibited; *, not expressed or insufficient information on the precise expression levels.

metabolism and embryonic development during early pregnancy (Singh et al., 2011). In mice, IGFBP-1 is detected in uterine epithelial cells and IGFBP-4 is the predominant IGFBP in stromal cells during the periimplantation phase. It has also been reported that IGFBP3 inhibits IGF1-induced DNA synthesis in the mouse endometrial stromal cells (Nayak and Giudice, 2003). IGFBPs including IGFBP1, IGFBP2, IGFBP3, and IGFBP4 are also expressed by human endometrial stromal cells upon differentiation (Giudice et al., 1993). As pregnancy progresses, IGFs and IGFBPs continue to play an important role in formation of the placenta. Inadequate production of IGFs or IGFBPs causes abnormal placentation (Davila et al., 2015; Nayak and Giudice, 2003).

Epidermal Growth Factor (EGF) Family

The EGF family of growth factors includes EGF, TGF- α , HB-EGF, amphiregulin, β -cellulin, epiregulin, and neuregulin. Many of these members are expressed in response to steroid hormones in rodent and human endometrium during the reproductive cycle and pregnancy. The EGF-like growth factors interact with receptor subtypes that belong to an ErbB gene family of tyrosine kinase receptors: *ErbB1* (EGFR), *ErbB2*, *ErbB3*, and *ErbB4*, which are known to be expressed in the uterine stromal cells. These receptors are structurally similar but differ in their ligand specificity and kinase activity. The expressions of EGF receptors are also regulated by steroid hormones. Studies have shown that during early pregnancy in mice, progesterone induces secretion of Indian hedgehog (IHH) by the uterine epithelial cells, which interact with their transmembrane Patched receptors (PTC) on the stromal cells to induce EGFR (Franco et al., 2010). Microarray analysis identified additional members of the EGF receptor family that are regulated by IHH, including *ErbB2/Her2*, *ErbB3/Her3*, and *ErbB4/Her4* (Franco et al., 2010). In vitro studies show that epithelial EGF via stromal EGFR induces stromal cell proliferation. Administration of steroid hormones, estrogen and progesterone, along with EGF, TGF- α increased the percentage of BrdU-labeled stromal cells. The stimulatory effect of EGF, TGF- α and steroid hormones on DNA replication in the uterine stromal cells was repressed by RG-13022, an inhibitor of the EGF receptor tyrosine kinase, suggesting that EGF–EGFR signaling is a critical driver of stromal cell

proliferation and differentiation. Consistent with these observations, it was found that mice lacking *Egfr* expression are severely subfertile. Pregnancy demise occurred shortly after embryo implantation due to defects in stromal cell proliferation and differentiation.

HB-EGF is a prominent growth factor of uterine receptivity to embryo implantation in both mice and humans. In mice, the soluble and the transmembrane form of HB-EGF are expressed exclusively at the implantation site prior to embryo attachment (Lim and Dey, 2009). Interestingly, the soluble form of HB-EGF increases the rate of human blastocyst hatching in vitro. Blastocysts from mice and human adhere to the epithelial cells expressing HB-EGF (Lim and Dey, 2009). Conditional ablation of HB-EGF expression in mouse uterine tissue markedly reduces the rate of implantation. Other growth factors including amphiregulin, beta-cellulin, epiregulin, neuregulin are expressed at the time of embryo implantation. However, due to their overlapping expression patterns around the implanting blastocyst at the time of attachment, mice with targeted disruptions of these growth factors do not exhibit implantation defects (Lim and Dey, 2009).

Fibroblast Growth Factors (FGFs)

Recent studies have shown that a subset of FGF family members including Fgf-1, -10, -18, and -21, are expressed by the stromal cells while the FGF receptors (FGFR) are expressed in the epithelium of rodent uteri (Li et al., 2011). Estrogen-mediated signaling by FGF–FGFR stimulates epithelial proliferation in the uterus. The FGFs via FGF receptors activate the extracellular-signal-regulated kinases 1 and 2 (ERK1/2) and/or PI3 kinase pathway to induce expression of cyclin D1. Cyclin D1 is required for progression of cell cycle during uterine epithelial proliferation. Progesterone mediated suppression of the FGFR–ERK1/2 pathway in uterine epithelia causes the epithelial cells to exit from the cell cycle and become “receptive” for embryo implantation (Li et al., 2011). Ablation of FGFR2 in the adult uterus render the female mice initially subfertile and later infertile with increasing parity (Filant et al., 2014).

Cytokines

Leukemia Inhibitory Factor (LIF)

Leukemia inhibitory factor (LIF) is a critical factor for embryo implantation (Stewart et al., 1992). It is a member of the IL-6 family and functions through the LIF receptor, which is associated with the signal transducer GP130. In rodent uterus, the expression of LIF is induced specifically in the glandular epithelium in response to a surge of estrogen immediately before implantation. Recent studies indicated that the conditional ablation of uterine epithelial estrogen receptor alpha in mice leads to a loss of LIF production by the glands, confirming the estrogen regulation of this factor (Kimber, 2005; Pawar et al., 2013). LIF regulates the remodeling of the uterine luminal epithelial cell–cell junctions at the site of implantation to facilitate embryo invasion. LIF secreted from the uterine glands binds to the LIF receptors on the luminal epithelial cells to activate STAT3 (signal transducer and activator of transcription 3). Epithelial STAT3, upon activation, suppresses the expression of the tight junction proteins (claudin-1, -2, -3), within the implantation window (Pawar et al., 2013). Additionally, LIF may be involved in the migration of uterine natural killer cells (uNK) during early pregnancy (Kimber, 2005).

In humans, LIF is expressed throughout the menstrual cycle with a striking increase in the mid- and late-secretory phase (days 18–28) and in early pregnancy. LIF is expressed primarily in the luminal and glandular epithelial cells. LIF-receptor mRNA is restricted to luminal epithelium throughout the cycle. LIF is detected in decidual leukocytes, which are abundant at the implantation site. Although the expression of *LIF* peaks at the time of implantation in humans, its role in this process is not clear. During mid-pregnancy, *LIF* and *LIF-receptor* have been detected in the decidua and chorionic villi of first trimester and term placenta (Kimber, 2005).

Interleukins

Interleukins are secretory cytokines, which are responsible for a variety of cellular functions. Although they are not direct targets of regulation by steroid hormones estrogen and progesterone, their actions are nevertheless important for endometrial functions in both mice and humans. Specifically, the expression of interleukin 11 (IL-11) rises transiently in the decidua upon embryo implantation, while that of its receptor, interleukin 11 receptor alpha (IL-11R α), is localized to this tissue. Consistent with this expression pattern, mice carrying a deletion of the gene encoding IL-11R α are infertile due to impaired decidual response. A recent study shows that inhibition of IL-11 function with an antagonist disrupts the proliferation and differentiation of stromal cells in pregnant mice and in human endometrial stromal cells (hESC) in vitro. In hESC, IL-11 and its receptor are believed to control mitotic expansion of hESCs obtained from early to mid-secretory stage endometrium (Dimitriadis et al., 2005).

Studies have shown that several interleukins such as IL-6, IL-9, IL-10, IL-11, IL-15, and IL-35 help create an immune-tolerant milieu that is essential for successful pregnancy. Many of these interleukins, (e.g., IL-6, IL-10, and IL-11) mediate cell–cell communication with the uterine stromal and embryonic cells via JAK-STAT pathways. IL-6-deficient mice exhibit reduced fertility and a decrease in viable implantation sites (Dimitriadis et al., 2005). During pregnancy, IL-10 is secreted by the uNK cells in the decidua as well as by the embryonic cells. It functions as an immune suppressive agent and a mediator of cross-talk between the fetal tissue and the uterus (Thaxton and Sharma, 2010). Studies in mice suggest that IL-11 signaling is required for decidual-specific maturation

of uNK cells. Interestingly, a recent study showed that IL-1 β , derived in part from infiltrating innate immune cells, affects stromal differentiation via effects on gap junction physiology in the human (Yu et al., 2017).

Transforming Growth Factor- β (TGF β)

Transforming growth factor beta (TGF β) 1–3 are founding members of the TGF β superfamily, which have profound effects on extracellular matrix (ECM) production, cell growth and differentiation. In the mouse uteri, individual TGF β isoforms are differentially expressed in epithelial and stromal cells during the reproductive cycle and early pregnancy. Similarly in the human endometrium, TGF β -1, -2, and -3 are expressed in both epithelial and stromal cells. Of the three, only TGF β -3 varies across the cycle, and, its expression increases in the glandular epithelium during the late secretory phase (Dimitriadis et al., 2005). The in vivo role of TGF β in the uterus remains elusive, partially because of the redundancy of the ligands and the lack of appropriate animal models as a result of the embryonic lethality in mice lacking TGF β ligands. In vitro treatment of preimplantation stage embryos with TGF β -1 increases total number of cells in expanded and hatching blastocysts. In addition, TGF β -1 increases the expression of oncofetal fibronectin, an anchoring trophoblast marker, indicating a potential role of TGF β in trophoblast adhesion during implantation. TGF β -1 also inhibits human trophoblast cell invasion by promoting the production of tissue inhibitor of metalloproteinases. Besides TGF β 1–3, several other TGF β superfamily ligands including bone morphogenetic proteins and nodal growth differentiation factor are also expressed in the uterus. Recent studies using tissue-specific knockout approaches have yielded new insights into this growth factor superfamily in uterine function and diseases (Li, 2014).

Colony Stimulating Factor (Csf) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

CSF via its receptor, c-fms, triggers JAK/STAT and/or PI3/AKT pathways to regulate cellular proliferation, differentiation and survival. It has been reported that in the human endometrium, estrogen upregulates CSF1 and GM-CSF during the proliferative phase and early secretory phase of the menstrual cycle. The uterine expression of these factors decline in the late secretory phase when progesterone begins to rise. In the human, CSF-1 protein is much higher in the pregnant than the non-pregnant uteri and is high in the placenta throughout gestation (Dimitriadis et al., 2005). CSF blocks the response of cytotoxic T lymphocyte precursors and protects the embryo against cytotoxic mechanisms.

In the mouse, there is a surge of GM-CSF following mating. This surge results in influx of leukocytes into the uterus. GM-CSF promotes cellular proliferation and differentiation that supports embryo growth including progression into the blastocyst stage and blastocyst hatching. It also regulates macrophage activity during pregnancy. GM-CSF null mice are not infertile. However, in the absence of uterine GM-CSF, there are abnormalities in the relative proportions of the different cell types contributing to the placenta resulting in gestational death of the fetuses (Dimitriadis et al., 2005).

Vascular Endothelial Growth Factor (VEGF)

VEGFs are a family of endothelial cell specific mitogens and are the main factors responsible for de novo blood vessel formation or vasculogenesis. The VEGF family consists of six members: VEGF-A, -B, -C, -D, -E and placental growth factor. These ligands bind to three cell surface receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and -3 that are type 3 receptor tyrosine kinases, all of which are expressed in the uterus. In both mice and humans, VEGF-A is the major angiogenic growth factor in the uterus. Although the expression of VEGF-A is regulated by both estrogen and progesterone in the uterus, decidual angiogenesis is mainly regulated by VEGF-A secreted from the progesterone receptor (PR)-expressing decidual cells (Kim et al., 2013). The highest level of VEGF-A expression is observed in the mid to late proliferative phase of the cycle.

In the mouse, the expressions of VEGF-A and VEGFR in the uterus dynamically change during estrus cycle and early pregnancy. During embryo implantation, VEGF-A regulates endometrial vascularization and vascular permeability, thereby rendering the endometrium “receptive” for embryo implantation. VEGF-A also regulates endothelial cell proliferation at implantation sites in rodents. Later in pregnancy, VEGF-A plays an important role in formation of the “vascular sinus folding” during placentation (Kim et al., 2013). Inadequate production of VEGF-A by the decidual cells causes abnormal placentation (Davila et al., 2015). A study using specific blockades of VEGFR indicated that VEGFR2 is critical for decidual angiogenesis, vascular remodeling, and pregnancy progression. While VEGFR1 signaling has no obvious effect, signaling via VEGFR3 moderately affects decidual angiogenesis (Douglas et al., 2009).

Uterine Pathologies Associated With the Dysregulation of Cytokines and Growth Factors

In response to steroid hormone signaling, growth factors and cytokines are expressed in different cellular compartment of the uterus. These factors then act in a paracrine fashion to regulate uterine physiology during the reproductive cycle as well as in pregnancy. Disruption of the endometrial stromal–epithelial crosstalk involving these cytokines and growth factors often results in altered uterine physiology leading to unsuccessful pregnancy outcomes. It has been shown that in pregnancies with intrauterine growth restriction, decreased expression of VEGFA and aberrant expression of angiopoietins are associated with angiogenic abnormalities during placentation (Kim et al., 2013). Studies have also shown that overproduction of VEGF receptor, sFLT1, in the placental trophoblast causes vascular dysfunction in preeclampsia and complications related to pregnancy (Fan et al., 2014).

Miscarriage remains one of the most prevalent obstetrical complications but little is known about the risk factors and etiology. Reduced expression of VEGFA has also been reported in endometrium of women with recurrent miscarriage. Elevated serum levels of certain cytokines such as IL-8 is associated with second-trimester miscarriages in women. Some cases of uterine pathology involving these cytokines, however, are much more complicated. It has been reported that elevated levels of IL-6 at the maternal–fetal interface are associated with fetal loss in mice. Similarly, increased levels of serum IL-6 was reported in women with first- or second-trimester miscarriage. However, another study showed reduced secretion of IL-6 in women with first-trimester miscarriage and altered decidual cell expression of IL-6 and its receptors in the placental bed of women with second-trimester miscarriage. Although a large number of cytokines are expressed in the uterus over the course of the reproductive cycle, functional redundancy among the cytokines makes dissecting the role of individual cytokines expressed in the uterus challenging (Lash and Ernerudh, 2015).

Aberrant growth factor signaling also adversely affects uterine physiology. As discussed earlier in this article, FGFs act in a paracrine manner via the FGF receptors to promote epithelial proliferation. Uncontrolled proliferation of the endometrial epithelium results in alterations of glandular architecture (shape and size) and an increase in endometrial gland-to-stroma ratio, leading to endometrial hyperplasia. Studies have shown that progesterone via its downstream target Hand- and neural crest derivatives-expressed protein 2 (HAND2) suppresses the production of several stromal FGFs curtailing epithelial proliferation (Jones et al., 2013; Li et al., 2011). It has also been reported that epigenetic silencing of *HAND2* is associated with endometrial hyperplasia and cancer (Jones et al., 2013), indicating that persistent activation of FGF signaling due to absence of *HAND2* affects normal uterine physiology.

Clinical Application

Assisted Reproductive Technology, IVF

Based on strong evidence from the animal and human data, GM-CSF became the first cytokine to be used in the human embryo cultures used for IVF procedures. Fluorescence in-situ hybridization analysis for 7 chromosomes (13, 16, 18, 21, 22, X and Y) was carried out in all blastomeres of embryos cultured until 8 cell stage with or without GM-CSF. Addition of GM-CSF at 2 ng/mL in the culture media resulted in 34.8% uniformly normal embryos, compared with 33.3% uniformly normal embryos in the absence of this cytokine (Agerholm et al., 2010). Today GM-CSF is widely used in the IVF clinics.

Although understanding the molecular biology of these cytokines has helped to advance the field of reproductive medicine, certain questions need to be addressed. For example, it is important to determine how the embryo's epigenome is influenced by manipulation of autocrine and exogenous cytokine signals. It is also important to determine whether maximizing autocrine signaling is sufficient to sustain optimal epigenetic parameters in IVF embryos.

Treatments of Uterine Pathologies

The majority of cases of endometrial hyperplasia are associated with compromised progesterone signaling that fails to inhibit epithelial proliferation. Understanding the growth factor signaling pathways that regulate uterine epithelial proliferation has helped to devise treatments for endometrial hyperplasia. Progesterone treatments have been successfully used to regress both simple and complex hyperplasia (Marra et al., 2014).

References

- Agerholm, I., Loft, A., Hald, F., Lemmen, J. G., Munding, B., et al. (2010). Culture of human oocytes with granulocyte-macrophage colony-stimulating factor has no effect on embryonic chromosomal constitution. *Reproductive Biomedicine Online*, 20, 477–484.
- Davila, J., Laws, M. J., Kannan, A., Li, Q., Taylor, R. N., et al. (2015). Rac1 regulates endometrial secretory function to control placental development. *PLoS Genetics*, 11, e1005458.
- Dimitriadis, E., White, C. A., Jones, R. L., & Salamonsen, L. A. (2005). Cytokines, chemokines and growth factors in endometrium related to implantation. *Human Reproduction Update*, 11, 613–630.
- Douglas, N. C., Tang, H., Gomez, R., Pytowski, B., Hicklin, D. J., et al. (2009). Vascular endothelial growth factor receptor 2 (VEGFR-2) functions to promote uterine decidual angiogenesis during early pregnancy in the mouse. *Endocrinology*, 150, 3845–3854.
- Fan, X., Rai, A., Kambham, N., Sung, J. F., Singh, N., et al. (2014). Endometrial VEGF induces placental sFLT1 and leads to pregnancy complications. *The Journal of Clinical Investigation*, 124, 4941–4952.
- Filant, J., DeMayo, F. J., Pru, J. K., Lydon, J. P., & Spencer, T. E. (2014). Fibroblast growth factor receptor two (FGFR2) regulates uterine epithelial integrity and fertility in mice. *Biology of Reproduction*, 90, 7.
- Franco, H. L., Lee, K. Y., Broaddus, R. R., White, L. D., Lanske, B., et al. (2010). Ablation of Indian hedgehog in the murine uterus results in decreased cell cycle progression, aberrant epidermal growth factor signaling, and increased estrogen signaling. *Biology of Reproduction*, 82, 783–790.
- Giudice, L. C., Irwin, J. C., Dsupin, B. A., Pannier, E. M., Jin, I. H., et al. (1993). Insulin-like growth factor (IGF), IGF binding protein (IGFBP), and IGF receptor gene expression and IGFBP synthesis in human uterine leiomyomata. *Human Reproduction*, 8, 1796–1806.
- Jones, A., Teschendorff, A. E., Li, Q., Hayward, J. D., Kannan, A., et al. (2013). Role of DNA methylation and epigenetic silencing of *HAND2* in endometrial cancer development. *PLoS Medicine*, 10, e1001551.
- Kim, M., Park, H. J., Seol, J. W., Jang, J. Y., Cho, Y. S., et al. (2013). VEGF-A regulated by progesterone governs uterine angiogenesis and vascular remodelling during pregnancy. *EMBO Molecular Medicine*, 5, 1415–1430.

- Kimber, S. J. (2005). Leukaemia inhibitory factor in implantation and uterine biology. *Reproduction*, 130, 131–145.
- Lash, G. E., & Emerudh, J. (2015). Decidual cytokines and pregnancy complications: Focus on spontaneous miscarriage. *Journal of Reproductive Immunology*, 108, 83–89.
- Li, Q. (2014). Transforming growth factor beta signaling in uterine development and function. *Journal of Animal Science and Biotechnology*, 5, 52.
- Li, Q., Kannan, A., DeMayo, F. J., Lydon, J. P., Cooke, P. S., et al. (2011). The antiproliferative action of progesterone in uterine epithelium is mediated by Hand2. *Science*, 331, 912–916.
- Lim, H. J., & Dey, S. K. (2009). HB-EGF: A unique mediator of embryo-uterine interactions during implantation. *Experimental Cell Research*, 315, 619–626.
- Marra, C., Penati, C., Ferrari, L., Cantu, M. G., Bargossi, L., & Fruscio, R. (2014). Treatment of simple and complex endometrial non-atypical hyperplasia with natural progesterone: Response rate to different doses. *Gynecological Endocrinology*, 30, 899–901.
- Nayak, N. R., & Giudice, L. C. (2003). Comparative biology of the IGF system in endometrium, decidua, and placenta, and clinical implications for foetal growth and implantation disorders. *Placenta*, 24, 281–296.
- Pawar, S., Starosvetsky, E., Orvis, G. D., Behringer, R. R., Bagchi, I. C., & Bagchi, M. K. (2013). STAT3 regulates uterine epithelial remodeling and epithelial–stromal crosstalk during implantation. *Molecular Endocrinology*, 27, 1996–2012.
- Singh, M., Chaudhry, P., & Asselin, E. (2011). Bridging endometrial receptivity and implantation: Network of hormones, cytokines, and growth factors. *The Journal of Endocrinology*, 210, 5–14.
- Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., et al. (1992). Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature*, 359, 76–79.
- Thaxton, J. E., & Sharma, S. (2010). Interleukin-10: A multi-faceted agent of pregnancy. *American Journal of Reproductive Immunology*, 63, 482–491.
- Yu, J., Berga, S. L., Zou, W., Yook, D. G., Pan, J. C., et al. (2017). IL-1 β inhibits connexin 43 and disrupts decidualization of human endometrial stromal cells through ERK1/2 and p38 MAP kinase. *Endocrinology*, 158(12), 4270–4285.

Cervix

Mala Mahendroo and Shanmugasundaram Nallasamy, University of Texas Southwestern Medical Center, Dallas, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

5 α reductase type 1 An enzyme that catalyzes the reduction of the double bond between carbons 4 and 5 of many steroid substrates. In the cervix, 5 α reductase type 1 converts the steroid progesterone to 5 α progesterone, a less active steroid.

20 α -hydroxysteroid dehydrogenase An enzyme that catalyzes the conversion of progesterone into its inactive form, 20 α -hydroxyprogesterone.

Adherens junction A junction which joins the actin cytoskeleton to the plasma membrane to form adhesive contacts between cells or between cells and extracellular matrix.

Aquaporins Cell membrane proteins which selectively permit water to pass into and out of the cell.

Desmosomes Adhesive intercellular junctions that mechanically integrate adjacent cells by coupling adhesive interactions mediated by desmosomal cadherins to the intermediate filament cytoskeletal network.

Exocervix The portion of the uterine cervix extending into the vagina and lined with stratified squamous epithelium.

Ehlers–Danlos syndrome Group of inherited disorders that affect the connective tissues that support the skin, bones, blood vessels, and many other organs and tissues.

Endocervix The mucous membrane lining the canal of the cervix uteri.

External os The opening of the uterine cervix into the vagina.

Extracellular matrix (ECM) Complex network of non-cellular portion of a tissue composed of proteins and polysaccharides that are secreted locally by cells and remain closely associated with them to provide structural, adhesive and biochemical signaling support.

Fibrillar collagen Polymerized, supramolecular collagen organized in fibrils.

Glycosaminoglycans Long, linear, charged polysaccharides that comprise repeating pairs of sugars, of which one is an amino sugar.

Internal os The opening of the cervix into the body of the uterus.

Lysyl oxidase An enzyme that participates in the formation of collagen polymers by facilitating oxidative deamination of peptidyl lysine residues on collagen monomers, to form crosslinks between collagen tripeptides.

Marfan syndrome A connective tissue multisystemic disorder characterized by skeletal changes, cardiovascular defects, and ocular defects. It is an autosomal dominant inheritance, caused by a mutation in the fibrillin-1 gene.

Mullerian ducts Paired ducts of mesodermal origin in the embryo giving rise in the female to the fallopian tubes, uterus, cervix, and upper portion of the vagina.

Papanicolaou screening test (Pap) A method of cervical screening used to detect potentially pre-cancerous and cancerous processes in the cervix.

Parity Number of times a female has given birth to offspring.

Pericellular matrix Extracellular matrix located on the outer surface of a cell.

Portio supravaginalis The part of the cervix of the uterus lying above the attachment of the vagina.

Portio vaginalis The part of the cervix within the vagina.

Proteoglycans Glycoproteins that consist of a core protein with one or more glycosaminoglycan chains.

Spinnbarkeit The elastic quality that is characteristic of mucus.

Tight junctions Type of cell-cell junction found in epithelia that forms a barrier that is impermeable to most soluble molecules in the space between adjacent epithelium. Major components of tight junctions are claudin and occludin proteins.

Anatomy

The cervix is a part of the female reproductive tract which connects the uterus with the vagina. The cervix is in the pelvic cavity, anterior to the rectum and posterior to the bladder and is supported by uterosacral and lateral ligaments. It receives its blood supply from branches of the uterine artery and vaginal arteries of the internal iliac arteries (Nott et al., 2016). The cervix is developed along with the uterus and vagina from Mullerian ducts (paramesonephric ducts) (Spencer et al., 2005). Fusion of the two Mullerian ducts vary between species resulting in species specific gross anatomy of the uterus and cervix. For example, humans have a single cervix and single uterine cavity; mice and cows have a single cervix that extends into two uterine horns while rabbits have two distinct cervixes each of which extends into a separate uterine horn (Fig. 1). While contiguous with both uterus and vagina, the cellular/

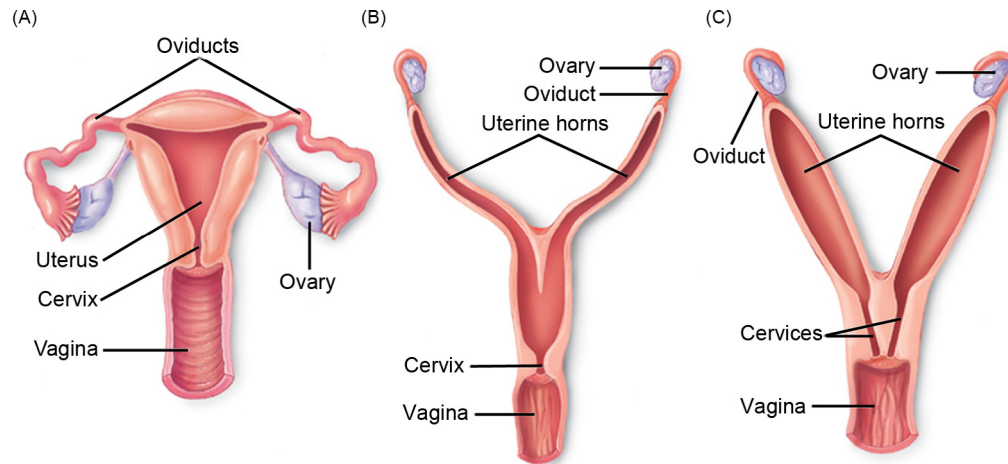


Fig. 1 Diagrammatic representation of species-specific variations in cervix and uterine architecture. (A) Single cervix opens into a single uterine cavity (e.g. women). (B) Single cervix opens into a bicornuate uterus (e.g. cow). (C) Two distinct cervical canals each of which separately extend into a uterine cavity (e.g. rabbits). The figure is adapted with permission from [Vodopich and Moore, 2001](#), © 2001 McGraw-Hill.

extracellular composition and functions are distinct from these compartments and vary between the non-pregnant and pregnant state. Consistent with its autonomy, cervical pathophysiology is an independent risk factor in the complications associated with pregnancy and birth ([Gee, 2009](#)).

There is great variation among species in length and diameter of the cervix, in the point of transition from columnar to squamous epithelia, the presence of epithelial glands and in the architecture of the epithelial mucosal folds. In humans, the cervix is small before puberty and increases in size with onset of puberty along with maturation of the epithelia and formation of glands ([Hafez, 1973](#)). In the non-pregnant adult woman, the dimensions of the cervix are 3 cm in length and 2 cm in diameter, though it can vary depending on age, parity and stage of menstrual cycle ([Nott et al., 2016](#); [Singer and Jordan, 2009](#)). Structurally, the cervix is divided into the upper portion, supravaginal portion (*portio supravaginalis*) and a lower portion (*portio vaginalis*). The central canal which connects the uterine cavity with the vaginal cavity is the inner cervical canal. This canal is lined by an epithelium and is termed the endocervix. The opening of the cervical canal at the uterine end is called the internal os and the opening at the vaginal end is called the external os ([Singer and Jordan, 2009](#)). The outer portion of the cervix extends into the vagina and is termed the ectocervix or exocervix. The parts of the cervix are depicted in [Fig. 2](#). Epithelial glands are present in the human but absent in mice. With respect to mucosal folds present in the endocervix, humans have branching folds on the surface, cows have four longitudinal annular rings arranged spirally, horses have no annular rings and pigs have corkscrew shaped projections ([Hafez, 1973](#)).

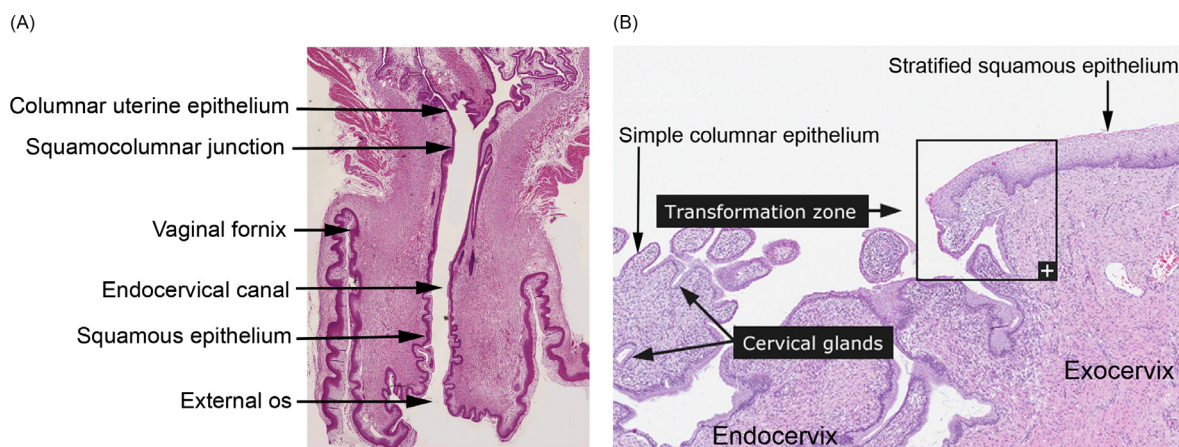


Fig. 2 Histology of the mouse and human cervix. (A) Longitudinal mouse cervical section stained with Hematoxylin and Eosin. The external os, endocervical canal, simple columnar uterine epithelium, squamocolumnar junction and squamous epithelium are indicated. (B) Human cervical tissue section stained with Hematoxylin and Eosin. Note that the human endocervix is lined with simple columnar epithelium and studded with cervical glands. The squamocolumnar junction (transformation zone) is distinct in human cervix. The exocervix is lined with stratified squamous epithelium. Image credit: Human Protein Atlas. Image available from Protein Atlas Dictionary - Normal tissue histology: Cervix, uterine (<http://www.proteinatlas.org/learn/dictionary/normal/cervix,+uterine+1>).

Functions

Non-pregnant state	1. Regulates motility of the sperms into the uterus 2. Allows outward flow of menstrual blood from the uterus into the vagina 3. Provides immune and barrier function of the reproductive tract
Pregnancy	1. Maintains pregnancy by withstanding the pressure generated by the growing fetus(es) and uterus 2. Provides immune and barrier function to protect the uterine compartment and fetus against environmental insults such as ascending infection
Parturition	Dilates sufficiently to facilitate birth process
Postpartum	Repositions and repairs itself to protect upper reproductive tract from external insults to ensure successive pregnancies. Regains structural integrity and mechanical strength.

Histology

Similar to any typical tubular organ, the cervix has stroma and epithelial compartments. During development, the Mullerian ductal epithelium proliferates antero-posteriorly to make two distinct epithelial cell types along the female reproductive tract. The uterus and upper portion of the cervix are lined with simple columnar epithelium and lower portion of the cervix and vagina are lined with stratified squamous epithelium. Hence, the cervix is comprised of two types of mucosal epithelium. The transition between these two cell types termed the squamocolumnar junction varies between species and stage of the cycle. In women, the endocervix and cervical glands are lined with simple columnar mucus secreting epithelium. In contrast, the ectocervix is lined with stratified squamous epithelium. In mice, only the uppermost portion of the endocervix harbors columnar epithelium while much of the endocervix and the ectocervix is comprised of squamocolumnar epithelium (Fig. 2). The proliferation and secretory behavior of the epithelium is highly variable depending on the stage of the cycle and pregnancy status (Singer and Jordan, 2009). The stromal compartment consists of multiple cell types and extracellular matrix (ECM). The cell types include fibroblasts, smooth muscle cells, and immune cells. The extracellular matrix is composed of fibrillar collagen, elastic fibers, glycosaminoglycans, proteoglycans and matricellular proteins. Both the epithelial and stromal compartments undergo structural changes depending on the stage of the cycle or pregnancy status (Timmons et al., 2010).

Epithelium

In Non-pregnancy

The cervical epithelium functions to provide a structural and an immunoprotective barrier as well as produce a cycle specific mucosal composition to regulate fertility. The epithelium produces junctional barrier proteins, cytokines, chemokines, antimicrobials, pattern recognition receptors and protease inhibitors to achieve these functions. Epithelial cell proliferation and the properties of their mucosal secretions undergoes significant changes during the menstrual cycle. These properties of mucus influence sperm motility and survival within the female reproductive tract thus influencing overall fertility (Martyn et al., 2014). The mucus is mostly composed of water, mucins, ions, antimicrobials and various other factors. Mucins are large glycosylated polymers that alter visco-elastic properties of the cervical mucus. Five different mucin proteins are known to be secreted by the cervical epithelia (Martyn et al., 2014). At the time of ovulation, the cervical mucus has low viscosity with marked spinnbarkeit and a high ionic concentration capable of forming a clear fern pattern. During this time, mucin polymers are arranged in such a way to permit sperm movement. The mucus turns highly viscous with low spinnbarkeit and exhibits no fern pattern at other stages of cycle to prevent sperm entry (Jequier, 2009) (Fig. 3). The functions of the cervical epithelia and its mucus are well conserved between species including human and mice.

In Pregnancy

The cervical epithelium serves as a physical barrier of protection, provides immune surveillance and modulates the local tissue steroid environment to maintain pregnancy or initiate cervical ripening for parturition (Timmons et al., 2010). Through pregnancy, the epithelium undergoes extensive proliferation followed by differentiation (Fig. 4). In the latter part of pregnancy, the upper layers of terminally differentiated epithelia are laden with mucin – secreting vacuoles. In mice, the thickness of this mucosal epithelium reaches a maximum at the time of birth on day 19 of gestation and regresses during the postpartum period. Mucus provides a physical and immune- barrier, while epithelial cell junctions such as tight junctions, adherens junctions and desmosomes are formed between adjacent cells in the pericellular space and serve to regulate the movement of molecules between cells (Timmons et al., 2010). Further, the barrier function of epithelium is complemented by the structure of the pericellular matrix and its constituents. In addition, the cervical epithelia play a role in tissue hydration through the expression of aquaporins. Similar to non-pregnancy, the epithelium at pregnancy also produces cytokines, chemokines, antimicrobials, pattern recognition receptors and proteases inhibitors to achieve its functions. While the cervical epithelia and the mucus it secretes have a similar function in most species,

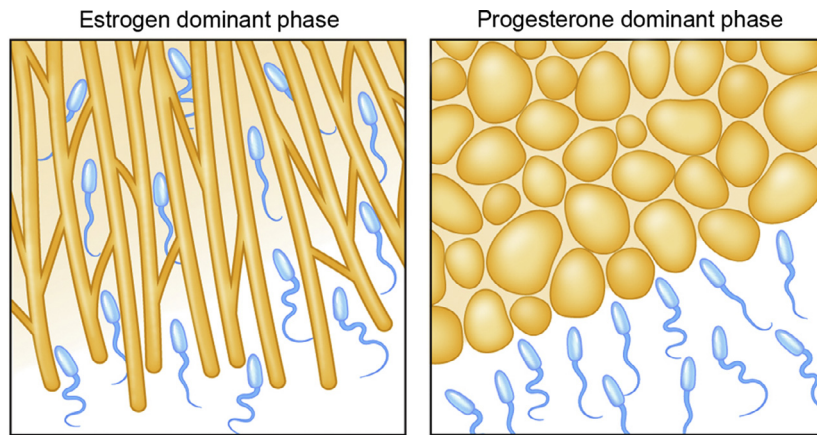


Fig. 3 Steroid hormones regulate the physical properties of cervical mucus to control sperm entry. During ovulation, an estrogen dominant phase, cervical mucus has a low viscosity with spinnbarkeit and mucin polymers (shown in yellow) are arranged in such a way to permit sperm movement into the upper reproductive tract. In contrast, the cervical mucus in progesterone dominant phases is highly viscous with low spinnbarkeit and mucin polymers form a barrier to prevent sperm entry.

primates including humans are unique in that during pregnancy, the cervical glands and epithelium secrete a thick mucus which fills the endocervical canal to form a plug. The cervical mucus plug is present till term and will be shed only around the time of parturition. Its unique composition provides physical as well as immunological barrier function (Becher et al., 2009).

Stromal Compartment

The stromal compartment is composed of fibroblasts, smooth muscle cells and a rich and complex ECM. The composition and structure of the ECM determines mechanical rigidity (Fig. 4). While not a highly-vascularized tissue, there are blood vessels in the stroma and cycle-specific changes in vascularity. The endocervix has numerous sensory nerve endings while the ectocervix has only few. In addition, the stroma is populated by innate and adaptive immune cells that are poised to respond to danger signals from the cervical epithelia or pathogens. The stromal compartment undergoes modest changes in hydration, vascularity and immune cell abundance and function in the non-pregnant state. During pregnancy, the stromal cell populations proliferate with increased tissue vascularity and hydration at term. Resident immune cells display an immunosuppressive phenotype during pregnancy. An influx of tissue monocytes just prior to birth and expression of markers of proinflammatory and immunotolerance/repair macrophages after birth suggest an important role of immune cells in postpartum repair of the cervix.

Remodeling of the Cervical Stroma in Pregnancy

From early pregnancy to the onset of labor, the cervix is transformed from a closed and rigid to a soft and compliant structure that opens sufficiently for fetal exit from the birth canal. Concurrent with the dynamic changes required to facilitate cervical compliance, the cervix must maintain its structural and mechanical integrity to protect the pregnancy until term. This dynamic process termed cervical remodeling can be divided into four overlapping phases termed softening, ripening, dilation, and postpartum repair (Word et al., 2007). In each phase, specific alterations in the synthesis, processing, and assembly of ECM components collectively orchestrate the gradual structural reorganization of the ECM that allows for the decline in mechanical strength of the cervix (Fig. 4). Key features that define each phase are well characterized in the mouse (Mahendroo, 2012). During softening, there is a gradual decline in stiffness of the cervix as compared to the non-pregnant cervix. It is a slow and incremental process and continues until late pregnancy. This softening process coincides with the period of uterine quiescence. In contrast to softening, the ripening phase is a rapid process and happens one day before delivery in the mouse. With onset of uterine contractions, the cervical canal undergoes dilation thus expanding to a maximal size to facilitate the delivery process. After birth, a remodeling process is initiated to allow recovery of the cervix back to the non-pregnant rigid and closed state to protect the reproductive tract from environmental insults.

Terminology used to assess cervical changes in pregnant women include softening, shortening, funnelling, effacement and dilation (Cunningham et al., 2013b; Myers et al., 2015). Softening can be assessed by digital exam while vaginal ultrasound allows visualization of cervical length from the internal to the external os (i.e. shortening) and the funnel shape the cervix assumes with progression of effacement and dilation. As the frequency, intensity, and duration of uterine contractions increases, the cervix becomes thinner by shortening the cervical canal in a process called effacement and dilatation. This coincides with the descending fetal head into the birth canal.

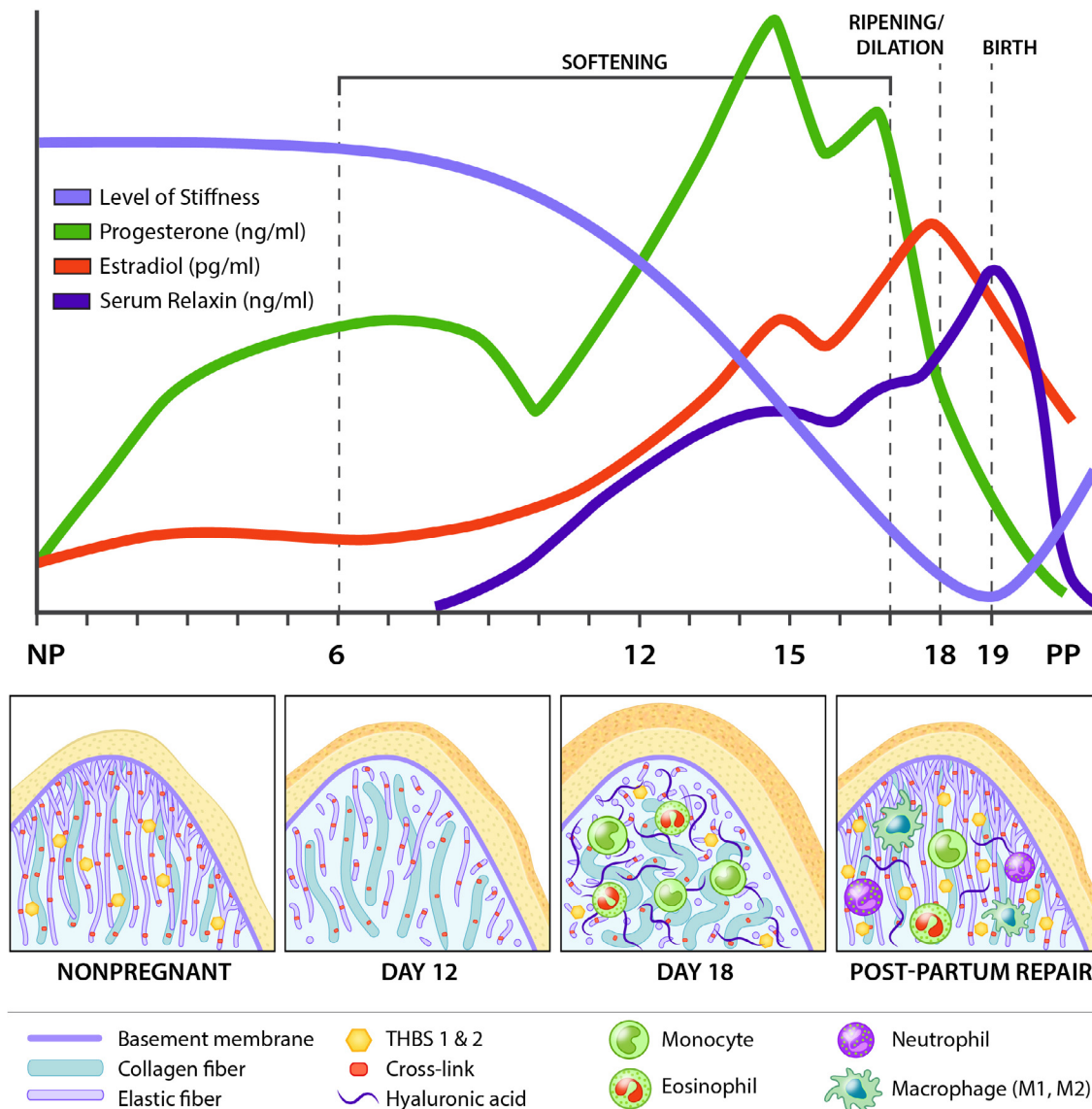


Fig. 4 Summary of the dynamic change in steroid hormones, tissue stiffness, ECM organization and immune cell populations that characterize the non-pregnant (NP), pregnant and postpartum (PP) cervix in mice. As depicted in the graph, the concentration of progesterone, estrogen and relaxin varies over the 19 day pregnancy in mice and is distinct during each phase of cervical remodeling. Cervical stiffness gradually declines during the softening phase of remodeling and reaches a maximal low at birth. The change in the mechanical properties of the cervix is determined in part by alterations in levels and ratios of progesterone, estrogen and relaxin. Lower panel: Schematic depicting changes in the cervical epithelium (shown in yellow) and the stroma on pregnancy days 12 and 18 and postpartum relative to the NP cervix. During pregnancy, the epithelium (shown in yellow) undergoes extensive proliferation and differentiation. Compared to NP, the number of epithelial layers is increased during pregnancy and the terminally differentiated epithelial layers (indicated in the darker yellow) are laden with mucus filled vacuoles that secrete mucus to provide physical as well as immunological protection. Hyaluronic acid synthesis along with junctional proteins in the late pregnancy epithelia are critical to maintain cervical barrier protection. Within the stroma region, collagen and elastic fibers undergo a dramatic reorganization during pregnancy. In the NP cervix, collagen and elastic fibers appear densely packed, linear and form parallel bundles. During pregnancy, the elastic fibers lose their branching pattern and collagen fibers have a reduced number of strength bearing crosslinks. Under the control of progesterone and estrogen, the synthesis, processing and assembly of collagen and elastic fibers is altered to allow the progressive decline in tissue stiffness required for cervical opening during birth. In addition, changes in the composition of the ECM during pregnancy (e.g. a decline in thrombospondin 1 and 2 (THBS 1 & 2) and increase in hyaluronic acid) also contribute to increased tissue compliance. Relative to the NP cervix, resident immune cells (not shown) are quiescent during pregnancy. In preparation for postpartum repair, monocytes and eosinophils infiltrate the cervix before parturition while neutrophils and macrophages (M1, proinflammatory macrophages; M2, tissue repair macrophages) are increased at birth. These immune cells function to clean up the disorganized ECM. During the PP repair phase the organization and structure of collagen and elastic fibers is recovered back to the NP state.

ECM remodeling

The fibrillar collagens, collagen I and III, are the predominant structural proteins in the cervix and significantly influence mechanical properties of the cervix (Mahendroo, 2012). The content of collagen does not change in the cervix presumably due to its coordinated regulation of synthesis and degradation. However, the synthesis and assembly of collagen fibers are modified early in pregnancy (softening) with reduced expression and activity of the enzyme lysyl oxidase (Nallasamy and Mahendroo, 2017). This enzyme forms cross-links on collagen and elastic fibers and the degree of cross-links correlates directly with mechanical strength and structure. Specifically, reduction in cross-link density of newly synthesized collagen in early pregnancy in parallel with collagen turnover is believed to alter tissue tensile properties to allow for the progressive increase in compliance in late pregnancy. In addition to modifications in processing of collagen fibers, reduction in the expression of matricellular proteins such as thrombospondin 1 and 2 (THBS 1 & 2) also influence collagen structure (Fig. 4). Elastic fibers also contribute to the cervical mechanical properties. They are predominantly localized in the subepithelial stromal region surrounding the endocervix and undergo remodeling during pregnancy (Nallasamy et al., 2017). Synthesis of the glycosaminoglycan, hyaluronan is elevated during cervical ripening in women, mice, rabbit and numerous other species. Hyaluronan is a hydrophilic molecule with proposed function in the ECM of the cervical stroma as well as in maintenance of barrier function of the cervical epithelia to protect against infection (Akgul et al., 2014).

Hormonal Regulation of Cervical Function

The functions of the non-pregnant and pregnant cervix are in part regulated by steroid hormones (e.g. estrogen, progesterone), and peptide hormones (e.g. relaxin) (Cunningham et al., 2013b; Mahendroo, 2012; Nallasamy et al., 2017; Sherwood, 2004). In most species estrogen and progesterone are synthesized in other tissues and enter the cervix to act through their receptors, estrogen receptor α and progesterone receptor respectively. These receptors are expressed in both epithelial and stromal cells. The fluctuation in the levels or ratio of estrogen and progesterone results in a change in the function of the cervical epithelium and stroma. For example, in the non-pregnant cervix, high levels of estrogen around the time of ovulation stimulate the secretion of cervical mucus suitable for sperm movement (Fig. 3). In contrast, high levels of progesterone produce mucus incompatible for sperm motility.

Within the cervical microenvironment during pregnancy, steroid hormone levels can be further regulated by the expression of enzymes that inactivate steroids (for example progesterone can be metabolized by steroid 5 α reductase type 1 and steroid 20 α hydroxysteroid dehydrogenase in the mice and 17 β -hydroxysteroid dehydrogenase type 2 in the human (Cunningham et al., 2013b)). In addition, relaxin synthesized in the ovary can act through its receptor, Rxfp1 to facilitate epithelial cell proliferation and ECM remodeling during cervical ripening (Sherwood, 2004). During pregnancy, elevations in the progesterone/estrogen ratio regulate epithelial cell proliferation, differentiation, mucosal layer formation and cervical mucus plug secretion. Concurrently, these hormones regulate the synthesis, processing, and assembly of stromal extracellular matrix constituents. These carefully timed molecular changes prepare the cervix for birth by gradually increasing the compliance of the cervix during the softening phase. Around the time of parturition, decline in the progesterone to estrogen ratio triggers a cascade of events to achieve cervical ripening and dilation and subsequently parturition.

Clinical Significance

Induction of Cervical Ripening for Labor

In some women, cervical ripening and onset of uterine contractions are not initiated by 40 weeks of gestation. In these cases, labor must be induced first by exogenous administration of prostaglandins to induce cervical ripening followed by oxytocin to induce uterine contractions and subsequently vaginal delivery. Prostaglandins are a group of compounds that induce cervical ripening presumably by increasing the activity of proteases that degrade the ECM to facilitate cervical ripening and dilation. While prostaglandins are sufficient to induce cervical ripening in women and animal models, studies in mice suggest they are not an essential mediator of cervical ripening in normal gestation (Timmons et al., 2014). They are key mediators of cervical ripening induced by infection. In addition to pharmacologic methods, induction of cervical ripening can be done using mechanical techniques. These include transcervical placement of a Foley catheter with or without extra amniotic saline infusion, hygroscopic cervical dilators, and membrane stripping (Cunningham et al., 2013a).

Cervix and Premature Birth

Premature birth, defined as birth less than 37 of a 40-week gestation, can be induced by numerous etiologies. Irrespective of cause, premature cervical remodeling is a common endpoint that usually precedes preterm labor (Romero et al., 2014; Vink and Feltoich, 2016). While the underlying cause for premature birth is unidentified in roughly 50% of premature births, known risk factors include infection, premature rupture of membranes, lifestyle factors (diet, stress, obesity, age, smoking), multiple fetuses and a previous preterm birth. In some cases of preterm birth, cervical dysfunction is the initiating cause of preterm birth. For example, preterm birth can result from cervical insufficiency, defined as premature cervical opening in the absence of any uterine contractions

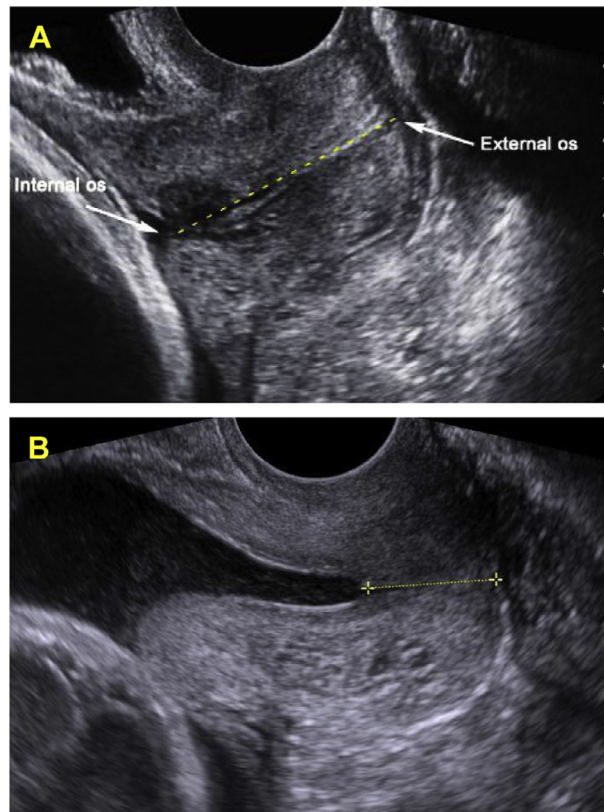


Fig. 5 Transvaginal ultrasonographic measurement of cervical length in women. (A) This image shows the length (*dotted yellow line*) and appearance of a normal cervix at 29 weeks of gestation. (B) This image of a cervix taken from a woman at 23 weeks of gestation shows funneling at the internal os and a prematurely reduced cervical length (*dotted yellow line*). This is an indicator of potential preterm birth risk. Photograph courtesy of Dr. Jodi Dashe, University of Texas Southwestern Medical Center, Dallas, TX.

and is due to structural defects in the cervical ECM. It is believed to be caused by acquired (e.g. surgery, injury) or intrinsic genetic defects in collagen and elastic fiber assembly (e.g. Ehlers–Danlos syndrome and Marfan syndrome) (Anum et al., 2009; Vink and Feltovich, 2016). In addition, premature cervical shortening as determined by vaginal ultrasound is a risk factor for premature birth (Fig. 5). A length less than 25 mm in women with a prior sPTB or less than 20 mm in unselected women based on the transvaginal ultrasound measurement is considered as a short cervix and currently is the best clinical indicator of premature cervical remodeling (Fig. 5) (Iams et al., 1996; Vink and Feltovich, 2016).

Human Papilloma Virus and Cervical Cancer

Cervical cancer is the most common gynecologic cancer in women. Most cervical cancers result from human papillomavirus (HPV) infection. HPV infection is a sexually transmitted infection. Chronic infection results in gradual transformation of cervical epithelia to precancerous lesions which eventually will form a cancer. The Papanicolaou screening test (Pap) provides accurate screening for neoplasia. Vaccination against the subtypes HPV-16 and HPV-18 reduces the incidence and persistence of infections and the incidence of HPV-mediated neoplasia (Hoffman et al., 2016).

Conclusion

The cervix plays a distinct and critical role in the non-pregnant and pregnant female to regulate fertility, maintain pregnancy and ensure safe delivery of a term fetus during parturition. The steroid hormones estrogen and progesterone are key mediators of the dynamic changes in the cervix. The peptide hormone relaxin also plays a role in cervical ripening in late pregnancy. During pregnancy, the uterine cervix undergoes an ECM reorganization of impressive scope and magnitude to transition the cervix from closed and rigid to one that is flexible to allow delivery of a term infant. Understanding this fascinating biological process will aid in prevention of premature birth, a leading cause of infant death globally.

References

- Akgul, Y., Word, R. A., Ensign, L. M., Yamaguchi, Y., Lydon, J., Hanes, J., & Mahendroo, M. (2014). Hyaluronan in cervical epithelia protects against infection-mediated preterm birth. *Journal of Clinical Investigation*, 124, 5481–5489.
- Anum, E. A., Hill, L. D., Pandya, A., & Strauss, J. F., III (2009). Connective tissue and related disorders and preterm birth: Clues to genes contributing to prematurity. *Placenta*, 30, 207–215.
- Becher, N., Adams Waldorf, K., Hein, M., & Ulbjerg, N. (2009). The cervical mucus plug: Structured review of the literature. *Acta Obstetrica et Gynecologica Scandinavica*, 88, 502–513.
- Cunningham, F. G., Leveno, K. J., Bloom, S. L., Spong, C. Y., Dashe, J. S., Hoffman, B. L., Casey, B. M., & Sheffield, J. S. (2013a). Induction and augmentation of labor. In *Williams obstetrics* (24th edn.). New York, NY: McGraw-Hill Education.
- Cunningham, F. G., Leveno, K. J., Bloom, S. L., Spong, C. Y., Dashe, J. S., Hoffman, B. L., Casey, B. M., & Sheffield, J. S. (2013b). Physiology of labor. In *Williams obstetrics* (24th edn.). New York, NY: McGraw-Hill Education.
- Gee, H. (2009). Mechanics, biochemistry and pharmacology of the cervix and labour. In *The cervix*. Blackwell Publishing Ltd.
- Hafez, E. S. E. (1973). The comparative anatomy of the mammalian cervix. In R. J. Blandau, & K. S. Moghissi (Eds.), *The biology of the cervix*. Chicago: University of Chicago Press.
- Hoffman, B. L., Schorge, J. O., Bradshaw, K. D., Halvorson, L. M., Schaffer, J. I., & Corton, M. M. (2016). Cervical cancer. In *Williams gynecology* (3rd edn.). New York, NY: McGraw-Hill Education.
- Iams, J. D., Goldenberg, R. L., Meis, P. J., Mercer, B. M., Moawad, A., Das, A., Thom, E., McNellis, D., Copper, R. L., Johnson, F., & Roberts, J. M. (1996). The length of the cervix and the risk of spontaneous premature delivery. *New England Journal of Medicine*, 334, 567–573.
- Jequier, A. M. (2009). Sperm transport in the human and mammalian cervix and genital tract: Its relation to fertility. In *The cervix*. Blackwell Publishing Ltd.
- Mahendroo, M. (2012). Cervical remodeling in term and preterm birth: Insights from an animal model. *Reproduction*, 143, 429–438.
- Martyn, F., McAuliffe, F. M., & Wingfield, M. (2014). The role of the cervix in fertility: Is it time for a reappraisal? *Human Reproduction*, 29, 2092–2098.
- Myers, K. M., Feltovich, H., Mazza, E., Vink, J., Bajka, M., Wapner, R. J., Hall, T. J., & House, M. (2015). The mechanical role of the cervix in pregnancy. *Journal of Biomechanics*, 48, 1511–1523.
- Nallasamy, S., & Mahendroo, M. (2017). Distinct roles of cervical epithelia and stroma in pregnancy and parturition. *Seminars in Reproductive Medicine*, 35, 190–200.
- Nallasamy, S., Yoshida, K., Akins, M., Myers, K., Iozzo, R., & Mahendroo, M. (2017). Steroid hormones are key modulators of tissue mechanical function via regulation of collagen and elastic fibers. *Endocrinology*, 158, 950–962.
- Nott, J. P., Bonney, E. A., Pickering, J. D., & Simpson, N. A. B. (2016). The structure and function of the cervix during pregnancy. *Translational Research in Anatomy*, 2, 1–7.
- Romero, R., Dey, S. K., & Fisher, S. J. (2014). Preterm labor: One syndrome, many causes. *Science (New York, N.Y.)*, 345, 760–765.
- Sherwood, O. D. (2004). Relaxin's physiological roles and other diverse actions. *Endocrine Reviews*, 25, 205–234.
- Singer, A., & Jordan, J. A. (2009). The functional anatomy of the cervix, the cervical epithelium and the stroma. In *The cervix*. Blackwell Publishing Ltd.
- Spencer, T. E., Hayashi, K., Hu, J., & Carpenter, K. D. (2005). Comparative developmental biology of the mammalian uterus. *Current Topics in Developmental Biology*, 68, 85–122.
- Timmons, B., Akins, M., & Mahendroo, M. (2010). Cervical remodeling during pregnancy and parturition. *Trends in Endocrinology & Metabolism*, 21, 353–361.
- Timmons, B. C., Reese, J., Socrate, S., Ehinger, N., Paria, B. C., Milne, G. L., Akins, M. L., Auchus, R. J., McIntire, D., House, M., & Mahendroo, M. (2014). Prostaglandins are essential for cervical ripening in LPS-mediated preterm birth but not term or antiprogesterone-driven preterm ripening. *Endocrinology*, 155, 287–298.
- Vink, J., & Feltovich, H. (2016). Cervical etiology of spontaneous preterm birth. *Seminars in Fetal and Neonatal Medicine*, 21, 106–112.
- Vodopich, D. S., & Moore, R. (2001). *Biology of Laboratory Manual*. New York: McGraw-Hill Education.
- Word, R. A., Li, X. H., Hnat, M., & Carrick, K. (2007). Dynamics of cervical remodeling during pregnancy and parturition: Mechanisms and current concepts. *Seminars in Reproductive Medicine*, 25, 69–79.

Cervix: Cell Biology

Takeshi Kurita, The Ohio State University, Columbus, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Structure and Function

The cervix is the muscular canal at the tapering end of the uterus. It can be divided into two histologically distinctive regions, endocervix and ectocervix/exocervix (**Fig.1**). The endocervix communicates with the uterine cavity via the internal orifice of the uterus (internal os), and the ectocervix opens into the upper vagina via the external orifice of the uterus (external os).

Endocervix

The endocervix is the upper proportion of cervix that is lined by a single folded layer of mucus-secreting columnar epithelium (**Fig.2**). The mucinous epithelium invaginates into the substance of the cervical stroma, forming the endocervical glands. These cervical glands secrete cervical mucus, a viscous fluid containing glycoproteins.

Cervical mucus

Cervical mucus is a hydrogel consisting of 90–95% water and a heterogeneous mixture of mucin glycoproteins. The physical properties and the biochemical composition of cervical mucus change during the menstrual cycle. When the ovulation approaches, cervical mucus becomes thinner and more watery and is so-called “egg white cervical mucus”, as it resembles raw egg white. Egg white cervical mucus is believed to be preferable mucus for conception, as it provides an easy pathway for the sperm to swim through.

The cervical mucus layer on the surface of the cervix works as barrier to prevent infection. Cell-surface (MUC1, MUC4 and MUC16) and gel-forming (MUC5B, MUC5AC, MUC6) mucins produced by endocervical epithelium contribute to the formation

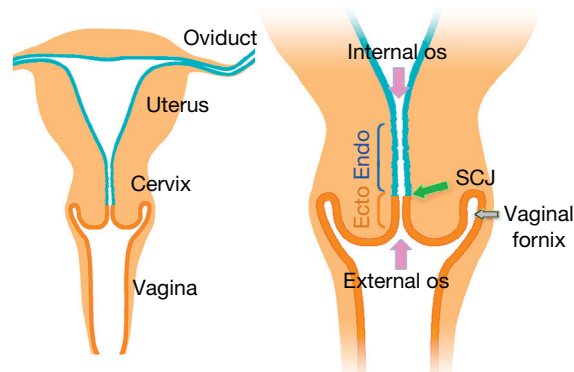


Fig. 1 Structure of Uterine Cervix.

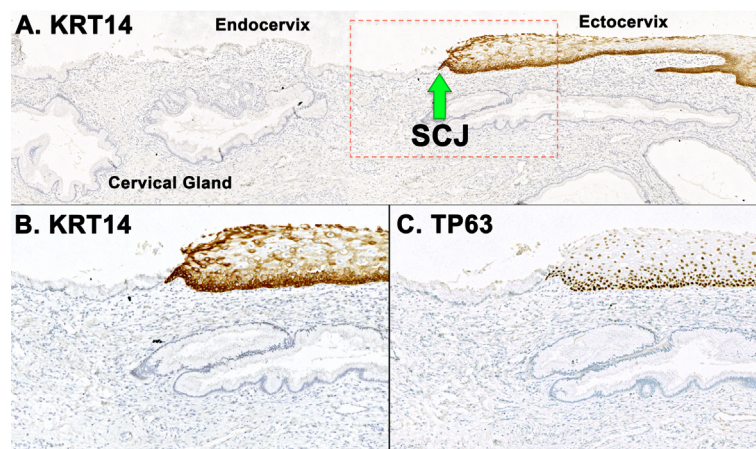


Fig. 2 Squamocolumnar Junction in Human Cervix. KRT14 and TP63 immunohistochemistry stained stratified squamous ectocervical epithelium. SCJ is indicated by an arrow.

of the mucus layer in the cervical canal (Andersch-Bjorkman et al., 2007). In addition, the quality of cervical mucus significantly influences the fertility, as interactions with cervical mucus affect the penetration of spermatozoa into the uterine cavity (Martyn et al., 2014). Cervical mucus also works as lubricant during sexual intercourse.

Ectocervix (Exocervix, Portio Vaginalis)

The ectocervix is the supravaginal portion of the cervix, which is lined by stratified squamous epithelium continuous with vaginal epithelium. In the multi-layered epithelium of ectocervix and vagina, new cells are formed in the most basal layers by mitotic division, and the daughter cells move upwards, gradually changing the morphology from a cuboidal to a more flattened shape. The formation, maturation and loss of cells occur continuously under the influence of systemic ovarian hormone levels. The stratified squamous epithelium of vagina and ectocervix is an important battlefield in defense against pathogenic infections. The most apical layers of the ectocervical epithelium are permeable to immunoglobulins due to absence of classical cell-cell adhesions. Instead, cells in the suprabasal and basal epithelial layers form an exclusionary barrier with high densities of tight junctions, adherens junctions and desmosomes (Blaskewicz et al., 2011). Hence, in ectocervical epithelium the mechanical barrier of basal/suprabasal layers in combination with the permeable apical layers containing large-molecule immunomediators constitutes the first line of defense against sexually-transmitted infections for the entire female reproductive system.

Squamocolumnar Junction (SCJ)

Simple columnar epithelium of endocervix and stratified squamous epithelium of ectocervix meet within the cervical canal. The boundary is called the squamocolumnar junction (SCJ) (Fig. 1). The boundary can be highlighted by markers for columnar and squamous epithelial cells. For example, cytokeratin 14/KRT14, a marker for squamous epithelium, is expressed in ectocervical epithelium but not in the mucinous epithelium of endocervix (Fig. 2A and B). In mice and rats, SCJ is located at the boundary between simple columnar uterine epithelium and stratified squamous ectocervical epithelium as these species lack an endocervix with mucinous glands.

Formation of SCJ

During development, the Müllerian ducts (MDs) undergo a dynamic transformation from simple tubes consisting of homogeneous epithelium and mesenchyme into distinct organs, namely the oviduct, uterus, cervix and vagina (Kurita, 2010). There is no epithelial boundary in the MD. The uniform MD epithelium (MDE) then differentiates into diverse cell types with unique functions in each organ. The key role of mesenchyme in the cell fate specification of MD derived organs was first demonstrated by Cunha in a classic tissue recombination study (Cunha, 1976). In the experiment, epithelial (E) and mesenchymal (M) tissues from the uterus (Ut) and ectocervix/vagina (Vg) of newborn mice were recombined, grown in adult female syngeneic mice as kidney transplants, and the epithelial morphology was analyzed one month later (Fig. 3). A series of studies utilizing this technique established that the organ-specific epithelial cell fate of the uterus and ectocervix/vagina is dictated by the underlying mesenchyme. This epithelial specification occurs within a specific developmental time window. Accordingly, the original position of the SCJ is determined by the differences in the inductive activity of underlying mesenchymal cells of endocervix and ectocervix.

Molecular mechanism of ectocervical differentiation

In the lower MD, the expression of Δ Np63, a N-terminal truncated isoform of p63 transcription factor (TP63, Fig. 2C), determines the cell fate of MDE to become squamous ectocervical/vaginal epithelium or columnar endocervical/uterine epithelium. The mesenchyme of ectocervix and vagina induces the expression of Δ Np63 in MDE by secreting paracrine factors, namely bone morphogenetic protein 4 (BMP4), activin A, fibroblast growth factor 7 (FGF7) and FGF10 (Terakawa et al., 2016) (Fig. 4). In MDE, FGF signaling is transduced by FGFR2IIIb/MAPK/ERK pathway, and BMP4 signaling is transduced by the canonical SMAD pathway. Activin A signaling is mediated by the expression of runt related transcription factor 1 (RUNX1), but molecular mechanism underlying induction of RUNX1 by activin A is unclear. While FGF, BMP and activin signaling pathways are essential for the activation of Δ Np63 in MDE, maintenance of Δ Np63 expression in ectocervical epithelium is independent of all three mesenchymal factors. Thus, the SCJ is maintained in the cervical epithelium independent of external signals.

Changes in the SCJ

In infancy and childhood, the SCJ locates at its original position, just above the external os, but it moves as influenced by hormones when ovaries become active. Ectropion or ectopy of endocervix is defined as the presence of endocervical epithelium on the ectocervix, below the external os. Endocervical ectropion is a physiological condition common for women of reproductive age. The degree of ectopy changes over the time and varies by individual. In some extreme cases, the columnar epithelium extends into the vaginal fornix. During menopause, the process of ectropion reverses, and the SCJ inverts to lie above the external os.

Transformation zone

The term "transformation zone" is sometimes used interchangeably with the SCJ, but it often refers to the wider area within which the SCJ moves. The SCJ is more specific to the boundary of epithelium, whereas the transformation zone includes underlying stroma as well. The transformation zone is clinically important because ~95% of cervical squamous cell carcinoma occur in this region.

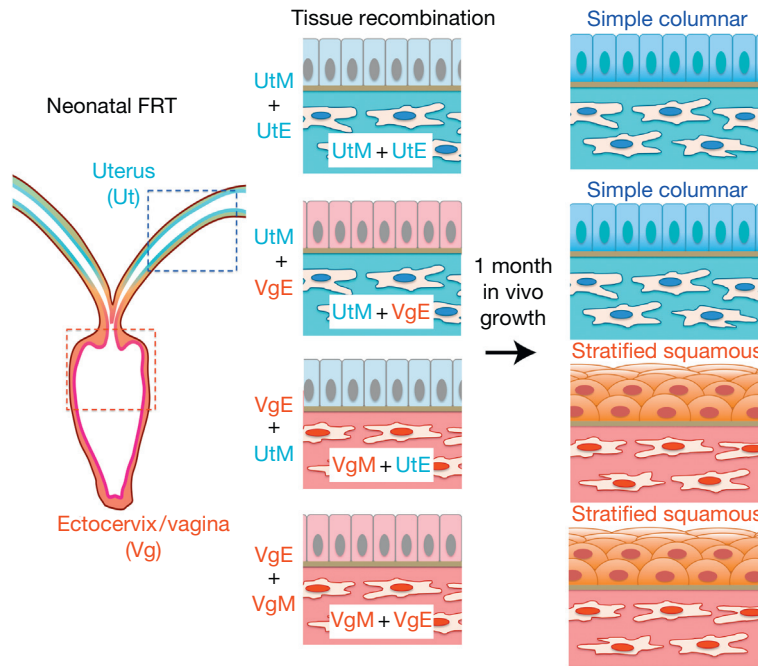


Fig. 3 Scheme of Tissue Recombination Experiment.

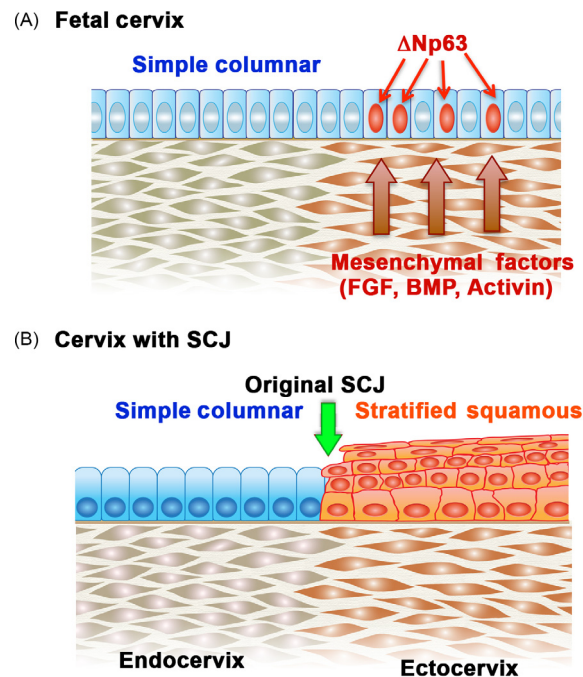


Fig. 4 Formation of SCJ in developing cervix.

Metaplastic Change

Endocervical squamous metaplasia

Squamous metaplasia of endocervix is defined by a presence of islands of squamous cells above the SCJ. It is a common physiological condition believed to be associated with the hormonal changes that occur during puberty in young girls. Although squamous metaplasia is often described as the transformation of columnar endocervical epithelium to squamous ectocervical-like epithelium, there is no experimental evidence for the conversion of epithelial cell type in the pathogenesis of endocervical squamous metaplasia. There is no risk of malignant transformation for squamous metaplasia. Nevertheless, the metaplastic change within the endocervix may increase the risk for human papilloma virus infection (Hwang et al., 2012), which is a risk factor for cervical cancer.

Ectocervical/vaginal adenosis

Ectocervical adenosis is a benign epithelial lesion defined as development of glandular (columnar) epithelium within the stratified squamous epithelium of the ectocervix (below the SCJ). Ectocervical adenosis often occurs in young women, but it usually disappears by menopause as squamous epithelium replaces adenotic cells. Since it is mostly asymptomatic, the accurate prevalence is unknown. There are three major epithelial histopathologies, endocervical, endometrial and tubal types. The condition is considered congenital, as it has been observed in fetuses and infants. Studies with genetically engineered mouse models demonstrated that ectocervical/vaginal adenosis occurs due to faulty epithelial cell fate decision, supporting the congenital nature of this condition. Its pathogenesis usually involves exposure to diethylstilbestrol or other estrogens (Laronda et al., 2012). However, idiopathic cases in women who had no history of in utero hormone exposure have also been reported. The incidence of ectocervical/vaginal adenosis in women who were exposed to diethylstilbestrol in utero (DES daughters) significantly varies between reports utilizing different detection methods (e.g. colposcopy versus histopathology). For instance, routine cytology screening detected vaginal adenosis in only a quarter of patients in which biopsy histology identified the presence of a lesion (Robboy et al., 1976). Some suspect de novo formation of adenosis in ectocervix and vagina of adult women. However, given the low detection sensitivity of routine colposcopy and cytology screenings for adenosis, the adenosis cases that appear to be de novo are likely due to an increased visibility of originally hidden small adenosis lesions by a reactive change to hormone treatments. There is no experimental data directly indicating if and how adenosis transforms to adenocarcinoma. The risk of adenocarcinoma is extremely small in women who have adenosis but no history of diethylstilbestrol exposure. Nevertheless, some doctors recommend careful screening and follow-up for women with adenosis.

Cervical Wall

The cervical wall consists of subepithelial fibrous stroma and smooth muscle. The concentration of smooth muscle cells in cervical stroma diminishes from the upper towards the lower part, where smooth muscle cells are scattered, forming very few bundles. Based on the morphology and distribution, it has been long believed that the cervical smooth muscle is non-functioning. However, a recent study demonstrated that the stromal wall around the internal os contains 50–60% smooth muscle cells that are circumferentially organized in the periphery of the cervical wall, suggesting that smooth muscle cells contribute to the tensile strength and firmness in the upper portion of the cervix (Vink et al., 2016). In contrast, the lower cervix around the external os contains only ~10% of smooth muscle cells randomly scattered in the stroma. Thus, the firmness of the lower cervix is primarily due to the rigid extracellular matrix (ECM) that is rich in collagen and elastic fibers. Fibrillar type I and III collagens are the major constituents of the ECM in cervix. Particularly, Type I collagen is the most abundant protein in cervical stroma. The circumferentially arranged collagen fibers give the cervix mechanical strength to withstand forces encountered in pregnancy. The cervical stroma undergoes dynamic remodeling involving rearrangement of collagen fibers during pregnancy.

Hormone action

The function of hormones in the human cervix is primarily assessed by the correlation between hormone levels and cervical functions. However, such approach does not distinguish the direct and indirect actions of different hormones that are present in the system at the time of analysis. Since manipulation of systemic hormone levels beyond the administration of hormone-containing medications is unethical, our knowledge of hormone actions on the cervix is mostly derived from studies with animal models. Particularly, genetically engineered mouse models have contributed significantly to our understanding. It should be noted that the hormone actions in model animals may not be identical to those in women, given the fundamental difference in the duration of reproductive cycle and gestation as well as anatomy of the cervix.

Hormone Actions on Endocervix

In pregnant women, endocervical glands increase in mass as endocervical epithelial cells proliferate. While the observation indicates the growth of endocervical epithelium is under the control of systemic hormones, the hormones that drive the proliferation of endocervical epithelial cells has not been determined. The absence of equivalent tissues between rodents and humans is one of many factors that impede the mechanistic studies of hormonal control on endocervical function. Cellular and biochemical changes in human endocervix during the menstrual cycle remain undetermined.

Hormone Actions on Ectocervix

In human ectocervix, the epithelial proliferation rate is highest in the parabasal layer. The proliferation rate peaks at luteal phase in menstrual cycle and during pregnancy (Konishi et al., 1991), suggesting the proliferation of ectocervical epithelium is stimulated by 17 β -estradiol and progesterone. In human and mouse ectocervices, the receptors for estrogen (ESR1) and progestin (PGR) are expressed in both epithelial and stromal cells. Mouse genetic studies demonstrated that the proliferation of stratified squamous epithelium in the cervix and vagina is regulated by estrogen through ESR1 in stromal cells (Buchanan et al., 1998). In contrast, progesterone stimulates proliferation of ectocervical epithelium through the epithelial PGR (Mehta et al., 2016). Since epithelial

ESR1 is required for the expression of PGR in the epithelium, ESR1 in both epithelial and stromal cells is required for the full growth response of ectocervical epithelium to estrogen and progestin. Similarly, differentiation and apoptosis of ectocervical/vaginal epithelial cells are regulated by estrogen and progestin through ESR1 and PGR in both epithelial and stromal cells.

Pregnancy Associated Changes

The cervix must undergo a dramatic process of remodeling to allow for passage of the fetus (Timmons et al., 2010). Three phases of prepartum cervical remodeling have been proposed (Word et al., 2007), softening, ripening and dilation. In this section, the molecular aspects of softening and ripening of cervix is described.

Softening

The initial softening phase is a slow, progressive process for reorganization of ECM. In women, the cervix begins to soften as early as 1 month of gestation. Softening of the cervix is characterized with a progressive increased turnover of the ECM including the dispersion and rearrangement of the collagen fibrillar network. At the same time, vascularity increases and leukocytes are recruited to the subepithelial stroma in this phase.

Ripening

Ripening of cervix is the phase of accelerated softening and begins prior to the onset of labor contractions and cervical dilation. In this phase, firmness of cervical ECM is reduced further by a decrease in collagen fiber alignment, degradation of collagen cross-linking and increase in water intercalate among the collagen fibers. These changes are considered as a part of inflammatory reactions, which are characterized as the increase in pro-inflammatory cytokines (e.g. interleukin-8, interleukin-6 and granulocyte colony-stimulating factor) and the influx of granulocytes, such as neutrophils and eosinophils. In addition, macrophages increase in cervical epithelium and stroma at labor. During the process of ripening, the cervix loses gross morphological characteristics such as endocervical glands and smooth muscles. How this morphological transformation occurs during ripening of cervix is not well understood.

Progesterone

The molecular mechanism that regulates the transition from softening to ripening in normal pregnancy is not fully elucidated. The reduced progesterone signal has been implicated in the initiation of the cervical ripening process, based on the decline of systemic progesterone prior to the onset of labor in many species, including rodents and some primates. However, some aspects of cervical ripening in women occur when progesterone concentrations remains at high levels. Thus, it is unclear if the reduced progesterone triggers the cervical ripening in term labor of women. Nevertheless, antiprogestins, such as mifepristone, can have a cervical ripening effect, and progesterone supplementation can reduce the rate of spontaneous preterm birth in women at high risk with a prior spontaneous preterm birth, indicating anti-ripening activity of progesterone.

Prostaglandin E2

Prostaglandin E2 (PGE2), a principal mediator of inflammation, plays a critical role in cervical ripening. Its biosynthesis in the cervix involves conversion of arachidonic acid to prostaglandin H2 (PGH2) by PGH2 synthase (PTGS2) and conversion of PGH2 to PGE2 by prostaglandin E synthase 2 (PTGES2). In preterm cervix, 15-hydroxyprostaglandin dehydrogenase (HPGD) is highly expressed repressing tissue PGE2 levels by converting PGE2 to inactive 15-keto PGE2. When term approaches, up-regulation of PTGS2 and down-regulation of HPGE concomitantly occur, increasing the local concentration of PGE2 in the cervix. PGE2 has been used as an effective medication to induce cervical ripening and labor.

Other ripening agents of cervix

Animal studies suggested that cervical ripening can be induced by other factors including estrogens, relaxin, nitric oxide donors, and hyaluronidase. However, these agents are shown to be ineffective in women, and thus not recommended for cervical ripening agents in labor induction.

References

- Andersch-Bjorkman, Y., Thomsson, K. A., Holmen Larsson, J. M., Ekerhovd, E., & Hansson, G. C. (2007). Large scale identification of proteins, mucins, and their O-glycosylation in the endocervical mucus during the menstrual cycle. *Molecular & Cellular Proteomics*, 6, 708–716.
- Blaskewicz, C. D., Pudney, J., & Anderson, D. J. (2011). Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia. *Biology of Reproduction*, 85, 97–104.
- Buchanan, D. L., Kurita, T., Taylor, J. A., Lubahn, D. B., Cunha, G. R., & Cooke, P. S. (1998). Role of stromal and epithelial estrogen receptors in vaginal epithelial proliferation, stratification, and cornification. *Endocrinology*, 139, 4345–4352.

- Cunha, G. R. (1976). Stromal induction and specification of morphogenesis and cytodifferentiation of the epithelia of the Müllerian ducts and urogenital sinus during development of the uterus and vagina in mice. *The Journal of Experimental Zoology*, 196, 361–370.
- Hwang, L. Y., Ma, Y., Shiboski, S. C., Farhat, S., Jonte, J., & Moscicki, A. B. (2012). Active squamous metaplasia of the cervical epithelium is associated with subsequent acquisition of human papillomavirus 16 infection among healthy young women. *The Journal of Infectious Diseases*, 206, 504–511.
- Konishi, I., Fujii, S., Nonogaki, H., Nanbu, Y., Iwai, T., & Mori, T. (1991). Immunohistochemical analysis of estrogen receptors, progesterone receptors, Ki-67 antigen, and human papillomavirus DNA in normal and neoplastic epithelium of the uterine cervix. *Cancer*, 68, 1340–1350.
- Kurita, T. (2010). Developmental origin of vaginal epithelium. *Differentiation*, 80, 99–105.
- Laronda, M. M., Unno, K., Butler, L. M., & Kurita, T. (2012). The development of cervical and vaginal adenosis as a result of diethylstilbestrol exposure in utero. *Differentiation*, 84, 252–260.
- Martyn, F., McAuliffe, F. M., & Wingfield, M. (2014). The role of the cervix in fertility: Is it time for a reappraisal? *Human Reproduction*, 29, 2092–2098.
- Mehta, F. F., Son, J., Hewitt, S. C., Jang, E., Lydon, J. P., Korach, K. S., & Chung, S. H. (2016). Distinct functions and regulation of epithelial progesterone receptor in the mouse cervix, vagina, and uterus. *Oncotarget*, 7, 17455–17467.
- Robboy, S. J., Friedlander, L. M., Welch, W. R., Keh, P. C., Taft, P. D., Barnes, A. B., Scully, R. E., & Herbst, A. L. (1976). Cytology of 575 young women with prenatal exposure to diethylstilbestrol. *Obstetrics and Gynecology*, 48, 511–515.
- Terakawa, J., Rocchi, A., Serna, V. A., Bottinger, E. P., Graff, J. M., & Kurita, T. (2016). FGFR2IIIb-MAPK activity is required for epithelial cell fate decision in the lower Mullerian duct. *Molecular Endocrinology*, 30, 783–795.
- Timmons, B., Akins, M., & Mahendroo, M. (2010). Cervical remodeling during pregnancy and parturition. *Trends in Endocrinology and Metabolism*, 21, 353–361.
- Vink, J. Y., Qin, S., Brock, C. O., Zork, N. M., Feltovich, H. M., Chen, X., Urie, P., Myers, K. M., Hall, T. J., Wapner, R., Kitajewski, J. K., Shawber, C. J., & Gallos, G. (2016). A new paradigm for the role of smooth muscle cells in the human cervix. *American Journal of Obstetrics and Gynecology*, 215, 478.e1–478.e11.
- Word, R. A., Li, X. H., Hnat, M., & Carrick, K. (2007). Dynamics of cervical remodeling during pregnancy and parturition: Mechanisms and current concepts. *Seminars in Reproductive Medicine*, 25, 69–79.

Further Reading

- Cunha, G. R., Cooke, P. S., & Kurita, T. (2004). Role of stromal-epithelial interactions in hormonal responses. *Archives of Histology and Cytology*, 67, 417–434.
- Gipson, I. K., Spurr-Michaud, S., Moccia, R., Zhan, Q., Toribara, N., Ho, S. B., Gargiulo, A. R., & Hill, J. A., 3rd (1999). MUC4 and MUC5B transcripts are the prevalent mucin messenger ribonucleic acids of the human endocervix. *Biology of Reproduction*, 60, 58–64.
- Goetzl, L. (2014). Methods of cervical ripening and labor induction: Pharmacologic. *Clinical Obstetrics and Gynecology*, 57, 377–390.
- Kobayashi, A., & Behringer, R. R. (2003). Developmental genetics of the female reproductive tract in mammals. *Nature Reviews. Genetics*, 4, 969–980.
- Kurita, T. (2011). Normal and abnormal epithelial differentiation in the female reproductive tract. *Differentiation*, 82, 117–126.
- Kurita, T., Mills, A. A., & Cunha, G. R. (2004). Roles of p63 in the diethylstilbestrol-induced cervicovaginal adenosis. *Development*, 131, 1639–1649.
- Kurita, T., & Nakamura, H. (2008). Embryology of the uterus. In J. D. Aplin, A. T. Fazleabas, S. R. Glasser, & L. C. Giudice (Eds.), *Endometrium* (2nd ed.). London, UK: Informa UK Ltd.
- Laronda, M. M., Unno, K., Ishi, K., Serna, V. A., Butler, L. M., Mills, A. A., Orvis, G. D., Behringer, R. R., Deng, C., Sinha, S., & Kurita, T. (2013). Diethylstilbestrol induces vaginal adenosis by disrupting SMAD/RUNX1-mediated cell fate decision in the Mullerian duct epithelium. *Developmental Biology*, 381, 385–916.
- Martyn, F., McAuliffe, F. M., & Wingfield, M. (2014). The role of the cervix in fertility: Is it time for a reappraisal? *Human Reproduction*, 29, 2092–2208.
- Moghissi, K. S. (1987). Cervical and uterine factors in infertility. *Obstetrics and Gynecology Clinics of North America*, 14, 887–904.
- Mossman, H. W. (1977). Comparative anatomy. In R. M. Wynn (Ed.), *Biology of The Uterus*. New York: Plenum Press.
- Mukonoweshuro, P., Oriwolo, A., & Smith, M. (2005). Audit of the histological definition of cervical transformation zone. *Journal of Clinical Pathology*, 58, 671.
- Mullen, R. D., & Behringer, R. R. (2014). Molecular genetics of Mullerian duct formation, regression and differentiation. *Sexual Development*, 8, 281–296.
- Reich, O., & Fritsch, H. (2014). The developmental origin of cervical and vaginal epithelium and their clinical consequences: A systematic review. *Journal of Lower Genital Tract Disease*, 18, 358–360.

Relevant Websites

- <http://www.mayoclinic.org/tests-procedures/cervical-mucus-method/basics/definition/prc-20013005>.
- <http://www.proteinatlas.org/learn/dictionary/normal/cervix,+uterine/anatomy+1>.
- <https://histologyblog.com/2013/01/20/histoquarterly-cervix>.

Vagina

Paweł Łaniewski and Melissa Herbst-Kralovetz, University of Arizona, Phoenix, AZ, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

16S rRNA sequencing A laboratory method used to identify and compare bacteria present within given samples.

Adventitia The outermost connective tissue covering of any organ.

Asymptomatic Exhibiting no symptoms of disease.

Commensal An organism that benefits from living in a close relationship with other species without harming it.

Competitive exclusion An ability of microorganism to effectively compete for resources in the microenvironment with other microorganisms.

Copulative Relating to sexual intercourse.

Dysbiosis Microbial imbalance resulted from a depletion of healthy bacteria and an overgrowth of harmful bacteria.

Dysuria Pain, discomfort or burning during urination.

Homeostasis A proper balance of host immune response under normal physiological conditions.

Dyspareunia Pain occurring just before, during or after sexual intercourse due to medical or psychological reasons.

Lamina propria A layer of connective tissue that lies under the epithelium.

Microbiome A combined genetic material of microorganisms inhabiting particular microenvironment.

Microbiota A community of microorganisms (bacteria, archaea, fungi and viruses) inhabiting particular microenvironment (e.g., body site).

Mucosa A mucous membrane consisted of the epithelium and lamina propria that lies various cavities in the body.

Parturition Childbirth.

Premenarche Occurring in the period before the first menstrual period (menarche).

Postpartum Occurring in the period following childbirth.

Pyknotic Relating to pyknosis (condensation and reduction in size of a cell or its nucleus).

Stratified squamous epithelium A tissue consisting of several layers of epithelial cells in which the surface cells are flattened (squamous).

Introduction

The vagina is a component of the lower female reproductive tract (FRT), which is located between the cervix of the uterus and external genitalia, primarily the vulva. The external opening of vagina may be surrounded and partially closed by a hymen. The vagina functions as a female copulative organ and is a part of the birth channel during parturition. It also functions as an excretory canal during menses. The vaginal wall contains abundant connective tissue and two layers of smooth muscle (an inner circular and an outer longitudinal layer). The adventitia, the outermost connective tissue, is rich in elastic fibers and contains nerves and blood vessels that blend with the connective tissue of the pelvis. The adventitia fixes the vagina to surrounding tissue structures. The vaginal mucosa is lined with nonkeratinized stratified squamous epithelium, supported by thick lamina propria. The vaginal epithelial cells produce glycogen, which is both housed in the epithelial cells and secreted into the vaginal lumen. Glycogen supports the growth of commensal vaginal bacteria, such as lactobacilli, which acidify the mucosa and protect it against infection with invading pathogens. When the vaginal microbiota are disrupted, dysbiosis may occur. The outcomes of dysbiosis are detailed herein. The lining of vaginal cavity is also altered by sex hormones during the menstrual cycle and throughout a woman's lifespan (Muhleisen and Herbst-Kralovetz, 2016).

Gross Anatomy

The vagina is a fibromuscular, collapsed tube, when it is in a relaxed state. The lateral walls of the vagina are more rigid than anterior and posterior walls. Because of this, the vagina has a H-shaped cross section in the central portion. The length and shape of the vagina varies between women with an average length of 6.3 cm (2.5 in) (Barnhart et al., 2006). The vagina is located within the female pelvis, anterior to the rectum and the anus, posterior to the urinary bladder and the urethra and laterally to the ureter and uterine artery. It lies at a 45 degree angle in relation to the perineum, and 90 degree angle in relation to the uterus. The vagina is attached to the pelvic walls by ligaments and endopelvic fascia, a system of connective tissue. Protrusion of the cervix into vagina forms vaginal fornices, that is, recesses of vaginal vault, divided into two lateral fornices; anterior fornix and posterior fornix. The

posterior fornix is the deeper recess and is adjacent to the rectouterine pouch, whereas the smaller anterior fornix adjoins the vesico-uterine pouch (Standing, 2008).

Functional Anatomy

The vagina functions as a female copulative organ. During intercourse, this organ assists in receiving and transporting semen to the uterus. The vagina also provides a birth canal for the delivery of a fetus from the uterus and serves as a channel for menstrual fluid and tissue to leave the body. These physiological functions of the vagina require both lubrication and an elasticity to allow for expansion. The vagina does not have secretory glands, instead most of the vaginal lubrication comes from transudation of plasma mixed with cervical gland secretions (Dutta, 2014). Lubrication significantly increases with sexual arousal due to vascular engorgement of the vagina. The vagina is also lubricated, however diminutively, by two Bartholin's glands located in the vestibule on either side. During sexual arousal and intercourse, the vagina expands in length and width, while the cervix retracts, the elastic walls of the vagina stretch and contract with support of pelvic muscles, specifically the pubococcygeus muscle. The elasticity of the vagina is related to the large number of elastic fibers in the connective tissue of the wall and the extensive venous plexus. These features also allow the vagina to greatly increase its diameter to accommodate a fetus during parturition. The vaginal mucosa also displays distinct morphological structures, including microfolds (vaginal rugae), which provide the vagina with additional surface area to expand and these structures also serve to interlock the cell layers and hold mucus in place (Fedele et al., 2006; Ludwig and Metzger, 1976). The vaginal stratified squamous epithelium also functions as a physical barrier, which protects the body against invading pathogens. The vaginal mucosa tolerates commensal microbiota, which also play an active role in maintaining homeostasis and host defense. The growth of these microbes is supported by glycogen produced and secreted by vaginal epithelial cells. The commensal lactobacilli utilize glycogen and produce lactic acid, which decreases the vaginal pH (O'Hanlon et al., 2013). The acidic microenvironment coupled with the antimicrobial compounds produced by lactobacilli protects the vaginal mucosa against microbial insults.

Vascular Supply, Lymphatics and Innervation

Blood is supplied to the vagina by the vaginal and uterine arteries, which are branches of the internal iliac artery. The vaginal arteries anastomose with a branch of uterine artery to form azygos arteries of the vagina, which run longitudinally on the anterior and posterior of the vagina. The vaginal wall is highly vascularized with both a venous and arterial plexus. Two main veins drain blood from the vagina, located on both sides. Ultimately blood is drained into the internal iliac veins (Standing, 2008).

Lymphatic drainage occurs through the iliac and superficial inguinal lymph nodes. The upper vagina drains into the external iliac lymph nodes. The posterior vagina drains into inferior gluteal, sacral and anorectal lymph nodes, then ultimately in the internal iliac nodes. The distal vagina drains into superficial inguinal nodes (Standing, 2008).

The vagina receives innervation primarily from the sympathetic and parasympathetic nerves derived from the uterovaginal nerve plexus and the pudendal nerve. The inferior fibers from the uterovaginal plexus, which lies on each supravaginal sides of the cervix, supply the upper part of the vagina. These fibers are derived from the inferior hypogastric plexus and the pelvic splanchnic nerves (from sacral spinal cord levels S2–4) (Standing, 2008). The lower vagina innervation is carried through the pudendal nerve, which carries sympathetic, but not parasympathetic fibers. These autonomic efferent nerves supply the vaginal smooth muscles and vascular muscles, penetrating the lamina propria and epithelium. Somatic nerve supply is mainly to the lower portion of the vagina and is carried by the pudendal nerve to the sacral spinal cord (Standing, 2008).

Hormonal Regulation

In premenopausal women, the vaginal epithelium is under the influence of hormonal changes during the menstrual cycle. During the proliferative phase of the menstrual cycle, high levels of estrogen increase the thickness of the epithelium, glycogen production and mucus secretion. The vaginal epithelium thickens through estrogen-stimulated epithelial proliferation, stratification, and cornification. The apical epithelial layers consist of large, polygonal-shaped cells that are distinctly flat (Fig. 1). These superficial cells are not nucleated or their nuclei are pyknotic. Moreover, the multilayered epithelial cells accumulate glycogen in the cytoplasm. High levels of glycogen promote the growth of lactobacilli and production of lactic acid, which protects the vaginal mucosa against infections (Nunn and Forney, 2016). Due to the production of lactic acid, the pH level decreases and is directly correlated with free glycogen levels in vaginal secretions (Mirmonsef et al., 2016). During the secretory phase of the menstrual cycle, when estrogen levels are low, the epithelial thickness decreases due to continuous epithelial shedding (desquamation). Moreover, glycogen is less abundant within cells, which may result in increased vaginal pH.

Hormones also influence the vaginal mucosa through a woman's lifespan (Table 1). Prior to puberty women produce little estrogen, which results in low levels of glycogen and high vaginal pH. The vaginal mucosa prior to puberty in premenarchal women is composed of a thin, stratified squamous epithelium with anucleated cells on the top that are covered with a thin layer of mucus. In reproductive age premenopausal women with high levels of estrogen, glycogen deposits in the epithelial cells and free glycogen are increased and available for lactobacilli to thrive. During the premenopausal stage the vaginal epithelium is thickest and covered by a thick layer of mucus. During menopause, the levels of estrogen drops, decreasing glycogen and the vaginal epithelium

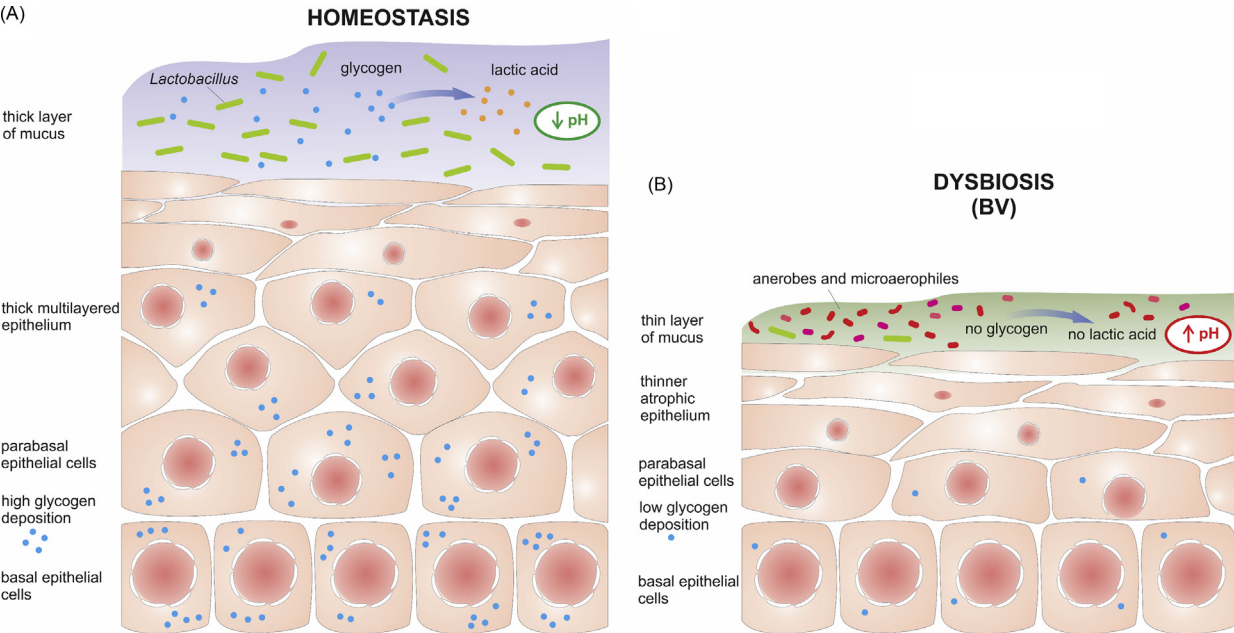


Fig. 1 A *Lactobacillus*-dominant vaginal microbiome promotes homeostasis, whereas a diverse non-*Lactobacillus*-dominant vaginal microbiome leads to dysbiosis. (A) High levels of glycogen, which is produced and secreted by the thick multilayered vaginal squamous epithelium, promotes lactobacilli growth. Lactobacilli produce lactic acid, which acidify the vaginal microenvironment, protects against infection, and maintain homeostasis. (B) Low levels of available glycogen can lead to depletion of lactobacilli and subsequent overgrowth of anaerobic and microaerophilic bacteria resulting in bacterial vaginosis (BV). The epithelium becomes thinner and atrophic. Lack of lactic acid results in increase in vaginal pH, and higher vulnerability to infections.

Table 1 Changes in the vaginal microenvironment during woman’s lifespan

Stage of life	Prior puberty/premenarchal	Reproductive age	Post menopause
Vaginal epithelium	Thin, dry	Multilayered, lubricated	Thin, dry/atrophic
Mucus	Thin layer	Thick layer	Thin layer
Estrogen level	Low	High	Low
Glycogen deposition	Low	High	Low
Microbiome	Diverse non- <i>Lactobacillus</i> dominant	<i>Lactobacillus</i> -dominant	Diverse non- <i>Lactobacillus</i> dominant
Vaginal pH	High (>4.5)	Low (<4.5)	High (>4.5)

resembles the prepuberty stage, with less layers and a thinner mucus layer (Muhleisen and Herbst-Kralovetz, 2016). The lack of estrogen and depletion of commensal lactobacilli following menopause impacts not only the vaginal epithelium, but also other layers of the vaginal tissue, including the loss of elasticity and rugae and ultimately leads to thinning of the vaginal walls. These microanatomical changes often culminate in vaginal symptoms associated with menopause (e.g., vulvovaginal atrophy, vaginal dryness), and can be restored by hormone replacement therapy.

Vaginal Microbiome

The vaginal microbiota (VMB) are defined as a community of commensal, symbiotic and pathogenic microorganisms that colonize the vagina. In the majority of healthy reproductive age women, the VMB is dominated by lactobacilli; although these bacterial communities can vary considerably between individuals and over time. Use of 16S rRNA sequencing revealed that the VMB can be classified according to the dominant bacterial species present. Five different community state types (CST) were proposed by Ravel et al. to characterize the VMB composition (Ravel et al., 2011). Four of these community types are dominated by *Lactobacillus* species. The most common CSTs, CST I, and CST III, are composed predominantly by *Lactobacillus crispatus* and *Lactobacillus iners*, respectively. VMB can also be dominated by *Lactobacillus gasseri* (CST II) and *Lactobacillus jensenii* (CST V). Women with these *Lactobacillus*-dominant VMB also exhibit low vaginal pH (typically <4.5). CST IV lacks a high proportion of lactobacilli and is defined as the diversity state dominated by anaerobic and microaerophilic bacteria and is correlated with higher vaginal pH

(>4.5). This heterogeneous CST can be divided into two sub-types: CST IV-A, which is characterized by a modest proportion of lactobacilli, along with low proportion of *Anaerococcus*, *Corynebacterium*, *Finegoldia*, or *Streptococcus*, and CST IV-B with high proportion of *Atopobium*, in addition to *Prevotella*, *Parvimonas*, *Sneathia*, *Gardnerella*, *Mobiluncus*, *Peptoniphilus* and other taxa (Gajer et al., 2012). Notably, several bacteria present in the CST IV-B are associated with bacterial vaginosis (BV). Interestingly, significant differences in the VMB composition between women of different ethnic groups are observed. The *Lactobacillus*-dominant VMB are widely prevalent in Asian and White/Caucasian women (80%–90%), however only 60%–70% of African American or Hispanic women have *Lactobacillus*-dominant VMB. The higher frequency of CST IV/BV in African American and Hispanic women may contribute to health disparities. However, it is unknown whether the differences in the VMB composition and rates of BV are related to socioeconomic, behavioral, environmental or genetic factors and this is an active area of investigation.

Not all VMB compositions are beneficial for the host. The diversity state type (CST IV), characterized by a depletion of lactobacilli and increase in anaerobic and microaerophilic bacteria, has been associated with adverse reproductive and obstetric health outcomes, including increased risk of acquiring sexually transmitted infections, pelvic inflammatory disease, endometritis, preterm birth, and spontaneous abortions (Martin and Marrazzo, 2016), whereas *Lactobacillus*-dominant VMB are associated with homeostasis and vaginal health (Fig. 1). Lactobacilli benefit the host by producing lactic acid, which acidifies the vaginal microenvironment and provides protection against genital infections. Furthermore, lactobacilli inhibit colonization of invading pathogens through competitive exclusion (i.e., an ability to effectively compete for resources in the vaginal microenvironment with other microorganisms). The protective effect of *Lactobacillus*-dominant VMB have also been attributed to hydrogen peroxide; however, production of this antimicrobial compound by lactobacilli in hypoxic conditions of the vagina remains controversial (O'Hanlon et al., 2013). Other metabolites (e.g., biogenic amines) produced by specific vaginal bacterial species can also impact the VMB structure, facilitating growth of anaerobes and microaerophiles and could further impact the local microenvironment (Nelson et al., 2015).

The VMB composition often changes over relatively short periods of time. Age, menstruation, estrogen level, smoking, intercourse, hygiene habits and other practices have been shown to significantly impact the VMB composition (Hickey et al., 2012). During the menstrual cycle the VMB structure is more stable when estrogen levels are high. Similarly, pregnant women, which also exhibit elevated levels of estrogen, have more stable VMB when compared to nonpregnant women (Romero et al., 2014). Furthermore, the VMB of pregnant women are more commonly dominated by *L. crispatus* and *L. iners*, and the diversity (non-*Lactobacillus*-dominant) state is rarely observed. The postpartum decrease in estrogen often results in less stability of the VMB structure (Nunn and Forney, 2016). Low levels of circulating estrogen prior to puberty and following menopause also results in the depletion of lactobacilli and the growth of diverse anaerobic and microaerophilic bacteria (Muhleisen and Herbst-Kralovetz, 2016).

Estrogen impacts the VMB composition through increasing the glycogen content in the vaginal epithelial cells. Glycogen, which is a storage form of glucose, has been shown to promote growth of lactobacilli (Mirmonsef et al., 2016). It is poorly understood how glycogen becomes available for lactobacilli in the vaginal lumen. Interestingly, vaginal lactobacilli are not capable of metabolizing glycogen, although these bacteria can utilize maltose and other glycogen breakdown products. Vaginal secretions contain α -amylase, an enzyme, which converts glycogen to maltose, maltotriose, maltopentaose, and maltodextrins that support lactobacilli growth (Spear et al., 2014). The origin of α -amylase in vaginal secretions remains unknown. However, it is hypothesized that α -amylase may be synthesized by other bacterial species co-inhabiting in the vagina with lactobacilli. In sum, both high levels of free glycogen and α -amylase are associated with *Lactobacillus*-dominant VMB.

Overall, *Lactobacillus*-dominant VMB are associated with vaginal health, and the disruption of the VMB appears to be a factor in the pathogenesis of several infectious diseases, including vaginitis and sexually transmitted infections. Host-vaginal microbiota interactions impact the local microenvironment through the production of soluble immune mediators (Doerflinger et al., 2014); these mechanisms influence outcomes related to vaginal health.

Disease

Primary lesions of the vagina, which are defined as a neoplasia that arises solely from the vagina, are relatively rare (1%–3% of gynecological malignancies) (Kumar et al., 2010). Most of these tumors (80%–85%) are squamous cell carcinomas, which are associated with high risk human papilloma virus (HPV) infections, similar to cervical cancer. Prior carcinomas of the cervix or vulva are also the greatest risk factors of vaginal squamous cell carcinoma. The Food and Drug Administration recommends HPV vaccination for girls and women before they become sexually active to prevent not only cervical cancer, but also vaginal cancer. Vaginal squamous cell carcinoma arises from the vaginal intraepithelial neoplasia (VAIN), a premalignant lesion characterized by the squamous cell dysplasia in the lining of vagina without invasion to the deeper tissue. This condition is generally asymptomatic; however, it can be detected by the presence of abnormal cells in the Papanicolaou test (Pap smear). This invasive cancer most often occurs in the posterior wall of the upper third of the vagina and metastasizes to the inguinal and iliac nodes. Adenocarcinoma, which arises from glandular cells of the vagina, is the second most common subtype (15%) of vaginal cancer. Other types include vaginal melanoma and two tumors mostly found in infants and children, that is, vaginal germ cell tumor, sarcoma botryoides. However, the most common vaginal malignancy is not the primary carcinoma, but metastatic squamous cell carcinoma from the cervix. Benign tumors of the vagina are also rare and include fibroepithelial (stromal) polyps, leiomyomas (smooth muscle neoplasia), and several cystic tumors (Kumar et al., 2010).

The majority of vaginal diseases are of microbial origin. Vaginitis is an inflammation of the vagina and possibly the vulva, which can result in itching or irritation, unusual discharge and pain or discomfort during intercourse (dyspareunia); however, some women exhibit no symptoms. Vaginitis is primarily caused by a disruption of normal vaginal microbiota (dysbiosis) or an infection with pathogenic bacteria, viruses or protozoan parasites. Other causes of vaginitis include the decrease in estrogen levels, for example, during menopause, breastfeeding or particular treatments that intend to decrease estrogen. The most common types of vaginitis include candidiasis, bacterial vaginosis (BV), aerobic vaginosis (AV), trichomoniasis, and atrophic vaginitis (Table 2). These entities can also co-occur, resulting in clinical and therapeutic implications.

Vulvovaginal candidiasis (VVC) is a fungal infection, which is due to an excessive growth of the yeast *Candida* in the vagina. This fungus resides in small amounts in the vagina and other parts of human body and is usually not associated with any signs of disease. The causes of *Candida* overgrowth are still not fully understood. The risk factors that have been associated with VVC include antibiotic use, diabetes, high levels of estrogen and genetic predispositions (Achkar and Fries, 2010). *Candida albicans* is the most common cause (76%–89%) of candidiasis in the vagina. However, infections with other *Candida* species (*Candida glabrata*, *Candida tropicalis*, *Candida krusei*, and *Candida parapsilosis*) have increased. These non-*Candida albicans* *Candida* (NCAC) species are now classified as emerging pathogens. The symptoms of VVC are itching, burning during urination (dysuria), dyspareunia and a thick, white, curdy, odorless vaginal discharge. Vulvovaginal inflammation (erythema and edema) can also occur, which results from leukocyte infiltration (Table 2).

BV is the most common vaginal infection and is characterized as a depletion of commensal lactobacilli and an overgrowth of anaerobic and microaerophilic bacteria, including *Gardnerella*, *Atopobium*, *Prevotella*, *Leptotrichia*, *Sneathia*, *Mobiluncus*, *Megasphaera*, and BV-associated bacteria (BVAB) 1–3 (Onderdonk et al., 2016). Importantly, BV has a high recurrence rate and is difficult to treat. Epidemiological studies strongly suggest that BV is sexually transmitted (Muzny and Schwebke, 2016). BV-associated bacteria can be found in male penile skin, urethra, and semen. Studies on women that have sex with women also showed that BV is associated with increased number of female partners or having a female partner with BV. It is still controversial whether new or recurring cases of BV are caused by a single species, for example, *Gardnerella vaginalis*, or a polymicrobial consortium of BV-associated bacteria (Muzny and Schwebke, 2016). Importantly, BV is associated with serious reproductive and obstetric sequelae, including preterm birth, spontaneous abortions, pelvic inflammatory disease, endometritis, increased risk of acquiring HIV and other sexually transmitted infections (Martin and Marrazzo, 2016). The symptoms of BV include a white or gray vaginal discharge with fishy odor and dysuria. Itching and vulvovaginal inflammation (irritation, redness, and swelling resulting from leukocyte infiltration) are uncommon; BV can be also asymptomatic. In clinical practice, BV is generally diagnosed using the Amsel criteria. Demonstrating three of the following four characteristics is considered to be necessary to diagnose BV: (1) high pH of vaginal fluid (>4.5), (2) thin homogenous vaginal discharge, (3) a fishy volatile amine odor when the discharge is treated with a potassium hydroxide solution (whiff test) and (4) a presence of squamous epithelial cells coated with bacteria (clue cells) in wet mount (Table 2). Alternatively, the Nugent score is used mostly in the research setting and is calculated by assessing the presence of large gram-positive rods (bacilli, *Lactobacillus* morphotype, scored 0–4), small gram-variable rods (coccobacilli, *Gardnerella* morphotype, scored 0–4) and curved gram-variable rods (*Mobiluncus* morphotype, scored 0–2) in vaginal secretion using Gram stain and microscopy. A total score between 0 and 3 is considered negative for BV, whereas score 4–6 is intermediate and score 7–10 is defined as BV (Nugent et al., 1991).

AV is characterized by a decrease in commensal lactobacilli and presence of dysbiotic VMB containing aerobic bacteria of intestinal origin, including *Escherichia coli*, staphylococci, streptococci and enterococci (Donders et al., 2017). These changes in VMB result in an increase in vaginal pH. Women with AV, in contrast to women with BV, exhibit increased levels of vaginal inflammation, characterized by leukocyte recruitment. Women with AV also present with a thin, reddish vaginal mucosa with possible erosions and ulceration, a thick mucoid discharge of yellow to green color and foul smell, and dyspareunia (Table 2). AV can be diagnosed using wet mount microscopy. Compared to wet mounts from women with BV, microscopic slides obtained from patients with AV show presence of leukocytes and immature or parabasal epithelial cells (small round epithelial cells with high nuclear to cytoplasmic ratio). Moreover, clue cells are not observed. The AV scoring system includes assessment of number of lactobacilli and coccoid bacteria, leukocytes (including toxic leukocytes showing lysosomal activity) and parabasal epithelial cells. Total scores represent, respectively, no AV (0–2), light (3–4), moderate (5–6) and severe AV (7–10) (Donders et al., 2017). An alternative diagnostic test for AV was proposed to be based on the activity of five enzymes in the vaginal secretions: hydrogen peroxidase, leukocyte esterase, sialidase, β -glucuronidase and coagulase, which indicate inflammation and the presence of particular bacterial species associated with AV or BV (Donders et al., 2017).

Trichomoniasis is a sexually transmitted infection (STI) caused by a protozoan parasite, *Trichomonas vaginalis*. The parasite preferentially infects the squamous vaginal epithelium; however, it can also reach the urethra and the endocervix (Menezes et al., 2016). Most of the infected women (70%) do not exhibit any symptoms. If symptoms occur, they include itching and soreness of the vagina and vulva, redness, dysuria, dyspareunia, discomfort in the lower abdomen and thin clear, white, yellowish or greenish, discharge with fishy smell (Table 2). Moreover, women with trichomoniasis may exhibit erythema and edema with petechial spots on the vaginal and cervical mucosa (strawberry cervix). Notably, trichomoniasis also increases the risk of HIV acquisition and is associated with adverse pregnancy outcomes (Menezes et al., 2016). Other sexually transmitted viral and bacterial agents that can cause vaginitis include human papilloma virus (HPV), herpes simplex virus (HSV), *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*.

Atrophic vaginitis, also known as vaginal atrophy or vulvovaginal atrophy (VAA), is an inflammation of the vagina and the vulva, which results from a decrease in circulating estrogen levels. In postmenopausal women the low levels of estrogen cause

Table 2 The most common types of vaginitis

Diagnosis	Etiology	Associated species	Inflammation (leukocyte infiltration)	Vaginal discharge	Vaginal pH
Normal	Healthy <i>Lactobacillus</i> -dominant VMB	<i>Lactobacillus crispatus</i> <i>Lactobacillus gasseri</i> <i>Lactobacillus jensenii</i> <i>Lactobacillus iners</i>	No	No	< 4.5 (normal)
Vulvovaginal candidiasis	Yeast infection	<i>Candida albicans</i> <i>Candida glabrata</i> <i>Candida tropicalis</i> <i>Candida krusei</i> <i>Candida parapsilosis</i>	Yes	Thick, white, curdy without odor	< 4.5 (normal)
Bacterial vaginosis	Overgrowth of anaerobic and microaerophilic bacteria	<i>Gardnerella vaginalis</i> <i>Atopobium vaginae</i> <i>Prevotella</i> <i>Leptotrichia</i> <i>Sneathia</i> <i>Mobiluncus</i> <i>Megasphaera</i> BVAB 1–3	No	Thin, homogenous, white or gray with fishy odor	> 4.5 (increased)
Aerobic vaginitis	Overgrowth of aerobic bacteria of intestinal origin	<i>Escherichia coli</i> <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i> <i>Streptococcus agalactiae</i> <i>Enterococcus faecalis</i>	Yes	Thick, mucoid, yellow to green with foul odor	> 4.5 (increased)
Trichomoniasis	Parasitic infection	<i>Trichomonas vaginalis</i>	No/Yes ^a	Thin, clear, white, yellowish or greenish with fishy odor	> 4.5 (increased)
Atrophic vaginitis	Low level of estrogen	—	Yes	No discharge	> 4.5 (increased)

^aAsymptomatic vs. symptomatic.

microanatomical changes of the vagina, which include thinning of the vaginal epithelium, shrinking of the vaginal walls, and decreased lubrication. VVA symptoms consist of vaginal dryness, soreness and dyspareunia that impact quality of life and sexual health. The VMB structure can also attribute to the severity of disease (Muhleisen and Herbst-Kralovetz, 2016). Women with more diverse VMB, which are not lactobacilli-dominant, exhibit more severe signs and symptoms, when compared to women with lactobacilli-dominant VMB. The VVA symptoms can be improved by hormone replacement therapy. Estrogen administration not only improves vaginal symptoms, but also promotes growth of lactobacilli and improves vaginal health. Probiotics have also been shown promise to restore a healthy vaginal microbiome; however, more robust clinical studies are needed to determine efficacy and safety of these novel therapeutics (Muhleisen and Herbst-Kralovetz, 2016).

References

- Achkar, J. M., & Fries, B. C. (2010). Candida infections of the genitourinary tract. *Clinical Microbiology Reviews*, 23, 253–273.
- Barnhart, K. T., Izquierdo, A., Pretorius, E. S., Shera, D. M., Shabbout, M., & Shaunik, A. (2006). Baseline dimensions of the human vagina. *Human Reproduction*, 21, 1618–1622.
- Doerflinger, S. Y., Throop, A. L., & Herbst-Kralovetz, M. M. (2014). Bacteria in the vaginal microbiome alter the innate immune response and barrier properties of the human vaginal epithelia in a species-specific manner. *Journal of Infectious Diseases*, 209, 1989–1999.
- Donders, G. G. G., Bellen, G., Grinceviciene, S., Ruban, K., & Vieira-Baptista, P. (2017). Aerobic vaginitis: No longer a stranger. *Research in Microbiology*. pii: S0923-2508(17)30086-4.
- Dutta, D. C. (2014). *DC Dutta's textbook of gynecology* (6th edn.). London: JP Medical Ltd.
- Fedele, L., Bianchi, S., Berlanda, N., Fontana, E., Raffaelli, R., Bulfoni, A., & Braidotti, P. (2006). Neovaginal mucosa after Vecchietti's laparoscopic operation for Rokitansky syndrome: Structural and ultrastructural study. *American Journal of Obstetrics and Gynecology*, 195, 56–61.
- Gajer, P., Brotman, R. M., Bai, G., Sakamoto, J., Schutte, U. M., Zhong, X., Koenig, S. S., Fu, L., Ma, Z. S., Zhou, X., Abdo, Z., Forney, L. J., & Ravel, J. (2012). Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine*, 4, 132ra52.
- Hickey, R. J., Zhou, X., Pierson, J. D., Ravel, J., & Forney, L. J. (2012). Understanding vaginal microbiome complexity from an ecological perspective. *Translational Research*, 160, 267–282.
- Ludwig, H., & Metzger, H. (1976). *The human female reproductive tract: A scanning electron microscopy atlas*. New York: Springer-Verlag.
- Kumar, R., Abbas, A., DeLancey, A., & Malone, E. (Eds.). (2010). *Robins and Contran pathologic basis of disease* (8th edn.). Philadelphia: Saunders.
- Martin, D. H., & Marrazzo, J. M. (2016). The vaginal microbiome: Current understanding and future directions. *Journal of Infectious Diseases*, 214(Suppl 1), S36–41.
- Menezes, C. B., Frasson, A. P., & Tasca, T. (2016). Trichomoniasis—Are we giving the deserved attention to the most common non-viral sexually transmitted disease worldwide? *Microbial Cell*, 3, 404–419.
- Mirmonsef, P., Hotton, A. L., Gilbert, D., Gioia, C. J., Maric, D., Hope, T. J., Landay, A. L., & Spear, G. T. (2016). Glycogen levels in undiluted genital fluid and their relationship to vaginal pH, estrogen, and progesterone. *PLoS One*, 11, e0153553.
- Muhleisen, A. L., & Herbst-Kralovetz, M. M. (2016). Menopause and the vaginal microbiome. *Maturitas*, 91, 42–50.
- Muzny, C. A., & Schwabke, J. R. (2016). Pathogenesis of bacterial vaginosis: Discussion of current hypotheses. *Journal of Infectious Diseases*, 214(Suppl 1), S1–5.
- Nelson, T. M., Borgogna, J. L., Brotman, R. M., Ravel, J., Walk, S. T., & Yeoman, C. J. (2015). Vaginal biogenic amines: Biomarkers of bacterial vaginosis or precursors to vaginal dysbiosis? *Frontiers in Physiology*, 6, 253.
- Nugent, R. P., Krohn, M. A., & Hillier, S. L. (1991). Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. *Journal of Clinical Microbiology*, 29, 297–301.
- Nunn, K. L., & Forney, L. J. (2016). Unraveling the dynamics of the human vaginal microbiome. *Yale Journal of Biology and Medicine*, 89, 331–337.
- O'Hanlon, D. E., Moench, T. R., & Cone, R. A. (2013). Vaginal pH and microbicidal lactic acid when lactobacilli dominate the microbiota. *PLoS One*, 8, e80074.
- Onderdonk, A. B., Delaney, M. L., & Fichorova, R. N. (2016). The human microbiome during bacterial vaginosis. *Clinical Microbiology Reviews*, 29, 223–238.
- Ravel, J., Gajer, P., Abdo, Z., Schneider, G. M., Koenig, S. S., Mcculle, S. L., Karlebach, S., Gorle, R., Russell, J., Tacket, C. O., Brotman, R. M., Davis, C. C., Ault, K., Peralta, L., & Forney, L. J. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of National Academy of Science U. S. A.*, 108(Suppl 1), 4680–4687.
- Romero, R., Hassan, S. S., Gajer, P., Tarca, A. L., Fadros, D. W., Nikita, L., Galuppi, M., Lamont, R. F., Chaemsaitong, P., Miranda, J., Chaiworapongsa, T., & Ravel, J. (2014). The composition and stability of the vaginal microbiota of normal pregnant women is different from that of non-pregnant women. *Microbiome*, 2, 4.
- Spear, G. T., French, A. L., Gilbert, D., Zariffard, M. R., Mirmonsef, P., Sullivan, T. H., Spear, W. W., Landay, A., Micci, S., Lee, B. H., & Hamaker, B. R. (2014). Human alpha-amylase present in lower-genital-tract mucosal fluid processes glycogen to support vaginal colonization by Lactobacillus. *Journal of Infectious Diseases*, 210, 1019–1028.
- Standing, S. (Ed.). (2008). *Gray's anatomy: The anatomical basis of clinical practice* (40th ed). London: Churchill Livingstone.

Further Reading

- Baker, J. M., Al-Nakkash, L., & Herbst-Kralovetz, M. M. (2017). Estrogen-gut microbiome axis: Physiological and clinical implications. *Maturitas*, 103, 45–53.
- Chase, D., Goulder, A., Zenhausern, F., Monk, B., & Herbst-Kralovetz, M. (2015). The vaginal and gastrointestinal microbiomes in gynecologic cancers: A review of applications in etiology, symptoms and treatment. *Gynecologic Oncology*, 138, 190–200.
- Herbst-Kralovetz, M. M., Pyles, R. B., Ratner, A. J., Sycuro, L. K., & Mitchell, C. (2016). New systems for studying intercellular interactions in bacterial vaginosis. *Journal of Infectious Diseases*, 214(Suppl 1), S6–S13.
- Hjelm, B. E., Berta, A. N., Nickerson, C. A., Arntzen, C. J., & Herbst-Kralovetz, M. M. (2010). Development and characterization of a three-dimensional organotypic human vaginal epithelial cell model. *Biology of Reproduction*, 82, 617–627.
- Łaniewski, P., Gomez, A., Hire, G., So, M., & Herbst-Kralovetz, M. M. (2017). Human three-dimensional endometrial epithelial cell model to study host interactions with vaginal bacteria and *Neisseria gonorrhoeae*. *Infection and Immunity*, 85, e01049-16.
- Martin, D. H. (2012). The microbiota of the vagina and its influence on women's health and disease. *The American Journal of the Medical Sciences*, 343, 2–9.
- Muzny, C. A., & Schwabke, J. R. (2016). Pathogenesis of bacterial vaginosis: Discussion of current hypotheses. *Journal of Infectious Diseases*, 214(Suppl 1), S1–5.
- Tachedjian, G., Aldunate, M., Bradshaw, C. S., & Cone, R. A. (2017). The role of lactic acid production by probiotic lactobacillus species in vaginal health. *Research in Microbiology*. pii: S0923-2508(17)30083-9.

Vagina: Cell Biology

Tomomi Sato and Taisen Iguchi, Yokohama City University, Yokohama, Japan
Sipra Mohapatra, Ehime University, Ehime, Japan

© 2018 Elsevier Inc. All rights reserved.

Embryology

In the 6th week of human embryonic development, the sexually undifferentiated embryo possesses two pairs of genital ducts, the Wolffian (mesonephric) and Müllerian (paramesonephric) duct. Only one of the ducts reaches complete development, depending upon the sex, i.e., if the embryo is supposed to become a female, the Müllerian ducts develop and vice versa. Initially, the Müllerian ducts are separate, but later, they fuse together to form the uterovaginal canal (Fig. 1); and this uterovaginal canal further extends towards the caudal opening called the urogenital sinus (Figs. 2 and 3). By the end of the 3rd month of embryonic development in human, the fusion septum of the two Müllerian ducts starts to recede, and a single uterovaginal canal is formed (Figs. 2 and 3). Proliferation of the endoderm at the urogenital sinus results in the sinovaginal bulb formation (Figs. 1A, 2A and B, 3A and B), which stretches from the urogenital sinus to the uterovaginal canal. Later the sinovaginal bulb (plate) expands and forms the major portions of the vaginal primordium. The upper one-third of the vagina originates from the uterovaginal canal (Müllerian duct), while the lower two-thirds develops from the endoderm of the sinovaginal bulb (urogenital sinus). On the other hand, the fibromuscular wall of the vagina evolves from the splanchnic mesenchyme that surrounds the epithelium of the developing vagina. The vaginal orifice is covered with a membrane called hymen, and this hymen marks the junction between sinovaginal plate and urogenital sinus (Figs. 2 and 3). Although the hymen ruptures in the perinatal period, a portion of it remains as a thin fold of tissue, located just within the vaginal entrance.

At the time of birth, the stratified squamous epithelium lining the vagina ranges from 6 to 10 cells in thickness. Although, the vaginal epithelium remains comparatively thin during childhood, it later thickens at puberty, due to the estrogenic stimulation. The epithelial thickness varies, depending upon the menstrual cycle, pregnancy, and atrophies. Interestingly, after menopause, the epithelial thickness reverts back to its initial thin prepubertal state.

In mice, the Müllerian duct formation and differentiation are well investigated. After the Wolffian duct formation, the progenitor cells associated with LIM (Lin11/Isl1/Mec3) class homeodomain transcription factor (LHX1) expression are formed in the mesonephric epithelium at the upper end of the urogenital ridge at embryonic day (E) 11.5. These Müllerian duct cells invaginate caudally into the mesonephros and then elongate along the Wolffian duct to the urogenital sinus derived from the endoderm. This elongation completes by E13.5. Wnt4 expression in the mesonephric mesenchyme is necessary for Müllerian duct cells invagination. While the upper Müllerian ducts are separated both right and left sides, elongated each Müllerian duct is joined and fused

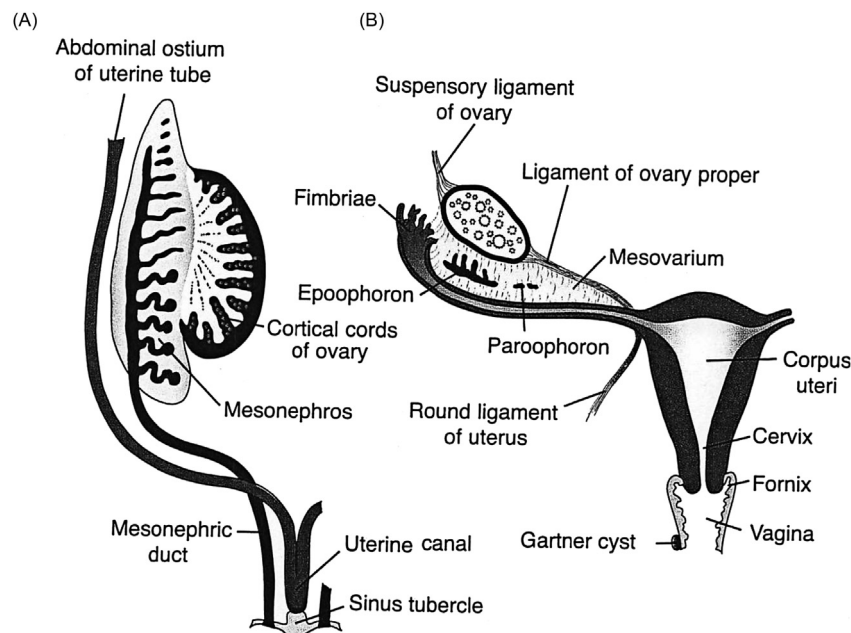


Fig. 1 (A) Genital ducts in the female at the end of the second month. Note the paramesonephric [Müllerian] tubercle and formation of the uterine canal. (B) Genital ducts after descent of the ovary. The only parts remaining from the mesonephric system are the epoophoron, paroophoron, and Gartner cyst. Note the suspensory ligament of the ovary, ligament of the ovary proper, and round ligament of the uterus. Reproduced with permission from Sadler, T. W. (ed.) (2015). *Langman's Medical Embryology* (13th edn.). Philadelphia: Wolters Kluwer.

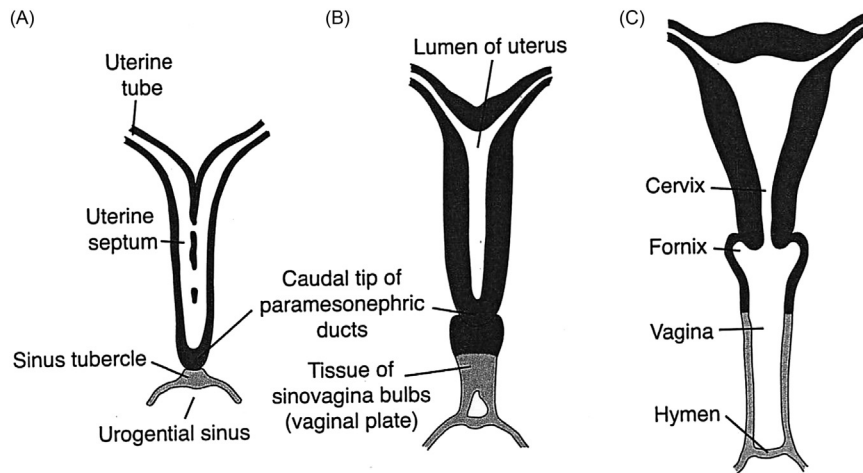


Fig. 2 Formation of the uterus and vagina. (A) 9 weeks. Note the disappearance of the uterine septum. (B) At the end of the 3rd month. Note the tissue of the sinovaginal bulbs. (C) Newborn. The fornices and the upper portion of the vagina are formed by vacuolization of the paramesonephric tissue, and the lower portion of the vagina is formed by vacuolization of the sinovaginal bulbs. Reproduced with permission from Sadler, T. W. (ed.) (2015). *Langman's Medical Embryology* (13th edn.). Philadelphia: Wolters Kluwer.

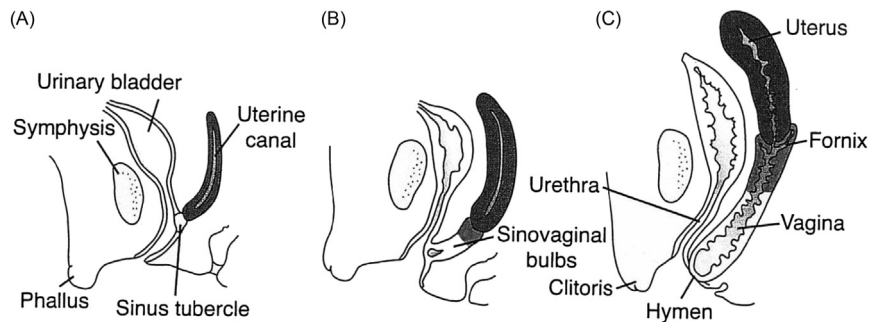


Fig. 3 Sagittal sections showing formation of the uterus and vagina at various stages of development. (A) 9 weeks. (B) End of third month. (C) Newborn. Reproduced with permission from Sadler, T. W. (ed.) (2015). *Langman's Medical Embryology* (13th edn.). Philadelphia: Wolters Kluwer.

after they cross over the Wolffian ducts. This fused caudal portion of the Müllerian duct develops into the upper three-fifth of the vagina, and the urogenital sinus develops into the lower vagina. After birth, the epithelium of the Müllerian vagina migrated towards the posterior, and the epithelium of the urogenital sinus remains in the vulva when the vagina is opened for puberty. Tissue recombination studies reveal that the each epithelium-specific phenotype can be induced by the local factors from the surrounding mesenchyme. Fibroblast growth factor 7/10, bone morphogenetic protein 4 and activin A can act as paracrine factors to stimulate vaginal epithelial cell differentiation. Thus this reflects that the epithelial cells have plasticity, however it is restricted to the first week of postnatal development.

Histology

The vaginal wall consists of the tunica mucosa or mucosa, tunica muscularis or muscularis, and the tunica adventitia or adventitia (Fig. 4).

Tunica Mucosa

The mucosa consists of the lining epithelium and the underlying lamina propria (connective tissue). A nonkeratinized stratified squamous epithelium shows 150–200 μm thick and can be classified into type II mucosa. Human vaginal epithelium is less prone to hormonal fluctuations during the menstrual cycle as compared to other species. In humans, during the follicular phase, when the estrogen level is at peak, the vaginal epithelial thickness rises up to 45 cells thick, while during the luteal phase, which is marked by low estrogen and high progesterone amount, the epithelial layer is reduced to about only 30 cells thickness (Fig. 5). The glycogen content of the epithelial cells is an important factor and is known to vary with the menstrual cycle. The epithelial cells are rich in glycogen during the mid-cycle, probably under the estrogenic influence, (Fig. 5), but the amount reduces significantly in the latter

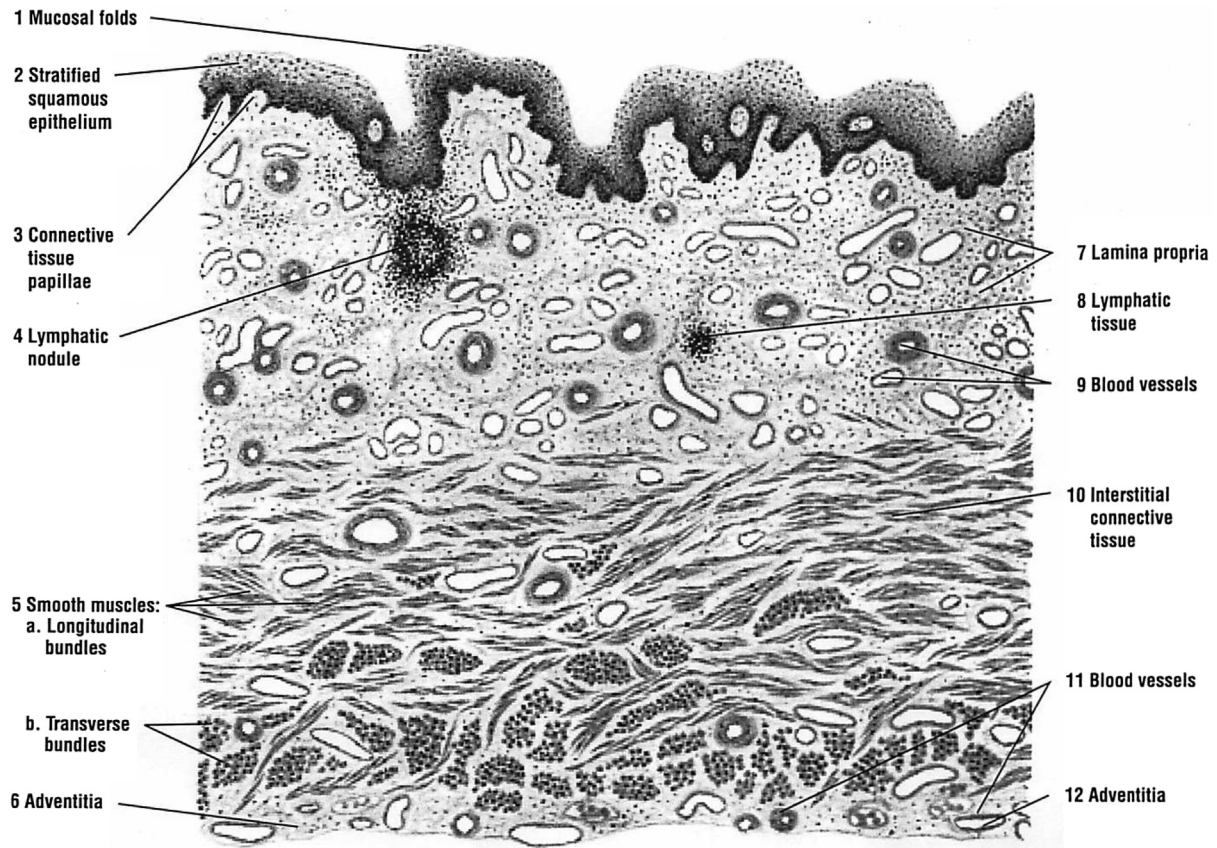


Fig. 4 Vagina (longitudinal section). Stain: hematoxylin and eosin. Low magnification. Reproduced with permission from Eroschenko, V. P. (ed.) (2013). *diFiore's Atlas of Histology* (12th edn.). Philadelphia: Wolters Kluwer.

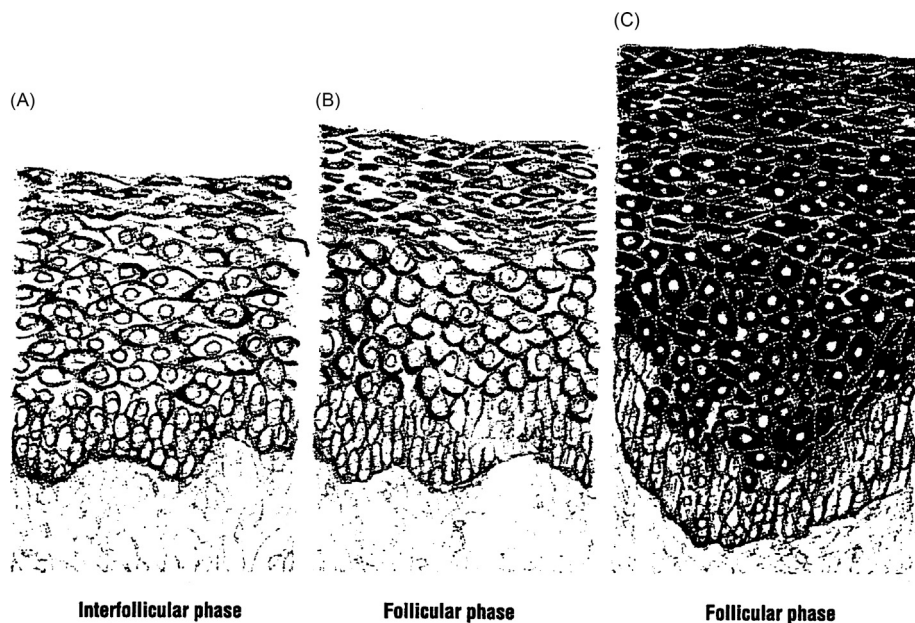


Fig. 5 Glycogen in human vaginal epithelium. Stain: Mancini iodine technique. Medium magnification. Reproduced with permission from Eroschenko, V. P. (ed.) (2013). *diFiore's Atlas of Histology* (12th edn.). Philadelphia: Wolters Kluwer.

phase of the cycle. The epithelial cells are interconnected together, primarily in the basal layers, by several mechanical adherent junctions and communication junctions called desmosomes and gap junctions, respectively. However, at the superficial layers, several tight junctions are present which form a sort of permeability barrier for the larger water-soluble molecules. The intercellular spaces between the epithelial cells which facilitate the unobstructed movement of blood-derived mononuclear leucocytes, especially the lymphocytes. The epithelial cells are also involved in the immune response of the vagina.

Toll-like receptors (TLR), important for the innate vaginal immunity, are expressed in the surface of vaginal epithelial cells. Epithelial cells produce molecules showing nonspecific antimicrobial activity. Defensins can bind negatively-charged bacterial cell surfaces and disrupt microorganism membrane. Secretory leukocyte protease inhibitor (SLPI) inhibits proteases and protects the epithelium from the bacteria and viruses action. Mannose-binding lectin (MBL) induces the direct lysis of bacteria by binding mannose residues, *N*-acetylglucosamine and fucose in the surface of microorganisms. Various immune cells are also distributed in the vagina. T cells, natural killer (NK) cells and macrophages/dendritic cells are dominant. Components of immune cells are similar in the vagina during the menstrual cycle unlike those in the uterus.

Vaginal epithelial cells secrete mucus, which consists of mucins, to protect the epithelial cells against infectious microorganisms. The amount of MUC5B protein in mucus and its glycosylation are increased during ovulation. In addition, the mucus structure is changed at the time of ovulation. Mucus in the vagina is known as sialoglycan-rich proteins, negatively charged hydrophilic carbohydrates. Sialoglycoproteins play roles in protecting from bacterial infection.

The lamina propria, which lies subjacent to the epithelium, is composed of dense connective tissue layers, with several elastic fibers running through it to the underlying muscles. Interestingly, the lamina propria gradually becomes less dense as it approaches the muscle. The lamina propria is composed of several large thin-walled veins (Fig. 4), bound together in a grid and giving the appearance of an erectile tissue. Since the lamina propria is devoid of any glands, the vagina obtains much of its lubrication from the glands of the uterine cervix. However, the rich vascularization of the vagina is also known to serve as a fluid source that helps the fluids to seep through the squamous epithelium during sexual stimulation. Vaginal elasticity is often related to the massive network of elastic fibers in the connective tissues of its wall and its extensive venous plexus.

Tunica Muscularis

The tunica muscularis layer is composed of numerous loosely arranged, spiraling bundles of smooth muscle cells (Fig. 4). The smooth muscles, in addition to the spiral bundles, are also comprised with several inner circular and outer longitudinal layers. A multitude of skeletal muscle fibers (bulbospongiosus muscle) are also present at the vaginal opening, forming a sphincter.

Tunica Adventitia

The thin layer of connective tissue that is rich in elastic fibers, and merges with the connective tissues of the urethra, rectum and anal canal is known as the tunica adventitia (Fig. 4). Countless nerve bundles travel through the adventitia in order to reach the muscle and mucosa layers.

Cytology

It is of paramount importance to understand the effect of various endogenous and exogenous hormones on the vaginal epithelium and the ovaries in order to get a comprehensive idea of their functioning in the female body.

Since the cellular differentiation takes place in the vaginal epithelium, the superficial epithelial cells differentiate continuously and are shed during the menstrual cycle, with the greatest desquamation happening at the latter part of the luteal phase and during menstruation. Vaginal smears are the best indicators (based on the cytology of cells) of the functional state of the ovaries (in regard to the secretion of hormones). Thus, the morphological studies of the cells collected from vaginal smears, especially the different types of cells from the most superficial layer of the epithelium, provides the most accurate information for clinical examination, such as the presence of leukocytes in case of early cancer detection (Pap smear; Papanicolaou and Traut, 1943).

The desquamation of superficial cells is more pronounced shortly before the onset of menstruation, particularly during the mid-cycle or premenstruation. Although, the superficial cells become relatively smoother during the first trimester of pregnancy, it later becomes highly fragmented in the postmenopausal phase. The vaginal smear shows signs of continuous exfoliation of single superficial cells, as the female ages. The size of superficial vaginal cells varies with age; larger cells being more profound in young women (17 years), while the smaller sized cells are found in older women (47 years). However, after menopause, the normal vaginal mucosa consists of a thin squamous epithelium with basal cells and no glycogen content.

In rodents, vaginal smears have been also used for microscopic examination to evaluate the estrous cycle (Fig. 6). Generally, the estrous cycle is separable into four stages, proestrus, estrus, metestrus, and diestrus. Three basic cell types are found in the vaginal smears, leukocytes, epithelial cells with smooth margins and keratinized cells. On the morning of the day at proestrus, high levels of estrogen stimulates the LH surge, resulting in ovulation. Epithelial cells, but not leukocytes, can be found in the vaginal smears. Estrogen stimulates the cornification and keratinization of vaginal epithelial cells, therefore, keratinized cells are dominantly found in the vaginal smears at estrus. After ovulation, keratinized cells are reduced but leukocytes are infiltrated at metestrus, while absence of keratinized cells and leukocytes dominance are detected at diestrus.

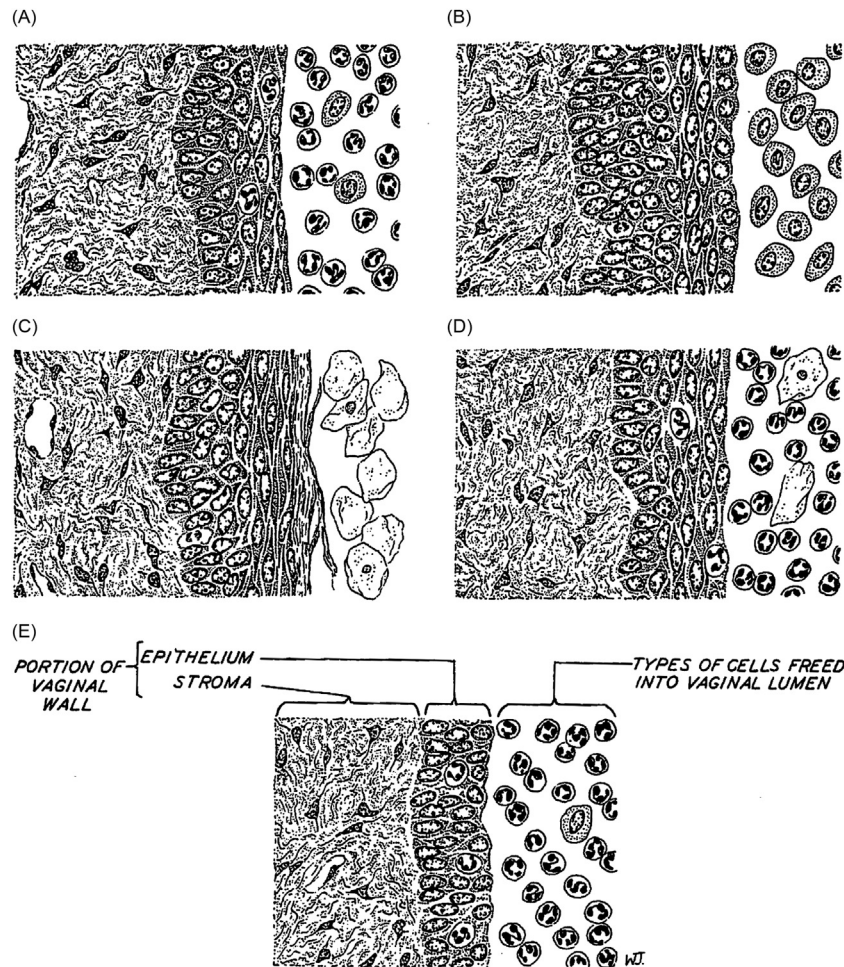


Fig. 6 Sections through the vaginal wall of the rat during different stages of the estrous cycle, showing the corresponding types of cells which appear in smears obtained from the vaginal lumen. (A) Diestrus. (B) Proestrus. (C) Estrus. (D) Metestrus. (E) Adult animal which had been oophorectomized for 6 months. Reproduced with permission from Turner, C. D. (ed.) (1966). *General Endocrinology* (4th edn.). Philadelphia: W.B. Saunders Company.

Hormonal Influence

The vaginal epithelium, during the functional reproductive years with an active sex life is under normal estrogen stimulation, and is thickened and slightly cornified but not keratinized in structure. However, during the proliferative phase (follicular stage) of the menstrual cycle, the estrogen level rises, the superficial epithelial cells become large and flattened with pyknotic nuclei, and the cytoplasm show increased acidophilia and glycogen accumulation. The estrogen stimulation peaks at midcycle and maximum epithelial thickness and glycogen accumulation is observed (Fig. 5B). At this stage, the lactic acid bacteria ferment the glycogen of desquamated cells to lactic acid, resulting in a decrease in pH of the vaginal fluid which is helpful in resisting infections. Although when the estrogen content decrease in the secretory phase (luteal) of the cycle, the epithelial layer gradually loses its thickness due to sloughing of surface cells. The appearance of the desquamated cells become irregular and elongated with folded and curled edges. The glycogen content of the cells also becomes less abundant; resulting in an increase in pH of vaginal fluid which enhances the chances of infections.

The administration of exogenous estrogen is an efficient therapeutic strategy for vaginal infections owing to its profound influence on the vaginal epithelium. On the contrary, excess estrogen causes hyperestrogenism which increases the thickness of the epithelial layer along with intense cornification and exfoliation of epithelial cells, and higher acidic pH of the vaginal fluid. Hypoestrogenism, is the reverse of hyperestrogenism, wherein increased pH of the vaginal fluid increases and epithelial atrophies are observed.

Ovarian hormones, 17β -estradiol (E2) and progesterone, can bind the specific nuclear receptor superfamily, estrogen receptor (ER) and progesterone receptor (PR). Mammalian ER has two distinct forms, ER α and ER β , and since ER α knockout mice do not respond to E2 stimulation, it emphasizes the essential roles of ER α in the reproductive tracts. In mice, ER α mRNA and protein are detected in the vaginal epithelium and stroma from the fetal age through the adult and its roles in the epithelium and stroma is

investigated by both tissue recombinant and knockout techniques. The vaginal epithelial cell proliferation stimulated by E2 is mediated through stromal ER α , but not by the epithelial ER α . Both epithelial and stromal ER α are necessary for E2 responsive epithelial cornification in the vagina. ER α in the vaginal epithelium is also necessary for the switch between E2-dependent cell proliferation and squamous cell differentiation. On the other hand, ER β mRNA and protein are also expressed in the vaginal epithelium during neonatal period, however, they decrease with age in mice. The role of ER β in reproductive tracts is considered to inhibit cell proliferative action stimulated by E2.

PR has two natural isoforms, PRA and PRB, which are transcribed from the same gene. PR mRNA and protein are detected both in the epithelium and stroma of the vagina, however, ovariectomy reduces epithelial PR in mice. E2 also directly induces PR expression in the vaginal epithelium and stroma. Progesterone stimulates epithelial cell differentiation but inhibits cell proliferation of the vaginal epithelium. Tissue recombination studies reveal that stromal PR, not the epithelial PR, is essential to mediate the inhibitory effect of progesterone on epithelial cell proliferation, especially upon E2 stimulation.

Additionally, the phosphatase and tensin homolog deleted from chromosome 10 (Pten), a tumor suppressor gene, plays a vital role in the epithelial cell homeostasis of the mouse vagina. Ovariectomy reduces the epithelial cell layers, and Pten is expressed in the suprabasal cells to inhibit abnormal cell proliferation and differentiation.

Innervation

The autonomic nervous system and the pudendal nerve innervates the vagina, while the parasympathetic supply to the vagina is through the pelvic splanchnic nerves (from sacral spinal cord levels S2–S4 in human) and the uterovaginal plexuses. The abdominal sympathetic trunks continues caudally into the pelvis, passes over the ala of sacrum and converges into the ganglion impar near the vertebral level S5. Two or three sacral splanchnic nerves supply the inferior hypogastric plexus, and mixed branches from the inferior hypogastric plexus connects the internal iliac artery to the vagina. Additionally, the pudendal nerve furnishes the utero-vaginal plexus with the general visceral efferent fibers.

The smooth and vascular smooth muscle of the vagina are supplied with several fibers from the utero-vaginal plexuses, which are autonomic efferent in nature and travel through and in the lamina propria. Some of these intraepithelial nerve fibers are quite discernable and appear as free nerve endings among the epithelial cells. Although some fibers are successful in penetrating the surface of the epithelium, most of them end among the basal two-thirds of the epithelium. These fibers are located mainly near the introitus of the vagina and are nociceptive in nature.

The lamina propria and tunica muscularis are composed of more dense nerve fibers than the epithelium, with the anterior vaginal wall having more fibers than the posterior wall. The anterior vaginal wall also has a lower threshold level to pain stimulation as compared to the posterior wall. The vaginal nerve fibers utilize a variety of classical (catecholamine and acetylcholine) and nonclassical neurotransmitters (neuropeptides and nitric oxide), especially the neuropeptides like neuropeptide Y (NPY), calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal polypeptide (VIP). All the above substances are not only abundant in the nerves close to arteries and capillaries in the connective tissue papilla (Fig. 4), several neuropeptides and nitric oxide are also known to influence the capillary permeability and blood flow. The higher VIP content in the vagina results in increased vaginal exudation (vaginal fluid production) rate and blood flow during sexual arousal, along with reduced postmenopausal effects. Vasodilators like CGRP and SP are usually associated with sensory nerves, while NPY is known to potentiate the vasoconstrictor activity of noradrenaline.

The pudendal nerves provides the lower vagina and presumably influences the somatic pain in the lower vagina.

Pathology

Bacteria inhabit the vaginal cavity are thought to be maintain vaginal homeostasis. Imbalance or disruptions of vaginal flora are associated with pelvic inflammatory disease, miscarriages, and prematurity. The bacteria *Lactobacillus* spp. is dominant in the normal flora of the vagina in most women. *Lactobacillus* is responsible for production of lactic acid from glycogen by fermentation, resulting in the maintenance of low pH of the vagina and protection against the growth of pathogenic bacteria. Levels of *Lactobacillus* are correlated with free glycogen levels in genital fluid. *Lactobacillus* in the vagina also produces hydrogen peroxidase, which is toxic to pathogens. Infections of viruses (herpes simplex virus and human papilloma virus), fungi (*Candida albicans*) and the bacterium result in diseases of the vagina, vaginitis. Addition to the low pH in the vagina by *Lactobacillus* spp., it is reported that bacteriocidal and bacteriostatic proteins (bacteriocins) can inhibit the growth of pathogens. Maternal vaginal microbiome also contributes to the intestinal colonization of neonates by exposure in the time of delivery. The bacterial composition of the vagina changes across the female lifespan, from prepuberty to postmenopausal women. Prepuberty and postmenopausal women show lower levels of *Lactobacillus* spp. Estrogen can stimulate the accumulation of glycogen in the vaginal mucosa and the growth of *Lactobacillus* spp., therefore, the vaginal microbiota is under the influence of sex hormones. Exogenous factors such as contraceptives, sexual intercourse, showers, deodorant products, antibiotics and medications can alter the composition of the vaginal microbiota as well as endogenous factors.

Müllerian duct anomalies are caused by disruption of normal development of the Müllerian ducts during embryogenesis. Failure of the Müllerian duct fusion developmentally results in duplication of the vagina, which may be associated with a transverse vaginal

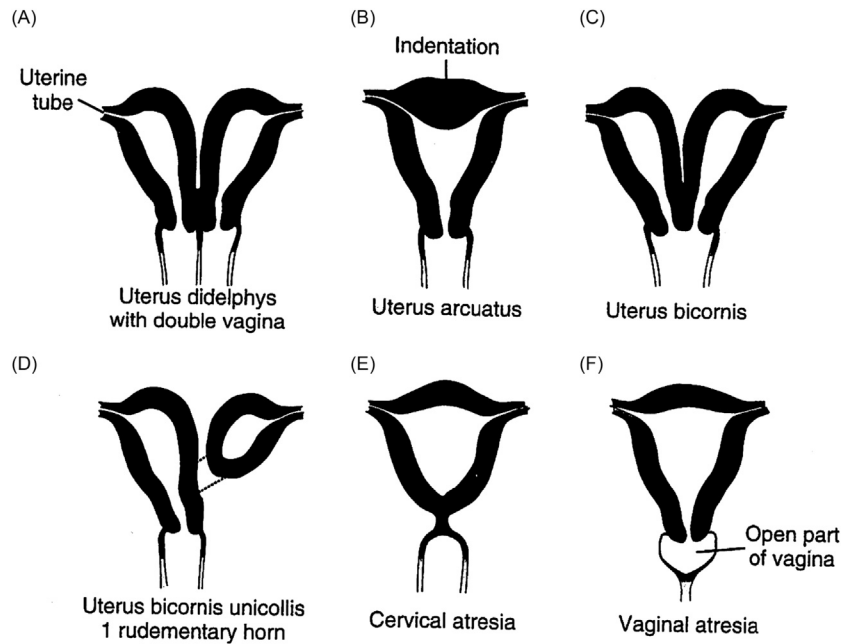


Fig. 7 Main abnormalities of the uterus and vagina, caused by persistence of the uterine septum or obliteration of the lumen of the uterine canal. Reproduced with permission from Sadler, T. W. (ed.) (2015). *Langman's Medical Embryology* (13th edn.). Philadelphia: Wolters Kluwer.

septum (Fig. 7). The partial or complete uterovaginal septum is caused by a failure of septum reabsorption. Lack of the development of the sinovaginal bulb or canalization of the bulb results in the absence of a vagina. Congenital absence of the uterus and two-third of the upper vagina, known as Mayer-Rokitansky-Küster-Hauser syndrome, is Müllerian agenesis. While it has not been revealed molecular mechanisms yet, involvement of several genes including *Hoxa* and *Wnt4* with Mayer-Rokitansky-Küster-Hauser syndrome has been reported. Faulty development of an urorectal septum results in an abnormal connection between the rectum and the vagina (rectovaginal fistula). Damage (e.g., during childbirth) to the supporting structures (Transverse cervical, pubocervical, and sacrocervical ligaments) of the uterine cervix, and thus the vagina, within the pelvic cavity can lead to prolapse of the vagina (exposure to the exterior).

Vaginal Cancer

Diethylstilbestrol (DES), a synthetic nonsteroidal estrogen, synthesized in 1938 has been used for the prevention of miscarriages as well as some of the later complications of pregnancy, since 1940. In 1971, Herbst et al. discovered the association of DES usage during pregnancy and the occurrence of vaginal clear cell adenocarcinoma in a small percentage of exposed daughters. Moreover, studies on mice, exposed prenatally and neonatally to exogenous estrogens, have revealed similar types of vaginal cancers in 1963–64 (Dunn and Green, 1963; Takasugi and Bern, 1964).

Prenatal exposure to DES and related drugs, causes vaginal and cervical adenocarcinoma in women. Several nonneoplastic abnormalities of the vagina and cervix, such as cervical ectropion (erosion and eversion), and vaginal adenosis, both of which are commonly accompanied by squamous metaplasia, as well as variety of gross malformations, were found in women after intra-uterine exposure to DES. Cervical ectropion, defined as the presence of glandular (columnar) epithelium or its mucinous products in the ectocervix, is a common phenomenon (40%–65%) in women who have not been exposed to DES. However, these lesions are encountered in over 95% of DES-exposed women. Vaginal adenosis is defined as the presence of glandular (columnar) epithelium or its mucinous products in the vagina. These lesions are noted in 3%–13% of unexposed women, and in 35%–90% of DES-exposed women.

Mice exposed prenatally or neonatally to estrogen including DES, developed squamous cell carcinoma in the vagina and/or cervical regions. Higher dosages of estrogen exposure during perinatal life induced ovary-independent vaginal cornification, lesions, and tumors. The squamous cell carcinoma induced by neonatal estrogen exposure was transplantable serially for many generations.

References

- Dunn, T. B., & Green, A. W. (1963). Cysts of the epididymis, cancer of the cervix, granular cell myoblastoma, and other lesions after estrogen injection in newborn mice. *Journal of the National Cancer Institute*, 31, 425–455.

- Papanicolaou, G., & Traut, H. (1943). Diagnosis of uterine cancer by the vaginal smear. *Yale Journal of Biology and Medicine*, 15, 924.
- Takasugi, N., & Bern, H. A. (1964). Tissue changes in mice with persistent vaginal cornification induced by early postnatal treatment with estrogen. *Journal of the National Cancer Institute*, 33, 855–865.

Further Reading

- Behr, S. C., Courtier, J. L., & Qayyum, A. (2012). Imaging of Müllerian duct anomalies. *Radiographics*, 32, E233–E250.
- Brauer, M. M., & Smith, P. G. (2015). Estrogen and female reproductive tract innervation: Cellular and molecular mechanisms of autonomic neuroplasticity. *Autonomic Neuroscience: Basic and Clinical*, 187, 1–17.
- Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal cytology of the laboratory rat and mouse: Review and criteria for the staging of the estrous cycle using standard vaginal smears. *Toxicologic Pathology*, 43, 776–793.
- Cunha, G. R., Cooke, P. S., & Kurita, T. (2004). Role of stromal-epithelial interactions in hormonal responses. *Archives of Histology and Cytology*, 67, 417–434.
- Druart, X. (2012). Sperm interaction with the female reproductive tract. *Reproduction in Domestic Animals*, 47, 348–352.
- Eroschenko, V. P. (Ed.). (2013). *diFiore's Atlas of Histology* (12th edn.). Philadelphia: Wolters Kluwer.
- Fawcett, D. W., & Jensch, R. P. (Eds.). (2002). *Concise Histology* (2nd edn.). London: Arnold.
- Krantz, K. E. (1959). The gross and microscopic anatomy of the human vagina. *Annals of the New York Academy of Sciences*, 83, 89–104.
- Kurita, T. (2011). Normal and abnormal epithelial differentiation in the female reproductive tract. *Differentiation*, 82, 117–126.
- Linhares, I. M., Giraldo, P. C., & Baracat, E. C. (2010). New findings about vaginal bacterial flora. *Revista Da Associacao Medica Brasileira*, 56, 370–374.
- Mullen, R. D., & Behringer, R. R. (2014). Molecular genetics of Müllerian duct formation, regression and differentiation. *Sexual Development*, 8, 281–296.
- Nakajima, T., Tanimoto, Y., Tanaka, M., et al. (2015). Neonatal estrogen receptor β is important in the permanent inhibition of epithelial cell proliferation in the mouse uterus. *Endocrinology*, 156, 3317–3328.
- Norris, D. O., & Carr, J. A. (Eds.). (2013). *Vertebrate Endocrinology* (5th edn.). London: Elsevier.
- Plant, T. M., & Zeleznik, A. J. (2015). *Knobil and Neill's physiology of reproduction* (4th edn.). Waltham: Academic Press.
- Rampersaud, R., Randis, T. M., & Ratner, A. J. (2012). Microbiota of the upper and lower genital tract. *Seminars in Fetal & Neonatal Medicine*, 17, 51–57.
- Sadler, T. W. (Ed.). (2015). *Langman's Medical Embryology* (13th edn.). Philadelphia: Wolters Kluwer.
- Watanabe, K., Kobayashi, Y., Banjo, K., et al. (2017). Recent advances in the molecular mechanisms of Mayer-Rokitansky-Küster-Hauser syndrome. *Biomedical Reports*, 7, 123–127.

EARLY PREGNANCY AND IMPLANTATION

Fertilization and the Signaling of Egg Activation

Takuya Wakai, Okayama University, Okayama, Japan

Aujan Mehregan and Rafael A Fissore, University of Massachusetts, Amherst, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

After receiving the LH signal, Graffian follicles in the ovary are induced to ovulate. The product of this ovulation, the female gamete, the oocyte, is collected in the oviduct. In all mammals, prior to ovulation, the oocyte is arrested in the cell cycle at the prophase stage of the first meiosis, which is also known as the germinal vesicle (GV stage). Following the LH surge, the oocyte resumes meiosis and progresses to the metaphase stage of the second meiosis, the MII stage, where it undergoes a second arrest and where fertilization takes place. Fertilization can be defined as the union of two haploid gametes, the spermatozoa and the oocyte, hereto referred to as egg, to restore the diploid state, form a zygote through the process of egg activation, and commence a series of mitotic divisions that results in cell differentiation and embryo development.

Fertilization takes place in a region of the oviduct named the ampulla. The initial interaction of the gametes is mediated by the glycoprotein components of the zona pellucida (ZP), ZP1–3 in the mouse and ZP1–4 in humans, in the egg, and an unknown cognate sperm receptor (Avella et al., 2013). The sperm then powers its way through the ZP entering the perivitelline space, which is the space between the ZP and the egg's plasma membrane (PM), the oolema. A sperm protein, Izumo, and several egg proteins including Juno, formerly Folr4, which is the receptor for Izumo, and CD9 and CD81, which are tetraspanins involved in the organization of the oolema, are required for the fusion of mammalian gametes (Grayson, 2015), although their precise role in the fusion process is unclear. Following fusion, the sperm triggers the first stage of embryo development, the zygote, through a process known as egg activation, which consists of a multilayered series of events that transforms this meiotic, haploid, and transcriptionally inactive cell into a diploid and mitotically competent early embryo. The events of activation are initiated by an increase in the intracellular concentration of free calcium (Ca^{2+}) in the egg (Stricker, 1999; Wakai et al., 2011). In this article, we will review the molecular events underlying the main steps of egg activation, the signaling mechanisms responsible for the increase in Ca^{2+} and the consequences on fertility from failure to initiate these responses.

Events of Egg Activation

Besides introducing the paternal genome, fertilization induces egg activation, which consists of a series of biochemical and structural changes that transform the female gamete into a zygote. These events include the release of cortical granules (CGs) and prevention of polyspermy, the resumption and completion of meiosis, and the progression into interphase with pronuclear (PN) formation (Florman and Fissore, 2015) (Fig. 1). In most species and in all mammals, an increase in the intracellular Ca^{2+} concentration is the universal signal for egg activation (Stricker, 1999). Downstream effectors in the egg decode the spatiotemporal information provided by the Ca^{2+} signal and transform it into the distinct cellular events of egg activation that will be described later (see also Wakai et al., 2011, p. 42).

Prevention of Polyspermy

At fertilization, eggs are exposed to multiple sperm but entry of more than one sperm causes polyploidy, polyspermy, which results in abnormal embryo development. To ensure fusion of a single sperm, the egg becomes rapidly intolerant to additional sperm. It accomplishes this by developing a block to polyspermy, which consists of the ZP and PM blocks (Avella et al., 2013). The relative contributions of these two blocks vary in different species, with the PM block unfolding earlier than the ZP block. The PM block prevents binding/fusion of additional sperm, as evidenced by the presence of unfused sperm within the perivitelline space. The underlying molecular mechanism of this block remains unknown, although it lacks the membrane depolarization component present in marine eggs (Igusa et al., 1983). Remarkably, the PM block is deployed after fusion of the gametes in MII eggs but not after ICSI, suggesting that fusion is essential for this process. The recently identified Izumo receptor, Juno, might be involved, as it is shed from the oolema only after spontaneous fertilization either in vivo or in vitro (Bianchi et al., 2014). Nevertheless, how it is released from the oolema and its action to prevent additional fusions require additional characterization.

The ZP block is intended to prevent additional sperm penetration and/or binding of the ZP. Several mechanisms have been proposed to account for this block, including those that might cause changes in sperm-binding residues or changes in the organization of the zona or of its components (Avella et al., 2013). The most established model, which is based on the observation that ZP2, one of the main components of the ZP, undergoes cleavage after fertilization thereby eliminating the site(s) of sperm binding

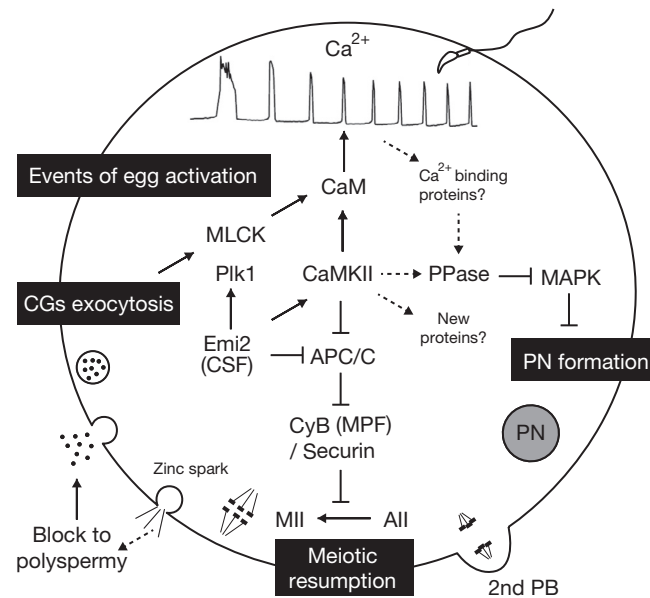


Fig. 1 Schematic of the sequence of events of egg activation following fertilization in mammals. A typical pattern of Ca^{2+} oscillations initiated by fertilization or by injection of $\text{PLC}\zeta$ cRNA (recordings were terminated prematurely). The Ca^{2+} signals are decoded by downstream protein kinases, including MLCK, CaMKII, MPF, MAPK, and Plk1. *Full arrows* denote molecules and pathways whose function have been demonstrated by inhibitors or genetic models, whereas *broken arrows* indicate tentative pathways and/or molecules whose participation/role requires additional confirmation. PPase, denotes phosphatase. Other abbreviations are explained in the body of the article. From Ducibella T, Fissore R. The roles of Ca^{2+} , downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Developmental Biology* 2008;315:257–279.

located on the N-terminal portion of the molecule. A component of the GC exudate, which is exocytosed into the perivitelline space following the Ca^{2+} oscillations, is responsible for this cleavage. Ovastacin has been identified as the CG's ZP2 protease, and eggs null for the *Astl* gene, which encodes for ovastacin, failed to cleave ZP2 and allowed continuous ZP binding and penetration (Burkart et al., 2012). In vivo, ovastacin action is inhibited by the liver-derived plasma protein fetuin-B, which is part of the cystatin superfamily and related to protease inhibitors, as eggs of *Fetub*^{-/-} mice showed preovulatory ZP2 cleavage that prevented sperm penetration of the ZP rendering these females infertile (Dietzel et al., 2013).

Little is known about the mechanism underlying the dynamics of CG exocytosis during egg activation, although their release requires Ca^{2+} release. Moreover, a Ca^{2+} and calmodulin (CaM)-dependent enzyme, myosin light chain kinase (MLCK2), and its direct target myosin II are involved in CG exocytosis, as their inhibition impairs CG release (Simerly et al., 1998). Given that MLCK is regulated by phosphorylation (Deng et al., 2005), the kinase(s) involved in its regulation need(s) to be determined. The role of molecules such as Rab3 and synaptotagmins on GC release also needs to be established. Another event that occurs in close association with CG release is the efflux of zinc, which is known as zinc sparks (Kim et al., 2011). The zinc sparks arise from zinc-loaded vesicles that have similar distribution to CGs. Its release may facilitate exit of MII and contribute to the polyspermy block, as ovastacin is a zinc-dependent enzyme and high concentrations of zinc in the perivitelline space induce changes in the organization of the ZP that prevent polyspermy (Que et al., 2017).

Exit of Meiosis and Resumption of the Cell Cycle

Most vertebrate oocytes undergo maturation and become arrested until fertilization at the MII stage of meiosis. Fertilization triggers completion of meiosis, establishment of euploidy and the start of embryonic cell cycles. The MII arrest is imposed by the action of the cytosstatic factor (CSF) (Masui and Markert, 1971). CSF constrains the activity of the anaphase promoting complex (APC/C), an E3 ubiquitin ligase (Tunquist and Maller, 2003), which is responsible for the ubiquitination and degradation of cyclin B (CycB) and of the separase's inhibitor securin that prevents sister chromatid separation (Madgwick et al., 2004). CycB and the cyclin-dependent kinase 1 (Cdk1, also known as cdc2) are the molecular components of the maturation-promoting factor (MPF) (Swenson et al., 1986), and inhibition of CycB degradation, which maintains high activity of MPF, makes possible the arrest at the MII stage (Masui and Markert, 1971). The endogenous meiotic inhibitor 2 (Emi2), a component of CSF (Tung et al., 2007), is the direct inhibitor of the APC/C (Sako et al., 2014), and exit of the MII arrest is brought about by SCF/proteasome-dependent degradation of Emi2 triggered by the fertilization-induced Ca^{2+} increase (Hansen et al., 2006). Although the precise molecular mechanisms in mammals remain to be elucidated, the degradation of Emi2 is caused by sequential phosphorylation events: the first one mediated by the Ca^{2+} -CaM-dependent protein kinase II (CaMKII) followed by a Polo-like kinase 1 (Plk1) phosphorylation (Jia et al., 2015). The activation of the APC/C reduces the levels of active MPF, which causes completion of the second meiotic division displayed as the extrusion of a second polar body and the progression into interphase with PN formation.

The Formation of the Pronuclei and DNA Synthesis

While eggs complete the second meiotic division following fertilization, the sperm DNA undergoes dynamic changes in chromatin structure. The sperm chromatin is tightly packaged due to its association with protamines, which are basic proteins with extensive disulfide bond formation. This association creates a toroid complex that results in transcriptional silencing (Balhorn, 1982), which must be reversed after fertilization. This process is coordinated by factors in the ooplasm such as glutathione, which reduces the protamine's disulfide bonds (Perreault et al., 1984), and by several proteins including the mammalian homologues of the chaperone protein nucleoplasmin (NPM), NPM1, NPM2, and NPM3, which assist with the removal of protamines and deposition of histones onto the paternal chromatin (Burns et al., 2003). Approximately 4–6 h after sperm entry, and almost simultaneously, a nuclear membrane encloses the decondensed male and female chromatin forming two PNs that contain the sets of haploid genomes. This process is dependent on ATP and GTP, and relies on the extensive egg reserves of endoplasmic reticulum membranes to generate the large surface area of the PNs (Byrne et al., 2009). Formation of PNs is associated with progressive inactivation of the MAPK pathway, which occurs 2–4 h later than the decline in MPF activity, although it is not known how MAPK influences the timing of PN formation (Gonzalez-Garcia et al., 2014). The protracted formation of PNs in mammalian zygotes may be necessary nevertheless to support the remodeling of the sperm chromatin, which may be halted or incomplete in cases of premature PN formation.

During the PN stage, both sets of chromatin undergo asynchronous DNA synthesis and differential epigenetic reprogramming including changes in DNA methylation, which the paternal genome experiences prior to and during the first DNA replication. In contrast, the female genome maintains a stable methylation level during the zygote stage, although it undergoes passive demethylation over subsequent cleavages (Wossidlo et al., 2010). The male PN is also hypomethylated and contains histone marks present in active promoters such as H3K4me3, whereas repressive histone methylation markers are mostly absent (Lepikhov et al., 2010). Conversely, these repressive histone methylation markers can be detected in the female PN. As DNA synthesis and epigenetic reprogramming proceeds, PNs undergo migration so that they become apposed. Actin microfilaments and microtubules are required for pronuclear apposition in mouse eggs, although in humans and other large domestic animals, pronuclear migration and apposition relies exclusively on microtubules (Payne et al., 2003, p. 65). The two nuclear envelopes break down without fusing, and the centrosome splits to organize a bipolar spindle. The condensed chromosomes of both male and female origin align along the spindle equator. A cleavage furrow forms at the first mitotic anaphase and telophase. The completion of mitosis gives rise to two embryonic cells, each containing a new diploid genome. For a more detailed discussion of other events of egg activation and fertilization such as recruitment of maternal RNAs, please refer to exhaustive reviews on the subject (Ducibella and Fissore, 2008; Florman and Fissore, 2015; Schultz and Kopf, 1995).

The Signal for Egg Activation

Ca²⁺ Triggers Development

An increase in the levels of free Ca²⁺ in the female gamete is the earliest and universal signal of egg activation. Work by Loeb and colleagues at the end of the nineteenth century associated egg activation with changes in the intracellular concentration of several ions (Loeb, 1899), and later studies demonstrated that the divalent cation Ca²⁺ was the responsible ion. Ca²⁺ is able to induce a multitude of cellular events because it alters the function of proteins by changing their charge and shape (Clapham, 2007). The actions of Ca²⁺ are also favored by: (1) the steep gradient between its intracellular and 10,000-fold greater extracellular concentration; and (2) the energy-dependent mechanisms that cells dedicate to restore intracellular Ca²⁺ levels to ~100 nM, which is the basal level in most cells (Clapham, 2007). Ca²⁺ ionophores, which can mobilize Ca²⁺ across membranes and along concentration gradients, are effective means of inducing egg activation (Steinhardt et al., 1974). Two common targets of Ca²⁺ in all cells, which are abundantly expressed in eggs, are CaM and the Ca²⁺-CaM-dependent kinase II, CaMKII (Ducibella and Fissore, 2008). In support of this signaling cascade, eggs lacking CaMKIIγ3, which is the isoform expressed in mammalian eggs, fail to exit the MII stage following fertilization despite having normal oscillations (Backs et al., 2010).

In all mammals, the initial activating Ca²⁺ signal occurs within a few minutes following fusion of the gametes (Jones et al., 1998), although in insects, where fertilization is dissociated from activation and where embryos can develop in the absence of a male genome, parthenogenesis, the presumed Ca²⁺ increase is induced by mechanical stimulation during transport through the female reproductive tract (Sartain and Wolfner, 2013). The advent of imaging methodologies allowed researchers to gain insights into the spatio-temporal features of Ca²⁺ responses during fertilization. Using the luminescent protein aequorin, it was first demonstrated that the single Ca²⁺ rise in fish and sea urchin eggs is explosive and propagates through the egg as a wave initiating from the point of sperm entry (Ridgway et al., 1977). Later studies using fluorescent probes demonstrated the same feature to characterize the first Ca²⁺ rise in mammals (Deguchi et al., 2000; Stricker, 1999). Nevertheless, in these species, the sperm-initiated Ca²⁺ responses continued at periodic intervals, also known as Ca²⁺ oscillations (Miyazaki et al., 1986), which occur every 10–40 min according to the species, and last for more than 4 h (Miyazaki and Ito, 2006; Wakai et al., 2011). The persistent oscillations are needed to support the events of egg activation, which necessitate different numbers of oscillations for their initiation, and whose completion requires more rises than for their initiation (Ducibella and Fissore, 2008). Eggs that age postovulation are an exception, as they might be activated by single Ca²⁺ rises (Whittingham and Siracusa, 1978). It is worth noting that completion of the first cell cycle in mammals demands in excess of 20 h, whereas in most other species this stage is completed in less than 120 min (Stricker, 1999).

The Ca^{2+} Toolkit

The fertilization signal depends on Ca^{2+} release from intracellular stores. To accumulate, release, restore and maintain Ca^{2+} levels both in the stores and in the cytoplasm, i.e., Ca^{2+} homeostasis, cells, including oocytes and eggs, use a set of complementary molecules collectively referred to as the Ca^{2+} toolkit (Fig. 2). The endoplasmic reticulum (ER) is the cell's main Ca^{2+} reservoir, and Ca^{2+} release from the ER underlies multiple cellular events in a wide array of cell types (reviewed in Berridge, 2009). Ca^{2+} release from the ER is mostly mediated by 1,4,5-inositol trisphosphate (IP_3) and its cognate receptor, IP_3R , which is the main Ca^{2+} release channel in nonmuscle cells. IP_3Rs function as tetramers and mammalian eggs predominantly express the type I isoform (Jellerette et al., 2000; Parrington et al., 1998). Technical limitations have thus far prevented the detection of IP_3 synthesis during fertilization in mammals, although such production has been detected during sea urchin and amphibian fertilization (Stith et al., 1993; Turner et al., 1984). Further, injection of IP_3 or of its analogues into mammalian eggs initiated Ca^{2+} oscillations (Miyazaki, 1988). Phospholipase C enzymes, PLCs (reviewed in Rebecchi and Pentyala, 2000), which are required for the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), have been detected in mammalian eggs at the transcript and/or protein levels (Dupont et al., 1996; Igarashi et al., 2007), although their contributions to Ca^{2+} oscillations are presently unknown, as specific inhibitors failed to disturb fertilization-associated oscillations. It has therefore been suggested that the PLC responsible for the oscillations is delivered by the sperm (see later). It is worthwhile noting however that one of the egg's PLC, $\text{PLC}\gamma$, is indispensable for Ca^{2+} release during sea urchin, ascidian, and *Xenopus* fertilization (reviewed in Runft et al., 2002).

The ER and IP_3R s undergo reorganization during oocyte maturation displaying a prominent cortical cluster distribution. This organization, together with the phosphorylation of IP_3R and accumulation of Ca^{2+} in the ER, enhance the function of IP_3R in preparation for fertilization (Ajduk et al., 2008; Wakai et al., 2011). IP_3R mediated Ca^{2+} release is required for the first Ca^{2+} wave and for the subsequent oscillations in the mouse and most likely in all mammals, as a functional blocking antibody against it prevented initiation of Ca^{2+} oscillations (Miyazaki et al., 1992) and egg activation (Xu et al., 1994); similar results were reported for other species (reviewed in Florman and Fissore, 2015). The IP_3R is particularly well suited to mediate the oscillations, as it is gated both by IP_3 and Ca^{2+} , where a high concentration of Ca^{2+} inhibits the channel (Iino, 1990). As oscillations persist, IP_3R undergoes a protracted degradation that depends on the same production of IP_3 that underlies the oscillations; this downregulation contributes to the slow down and eventual termination of the oscillations (Jellerette et al., 2000).

For oscillations to persist, Ca^{2+} levels must be returned to baseline after each rise and Ca^{2+} influx be promoted, as oscillations cease prematurely in conditions devoid of external Ca^{2+} (Igusa and Miyazaki, 1983). Cells use several molecules to maintain basal Ca^{2+} levels including the sarco-endoplasmic reticulum Ca^{2+} ATPases (SERCAs), which uptake Ca^{2+} into the ER, PM Ca^{2+} ATPases (PMCA), and $\text{Na}^+/\text{Ca}^{2+}$ exchangers, which extrude/exchange Ca^{2+} out of the cell (Berridge, 2002). These mechanisms are functional in mammalian eggs, as their pharmacological manipulation altered and/or terminated oscillations (reviewed in Wakai et al., 2011, p. 520; Miao and Williams, 2012). Cells also use a variety of PM channels to influx Ca^{2+} (Clapham, 2007, p. 499). Mouse eggs express voltage-gated Ca^{2+} (VGC) channels as well as components of the store-operated Ca^{2+} entry mechanism, although mice null for these channels are fertile (Bernhardt et al., 2017). Mammalian eggs also express members of the TRP family

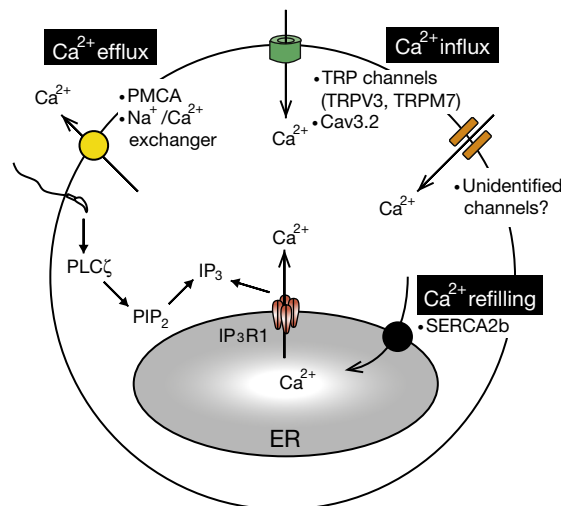


Fig. 2 The fertilization Ca^{2+} toolkit. A series of molecules are required by oocytes and eggs to initiate and maintain Ca^{2+} oscillations and homeostasis. At fertilization, the sperm delivers a $\text{PLC}\zeta$, which hydrolyzes PIP_2 into IP_3 and diacylglycerol (DAG). IP_3 binds its receptor, IP_3R , causing Ca^{2+} release from the ER. Following Ca^{2+} release, part of the elevated free Ca^{2+} is removed to the extracellular media by the action of the PM Ca^{2+} pump PMCA and/or of a $\text{Na}^+/\text{Ca}^{2+}$ exchanger. The SERCA pump reuptakes part of the elevated free Ca^{2+} from the ooplasm into the ER stores, thereby refilling them. To maintain Ca^{2+} oscillations and refill the stores, eggs need Ca^{2+} influx from the extracellular milieu. To accomplish this, a variety of PM channels mediate Ca^{2+} influx. In mouse oocytes and eggs, TRP and/or Cav3.2 T-type channels are proposed to mediate the majority of this influx, although the PM channels that mediate Ca^{2+} influx during maturation and fertilization in other species remain to be fully identified.

of ion channels but their contributions to Ca^{2+} oscillations during fertilization remain undetermined, although TRPV3 is required for SrCl_2 influx (Carvacho et al., 2013), which in mouse eggs is used as a common parthenogenetic procedure, and TRPM7 is required for early embryo development (Jin et al., 2012). Therefore, mouse eggs possess a complete tool kit, although the role and specific molecules involved in the regulation of each of parameter of Ca^{2+} homeostasis remains to be elucidated.

Sperm-Specific PLC ζ

The long-lasting oscillations of mammalian fertilization require a mechanism(s) and/or molecule capable of remaining active for extended period of time. The demonstration that injection of soluble extracts from sea urchin and ascidian sperm, sperm factors (SF), into homologous eggs triggered activation events similar to those of fertilization (Dale, 1988) led to the hypothesis that a sperm component(s) could initiate the oscillations after diffusing into the ooplasm. In support of this notion, injection of mammalian SFs into eggs replicated the events of fertilization and induced oscillations (Swann, 1990). Analysis of the SFs revealed the active component(s) was a protein moiety of $\sim 40\text{--}100$ kDa MW that displayed PIP_2 hydrolyzing activity, which was highly sensitive to Ca^{2+} and therefore capable of operating at basal intracellular Ca^{2+} levels (reviewed in Swann and Lai, 2016). Studies later identified a novel sperm-specific PLC, PLCZeta1 or ζ , as the active component of mammalian SFs (Saunders et al., 2002). Consistent with this, PLC ζ was present in the active SF fractions, its removal abrogated the activity of the extracts, and injection of its cRNAs induced sperm-like oscillations (Kurokawa et al., 2005; Saunders et al., 2002). Injection of recombinant PLC ζ induced similar responses (Yoda et al., 2004). The localization of PLC ζ in the equatorial and/or postacrosomal region of the sperm head is consistent with its rapid transfer into the ooplasm after gamete fusion (Fujimoto et al., 2004; Yoon and Fissore, 2007). Finally, the structure of PLC ζ is similar to that of other PLCs, although it lacks several regulatory domains found in other PLCs including a pleckstrin homology domain, which renders it soluble (Saunders et al., 2002; reviewed in Nomikos et al., 2013, p. 80). Therefore, this novel sperm-specific PLC ζ is endowed with unique properties that makes it specially suited to initiate and maintain oscillations in eggs; it may also underlie egg activation in other vertebrates species such as certain fish and birds (Coward et al., 2011; Ito et al., 2011).

Infertility and Egg Activation

Intracytoplasmic Sperm Injection (ICSI)

The injection of sperm into the ooplasm bypassing fusion of the gametes, which is known as ICSI, was initially developed to study fertilization in mammals. It was later applied in the clinic to overcome male infertility, many cases of which until then had poor prognosis. It has had unprecedented success, and today nearly 50% of the live births reported by fertilization clinics are the result of this procedure (reviewed in Neri et al., 2014). The application of ICSI has now been extended to nearly all other mammals, where mostly results in efficient production of offspring. Remarkably, the success of this technique has served to prove the concept that a sperm component is responsible for egg activation and of initiation of the Ca^{2+} oscillations in mammals, as bypassing the interaction and fusion of the gametes' membrane does not affect egg activation and/or embryo development. Consistent with this, follow up studies found that ICSI initiated Ca^{2+} oscillations very similar to that of fertilization (Nakano et al., 1997). Therefore, ICSI has wide applications both in the clinic as well as in the laboratory.

Infertility and PLC ζ

Fertilization by ICSI offers advantages over conventional fertilization in mammals in that it is possible to know the time of sperm entry as well as achieve synchronous fertilization. Further, unlike in vitro fertilization, IVF, where a wide array of sperm defects, ranging from motility, to interaction and/or penetration of the zona pellucida and/or defects in fusion, can underlie the failure of fertilization, only a few sperm deficiencies can undermine fertilization by ICSI; these defects are mostly related to failure of release and/or function of the egg-activating stimulus. In agreement with this, only $\sim 3\%$ of ICSI cases fail fertilization, and for the most part they all display a uniform phenotype, which is failure of egg activation. This was the case with the unusually high numbers of fertilization failures following ICSI with globozoospermic sperm, which are missing the acrosome. Later studies showed that these sperm lack or have reduced levels of PLC ζ (Yoon and Fissore, 2007), and this defect is associated with loss of expression of a novel gene, Dpy19L2, which is required for the organization of the sperm's perinuclear theca, the site of PLC ζ localization (Escoffier et al., 2015). Other cases of fertilization failure or reduced rates of fertilization are associated with specific mutations within different domains of PLC ζ (Escoffier et al., 2016; Kashir et al., 2012). Therefore, analysis of cases of fertilization failure after ICSI combined with whole exome sequencing and mutational studies have yielded insights into the mechanism of mammalian fertilization, and this approach promises to aid in uncovering the mechanism(s) that regulate the function of PLC ζ both in the sperm and in the ooplasm, which are presently unknown.

Genetic Models

The ultimate demonstration that a molecule is required for fertilization and/or initiation of development is to examine how its elimination impacts the fertility of the individual or of its progeny. On occasions this approach fails, as elimination of a gene results

in embryonic lethality or, alternatively, in abrogation of spermatogenesis. Using the CRISPR-Cas technology, researchers generated mice devoid of PLC ζ and with normal spermatogenesis (Hachem et al., 2017). The analyses of these males showed PLC ζ null sperm are unable to mount normal Ca²⁺ oscillations, which compromises the progression of all events of egg activation, especially the blocks to polyspermy. Lastly, these males were subfertile but not sterile, as females paired with them produced smaller litters at greater intervals. These results raise important questions, such as are mice the exception in producing offspring without a normal activation stimulus? What are the effects on the offspring's behavior and life expectancy? Ultimately, these PLC ζ null males are a great tool to ascertain the role of Ca²⁺ and downstream molecules in egg activation and initiation of development. It is noteworthy that a knockout mouse line was recently generated for PAWP, a molecule also suggested to be involved in egg activation. Elimination of this protein had no consequences on fertility or spermatogenesis, suggesting that its function in fertility is dispensable (Satouh et al., 2015).

Outlook

Great progress has been made over the last 30 years in understanding the mechanisms of fertilization and egg activation. Some of these findings have been successfully translated into the clinic for the treatment of infertility. Nevertheless, some important questions remain. For example, what molecule(s) mediate(s) gamete fusion in mammals? Or, how necessary are Ca²⁺ oscillations for normal embryo development in vivo? What are the PM channel(s) that mediate Ca²⁺ influx? Answers to these questions will be the basis for the design of novel, non-hormonal methods of contraception, better egg activation procedures, and culture media for oocyte maturation and embryo development. Over the next decades we expect the progress to accelerate, aided by the ability to more easily edit the mammalian genome and advances in imaging technologies. These discoveries should assist in the treatment of infertility, production of animals with better productive and reproductive profiles and resistance to disease, and in the preservation of endangered species.

Acknowledgments

The authors would like to thank Changli He, Goli Ardestani, and previous members of the Fissore lab for their research contributions and helpful discussions that shaped part of this article. This work was supported in part by funds from the National Institute of Child Health and Human Development (HD051872) and NIFA to RAF. Due to space constraints, we apologize to those authors whose worthy contributions have not been mentioned.

References

- Ajduk, A., Malagocki, A., & Maleszewski, M. (2008). Cytoplasmic maturation of mammalian oocytes: Development of a mechanism responsible for sperm-induced Ca²⁺ oscillations. *Reproductive Biology*, 8, 3–22.
- Avella, M. A., Xiong, B., & Dean, J. (2013). The molecular basis of gamete recognition in mice and humans. *Molecular Human Reproduction*, 19, 279–289.
- Backs, J., Stein, P., Backs, T., Duncan, F. E., Grueter, C. E., McAnally, J., Qi, X., Schultz, R. M., & Olson, E. N. (2010). The gamma isoform of CaM kinase II controls mouse egg activation by regulating cell cycle resumption. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 81–86.
- Balhorn, R. (1982). A model for the structure of chromatin in mammalian sperm. *The Journal of Cell Biology*, 93, 298–305.
- Bernhardt, M. L., Padilla-Banks, E., Stein, P., Zhang, Y., & Williams, C. J. (2017). Store-operated Ca²⁺ entry is not required for fertilization-induced Ca²⁺ signaling in mouse eggs. *Cell Calcium*, 65, 63–72.
- Berridge, M. J. (2002). The endoplasmic reticulum: A multifunctional signaling organelle. *Cell Calcium*, 32, 235–249.
- Berridge, M. J. (2009). Inositol trisphosphate and calcium signalling mechanisms. *Biochimica et Biophysica Acta*, 1793, 933–940.
- Bianchi, E., Doe, B., Goulding, D., & Wright, G. J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature*, 508, 483–487.
- Burkart, A. D., Xiong, B., Baibakov, B., Jimenez-Movilla, M., & Dean, J. (2012). Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *The Journal of Cell Biology*, 197, 37–44.
- Burns, K. H., Viveiros, M. M., Ren, Y., Wang, P., DeMayo, F. J., Frail, D. E., Eppig, J. J., & Matzuk, M. M. (2003). Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science*, 300, 633–636.
- Byrne, R. D., Zhendre, V., Larijani, B., & Poccia, D. L. (2009). Nuclear envelope formation in vitro: A sea urchin egg cell-free system. *Methods in Molecular Biology*, 464, 207–223.
- Carvacho, I., Lee, H. C., Fissore, R. A., & Clapham, D. E. (2013). TRPV3 channels mediate strontium-induced mouse-egg activation. *Cell Reports*, 5, 1375–1386.
- Clapham, D. E. (2007). Calcium signaling. *Cell*, 131, 1047–1058.
- Coward, K., Ponting, C. P., Zhang, N., Young, C., Huang, C. J., Chou, C. M., Kashir, J., Fissore, R. A., & Parrington, J. (2011). Identification and functional analysis of an ovarian form of the egg activation factor phospholipase C zeta (PLCzeta) in pufferfish. *Molecular Reproduction and Development*, 78, 48–56.
- Dale, B. (1988). Primary and secondary messengers in the activation of ascidian eggs. *Experimental Cell Research*, 177, 205–211.
- Deguchi, R., Shirakawa, H., Oda, S., Mohri, T., & Miyazaki, S. (2000). Spatiotemporal analysis of Ca(2+) waves in relation to the sperm entry site and animal-vegetal axis during Ca(2+) oscillations in fertilized mouse eggs. *Developmental Biology*, 218, 299–313.
- Deng, M., Williams, C. J., & Schultz, R. M. (2005). Role of MAP kinase and myosin light chain kinase in chromosome-induced development of mouse egg polarity. *Developmental Biology*, 278, 358–366.
- Dietzel, E., Wessling, J., Floehr, J., Schafer, C., Ensslen, S., Denecke, B., Rosing, B., Neulen, J., Veitinger, T., Spehr, M., Tropartz, T., Tolba, R., Renne, T., Egert, A., Schorle, H., Gottenbusch, Y., Hildebrand, A., Yiallouris, I., Stocker, W., Weiskirchen, R., & Jahnke-Dechent, W. (2013). Fetuin-B, a liver-derived plasma protein is essential for fertilization. *Developmental Cell*, 25, 106–112.
- Ducibella, T., & Fissore, R. (2008). The roles of Ca²⁺, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Developmental Biology*, 315, 257–279.

- Dupont, G., McGuinness, O. M., Johnson, M. H., Berridge, M. J., & Borgese, F. (1996). Phospholipase C in mouse oocytes: characterization of beta and gamma isoforms and their possible involvement in sperm-induced Ca^{2+} spiking. *The Biochemical Journal*, 316(Pt. 2), 583–591.
- Escoffier, J., Yassine, S., Lee, H. C., Martinez, G., Delaroche, J., Coutton, C., Karaouzene, T., Zouari, R., Metzler-Guillemain, C., Pernet-Gallay, K., Hennebicq, S., Ray, P. F., Fissore, R., & Arnoult, C. (2015). Subcellular localization of phospholipase C ζ in human sperm and its absence in DPY19L2-deficient sperm are consistent with its role in oocyte activation. *Molecular Human Reproduction*, 21, 157–168.
- Escoffier, J., Lee, H. C., Yassine, S., Zouari, R., Martinez, G., Karaouzene, T., Coutton, C., Kherraf, Z. E., Halouani, L., Triki, C., Nef, S., Thierry-Mieg, N., Savinov, S. N., Fissore, R., Ray, P. F., & Arnoult, C. (2016). Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. *Human Molecular Genetics*, 25, 878–891.
- Florman, H., & Fissore, R. A. (2015). *Fertilization in mammals, Knobil and Neill's Physiology of Reproduction: Two-volume set*. Elsevier.
- Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C., Izumi, T., & Perry, A. C. (2004). Mammalian phospholipase C ζ induces oocyte activation from the sperm perinuclear matrix. *Developmental Biology*, 274, 370–383.
- Gonzalez-Garcia, J. R., Bradley, J., Nomikos, M., Paul, L., Machaty, Z., Lai, F. A., & Swann, K. (2014). The dynamics of MAPK inactivation at fertilization in mouse eggs. *Journal of Cell Science*, 127, 2749–2760.
- Grayson, P. (2015). Izumo1 and Juno: The evolutionary origins and coevolution of essential sperm–egg binding partners. *Royal Society Open Science*, 2, 150296.
- Hachem, A., Godwin, J., Ruas, M., Lee, H. C., Ferrer Buitrago, M., Ardestani, G., Bassett, A., Fox, S., Navarrete, F., de Sutter, P., Heindryckx, B., Fissore, R., & Parrington, J. (2017). PLC ζ is the physiological trigger of the Ca^{2+} oscillations that induce embryogenesis in mammals but conception can occur in its absence. *Development*, 144, 2914–2924.
- Hansen, D. V., Tung, J. J., & Jackson, P. K. (2006). CaMKII and polo-like kinase 1 sequentially phosphorylate the cytosolic factor Emi2/XErp1 to trigger its destruction and meiotic exit. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 608–613.
- Igarashi, H., Knott, J. G., Schultz, R. M., & Williams, C. J. (2007). Alterations of PLC β 1 in mouse eggs change calcium oscillatory behavior following fertilization. *Developmental Biology*, 312, 321–330.
- Igusa, Y., & Miyazaki, S. (1983). Effects of altered extracellular and intracellular calcium concentration on hyperpolarizing responses of the hamster egg. *The Journal of Physiology*, 340, 611–632.
- Igusa, Y., Miyazaki, S., & Yamashita, N. (1983). Periodic hyperpolarizing responses in hamster and mouse eggs fertilized with mouse sperm. *The Journal of Physiology*, 340, 633–647.
- Iino, M. (1990). Biphasic Ca^{2+} dependence of inositol 1,4,5-trisphosphate-induced Ca release in smooth muscle cells of the guinea pig taenia caeci. *The Journal of General Physiology*, 95, 1103–1122.
- Ito, J., Parrington, J., & Fissore, R. A. (2011). PLC ζ and its role as a trigger of development in vertebrates. *Molecular Reproduction and Development*, 78, 846–853.
- Jellerette, T., He, C. L., Wu, H., Parys, J. B., & Fissore, R. A. (2000). Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. *Developmental Biology*, 223, 238–250.
- Jia, J. L., Han, Y. H., Kim, H. C., Ahn, M., Kwon, J. W., Luo, Y., Gunasekaran, P., Lee, S. J., Lee, K. S., Kyu Bang, J., Kim, N. H., & Namgoong, S. (2015). Structural basis for recognition of Emi2 by Polo-like kinase 1 and development of peptidomimetics blocking oocyte maturation and fertilization. *Scientific Reports*, 5, 14626.
- Jin, J., Wu, L. J., Jun, J., Cheng, X., Xu, H., Andrews, N. C., & Clapham, D. E. (2012). The channel kinase, TRPM7, is required for early embryonic development. *Proceedings of the National Academy of Sciences of the United States of America*, 109, E225–233.
- Jones, K. T., Soeller, C., & Cannell, M. B. (1998). The passage of Ca^{2+} and fluorescent markers between the sperm and egg after fusion in the mouse. *Development*, 125, 4627–4635.
- Kashir, J., Konstantinidis, M., Jones, C., Heindryckx, B., De Sutter, P., Parrington, J., Wells, D., & Coward, K. (2012). Characterization of two heterozygous mutations of the oocyte activation factor phospholipase C ζ (PLC ζ) from an infertile man by use of minisequencing of individual sperm and expression in somatic cells. *Fertility and Sterility*, 98, 423–431.
- Kim, A. M., Bernhardt, M. L., Kong, B. Y., Ahn, R. W., Vogt, S., Woodruff, T. K., & O'Halloran, T. V. (2011). Zinc sparks are triggered by fertilization and facilitate cell cycle resumption in mammalian eggs. *ACS Chemical Biology*, 6, 716–723.
- Kurokawa, M., Sato, K., Wu, H., He, C., Malcuit, C., Black, S. J., Fukami, K., & Fissore, R. A. (2005). Functional, biochemical, and chromatographic characterization of the complete $[\text{Ca}^{2+}]_i$ oscillation-inducing activity of porcine sperm. *Developmental Biology*, 285, 376–392.
- Lepikhov, K., Wossidlo, M., Arand, J., & Walter, J. (2010). DNA methylation reprogramming and DNA repair in the mouse zygote. *The International Journal of Developmental Biology*, 54, 1565–1574.
- Loeb, J. (1899). On the nature of the process of fertilization and the artificial eggs of the sea urchin. *The American Journal of Physiology*, 3, 135–138.
- Madgwick, S., Nixon, V. L., Chang, H. Y., Herbert, M., Levasseur, M., & Jones, K. T. (2004). Maintenance of sister chromatid attachment in mouse eggs through maturation-promoting factor activity. *Developmental Biology*, 275, 68–81.
- Masui, Y., & Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *The Journal of Experimental Zoology*, 177, 129–145.
- Miao, Y. L., & Williams, C. J. (2012). Calcium signaling in mammalian egg activation and embryo development: The influence of subcellular localization. *Molecular Reproduction and Development*, 79, 742–756.
- Miyazaki, S. (1988). Inositol 1,4,5-trisphosphate-induced calcium release and guanine nucleotide-binding protein-mediated periodic calcium rises in golden hamster eggs. *The Journal of Cell Biology*, 106, 345–353.
- Miyazaki, S., & Ito, M. (2006). Calcium signals for egg activation in mammals. *Journal of Pharmacological Sciences*, 100, 545–552.
- Miyazaki, S., Hashimoto, N., Yoshimoto, Y., Kishimoto, T., Igusa, Y., & Hiramoto, Y. (1986). Temporal and spatial dynamics of the periodic increase in intracellular free calcium at fertilization of golden hamster eggs. *Developmental Biology*, 118, 259–267.
- Miyazaki, S., Yuzaki, M., Nakada, K., Shirakawa, H., Nakanishi, S., Nakada, S., & Mikoshiba, K. (1992). Block of Ca^{2+} wave and Ca^{2+} oscillation by antibody to the inositol 1,4,5-trisphosphate receptor in fertilized hamster eggs. *Science*, 257, 251–255.
- Nakano, Y., Shirakawa, H., Mitsuhashi, N., Kuwabara, Y., & Miyazaki, S. (1997). Spatiotemporal dynamics of intracellular calcium in the mouse egg injected with a spermatozoon. *Molecular Human Reproduction*, 3, 1087–1093.
- Neri, Q. V., Lee, B., Rosenwaks, Z., Machaca, K., & Palermo, G. D. (2014). Understanding fertilization through intracytoplasmic sperm injection (ICSI). *Cell Calcium*, 55, 24–37.
- Nomikos, M., Kashir, J., Swann, K., & Lai, F. A. (2013). Sperm PLC ζ : from structure to Ca^{2+} oscillations, egg activation and therapeutic potential. *FEBS Letters*, 587, 3609–3616.
- Parrington, J., Brind, S., De Smedt, H., Gangeswaran, R., Lai, F. A., Wojcikiewicz, R., & Carroll, J. (1998). Expression of inositol 1,4,5-trisphosphate receptors in mouse oocytes and early embryos: the type I isoform is upregulated in oocytes and downregulated after fertilization. *Developmental Biology*, 203, 451–461.
- Payne, C., Rawe, V., Ramalho-Santos, J., Simply, C., & Schatten, G. (2003). Preferentially localized dynein and perinuclear dyactin associate with nuclear pore complex proteins to mediate genomic union during mammalian fertilization. *The Journal of Cell Science*, 116, 4727–4738.
- Perreault, S. D., Wolff, R. A., & Zirkin, B. R. (1984). The role of disulfide bond reduction during mammalian sperm nuclear decondensation in vivo. *Developmental Biology*, 101, 160–167.
- Que, E. L., Duncan, F. E., Bayer, A. R., Philips, S. J., Roth, E. W., Bleher, R., Gleber, S. C., Vogt, S., Woodruff, T. K., & O'Halloran, T. V. (2017). Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy. *Integrative Biology: Quantitative Biosciences From Nano to Macro*, 9, 135–144.
- Rebecchi, M. J., & Pentyala, S. N. (2000). Structure, function, and control of phosphoinositide-specific phospholipase C. *Physiological Reviews*, 80, 1291–1335.
- Ridgway, E. B., Gilkey, J. C., & Jaffe, L. F. (1977). Free calcium increases explosively in activating medaka eggs. *Proceedings of the National Academy of Sciences of the United States of America*, 74, 623–627.

- Runft, L. L., Jaffe, L. A., & Mehlmann, L. M. (2002). Egg activation at fertilization: where it all begins. *Developmental Biology*, 245, 237–254.
- Sako, K., Suzuki, K., Isoda, M., Yoshikai, S., Senoo, C., Nakajo, N., Ohe, M., & Sagata, N. (2014). Emi2 mediates meiotic MII arrest by competitively inhibiting the binding of Ube2S to the APC/C. *Nature Communications*, 5, 3667.
- Sartain, C. V., & Wolfner, M. F. (2013). Calcium and egg activation in Drosophila. *Cell Calcium*, 53, 10–15.
- Satouh, Y., Nozawa, K., & Ikawa, M. (2015). Sperm postacrosomal WW domain-binding protein is not required for mouse egg activation. *Biology of Reproduction*, 93, 94.
- Saunders, C. M., Larman, M. G., Parrington, J., Cox, L. J., Royse, J., Blayney, L. M., Swann, K., & Lai, F. A. (2002). PLC zeta: A sperm-specific trigger of Ca^{2+} oscillations in eggs and embryo development. *Development*, 129, 3533–3544.
- Schultz, R. M., & Kopf, G. S. (1995). Molecular basis of mammalian egg activation. *Current Topics in Developmental Biology*, 30, 21–62.
- Simerly, C., Nowak, G., de Lanerolle, P., & Schatten, G. (1998). Differential expression and functions of cortical myosin IIA and IIB isotypes during meiotic maturation, fertilization, and mitosis in mouse oocytes and embryos. *Molecular Biology of the Cell*, 9, 2509–2525.
- Steinhardt, R. A., Epel, D., Carroll, E. J., Jr., & Yanagimachi, R. (1974). Is calcium ionophore a universal activator for unfertilized eggs? *Nature*, 252, 41–43.
- Stith, B. J., Goalstone, M., Silva, S., & Jaynes, C. (1993). Inositol 1,4,5-trisphosphate mass changes from fertilization through first cleavage in *Xenopus laevis*. *Molecular Biology of the Cell*, 4, 435–443.
- Stricker, S. A. (1999). Comparative biology of calcium signaling during fertilization and egg activation in animals. *Developmental Biology*, 211, 157–176.
- Swann, K. (1990). A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development*, 110, 1295–1302.
- Swann, K., & Lai, F. A. (2016). Egg activation at fertilization by a soluble sperm protein. *Physiological Reviews*, 96, 127–149.
- Swenson, K. I., Farrell, K. M., & Ruderman, J. V. (1986). The clam embryo protein cyclin A induces entry into M phase and the resumption of meiosis in *Xenopus* oocytes. *Cell*, 47, 861–870.
- Tung, J. J., Padmanabhan, K., Hansen, D. V., Richter, J. D., & Jackson, P. K. (2007). Translational unmasking of Emi2 directs cytostatic factor arrest in meiosis II. *Cell Cycle*, 6, 725–731.
- Tunquist, B. J., & Maller, J. L. (2003). Under arrest: Cytostatic factor (CSF)-mediated metaphase arrest in vertebrate eggs. *Genes & Development*, 17, 683–710.
- Turner, P. R., Sheetz, M. P., & Jaffe, L. A. (1984). Fertilization increases the polyphosphoinositide content of sea urchin eggs. *Nature*, 310, 414–415.
- Wakai, T., Vanderheyden, V., & Fissore, R. A. (2011). Ca^{2+} signaling during mammalian fertilization: Requirements, players, and adaptations. *Cold Spring Harbor Perspectives in Biology*, 3.
- Whittingham, D. G., & Siracusa, G. (1978). The involvement of calcium in the activation of mammalian oocytes. *Experimental Cell Research*, 113, 311–317.
- Wossidlo, M., Arand, J., Sebastiano, V., Lepikhov, K., Boiani, M., Reinhardt, R., Scholer, H., & Walter, J. (2010). Dynamic link of DNA demethylation, DNA strand breaks and repair in mouse zygotes. *The EMBO Journal*, 29, 1877–1888.
- Xu, Z., Kopf, G. S., & Schultz, R. M. (1994). Involvement of inositol 1,4,5-trisphosphate-mediated Ca^{2+} release in early and late events of mouse egg activation. *Development*, 120, 1851–1859.
- Yoda, A., Oda, S., Shikano, T., Kouchi, Z., Awaji, T., Shirakawa, H., Kinoshita, K., & Miyazaki, S. (2004). Ca^{2+} oscillation-inducing phospholipase C zeta expressed in mouse eggs is accumulated to the pronucleus during egg activation. *Developmental Biology*, 268, 245–257.
- Yoon, S. Y., & Fissore, R. A. (2007). Release of phospholipase C zeta and $[\text{Ca}^{2+}]_i$ oscillation-inducing activity during mammalian fertilization. *Reproduction*, 134, 695–704.

Mammalian Gestational Strategies: Embryonic Diapause and Pseudopregnancy

Bruce D Murphy, Université de Montréal, St-Hyacinthe, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

The key to evolutionary success in any lifeform is its ability to successfully reproduce and pass its genetic information on to succeeding generations. It is for this reason that evolution has favored strategies that maximize reproductive success. One of these is viviparity, the capacity for the development of the embryo and the fetus to occur within the body of the mother. While not restricted to mammals, viviparity is a strategy that accounts for much of the evolutionary success of mammals (Bainbridge, 2014). Widespread adaptation of variation in reproductive anatomy, physiology, and behavior further accounts for the survival of diverse members of the Class *Mammalia*. Two of the strategies that have contributed to this success are pseudopregnancy and embryonic diapause, the latter also known as delayed implantation.

Pseudopregnancy

Pseudopregnancy, also known as pseudocyesis, can be defined as a condition that resembles pregnancy, but in the absence of a fetus incubating in the uterus. In nature, it will reduce the frequency of pregnancy for a species and is seemingly contrary to the concept that maximum reproduction assures evolutionary success. For this reason, in most species, it is a relatively rare phenomenon. In experimental contexts, pseudopregnancy can be readily induced, and these models have provided valuable insight into reproductive function, in particular to the understanding of signals to register presence of the embryo in the uterus. Examples from two orders of mammals, *Rodentia* and *Carnivora*, have been chosen to illustrate the physiology of pseudopregnancy.

Rodents

Laboratory species of rodents, including the mouse, rat, and hamster, ovulate spontaneously and undergo repeated 4-day estrous cycles if not bred. The luteal phase, that is, the life span of the corpus luteum, comprises metestrus and diestrus and is but 2 days in unbred animals. If the animals are mated during this time, the luteal phase is extended until placental steroid hormone secretion, primarily progesterone, takes over the support of pregnancy (Ben-Zimra et al., 2002). The key event in conversion of the short-lived corpus luteum of the cycle to the longer surviving corpus luteum of pregnancy is the initiation of daily peaks of prolactin secretion from the pituitary (Fig. 1). In the mouse and rat, these occur twice daily, while a single peak characterizes the hamster signal. If these animals are mated to an infertile male, the corpus luteum and thus a state of pseudopregnancy will persist for 7–10 days. Then the well-known uterine secretion of prostaglandin F₂ α , produced by the endometrium in the absence of the embryo, will induce luteal demise. When the embryo is present, the trophoblast in the developing placenta secretes a prolactin-like hormone, placental lactogen-1 α that takes the place of prolactin and maintains the corpus luteum (Soares, 2004). If not, there is rapid loss of the capacity to synthesize progesterone, and eventually, there is structural regression of the corpus luteum. The hormonal profiles that demonstrate the differences among the cycling, pseudopregnant, and pregnant rat are depicted in Fig. 1.

Carnivores

Pseudopregnancy occurs in both felids and canids, and the best-studied models are the domestic cat and dog. In both, the duration of the luteal phase differs between the pseudopregnant and pregnant animals. In the domestic cat, ovulation is induced by copulation, and the consequent pregnancy supported by the corpus luteum has a duration of approximately 65 days. Circulating progesterone in pregnant animals increases through the first 25 days following mating and then begins to decline. The support of gestation is supplemented from the placenta, but luteal progesterone is essential to prevent abortion (Verstegen et al., 1993). If the queen is mated with a sterile male, a condition of pseudopregnancy ensues with functional corpora lutea that begin to regress approximately 20 days later, and luteal regression is complete by 40 or so days (Jewgenow et al., 2012). Administration of dopamine agonists at 30 days after mating effectively reduces prolactin secretion in the pregnant female cat, with the consequence of reduced progesterone synthesis, and abortion within a few days (Eilts, 2002). This indicates that prolactin is the principal luteotropic signal and is therefore expected to be responsible for the maintenance of the corpus luteum of pseudopregnancy. The embryonic signal that prevents luteal regression in the pregnant animal has not been discovered (Bazer, 2015).

Pregnancy and pseudopregnancy in the domestic dog have been studied in more detail than in other carnivore species, and much of what we know is due to the pioneering work of Concannon (2011). In contrast to the cat, the dog ovulates spontaneously, and pseudopregnancy is a common condition. It is often accompanied by mammary development and behavioral changes that mimic pregnancy, and lactation can be induced (Gobello et al., 2001). As with the cat, the luteal dynamics and progesterone profiles differ between pregnancy and pseudopregnancy, but not in the same manner. In the dog, the corpus luteum is required for the duration of pregnancy, as ovariectomy at any stage results in abortion. During pseudopregnancy, the canine corpus luteum has lower

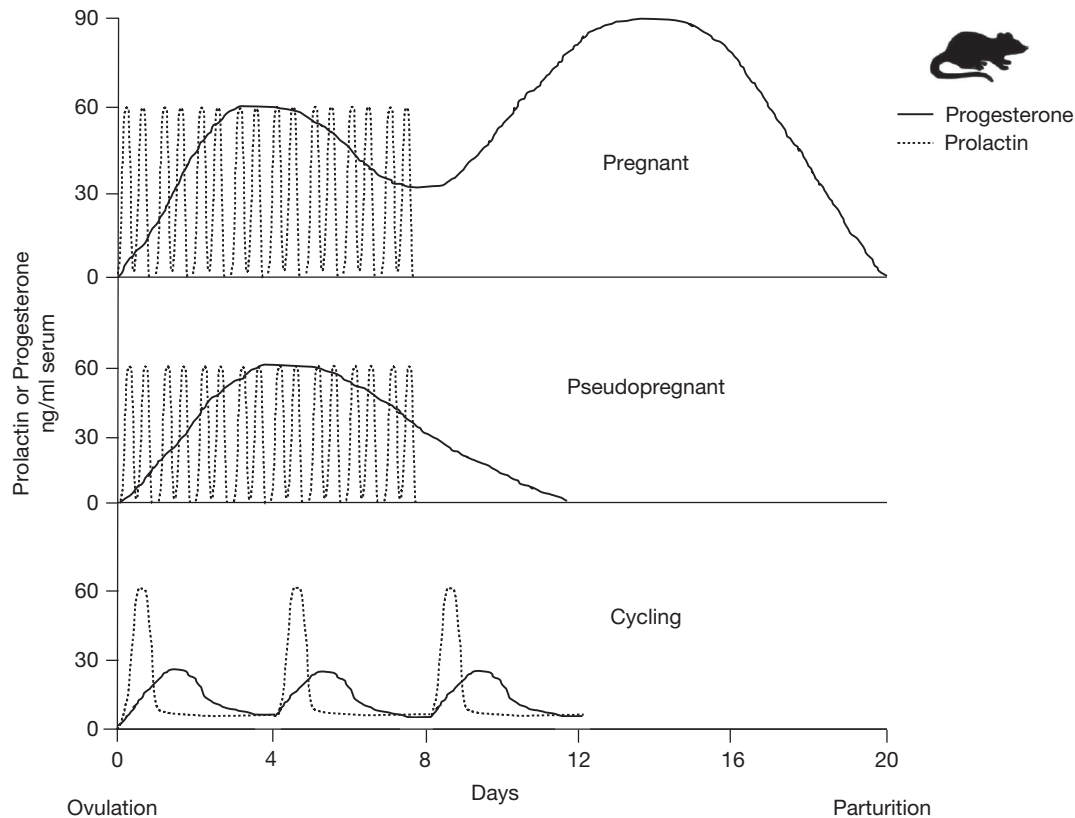


Fig. 1 Comparison of circulating profiles of the pituitary hormone, prolactin, and the ovarian hormone, progesterone, during cycling, unmated, pseudopregnant, and pregnant laboratory rats. Modified from Ben-Zimra, M., M. Koler, N. Melamed-Book, J. Arensburg, A. H. Payne, and J. Orly. (2002). Uterine and placental expression of steroidogenic genes during rodent pregnancy. *Molecular and Cellular Endocrinology* **187** (1–2), 223–231.

progesterone output relative to its pregnant counterpart (Concannon, 2011), but it can persist for weeks or more beyond the normal 60 or so day luteal phase in the pregnant animal (Kowalewski et al., 2015). The canine corpus luteum appears independent of gonadotropin influence for the first 25 days after ovulation, but for the remainder of gestation, the pituitary hormones, prolactin (PRL) and luteinizing hormone (LH), are required to maintain it (Concannon, 2011). Recent studies have shown that luteal expression of the genes for both LH and PRL receptors occurs in the pregnant bitch at parturition, but similar changes do not occur in the pseudopregnant animal, indicating different mechanisms of regulation between the two conditions (Pagnano Derussi et al., 2017). The hormonal patterns in canine pregnancy and pseudopregnancy are depicted in Fig. 2.

Evolutionary Significance of Pseudopregnancy

A tenet of evolution dictates that species survival depends on reproductive success, and the normal status of fertile females is pregnancy or lactation during the breeding season. Thus, it is unlikely in most species that pseudopregnancy is a common occurrence. In contrast, in wild canids, a condition known as cooperative breeding has been described, where only the dominant female of a group or pack whelps and pseudopregnancy is obligate in subordinate females (Asa and Valdespino, 1998). The expected evolutionary advantage is that lactating pseudopregnant members of a group can then participate in nursing and nurturing young, increasing their chances for survival.

Embryonic Diapause

Mammals of diverse phyletic provenance display another reproductive strategy, known as embryonic diapause or delayed implantation. This term describes the capacity of the embryo to undergo a reversible arrest, rather than proceed in the course of continual development that characterizes embryogenesis in most species (Fenelon et al., 2014). With very few exceptions, this arrest is visited on the embryo at the blastocyst stage of development (Fig. 3), and the proximal temporal regulation is imposed by the uterine environment. Embryonic diapause has been identified in over 130 mammalian species from 10 different mammalian orders. Diapause in mammals occurs in two forms, facultative diapause, where it occurs during pregnancy under certain conditions in

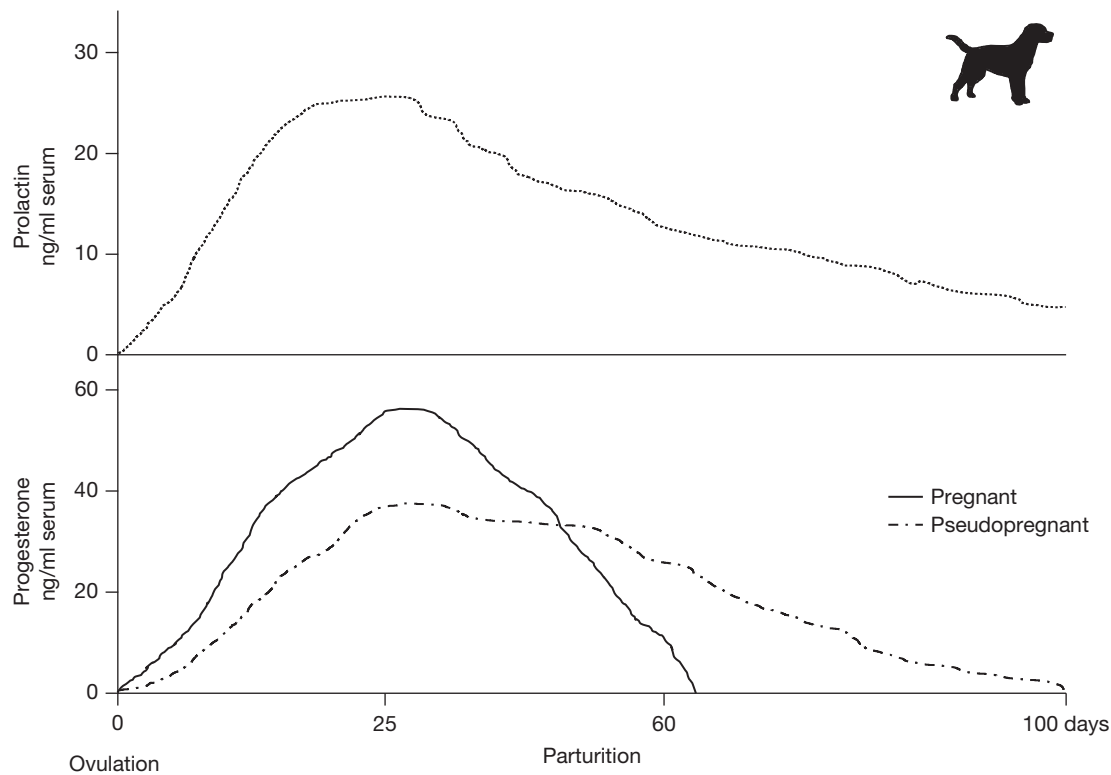


Fig. 2 Hormonal profiles of pregnancy and pseudopregnancy in the domestic dog during pregnancy and pseudopregnancy. Modified from Concannon, P. W. (2011). Reproductive cycles of the domestic bitch. *Animal Reproductive Science* **124** (3–4), 200–210.

some species, but not necessarily in all gestations (Table 1). The second form is known as obligate diapause, in that it is a component of every gestation in a species. The division is not absolute, as some marsupial species display a combination of the two types of diapause, where lactational delay is further extended by seasonal photoperiodic cues. The distribution, characteristics, and regulation of each of these two forms of diapause are discussed later. Although the range of external mechanisms and processes acting on the uterine environment during this time can differ significantly among species, many of the molecular controls of embryonic diapause appear to have been conserved (Fenelon et al., 2014).

Facultative Diapause

Facultative diapause, also known as lactational diapause, is common in species of rodents that experience a postpartum estrus. In this case, the female will mate soon after birth of its litter and then will be suckling one generation of young, while the next is developing in the uterus. The energetic stress of lactation results in arrest in the development of the embryonic generation at the blastocyst stage. In the mouse, the best-studied species with respect to lactational diapause, the embryo of 30–40 cells hatches from the zona pellucida on the fourth day after mating, followed by proliferation to approximately 130 cells over the following 72 h (Fig. 3) (Lopes et al., 2004). The blastocyst is then in a dormant state, incapable of implantation. A small number of cells in both the trophoblast and the inner cell mass of the dormant blastocyst undergo autophagy, and this autophagy is essential for recycling of nutrients to the remaining cells (Lee et al., 2011).

The ovary serves as the proximal regulator of embryonic diapause in the mouse and rat (Fenelon et al., 2014). When gestation is not interrupted by diapause, progesterone secreted by the corpus luteum is essential for the early development and preimplantation survival of the embryo. During the morning of the fourth day following mating, there is a brief peak in the secretion of estrogen from the ovary that induces implantation. The cellular provenance of this secretion remains unknown. It has been shown by serial blood sampling and by generations of experimentation that this brief estrogen surge is the indispensable signal, without which diapause ensues (Fenelon et al., 2014). The endocrine mechanism that induces and maintains diapause in the rat and mouse is the secretion of prolactin associated with lactation, which then prevents the surge of estrogen that induces implantation (Renfree, 2015). The role of estrogen appears to be multiple and includes direct effects on both the uterus and the embryo. The former includes large-scale changes in gene expression by up- or downregulation of hundreds of genes (Lopes et al., 2004). Among the first to be identified by targeted deletion was leukemia inhibitory factor (*Lif*), followed by prostaglandin synthesis-2 (*Ptgs2*) (Lee et al., 2007). Noninclusive lists of uterine genes essential to implantation in the mouse can be found in reviews by Cha and Dey (2015) and Lee et al. (2007). The estrogen episode has the effect of awakening the dormant embryo, either directly or through uterine

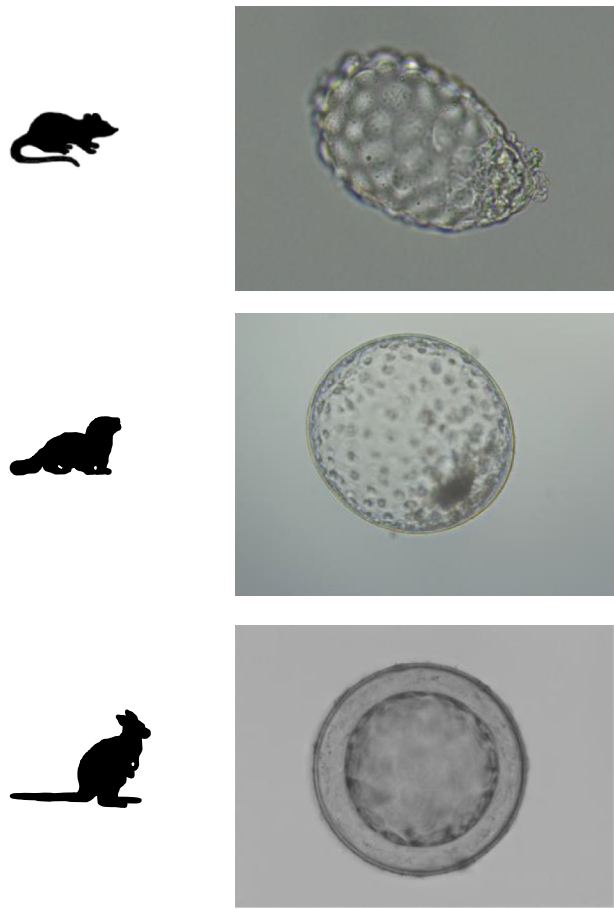


Fig. 3 Diapause occurs at the blastocyst stage of development in species where it is present. In the mouse, diapause follows hatching of the embryo from the zona pellucida 4 days after mating. In the mink, the blastocyst forms in the oviduct at 6 days after induced ovulation. In the wallaby, the unilaminar (no inner cell mass) blastocyst forms at 6 days after ovulation.

Table 1 Comparison of the characteristics of the two types of diapause, facultative or lactational and obligate

Facultative diapause	Obligate diapause
Induced by lactational or other metabolic stress	Present in every gestation of a species
Usually brief in rodents	Most commonly regulated by seasonal changes in photoperiod
Months of duration in marsupials	Duration of months in most species
Induced by prolactin from the pituitary	Common in Ursid and Mustelid carnivores where prolactin terminates diapause.
	Also found in the Roe deer and armadillos.

intermediates. The rapid effects include the formation of multivesicular bodies in the trophoblast cells and removal of accumulated autophagosomes (Lee et al., 2011), along with a burst of cell proliferation and differentiation of the trophoblast to acquire the invasive properties required for implantation (Bedzhov et al., 2014).

In addition to rodents, more than 35 marsupial species across five orders display lactationally induced diapause (Fenelon et al., 2014). The best-studied species is the tammar wallaby (*Macropus eugenii*), where the joey is born and makes its way to the mammary gland in the maternal pouch. Parturition is accompanied by postpartum estrus, and fertilization results in a new embryo that is soon arrested at the blastocyst stage, approximately 80 cells (Fig. 3). This blastocyst differs from the Eutherian model in that it is surrounded by a shell coat and is unilaminar, that is, there is no defined inner cell mass (Fenelon et al., 2014).

The endocrine events associated with lactation are the key regulators of marsupial facultative diapause, as departure of the joey from the pouch results in reactivation of the dormant embryo. In the tammar wallaby, the first changes in the embryo can be seen after 4 days. This is followed by embryo expansion some 4 days later and attachment to the endometrium after 18 days (Fenelon et al., 2014). During diapause, the corpus luteum is in an immature state, with only a basal level of secretion of the progesterone signal that is essential for the termination of diapause. Removal of the pituitary or blockade of prolactin secretion by dopamine

agonists results in increased progesterone secretion within 5 days (Renfree, 2015). While less is known about the uterine regulation of diapause in this species, a recent study has implicated epidermal growth factors as a conserved signal for termination of diapause (Fenelon et al., 2017b).

Obligate Diapause

The occurrence of a developmental arrest can be found in every gestation of a number of species, as diverse as the roe deer and the armadillo. Obligate diapause has been best studied in carnivores, where it can be found in more than 50 species in five orders (Fenelon et al., 2017a). The uncoupling of mating and parturition allows for mating during a favorable season, usually spring or fall, and parturition at a time optimal for neonate survival, usually spring. This gamut includes bears, wolverines, and aquatic species. The embryos of obligate diapause are uniformly in the blastocyst stage of development, but the number of cells present in the embryo usually far exceeds the complement found in rodent facultative diapause (Fig. 3) (Lopes et al., 2004). In the roe deer (*Capreolus capreolus*), for example, the blastocyst can be as large as 3 mm in diameter with more than 500 cells, while it is 1 mm in the nine-banded armadillo (*Dasypus novemcinctus*), with approximately 600 cells (Lopes et al., 2004).

The species that have been more extensively studied include the American mink (*Neovison vison*), the Western spotted skunk (*Spilogale gracilis*), and the European badger (*Meles meles*). In the former two of these species, the principal environmental regulator of obligate diapause has been definitively shown to be the seasonal change in photoperiod with increasing daylength, after the vernal equinox. This regulation is mediated through pineal secretion of melatonin (Fenelon et al., 2017a). The situation is opposite in seals, where implantation occurs during times of decreasing photoperiod (Atkinson, 1997). Diapause occurs in bears and its termination by implantation happens near the winter solstice, while the animals are in hibernation. There exists little information about regulation of diapause in these species, most likely because they are not a tractable model for study.

The photoperiodic effects are mediated through the hypothalamus–pituitary system (Fenelon et al., 2017a). In contrast to the facultative model of diapause, where prolactin is inhibitory to implantation, in the mustelid carnivores, prolactin plays a stimulatory role. In the members of the mustelid family that have been studied, the corpus luteum that develops following ovulation is small and actually regresses during the period of diapause (Mead, 1993). Prolactin is the essential signal for termination of diapause in that it induces massive increases in the secretion of progesterone and is the sole factor required for reactivation of the corpus luteum and consequent implantation (Fenelon et al., 2017a). The abundance of the prolactin receptor transcript in the mink corpus luteum is low during diapause and increases rapidly during reactivation. A concomitant increase in the expression of key steroidogenic enzymes also occurs. Although the embryo of the mink remains in diapause for around 1–2 weeks and the embryo of the spotted skunk remains in diapause for 6 months, the dynamics of luteal function are similar (Mead, 1993). In many carnivores, the progesterone profiles during diapause and reactivation, as determined by noninvasive evaluation of feces or urine, or indirectly by monitoring of body temperature, have been found to follow the pattern of a period of low progesterone secretion, followed by large increases associated with implantation (Fenelon et al., 2017a).

The proximal site for control of obligate diapause is the uterus, and two possible regulatory regimes can be envisioned. In the first, the uterine environment actively inhibits the development of the embryo beyond the blastocyst stage by secretion of one or more factors that interfere with the progression of embryogenesis. The second possibility is that the uterine environment lacks the appropriate constituents to support continued development. Available information support the latter hypothesis, in that reciprocal transplants of arrested blastocysts from a diapause species to a closely related species that does not display diapause results in reactivation of the embryo and implantation (Chang, 1968). Further, incubation of arrested blastocysts with immortalized uterine stroma can result in reactivation of development (Fenelon et al., 2017a).

Studies of uterine candidate genes associated with termination of obligate diapause showed that prostaglandin synthesis, and particularly, prostaglandins of the E-series were expressed by the uterus at the time of implantation, along with leukemia inhibitory factor (LIF) as an essential factor for rodent implantation (Fenelon et al., 2017a). Global gene analysis revealed that there are multiple genes that are upregulated or newly transcribed associated with activation of the embryo (Lefevre et al., 2011a). Gene ontology analysis focused on secretory factors and validation studies identified growth factors, as well as factors associated with the initiation and maintenance of the cell cycle (Lefevre et al., 2011a). It was discovered that a cluster of genes that regulate the synthesis and metabolism of the polyamines were likewise upregulated and associated with embryo activation (Lefevre et al., 2011b). In subsequent studies, it was shown that the polyamine, putrescine, can induce activation of the embryo from a diapause state and support its further development in vitro (Fenelon et al., 2017a). Polyamines play multiple roles in cell function, among these are the stimulation of gene expression, cell proliferation and tissue growth (Lefevre et al., 2011c). These are effects consistent with the reactivation of the dormant blastocyst. These findings argue that the basis of embryonic arrest is the absence of stimulation by polyamines and other uterine factors.

Evolution of Diapause

The phenomenon of embryo arrest can be found in both the animal and plant kingdoms, and as noted above, a wide range of mammals either display or can be induced to display this characteristic. Sandell (Sandell, 1990) hypothesized that diapause evolved independently multiple times, in both its facultative and obligate manifestations. This conclusion is based on the presence or

absence of diapause in congeneric species such as the ermine (*Mustela erminea*) and the least weasel (*Mustela nivalis*), a species that displays no diapause. In the spotted skunk, the western species (*Spilogale gracilis*) displays obligate diapause and its eastern counterpart, (*Spilogale putorius*), does not (Mead, 1993). The theory of convergent evolution of diapause is further supported by its manifestation in a wide variety of species, including armadillos, at least one mole, three species of bats, and an ungulate. These diverse species display considerable divergence in placentation, postimplantation embryogenesis, and other reproductive traits. As can be seen in the model species, the diversity of regulatory mechanisms further argues for independent evolution of diapause. Prolactin maintains diapause (marsupials), terminates diapause (mustelids), or has no effect (rodents). The ovaries are essential for implantation in the model species, but, remarkably, in the nine-banded armadillo, embryo reactivation and implantation are induced by ovariectomy (Buchanan et al., 1956).

Arguing for a single evolutionary event in mammals is the conservation of multiple mechanisms. For example, the transcriptional repressors coded by the muscle segment homeobox genes (MSX) are expressed in uterine stroma during diapause and are rapidly downregulated when the arrested blastocyst is reactivated (Cha et al., 2013). MSX1 is present in the mouse uterus during facultative delay, and in its absence, diapause and implantation do not occur. MSX1 is present in the stroma of the mink uterus during diapause, and MSX2 can be found in the uterus of the tammar wallaby (Cha et al., 2013). Likewise, the expression of epidermal growth factor (EGF) and heparin-binding EGF (HBEGF) and their receptors in the uterus are upregulated at the termination of diapause and reactivation of the embryo in both the wallaby and the mink (Fenelon et al., 2017a). Polyamine deprivation in vivo induces a state of diapause in both the mouse (Fenelon and Murphy, 2017) and the mink (Fenelon et al., 2017a). Facultative diapause, by its very nature, is inducible by metabolic stress and can be present in both rodents and marsupials. But can diapause be induced in species where it appears never to occur during normal pregnancy? In a single study, Ptak et al. (Ptak et al., 2012) induced diapause in sheep, a nondiapause species, by transfer of the embryos to the uterus of a mouse where the diapause condition had been experimentally induced by ovariectomy and hormone treatment. When the sheep embryos were transplanted back to the uteri of the ewes, live lambs were born. Thus, the hypothesis of a single evolutionary event remains viable and is perhaps the best explanation for the occurrence of diapause in mammals.

References

- Asa, C., & Valdespino, C. (1998). Canid reproductive biology: An integration of proximate mechanisms and ultimate causes. *American Zoologist*, 38, 251–259.
- Atkinson, S. (1997). Reproductive biology of seals. *Reviews of Reproduction*, 2(3), 175–194.
- Bainbridge, D. R. (2014). The evolution of pregnancy. *Early Human Development*, 90(11), 741–745.
- Bazer, F. W. (2015). History of maternal recognition of pregnancy. *Advances in Anatomy, Embryology, and Cell Biology*, 216, 5–25.
- Bedzhov, I., Leung, C. Y., Bialecka, M., & Zernicka-Goetz, M. (2014). In vitro culture of mouse blastocysts beyond the implantation stages. *Nature Protocols*, 9(12), 2732–2739.
- Ben-Zimra, M., Koler, M., Melamed-Book, N., Arensburg, J., Payne, A. H., & Orly, J. (2002). Uterine and placental expression of steroidogenic genes during rodent pregnancy. *Molecular and Cellular Endocrinology*, 187(1–2), 223–231.
- Buchanan, G. D., Enders, A. C., & Talmage, R. V. (1956). Implantation in armadillos ovariectomized during the period of delayed implantation. *Journal of Endocrinology*, 14(2), 121–128.
- Cha, J. M., & Dey, S. K. (2015). Reflections on rodent implantation. *Advances in Anatomy, Embryology, and Cell Biology*, 216, 69–85.
- Cha, J., Sun, X., Bartos, A., Fenelon, J., Lefevre, P., Daikoku, T., Shaw, G., Maxson, R., Murphy, B. D., Renfree, M. B., & Dey, S. K. (2013). A new role for muscle segment homeobox genes in mammalian embryonic diapause. *Open Biology*, 3(4), 130035.
- Chang, M. C. (1968). Reciprocal insemination and egg transfer between ferrets and mink. *Journal of Experimental Zoology*, 168(1), 49–59.
- Concannon, P. W. (2011). Reproductive cycles of the domestic bitch. *Animal Reproductive Science*, 124(3–4), 200–210.
- Eilts, B. E. (2002). Pregnancy termination in the bitch and queen. *Clinical Techniques in Small Animal Practice*, 17(3), 116–123.
- Fenelon, J. C., & Murphy, B. D. (2017). Inhibition of polyamine synthesis causes entry of the mouse blastocyst into embryonic diapause. *Biology of Reproduction*. <https://doi.org/10.1093/biolre/iox060>.
- Fenelon, J. C., Banerjee, A., & Murphy, B. D. (2014). Embryonic diapause: Development on hold. *International Journal of Developmental Biology*, 58(2–4), 163–174.
- Fenelon, J. C., Lefevre, P. L., Banerjee, A., & Murphy, B. D. (2017a). Regulation of diapause in carnivores. *Reproduction in Domestic Animals*, 52(Suppl 2), 12–17.
- Fenelon, J. C., Shaw, G., Frankenberg, S. R., Murphy, B. D., & Renfree, M. B. (2017b). Embryo arrest and reactivation: Potential candidates controlling embryonic diapause in the tammar wallaby and minkdogger. *Biology of Reproduction*, 96(4), 877–894.
- Gobello, C., de la Sota, R. L., & Goya, R. G. (2001). A review of canine pseudocyesis. *Reproduction in Domestic Animals*, 36(6), 283–288.
- Jewgenow, K., Amelkina, O., Painer, J., Goritz, F., & Dehnhard, M. (2012). Life cycle of feline corpora lutea: Histological and intraluteal hormone analysis. *Reproduction in Domestic Animals*, 47(Suppl 6), 25–29.
- Kowalewski, M. P., Gram, A., Kautz, E., & Graubner, F. R. (2015). The Dog: Nonconformist, not only in maternal recognition signaling. *Advances in Anatomy, Embryology, and Cell Biology*, 216, 215–237.
- Lee, K. Y., Jeong, J. W., Tsai, S. Y., Lydon, J. P., & DeMayo, F. J. (2007). Mouse models of implantation. *Trends in Endocrinology and Metabolism*, 18(6), 234–239.
- Lee, J. E., Oh, H. A., Song, H., Jun, J. H., Roh, C. R., Xie, H., Dey, S. K., & Lim, H. J. (2011). Autophagy regulates embryonic survival during delayed implantation. *Endocrinology*, 152(5), 2067–2075.
- Lefevre, P. L., Palin, M. F., Beaudry, D., Dobias-Goff, M., Desmarais, J. A., et al. (2011a). Uterine signaling at the emergence of the embryo from obligate diapause. *American Journal of Physiology. Endocrinology and Metabolism*, 300(5), E800–E808.
- Lefevre, P. L., Palin, M. F., Chen, G., Turecki, G., & Murphy, B. D. (2011b). Polyamines are implicated in the emergence of the embryo from obligate diapause. *Endocrinology*, 152(4), 1627–1639.
- Lefevre, P. L., Palin, M. F., & Murphy, B. D. (2011c). Polyamines on the reproductive landscape. *Endocrine Reviews*, 32(5), 694–712.
- Lopes, F. L., Desmarais, J. A., & Murphy, B. D. (2004). Embryonic diapause and its regulation. *Reproduction*, 128(6), 669–678.
- Mead, R. A. (1993). Embryonic diapause in vertebrates. *Journal of Experimental Zoology*, 266(6), 629–641.
- Pagnano Derussi, A., Meinsohn, M.-C., Destro, F., Gaitolini, C., Volpato, R., et al. (2017). Variation in the gene profile of the principal hormonal receptors in cyclic and gestational canine diestrus. In *Proceedings of Society for the Study of Reproduction*.
- Ptak, G. E., Tacconi, E., Czernik, M., Toschi, P., Modlinski, J. A., & Loi, P. (2012). Embryonic diapause is conserved across mammals. *PLoS ONE*, 7(3).
- Renfree, M. B. (2015). Embryonic diapause and maternal recognition of pregnancy in diapausing mammals. *Advances in Anatomy, Embryology, and Cell Biology*, 216, 239–252.

- Sandell, M. (1990). The evolution of seasonal delayed implantation. *Quarterly Review of Biology*, 65(1), 23–42.
- Soares, M. J. (2004). The prolactin and growth hormone families: Pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reproductive Biology and Endocrinology*, 2, 51.
- Verstegen, J. P., Oncin, K., Silva, L. D., Wouters-Ballman, P., Delahaut, P., et al. (1993). Regulation of progesterone during pregnancy in the cat: Studies on the roles of corpora lutea, placenta and prolactin secretion. *Journal of Reproduction and Fertility. Supplement*, 47, 165–173.

Further Reading

- Edwards, H. E., & Wynne-Edwards, K. E. (1994). Spontaneous termination of an induced pseudopregnancy in the Djungarian hamster, *Phodopus campbelli*. *Hormones and Behavior*, 28(2), 165–180.
- Egli, M., Leeners, B., & Kruger, T. H. (2010). Prolactin secretion patterns: Basic mechanisms and clinical implications for reproduction. *Reproduction*, 140(5), 643–654.
- Fenelon, J. C., Banerjee, A., & Murphy, B. D. (2014b). Embryonic diapause: Development on hold. *The International Journal of Developmental Biology*, 58(2–4), 163–174.
- Fenelon, J. C., Lefevre, P. L., Banerjee, A., & Murphy, B. D. (2017c). Regulation of diapause in carnivores. *Reproduction in Domestic Animals*, 52(Suppl 2), 12–17.
- Kowalewski, M. P., Gram, A., Kautz, E., & Graubner, F. R. (2015b). The Dog: Nonconformist, not only in maternal recognition signaling. *Advances in Anatomy, Embryology and Cell Biology*, 216, 215–237.
- Renfree, M. B. (2015b). Embryonic diapause and maternal recognition of pregnancy in diapausing mammals. *Advances in Anatomy, Embryology, and Cell Biology*, 216, 239–252.

Pregnancy Recognition Signals With an Emphasis on Ruminants

R Michael Roberts and Toshihiko Ezashi, University of Missouri, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Maternal Recognition of Pregnancy

In placental mammals, the ovarian follicular structure remaining after ovulation of an oocyte in the mature female transforms into a structure called the corpus luteum (CL), whose major role is to produce the hormone of pregnancy, progesterone. If the oocyte does not become fertilized, the CL (or CLs in the case of species that ovulate more than a single egg) regresses, and a new ovarian cycle begins. However, if fertilization is successful and if the pregnancy is to prosper, with some exceptions, such as in the bitch where the length of the luteal phase of the estrous cycle approximates the length of gestation, the functional lifespan of the CL must be increased (Bazer, 2015). This extension is necessary to provide continued production of progesterone, a steroid hormone whose action on the mucosal surface of the uterus (the endometrium) converts it to a state that allows the conceptus to attach to the uterine wall and subsequently to develop until it can survive outside the uterine environment. Although an ovarian supply of progesterone is not necessarily essential throughout pregnancy, because, the placenta itself can often be an alternative and sometimes necessary source of the steroid, it is invariably crucial in the early stages. In those few species of eutherian mammal where maternal recognition of pregnancy has been studied, it is the extra-embryonic trophoblast tissue that is the major player preventing luteal regression and ensuring conceptus survival, although the means by which this is achieved varies markedly across taxa and is understood in detail for only a few. Here we describe the discovery, function, and evolution of interferon-tau (IFNT), the anti-luteolytic factor responsible for luteal rescue in the Ruminantia, a sub-order within the Artiodactyla that includes the families Bovidae, Cervidae, Antilocapridae, Giraffidae (Leaman and Roberts, 1992), and, based on their close phylogenetic position to the Bovidae, the Moschidae (musk deer). As far as is known, no species outside this taxonomic grouping possesses a family of genes orthologous to the *IFNT*. Moreover, it is unclear whether any other mammalian grouping exploits IFN signaling pathways to accomplish CL rescue, although the conceptuses of some species, most notably the pig, do release both a Type I IFN (interferon-delta; IFND) and a Type II IFN (interferon-gamma; IFNG). However, more is probably known about maternal recognition of pregnancy in the ruminants than in any other mammal, including the human (and other primates) and laboratory animal species, such as the mouse, rat, and guinea pig.

It is well established that luteal regression in cattle and sheep, and most likely in all sub-primate species, is caused by the release of prostaglandin F₂-alpha (PGF) by the uterine endometrium towards the end of the ovarian cycle. In ruminants, at least, it is generally believed that it is the pulsatile pattern of PGF discharge rather than the threshold concentration achieved that is most critical in causing CL demise. In an animal that is pregnant, the conceptus rescues a CL hovering on the point of regression by dampening the production and/or pulsatile nature of PGF output from the endometrium. By the late 1970s it was clear that the active factor was proteinaceous in nature and produced by the conceptus for a few days during the short period in which it elongates from a sphere to a thread-like form that occupies both uterine horns (Roberts et al., 1992). Extracts of these tissues were sufficient to extend the lifespan of an ovine CL from a few days to several weeks although this state of pseudopregnancy never corresponded to the length of a normal gestation, which is approximately 140 days. Clearly other factors are required to reinforce the initial extension brought about by the trophoblast extracts, and these remain uncharacterized to this day.

The history of events that led to the characterization of the protein we now know as IFNT has been extensively reviewed (Roberts et al., 1992; Bazer and Thatcher, 2017; Ealy and Woodridge, 2017). Although Martal and colleagues partially purified a low Mr. protein (trophoblastin) from extracts of sheep conceptuses prepared at the time the conceptuses were releasing the anti-luteolytic factor, it was not fully characterized; nor was it purified in sufficient quantities to determine whether it was capable of extending luteal lifespan when injected into the uterine lumen of a non-pregnant ewe. A group of scientists at the University of Florida took a slightly different approach (Reviewed by Roberts et al., 1992). Reasoning that the protein must be secreted in relatively large quantities, they flushed d 14–17 ovine conceptuses from the uterine tract and cultured them for up to 48 h under serum free conditions in presence of radioactive amino acids. Through use of two-dimensional gel electrophoresis they noted a dominant secretory product consisting of 3–4 polypeptides of slightly acidic pI and Mr. about 18,000. Parallel studies performed on bovine conceptuses revealed a similar group of polypeptides of slightly higher Mr., which we now know to be glycosylated. The ovine product proved relatively simple to purify in sufficient amounts to demonstrate it could extend the estrous cycle of non-pregnant ewes. Antisera were also produced, which later allowed a bacteriophage cDNA expression library prepared from ovine conceptus mRNA to be screened and cDNAs to be cloned and sequenced. The inferred amino acid sequences provided a totally surprising result. The antiluteolytic factor, until then named variously as protein-X, ovine trophoblast protein 1 (oTP1) and trophoblastin, was clearly a Type I interferon (IFN), later officially called ovine IFNT-tau (ovIFNT). This IFN was structurally similar to, although clearly distinct from, interferon-alpha (IFNA) and interferon-beta (IFNB) and most resembled members of a lesser known family of Type I IFN, now named IFN-omega (IFNW). Two other groups subsequently confirmed the co-identity of trophoblastin and oTP1 and their close similarity to Type I IFN by molecular cloning techniques (Reviewed by Bazer and Thatcher, 2017). A structurally similar bovine IFN (boIFNT) was similarly identified after cloning its transcript from a bovine conceptus cDNA library.

The Anti-Luteolytic Action of IFNT

The cloning of the cDNAs for ovIFNT and boIFNT permitted the recombinant proteins to be produced in relatively unrestricted amounts in *E. coli* and yeast, and to be tested extensively in animals. However, even with the ready availability of the active proteins, it is still not completely clear how IFNT controls PGF production and release in order to protect the CL. What is apparent is that IFNT mainly acts locally, i.e. in a paracrine manner, on the endometrial epithelium to alter the expression of a wide array of genes, including the down-regulation of the gene for the oxytocin receptor (OXTR) (Reviewed by Hansen et al., 2017). The latter is usually up-regulated towards the end of the ovarian cycle in response to rising levels of estrogen. Evidence in sheep but not in cattle (Hansen et al., 2017) is consistent with the idea that towards the end of the second week of pregnancy IFNT inhibits transcription of *ESR1*, the gene encoding ESR1 (estrogen receptor 1), thereby preventing the estrogen-induced rise in OXTR. In absence of IFNT, oxytocin from either the pituitary or the CL binds to the uterine OXTR to initiate the release of small pulses of PGF into uterine venous blood. PGF then moves from the uterine vein to the ovarian artery by a countercurrent mechanism and triggers the release of additional amounts of oxytocin from the CL, which in turn stimulates further pulses of PGF by the uterine endometrium, thereby creating a positive feedback loop and ultimately causing luteolysis. IFNT may also decrease PGF production by down-regulating other key genes, including *PTGS2*, whose translation product catalyzes a rate limiting step in prostaglandin synthesis (Chen et al., 2006).

Like other Type I IFN, IFNT is a potent cytokine acting through the Type I IFN receptor, a complex of two subunits (IFNAR1 and IFNAR2), abundant on the uterine epithelium of the ewe. As with IFNA and IFNB, it directly regulates large numbers of what are often collectively called interferon-stimulated genes (ISGs), many of which are protective against virus pathogens (Chen et al., 2006). The fact that some IFNT appears to escape the pregnant uterus and enter the maternal blood stream suggests that it may act, not just locally, but in a far more systemic, endocrine manner, whose reach can be traced by following the induction of ISGs outside the immediate confines of the reproductive tract (Hansen et al., 2018). Indeed, one target is the ovary (Chen et al., 2006) where IFNT may induce luteal resistance to PGF (Reviewed by Hansen et al., 2017). The temporal induction of ISGs, especially ones that are secreted, may also provide an opportunity to test whether an animal is pregnant long before other tests are effective. Such tests, although not fully developed for commercial use, could be of particular value in the dairy and meat industries and also in zoos and conservation programs provided that they become reliable and not flawed by an abundance of false positives.

In summary, IFNT is released by the trophectoderm of the elongating conceptus before it attaches firmly to the uterine wall and is a crucial factor in the phenomenon of maternal recognition of pregnancy in ruminants by protecting the CL from PGF-induced destruction. It appears to accomplish this action in a number of ways: in an exocrine fashion by attenuating the release of luteolytic pulses of PGF from the uterine endometrium through control of OXTR concentrations, by down-regulating expression of endometrial *PTGS2* and other enzymes associated with PGF biosynthesis, and by a little understood, endocrine effects outside the uterine endometrium CL in ovary.

Expression of IFNT During Conceptus Development and Transcriptional Control of the IFNT Gene

Ovine (ov)IFNT production takes place largely between d 13–21 of pregnancy, with the peak at d 15, when a single cultured conceptus, which can be several cm in length, can produce as much as 0.5 mg of IFNT in 24 h (Roberts et al., 1992). These amounts are huge compared to any other known system known to generate a type I IFN, including virus-exposed leukocytes, especially as the K_d for the IFN receptor is in the region of 10^{-11} M (Reviewed by Hansen et al., 2017). These observations lend further credence to the premise that IFNT may need to target sites outside the reproductive tract as well as the uterine epithelium. A high rate of production is probably also important to trigger responses around the critical d 13 stage of development when the CL is on the point of regressing and when the tiny conceptus must exert an influence on both uterine horns if the pregnancy is to be maintained.

Although IFNT mRNA can be readily detected in ovine and bovine blastocysts, peak transcriptional activity is reached when the blastocysts have expanded and are just about to elongate, i.e. at about d 13 in the sheep, and ends during the period when the trophoblast forms a firm attachment with the uterine epithelium (Reviewed by Roberts et al., 1992). At all stages of development IFNT expression is confined strictly to the trophectoderm and silent in the inner cell mass and extraembryonic endoderm (Farin et al., 1989). In all these events relating to protein production and gene expression, similar outcomes are observed in cattle as in sheep, except events are delayed by two to three days. Expression in other ruminants can be inferred to be matched to the same elongation phase of development.

As discussed later, one feature that sets IFNT apart from other Type I IFN is their constitutive expression in trophectoderm and nowhere else, plus their lack of viral inducibility. There are no conserved viral response elements upstream of their transcription start sites. Instead, expression appears to be governed primarily by two regions (Leaman et al., 1994). One is a distal A/T rich element situated approximately –358 to –322 bp from the transcription start site, which has not been studied in detail. The second, more dominant one, is in a more proximal region (–91 to –69). Both of these regions form strong associations with nuclear proteins in electrophoretic mobility shift assays performed with extracts prepared from elongating ovine and bovine trophoblast. These complexes disappear as the IFNT become downregulated. Moreover, the removal of these enhancer regions, especially the proximal one, significantly reduces promoter activity.

Ezashi et al., used four tandem copies of the –91 to –69 region of the IFNT promoter as a 'bait' (or target) in a yeast single-hybrid system to screen for putative transcriptional regulators represented in a cDNA library prepared from day 13 elongating ovine conceptuses (Reviewed by Ezashi and Imakawa, 2017). The transcription factor ETS2 bound this bait sequence, which happens to contain a close-to-consensus ETS2 binding site with a central C/AGGAA/T core motif. The ETS2-binding motif is part of what appears to be a more complex enhancer with an adjacent AP1-binding site. Additionally, ETS2 is activated by the RAS/mitogen

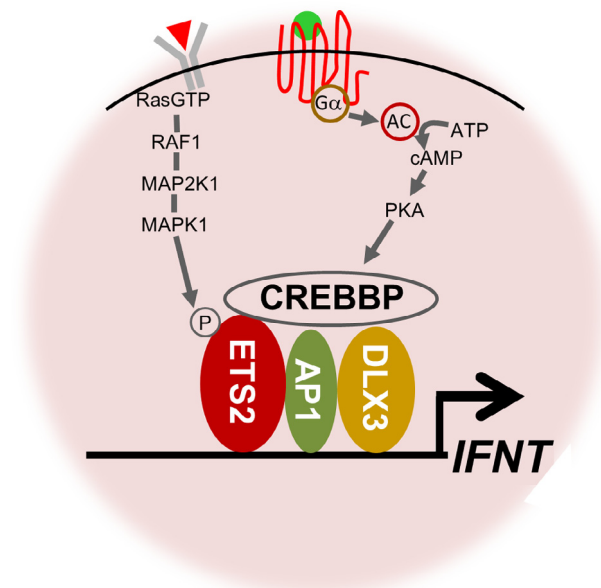


Fig. 1 A speculative model for *IFNT* transcriptional control in trophoblast during the stage of conceptus elongation. Transcriptional regulators ETS2, AP1, and DLX3 interact with the *IFNT* 5' UTR proximal region. Growth factors released from maternal endometrium activate MAP kinase and PKA signaling pathways and increase *IFNT* transcription through induction and/or activation of transcription factors and coactivators that associate with the ETS2-enhancer region. Modified from Ezashi, T., Imakawa, K. (2017). Transcriptional control of IFNT expression. *Reproduction* **154**, F1–F11.

activated protein (MAP) kinase pathway and protein kinase A (PKA) pathway through the coactivator CREBBP, making it responsive to external factors present in the maternal uterine environment, such as the cytokine CSF1, that operate through RAS-GTP coupled receptors (Fig. 1) (Ezashi and Roberts, 2004). Finally, a series of additional transcription factors that, like DLX3, have been implicated in the emergence and maturation of the trophoblast lineage may operate in combination with ETS2 and AP1 to synchronize the remarkably rapid up-regulation and almost equally fast down-regulation of *IFNT* in the trophoblast, events that occur over the course of only about a week (Reviewed by Ezashi and Imakawa, 2017). These interactions are summarized in Fig. 1.

What Distinguishes IFNT From Other Type I IFN?

In the previous section we described some of the unique features of the IFNT, namely the lack of viral response elements within their proximal 5' UTR regions and their constitutive and localized expression to trophoblast. Despite these unusual attributes, the biological activities of IFNT are not very different from those of other type I IFN. For example, they bind to the same Type I IFNAR1/IFNAR2 receptor system with high affinity, and, as a consequence, are powerful antiviral, anti-proliferative, and immunomodulatory agents (Reviewed by Bazer and Thatcher, 2017; Roberts et al., 1992). These actions appear to be mediated primarily by the same JAK-STAT1/STAT2-IRF1 signal transduction pathway utilized by the other Type I IFN (Stewart et al., 2002) and cause a similar up-regulation of a common set of interferon-responsive genes (IRGs) (Hansen et al., 2017). Indeed, IFNA, when injected into the uteri of non-pregnant cattle, is also able to induce an extension of the estrous cycle comparable in length to that achieved with boIFNT (Roberts et al., 1992). Finally the six-amino acid (aa) sequence at the carboxyl tail of the 172 IFNT, which distinguishes these proteins from the 166 aa IFNA, appears to have little significance in terms of biological potency (Ealy et al., 1998).

One conclusion that might be drawn from these data is that the exceptionality of the IFNTs does not reside in their biological and structural properties. Instead, their uniqueness relates to where they are expressed, the timing of their expression, and the quantities in which they are produced. These are features most likely conferred by powerful selection for changes in nucleotide sequences outside the open reading frames of their genes.

However, over the course of the past 20 years there have been a series of reports that ovIFNT, in particular, is anti-inflammatory and somehow less toxic than other type I IFN. It has been claimed for example that this reagent might have therapeutic value in treating autoimmune diseases, such as multiple sclerosis, obesity, and even HIV AIDS (Bazer and Thatcher, 2017). To the authors' knowledge none of these claims have been vindicated in clinical trials. Nor has there been extensive laboratory follow-up on the initial reports.

Evolution of the IFNT

It quickly became apparent from the nucleotide sequences of transcripts and cloned genes, as well as from genomic Southern blotting, that cattle, sheep, and most likely other pecoran ruminants possessed more than a single IFNT gene

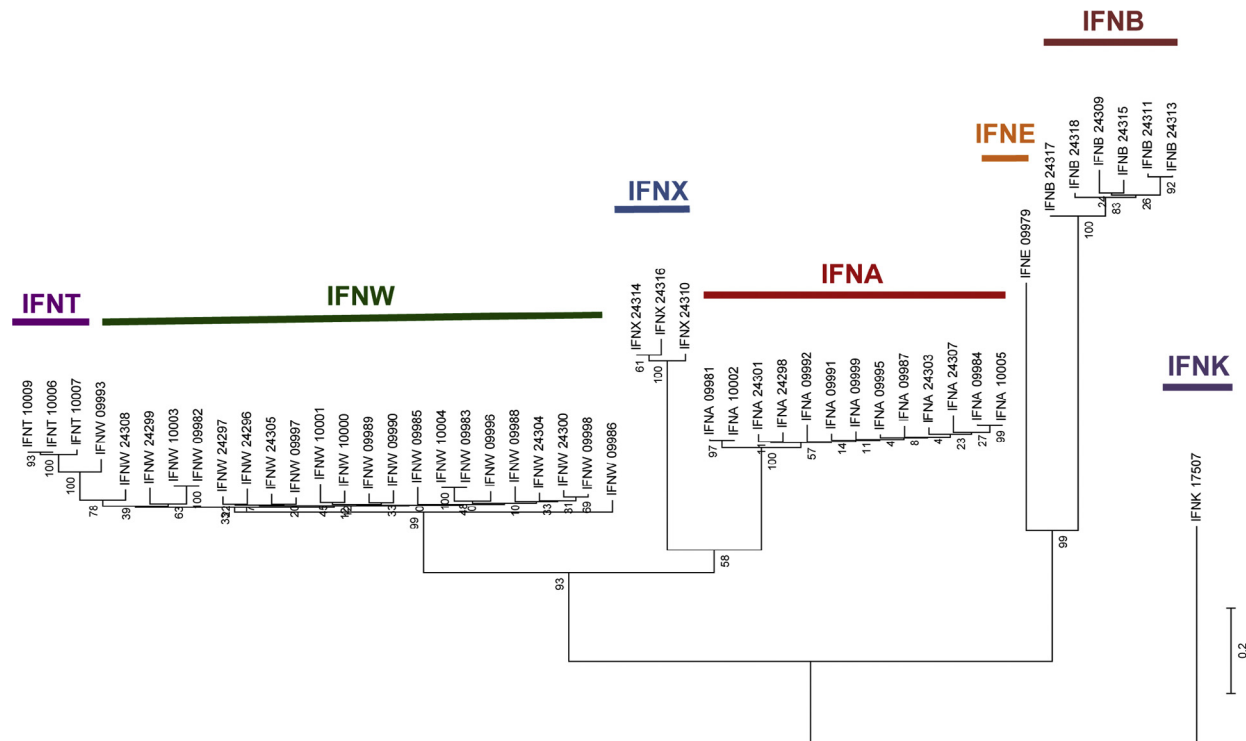


Fig. 2 Phylogenetic Tree of the Bovine Type I IFN. The evolutionary history of bovine Type I *IFN* was inferred by using the Neighbor-Joining (NJ) method. The tree was rooted to *IFN-kappa* (*IFNK*) and calculations were based on uniform rates of change for all sites.

(Leaman and Roberts, 1992). How many such genes are actually present in a particular genome remains unclear, however, because recently spawned tandem repeats occurring via either gene duplication events or by gene conversion are difficult to distinguish. Additionally, numerous sequence variants have been detected by cloning RNA from individual conceptuses. For example 26 such sequences have been cloned from cattle conceptuses (Ealy and Woolridge, 2017). Some of these transcripts may represent allelic variants, others novel genes. There are also indications that different breeds within a species and even individual animals within a breed may vary in the number and nature of *IFNT* they possess. The sequenced genome of a single Hereford bull revealed only three *IFNT* (Fig. 2), but none of these had exact sequence matches to the transcript sequences, including the two commonest, present in data bases (Walker and Roberts, 2009). Fig. 2 also illustrates the extraordinary recent expansion of the IFNW family in cattle, and defines a previously unknown family, the IFNX, whose function is unknown but which emerged well before the *IFNT* and *IFNW*.

Despite this uncertainty about gene number, calculations based on nucleotide substitution rates indicate that the *IFNT* and their nearest relatives, the *IFNW*, which also consist of a 195 aa codon open reading frame, diverged approximately 35 million years ago in the lineage leading to present day cattle, deer, giraffes, and their relatives. As a consequence, the *IFNT* do not occur outside of the ruminant, even-toed ungulates. The evolution of the *IFNT* from a progenitor *IFNW*, which was most likely involved in responses to pathogens, to a non-virally inducible placental hormone necessary for pregnancy recognition must have coincided with the acquisition of powerful trophoblast-specific enhancer elements in its genes. This unique mechanism for trophoblast signaling appears to have evolved, therefore, in parallel with the distinctive, evolutionarily advanced, non-invasive, synepitheliochorial placentation that characterizes pecoran ruminant species.

References

- Bazer, F. W. (2015). History of maternal recognition of pregnancy. *Advances in Anatomy, Embryology and Cell Biology*, 216, 5–25.
- Bazer, F. W., & Thatcher, W. W. (2017). Chronicling the discovery of interferon tau (vol. 154). Reproduction.
- Chen, Y., Green, J. A., Antoniou, E., et al. (2006). Effect of interferon-tau administration on endometrium of nonpregnant ewes: a comparison with pregnant ewes. *Endocrinology*, 147, 2127–2137.
- Ealy, A. D., Alexenko, A. P., Keisler, D. H., & Roberts, R. M. (1998). Loss of the signature six carboxyl amino acid tail from ovine interferon-tau does not affect biological activity. *Biology of Reproduction*, 58, 1463–1468.
- Ealy, A. W., & Woolridge, D. (2017). The evolution of IFNT (vol. 154). Reproduction.
- Ezashi, T., & Imakawa, K. (2017). Transcriptional control of IFNT expression (vol. 154). Reproduction.
- Ezashi, T., & Roberts, R. M. (2004). Regulation of interferon-tau (IFN-tau) gene promoters by growth factors that target the Ets-2 composite enhancer: a possible model for maternal control of IFN-tau production by the conceptus during early pregnancy. *Endocrinology*, 145, 4452–4460.

- Farin, C. E., Imakawa, K., & Roberts, R. M. (1989). In situ localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. *Molecular Endocrinology*, 3, 1099–1107.
- Hansen, T. R., Sinedino, L. D. P., & Spencer, T. E. (2017). Paracrine and endocrine actions of interferon tau (IFNT) (vol. 154). *Reproduction*.
- Leaman, D. W., Cross, J. C., & Roberts, R. M. (1994). Multiple regulatory elements are required to direct trophoblast interferon gene expression in choriocarcinoma cells and trophectoderm. *Molecular Endocrinology*, 8, 456–468.
- Leaman, D. W., & Roberts, R. M. (1992). Genes for the trophoblast interferons in sheep, goat, and musk ox and distribution of related genes among mammals. *Journal of Interferon Research*, 12, 1–11.
- Roberts, R. M., Cross, J. C., & Leaman, D. W. (1992). Interferons as hormones of pregnancy. *Endocrinology Reviews*, 13, 432–452.
- Stewart, M. D., Choi, Y., Johnson, G. A., et al. (2002). Roles of Stat1, Stat2, and interferon regulatory factor-9 (IRF-9) in interferon tau regulation of IRF-1. *Biology of Reproduction*, 66, 393–400.
- Walker, A. M., & Roberts, R. M. (2009). Characterization of the bovine type I IFN locus: rearrangements, expansions, and novel subfamilies. *BMC Genomics*, 10, 187.

Chorionic Gonadotropin

Michael Strug, Michigan State University, Grand Rapids, MI, United States; and Spectrum Health, Grand Rapids, MI, United States
Asgerally Fazleabas, Michigan State University, Grand Rapids, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

α -SMA α -Smooth muscle actin
ART Assisted reproductive technologies
CG Chorionic gonadotropin
CL Corpus luteum
cAMP 3',5'-Cyclic adenosine monophosphate
ERK1/2 Extracellular signal-regulated protein kinases 1 and 2
FSH Follicle stimulating hormone
G_s Stimulatory G-protein
GTD Gestational trophoblastic disease
hCG Human chorionic gonadotropin
h-hCG Hyperglycosylated human chorionic gonadotropin
IVF In vitro fertilization
LH Luteinizing hormone
LHCGR LH/CG receptor
LIF Leukemia inhibitory factor
PAPP-A Pregnancy-associated plasma protein-A
PCOS Polycystic ovary syndrome
PGR Progesterone receptor
PKA Protein kinase A
PLC Phospholipase C
PGE₂ Prostaglandin E₂
TSH Thyroid stimulating hormone
TGF β Transforming Growth Factor β
uNK Uterine natural killer

Introduction and Historical Perspectives

The highly-coordinated series of events leading to the establishment of pregnancy are largely orchestrated by the embryo. The presence of a developing embryo ensures adequate progesterone synthesis from the corpus luteum (CL) initially which primes the maternal reproductive tract to both transport and receive the implanting embryo. Additionally, the embryo produces signals that directly promote uterine receptivity. In primates, the principal embryonic signal driving these central processes is Chorionic Gonadotropin (CG).

Our understanding of CG's functional role as a gonadotropin began long before the isolation, purification and characterization of this secreted hormone ([Bahl, 1969](#)). Placental tissue, a primary source of human CG (hCG) production, was described to stimulate genital tract growth and ovulation in rodents as early as 1910 ([Cole, 2010](#)). In a landmark discovery, Ascheim and Zondek developed the first clinical pregnancy test utilizing this functional role of CG ([Ascheim and Zondek, 1928](#)). Treatment of mice with urine from pregnant women induced ovarian follicular development with ovulation resulting in the presence of macroscopically enlarged ovaries with hemorrhages. The name Chorionic Gonadotropin was derived from the latin word for its source of origin, *chordata* or afterbirth, and its functional role stimulating gonads, or gonadotropin ([Cole, 2010](#)). CG is unique to humans and anthropoid primates, and although an equine CG exists, this chapter will focus on the role of CG in human and non-human primate reproduction. Since its discovery, CG has been determined to drive key physiologic functions during the establishment of pregnancy and, more recently, its potentially significant clinical utility in Reproductive Medicine has been under investigation.

Sources, Structure and Isoforms

The placenta and its embryonic derivatives, specifically trophoblast cells, represent the primary source of hCG, although 8-cell stage embryos have been described to produce hCG prior to blastocyst development ([Banerjee and Fazleabas, 2010](#); [Cunningham et al.,](#)

Table 1 hCG isoforms: source of production, structural differences and global function (adapted from Choi and Smitz, 2014; Cole, 2010)

<i>Isoform</i>	<i>Predominant Source</i>	<i>Structural Differences from hCG</i>	<i>Global functions</i>
hcG	Fused and differentiated syncytiotrophoblast cells	N/A	Lutotropic and antiluteolytic actions for progesterone secretion Endometrial epithelial cell secretory function Endometrial stromal cell survival Myometrial growth and relaxation during pregnancy Angiogenesis and recruitment of uNK cells for uterine vascular remodeling Differentiation of cytotrophoblast cells Testicular production of testosterone in the male fetus Promotes growth and invasion of cytotrophoblast cells for embryo implantation
H-hCG	Extravillous cytotrophoblast cells and choriocarcinoma cells	Longer, more complex oligosaccharide side chains	Promotes cancer growth and invasiveness
Free β -hCG	Advanced nontrophoblastic malignancies	Hyperglycosylated free β -subunit and lacks α -subunit	Mimics LH function to promote ovarian follicle development, ovulation and luteinization
Pituitary hCG	Anterior pituitary gonadotrophic cells	Sulfated rather than sialylated oligosaccharides	

2013). CG is a member of a family of hetero-dimeric glycoprotein hormones comprised of luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH). The structure of these glycoprotein hormones consists of an identical α subunit, and they differ largely based on their β subunits (Srisuparp et al., 2001). The shared α subunit is encoded by a single gene *CGA*, which produces a 92-amino acid residue glycoprotein with two N-linked oligosaccharides. Within the aforementioned group of glycoprotein hormones, CG and LH contain the highest degree of homology, and it is likely that CG, found only in equine and primate species, is an evolutionary derivative of LH present in all species (Bulun, 2011; Choi and Smitz, 2014). Genes for the CG and LH β subunits are found within a cluster of seven similar sequences, where four encode the CG β subunit (*CGB*, *CGB5*, *CGB7*, and *CGB8*), one encodes the LH β subunit (*LHB*), and the remaining two are pseudogenes (*CGB1*, *CGB2*). The CG β subunit consists of 145-amino acid residues with two N-glycosylation and four O-glycosylation sites, while LH undergoes cleavage resulting in a 121-amino acid sequence. The presence of multiple β subunit genes, enhanced promoter expression patterns and post-translational modifications favor increased levels and stability of CG in circulation. Overall, these evolutionarily developed features ensure CG is rapidly induced and maintained during the establishment of pregnancy.

Four well-characterized isoforms of hCG have been described: (1) hCG, (2) hyperglycosylated hCG (h-hCG), (3) free β -hCG, and (4) pituitary hCG (Choi and Smitz, 2014). These hCG isoforms differ in their biochemical and physiologic properties along with their source of synthesis (Table 1). hCG represents the predominant isoform during pregnancy and is produced by fused and differentiated syncytiotrophoblast cells of the developing chorionic villi of the placenta (Cole, 2010). Syncytiotrophoblast cells are placental epithelial cells in direct communication with the maternal endometrial vasculature at the maternal-fetal interface. hCG is the most well-characterized of hCG isoforms and coordinates traditional functions important for promoting CL secretion of steroid hormones to support the establishment of pregnancy while preventing its regression. These mechanisms along with embryonic and additional maternal responses to hCG will be further described in Section IV. H-hCG, as its name implies, contains longer, more complex oligosaccharide side chains as compared to hCG. H-hCG is produced by root or extravillous cytotrophoblast cells. Its expression peaks during the first trimester, exceeding that of hCG, and subsequently declines during the remainder of pregnancy. As a result of the additional sugar moieties, typical protein folding is altered exposing additional receptor binding regions, including the TGF β receptor, and functional roles (Cole, 2010). Functions of h-hCG are primarily autocrine on cytotrophoblast cells, promoting growth and invasion while preventing apoptosis, which is critical during implantation and placentation. However, tight regulation of h-hCG expression is necessary to prevent pathological invasiveness. For example, choriocarcinoma cells secrete high levels of h-hCG contributing to their malignant potential. Free β -hCG are hyperglycosylated hCG β -subunits, lacking α subunits. While found at low levels during pregnancy, elevations of free β -hCG are present in the setting of advanced stage nontrophoblastic malignancies (Cole and Butler, 2008). Without an α subunit, free β -hCG does not activate canonical hCG signaling through the LH/CG receptor but likely promotes cancer development and invasiveness through non-canonical pathways described for h-hCG (Cole, 2009). Lastly, pituitary hCG is secreted by anterior pituitary gonadotrophic cells and consists of sulfated rather than sialylated oligosaccharides, resulting in its much shorter half-life than hCG (Cole, 2010). Pituitary hCG secretion occurs in a menstrual cycle-dependent fashion mirroring LH in both pattern and function, promoting ovarian follicle development, ovulation and luteinization with progesterone secretion.

LH/CG Receptor Structure, Localization and its Downstream Signaling

Both LH and hCG exert their canonical signaling through a shared receptor, the LH/CG receptor (LHCGR). The LHCGR is a 675-amino acid member of the glycoprotein hormone receptor subfamily within the superfamily of G-protein-coupled receptor

(GPCR)/seven-transmembrane domain receptors superfamily (McFarland et al., 1989). TSH and FSH receptors are classified within the same subfamily, consistent with the structural similarities of these glycoproteins with LH and CG. The LHCGR contains a large N-terminal extracellular domain with many leucine-rich repeats and sites for N-linked glycosylation involved in protein-protein interactions. The remainder of the receptor consists of seven-transmembrane domains and a C-terminal domain, which initiates signal transduction upon ligand binding to the extracellular domain. Interestingly, the LHCGR is encoded by 11 exons, of which the first 10 contribute to producing the large extracellular domain while the final exon generates the endodomain.

LHCGR expression has been studied in both gonadal and extra-gonadal (reproductive and non-reproductive) tissues. In the ovaries, LHCGR is expressed by multiple cell types including luteal, thecal, interstitial and granulosa cells. LHCGR is temporally expressed in the ovary during the ovarian cycle (Choi and Smitz, 2014). Following the LH surge, LHCGR levels decrease prior to ovulation and are subsequently induced during the mid-luteal phase. Finally, LHCGR levels decline along with CL regression. In the human endometrium, full length LHCGR is expressed during the proliferative and secretory phase, while a truncated form predominates in the decidua during early pregnancy (Banerjee and Fazleabas, 2010). Prior to pregnancy in the baboon, LHCGR expression is reduced in the proliferative phase but is induced in luminal and glandular epithelium during the secretory phase. During early pregnancy in the baboon, LHCGR localizes to the endometrial stromal cells surrounding the spiral arteries, associated with the onset of decidualization. Subsequently, LHCGR expression decreases as pregnancy progresses in the mature decidua, which parallels a decline in peripheral serum CG levels. Lastly, control of LHCGR signaling is mediated by both receptor desensitization due to conformational changes following continuous ligand binding and synthesis of an hCG/LH receptor binding protein with *endo*- and *exo*-nuclease activity leading to degradation of receptor mRNA (Cole, 2010).

Downstream LHCGR signaling is cell-type specific. In the ovary, ligand binding initiates a receptor conformational change resulting in activation of the classically-described stimulatory G-protein (G_s) (Cole, 2010). Subsequently, the G_s induces adenylate cyclase activity, yielding 3',5'-cyclic adenosine monophosphate (cAMP). Lastly, cAMP production initiates downstream signaling by protein phosphorylation and gene transcription, through activation of protein kinase A (PKA) and the cAMP responsive element, respectively. This signaling pathway is associated with classical effector pathways of CG, including transcription of cholesterol side-chain cleavage enzyme for progesterone secretion in luteal cells. Activation of alternate signaling pathways have also been described (Cole, 2010). For example, LHCGR signaling through phospholipase C (PLC) activation catalyzes phosphoinositide and production of secondary messengers inositol triphosphate and diacylglycerol. Significantly higher LH or CG levels, such as during the pre-ovulatory LH peak or in pregnancy respectively, favors the PLC pathway. Lastly, LHCGR activation mediates signaling through the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) and AKT pathways independent of PKA. This is particularly relevant in the setting of endometrial epithelial cells, which contain undetectable levels of cAMP following CG stimulation, and, rather, production of targets cyclooxygenase 2 (COX2) and prostaglandin E_2 (PGE₂) are mediated by ERK1/2 (Srisuparp et al., 2003).

While LH and CG both bind to the LHCGR, there are ligand-specific differences in receptor activation and downstream signaling (Choi and Smitz, 2014). CG acts as a super-agonist binding the LHCGR with greater affinity compared to LH. Downstream cAMP production is significantly greater in response to CG versus LH treatment. Alternatively, treatment of human granulosa cells with recombinant LH induces ERK1/2 and AKT signaling to a greater extent than CG. Additionally, the temporal stimulation of LHCGR by different ligand impacts cell response. For instance, chronic treatment with LH favors growth of granulosa cells in culture while chronic CG exposure impairs this process. Overall, the evolutionarily developed structural differences in LH versus CG, described in Section II, likely contribute to their distinct LHCGR binding capacity and downstream signaling.

Embryonic and Maternal Responses to Chorionic Gonadotropin

It is well established that CG functions to rescue the CL from regression during the establishment of pregnancy, which maintains progesterone production for embryo implantation. Spontaneous abortion, or pregnancy loss, will occur in the absence of this CL-derived progesterone. CG maintains the CL through a luteotropic role and as an inhibitor of luteolysis until the luteal-placental shift occurs (Srisuparp et al., 2001). Luteotropic mechanisms coordinated by CG include induction of genes responsible for steroidogenesis, such as steroidogenic acute regulatory protein, cytochrome P450 cholesterol side-chain cleavage, and 3 β -hydroxysteroid dehydrogenase. Anti-luteolytic properties of CG include maintenance of luteal blood flow, prevention of apoptosis, and regulation of tissue remodeling and macrophage recruitment.

While CG's role in maintaining CL progesterone production is well established, its secretion persists throughout pregnancy beyond the point where it is no longer required for progesterone secretion. Therefore, extra-gonadal functions for CG have been studied in depth (Fig. 1). Among these roles, CG coordinates the establishment of the maternal-fetal interface through directly regulating key events in both the uterus and the placenta (Cole, 2010; Srisuparp et al., 2001). Prior to embryo implantation, CG promotes endometrial receptivity, which was characterized using a baboon model of simulated pregnancy (Banerjee and Fazleabas, 2010; Fazleabas et al., 1999).

CG directly modulates endometrial compartment-specific functions before, during and after implantation (Banerjee and Fazleabas, 2010; Fazleabas et al., 1999). In the luminal epithelium, CG promotes the "epithelial plaque" reaction, characterized by hyperplasia and differentiation of epithelial cells at the implantation site. In the glandular epithelium, CG promotes the expression of factors that provide a favorable microenvironment for implantation. These factors include leukemia inhibitory factor (LIF), glycodelin and COX2. LIF regulates trophoblast cell adhesion and may be important for embryo invasion and placental development. Glycodelin, one of the most abundant glycoproteins present in the uterus during implantation, plays an important role in

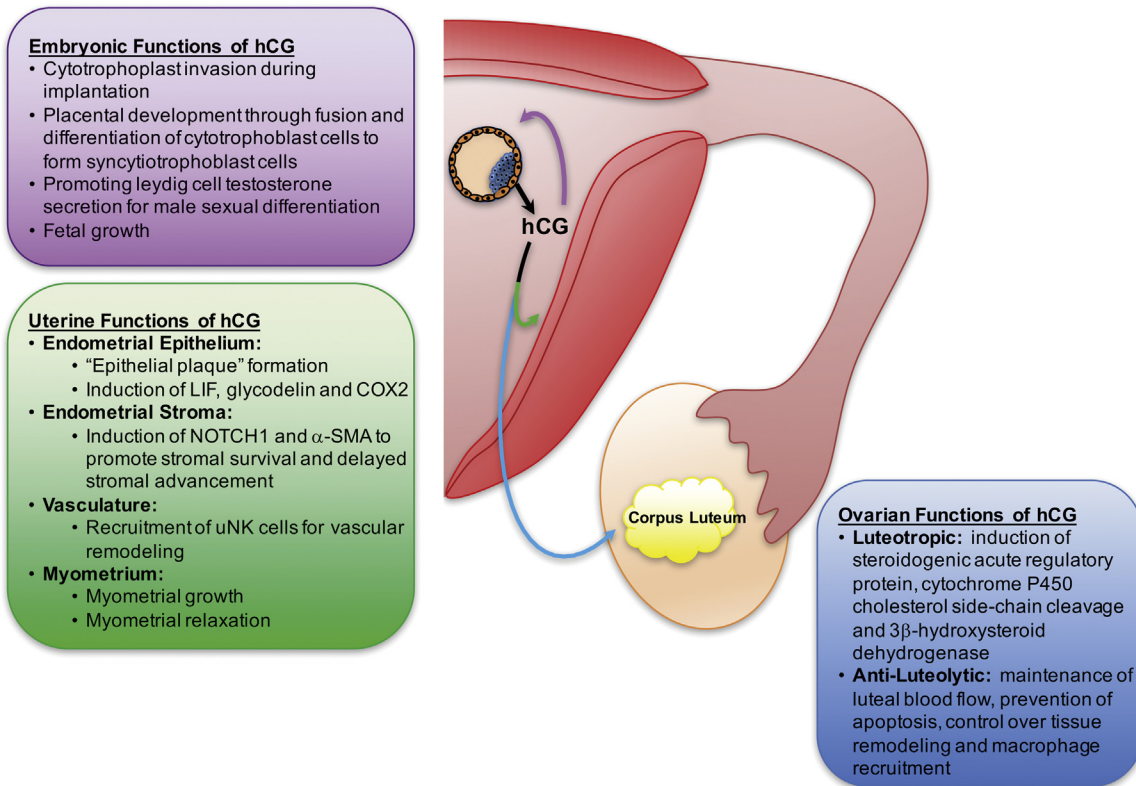


Fig. 1 Gonadal and extra-gonadal roles of hCG.

immunosuppression and preventing fetal allograft rejection. COX2, an upstream regulator of prostaglandin synthesis, has been shown to produce PGE₂, which in part mediates vascular permeability for implantation.

In the endometrial stroma, CG induces α -smooth muscle actin (α -SMA) expression and delays stromal advancement, theoretically extending the window of implantation. α -SMA is initially expressed in endometrial stromal cells as part of the differentiation process from stromal fibroblasts towards their secretory phenotype. Upstream of α -SMA, NOTCH1 is induced by CG and coordinates pro-survival signaling in the endometrial stroma for decidualization (Afshar et al., 2012; Banerjee and Fazleabas, 2010; Strug et al., 2016). Interestingly, many of these described roles for CG throughout the endometrium requires synergism with progesterone receptor signaling (PGR) (Banaszak et al., 2000).

CG regulates mechanisms responsible for development of uterine vasculature and maintaining myometrial quiescence during pregnancy (Cole, 2010). LHCG is expressed on uterine spiral arteries to directly stimulate angiogenesis. Additionally, CG promotes recruitment of uterine natural killer (uNK) cells, responsible in part for remodeling the uterine vasculature. In the uterine myometrium, LHCG expression promotes growth to parallel that of the developing fetus. Also, CG signaling promotes myometrial relaxation through calcium channel modulation. Interestingly, the levels of hCG decline towards the end of pregnancy coinciding with increasing uterine contractions.

Lastly, CG exerts autocrine effects on the developing fetus and developing placenta (Cunningham et al., 2013). h-hCG, as mentioned previously, promotes cytotrophoblast invasion during implantation. CG directly acts to promote fusion of cytotrophoblast cells, which differentiate into syncytiotrophoblast cells during placentation. hCG plays a role in male sexual differentiation through stimulating fetal testicular development, by acting as a substitute for LH to promote leydig cell function and secretion of testosterone. While hCG levels are significantly lower in fetal circulation, there is a role for CG signaling in fetal growth. hCG coordinates umbilical cord growth, and LHCG is expressed in multiple fetal organs, such as the kidney and liver. Further, its expression is essentially absent in the mature, adult organs, suggesting a putative role for hCG in fetal growth.

Clinical Implications of CG and Future Perspectives

As described in "Introduction and Historical Perspectives" section, hCG has served as a clinical marker of pregnancy in women long before it was molecularly characterized. Today, hCG is an important marker for diagnosis of normal and pathologic pregnancy (Stenman et al., 2006). In many cases, patients identify pregnancy using reliable over the counter pregnancy tests. These tests use immunochromatography to provide semi-quantitative measurement of hCG metabolite hCG β core fragment from urine, detecting levels from 25 to 50 mIU/mL. However, the diagnosis of pregnancy is more accurately determined through quantitative

measurements using serum. These tests utilize the sandwich enzyme linked immunoassay (ELISA) method for direct measurement against pre-determined standards. hCG serum levels rise, doubling approximately every two days and peak by 10 weeks in pregnant women. After the hCG peak, serum levels slightly decline and plateau between 13 and 15 weeks gestation followed by a slight increase until 30–33 weeks (Stenman et al., 2006). Subsequently, hCG levels decrease until term, which may contribute to a reduction in myometrial relaxation and favor uterine contractions.

Clinically, doubling of hCG is predictive of successful embryo implantation, is particularly useful in the setting of patients undergoing treatment for infertility. Inappropriately rising levels of hCG or plateauing levels may indicate abnormal implantation, such as ectopic pregnancy or inevitable pregnancy loss. Typically, an intrauterine sac is visible on transvaginal ultrasound when serum hCG levels reach 1500–2000 mIU/mL, but failure to visualize a gestational sac at the “discriminatory zone” of 6000–7000 mIU/mL suggests an ectopic pregnancy. Following pregnancy loss, persistently elevated hCG levels may indicate multiple gestations or gestational trophoblastic disease (GTD). GTD refers to a wide range of pathologic pregnancies, ranging from molar pregnancy to choriocarcinoma. Serial measurement of hCG and hCG β fragment is performed for surveillance of GTD progression and for determining effectiveness of therapeutic management in affected patients. As mentioned earlier, measurement of hCG and its isoforms are clinically useful for detection of germ cell and non-trophoblastic malignancies.

During pregnancy, physicians may determine fetal aneuploidy risk through hCG measurement in combination with other markers (Stenman et al., 2006). Fetal aneuploidies include embryos with abnormal chromosome numbers, such as Down’s syndrome (Trisome 21). First trimester screening is performed between 11 and 13 weeks gestation and measures the levels of free β -hCG and pregnancy-associated plasma protein-A (PAPP-A) in combination with a specific ultrasonographic measurement, termed nuchal translucency. Second trimester screening, referred to as the “quad screen”, is performed between 15 and 18 weeks of pregnancy and measures four specific markers: free β -hCG, inhibin-A, estriol and α -fetoprotein. While these tests alone do not diagnose fetal aneuploidy, they provide a probability risk profile, which may prompt further diagnostic testing using cell-free DNA, chorionic villous sampling or amniocentesis.

Dysfunctional hCG signaling is associated with reproductive disorders affecting fertility (Choi and Smits, 2014). Although rare, polymorphisms in the hCG β -subunit gene are associated with recurrent miscarriage. Mutations in the LHCGR gene result in developmental defects with reproductive consequences, including pseudohermaphroditism and infertility. Additionally, increased risk for Polycystic Ovarian Syndrome (PCOS), associated with ovulatory defects and sub-fertility, has been associated with polymorphisms in the LHCGR gene. Endometriosis is a prevalent condition where endometrial tissue implants outside of the uterus and approximately half of women with this condition are infertile. In a well-established baboon model of endometriosis, treatment with hCG is associated with a blunted endometrial response (Sherwin et al., 2010). Specifically, endometriosis impaired the classical hCG-driven epithelial plaque formation and stromal α -SMA induction. These findings suggest an important mechanism for infertility in women with this debilitating disease.

Use of hCG in the management of infertile patients has been an important tool in assisted reproductive technologies (ART) and its expanded clinical use is currently being investigated for improving success rates. For many years, purified urinary hCG, and more recently recombinant hCG, has been used to induce the final stages of oocyte maturation and ovulation following ovarian stimulation as part of in vitro fertilization (IVF) protocols (Stenman et al., 2006). Additionally, systemic hCG may be administered following ovulation in IVF cycles for luteal support, with the intention of counteracting the negative impact of elevated estradiol levels from ovarian stimulation on endometrial function, which extends the implantation window. A role for hCG in priming the endometrium for embryo implantation and decidualization has been clearly presented (Afshar et al., 2012; Banerjee and Fazleabas, 2010; Fazleabas et al., 1999; Strug et al., 2016). More recently, it was shown that free β -hCG levels in embryo culture media positively correlate with implantation rates in women undergoing IVF treatment for infertility (Xiao-Yan et al., 2013).

Several studies have been performed to determine a therapeutic role for intrauterine hCG administration prior to embryo transfer in IVF cycles (Osman et al., 2016). While some studies have shown a positive effect on clinical pregnancy rates in infertile women, other studies have shown no impact on markers of IVF success. Clinical studies in women receiving intrauterine hCG following oocyte donation did show induction of classically-described endometrial hCG markers along with reduction in endometrial stromal advancement associated with controlled ovarian stimulation (Strug et al., 2016). Therefore, women undergoing ART may benefit from this therapy to counteract endometrial dyssynchrony from ovarian stimulation. Also, intrauterine administration of hCG may compensate for the lack of early hCG secretory functions with more blastocyst embryo transfers performed in IVF cycles. Altogether, hCG represents an evolutionarily developed messenger which establishes the initial stages of embryonic-maternal communication to support pregnancy. In addition to its important physiologic roles in pregnancy, hCG has served as a landmark tool for clinical diagnosis of normal and pathologic pregnancy. Lastly, its therapeutic potential in treatment of infertility remains an important topic of study to this date.

References

- Afshar, Y., Miele, L., & Fazleabas, A. T. (2012). Notch1 is regulated by chorionic gonadotropin and progesterone in endometrial stromal cells and modulates decidualization in primates. *Endocrinology*, 153, 2884–2896.
- Aschheim, S., & Zondek, B. (1928). Die Schwangerschaft diagnose aus dem Harn durch Nachweis des Hypophysenvorderlappenhormons. *Klinische Wochenschrift*, 7, 1404.
- Bahl, O. P. (1969). Human chorionic gonadotropin. I. Purification and physicochemical properties. *The Journal of Biological Chemistry*, 244, 567–574.
- Banaszak, S., Brudney, A., Donnelly, K., Chai, D., CHWALISZ, K., & FAZLEABAS, A. T. (2000). Modulation of the action of chorionic gonadotropin in the baboon (*Papio anubis*) uterus by a progesterone receptor antagonist (ZK 137. 316). *Biology of Reproduction*, 63, 820–825.

- Banerjee, P., & Fazleabas, A. T. (2010). Endometrial responses to embryonic signals in the primate. *The International Journal of Developmental Biology*, 54, 295–302.
- Bulun, S. E. (2011). Physiology and pathology of the female reproductive axis. In S. Melmed, K. S. Polonsky, P. R. Larsen, & H. M. Kronenberg (Eds.), *Williams textbook of endocrinology*. Philadelphia, PA: Elsevier Saunders.
- Choi, J., & Smitz, J. (2014). Luteinizing hormone and human chorionic gonadotropin: origins of difference. *Molecular and Cellular Endocrinology*, 383, 203–213.
- Cole, L. A. (2009). New discoveries on the biology and detection of human chorionic gonadotropin. *Reproductive Biology and Endocrinology*, 7, 8.
- Cole, L. A. (2010). Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology*, 8, 102.
- Cole, L. A., & Butler, S. A. (2008). Hyperglycosylated human chorionic gonadotropin and human chorionic gonadotropin free beta-subunit: tumor markers and tumor promoters. *The Journal of Reproductive Medicine*, 53, 499–512.
- Cunningham, F. G., Leveno, K. J., Bloom, S. L., Spong, C. Y., Dashe, J. S., Hoffman, B. L., Casey, B. M., & Sheffield, J. S. (2013). Implantation and placental development. In *Williams obstetrics*, 24e. New York, NY: McGraw-Hill Education.
- Fazleabas, A. T., Donnelly, K. M., Srinivasan, S., Fortman, J. D., & Miller, J. B. (1999). Modulation of the baboon (*Papio anubis*) uterine endometrium by chorionic gonadotropin during the period of uterine receptivity. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 2543–2548.
- McFarland, K. C., Sprengel, R., Phillips, H. S., Kohler, M., Rosembliit, N., Nikolics, K., Segaloff, D. L., & Seeburg, P. H. (1989). Lutropin-choriogonadotropin receptor: An unusual member of the G protein-coupled receptor family. *Science*, 245, 494–499.
- Osman, A., Pundir, J., Elsherbini, M., Dave, S., El-Toukhy, T., & Khalaf, Y. (2016). The effect of intrauterine HCG injection on IVF outcome: a systematic review and meta-analysis. *Reproductive Biomedicine Online*, 33, 350–359.
- Sherwin, J. R., Hastings, J. M., Jackson, K. S., Mavrogianis, P. A., Sharkey, A. M., & Fazleabas, A. T. (2010). The endometrial response to chorionic gonadotropin is blunted in a baboon model of endometriosis. *Endocrinology*, 151, 4982–4993.
- Srisuparp, S., Strakova, Z., Brudney, A., Mukherjee, S., Reierstad, S., Hunzicker-Dunn, M., & Fazleabas, A. T. (2003). Signal transduction pathways activated by chorionic gonadotropin in the primate endometrial epithelial cells. *Biology of Reproduction*, 68, 457–464.
- Srisuparp, S., Strakova, Z., & Fazleabas, A. T. (2001). The role of chorionic gonadotropin (CG) in blastocyst implantation. *Archives of Medical Research*, 32, 627–634.
- Stenman, U. H., Tiitinen, A., Alfthan, H., & Valmu, L. (2006). The classification, functions and clinical use of different isoforms of HCG. *Human Reproduction Update*, 12, 769–784.
- Strug, M. R., Su, R., Young, J. E., DODDS, W. G., Shavell, V. I., Diaz-Gimeno, P., Ruiz-Alonso, M., Simon, C., Lessey, B. A., Leach, R. E., & Fazleabas, A. T. (2016). Intrauterine human chorionic gonadotropin infusion in oocyte donors promotes endometrial synchrony and induction of early decidual markers for stromal survival: a randomized clinical trial. *Human Reproduction*, 31, 1552–1561.
- Xiao-Yan, C., Jie, L., Dang, J., Tao, L., Xin-Ru, L., & Guang-Lun, Z. (2013). A highly sensitive electrochemiluminescence immunoassay for detecting human embryonic human chorionic gonadotropin in spent embryo culture media during IVF-ET cycle. *Journal of Assisted Reproduction and Genetics*, 30, 377–382.

Uterine Receptivity: The Status of Uterus for Implantation

Jinhua Lu, Shuangbo Kong, and Haibin Wang, Xiamen University, Xiamen, Fujian, People's Republic of China; and Medical College of Xiamen University, Xiamen, Fujian, People's Republic of China

© 2018 Elsevier Inc. All rights reserved.

The Concept of Uterine Receptivity

In mammals, one of the most fascinating tissues is the uterus, whose major function is to accept implantation-competent blastocyst during a relatively short period defined as “the window of implantation.” Correspondingly, the window of uterine receptivity is defined as the limited time when the uterus is suitable for embryo implantation. This concept was first raised and established by the studies employing the embryo transfer technique in the 1960s (Dickmann and Noyes, 1961). In rats, while preimplantation embryos on day 4 of pregnancy (day 1 = vaginal plug) showed severe damage 9 h after transfer into a day 5 uterus, blastocysts obtained from pregnant mice at day 5 can implant normally after transfer to either day 4 or day 5 uteri, but not in the uteri beyond day 5 of pregnancy or pseudopregnancy. These findings suggest that the uterus is not constantly receptive to blastocysts and embryo implantation could occur only in a limited period. This notion was further confirmed by the experiments conducted in mouse (Paria et al., 1993). On the basis of these previous findings, the uterine sensitivity to implantation-competent blastocysts is classically divided into three stages: prereceptive, receptive, and refractory phase. During the prereceptive stage, the uterus is favorable for embryo development but not suitable for implantation, while the uterus can initiate implantation when there are competent blastocysts at the receptive stage. During the refractory stage, however, implantation-competent blastocysts could not implant in the uterus, and the uterus is adverse to the survival and development of blastocyst (Wang and Dey, 2006). In mice, the uterus on days 1–3 of pregnancy is conventionally considered to be in the prereceptive phase. On day 4 of pregnancy, the uterus becomes fully receptive following the priming actions of ovarian progesterone and preimplantation estrogen, whereas the uterus fails to initiate implantation by late day 5. Similarly, the first 7 days of the secretory stage in the menstrual cycle is considered as the prereceptive stage in humans, 7–10 days after ovulation, is considered as the receptive stage, and the rest of the secretory stage is defined as the nonreceptive stage (Wang and Dey, 2006). It has been generally accepted that uterine receptivity is one of the pivotal events that determine the success of pregnancy, since implantation-competent blastocyst only implants in the uterus at the receptive state and any disturbance to uterine receptivity would lead to compromised pregnancy outcomes, including retarded embryo development and pregnancy failure.

The Uterine Preparation for Receptivity

The uterine tissue is composed of three major layers: the outer muscle layer, the inner luminal epithelium, and the stromal bed in between. Actually, changes in the uterus during pregnancy are hormone-dominant events, controlled by the steroid hormones from the ovaries. In essence, synchronization of the two major ovarian steroid hormones, estrogen and progesterone, directs the uterus into the receptive state, which exhibits the morphological and functional changes in the uterine epithelial and stromal cells (Wang and Dey, 2006).

Morphological Changes for Uterine Receptivity

Microvilli and pinopodes

The uterine lumen is lined by a polarized epithelium overlying the underlying stroma and myometrium. One of the morphological changes of the luminal epithelium that marks the uterine transition from prereceptive state to receptive state is the retraction of apical microvilli on the apical side of the luminal epithelial cells. These microvilli were the focus of many early ultrastructural studies and underwent dynamic changes in appearance in response to ovarian hormones. With progesterone alone, short regular microvilli are characteristically present, whereas estrogen alone results in long thin regular microvilli. Under the influence of either hormone alone, changes in the apical plasma membrane are mostly limited to alterations in the height and frequency of microvilli. However, when progesterone and estrogen act together leading to uterine receptivity for embryo implantation during the periimplantation period, the apical plasma membrane of uterine epithelial cells undergoes a more marked structural change during the several days of early pregnancy. More specifically, it gradually loses regular microvilli and becomes very flat. The apical plasma membrane of uterine epithelial cells from a microvillus to the flattened profile is a morphological sign of uterine receptivity in many species, since the uterus that lacks the apical membrane flattening is unable to support embryo implantation. Moreover, it has been established that regular microvilli begin to return to the apical plasma membrane very soon after the period of uterine receptivity, further indicating the close association between the membrane changes and uterine receptivity (Murphy, 2004).

Another morphological change of the luminal epithelium is the presence of large apical protrusions, marking the uterine transition from prereceptive state to receptive state. This structure was first discovered in rats and mice by traditional electron microscopy and was named as a “pinopode” because of its pinocytotic function (Nilsson, 1958). In rats, it has been shown that the development of pinopodes synchronizes with the window of uterine receptivity, since the number of pinopode increases on day 4 of pregnancy and becomes more abundant on day 5 when the uterus enters the receptive phase and decreases rapidly during the

postimplantation period. Pinopodes, the bulbous cytoplasmic protrusion projections on the apical surface of the luminal epithelium, appear only during the receptive phase. Therefore, these pinopodes are the best-studied ultrastructural markers of uterine receptivity, which are believed to be helpful in the attachment of the blastocyst to the surface of luminal epithelium. Further studies revealed that the dynamics of pinopodes in the uterus are under the control of ovarian steroid hormones. Actually, the appearance of pinopodes was found to be strictly progesterone-dependent. As to the estrogen, it is dose-dependent. While treatment with high doses of estradiol together with progesterone before the development of pinopodes inhibited pinopode formation, low doses of estradiol did not interfere with the process until the fourth day of treatment. Moreover, when estradiol was given as a single injection after pinopode formation, both doses of estrogen were equivalent in inducing the regression of pinopodes 48–72 h later (Martel et al., 1991). These findings demonstrate the similar hormonal conditions for both pinopode formation and the attainment of uterine receptivity, further confirming that the appearance of pinopodes is a well-defined histological marker for uterine receptivity. However, it is still questionable about whether human pinopodes are clinically useful to delineate the period of endometrial receptivity, since pinopodes are present throughout the luteal phase of the menstrual cycle. However, there are evidences that pinopodes are most prominent during the putative implantation window, suggesting that the pinopodes in human endometrium are at least helpful to the determination of uterine receptivity.

Luminal closure

Luminal closure, defined as the closure of uterine lumen during embryo apposition prior to attachment, is another morphological landmark for uterine receptivity. This event supports a closer contact between the luminal epithelium and the blastocysts, which is essential for appropriate blastocyst apposition and subsequent attachment. In rodents, a generalized stromal edema under the influence of ovarian steroid hormones leads to the closure of uterine luminal epithelium (Wang and Dey, 2006). And progesterone has been demonstrated to be essential for the luminal closure, since uterine luminal closure failed to occur in mice missing FK506-binding protein 4 (FKBP52), a cochaperone for full functions of progesterone receptor (PR) in the uterus. However, the occurrence of luminal closure does not require the presence of blastocysts, since this phenomenon could also be observed in both the pregnant and pseudopregnant uteri (Wang and Dey, 2006). Moreover, it has been shown that the secretion and reabsorption of uterine fluid is important for the uterine luminal closure and these processes are at least under the control of two major gatekeepers: cystic fibrosis transmembrane conductance regulator and epithelial Na⁺ channel (Tu et al., 2014).

Functional Changes for Uterine Receptivity

During the establishment of uterine receptivity, functional changes are mediated by several factors such as adhesion molecules, cytokines, and homeotic proteins. Many of these molecules have been identified as potential markers of uterine receptivity. For example, the glycoproteins expressed in the luminal epithelium are thought to function as a uterine barrier that inhibits the interaction between the blastocyst and luminal epithelium at the time of attachment. Unmasking of these glycoproteins at the implantation site correlates with increased blastocyst adhesion to the uterus (Dey et al., 2004). MUC1, a mucin-type glycoprotein, is integrally located in the apical plasma membrane of the luminal epithelium before implantation, whereas its expression is timely downregulated during the receptive period, in agreement with the view that glycoproteins act as uterine barrier that inhibits implantation. In humans, on the other hand, the expression of MUC1 remains at high levels during the implantation window, which seems to be contradictory to the antiadhesion function of MUC1. One explanation is that the embryo utilizes MUC1-associated glycans, which has been demonstrated in rabbit implantation. Actually, in vitro experiment using human blastocyst and endometrial epithelial cells indicates that the embryo could induce paracrine degeneration of epithelial-expressed MUC1 at the implantation site. Thus, it appears that MUC1 must be locally removed at the implantation site prior to successful blastocyst attachment in both human and animal models.

The Embryonic Contribution to Uterine Receptivity

Embryo implantation involves the implantation-competent embryo and the uterus at the receptive state. In addition to uterus itself, the embryo also plays an important role in determining the implantation window. Although it is clear that the states of the blastocyst determined the implantation window (Paria et al., 1993), there is a long-standing quest for specific embryo-derived molecules and signaling pathways that can functionally influence the states of the uterus, especially the receptive state of the uterus. One such important molecule is heparin-binding EGF-like growth factor (HB-EGF), encoded by Hbegf gene. Global gene analysis of the blastocysts with differential competency for implantation revealed that HB-EGF is significantly upregulated during blastocyst activation (Hamatani et al., 2004), suggesting that this molecule might be important for embryo implantation. This was confirmed by the experiments employing the blastocyst-size Affi-gel beads presoaked with HB-EGF protein. When these beads were transferred intraluminally into a pseudopregnant uterus, they can induce the expression of HB-EGF in uterine cells surrounding the beads and increase vascular permeability, similar to the physiological changes induced by normal blastocysts (Hamatani et al., 2004). Interestingly, previous studies demonstrated that HB-EGF is also expressed in the luminal epithelium at the site of blastocyst apposition approximately 6 h prior to blastocyst implantation in mice, with increasing expression of its receptors ErbB1 and ErbB4 and ligand-receptor binding activities observed in the blastocysts that are competent for implantation. Signaling by HB-EGF back to the embryo, in turn, activates blastocyst differentiation required for embryo adhesion during subsequent attachment and invasion. These observations

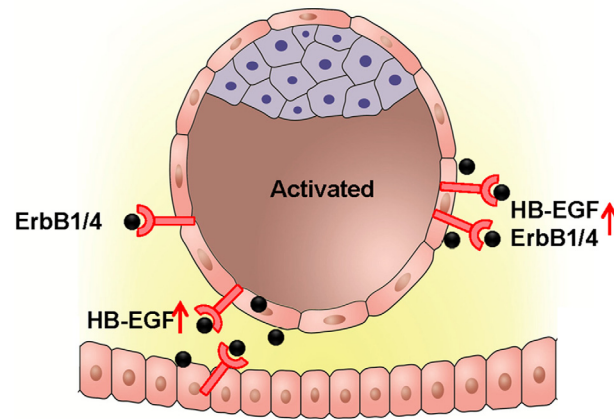


Fig. 1 The autoinduction loop of HB-EGF/ErbB4 between the implantation-competent blastocyst and the receptive uterus.

also suggest that HB-EGF conducts an autoinduction loop between the implanting blastocyst and the uterus via paracrine and juxtacrine signaling (Fig. 1). In human, HB-EGF is highly expressed in the receptive endometrium, and its receptor ErbB4 is localized on the surface of the trophoblast in periimplantation blastocysts (Chobotova et al., 2002), indicating that HB-EGF/ErbB4 signaling also mediates the trophoblast–uterine epithelium interaction during implantation in humans.

The Hormonal Control of Uterine Receptivity

Pregnancy is a hormone-dominant process, including the establishment of uterine receptivity, a key event and stage during normal pregnancy. The hormones that direct the acquisition of uterine receptivity are mainly the ovarian steroids estrogen and progesterone, although the hormonal requirements for uterine receptivity are species-dependent. While Progesterone is essential for the establishment of uterine receptivity in nearly all the mammals studied so far, the requirements for ovarian estrogen are species-specific. For instance, estrogen is essential for the acquisition of uterine receptivity in the rat and mouse, while maternal estrogen is not required for implantation in some species such as rabbit, hamster, pig, and guinea pig, with the explanation that blastocysts in these species have the ability to synthesize estrogen, which may contribute to the activation of implantation process. In other species, such as nonhuman primates and the human, the functions of estrogen during implantation remain inconclusive (Wang and Dey, 2006).

Estrogen is essential for uterine receptivity in the progesterone-primed uterus in mice. On day 1 of pregnancy in mice, under the influence of preovulatory ovarian estrogen, the uterine epithelial cells undergo extensive proliferation that to some extent continue through day 2. Rising progesterone levels secreted from the newly formed corpora luteum initiate the proliferation of uterine stromal cells from day 3 onward. On the morning of day 4, when the uterus is at the prereceptive stage, the production of a small amount of estrogen is crucial for the uterus to attain receptivity. At this time, the uterine epithelial cells gradually lose their polarity, and the apical plasma membranes of the epithelial cells become smooth and flattened at the site of blastocyst apposition. Ovariectomy immediately before the preimplantation estrogen secretion on day 4 and daily supplementation of progesterone beginning on day 5 result in blastocyst dormancy and inhibition of implantation, whereas a single injection of physiological levels of 17 β -estradiol can induce the uteri from the neutral phase into the receptive state and renders the reactivation of blastocyst implantation (Zhang et al., 2013b). Based on these hormone profiles during the preimplantation period, exogenous estrogen and progesterone can also confer a receptive-stage uterus in ovariectomized mice.

In the uteri, estrogen and progesterone function mainly through nuclear estrogen receptors (ER) and PRs, respectively. Both the receptors have two main isoforms, known as ER α and ER β for estrogen and PRA and PRB for progesterone. Pharmacological and genetic evidences have revealed the requirements of ER and PR for the preparation of uterine receptivity. For example, administration of antagonists for either ER or PR before implantation efficiently abolished the establishment of uterine receptivity. Moreover, previous works using knockout mice for ER or PR have also demonstrated their differential functions during uterine physiology. The uterus with *Esr1* (gene encodes ER α) deletion is hypoplastic and unable to support implantation, whereas the uterus deficient for *Esr2* (gene encodes ER β) retains biological functions that allow for normal implantation, indicating the essential role of ER α during implantation. As to the PR, while mice with *Pgr* (gene encodes both the PRA and PRB with different promoters) deletion are infertile with defective ovarian and uterine functions, PRB-deficient females are fertile with normal ovarian and uterine responses, indicating that the functions of progesterone in uteri are primarily mediated by PRA (Zhang et al., 2013a).

The Establishment of Uterine Receptivity: Cell–Cell Interactions

Embryo implantation is a dynamic developmental event that involves a series of physical and physiological interactions among the blastocyst trophoblast and various endometrial cell types. Similarly, the establishment of uterine receptivity also involves

multiple cell–cell interactions between the luminal and glandular epithelium and the stromal cells, which are under the primary influence of ovarian steroids estrogen and progesterone (Zhang et al., 2013b).

The Uterine Epithelial–Stromal Interaction

As previously mentioned, synchronization of estrogen and progesterone directs the uterus into a receptive state that is accompanied by obvious morphological and functional changes in the epithelium (Zhang et al., 2013a). The interactions of ovarian progesterone and estrogen during uterine cell proliferation and differentiation are summarized in Fig. 2 and are discussed later.

Estrogen binds stromal ER α to stimulate the proliferation of uterine epithelium via paracrine factors

ER is expressed in both epithelial and stromal cells of the adult uteri. It was initially assumed that estrogen acts directly through the ER in the corresponding compartments. However, evidences from the experiments employing ESR1 knockout mouse models and stromal–epithelial separation/recombination systems (Cunha, 2008) demonstrated that estrogen could not stimulate epithelial proliferation in genetically recombined uterine tissue that lacks stromal ER α , even in the presence of epithelial ER α . The tissue-specific knockout techniques render the selective deletion of ESR1 in the uterine epithelium and further proved that stromal ER α is responsible for estrogen-induced epithelial proliferation. As to how the estrogen–ER α activity in the stroma induces the epithelial proliferation, paracrine actions of polypeptide growth factors are believed to be essential for the uterine response to estrogens, such as insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), or transforming growth factor α . Specifically, IGF-1 is an important growth factor that could be induced and activated immediately in the uterine stroma upon treatment with estrogen. It is necessary for estrogen-induced uterine epithelial proliferation through IGF-1 receptor in the luminal epithelium. Moreover, stromal ER α is also sufficient for estrogen-induced downregulation of PR in the uterine epithelial cells.

Differentiation of uterine epithelial cells requires both epithelial and stromal ER α

Although it is the uterine stromal ER α not the epithelial ER α that is indispensable for estrogen-induced epithelial proliferation, the epithelial ER α is also essential for complete biological functions in the uteri, since selective deletion of uterine epithelial ESR1 resulted in compromised increase of uterine weight induced by estrogen and led to epithelial apoptosis after initial proliferation. Further evidence revealed that both the stromal and epithelial ER α are required for the functional differentiation of uterine epithelium, which could be indicated by such secretory proteins as lactoferrin, complement component C3, and MUC1 (Buchanan et al., 1999).

Progesterone functions through stromal PR to antagonize the epithelial proliferative response to estrogen and induce stromal proliferation

The phenotypes of uteri with Pgr-null mutation are similar to that of the uteri in the ovariectomized mice exposed to prolonged estrogen treatment. This finding revealed an essential role of PR in the uterus. Furthermore, recombination experiments using uterine tissue from Pgr-null mice demonstrated that stromal PR is required for progesterone to decrease the proliferation-promoting effect of estrogen on the uterine epithelial cells and promote the proliferation of stromal cells. And numerous molecules have been identified to mediate progesterone–PR signaling, such as immunophilin FK506-binding protein 4 (FKbp52), chicken ovalbumin upstream promoter-transcription factor II, the basic helix–loop–helix transcription factor, and heart and neural crest derivatives expressed transcript 2 (Hand2).

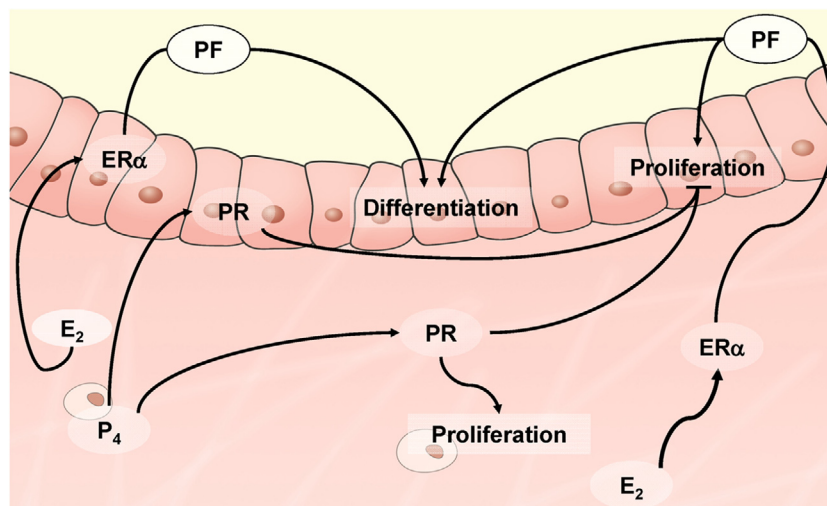


Fig. 2 The uterine epithelial–stromal interactions mediated by the ovarian progesterone and estrogen and their respective receptors.

Epithelial PR is also involved in stromal–epithelial cross talk

Despite the well-established concept that stromal PR mediates the antagonistic functions of progesterone on the epithelial proliferative response to estrogen, the important roles of epithelial PR in uterine biology were largely ignored. A recent study with Pgr deficiency in the uterine epithelial cells has demonstrated that epithelial PR is essential for epithelial expression of Indian hedgehog, which participates in the uterine stromal–epithelial cross talk. The absence of epithelial Pgr results in complete failure of pregnancy due to impaired uterine receptivity. This finding clearly demonstrated that epithelial PR is indispensable for the stromal–epithelial interactions during the establishment of normal uterine receptivity.

The Uterine Glandular–Epithelial Interaction

All mammalian uteri contain glands in the endometrium that synthesize and secrete substances essential for the survival and development of the conceptus. It has been shown that uterine glands and their paracrine-acting secretions have important biological roles in the establishment of uterine receptivity and blastocyst implantation (Kelleher et al., 2016). Of the substances secreted by uterine glands, LIF is one of the most important molecules, since LIF null mutation in mice led to infertility, defective uterine receptivity, and failed blastocyst implantation. The LIF is secreted into the uterine lumen where it binds the LIF-receptor complex composed of LIF receptor (LIFR) and gp130. The overlapping expression pattern of LIFR and gp130 in the luminal epithelium provides robust evidence for the paracrine nature of glandular LIF acting on uterine epithelial cells. Failure of gp130 activation in the luminal epithelium in LIF null mice reinforces the notion that LIF-driven glandular–luminal epithelial interaction is essential for normal uterine receptivity and blastocyst implantation. Although LIF has a well-recognized role to establish receptivity of the luminal epithelium for implantation, the role of many other genes expressed in the uterine glands has not been established. Moreover, future studies are needed to determine the role of the uterine glands and their secretions in paracrine interactions with the luminal epithelial cells.

The Flexibility of Uterine Receptivity

Since the uterine receptivity is determined by the ovarian steroid hormones estrogen and progesterone, the states of the uterine receptivity could be changed by the manipulation of the hormones estrogen and progesterone (Zhang et al., 2013a).

Estrogen Is Critical for Specifying the Duration of Uterine Receptivity

In rodents, estrogen is essential for the preparation of a progesterone-primed uterus to the receptive state. Ovariectomy conducted before preimplantation estrogen secretion on the morning of day 4 results in blastocyst dormancy and inhibition of implantation, also known as delayed implantation. This neutral uterine phase can be maintained by continued progesterone treatment but is terminated by estrogen injection. The impacts of different doses of estrogens on the length of implantation window have been explored using this delayed implantation model. For instance, estrogen at a low threshold level extends the window of uterine receptivity, whereas physiological higher levels of estrogen rapidly shut off the implantation window, transforming the uterus into a refractory state. The views that high levels of estrogen are detrimental to implantation are further supported by the findings that ovarian hyperstimulation leads to implantation failure and embryo resorption. In humans, the life span of fully developed pinopodes lasts maximally 48 h, suggesting a transient cell state in the receptive endometrium. Following ovarian stimulation with clomiphene citrate and human chorionic gonadotropin, pinopodes formed 1–2 days earlier than that in the natural cycles. Early pinopode formation caused by ovarian stimulation may suggest the shifting of the window for receptivity. Based on these findings, it is reasonable to speculate that reduced implantation in the cycles of in vitro fertilization could be due to asynchrony between the receptive endometrium and implantation-competent blastocyst that result from exposure to excessive estrogen.

Progesterone Supplementation Extends the Window of Uterine Receptivity

In mice, blastocysts can initiate implantation out of the normal “window” of uterine receptivity. For example, blastocysts can still initiate implantation when transferred on day 5 of pseudopregnancy, but implantation will not occur when normal blastocysts are transferred into day 6 pseudopregnant uteri. Exogenous progesterone supplementation can prolong the implantation window to day 6. However, deferred embryo implantation beyond the normal “window” of uterine receptivity leads to embryonic demise before birth in mice and is often associated with higher risk of early pregnancy losses in humans (Wang and Dey, 2006).

References

- Buchanan, D. L., Setiawan, T., Lubahn, D. B., Taylor, J. A., Kurita, T., Cunha, G. R., & Cooke, P. S. (1999). Tissue compartment-specific estrogen receptor- α participation in the mouse uterine epithelial secretory response. *Endocrinology*, 140, 484–491.
- Chobotova, K., Spyropoulou, I., Carver, J., Manek, S., Heath, J. K., Gullick, W. J., Barlow, D. H., Sargent, I. L., & Mardon, H. J. (2002). Heparin-binding epidermal growth factor and its receptor ErbB4 mediate implantation of the human blastocyst. *Mechanisms of Development*, 119, 137–144.

- Cunha, G. R. (2008). Mesenchymal-epithelial interactions: past, present, and future. *Differentiation*, 76, 578–586.
- Dey, S. K., Lim, H., Das, S. K., Reese, J., Paria, B. C., Daikoku, T., & Wang, H. (2004). Molecular cues to implantation. *Endocrine Reviews*, 25, 341–373.
- Dickmann, Z., & Noyes, R. W. (1961). The zona pellucida at the time of implantation. *Fertility and Sterility*, 12, 310–318.
- Hamatani, T., Daikoku, T., Wang, H., Matsumoto, H., Carter, M. G., Ko, M. S., & Dey, S. K. (2004). Global gene expression analysis identifies molecular pathways distinguishing blastocyst dormancy and activation. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 10326–10331.
- Kelleher, A. M., Burns, G. W., Behura, S., Wu, G., & Spencer, T. E. (2016). Uterine glands impact uterine receptivity, luminal fluid homeostasis and blastocyst implantation. *Scientific Reports*, 6, 38078.
- Martel, D., Monier, M. N., Roche, D., & Psychoyos, A. (1991). Hormonal dependence of pinopode formation at the uterine luminal surface. *Human Reproduction*, 6, 597–603.
- Murphy, C. R. (2004). Uterine receptivity and the plasma membrane transformation. *Cell Research*, 14, 259–267.
- Nilsson, O. (1958). Ultrastructure of mouse uterine surface epithelium under different estrogenic influences. 2. Early effect of estrogen administered to spayed animals. *Journal of Ultrastructure Research*, 2, 73–95.
- Paria, B. C., Huet-Hudson, Y. M., & Dey, S. K. (1993). Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proceedings of the National Academy of Sciences of the United States of America*, 90, 10159–10162.
- Tu, Z., Ran, H., Zhang, S., Xia, G., Wang, B., & Wang, H. (2014). Molecular determinants of uterine receptivity. *International Journal of Developmental Biology*, 58, 147–154.
- Wang, H., & Dey, S. K. (2006). Roadmap to embryo implantation: clues from mouse models. *Nature Reviews. Genetics*, 7, 185–199.
- Zhang, S., Kong, S., Lu, J., Wang, Q., Chen, Y., Wang, W., Wang, B., & Wang, H. (2013a). Deciphering the molecular basis of uterine receptivity. *Molecular Reproduction and Development*, 80, 8–21.
- Zhang, S., Lin, H., Kong, S., Wang, S., Wang, H., Wang, H., & Armant, D. R. (2013b). Physiological and molecular determinants of embryo implantation. *Molecular Aspects of Medicine*, 34, 939–980.

Epidermal Growth Factor Signaling in Embryo-Uterine Interactions During Implantation

Hyunjung Jade Lim, Konkuk University, Seoul, Korea

Haengseok Song, CHA University, Seongnam, Korea

© 2018 Elsevier Inc. All rights reserved.

Introduction: A General Scheme of Growth Factor-Receptor Interaction

Growth factors are ligands that interact with cognate receptor(s) to activate intracellular signaling cascades. While most ligands utilize specifically fitted receptors for interactions, downstream conveyance of signals at the intracellular level often merge to regulate molecular events such as transcription and translation. Diverse changes subsequently occurring at subcellular levels are manifested as growth responses; thus, cells may proliferate, change shape, express specific genes to differentiate, or move. In a simplified version of a growth factor-receptor interaction, a factor, the ligand, approaches either in a secreted or in a juxtaposed form anchored in the membrane of another cell (Massagué and Pandiella, 1993). The target cell expresses the cognate receptor: secreted ligands can be reached via extracellular fluids but juxtaposed ligands require the presence of target cells within near reach. In many cases, receptors form multimers for the division of labor, i.e., one receptor directly interacts with a ligand, while the other receptor triggers an intracellular signaling cascade. For a specific family of growth factors, ligands often show promiscuity towards different receptors. Receptors generally undergo phosphorylation at certain residues on the cytoplasmic tail, triggering the recruitment of intracellular signaling mediators and leading to activation of intracellular signaling pathways (Citri and Yarden, 2006). Thus, the presence of specific ligands and cognate receptors at the same time within a reachable range, along with the right type of secondary signaling cascade, determines whether growth factor signals propagate or halt at a specific cellular context.

EGF: The First Evidence

Since the discovery of epidermal growth factor (EGF) in the submaxillary gland of mice in 1962 and the Nobel committee's recognition of the versatile actions of growth factors in 1986, the story behind the study of growth factors has been dramatic and expansive. As uterine preparation for implantation is accompanied by growth and differentiation of both embryonic and uterine cells, it was an expected course of investigation to look for expression and functions of newly discovered growth factors. EGF first entered the scene of scrutiny in the field of uterine biology because its function in the epithelial proliferation of other tissues was clearly pertinent to the implantation process involving close contact between a blastocyst and well-prepared uterine epithelium. Ovarian steroid hormones are seminal regulators of various aspects of uterine function (Huet-Hudson et al., 1989). Regulated expression of certain factors by ovarian steroid hormones therefore provides clues into understanding their roles. Early studies showed that isolated uterine epithelial cells proliferate in response to EGF (Tomooka et al., 1986) and that EGF expression is influenced by estrogen (Huet-Hudson et al., 1990). Thus, EGF became one of the first candidate mediators of estrogen activity in embryo implantation.

In initial investigations of EGF in uterine biology, several biochemical and molecular techniques were frequently used: expression of ligands and receptors at the mRNA and protein levels in vivo, receptor binding with radioactive isotope-labeled ligands using tissue extracts, and receptor phosphorylation (DiAugustine et al., 1988; Huet-Hudson et al., 1990; Lin et al., 1988). When gene-targeted mouse models became available in the 1990s, previous results demonstrating the involvement of growth factors in embryo-uterine interactions were confirmed (Lim and Dey, 2009).

Introducing the EGF Family

The family of EGF-like growth factors includes EGF, transformation growth factor alpha (TGF α), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, neuregulins, and epigen (Schneider and Yarden, 2014). These ligands were first produced in their membrane-anchored precursor forms and later proteolytically processed and secreted (Massagué and Pandiella, 1993). Both membrane-anchored and processed forms possess biological activities and functions. The EGF family of growth factors recognizes a set of receptor tyrosine kinases known as ErbBs. There are four ErbB receptors: ErbB1 (EGF receptor, EGFR), ErbB2, ErbB3, and ErbB4. They share a common structural feature bearing kinase activity which auto-phosphorylates their tyrosine residue, but differ in ligand specificity and kinase activity. Upon ligand binding, ErbB receptors form homo- or heterodimers and, similarly to other growth factor receptors, convey this signal to the intracellular compartment with kinase activity. Specific tyrosine residues on the cytoplasmic tails of each receptor are phosphorylated and intracellular signaling mediators are recruited (Citri and Yarden, 2006). There are exceptions to this standardized depiction: the ErbB2 receptor does not directly interact with a ligand and the ErbB3 receptor shows impaired kinase activity.

Multiple EGF-like ligands are expressed in uterine cells and blastocysts around the time of implantation. Table 1 summarizes the results from various investigators, focusing on uterine expression at the time of implantation in mice. Many EGF-like factors are expressed in similar spatial and temporal patterns. At the time of the blastocyst attachment reaction, HB-EGF, amphiregulin,

Table 1 Studies on the expression and potential roles for EGF-like ligands and their receptors at the time of implantation in mice^a

	<i>Uterine expression</i>	<i>Gene-targeted mouse model</i>	<i>References</i>
EGF	Present	Female knockout mice are fertile	Huet-Hudson et al., 1990; Luetkeke et al., 1999
TGF α	Present	Female knockout mice are fertile	Das et al., 1997; Luetkeke et al., 1999
HB-EGF	Present	Uterine specific deletion leads to compromised fertility	Das et al., 1994a; Xie et al., 2007
Amphiregulin	Present	Female knockout mice are fertile	Das et al., 1995; Luetkeke et al., 1999
Epiregulin	Present	Female knockout mice are fertile	Das et al., 1997; Lee et al., 2004
Betacellulin	Present	–	Das et al., 1997
Neuregulins (multiple forms)	Some isoforms expressed in the subepithelial stroma near implanting blastocyst	Fertility issues were not reported	Reese et al., 1998; Brown et al., 2004
Epigen	–	Female knockout mice are fertile	Dahlhoff et al., 2013
ErbB1 (EGFR)	Expressed in the subepithelial stroma near implanting blastocyst	Early developmental defects; knockout blastocysts implant normally	Das et al., 1994a,b; Lim et al., 1998; Wong, 2003
ErbB2	Widespread in all epithelial cells and some in stromal cells	Early developmental defects; knockout blastocysts implant normally	Lim et al., 1997; Erickson et al., 1997; Brown et al., 2004
ErbB3	Widespread expression both in epithelial and some in stromal cells	Early developmental defects; knockout blastocysts implant normally	Erickson et al., 1997; Lim et al., 1998; Brown et al., 2004
ErbB4	Not expressed near implanting blastocyst	Early developmental defects; knockout blastocysts implant normally	Lim et al., 1998; Gassmann et al., 1995

^aReferences on gene-targeted mouse models are given in the Further Reading section.

betacellulin, epiregulin, and neuregulin are expressed in the luminal epithelium and/or subepithelial stromal cells just surrounding the blastocyst. Information regarding the presence of ligands in secreted or membrane-anchored form, availability of the proper receptors, evidence of actual ligand-receptor interactions in the context of embryo implantation, and pregnancy-related phenotypes in knockout mouse models would provide clues for deciphering the roles for these factors in embryo implantation. While many of these ligands exhibit implantation-specific expression patterns and possess bioactivity for phosphorylating ErbB receptors (Lim et al., 1998; Lim et al., 1997), triple-compound knockout mice of EGF-TGF α -amphiregulin do not show implantation defects (Luetkeke et al., 1999). Overlapping expression thus suggests possible redundancy in functions. One EGF-like ligand, HB-EGF, satisfies all the above conditions. In this sense, HB-EGF deserves full attention among EGF-like ligands for its outstanding role in embryo implantation, and will be reviewed in the following section as a crucial mediator of implantation.

In contrast to the redundant roles of EGF-like ligands, gene deletion models of a single ErbB receptor leads to severe developmental defects, precluding the investigation of specific pregnancy phenotypes in adult female mice (Table 1). From an embryonic perspective of implantation, however, the absence of one ErbB gene in blastocysts does not hinder implantation of deficient embryos.

HB-EGF in Embryo Implantation

Near the time of embryo implantation, the uterus becomes edematous in the stroma, which contributes to closing of the uterine lumen. This closure is necessary for the close encounter of the blastocyst to the receptive uterine epithelium. When these components are close to each other, juxtacrine or paracrine ligand-receptor interactions become more effective. HB-EGF is unique in that its expression precedes the actual attachment reaction by several hours: its mRNA is first expressed at around 4 PM on day 4 of pregnancy just around blastocysts in the uterus (Das et al., 1994b). The presence of a blastocyst is required for this expression. Although the attachment reaction is many hours away, the luminal closure bringing the blastocyst near the luminal epithelium occurs at this point under hormonal influence. When blastocysts are brought closer, the membrane-anchored form of HB-EGF on the luminal epithelium can initiate juxtacrine interactions with ErbB receptors on blastocysts. Like other EGF-like factors, HB-EGF is initially produced in a membrane-anchored form, which can function in a juxtacrine manner with nearby ErbB receptors. When the transmembrane form (HB-EGFTM) is enzymatically processed for shedding into the extracellular space, it is now a mature form that functions in a paracrine manner. For implantation, both forms of HB-EGF appear to be important. The paracrine form of HB-EGF effectively increases embryonic growth and hatching in vitro when added to 8-cell stage embryos (Das et al., 1994a). In contrast, HB-EGFTM functions as an adhesion molecule, providing a tight connection between implantation-competent blastocyst and cells expressing cognate receptors. This was demonstrated by using 32D cells, a bone marrow-derived cell line, engineered to express HB-EGFTM on the surface. When HB-EGFTM-expressing cells were co-incubated with blastocysts, they adhered only to implantation-competent blastocysts.

HB-EGF exhibits dual specificity towards ErbB1 and ErbB4 and is capable of binding to heparan sulfate proteoglycans. All of these potential receptors are available for interaction during the early implantation phase (Lim and Dey, 2009). In the search to

identify the functional receptor for HB-EGF, the *Pseudomonas* exotoxin (PE)-conjugated system was devised (Paria et al., 1999). In this experiment, HB-EGF-PE or TGF α -PE was treated to day 4 normal or dormant blastocysts, and the cytotoxic effects were the greatest in HB-EGF-PE-treated day 4 blastocysts. In addition, HB-EGF-PE was toxic to most *Egfr*^{-/-} blastocysts. This observation also suggests that HB-EGF utilizes multiple receptors for its action on blastocysts. Although heparan sulfate proteoglycans can interact with HB-EGF, it is not considered the receptor for HB-EGF during implantation.

Functionally, HB-EGF is responsible not only for the attachment reaction, but also for the multiple uterine changes occurring during the early phase of implantation. Cellular and molecular changes provoked by a local factor can be examined in a simplified model of implantation using beads that are approximately the size of blastocysts. When HB-EGF-soaked beads were transferred to pseudopregnant uteri, they triggered a blue dye reaction, indicating increased vascular permeability occurring during the early implantation phase. This treatment also induced expression of HB-EGF itself in the luminal epithelium and differentiation of stromal cells into decidual cells (Paria et al., 2001). Consistent with these results, mice with uterine-specific deletion of the HB-EGF gene show compromised implantation (Xie et al., 2007). In summary, HB-EGFTM in the uterine luminal epithelium appears to interact with multiple receptors including EGFR and ErbB4 on implantation-competent blastocysts and contributes to the establishment of early implantation-related changes.

Other EGF-Like Ligands

Along with HB-EGF, amphiregulin, betacellulin, epiregulin, and neuregulins are other EGF-like ligands that are expressed near implanting blastocysts. Overlapping expression patterns of these ligands suggest that specific compensatory mechanisms may be operative (Das et al., 1997). As described above, amphiregulin deficiency does not cause any implantation-related defects, but amphiregulin is worth mentioning in the context of implantation biology because of two unique features. While most of these ligands are expressed at the time of the attachment reaction and persist as implantation proceeds, amphiregulin expression disappears immediately after the attachment reaction (Das et al., 1995). Additionally, amphiregulin is a rare example of progesterone-regulated gene expressed only in the uterine epithelium. Thus, amphiregulin expression is a widely used monitoring tool of progesterone responsiveness in the uterus of many gene-targeted mouse models with uterine defects.

Molecular Effects of HB-EGF-ErbB Signaling

We next describe the molecular events triggered by the HB-EGF-ErbB interaction during implantation. Both uterine epithelial and trophoblast cells near the time of implantation express HB-EGF and ErbB receptors. Thus, autocrine, paracrine, and juxtacrine interactions are all possible. Several studies have provided valuable clues to understanding the changes occurring when HB-EGF binds to its cognate receptors on target cells during implantation. HB-EGF binds to mouse blastocysts and opens specific Ca²⁺ channels, causing influx from the extracellular space. This effect is mediated by embryonic ErbB4, which is translocated to the apical surface of trophoblast cells at the relevant time. Intracellular Ca²⁺ accumulates in trophoblast cells by various means and then activates other intracellular signaling molecules, such as calmodulin and protein kinase C. The molecular and cellular changes mediated by HB-EGF-ErbB interactions promote trophoblast differentiation into an adhesion- and invasion-competent stage (Wang et al., 2000). When assessing trophoblast differentiation in vitro, one can use the outgrowth culture system for qualitative and quantitative evaluation. When a blastocyst is added to a culture well coated with an adhesion-promoting agent, trophoblastic cells attach and “outgrow” on the surface. The area occupied by growing trophoblast cells is measured to test the effectiveness of certain added agents as implantation-promoting factors. In this assay system, HB-EGF scores high. Thus, HB-EGF triggers molecular and cellular changes that closely resemble initial implantation in vivo. HB-EGF signaling also cross-talks with another signaling pathway in promoting blastocyst differentiation. Lysophosphatidic acid is a lipid signaling mediator with multiple functions in various physiological contexts, including embryo implantation (Ye and Chun, 2010). When added to blastocysts in vitro, lysophosphatidic acid induces accumulation of HB-EGF on the embryonic surface and promotes blastocyst outgrowth (Liu and Armant, 2004).

Now we will look into the second mechanism of HB-EGF action from the uterine perspective. One of the changes provoked by HB-EGF-soaked beads in the uterus is decidualization (Paria et al., 2001). The mechanism underlying this observation was examined in cultured uterine stromal cells. Here, HB-EGF treatment of cultured stromal cells causes characteristic changes in decidualization, i.e., proliferation, polyploidy, and binucleation. This effect was shown to be mediated by cyclin D3, a cell cycle molecule abundant in the uterine stroma (Tan et al., 2004).

Collectively, the above series of investigations verify the versatile involvement of this factor from the aspects of embryo-uterine interactions that further propagate into later stages of implantation (Fig. 1).

Conserved Roles for HB-EGF in Implantation: A Comparative Perspective

As presented above, extensive studies of mice have indicated that HB-EGF is a crucial mediator of embryo-uterine interactions during implantation. The mouse is often the most suitable system for functional validation of specific molecules because of the availability of various genetically modified model systems. Thus, initial findings from mice are tested further in different animal

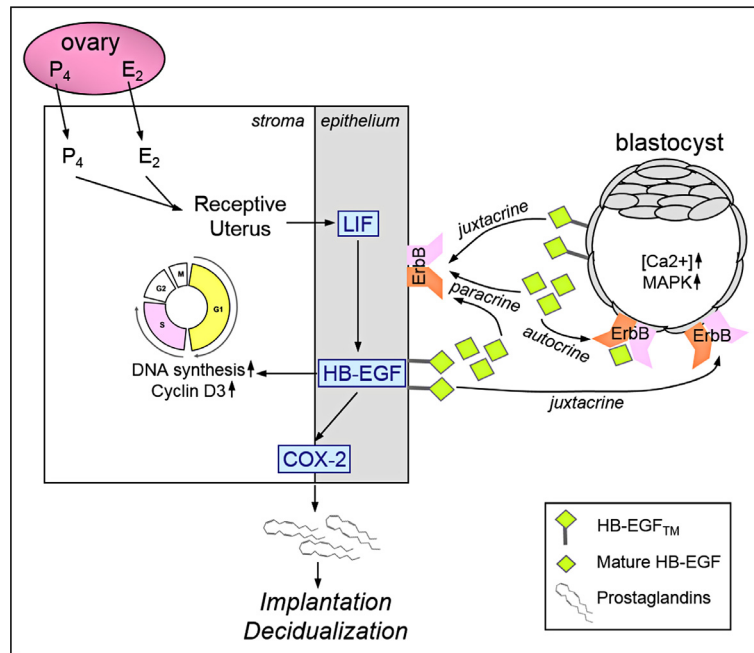


Fig. 1 A schematic landscape of signaling by HB-EGFTM and mature HB-EGF. HB-EGF takes advantage of autocrine, paracrine, and juxtacrine modes of signaling to regulate both embryonic and uterine functions. Reproduced from Lim, H. and Dey, S. (2009). HB-EGF: A unique mediator of embryo-uterine interactions during implantation. *Experimental Cell Research*, 315, 619–626, with permission.

models, and these efforts help solidify the original idea and build a hypothesis of conserved functions across species. In this sense, expression of HB-EGF was examined in the endometrium of species such as rabbit, hamster, cow, baboon, and human.

Rabbit blastocysts initiate attachment on day 7 of pregnancy. At this time, HB-EGF is induced at the site where the blastocyst initially contacts the uterine epithelium. In pigs, multiple forms of HB-EGF are produced and secreted into the uterine lumen. In cows exhibiting blastocyst elongation, HB-EGF is also detected on day 13 of pregnancy. The baboon endometrium is another site of HB-EGF expression: herein, its expression is under the regulation of progesterone and chorionic gonadotropin. The implantation process in hamsters, unlike in mice, depends mainly on progesterone and does not appear to require ovarian estrogen. Although the hormonal requirement for implantation is distinct in mice and hamsters, the pattern of HB-EGF induction at their implantation sites are analogous; both species require the presence of a blastocyst. In both mouse and hamster uteri, however, estrogen is a strong inducer of HB-EGF expression in the luminal epithelium. Based on these observations, it appears that multiple factors are involved in HB-EGF expression, but the implantation-specific pattern of induction is under strict control of the blastocyst.

Relevant references regarding the potential roles of HB-EGF during implantation in other species are cited in a review article by Lim and Dey (2009). With respect to human studies, refer to the article written by Armant et al. (2015) and references therein.

References

- Armant, D. R., Fritz, R., Kilburn, B. A., et al. (2015). Reduced expression of the epidermal growth factor signaling system in preeclampsia. *Placenta*, 36, 270–278.
- Brown, N., Deb, K., Paria, B. C., Das, S. K., & Reese, J. (2004). Embryo-uterine interactions via the neuregulin family of growth factors during implantation in the mouse. *Biology of Reproduction*, 71, 2003–2011.
- Citri, A., & Yarden, Y. (2006). EGF-ERBB signalling: Towards the systems level. *Nature Reviews Molecular Cell Biology*, 7, 505–516.
- Dahlhoff, M., Schäffer, M., Wolf, E., & Schneider, M. R. (2013). Genetic deletion of the EGFR ligand epigen does not affect mouse embryonic development and tissue homeostasis. *Experimental Cell Research*, 319, 529–535.
- Das, S. K., Chakraborty, I., Paria, B. C., et al. (1995). Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. *Molecular Endocrinology*, 9, 691–705.
- Das, S. K., Das, N., Wang, J., et al. (1997). Expression of betacellulin and epiregulin genes in the mouse uterus temporally by the blastocyst solely at the site of its apposition is coincident with the “window” of implantation. *Developmental Biology*, 190, 178–190.
- Das, S. K., Tsukamura, H., Paria, B. C., Andrews, G. K., & Dey, S. K. (1994a). Differential expression of epidermal growth factor receptor (EGF-R) gene and regulation of EGF-R bioactivity by progesterone and estrogen in the adult mouse uterus. *Endocrinology*, 134, 971–981.
- Das, S. K., Wang, X. N., Paria, B. C., et al. (1994b). Heparin-binding EGF-like growth factor gene is induced in the mouse uterus temporally by the blastocyst solely at the site of its apposition: a possible ligand for interaction with blastocyst EGF-receptor in implantation. *Development*, 120, 1071–1083.
- DiAugustine, R. P., Petrusz, P., Bell, G. I., et al. (1988). Influence of estrogens on mouse uterine epidermal growth factor precursor protein and messenger ribonucleic acid. *Endocrinology*, 122, 2355–2363.
- Erickson, S. L., O’Shea, K. S., Ghaboosi, N., & Loverro, L. (1997). ErbB3 is required for normal cerebellar and cardiac development: A comparison with ErbB2-and heregulin-deficient mice. *Development*, 124, 4999–5011.
- Gassman, M., Casagrande, F., & Orioli, D. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature*, 378, 390–394.

- Huet-Hudson, Y. M., Andrews, G. K., & Dey, S. K. (1989). Cell type-specific localization of c-myc protein in the mouse uterus: modulation by steroid hormones and analysis of the periimplantation period. *Endocrinology*, 125, 1683–1690.
- Huet-Hudson, Y. M., Chakraborty, C., De SK, S. Y., Andrews, G. K., & Dey, S. K. (1990). Estrogen regulates the synthesis of epidermal growth factor in mouse uterine epithelial cells. *Molecular Endocrinology*, 4, 510–523.
- Lee, D., Pearsall, R. S., Das, S., et al. (2004). Epiregulin is not essential for development of intestinal tumors but is required for protection from intestinal damage. *Molecular and Cellular Biology*, 24, 8907–8916.
- Lim, H., Das, S. K., & Dey, S. K. (1998). erbB genes in the mouse uterus: cell-specific signaling by epidermal growth factor (EGF) family of growth factors during implantation. *Developmental Biology*, 204, 97–110.
- Lim, H., & Dey, S. (2009). HB-EGF: A unique mediator of embryo-uterine interactions during implantation. *Experimental Cell Research*, 315, 619–626.
- Lim, H., Dey, S. K., & Das, S. K. (1997). Differential expression of the ErbB2 gene in the periimplantation mouse uterus: Potential mediator of signaling by epidermal growth factor-like growth factors. *Endocrinology*, 138, 1328–1337.
- Lin, T. H., Mukku, V. R., Verner, G., Kirkland, J. L., & Stancel, G. M. (1988). Autoradiographic localization of epidermal growth factor receptors to all major uterine cell types. *Biology of Reproduction*, 38, 403–411.
- Liu, Z., & Armant, D. R. (2004). Lysophosphatidic acid regulates murine blastocyst development by transactivation of receptors for heparin-binding EGF-like growth factor. *Experimental Cell Research*, 296, 317–326.
- Luetkeke, N. C., Qiu, T. H., Fenton, S. E., et al. (1999). Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development*, 126, 2739–2750.
- Massagué, J., & Pandiella, A. (1993). Membrane-anchored growth factors. *Annual Review of Biochemistry*, 62, 515–541.
- Paria, B. C., Elenius, K., Klagsbrun, M., & Dey, S. K. (1999). Heparin-binding EGF-like growth factor interacts with mouse blastocysts independently of ErbB1: A possible role for heparan sulfate proteoglycans and ErbB4 in blastocyst implantation. *Development*, 126, 1997–2005.
- Paria, B. C., Ma, W., Tan, J., et al. (2001). Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. *Proceedings of the National Academy of Sciences USA*, 98, 1047–1052.
- Reese, J., Brown, N., Das, S. K., & Dey, S. K. (1998). Expression of neu differentiation factor during the periimplantation period in the mouse uterus. *Biology of Reproduction*, 58, 719–727.
- Schneider, M. R., & Yarden, Y. (2014). Structure and function of epigen, the last EGFR ligand. *Seminars in Cell and Developmental Biology*, 28, 57–61.
- Tan, Y., Li, M., Cox, S., et al. (2004). HB-EGF directs stromal cell polyploidy and decidualization via cyclin D3 during implantation. *Developmental Biology*, 265, 181–195.
- Tomooka, Y., DiAugustine, R. P., & McLachlan, J. A. (1986). Proliferation of mouse uterine epithelial cells in vitro. *Endocrinology*, 118, 1011–1018.
- Wang, J., Mayernik, L., Schultz, J. F., & Armant, D. R. (2000). Acceleration of trophoblast differentiation by heparin-binding EGF-like growth factor is dependent on the stage-specific activation of calcium influx by ErbB receptors in developing mouse blastocysts. *Development*, 127, 33–44.
- Wong, R. W. (2003). Transgenic and knock-out mice for deciphering the roles of EGFR ligands. *Cellular and Molecular Life Sciences*, 60, 113–118.
- Xie, H., Wang, H., Tranguch, S., et al. (2007). Maternal heparin-binding-EGF deficiency limits pregnancy success in mice. *Proceedings of the National Academy of Sciences USA*, 104, 18315–18320.
- Ye, X., & Chun, J. (2010). Lysophosphatidic acid (LPA) signaling in vertebrate reproduction. *Trends in Endocrinology and Metabolism*, 21, 17–24.

Further Reading

- Cha, J., Lim, H., & Dey, S. K. (2015). Embryo Implantation. In T. M. Plant, & A. J. Zelenik (Eds.), *Knobil and Neill's Physiology of Reproduction* (4th edn.). Amsterdam: Elsevier.
- Jessmon, P., Leach, R. E., & Armant, D. R. (2009). Diverse functions of HBEGF during pregnancy. *Molecular Reproduction and Development*, 76, 1116–1127.

Leukemia Inhibitory Factor

Colin L Stewart, Institute of Medical Biology, Singapore, Singapore

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blastocyst The stage at which the mammalian embryo will implant into the uterus. The *blastocyst* consists of the *inner cell mass*, a group of cells that will form the embryo proper and is enclosed by the hollow epithelial layer or sphere of cells, the *trophoblast*. The trophoblast initially mediates interactions between the developing embryo and the uterus. After implantation, the trophoblast, together with cells from the embryo, and uterus forms the placenta.

Decidua The cellular structure produced by extensive proliferation and differentiation of the uterine stroma (decidualization). It also contains many blood cells derived from the bone marrow. The decidua is formed after implantation and surrounds and supports the early growth and development of the implanting embryo. In mammals, where the trophoblast invades the uterus, such as in mice, the decidua restricts trophoblast growth and invasion.

Delayed implantation A physiological state that can be induced in about 40 mammalian species, such as mice, rats, and Roe deer. Blastocyst implantation is inhibited and embryo development arrested. By delaying implantation mammals regulate the duration of pregnancy, thus ensuring the young are born at the most optimal time and conditions for their survival.

Embryonic stem (ES) cells Developmentally totipotent cells derived from the mouse embryo's inner cell mass (ICM). These cells are cultured in vitro and can differentiate into many other cell types or produce entire mice when reintroduced into blastocysts, that are then transferred back into the uterus to develop to term. They have been primarily used to derive lines of mice ("knockout" mice) carrying mutations in any gene of interest.

Gene targeting A powerful technique used to introduce mutations into specific genes in ES cells grown in vitro. The ES cells are used to derive mice carrying the mutated gene so that its function can be studied in the whole animal.

Luminal epithelium The single layer of cells lining the cavity or lumen of the uterus. This tissue is central to regulating when blastocyst implantation occurs and in stimulating the underlying stromal cells to decidualize.

Introduction

In mammals, the attachment and then implantation of the developing embryo into the uterus is a unique, essential and a defining feature of their reproduction. Following fertilization, the zygote moves down the reproductive tract and undergoes a series of cleavage divisions that result in the formation of the multicellular blastocyst at the point the embryo moves from the fallopian tube into the uterus. As typified by the mouse and human, the blastocyst comprises some 60–100+ cells consisting of two types: the trophoblast, which will function as an early placenta, and the inner cell mass (ICM), which will form the embryo proper. For the blastocyst to survive, it must establish physical contact with the uterine tissues for it to receive nutrients to support its continued growth and development. In the uterus, the blastocysts attach to the luminal epithelium (LE), a single cell layered epithelium that lines the uterine lumen. Following attachment, the extent to which the embryonic trophoblast then invades the uterine tissues varies between species, with mouse and human embryos extensively invading the uterus, whereas in pigs and horses the embryo remains attached to the LE but does not invade.

An essential feature to successful implantation, is that the development of the pre-implantation embryo coordinates with the cellular and physiological changes occurring in the uterus in preparation for implantation during each reproductive cycle. Any asynchrony between the preparation of the uterus and embryonic development results in implantation failure and embryo loss.

The factors primarily driving the cellular changes in the uterus in its preparation to receive a blastocyst are the ovarian steroid hormones, estrogen (E2) and progesterone (P4). In mice, E2 release from the ovary stimulates extensive cell proliferation initially in the luminal (LE) and glandular epithelia (GE). Thereafter rising levels of P4 from the ovarian corpora lutea inhibit the E2 stimulated epithelial proliferation, and initiate a switch to stromal proliferation, together with the induction of cellular differentiation within the different uterine compartments. Most of these E2 and P4 mediated effects are now known to be due to the steroid hormones inducing the expression a variety of locally produced growth factors, including cytokines and their receptors that activate a variety of signaling pathways that then act on the different cell types in either a paracrine or autocrine manner (Finn and Martin, 1974). A key cytokine in the implantation process is Leukemia Inhibitory Factor (LIF) which is essential to regulating uterine receptivity and responsiveness to a potential implanting blastocyst (Stewart et al., 1992).

The Molecular Biology of LIF and Its Receptor

Leukemia inhibitory factor (LIF) is a secreted monomeric, glycosylated protein with a molecular weight (MW) of 32–62 kDa. It is initially synthesized as a 202 amino acid precursor that is then reduced to 180 amino acids by cleavage of a 22 amino acid peptide

from its N-terminus. The LIF protein is folded into a 4 α helix bundle with the molecular weight of the unglycosylated protein being 20 kDa. Though heavily glycosylated, glycosylation is apparently nonessential to LIF's biological activity. LIF is encoded by a single, 6 kb gene, consisting of three exons. The gene is transcribed as a 4.8-kb mRNA transcript, of which –3.2 kb at the 3' end is untranslated. In the human, the chromosomal locus of the LIF gene is 22q12.2 and in the mouse it maps to chromosome 11, band A1. LIF is a member of the interleukin-6 (IL-6) family of cytokines, that include IL-6 and 11, oncostatin M (OSM), cardiotrophin (CT), ciliary neurotrophic factor (CNTF) and cardiotrophin like cytokine (CLC). These factors share overlapping, as well as unique functions on many different tissues as they all utilize the transmembrane protein gp130 as a component of their specific receptors (Nicola and Babon, 2015).

LIF Receptor- β and gp130

LIF binds to the cell's surface via a heterodimeric high-affinity cytokine receptor consisting of two transmembrane proteins, the LIF receptor beta (LIFR β) and gp130. The human LIFR β precursor is a single pass transmembrane protein of 1097 amino acids, and maps to chromosome 5p13.1 and in the mouse to the proximal region of chromosome 15. At least three transcripts are transcribed from the gene in adult murine and human tissues. The largest transcript is 10–11 kb, and both it and the 5 kb transcript code for the full-length transmembrane protein. The other shorter form of 3 kb is generated by alternate splicing with the introduction of a novel stop codon truncating the mRNA, resulting in the synthesis of a soluble form of the receptor lacking both the transmembrane and intracellular domains. During pregnancy, synthesis of all three forms increases in the adult female liver resulting in elevated levels of the soluble form of the receptor appearing in the circulatory system which then peak at midgestation. The function of the soluble form is unclear, although it may act as an antagonist by binding to and sequestering LIF from the trans-membrane form of the receptor. Mutations in the LIFR β gene result the rare disease, Stuve-Wiedemann/Schwartz-Jampel syndrome that cause bone abnormalities and a dysfunctional autonomic nervous system that affects breathing and temperature regulation.

The other component of the high-affinity LIF receptor, gp130 (or IL6ST), is a 918-amino acid glycoprotein, with a 22-amino acid transmembrane domain and a 277-amino acid intracellular domain. This produces a protein with a predicted MW of 101 kDa, with glycosylation increasing the size to 130 kDa. The locus encoding gp130 maps to chromosome 5q11.2, which is homologous to the murine locus on Chr13. A pseudogene in humans is located on Chr17. In humans the gene is transcribed as a single 8.5 kb transcript, whereas in the mouse two transcripts of 7 and 10 kb are detected (Nicola and Babon, 2015).

LIF receptors are found in many different tissues including the liver, central nervous system, macrophages, bone, kidney, placenta, ICM of the blastocyst and the uterine epithelia. The binding of LIF to the LIFR β results in the non-covalent association of the complex with gp130 to create the high-affinity heterodimeric receptor complex (Fig. 1). In most cell types the formation of the high-affinity complex between LIF, LIFR β , and gp130 is required for signal transduction, although there is evidence that the LIFR β can function as a homodimer, without gp130, at inhibiting embryonic stem (ES) cell differentiation. The heterodimeric receptor complex itself has no intrinsic tyrosine kinase activity. However the cytoplasmic domains of LIFR β -gp130-LIF trimeric complex associate with at least three members of the Janus (JAK) kinase family, in particular, JAK1, JAK2, and TYK2. Activation of the JAKs, especially JAK1, is by transphosphorylation with one JAK1 member, that is attached to the cytoplasmic chain of LIFR β -gp130 dimer, phosphorylating another JAK1 bound to the other chain. The activated JAK kinases then stimulate, by phosphorylation, of at least three signaling pathways, the STAT pathway, p21ras/MAP kinase pathway, and PI-3 kinase pathway. Of these three pathways, it is the activation of the STAT pathway that is of most significance in the uterus (Fig. 1).

Phosphorylation of the bound JAKs recruits the STAT (signal transducer and activator of transcription) factors to the LIFR β -gp130-JAK1 complex. The STATs are a family of latent transcription factors present in the cytoplasm. Within the family, STATs 1, 3 and 5 are activated by gp130 cytokines, of which STAT3 is the predominant form with regard to LIF signaling in the uterus. The binding of STAT3 to the receptor complex results in its phosphorylation by JAK1. In turn the phosphorylated STAT3 forms a dimer which then translocates to the nucleus where it transcribes genes. Among the genes that STAT3 induces is SOCS3, which inhibits JAK/STAT signaling by binding to both gp130 and JAK1, and so acts in a negative feedback loop, shutting down the JAK/STAT3 and MAP kinase pathways (Fig. 1) (Nicola and Babon, 2015).

LIF and Female Reproduction

LIF was initially discovered as being a factor that simultaneously induced the differentiation and inhibited the proliferation of the myeloid leukemia cell line M1. Subsequently LIF was shown to be necessary to maintain cultured mouse embryonic stem cells (ES cells) in an undifferentiated state revealing a potential role in regulating mouse embryonic development (Williams et al., 1988). ES cells are the pluripotent stem cells established from the inner cell mass (ICM) of the mouse blastocyst and have been extensively used to both study mammalian development and as a vehicle for the modification of the mouse's genome. In mouse blastocysts, LIF transcripts are found in the trophoblast of the blastocyst, whereas the LIFR β and gp130 are detected in the ICM. In blastocysts, LIF expression in the trophoblast may act to maintain the cells of the ICM during implantational delay (embryonic diapause), a state in which, in some mammals, blastocyst growth is arrested, with cell proliferation and development ceasing in the embryo (Nichols et al., 2001). Such a strategy has evolved in some mammals to as to regulate the birth of the embryos to a time where food and resources are maximally available to the mother, so she can support the newborn's postnatal growth (Renfree and Shaw, 2000).

During post-implantation embryogenesis LIF is undetectable in the embryo proper, whereas the placenta and developing skeletal system both express the LIFR β , as does the developing central nervous system (CNS). Constitutive deletion of the LIFR β results

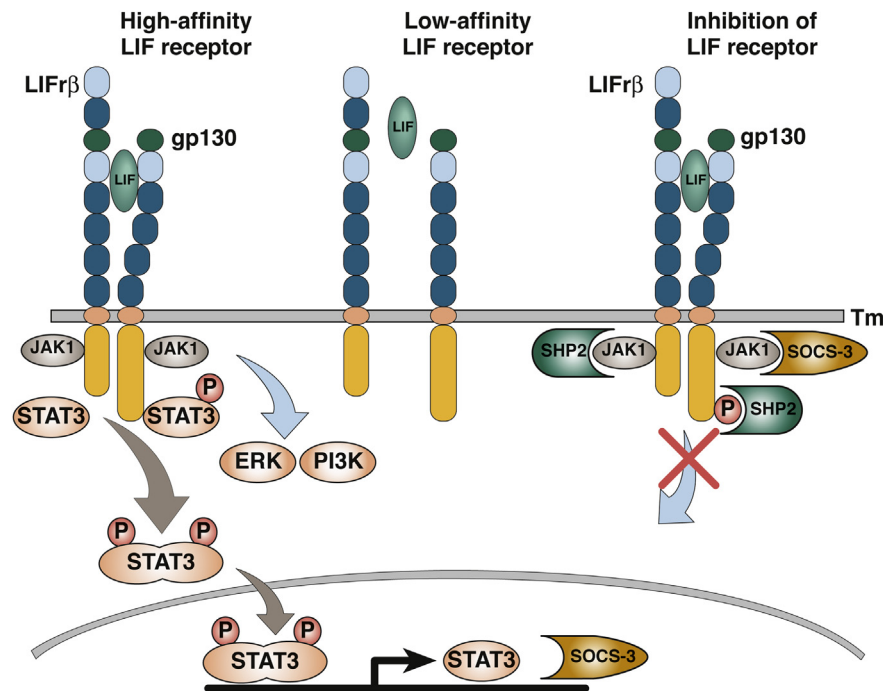


Fig. 1 Structure of the LIF receptor and activation of the JAK-STAT3 signaling pathway. The soluble cytokine LIF binds to the low affinity LIFRβ-gp130 (centre) making them dimerize to form the high affinity LIFRβ-gp130-LIF complex (left panel). This recruits the JAK kinases (Jak1) which autophosphorylate each other and in turn recruit the transcription factor STAT3. STAT3 is phosphorylated, dimerizes and enters the nucleus. The LIFRβ-gp130-LIF complex can also activate the ERK and PI3K pathways as well but these do not appear to be important in regulating LIF signaling during implantation. The STAT3 phosphorylated dimer binds to the promoters of various genes including SOCS3, initiating their transcription. SOCS3 protein can bind to the LIFRβ-gp130-LIF complex and block JAK kinase activity, so inhibiting the LIF mediated STAT3 signaling pathway (right panel).

in these tissues developing abnormalities that result in embryonic lethality either in utero, due to abnormal placental development or shortly after birth and correlates with defective development of the brain (Cheng et al., 2017; Ware et al., 1995). During postnatal development, LIF expression reappears in many tissues, as do the LIFRβ and gp130 receptors in many different adult tissues, such as the liver. Intriguingly, however LIF null embryos can develop into normal mice, suggesting that some other ligand, that utilizes the LIFRβ, is produced in the placenta and CNS during embryogenesis and is necessary for normal development of these tissues.

In adults, LIF is most highly expressed in the uterus, albeit transiently. In mice, uterine expression of LIF is biphasic, with the first peak occurring at ovulation (Day 0–1). Following mating, LIF expression declines but then rises again, starting on the morning of the fourth day of pregnancy (D4) that peaks around midday, before falling again to basal levels that persist throughout the rest of pregnancy (Bhatt et al., 1991). In the human uterus, LIF is first detected around Day 18 in the postovulatory phase of the menstrual cycle and persists until the end of the cycle. Unlike the mouse, there is no indication that LIF is expressed at the time of ovulation in women. In both the mouse and human uterus, LIF transcripts and protein are localized to the epithelium of the glandular endometrial glands (GE), and in the human LIF transcripts are also found in the LE (Cullinan et al., 1996). In women, LIF protein is detected in uterine washings, indicating that LIF is secreted into the uterine lumen. It is of significance that in both humans and rodents uterine levels of LIF peak at the exact time that the uterus becomes receptive to the embryo. Similar increases in LIF expression around the time of embryo implantation have been reported in many other species including the rabbit, hamster, rat, pig, cow, rhesus monkey, and western spotted skunk, indicating that this pattern of expression is a widespread among mammals (Cheng et al., 2002).

In mice the uterine expression of LIF is under maternal control and is not influenced by the presence or absence of embryos. Expression is detected on D4 in pseudopregnant mice that are physiologically primed for pregnancy, but have no embryos. LIF is not detected in mice during lactationally induced implantational delay, in which embryos are present in the uterus, but implantation is arrested, with the blastocysts entering a state of growth arrest and physiological dormancy. Once delay is broken, by weaning or removal of the suckling pups, LIF expression is induced and the dormant blastocysts are reactivated and implant (Chen et al., 2000).

In ovariectomized mice, where there is no production of endogenous E2 and P4 (due to the absence of the ovaries), uterine LIF expression can be induced by E2 injection, whereas injected P4 alone has no effect. In contrast, in rabbits, LIF expression is induced by P4 but not by E2. This reflects the differences in hormonal regulation in the rabbit, in which E2 is not required for the induction of implantation. LIF expression may be directly regulated by the action of the steroid hormone receptors because its promoter contains steroid response elements located upstream of the transcription start site. In transfection studies, the human LIF promoter

linked to a reporter gene is activated when cells are treated with P4. In both mice and humans, E2 stimulates the estrogen receptor alpha with transcription of the LIF gene being co-regulated with another transcription factor, the tumor suppressor factor p53 (Hu et al., 2007).

LIF Is Essential for Blastocyst Implantation

The coincidence between LIF expression in the uterus with the onset of embryo implantation, suggested that LIF might be an essential factor regulating this process. Direct evidence for this was established by the derivation of mice in which the LIF gene was mutated so that it was no longer functional.

Mice lacking a functional LIF gene develop to adulthood, although this can be influenced by strain background. Homozygous LIF-deficient males are fertile, however, homozygous females never produce any offspring despite repeated mating to males of proven fertility. In normal mice, by the fourth day of pregnancy, the blastocysts are distributed along the length of the uterine lumen. The mechanism by which this distribution and positioning is accomplished is not understood but may involve contractions of the uterine musculature, since there is no evidence for the existence of specific attachment sites in the uterus. During this time the cross-sectional shape of the lumen changes to a slit, with the top of the lumen orientated to the mesometrial side that is attached to connective tissue, fat, and the blood vessels supplying the uterus. The blastocysts are always located at the opposite (antimesometrial) side of the lumen and orientated such that the ICM faces the mesometrial side (Fig. 2A). The blastocysts are juxtaposed to the LE, with the LE almost entirely surrounding the blastocyst. However, at this stage, no firm attachment is established between the embryo and uterus as the blastocysts can be readily flushed from the uteri.

LIF deficient females ovulate eggs at normal numbers. Subsequently the eggs are fertilized, develop to the blastocyst stage and position themselves correctly along the length of the uterus in a manner that doesn't differ from normal LIF positive females. The phase at which the blastocysts are distributed along the uterus is referred to as the appositional stage. In normal mice, around the middle of the fourth day of pregnancy, full attachment or implantation commences with the luminal epithelium adjacent to the trophoblast being phagocytosed by the trophoblast resulting in the trophoblast coming into direct contact with the underlying stromal cells. By the fifth day the stromal cells, adjacent to the site of blastocyst attachment, have started to proliferate and initiate extensive morphological changes, that include an increase in size, and polyploidization, all of which leads to the formation of the primary deciduum. Decidua formation is accompanied by a localized increase in vascular capillary permeability and edema. Together, these changes produce a tissue that nourishes and supports the growth of the early post-implantation embryo, as well as restricting trophoblast invasion.

In LIF deficient females there is no evidence that the trophoblast invades the LE. Neither can any stage of decidua formation be induced, even after using different artificial procedures to induce decidualization. This revealed that LIF is not only required to initiate blastocyst implantation/invasion but is also required for the uterus to decidualize. This failure of the LIF deficient uteri to both allow blastocyst implantation and decidualization was not due to the uterus undergoing the appropriate patterns of cell proliferation and differentiation in preparation for implantation, since the LIF deficient uteri, when treated with E2 and P4, undergo normal patterns of cell proliferation and differentiation. Furthermore, post-ovulatory P4 levels increased at the same rate and to the same extent in the LIF-deficient mice as in normal females.

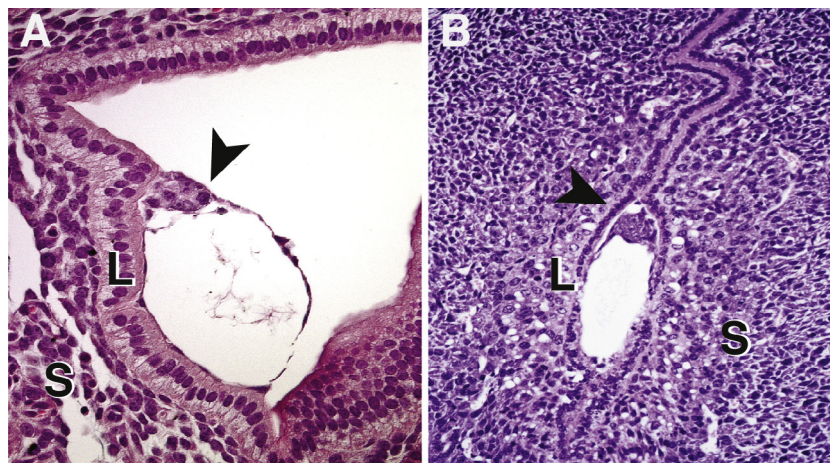


Fig. 2 Blastocysts in the murine uterus prior to implantation and within 24 h after the onset of implantation. (A) A blastocyst on D4 of gestation (arrow head) lies against the single cell layer of the luminal epithelium (L). Underneath the luminal epithelium is the stroma (S). The arrow head points to the inner cell mass (ICM) of the blastocyst that will form the embryo. The ICM is enclosed within the thin walled sphere of the trophoblast. (B) A blastocyst (arrow head) beginning to implant into the uterus. Note how the cellular density of the stroma (S) has increased as in forms the decidua. The luminal epithelium (L) is being phagocytosed by the trophoblast at the sides of the implanting blastocyst.

Conclusive evidence that the loss of LIF primarily affected the uterus was shown by the surgical transplantation of unimplanted LIF-deficient blastocysts (recovered from LIF deficient mothers mated to LIF deficient males) to the uteri of WT pseudopregnant recipients. In the pseudopregnant females the LIF deficient blastocysts implanted and developed normally to term. In the reciprocal procedure, transfer of WT blastocysts to pseudopregnant LIF-deficient females did not result in the implantation of the WT blastocysts in the LIF-deficient females. This revealed that the failure of implantation was due to a uterine defect rather than a defect in the blastocysts. This was further supported by the injection of recombinant LIF protein into pregnant LIF deficient females on the fourth day of pregnancy, which resulted in the blastocysts implanting and developing to term. Sterility in the LIF-deficient females is therefore due to an otherwise normal uterus not receiving the necessary signal (LIF) that both induces implantation and decidualization (Chen et al., 2000, Stewart et al., 1992).

The Uterine Luminal Epithelium Is the Target for LIF

The LIFR β and gp130 are expressed in both the GE and LE of human uteri. In the mouse, LIFR β levels in the LE progressively increase from the second day after mating and peak around D4 with the receptor being localized to the apical and basal sides of the LE. In contrast, gp130 is only transiently expressed in the LE around D4 and is not detected in the GE (Figs. 3A and B). In both mice and women there is no indication that LIFR β or gp130 are expressed at significant levels in the uterine stromal cells, with the possible exception of some hematopoietic cells such as macrophages or neutrophils. In the murine uterus, STAT3 is strongly expressed in the

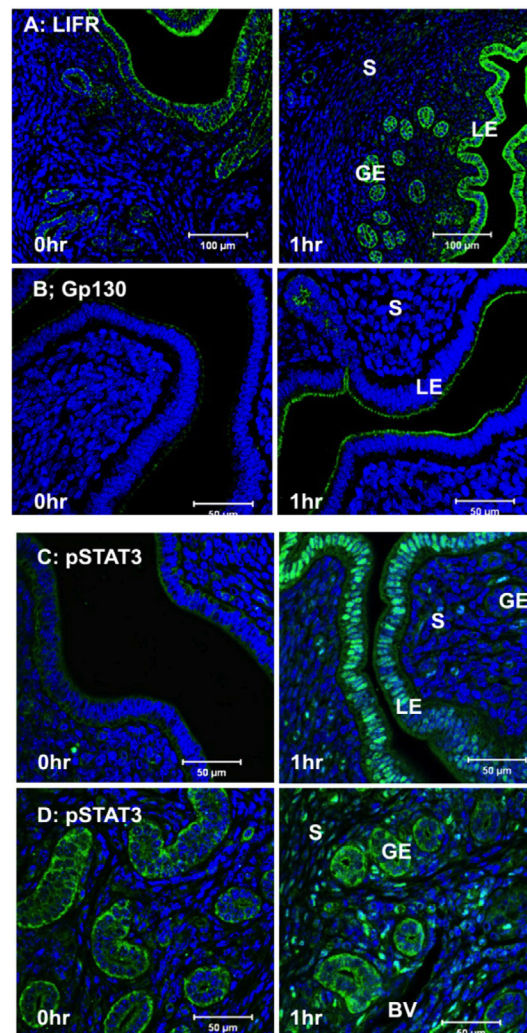


Fig. 3 STAT3 signaling by the LIFR β -gp130-LIF complex in the Luminal epithelium of the mouse. (A) The LIFR β (green) is expressed in the luminal (LE) and glandular epithelia (GE) but not in the stroma (S). (B) Gp130 is only expressed at the apical surface of the LE. (C) Phosphorylated STAT3 (pSTAT3) translocates to the nuclei of the LE 1 h after LIF binding to the receptor complex. (D) However STAT3 though expressed in the GE does not translocate to the nuclei.

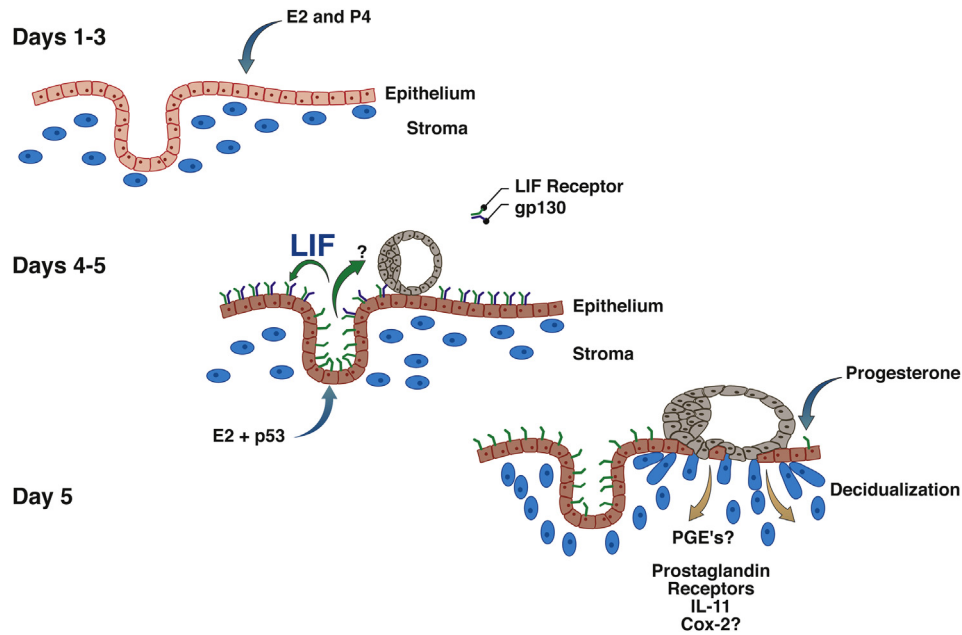


Fig. 4 A model depicting the progression of molecular changes in the murine uterus that lead to blastocyst implantation. On days 1–3 after mating the uterine epithelia and stroma undergo cell proliferation and differentiation that is driven by the ovarian steroid hormones estrogen (E2) and progesterone (P4). On Day 4 the blastocyst are in the uterus juxtaposed the epithelium (as shown in Fig. 3A). A transient increase in E2 (nidatory) that presumably activates the estrogen receptor and together with the p53 transcription factor induce LIF expression in the endometrial glands. LIF is secreted into the uterine lumen where it binds to the LIFR β -gp130 receptor complex on the surface of the LE (Fig. 2A and B). The binding of LIF to the LIFR β -gp130 complex activates STAT3 (Fig. 2C) that moves into the nuclei of the LE where it induces/represses the expression of some ~300 genes. By D5 the blastocyst has started to breakdown the LE and invade the stroma. The stroma undergoes decidualization that involves a range of cellular and molecular changes as seen in Fig. 3B and includes the expression of the LIF related cytokine IL-11 and the inflammation inducing factors such as prostaglandins (PGEs).

cytoplasm of the LE. Activation of the JAK-STAT pathway *in vivo* by LIF injection into mice, or when the LE was isolated as cell sheets from the uterus and then treated with LIF, revealed that STAT3 was phosphorylated and translocated to the nuclei of the LE (Fig. 3C and D). In addition, genetic ablation of either STAT3 or LIFR β specifically in the LE, as well as the mutation of the cytoplasmic domain of gp130, all result in an unresponsive uterus in which blastocysts could not implant.

LIF stimulation of the LIFR β /gp130 receptor, results in STAT3 activation and nuclear translocation within 15 min of LIF treatment. STAT3 translocation to the nucleus increases the levels in expression of some 250+ genes in addition to a further 50 genes whose expression decreases within 1 h after LIF treatment. This culminates in the activation or inhibition of numerous biological pathways in the uterus, involving signaling, the activation of 40+ transcription factors in the LE, changes in cell adhesion, cellular organization, immune status, metabolism, the secretion of other growth factors, cytokines and chemokines. The result of all these changes is that the blastocysts that are juxtaposed to the apical side of the LE start to implant (Rosario et al., 2014).

A summary for the action of LIF is proposed in Fig. 4, where LIF, secreted from the endometrial gland, binds to specific receptors on the LE. In turn this stimulates the epithelium to respond to the already closely apposed blastocyst by allowing trophoblast invasion of the uterus and for a second signal(s) to induce the underlying stromal cells to decidualize. However, given the extensive changes in gene expression that are induced in the LE by LIF, it is still necessary to identify the molecular factor(s) that directly initiate blastocyst activation/trophoblast invasion and which signaling pathway/factor(s), originating from the LIF stimulated LE initiate stromal decidualization.

Conclusions and Prospects

Cellular proliferation and differentiation in the uterus during the reproductive cycle, as well as embryo implantation, are primarily regulated by the steroid hormones E2 and P4, which in turn are regulated by the hormones of the hypothalamic–pituitary axis. However, it is now established that the complex cellular events at implantation are not only regulated by a few hormones secreted into the circulatory system but also by a cascade of locally produced peptide growth factors (cytokines, growth factors) and other small molecules, such as prostaglandins. This multitude of factors act to “amplify” the cellular and tissue responses to estrogen and progesterone by regulating the expression of a large number of genes through different signal transduction pathways and transcription factors. An increasing understanding of just which factors are crucial, such as LIF, how they interact with each other, and their control of gene expression in the uterus, are providing new opportunities to potentially regulate the fertility of many

mammals. In humans, a more pressing need is to gain a better understanding to some of the causes of “unexplained” infertility in women. There is now some evidence indicating that infertility in women due to defects at implantation is associated with disruption to the LIF regulated JAK-STAT pathway (Choi et al., 2016, Kang et al., 2009).

References

- Bhatt, H., Brunet, L. J., & Stewart, C. L. (1991). Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 11408–11412.
- Chen, J. R., Cheng, J. G., Shatzer, T., Sewell, L., Hernandez, L., & Stewart, C. L. (2000). Leukemia inhibitory factor can substitute for nidatory estrogen and is essential to inducing a receptive uterus for implantation but is not essential for subsequent embryogenesis. *Endocrinology*, 141, 4365–4372.
- Cheng, J., Rosario, G., Cohen, T. V., Hu, J., & Stewart, C. L. (2017). Tissue-specific ablation of the LIF receptor in the murine uterine epithelium results in implantation failure. *Endocrinology*, 158, 1916–1928.
- Cheng, J. G., Rodriguez, C. I., & Stewart, C. L. (2002). Control of uterine receptivity and embryo implantation by steroid hormone regulation of LIF production and LIF receptor activity: Towards a molecular understanding of “the window of implantation”. *Reviews in Endocrine & Metabolic Disorders*, 3, 119–126.
- Choi, Y., Kim, H. R., Lim, E. J., Park, M., Yoon, J. A., Kim, Y. S., Kim, E. K., Shin, J. E., Kim, J. H., Kwon, H., Song, H., & Choi, D. H. (2016). Integrative analyses of uterine Transcriptome and Micronaome reveal compromised LIF-STAT3 signaling and progesterone response in the endometrium of patients with recurrent/repeated implantation failure (Rif). *PLoS One*, 11, E0157696.
- Cullinan, E. B., Abbondanzo, S. J., Anderson, P. S., Pollard, J. W., Lessey, B. A., & Stewart, C. L. (1996). Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 3115–3120.
- Finn, C. A., & Martin, L. (1974). The control of implantation. *Journal of Reproduction and Fertility*, 39, 195–206.
- Hu, W., Feng, Z., Teresky, A. K., & Levine, A. J. (2007). P53 regulates maternal reproduction through LIF. *Nature*, 450, 721–724.
- Kang, H. J., Feng, Z., Sun, Y., Atwal, G., Murphy, M. E., Rebbeck, T. R., Rosenwaks, Z., Levine, A. J., & Hu, W. (2009). Single-nucleotide polymorphisms in the P53 pathway regulate fertility in humans. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 9761–9766.
- Nichols, J., Chambers, I., Taga, T., & Smith, A. (2001). Physiological rationale for responsiveness of mouse embryonic stem cells to Gp130 cytokines. *Development*, 128, 2333–2339.
- Nicola, N. A., & Babon, J. J. (2015). Leukemia inhibitory factor (LIF). *Cytokine & Growth Factor Reviews*, 26, 533–544.
- Renfree, M. B., & Shaw, G. (2000). Diapause. *Annual Review of Physiology*, 62, 353–375.
- Rosario, G. X., Hondo, E., Jeong, J. W., Mutalif, R., Ye, X., Yee, L. X., & Stewart, C. L. (2014). The LIF-mediated molecular signature regulating murine embryo implantation. *Biology of Reproduction*, 91, 66.
- Stewart, C. L., Kaspar, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F., & Abbondanzo, S. J. (1992). Blastocyst implantation depends on maternal expression of Leukaemia inhibitory factor [see comments]. *Nature*, 359, 76–79.
- Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S. A., Glianiak, B. C., McKenna, H. J., Papayannopoulou, T., Thoma, B., & Et, A. (1995). Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. *Development*, 121, 1283–1299.
- Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., & Gough, N. M. (1988). Myeloid Leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. *Nature*, 336, 684–687.

Interferons

Troy L Ott and Alan D Ealy, Pennsylvania State University, University Park, PA, United States; and Virginia Tech University, Blacksburg, VA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The term interferon (IFN) was first used by Isaacs and Lindenmann in 1957 to describe substances secreted by chicken chorioallantoic membranes exposed to influenza virus. The soluble interferon secreted into the culture medium rendered other membrane cultures resistant to virus challenge. This effect was termed virus “interference,” and served as the origin of the name interferon. More than 60 years later it is clear that interferons are members of the large cytokine family of evolutionarily conserved pleiotropic regulators of cellular function. Mammalian IFN are broadly classified into Type I, Type II, and Type III (Bayer et al., 2016). Type I IFN include alpha (IFNA), beta (IFNB), delta (IFND), epsilon (IFNE), tau (IFNT), and omega (IFNW). The Type II IFN class is represented by a single member, termed IFN gamma (IFNG), and the Type III IFN class contains at least three members that are known either as IFN lambda (IFNL) 1, 2, and 3 or as interleukin (IL) 29, 28A, and 28B. The roles of IFN are perhaps best described as components of the innate arm of the immune system and serve as early responders to invading pathogens. Interferons play a key role in reducing pathogen replication, and regulating immune responses to infection (Snell et al., 2017). However, a subclass of Type I IFN is now known to serve as vital controllers of early signaling between the developing embryo (conceptus) and the uterine endometrium in ruminant ungulates (Bazer and Thatcher, 2017). It is this role of interferons that will be reviewed here.

Evolution of Mammalian Interferons

The Type III IFNs are the most ancient class of IFN. They exist in birds, reptiles, and mammals. The Type I and II IFNs emerged after separation of mammals from other animals. The Type I IFN have fascinated scientists and clinicians for the past 60+ years and they were the first members of the family recognized to possess antiviral activity. Also, they have been long-recognized to contain different biological potencies against different pathogens. Some Type I IFN are general, broad spectrum immediate responders to pathogens while others contain more specialized functions, with some being expressed in specific cell types and reacting only with specific types of viruses (Ealy and Wooldridge, 2017). Multiple Type I IFN genes exist in mammals. For example, ruminant species (cattle, sheep, goats) that contain abundant and diverse rumen bacterial populations likely require a very robust immune system. Not surprisingly, cattle contain genes that encode 13 distinct IFNA, 6 IFNB, 24 IFNW, and between 3 and 6 IFNT (Walker and Roberts, 2009). Interferon gene duplication likely reflects the need for a variety of IFN to respond to a wide range of pathogens. The presumption is that as species emerged, they continued to duplicate and mutate IFN genes in their genomes in ways that improved their survivability. These mutations and duplications then provided genetic variation that likely facilitated the evolution of IFN signaling in the early communication between the conceptus and the uterine endometrium.

At least two Type I IFNs developed biological activities outside of the innate immune response. Both are produced exclusively by the developing placenta in a subset of even-toe hooved mammals (i.e., artiodactyla). In the pig, IFND has been studied as a potential facilitator of early pregnancy, although its specific function during pregnancy has not been elucidated in this species (Sang et al., 2014). Also, IFND is not found in all artiodactyla, which further complicates discovering its function. The second Type I IFN was identified in early placental cells of conceptuses from ruminant species (cattle, sheep, goats, bison, giraffe, yak, deer). The evolution of this IFN family is well-established (Ealy and Wooldridge, 2017). It emerged after ruminants diverged from other mammals approximately 36 million years ago. Since then, several duplication, rearrangement, and conversion events have produced multiple genes in each species. Cattle, for example, contain at least three and likely as many as six distinct IFNT genes. Several polymorphic forms of these genes are present in cattle, sheep, and goats. The reasons why multiple genes exist for IFNT in ruminants remains unclear. However, it is clear that the proteins expressed from these genes contain different biological activities, leading to speculation that multiple protein variants may be needed to fully activate the various physiological mechanisms needed for pregnancy to succeed.

The current theory for the origin of the IFNT gene is that it formed from an ancestral IFNW gene soon after ruminants diverged from other artiodactyla (Ealy and Wooldridge, 2017). Interferon tau and IFNW proteins have approximately 70% amino acid identity, establishing them as more closely related than the other Type I IFN. The evolutionary relatedness of IFNT to IFNA, IFNB and IFNW is shown in Fig. 1 (Ealy and Wooldridge, 2017). Clustering of each main IFN class is evident, and the IFNT cluster closer to the IFNW than the others. There also is a clear multiplication of IFNT genes after their divergence from other Type I IFN. Although IFNT contains a distinctive primary amino acid sequence, these differences in primary sequence do not prevent IFNT from sharing similar secondary and tertiary structure with other Type I IFNs. This enables IFNT to bind and activate the same receptors used by other Type I IFNs, but also likely accounts for differences in biological activity between Type I IFN. The emergence of IFNT likely occurred following at least one and potentially several mutation events. One noteworthy recombination event modified the 400-base sequence upstream of the translational start site so that the newly formed gene would be under the control of a trophectoderm-specifying promoter/enhancer. This permits IFNT genes to be expressed exclusively by the trophectoderm during

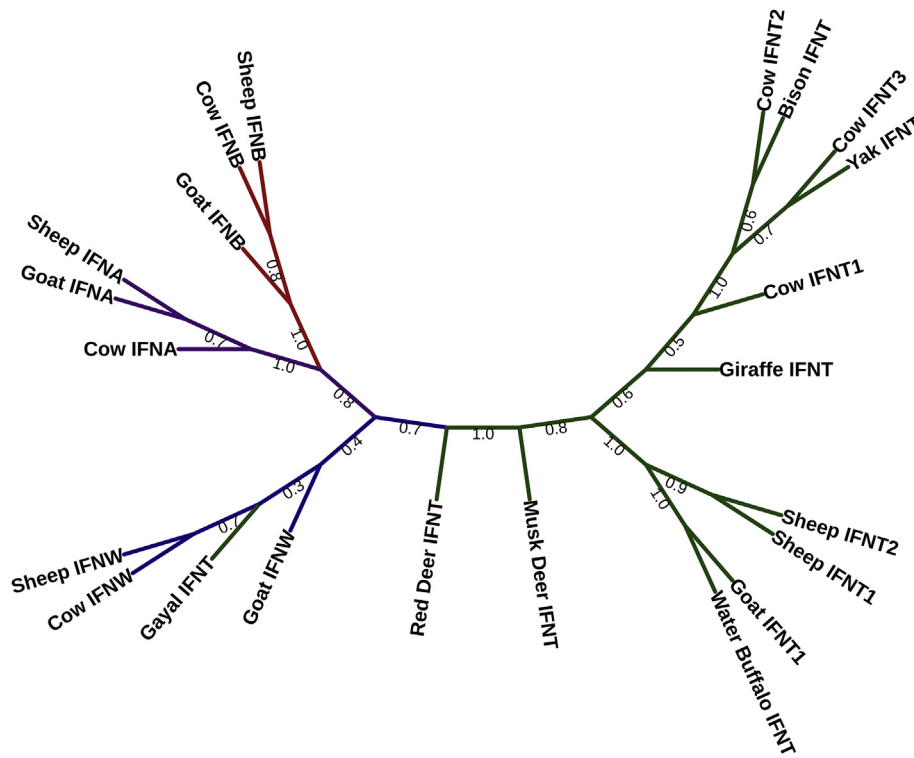


Fig. 1 Phylogenetic analysis of nucleotide coding sequences from representative *IFNA*, *IFNB*, *IFNW*, and *IFNT* genes in various ruminant species. Genus/species: *Bison bison* (Bison, American), *Bos frontalis* (Gaur, Mithun, Gayal), *Bos grunniens* (Yak, domestic), *Bos taurus* (Cow), *Bubalus bubalis* (Water buffalo), *Capra hircus* (Goat), *Cervus elephus* (Red deer), *Giraffa camelopardalis* (Giraffe), *Moschus berezovskii* (Musk deer, dwarf), and *Ovis aries* (Sheep). From Ealy and Wooldridge (2017). *Reproduction* **154**, F1–F10. <https://doi.org/10.1530/REP-17-0292>.

early pregnancy and renders them poorly inducible by virus (Ealy and Wooldridge, 2017). The IFNT also contains a unique 3' UTR region, but the functional significance of this region is not clear.

Discovery of Interferons' Role in Pregnancy

A role for interferons in pregnancy was established by several groups in the late 1960s and early 1970s. These groups were attempting to characterize signaling between the developing conceptus (embryo and associated membranes) and the uterine endometrium in ruminants (Bazer and Thatcher, 2017). Early work showed that a heat labile component of conceptus secretory proteins was able to extend corpus luteum (CL) function (inhibit luteolysis) when infused into the uterine lumen during a key stage of early pregnancy. Initially called Trophoblastin, Protein X and Trophoblast Protein-1, purified protein was prepared and sequenced leading to the identification of a unique, Type I, interferon designated interferon tau (IFNT; tau for trophoblast, its tissue of origin).

Interferon Signaling and Interferon Stimulated Genes

Type I IFN signal by binding the Type I IFN receptor, which consists of two subunits, IFNAR1 and IFNAR2. To date, there is no evidence for a distinct receptor mediating Type I IFN action in the uterus, nor is there evidence that response to IFN during early pregnancy is regulated by changes in receptor abundance on the endometrium (Hansen et al., 2017). Following binding to the Type I receptor, Janus kinase members JAK1 and TYK2 are reciprocally trans-phosphorylated to activate downstream signaling involving signal transducers and activators of transcription (STAT) proteins (Snell et al., 2017). Depending on the cell type and IFN bound, this can involve combinations of STAT1–6 that homo- or hetero-dimerize and act as transcription factors. The canonical Type I IFN STAT signaling pathway involves a heterodimer of STAT1 and STAT2 that translocates to the nucleus and binds interferon stimulated response elements (ISRE) on interferon stimulated genes to regulate transcription. Early transcribed genes include interferon regulatory factor 1 (IRF1) and IRF2. Interferon regulatory factor 1 induces transcription of other interferon stimulated genes (ISG), while IRF2 binds the ISRE and acts as a repressor of ISG thus serving as an “off” switch for IFN action. There are greater than 250 genes known to be activated by Type I IFN signaling. Among them are a large group of genes involved in the innate arm of the immune system, including myxovirus resistance 1 (MX1) and MX2, 2'5' oligoadenylate synthetase (OAS) and interferon stimulated gene 15 (ISG15). Following receptor binding, the combination of IRF2 action and internalization of the IFN receptor combine to limit Type I IFN action (Fig. 2).

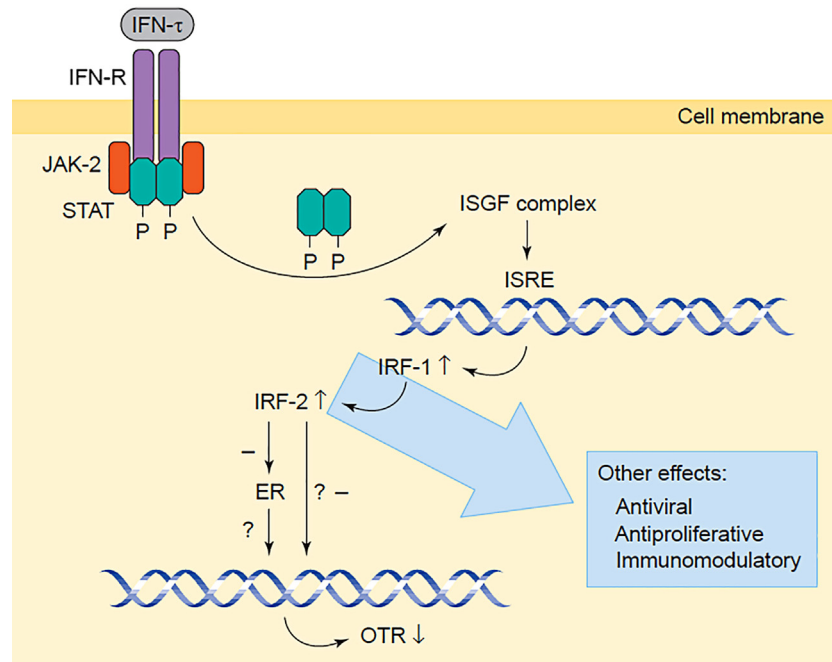


Fig. 2 Schematic of IFNT signaling pathways. Reproduced from Demmers, K. J., Derecka, K. and Flint, A. (2001). Trophoblast interferons and pregnancy. *Reproduction* **121**, 141–149.

Similarly, IFNG, the lone Type II IFN signals via the JAK/STAT pathway. Receptor binding stimulates phosphorylation and activation of STAT1, which forms homodimers that bind gamma activated sequence (GAS) elements in promoters of IFNG-stimulated genes (Gough et al., 2008).

Interferon in Pregnancy

Interferon in ruminant pregnancy: Interferon tau is now well-accepted as the key signaling molecule initiating a cascade of biochemical events that culminate in rescue of the corpus luteum and maintenance of pregnancy (Bazer and Thatcher, 2017). Interferon tau is produced by the conceptus trophoderm during a defined period between 2 and 4 weeks of early pregnancy. Early studies showed that purified native or recombinant IFNT extend the function of the corpus luteum (CL) when infused into the uterine lumen. However, infusion of IFNT usually only extended CL function for several days or a few weeks, and never the entire length of a normal gestation. Therefore, additional conceptus signals are undoubtedly required to maintain the CL for the full length of gestation. The identity of these signals remains unknown, but is an area of active investigation. The proximal effect of IFNT is to alter the pattern of prostaglandin $F_{2\alpha}$ (PGF) production and/or release from the endometrium to block CL regression. This effect is mediated by IFNT suppression of endometrial expression of oxytocin receptors. Production of IFNT by the conceptus is transitory and must precede the onset of pulsatile secretion of PGF that is responsible for CL regression (referred to as an antiluteolytic mechanism). In domestic ruminants, conceptus production of IFNT continues for 1–2 weeks and then ceases (Bazer and Thatcher, 2017) (Fig. 3).

Interferon in porcine pregnancy: Interferon tau is not produced by the pig placenta. However, at least two other IFN are produced. These are IFND and the Type II IFN, IFNG. Neither IFN, alone or together, are considered to be an antiluteolytic signal in pigs (Mathew et al., 2016). However, these two IFN, along with conceptus estrogen and other cytokines are thought to promote uterine receptivity, uterine secretion and to modulate immune cells in the endometrium during early pregnancy.

Interferon in rodent pregnancy: There is no evidence that an IFNT gene exists in rodents or any species outside of the ruminants. Rodent conceptuses produce antiviral agents, but immunoneutralization studies indicate that this activity is not caused by IFNA or IFNB (Cross et al., 1990). IFNG may be responsible for this activity. It is produced by post-implantation mouse placentae (Chen et al., 1994). Also, the IFN-responsive protein, ISG15, is highly expressed during early pregnancy in the mouse uterus and is covalently linked to other proteins in a process referred to as ISGylation. This reaction is thought to alter protein half-life or function in ways that facilitate implantation and/or serve as a defense to infection during pregnancy (Hansen et al., 2017).

Interferon in human pregnancy: Human placental tissue has the capacity to produce IFNA, IFNB, IFNW, and IFNL as early as the first trimester (Bayer et al., 2016). In contrast to ruminants, however, there is no evidence that IFN play a role in rescuing CL function. The human conceptus produces a placental gonadotropin, chorionic gonadotropin, that acts directly on the CL via the circulation to support progesterone production. IFN production by the human placenta is thought to largely play a role in inhibiting maternal to fetal viral infection and to enhance adaptive immunity (Racicot and Mor, 2017).

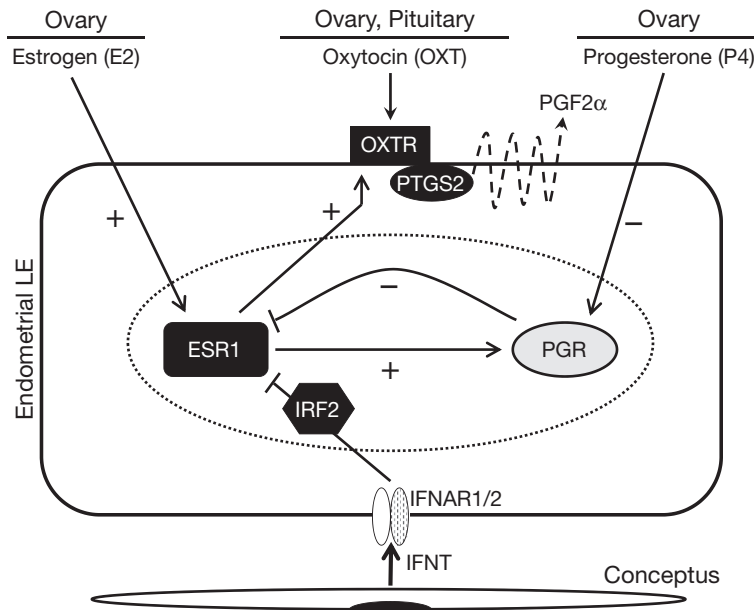


Fig. 3 IFN tau model for signaling MRP in ruminants. From Hansen, T. R., Sinedino, L. D. and Spencer, T. E. (2017). Paracrine and endocrine actions of interferon tau (IFNT). *Reproduction* **154**, F45–F59. <https://doi.org/10.1530/REP-17-0315>.

Summary

Interferons are a large family of related cytokines first identified by their ability to confer resistance to viral infections. They are firmly established as components of the innate arm of the immune system providing rapid and broad protection against a wide variety of invading pathogens. Of the three subfamilies, Type I IFN show the greatest diversity with over 20 family members. Interestingly, one member of this class, interferon tau, evolved to function as a signaling molecule between the early conceptus and the maternal uterus in ruminant ungulates. This interferon, IFNT, is necessary and sufficient to rescue the function of the corpus luteum during early pregnancies in these species. In addition, it is clear that other IFN family members, including IFNA, IFNB, IFNG, and IFND, are produced by placentae of rodents, humans, and swine. Their functions are less well-understood, but they likely play roles in modulating the immune function at the fetal–maternal interface and protecting the fetus against infection by maternal pathogens.

References

- Bayer, A., Lennemann, N. J., Ouyang, Y., Bramley, J. C., Morosky, S., De Azevedo Marques, E. T., Jr., Cherry, S., Sadovsky, Y., & Coyne, C. B. (2016). Type III Interferons produced by human placental trophoblasts confer protection against Zika virus infection. *Cell Host & Microbe*, *19*, 705–712.
- Bazer, F. W., & Thatcher, W. W. (2017). Chronically the discovery of interferon-tau. *Reproduction*, F11–F20. <https://doi.org/10.1530/REP-17-0257>.
- Chen, H.-L., Kamath, R., Place, J. L., Russel, S. W., & Hunt, J. S. (1994). Expression of the interferon-gamma/receptor gene in mouse placentas is related to stage of gestation and is restricted to specific subpopulations of trophoblast cells. *Placenta*, *15*, 109–121.
- Cross, J. C., Farin, C. E., Sharif, S. F., & Roberts, R. M. (1990). Characterization of the antiviral activity constitutively produced by murine conceptuses: Absence of placental mRNAs for interferon alpha and beta. *Molecular Reproduction and Development*, *26*, 122–128.
- Ealy, A. D., & Wooldridge, L. K. (2017). The evolution of interferon-tau. *Reproduction*, *154*, F1–F10. <https://doi.org/10.1530/REP-17-0292>.
- Gough, D., Levy, D. E., Johnstone, R. W., & Clarke, C. J. (2008). IFNγ signaling—Does it mean JAK–STAT? *Cytokine & Growth Factor Reviews*, *19*(2008), 383–394.
- Hansen, T. R., Sinedino, L. D., & Spencer, T. E. (2017). Paracrine and endocrine actions of interferon tau (IFNT). *Reproduction*, *154*, F45–F59. <https://doi.org/10.1530/REP-17-0315>.
- Mathew, D. J., Lucy, M. C., & Geisert, R. D. (2016). Interleukins, interferons, and establishment of pregnancy in pigs. *Reproduction*, *151*, R111–R122. <https://doi.org/10.1530/REP-16-0047>.
- Racicot, K., & Mor, G. (2017). Risks associated with viral infections during pregnancy. *Journal of Clinical Investigation*, *127*(5), 1591–1599. <https://doi.org/10.1172/JCI87490>.
- Sang, Y., Bergkamp, J., & Blecha, F. (2014). Molecular evolution of the porcine type I interferon family: Subtype-specific expression and antiviral activity. *PLoS One*, *9*(11), e112378. <https://doi.org/10.1371/journal.pone.0112378>.
- Snell, L. M., McGaha, T. L., & Brooks, D. G. (2017). Type I interferon in chronic virus infection and cancer. *Trends in Immunology*, *38*(8), 542–557. <https://doi.org/10.1016/j.it.2017.05.005>.
- Walker, A. M., & Roberts, R. M. (2009). Characterization of the bovine type I IFN locus: Rearrangements, expansions, and novel subfamilies. *BMC Genomics*, *10*, 187.

Further Reading

- Demmers, K. J., Derecka, K., & Flint, A. (2001). Trophoblast interferons and pregnancy. *Reproduction*, *121*, 141–149.
- Ezashi, T., & Imakawa, K. (2017). Transcriptional control of IFNT expression. *Reproduction*, *154*, F21–F31. <https://doi.org/10.1530/REP-17-0330>.

- Forde, N., & Lonergan, P. (2017). Interferon-tau and fertility in ruminants. *Reproduction*, 154, F33–F43. <https://doi.org/10.1530/REP-17-0432>.
- Ott, T. L., & Gifford, C. A. (2010). Effects of early conceptus signals on circulating immune cells: Lessons from domestic ruminants. *American Journal of Reproductive Immunology*, 64, 245–254. <https://doi.org/10.1111/j.1600-0897.2010.00912.x>.
- Roberts, R. M. (2017). 30 years on from the molecular cloning of interferon-tau. *Reproduction*, 154, E1–E2. <https://doi.org/10.1530/REP-17-0585>.

Trophoblast

Michael J Soares, University of Kansas Medical Center, Kansas City, KS, United States; and Children's Mercy Kansas City, Kansas City, MO, United States

Kaela M Varberg, University of Kansas Medical Center, Kansas City, KS, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Trophoblast cells are epithelial cells that contribute to the specialized functions of the placenta. They are essential for viviparity, allowing for growth and development of the fetus within the confines of the uterus. The term trophoblast originated with the noted Dutch embryologist, Ambrosius A.W. Hubrecht, during his efforts to describe placental development in the hedgehog, *Erinaceus europaeus* (Hubrecht, 1889; Pijnenborg and Vercruysse, 2013). Selection of the name was based on the perceived nutritive role of the cell population supporting embryonic development within the female reproductive tract. Unlike most organ systems the placenta exhibits greater anatomical diversity among species and thus comparative placentation is plagued with species-specific nomenclature. Nonetheless, trophoblast is a term that has endured and in its many formulations has been used to describe cellular constituents of the placenta. In this article, we discuss this vital cell lineage, addressing its developmental origin, specializations, and critical roles in placenta function. Our focus is on trophoblast cells that contribute to hemochorial placentation, a process observed in several species including human, mouse, and rat, with some limited information regarding ruminant trophoblast cells.

Developmental Origin of Trophoblast Cells

Trophoblast cells arise as the first landmark differentiation event during embryonic development. Fertilization reconstitutes a diploid chromosomal composition through fusion of a haploid sperm with a haploid egg, forming a one-cell embryo and activating cell division. As the embryo expands in size and cell number, niches change, and cells on the outer surface of the embryo are directed to a trophoblast cell fate, which fundamentally redirects their functional properties and developmental potential. At the blastocyst stage, embryonic cells are partitioned into two compartments: the outer trophoblast (early trophoblast cells) and a cluster of cells it surrounds referred to as the inner cell mass (Fig. 1). Further trophoblast cell lineage development is stimulated through interactions with the inner cell mass and its derivatives as well as the maternal uterine environment.

Molecular Regulation of Trophoblast Cell Lineage Development

Considerable mechanistic insights underlying trophoblast cell development have been derived from experimentation with rodent models. Such efforts have been spurred by the capacity for in vivo experimental mutagenesis, especially in the mouse, and the investigation of in vitro trophoblast cell culture models with stem cell properties (Faria and Soares, 1991; Tanaka et al., 1998; Asanoma et al., 2011). Key components of signaling pathways have been defined and include transcriptional regulators required for development of the trophoblast cell lineage (Pfeffer and Pearton, 2012; Latos and Hemberger, 2016). TEAD4, CDX2, EOMES, GATA3, TFAP2C, and several more transcription factors have been shown to be vital to the establishment of the trophoblast lineage. Expansion of the trophoblast lineage is driven by fibroblast growth factor and transforming growth factor beta (Activin/Nodal) signals secreted from the inner cell mass and its derivatives, which act on neighboring trophoblast stem and progenitor cell populations. These efforts provided the basis for developing conditions for inducing trophoblast stem cell development from mouse embryonic fibroblasts (Benchetrit et al., 2015; Kubaczka et al., 2015). Introduction of a core group of three transcription factors (EOMES, GATA3, and TFAP2C) into fibroblasts activated the trophoblast stem cell program. The resulting induced-trophoblast stem cells behave like trophoblast stem cells isolated from mouse embryos, including: (i) transcriptomic and epigenomic profiles, (ii) in vitro differentiation into specialized trophoblast lineages, and (iii) chimeric contributions to the developing placenta when re-introduced into early embryos. Although, this collective mechanistic information has illuminated our understanding of the derivation of the mouse trophoblast lineage, it is evident that this process lacks complete conservation across mammals (Berg et al., 2011; Niakan and Eggan, 2013; Blakely et al., 2015).

Insights into the regulation of human trophoblast cell lineage development have lagged. Human trophoblast stem cell cultures comparable to rodent in vitro models have not been established and ethical constraints prevent in vivo genetic manipulation strategies. An assortment of different in vitro human trophoblast cell culture systems has expanded our understanding of trophoblast cell development but each has limitations (Lee et al., 2016). A serendipitous finding by Thomson and colleagues working with human embryonic stem cells provided a potential in vitro model system for trophoblast cell lineage investigation (Xu et al., 2002). Human embryonic stem cells exposed to bone morphogenetic protein 4 differentiate into cells with a trophoblast cell phenotype, representing an in vitro model system suitable for dissecting potential molecular mechanisms controlling human trophoblast cell lineage development (Xu et al., 2002; Ezashi et al., 2012; Li and Parast, 2014). It has been proposed, that the resulting differentiated cell population reflects an early stage of trophoblast development. However, validation of such a hypothesis has been problematic due to the limited availability of adequate in vivo reference material.

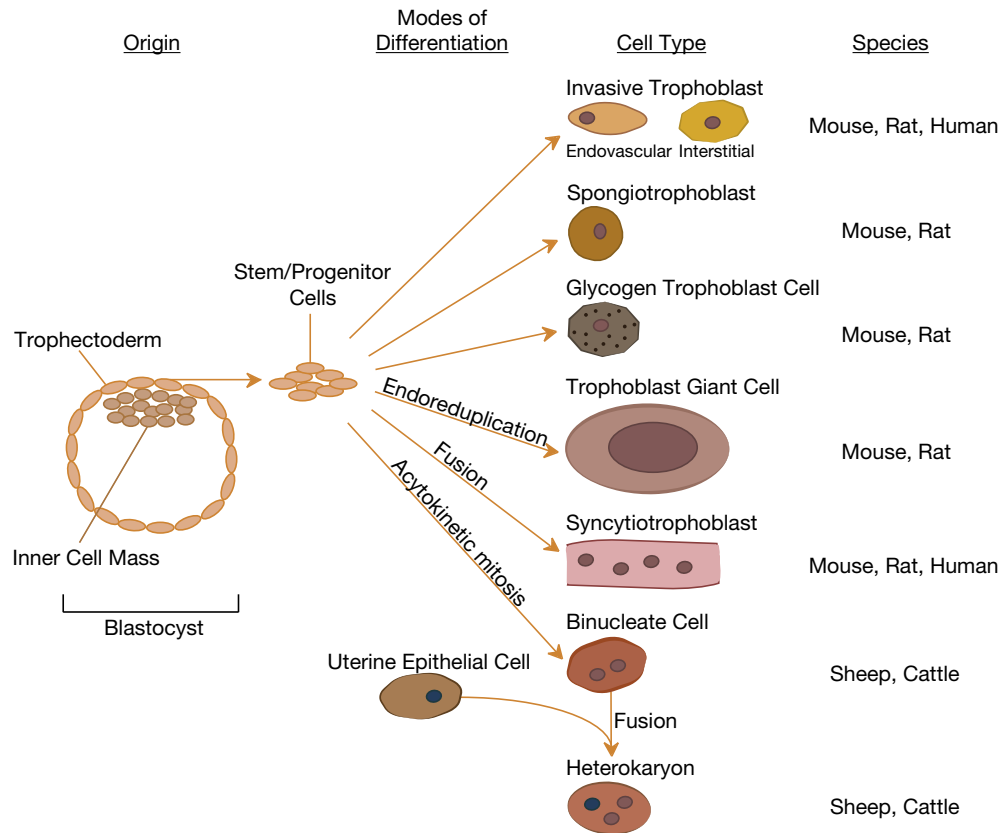


Fig. 1 Schematic presentation of modes of trophoblast cell differentiation. Trophoblast stem and progenitor cells arise from trophectoderm of the blastocyst and give rise to differentiated cell lineages. Differentiated cell types include: invasive trophoblast (endovascular and interstitial), spongiotrophoblast, glycogen trophoblast cells, trophoblast giant cells, syncytiotrophoblast, and binucleate cells. Trophoblast giant cells, syncytiotrophoblast, and binucleate cells are formed through specific modes of differentiation (endoreduplication, fusion, and acytokinetic mitosis respectively) as indicated by the labeled arrows. Binucleate cells fuse with uterine epithelial cells to form trinucleate heterokaryons. The species in which the differentiated cell types exist are indicated to the right of each cell type.

Trophoblast Cell Specialized Functions

Viviparity is a successful reproductive strategy; however, it poses significant challenges. The developing embryo/fetus needs access to nutrients and protection from the adversities of the maternal environment. Trophoblast cells engineer essential solutions to meet these needs and facilitate adaptation of the embryo/fetus to intrauterine life. Acquisition of specialized trophoblast cell functions are accomplished through multilineage cell differentiation, which is to say that not all trophoblast cells are alike in form or function. Trophoblast cells differentiate from trophoblast stem cell populations and become specialized to address the critical functions of the placenta which include: (i) acquisition of nutrients from the mother and delivery to the fetus; (ii) protection from adversity associated with exposure to the maternal environment.

Trophoblast cells acquire several specializations to precondition the uterus in support of embryonic/fetal development. They target the uterine parenchyma and especially the uterine vasculature where they transform uterine arteries through disorganization of associated smooth muscle and replacement of arterial endothelium (Kaufmann et al., 2003; Pijnenborg et al., 2006). These efforts are carefully orchestrated through acquisition of properties facilitating trophoblast cell movement, dissolution of extracellular matrices, invasion into the uterine parenchyma and vasculature, and endothelial cell mimicry (Harris, 2010). In doing so, subpopulations of trophoblast cells transform uterine vascular conduits from high resistance and low capacity vessels to low resistance and high capacity vessels (Harris, 2010). Thus, attenuating flow rates and the subsequent damaging effects of shear stress on the uterine endothelium and placenta. Trophoblast cells are also capable of facilitating fetal nutrient delivery through two mechanisms: (i) production of hormones that sustain the gestational state and redirect maternal metabolism (Porter et al., 1982; Newbern and Freemark, 2011; Carter, 2012); (ii) expression and activities of nutrient transporters (Carter, 2012; Dilworth and Sibley, 2013; Diaz et al., 2014).

Cellular mechanisms underlying fetal protection are fundamental properties of trophoblast cells. Importantly, trophoblast cells help modulate the maternal immune system by evading and abating the triggers of immune rejection (Erlebacher, 2013) and providing an essential barrier function (Burton et al., 2016). Specialized trophoblast cells prevent adverse exposures through the

biotransformation of potentially harmful agents and facilitate their exodus via efflux transporters (Hakkola et al., 1998; Joshi et al., 2016).

One of the most important properties of the placenta is its plasticity. Plasticity is achieved through marshaling trophoblast stem and progenitor cell populations to expand and/or differentiate along pathways conducive to maximize adaptive abilities (Soares et al., 2014).

Collectively, trophoblast cell specializations provide the placenta with functionality ensuring successful in utero survival and growth of the embryo and fetus.

Trophoblast Cell Differentiation

There is an assortment of differentiated trophoblast cell types (Table 1 and Fig. 1). Trophoblast cells are specialized to control early stages of embryonic development by interacting with maternal uterine structures to establish the interface with the fetal vascular conduit. Some differentiated cell types define specific features of placentation and are characteristic of a species.

Trophectoderm

Trophectoderm corresponds to the outer cell layer of the mammalian blastocyst-stage embryo and represents the source of stem and progenitor cells for further trophoblast development. Two additional key functions are ascribed to trophoctoderm: (i) facilitating interactions with uterine epithelium at embryo implantation; (ii) creating a protective environment for inner cell mass/epiblast development (Biggers et al., 1988; Aplin and Ruane, 2017).

Differentiated Trophoblast Lineages: Mouse and Rat

The placenta of the mouse and rat are organized into two compartments: (i) junctional zone; (ii) labyrinth zone (Soares et al., 2012).

The junctional zone represents the interface with the maternal environment and is composed of several differentiated trophoblast cell types, including: trophoblast giant cells, glycogen trophoblast cells, spongiotrophoblast cells, and invasive trophoblast cells. Trophoblast giant cell development is dependent upon the transcription factor HAND1 (Riley et al., 1998) and arises through endoreduplication, a process where DNA synthesis continues while mitosis is interrupted, resulting in the formation of large polyploid/polytene cells. CDKN1C (p57kip2), a cyclin-dependent kinase inhibitor, has been implicated in the process of endoreduplication (Ullah et al., 2008). The trophoblast giant cell is prominently situated at the uterine-placental boundary and is the major endocrine cell of the placenta, possessing the capacity to produce both steroid and peptide hormones (Soares et al., 1996). Trophoblast giant cells are also located elsewhere in the placenta. Their positioning within the placentation site

Table 1 Differentiated trophoblast cell types

Cell type	Species ^a	Placentation site location	Principal function(s)
Trophectoderm	Mouse, rat, human, sheep, and cattle	Outer cellular layer of blastocyst	Embryo implantation; inner cell mass/epiblast protection; source of trophoblast stem and progenitor cells
Trophoblast giant cell	Mouse, rat	Junctional zone: uterine-placental interface (primary), labyrinth zone (secondary)	Endocrine activity: steroid and peptide hormonogenesis
Glycogen trophoblast cell	Mouse, rat	Junctional zone (transient)	Energy reserve
Spongiotrophoblast cell	Mouse, rat	Junctional zone	Endocrine activity: peptide hormonogenesis
Endovascular invasive trophoblast cell	Mouse, rat, and human	Intrauterine: intravascular; extravillous compartment (human)	Uterine spiral artery restructuring
Interstitial invasive trophoblast cell	Mouse, rat, and human	Intrauterine: extravascular; extravillous compartment (human)	Placental anchor; uterine stroma remodeling
Syncytiotrophoblast	Mouse, rat, and human	Labyrinth zone (mouse, rat); villous compartment (human)	Barrier/transport (mouse, rat, human); endocrine activity: steroid and peptide hormonogenesis (human)
Uninucleate trophoblast cell	Sheep, cattle	Cotyledon	Endocrine activity: interferon tau; source of trophoblast stem and progenitor cells
Binucleate trophoblast cell	Sheep, cattle	Cotyledon	Endocrine activity: steroid and peptide hormonogenesis
Trinucleate cell	Sheep, cattle	Uteroplacental interface; uterine epithelial cell-binucleate cell heterokaryon	Endocrine activity: steroid and peptide hormonogenesis

^aDifferentiated trophoblast cell types found in mouse, rat, human, sheep, and cattle.

influences their behavior (Soares et al., 1996; Simmons et al., 2007). Glycogen trophoblast cells appear transiently during the last week of gestation and are conspicuous in their accumulation of glycogen and thus, at least in part, serve as a placental energy reserve (Coan et al., 2006). There is some thought that glycogen trophoblast cells migrate out of the junctional zone and infiltrate the uterine decidua; however, precise lineage tracing data is lacking. Spongiotrophoblast cells contribute to the endocrine functions of the placenta. They actively secrete a range of peptide hormones, including members of the expanded prolactin family (Soares, 2004). Finally, the junctional zone serves as a reservoir for invasive trophoblast cells that infiltrate the uterine parenchyma and coordinate remodeling of the uterine arterial vasculature (Ain et al., 2003). Until midgestation, the movement of trophoblast cells is similar in the mouse and rat. Endovascular invasive trophoblast cells enter the vasculature and are confined to the decidua. After midgestation, interstitial invasive trophoblast cells move into the uterus. In the mouse, interstitial invasive trophoblast cells are restricted in the depth of their invasion to the decidua, whereas in the rat both endovascular and interstitial invasive trophoblast cells extend beyond the decidua and deep into the uterine mesometrial compartment (Ain et al., 2003; Soares et al., 2012). Hypoxia/hypoxia inducible factor signaling is an effective activator of the invasive trophoblast cell lineage (Chakraborty et al., 2011, 2016). Furthermore, the junctional zone and its cellular constituents exhibit robust responses to genetic and environmental manipulations (Konno et al., 2007; Rosario et al., 2008; Soares et al., 2012).

The labyrinth zone is positioned at the fetal interface and consists of trophoblast progenitor cells that fuse to form multinucleated syncytiotrophoblast or as gestation progresses differentiate into trophoblast giant cells via endoreduplication (Walentin et al., 2016). Labyrinthine syncytiotrophoblast comprise two layers (syncytiotrophoblast layers I and II; Walentin et al., 2016), which form the barrier that regulates the entry of solutes into the fetal vasculature and possesses a robust complement of transporters (Georgiades et al., 2002; Dilworth and Sibley, 2013). The syncytiotrophoblast layers are contiguous, with syncytiotrophoblast layer I located proximal to trophoblast cells lining the maternal blood space and syncytiotrophoblast layer II situated next to the fetal vasculature. Syncytiotrophoblast arise through a process of cell fusion orchestrated by endogenous retroviral envelope genes referred to as syncytin-A (SYNA) and syncytin-B (SYNB; Dupressoir et al., 2012). SYNA drives the fusion of syncytiotrophoblast layer I, while SYNB promotes the fusion of syncytiotrophoblast layer II (Dupressoir et al., 2011). The glycosylphosphatidylinositol-anchored cell surface protein LY6E is a candidate receptor for the fusogenic properties of SYNA (Bacquin et al., 2017). Trophoblast giant cells appear in the labyrinth zone during the last week of gestation where they line maternal blood spaces adjacent to syncytiotrophoblast layer I and exhibit distinctive endocrine properties (Soares et al., 1996; Simmons et al., 2007).

Differentiated Trophoblast Lineages: Sheep and Cattle

Placentation in sheep and cattle is classified as synepitheliochorial, which differs from hemochorial placentation found in the mouse, rat, and human (Wooding and Burton, 2008; Roberts et al., 2016). The synepitheliochorial placenta consists of multiple placentation sites (placentomes) dispersed throughout the uterus. Each placentome is subdivided into trophoblast-rich cotyledon tissue and an adjacent caruncle composed of uterine tissue. This type of placentation exhibits a maximal number of maternal and extraembryonic cell layers constituting the placental barrier and an unusual differentiated cell type, the binucleate cell (Wooding, 1992). Binucleate cells arise from uninucleate (also called mononucleate) trophoblast cell progenitors through a process referred to as acytokinetic mitosis. During acytokinetic mitosis, uninucleate trophoblast cells undergo DNA synthesis and karyokinesis, without proceeding to cytokinesis (Klisch et al., 1999). Binucleate cells are migratory, secrete both steroid and peptide hormones, and are capable of fusing with uterine epithelial cells to form trinucleate cells consisting of binucleate trophoblast cell-uterine epithelial cell heterokaryons (Wooding, 1992).

Differentiated Trophoblast Lineages: Human

The early human implantation site is characterized by the formation of a trophoblast syncytium (Huppertz, 2008). This structure, which presumably arises through cell fusion of trophoblast cells, envelopes the developing embryo and is embedded within the uterine parenchyma. The early trophoblast syncytium provides a potentially vital role in the establishment of pregnancy. Syncytiotrophoblast of early placentation has been an ethical challenge to investigate in the human. Alternatively, the guinea pig exhibits parallels in early trophoblast development (Enders and Schlafke, 1969) and may represent a useful model for examining the biology of this early stage of hemochorial placental development.

Trophoblast cells of the mature human placenta can be partitioned into two major compartments: (i) extravillous; (ii) villous (Maltepe and Fisher, 2015).

Extravillous trophoblast cells consist of invasive trophoblast cells and their progenitors, which are situated within extravillous trophoblast cell columns (Velicky et al., 2016); structures analogous to the junctional zone of the rodent placenta. Invasive trophoblast cells are further classified based on their entry route into the uterine parenchyma. As described above, interstitial invasive trophoblast cells infiltrate between uterine vasculature, whereas endovascular invasive trophoblast cells migrate into and replace the endothelium of uterine spiral arteries. Human invasive trophoblast cells exhibit functionality analogous to invasive trophoblast cells of the mouse and rat (Pijnenborg et al., 1981, 2006; Soares et al., 2014). Several signaling pathways have been implicated in the regulation of extravillous trophoblast cell development and function (Knöfler, 2010).

Villous trophoblast cells consist of progenitors referred to as cytotrophoblast, which fuse to form syncytiotrophoblast (Aplin, 2010). Cytotrophoblast are most abundant during early phases of gestation and decline in number as gestation advances. They

are located beneath the syncytiotrophoblast layer, which borders maternal blood spaces. Syncytiotrophoblast represent both the primary barrier regulating transport between maternal and fetal vascular compartments and, also the principal source of steroid and peptide hormones produced by the human placenta. Syncytialization is driven by the endogenous retroviral envelope gene called syncytin-2 (ERVFRD-1). ERVFRD-1 is expressed on a subset of cytotrophoblast and acts through its receptor, MFSD2A, which is located on the syncytiotrophoblast cell surface (Lavialle et al., 2013). Another endogenous retroviral gene product referred to as syncytin-1 (ERVW-1) may contribute to the fusion process through its receptor, ASCT2 (Lavialle et al., 2013). The syncytins are also notable because they are regulated by GCM1 (Yu et al., 2002; Liang et al., 2010), a transcription factor pivotal to labyrinthine development in the mouse (Anson-Cartwright et al., 2000). Although, rodent and human syncytins are endogenous retroviral envelope genes that share overlapping functional properties, they evolved independently and are not orthologous genes (Dupressoir et al., 2012).

Final Thoughts

Trophoblast cells are an intriguing and unique cell lineage. The range of cellular functions originating from a relatively small number of trophoblast cell differentiation events is formidable. These specialized trophoblast cells single-handedly redirect maternal physiology to support the survival and growth of the fetus. A portion of this remarkable functionality is, undoubtedly, related to the extraordinary attributes of polyploid and multi-nucleated cells and their abilities to adapt to adversity (Oren-Suissa and Podbilewicz, 2010; Van de Peer et al., 2017). However, this is not the entire explanation because not all mammalian species possess these unique trophoblast cell populations (Wooding and Burton, 2008).

Gaps exist in understanding the precise molecular mechanisms controlling trophoblast cell development. These deficits arise, in part, from flawed approaches used to investigate trophoblast cells. It is important to appreciate that in vitro models are artificial paradigms and reflect cellular “potential” but not necessarily true physiological behavior. Nonetheless, in vitro systems can be powerful when combined with in vivo research strategies. Mouse and rat trophoblast stem cells can be effectively used to develop hypotheses regarding processes controlling trophoblast cell development. These hypotheses can then be tested in vivo using experimentally manipulated mouse or rat models. The rat is especially valuable in elucidating mechanisms controlling the invasive trophoblast cell lineage and uterine spiral artery remodeling. Limitations in current human trophoblast cell culture systems and ethical constraints with in vivo human trophoblast cell analyses present obstacles in understanding human trophoblast cell development. Efforts to identify and focus on “conserved” regulatory pathways are necessary to gain insights regarding human trophoblast cells. However, it is important to appreciate that conservation is not complete and species-specific elements characterize trophoblast cell biology and placentation.

In closing, trophoblast cell biology is integral to understanding placental development and pregnancy-related diseases. The process of trophoblast cell specialization is sensitive to intrinsic and extrinsic environmental challenges. Final construction of each placenta is unique and reflects a progression of adaptations responsive to signals emanating from both the mother and fetus. The nature of this dialog is dynamic and continually changes as pregnancy proceeds. Placental plasticity is a measure of the ability of trophoblast stem and progenitor cell populations to adapt and can be viewed as an important indicator of a healthy placenta. Placental dysfunction is linked to failures in the expansion and differentiation of trophoblast stem and progenitor cell populations and can originate from aberrations in communication with maternal and fetal environments or the consequence of intrinsic defects in the life history of trophoblast cells.

Acknowledgements

The authors would like to recognize past and current laboratory members and acknowledge research support from the National Institutes of Health (HD020676, HD079363, HD082535) and the Sosland Foundation.

References

- Ain, R., Canham, L. N., & Soares, M. J. (2003). Gestation stage-dependent intrauterine trophoblast cell invasion in the rat and mouse: Novel endocrine phenotype and regulation. *Developmental Biology*, 260, 176–190.
- Anson-Cartwright, L., Dawson, K., Holmyard, D., et al. (2000). The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nature Genetics*, 25, 311–314.
- Aplin, J. D. (2010). Developmental cell biology of human villous trophoblast: Current research problems. *International Journal of Developmental Biology*, 54, 323–329.
- Aplin, J. D., & Ruane, P. T. (2017). Embryo–epithelium interactions during implantation at a glance. *Journal of Cell Science*, 130, 15–22.
- Asanoma, K., Rumi, M. A. K., Kent, L. N., et al. (2011). FGF4-dependent stem cells derived from rat blastocysts differentiate along the trophoblast lineage. *Developmental Biology*, 351, 110–119.
- Bacquin, A., Bireau, C., Tanguy, M., et al. (2017). A cell fusion-based screening method identifies glycosylphosphatidylinositol-anchored protein Ly6e as the receptor for mouse endogenous retroviral envelope syncytin-A. *Journal of Virology*, 91(18), e00832–17.
- Benchetrit, H., Herman, S., van Wietmarschen, N., et al. (2015). Extensive nuclear reprogramming underlies lineage conversion into functional trophoblast stem-like cells. *Cell Stem Cell*, 17, 543–556.
- Berg, D. K., Smith, C. S., Pearton, D. J., et al. (2011). Trophoblast lineage determination in cattle. *Developmental Cell*, 20, 244–255.
- Biggers, J. D., Bell, J. E., & Benos, D. J. (1988). Mammalian blastocyst: Transport functions in a developing epithelium. *American Journal of Physiology*, 255, C419–C432.
- Blakely, P., Fogarty, N. M. E., del Valle, I., et al. (2015). Defining the three cell lineages of the human blastocyst by single-cell RNA-seq. *Development*, 142, 3151–3165.

- Burton, G. J., Fowden, A. L., & Thornburg, K. L. (2016). Placental origins of chronic disease. *Physiological Reviews*, 96, 1509–1565.
- Carter, A. M. (2012). Evolution of placental function in mammals: The molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. *Physiological Reviews*, 92, 1543–1576.
- Chakraborty, D., Rumi, M. A. K., Konno, T., & Soares, M. J. (2011). Natural killer cells direct hemochorial placentation by regulating HIF-dependent trophoblast lineage decisions. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 16295–16300.
- Chakraborty, D. C., Cui, W., Rosario, G. X., et al. (2016). HIF-KDM3A-MMP12 regulatory circuit ensures trophoblast plasticity and placental adaptations to hypoxia. *Proceedings of the National Academy of Sciences of the United States of America*, 113, E7212–E7221.
- Coan, P. M., Conroy, N., Burton, G. J., & Ferguson-Smith, A. C. (2006). Origin and characteristics of glycogen cells in the developing murine placenta. *Developmental Dynamics*, 235, 3280–3294.
- Diaz, P., Powell, T. L., & Jansson, T. (2014). The role of placental nutrient sensing in maternal-fetal resource allocation. *Biology of Reproduction*, 91, 82.
- Dilworth, M. R., & Sibley, C. P. (2013). Transport across the placenta of mice and women. *Placenta*, 34(Supplement), S34–S39.
- Dupressoir, A., Vernochet, C., Harper, F., et al. (2011). A pair of co-opted retroviral envelope syncytin genes is required for formation of the two-layered murine placental syncytiotrophoblast. *Proceedings of the National Academy of Sciences of the United States of America*, 108, E1164–E1173.
- Dupressoir, A., Lavalie, C., & Heidmann, T. (2012). From ancestral infectious retroviruses to bona fide cellular genes: Role of the captured syncytins in placentation. *Placenta*, 33, 663–671.
- Enders, A. C., & Schlafke, S. (1969). Cytological aspects of trophoblast-uterine interaction in early implantation. *The American Journal of Anatomy*, 125, 1–29.
- Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annual Review of Immunology*, 31, 387–411.
- Ezashi, T., Telugu, B. P., & Roberts, R. M. (2012). Model systems for studying trophoblast differentiation from human pluripotent stem cells. *Cell and Tissue Research*, 349, 809–824.
- Faria, T. N., & Soares, M. J. (1991). Trophoblast cell differentiation: Establishment, characterization, and modulation of a rat trophoblast cell line expressing members of the placental prolactin family. *Endocrinology*, 129, 2895–2906.
- Georgiades, P., Ferguson-Smith, A. C., & Burton, G. J. (2002). Comparative developmental anatomy of the murine and human definitive placentae. *Placenta*, 23, 3–19.
- Hakkola, J., Pelkonen, O., Pasanen, M., & Raunio, H. (1998). Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: Role in intrauterine toxicity. *Critical Reviews in Toxicology*, 28, 35–72.
- Harris, L. K. (2010). Trophoblast-vascular cell interactions in early pregnancy: How to remodel a vessel. *Placenta*, 31(Supplement A), S93–S98.
- Hubrecht, A. A. W. (1889). Studies in mammalian embryology. I. The placentation of *Erinaceus europaeus*, with remarks on the phylogeny of the placenta. *Quarterly Journal of Microscopical Science*, 30, 283–404.
- Huppertz, B. (2008). The anatomy of the normal placenta. *Journal of Clinical Pathology*, 61, 1296–1302.
- Joshi, A. A., Vaidya, S. S., St-Pierre, M. V., et al. (2016). Placental ABC transporters: Biological impact and pharmaceutical significance. *Pharmaceutical Research*, 33, 2847–2878.
- Kaufmann, P., Black, S., & Huppertz, B. (2003). Endovascular trophoblast invasion: Implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biology of Reproduction*, 69, 1–7.
- Klisch, K., Hecht, W., Pfarrer, C., et al. (1999). DNA content and ploidy level of bovine placental trophoblast giant cells. *Placenta*, 20, 451–458.
- Knöfler, M. (2010). Critical growth factors and signalling pathways controlling human trophoblast invasion. *International Journal of Developmental Biology*, 54, 269–280.
- Konno, T., Rempel, L. A., Arroyo, J. A., & Soares, M. J. (2007). Pregnancy in the Brown Norway rat: A model for investigating the genetics of placentation. *Biology of Reproduction*, 76, 709–718.
- Kubaczka, C., Sennar, C. E., Cierlitz, M., et al. (2015). Direct induction of trophoblast stem cells from murine fibroblasts. *Cell Stem Cell*, 17, 557–568.
- Latos, P. A., & Hemberger, M. (2016). From the stem of the placental tree: Trophoblast stem cells and their progeny. *Development*, 143, 3650–3660.
- Lavalie, C., Cornelis, G., Dupressoir, A., et al. (2013). Paleovirology of “syncytins”, retroviral env genes exapted for a role in placentation. *Philosophical Transactions of the Royal Society B*, 368, 20120507.
- Lee, C. Q., Gardner, L., Turco, M., et al. (2016). What is trophoblast? A combination of criteria define human first-trimester trophoblast. *Stem Cell Reports*, 6, 257–272.
- Li, Y., & Parast, M. M. (2014). BMP4 regulation of human trophoblast development. *International Journal of Developmental Biology*, 58, 239–246.
- Liang, C. Y., Wang, L. J., Chen, C. P., et al. (2010). GCM1 regulation of the expression of syncytin 2 and its cognate receptor MFSD2A in human placenta. *Biology of Reproduction*, 83, 387–395.
- Maltepe, E., & Fisher, S. J. (2015). Placenta: The forgotten organ. *Annual Review of Cell and Developmental Biology*, 31, 523–552.
- Newbern, D., & Freemark, M. (2011). Placental hormones and the control of maternal metabolism and fetal growth. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 18, 409–416.
- Niakan, K. K., & Eggen, K. (2013). Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. *Developmental Biology*, 375, 54–64.
- Oren-Suissa, M., & Podbilewicz, B. (2010). Evolution of programmed cell fusion: Common mechanisms and distinct functions. *Developmental Dynamics*, 239, 1515–1528.
- Pfeffer, P. L., & Pearson, D. J. (2012). Trophoblast development. *Reproduction*, 143, 231–246.
- Pijnenborg, R., & Vercruysse, L. (2013). A.A.W. Hubrecht and the naming of the trophoblast. *Placenta*, 34, 314–319.
- Pijnenborg, R., Robertson, W. B., Brosens, I., & Dixon, G. (1981). Trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. *Placenta*, 2, 71–91.
- Pijnenborg, R., Vercruysse, L., & Hanssens, M. (2006). The uterine spiral arteries in human pregnancy: Facts and controversies. *Placenta*, 27, 939–958.
- Porter, D. G., Heap, R. B., & Flint, A. P. F. (1982). Endocrinology of the placenta and the evolution of viviparity. *Journal of Reproduction and Fertility. Supplement*, 31, 113–138.
- Riley, P., Anson-Cartwright, L., & Cross, J. C. (1998). The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. *Nature Genetics*, 18, 271–275.
- Roberts, R. M., Green, J. A., & Schulz, L. C. (2016). The evolution of the placenta. *Reproduction*, 152, R179–R189.
- Rosario, G. X., Konno, T., & Soares, M. J. (2008). Maternal hypoxia activates endovascular trophoblast cell invasion. *Developmental Biology*, 314, 362–375.
- Simmons, D. G., Fortier, A. L., & Cross, J. C. (2007). Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. *Developmental Biology*, 304, 567–578.
- Soares, M. J. (2004). The prolactin and growth hormone families: Pregnancy-specific hormones/cytokines at the maternal-fetal interface. *Reproductive Biology and Endocrinology*, 2, 51.
- Soares, M. J., Chapman, B. M., Rasmussen, C. A., et al. (1996). Differentiation of trophoblast endocrine cells. *Placenta*, 17, 277–289.
- Soares, M. J., Chakraborty, D., Rumi, M. A. K., Konno, T., & Renaud, S. J. (2012). Rat placentation: An experimental model for investigating the hemochorial maternal-fetal interface. *Placenta*, 33, 233–243.
- Soares, M. J., Chakraborty, D., Kubota, K., Renaud, S. J., & Rumi, M. A. K. (2014). Adaptive mechanisms controlling uterine spiral artery remodeling during the establishment of pregnancy. *International Journal of Developmental Biology*, 58, 247–259.
- Tanaka, S., Kunath, T., Hadjantonakis, A.-K., Nagy, A., & Rossant, J. (1998). Promotion of trophoblast stem cell proliferation by FGF4. *Science*, 282, 2072–2075.
- Ullah, Z., Kohn, M. J., Yagi, R., et al. (2008). Differentiation of trophoblast stem cells into giant cells is triggered by p57/Kip2 inhibition of CDK1 activity. *Genes and Development*, 22, 3024–3036.
- Van de Peer, Y., Mizrachi, E., & Marchal, K. (2017). The evolutionary significance of polyploidy. *Nature Reviews Genetics*, 18, 411–424.
- Velicky, P., Knöfler, M., & Pollheimer, J. (2016). Function and control of human invasive trophoblast subtypes: Intrinsic vs. maternal control. *Cell Adhesion and Migration*, 10, 154–162.

- Walentin, K., Hinze, C., & Schmidt-Ott, K. M. (2016). The basal chorionic trophoblast cell layer: An emerging coordinator of placenta development. *BioEssays*, 38, 254–265.
- Wooding, F. B. P. (1992). The synepitheliochorial placenta of ruminants: Binucleate cell fusions and hormone production. *Placenta*, 13, 101–113.
- Wooding, F. B. P., & Burton, G. J. (2008). *Comparative placentation: Structures, functions and evolution*. Berlin: Springer-Verlag.
- Xu, R.-H., Chen, X., Li, D. S., et al. (2002). BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nature Biotechnology*, 20, 1261–1264.
- Yu, C., Shen, K., Lin, M., et al. (2002). GCMa regulates the syncytin-mediated trophoblastic fusion. *Journal of Biological Chemistry*, 277, 50062–50068.

Decidua

Joanne Muter, University of Warwick, Coventry, United Kingdom

Jan J Brosens, University of Warwick, Coventry, United Kingdom; and University Hospitals Coventry and Warwickshire, Coventry, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

Decidualization denotes the transformation of the endometrial stroma into the decidual matrix that supports embryo implantation and subsequent placenta formation. This process is foremost characterized by the differentiation of endometrial stromal cells (EnSCs) into secretory decidual cells (Gellersen and Brosens, 2014). Decidualization only occurs in species where the trophoblast breaches the luminal endometrial epithelium and invades maternal tissues. The depth of decidual transformation is determined by the degree of placental trophoblast invasion (Ramsey et al., 1976). In most mammals, decidualization is initiated upon embryo implantation. However, in a handful of species, including humans, Old World monkeys, some bats, elephant shrew, and spiny mouse, decidualization is “spontaneous,” meaning that it is initiated independently of an implanting embryo during the midluteal phase of each cycle (Emera et al., 2012). Once triggered, the decidual phenotype is strictly dependent on sustained progesterone signaling. In the absence of pregnancy, falling ovarian progesterone production triggers a cascade of inflammatory events in the decidualizing endometrium, which upon recruitment and activation of leucocytes becomes irrevocable and leads to partial tissue destruction, bleeding and menstrual shedding. Hence, spontaneous decidualization is inextricably linked to cyclic menstruation; and the term “decidua,” derived from the Latin verb “decidere” (meaning to fall off, to detach, or to die), aptly captures the nature of the process.

Although decidual transformation of EnSCs starts during the midluteal phase of the cycle, characteristic morphological changes are apparent only at the onset of the late-luteal phase, that is approximately 9–10 days after the postovulatory rise in circulating progesterone levels (Fig. 1). Decidualizing cells lose their fibroblastic appearance and become enlarged. They are further characterized by rounding of the nucleus, enlargement of the rough endoplasmic reticulum and Golgi apparatus, and accumulation of glycogen and lipid droplets (Gellersen and Brosens, 2014). Because of this expansion of the cellular secretory machinery, many genes that are constitutively expressed in epithelial cells are induced in EnSCs upon decidualization. The lag-period between ovulation and the onset of decidual process reflects the fact that transcriptional activation of decidual genes is strictly dependent on a sharp rise in intracellular production of the second messenger cyclic adenosine monophosphate (cyclic AMP) during the luteal phase. Cyclic AMP activates protein kinase A and induces the expression of decidual-specific transcription factors, such as the Forkhead box protein O1 (FOXO1), HOXA10 (Homeobox A10) and HOXA11, CCAAT-enhancer-binding proteins (C/EBPs). Once the decidual process is initiated, the liganded progesterone receptor (PGR) physically binds these core decidual-specific transcription factors, thus maintaining and amplifying the expression of differentiation genes, including *PRL* (coding prolactin) and *IGFBP1* (insulin-like growth factor-binding protein 1) (Gellersen and Brosens, 2014).

Typically, decidual transformation starts with EnSCs around the spiral arteries and underlying the luminal epithelium. Short- and long-range cytokines and morphogens then control the spatiotemporally progression of the decidual process, which in pregnancy encompasses the entire endometrium. It is important to note that decidualization of the stroma occurs in concert with profound changes in glandular gene expression and influx of immune cells, especially uterine natural killer (uNK) cells and, to

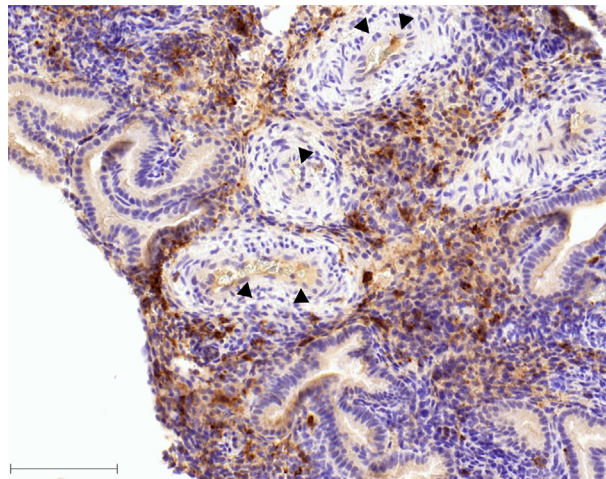


Fig. 1 Immunohistochemistry for CD56 (brown) and hematoxylin in human endometrium from the midluteal phase. Note decidual transformation of EnSCs around the spiral arteries (arrows). Scale bar = 100 μ m.

a lesser extent, macrophages. For an in-depth description of the molecular drivers of decidualization, we refer the reader to a recent review (Gellersen and Brosens, 2014). Here we focus on the emerging functions of the decidual process that enables the endometrium to transit from a cycling to a gestational tissue.

Ontogeny of Spontaneous Decidualization

Decidualization evolved in the stem lineage of Eutherian (placental) mammals. It bestows essential traits onto the uterus that enables controlled trophoblast invasion and confers maternal immune tolerance of the antigenically distinct fetus. These functions in turn underpin the formation of a hemochorial placenta and ensure effective maternal–fetal communication. Evolutionary, EnSCs acquired the ability to accommodate an invading conceptus upon genomic integration of transposable elements (TEs) enriched in *cis*-regulatory elements that co-opted specific transcription factors to drive decidual gene expression (Lynch et al., 2011).

As aforementioned, decidualization is triggered by the implanting embryo in all but menstruating mammals. However, the ability of human EnSCs to decidualize in response to maternal cues is not innate but acquired (Brosens et al., 2017). For example, the fetal human endometrium is exposed during gestation to progressively rising plasma concentrations of unbound estrogens and progesterone, which at birth are several-fold higher when compared to the maternal circulation. Circulating progesterone levels drop very rapidly in the newborn yet overt vaginal bleeding is uncommon, affecting approximately 5% of neonates. Further, an autopsy study on 169 newborn girls demonstrated inactive or weakly proliferative endometrium in 68% of term babies and secretory glandular changes in 27% of newborns. Evidence of decidualization of the stroma or menstrual changes was observed in only 5% of cases.

The lack of neonatal menstruation in most newborns suggests that endometrium only acquires the ability to decidualize around the menarche. The process that renders the endometrium permissive to decidualization is as yet unclear, although there are a number of pointers. In nonmenstruating mammals, decidualization is readily triggered in hormonally primed animals by exogenous stress signals, including endometrial scratching or exposure of the uterine cavity to oil. An emerging line of thought is that rapid estrogen-dependent growth during the follicular phase causes replicative exhaustion of a subpopulation of endometrial cells, which then produce an endogenous stress signal in response to acute senescence during the midluteal phase (Brosens et al., 2017). Cellular senescence is characterized by permanent cell cycle exit and secretion of a host of inflammatory mediators, commonly referred to as senescence-associated secretory phenotype. Several lines of circumstantial evidence support this hypothesis including the fact that the menarche is preceded by a prolonged rise in estrogen levels and sustained uterine growth. Further, telomere lengths of endometrial cells are shortest during the midluteal phase in the cycle, indicative of replication stress. Furthermore, permanent cell cycle exit at G0/G1 during the midluteal phase is a prerequisite not only for cellular senescence but also for terminal differentiation of EnSCs into decidual cells. Clinically, it is well established clinically that rapid estrogen-dependent endometrial growth during the proliferative phase is necessary for successful embryo implantation (Brosens et al., 2017).

Decidualization: A Multistep Process

Decidualization is not an all or nothing process as is often assumed. Instead, differentiating EnSCs transit through distinct phases (Fig. 2) each of which bestows unique functions onto the endometrium that are essential for successful implantation (Gellersen and Brosens, 2014).

Analysis of EnSCs in culture demonstrated that the decidual pathway starts with a burst of reactive oxygen species (ROS) and secretion of a host of chemokines and other inflammatory mediators (Al-Sabbagh et al., 2011; Lucas et al., 2016; Salker et al., 2012), many of which are involved in recruitment and activation of innate immune cells. Feedback pathways ensure that the inflammatory decidual response is self-limiting, lasting between 2 and 4 days. The second decidual phase is characterized by simultaneous downregulation of various chemokines and inflammatory mediators (Al-Sabbagh et al., 2011; Lucas et al., 2016). During this anti-inflammatory phase, decidual cells are increasingly connected and form a matrix through gap and tight junctions. In concert, decidual cells express high levels of 11 β -hydroxysteroid dehydrogenase type 1, an enzyme that converts inert cortisone to active cortisol (Kuroda et al., 2013). This cortisol gradient in the decidua likely contributes to the protection of the invading allogeneic fetal trophoblast against a maternal immune response. Further, by silencing chemokine expression, decidual cells actively prevent effector T cells from entering the feto-maternal interface (Erlebacher, 2013). The third and final phase involves the resolution of the decidual phenotype, triggered in a nonconception cycle by falling progesterone levels. In pregnancy, senescence is thought to drive decidual inflammation. During this phase, secretion of inflammatory factors and matrix metalloproteinases sets in motion a cascade of events that either results in menstruation or contributes to the onset of parturition (Gellersen and Brosens, 2014). In recent years, new insights into the functional importance of this triphasic decidual pathway have emerged.

The Window of Implantation

A limited implantation window is critical as it synchronizes two parallel but initially independent processes: embryo development and endometrial maturation. In mice, a single endocrine signal not only functionally switches a progesterone-primed, prereceptive endometrium to a receptive state but also activates the “dormant” preimplantation embryo. This obligatory maternal implantation

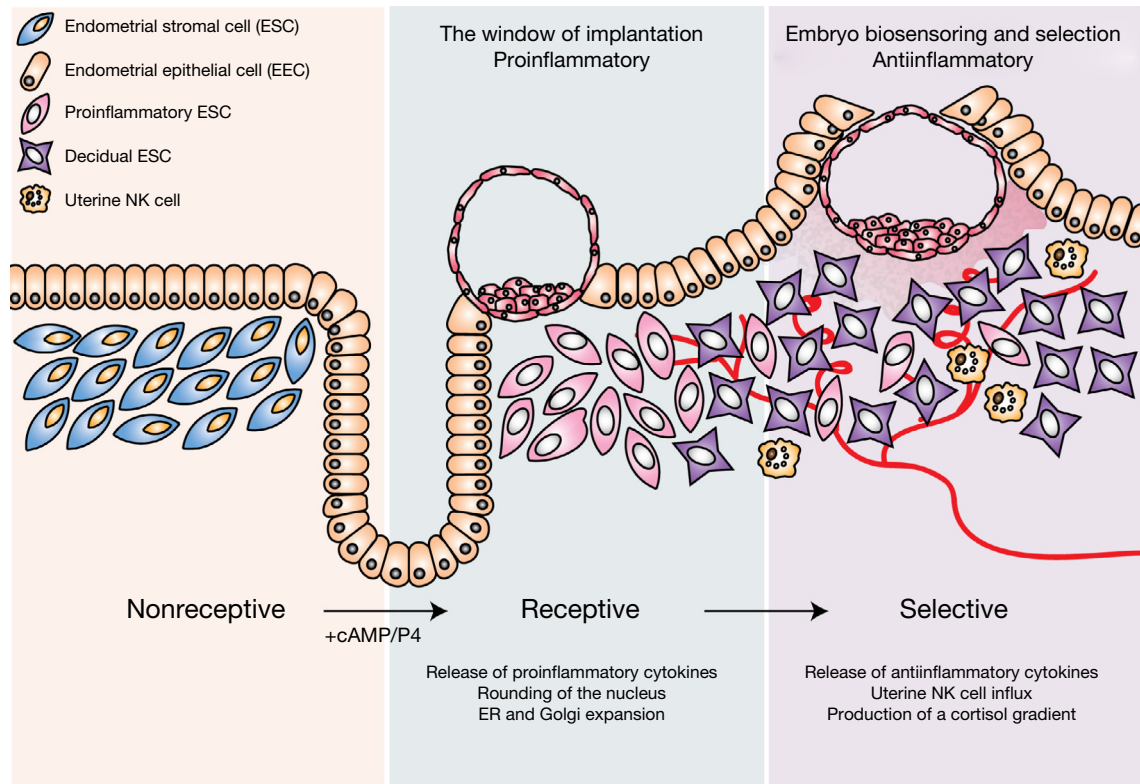


Fig. 2 Schematic diagram of the distinct phases upon spontaneous decidualization, rendering the endometrium first receptive to embryo implantation and selective for embryos of different developmental potential. For full details, see text.

signal consists of a transient rise in postovulatory estradiol production. By contrast, there is no evidence that the midluteal implantation window in human endometrium is controlled by a nidatory estradiol surge, perhaps reflecting that synchronized implantation of multiple human embryos is neither required nor desirable. Instead, compelling experimental evidence suggests that the initial autoinflammatory decidual response renders the endometrium receptivity for embryo implantation. For example, exposure of the mouse uterus to proinflammatory secretome of decidualizing human EnSCs induces the expression of evolutionarily conserved implantation genes, including *Wnt4*, leukemia inhibitory factor (*Lif*), and bone morphogenetic protein 2 (*Bmp2*), and enables efficient implantation of *in vitro* cultured mouse blastocysts (Salker et al., 2012). Furthermore, human blastocysts degrade when cultured in medium conditioned by undifferentiated EnSCs but not decidual cells, suggesting that the endometrium is actively embryotoxic prior to decidualization (Peter Durairaj et al., 2017).

Embryo Biosensing and Selection

Human embryos are characterized by intrinsic genomic instability (McCoy et al., 2015). Meiotic chromosomal aneuploidies correlate strongly with maternal age and disproportionally affect smaller maternal chromosomes. By contrast, chaotic cell divisions caused by spindle abnormalities in early cleavage stage embryos result in complex mitotic aneuploidies that are independent of maternal age, frequently involve larger chromosomes, and are not biased towards either maternal or paternal homologs. As a consequence of mitotic disjunction during the cleavage stages, most—if not all—human preimplantation embryos harbor aneuploid blastomeres. While some chromosomal errors may lead to early embryonic arrest, complex mosaic aneuploidies remain prevalent at the blastocyst stage.

The human endometrium is adapted to deal with this diversity in embryo quality. In fact, the coupling of spontaneous decidualization to menstrual shedding could be viewed as an ingenious solution to deal with chaotic and potentially highly invasive blastocysts. First, with menstruation being the default position, an effective and safe “disposal system” for aberrant embryos is built into the cycle. Second, a significant lag-period exists between implantation and sufficient progesterone production by the emerging placenta. This lag-period makes it incumbent on the implanting embryo to produce sufficient “fitness” signals, such as human chorionic gonadotropin (hCG), to avoid corpus luteum failure and inevitable rejection. Furthermore, decidual cells have emerged as exquisite biosensors of embryo quality and mount a tailored response to an implanting blastocyst that either supports further development or results in a stress response that facilitates early disposal (Brosens et al., 2014).

Different experimental models have highlighted the particular aptness of decidualizing cells in sensing poor quality human embryos. In coculture, decidualizing EnSCs respond to a developmentally compromised blastocyst by silencing the secretion of

multiple implantation factors, including interleukin-1 β and Heparin-binding EGF-like growth factor (HB-EGF). By contrast, undifferentiated EnSCs appear impervious to embryonic signals, irrespective of developmental potential. Furthermore, genome-wide expression profiling revealed a dramatic up-regulation of implantation and metabolic genes in mouse uteri transiently exposed to spent medium of human IVF embryos that resulted in ongoing pregnancies following transfer. By contrast, culture medium conditioned by low-quality human embryos triggered a stress response in the mouse uterus akin to the response observed in primary decidualizing EnSCs (Brosens et al., 2014).

When extrapolated to the *in vivo* situation, these observations strongly suggest that human embryos are subjected to both positive and negative selection pressures at implantation. Efficient biosensing and negative selection of poor quality embryos at implantation results in preclinical attrition. However, lack of negative selection increases the risk of implantation of developmentally compromised embryos and early pregnancy loss. Similarly, failure of positive selection results in high-quality embryos developing in an unsupportive or hostile maternal environment, which arguably predisposes for fetal miscarriage. The clinical hallmark of persistent lack of embryo selection at implantation is superfertility, defined by a mean time to pregnancy of three cycles or less. The estimated incidence of superfertility in the general population is 3%. By contrast, 40% of patients suffering recurrent pregnancy loss (RPL), defined here as three consecutive miscarriages, are reportedly superfertile. The incidence is even higher in women who experienced five or more miscarriages (Salker et al., 2010).

The mechanism that imparts responsiveness of decidual cells to soluble signals from developmentally competent and incompetent embryos is poorly understood. Nevertheless, *HSPA8* has been identified as the most downregulated decidual gene in differentiating EnSC cultures exposed to the secretome of developmentally impaired human embryos (Brosens et al., 2014). *HSPA8* encodes heat shock 70 kDa protein 8 (HSC70), a key regulator of cellular protein homeostasis. HSC70 levels increase upon decidualization in parallel with expansion of the endoplasmic reticulum that drives the secretory phenotype of differentiating EnSCs. Hence, the importance of this molecular chaperone increases as decidual cells expand their secretory machinery, which may explain why undifferentiated endometrial fibroblasts are not bequeathed with a biosensor function.

Decidual Resolution and Menstruation

Falling progesterone levels in a nonconception cycle reactivates the inflammatory decidual phenotype, harbinger of menstrual shedding of the superficial endometrial layer. Women now experience in excess of 400 cycles of menstrual shedding and scar-free regeneration during the reproductive years. However, there are two consequences of cycling endometrium shedding and regeneration that tend to be overlooked. First, cell turnover in cycling endometrium is very high. Not surprisingly, the human endometrium is rich in glandular progenitor cells and mesenchymal stem-like cells (MSC), which reside not only in the basal endometrium but also in a perivascular niche around the spiral arteries. Endometrial MSC are identified by specific cell surface markers, such as Sushi Domain containing 2 (SUSD2) or coexpression of CD146 and platelet-derived growth factor receptor beta (PDGFR β) (Gellersen and Brosens, 2014). Apart from MSC, the endometrial stromal compartment consists of other distinct subpopulations that are defined either by the stage of lineage commitment (e.g., transit amplifying cells, mature progeny, and presenescent cells) or topology (e.g., perivascular EnSCs). Growing evidence indicates that response to decidualogenic signals differs profoundly between subpopulations of EnSCs. For example, perivascular EnSCs are relatively quiescent in an undifferentiated state but become a dominant source of chemokines and cytokines upon decidualization (Murakami et al., 2014). Thus, the spatiotemporal control of decidual response is governed by distinct responses in various EnSC subpopulations. A second important consequence of menstruation is that spontaneous decidualization is an iterative process. As the abundance of different EnSC subpopulations likely fluctuates from cycle to cycle, the quality of the decidual response may equally vary in different cycles. Consequently, cyclic menstruation and renewal renders the endometrium intrinsically dynamic and arguably capable of adapting in response to reproductive failure.

Decidual Cells: Instructors of Local Immune Cells

As aforementioned, spontaneous decidualization of EnSCs during the luteal phase of the cycle occurs in parallel with recruitment of innate immune cells, predominantly CD56^{bright}/CD16⁻ uNK cells. During the late-luteal phase, as many as 30%–50% of cells in the endometrial stroma are uNK cells. Compared to their circulating (CD56^{dim}/CD16⁺) counterparts, uNK are less cytotoxic but more proangiogenic; and exert an evolutionarily conserved role in orchestrating vascular adaptation and trophoblast invasion during pregnancy.

Decidual cells play a pivotal role in instructing uNK cells; meaning that they provide the necessary cues that enable these cells to carry out their specialist functions in pregnancy. For example, conditioned medium from decidual cells supplemented with interleukin-15 and stem cell factor convert peripheral NK cells into uNK-like cells. Coculture with decidual stromal cells also convert CD34⁺ hematopoietic precursors into phenotypic uNK cells. Further, when injected into immunocompromised pregnant mice, induced uNK-like cells migrate to the uterus and improve placental perfusion. Thus, decidual cells not only protect the semiallogenic conceptus against a maternal immune response through local cortisol production and by actively excluding T-cells, they are also instrumental in adapting innate immune cells for pregnancy (Brosens et al., 2017).

Decidual Autonomy in Early Pregnancy

In early pregnancy, the conceptus is embedded in a matrix of decidual cells. As the process of interstitial and endovascular trophoblast invasion commences, the embryo–maternal interface is increasingly subjected to intense tissue remodeling and exposed to profound changes/fluctuations in oxygen tension associated with vascular remodeling. Decidual cells are programmed to resist a range of environmental stress signals, thus ensuring survival of the conceptus. Several molecular mechanisms underpin this quasiautonomous state of decidua prior to the onset of placental perfusion around 10 weeks of pregnancy (Fig. 3).

Cessation of Circadian Rhythms

An early event that signals autonomy of decidualizing EnSCs is cessation of circadian gene expression. Circadian oscillations are predicated on transcriptional–translational feedback loops that balance the activities of the transcriptional activators CLOCK and BMAL1 and repressors encoded by *PER* and *CRY* genes. An early event in the decidual pathway is loss of the clock protein Period 2 (PER2), which in turn silences circadian gene expression in differentiating EnSCs (Muter et al., 2015). As the implanting conceptus is also aperiodic, cessation of circadian oscillations in surrounding decidual cells is arguably essential for optimal embryo–maternal interactions. However, PER2 differs from other core clock proteins in that it exhibits several structural features of steroid receptor coregulators. Further, PER2 knockdown arrests EnSCs at G2/M and results in a grossly disorganized decidual response. Thus, regulation of PER2 expression across the cycle synchronizes endometrial proliferation with initiation of aperiodic decidual gene expression. The importance of transition is underscored by the observation that endometrial *PER2* transcript levels in the midluteal phase of the cycle correlate inversely with the number of previous miscarriages in RPL patients (Muter et al., 2015).

Oxidative Stress Resistance

ROS, including superoxide anion radical, hydrogen peroxide, and the hydroxyl radical, are highly reactive, diffusible, and ubiquitous molecules that are generated as inevitable byproducts of aerobic respiration and metabolism. Mammalian cells possess multiple mechanisms to remove ROS, including endogenous enzymatic and dietary nonenzymatic antioxidants. Detoxification of superoxide by superoxide dismutase enzymes converts it to hydrogen peroxide. Catalase and glutathione peroxidase further degrade hydrogen peroxide to produce water as the end product. Physiological levels of ROS are required to ensure proper functioning of different biological pathways and to maintain tissue homeostasis. However, excessive levels of ROS cause irreversible damage to DNA, proteins, and lipids, ultimately resulting in cell death.

Decidual cells are remarkably resistant to oxidative cell death compared to their undifferentiated progenitor cells (Gellersen and Brosens, 2014). This resistance is partly accounted for by the induction of various free radical scavengers, most notably superoxide dismutase 2, monoamine oxidases A and B, thioredoxin, glutaredoxin, and peroxiredoxin. Several genes coding free radical

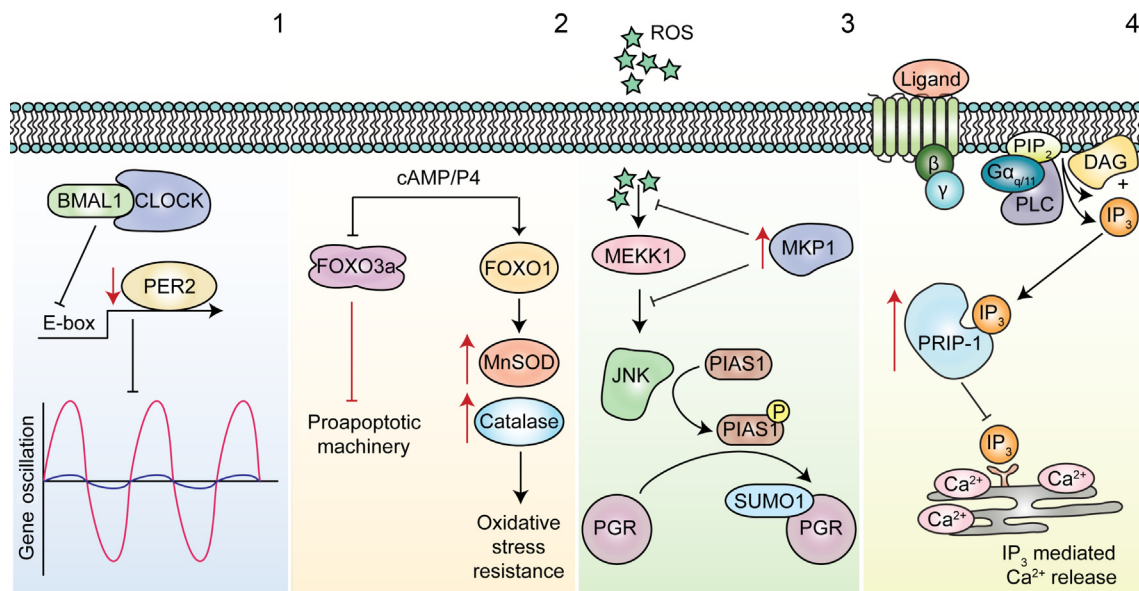


Fig. 3 Illustration of molecular mechanisms of decidual autonomy in early pregnancy. For full details, see text. (1) Cessation of circadian rhythms, (2) oxidative stress resistance, (3) uncoupling of stress signaling and SUMOylation, and (4) silencing of phospholipase C signaling. MnSOD, manganese superoxide dismutase; MEKK1, MAPK kinase kinase 1; PIAS1, protein inhibitor of activation STAT-1; PIP2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol.

scavengers are transcriptionally controlled by FOXO1, a core decidual transcription factor. However, a second mechanism contributes to the exceptional ROS resistance in decidual cells. Exposure of undifferentiated EnSCs to exogenous free radicals rapidly activates the c-Jun NH-terminal kinase (JNK) stress signaling pathway and upregulates another forkhead protein, FOXO3a, which in turn triggers cell death. However, decidual cells strongly upregulate mitogen-activated protein (MAP) kinase phosphatase-1 (MKP-1), which not only silences the JNK pathway but also inhibits FOXO3a expression. Hence, while undifferentiated HESCs are programmed to self-destruct in response to oxidative stress signals, this mechanism is firmly disabled upon differentiation into decidual cells (Gellersen and Brosens, 2014).

Uncoupling of Stress Signaling and SUMOylation

A striking adaptation in decidual cells involves the small ubiquitin related modifier (SUMO) conjugation/deconjugation pathway. SUMOylation, an important posttranslational protein modification that affects a large number of substrates, is catalyzed through a sequence of enzymatic reactions. This reversible posttranslational protein modification regulates numerous cellular processes, including cell signaling, nuclear transport, transcription, and DNA replication and repair. Nuclear receptors, including PGR, are important SUMO targets. The transcriptional activity of PGR in response to progesterone binding is strongly repressed upon SUMOylation. Interestingly, decidualization is associated with global cellular hypoSUMOylation, effected by altered expression of SUMO-specific ligases and proteases. Various stress signals convert on the SUMO pathway, including ROS, hypoxia, heat shock, and genotoxic stress. However, induction of MKP1 and silencing of the JNK pathway ensure that decidual cells do not exhibit a hyperSUMOylation response when exposed to exogenous stress signals (Gellersen and Brosens, 2014). Importantly, this means that progesterone signaling is unimpeded in the decidua under the oxidative stress conditions imposed by pregnancy.

Silencing of Phospholipase C Signaling

Further decidual autonomy is achieved via induction of phospholipase C (PLC)-related catalytically inactive protein 1 (PRIP-1). PRIP-1 is a progesterone-inducible scaffold protein that uncouples PLC activation downstream of G_q -protein-coupled receptors from intracellular Ca^{2+} release by attenuating inositol trisphosphate (IP3) signaling (Muter et al., 2016). As many external signals are transduced by G_q -protein-coupled receptors, strong induction PRIP-1 is a remarkably simple solution to ensure decidual autonomy. Furthermore, by chelating IP3, PRIP-1 also limits Ca^{2+} release from the expanding endoplasmic reticulum, thereby safeguarding decidual cells against excessive Ca^{2+} accumulation in the mitochondrial matrix, permeabilization of the mitochondrial outer membrane, and cell death caused by leakage of apoptosis factors from the intermembrane space.

Clinical Perspective

Failure of the endometrium to mount a decidual response inevitably leads to implantation failure whereas a prolonged and disordered autoinflammatory decidual response is linked to out-of-phase implantation, impaired embryo biosensing, and RPL. Clinically, implantation failure is the rate-limiting step in assisted reproductive technologies whereas miscarriage is the most common complication of pregnancy. The incidence of clinical miscarriage after 6 weeks of pregnancy is approximately 15%. When preclinical losses are taken in account, the incidence of early pregnancy attrition is estimated to be between 30% and 50%. Numerous gene ablation studies in mice have identified key signals, pathways, and downstream transcription factors that are indispensable for decidualization (Gellersen and Brosens, 2014). However, clinical translation of this information into either new treatment or endometrial biomarkers predictive of reproductive failure has so far be elusive.

So, what are the roadblocks? An obvious hurdle is that human blastocysts, unlike their murine counterparts, are chromosomally diverse. Because of intrinsic mosaicism, it remains difficult, if not impossible, to predict accurately the developmental potential of an individual embryo, even following preimplantation genetic screening. Consequently, it is often not possible to ascertain retrospectively if implantation failure or early pregnancy loss was caused by an embryonic, a maternal factor, or a combination. In general, the likelihood of an underlying endometrial defect compromising nidation or development of a competent embryo increases with each additional failure. Another hurdle, perhaps less appreciated, is that spontaneous decidualization and cyclic menstruation renders the endometrium intrinsically dynamic (Lucas et al., 2016). In other words, analysis of the endometrium in a given cycle may not necessarily be predictive of implantation competence in a subsequent cycle.

Yet, these perceived hurdles—embryo diversity and endometrial plasticity—also belie the clinical observation that the cumulative live birth rate for most women is high, even after multiple implantation failures or miscarriages. For example, several randomized double-blind placebo control studies reported live births rates of 65% or more in patients with three consecutive miscarriages who were assigned to the placebo group. Even after five consecutive miscarriages, the likelihood of a live birth in a subsequent pregnancy remains in excess of 50%. Similar high cumulative live birth rates have been reported following implantation failure in IVF. Hence, it is as paramount to understand the mechanisms that enable a successful pregnancy to occur after multiple failures as it is to define the primary cause of reproductive failure. The discovery of specific endometrial defects associated with RPL, such as stem cell deficiency (Lucas et al., 2016), is opening up the possibility of prepregnancy screening of women at risk of reproductive failure. Effective interventions, however, will require a more in-depth understanding of the mechanisms that control endometrial homeostasis, and by extension the decidual pathway, from cycle to cycle.

References

- Al-Sabbagh, M., Fusi, L., Higham, J., Lee, Y., Lei, K., Hanyaloglu, A. C., Lam, E. W., Christian, M., & Brosens, J. J. (2011). NADPH oxidase-derived reactive oxygen species mediate decidualization of human endometrial stromal cells in response to cyclic AMP signaling. *Endocrinology*, 152(2), 730–740.
- Brosens, J. J., Salker, M. S., Teklenburg, G., Nautiyal, J., Salter, S., Lucas, E. S., Steel, J. H., Christian, M., Chan, Y. W., Boomsma, C. M., Moore, J. D., Hartshorne, G. M., Sucurovic, S., Mulac-Jericevic, B., Heijnen, C. J., Quenby, S., Koerkamp, M. J., Holstege, F. C., Shmygol, A., & Macklon, N. S. (2014). Uterine selection of human embryos at implantation. *Scientific Reports*, 4(3894), 1–8.
- Brosens, I., Muter, J., Gargett, C., Puttemans, P., Benagiano, G., & Brosens, J. J. (2017). The impact of uterine immaturity on obstetrical syndromes during adolescence. *American Journal of Obstetrics and Gynecology*. Epub ahead of print.
- Emera, D., Romero, R., & Wagner, G. (2012). The evolution of menstruation: A new model for genetic assimilation: Explaining molecular origins of maternal responses to fetal invasiveness. *BioEssays*, 34(1), 26–35.
- Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annual Review of Immunology*, 31, 387–411.
- Gellersen, B., & Brosens, J. J. (2014). Cyclic decidualization of the human endometrium in reproductive health and failure. *Endocrine Reviews*, 35(6), 851–905.
- Kuroda, K., Venkatakrishnan, R., Salker, M. S., Lucas, E. S., Shaheen, F., Kuroda, M., Blanks, A., Christian, M., Quenby, S., & Brosens, J. J. (2013). Induction of 11 β -HSD 1 and activation of distinct mineralocorticoid receptor- and glucocorticoid receptor-dependent gene networks in decidualizing human endometrial stromal cells. *Molecular Endocrinology*, 27(2), 192–202.
- Lucas, E. S., Dyer, N. P., Murakami, K., Lee, Y. H., Chan, Y. W., Grimaldi, G., Muter, J., Brighton, P. J., Moore, J. D., Patel, G., Chan, J. K., Takeda, S., Lam, E. W., Quenby, S., Ott, S., & Brosens, J. J. (2016). Loss of endometrial plasticity in recurrent pregnancy loss. *Stem Cells*, 34(2), 346–356.
- Lynch, V. J., Leclerc, R. D., May, G., & Wagner, G. P. (2011). Transposon-mediated rewiring of gene regulatory networks contributed to the evolution of pregnancy in mammals. *Nature Genetics*, 43(11), 1154–1159.
- McCoy, R. C., Demko, Z. P., Ryan, A., Banjevic, M., Hill, M., Sigurjonsson, S., Rabinowitz, M., & Petrov, D. A. (2015). Evidence of selection against complex mitotic-origin aneuploidy during preimplantation development. *PLoS Genetics*, 11(10), e1005601.
- Murakami, K., Lee, Y. H., Lucas, E. S., Chan, Y. W., Durairaj, R. P., Takeda, S., Moore, J. D., Tan, B. K., Quenby, S., Chan, J. K., Gargett, C. E., & Brosens, J. J. (2014). Decidualization induces a secretome switch in perivascular niche cells of the human endometrium. *Endocrinology*, 155(11), 4542–4553.
- Muter, J., Lucas, E. S., Chan, Y. W., Brighton, P. J., Moore, J. D., Lacey, L., Quenby, S., Lam, E. W., & Brosens, J. J. (2015). The clock protein period 2 synchronizes mitotic expansion and decidual transformation of human endometrial stromal cells. *The FASEB Journal*, 29(4), 1603–1614.
- Muter, J., Brighton, P. J., Lucas, E. S., Lacey, L., Shmygol, A., Quenby, S., Blanks, A. M., & Brosens, J. J. (2016). Progesterone-dependent induction of phospholipase C-related catalytically inactive protein 1 (PRIP-1) in Decidualizing human endometrial stromal cells. *Endocrinology*, 157(7), 2883–2893.
- Peter Durairaj, R. R., Aberkane, A., Polanski, L., Maruyama, Y., Baumgarten, M., Lucas, E. S., Quenby, S., Chan, J. K., Raine-Fenning, N., Brosens, J. J., Van de Velde, H., & Lee, Y. H. (2017). Deregulation of the endometrial stromal cell secretome precedes embryo implantation failure. *Molecular Human Reproduction*, 23(7), 478–487.
- Ramsey, E. M., Houston, M. L., & Harris, J. W. (1976). Interactions of the trophoblast and maternal tissues in three closely related primate species. *American Journal of Obstetrics and Gynecology*, 124(6), 647–652.
- Salker, M., Teklenburg, G., Molokhia, M., Lavery, S., Trew, G., Aojanepong, T., Mardon, H. J., Lokugamage, A. U., Rai, R., Landles, C., Roelen, B. A., Quenby, S., Kuijk, E. W., Kavelaars, A., Heijnen, C. J., Regan, L., Macklon, N. S., & Brosens, J. J. (2010). Natural selection of human embryos: Impaired decidualization of endometrium disables embryo-maternal interactions and causes recurrent pregnancy loss. *PLoS One*, 5(4), e10287.
- Salker, M. S., Nautiyal, J., Steel, J. H., Webster, Z., Sucurovic, S., Nicou, M., Singh, Y., Lucas, E. S., Murakami, K., Chan, Y. W., James, S., Abdallah, Y., Christian, M., Croy, B. A., Mulac-Jericevic, B., Quenby, S., & Brosens, J. J. (2012). Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. *PLoS One*, 7(12), e52252.

Human Placentation

Berthold Huppertz, Medical University of Graz, Graz, Austria

© 2018 Elsevier Inc. All rights reserved.

The Human Placenta

Already the early Egyptians have recognized and venerated the human placenta. In ancient Greece, Diogenes of Apollonia (about 480 BCE) was the first to describe the placenta as being responsible for fetal nutrition. Only during Renaissance times (1559) Realdus Columbus started to use the term placenta, which was taken from the Latin root for a flat cake.

Development of the Human Placenta

Prelacunar Phase (Days 5–8 after Fertilization)

The trophoblast is the first cell line that differentiates in an embryo. It develops at day 5 after fertilization (day 5 post conception, p.c.), leading to the formation of the blastocyst. The trophoblast cells of the blastocyst organize themselves as a ball-shaped cover (*trophectoderm*), surrounding the blastocyst cavity and the inner cell mass (*embryoblast*). Later during pregnancy, the trophoblast only resides within the placenta, while the cells of the embryoblast will form the whole embryo proper plus specific tissues within the placenta.

The blastocyst adheres to the uterine luminal epithelium at day 6–7 p.c., inducing further differentiation of the trophoblast. Those trophoblast cells in direct contact with the uterine epithelium fuse with each other and develop into the first multinucleated *syncytiotrophoblast*. In the human, the syncytiotrophoblast seems to be the layer that enables penetration of the uterine epithelium and invasion of the embryo into the decidual connective tissues.

The blastocyst has completely passed the uterine epithelium at day 7–8 p.c. The trophoblast grows massively during this process, becomes multi-layered and completely surrounds the embryo that does not come into direct contact with maternal tissues. The multinucleated syncytiotrophoblast at the outside of the placenta directly contacts maternal cells and tissues, while the mononucleated *cytotrophoblasts* face towards the embryo.

All the above stages and phases of differentiation and development occur prior to the development of fluid-filled spaces within the syncytiotrophoblast. This is why this phase is termed *prelacunar phase* (Benirschke et al., 2006).

Lacunar Phase (Days 8–12 after Fertilization)

The syncytiotrophoblast forms a growing number of fluid-filled spaces, starting around day 8–9 p.c. This defines the start of the *lacunar phase* (Benirschke et al., 2006). Due to flowing together of these spaces a large single fluid-filled space develops, the *intervillous space*. Between the trophoblast on the embryonic side and the trophoblast facing maternal tissues this fluid-space develops, only leaving a number of trophoblast branches (trabeculae) bridging the two sides of trophoblast.

At the end of the lacunar phase, at day 12 p.c., the embryo has invaded deeper into the uterine wall and is now completely embedded into decidual tissues. At that time the embryo adds a new tissue layer of mesenchymal cells (*extra-embryonic mesoderm*) underneath the two types of trophoblast, the mononucleated cytotrophoblasts and the multinucleated syncytiotrophoblast.

All the above processes of development have subdivided the placenta into different compartments:

1. The cytotrophoblasts at the embryonic side plus the mesoderm cells will develop into the *chorionic plate*.
2. The trabeculae will develop into *anchoring villi* that adhere the placenta proper to the uterine wall.
3. Side branches of the trabeculae will develop into *floating villi*.
4. The fluid-filled spaces villi develop into the intervillous space; the latter will be the chamber for maternal blood during hemotrophic nutrition of the fetus.
5. The part of the trophoblast at the maternal side of the placenta plus cellular and acellular components of the decidua will develop into the *basal plate*.

Early Villous Phase (Days 13–28 after Fertilization)

During this phase, first placental villi develop as the precursors of the specific placental villi later in pregnancy (Benirschke et al., 2006). Proliferation of cytotrophoblasts at the embryonic side of the placenta pushes a set of these cells into the syncytial trabeculae. The latter structures are finally fully penetrated by mononucleated cells that reach the maternal side of the syncytiotrophoblast at day 14 p.c. Further proliferation of cytotrophoblasts within the trabeculae leads to the development of syncytial protrusions and finally side branches filled with cytotrophoblasts (*primary villi*, day 13 p.c.).

Mesenchymal cells of the extra-embryonic mesoderm follow the cytotrophoblasts and penetrate the trabeculae as well as the primary villi, resulting in the generation of *secondary villi* with two trophoblast layers outside and a mesenchymal core.

Finally, at about day 18–20 p.c., new vessels form based on hemangioblastic precursor cells within the villous mesenchyme (*vascularization*) (Demir et al., 2004). These first placental vessels give rise to the development of *tertiary villi* (Fig. 1).

Due to the fact that placental villi develop from single trabeculae, they are organized in *villous trees* (with the trabecula becoming the stem villus of the tree). These villous trees become the major spherical units of the placenta and are termed lobules or *placentomes*. The side branches of the trabeculae, the placental villi, continuously branch resulting in multiple branching sites from the stem villus to the outermost floating and freely ending villi in the intervillous space.

Villous Phase (Day 28 after Fertilization to End of Pregnancy)

Growth of placental villi occurs following a uniform principle. On the surface of larger villi the syncytiotrophoblast protrudes and forms *syncytial sprouts* (Fig. 1). Underneath these sprouts, increased proliferation of cytotrophoblasts pushed the daughter cells into the sprouts (primary villi). Then mesenchymal cells follow (secondary villi) and finally blood vessels reach the villi (tertiary villi) starting from already existing vessels in the larger villus (*angiogenesis*) (Benirschke et al., 2006).

This uniform way of villous development results in a general layout of such villi with a specific set of layers, cells and tissues.

Villous layers, cells and tissues

Villous cytotrophoblast

Morphologically, *villous cytotrophoblasts* are characterized by their roundish shape in histological sections (Fig. 1). Their nuclei are large and present only minor amounts of heterochromatin. During the first trimester a complete layer of villous trophoblasts can be found underneath the syncytiotrophoblast (Benirschke et al., 2006). A basement membrane is separating this layer from the villous stroma. Throughout pregnancy the villous core is growing faster than this layer of cells resulting in a seemingly reduction of cells. At term the villous cytotrophoblasts can be found as single cells lying underneath the syncytiotrophoblast, a complete layer is no longer present.

The continuous increase in the number of villous cytotrophoblasts during pregnancy can be seen from cell numbers over gestation: about 1×10^9 cells at 13–16 weeks and about 6×10^9 cells at 37–41 weeks (Benirschke et al., 2006). The rate between proliferation of villous cytotrophoblast and syncytial fusion with the overlying syncytiotrophoblast remains quite constant over gestation. The relation between villous cytotrophoblast nuclei and syncytial nuclei is 1:9, irrespective whether this is counted at 13–16 weeks of gestation or at 37–41 weeks (Mayhew et al., 1999).

Villous cytotrophoblasts do not direct contact to maternal blood. If focal damage of the syncytiotrophoblast occurs, the hole is filled with *fibrin-type fibrinoid* (a maternal blood clot product) covering the exposed cytotrophoblasts (Benirschke et al., 2006).

Syncytiotrophoblast

As the outermost layer of all villi, the syncytiotrophoblast covers all placental villi as one single layer covering all villi of a single placenta. This layer is a true syncytium, developed and maintained by fusion of mononucleated cells and without any lateral cell borders (Benirschke et al., 2006). The syncytiotrophoblast is easily identifiable during the first half of pregnancy (Fig. 1) and is characterized by a continuous row of nuclei. During the second half of pregnancy the syncytiotrophoblast becomes thinner to reduce the diffusion distance between maternal and fetal blood. Also the syncytial nuclei are now ordered in groups at specific sites to allow maximal thinning of the syncytiotrophoblast at sites of direct contact of placental blood vessels with the trophoblast layer.

As maintenance and growth of the syncytiotrophoblast are based on syncytial fusion with the underlying cytotrophoblasts, syncytial nuclei are introduced into this layer by fusion, show different levels of aging and do no longer show any signs of

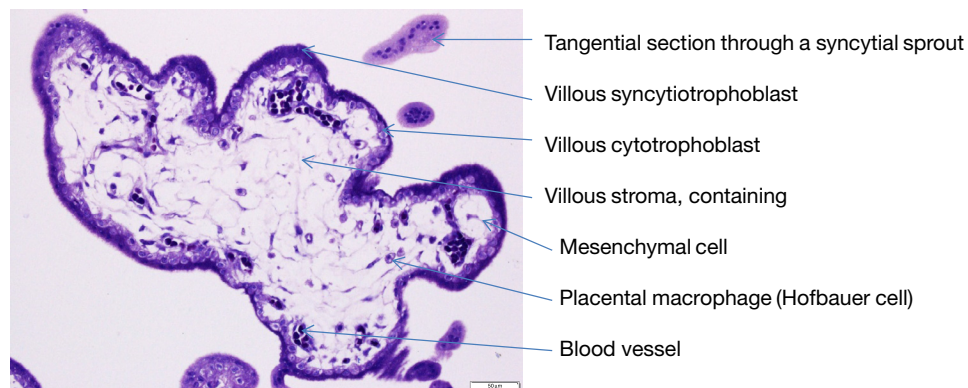


Fig. 1 Cross section through a mesenchymal villus at 5–6 weeks of gestation. This villus comprises all the typical tissues and cells characteristic for a first trimester villus: a cover by the syncytiotrophoblast, a complete layer of villous cytotrophoblasts, and a villous stroma with mesenchymal cells, Hofbauer cells and blood vessels. On the upper right a tangential section through a syncytial sprout can be seen pointing to further growth of the villous trees.

proliferative activity. Directly after fusion the syncytial nuclei exhibit a large and ovoid shape, then during the 3–4 weeks of staying within the syncytiotrophoblast they become smaller and denser. Finally, they show clear signs of late apoptosis with envelope convolution, increased packing density and increased heterochromatinization (Huppertz, 2010). The aged nuclei accumulate at specific sites (often at the tips of villi), are packed into protrusions of the syncytial apical membrane (*syncytial knots*) and are released into the maternal circulation as apoptotic corpuscular structures (Benirschke et al., 2006; Huppertz, 2010). Interestingly, signs of late apoptosis are extremely rare in the villous cytotrophoblast but may well occur in those cytotrophoblasts that fail to undergo syncytial fusion (Longtine et al., 2012).

The syncytiotrophoblast is the site with the highest metabolic and endocrine activity in the placenta. The main function of this layer is the materno-fetal (and feto-maternal) transfer and its control, including (Benirschke et al., 2006):

- Active transport of amino acids and electrolytes such as sodium and calcium,
- Facilitated transport of glucose,
- Diffusion of water and gases,

as well as

- Synthesis of a variety of hormones such as β -hCG (beta-subunit of human chorionic gonadotropin) and
- Catabolism and resynthesis of proteins and lipids.

Villous trophoblast turnover

Each epithelium shows proliferation at the basal side and extrusion of aged material at the apical side. Also the villous trophoblast exhibits this phenomenon of continuous turnover, which comprises the following steps (Huppertz et al., 1998; Huppertz, 2010):

- Proliferation of a subset of cytotrophoblast progenitor cells.
- Differentiation of post-proliferative mononucleated daughter cytotrophoblasts within 2–3 days.
- Syncytial fusion of finally differentiated cytotrophoblasts with the overlying syncytiotrophoblast.
- Further differentiation and maturation of cellular components and organelles within the syncytiotrophoblast, including nuclei (3–4 weeks).
- Aging and late apoptosis at specific sites of the syncytiotrophoblast.
- Packing of aged material into syncytial knots, mostly known for aged nuclei.
- Syncytial knots are extruded into the maternal circulation (Huppertz et al., 1998; Benirschke et al., 2006).

During gestation, a large number of such syncytial knots is detached from the syncytiotrophoblast and released into the maternal circulation. In late gestation up to 150,000 such corpuscular structures or 2–3 g of trophoblast material have been estimated to enter the maternal circulation each day (Huppertz et al., 1998; Benirschke et al., 2006). Since most of these multinucleated structures become lodged in the capillary bed of the lungs, they are extremely rare in arterial or peripheral venous blood of pregnant women.

Placental mesenchyme

The mesenchymal stroma within the core of placental villi (Fig. 1) is composed of a population of fixed and moving connective tissue cells including (Benirschke et al., 2006):

- Mesenchymal cells (Fig. 1) and fibroblasts in different stages of differentiation up to myofibroblasts.
- Placental macrophages (*Hofbauer cells*). Placental macrophages (Fig. 1) differentiate from progenitor cells within the mesenchyme of placental villi even prior to onset of blood flow between embryo and placenta. Afterwards, placental macrophages are also recruited from blood-derived monocytes.
- Placental vessels with smooth muscle cells and endothelial cells including arteries, veins, capillaries and sinusoids. During the first trimester of pregnancy, placental vessels (Fig. 1) are not surrounded by a basement membrane, which only develops afterwards. The endothelium of placental capillaries acts as passive filter and reduces the intercellular transfer of molecules (Eaton et al., 1993).

Villous types

During pregnancy five specific types of villi develop and can be distinguished based on their diameter/perimeter, stromal characteristics, vessel structures and functions (Benirschke et al., 2006; Baergen, 2011) (Fig. 2). Each stage of pregnancy can be defined by a specific subset of villous types.

Mesenchymal villi

Mesenchymal villi are the first type of villi that develops as soon as the first tertiary villi have evolved at around day 20 p.c. Hence, mesenchymal villi are the forerunners of all other villous types. During the first and second trimester mesenchymal villi differentiate into immature intermediate villi, while during the third trimester they directly differentiate into stem villi. Early during pregnancy mesenchymal villi are the dominant villous type, while at term only about 1% of the villous volume can be attributed to mesenchymal villi.

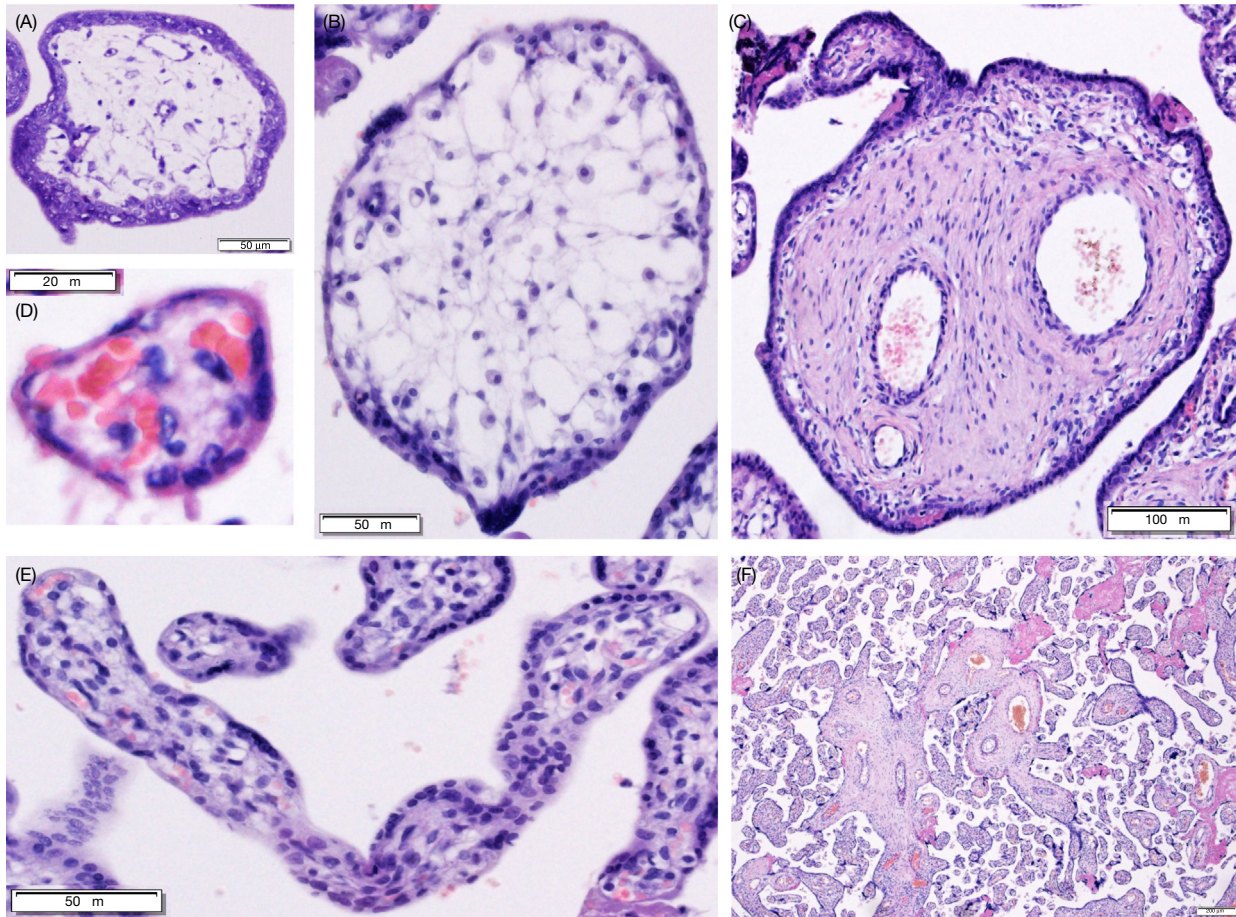


Fig. 2 The five villous types in a human placenta: (A) mesenchymal villus, (B) immature intermediate villus, (C) stem villus, (D) terminal villus, and (E) mature intermediate villus. (F) Gives an overview of a cross section through a term placenta with centrally located stem villi surrounded by mature intermediate villi and terminal villi.

Mesenchymal villi have diameters between 40 and 80 μm and show two-layered villous trophoblast coverage with the syncytiotrophoblast and a clear layer of villous cytotrophoblast (Fig. 2A). Their stromal core is characterized by only few fibers such as collagen type I, few fibroblasts and Hofbauer cells and first small vessels. At the same time the core is full of undifferentiated mesenchymal cells. Mesenchymal villi are the villous type with the highest proliferation rates, mostly due to massive proliferation of mesenchymal cells and villous cytotrophoblasts.

Immature intermediate villi

Immature intermediate villi, the first villous type differentiating from mesenchymal villi, start to show up starting at about week 8 of gestation (post menstruation, p.m.). This villous type is prevailing between weeks 14–20, while at term only about 5% of the villous volume can be attributed to immature intermediate villi. This villous type is much larger than mesenchymal villi with diameters between 100 and 300 μm . Their stromal core shows a loose reticular connective tissue with little extracellular matrix, small blood vessels and large numbers of Hofbauer cells. The specific characteristic of immature intermediate villi is the presence of stromal channels (Benirschke et al., 2006) (Fig. 2B). These channels are fluid-filled spaces along the roll axis of the villi and do not have any known connection to blood vessels. They are surrounded by fibroblasts but lack any relation to endothelial cells. Hofbauer cells are present in these channels and hence can easily be identified in these villi.

Stem villi

Stem villi develop from further differentiation of immature intermediate villi or directly differentiate from mesenchymal villi. Stem villi show up at about week 18–20 p.m. and are the largest villi with diameters of 100 μm to some millimeters. Due to their size about 20%–25% of the villous volume at term can be attributed to this villous type. Stem villi function as mechanical stabilization of the whole villous tree, similar to the trunk of a tree.

The villous trophoblast of stem villi is quite thin with low numbers of villous cytotrophoblasts. The villous stroma is filled with only few fibroblasts and Hofbauer cells but lots of fibers such as collagen type I and other matrix components to stabilize this type of

villi. The large stem villi (Fig. 2C) are characterized by two centrally located vessels (artery and vein) surrounded by a system of extravascular contractile cells that locally regulate vessel diameters in the absence of nerve cells in the placenta.

Mature intermediate villi

At the beginning of the third trimester (about week 24 p.m.) mesenchymal villi differentiate into *mature intermediate villi*, the latter with diameters between 80 and 120 μm . At term about 25% of the villous volume can be attributed to mature intermediate villi. This villous type displays long and slender villi, a loose connective tissue, often marginal small vessels and some terminal arterioles and venules (Fig. 2E). Mature intermediate villi serve as source for terminal villi and connect terminal villi with stem villi.

Terminal villi

Terminal villi are the last type of villi that develops during pregnancy appearing at about week 27 of gestation. This villous type does not differentiate from a forerunner but rather develops from protrusions of mature intermediate villi. Within these intermediate villi an increased longitudinal growth of vessels results in protrusions of vessels covered by villous trophoblast, terminal villi (Fig. 2D).

The function of terminal villi is the direct exchange between mother and fetus across the placental barrier. The placental barrier is the tissues between maternal and fetal blood that need to be crossed to enable exchange. This barrier comprises the syncytiotrophoblast, sometimes also villous cytotrophoblasts, the trophoblastic basement membrane, sometimes villous stroma, the endothelial basement membrane and endothelial cells of the placental blood vessels. In a best-case scenario the placental barrier consists of the syncytiotrophoblast, the merged basement membranes of trophoblast and endothelium and the endothelium (termed *vasculosyncytial membrane*). Here, the thickness of the barrier may decrease down to 0.5–2 μm .

Terminal villi show a length of about 200 μm with diameters between 50 and 100 μm . In a term placenta about 40% of villous volume can be attributed to this type of villi. Capillaries within terminal villi often show extension of their diameters and are referred to as sinusoids. Due to their function as exchange villi, sometimes more than 50% of the stromal cross section is dominated by vessels (capillaries and sinusoids).

Interestingly, the number of terminal villi follows their microenvironment within the placenta. In cases with maternal hypoxia (chronic anemia or pregnancy at high altitude) less oxygen reaches the placenta. The third trimester placenta senses and reacts on this altered environment by increasing the number of terminal villi to increase exchange surface area. On the other hand, cases with growth restricted babies and reduced blood flow in the umbilical arteries leads to an increase in placental oxygen concentrations (Sibley et al., 2002) followed by regression and thus decreased numbers of terminal villi.

Extravillous Trophoblast

Cytotrophoblasts that penetrate the trabeculae finally reach the decidual tissues where they come into direct contact with maternal cells. The mesenchymal cells that subsequently penetrate the trabeculae, however, do not reach maternal tissues (Benirschke et al., 2006). This difference in depth of penetration results in the formation of multiple layers of cytotrophoblasts at the tips of the trabeculae, which then turn into anchoring villi. These trophoblast layers are referred to as *trophoblastic cell columns* (Benirschke et al., 2006) (Fig. 3). Within the cell columns only those cytotrophoblasts remain in a proliferative state that are in direct contact to the basement membrane separating trophoblasts from the mesenchymal core of the anchoring villi. Their proliferative pressure pushes cytotrophoblasts down the columns where they finally reach and start actively invading maternal tissues.

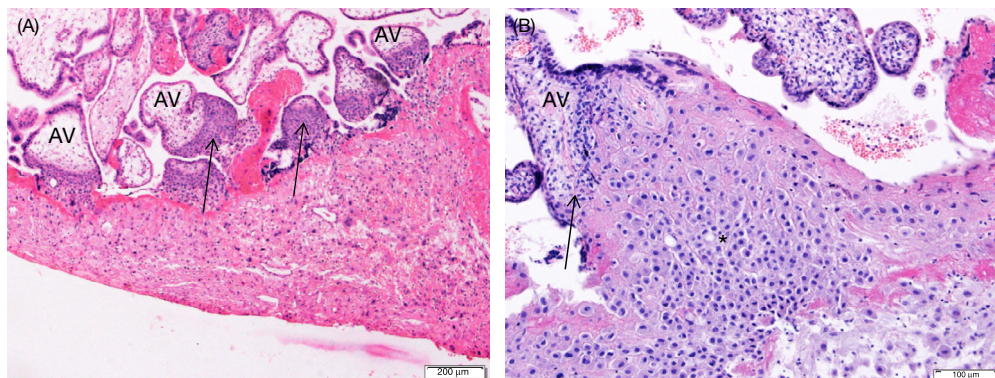


Fig. 3 Trophoblast cell columns. (A) The invasive front of a placenta at 15 weeks of gestation. Multiple cell columns (arrows) attached to anchoring villi (AV) can be seen from which extravillous trophoblasts migrate into the decidual stroma. (B) A cell column of a human placenta at 28 weeks of gestation. The anchoring villus (AV) carries a trophoblastic cell columns with only very few rows of cells (arrow). Below the cell columns multiple interstitial trophoblasts can be found belonging to the type of the large, polygonal cells.

A general feature of all extravillous trophoblasts is the expression of the human leukocyte antigen-G (HLA-G), which differentiates them from the villous trophoblast (McMaster et al., 1995). HLA-G is used as specific marker of extravillous trophoblasts especially in immunohistochemical studies. Another general feature of invading extravillous trophoblasts is the absence of any proliferative activity once these cells have actively started to invade maternal tissues. This is one of the major differences compared to invading tumor cells.

Invasion of extravillous trophoblast is not restricted to early placental development but occurs throughout pregnancy (Fig. 3A and B). The part of the uterine wall below the placenta and invaded by extravillous trophoblasts is termed *placental bed*. The part of the decidua within the placental bed is referred to as *decidua basalis*, while the non-invaded rest of the decidua is termed *decidua parietalis*. The part of the decidua basalis that is delivered with the placenta has been termed *basal plate*. Finally, the part of the decidua that is present in the *fetal membranes*, the *chorion laeve*, is termed *decidua capsularis*.

Trophoblast invasion serves a number of functions that are fulfilled by a respective number of different trophoblast subpopulations (Fig. 4).

Interstitial Trophoblast

Starting within the trophoblastic cell columns, *interstitial trophoblasts* invade into the tissues of the decidua basalis and the inner third of the myometrium within the placental bed (Figs. 3 and 4). Hence, interstitial trophoblast is the source of all other subpopulations of extravillous trophoblast. Besides serving as source for other trophoblast populations, the main function of interstitial trophoblasts is to adhere the placenta to the uterine wall (Benirschke et al., 2006).

On their way through the uterine interstitium interstitial trophoblasts secrete a specific set of extracellular matrix components, called *matrix-type fibrinoid* (Kaufmann et al., 1996), which seems to function as a glue to anchor the placenta to the uterine wall.

The population of interstitial trophoblasts is morphologically and functionally very heterogeneous, and different phenotypes can be distinguished. These phenotypes differ regarding invasive behavior, contact to maternal cells and secretion of matrix components. So far, a molecular basis for these differences has not been established; however, respective attempts to explain differences in trophoblast phenotypes have been made in the mouse (Simmons et al., 2007).

Typically, three different phenotypes can be distinguished within the population of the interstitial trophoblast:

Large, Polygonal Cells

These large, polygonal cells display a large nucleus, are irregularly formed and can be clearly stained for HLA-G and cytokeratin, while they are always negative for proliferation markers such as Ki-67. This phenotype secretes most of the matrix-type fibrinoid and can be found embedded in their own matrix. Hence, contact to maternal cells is rare. The above properties of the cells indicate that the cells are not very invasive, if at all. These cells can be found in the uterus even decades after the last pregnancy.

In early pregnancy (weeks 9–12) about 45% of all interstitial trophoblasts belong to this phenotype. This portion increases throughout gestation with 69% at 16–24 weeks and 89% at 31–39 weeks (Benirschke et al., 2006) (Fig. 3B). This shift in phenotype indicates that trophoblast invasion takes place throughout pregnancy, but is clearly reduced towards term.

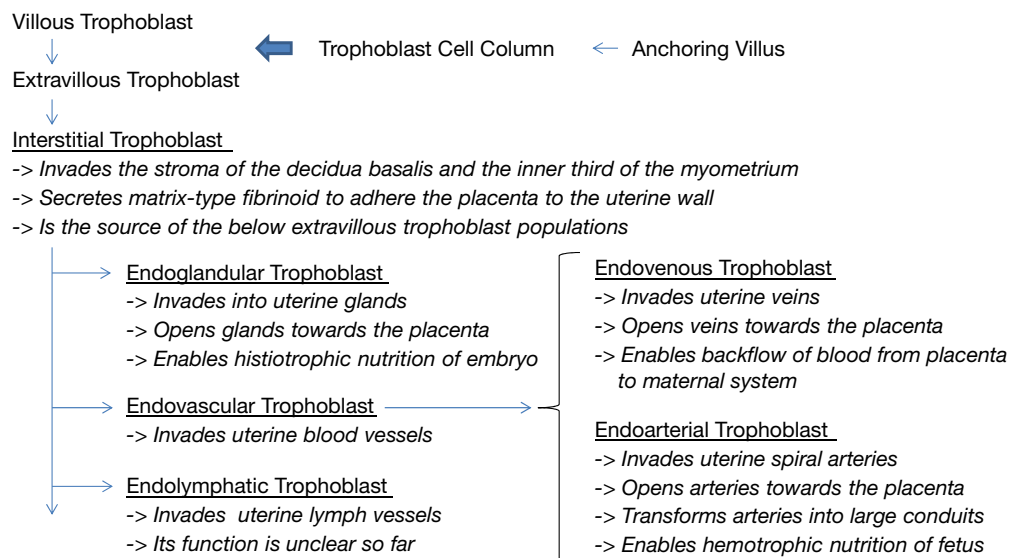


Fig. 4 Subtypes of extravillous trophoblast. Starting with the transformation of villous trophoblasts into extravillous trophoblasts within trophoblastic cell columns, various types of extravillous trophoblast populations can be described.

Small, Spindle-Shaped Cells

These small, spindle-shaped cells large, polygonal cells display small and oval nuclei, are long and slender and can be moderately stained for HLA-G and cytokeratin, while they are always negative for proliferation markers such as Ki-67. This phenotype secretes only small amounts of matrix-type fibrinoid and comes into direct contact to maternal cells. The above properties of the cells indicate that these cells are highly invasive.

In early pregnancy (weeks 9–12) about 55% of all interstitial trophoblasts belong to this phenotype. This portion decreases throughout gestation with 31% at 16–24 weeks and 11% at 31–39 weeks (Benirschke et al., 2006). This shift in phenotype again indicates reduction of trophoblast invasion during pregnancy.

Multinucleated Giant Cells

The difference compared to the above phenotypes is that this phenotype exhibits more than one and up to ten nuclei. These nuclei are irregularly shaped and display varying sizes. These giant cells are much larger than the other interstitial trophoblasts with diameters between 50 and 100 μm (Benirschke et al., 2006). The cells can only hardly be stained for HLA-G and cytokeratin, while they are again negative for proliferation markers such as Ki-67.

Although syncytial fusion of extravillous trophoblasts has never been observed, it is generally accepted that this phenotype is generated by fusion of mononucleated interstitial trophoblasts. This phenotype can mostly be found at the border between decidua and myometrium, while its portion is very low compared to the other phenotypes.

Endoglandular Trophoblast

During the last couple of years the picture of trophoblast invasion has changed dramatically. Until few years ago only invasion into spiral arteries has been the only accepted invasive route of trophoblasts into luminal structures. Today, it has become clear that all luminal structures of the placental bed are invaded by extravillous trophoblasts (Moser et al., 2010, 2015) (Fig. 4).

Already 15 years ago, secretion products of uterine glands have been described in the intervillous space of first trimester placentas (Burton et al., 2002). However, there was no description of any mechanism how these products reach the placenta as uterine glands normally open towards the uterine cavity. Only the description of a directed invasion of uterine glands by extravillous trophoblasts opened the way for a mechanistic correlation (Moser et al., 2010). Today it is clear that *endoglandular trophoblast* erodes and opens uterine glands towards the intervillous space, enabling histiotrophic nutrition of the embryo during the first trimester of pregnancy (Moser et al., 2015; Moser and Huppertz, 2017). Infiltration of uterine glands, displacement of glandular epithelial cells and invasion into the lumen of glands enable opening of the glands and directing them towards the intervillous space of the placenta (Moser and Huppertz, 2017) (Fig. 5).

Endovascular Trophoblast

Endovascular trophoblast is an old term that needs revision. So far it has been used for those extravillous trophoblasts invading into uterine spiral arteries as these vessels have been the only blood vessels where invasion has been described. Today it has become clear

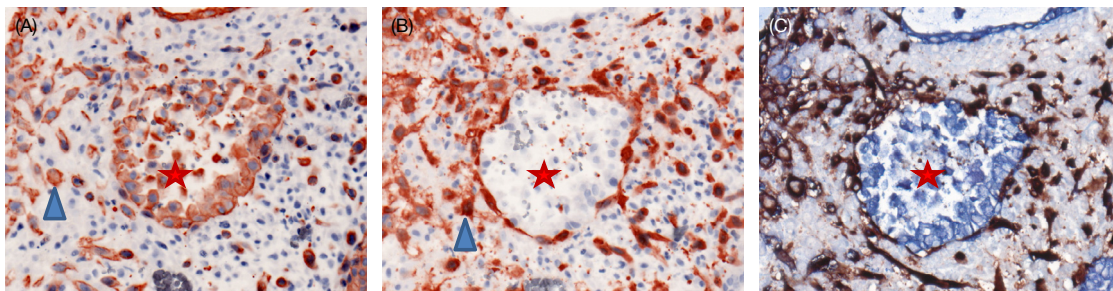


Fig. 5 Invasion of a uterine gland by endoglandular trophoblasts. The images show staining of extravillous trophoblasts for cytochrome 7 (A, C) and for HLA-G (B, C). (C) Shows double staining for both antigens. Staining for cytochrome 7 and HLA-G reveals staining of interstitial trophoblasts in the decidua (blue triangle in A, B) as well as staining of endoglandular trophoblasts invading the gland. Image (A) reveals that staining for cytochrome 7 does not enable to differentiate between staining of glandular epithelial cells and endoglandular trophoblasts, while this is easily possible with staining for HLA-G (B). The red star (A–C) displays the problems arising from single staining for cytochrome 7. The cytochrome 7 stain (A) suggests invasion into a spiral artery with plugging of this vessel by trophoblasts. Respective similar images have been published over the last centuries claiming invasion into spiral arteries. The HLA-G stain (B) reveals that the cells “plugging the vessel” are indeed not at all endovascular trophoblasts but rather detached glandular epithelial cells. The double stain (C) clearly identifies this structure as a uterine gland invaded by endoglandular trophoblasts. Original magnification: $\times 200$. Images courtesy of Dr. Gerit Moser, Medical University of Graz, Austria.

that not only uterine arteries but also uterine veins are invaded by extravillous trophoblasts. Hence, this part of the nomenclature needs revision (Moser and Huppertz, 2017).

Endoarterial Trophoblast

A specific population of extravillous trophoblasts invades towards uterine spiral arteries, percolates the walls of these vessels, displaces the endothelial lining and finally reaches the lumen of the arteries (Kaufmann et al., 2003). This *endoarterial trophoblast* erodes, opens and transforms uterine spiral arteries to enable hemotrophic nutrition during the second and third trimester of pregnancy.

The main function of endoarterial trophoblasts is not only opening of the spiral arteries but most of all their transformation into large conduits that have lost the maternal control on their contractility. It needs to be clarified that widening of these vessels takes place at the very end of the arteries towards the placenta.

Transformation of spiral arteries can be divided into three parts:

1. Maternal factors induce first changes of the uterine spiral arteries very early in pregnancy prior to trophoblasts reaching such vessels. The vessels show reduced organization of vascular smooth muscle cells, altered morphology of endothelial cells and first signs of dilation. It seems as if maternal immune cells trigger these early changes. They accumulate within the decidua in close vicinity to spiral arteries and play an active role in transforming these vessels (Benirschke et al., 2006).
2. After disorganization of the vessel wall and first slight dilation of the vessel, endoarterial trophoblasts percolate from the decidual stroma into the vessel wall. During the very first invasion endoarterial trophoblasts reach the lumen and form plugs to block blood flow towards the placenta (Weiss et al., 2016).
3. Later, endoarterial trophoblasts infiltrate deeper regions of the vessels up to the myometrial portion of the arteries. This results in a further dilation of the vessels, finally reaching a multiple of the original diameter. The reduced activity of smooth muscle cells plus the loss of elastic fibers in the vessel wall further contribute to the dilation effect (Benirschke et al., 2006).

Endovenous Trophoblast

Invasion of extravillous trophoblasts into uterine veins has been described for the first time only very recently by Moser et al., 2017. These authors termed this population of extravillous trophoblast *endovenous trophoblast*. This type of trophoblasts erodes and opens uterine veins within the placental bed to allow backflow of maternal blood from the placenta during pregnancy. This backflow is needed throughout pregnancy and covering histiotrophic nutrition of the embryo as well as hemotrophic nutrition of the fetus (Moser et al., 2017).

The identification of the endovenous trophoblast was already overdue. For decades, invasion of extravillous trophoblast was only accepted to take place into spiral arteries but no other luminal structure in the placental bed. However, if maternal blood flow from uterine arteries into the placenta is established at the beginning of the second trimester, there is the urgent need to have established a respective backflow of blood into the maternal vascular system. Hence, mechanistically, invasion of uterine veins was needed – but never described until 2017 (Moser et al., 2017; Windsperger et al., 2017).

While invasion of spiral arteries comprises invasion, opening and transformation including widening of the vessels, invasion into uterine veins only needs opening and no transformation. Since there is a plasma flow through the endoarterial plugs within the spiral arteries already during the first trimester of pregnancy (Jauniaux et al., 2000), backflow into the maternal system and thus endovenous trophoblast invasion needs to be established very early in pregnancy.

Endolymphatic Trophoblast

Very recently, also invasion into uterine lymph vessels has been described (Windsperger et al., 2017; He et al., 2017). The role of this invasion of *endolymphatic trophoblast* is not clear yet. Maybe, extravillous trophoblasts are much more invasive than thought of so far. They may simply erode and invade any luminal structure that can be found in their invasive pathway.

References

- Baergen, R. N. (2011). *Manual of pathology of the human placenta* (2nd edn.). Springer.
- Benirschke, K., Kaufmann, P., & Baergen, R. N. (2006). *Pathology of the human placenta* (5th edn.). Springer.
- Burton, G. J., Watson, A. L., Hempstock, J., Skepper, J. N., & Jauniaux, E. (2002). Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *Journal of Clinical Endocrinology and Metabolism*, 87, 2954–2959.
- Demir, R., Kayisli, U. A., Seval, Y., Celik-Ozenci, C., Korgun, E. T., Demir-Weusten, A. Y., & Huppertz, B. (2004). Sequential expression of VEGF and its receptors in human placental villi during very early pregnancy: Differences between placental vasculogenesis and angiogenesis. *Placenta*, 25, 560–572.
- Eaton, B. M., Leach, L., & Firth, J. A. (1993). Permeability of the fetal villous microvasculature in the isolated perfused term human placenta. *Journal of Physiology*, 463, 141–155.
- He, N., van Iperen, L., de Jong, D., Szuhai, K., Helmerhorst, F. M., van der Westerlaken, L. A. J., & Chuva de Sousa Lopes, S. M. (2017). Human extravillous trophoblasts penetrate decidual veins and lymphatics before remodeling spiral arteries during early pregnancy. *PLoS One*, 12, e0169849.
- Huppertz, B., Frank, H. G., Kingdom, J. C., Reister, F., & Kaufmann, P. (1998). Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. *Histochemistry and Cell Biology*, 110, 495–508.
- Huppertz, B. (2010). IFPA Award in Placentology Lecture: Biology of the placental syncytiotrophoblast—myths and facts. *Placenta*, 31(Suppl), S75–S81.
- Jauniaux, E., Watson, A. L., Hempstock, J., Bao, Y. P., Skepper, J. N., & Burton, G. J. (2000). Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *American Journal of Pathology*, 157, 2111–2122.
- Kaufmann, P., Black, S., & Huppertz, B. (2003). Endovascular trophoblast invasion: Implications for the pathogenesis of intrauterine growth retardation and preeclampsia. *Biology of Reproduction*, 69, 1–7.

- Kaufmann, P., Huppertz, B., & Frank, H. G. (1996). The fibrinoids of the human placenta: Origin, composition and functional relevance. *Annals of Anatomy*, 178, 485–501.
- Longtine, M. S., Chen, B., Odibo, A. O., Zhong, Y., & Nelson, D. M. (2012). Caspase-mediated apoptosis of trophoblasts in term human placental villi is restricted to cytotrophoblasts and absent from the multinucleated syncytiotrophoblast. *Reproduction*, 143, 107–121.
- Mayhew, T. M., Leach, L., McGee, R., Ismail, W. W., Myklebust, R., & Lammiman, M. J. (1999). Proliferation, differentiation and apoptosis in villous trophoblast at 13–41 weeks of gestation (including observations on annulate lamellae and nuclear pore complexes). *Placenta*, 20, 407–422.
- McMaster, M. T., Librach, C. L., Zhou, Y., Lim, K. H., Janatpour, M. J., DeMars, R., Kovats, S., Damsky, C., & Fisher, S. J. (1995). Human placental HLA-G expression is restricted to differentiated cytotrophoblasts. *Journal of Immunology Baltimore*, 154, 3771–3778.
- Moser, G., Gauster, M., Orendi, K., Glasner, A., Theuerkauf, R., & Huppertz, B. (2010). Endoglandular trophoblast, an alternative route of trophoblast invasion? Analysis with novel confrontation co-culture models. *Human Reproduction*, 25, 1127–1136.
- Moser, G., Weiss, G., Gauster, M., Sundl, M., & Huppertz, B. (2015). Evidence from the very beginning: Endoglandular trophoblasts penetrate and replace uterine glands in situ and in vitro. *Human Reproduction*, 30, 2747–2757.
- Moser, G., Weiss, G., Sundl, M., Gauster, M., Siwetz, M., Lang-Olrip, I., & Huppertz, B. (2017). Extravillous trophoblasts invade more than uterine arteries: Evidence for the invasion of uterine veins. *Histochemistry and Cell Biology*, 147, 353–366.
- Moser, G., & Huppertz, B. (2017). Implantation and extravillous trophoblast invasion: From rare archival specimens to modern biobanking. *Placenta*. <https://doi.org/10.1016/j.placenta.2017.02.007>.
- Sibley, C. P., Pardi, G., Cetin, I., Todros, T., Piccoli, E., Kaufmann, P., Huppertz, B., Bulfamante, G., Cribiu, F. M., Ayuk, P., Glazier, J., & Radaelli, T. (2002). Pathogenesis of intrauterine growth restriction (IUGR)—conclusions derived from a European Union Biomed 2 Concerted Action project 'Importance of Oxygen Supply in Intrauterine Growth Restricted Pregnancies'—a workshop report. *Placenta*, 23(Suppl A), S75–S79.
- Simmons, D. G., Fortier, A. L., & Cross, J. C. (2007). Diverse subtypes and developmental origins of trophoblast giant cells in the mouse placenta. *Developmental Biology*, 304, 567–578.
- Weiss, G., Sundl, M., Glasner, A., Huppertz, B., & Moser, G. (2016). The trophoblast plug during early pregnancy: A deeper insight. *Histochemistry and Cell Biology*, 146, 749–756.
- Windsperger, K., Dekan, S., Pils, S., Golletz, C., Kunihs, V., Fiala, C., Kristiansen, G., Knöfler, M., & Pollheimer, J. (2017). Extravillous trophoblast invasion of venous as well as lymphatic vessels is altered in idiopathic, recurrent, spontaneous abortions. *Human Reproduction*, 32, 1208–1217.

Placentation in the Mouse

Bryony V Natale and David RC Natale, University of California San Diego, La Jolla, CA, United States

© 2018 Elsevier Inc. All rights reserved.

General Introduction

Gestation in the mouse has a range of 19–22 days, with the fertilized one-cell zygote called embryonic day (E) 0.5, the first step of placentation beginning at ~E 4.0 and the functional placenta formed by E 10.5. Though strain dependent, the average litter size in the mouse is 10–12 pups, with an equal number of placentas. The placenta is essential to maintaining a healthy pregnancy and develops as a multipurpose organ. Its purposes include endocrine function that controls maternal adaptations to maintain pregnancy, facilitating nutrient, and oxygen delivery to the embryo/fetus as well as the removal of waste. Trophoblast cells are derived from the embryo and are unique to, and comprise most of the placenta. The mouse placenta, when fully developed has a discoid shape and is hemochorial, meaning that trophoblast cells come in direct contact with maternal blood. At the gross anatomical level, the mouse placenta is comprised of three layers—maternal decidua and two trophoblast-derived layers, the junctional zone (JZ) and labyrinth layer. The mouse, which is commonly used as a laboratory model, has contributed to our understanding of embryonic and placental development. The goal of this article is to review the current understanding of placentation in the mouse including basic development, structure, composition, function, and recent advances in experimental approaches used in the field.

Implantation

The mouse placenta is comprised of many differentiated subtypes of trophoblast cells, which have their own specific function and are restricted to specific placental layers. All trophoblast subtypes are derived from the trophoblast of the blastocyst. After the oocyte is fertilized, the one-cell zygote undergoes several rounds of cleavage divisions, progressing through the 2-, 4-, and 8-cell stages (Fig. 1). During this time, the cells are referred to as blastomeres and are totipotent, meaning that they have the potential to become placenta, yolk sac or embryo proper. It is at the 16-cell, morula stage that the first differentiation event occurs where the totipotent blastomeres diverge to become either trophoblast or inner cell mass (ICM), thereby, facilitating transition to the early blastocyst stage (Fig. 1). The trophoblast gives rise to the trophoblast components of both the placenta and the parietal yolk sac, while the ICM gives rise to the primitive endoderm that becomes the parietal and visceral yolk sacs and the epiblast, that becomes the embryo proper. The Hippo/Lats signaling pathway (Nishioka et al., 2009; Rayon et al., 2016) has been shown to be essential for trophoblast differentiation as it regulates expression of the transcription factors Tead4 and Cdx2, that are required for trophoblast specification. This is followed by a second differentiation event where the cells of the ICM become either epiblast or primitive endoderm (Fig. 1). The trophoblast is the first differentiated cell type of the mouse embryo. It is an epithelial cell type and forms a monolayer of cells that surrounds the embryo at the blastocyst stage. The portion that covers the ICM is called polar trophoblast, and will contribute to the placenta, while the remainder is referred to as the mural trophoblast and contributes to the yolk sac tissues. It is trophoblast that enables embryo implantation and triggers the uterine remodeling that leads to an increase in blood, oxygen, and nutrient supplies. The first step of placentation occurs at ~E 4.0, when the embryo hatches from the zona pellucida (ZP) and the maternal endometrial stromal tissue undergoes decidualization, forming decidua and

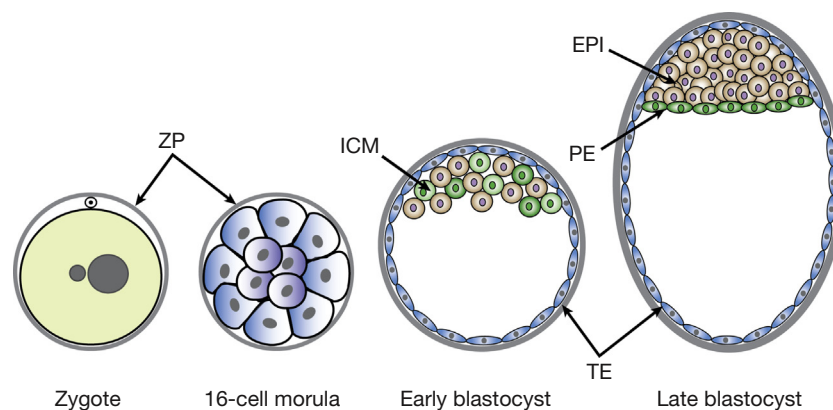


Fig. 1 The progression from zygote to blastocyst stage embryo during the first ~4 days of development. Following fertilization, the oocyte and sperm form a one-cell zygote. At the 16-cell morula stage, the first differentiation event occurs leading to specification of blastomeres to become either trophoblast (TE, blue) or inner cell mass (ICM) (purple). The second differentiation event begins at the blastocyst stage where ICM cells become either primitive endoderm (PE, green) or epiblast (EPI, brown). At the late blastocyst stage, just prior to implantation, the trophoblast that covers the ICM is called polar trophoblast, while the remaining TE is mural; the epiblast and primitive endoderm are defined and the blastocyst begins to “hatch” from its zona pellucida (ZP) to begin implantation.

allowing for implantation. The embryo implants (E 4.5) in a process governed by signaling pathways, including WNT signaling, between the trophoblast layer of the embryo and the maternal uterine endometrial epithelium, and through the physical interactions between the two, as the blastocysts attaches to the uterine epithelium. At implantation, the embryo is composed of the trophoblast cells that will contribute to the placenta, the primitive endoderm that will contribute to the yolk sac and the epiblast that will form the embryo proper. At this time, the mural trophoblast at the abembryonic pole stop proliferating and differentiate to form primary trophoblast giant cells (TGCs; Fig. 2A). Primary TGCs are the first terminally differentiated trophoblast subtype, and are highly polyploid due to endoreduplication. This modified cell cycle, in which DNA is continually replicated without any intervening mitosis, is a characteristic of TGCs and can result in DNA content as high as 1024N in these cells, which are also highly invasive and display phagocytic ability. Implantation occurs via the embryo's lateral sides, through uterine epithelial/TGC interaction as they attach to and proceed to invade the decidualizing uterine stroma.

Extraembryonic Ectoderm and Ectoplacental Cone

Following implantation, the polar trophoblast that overlie the ICM, proliferate on the inner side of the blastocyst to form the extraembryonic ectoderm (ExE). This growth then presses down on the epiblast of the ICM which stimulates development of the yolk sac and the proamniotic cavity (Fig. 2A). These proliferating cells are referred to as trophoblast stem (TS) cells and are dependent on fibroblast growth factor 4 (FGF4) (Murohashi et al., 2010; Rappolee et al., 1994) and Nodal (Guzman-Ayala et al., 2004) signaling from the developing embryo epiblast to remain undifferentiated and proliferative. This population is identifiable, histologically, by the expression of TS cell markers (*Cdx2*, *Esrrb*, *Eomes*). As the distance from the epiblast increases, the concentration of FGF4 decreases, causing TS cells to differentiate. These differentiating trophoblast cells form the ectoplacental cone, which as it grows, protrudes into the uterine lumen (Cross et al., 2006) and is considered the first sign of placental development. Both the ExE and ectoplacental cone can be identified in histological sections of the placenta at ~E 5.5–E 6.5. The ectoplacental cone is separated from the epiblast by the ExE and though the inner cells of the ectoplacental cone continue to proliferate and display characteristics of undifferentiated cells, these cells, are no longer TS cells, rather they are self-renewing progenitors that will give rise to the JZ-specific trophoblast lineage later in development. These cells can be identified by *Ascl2*, in histological sections. Simultaneously, the trophoblast cells of the ectoplacental cone that have move outward from the highly proliferative center of the ectoplacental cone and further from the source of FGF4, start to differentiate. These outer cells become bigger and more distant from each other with cytoplasmic projections that allow them to maintain contact with other cells. This process occurs as they become terminally differentiated, nonproliferating, polyploid, invasive secondary TGCs (Fig. 2B). These cells are called the Parietal TGC's and separate the embryonic tissue from the maternal decidua and endothelial cells. They play a role in mesometrial implantation, interaction with the maternal decidua, secretion of regulatory hormones and angiogenesis. TGCs are responsible for opening endometrial capillaries, allowing for blood to percolate in the spaces between the cells, thereby creating blood sinuses.

Chorioallantoic Attachment

Chorioallantoic attachment, marks the beginning of the morphological events that give rise to the fetal blood vessels of the placenta. This process begins, at ~E 7–E 7.5, when one side of the ExE folds within the proamniotic cavity (Fig. 2B) and joins with the ExE on

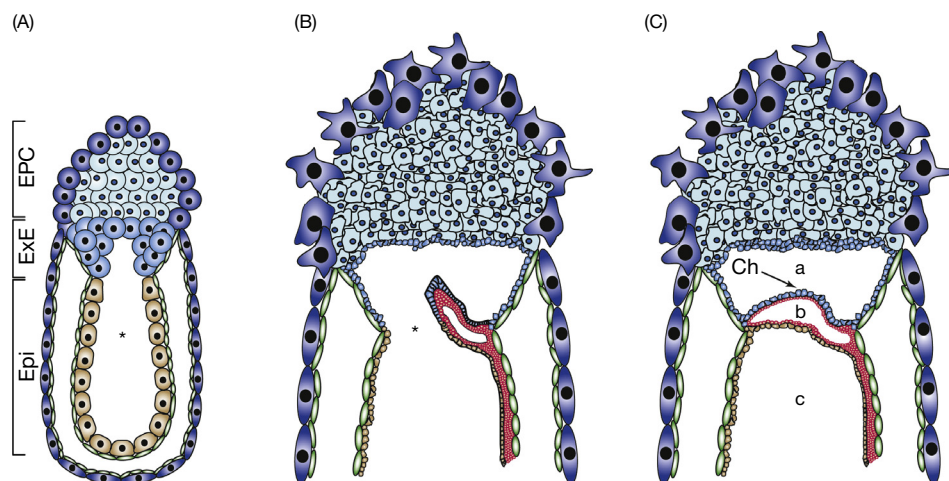


Fig. 2 The placental development after implantation, between ~E 6.0 and E 7.5. (A) Mural TE differentiate to primary TGCs (dark blue), polar TE forms extraembryonic ectoderm (ExE) which proliferates to expand ExE and also lead to formation of the EPC. The ExE and EPI surround the proamniotic cavity (*). (B) As development proceeds to ~E 7.0, the TGCs surrounding the EPC become larger and more spread out with cytoplasmic projections (dark blue). (C) By E 7–E 7.5, the chorion (Ch) has formed, leading to the division of the proamniotic cavity into the ectoplacental cavity (A), the exocoelom (B), and the amniotic cavity (C). The chorion is composed of a single layer of mesothelium (red) and a single layer of trophoblast, which include trophoblast stem (TS) cells (blue).

the opposite side, thus subdividing the proamniotic cavity and creating the ectoplacental cavity, which is in contact with the ectoplacental cone, and the amniotic cavity, which is in contact with the epiblast, from which the fetus is formed. As the ExE folds, a cavity is formed, thus creating a third space, the chorionic cavity or exocoelom (Fig. 2C). Trophoblast cells line the ectoplacental cavity whereas a layer of extraembryonic mesoderm cells derived from the embryo lines the exocoelom. As a result, the structure that separates the exocoelom from the ectoplacental cavity is composed two layers of cells, a single layer of trophoblast cells, derived from the ExE and a single layer of mesoderm, referred to as chorionic mesothelium. Together, these two layers of cells make up the chorion (Fig. 2C). At ~E 7.5–E 8.0, extraembryonic mesoderm of the exocoelom proliferates to form the allantois, which grows up from the posterior of the epiblast and contacts the basal surface of the chorion (Fig. 3A,B). This process is called chorioallantoic attachment and leads to the formation of the chorionic plate and is a critical event in the formation of the placenta as it precedes and triggers the differentiation of trophoblast cells that are required for the onset of branching morphogenesis. These events mark the beginning of labyrinth formation that leads to the development of the fetal vascular network of the placenta.

Following formation of the chorion and at the time of chorioallantoic attachment, the trophoblast stem cells of the ExE persist, however the closing of the ectoplacental cavity coincides with downregulation of trophoblast stem cell markers in chorionic trophoblast. The closing of the ectoplacental cavity brings the basal surface of the ectoplacental cone in contact with the central surface of the chorion and closes from the center toward the edges. The downregulation of trophoblast stem cell genes matches this pattern, such that by the time this is almost complete, they remain expressed only at the edges of the chorion, only where it is not yet in contact with the ectoplacental cone (Fig. 3B). Following closure of the ectoplacental cavity, the chorion, which was composed of a single layer of trophoblast and a layer of mesoderm cells, starts to expand through proliferation and some contribution from the ectoplacental cone, resulting in multiple layers of trophoblast cells (Fig. 3C).

Branching Morphogenesis and Development of the Labyrinth

The labyrinth layer is responsible for the nutrient, waste, and gas exchange between the mother and fetus and its development begins with the onset of branching morphogenesis that is triggered by chorioallantoic attachment. An important marker of the first step in this process is the expression of the transcription factor, *Gcm1*. Expression of this gene precedes chorioallantoic attachment as it is detectable at ~E 7.5. It is expressed in distinct clusters of 3–4 trophoblast cells that are evenly distributed across the basal layer

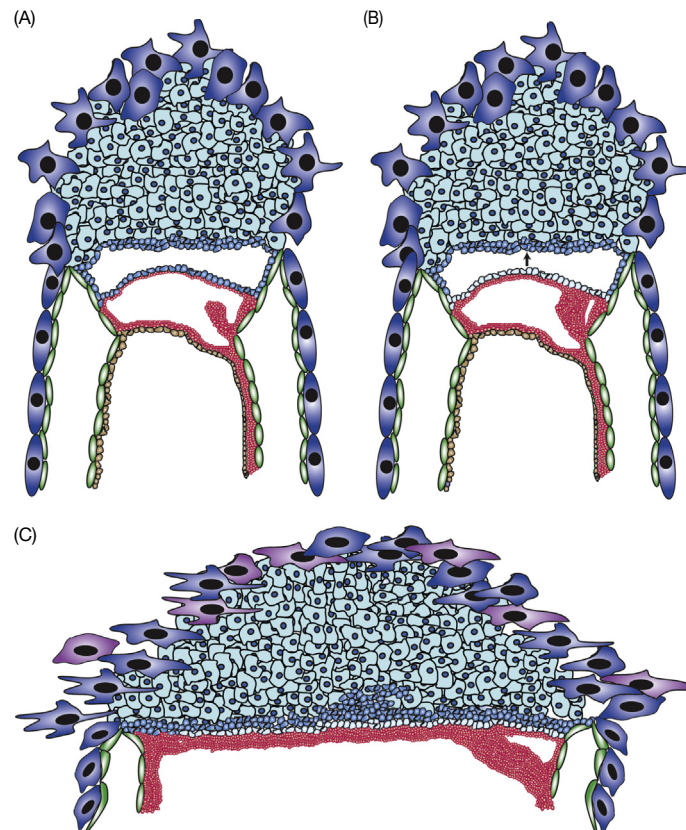


Fig. 3 The placental development between ~E 7.5 and E 8.0. (A) Following formation of the chorion, the allantois (mesoderm, red) begins to grow up and toward the basal surface of the chorion. (B) When the allantois contacts the chorion, it spreads across the basal surface. At this point, the ectoplacental cavity begins to close from the centre (arrow) and the chorionic trophoblast (white) contact the trophoblast of the basal surface of the EPC (royal blue, arrow). (C) After closure of the ectoplacental cavity, TS markers are downregulated, the chorion expands through proliferation in preparation for branching morphogenesis.

chorion and its expression is significant because it defines the future sites of branching morphogenesis (Fig. 4A). Following attachment of the allantois, trophoblast cells at these sites differentiate and invaginate toward the ectoplacental cone, thereby forming branch points from which the fetal capillary network will continue to develop (Anson-Cartwright et al., 2000) (Fig. 4A–E).

The mouse labyrinth is triepitheliochorial, meaning there are three distinct layers of differentiated trophoblast cells that separate maternal from fetal blood. Additionally, there is an endothelial cell layer that defines the fetal capillaries. These cells are organized with sinusoidal TGCs in direct contact with and lining the maternal blood spaces (MBS) and immediately adjacent to this layer, are two syncytiotrophoblast (SynT) layers, designated SynT-I and SynT-II, respectively (Fig. 5D(ii) and (iii), respectively). The final layer is endothelial cells that form the fetal capillaries and sit between SynT-II and fetal blood (Fig. 4C–D).

When branching morphogenesis begins, the chorion is composed of proliferating progenitor cells that are positioned to give rise to the organized structure of the mature labyrinth (Fig. 5A). As invagination of the trophoblast proceeds, fetal mesoderm from the

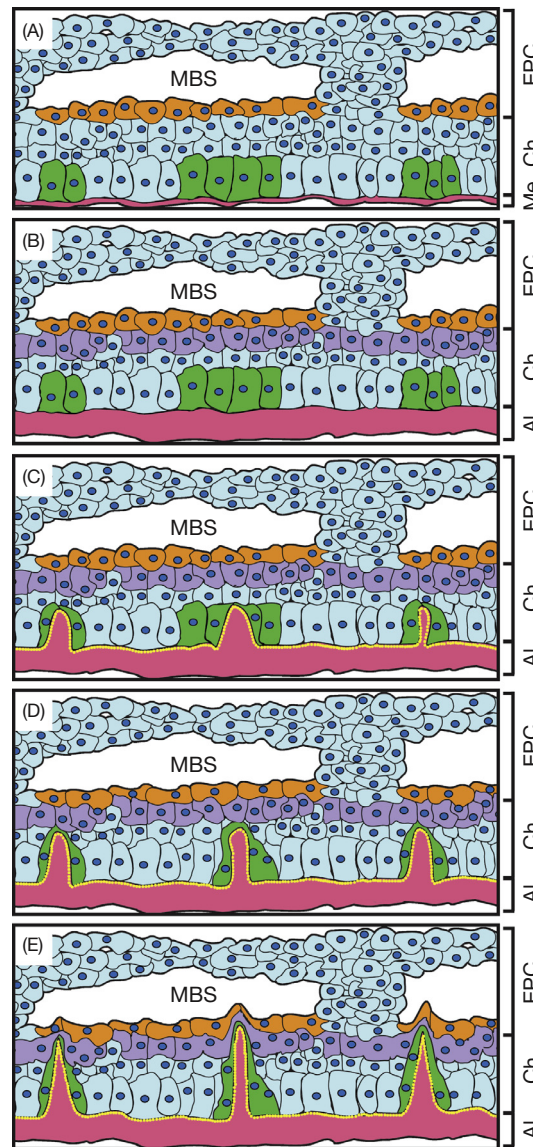


Fig. 4 The stages of the initiation of branching morphogenesis. (A) Prior to chorioallantoic attachment, subsets of cells in the chorion express the transcription factor *Gcm1* (green), which defines future branch points. Expression of *Hand1* identifies progenitors of the S-TGCs (orange) (B) At the time of chorioallantoic attachment, progenitors of the SynT-I cells can also be identified by the expression of specific marker genes (purple). (C) As branching is initiated, *Gcm1*⁺ trophoblast elongate and thin and invaginate toward the developing labyrinth. Mesoderm cells, including differentiating endothelial cells (yellow) move into these developing branches. (D) As the branches develop and endothelial cells interact with trophoblast, the *Gcm1*⁺ cells begin to fuse and differentiate into SynT-II. (E) Branches continue to grow, pushing toward the SynT-I and S-TGC progenitors (purple, orange, respectively). As the *Gcm1*⁺ cells contact the SynT-I cells, the SynT-I cells fuse along their lateral borders. These two cell layers continue to be pushed up, coming in contact with the overlying S-TGC progenitor cells and the branches continue to develop into maternal blood spaces (MBS). Me, mesothelium; Al, Allantois; Ch, chorion; EPC, ectoplacental cone.

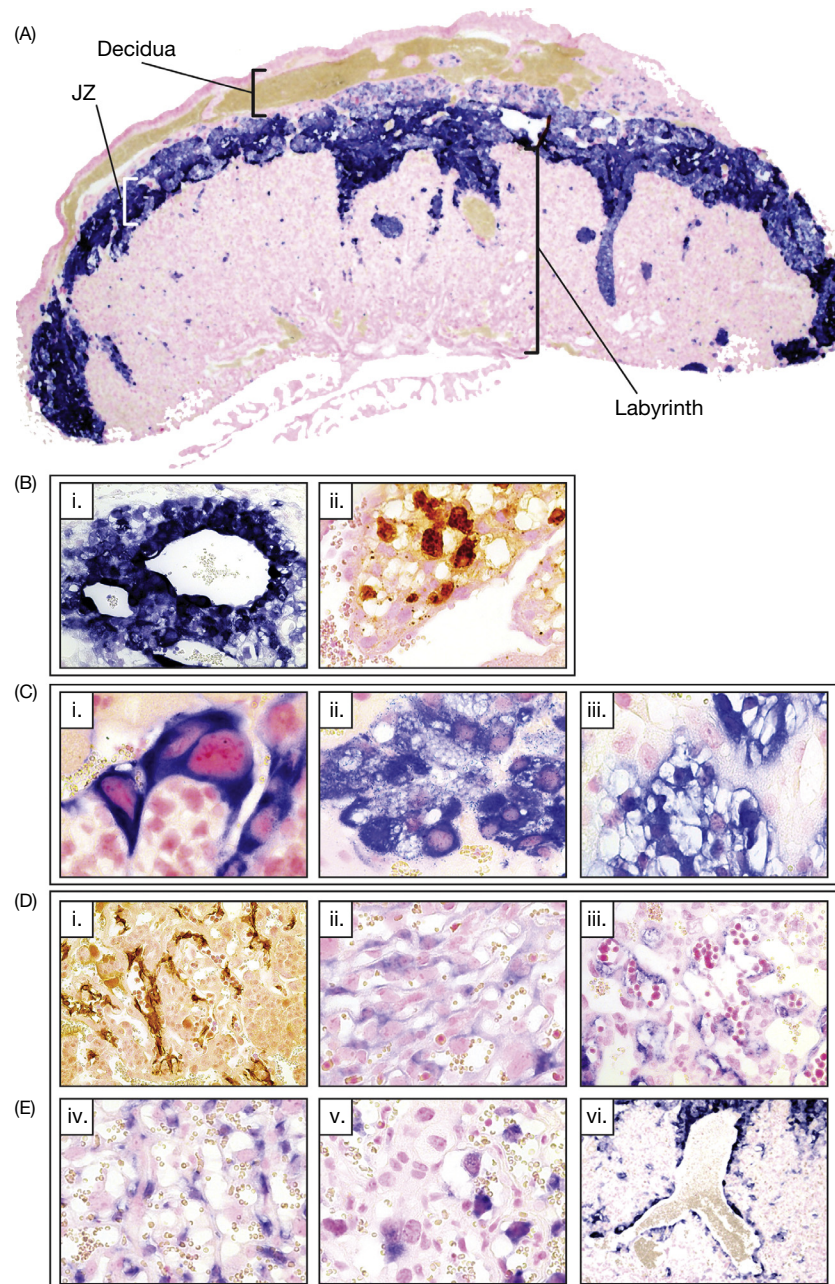


Fig. 5 A low magnification image of a histological section of the mature mouse placenta that highlights the gross morphology and below, high magnification images of individual trophoblast subtypes that comprise it. (A) The mouse placenta is composed of three layers; the decidua, the junctional zone (JZ), and the labyrinth. The JZ is highlighted by the expression of the gene *Tpbpa* (blue), which also identified glycogen trophoblast that have migrated into the decidua. (B) SpA-TGCs and maternal uterine natural killer (uNK) cells are found in the decidua surrounding maternal spiral arterioles (i, ii, respectively). (C) Differentiated trophoblast cells found in the JZ include S-TGCs (i), spongiotrophoblast (ii), and glycogen trophoblast (iii). (D) Many different cells contribute to the labyrinth layer including pericytes (i), SynT-I and -II (ii and iii, respectively), endothelial cells (iv), S-TGCs (v), and C-TGCs (vi). All of the trophoblast subtypes and maternal cells that are found in each layer can be identified by the expression of specific genes as shown here by in situ hybridization (B(i); C(i, ii, iii); D(ii, iii, iv, v, iv)) and/or immunohistochemistry (B ii, D i). Magnification: A, 40 ×; B(i), 400 ×; B(ii), 1600 ×; C(i, ii, iii), 1600 ×; D(i), 400 ×; D(ii, iii, iv, iv), 1600 ×; D(vi), 200 ×.

allantois fills in these branch points and development of fetal capillaries follows. *Gcm1*⁺ cells are the progenitors of the SynT-II layer. When fetal endothelial cells (Fig. 5D (iv)) come in direct contact with these predefined branch-points, the *Gcm1*⁺ cells differentiate into the SynT-II cells by thinning and fusing with each other, creating a multinucleated, continuous syncytium (Fig. 4A–D). This occurs after chorioallantoic attachment and the onset of branching. As the branch points elongate the SynT-II cells contact a layer of *SynA*⁺-SynT-I progenitors. The subsequent fusion of these SynT-I progenitors results in formation of the SynT-I layer (Fig. 4B–E). Immediately adjacent to the SynT-I progenitors/SynT-I layer, is the population of trophoblast cells that are

in direct contact with maternal blood sinuses (Fig. 4A–E). These trophoblast cells are proposed to be the progenitors of and differentiate into the S-TGCs (Fig. 5D(v)) and as branching morphogenesis continues, they become the third layer of trophoblast that separates maternal from fetal blood. *Hand1* is required for their differentiation. In addition to the three layers of differentiated trophoblast and the layer of fetal endothelial cells, there is a pericyte population that is derived from fetal mesoderm (Fig. 5D(i)). It is thought that their function is to help direct branching of the fetal capillaries. Interactions between S-TGCs, SynT, endothelial cells, and pericytes are important in fetal branching and for controlling the proliferation of endothelial cells, pericytes, and trophoblast progenitor cells that support the growth and development of the labyrinth. Although the pericyte/trophoblast and pericyte/endothelial relationships are not absolutely defined,

The basic structure of the mature placenta is formed by ~E 10.5 at which time, it is functional in its exchange of nutrients between maternal and fetal blood. During the period of labyrinth development from E 8.5 to E 12.5, growth and increases in cell number are supported by labyrinth-specific trophoblast progenitor cells, which are identified by high levels of expression of the cell adhesion molecule, *Epcam* (Ueno et al., 2013). *Epcam*-expressing trophoblast cells are localized to the chorionic plate and the associated lower regions of the labyrinth. The labyrinth of the placenta continues to grow through cell proliferation and increases in trophoblast number until ~E 12.5. After this time, markers of progenitor populations and proliferation of trophoblast cells are greatly reduced. Between E 12.5 and E 16.5, the volume of the labyrinth increases as the complexity of the vascular network increases. During this period, fetal endothelial cells continue to proliferate however trophoblast cells are thought to increase in size rather than number to support this growth.

Junctional Zone Expansion

The JZ is formed through the proliferation and differentiation of trophoblast cells in the ectoplacental cone. As the labyrinth forms, the cells of the ectoplacental cone proliferate to form a layer of differentiated trophoblast that sit between the labyrinth and the maternal decidua. In addition to blanketing the labyrinth, “fingers” of JZ (both spongiotrophoblast and glycogen trophoblast) are found reaching into the labyrinth (Fig. 5A), which are hypothesized to physically support the labyrinth and through paracrine interactions.

Found within the JZ, are five trophoblast subtypes: spongiotrophoblast, glycogen trophoblast, and the TGC populations, made up of parietal-TGCs, canal-TGCs or spiral artery-associated-TGCs (Fig. 5C(ii, iii, i), D(vi), B(i), respectively). Each of these three subtypes of TGC is distinct in their location within the placenta, the genes that they express and the time at which they differentiate. Based on these characteristics, they are proposed to have distinct functions. As previously noted, parietal-TGCs are the first TGC subtype to differentiate and as the placenta develops, they line the JZ on the decidua side, forming a border that separates the fetal-derived placental from the maternal decidua. The canal-TGCs are found lining the large blood canals that deliver maternal blood from the maternal spiral arterioles in the decidua, through the JZ and down through to the base of the labyrinth (Fig. 5D(vi)), while the spiral artery-associated-TGCs migrate into the decidua and associate with the maternal spiral arterioles (Fig. 5B(i)). The spiral artery-associated-TGCs play an important role in modifying the maternal spiral arterioles that deliver maternal blood to the placenta. Through interactions with maternal uterine natural killer (uNK) cells (Fig. 5B(ii)), remodeling of the extracellular matrix, smooth muscle, and endothelial cell layers occurs. This remodeling, results in low resistance vessels that are lined by spiral artery-associated-TGCs in the decidua, just proximal to their entry into the JZ. Spongiotrophoblast and glycogen trophoblast cells are found within the JZ in clusters and are clearly discernible by E 12.5. Spongiotrophoblast appear dense and compact with slightly larger nuclei than glycogen trophoblast whereas glycogen trophoblast, appear as clusters of “foamy” cells, with clearly identifiable nuclei and seemingly empty cytoplasm, due to the presence of large vacuoles storing glycogen, from which they derive their name. Spongiotrophoblast remain within the JZ and increase in number modestly over gestation, however glycogen trophoblast cells increase significantly in number followed by a migration out of the JZ and into the decidua (migration visible in Fig. 5A) to associate with maternal spiral arterioles. The purpose of this migration is unknown, however, between E 16.5 and E 18.5, the number of glycogen trophoblast are significantly decreased and it is proposed that they release their stored glycogen as energy to support the rapid increase in fetal growth during this time.

Blood Flow and Nutrient Transfer

For the placenta to function efficiently, the maternal and fetal blood must come in close approximation. Maternal blood is delivered to the placenta through the uterine artery to the maternal spiral arterioles; pass through the decidua and into the placenta. As described above, these spiral arterioles undergo remodeling that result in low resistance vessels, which are lined by spiral artery-associated-TGCs (Fig. 6). The spiral arterioles feed into large canals that deliver blood directly to the bottom of the labyrinth. These canals are lined by canal-TGCs. Maternal blood then percolates up through the labyrinth, coming in direct contact with the sinusoidal-TGCs that line the MBS in this layer. This is the site of nutrient and gas exchange. From these spaces, the now deoxygenated blood flows through small channels lined by channel-TGCs and into large lakes called lacunae that empty into uterine veins and returned to maternal circulation. These lakes are lined by parietal TGCs. From entry into the placenta, until exit, the maternal blood is in constant contact with one of five TGC subtypes, in order: spiral artery, canal, sinusoidal, channel, and parietal (Fig. 6). As a result, TGCs are ideally situated to secrete factors directly into maternal blood as it enters the placenta, while it is in the labyrinth and as it exits to return to maternal circulation. Therefore, these factors have the potential to act locally on

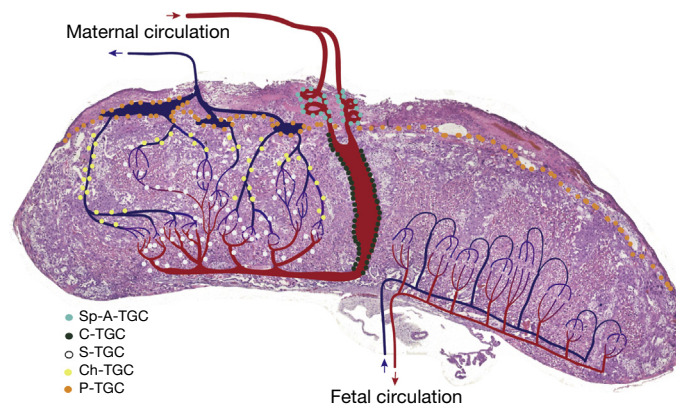


Fig. 6 A cartoon image depicting the maternal circulation (*on the left*) and the fetal circulation (*on the right*), over top of a developed mouse placenta. As maternal blood enters the placenta, the system is lined with trophoblast giant cells (TGC). The fetal system is restricted to the labyrinth layer. Maternal blood enters the placenta oxygenated (*red*) and nutrient rich, and leaves deoxygenated (*blue*), while fetal blood enters the placenta deoxygenated and leaves oxygenated and nutrient rich. *Sp-A-TGC*, Spiral Artery-TGC; *C-TGC*, Canal-TGC; *S-TGC*, Sinusoidal-TGC; *Ch-TGC*, Channel-TGC; *P-TGC*, Parietal-TGC.

trophoblast cells within the placenta, as well as in an endocrine manner by influencing maternal physiology through circulation in the maternal blood.

The deoxygenated fetal blood enters the placenta through the umbilical artery at the chorionic plate, where the vessel branches and blood is delivered to endothelial lined fetal capillaries within the labyrinth. Within the labyrinth layer, the structure and organization of fetal capillaries provides a massive surface area to facilitate nutrient and gas exchange. This is further enhanced by the close opposition of the maternal and fetal blood spaces, and the relative short distance across the four cell layers, which is referred to as the interhemal membrane. As gestation proceeds and the fetal capillary network of the labyrinth continues to develop, it becomes more complex and the interhemal membrane becomes thinner, measuring as small as 4–10 μm in places toward the end of gestation. This promotes nutrient transfer in two ways: (1) it provides an increased surface area and (2) a thinner interhemal membrane means a reduction in the distance separating maternal and fetal blood. Oxygen and CO_2 diffuse across the interhemal membrane freely and the uptake and transfer between maternal and fetal blood of amino acids, glucose and other molecules required for fetal growth and development, is facilitated by numerous transport proteins in the trophoblast cells. Fetal blood leaves the placenta (at the chorionic plate), through the umbilical vein in the umbilical cord to return oxygenated, nutrient rich blood to the fetus (**Fig. 6**).

The trophoblast cells of the labyrinth layer also act as a barrier to pathogens, drugs, and other molecules from the dam that may be detrimental to fetal health. Labyrinth trophoblast cells metabolize maternal glucocorticoids from their active to inactive form through the expression of 11 β HSD2 in the SynT. This activity is important to limit fetal exposure to glucocorticoids during gestation, which can cause growth restriction at early stages. Toward the end of pregnancy, glucocorticoid metabolism is altered, as increased levels are required for fetal lung development, prior to parturition.

Endocrine Function

In addition to nutrient/gas exchange, metabolism, and transport, the placenta plays a critical role as an endocrine organ during pregnancy. In this capacity, trophoblast cells produce a vast array of secreted factors that include: peptide and steroid hormones, growth factors, and cytokines (Evain-Brion, 1999; Soares et al., 1996; Solomon, 1988). Among their many functions, these factors act to: support and maintain pregnancy, modulate maternal physiology to adapt to pregnancy, control the onset of parturition and support embryonic/fetal development and growth. Mouse trophoblast do not make the human chorionic gonadotropin (hCG) that is seen in primate trophoblast. Instead the mouse TGCs, glycogen trophoblast, and spongiotrophoblast all express placental lactogens and prolactin-related proteins in cell-specific patterns throughout gestation. These proteins signal through the prolactin receptor and early in gestation, expression by TGCs acts to maintain progesterone production by the corpus luteum in the ovary which is essential for maintenance of pregnancy. Genes in this family have also been shown to regulate angiogenesis and likely have many as-of-yet unidentified roles throughout gestation.

Placentation as a Model for Human Diseases

The gross anatomy of the mouse and human placenta is quite different though functionally, they are very similar. Many of the genes and pathways that are important in mouse placental development and function, are conserved in the human. For this reason, studying placentation in the mouse has been very useful in understanding how human placental development is controlled, making it a popular model. Advantages of using the mouse include the knowledge of mouse genetics and the antibodies and experimental resources available as a result. The physiology of mice is well understood and they are relatively easy and inexpensive to obtain and

house, especially when compared to large animal or primate models. Mice have a short gestation, reach sexual maturity quickly and reproduce easily.

There are differences that do exist that should be considered, including the fact that in studying mouse pregnancy, there are multiple fetoplacental units which are linked by maternal blood flow and can therefore interact and influence each other. Additionally, maternal physiology is slightly different in litter-bearing species with respect to maternal adaptation to pregnancy. Finally, in the mouse, fetal development at the time of birth is immature with respect to human and therefore, placental function later in gestation may be slightly different. Despite these differences, the mouse is still a powerful model and perhaps most significantly, because the mouse genome can be targeted and using transgenic approaches, manipulated to misexpress, overexpress or eliminate completely, the expression of almost any gene.

Investigators have used transgenic approaches to manipulate the expression of targeted genes (i.e., knock out or knock in) to gain insight into their role in placental development or placental function later in gestation. At the time of writing, over 200 transgenic mouse models have been described with a phenotype related to placental development or function and thereby, detrimental to fetal health and growth. Advances in transgenic technologies have allowed experiments to be conducted in a cell-specific manner. These technologies include the Cre/LoxP approach, which enables experiments to be conducted in a cell-specific manner and/or with the ability to manipulate the timing of gene expression. The use of lentiviral vectors allows for the specific targeting of the trophoblast cells in the placenta, by exposing only the outer trophoctoderm layer of the embryo to nonreplicating lentiviral particles that contain either expression constructs or shRNAs to overexpress or knockdown expression of a specific gene.

Placental insufficiency can lead to fetal growth restriction or fetal demise and has been widely studied in the mouse. Both transgenic mouse and surgical models have been used to understand placental insufficiency and compromised placental environments. In the RUPP (reduced uteroplacental perfusion pressure) model, the uterine arteries are surgically ligated late in gestation to reduce blood flow to the fetoplacental units. This model, results reliably in fetal growth restriction and mimics symptoms of preeclampsia, including maternal hypertension, and proteinuria (Fushima et al., 2016). Other described models include: exposing pregnant mice to alcohol, cigarette smoke, hypoxia, environmental toxicants, altered diet (low calorie, high calorie, low protein, high fat), maternal diabetes, and have been shown to influence placental function and/or development as well as fetal growth. Identifying both the similarities and the differences in the altered placental function or fetal growth allows for a better understanding of placental development and function as it relates to placental pathologies. Through the use and study of mouse models, scientists gain an understanding of human placental pathologies that cannot be investigated in the same depth and detail through the study of human tissue. In conclusion, understanding mouse placentation is not only important for the purpose of placenta biology, but rather should be additionally considered in all mouse models that include genetic manipulation, as poor placental health or compromised placental circulation may affect the developing fetus. In addition to the immediate health impact on the fetus, the placenta environment is considered a factor in fetal programming of adult health, and thus a placental assessment should be included in all phenotypic assessment of mouse transgenic models.

References

- Anson-Cartwright, L., Dawson, K., Holmyard, D., Fisher, S. J., Lazzarini, R. A., & Cross, J. C. (2000). The glial cells missing-1 protein is essential for branching morphogenesis in the chorioallantoic placenta. *Nature Genetics*, 25, 311–314.
- Cross, J. C., Nakano, H., Natale, D. R. C., Simmons, D. G., & Watson, E. D. (2006). Branching morphogenesis during development of placental villi. *Differentiation*, 74(7), 393–401.
- Evain-Brion, D. (1999). Maternal endocrine adaptations to placental hormones in humans. *Acta Paediatrica. Supplement*, 88(428), 12–16.
- Fushima, T., Sekimoto, A., Minato, T., Ito, T., Oe, Y., Kisu, K., Sato, E., Funamoto, K., Hayase, T., Kimura, Y., Ito, S., Sato, H., & Takahashi, N. (2016). Reduced uterine perfusion pressure (RUPP) model of preeclampsia in mice. *PLoS One*, 11(5), e0155426.
- Guzman-Ayala, M., Ben-Haim, N., Beck, S., & Constam, D. B. (2004). Nodal protein processing and fibroblast growth factor 4 synergize to maintain a trophoblast stem cell microenvironment. *Proceedings of the National Academy of Sciences of the United States of America*, 101(44), 15656–15660.
- Murohashi, M., Nakamura, T., Tanaka, S., Ichise, T., Yoshida, N., Yamamoto, T., Shibuya, M., Schlessinger, J., & Gotoh, N. (2010). An FGF4-FRS2alpha-Cdx2 axis in trophoblast stem cells induces Bmp4 to regulate proper growth of early mouse embryos. *Stem Cells*, 28(1), 113–121.
- Nishioka, N., Inoue, K.-i., Adachi, K., Kiyonari, H., Ota, M., Ralston, A., Yabuta, N., Hirahara, S., Stephenson, R. O., Ogonuki, N., Makita, R., Kurihara, H., Morin-Kensicki, E. M., Nojima, H., Rossant, J., Nakao, K., Niwa, H., & Sasaki, H. (2009). The hippo signaling pathway components lats and yap pattern Tead4 activity to distinguish mouse trophoctoderm from inner cell mass. *Developmental Cell*, 16(3), 398–410.
- Rappolee, D. A., Basilico, C., Patel, Y., & Werb, Z. (1994). Expression and function of FGF-4 in peri-implantation development in mouse embryos. *Development*, 120, 2259–2269.
- Rayon, T., Menchero, S., Rollan, I., Ors, I., Helness, A., Crespo, M., Nieto, A., Azuara, V., Rossant, J., & Manzanares, M. (2016). Distinct mechanisms regulate Cdx2 expression in the blastocyst and in trophoblast stem cells. *Scientific Reports*, 6, 27139.
- Soares, M. J., Chapman, B. M., Rasmussen, C. A., Dai, G., Kamei, T., & Orwig, K. E. (1996). Differentiation of trophoblast endocrine cells. *Placenta*, 17, 277–289.
- Soloman, S. (1988). The placenta as an endocrine organ. In E. Knobil, & J. Neill (Eds.), *The Physiology of Reproduction* (pp. 2085–2091). New York: Raven Press.
- Ueno, M., Lee, L. K., Chhabra, A., Kim, Y. J., Sasidharan, R., Van Handel, B., Wang, Y., Kamata, M., Kamran, P., Sereti, K.-I., Ardehali, R., Jiang, M., & Mikkola, H. K. A. (2013). C-met-dependent multipotent labyrinth trophoblast progenitors establish placental exchange interface. *Developmental Cell*, 27(4), 373–386.

Domestic Animal Placentation

Gregory A Johnson, Texas A&M University, College Station, Texas, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Pregnancies in the livestock species, here represented by pigs, sheep and cows, share the key events of early embryo development, conceptus (embryo/fetus and associated placental membranes) elongation, pregnancy recognition, implantation, and establishment of functional placentation.

The early stages of embryo development in pigs, sheep and cows proceed in a manner similar to other mammalian species (Fig. 1). After fertilization within the oviduct, the zygote undergoes the first cleavage division to form the 2-cell embryo, and cleavage divisions continue through the 8–16 cell stage, when transcriptome activation occurs. These divisions culminate in formation of the solid mass of cells, called the morula, that remains encased in the zona pellucida of the original egg. Embryos remain in the oviduct before entering the uterus at 60–72 h in pigs, 72 h in ewes, and 72–96 h in cows. Embryos of most species fail to develop beyond the early blastocyst stage if confined to the oviduct, due perhaps to the absence of critical factors required for embryonic development that are supplied by the uterus. The developing embryo next forms the blastocyst. At this point the pluripotent blastomeres begin to differentiate into the inner cell mass (ICM) and trophoblast to begin the cell lineages that will eventually become the embryo/fetus proper (primitive ectoderm, mesoderm and endoderm), and the placental chorion, respectively, and are collectively termed the conceptus (Bazer et al., 2005).

The blastocyst hatches out of the zona pellucida and increases in size before undergoing a rapid morphological transition called elongation (Fig. 1). The small spherical conceptus grows into a long tubular form with the trophoblast and endoderm cells migrating towards the ICM to form the elongation zone. The tubular conceptus then begins to increase in length and decrease in diameter due to the migration of trophoblast and endoderm cells towards the ends of the conceptus to produce the filamentous form. Conceptuses elongate from spherical to filamentous forms primarily due to trophoblast remodeling. The next phase of growth is very rapid and is dependent primarily on cell proliferation to form the mature filamentous conceptus. During the early elongation period, the conceptus remains free-floating and dependent on the uterus for nutrients. Conceptus elongation provides increased surface area for nutrient exchange between the conceptus and uterus and helps inhibit luteolysis for pregnancy recognition (Bazer and Johnson, 2014).

The peri-implantation conceptuses of pigs, sheep, and cows secrete pregnancy recognition signals that act in a paracrine manner on the uterus to exert antiluteolytic effects that prevent uterine release of luteolytic prostaglandin $F_{2\alpha}$ (PGF) to maintain the corpus luteum for production of progesterone, the hormone of pregnancy (Fig. 2). This event is critical for a successful pregnancy. During the period of pregnancy recognition, progesterone down-regulates expression of progesterone receptors in the uterine luminal (LE) and glandular epithelium (GE). The loss of progesterone receptor expression by these epithelia appears to be prerequisite for progesterone to stimulate production and secretion of histotroph, a mixture of hormones, growth factors, nutrients, and other substances required for growth and development of the conceptus and implantation (Bazer et al., 2012).

The process of implantation is highly synchronized, requiring reciprocal secretory and physical interactions between a developmentally competent conceptus and the uterus during a restricted period of the uterine cycle termed the “window of receptivity.” The initial interactions between apical surfaces of uterine LE and trophoblast begin with sequential phases i.e., nonadhesive or precontact, apposition, and adhesion, and conclude with formation of a placenta that supports fetal-placental development throughout pregnancy (Fig. 3). Conceptus attachment first requires loss of antiadhesive molecules in the glycocalyx of uterine LE, comprised largely of mucins that sterically inhibit attachment. This results in “unmasking” of molecules, including selectins and galectins, which contribute to initial attachment of trophoblast to uterine LE. These low affinity contacts are then replaced by a repertoire of adhesive interactions between integrins and maternal extracellular matrix (ECM) molecules that appear to be the dominant contributors to stable adhesion at implantation. Implantation can be defined as the beginning of placentation (Johnson et al., 2014).

Considerable variability exists relative to histogenesis and organization of the placenta; however, trophoblast interactions with maternal uterus remain extensive in all species studied, and this allows for the close juxtaposition of the microcirculatory systems of the uterus and placenta for the transport of nutrients from the mother to the embryo/fetus. Pigs have a true epitheliochorial placenta in which there is no displacement or invasion of the maternal uterine tissues. Ruminants have synepitheliochorial placentae in which binucleate trophoblast cells migrate, invade, and fuse with uterine LE to form multinucleated syncytia in aglandular areas of the uterus where placentomes form. In both epitheliochorial and synepitheliochorial placentation, the conceptus remains within the uterine lumen throughout gestation (Bazer et al., 2012).

Early Pregnancy, Implantation, and Placentation in Pigs

The pig embryo moves from the oviduct into the uterus 60–72 h after onset of estrus, and reaches the blastocyst stage by day 5. The spherical 0.5–1 mm diameter blastocyst sheds the zona pellucida between days 6 and 7 and expands to a 2–6 mm diameter by day 10. At this stage development of pig embryos diverges dramatically from rodents or primates, and the presumptive placental

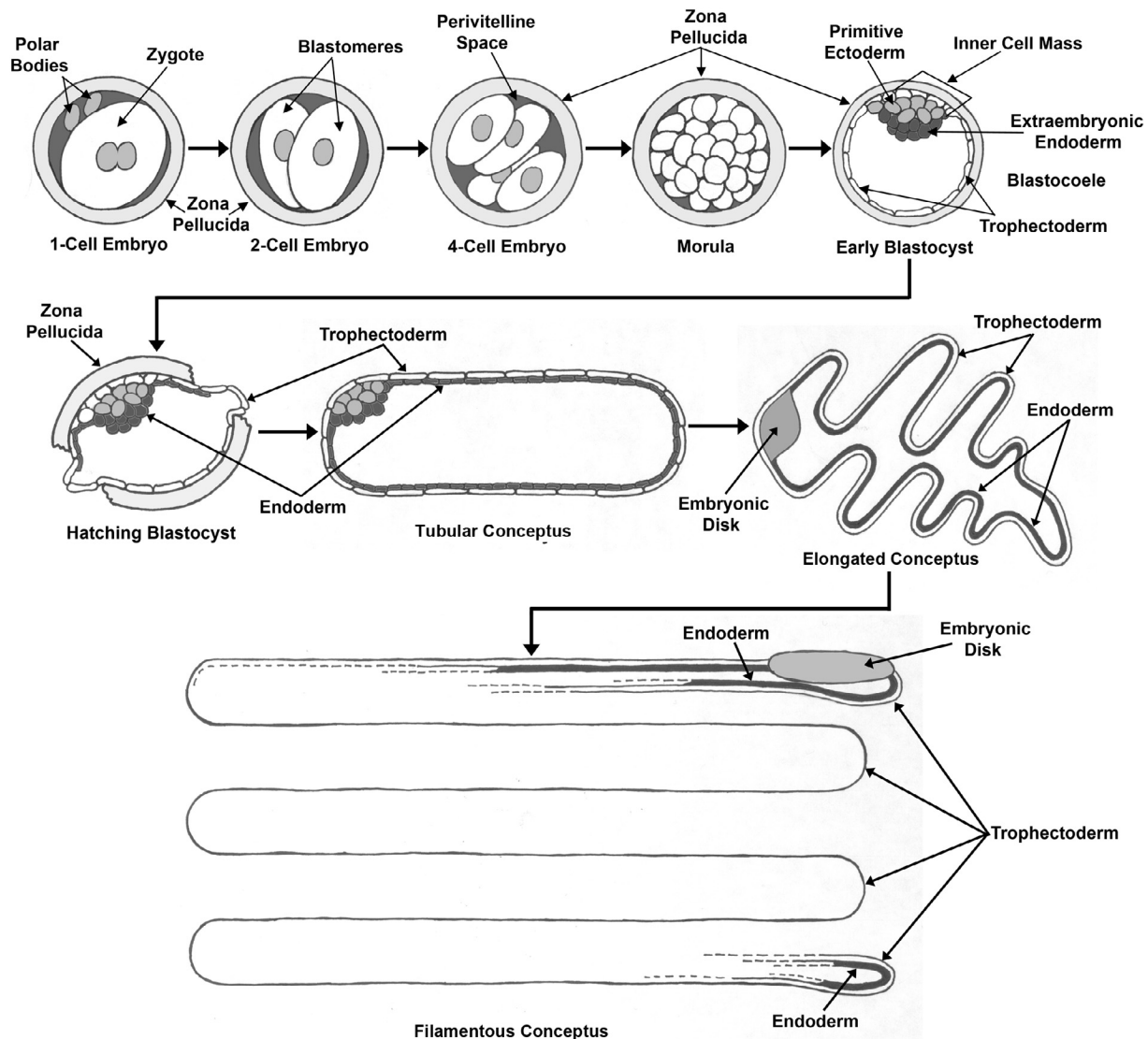


Fig. 1 Early embryonic development generalized for pigs, sheep and cows. Adapted from Bazer, F. W., Johnson, G. A., and Spencer, T. E. (2005). Growth and development: Pre-implantation embryo. In: *Encyclopedia of animal science*, (eds.) Pond, W. G. and Bell, A. W. NY.: Marcel Dekker, Inc., Vol. 1, No. 11–13, pp. 1–3.

membranes (trophoblast and endoderm) elongate rapidly to a filamentous form by day 16. Blastocysts expand at about 0.25 mm/h from the early spherical blastocyst stage to the 4–9 mm diameter spherical blastocyst stage. Then a remarkable increase in the rate of elongation to 30–45 mm/h from the 10 mm blastocyst to the 150–200 mm long filamentous conceptus occurs within a few hours. A dense band of cells (the elongation zone) composed of both endoderm and trophoblast extends from the ICM to the tip of the ovoid blastocyst on day 10. After formation of the elongation zone, there is further rapid elongation of the 100–200 mm long conceptus to a conceptus of 800–1000 mm in length by day 16 of pregnancy mediated through alterations in microfilaments and junctional complexes of trophoblast cells and formation of filopodia by endodermal cells. This last period of elongation involves cellular hyperplasia and each conceptus within the litter achieves maximum surface area for contact between trophoblast and uterine LE to facilitate uptake of nutrients from uterine LE and uterine GE, which increase coincidentally with elongation of the conceptuses (Bazer and Johnson, 2014).

Available evidence indicates estradiol is the pregnancy recognition signal in pigs. Estradiol, produced by day 10–12 pig conceptuses, redirects uterine LE secretion of PGF. In the absence of estradiol, PGF is secreted into the uterine venous drainage (endocrine secretion), where it has access to the corpus luteum and causes luteolysis. In the presence of estradiol, PGF is secreted into the uterine lumen, where it may be converted to prostaglandin E2 or to its inactive metabolite, 13,14-dihydro-15-keto-PGF, by the uterine endometrium and/or conceptus. The result is maintenance of progesterone production by the corpus luteum, and progesterone then down-regulates progesterone receptor in the uterine LE by day 10 and in the uterine GE by day 12 of pregnancy. A major role for both estradiol and progesterone is to stimulate the production and secretion of histotroph that supports conceptus

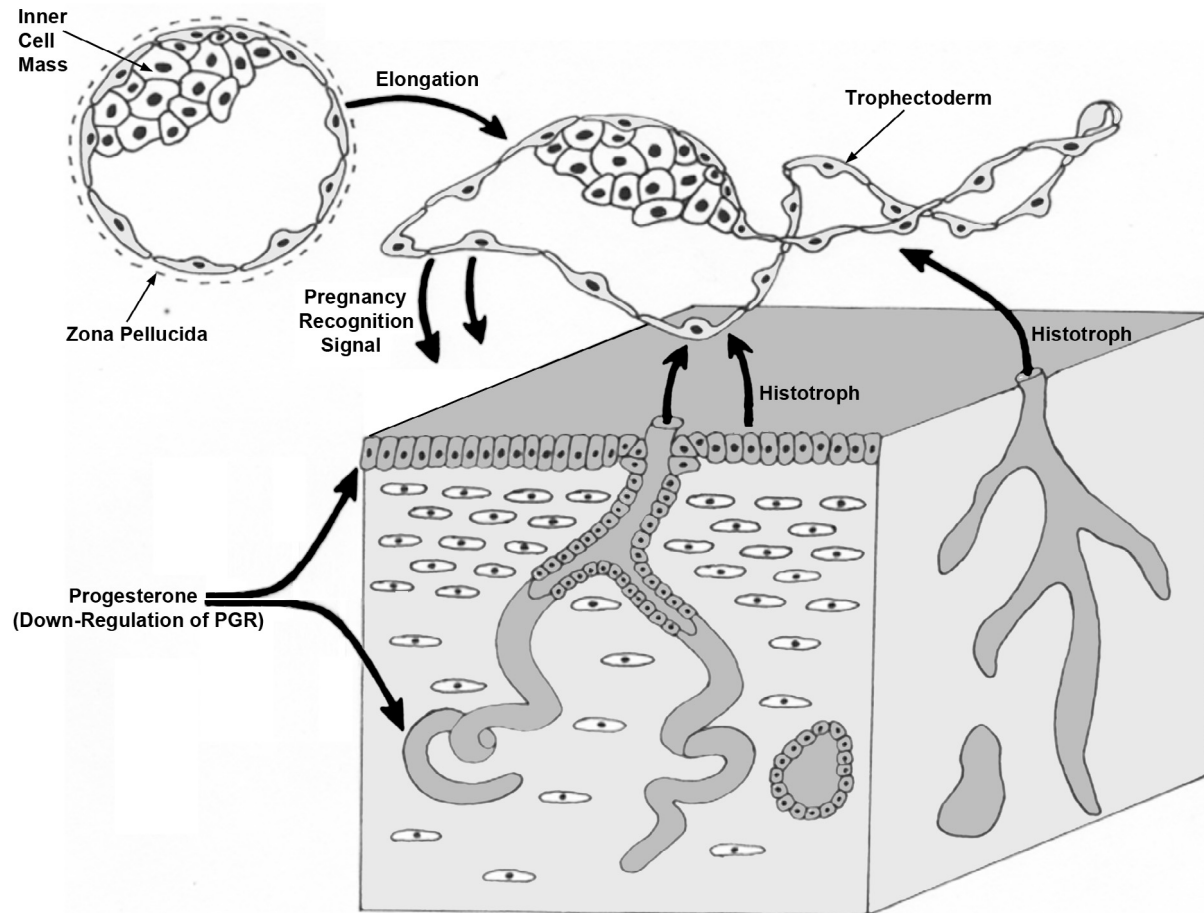


Fig. 2 Pregnancy recognition and production of histotroph generalized for pigs, sheep and cows. Adapted from Spencer, T. E., Johnson, G. A., Bazer, F. W., and Burghardt, R. C. (2004). Implantation mechanisms: Insights from the sheep. *Reproduction* 128:656–668.

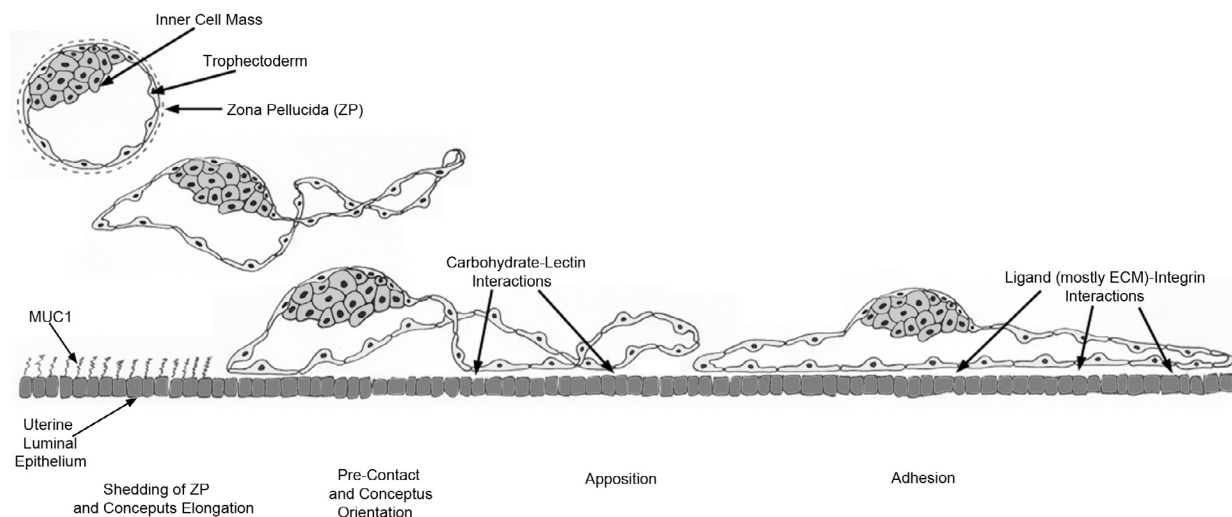


Fig. 3 Implantation cascade generalized for pigs, sheep and cows. Adapted from Geisert, R. D., Johnson, G. A., and Burghardt, R. C. (2015). Implantation and establishment of pregnancy in the pig. In: Regulation of implantation and establishment of pregnancy in mammals: Tribute to 45 year anniversary of Roger V. Short's "Maternal Recognition of Pregnancy". (eds.) Geisert, R. D. and Bazer, F. W. pp. 137–164.

development and implantation. Histotroph secretion is coincident with down-regulation of progesterone receptors in LE, and estradiol and progesterone induce expression and secretion of histotroph from the uterine LE and GE, respectively (Bazer and Johnson, 2014).

The term “implantation” is somewhat a misnomer for the pig, but nevertheless, it is used to describe the initial stages of placentation in the livestock species. Pigs demonstrate true epitheliochorial placentation in which there is no displacement or invasion of the maternal tissues and the conceptus remains within the uterine lumen throughout gestation. Throughout implantation, days 13–26 of pregnancy in pigs, the glycocalyx that extends from the apical surface of the uterine LE is thicker than the glycocalyx at the surface of trophoblast. On days 13 and 14 the uterine LE develops protrusions that become enclosed by caps of trophoblast cells that serve to physically immobilize the conceptus, and by day 14 there is close apposition between the apical plasma membranes of trophoblast and uterine LE cells. Interdigitating microvilli form between these plasma membranes on days 15 and 16, and then the interface becomes increasingly complex over days 15–20. This transition is characterized by the development of apical domes on the LE that are closely related to the trophoblast, and provide long cytoplasmic extensions into a luminal space between the apical domes. Finally, adhesion transitions into placentation through ever-increasing development of interdigitating microvilli between the uterine LE and trophoblast by day 26 of gestation (Dantzer 1985).

In epitheliochorial placentation, separation of fetal and maternal blood is always maintained by an elaborate array of endometrial and extraembryonic fetal tissues that represent a potential barrier to hemotrophic (nutrients carried in the blood) nutrient transport from the mother to fetus. Thus, the interhaemal distance in pigs must be minimized. This is initially accomplished through degradation of much of the connective tissue separating the uterine LE and trophoblast/chorion (referred to as trophoblast throughout this paragraph) from their underlying capillary beds resulting in the blood vessels actually indenting into the basal surfaces of uterine LE and trophoblast cells. Surface area of contact between the uterus and placenta is increased through interdigitation of microvilli between uterine LE and trophoblast. However, between day 25 and day 30, extensive remodeling of the uterine-placental interface to form chorionic (placental) ridges and corresponding endometrial invaginations results in folding that further increases the area of uterine-placental association across the entire placenta, except at the openings of uterine glands (Fig. 4). In addition to having uterine LE closely apposed to the trophoblast, there are specialized chorionic epithelial cells at the openings of the mouths of uterine glands where the trophoblast never fuses with the uterine LE, but forms a pocket referred to as an areola. Histotroph from the uterine GE is delivered into these areolae and is absorbed and transported across the chorion by fluid

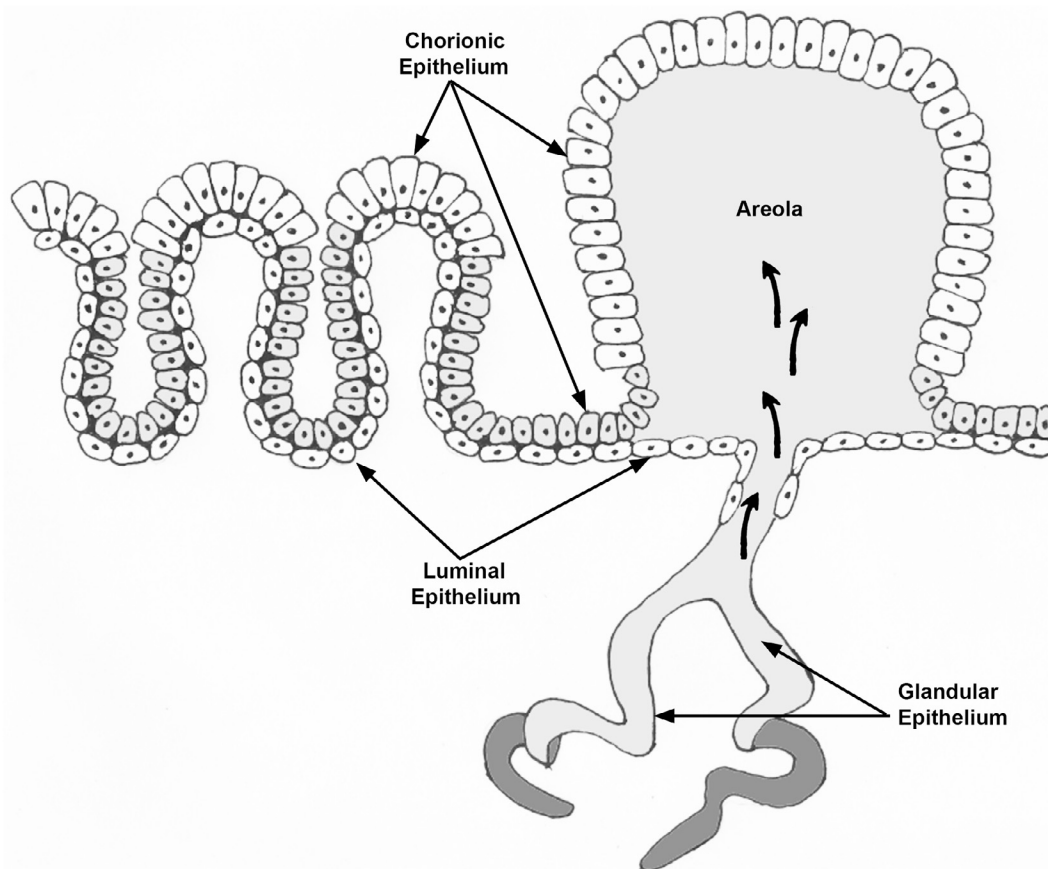


Fig. 4 The uterine-placental interface of mature placentation in the pig illustrating folding for hemotrophic and an areola for histotrophic support of the fetus.

phase pinocytosis for release into the placental circulation. The placenta of each piglet in a litter has about 2500 areolae and their number correlates with fetal weight. The trophoctoderm cells that line the areolae and line the tops of the placental ridges of the folds are tall columnar epithelia, well suited for transport of nutrients across the epithelial barrier (Song et al., 2010).

Early Pregnancy, Implantation, and Placentation in Sheep and Cows

Sheep and cows share many features of early embryonic development and implantation with pigs. The morula enters the uterus on day 4 postfertilization in sheep (days 4–6 in cows), and the blastocyst is formed by day 6. Before sheep and cow blastocysts develop into a conceptus, they “hatch” from the zona pellucida on days 6–7 or 9–10, respectively. Sheep blastocysts are spherical on day 4 (0.14 mm) and day 10 (0.4 mm), elongate to the filamentous form between days 12 (33 mm) and 15 (150–190 mm), and extend through the uterine body into the contralateral uterine horn by days 16–17 of pregnancy. Cow blastocysts are spherical on days 12–14 (2 mm on day 13), elongate to 60 mm by day 16, and reach 200 mm by day 19. In sheep, the filamentous conceptus is closely associated with the uterine LE and appears to be immobilized within the uterine lumen by day 14, although the conceptus can still be recovered intact from the uterus by lavage with only superficial damage. Apposition begins near the inner cell mass, and spreads towards the ends of the elongated conceptus, and by day 16 (sheep) and day 19 (cows), the trophoctoderm is firmly attached to the uterine LE with significant interdigitation between the microvilli on uterine LE and trophoctoderm cells. As sheep and cow conceptuses elongate and attach to the uterine wall, they synthesize and secrete interferon (IFN) τ , the signal for maternal recognition of pregnancy, from day 10 to day 21 in sheep, peaking on day 14 in sheep and day 20 in cows. IFN τ acts on the uterine LE and superficial GE to inhibit transcription of the estrogen receptor α gene. This precludes estrogen receptor α from stimulating an increase in oxytocin receptors in uterine LE, thereby preventing oxytocin from inducing release of luteolytic pulses of PGF. The result is maintenance of the corpus luteum, the source of progesterone required for the production of histotroph for a successful pregnancy (Spencer and Hansen, 2015).

In contrast to the pig, sheep and cows demonstrate synepitheliochorial placentation in which limited fusion of trophoctoderm with uterine LE occurs. Two morphologically and functionally distinct cell types, mononucleate trophoctoderm cells and binucleate trophoblast giant cells (BNCs), are present in the trophoctoderm of ruminant placentae (Fig. 5). The mononucleate cells constitute

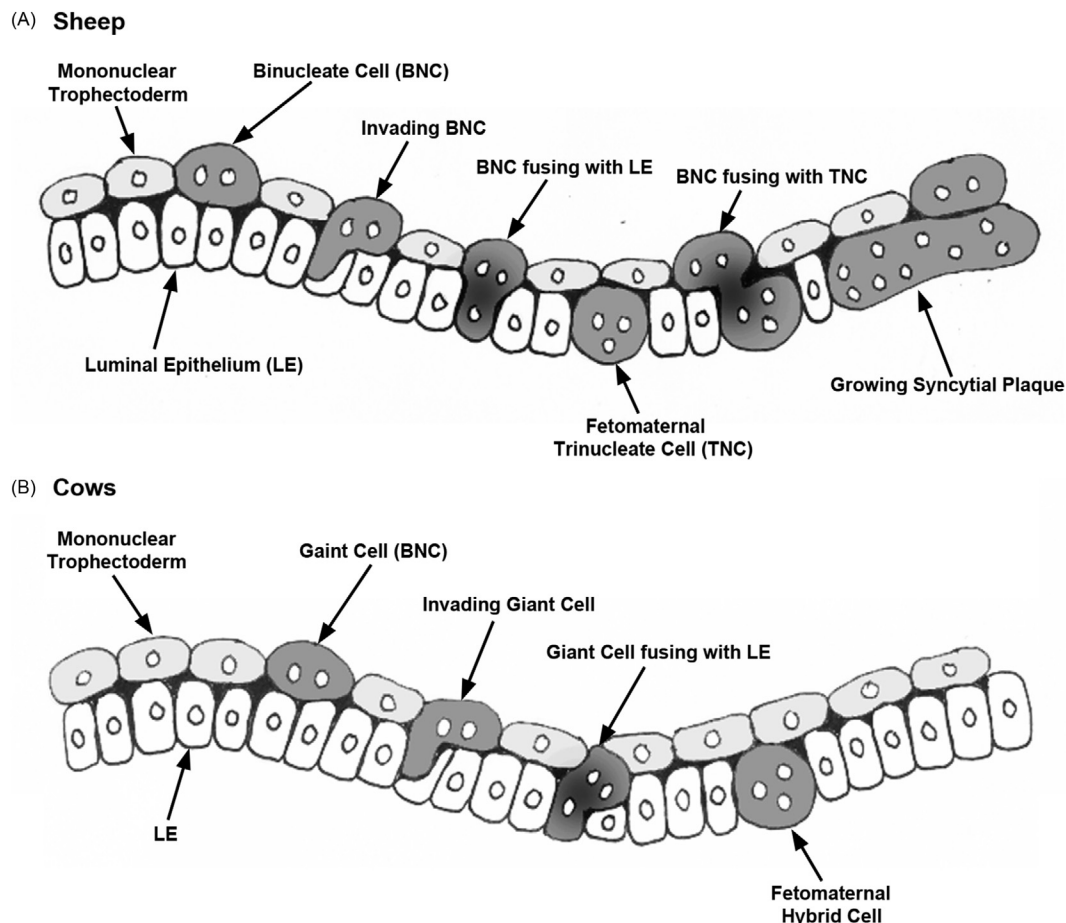


Fig. 5 (A) Syncytialization at the uterine-placental interface of sheep. (B) Syncytialization at the uterine-placental interface of cows.

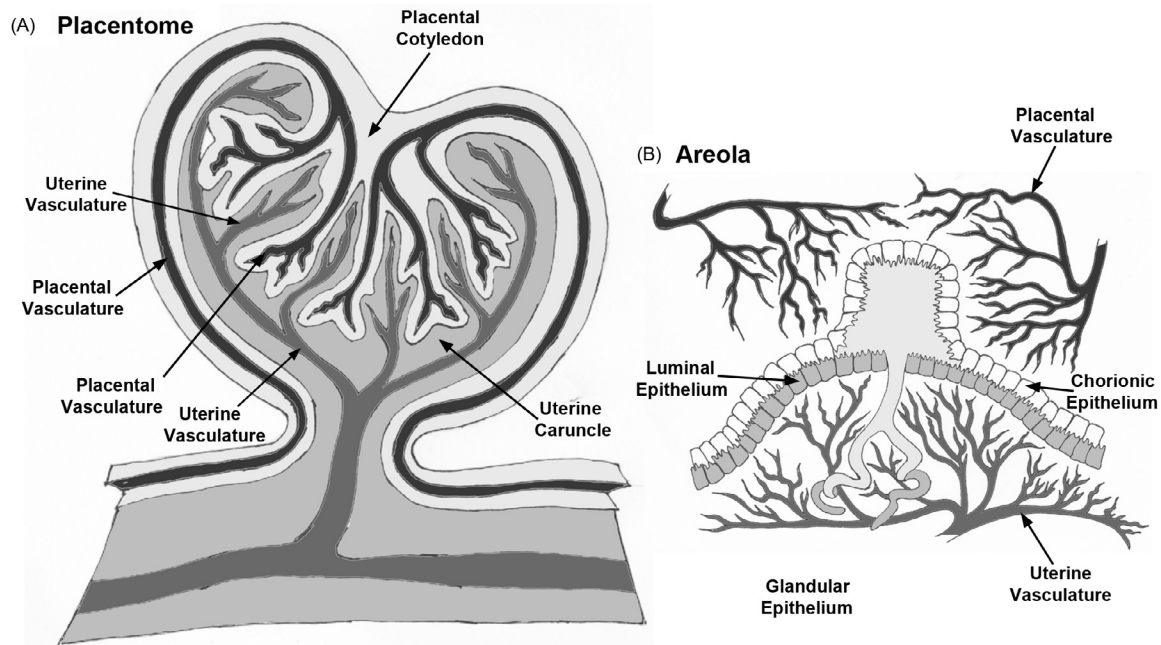


Fig. 6 (A) Structure of a placentome for hemotrophic support of fetal development. (B) Structure of an areola for histotrophic support of fetal development.

the majority of the trophoblast cells and BNCs begin to differentiate from the mononucleate trophoblast cells in concert with trophoblast outgrowth during conceptus elongation. BNCs first appear between days 14 and 16, and days 17 and 19 of gestation in sheep and cow conceptuses, respectively, and comprise 15%–20% of the trophoblast during the apposition and attachment phases of implantation. BNCs migrate and fuse with individual uterine LE cells to form trinucleate syncytial cells (beginning about day 16 of pregnancy in sheep, day 20 of pregnancy in cows), thereby assimilating the LE. The syncytia of sheep subsequently enlarge through continued BNC migration and fusion to form syncytial plaques (Fig. 5A). The syncytial plaques are conceptus-maternal hybrid cells that are composed of uterine LE and BNCs, and they eventually form the epithelial interface between uterine and placental tissues within the placentome (described in the next paragraph). In sheep, the syncytial plaques are a consistent feature in the placentomes throughout pregnancy. In cattle, BNC-uterine LE fusion to form syncytia is limited to the production of only transient trinucleate syncytial cells throughout pregnancy (Fig. 5B) (Wooding, 1984).

Following successful elongation of the conceptus, trophoblast outgrowth, and implantation, the placentae of sheep and cows, organize into placental and interplacental regions (Fig. 6). During placental development, highly branched villous placental folds, termed cotyledons, initially form by day 30 of gestation in sheep. Cotyledonary chorioallantoic villi lined by syncytial plaques then begin to protrude into crypts in the maternal endometrial caruncular tissue (aglandular areas of endometrium consisting of stroma covered by a single layer of uterine LE), resulting in extensive interdigitation of endometrial and placental tissues by day 40. Placentomes provide a conduit for hemotrophic nutrition to the fetus where maternal and placental blood vessels are in close proximity for exchanging oxygen and micronutrients, and there is a close correlation between placental mass and birth weight of the fetus. In contrast, interplacental areas exhibit epitheliochorial attachment of uterine LE to trophoblast, and contain areolae that take up histotroph secreted by the uterine GE for transport to placental vasculature that rings the areola (Wooding and Burton, 2008).

References

- Bazer, F. W., & Johnson, G. A. (2014). Pig blastocyst-uterine interactions. *Differentiation*, 87, 52–65.
- Bazer, F. W., Johnson, G. A., Song, G., & Wu, G. (2012). Pregnancy recognition signaling, fetal-placental development and prenatal fetal programming. In S. A. Blanco, & A. B. Buines (Eds.), *Encyclopedia of life support systems (EPLSS)*, developed under the auspices of the UNESCO animal reproduction in livestock. Oxford, UK: Eolss Publishers.
- Bazer, F. W., Johnson, G. A., & Spencer, T. E. (2005). Growth and development: Pre-implantation embryo. In W. G. Pond, & A. W. Bell (Eds.), vol. 1. *Encyclopedia of animal science* (pp. 1–3). NY: Marcel Dekker, Inc. No. 11–13.
- Dantzer, V. (1985). Electron microscopy of the initial stages of placentation in the pig. *Anatomy and Embryology*, 172, 281–293.
- Johnson, G. A., Burghardt, R. C., & Bazer, F. W. (2014). Osteopontin: A leading candidate adhesion molecule for implantation in pigs and sheep. *Journal of Animal Science and Biotechnology*, 5, 56.
- Song, G., Bailey, D. W., Dunlap, K. A., Burghardt, R. C., Spencer, T. E., Bazer, F. W., & Johnson, G. A. (2010). Cathepsin B, Cathepsin L and Cystatin C in the porcine uterus and placenta: Potential roles in endometrial/placental remodeling and in fluid-phase transport of proteins secreted by uterine epithelia across placental areolae and neonatal gut. *Biology of Reproduction*, 82, 854–864.

- Spencer, T. E., & Hansen, T. R. (2015). Implantation and establishment of pregnancy in ruminants. In R. D. Geisert, & F. W. Bazer (Eds.), *Regulation of implantation and establishment of pregnancy in mammals: Tribute to 45 year anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"* (pp. pp. 105–135).
- Wooding, F. B. (1984). Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. *The American Journal of Anatomy*, 170(2), 233–250.
- Wooding, F. B., & Burton, G. J. (2008). *Comparative placentation: Structure, function and evolution. Chapter 6, Synepitheliochorial placentation: Ruminants (ewe and cow)* (pp. 133–144). Heidelberg: Springer-Verlag.

Further Reading

- Geisert, R. D., Johnson, G. A., & Burghardt, R. C. (2015). Implantation and establishment of pregnancy in the pig. In R. D. Geisert, & F. W. Bazer (Eds.), *Regulation of implantation and establishment of pregnancy in mammals: Tribute to 45 year anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"* (pp. pp. 137–164).
- Spencer, T. E., Johnson, G. A., Bazer, F. W., & Burghardt, R. C. (2004). Implantation mechanisms: Insights from the sheep. *Reproduction*, 128, 656–668.

Comparative Placentation-Mammals

Günter P Wagner, Yale University, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Placentation: Consolidating the Fetal–Maternal Relationship

Placentation is the establishment of a physical connection between the maternal and the fetal organisms inside the body of the mother (Ramsey, 1982; Mossman, 1987; Wooding and Burton, 2008). The placenta is thus a composite organ consisting of fetal and maternal tissues, although the terminology varies between authors. Some authors prefer to only call the fetal component “placenta” while others include the fetal-maternal interface together with the fetal placental structures. Given the high degree of integration between the fetal and maternal organisms during placental development it seems biologically more meaningful to include both the fetal and the maternal components in a discussion of placentation. This is particularly appropriate when fetal and maternal cells are highly intermixed, as it is the case in humans and apes.

The manifest purpose of placentation is maternal provisioning of nutrients and oxygen to the fetus and also waste removal from the embryo or the fetus (Wooding and Burton, 2008). Placentation is thus the major alternative to provisioning the developing embryo through yolk. The evolution of placentation is thus correlated with a decrease in the amount of yolk provided to the oocyte (egg cell), and in mammals even the loss of genes for egg yolk genes (Brawand et al., 2008). Somewhat intermediate ways of maternal provisioning between yolk and placentation also exist. For example in some sharks (mackerel sharks, Lamniformes) the fetus develops inside the mother and is nourished by yolk rich eggs that the mother continues to ovulate and which are eaten by the fetus during intra-uterine growth (Hamlett, 1989). Even fratricide has been reported where one of the growing shark fetuses eats his/her brothers and sisters as a source of nutrition, as found in Sandtiger sharks (*Carcharias taurus*). Less dramatic forms of nonplacental maternal provisioning consist of uterine secretions that are either taken up through the eggshell or eaten by the fetus as in the false cat sharks (Pseudotriakidae) and viviparous salamanders (e.g., *Salamandra maculatus*).

Placentation evolved many times, both within the vertebrates as well as invertebrates. For instance some of the so-called velvet worms (Onychophora, Para-arthropoda, i.e., relatives of arthropods) have a placenta (Anderson and Manton, 1972). Within vertebrates, placentation is found in all major groups, with the exception of jawless vertebrates, lampreys and hagfish. The latter’s lack of placentas may simply reflect the small number of species alive today rather than a biological constraint against evolving placentation. The fetal and the parental structures involved in placentation vary. Briefly, in sharks the placentas develop from apposition of the yolk sack to the oviduct of the mother (Hamlett, 1989); in teleosts placentation can occur either within the ovary itself (Wourms, 1981), or within a skin pouch of the father (seahorses and pipefish, Syngnathidae) (Stolting and Wilson, 2007). In reptiles, placentation is particularly frequent in squamates (lizards and snakes) (Blackburn and Flemming, 2009; Pyron and Burbrink, 2015) but absent among extant archosaurs (crocodilians and birds). Variation of mammalian placentation will be discussed in greater detail below.

The Fetal–Maternal Relationship in Mammals

In mammals, the only maternal space of placentation is the uterus. This is not self-evident when one compares mammalian placentation with the variety places that accommodate placentation in teleost fishes (see above). The uterus is homologous to the shell gland of egg laying (oviparous) reptiles and monotremes (Lombardi, 1998). It shares its principle structure with the shell gland consisting of a smooth muscle myometrium, lined internally by the so-called endometrium. The endometrium consists of an epithelium that continues into endometrial glands and an endometrial stoma. The stroma is made up of endometrial fibroblasts, macrophages and natural killer cells, as well as blood vessels and capillaries. Of these endometrial cells the luminal epithelium, the endothelium of the blood vessels, the maternal blood and the endometrial stromal fibroblasts are the main maternal contributions to the fetal-maternal interface. The epithelium can be a permanent structure of the F-M interface or can be eroded during the process of implantation. In the latter case the F-M interface is principally formed by endometrial fibroblasts which then differentiate into a quasi-epithelial cell type, called decidual stromal cell.

The term decidua originally signified the parts of the uterine lining that is extruded together with the placenta, after the neonate has been born, i.e., the tissue of the mother that gets “lost” during childbirth (decidua = gr. “falling off”). Over the centuries, the term has changed in meaning, and now often refers to the differentiated form of endometrial fibroblasts, which are a specialized cell type that evolved to accommodate and contain the invading fetal portion of the placenta. Consequently decidual cells are not found in mammals that have a noninvasive placenta, e.g., cows, sheep, horses etc.

The fetal contribution to the placenta, or the “placenta proper” according to some authors, develops from extra embryonic tissues of the embryo in mammals and reptiles. There are four principal kinds of extra-embryonic membranes we need to distinguish in an amniote embryo: the chorion, the amnion, the yolk sac and the allantois (Fig. 1). The topological relationships among these membranes is variable and cannot be adequately reviewed here. The interested reader is encouraged to consult the short but very clear and accurate summary by Elizabeth Ramsey (Ramsey, 1982).

In brief, the chorion is the tissue layer that surrounds the embryo and all the deeper lying extraembryonic membranes (Fig. 1). The cells of the chorion are also sometimes called trophoblast cells since in eutherian development the outermost layer of cells in

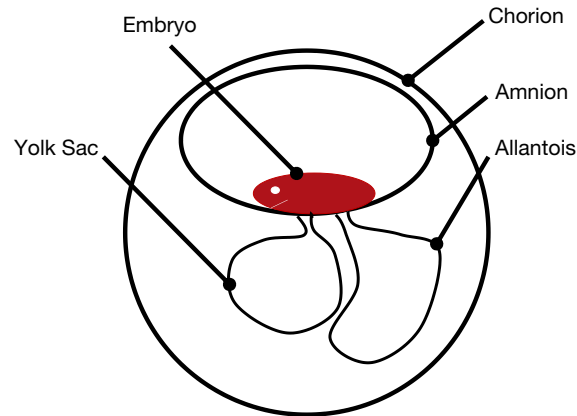


Fig. 1 The topological relationships among the principal extraembryonic tissues of an amniote vertebrate embryo.

the early embryo is called the trophoblast. The amnion is a bubble surrounding the embryo, and the yolk sac and the allantois are growing out of the belly of the embryo from the embryonic gut, the endoderm. In all vertebrates with yolk rich eggs the yolk sac, naturally, covers the yolk of the egg and is the principle conduit through which the embryo can access the nutrients stored in the egg yolk. In spite of its yolk related origin, a structure homologous to the yolk sac of reptiles, birds and fishes, is also found in eutherian mammals in spite of the fact that there is no yolk in a eutherian egg cell (Ramsey, 1982). Eutherian mammals have no yolk in their egg cells, because they evolved a complete reliance on nutrient transfer from the mother through the placenta. Never the less a structure homologous to the yolk sac develops which transiently can contribute to the placenta, and has a role in blood development. Finally, the allantois originated as a container for the fetal urine and also serves as a structure of gas exchange through the egg shell. Is also grows out of the belly of the embryo and is also found in the same space as the yolk sac (Fig. 1). The intra-embryonic portion of the allantois later develops into the urinary bladder.

The fetal placental structures in mammals, as well as in reptiles, derive from either the yolk sac or the allantois or both. The contribution of either to the placenta changes during evolution, with a dominant contribution from the yolk sac in earlier branching lineages of mammals (Tyndale-Biscoe and Renfree, 1987; Freyer et al., 2003), and a stronger contribution of the allantois in more derived (later evolved) forms. This shift from yolk sac to allantois derived placental structures does not only occur during evolution, but can also happen during development, where earlier in fetal life the yolk sac may dominate and gradually being replaced by structured derived from the allantois.

In mammals, the placenta is the most variable organ between species. For that reason, a number of complementary classification systems have been developed, the main ones are based on the degree of intimacy between the fetal and the maternal tissues, the fetal structure forming the placenta, and the morphology of the fetal part of the placenta itself.

A broadly used classification is based on the number of tissue layers that separate the fetal and the maternal blood compartment (Grosser, 1927). The principal differences among placental types arise from the number of maternal tissue layers removed during implantation. The least invasive placentation is called the epithelio-chorial placenta. Its name derives from the name of the maternal and the fetal tissue that come into contact: the luminal epithelium of the uterus (maternal) and the chorion of the fetus (Fig. 2A). This form of placentation is found in many marsupials and in a large group of eutherian mammals, represented by the hoofed animals, like pig, cow, horses and their relatives, including whales and dolphins. Some smaller groups of eutherians also have

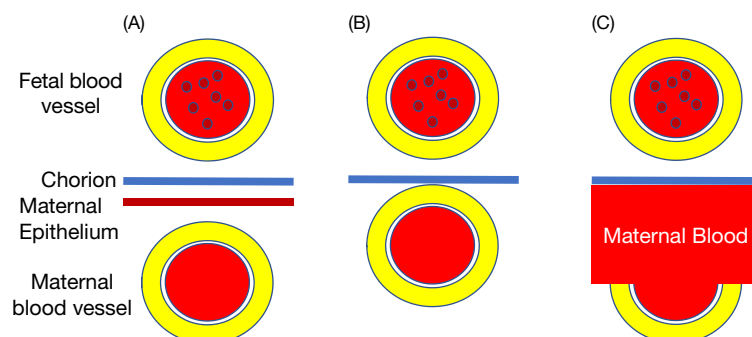


Fig. 2 The three main forms of the fetal-maternal interface classified according to Grosser (1927). (A) Epitheliochorial placenta, the chorion of the fetus attaches to the luminal epithelium of the uterus. (B) Endotheliochorial placenta, the embryo erodes the luminal epithelium and the chorion is in contact with the maternal blood vessels. (C) Hemochorial placenta, the maternal epithelium and blood vessels are eroded and the fetal chorion is bathed in maternal blood.

epithelia-chorial placenta, such as the Strepsirhini (lemurs, galagos and lorises) and dugongs. These groups, marsupials, hoofed animals, Strepsirhini and dugongs are distantly related to each other and thus their epithelio-chorial placenta is independently evolved.

A more invasive form of placentation is called the endothelio-chorial placenta, where during implantation the uterine epithelium is eroded as is part of the stroma and the chorion of the fetus is only separated from the maternal blood by the lining of the blood vessels, the endothelium (Fig. 2B). This form of placentation is found in carnivores, cats and dogs and their relatives, as well as elephants, the armadillo and many bats.

The most invasive placenta is the hemochorial placenta, where the fetal cells of the chorion are directly in contact with the maternal blood after the uterine epithelium and the blood vessels have been partially destroyed by the invading conceptus (Fig. 2C). This form of placentation is wide spread among eutherian mammals, including the rodents and humans.

This formal classification of the FM interface hides a large amount of biological differences that exist within each category. Epithelia-chorial placentae can either consist of an attachment of the chorion to the maternal epithelium, but more intimate forms also exist. For instance, in the opossum the cells of the chorion interdigitate between the maternal epithelial cells, rather than just attach to the surface of the epithelium (Freyer et al., 2002). Furthermore, it is possible that the chorial and the maternal cells fuse, forming cells that contain both maternal and fetal cell nuclei. This has been documented in sheep and the distantly related bandicoots (Padykula and Taylor, 1976), a marsupial. Even among the hemochorial placentae, the nature of the fetal invasion can vary greatly between species (see below). For instance the human and ape placenta includes not only a partial destruction of the endometrium, including the blood vessels, but also a replacement of the maternal endothelium (the inner lining of the blood vessels) of the spiral arteries as well as migratory cells, so-called extravillous trophoblast cells, that invade as far as the myometrium.

The second aspect of the placenta that can be used to classify a mammalian placenta is the fetal tissue contributing to the definite placental structure. As mentioned above, there are three principal fetal tissues that contribute to the placenta: the chorion, the yolk sack and the allantois. In most forms of placentation the outermost layer is formed by the chorion, often also called the trophoblast. The inner tissue layers of the placenta can either be formed by the yolk sack or the allantois, and thus we can distinguish between chorio-vitelline placenta made up from the chorion and the yolk sack, and a chorio-allantoic placenta. The chorio-vitelline placenta is dominant in marsupials and some eutherians, like the rabbit (Freyer and Renfree, 2009). In contrast, most eutherians have a chorioallantoic placenta (Ramsey, 1982). An exception to the rule that the outermost layer of the fetal placenta is composed of chorion cells is the so-called “inverted” yolk sack placenta. In this case the chorion and part of the yolk sack is eroded so that the fetus contacts the mother with the inner surface of the yolk-sack, which leads to a situation where the yolk-sack tissue seems to lie “outside” the chorion. This special form of placentation is found in rabbits and most rodents (Ramsey, 1982).

A peculiarity of the mammalian fetal-maternal interface is that cells in this part of the body have a tendency to fuse and form of either syncytial structures, i.e., structures where multiple nuclei are found in the same continuous cytoplasmatic body. The most frequent form, also found in humans, is where the fetal surface of the placenta is made of fused trophoblast cells, the so-called syncytio-trophoblast. Cell fusion can also occur between maternal and fetal cells, leading to cells containing both, fetal and maternal nuclei, so-called heterokaryons. The latter has been described in some marsupials (bandicoots) and in sheep. This tendency to cell fusion is driven by the expression of retroviral envelop proteins called syncytins (Huppertz et al., 2006), which probably are expressed at the placenta because they also ameliorate the maternal immune response (Mi et al., 2000).

Finally, the oldest classification of placentas is based on the overall morphology of the term placenta, i.e., the morphology of the placenta at the end of pregnancy (Fig. 3). The morphological classification is based on the external shape of the chorioallantoic part of the placenta. In the mature placenta the surface area is increased by folds or projections (villi) according to the same principle as other metabolic exchange surfaces increase in surface area (villi in the gut for instance). The morphological classification is then based on the distribution of these folds and projections. If these structures are distributed all over the surface one talks about a diffuse placenta. This form is for instance found in the pig and the horse. In the cotyledonary placenta the folds or villi are concentrated into a small number of groups, called cotyledones or placentomes. This form is found in ruminants, like the cow. If the projections are located in a belt around the fetus the placenta is called “zonary” and is characteristically found in carnivores, e.g., cats and dogs with exceptions, like the bear. Finally, if the exchange surface is concentrated in one larger disc the placenta is called discoidal. This form is found in humans and other anthropoid primates (new world monkeys, old world monkeys and apes), rodents (mice and rats) as well as bears.

The Evolving Fetal–Maternal Relationship in Mammals

A simple listing of the various forms of placentation as summarized above is highly confusing, since it does not distinguish between ancestral and evolved forms of placentation, which formally would fall into the same class of placentas but hide the often fundamentally different biology behind them. For that reason, it is important to briefly summarize the evolution of the fetal–maternal relationship in order to put these descriptive categories into context.

Living mammals consist of three major groups, monotremes, marsupials and eutherians (Fig. 4), the latter are also sometimes called “placental mammals” which is a misnomer because marsupials also have a placenta, though often a very brief and simple one. Among the three groups, the marsupials and eutherians are more closely related to each other than each of them are to monotremes (Fig. 3). For that reason, they are collectively called Theria. Monotremes are a small group of animals that, in many respects,

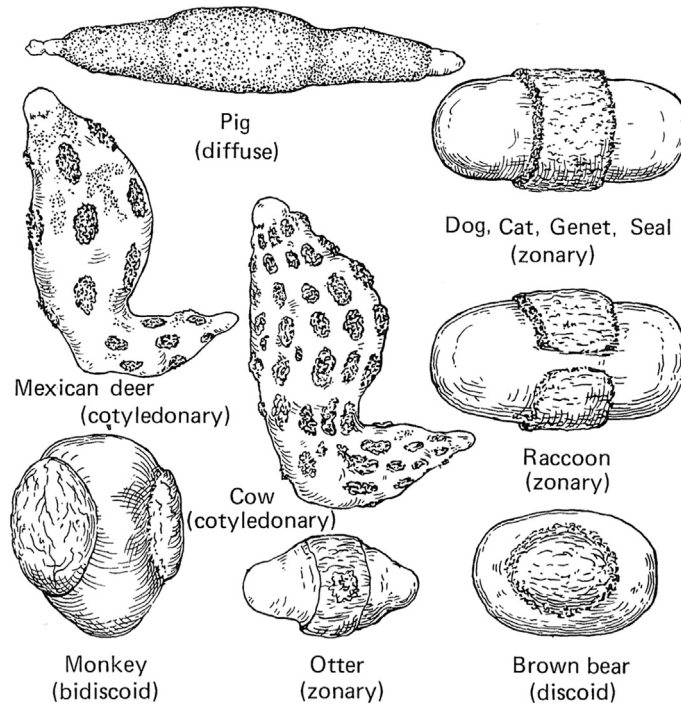


Fig. 3 Classification of mammalian placentas based on their gross morphological features. The distinctions are based on the shape and distribution of the specialized exchange structures on the surface of the fetal membrane.

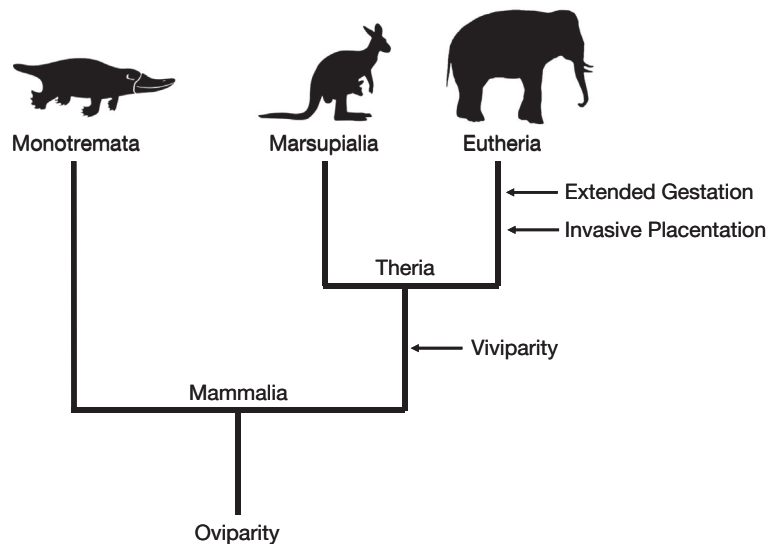


Fig. 4 The major groups of mammals and the main stages in the evolution of mammalian female reproductive mode.

represent a mosaic of “reptilian” and therian characteristics. They consist of 14 extant species that come in two types, the echidnas and the platypus (Griffiths, 1978). They are also known as the “egg laying mammals.”

The differences in female reproductive biology between the three main groups of mammals can be read as a stepwise progression towards the more “advanced” eutherian form of reproduction. This view is certainly a simplification, since each lineage acquired their own characteristics and can thus not be considered generally “primitive” in any generalized way. Never the less, it is still true that some groups have retained ancestral features that underwent transformation and innovation in other groups. For instance, focusing on placentation, it is true that eutherians have a more elaborated fetal maternal communication, and the monotremes less so. In brief, monotremes lay eggs and incubate them until hatching, remotely similar to reptilian reproduction. In marsupials the early embryo remains in the shell coat (colloquially called “egg shell”) for much of its intra-uterine development but then “hatch” within the uterus and attach to the uterine endometrium to form an epithelio-chorial placentation (Tyndale-Biscoe and Renfree,

1987). Attachment can be brief, like in the opossum (2–3 days) or somewhat extended, like in the more derived wallabies (8 days) (Selwood, 2000; Griffith et al., 2017), but most of the “fetal” growth phase is happening outside the uterus attached to the maternal nipple. The term fetal seems appropriate for marsupial neonates because of their early stage of development. For instance the new born marsupials generally do not have hind limbs. They only develop and grow during the extended postnatal nursing phase. Finally, the eutherian mammals are characterized by a more extended gestation and a complete reliance on placental nutrition during fetal development. Below we will give a slightly more detailed description of the fetal–maternal relationship for each of the three groups.

Monotremes

All mammals have much smaller eggs compared to their reptilian sisters (Griffiths, 1978; Renfree and Shaw, 2002). The eggs are smaller because they are endowed with less yolk than bird and reptilian eggs. This also applies to the otherwise more “reptilian” monotremes, which do lay eggs rather than giving birth to live neonates. Never the less, already in monotremes there is a partial reliance on uterine provisioning from the mother, where the conceptus takes up uterine secretions and increases substantially in size before the egg shell is formed around them. For that reason some researchers consider monotremes as having a placenta, although an odd form of placentation. The odd thing about them is that the eggshell is formed after a phase of placental nutrient transfer rather than the other way around as in marsupials. After laying the eggs are incubated for about 10 days, either in a depression of the abdomen or even a posterior facing pouch. After hatching the young are licking milk secretions out of the fur of the mother for up to 6 months. Monotremes have milk glands but no nipples, so that the milk is seeping into the fur, where it is taken up by the young monotremes. Hence monotremes, even though they superficially appear reptile like in their reproductive biology, they are far from a mere copy of the ancestral reptilian condition.

Marsupials

As mentioned above, marsupial reproduction is in some respects between the monotreme and the eutherian phenotypes, but this view is inaccurate because of the diversity of marsupials and their reproduction. There are 334 recognized extant species of marsupials (Hunsaker, 1977), which evolved a wide variety of reproductive modes (Tyndale-Biscoe and Renfree, 1987). The deepest split within the marsupial tree is that between the American opossums and the Australian marsupials which make up 70% of the species diversity (Beck, 2008; Meredith et al., 2008). In the comparison between the opossums (Ameridelphia) and the Australian marsupials (Australidelphia) the opossums are more uniform and apparently more ancestral in many of their reproductive traits. The arguably ancestral characteristics include ovulation of multiple eggs, lack of maternal recognition of pregnancy, and a very short attachment phase (only 2 days in the gray short tailed opossum, *Monodelphis domestica*). In *Monodelphis*, the placental attachment leads to an inflammatory reaction on the side of the mother, which, in contrast to the situation in eutherians (see below), is increasing until it ends in parturition (Griffith et al., 2017). In contrast, the Australidelphia are more diverse and include groups which ovulate only a single egg and have definite recognition of pregnancy and a longer attachment phase, e.g., kangaroos and wallabies (Tyndale-Biscoe and Renfree, 1987).

In marsupials the conceptus spends a long time in the uterus surrounded in a shell coat, and only late in gestation dissolve it and attach to the lining of the uterus initiating a brief period of placentation. All placentation in marsupials is epitheliochorial, in the sense that there is no sustained contact between the trophoblast of the fetus and the connective tissue of the uterine endometrium. Nevertheless, the fetus seems to “attack” the uterine epithelium and, in opossum for instance, the trophoblast cells can penetrate between the epithelial cells of the luminal epithelium (Freyer et al., 2002). In rare occasions the trophoblast even can breach the basal membrane of the epithelium, but only very briefly before birth. In bandicoots (*Perameles*) the fetal trophoblast cells and the maternal uterine epithelial cells fuse and form a so-called “heterokaryons,” that are cells that contain nuclei from two different individuals, the fetus and the mother (Padykula and Taylor, 1976).

Placental structures are predominantly yolk sac derived with no contribution from the allantois. Only three families of marsupials have been reported to develop a choiroallantoic placenta, in addition to the yolk sac placenta. These are wombats, the koalas (Phalcolarctidae) and bandicoots (Peramelidae) (Freyer et al., 2003).

Eutherians

The pregnancy and placentation of eutherians is characterized by an earlier attachment and implantation of the blastocyst and a much longer sustained integration of fetal and maternal tissues (placentation) than in marsupials. That is to say that the absolute length of gestation is not necessarily longer, for instance the house (laboratory) mouse with 19–21 days of gestation has a shorter gestation time than wallaby with 28 days, but it is true that the gestation length of some eutherians can be much longer than any marsupial, up to 22 months as in elephants. Hence eutherians have evolved the ability to sustain fetal attachment and implantation for a very long time. This feature is associated with a much more intimate relationship between the fetus and the mother, although the exact anatomical and histological structures involved are highly variable between eutherian groups. Also, eutherians rely much more on allantois derived placental structures than marsupials. When yolk sack contributions to the placenta do occur, they play a much smaller role than they do in marsupials. Placentation through the yolk sack are usually found in early stages of pregnancy.

Contrary to the consensus in the 20th century, molecular mammalian phylogenies together with ancestral character state reconstructions have shown that the most recent common ancestor of eutherian likely had invasive placentation (Mess and Carter, 2006; Wildman et al., 2006; Martin, 2008; Elliot and Crespi, 2009). The classical model was based on the intuitively appealing model that epithelio-chorial → endothelio-chorial → hemo-chorial placentation seems like a natural progression. While this progression may have happened in the stem lineage of eutherian mammals, the most recent common ancestor of crown eutherians most likely had a form of invasive placentation. The exact form of placentation in the ancestor is not clear but the consensus view is that it was not epithelio-chorial. This model implies that the eutherian groups with less invasive placentation, epitheliochorial placenta, as seen in hoofed animals and their relatives, as well as galagos and dugongs must have evolved a less invasive placentation secondarily.

Even though hoofed animals together with their relatives (whales and dolphins) are classified as having an epithelio-chorial placenta and are thus grouped together with marsupials, the cow, horse and sheep placentas are not just a re-evolution of the transient, noninvasive placenta found in marsupials. Reevolving a less invasive placentation type, the so-called “ungulates” have evolved a number of unique specializations that make their placenta quite complex. As examples we will briefly review the situation in cows.

The uterus of the cow and related animals has specialized structures in their endometrium called caruncles. These are areas where the endometrial surface is highly folded forming either a convex or a concave structure. During pregnancy the trophoblast of the fetus forms projections that inter-digitate with the caruncular folds and thus forms a structure called cotyledon. Together the caruncle and the cotyledon form a placentome, which is the primary locus of exchange between the mother and the fetus. The number and size of placentomes varies between species between three to eight large ones in deer to as many as 150 in cows, goats and sheep. Within the placentome the uterine and fetal epithelia separate in certain places and accumulate stagnant maternal blood. These areas are called “hemophagous” zones because the trophoblast cells in these zones specialize in phagocytosing red blood cells and digest them to harvest the iron for the generation of fetal blood. Another cellular specialization of the trophoblast in the cotyledon is the development of binuclear cells (BNC) which play a role in the hormonal communication between the fetus and the mother. The BNC fuse with epithelial cell of the endometrium forming transient tri-nuclear cells. With the TNC the trophoblast cells can deliver hormones directly into the maternal endometrium without having to be taken up or let through by the luminal epithelium.

The areas of the trophoblast between the cotyledons have additional specializations, called areole. Areole form opposite to the openings of the uterine glands and are specialized for the resorption of the secretions of the uterine glands. These secretions are called “uterine milk” and is an alternative pathway for passing nutrients and antibodies from the mother to the fetus. The other pathway, of course, is the diffusion or active transport from the maternal blood to the fetal circulation at the placentome.

Hemochorial placentation, the primitive condition in eutherians, can be quite differently realized in different lineages. In most cases the development of a hemochorial placenta is accomplished through broad destruction of the endometrium and the maternal blood vessels. There are, however, exceptions. For instance placentation in armadillo and ant eaters is classified as hemochorial, which is technically correct as the placental villi are floating directly in the maternal blood. But this configuration is achieved with minimal tissue destruction. The placenta penetrates the endometrium at focal places, the villi grow down into the endometrium and finally ramify within materially provided blood sinuses. The armadillo endometrium has performed enlargements of the blood vessels at the fundus of the uterus. This mode of hemochorial placentation is quite different from the way hemochorial placentation is achieved in primates or rodents.

References

- Anderson, D. T., & Manton, S. M. (1972). Studies on onychophora VIII. Relationship between embryos and oviduct in viviparous placental onychophorans *epiperipatus-trinidadensis* Bouvier and *Macroperipatus-torquatus* (Kennel) from Trinidad. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences*, 264(860), 161.
- Beck, R. M. D. (2008). A dated phylogeny of marsupials using a molecular supermatrix and multiple fossil constraints. *Journal of Mammalogy*, 89(1), 175–189.
- Blackburn, D. G., & Fleming, A. F. (2009). Morphology, development, and evolution of fetal membranes and placentation in squamate reptiles. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 312(6), 579–589.
- Brawand, D., Wahli, W., & Kaessmann, H. (2008). Loss of egg yolk genes in mammals and the origin of lactation and placentation. *PLoS Biology*, 6(3), e63.
- Elliot, M. G., & Crespi, B. J. (2009). Phylogenetic evidence for early hemochorial placentation in eutheria. *Placenta*, 30(11), 949–967.
- Freyer, C., & Renfree, M. B. (2009). The mammalian yolk sac placenta. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 312(6), 545–554.
- Freyer, C., Zeller, U., & Renfree, M. B. (2002). Ultrastructure of the placenta of the tammar wallaby, *Macropus eugenii*: Comparison with the grey short-tailed opossum, *Monodelphis domestica*. *Journal of Anatomy*, 201(2), 101–119.
- Freyer, C., Zeller, U., & Renfree, M. B. (2003). The marsupial placenta: A phylogenetic analysis. *Journal of Experimental Zoology Part A-Comparative Experimental Biology*, 299A(1), 59–77.
- Griffith, O. W., Chavan, A. R., Protopapas, S., Maziarz, J., Romero, R., & Wagner, G. P. (2017). Embryo implantation evolved from an ancestral inflammatory attachment reaction. *Proceedings of the National Academy of Sciences of the United States of America*, 114(32), E6566–E6575.
- Griffiths, M. (1978). *The biology and monotremes*. San Diego, CA: Academic Press.
- Grosser, O. (1927). *Fruhentwicklung, Eihautbildung und Placentation des Menschen und der Säugetiere*. München: J. F. Bermann.
- Hamlett, W. C. (1989). Evolution and morphogenesis of the placenta in sharks. *Journal of Experimental Zoology*, 252, 35–52.
- Hunsaker, D. (1977). *The biology of marsupials*. New York: Academic Press.
- Huppertz, B., Bartz, C., & Kokozidou, M. (2006). Trophoblast fusion: Fusogenic proteins, syncytins and ADAMs, and other prerequisites for syncytial fusion. *Micron*, 37(6), 509–517.
- Lombardi, J. (1998). *Comparative vertebrate reproduction*. Boston: Kluwer Academic Publishers.
- Martin, R. D. (2008). Evolution of placentation in primates: Implications of mammalian phylogeny. *Evolutionary Biology*, 35(2), 125–145.
- Meredith, R. W., Westerman, M., Case, J. A., & Springer, M. S. (2008). A phylogeny and timescale for marsupial evolution based on sequences for five nuclear genes. *Journal of Mammalian Evolution*, 15(1), 1–36.

- Mess, A., & Carter, A. M. (2006). Evolutionary transformation of fetal membrane characters in Eutheria with special reference to Afrotheria. *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 306B, 140–163.
- Mi, S., Lee, X., Li, X., Veldman, G. M., Finnerty, H., Racie, L., LaVallie, E., Tang, X. Y., Edouard, P., Howes, S., Keith, J. C., Jr., & McCoy, J. M. (2000). Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature*, 403(6771), 785–789.
- Mossman, H. W. (1987). *Vertebrate fetal membranes*. New Brunswick, NJ, Rutgers: University Press.
- Padykula, H. A., & Taylor, J. M. (1976). Ultrastructural evidence for loss of the trophoblastic layer in the chorioallantoic placenta of Australian bandicoots (Marsupialia: Peramelidae). *The Anatomical Record*, 186(3), 357–385.
- Pyron, R. A., & Burbrink, F. T. (2015). Contrasting models of parity-mode evolution in squamate reptiles. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 324, 467–472.
- Ramsey, E. (1982). *Placenta*. Praeger Inc: Santa Barbara, CA.
- Selwood, L. (2000). Marsupial egg and embryo coats. *Cells, Tissues, Organs*, 166(2), 208–219.
- Stolting, K. N., & Wilson, A. B. (2007). Male pregnancy in seahorses and pipefish: Beyond the mammalian model. *BioEssays*, 29(9), 884–896.
- Tyndale-Biscoe, C. H., & Renfree, M. B. (1987). *Reproductive physiology of marsupials*. Cambridge, UK: Cambridge University Press.
- Wildman, D. E., Chen, C., Erez, O., Grossman, L. I., Goodman, M., & Romero, R. (2006). Evolution of the mammalian placenta revealed by phylogenetic analysis. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 3203–3208.
- Wooding, F. B. P., & Burton, G. J. (2008). *Comparative placentation*. Berlin and Heidelberg: Springer.
- Wourms, J. P. (1981). Viviparity—The maternal-fetal relationship in fishes. *American Zoologist*, 21(2), 473–515.

Further Reading

- Ramsey, E. (1982). *Placenta*. Santa Barbara, CA: Praeger Inc.
- Renfree, M., & Shaw, G. (2002). *Reproduction in monotremes and marsupials*. *Encyclopedia of life sciences*. New York: Wiley and Sons.
- Wooding, F. B. P., & Burton, G. J. (2008). *Comparative placentation*. Berlin and Heidelberg: Springer.

Relevant Websites

- http://www.elasmo-research.org/education/topics/lh_rep_modes.htm.
- <http://www.departments.bucknell.edu/biology/resources/msw3/browse.asp>.
- <http://placentation.ucsd.edu/index.html>.

Uterine Natural Killer (NK) Cells

Ashley Moffett, University of Cambridge, Cambridge, United Kingdom

Martin A Ivarsson, University of Cambridge, Cambridge, United Kingdom; and Center for Infectious Medicine, Karolinska Institutet, Stockholm, Sweden

© 2018 Elsevier Inc. All rights reserved.

Glossary

Decidua The mucosal lining during pregnancy

EVT Extra villous trophoblast, invasive in nature

GOS The great obstetric syndromes, collection of syndromes caused by poor placentation

HLA Human leukocyte antigen

KIR Killer immunoglobulin-like receptor

Preeclampsia A pregnancy complication presenting with diverse symptoms including hypertension

Introduction

The presence of unusual granulated cells in the uterine mucosa has been recognized for > 100 years ([Marchand, 1895](#)). The cells were first believed to originate from stromal cells but, with the advent of immunohistochemistry, it was realized that they belong to the hematopoietic lineage ([Bulmer and Sunderland, 1984](#)). Uterine NK cells are not simply NK cells found in the uterus. NK cells in the peripheral blood (pbNK cells) were identified 40 years ago and were named because of their innate and *natural* ability to kill certain leukemic cell lines without the prior sensitization needed for killing by cytotoxic CD8⁺ T cells. Uterine NK cells however, are poor killers of the same cell lines unless they are first activated in vitro ([King et al., 1989a](#)). It seems likely that uNK cells belong to the family of innate lymphoid cells (ILCs) that includes blood and tissue NK cells. ILCs primarily perform regulatory functions via release of mediators. Regrettably, a view that uterine NK cells can kill the embryo, and therefore need suppressing for a successful pregnancy to occur, has become widespread amongst many clinicians. Uterine NK cells are only in contact with placental trophoblast cells and not with somatic cells of the embryo and are unable to kill trophoblast without prior activation in vitro. If uNK cells were discovered today they would have been given a less tainted name, for example, uterine innate lymphoid cells.

Uterine NK cells are relevant to study because:

- they are the major population of lymphocytes present in the secretory endometrium at the time of implantation and in early decidua (the uterine lining in pregnancy) during the first trimester
- studies indicate they produce soluble products that act to modulate the walls of the uterine spiral arteries
- they are in close contact with trophoblast cells during placentation ([Fig. 1](#))
- genetic variants of NK receptors and their ligands have been linked to pregnancy complications and birth weight

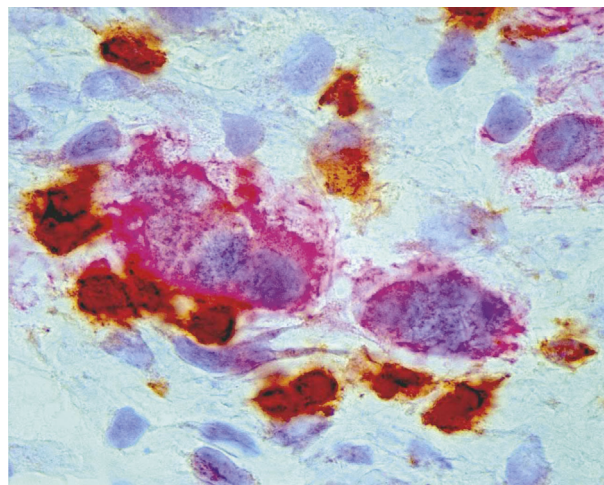


Fig. 1 Immunohistochemistry of human implantation site. HLA-G⁺ extra villous trophoblast (EVT) cells (*pink*) and CD56⁺ decidual NK cells (*brown*) are found in close proximity. Courtesy of Lucy Gardner.

Most research on uNK cells focuses on their role in implantation. The invasion of trophoblast cells into the decidua during placentation in the early stages of pregnancy is important to establish the supply of blood and nutrients to the developing conceptus. During this process of arterial transformation, extravillous trophoblast (EVT) cells home to the spiral arteries resulting in destruction of the smooth muscle cells of the media to be replaced by “fibrinoid” material (Loke and King, 1997). Endovascular EVT cells move down the arterial lumen so that they are lined by trophoblast and not by endothelial cells. Deep invasion into the inner part of the myometrium are characteristics of a normal pregnancy (Brosens et al., 2011). However, the highly invasive nature of EVT cells clearly needs to be regulated since implantation on a scar from a previous cesarean section, or in the fallopian tube, results in trophoblast cells invading unhindered (placenta percreta) (Moffett and Colucci, 2015). Thus, the decidua has a unique capability to control and regulate the invasion. This control can also be too efficient as is the case in conditions such as preeclampsia and fetal growth restriction, where spiral artery remodeling is insufficient (Brosens et al., 2011). What components of the decidua regulate trophoblast invasion? One candidate is the uNK cells that are found in high abundance in the decidua. This article will summarize what is known about uNK cell involvement in human reproduction.

Characteristics of Uterine NK Cells

Nonpregnant Endometrium

Uterine NK cells are lymphocytes that lack markers for T and B cells and do not express clonotypic receptors that have undergone somatic gene rearrangement, but express the cardinal NK cell marker CD56 (Moffett-King, 2002). In the nonpregnant uterus, uNK cells are found both in the deeper basalis layer and in the superficial functionalis layer of the endometrium (King et al., 1991). In the estrogen-dominated proliferative phase, CD56⁺ uNK are relatively small and agranular. Following ovulation and the formation of the progesterone-secreting corpus luteum, uNK cells proliferate vigorously and become larger and granulated. Classical uterine NK cells are absent both before menarche and after the menopause. This suggests a hormonal dependence, but uNK cells do not express receptors for estrogen or progesterone. Such receptors are however found on uterine stromal cells and, in response to progesterone, the stromal cells produce interleukin 15 (IL-15), a cytokine known to induce NK cell proliferation. If no embryo implants the corpus luteum involutes and progesterone levels wane, leading to a decrease in IL-15. Uterine NK cells in the late secretor phase just before menstrual breakdown undergo nuclear fragmentation reminiscent of dying cells (King et al., 1989b). However uNK cells from menstrual blood can be used as a source of uNK cells and are viable (Ivarsson et al., 2016). More analysis is needed on the exact differences in phenotype and function between such uNK cells and those isolated from other time-points during the menstrual cycle.

During Pregnancy

If an embryo implants, the corpus luteum (and eventually the placenta) continues to produce progesterone and IL-15 levels are maintained permitting uNK cells to continue to proliferate and become even more abundant. Whether there are differences in the density and morphology of uNK in the decidua basalis where implantation takes place, compared to the decidua parietalis that lines the rest of the uterus is suspected but not well-documented. Uterine NK cells can however be identified throughout the decidua and are also always present in ectopic foci of decidua such as in endometriotic lesions and in tubal pregnancy (Bulmer et al., 2009).

The depth of trophoblast invasion differs greatly between species, ranging from no invasion (epitheliochorial placentation), to the very invasive placentation (hemochorial placentation) seen in humans. Only species with invading trophoblast cells have decidua and uNK cells, and a more invasive placentation correlates with increased decidualization of the uterine mucosa, again suggesting uNK cells perform a regulatory role to ensure a balanced invasion takes place (Moffett and Loke, 2006). During the first trimester of pregnancy the uNK cells persist in high numbers but towards term the number decreases with only a few cells still present. Uterine NK cells often have a distinct reniform shape and contain fewer but larger granules than pbNK cells. In spite of their granule content uNK cells are poor killers when measured in classic NK cell assays, as well as when they are cultured with trophoblast cells. Moreover, no reliable evidence of trophoblast killing by uNK cells *in vivo* has been reported. Instead regulatory functions during implantation are believed to be more important (discussed more below).

Detailed Phenotype of uNK as Defined by Flow Cytometry

Using modern flow cytometry techniques and different markers, the cells can be identified as CD45⁺ cells expressing CD56 in the absence of lineage markers (CD3, CD19, and CD14) for other leukocytes. Although both uNK cells and pbNK cells express CD56, uNK cells are CD56^{superbright} whereas pbNK cells can be either CD56^{bright} or CD56^{dim} (Moffett-King, 2002). Furthermore, uNK cells express combinations of NK cell receptors NKG2A, NKG2C, NKG2D, KIRs, LILRB1 and tissue-residency markers such as CD9, CD103, and CD49a. The activating receptors CD16 and DNAM-1 are found only on pbNK cells and not on uNK cells (Table 1). Some markers expressed by both uNK cells and pbNK cells differ in function. On pbNK cells the natural cytotoxicity receptors (NCRs) NKp30, NKp44 and NKp46 are all activating, but on uNK cells NKp30 is expressed as an inhibitory variant and NKp44 is only upregulated after activation *in vitro*. This NK receptor profile probably explains why uNK cells are poorly cytotoxic in cell–cell interactions. Similarly, adhesion receptor 2B4 is the same on the cell surface of pbNK cells and uNK cells, but it is missing its intracellular activating adaptor molecule (SAP) in uNK cells (Jabrane-Ferrat and Siewiera, 2014). In terms of granule content both uNK cells and pbNK cells (in particular CD56^{dim} pbNK cells) contain granules with similar types of preformed cytolytic molecules including perforin, granzymes, and granzylins. The functions of these granular molecules are unknown in uNK cells

Table 1 Summary of key surface markers and functional features that distinguish resting uterine NK cells from peripheral blood NK cell subsets

Receptor/property	uNK cells	pbNK cells	
CD56	Superbright	Bright (10% of NK cells)	Dim (90% of NK cells)
CD16	—	—	+
KIR	++/—	—	+/—
LILRB1	+/—	—	+/—
NKG2A	+	+	+/—
NKG2C	Low	+/—	+/—
CD9	+	—	—
CD103	+	—	—
CD49a	+	—	—
CD69	+	—	—
DNAM-1	—	+	+
CD57	—	—	+/—
Cytotoxic	—	+	+++
Cytokine production	+	++	+

but do indicate that they have the potential to kill if, for example, any NKG2D ligands were pathologically expressed by trophoblast. Gene expression arrays have identified several hundred genes that are differentially expressed between uNK cells and pbNK cell subsets (Koopman et al., 2003). Only recently have modern cytometry tools been implemented to study uNK cells and much remains to be understood about their phenotype and function in the nonpregnant and pregnant uterus.

Uterine NK Cell Development and Differentiation

Peripheral blood NK cells progenitors can be found in bone marrow as well as in secondary lymphoid tissue and liver. How uNK cell development is related to the development of pbNK cells is still unknown but three nonmutually exclusive theories have been proposed: First, a uNK cell precursor may be recruited from the blood which, upon entering the endometrium, differentiates into uNK cells. Second, a tissue-resident uNK cell precursor (possibly similar to the pbNK cell progenitor) may exist in the basal layer. This precursor would remain in the tissue throughout the menstrual cycle and respond to the sex hormones including progesterone, via IL-15 produced by stromal cells. Finally, pbNK cells may be recruited to the endometrium and decidua and there undergo changes in phenotype and function. More studies are needed before a consensus model of uNK cell development can be presented but whatever their origin, uNK cells do proliferate and differentiate in the unique hormonally induced microenvironment of the uterine mucosa (Vacca et al., 2011).

The Function of Uterine NK Cells

Nonpregnant Endometrium

What initiates menstruation is not fully understood but the first change in the morphology of the endometrium just prior to breakdown is the particular nuclear appearance of uNK cells. The nuclei are normally reniform but appear to fragment giving a superficial similarity to neutrophils—this is why they were called “endometrial granulocytes.” Because of their hormonal dependence and presence in the mucosa only during reproductive life, uNK cells are most likely involved in mucosal homeostasis, rather than in immune defense. (Classical T and B cells responses to infections can occur in the endometrium. The pathology of endometritis is characterized by the presence of abundant plasma cells and expansion of the small lymphoid follicles in the basal layer into germinal centers. Systemic infection by Mycobacteria TB can also result in classical endometrial granulomas.) It is thus possible that the angiogenic factors that uNK cells produce maintain the integrity of the arterial walls and when this is lost premenstrually breakdown ensues (King et al., 1989b). If pregnancy occurs the decidualization process of stromal cells that begins during the luteal phase of the menstrual cycle in the periarial regions (Streeter’s columns) continues. Part of the decidualization process is the change in the appearance of the media with loosening of the thick-walled muscle layer. The encircling of the arteries by uNK and their production of angiogenic mediators makes it likely that they contribute to the initial changes in the blood flow through these spiral arteries. Furthermore, uNK are also abundant beneath the basement membrane of the glands and secretion of factors such as epidermal growth factor (EGF) may contribute to the hypersecretory pregnant state of the glands and the stage of histiotrophic nutrition before the hemochorial placenta is established.

KIR and HLA Genes in Reproduction

If maternal immune cells do recognize and respond to the implanting trophoblast the receptor/ligand interactions are crucial to establish. The products of HLA class I and class II genes are dominant ligands for both NK and T cells and function for them to

survey the health of cells. The HLA genes are highly variable between individuals and encode proteins expressed on the cell surface of most cells in the body. However, in reproduction HLA class I molecules seem to serve an additional purpose. Villous cyto- and syncytiotrophoblast cells that are in contact with maternal blood circulating through the intervillous space are all devoid of HLA molecules. They also lack other ligands for immune cell receptors and adhesion proteins, and are thus inert to the immune cells of the maternal blood. In contrast, the invading EVT cells display a selective range of HLA molecules: no HLA class II nor HLA-A or B molecules, but high levels of HLA-C, and the nonclassical HLA-E and G molecules (Moffett-King, 2002). HLA-E and G genes do not vary much between individuals (oligomorphic), whereas HLA-C genes are highly variable or polymorphic with at least one thousand alleles that encode several hundred functional proteins. The receptors that bind to HLA-C are members of the Killer Immunoglobulin-like gene family (KIR) that are mainly expressed by both pbNK and uNK. The specificity of KIR for HLA-C depends on a dimorphism of two amino acids in the HLA-C sequence, which creates the C1 + HLA-C and C2 + HLA-C groups. KIR can impart either an activating or inhibitory signal to the NK cell depending on the length of the cytoplasmic tail, Long or Short. Inhibitory KIR2DL1 and activating KIR2DS1 bind C2 + HLA-C molecules, whereas inhibitory KIR2DL2/3 bind C1 + HLA-C. One set of HLA genes is inherited from each parent and a given individual therefore can be homozygous or heterozygous for C1 + or C2 + HLA-C. The KIR gene complex is also highly individual both in terms of gene numbers and allelic variation. The genes are inherited as two main KIR haplotypes, KIR A and KIR B, that can be used in genetic epidemiological studies to study disease associations. KIR A haplotypes contain only inhibitory KIRs (except KIR2DS4), whereas KIR B haplotypes contain additional activating KIRs (Fig. 2). Similar to HLA-C, an individual inherits KIR genes from both parents, and can therefore be KIR AA, AB, or BB. NK cells in KIR AA women will thus have no activating KIR compared to KIR AB or BB women. Because trophoblast cells are fetal in origin and will contain a mix of maternal and paternal genes, each pregnancy will be unique in terms of maternal KIR and fetal HLA-C genotypes (Moffett and Colucci, 2015).

When the KIR and C1 + or C2 + HLA-C genetic variants in mothers and their babies are genotyped and compared between those with normal pregnancies and those with pregnancy disorders, a role for uNK cells emerges. The Great Obstetric Syndromes (GOS) define pregnancy disorders associated with defective placentation, including preeclampsia (PE), stillbirth and fetal growth restriction (FGR), conditions associated with low birth weight. If the mother has a KIR AA genotype (meaning no activating KIR including the KIR2DS1 gene is present, Fig. 2), and the fetus carries a C2 + HLA inherited from the father, the pregnancy is at increased risk for PE (Fig. 3). Mothers carrying at least one KIR B haplotype (with the activating KIR2DS1 gene) are more likely to be protected from PE and FGR. In fact, the presence of a KIR2DS1 gene in combination with the fetus carrying paternal C2 + HLA increases the birth weight on average 200 g. This can be compared to the 50 g difference in birth weight seen between boys and girls. However, mothers with babies who are very large are at increased risk of obstructive labor, fistulae formation and fetal hypoxia. Notably, KIR BB mothers that carry multiple copies of KIR2DS1 are over-represented in pregnancies with such complications (Moffett et al., 2015).

How can these genetic findings be translated into uNK functions at the placental-uterine interface in the first trimester? If uNK cells are stimulated through KIR2DS1 in vitro, increased production of a range of soluble factors including granulocyte macrophage colony-stimulating factor (GM-CSF) occurs. EVT cells express the receptor for GM-CSF and when primary EVT cells are exposed to

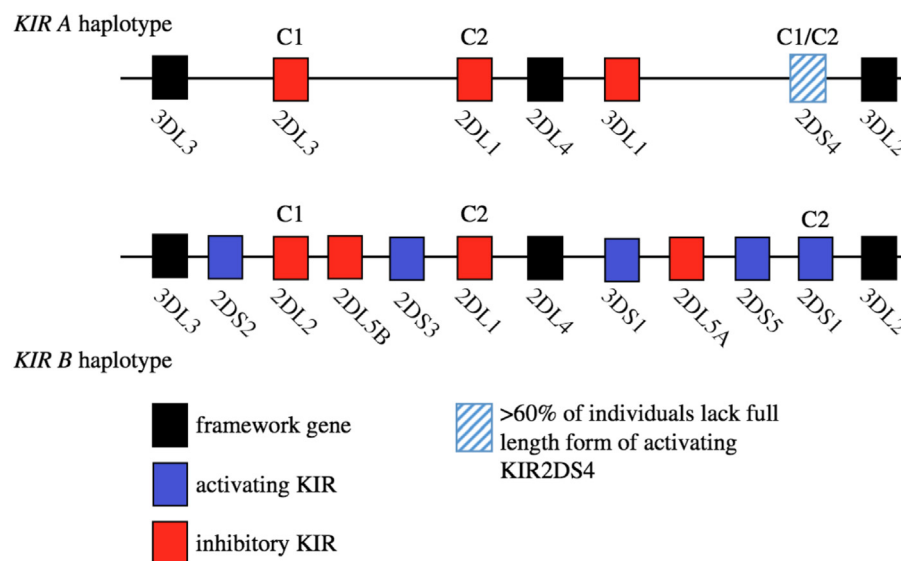


Fig. 2 Representative KIR A and B haplotypes of the KIR gene family, together with their known HLA-C binding specificities (C1 and C2) depicted above their cognate KIR receptors. KIR2DS4 binds a limited number of C1 and C2 allotypes. Activating KIR are shown as blue boxes, inhibitory KIR are shown in red. Framework KIR genes that are present in all haplotypes are shown in black. Note that many of the KIR shown on the B haplotype are not present in all individuals. Reproduced from Moffett, A., Hiby, S. E. and Sharkey, A. M. (2015). The role of the maternal immune system in the regulation of human birthweight. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **370** (1663).

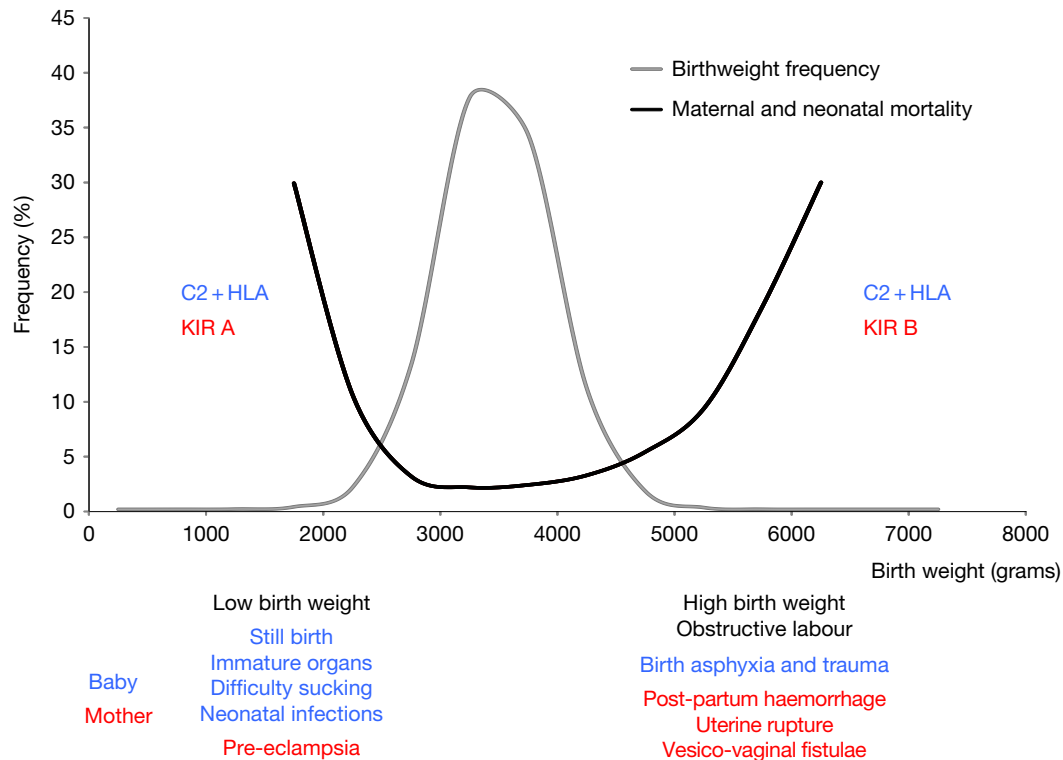


Fig. 3 Presence of KIR B is associated with increased birthweight. Distribution of birthweights in the Norwegian MoBa cohort correlates with increased neonatal morbidity. Adaptation of figure from Moffett, A., Hiby, S. E. and Sharkey, A. M. (2015). The role of the maternal immune system in the regulation of human birthweight. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* **370** (1663).

GM-CSF they are attracted towards its source (Xiong et al., 2013). This offers proof of principle for how activating KIR2DS1 genes influence trophoblast behavior (invasion) in a favorable manner: In the presence of C2 + HLA on EVT cells, uNK cells expressing KIR2DS1 can bind to it and be activated. By contrast, in KIR AA mothers (with no KIR2DS1), this interaction cannot take place and the EVT cells are more likely to invade insufficiently. Importantly, many women with KIR AA genotypes do reproduce normally meaning activating KIRs are not essential for human reproduction. Instead, it is possible other activating receptors yet to be identified are expressed by all uNK cells, and that this basal activation is triggered by interaction between any uNK cell and EVT cells. This basal activation may contribute to the general EVT invasion, on top of which the KIRs fine tune and balance the degree of invasion.

KIRs are expressed both on pbNK cells in the peripheral blood of women, and on uNK cells. The KIR repertoire on uNK cells is different from that on pbNK cells, both in the nonpregnant uterus (Ivarsson et al., 2016) and in the pregnant uterus (Sharkey et al., 2015) with a much higher proportion of CD56⁺ uNK expressing KIR specific for HLA-C (KIR2DL1/S1 and KIR2DL2/L3) than found on pbNK in the same woman. Moreover, the KIR repertoire on pbNK cells is stable with time, and the repertoire on uNK cells is recapitulated each menstrual cycle. This indicates that each woman follows a unique uNK cell KIR acquisition program. How the uNK cell KIR repertoire in the nonpregnant uterus is related to that on uNK cells in the decidua during early pregnancy remains unknown but is relevant to study because of the implications the KIR receptors have, as discussed above.

Other receptors may also be involved in interaction of uNK with EVT. Similar to KIR, LILRB1, expressed by < 40% of uNK cells, is a strong inhibitory receptor that binds all HLA class I molecules and has a particularly strong affinity for the homodimer of HLA-G that is uniquely expressed by EVT cells (Jabrane-Ferrat and Siewiera, 2014). The inhibitory NKG2A receptor is highly expressed by up to 95% of uNK cells, and so is its activating counterpart NKG2C, albeit at much lower levels (Ivarsson et al., 2016). They both bind the nonclassical HLA-E ligand, which is expressed on EVT, where it forms a ligand for NKG2A/C. Up to 10% of some human populations lack a functional NKG2C gene, which may influence the function of uNK cells and in turn have consequences for pregnancy outcome similar to that seen for KIR2DS1 absence.

KIR receptors expressed on NK cells in other tissues and blood function in the detection and elimination of virally infected cells. Particular KIR/HLA genetic variants associate with resistance/susceptibility to a range of viruses. In general individuals with a combination of a KIR AA and C1 + HLA-C genotype are better at controlling viral infections. This is in contrast with protection from GOS associated with a maternal KIR B and fetal C2 + HLA-C genotype. This has led to the idea that the great variation in these two genetic families, KIR and HLA—known to be outliers in the extent of polymorphism—is balanced by the need for successful reproduction and survival from infection: If a virus epidemic strikes a population, individuals with C1 + HLA-C and KIR AA genotypes will be more likely to survive to reproductive age. Once the epidemic is over individuals with C2 + HLA-C and KIR B genotypes will be

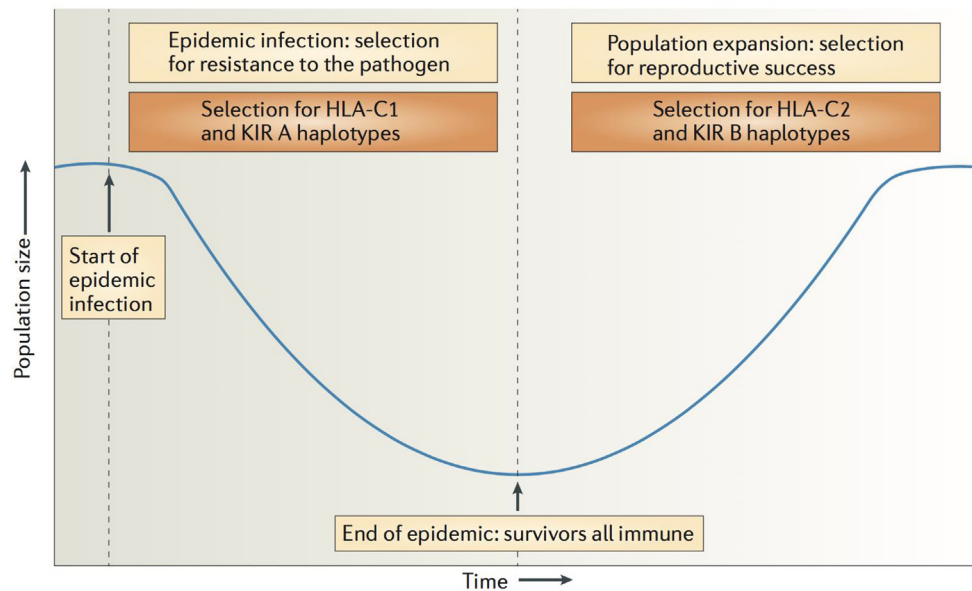


Fig. 4 Model for the maintenance of KIR A and B haplotypes and HLA-C1 and C2 epitopes in human populations. With the onset of an epidemic with a lethal virus individuals with the protective C1 + HLA-C (HLA-C1) and KIR A haplotypes will be more resistance to infection, leading to selection of such individuals. At the end of the epidemic the survivors that carry C2 + HLA-C (HLA-C2) and KIR B will have larger and more robust progeny resulting in selection of HLA-C1 and KIR B. Adaptation of figure from Parham, P. and Moffett, A. (2013). Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nature Reviews Immunology* **13** (2), 133–144.

able to reproduce better and will increase in frequency, until the next epidemic when the process may start again. Through human evolution this would result in the presence of C1 + and C2 + HLA-C, and KIR A and KIR B haplotypes individuals in all human populations, and this is indeed what is found (Parham and Moffett, 2013) (Fig. 4).

NK Cells and Fertility Tests

Many reports claim NK cells are dangerous in pregnancies and need suppressing to avoid killing the embryo but there is limited evidence for this view. As illustrated above a degree of uNK cell activity seems to be beneficial for pregnancy since it includes release of factors beneficial for implantation. Furthermore, uNK are phenotypically and functionally quite distinct from pbNK. Nevertheless, commercial blood tests that measure “NK cell numbers and activity” are offered to women at private infertility clinics. Women are then treated with a range of immunosuppressive drugs aimed at “suppressing” uNK activity. Future studies that link genetic, phenotypic, and functional data on uNK cells in large cohorts are needed if any form of useful NK cell tests are going to see the light of day (Moffett and Shreeve, 2015). Our understanding of their exact functional role in normal or disordered pregnancies is still very limited.

Summary and Future Perspectives

Since their discovery uNK cells have been suggested to influence pregnancy outcome via release of factors that act on the uterine arteries as well as invading trophoblast cells. Receptors expressed by uNK that have ligands on invading trophoblast cells have also been identified. Data supporting the importance of this interaction primarily comes from genetic studies, and insights into the cellular mechanisms are only beginning to emerge. The needed studies are limited by access to relevant study material such as endometrium and decidual tissue, and trophoblast cells. Acquiring primary trophoblast cells in sufficient numbers for in vitro studies is a limitation. A major stumbling block in the field is the lack of trophoblast stem cells in humans although these do exist in mice. The ability to perform in vitro studies is thus severely limited. The great heterogeneity afforded by KIR and HLA genes makes cohort studies of different tissue donors critical. Close collaborations between clinicians and immunologists are needed for this, as well as standardized methods to isolate and study uNK cells.

References

- Brosens, I., et al. (2011). The “great obstetrical syndromes” are associated with disorders of deep placentation. *American Journal of Obstetrics and Gynecology*, 204(3), 193–201.
- Bulmer, J. N., & Sunderland, C. A. (1984). Immunohistological characterization of lymphoid cell populations in the early human placental bed. *Immunology*, 52(2), 349–357.
- Bulmer, J. N., Williams, P. J., & Lash, G. E. (2009). Immune cells in the placental bed. *International Journal of Developmental Biology*, 54(2–3), 281–294.

- Ivarsson, M. A., et al. (2016). Composition and dynamics of the uterine NK cell KIR repertoire in menstrual blood. *Mucosal Immunology*, 10(2), 322–331.
- Jabrane-Ferrat, N., & Siewiera, J. (2014). The up side of decidual natural killer cells: New developments in immunology of pregnancy. *Immunology*, 141(4), 490–497.
- King, A., Birkby, C., et al. (1989a). Early human decidual cells exhibit NK activity against the K562 cell line but not against first trimester trophoblast. *Cellular Immunology*, 118(2), 337–344.
- King, A., Wellings, V., et al. (1989b). Immunocytochemical characterization of the unusual large granular lymphocytes in human endometrium throughout the menstrual cycle. *Human Immunology*, 24(3), 195–205.
- King, A., et al. (1991). CD3-leukocytes present in the human uterus during early placentation: Phenotypic and morphologic characterization of the CD56++ population. *Developmental Immunology*, 1(3), 169–190.
- Koopman, L. A., et al. (2003). Human decidual natural killer cells are a unique NK cell subset with immunomodulatory potential. *Journal of Experimental Medicine*, 198(8), 1201–1212.
- Loke, Y. W., & King, A. (1997). Immunology of human placental implantation: Clinical implications of our current understanding. *Molecular Medicine Today*, 3(4), 153–159.
- Marchand, F. (1895). Über die sogenannten dezidualen Geschwulste im Anschluß an normale Gebert, Blasenmole und Extrauterinschwangerschaft. *Msschr Geburtsh Gynak*, 419, 1–16.
- Moffett, A., & Colucci, F. (2015). Co-evolution of NK receptors and HLA ligands in humans is driven by reproduction. *Immunological Reviews*, 267(1), 283–297.
- Moffett, A., & Loke, C. (2006). Immunology of placentation in eutherian mammals. *Nature Reviews Immunology*, 6(8), 584–594.
- Moffett, A., & Shreeve, N. (2015). First do no harm: Uterine natural killer (NK) cells in assisted reproduction. *Human Reproduction (Oxford, England)*, 30(7), 1519–1525.
- Moffett, A., Hiby, S. E., & Sharkey, A. M. (2015). The role of the maternal immune system in the regulation of human birthweight. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 370(1663).
- Moffett-King, A. (2002). Natural killer cells and pregnancy. *Nature Reviews Immunology*, 2(9), 656–663.
- Parham, P., & Moffett, A. (2013). Variable NK cell receptors and their MHC class I ligands in immunity, reproduction and human evolution. *Nature Reviews Immunology*, 13(2), 133–144.
- Sharkey, A. M., et al. (2015). Tissue-specific education of Decidual NK cells. *Journal of Immunology*, 195(7), 3026–3032.
- Vacca, P., et al. (2011). Origin, phenotype and function of human natural killer cells in pregnancy. *Trends in Immunology*, 32(11), 517–523.
- Xiong, S., et al. (2013). Maternal uterine NK cell-activating receptor KIR2DS1 enhances placentation. *Journal of Clinical Investigation*, 123(10), 4264–4272.

PREGNANCY

Endocrinology of Pregnancy

Megan S Johnson, Daniel L Jackson, and Danny J Schust, University of Missouri, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Beginning in the early first trimester, maternal physiology undergoes significant alterations. These adaptive changes must satisfy the fetal requirements for life while sustaining the maternal host. The placenta orchestrates many of these adaptations and is the site of complex interactions between the maternal and fetal endocrine systems. Not merely a conduit for the transfer of oxygen, nutrients, and waste products, the placenta functions dynamically as a new, transient endocrine organ as it utilizes precursors and substrates from the maternal and fetal compartments to synthesize new hormones (Pasqualini and Chetrite, 2016). Thus, the mother, placenta, and fetus express and respond to physiologic needs using an assembly of complex, bidirectional hormonal cascades as the mode of communication. In turn, the maternal–placental–fetal unit influences every endocrine axis throughout pregnancy, from conception to parturition. Many of these changes have direct implications in terms of prenatal care.

Establishment of Early Pregnancy

Maternal sex steroids alter the structure and function of the endometrium prior to implantation. Specifically, the endometrium undergoes cyclic changes in response to maternal estrogen and progesterone that enhance its receptivity to implantation in the early luteal phase of the menstrual cycle. If implantation occurs, the placenta and corpus luteum contribute changes essential to the maintenance of pregnancy.

Human chorionic gonadotropin (hCG) is the hormone used clinically to test for pregnancy. hCG is produced by the syncytiotrophoblast of the developing placenta and is responsible for maintaining the corpus luteum. The corpus luteum in turn produces progesterone to support development of the placenta. Early collapse of the corpus luteum leads to a drop in progesterone levels and pregnancy loss, whereas exogenous progesterone supplementation can prevent spontaneous abortion in select cases. It is for this reason that women with IVF pregnancies, in which the corpus lutei have been disrupted, require progesterone supplementation to maintain pregnancy until the 10th week of gestation. By this point, the placenta has predictably taken over progesterone production, and supplementation to recapitulate corpus luteal support is no longer required. A transient drop in maternal serum progesterone levels demonstrates this luteal–placental shift. Following this transition, the placenta, a new, temporary endocrine organ, becomes the primary source of pregnancy hormones, utilizing maternal and fetal precursors to produce an extensive array of proteins and steroids.

Placental Hormones

Human Chorionic Gonadotropin

Placental hCG shares structural homology with the pituitary hormones LH, FSH, and TSH. These protein hormones share a common alpha subunit but have distinct beta subunits that confer receptor specificity and thus biologic function. Still, hCG can also weakly interact with the receptors for these other pituitary protein hormones. Its luteotrophic effects can be seen in the stimulation of LH receptors of the corpus luteum, leading to progesterone production. Additionally, as hCG peaks in the first trimester, its binding to TSH receptors and subsequent thyroid stimulation leads to negative feedback that, in turn, causes a transient decrease in TSH levels.

As previously mentioned, embryonic production of hCG is crucial to early pregnancy. The early, rapid production of hCG coupled with its relatively long half-life (24 h) leads to a predictable, logarithmic increase in maternal serum levels of this hormone in normal pregnancy. The beta subunit of hCG can be detected in maternal urine or serum within 6–12 days following conception. Pregnancies that are outside the uterus (ectopic) or destined to end in miscarriage produce lower than normal levels of hCG, which manifests as a less than expected increase over a fixed, generally 48 h, time frame. Measurement of the beta subunit of hCG in maternal serum is frequently clinically useful. For instance, serial measurement of quantitative beta HCG (β hCG) is used in clinical practice to differentiate between viable intrauterine pregnancies and nonviable pregnancies, including ectopic pregnancies. Conversely, placental disorders such as hydatidiform moles tend to present with quantitative β hCG levels as much as 10-fold higher than the upper limit of that seen in normal pregnancies. In cases of hydatidiform moles or other

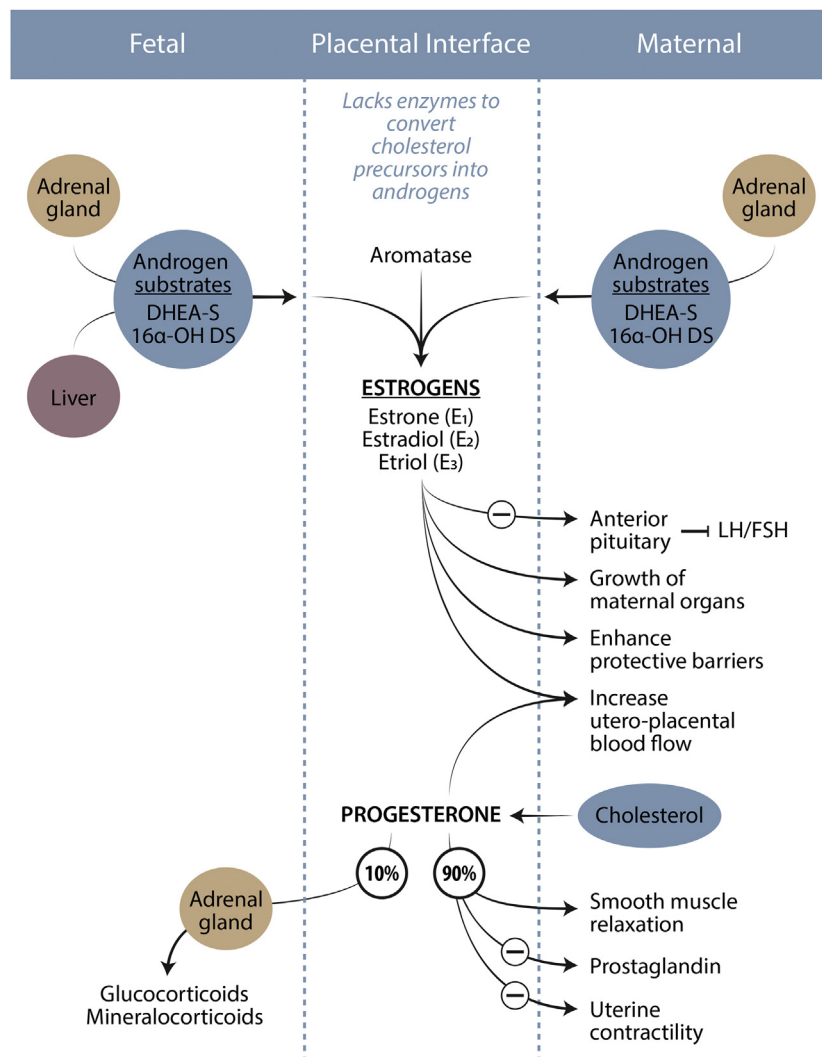


Fig. 1 Steroid hormone production by the maternal–placental–fetal unit. *DHEA-S*, Dehydroepiandrosterone sulfate; *16-OH-DS*, 16-Hydroxyl-dehydroepiandrosterone sulfate; *LH*, Luteinizing hormone; *FSH*, Follicle stimulating hormone.

trophoblastic disease, excessive β hCG levels can result in clinical thyrotoxicosis and thyroid storm due to the excessive cross-stimulation by hCG of TSH receptors.

Progesterone

Placental production of progesterone utilizes maternal cholesterol precursors (Fig. 1). The placenta lacks the enzymes (17 α -hydroxylase and 17,20-lyase) necessary to further convert progesterone to androgens, so the majority of progesterone is released from the placenta without further modification. Less than 10% of placental progesterone is transferred to the fetal compartment where it is converted to glucocorticoids and mineralocorticoids in the fetal adrenal glands. The remaining majority of placental progesterone (>90%) is released into the maternal circulation. Increased maternal progesterone leads to smooth muscle relaxation, affecting a wide range of maternal systems. Perhaps the most significant effect is the increased peripheral vasodilatation and resulting decrease in systemic vascular resistance (SVR) that occurs in normal pregnancy. This decrease in SVR is necessary to accommodate the expected 50% increase in maternal blood volume and is also associated with increased maternal cardiac output. Increases in maternal blood volume in pregnancy give delivering mothers a reserve to accommodate postpartum bleeding without cardiovascular collapse and could not be tolerated without the associated vasodilatation afforded by progesterone. Progesterone-mediated smooth muscle relaxation also contributes to ureteral dilatation, increasing risk of urinary tract infections, and esophageal sphincter relaxation, which, in combination with upward displacement of abdominal contents by the enlarging uterus, can lead to heartburn and gastroesophageal reflux disease (GERD). In the gallbladder, decreased smooth muscle contractility delays emptying and increases risk of maternal gallstones, while delayed bowel motility contributes to the nausea and constipation of pregnancy. Progesterone also

maintains uterine quiescence in human pregnancy by decreasing the expression of a number of genes related to myometrial contraction. Prostaglandin production is also inhibited by progesterone. Interestingly, a decrease in progesterone activity (and an increase in estrogen receptor expression) near term leads to parturition. Thus, progesterone supplementation in women at high risk of preterm birth delays parturition by preventing uterine contractions and maintaining cervical competence. Progesterone is therefore essential to many of the underlying physiologic changes necessary to support pregnancy and delivery. An understanding of these physiologic changes and their impact on maternal disease processes is essential to providing medical care for pregnant women.

Estrogen

Maternal serum estrogen levels rapidly increase early in pregnancy and peak at term, and the placenta is the major source of production. As mentioned above, the placenta lacks the necessary enzymes to convert cholesterol precursors into androgens. In a dramatic demonstration of maternal–fetal–placental interplay, androgens are supplied to the placenta from both the maternal and fetal adrenal glands and the fetal liver (Fig. 1). Placental aromatase then converts these androgen substrates (DHEA-S, 16-OH-DS) into estrogens, namely estrone, estradiol, and estriol. Estriol is essentially unique to pregnancy and serves as the major estrogen in maternal circulation during gestation. Because of this unique physiologic role and the fact that it is produced from fetal precursors, estriol has been studied as a surrogate marker of fetal well-being. Estriol levels are also used clinically as a component of prenatal screening for fetal aneuploidy.

Estrogens serve numerous physiologic roles during pregnancy. Through negative feedback on the anterior pituitary, estrogen ensures maternal resources will be devoted to the current pregnancy by suppressing FSH and LH production, thereby preventing development of additional ovarian follicles. Estrogen's major role in pregnancy is that of a trophic factor for both maternal and fetal tissues. Importantly, estrogens are responsible for growth of maternal organs, for example, enlargement of the uterus, expansion of pancreatic beta cell mass, and development of lactiferous glands and ducts in the breasts. Estrogens enhance several protective barriers in the pregnant woman, including formation of the "mucus plug" through cervical gland hypertrophy and mucin secretion, and reductions in vaginal pH through estrogen-mediated proliferation of vaginal lactobacilli. In concert with progesterone, estrogens increase uteroplacental blood flow through vascular proliferation, remodeling, and vasodilation, thus creating the low-resistance system crucial to ensuring adequate transfer of oxygen and nutrients to the growing fetus. Estrogens' trophic effects extend to fetal tissues as well, facilitating the maturation of fetal lung, liver, intestines, and CNS.

Human Placental Lactogen

Human placental lactogen (hPL) is a placental protein hormone secreted by the syncytiotrophoblast that rises steadily and peaks at 34 weeks of gestation. hPL shares structural homology with growth hormone (GH) and prolactin and can bind both receptors. In concert with human placental growth hormone (hPGH), hPL modulates maternal metabolism to meet the energy requirements of the developing fetus. Specifically, hPL and hPGH induce peripheral insulin resistance and thereby reduce maternal glucose utilization, while concomitantly increasing maternal lipolysis. The net result is an increased maternal reliance on fatty acids and ketones, leaving an abundant supply of glucose for the fetus. Increased fatty acids and ketones in the maternal circulation also serve as substrates for steroid hormone production by the placenta. Interestingly, insulin resistance and compensatory hyperinsulinemia resolve quickly after delivery, further affirming that these changes in maternal metabolism are driven specifically by diabetogenic placental hormones.

Thyroid Physiology During Pregnancy

Maternal Thyroid Physiology

Thyroid physiology changes dramatically in normal pregnancies. Under the influence of estrogen, the maternal liver increases production of thyroid-hormone-binding globulin (TBG), which sequesters free T3 and T4. This initiates a compensatory stimulation of thyroid hormone production driven by the hypothalamic–pituitary axis, and the thyroid gland enlarges by 15%–30%. As a result of these changes, total (free and TBG-bound) serum T3 and T4 levels increase in pregnancy, while free T3 and T4 remain unchanged or only marginally decrease. In the first trimester, the increased thyroid hormone production is uniquely driven by stimulation of the thyroid gland by the alpha subunit of placental hCG, which is structurally similar to TSH (Fig. 2). Following the 1st trimester, TSH steadily increases through gestation and peaks at term. Maternal peripheral thyroid hormone metabolism is also enhanced in pregnancy. The placenta expresses type II and type III deiodinases that, respectively, convert T4 into T3 and T3 into reverse T3. This process is essential to provide the necessary thyroxine for normal fetal brain development in the first trimester prior to the onset of fetal thyroid function.

Thyroid dysfunction is one of the most common complications of pregnancy and adversely affects fetal and maternal well-being, and an understanding of maternal thyroid physiology is essential to proper management.

Hypothyroidism in Pregnancy

Hypothyroidism affects about 2% of pregnancies and can result from iodine deficiency, autoimmune disease, drug induced hypothyroidism, or treatment for hyperthyroidism. The most common cause of hypothyroidism worldwide is iodine deficiency,

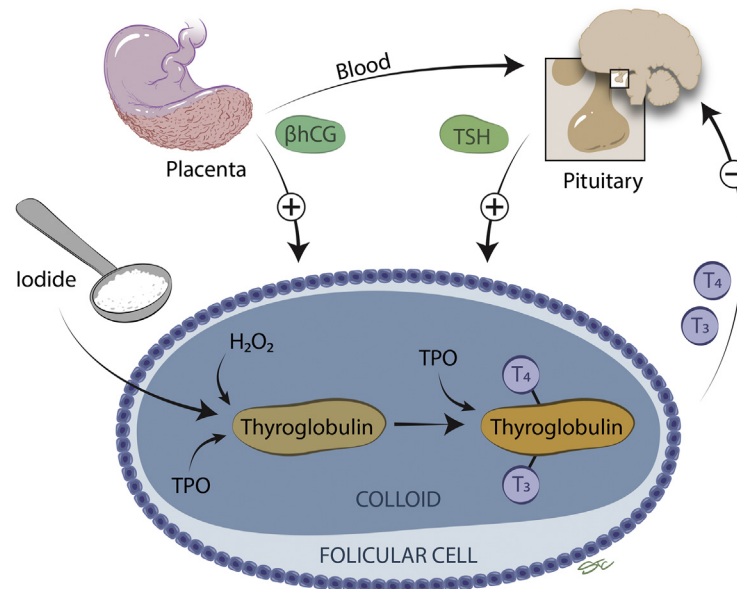


Fig. 2 Thyroid physiology during pregnancy. *bhCG*, Beta human chorionic gonadotropin; H_2O_2 , Hydrogen peroxide; *TPO*, Thyroid peroxidase; T_3 , Triiodothyronine; T_4 , Tetraiodothyronine; *TSH*, thyroid stimulating hormone.

although in the developed world where dietary iodine deficiency is rare, Hashimoto's disease is the most common underlying etiology. Hypothyroidism is diagnosed by elevated serum levels of TSH and low levels of T_4 (free or total corrected for pregnancy).

Normal fetal growth and development are dependent upon maternal thyroid hormone production and transport. While maternal TSH does not cross the placenta, maternal thyroxine does cross the placenta. In early gestation, maternal euthyroid status needs to be maintained to ensure that sufficient thyroid hormone, which is critical for the developing fetal brain, reaches the fetus. The fetal brain expresses thyroid hormone (TH) receptors in the first trimester. This is prior to the initiation of fetal thyroxine synthesis, which occurs at around 14 weeks of gestation. The critical period for brain development is therefore occurring during a time when thyroid hormone is exclusively supplied via the placenta from maternal-derived sources. Untreated maternal hypothyroidism is associated with decreased infant mental and motor function scores as well as lower IQ scores in childhood, and severe maternal hypothyroidism can lead to congenital cretinism characterized by growth failure and fetal cognitive and neuropsychological deficits. In fact, iodine deficiency, which affects > 30% of the global population, is the world's leading cause of preventable intellectual disabilities. Apart from fetal requirements, adequate thyroid function appears to also be necessary for healthy pregnancy. Pregnancies complicated by hypothyroidism are at risk for a number of adverse outcomes including preterm birth, preeclampsia, maternal anemia, placental abruption, low birth weight (Tong et al., 2016), and postpartum hemorrhage. In addition, antithyroid antibodies, such as those found in autoimmune thyroid disease, have been associated with early pregnancy loss and may even be a cause of recurrent pregnancy loss in some women (Smith and Schust, 2011).

Early diagnosis and management of hypothyroidism can minimize the risk of poor outcomes. While a recent, large metaanalysis demonstrated that universal thyroid function screening did not impact maternal and neonatal outcomes (Spencer et al., 2015), clinicians should have a low threshold for initiating testing in pregnant women with symptoms of hypothyroidism or a personal or family history of autoimmune endocrine disease. Unfortunately, the cluster of nonspecific symptoms that can accompany subclinical and even overt hypothyroidism (fatigue, lethargy, weight gain, cold intolerance, constipation, enlarged thyroid, hair loss, brittle nails, and dry skin) overlaps considerably with many normal pregnancy symptoms. The thyroid function of women with preexisting hypothyroidism should be tested at least each trimester, as replacement requirements increase throughout pregnancy and should be adjusted accordingly. Initial testing should include TSH levels, with free T_3 levels if TSH is found to be abnormal. Correct interpretation of thyroid function studies in pregnancy requires a thorough understanding of the normal physiologic changes that occur over the course of gestation. The recommended treatment for maternal hypothyroidism is oral levothyroxine.

Hyperthyroidism in Pregnancy

Hyperthyroidism, while less common in pregnancy than hypothyroidism, also places a burden on maternal physiology and can cause adverse pregnancy outcomes. Overt and subclinical hyperthyroidism complicate 1% of pregnancies. Prompt recognition and treatment are critical to prevent maternal and neonatal morbidity but can be complicated by the overlap of normal pregnancy symptoms and those of hyperthyroidism, including nausea/vomiting, heat intolerance, increased appetite, insomnia, fatigue, irritability or anxiety, and tachycardia. Weight loss, hypertension, tremor, eyelid lag, proptosis, and thyroid enlargement are symptoms

less common in pregnancy that may more directly suggest hyperthyroidism. Hyperthyroidism is characterized by a suppressed TSH and elevated levels of thyroxine. As in hypothyroidism, unadjusted serum free T3 and T4 levels, or total T3 and T4 levels corrected for pregnancy can be used for diagnostic purposes.

Subclinical hyperthyroidism in pregnancy is characterized by suppressed circulating TSH levels with free T3 and T4 in normal pregnancy ranges. A strong association between subclinical hyperthyroidism and adverse pregnancy outcomes has not been definitively established, though the presence of antithyroid antibodies in euthyroid women may be associated with early pregnancy loss (Thangaratinam et al., 2011). However, overt hyperthyroidism, with low TSH and high free T3 and T4 levels outside the normal pregnancy ranges, places pregnancies at risk for preterm labor, fetal growth restriction, stillbirth, preeclampsia, and maternal heart failure. Furthermore, fetal thyrotoxicosis caused by transplacental transfer of thyroid-stimulating antibodies may manifest as fetal tachycardia, fetal heart failure, nonimmune hydrops, and even fetal death.

By far the most common cause of hyperthyroidism in pregnancy is Graves' disease, which accounts for 90%–95% of cases and is mediated by maternal thyroid receptor-activating antibodies. It is important to distinguish Graves' disease from transient hyperthyroidism in early pregnancy, which does not require treatment. Transient hyperthyroidism of pregnancy results from hCG-mediated thyroid receptor activation. Although historically an evaluation for hyperthyroidism has been part of the evaluation for hyperemesis gravidarum, current guidelines do not support testing for hyperthyroidism in the setting of hyperemesis unless other signs or symptoms of hyperthyroidism are present. Treatment of transient hCG driven hyperthyroidism has not been shown to benefit maternal or fetal outcomes or improve symptoms of hyperemesis. Gestational trophoblastic disease can cause hyperthyroidism due to extremely high levels of hCG and cross-reactivity with the thyroid hormone receptor. Other uncommon causes of hyperthyroidism in pregnancy include thyroid nodules, toxic multinodular goiter, exogenous thyroid hormone exposure, thyroid cancer, ovarian teratomas with ectopic thyroid tissue (struma ovary), and pituitary tumors.

Regardless of the cause, overt hyperthyroidism and thyrotoxicosis require treatment to prevent maternal and neonatal morbidity. Thionamides prevent thyroid hormone synthesis by inhibiting thyroid peroxidase-mediated iodination of thyroglobulin tyrosine residues and are the mainstay of therapy in pregnancy. Both propylthiouracil (PTU) and methimazole cross the placenta and can cause congenital anomalies and fetal hypothyroidism, so in general the lowest dose of medication effective in treating maternal symptoms and resulting in biochemical maternal euthyroidism should be used. In particular, fetal exposure to methimazole in the first trimester is associated with fetal malformations such as choanal and esophageal atresia, facial anomalies, and aplasia cutis. For this reason, PTU, which further treats hyperthyroidism through inhibition of peripheral conversion from T4 to T3, is the recommended therapy during the first trimester. However, transition to methimazole is recommended for the second and third trimester to avoid the rare but serious risk of PTU-induced maternal hepatotoxicity. Both thionamides can cause leukopenia and, rarely, acute onset of agranulocytosis. Therefore, any evidence of maternal or fetal infection warrants a complete workup. Of note, radioactive ablation of the thyroid with I-131 is a mainstay of treatment in the nonpregnant population. This approach is absolutely contraindicated in pregnancy as it results in destruction of the fetal thyroid.

Women with overt hyperthyroidism are at risk for thyroid storm, which is a life threatening acute exacerbation of hyperthyroidism that can be triggered by infection, anemia, preeclampsia, labor, and vaginal or cesarean delivery. Thyroid storm is marked by a hypermetabolic state causing dysfunction in the following systems: thermoregulatory (hyperthermia), central nervous system (agitation, delirium, psychosis, coma), gastrointestinal (nausea, vomiting, diarrhea), hepatic (jaundice), and cardiovascular (tachycardia, pulmonary edema, arrhythmia). Immediate recognition and treatment is critical, and transfer to an intensive care unit and immediate consultation with physicians experienced in this condition can be life saving for both the mother and fetus. In crisis situations, high-dose thionamide therapy is initiated first, and iodine can be added after at least 1 h of stabilization to prevent release of T4. Beta blockers are often utilized to control maternal heart rate and further inhibit peripheral conversion of T4 to T3. Corticosteroids may also reduce this conversion and are thus an additional adjunct therapy. Delivery for nonreassuring fetal status should be delayed until the maternal condition has stabilized, as delivery can exacerbate thyroid storm.

Hypothalamic–Pituitary (–Adrenal) Axis Physiology

The anterior pituitary can double or triple in size during pregnancy. The increased mass is largely due to lactotroph hyperplasia and hypertrophy. Increases in maternal estrogens drive increased lactotroph production of prolactin, the levels of which rise throughout pregnancy. In contrast, pituitary somatotroph and gonadotroph size decreases in pregnancy, likely via negative feedback from placental-derived sex steroids; corticotroph and thyrotroph sizes remain unchanged. Pregnancy-related adaptations in pituitary function drive a number of alterations in HPA physiology.

Cortisol

Cortisol production and secretion from the adrenal cortex is stimulated by adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH is, in turn, under the control of corticotropin-releasing hormone (CRH) from the hypothalamus. Total and free plasma cortisol levels increase considerably during pregnancy, driven primarily by estrogen-mediated increases in hepatic production of cortisol-binding globulin. Increased pituitary ACTH secretion as well as increased adrenal responsiveness to ACTH also

contribute to the enhanced rate of cortisol synthesis. Additional CRH and ACTH are produced by the placenta. In contrast to the nonpregnant state in which cortisol exhibits negative feedback on CRH secretion from the hypothalamus and ACTH production in the pituitary, cortisol stimulates placental CRH in a feed-forward mechanism that continues throughout pregnancy. Although the resulting pregnancy-associated generation of increasingly elevated plasma free cortisol can reach levels approaching those seen in Cushing syndrome, the normal diurnal pattern of cortisol is maintained, and women do not typically exhibit symptoms of hypercortisolism. Interestingly, CRH interactions appear to play a role in determining timing of parturition ([Wadhwa et al., 1998](#)). Higher levels of maternal cortisol have been associated with increased risk of preterm delivery.

Aldosterone

Estrogen stimulates hepatic production of renin substrates, which initiates an upregulation of the renin–angiotensin–aldosterone system (RAAS). The subsequent rise in angiotensin II leads to an 8- to 10-fold increase in adrenal aldosterone production and a corresponding increase in maternal plasma aldosterone levels. These changes increase sodium and water retention and support maternal blood volume expansion. Consequent maternal hypertension is avoided due to decreased vascular sensitivity to angiotensin II. However, in the setting of gestational hypertension and preeclampsia, vascular resistance to angiotensin II does not occur and maternal hypertension ensues.

Androgens

The maternal adrenal gland is an important source of androgens in pregnancy, and adrenal production of DHEA and DHEA-sulfate doubles compared to the nonpregnant state ([Makieva et al., 2014](#)). Despite this, maternal serum levels remain at nonpregnant levels; the placenta utilizes the additional maternal androgens as substrates in estrogen production. Total circulating maternal testosterone levels rise in pregnancy, with the majority being bound by sex hormone binding globulin. The fetus serves as an additional source of DHEA, and thereby contributes to placental estrogen synthesis.

Hormonal Control of Glucose Regulation

Fetal Fuel Requirements Drive Alterations in Maternal Metabolism

Maternal glucose is the primary energy source for the fetus, and the maternal host is the sole provider of that fuel. Glucose transport to the fetus across the placenta is via facilitated diffusion through the sodium-independent transporter protein Glucose transporter 1. Beginning early in pregnancy, fetal metabolic requirements drive alterations in maternal insulin secretion and glucose metabolism. Notable changes include relative maternal hyperinsulinemia, insulin resistance, fasting hypoglycemia, postprandial hyperglycemia, and increased maternal plasma lipid concentrations. The net result is a shift in maternal metabolism toward fatty acid utilization, thereby preserving glucose for fetal use.

Maternal pancreatic beta cell mass expands by approximately 50% in pregnancy. Beta cell hyperplasia and hypertrophy are stimulated by estrogen, progesterone, and human placental lactogen and result in increased insulin secretory capacity. While basal insulin concentrations remain stable, secretion in response to food increases, resulting in a net hyperinsulinemic state in the pregnant mother. This is accompanied by relative insulin resistance and thus decreased insulin-mediated glucose transport to maternal tissues. Insulin resistance is mediated primarily by the placental hormones hPL and hPGH. Increased maternal cortisol, TNF- α , leptin, and decreased adiponectin also contribute to increased maternal insulin resistance. These physiologic changes confer insulin resistance through decreased insulin receptor tyrosine kinase autophosphorylation and a decrease in insulin signal transduction ([Friedman et al., 1999](#)). This is evidenced by altered expression of the insulin receptor downstream signaling molecules IRS-1, IRS-2, PI3-K, and GLUT-4 in women with gestational diabetes ([Colomiere et al., 2010](#)).

The net result of these changes is a unique physiologic state driven by placental hormones. Maternal insulin resistance increases, leading to increased peak glucose levels following meals. This is coupled with accelerated maternal lipolysis and increased maternal utilization of triglycerides, cholesterol and fatty acids. The end result is increased availability of glucose as an energy source for the fetus and placenta.

Gestational Diabetes

Insulin resistance peaks in the third trimester of pregnancy, and if the increase in insulin secretion is not commensurate, gestational diabetes (GDM) develops. Put another way, GDM manifests when increased maternal insulin secretion is not sufficient to counter the increased insulin resistance caused by placental hormones. Multiple sets of diagnostic criteria are used to diagnose GDM, and depending on whether one step or two step screening is used, the incidence in pregnancy ranges from 3% to 25%. Prevalence also varies according to ethnicity and increases with increasing maternal age. Women with GDM are at increased risk of urinary tract infections, pyelonephritis, hypertension, and preeclampsia. Pregnancies of women with GDM are also at risk of polyhydramnios, preterm delivery, and cesarean delivery. Fetal risks include macrosomia, intrauterine growth retardation (IUGR), stillbirth, birth injury, and, with poor glycemic control near the time of delivery, neonatal acidemia and hypoglycemia. Treatment of GDM reduces the risk of macrosomic infants, serious perinatal complications, and maternal hypertensive disorders ([Crowther et al., 2005](#); [Landon](#)

et al., 2009). Nutritional and lifestyle modifications are critical to this goal and can be effective interventions, but early transition to pharmacologic therapies is advised if dietary changes and exercise do not adequately control blood glucose values consistently. The American Diabetes Association and the American Congress of Obstetricians and Gynecologists recommend insulin over oral hypoglycemic agents as the first line treatment for gestational diabetes mellitus (GDM). The reason for this recommendation is that oral hypoglycemic agents such as metformin and glyburide cross the placenta, while insulin does not. There is some data to suggest that in utero exposure to oral hypoglycemics confers additional fetal risks beyond those seen in GDM. Neonatal outcomes are also improved with insulin use as compared to oral hypoglycemics. Women with GDM have up to 70% increased risk of type 2 diabetes mellitus (T2DM) later in life, with the common mechanism likely being increased baseline insulin resistance. Thus, women who had GDM should undergo postpartum screening for abnormal glucose metabolism.

Preexisting Diabetes in Pregnancy

Because the complications of GDM detailed above are related to suboptimal glycemic control, pregnancies in women with preexisting diabetes have similar risks. However, due to the earlier fetal exposure to potentially elevated glucose levels during embryogenesis, preexisting diabetes carries additional risks of spontaneous abortion and congenital anomalies of the cardiac and central nervous systems. As the diabetogenic placental hormones facilitate an environment of insulin resistance, insulin requirements for women with preexisting diabetes increase throughout pregnancy, and many women on oral hypoglycemic agents prior to pregnancy will need to transition to insulin therapy. Close blood sugar monitoring and aggressive pharmacological management is necessary to prevent complications and optimize conditions for delivery. An ultrasonographic assessment of fetal morphology at 20 weeks and fetal echocardiography at 24 weeks of gestation can detect most major fetal anomalies, and antepartum fetal surveillance recommendations are the same as in GDM. It is also noteworthy that the increased lipolysis and catabolism of fatty acids that occur in normal pregnancy predispose pregnant type I diabetics to the development of diabetic ketoacidosis (DKA), a life-threatening condition characterized by acidemia, hyperglycemia and profound dehydration and electrolyte abnormalities.

Maternal diabetes accounts for a substantial portion of stillbirth in the developed world. As such, women with pregestational diabetes and women with gestational diabetes requiring medical treatment undergo serial antenatal testing in the third trimester in an attempt to prevent stillbirth. Due to the increased risk of stillbirth, delivery is typically recommended at 39 weeks of gestation, with earlier delivery indicated in the setting of poor glycemic control.

Conclusion

Starting with early gestation and continuing through parturition, the maternal endocrine system undergoes profound alterations to facilitate implantation, growth, development, and delivery of a healthy neonate. These changes are generally beneficial to the developing fetus, but in the presence of underlying maternal comorbidities can occasionally be maladaptive. A thorough understanding of maternal endocrine changes is essential to the provision of quality prenatal care with the goal of optimizing maternal and fetal outcomes.

References

- Colomiere, M., Permezel, M., & Lappas, M. (2010). Diabetes and obesity during pregnancy alter insulin signaling and glucose transporter expression in maternal skeletal muscle and subcutaneous adipose tissue. *Journal of Molecular Endocrinology*, 44(4), 213–223.
- Crowther, C. A., Hiller, J. E., Moss, J. R., McPhee, A. J., Jeffries, W. S., Robinson, J. S., & Australian Carbohydrate Intolerance Study in Pregnant Women (ACHOIS) Trial Group. (2005). Effect of treatment of gestational diabetes mellitus on pregnancy outcomes. *New England Journal of Medicine*, 352(24), 2477–2486.
- Friedman, J. E., Ishizuka, T., Shao, J., Huston, L., Highman, T., & Catalano, P. (1999). Impaired glucose transport and insulin receptor tyrosine phosphorylation in skeletal muscle from obese women with gestational diabetes. *Diabetes*, 48(9), 1807–1814.
- Landon, M. B., Spong, C. Y., Thom, E., Carpenter, M. W., Ramin, S. M., Casey, B., Wapner, R. J., Varner, M. W., Rouse, D. J., Thorp, J. M., Jr., Sciscione, A., Catalano, P., Harper, M., Saade, G., Lain, K. Y., Sorokin, Y., Peaceman, A. M., Tolosa, J. E., Anderson, G. B., & Eunice Kennedy Shriver National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. (2009). A multicenter, randomized trial of treatment for mild gestational diabetes. *New England Journal of Medicine*, 361(14), 1339–1348.
- Makieva, S., Saunders, P., & Norman, J. (2014). Androgens in pregnancy: Roles in parturition. *Human Reproduction Update*, 20(4), 542–559.
- Pasqualini, J. R., & Chetrite, G. S. (2016). The formation and transformation of hormones in maternal, placental and fetal compartments: Biological implications. *Hormone Molecular Biology and Clinical Investigation*, 27(1), 11–28.
- Smith, M. L., & Schust, D. J. (2011). Endocrinology and recurrent early pregnancy loss. *Seminars in Reproductive Medicine*, 29(6), 482–490.
- Spencer, L., Bubber, T., Bain, E., & Middleton, P. (2015). Screening and subsequent management for thyroid dysfunction pre-pregnancy and during pregnancy for improving maternal and infant health. *Cochrane Database of Systematic Reviews*, 9, CD011263.
- Thangaratnam, S., Tan, A., Knox, E., Kilby, M. D., Franklin, J., & Coomarasamy, A. (2011). Association between thyroid autoantibodies and miscarriage and pre-term birth: A meta-analysis of the evidence. *British Medical Journal*, 342, d2616.
- Tong, Z., Xiaowen, Z., Baomin, C., Aihua, L., Yingying, Z., Weiping, T., & Zhongyan, S. (2016). The effect of subclinical maternal thyroid dysfunction and autoimmunity on intrauterine growth restriction: A systematic review and meta-analysis. *Medicine (Baltimore)*, 95(19), e3677.
- Wadhwa, P. D., Porto, M., Garite, T. J., Chic-DeMet, A., & Sandman, C. A. (1998). Maternal corticotropin-releasing hormone levels in the early third trimester predict length of gestation in human pregnancy. *American Journal of Obstetrics and Gynecology*, 179(4), 1079–1085.

Further Reading

- Clark, S. M., Saade, G. R., & Snodgrass, W. R. (2006). Pharmacokinetics and pharmacotherapy of thionamides in pregnancy. *Therapeutic Drug Monitoring*, 28(4), 477–483.
- Committee on Practice Bulletins – Obstetrics. (2017). Gestational diabetes mellitus: Practice bulletin no. 180. *Obstetrics and Gynecology*, 130, e17–e31.
- Committee on Practice Bulletins – Obstetrics. (2015). Thyroid disease in pregnancy: Practice bulletin no. 148. *Obstetrics and Gynecology*, 125, 996–1005.
- Reis, F. M., Fadalti, M., Florio, P., & Petraglia, F. (1999). Putative role of placental corticotropin-releasing factor in the mechanisms of human parturition. *Journal of the Society for Gynecologic Investigation*, 6(3), 109–119.
- Resnik, R., Creasy, R., Iams, J., Lockwood, C., Moore, T., & Greene, M. (2013). *Creasy and Resnik's maternal fetal medicine: Principles and practice* (7th edn.). Philadelphia: Saunders Elsevier.

Fetal Maternal Unit

Marisol Castillo-Castrejon, Thomas Jansson, and Theresa L Powell, University of Colorado, Aurora, CO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Pregnancy leads to extensive changes in maternal physiology, which promote metabolic, cardiovascular and immunological adaptations critical for maintaining pregnancy and supporting fetal growth. The placenta is a transitory fetal organ that serves as the primary interface between the mother and the fetus, establishing an immunological barrier and performing critical functions such as production of pregnancy-related hormones and transport of nutrients and oxygen from the mother to the developing fetus. This chapter discusses the fetal-maternal unit by describing placental structure among mammalian species and concisely reviewing placental endocrine and transfer functions. The importance of the fetal membranes and decidua, which represent other sites where maternal and fetal tissues interact, will also be discussed.

Placental Unit

The placenta is a complex, transient organ that constitutes the primary interface between the mother and the fetus throughout pregnancy. It is formed after the implantation of the embryo in the uterine lining. Placental development involves complex cellular and molecular interactions between maternal and embryonic tissues such as cell proliferation and migration and immunomodulation. In mammals, placental morphology and structure varies but its function remains essentially the same in all species. The placenta performs a number of critical physiological, endocrinological and immunological functions, which are important to establish and maintain an optimal intrauterine environment for the development and growth of the fetus.

Diversity of the placental structure

The establishment of an intimate contact between mother and embryo in early pregnancy is followed by critical steps of placental development with varying timing between species. The structure of the placenta is also dependent on the anatomical structure of the uterus which differs among mammalian species. There are three main types of uteri: (a) double uterus: with two cervixes and two independent uterine horns, found in rodents; (b) bicornuate uterus: with one cervix, one uterine body and two communicating uterine horns, found in ruminants, and carnivores; (c) simplex uterus: with one cervix and one uterine body without uterine horns, found in primates and bats (Chavatte-Palmer and Tarrade, 2016).

The placenta can be classified based on morphology, histological structure and interdigitation of the feto-maternal interface (Fig. 1) (Furukawa, S. et al. (2014), Enders and Blankenship, 1999). Based on gross shape or on the distribution of the feto-maternal exchange surface area that covers the chorionic sac enclosing the fetus, four main types of placenta are recognized: (i) diffuse: covers the entire surface of the uterine epithelium, found in pigs and horses; (ii) multi-cotyledonary: characterized by distinct placental regions or cotyledons occupying the entire surface of the endometrium, found in ruminants; (iii) zonary: feto-maternal exchange zone that forms a belt around the chorionic sac, found in carnivores; (Sobrevia, L. et al.) bidiscoid/discoid: characterized by a single or double disc where the maternal-fetal exchange area is confined to a circular or oval area, found in primates, rodents and humans (Chavatte-Palmer and Tarrade, 2016).

Based on the histology of the feto-maternal interface, the three main placental types are epitheliochorial, endotheliochorial and hemochorial. *Epitheliochorial interface* is the most superficial and lacks significant invasion of the uterine lining by trophoblast cells. No loss or invasion of the maternal tissues occurs and no cell layers are removed. There are three maternal layers (vascular endothelium, uterine connective tissue, uterine epithelium) and three fetal layers (vascular endothelium, fetal connective tissue and trophoblast) that separate maternal and fetal blood supplies. In the *endotheliochorial interface*, two maternal cell layers (uterine epithelium and connective tissue) are removed after implantation. The maternal vascular endothelium remains and comes into direct contact with the trophoblast cells, fetal connective tissue and fetal endothelium. The *hemochorial placenta*, is the most invasive type and all three cell maternal layers (vascular endothelium, uterine connective tissue and uterine epithelium) disappear, allowing direct contact between the maternal blood and the syncytiotrophoblast. Hemochorial placenta can also be classified based on the number of trophoblast layers; namely hemodichorial (two trophoblast layers) which is the case for rabbits and hemotrichorial (three trophoblast cell layers) in rats and mice. The human placenta is hemochorial, where a single trophoblast layer (the syncytiotrophoblast) constitutes the primary barrier between maternal blood in the intervillous space and the fetal capillary endothelium.

The number of cell layers in the feto-maternal interface influences placental transfer efficiency for example, the ability of molecules to cross the placenta in either direction is affected by the distance or thickness of the cellular barrier between maternal and fetal blood. An invasive type of placenta such as the hemochorial placenta of rodents and primates, with a minimal number of cell layers between maternal and fetal circulations, is characterized by an efficient exchange.

Placentas are also classified based on their interdigitation: (i) folded: is the simplest form and is characterized by small folds that interlock with corresponding endometrial folds (Hafez, 2017); (ii) lamellar: a more extensive array of branched folds that increase adherence and facilitate exchange by increasing the surface area; (iii) villous: trophoblast projections form tree-like highly branched

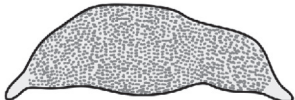
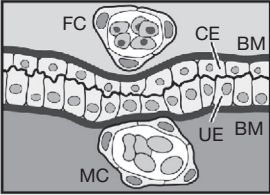
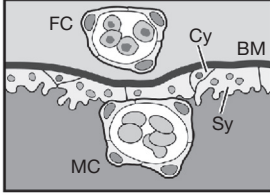
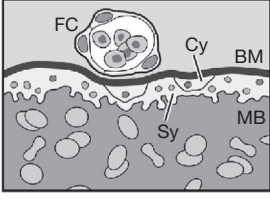
Representative Species	Pregnancy Length (Days)	Placental Morphology	Placental Interdigitation	Feto-Maternal Interface
	308-336	 Diffuse Cover over the entire surface of the uterine luminal epithelium with formation of folds/villi.	 Horse (a)	 Epitheliochorial Most superficial placenta with lack of significant invasion of the maternal tissues. No maternal layers or connective tissue are removed. Three maternal and three fetal layers are present. Synepitheliochorial is used to describe to placenta of ruminants. Trophoblasts (binucleate cells) fuse with a single uterine epithelial cell, giving rise to trinucleate cells or multinucleate structures of mixed fetal and maternal origin.
	115-122		 Pig (b) (a) Villous Chorion forms tree-like villi which interdigitate into corresponding endometrial folds. (b) Folded Chorion forms simple small folds that interlock with corresponding endometrial folds.	
	279-287	 Multi/Cotyledonary Characterized by distinct placental regions of the endometrium, also known as caruncles. These caruncles are protuberances in the uterine wall with vascular adaptations to establish fetomaternal interface.	 Villous Chorion forms tree-like villi which interdigitate into corresponding endometrial folds.	
	152			
	150			
	504-616	 Zonary Intimate interdigitating contact zone that forms a belt around the chorionic sac.	 (a)	 Endotheliochorial Maternal uterine epithelium and connective tissue disappear after implantation. Fetal chorionic epithelium comes into direct contact with maternal endometrium epithelium.
	93-112		 (b)	
	58-68		(a) Lamellar More extensive array of branched folds that increase adherence and facilitate exchange. (b) Labyrinthine Maternal blood circulates between the syncytiotrophoblast.	
	259-280	 Bidiscoid/Discoide Characterized by single or double disc. Interaction is confined to the circular area.	 Villous Chorion forms tree-like villi which interdigitate into corresponding endometrial folds.	 Hemochorial The most invasive. Maternal connective tissue layers disappear leading to direct contact between chorionic epithelium and maternal blood.
	134-243			
	31			
	21			

Fig. 1 Classification of the placenta by morphology, interdigitation and interface histology in *representative species*. FC, fetal capillary; CE, chorionic epithelium; BM, basement membrane; UE, uterine epithelium; MC, maternal capillary; Cy, cytotrophoblast; Sy, syncytiotrophoblast; MB, maternal blood.

villi which interdigitate into endometrial folds; (Sobrevia, L. *et al.*) labyrinthine: is the most structurally elaborate type, with trophoblast projections interconnected in a web-like structure, leaving narrow channels for maternal blood flow.

Functions of the placenta

Endocrine

The placenta secretes hormones and other signaling molecules to the maternal circulation, which are critical for the establishment and maintenance of pregnancy by regulating the maternal physiological adaptations to pregnancy, and to the fetus, which promote fetal development and growth (Costa, 2016). In addition, placental hormones are believed to play a role not only in placental development but also the initiation of labor and breast feeding (Costa, 2016). Abnormal production of placental hormones may contribute to the development of pregnancy complications such as preeclampsia. Hormones are synthesized and secreted by the trophoblast and its concentration vary widely between species. The major source of hormone production is the syncytiotrophoblast — a differentiated multinucleated subtype of trophoblast with no proliferative capacity and in intimate contact with maternal blood in the case of hemochorial placentas.

Steroids hormones are one important class of molecules secreted by the placenta. Progesterone, synthesized from maternal cholesterol in the syncytiotrophoblast mitochondria, is required for successful embryo attachment and implantation and essential to maintain pregnancy (Halasz and Szekeres-Bartho, 2013). Progesterone contributes to immune-tolerance by suppressing the maternal cell-mediated immunity to prevent rejection of the fetus, inhibits myometrial contractility and promotes uterine quiescence throughout pregnancy (Romero *et al.*, 2014, Ruddock *et al.*, 2008). Circulating maternal progesterone concentrations increase throughout pregnancy and decrease following labor and placental delivery. In addition, progesterone prepares the mammary gland for lactation by enhancing its proliferation and prevents lactation until labor.

Estrogens such as estrone (E₁), 17 β -estradiol (E₂), estriol (E₃) and esterol (E₄) are synthesized by the placenta. Maternal serum estrogen concentrations increase profoundly during pregnancy, peaking at term. Estradiol is the most abundant estrogen, which has an array of functions in pregnancy, such as promoting receptor-mediated uptake of LDL cholesterol for placental steroid production, increasing uteroplacental blood flow and preparing breast tissue for lactation by increasing prolactin synthesis and secretion (Corcoran *et al.*, 2014). In addition, estradiol is involved in labor as it promotes myometrial contractility. Moreover, estradiol stimulates the proliferation of the mammary gland preparing this tissue for breastfeeding and it is involved in maternal metabolic changes such as promoting insulin biosynthesis, hyperlipidemia, fat storage and inhibits lipolysis (Hur *et al.*, 2017).

The placenta secretes polypeptide hormones such as human chorionic gonadotropin (hCG), which is detectable in maternal blood as early as two days after implantation and is utilized in most common pregnancy tests. There are five independent variants of hCG, they all share a common α -subunit and a distinct β -subunit that confers specificity. hCG has multiple functions during pregnancy such as promotion of angiogenesis in the uterine vasculature, maintaining quiescence of the myometrium and contributing to the immunotolerance at the fetal-maternal interface (Fournier *et al.*, 2015). The syncytiotrophoblast is the main source of hCG. A unique function of hCG is to maintain the secretion of progesterone and estradiol from the corpus luteum during the first 6 weeks of pregnancy. In addition, hCG promotes the fusion and differentiation of cytotrophoblast to syncytiotrophoblast cells and thus generating the villous trophoblastic layer that characterizes the hemochorial placentation in humans (Cole, 2012). Total hCG concentration in the maternal circulation increases rapidly during the first trimester, reaches highest concentration at 10 weeks of gestation and declines slowly until term.

Human placental lactogen (hPL), also known as chorionic somatomammotropin, is a polypeptide hormone that is mainly synthesized by the syncytiotrophoblast. It is detected in maternal circulation after 3–6 weeks of gestation, increases throughout gestation and disappears after placental delivery. The main functions of hPL are the regulation of maternal lipid and carbohydrate metabolism and it may contribute to the insulin resistance emerging in mid to late gestation to ensure nutritional support for the developing fetus and maintain metabolic homeostasis in the pregnant mother (Newbern and Freemark, 2011).

Placental growth hormone (Than *et al.*, 2014.) is a single-chain peptide hormone, structurally related to pituitary growth hormone, hCG and prolactin. Up to the first 15 to 20 weeks of pregnancy, pituitary growth hormone is the main form of growth hormone present in the maternal circulation. From mid gestation until term, placental GH, synthesized by the syncytiotrophoblast, gradually replaces pituitary GH (Newbern and Freemark, 2011). Secretion of placental GH is regulated in part by placental size and maternal glucose. The primary function of GH is to ensure nutrient availability to the fetus by stimulating gluconeogenesis and lipolysis in the maternal compartment. Moreover, placental GH functions as an insulin antagonist and decreases maternal glucose utilization (Feldt-Rasmussen and Mathiesen, 2011).

The levels of adipocyte-derived signaling molecules such as leptin, resistin, adiponectin and pro-inflammatory cytokines have been reported to be associated to various pregnancy disorders and might affect the materno-fetal interface. Leptin appears to modulate female reproductive physiology at multiple levels including centrally via the hypothalamic-pituitary-gonadal axis, and by peripheral and local regulation. Leptin is mainly secreted by adipocytes, has important roles in regulation of body weight, appetite and energy homeostasis and its plasma levels are positively correlated to the amount of body fat. In pregnancy, leptin is also synthesized by the syncytiotrophoblast and released into both maternal and fetal circulations. Maternal circulating levels of leptin increase significantly in early pregnancy, reaching 30% higher levels at 12 weeks of gestation compared with pregravid women. Maternal leptin levels then peak in late second or early third trimester and decreases to pregravid concentrations immediately after delivery (Hauguel-De Mouzon *et al.*, 2006). Maternal body mass index is positively associated with leptin concentrations during pregnancy (Jansson *et al.*, 2008). It has been proposed that leptin released from the placenta may modulate maternal metabolism and partition

of nutrients to the fetus. The human placenta expresses leptin receptors, suggesting that leptin may regulate placental function. Indeed, leptin may act in a paracrine or endocrine manner to modulate angiogenesis and immunomodulation (Perez-Perez et al., 2013, Herrid et al., 2014), stimulate the secretion of hCG and IL-6 by human trophoblast cells in culture (Cameo et al., 2003), promote trophoblast invasion and activate placental nutrient transporters. Based on these observations, it has been speculated that high maternal leptin levels may be a link between maternal obesity and fetal overgrowth (Jansson et al., 2003).

Contrary to leptin, adiponectin promotes insulin sensitivity, has anti-inflammatory and anti-atherogenic properties and adiponectin levels are negatively correlated with body fat. In normal pregnancies, maternal serum adiponectin increases during first trimester and subsequently undergoes a modest decrease in maternal serum levels in the second and third trimester. Serum levels of maternal adiponectin are inversely correlated with birth weight and low maternal adiponectin is associated with increased risk of delivering large for gestational age infants by obese pregnant women or with gestational diabetes (Jansson et al., 2008). Adiponectin is secreted by maternal adipose tissue and modulates placentation by stimulating trophoblast migration and differentiation via specific receptors. Whereas adiponectin promotes insulin sensitivity in skeletal muscle and liver, recent findings show that adiponectin inhibits insulin signaling in the placenta, thereby reducing insulin-stimulated amino acid transport and subsequently decreasing fetal growth. Adiponectin does not cross the placenta and fetal adiponectin is positively associated to fetal adiposity and growth (Aye et al., 2013). In addition, adiponectin might promote trophoblast cells evasion of the maternal immune system by increasing the expression of cell surface molecules involved in the downregulation of maternal immune response to fetal antigens (D'ippolito et al., 2012).

Resistin is a signaling molecule induced during adipogenesis and is positively correlated with obesity. During pregnancy, maternal serum levels of resistin in early pregnancy are similar to those of non-pregnant women but increases significantly during the third trimester. Resistin is produced by the placenta, mainly by the syncytiotrophoblast. *In vitro* models using primary human cytotrophoblast cells and choriocarcinoma cell lines, demonstrated that resistin can modulate placental function by modifying the expression and activity of glucose transporters in placental cells. In low concentrations, resistin enhances glucose uptake and at high concentrations, it impairs this process (Di Simone et al., 2009). In addition, resistin has been linked to trophoblast invasiveness by upregulating the secretion and activity of metalloproteinases (Mu et al., 2006). Syncytiotrophoblast cells also synthesize other adipocyte-derived proteins such as visfatin, fibroblast growth factor (FGF21), placental protein 13 (PP13) and pregnancy-associated plasma protein A (PAPP-A) that may contribute to the establishment and maintenance of pregnancy (Than et al., 2014) (Campos et al., 2008) (Mesdaghi-Nia et al., 2016).

In conclusion, the placenta synthesizes and secretes numerous hormones and peptides that are critical for the regulation of the different stages of human pregnancy, ranging from, implantation to labor and also includes promoting maternal physiological adaptations to pregnancy and the initiation of breastfeeding. Deficient or altered production of these factors may alter placental development and function.

Transport

The human placenta is a highly specialized organ that transports nutrients from mother to fetus as well as removes waste products from the gestational compartment. Exchange between the mother and fetus relies on the highly differentiated barrier between the maternal and fetal circulations. Placental capacity for transport of nutrients increases with fetal demand as gestation progresses. Placental transport involves cellular mechanisms that participate in the delivery of macronutrients (glucose, amino acids and lipids), water, ions, vitamins, minerals and oxygen. All of these molecules reach fetal blood by traversing the placental barrier using a variety of mechanisms: simple diffusion, facilitated diffusion, active transport or endocytosis/vesicular transport. Vectorial transport across the placental barrier is established through the polarized distribution specific transporters in the syncytiotrophoblast microvillous membrane facing the maternal circulation and the basal membrane facing the fetal capillary. Details of the transport functions in the placenta are provided in the chapters *Placental transfer* and *Placental nutrient transport*.

Imprinted genes in the placenta

Most mammalian cells express their autosomal genes from two parental alleles. However, at some loci the allele inherited from one parent is suppressed which results in imprinted or monoallelic parent-of-origin specific expression. Imprinted genes exhibit expression differences in an allele-specific manner depending on parent of origin, usually resulting in the complete or partial silencing of one allele and very high expression of the other allele. Pregnancy involves a cooperative interaction between mother and fetus, and through a variety of maternal physiological adaptations the fetal demand for resources to support its growth are met. Resource allocation during pregnancy results in an inherent conflict between the mother and her fetus who carries the paternal allele. Although the mother benefits by the transmission of her genes to future generations, she has an interest in limiting the current fetus to retain resources for subsequent pregnancies. Limiting allocation of resources to the fetus is more relevant when the resources are limited (Fowden and Moore, 2012). The importance of conflict in nutrient allocation may vary between species and is proportional to the maternal weight that is utilized to support pregnancy. Maternal-fetal conflict results in differential expression of maternally and paternally inherited alleles in the fetus and placenta. Paternal inherited genes promote placental and fetal growth while maternally expressed genes have a limiting effect. Imprinted genes often impact resource allocation by the placenta. There are over one hundred confirmed imprinted genes in mice and approximately 50 of these maintain their imprinted status in humans and are expressed in embryonic, fetal and early postnatal stages of development. Recent research has supported the idea that genomic imprinting in the placenta may be an adaptive mechanism permitting plasticity of function in response to changing environmental conditions during

gestation (Wang et al., 2013). As imprinted genes regulate placental development and fetal growth, its implications in complicated pregnancies including low birth weight, intrauterine growth restriction, gestational diabetes and preeclampsia remain poorly understood (Cleaton et al., 2014) (Monk, 2015).

Fetal Unit

Fetal Membranes

Fetal membranes are extra-embryonic tissues, genetically identical to the fetus, which encircle the fetus and form a maternal-fetal interface. There are different morphological types of fetal membranes represented among the vertebrates. In humans, fetal membranes at term have an area of approximately 1000–1200 cm², where 30% overlay the placenta and the remaining 70% interact with the maternal tissues. The maintenance of the fetal membrane integrity is critical to the success of pregnancy where sufficient strength and elasticity of the tissues enables the tissue to double in size and support vigorous fetal movements, particularly toward the end of the pregnancy. Fetal membranes consist of several cell types including epithelial, mesenchymal and trophoblasts, which are embedded in an extracellular matrix that imparts tensile strength. Fetal membranes are multilayered tissues formed by two principal layers: the amnion and the chorion. The amnion is in direct contact with the fetus and is composed of five different layers: a single layer of epithelial cells, a basal membrane, the compact layer, the fibroblast layer and the intermediate or spongy layer. The chorion consists of a layer of connective tissue, a basal membrane and a region containing trophoblasts, which by term is firmly adhered to the maternal decidual tissue (Bryant-Greenwood, 1998). The decidua, a maternal tissue discussed below, is composed of differentiated stromal cells surrounded by an extracellular matrix that fuses with the chorion over the course of pregnancy. Collagens are the major structural components of the fetal membranes, where the major tensile strength is provided by the interstitial collagen type I and III, together with smaller amounts of types V, VI and VII. The structural integrity of fetal membranes is due to a precise balance of synthesis and degradation of collagens.

Fetal membranes undergo physical and biochemical changes during the process of normal labor, including increased the collagenolytic activity and apoptosis favoring the loss of structure, leading to the rupture of membranes necessary for the delivery of the fetus (Vadillo-Ortega and Estrada-Gutierrez, 2005). In addition, an inflammatory microenvironment is created within the fetal membranes during parturition, characterized by specific leukocyte subsets and the secretion of autocrine and paracrine mediators. Choriodecidual cells and infiltrated leukocytes secrete pro-inflammatory cytokines, prostaglandins and matrix metalloproteases during the onset of labor which may lead to spontaneous labor, the rupture of the membranes and eventually the delivery of the fetus (Gomez-Lopez et al., 2013, Cleaton et al., 2014).

Fetal membranes in humans are not vascularized which limits their function as an exchange surface for nutrients between mother and fetus. However, steroids and prostaglandins are secreted by the fetal membranes, allowing for paracrine signaling between the maternal and fetal compartments. Glucocorticoids are involved in fetal maturation and the initiation of labor (Myatt and Sun, 2010). In addition, fetal membranes can synthesize, store and secrete a wide variety of cytokines that can regulate and initiate local inflammatory responses believed to be involved in the physiology of normal parturition and in the pathology of premature delivery associated to intrauterine infection (Chavatte-Palmer and Tarrade, 2016). The maternal-fetal interface of the fetal membranes is a unique site that requires modulation of the local immune response to the allogenic fetus, while maintaining a defensive barrier against infectious agents. Several reports have evaluated human fetal membrane responses to entire bacteria and their products by using in vitro models to investigate intrauterine infection. Fetal membranes are capable of responding to bacterial products by secreting pro-inflammatory cytokines such as IL-1 β , TNF- α , and IL-6. Epithelial amnion cells react to infection by secreting IL-6 and IL-18. In vitro models suggest that in the presence of infection, the mechanisms leading to the rupture of the fetal membranes appear to be dependent on the nature of the infecting microorganism (Di Simone et al., 2009).

Maternal Unit

Decidua

The decidua is a transient layer that is localized between the fetal membranes and the myometrium and may play a role in the cross-talk between maternal and fetal compartments. It is formed during the secretory phase of the menstrual cycle as a result of elevated ovarian hormones such as estrogen and progesterone. The decidua is composed of glands, immune cells, blood and lymph vessels, and decidual stromal cells (DSCs) and fetal extravillous trophoblast (Mori et al., 2016). The interaction of the maternal leukocyte populations in the decidua with fetal trophoblasts is crucial at the time of placentation and for the success of pregnancy. Therefore, any disturbance in the decidualization process and/or maternal-fetal crosstalk could lead to pregnancy complications such as implantation failure and pregnancy loss (Sharma, S. et al., 2016).

There are three major process in which maternal-fetal cross-talk is essential for the maintenance of pregnancy: a) the establishment of immune tolerance, b) regulation of the trophoblast invasion, and c) remodeling of the spiral arteries (Romero et al., 2014). Defects in trophoblast invasion are associated with severe pregnancy complications affecting both mother and fetus. Cytokines and growth factors are key molecules involved in the regulation of trophoblast invasion and act either to stimulate such as IL-1 β , IL-6, IL-11, IL-8, RANTES, and IP-10 or inhibit; IL-10, IL-12 and vascular endothelial growth factor (VEGF) (Sharma et al., 2016).

Given their anatomical position, decidual cells are likely to be the first cells of the feto-maternal interface to be in contact and respond to pathogens and establish the immunotolerance mechanisms towards fetal antigens (Anders et al., 2017). There are

several critical windows in which the maternal immune system may be challenged with fetal alloantigens at the decidua: i) during implantation, ii) when extravillous trophoblast invade and remodel the maternal spiral arteries, iii) when the syncytiotrophoblast turnover sheds microvesicles, nanoparticles, microparticles and exosomes into maternal circulation. The female immune system undergoes constant transformation and adaptation beginning with each menstrual cycle and continuing through gestation with fertilization and implantation. Changes in the immune cell populations, secretory profiles and the immune effects of pregnancy-associated hormones in the decidua are some of the maternal adaptations allowing for immunotolerance (Hyde and Schust, 2016).

The establishment of the fetal-maternal unit is fundamental for the establishment and maintenance of pregnancy. This maternal-fetal interface serves two principal functions: (1) to act as an interface between the mother and fetus and allow nutrient and oxygen transfer and waste products removal, a function mainly orchestrated by the placenta; and (2) to protect the fetus from the extrauterine environment until it is fully developed, a function served by the fetal membranes and decidua. Although the structure and some functions of the fetal-maternal unit may vary between species, the endocrine and transport functions in the unit remain consistent. Abnormal establishment of the fetal-maternal unit may be involved in the pathogenesis of impaired implantation, recurrent miscarriages, intrauterine growth restriction and preeclampsia.

References

- Anders, A. P., Gaddy, J. A., Doster, R. S., & Aronoff, D. M. (2017). Current concepts in maternal-fetal immunology: Recognition and response to microbial pathogens by decidual stromal cells. *American Journal of Reproductive Immunology*, 77, 1–8.
- Aye, I. L., Powell, T. L., & Jansson, T. (2013). Review: Adiponectin—the missing link between maternal adiposity, placental transport and fetal growth? *Placenta. Suppl.*, 34, S40–45.
- Bryant-Greenwood, G. D. (1998). The extracellular matrix of the human fetal membranes: Structure and function. *Placenta*, 19, 1–11.
- Cameo, P., Bischof, P., & Calvo, J. C. (2003). Effect of leptin on progesterone, human chorionic gonadotropin, and interleukin-6 secretion by human term trophoblast cells in culture. *Biology of Reproduction*, 68, 472–477.
- Campos, D. B., Palin, M. F., Bordignon, V., & Murphy, B. D. (2008). The 'beneficial' adipokines in reproduction and fertility. *International Journal of Obesity*, 32, 223–231.
- Chavatte-Palmer, P., & Tarrade, A. (2016). Placentation in different mammalian species. *Annales d'Endocrinologie (Paris)*, 77, 67–74.
- Cleaton, M. A., Edwards, C. A., & Ferguson-Smith, A. C. (2014). Phenotypic outcomes of imprinted gene models in mice: Elucidation of pre- and postnatal functions of imprinted genes. *Annual Review of Genomics and Human Genetics*, 15, 93–126.
- Cole, L. A. (2012). hCG, The wonder of today's science. *Reproductive Biology and Endocrinology*, 10, 24.
- Corcoran, J. J., Nicholson, C., Sweeney, M., Charnock, J. C., Robson, S. C., Westwood, M., & Taggart, M. J. (2014). Human uterine and placental arteries exhibit tissue-specific acute responses to 17beta-estradiol and estrogen-receptor-specific agonists. *Molecular Human Reproduction*, 20, 433–441.
- Costa, M. A. (2016). The endocrine function of human placenta: An overview. *Reproductive Biomedicine Online*, 32, 14–43.
- Di Simone, N., Di Nicuolo, F., Marzoni, D., Castellucci, M., Sanguinetti, M., D'Ippolito, S., & Caruso, A. (2009). Resistin modulates glucose uptake and glucose transporter-1 (glut-1) expression in trophoblast cells. *Journal of Cellular and Molecular Medicine*, 13, 388–397.
- D'Ippolito, S., Tersigni, C., Scambia, G., & Di Simone, N. (2012). Adipokines, an adipose tissue and placental product with biological functions during pregnancy. *Biofactors*, 38, 14–23.
- Enders, A. C., & Blankenship, T. N. (1999). Comparative placental structure. *Advanced Drug Delivery Reviews*, 38, 3–15.
- Feldt-Rasmussen, U., & Mathiesen, E. R. (2011). Endocrine disorders in pregnancy: Physiological and hormonal aspects of pregnancy. *Best Practice & Research Clinical Endocrinology Metabolism*, 25, 875–884.
- Fournier, T., Guibourdenche, J., & Evain-Brion, D. (2015). Review: Hcgs: Different sources of production, different glycoforms and functions. *Placenta*, 36(Suppl. 1), S60–65.
- Fowden, A. L., & Moore, T. (2012). Maternal-fetal resource allocation: Co-operation and conflict. *Placenta*, 33(Suppl. 2), e11–15.
- Furukawa, S., Kuroda, Y., & Sugiyama, A. (2014). A comparison of the histological structure of the placenta in experimental animals. *Journal of Toxicology Pathology*, 27, 11–18.
- Gomez-Lopez, N., Vega-Sanchez, R., Castillo-Castrejon, M., Romero, R., Cobeiro-Areola, K., & Vadillo-Ortega, F. (2013). Evidence for a role for the adaptive immune response in human term parturition. *American Journal of Reproductive Immunology*, 69, 212–230.
- Hafez, S. (2017). Comparative placental anatomy. Divergent structures serving a common purpose. *Progress in Molecular Biology and Translational Sciences*, 145, 1–28.
- Halasz, M., & Szekeres-Bartho, J. (2013). The role of progesterone in implantation and trophoblast invasion. *Journal of Reproductive Immunology*, 97, 43–50.
- Hauguel-De Mouzon, S., Lepercq, J., & Catalano, P. (2006). The known and unknown of leptin in pregnancy. *American Journal of Obstetrics and Gynecology*, 194, 1537–1545.
- Herrid, M., Palanisamy, S. K., Ciller, U. A., Fan, R., Moens, P., Smart, N. A., & McFarlane, J. R. (2014). An updated view of leptin on implantation and pregnancy: A review. *Physiological Research*, 63, 543–557.
- Hur, J., Cho, E. H., Baek, K. H., & Lee, K. J. (2017). Prediction of gestational diabetes mellitus by unconjugated estriol levels in maternal serum. *International Journal of Medical Sciences*, 14, 123–127.
- Hyde, K. J., & Schust, D. J. (2016). Immunologic challenges of human reproduction: An evolving story. *Fertility and Sterility*, 106, 499–510.
- Jansson, N., Greenwood, S. L., Johansson, B. R., Powell, T. L., & Jansson, T. (2003). Leptin stimulates the activity of the system a amino acid transporter in human placental villous fragments. *The Journal of Clinical Endocrinology & Metabolism*, 88, 1205–1211.
- Jansson, N., Nilsfelt, A., Gellerstedt, M., Wennergren, M., Rossander-Hulthen, L., Powell, T. L., & Jansson, T. (2008). Maternal hormones linking maternal body mass index and dietary intake to birth weight. *The American Journal of Clinical Nutrition*, 87, 1743–1749.
- Mesdaghi-Nia, E., Behrashi, M., Saeidi, A., Abedzadeh Kalahroodi, M., & Sehat, M. (2016). Association between papp-a and placental thickness. *International Journal of Reproductive Biomedicine*, 14, 421–426.
- Monk, D. (2015). Genomic imprinting in the human placenta. *American Journal Obstetrics & Gynecology*, 213, S152–162.
- Mori, M., Bogdan, A., Balassa, T., Csabai, T., & Szekeres-Bartho, J. (2016). The decidua-the maternal bed embracing the embryo-maintains the pregnancy. *Seminars in Immunopathology*, 38, 635–649.
- Mu, H., Ohashi, R., Yan, S., Chai, H., Yang, H., Lin, P., Yao, Q., & Chen, C. (2006). Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. *Cardiovascular Research*, 70, 146–157.
- Myatt, L., & Sun, K. (2010). Role of fetal membranes in signaling of fetal maturation and parturition. *The International Journal of Developmental Biology*, 54, 545–553.
- Newbern, D., & Freemark, M. (2011). Placental hormones and the control of maternal metabolism and fetal growth. *Current Opinion in Endocrinology, Diabetes and Obesity*, 18, 409–416.
- Perez-Perez, A., Maymo, J., Gambino, Y., Guadix, P., Duenas, J. L., Varone, C., & Sanchez-Margalet, V. (2013). Insulin enhances leptin expression in human trophoblastic cells. *Biology of Reproduction*, 89, 20.

- Romero, R., Yeo, L., Chaemsaitong, P., Chaiworapongsa, T., & Hassan, S. S. (2014). Progesterone to prevent spontaneous preterm birth. *Seminars in Fetal & Neonatal Medicine*, 19, 15–26.
- Ruddock, N. K., Shi, S. Q., Jain, S., Moore, G., Hankins, G. D., Romero, R., & Garfield, R. E. (2008). Progesterone, but not 17-alpha-hydroxyprogesterone caproate, inhibits human myometrial contractions. *American Journal of Obstetrics and Gynecology*, 199(391), e391–397.
- Sharma, S., Godbole, G., & Modi, D. (2016). Decidual control of trophoblast invasion. *American Journal of Reproductive Immunology*, 75, 341–350.
- Than, N. G., Balogh, A., Romero, R., Karpati, E., Erez, O., Szilagyi, A., Kovalszky, I., Sammar, M., Gizurarson, S., Matko, J., Zavodszky, P., Papp, Z., & Meiri, H. (2014). Placental protein 13 (pp13) - a placental immunoregulatory galectin protecting pregnancy. *Front Immunol*, 5, 1–25.
- Vadillo-Ortega, F., & Estrada-Gutierrez, G. (2005). Role of matrix metalloproteinases in preterm labour. *British Journal of Obstetrics and Gynaecology*, 112(Suppl. 1), 19–22.
- Wang, X., Miller, D. C., Harman, R., Antczak, D. F., & Clark, A. G. (2013). Paternally expressed genes predominate in the placenta. *Proceedings of the National Academy of Sciences of United States of America*, 110, 10705–10710.

Further Reading

- Behnia, F., Sheller, S., & Menon, R. (2016). Mechanistic differences leading to infectious and sterile inflammation. *American Journal of Reproductive Immunology*, 75, 505–518.
- Estrada-Gutierrez, G., Gomez-Lopez, N., Zaga-Clavellina, V., Giono-Cerezo, S., Espejel-Nunez, A., Gonzalez-Jimenez, M. A., Sosa, S. E. Y., Olson, D. M., & Vadillo-Ortega, F. (2010). Interaction between pathogenic bacteria and intrauterine leukocytes triggers alternative molecular signaling cascades leading to labor in women. *Infection and Immunity*, 78, 4792–4799.
- Gomez-Lopez, N., Laresgoiti-Servitje, E., Olson, D. M., Estrada-Gutierrez, G., & Vadillo-Ortega, F. (2010). The role of chemokines in term and premature rupture of the fetal membranes: A review. *Biology of Reproduction*, 82, 809–814.
- Lash, G. E. (2015). Molecular cross-talk at the feto-maternal interface. *Cold Spring Harbor Perspectives in Medicine*, 5, 1–15.
- Sobrevia, L., Salsoso, R., Fuenzalida, B., Barros, E., Toledo, L., Silva, L., Pizarro, C., Subiabre, M., Villalobos, R., Araos, J., Toledo, F., Gonzalez, M., Gutierrez, J., Farias, M., Chiarello, D. I., Pardo, F., & Leiva, A. (2016). Insulin is a key modulator of fetoplacental endothelium metabolic disturbances in gestational diabetes mellitus. *Frontiers in Physiology*, 7, 119.

Fetal Endocrinology

Sean W Limesand and Melissa A Davis, University of Arizona, Tucson, AZ, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction to Fetal Endocrinology

The fetal hormone milieu is largely independent of maternal hormone because the placenta creates an impermeable barrier for most hormones, which provides distinct endocrine regulation in the maternal and fetal compartments. The initial information presented in this article describes general principles of endocrinology that all endocrinologists consider in adults. For example, hormone synthesis, secretion, transport, receptor binding, and clearance are important processes for endocrine systems and complications in any one of these processes will result in endocrine pathologies. In the fetus, the formation and maturation of the endocrine systems during development must be considered. Maturation of the endocrine system occurs after the formation of the gland because, even though synthesis of a hormone has begun, the endocrine tissues must also develop the responsiveness to secrete the hormone. Similar maturation processes also take place at target tissues, which need to express receptors and intracellular signaling proteins to receive and interpret the message conveyed by the hormone. Consideration of maturation during development involves an additional layer of complexity for understanding fetal endocrinology and also indicates a point for adaptive programming to various clinically important situations like placental insufficiency and intrauterine growth restriction.

Principles of Endocrinology

The classical definition of endocrinology states that hormones are chemical substances produced by specialized tissues and secreted into the blood, where they are carried to target organs to elicit a biological response. Investigations by physiologists, biochemists, and clinicians have shown that the classic definition, while appropriate for some endocrine systems, was restrictive because not all hormones are produced in specialized glands that are distinct from the target organ. These limitations have led to a broader definition: a hormone is a chemical substance that carries information between two or more cells. In this capacity, the endocrine system resembles the nervous system and is one of the two distinct mechanisms involved in communication within the body to maintain homeostasis by coordinating physiological interactions. These roles become apparent in several endocrine-related diseases such as diabetes and hypothyroidism.

Chemical Nature of Hormones

The chemical nature of hormones is highly variable, but in general hormones are classified into by four broad categories. These categories are lipids, proteins, peptides, and modified amino acids. Lipid hormones include steroids or vitamin D derived from cholesterol, and prostaglandins derived from arachidonic acid. Lipid hormones are usually hydrophobic or unable to dissolve in water. Protein and peptide hormones are synthesized in cells from amino acids and stored in granules until the cell is stimulated to release them into the extracellular space. Some protein hormones are glycosylated, which will further stabilize them after release, increasing their half-life by slowing their clearance compared to nonglycosylated proteins or peptides. Proteins and peptides are often hydrophilic allowing them to freely dissolve in water. Modified amino acids are produced biochemically in the cell from tyrosine or tryptophan. The solubility of these hormones in water is dependent on the modifications. Catecholamines and thyroid hormones are both derived from tyrosine. Catecholamine, epinephrine and norepinephrine, are hydrophilic and store in intracellular secretory vesicles, whereas thyroid hormones are hydrophilic.

Based on the chemical nature of hormones predictions can be made about their synthesis, storage, transport, and receptors. Hydrophilic hormones are more easily stored in intracellular compartments, and once released will interact with receptors on the plasma membrane. In contrast, hydrophobic hormones require protein chaperones to transport them through the blood and are free to diffuse across the plasma membrane to bind to intracellular receptors that act as ligand mediated transcription factors to initiate the cell response.

Biological Activity of Hormones

Autocrine, paracrine, and endocrine regulation of signaling describe the proximity between the endocrine cell and target tissue. Autocrine refers to a chemical-produced response in the same cell from which it was secreted. Paracrine describes a hormone that provokes a response in local, neighboring cell that is distinct from the cell secreting the hormone. Endocrine signaling is long-distance communication often using the blood for its distribution. Autocrine, paracrine, and endocrine communication allow for coordination of fetal development and preparation for postnatal life.

Hormones work in an induced fit, lock-and-key manner with the hormone being the ligand or in this scenario the key and the lock being a specific receptor opening the door of communication. There are intracellular receptors (nuclear) and cell membrane receptors (membrane-bound). Lipid-soluble hormones, like steroid hormones, diffuse directly across cell membranes and bind to intracellular receptors in the cytoplasm or nucleus. Receptor-hormone complex then bind to specific elements on the DNA to initiate transcription of RNA and increase translation of proteins. Water-soluble hormones, like amine, peptide, and protein hormones, utilize cell membrane receptors and second messenger signaling pathways because they cannot diffuse through the lipid

bilayer. A common second messenger used by hormones is cyclic adenosine monophosphate (cAMP). In this pathway, hormones bind to the membrane receptor (this is referred to as a G-protein coupled receptor), which cause a conformational change that activates a G-protein. The active G-protein then initiates adenylyl cyclase to catalyze the conversion of adenosine triphosphate to cAMP. This second messenger, cAMP, binds to the regulatory subunit of protein kinase A causing the release of active catalytic subunits to phosphorylate other intracellular proteins and elicit the biological response. Second messenger pathways allow for the amplification of the hormone signal leading to a faster, more efficient response. Whether intracellular or cell membrane-bound receptors are utilized, hormones are able to elicit specific and calculated responses to stimuli. These activities like the production of the hormone must mature during development.

Regulation of Hormone Secretion

Hormone concentrations are typically determined by rate of production, rate of delivery, and rate of degradation and elimination. Rate of production is controlled through feedback mechanisms, which allows the endocrine system to be finely tuned. Hormone secretion is predominantly regulated through feedback loops that can provide negative or positive feedback. Negative feedback is the most common method of homeostasis and results from either direct or indirect inhibition of hormone secretion. Positive feedback occurs when a hormone stimulates its release, reinforcing its secretion, which is rare and requires a terminal event. For example, delivery of the baby circumvents further secretion of oxytocin. Feedback loops allow for tight control of hormone secretion to maintain homeostasis within the system.

Neuroendocrinology: Hypothalamic–Pituitary Complex

The hypothalamus and pituitary are two separate organs that work together as the “command center” for many endocrine systems. Arising from ectoderm and neuroectoderm, the pituitary gland is present by 12 weeks of gestation, and the primary plexus of the pituitary portal blood vascular system begins development at 14 weeks being fully developed by 21 weeks in humans. The pituitary is located immediately beneath the hypothalamus, which contains measurable releasing hormones as early as 8 weeks of gestation. Four primary regions of the hypothalamus are the preoptic, supraoptic, tuberal, and mammillary (Porter, 1998). These regions are responsible for the regulation of blood pressure, energy metabolism, reproduction, body temperature, stress response, and circadian rhythm in the adult. Some hypothalamic hormones are synthesized and transported to the posterior pituitary gland, while others serve as releasing hormones that regulate secretion of anterior pituitary hormones. The hypothalamic–hypophyseal portal system connects a series of capillary beds that allows hypothalamic regulation of the anterior pituitary. Releasing hormones include: corticotrophin releasing hormone (CRH), thyrotrophin releasing hormone (TRH), gonadotrophin releasing hormone (GnRH), growth hormone releasing hormone (GHRH), and prolactin inhibitory hormone (dopamine). The hypothalamus also secretes somatostatin, which inhibits GH, and dopamine, which stimulates GH inhibits prolactin (PRL) secretion (Porter, 1998) (Table 1).

During development, the hypothalamus is vulnerable to genetic modulation and is influenced by maternal factors like undernutrition, obesity, and diabetes. The hypothalamus is known to regulate satiety as well as energy and peripheral glucose homeostasis in adults. Since the hypothalamus is sensitive to metabolic changes, negative environmental exposures, like elevated glucose, in utero can leave a lasting imprint on gene expression resulting in neurodevelopmental insufficiencies or increased risk of neurobehavioral issues (Omoy, 2011). Due to maternal over- or undernutrition, programming of hypothalamic genes like pro-opio-melanocortin and neuropeptide Y can lead to altered food consumption and glucose homeostasis. In growth restricted fetal baboons, it has been shown that appetitive neuropeptide balance is altered to promote increased appetite drive postnatally. Interestingly, the second half of pregnancy seems to be the critical window for programming of the hypothalamus in regards to food intake (Cun et al., 2013).

Pituitary Hormones: Adenohypophysis (Anterior)

The adenohypophysis consists of the anterior pituitary gland, also known as the pars distalis, and the much smaller pituitary stalk, pars tuberalis. The anterior pituitary secretes seven different hormones. The ability for the anterior pituitary to secrete so many different hormones is due to the various endocrine cell types that comprise the gland (Pocock et al., 2013). Somatotrophs make up the majority (50%) of the cell population and secrete GH. Its function is primarily anabolic, promoting protein synthesis and accretion both directly and indirectly. Thyrotrophs (5%) secrete thyroid-stimulating hormone (TSH), which promotes thyroid hormone secretion. Thyrotroph and somatotroph differentiation occurs by 10 weeks of gestation with lactotrophs differentiating last at 16 weeks. Lactotrophs (15–20%) secrete PRL by 16 weeks of gestation, which is known to play a significant role in lung maturation and peak in concentration in late gestation. Gonadotrophs (10%) secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are synthesized by 9 weeks of gestation and reach their highest concentration mid-gestation. Corticotrophs (15–20%) primarily secrete adrenocorticotrophic hormone (ACTH) but can also secrete melanocyte-stimulating hormone (MSH). By 9 weeks of gestation, ACTH is detectable with the primary role being to stimulate adrenal glucocorticoid synthesis and regulate metabolism and stress response (Pocock et al., 2013). The hypothalamus controls secretion of FSH, LH, ACTH, MSH, and TSH through releasing hormones (Kota et al., 2013). PRL is primarily regulated through the inhibitory effect of dopamine. The hypothalamus also controls GH through both GHRH and somatostatin, which is also known as growth hormone-inhibiting hormone.

Table 1 Summary of the development of endocrine systems

<i>Hormone</i>	<i>Formation</i>	<i>Endocrine gland</i>
Coricotrophin releasing hormone (CRH)	9 weeks	Hypothalamus
Thyrotrophin releasing hormone (TRH)	8 weeks	Hypothalamus
Gonadotrophin releasing hormone (GnRH)	9 weeks	Hypothalamus
Prolactin inhibitory hormone (dopamine)	10 weeks	Hypothalamus
Somatostatin (GHRH)	10 weeks	Hypothalamus
Growth hormone (GH)	10 weeks	Anterior pituitary
Thyroid-stimulating hormone (TSH)	10 weeks	Anterior pituitary
Prolactin	16 weeks	Anterior pituitary
Follicle-stimulating hormone (FSH)	9 weeks	Anterior pituitary
Luteinizing hormone (LH)	9 weeks	Anterior pituitary
Adrenocorticotrophic hormone (ACTH)	9 weeks	Anterior pituitary
Melanocyte-stimulating hormone (MSH)	9 weeks	Anterior pituitary
Arginine vasopressin (AVP)	10 weeks	Posterior pituitary
Oxytocin	11 weeks	Posterior pituitary
Dehydroepiandrosterone sulfate (DHEA-S)	9 weeks	Adrenal cortex
Glucocorticoids (Cortisol)	12 weeks	Adrenal cortex
Catecholamines (norepinephrine)	7 weeks	Adrenal medulla
Testosterone	8 weeks	Gonads (male)
Estrogen	10 weeks	Gonads (female)
Thyroxine (T ₄)	12 weeks	Thyroid gland
Parathyroid hormone (PTH)	10 weeks	Parathyroid glands
Insulin	10 weeks	Pancreas
Glucagon	10 weeks	Pancreas
Somatostatin	10 weeks	Pancreas
Pancreatic polypeptide	10 weeks	Pancreas
Insulin-like growth factors (IGF)	9 weeks	Liver

Throughout gestation, fetal GH concentrations fluctuate with the highest concentrations being at mid-gestation due to the formation of the somatotrophs and subsequent maturation of the pituitary feedback control systems to regulate GH secretion in the third trimester. In contrast to GH, PRL concentration will remain low until after 25 weeks of gestation peak at term, showing autonomous secretion of PRL is limited early in lactotrophs (Kota et al., 2013). Postnatally, GH acts through receptors in the liver and other tissues to stimulate insulin-like growth factor 1 (IGF-1) and IGF-2, albeit to a lesser degree. In the fetus, the GH receptors in the liver are low suggesting they are less responsive to GH from the fetal pituitary. Additionally, high placental lactogen concentrations in the fetus may saturate GH receptors even though the affinity of placental lactogen is lower than GH for the GH receptors (Gluckman and Pinal, 2002). One additional piece of evidence suggesting that the action of pituitary GH in the fetus is not effective at regulating fetal growth is that the growth of an anencephalic fetus is nearly normal. Similarly, PRL receptors are present in several fetal tissues and may be important for adipogenesis, but crosstalk from placental lactogen is also expected with PRL receptors.

Pituitary Hormones: Neurohypophysis (Posterior)

The neurohypophysis consists of hypothalamic neurons that extend through the median eminence and terminate the posterior pituitary. The magnicellular neurons of the supraoptic and paraventricular nuclei secrete oxytocin and arginine vasopressin (AVP). Oxytocin synthesis begins by 11 weeks and increases just prior to parturition. Oxytocin is known for its role in parturition and lactation as well as social behaviors; however, its role in fetal development is less understood. AVP production begins by 10 weeks of gestation (Kota et al., 2013). AVP, or antidiuretic hormone, regulates blood volume and osmolality via renal tubule water reabsorption (V₂ receptors) and vasoconstriction (V_{1a} receptors) in adults. In fetal sheep, responsiveness to volume and osmolar stimuli is well developed in late gestation. AVP concentrations increase in response to hypoxia in the sheep fetus, which suggest that AVP functions as a fetal stress-responsive hormone. The vasopressor actions of AVP may be critical activities during fetal hypoxia or hemorrhage. Not surprisingly, AVP has little effect placental blood flow because restriction of oxygen and nutrient transfer from the mother to the fetus caused by restricted flow is expected to lead to greater hypoxic stress.

The hypothalamic–pituitary complex has specific target organs including adrenal glands, gonads, and thyroid gland, coordinating important events in fetal development. These specific endocrine axes are discussed below.

Hypothalamic–Pituitary–Adrenal Axis

The hypothalamic–pituitary–adrenal axis controls stress response by intertwining the central nervous system and endocrine system. Adrenal glands, which sit above the kidney, begin developing by week 4 of gestation. Fetal adrenal glands have a cortex, which is

able to produce three types of steroid hormones: androgens (fetal zone), glucocorticoids (transitional zone), and mineralocorticoids (definitive zone). The cortex is steroidogenically active by 9–12 weeks, producing primarily the androgen dehydroepiandrosterone sulfate (DHEA-S). The relatively high and sustained production of DHEA-S from adrenal glands and conversion to 16 α -hydroxy-DHEA-S in the fetal liver forms the maternal–fetal–placental unit (Kota et al., 2013). Thereby, adrenal glands provide substrates for estradiol and estrone production in the placenta to overcome the lack of 17 α -hydroxylase/17,20-lyase expression in the trophoblast cells. Placental human chorionic gonadotrophin plays a role in early growth but maintenance and growth after 15 weeks becomes dependent on ACTH, demonstrating maturation of the axis (Ng, 2000). The fetal adrenal function is largely geared to produce inactive steroidogenic products.

The majority of fetal glucocorticoids (cortisol) in circulation are secreted from the adrenal glands with the rest coming from placental transfer. Metabolic clearance rates of cortisol to cortisone are rapid and raise the inactive cortisone concentrations in fetal circulation. This inactivation process may be critical to protect the fetus from catabolic effects of cortisol. The fetal hypothalamic–pituitary–adrenal axis progressively matures after mid gestation and CRH–ACTH stimulation of glucocorticoid secretion develops its negative feedback regulation (Ng, 2000). Glucocorticoid receptors are members of the nuclear receptor family, and are present in most fetal tissues after mid-gestation and play important roles in fetal development. In late gestation, there is a cortisol surge, which is crucial to mature fetal organs and prepares the fetus for delivery. In addition to cortisol production by adrenal glands, tissue-specific expression of 11-ketosteroid reductase increases in fetal lung and liver, which promotes the conversion of cortisone to cortisol locally to induce maturation.

Although glucocorticoids are necessary for normal brain development, inappropriate exposure to excess glucocorticoids can have a lasting impact postnatally. The hypothalamic–pituitary–adrenal axis is susceptible to permanent adaptation from prenatal stressors, which is associated with increased maternal stress (Fowden and Forhead, 2004). Fetal adaptations to maternal stress are dependent on both timing of insult during gestation and duration of the insult. In guinea pigs, maternal stress on days 50–52 of gestation proved to be a critical developmental window because these offspring have elevated basal adrenocortical activity compared to offspring stressed at later ages that displayed normal function. A similar study in nonhuman primates generated the same manifestation of elevated basal adrenocortical activity, but no critical window of programming was evident. Some rodent and porcine studies suggest that hypothalamic–pituitary–adrenal axis programming is sex-specific, with females being more vulnerable to adaptations. Although many studies have been done in various species, little research has been done in humans. In children of mothers that presented with stress and anxiety, salivary cortisol concentrations were elevated at 5 years of age. This is similar to female piglets that were exposed to maternal social stress in utero exhibiting elevated salivary cortisol in response to social stress (Kapoor et al., 2006). Mechanisms of maternal stress have been proposed such as placental CRH stimulation of fetal hypothalamic–pituitary–adrenal axis and maternal elevation in catecholamines leading to abnormal uterine blood flow, which stimulates the fetal hypothalamic–pituitary–adrenal axis. Chronic activation of the hypothalamic–pituitary–adrenal axis postnatally can lead to long-term consequences like psychological dysfunction, as well as metabolic and cardiovascular disease.

Hypothalamic–Pituitary–Gonadal Axis

The hypothalamic–pituitary–gonadal axis controls sexual development and reproduction. Gonadotrophs in the anterior pituitary secrete FSH and LH in response to GnRH. Due to inhibitory action of testosterone in male fetuses, circulating LH and FSH concentrations are generally low with the peak concentrations occur between 14 and 21 weeks of gestation. In female fetuses, LH and FSH concentrations are higher than males. The ratio of FSH/LH is also higher in females compared to males in utero. FSH and LH concentrations decline late in gestation due to the development of neuroendocrine responsiveness to negative gonadal feedback. Near term, pituitary hormones are needed for granulosa cell and follicular proliferation. The ovary also produces inhibin in utero though significantly less than males, which could also explain higher gonadotropin levels. Fetal sexual differentiation does not depend on gonadotropins from the pituitary but on the presence of the *SRY* (sex-determining region on the Y) gene and anti-Müllerian hormone. The hypothalamic–pituitary–gonadal axis continues to be fine-tuned during the neonatal period and throughout postnatal development, with most of the reproductive maturation occurring during puberty.

It is known that adverse in utero exposure to testosterone can produce reproductive (infertility) and metabolic deficiencies (obesity and insulin resistance) into adulthood. This has been reproduced in rats, nonhuman primates, and sheep. Exposure to testosterone between 30 and 60 days of gestation in fetal sheep resulted in growth restriction and consequent reductions in birth weight. Females also exhibited catch-up growth postnatally while males did not. Early pregnancy appears to be the critical window for fetal programming, which is concerning due to fetal exposure possibilities like continued contraceptive steroid use, unintentional environmental exposure, use of anabolic steroids, or from maternal diseases like polycystic ovarian syndrome, which increase circulating androgens (Manikkam et al., 2004). This knowledge allows growth restriction and catch-up growth to be a potential indicator for adult disease.

Hypothalamic–Pituitary–Thyroid Axis

The thyroid gland forms around week seven of gestation making it one of the first endocrine glands. It originates from the primitive pharynx, which forms the majority of the gland (median thyroid), as well as the neural crest, which gives rise to the rudimentary lateral thyroid. The thyroid gland undergoes three histologically distinct stages of growth and differentiation: precolloid (7–10 weeks), colloid (10–11 weeks), and follicular (11 weeks). By week 12, the thyroid is functional and is critical for neural development and metabolism stimulation. The thyroid is fully functional by 16–18 weeks of gestation and continues to expand throughout gestation. Prior to fetal thyroid development, the fetus is dependent on maternal thyroid hormone for brain

development. The thyroid gland synthesizes thyroxine (T_4), which is a prohormone because triiodothyronine (T_3) is normally produced at the target tissues by 5'-monodeiodinase. TSH secretion begins by 10 weeks of gestation and reaches adult concentrations between 26 and 36 weeks. In early development, TSH likely serves to regulate T_4 secretion through negative feedback by T_4 versus TRH stimulation. At the thyroid gland, TSH promotes follicular growth as well as thyroid hormone synthesis and secretion. When T_3 and T_4 concentrations are too low, TSH production is increased. Once appropriate concentrations are reached, TSH production is decreased. Late in gestation, T_4 and T_3 concentrations are increased resulting in low TSH; however, after birth, TSH, T_4 , and T_3 concentrations increase dramatically. Though active during late fetal development, the hypothalamic–pituitary–thyroid axis will not fully mature until postnatal life (Forhead and Fowden, 2014).

The impact of thyroid hormones in human fetal development is not fully understood. Although in fetal sheep growth and bone maturation are affected by hypothyroidism, human fetal development does not appear to be drastically impacted, possibly due to sufficient maternal thyroid delivery across the placenta. In the fetal sheep, hypothyroidism results in asymmetric growth restriction with muscle mass reduction as a primary consequence, along with other secondary development abnormalities. In particular hypothyroidism in fetal sheep shows deficiencies in maturation of the lung and cardiovascular systems. Fetal thyroid hormones are able to modulate accretion and differentiation of tissue as well as metabolic processes, promoting fetal development (Fowden and Forhead, 2004).

Parathyroid Glands and Calcium Homeostasis

Parathyroid glands arise from the third and fourth pharyngeal pouches (inferior and superior). In humans, parathyroid hormone (PTH) secretion begins by 10 weeks of gestation. PTH is produced from chief cells, and it controls plasma calcium concentration and counters calcitonin from the parafollicular cells in the thyroid gland. In the fetus, both endocrine systems are functional during the second and third trimesters. Receptors for PTH can be found in bone, the kidneys, and the gastrointestinal tract. There are five main physiological impacts of PTH: increased gastrointestinal calcium absorption, stimulation of osteoclasts to free calcium and phosphate from bone, increased calcium reabsorption but decreased phosphate reabsorption by the kidneys, and stimulates calcitriol (vitamin D) production in the kidneys. In sheep fetal calcium concentrations are greater than the mother, which indicates there is active placental transport from maternal circulation. The parathyroid hormone-related protein (PTHrP) is a regulatory protein expressed during organogenesis (Manley, 2015). Studies in various mouse models and humans suggest that PTH and PTHrP are required for fetal bone development and critical for regulation of serum minerals. PTHrP, and possibly PTH, is necessary to stimulate the placental transfer of calcium and magnesium. Loss of either hormone is known to result in hypocalcemia with the most significant hypocalcemia occurring with the loss of both PTH and PTHrP (Kovacs, 2014).

Endocrine Pancreas and Glucose Homeostasis

Mature pancreatic islets consist of insulin-secreting β -cells, glucagon-secreting α -cells, somatostatin-secreting δ -cells, and pancreatic polypeptide-secreting F-cells. β -cell proliferation starts 8 weeks into gestation in humans and continues throughout gestation with primitive islets forming around 11 weeks of gestation. Since insulin does not cross the placenta, fetal β -cells synthesize and secrete insulin and become responsive to glucose and other secretagogues around mid-gestation (Pictet et al., 1972). In sheep, β -cell responsiveness to glucose and arginine increases significantly after mid-gestation, showing β -cell maturation parallels the maturation of insulin action. In the fetus, insulin is a potent growth-promoting hormone, and umbilical cord plasma insulin concentrations are positively correlated to birth weight as well as glucose concentrations. Glucagon counteracts insulin action and promotes gluconeogenesis in the liver. In the sheep fetus, maternal starvation or administration of exogenous glucagon will stimulate hepatic glucose production. However, this appears to require an upregulation of enzymes in hepatocytes because normally fetal gluconeogenesis is minimal. Fetal development of the pancreas is critical for appropriate glucose homeostasis, while disrupted development can lead to metabolic disease as an adult.

Fetal Growth Regulation by Insulin-like Growth Factors

The fetal liver and placenta produce IGFs to stimulate fetal growth. IGF-1 and IGF-2 promote cell division, differentiation, and migration with IGF-2 being dominant in embryonic growth and IGF-1 becoming dominant later in gestation and postnatally. Mice that lack IGF-2 and IGF-1 receptors have significant growth restriction compared to mice with mutations of the IGF-1 gene and IGF-1 receptors. In mice models with specific liver IGF-1 deletion, IGF-1 concentrations were significantly reduced and peripheral insulin action was impaired. In late gestation, IGF-1 concentrations correlate directly to birth and placental weights. Fetal IGF-1 production is influenced by glucose and insulin concentrations, while IGF-2 is only governed by glucose. Placental IGF-2 is detected at 6 weeks of gestation and is thought to regulate placental growth, through nutrient availability, and aid in maintenance of pregnancy. The placenta is also able to secrete IGF-1 into fetal circulation. Unlike postnatal growth, fetal IGF-1 concentrations are not dependent on pituitary GH, owing to the high placental lactogen concentrations. Decreased IGF-1, insulin and T_4 are associated with a decline in fetal nutrient availability and growth restriction. Alternately, overexpression of the IGF-2 gene is known to cause overgrowth in mice models (Fowden and Forhead, 2004). Therefore, IGFs play an important role in nutrient and substrate availability to the growing fetus.

Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) affects up to 10% of all pregnancies and is often caused by placental insufficiency. Placental insufficiency is the failure of the placenta to deliver adequate oxygen and nutrients to maintain fetal growth rates, which causes the fetus to become hypoxemic and hypoglycemic. In order to resolve the conditions of placental insufficiency, the fetus diverts its nutrients to critical tissues causing adaptations in the body's structure, function, and metabolism. Observational data in humans indicates that developmental adaptations in both insulin secretion and insulin sensitivity to IUGR result in lifelong complications that increase the risk of developing glucose intolerance, diabetes, and cardiovascular disease.

Chronic low glucose and oxygen concentrations associate with placental insufficiency-induced IUGR lower plasma insulin concentrations, reduce β -cell mass, and impair β -cell function (Limesand and Rozance, 2017). In sheep fetuses with placental insufficiency and IUGR, body-weight specific glucose utilization rates, measured with tracer methodologies, are normal even though plasma insulin concentrations are substantially less than age-match control fetuses. This leads to the conclusion that IUGR fetuses have greater insulin sensitivity. However, these same IUGR fetuses have lower rates of umbilical (fetal) glucose uptake, which requires the IUGR fetuses to make a substantial portion of the glucose to maintain their glucose utilization rates. This suggests that the IUGR fetus develops a specific endocrine profile that promotes gluconeogenesis but also produces adaptive changes in skeletal muscle to enhance nonoxidative glucose uptake. Furthermore, the portion of noninsulin sensitive tissues, for example neuronal tissues, may also partially explain the near normal glucose utilization rates because IUGR fetuses tend to grow asymmetrically and have greater brain to liver ratios. These alterations in metabolism and asymmetric growth are influenced by endocrine actions.

Fetal responses to hypoxemia and hypoglycemia elevate norepinephrine and epinephrine. High catecholamines have been shown to slow fetal growth independent of other IUGR conditions and promote asymmetric growth (Limesand and Rozance, 2017). In fetal sheep, the sustained high catecholamine concentrations account for approximately 50% of the growth restriction. Catecholamines act via the adrenergic receptors (AR), which are G-protein coupled receptors. Adrenergic stimulation is tissue-specific due to the localized expression of the nine different isoforms of the AR. Catecholamines regulate blood glucose concentrations and under stressful conditions stimulate glycogenolysis and gluconeogenesis in the liver, but also act on the skeletal muscle to increase lactate release to provide gluconeogenic substrates to the liver. Sustained exposure to β 2-AR agonists increase nonoxidative glucose uptake, which indicates that in the IUGR fetus with chronically high concentrations of catecholamines cause the increased uptake but decrease oxidative metabolism of glucose in skeletal muscle. This will support higher lactate release with the appearance of greater insulin sensitivity for glucose utilization. Catecholamines also inhibit insulin secretion (α ₂-AR), thereby lowering plasma insulin concentration to allow hepatic glucose production, preferentially channeling glucose to the brain. The interplay between catecholamines and insulin are proposed to support the Cori cycle, also known as the Lactic acid cycle in IUGR fetuses (Fig. 1).

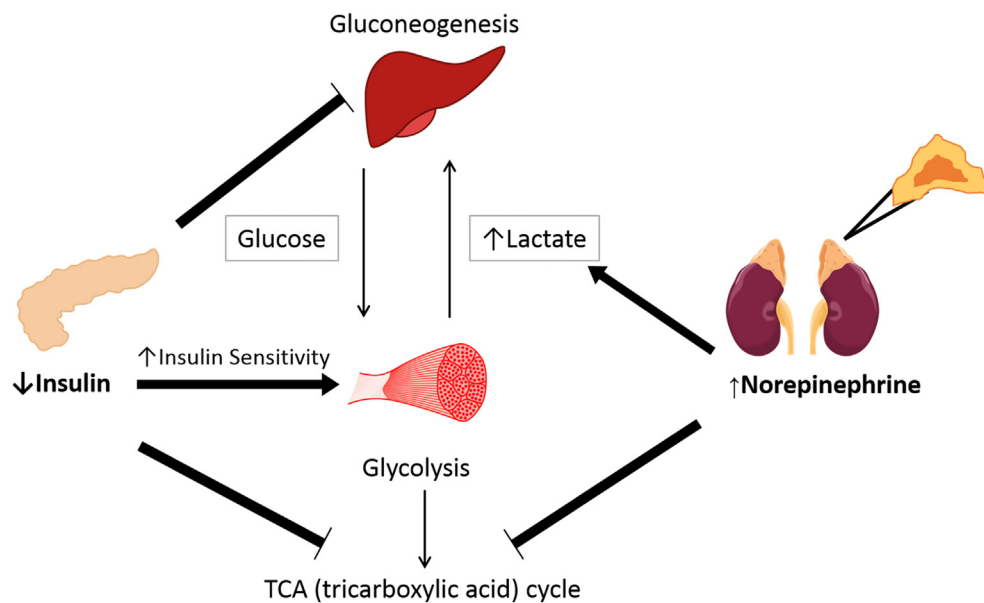


Fig. 1 Schematic for endocrine regulation for glucose production in the IUGR fetus. The Cori cycle is proposed in IUGR fetuses because the increase in norepinephrine, the primary fetal catecholamine, stimulates lactate release and inhibits oxidative phosphorylation in skeletal muscle. Catecholamines also inhibit insulin secretion to decrease oxidative metabolism in skeletal muscle, but the muscle counteracts the low insulin concentrations by increasing glucose uptake appearing to be more insulin sensitive. The low insulin concentrations limits its ability to suppress hepatic gluconeogenesis, which is enhanced by high catecholamines.

Epidemiological studies show that fetal adaptations to IUGR lead to β -cell dysfunction after parturition and increase the odds of developing coronary heart disease, type 2 diabetes, stroke, and hypertension with advanced age. In various animal models of IUGR (guinea-pig, rat, sheep), the postnatal offspring experienced obesity, insulin resistance, glucose intolerance and/or hypertension (Fowden and Forhead, 2004). These parallels allow for IUGR to be studied across a range of animal models working toward an effective treatment.

Summary

Fetal endocrinology plays a significant role in preparing the fetus for postnatal life and regulates several aspects of development and growth. The concept that physiological processes can be modulated during critical windows of development is a theme seen throughout the article. It is important to elucidate if these adaptations have lasting impact on the fetus in postnatal life. Because this review focused primarily on the role hormones in fetal growth, development, and metabolic homeostasis, other aspects of fetal endocrinology may not have been addressed.

References

- Cun, L., McDonald, T. J., Guoyao, W., Nijland, M. J., & Nathanielsz, P. W. (2013). Intrauterine growth restriction alters term fetal baboon hypothalamic appetitive peptide balance. *Journal of Endocrinology*, 217, 275–282.
- Forhead, A. J., & Fowden, A. L. (2014). Thyroid hormones in fetal growth and parturition maturation. *Society for Endocrinology*, 221, R87–R103.
- Fowden, A. L., & Forhead, A. J. (2004). Endocrine mechanisms of intrauterine programming. *Reproduction*, 127, 515–526.
- Gluckman, P. D., & Pinal, C. S. (2002). Maternal-placental-fetal interactions in the endocrine regulation of fetal growth. *Endocrine*, 19, 81–89.
- Kapoor, A., Dunn, E., Kostaki, A., Andrews, M. H., & Matthews, S. G. (2006). Fetal programming of the hypothalamo-pituitary-adrenal function: Prenatal stress and glucocorticoids. *The Journal of Physiology*, 572, 31–44.
- Kota, S. K., Gayatri, K., Jammula, S., Meher, L. K., Kota, S. K., Krishna, S., & Modi, K. D. (2013). Fetal endocrinology. *Indian Journal of Endocrinology and Metabolism*, 17, 568–579.
- Kovacs, C. S. (2014). Bone development and mineral homeostasis in the fetus and neonate: Roles of the calciotropic and phosphotropic hormones. *Physiological Reviews*, 94, 1143–1218.
- Limesand, S. W., & Rozance, P. J. (2017). Fetal adaptations in insulin secretion results from high catecholamines during placental insufficiency. *Journal of Physiology*, 595(15), 5103–5113.
- Manikkam, M., Crespi, E. J., Doop, D. D., Herkimer, C., Lee, J. S., Yu, S., Brown, M. B., Foster, D. L., & Padmanabhan, V. (2004). Fetal programming: Prenatal testosterone excess leads to fetal growth retardation and postnatal catch-up growth in sheep. *Endocrinology*, 145, 790–798.
- Manley, N. (2015). Chapter 2: Embryology of the parathyroid glands. In M. L. Brandi, & E. M. Brown (Eds.), *Hypoparathyroidism* (pp. 11–18). New York: Springer.
- Ng, P. C. (2000). The fetal and neonatal hypothalamic–pituitary–adrenal axis. *Archives of Disease in Childhood—Fetal and Neonatal Edition*, 82, F250–F254.
- Ornoy, A. (2011). Prenatal origin of obesity and their complications: Gestational diabetes, maternal overweight and the paradoxical effects of fetal growth restriction and macrosomia. *Reproductive Toxicology*, 32, 205–212.
- Pictet, R., Rutter, W. J., & Geiger, S. R. (1972). Development of the embryonic endocrine pancreas. In R. O. Greep, E. B. Astwood, D. F. Steiner, N. Fienkel, & S. R. Geiger (Eds.), *Handbook of physiology: A critical comprehensive presentation of physiological knowledge and concepts* (pp. 25–66). Washington, DC: American Physiological Society.
- Pocock, G., Richards, C. D., & Richards, D. A. (2013). Chapter 15: The pituitary gland and hypothalamus. In G. Pocock (Ed.), *Human physiology* (pp. 256–270). New York: Oxford University Press.
- Porter, T. E. (1998). Development and function of the fetal endocrine system. In F. W. Bazer (Ed.), *Endocrinology of pregnancy (contemporary endocrinology)* (pp. 387–406). New York: Humana Press.

Placental Endocrinology

Eugene D Albrecht, University of Maryland School of Medicine, Baltimore, MD, United States
Gerald J Pepe, Eastern Virginia Medical School, Norfolk, VA, United States

© 2018 Elsevier Inc. All rights reserved.

Protein Hormones

Human Chorionic Gonadotrophin (hCG)

Structure and synthesis

hCG is a glycosylated protein composed of α and β subunits and exhibits a molecular weight of 37,180. hCG- α is comprised of 92 amino acids and is located on chromosome 6q14-q21. hCG- β is comprised of 145 amino acids and is structurally similar to the β -subunit of pituitary luteinizing hormone (LH), differing only in the terminal 28 amino acids. The β -subunit of hCG is encoded by a cluster of nonallelic genes (BhCG 1–8) located on chromosome 19q13.32. There are five variants of hCG, including hyperglycosylated hCG (hCG-H) synthesized by extravillous cytotrophoblasts (EVT) and sulphated hCG produced by the pituitary. hCG is produced by the placental villous cytotrophoblasts prior to week 6 of human pregnancy and by the syncytiotrophoblast thereafter. hCG is detectable in maternal blood 1–3 days after implantation, exhibits peak levels at 8–12 weeks of gestation, then declines and remains relatively constant thereafter (Fig. 1). Maternal serum hCG levels are predictive of onset of pregnancy and complications of pregnancy. For example, hCG levels are increased in women who develop hypertension, preeclampsia and carry a Down syndrome fetus, reduced in pregnancies complicated by fetal growth restriction (FGR), and may also serve as a predictor of pregnancy outcome, e.g., preterm birth and fetal demise.

cAMP, which stimulates protein kinase A (PKA) and phosphorylation of CREB(s) essential for binding to cAMP response elements (CRE), increases hCG α and β . The human placenta produces a gonadotrophin-releasing hormone (GnRH) which stimulates hCG production. Numerous other factors, including growth factors and steroid hormones, may also modulate hCG expression by trophoblast cells, although our understanding of the physiological role of these other factors is incomplete.

Function

The most well established action of hCG is regulation of the maintenance and function of the corpus luteum in early pregnancy. hCG binds to the LH/CG receptor to maintain the life span of, promote angiogenesis within, and stimulate progesterone and

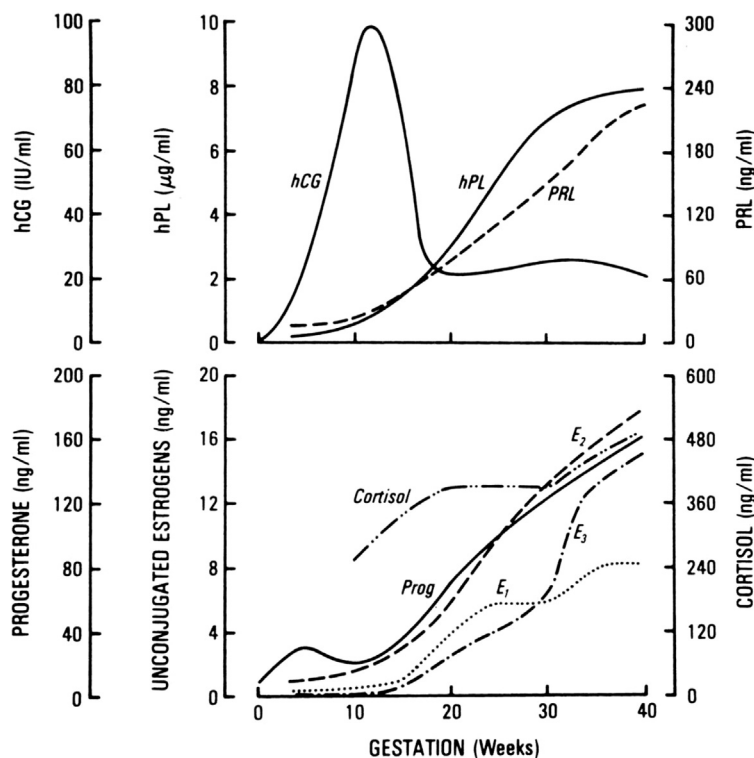


Fig. 1 Serum concentrations of human chorionic gonadotropin (hCG), human placental lactogen (hPL), prolactin (PRL), cortisol, progesterone (Prog), estrone (E₁); estradiol (E₂) and estriol (E₃) during pregnancy From Rebar, R. W. (1999). The breast and the physiology of lactation. In: Creasy, R.W., Resnik, R. (eds.). Maternal-fetal medicine. 4th edn., pp. 106–121. Philadelphia, PA: WB Saunders Co.

estradiol synthesis by the corpus luteum. hCG stimulates luteal progesterone and estradiol production by increasing low-density lipoprotein (LDL) receptor-mediated uptake of cholesterol substrate and increasing the expression of key enzymes, including P-450 cholesterol side-chain cleavage (P-450_{scc}), 3 β -hydroxysteroid dehydrogenase (3 β -HSD) and aromatase (P-450_{arom}), required for steroidogenesis. hCG also develops and stimulates testosterone production by the fetal testes in the first trimester, a process essential for differentiation of the internal duct system and external genitalia in the male fetus. hCG also stimulates relaxin production by the corpus luteum, which may prevent regression of luteal cells and elicit vascular effects in other organs.

Recent studies indicate that hCG has more widely different functions. Thus, hCG produced by the blastocyst promotes decidualization and endometrial receptivity for implantation and interleukin-1 (IL-1) signaling in endometrial stromal cells leading to increased angiogenesis, thereby coordinating embryonic signaling with endometrial function to enhance embryonic growth. hCG also appears to promote immune tolerance by attracting T regulatory cells to the maternal-fetal interface and binds to its receptor expressed on villous cytotrophoblast to enhance fusion of these cells and thus syncytiotrophoblast formation and placental development. hCG reduces oxytocin-stimulated uterine myometrial smooth muscle contraction by activating large conductance calcium-activated potassium channels and decreasing intracellular levels of cyclic nucleotides that regulate contractility. Whether the decline in hCG levels late in gestation facilitates oxytocin-induced myometrial contractions remains to be determined.

hCG action involves activation of the hCG receptor and induction of cAMP and protein kinase A (PKA). cAMP-induced PKA signaling involves binding of the regulatory subunits of PKA to A-kinase anchoring proteins (AKAPs) expressed in the placenta. Such binding ensures subcellular compartmentalization of PKA and thus spatial and temporal regulation of PKA signaling events. The spatiotemporal regulation of PKA signaling by hCG appears to underpin its action on placental function. Finally, although hCG and LH act on the same receptor, intracellular signaling may be quantitatively and/or qualitatively different, e.g., hCG a more potent stimulator of cAMP production.

Placental Lactogen (PL)

Structure and expression

PL, also designated chorionic somatomammotropin (CS), is a member of the growth hormone (GH) family which also consists of pituitary GH (GH-N) and placental GH variant (GH-V). Human PL (hPL) is a single-chain polypeptide with 191 amino acids and a molecular weight of 22,279. PL, GH and prolactin exhibit structural homology. Three genes, CSH-1, CSH-2 and CS-L, encode PL and exhibit 94–98% sequence homology in their coding and flanking regions. Members of the CCAAT/enhancer-binding protein (C/EBP) and ETs family of transcription factors differentially modulate CSH1 and CSH-2 by regulating promoter/enhancer complexes and/or chromatin modification and thus may contribute to expression of CS in the placenta and lack of expression in the pituitary.

PL is synthesized by placental villous trophoblasts and differentiation of cytotrophoblasts into the syncytiotrophoblast during the first trimester of human pregnancy result in an exponential upregulation of PL production (**Fig. 1**). Maternal serum PL levels correlate directly with placental mass and approximate 5–10 μ g/mL near term. Because PL appears to have a role in modulating intermediary metabolism within the mother the effects of components of intermediary metabolism on PL synthesis have been investigated. Prolonged fasting increases and hyperglycemia decreases the secretion of PL, while short-term alterations in glucose have little impact. Moreover, high density lipoprotein (HDL) stimulates PL secretion by human trophoblast cultures. Growth factors, GH-releasing hormone (GHRH), steroids, and neurotransmitters either stimulated, inhibited, or had no effect on PL release in vitro. Thus, a clear understanding of the physiological regulation of PL secretion has not been established, possibly resulting from the different experimental approaches employed.

Function

Since PL, GH and prolactin share similar structure, and PL binds with high affinity to prolactin and GH receptors, it is not surprising that PL has effects on intermediary metabolism, growth, and lactogen function. Thus, PL stimulates pancreatic islet beta cell proliferation and insulin secretion and is necessary for pancreatic beta cell development. PL activates the Akt and MAPK signaling pathway, phosphorylation of JAKs/STATs, and expression of homeobox transcription factors. It has been suggested that lactogenic hormones maintain beta cell mass and function under conditions of stress and nutrient deprivation to protect against the development of gestational diabetes ([Arumugam et al., 2008](#)). During fasting PL reduced insulin sensitivity and induced glucose intolerance at mid to late pregnancy. The latter effects along with an increase in lipolysis may promote utilization of free fatty acids as a source of energy for the mother, while sparing glucose as a primary source of energy for the fetus. PL also may have a role in promoting fetal growth, since PL stimulated amino acid uptake by human fetal fibroblasts and skeletal muscle, effects mediated by IGF-I. PL also stimulated human mammary gland epithelial cells and ductal growth and lactogenesis.

GH-V is a glycosylated protein which differs from GH-N by 15 amino acids. GH-V is synthesized by the syncytiotrophoblast and maternal circulating levels progressively increase with advancing pregnancy. GH-V binds to the prolactin and GH receptors and exhibits somatotrophic and metabolic effects and may also modulate insulin action during pregnancy. PL and GH-V appear to be differentially expressed in association with altered fetal growth, possibly as consequence of polymorphisms in the locus control region and binding of regulatory transcription factors to the promoter. Thus fetal growth restriction (FGR) is associated with lower placental PL and GH, but not GH-V expression, while PL levels are greater in pregnancies with large for gestational age babies. It has been suggested that the divergent regulation of PL and GH-V may provide adaptive maternal and fetal benefits, in which changes in PL and GH-V induced by placental insufficiency alter the production of insulin and/or IGF-1, leading to decreased or increased fetal

growth (Newbern and Freemark, 2011). Thus, PL may act in concert with prolactin, GH-N, and other growth factors (e.g., IGFs) in regulating metabolic adjustments and fetal growth during human pregnancy.

Placental Growth Factors

The human placenta synthesizes numerous growth factors the most physiologically relevant of which are insulin-like growth factor (IGF)-I and -II, transforming growth factor β (TGF β), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), and endocrine gland (EG)-VEGF.

IGF-I and -II

IGF-I and IGF-II are structurally similar to insulin, approximate 7600 kDa and are comprised of α and β chains. The IGF-I gene is located on chromosome 12q22–24.1 and transcription is governed by two promoters. The IGF-II gene is located adjacent to the H19 gene on chromosome 11p15.5 and both genes are imprinted. Imprinted genes that are paternally expressed, such as IGF-II, promote fetal growth, whereas those maternally expressed, e.g., H19, are growth suppressors. Loss of imprinting of the IGF-II gene is extensive in placentas of pregnancies with FGR. Circulating IGF-I and -II are bound to binding proteins (IGFBP-1-6) that control the availability, stability and activity of the IGFs. IGFBP-3 binds 70%–80% of total maternal serum IGF-I and IGF-II. The physiologic actions of IGFs are controlled by IGF-I and IGF-II receptors. The IGF-I receptor binds IGF-I > IGF-II > insulin and the IGF-II receptor binds IGF-II > IGF-I but not insulin. IGF receptor signaling involves receptor autophosphorylation and activation of PI3K, ERK/MAPK and/or Akt.

IGF-I is expressed primarily in the placental syncytiotrophoblast, while IGF-II is expressed in placental cytotrophoblasts, syncytiotrophoblast, extravillous trophoblasts (EVT) and fibroblasts. Placental expression and maternal serum levels of IGF-I and IGF-II increase with advancing human pregnancy. Although the regulation of placental IGF expression is incompletely understood, placental expression and serum levels of GH-V and IGF-I are positively correlated, suggesting that GH-V is a primary regulator of placental IGF-I and supporting a role for GH-V in promoting fetal growth via regulation of IGF-I.

IGF-I and IGF-II appear to have important roles in promoting placental and fetal growth (Murphy et al., 2006; Sferruzzi-Perri et al., 2017). Thus, mice with deletions of IGF-I and/or IGF-II have small pups and IGF-I-deleted mice die before reaching adulthood. Although placental growth is normal in IGF-I knockouts, placentas are small and hypoplastic in IGF-II-null animals. Administration of IGF-I to growth-restricted fetal sheep and guinea pigs restored growth (Sferruzzi-Perri et al., 2013) via up-regulating placental nutrient transporters. Moreover, severe FGR occurs in human infants with a mutation of the IGF-I receptor. Collectively, it appears that IGF-I has a role in fetal growth and development, while IGF-II is more important for placental development. Along with their anabolic effects, the IGFs may promote fetal-placental growth by proliferation of the placental syncytia and uterine artery remodeling to promote maternal-fetal exchange. Importantly, IGF-I and II do not act alone and a complex regulatory interrelationship between the IGFs and insulin, thyroid hormone, cortisol, leptin, and nutritional drivers, appear to control placental and fetal growth (Fig. 2).

TGF β

TGF β s are members of a superfamily of cytokines that includes activins, inhibins, and bone-morphogenic proteins. Three TGF β isoforms, TGF β -1, -2 and -3, are encoded by three different genes. TGF β -1 is expressed primarily in the villous placenta, TGF β -2 in the decidua and TGF β -3 in villous and extravillous trophoblasts. TGF β members bind to the type I and II receptors, thereby activating serine/threonine kinase activity. The TGF β family may play a role in fetal growth, directly or indirectly via regulating placental function. TGF β -1 and -3, as mediated by connective tissue growth factor (Cheng et al., 2017) and the TGF β -binding proteoglycan decorin, act in a paracrine manner to suppress EVT invasion. TGF β -1 and -3 expression is increased with preeclampsia and exacerbates placental dysfunction and the associated complications of adverse pregnancy.

Activin is composed of subunits β_A and β_B that dimerize to form activin A, B and AB. Inhibin is comprised of α and β subunits forming inhibin A and B. Activin A and inhibin A are expressed in the syncytiotrophoblast, cytotrophoblast and decidua and maternal serum levels increase with advancing gestation. Activin A and inhibin B act in a para/autocrine manner to regulate cell proliferation and differentiation, and roles for placental development, vascular adaptation of pregnancy and onset of labor have been proposed. Activin A and B may modulate inflammatory processes and immunity during pregnancy by regulating production of interleukin, neutrophils, monocytes, and lymphocytes. Activin A levels are elevated and may participate in intra-amniotic infection and preterm birth.

EGF

EGF is a member of the EGF superfamily comprised of heparin-binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen, neuregulins 1–6 and TGF α . Each EGF member activates four receptors termed HER-1-4. Receptor activation is complex and controlled by spatiotemporal expression, post-translational processing and ectodomain shedding of the ligands. EGF receptors are expressed in cytotrophoblasts, syncytiotrophoblast and decidua. Maternal serum EGF levels are highest in the first trimester. EGF plays a role in placental development since EGF-null mice are growth-retarded, HER-null mice die in utero due to abnormal placental development, and the EGF receptor is altered in human placentas of intrauterine growth restricted (IUGR) pregnancies. Moreover, EGF promotes trophoblast proliferation and differentiation and inhibits trophoblast apoptosis. HB-EGF is expressed by the placenta throughout human pregnancy, but expression was negligible in pregnancies complicated by preeclampsia and FGR. HB-EGF stimulates EVT proliferation, migration and invasion.

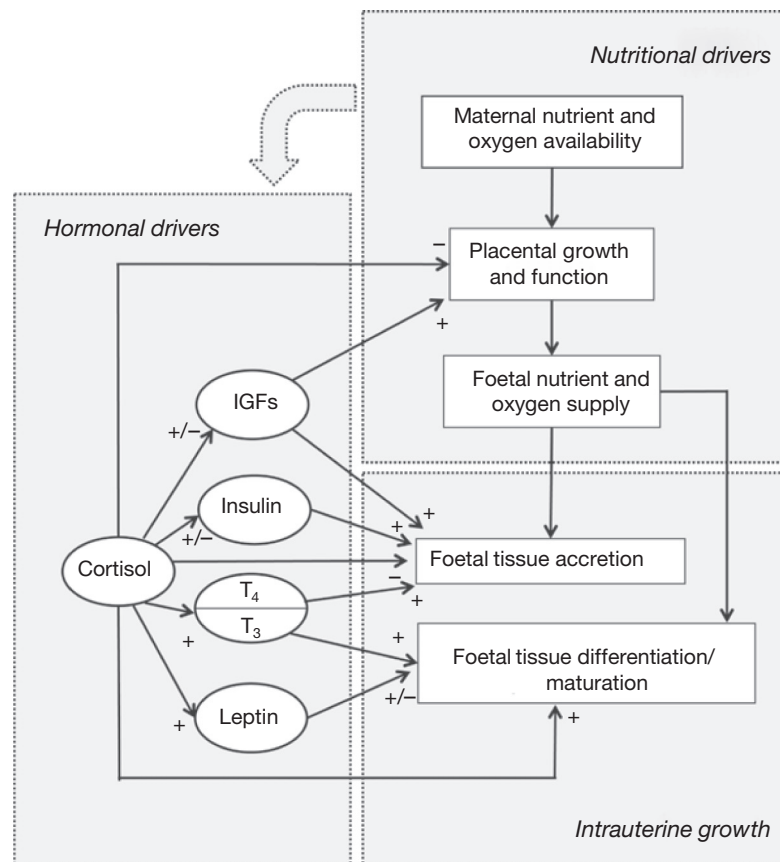


Fig. 2 Proposed interactions of hormonal and nutritional drivers in the control of intrauterine growth. From Sferruzzi-Perri, A. N, Vaughan, O. R., Forhead, A. J., Fowden, A. L. (2013). Hormonal and nutritional drivers of intrauterine growth. *Current Opinion in Clinical Nutrition and Metabolic Care* 16, 298–309

VEGF

The VEGF superfamily is comprised of VEGF-A (known as VEGF), -B, -C, -D, -E and placental growth factor (PlGF). VEGF is encoded from a single gene organized as eight exons and alternative splicing produces 121, 145, 165, 189 and 206 amino acid isoforms. VEGF is a heparin-binding glycoprotein of 45 kDa and a highly potent endothelial cell mitogen that stimulates vascular permeability and consequently new blood vessel formation via angiogenesis. Although new vessel growth and maturation are highly complex processes, VEGF has a critical early rate-limiting role. Thus, VEGF-null mice exhibit vascular defects in embryonic tissues and embryonic lethality.

VEGF binds to the fms-like kinase (Flt-1 or type I) and kinase-insert domain (KDR/Flk-1 or type II) transmembrane tyrosine kinase receptors (Fig. 3, Shibuya, 2013). Both receptors are required for vascular development as Flt-1 or KDR/Flt-1 defective mice die in utero. VEGF-A binds to both receptors and coreceptor neuropilin-1, VEGF-B only binds to the Flt-1 receptor which also binds PlGF. VEGF-C and D bind to Flt-4 or type III and stimulate lymph angiogenesis, while VEGF-E is a KDR/Flk-1 agonist. Flt-1 signaling is important for tumor growth, metastasis and chronic inflammation. A soluble truncated form of the Flt-1 receptor (sFlt-1) which lacks tyrosine kinase signaling, sequesters VEGF and PlGF thereby preventing VEGF interaction with its receptors. VEGF, PlGF and the respective receptors are expressed by placental cytotrophoblasts, syncytiotrophoblast, inner villous stromal cells and decidua cells. VEGF expression in villous cytotrophoblasts and vessel density within the inner villous compartment increase with advancing pregnancy.

Although unproven, VEGF-A may also promote migration and invasion of EVT. Placental expression and serum levels of VEGF and PlGF are lower and the sFlt-1 receptor which sequesters VEGF higher in human preeclampsia and IUGR, adverse pregnancy conditions underpinned by deficient EVT invasion and remodeling of the uterine arteries. Since the Flt-1 promoter contains a hypoxia response element (HRE), the increase in placental hypoxia induced by improper uterine artery remodeling with preeclampsia may account for the increase in expression of placental sFlt-1, which disrupts renal glomerular endothelial cell permeability resulting in proteinuria, a major symptom of preeclampsia. PlGF is expressed as four isoforms, PlGF-1 to -4, as a result of alternative splicing. PlGF may also promote EVT migration/invasive capacity.

EG-VEGF

EG-VEGF is a growth factor expressed only in steroid-producing endocrine glands, that is, ovary, testis, adrenal and placenta. EG-VEGF is mitogenic for endocrine gland endothelial cells and like VEGF induces fenestrations in these cells. However,

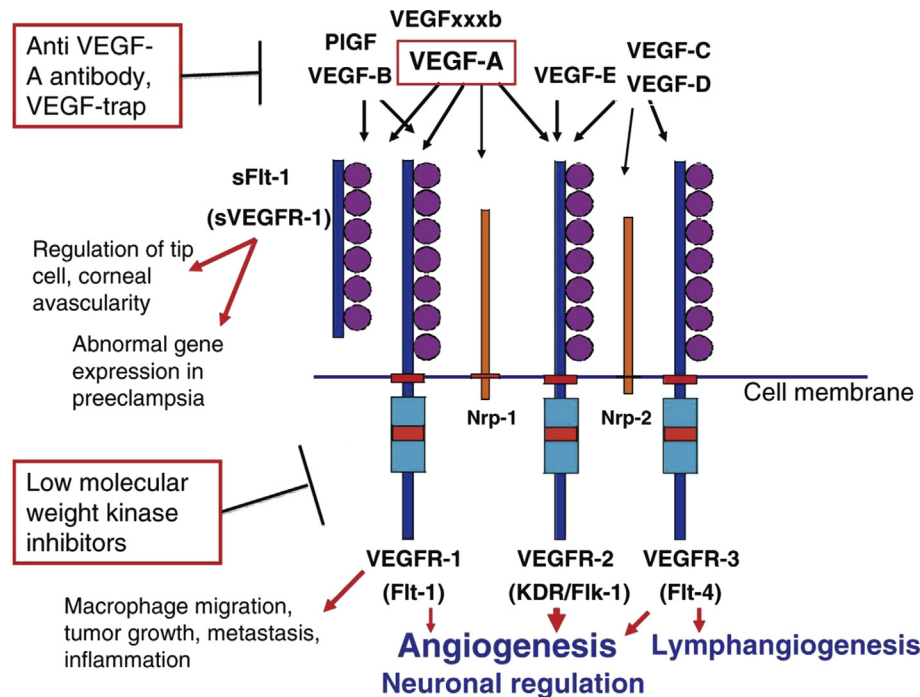


Fig. 3 The VEGF and VEGF receptor (R) system. VEGF-A acting via the VEGFR-1 has a central role in angiogenesis, inflammation, and the phenotypes of cancer. Pro-angiogenic signals are also generated from VEGFR-2, whereas sFlt-1 underlies physiological corneal avascularity and symptoms of preeclampsia. Nrp co-receptors for VEGFs increase the affinity between VEGF and its receptors. VEGFxxx, an alternatively processed variant form of VEGF-A, is suggested to be an endogenous competitor against VEGF-A. From Shibuya, M. (2013). Vascular endothelial growth factor and its receptor system: Physiological functions in angiogenesis and pathological roles in various diseases. *Journal of Biochemistry* **153**, 13–19.

EG-VEGF shares no structural homology with VEGF, but rather is structurally related to a family of peptides termed prokineticins, e.g., prokineticin-2 (PROK2). The gene for EG-VEGF is located on chromosome 1p21 and two G-coupled receptors (PROKR1 and PROKR2) bind PROK1 and PROK2 and mediate a cellular response dictated by G-proteins.

EG-VEGF and its receptors are expressed by the placental syncytiotrophoblast and cytotrophoblasts and maternal serum EG-VEGF levels peak in the first trimester then decline in a pattern very similar to that of hCG. Moreover, hCG increases placental EG-VEGF and PROKR2 expression as modulated via CRE-1 and -2. It appears that EG-VEGF suppresses invasive capacity of trophoblasts, although it is unknown whether EG-VEGF plays a physiologic role in this process or exacerbates the placental dysfunction characteristic of adverse conditions of pregnancy, e.g., preeclampsia.

Thus, several of the placental growth factors, acting in an autocrine and/or paracrine manner, appear to control placental villous and extravillous trophoblast function, including EVT migration and invasion. Future research in this area of placental biology is needed to establish the interactive role the placental growth factors have in vivo in regulating human placental development and function.

Steroid Hormones

Estrogens

Synthesis and regulation

The placenta becomes the primary source of estrogen at approximately 8 weeks of human gestation, and serum estrone (E_1), estradiol (E_2) and estriol (E_3) levels progressively increase thereafter (Fig. 1). The placenta lacks the 17α -hydroxylase/ $17-20$ -lyase ($P450_{C17}$) gene required for de novo production of estrogen. However, the fetal and to some extent the maternal adrenal cortex produces C_{19} steroids, particularly dehydroepiandrosterone (DHEA), that are aromatized via $P450_{arom}$ to the estrogens. In the fetal adrenal cortex, DHEA is synthesized de novo from LDL cholesterol, then sulfurylated and transferred to the placenta as DHEAS or, after 16-hydroxylation, as 16-OH-DHEAS (Fig. 4). DHEAS is converted to DHEA and 16-OH-DHEAS to 16 OH-DHEA via a placental sulphatase. DHEA is converted to Δ^4 -androstenedione via 3β -HSD, and an $P450_{arom}$ enzyme encoded by the CYP19A1 gene aromatizes Δ^4 -androstenedione to estrone, testosterone to estradiol, and 16-OH-DHEA to estriol. The interconversion of estrone and estradiol and of Δ^4 -androstenedione and testosterone is catalyzed by the 17β -hydroxysteroid oxidoreductase (17β -HSD). The fetal adrenal cortex by synthesizing DHEA and other C_{19} -steroids has a major role in regulating placental estrogen production. Fetal pituitary ACTH and corticotrophin releasing hormone (CRH) secreted by the placenta stimulate fetal adrenal DHEAS production, and thus indirectly placental estrogen formation. Moreover, cAMP stimulates expression of the $P450_{arom}$ enzyme.

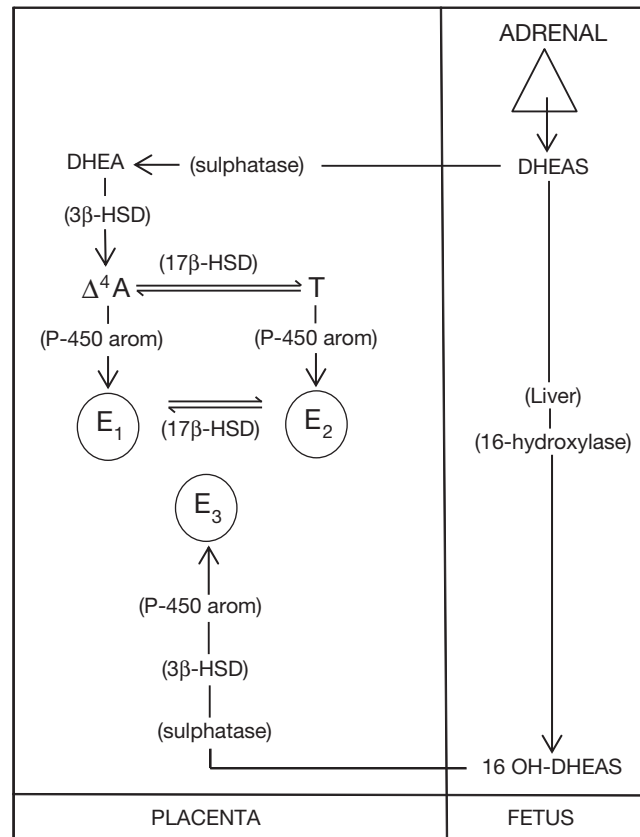


Fig. 4 Estrogen biosynthesis in the fetal-placental unit during human pregnancy. Dehydroepiandrosterone (DHEA); DHEA sulfate (DHEAS); Δ^4 -androstenedione (Δ^4 A); testosterone (T); estrone (E_1); estradiol (E_2); estriol (E_3).

Function

Estrogen has significant roles in preparing the endometrium for implantation, the cascade of events leading to the onset of labor, the maternal cardiovascular changes of pregnancy and preparing the breast for lactation. The placental trophoblast also expresses estrogen receptor α and β , providing a mechanism for estrogen action within this tissue. Thus, the differentiation of human villous cytotrophoblasts into the syncytiotrophoblast is stimulated by estradiol, an effect possibly mediated by leptin. Extravillous trophoblast (EVT) migration to and invasion and remodeling of the uterine spiral arteries, which is extensive during the first trimester of human pregnancy, transforms these arteries into low-resistance vessels to promote blood flow to the placenta and thus development of the fetus. Prematurely elevating estrogen levels in early baboon pregnancy suppresses trophoblast VEGF expression and remodeling of the uterine spiral arteries. It has been proposed (Albrecht and Pepe, 2010), therefore, that the low levels of ovarian estrogen in early pregnancy promote VEGF expression and EVT remodeling of the uterine arteries (Fig. 5), whereas the normal rise in estrogen thereafter suppresses and thus controls the extent to which the uterine vessels are remodeled. This process translates to the human, since the elevated levels of estradiol induced in IVF human pregnancy increase the risk of developing preeclampsia, which is underpinned by a defect in uterine artery remodeling.

During the second half of pregnancy, estrogen stimulates placental receptor-mediated uptake of LDL as cholesterol substrate for and activity of the P450_{scc} enzyme catalyzing the synthesis of progesterone. Moreover, estrogen regulates transplacental cortisol-cortisone metabolism and the levels of cortisol arriving in the fetus, thereby controlling onset of fetal pituitary-adrenal ACTH secretion (Pepe and Albrecht, 1995). Estrogen also downregulates growth of and synthesis of C₁₉-steroid estrogen precursors by the fetal zone of the fetal adrenal cortex, to control estrogen levels within a physiological range. Estrogen also increases fetal pituitary ACTH secretion during ovine pregnancy, and thus the interplay of placental estrogen and the fetal pituitary-adrenal axis may have a role in the initiation of labor. In addition, estrogen promotes formation of the stockpile of healthy ovarian follicles within the human and nonhuman primate fetus that is critical for timely onset of puberty and reproductive function after birth.

Estrogen-deprived pregnant baboons, P450_{arom} or ER α knock-out mice, and P450_{arom}-null humans exhibit a disruption in testis morphology, germ cell, efferent ductile and epididymal development and/or infertility, showing that estrogen also has an essential role in promoting fetal testis development and onset of fertility. Finally, estrogen deprivation in human and baboon pregnancy results in insulin insensitivity and glucose intolerance in the offspring, indicating that estrogen programs mechanisms within the fetus required for normal insulin action after birth.

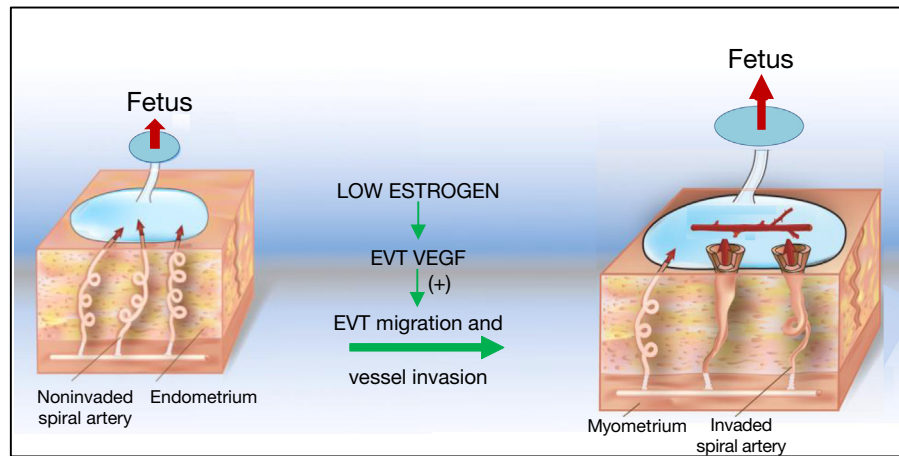


Fig. 5 Proposed role of low estrogen levels in early primate pregnancy in promoting VEGF expression and extravillous trophoblast (EVT) migration, invasion and remodeling of the uterine spiral arteries.

Progesterone

The placenta also becomes the primary source of progesterone at approximately 8 weeks of human pregnancy and levels increase to 150–175 ng/mL at term (Fig. 1). Although *de novo* synthesis of cholesterol is limited, the receptor-mediated uptake of LDL cholesterol by the placental syncytiotrophoblast accounts for the major proportion of placental progesterone production. Cholesterol is converted to pregnenolone via the P450_{scc} enzyme (CYP11A1 gene) within the mitochondria. P450_{scc} functions as a terminal oxidase, in which electrons from NADPH are accepted by an adrenodoxin reductase flavoprotein that transfers the electrons to an iron–sulfur protein, adrenodoxin, that donates the electrons to cytochrome P450_{scc}. The conversion of pregnenolone to progesterone is catalyzed by 3 β -HSD, which is in excess, while the reduction of adrenodoxin is rate-limiting for progesterone synthesis. cAMP chronically stimulates expression/activity of P450_{scc}, adrenodoxin, and LDL receptors in a dose-dependent manner in human cytotrophoblasts, an effect that requires the catalytic unit of protein kinases which may regulate gene transcription.

Progesterone has an essential role in promoting endometrial decidualization, implantation, and glandular function in the early establishment of pregnancy. Progesterone signaling through progesterone receptor isoforms A and B, as modulated by COUP-TFII nuclear receptor and Hand 2 transcription factor, is critical for decidualization and implantation. Progesterone also suppresses the maternal immune system and inflammatory processes at the maternal-fetal interface to promote implantation of the conceptus, and pregnancy maintenance. Progesterone has a pivotal role in repressing myometrial contractility and labor throughout pregnancy. Finally, progesterone is involved in lobuloalveolar proliferation and growth of the mammary gland, but suppresses lactation until after delivery when placental progesterone is lost.

Placental Hypothalamic-Like Neuropeptide and Peptide Hormones

The placenta produces key components of the hormonal systems that characterize the hypothalamic-pituitary axis, including corticotrophic hormone (CRH), urocortin (Ucn), and gonadotropin-releasing hormone (GnRH). The placenta also synthesizes several neuropeptides, e.g., kisspeptin, neuropeptide Y (NPY), oxytocin, neurokinin B (NKB), and vasoactive peptides, e.g., endothelin-1 (ET), adrenomedullin (AM), calcitonin gene-related peptide (CGRP), and parathyroid hormone related peptide (PTHrP) that modulate other hormone systems important to successful pregnancy (Petraglia et al., 2010).

CRH and Ucn

The CRH/Ucn family of peptides is produced in the placental trophoblasts and include CRH, Ucn, Ucn2 and Ucn3. CRH and the Ucn bind to a CRH-binding protein (CRHBP) also synthesized in the placenta. In contrast to the increase in maternal serum levels of CRH with advancing gestation, CRHBP levels decline sharply near the end of gestation, thereby enhancing the bioavailability of CRH and Ucn. CRH and Ucn bind to the CRHR1 and CRHR2 G-protein-coupled receptors expressed in the trophoblast, myometrium, amnion and chorion. Glucocorticoids, estrogen, norepinephrine and prostaglandins increase and progesterone suppresses placental CRH production.

Placental CRH stimulates fetal pituitary ACTH expression and thus fetal adrenal DHEAS and cortisol production in late gestation. CRH may also act directly on placental trophoblast to promote estrogen and decrease progesterone biosynthesis and play a role in coordinating metabolic and neuroendocrine signals between the mother and fetus (Alcantara-Alonso et al., 2017). Since the elevation in CRH is more rapid in women who deliver preterm and slower in those who deliver late, it has been proposed that

CRH acts as a biologic clock to control the length of gestation and thus the timing of birth. Ucn2 acting via CRHR2 regulates the signaling pathway, myosin light chain phosphorylation, and calcium-activated potassium channels in the human myometrium, which would promote labor activity.

Maternal serum CRH levels are increased and CRHBP decreased in pregnancies complicated by preeclampsia and preterm labor and thus their levels may be predictive of these conditions. CRH in high level can also act on vascular smooth muscle to increase peripheral vasodilation and thus lower arterial blood pressure and vascular tone within the uteroplacental circulation. CRH and Ucn stimulate placental pro-opiomelanocortin (POMC) processing and thus expression of ACTH, β -endorphin and melanocyte stimulating hormone (α -MSH). The placenta also synthesizes the opioid peptides, enkephalin, dynorphins 1–8 and 1–13 and β -endorphin, although the specific role of these opioids on placental function remains to be elucidated.

GnRH

GnRH, typically expressed by the hypothalamus, is also synthesized as isoforms GnRH-I and -II by the human placental trophoblast. Both isoforms signal via activation of the GnRH receptor-1 which is localized to cytotrophoblasts, syncytiotrophoblast and the decidua. Although levels of GnRH remain relatively constant during gestation, placental GnRH-1 expression mirrors that of β hCG. As in the hypothalamus, depolarizing and cAMP stimulatory agents, activin A, and estrogen stimulate placental GnRH secretion. Placental GnRH, notably GnRH-1, is a major regulator of β hCG. GnRH may also have a role in decidualization in early pregnancy, since it induced apoptosis in human decidua stromal cells, and in EVT invasion via modulation of matrix metalloproteinases.

Kisspeptin

Kisspeptins comprise a family of proteins derived from the Kiss-1 gene which when transcribed yields Kiss-1, which is further transformed to additional kisspeptins. Kiss-1 is expressed by the placental syncytiotrophoblast, cytotrophoblasts and decidua. The kisspeptin G protein coupled receptor, GPR54, is expressed in the human hypothalamus and the placenta. Placental Kiss-1 and receptor levels are elevated in the first trimester and in preterm and preeclampsia pregnancies, while plasma kisspeptin levels show a progressive rise during normal pregnancy.

Kisspeptin affects GnRH neuronal excitability and expression and thus influences the hypothalamic-pituitary-gonadal axis and puberty onset. Subjects with GPR54 null mutation exhibit idiopathic hypogonadotropic hypogonadism. Kiss-1 also inhibits trophoblast migration and placental trophoblast kisspeptin expression is low in women with a history of recurrent miscarriage.

NPY

NPY is expressed in the decidua, fetal membranes and placental trophoblast, as well as the central and peripheral nervous system. Maternal serum NPY levels are relatively constant during pregnancy. The two NPY receptor subtypes, NPY1R and NPY3R, are localized to placental syncytiotrophoblast microvilli. NPY appears to stimulate placental CRH synthesis and release by modulating the phospholipase C/inositol triphosphate receptor axis and calcium calmodulin kinase II activity. NPY may also stimulate food intake, inhibit insulin release, modulate gastrointestinal motility and events leading to labor, although its roles in these processes are not clearly defined.

Oxytocin

In addition to its synthesis within the hypothalamus, oxytocin is also produced in placental syncytiotrophoblast, decidua, amnion and chorion. Because oxytocin levels are much greater in the placenta than in the posterior pituitary, the placenta appears to be a major source of maternal circulating oxytocin. Maternal serum oxytocin concentrations are highest at night coinciding with nocturnal spontaneous uterine activity and labor. Estrogen appears to be a major regulator of oxytocin expression, particularly in decidua and chorion. Placental oxytocin expression is also increased by placental CRH, activin and prostaglandins, factors integral to the cascade of events leading to parturition. The oxytocin receptor is expressed in the myometrium, decidua and chorion leavies, where levels are increased by estrogen and decreased by progesterone and may have a role in the onset of labor.

NKB

The placenta also expresses NKB, which belongs to the tachykinin family of signaling peptides, such as substance P, that function in the central nervous system as excitatory neurotransmitters for nociception. The G-protein-linked NKB receptors (NKBR 1–3) are expressed in human placenta and umbilical vein endothelial cells. NKB or NKB receptor-3 mutation causes normosmic isolated hypogonadotropic hypogonadism, indicating the role of NKB in human reproductive function and development. NKB also modulates water homeostasis, pulmonary inflammation, cognition, and placental vasodilation via a receptor-mediated mechanism independent of nitric oxide.

ET

ET is synthesized as three isoforms, ET-1-3, expressed by placental endothelial cells, villous trophoblast, amniotic epithelium, as well as fetal-placental vessels. ET-1 is a potent vasoconstrictor of the human placental vasculature and thus appears to play a role in control of uteroplacental blood flow. Placental ET-1 expression and circulating levels are higher in women with preeclampsia.

AM

AM is a 52 amino acid peptide expressed in syncytiotrophoblast, villous cytotrophoblast, EVT and the decidua. Maternal AM levels are 3-fold higher in pregnant than nonpregnant women and increase during the course of human gestation. AM null mutation in mice results in fetal death at midgestation caused by cardiovascular defects. Administration of an AM antagonist caused fetal growth restriction and defective blood vessel development in the placenta, suggesting a role in placental vascularization.

CGRP

CGRP is a 37 amino acid neuropeptide expressed in the placental syncytiotrophoblast, villous vascular endothelial cells, and decidua. CGRP is secreted into the maternal circulation where levels progressively increase with advancing gestation. CGRP binds to a G protein-coupled receptor, which is modified by receptor activity modifying proteins (RAMP-1-3) and is localized to the placental syncytiotrophoblast microvillous and basal membranes, and the myometrium. CGRP receptor-null mice exhibit cardiovascular defects and embryonic death at midgestation, indicating the importance of CGRP for fetal development. A major function of CGRP in pregnancy appears to be modulation of uteroplacental vasoactive tone. Moreover, CGRP promotes human vascular endothelial cell proliferation and migration and capillary-like tube formation, suggesting that CGRP has a role in placental angiogenesis and neovascularization.

PTHrP

PTHrP is a 141 amino acid protein which shares sequence homology with PTH and is enzymatically processed to PTHrP 1–34 and 67–86. PTHrP1–34 is expressed in the syncytiotrophoblast and PTHrP 67–86 in placental villous capillary endothelial cells. Maternal serum levels of PTHrP are elevated in pregnancy particularly in late gestation. PTHrP binds to the PTH receptor expressed in the placenta and myometrium. PTHrP-deficient mice exhibit a reduced maternal-fetal calcium gradient, skeletal dysplasia and die at birth. PTHrP also elicits a potent vasodilatory effect on the uteroplacental circulation and inhibition of uterine contractility.

Adipokines

Adipokines comprise a group of peptides, including leptin, adiponectin, resistin and ghrelin, that play an important role in the metabolic adaptations of human pregnancy, notably the increase in maternal insulin resistance with advancing gestation. Although originally isolated from adipose, adipokines are also expressed by the placenta.

Leptin

Leptin is a 146 amino acid protein that regulates food intake, energy expenditure and metabolism and thus body weight. Leptin action is modulated by the leptin receptor, a member of the cytokine receptor superfamily. Differential splicing of the leptin receptor results in four isoforms characterized by the length and sequence of the cytoplasmic terminal and classified as long (HLR_L) or short (HLR_S). In addition to maternal tissues, amnion, chorion and umbilical vasculature express HLR_L and HLR_S.

Leptin is expressed by the placental syncytiotrophoblast and cytotrophoblast as well as adipose. Maternal serum leptin concentrations rise throughout pregnancy, peaking during the second and third trimester. Placental leptin expression appears to be regulated by the hormonal *milieu* of pregnancy and not by trophoblast mass. Leptin is encoded by the *lep* gene located on chromosome 7q31 and transcription in adipose is increased by estradiol, glucocorticoids, and insulin and decreased by β -adrenergic agonists. Consensus half sites for estrogen receptor and a hypoxia response element in the promoter contribute to leptin transcription. Tissue-specific regulation of leptin expression is also known to be epigenetically mediated through DNA methylation at a tissue-specific region in the *lep* gene promoter. The degree of methylation of the *lep* gene in the placenta may play an important role in leptin expression in normal pregnancy, as well as in pregnancies complicated by FGR, diabetes and preeclampsia.

In addition to regulation of satiety, leptin has other metabolic functions, including the suppression of pancreatic β cell insulin secretion, enhancement of adipocyte glucose utilization and lipolysis, and increase in insulin sensitivity. Importantly, the primary physiologic role of leptin to suppress food intake is reduced during pregnancy, apparently to assure nutrient availability for fetal growth and maternal metabolic homeostasis (Schanton et al., 2017). The hyperphagia of pregnancy is caused by leptin resistance and disruption of the leptin receptor signaling pathway in the hypothalamus and/or changes in placental PL secretion. In addition to effects on maternal tissues, leptin modulates aspects of placental function, including stimulation of trophoblast proliferation and syncytiotrophoblast amino acid transport activity critical to fetal growth.

Adiponectin

Adiponectin is a 248 amino acid protein that enhances insulin sensitivity and reduces hepatic glucose production. Although this metabolic hormone is abundantly produced by adipose, the placenta also expresses adiponectin. The full length protein (fAD) can multimerize to form several low, medium and high molecular weight complexes. The adiponectin receptors, ADIPO-R1 and -R2, are expressed in multiple tissues, including skeletal muscle, liver and the placenta. In humans, maternal serum adiponectin

levels are elevated very early in gestation and then decline and remain relatively constant throughout the remainder of pregnancy. In contrast, adiponectin levels in the fetus are markedly increased and correlated with gestational age. In addition to enhancing insulin sensitivity, adiponectin may have a role in promoting fetal growth, and stimulating trophoblast hCG expression.

Resistin

Resistin is comprised of two 92 amino acid disulfide-linked polypeptides and expressed by placental syncytiotrophoblast. Maternal serum resistin levels are relatively constant in the first and second trimester, then increased by the third trimester. In conjunction with placental lactogen, resistin is thought to play an important role in the marked increase in insulin resistance with advancing gestation. The factors controlling expression of the gene encoding resistin, which is localized to chromosome 19p13.2, remain to be elucidated.

Ghrelin

Ghrelin is a 28 amino acid peptide hormone produced by the intestine, pancreas, kidneys and the placenta. Once in blood, ghrelin is deacylated by enzymes to form des-acyl-ghrelin (DAG) which accounts for 90% of the hormone circulating in plasma. DAG acting via pathways yet to be identified stimulates adipogenesis. Importantly, hyperglycemia and insulin resistance are independently associated with a decrease in ghrelin expression. Maternal serum ghrelin levels and placental expression are highest in early-midgestation then decline thereafter. The factors regulating placental ghrelin expression are unknown, however, women carrying the 51Q allele of the ghrelin gene have a fivefold lower risk of developing gestational diabetes. The protective effect of 51Q may reflect decreased levels of ghrelin which would facilitate increased pancreatic insulin release.

Thus, with advancing gestation placental expression and maternal serum levels of leptin and resistin increase, while adiponectin and ghrelin levels decrease, a pattern of expression that would promote maternal satiety and decrease insulin sensitivity to ensure adequate maternal nutrient (e.g., glucose) supply to the fetus for growth and maternal adipose deposition for metabolic homeostasis. Leptin and adiponectin may also regulate aspects of trophoblast function, including expression and activity of nutrient transporters important to fetal growth. Moreover, placental expression and maternal serum concentrations of leptin are elevated in pregnancies complicated by preeclampsia, particularly in the presence of a small fetus, apparently as a compensatory response to an under-perfused placenta.

In summary, the placenta produces a wide range of protein and steroid hormones, as well as hypothalamic-like neuropeptides and adipokines that have important roles in regulating pregnancy maintenance, placental and maternal vascular development, fetal growth, and metabolic pathways that control maternal and fetal homeostasis and mechanisms that coordinate maturation of fetal organ systems important for function in adulthood. A major challenge in future investigation is to establish how these placental peptide and steroid hormones interact to control these critically important aspects during pregnancy and prenatal and postnatal development.

Acknowledgments

Portions of the material presented in this chapter were funded by the National Institutes of Health Research Grants R01 HD93070 and R01 DK93950. The authors gratefully acknowledge the assistance of Wanda James with the computer word processing preparation of this chapter.

References

- Albrecht, E. D., & Pepe, G. J. (2010). Estrogen regulation of placental angiogenesis and fetal ovarian development during primate pregnancy. *The International Journal of Developmental Biology*, 54, 397–408.
- Alcantara-Alonso, V., Panetta, P., de Gortari, P., & Grammatopoulos, D. K. (2017). Corticotropin-releasing hormone as the homeostatic rheostat of feto-maternal symbiosis and developmental programming in utero and neonatal life. *Front Endocrinol (Lausanne)*, 8, 161.
- Arumugam, R., Horowitz, E., Lu, D., et al. (2008). The interplay of prolactin and the glucocorticoids in the regulation of beta-cell gene expression, fatty acid oxidation, and glucose-stimulated insulin secretion: Implications for carbohydrate metabolism in pregnancy. *Endocrinology*, 149, 5401–5404.
- Cheng, J. C., Chang, H. M., & Leung, P. C. K. (2017). TGF- β 1 inhibits human trophoblast cell invasion by upregulating connective tissue growth factor expression. *Endocrinology*, 158, 3620–3628.
- Murphy, V. E., Smith, R., Giles, W. B., & Clifton, V. L. (2006). Endocrine regulation of human fetal growth: The role of the mother, placenta, and fetus. *Endocrine Reviews*, 27, 141–169.
- Newbern, D., & Freemark, M. (2011). Placental hormones and the control of maternal metabolism and fetal growth. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 18, 409–416.
- Pepe, G. J., & Albrecht, E. D. (1995). Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocrine Reviews*, 16, 608–648.
- Petraglia, F., Imperatore, A., & Challis, J. R. (2010). Neuroendocrine mechanisms in pregnancy and parturition. *Endocrine Reviews*, 31, 783–816.
- Schanton, M., Maymó, J. L., Pérez-Pérez, A., Sanchez-Margalet, V., & Varone, C. (2017). Involvement of leptin in the molecular physiology of the placenta. *Reproduction*, REP-17-0512 [Epub ahead of print].

- Sferruzzi-Perri, A. N., Vaughan, O. R., Forhead, A. J., & Fowden, A. L. (2013). Hormonal and nutritional drivers of intrauterine growth. *Current Opinion in Clinical Nutrition and Metabolic Care*, 16, 298–309.
- Sferruzzi-Perri, A. N., Sandovici, I., Constancia, M., & Fowden, A. L. (2017). Placental phenotype and the insulin-like growth factors: Resource allocation to fetal growth. *The Journal of Physiology*, 595, 5057–5093.
- Shibuya, M. (2013). Vascular endothelial growth factor and its receptor system: Physiological functions in angiogenesis and pathological roles in various diseases. *Journal of Biochemistry*, 153, 13–19.

Further Reading

- Rebar, R. W. (1999). The breast and the physiology of lactation. In R. W. Creasy, & R. Resnik (Eds.), *Maternal-fetal medicine* (4th edn., pp. 106–121). Philadelphia, PA: WB Saunders Co.

Placental Lactogen (CSH)

Russell V Anthony and Kimberly M Jeckel, Colorado State University, Fort Collins, CO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

While the importance of pituitary-derived growth hormone (GH) and prolactin (PRL) in postnatal growth and lactogenesis is well documented, their role in fetal growth and development has been more controversial. The lack of effect of anencephaly or congenital absence of the pituitary gland on human fetal growth rate led to the conclusion that fetal development occurs independent of fetal pituitary-derived GH. Yet, infants with GH receptor (GHR) dysfunction exhibit clinical in utero growth restriction, suggesting that GH or a GH “analog” may have actions within fetal tissues. Indeed, members of the GH/PRL gene family are produced by the placenta, which may have common functions providing sufficient redundancy to enhance the likelihood of an optimal pregnancy outcome.

Of the members of the ancestral GH/PRL gene family transcribed in the placenta, the placental lactogens (PL) or chorionic somatomammotropin (CSH) is produced by most eutherian mammals, with the notable exception of the horse and pig. Human CSH was initially purified by two groups in 1961 and 1962 and was later shown to exhibit $\approx 85\%$ amino acid sequence identity with human GH. By contrast, nonprimate CSHs appear to have evolved from the ancestral PRL gene. Placental and circulating concentrations of CSH are reduced in human and sheep intrauterine growth-restricted (IUGR) pregnancies. However, whether this is a cause or an effect of IUGR has not been determined, and as will be discussed, the exact function of CSH in any species has not been fully elucidated. For obvious ethical reasons, a thorough examination of the function of CSH in human pregnancy is not feasible, predicating the need for relevant animal models.

Gene Organization and Biochemical Characteristics

Human CSH evolved from the ancestral GH gene, explaining its 87% amino acid sequence homology with GH, and the genes encoding CSH reside within a five gene cluster on chromosome 17 (Walker et al., 1991). This gene cluster includes, in order, *GH1* (GH-N), *CSHL1* (CS-L or PL1), *CSH1* (CS-A or PL4), *GH2* (GH-V), and *CSH2* (CS-B or PL3). *GH1* is expressed by somatotropes of the anterior pituitary, whereas *GH2* is expressed by syncytiotrophoblast cells of the placenta and is secreted only into the maternal circulation and not the fetal circulation. Of the CSH genes, both *CSH1* and *CSH2* are actively transcribed by the syncytiotrophoblasts, and the proteins encoded by these genes only differ by one amino acid within the signal peptide, such that the mature secreted CSH1 and CSH2 are identical nonglycosylated proteins. By contrast, *CSHL1* is often considered a pseudogene due to complex transcript splicing events, predicting that the majority of processed *CSHL1* mRNA is nonfunctional. While the “upstream” flanking regions of *GH1*, *CSH1*, and *CSH2* genes share significant sequence homology, there are specific differences that appear to impede the transcription of the CSH genes by pituitary somatotropes. Syncytiotrophoblast-specific transcriptional regulation of *CSH1* and *CSH2* appears to reside with enhancer elements located distal to the *CSH2* gene, and the proteins binding these enhancer elements may be required for nucleation of the preinitiation complex leading to trophoblast-specific transcription (Walker et al., 1991). More recent evidence indicates transcription of these placental genes is enhanced when the locus control region and the local enhancer and promoter elements undergo DNA demethylation and histone hyperacetylation.

All nonprimate CSH genes appear to have evolved from the ancestral PRL gene rather than the GH gene, and as such, the genes encoding nonprimate CSH are located on the same chromosome as PRL. With mice and rats (Soares et al., 2007), each express two different PLs/CSHs: PL-I (*Prl3d*, *Csh1*) and PL-II (*Prl3b*, *Csh2*). There are at least three distinct *Prl3d* genes and one *Prl3b* gene, located on the same chromosome as *Prl*, as well as several proliferin (*Plf*) genes and approximately 20 PRL-like protein genes expressed by the placenta. Interestingly, all three rodent PL-I (Prl3d) are glycoproteins, whereas PL-II (Csh2) is not glycosylated. As with rodents, CSH in cattle and sheep also evolved from the ancestral PRL gene. In cattle, there is a single CSH (*CSH2*) gene located on chromosome 23 with the PRL gene and a number of genes encoding prolactin-related proteins (*PRP*) produced by the placenta. Sheep are similar in that there is a single CSH (*CSH*) gene located on chromosome 20 with the PRL gene, and several *PRP* genes expressed by the placenta. Cattle and sheep CSHs (Anthony et al., 1995) share $\approx 50\%$ amino acid sequence identity with their respective PRL and only $\approx 24\%$ with their respective GH. Cattle CSH exists as multiple glycosylated isoforms, whereas sheep CSH exists as a single nonglycosylated polypeptide. Furthermore, cattle and sheep CSHs are only $\approx 66\%$ identical in their amino acid sequences, whereas the amino acid identity for GH or PRL between these species is $\approx 99\%$. The divergence in the structure of cattle and sheep CSHs could infer adaptive evolutionary pressure on these genes expressed by the placenta.

Secretion and Receptors

Human CSH is detectable in the placenta by 18 days of gestation (dGA), can be detected in maternal circulation by 21 dGA, and increases exponentially during the first trimester and then more gradually until term, reaching values of 5–10 $\mu\text{g/mL}$ in maternal circulation (Fig. 1). In contrast to placental-derived GH (GH2), CSH is secreted into the fetal circulation, and while the available

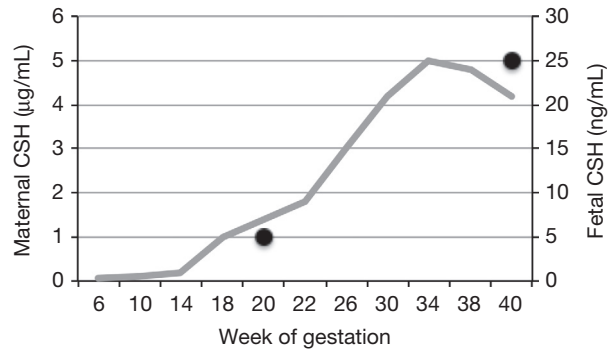


Fig. 1 Human maternal and fetal plasma CSH concentrations. Maternal plasma CSH is represented by the *solid gray line*, and fetal plasma CSH is represented by the *black circles*. Information derived from Handwerger (1991) and Handwerger and Freemark (2000).

data is minimal, the CSH concentration in fetal plasma at 20 weeks of gestation is approximately 5 ng/mL and at term is approximately 20–30 ng/mL. Prior to week 6 of gestation, the cytotrophoblasts of the developing placenta appear to be the site of CSH production, whereas during the remainder of gestation, the syncytiotrophoblasts are clearly the site of CSH production. The amount of CSH mRNA within individual syncytiotrophoblasts does not appear to change during the course of pregnancy, but the amount of syncytiotrophoblast tissue within the placenta increases as gestation progresses, which could explain the fivefold increase in CSH mRNA concentration between the first and last trimester. From these data, one may infer that CSH is constitutively expressed following syncytiotrophoblast formation, and its synthesis may not be subject to a great deal of regulation. However, a variety of growth factors, secretagogues and metabolic regulators, have been implicated in the regulation of CSH secretion, but no clear regulatory control has been established. As noted earlier, there are several reports demonstrating significant reductions in the concentration of CSH in maternal circulation of IUGR pregnancies. As yet, there is no direct evidence in the human to decipher whether or not this is just a function of reduced placental mass associated with IUGR or is playing a more causative role.

A unique CSH receptor has yet to be isolated or structurally characterized in humans. While CSH shares $\approx 85\%$ amino acid sequence identity with pituitary-derived GH (GH1), CSH exhibits low affinity for the GHR, but both CSH and GH exhibit high-affinity binding to the PRL receptor (PRLR). Interestingly, both CSH and GH bind the PRLR with greater affinity than does PRL itself, whereas PRL does not bind the GHR. In maternal tissues, it may well be that CSH exerts its actions through either the GHR or PRLR or both. However, in human fetal tissues, the limited available studies lend credence to the likelihood of a “unique” CSH receptor. Specific binding of GH to fetal liver microsomes has been demonstrated, but no specific binding was apparent with fetal skeletal muscle microsomes. There was specific binding of CSH to microsomes derived from both fetal tissues. Furthermore, the GH binding sites in the fetal liver exhibited sixfold greater affinity for GH than for CSH, and the CSH binding sites exhibited a ninefold greater affinity for CSH than for GH. The differences in relative affinity for GH and CSH with fetal liver microsomes, coupled with the lack of specific GH binding to fetal skeletal muscle microsomes, suggest that their respective binding sites or receptors are different. This conclusion is supported by the fact that GHR can be immunolocalized in the fetal liver, but not fetal skeletal muscle.

With mice and rats, during essentially the middle third of gestation, Prl3d is produced by polyploid trophoblast giant cells of the developing placenta, followed by the expression and secretion of Prl3b by trophoblast giant cells during the last third of gestation. Both Prl3d and Prl3b are PRLR receptor agonists. In domestic ruminants, CSH is produced by chorionic binucleate cells (BNCs). These are thought to be derived from endoreduplication without cytokinesis of the chorionic epithelium and begin to arise within the trophoctoderm layer during conceptus elongation during the establishment of pregnancy. During most of gestation, BNC comprise approximately 25% of the chorionic epithelium, and at any given point in time, approximately 20%–25% of the BNCs are actively migrating to fuse with the maternal–fetal syncytium. In domestic ruminants, the syncytium initially arises from the fusion of BNC with maternal uterine epithelial cells, forming syncytial plaques. However, as gestation progresses in cattle, the maternal–fetal syncytium is lost, with reestablishment of the maternal uterine epithelium. Consequently, BNCs fuse with maternal epithelial cells, forming trinucleated cells. By contrast, sheep BNCs continue to migrate and fuse with the established syncytium, and as pregnancy progresses, the uterine luminal epithelium is lost, and the syncytium becomes predominantly of “fetal origin.” The difference between these two ruminant species in the retention of a functional maternal–fetal syncytium is also reflected in the concentration of CSH in both maternal and fetal circulation. In cattle, the concentration of CSH in maternal blood is quite low, with values typically not exceeding 2 ng/mL throughout gestation. By contrast, sheep CSH has similar maternal concentration profiles with human CSH, increasing steadily throughout gestation (Fig. 2), and becomes quite variable during late gestation, often reaching concentrations exceeding 1 µg/mL (Kappes et al., 1992). The concentration of maternal sheep CSH is correlated to placental CSH mRNA concentrations, and the gestational increase is reflective of increasing total BNC in the sheep placenta; as placental mass plateaus during the second third of gestation, the placenta continues to restructure by adding proportionately more chorionic epithelium. In cattle, fetal concentrations of CSH are greatest (25–30 ng/mL) at the end of the first trimester and decline to 5–10 ng/mL near term. In sheep, fetal CSH concentrations increase during the first two-thirds of gestation but then plateau at concentrations of 30–60 ng/mL during late gestation (Fig. 2). The plateau in fetal concentrations can be misinterpreted, as the entry rate kinetics into the umbilical circulation increases during this time frame but is offset by increasing fetal blood volume.

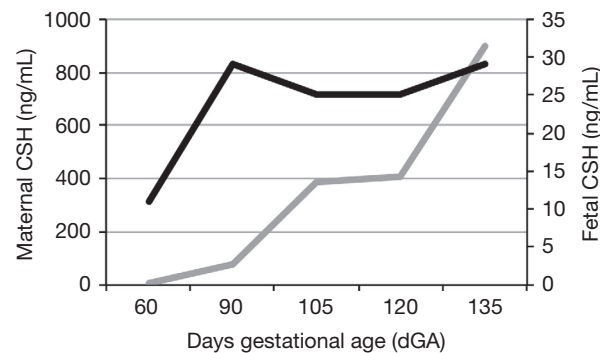


Fig. 2 Maternal and fetal plasma CSH concentrations in sheep. Maternal plasma CSH is represented by the solid gray line, and fetal plasma CSH is represented by the solid black line. Information derived from Kappes et al. (1992).

As noted earlier, rodent Prl3d and Prl3b are PRLR agonists, but the exact receptor through which ruminant CSHs elicit their actions is less clear. GHR and PRLR radioreceptor assays were the basis for monitoring the purification of ruminant CSH, yet in competitive binding assays, GH has greater affinity for the GHR than does CSH, as is also the case with PRL and the PRLR. Both cattle and sheep CSHs have been shown to specifically bind the GHR, but they do so in a 1:1 stoichiometry rather than the 1:2 stoichiometry of binding that GH and GHR exhibit. Dimerization of the GHR (i.e., 1:2 stoichiometry of binding) is required to elicit downstream signaling from this receptor, and the evidence at hand suggests that ruminant CSH and its 1:1 binding of the GHR is acting as a functional antagonist to GH. However, cattle and sheep CSHs have been shown in vitro to heterodimerize the GHR and PRLR, activating downstream signaling. The heterodimerization of the GHR and PRLR may well account for CSH actions, at least within maternal tissues. As noted with human CSH, there is evidence within the fetus for a “unique” or specific CSH receptor. In fetal sheep, while *GHR* mRNA is transcribed throughout most of gestation in the fetal liver, the transcript is derived from one of several alternate exon 1’s, all of which encode an autonomous short-open reading frame upstream of the normal “start” codon, which resides in exon 2. Consequently, it is unlikely that a functional GHR is translated, until near term, when fetal cortisol appears to induce “normal” splicing of exon 1A in the *GHR* transcript. Additionally, while the *PRLR* gene is transcribed in the fetal liver, its expression is quite low. More importantly, saturation analysis of fetal sheep liver microsomes demonstrated specific saturable binding kinetics for CSH from 60 dGA through near term but no specific saturable binding kinetics for either GH or PRL. If CSH exerts its actions through the heterodimerization of GHR and PRLR in sheep fetal liver, that would require both GHR and PRLR to exist in the fetal liver and should have provided for specific saturable binding of GH and PRL. It would therefore appear that at least within the human and sheep fetus, CSH may exert its actions through a structurally characterized receptor.

Biological Actions

While we have known of the existence of CSH for nearly 60 years, as yet, the exact biological actions of CSH have yet to be clearly defined. Especially within maternal circulation in humans and sheep, the concentration of CSH is extremely high relative to the reported binding affinity for any receptor, such that infusion of CSH into maternal circulation is not likely to yield tangible/interpretable results. This is also somewhat true within the fetus as well, but at least one study demonstrated discernible effects from chronic infusion of CSH into the sheep fetus. CSH in maternal circulation is depressed in both human and sheep IUGR pregnancies, but it has not been ascertained whether this is a mediator of IUGR or simply an effect of reduced placental mass. There are case reports of patients with deletions within the human *CSH1-GH2-CSH2* gene cluster, with varying neonatal outcomes, both supporting and refuting an important role of CSH during pregnancy. In many cases, the phenotype was not adequately characterized, and it is not known if other hormones may have been upregulated in a compensatory fashion. While homologous recombination has been in use to generate specific gene knockouts in mice for about 30 years, as yet, neither Prl3d nor Prl3b have been functionally ablated to determine the resulting phenotype. The recent use (Baker et al., 2016) of in vivo RNA interference of CSH in sheep could provide the means to directly assess the function of CSH in vivo.

Maternal Effects

While responses to CSH in maternal circulation likely vary between humans, rodents, and ruminants and may reflect the receptor(s) through which it acts, CSH has been implicated in modifying maternal metabolism, ovarian steroidogenesis, and mammaryogenesis. The most abundant evidence, derived from both humans and ruminants, is in support of CSH effects on maternal metabolism and will be the focus of this discussion. During the third trimester of human pregnancy, when maternal CSH concentrations are quite high, basal- and glucose-stimulated insulin secretion increases, some tissues become insulin resistant, glucose tolerance is impaired, lipid mobilization is increased, and maternal concentrations of insulin-like growth factor 1 (IGF-1) increase markedly, leading Handwerger (1991) to postulate that these changes in maternal metabolism may result from CSH actions (Fig. 3). All of these

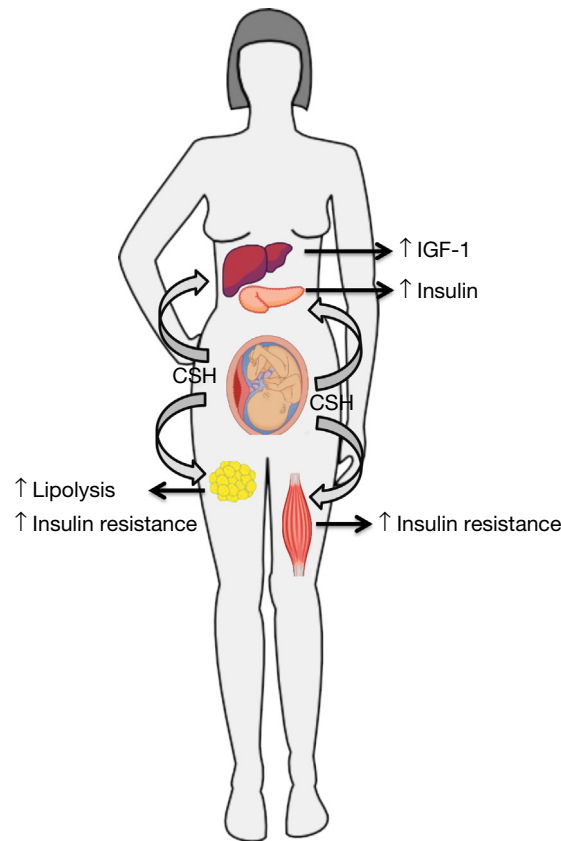


Fig. 3 Predicted biological actions of CSH within the mother. Representation of the various hypothesized effects of CSH within the maternal system during pregnancy. These include the stimulation of IGF-1 and insulin secretion, enhanced peripheral insulin resistance, and increased lipolysis. Conceptually adapted from Handwerger, S. (1991). Clinical counterpoint: the physiology of placental lactogen in human pregnancy. *Endocrine Reviews* 12, 329–336.

changes in maternal metabolism are thought to provide more glucose for placental transport and utilization by the fetus as its primary energy source.

In vitro-derived data from rats and humans support the hypothesis that CSH promotes maternal insulin resistance and β -cell proliferation, with CSH augmenting insulin secretion from adult human pancreatic islets. However, the ability of human CSH to augment glucose-stimulated insulin secretion has varied among experiments, as has the impact of CSH on glucose tolerance. The variability in the response to CSH has also been observed in sheep. An 8 h infusion of sheep CSH into fasting pregnant ewes resulted in reduced plasma glucose and elevated plasma insulin, whereas a 36 h infusion resulted in elevated plasma glucose and reduced plasma insulin. A short-term (12 h) immunoneutralization of maternal CSH did not impact glucose utilization, but did increase plasma insulin concentrations. These three reports differ in their experimental approach, the purity of CSH used and gestational age, making it difficult at best to draw conclusions. In cattle, administration of CSH during pregnancy had no effect on maternal plasma glucose or insulin. With the recent CSH-deficient sheep pregnancies, Baker et al. (2016) reported no effect on maternal glucose concentrations, but did observe a 57% increase in maternal insulin concentrations and a 50% increase in the insulin/glucose ratio. These results agree with the short-term PL immunoneutralization study, both countering the hypothesis of CSH augmenting maternal insulin resistance. Unfortunately, none of these studies exploited the advantage of pregnant sheep as an experimental model, that being the ability to chronically catheterize both maternal and fetal vasculature, and conduct steady-state physiological assessment of insulin/glucose homeostasis. Maternal hyperinsulinemic-euglycemic clamp studies in control and CSH-deficient pregnancies would provide a steady-state well-controlled assessment of CSHs impact on maternal insulin sensitivity.

During late gestation, there is increased lipolysis, providing free fatty acids (FFA) to be used as a maternal energy source during fasting or for building energy stores during nonfasting periods. Studies examining the effect of CSH on lipid metabolism in humans generally support the concept that maternal CSH is lipolytic (Fig. 3). Human CSH increases basal lipolysis in vitro, and administration of CSH in vivo increased the circulating concentrations of FFA, ketones, and glycerol, although these in vivo effects have not been observed in all studies. Esterification of FFA into triglycerides within human adipose is promoted by insulin-stimulated glucose uptake, and this effect is enhanced by treating adipose tissue with CSH in vitro. As with the impact of sheep CSH administration on insulin and glucose homeostasis, there are contrasting results on the impact of CSH on circulating FFA. The short-term

CSH immunoneutralization studies in sheep resulted in a tendency for reduced plasma FFA, agreeing with the potential lipolytic effects of human CSH. However, treating adipose tissue obtained from pregnant sheep with CSH did not result in increased lipolysis. Administration of CSH to pregnant cattle did not impact plasma FFA. While interpreting the effects of CSH on maternal lipolysis and/or lipid deposition in adipose across these species, it should be kept in mind that cattle and sheep are ruminants, and while lipid metabolism pathways are similar with humans, the composition and concentration of circulating FFAs can be quite different between humans and ruminants.

While maternal plasma IGF-1 increases during late gestation when CSH concentrations are high in human maternal plasma, there is no direct evidence that human CSH in fact induces IGF-1 synthesis and secretion, and it may well be that the increase in IGF-1 results from increased concentrations of placental GH (CSH2; GH-V). In cattle, administration of CSH during pregnancy resulted in increased maternal plasma IGF-1 and insulin-like growth factor 2 (IGF-2). However, liver *IGF-1* mRNA concentrations in pregnant ewes were not impacted by a 7-day administration of CSH during the last third of gestation. This lack of effect of CSH administration on liver *IGF-1* expression in pregnant sheep is supported by the lack of effect on maternal liver *IGF-1*, *IGF-2*, IGF-binding protein (*IGFBP1*, *IGFBP2*, and *IGFBP3*) mRNA concentrations, and IGF-1 concentrations in maternal blood in CSH-deficient pregnancies (Baker et al., 2016). The absence of response in sheep, contrasted with a significant enhancement in maternal IGF-1 in cattle, may be influenced by the relative concentrations of CSH in maternal circulation (high in sheep and low in cattle), such that pregnant cattle might respond more robustly to maternal CSH administration due to the low endogenous CSH concentrations. As discussed later, there is considerably more evidence of CSH stimulating IGF production within the fetus.

Fetal Effects

There has been considerable interest in potential CSH anabolic effects in the fetus (Fig. 4), at least in part due to the concept that fetal GH may not be exerting the same growth-promoting effects that it does during postnatal life. This concept was augmented with the demonstration that sheep CSH stimulated ornithine decarboxylase activity, amino acid uptake, IGF-2 secretion, and glycogen synthesis by fetal rat tissues, albeit recognizing that sheep CSH would not have been activating its normal receptor in these studies. However, the effect of sheep CSH on fetal hepatocyte incorporation of glucose into glycogen has since been confirmed using fetal sheep hepatocytes. Furthermore, human CSH has been shown to stimulate uptake of amino acids, DNA synthesis, and IGF-1 production by human fetal fibroblasts, hepatocytes, and myoblasts. With all of these in vitro endpoints, while CSH acted in

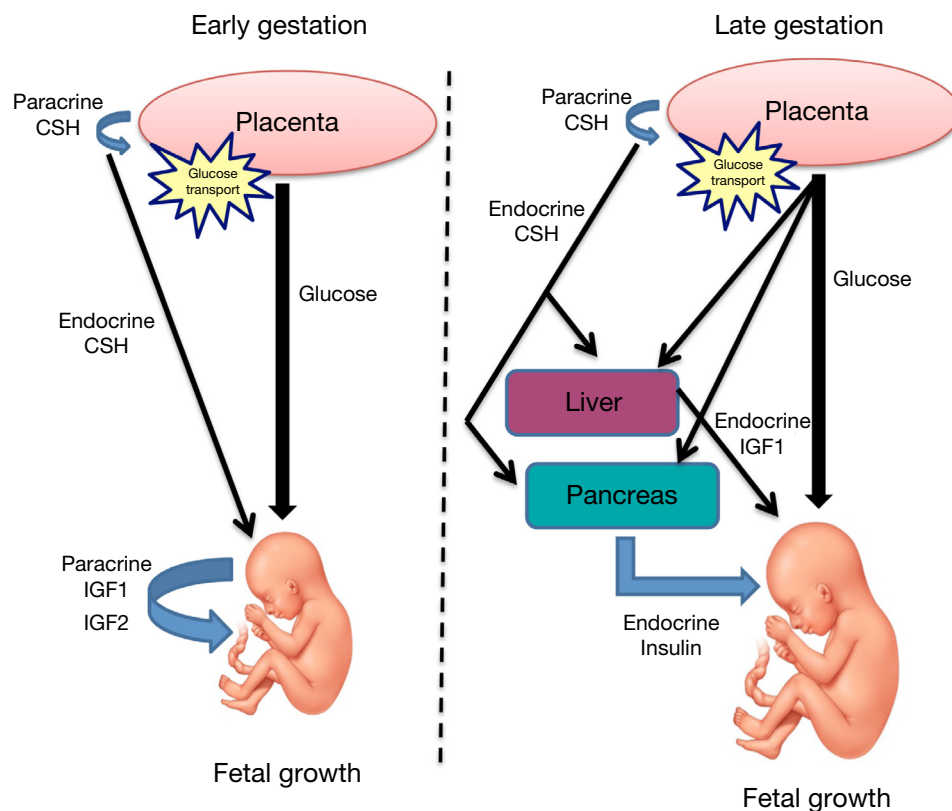


Fig. 4 Predicted biological actions of CSH on the placenta and fetus. Representation of hypothesized effects of CSH on the fetus and placenta. Long-held hypotheses (Handwerger, 1991) on CSH stimulation of fetal IGF-1 and insulin during late gestation were supported by the recent results of Baker et al. (2016), which also revealed impacts on placental growth and function.

a dose-responsive fashion, in general, GH was without or had minimal effect, as compared with CSH. In vivo, a 14-day infusion of CSH into the sheep fetus resulted in greater plasma IGF-1 and fetal liver glycogen accumulation, supporting the conclusions drawn from the previous in vitro experiments. The strongest in vivo support for the concept that CSH could have direct growth-promoting effects in the fetus was provided by the recent CSH-deficient sheep pregnancies reported by Baker et al. (2016). Near-term (135 dGA) fetal weight was 32% less in the CSH-deficient pregnancies and was associated with an 83% reduction in fetal liver IGF-1 mRNA concentrations and a 62% reduction in umbilical artery IGF-1 concentrations. Additionally, reductions in fetal liver IGF-2 (71%), IGFBP2 (74%), and IGFBP3 (81%) mRNA concentrations were reported for the near-term CSH-deficient pregnancies.

It had also been hypothesized (Handwerger and Freemerk, 2000) that CSH could stimulate fetal insulin production. This hypothesis was based on the report that human fetal pancreatic explants treated with CSH exhibited significantly greater insulin content and glucose-stimulated insulin release. This in vitro study is supported by the 49% reduction in umbilical artery insulin concentrations observed in near-term CSH-deficient sheep pregnancies (Baker et al., 2016). However, definitive assessment of the impact of CSH deficiency on fetal pancreas development, insulin secretion, and insulin sensitivity awaits in vivo determination of umbilical uptakes and utilization of glucose, steady-state concentrations of fetal insulin, in vivo and in vitro fetal islet insulin secretion, and measurements of islet size, structure, and β -cell mass.

The sheep CSH-deficient pregnancies (Baker et al., 2016) also revealed a previously unrecognized effect of CSH, that being placental growth and function. The placentas of CSH-deficient pregnancies were 52% smaller near term (135 dGA), which likely also contributed to the IUGR observed in these pregnancies. However, we (Jeckel and Anthony) have unpublished data demonstrating that significant IUGR is apparent at the end of the first third of gestation (50 dGA) in CSH-deficient sheep pregnancies. At this stage of gestation (50 dGA), placental size was not significantly reduced, although there were significant reductions in the expression of glucose transporters and IGF, with even greater reductions in the expression of IGFs and glucose transporters near term (135 dGA). While the significance of these findings awaits steady-state assessment of placental glucose transport capacity in vivo and experiments alluded to earlier, we hypothesize (Fig. 4) that during early to midgestation CSH may impact fetal growth by stimulating placental glucose transfer capacity but that during the latter half of gestation, it has additional direct anabolic effects on the fetus by stimulating hepatic IGF-1 production, β -cell proliferation, and insulin production.

References

- Anthony, R. V., Pratt, S. L., Liang, R., & Holland, M. D. (1995). Placental-fetal hormonal interactions: impact on fetal growth. *Journal of Animal Science*, 73, 1861–1871.
- Baker, C. M., Goetzmann, L. N., Cantlon, J. D., et al. (2016). Development of ovine chorionic somatomammotropin hormone-deficient pregnancies. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 310, R837–R846.
- Handwerger, S. (1991). Clinical counterpoint: the physiology of placental lactogen in human pregnancy. *Endocrine Reviews*, 12, 329–336.
- Handwerger, S., & Freemerk, M. (2000). The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. *Journal of Pediatric Endocrinology & Metabolism*, 13, 343–356.
- Kappes, S. M., Warren, W. C., Pratt, S. L., Liang, R., & Anthony, R. V. (1992). Quantification and cellular localization of ovine placental lactogen messenger ribonucleic acid expression during mid- and late-gestation. *Endocrinology*, 131, 2829–2838.
- Soares, M. J., Konno, T., & Khorshed Alam, S. M. (2007). The prolactin family: effectors of pregnancy-dependent adaptations. *Trends in Endocrinology and Metabolism*, 18, 114–121.
- Walker, W. H., Fitzpatrick, S. L., Barrera-Saldana, H. A., Resendez-Perez, D., & Saunders, G. F. (1991). The human placental lactogen genes: structure, function, evolution and transcriptional regulation. *Endocrine Reviews*, 12, 316–328.

Pregnancy Associated Glycoproteins

Jonathan A Green and Madison E Hennessy, University of Missouri, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Overview

Pregnancy-Associated Glycoproteins (PAGs) are products of a gene family expressed by placental trophoblasts of mammals in the Cetartiodactyla order (Wallace et al., 2015). PAGs are thought to have arisen from duplication and subsequent expansion of a pepsin F-like ancestral gene, that itself appears to have been a duplication of Pepsin A (Hughes et al., 2003). The PAGs, pepsin F and pepsin A all belong to a class of proteolytic enzymes known as aspartic proteinases. The products of the PAG gene family are abundantly expressed by placental trophoblasts, and the expression of individual PAGs does change during the course of pregnancy (Green et al., 2000; Touzard et al., 2013). In ruminant ungulates, PAG expression is often cell-specific. Most members of the PAG family in ruminants are expressed predominantly in giant binucleated trophoblast cells (GBCs). Because of their production by GBCs, many of these PAGs can enter the blood circulation of pregnant females. There are other ruminant PAGs that are expressed in only mononucleated trophoblasts or are localized to both trophoblast populations. Transcripts for some PAGs expressed in mononucleated trophoblasts are detectable early in conceptus development and remain detectable in placenta until term, even though relative transcript abundance often varies throughout gestation (Green et al., 2000; Patel et al., 2004; Touzard et al., 2013).

Several sequenced genomes are available for cetartiodactyls, which provides an opportunity to evaluate the number and type of PAGs across the even-toed ungulates. Based on available genome data, the number of PAGs present in each suborder varies from none or a few (in camelids and cetaceans), to more than thirty in the small ruminants. Although the PAGs have been studied extensively for the past three decades, there is no known function for this expansive family. The following chapter summarizes some of the salient features of this gene family expressed by cattle, sheep, swine and related species.

PAGs Are Aspartic Proteinases

The PAGs belong to the aspartic proteinase group of enzymes. They appear to have arisen by duplication and expansion of a pepsin F-like gene soon after, or around the same time, that the Cetartiodactyla order arose (Fig. 1) (Hughes et al., 2000). They share many common features with other mammalian aspartic proteinases including a 9 exon and 8 intron gene organization and a bi-lobed structure that forms a cleft used for binding peptide substrates (Ganguly and Prasad, 2012). The name for these proteinases arises from the presence of two aspartic acids that participate directly in the catalytic mechanism. These aspartates are contained within each lobe of the protein and they reside alongside other highly conserved residues. The consensus sequence around each aspartate is hydrophobic-hydrophobic-Asp-Thr-Gly-Ser/Thr-Ser/Thr. These aspartates participate in peptide bond cleavage by orientating and stabilizing a water molecule that is complexed between them; it is the water molecule itself that acts as a nucleophile during the hydrolysis of the peptide bond of the bound substrate (Dunn, 2002). Most PAGs possess all the structural features of aspartic proteinases and proteolytic activity has been characterized for some of them (Telugu and Green, 2008; Telugu et al., 2010). However, there are a few that are highly likely to not have proteolytic activity because they either lack one of the catalytic aspartates or they contain atypical residues within the highly conserved sequences flanking them (Green et al., 2000; Xie et al., 1991). However, molecular modeling suggests that those PAGs predicted to lack proteolytic activity still possess the overall conformation of aspartic proteinases (Telugu and Green, 2008). In other words, any lack of activity does not seem to be associated with drastic alterations in the overall ability to bind peptide sequences via the binding cleft.

PAGs Are Restricted to the Cetartiodactyla Order

The PAG gene family is restricted to the Cetartiodactyla order. Animals within this order include swine, giraffes, cattle, bison, sheep, whales, and related species. When conducting a phylogenetic analysis of all known PAGs in public databases, there are currently over 250 total PAG genes present. Fig. 1 represents a simplified phylogenetic tree that illustrates the relationship between PAGs and other mammalian aspartic proteinases. Based on current genome sequencing results, PAG genes are most prevalent in ruminant species. In other species of the Cetartiodactyla order the number of PAG genes is smaller. For example, the number of total PAG genes present per species ranges from two genes represented in the genome builds of some whale species, to over thirty known PAG genes in many of the Bovidae (Telugu et al., 2009). It is notable that, when comparing across the different suborders (Suidae, Cetacea, Ruminantia), there is little indication that individual PAGs are conserved across the order. In other words, there are no obvious orthologs across the suborders, and all the PAGs represented by each suborder cluster together in phylogenetic analyses. It is not clear whether that pattern is due to expansion of PAGs in these species after their split from a common ancestor or due to homogenization of PAG genes in these suborders. There is also evidence that certain parts of these proteins (mainly surface loops) are evolving rapidly (Hughes et al., 2000).

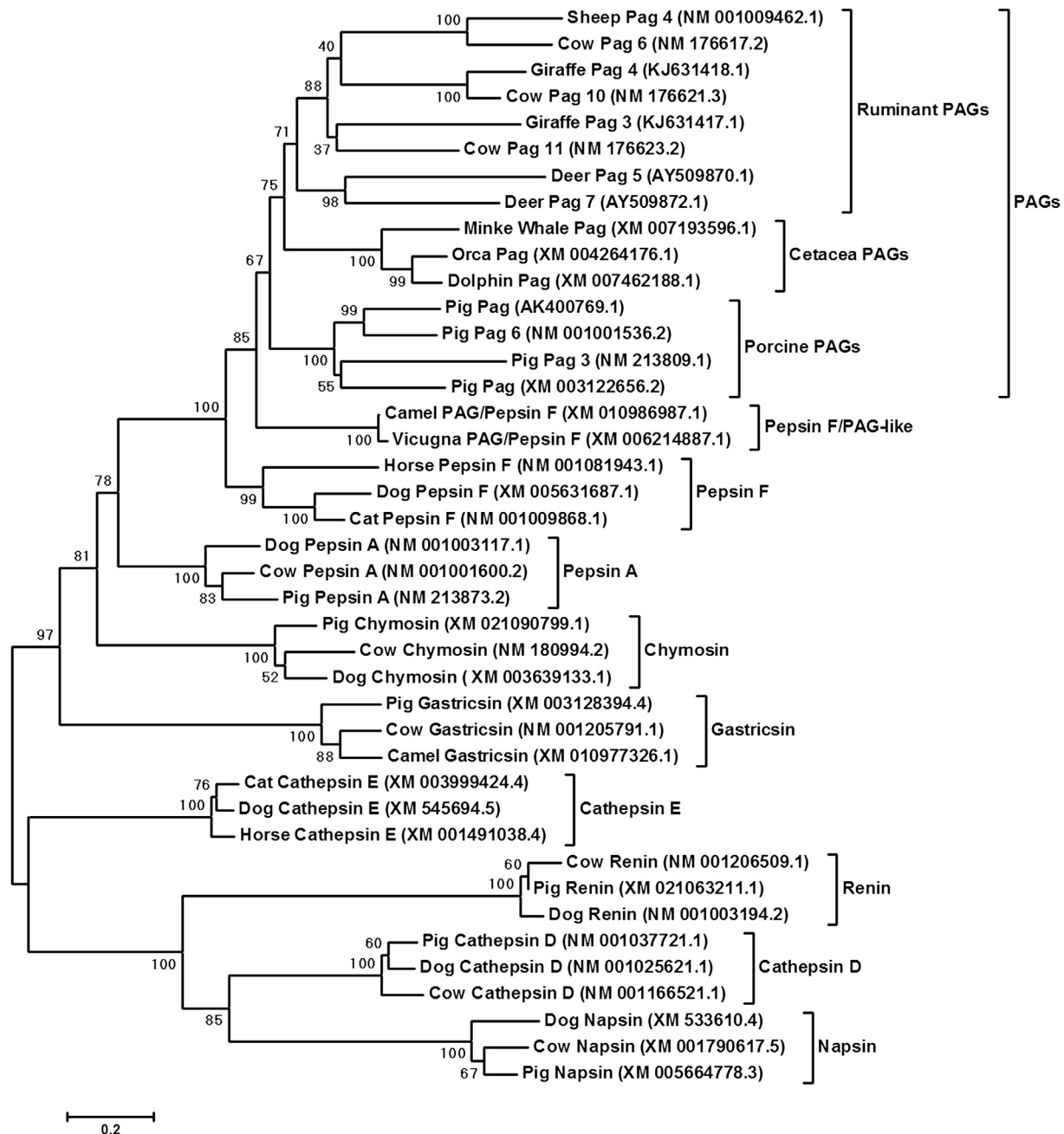


Fig. 1 Evolutionary relationships among representative PAGs and mammalian aspartic proteinases. The PAGs belong to the aspartic proteinase family of enzymes. They seem to have arisen by an ancestral duplication of the pepsin F gene, which itself arose from an ancestral pepsin A gene. The PAGs have undergone extensive expansions and refinements within the suborders of the Cetartiodactyla. The phylogenetic tree displayed was inferred by using the Minimum Evolution method. Results from bootstrap tests (percentages from 1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances which are in the units of the number of amino acid substitutions per site. The evolutionary analyses were conducted in MEGA7 (www.megasoftware.net).

The diversity in the number of PAG genes, their lack of orthology and/or their rapid evolution is suggestive that the functional role of PAGs is likely to differ across species within the suborders of the Cetartiodactyla. It is worth noting that all cetartiodactyls possess syn/epitheliochorial placentas that are adaptive forms that are distinct from the ancestral endotheliochorial or hemochorial forms (Wildman et al., 2006). Presumably, the PAGs are performing functions that are important for these unique placental forms. Speculations about those possibilities are elaborated upon below.

PAGs Are Expressed by the Placenta

The PAGs are products of trophoblast cells of the placenta (Fig. 2) (Wallace et al., 2015). The presence of PAGs in the placenta has been reported in pigs and several other cetartiodactyls. The expression of PAGs during development of the conceptus and placenta is best understood in cattle. In this species, the phylogenetically more ancient bovine PAGs (ones that represent the forms that arose earliest in the Cetartiodactyla order) are usually found to be expressed mainly by mononucleated trophoblasts although some are present in both mononucleated and GBCs (Fig. 2). The largest subgrouping of bovine PAGs have more recent origins and these are generally found to be restricted in their localization to GBCs (Green et al., 2000). These cells are particularly notable because of their capacity to fuse with nearby uterine epithelial cells to form a syncytial fetal-maternal cell type (Wooding, 1982). Upon fusion, the secretory granules within the giant trophoblasts move toward the basal side of the uterine epithelium. The contents of the secretory granules are then released via exocytosis toward the uterine connective tissue (Wooding, 1992). Since most PAGs are components of the GBCs granules, they ultimately are delivered to maternal tissues and can accumulate in maternal blood, milk and urine (Wallace et al., 2015). Most of what is understood about PAG localization and delivery to the maternal system is from examining placentomes (consisting of fetal cotyledons and maternal caruncles). Currently, little is known about PAG expression in inter-placentomal membranes.

Ruminant PAGs can Be Used to Evaluate Pregnancy Status

Bovine PAGs first become detectable in the maternal blood circulation around day 25 of pregnancy, which is soon after GBCs first form (Sasser et al., 1986; Wathes and Wooding, 1980; Zoli et al., 1992). Circulating concentrations rise steadily throughout pregnancy and peak around the time of parturition (Green et al., 2005; Sasser et al., 1986; Zoli et al., 1992). The appearance of these placental products in the maternal bloodstream of ruminants made these proteins useful markers for the detection of pregnancy in bred females. Indeed, multiple commercial assays have been developed and marketed for this purpose. In general these assays for bovine PAGs are able to detect pregnant animals at day 30 with 95% accuracy or greater. PAG assays represent an economical and efficient way to detect pregnancy relatively early in cattle as well as in sheep, deer and other ruminant ungulates (Huang et al., 1999; Melo De Sousa et al., 2003; Ranilla et al., 1994). In addition to PAGs' utility in detecting pregnancy, assessing PAG levels also

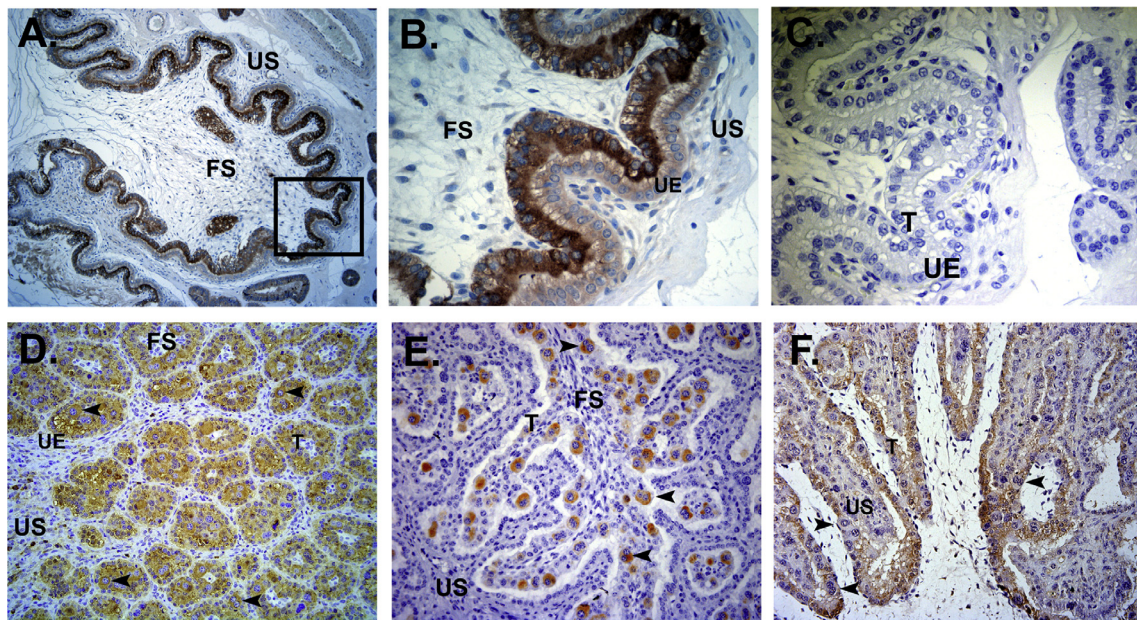


Fig. 2 Localization of PAGs at the placenta-uterine interface of pigs and cattle. Panels A, B and C are day 35 pig placenta. Panels A (100 $\times$) and B (400 $\times$) show immunohistochemical staining of trophoblasts obtained by using a porcine PAG2 antibody. Panel C (400 $\times$) is an unstained section stained with hematoxylin and eosin. The boxed area in panel A is the region shown in panel B. Panels D, E and F are bovine placental sections stained with different bovine PAG antibodies to illustrate the distinct expression patterns of PAGs in cattle and related ruminant ungulates. Panel D (200 $\times$) is a day 45 placental section stained with a bovine PAG2 antibody. This antibody stained both mononucleated and binucleated giant trophoblasts. Panel E (200 $\times$) is a day 75 placental section stained with a bovine PAG monoclonal antibody that reacts predominantly with PAG4 and PAG6. This antibody staining is restricted to binucleated giant trophoblasts. Panel F (200 $\times$) is a day 90 placental section stained with a bovine PAG8 antibody. This antibody stained mononucleated trophoblasts, but exhibited little or no staining of binucleated giant trophoblasts. FS, fetal stroma; US, uterine stroma; UE, uterine epithelium; T, trophoblast; arrowheads indicate binucleated trophoblast giant cells in bovine placentomes.

appears to be a way to predict future pregnancy loss in pregnant cattle. Several research teams have reported that circulating PAG levels are often reduced early in pregnancy in animals that will later experience fetal loss (Breukelman et al., 2012; Giordano et al., 2012; Humblot, 2001; Pohler et al., 2013; Pohler et al., 2016). Presumably, the lower circulating PAGs reflect a compromised placenta that is incapable of normal production of secretory proteins and that ultimately fails to adequately support the fetus (Pohler et al., 2013; Wallace et al., 2015). Over time, as these assays are perfected and more antibodies are developed to detect specific ruminant PAGs, they may provide improved diagnostic tools for assessing both the presence and likelihood of a successful outcome for a pregnancy.

Potential Functions of PAGs

The PAGs have been studied for more than three decades and there has been much speculation regarding the function of PAGs in pregnancy. The particular functions attributed to them generally hinges upon their presence at the placenta-uterine interface and their presence in the maternal milk and blood. The proposed functions have focused on putative roles in tissue remodeling, immunomodulation of the maternal immune system and/or luteotrophic actions on the ovary.

Many PAGs accumulate at the placenta-uterine interface. In pigs, which lack the fusogenic trophoblasts cells present in ruminants, the majority of PAGs that are secreted by trophoblasts do not enter the maternal circulation. Instead, they remain at the microvillar interface between the trophoblasts and the uterine epithelia (Majewska et al., 2006; Wooding et al., 2005). In cattle, those PAGs that are produced by mononucleated trophoblasts also appear to remain predominantly at the trophoblast-uterine interface (Wallace et al., 2015; Wooding et al., 2005). Wooding and colleagues suggested that catalytically active PAGs at the interface could have general or individualized capacity to proteolytically process other proteins present at that location, such as latent growth factors. Other possibilities for PAGs at the interface involve roles serving a structural purpose. It was speculated that PAGs may be acting as protein-binding proteins or as adhesion molecules that allow for docking of other proteins to the interface. The substrate binding cleft and/or the carbohydrate moieties displayed on their surface could permit interactions with other proteins (e.g. lectins) (Klisch et al., 2008; Wooding et al., 2005). If PAGs served as adhesion molecules, even enzymatically active PAGs could conceivably serve this purpose provided the pH at the microvillar junction is not so low as to stimulate their activity (most aspartic proteinases have greatest activity at low pH). Indeed, in this scenario, a pH drop, such as around parturition, could activate PAGs to promote cleavage of protein-protein connections between the placenta and uterus.

For those PAGs that are found in the maternal bloodstream, it was suggested that they may be modulating the maternal immune system. In regards to such an immunological role, PAGs are known to influence certain immune progenitor cell responses; conversely they may also aid in dampening the maternal immune response around the time of parturition (Dosogne et al., 2000; Hoebe et al., 1999; Hoebe et al., 2000). For example, some PAGs are able to bind to uterine serpins (Mathialagan and Hansen, 1996); increase the chemokine, GCP2, from endometrial cells (Austin et al., 1999); decrease hemopoietic cell proliferation, and modulate polymorphonuclear neutrophil leukocyte activity. Though many of the processes above are not directly related, PAGs may be able to modulate each process via similar mechanisms, including possible generation of ligands by proteolytic cleavage, interaction with PAG 'receptors' and by interacting with other proteins by their unusual glycosylation moieties (Klisch et al., 2008; Wallace et al., 2015; Wooding et al., 2005).

Another interesting possibility is that ruminant PAGs may play roles as luteotropic agents. They can elevate the luteotrophic prostaglandin, PGE₂, relative to that of the luteolytic prostaglandin PGF_{2α} (Weems et al., 2007; Weems et al., 2003; Weems et al., 1998). On the surface, this role is quite distinct from the functional hypotheses related to immunomodulation. However, it is worth noting that PGE₂ itself can also modulate immunological actions, which might be suggestive that the immune and luteo-protective actions attributed to PAGs may be due to overlapping signaling systems (Emond et al., 1998).

Summary

Pregnancy-Associated Glycoproteins (PAGs) are a gene family expressed by the placenta of species within the Cetartiodactyla order. They are members of the aspartic proteinase group of enzymes that arose from a series of gene duplication events. They are most closely related to pepsin F and pepsin A. The PAGs share the same bi-lobed structure that is typical of aspartic proteinases. However, some PAGs have amino acid substitutions in the active site of the molecule that may render them catalytically inactive. Genomic and cDNA sequencing has revealed the presence of numerous PAG members, especially within the Ruminantia suborder. In ruminants, their detection in the blood of pregnant females has revealed that they can serve as useful circulating markers of pregnancy.

The functions of PAGs are not known. However, they have been extensively studied and several models for PAG function have been proposed. Further research is needed to determine a specific role for these proteins, but as of yet, their transcriptional abundance and complexity suggests they are extremely important in the processes related to placentation in cetartiodactyls.

References

- Austin, K. J., King, C. P., Vierk, J. E., Sasser, R. G., & Hansen, T. R. (1999). Pregnancy-specific protein b induces release of an alpha chemokine in bovine endometrium. *Endocrinology*, 140, 542–545.
- Breukelman, S. P., Perényi, Z., Taverne, M. a. M., et al. (2012). Characterisation of pregnancy losses after embryo transfer by measuring plasma progesterone and bovine pregnancy-associated glycoprotein-1 concentrations. *The Veterinary Journal*, 194, 71–76.
- Dosogne, H., Massart-Leen, A. M., & Burvenich, C. (2000). Immunological aspects of pregnancy-associated glycoproteins. *Advances in Experimental Medicine and Biology*, 480, 295–305.
- Dunn, B. M. (2002). Structure and mechanism of the pepsin-like family of aspartic peptidases. *Chemical Reviews*, 102, 4431–4458.
- Emond, V., Fortier, M. A., Murphy, B. D., & Lambert, R. D. (1998). Prostaglandin e2 regulates both interleukin-2 and granulocyte- macrophage colony-stimulating factor gene expression in bovine lymphocytes. *Biology of Reproduction*, 58, 143–151.
- Giordano, J. O., Guenther, J. N., Lopes, G., & Fricke, P. M. (2012). Changes in serum pregnancy-associated glycoprotein, pregnancy-specific protein b, and progesterone concentrations before and after induction of pregnancy loss in lactating dairy cows. *Journal of Dairy Science*, 95, 683–697.
- Green, J. A., Parks, T. E., Avale, M. P., et al. (2005). The establishment of an elisa for the detection of pregnancy-associated glycoproteins (pags) in the serum of pregnant cows and heifers. *Theriogenology*, 63, 1481–1503.
- Green, J. A., Xie, S., Quan, X., et al. (2000). Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. *Biology of Reproduction*, 62, 1624–1631.
- Hoeben, D., Burvenich, C., Massart-Leen, A. M., et al. (1999). In vitro effect of ketone bodies, glucocorticosteroids and bovine pregnancy-associated glycoprotein on cultures of bone marrow progenitor cells of cows and calves. *Veterinary Immunology and Immunopathology*, 68, 229–240.
- Hoeben, D., Monfardini, E., Opsomer, G., et al. (2000). Chemiluminescence of bovine polymorphonuclear leucocytes during the periparturient period and relation with metabolic markers and bovine pregnancy-associated glycoprotein. *The Journal of Dairy Research*, 67, 249–259.
- Huang, F., Cockrell, D. C., Stephenson, T. R., Noyes, J. H., & Sasser, R. G. (1999). Isolation, purification, and characterization of pregnancy-specific protein b from elk and moose placenta. *Biology of Reproduction*, 61, 1056–1061.
- Hughes, A. L., Green, J. A., Garbayo, J. M., & Roberts, R. M. (2000). Adaptive diversification within a large family of recently duplicated, placentally-expressed genes. *Proceedings of the National Academy of Sciences*, 97, 3319–3323.
- Hughes, A. L., Green, J. A., Piontkivska, H., & Roberts, R. M. (2003). Aspartic proteinase phylogeny and the origin of pregnancy-associated glycoproteins. *Molecular Biology and Evolution*, 20, 1940–1945.
- Humblot, P. (2001). Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. *Theriogenology*, 56, 1417–1433.
- Klisch, K., Jeanrond, E., Pang, P.-C., et al. (2008). A tetraantennary glycan with bisecting n-acetylglucosamine and the sda antigen is the predominant n-glycan on bovine pregnancy-associated glycoproteins. *Glycobiology*, 18, 42–52.
- Majewska, M., Panasiwicz, G., Majewski, M., & Szafrńska, B. (2006). Localization of chorionic pregnancy-associated glycoprotein family in the pig. *Reproductive Biology*, 6, 205–230.
- Mathialagan, N., & Hansen, T. R. (1996). Pepsin-inhibitory activity of the uterine serpins. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 13653–13658.
- Melo De Sousa, N., Zongo, M., Pitala, W., et al. (2003). Pregnancy-associated glycoprotein concentrations during pregnancy and the postpartum period in azawak zebu cattle. *Theriogenology*, 59, 1131–1142.
- Patel, O. V., Yamada, O., Kizaki, K., et al. (2004). Quantitative analysis throughout pregnancy of placental and interplacental expression of pregnancy-associated glycoproteins-1 and -9 in the cow. *Molecular Reproduction and Development*, 67, 257–263.
- Pohler, K. G., Geary, T. W., Johnson, C. L., et al. (2013). Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. *Journal of Animal Science*, 91, 4158–4167.
- Pohler, K. G., Pereira, M. H. C., Lopes, F. R., et al. (2016). Circulating concentrations of bovine pregnancy-associated glycoproteins and late embryonic mortality in lactating dairy herds. *Journal of Dairy Science*, 99, 1584–1594.
- Ranilla, M., Sulon, J., Carro, M., Mantecón, A., & Beckers, J. (1994). Plasmatic profiles of pregnancy-associated glycoprotein and progesterone levels during gestation in churra and merino sheep. *Theriogenology*, 42, 537–545.
- Sasser, R. G., Ruder, C. A., Ivani, K. A., Butler, J. E., & Hamilton, W. C. (1986). Detection of pregnancy by radioimmunoassay of a novel pregnancy- specific protein in serum of cows and a profile of serum concentrations during gestation. *Biology of Reproduction*, 35, 936–942.
- Telugu, B. P., & Green, J. A. (2008). Characterization of the peptidase activity of recombinant porcine pregnancy-associated glycoprotein-2. *Journal of Biochemistry*, 144, 725–732.
- Telugu, B. P., Walker, A., & Green, J. (2009). Characterization of the bovine pregnancy-associated glycoprotein gene family—Analysis of gene sequences, regulatory regions within the promoter and expression of selected genes. *BMC Genomics*, 10, 185.
- Telugu, B. P. V. L., Palmier, M. O., Van Doren, S. R., & Green, J. A. (2010). An examination of the proteolytic activity for bovine pregnancy-associated glycoproteins 2 and 12. *Biological Chemistry*, 391, 259–270.
- Touzard, E., Renaud, P., Dubois, O., et al. (2013). Specific expression patterns and cell distribution of ancient and modern pag in bovine placenta during pregnancy. *Reproduction*, 146, 347–362.
- Wallace, R. M., Pohler, K. G., Smith, M. F., & Green, J. A. (2015). Placental pags: Gene origins, expression patterns, and use as markers of pregnancy. *Reproduction*, 149, R115–R126.
- Wathes, D. C., & Wooding, F. B. P. (1980). An electron microscopic study of implantation in the cow. *The American Journal of Anatomy*, 159, 285–306.
- Weems, Y. S., Kim, L., Humphreys, V., Tsuda, V., & Weems, C. W. (2003). Effect of luteinizing hormone (lh), pregnancy specific protein b (pspb), or arachidonic acid (aa) on ovine endometrium of the estrous cycle or placental secretion of prostaglandins e2 (pge2) and f2alpha (pgf2alpha) and progesterone in vitro. *Prostaglandins & Other Lipid Mediators*, 71, 55–73.
- Weems, Y. S., Kim, L., Humphreys, V., et al. (2007). Effect of luteinizing hormone (lh), pregnancy-specific protein b (pspb), or arachidonic acid (aa) on secretion of progesterone and prostaglandins (pg) e (pge; pge1) and pge(2) and f(2alpha) (pgf2alpha) by ovine corpora lutea of the estrous cycle or pregnancy in vitro. *Prostaglandins & Other Lipid Mediators*, 84, 163–173.
- Weems, Y. S., Lammoglia, M. A., Vera-Avila, H. R., et al. (1998). Effects of luteinizing hormone (lh), pge2, 8-epi-pge1, 8-epi-pgf2alpha, trichosanthin and pregnancy specific protein b (pspb) on secretion of prostaglandin (pg) e (pge) or f2alpha (pgf2alpha) in vitro by corpora lutea (cl) from nonpregnant and pregnant cows. *Prostaglandins & Other Lipid Mediators*, 55, 359–376.
- Wildman, D. E., Chen, C., Erez, O., et al. (2006). Evolution of the mammalian placenta revealed by phylogenetic analysis. *PNAS*, 103, 3203–3208.
- Wooding, F. B. P. (1982). The role of binucleate cell in ruminant placental structure. *Journal of Reproduction and Fertility*, 31, 31–39.

- Wooding, F. B. P. (1992). Current topic: The synepitheliochorial placenta of ruminants: Binucleate cell fusions and hormone production. *Placenta*, 13, 101–113.
- Wooding, F. B. P., Roberts, R. M., & Green, J. A. (2005). Light and electron microscope immunocytochemical studies of the distribution of pregnancy-associated glycoproteins (pags) throughout pregnancy in the cow: Possible functional implications. *Placenta*, 26, 807–827.
- Xie, S. C., Low, B. G., Nagel, R. J., et al. (1991). Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. *Proceedings of the National Academy of Sciences of the United States of America*, 88, 10247–10251.
- Zoli, A. P., Guilbault, L. A., Delahaut, P., Ortiz, W. B., & Beckers, J. F. (1992). Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: Its application for pregnancy diagnosis. *Biology of Reproduction*, 46, 83–92.

Adrenomedullin in Female Reproduction and Pregnancy

Margeaux Wetendorf and Kathleen M Caron, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

© 2018 Elsevier Inc. All rights reserved.

Adrenomedullin Signaling in Female Reproduction

Introduction

Peptide hormones are potent governors of reproductive processes with a versatile display of multi-factorial functions in a variety of tissues (Hinson et al., 2000). Peptides mirroring hormone-like properties retain the ability to pass through multiple cell membranes and easily collect in blood or lymph circulation awaiting transport to multiple tissues. One of these protein families, the calcitonin gene-related peptide (CGRP) family of conserved ligand peptides, was identified to exhibit a myriad of functions including the ability to control blood pressure and aid in neurotransmission (Hinson et al., 2000). This family is comprised of CGRP, calcitonin, amylin, adrenomedullin (ADM), and intermedin (IDM/ADM2). Although similar in structure, these peptides differ in functionality and receptor preference. CGRP, ADM and IMD ligands actively signal via a membrane-integrated G protein-coupled receptor called calcitonin receptor-like receptor or CLR, when the receptor is associated with one of three conjugate CLR interacting proteins, designated receptor activity-modifying proteins or RAMPs to elicit downstream functions (Gibbons et al., 2007). Thus, initiation of the CGRP signaling cascade is dependent on coordinate expression of the ligand, receptor, and receptor modifier in a particular target tissue. Activation of the CGRP ligand–CLR–RAMP complex elicits multiple second messenger signaling cascades including nitric oxide (NO) synthase, cAMP, and MAPK pathways (Gibbons et al., 2007), all of which are ubiquitous pathways, universally expressed in all tissues. These second messenger signaling pathways control cellular proliferation, differentiation, vasodilation, and metabolism of multiple tissues.

Within the CGRP family, ADM exhibits broad expression and function in a variety of tissues, with a strong role in cardiovascular and lymphatic vessel growth and homeostasis. ADM was identified in 1993 after Kitamura et al. identified a peptide exhibiting vasoactive properties expressed heavily in an adrenal pheochromocytoma, coining it “adrenomedullin” (Kitamura et al., 1993). Now, nearly a quarter of a century later, literature citations of ADM function range in the thousands, pertaining to multiple fields with reported expression in every organ system of the human body (Hinson et al., 2000). Due to its broad expression and function, it is of no surprise that ADM signaling is highly conserved across many species (Chang and Hsu, 2013). Besides being a potent vasodilator and important effector in lymphatic and cardiovascular development, ADM exhibits nontraditional functions as an anti-bacterial and antiinflammatory factor, combatting infections in the blood and surface of the skin, despite its short half-life of 23 min within the human body (Hinson et al., 2000). Additionally, ADM is highly expressed in the female reproductive tract, responsive to the ovarian steroid hormones, and has exhibited a strong role in the initiation and maintenance of a pregnancy (Lenhart and Caron, 2012). This article will discuss the role of ADM in multiple processes of pregnancy and in the initiation of or association with several reproductive-related diseases.

Of the CGRP ligands, ADM is the most widely studied in the field of female reproduction, exhibiting critical functions in the initiation, maintenance, and completion of pregnancy. Although expressed in the adrenal gland and particularly enriched in most vascular endothelial cells (Hinson et al., 2000), ADM also exhibits robust expression in female reproductive tissues including the uterine endometrium, ovary, oviduct, cervix, vagina, placenta, as well as fetal membranes including trophoblast cells (Lenhart and Caron, 2012). Furthermore, ADM is secreted by both maternal and fetal compartments of the placenta during pregnancy (Lenhart and Caron, 2012), resulting in approximately a 3–5 fold increase in serum ADM levels observed in pregnant women compared to nonpregnant women (Di Iorio et al., 1998). ADM is enriched in fetal-derived trophoblast cells in both human (6–10 weeks of pregnancy) and mouse (day 9.5) at the onset of pregnancy, with cognate receptors expressed on maternal tissues to elicit autocrine and paracrine control of pregnancy (Di Iorio et al., 1998, 2003). Expression of ADM alone is not sufficient to activate the ADM signaling pathway. ADM with its receptor, CLR, and receptor-modifying protein, RAMP2 or RAMP3, are required for downstream signaling (Gibbons et al., 2007). Indeed, ADM and its signaling components, CLR and RAMP2/3, are all responsive to the presence of the ovarian steroid hormones and are present in the uterus and uterine vasculature (Chang and Hsu, 2013). Thus, coordinate expression within maternal and fetal tissues of ADM, CLR, and RAMPs is required for successful ADM signaling during pregnancy.

Aberrant expression of ADM in the fetal or maternal tissues is often indicative of diseased conditions such as fetal growth restriction (FGR), preeclampsia, and other reproductive-associated diseases (Lenhart and Caron, 2012). Although a working foundational knowledge of ADM signaling exists, there is still a lack of understanding regarding the mechanisms following local ADM signaling activation in the uterus and placenta. Further investigation of ADM signaling in the reproductive tract is required to understand this complex signaling pathway. In the following paragraphs of this article, the known and potential roles of ADM in the female reproductive tract throughout every step of pregnancy will be explored. Finally, the involvement of ADM signaling in the provocation of female reproductive-associated disease and the potential for clinical therapeutic use will be described.

Adrenomedullin Function in Pregnancy

Ovulation

Upon prompting from the hypothalamic–pituitary axis, the ovaries control the appropriate secretion of the steroid hormones and at the onset of pregnancy, govern the timely release of the developed oocyte into the fallopian tube (Wang and Dey, 2006). During

menses, circulating ADM protein increases during the follicular phase of ovulation, while decreasing at the luteal phase, coinciding with the increase in ovulatory estrogen production (Di Iorio et al., 2003). Additionally, circulating ADM levels increase dramatically with the luteinizing hormone surge, responsible for ovulation and release of the developing oocyte (Di Iorio et al., 2003). Indeed, ADM levels are increased in ovarian follicular fluid extracts, providing a potential role for ADM in the maturation and release of a developed egg (Di Iorio et al., 2003, Chang and Hsu, 2013). Experiments in the rat have also identified a potential role of ADM in the promotion of progesterone ligand, the ovarian hormone required for pregnancy, in the ovarian granulosa cells via cAMP second messenger signaling (Di Iorio et al., 2003). Therefore, ADM is sensitive to ovulatory estrogen secretions and may potentiate oocyte maturation and aid in the production of progesterone ligand within the ovary.

Conception and Embryo Transport

At the onset of human conception, an egg is released from the ovary and travels down the fallopian tube at which it is intercepted by sperm. The fallopian tube is not just a means of delivery for the embryo to the uterus, but functions as a perfect environment for conception to occur and for the embryo to appropriately advance in development (Ezzati et al., 2014). Ciliated cells lining the fallopian tube beat the embryo in a timely manner to the receptive uterus. The uterus is receptive to the implanting embryo for a narrow time frame, coined “the window of receptivity” (Wang and Dey, 2006). This window occurs in both mice, at day 4 of pregnancy, and in women, at day 9 of pregnancy. It is during this unique time in which the uterus is prepared to receive the developed embryo from the fallopian tube (Wang and Dey, 2006). Late arrival of the embryo results in embryo destruction, while early embryos await the receptive time point. ADM is expressed within the endothelial cells lining the murine oviduct and human fallopian tube (Chang and Hsu, 2013). Additionally, ADM promotes the cilia beat frequency resulting in timely delivery of the fertilized embryo to the receptive uterus in the rat (Liao et al., 2011). The presence of sperm within the fallopian tube also enhances ADM levels, resulting in increased ciliary beating (Fazeli et al., 2004). Thus, ADM not only regulates ovulation and conception, but also controls the duration of time the embryo stays within the fallopian tube, resulting in a well-timed delivery of the developed embryo to the uterus.

Embryo Implantation

The establishment of a receptive uterine state is a complex process involving molecular crosstalk between both the uterine epithelial and stromal compartments. The ovarian steroid hormones govern this process of early pregnancy initiation and maintenance (Wang and Dey, 2006). Interestingly, ADM exhibits estrogen response elements in its promoter region (Watanabe et al., 2006) and is coordinately upregulated in pregnancy or by administration of a progestin (Hinson et al., 2000). Thus, ADM is hormonally responsive and involved in multiple processes of pregnancy. Upon expression of estrogen, the uterine epithelium proliferates and forms endometrial glands responsible for secreting critical growth factors required for embryo implantation (Wang and Dey, 2006). As early pregnancy progresses, the uterine epithelium is exposed to a predominance of progesterone ligand resulting in multiple morphological changes to the epithelial cells, allowing for reception of the embryo (Wang and Dey, 2006). Epithelial cells change in polarity and form bleb-like structures called uterine pinopodes on their outer surface facing the uterine lumen as potential “homing beacons” for implanting embryos (Quinn and Casper, 2009). These pinopodes are hypothesized to express adhesion molecules and aid in the attachment and integration of the implanting embryo on the epithelial surface (Quinn and Casper, 2009). Concurrently, the ADM signaling components, CLR, RAMP2, and RAMP3 exhibit expression in the luminal epithelium just prior to the time of implantation (Li et al., 2008). Single allelic ablation of ADM resulted in reduced litter size and a reduction of pinopode number, confirming its integral role in the attachment and implantation of embryos (Li et al., 2006). Indeed, increased expression of ADM restored litter size in the murine model (Li et al., 2013). Furthermore, exogenous administration of ADM to pseudopregnant dams, (female mice mated to vasectomized mice), receiving blastocyst transfers, caused a higher embryo receptivity rate and improved embryo spacing compared to normal blastocyst transfers in wild type mice (Matson et al., 2017). Thus, ADM promotes embryo reception rate and implantation spacing, making it a strong therapeutic candidate for in vitro fertilization. ADM also promotes water retention within the stroma at the time of implantation (Matson et al., 2017), which may aid in ionic balance and fluid homeostasis within the inter-luminal space, factors speculated to be critical for embryo receptivity (Liu et al., 2014). Additionally, a surge of estrogen initiates the time of implantation, coordinately promoting ADM expression and RAMP3 expression in the uterine endometrium, the stroma, and the blastocyst at the time of implantation (Lenhart and Caron, 2012). This surge likely supports the implantation of the embryo while simultaneously preparing the stroma for increased proliferation and angiogenesis. Thus, ADM regulates pinopode formation, embryo receptivity rate and spacing, and ionic balance responsible for the preparation of the receptive uterine epithelium for the attaching embryo. Further studies are required to understand the extensive functions of ADM at the implantation surface, many of which are unknown.

Decidualization

After the embryo implants into the uterine epithelium, the stromal decidual reaction begins, an extensive process also known as decidualization. Decidualization encompasses a wave of differentiation and proliferation of the stromal cells into epithelial-like cells, with a marked response in angiogenesis, important for the appropriate growth and support of the embryo. Although ADM is expressed in the epithelial uterine compartment and in the postimplantation decidual stromal cells surrounding the attached

embryo (Li et al., 2006), there is limited knowledge regarding the role of ADM in the decidualization process. Based on its extensive expression pattern and known functions, ADM likely enhances the vascular permeability and angiogenesis surrounding the developing embryo (Yallampalli et al., 2014). Therefore, ADM exhibits a potential role in the promotion of blood flow and delivery of necessary nutrients before the development of the placenta.

Placentation

After implantation and decidualization, cells from the fetal trophoblast migrate and invade through the maternal tissue in order to establish a vascular conduit between fetal and maternal networks (Lenhart and Caron, 2012). These invaded trophoblast cells recruit specialized immune cells known as uterine natural killer (uNK) cells to the site of placental formation, the maternal spiral arteries (Smith et al., 2009). The recruitment of uNK cells is strongly dependent on the level of ADM secretion by the trophoblast cells (Li et al., 2006, 2013). Within genetically engineered mouse models, increased expression of ADM increases the uNK cell population, while heterozygous ablation of ADM results in decreased recruitment of uNK cells (Li et al., 2006, 2013). The presence of uNK cells is necessary for the induction of matrix metalloproteinases (MMPs) and other growth factors, such as vascular endothelial growth factor, causing the apoptosis of vascular smooth muscle cells and eventual remodeling of the maternal spiral arteries (Lenhart and Caron, 2012). After invasion of the trophoblast cells, expression of ADM, and recruitment of uNK cells, the maternal spiral arteries are remodeled from low-capacitance, high pressure vessels into high-capacitance, low pressure vessels allowing for the maximum exchange of nutrients between mother and baby (Lenhart and Caron, 2012). Since the placenta is not autonomously innervated, vasoactive agents and downstream endothelial cell remodelers are required for this structural remodeling of the spiral arteries and development of the placenta as well as the control of systemic blood pressure and vascular flow (Di Iorio et al., 2003). This is performed by activation of second messenger signaling cascades downstream of ADM such as the NO pathway, resulting in low vascular resistance, and the cAMP pathway, allowing for the rebuilding of vascular walls (Di Iorio et al., 2003). Therefore, invading fetal trophoblast cells initiate the remodeling of the maternal spiral arteries by the recruitment of specialized immune cells and activation of second messenger signaling via ADM. These immune cells further recruit vasoactive growth factors and remodeling enzymes, resulting in the formation of the placenta and efficient exchange of nutrients, gasses, and waste between mother and baby.

It is of utmost importance that the placental maternal arteries be remodeled into large, high-capacitance vessels, as inappropriate spiral artery remodeling can result in systemic maternal hypertension and risk for FGR (Chaiworapongsa et al., 2014). FGR is a serious condition for the infant that can lead to multiple developmental abnormalities (Sharma et al., 2016). Upon ablation of a single allele of ADM within mice, pregnant females exhibit a reduced litter size due to FGR (Li et al., 2006). Placentas from embryos exhibiting FGR had shallow placentas with fragile maternal and fetal neighboring components and a dysregulated decidual boundary (Li et al., 2006), defects often observed in women with preeclampsia, a severe hypertensive pregnancy disorder. Additionally, genetic rescue of ADM expression crossed onto the ADM ablated mice rescued the FGR and reduction in litter size (Li et al., 2013). Thus, ADM expression is necessary for placental development resulting in the appropriate exchange of gasses and nutrients between the fetal and maternal tissues. Additional investigation is required to identify the factors affecting ADM signaling and how these complex mechanisms communicate leading to the successful establishment of a healthy placenta.

Parturition

After completion of placentation, the fetus grows until the time of delivery or parturition. Parturition, much like many processes of pregnancy, is regulated by a myriad of factors ultimately resulting in contraction of the uterine muscles or myometrium and ripening of the cervical tissue (Mendelson, 2009). Multiple hormones, cytokines, growth factors, and inflammatory molecules are important for preparing the uterine myometrium for parturition (Mendelson, 2009, Di Iorio et al., 2003). Before the onset of muscle contraction, inflammatory molecules and cytokines are recruited to the uterus, causing the upregulation of the NFκB pathway and inhibition of the progesterone signaling pathway (Mendelson, 2009). Through this synergistic action, the myometrium is prepared for physical contraction, yet dependent on intracellular stores of Ca^{+2} ions. ADM is present within the smooth muscle cells of the uterus with increased expression in pregnant uterus versus nonpregnant uterus (Di Iorio et al., 2003). The presence of ADM in the uterus is hypothesized to quiet the uterine tissue through elevated cAMP signaling (Di Iorio et al., 2003). However, ADM may also regulate uterine quiescence via other contractile factors and additional second messenger signaling cascades. Indeed, ADM was identified to suppress uterine myometrial contractility in a concentration-dependent manner using rat tissue contractility studies (Di Iorio et al., 2003). Despite this convincing evidence, ADM is consistently upregulated in amniotic fluid from women undergoing preterm birth and in plasma prior to cervical ripening at the onset of labor (Di Iorio et al., 2003). Although expression of ADM at the time of labor is contrary to the quiescent hypothesis, the increased expression may be a direct result of the surge in immune cell population present at the time of labor. Because ADM functions as a circulating antiinflammatory compound, it is induced in the presence of diseased or inflamed states, providing a potential explanation for the sharp increase in ADM before the initiation of labor. Additionally, ADM upregulation was proposed to initiate NO, relaxin, and prostaglandin signaling cascades required at the time of cervical ripening (Di Iorio et al., 2003). Therefore, ADM signaling functions as a uterine relaxant during uterine quiescence, but can be upregulated at the onset of labor in the presence of increased immune cells or at the start of cervical ripening. In conclusion, the involvement of ADM signaling in the maintaining of a pregnancy and initiation of parturition is complex and requires further investigation to determine the downstream signaling mechanisms affecting uterine contraction.

Adrenomedullin in Reproductive-Associated Disease

As ADM is an important regulator of the initiation and maintenance of pregnancy, it is of no surprise that modifications in ADM expression can result in severe consequences to the female reproductive tract and future fecundity. Due to known ADM functions, reduction in ADM expression is indicative of decreased second messenger signaling via cAMP, NO, and MAPK pathways which can have devastating effects on the progression of pregnancy. However, increased circulating amounts of ADM can describe a variety of different situations. High circulating or local ADM expression may be helpful in some reproductive situations, yet it also exhibits upregulation to balance a diseased state, as is observed in patients with sepsis (Lenhart and Caron, 2012). Thus, the function of ADM signaling in reproductive-associated disease is complex and the current understanding of its role in the maintaining of reproductive health will be outlined in the following paragraphs.

Recurrent Implantation Failure

For women deemed infertile, defined as a failure to become pregnant after 1 year, various treatments can be administered to promote pregnancy. These treatments can vary from fertility drugs, hormone supplements, and potentially in vitro fertilization (IVF), which currently has a success rate of 50% (Timeva et al., 2014). Women who undergo multiple rounds of IVF and still fail to become pregnant are diagnosed with recurrent implantation failure (RIF). Women with RIF exhibit much difficulty becoming pregnant, often requiring highly technical variations of IVF and/or strong implantation therapeutic drugs (Timeva et al., 2014). Recently, an IVF clinical trial consisting of infertile women with and without RIF was performed to correlate the number of pinopodes with embryo receptivity success rate (Qiong et al., 2017). They were able to discern a positive correlation between pinopode number and successful embryo implantation. Furthermore, women with RIF exhibited poor receptivity rates with severely low pinopode numbers (Qiong et al., 2017). Thus, increased pinopode number is likely a valuable indicator of successful implantation. Interestingly, genetic reduction of ADM results in fewer pinopodes (Li et al., 2008) and treatment of wild type mice with ADM at the time of implantation dramatically increases pinopode formation (Matson et al., 2017). Additionally, exogenous administration of ADM with developed blastocysts in wild type mice prepared for pregnancy dramatically enhanced embryo receptivity success. Furthermore, the implantation spacing of the embryos was improved compared to vehicle treated wild type mice (Matson et al., 2017). Thus, ADM promotes the formation of pinopodes and enhances implantation success and embryo spacing. Therefore, ADM could be used as a powerful therapeutic intervention for women suffering from RIF.

Tubal Ectopic Pregnancy

Improper ciliary beating or oviductal muscle contraction can result in a rare pregnancy complication, known as tubal ectopic pregnancy (Horne and Critchley, 2012). In this condition, the embryo fails to travel down the fallopian tube in a timely manner and implants into the fallopian tube instead of the uterus. This aberrant implantation can lead to rupture and massive internal bleeding usually requiring surgery (Horne and Critchley, 2012). If not immediately remedied, ectopic pregnancies can lead to severe internal bleeding that can be deadly. As described previously, ADM is expressed in the endothelial cells lining the fallopian tube and is known to promote ciliary beat frequency for embryo delivery. In a recent study, the role of ADM in the formation of tubal ectopic pregnancies was examined (Liao et al., 2012). Interestingly, ADM was decreased in oviductal tissue obtained from human patients with tubal ectopic pregnancies. Although further investigation is required to understand the full scope of ADM function in the oviduct, these studies provide a potential mechanism for the effect of decreased ciliary beat by insufficient ADM in the instigation of tubal pregnancy.

Endometriosis

Endometriosis, or the presence of the endometrium outside of the uterus, occurs in up to 10% of the US population (Kim et al., 2013). Endometriotic lesions are hypothesized to generate from retrograde menstrual flow out of the fallopian tube into the surrounding peritoneal cavity (Kim et al., 2013). These uterine tissues can attach to ovaries or other interior organs and as uterine tissue, are responsive to the systemic expression of the ovarian steroid hormones, especially estrogen secretion (Kim et al., 2013). The endometriotic lesions often develop resistance to progesterone, making them difficult to treat, resulting in further growth and discomfort (Kim et al., 2013). The role of ADM in endometriosis is relatively unknown with few papers commenting on ADM expression or function in endometriosis. The limited papers from endometriotic patient samples report ADM expression is increased in follicular fluid after a round of IVF (Singh et al., 2016) and also upregulated in peripheral leukocytes from blood samples (Gentilini et al., 2011). Being estrogen responsive, endometriotic lesions exhibit proinflammatory and pro-proliferative qualities. Thus, ADM is speculated to be increased within local and systemic environments in these patients for a variety of reasons: 1.) ADM is upregulated in the presence of increased estrogen signaling, 2.) ADM increases upon abundance of circulating inflammatory markers to quiet the inflammation, 3.) ADM is often an upstream effector of pro-proliferative and proangiogenic pathways observed in growing endometriotic lesions. Therefore, besides being upregulated in blood samples, ADM is also likely increased in endometriotic tissues. ADM may exhibit a significant role in the exacerbation of this reproductive-associated disease, yet further inquiry is required to assess its function in the formation and maintenance of endometriotic lesions.

Preeclampsia

The appropriate formation of the placenta is critical for the progression of pregnancy and nourishment of the growing fetus. Abnormal placentation can result in fetal and maternal complications that can be lethal or can lead to future cardiovascular disease. Since nutrient exchange occurs at the placenta, aberrant placental development is integrally linked with FGR as it is often inevitable for the fetus to obtain the nutrients required for appropriate development. Currently preeclampsia is responsible for over 50,000 maternal deaths and FGR accounts for around 50% of stillbirths worldwide per year (Sibley, 2017). Thus, understanding the mechanisms that lead to improper formation of the placenta is absolutely critical for the treatment of this disease.

As mentioned previously, ADM is secreted by both the fetal and maternal tissues, promoting the efficient invasion of the fetal trophoblasts through the maternal tissue and initiating the recruitment of the uNK cells (Lenhart and Caron, 2012). The uNK cells recruit growth factors and cytokines responsible for the efficient remodeling of the spiral arteries into high-capacitance vessels for maximum transport of nutrients to the fetus (Lenhart and Caron, 2012). Ablation of ADM in a mouse model causes preeclamptic-like conditions including reduced litter number due to FGR from abnormal placental development (Li et al., 2006). These defects are rescued by inducing a genetic increase of ADM in the entire mouse (Li et al., 2013), providing strong evidence for the role of ADM in placental formation and protection of preeclampsia. Similarly, infusion of ADM in a hypertensive pregnant rat model reduced hypertension and fetal loss without posing negative effects on systemic blood pressure or fetal survival in wildtype animals (Di Iorio et al., 2003). However, confirming this protective relationship in preeclamptic women has proven challenging. Levels of circulating ADM are often difficult to detect due to its short half-life and physical characteristics and binding capabilities making it difficult to assay (Lenhart and Caron, 2012). However, Matson et al. described a new assay to measure the cleaved propeptide portion of ADM, which is stoichiometrically equivalent to the amount of ADM peptide in circulating plasma (Matson et al., 2014). Not surprisingly, the propeptide of ADM is strongly decreased in circulating plasma from women with severe preeclampsia compared to normotensive women (Matson et al., 2014). Thus, increased ADM levels strongly correlate with a normotensive pregnancy. With this known, further investigation is needed to identify new therapies targeting the upregulation of ADM during early pregnancy to ensure appropriate placental development.

Endometrial Cancer

Although hormone responsive and often successfully treated by therapy or surgery, endometrial cancer is the most common reproductive-associated disease in the United States (Kim et al., 2013). There are two major types of endometrial cancer (Kim et al., 2013). The first is estrogen dependent and is often a low-grade cancer with a favorable prognosis, often resulting from chronic estrogen supplements without counterbalancing progesterone supplementation (Kim et al., 2013). The second type is a rare, estrogen independent, high-grade cancer, with a poor prognosis. Within endometrial adenocarcinoma cells, ADM treatment caused hypoxia-induced apoptotic resistance by promoting expression of the oncogene and apoptosis protector, BCL-2 (Oehler et al., 2001). Other studies have identified ADM acting synergistically with VEGF to promote angiogenesis and proliferation in human endometrial cancer biopsies and endometrial adenocarcinoma cell lines (Evans et al., 2012). This is unsurprising as ADM is often induced by hypoxic conditions to promote vasculogenesis (Lenhart and Caron, 2012). In addition to furthering endothelial cell growth in the blood and lymph vasculature, ADM also promotes cellular permeability which may result in metastasis (Karpnich et al., 2011). Thus, ADM signaling may worsen endometrial cancer and metastasis. Utilization of a combined therapy to suppress ADM and estrogen signaling pathways may prove beneficial in the treatment of endometrial cancer.

ADM As a Therapy and Biomarker in Human Reproductive-Associated Disease

The structure and function of ADM as a circulating peptide in all vasculature validate ADM as an obligatory therapeutic target for a variety of human diseases. As a stabilizer for inflammatory disease states and a natural antibiotic, ADM is a promising candidate as an antiinflammatory drug for diseases such as sepsis and colitis (Gibbons et al., 2007). However, since ADM is a potent vasodilator, there can be severe risks systemically with elevated circulating ADM levels. As an alternative, a modified form of ADM or locally administered upstream activator of ADM may be used to circumvent the vasodilating properties and short half-life of the original compound. Recently, ADM has been tested in clinical trials for treatment of inflammatory bowel disease (Kato and Kitamura, 2015). In addition, there are currently high expectations for clinical trial testing of ADM administration during the IVF procedure to enhance embryo receptivity rates. By administering ADM with IVF to women suffering from RIF, the embryo implantation success rates may be significantly improved (Matson et al., 2017). Thus, despite the side effect of systemic vasodilation, ADM is still a promising candidate for IVF treatment and the prevention of inflammatory disease and infection.

Preeclampsia is a devastating hypertensive pregnancy disorder resulting in rampant fetal and maternal death worldwide. Currently, there is no cure for this disease, nor is there an easy means of detecting preeclamptic patients early. Recently, an assay was utilized to measure total circulating ADM plasma levels by measuring the stoichiometrically similar cleaved propeptide portion of ADM (Matson et al., 2014). The physical characteristics of ADM provide a difficult means to attain an accurate measurement of circulating ADM. Measuring the propeptide form circumvents these obstacles, resulting in an accurate measurement of circulating ADM. By measuring propeptide levels in circulating plasma from normotensive and severely preeclamptic patients, propeptide levels were robustly decreased in severe preeclamptic patients compared to normotensive patients (Matson et al., 2014). Therefore,

if implemented early, propeptide levels could be an accurate predictor of risk for preeclampsia. Currently few assays exist to diagnose preeclampsia and these methods are late and not helpful for women suffering from this disease. Thus, further investigation is required to identify and implement new biomarkers for early diagnosis of preeclampsia.

Conclusion

ADM is a robust circulating peptide hormone with multiple functions in cardiovascular and lymphatic development and reproductive processes. ADM is a model example of the functional diversity represented by peptide hormone families, especially the CGRP family. The knowledge base of ADM and especially its function in pregnancy has grown exponentially in the last 10 years. However, due to the extreme complexity of ADM signaling in the promotion of embryo delivery and attachment, the vascularization and restructuring of the maternal decidua and spiral arteries, and the control of fine-tuned signaling mechanisms at the onset of labor, further understanding is still needed. Deepened understanding of the pathways that communicate with the ADM signaling pathway will benefit the development of extensive therapies to treat reproductive-associated disease as well as establish new biomarkers for early diagnosis of these devastating diseases. Finally, in the expectant future, ADM or modified versions of ADM will be implemented more commonly as a therapeutic in the treatment of inflammatory disease or infection and in the enhancement of IVF treatment.

References

- Chaiworapongsa, T., Chaemsaitong, P., Yeo, L., & Romero, R. (2014). Pre-eclampsia Part 1: Current understanding of its pathophysiology. *Nature Reviews. Nephrology*, 10, 466–480.
- Chang, C. L., & Hsu, S. Y. (2013). Roles of CLR/RAMP receptor signaling in reproduction and development. *Current Protein & Peptide Science*, 14, 393–406.
- Di Iorio, R., Marinoni, E., & Cosmi, E. V. (1998). New peptides, hormones and parturition. *Gynecological Endocrinology*, 12, 429–434.
- Di Iorio, R., Marinoni, E., Letizia, C., & Cosmi, E. V. (2003). Adrenomedullin in perinatal medicine. *Regulatory Peptides*, 112, 103–113.
- Evans, J. J., Chittcholtan, K., Dann, J. M., Guilford, P., Harris, G., Lewis, L. K., Nagase, J., Welkamp, A. A., Zwerus, R., & Sykes, P. H. (2012). Adrenomedullin interacts with VEGF in endometrial cancer and has varied modulation in tumours of different grades. *Gynecologic Oncology*, 125, 214–219.
- Ezzati, M., Djahanbakhch, O., Arian, S., & Carr, B. R. (2014). Tubal transport of gametes and embryos: A review of physiology and pathophysiology. *Journal of Assisted Reproduction and Genetics*, 31, 1337–1347.
- Fazeli, A., Affara, N. A., Hubank, M., & Holt, W. V. (2004). Sperm-induced modification of the oviductal gene expression profile after natural insemination in mice. *Biology of Reproduction*, 71, 60–65.
- Gentilini, D., Perino, A., Vignani, P., Chiodo, I., Cucinella, G., Vignali, M., Di Blasio, A. M., & Busacca, M. (2011). Gene expression profiling of peripheral blood mononuclear cells in endometriosis identifies genes altered in non-gynaecologic chronic inflammatory diseases. *Human Reproduction*, 26, 3109–3117.
- Gibbons, C., Dackor, R., Dunworth, W., Fritz-Six, K., & Caron, K. M. (2007). Receptor activity-modifying proteins: RAMPing up adrenomedullin signaling. *Molecular Endocrinology*, 21, 783–796.
- Hinson, J. P., Kapas, S., & Smith, D. M. (2000). Adrenomedullin, a multifunctional regulatory peptide. *Endocrine Reviews*, 21, 138–167.
- Horne, A. W., & Critchley, H. O. (2012). Mechanisms of disease: the endocrinology of ectopic pregnancy. *Expert Reviews in Molecular Medicine*, 14, e7.
- Karpinich, N. O., Hoopes, S. L., Kechele, D. O., Lenhart, P. M., & Caron, K. M. (2011). Adrenomedullin function in vascular endothelial cells: Insights from genetic mouse models. *Current Hypertension Reviews*, 7, 228–239.
- Kato, J., & Kitamura, K. (2015). Bench-to-bedside pharmacology of adrenomedullin. *European Journal of Pharmacology*, 764, 140–148.
- Kim, J., Kurita, T., & Bulun, S. E. (2013). Progesterone action in endometrial cancer, endometriosis, uterine fibroids, and breast cancer. *Endocrine Reviews*, 34, 130–162.
- Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., & Eto, T. (1993). Adrenomedullin: A novel hypotensive peptide isolated from human pheochromocytoma. *Biochemical and Biophysical Research Communications*, 192, 553–560.
- Lenhart, P. M., & Caron, K. M. (2012). Adrenomedullin and pregnancy: Perspectives from animal models to humans. *Trends in Endocrinology and Metabolism*, 23, 524–532.
- Li, M., Yee, D., Magnuson, T. R., Smithies, O., & Caron, K. M. (2006). Reduced maternal expression of adrenomedullin disrupts fertility, placentation, and fetal growth in mice. *The Journal of Clinical Investigation*, 116, 2653–2662.
- Li, M., Wu, Y., & Caron, K. M. (2008). Haploinsufficiency for adrenomedullin reduces pinopodes and diminishes uterine receptivity in mice. *Biology of Reproduction*, 79, 1169–1175.
- Li, M., Schwerbrock, N. M., Lenhart, P. M., Fritz-Six, K. L., Kadmiel, M., Christine, K. S., Kraus, D. M., Espenschied, S. T., Willcockson, H. H., Mack, C. P., & Caron, K. M. (2013). Fetal-derived adrenomedullin mediates the innate immune milieu of the placenta. *The Journal of Clinical Investigation*, 123, 2408–2420.
- Liao, S. B., Ho, J. C., Tang, F., & O, W. S. (2011). Adrenomedullin increases ciliary beat frequency and decreases muscular contraction in the rat oviduct. *Reproduction*, 141, 367–372.
- Liao, S. B., Li, H. W., Ho, J. C., Yeung, W. S., Ng, E. H., Cheung, A. N., Tang, F., & O, W. S. (2012). Possible role of adrenomedullin in the pathogenesis of tubal ectopic pregnancy. *The Journal of Clinical Endocrinology and Metabolism*, 97, 2105–2112.
- Liu, X. M., Zhang, D., Wang, T. T., Sheng, J. Z., & Huang, H. F. (2014). Ion/water channels for embryo implantation barrier. *Physiology (Bethesda)*, 29, 186–195.
- Matson, B. C., Corty, R. W., Karpinich, N. O., Murtha, A. P., valdar, W., Grotegut, C. A., & Caron, K. M. (2014). Midregional pro-adrenomedullin plasma concentrations are blunted in severe preeclampsia. *Placenta*, 35, 780–783.
- Matson, B. C., Pierce, S. P., Espenschied, S. T., Holle, E., Sweatt, I. H., Davis, E. S., Tarran, R., Young, S. L., Kohout, T. A., Van Duin, M., & Caron, K. M. 2017. Adrenomedullin improves fertility and promotes pinopodes and cell junctions in the peri-implantation endometrium. *Biology of Reproduction*, Accepted Advance Article.
- Mendelson, C. R. (2009). Minireview: Fetal-maternal hormonal signaling in pregnancy and labor. *Molecular Endocrinology*, 23, 947–954.
- Oehler, M. K., Norbury, C., Hague, S., Rees, M. C., & Bicknell, R. (2001). Adrenomedullin inhibits hypoxic cell death by upregulation of Bcl-2 in endometrial cancer cells: A possible promotion mechanism for tumour growth. *Oncogene*, 20, 2937–2945.
- Qiong, Z., Jie, H., Yonggang, W., Bin, X., Jing, Z., & Yanping, L. (2017). Clinical validation of pinopode as a marker of endometrial receptivity: A randomized controlled trial. *Fertility and Sterility*, 108, 513–517.
- Quinn, C. E., & Casper, R. F. (2009). Pinopodes: a questionable role in endometrial receptivity. *Human Reproduction Update*, 15, 229–236.
- Sharma, D., Farahbakhsh, N., Shastri, S., & Sharma, P. (2016). Intrauterine growth restriction - part 2. *The Journal of Maternal-Fetal & Neonatal Medicine*, 29, 4037–4048.
- Sibley, C. P. (2017). Treating the dysfunctional placenta. *Journal of Endocrinology*, 234, R81–R97.

- Singh, A. K., Dutta, M., Chattopadhyay, R., Chakravarty, B., & Chaudhury, K. (2016). Intrafollicular interleukin-8, interleukin-12, and adrenomedullin are the promising prognostic markers of oocyte and embryo quality in women with endometriosis. *Journal of Assisted Reproduction and Genetics*, 33, 1363–1372.
- Smith, S. D., Dunk, C. E., Aplin, J. D., Harris, L. K., & Jones, R. L. (2009). Evidence for immune cell involvement in decidual spiral arteriole remodeling in early human pregnancy. *The American Journal of Pathology*, 174, 1959–1971.
- Timeva, T., Shterev, A., & Kyurkchiev, S. (2014). Recurrent implantation failure: the role of the endometrium. *The Journal of Reproduction & Infertility*, 15, 173–183.
- Wang, H., & Dey, S. K. (2006). Roadmap to embryo implantation: Clues from mouse models. *Nature Reviews. Genetics*, 7, 185–199.
- Watanabe, H., Takahashi, E., Kobayashi, M., Goto, M., Krust, A., Chambon, P., & Iguchi, T. (2006). The estrogen-responsive adrenomedullin and receptor-modifying protein 3 gene identified by DNA microarray analysis are directly regulated by estrogen receptor. *Journal of Molecular Endocrinology*, 36, 81–89.
- Yallampalli, C., Chauhan, M., Endsley, J., & Sathishkumar, K. (2014). Calcitonin gene related family peptides: Importance in normal placental and fetal development. *Advances in Experimental Medicine and Biology*, 814, 229–240.

Placental Angiogenesis

Lawrence P Reynolds, Anna T Grazul-Bilska, and Pawel P Borowicz, North Dakota State University, Fargo, ND, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Angiogenesis The formation of a new vascular bed from a pre-existing one.

Capillary The smallest blood vessel (approximately 5 μm in diameter) consisting of an inner layer of flattened (squamous) epithelial cells, called endothelial cells, and an outer layer of vascular smooth muscle-like cells, called pericytes. The capillaries are the site of exchange of nutrients, respiratory gases, and metabolic wastes between the tissues and circulatory system, whereas the pericytes are thought to regulate endothelial function.

Circulatory system The system that distributes blood to the body's organs and tissues and returns the blood and tissue fluid to the heart—includes the heart, arteries, veins and lymphatics—also called the vascular system.

Compromised pregnancy Any pregnancy in which fetal or placental growth, or both, are abnormal.

Endothelial cells The specialized, flattened (squamous) epithelial cells that line, or form the inner layer, of the entire circulatory system including the heart (where they are called endocardial cells, or the cells making up the endocardium).

Endothelium The inner layer of all blood vessels, made up of endothelial cells. The endothelium comprises a single, complete layer of cells.

Metabolic wastes By-products of metabolism which cannot be used by the body or are even toxic and must therefore be eliminated—these include metabolic water (which is an end-product of aerobic metabolism), carbon dioxide, and ammonia.

Metabolism The processes involved in supply of energy to support cellular activities including accumulation (accretion, anabolism) of materials and destruction (catabolism) of materials including the processes involved in detoxification and excretion. Metabolism can be oxidative (requiring oxygen, also called aerobic) or anaerobic (oxygen independent).

Nutrients A nutritive, nutritious substance required to remain healthy—usually divided into macronutrients (nutrients required in relatively large quantities), which includes carbohydrates, proteins, and fats, and micronutrients (nutrients required in relatively small quantities), which includes minerals (e.g., calcium, potassium, iron, selenium, zinc, etc.) and vitamins.

Pericytes Vascular smooth muscle-like cells that are one of the cell types making up and critical to normal function of capillaries—these exist outside of the endothelium.

Respiratory gases Gases (oxygen, carbon dioxide, nitric oxide) involved in respiration, which includes the processes involved in supplying oxygen to the tissues and cells to support metabolism and removing carbon dioxide which is a product of metabolism.

Vascular bed Also called the microcirculation—composed of arterioles (the smallest arteries), capillaries, and venules (the smallest veins) within a tissue (such as the placenta). It provides for exchange of nutrients, respiratory gases (e.g., O_2 and CO_2), and metabolic wastes (e.g., urea) between the tissues and the circulatory system.

Vasculogenesis The de novo formation of a new vascular bed in situ, as happens during early embryogenesis and placentation.

Introduction

Growth of vascular beds, which includes capillaries, venues and arterioles, is termed angiogenesis, is essential for growth and development of tissues because the capillaries are the site of exchange of nutrients, respiratory gases, and metabolic wastes between the tissues and circulatory system. Angiogenesis begins with formation of new capillaries and culminates in a new microcirculatory bed. As shown in **Fig. 1**, the initial component of angiogenesis, capillary proliferation, consists of:

1. Production of proteases by the existing capillary (endothelial) cells, which results in breakdown of their basement membrane (basal lamina).
2. Once the basement membrane is broken down, the endothelial cells of the existing capillaries proliferate and then migrate towards the angiogenic stimulus, which might be low tissue oxygen (hypoxia) or an angiogenic growth factor.
3. The new vascular tubes begin to form a lumen so that blood can begin circulating through them.
4. The newly formed capillaries form their own basement membrane and recruit vascular smooth muscle-like cells, pericytes, which are the final steps in maturation of the newly formed capillary bed.

The term angiogenesis, which is derived from Greek *angei*, vessel, and *gignesthai*, to be born, was first coined in 1935 by Arthur T. Hertig in his study of blood vessel development in the early placenta of humans and macaques ([Hertig, 1935](#)). This is appropriate, as angiogenesis not only supports growth and development of the placenta, but also, as we will discuss below, is critical for the large increase in placental blood flow that occurs during pregnancy and provides metabolic support to the growing fetus.

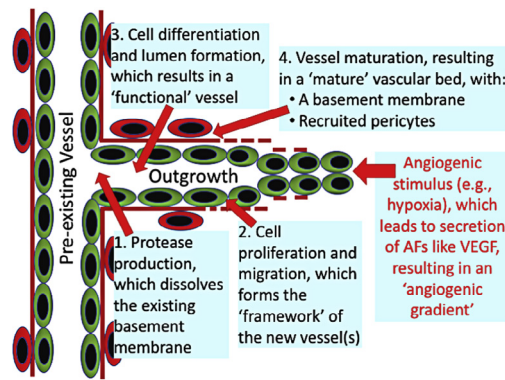


Fig. 1 The process of angiogenesis. In response to an angiogenic stimulus and secretion of angiogenic factors (AFs): (1) the endothelial cells (green ovals) of the existing capillary vessel produce and release proteases that dissolve the existing basement membrane (red line); (2) once the basement membrane is broken down the endothelial cells of the existing capillaries proliferate and then migrate towards the angiogenic stimulus—the direction of migration is based on the “angiogenic gradient”; (3) the new endothelial tubes begin to form a lumen so that blood can begin circulating through them; and (4) the newly formed capillaries form their own basement membrane and also recruit vascular smooth muscle-like cells, pericytes (red ovals), which are the final steps in maturation of the newly formed capillary bed.

Because angiogenesis supports tissue growth, it is seen primarily in tissues during pre- and post-natal development, whereas the vascular beds of adults are quite stable. In fact, when it does occur in adults, angiogenesis is often associated with pathological conditions, such as tumors, retinopathies, hemangiomas, etc. The exception is that angiogenesis occurs in adults during wound healing, and also in tissues that undergo cycles of growth and regression including those of the reproductive system—for example, ovaries, uterus, testis, and placenta.

The vascular bed, also called the microcirculation, is composed of arterioles (the smallest arteries), capillaries, and venules (the smallest veins) and is located within the tissue itself. Because the vascular bed provides for exchange of nutrients, respiratory gases (e.g., O_2 and CO_2), and metabolic wastes (e.g., urea) between the tissues and the circulatory system, its size is proportional to the metabolic requirements of the tissue. For example, as the metabolic rate of the tissue increases, so does the density of capillaries within that tissue. Conversely, as tissue oxygen levels increase, angiogenesis decreases, and vice versa. That is, low tissue oxygen, which is not sufficient to support the metabolic needs of the tissue, not only is a signal for dilation of blood vessels (and hence increased blood flow) but also is a primary “signal” for increased capillary growth, or angiogenesis (Fig. 1).

Placental Angiogenesis and Blood Flow During Normal Pregnancy

Both uterine blood flow, which supplies the entire gravid uterus (the uterine tissue, the placenta, and the fetus), and umbilical blood flow, which supplies the fetus and the fetal part of the placenta, increase dramatically during normal pregnancy. During the last 40% of pregnancy in humans, for example, uterine blood flow increases by 2.5-fold and umbilical blood flow by 5.5-fold (Fig. 2). Similarly, during the last 50% of pregnancy in cattle and sheep, uterine blood flow increases by 3.0- to 4.5-fold, whereas umbilical blood flow increases by 19-fold (Fig. 2). Conversely, the extraction rate per unit of blood, which is also known as the arterial-venous difference, increases less than 1-fold over the same period. Thus, for example, the 10- to 20-fold increase in oxygen (O_2) consumption by the gravid uterus and fetus (Fig. 3A) can be accounted for primarily by the increases in uterine and umbilical blood flows (Figs. 2 and 3B). This is also true for other nutrients including glucose and amino acids. The increase in umbilical blood flow keeps pace with fetal growth, such that umbilical blood flow and O_2 consumption per unit of fetal weight remain relatively constant both across stage of gestation within a species and between species.

Because capillary beds are arranged as a parallel rather than a serial system, blood flow to any tissue is proportional to the total cross sectional area of the capillary bed, which dictates resistance to flow. Thus, to a large extent, the large increase in blood flow to the placenta (i.e., uterine and umbilical blood flows) depends on growth of the placental vascular beds—that is, on placental angiogenesis.

To examine the pattern of placental angiogenesis, we chose to use sheep as a model, because the type of placenta, called cotyledonary, allowed us to examine both uterine, or maternal placental (also termed endometrial or caruncular), and fetal placental (also termed chorioallantoic or cotyledonary) angiogenesis. We accomplished this by cannulating the uterine artery or the umbilical artery and then perfusing the maternal or fetal placental vascular beds with a fixative before embedding the placental tissues in paraffin and sectioning by microtomy. Initially we stained the tissue sections using a histochemical method, called periodic acid-Schiff's reagent, that stained the capillary basement membranes and then quantified vascular development via image analysis. We subsequently have used more sophisticated methods, including immunofluorescence and confocal microscopy, to examine the pattern of placental angiogenesis.

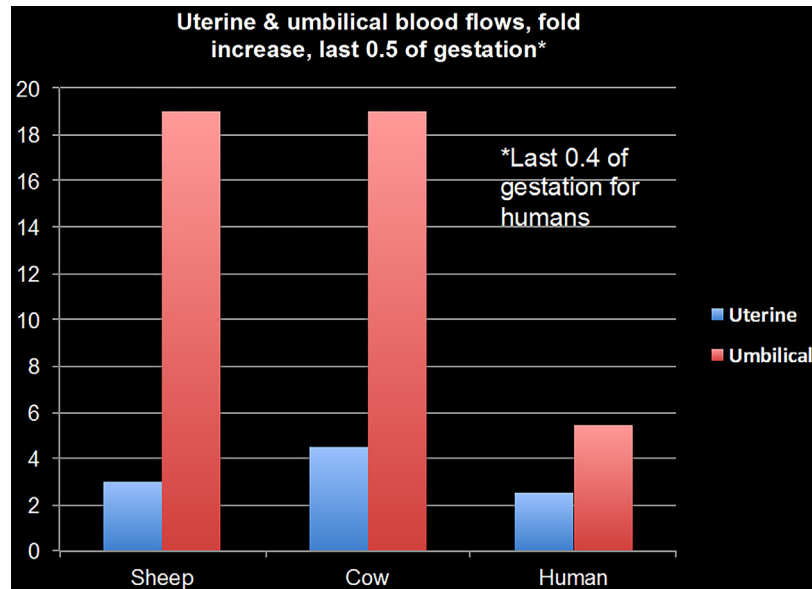


Fig. 2 Fold increase in uterine and umbilical blood flows during the last 0.4–0.5 (40%–50%) of pregnancy in sheep, cow, and human. Data are expressed as fold-increase compared with the level at 50% or 60% of pregnancy. Data taken from Meschia, G. (1983). Circulation to female reproductive organs. In Shepherd, J.T. and Abboud, F.M. (eds.) *Handbook of physiology*, Sec 2, Vol III, Part 1. pp 241–69. Bethesda, MD: American Physiological Society; Rosenfeld, C.R., Morriss, F.H., Makowski, E.L., Meschia, G. and Battaglia, F.C. (1974). Circulatory changes in the reproductive tissues of ewes during pregnancy. *Gynecological Investigation* 5, 252–268; Reynolds, L.P., Ferrell, C.L., Robertson, D.A. and Ford S.P. (1986). Metabolism of the gravid uterus, foetus and uteroplacenta at several stages of gestation in cows. *Journal of Agricultural Science, Cambridge* 106, 437–444 and Konje, J.C., Howarth, E.S., Kaufmann, P. and Taylor, D.J. (2003) Longitudinal quantification of uterine artery blood volume flow changes during gestation in pregnancies complicated by intrauterine growth restriction. *British Journal of Obstetrics and Gynaecology* 110, 301–305.

What we found was that although the maternal placental capillary bed grows and develops extensively throughout pregnancy, growth of the fetal placental capillary bed is even more extensive. This mirrors the larger percentage increase in umbilical compared with uterine blood flow depicted in **Fig. 2**. Moreover, in the sheep, the pattern of vascular growth differs dramatically between the maternal and fetal placenta. In the maternal placenta, capillaries become much larger in diameter as gestation progresses, but the increase in capillary density (whether one measures area, number, or surface density—that is, capillary area, number, or surface area per unit of tissue volume) is only modest, on the order of 1.5- to 3-fold (**Fig. 4A and B**). Conversely, in the fetal placenta the capillaries become smaller as gestation progresses, but the capillary density increases from 6- to 12-fold for capillary area, number, and surface area (**Fig. 4A and B**). We have verified this difference in the pattern of capillary growth using a method called ‘vascular casting’ and scanning electron microscopy (**Fig. 4B**). This pattern of capillary growth would dictate a low-resistance, low-velocity flow of blood through the maternal placental tissue, which we have termed ‘irrigation’ flow, and is ideal for nutrient delivery. On the other hand, the pattern of capillary growth of the fetal placental vascular bed results in a large surface area, which is optimal for nutrient transport.

Is this pattern of placental vascular growth unique to ruminants like sheep, or is it similar among mammals? The mouse placenta develops a very similar vascular architecture (Adamson et al., 2002), and the human placenta is probably the ultimate irrigation flow system, as in primates the fetal membranes digest away a large part of the uterine wall, such that the fetal villi, or cotyledons, are suspended in a pool of slowly percolating maternal blood (**Fig. 5**).

Because angiogenesis is also critical to support growth of tumors, the factors that are involved in the angiogenic process are well studied. Just as for tumors and other tissues, it appears that placental angiogenesis is dependent on production of angiogenic factors by the placenta itself, including vascular endothelial growth factor (VEGF) and nitric oxide (NO) (**Fig. 6**). Other growth factors and cytokines, such as the chemokine family, also may be involved. The involvement of nitric oxide is important, as it also serves as a major vasodilator, and therefore may coordinate placental vascular growth with placental blood flow to meet the metabolic demands of fetal and placental growth.

Placental Angiogenesis and Blood Flow During Compromised Pregnancy

The sheep is a widely used model for study of pregnancy complications for numerous reasons:

1. Their physiology of pregnancy is well studied.
2. They have primarily singleton and twin pregnancies.

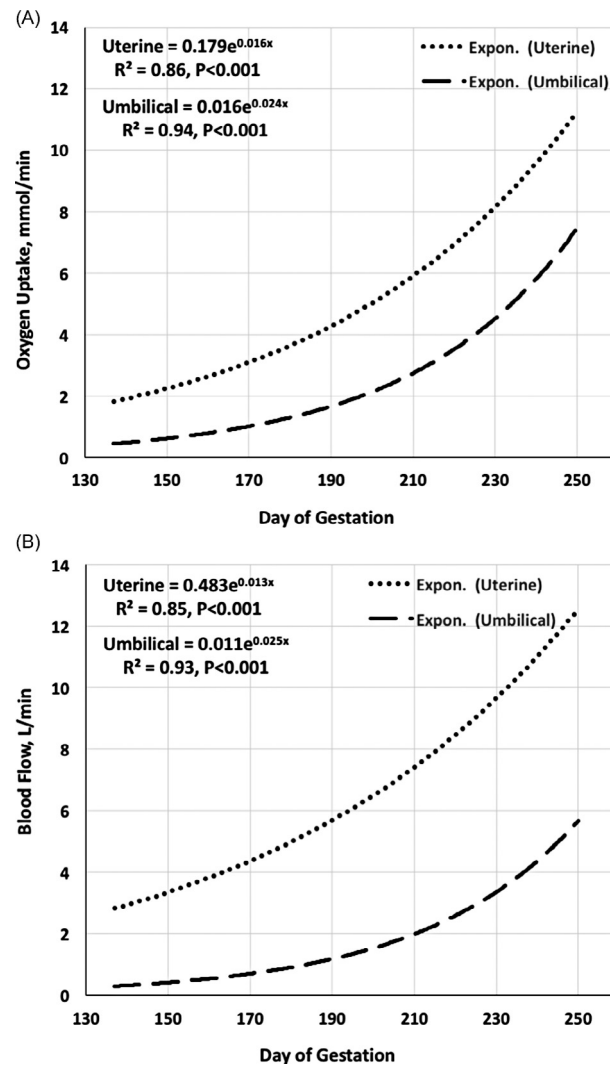


Fig. 3 Uterine and umbilical (A) oxygen uptakes and (B) blood flows during the last 0.5 (50%) of pregnancy in cows. Data taken from Reynolds, L.P., Ferrell, C.L., Robertson, D.A. and Ford S.P. (1986). Metabolism of the gravid uterus, foetus and uteroplacenta at several stages of gestation in cows. *Journal of Agricultural Science, Cambridge* 106, 437–444 and were determined in vivo. Data are similar for other mammals.

3. They have a relatively long gestation (145–150 days).
4. The gravid uterus can be chronically instrumented (e.g., with blood flow probes).
5. Large amounts of tissue can be collected from the gravid uterine tissue, including the placenta.
6. One can examine multiple stages of pregnancy.
7. Compromised pregnancies exhibit complications similar to those of humans.
8. The structure and function of the placental blood vessels is similar to that of other mammals, including humans.

Using various sheep models of compromised pregnancy, we and others have shown that placental angiogenesis is altered, resulting in reduced placental vascular development, or vascularity, in association with reduced maternal placental (gravid uterine) and fetal placental (umbilical) blood flows. These models of compromised pregnancy include overfed adolescents, multiple pregnancies (specifically singletons vs. triplets), heat stress, underfed adults and adolescents, maternal age (adolescent vs. adult pregnancies), and maternal genotype (Table 1). These observations emphasize the close relationship between placental angiogenesis and placental blood flows. Similar observations of altered placental vascular development in growth-restricted fetuses have been observed for humans (Fig. 7).

Perhaps the most compromised of all pregnancies are those resulting from the use of assisted reproductive technologies (ART), including embryos derived from in vitro fertilization procedures or from cloning. For example, after transfer of in vitro-fertilized embryos in humans and livestock only around 40% or less survive to birth. Because of this we and others have used ART pregnancies

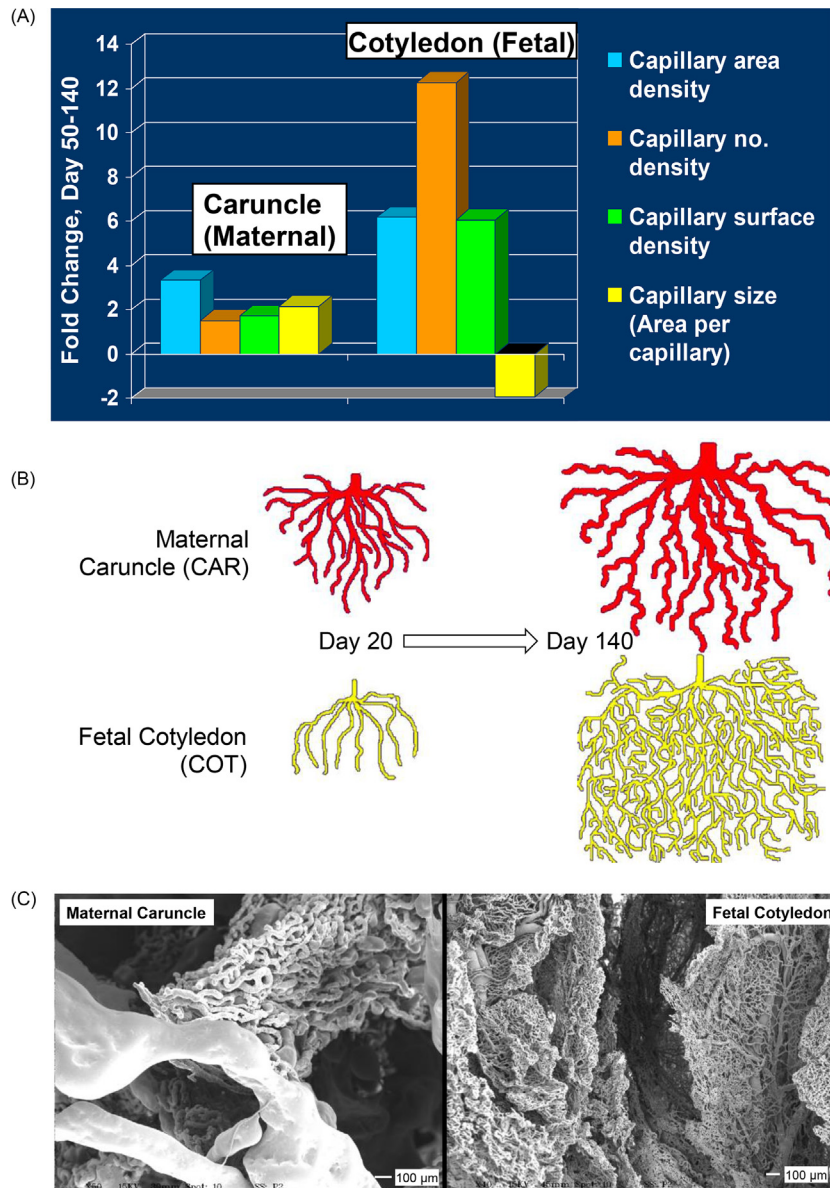


Fig. 4 (A) Changes in capillary area, number, and surface densities of maternal placenta (caruncle) and fetal placenta (cotyledon) from day 20 to 140 of gestation (length of gestation 145–150 days) in sheep; (B) Empirical model of the pattern of capillary growth of the maternal caruncle and fetal cotyledon based on the data in (A); and (C) Scanning electron micrographs of vascular casts of the maternal caruncle and fetal cotyledon on day 90 of gestation—note how similar the micrographs look to the empirical model presented in (B). Modified from (A and B) Borowicz, P.P., Arnold, D.R., Johnson, M.L., Grazul-Bilska, A.T., Redmer, D.A. and Reynolds, L.P. (2007). Placental growth throughout the last two-thirds of pregnancy in sheep: Vascular development and angiogenic factor expression. *Biology of Reproduction* 76, 259–267 and (C) Hafez, S., Borowicz, P., Reynolds, L. and Redmer, D. (2010) Maternal and fetal microvasculature in sheep placenta across several stages of gestation. *Journal of Anatomy* 216, 292–300.

as models for not only highly compromised pregnancies but also to determine whether any vascular defects were observed very early in pregnancy. As shown in [Table 2](#), both in vitro fertilized and cloned (parthenogenetic, meaning in vitro activated to develop but with only maternal genes) embryos from very early in pregnancy (day 22 after mating) showed many defects in growth as well as altered placental vascularization, angiogenic factor expression, DNA methylation, steroid receptor expression, and markers of diapause (embryonic quiescence). These data support the concept that placental vascular defects begin very early in pregnancy and result in poor fetal growth and development.

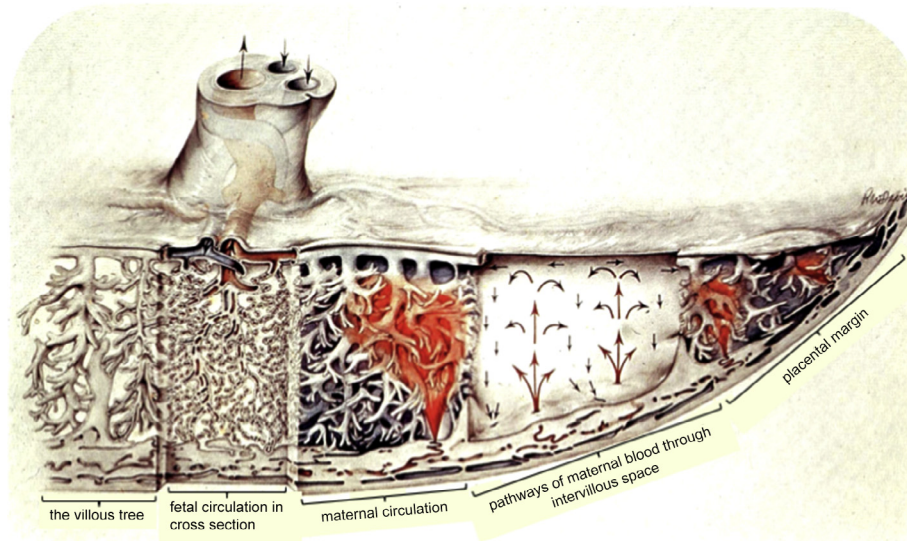


Fig. 5 Model of the placental circulation in humans—note that the fetal villi are suspended in a pool of maternal blood—the maternal arterioles are open-ended and ‘spurt’ blood into the maternal blood spaces. From Ramsey, E.M. and Donner, M.W. (1980). Placental vasculature and circulation: Anatomy and Physiology. Fort Worth, TX: Saunders College Publishing/Harcourt Brace.

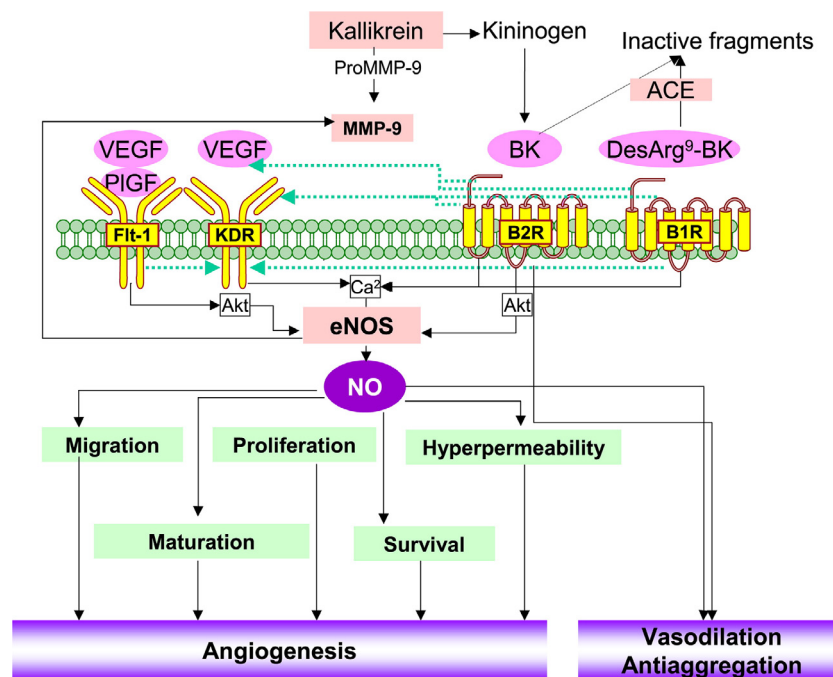


Fig. 6 Proposed role of the VEGF and kallikrein-kinin systems and the eNOS-NO pathway in placental angiogenesis, vasodilation, and platelet anti-aggregation. Taken from Valdés, G. and Corthorn, J. (2011). Review: The angiogenic and vasodilatory utero-placental network. Placenta. 32 Suppl 2, S170–S175.

Conclusions

Although it seems that placental vascular defects, which probably have their origins very early in pregnancy, are major factors contributing to poor fetal growth and development, and perhaps even have long-term consequences for the offspring, we have much to learn about the mechanisms that determine not only poor placental angiogenesis but also how that impacts fetal growth and development.

Table 1 Fetal and placental weights, placental vascularity, and maternal placental (gravid uterine) and fetal placental (umbilical) blood flows in various models of compromised pregnancy in sheep^a

Model	Fetal wt	Placental wt	Placental vascularity	Gravid Ut blood flow	Umbilical blood flow
Overfed adolescent	–20%–40%	–20–45%	–31%	–36%	–37%
Multiple pregnancy	–30%	–37%	–30%	–23%	–
Heat-stressed adult	–42%	–51%	–	–26%	–60%
Underfed adult	–12%	–	–14%	–25%	NSE
Underfed adolescent	–17%	NSE	–20%	–	–
Adolescent vs. adult	–16%	–26%	–24%	–	–
Maternal genotype (adult only)	–44%	–28%	–33%	–	–

^aAll data from late pregnancy (days 130–140; 0.88–0.95 of gestation).

Taken from Reynolds, L.P., Caton, J.S., Redmer, D.A., Grazul-Bilska, A.T., Vonnahme, K.A., Borowicz, P.P., Luther, J.S., Wallace, J.M., Wu, G. and Spencer, T.E. (2006). Topical Review: Evidence for altered placental blood flow and vascularity in compromised pregnancies. Invited review. Journal of Physiology 572, 51–58.

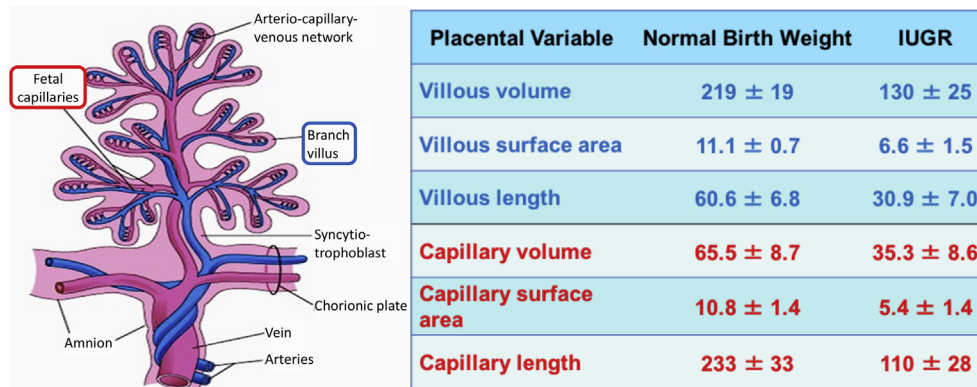


Fig. 7 Placental growth and vascular development in normal and intrauterine growth restricted (IUGR) pregnancies. The IUGR pregnancies were of unknown cause. The placental variables in blue in the table correspond to the placental branch villus structure in the figure on the left, whereas those in red in the table correspond to the villus (fetal) capillaries in the figure. Data in the table from Mayhew, T.M., Charnock-Jones, D.S. and Kaufmann, P. (2004) Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies. Placenta 25, 829–833 and figure from Quizlet, Inc.

Table 2 Fetal growth and placental vascularization, expression of gap junction connexins, angiogenic factors, steroid receptors, and diapause markers, and global DNA methylation, in naturally mated-embryo transfer (NAT-ET), in vitro fertilized (IVF), and cloned, in vitro activated (IVA; parthenogenetic, meaning only maternal genes) sheep embryos.^a

Process	Factor	NAT-ET group		IVF group		IVA group	
		Maternal placenta	Fetal placenta	Maternal placenta	Fetal placenta	Maternal placenta	Fetal placenta
Vascularization ⁵	Blood vessel number	↓	—	↓	↓	↓	↓
	Average size of capillary	—	NP	↓	NP	↓	NP
Tissue growth ⁶	Length of fetus	NA	↓	NA	↓	NA	↓
	Labeling index	↓	↓	↓	↓	↓	↓
Gap junctional connexins mRNA	C × 26	—	—	—	—	—	↓
	C × 32	↓	—	↓	—	↓	—
	C × 37	—	—	—	—	—	—
	C × 43	—	—	—	—	—	↓

(Continued)

Table 2 Fetal growth and placental vascularization, expression of gap junction connexins, angiogenic factors, steroid receptors, and diapause markers, and global DNA methylation, in naturally mated-embryo transfer (NAT-ET), in vitro fertilized (IVF), and cloned, in vitro activated (IVA; parthenogenetic, meaning only maternal genes) sheep embryos.^a—cont'd

Process	Factor	NAT-ET group		IVF group		IVA group	
		Maternal placenta	Fetal placenta	Maternal placenta	Fetal placenta	Maternal placenta	Fetal placenta
Angiogenic factors mRNA expression	VEGF ²	↓ ³	↓	—	↓	↓	↓
	FLT1	↓	↓	↓	↓	↓	↓
	KDR	—	—	—	—	—	—
	PGF	↓	↓	—	↓	—	—
	NP1	—	↓	—	↓	—	↓
	NP2	—	—	—	↓	—	↓
	ANGPT1	↓	↓	—	↓	—	↓
	ANGPT2	—	↓	—	↓	—	↓
	TEK	↓	—	↓	—	↓	—
	NOS3	—	↓	—	—	—	↓
	GUCY1B3	—	—	—	—	—	—
	HIF1A	—	↓	—	—	—	↓
	FGF2	—	↓	—	↓	—	—
	FGFR	—	↓	—	↓	—	—
	DNMT1 mRNA	—	—	—	—	—	↑
	DNMT3a mRNA	↓	—	↓	—	↓	—
Global DNA methylation	DNMT3b mRNA	—	—	—	—	—	—
	5mC ⁴	NP	—	NP	↑	NP	↑
	Nuclear P4	↓	—	↓	—	↓	—
	Membrane P4 alpha	—	—	—	—	—	—
Steroid receptor mRNA expression	Membrane P4 beta	—	—	—	—	—	—
	Membrane P4 gamma	—	—	—	—	—	—
	Nuclear E alpha	↓	—	↓	↑	↓	↑
	Nuclear E beta	—	—	—	—	—	—
	Membrane E (GPR30)	—	↑	—	↑	—	—
	BTG1	—	↑	↑	↑	↑	↑
mRNA expression of diapause markers	IGF2R	—	↑	—	↑	↑	↑

^aAll groups compared with normal embryos from natural mating.Taken from Reynolds, L.P., Borowicz, P.P., Palmieri, C. and Grazul-Bilska, A.T. (2014). Placental vascular defects in compromised pregnancies: Effects of assisted reproductive technologies and other maternal stressors. In Zhang, L. and Ducsay, C.A. (eds.) *Advances in fetal and neonatal physiology (Advances in Experimental Medicine and Biology 814)* pp. 193–204. New York: Springer Science+Business Media.

For example, as shown in [Table 2](#), even embryos derived in vivo from natural pregnancies, when subjected to what we thought was the fairly ‘mild’ insult of embryo transfer (flushing from the FSH-treated donor’s uterus and transfer to the recipient’s uterus, which takes only about 20–30 min, during which the embryo is in vitro), show many of the same defects as in vitro fertilized or cloned embryos. At the very least, this tells us that our in vitro conditions are not optimal. In fact, several research groups have now shown that culturing embryos in the presence of natural oviductal secretions (and fertilization and early embryonic development take place in the oviduct) can improve the outcome of in vitro fertilization. This is very interesting, as the oviductal fluid contains a very complex mixture of oviduct- and serum-derived factors that support fertilization and early embryonic development.

In addition, we must investigate whether defects in placental angiogenesis affect fetal growth and development in many or only a few mammalian species. At the very least, we know that sheep (and probably other ruminants like cows) and humans exhibit similar defects in placental angiogenesis in pregnancies in which fetal and placental growth are compromised.

Lastly, we need to continue to pursue therapeutic (e.g., sildenafil) and management (e.g., improved maternal nutrition) strategies to improve placental angiogenesis and thereby fetal growth and development, as these problems, which probably also contribute to miscarriage (also termed spontaneous abortion), have major socioeconomic consequences.

References

- Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C., & Cross, J. C. (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Developmental Biology*, 250, 358–373.
- Hertig, A. T. (1935). Angiogenesis in the early human chorion and in the primary placental of the macaque monkey. *Contributions to Embryology*, 146, 37–89.
- ## Further Reading
- Bairagi, S., Quinn, K. E., Crane, A. R., Ashley, R. L., Borowicz, P. P., Caton, J. S., Redden, R. R., Grazul-Bilska, A. T., & Reynolds, L. P. (2016). Maternal environment and placental vascularization in domestic ruminants. Invited review *Theriogenology*, 86, 288–305.
- Borowicz, P. P., Hafez, S., Redmer, D. A., & Reynolds, L. P. (2008). Methods for evaluating uteroplacental angiogenesis and their application using animal models. In D. Cheresch (Ed.), *Methods in enzymology*, Vol. 445, *Angiogenesis, in vivo, Part B* (pp. 229–253). New York: Elsevier.
- Redmer, D. A., Wallace, J. M., & Reynolds, L. P. (2004). Effects of nutrient intake during pregnancy on fetal and placental growth and vascular development. Invited review *Domestic Animal Endocrinology*, 27, 199–217.
- Reynolds, L. P., Killilea, S. D., & Redmer, D. A. (1992). Angiogenesis in the female reproductive system. Invited review *FASEB Journal*, 6, 886–892.
- Reynolds, L. P., & Redmer, D. A. (2001). Mini-review: Angiogenesis in the placenta. Invited review *Biology of Reproduction*, 64, 1033–1040.
- Reynolds, L. P., Grazul-Bilska, A. T., & Redmer, D. A. (2002). Angiogenesis in the female reproductive system: Pathological implications. Invited review *International Journal of Experimental Pathology*, 83, 151–164.
- Reynolds, L. P., Borowicz, P. P., Caton, J. S., Vonnahme, K. A., Luther, J. S., Buchanan, D. S., Hafez, S. A., Grazul-Bilska, A. T., and Redmer, D. A. (2010). Utero-placental vascular development and placental function: An update. Invited review. *International Journal of Developmental Biology*, Special Issue Placental Developmental Biology, Hunt, J. S. and Thornburg, K. L. (eds.) 54, 355–366.
- Reynolds, L. P., Vonnahme, K. A., Lemley, C. O., Redmer, D. A., Grazul-Bilska, A. T., Borowicz, P. P. and Caton J. S. (2013). Maternal stress and placental vascular function and remodeling. Invited review. *Current Vascular Pharmacology, Hot Topic Issue 'Uteroplacental Circulation and Fetal Vascular Function and Development'*, Zhang, L. (guest editor) 11, 564–593.
- Borowicz, P. P., Arnold, D. R., Johnson, M. L., Grazul-Bilska, A. T., Redmer, D. A., & Reynolds, L. P. (2007). Placental growth throughout the last two-thirds of pregnancy in sheep: Vascular development and angiogenic factor expression. *Biology of Reproduction*, 76, 259–267.
- Hafez, S., Borowicz, P., Reynolds, L., & Redmer, D. (2010). Maternal and fetal microvasculature in sheep placenta across several stages of gestation. *Journal of Anatomy*, 216, 292–300.
- Konje, J. C., Howarth, E. S., Kaufmann, P., & Taylor, D. J. (2003). Longitudinal quantification of uterine artery blood volume flow changes during gestation in pregnancies complicated by intrauterine growth restriction. *British Journal of Obstetrics and Gynaecology*, 110, 301–305.
- Mayhew, T. M., Charnock-Jones, D. S., & Kaufmann, P. (2004). Aspects of human fetoplacental vasculogenesis and angiogenesis. III. Changes in complicated pregnancies. *Placenta*, 25, 829–833.
- Meschia, G. (1983). Circulation to female reproductive organs. In J. T. Shepherd, & F. M. Abboud (Eds.), *Handbook of physiology* (vol. III, pp. 241–269). Bethesda, MD: American Physiological Society. sec 2, Part 1.
- Ramsey, E. M., & Donner, M. W. (1980). *Placental vasculature and circulation: Anatomy and physiology*. Fort Worth, TX: Saunders College Publishing/Harcourt Brace.
- Reynolds, L. P., Ferrell, C. L., Robertson, D. A., & Ford, S. P. (1986). Metabolism of the gravid uterus, foetus and uteroplacenta at several stages of gestation in cows. *Journal of Agricultural Science, Cambridge*, 106, 437–444.
- Reynolds, L. P., Caton, J. S., Redmer, D. A., Grazul-Bilska, A. T., Vonnahme, K. A., Borowicz, P. P., Luther, J. S., Wallace, J. M., Wu, G., & Spencer, T. E. (2006). Topical review: Evidence for altered placental blood flow and vascularity in compromised pregnancies. *Journal of Physiology*, 572, 51–58. Invited review.
- Reynolds, L. P., Borowicz, P. P., Palmieri, C., & Grazul-Bilska, A. T. (2014). Placental vascular defects in compromised pregnancies: Effects of assisted reproductive technologies and other maternal stressors. In L. Zhang, & C. A. Ducey (Eds.), *Advances in Experimental Medicine and Biology*: 814. *Advances in Fetal and neonatal physiology* (pp. 193–204). New York: Springer Science+Business Media.
- Rosenfeld, C. R., Morriss, F. H., Makowski, E. L., Meschia, G., & Battaglia, F. C. (1974). Circulatory changes in the reproductive tissues of ewes during pregnancy. *Gynecological Investigation*, 5, 252–268.
- Valdés, G., & Corthorn, J. (2011). Review: The angiogenic and vasodilatory utero-placental network. *Placenta*, 32(Suppl 2), S170–S175.

Placental Transfer

Thomas Jansson, University of Colorado, Aurora, CO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Of all the diverse functions performed by the human placenta, transfer of oxygen, ions, and nutrients is of particular importance for normal fetal growth and development. This article will briefly review the pathways for placental transfer, discuss the key differences between nonmediated and mediated transport and outline the principal factors influencing placental transfer. Subsequently, we will summarize key characteristics of transfer of oxygen, water, and ions across the human placental barrier.

Pathways of Placental Transfer

Solute transfer across epithelia can occur by *paracellular* (i.e., via water-filled channels) or *transcellular* pathways. In contrast to classical epithelia, such as in the kidney and intestine, the transporting epithelium of the human placenta (the syncytiotrophoblast) lacks intercellular spaces with tight junctions. However, physiological evidence, including a correlation between the rate of transplacental flux of a certain solute and its diffusion coefficient in water, suggests that paracellular transfer across the human placenta takes place via transtrophoblastic channels. Moreover, the existence of transtrophoblastic channel is supported by the transfer of large molecules such as alpha-fetoprotein across placental cotyledons perfused in vitro and the observation that fetal blood cells are present in the maternal circulation in vivo. However, the total area occupied by these putative water-filled channels across the human placental barrier is likely to be very small because the syncytiotrophoblast is able to maintain substantial maternal–fetal concentration differences for a large number of solutes including amino acids and calcium. As a consequence, the predominant pathway for transfer across the human placental barrier is transcellular, largely mediated by transporter proteins expressed in the syncytiotrophoblast plasma membranes. The morphological structures corresponding to the transtrophoblastic channels supported by the functional data have yet to be identified. The existence of trophoblast denudations associated with fibrin deposits supports the idea that breaks in the syncytial barrier opens temporary water-filled pathways between the maternal and fetal circulations, which may constitute the morphological correlate to the postulated transtrophoblastic channels (Brownbill et al., 1995).

Nonmediated and Mediated Transport

Placental transfer can be *nonmediated* (not involving any form of specific transport mechanism) or *mediated* (dependent on specific transport mechanisms). The rate of nonmediated transfer is determined by the physical and chemical properties of the molecules, including the charge, size, lipid solubility, and degree of protein binding. Specifically, a solute with high electrical charge, molecular weight and degree of protein-binding and low lipid solubility is expected to cross the placental barrier very slowly, if at all. The nonmediated placental transfer of pharmaceutical drugs is determined by similar factors. For example, in the class of anticoagulants, the placental barrier is virtually impermeable to heparin because of its high molecular weight and charge, whereas orally active drugs such as dicumarol crosses the placental barrier without major restriction because of its relatively low molecular weight (Bajoria and Contractor, 1992). However, the transfer of drugs across the human placenta is influenced by a number of factors beyond physical–chemical properties because many drugs are subjected to mediated uptake, metabolism by cytochrome 450 enzymes and/or extrusion from the syncytiotrophoblast by efflux pumps such as P-glycoprotein (Syme et al., 2004).

The syncytiotrophoblast represents the primary barrier for movement of most solutes from the maternal to the fetal circulations (Fig. 1). Specifically, transfer across the two polarized plasma membranes of the syncytiotrophoblast, the apical or microvillous plasma membrane (MVM) directed to maternal blood in the intervillous space and the basal plasma membrane (BM) facing the fetal capillaries constitutes the limiting step. Maternal fetal-exchange of most nutrients and ions occurs by mediated transfer, largely mediated by transporter proteins expressed in the syncytiotrophoblast plasma membranes. *Facilitated transport* (or *facilitated diffusion*) is defined as a mediated transport not requiring energy expenditure, as exemplified by placental glucose transfer, which is mediated by facilitative glucose transporters expressed in the syncytiotrophoblast MVM and BM. Transplacental transport may also be facilitated by the expression of receptors in the MVM (e.g., the transferrin receptor, which binds transferrin–iron complex in maternal blood thereby facilitating the placental uptake of iron) or binding proteins in the syncytiotrophoblast cytosol (such as fatty acid binding proteins). *Active transport* involves energy consumption, either directly (primary active transport) or indirectly (secondary/tertiary active transport). An example of primary active transport in the placenta is calcium efflux across the BM mediated by Ca^{2+} -ATPase. Moreover, Na^{+} -dependent amino acid transport represents an example of a secondary active transport mechanism.

Endocytosis/transcytosis plays an important role in the transplacental transfer of IgG, iron, and lipoproteins (Fuchs and Ellinger, 2004) and represents a specialized form of transport, which may be nonmediated or mediated. The endocytotic/transcytotic process is initiated by the formation of an intracellular vesicle through the invagination of the plasma membrane on one side of the syncytiotrophoblast. Vesicles may then be transported to the opposite side of the cell where the vesicle content is released into the extracellular space following fusion of the vesicles with the plasma membrane. Endocytosis/transcytosis is nonmediated when vesicles

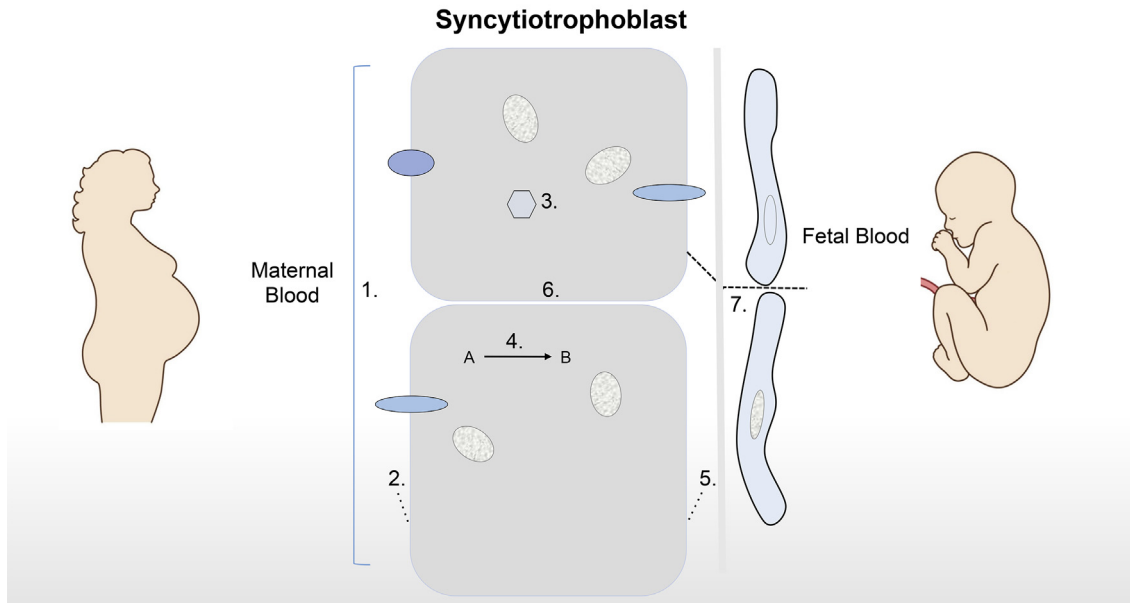


Fig. 1 Key properties of the placental barrier influencing transfer rate. See text for details. (1) Total effective surface area; (2) Characteristics of the syncytiotrophoblast microvillous plasma membrane (MVM), including passive properties such as lipid composition and fluidity, and expression of transporters, channels, enzymes and receptors; (3) Cytosolic binding proteins; (4) Metabolic interconversion or catabolism in the cytoplasm; (5) Characteristics of the syncytiotrophoblast basal plasma membrane (BM) including passive properties such as lipid composition and fluidity, and expression of transporters, channels, enzymes, and receptors; (6) Transtrophoblastic channels; (7) Diffusion across basement membrane, villous core and capillary endothelium.

incorporate fluid and any dissolved solute (fluid-phase endocytosis) and subsequently are transferred across the syncytium as a consequence of the random motion affecting all particles suspended in a fluid. Mediated endocytosis, on the other hand, involves the binding of ligands to plasma membrane receptors, which initiates the invagination of the plasma membrane and vesicle transfer across the syncytiotrophoblast cytosol may be facilitated by specific components of the cytoskeleton.

Flow-Limited and Diffusion-Limited Transfer

Placental transfer can be limited by the diffusion across the placental barrier (“diffusion-limited” transport) and/or by the rate of uteroplacental and umbilical blood flows (“flow-limited” transport). For example, the transfer of nutrients, which in most cases is mediated by transporters, binding proteins and/or receptors expressed in the syncytiotrophoblast plasma membranes or cytosol, is predominantly diffusion-limited. In contrast, placental transfer of oxygen is blood flow limited because it is a small molecule with high lipid solubility, which rapidly diffuses across the syncytiotrophoblast and fetal capillary endothelium. This is the reason why short-term reductions in placental blood flows affect the transfer of oxygen more than nutrient transport.

Factors Determining the Rate of Placental Transfer

The driving forces and the properties of the placental barrier are the two primary factors governing the rate of placental transfer. The relative importance of these two factors in determining transfer rate is largely dependent on the molecule in question. This section will provide a general discussion of these factors.

The main driving forces for nonmediated transfer and for facilitated diffusion are differences in concentrations and electrical potential, albeit potential differences affect only charged molecules. In addition, osmotic and hydrostatic pressure differences constitute the driving forces governing the movement of water across the placental barrier. Bulk water through transtrophoblastic channels will also carry solutes. Finally, hydrolysis of ATP is the driving force for active transport.

The transplacental potential difference is generated by active ion transport. In contrast to many animals, the maternal-fetal potential difference in the human is small, -2.7 mV (fetal negative) in mid-gestation and close to zero at term (Mellor et al., 1969). However, this potential difference is measured across the entire placental barrier and larger potential differences across individual components of the barrier constitutes powerful driving forces for charged molecules. For example, the *in vitro* potential difference across the MVM has been reported to be -32 mV in early first trimester and -21 mV at term (Birdsey et al., 1997).

The barrier properties that influence the rate of placental transfer are illustrated in [Fig. 1](#). The available effective exchange surface area is a key determinant of net transfer. Changes in exchange area can be due to structural factors, which may alter the total area available for exchange, or impaired blood flow in the umbilical or uteroplacental circulation removing a section of the placental barrier from effective exchange. Lipid composition, membrane fluidity and other passive characteristics of the MVM (#2 in [Fig. 1](#)) and BM (#5) are factors of particular importance influencing nonmediated transport. The expression and activity of transporters (e.g., amino acid transporters), channels, enzymes (for example MVM lipoprotein lipase) and receptors (e.g., the Fc receptor in MVM, mediating uptake of IgG) in the two polarized plasma membranes of the syncytiotrophoblast are critical for mediated transport. Indeed, for most solutes the transfer across the syncytiotrophoblast MVM and/or BM are the rate-limiting and actively regulated step in maternal-fetal exchange. The mediated transfer of some molecules, including fatty acids and calcium, requires the presence of a cytosolic binding protein (#3). Not all solutes transferred across the placenta are inert but are subjected to metabolism, including glucose and amino acids, in the syncytiotrophoblast (#4), which represent an additional property of the placental barrier that influences the overall transfer rate. Molecules transferred across the BM must move across the basement membrane, villous core and capillary endothelium (#7) to reach fetal blood in the fetal placental capillaries.

Placental Oxygen Transfer

Oxygen is critical for oxidative metabolism in the placenta and fetus, generating ATP for energy requiring processes such as active transport and protein synthesis. In addition, oxygen availability regulates placental growth, trophoblast gene expression, and invasion. Fetal hypoxemia in, for example, the sheep ([Jacobs et al., 1988](#)) is associated with fetal growth restriction, however the cause-and-effect relationship between limitation in oxygen supply and reduced fetal growth is unclear because interventions resulting in fetal hypoxemia alter a multitude of other factors that could be the cause of the growth restriction. However, more conclusive evidence for a mechanistic link between hypoxia and restricted fetal growth has been obtained in the chick embryo, a model in which the effects of changes in oxygenation on the fetus can be investigated in isolation ([Giussani et al., 2007](#)).

Although direct evidence in the human is lacking, placental oxygen transfer is believed to be limited by blood flows rather than by diffusion across the barrier. This model is primarily based on studies in the sheep (reviewed in [Carter, 1989](#)) demonstrating that there is a linear correlation between placental blood flow and fetal oxygen delivery following acute reductions in either umbilical or uterine blood flow. It is notable, however, that despite the decrease in oxygen delivery fetal oxygen consumption was maintained by increased umbilical oxygen extraction. This homeostatic mechanism is highly beneficial in response to physiological short-term reductions in uterine blood flow, which occur in labor and during physical exercise.

In response to chronic reductions in uterine or umbilical blood flows, however, fetal oxygen consumption is decreased and fetal growth is restricted.

Because of the flow limitation of oxygen transfer, the geometry of placental blood flows affects the efficiency of oxygen transport across the placental barrier (reviewed in [Faber and Thornburg, 1983](#)). The possible types of geometric arrangement of blood flows on either side of the placental barrier fall into a number of partly overlapping categories or models, which may explain differences in oxygen transfer efficiencies between species. The counter-current exchanger, in which blood in the fetal and maternal circulations flow in opposite directions in vessels running parallel to each other, represents the most efficient geometric arrangement. The concurrent exchanger, where fetal and maternal blood flows in the same direction in vessels that are arranged in parallel, is less efficient than the counter-current arrangement. The human placenta has a “multivillous” geometry of placental blood flows, with efficiency in between the counter-current and the concurrent model.

With maternal arterial pO_2 at approximately 100 mmHg and pO_2 in the umbilical vein in the range of 34–41 mmHg at the end of pregnancy, there is a large gradient facilitating maternal-fetal oxygen transfer. A higher hemoglobin concentrations in fetal blood, resulting in a significantly higher oxygen carrying capacity, and a higher affinity of fetal (HbF) hemoglobin for oxygen as compared to maternal hemoglobin (HbA) constitute other factors promoting the movement of oxygen from the maternal to the fetal circulation.

In vascular beds such as in the brain and kidney there is extensive auto-regulation and muscle is characterized by reactive hyperemia, which are processes that adapt blood flow in response to changes in either oxygen delivery or demand. Because the placental vasculature does not exhibit auto-regulation or reactive hyperemia, short-term regulation of placental oxygen transfer is largely limited to changes in fetal oxygen extraction as discussed above. As an important example of long-term regulation of placental oxygen transfer, fetal polycythemia (increased hematocrit) develops in response to chronic hypoxemia.

Placental Transfer and the Maintenance of Fetal Acid–Base Balance

For maintenance of its acid–base balance the fetus is entirely dependent on placental transfer of carbon dioxide, protons, and lactate ([Fig. 2](#)).

Carbon dioxide

Fetal and placental oxidative metabolism results in the generation of large amounts of carbon dioxide, characterized as a “respiratory acid” because it is in equilibrium with carbonic acid that can rapidly produce protons. CO_2 is eliminated across the placental barrier predominantly via diffusion of dissolved gas, which is rapid given the high lipid solubility of carbon dioxide. One of the characteristic maternal physiological adaptations to pregnancy is hyperventilation and as a result arterial pCO_2 is lower in pregnancy (between 26 and 34 mmHg) as compared to the nonpregnant woman (38–42 mmHg). With umbilical vein pCO_2 at term in

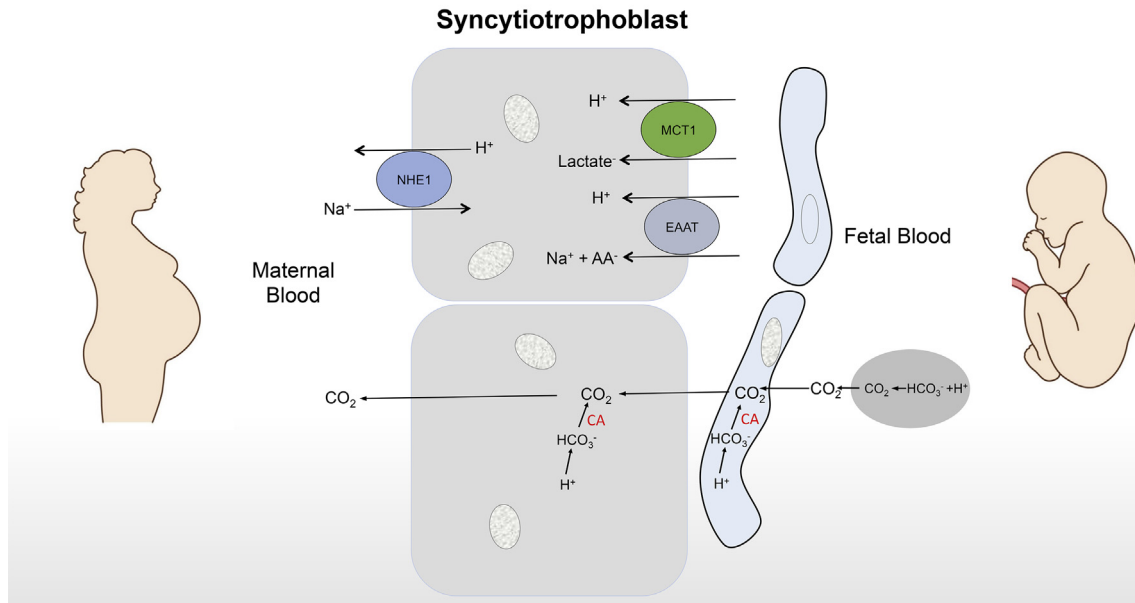


Fig. 2 Placental transport of carbon dioxide, protons, and lactate. Carbon dioxide rapidly diffuses across the placental barrier as dissolved gas. Metabolically produced protons are eliminated across the placenta, mediated by MCT1 H^+ /lactate co-transport in the BM, followed by extrusion of protons in exchange for sodium across the MVM and mediated by the sodium-proton exchanger (NHE). Transporters for excitatory amino acid transporters (EAAT), which co-transport Na^+ , H^+ and anionic amino acids, may contribute to the uptake of protons across the BM. CA, carbonic anhydrase.

the range of 38 and 45 mmHg, there is a substantial diffusion gradient across the placental barrier promoting fetal-maternal CO_2 transfer. CO_2 is predominantly present as H^+ and bicarbonate ions in blood and is converted at the placental barrier to CO_2 , catalyzed by the action of carbonic anhydrase expressed in erythrocytes, endothelial and trophoblast cells (Muhlhauser et al., 1994). A respiratory acidosis develops in response to acute reductions in uteroplacental and/or umbilical blood flows, however this acid-base balance disturbance is rapidly normalized following re-establishment of adequate blood flows.

Protons

A net production of protons by fetal metabolism cannot be eliminated by transport of CO_2 across the placental barrier but require transfer of protons from the fetal to the maternal circulation or movement of bicarbonate from the mother to the fetus. The latter process is unlikely to be important given that transport mechanisms mediating bicarbonate transfer from the maternal to the fetal circulation has yet to be identified.

The human sodium-proton exchanger (NHE, SLC9) gene family is comprised of nine transporter isoforms, NHE1–9, most of which mediate sodium-dependent transport of protons out of the cell. NHE1 is the predominant isoform in the MVM, which, with some contribution from NHE 2 and 3, exports protons from the cytosol of the syncytium into maternal blood in the intervillous space (Fig. 2), thereby playing a critical role in the maintenance of syncytiotrophoblast pH (Powell and Illsley, 1996). Placental NHE activity is regulated by a wide range of factors (Sibley et al., 2002), including epidermal growth factor, sphingosine-1-phosphate, aldosterone and cortisol, which all have been reported to activate the transporter in term villous fragments.

Lactate levels are significantly higher in the fetal circulation than in maternal blood and lactate is used as a source of energy by the fetal heart, brain, and skeletal muscle. The placenta serves both as a net producer of lactate under normoxia and as a site of clearance of fetal lactate when produced in excess during fetal hypoxia. Members of the monocarboxylate transporter (MCT) family (SLC19) represent the main transporters of lactate by mediating H^+ /lactate co-transport. MCT4 is highly expressed in the MVM, whereas MCT1 is believed to be the main lactate transporter in the BM.

Fig. 2 provides a simplified model of the current understanding of the main mechanisms involved in placental regulation of fetal and placental pH. CO_2 readily diffuses across the placental barrier as dissolved gas. Metabolically produced protons are transported across the BM by the H^+ /lactate co-transporter MCT 1 and subsequently transferred across the MVM mediated by NHE1. Excitatory amino acid transporters (EAAT), mediating the cellular uptake of anionic amino acids coupled to the co-transport of Na^+ and H^+ may also contribute to the transport of protons from the fetal compartment into the syncytiotrophoblast.

Placental Transfer of Sodium, Potassium, and Chloride

Sodium is the predominant extracellular cation, a main contributor to crystalloid osmotic pressure and an important determinant of extracellular volume. In classical epithelia, such as in the kidney, sodium enters the cell passively across the apical plasma

membrane by a wide range of mechanisms, followed by active transport across the basolateral membrane mediated by $\text{Na}^+\text{K}^+\text{-ATPase}$. Although syncytiotrophoblast sodium handling is organized according to the same general principles, the placental transporting epithelium also has unique properties with respect to sodium transport.

Na^+ -dependent co-transporters expressed in the MVM utilize the inwardly directed sodium gradient to drive the uptake of, for example, amino acids, phosphate, biotin, and succinate from the intervillous space into the syncytiotrophoblast or the efflux of protons via exchange mechanisms. Low syncytiotrophoblast intracellular sodium concentrations are maintained by $\text{Na}^+\text{K}^+\text{-ATPase}$, which, in contrast to the transporting epithelium in the kidney and intestine, is expressed and active in both BM (Whitsett and Wallick, 1980; Johansson et al., 2000) and MVM (Johansson et al., 2000) of the human syncytiotrophoblast.

Potassium, the predominant intracellular cation, is actively transported into the cell mediated by $\text{Na}^+\text{K}^+\text{-ATPase}$. Energized by the large outwardly directed gradient, potassium exit the syncytium passively through an array of different potassium channels present in the MVM and BM. The mechanisms for transplacental K^+ transport remain elusive because the identity of the channels participating in K^+ transfer across the placenta, the polarization of potassium channels to the MVM and BM and which are primarily involved in cellular potassium homeostasis are largely unknown.

Chloride is the most abundant extracellular anion, which is transferred across plasma membranes mediated by multiple mechanisms including co-transport with Na^+ (uptake) or K^+ (efflux), exchange with bicarbonate or through channels. The anion exchanger 1 (AE1, SLC4A1) is expressed and active in the MVM throughout gestation but not in the BM. Additional chloride transporters that have been shown to be active in the trophoblast include a high conductance (maxi) chloride channel, intermediate DPC-inhibitable, small PKA/ATP/cAMP activated chloride channels, CL15, CFTR and MDR1 P-glycoprotein. However, the physiological role of these chloride transporters and channels remains elusive and how they mediate transcellular chloride transport is not well understood. Because the concentration and electrical gradients favor passive chloride movement in the fetal-maternal direction, net transplacental chloride transport cannot be mediated by passive transport.

Water Transport

It has been estimated that the human fetus accumulates 22 mL of water per day close to term and because only around 20% of this can be accounted for metabolically produced water by the fetus; most of the water must be transferred from the maternal circulation (Stulc, 1997). The driving forces and pathways for net water movement from the mother to the fetus remain to be fully established. However, reported values for pressures in the umbilical vein and intervillous space demonstrate that hydrostatic pressure differences across the human placenta in vivo promote transfer of water in the fetal-maternal direction. Moreover, the colloidal osmotic pressure is slightly higher in the maternal than in the fetal circulation, establishing another driving force moving water towards the mother. As a consequence, net transplacental movement of water must be driven by an osmotic gradient of solutes.

Intriguingly, investigators have not been able to demonstrate such a gradient in vivo. Studies demonstrating significant water permeability of the syncytiotrophoblast plasma membranes (Ilsley and Verkman, 1986; Jansson and Ilsley, 1993) provide one possible explanation because this suggests that a net water accumulation of 15–20 mL/day could easily be accounted for by a solute gradient across the placental barrier small enough to be within the margin of error of standard osmolarity measurements (Jansson and Ilsley, 1993). In line with a model that has been proposed for the rat placenta (Stulc, 1997), available data suggests that actively transported solutes into the fetal side of the human placental barrier generate a small osmotic gradient, which promotes water movement via a transcellular pathway. Most of this water then returns to the maternal circulation via transtrophoblastic channels driven by the hydrostatic and colloid osmotic pressure gradients.

Cells expressing water channels, or aquaporins (AQP), in the plasma membranes have a water permeability that is 2–50 fold higher than cells lacking water channels. There are at least 13 mammalian AQP isoforms, some of which also function as glycerol transporters, including AQP3, 7, and 9. Moreover, AQP9 transports amino acids and sugars in addition to water and AQP8 also mediates the transport of ammonium. Although AQP3, 8, and 9 are expressed at the RNA and protein level in the human syncytiotrophoblast, the functional role of these aquaporins in the syncytiotrophoblast remains to be determined because efforts to demonstrate AQP mediated water permeation across isolated syncytiotrophoblast MVM and BM have been unsuccessful (Jansson and Ilsley, 1993). It has been proposed that placental aquaporins are important for solute transport because all three aquaporins expressed in the syncytiotrophoblast either transport glycerol (AQP3 and 9) or ammonium (AQP8) in addition to water and (Damiano et al., 2001).

Calcium Transport

Total accretion of calcium in the human fetus is 30 g across gestation, the majority occurs in the third trimester when fetal bone mineralization occurs. As in other cells, calcium is a critical intracellular messenger in the syncytiotrophoblast, which necessitates that cytosolic calcium concentrations are maintained at levels that are four magnitudes lower than extracellular concentrations. The transfer of calcium across the syncytiotrophoblast takes place in three distinct steps (reviewed in Belkacemi et al., 2005). Following Ca^{2+} entry into the cell across MVM, calcium moves through the cytosol associated with calcium binding proteins and subsequently Ca^{2+} ATPase mediates the active transport of the ion across BM.

Members of the transient receptor potential (TRP) gene family, in particular TRPV5 and 6, are calcium-selective channels functioning as apical calcium entry mechanisms in transporting epithelia. TRPV6 is highly expressed in the human placenta and functional in cultured primary human trophoblast (PHT) cells (Moreau et al., 2002). Store operated channels (SOC), which are

calcium channels in the plasma membrane activated by depletion of intracellular calcium stores, may serve as an additional type of MVM entry mechanism because SOCs are expressed and functional in syncytiotrophoblast of term villous fragments (Clarson et al., 2003). Moreover, ATP promotes calcium influx into trophoblast cells mediated by interaction with the P2 purinergic receptor family. In addition to these calcium-selective channels, a range of nonselective cation channels, such as Polycystin-2, have been reported to be expressed and active in the MVM, which also may contribute to Ca^{2+} entry into the syncytiotrophoblast.

Following calcium entry into the cytoplasm of the syncytiotrophoblast, the ion is rapidly stored in intracellular compartments or bound to calcium-binding proteins including calmodulin and calbindin. Sequestration of calcium into the endoplasmic reticulum allows for regulation of cytosolic calcium concentrations by uptake to and release from this store in response to DAG-PKC-IP3 signaling. Intracellular calcium binding proteins transport calcium through the cytoplasm while maintaining very low intracellular concentrations of free calcium. Whereas calbindin- D_{9k} has been found to be an important calcium binding protein in the rat placenta, the identity of the key calcium binding proteins in the human syncytiotrophoblast remains to be fully established.

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger and the calcium pump (or Ca^{2+} ATPase) represent the two fundamental mechanisms by which Ca^{2+} is actively transported out of the cell against a large electrochemical gradient. Whereas $\text{Na}^+/\text{Ca}^{2+}$ exchange does not play a major role in Ca^{2+} efflux from the human syncytiotrophoblast, the plasma membrane Ca^{2+} ATPase (PMCA) is highly expressed and functional in the BM. Of the four PMCA isoforms (PMCA1–4), PMCA1 and 4 are ubiquitously expressed, including in the syncytiotrophoblast basal plasma membrane (Strid and Powell, 2000). The activity of the BM calcium pump was found to increase significantly in the third trimester suggesting an increased transport capacity during the critical period of rapid mineralization of the fetal skeleton (Strid and Powell, 2000).

The mechanisms regulating placental calcium transport are only beginning to be determined. The role of parathyroid hormone (PTH), calcitonin and 1,25-dihydroxycholecalciferol (vitamin D) in the regulation placental calcium transport has been studied in animal models (reviewed in Stulc, 1997). These studies have revealed that vitamin D stimulates and calcitonin inhibits placental calcium transport in the sheep but not in rat. However detailed studies of the influence of the three main calcium-regulating hormones on calcium handling in the human placenta are largely lacking. Parathyroid hormone related peptide (PTHrP) has emerged as the primary hormonal regulator of placental calcium transport. PTHrP is synthesized by a range of fetal tissues including the parathyroid glands, as a 141 amino acid pro-hormone. Subsequently the pro-hormone is cleaved into several fragments of which a mid molecule fragment PTHrP-(38–94) is of particular importance for the regulation of placental calcium transport.

A fragment (67–86) of the PTHrP mid-molecule was reported to maintain the calcium gradient across the sheep placenta. This observation was subsequently confirmed using the highly conserved mid-region secretory fragment of PTHrP (PTHrP (38–94) amide). Moreover, homozygous deletion of the PTHrP gene in mice resulted in decreased placental transport of calcium and dissipation of the normal Ca^{2+} concentration gradient across the placental barrier (fetal Ca^{2+} concentrations > maternal). PTHrP (38–94) activates human placental Ca^{2+} ATPase (Strid et al., 2002), suggesting that fetal PTHrP regulates placental calcium transport also in pregnant women.

References

- Bajoria, R., & Contractor, S. F. (1992). Transfer of heparin across the human perfused placental lobule. *The Journal of Pharmacy and Pharmacology*, 44, 952–959.
- Belkacemi, L., Bedard, I., Simoneau, L., & Lafond, J. (2005). Calcium channels, transporters and exchangers in placenta: A review. *Cell Calcium*, 37, 1–8.
- Birdsey, T. J., Boyd, R. D. H., Sibley, C. P., & Greenwood, S. L. (1997). Microvillous membrane potential (E_m) in villi from first trimester human placenta: comparison to E_m at term. *The American Journal of Physiology*, 273, R1519–R1528.
- Brownbill, P. D., et al. (1995). Mechanisms of alpha-fetoprotein transfer in the perfused human placental cotyledon from uncomplicated pregnancy. *The Journal of Clinical Investigation*, 96, 2220–2226.
- Carter, A. M. (1989). Factors affecting gas transfer across the placenta and the oxygen supply to the fetus. *Journal of Developmental Physiology*, 12(6), 305–322.
- Clarson, L. H., Roberts, V. H., Hamark, B., Elliott, A. C., & Powell, T. L. (2003). Store-operated Ca^{2+} entry in the first trimester and term human placenta. *The Journal of Physiology*, 550, 515–528.
- Damiano, A., Zotta, E., Goldstein, J., Reisin, I., & Ibarra, C. (2001). Water channel proteins AQP3 and AQP9 are present in the syncytiotrophoblast of human term placenta. *Placenta*, 22, 776–781.
- Faber, J. J., & Thornburg, K. L. (1983). Placental physiology: *Structure and function of fetomaternal exchange*. New York: Raven Press.
- Fuchs, R., & Ellinger, I. (2004). Endocytic and transcytotic processes in villous syncytiotrophoblast: Role in nutrient transport to the human fetus. *Traffic*, 5, 725–738.
- Giussani, D. A., Salinas, C. E., Villena, M., & Blanco, C. E. (2007). The role of oxygen in prenatal growth: Studies in the chick embryo. *The Journal of Physiology*, 585(Pt 3), 911–917.
- Illsley, N. P., & Verkman, A. S. (1986). Serial permeability barriers to water transport in human placental vesicles. *The Journal of Membrane Biology*, 94, 267–278.
- Jacobs, R., Robinson, J. S., Owens, J. A., Falconer, J., & Webster, M. E. D. (1988). The effect of prolonged hypobaric hypoxia on growth of fetal sheep. *Journal of Developmental Physiology*, 10, 97–112.
- Jansson, T., & Illsley, N. P. (1993). Osmotic water permeabilities of human placental microvillous and basal membranes. *The Journal of Membrane Biology*, 132, 147–155.
- Johansson, M., Jansson, T., & Powell, T. L. (2000). Na^+/K^+ -ATPase is distributed to microvillous and basal membrane of the syncytiotrophoblast in the human placenta. *The American Journal of Physiology*, 279, R287–R294.
- Mellor, D. J., Cockburn, F., Lees, M. M., & Blagden, A. (1969). Distribution of ions and electrical potential differences between mother and fetus in the human at term. *The Journal of Obstetrics and Gynaecology of the British Commonwealth*, 76, 993–998.
- Moreau, R., et al. (2002). Calcium uptake and calcium transporter expression by trophoblast cells from human term placenta. *Biochimica et Biophysica Acta*, 1564(2), 325–332.
- Muhlhauser, J., et al. (1994). Immunohistochemistry of carbonic anhydrase in human placenta and fetal membranes. *Histochemistry*, 101, 91–98.
- Powell, T. L., & Illsley, N. P. (1996). A novel technique for studying cellular function in human placenta: Gestational changes in intracellular pH regulation. *Placenta*, 17, 661–668.

- Sibley, C. P., et al. (2002). Regulation of placental transfer: The Na^+/H^+ exchanger—A Review. *Placenta*, (Supplement A), S39–S46.
- Strid, H., Care, A., Jansson, T., & Powell, T. (2002). Parathyroid hormone-related peptide (38-94) amide stimulates ATP-dependent calcium transport in the basal plasma membrane of the human syncytiotrophoblast. *The Journal of Endocrinology*, 175, 517–524.
- Strid, H., & Powell, T. L. (2000). ATP-dependent Ca^{2+} transport is upregulated during third trimester in human syncytiotrophoblast basal membranes. *Pediatric Research*, 48, 58–63.
- Stulc, J. (1997). Placental transfer of inorganic ions and water. *Physiological Reviews*, 77, 805–836.
- Syme, M. R., Paxton, J. W., & Keelan, J. A. (2004). Drug transfer and metabolism by the human placenta. *Clinical Pharmacokinetics*, 43, 487–514.
- Whitsett, J., & Wallick, E. (1980). ^3H -ouabain binding and Na^+/K^+ ATPase activity in human placenta. *The American Journal of Physiology*, 238, E38–E45.

Placental Nutrient Transport

Laura B James-Allan, Theresa Powell, and Thomas Jansson, University of Colorado, Aurora, CO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The placenta constitutes the primary interface between the mother and fetus with a multitude of functions, including nutrient and respiratory gas transport, waste removal, providing an immunological barrier, as well as acting as an endocrine organ. The placenta produces and secretes a wide range of hormones, cytokines, and growth factors. The syncytiotrophoblast (ST) functions both as a barrier and the transporting epithelium of the placenta mediating vectorial transport of a wide range of molecules. At term, the fetal and maternal circulations are separated by two cell layers: the ST and the fetal capillary endothelium. Fetal placental capillaries are nonfenestrated and are linked to neighboring endothelial cells (EtC) by junctional complexes, allowing relatively unrestricted intercellular passage of small molecules such as glucose and amino acids, but restricting transfer of larger molecules, including immunoglobulins. These larger molecules must be transported across the EtC via vesicular transport. Thus, ST acts as the primary barrier between the maternal and fetal circulations, thereby controlling placental transfer of small solutes. The ST is made up of a multinucleated continuous layer of specialized epithelial cells that line the placental villous tree vascular structure. The ST consists of two polarized plasma membranes: the fetal facing basal plasma membrane (BM) adjacent to the fetal capillaries (FC) and the maternal facing apical brush border or microvillus plasma membrane (MVM) which is in direct contact with maternal blood (Fig. 1). The MVM has a large surface area to allow for rapid transport of nutrients from the maternal circulation in to the ST, nutrients are subsequently transported across the BM in order to reach the fetus.

A wide array of transporters are expressed in the plasma membranes of the ST and the polarized localization of transporters with differing characteristics to the two membrane surfaces allows for vectorial transport across the barrier. Placental nutrient transport is a critical determinant of fetal growth. Fetal overgrowth and intra-uterine growth restriction (IUGR) are two important pregnancy complications associated with an increased perinatal morbidity and mortality, as well as an increased risk of developing cardiovascular and metabolic disease later in life. Both of these conditions represent pathological fetal growth and are associated with distinct changes in placental nutrient transport. In IUGR, a range of placental nutrient and ion transporters are downregulated, whereas fetal overgrowth is associated with an increased placental nutrient transport capacity. These observations in women, together with cause-and-effect experiments in animal models, have led the suggestion that changes in placental nutrient transport directly contribute to or are causative in the development of IUGR and fetal overgrowth. Thus, unraveling the mechanisms and regulation of placental nutrient transport is critical for our understanding of the pathophysiology of important pregnancy complications and may provide new fundamental knowledge that can be used to develop novel intervention strategies.

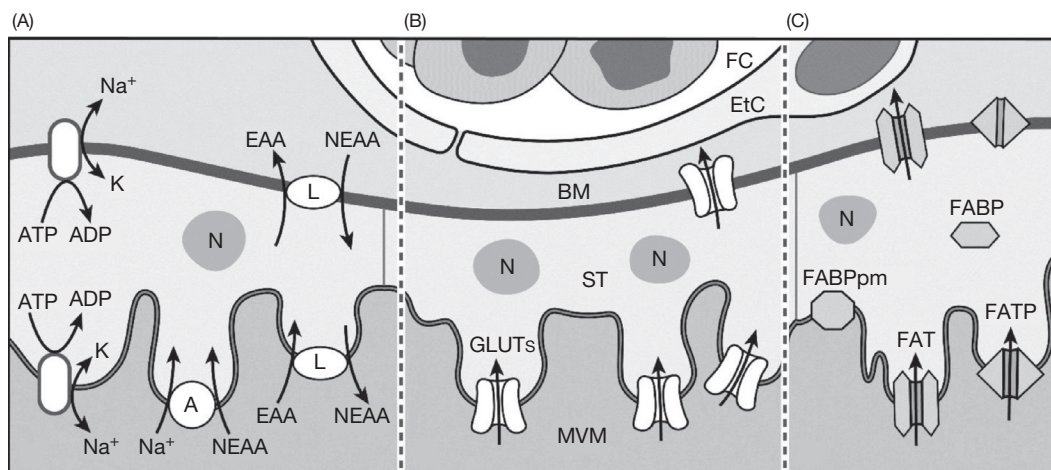


Fig. 1 The syncytiotrophoblast (ST) constitutes the transporting epithelium in the human placenta and is characterized by the fetal facing basal plasma membrane (BM) adjacent to the fetal capillaries (FC) and the maternal facing microvillus plasma membrane (MVM). Fetal placental capillaries allow unrestricted movement of small molecules through junctional complexes in endothelial cells (EtC). (A) Amino acids are delivered to the fetus by membrane bound transporters, such as system A which is predominantly found on the MVM plasma membrane and transports neutral amino acids, and system L transporters, which exchanges essential for nonessential amino acids on both the MVM and BM. (B) Glucose is transported from maternal blood to the fetus by GLUT transporters on the MVM and BM, with greater expression on the maternally facing MVM. (C) Fatty acids from the maternal circulation are transported in to the ST by fatty acid transporters (FAT), fatty acid transporter proteins (FATP), and placental specific plasma membrane fatty acid binding protein (FABPpm). Fatty acid binding proteins (FABP) are localized to the ST cytosol, with FAT and FATP on the BM transporting fatty acids from the ST to the fetus.

Amino Acids

Amino acids are essential for the growth of the fetus because they are required for protein synthesis, energy production, oxidative metabolism, and biosynthetic pathways. Amino acids are delivered to the fetus through transporters on both ST membranes of the placenta (Fig. 1A). Amino acid concentrations are higher in the fetal compartment compared to maternal plasma, indicating that amino acids are actively transported from maternal blood to the fetus via the placenta. The amino acid transport process utilizes energy and there are two energy dependent transporters, accumulative transporters and exchangers, as well as facilitated efflux transporters that do not require energy. Accumulative transporters mediate the uptake of amino acids against their concentration gradients, often energized by the co-transport of extracellular sodium. These transporters establish high intracellular concentrations of nonessential amino acids and efflux of these amino acids provides a driving force for uptake of essential amino acids from the maternal circulation by exchange transporters. Accumulative transporters are predominantly found in the MVM of the ST, however some are also present in the BM, but to a lesser extent. Amino acid exchangers transport one amino acid across the ST in exchange for another amino acid. This changes the composition of the amino acid pool without altering the total concentration. Exchangers are found on both the MVM and BM membranes, and are specific for amino acids that are not transported by accumulative or facilitative transporters. Facilitated transporters are primarily located on the BM, where they allow transport of specific amino acids down their concentration gradient, therefore typically mediating amino acid efflux into the fetal circulation.

Amino acid transporters can be categorized using a variety of characteristics, including substrate specificity and dependence on, for example, pH and charge and the presence of sodium and chloride. More than 20 different amino acid transporter systems have been described in the human placenta, some of which are listed in Table 1. The most well studied amino acid transporters found in the placenta are system A and system L.

System A

The system A transporter is a Na^+ dependent accumulative transporter, transporting small zwitterionic, nonessential neutral amino acids, including alanine, glycine, and serine. The system A transporter is made up of three homologous isoforms, SNAT1, SNAT2, and SNAT4, which are encoded by the *SLC38* gene family, *SLC38A1*, *SLC38A2*, and *SLC38A4*, respectively. SNAT1 and SNAT2 are functionally similar, whereas SNAT4 has a lower affinity for neutral amino acids. All three isoforms are expressed in the plasma membranes of the ST and are highly polarized to the MVM. The expression of these isoforms differs throughout gestation; SNAT4 is highly expressed during the first trimester, whereas SNAT1 plays a more important role in system A activity in the term placenta (Desforges et al., 2009, 2010). MVM system A activity increases between the first trimester and term, matching the increasing needs of the growing fetus (Mahendran et al., 1994). System A activity leads to high intracellular concentration of nonessential amino acids, which are exchanged for extracellular essential amino acids via system L. Therefore, system A is critical for the transport of both nonessential and essential amino acids.

Activation of mechanistic target of rapamycin (mTOR) promotes system A activity by regulation at the post translational level, involving increased plasma membrane trafficking of the SNAT2 isoform (Rosario et al., 2013). In addition, a range of hormones and growth factors, such as IGF-I, insulin, and leptin, which are upstream regulators of mTOR, are positive regulators of system A (Jansson et al., 2003; Jones et al., 2009; Kniss et al., 1994). Conversely, system A is inhibited by a variety of factors, such as hypoxia, IL-1 β , and growth hormone (Ericsson et al., 2005; Nelson et al., 2003; Thongsong et al., 2005).

Table 1 Key amino acid transport systems in the human term placenta.

System	Characteristics	Preferred substrates	Distribution
<i>Na^+-dependent</i>			
System A	Transports small, zwitterionic, nonessential amino acids. Accumulative transporter	Glycine, serine, alanine, cysteine, glutamine, histidine, asparagine, MeAIB	Microvillus plasma membrane (MVM) Basal plasma membrane (BM) (low abundance)
System ASC	Exchanger	Alanine, serine, cysteine, threonine	BM
System β	Specific for β -amino acids Chloride dependent	B-alanine, taurine	MVM
System X_{AG}^-	Accumulative transporter K^+ -dependent	Glycine, aspartate, cysteine	MVM BM
<i>Na^+-independent</i>			
System L	Transports nonessential branched chain and aromatic amino acids Exchanger transporter	Leucine, phenylalanine, histidine, methionine, leucine, valine, isoleucine	MVM BM
System y^+	Specific for cationic amino acids Uniporter	Arginine, lysine, histidine	MVM BM
System $\text{y}^+ \text{L}$	Transports cationic amino acids Exchanger transporter	Lysine, arginine, histidine, methionine, leucine	MVM BM

System L

System L is a Na^+ —independent transporter of essential amino acids, including L-leucine and L-phenylalanine. System L activity has been demonstrated in both the MVM and BM of the ST, exchanging nonessential amino acids for predominantly essential amino acids. This transporter is a homodimer, made up of a light chain protein, L-type Amino acid Transporter, LAT1 (*SLC7A5*) or LAT2 (*SLC7A8*), covalently linked to a heavy chain transmembrane protein, 4F2hc/CD98 (*SLC3A2*). Protein expression of LAT1 and 4F2hc increases in the placenta from mid-gestation to term (Okamoto et al., 2002). Both LAT1 and LAT2 are expressed in the ST; LAT1 is primarily found in the MVM whereas LAT2 mainly in the BM, although it can also be expressed in the MVM (Kudo and Boyd, 2001; Okamoto et al., 2002). LAT1 and LAT2 both contribute to system L activity in the term placenta, however LAT1 is primarily involved in the uptake of larger neutral amino acids on the maternally facing MVM, whereas LAT2, being prominent on the BM, is responsible for the exchange of nonessential amino acids in the fetal compartment for essential amino acids in the ST cytoplasm (Gaccioli et al., 2015).

The regulation of system L is not as well characterized, however it is known that activity is not regulated by the same factors as system A. Increased intracellular calcium, and activation of protein kinase C and mTOR signaling stimulate system L activity (Okamoto et al., 2002; Roos et al., 2007).

Glucose

Glucose is the main energy source for the placenta and the fetus. As fetal gluconeogenesis is minimal, the fetus is dependent on placental supply of glucose from the maternal circulation. Glucose transport across the ST is carried out by a family of glucose transporters (GLUT) which mediate stereo-specific, sodium-independent facilitated diffusion of glucose down a concentration gradient (Fig. 1B). The maternal circulation has a higher concentration of glucose compared to the fetus, resulting in a net maternal-fetal diffusion gradient for glucose across the ST. This allows for adequate glucose consumption by the placenta, while maintaining a gradient between the interior of the ST and the fetal capillary to allow for net transport to the fetus. GLUTs are expressed in both plasma membranes of the ST, although the expression of GLUTs is highest in the MVM. Glucose uptake across the MVM is believed to be very rapid because of the high abundance of GLUTs in combination with the large surface area of the MVM. The BM, with its lower abundance of GLUT and smaller surface area, is thought to be the rate limiting step in transplacental glucose transport at term.

GLUT1 (*SLC2A1*), the ubiquitous isoform of the glucose transporter family, is the primary glucose transporter in the human placenta at term. Its expression increases over gestation and is present in both ST plasma membranes, with a threefold higher expression in the MVM than BM at term (Jansson et al., 1993). GLUT3 (*SLC2A3*) is highly expressed in the ST in early pregnancy and decreases across gestation (Brown et al., 2011). Corticotropin-releasing hormone (CRH) secreted locally by the ST modulates GLUT1 and GLUT3; CRH levels positively correlate with increasing gestation and therefore are thought to upregulate the expression of GLUT1 and downregulate GLUT3 expression. The insulin-sensitive glucose transporters GLUT4 (*SLC2A4*) and GLUT12 (*SLC2A12*) have been detected in the ST in early gestation placenta, with decreasing expression in late gestation (Ericsson et al., 2005; Gude et al., 2003). Insulin receptors are believed to be more highly expressed in the ST in early gestation compared to term, which correlates with the expression of both GLUT4 and GLUT12 expression (Desoye et al., 1994). This suggests that insulin may be driving the recruitment and/or activation of GLUT4 and GLUT12 transporters during early gestation and therefore playing a primary role in placental glucose uptake in the first trimester of pregnancy. The placenta also expresses GLUT8 (*SLC2A8*), GLUT9 (*SLC2A9*), and GLUT10 (*SLC2A10*) (Bibee et al., 2011; Dawson et al., 2001; Doege et al., 2000). The placenta at term expresses both splice variants of GLUT9, with GLUT9a found on the BM and GLUT9b primarily on the MVM (Bibee et al., 2011). GLUT9 is known to transport fructose as well as glucose, therefore GLUT9 in the placenta may function to transport fructose as well as glucose. However, the functional role of GLUT 8–10 in term placental remains to be established.

Fatty Acids

Fatty acids are essential for the growth of the fetus and development of specific organs and tissues. An ample fetal supply of essential fatty acids (EFA) and long chain polyunsaturated fatty acids (LC-PUFA) are particularly important in the third trimester, when the fetal demand for nutrients is at its peak. Fatty acid concentrations in the maternal plasma increase during pregnancy due to physiological hyperlipidaemia. EFA, such as linoleic acid (LA, 18:2n-6) and α -linolenic acid (ALA, 18:3n-3) are only obtained from the diet. Placental transfer of PUFAs, mainly arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), to the fetus in the third trimester is critical for normal development of the brain and retina. This is because the fetus is able to synthesize saturated and monounsaturated fatty acids from glucose and ketone bodies, however, neither the placenta nor the fetus can produce EFA or the n-6 and n-3 LCPUFA derivatives, which must be derived from the maternal circulation. The placenta preferentially transfers LC-PUFAs versus other types of fatty acids; this selective LC-PUFA transfer to the fetal circulation is termed “biomagnification.” Fatty acids that are transported across the placenta originate from the maternal circulation, either as nonesterified fatty acids (NEFAs) or esterified fatty acids in triglycerides (TGs) that are carried by lipoproteins, with subsequent metabolism within the placenta.

Triglycerides in maternal lipoproteins are made available for placental transfer via hydrolysis into NEFAs. This is an enzymatic process that requires lipase enzymes. There are two main lipases found in the placenta: lipoprotein lipase (LPL) and endothelial lipase (EL) which are both capable of hydrolysing lipoprotein TGs and phospholipids. LPL and EL are found on both ST plasma membranes, with LPL localized primarily to the MVM and EL also present in the luminal plasma membrane of fetal capillary EtC. LPL primarily hydrolyses TGs, whereas EL predominantly hydrolyses phospholipids, with a small amount of TG activity. LPL activity increases throughout gestation, with higher activity at term compared to the first trimester. Placental LPL activity is regulated by hormones such as estradiol, insulin, IGF-1, and cortisol, however activity is not regulated by pro-inflammatory cytokines, as in other tissues (Magnusson-Olsson et al., 2006). EL expression changes during pregnancy; term placenta expresses higher levels of EL than in the first trimester. Once taken up NEFAs can be re-esterified and deposited as TGs or phospholipids in the cytosol of the ST and then hydrolysed at a later time to be released to the fetal circulation.

Fatty acids can be transported to the fetus via simple diffusion or by facilitated diffusion mediated by transport proteins in the ST of the placenta. A range of proteins implicated in fatty acid transport, including fatty acid translocase (FAT)/CD36, placental specific plasma membrane FA binding protein (pFABPpm), fatty acid transport proteins (FATP), and intracellular fatty acid binding proteins (FABP) are expressed in the ST (Fig. 1C). FATP are a family of integral membrane proteins consisting of six related members, of which five are present in the human placenta. They are believed to be important for the cellular uptake of long-chain fatty acids. FATP1–4 (*SCL27A1–4*) and FATP6 (*SLC27A6*) are expressed in the human placenta, with FATP2, FATP4, and FATP6 being more highly expressed at the mRNA level. FATP1 was first identified in the human placenta, and is expressed in both the MVM and BM. The mRNA expression of FATP1 and FATP4 correlates with levels of maternal DHA (Larque et al., 2006). The especially strong positive correlation of FATP4 with fetal DHA phospholipid levels suggests that FATP4 has an important role in the transfer of LC-PUFAs.

pFABPpm is the only placenta specific fatty acid transporter and is exclusively expressed on the MVM of the ST. Essential FA and LC-PUFA preferentially bind to FABPpm, consistent with the possibility that this transporter is involved in transfer of these fatty acids across the placenta (Campbell et al., 1997). FAT is a glycosylated protein that has been identified on both placental MVM and BM membranes (Campbell et al., 1998). It has multiple ligands, including FFA, collagen, and oxidized low-density lipoproteins. Little is known regarding its function in the placenta, however it is believed to be involved in placental FA transfer.

FABPs are a family of proteins that are expressed in a diverse range of tissues; FABP1, FABP3, FABP4, and FABP5 have all been shown to be expressed, at the mRNA and protein level, in the human placenta at term (Biron-Shental et al., 2007; Campbell et al., 1998). FABP are found within the cytosol of the ST where they traffic fatty acids to sites within the cell for esterification, beta-oxidation or transport to the fetus. Hypoxia enhances the expression of FABP1, 3, and 4, suggesting that FABP supports the transfer of fatty acids in hypoxic conditions. PPAR γ increases the expression of FABP1 and FABP4 (Biron-Shental et al., 2007).

Placental Transport and Fetal Growth

IUGR and fetal overgrowth are both associated with higher perinatal morbidity and mortality as well as an increased risk of cardiovascular and metabolic disease later on in life. When the maternal supply line is unable to deliver adequate amounts of nutrients and oxygen to the placenta, which includes conditions such as compromised utero-placental blood flow, high altitude hypoxia and maternal undernutrition, hormonal (such as low insulin, leptin and IGF-I, and high adiponectin and cortisol) and biochemical signals (such as hypoxia) downregulate key placental functions such as nutrient transport, directly contributing to fetal growth restriction. In contrast, when the maternal supply line delivers an excessive amount of nutrients, such as in maternal obesity and diabetes, signals (including high insulin, leptin and IGF-I and low circulating levels of adiponectin) cause an upregulation of placental nutrient transport, contributing to increased fetal growth. Consequently, the placenta is key in serving as a nutrient sensor and regulating placental nutrient transporters in the ST, summarized in Table 2.

IUGR

IUGR can result from a variety of causes, including placental insufficiency and maternal undernutrition. IUGR is associated with a reduction in nutrient transport and oxygen supply to the fetus, however the mechanisms are still not fully understood. Both utero-placental and umbilical blood flows are typically reduced in IUGR, at least in severe cases, however this is unlikely to fully explain the decreased placental nutrient transfer in this condition.

Fetal nutrient availability is decreased in IUGR. For example, umbilical vein concentrations of essential amino acids are reduced in growth restricted fetuses (Cetin et al., 1992), which is believed to be due to a downregulation of placental amino acid transporters, including reduced activity of the system A amino acid transporter in the MVM in IUGR (Jansson et al., 2002). System L activity is also reduced in both the MVM and BM in IUGR, changes that are believed to contribute to a decreased placental transfer of essential amino acids in IUGR (Jansson et al., 1998). Amino acids are one of the key stimulators of the secretion of fetal insulin, which is one of the primary growth hormones in fetal life. Therefore, a reduction in fetal amino acid availability can directly lead to a decrease in fetal growth. Many IUGR fetuses are hypoglycaemic. However, this appears not to be due to changes in placental glucose transport as the activity and expression of GLUT1, the main glucose transporter in the placenta, is unaltered in MVM and BM of IUGR placentas. GLUT3 protein expression has been reported to be increased in placenta from IUGR pregnancies (Janzen et al., 2013), however the functional consequences remain to be established.

Table 2 Alterations in placental nutrient transporter expression and activity in pregnancy complications

Nutrient transporter	IUGR	Obesity	Type 1 diabetes	GDM
Amino acids				
System A	↓ Activity in MVM – Activity in BM	↑ Activity in MVM ↑ SNAT1 expression ↑ SNAT2 expression	↑ Activity in MVM – Activity in BM	↑ Activity in MVM – Activity in BM
System L	↓ Activity in MVM ↓ Activity in BM – Activity in MVM – Activity in BM	– Activity in MVM – Activity in BM – Activity in MVM – Activity in BM	– Activity in MVM – Activity in BM – Activity in MVM ↑ Activity in BM	↑ Activity in MVM – Activity in BM – Activity in MVM – Activity in BM
Glucose				
GLUT1	– Expression in MVM	– Expression in MVM	– Expression in MVM	↑ ↓ mRNA and protein expression dependent on maternal BMI and treatment
	– Expression in BM	↑ Expression in BM	↑ Expression in BM	
GLUT3	↑ Expression in MVM	↑ Expression in placental membranes		
GLUT4	↓ Expression in placental membranes	↑ Expression in placental membranes		
Fatty acids				
	↓ LPL activity in MVM	↑ FAT/CD36 mRNA expression in MVM	↑ EL expression	↑ LPL expression and activity
		↑ LPL expression and activity	↑ LPL activity	
			↑ FABP1, -4, -5 expression	↑ FABP1, -4, -5 expression
		↓ FATP4 expression ↓ FABP1 and FABP3 expression ↑ FATP2 expression in BM		

↑ Increase; ↓ Decrease; – No change.

Fatty acids are essential for the growth of the fetus as they are an important energy source and structural components of cell membranes. IUGR newborns have reduced subcutaneous fat depots, which may be due to decreased *de novo* fetal lipid synthesis and/or reduced placental transport of fatty acids. MVM LPL activity is reduced in preterm IUGR compared to preterm gestational age-matched controls (Magnusson *et al.*, 2004). This alteration may contribute to the reduced birth weight and minimal adipose tissue deposition observed in IUGR.

Obesity

The number of obese women of reproductive age has increased markedly in the past 30 years, paralleling the obesity “epidemic” in the general population. Maternal obesity affects both the placenta and the fetus, sometimes leading to fetal overgrowth and the subsequent delivery of large-for-gestational age or macrosomic (>4000 g) babies. Infants born to obese mothers have an increased risk of hyperinsulinaemia and hypoglycaemia in the neonatal period and are more likely to develop childhood obesity and metabolic disorders and obesity, cardiovascular, and metabolic disease in adult age. Animal models have demonstrated that the complications linked to maternal obesity in pregnancy are associated with dysregulation of placental nutrient transport (Gaccioli *et al.*, 2013).

There is a positive correlation between maternal BMI and infant birth weight, suggesting that the metabolic milieu in maternal obesity promotes placental nutrient transport. Birth weight has been positively correlated with umbilical vein glucose and insulin levels, as well as fetal hyperglycaemia. BM GLUT1 protein expression has been reported to be positively correlated to birth weight in maternal obesity however, there was no correlation in GLUT9 protein expression in ST plasma membranes and birth weight (Acosta *et al.*, 2015). An increase in the expression of BM GLUT1 is expected to increase glucose delivery to the fetus, thereby promoting fetal growth.

MVM System A activity has been shown to be increased in obese mothers delivering large babies (Jansson *et al.*, 2013). In addition, MVM protein expression of the System A isoform SNAT2 was positively correlated to early pregnancy BMI and birth weight. This suggests that upregulation of specific placental amino acid transporters may contribute to fetal overgrowth in obese women.

Placental expression of FAT/CD36 mRNA and protein and activity of the enzyme LPL are increased in pregnancies complicated by maternal obesity compared to pregnancies of normal weight women (Dube *et al.*, 2012). LPL hydrolyses triglyceride rich lipoproteins, making fatty acids available for uptake into the placenta across the MVM and activation of LPL may promote increased placental transfer of fatty acids. However, Dube and co-workers reported that maternal obesity without fetal overgrowth is associated with lower placental protein and mRNA expression of FATP4, FABP1, and lower FABP3 protein expression, and the reasons for this are unknown (Dube *et al.*, 2012). In contrast, Lager *et al.* reported BM FATP2 protein expression is increased in association to

maternal obesity (Lager et al., 2016). Although the precise role of placental fatty acid transporters in maternal-fetal lipid transport remains to be established, increases fatty acid transporter expression in pregnancies complicated by maternal obesity may lead to an enhanced transfer of fatty acids to the fetus.

Diabetes

The number of women with diabetes in pregnancy is increasing globally. Gestational diabetes mellitus (GDM), which makes up the majority of diabetes cases in pregnancy, and type 1 diabetes both lead to increased risk of perinatal morbidity and mortality, and are associated with a susceptibility to develop diabetes and GDM later in life. Maternal hyperglycaemia and hyperinsulinaemia are associated with fetal overgrowth and often large for gestational age babies, increasing the risk of delivery complications and birth injury. Accelerated fetal growth is believed to be caused by maternal hyperglycaemia, which results in increased fetal glucose levels, stimulating fetal insulin production. Insulin is the major growth hormone in fetal life; therefore increases in fetal insulin can lead to accelerated fetal growth. However, birth weight is not positively correlated with maternal glucose levels in type 1 diabetes or GDM, suggesting that there are other contributing factors leading to fetal overgrowth. An increased fetal availability of other nutrients than glucose, including fatty acids, and amino acids, which are potent stimulators of insulin, may promote fetal growth in pregnancies complicated by maternal diabetes.

In type 1 diabetes system A, but not system L, activity is increased in the MVM of the ST, consistent with an increased capacity of the ST to transfer amino acids to the fetus (Jansson et al., 2002). Type 1 diabetes is also associated with an increase in GLUT1 protein expression and glucose uptake in the BM (Jansson et al., 1999). Birth weight is positively correlated with GLUT1 expression in the BM from placentas of type 1 diabetics. As the BM acts as the rate-limiting step in glucose transport in the ST, an increase in expression and activity of glucose transport at this membrane could lead to an increase in the delivery of glucose to the fetus. Infants of mothers with diabetes have increased fat mass, which may be due to an increased de novo fetal lipid synthesis from glucose or be caused by an increase in lipid transport from the mother to the fetus. Consistent with the latter hypothesis, placental triglyceride concentrations, EL gene expression and LPL activity have been reported to be increased in type 1 diabetes (Lindegaard et al., 2006; Magnusson et al., 2004). Moreover, FABP protein expression in placentas from women with type 1 diabetes is also increased (Magnusson et al., 2004; Radaelli et al., 2009). This suggests that in type 1 diabetes there is an increase in lipid uptake and transport by the placenta, contributing to the increased fetal body fat mass in these infants.

The available data on placental nutrient transport in GDM is inconsistent, possibly due to the limited number of study subjects included in most studies and the lack of full consideration of a range of cofounders including the influence of diverse treatment regimens and maternal obesity. In nonobese women with insulin-controlled GDM, placental GLUT1 mRNA and protein expression was increased and GLUT4 mRNA and protein expression was decreased compared to nonobese diet controlled GDM women. In obese women with insulin-controlled GDM placental GLUT4 mRNA expression was lower than obese women with diet controlled GDM. These observations highlight that maternal BMI and the mode of treatment for GDM are associated with differences in the pathophysiology of this disease (Colomiere et al., 2009). System A activity is upregulated in the MVM in women with GDM with or without LGA babies when compared to healthy controls, whereas in healthy women with LGA babies system A activity in the MVM was unaltered. This suggests that there is an increased uptake of neutral amino acids into the ST, allowing for an increased delivery to the fetus. System L activity is also increased in the MVM in GDM pregnancies with LGA babies, whereas there is no change in system L activity in type 1 diabetes with LGA. As in type 1 diabetes, there is an upregulation of placental FABP protein expression in GDM (Magnusson et al., 2004; Radaelli et al., 2009). In term placentas from normal weight women or overweight/obese women with GDM, LPL expression, and activity are increased compared to normal controls (Dube et al., 2012). However, in a study that of insulin-treated GDM women only, LPL expression was downregulated (Radaelli et al., 2009). Dysregulation of placental lipid transport and metabolism, regardless of maternal BMI and GDM treatment, are thought to be playing a key role in the fetal fat accumulation and LGA babies that are more frequent in GDM pregnancies.

References

- Acosta, O., Ramirez, V. I., Lager, S., Gaccioli, F., Dudley, D. J., Powell, T. L., & Jansson, T. (2015). Increased glucose and placental Glut-1 in large infants of obese nondiabetic mothers. *American Journal of Obstetrics and Gynecology*, 212(227), e221–227.
- Bibee, K. P., Illsley, N. P., & Moley, K. H. (2011). Asymmetric syncytial expression of Glut9 splice variants in human term placenta and alterations in diabetic pregnancies. *Reproductive Science*, 18, 20–27.
- Biron-Shental, T., Schaiff, W. T., Ratajczak, C. K., Bildirici, I., Nelson, D. M., & Sadovsky, Y. (2007). Hypoxia regulates the expression of fatty acid-binding proteins in primary term human trophoblasts. *American Journal of Obstetrics and Gynecology*, 197, 516.e1–516.e6.
- Brown, K., Heller, D. S., Zamudio, S., & Illsley, N. P. (2011). Glucose transporter 3 (Glut3) protein expression in human placenta across gestation. *Placenta*, 32, 1041–1049.
- Campbell, F. M., Bush, P. G., Veerkamp, J. H., & Dutta-Roy, A. K. (1998). Detection and cellular localization of plasma membrane-associated and cytoplasmic fatty acid-binding proteins in human placenta. *Placenta*, 19, 409–415.
- Campbell, F. M., Clohessy, A. M., Gordon, M. J., Page, K. R., & Dutta-Roy, A. K. (1997). Uptake of long chain fatty acids by human placental Choriocarcinoma (Bewo) cells: Role of plasma membrane fatty acid-binding protein. *Journal of Lipid Research*, 38, 2558–2568.
- Cetin, I., Marconi, A. M., Corbetta, C., Lanfranchi, A., Baggiani, A. M., Battaglia, F. C., & Pardi, G. (1992). Fetal amino acids in normal pregnancies and in pregnancies complicated by intrauterine growth retardation. *Early Human Development*, 29, 183–186.
- Colomiere, M., Permezel, M., Riley, C., Desoye, G., & Lappas, M. (2009). Defective insulin signaling in placenta from pregnancies complicated by gestational diabetes mellitus. *European Journal of Endocrinology*, 160, 567–578.

- Dawson, P. A., Mychaleckyj, J. C., Fossey, S. C., Mihic, S. J., Craddock, A. L., & Bowden, D. W. (2001). Sequence and functional analysis of Glut10: A glucose transporter in the type 2 diabetes-linked region of chromosome 20q12-13.1. *Molecular Genetics and Metabolism*, 74, 186–199.
- Desforges, M., Greenwood, S. L., Glazier, J. D., Westwood, M., & Sibley, C. P. (2010). The contribution of Snat1 to system a amino acid transporter activity in human placental trophoblast. *Biochemical and Biophysical Research Communications*, 398, 130–134.
- Desforges, M., Mytett, K. J., Jones, R. L., Greenwood, S. L., Westwood, M., Sibley, C. P., & Glazier, J. D. (2009). The Snat4 isoform of the system a amino acid transporter is functional in human placental microvillous plasma membrane. *Journal of Physiology*, 587, 61–72.
- Desoye, G., Hartmann, M., Blaschitz, A., Dohr, G., Hahn, T., Kohnen, G., & Kaufmann, P. (1994). Insulin receptors in Syncytiotrophoblast and fetal endothelium of human placenta. Immunohistochemical evidence for developmental changes in distribution pattern. *Histochemistry*, 101, 277–285.
- Doege, H., Schurmann, A., Bahrenberg, G., Brauers, A., & Joost, H. G. (2000). Glut8, a novel member of the sugar transport facilitator family with glucose transport activity. *The Journal of Biological Chemistry*, 275, 16275–16280.
- Dube, E., Gravel, A., Martin, C., Desparois, G., Moussa, I., Ethier-Chiasson, M., Forest, J. C., Giguere, Y., Masse, A., & Lafond, J. (2012). Modulation of fatty acid transport and metabolism by maternal obesity in the human full-term placenta. *Biological of Reproduction*, 87(14), 1–11.
- Ericsson, A., Hamark, B., Jansson, N., Johansson, B. R., Powell, T. L., & Jansson, T. (2005). Hormonal regulation of glucose and system a amino acid transport in first trimester placental villous fragments. *American Journal of Physiology Regulatory Integrative and Comparative Physiology*, 288, R656–662.
- Ericsson, A., Hamark, B., Powell, T. L., & Jansson, T. (2005). Glucose transporter isoform 4 is expressed in the Syncytiotrophoblast of first trimester human placenta. *Human Reproduction*, 20, 521–530.
- Gaccioli, F., Aye, I. L., Roos, S., Lager, S., Ramirez, V. I., Kanai, Y., Powell, T. L., & Jansson, T. (2015). Expression and functional characterisation of system L amino acid transporters in the human term placenta. *Reproductive Biology and Endocrinology*, 13, 57.
- Gaccioli, F., Lager, S., Powell, T. L., & Jansson, T. (2013). Placental transport in response to altered maternal nutrition. *Journal of Developmental Origins of Health and Disease*, 4, 101–115.
- Gude, N. M., Stevenson, J. L., Rogers, S., Best, J. D., Kalonis, B., Huisman, M. A., Erwich, J. J., Timmer, A., & King, R. G. (2003). Glut12 expression in human placenta in first trimester and term. *Placenta*, 24, 566–570.
- Jansson, T., Ekstrand, Y., Bjorn, C., Wennergren, M., & Powell, T. L. (2002). Alterations in the activity of placental amino acid transporters in pregnancies complicated by diabetes. *Diabetes*, 51, 2214–2219.
- Jansson, N., Greenwood, S. L., Johansson, B. R., Powell, T. L., & Jansson, T. (2003). Leptin stimulates the activity of the system a amino acid transporter in human placental villous fragments. *Journal of Clinical Endocrinology and Metabolism*, 88, 1205–1211.
- Jansson, N., Rosario, F. J., Gaccioli, F., Lager, S., Jones, H. N., Roos, S., Jansson, T., & Powell, T. L. (2013). Activation of placental Mtor signaling and amino acid transporters in obese women giving birth to large babies. *Journal of Clinical Endocrinology and Metabolism*, 98, 105–113.
- Jansson, T., Scholtbach, V., & Powell, T. L. (1998). Placental transport of leucine and lysine is reduced in intrauterine growth restriction. *Pediatric Research*, 44, 532–537.
- Jansson, T., Wennergren, M., & Illsley, N. P. (1993). Glucose transporter protein expression in human placenta throughout gestation and in intrauterine growth retardation. *The Journal of Clinical Endocrinology and Metabolism*, 77, 1554–1562.
- Jansson, T., Wennergren, M., & Powell, T. L. (1999). Placental glucose transport and Glut 1 expression in insulin-dependent diabetes. *American Journal of Obstetrics and Gynecology*, 180, 163–168.
- Jansson, T., Ylven, K., Wennergren, M., & Powell, T. L. (2002). Glucose transport and system a activity in Syncytiotrophoblast microvillous and basal plasma membranes in intrauterine growth restriction. *Placenta*, 23, 392–399.
- Janzen, C., Lei, M. Y., Cho, J., Sullivan, P., Shin, B. C., & Devaskar, S. U. (2013). Placental glucose transporter 3 (Glut3) is up-regulated in human pregnancies complicated by late-onset intrauterine growth restriction. *Placenta*, 34, 1072–1078.
- Jones, H. N., Jansson, T., & Powell, T. L. (2009). IL-6 stimulates system a amino acid transporter activity in trophoblast cells through Stat3 and increased expression of Snat2. *American Journal of Physiology Cell Physiology*, 297, C1228–1235.
- Kniss, D. A., Shubert, P. J., Zimmermann, P. D., Landon, M. B., & Gabbe, S. G. (1994). Insulinlike growth factors. Their regulation of glucose and amino acid transport in placental trophoblasts isolated from first-trimester chorionic villi. *Journal of Reproductive Medicine*, 39, 249–256.
- Kudo, Y., & Boyd, C. A. R. (2001). Characterisation of L-tryptophan transporters in human placenta: A comparison of brush border and basal membrane vesicles. *The Journal of Physiology*, 531, 405–416.
- Lager, S., Ramirez, V. I., Gaccioli, F., Jang, B., Jansson, T., & Powell, T. L. (2016). Protein expression of fatty acid transporter 2 is polarized to the trophoblast basal plasma membrane and increased in placentas from overweight/obese women. *Placenta*, 40, 60–66.
- Larque, E., Krauss-Etschmann, S., Campoy, C., Hartl, D., Linde, J., Klingler, M., Demmelair, H., Cano, A., Gil, A., Bondy, B., & Koletzko, B. (2006). Docosahexaenoic acid supply in pregnancy affects placental expression of fatty acid transport proteins. *The American Journal of Clinical Nutrition*, 84, 853–861.
- Lindegaard, M. L., Damm, P., Mathiesen, E. R., & Nielsen, L. B. (2006). Placental triglyceride accumulation in maternal type 1 diabetes is associated with increased lipase gene expression. *Journal of Lipid Research*, 47, 2581–2588.
- Magnusson, A. L., Waterman, I. J., Wennergren, M., Jansson, T., & Powell, T. L. (2004). Triglyceride hydrolase activities and expression of fatty acid binding proteins in the human placenta in pregnancies complicated by intrauterine growth restriction and diabetes. *The Journal of Clinical Endocrinology and Metabolism*, 89, 4607–4614.
- Magnusson-Olsson, A. L., Hamark, B., Ericsson, A., Wennergren, M., Jansson, T., & Powell, T. L. (2006). Gestational and hormonal regulation of human placental lipoprotein lipase. *Journal of Lipid Research*, 47, 2551–2561.
- Mahendran, D., Byrne, S., Donnai, P., D'Souza, S. W., Glazier, J. D., Jones, C. J., & Sibley, C. P. (1994). Na⁺ transport, H⁺ concentration gradient dissipation, and system a amino acid transporter activity in purified microvillous plasma membrane isolated from first-trimester human placenta: Comparison with the term microvillous membrane. *American Journal of Obstetrics and Gynecology*, 171, 1534–1540.
- Nelson, D. M., Smith, S. D., Furesz, T. C., Sadovsky, Y., Ganapathy, V., Parvin, C. A., & Smith, C. H. (2003). Hypoxia reduces expression and function of system a amino acid transporters in cultured term human trophoblasts. *American Journal of Physiology. Cell Physiology*, 284, C310–315.
- Okamoto, Y., Sakata, M., Ogura, K., Yamamoto, T., Yamaguchi, M., Tasaka, K., Kurachi, H., Tsurudome, M., & Murata, Y. (2002). Expression and regulation of 4f2hc and Hlat1 in human trophoblasts. *American Journal of Physiology. Cell Physiology*, 282, C196–204.
- Radaelli, T., Lepercq, J., Varastehpour, A., Basu, S., Catalano, P. M., & Hauguel-De Mouzon, S. (2009). Differential regulation of genes for Fetoplacental lipid pathways in pregnancy with gestational and type 1 diabetes mellitus. *American Journal of Obstetrics and Gynecology*, 201, 209.e201–209.e210.
- Roos, S., Jansson, N., Palmberg, I., Saljo, K., Powell, T. L., & Jansson, T. (2007). Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. *The Journal of Physiology*, 582, 449–459.
- Rosario, F. J., Kanai, Y., Powell, T. L., & Jansson, T. (2013). Mammalian target of rapamycin Signalling modulates amino acid uptake by regulating transporter cell surface abundance in primary human trophoblast cells. *The Journal of Physiology*, 591, 609–625.
- Thongsong, B., Subramanian, R. K., Ganapathy, V., & Prasad, P. D. (2005). Inhibition of amino acid transport system a by interleukin-1beta in trophoblasts. *Journal of the Society for Gynecologic Investigation*, 12, 495–503.

Immunology of Pregnancy

Margaret G Petroff and Sean L Nguyen, Michigan State University, East Lansing, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Allogeneic In transplantation biology, this term refers to tissues transplanted within the same species but between genetically dissimilar donor and recipient. This dissimilarity introduces donor *alloantigens* into the host, and therefore is recognized as foreign by the host immune system. This is in contrast to *syngeneic* grafts, which are genetically identical to that of the host and therefore are not rejected.

Blood group antigens Antigens found mainly on the surface of blood cells that are capable of being recognized as foreign by the immune system. In humans, there are different blood group systems; in most cases, the antigenic residue resides within carbohydrate residues of proteins or lipids. Blood group antigen systems include Rhesus, ABO, and Kell antigens.

Clonal expansion Rapid replication of a lymphocyte after its encounter with cognate antigen that gives rise to multiple progeny with the same antigen specificity.

Complement A group of evolutionarily conserved serum proteins of the innate immune system that is activated in a cascade manner, whose products act to promote phagocytosis, enhance inflammation, and to eliminate pathogens by forming pores on the pathogen membrane surface.

Costimulatory molecule Membrane proteins (B7 and CD40) that are expressed on the surface of activated antigen presenting cells that provide a required second signal for T cell activation; the first stimulatory signal comes from MHC-T cell receptor ligation.

Cross presentation Process by which dendritic cells internalize and degrade extracellular proteins or pathogens, and shuttle resulting peptide antigens to the MHC class I molecules for cell surface presentation. This is a specialized function of dendritic cells that other cells have only limited capacity to perform.

Decidualization A highly specialized differentiation response to implantation of the conceptus, in which endometrial stromal cells transform into enlarged hormone-producing cells that will ultimately surround the implanted embryo.

Effector lymphocytes Activated T and B cells following expansion and differentiation in response to pathogen, and that have gained ability to promote pathogen clearance. Different subsets of lymphocytes have different effector functions. T helper cells are the earliest to respond to pathogen in an adaptive immune response, not only because they can respond to antigen presented by professional antigen presenting cells, such as dendritic cells, but also because they produce the required “help” to other lymphocytes. This help occurs in the form of cytokines that are needed for differentiation of either cytotoxic T cells or B cells that recognize antigens related to that of the T helper cell. Whether cytokine help is provided to cytotoxic T cells or B cells depends on the type of antigen/pathogen as well as the surrounding environment. The effector function of cytotoxic T cells is to kill infected target cells, while the effector function of activated B cells, or plasma cells, is to produce antibodies, which in turn neutralize toxin, kill pathogens, or stimulate complement-mediated cytotoxicity.

Extracellular vesicles There are three broad classes of extracellular vesicles that differ according to their origin, size and molecular contents. Exosomes are the smallest of these, ranging 50–150 nm in diameter. These vesicles arise from the inward budding of the limiting membrane of late endosomes, which form intraluminal vesicles (ILV). Subsequent fusion of the endosome with the plasma membrane causes release of the ILV. Larger microvesicles (MV), which arise from budding of the plasma membrane, represent the second class, ranging from 50 to 1000 nm. Finally, apoptotic bodies arise from the blebbing cell membranes of cells undergoing programmed cell death. The anatomy of the outer syncytiotrophoblast layer of placenta and its direct contact with maternal blood allows for placental vesicle release into the blood and interaction with the maternal immune system.

Haplotype A group of linked allelic genes that is inherited as a unit from a parent. For example, humans have multiple polymorphic HLA genes, and they inherit two sets, or haplotypes, of these genes, one from each parent; this is similarly true for KIR genes. One KIR haplotype is designated *A*, and another haplotype *B*, then a person may have a combined haplotype of *KIRAA*, *AB*, or *BB*.

Homograft Tissue transplanted from a donor to a recipient of the same species. This is in contrast to *xenograft*, which is tissue transplanted from a donor to a recipient of a different species. A homograft can be either *allogeneic* (between genetically different individuals) or *syngeneic* (between genetically identical individuals, such as between identical twins or mice of the same strain). Xenografts are always allogeneic.

Killer inhibitory receptors Polyallelic transmembrane receptors present on the surface of natural killer cells, whose ligands are class Ia and class Ib MHC molecules. Ligation of KIR by MHC can inhibit the cytotoxic function of NK cells, or, in uterine NK cells, promote cytokine function.

Major histocompatibility complex (MHC) A group of genes that encode cell surface proteins responsible for mediating recognition of endogenous and exogenous peptides by the T lymphocytes. In humans, MHC molecules called human leukocyte antigens (HLA); thus the two terms are synonymous. MHC class I molecules are expressed on nearly all cells, and

present peptides derived from intracellular proteins; this group can be further broken down into MHC class Ia (classical) and MHC class Ib (nonclassical) molecules. Class Ia MHC molecules are expressed by nearly all nucleated cells, with notable of red blood cells and placental trophoblast cells. These proteins are highly polymorphic and function primarily to present pathogen-derived peptides to cytotoxic T cells the T cell receptor. Dendritic cells also use class Ia proteins to present peptides from extracellular material and pathogens, using a process called *cross-presentation*. Class Ib molecules have limited polymorphism, are expressed by trophoblast cells, and serve as ligands for activating and/or inhibitory receptors on innate and adaptive immune cells. MHC class II molecules are expressed primarily by antigen presenting cells and display peptides derived from extracellular material to helper T cells.

Minor histocompatibility antigen Antigenic peptides derived from proteins of polyallelic genes that can be presented by MHC class I and/or II molecules. Allelic differences can arise as single nucleotide polymorphisms, insertions, deletions, or coding on the Y chromosome. These antigens can be responsible for chronic rejection of transplants, even when tissues are matched for class I and class II MHC. An example of a minor antigen is the SMCY antigen, which derives from a gene encoded on the Y chromosome. While an X homolog of this gene exists (SMCX), differences in amino acid sequence of the Y-encoded allele of the gene gives rise to several class I and class II-restricted peptide antigens. Since females lack SMCY, their T cells will recognize the protein as foreign, provided that they possess the appropriate MHC.

Naïve T cells Mature T lymphocytes that have not encountered cognate antigen and have the potential to become activated once stimulated by antigen presenting cells.

Regulatory T cells A subpopulation of T cells that maintains tolerance to antigens derived from self-proteins. These cells are critical for preventing autoimmune disease. Regulatory T cells can be formed during T cell development in the thymus, or can be induced from mature T cells in the periphery. In pregnancy, it is the latter type that is thought to be critical for maternal tolerance to the fetus.

Uterine natural killer cells These cells are related to classical natural killer cells, which serve in the innate immune system to target and kill virally infected cells. Uterine natural killer cells, however, exhibit a different immunophenotype, and can be identified by their high level of CD56 and absent expression of CD16; classical NK cells are double positive for CD56 and CD16. Uterine NK cells use killer inhibitory receptors (KIR) to bind class Ib MHC molecules on trophoblast cells and enhance production of proangiogenic cytokines. Uterine NK cells constitute about 40% of total cells, 70% of leukocytes in the decidua; thus, their importance has long been regarded as high.

Introduction

Pregnancy is often compared to an *allogeneic* or semiallogeneic graft because of paternal genetic contribution, which is immunologically distinct from the mother, to the fetus. In 1953, Sir Peter Medawar, a zoologist at the University of Oxford during World War II, was recruited to help solve the problem of failing skin grafts on account of victims of the war. Having only uncertain prior experimental evidence of others to go on, Medawar performed a series of experiments that confirmed *homograft* rejection as an immunological event. In doing so, he became the preeminent leader in the field, receiving the Nobel Prize for his work in 1960.

Medawar's musings famously included the question of how the fetus survives in utero despite its allogeneicity in relation to the mother (Medawar, 1953). From the viewpoint of transplantation, the genetically distinct fetus should elicit an immune rejection response, but yet develops unhindered during normal pregnancy. Since then, studies have improved our understanding of maternal immune adaptation to pregnancy. Here, we review the immunological events that occur in pregnant women, placing this into context with the innate and adaptive immune responses to infection and transplantation (Table 1). Particular attention is paid

Table 1 Spectrum of immune responses to self and non-self

Immune response to	Self	Autograft	Pregnancy	Self: injury	Self: autoimmune disease	Allograft	Pathogen
Source of antigens	None	None	Paternal alloantigens	None	Self cells and tissues	Alloantigens	Pathogen antigens
Degree of difference from self	None	None	Semi-foreign	None	None	Foreign	Foreign
Initial tissue damage	No	Yes	Minimal/controlled	Yes	Possible	Yes	Yes
Danger signals	No	DAMPs	Possible in abnormal pregnancy	Injury: DAMPs	Possible DAMPs	DAMPs	PAMPs
Type of immune response	None	None	Innate and Adaptive	Innate	Innate and Adaptive	Innate and Adaptive	Innate and Adaptive
Immune outcome	Tolerance	Tolerance	Tolerance	Wound healing	Rejection	Rejection	Clearance

Different sources of self and foreign antigens elicit different types of immune responses, which can be explained at least partially by the source of antigens and presence of tissue damage and danger. The *green-red bar* indicates severity of immune response, starting from tolerance (*green*) to full immunity and clearance (*red*).

to the means by which each elicits the immune system, and why foreign organisms and transplanted tissue incite immunity, whereas the fetus also establishes both tolerance and collaboration with maternal immune cells to promote placentation and fetal growth.

Overview of Innate and Adaptive Immunity

The cells of the immune system, collectively called leukocytes, can be classified into the innate and adaptive branches. Some leukocytes functionally bridge these two arms by responding to innate signals and in turn, activating the adaptive cells. Innate immune cells include macrophages, neutrophils, eosinophils, basophils, and mast cells. Macrophages and neutrophils specialize in ingestion (phagocytosis) and destruction of foreign organisms. The innate immune system is more ancient than adaptive immunity, and as such, their response is limited in diversity and specificity. Nonetheless, they home to damaged tissues very rapidly whether or not pathogens are present. Further, they arrive armed with presynthesized, ready to be released effector molecules. The lifespan of innate immune cells is short: once they have discharged their defenses, the cells die.

The adaptive immune system, in contrast, is governed by T and B lymphocytes and is slower to respond because it requires days for cells to proliferate and differentiate prior to effecting their function. Proliferation by *clonal expansion* is necessary because they sense pathogens through receptors that are highly specific to discrete pathogen-specific antigens, and lymphocytes of individual antigenic specificity (clonality) circulate at exceedingly low numbers. Antigenic stimulation, together with the necessary *costimulation*, causes antigen-specific lymphocytes expand many-fold and differentiate into *effector lymphocytes*, which are then ready to eliminate pathogen. The low frequency of individual lymphocyte clones, as well as the manner in which their antigen receptors recombine during lymphocyte development, permits an extraordinary range of lymphocyte diversity within the circulation. Thus, the adaptive immune system is able to canvas the body for an enormous array of pathogens simultaneously. Effector T and B cells are short-lived; however, unlike cells of innate immunity, they retain “memory”: after responding to their antigen, T and B cells form daughter cells that mount stronger, faster recall responses upon re-encounter with the same antigen, curbing or preventing recurrent disease with the same pathogen.

Bridging the adaptive and innate immune systems are dendritic cells (DC). These cells employ multiple mechanisms of ingestion (phagocytosis, pinocytosis, and macropinocytosis), and in their immature state, sample their environment continuously. Dendritic cells can sense the presence of pathogens, and respond vigorously to pathogenic stimuli. The mechanisms of degradation and processing of ingested products is highly specialized and designed to relay information about the products to the adaptive immune system. In this way, DC are uniquely able to prime *naïve T cells*, thus initiating an immune response.

Self, Non-Self, and Danger

To understand the nature of the maternal immune system’s response to the semi-allogeneic fetus, it is helpful to have an understanding of how it responds to both pathogens and to transplanted tissue. Fundamentally, the immune system’s mission is to defend the body from organisms that cause disease. Although this task can be further reduced conceptually as defense against anything that is non-self, in reality, it is only when harm or danger is imminent that the immune system needs to deploy its defenses (Fig. 1). It is thus imperative that mechanisms be in place by which both *foreignness* and *imminent damage* are identified (Matzinger, 2002). Clearly, the immune system should leave normal-self alone at minimum, as it should certain foreigners like the fetus and, ideally, transplanted organs. At best, it should promote the establishment and growth of self, the fetus, and transplants.

On the surface, the early events of infection, transplantation, and pregnancy parallel each other, in that all involve entry of a foreigner past a first line of immune defense (e.g., an epithelium) (Table 1). Upon breach of this barrier, immune cells must immediately determine whether or not danger is imminent. To this end, the cells employ pattern recognition receptors (PRR), which are cell surface-associated or intracellular receptors whose ligands derive from damaged cells, pathogens, or both. Damaged cells release danger signals known as DAMPs (danger-associated molecular patterns); these are molecules that normally reside and function intracellularly, but whose extracellular release alerts immune cells that damage has been done. DAMPs include high-mobility group box 1 (HMBG1), heat shock proteins (HSPs), and low-density lipoprotein (LDL). In the absence of pathogens, a DAMP-initiated inflammatory response normally resolves; exceptions may occur in cases of autoimmune disease.

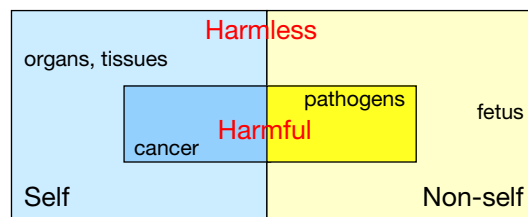


Fig. 1 Self, non-self, and harm. At the most basic level, the function of the immune system can be distilled down to recognition of self vs. non-self (light blue, light yellow). Critically, the immune system must also be able to sense harm, or imminent danger. Thus, some “self” can be harmful (cancer), and some non-self, harmless (fetus). The immune system distinguishes harmful and harmless through its use of pattern recognition receptors. Adapted from Matzinger, P. (2002). The danger model: A renewed sense of self. *Annual Review of Immunology*, 12, 991–1045.

If pathogenic organism(s) are also introduced, on the other hand, pathogen-derived danger signals (PAMPs) assume a pivotal role. PAMPs are not highly specific to different pathogens, but instead are evolutionarily conserved such that the same leukocyte-associated PRR can recognize many different pathogens. Examples include lipopolysaccharide (LPS) of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria, and double-stranded RNA of certain viruses. PAMPs induce strong inflammatory responses, and importantly, vertebrate cells lack these molecules. Thus, the interaction of PAMPs with PRRs alerts immune cells to the presence of both foreignness and danger.

At this stage, DC assume a pivotal role. Ligand of PRRs by PAMPs or DAMPs on DC induces dramatic changes that result in “maturation” of the cells, which involves down-regulation of phagocytosis and upregulation of *major histocompatibility complex* (MHC) and costimulatory molecules, *antigen presentation*, and subsequent stimulation of T cells. Antigen presentation involves the partial degradation of protein antigens creating peptides that are then complexed with MHC and presented to T cells. When T helper cells recognize peptide through its T cell receptor (TCR), together with appropriate costimulation from DC, they respond by clonal expansion and cytokine production, in turn activating cytotoxic T and/or B cells for killing activity and antibody production, respectively. Altogether, at least three signals are required to produce T lymphocyte priming and activation: the “danger” signal provided through PRR, MHC/foreign peptide ligand of TCR, and costimulation. Absent any one of these, inflammation will resolve, and antigen-specific tolerance will ensue.

Immune Response in Transplantation

With an understanding of how pathogens activate immunity, we can now consider a situation closer in analogy to the fetus, that is, a transplanted organ. Tissue and organ grafting is performed for the patients *benefit*, so why is graft rejection such a problem, and what molecular mechanisms account for graft rejections? Which of these mechanisms could be problematic for pregnancy?

Even with the best available surgical technique, transplantation iatrogenically induces tissue damage associated with excision—that is, removal of an organ and consequent ischemia/reperfusion and cell death. Because of this, transplanted tissues release extensive amounts of DAMPs, providing an abundance of the signals that induce maturation of both host and donor DC. If the patient receives his/her own tissue of that of an identical twin, and assuming the absence of pathogens, no foreign material is introduced, inflammation resolves, and the tissue heals and is accepted. However, if the donor tissue derives from an allogeneic individual, many foreign proteins are introduced. The presence of both danger signals associated with the surgical process and foreign antigens cause the immune system to “mistake” the transplanted tissue as dangerous, setting the stage for activation of an adaptive immune response and allograft rejection.

The nature of the rejection response depends what types of mismatched antigens occur between donor and recipient. For example, if ABO *blood group antigens* are not correctly matched between donor and recipient, preexisting antibodies (produced because of prior exposure to similar antigens associated with plants, viruses, and bacteria), together with *complement*, can cause hyperacute graft rejection, which takes place within only minutes of transplantation. Because of the dangers of hyperacute rejection, matching of blood group antigens is mandatory in tissue transplantation.

The next major barrier to graft acceptance is differences in the highly polymorphic MHC molecules between donor and recipient. A large proportion—1%–10%—of mature T cells in a given person or animal cross react to foreign (allogeneic) MHC molecules. Though the molecular basis for this alloreactivity is not well understood, it is a major clinical problem in transplantation, causing acute rejection of transplanted organs over days or weeks. Because of this, “tissue typing,” or matching of MHC allotypes between donor and recipient, is also indispensable for successful transplantation. In humans, six MHC molecules are typed for in the clinic: three class I molecules, human leukocyte antigen (HLA)-A, HLA-B, HLA-C; and three class II molecules: HLA-DP, HLA-DQ, and HLA-DR. Although matching for all six antigens often proves a significant challenge, the relatively low number makes it feasible.

The last immunological hurdle to successful transplantation, is more difficult to overcome and involves differences in *minor histocompatibility antigens* between donor and recipient. Minor antigens derive from normal cellular proteins, and can elicit antigenicity because of allelic differences between individuals. Since T cells cannot sense antigenic proteins directly, minor antigens are presented in the context of class I and/or class II MHC molecules, similar to pathogens. In transplantation, donor DC that accompany the graft can present minor antigens directly; if the antigen is lacking and thus foreign to the recipient, they will be recognized in a T cell-dependent, MHC-restricted manner. Alternatively, donor minor antigens can be *cross-presented* by recipient DC. T cells There are more than 60 known minor antigens, and likely hundreds or even thousands more are yet undiscovered—far too many to match in the clinic. Although lymphocyte reactions to minor antigens are generally weaker and slower than those against MHC, they are responsible for chronic rejection of grafts, often necessitating long-term immunosuppressant drug treatment of recipients.

Pregnancy: Invasion, Damage, and Antigens

How do each of these components of immune activation and transplant rejection relate to pregnancy? During implantation and placental development, pregnancy too, involves breach of an epithelial barrier, production of inflammatory signals, and introduction of foreign antigen. However, the maternal immune and reproductive systems together with the fetal–placental unit employ extensive strategies to ensure robust tolerance to gestational antigens. Unlike the events of transplantation and infection, which involve immunity-driven clearance of foreign material, the immunological events of normal pregnancy support implantation, placentation, and tolerance to fetal antigen.

Coitus. From the start, the maternal immune reaction in pregnancy differs from that of microbes and transplants. In men, anti-sperm antibodies that negatively impact fertility can be elicited as a result of vasectomy, injury, or infection to the genital tract. Immunity to sperm occurs in 1%–2% of women, but interferes with fertility only rarely. Key differences include danger signals and location: leakage of sperm, together with DAMPs into the blood in men, as opposed to lack of DAMPs and deposition of sperm and seminal fluid into the vaginal canal and cervix in women.

Coitus induces an inflammatory reaction in the cervix, an event that not only represents the first introduction of paternal antigens into the female reproductive tract, but also the incipient events of immune tolerance to gestational antigens (Robertson and Sharkey, 2016). Studies first performed in mice and later confirmed in women demonstrated that coitus induces neutrophil, macrophage, and DC accumulation, as well as production of proinflammatory cytokines and chemokines. Neutrophils ingest excess sperm, microbes, and debris. Seminal fluid, independently of sperm or the physical act of coitus, is responsible for the inflammatory reaction, mediated by bioactive factors including prostaglandins, growth factors, and cytokines.

Although sperm lacks MHC molecules, other cellular and soluble seminal fluid constituents contain both MHC and minor antigens. Seminal fluid antigens enter the vaginal, cervical, and endometrial mucosa, where they are presented by local DC. This early reaction primes maternal T cells, and may culminate in generation of *regulatory T cells* (T_{Reg}), which vitally contribute to fetal–maternal tolerance (Moldenhauer et al., 2009; Aluvihare et al., 2004). Indeed, several studies show that repeated exposure to seminal fluid, even in the absence of pregnancy, protects against preeclampsia—a phenomenon that may be explained by additive tolerogenic effects of seminal fluid in women. Clearly, neither exposure to seminal fluid nor the associated inflammatory response is required for pregnancy, but data suggest improved pregnancy outcome with seminal fluid exposure, for example, when couples engage in intercourse around the time of in vitro fertilization. Thus, while an immune response to sperm or seminal fluid can sometimes occur, the sequence of events at the inception of pregnancy ensure tolerization rather than immunization.

Implantation: Breach of the uterine epithelial barrier. Once fertilization occurs and a blastocyst has formed, the semi-foreign embryo is positioned for implantation. At the blastocyst stage, embryonic trophoblast cells attach to and invade the endometrium, breaching the epithelium and eliciting prostaglandins, leukotrienes, histamines, cytokines, and chemokines, and vascular leakage—in other words, inflammation. However, the ensuing response is atypical: together with prerequisite hormonal priming of the uterus, the implanting embryo triggers *decidualization*. Remarkably, the similarity of implantation to wound induction is such that “artificial” decidualization in the absence of an embryo can be stimulated by mild mechanical abrasion or “scratching” of the endometrium in an experimental setting in mice; endometrial scratching may improve uterine receptivity in some women undergoing assisted reproduction. Although maternal endometrial cells die in the process of implantation, they do so via apoptosis and local macrophages rapidly clear the dead cells. Thus, cellular damage, danger signals, and DAMPs are kept to a minimum.

While macrophages and T cells are present in steady numbers prior to and during decidualization, a unique population of granular leukocytes called *uterine natural killer* (uNK) cells proliferate to high numbers during decidualization. Animal studies have shown that both decidualization and pregnancy will proceed in the absence of these cells; however, establishment of the maternal blood supply to the placenta, often referred to as spiral artery remodeling, is impaired (Zhang et al., 2011). Uterine NK cells encircle spiral arteries prior to their transformation in the first trimester of gestation, and help modify the arteries by secreting angiogenic factors, cytokines, and chemokines, and recruiting trophoblast cells towards the vessels they will ultimately line. A unique feature of hemochorial placentation, vascular remodeling coincides with completion of placental maturation, in time for commencement of rapid fetal growth. Failure of remodeling is associated with several pathologies of pregnancy, including preeclampsia and fetal intrauterine growth restriction.

Immature DC are also present in the developing decidua. Although these cells can be activated in vivo, and potently present antigen in vitro, they appear not to do so effectively in vivo (Collins et al., 2009). Instead, uterine DC play an important role in decidualization. Without DC, pregnancy fails around the time of implantation due defective decidualization (Plaks et al., 2008). Thus, like uterine NK cells, uterine DC have unique and indispensable functions in pregnancy.

Intertwined with the process of maternal spiral artery remodeling is invasion of trophoblast cells. As the blastocyst encases itself within the decidua, its trophoblast cells give rise to the major placental trophoblast cell populations that define the physical, and therefore, immunological boundaries between the mother and fetus. Invasive extravillous trophoblast cells reside in close juxtaposition with a plethora of maternal immune cells, participating with uNK cells to achieve spiral artery remodeling. A second population, the syncytiotrophoblast, which will ultimately constitute the outermost layer of the chorionic villi in the mature placenta, is bathed in maternal blood starting around 13 weeks (i.e., upon establishment of maternal blood supply to the placenta), and throughout the remainder of gestation. The syncytiotrophoblast consists of a continuous multinucleated layer covering the entire placental surface. The syncytiotrophoblast–maternal blood interface is enormous, covering as much as 12 m² at term (Benirschke and Kaufmann, 2000).

One of Medawar’s postulates as to why the semiallogeneic fetus is not rejected by the maternal immune system was that the fetus is physically separated from the mother. This is partially correct, as the trophoblast serves as an epithelial barrier separating mother and fetus. Importantly, however, paternally derived “foreign” antigens are expressed by both the fetus and the placenta, including the border trophoblast populations. Furthermore, trophoblast cells release an abundance of *extracellular vesicles* directly into the maternal blood—potentially expanding the area of maternal–fetal contact exponentially as these vesicles circulate throughout the mother’s body. Collectively, this intimate maternal–fetal contact raises important, poorly understood questions of how the same proteins that are of significance in transplantation, exposed and released in such abundance from the placenta, are dealt with in pregnancy.

Blood group antigens. Blood group antigens are a significant clinical concern in pregnancy. Because AB antigens are minimally expressed by fetal erythrocytes, disease caused by these antigens is rare. However, the RhD antigen is of importance in cases of

an RhD-positive father and an RhD-negative mother. Here, the fetus may inherit the antigen and prime the mother's immune system, especially at birth, when the placenta hemorrhages during separation from the uterine wall. Then, the mother can be immunized by fetal RhD, forming cytotoxic IgG antibodies. While this may not be an issue for the first pregnancy, subsequent pregnancies with RhD-positive fetuses are at significant risk for hemolytic disease of the newborn—a condition analogous to antibody-mediated graft rejection. Fortunately, preventative treatment with Rho(D) immune globulin is effective.

MHC. At first glance, the paternally inherited MHC might seem problematic, particularly as the placenta is continually exposed to maternal immune cells. However, syncytiotrophoblast cells strictly regulate MHC expression, repressing all polymorphic class I and class II MHC. This precludes trophoblast cells from becoming targets of alloreactive maternal T cells. Some women generate antibodies against paternal MHC during pregnancy, possibly due to passage of fetal (MHC-expressing) cells into the mother. Still, these anti-fetal MHC antibodies appear to be innocuous.

Invasive extravillous trophoblast cells in the decidua express only certain MHC molecules: the non-polymorphic class Ib molecules, HLA-E, -F, and -G, and the class Ia molecule, HLA-C. These molecules serve as ligands for receptors on uterine NK cells. Specifically, HLA-E is a ligand for the inhibitory receptor, CD94/NKG2A, a receptor expressed on a subset of uNK cells that inhibits NK cell cytotoxicity, possibly protecting trophoblast cells in this respect (King et al., 2000). Little is known about the function of HLA-F, but this protein may also serve as a ligand for uNK cells.

HLA-G is uniquely expressed on extravillous trophoblast cells. Alternative splicing of HLA-G mRNA generates surface-bound (HLA-G1–4) and soluble (HLA-G5–7) isoforms, which have differential abilities to interact with its receptors: the NK and CD8 T cell inhibitory receptor LILRB1, the inhibitory monocytes receptor LILRB2, and the activating NK receptor KIR2DL4. HLA-G plays a role in maternal-fetal tolerance due to its ability to inhibit T cell proliferation and cytotoxicity and inhibit maturation of DC (Hunt et al., 2005). Its action on NK cells, on the other hand, may stimulate production of pro-angiogenic factors and thus may play role in spiral artery remodeling (Rajagopalan and Long, 2012).

HLA-C has a complex but important relationship with uNK cells. HLA-C is more polymorphic than the non-classical HLAs, having hundreds of alleles. This may have functional implications in terms of T cells that home to the maternal-fetal interface; additionally, HLA-C may be important in pathogen defense by presentation of pathogen antigens.

Uterine NK cells also interact with HLA-C. There are two functional groups of HLA-C alleles, HLA-C1 and HLA-C2, distinguished by the identities of two amino acids at positions 77 and 80, and which differ in utilization of *killer inhibitory receptors* (KIR). KIR are also polymorphic, and the combination of HLA-C and KIR *haplotypes* expressed by fetal trophoblast cells and maternal uNK cells, respectively, likely determines the balance of activating and inhibitory signals the uNK cell receives from the trophoblast, and in turn, how the uNK cells function. Genetic association studies have shown that an overall balance towards activating HLA-C and KIR haplotypes favor successful pregnancy outcomes, while inhibitory haplotypes predispose women to pregnancy disorders (Parham and Guethlein, 2010). Functionally, the activated uNK phenotype is hypothesized to drive these cells towards secretion of trophic and pro-angiogenic growth factors, chemokines, and cytokines. The inhibitory phenotype, in contrast, may constrain these progestational functions, and may disfavor angiogenesis and spiral artery remodeling.

Minor histocompatibility antigens. Minor antigens include “male” antigens encoded on the Y chromosome and others that are autosomally encoded. Unlike MHC molecules, many minor antigens are highly expressed by placental trophoblast cells (Holland et al., 2012). Further, they are cross-presented by maternal antigen presenting cells in much the same way as transplanted tissue antigens are by recipient DC (Erlebacher et al., 2007). Antigens gain access to maternal antigen presenting cells in lymphoid organs as they are released from the placenta into the maternal circulation, either as free or as vesicle-associated proteins. This allows priming of maternal T cells, which persist as memory cells *in vivo* for many years following pregnancy (James et al., 2003; van Halteren et al., 2009).

Despite the ability of T cells to respond to fetal antigen, the expansion of these T cells is blunted. They do not gain full effector function, neither killing fetal cells nor promoting production of cytotoxic antibodies. This is likely explained a number of factors, perhaps most importantly, including a lack of danger signals/DAMPs and thus lack of DC maturation (Bonney and Matzinger, 1998); expansion of regulatory T (T_{Reg}) cells during pregnancy; and lack of T cell access to the maternal-fetal interface due to epigenetic silencing of decidual chemokine genes (Nancy et al., 2012). Possibly, the manner in which fetal antigens are presented during pregnancy also contribute to the blunted response, but little is known about this potential mechanism.

Critical Factors in Preventing Maternal Immune Attack of the Fetus

Although there are a number of clinical syndromes, such as preterm birth and preeclampsia, that have immunological components, these involve inflammation rather than fetal antigen-specific lymphocytes. Despite some claims and/or loose usage of terminology, evidence that these syndromes represent “fetal allograft rejection” is sparse. A classical scientific approach to determining whether a factor is critical for a physiological/pathological process is ablation/replacement; this approach has been used in reproductive immunology, and occasionally, reports of “essential mediators” of maternal-fetal tolerance are reported. However, these reports are rarely upheld, probably because of robust and redundant mechanisms of tolerance.

A notable exception appears to be the requirement for regulatory T cells for maternal-fetal tolerance, as alluded to in preceding sections. This was first shown in an elegant study in which lymphocyte-deficient pregnant mice were reconstituted with either a complete complement of T cells, or with all T cells except T_{Reg} cells (Aluvihare et al., 2004). Here, the authors observed mid-gestation fetal loss in allogeneic, but not syngeneic pregnancies, suggesting that fetal loss is related to expression of paternally-inherited alloantigens. T_{Reg} cells in pregnancy expand due to both hormone- and antigen-dependent mechanisms;

moreover, during subsequent pregnancies, antigen-specific T_{Reg} cells expand more rapidly and to a greater degree than in first pregnancies, suggesting that these cells, like conventional T cells, possess memory (Rosenblum et al., 2016). T_{Reg} cells also expand during pregnancy in women, and reduced expansion is associated with adverse pregnancy outcome. While a number of studies have confirmed that T_{Reg} are critical for pregnancy, little is known about their mechanism of action; important open questions include which effector cells are controlled by pregnancy-induced T_{Reg}, and whether antigen-specificity and/or cell–cell contact is required for T_{Reg} function during pregnancy.

Summary and Future Directions

Key differences exist between a transplanted organ and the fetus during pregnancy that help explain Medawar's original question of how the fetus evades maternal immune rejection. Differences in introduction and establishment of embryonic and fetal growth in ways that avoid generation of "danger" signals, promote generation of suppressive T_{Reg} cells, and preclude access of dangerous lymphocytes to the maternal–fetal interface are likely essential. The past 60 or more years has seen leaps forward in our understanding of the biology of pregnancy and immunology, yet many important questions remain unanswered. The contributions of the immune system to pathologies of pregnancy remain enigmatic, and the insight to apply our knowledge in the clinic is of paramount importance.

Looking forward, we can bring the lessons of the past decades of research together with emerging questions, while taking advantage of the newest technical advances. For example, recent work has raised much interest in the role of placental extracellular vesicles and cell-free fetal DNA in modulating the maternal immune system. Already abundant in maternal blood in normal pregnancy, placental vesicles are even more copious in abnormal pregnancy, contain altered cargo, and differentially affect target cells. Our understanding of the role of these cells in modulating innate and adaptive immune cells in normal pregnancy is in its infancy, as is how they may affect disease. The discovery of cell-free fetal DNA has seen rapid translation as a clinical tool for noninvasive prenatal testing; however, like extracellular vesicles, their role in maternal biology and immunology is poorly understood.

There also exist a number of well-documented but poorly understood phenomena. For example, B cells, the precursors to antibody-producing plasma cells, are dramatically altered in pregnancy as evidenced by pregnancy-associated shrinkage of the bone marrow (where B cells develop), changes in B cell surface markers, and shifts in antibody production. Similarly, the thymus, where T cells develop, undergoes dramatic hormone-mediated changes during pregnancy, but there are almost no explanations of its importance.

It is likely that the solutions to these and other hanging questions will bring great insight into how the maternal immune system responds to the fetus in pregnancy. Continued collaboration between basic and clinical scientists will bring answers, and more importantly, novel strategies to manage clinical problems of not only pregnancy, but also diseases with immunological components such as autoimmune disease, tissue and organ transplantation, and cancer.

References

- Aluvihare, V. R., Kallikourdis, M., & Betz, A. G. (2004). Regulatory T cells mediate maternal tolerance to the fetus. *Nature Immunology*, 5, 266–271.
- Benirschke, K., & Kaufmann, P. (2000). *Pathology of the human placenta*. New York: Springer.
- Bonney, E. A., & Matzinger, P. (1998). Much IDO about pregnancy. *Nature Medicine*, 4, 1128–1129.
- Collins, M. K., Tay, C.-S., & Erlebacher, A. (2009). Dendritic cell entrapment within the pregnant uterus inhibits immune surveillance of the maternal/fetal interface in mice. *The Journal of Clinical Investigation*, 119, 2062–2073.
- Erlebacher, A., Vencato, D., Price, K. A., et al. (2007). Constraints in antigen presentation severely restrict T cell recognition of the allogeneic fetus. *The Journal of Clinical Investigation*, 117, 1399–1411.
- Holland, O. J., Linscheid, C., Hodes, H. C., Nauser, T. L., Gilliam, M., Stone, P., Chamley, L. W., & Petroff, M. G. (2012). Minor histocompatibility antigens are expressed in syncytiotrophoblast and trophoblast debris: Implications for maternal alloreactivity to the fetus. *American Journal of Pathology*, 180, 256–266.
- Hunt, J. S., Petroff, M. G., McIntire, R. H., & Ober, C. (2005). HLA-G and immune tolerance in pregnancy. *FASEB Journal*, 19, 681–693.
- James, E., Chai, J. G., Dewchand, H., et al. (2003). Multiparity induces priming to male-specific minor histocompatibility antigen, HY, in mice and humans. *Blood*, 102, 388–393.
- King, A., Allan, D. S., Bowen, M., et al. (2000). HLA-E is expressed on trophoblast and interacts with CD94/NKG2 receptors on decidual NK cells. *European Journal of Immunology*, 30, 1623–1631.
- Matzinger, P. (2002). The danger model: A renewed sense of self. *Annual Review of Immunology*, 12, 991–1045.
- Medawar, P. (1953). Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrates. *Symposia of the Society for Experimental Biology*, 7, 320–338.
- Moldenhauer, L. M., Diener, K. R., Thring, D. M., et al. (2009). Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy. *Journal of Immunology*, 182, 8080–8093.
- Nancy, P., Tagliani, E., Tay, C. S., et al. (2012). Chemokine gene silencing in decidual stromal cells limits T cell access to the maternal–fetal interface. *Science*, 336, 1317–1321.
- Parham, P., & Guethlein, L. A. (2010). Pregnancy immunogenetics: NK cell education in the womb? *The Journal of Clinical Investigation*, 120, 3801–3804.
- Plaks, V., Birnberg, T., Berkutzi, T., et al. (2008). Uterine DCs are crucial for decidua formation during embryo implantation in mice. *The Journal of Clinical Investigation*, 118, 3954–3965.
- Rajagopalan, S., & Long, E. O. (2012). KIR2DL4 (CD158d): An activation receptor for HLA-G. *Frontiers in Immunology*, 3, 258.
- Robertson, S. A., & Sharkey, D. J. (2016). Seminal fluid and fertility in women. *Fertility and Sterility*, 106, 511–519.
- Rosenblum, M. D., Way, S. S., & Abbas, A. K. (2016). Regulatory T cell memory. *Nature Reviews Immunology*, 16, 90–101.
- van Halteren, A. G., Jankowska-Gan, E., Joosten, A., et al. (2009). Naturally acquired tolerance and sensitization to minor histocompatibility antigens in healthy family members. *Blood*, 114, 2263–2272.
- Zhang, J., Chen, Z., Smith, G. N., & Croy, B. A. (2011). Natural killer cell-triggered vascular transformation: Maternal care before birth? *Cellular and Molecular Immunology*, 8, 1–11.

Yolk Sac

Daoyin Dong and Peixin Yang, University of Maryland School of Medicine, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The yolk sac is the first fetal membrane to be formed in all mammals and is the oldest of the extra-embryonic membranes. It is a bilayer structure of mesoderm- and endoderm-derived cell layers. The human embryo retains a yolk sac, which goes through primary and secondary phases of development. Human yolk sac has been considered to be a vestigial organ at the beginning of its formation, an evolutionary remnant based on the fact that it does not contain any yolk. Later in development, yolk sac has been shown to deliver nutrients, immunoglobulins and oxygen to the embryo due to an absence of maternal intervillous circulation in the first 12 weeks of pregnancy (Exalto, 1995). Currently, yolk sac is suggested to contribute to erythropoiesis, hematopoiesis, vasculogenesis and subsequently organogenesis. Recent studies demonstrate the role of yolk sac in diabetic vasculopathy, which is associated with maternal diabetes-induced birth defects.

Yolk Sac Development

The development and destiny of the yolk sac vary greatly across mammals and developmental stages, even within the same species (Carter, 2016). There are three different principal patterns of yolk sac development (Carter and Enders, 2016). In the first, yolk sac is used to support the early embryo in gestation. A vascular yolk sac is attached to the chorion, named choriovitelline placentation. Subsequently, the yolk sac is removed from the chorion by expansion of the exocoelom and allantois, and fetal development is supported by choriovitelline placentation. This occurs in pigs, horses and many other mammals (Carter, 2016; Carter and Enders, 2016).

The second pattern of yolk sac development is typical in rodents, insectivores, and lagomorphs. At the blastocyst stage, the extra-embryonic endoderm appears. Following blastocyst attachment, endoderm cells migrate around the inner surface of the trophoblast and the blastocyst cavity becomes the primary yolk sac. Trophoblast everts the epiblast into the yolk sac cavity by proliferation to form the ectoplacental cone. The space at the centre of the epiblast is called pro-amnion (Fig. 1). It will divide into the ectoplacental cavity, exocoelom and amniotic cavity following the appearance of the extra-embryonic mesoderm (Rossant and Cross, 2001) (Fig. 1). Subsequent events known as ventral folding morphogenesis lead to the enclosure of the embryo by the yolk sac and amnion, which includes several concurrent rearrangements (turning and axial rotation of the embryo). During this process, the allantois moves so that the umbilical cord is placed in the midline (Gavrilov and Lacy, 2013) (Fig. 2).

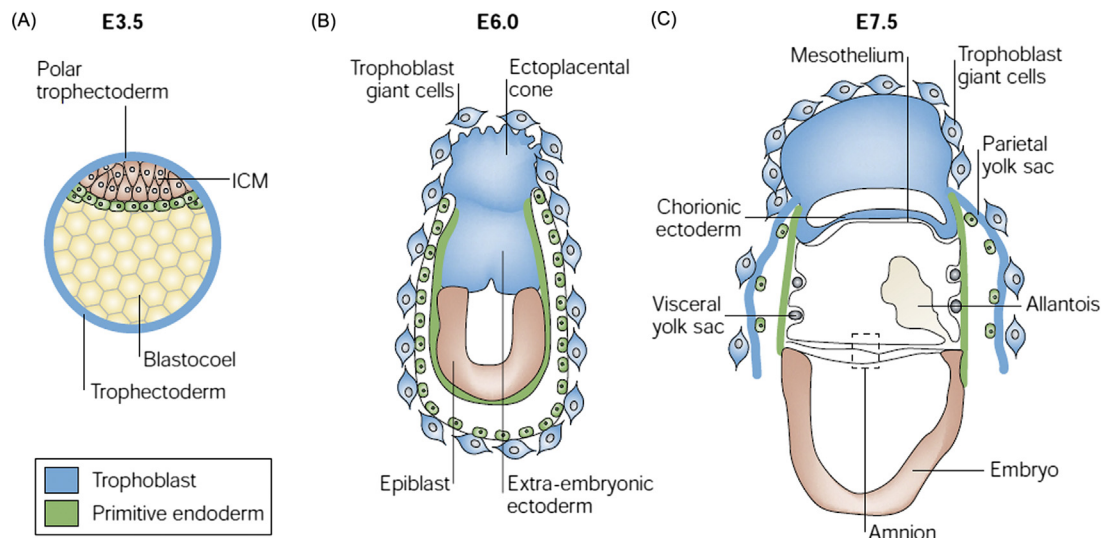


Fig. 1 Early development of the mouse embryo from E3.5 to E12.5. (A) The blastocyst consists of the outer and specialized trophectodermal epithelium and the inner cell mass (ICM). Trophectodermal epithelium pumps fluid internally to form the blastocoelic cavity and ICM lies to one side of the cavity. (B) The trophectoderm cells overlying the ICM (polar trophectoderm) continue to proliferate and expand to form extra-embryonic ectoderm and the ectoplacental cone. The trophectoderm cells distal from the ICM continue to form trophoblast giant cells. The endoderm adjacent to epiblast is the visceral endoderm; it will eventually contribute to the yolk sac. (C). The central cavity divides into the ectoplacental cavity, exocoelom and amniotic cavity following the appearance of the extraembryonic mesoderm. Reprinted from Rossant, J., and Cross, J. C. (2001). Placental development: lessons from mouse mutants. *Nature Reviews. Genetics* 2 (7), 538-48, <https://doi.org/10.1038/35080570>, with permission of Springer Nature.

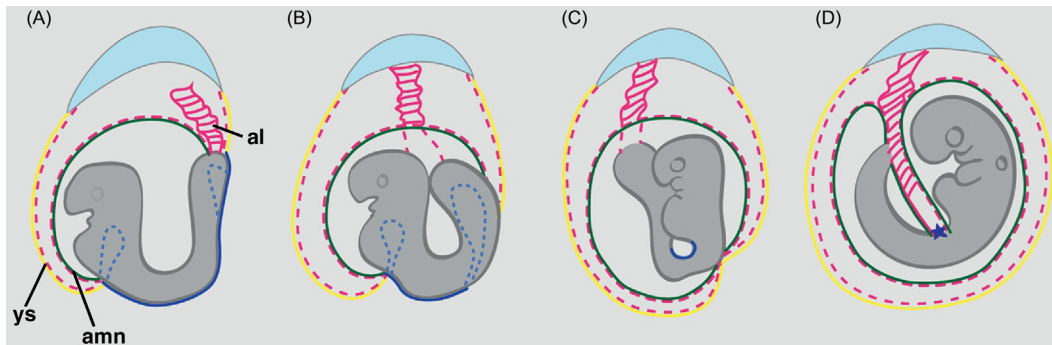


Fig. 2 Ventral folding morphogenesis in the mouse. Upon completion of embryo body turning, the embryo is embedded in the extra-embryonic membranes yolk sac (ys) and amnion (amn). The movement of allantois (al) places the umbilical cord (d) in the midline, at the meeting point of the original anterior and posterior connection sites between the amnion and embryo (* in D). (A) 8–10 somites; (B) 10–12 somites; (C) 12–14 somites; (D) 15–20 somites. Reprinted from Gavrilov, S., and Lacy, E. (2013). Genetic dissection of ventral folding morphogenesis in mouse: Embryonic visceral endoderm-supplied BMP2 positions head and heart. *Current Opinion in Genetics and Development* 23 (4), 461–469, <https://doi.org/10.1016/j.gde.2013.04.001>, with permission from Elsevier.

The third pattern of yolk sac characterizes human and haplorhine primates. The human yolk sac goes through two developmental stages: a primary yolk sac and a secondary yolk sac. A primary yolk sac is formed by extraembryonic endoderm and trophoblast and presents at implantation by proliferation and differentiation of primitive endoderm cells into visceral and parietal endoderm (Fig. 3). The primary yolk sac will break up into small vesicles, and the secondary yolk sac will form from the remnants of the primary yolk sac (Palis and Yoder, 2001) (Fig. 3). The secondary yolk sac, which is not in contact with the trophoblast, serves an important function in embryonic metabolism and maternal-fetal exchange in the first trimester in the human. In the second

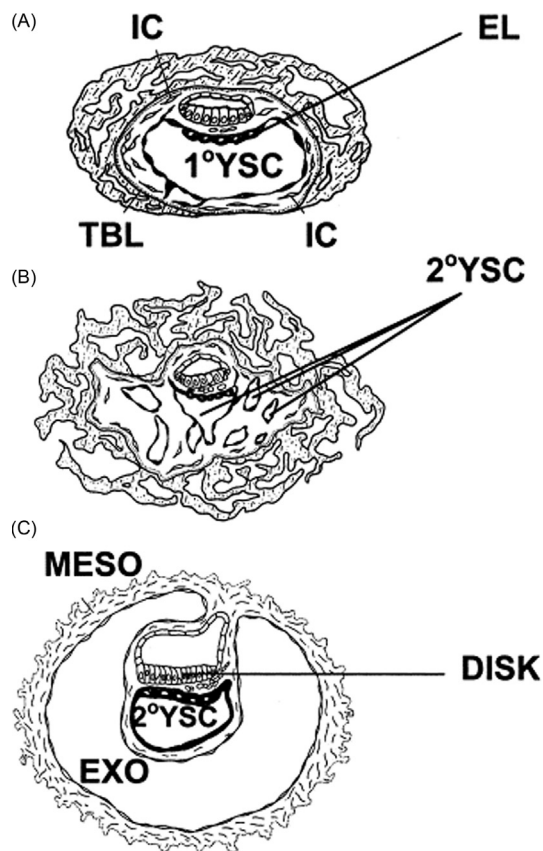


Fig. 3 Stages of human yolk sac formation. A. The primary yolk sac (1°YSC) structure in the human implantation site at stage 5c (day 9–12). IC, intermediate cells; EL, endodermal layer; TBL, trophoblastic basal lamina. (B) The fragmentation of primary yolk sac and formation of secondary yolk sac (2°YSC). (C) The secondary yolk sac structure at stage 7 (day 16). The second yolk sac has expanded to the edge of the embryonic disk. The exocoelom (EXO) is completely lined by a mesothelium (MESO). Reprinted from Palis, J., and Yoder, M. C. (2001). Yolk-sac hematopoiesis: The first blood cells of mouse and man. *Experimental hematology* 29 (8), 927–936, with permission from Elsevier.

trimester, expansion of the amnion results in disappearance of the extraembryonic coelom and regression of the yolk sac. Finally, the yolk sac is absent at term (Carter, 2016; Carter and Enders, 2016).

Blood Islands and Hematopoiesis

A role of yolk sac in hematopoietic and vascular induction was demonstrated by classic studies in the chick embryo, and this was supported by that Gata4-deficient embryonic stem cells showing reduced primitive erythropoiesis (Belaoussoff et al., 1998). It has been suggested that the yolk sac blood islands are the first site of hematopoiesis and vascular development in the vertebrate embryo (Baron et al., 2012; Ferkowicz and Yoder, 2005; Golub and Cumano, 2013; Keller et al., 1999; Mikkola and Orkin, 2006; Palis and Yoder, 2001; Tavian and Peault, 2005). Yolk sac blood islands are defined as “a cluster of primitive erythroblasts surrounded by an endothelial covering and nestled between the outer visceral endoderm and inner mesothelial cell layers comprising the yolk sac” (Ferkowicz and Yoder, 2005). Light and electron microscope studies in the mouse have observed the details of the morphological changes that occur during development of the blood islands region (Palis and Yoder, 2001) (Fig. 4). In the mouse, presumptive blood islands arise from mesodermal cell masses or angioblastic cords, which form from proximal mesodermal cells adjacent to visceral yolk sac between E7.0-E7.5 (Palis and Yoder, 2001) (Fig. 4). Blood islands are surrounded by visceral endodermal cells, which face the yolk sac cavity, and the single layer of mesoderm-derived cells, which faces the exocoelomic cavity (Ferkowicz and Yoder, 2005) (Fig. 4). Over the next 24 h, the outer layer of the blood island cell forms a spindle shape, and then these cells differentiate into endothelial cells (Palis and Yoder, 2001) (Fig. 4). The inner cells of blood islands lose their intercellular attachments to differentiate into primitive erythroblasts (Palis and Yoder, 2001) (Fig. 4).

The close spatial and temporal association between blood cells and endothelial cells in the yolk sac blood islands has led to the concept of a common progenitor termed the “hemangioblast” (Baron et al., 2012). Studies from differentiating mouse and human embryonic stem cells support this concept (Choi et al., 1998; Zambidis et al., 2005). Blast colony-forming cells (BL-CFCs) were hypothesized to be the in vitro equivalent of the hemangioblast (Choi et al., 1998). However, the majority of BL-CFCs were not found in the yolk sac but in the posterior primitive streak (Huber et al., 2004). In addition, the BL-CFCs are not strictly bipotential (Tober et al., 2007). Except for primitive and definitive hematopoietic cell types (Tober et al., 2007), they can also differentiate into smooth muscle in vitro (Ema et al., 2003). Therefore, it remains unknown whether an equivalent of the hemangioblast exists in vivo.

There are three distinct waves for hematopoiesis in the mouse (Golub and Cumano, 2013; Jagannathan-Bogdan and Zon, 2013; McGrath and Palis, 2005; Perdiguerro and Geissmann, 2016; Yoder, 2014). In the first wave, the yolk sac blood islands give rise to primitive erythroid progenitors, which are the first hematopoietic cells, from embryonic day 7.0 (E7.0) to E9.0 (Palis, 2014). These cells produce large and nucleated red blood cells that express embryonic hemoglobin molecules in a process known as primitive hematopoiesis. They are well known to be primitive erythroblasts because of their distinguishing features compared to adult red blood cells. Hematopoietic stem cells and lymphoid cells are not produced from the first wave of hematopoiesis. The second wave of hematopoiesis begins at E8.25 (Yoder, 2014). The erythromyeloid progenitors are first produced from vascular hemogenic endothelium as clusters of cells within yolk sac blood island capillaries. They then leave the blood islands, enter the systemic circulation and seed the fetal liver at E10.0. Within fetal liver, they differentiate into red blood cell populations that express adult-type hemoglobin molecules. Hemogenic endothelial cells of the yolk sac and the embryo proper also differentiate into B and T lymphoid progenitors (Boiers et al., 2013; Yoshimoto et al., 2012). The second wave is suggested to be definitive hematopoiesis because

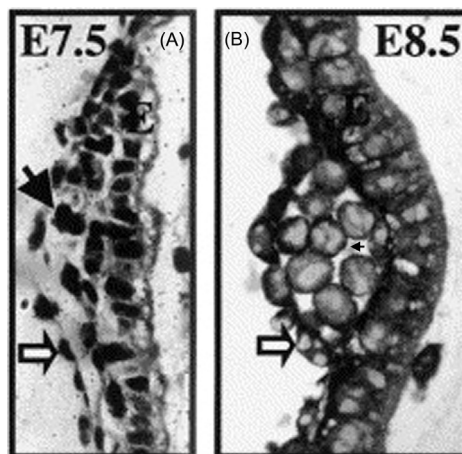


Fig. 4 Yolk sac blood islands formation in the mouse. (A) At E7.5, blood islands form between endoderm cells (E) and mesothelial cells (open arrow). Blood islands develop from undifferentiated mesoderm cells (closed arrow). (B) At E8.5, blood islands contain primitive hematopoietic cells (closed arrow) surrounded by endothelial cells (open arrow) adjacent to visceral endoderm cells (E). Reprinted from Palis, J., and Yoder, M. C. (2001). Yolk-sac hematopoiesis: The first blood cells of mouse and man. *Experimental hematology* 29 (8), 927–936, with permission from Elsevier.

erythromyeloid progenitors produce blood cells with adult-type characteristics and functions. However, this wave does not produce adult-type hematopoietic stem cells (Yoder, 2014). The third wave of hematopoiesis commences at E10.5 with the appearance of hematopoietic stem cells in the aorto-gonad-mesonephros (AGM) region of the embryo and in other arterial vessels of the embryo, yolk sac and placenta at E11.0 (Swiers et al., 2013). In this wave, hematopoietic stem cells produce all the cell types of the multi-lineage hematopoietic hierarchy (Yoder, 2014).

Vasculogenesis

Vasculogenesis is the de novo formation of a network of blood vessels (Garcia and Larina, 2014; Goldie et al., 2008; Jones et al., 2006; Patan, 2004; Patan, 2000). The blood islands located in the proximal extraembryonic yolk sac in the mouse comprise hematopoietic cells surrounded by endothelial cells (Jones et al., 2006) (Fig. 5). These endothelial cells of the blood islands, which expand from a thin band of endothelial and hematopoietic cells, migrate distally into the yolk sac, forming a simple capillary network that covers the yolk sac. This network is known as the primitive capillary plexus (Garcia and Larina, 2014) (Fig. 5). During vasculogenesis, the capillary plexus is initially established by activation of the vascular endothelial growth factor (VEGF) signaling pathway (Goldie et al., 2008; Jeltsch et al., 2013). Vascular endothelial receptor-2 (VEGFR2/flk1) has been identified as a regulator of de novo vasculogenesis and is vital for the development of both the primitive capillary plexus and blood cells during embryogenesis (Goldie et al., 2008; Jeltsch et al., 2013).

Following the establishment of the primitive capillary plexus in the surface of the yolk sac at E8.5, the honeycomb-like vascular network remodels to meet the demands of the expanding yolk sac and the growing embryo as it undergoes rapid morphological changes (Garcia and Larina, 2014). The established vessels expand and increase in diameter and small vessels fuse to form the large major vessels of the embryo and yolk sac. Concurrently, nascent vessels sprout into new, small capillary beds from existing larger vessels. This morphogenetic process is known as vascular remodeling, which occurs between E8.5 and E9.5, and results in a more hierarchical vascular tree (Garcia and Larina, 2014) (Fig. 5). This process is vital for the establishment of the cardiovascular system and support for the growth of the embryo. The viability of the embryo is dependent on the correct vascular remodeling. It has been demonstrated that the Tie family of receptors and ligands are critical molecular mediators for vascular remodeling and initial establishment of the capillary plexus during vasculogenesis (Jeltsch et al., 2013; Jones et al., 2001). Targeted deletion of Tie receptors in mice leads to impairments of vascular development, including yolk sac vascular remodeling (Sato et al., 1995). Four ligands have been identified: angiopoietins 1–4 (Jeltsch et al., 2013; Jones et al., 2001). They regulate vascular remodeling in the mouse embryo by binding to Tie receptors (Jeltsch et al., 2013). Besides Tie family receptors and ligands, members of the transforming growth factor β superfamily (TGF β), including TGFs, bone morphogenetic proteins (BMPs), and activins are molecular regulators of

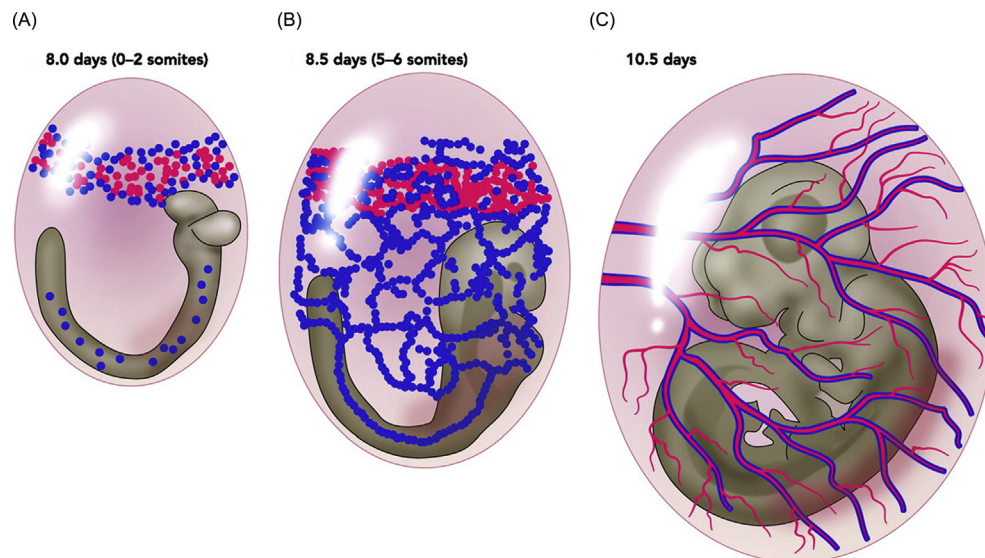


Fig. 5 Yolk sac vasculogenesis in the mouse. (A) The blood islands located in the proximal extraembryonic yolk sac comprise hematopoietic cells (red) surrounded by endothelial cells (blue). (B) Endothelial cells (blue) of the blood islands expand from a thin band of endothelial and hematopoietic cells (red) and migrate distally into the yolk sac, forming a simple capillary network that covers the yolk sac. The expansion of endothelial cells (blue) from the blood islands occurs earlier than the expansion of hematopoietic cells (red). (C) Following the establishment of primitive capillary plexus in the surface of the yolk sac at E8.5, the honeycomb-like vascular network expands and increases in diameter while small vessels fuse to form the large major vessels of the embryo and yolk sac, resulting in a more hierarchical vascular tree. Reprinted from Jones, E. A., le Noble, F., and Eichmann, A. (2006). What determines blood vessel structure? Genetic prespecification vs. hemodynamics. *Physiology* **21**, 388–395, 10.1152/physiol.00020.2006.

yolk sac vascular remodeling and angiogenesis (Kubis and Levy, 2003). It has been demonstrated that knockout of TGF β family members is lethal at E10.5-E11.5, along with unremodeled yolk sac vasculature, defective endothelial and hematopoietic differentiation, and weak vessels (Garcia and Larina, 2014; Larsson et al., 2001). In addition, it has been demonstrated that Hedgehog and Hippo signalling are required for murine yolk sac vasculogenesis (Byrd et al., 2002; Morin-Kensicki et al., 2006).

Diabetic Vasculopathy

It is well known that maternal diabetes is an independent risk factor for structural birth defects, including congenital heart defects (CHDs) and neural tube defects (NTDs) (Dong et al., 2016; Yang et al., 2015). Data from the National Birth Defects Prevention Study shows that the incidence for newborn NTDs and CHDs are up to 10 times more frequent in women with maternal diabetes compared to women who never had diabetes or who developed diabetes late in pregnancy (Correa et al., 2008). Experimental evidence has demonstrated that maternal diabetes-induced birth defects are associated with the failure of yolk sac vasculogenesis, which is also called vasculopathy (Dong et al., 2016). Morphological scores are assigned to individual vasculature to characterize the adverse effect of hyperglycemia on the yolk sac vasculogenesis (Yang et al., 2008). Using the rating system, it has been demonstrated that hyperglycemia induces significantly lower yolk sac vasculature scores compared to the euglycemic group (Yang et al., 2008). Meanwhile, yolk sac vasculature scores are inversely correlated with embryonic malformations (Yang et al., 2008).

The mouse experiments revealed the abnormal and arrested development of the yolk sac vasculature in the E7.5 embryo, which results in congenital malformations in a wide range of organs and tissues as well as embryonic lethality under maternal diabetic conditions (Nath et al., 2004; Pinter et al., 2001; Pinter et al., 1999; Pinter et al., 1986; Reece et al., 1994; Yang and Reece, 2011). In cultured mouse embryos, exposure to excess glucose leads visceral yolk sac capillaries and vitelline vessels to be sparse, patchy, and not uniformly located (Pinter et al., 1986). In the maternal diabetic mouse model, hyperglycemia causes alteration and disruption of cellular structures of blood vessels in the yolk sac (Pinter et al., 2001; Pinter et al., 1999). Various and profound abnormalities of yolk sac vasculature are observed, including complete lack of vasculogenesis with no apparent arborization or distinction of arteries and veins (Pinter et al., 1997; Pinter et al., 2001; Pinter et al., 1999; Yang et al., 2008) (Fig. 6). The mechanical studies

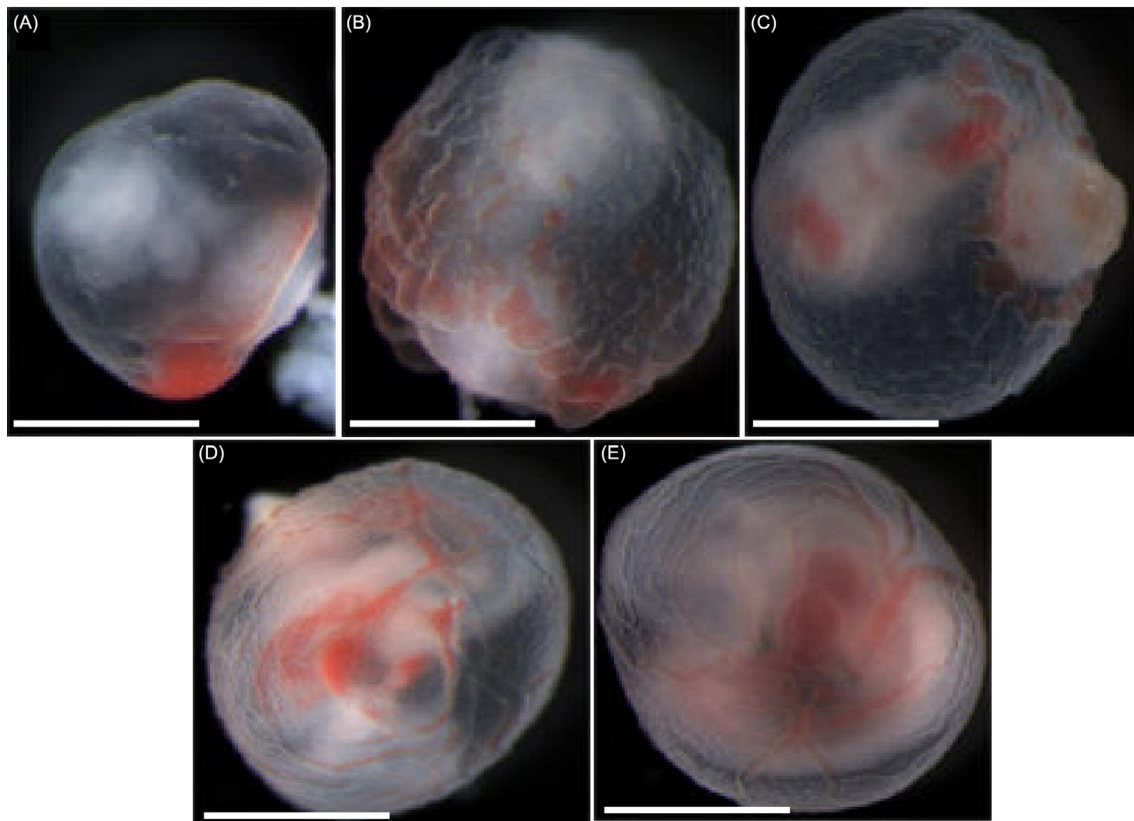


Fig. 6 Diabetic vasculopathy in the mouse. Morphological changes of yolk sac vasculogenesis exposed to high glucose (A–D: 500 mg/dL) and normal glucose (E: 150 mg/dL) in cultured embryos. (A)–(E). The yolk sac with a morphological score 0–4. 0 is the worst and 4 is the normal. Scale bar: 1 mm. Reprinted from Yang, P., Zhao, Z., and Reece, E. A. (2008). Blockade of c-Jun N-terminal kinase activation abrogates hyperglycemia-induced yolk sac vasculopathy in vitro. *American Journal of Obstetrics and Gynecology* **198** (3), 321 e1–7, <https://doi.org/10.1016/j.ajog.2007.09.010>, with permission from Elsevier.

show that vascular endothelial cells are the primary target of hyperglycemia (Pinter et al., 1997; Pinter et al., 1999). Hyperglycemia adversely affects the function of endothelial cells, including increased apoptosis, decreased proliferation and differentiation through endothelial cell-dependent molecular pathways: hypoxia-inducible factor-1 (HIF-1); nitric oxide (NO) and nitric oxide synthase (NOS); mitogen-activated protein kinases (MAPKs); and redox sensitive thioredoxin-1 (Dong et al., 2016).

The popular intervention of preventing maternal diabetes-induced birth defects is rigorous glycemic control by antidiabetic drugs and lifestyle alterations (Gabbay-Benziv et al., 2015). However, continuous euglycemic control is difficult to maintain (Reece, 2012). Fortunately, reports have shown the potential for developing therapeutics to treat maternal diabetes-induced birth defects through small molecular targeting of critical sites. Blockage of JNK1/2 activation by JNK1/2-specific inhibitor SP60025 restores hyperglycemia-induced vasculopathy and reduces malformation rates in cultured embryos (Yang et al., 2008). Epigallocatechin-3-gallate (EGCG), a natural antioxidant purified from green tea, also shows a preventive effect on hyperglycemia-induced vasculopathy and congenital malformations (Yang and Li, 2010). In addition, supplementation of thioredoxin, an endogenous antioxidant protein, completely reverses hyperglycemia-induced vasculopathy and congenital malformations by restoring HIF-1 α levels in the yolk sac (Yang and Reece, 2011).

Conclusions and Future Directions

This chapter has summarized what is currently understood about the development and function of yolk sac as well as its association with maternal diabetes-induced birth defects. Although many valuable studies support the claim of yolk sac as the origin of the first blood cells, many unanswered questions remain. Because of ethical issues and difficulty in acquiring human yolk sac samples, most studies on yolk sac development and function are performed by using the mouse model. However, it is unclear whether mechanical studies on mouse yolk sac are relevant to human yolk sac because of differences between human and mouse in yolk sac development and shape. The mechanism underlying the yolk sac's extreme sensitivity to hyperglycemia exposure, which leads to diabetic vasculopathy, is still elusive. More studies are needed to investigate therapeutic interventions that target yolk sac to prevent birth defects induced by maternal diabetes.

References

- Baron, M. H., Isern, J., & Fraser, S. T. (2012). The embryonic origins of erythropoiesis in mammals. *Blood*, 119(21), 4828–4837. <https://doi.org/10.1182/blood-2012-01-153486>.
- Belaoussoff, M., Farrington, S. M., & Baron, M. H. (1998). Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. *Development*, 125(24), 5009–5018.
- Boiers, C., Carrelha, J., Lutteropp, M., Luc, S., Green, J. C., Azzoni, E., Woll, P. S., Mead, A. J., Hultquist, A., Swiers, G., Perdiguero, E. G., Macaulay, I. C., Melchior, L., Luis, T. C., Kharazi, S., Bouriez-Jones, T., Deng, Q., Ponten, A., Atkinson, D., Jensen, C. T., Sitnicka, E., Geissmann, F., Godin, I., Sandberg, R., de Bruijn, M. F., & Jacobsen, S. E. (2013). Lymphomyeloid contribution of an immune-restricted progenitor emerging prior to definitive hematopoietic stem cells. *Cell Stem Cell*, 13(5), 535–548. <https://doi.org/10.1016/j.stem.2013.08.012>.
- Byrd, N., Becker, S., Maye, P., Narasimhaiah, R., St-Jacques, B., Zhang, X., McMahon, J., McMahon, A., & Gabel, L. (2002). Hedgehog is required for murine yolk sac angiogenesis. *Development*, 129(2), 361–372.
- Carter, A. M. (2016). IFPA senior award lecture: Mammalian fetal membranes. *Placenta*, 48(Suppl 1), S21–S30. <https://doi.org/10.1016/j.placenta.2015.10.012>.
- Carter, A. M., & Enders, A. C. (2016). Placentation in mammals: Definitive placenta, yolk sac, and paraplacenta. *Theriogenology*, 86(1), 278–287. <https://doi.org/10.1016/j.theriogenology.2016.04.041>.
- Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., & Keller, G. (1998). A common precursor for hematopoietic and endothelial cells. *Development*, 125(4), 725–732.
- Correa, A., Gilboa, S. M., Besser, L. M., Botto, L. D., Moore, C. A., Hobbs, C. A., Cleves, M. A., Riehle-Colarusso, T. J., Waller, D. K., & Reece, E. A. (2008). Diabetes mellitus and birth defects. *American Journal of Obstetrics and Gynecology*, 199(3), 237–239. <https://doi.org/10.1016/j.ajog.2008.06.028>.
- Dong, D., Reece, E. A., Lin, X., Wu, Y., AriasVilela, N., & Yang, P. (2016). New development of the yolk sac theory in diabetic embryopathy: Molecular mechanism and link to structural birth defects. *American Journal of Obstetrics and Gynecology*, 214(2), 192–202. <https://doi.org/10.1016/j.ajog.2015.09.082>.
- Ema, M., Faloony, P., Zhang, W. J., Hirashima, M., Reid, T., Stanford, W. L., Orkin, S., Choi, K., & Rossant, J. (2003). Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. *Genes & Development*, 17(3), 380–393. <https://doi.org/10.1101/gad.1049803>.
- Exalto, N. (1995). Early human nutrition. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 61(1), 3–6.
- Ferkowicz, M. J., & Yoder, M. C. (2005). Blood island formation: Longstanding observations and modern interpretations. *Experimental Hematology*, 33(9), 1041–1047. <https://doi.org/10.1016/j.exphem.2005.06.006>.
- Gabbay-Benziv, R., Reece, E. A., Wang, F., & Yang, P. (2015). Birth defects in pregestational diabetes: Defect range, glycemic threshold and pathogenesis. *World Journal of Diabetes*, 6(3), 481–488. <https://doi.org/10.4239/wjcd.v6.i3.481>.
- Garcia, M. D., & Larina, I. V. (2014). Vascular development and hemodynamic force in the mouse yolk sac. *Frontiers in Physiology*, 5, 308. <https://doi.org/10.3389/fphys.2014.00308>.
- Gavrilov, S., & Lacy, E. (2013). Genetic dissection of ventral folding morphogenesis in mouse: Embryonic visceral endoderm-supplied BMP2 positions head and heart. *Current Opinion in Genetics & Development*, 23(4), 461–469. <https://doi.org/10.1016/j.gde.2013.04.001>.
- Goldie, L. C., Nix, M. K., & Hirschi, K. K. (2008). Embryonic vasculogenesis and hematopoietic specification. *Organogenesis*, 4(4), 257–263.
- Golub, R., & Cumano, A. (2013). Embryonic hematopoiesis. *Blood Cells, Molecules & Diseases*, 51(4), 226–231. <https://doi.org/10.1016/j.bcmd.2013.08.004>.
- Huber, T. L., Kouskoff, V., Fehling, H. J., Palis, J., & Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature*, 432(7017), 625–630. <https://doi.org/10.1038/nature03122>.
- Jagannathan-Bogdan, M., & Zon, L. I. (2013). Hematopoiesis. *Development*, 140(12), 2463–2467. <https://doi.org/10.1242/dev.083147>.
- Jeltsch, M., Leppanen, V. M., Saharinen, P., & Alitalo, K. (2013). Receptor tyrosine kinase-mediated angiogenesis. *Cold Spring Harbor Perspectives in Biology*, 5(9). <https://doi.org/10.1101/cshperspect.a009183>.
- Jones, E. A., le Noble, F., & Eichmann, A. (2006). What determines blood vessel structure? Genetic prespecification vs. hemodynamics. *Physiology*, 21, 388–395. <https://doi.org/10.1152/physiol.00020.2006>.

- Jones, N., Iijin, K., Dumont, D. J., & Alitalo, K. (2001). Tie receptors: New modulators of angiogenic and lymphangiogenic responses. *Nature Reviews. Molecular Cell Biology*, 2(4), 257–267. <https://doi.org/10.1038/35067005>.
- Keller, G., Lacaud, G., & Robertson, S. (1999). Development of the hematopoietic system in the mouse. *Experimental Hematology*, 27(5), 777–787.
- Kubis, N., & Levy, B. I. (2003). Vasculogenesis and angiogenesis: Molecular and cellular controls. Part 1: Growth factors. *Interventional Neuroradiology: Journal of Peritherapeutic Neuroradiology, Surgical Procedures and Related Neurosciences*, 9(3), 227–237. <https://doi.org/10.1177/159101990300900301>.
- Larsson, J., Goumans, M. J., Sjostrand, L. J., van Rooijen, M. A., Ward, D., Leveen, P., Xu, X., ten Dijke, P., Mummery, C. L., & Karlsson, S. (2001). Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. *The EMBO Journal*, 20(7), 1663–1673. <https://doi.org/10.1093/emboj/20.7.1663>.
- McGrath, K. E., & Palis, J. (2005). Hematopoiesis in the yolk sac: More than meets the eye. *Experimental Hematology*, 33(9), 1021–1028. <https://doi.org/10.1016/j.exphem.2005.06.012>.
- Mikkola, H. K., & Orkin, S. H. (2006). The journey of developing hematopoietic stem cells. *Development*, 133(19), 3733–3744. <https://doi.org/10.1242/dev.02568>.
- Morin-Kensicki, E. M., Boone, B. N., Howell, M., Stonebraker, J. R., Teed, J., Alb, J. G., Magnuson, T. R., O'Neal, W., & Milgram, S. L. (2006). Defects in yolk sac vasculogenesis, chorioallantoic fusion, and embryonic axis elongation in mice with targeted disruption of Yap65. *Molecular and Cellular Biology*, 26(1), 77–87. <https://doi.org/10.1128/MCB.26.1.77-87.2006>.
- Nath, A. K., Enciso, J., Kuniyasu, M., Hao, X. Y., Madri, J. A., & Pinter, E. (2004). Nitric oxide modulates murine yolk sac vasculogenesis and rescues glucose induced vasculopathy. *Development*, 131(10), 2485–2496. <https://doi.org/10.1242/dev.01131>.
- Palis, J. (2014). Primitive and definitive erythropoiesis in mammals. *Frontiers in Physiology*, 5, 3. <https://doi.org/10.3389/fphys.2014.00003>.
- Palis, J., & Yoder, M. C. (2001). Yolk-sac hematopoiesis: The first blood cells of mouse and man. *Experimental Hematology*, 29(8), 927–936.
- Patan, S. (2000). Vasculogenesis and angiogenesis as mechanisms of vascular network formation, growth and remodeling. *Journal of Neuro-Oncology*, 50(1–2), 1–15.
- Patan, S. (2004). Vasculogenesis and angiogenesis. *Cancer Treatment and Research*, 117, 3–32.
- Perdiguerro, E. G., & Geissmann, F. (2016). The development and maintenance of resident macrophages. *Nature Immunology*, 17(1), 2–8. <https://doi.org/10.1038/ni.3341>.
- Pinter, E., Barreuther, M., Lu, T., Imhof, B. A., & Madri, J. A. (1997). Platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) tyrosine phosphorylation state changes during vasculogenesis in the murine conceptus. *The American Journal of Pathology*, 150(5), 1523–1530.
- Pinter, E., Haigh, J., Nagy, A., & Madri, J. A. (2001). Hyperglycemia-induced vasculopathy in the murine conceptus is mediated via reductions of VEGF-A expression and VEGF receptor activation. *The American Journal of Pathology*, 158(4), 1199–1206.
- Pinter, E., Mahooti, S., Wang, Y., Imhof, B. A., & Madri, J. A. (1999). Hyperglycemia-induced vasculopathy in the murine vitelline vasculature: Correlation with PECAM-1/CD31 tyrosine phosphorylation state. *The American Journal of Pathology*, 154(5), 1367–1379. [https://doi.org/10.1016/S0002-9440\(10\)65391-6](https://doi.org/10.1016/S0002-9440(10)65391-6).
- Pinter, E., Reece, E. A., Leranthe, C. Z., Sanyal, M. K., Hobbins, J. C., Mahoney, M. J., & Naftolin, F. (1986). Yolk sac failure in embryopathy due to hyperglycemia: Ultrastructural analysis of yolk sac differentiation associated with embryopathy in rat conceptuses under hyperglycemic conditions. *Teratology*, 33(1), 73–84. <https://doi.org/10.1002/tera.1420330110>.
- Reece, E. A. (2012). Diabetes-induced birth defects: What do we know? What can we do? *Current Diabetes Reports*, 12(1), 24–32. <https://doi.org/10.1007/s11892-011-0251-6>.
- Reece, E. A., Pinter, E., Homko, C., Wu, Y. K., & Naftolin, F. (1994). The yolk sac theory: Closing the circle on why diabetes-associated malformations occur. *Journal of the Society for Gynecologic Investigation*, 1(1), 3–13.
- Rossant, J., & Cross, J. C. (2001). Placental development: Lessons from mouse mutants. *Nature Reviews. Genetics*, 2(7), 538–548. <https://doi.org/10.1038/35080570>.
- Sato, T. N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., & Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature*, 376(6535), 70–74. <https://doi.org/10.1038/376070a0>.
- Swiers, G., Baumann, C., O'Rourke, J., Giannoulatos, E., Taylor, S., Joshi, A., Moignard, V., Pina, C., Bee, T., Kokkalis, K. D., Yoshimoto, M., Yoder, M. C., Frampton, J., Schroeder, T., Enver, T., Gottgens, B., & de Bruijn, M. F. (2013). Early dynamic fate changes in haemogenic endothelium characterized at the single-cell level. *Nature Communications*, 4, 2924. <https://doi.org/10.1038/ncomms3924>.
- Tavian, M., & Peault, B. (2005). Embryonic development of the human hematopoietic system. *The International Journal of Developmental Biology*, 49(2–3), 243–250. <https://doi.org/10.1387/ijdb.041957mt>.
- Tober, J., Koniski, A., McGrath, K. E., Vernishetti, R., Emerson, R., de Mesy-Bentley, K. K., Waugh, R., & Palis, J. (2007). The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood*, 109(4), 1433–1441. <https://doi.org/10.1182/blood-2006-06-031898>.
- Yang, P., & Li, H. (2010). Epigallocatechin-3-gallate ameliorates hyperglycemia-induced embryonic vasculopathy and malformation by inhibition of Foxo3a activation. *American Journal of Obstetrics and Gynecology*, 203(1), 75–76. <https://doi.org/10.1016/j.ajog.2010.02.008>.
- Yang, P., & Reece, E. A. (2011). Role of HIF-1alpha in maternal hyperglycemia-induced embryonic vasculopathy. *American Journal of Obstetrics and Gynecology*, 204(4), 332–337. <https://doi.org/10.1016/j.ajog.2011.01.012>.
- Yang, P., Reece, E. A., Wang, F., & Gabbay-Benziv, R. (2015). Decoding the oxidative stress hypothesis in diabetic embryopathy through proapoptotic kinase signaling. *American Journal of Obstetrics and Gynecology*, 212(5), 569–579. <https://doi.org/10.1016/j.ajog.2014.11.036>.
- Yang, P., Zhao, Z., & Reece, E. A. (2008). Blockade of c-Jun N-terminal kinase activation abrogates hyperglycemia-induced yolk sac vasculopathy in vitro. *American Journal of Obstetrics and Gynecology*, 198(3), 321–327. <https://doi.org/10.1016/j.ajog.2007.09.010>.
- Yoder, M. C. (2014). Inducing definitive hematopoiesis in a dish. *Nature Biotechnology*, 32(6), 539–541. <https://doi.org/10.1038/nbt.2929>.
- Yoshimoto, M., Porayette, P., Glosson, N. L., Conway, S. J., Carlesso, N., Cardoso, A. A., Kaplan, M. H., & Yoder, M. C. (2012). Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. *Blood*, 119(24), 5706–5714. <https://doi.org/10.1182/blood-2011-12-397489>.
- Zambidis, E. T., Peault, B., Park, T. S., Bunz, F., & Civin, C. I. (2005). Hematopoietic differentiation of human embryonic stem cells progresses through sequential hemoendothelial, primitive, and definitive stages resembling human yolk sac development. *Blood*, 106(3), 860–870. <https://doi.org/10.1182/blood-2004-11-4522>.

Further Reading

- Carter, A. M. (2016 Dec). IFPA senior award lecture: Mammalian fetal membranes. *Placenta*, 48(Suppl 1), S21–S30.
- Carter, A. M., & Enders, A. C. (2016). Placentation in mammals: Definitive placenta, yolk sac, and paraplacenta. *Theriogenology*, 86(1), 278–287.
- Dong, D., Reece, E. A., Lin, X., Wu, Y., AriasVilella, N., & Yang, P. (2016 Feb). New development of the yolk sac theory in diabetic embryopathy: Molecular mechanism and link to structural birth defects. *American Journal of Obstetrics and Gynecology*, 214(2), 192–202.
- Godin, I., & Cumano, A. (2006). *Hematopoietic stem cell development*. New York: Springer.
- Jagannathan-Bogdan, M., & Zon, L. I. (2013 Jun 15). Hematopoiesis. *Development*, 140(12), 2463–2467.
- Lucitti, J. L., Jones, E. A. V., Huang, C., Chen, J., Fraser, S. E., & Dickinson, M. E. (2014). Vascular remodeling of the mouse yolk sac requires hemodynamic force. *Frontiers in Physiology*, 5, 308.
- Nogales, F. F. (1993). *The Human Yolk Sac and Yolk Sac Tumors*. Heidelberg: Springer.
- Yoder, M. C. (2014 Jun). Inducing definitive hematopoiesis in a dish. *Nature Biotechnology*, 32(6), 539–541.

Relevant Websites

<https://discovery.lifemapsc.com/>.
<http://study.com/academy/lesson/yolk-sac-in-humans-function-definition-measurement.html>.
<https://www.ehd.org/index.php>.
<https://www.britannica.com/science/yolk-sac>.
<http://www.sciencedirect.com/topics/neuroscience/blood-island-of-umbilical-vesicle>.

Allantois

Fuller W Bazer and Gregory A Johnson, Texas A&M University, College Station, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Allantoic membrane The allantoic membrane of the placenta forms as an evagination of the hindgut to form a sac filled with allantoic fluid composed of water, urine, nutrients (e.g., amino acids, glucose, vitamins, and minerals), growth factors, hormones, and enzymes that support growth and development of the conceptus.

Conceptus refers to the embryo/fetus and its associated placental membranes.

Placenta refers to the amnion, allantois, and chorion that collectively form the unit for the exchange of nutrients and gases between the maternal and fetal–placental vascular systems.

Umbilicus is the stalk connecting the fetus to the placenta. It contains major blood vessels and the urachus that connects the fetal bladder and allantoic sac.

Urachus is a duct that transports the contents of the bladder into the allantoic sac. This fluid from the bladder includes water, urea, proteins, nutrients, and other molecules that are cleared by the fetal kidney into the bladder.

Introduction

The allantoic membrane forms as an evagination of the hindgut (endoderm) as a diverticulum covered by mesoderm (splanchnopleure) (see Fig. 1). The allantoic membrane dilates and extends outside the embryo/fetus except for attachment by the umbilical stalk that contains the urachus for communication between the fetal bladder and allantoic sac. The allantoic membrane acquires an extensive blood supply from caudal branches of the aorta that break up into an extensive vascular net. The allantois and chorion fuse early in gestation so that the vascular system of the allantoic membrane supplies the entire chorioallantoic placenta.

The placental membranes of most subprimate mammals are of the chorioallantoic type that includes the yolk sac, amnion, allantois, and chorion (Perry, 1981). The yolk sac develops from about day 14, is maximum in size by about day 18, and becomes

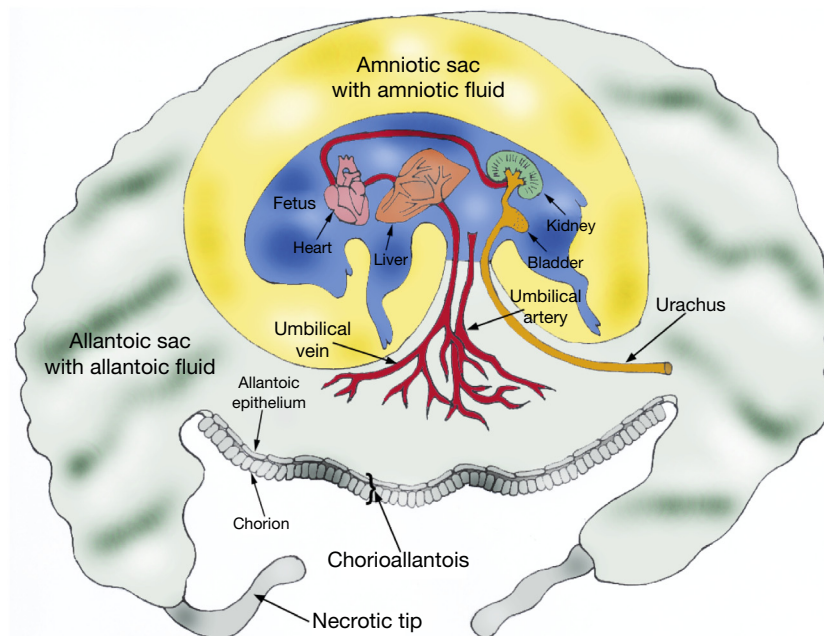


Fig. 1 Diagram of the fetal membranes depicting outgrowth of the allantoic membrane from the hindgut. The drawing indicates that the vasculature of the placenta resides in the allantois. Water, sugars, amino acids, and proteins are cleared from the fetal–placental circulation by the kidney into the bladder, and the contents of the bladder enter the allantoic sac via the urachus. This allows most components of the so-called urine, such as glucose, fructose, and amino acids, to be recycled. That is, those nutrients and many of the proteins can reenter the fetal–placental circulation to nourish the fetal–placental tissues. Thus, the allantoic sac is a major reservoir of nutrients rather than a reservoir for wastes as claimed in many textbooks.

vestigial after day 20 of pregnancy. Soon after the hindgut of the embryo is established, a diverticulum known as the allantois develops from the splanchnopleure. Thus, the allantoic membrane forms as a diverticulum of the hindgut (endoderm) covered by mesoderm (splanchnopleure). The allantoic membrane dilates and extends outside the embryo/fetus except for attachment by the umbilical stalk that contains the urachus for communication between the allantoic sac and fetal bladder (Bazer, 1989).

The porcine allantoic epithelium is a simple columnar epithelium with angular cells and a fine foamy cytoplasm containing large amounts of glycogen and small lipid granules (Naaktgeboren et al., 1975). The glycogen disappears in late gestation. The allantoic epithelium of pigs is dimorphic granular cells and mitochondria-rich cells with the ratio of granular cells predominating at a ratio of about 40:1. The granular cells may release proteoglycans, which provide a mucous covering of the epithelium. These cells are asymmetrical due to a thickening of the apical plasmalemma; however, the significance of the asymmetry is not known. Similarly, the function of the mitochondria-rich cells is unknown, but they are rich in cAMP and may also play a role in the regulation of water and solute flux. The allantoic sac was originally considered a reservoir for fetal urine, and the mesonephric glomeruli were thought to be the source of allantoic fluid (Bazer and First, 1983; Buhi et al., 1983). However, it is now well established that the urinary system does not make water, but only redistributes available water that must be derived from the maternal system. Water may enter the allantoic sac from the embryo/fetus via the kidney, bladder, and urachus, or water may be transported directly across the chorioallantois into the allantoic sac (Gresham et al., 1972). Ligation of the urachus in sheep fetuses between days 85 and 127 of gestation results in the disappearance of allantoic fluid, which indicates that water and other molecules enter the allantoic sac via the urinary system of the fetus.

Functions of the Allantoic Membrane

The allantoic membrane acquires an extensive blood supply from caudal branches of the aorta that break up into an extensive vascular network. The allantois and chorion fuse early in gestation so that the vascular system of the allantoic membrane supplies the entire chorioallantoic placenta. In the pig, the allantoic membrane does not expand to fill the entire chorionic vesicle; therefore, the extreme ends of the chorion become necrotic due to the lack of a vascular supply from the allantoic membrane (see Fig. 1). The allantoic membrane forms a sac and accumulates allantoic fluid, which is of both maternal and fetal (urine) origin. The components of allantoic fluid include water, glucose, fructose, amino acids, electrolytes, proteins, hormones, and growth factors, which accumulate for immediate recycling into the fetal circulation (e.g., fetal transferrin), for use as metabolic substrates (e.g., glucose and fructose), or for synthesis of peptides and proteins (amino acids) by the conceptus. The allantoic membrane is derived from splanchnopleure (mesoderm and endoderm) and can transport nutrients into the fetal circulation.

The transports of water, electrolytes, and nutrients into the allantoic sac are influenced by the presence of aquaporins, sex steroids (estrogen and progesterone), and extracellular matrix proteins such as secreted phosphoprotein 1 (also known as osteopontin) (see Bazer, 1989). Transport of water into the allantois expands the allantoic sac, which brings the allantoic membrane into apposition with the chorion and then the chorioallantoic placenta into intimate contact with the maternal uterine endometrium. Temporal changes in allantoic fluid volume are accompanied by essentially identical changes in the total amounts of glucose and fructose in the allantoic sac of sheep and swine. Total protein in allantoic fluid of sheep increases throughout gestation, which is consistent with evidence that secretory activity of uterine endometrial glands increases throughout gestation. Secretions of the uterine glands are transported into the fetal-placental circulation via the areolae and enter the allantoic sac via the urachus if not metabolized by the fetus. Total protein in allantoic fluid of pigs increases from day 20 to day 75 of gestation and then decreases to term (day 114 of gestation). This is true for proteins in general and for progesterone-induced proteins such as uteroferrin (also known as acid phosphatase type 5 and tartrate-resistant acid phosphatase). Uteroferrin is secreted by the uterine glandular epithelium in response to progesterone, transported into the fetal circulation, and cleared into the allantoic sac if not taken up by reticuloendothelial cells of the fetal liver. The role of uteroferrin is to transport iron from the uterine endometrial glands to the fetal-placental unit, and there is evidence that it stimulates hematopoiesis (Bazer et al., 1991; Ying et al., 2014).

Humans Lack an Allantoic Membrane

In humans, the allantoic membrane does not form a sac filled with allantoic fluid (Perry, 1981). Rather, the allantoic membrane is represented by an endodermal outgrowth from the hindgut that fuses with the chorion and develops blood vessels, thus participating in the formation of the umbilical cord.

References

- Bazer, F. W. (1989). Allantoic fluid: Regulation of volume and composition. In R. A. Brace (Ed.), *Fetal and neonatal body fluids* (pp. 135–157). Cornell, NY: Perinatology Press.
- Bazer, F. W., & First, N. L. (1983). Pregnancy and parturition. *Journal of Animal Science*, 57(Suppl 2), 425–459.
- Bazer, F. W., Worthington-White, D., Fliss, M. F. V., & Gross, S. (1991). Uteroferrin: A progesterone-induced hematopoietic growth factor of uterine origin. *Experimental Hematology*, 19, 910–915.
- Buhi, W. C., Ducsay, C. A., Bartol, F. F., Bazer, F. W., & Roberts, R. M. (1983). A function of the allantoic sac in the metabolism of uteroferrin and maternal iron by the fetal pig. *Placenta*, 4, 455–470.

- Gresham, E. L., Rankin, J. H. G., Makowski, E. L., Meschia, G., & Battaglia, F. C. (1972). An evaluation of fetal renal function in a chronic sheep preparation. *Journal of Clinical Investigation*, 51, 149–156.
- Naaktgeboren, C., DeVries, I. J., Stegeman, J. H. J., Kok, K., & Beelen, R. (1975). Developmental influences on the composition of fetal fluids in sheep, with special reference to dysmaturity. *Zeitschrift Fur Tierzucht Und Zuchtungs Biologie*, 92, 51–56.
- Perry, J. S. (1981). The mammalian fetal membranes. *Journal of Reproduction and Fertility*, 62, 321–335.
- Ying, W., Bazer, F. W., & Zhou, B. (2014). Uteroferrin enhances fetal erythropoiesis at terminal stages. *Endocrinology*, 155, 4521–4530.

The Amnion and Amniotic Fluid

Aisling Murphy and Brian Koos, University of California, Los Angeles, Los Angeles, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Throughout gestation, the amniotic fluid (AF) not only protects the fetus from trauma, but also permits normal fetal growth and movement, and prevents compression of the umbilical cord. Its bacteriostatic properties defend against infection and analysis of AF enables prenatal genetic diagnosis. AF also provides an acoustic interface that facilitates fetal ultrasound imaging. Disorders of amniotic fluid volume are among the most common obstetric complications and arise from a diverse number of underlying pathological processes. This chapter reviews amnion embryology, AF volume regulation and clinical disorders of amniotic fluid volume.

Embryology

Humans belong to amniotes, a large vertebrate subgroup with a fluid-containing membrane (amnion) encapsulating the embryo. The development of an amnion enabled our remote tetrapod ancestors to lay their eggs on land rather than in water, and therefore ranks as among the most important evolutionary developments (Hickman et al., 2013).

The human blastocyst contains an outer layer of trophoctodermal cells and an inner cell mass. Before implantation, the inner cell mass differentiates into two layers. Epiblast, the outer upper layer, is the morphologic precursor to the amnion, which ultimately encompasses the entire embryo and surrounding fluid. The hypoblast underlying the epiblast forms the endodermal lining of the yolk sac. Developing ventral to the epiblast, the extra-embryonic mesoderm becomes the inner layer of the chorionic membrane (Fig. 1; Carlson, 2014). The trophoctodermal cells of the cytotrophoblast and syncytiotrophoblast form the outer layer.

The enlarging amnion completely surrounds the embryo upon merging with the body stalk, a precursor of the umbilical cord. The amnion forms the epithelial layer of the umbilical cord. Surrounding the amnion is the chorionic cavity or extra-embryonic coelom, which is formed within the extra-embryonic mesoderm (Fig. 2; Carlson, 2014). In early gestation, the coelomic cavity is filled with fluid, but at the end of the first trimester (12–14 weeks' gestation) the enlarging amnion fuses with the chorion, obliterating the coelomic cavity.

Amniotic Fluid Circulation in Early Gestation

The coelomic cavity, (secondary) yolk sac, and amniotic cavity are present in the first-trimester. Coelomic fluid (CF) has a similar composition to maternal plasma, suggesting that CF is an ultrafiltrate of maternal plasma (Jauniaux and Gulbis, 2000).

The very early origin of amniotic fluid is CF rather than the fetus, since AF is present in anembryonic gestations (Seeds, 1980). Later on in the first trimester, the compositions of CF and AF differ significantly (Campbell et al., 1992, 1993; Jauniaux and Gulbis, 2000), and at this time transudation of fluid and nutrients across the non-keratinized epithelium appears to be a major source of AF (Jauniaux et al., 1994). The magnitude of contribution from the maternal circulation across the chorion and amnion (transmembranous pathway) in the first trimester is unknown.

Amniotic Fluid Circulation in Late gestation

Fetal Urine

Fetal urine contributes to AF volume when the kidneys begin to function by the end of the first trimester. Fetal urine becomes the major source of AF once fetal skin is keratinized in the second trimester. The volume of urine production has been estimated using ultrasound assessment of fetal bladder volume. These data suggest that fetal urine output increases from 110 mL/kg fetal weight/day at 25 weeks up to approximately 200 mL/kg fetal weight/day at term (Rabinowitz et al., 1989; Fägerquist et al., 2001). Fetal urine production decreases after 40 weeks' gestation (Abramovich et al., 1979; Wladimiroff and Campbell, 1974).

Fetal Lung Fluid

Another major source of AF is the secretion of pulmonary fluids. Based on animal studies, fetal lungs excreted about 60–100 mL/kg fetal weight/day (Gilbert and Brace, 1993). About half of the volume flows into the amniotic cavity, while the other portion is swallowed.

Fetal lung fluid differs from urine in having a higher chloride content, and lower concentration of protein and bicarbonate. Pulmonary phospholipids are present, particularly in late gestation. These phospholipids are critical for postnatal lung function, and levels in AF can provide a risk assessment for neonatal respiratory distress syndrome.

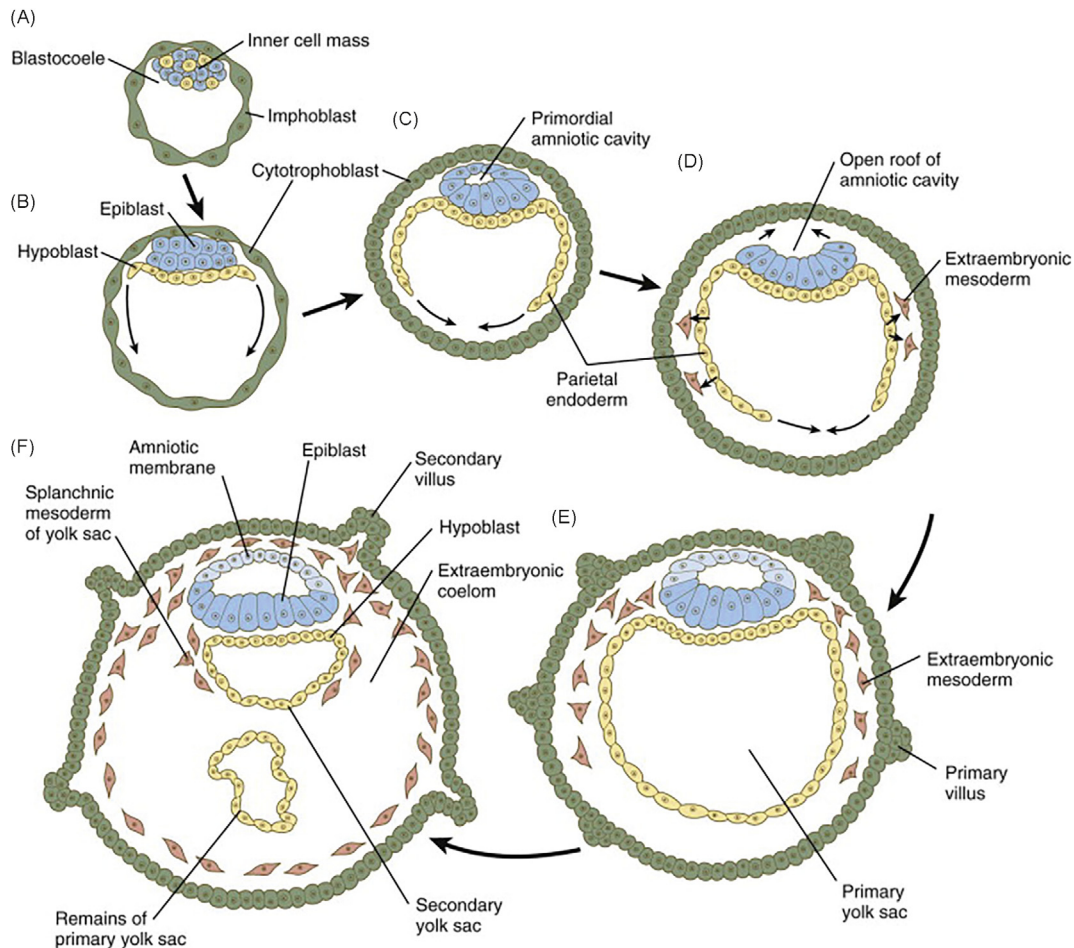


Fig. 1 (A) Late blastocyst. (B) Beginning of implantation at 6 days. (C) Implanted blastocyst at 7.5 days. (D) Implanted blastocyst at 8 days. (E) Embryo at 9 day. (F) Late second week. Reproduced from Carlson, B. (2014). *Formation of germ layers and early derivatives. Human embryology and developmental biology* (5th edn.) Elsevier (Chapter 5).

Lung fluid secretion is essential for normal lung development. Cortisol and catecholamines lower lung fluid production. Neonates who have undergone the forces of vaginal delivery have higher levels of these hormones than those delivered by cesarean. The higher hormone concentrations enhance clearance of lung fluid as the fetus transitions to postnatal life and likely account for the lower rates of transient tachypnea in vaginally delivered newborns.

Very little amniotic fluid (< 2 mL) is normally exchanged per single breathing movement. But the train of breaths in a breathing episode increases lung volume, and this expansion contributes to lung growth (Harding and Hooper, 1996). Hypoxia normally inhibits breathing movements, but severe hypoxia/asphyxia can elicit intermittent, strong gasping efforts. Large transthoracic pressure gradients produced by isolated gasps can result in a net inflow of amniotic fluid. This phenomenon is supported by autopsies of stillborn infants revealing meconium in the pulmonary tissue (Brown and Gleicher, 1981) and by the finding of meconium aspiration syndrome in neonates without postnatal aspiration (Byrne and Gau, 1987; Sarno Jr et al., 1990).

Intramembranous Pathway

The intramembranous pathway involves absorption of AF across the vasculature of the chorionic plate into the fetal circulation. The amount of fluid passing through this pathway is highly variable, ranging from 200 to 500 mL per day (Wintour and Shandley, 1993) (Fig. 3).

Transmembranous Pathway

The transmembranous pathway involves fluid passage through fetal membranes to the maternal blood in capillaries within the uterine wall. This pathway has a minimal effect of AF volume with only about 10 mL/day transferred at term (Gilbert and Brace, 1993; Seeds, 1980).

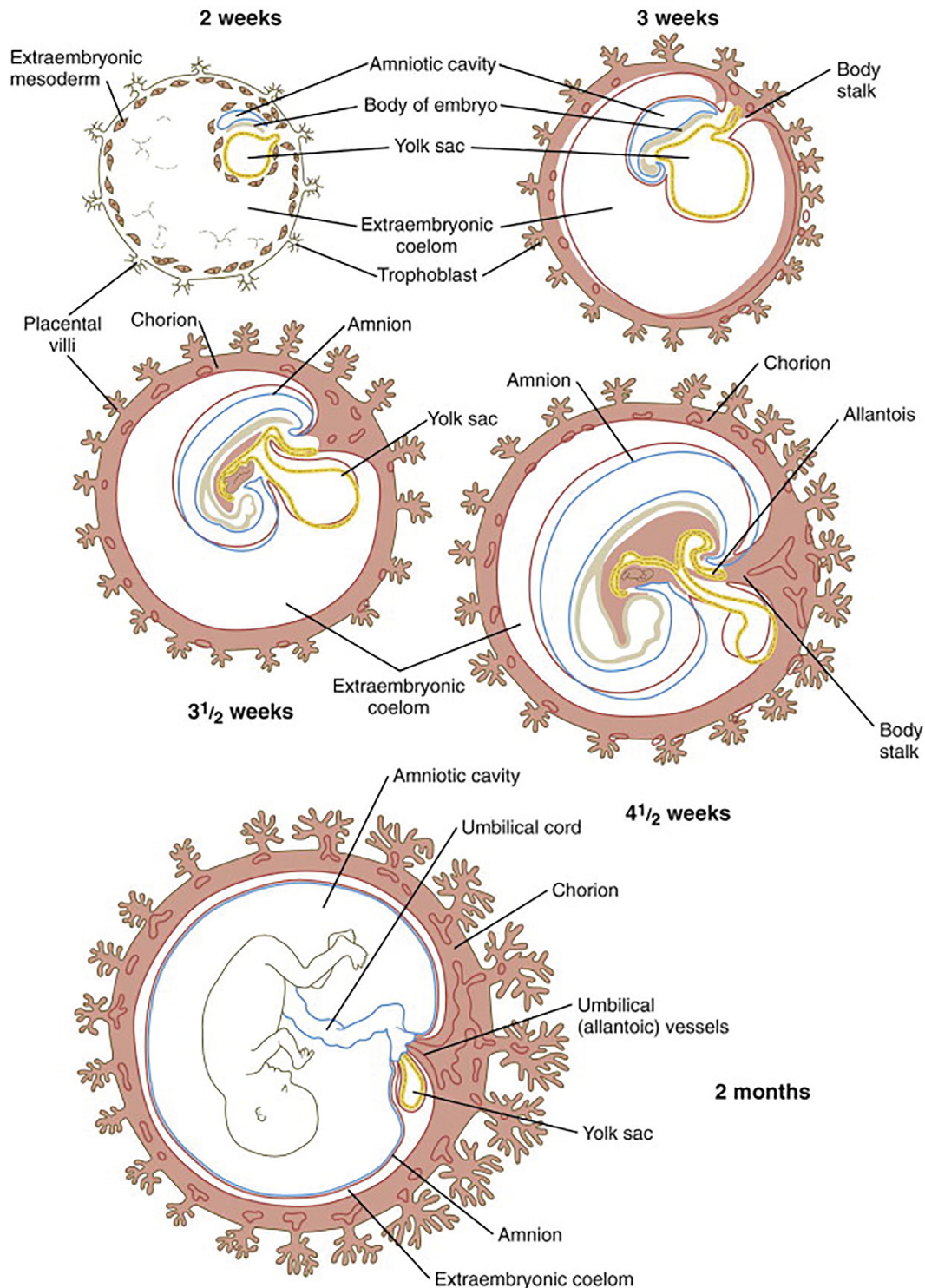


Fig. 2 Origins of the major extraembryonic tissues. Reproduced from Carlson, B. (2014). Formation of germ layers and early derivatives. *Human embryology and developmental biology* (5th edn.) (Chapter 5).

Fetal Swallowing

Fetal swallowing is the predominant AF outflow, about 200–250 mL/kg fetal weight/day (Abramovich et al., 1979). Although swallowing activity can be observed in late first trimester, these movements become well-coordinated with behavioral states only in later gestation (Grassi et al., 2005). Dipsogenic (thirst) and orexigenic (appetite) pathways stimulate swallowing activity in near-term fetal sheep (Xu et al., 2001; El-Haddad et al., 2004). Fetal swallowing is also augmented by increased volume of AF, decreased

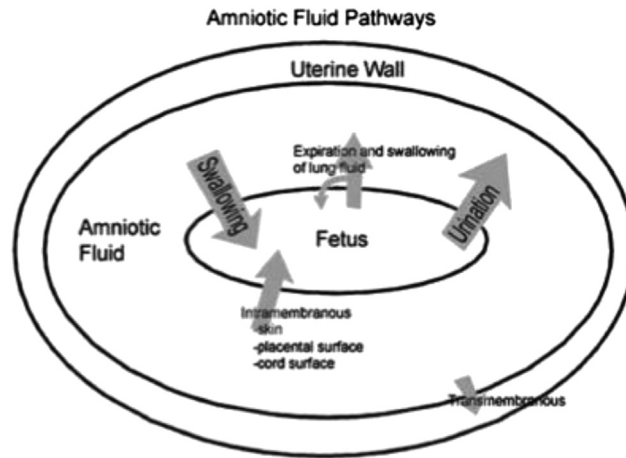


Fig. 3 Amniotic fluid pathways. Reproduced from Underwood, M. A., Gilbert, W. M. and Sherman, M. P. (2005). Amniotic fluid: Not just fetal urine anymore. *Journal of Perinatology* 25(5), 341–348.

AF osmolarity and elevated fetal plasma osmolarity (Moore, 2010). On the other hand, fetal swallowing diminishes in response to oligohydramnios, hypotension, and hypoxia (Brace et al., 1994; Sherman et al., 1991).

Fetal CNS lesions (e.g., anencephaly) and some neuromuscular disorders can elicit polyhydramnios by inhibiting fetal swallowing. Likewise, conditions interrupting passage of AF through the gastrointestinal tract (e.g., esophageal or duodenal atresia) can also cause polyhydramnios. In obstruction cases without polyhydramnios, AF volume is likely normalized via the intramembranous pathway.

Composition of Amniotic Fluid

Early Gestation (First Trimester)

AF is isotonic with fetal plasma with very low protein concentrations except for α -fetoprotein. AF is a milieu of low oxygen tension. High levels of polyols are present, suggesting activity of the polyol pathway, which is involved in generating NAD⁺ from NADH. Given the low oxygen tensions of embryonic tissues, increased NADH production allows glycolysis to continue, while limiting lactate generation and fall in pH (Jauniaux et al., 2005). This pathway is also involved in the production of precursors for nucleic acids, important in rapidly dividing tissues.

Later Gestation (Second and Third Trimesters)

With keratinization of fetal skin, AF approximates urine in composition, containing carbohydrates, proteins, amino acids, and lipids, as well as the electrolytes, enzymes, and hormones (Underwood et al., 2005). Amino acids levels drop across gestation and are lower than serum levels, with the exception of taurine (Reid et al., 1971; Kerr and Kennan, 1969; Cockburn et al., 1970). In the ovine fetus, swallowed AF amino acids appear important in the development of the intestinal mucosa (Kwon et al., 2003). Swallowed AF lipid and carbohydrates unlikely contribute significantly to fetal nutrition, and nutritional supplementation via AF does not improve weight-gain in experimental fetal growth restriction (Buchmiller et al., 1994).

AF contains multiple growth factors including epidermal growth factor, transforming growth factor alpha, transforming growth factor beta-1, insulin-like growth factor I, erythropoietin, and granulocyte colony stimulating factor. These substances appear to have significant trophic effects in the intestinal mucosa (Underwood et al., 2005). Antimicrobial substances are also present in AF, such as α -defensins [HNP1–3] and lactoferrin (Underwood et al., 2005).

Amniotic Fluid Volume

Based on dye dilution studies, AF volume normally rises progressively from early pregnancy to about 32–34 weeks and thereafter declines until delivery (Brace and Wolf, 1989). The normal range of AF across gestation is quite broad. The decline in AF volume may involve relative changes in swallowed fluid relative urine production as well as alterations in fluid transfer across the intramembranous pathway (Fig. 4).

Regulation of Amniotic Fluid Volume

AF volume changes little from day to day, despite the daily fluid turnover. Thus, AF volume is regulated. Fetal sheep studies indicate that the intramembranous pathway is the primary controller of AF volume (Brace and Cheung, 2014). Putative components of this

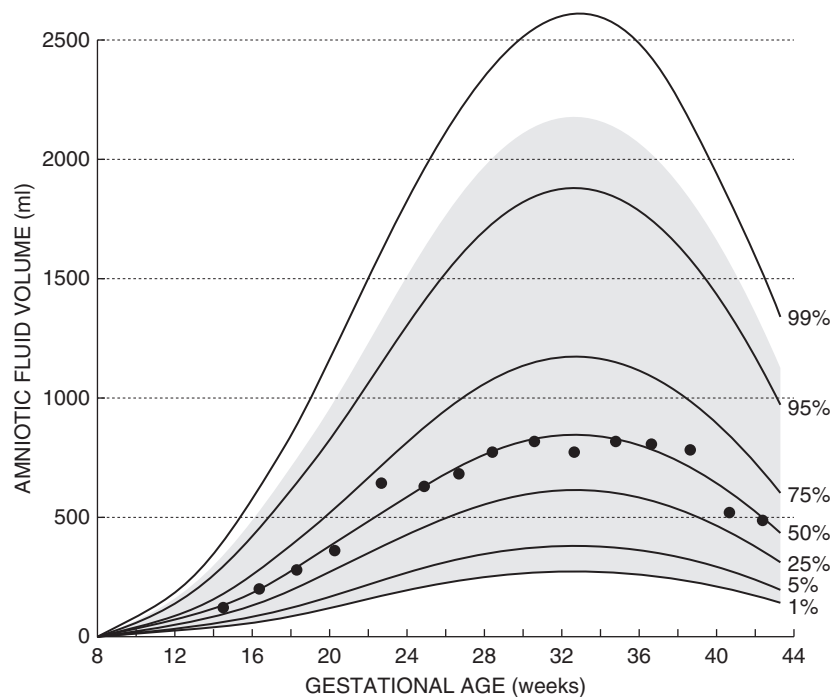


Fig. 4 Amniotic fluid volumes as a function of gestational age. Shaded area covers 95% confidence interval. Reproduced from Brace, R. A., Wolf, E. J. (1989). Normal amniotic fluid volume changes throughout pregnancy. *American Journal of Obstetrics and Gynecology* **161**, 382.

regulatory pathway include chorioamnion vascular endothelial growth factor (Matsumoto et al., 2001; Feng et al., 1999; Cheung et al., 2010; Shandley et al., 1997), transmembrane water channels (aquaporins) (Brace and Wolf, 1989; Mann et al., 2006; Beall et al., 2007) absorption inhibitors in fetal urine, intra-amniotic prostaglandin E₂ via its stimulation of vascular endothelial growth factor production in the fetal membranes and membrane stretch via a negative feedback effect (Brace and Cheung, 2014).

Assessment of Amniotic Fluid Volume

Estimation of AF volume involves ultrasound measurement of the maximum vertical pocket or the four quadrant sum of the deepest vertical pockets, the amniotic fluid index (AFI). Excessive AF volume (polyhydramnios) is generally identified with a single pocket > 8 cm or AFI > 25 cm with low volumes (oligohydramnios) having a single pocket < 2 cm or AFI < 5 cm. These extremes of AF volume have important clinical implications.

Polyhydramnios. Complicating about 1% of pregnancies, polyhydramnios is associated with variety of conditions including maternal diabetes mellitus, malformations which may inhibit fluid passage through the gastrointestinal tract, neuromuscular impairments which may inhibit fetal swallowing, and fetal anemia (Table 1). About 40% of cases are unexplained. Polyhydramnios increases the risk of a number of complications, including preterm labor, cesarean delivery, stillbirth, cord prolapse, and postpartum hemorrhage (Biggio Jr et al., 1999; Pilliod et al., 2015). The enlarged uterus can also cause shortness of breath in the mother.

Oligohydramnios. The progressive fall in AF after about 32 weeks increases the prevalence of oligohydramnios, particularly after 41 weeks' gestation. The normal decline in AF volume is likely due to changes in fluid transfer via the intramembranous pathway. Oligohydramnios can also accompany premature (before onset of contractions) rupture of the membranes, fetal growth restriction, renal disorders, and chronic disruptions in placental function. Early onset, severe oligohydramnios greatly increases risk of pulmonary dysplasia and compression abnormalities of the fetus. Preterm (< 37 weeks) premature rupture of the membranes only complicates 1%–2% of pregnancies but is a major cause of preterm birth and its adverse sequelae. Even at term, oligohydramnios increases fetal morbidity and mortality (Shipp et al., 1996; Sarno Jr et al., 1990; Chauhan et al., 1999) (Table 2).

Summary

The amnion arises from the epiblast cells of the blastocyst and grows to surround the developing embryo, creating a fluid-filled cavity. Thus, throughout prenatal life, humans are surrounded by AF. This fluid serves many functions critical for prenatal development. In very early gestation AF appears to arise from the nearby coelomic cavity. As the fetus grows, the transudation of fluid across the fetal skin becomes an important source of fluid. In second trimester, after keratinization of the fetal skin, fetal urine becomes the

Table 1 Causes of polyhydramnios

Idiopathic
Fetal macrosomia
Maternal diabetes
Gastrointestinal anomalies, e.g., cleft palate, esophageal atresia, duodenal atresia, annular pancreas, jejunal atresia, volvulus, gastroschisis
CNS lesions/musculoskeletal disorders, e.g., anencephaly, hydranecephaly, microcephaly, encephalocele, myoclonic dystrophy, Pena-Shokeir syndrome, fetal akinesia syndrome
Obstructive chest lesions, e.g., congenital diaphragmatic hernia, CPAM
Hydrops/fetal high output cardiac failure—isoimmunization, congenital cardiac lesions, fetal arrhythmias, TORCH infections, Alpha thalassemia, fetal anemia, sacrococcygeal teratoma, chorioangioma
Twin to twin transfusion syndrome
Aneuploidy—trisomy 18, trisomy 21
Skeletal dysplasias—osteogenesis imperfecta, thanatophoric dysplasia
Renal disorders—unilateral uteropelvic junction obstruction, Bartter syndrome

Table 2 Causes of oligohydramnios

Rupture of membranes
Placental insufficiency/fetal growth restriction/placental abruption
Postterm pregnancy
Fetal urinary tract anomalies, e.g., renal agenesis, multicystic dysplastic kidneys, polycystic kidneys, fetal bladder outflow tract obstruction, bilateral ureteropelvic junction obstruction
Maternal medications, e.g., prostaglandin synthetase inhibitors, angiotensin converting enzyme inhibitors
Chromosomal anomalies
Other causes, e.g., donor twin in twin to twin transfusion syndrome, fetal demise

primary source of AF, with a lesser contribution from lung fluid. Fluid is removed from the cavity by fetal swallowing and via absorption back into the fetal circulation, known as the intramembranous pathway.

AF volume normally rises to a peak in the early third trimester, and then subsequently declines. Estimation of AF volume can be made by use of prenatal ultrasound and disorders of AF volume are common obstetric complications.

References

- Abramovich, D. R., Garden, A., Jandial, L., et al. (1979). Fetal swallowing and voiding in relation to hydramnios. *American Journal of Obstetrics and Gynecology*, 54, 15.
- Beall, M. H., et al. (2007). Placental and membrane aquaporin water channels: Correlation with amniotic fluid volume and composition. *Placenta*, 28(5–6), 421–428.
- Biggio, J. R., Jr., Wenstrom, K. D., Dubard, M. B., & Cliver, S. P. (1999). Hydramnios prediction of adverse perinatal outcome. *Obstetrics and Gynecology*, 94(5 Pt 1), 773–777.
- Brace, R. A., & Cheung, C. Y. (2014). Regulation of amniotic fluid volume evolving concepts. In L. Zhang, & C. A. Ducey (Eds.), *Advances in experimental medicine and biology* 814 *Advances in fetal and neonatal physiology*. New York: Springer Science + Business Media. https://doi.org/10.1007/978-1-4939-1031-1_5.
- Brace, R. A., & Wolf, E. J. (1989). Normal amniotic fluid volume changes throughout pregnancy. *American Journal of Obstetrics and Gynecology*, 161, 382.
- Brace, R. A., Wiodek, M. E., Cock, M. L., & Harding, R. (1994). Swallowing of lung liquid and amniotic fluid by the ovine fetus under normoxic and hypoxic conditions. *American Journal of Obstetrics and Gynecology*, 171(3), 764–770.
- Brown, B. L., & Gleicher, N. (1981). Intrauterine meconium aspiration. *Obstetrics and Gynecology*, 57(1), 26–29.
- Buchmiller, T. L., Kim, C. S., Chopourian, H. L., & Fonkalsrud, E. W. (1994). Transamniotic fetal feeding: Enhancement of growth in a rabbit model of intrauterine growth retardation. *Surgery*, 116, 36–41.
- Byrne, D. L., & Gau, G. (1987). In utero meconium aspiration: An unpreventable cause of neonatal death. *British Journal of Obstetrics and Gynaecology*, 94(8), 813–814.
- Campbell, J., Wathen, N., Macintosh, M., Cass, P., Chard, T., & Mainwaring Burton, R. (1992). Biochemical composition of amniotic fluid and extraembryonic coelomic fluid in the first trimester of pregnancy. *British Journal of Obstetrics and Gynaecology*, 99(7), 563.
- Campbell, J., Wathen, N., Perry, G., Soneji, S., Sourial, N., & Chard, T. (1993). The coelomic cavity: An important site of materno-fetal nutrient exchange in the first trimester of pregnancy. *British Journal of Obstetrics and Gynaecology*, 100(8), 765.
- Carlson, B. (2014). Human embryology and developmental biology. In *Formation of germ layers and early derivatives* (5th edn). New York: Elsevier. Chapter 5.
- Chauhan, S. P., Sanderson, M., Hendrix, N. W., Magann, E. F., & Devoe, L. D. (1999). Perinatal outcome and amniotic fluid index in the antepartum and intrapartum periods: A meta-analysis. *American Journal of Obstetrics and Gynecology*, 181(6), 1473–1478.
- Cheung, C. Y., Li, S., Chen, D., & Brace, R. A. (2010). Regulation of caveolin-1 expression and phosphorylation by VEGF in ovine amnion cells. *Reproductive Sciences*, 17, 1112–1119.
- Cockburn, F., Robins, S. P., & Forfar, J. O. (1970). Free amino-acid concentrations in fetal fluids. *British Medical Journal*, 3(5725), 747–750.
- El-Haddad, M. A., Desai, M., Gayle, D., & Ross, M. G. (2004). In utero development of fetal thirst and appetite: Potential for programming. *Journal of the Society for Gynecologic Investigation*, 11(3), 123–130.
- Fägerquist, M., Fägerquist, U., Odén, A., & Blomberg, S. G. (2001 Feb). Fetal urine production and accuracy when estimating fetal urinary bladder volume. *Ultrasound in Obstetrics & Gynecology*, 17(2), 132–139.
- Feng, D., Nagy, J. A., Pyne, K., Hammel, I., Dvorak, H. F., & Dvorak, A. M. (1999). Pathways of macromolecular extravasation across microvascular endothelium in response to VPF/VEGF and other vasoactive mediators. *Microcirculation*, 6(1), 23–44.

- Gilbert, W. M., & Brace, R. A. (1993). Amniotic fluid volume and normal flows to and from the amniotic cavity. *Seminars in Perinatology*, 17, 150–157.
- Grassi, R., Farina, R., Fioriani, I., Amodio, F., & Romano, S. (2005). Assessment of fetal swallowing with gray-scale and color Doppler sonography. *AJR. American Journal of Roentgenology*, 185(5), 1322–1327.
- Harding, R., & Hooper, S. B. (1996). Regulation of lung expansion and lung growth before birth. *Journal of Applied Physiology (Bethesda, MD: 1985)*, 81(1), 209–224.
- Amniote origins and nonavian reptiles. In Hickman, C., Roberts, L., Keen, S., et al. (Eds.), *Integrated principles of zoology* (16th edn), (2013). Boston, MA: McGraw-Hill. Chapter 28.
- Jauniaux, E., & Gulbis, B. (2000). Fluid compartments of the embryonic environment. *Human Reproduction Update*, 6(3), 268–278.
- Jauniaux, E., Gulbis, B., Jurkovic, D., et al. (1994). Relationship between protein levels in embryological fluids and yolk sac size during early human pregnancy. *Human Reproduction*, 9, 1175–1179.
- Jauniaux, E., Hempstock, J., Teng, C., Battaglia, F. C., & Burton, G. J. (2005). Polyol concentrations in the fluid compartments of the human conceptus during the first trimester of pregnancy: Maintenance of redox potential in a low oxygen environment. *The Journal of Clinical Endocrinology and Metabolism*, 90(2), 1171–1175.
- Kerr, G. R., & Kennan, A. L. (1969). The free amino acids of amniotic fluid during pregnancy of the rhesus monkey. *American Journal of Obstetrics and Gynecology*, 105(3), 363–367.
- Kwon, H., Wu, G., Bazer, F. W., & Spencer, T. E. (2003). Developmental changes in polyamine levels and synthesis in the ovine conceptus. *Biology of Reproduction*, 69, 1626–1634.
- Mann, S. E., Dvorak, N., Gilbert, H., & Taylor, R. N. (2006). Steady-state levels of aquaporin 1 mRNA expression are increased in idiopathic polyhydramnios. *American Journal of Obstetrics and Gynecology*, 194, 884–887.
- Matsumoto, L. C., Bogic, L., Brace, R. A., & Cheung, C. Y. (2001). Fetal esophageal ligation induces expression of vascular endothelial growth factor messenger ribonucleic acid in fetal membranes. *American Journal of Obstetrics and Gynecology*, 184(2), 175–184.
- Moore, T. R. (2010). Amniotic fluid dynamics reflect fetal and maternal health and disease. *Obstetrics and Gynecology*, 116(3), 759–765.
- Pilliod, R. A., Page, J. M., Burwick, R. M., Kaimal, A. J., Cheng, Y. W., & Caughey, A. B. (2015). The risk of fetal death in nonanomalous pregnancies affected by polyhydramnios. *American Journal of Obstetrics and Gynecology*, 213(3), 410.e1–6, Epub 2015 May 14.
- Rabinowitz, R. I., Peters, M. T., Vyas, S., Campbell, S., & Nicolaides, K. H. (1989). Measurement of fetal urine production in normal pregnancy by real-time ultrasonography. *American Journal of Obstetrics and Gynecology*, 161(5), 1264–1266.
- Reid, D. W., Campbell, D. J., & Yakymyshyn, L. Y. (1971). Quantitative amino acids in amniotic fluid and maternal plasma in early and late pregnancy. Preliminary report. *American Journal of Obstetrics and Gynecology*, 111(2), 251–258.
- Sarno, A. P., Jr., Ahn, M. O., & Phelan, J. P. (1990). Intrapartum amniotic fluid volume at term. Association of ruptured membranes, oligohydramnios and increased fetal risk. *The Journal of Reproductive Medicine*, 35(7), 719–723.
- Seeds, A. E. (1980). Current concepts of amniotic fluid dynamics. *American Journal of Obstetrics and Gynecology*, 138, 575–586.
- Shandley, L., Alcorn, D., & Wintour, E. M. (1997). Ovine amniotic and allantoic epithelia across gestation. *The Anatomical Record*, 248, 542–553.
- Sherman, D. J., Ross, M. G., Day, L., Humme, J., & Ervin, M. G. (1991). Fetal swallowing: Response to graded maternal hypoxemia. *Journal of Applied Physiology (Bethesda, MD: 1985)*, 71(5), 1856–1861.
- Shipp, T. D., Bromley, B., Pauker, S., Frigoletto, F. D., Jr., & Benacerraf, B. R. (1996). Outcome of singleton pregnancies with severe oligohydramnios in the second and third trimesters. *Ultrasound in Obstetrics & Gynecology*, 7(2), 108–113.
- Underwood, M. A., Gilbert, W. M., & Sherman, M. P. (2005). Amniotic fluid: Not just fetal urine anymore. *Journal of Perinatology*, 25(5), 341–348.
- Wintour, E. M., & Shandley, L. (1993). Effects of fetal fluid balance on amniotic fluid volume. *Seminars in Perinatology*, 17, 158–172.
- Wladimiroff, J. W., & Campbell, S. (1974). Fetal urine-production rates in normal and complicated pregnancy. *Lancet*, 1, 151.
- Xu, Z., Nijland, M. J., & Ross, M. G. (2001 May). Plasma osmolality dipsogenic thresholds and c-fos expression in the near-term ovine fetus. *Pediatric Research*, 49(5), 678–685.

Further Reading

- Sunoo, C., Kosasa, T. S., & Hale, R. W. (1989). Meconium aspiration syndrome without evidence of fetal distress in early labor before elective cesarean delivery. *Obstetrics and Gynecology*, 73(5 Pt1), 707–709.

Nutrition and Pregnancy Outcomes

Christian J Bellissimo, McMaster University, Hamilton, ON, Canada

Mark H Vickers, University of Auckland, Auckland, New Zealand

Deborah M Sloboda, McMaster University, Hamilton, ON, Canada

© 2018 Elsevier Inc. All rights reserved.

Glossary

Adiposity rebound The point during childhood at which body fatness declines to its minimum point, before rising again into adulthood. The age of adiposity rebound is typically 3–7 years of age.

Body mass index (BMI) A classification measure used for defining an individual as underweight, normal weight, overweight or obese; calculated as body weight (kg)/height (m)².

Epigenetics The study of environmental influences on a phenotypic outcome through modulation of gene expression by way of post-transcriptional regulation of mRNA, post-translational modifications to DNA associated proteins, and/or chemical modification of DNA without changing the underlying genetic code.

Hypoxia Deficiency in the amount of oxygen available to a tissue, usually induced either by lack of oxygen reaching tissues, or by increased metabolic demand beyond what can be sustained by oxidative metabolism.

Methyl donor Molecules which partake in enzymatic transfer of a methyl (–CH₃) group to another recipient molecule (i.e., DNA).

Non-communicable disease (NCD) A chronic medical condition that is not caused by an infectious agent and is thereby not transmissible from one individual to another.

Oxidative stress A state of imbalance in which cells produce free radicals (including reactive oxygen species) at rates faster than they can be metabolized/detoxified. This results in an accumulation of reactive intermediates, imbalance in cellular redox state, and damage to cellular components, including nucleic acids and organelles.

Ponderal index (PI) A measure of whole body adiposity or thinness; calculated as body weight (kg)/height (m)³.

Socioeconomic status (SES) A sociological measure of a person's or family's social and economic position relative to others. The main factors considered when determining SES are income, level of education, and occupation.

The metabolic syndrome A cluster of symptoms indicative of risk for the development of serious, life-threatening health complications. Symptoms include central obesity, hypertriglyceridemia, reduced high-density lipoprotein levels, hypertension, and elevated fasting blood glucose levels. Possessing three or more of these symptoms qualifies a diagnosis of this syndrome.

Nutrition, Pregnancy, and Disease—A Developmental Origins Hypothesis

Adequate nutrition during pregnancy has long been appreciated for its importance in promoting fetal growth and development up to the time of birth. It is only within the last three decades, however, that the impacts of periconceptional nutrition on embryonic and fetal development have emerged as key determinants of later life health and chronic disease risk. Some of the first evidence to demonstrate the importance of the intrauterine environment in mediating fetal growth and postnatal outcomes came from embryo transfer and artificial insemination experiments, in both humans and other mammals. These studies showed that fetal size at birth was more closely related to the size of the mother carrying the fetus, rather than paternal size, or size of the embryo donor (Brooks et al., 1995; Walton and Hammond, 1938). This evidence strongly supported the prevailing concept of maternal constraint, where maternal size, available nutrient stores, and energy demand, as opposed to genetics, strongly influence fetal growth trajectory in utero and impacted on postnatal phenotype.

More recently, an extended role for nutrition during pregnancy has been established. It is now known that maternal nutrition before and during pregnancy and its impact on fetal development determine not only size at birth, but also long-term growth trajectory and health potential across the life course, a theory known as the Developmental Origins of Health and Disease (DOHaD). Pioneering work in this field came from epidemiologist and physician, Dr. David Barker, where in a nationwide study of mortality rates from cardiovascular disease (CVD) in England and Wales, Barker saw that the areas with the highest incidence of neonatal and infant mortality in the early 20th century were the same areas with the highest incidence of death from CVD > 60 years later (Barker and Osmond, 1986). This finding contrasted with the notion that heart disease was a disease of affluence and nutritional excess, and not due to low socioeconomic status (SES). The findings from this study led Dr. Barker to hypothesize that predispositions to *non-communicable disease* (NCDs) or chronic diseases such as CVD, are established based on the early life environment, including those conditions found in utero. In a subsequent study, Barker and his colleague Charles Nicholas Hales demonstrated that the risk for developing Type 2 diabetes was inversely related to a child's birth weight, a surrogate measure for intrauterine growth and nutrition (Hales et al., 1991, Fig. 1A). Follow up studies in European, North American, and Asian populations have confirmed these same associations (Hales and Barker, 2001). One of the largest, The American Nurses Study, examined the risk of later life CVD

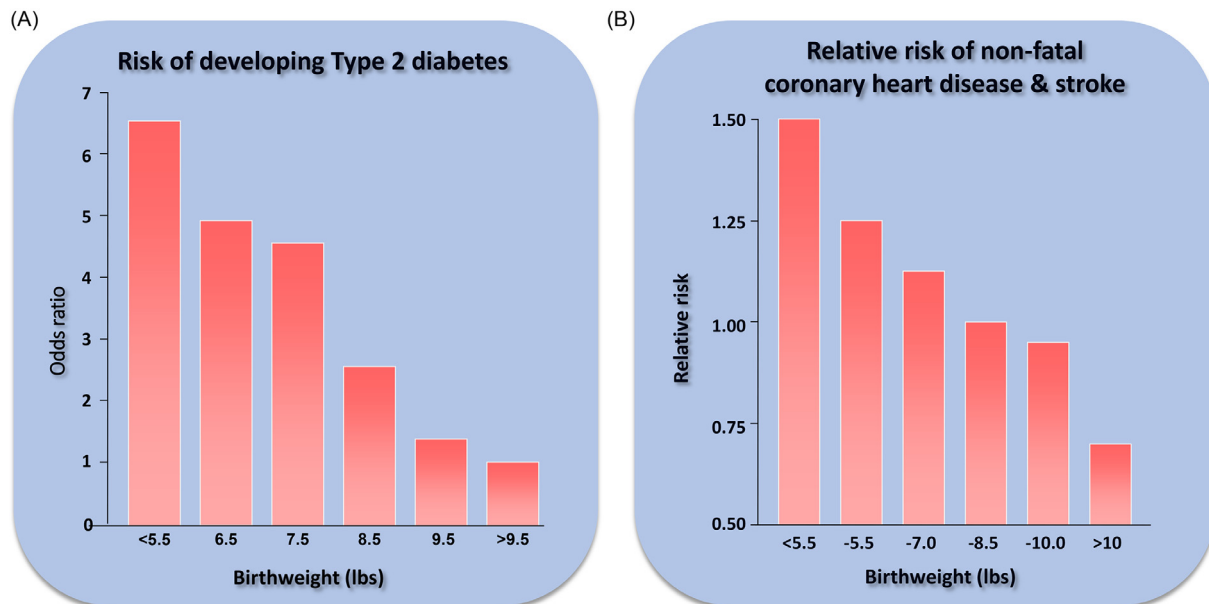


Fig. 1 Low birth weight is associated with increased risk of later life cardiometabolic disease in two separate birth cohorts. (A) In a study of >800 men over the age of 64 Barker et al. discovered a significant, inverse association of birthweight with risk of developing diabetes later in life. Of note, risk increases with on the far end of the spectrum. (B) The American Nurses study, consisting of a cohort of >70,000 women, demonstrated a similar trend to that observed in Hertfordshire, between birth weight and relative risk of developing coronary heart disease. (A) Data adapted from Hales, C.N., Barker, D.J., Clark, P.M., Cox, L.J., Fall, C., Osmond, C. and Winter, P.D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ* **303**, 1019–1022. (B) Data adapted from Rich-Edwards, J.W., Stampfer, M.J., Manson, J.E., Rosner, B., Hankinson, S.E., Colditz, G.A., Willett, W.C., Hennekens, C.H., (1997). Birth weight and risk of cardiovascular disease in a cohort of women followed up since. *BMJ* **315**, 396–400.

in over 70,000 female participants for whom birth records were available. Here, the inverse relationship that was reported in Hertfordshire was similarly observed (Fig. 1B) and was not weakened by controlling for SES or adult lifestyle (Rich-Edwards et al., 1997).

Since the initial Barker data, numerous studies investigating the DOHaD hypothesis have reported associations between birth weight and neonatal and infant body mass index (BMI), ponderal index (PI), and placental size and morphology, with physiological and disease outcomes including glucose intolerance, insulin resistance, Type 2 diabetes, hypertension, obesity, precocious puberty, allergy, and asthma. As a part of these observations, when intrauterine growth restriction (IUGR, also known as fetal growth restriction (FGR)) occurs, the development of certain organs, primarily the brain, is spared at the expense of other tissues including muscle, liver, and pancreas. It has been proposed that the fetus adapts in utero by adjusting set points of *metabolic homeostasis* to respond to the perceived nutrient availability in the postnatal environment. In doing so, the fetus alters its metabolism to make the best of use of the limited nutrient supply, a theory termed by Barker and his colleague Charles Nicholas Hales as the *Thrifty Phenotype Hypothesis* (Hales and Barker, 2001).

As a part of adopting a “thrifty phenotype”, it has been suggested that the fetus makes predictive adaptive responses (PARs); processes that lead to changes in the phenotype of the developing individual not for immediate survival, but in expectation of future advantage in the postnatal environment. In the case of nutrient restriction, some have hypothesized that the fetus sacrifices growth of certain organs (by limiting cellular division) thereby reducing overall metabolic demand. For example, a decrease in muscle fiber number, with concomitant decreases to muscle insulin sensitivity, will spare glucose uptake. Similarly, a reduction in fetal pancreatic insulin-producing β -cells would spare stimulation of glucose uptake in insulin sensitive tissues. These functional adaptations make glucose, the primary fetal energy substrate, more available to tissues such as the brain—an organ that is less evolutionarily dispensable (Hales and Barker, 2001). In a sense, the fetus becomes metabolically programmed to respond appropriately to the perceived nutrient poor environment outside of the womb. While these adaptations may confer an advantage if the accessibility of nutrients in the intrauterine environment is reflective of the actual postnatal environment, if a mismatch between pre- and postnatal occurs regarding perceived and actual nutrient availability, these responses (i.e., decreased insulin sensitivity or production) may be maladaptive and lead to progressive, chronic metabolic disease (i.e., Type 2 diabetes).

Several epidemiological and experimental studies have shown that pregnancy outcomes and associated programming effects apply to all facets of nutritional balance. Below we outline the evidence of how nutritional imbalance during pregnancy impacts fetal and placental growth, pregnancy outcomes, and later life adult disease risk.

Maternal Nutrient Restriction and Later Life Disease Risk

Much of our understanding of how nutrition, especially undernutrition, during pregnancy influences offspring health throughout the life course comes from epidemiological studies utilizing large birth cohorts exposed to nutritional challenges during pregnancy due to war, natural disaster, or political unrest. By comparing data collected from these women and their offspring, to those residing in the same geographic area before or after the period of disaster, epidemiologists have been able to use these “natural experiments” to test the effects of maternal malnutrition on pregnancy outcomes and later life disease risk in humans.

Epidemiological Evidence

The Dutch Hunger Winter of 1944–1945 has provided a wealth of information in this regard. During the second World War, German occupation of The Netherlands led to a complete blockade of food and fuel resulting in a five-month period from December 1944 to April 1945 in which the average individual caloric intake varied from 400 to 800 kcal per day. Although this restriction was rapidly lifted in May 1945 with liberation by the Allied forces, this short but severe famine period allowed the examination of pregnant women based on their window of exposure to caloric restriction. Several important observations were made from studies of the Dutch famine cohort, the most important being that fetal growth and the long-term effects of maternal nutrient restriction were dependent on the timing of exposure. Offspring born to mothers exposed exclusively during early gestation were born with a normal birth weight, but as adults, had an atherogenic lipid profile, obesity, early onset coronary heart disease, increased reactivity to stress, and higher rates of breast cancer. In contrast, offspring exposed later in pregnancy were born low birth weight (LBW) with fewer adult physiological complications. Critically, all exposed adult offspring demonstrated impaired glucose tolerance and insulin resistance, independent of timing of exposure, suggesting metabolic regulation is particularly vulnerable to changes in prenatal nutrient intake ([Roseboom et al., 2006](#)). These findings were some of the first to offer insight into the later life consequences of nutrient deprivation for pregnancy outcomes and later life metabolic disease.

The Dutch Hunger Winter was not the only famine cohort to be studied. The Siege of Leningrad (now St. Petersburg) by German forces in the second World War lasted from September 1941 to January 1944 and prevented food rations from entering the city for 872 days. It is estimated that 30%–40% of the population died of starvation, and during the height of the famine the average bread ration contained 300 calories with negligible protein content. Between 1941 and 1942 the total number of live births dropped from 3056 to 468, and neonatal mortality nearly doubled. Many newborns during this time were born well below the normal weight range, and had no sustained weight gain before being discharged from the hospital after birth, with some even losing weight further ([Antonov, 1947](#)). No association between maternal nutrient deprivation during pregnancy and later life metabolic disease in offspring has been demonstrated in this cohort ([Rotar et al., 2015](#); [Stanner et al., 1997](#)). While some have pointed to this as evidence against the DOHaD hypothesis, others see it as supportive evidence that timing of nutritional challenge and mismatch between pre- and postnatal environments are key drivers behind developing later life metabolic disease. Where the Dutch Hunger Winter was very short and saw rapid restoration of normal nutrient availability at the end of the blockade, the Leningrad Siege in contrast lasted almost 3 years, and saw a period of continued malnutrition for years following the end of The Siege. Offspring born during The Siege would have had a high level of congruency between nutritional deprivation in utero and in early childhood, whereas offspring of the Dutch cohort would have had a high level of mismatch between the fetal and infant life. It is possible that the level of compatibility between the early life and adult environment would determine whether the adaptations made during fetal life would be suitable for life outside of the womb, and thus dictate whether metabolic disease ensued.

In support of this theory of mismatch, in the Chinese Great Leap Forward Famine cohort of 1959–1961—a famine of similar length to Leningrad—programming effects were dependent on whether individuals lived in rural or urban areas after the end of the famine. Those offspring who were exposed to famine and lived in urban areas, where food was more accessible in greater quantities, were more prone to developing hypertension compared to their counterparts living in rural areas, where food availability was similar to what was predicted in utero ([Wu et al., 2017](#)). The disparity between these groups regarding exposure and postnatal nutrient availability highlights the interaction between fetal adaptations and the postnatal environment, and how a mismatch can result in postnatal disease risk ([Duque-Guimarães and Ozanne, 2013](#)). A second important observation to come from this cohort was that some outcomes were dependent on offspring sex. Males exposed to famine during pregnancy were more likely than females to develop non-alcoholic fatty liver disease (NAFLD) ([Chen et al., 2016](#)). In contrast, more females were born to mothers exposed in mid-to-late pregnancy ([Song, 2014](#)). These data might suggest that male fetuses were more sensitive to nutrient deprivation in the womb, and less equipped to adapt to poor nutrition.

Investigations of other cohorts support these observations. In a study of over 13,000 men and women born in Helsinki, Finland from 1934 to 1944 it was demonstrated that babies with a low ponderal index (PI, a measure of thinness at birth) and low BMI at 2 years of age, showed an early *adiposity rebound*, and that these children had a high BMI at 11 years of age ([Barker et al., 2009](#)). It is suggested that a low PI might indicate a forfeiture of muscle mass in favor of other organs (such as the brain), and that accelerated postnatal “catch-up” growth early in infant life is associated with a decreased ratio of lean muscle mass to fat mass. It is this combination of factors that is thought to contribute to the development of the *metabolic syndrome* in adulthood ([Eriksson et al., 1999, 2003](#)).

Adequate availability of macronutrients is not the only factor important during pregnancy. Epidemiological studies have found that the *balance* between calories derived from specific macronutrients during gestation can be equally as important. In a cohort of

253 men and woman born in the Aberdeen Maternity Hospital, it was demonstrated that an imbalance between calories consumed from animal protein and those from carbohydrate (i.e., high-carb, low-protein, or low-carb, high-protein diet) tended to decrease placental size and was associated with hypertension in later life (Campbell et al., 1996). As the fetus is entirely dependent upon the placenta for transport of nutrients, altered placental growth and function because of poor maternal nutrition is as equally important as, and likely responsible for, changes in fetal growth. Indeed, several studies have shown relationships between maternal fat mass, and diet with placental morphology and function (Alwaseel et al., 2010; Godfrey and Barker, 1995; Huppertz et al., 2006; Roseboom et al., 2011; Winder et al., 2011). Taken together, these studies indicate that poor maternal nutrition impairs the number of cells present in, and the function of metabolic organs, which have long term consequences for the health and longevity of the offspring in adult life.

While famine cohorts offer insight into associations between poor nutrition and outcome, they do little to uncover the cellular and biochemical mechanism(s) controlling fetal adaptation to changes in nutrition. Proxy measures such as birth weight, BMI and ponderal index are poor informants of intrauterine growth patterns and organ development. Many of the observed effects are prone to survivor bias, where only those with a strong enough constitution to survive the effects of gestational famine make up the study population, which could lead to misrepresentation of the severity of effects. This is where animal models have been invaluable in uncovering the mechanisms underlying nutrient deprivation.

Experimental Evidence

Animal models of nutrient restriction prior to and during pregnancy, and into lactation, have allowed researchers to isolate specific biological metabolic programming mechanisms. The most commonly used organisms in these studies have been rodents, guinea pigs, and sheep, although studies in non-human primates are increasing. While there are many similarities among mammalian pregnancies, notable differences exist regarding the number of offspring in a single pregnancy, the metabolic adaptations made during pregnancy, and the type of placentation. As such, these factors must be considered when selecting an animal model and interpreting the applicability of data to human populations.

Caloric restriction models

Many models of maternal undernutrition during pregnancy have examined mild, moderate, or severe caloric restriction (~70%, ~50%, and ~30% of ad libitum caloric intake, respectively) before, for part of, or the entirety of pregnancy to recapitulate adaptations made by the fetus in the context of human dietary restriction. Consistent with human observations, 50% caloric restriction during the latter half of pregnancy in rats produced growth restricted offspring, which display catch up growth postnatally, decreased pancreatic beta-cell mass in infancy and continued impairment of insulin production into adulthood (Garofano et al., 1997). In a similar model in which 50% dietary restriction was imposed for the entire pregnancy, the same trends in neonatal and adult weights were observed, and it was found that intra-abdominal fat mass was increased, with a concomitant reduction to lean body mass (Suzuki et al., 2010). Interestingly, even a modest reduction of 20% global caloric restriction has been shown to decrease offspring insulin sensitivity, an effect thought to be mediated by defects in insulin receptor signaling (Palou et al., 2012), which has even been recapitulated in human studies of men born with a low birth weight (Ozanne et al., 2005). Similar outcomes have been shown in insulin activity and blood pressure regulation in Guinea pig models, consistent with human observations (Cañas et al., 2017; Elias et al., 2016). Caloric restriction during pregnancy in rats has been shown to induce maternal-derived long chain polyunsaturated fatty acids mobilization from adipose and even neural tissue to protect the brain growth of the fetus (Agale et al., 2010). Caloric restriction also compromises reproduction function and has been proposed to result in early ovarian aging and reduced fertility in female offspring (Chan et al., 2015).

In contrast to rodent studies, caloric restriction in sheep at any point of gestation, appears to have negligible effects on birth weight (Fainberg et al., 2013; Gardner et al., 2004; Sébert et al., 2009). Global nutrient restriction during the first 30 days of pregnancy (where full gestation is 147–150 days), does not change birth weight or postnatal weight gain up to 1 year, but does impair baroreflex response, a key homeostatic mechanism in regulating blood pressure, indicating a predisposition to developing cardiovascular dysfunction in offspring (Gardner et al., 2004). While birth weight generally remains unaffected in sheep, lipid deposition has been observed in visceral tissues, including the heart (Ojha et al., 2015), and maternal undernutrition appears to make offspring more susceptible to the negative impacts of an obesogenic diet in adulthood (Chan et al., 2009; Fainberg et al., 2013; Hyatt et al., 2011).

Protein restriction

The Aberdeen cohort studies highlighted that a balance between macronutrients, especially protein and carbohydrates is as equally important for proper fetoplacental development as adequate calorie intake. In this regard, animal models have also used isocaloric, low protein diets to examine the effects of nutrient imbalance on long term disease risk. Pregnant rats have served as the most prominent model showing that low protein diets during pregnancy (50%–70% reduction in protein) result in several postnatal phenotypes that mirror observations in human famine cohorts. Maternal dietary protein restriction consistently impairs growth, reducing birthweight and cardiovascular and renal function (Gao et al., 2013; Hoppe et al., 2007; Koshy et al., 1975; Lim et al., 2010; Rosario et al., 2011). Hypertension in offspring appears to be associated with changes in the number of cardiomyocytes and nephrons, the functional units of the heart and kidney. Like caloric restriction, protein deprivation also induces insulin resistance in offspring (Duque-Guimarães and Ozanne, 2013).

Impacts on the placenta

While observations in fetal outcomes are important in determining how changes in nutrient intake mediate long term health and disease, perhaps of more importance is how these adaptive changes come about. The placenta is a conduit between the maternal and fetal environments. It is generally agreed that changes in placental growth, development, and function in the face of altered nutrient availability likely initiate many of the fetal adaptations that accompany offspring disease outcomes. Both pre-conceptional and gestational undernutrition, impaired placental blood flow, and reduced nutrient availability restrict transplacental nutrient transport to the fetus, a condition known as placental insufficiency. Indeed, placentae of pregnancies complicated by IUGR have increased vascular resistance, decreased surface area available for nutrient transfer, and decreased expression of amino acid and glucose transporters (Mayhew, 2003; Regnault et al., 2005; Rosario et al., 2011). This has been demonstrated in both human and experimental animal models of caloric and protein restriction.

In addition to providing an interface for nutrient and waste exchange, the placenta also serves as a barrier. Maternal glucocorticoids restrict fetal growth (Seckl, 1997). Placental 11 β -hydroxysteroid dehydrogenase Type 2 (11 β -HSD2) serves as an enzymatic barrier that converts bioactive cortisol into an inactive keto metabolite, cortisone (Seckl, 1997). Studies in both humans and other mammalian models have demonstrated that this barrier is disrupted during IUGR, resulting in increased fetal exposure to maternally derived corticosteroids, retarding growth, impairing nutrient transport, and contributing to postnatal offspring disease outcomes (Belkacemi et al., 2011; Connor et al., 2009; McTernan et al., 2001).

While science has made great advances in understanding the signals that mediate maternal-fetal interactions and long-term disease risk, our understanding is far from complete. While some animal data are consistent with human observations, undernutrition does not induce low birthweight or a dysfunctional adult phenotype in all models. This disparity is likely the result of how and when a nutritional challenge is applied, the length of the restriction, the species used and the methods by which phenotypic outcomes are evaluated. While discrepancies do exist, together this body of evidence supports a causal role for early life nutrient-mediated postnatal insulin resistance and impaired hepatic and pancreatic function in an effort to confer improved postnatal fitness, but in many circumstances, predisposing offspring to NCDs including Type 2 diabetes and obesity later life.

Micronutrient Deficiencies

Macronutrients are not the only nutritional factors influencing pregnancy outcomes. Proper intake and supplementation of essential vitamins and minerals, collectively known as micronutrients, are critical for a healthy pregnancy. Among the numerous micronutrients supplied through our diet, there are six that are closely monitored and/or supplemented during pregnancy to ensure appropriate fetal development and maternal health during pregnancy. These include: iodine, iron, vitamin A, folate (vitamin B9), vitamin B12, and vitamin D. Iodine is normally supplemented during pregnancy to promote adequate levels of transplacental transfer of maternal thyroxine to the fetus, to aid in proper neurological development. Iron supplementation, along with vitamin B12 and folate, prevent anemia and ensure adequate red blood cell production. Vitamin A contributes to retinoic acid metabolism, which is imperative for proper cellular differentiation in early development, including formation of neural and cardiac tissues (Bailey et al., 2015). Micronutrient deficiencies (MNDs) can have long-lasting, even permanent consequences for fetal, infant, and adult health, including impaired uteroplacental blood flow, neural defects, changes to metabolism and altered whole body adiposity (Rao et al., 2012). Iodine, iron, vitamin A, and folate deficiencies are among the leading causes of MNDs globally, and primarily affect populations in low-to-middle income countries (Bailey et al., 2015).

Iron

Iron deficiency is the most common MND worldwide, affecting an estimated 2 billion people. Iron deficiency causes anemia, due to lack of iron available for formation of hemoglobin, and is particularly common in pregnant women because of increased demand for iron for the development of the fetoplacental circulatory system (Bailey et al., 2015). Maternal iron deficiency is associated with low birth weight, preterm birth, maternal hemorrhage, and impaired immune system development in neonates (Cornock et al., 2013; Fu et al., 2012). It is routine to supplement dietary intake of iron to avoid these complications. In rat models, iron deficiency during pregnancy induces placental *hypoxia* and *oxidative stress*, which has growth restricting effects (Toblli et al., 2012). Maternal iron deficiency prior to and during pregnancy in mice decreases the number of live pups at birth, fetal body weight, and crown-rump length (Hubbard et al., 2013). Alterations to fetal mouse and postnatal rat, guinea pig, and human brain and liver fat metabolism have also been observed in response to prenatal iron deficiency and anemia (Rao et al., 2012). While mechanisms by which iron deficiency and maternal anemia remain under studied, it is undeniable that maternal iron status is critical for short and long-term health outcomes for the next generation.

Folate

Folate is found abundantly in green leafy vegetables and legumes. Once consumed, folate is converted to tetrahydrofolate (THF) which, along with vitamin B12, are key co-factors in one-carbon metabolism, modulating cellular processes including DNA synthesis and *epigenetic* modifications, including DNA methylation. As such, folate intake is critical for maintaining appropriate methylation and control of gene expression throughout development and postnatal life. Folate deficiency during pregnancy results

in macrocytic or megaloblastic anemia, and failure of embryonic neural tube closure, resulting in neural tube defects (NTDs) (Bailey et al., 2015). The incidence of NTDs decreased drastically with the introduction of folic acid fortification and supplementation during pregnancy, where it is recommended that pregnant women receive an additional 400 µg per day of folate. Unfortunately, no reliable estimates exist of how many women at high risk of pregnancy complications experience folate acid deficiency (Bailey et al., 2015).

Experimental evidence from mouse and rat models has suggested that folate also plays an important role in decidual angiogenesis. Folate deficiency prior to pregnancy in mice causes impaired decidual apoptosis, preventing decidual remodeling for embryonic implantation and impairments in the generation of maternal blood sinuses (Li et al., 2015b; Liao et al., 2015). This could have several implications for both the mother and child, including pre-eclampsia. Clinically, folate deficiency has been associated with IUGR and preterm delivery (Singh et al., 2015). The role of folate (in)sufficiency does not end with the mother, however. Recently, paternal folate deficiency, among other factors, has been implicated in fetal development, pregnancy outcomes, and disease outcomes (Lambrot et al., 2013). Paternal folate deficiency is hypothesized to alter sperm epigenome and impair proper expression of key genes involved in placentation and placental function. As previously mentioned, placental insufficiency is a prominent cause of growth restriction and metabolic programming outcomes, as seen in cases of macronutrient restriction. The extent to which paternal nutrient challenges influence pregnancy outcomes is highly debated. Only time and more experimental work will tell how much a father's nutritional status can impact a child before they are born.

Maternal Nutrient Excess

While negative energy balance and famine have clear associations with harmful outcomes at birth and postnatally, a positive energy balance, in the form of excess calorie intake, weight gain, and obesity, have equally adverse effects on maternal metabolism and on pregnancy outcomes. Overweight and obesity are often accompanied by increased levels of circulating pro-inflammatory cytokines, insulin resistance, glucose intolerance, and increased circulating levels of gut-derived bacterial endotoxins in the mother. Additionally, inflammation is also manifest within metabolic tissues of obese individuals including the liver, adipose, and pancreas, marked by increased infiltration of immune cells and in situ pro-inflammatory cytokine production.

Epidemiological Evidence

Excess adiposity during pregnancy (EADP) has become a worldwide concern in recent years, especially in Westernized countries. Globally, 39% of adults are overweight and 13% are considered obese (WHO | Health in 2015). This widespread epidemic has resulted in nearly 1 in every 2 women entering pregnancy overweight, or gaining more weight than is recommended throughout gestation (Dudenhausen et al., 2015). Overweight women (BMI > 24.9) are at an increased risk of developing gestational diabetes mellitus, preeclampsia, macrosomia, and delivering by C-section. Women considered morbidly obese (BMI > 40.0) are at an even higher risk of these same complications, being five times as likely than lean women to develop pre-eclampsia and three times as likely to deliver a stillborn child. Furthermore, children of obese women are over three times as likely to die in the neonatal period (Cedergren, 2004).

The consequences of nutritional excess during gestation do not end in early neonatal life, however. EADP can induce several postnatal effects, much like the effects of undernutrition, with implications for long term offspring health. Maternal obesity is the strongest predictor of offspring obesity and the metabolic syndrome (Ong et al., 2015; Patro et al., 2013). Insulin resistance, Type 2 diabetes, hypertension, and cardiovascular disease also accompany these outcomes. Maternal overweight and obesity are associated not only with giving birth to a large for gestational age baby, but also with preterm and/or IUGR neonates (Higgins et al., 2011; Radulescu et al., 2013). While this may seem counterintuitive, some evidence from humans and animals suggest that despite consuming excess calories, a poor quality of diet and lack of physical activity can, in some cases, impair embryo placentation and disrupt maternal uterine blood flow, restricting fetal access to maternal (micro)nutrient stores, resulting in growth restriction (Fig. 2). This has been an area of intense investigation using animal models.

Experimental Models

Like models of undernutrition, models of maternal nutrient excess have primarily used sheep and rodents, with the latter including many studies of diet-induced obesity in mice and rats. In general, three dietary models have been primarily used: Western-style diets (WS, ~40% kcal from simple sugar, ~40% kcal from animal fat), high fat diet (HF, 45%–60% kcal from animal fat) and high fructose diets (HFr, 50%–70% kcal from fructose). Several studies use these obesogenic diets to prime mothers prior to pregnancy to mimic the overweight phenotype of women with high pre-pregnancy BMI. These studies have shown that maternal obesity programs offspring obesity independent of the offspring's postnatal diet, cementing the role for in utero exposures as a key for the development of later life metabolic disease (Howie et al., 2009). In rodent models, it has been demonstrated that offspring exposed to an adverse environment in the form of maternal obesity leads to IUGR in some but not all cases (Howie et al., 2013).

Diets supplemented with fructose, high fructose corn syrup (HFCS), or common sweeteners used in both food and beverages have also been shown to have adverse effects when consumed in excess. Studies have demonstrated that excess maternal intake of fructose, unlike its structural isomer glucose, does not promote insulin and leptin secretion, resulting in calorie consumption

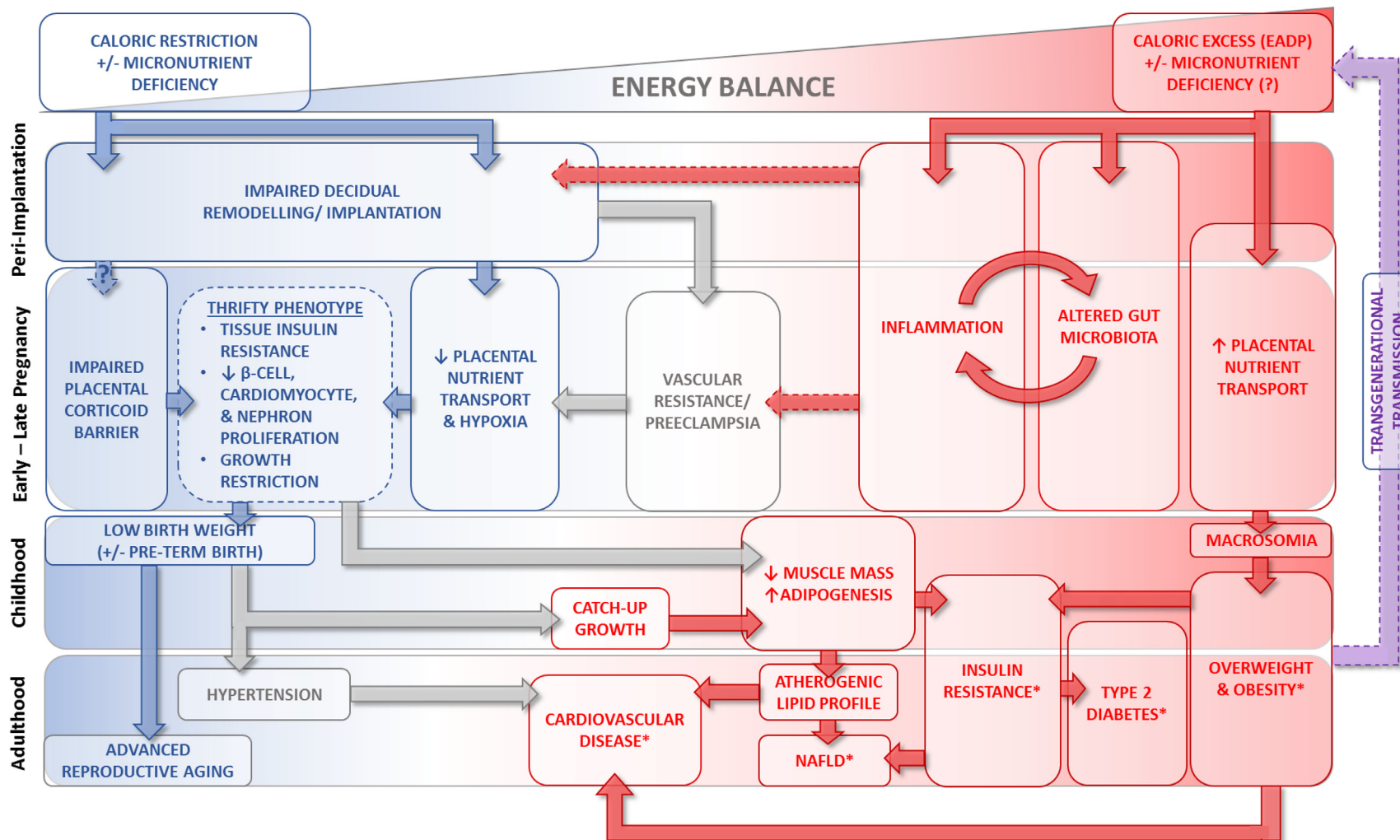


Fig. 2 A constellation of nutritional factors impact cardiometabolic programming of offspring disease. Maternal energy balance and dietary quality directly impact placentation and concomitant nutrient transport to the fetus, with downstream consequences for fetal metabolic adaptation to perceived postnatal nutritional availability. Adaptations made in the womb interact with the postnatal nutritional environment to drive development of chronic disease. Metabolic disease, including obesity and type 2 diabetes may act as stimuli driving the production of transgenerational programming effects. *Denotes features of the metabolic syndrome.

without promoting a feeling of fullness (Sloboda et al., 2014). Furthermore, excess fructose is preferentially used as a lipogenic substrate, resulting in conversion to lipids and storage in adipose tissue (Sloboda et al., 2014). Excess fructose consumption results in hyperinsulinemia, hypertriglyceridemia, hypertension, non-alcoholic fatty liver disease (NAFLD), and leptin resistance in rat models (Goran et al., 2013). While many studies use supraphysiological levels (> 50% of total caloric intake) of fructose to induce obesity, physiologically relevant intakes (i.e., 10% fructose solutions in drinking water or 20% of total caloric intake) have also been shown to induce maternal hyperinsulinemia, hypertriglyceridemia, altered liver metabolism, and impaired maternal and fetal leptin signaling (Goran et al., 2013; Sloboda et al., 2014). As might be expected, these changes in maternal physiology during pregnancy are associated with changes to neonate metabolism, with or without alterations to birth weight and placental growth. Despite obvious detrimental effects, there is a paucity of data examining the severity of programmed effects and the efficacy of intervention beyond the neonatal period.

Placental growth, hypoxia, and inflammation

As demonstrated in undernutrition models, many of the contributing factors underpinning the programming of disease risk are believed to originate with altered placental function. Studies of human term placentae as well as those in animal models have indicated several common placental phenotypic characteristics including altered vascularization and growth, inflammation, and placental hypoxia.

Vascularization of the placenta, invasion into the maternal decidua, and proliferation of trophoblast tissue are of great importance in ensuring adequate energy supply and waste exchange throughout the course of pregnancy. In an ovine model of nutrient excess during pregnancy placental vascularization in early to mid-gestation was impaired, resulting in reduced placental size in late gestation (Redmer et al., 2009). In a mouse model of maternal obesity during pregnancy, it was found that term placentae (gestational day 18) had decreased levels of mTOR signaling, a key signaling cascade in cellular growth, proliferation, and invasion as well as nutrient sensing (Lager et al., 2014). Placental mTOR levels are decreased in models of undernutrition as well, highlighting the importance of this pathway in modulating placental growth and the growth capacity of the fetus.

Inflammation, a common hallmark of obesity, has been seen in human, ovine, and rodent placentae from pregnancies complicated by nutrient excess and/or obesity. These placentae show increased immune cell infiltration, expression of pro-inflammatory markers (interleukins (IL) 1, 6, 8, and tumor necrosis factor (TNF)), or changes to immune cell phenotype (Challier et al., 2008; Zhu et al., 2010). Placental and decidual tissues contain within them several innate immune cell populations responsible for remodeling vasculature during placentation. Increased exposure of these cells to inflammatory cytokines may impair their acquisition of phenotypes promoting tissue remodeling to an inappropriate pro-inflammatory state, impairing proper embryo implantation and subsequent fetoplacental growth throughout gestation (Hemberger, 2013). Increased exposure to inflammation can have deleterious effects on both fetal growth and postnatal metabolic homeostasis (Ingvorsen et al., 2015). Moreover, many cytokines modulate placental nutrient uptake and can cross from maternal to fetal circulation which promote fatty acid uptake, and mobilization of maternal glucose to the fetoplacental unit (Segovia et al., 2014). As such, innate immune cell invasion and their phenotype in the placenta, and cytokine exposure in utero likely represent potent programming stimuli of postnatal metabolic homeostasis (Fig. 2).

Maternal Gut Microbial Health—A Novel Programming Factor

Increasing evidence suggests that the processing of nutrients by intestinal (gut) commensal bacteria has implications for offspring development. The human gut is a reservoir for trillions of commensal organisms commonly referred to as our microbiota. These microbes, the genes they encode and their products are collectively referred to as our gut microbiome. The microbiota plays several key roles in both host immunity and metabolism. Gut bacteria function to produce essential micronutrients including vitamin K2, biotin, and folate, they metabolize indigestible dietary fibers, and produce short chain fatty acids which modulate a variety of host metabolic functions such as gastrointestinal motility, hormone secretion, as well as gut immune cell function and epithelial barrier integrity (Macpherson and Harris, 2004; O'Hara and Shanahan, 2006). Experimental evidence from mice lacking any commensal organisms, (germ free (GF) animals), suggests that GF mice require a higher caloric intake to maintain the same body weight as their colonized counterparts, highlighting the importance of commensal microbes in mediating proper nutrient absorption and metabolism (Bäckhed et al., 2004).

The microbiota can also contribute to disease pathophysiology and one area of primary interest is the development and maintenance of chronic low grade systemic inflammation seen in obese individuals. Significant shifts occur in the composition of the gut microbiome in the context of obesity (Albenberg and Wu, 2014). Obesity-induced shifts in the microbiota are often accompanied by elevated levels of bacterial component including LPS (Cani et al., 2008; Erridge et al., 2007). LPS induces inflammation through the activation of innate immune receptor signaling pathways leading to production of pro-inflammatory cytokines. Increased circulating LPS in the context of obesity, is likely the result of decreased intestinal barrier function and increased permeability to bacterial components.

Recent evidence suggests that host-microbial interactions may mediate maternal metabolic adaptations made during pregnancy to support the growth of the developing embryo and fetus. In a landmark paper by Koren et al. (2012), it was demonstrated that the composition of the gut microbiota changes over the course of pregnancy, and that changes in third trimester composition can induce increased intestinal inflammation, adiposity, and insulin resistance, as compared to first trimester, when transplanted

into germ free mice. These changes are consistent with the progressive metabolic adaptations made by women throughout normal pregnancy. Following this work, [Gohir et al. \(2015\)](#) showed that the native microbial communities in mice undergo shifts during normal pregnancy, and that the specific changes that occur are dependent on the maternal peri-conceptual diet. Given the known role of the microbiome in modulating host metabolism, immunity, and inflammation, it is likely that this area of research will provide novel insights into the mechanisms by which diet influences pregnancy outcomes and offspring disease risk, providing new avenues for intervention during pregnancy to improve outcomes for both mother and child.

Nutritional Interventions to Reverse or Ameliorate Impacts of Maladaptive Early Life Nutritional Programming

The programming of postnatal disease through adaptations that occur early in life was once considered to result in irreversible changes in developmental trajectory via permanent resetting of the homeostatic mechanisms involved in growth and metabolism. However, it has now been shown, at least in animal models, that these effects can be reversed via targeted nutritional interventions during critical early life windows of developmental plasticity.

Methyl Donors

There has been increasing attention given to *epigenetic* processes as likely mediators of gene expression with changes in DNA methylation, histone modification and miRNAs in offspring all shown to arise as a consequence of early life nutritional insults. Maternal dietary *methyl donor* supplementation including the addition of choline to the maternal diet for example, in the setting of low protein (MLP) exposure, can prevent the increases in adiposity and blood pressure observed in offspring of non-supplemented mothers ([Bai et al., 2012](#)). Maternal glycine supplementation has been similarly shown to prevent increased systolic blood pressure in MLP offspring ([Jackson et al., 2002](#)), by correcting the vascular dysfunction that arises due to protein restriction ([Brawley et al., 2004](#)). Folic acid supplementation can also prevent hypomethylation of hepatic gene expression in offspring (including peroxisomal proliferator-activated receptor (PPAR)-alpha and glucocorticoid receptor (GR) genes) ([Lillicrop et al., 2005](#)).

Taurine

Maternal taurine, a sulfonated amino acid, supplementation in the MLP rat can normalize the vascularization of the fetal endocrine pancreas ([Boujendar et al., 2003](#)) and can partially prevent the adverse effects associated with protein restriction on pancreatic β -cell function and insulin sensitivity ([Boujendar et al., 2002](#); [Cherif et al., 1998](#); [Tang et al., 2013](#)). Since mitochondrial dysfunction may predispose to the development of Type 2 diabetes in adulthood, taurine deficiency may be a key causative factor, since taurine supplementation has been shown to restore low protein-induced changes in pancreatic islet mitochondria ([Lee et al., 2011](#)). In the setting of maternal obesogenic diets, fructose-induced metabolic and inflammatory dysregulation in offspring can also be partially reversed via maternal taurine supplementation ([Li et al., 2015a](#)). Similarly, in the setting of a maternal HF diet, supplementation with taurine results in beneficial changes in hepatic markers of inflammation and lipid metabolism in offspring. However, in this case, taurine supplementation resulted in hepatic pro-inflammatory phenotype in the mothers and thus may suggest a possible maternal “trade-off” to protect the neonate. Further, despite taurine conferring some protection against programming-related disorders in offspring of obese mothers, there was a significant increase in neonatal mortality in neonates born to taurine supplemented control mothers, indicative of possible adverse effects of taurine in the setting of normal pregnancy ([Li et al., 2013](#)).

Linoleic Acid

In rats, conjugated linoleic acid (CLA), an omega 6 fatty acid, has shown efficacy in reversing metabolic disorders in offspring born to mothers fed a HF diet. Developmental programming of endothelial dysfunction, hypertension, impaired insulin sensitivity, skeletal muscle atrophy, and inflammation in male rat offspring was partially improved by maternal CLA supplementation ([Gray et al., 2015](#); [Pileggi et al., 2016](#); [Segovia et al., 2015](#)). Which also ameliorates maternal HF diet-induced programming of inflammatory gene expression in the gut of rat offspring ([Reynolds et al., 2015](#)).

Micronutrient Supplementation

Given that maternal obesity results in oxidative stress the use of antioxidants as a reversal agent has also been investigated. In HF fed rats supplemented with antioxidants (including vitamins A, C, and E, and selenium), adiposity was decreased and glucose tolerance normalized as compared to non-supplemented mothers ([Sen and Simmons, 2010](#)). Dietary intake of the antioxidant coenzyme Q10 has also been shown to ameliorate programmed disorders in the rat. In maternal low protein diets Q10 supplemented offspring have reduced oxidative stress, inflammation and insulin levels compared to non-supplemented counterparts ([Tarry-Adkins et al., 2015, 2016](#)). Polyphenols including resveratrol decrease oxidative stress where in the setting of a MLP diet, resveratrol partially prevented alterations in maternal, placental and offspring markers related to oxidative stress and metabolic function ([Vega et al., 2016](#)).

Considerations and Future Directions

The experimental interventions trialed to date, does not represent a “one size fits all”. Taurine supplementation for example, may ameliorate metabolic defects in the setting of nutritional adversity but in a normal pregnancy may have deleterious effects on both mother and offspring, possibly via hypoglycemic effects of taurine (Li et al., 2013). Similarly, maternal folic acid supplementation intakes has been associated with increased incidence of respiratory impairment, and increased folate status during pregnancy has been linked to insulin resistance in children (Burdge and Lillycrop, 2012). Given the high proportion of women that take nutritional supplements during pregnancy, the evidence surrounding maternal methyl donor and vitamin supplements, one carbon metabolism and altered DNA methylation patterns would suggest that more attention needs to be given to the safety and potential long-term effects of such supplements on later offspring outcomes (Vanhees et al., 2014). Thus, identifying those at potential risk is an important consideration when nutritional intervention strategies are implemented as in some settings have the potential to induce harm.

Concluding Remarks

The last 30 years have provided undeniable evidence that maternal nutrition before, during, and shortly after pregnancy can have a profound impact on next generation health, well beyond fetal and neonatal life. Proper caloric intake, balanced macronutrient consumption, and micronutrient sufficiency are of utmost importance in ensuring not only the proper development of the fetus, but also in attenuating risk of chronic disease risk in later life. While beyond the scope of this article, it is worth noting that many of the negative cardiometabolic impacts of fetal programming are not confined to a single generation. As the fetus develops, so do its germs cells, which will give rise to gametes that form grand-offspring. As such, more than one generation is exposed at a time to the effects of maternal malnutrition in utero. What is more, many of the metabolic consequences for which the fetus is primed in later life (i.e., obesity and diabetes) may act as programming stimuli on subsequent generations as well. This, along with heritable changes in epigenetic marks are thought to be responsible for multi- and transgenerational metabolic programming effects. Malnutrition during pregnancy is a single, albeit powerful, factor in a constellation of interacting stimuli which can be deleterious to fetal development and increase later life disease risk, including maternal stress, smoking, recreational drug use, alcohol consumption, prescription medication use, and environmental toxicant exposure. Finally, it has been demonstrated that fetal programming of metabolic disorders is not an entirely irreversible process, with several interventions offering promising outcomes for offspring health. To date, in human populations at least, adequate and balanced nutrition during preconception, gestation, and the early neonatal period undoubtedly remain the best, most efficient way to ensure a positive contribution of maternal nutrition to perinatal and adult health. In cases where programming effects are unavoidable, future research into understanding the complex mechanisms underlying fetal adaptations to maternal nutrition will be invaluable in the development of targeted therapies that can be applied both during pregnancy and in early life to attenuate undesirable health outcomes.

References

- Agale, S., Kulkarni, A., Ranjekar, P., & Joshi, S. (2010). Maternal caloric restriction spares fetal brain polyunsaturated fatty acids in Wistar rats. *Brain and Development*, 32, 123–129. <https://doi.org/10.1016/j.braindev.2008.12.001>.
- Albenberg, L. G., & Wu, G. D. (2014). Diet and the intestinal microbiome: Associations, functions, and implications for health and disease. *Gastroenterology*, 146, 1564–1572. <https://doi.org/10.1053/j.gastro.2014.01.058>.
- Alwasel, S. H., Abotalib, Z., Aljarallah, J. S., Osmond, C., Alkharaz, S. M., Alhazza, I. M., Badr, G., & Barker, D. J. P. (2010). Changes in placental size during Ramadan. *Placenta*, 31, 607–610. <https://doi.org/10.1016/j.placenta.2010.04.010>.
- Antonov, A. N. (1947). Children born during the Siege of Leningrad in 1942. *The Journal of Pediatrics*, 30, 250–259.
- Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Koh, G. Y., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 15718–15723. <https://doi.org/10.1073/pnas.0407076101>.
- Bai, S. Y., Briggs, D. I., & Vickers, M. H. (2012). Increased systolic blood pressure in rat offspring following a maternal low-protein diet is normalized by maternal dietary choline supplementation. *Journal of Developmental Origins of Health and Disease*, 3, 342–349. <https://doi.org/10.1017/S2040174412000256>.
- Bailey, R. L., West, K. P., & Black, R. E. (2015). The epidemiology of global micronutrient deficiencies. *Annals of Nutrition and Metabolism*, 66(Suppl. 2), 22–33. <https://doi.org/10.1159/000371618>.
- Barker, D. J., & Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*, 1, 1077–1081.
- Barker, D. J. P., Osmond, C., Kajantie, E., & Eriksson, J. G. (2009). Growth and chronic disease: Findings in the Helsinki Birth Cohort. *Annals of Human Biology*, 36, 445–458. <https://doi.org/10.1080/03014460902980295>.
- Belkacemi, L., Jelks, A., Chen, C.-H., Ross, M. G., & Desai, M. (2011). Altered placental development in undernourished rats: Role of maternal glucocorticoids. *Reproductive Biology and Endocrinology*, 9, 105. <https://doi.org/10.1186/1477-7827-9-105>.
- Boujendar, S., Arany, E., Hill, D., Remacle, C., & Reusens, B. (2003). Taurine supplementation of a low protein diet fed to rat dams normalizes the vascularization of the fetal endocrine pancreas. *The Journal of Nutrition*, 133, 2820–2825.
- Boujendar, S., Reusens, B., Merezak, S., Ahn, M.-T., Arany, E., Hill, D., & Remacle, C. (2002). Taurine supplementation to a low protein diet during foetal and early postnatal life restores a normal proliferation and apoptosis of rat pancreatic islets. *Diabetologia*, 45, 856–866. <https://doi.org/10.1007/s00125-002-0833-6>.
- Brawley, L., Torrens, C., Anthony, F. W., Itoh, S., Wheeler, T., Jackson, A. A., Clough, G. F., Poston, L., & Hanson, M. A. (2004). Glycine rectifies vascular dysfunction induced by dietary protein imbalance during pregnancy. *The Journal of Physiology*, 554, 497–504. <https://doi.org/10.1113/jphysiol.2003.052068>.
- Brooks, A. A., Johnson, M. R., Steer, P. J., Pawson, M. E., & Abdalla, H. I. (1995). Birth weight: Nature or nurture? *Early Human Development*, 42, 29–35.
- Burdge, G. C., & Lillycrop, K. A. (2012). Folic acid supplementation in pregnancy: Are there devils in the detail? *The British Journal of Nutrition*, 108, 1924–1930. <https://doi.org/10.1017/S0007114512003765>.

- Campbell, D. M., Hall, M. H., Barker, D. J., Cross, J., Shiell, A. W., & Godfrey, K. M. (1996). Diet in pregnancy and the offspring's blood pressure 40 years later. *British Journal of Obstetrics and Gynaecology*, 103, 273–280.
- Cañas, D., Herrera, E. A., García-Herrera, C., Celentano, D., & Krause, B. J. (2017). Fetal growth restriction induces heterogeneous effects on vascular biomechanical and functional properties in guinea pigs (*Cavia porcellus*). *Frontiers in Physiology*, 8, 144. <https://doi.org/10.3389/fphys.2017.00144>.
- Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M., & Burcelin, R. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes*, 57, 1470–1481. <https://doi.org/10.2337/db07-1403>.
- Cedergren, M. I. (2004). Maternal morbid obesity and the risk of adverse pregnancy outcome. *Obstetrics and Gynecology*, 103, 219–224. <https://doi.org/10.1097/01.AOG.0000107291.46159.00>.
- Challier, J. C., Basu, S., Bintein, T., Minium, J., Hotmire, K., Catalano, P. M., & Hauguel-de Mouzon, S. (2008). Obesity in pregnancy stimulates macrophage accumulation and inflammation in the placenta. *Placenta*, 29, 274–281. <https://doi.org/10.1016/j.placenta.2007.12.010>.
- Chan, K. A., Bernal, A. B., Vickers, M. H., Gohir, W., Petrik, J. J., & Sloboda, D. M. (2015). Early life exposure to undernutrition induces ER stress, apoptosis, and reduced vascularization in ovaries of adult rat offspring. *Biology of Reproduction*, 92, 110. <https://doi.org/10.1095/biolreprod.114.124149>.
- Chan, L. L. Y., Sébert, S. P., Hyatt, M. A., Stephenson, T., Budge, H., Symonds, M. E., & Gardner, D. S. (2009). Effect of maternal nutrient restriction from early to midgestation on cardiac function and metabolism after adolescent-onset obesity. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 296, R1455–R1463. <https://doi.org/10.1152/ajpregu.91019.2008>.
- Chen, J.-P., Peng, B., Tang, L., Sun, R., Hu, S., Wen, X.-Y., Que, P., & Wang, Y.-H. (2016). Fetal and infant exposure to the Chinese famine increases the risk of fatty liver disease in Chongqing, China. *Journal of Gastroenterology and Hepatology*, 31, 200–205. <https://doi.org/10.1111/jgh.13044>.
- Cherif, H., Reusens, B., Ahn, M. T., Hoet, J. J., & Remacle, C. (1998). Effects of taurine on the insulin secretion of rat fetal islets from dams fed a low-protein diet. *The Journal of Endocrinology*, 159, 341–348.
- Connor, K. L., Challis, J. R. G., van Zijl, P., Rumball, C. W., Alix, S., Jaquiere, A. L., Oliver, M. H., Harding, J. E., & Bloomfield, F. H. (2009). Do alterations in placental 11beta-hydroxysteroid dehydrogenase (11betaHSD) activities explain differences in fetal hypothalamic-pituitary-adrenal (HPA) function following periconceptual undernutrition or twinning in sheep? *Reproductive Sciences*, 16, 1201–1212. <https://doi.org/10.1177/1933719109345162>.
- Cornock, R., Gambling, L., Langley-Evans, S. C., McArdle, H. J., & McMullen, S. (2013). The effect of feeding a low iron diet prior to and during gestation on fetal and maternal iron homeostasis in two strains of rat. *Reproductive Biology and Endocrinology*, 11, 32. <https://doi.org/10.1186/1477-7827-11-32>.
- Dudenhausen, J. W., Grünebaum, A., & Kirschner, W. (2015). Prepregnancy body weight and gestational weight gain-recommendations and reality in the USA and in Germany. *American Journal of Obstetrics and Gynecology*, 213, 591–592. <https://doi.org/10.1016/j.ajog.2015.06.016>.
- Duque-Guimarães, D. E., & Ozanne, S. E. (2013). Nutritional programming of insulin resistance: Causes and consequences. *Trends in Endocrinology and Metabolism*, 24, 525–535. <https://doi.org/10.1016/j.tem.2013.05.006>.
- Elias, A. A., Ghaly, A., Matuszewski, B., Regnault, T. R. H., & Richardson, B. S. (2016). Maternal nutrient restriction in guinea pigs as an animal model for inducing fetal growth restriction. *Reproductive Sciences*, 23, 219–227. <https://doi.org/10.1177/1933719115602773>.
- Eriksson, J. G., Forsén, T., Tuomilehto, J., Osmond, C., & Barker, D. J. P. (2003). Early adiposity rebound in childhood and risk of Type 2 diabetes in adult life. *Diabetologia*, 46, 190–194. <https://doi.org/10.1007/s00125-002-1012-5>.
- Eriksson, J. G., Forsén, T., Tuomilehto, J., Winter, P. D., Osmond, C., & Barker, D. J. (1999). Catch-up growth in childhood and death from coronary heart disease: Longitudinal study. *BMJ*, 318, 427–431.
- Erridge, C., Attina, T., Spickett, C. M., & Webb, D. J. (2007). A high-fat meal induces low-grade endotoxemia: Evidence of a novel mechanism of postprandial inflammation. *The American Journal of Clinical Nutrition*, 86, 1286–1292.
- Fainberg, H. P., Sharkey, D., Sebert, S., Wilson, V., Pope, M., Budge, H., & Symonds, M. E. (2013). Suboptimal maternal nutrition during early fetal kidney development specifically promotes renal lipid accumulation following juvenile obesity in the offspring. *Reproduction, Fertility, and Development*, 25, 728–736. <https://doi.org/10.1071/RD12037>.
- Fu, J., Yang, A., Ma, Y., Liu, M., Zhang, L., Wang, Y., & Liu, L. (2012). The effect of fetal and early postnatal iron deficiency on iron metabolism in adult rats. *Biological Trace Element Research*, 149, 412–418. <https://doi.org/10.1007/s12011-012-9447-0>.
- Gao, H., Yallampalli, U., & Yallampalli, C. (2013). Gestational protein restriction increases angiotensin II production in rat lung. *Biology of Reproduction*, 88, 64. <https://doi.org/10.1095/biolreprod.112.103770>.
- Gardner, D. S., Pearce, S., Dandrea, J., Walker, R., Ramsay, M. M., Stephenson, T., & Symonds, M. E. (2004). Peri-implantation undernutrition programs blunted angiotensin II evoked baroreflex responses in young adult sheep. *Hypertension*, 43, 1290–1296. Dallas Tex 1979 <https://doi.org/10.1161/01.HYP.0000126991.67203.7b>.
- Garofano, A., Czernichow, P., & Bréant, B. (1997). In utero undernutrition impairs rat beta-cell development. *Diabetologia*, 40, 1231–1234. <https://doi.org/10.1007/s001250050812>.
- Godfrey, K. M., & Barker, D. J. (1995). Maternal nutrition in relation to fetal and placental growth. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 61, 15–22.
- Gohir, W., Whelan, F. J., Surette, M. G., Moore, C., Schertzer, J. D., & Sloboda, D. M. (2015). Pregnancy-related changes in the maternal gut microbiota are dependent upon the mother's periconceptual diet. *Gut Microbes*, 6, 310–320. <https://doi.org/10.1080/19490976.2015.1086056>.
- Goran, M. I., Dumke, K., Bouret, S. G., Kayser, B., Walker, R. W., & Blumberg, B. (2013). The obesogenic effect of high fructose exposure during early development. *Nature Reviews. Endocrinology*, 9, 494–500. <https://doi.org/10.1038/nrendo.2013.108>.
- Gray, C., Vickers, M. H., Segovia, S. A., Zhang, X. D., & Reynolds, C. M. (2015). A maternal high fat diet programmes endothelial function and cardiovascular status in adult male offspring independent of body weight, which is reversed by maternal conjugated linoleic acid (CLA) supplementation. *PLoS One*, 10, e0115994. <https://doi.org/10.1371/journal.pone.0115994>.
- Hales, C. N., & Barker, D. J. (2001). The thrifty phenotype hypothesis. *British Medical Bulletin*, 60, 5–20.
- Hales, C. N., Barker, D. J., Clark, P. M., Cox, L. J., Fall, C., Osmond, C., & Winter, P. D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. *BMJ*, 303, 1019–1022.
- Hemberger, M. (2013). Immune balance at the foeto-maternal interface as the fulcrum of reproductive success. *Journal of Reproductive Immunology*, 97, 36–42. <https://doi.org/10.1016/j.jri.2012.10.006>.
- Higgins, L., Greenwood, S. L., Wareing, M., Sibley, C. P., & Mills, T. A. (2011). Obesity and the placenta: A consideration of nutrient exchange mechanisms in relation to aberrant fetal growth. *Placenta*, 32, 1–7. <https://doi.org/10.1016/j.placenta.2010.09.019>.
- Hoppe, C. C., Evans, R. G., Bertram, J. F., & Moritz, K. M. (2007). Effects of dietary protein restriction on nephron number in the mouse. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 292, R1768–R1774. <https://doi.org/10.1152/ajpregu.00442.2006>.
- Howie, G. J., Sloboda, D. M., Kamal, T., & Vickers, M. H. (2009). Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet. *The Journal of Physiology*, 587, 905–915. <https://doi.org/10.1113/jphysiol.2008.163477>.
- Howie, G. J., Sloboda, D. M., Reynolds, C. M., & Vickers, M. H. (2013). Timing of maternal exposure to a high fat diet and development of obesity and hyperinsulinemia in male rat offspring: Same metabolic phenotype, different developmental pathways? *Journal of Nutrition and Metabolism*, 2013. <https://doi.org/10.1155/2013/517384>.
- Hubbard, A. C., Bandyopadhyay, S., Wojczyk, B. S., Spitalnik, S. L., Hod, E. A., & Prestia, K. A. (2013). Effect of dietary iron on fetal growth in pregnant mice. *Comparative Medicine*, 63, 127–135.
- Huppertz, B., Burton, G., Cross, J. C., & Kingdom, J. C. P. (2006). Placental morphology: From molecule to mother—A dedication to Peter Kaufmann—A review. *Placenta*, 27(Suppl A), S3–S8. <https://doi.org/10.1016/j.placenta.2006.01.007>.

- Hyatt, M. A., Gardner, D. S., Sebert, S., Wilson, V., Davidson, N., Nigmatullina, Y., Chan, L. L. Y., Budge, H., & Symonds, M. E. (2011). Suboptimal maternal nutrition, during early fetal liver development, promotes lipid accumulation in the liver of obese offspring. *Reproduction*, 141, 119–126. Camb. Engl. <https://doi.org/10.1530/REP-10-0325>
- Ingvorsen, C., Brix, S., Ozanne, S. E., & Højlund, L. I. (2015). The effect of maternal inflammation on foetal programming of metabolic disease. *Acta Physiologica (Oxford, England)*, 214, 440–449. <https://doi.org/10.1111/apha.12533>
- Jackson, A. A., Dunn, R. L., Marchand, M. C., & Langley-Evans, S. C. (2002). Increased systolic blood pressure in rats induced by a maternal low-protein diet is reversed by dietary supplementation with glycine. *Clinical Science (London, England)*, 103, 633–639, 1979. <https://doi.org/10.1042/>
- Koren, O., Goodrich, J. K., Cullender, T. C., Spor, A., Laitinen, K., Bäckhed, H. K., Gonzalez, A., Werner, J. J., Angenent, L. T., Knight, R., Bäckhed, F., Isolauri, E., Salminen, S., & Ley, R. E. (2012). Host remodeling of the gut microbiome and metabolic changes during pregnancy. *Cell*, 150, 470–480. <https://doi.org/10.1016/j.cell.2012.07.008>
- Koshy, T. S., Sara, V. R., King, T. L., & Lazarus, L. (1975). The influence of protein restriction imposed at various stages of pregnancy on fetal and placental development. *Growth*, 39, 497–506.
- Lager, S., Samuëlsson, A., Taylor, P. D., Poston, L., Powell, T. L., & Jansson, T. (2014). Diet-induced obesity in mice reduces placental efficiency and inhibits placental mTOR signaling. *Physiological Reports*, 2. <https://doi.org/10.1002/phy2.242>
- Lambrot, R., Xu, C., Saint-Phar, S., Chountalos, G., Cohen, T., Paquet, M., Suderman, M., Hallett, M., & Kimmins, S. (2013). Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. *Nature Communications*, 4. <https://doi.org/10.1038/ncomms3889>
- Lee, Y. Y., Lee, H.-J., Lee, S.-S., Koh, J. S., Jin, C. J., Park, S.-H., Yi, K. H., Park, K. S., & Lee, H. K. (2011). Taurine supplementation restored the changes in pancreatic islet mitochondria in the fetal protein-malnourished rat. *The British Journal of Nutrition*, 106, 1198–1206. <https://doi.org/10.1017/S0007114511001632>
- Li, M., Reynolds, C. M., Sloboda, D. M., Gray, C., & Vickers, M. H. (2015a). Maternal taurine supplementation attenuates maternal fructose-induced metabolic and inflammatory dysregulation and partially reverses adverse metabolic programming in offspring. *The Journal of Nutritional Biochemistry*, 26, 267–276. <https://doi.org/10.1016/j.jnutbio.2014.10.015>
- Li, M., Reynolds, C. M., Sloboda, D. M., Gray, C., & Vickers, M. H. (2013). Effects of taurine supplementation on hepatic markers of inflammation and lipid metabolism in mothers and offspring in the setting of maternal obesity. *PLoS One*, 8, e76961. <https://doi.org/10.1371/journal.pone.0076961>
- Li, Y., Gao, R., Liu, X., Chen, X., Liao, X., Geng, Y., Ding, Y., Wang, Y., & He, J. (2015b). Folate Deficiency Could Restrain Decidual Angiogenesis in Pregnant Mice. *Nutrients*, 7, 6425–6445. <https://doi.org/10.3390/nu7085284>
- Liao, X. G., Li, Y. L., Gao, R. F., Geng, Y. Q., Chen, X. M., Liu, X. Q., Ding, Y. B., Mu, X. Y., Wang, Y. X., & He, J. L. (2015). Folate deficiency decreases apoptosis of endometrium decidual cells in pregnant mice via the mitochondrial pathway. *Nutrients*, 7, 1916–1932. <https://doi.org/10.3390/nu7031916>
- Lillycrop, K. A., Phillips, E. S., Jackson, A. A., Hanson, M. A., & Burdge, G. C. (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *The Journal of Nutrition*, 135, 1382–1386.
- Lim, K., Zimanyi, M. A., & Black, M. J. (2010). Effect of maternal protein restriction during pregnancy and lactation on the number of cardiomyocytes in the postproliferative weanling rat heart. *Anatomical Record*, 293, 431–437. Hoboken NJ 2007. <https://doi.org/10.1002/ar.21084>
- Macpherson, A. J., & Harris, N. L. (2004). Interactions between commensal intestinal bacteria and the immune system. *Nature Reviews. Immunology*, 4, 478–485. <https://doi.org/10.1038/nri1373>
- Mayhew, T. M. (2003). Changes in fetal capillaries during preplacental hypoxia: Growth, shape remodelling and villous capillarization in placentae from high-altitude pregnancies. *Placenta*, 24, 191–198.
- McTernan, C. L., Draper, N., Nicholson, H., Chalder, S. M., Driver, P., Hewison, M., Kilby, M. D., & Stewart, P. M. (2001). Reduced placental 11beta-hydroxysteroid dehydrogenase Type 2 mRNA levels in human pregnancies complicated by intrauterine growth restriction: An analysis of possible mechanisms. *The Journal of Clinical Endocrinology and Metabolism*, 86, 4979–4983. <https://doi.org/10.1210/ajem.86.10.7893>
- O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO Reports*, 7, 688–693. <https://doi.org/10.1038/sj.embor.7400731>
- Ojha, S., Symonds, M. E., & Budge, H. (2015). Suboptimal maternal nutrition during early-to-mid gestation in the sheep enhances pericardial adiposity in the near-term fetus. *Reproduction, Fertility, and Development*, 27, 1205–1212. <https://doi.org/10.1071/RD14007>
- Ong, K. K., Kennedy, K., Castañeda-Gutiérrez, E., Forsyth, S., Godfrey, K. M., Koletzko, B., Latulippe, M. E., Ozanne, S. E., Rueda, R., Schoemaker, M. H., van der Beek, E. M., van Buuren, S., & Fewtrell, M. (2015). Postnatal growth in preterm infants and later health outcomes: A systematic review. *Acta Paediatrica*, (104), 974–986. Oslo Nor. 1992. <https://doi.org/10.1111/apa.13128>
- Ozanne, S. E., Jensen, C. B., Tingey, K. J., Storgaard, H., Madsbad, S., & Vaag, A. A. (2005). Low birthweight is associated with specific changes in muscle insulin-signalling protein expression. *Diabetologia*, 48, 547–552. <https://doi.org/10.1007/s00125-005-1669-7>
- Palou, M., Konieczna, J., Torrens, J. M., Sánchez, J., Priego, T., Fernandes, M. L., Palou, A., & Picó, C. (2012). Impaired insulin and leptin sensitivity in the offspring of moderate caloric-restricted dams during gestation is early programmed. *The Journal of Nutritional Biochemistry*, 23, 1627–1639. <https://doi.org/10.1016/j.jnutbio.2011.11.005>
- Patro, B., Liber, A., Zalewski, B., Poston, L., Szajewska, H., & Koletzko, B. (2013). Maternal and paternal body mass index and offspring obesity: A systematic review. *Annals of Nutrition & Metabolism*, 63, 32–41. <https://doi.org/10.1159/000350313>
- Pileggi, C. A., Segovia, S. A., Markworth, J. F., Gray, C., Zhang, X. D., Milan, A. M., Mitchell, C. J., Barnett, M. P. G., Roy, N. C., Vickers, M. H., Reynolds, C. M., & Cameron-Smith, D. (2016). Maternal conjugated linoleic acid supplementation reverses high-fat diet-induced skeletal muscle atrophy and inflammation in adult male rat offspring. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 310, R432–439. <https://doi.org/10.1152/ajpregu.00351.2015>
- Radulescu, L., Munteanu, O., Popa, F., & Cirstoiu, M. (2013). The implications and consequences of maternal obesity on fetal intrauterine growth restriction. *Journal of Medicine and Life*, 6, 292–298.
- Rao, K. R., Padmavathi, I. J. N., & Raghunath, M. (2012). Maternal micronutrient restriction programs the body adiposity, adipocyte function and lipid metabolism in offspring: A review. *Reviews in Endocrine and Metabolic Disorders*, 13, 103–108. <https://doi.org/10.1007/s11154-012-9211-y>
- Redmer, D. A., Luther, J. S., Milne, J. S., Aitken, R. P., Johnson, M. L., Borowicz, P. P., Borowicz, M. A., Reynolds, L. P., & Wallace, J. M. (2009). Fetoplacental growth and vascular development in overnourished adolescent sheep at day 50, 90 and 130 of gestation. *Reproduction*, 137, 749–757. <https://doi.org/10.1530/REP-08-0516>
- Regnault, T. R. H., Friedman, J. E., Wilkening, R. B., Anthony, R. V., & Hay, W. W. (2005). Fetoplacental transport and utilization of amino acids in IUGR—A review. *Placenta*, 26(Suppl A), S52–S62. <https://doi.org/10.1016/j.placenta.2005.01.003>
- Reynolds, C. M., Segovia, S. A., Zhang, X. D., Gray, C., & Vickers, M. H. (2015). Maternal high-fat diet-induced programming of gut taste receptor and inflammatory gene expression in rat offspring is ameliorated by CLA supplementation. *Physiological Reports*, 3. <https://doi.org/10.14814/phy2.12588>
- Rich-Edwards, J. W., Stampfer, M. J., Manson, J. E., Rosner, B., Hankinson, S. E., Colditz, G. A., Willett, W. C., & Hennekens, C. H. (1997). Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *BMJ*, 315, 396–400.
- Rosario, F. J., Jansson, N., Kanai, Y., Prasad, P. D., Powell, T. L., & Jansson, T. (2011). Maternal protein restriction in the rat inhibits placental insulin, mTOR, and STAT3 signaling and down-regulates placental amino acid transporters. *Endocrinology*, 152, 1119–1129. <https://doi.org/10.1210/en.2010-1153>
- Roseboom, T., de Rooij, S., & Painter, R. (2006). The Dutch famine and its long-term consequences for adult health. *Early Human Development*, 82, 485–491. <https://doi.org/10.1016/j.earhumdev.2006.07.001>
- Roseboom, T. J., Painter, R. C., de Rooij, S. R., van Abeelen, A. F. M., Veenendaal, M. V. E., Osmond, C., & Barker, D. J. P. (2011). Effects of famine on placental size and efficiency. *Placenta*, 32, 395–399. <https://doi.org/10.1016/j.placenta.2011.03.001>
- Rotar, O., Moguchala, E., Boyarinova, M., Kolesova, E., Khromova, N., Freylikhman, O., Smolina, N., Solntsev, V., Kostareva, A., Konradi, A., & Shlyakhto, E. (2015). Seventy years after the siege of Leningrad: Does early life famine still affect cardiovascular risk and aging? *Journal of Hypertension*, 33, 1772–1779. discussion 1779. <https://doi.org/10.1097/HJH.0000000000000640>

- Sébert, S. P., Hyatt, M. A., Chan, L. L. Y., Patel, N., Bell, R. C., Keisler, D., Stephenson, T., Budge, H., Symonds, M. E., & Gardner, D. S. (2009). Maternal nutrient restriction between early and midgestation and its impact upon appetite regulation after juvenile obesity. *Endocrinology*, 150, 634–641. <https://doi.org/10.1210/en.2008-0542>.
- Seckl, J. R. (1997). Glucocorticoids, feto-placental 11 beta-hydroxysteroid dehydrogenase Type 2, and the early life origins of adult disease. *Steroids*, 62, 89–94.
- Segovia, S. A., Vickers, M. H., Gray, C., & Reynolds, C. M. (2014). Maternal obesity, inflammation, and developmental programming. *BioMed Research International*, 2014, 418975. <https://doi.org/10.1155/2014/418975>.
- Segovia, S. A., Vickers, M. H., Zhang, X. D., Gray, C., & Reynolds, C. M. (2015). Maternal supplementation with conjugated linoleic acid in the setting of diet-induced obesity normalises the inflammatory phenotype in mothers and reverses metabolic dysfunction and impaired insulin sensitivity in offspring. *The Journal of Nutritional Biochemistry*, 26, 1448–1457. <https://doi.org/10.1016/j.jnutbio.2015.07.013>.
- Sen, S., & Simmons, R. A. (2010). Maternal antioxidant supplementation prevents adiposity in the offspring of Western diet-fed rats. *Diabetes*, 59, 3058–3065. <https://doi.org/10.2337/db10-0301>.
- Singh, M. D., Thomas, P., Owens, J., Hague, W., & Fenech, M. (2015). Potential role of folate in pre-eclampsia. *Nutrition Reviews*, 73, 694–722. <https://doi.org/10.1093/nutrit/nuv028>.
- Sloboda, D. M., Li, M., Patel, R., Clayton, Z. E., Yap, C., & Vickers, M. H. (2014). Early life exposure to fructose and offspring phenotype: Implications for long term metabolic homeostasis. *Journal of Obesity*, 2014. <https://doi.org/10.1155/2014/203474>.
- Song, S. (2014). Malnutrition, sex ratio, and selection: A study based on the great leap forward famine. *Human Nature*, 25, 580–595. Hawthorne N <https://doi.org/10.1007/s12110-014-9208-1>.
- Stanner, S. A., Bulmer, K., Andrés, C., Lantseva, O. E., Borodina, V., Poteen, V. V., & Yudkin, J. S. (1997). Does malnutrition in utero determine diabetes and coronary heart disease in adulthood? Results from the Leningrad siege study, a cross sectional study. *BMJ*, 315, 1342–1348.
- Suzuki, M., Shibamura, M., & Kimura, S. (2010). Effect of severe maternal dietary restriction on growth and intra-abdominal adipose tissue weights in offspring rats. *Journal of Nutritional Science and Vitaminology (Tokyo)*, 56, 293–298.
- Tang, C., Marchand, K., Lam, L., Lux-Lantos, V., Thyssen, S. M., Guo, J., Giacca, A., & Arany, E. (2013). Maternal taurine supplementation in rats partially prevents the adverse effects of early-life protein deprivation on β -cell function and insulin sensitivity. *Reproduction*, 145, 609–620. Camb. Engl. <https://doi.org/10.1530/REP-12-0388>.
- Tarry-Adkins, J. L., Fernandez-Twinn, D. S., Hargreaves, I. P., Neergheen, V., Aiken, C. E., Martin-Gronert, M. S., McConnell, J. M., & Ozanne, S. E. (2016). Coenzyme Q10 prevents hepatic fibrosis, inflammation, and oxidative stress in a male rat model of poor maternal nutrition and accelerated postnatal growth. *The American Journal of Clinical Nutrition*, 103, 579–588. <https://doi.org/10.3945/ajcn.115.119834>.
- Tarry-Adkins, J. L., Fernandez-Twinn, D. S., Madsen, R., Chen, J.-H., Carpenter, A., Hargreaves, I. P., McConnell, J. M., & Ozanne, S. E. (2015). Coenzyme Q10 prevents insulin signaling dysregulation and inflammation prior to development of insulin resistance in male offspring of a rat model of poor maternal nutrition and accelerated postnatal growth. *Endocrinology*, 156, 3528–3537. <https://doi.org/10.1210/en.2015-1424>.
- Toblli, J. E., Cao, G., Oliveri, L., & Angerosa, M. (2012). Effects of iron deficiency anemia and its treatment with iron polymaltose complex in pregnant rats, their fetuses and placentas: Oxidative stress markers and pregnancy outcome. *Placenta*, 33, 81–87. <https://doi.org/10.1016/j.placenta.2011.11.017>.
- Vanhees, K., Vonhögen, I. G. C., van Schooten, F. J., & Godschalk, R. W. L. (2014). You are what you eat, and so are your children: the impact of micronutrients on the epigenetic programming of offspring. *Cellular and Molecular Life Sciences*, 71, 271–285. <https://doi.org/10.1007/s00018-013-1427-9>.
- Vega, C. C., Reyes-Castro, L. A., Rodríguez-González, G. L., Bautista, C. J., Vázquez-Martínez, M., Larrea, F., Chamorro-Cevallos, G. A., Nathanielsz, P. W., & Zambrano, E. (2016). Resveratrol partially prevents oxidative stress and metabolic dysfunction in pregnant rats fed a low protein diet and their offspring. *The Journal of Physiology*, 594, 1483–1499. <https://doi.org/10.1113/JP271543>.
- Walton, A., & Hammond, J. (1938). The maternal effects on growth and conformation in shire horse-shetland pony crosses. *Proceedings of the Royal Society of London - Series B: Biological Sciences*, 125, 311–335. <https://doi.org/10.2307/82213>.
- WHO | Health in 2015. (2015WHOHealthin.). from MDGs to SDGs [WWW Document]. WHO <http://www.who.int/gho/publications/mdgs-sdgs/en/>. (Accessed 2 February 2017).
- Winder, N. R., Krishnaveni, G. V., Veena, S. R., Hill, J. C., Karat, C. L. S., Thornburg, K. L., Fall, C. H. D., & Barker, D. J. P. (2011). Mother's lifetime nutrition and the size, shape and efficiency of the placenta. *Placenta*, 32, 806–810. <https://doi.org/10.1016/j.placenta.2011.09.001>.
- Wu, L., Feng, X., He, A., Ding, Y., Zhou, X., & Xu, Z. (2017). Prenatal exposure to the Great Chinese Famine and mid-age hypertension. *PLoS One*, 12, e0176413. <https://doi.org/10.1371/journal.pone.0176413>.
- Zhu, M. J., Du, M., Nathanielsz, P. W., & Ford, S. P. (2010). Maternal obesity up-regulates inflammatory signaling pathways and enhances cytokine expression in the mid-gestation sheep placenta. *Placenta*, 31, 387–391. <https://doi.org/10.1016/j.placenta.2010.02.002>.

Adaptations to Pregnancy

Kent L Thornburg and Amy M Valent, Oregon Health & Science University, Portland, OR, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Alveolus A hollow cavity found in the [lung](#) where gas is exchanged between air and blood.

Angiotensin receptors A class of receptors that bind to the hormone [angiotensin II](#).

Corpus luteum The temporary mini-organ on the ovary left behind after ovulation. It generates hormones that are important in pregnancy.

Cytochrome P-450 [Proteins](#) used in the metabolism of a variety of chemical compounds.

Decidua The decidua is the portion of the [uterine](#) lining shed during menstruation and important during [pregnancy](#).

Deiodinases Protein enzymes that remove an iodine from a thyroid hormone molecule.

Epigenetics Heritable changes in gene activity without changes in gene sequence.

Hematopoietic The formation of blood cellular components.

Hemodynamic The forces that regulate the flow of blood through the body.

Human chorionic gonadotropin A [hormone](#) produced by the [placenta](#) after [implantation](#).

Macronutrients The primary types of food containing carbon molecules that are used for fuel and chemical reactions in the body, including proteins, fats and carbohydrates.

Macrosomia Excessive birth weight on a newborn—typically defined as more than 4.0 kg at birth.

Microbiome The entire collection of microorganisms in a specific niche, such as the human intestine.

Peptide Short chains of [amino acids](#).

Polycythemia An excessive number of red blood cells in the circulation.

Preeclampsia A serious pregnancy complication characterized by high blood pressure and protein in the urine.

Nomenclature

$\dot{Q} = (P_{ao} - P_{ra}) / TPR$ Blood flow ($\dot{Q}$) is equal to the difference in blood pressure between the aorta (P_{ao}) and the right atrium (P_{ra}) divided by the total peripheral resistance (TPR).

$GFR = (P_{art} - \pi_{gc}) K_f$ The glomerular filtration rate (GFR) is the rate that blood is filtered by the glomeruli, the filtering units of the kidney, and is equal to the difference between arterial pressure (P_{art}) and osmotic forces (π_{gc}) caused by large molecules in plasma times the permeability coefficient (K_f) of the glomerular membrane.

$\dot{V}_E / \dot{V}_{O_2}$ The amount of pulmonary gas expired per minute ($\dot{V}_E$) divided by the rate that the body uses oxygen ($\dot{V}_{O_2}$), a measure of the efficiency of respiration.

Introduction

Female mammals have made physiological adaptations to their organ systems to provide a healthy environment for offspring as they grow in the uterus until birth. Raising offspring inside the body has its advantages. Nutrients for the embryo and fetus are derived from the mother's diet and from her tissue stores to provide a constant flow of nutrients across the placenta. This is in contrast to vertebrates that lay eggs where offspring are more vulnerable to predators. In addition, offspring can grow larger in the womb before being exposed to the external environment than would be possible in an egg. For example, a baby blue whale weighs about 3 tons at birth and is quite mature. It stretches the imagination to envision the size of an egg required to provide the space and total nutrients to care for such a fetus. The process of laying an appropriate sized egg might compromise the mother whale's wellbeing. In spite of advantages, carrying offspring in a uterus exacts a high physiological price for mothers to maintain the pregnancy.

Most of our knowledge regarding the physiology of pregnancy has come in the last 75 years but generalizations about changes in physiology must now be updated based on modern pregnancies. In the last quarter century, the physiology of pregnancy has changed dramatically because of the epidemic of obesity and diabetes mellitus. Patients with these conditions are now so commonplace they are no longer considered extraordinary in clinics where pregnant women are cared for and delivered. Obesity in pregnancy leads to risks for a number of complications and poor outcomes for offspring. The risks elevate as maternal fat mass increases (Yu et al., 2006). The condition of obesity leads to elevated risks for recurrent miscarriage, operative delivery, and preeclampsia. Offspring risks include perinatal mortality, obesity in later life (Yu et al., 2006) and congenital defects. The Institute of Medicine has made recommendations regarding weight gain during pregnancy because elevated weight gain has medical consequences (Fig. 1) (Nationalacademics.org,

Pregnancy Weight Gain Guidelines

Pre-pregnancy Body Mass Index (BMI)	Underweight <18.5	Normal Healthy 18.5 to 24.9	Overweight 25 to 29.9	Obese >30
	Recommended Weight Gain Range			
Pounds	28-40	25-35	15-25	11-20
Kilograms	12.5-18	11.5-16	7.5-11.5	5-9

Fig. 1 Guidelines for weight gain during pregnancy issued by the Institute of Medicine and the National Research Council (2009). IOM and NRC has recently issued new guidelines for weight gain during pregnancy. These guidelines were developed considering short- and longer-term outcomes for offspring. From Rasmussen, K. M. and Yaktine, A. L. (eds.) (2009). *Institute of Medicine. Weight gain during pregnancy: Reexamining the guidelines*. Washington, DC: National Academy Press. NATIONALACADEMICS.ORG 2013. Implementing guidelines on weight gain and pregnancy. <https://www.nap.edu/catalog/18292/implementing-guidelines-on-weight-gain-and-pregnancy>.

2013). Likewise, being born to a diabetic mother includes higher rates of preeclampsia, cesarean section, macrosomia, and neonatal hypoglycemia and polycythemia. Babies born to diabetic mothers are “programmed” to be vulnerable for childhood or adolescent obesity, type 2 diabetes mellitus, and heart disease (Simeoni and Barker, 2009).

Carrying a fetus to term exacts a physiological price on the mother. She undergoes profound changes in her organ systems and her organs function differently. Providing adequate energy and specific nutrients for the baby is a difficult task. It requires a healthy mother and a healthy placenta. It has become evident in recent years that the growth of fetuses is limited by placental capacity as well as by the mother’s ability to deliver nutrients to the placenta (Thornburg et al., 2000, 2015).

Metabolism

The basal metabolic rate (BMR) is the quantity of calories burned by the whole body per unit time at rest. It increases by some 30% over the course of pregnancy from about 1300 kcal/day to 1700 kcal/day (Butte et al., 2004). However, the relative and absolute change in BMR depends strongly on the pre-pregnancy body mass index (BMI = body weight/height²) and weight gain over gestation. This is shown in Fig. 2 (Butte et al., 2004). BMR was found to correlate with body weight, fat free mass, fat mass, cardiac output, maternal plasma insulin-like growth factor-1 concentration and thyroid hormone (T₃) concentration as well as fetal body mass. As individual organs adapt to their new role in supporting the pregnancy, their oxygen consumption rises. Thus, basal oxygen consumption increases most in the uterus to support the fetus but oxygen utilization rates are also increased in breast, kidney, heart, and respiratory muscles. As expected, total oxygen consumption in pregnancy increases in parallel to BMR and by some 50 mL O₂/min by term. Much of the energy cost is expended for augmenting ATP production. Extra energy is also required for generating heat, cellular processes, and to support added organ activity in kidney, heart, and breast.

Diet

Unlike pregnant bears, human females must consume food during pregnancy in order to provide for the nutrient needs of their offspring. Tissue turnover from a woman’s body is also important in providing nutrients. Thus, in early pregnancy when many

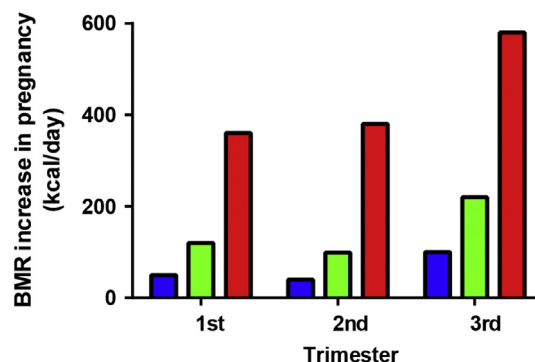


Fig. 2 Basal metabolic rate in pregnant women by trimester and Body Mass Index (BMI, weight/height²). Blue bars = low BMI; green bars = medium BMI; red bars = high BMI. Data from Butte, N. F., Wong, W. W., Treuth, M. S., Ellis, K. J. and O’Brian Smith, E. (2004). Energy requirements during pregnancy based on total energy expenditure and energy deposition. *American Journal of Clinical Nutrition* 79, 1078–1087.

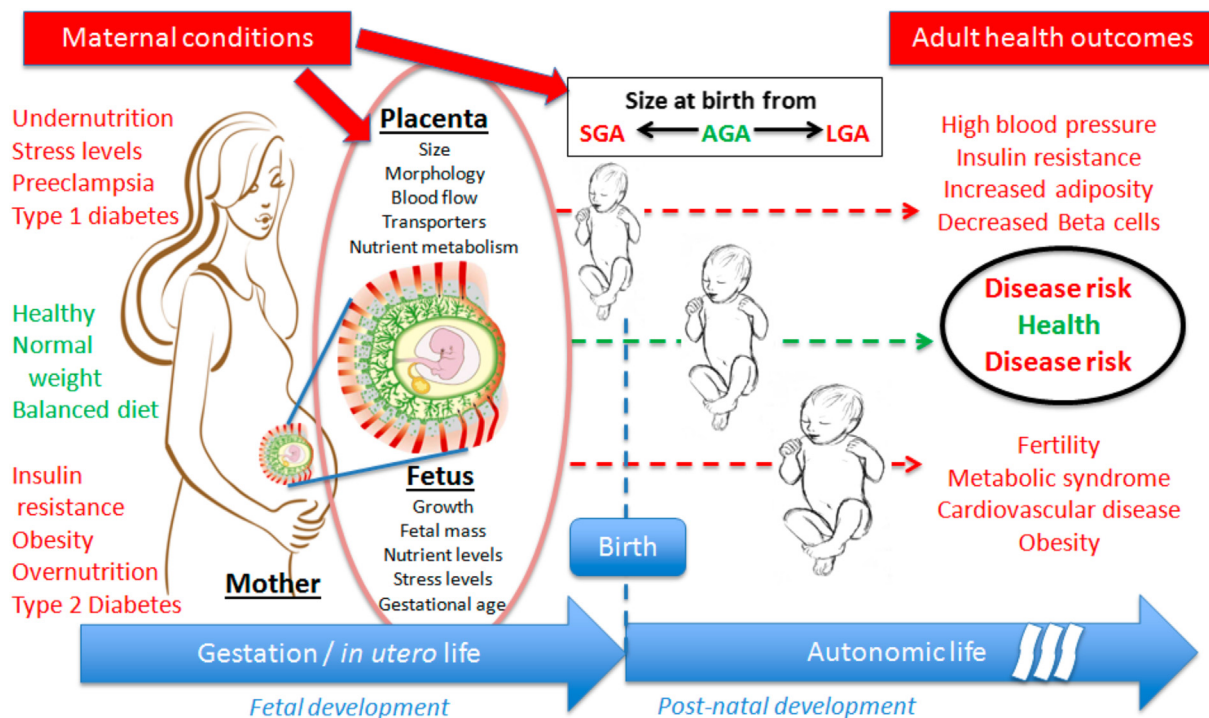


Fig. 3 Showing the maternal conditions that influence the health of the fetus and the outcomes suffered by the fetus when it reaches adulthood. From Chavatte-Palmer, P., Tarrade, A. and Rousseau-Ralliard, D. (2016). Diet before and during pregnancy and offspring health: The importance of animal models and what can be learned from them. *International Journal of Environmental Research and Public Health*, 13, with permission.

women become nauseated after eating food (hyperemesis or morning sickness), embryos are little affected if the mother had a healthy body preceding pregnancy. Bears on the other hand must gain all their weight before pregnancy so that they can hibernate over the entire course of gestation and provide nourishment to offspring in the uterus solely from maternal tissue stores.

One of the most profound discoveries made over the past quarter century is the role of maternal diet in affecting offspring health. While we know that the numbers and types of intestinal bacteria (microbiome) change during pregnancy, we do not know the degree to which individual components of the diet affect the microbe population in women who are pregnant. There is increasing evidence that bacteria live in the placenta and intestine of the fetus too. Preconception nutrition is also important because it provides nutrient stores for a woman that will become available during pregnancy. A healthy balanced diet or a “prudent diet” before and during pregnancy is associated with lower rates of cardiovascular disease and osteoporosis in offspring. See Fig. 3 (Chavatte-Palmer et al., 2016). The nourishment of eggs and sperm long before conception is important in offspring health. In spite of this new knowledge, little is known about how most nutrients actually affect fetal growth and development.

As for a non-pregnant woman, calories and nutrients are important for the pregnant mother to provide for the fetus and maternal tissues. Not only does the mother need a variety of nutrients for metabolism but foods high in methyl groups, like leafy green vegetables, are important for DNA and histone methylation, two “epigenetic” mechanisms by which gene expression is modified by diet. Specific nutrients or lack thereof, appear to impart epigenetic changes in tissues of both the mother and the fetus. Detrimental changes in gene expression may lead to disease vulnerability in offspring. Balanced macronutrient intake by the mother provides fuel for the baby as well as organic molecules that provide building blocks for organ development. Pregnant women need a balanced high fiber diet composed of whole grains, fruit, vegetables, nut, legumes and healthy oils, including vegetables and/or fish that contain omega three fatty acids (Fig. 4) (University, 2015). The omega three fatty acids, as found in most fish, are required for normal brain, retina and cardiovascular development. Some specific nutrients, like essential amino acids and fatty acids, are required for normal development because they have specific roles in building fetal organs.

Diet quality can be evaluated using the Healthy Eating Index, the Alternative Healthy Eating Index, and/or the Dietary Inflammation Index (Shivappa et al., 2014). Women who had a low-fat mass at conception generally gain more weight than women who had a large fat mass. Maternal diet also depends on the sex of the baby. Women carrying boys consume more calories per day from all macronutrient sources and gain more weight. Boys grow faster than girls and thus weigh more at birth. They do so while investing less in their placenta. Thus, boys have relatively smaller but more efficient placentas and a low fetal to placental weight ratio at birth. The consequence is that boys are more vulnerable to nutrient deprivation in the womb than are girls. The ratio of boys to girls being born during famine conditions can be reduced as boys are less likely to survive under such adverse conditions.

My Pregnancy Plate

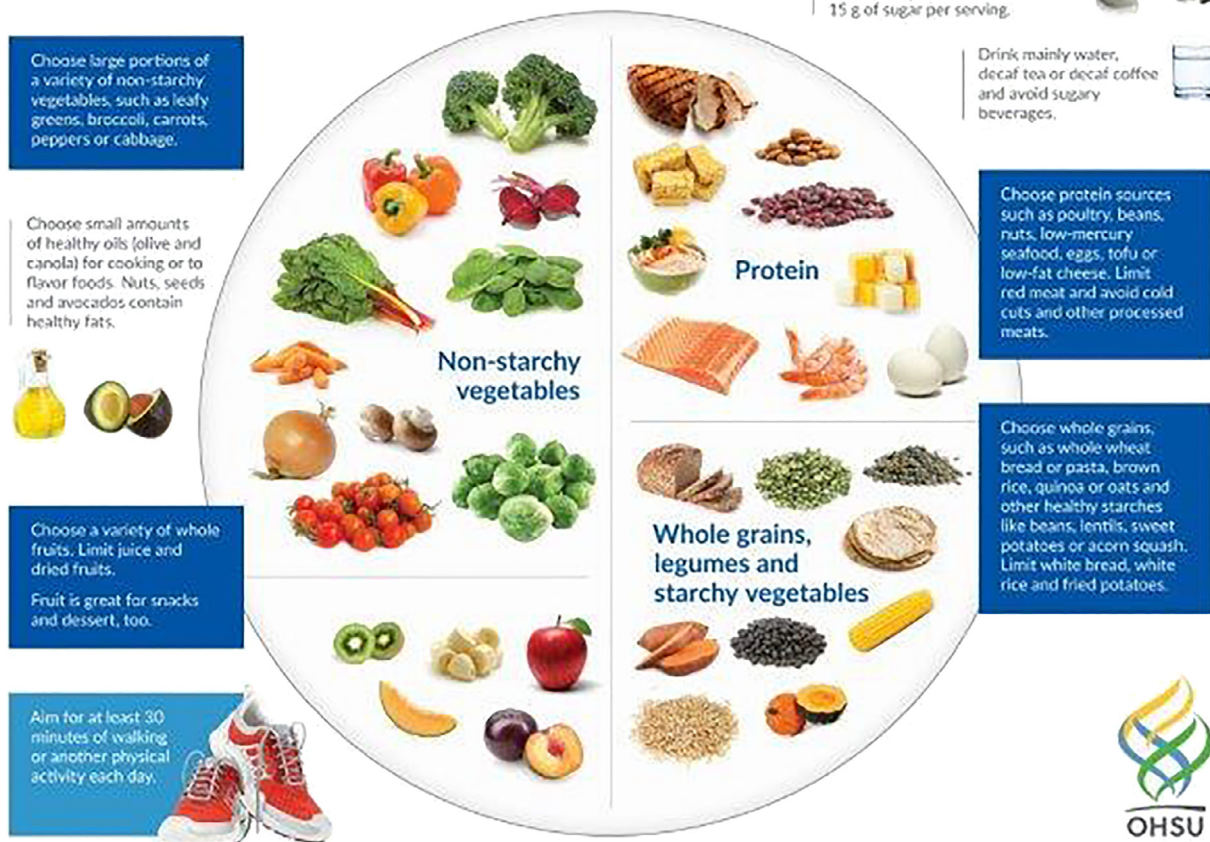


Fig. 4 My pregnancy plate is a guide for pregnant women (or women planning to be pregnant) to assist in eating an optimal diet. Women should strive to eat from each plate quadrant during her main meals throughout the day. From OHSU Website: <http://www.ohsu.edu/blogs/doernbecher/files/2013/04/ohsu-my-pregnancy-plate.pdf>.

Heart and Blood Vessels

Of all the systems that change in response to pregnancy, changes in the cardiovascular system are the most profound. Adaptations are related to providing increases in blood flow to the kidney and uterus and having a vascular reserve in case of hemorrhage, especially at the time of delivery. Thus, a number of cardiovascular adjustments are found in normal pregnancies and are listed in **Table 1**. A recent meta-analysis of cardiovascular patterns confirms the patterns reported in the literature and in the table.

Table 1 Cardiovascular changes that occur in pregnancy

<i>Increased</i>	<i>Decreased</i>
Plasma volume	Pulmonary vascular resistance
Extracellular volume	Systemic vascular resistance
Red blood cell production rate	Hematocrit
Total red blood cell mass	Systemic arterial blood pressure
Uterine blood flow	Plasma albumin concentration
End diastolic dimension	Colloid osmotic pressure
Stroke volume	Arterial hydrogen ion concentration
Heart rate	Arterial carbon dioxide tension
Arterial oxygen tension	Platelet concentration
Venous capacitance	
Most clotting factors (fibrinogen, Factor VII)	

Blood Volume

Normal pregnancies are characterized by an increase in blood volume, with a less robust increase in red blood cell production rate which leads to a decrease in hematocrit. Thus, normal pregnant women are relatively anemic during pregnancy. While the low hematocrit is often described as caused by “hemodilution,” this cannot be the case. Rather, the set point for maintaining red cell mass appears to be lower during pregnancy. This makes sense from a hemodynamic point of view. The lowered red cell concentration lowers viscosity, improves flow through microvessels and lessens the systolic load for the left ventricle. Oxygen delivery to the uterus is maintained or augmented by the increase in cardiac output and blood flow to the uterus. Red blood cell production is augmented enormously during pregnancy while the life span of individual red cells is shortened. Under the influence of estrogen, progesterone, human placental lactogen and prolactin, hematopoietic stem cells take up residence in the spleen in addition to their normal home in bone marrow in pregnant mice which allows expansion of red cell production. This is likely to occur in human pregnancy also since the spleen is enlarged over the course of gestation.

Blood volume expansion of up to 50% begins early in pregnancy and is a requirement for a normal pregnancy. By 12 weeks of gestation plasma volume has increased by some 14% (Bernstein et al., 2001). Failure to expand blood volume during pregnancy has negative consequences for the fetus. One of the most profound changes is compromised blood flow to the uterus itself. Uterine blood flow is thought to increase 7- to 10-fold over the course of normal pregnancy. Doppler studies have shown that uterine flow velocity increases some 7-fold in normal humans as gestation proceeds. However, blood flow to the uterus may be compromised in mothers who do not expand their blood volume appropriately. In addition, mothers with hypertension may suffer similar decreases in uterine blood flow and reduced fetal growth. These conditions often lead to fetal growth restriction.

Heart

To provide greater blood flow to needy organs in a normal pregnancy, cardiac output is increased by augmenting both stroke volume and heart rate. Stroke volume is the volume of blood ejected from the heart each beat. Thus, the heart of the pregnant woman enlarges and the myocardium remodels so that stiffness is reduced. This remodeling is a major modification of the basic mechanical properties of the heart muscle; these changes mostly resolve in the weeks after delivery. Thus, during pregnancy, the chamber volumes are larger at any given transmural pressure than they were before the pregnancy. Fig. 5 (Poppas et al., 1997; Hunter and Robson, 1992) shows changes in stroke volume and cardiac output over the course of gestation. Fig. 6 (Poppas et al., 1997; Hunter and Robson, 1992) shows changes heart rate. Changes in mean arterial blood pressure and total peripheral resistance are shown in Fig. 7 (Poppas et al., 1997; Hunter and Robson, 1992). These changes are stimulated by increases in estrogen (Giraud et al., 1993). The timing of changes of hemodynamic factors can also be seen in the figures. Noteworthy is the fact that the stroke volume has already risen by 6–8 weeks post conception before the embryo has significant oxygen demands.

The quantitative aspects of these relationships can be visualized in the vascular adaptation of Ohm’s law in appropriate units. The cardiac output, $\dot{Q}$, is the flow per minute coming from the heart and is equal to the driving pressure, ΔP (P_{aorta} minus $P_{\text{right atrium}}$) divided by the total peripheral resistance (TPR). In other words,

$$\dot{Q} = (P_{\text{ao}} - P_{\text{ra}}) / \text{TPR}$$

Of the parameters in this equation, it is clear that the primary mechanism of increased cardiac output is the drop in total vascular resistance because ΔP decreases as blood pressure in the aorta is decreased. Stroke volume is increased, in part, because the heart chambers become larger at the same filling pressure and because the arterial blood pressure decreases and the arteries become more compliant. This makes it easier for the heart to pump a large volume of blood each beat.

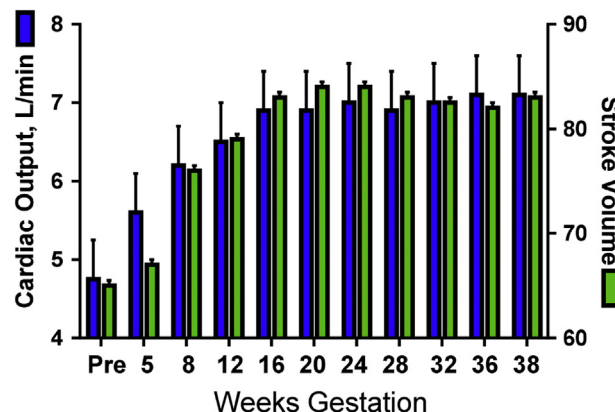


Fig. 5 Stroke volume and cardiac output across the course of gestation. Blue bars = cardiac output (L/min). Green bars = stroke volume. Data from Hunter, S. and Robson, S. C. (1992). Adaptation of the maternal heart in pregnancy. *British Heart Journal* **68**, 540–543.

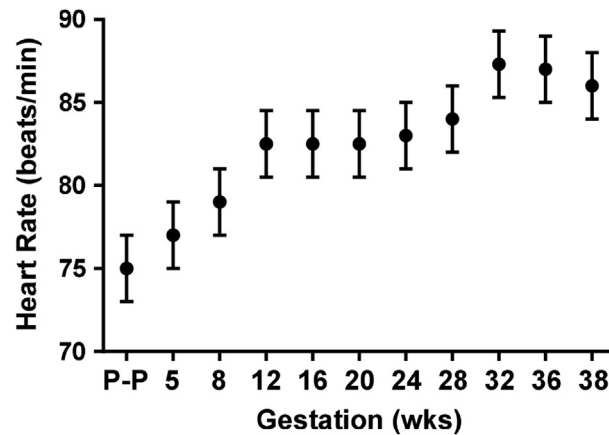


Fig. 6 Heart rate across the course of gestation. Data from Hunter, S. and Robson, S. C. (1992). Adaptation of the maternal heart in pregnancy. *British Heart Journal* **68**, 540–543.

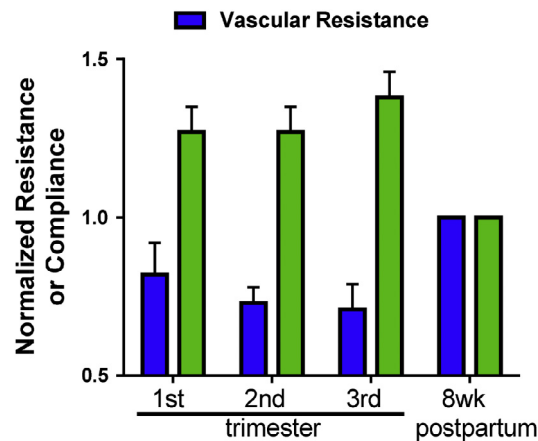


Fig. 7 Changes in mean arterial pressure and arterial compliance across pregnancy normalized to these parameters at 8 weeks post-partum. Blue bar = vascular resistance; green bars = arterial compliance. Data from Poppas, A., Shroff, S. G., Korcarz, C. E., Hibbard, J. U., Berger, D. S., Lindheimer, M. D. and Lang, R. M. (1997). Serial assessment of the cardiovascular system in normal pregnancy. Role of arterial compliance and pulsatile arterial load. *Circulation* **95**(10), 2407–2415.

Blood Vessels

Total peripheral vascular resistance is decreased with pregnancy. This involves two processes, remodeling of the arterial and arteriolar walls and active vasodilation of arterioles. The degree to which veins are remodeled, or made more compliant, in response to the changing hormonal environment is controversial. Because venous distension in the legs, varicose veins, hemorrhoids and ankle swelling are common complaints in pregnant women, but not before conception or after delivery, it is obvious that veins are more compliant as an adaptation to pregnancy (Edouard et al., 1998). Edouard et al. (1998) have argued that veins in the lower extremities are more affected than those in upper extremities. This remains to be seen. What is not controversial is the increase in a number of vasoactive agents in the maternal circulation. These include estrogen, nitric oxide, relaxin, and calcitonin gene-related peptide. It has been recognized for decades that the blood pressure elevating effects of angiotensin II (Ang II) are attenuated during pregnancy. This is likely due to a decrease in the ratio of type 1 angiotensin receptors (AT1R) to type 2 (AT2R) expressed in vascular smooth muscle. It is also true that arteries in pregnant animals are less constricted by alpha adrenergic agonists than in non-pregnant animals.

Hormones

There are several hormonal changes that accompany the more obvious hemodynamic changes during pregnancy. Progesterone is a key pregnancy-sustaining hormone. After 6–10 weeks gestation, the placenta is the major source of progesterone, producing up to 250 mg/day (Simpson and MacDonald, 1981). Estrogen and relaxin are critical hormones for the cardiovascular adaptations in pregnancy. They promote nitric oxide production. Relaxin, produced by the corpus luteum, placenta, and decidua, promotes

increased cardiac output and systemic arterial compliance and overall reduction in peripheral vascular resistance. Estrogens stimulate the synthesis of angiotensinogen by the liver, augmenting the renin increase observed in early pregnancy. Maternal plasma levels of atrial natriuretic peptide (ANP) and angiotensin II (Ang II) are elevated in pregnancy. ANP levels are elevated in response to atrial stretch as is evident by ANP increases in plasma when a woman is in the supine position. The effect is especially evident in the first trimester. Plasma levels of Ang II on the other hand, are more than doubled in the pregnant state. Although there is activation of the renin–angiotensin–aldosterone system early in pregnancy, which under non-pregnant conditions would cause vasoconstriction, there is a counterbalancing effect by progesterone, which has vasodilatory effects and is a potent aldosterone antagonist. These hormones promote vasodilation and sodium filtration on balance with the sodium-retaining properties of aldosterone and vasoconstrictive properties of Ang II.

Maternal thyroid hormone affects the development of the fetus. Human chorionic gonadotropin stimulates increases in thyroxine in maternal plasma early in pregnancy. Increased maternal plasma estrogen concentrations in early pregnancy stimulate an increase in thyroid hormone binding globulin which increases the capacity of maternal plasma to carry thyroid hormone. As term approaches, increased expression of deiodinases in the fetus under the influence of rising cortisol levels leads to an increase in fetal tri-iodo-L-thyronine (T3), the active form of thyroid hormone. Elevations in plasma and tissue levels of T3 lead to suppression of cardiomyocyte proliferation, maturation of the myocardium and pancreas. Thus, the mother and fetus work together to provide for the thyroid hormone needs of the developing embryo and fetus.

Kidney

The kidney undergoes enormous changes in response to pregnancy. Like the cardiovascular system, renal changes begin early in the pregnancy. The organs themselves change physically with an increase in length and volume under the internal influence of increased vascularity, interstitial volume and nephron hypertrophy (Lindheimer and Katz, 1992).

There are a host of physiological changes that underlie renal function in pregnancy. One of the most profound is glomerular filtration rate (GFR). GFR increases and peaks at 50% over non-pregnant values by the end of the first trimester (Baylis, 1999). The filtration rate of the glomeruli, when measured by inulin clearance, reaches up to 180 mL/min on average during pregnancy. The effective renal plasma flow also increases approximately 50% more than pre-pregnancy values by the third trimester.

Functional changes in the nephron during pregnancy include increases in urinary non-albumin protein excretion up to 260 mg/day. Urinary protein loss is considered normal if it remains below the arbitrary 300 mg/day. While some albumin is excreted in urine in normal pregnancies where <29 mg/day is considered the threshold for normal, the amount is amplified in women with preeclampsia. Loss of glucose in urine during pregnancy is also a normal condition, resulting from impaired glucose resorption in the collecting tubule and loop of Henle. Increased amounts of selective amino acids along with other nutrients are also lost in urine during a normal pregnancy.

The filtered load of sodium increases with increases in GFR during pregnancy. However, this is overcome by an augmented reabsorption of sodium by the renal tubule and increased secretion of aldosterone, deoxycorticosterone, and estrogen. The excess absorption of sodium ion is accompanied by retention of 1–2 L of water in the extracellular fluid volume. However, renin release and associated plasma renin activity are also increased during pregnancy due, in part, to decreases in arterial blood pressure and activation of the sympathetic nervous system as well as hormone actions including aldosterone.

There are many questions regarding functional changes in the kidney that remain unanswered. These include, what drives hemodynamic changes in the kidney, what mechanisms underlie increases in GFR, what regulates release of protein in the urine, and which vasoactive mediators that rise in concentration during pregnancy have a specific role in the kidney.

As mentioned, GFR and effective renal plasma flow increase in the pregnant woman. The same is true for most, if not all, mammals. In equation form, the forces that drive GFR are the difference between the transmural hydrostatic pressure across the glomerular capillary wall (ΔP) and the oncotic pressure (π_{cg}) in plasma.

$$GFR = (P_{art} - \pi_{cg})K_f$$

The quantification of filtration rate requires multiplication by a filtration constant (K_f) that represents both the hydraulic conductivity or “permeability” and the surface area of the glomerular membrane. Mathematical models of glomerular function suggest that hydrostatic pressure does not change appreciatively as blood travels down the capillary. However, the oncotic pressure does change as fluid is lost from the plasma as it moves down the capillary. An increase in renal plasma flow slows the increase in π_{cg} , maintaining the ΔP gradient and increasing the amount of fluid filtered. Thus, it appears that increased blood flow in the capillary is the primary driver of the increase in GFR in the pregnant state.

Respiration

There are a number of adaptations to pregnancy that improve the oxygenation levels in blood. The rate at which gas moves through the lung increases by 50% during pregnancy. Minute ventilation, $\dot{V}_E$ and alveolar ventilation, $\dot{V}_A$ are both increased in pregnant women. The increase in $\dot{V}_E$ is driven primarily by increased basal metabolic rate and plasma progesterone which affect the sensitivity of carbon dioxide in the respiratory centers in the brain stem. But other factors including plasma osmolality and concentrations of

ang II and arginine vasopressin also contribute (Heenan et al., 2001). Increased progesterone concentrations during the menstrual cycle are large enough to change the medullary set point for arterial P_{CO_2} in normal cycling women. In other words, women hyperventilate during the luteal phase of the cycle when progesterone levels are high, relative to the follicular phase when they are low. The same is true in experimental animals. Administration of progesterone to a rodent will cause an increase in alveolar ventilation even in male rodents. Women have a partial pressure of carbon dioxide in arterial blood of about 40 mmHg in menopausal years, about the same as for men. However, pregnant women have an increased respiratory drive that lowers their arterial gas pressure of carbon dioxide to about 32 mmHg or lower. This happens in spite of their increased metabolism and the production of CO_2 by the conceptus. Oxygen tensions in alveolar gas and arterial blood are determined by the difference between inspired partial pressure of oxygen and the partial pressure of carbon dioxide in alveolar gas. Thus increasing ventilation drives down the carbon dioxide tensions in the alveolar gas and increased pressure of oxygen in the lung. This increases the oxygen pressure in the arterial blood of the pregnant woman. The end result is that peripheral arterial PO_2 exceeds 100 mmHg at sea level, higher than in the non-pregnant state.

There are mechanical changes that occur in lung and chest wall as pregnancy proceeds. Tidal volume is increased by 40% early in pregnancy and is maintained. Functional residual capacity is decreased by some 20% as are its component volumes, expiratory reserve volume and residual volume. Total lung capacity is decreased by a few per cent. The forces at work include an increased respiratory drive which increases the depth of breathing and increases alveolar ventilation at normal breathing rates, and the reductions in functional residual capacity that occurs in response to the space occupying growing uterus against the diaphragm. These adaptations cause a mild respiratory alkalosis is normal in pregnancy. Oxygen levels in the arterial blood are maintained for the fetus, and low carbon dioxide levels in maternal blood favor diffusion of CO_2 out of the fetal compartment. The price the mother pays is more labored breathing which many women notice especially late in gestation.

Exercise

Exercise during pregnancy has not only proven to be safe, but it is beneficial to the mother, fetus, and placenta (Anon, 2006). Exercise improves cardiovascular function, suppresses pregnancy weight gain, decreases musculoskeletal discomfort, reduces muscle cramps and lower limb edema, improves mood stability, decreases preterm birth, increases neonatal lean body mass, and expands placenta volume. Many of the physiological aspects of exercise in pregnancy are similar to those found in non-pregnant women. Most of the physiological responses to exercise during pregnancy that are distressing are found in women who were sedentary before pregnancy or in fit women late in their third trimester. As mentioned above, heart rate and alveolar ventilation rate are higher in pregnant women than in non-pregnant women. Thus for any given exercise work load these parameters remain higher for pregnant women. The ventilatory equivalent for oxygen ($\dot{V}_E/\dot{V}_{\text{O}_2}$) is higher in pregnant women at similar non-pregnant workloads. In late gestation, pregnant women may have lower concentrations of glucose during and following prolonged exercise. In addition, pregnant women maintain a lower body temperature and higher blood pH levels than non-pregnant women.

For obese pregnant women, the response to exercise has not been well studied and the few existing studies are contradictory. It is known that in non-obese women, the maximal oxygen consumption ($\dot{V}_{\text{O}_{2\text{max}}}$) declines as a function of gestational weight gain when expressed per unit of body mass. In all normal men and women, a net lactate production occurs during graded exercise at the work load when they cross the so-called “ventilatory threshold.” At workloads beyond the threshold, ventilation rate trajectory increases more dramatically. For reasons that are not completely known, the peak blood lactate levels in women who are pregnant are lower than in non-pregnant women (Heenan et al., 2001). Possible explanations include greater dilution by the expanded blood and extracellular fluid volume in pregnancy and lactate consumption by the fetus. The latter makes sense because the fetal heart and kidney use lactate as their preferred fuel. Another fact is that the capacity for making lactate appears to be compromised in pregnant women.

Placenta

Data from several sources have shown that the shape of the placenta is a marker for disease risk in offspring. The more that placental shape veers from being round, the higher the risk for cardiovascular disease, preeclampsia, and some cancers. It appears that placental size and shape are most predictive of offspring health if maternal phenotype is taken into account. For example, babies born to shorter than average women with very small or very large placentas are more vulnerable for acquiring disease in adulthood than those born to taller women.

Drug Metabolism

Drugs are carried in the blood and metabolized differently in women who are pregnant. Oral drugs may reach the blood stream more slowly because of reduced gut motility believed to be due to elevated plasma progesterone levels. Once in the blood stream drugs dilute into a larger volume of distribution because of the expanded extracellular volume. In addition, the concentrations of plasma protein to which many drugs are normally bound are reduced in pregnancy. Thus, unbound concentrations of drugs may be

increased in the blood compared to the non-pregnant state. On balance, the increased concentration of steroid hormones in the blood may displace drugs from their binding sites on circulating proteins and lead to a reduced biological half-life of the drug. Thus, each drug has to be considered independently because of different protein binding properties.

Obese women who are pregnant will have a higher fat mass in which lipid soluble drugs will distribute. However, this has been little studied in women who have a high fat mass, either in or outside of pregnancy. This should be taken into account when drugs are administered. On the other hand, hormones of pregnancy like estrogen and progesterone and their cousins will induce the expression of some members of the cytochrome P-450 system while other members will be inhibited. The take home message is that pregnancy changes the pharmacokinetics of most drugs. Many of these changes can be directly accounted for by changes in metabolic activity in the liver. Another reason that pregnant women may metabolize drugs differently than non-pregnant women is that drugs may be metabolized by the placenta or will cross the placenta and be metabolized by the fetus. Contrary to popular opinion, all drugs cross the placenta in detectable amounts. Large lipid insoluble molecules cross the placenta very slowly by diffusion. Highly lipid soluble molecules cross the placenta by diffusion very rapidly because they instantly cross the lipid bilayer of the maternal facing placental membrane and thus have the entire surface area of the placenta available for diffusion. The placenta has its own molecular system to de-toxify some molecules and this helps protect the fetus from further damage.

Conclusion

Nearly every organ of a woman's body is modified by the changes in the hormonal environment found during pregnancy. The cardiovascular system is dramatically modified in favor of delivering oxygen and nutrients to the fetus through augmented blood flow to the uterus. The increased rate and depth of breathing ensure better oxygenation of blood. Physical exercise is beneficial to both mother and baby. Yet, there are many aspects of pregnancy changes that we do not understand. These include changes in individual organs like pancreas, spleen, brain, and placenta that have not been thoroughly investigated.

References

- Anon. (2006). Impact of physical activity during pregnancy and postpartum on chronic disease risk. *Medicine and Science in Sports and Exercise*, 38, 989–1006.
- Baylis, C. (1999). Glomerular filtration rate in normal and abnormal pregnancies. *Seminars in Nephrology*, 19, 133–139.
- Bernstein, I. M., Ziegler, W., & Badger, G. J. (2001). Plasma volume expansion in early pregnancy. *Obstetrics and Gynecology*, 97, 669–672.
- Butte, N. F., Wong, W. W., Treuth, M. S., Ellis, K. J., O'brian, S., & E. (2004). Energy requirements during pregnancy based on total energy expenditure and energy deposition. *The American Journal of Clinical Nutrition*, 79, 1078–1087.
- Chavatte-Palmer, P., Tarrade, A., & Rousseau-Ralliard, D. (2016). Diet before and during pregnancy and offspring health: The importance of animal models and what can be learned from them. *International Journal of Environmental Research and Public Health*, 13.
- Edouard, D. A., Pannier, B. M., London, G. M., Cuhe, J. L., & Safar, M. E. (1998). Venous and arterial behavior during normal pregnancy. *The American Journal of Physiology*, 274, H1605–H1612.
- Giraud, G. D., Morton, M. J., Davis, L. E., Paul, M. S., & Thornburg, K. L. (1993). Estrogen-induced left ventricular chamber enlargement in ewes. *The American Journal of Physiology*, 264, E490–6.
- Heenan, A. P., Wolfe, L. A., & Davies, G. A. (2001). Maximal exercise testing in late gestation: Maternal responses. *Obstetrics and Gynecology*, 97, 127–134.
- Hunter, S., & Robson, S. C. (1992). Adaptation of the maternal heart in pregnancy. *British Heart Journal*, 68, 540–543.
- Lindheimer, M. D., & Katz, A. I. (1992). Renal physiology and disease in pregnancy. In D. W. Seldin, & G. Giebisch (Eds.), *The kidney—Physiology and pathophysiology*. New York: Raven Press.
- Nationalacademics.org 2013. Implementing guidelines on weight gain and pregnancy. <https://www.nap.edu/catalog/18292/implementing-guidelines-on-weight-gain-and-pregnancy>.
- Poppas, A., Shroff, S. G., Korcarz, C. E., Hibbard, J. U., Berger, D. S., Lindheimer, M. D., & Lang, R. M. (1997). Serial assessment of the cardiovascular system in normal pregnancy. Role of arterial compliance and pulsatile arterial load. *Circulation*, 95, 2407–2415.
- Shivappa, N., Steck, S. E., Hurley, T. G., Hussey, J. R., & Hebert, J. R. (2014). Designing and developing a literature-derived, population-based dietary inflammatory index. *Public Health Nutrition*, 17, 1689–1696.
- Simeoni, U., & Barker, D. J. (2009). Offspring of diabetic pregnancy: Long-term outcomes. *Seminars in Fetal & Neonatal Medicine*, 14, 119–124.
- Simpson, E. R., & Macdonald, P. C. (1981). Endocrine physiology of the placenta. *Annual Review of Physiology*, 43, 163–188.
- Thornburg, K. L., Jacobson, S. L., Giraud, G. D., & Morton, M. J. (2000). Hemodynamic changes in pregnancy. *Seminars in Perinatology*, 24, 11–14.
- Thornburg, K. L., Bagby, S. P., & Giraud, G. D. (2015). Maternal adaptations to pregnancy. *Knobil and Neill's Physiology of Reproduction*, 2, 1927–1955.
- UNIVERSITY, O. H. S. U. 2015. My pregnancy plate. <http://www.ohsu.edu/blogs/doernbecher/files/2013/04/ohsu-my-pregnancy-plate.pdf>.
- Yu, C. K., Teoh, T. G., & Robinson, S. (2006). Obesity in pregnancy. *BJOG*, 113, 1117–1125.

Relaxin

Ashley F George and Carol A Bagnell, Rutgers University, New Brunswick, NJ, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Corpus luteum A transient, hormone-secreting ovarian structure that develops from a ruptured follicle after ovum release but degenerates unless pregnancy occurs.

Decidualization Differentiation of the endometrium in preparation for pregnancy in rodents and primates.

Implantation The process of attachment of the early embryo to the maternal uterine wall.

Paracrine Type of cell-to-cell communication whereby a cell produces a signal that targets nearby cells.

Placenta Temporary endocrine organ of pregnancy that allows for transport of nutrients, gasses, and waste between mother and fetus.

Pubic symphysis Cartilaginous joint connecting the left and right pubic bones.

Relaxin A 6-kDa polypeptide hormone secreted primarily by corpora lutea that promotes reproductive tract growth and connective tissue remodeling in preparation for birth.

Introduction

Relaxin was discovered more than 90 years ago by Frederick Hisaw who found that serum from pregnant guinea pigs promoted relaxation of the interpubic symphysis of virgin guinea pigs. Relaxin was later isolated from the sow corpus luteum and identified as a hormone of pregnancy that relaxes uterine smooth muscle and connective tissue of the reproductive tract during the latter half of pregnancy in preparation for birth, hence its name. Subsequently, research to isolate, purify, and characterize relaxin from reproductive tissues in multiple species led to an understanding of the structure, chemistry, and biological actions of the hormone. A new chapter in our understanding of relaxin opened up in the late 1990s and early 2000s with the discovery of relaxin family peptide (RXFP) receptors and ligands, the generation of relaxin and relaxin receptor null animals, and the production of recombinant human (H2) relaxin. These discoveries provided important information about relaxin target tissues and mechanisms of action and revealed new roles for the hormone that extended beyond the female reproductive tract. Relaxin is now recognized to target both reproductive and nonreproductive tissues, in males and females. This hormone acts as a neuropeptide in the central nervous system, as an antifibrotic agent in tissue remodeling and as a vasodilator in the cardiovascular system. Relaxin has a wide variety of physiological roles in pregnancy that are species dependent. The targets of relaxin include the pubic symphysis, uterus, cervix, vagina, and mammary glands; and relaxin is a key modulator of cardiovascular changes associated with pregnancy. This chapter focuses on the structure, sources, and biological actions of relaxin during the cycle, in the early embryo, during implantation, and in early pregnancy as well as the impact of relaxin on reproductive tract remodeling and on the cardiovascular system in late pregnancy.

Relaxin and the Relaxin Receptor

Relaxin Structure

Relaxin is a member of the relaxin family of peptide hormones, with structural similarity to insulin and insulin-like growth factors. It is synthesized as a single chain, preprorelaxin, whose signal A-peptide is cleaved to form prorelaxin. The C-chain of prorelaxin is subsequently targeted by convertases to form the native 6-kDa relaxin hormone, consisting of an A and B chain connected by two disulfide bonds and another intra-A chain disulfide bond. In humans and higher primates, there are three relaxin genes (*RLN1*, *RLN2*, *RLN3*), which generate H1, H2, and H3 relaxin peptides, respectively. In rodents, there are two forms of the gene (*Rln1*, *Rln3*) while other mammals have one (*Rln1*) with the exception of ruminants (e.g., sheep and cattle) that lack a *Rln1* gene. The human *RNL2* gene and the *Rln1* gene in other species generate the form of relaxin protein detected in the circulation during pregnancy. While the amino acid sequence of relaxin differs markedly across species, the receptor binding region of the B-chain, amino acids 13–20 (Arg-X-X-X-Arg-X-X-Ile/Val), is highly conserved and required for biological actions of relaxin.

Relaxin Receptor and Mechanism of Action

Given relaxin's structural similarity to insulin, it was thought that relaxin receptors would be similar to the tyrosine kinase-containing receptors that bind insulin and insulin-like growth factors. However, the long-awaited discovery of the relaxin receptor in 2002 revealed it to be a guanine nucleotide binding protein (G-protein)-coupled receptor, related to the family of receptors for

glycoprotein hormones including follicle stimulating hormone, luteinizing hormone, and thyroid stimulating hormone. As illustrated in Fig. 1, the relaxin receptor, now identified as the relaxin family peptide (RXFP)1 receptor, consists of a large extracellular domain containing 10 leucine-rich repeats (LRR) important for high-affinity binding of relaxin and a low-density lipoprotein a (LDLa) module needed for signaling. A lower affinity relaxin binding site is found in the extracellular loops (ECL) of the seven-transmembrane domain that is linked to a C-terminal endodomain. Unlike the heterogeneity of relaxin proteins across species, sequence analysis of RXFP1 receptors shows a high degree of conservation. For example, human and rodent orthologues of RXFP1 show greater than 90% sequence similarity.

The RXFP1 receptor has a wide tissue distribution. Both mRNA and protein for RXFP1 have been identified in female reproductive tissues including the ovary, uterus, cervix, vagina, placenta, and mammary gland as well as in males, in the prostate and testes. In addition, nonreproductive tissue targets expressing RXFP1 receptor include parts of the brain, heart, kidney, liver, lung, and blood vessels. Studies on the mechanism of relaxin action are complicated by the variety of signaling pathways activated in different cell types by the hormone including cyclic adenosine monophosphate (cAMP), mitogen-activated protein kinase, tyrosine kinase, and nitric oxide (NO) pathways. Relaxin activates the adenylate cyclase-cAMP-protein kinase A (PKA) system in reproductive tissues including the mouse interpubic ligament, rat myometrium, and human endometrium. Studies in uterine stromal cells indicate that the cAMP response to RXFP1 activation involves the protein kinase C pathway and tyrosine kinase activity, unlike other G-protein coupled receptors. In addition, there is evidence that actions of relaxin in the heart and blood vessels, as well as the anti-fibrotic effects of relaxin in tissue remodeling, involve the NO synthase-NO-cyclic guanine monophosphate (cGMP) pathway. Taken together, these studies indicate that in response to relaxin, a variety of signaling pathways may be activated including increases in phosphorylation of multiple kinases that may be target tissue-dependent.

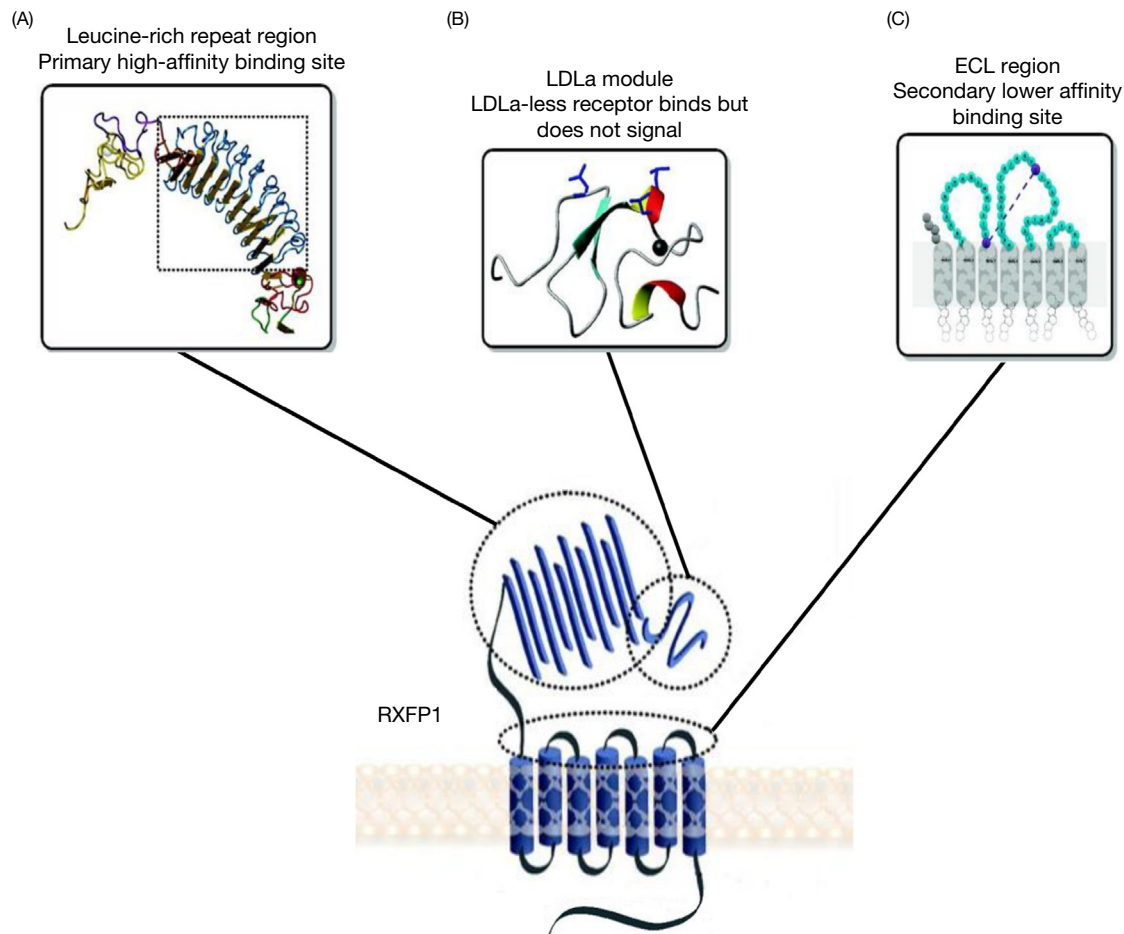


Fig. 1 Structural features of the relaxin family peptide receptor RXFP1. The RXFP1 receptor consists of a large extracellular domain connected to a transmembrane domain via a hinge region. The extracellular domain consists of (A) a leucine-rich repeat (LRR) region that contains the primary high-affinity binding site for relaxin. Connected to the LRR region is (B) an NH₂-terminal, low-density lipoprotein receptor type A (LDLa) module, which is required for signaling. (C) The extracellular loops (ECL) of the transmembrane domain contain a lower affinity binding site for the peptide. Adapted with permission from Bathgate, R.A.D., Halls, M.L., van der Westhuizen, E.T., Callander, G.E., Kocan, M., and Summers, R.J. (2013). Relaxin family peptides and their receptors. *Physiological Reviews* **93**, 405–480.

Sources and Secretion of Relaxin

Ovarian Relaxin

While sources of relaxin are species-specific, relaxin is a product of the corpus luteum in most mammalian species. The corpus luteum of pregnancy is a rich source of relaxin in pigs, rodents, and humans. There is also evidence that relaxin is expressed in large, granulosa cell-derived luteal cells of the corpus luteum of the cycle in these species. Relaxin protein and/or transcripts have also been identified in theca interna cells of developing follicles in both humans and pigs. Also, gonadotropin-induced luteinization of both theca interna cells and granulosa cells is linked to increased relaxin production. Human granulosa-lutein cells collected during *in vitro* fertilization protocols produce relaxin in culture.

Placental Relaxin

The placenta is also a source of relaxin during the second half of pregnancy for many mammals including dogs, cats, rabbits, hamsters, horses, and humans. An increase in circulating relaxin in dogs and cats is used as a diagnostic aid for pregnancy detection based on the increase in systemic relaxin produced by the placenta within 3–4 weeks after mating. In the mare, microcotyledons of the placenta express relaxin transcripts after the first trimester of pregnancy and secrete relaxin into the circulation. Systemic relaxin levels decline in mares with placental insufficiency or other placental disorders. In addition, there is evidence that the early equine embryo expresses relaxin transcripts, although secretion of relaxin has not been verified. In women, evidence for both H1 and predominantly H2 gene expression has been found in decidual and placental tissues. The uterus is a primary source of relaxin in pregnant guinea pigs, and there is evidence for relaxin production by the uterine epithelium in both rats and pigs. Relaxin is also detectable in milk of several species including rats, dogs, pigs, and humans. The detection of higher concentrations of relaxin in milk than in the maternal circulation suggests that relaxin is either produced locally by the mammary gland or made elsewhere and concentrated in milk. Detection of relaxin protein and/or relaxin transcripts in mammary tissues of rats, guinea pigs, pigs, and women supports the idea of a local source for relaxin in milk.

Relaxin Secretion

Relaxin levels are low to undetectable in the circulation of nonpregnant animals. In pregnancy, relaxin circulates in the blood of all mammals, but the pattern of relaxin secretion differs markedly across species. For example, in pregnant pigs, relaxin is detectable at low levels in the circulation and marked by a large prepartum surge (over 100 ng/mL) just prior to parturition. In rodents, serum relaxin levels are relatively constant until the end of pregnancy, when there is a marked surge in relaxin secretion just before parturition. In the mare, serum relaxin rises in the first trimester of pregnancy and remains elevated until foaling. Following delivery of the placenta, relaxin levels are undetectable in the circulation of the postpartum mare suggesting that the placenta is the main source of circulating relaxin in the horse. In contrast, in humans and other primates, serum relaxin is highest in early pregnancy and peaks (1 ng/mL) near the end of the first trimester, after which relaxin levels decline and remain low for the duration of pregnancy. In pregnant women, luteal relaxin is the source of systemic relaxin as illustrated in ovum-donation pregnancies in which circulating relaxin is not detectable in the absence of functioning ovaries. In contrast, human placental/decidual relaxin is thought to act locally in a paracrine manner, playing an important role in controlling fetal membrane remodeling at the time of parturition.

Biological Actions of Relaxin in Female Reproduction

Biological actions of relaxin differ markedly across species, as evident in [Table 1](#). During the cycle and in early pregnancy, relaxin actions include ovarian follicular growth and remodeling, implantation and decidualization, and myometrial quiescence. Later in pregnancy, a role for relaxin has been proposed in uterine growth, remodeling the birth canal and fetal membranes in preparation for parturition, and mammary gland development. Additionally, relaxin is involved in changes in the cardiovascular system and in fluid homeostasis as pregnancy progresses.

Follicular Growth and Remodeling

While relaxin is best known for its actions during the latter half of pregnancy, the expression of ovarian relaxin in nonpregnant animals during the estrous cycle supports a role in ovarian function. Although systemic relaxin is not detectable during the follicular phase of the cycle in any species, relaxin levels in follicular fluid increase with follicular size in pigs, suggesting local production and action of relaxin in the follicle. Relaxin has been implicated in follicular growth in pigs, humans, and rats. In pigs, low doses of relaxin promote the growth of granulosa and theca cells *in vitro*. Consistent with these observations, relaxin binding sites have been localized in both granulosa and theca cells of developing porcine follicles. Given the importance of proteolytic remodeling for follicular wall rupture, a paracrine role for relaxin in ovulation was suggested based on evidence in several species. Relaxin increases secretion of proteolytic enzymes, including plasminogen activator and collagenase, in rat follicular cell cultures. In the *in vitro* perfused rat ovary, human relaxin-2 induces ovulation. Human granulosa cells from all follicle sizes express the RXFP1 receptor, and incubation with relaxin increases vascularization of human follicular tissues. On the other hand, there is no evidence

Table 1 Biological Actions of Relaxin in Female Reproduction

<i>Tissue</i>	<i>Rat</i>	<i>Mouse</i>	<i>Pig</i>	<i>Human/ Nonhuman primate</i>
Ovary	Follicular growth Ovulation		Follicular growth	Follicular growth Vascularization
Uterus	Uterine growth Reduced collagen density Vasodilation Inhibition of myometrial contractions Decreased duration of labor	Uterine growth Reduced collagen density Vasodilation Inhibition of myometrial contractions	Uterine growth Tissue remodeling Inhibition of myometrial contractions Decreased duration of labor	Decidualization Angiogenesis Increased endometrial thickness
Pubic symphysis		Fibrocartilage to a flexible ligament		
Cervix/vagina	Growth and remodeling	Growth and remodeling	Growth and remodeling	
Mammary gland/ nipple	Required for nipple development	Required for nipple development	Mammary parenchymal tissue growth	
Vascular adaptations	In pregnancy: Vasodilation, increased cardiac output, and plasma volume Reduced systemic and renal resistance Decline in plasma osmolality	In pregnancy: Decline in plasma osmolality		Vasodilation in systemic and renal circulation Increased cardiac index ^a Decreased vascular resistance ^a

^aRelaxin's effects tested in patients with heart failure only.

Data summarized from work reviewed by Sherwood, O.D. (2004). Relaxin's physiological roles and other diverse actions. *Endocrine Reviews* **25**, 205–234 and Conrad, K.P. (2011). Maternal vasodilation in pregnancy: the emerging role of relaxin. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology* **301** (2), R267–R275.

for a role for relaxin in follicular growth or ovulation in mice. Administration of relaxin failed to increase mouse preantral follicular growth. In addition, studies in both relaxin-null and RXFP1 knockout mice confirm that both groups of mice are fertile and litter sizes are comparable to wild-type controls. Taken together, these studies reinforce the observation that relaxin's action differs across species. Further research is needed to confirm a role for relaxin in ovulation.

Implantation

Evidence for a role for relaxin in implantation comes from studies in nonhuman primates and women. Here, highest circulating levels of relaxin occur in the first trimester of pregnancy in comparison to other species, including rodents, pigs, and horses, in which relaxin levels are maximal at the end of pregnancy. Administration of relaxin and estrogens to ovariectomized, nonpregnant marmoset monkeys increased endometrial thickness and angiogenesis. Similar results were observed following relaxin treatment in a model of early pregnancy in the rhesus monkey and during peri-implantation in the cynomolgus macaque. In addition, relaxin targets RXFP1 receptors expressed in human endometrial cells to increase production of hormones and growth factors involved in decidualization and angiogenesis, important for implantation. Decidualization of the endometrial stroma creates a receptive environment for blastocyst implantation, and there is evidence that relaxin is important for initiating this process in nonhuman primates and in women. The decidual response is characterized by increased expression of two biological markers: insulin-like growth factor binding protein 1 and prolactin. Relaxin is produced by human decidual cells and can induce decidualization of endometrial stromal in culture. Additionally, administration of relaxin in the rhesus monkey resulted in increased resident lymphocytes and maintained connective tissue integrity in the endometrium, processes necessary for establishment of pregnancy. Taken together, these studies show effects of relaxin on the human and nonhuman primate endometrium of early pregnancy that support a role for relaxin in implantation in these species. However, clinical observations in women indicate that implantation and pregnancy still occur in the absence of a corpus luteum or detectable serum relaxin. Whether locally produced relaxin can support implantation is unknown, and confirmation of paracrine effects of relaxin in promoting implantation await further study.

Uterine Growth and Remodeling

Multiple studies have reported uterotrophic effects of relaxin in nonpregnant rodents, pigs, and primates. Growth-promoting effects of relaxin include water imbibition as well as increased DNA content and proteins involved in structural changes in the uterus such as collagen, gap junction proteins, and matrix metalloproteinases. In prepubertal pigs, uterotrophic effects of relaxin include increases in uterine dry weight, protein and DNA content. In rats and mice, relaxin treatment increases endometrial thickness, reduces densely packed collagen fibers, and dilates uterine blood vessels. In a similar manner, the vagina is a target for the growth-promoting actions of relaxin in rodents and pigs. Concurrent with uterine growth is a relaxin-induced increase in endometrial angiogenesis in rhesus and marmoset monkeys, and an increase in vascular endothelial growth factor (VEGF) by human endometrial stromal cells has been reported. In human endometrial cells, the angiogenic response to relaxin is menstrual-cycle specific in that incubation with relaxin increases VEGF secretion in the secretory phase but not in the proliferative phase. The angiogenic effects of exogenous relaxin were

demonstrated in clinical trials for scleroderma in which women who were treated for 24 weeks with relaxin experienced an increased incidence of irregular and/or heavier-than-normal menstrual bleeding compared to placebo-treated women.

These results led to the hypothesis that relaxin induces uterine growth during pregnancy to accommodate the growing fetus. In support of this hypothesis, relaxin deficiency in early pig pregnancy resulted in a 30% reduction in uterine wet weight compared to controls. Further, relaxin increased uterine growth during pig pregnancy. In the pig, VEGF is produced by endometrial stromal cells *in vitro*, and relaxin increased VEGF secretion from endometrium collected on days 10–12 of pregnancy. In contrast, relaxin is not required for uterine growth during rodent pregnancy. Relaxin immunoneutralization in rats during the second half of pregnancy did not affect uterine weight at term. Likewise, relaxin knockout mice had similar uterine weights throughout pregnancy as compared to wild-type controls.

Myometrial Quiescence

Relaxin decreases myometrial contractility in pregnancy, but this effect is species-specific. In rodents and pigs, relaxin inhibits spontaneous and oxytocin-induced smooth muscle contractions conducive to implantation and preventing premature delivery. Rats ovariectomized in early pregnancy and supplemented with ovarian steroids exhibited increased uterine contractile frequency compared with controls. When hormone replacement therapy included relaxin, uterine contractile activity returned to normal. Further, data in rats indicate that relaxin's effects on the myometrium may also contribute to the spacing of embryos. *In vitro* experiments in rats and pigs suggest that progesterone increases myometrial sensitivity to relaxin, synergistically reducing uterine contractions. In rats, relaxin attenuates myometrial contractions partially through activation of cAMP/PKA signaling. In humans, relaxin does not inhibit spontaneous or oxytocin-induced myometrial contractility, although human myometrial cells *in vitro* do generate cAMP in response to relaxin via RXFP1 signaling (as in other species), suggesting that myometrial quiescence is stimulated by signaling factors downstream of cAMP.

The physiological role of the prepartum surge in relaxin to maximal levels in rats, mice, and pigs just prior to parturition is not well understood. It has been suggested that relaxin helps to coordinate contractions at the onset of delivery in these species but relaxin-deficient rodents do not have altered timing of labor onset. Several studies have determined that relaxin does play a role in the duration of delivery and live birth rates. In pigs and rats made relaxin-deficient in the second half of pregnancy, duration of labor increased and live birth rates decreased compared with controls.

Connective Tissue Remodeling of the Pubic Symphysis and Cervix

In guinea pigs, mice, and bats the pubic symphysis transforms from fibrocartilage to a flexible ligament prior to parturition. Relaxin's ability to remodel connective tissue of the guinea pig pubic symphysis of pregnancy led to discovery of the hormone in 1926, and similar effects in mice supported the development of a mouse interpubic ligament bioassay for relaxin. As expected in relaxin-null mice, interpubic ligament length and weight are reduced in comparison to wild-type mice. The significance of relaxin-induced pubic symphysis remodeling in late pregnancy may be species dependent. Studies show that the substantial increase in interpubic ligament length observed in late-pregnant mice is not required for delivery. Parturition in relaxin knockout mice is normal, and they deliver their pups as rapidly as their wild-type littermates. Relaxin-induced interpubic ligament elasticity in guinea pigs and mice is estrogen-dependent, but this pelvic joint transformation does not occur in all species late in pregnancy. There are reports of pelvic joint laxity in women during the first half of pregnancy when relaxin levels are elevated but whether endogenous relaxin contributes to pelvic joint laxity in pregnant women is unknown.

Cervical growth and softening are well-known effects of relaxin in late-pregnant rats and pigs. Relaxin-induced cervical growth during late pregnancy involves an increase in both weight and size of the cervix. Studies show that relaxin-induced proliferation of epithelial and stromal cells, as well as inhibition of cervical cell apoptosis, are responsible for these trophic effects. The characteristic increase in cervical elasticity and softening near the end of pregnancy in rats and pigs involves dispersion of collagen fibers and remodeling of the extracellular matrix that changes the tensile strength of the cervix. In fact, these cervical modifications failed to appear following ovariectomy of pregnant pigs and rats but returned following relaxin replacement therapy. The cervix in relaxin-deficient rats, pigs, and knockout mice remains small and firm, with collagen fiber bundles failing to disperse. Cervical softening is estrogen-dependent in rats but not in pigs. Debate exists about whether relaxin mediates human cervical softening. While cervical softening can be induced in women with direct application of pig relaxin to the cervix, similar results were not observed in clinical trials using recombinant human relaxin-2. However, in women there is evidence for a role for decidual relaxin acting locally, in a paracrine manner, to stimulating proteolytic enzyme degradation of fetal membranes near the time of delivery.

Mammary Gland and Nipple Development

Relaxin promotes mammary gland development during pregnancy in several species. Its major effect in pigs is on the mammary parenchyma while in rodents nipple development is affected. In pigs, mammary gland development begins around day 80 of pregnancy and continues until parturition, corresponding to increasing systemic estrogen and relaxin levels. Relaxin replacement in ovariectomized pigs stimulated mammary gland parenchyma growth through reduction in collagen fiber bundles. Similar effects on mammary collagen fiber bundles were observed in rats and mice, where relaxin was also shown to reduce the length of elastin

fibers and increase the cross-sectional area of arterioles. Relaxin-binding sites have been identified in mammary tissues of rats, pigs, and humans.

Studies in relaxin-null mice and in rats made relaxin-deficient by immunoneutralization revealed that the hormone is required for development of mammary nipples, which occurs in the latter half of pregnancy. The mammary glands of relaxin and RXFP1 knockout mice are normal and produce milk, but these dams have such small nipples at term that pups are unable to suckle and die. In contrast, the nipples of relaxin-deficient pigs are fairly well developed at term and nursing pigs can obtain milk. Given that relaxin is detectable in milk of several species in levels greater than observed in maternal circulation, it is possible that relaxin is acting in an autocrine or paracrine fashion on mammary tissues during late pregnancy.

Maternal Vascular Adaptations

Studies indicate that relaxin plays an important role in regulating cardiovascular changes during pregnancy. This includes pregnancy-related increases in plasma volume, cardiac output, and vasodilation as well as reductions in systemic vascular resistance and blood pressure. In nonpregnant rats, relaxin administration results in cardiovascular changes similar to those observed in pregnancy, including increased cardiac output, decreased systemic vascular resistance, and increased renal filtration rate and plasma flow. In pregnant rats made relaxin-deficient early in pregnancy, maternal vascular adaptations fail to occur at mid-pregnancy. Further, in relaxin-deficient, pregnant rats, uterine blood vessel elasticity is reduced and renal and systemic vascular resistance is increased, similar to nonpregnant rats. In humans, similar maternal vascular changes also occur during the first trimester, when relaxin levels are maximal.

Relaxin is a potent vasodilator of blood vessels in both reproductive and nonreproductive tissues; however, this effect is vessel-specific. For example, in humans, in vitro relaxin treatment relaxed precontracted gluteal arteries but did not affect small pulmonary resistance arteries, uterine myometrial arteries, or placental stem villus arteries. In addition, the impact of relaxin on blood vessels includes both rapid and sustained vasodilatory responses. The rapid response to relaxin in dilating blood vessels involves RXFP1 binding, PI-3 kinase activation, and NO production. The slower, sustained vasodilatory response to relaxin involves generation of NO, matrix metalloproteinase production, and endothelin receptor binding. Of note is that these vasodilatory effects of relaxin, together with actions to increase cardiac output, provide the basis for clinical trials testing the efficacy of relaxin in treatment of cardiac failure.

During pregnancy in humans and rodents, a decline in plasma osmolality occurs without a concomitant decrease in antidiuretic hormone, thereby reducing the osmotic threshold. Studies in rats and mice suggest that relaxin plays a role in this adaptation during pregnancy. Reduced osmolality in the second half of pregnancy in rats and mice corresponds with an increase in circulating relaxin in this period. Plasma osmolality declines following relaxin infusion in nonpregnant rats, although there was no reduction in osmolality in relaxin-deficient pregnant rats. Whether relaxin plays a role in the reduction of plasma osmolality in human pregnancy is not clear. Plasma osmolality declines in women with ovum-donation pregnancies in which circulating relaxin is undetectable. Relaxin's effects on plasma osmolality during pregnancy in the rat may be due, in part, to alterations in water consumption. During late pregnancy, drinking increases in rats. Relaxin treatment by intravenous or intracerebroventricular injection results in water consumption within minutes in nonpregnant rats. Further, relaxin removal in rats via passive immunization during the second half of pregnancy reduces drinking frequency.

Conclusions

The diverse actions of relaxin in reproductive tissues across a variety of species supports its characterization as a pleotropic hormone. Advances in identifying the sources and biological actions of relaxin in female reproduction reviewed here preceded the discovery of the cognate receptor for relaxin in 2002. Subsequently, studies in relaxin-deficient animals have shed new light on the biological actions of relaxin in both reproductive and nonreproductive tissues. The widespread tissue distribution of the RXFP1 receptor has led to investigation of new roles for relaxin in the brain and cardiovascular system, as well as in fibrosis and wound healing. In terms of reproduction, fundamental questions about the control of relaxin synthesis, its mechanism of action, and significance of local vs. systemic relaxin production and action in reproductive tissues will continue to be important areas of investigation.

Further Reading

- Anand-Ivell, R., & Ivell, R. (2014). Regulation of the reproductive cycle and early pregnancy by relaxin family peptides. *Molecular and Cellular Endocrinology*, 382, 472–479.
- Bathgate, R. A. D., Halls, M. L., van der Westhuizen, E. T., Callander, G. E., Kocan, M., & Summers, R. J. (2013). Relaxin family peptides and their receptors. *Physiological Reviews*, 93, 405–480.
- Bergfelt, D. R., Peter, A. T., & Beg, M. A. (2014). Relaxin: A hormonal aid to diagnose pregnancy status in wild mammalian species. *Theriogenology*, 82, 1187–1198.
- Conrad, K. P. (2011). Maternal vasodilation in pregnancy: the emerging role of relaxin. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*, 301(2), R267–R275.
- Halls, M. L., Bathgate, R. A. D., Sutton, S. W., Dschietzig, T. B., & Summers, R. J. (2015). International Union of Basic and Clinical Pharmacology. XCV. Recent advances in the understanding of the pharmacology and biological roles of relaxin family peptide receptors 1–4, the receptors for relaxin family peptides. *Pharmacological Reviews*, 67, 389–440.
- Sherwood, O. D. (2004). Relaxin's physiological roles and other diverse actions. *Endocrine Reviews*, 25, 205–234.

Oxytocin

Richard Ivell, University of Nottingham, Sutton Bonington, United Kingdom

Mike Ludwig, University of Edinburgh, Edinburgh, United Kingdom

Rachel M Tribe, Kings College London, London, United Kingdom

Ravinder Anand-Ivell, University of Nottingham, Sutton Bonington, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Structure and Evolution

Oxytocin is a small peptide hormone comprising just nine amino acids arranged in a cyclic form (**Fig. 1**) held together by a single cystine sulphhydryl bridge between amino acids 1 and 7. Together with the modified C-terminal amino acid, this gives it a resistance to rapid degradation in the blood stream as well as providing a unique three-dimensional structure which complements the hormone-binding pocket of its receptor (see later). It also offers a specific surface with which to interact with its binding protein, neurophysin 1. In all mammals oxytocin is very closely associated with the structurally related hormone vasopressin. Both derived by gene duplication from a common invertebrate ancestral hormone, probably much like the molecule annetocin in worms. Both vasopressin and oxytocin have retained aspects of their ancestral physiology: annetocin is responsible for the contractility and function of the mesonephric duct linking the coelomic cavity with the outside, thereby helping to convey gametes out of the body and maintaining osmotic balance. In modern mammals, oxytocin is still linked with the contraction of the uterus and the vas deferens, and vasopressin with maintaining osmotic balance in the kidneys. However, during evolution, both hormones have also acquired a number of other functions, which have led to increasing subtlety in their regulation as well as in the expression and regulation of their specific receptors.

One of the more interesting aspects in the evolution of oxytocin is the way in which the gene which encodes it is strongly related to the gene for vasopressin. In all vertebrates, both genes are separated by only a few nucleotides from one another. Most genes following gene duplication tend to diverge both spatially across the genome and in terms of their sequence. The opposite is true for oxytocin and vasopressin. One explanation is that by being so closely linked they can more easily exchange genetic information

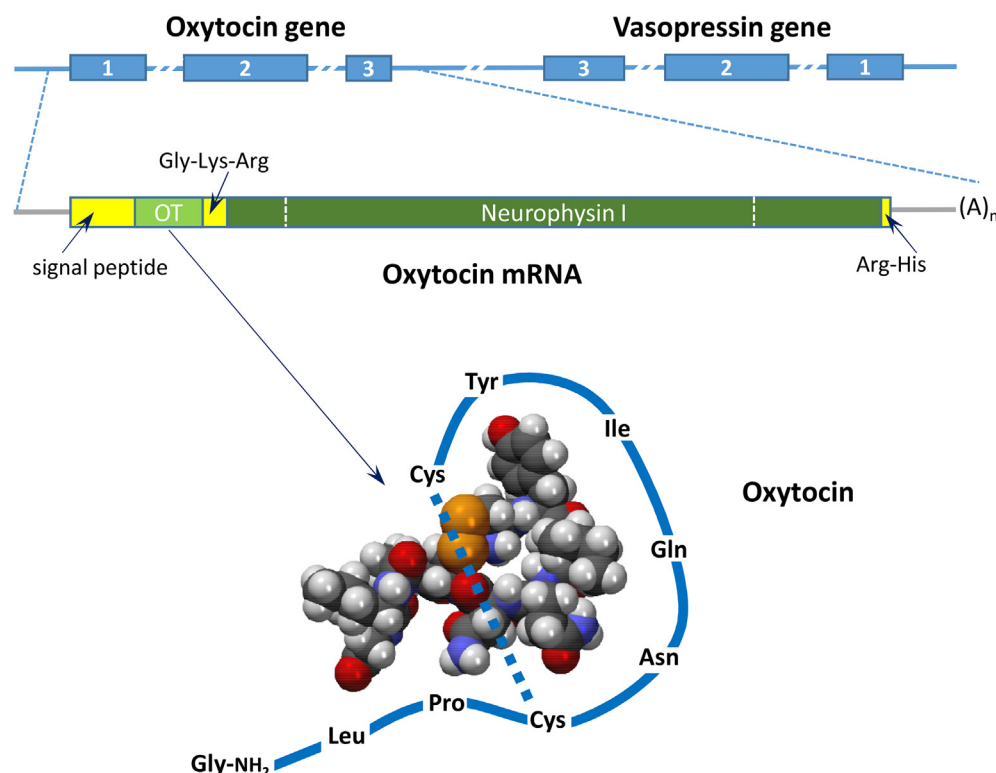


Fig. 1 Schematic diagram to illustrate the structure of the oxytocin gene and its derived mRNA transcript. The oxytocin mRNA is translated to yield a single polypeptide precursor comprising in order a signal peptide to aid translocation to the cellular secretory apparatus, and the oxytocin peptide itself, followed by the tripeptide Gly-Lys-Arg which acts as a cleavage signal for intracellular enzymes as well as a donor of an amide group the C-terminal end of the oxytocin peptide. This is followed by the larger neurophysin 1 carrier protein. The lower panel shows a three-dimensional representation of the oxytocin peptide indicating how the cysteine bridge between amino acids 1 and 6 maintains a rigid shape to the molecule.

with each other in a process called gene conversion, which encourages the encoded proteins to be more similar to one another and conserved.

Like vasopressin, oxytocin is encoded as part of what is called a polyprotein, comprising more than one functional unit (Ivell and Richter, 1984a). Besides oxytocin, the gene also encodes a larger cysteine-rich polypeptide called neurophysin 1, which acts as a carrier protein for oxytocin. The vasopressin gene similarly encodes not only vasopressin but also neurophysin 2. The two carrier proteins are very similar and both can bind their associated peptide hormones with high affinity. Oxytocin mRNA encodes a pre-pro-polyprotein (Fig. 1), which because of an N-terminal signal peptide is first translocated from the ribosome into the lumen of the endoplasmic reticulum, where this is removed to make the pro-polyprotein; this is then transferred to the Golgi apparatus and finally to acid-rich secretory vesicles. During this process specific proteolytic enzymes separate the oxytocin from the neurophysin and ensure transamidation of the oxytocin C-terminus, although the oxytocin remains associated with its neurophysin carrier protein until released from the cell.

Oxytocin Expression—Cells, Tissues, and Organs

The principal site of oxytocin synthesis is within the large magnocellular neuroendocrine neurones (MCNs) of the hypothalamus within the brain. These MCNs are arranged symmetrically close to the third ventricle in two groups (Fig. 2), the supraoptic nucleus (SON) and the paraventricular nucleus (PVN), and can produce either oxytocin or vasopressin. The MCNs, of which there are only a few thousand in both nuclei, are characterized by long axons which project downwards out of the hypothalamus, along the pituitary stalk, with endings from which the hormone is released within the posterior lobe of the pituitary gland, also called the neurohypophysis (Fig. 2). In addition, secretory granules containing oxytocin may also be released from the cell bodies and especially dendrites within the hypothalamus, often projecting to more distant parts of the brain (Pow and Morris, 1989). Moreover, the PVN also contains smaller, so-called parvocellular neurones which express oxytocin and project elsewhere in the hypothalamus or to the brainstem and spinal cord (Armstrong, 1995; Brown et al., 2013).

The MCNs are responsible for the oxytocin which is released from the posterior pituitary in a pulsatile fashion and is responsible for the key oxytocin-dependent physiologies of uterine contraction at birth, the milk let-down response during lactation, and smooth muscle contraction of the male tract during copulation (see below). However, oxytocin is also produced locally within

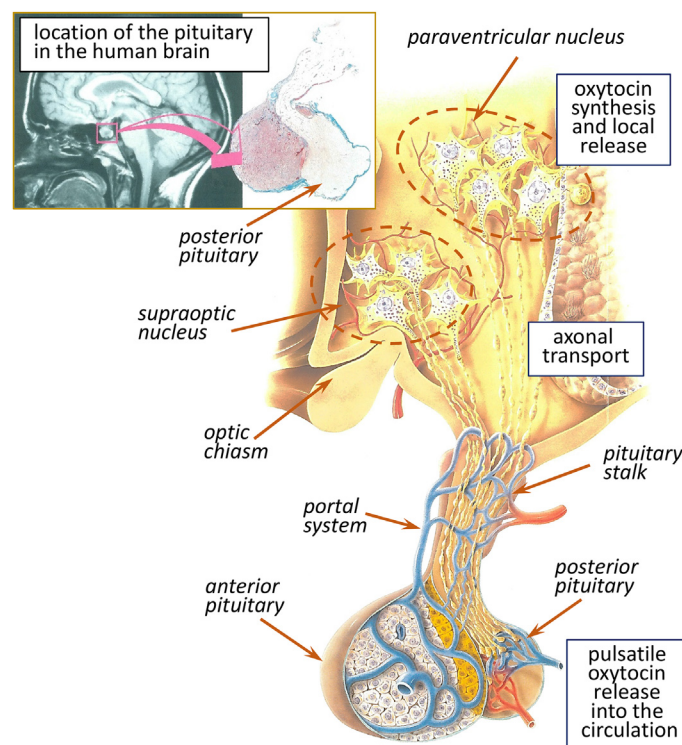


Fig. 2 Diagram to show the location of the pituitary gland on the underside of the human brain, beneath the hypothalamic region (inset). The paraventricular nucleus (PVN) and the supraoptic nucleus (SON) each comprising several thousand magnocellular neuroendocrine cells (MCN), are indicated by ovals. Their beaded axons extend through the pituitary stalk to end in the posterior pituitary where oxytocin is released into the bloodstream. The anterior pituitary and associated hypophyseal portal system are also illustrated. Modified from Breckwoldt M, Neumann F, Brauer H (1991) *Exempla endokrinologica*, Band 1, Medical Service Munchen, Germany, pp. 1–173.

several organs and tissues where it appears to serve paracrine regulatory roles. These include the testes, follicular and luteal cells in the ovaries (see below), the thymus, placenta and probably other tissues (Ivell and Russell, 1995).

Regulation of the Oxytocin Gene

The gene for oxytocin is located on chromosome 20p12.2 in humans and is very small comprising altogether only about 1000 nucleotides made up of three exons and two intervening sequences (Fig. 1). Particularly the region of exon 2, encoding most of the neurophysin moiety, is highly homologous with the equivalent region of the vasopressin gene. In terms of regulation, there is still relatively little known about the region responsible for determining cell specificity, although experiments in transgenic mice indicate that this resides within only a few thousand nucleotides from the transcription start site. In vivo, the oxytocin gene can be modulated by circulating steroid hormones, in particular estradiol and testosterone seem able to cause an up-regulation of the gene, for example to increase synthesis and secretion of oxytocin in late pregnancy. Whilst some of this effect may be due to indirect effects of the steroids on the cells which make oxytocin, in vitro studies have shown that activation of estrogen and androgen receptors can directly increase the transcriptional activity of the oxytocin gene. However, this is mediated in an unconventional way, which does not require a direct interaction between the steroid receptor and the oxytocin gene promoter region. Instead, it has been shown that activated steroid receptors work through another transcription factor called steroidogenic factor 1, which interacts with a specific responsive element at –160 nucleotides upstream of the transcription start site (Ivell et al., 2014). In fact, mutation of the DNA-binding domain of a steroid receptor such that it can no longer interact with DNA does not prevent the steroid-dependent up-regulation of the oxytocin gene. Moreover, there is no potential responsive element within several thousand nucleotides of the transcription start site which exhibits the potential to directly interact with a steroid receptor. This non-classical activation of the oxytocin gene by steroid receptors also allows for regulation by selective estrogen receptor modulators (SERMs), such as tamoxifen which, at least in an in vitro cell system, stimulates the oxytocin gene, rather than being an antagonist as in the classical estrogen-dependent pathway (Ivell et al., 2014).

Oxytocin Synthesis and Secretion

The MCNs are highly active cells receiving informational input from a wide range of brain regions, including the hypothalamus, the limbic region, and the brainstem (Leng et al., 1999). Mostly such inputs are either excitatory or inhibitory, employing glutamate or GABA neurotransmitters, respectively, particularly where these derive from within the hypothalamus. Here there is direct input from the arcuate, suprachiasmatic and tuberomammillary nuclei. Additionally MCNs express receptors for a wide range of peptides and other neurotransmitters, such as serotonin, dopamine or acetylcholine (Brown et al., 2013). Neuronal input also comes from more distant regions such as the subfornical organ or the organum vasculosum of the lamina terminalis. These are so-called circumventricular organs which lack a blood-brain barrier, hence allowing reception of many informational molecules in the blood, including other hormones. There are also significant projections to the MCNs from the nucleus tractus solitarius and ventrolateral medulla, which receive signals from peripheral tissues involving the vagus nerve. This informational route includes the important sensory afferents from the mammary glands and the uterus, as well as coordinating appetite-regulating peptides such as cholecystokinin (CCK) from the gastrointestinal tract or cytokine immune signals.

Within the MCNs oxytocin is packaged into secretory vesicles. These can be transported to or released from any part of the neurones, including axon swellings and undilated axons, and also from the dendrites and the cell bodies themselves (Morris and Pow, 1991). It is important to distinguish between oxytocin released axonally from the posterior pituitary and that which is released within the brain, mostly via dendrites. Such secretion is regulated differentially. Whereas release from axon terminals occurs largely via electrical action potentials (spikes), that from dendrites or from cell bodies is consequent on intracellular Ca^{2+} release from stores in the endoplasmic reticulum (Ludwig and Leng, 2006).

Although the neurosecretion of oxytocin or vasopressin is the main role of MCNs, they also release, particularly within the hypothalamus, many other signaling molecules, including the peptides CCK, dynorphin, galanin, apelin and secretin. They can also generate NO via nitric oxide synthase, as well as endocannabinoids and adenosine. These local informational molecules serve in an autocrine/paracrine way to orchestrate and modulate MCN function.

Release of oxytocin from dendrites does not necessarily occur synchronously with axonal release. Such responses may be to the same stimulus, but on a different time-scale, or dendritic release may be completely independent of axonal release from the pituitary. For example, in lactating rats oxytocin is secreted within the SON before it is secreted from the pituitary; in contrast, upon hyperosmotic stimulation the sequence is reversed. One role for oxytocin released within the hypothalamus appears to be to act as part of a feedback loop auto-regulating MCN electrical function (Ludwig and Leng, 2006; Ludwig and Stern, 2015). Dendritic oxytocin causes release by the MCNs of intracellular Ca^{2+} independently of electrical activity, thereby priming the cells for subsequent oxytocin release by moving secretory vesicles close to the cell surface. This priming effect serves to amplify and co-ordinate the dendritic release of oxytocin and hence the overall activity of the MCNs within a nucleus.

Oxytocin Receptor, Expression and Regulation

Although the oxytocin peptide and its effects were known for many years, it was the work of the Japanese scientist Tadashi Kimura which in 1992 finally led to the identity and structure of the oxytocin receptor (Kimura et al., 1992). The oxytocin receptor belongs to the class A group of 7-transmembrane domain G-protein-coupled receptors. It has only a short N-terminal extracellular domain and the hormone effectively interacts with all of the exposed extracellular regions of the receptor. These regions may also be differentially glycosylated though whether this is important for regulation is not known. Once the hormone has bound, a conformational change in the receptor leads to activation of an inhibitory G-protein and hence a PI3-kinase-linked signaling pathway leading to intracellular Ca^{2+} release. Importantly, the oxytocin receptor responds to activation by oxytocin by rapid desensitization and internalization as is typical for most hormones which are secreted in a pulsatile fashion (Arrowsmith and Wray, 2014). Up regulation of the oxytocin receptor system in a cell either requires a delay in the desensitization process or simply an increase in the expression of the oxytocin receptor gene. This is what happens, for example, at term of labor in women when oxytocin receptor gene transcripts appear to be increased almost 100-fold. However, the responsiveness of the oxytocin receptor may also be modulated by changing the properties of the cell membranes in which it is anchored. For example, increasing the rigidity of the plasma membrane by increasing its cholesterol and possibly also its steroid (e.g., 17OH-progesterone) content attenuates the intracellular response (Gimpl et al., 2002).

There is a single gene encoding the oxytocin receptor, located on chromosome 3p25 in the human, which is highly homologous across species. The gene comprises three exons. In spite of the importance of the oxytocin receptor for the induction of labor and mammary function, surprisingly little is known about the transcriptional regulation of the oxytocin gene. It is certainly regulated by steroids such as estrogens, though probably in a non-classical manner, whereby activated estrogen receptors act indirectly via other transcription factors within the upstream promoter region of the gene. Other specific transcription factors such as MafK are also involved, acting within an intron of the receptor gene. Moreover, DNA methylation within different regions of the gene also appears to correlate with cell specificity of expression. To date, however, we still have very little understanding of the precise molecular mechanisms invoked when the receptor is either up- or down-regulated (Kimura et al., 2013). Our lack of understanding is exacerbated by the evident physiological redundancy in some functions, so that, for example, oxytocin receptor knockout mice appear to undergo parturition normally.

Oxytocin, Uterine Contraction and Parturition

Oxytocin is one of the most potent uterotonic factors known, a property which is used today in the induction of birth (see below). Shortly before the end of pregnancy (term), as estrogen concentrations rise and there is functional progesterone withdrawal, oxytocin receptors are up-regulated in the myometrial smooth muscle layers of the uterus, and become sensitive to the increasing pulses of oxytocin coming from the posterior pituitary and released locally from reproductive tissues (Summerlee, 1981; Russell et al., 2003). Labor and birth represent a series of positive feedback loops whose resultant has to be the expulsion of the uterine contents. Part of this involves the induction in term myometrium of local prostaglandin-F2 α expression, which like oxytocin causes smooth muscle contractility by mobilizing intracellular Ca^{2+} stores. The influence of factors which have promoted myometrial quiescence, such as the hormone relaxin in most species (though not in humans) and progesterone decreases, as is the placental production of the oxytocin-degrading enzyme oxytocinase. Uterine contractility, along with fetal movements, elicits the *Ferguson reflex*, whereby neuronal afferents from the cervix via the vagal system result in further activation of the MCNs in the hypothalamus.

In rats and other species, oxytocinergic MCN give rise to intense synchronized bursts of electrical activity which cause the release of increasingly large secreted pulses of oxytocin (Fig. 3). Pulsatility is important since a continuous and sustained secretion of oxytocin would not only quickly deplete cellular stores of the hormone, but would lead to a desensitization of the oxytocin receptors and a slowing of the birth process. This activation of the MCNs also leads to an increase in the dendritic secretion of oxytocin, which, as mentioned above, would effectively influence neuronal plasticity to further orchestrate and increase the oxytocinergic function of the magnocellular nuclei. High prevalent estrogen levels would also act to stimulate oxytocin gene transcription in the MCN, presumably via estrogen receptor- β , which is known to be expressed in these cells. Interestingly, in cows at term additional oxytocin is produced in pulses by the ovarian corpus luteum which otherwise is relatively quiescent during the second half of gestation.

As in other aspects of reproductive physiology, there is a degree of redundancy in this system. While transgenic mice in which either oxytocin or its receptor have been inactivated still appear to give birth normally, lack of the prostaglandin-synthesizing enzyme COX2 does lead to a disruption of labor and birth, apparently due to a down-regulation of the oxytocin receptor in the uterus. Moreover, the oxytocin-deficient mice do appear to have lost some of the circadian control of birth, suggesting that the oxytocin system is still critical for the precise timing of the birth process (Ratajczak and Muglia, 2008).

Within the hypothalamus there are further adaptations to pregnancy and birth. Largely based on studies in rats it has been shown that in the latter half of pregnancy there is an up-regulation of the κ -opioid dynorphin within the MCNs. This feeds back on the MCNs to suppress electrical activity and maintain the pool of stored oxytocin, and is supported by increased GABAergic activity to further suppress MCN activity. With the onset of uterine contractions, noradrenergic neurones which are stimulated by the vagus system, together with the co-expressed μ -opioid enkephalin, directly activate the MCNs, leading to a release of both axonal and

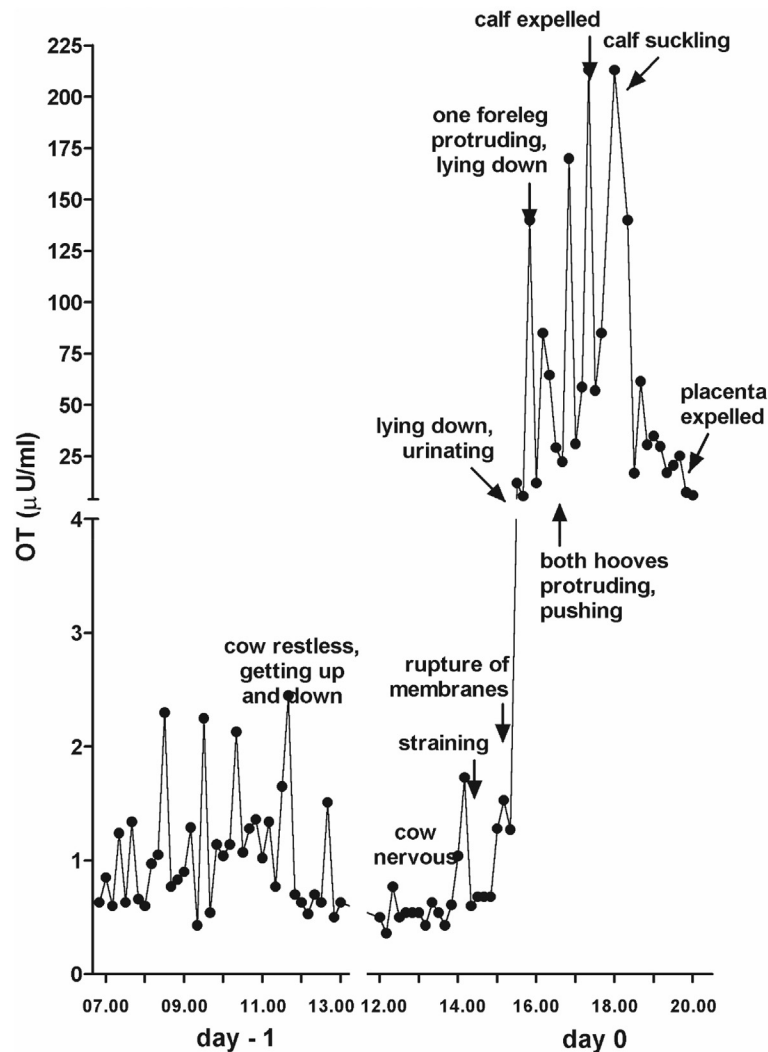


Fig. 3 Pulsatile appearance of oxytocin in the blood of a pregnant cow in the day prior to and of parturition, as indicated. Oxytocin was measured every 15 min and corresponding behavior was noted. From Fuchs AR, Ivell R, Ganz N, Fields MJ, Gimenez T (2001) Secretion of oxytocin in pregnant and parturient cows: corpus luteum may contribute to plasma oxytocin at term. *Biol Reprod.* 65:1135–1141, with permission.

dendritic oxytocin (Leng et al., 2008; Hatton and Wang, 2008). The latter then causes an amplification of the MCN response (see above) and uterine contractility accelerates.

It is important to note that oxytocin is not only involved in the expulsion of the fetus at birth, but also subsequently of the placenta, and then critically ensures the contraction of the empty uterus and constriction of blood vessels to prevent undue blood loss. Therapeutically, oxytocin may be given to support this process, if not before.

Oxytocin and Lactation

Lactation naturally follows birth, and thus takes advantage of the already active and primed oxytocinergic MCNs. Although not involved in mammary gland development, nor in milk synthesis and secretion, transgenic mouse studies have shown that oxytocin is essential for the so-called *milk let-down reflex*: hypothalamic oxytocin is released from the posterior pituitary in response to suckling at the nipples, causing contraction of the smooth muscle cells surrounding the mammary alveoli and release of milk. As in the uterus, oxytocin receptors are increasingly expressed in the myoepithelial cells of the developing alveoli towards the end of pregnancy, and their responsiveness to oxytocin also increases. Studies in rats indicate that there are short bursts of electrical activity every 5–15 min in the MCN leading to pulses of oxytocin release from the posterior pituitary and release of milk to the suckling pups (Lincoln and Wakerley, 1974), even though these are persistently attached to the nipples for much longer periods. This appears to be a common feature in all mammals and recent imaging in humans has shown a similar intermittent milk-release in response to continuous suckling. As for uterine contractility, oxytocin receptors in the mammary gland appear to become desensitized if

exposed to continuous oxytocin delivery, and seem to require relatively high concentrations of oxytocin within a pulse, together indicating that such burst pulsatility is both essential and efficient.

Oxytocin and Natriuresis

The oxytocin-producing MCNs, like those making vasopressin, are also osmoreceptors and respond to increased osmotic pressure by secretion of oxytocin. But unlike in uterine contractility and lactation here oxytocin seems to be produced in a more sustained fashion with circulating levels increasing but without evident pulsatility. In animal models it has been shown that oxytocin can directly stimulate the kidney to increase Na^+ excretion (natriuresis), presumably via oxytocin receptors, though possibly also via vasopressin V1 receptors with which oxytocin can weakly interact. However, much of this effect in vivo appears to be due to the stimulation of cardiac atrionatriuretic peptide secretion, which in turn influences the kidney. There appears to be some species specificity in the natriuretic response, with humans and dogs exhibiting little effect compared to rodents.

Oxytocin and Satiety

It is known that cholecystokinin (CCK) can stimulate hypothalamic oxytocin secretion via CCK receptors on vagal efferents from the stomach. Since CCK is produced by the duodenum following a meal, these vagal actions evidently mediate a satiety reflex. Then experimentally it has been shown in rats that injection of oxytocin into the brain can suppress appetite. Taken together these findings strongly suggest that CCK can reduce appetite by activating the release of dendritic oxytocin in the brain.

Oxytocin and Copulation

The oxytocin-dependent induction of smooth muscle contractility in the reproductive system is not limited to female mammals. Also in male mammals there is good evidence that pulses of oxytocin can be measured accompanying the contraction of smooth muscle cells in the epididymis and vas deferens associated with ejaculation (Ivell et al., 1997). Nothing is known, however, about the mechanisms involved in inducing such oxytocin pulsatility. This has been shown in rodents, bulls and humans. Additionally, it is believed that oxytocin produced locally within the testes is responsible for the slow peristaltic contractility of the seminiferous tubules associated with sperm release from the seminiferous epithelium. Centrally within the hypothalamus, oxytocin also appears to promote penile erection (Argiolas and Melis, 2013), though whether this is due to dendritic release of oxytocin from MCNs or from parvocellular oxytocinergic neurones in the PVN is not clear.

Oxytocin and the Maternal Recognition of Pregnancy

Although it has been shown that oxytocin is made locally at low concentration within the ovary of several mammalian species, including humans, monkeys, pigs and rodents, only in ruminants such as cows and sheep is this expression amplified to assume the function of a novel endocrine system. In cows, oxytocin is synthesized in large amounts by the granulosa cells of the growing antral follicle shortly before ovulation (Ivell and Richter, 1984b). After the oocyte is released, these cells differentiate further to become the large luteal cells of the new corpus luteum. Both oxytocin mRNA and peptide continue to be accumulated within these luteal cells throughout the luteal phase of the oestrous cycle with only limited amounts being secreted into the blood stream in a pulsatile fashion. The majority of oxytocin is only released towards the end of the oestrous cycle upon stimulation by the prostaglandin- $\text{F}_{2\alpha}$ secreted at that time by the uterine epithelium and only if there is no pregnancy (Fig. 4). This prostaglandin- $\text{F}_{2\alpha}$ also causes the induction of the process known as luteolysis, whereby the corpus luteum, which up until now has been making all of the progesterone of the female cycle, involutes to become a small corpus albicans and progesterone production ceases. If there is a pregnancy, luteolysis must be prevented so that the corpus luteum can continue to make the progesterone essential for supporting the pregnant uterus and early embryo.

The process by which this happens is called the *maternal recognition of pregnancy* (MRP) and in ruminants the oxytocin–oxytocin receptor system plays a central part (Hansen et al., 1999). Firstly, the oxytocin released from the corpus luteum activates oxytocin receptors on the endometrial epithelium of the uterus (Fig. 4), these in turn cause the activation of prostaglandin-producing enzymes, such as cyclooxygenase 2 (COX2), as well as ensuring that the end-product is prostaglandin- $\text{F}_{2\alpha}$ rather than the related prostaglandin- E_2 which has opposing effects on cells. If a pregnancy occurs, the early ruminant embryo begins to produce a peptide called interferon-tau in large amounts already by day 16 of the luteal phase and reaching a maximum at around the time when luteolysis would normally be occurring at the end of the non-pregnant cycle. This interferon interacts with interferon receptors also on the uterine epithelial cells, which in turn suppress the expression of the oxytocin receptors on the same cells, and therefore effectively interrupts the positive feedback loop between ovarian oxytocin production and uterine prostaglandin- $\text{F}_{2\alpha}$ expression. The precise mechanisms how interferon achieves the suppression of the oxytocin receptors is still unclear. In sheep it appears

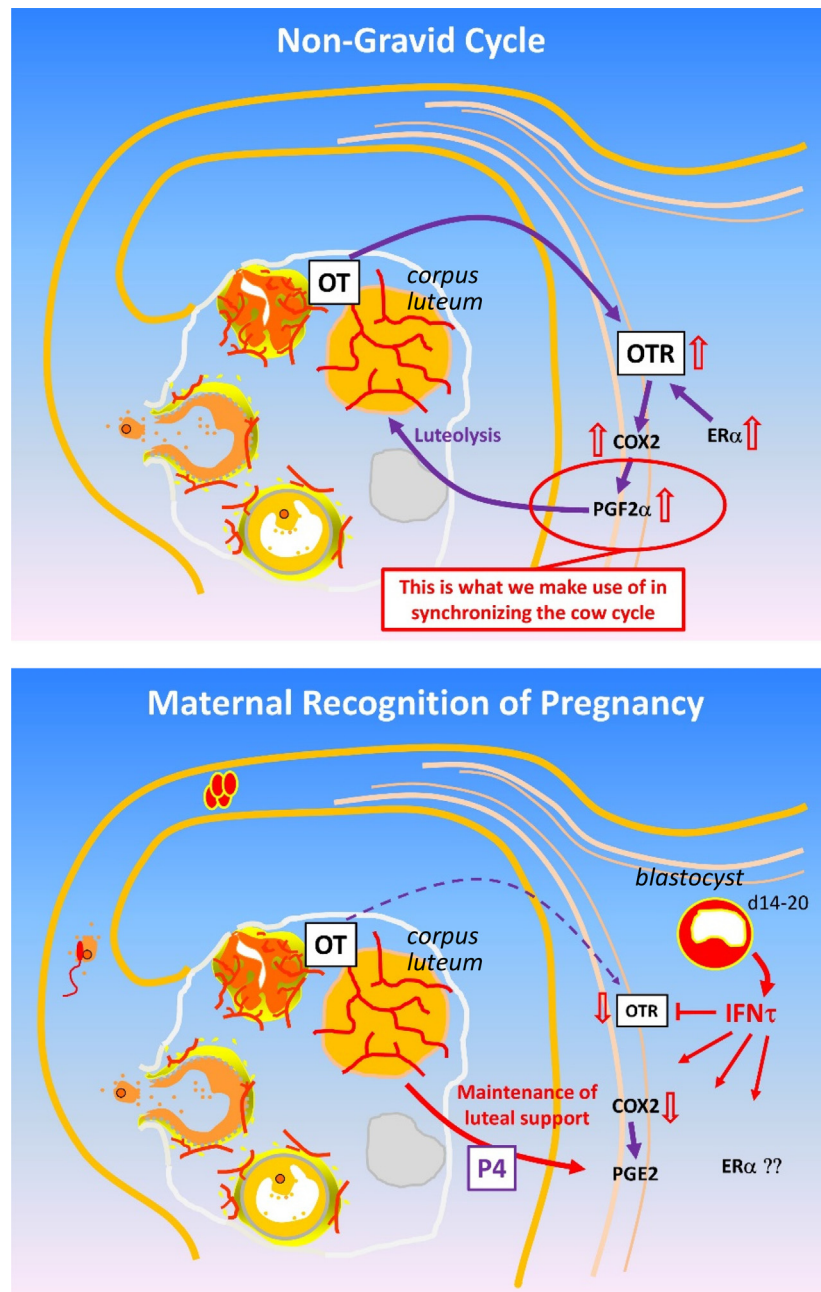


Fig. 4 Schematic diagram (upper panel) to illustrate the oxytocin-dependent feedback loop between the corpus luteum of the ovary and the uterine lining at the end of the non-pregnant oestrous cycle in cows (see text for details). The indicated PGF2 α , which causes luteolysis, is mimicked pharmacologically when oestrous cycles are artificially terminated before the normal time by injection of the drug cloprostenol (a PGF2 α analogue) in order to induce precisely timed cycles for artificial reproduction in a process called synchronization. Lower panel: when an egg is fertilized, the resulting early embryo (blastocyst) begins to produce the trophoblast hormone interferon-tau (IFN τ) which, acting on interferon receptors, causes the down-regulation of the oxytocin receptor in the uterine lining. At the same time it switches prostaglandin production from the luteolytic PGF2 α to the luteotrophic PGE2.

that this may involve the suppression of estrogen receptors which support the expression of the uterine oxytocin receptors. In dairy cows, it would appear that interferon-tau specifically induces in the epithelial cells so-called interferon-responsive factors which act directly to inhibit the gene encoding the oxytocin receptor. In addition to this down-regulation of the oxytocin receptor, the interferon also appears to cause a switch in prostaglandin production from prostaglandin-F2 α to prostaglandin-E2. In this way luteolysis is prevented and pregnancy continues to be supported by ovarian steroids.

Although there may be a role for oxytocin and oxytocin receptors in endometrial function in other species, only in ruminants do we see this high ovarian expression of oxytocin and explicit involvement in MRP.

Oxytocin and the HPG Axis

Oxytocin not only has effects directly on receptors within the reproductive organs themselves, but it also appears to be able to influence the hypothalamo-pituitary-gonadal (HPG) axis (Evans, 2002). Activation of oxytocin receptors on the gonadotropin-producing cells of the anterior pituitary causes activation of phosphoinositide-3-kinase and intracellular Ca^{2+} release, together therefore interacting with the GnRH-induced signaling activity in the same cells. Whether this oxytocin *in vivo* arrives like GnRH via the portal system from the hypothalamus or comes from the posterior pituitary is not known, but it does suggest that the hormones of the HPG axis can be modulated at times of high oxytocin expression, for example at the end of pregnancy or during lactation.

Behavioral Effects of Oxytocin

Since oxytocin is largely unable to cross the blood–brain barrier, the various behavioral effects of oxytocin must be due to centrally released hormone, possibly from the dendrites of the MCNs. Moreover, such effects do not correlate with levels of oxytocin measured in blood, and these do not reflect the levels in cerebrospinal fluid.

There is no doubt that central oxytocin is responsible for a range of maternal and affiliative behaviors (Ivell and Russell, 1995; Hammock and Young, 2006; Leng et al., 2008; Neumann and Landgraf, 2012). For example, in sheep oxytocin induces the bonding of a new-born lamb with its mother. In the female prairie vole it promotes monogamous pair-bonding. It is generally described as “anti-anxiolytic,” encouraging more exploratory and olfactory behavior in laboratory animals. It is also linked to sexual responsiveness and behavior in female and male rats, respectively. Finally, it appears to be released centrally in response to food rewards and satiety.

There have been numerous claims of similar effects in humans, though possibilities for therapeutic approaches using oxytocin in certain mental disorders are very limited due to the difficulty of targeting the brain for a peptide which cannot cross the blood–brain barrier. Although it has been proposed that oxytocin applied as a nasal spray might circumvent this difficulty by directly reaching the brain across the nasal mucosa, the findings from such studies are not without substantial controversy (Leng and Ludwig, 2016).

Oxytocin Pharmacology and Clinical Use

The main pharmacological use of oxytocin to date has been in the field of obstetrics. The potential of oxytocin for stimulating uterine contractions can be traced back to the early experiments of Sir Henry Dale (1906) and the founder of the Royal College of Obstetricians and Gynecologists, Professor William Blair Bell (1909) who used extracts of the infundibular body (the pituitary stalk connecting the hypothalamus to the pituitary) to stimulate rabbit uterus contraction *in vitro*. Blair Bell first employed oxytocin to increase blood pressure and induce uterine contractions. The effect on the uterus was more profound and maintained than seen for blood pressure so Blair Bell starting using it to treat atonic uterus and postpartum hemorrhage. Following this, and the sequencing and synthesis of oxytocin in 1953, the use of synthetic oxytocin (Brand name, Syntocinon) for induction and augmentation of labor became embedded in clinical practice. Oxytocin was also used in combination with ergometrine to manage the third stage of labor (delivery of the placenta) and prevention of postpartum hemorrhage. However, the introduction into mainstream clinical practice was not based on any randomized clinical trial of syntocinon versus placebo, rather by consensus following a large case series of 1000 primigravida pregnancies in which 120 were given an intravenous infusion of oxytocin (from 5 drops to 50 mU per minute) as part of an active management strategy (O’Driscoll et al., 1969). In the United Kingdom, standard (low dose) protocols recommended by the National Institute of Clinical Excellence guidelines are widely implemented. Synthetic oxytocin is delivered as a continuous infusion with incremental rises in concentration every 30 min until adequate contractions are achieved (2–32 mU/min). In the United States and other countries, there is less uniformity in the protocols employed leading to concerns about the high concentrations used. With a half-life reported between 3 and 20 min, synthetic oxytocin use can be associated with uterine hyperstimulation, fetal distress, operative delivery and postpartum hemorrhage. Continuous exposure to oxytocin has also been reported to down-regulate the oxytocin receptors in myometrial muscle and in theory could predispose women to postpartum hemorrhage due to a refractory response to ergometrine/oxytocin treatments. As a result, the use of pulsatile rather than continuous infusion of synthetic oxytocin for induction and augmentation of labor has been proposed. The most recent randomized clinical trial (Tribe et al., 2012) reported that pulsatile oxytocin resulted in as many vaginal births as a continuous infusion with less uterine over-stimulation or reports of “suspected fetal distress.” The pulsatile infusion delivered 1/6th of the dose of oxytocin to achieve the same clinical outcomes which suggests current protocols could be modified. Given the potentially worrying side-effects of synthetic oxytocin, this could benefit women and provide a slower and more natural labor. In contrast, however, others argue that there is a need to introduce higher dose protocols to expedite labor and trials are ongoing.

The involvement of oxytocin in labor and delivery has also led to the hypothesis that inhibition of uterine oxytocin receptors can prevent preterm labor. Atosiban, a mixed oxytocin/vasopressin receptor antagonist has been introduced as a tocolytic in Europe as a first choice treatment for threatened preterm labor, although it failed to gain FDA approval for use in the United States.

Meta-analysis suggests that although atosiban has fewer side effects than other tocolytics, it is no better than placebo for important clinical outcomes such as gestation at delivery and neonatal wellbeing.

Other more selective oxytocin receptor antagonists have been developed. These include barusiban (for preterm labor, but not found to be effective), epelsiban (for treatment of premature ejaculation and to enhance myo/blastocyst implantation in IVF), L-368,899 (social behavior and pair bonding), L-371,257 (preterm labor, not yet tested) and retosiban (preterm labor, under investigation).

Alternative Clinical Uses for Oxytocin

The development of intranasal applicators has opened up the possibility of oxytocin being used in other conditions such as postpartum depression, autism, social dysfunction and anxiety, Parkinson's, schizophrenia, and sleep apnoea.

In general, intranasal administration appears to have few side effects and be safe to use in vulnerable as well as healthy individuals. In studies of postpartum depression, there have been very few significant findings compared to placebo. Some women have reported a sensation of "calmness" with oxytocin, but also negative effects such as rating baby-crying as more urgent and needing more harsh care-giving strategies have led to caution with implementing oxytocin as a treatment. With regard to social behaviors, there is ongoing debate as to whether oxytocin will provide a consistent panacea for all individuals (Leng and Ludwig, 2016). Continued research is needed that considers phenotype, optimal dosing and potential interactions with other neurotransmitters.

References

- Argiolas, A., & Melis, M. R. (2013). Neuropeptides and central control of sexual behaviour from the past to the present: A review. *Progress in Neurobiology*, 108, 80–107.
- Armstrong, W. E. (1995). Morphological and electrophysiological classification of hypothalamic supraoptic neurons. *Progress in Neurobiology*, 47, 291–339.
- Arrowsmith, S., & Wray, S. (2014). Oxytocin: Its mechanism of action and receptor signalling in the myometrium. *Journal of Neuroendocrinology*, 26, 356–369.
- Brown, C. H., Bains, J. S., Ludwig, M., & Stern, J. E. (2013). Physiological regulation of magnocellular neurosecretory cell activity: Integration of intrinsic, local and afferent mechanisms. *Journal of Neuroendocrinology*, 25, 678–710.
- Evans, J. J. (2002). Peptides interact in gonadotrophin regulation. *Archives of Physiology and Biochemistry*, 110, 154–161.
- Gimpl, G., Wiegand, V., Burger, K., & Fahrenholz, F. (2002). Cholesterol and steroid hormones: Modulators of oxytocin receptor function. *Progress in Brain Research*, 139, 43–55.
- Hammock, E. A., & Young, L. J. (2006). Oxytocin, vasopressin and pair bonding: Implications for autism. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 361, 2187–2198.
- Hansen, T. R., Austin, K. J., Perry, D. J., Pru, J. K., Teixeira, M. G., & Johnson, G. A. (1999). Mechanism of action of interferon-tau in the uterus during early pregnancy. *Journal of Reproduction and Fertility. Supplement*, 54, 329–339.
- Hatton, G. I., & Wang, Y. F. (2008). Neural mechanisms underlying the milk ejection burst and reflex. *Progress in Brain Research*, 170, 155–166.
- Ivell, R., & Richter, D. (1984a). Structure and comparison of the oxytocin and vasopressin genes from the rat. *Proceedings of the National Academy of Sciences of the United States of America*, 81, 2006–2010.
- Ivell, R., & Richter, D. (1984b). The gene for the hypothalamic peptide hormone oxytocin is highly expressed in the bovine corpus luteum: Biosynthesis, structure and sequence analysis. *The EMBO Journal*, 3, 2351–2354.
- Ivell, R., & Russell, J. (1995). *Oxytocin: Cellular and molecular approaches in medicine and research*. New York: Plenum Press, 1–673.
- Ivell, R., Balvers, M., Rust, W., Bathgate, R., & Einspanier, A. (1997). Oxytocin and male reproductive function. *Advances in Experimental Medicine and Biology*, 424, 253–264.
- Ivell, R., Dai, Y., Mann, N., & Anand-Ivell, R. (2014). Non-classical mechanisms of steroid sensing in the ovary: Lessons from the bovine oxytocin model. *Molecular and Cellular Endocrinology*, 382, 466–471.
- Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., & Okayama, H. (1992). Structure and expression of a human oxytocin receptor. *Nature*, 356, 526–529.
- Kimura, T., Ogita, K., Kumasawa, K., Tomimatsu, T., & Tsutsui, T. (2013). Molecular analysis of parturition via oxytocin receptor expression. *Taiwanese Journal of Obstetrics & Gynecology*, 52, 165–170.
- Leng, G., & Ludwig, M. (2016). Intranasal oxytocin: Myths and delusions. *Biological Psychiatry*, 79, 243–250.
- Leng, G., Brown, C. H., & Russell, J. A. (1999). Physiological pathways regulating the activity of magnocellular neurosecretory cells. *Progress in Neurobiology*, 57, 625–655.
- Leng, G., Meddle, S. L., & Douglas, A. J. (2008). Oxytocin and the maternal brain. *Current Opinion in Pharmacology*, 8, 731–734.
- Lincoln, D. W., & Wakerley, J. B. (1974). Electrophysiological evidence for the activation of supraoptic neurones during the release of oxytocin. *The Journal of Physiology*, 242, 533–554.
- Ludwig, M., & Leng, G. (2006). Dendritic peptide release and peptide-dependent behaviours. *Nature Reviews. Neuroscience*, 7, 126–136.
- Ludwig, M., & Stern, J. (2015). Multiple signalling modalities mediated by dendritic exocytosis of oxytocin and vasopressin. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 370, 20140182.
- Morris, J. F., & Pow, D. V. (1991). Widespread release of peptides in the central nervous system: Quantitation of tannic acid-captured exocytoses. *The Anatomical Record*, 231, 437–445.
- Neumann, I. D., & Landgraf, R. (2012). Balance of brain oxytocin and vasopressin: Implications for anxiety, depression, and social behaviors. *Trends in Neurosciences*, 35, 649–659.
- O'Driscoll, K., Jackson, R. J. A., & Gallagher, J. T. (1969). Prevention of prolonged labour. *British Medical Journal*, 2, 477–480.
- Pow, D. V., & Morris, J. F. (1989). Dendrites of hypothalamic magnocellular neurons release neurohypophysial peptides by exocytosis. *Neuroscience*, 32, 435–439.
- Ratajczak, C. K., & Muglia, L. J. (2008). Insights into parturition biology from genetically altered mice. *Pediatric Research*, 64, 581–589.
- Russell, J. A., Leng, G., & Douglas, A. J. (2003). The magnocellular oxytocin system, the fount of maternity: Adaptations in pregnancy. *Frontiers in Neuroendocrinology*, 24, 27–61.
- Summerlee, A. J. (1981). Extracellular recordings from oxytocin neurones during the expulsive phase of birth in unanaesthetized rats. *The Journal of Physiology*, 321, 1–9.
- Tribe, R. M., Crawshaw, S. E., Seed, P., Shennan, A. H., & Baker, P. N. (2012). Pulsatile versus continuous administration of oxytocin for induction and augmentation of labor: Two randomized controlled trials. *American Journal of Obstetrics and Gynecology*, 206, 230.e1–230.e8.

Further Reading

- Breckwoldt, M., Neumann, F., & Brauer, H. (1991). *Exempla endocrinologica, Band 1* (pp. 1–173). Germany: Medical Service Munchen.
- Fuchs, A. R., Ivell, R., Ganz, N., Fields, M. J., & Gimenez, T. (2001). Secretion of oxytocin in pregnant and parturient cows: Corpus luteum may contribute to plasma oxytocin at term. *Biology of Reproduction*, 65, 1135–1141.
- Telgmann, R., Bathgate, R. A. D., Jaeger, S., Tillmann, G., & Ivell, R. (2003). The transcriptional regulation of the bovine oxytocin receptor gene. *Biology of Reproduction*, 68, 1015–1026.

Pregnancy Complications (FGR, Preeclampsia)

Berthold Huppertz, Medical University of Graz, Graz, Austria

© 2018 Elsevier Inc. All rights reserved.

Introduction

The development of the fetus within the maternal uterus is strongly determined by fetal genetic factors as well as factors derived from the mother and from the placenta as mediator between the two individuals. Using its intrinsic growth potential the fetus should be able to reach appropriate growth as a healthy fetus. However, only a fully functioning placenta in direct interaction with the mother can provide the respective nutritional and gaseous support of the fetus. This needs to be directly linked to the physiological adaptations of the maternal system during pregnancy. Any major alteration of this interactive interplay may result in pregnancy pathologies including fetal growth restriction (FGR) and preeclampsia.

Definitions and Features

Fetal Growth Restriction

Fetal or intra-uterine growth restriction (FGR/IUGR) remains one of the leading causes of perinatal mortality and morbidity of the baby, distressing nearly 5% of all pregnancies (Bernstein & Gabbe 1996). Perinatal mortality dramatically increases with reducing newborns' weight: A baby born with <2500 g of weight at term has a 5–30 times higher risk for perinatal mortality, while the risk for a baby born with <1500 g of weight is 70–100 times greater.

Although the terms FGR/IUGR and SGA (small-for-gestational age) are often used as synonyms, there is a clear difference between the two. By definition, both terms include newborns with a birth weight below the 10th percentile (Bernstein & Gabbe 1996). The term FGR includes babies that have not been able to fully utilize their genetic growth potential but due to various reasons ended up growth restricted. By contrast, the term SGA includes FGR babies as well as babies that have used their genetic potential but are genetically small and only reach a birth weight up to the 10th percentile. Thus, FGR is a subset of SGA rather than a synonym. Looking at the babies with a birth weight below the 10th percentile, all of these babies belong to the SGA group, while only about 30% of these newborns are growth restricted and hence belong to the FGR group.

Typical features of FGR in contrast to SGA are changes in blood flow of maternal and fetal blood vessels to the placenta, hence uterine and umbilical arteries, respectively (Cetin & Alvino 2009). Alterations of maternal blood flow towards the placenta can be visualized in the uterine arteries by looking at the presence of notches and an increased pulsatility index in Doppler ultrasound. These changes have been described being caused by insufficient invasion of extravillous trophoblast into spiral arteries. Thus, incomplete invasion of these vessels by endoarterial trophoblasts (Moser & Huppertz 2017) results in the fact that these vessels stay narrow and under maternal vasomotor control. Alterations of the fetal blood flow towards the placenta are other typical features of FGR and show an increased systole/diastole (S/D) ratio in the umbilical arteries. Variations of the theme include FGR cases with still preserved end diastolic flow (PEDF) in the umbilical arteries, absent end diastolic flow velocity in these arteries (AEDF) or even reversed end diastolic flow (REDF) (Sibley et al. 2002). One possibility to explain these changes is an increased peripheral resistance within placental vessels, maybe due to malformation of the placental villous trees or increased blood flow and pressure in the intervillous space (Burton et al. 2009).

Preeclampsia

Despite massive scientific and clinical efforts, the etiology of preeclampsia still remains obscure in a variety of aspects and thus preeclampsia stays as the pathology of hypotheses even today. It is generally accepted that the placenta rather than the fetus is the driver for the development of preeclampsia, while multiple other factors influencing the development of preeclampsia are under discussion.

Just as FGR, also preeclampsia belongs to the group of major reasons for maternal as well as fetal and neonatal morbidity and mortality. Worldwide, 2–8% of all pregnancies are affected by preeclampsia (ACOG 2013). This pregnancy specific syndrome induces long-term effects for mothers and children. As an example, women who experienced preeclampsia show a higher risk to develop cardiovascular diseases later in life (Bellamy et al. 2007).

The definition of preeclampsia includes diagnosis of hypertension and proteinuria in a so far normotensive woman (ACOG 2013). The diagnosis of hypertension is defined as developing systolic blood pressure of ≥ 140 mmHg, and/or diastolic blood pressure of ≥ 90 mmHg after 20 weeks of gestation, measured two times at least 4 h apart. Proteinuria is defined as ≥ 0.3 g protein in 24 h urine, or a protein to creatinine ratio of ≥ 3 (ACOG 2013). These two features are the typical criteria to define preeclampsia. However, especially in the absence of proteinuria other criteria may become important, including thrombocytopenia ($< 100,000$ platelets μL^{-1} blood), elevated levels (factor > 2) of liver transaminases in blood, elevated levels (> 1.1 mg/dL) of serum creatinine (indicating kidney insufficiency) as well as cerebral or visual disturbances or pulmonary edema (ACOG 2013).

The final clinical syndrome of preeclampsia is a maternal syndrome, while the baby in the womb may suffer as well from FGR or may show a completely normal development and growth. Hence, it is the combination of maternal response and susceptibility to factors derived from the placenta that defines time of onset, severity and progression of preeclampsia. Consequently, all three players, mother, fetus and placenta, including their interplay need to be considered when evaluating the etiology of preeclampsia.

Definition of preeclampsia subtypes

Due to the different times of onset and effects on mother and child, preeclampsia has been grouped into different subtypes. The two main types are early-onset preeclampsia with clinical symptoms forcing delivery before 34 weeks of gestation and mostly associated with a growth restricted baby and late-onset preeclampsia with delivery of the baby at or after 34 weeks of gestation and mostly associated with a normally grown baby. Looking at these two subtypes in more detail, further differences, other than time of delivery, can be identified:

1. Shallow invasion of extravillous trophoblast – mostly in the early-onset type;
2. Changes in perfusion of the placenta with maternal blood – mostly in the early-onset type;
3. Changes in the fetoplacental vasculature plus changes of fetal blood flow to essential organs – mostly in the early-onset type; and
4. Development of fetal growth restriction – mostly in the early-onset type.

Interestingly, the two subtypes do not differ in their features that directly define preeclampsia (hypertension and proteinuria) but rather differ in features related to fetal growth ([Huppertz 2008](#)).

The subtype of late-onset preeclampsia

Worldwide, the subtype of late-onset preeclampsia comprises >80% and up to 95% of all preeclampsia cases. In such cases delivery is preterm or at term (>34 weeks), while the babies and the placentas of such cases mostly show normal growth trajectories and are not growth restricted. The uterine arteries and thus maternal blood flow patterns towards the placenta are mostly normal as well. Doppler waveforms of the uterine arteries show no changes or a non-significant increase of the pulsatility index. In line with the normal growth rate of the baby are the non-altered blood flow parameters of the umbilical arteries from the fetus towards the placenta.

The above description shows that this subtype of preeclampsia is purely a maternal problem while the babies are mostly fine. The question remains whether a defect of the placenta needs to be involved in the etiology of this subtype or whether it is just the mother who responds inappropriately to a normally functioning placenta. It may be a combination of both as it has been shown that pregnant women with placentas that display enlarged mass and/or surface area (like in diabetes mellitus, multiple pregnancies, anemia, or high altitude) are at an increased risk to develop the late-onset subtype of preeclampsia.

The subtype of early-onset preeclampsia

Only a small proportion of 5–20% of all preeclampsia cases, depending on the statistics, belongs to the early-onset type of preeclampsia. This type of preeclampsia is responsible for the majority of preeclampsia-related fetal and maternal morbidity and mortality in the developed world ([Chappell et al. 2008](#)).

Different to the late onset type, most of the cases of the early-onset type show typical features in clinical routine. A feature that became a matter of particular interest was the change of blood flow within the maternal uterine arteries as described above for FGR. Another regular feature of the early onset cases is abnormal blood flow in the umbilical arteries, again as described above for FGR. Hence, clinically, this type of preeclampsia is characterized by a mixture of symptoms not directly related to preeclampsia. This, of course, has led to major speculations and interpretations over the last decades.

The Combination of Preeclampsia and FGR

The features listed above for early-onset preeclampsia in clinical routine are not specific for this type of preeclampsia. Interestingly, most early-onset preeclampsia cases are associated with FGR. Thus, the typical features of FGR (fetal growth restriction in combination with alterations of uterine and umbilical blood flow) are simply used to define early onset preeclampsia. This raises the question whether the clinically relevant features of early-onset preeclampsia cases (excluding hypertension and proteinuria) are caused by preeclampsia or are features that are due to the combined and overlying presence of FGR ([Huppertz 2008](#)). The combination of FGR and early-onset preeclampsia makes such cases so dangerous for mothers and babies and hence important for clinical intervention.

Disproof of the most Popular Hypothesis on the Etiology of Preeclampsia

Interestingly, a single hypothesis on the etiology of preeclampsia is present in literature since decades. This hypothesis is based on shallow invasion of extravillous trophoblast and subsequent failure of these cells to transform uterine spiral arteries into large bore conduits. It is of particular interest that this hypothesis is still present as it has been disproved by a number of publications already. Regardless of its disproval this hypothesis is still a major basis for scientific studies. This shows the importance of a critical view on hypotheses and that scientific falsification of a hypothesis does not stop its political use.

Since this hypothesis is still spread today, the reader can find a short summary of the chronology of events within this hypothesis below:

1. First trimester: A so far unknown deleterious effect on the extravillous trophoblast;
2. First and second trimester: Shallow invasion of extravillous trophoblasts with a clearly reduced infiltration of spiral arteries;
3. Second trimester: Reduced flow of maternal blood into the intervillous space of the placenta;
4. Second and third trimester: Placental hypoxia and/or waves of hypoxia followed by reoxygenation of the placenta;

5. Second and third trimester: Hypoxic damage of the villous trophoblast;
6. (Second and) third trimester: Release of placental factors from the syncytiotrophoblast (such as soluble Fms-like tyrosine kinase-1, sFlt-1, and placental growth factor, PlGF) into maternal blood;
7. (Second and) third trimester: Maternal inflammatory reactions due to the above placental factors and development of clinical symptoms of the mother.

This has been a conclusive hypothesis 10 years ago, while today a number of aspects have been disproved. Two aspects of this hypothesis will be further described below with a critical appraisal of their current scientific value.

Preeclampsia and Shallow Invasion of the Trophoblast

Of course, early-onset preeclampsia is clinically much more relevant than late-onset preeclampsia. Even if the symptoms of preeclampsia (hypertension, proteinuria) can be severe in both types, in late-onset cases immediate delivery is easy, while this may not be the case in early-onset cases with deliveries at <34 weeks of gestation. Additionally, fetal growth restriction mostly occurs in early-onset cases, putting another burden on the clinical management of these pregnant mothers. Hence, early-onset preeclampsia as the clinically more relevant type of preeclampsia has also attracted more scientific studies. And the clinically relevant features of early-onset preeclampsia including FGR have then been transferred to all preeclampsia cases.

However, as listed in **Definition of Preeclampsia Subtypes**, shallow invasion is a feature of early-onset rather than late-onset preeclampsia; hence it is present in <20% of all preeclampsia cases. Regardless, in literature this fact seems to be ignored as in nearly all studies shallow invasion is claimed as the main event for the etiology of preeclampsia in general. However, this may explain only <20% of all preeclampsia cases, while the remaining majority remains unexplained.

It is important to note that the cascade of events described for early-onset preeclampsia (1. shallow invasion of extravillous trophoblast; 2. reduced perfusion of the placenta with maternal blood; 3. fetal growth restriction) is exactly the cascade of events occurring in cases with pure fetal growth restriction without preeclampsia and without any clinical symptoms of the mother. Hence, it needs to be critically viewed whether shallow trophoblast invasion is really a feature of preeclampsia or just a coincidence in a specific subset of cases.

The idea of overlapping symptoms of two different syndromes is supported by studies using uterine artery Doppler to predict preeclampsia and/or FGR. In a study assessing uterine artery blood flow at 11+0 to 13+6 weeks by Doppler ultrasound the detection rate for early onset preeclampsia was only 40% at a 10% false positive rate (Nicolaidis et al. 2006). In another study assessing uterine artery Doppler at 11–14 weeks of gestation, the sensitivity to predict early-onset preeclampsia was 33.3%, while for all cases of preeclampsia (early and late-onset) the sensitivity decreased to 21%. However, using the same cohort of women, the sensitivity to detect early-onset FGR was 100% (Pilalis et al. 2007).

The above mentioned studies plus additional studies cast doubts on a direct and causal relationship between shallow trophoblast invasion and the etiology of preeclampsia. This dysregulation of the extravillous trophoblast seems to be directly related to idiopathic cases of fetal growth restriction. If this is true, then the above mentioned “specific” features of early-onset preeclampsia are simply due to FGR. This would further imply that there are no specific features of early-onset preeclampsia compared to late-onset preeclampsia – besides the earlier time point of delivery and the co-occurrence with FGR.

Preeclampsia, Reduced Placental Perfusion and Placental Hypoxia

During the first trimester of pregnancy the placenta is perfused by maternal plasma rather than maternal blood with blood cells. Hence, only physically solved oxygen is transported into the placenta, allowing oxygen concentrations of below 20 mmHg (Jauniaux et al. 2000). This oxygen concentration is normoxic for this organ and this period of pregnancy and should not be called hypoxia, which is pathologically low oxygenation of tissues and organs. Only with the start of blood flow into the placenta at the beginning of the second trimester the intra-placental oxygen concentration rises to values of about 60 mmHg (Jauniaux et al. 2000). If this increase in oxygenation starts too early, loss of placental mass or even spontaneous abortion may be the consequence (Jauniaux et al. 2003).

At the same time it needs to be considered that the tissues of the maternal uterus are normally perfused with arterial blood throughout pregnancy, hence with about 90 mmHg in arterial blood and about 40 mmHg in venous blood (Jauniaux et al. 2000). There is no or only very little changes of oxygenation of these tissues during pregnancy; hence, trophoblast invasion always takes place into well oxygenated uterine tissues (Huppertz et al. 2009). Thus, shallow invasion does not change oxygenation of the uterine placental bed but changes the way maternal blood flows into the intervillous space of the placenta.

After the steep increase in placental oxygenation at the beginning of the second trimester a slight decrease until term has been observed. The oxygen concentrations during the second and third trimester that have been measured are between 35 mm and 50 mmHg (Schaaps et al. 2005, Sibley et al. 2002, Kakogawa et al. 2010). Continuous oxygenation of the placenta is facilitated via widening and reorganization of the uterine spiral arteries that have been opened towards the intervillous space of the placenta by invading extravillous trophoblasts. It is important to note that the widening is mostly executed at the very placental end of the vessels leading to a funnel-shaped opening of the arteries towards the placenta.

At the same time the blood volume flowing into the placenta needs to be increased due to the growing needs of the fetus. Between week 20 and week 38 the uterine blood flow increases from about 450 mL min⁻¹ to nearly 1000 mL min⁻¹ (Konje et al. 2001).

What is the effect of the funnel-shaped transformation of the uterine spiral arteries?

Blood flow velocity through the uterine spiral arteries is relatively constant during the second half of pregnancy with values between 33 cm s^{-1} and 50 cm s^{-1} (Bahlmann et al. 2012). Burton et al. (2009) have used assumption on length and diameter of vessels, total blood volume and other measures to calculate the velocity of blood flowing into the placenta. The funnel-shaped opening of the vessels reduces the flow of maternal blood into the placenta to values of about 10 cm s^{-1} while it does not significantly change the total blood volume flowing into the placenta (Burton et al. 2009). This low velocity is essential to maintain the fragile construction of the villous trees, to allow distribution of blood through the narrow passages between the floating villi and to maintain the connection between anchoring villi and basal plate.

What is the effect of shallow trophoblast invasion?

Burton et al. (2009) have calculated that blood flow from opened but not widened spiral arteries into the placenta shows a velocity of $1\text{--}2 \text{ m s}^{-1}$ and thus is 10–20 times faster than in normal pregnancy. This has major deleterious effects on the placenta proper, which are not related to changes in oxygenation and oxygen concentrations. The increased blood flow velocity induces damages on the villous surface leading to increased deposition of fibrin-type fibrinoid, leads to detachment of anchoring villi from the basal plate and thus reduces the number of extravillous trophoblasts, and results in alterations of the whole villous tree arrangement. This in turn may well lead to reduced nutritional and gaseous support of the fetus and hence fetal growth restriction.

Does this lead to reduced placental perfusion and placental hypoxia?

Surprisingly, hundreds of publications use both, reduced placental perfusion and placental hypoxia, as basis for their studies. At the same time, up to now there is not a single measurement showing any data and proof for both events. Looking at placental oxygenation in preeclampsia and FGR, only a few data from indirect measurements have been published:

(1) The pO_2 in blood samples of the uterine veins prior to cesarean section has been used as a surrogate for the pO_2 in the placenta (Sibley et al. 2002). Based on the calculations of Schaaps et al. (2005) there is a fixed relation between the uterine vein pO_2 and the placental pO_2 , with the venous pO_2 being 1.5 times higher than the placental pO_2 . Respective measurements in blood from uterine veins yielded a mean value of 63 mmHg for FGR cases, significantly higher than the 50 mmHg in the control cases (delivery between week 24 and 36 of gestation for both groups). For the controls the placental pO_2 can be calculated to be 33 mmHg and for the FGR group 42 mmHg, 1.3-times higher than the pO_2 of the control cases (Sibley et al. 2002).

(2) Also measurements of the concentration of oxygenated hemoglobin (termed tissue oxygenation index; Kawamura et al. 2007, Kakogawa et al. 2010) have been used as surrogate for the placental tissue pO_2 . The tissue oxygenation index of the placenta was 71% in healthy pregnant women, 78% in cases with FGR and 80% in cases with early-onset preeclampsia and FGR (Kawamura et al. 2007, Kakogawa et al. 2010). The transfer of these values to placental pO_2 values results in 30 mmHg for normal pregnant women, 49 mmHg for FGR cases and 54 mmHg for cases with early-onset preeclampsia and FGR.

Taken together, all the above studies showed increased levels of the placental pO_2 in cases with preeclampsia and/or FGR. There is not a single publication showing measurements that proof placental hypoxia. Thus, the combination of the calculations on flow velocity and the surrogate measurements of the placental pO_2 provide information on increased oxygenation levels in cases with shallow trophoblast invasion. However, there is no basis for a hypothesis that uses with placental hypoxia as a major step in its reasoning.

New Explanations of the Etiology of Preeclampsia

As described above, the placenta is essential for the development of preeclampsia. At the same time, the placenta cannot be looked at on its own but needs to be put into a larger context with its environment. Only the interaction between placenta, release of placental factors and particles into the maternal circulation and finally the response of the mother on these factors determines whether the clinical symptoms of preeclampsia develop or not. Hence, each site and player needs to be taken into account to identify whether clinical symptoms become relevant (Huppertz, 2008).

Based on the close interactions between maternal and feto-placental factors during pregnancy, at least three different scenarios can be developed. These scenarios can explain the different timing, the different severities and the involvement of the fetus in preeclampsia and FGR as well as the long-term effects on mother and child.

Scenario 1: A Healthy Mother Meets a Placenta with a Dysfunctioning Villous Trophoblast - > Preeclampsia without FGR

During normal pregnancy, the syncytiotrophoblast releases factors via controlled secretion and apoptotic processes, the latter releasing syncytial knots into maternal blood (Huppertz et al. 1998, Huppertz 2008, 2010). In this scenario a dysfunctional syncytiotrophoblast additionally releases subcellular fragments due to necrotic and aponecrotic processes (Huppertz 2008, 2010). This results in disposal of subcellular micro- and nanoparticles (Johansen et al. 1999) that systemically activate the maternal endothelium and in the long run damage the maternal vascular system (Goswami et al. 2006). A malfunction of the placenta and especially of the syncytiotrophoblast results in a continuous release of factors damaging the maternal endothelium and finally leading to the clinical symptoms of preeclampsia. At the same time, the extravillous trophoblast is not affected and hence transformation of spiral arteries, perfusion of the placenta and growth of the fetus should be normal (Fig. 1).

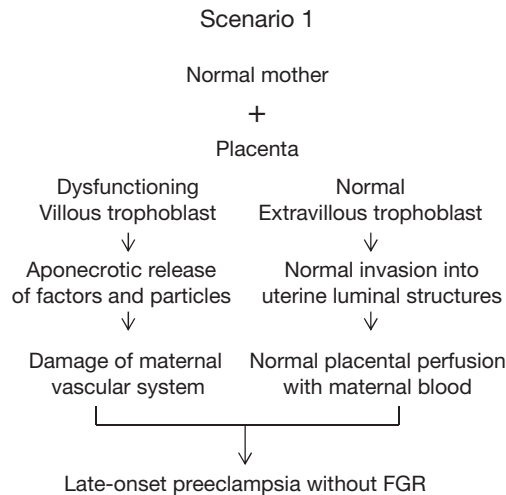


Fig. 1 Sequence of events in cases with a healthy mother and a placenta with a dysfunctional villous trophoblast but normal extravillous trophoblast. This sequence most probably results in late-onset preeclampsia without FGR.

In this scenario, a pathological placenta with a dysfunctional syncytiotrophoblast releases particles and factors into maternal blood that not only activate but also harm the vasculature of the mother throughout pregnancy. The magnitude of damage can be directly related to placental surface area: The larger the surface area (e.g. in gestational diabetes, in multiple pregnancies or pregnancies at high altitude), the larger the amount of non-apoptotically released factors. On the maternal site the compensatory defense systems will be overloaded when the amount of placental factors reaches a specific threshold, resulting in an even faster damage of the endothelium.

If the defense systems of the mother can withstand the damaging effects of the placental particles for a longer time period, late-onset preeclampsia may develop. However, if the overload is too extreme, early decompensation may occur, leading to early-onset preeclampsia. In both scenarios the pathological changes of the placenta may not affect the nutritional and gaseous supply of the fetus too much, which leads to the delivery of a normally grown baby without growth restriction. Predictive markers of preeclampsia such as the angiogenic markers PlGF and sFlt-1 cannot predict such cases of preeclampsia as the maternal endothelium is affected only late in pregnancy. Furthermore, such markers may be more related to FGR than to preeclampsia (Nicolaidis et al. 2006, Pilalis et al. 2007). At the same time, markers specific for the syncytiotrophoblast such as placental protein 13 (PP13) could be used to identify these cases of preeclampsia, maybe already in the first trimester of pregnancy (Huppertz et al. 2008). This scenario may not lead to any harmful effects later in life for both, mother and child.

Scenario 2: A Healthy Mother Meets a Placenta with a Dysfunctional Extravillous Trophoblast - > FGR

If the placental defect is present in the extravillous rather than the villous trophoblast, the release of factors from the syncytiotrophoblast is unaffected and normal. In this scenario invasion of extravillous trophoblast is affected leading to inadequate transformation of uterine luminal structures (Fig. 2).

So far, only reduced infiltration of uterine spiral arteries has been documented. However, very recently it has been shown that extravillous trophoblasts invade into all luminal structures of the uterus. Uterine glands are the first structures that are invaded by endoglandular trophoblast; it already takes place during the first days of implantation (Moser et al. 2010, Moser & Huppertz 2017). Then invasion into uterine vessels occurs to allow flow of plasma to the placenta via invaded spiral arteries (endoarterial trophoblast) and draining of the plasma from the placenta back into the maternal vascular system by invaded uterine veins (endovenous trophoblast) (Moser et al. 2017, Windsperger et al. 2017). There is also invasion into the lymph vessels (endolymphatic trophoblast) of the placental bed, although its function is unclear so far (Windsperger et al. 2017).

The effect of insufficient invasion into glands and veins has not been studied so far. Insufficient invasion into glands by reduced endoglandular trophoblast invasion may well result in reduced histiotrophic nutrition of the embryo and thus growth restriction very early in pregnancy. Also, insufficient invasion into uterine veins by reduced endovenous trophoblast invasion may result in inadequate draining of the maternal plasma from the placenta early in pregnancy and also in inadequate draining of maternal blood later on pregnancy. Both will have deleterious effects on the flow of maternal blood through the placenta and hence supply of the embryo/fetus.

Coming back to spiral arteries where the effects of insufficient invasion by endoarterial trophoblasts has been widely studied. As outlined above, the results of inadequate invasion of spiral arteries is not reduced blood flow into the placenta and is not placental hypoxia. Rather, it is increased flow velocities of blood into the placenta leading to damage of the placental villous tree as well as increased peripheral resistance within the small and fragile placental vessels. This increased peripheral resistance may well result in alterations of perfusion of the placenta and thus to malnutrition of the fetus.

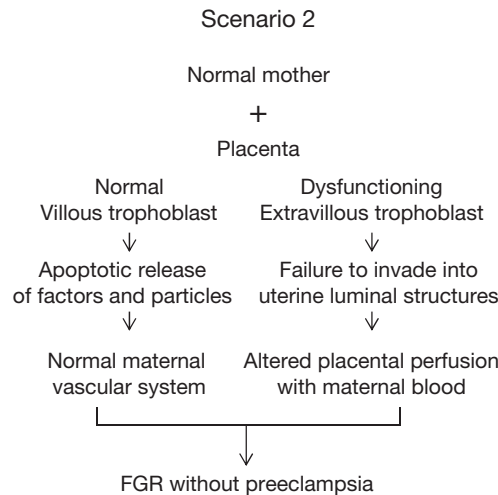


Fig. 2 Sequence of events in cases with a healthy mother and a placenta with a dysfunctioning extravillous trophoblast but normal villous trophoblast. This sequence most probably results in FGR without preeclampsia.

The changes in blood flow from the fetus to the placenta can be visualized by ultrasound of the umbilical arteries. Focusing on the flow pattern at the end of the diastole, mild form of FGR are associated with altered but still preserved flow at the end of diastole (preserved end diastolic flow, PEDF). In more severe cases, flow at the end of diastole may be absent (absent end diastolic flow, AEDF) or may even flow backwards (reversed end diastolic flow, REDF).

The combination in damage of the villous tree plus altered fetal perfusion of the placenta can easily lead to reduced nutritional supply of the fetus, which may result in a hypoxic fetus. Since the maternal oxygen supply into the placenta is mostly unchanged but less oxygen is transferred from the placenta to the fetus, the intraplacental oxygen level rises compared to normal pregnancy. The result is a hypoxic fetus with a hyperoxic placenta, as has been described in such cases (Sibley et al. 2002, Kawamura et al. 2007, Kakogawa et al. 2010).

This scenario may not lead to any harmful effects later in life for the mother; however, it may well induce long term effects on the child.

Scenario 3: A Predisposed Mother Meets a Normal Placenta - > Preeclampsia without FGR

In this scenario, the placental syncytiotrophoblast releases factors and particles in a normal way, that is via controlled secretion and apoptosis (Huppertz et al. 1998) (Fig. 3). This time, the mother shows a defect in her defense systems, which may include the endothelium, the whole vascular system, the scavenging systems or one or more specific signaling pathways to regulate blood pressure and/or kidney function. In such cases the mother may already be known to have predisposing diseases such as chronic hypertension or renal disease or may show subclinical alterations that have not been diagnosed before.

Depending on the extent of the maternal dysfunction, an overload of the scavenging systems or damage of the vascular system may occur early or late in pregnancy. The overload/damage is also dependent on the number of particles and factors released from the normal placenta. Hence, increased surface area of the placenta as present in multiple pregnancies or pregnancies at high altitude may foster the development of clinical symptoms.

The fetus should not be affected since the placenta shows normal growth trajectories; hence the baby should be delivered as a normally grown baby (no growth restriction). In this scenario, early changes of predictive markers like PlGF and sFlt-1 are mostly absent prior to onset of symptoms. Also placenta specific markers like PP13 may not enable early prediction of such cases as the placenta is normally developed.

In this scenario the women already suffer from clinical or subclinical defects of pathways affecting their vascular system. Hence, they are the ones with a high risk to develop cardiovascular diseases later in life, while the child should be unaffected.

Scenario 4: A Predisposed Mother Meets a Dysfunctioning Placenta - > Preeclampsia with or without FGR

This scenario includes the most severe cases since the combination of defects on both sites will result in deleterious effects for both, mother and child.

Scenario 4A: Placenta with dysfunctioning villous trophoblast

This subpopulation shows malfunction of the syncytiotrophoblast but not the extravillous trophoblast (Fig. 4). Hence, release of necrotic and aponecrotic particles and factors will take place with no alterations of blood flow towards the placenta. Since in these cases the maternal system is already defective, the systemic deterioration of the maternal system induced by the altered release from the syncytiotrophoblast will be fast. Hence, this subpopulation will result in cases with early-onset preeclampsia without fetal growth restriction. Due to the defect in the syncytiotrophoblast, predictive markers specific for the placenta, e.g. PP13, should easily predict such cases, while angiogenic markers such as PlGF and sFlt-1 may miss these cases.

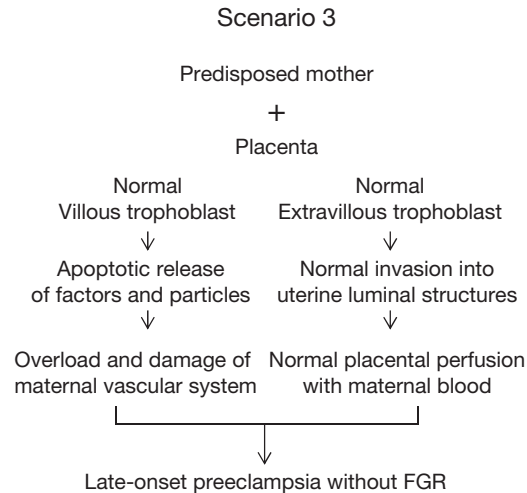


Fig. 3 Sequence of events in cases with a predisposed mother and a normal placenta. This sequence most probably results in late-onset preeclampsia without FGR.

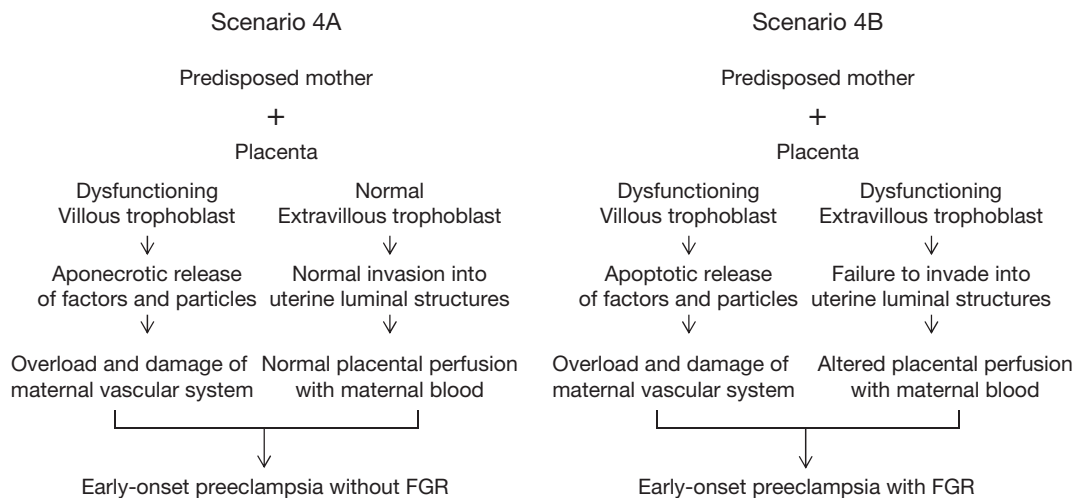


Fig. 4 4A: Sequence of events in cases with a predisposed mother and a placenta with a dysfunctioning villous trophoblast but normal extravillous trophoblast. This sequence most probably results in early-onset preeclampsia without FGR. 4B: Sequence of events in cases with a healthy mother and a placenta with a dysfunctioning villous trophoblast and a dysfunctioning extravillous trophoblast. This sequence most probably results in early-onset preeclampsia with FGR.

Scenario 4B: Placenta with dysfunctioning villous and extravillous trophoblast

This subpopulation shows malfunction of the syncytiotrophoblast as well as the extravillous trophoblast (Fig. 4). Hence, release of necrotic and aponecrotic particles and factors will take place with further alterations of blood flow towards the placenta. The damage due to the altered perfusion of the placenta may further enhance the damage of the syncytiotrophoblast and hence increase the release of particles from the placenta (Huppertz 2011). In combination with the already present damage of the maternal defense system, the damage of the maternal vascular system will develop early (early-onset preeclampsia), while the fetus will suffer from impaired growth as well (fetal growth restriction). Such cases can be predicted by placenta specific (Chafetz et al. 2007) as well as angiogenic markers (Schaarschmidt et al. 2013).

In this scenario, women in both subpopulations are predisposed and will most probably suffer from developing high risk for cardiovascular and other diseases later in life. In the second subpopulation with FGR, also the children show fetal programming and an increased risk to develop morbidities later in life (Longtine & Nelson 2011).

Conclusions

The etiologies of preeclampsia and FGR are still obscure and need further fruitful experiments and discussion. The list of new explanations clearly displays that the origin of both syndromes has not been identified yet. New routes of trophoblast invasion have been identified opening the way to new perspectives in thinking of new hypotheses. It has become obvious that the close

interplay between mother, placenta and fetus needs more attention as looking at only one site cannot explain the whole interactive setting.

References

- Bahlmann, F., Fittschen, M., Reinhard, I., Wellek, S., & Steiner, E. (2012). Reference values for blood flow velocity in the uterine artery in normal pregnancies from 18 weeks to 42 weeks of gestation calculated by automatic Doppler waveform analysis. *Ultraschall in der Medizin*, 33, 258–264.
- Bellamy, L., Casas, J. P., Hingorani, A. D., & Williams, D. J. (2007). Pre-eclampsia and risk of cardiovascular disease and cancer in later life: A systematic review and meta-analysis. *British Medical Journal*, 335, 974–977.
- Bernstein, I., & Gabbe, S. G. (1996). Intrauterine growth restriction. In S. G. Gabbe, J. R. Niebyl, J. L. Simpson, G. J. Annas, et al. (Eds.), *Obstetrics: normal and problem pregnancies* (3rd ed., pp. 863–886). New York: Churchill Livingstone.
- Burton, G. J., Woods, A. W., Jauniaux, E., & Kingdom, J. C. (2009). Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. *Placenta*, 30, 473–482.
- Cetin, I., & Alvino, G. (2009). Intrauterine growth restriction: Implications for placental metabolism and transport. A Review. *Placenta*, 30(Suppl a), S77–82.
- Chafetz, I., Kuhnreich, I., Sammar, M., Tal, Y., Gibor, Y., Meiri, H., Cuckle, H., & Wolf, M. (2007). First-trimester placental protein 13 screening for preeclampsia and intrauterine growth restriction. *American Journal of Obstetrics and Gynecology*, 197, 35.e1–35.e7.
- Chappell, L. C., Enye, S., Seed, P., Briley, A. L., Poston, L., & Shennan, A. H. (2008). Adverse perinatal outcomes and risk factors for preeclampsia in women with chronic hypertension: A prospective study. *Hypertension*, 51, 1002–1009.
- Goswami, D., Tannetta, D. S., Magee, L. A., Fuchisawa, A., Redman, C. W., Sargent, I. L., & von Dadelszen, P. (2006). Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction. *Placenta*, 27, 56–61.
- Huppertz, B. (2008). Placental origins of preeclampsia: Challenging the current hypothesis. *Hypertension*, 51, 970–975.
- Huppertz, B. (2010). Biology of the placental syncytiotrophoblast - myths and facts. *Placenta*, 31(Suppl), S75–S81.
- Huppertz, B. (2011). Trophoblast differentiation, fetal growth restriction and preeclampsia. *Pregnancy Hypertension*, 1, 79–86.
- Huppertz, B., Frank, H. G., Kingdom, J. C., Reister, F., & Kaufmann, P. (1998). Villous cytotrophoblast regulation of the syncytial apoptotic cascade in the human placenta. *Histochemistry and Cell Biology*, 110, 495–508.
- Huppertz, B., Sammar, M., Chafetz, I., Neumaier-Wagner, P., Bartz, C., & Meiri, H. (2008). Longitudinal determination of serum PP13 during development of preeclampsia. *Fetal Diagnosis and Therapy*, 24, 230–236.
- Huppertz, B., Gauster, M., Orendi, K., König, J., & Moser, G. (2009). Oxygen as modulator of trophoblast invasion. *Journal of Anatomy*, 215, 14–20.
- Jauniaux, E., Watson, A. L., Hempstock, J., Bao, Y. P., Skepper, J. N., & Burton, G. J. (2000). Onset of maternal arterial blood flow and placental oxidative stress; a possible factor in human early pregnancy failure. *American Journal of Pathology*, 157, 2111–2122.
- Jauniaux, E., Greenwold, N., Hempstock, J., & Burton, G. J. (2003). Comparison of ultrasonographic and Doppler mapping of the intervillous circulation in normal and abnormal early pregnancies. *Fertility and Sterility*, 79, 100–106.
- Johansen, M., Redman, C. W., Wilkins, T., & Sargent, I. L. (1999). Trophoblast deportation in human pregnancy - its relevance for pre-eclampsia. *Placenta*, 20, 531–539.
- Kakogawa, J., Sumimoto, K., Kawamura, T., Minoura, S., & Kanayama, N. (2010). Noninvasive monitoring of placental oxygenation by near-infrared spectroscopy. *American Journal of Perinatology*, 27, 463–468.
- Kawamura, T., Kakogawa, J., Takeuchi, Y., Takani, S., Kimura, S., Nishiguchi, T., Sugimura, M., Sumimoto, K., & Kanayama, N. (2007). Measurement of placental oxygenation by transabdominal near-infrared spectroscopy. *American Journal of Perinatology*, 24, 161–166.
- Konje, J. C., Kaufmann, P., Bell, S. C., & Taylor, D. J. (2001). A longitudinal study of quantitative uterine blood flow with the use of color power angiography in appropriate for gestational age pregnancies. *American Journal of Obstetrics and Gynecology*, 185, 608–613.
- Longtine, M. S., & Nelson, D. M. (2011). Placental dysfunction and fetal programming: The importance of placental size, shape, histopathology, and molecular composition. *Seminars in Reproductive Medicine*, 29, 187–196.
- Moser, G., & Huppertz, B. (2017). Implantation and extravillous trophoblast invasion: From rare archival specimens to modern biobanking. *Placenta*. <https://doi.org/10.1016/j.placenta.2017.02.007>.
- Moser, G., Gauster, M., Orendi, K., Glasner, A., Theuerkauf, R., & Huppertz, B. (2010). Endoglandular trophoblast, an alternative route of trophoblast invasion? Analysis with novel confrontation co-culture models. *Human Reproduction*, 25, 1127–1136.
- Moser, G., Weiss, G., Sundl, M., Gauster, M., Siwetz, M., Lang-Olip, I., & Huppertz, B. (2017). Extravillous trophoblasts invade more than uterine arteries: Evidence for the invasion of uterine veins. *Histochemistry and Cell Biology*, 147, 353–366.
- Nicolaidis, K. H., Bindra, R., Turan, O. M., Chafetz, I., Sammar, M., Meiri, H., Tal, J., & Cuckle, H. S. (2006). A novel approach to first-trimester screening for early pre-eclampsia combining serum PP-13 and Doppler ultrasound. *Ultrasound in Obstetrics and Gynecology*, 27, 13–17.
- Pilalis, A., Souka, A. P., Antsaklis, P., Basayiannis, K., Benardis, P., Haidopoulos, D., Papantoniou, N., Mesogitis, S., & Antsaklis, A. (2007). Screening for pre-eclampsia and small for gestational age fetuses at the 11-14 weeks scan by uterine artery Dopplers. *Acta Obstetrica et Gynecologica Scandinavica*, 86, 530–534.
- Schaaps, J. P., Tsatsaris, V., Goffin, F., Brichant, J. F., Delbecq, K., Tebache, M., Collignon, L., Retz, M. C., & Foidart, J. M. (2005). Shunting the intervillous space: New concepts in human uteroplacental vascularization. *American Journal of Obstetrics and Gynecology*, 192, 323–332.
- Schaarschmidt, W., Rana, S., & Stepan, H. (2013). The course of angiogenic factors in early- vs. late-onset preeclampsia and HELLP syndrome. *Journal of Perinatal Medicine*, 41, 511–516.
- Sibley, C. P., Pardi, G., Cetin, I., Todros, T., Piccoli, E., Kaufmann, P., Huppertz, B., Bulfamante, G., Cribiu, F. M., Ayuk, P., Glazier, J., & Radaelli, T. (2002). Pathogenesis of intrauterine growth restriction (IUGR)-conclusions derived from a European Union biomed 2 concerted action project 'Importance of oxygen supply in intrauterine growth restricted Pregnancies'-a workshop report. *Placenta*, 23(Suppl a), S75–S79.
- Windsperger, K., Dekan, S., Pils, S., Golletz, C., Kunihs, V., Fiala, C., Kristiansen, G., Knöfler, M., & Pollheimer, J. (2017). Extravillous trophoblast invasion of venous as well as lymphatic vessels is altered in idiopathic, recurrent, spontaneous abortions. *Human Reproduction*. <https://doi.org/10.1093/humrep/dex058>.

Further Reading

- ACOG Practice Bulletin. (2002). Diagnosis and management of preeclampsia and eclampsia. Number 33, 2002. American College of Obstetricians and Gynecologists. ACOG Committee on obstetric practice. *International Journal of Gynaecology and Obstetrics*, 77, 67–75.
- Moser, G., Weiss, G., Gauster, M., Sundl, M., & Huppertz, B. (2015). Evidence from the very beginning: Endoglandular trophoblasts penetrate and replace uterine glands in situ and in vitro. *Human Reproduction*, 30, 2747–2757.

Parturition, Labor, Delivery

Peyvand Amini and Sam Mesiano, Case Western Reserve University, Cleveland, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The success of pregnancy (i.e., birth of a healthy baby to a healthy mother) depends on multiple biological events and processes including: (1) appropriate interaction at the fetal/maternal interface to alter maternal physiology such that the allogenic embryo can implant to establish pregnancy and is not rejected by the maternal immune system; (2) a specific and ordered sequence of events in the embryo/fetal development program; (3) the nutritional, metabolic, and physical needs of the conceptus are satisfied as pregnancy advances; and (4) the appropriate timing and process of parturition so that birth occurs when the fetus is sufficiently mature to survive outside the uterus. This article addresses the process and timing of parturition and how this critical event in the reproductive cycle of viviparous species is controlled.

The Process of Parturition

The process of parturition involves: (1) transformation of the myometrium (smooth muscle of the uterine wall) from a quiescent and relaxed state to a highly contractile and excitable state, wherein it contracts forcefully and rhythmically to produce the contractions of labor and become the engine for birth; (2) remodeling of the extracellular matrix (ECM) in the uterine cervix such it transforms from a closed, rigid and unyielding structure, to a softened structure that distends in response to increased intrauterine pressure to open the uterine outlet (gateway for birth) allowing the conceptus (fetus and placenta) to be moved by uterine contractions into the vaginal canal; and (3) weakening and rupture of the fetal membranes (amnion and chorion) to release the fetus from the amniotic sack. These events are usually coordinated and occur at a time in pregnancy referred to as term, when fetal maturation is sufficient for it to survive as a neonate, and maternal physical, metabolic and behavioral processes are conducive to the parturition process and nurturing the neonate. Human term is between week 37 and 40 of gestation.

The Myometrium

For most of pregnancy the myometrium is relaxed, quiescent and compliant, and grows mainly by stretch-induced cellular hypertrophy of myometrial cells to accommodate the growing conceptus. The initial phase of parturition involves a phenotypic transformation whereby myometrial cells gain the capacity to contract forcibly, rhythmically and coordinately, and become more responsive to uterotonic hormones (factors that directly induce uterine contraction) such as oxytocin (OT) and prostaglandin- $F_{2\alpha}$ (PGF $_{2\alpha}$). This transformation involves increased expression of a specific cohort of genes encoding factors collectively referred to as contraction-associated proteins (CAPs). Some important CAPs include connexin-43 (CX43) that forms gap-junctions between myometrial cells, allowing rapid electrical, and chemical signaling between adjacent cells to permit synchronous contractions, receptors for stimulatory uterotonins (e.g., OT and PGF $_{2\alpha}$ receptors) leading to increased responsiveness to hormones that stimulate uterine contraction, and ion channels that decrease the resting membrane potential to increase excitability by lowering the threshold for contraction.

The Cervix

Although myometrial contractions move the fetus into the lower uterine segment, birth cannot occur unless the uterine cervix—a cylindrical extension of lower uterine segment that has a central canal connecting the uterus with the upper end of the vagina—dilates. For most of pregnancy the cylindrical wall of the cervix is composed of a dense and rigid fibrous ECM, which is unyielding and maintains a narrow canal that resists tensile forces generated by myometrial contractions and increased intrauterine pressure. In the nonpregnant state the cervical canal, albeit narrow, is open to allow menstrual discharge and entry of sperm. However, during pregnancy the canal is occluded by a mucous plug that separates the uterine and vaginal cavities. The mucous plug is usually discharged late pregnancy when the cervical canal dilates.

Parturition involves remodeling of the cervical wall ECM such that it softens and dilates in response to upward circumferential force from myometrial contractions, increased intrauterine pressure and the force of gravity. Changes in the biomechanical properties of the cervix begin before the onset of active labor and can be divided into three stages: softening, ripening, and dilation. Softening of the cervix occurs by restructuring of the collagen architecture, and increases progressively after mid-gestation, causing gradual thinning of the cervical wall and shortening of the cervical canal (i.e., distance between the uterine and vaginal openings). Although the tensile strength of the cervix is maintained during the softening phase, the rate of softening and cervical length decrease, which can be monitored by ultrasonography, may affect the timing of parturition since cervical shortening at mid gestation is a strong predictor of preterm birth. In contrast, cervical dilation occurs at parturition and within hours during active labor. This dramatic biomechanical change significantly decreases the capacity of the cervix to resist tensile force, and consequently the cervical canal dilates in response to the contractions of labor and simultaneous downward pressure from the fetus (usually the fetal

head). Eventually the dilation becomes sufficient to allow myometrial contractions to force the conceptus into the vaginal canal and subsequent birth. After birth the cervix remodels to reestablishes the rigid ECM architecture and narrow canal.

Fetal Membranes and Decidua

Human parturition requires rupture of the fetal membranes. The fetal membranes comprise the amnion membrane and the adjacent chorion and maternal decidua (the endometrium of pregnancy). In most women experiencing parturition at term, membrane rupture follows the initiation of myometrial contractions. However, in some women (~10%) fetal membrane rupture occurs without contractions and sometimes preterm. Interestingly, approximately 30% of women experiencing preterm parturition have preterm premature rupture of membranes without myometrial contractions suggesting a fetal membrane/decidua component in the etiology and pathophysiology of preterm parturition.

At parturition fetal membranes adjacent to the cervix separates from the decidua, which leads to the release of fetal fibronectin, which is normally located in the chorion–decidual contact zone. In this context, fetal fibronectin in the cervical-vaginal fluid serves as a biomarker for term and preterm parturition. In addition a mechanically weak zone develops in the membranes overlying the cervix which eventually becomes the site of membrane rupture. The weakening is associated with inflammation and inflammatory cytokines at the chorion–decidua interface, and may be a mechanism by which inflammation in the cervix induces rupture of the adjacent fetal membrane.

Thus, the process of parturition involves activation of the uterine musculature to produce the forceful contractions of labor, remodeling of ECM in the cervix to open the gateway for birth, and weakening of the fetal membranes to release the fetus from the amniotic sack. A key to unraveling the physiology and timing of parturition is to understand how these processes are controlled.

The Hormonal Control of Parturition

Specific biomechanical changes in each of the uterine compartments (myometrium, cervix, and amnion–chorion–decidua) are controlled by a complex interplay of hormonal (especially the steroid hormones progesterone and estradiol) interactions between the maternal and fetal tissues. The process of parturition is an inflammatory event that involves marked tissue-level inflammation within the myometrium, cervix, and decidua, characterized by activation of resident and infiltrating immune cells (e.g., macrophages, neutrophils, dendritic cells) and increased local levels of pro-inflammatory cytokines, including $\text{PGF}_{2\alpha}$ and chemokines (e.g., interleukin-8) that further stimulate inflammation to produce a tissue-level positive-feedback inflammatory state. For most of pregnancy the balance of regulatory signals favors inhibition of inflammation, myometrial quiescence, cervical closure, and the mechanical integrity of the fetal membranes. Parturition is induced by a switch in the balance to favor tissue-level inflammation and the production of uterotonic hormone that promote myometrial contraction and cervical softening and effacement and membrane weakening.

Hormonal Control of the Gravid Uterus

Growth and function—especially contractility and vascularity—of the gravid uterus are primarily controlled by the uterotrophic steroid hormones progesterone and estrogen (mainly estradiol). With respect to parturition these hormones affect myometrial contractility, the biomechanical integrity of the uterine cervix, and the inflammatory state of the decidua.

As its name implies, progesterone is a pro-gestation hormone; it is essential for the establishment and maintenance of pregnancy. For most of pregnancy progesterone blocks parturition by promoting myometrial relaxation and cervical rigidity and closure, and decreasing uterine cell responsiveness to pro-inflammatory/pro-labor stimuli. In contrast, it is generally considered that estrogen opposes progesterone by stimulating biochemical and physical changes in the myometrium, cervix, and fetal membranes that favors labor. During most of pregnancy labor-blocking and antiinflammatory actions of progesterone dominate. Parturition is induced when the actions of progesterone are withdrawn, which permits increased influence of estrogen and other pro-labor/pro-inflammatory hormones to induce parturition.

The hormonal control of parturition also involves uterotonic hormones that directly induce or repress myometrial contraction. The most notable are PGs (mainly $\text{PGF}_{2\alpha}$) and OT that directly stimulate myometrial contraction. PGs also promote cervical ripening. PG synthesis by the amnion, chorion, decidua, and myometrium increases during the prelude to, and in association with, the onset of labor. The increase in PGs is also due to increased synthesis by resident and infiltrating leukocytes into the myometrium, cervix, and decidua as part of the tissue-level inflammation that occurs during parturition. The central role of PGs in the control of parturition is reflected by clinical studies showing that administration of PGE_2 or $\text{PGF}_{2\alpha}$ to women at any stage of pregnancy induces labor by stimulating myometrial contractions and cervical ripening, and that inhibition of PG biosynthesis with aspirin, indomethacin, or specific PG synthase inhibitors suppresses labor and prolongs gestation.

OT, a 9-amino acid peptide secreted by the posterior pituitary, is a potent stimulator of uterine contraction and is routinely administered to augment the contractions of labor and treat postpartum hemorrhage. Vaginal distention is a principal stimulus for the release of pituitary OT via a neurologic feedback mechanism known as the Ferguson reflex. Thus, during the latter stages of parturition a positive feedback neuroendocrine interaction occurs whereby distention of the vaginal canal induced release of OT from the posterior pituitary which promotes myometrial contraction leading to further vaginal distention. As OT in the maternal

circulation does not increase prior to the latter stages of active labor, it is not generally considered to be involved in the initiation of parturition, but instead is thought to be a key stimulatory uterotonic required for the progression and completion of labor and delivery. OT effects on myometrial cells are mediated by the OT receptor (OXTR), whose abundance in myometrium increases gradually toward the end of pregnancy and rises significantly in association with the onset of labor. Expression of the OXTR by myometrial cells is inhibited by progesterone and increased by estrogen. Thus, induction of OT responsiveness by increased OXTR expression may be the principal mechanism underlying the uterotonic actions of pituitary OT in the pregnancy uterus. After birth, pituitary OT promotes lactation and stimulates the tonic contraction of the myometrium needed for postpartum uterine hemostasis.

The Progesterone Withdrawal Trigger for Parturition

A common characteristic among viviparous species is that progesterone is essential for pregnancy maintenance and its withdrawal is a key trigger for parturition. In most species parturition at term is initiated by a prepartum decrease in circulating progesterone levels (referred to as systemic progesterone withdrawal), and in all species interventions that disrupt progesterone synthesis or action induce parturition at all stages of pregnancy. Thus, progesterone blocks parturition and removal of the progesterone block alone is sufficient to initiate the full cascade leading to delivery. The mechanism and control of progesterone withdrawal is therefore central to the hormonal control of parturition.

In most species parturition is triggered by a systemic progesterone withdrawal. For example, in mice progesterone is supplied by the maternal corpus luteum (CL) and the progesterone withdrawal trigger for parturition is caused by hormonal signals from the uterus that induce luteolysis. In sheep progesterone is produced by the placenta and parturition is caused by hormonal signals from the fetus, mainly in the form of cortisol, that decreases placental progesterone secretion. In women parturition is induced at any stage of pregnancy by disruption of progesterone activity [e.g., by administration of RU486, a progesterone receptor (PR) antagonist]. However, human parturition at term or preterm is not associated with decrease in systemic progesterone. Instead, circulating levels of progesterone (and estrogens) are high for most of human pregnancy and during labor and delivery. Despite persistently elevated levels of progesterone throughout pregnancy, prophylactic progestin supplementation is used as a therapy to prevent preterm birth. The effectiveness of this treatment, however, is controversial since any benefits are detected only in a relatively small subset of women with an elevated risk for preterm birth due to a previous preterm birth or a short cervix. Thus, a deficiency in progesterone is not likely to be the underlying cause for preterm birth and decreased progesterone availability to cell targets in the uterus is not a mechanism for normal term parturition. To explain this conundrum, it is hypothesized that human parturition is triggered by a functional progesterone withdrawal, whereby uterine cells become refractory to progesterone actions. One mechanism by which this occurs is via changes in progesterone receptor (PR) signaling in uterine cells ([Mesiano et al., 2002](#)).

The capacity for progesterone to sustain pregnancy and block parturition is dependent on the expression and activity of the nuclear PRs in uterine cells where they function as ligand-activated nuclear transcription factors. The human PR exists as two major isoforms: the full length PR-B, and the truncated (by 164 N-terminal amino acids) PR-A. Responsiveness to progesterone is dependent on the combined actions of PR-A and PR-B. Studies in term myometrium and myometrial cell lines suggest that functional progesterone withdrawal is caused by differential activity of the PR isoforms such that pro-gestational actions of progesterone are mediated by PR-B and this effect is inhibited by repressive actions of PR-A that decreases the transcriptional activity of PR-B ([Mesiano et al., 2002](#)). Other mechanisms for functional progesterone withdrawal include increased expression of PR-B co-repressors ([Condon et al., 2003](#)), inhibition of PR activity by other transcription factors ([Kalkhoven et al., 1996](#)), and reduced binding of PRs to DNA ([Nadeem et al., 2016](#)). Progesterone also may become inactivated in uterine cells before it interacts with the PRs by its metabolism to an inactive form causing the PRs to become unliganded and therefore inactive ([Nadeem et al., 2016](#)). Data also suggest that PR signaling in myometrial cells is modulated by specific micro RNAs ([Williams et al., 2012](#)). Thus, human parturition may involve multiple redundant processes to cause PR-mediated functional progesterone withdrawal and local inactivation of progesterone ([Fig. 1](#)). The hormonal interactions that control progesterone withdrawal, whether it be functional, systemic or local, are key regulatory factors in the hormonal control of parturition.

Inflammatory Triggers for Parturition

It is now clear that parturition is an inflammatory process and that it can be triggered by inflammatory stressors leading to tissue-level inflammation in the myometrium, cervix, and decidua. This implies that inflammation is upstream of progesterone withdrawal in the hormonal control of parturition. Implicit in the hypothesis that labor is induced by inflammation is the notion that pregnancy is maintained if inflammation is inhibited. Progesterone acting via the PRs exerts antiinflammatory actions in uterine cells and decreases responsiveness to pro-inflammatory/pro-labor stimuli suggesting that progesterone sustains pregnancy by blocking inflammatory triggers for parturition ([Amini et al., 2016](#); [Tan et al., 2012](#); [Hardy et al., 2006](#)). In myometrial cells anti-inflammatory effects of progesterone are mediated mainly by PR-B and antiinflammatory effects of PR-B are inhibited by PR-A consistent with PR-A-mediated functional progesterone withdrawal. Interestingly, the capacity for PR-A to inhibit the antiinflammatory activity of PR-B in myometrial cells is induced by pro-inflammatory stimuli ([Amini et al., 2016](#); [Peters et al., 2017](#)). This effect is consistent with the concept that inflammation is upstream of functional progesterone withdrawal in the trigger mechanism for human parturition.

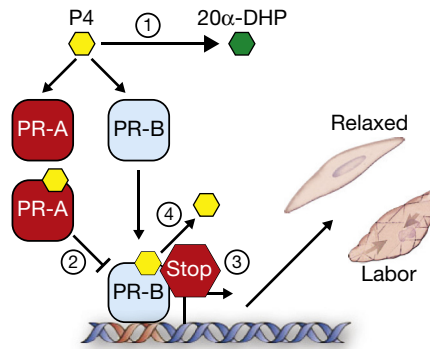


Fig. 1 Possible mechanisms for progesterone withdrawal in human parturition. Actions of progesterone could be eliminated to trigger parturition by: (1) metabolism of progesterone (P4) to an inactive form (e.g., 20α -dihydroprogesterone; 20α -DHP); (2) isoform switching and activation of PR-A transrepression of PR-B; (3) interaction of PRs with other transcriptional regulators that block PR-B activity at specific gene promoters; and (4) uncoupling of progesterone from the PR.

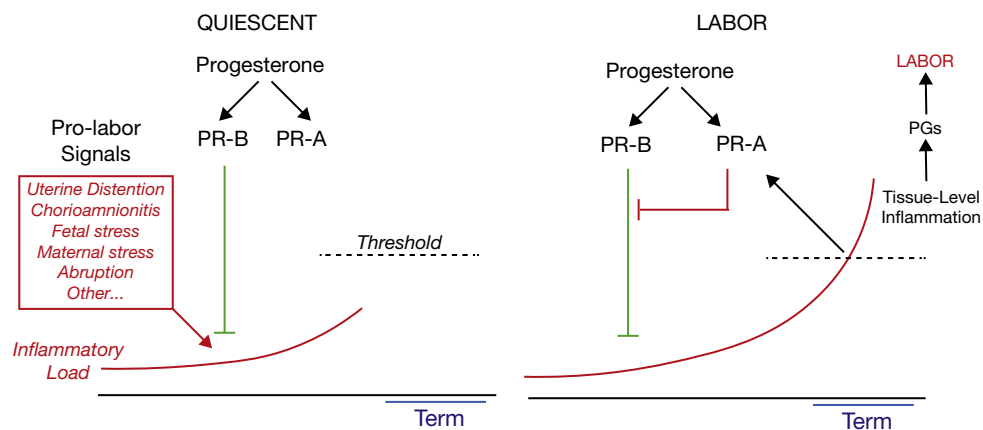


Fig. 2 Model to explain the functional interaction between pro-labor inflammatory stressors and PR-A-mediated functional progesterone withdrawal in the induction of human parturition. The net effect of multiple pro-labor signals places an inflammatory load on the gravid uterus that gradually increases with advancing gestation. For most of pregnancy response of uterine cells to inflammatory load is controlled (mainly surpassed) by progesterone via PR-B. An inflammatory load threshold exists above which the trans-repressive activity of PR-A is induced which inhibits the anti-inflammatory action of progesterone mediated by PR-B. This causes tissue-level inflammation in the uterine compartments that increases local PG levels that transform the uterus to the labor state.

The functional interaction between inflammation and progesterone/PR signaling suggest a model for the hormonal control of parturition (Fig. 2). It is expected that the uterus is exposed to multiple pro-inflammatory/pro-labor stimuli throughout pregnancy. Some are intrinsic to the normal maternal/fetal physiology of pregnancy (e.g., uterine distention, fetal maturation, placental senescence, maternal metabolic stress), while others are extrinsic (e.g., intrauterine infection, maternal environmental stress). The stressors impart on the pregnancy uterus a net inflammatory load that increases with advancing gestation. It is proposed that an inflammatory load threshold exists, which when surpassed, triggers parturition via PR-mediated functional progesterone withdrawal. Decreased antiinflammatory actions of progesterone could then create a positive-feedback inflammatory state within the uterine tissues leading to local production of PGs that promote labor. The increased incidence of preterm birth associated with infection/inflammation and fetal/maternal stress supports this concept. The paradigm predicts that altering the inflammatory load trajectory and/or functional progesterone withdrawal threshold may be effective strategies to prevent preterm birth.

Estrogen Stimulation of Parturition

Estrogens (mainly estradiol) have the opposite effects of progesterone on the gravid uterus by promoting its transition from quiescence to labor. Mechanisms by which estrogen promotes parturition include: (1) increasing responsiveness of myometrial cells to OT and PGs by increasing cognate receptor levels; (2) promoting synchronization of contractions by increasing expression of CX43 that leads to formation of functional gap junctions between myometrial cells, and (3) increasing expression of proteolytic enzymes by cervical fibroblasts and decidual cells to promote cervical ripening and membrane rupture, respectively. In most species pro-labor estrogenic actions are induced by increased estradiol levels during the prelude to labor and in conjunction with systemic

progesterone withdrawal. This does not occur on women. Instead, the estrogen milieu of human pregnancy is high throughout pregnancy and persists during labor and delivery. The reason for this is that the human placenta produces estrogens (estradiol, estrone, estriol, and estetrol) throughout pregnancy. The high circulating estrogen levels suggest that the uterine tissues are refractory to pro-labor estrogenic actions for most of pregnancy, and that parturition involves increased responsiveness of uterine cells to estrogens (i.e., estrogen activation). Increased estrogen responsiveness at parturition appears to be coordinated with functional progesterone withdrawal via a reciprocal interaction between progesterone/PR signaling and estrogen/ER signaling. In general, progesterone decreases estrogen responsiveness by decreasing ER expression and estrogen increases progesterone responsiveness by increasing PR expression. Thus, human parturition may involve a functional estrogen activation that is coordinated with, and possibly secondary to, functional progesterone withdrawal. In this context, interaction between the ER and PR systems in uterine cells and the regulation of this process by pro-labor/pro-inflammatory stimuli may be of paramount importance for the control of human parturition.

The Timing of Parturition

Parturition before term increases mortality and morbidity risk for the neonate due to insufficient in utero development and functional immaturity of organ systems needed to survive outside of the uterus and independent of the placenta. Parturition after term increases mortality and morbidity risk for the mother and neonate due to fetal overgrowth and the associated problems with labor and delivery. In this context the hormonal control of parturition is expected to involve signals linking birth timing with fetal maturation such that birth occurs only after the fetus is prepared for extrauterine life and before it becomes too large to pass through the birth canal. This hormonal signaling is apparent in the sheep in which parturition is induced by increased activity of the fetal hypothalamic–pituitary–adrenal (HPA) axis that produces a surge of cortisol during the week before parturition (Liggins, 1994). Cortisol increases the metabolism of progesterone to estradiol in the sheep placenta leading to a systemic decrease in maternal progesterone and a concomitant increase in estradiol that induces changes in the uterine tissue to initiate parturition. Importantly, cortisol from the fetal HPA axis also promotes the functional maturation of fetal organ systems, especially the lungs, that the neonate will need after birth. Thus, in sheep the timing of parturition is linked to fetal maturation via activity of the fetal HPA axis. The effect of cortisol on the maturation of organ systems in fetus appears to be conserved in multiple species including humans (Young et al., 2010). Glucocorticoid promotion of fetal lung maturation is especially important because the inability to exchange gases due to pulmonary immaturity (i.e., respiratory distress syndrome due to lack of surfactant) is the leading cause of neonatal morbidity and mortality among preterm infants. This is the underlying rationale for the practice of glucocorticoid therapy (usually with synthetic glucocorticoids that cross the placenta) to women presenting with preterm labor in an effort to stimulate fetal organ maturation (especially surfactant production by the lungs) to increase neonatal competency if preterm birth occurs. However, unlike the sheep, cortisol production by the fetal HPA is not involved in the timing of human parturition.

In most species parturition is induced by maternal stressors which represents a mechanism by which the mother terminates pregnancy as a survival response. Normal term birth is preceded by increased inflammation in the myometrium, decidua and cervix and preterm birth is commonly associated with intrauterine infection, clinically silent upper genital tract infection, and bacterial vaginosis. This suggests that maternal stress, at least in the form of inflammatory stimuli impacting the uterine tissues, is a trigger for parturition and is consistent with the inflammatory load hypothesis (Fig. 2). The concept is supported by animal studies showing that administration of pro-inflammatory cytokines or factors that induce a pro-inflammatory response, initiate parturition.

The timing of parturition can also be considered from an evolutionary perspective. Natural selection favors traits that increase reproductive efficiency. In viviparous species this affects pregnancy and parturition to optimize factors such as maternal habitus, fetal size, litter size, uterine shape, and nutrient access. The timing of parturition could decrease reproductive efficiency if the fetus is born too early, which would compromise neonatal survival, and if it is born too late, which would compromise maternal and fetal survival if the fetus is too large to pass through the pelvic outlet. The former causes loss of a pregnancy, while the latter causes loss of a female's future pregnancies. It is reasonable, therefore, that natural selection favored a combination of maternal and fetal triggers for parturition. This effect is reflected in the diversity and specificity of parturition timing across viviparous species. In general, the mechanism by which parturition is controlled and timed in a particular species is suited to its overall reproductive strategy and selected through evolution to optimize reproductive efficiency. Consequently, the timing of birth relative to fetal/neonatal maturation varies greatly. Some species (e.g., marsupial, rodent) give birth to highly altricial neonates that are helpless and require considerable nurturing, while others (e.g., ovine, bovine, some primates) give birth to precocial neonates that can run with the herd soon after birth. In general primates give birth to precocial neonates. The human neonate, however, although being a primate, is born highly altricial. One explanation for this is that the timing of hominid birth was altered by natural selection to favor encephalization that caused a marked increase in brain size especially during fetal development. The fossil record shows that hominid brain size began increasing around 1 million years ago and has remained constant for at least the last 100,000 years. This was likely associated with increase brain and head growth during fetal development that eventually would lead to cephalo-pelvic disproportion at birth. Thus, the advantages of encephalization would have been balanced by the costs of neonatal and maternal loss due to difficulties at birth. Consequently, human pregnancy has significantly less obstetric capacity–space between the infant head and maternal pelvis–compared to other extant primates (Rosenberg and Trevathan, 2001, 2002). To solve the problem of cephalo-pelvic disproportion at birth, hominid evolution is thought to have favored traits that shortened pregnancy length so that the fetus is born before its head becomes larger than the obstetric capacity. A consequence of this selection would

be that the human neonate is less mature at birth than other primates; which indeed is the case. However, as natural selection operates at the population level over multiple generations rather than the individual, the mechanism for human birth timing, although not be ideal, is sufficient to provide an overall advantage. This is reflected by the relatively high incidence of pregnancy failure (compared with other species) and the general difficulty of human parturition due mainly to the large fetal head. Thus, the evolutionary biology of parturition highlights the variability in the physiologic mechanisms that control the parturition process and its timing. Although certain traits, such as progesterone withdrawal and inflammation as triggers for parturition appear to be conserved across viviparous species, differences in the mechanism and relative importance of those processes explain much of the comparative biology of parturition.

References

- Amini, P., Michniuk, D., Kuo, K., Yi, L., et al. (2016). Human parturition involves progesterone receptor-A phosphorylation at serine-345 in myometrial cells. *Endocrinology*, 157(11), 4434–4445.
- Condon, J. C., Jeyasuria, P., Faust, J. M., Wilson, J. W., & Mendelson, C. R. (2003). A decline in the levels of progesterone receptor coactivators in the pregnant uterus at term may antagonize progesterone receptor function and contribute to the initiation of parturition. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 9518–9523.
- Hardy, D. B., Janowski, B. A., Corey, D. R., & Mendelson, C. R. (2006). Progesterone receptor plays a major antiinflammatory role in human myometrial cells by antagonism of nuclear factor-kappaB activation of cyclooxygenase 2 expression. *Molecular Endocrinology*, 20, 2724–2733.
- Kalkhoven, E., Wissink, S., van der Saag, P. T., & van der Burg, B. (1996). Negative interaction between the RelA(p65) subunit of NF-kappaB and the progesterone receptor. *The Journal of Biological Chemistry*, 271, 6217–6224.
- Liggins, G. C. (1994). The role of cortisol in preparing the fetus for birth. *Reproduction, Fertility, and Development*, 6, 141–150.
- Mesiano, S., Chan, E. C., Fitter, J. T., et al. (2002). Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor expression in the myometrium. *The Journal of Clinical Endocrinology and Metabolism*, 87, 2924–2930.
- Nadeem, L., Shynlova, O., Matysiak-Zablocki, E., et al. (2016). Molecular evidence of functional progesterone withdrawal in human myometrium. *Nature Communications*, 7, 11565.
- Peters, G. A., Yi, L., Skomorovska-Prokvolit, Y., et al. (2017). Inflammatory stimuli increase progesterone receptor- α stability and transrepressive activity in myometrial cells. *Endocrinology*, 158(1), 158–169.
- Rosenberg, K. R., & Trevathan, W. R. (2001). The evolution of human birth. *Scientific American*, 285, 72–77.
- Rosenberg, K. R., & Trevathan, W. R. (2002). Birth, obstetrics and human evolution. *BJOG*, 109, 1199–1206.
- Tan, H., Yi, L., Rote, N. S., Hurd, W. W., & Mesiano, S. (2012). Progesterone receptor-A and -B have opposite effects on proinflammatory gene expression in human myometrial cells: Implications for progesterone actions in human pregnancy and parturition. *The Journal of Clinical Endocrinology and Metabolism*, 97, E719–30.
- Williams, K. C., Renthal, N. E., Condon, J. C., Gerard, R. D., & Mendelson, C. R. (2012). MicroRNA-200a serves a key role in the decline of progesterone receptor function leading to term and preterm labor. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 7529–7534.
- Young, I. R., Renfree, M. B., Mesiano, S., et al. (2010). The comparative physiology of parturition in mammals: Hormones and parturition in mammals. In D. Norris, & K. Lopez (Eds.), *Hormones and reproduction in vertebrates*. London: Academic Press.

The Intrauterine Position Phenomenon

Frederick S vom Saal, University of Missouri-Columbia, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

AGD Ano-genital distance is the length of the space between the caudal aspect of the genital tubercle (penis in males) and the anterior aspect of the anus.

5 α -DHT 5 α -dihydrotestosterone.

EDCs Endocrine disrupting chemicals.

E₂ Estradiol.

IUP Position in the uterus of a fetus relative to siblings of the same or opposite sex.

T Testosterone.

Background

The focus of this article will be on the effects of very small differences in the sex steroids estradiol and testosterone on male and female fetal development due to a random developmental event, namely, whether embryos implant adjacent to male or female fetuses when there are multiple embryos in a uterus, which I referred to as the intrauterine position phenomenon (IUP) (vom Saal, 1981). This phenomenon results from the diffusion of sex hormones across the amniotic and chorionic placental membranes via amniotic fluid into the blood of adjacent fetuses (Fig. 1). To produce animals from known intrauterine positions, females are time mated, and just prior to parturition, pups are Cesarean delivered. Fetal location in the uterus is recorded, animals are individually marked for subsequent identification and raised by foster mothers for examination of phenotypic consequences during postnatal life.

The intrauterine position phenomenon has revealed the exquisite sensitivity of mammalian fetuses to very small perturbations in sex hormones, with significant consequences for subsequent life history (reviewed in vom Saal, 1981, 1989; vom Saal et al., 1999; vom Saal, 2016). This phenomenon provides the only mammalian model for relating postnatal traits to concentrations of endogenous hormones to which individuals are exposed at variable levels during the fetal period of sexual differentiation. Results from studies using this naturally occurring model system in litter-bearing species have provided insights concerning the consequences of individual differences in steroid concentrations during sexual differentiation that likely apply to all mammals, as revealed by significant effects of IUP identified in studies of human twins. In general, when there is a sex difference in a trait, a female fetus positioned next to males in utero has traits that are significantly more masculinized relative to female siblings that are next to only other female fetuses in utero. The effects on males of being adjacent to male or female fetuses is more complicated due to the fact that both testosterone and estradiol regulate specific aspects of masculinization (vom Saal, 2016).

Sexual differentiation in female mammals is less well understood than in males in terms of hormonal influences, although research has shown that exposure to testosterone and estrogenic hormones, drugs and environmental chemicals during development can lead to significant changes in organ function and disease in females as well as males. Importantly, fetal sexual differentiation is not just regulated by testosterone, although it is clearly the primary factor in masculinization, and for some aspects of sexual differentiation in males and females estradiol, other hormones (Gupta and Goldman, 1986; Zambrano et al., 2014), and environmental factors (Gore et al., 2015) have significant effects.

In mammals, sexual differentiation in males begins shortly after the onset of differentiation of the embryonic gonads into testes, after which testosterone secreted by Leydig cells initiates the process of masculinization. Sexual differentiation occurs primarily during prenatal life in mammals with a long gestation, as opposed to short-gestation species such as mice and rats where this process spans both prenatal and neonatal life. Thus, the impact of IUP on specific aspects of sexual differentiation (masculinization vs. defeminization) and other physiological processes varies between species. Specifically, sexual differentiation in mice and rats begins during the last few days of fetal life and continues into the early neonatal period (vom Saal, 2016). Due to the fact that sexual differentiation begins during the first trimester of pregnancy in humans, there is currently no method for assessing the levels of hormones in the blood of a human fetus during sexual differentiation, thus precluding examining the consequences of variability in gonadal steroid levels during fetal life for postnatal traits. Available evidence indicates that levels of compounds in amniotic fluid and blood are not the same (vom Saal et al., 2014), and thus amniotic fluid levels of hormones (determined by amniocentesis) cannot accurately identify the bioactive concentration of hormones in blood.

Differences in phenotype due to IUP were initially attributed only to differences in serum testosterone, thus, male and female fetuses positioned between female fetuses in early publications were referred to as 0M males (not next to a male fetus) rather than 2F males. However, findings concerning the effects of IUP on the male reproductive system led to experimental studies identifying that IUP effects were mediated by differences in serum estradiol as well as testosterone. Given the very small differences in free serum

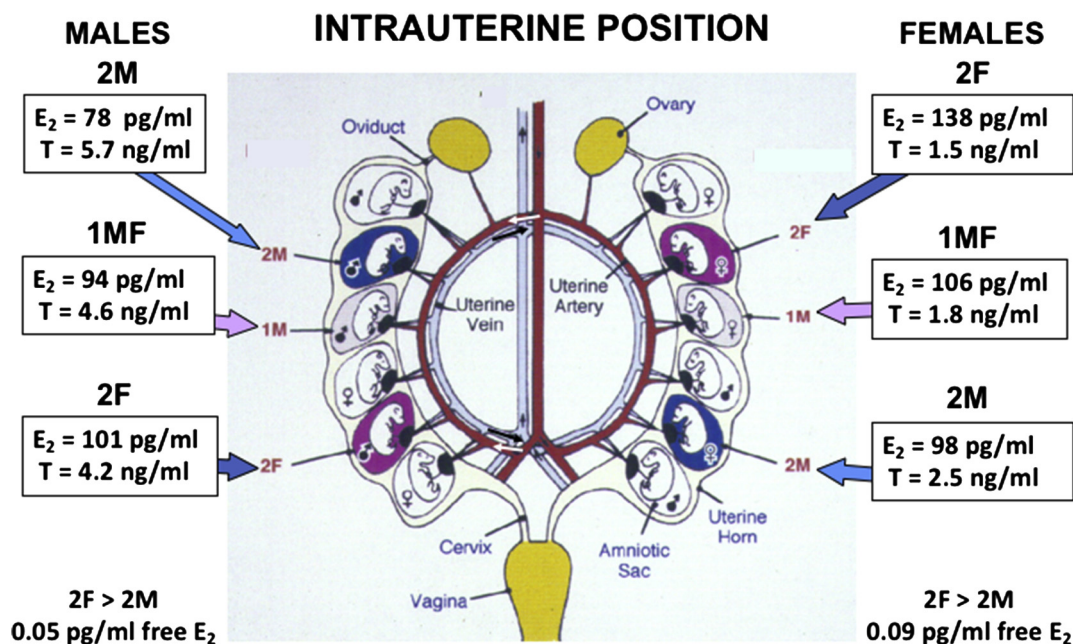


Fig. 1 Total serum concentrations of testosterone (T) and estradiol (E₂) on gestation day 18 (1 day prior to parturition) are shown for male and female CF-1 mouse fetuses from different intrauterine positions. Since only 0.22% of estradiol is not bound to plasma proteins (both albumin, with high capacity but low affinity, and high-affinity alphafetoprotein produced by the fetal liver) on gestation day 18 (vom Saal et al., 1997), the difference in free serum estradiol concentration between 2M and 2F female fetuses is calculated to be 0.09 pg mL⁻¹ (corresponding to a 40 pg mL⁻¹ difference in total serum estradiol). The difference in free serum estradiol concentration between 2M and 2F mouse males is 0.05 pg mL⁻¹, corresponding to a 23 pg mL⁻¹ difference in total serum estradiol. There are no high affinity testosterone binding proteins in fetal rat or mouse serum, and the fraction of serum testosterone not bound to albumin is free (vom Saal et al., 1992). Arrows in the arteries and veins indicate the direction of blood flow. Based on data in vom Saal, F. S. (1989). Sexual differentiation in litter-bearing mammals: Influence of sex of adjacent fetuses in utero. *Journal of Animal Science* **67**, 1824–1840; vom Saal, F. S. and Dhar, M. G. (1992). Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: Implications for transport of steroids between fetuses. *Physiology and Behavior* **52**, 163–171; Even, M. D., Dhar, M. G. and vom Saal, F. S. (1992). Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. *Journal of Reproduction and Fertility* **96**, 709–716.

estradiol between 2M and 2F fetuses of the same sex, it is easy to understand that there was skepticism 40 years ago concerning the hypothesis that these small differences in serum estradiol might account for some of the significant differences in phenotype related to IUP.

Testosterone can act directly in some target tissues or it can serve as a pro-hormone and act in other tissues after conversion to the more potent androgen 5 α -dihydrotestosterone (5 α -DHT) by the enzyme 5 α -reductase, or after conversion to estradiol by the enzyme aromatase. Gonadal steroids permanently influence 5 α -reductase (Type 2) and aromatase activity during differentiation of accessory reproductive organs (different isoforms of 5 α -reductase are in other tissues). In addition, estradiol regulates the expression of androgen receptors in the developing prostatic region of the urogenital sinus, but not in the organs that form from the Wolffian ducts (Taylor et al., 2011; vom Saal, 2016).

During critical periods in fetal life hormonal signals (endocrine and paracrine) regulate the differentiation of cells and thus the future functioning of organ systems. These hormonal signals operate at very low concentrations and low receptor occupancy. The endocrine system evolved to be responsive to very small changes in free serum hormone concentrations and to operate in a portion of the receptor binding range of hormone concentration that is nearly linear between hormone concentration and receptor occupancy, which is below 10% occupancy of total receptors (Welshons et al., 2003). An important point is that for lipophilic sex hormones, it is the free concentration in blood that determines bioactivity (Nagel et al., 1999).

An unexpected consequence of findings concerning the intrauterine position phenomenon was that they led to the prediction, now confirmed, that exposure during development to chemicals in the environment that interfere with hormones, such as testosterone and estradiol, known as endocrine disrupting chemicals (EDCs), might result in subsequent adverse health effects at much lower levels of exposure than were being examined by toxicologists conducting regulatory guideline studies for use in risk assessments (Colborn et al., 1996; Vandenberg et al., 2012; vom Saal, 2016).

Life-History is Impacted by the Intrauterine Position (IUP) Phenomenon

There are a large number of studies showing that naturally occurring variation in the blood levels of testosterone and estradiol in mouse, rat and gerbil fetuses, due to being positioned in utero between male fetuses (2M fetuses) or between female fetuses (2F

fetuses), leads to phenotypic differences that impact life history (vom Saal, 1989, 2016; Ryan and Vandenberg, 2002). Numerous studies have identified neuro-behavioral, reproductive and metabolic traits that are related to proximity to another fetus of the same or opposite sex, including in human twins (vom Saal, 2016).

During the fetal period of sexual differentiation, male fetuses have higher serum concentrations of testosterone than females, while in some species, such as house mice (*Mus musculus domesticus*), female fetuses have higher serum concentrations of estradiol than males (Fig. 1); there is also similar evidence in for a sex difference in serum estradiol in human fetuses during the second trimester when sexual differentiation is occurring (Reyes et al., 1974). Near the end of gestation in rhesus monkeys, female fetuses have almost twofold higher concentrations of estradiol than male fetuses (reviewed in vom Saal, 1989). Whereas 2M mouse fetuses (male or female) have significantly greater plasma concentrations of testosterone, 2F fetuses have significantly higher serum concentrations of estradiol relative to other fetuses of the same sex; animals next to a single male or female fetus have intermediate serum levels of testosterone and estradiol (Fig. 1); this is also the case for rats (Timms et al., 2002) and Mongolian gerbils (Clark et al., 1991). We showed that the intrauterine position phenomenon is due to transport of steroids via diffusion through the amniotic fluid and then across the chorionic and amniotic membranes separating adjacent fetuses (Even et al., 1992). A critical aspect of the intrauterine position phenomenon is that the positioning of fetuses in the uterus has been shown to be a random event (vom Saal, 1981), thus precluding the possibility that there are systematic genetic differences between fetuses that develop between males versus those that develop between females. However, genotype significantly impacts the functioning of the endocrine system during sexual differentiation, and phenotypic differences due to IUP are not found in inbred mouse strains, but have been reported in a number of outbred stocks (Svare et al., 1984; Ryan and Vandenberg, 2002). The molecular basis for the loss of IUP effects due to inbreeding have not been determined.

Another interesting phenomenon in rats and mice is that blood flow in the uterine artery and vein is bi-directional (Fig. 1), due to the fact that blood enters the uterine loop artery at both the cranial end from the descending aorta and the caudal end from the internal iliac artery; the uterine vein transports blood bi-directionally as well (vom Saal and Dhar, 1992; Even et al., 1994; Coe et al., 2008). The bi-directional blood flow in the rodent uterus leads to fetuses in the middle of a uterine horn being smaller than fetuses at either end due to reduced blood flow at the center of the uterine artery. While uterine blood flow impacts fetal growth, the transport of sex hormones between adjacent fetuses in rodents is unrelated to the direction of blood flow in the uterine artery and vein (Even et al., 1992).

Effects of IUP in Females

Experimental Animal Studies

Table 1 lists the numerous effects of developing between male or female fetuses that have been documented in female mice; many of these effects have also been found in gerbils, as well as in some studies with rats and rabbits. Effects due to IUP include genital morphology, timing of puberty, length of estrous cycles, age at cessation of fertility, sex ratio of litters, production of and response to pheromonal cues, sexual attractiveness to males, sexual receptivity when mounted, maternal behavior, postpartum aggression (defense of pups), interfemale aggression, size of home range, daily activity level, and body weight; most of these effects have previously been reviewed (vom Saal, 1981, 1989, 2016; vom Saal et al., 1999; Ryan and Vandenberg, 2002).

Differences between 2F and 2M female mice are what would be predicted. The effects of elevated testosterone during sexual differentiation on 2M females is consistent with a shorter period of fertility and longer estrous cycles in female rats and mice administered testosterone during early postnatal sexual differentiation, which is referred to as the delayed anovulatory syndrome (Mobbs et al., 1985). Androgenized 2M female mice, gerbils and rats show enlarged ano-genital distance (AGD), which is a well-known bioassay for fetal testosterone levels in males as well as females in rodents (Clemens et al., 1978; vom Saal and Bronson, 1978; Ryan and Vandenberg, 2002), rabbits (Banszegi et al., 2009), as well as in humans (Barrett et al., 2013). Reduced androgen exposure in male fetuses and shorter ano-genital distance predicts reduced adult semen quality in men (Mendiola et al., 2011). The difference in AGD observed at birth due to IUP persists into adulthood in mice (vom Saal and Bronson, 1978; Ryan and Vandenberg, 2002), and is eliminated in rats by maternal treatment with the antiandrogen Flutamide during pregnancy (Clemens et al., 1978). In swine, females born in litters with a high proportion of males had a longer AGD relative to females in litters with a low proportion of males (Drickamer et al., 1997).

An interesting aspect of IUP findings is that models predict that IUP in females may provide a basis for population cycles (irruptions and crashes) in small mammals, whereas most prior research had focused (unsuccessfully) on rapid changes in gene frequency as the mediator of irruptions and crashes in small mammal populations (vom Saal, 1984; Cowell et al., 1998).

Fetal differentiation of the mammary gland in mice is influenced by intrauterine position, with 2M females showing an increase in mammary gland duct development relative to 2F females on gestation day 18. An increase in mammary gland density is associated with mammary gland cancer later in life (Vandenberg et al., 2007).

Sex ratio of litters in Mongolian gerbils and mice varies as a function of prior intrauterine position of a female. 2M females produce male-biased litters and 2F females produce female-biased litters, although 2M and 2F females (mice and gerbils) have the same number of pups, so this phenomenon is not due to differential mortality of males and females in utero (Vandenberg and Huggett, 1994; Clark and Galef, 1995). Thus, 2M females produce more offspring that develop next to male fetuses relative to 2F females, which are more likely to produce offspring that develop next to female fetuses. This is an example of multigenerational transmission of phenotype through a novel, but still undetermined, mechanism.

Table 1 Effects on female mice (and in most cases also gerbils, rats, and rabbits) of developing in utero between female fetuses (2F) and male fetuses (2M)

<i>Effect of IUP</i>	<i>2F Females</i>	<i>2M Females</i>
Serum steroid concentrations during fetal life		
Testosterone		↑
Estradiol	↑	
Adult serum progesterone concentrations		↑
Postnatal morphology		
Perineum: ano-genital distance		↑
Body weight		↑
Mammary gland ducts		↑
Adult reproductive performance		
Earlier puberty	↑	
Shortest most regular estrous cycles	↑	
Longest most irregular estrous cycles		↑
Reproductive aging: longer period of fertility	↑	
Sexual attractiveness to males	↑	
Increased sexual activity (receptivity)	↑	
Female-biased sex ratio of litters	↑	
Male-biased sex ratio of litters		↑
Adult behavior		
Aggression		
Postpartum defense of pups		↑
Aggression toward other females		↑
Territorial/scent marking		↑
Size of home range		↑
Maternal behaviors	↑	
Daily activity	↑	
Enzyme and gene activity		
Preputial gland β glucuronidase activity		↑
Hepatic 7 α -hydroxylase and 5 α -reductase activity	↑	
ER α mRNA in hypothalamic ventromedial nucleus		↑

↑ indicates an increase associated with fetal intrauterine position. Not all effects were seen in all species. In general, 2M females show a phenotype more similar to males for traits that are sexually dimorphic.

Epidemiological Studies of IUP Effects in Human Females

The first evidence for an effect on women of developing with an opposite-sex versus same-sex twin was that developing with an opposite-sex twin significantly masculinized otoacoustic emissions, which are sounds produced by the motion of the cochlea's sensory hair cells (McFadden, 1993). This finding is particularly interesting because otoacoustic emissions represent a sexually dimorphic trait established during fetal life that does not change as a function of postnatal experience. McFadden reported that overall, males show fewer emissions than females, and females with an opposite-sex twin showed fewer emissions than same-sex twin females. Another interesting morphological trait that is significantly masculinized in females that develop with an opposite-sex twin is the eruption patterns of the incisors, upper first molars, and lower canines. These eruption patterns were found to be significantly delayed (masculinized) in opposite-sex twin girls relative to same-sex twin girls (Heikkinen et al., 2013).

A novel study described effects on human females of developing in utero with a male versus a female co-twin by examining historical data from Finland concerning age at marriage and lifetime production of offspring in women who lived during the 18th and 19th centuries (Lummaa et al., 2007). Women born with an opposite-sex twin (whether or not the male died at birth) married later and produced fewer offspring relative to females born with a same-sex twin. The consequence for women born with in utero proximity to a male or female twin was remarkably similar to effects of intrauterine position in mice on lifetime fecundity (Table 1): 2M female mice show later onset of puberty, earlier onset of the cessation of producing live pups, longer, and more irregular estrous cycles, are less sexually attractive and less receptive to males relative to 2F females (vom Saal, 1989). Mertice Clark and colleagues have reported similar findings for Mongolian gerbils in a large number of studies, many reviewed in (Ryan and Vandenberg, 2002). In swine, females born in litters in the highest tertile for proportion of males in the litter also had reduced fecundity relative to females in the lowest tertile for proportion of males (Drickamer et al., 1997).

In contrast to the findings by Lummaa, a study of contemporary young Finnish women did not find significant effects of developing with an opposite-sex twin on the timing of puberty or fertility between age 15 and 28 (Rose et al., 2002). These findings are consistent with another report of no effect on female fertility of developing in utero with an opposite-sex twin in contemporary populations in Australia, the Netherlands, and the United States (Medland et al., 2008). My hypothesis (vom Saal, 2016) is that the absence of significant findings in current studies of IUP effects in humans could be due to world-wide exposure during

development to environmental pollutants referred to as endocrine disrupting chemicals (EDCs). Differential exposure to EDCs today versus prior to the chemical revolution in the mid-20th century, might interfere with the differences in fertility identified by Lummaa. EDCs have been proposed by the Endocrine Society to be a factor in changes in fetal development, fertility, as well as the incidence of many diseases that have markedly increased since the mid-20th century (Gore et al., 2015).

Neuro-behavioral effects of IUP in women

Sexually dimorphic behaviors have been studied in men and women in relation to whether they had a same-sex or opposite-sex twin. Sensation seeking is a sexually dimorphic personality trait involving varied, novel and intense sensations, with males scoring higher on this trait than females. People who are sensation or thrill seekers tend to engage in risk-taking behaviors such as gambling, drinking, smoking, and illicit drug use. The impact on women of developing in utero with an opposite-sex twin on sensation seeking behavior has been examined in studies with similar results (Resnick et al., 1993; Slutske et al., 2011). Women who developed in utero with an opposite-sex twin had significantly higher sensation seeking scores than females from same-sex twin pairs, suggesting masculinization of this trait due to exposure to elevated testosterone from the male co-twin. Importantly, the psychosocial hypothesis that this could be related to having a near-in-age brother was ruled out as a factor in this finding, similar to the findings by Lummaa (Slutske et al., 2011).

In another study, a sex difference in aggression proneness was observed, and girls with an opposite-sex twin showed a more masculine pattern of aggression proneness than girls with a same-sex twin (Cohen-Bendahan et al., 2005). It is well documented that across many types of mammals studied (rodents, monkeys, humans), play behavior is influenced by fetal testosterone exposure. This led to a study comparing play behavior in same-sex and opposite-sex twin 3–8-year-old girls, but no significant differences were observed (Henderson and Berenbaum, 1997).

Effects of Intrauterine Position in Males

Experimental Animal Studies

Table 2 lists the numerous effects of IUP on morphology, physiology, and behavior in male mice and gerbils (intermale aggression, scent/urine marking, sexual behavior, attractiveness to females, parental behavior/infanticide, size and functioning of reproductive organs, size of home range, and daily activity level) that have previously been reviewed (Ryan and Vandenberg, 2002). These

Table 2 Effects on male mice (and in most cases also gerbils, rats, and rabbits) of developing in utero between female fetuses (2F) and male fetuses (2M)

Effect of IUP	2F Males	2M Males
Serum steroid concentrations during fetal life		
Testosterone		↑
Estradiol	↑	
Serum steroid concentrations in adulthood		
Testosterone		↑
Estradiol	↑	
Postnatal morphology		
Perineum: ano-genital distance		↑
Body weight		↑
Size of seminal vesicles		↑
Size of the prostate	↑	
Adult behavior		
Size of home range		↑
Aggression		
Aggression toward other males		↑
Aggression toward pups (infanticide)	↑	
Parental behavior toward pups		↑
Sexual activity	↑	
Daily activity	↑	
Enzyme activity		
Preputial gland β glucuronidase activity		↑
Seminal vesicle and prostate 5 α -reductase		↑
Hepatic NADPH cytochrome <i>c</i> reductase activity		↑
Prostate androgen receptors	↑	

↑ indicates an increase associated with fetal intrauterine position. Not all effects were seen in all species. An increase in 2F males compared to 2M males suggests developmental effects on some outcomes of elevated fetal serum estradiol, which has been experimentally demonstrated for the brain/behavior, and prostate.

findings demonstrate that in male as well as female rodents, small changes in serum levels of steroids can alter neuro-behavioral and reproductive system development. In swine, there is evidence for an increase in aggression and dominance status in 2M males relative to 2F males when competing for food, which led to increased weight gain in 2M relative to 2F adult boars; however, when fed individually, there was no difference in body weight due to prior IUP (Rohde Parfet et al., 1990). The few studies involving human twins that have sought to determine effects on males born with an opposite-sex twin have not reported any significant findings.

An important issue is that 2M and 2F male mice differ in their serum estradiol as well as testosterone concentrations, and some effects of IUP have been shown to be due to differences during fetal life in serum estradiol, while other effects appear to be related to fetal serum levels of testosterone (Fig. 1).

Association of IUP with altered serum testosterone in adulthood in male Mongolian gerbils

The random pulsatile nature of testosterone secretion in adult male mice renders the measurement of testosterone in a single blood sample essentially meaningless. However, we did measure serum testosterone in 2M and 2F male Mongolian gerbils, since they do not show evidence of the release of testosterone in large episodic pulses. Gonadally intact 2M male gerbils had elevated serum testosterone relative to the 2F male siblings (Clark et al., 1992). In gerbils, treatment via a subcutaneous Silastic capsule with the same dose of testosterone in castrated 2M and 2F males eliminated the difference in serum testosterone between intact 2M and 2F males, but there were still differences in scent marking (2M > 2F) due to IUP (Clark et al., 1993). These findings show that the secretion of testosterone in adult males can be impacted by the hormonal environment during fetal life, and there is a large literature that estrogenic and antiandrogenic environmental endocrine disrupting chemicals can lead to altered testosterone secretion and spermatogenesis in males (vom Saal, 2016).

Association of IUP with altered 5 α -reductase activity in seminal vesicles of male mice

The finding that a 2F male mouse fetus reliably has the greatest serum concentrations of estradiol and the lowest serum concentrations of testosterone relative to other male fetuses initially led to the prediction that 2F males would show a postnatal phenotype typical of fetal androgen deficiency. Analysis of the adult seminal vesicles, that differentiate from the distal portion of the embryonic Wolffian ducts, confirmed this prediction. We weighed the blotted seminal vesicles from gonadally intact 2M and 2F adult mice and found that the seminal vesicles from 2M males were significantly larger (Nonneman et al., 1992). We then measured seminal vesicle 5 α -reductase activity in intact and castrated + testosterone-treated 2M and 2F males and found that 2F males had significantly lower 5 α -reductase activity and smaller seminal vesicles relative to 2M males, even when castrated and implanted with a capsule containing testosterone (vom Saal, 2016). The effect of IUP on seminal vesicles was thus not mediated by possible differences due to IUP in serum testosterone in adulthood. In adult mice seminal vesicle fluid influences sperm motility, and removal of the seminal vesicles decreases fertility (Peitz, 1988); seminal vesicle fluid plays a role in semen coagulation, sperm motility, stability of sperm chromatin and suppression of the immune activity in the female reproductive tract (Gonzales, 2001).

Association of IUP with altered fetal prostate gland genesis and prostatic androgen receptors in adulthood in male mice

In contrast to the finding that 2F males had smaller seminal vesicles relative to 2M males, the finding that 2F male mice (Nonneman et al., 1992) and rats (Timms et al., 1999) had larger prostates than 2M males was initially considered implausible. One reason for this was that male rodents treated with high, supra-physiological doses of estrogen during fetal/neonatal life are markedly demasculinized, including having a small prostate (vom Saal et al., 1997). However, these findings emphasize the importance of hormone dose and led to what is now known in toxicology as the “low dose hypothesis.” The key issue is that endogenous estradiol and chemicals that can mimic or interfere with endogenous estradiol or testosterone can have effects at extremely low dose that are opposite to effects observed at high, supra-physiological toxic doses, resulting in inverted U dose-response relationships (Vandenberg et al., 2012).

An interesting finding was that adult 2F male mice that had enlarged prostates also had elevated prostate androgen receptors (about a three- to fourfold difference) but reduced 5 α -reductase activity (by about one-third) relative to 2M males (Nonneman et al., 1992). It thus appears that the increase in androgen receptors is the dominant factor that impacted prostate gland genesis and adult prostate size. In the seminal vesicles, 5 α -reductase was reduced in 2F males but there was no difference between 2M and 2F males in seminal vesicle androgen receptor number (Nonneman et al., 1992). Taken together, these data confirm numerous other findings that during fetal life, gonadal steroids “program” the activity of steroid metabolizing enzymes and steroid receptors in target tissues, with life-time consequences.

Summary

The effects of IUP in litter-bearing animals have predicted significant effects in women born with an opposite-sex twin in comparison to women born with a same-sex twin, while effects on the male siblings in these twin studies have not been found in the very few studies that have included examination of males. Importantly, there is consistency of findings regarding the effects of IUP on a wide variety of traits in both males and females in litter-bearing mammals (mice, gerbils, rats, rabbits, and swine). The IUP phenomenon has significantly impacted our understanding of the exquisite sensitivity of fetuses to extremely small changes in gonadal steroids during the critical period of sexual differentiation, and that the very small differences in testosterone and estradiol can alter the life history of both males and females.

References

- Banszegi, O., Altbacker, V., & Bilko, A. (2009). Intrauterine position influences anatomy and behavior in domestic rabbits. *Physiology & Behavior*, 98, 258–262.
- Barrett, E. S., Parlett, L. E., Sathyanarayana, S., et al. (2013). Prenatal exposure to stressful life events is associated with masculinized anogenital distance (agd) in female infants. *Physiology & Behavior*, 114–115, 14–20.
- Clark, M. M., Bishop, A. M., vom Saal, F. S., & Galef, B. G., Jr. (1993). Responsiveness to testosterone of male gerbils from known intrauterine positions. *Physiology & Behavior*, 53, 1183–1187.
- Clark, M. M., Crews, D., & Galef, B. G., Jr. (1991). Concentrations of sex steroid hormones in pregnant and fetal mongolian gerbils. *Physiology & Behavior*, 49, 239–243.
- Clark, M. M., & Galef, B. G., Jr. (1995). A gerbil dam's fetal intrauterine position affects the sex ratios of litters she gestates. *Physiology & Behavior*, 57, 297–299.
- Clark, M. M., vom Saal, F. S., & Galef, B. G. (1992). Intrauterine positions and testosterone levels of adult male gerbils are correlated. *Physiology & Behavior*, 51, 957–960.
- Clemens, L. G., Gladue, B., & Coniglio, L. (1978). Prenatal endogenous androgenic influences on masculine sexual behavior and genital morphology in male and female rats. *Hormones and Behavior*, 10, 40–53.
- Coe, B. L., Kirkpatrick, J. R., Taylor, J. A., & vom Saal, F. S. (2008). A new 'crowded uterine horn' mouse model for examining the relationship between foetal growth and adult obesity. *Basic & Clinical Pharmacology & Toxicology*, 102, 162–167.
- Cohen-Bendahan, C. C., Buitelaar, J. K., van Goozen, S. H., et al. (2005). Is there an effect of prenatal testosterone on aggression and other behavioral traits? A study comparing same-sex and opposite-sex twin girls. *Hormones and Behavior*, 47, 230–237.
- Colborn, T., Dumanosk, D., & Meyers, J. P. (1996). *Our stolen future: Are we threatening our fertility, intelligence, and survival? A scientific detective story*. New York: Dutton.
- Cowell, L. G., Crowder, L. B., & Kepler, T. B. (1998). Density-dependent prenatal androgen exposure as an endogenous mechanism for the generation of cycles in small mammal populations. *Journal of Theoretical Biology*, 190, 93–106.
- Drickamer, L. C., Arthur, R. D., & Rosenthal, T. L. (1997). Conception failure in swine: Importance of the sex ratio of a female's birth litter and tests of other factors. *Journal of Animal Science*, 75, 2192–2196.
- Even, M. D., Dhar, M. G., & vom Saal, F. S. (1992). Transport of steroids between fetuses via amniotic fluid in relation to the intrauterine position phenomenon in rats. *Journal of Reproduction and Fertility*, 96, 709–716.
- Even, M. D., Laughlin, M. H., Krause, G. F., & vom Saal, F. S. (1994). Differences in blood flow to uterine segments and placentae in relation to sex, intrauterine location and side in pregnant rats. *Journal of Reproduction and Fertility*, 102, 245–252.
- Gonzales, G. F. (2001). Function of seminal vesicles and their role on male fertility. *Asian Journal of Andrology*, 3, 251–258.
- Gore, A. C., Chappell, V. A., Fenton, S. E., et al. (2015). EDC-2: The endocrine society's second scientific statement on endocrine-disrupting chemicals. *Endocrine Reviews*, 36, E1–E150.
- Gupta, C., & Goldman, A. S. (1986). The arachidonic acid cascade is involved in the masculinizing action of testosterone on embryonic external genitalia in mice. *Developmental Biology*, 83, 4346–4349.
- Heikkinen, T., Harila, V., Tapanainen, J. S., & Alvesalo, L. (2013). Masculinization of the eruption pattern of permanent mandibular canines in opposite sex twin girls. *American Journal of Physical Anthropology*, 151, 566–572.
- Henderson, B. A., & Berenbaum, S. A. (1997). Sex-typed play in opposite-sex twins. *Developmental Psychobiology*, 31, 115–123.
- Lummaa, V., Pettay, J. E., & Russell, A. F. (2007). Male twins reduce fitness of female co-twins in humans. *Proceedings of the National Academy of Sciences*, 104, 10915–10920.
- McFadden, D. (1993). A masculinizing effect on the auditory systems of human females having male co-twins. *Proceedings of the National Academy of Sciences*, 90, 11900–11904.
- Medland, S. E., Loehlin, J. C., Willemsen, G., et al. (2008). Males do not reduce the fitness of their female co-twins in contemporary samples. *Twin Research and Human Genetics*, 11, 481–487.
- Mendiola, J., Stahlhut, R. W., Jorgensen, N., et al. (2011). Shorter anogenital distance predicts poorer semen quality in young men in rochester, new york. *Environmental Health Perspectives*, 119, 958–963.
- Mobbs, C. V., Kannegieter, L. S., & Finch, C. E. (1985). Delayed anovulatory syndrome induced by estradiol in female c57bl/6j mice: Age-like neuroendocrine, but not ovarian, impairments. *Biology of Reproduction*, 32, 1010–1017.
- Nagel, S. C., vom Saal, F. S., & Welshons, W. V. (1999). Developmental effects of estrogenic chemicals are predicted by an in vitro assay incorporating modification of cell uptake by serum. *The Journal of Steroid Biochemistry and Molecular Biology*, 69, 343–357.
- Nonneman, D. J., Ganjam, V. K., Welshons, W. V., & Vom Saal, F. S. (1992). Intrauterine position effects on steroid metabolism and steroid receptors of reproductive organs in male mice. *Biology of Reproduction*, 47, 723–729.
- Peitz, B. (1988). Effects of seminal vesicle fluid components on sperm motility in the house mouse. *Journal of Reproduction and Fertility*, 83, 169–176.
- Resnick, S. M., Gottesman, I. I., & McGue, M. (1993). Sensation seeking in opposite-sex twins: An effect of prenatal hormones? *Behavior Genetics*, 23, 323–329.
- Reyes, F. I., Boroditsky, R. S., Winter, J. S., & Faiman, C. (1974). Studies on human sexual development. II. Fetal and maternal serum gonadotropin and sex steroid concentrations. *The Journal of Clinical Endocrinology and Metabolism*, 38, 612–617.
- Rohde Parfet, K. A., Lamberson, W. R., Rieke, A. R., et al. (1990). Intrauterine position effects in male and female swine: Subsequent survivability, growth rate, morphology and semen characteristics. *Journal of Animal Science*, 68, 179–185.
- Rose, R. J., Kaprio, J., Winter, T., et al. (2002). Femininity and fertility in sisters with twin brothers: Prenatal androgenization? Cross-sex socialization? *Psychological Science*, 13, 263–267.
- Ryan, B. C., & Vandenbergh, J. G. (2002). Intrauterine position effects. *Neuroscience and Biobehavioral Reviews*, 26, 665–678.
- Slutske, W. S., Bascom, E. N., Meier, M. H., et al. (2011). Sensation seeking in females from opposite- versus same-sex twin pairs: Hormone transfer or sibling imitation? *Behavior Genetics*, 41, 533–542.
- Svare, B., Kinsley, C. H., Mann, M. A., & Broida, J. (1984). Infanticide: Accounting for genetic variation in mice. *Physiology & Behavior*, 33, 137–152.
- Taylor, J. A., Richter, C. A., Ruhlen, R. L., & vom Saal, F. S. (2011). Estrogenic environmental chemicals and drugs: Mechanisms for effects on the developing male urogenital system. *The Journal of Steroid Biochemistry and Molecular Biology*, 127, 83–95.
- Timms, B. G., Petersen, S. L., & vom Saal, F. S. (1999). Prostate gland growth during development is stimulated in both male and female rat fetuses by intrauterine proximity to female fetuses. *The Journal of Urology*, 161, 1694–1701.
- Timms, B. G., Peterson, R. E., & vom Saal, F. S. (2002). 2,3,7,8-tetrachlorodibenzo-p-dioxin interacts with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses. *Toxicological Sciences*, 67, 264–274.
- Vandenberg, L. N., Colborn, T., Hayes, T. B., et al. (2012). Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*, 33, 378–455.
- Vandenberg, L. N., Maffini, M. V., Wadia, P. R., et al. (2007). Exposure to environmentally relevant doses of the xenoestrogen bisphenol-a alters development of the fetal mouse mammary gland. *Endocrinology*, 148, 116–127.
- Vandenbergh, J. G., & Huggett, C. L. (1994). Mother's prior intrauterine position affects the sex ratio of her offspring in house mice. *Proceedings of the National Academy of Sciences*, 91, 11055–11059.
- vom Saal, F. S. (1981). Variation in phenotype due to random intrauterine positioning of male and female fetuses in rodents. *Journal of Reproduction and Fertility*, 62, 633–650.
- vom Saal, F. S. (1984). The intrauterine position phenomenon: Effects on physiology, aggressive behavior and population dynamics in house mice. *Progress in Clinical and Biological Research*, 169, 135–179.

- vom Saal, F. S. (1989). Sexual differentiation in litter-bearing mammals: Influence of sex of adjacent fetuses in utero. *Journal of Animal Science*, 67, 1824–1840.
- vom Saal, F. S. (2016). Triennial reproduction symposium: Environmental programming of reproduction during fetal life: Effects of intrauterine position and the endocrine disrupting chemical bisphenol a. *Journal of Animal Science*, 94, 2722–2736.
- vom Saal, F. S., & Bronson, F. H. (1978). In utero proximity of female mouse fetuses to males: Effect on reproductive performance during later life. *Biology of Reproduction*, 19, 842–853.
- vom Saal, F. S., Clark, M. M., Galef, B. G., et al. (1999). The intrauterine position (IUP) phenomenon. In E. Knobil, & J. Neill (Eds.), *vol. 2. Encyclopedia of reproduction* (pp. 893–900). New York: Academic Press.
- vom Saal, F. S., & Dhar, M. G. (1992). Blood flow in the uterine loop artery and loop vein is bidirectional in the mouse: Implications for transport of steroids between fetuses. *Physiology & Behavior*, 52, 163–171.
- vom Saal, F. S., Montano, M. M., & Wang, M. H. (1992). Sexual differentiation in mammals. In T. Colborn, & C. Clement (Eds.), *21. Chemically induced alterations in sexual and functional development: The wildlife/human connection* (pp. 17–83). Princeton: Princeton Scientific Publishing Co.
- vom Saal, F. S., Timms, B. G., Montano, M. M., et al. (1997). Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proceedings of the National Academy of Sciences*, 94, 2056–2061.
- vom Saal, F. S., Vandevoort, C. A., Taylor, J. A., et al. (2014). Bisphenol a (bpa) pharmacokinetics with daily oral bolus or continuous exposure via silastic capsules in pregnant rhesus monkeys: Relevance for human exposures. *Reproductive Toxicology*, 45, 105–116.
- Welshons, W. V., Thayer, K. A., Judy, B. M., et al. (2003). Large effects from small exposures. I. Mechanisms for endocrine-disrupting chemicals with estrogenic activity. *Environmental Health Perspectives*, 111, 994–1006.
- Zambrano, E., Guzman, C., Rodriguez-Gonzalez, G. L., et al. (2014). Fetal programming of sexual development and reproductive function. *Molecular and Cellular Endocrinology*, 382, 538–549.

COMPARATIVE MAMMALIAN FEMALE REPRODUCTION

Metatheria: Marsupials

Marilyn B Renfree and Geoffrey Shaw, The University of Melbourne School of BioSciences, Victoria, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Altricial young A new born young that is relatively undeveloped at birth, although particular features may be more advanced.

Embryonic diapause A regulated, reversible slowing or halt in embryonic development at the blastocyst stage.

Macropodid A member of the kangaroo and wallaby family of marsupials.

Pouch An abdominal fold of skin around the mammary areas on a marsupial.

Semelparity Having a single breeding cycle in a lifetime.

Introduction

Therian mammals (**Fig. 1**) consist of the marsupials and eutherian mammals that diverged from each other about 160 million years ago. There are about 340 living species (**Table 1**), representing about 6% of the world's mammalian fauna. Extant marsupials are found in Australasia and South America, with only one species, the opossum, extending into North America. They are a diverse group occupying almost every ecological niche from the tropical rainforest to alpine to desert, with aquatic, arboreal including gliders, and terrestrial herbivores, omnivores and carnivores (**Tyndale-Biscoe and Renfree, 1987**).



Fig. 1 Tammar wallaby (*Macropus eugenii*) female with a large, nearly weaned pouch young.

Table 1 Marsupial orders

<i>Marsupial Families</i>	<i>No. of species</i>
Didelphidae: American opossums	103 species
Caenolestidae: Shrew opossums	7 species
Microbiotheridae: Monito del Monte	1 species
Dasyuridae: Native cats, marsupial mice	75 species
Peramelidae: Bandicoots	19 species
Burramyidae: Pygmy possums	5 species
Acrobatidae:	2 species
Tarsipedidae: Honey possum	1 species
Petauridae: Gliding possums	11 species
Pseudocheiridae: Ringtail possums	17 species
Phalangeridae: Possums	28 species
Phascolarctidae: Koala	1 species
Vombatidae: wombats	3 species
Macropodidae: kangaroos, wallabies and rat kangaroos	65 species

**Fig. 2** Neonatal tammar wallaby attached to a teat in the pouch.

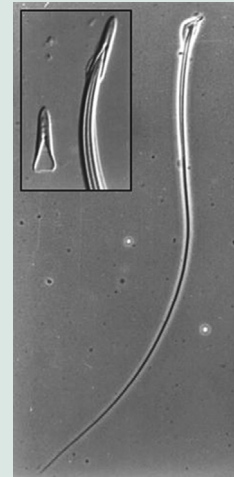
Marsupials are most clearly distinguished from the eutherian mammals by their mode of reproduction (Renfree, 2006). The new-born young are altricial, relatively under-developed, born at a stage when most eutherians are still fetuses in the uterus. Those features essential for post-natal survival, such as a digestive tract, respiratory system, and functional forelimbs needed to climb to the pouch, are disproportionately well developed at birth (Fig. 2). However, by the end of the long lactation, marsupial young are as well developed as equivalent eutherian mammals at weaning. Their reproduction has many remarkable features (Box 1).

Reproductive Anatomy

A difference between eutherian mammals and marsupials in the early development of the urogenital ducts in the embryo has had profound effects on the anatomy of the adult male and female reproductive tracts. In marsupials, the ureters enter the bladder medially. In females, this keeps the paired female genital ducts apart, while in eutherians they enter laterally so that the paired genital ducts can fuse to form a single vagina (Fig. 3). As a result, female marsupials have a complex vaginal structure with paired lateral vaginae and a new connective tissue strand medially which forms the birth canal or median vagina (Figs. 3 and 4(a)).

Box 1 Marsupial record holders

In the mammalian reproductive Olympiads marsupials hold many records. The diminutive (8–10 g) honey possum, *Tarsipes rostratus* (left), has the most gold medals. Almost five percent of their body is testes (the human equivalent is a man with two footballs in his scrotum!). Perhaps they need this size to accommodate the largest of mammalian sperm –360 μm long (right). Despite these 2 records, females are socially dominant, yet they give birth to the smallest mammalian baby, weighing in at less than 5 mg. They may have the shortest period of organogenesis (conclusive data is not available), but the shortest established period of organogenesis in any mammal is 64 h in another marsupial species, the Tasmanian devil, *Sarcophilus harrisii*, at 64h (Fig.10). Another marsupial the bandicoot, may have the shortest recorded gestation length –12.5 days (Fig.10). As more is discovered about these remarkable mammals there are bound to be more records broken.



Source: Left panel, M.B. Renfree; Right panel, J.M. Cummins.

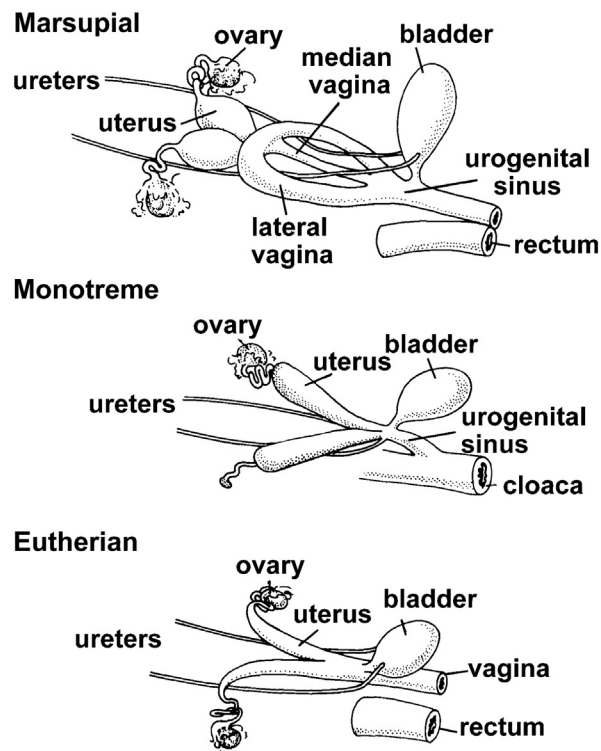


Fig. 3 Comparison of the urogenital tracts of marsupials, monotremes and eutherians. Note the relative location of the ureters, which prevents the fusion of the embryonic paired reproductive ducts to form a single vagina as in eutherians. The vaginae fuse cranially to form the anterior vaginal expansion, and a connective tissue strand between this and the urogenital sinus opens to form the median vagina, or birth canal, at first birth. Redrawn from Renfree, M.B., 1993. Ontogeny, genetic control and phylogeny of female reproduction in monotreme and therian mammals. In: Szalay, F.S., Novacek, J.J., McKenna, M.C. (Eds.), *Mammal Phylogeny*. Springer-Verlag, New York. pp. 4–20.

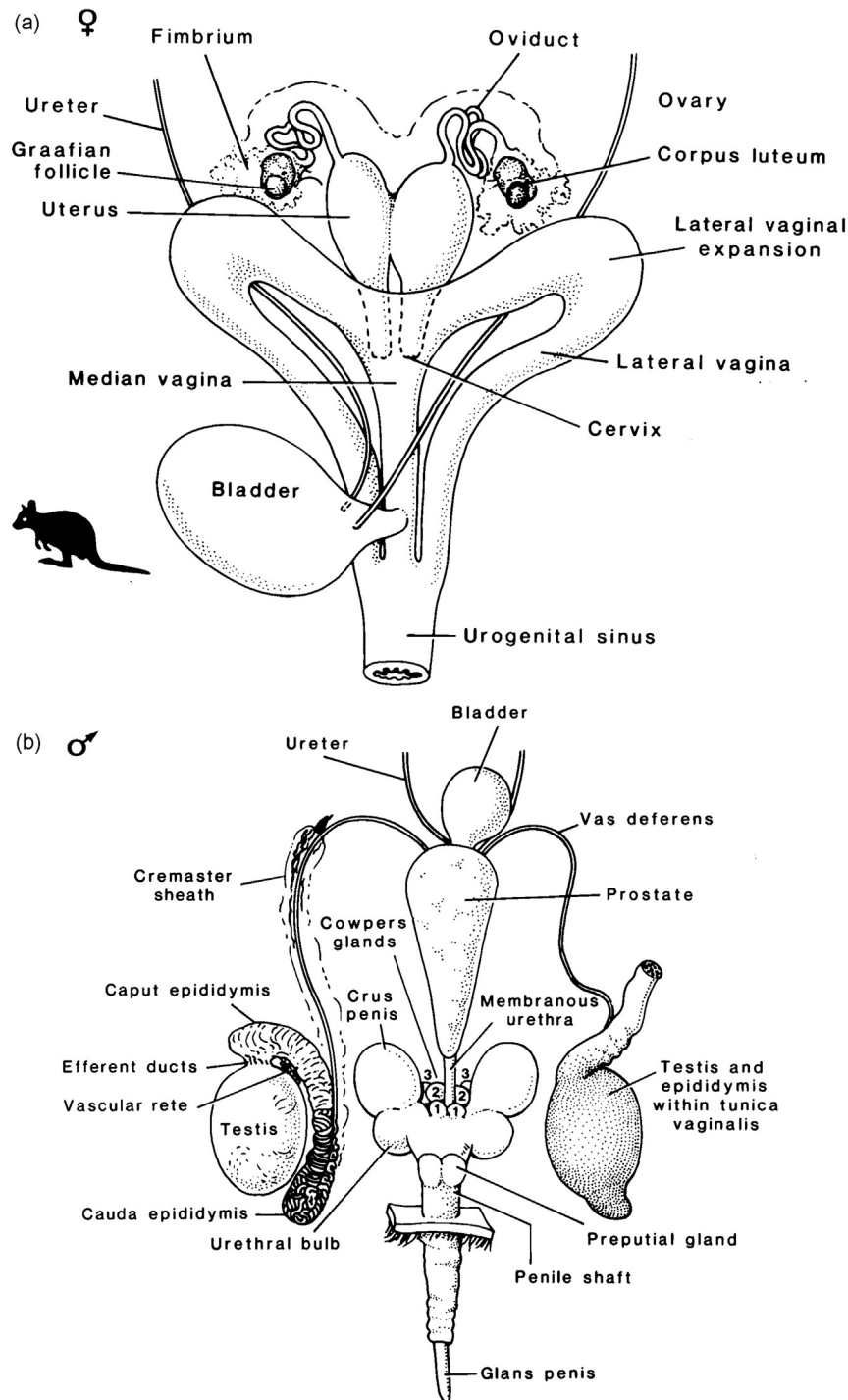


Fig. 4 Urogenital tracts of typical male and female marsupials. In females (a) the two uteri are completely separate, and have separate cervixes opening into the anterior vaginal expansion. Male (b) lacks seminal vesicles and coagulating glands, but has a large prostate and Cowper's glands. In most species the adult testes lie in a pendulous scrotum on the abdomen cranial to the penis, which when erect extends from the common urogenital opening. Redrawn from Renfree, M.B., 1994. Endocrinology of pregnancy, parturition and Lactation of marsupials. In: G.E. Lamming (Ed.), *Marshall's Physiology of Reproduction*, fourth ed. vol. 3: Pregnancy and lactation. Churchill Livingstone Edinburgh, London. pp. 677–766.

The characteristic that gives marsupials their name, the pouch, is not found in all marsupials. In no species does the male have a pouch, and pouches are also absent in females of some didelphids, caenolestids (South American) and dasyurids (Australian).

Male reproductive anatomy is similar to that of eutherians and the testis descends to the scrotum in most species, but the scrotum is always cranial to the penis (Fig. 4(b)). There are no seminal vesicles, ampullae or coagulating glands, but the vasa

deferentia enter into the anterior end of a large carrot-shaped prostate. The sperm undergo maturation in the epididymis. In the American marsupials, with the exception of one species, *Dromiciops*, the spermatozoa form into pairs in the epididymis by intimate association of the acrosomes. Sperm pairs separate in the oviduct and fertilisation is achieved by a single sperm. All Australian species have single sperm.

In marsupials there is a single external urogenital opening which is similar in appearance in both males and females. However, the female opening is not a cloaca, and there are two sphincters, one for the urogenital opening and the other for the rectum. Unlike in eutherians, in which the scrotum differentiates through fusion of the labio-scrotal folds behind the phallus, the marsupial scrotum forms on the abdomen anterior to the phallus. The pouch and scrotum are probably not homologous but arise from adjacent morphogenetic fields on the abdomen. Some marsupials such as the marsupial mole have inguinal testes, but none are testicond.

Patterns of Reproduction

Most marsupials are seasonal breeders, with reproduction timed so that the emergence of the young from the pouch coincides with the most favourable time of year. Photoperiod, nutrition, temperature and other factors may play a role. Some small dasyurid species are semelparous and the males die off after the breeding season (Fig. 5).

Marsupial reproductive patterns fall into 4 major groups.

- Group 1: Polyovular, polyoestrous species in which gestation occupying less than 60% of the oestrous cycle coinciding with the luteal phase. Oestrus and ovulation are suppressed during lactation (e.g., possums).
- Group 2: Polyovular, polyoestrous, with ultra-short gestation occupying less than the luteal phase which is prolonged into lactation (e.g., bandicoots, opossums).
- Group 3: Monoovular, polyoestrous species with gestation extending into the follicular phase, and gestation occupying 94%–109% of the oestrous cycle length. Postpartum oestrous occurs with lactation controlled diapause in most species (kangaroos, wallabies and rat-kangaroos).
- Group 4: Polyovular, polyoestrous with a very prolonged pre-luteal phase and gestation which includes a long period of embryonic diapause after *post partum* oestrus (e.g., honey possum, some small possums).

In all species, pregnancy is short relative to the length of lactation. The marsupial oestrous cycle varies from 22–42 days (mean across known species 28 days) (Fig. 6). The life of the corpus luteum is not prolonged by the presence of the conceptus, and because the length of gestation is shorter than the oestrous cycle in all but one species (the swamp wallaby, *Wallabia bicolor*), anatomical and endocrinological changes of pregnancy and the oestrous cycle are similar. This situation in marsupials gave rise to the term “pseudopregnancy”. This term was subsequently adopted by reproductive biologists in the 1920s to refer to the condition that follows sterile mating in the rabbit and rat. In most species, the young are born towards the end of the luteal phase so that the mothers return to oestrus is suppressed by the sucking of the neonate. By contrast, in the macropodids (kangaroos and wallabies) the single young is born at the end of the pro-oestrous phase and ovulation and fertilisation occur *post partum*. The resulting conceptus

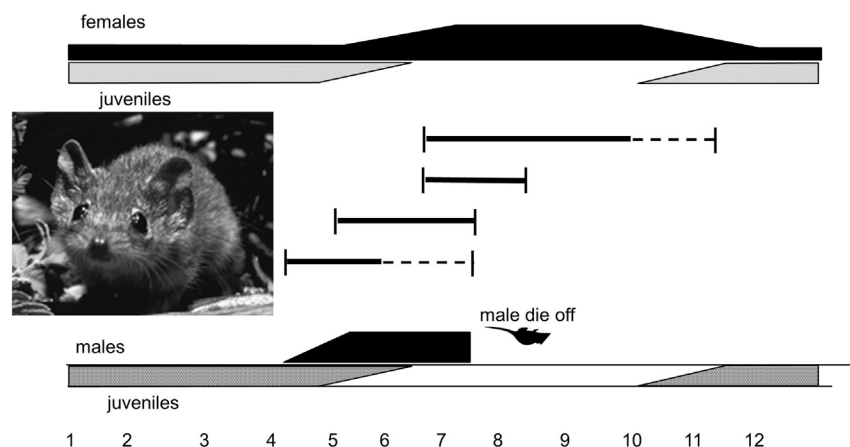


Fig. 5 Semelparity is a unique marsupial life-history strategy seen in some dasyurid species. The best known example is *Antechinus stuartii*. The young are weaned in summer (the timing varies in different locations in Australia), becoming sexually mature in late autumn. Over the next 3 months the males become increasingly territorial, and much fighting and agonistic behaviour occurs as females enter their breeding season. By the end of the breeding season, the males almost all die of stress related diseases. So that no males are alive by the time the females are having their litters. For about 2 months the young are permanently attached to the teats, and then for a further 2 months are left in a nest whilst their mother forages. The young are then weaned, and they disperse, becoming sexually mature before the next annual breeding cycle. Few females survive to breed for more than 2 years.

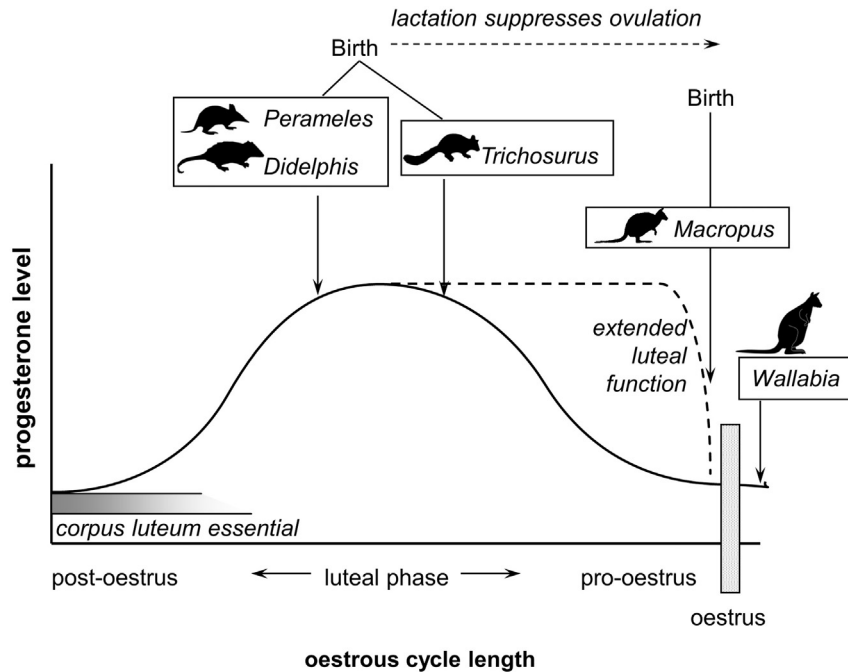


Fig. 6 Summary of the patterns of oestrous cycle and gestation in marsupials. In most species gestation is much shorter than the oestrous cycle, and the sucking stimulus from the newborn young in the pouch inhibits ovulation until after weaning. In most macropodids pregnancy is almost as long as the oestrous cycle, and the sucking stimulus does not suppress oestrus which can occur a few hours after birth. The resulting conceptus enters diapause, preventing problems of pouch occupancy by 2 young of different ages.

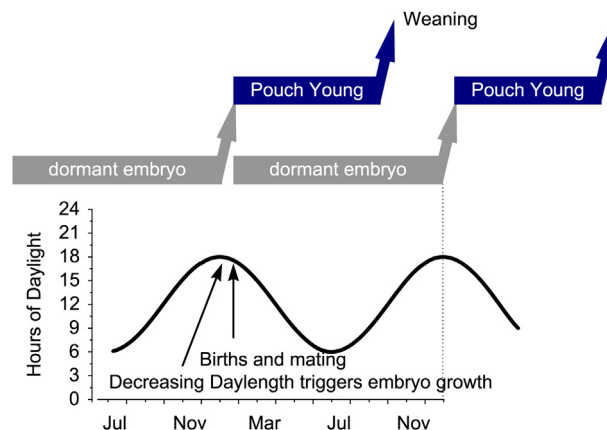


Fig. 7 Embryonic diapause in the tammar wallaby. In the breeding season – between the summer solstice and winter solstice – a sucking stimulus acts via a neural pathway to increase prolactin secretion. In the non-breeding season photoperiodic signals acting via melatonin produced in the pineal regulate prolactin. High prolactin levels prevent the CL from secreting progesterone. Removal of the sucking or seasonal inhibition, or treatment with prolactin inhibiting drugs allows progesterone to rise, stimulating the uterine secretions which allow the embryo to reactivate.

develops to an 80–100 cell blastocyst and then enters a dormant state known as embryonic diapause (Fig. 7). Embryonic diapause also occurs in some small possums.

Diapause is maintained by lactation, which stimulates prolactin release from the pituitary, which in turn inhibits the development of the corpus luteum. When the young weans, or if the young is prematurely lost, prolactin falls allowing the CL to reactivate. Luteal progesterone then stimulates the uterus to reactivate the embryo (see Fig. 7). The corpus luteum, although held in quiescence by prolactin itself, prevents a new ovulation in the opposite ovary.

Pregnancy

The endocrinology of reproduction in marsupials is broadly similar to that of eutherian mammals, but with some notable differences. The CL functions autonomously, and does not require pituitary support through pregnancy, although the pituitary is needed to supply gonadotrophins for follicular development. The corpus luteum secretes progesterone throughout the gestation period, but in the 3 species in which it has been investigated, progesterone is not needed for the maintenance of pregnancy after blastocyst expansion has been initiated, even though there little or no placental progesterone secretion. However it is needed for birth (see section **Parturition**) (Renfree and Shaw, 1996). In the macropodids, reactivation from diapause is associated with a rise in plasma progesterone concentrations with a peak about day 5 (depending on species) after removal of the pouch young (Renfree and Shaw, 2000, 2014; Renfree and Fenelon, 2017). Progesterone levels then drop, rising again to a plateau between about day 10 and 26, with a precipitous fall at birth.

Embryology

Marsupial oocytes are relatively large compared to those of eutherian ranging from about 150 to 250 μm diameter. As well as the primary plasma membrane the egg has a zona pellucida secreted in the developing follicle, and by tertiary membranes – the mucoid coat and a keratinous shell membrane secreted by the oviduct and uterus (Fig. 8). Sperm are rapidly transported up the lateral vaginae, not the median vagina, and reach the ampulla within 2–5 h. Fertilisation occurs in the oviduct, and passage to the uterus is rapid (less than 48 h).

Cleavage proceeds, without a morula stage, to the formation of a unilaminar blastocyst without an inner cell mass (Frankenberg *et al.*, 2013, 2016). Formation of the primitive streak occurs on the surface of the embryonic vesicle in a manner akin to the avian embryo. Duration of organogenesis is short and relatively constant (Figs. 9 and 10), even in species of widely differing body masses, ranging from 4 days in the dasyurid *Antechinus* to about 10 days in macropodids.

Molecular studies of early development in marsupials provide an interesting contrast to eutherian mammals. Whilst the blastocyst lacks an inner cell mass, distinct subpopulations of cells become distinguished by upregulation of lineage specific transcription factors such as POU2 and POU5F1 to form a cell population with similar developmental fate to the inner cell mass of eutherians. Patterns of expression and localisation of CDX2, YAP and WWTR1 differed significantly from the mouse but had similarities with humans and cattle (Frankenberg *et al.*, 2013, 2016). These studies are revealing considerable plasticity in the mechanisms underlying early lineage specification in mammals.

Marsupials are placental mammals with well-developed placental exchange mechanisms, although these are only established in about the last third of gestation (Renfree, 2010; Guernsey *et al.*, 2017). Most marsupials have a choriovitelline (yolk sac) placenta but some have an invasive chorioallantoic placenta as well. Four major placental types have been recognised ranging from those in which the allantois remains closed in the folds of the yolk sac (kangaroos, wallabies, possums and opossums), to those in which the



Fig. 8 Marsupial egg coats. The zona pellucida forms in the follicle. The mucoid coat and shell are laid down in the oviduct and the uterus. Sperm are visible embedded in the mucoid coat.

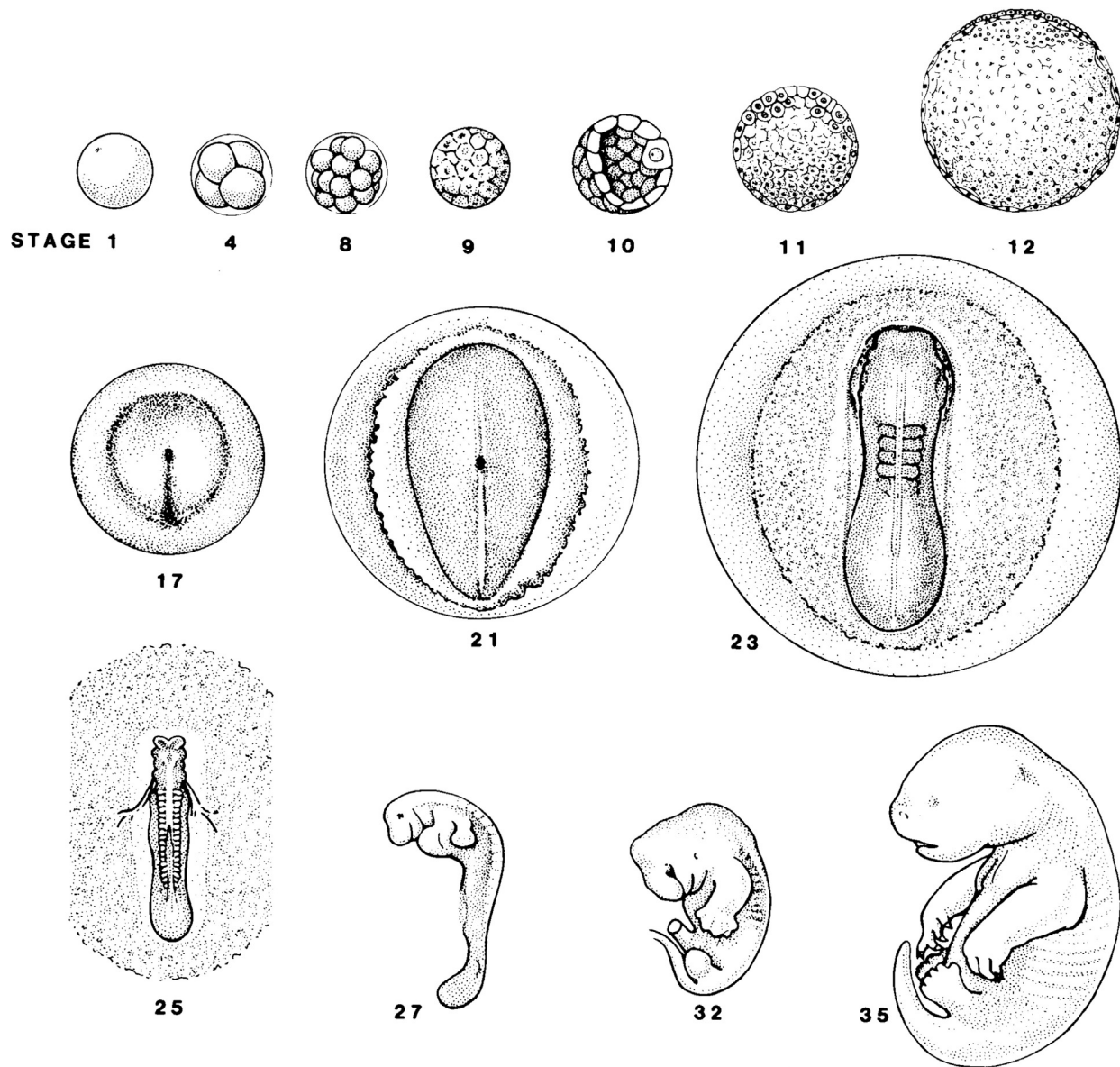


Fig. 9 Representative stages in the embryology of the opossum. Note the lack of an inner cell mass, superficial development, and the relative development of the forelimbs of the term fetus (stage 35). (Reproduced from Renfree, M.B., 1994. Endocrinology of pregnancy, parturition and Lactation of marsupials. In: G.E. Lamming (Ed.), Marshall's Physiology of Reproduction, fourth ed. vol. 3: Pregnancy and lactation. Churchill Livingstone Edinburgh, London. pp. 677–766).

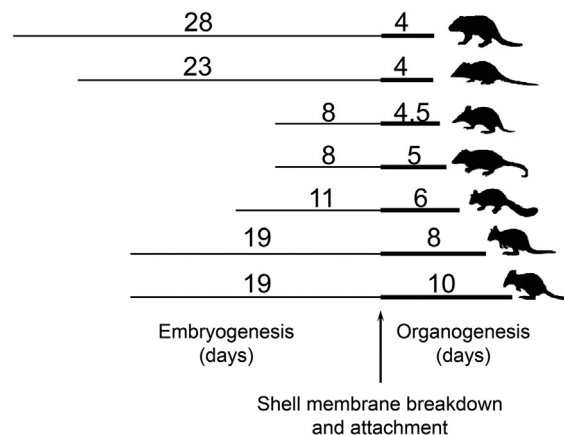


Fig. 10 Timing of embryogenesis and organogenesis in some marsupials. The species represented are (top to bottom) Tasmanian devil, Antechinus, bandicoot, American opossum, brush tail possum, tammar wallaby, potoroo.

allantois forms a discoid with a long umbilical cord in addition to the choriovitelline placenta. All bandicoots have this invasive placentation in which maternal and fetal cells fuse to form a syncytium. The placenta maintains a nutritive, excretory and metabolic function during gestation. As in eutherian mammals, genomic imprinting of several critical growth factor genes is essential for proper placental function and embryonic development. While the placenta of the tammar wallaby has minimal steroid synthetic activity, it does have considerable capacity for prostaglandin synthesis and also expresses other hormones including prolactin, relaxin, LH β , GH, GH-R and IGF2. The compositions of the yolk sac, amniotic and allantoic fluids are unique to each compartment, and the yolk sac contains high concentrations of cortisol at term.

Parturition

Despite the small size, a fetal signal is necessary to trigger birth as in many eutherians. The mechanisms involved have been investigated in very few species, but the best known is the tammar wallaby (Fig. 11; Renfree, 1994; Shaw and Renfree, 2006).

Labour is brief, and coincides with the onset of a characteristic parturient behaviour which is controlled by prostaglandins (Fig. 12). After the fetuses are expelled from the urogenital sinus they climb rapidly up to the pouch (or abdomen in pouchless forms), where they attach to a teat.

The neonate is altricial. Many organs are poorly developed, but the forelimbs, mouth, digestive tract and lungs are comparatively well developed and functional to permit the neonate to climb to the teat, attach, and digest milk (Fig. 2).

Lactation

Lactation is relatively prolonged due to the immaturity of the neonate. Three phases of lactation are recognised, Stages I and II being the equivalent of lactogenesis stage I of Eutheria. There are dynamic changes in milk composition throughout the whole of lactation that correlate with changes in needs of the young and ultimately control its growth and development.

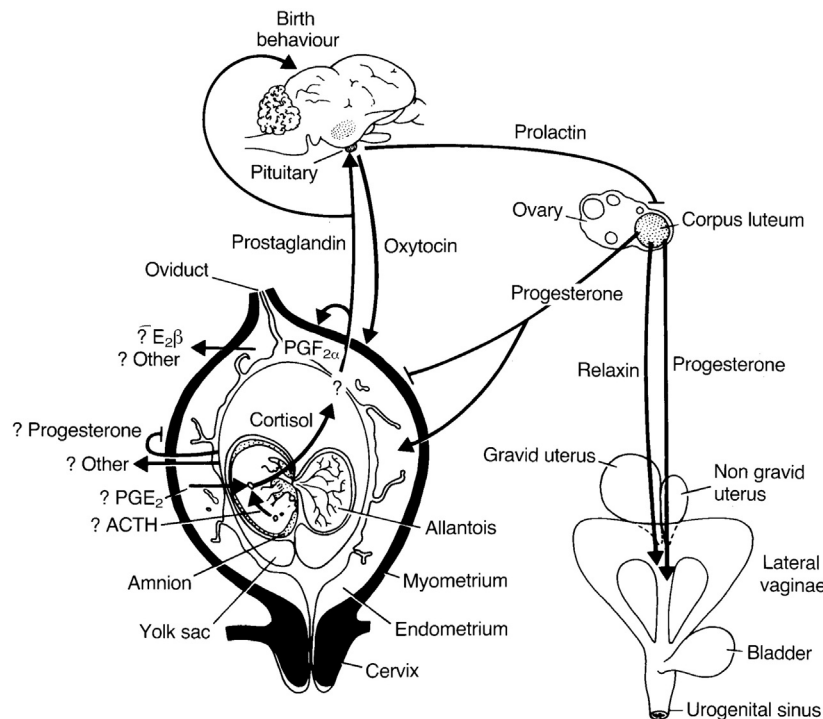


Fig. 11 Hypothetical model for the endocrine control of parturition in the tammar. Near term prostaglandin production in the endometrium and placenta increases. There is also a sharp rise in myometrial receptors for oxytocin. Relaxin, together with progesterone and possibly prostaglandins cause cervical softening and preparation of the birth canal. An increase in cortisol production by the fetal adrenal may stimulate placental or uterine PG production may initiate contractions. PG also acts centrally to initiate parturient behaviour, and induce release of oxytocin (or mesotocin) which further stimulates uterine contractions. The interactions between these hormones sets up a positive feedback cycle that ensures rapid evacuation of the uterus and delivery of the fetus. (Reproduced from Renfree, M.B., 1994. Endocrinology of pregnancy, parturition and Lactation of marsupials. In: G.E. Lamming (Ed.), *Marshall's Physiology of Reproduction*, fourth ed. vol. 3: Pregnancy and lactation. Churchill Livingstone Edinburgh, London. pp. 677–766).



Fig. 12 Tammar wallaby in the characteristic birth posture which is adopted minutes before delivery. The young must climb from the urogenital opening to the pouch in order to survive (photo by G. Shaw).

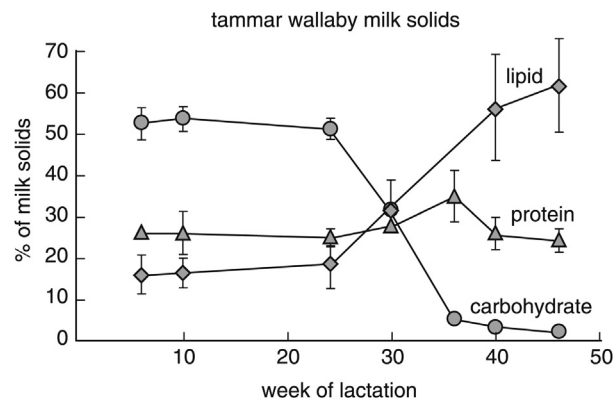


Fig. 13 Changes in milk composition through lactation in a marsupial. In many species of macropodid the decrease in sucking in late lactation terminates diapause, so a new young may be born and receive milk rich in carbohydrate from one gland, whilst the young at foot is getting milk rich in lipid and low in carbohydrate from an adjacent gland. This is known as concurrent asynchronous lactation. Redrawn from Green & Merchant, in Tyndale-Biscoe, C.H., Janssens, P.A. (Eds.), 1988. *The Developing Marsupial: Models for Biomedical Research*. Springer Verlag, Berlin.

In early lactation the milk has a high protein, low fat composition, while later in lactation there is a high fat, low protein milk (Fig. 13). The particular proteins, amino acids, fats and carbohydrates are all precisely regulated, and in some of the continuously breeding kangaroos, adjacent mammary glands supporting a small new young and a large young at foot produce milk of different compositions, a phenomenon known as concurrent asynchronous lactation.

Studies in which young of various ages were cross-fostered to mothers at different stages of lactation have shown the critical importance of synchrony between developmental stage of the young and the milk composition it receives. Transferring a young to a mother at a later stage of lactation can accelerate both growth and development (Menzies *et al.*, 2012; Hetz *et al.*, 2015). Transfer to an earlier stage of lactation retards growth.

Milk contains many bioactive components including PDGF, IGFBP5, IGFBP1 and EGFL6. Cross fostering studies have highlighted the importance of milk bioactives in development of organs including lungs and stomach.

Development

The immaturity of the new-born marsupial makes it an excellent model for some developmental studies (Pask and Renfree, 2010; Graves and Renfree, 2013; Grant *et al.*, 2012; Renfree *et al.*, 2011). One that has received substantial attention is sexual differentiation, which occurs mostly post-natally. Most aspects are similar to eutherians. They have a homologue of *SRY*, the testis determining gene on the Y chromosome. The differentiating testes secrete AMH and androgens which control virilisation. A notable exception is in the control of the pouch, mammary glands and scrotum (Renfree *et al.*, 2014a). In the tammar wallaby, where most information is available, mammary anlagen develop only in females and scrotal bulges form only in males. These sexually dimorphic structures form at least 4 days before gonadal differentiation, independent of gonadal hormones, as a result of as yet unidentified gene or genes on the X-chromosome. The presence of one X leads to scrotal differentiation, whilst possession of 2 X chromosomes leads to mammary gland (and pouch formation in pouched marsupials) (Figs. 13 and 14). Thus an XO marsupial

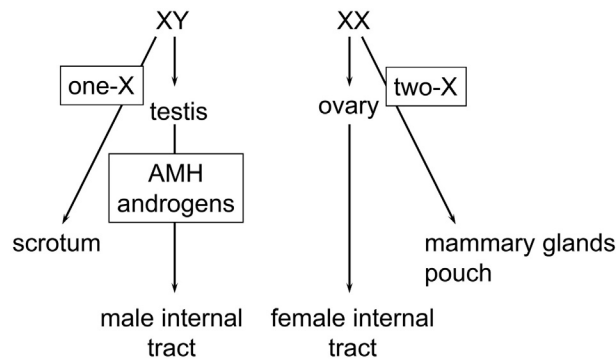


Fig. 14 Control of sexual differentiation in marsupials. As in eutherian mammals, the Y-chromosomal gene SRY induces the gonads to form testes and secrete MIS and testosterone, which virilise the internal reproductive tract. In the absence of a Y-chromosome, female gonads form into ovaries, which produce neither testosterone nor MIS, allowing a female internal tract to form. However, the sex-specific differentiation of the mammary anlagen and scrotum is not dependent on SRY nor gonadal hormones. Instead it is regulated by the number of X chromosomes present.

intersex has streak gonads, female internal genitalia, but an empty scrotum rather than a pouch and mammary glands, and an XXY marsupial intersex has a pouch and a penis but no scrotum.

The development of the internal genitalia and phallus is dependent on androgens but the differentiation of gonads, prostate and phallus occurs post-natally and separated in time, making them amenable to experimental manipulation. This allowed endocrine studies that revealed a new synthetic pathway for androgen in tammar wallabies that was subsequently found to be important in sexual development in other mammals including humans and clarifies previously unexplained cases of virilisation in girls with congenital adrenal hypoplasia and 21-hydroxylase deficiency (Wilson *et al.*, 2003).

The postnatal gonadal differentiation has allowed investigation of sex reversal in mammals (Renfree *et al.*, 2014b). In male tammar treated with oestrogen before testicular differentiation starts, both male somatic and germ cells can be sex reversed. Exogenous androgen treatment and castration of developing young at various stages demonstrated that virilisation depends on hormonal imprinting. Androgen action in specific windows of sensitivity preceding the start of morphological differentiation by days or weeks are needed for virilisation of the Wolffian ducts, prostate and especially phallus. Without androgens in these critical windows, for example after castration, virilisation fails leading to poor differentiation and in the case of the phallus, hypospadias. Androgen treatment of females through these windows of sensitivity leads to masculinisation.

X-chromosome inactivation also differs from that in Eutheria, in which X-inactivation is random in the fetus, but the paternal X is inactivated in the trophoblast. In marsupials, the paternal X is inactivated preferentially in both trophoblast and fetus, although the inactivation is tissue specific and leaky. The marsupial X-inactivation gene is a long non-coding RNA analogous to the eutherian *XIST* named *RSX* (RNA-on-the-silent-X). *RSX* is expressed only in females and, like *XIST*, coats the inactive X.

Summary

Marsupials are true mammals that have a distinctive reproductive physiology and developmental pathway. Their reproduction is characterised by short gestation with a fully functional placenta, delivering altricial neonates that depend on a long and sophisticated lactation. Females have a characteristic paired reproductive system and in males the scrotum is cranial not caudal to the penis. In most species of macropodids, and some possums there may be a period of embryonic diapause at the blastocyst stage. Embryonic diapause and early embryogenesis provide fascinating contrasts to eutherians, that are providing new clues to early development. Organogenesis is compressed into the last few days of gestation. The neonate is altricial, and undergoes prolonged development during a complex lactation, in most species held in a pouch. Studies of the control of milk production are leading to new insights into local regulation of mammary physiology, neonatal programming, and obesity. Striking differences in the timing of development have proved especially useful, and led to the demonstration that some aspects of somatic sexual differentiation are independent of gonadal hormones, but rather result from a direct effect of the sex-chromosomal genes. Genetic and genomic studies in marsupials are illuminating the evolution of fundamental processes such as genomic imprinting and X-inactivation that are so essential to mammalian reproduction and development. Because of their unique features, marsupials will continue to make a great contribution to the understanding of reproduction in all mammals.

References

- Frankenberg, S. R., de Barros, F. R. O., Rossant, J., & Renfree, M. B. (2016). The mammalian blastocyst. *Wiley Interdiscip Rev Dev Biol*, 5, 210–213.
- Frankenberg, S. R., Shaw, G., Freyer, C., Pask, A. J., & Renfree, M. B. (2013). Early cell lineage specification in a marsupial: A case for diverse mechanisms among mammals. *Development*, 140, 965–975.

- Grant, J., Mahadevaiah, S. K., Pavell, I. L., et al. (2012). *Rsx* is a metatherian RNA with *Xist*-like properties in X-chromosome inactivation. *Nature*, 487, 254–258.
- Graves, J. A. M., & Renfree, M. B. (2013). Marsupials in the age of genomics. *Annu Rev Genomics Hum Genet*, 14, 393–420.
- Guernsey, M. W., Chuong, E. B., Cornelis, G., Renfree, M. B., & Baker, J. C. (2017). Molecular conservation of marsupial and eutherian placentation and lactation. *eLife*, 6.
- Hetz, J. A., Menzies, B. R., Shaw, G., et al. (2015). Growth axis maturation is linked to nutrition, growth and developmental rate. *Mol Cell Endocrinol*, 411, 38–48.
- Menzies, B. R., Shaw, G., Fletcher, T. P., Pask, A. J., & Renfree, M. B. (2012). Maturation of the growth axis in marsupials occurs gradually during post-natal life and over an equivalent developmental stage relative to eutherian species. *Mol Cell Endocrinol*, 349, 189–194.
- Pask, A. J., & Renfree, M. B. (2010). Molecular regulation of marsupial reproduction and development. In J. E. Deakin, P. D. Waters, & J. A. M. Graves (Eds.), *Marsupial Genetics and Genomics* (pp. 285–316). Dordrecht, Heidelberg, London, New York: Springer.
- Renfree, M. B. (1994). Endocrinology of pregnancy, parturition and lactation of marsupials. In G. E. Lamming (Ed.), *Marshall's Physiology of Reproduction, fourth ed. vol. 3: Pregnancy and lactation* (pp. 677–766). Edinburgh, London: Churchill Livingstone.
- Renfree, M. B. (2006). Life in the pouch: Womb with a view. *Reprod Fertil Dev*, 18, 721–734.
- Renfree, M. B. (2010). Review: Marsupials: Placental mammals with a difference. *Placenta*, 31(Suppl), S21–S26.
- Renfree, M. B., Chew, K. Y., & Shaw, G. (2014a). Hormone-independent pathways of sexual differentiation. *Sex Dev*, 8, 327–336.
- Renfree, M. B., Chew, K. Y., & Shaw, G. (2014b). Inducing sex reversal of the urogenital system of marsupials. *Differentiation*, 87, 23–31.
- Renfree, M. B., & Fenelon, J. C. (2017). The enigma of embryonic diapause. *Development*, 144, 3199–3210.
- Renfree, M. B., Papenfuss, A. T., et al. (2011). Genome sequence of an Australian kangaroo, *Macropus eugenii*, provides insight into the evolution of mammalian reproduction and development. *Genome Biol*, 12, R81.
- Renfree, M. B., & Shaw, G. (1996). Reproduction of a marsupial: From uterus to pouch. *Anim Reprod Sci*, 42, 393–404.
- Renfree, M. B., & Shaw, G. (2000). Diapause. *Annu Rev Physiol*, 62, 353.
- Renfree, M. B., & Shaw, G. (2014). Embryo-endometrial interactions during early development after embryonic diapause in the marsupial tammar wallaby. *Int J Dev Biol*, 58, 175–181.
- Shaw, G., & Renfree, M. B. (2006). Parturition and perfect prematurity: Birth in marsupials. *Aust J Zool*, 54, 139–149.
- Tyndale-Biscoe, C. H., & Renfree, M. B. (1987). *Reproductive Physiology of Marsupials* (p. 476). Cambridge, UK: Cambridge University Press.
- Wilson, J. D., Leihy, M. W., Shaw, G., & Renfree, M. B. (2003). Unsolved problems in male physiology: Studies in a marsupial. *Mol Cell Endocrinol*, 211, 33–36.

Pig

Rodney D Geisert and Matthew C Lucy, University of Missouri, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Altrenogest A synthetic progestogen used to synchronize estrus in gilts.

Anestrous sow A sow that fails to come back into estrus after weaning.

Bicornuate uterus Uterus in which there are two long uterine horns (150–200 cm) connected by a common short uterine body (1 cm).

Blastocyst Early stage of embryo development containing an outer trophoblast with inner cell mass and fluid filled center called the blastocoel.

Conceptus Embryo and its extraembryonic placental membranes.

Corpus luteum (CL) Ovarian structure that is formed from the follicle after ovulation that synthesizes and secretes progesterone.

Cystic follicle Large follicle on the ovary that is incapable of ovulation.

Dystocia Difficult birth caused by a large or badly positioned fetus.

Epitheliochorial placentation A noninvasive type of placental attachment in which microvilli of placenta (chorion) and uterine surface epithelium interlock superficially at the maternal-fetal interface in the uterus.

Estrus Period of the estrous cycle when females are sexually active and receptive to mating by the male.

Farrowing rate Percentage of gilts or sows that become pregnant and farrow a litter after insemination.

Follicle Ovarian structure that contains the developing oocyte (egg) and also synthesizes and secretes estradiol to bring the pig into estrus.

Gilt Young female pig.

Luteolysis Functional and structural regression of the corpus luteum causing a decrease in blood progesterone concentrations.

Maternal recognition of pregnancy Period of early pregnancy when a chemical signal(s) produced by the developing conceptus prevents the regression of the corpus luteum to maintain the corpus luteum beyond the normal length of the estrous cycle.

Mesometrial The region of the uterus where the broad ligament containing maternal blood vessels attaches and enters into the uterus.

Morula Early embryo stage of development where multiple blastomeres form a solid ball of cells contained within the zona pellucida.

P.G. 600 A combination of a pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) that is used to bring prepubertal gilts and postpartum sows into estrus.

Polyestrous Females having multiple estrous cycles throughout the year.

Polytocous Females that give birth to multiple offspring at parturition.

Psuedopregnancy Condition of female where the corpus luteum lifespan is extended beyond the length of the estrous cycle without the presence of a fetus.

Puberty Period of adolescence when a male or female is first able to release gametes.

Sow Adult female pig that has given birth.

Introduction

The domestic pig (*Sus scrofa domesticus*) is a polyestrous, polytocous farm animal species. Modern breeds of domestic pigs typically breed year-round. The European wild boar and feral pigs, however, can display a seasonal (July to September) anestrus related to photoperiod and nutrient availability in their natural (wild) environment. General characteristics of reproduction for the domestic pig are presented in **Table 1**. Gilts (young female pigs before first farrowing) from commercial maternal lines reach puberty at 5–7 months of age and farrow their first litters when they are approximately 1 year old. The length of estrous cycle for gilts and sows (mature females that have given birth) is approximately 21 days (range 18–22 days) with estrus (standing heat) lasting from 24 to 72 h. Ovulation occurs approximately 30–40 h after the initiation of estrus. Gilts and sows are typically inseminated by either hand mating with a boar or by artificial insemination (AI) on the first and second day of estrus.

Gilts and sows ovulate from 20 to 30 ovarian follicles that are 8–10 mm in diameter when mature. Oocytes are released from the ovulatory follicles and transported into the oviduct for fertilization. The cervix of the pig has thick alternating interdigitating pads that lock around the spiral corkscrew shaped glans penis of the boar during mating (**Fig. 1**). Pipettes used for artificial insemination

Table 1 Characteristics of female reproduction in the domestic pig

Age at puberty	5–7 months
Estrous cycle length	18–22 days
Duration of estrus	24–72 h
Time of ovulation	30–40 h after the initiation of estrus; two-thirds to three-quarters of the time through estrus
Number of follicles ovulated	20–30
Gestation length	114–117 days
Age at first farrowing	12 months
Farrowing rate	>85%
Litter size	8–18
Still born rate	<7%
Lactation length	21–24 days
Weaning percentage (weaned/ live born)	>90%
Number of farrowings per sow per year	2.3–2.4
Number of piglets per sow per year	25–30
Number of litters before culling	3–4

(AI) lock into the spiraling cervical rings of the pig to replicate the anatomy of the boar penis. Compared with other farm species, the boar produces an extremely large volume of semen. Semen is diluted and used fresh for AI in pigs. The semen is infused into the cervix or uterus in a relatively large volume over a 5–10 min period.

A high percentage of pigs become pregnant after breeding. The uterus of the pig has a short uterine body (1 cm) with long uterine horns (150–200 cm) that support the extensive placentation of the conceptus (**Fig. 1**). Gestation is approximately 114 to 117 days and litter sizes are typically 8–18 piglets per litter with an average of approximately 12 piglets per litter. Sows are weaned 21–24 days after farrowing. After weaning sows return to estrus within 1 week and they are AI to reestablish pregnancy. The production cycle for

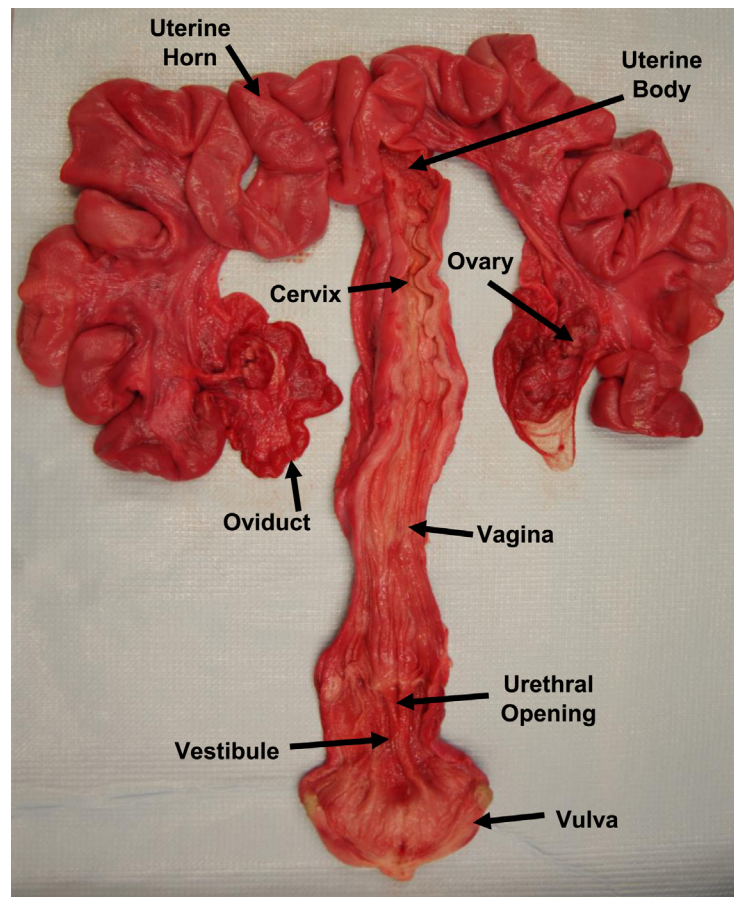


Fig. 1 Picture of pig bicornuate reproductive tract. Drawing by Lydia Michel and Sarah Petty.

a sow, therefore, is approximately 142 days (gestation+lactation+return to estrus). A highly productive sow, therefore, can farrow nearly 2.5 litter per year and produce over 30 piglets per year.

Puberty and the Estrous Cycle

Puberty

Gilts are born with an immature ovary that contains primordial, primary and secondary follicles. Ovarian follicular development depends on gonadotropins (follicle stimulating hormone (FSH) and luteinizing hormone (LH)) that arise from the anterior pituitary under the control of gonadotropin-releasing hormone (GnRH). Circulating concentrations of FSH are elevated until 10 weeks of age and provide an initial stimulus for ovarian follicular development. There is a gradual increase in the number of LH pulses per day with increasing age. The negative feedback effect of estradiol on LH secretion is also reduced over time. The ovary grows and there are successively larger ovarian follicles that secrete greater amounts of estradiol that in turn stimulate growth of the uterus. When ovarian follicles achieve a critical threshold of circulating estradiol then there is an LH surge that triggers first ovulation (puberty) at 5–7 months of age (80–120 kg of body weight). The age at puberty can be influenced by breed, season of the year, nutrition, social environment, and exposure to the boar. A delay in puberty has been observed in the summer months and also when young pigs are confined and crowded in indoor pens. Mixing gilts from different pens or transporting gilts in a truck or trailer can advance the age at puberty if gilts have adequate nutrition. Daily exposure of gilts to boars has a direct effect on advancing puberty in gilts that are between 135 and 160 day of age. Stimulation of puberty by the boar occurs through physical contact, vocalization, and secretion of musk-smelling compounds, 16-androstenes, that give the typical odor of mature boars. Boar odor provides signals to the female for inducing cyclicity and also the strong lordosis response during estrus. The first pubertal estrus in the gilt is generally less fertile with a lesser ovulation rate compared with subsequent estrous cycles. To achieve larger litters, therefore, gilts are typically inseminated at their second or third estrus.

Puberty can be advanced by treating gilts with a combination of pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) which is marketed under the trade name P.G. 600 (Merck Animal Health; Summit, New Jersey, United States). Prepubertal gilts should be older than 5.5 months of age before treatment. Gilts will show estrus 4–10 days after treatment (average=7 days). Gilts can be inseminated at their first estrus after P.G. 600 or can be inseminated at the next estrus which will increase farrowing rate and litter size.

Estrous Cycle

In domestic farm animals, the estrous cycle is defined as the number of days from initiation of behavior estrus expression to onset of a subsequent estrus. Endocrine changes during the estrous cycle are presented in Fig. 2. There are four stages of the estrous cycle: proestrus, estrus, metestrus, and diestrus. During *proestrus*, circulating concentrations of progesterone decline rapidly. The decrease in progesterone (regression of the corpus luteum) is caused by the episodic release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterine endometrium. The regression of the CL and concomitant decline in circulating progesterone is associated with an increase in the frequency of LH pulses that initiates a wave of follicular development. The development of large ovarian follicles increases the circulating concentrations of estradiol and inhibin. The increases in both estradiol and inhibin cause a decrease in circulating FSH

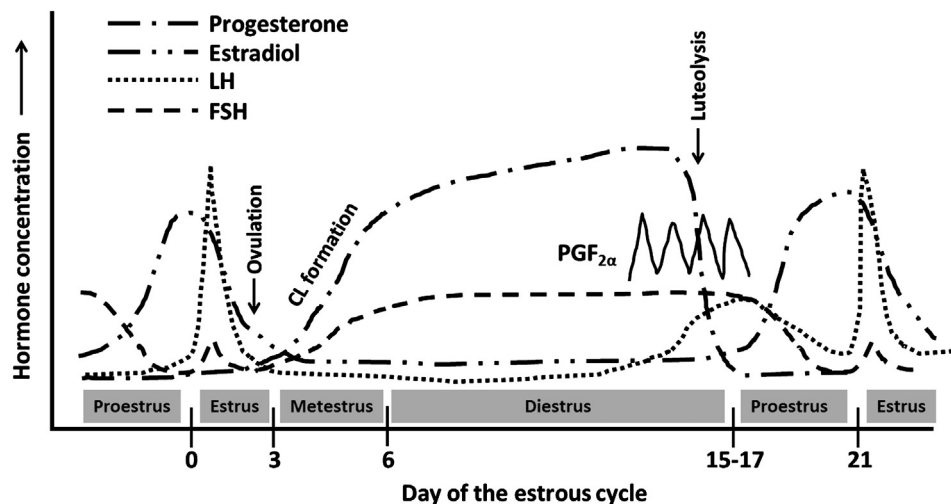


Fig. 2 Diagram of circulating hormone concentrations during the estrous cycle of the pig.

when the pig approaches estrus. High circulating concentrations of estradiol bring pigs into *estrus*; the next phase of the estrous cycle. Pigs display lordosis behavior or “standing heat” when they are in estrus meaning that they will stand still with erect ears when mounted by the boar or following the application of back pressure by a person. Other signs of estrus include swelling and reddening of the vulva, vocalization (estrual grunts), mounting other female pigs and boar-seeking behavior. In response to estradiol, the cervix becomes rigid and the uterine horns are tightly coiled.

The duration of estrus tends to be shorter in gilts (24–48 h) compared with mature sows (24–96 h). Circulating estradiol reaches peak concentrations near the onset of estrus and triggers the LH surge. The LH surge may occur before, during or after the initiation of estrus. High circulating LH concentrations last for about 10 h during the LH surge. Ovulation occurs during a 1–3 h period approximately 30–35 h after the LH surge. In general, ovulation occurs when the estrus period is 70% complete. The fact that the LH surge and ovulation is poorly timed with the onset of estrus makes the timing of AI difficult. Gilts and sows, therefore are typically inseminated two times during estrus and may be inseminated a third time if they are in estrus on day 3. A new animal drug named “OvuGel” (JBSUnited Animal Health; Sheridan, Indiana, United States) was recently introduced. OvuGel is a GnRH agonist that causes an LH surge with ovulation 40–48 h after treatment. OvuGel can be used in a program for single fixed time AI in sows after weaning.

Following ovulation, the granulosa cells and theca cells of the follicle wall become luteinized and form corpora lutea (CL). Initially blood fills the lumen of the ruptured follicle to form the corpus hemorrhagicum (CH) and the CH is eventually replaced by the CL. The *metestrus* phase of the estrous cycle follows as the CL grows, fills the ruptured follicle cavity of the CH, and increases its synthesis of progesterone. The presence of multiple CL (8–11 mm in diameter) on each ovary results in a steady increase in circulating progesterone. *Diestrus*, the period of maximal circulating progesterone follows metestrus and continues for approximately 2 weeks during the estrus cycle. Concentrations of progesterone greater than 30–40 ng/mL are achieved during diestrus. The episodic release of PGF_{2α} from the uterine endometrium and the regression of the CL defines the end of diestrus and beginning of the proestrus phase which leads back to estrus.

Synchronization of Estrus

Reproductive management of farm species typically involves protocols that are used to synchronize estrus in a group of females. Synchronization of estrus is done to reduce the time and labor associated with estrous detection and to facilitate the use of AI. Although exogenous PGF_{2α} is commonly used to regress the CL in cattle, it is not routinely used to synchronize estrus in gilts or sows because the CL does not respond to exogenous PGF_{2α} until after day 12 of the estrous cycle or pregnancy. Estrus in gilts can be synchronized by feeding altrenogest (a progestin) marketed under the trade name Matrix® (Merck Animal Health, Intervet Inc.; Summit, New Jersey, United States) at a dose of 15 mg/day for 14 day. The altrenogest suppresses follicular development so that a synchronous wave of follicular growth occurs in a group of gilts after feeding is stopped. Gilts typically express estrus 4–9 day after the last day of altrenogest feeding.

Breeding

Estrous detection is typically done twice daily using a boar. Gilts or sows that show an interest in the boar and that stand with erect ears when pressure is applied to the back are considered in estrus. Estrus lasts 2–3 days. The reproductive tract of the pig lacks a fornix vagina (blind pocket around the cervical opening) that is present in a number of other farm species (cow, mare and ewe) (Fig. 1). Hand mating of boars to sows or AI of gilts and sows is quite easily accomplished because the fornix vagina is not present. Hand mating or AI is usually done 12–24 h after first detection of estrus and then repeated on the next day. Gilts or sows that are in heat for a third day may be inseminated three times (once for each day in estrus). Artificial insemination is very typically done on swine farms because it conserves semen (allowing one boar to breed many females) and enables producers to purchase semen from superior boars for use on farm. When AI is used, semen is diluted to a volume of 50 mL in extender and a total of 2–3 billion sperm are used for each breeding. A plastic AI pipette attached to a bag of liquid semen is inserted into the vagina and threaded or inserted into the cervical rings. The semen is then slowly infused into the cervix over 5–10 min. Some AI pipettes lock into the cervix and a second catheter is fed through the cervix and into the uterine body for insemination. Intrauterine insemination catheters enable the use of one-half the number of sperm cells per insemination without compromising farrowing rate or litter size. A high percentage of oocytes are fertilized and most gilts or sows become pregnant after breeding (typically >85% of those inseminated). The expectation is that breeding will yield both a high percentage of pregnancies and also a large litter size (>11 piglets per litter).

Commercially raised females can exhibit a summertime decline in breeding efficiency (seasonal infertility) which is explained by the combined effect of heat stress and photoperiod. Typical signs of seasonal infertility are anestrus, weak or irregular expression of estrus, irregular estrous cycles, reduced farrowing rates, increased abortion rates, and depressed litter size. There are methods to mitigate heat stress on farm. For example, evaporative cooling systems will decrease the ambient temperature by 5–7 °C inside swine barns, depending on the outside temperature and relative humidity. These systems will reduce but not entirely eliminate the impact of seasonal infertility on the reproductive efficiency of swine farms.

Early Embryonic Development

Following ovulation, the sticky cumulus cells surrounding the oocytes assist adhesion and transport to the ampullary-isthmic junction (site of fertilization) of the oviduct through contraction of cilia lining the fimbria and ampulla luminal epithelium. Follicular growth and the release of estradiol stimulates oviductal contractions to move the oocytes to the site of fertilization. Estradiol stimulates a physical constriction of the isthmus lumen which restrains further movement until the uterus becomes receptive for embryo development. Fertilized ova undergo their first cleavage division approximately 24 h after fertilization. Cleavage continues with 4–8 cell embryos being rapidly transported into the uterine horns following progesterone induced relaxation and widening of the isthmus luminal diameter (Fig. 3). The embryos continue to divide near the tips of the uterine horns, developing into morula by Day 5 of gestation. The loose outer cells of the morula undergo compaction which is defined as the formation of tight cellular junctions. Polarization of the outer trophoblast allows sodium pumps located on the basal cell border to transport fluid into the center to form the blastocoel. The presence of the blastocoel is the defining characteristic of the blastocyst embryo which is found on Day 6–7 of gestation. Blastocysts continue to grow within the zona pellucida until production of enzymes from both the uterus and blastocyst and the physical expansion and contraction of the blastocyst stimulate hatching; the process through which the embryo breaks through the surrounding zona pellucida. Hatching occurs on Day 8 of gestation (Fig. 3).

From Days 8–12 of gestation, blastocysts begin to migrate down and between the uterine horns to equidistantly space themselves throughout uterus. Early uterine migration of blastocysts from the tip of the uterine horns is stimulated by blastocyst synthesis of prostaglandins that induce the release of histamine from the endometrium to stimulate local myometrial contractions. The spherical conceptuses continue to grow, migrate and complete spacing within the uterine horns through the production of estrogens (Fig. 4(A)). Conceptus migration and trophoblast expansion is essential because the pig has both a local and systemic pathway for PGF_{2α}-induced luteolysis (CL regression) which would reduce progesterone and terminate the pregnancy. The pig requires at least two conceptuses in each uterine horn to prevent luteolysis and maintain pregnancy.

The spherical conceptuses (Fig. 4(A)) steadily grow and increase in diameter from Day 10 (2–5 mm diameter) to 12 (8–10 mm diameter) of gestation. Once reaching an approximate 10 mm spherical diameter, conceptuses undergo a rapid transition in morphology to a thin filamentous tread-like structure measuring ~100 mm in length (Fig. 4(B)). The rapid morphological change during conceptus elongation occurs within approximately 1 h and results from massive cellular remodeling and movement rather than cellular growth and proliferation. Pig conceptuses synthesize a unique form of interleukin-1β (IL1B2) that is involved in regulating the rapid elongation of the conceptus. Gene edited *IL1B2* null embryos do not elongate. IL1B2 is a proinflammatory cytokine that stimulates a localized increase in blood flow to the uterus and regulates many endometrial responses to stimulate implantation and the maternal immune system.

Conceptus elongation provides the placental surface area for nutrient uptake that is essential for continued embryonic and fetal development to term. The pig endometrial surface is not a flat, smooth surface but has a substantial amount of primary and secondary folding (Fig. 4(C)). Conceptuses follow the folds and attach along the mesometrial border of the uterine horns.

Establishment of Pregnancy

Rapid conceptus elongation not only provides the surface area for later placental development and growth, but provides the mechanism to deliver conceptus estrogen (maternal recognition signal) across the endometrium to block luteolysis and maintain pregnancy. Two periods of conceptus estrogen production and release are involved in maintaining the CL throughout pregnancy in the pig. Conceptuses release estrogen during the process of elongation on Day 12 of gestation. This short pulse of estrogen production (1–2 days) extends the lifespan of the CL to 25–30 days. Although conceptus elongation normally occurs between Day 11–12, the window for elongation and uterine estrogen stimulation for establishment of pregnancy in the pig extends from Day 11–14 of gestation. A second sustained release of estrogen occurs during period of attachment and placental formation from Day 16–25 of gestation. The short (Day 12) and prolonged (Day 16–25) release of estrogen is essential for CL maintenance to term. Treatment

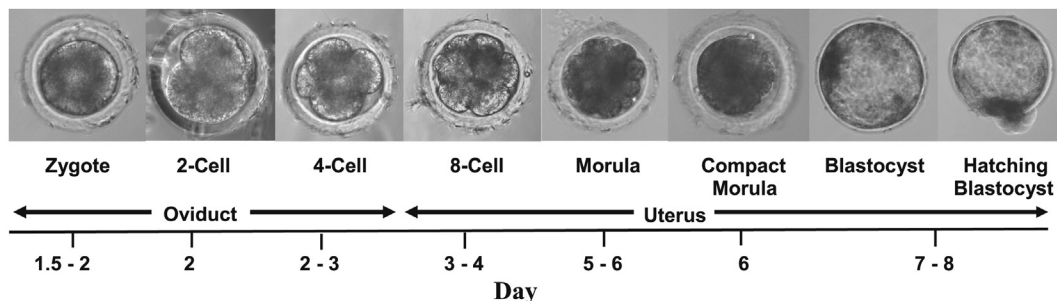


Fig. 3 Cleavage and early development of pig embryos from fertilization until blastocyst hatching. Day 0=time of ovulation.

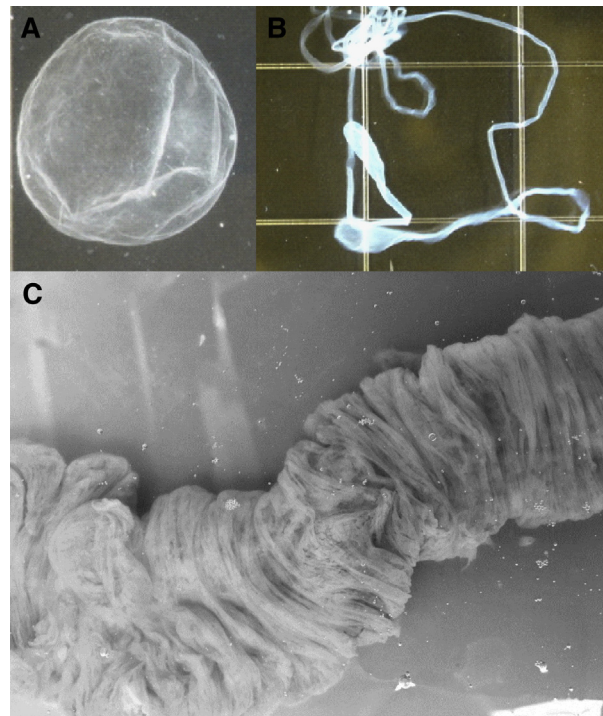


Fig. 4 Conceptus development from Day 11–12 of gestation. (A) Spherical conceptus (5 mm diameter) on Day 11 of gestation. (B) Elongated conceptus (100 mm) on Day 12 of gestation. (C) Segment of uterine horn demonstrating the multiple folding of the endometrium during placentation in the pig.

of cyclic gilts or sows with estrogen or environmental exposure to estrogenic compounds such as aflatoxins induces pseudopregnancy (extended lifespan of CL without conceptuses present) which can range from 28 to 110 days.

Conceptus secreted estrogens do not function to prevent luteolysis through inhibition of endometrial $\text{PGF}_{2\alpha}$ synthesis and release as occurs in a number of large farm animal species. In the pregnant pig, estrogen alters endometrial $\text{PGF}_{2\alpha}$ movement to redirect the $\text{PGF}_{2\alpha}$ away from the CL. The $\text{PGF}_{2\alpha}$ is sequestered into the uterine lumen and metabolized rather than being transported into the uterine capillaries to induce luteolysis as occurs during the estrous cycle. Pig conceptuses secrete PGE which also assists in sequestering $\text{PGF}_{2\alpha}$ into the uterine lumen and stimulates CL progesterone production and maintenance during early gestation.

Placental Attachment and Development

Evenly spaced, elongated conceptuses do not overlap and developing placenta do not fuse together during gestation. The conceptuses continue to grow and expand throughout the uterine horns growing from 100 mm to 500 mm length from Day 15–30 of gestation. The placenta will continue to grow reaching lengths of approximately 800–1000 mm by Day 70 of gestation. The pig has a noninvasive, epitheliochorial type of placentation which the chorion attaches to the surface epithelium along the folds of endometrium (Fig. 5(A)). The multiple primary and secondary folds of the endometrial lining allow the meter-long diffuse placenta (Fig. 5(B)) of each fetus to occupy a uterine area approximately one-third of its length (Fig. 5(A)). The endometrium of the pig contains thousands of uterine glands (Fig. 5(B)) to supply the nutrients needed for continued development to term. With regression of the yolk sac on Day 15 of gestation, complete attachment of the chorion to the endometrial surface epithelium occurs through expansion of the allantois from the hindgut of the developing embryo on Day 15. The fluid filled allantois expands throughout the chorion reaching a volume of 200–300 mL by Day 30 of gestation. Fluid in the allantois declines from Day 32–45 but a second increase in volume to 300–400 mL occurs and peaks on approximately Day 60 of gestation. The allantoic fluid declines from its peak volume on Day 60 to approximately 50–80 mL on Day 100 of gestation. The allantois serves as a reservoir for nutrients (proteins, amino acids, carbohydrates and ions) absorbed from the uterine glands by the overlying chorionic areola (Fig. 5(B)). The allantois may not expand to the extreme ends of the chorion when tips of placental membrane of individual fetuses make contact and ends fold over each other. The lack of placental blood supply from the allantois to the tip of the chorion results in the formation of brownish necrotic tips (Fig. 6). Placental length (800–1000 mm) and weight (200–250 g) of pigs continue to increase until approximately 70 days of gestation where it plateaus until parturition at 114–117 days of gestation.

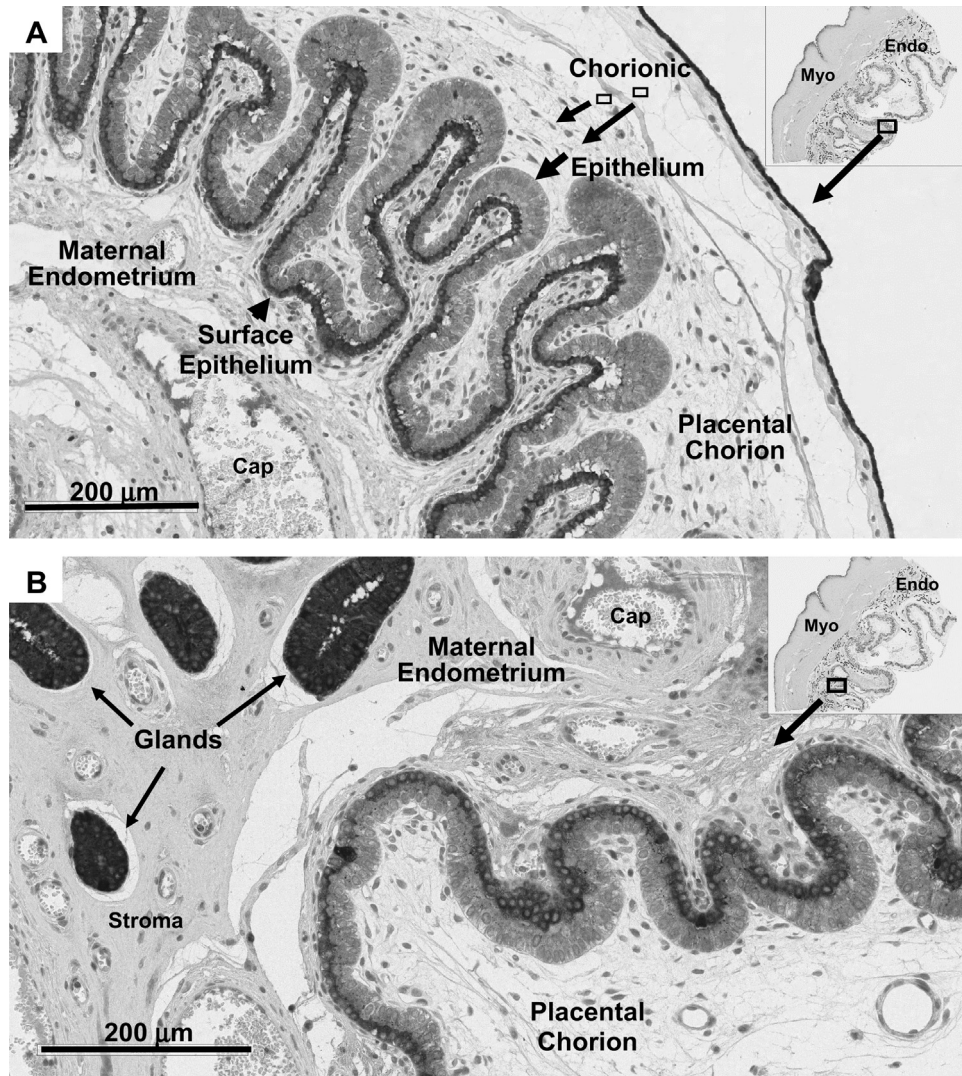


Fig. 5 Histological section of the epitheliochorial placenta in the pig at Day 30. (A) Attachment of the chorion to the luminal epithelium of the endometrium (note the intense folding). (B) Uterine glands that provide nutrients for fetal and placental growth throughout pregnancy. (Insert provides the uterine location for magnification of A and B). Cap, capillary; Endo, endometrium; Myo, myometrium.

Endocrinology of Pregnancy and Parturition

Corpora lutea do not regress during pregnancy because the conceptus prevents the secretion of $\text{PGF}_{2\alpha}$ into the uterine vasculature. There is a slight decline in circulating progesterone concentrations after Day 14 until term. The decline in circulating progesterone is explained by the steroid metabolism by the uterus and conceptus during pregnancy. Maintenance of circulating progesterone concentrations throughout gestation is totally dependent on the CL. Regression of the CL during pregnancy leads to abortion of the pregnancy.

Circulating concentrations of unconjugated and conjugated estrogens such as estrone sulfate increase from Day 16 to peak levels between Day 23 and 30 of gestation. The circulating concentrations then decline to low levels by Day 45 before slowly increasing to attain a second peak on the day before parturition. The fetoplacental unit is the primary source of circulating estrogen during pregnancy. Increases in estrogen follow the steroidogenic activity of the pig placenta and change in placental allantoic volume throughout gestation.

Parturition occurs on Day 114–117 of gestation in the pig. The release of glucocorticoids from the maturing fetal adrenal is associated with the initiation of parturition in the pig. The glucocorticoids stimulate the release of $\text{PGF}_{2\alpha}$ from the endometrium which in turn causes a rapid decline in circulating progesterone concentrations 1–2 day before parturition. There is the release of prolactin from the anterior pituitary and relaxin from the CL at this time as well. The release of prolactin stimulates mammary growth and lactogenesis. Along with relaxin, placental estrogen softens and dilates the cervix. The $\text{PGF}_{2\alpha}$ stimulates oxytocin release that increases myometrial activity that is essential for delivery of the piglets through the softened cervical canal. Sows can be induced

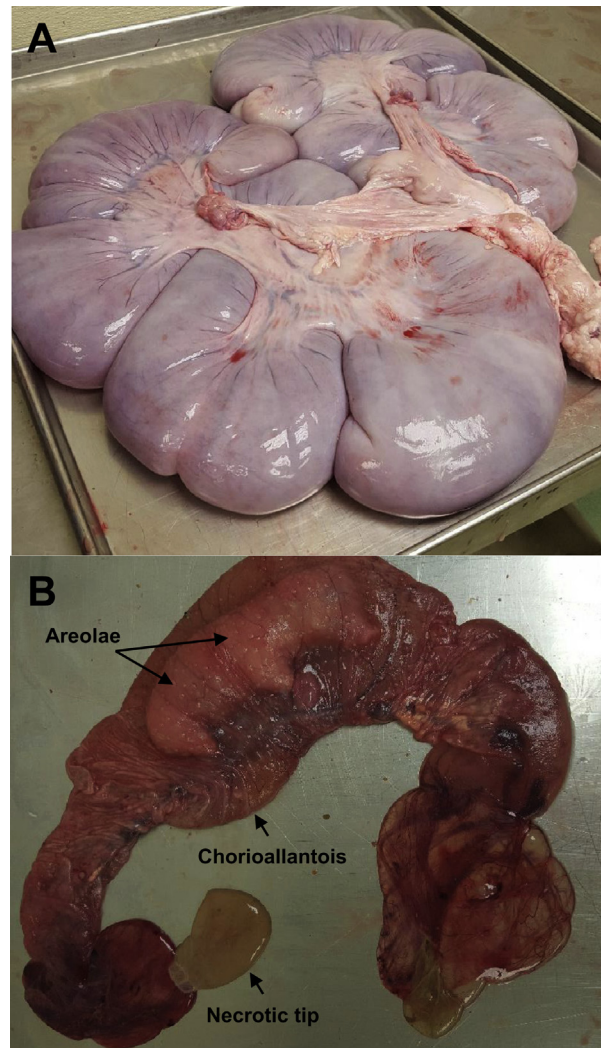


Fig. 6 Pregnant uterus (A) and fetal/placenta (B) collected on Day 60 of gestation.

to farrow after 110 of gestation. Before this time, induction of parturition results in a high percentage of stillborn piglets. Administration of exogenous $\text{PGF}_{2\alpha}$ (Lutalyse®, Zoetis Animal Health, Parsippany, New Jersey, United States) is an effective method to induce parturition after day 110. Delivery typically occurs within 2 days after treatment.

Sows are moved into farrowing rooms that are equipped with specially designed “farrowing crates” a few days before expected farrowing. The farrowing crates are designed to minimize the number of piglets that are crushed by the sow when she lays down. Farrowing in the sow typically occurs over a 2–5 h period. A typical interval between the delivery of each individual piglet is 10–15 min. Piglets are delivered randomly between the uterine horns. A typical delivery involves live born pigs, stillborn pigs, and mummies. Mummified pigs are brown in color and died at some time during gestation after there was skeletal development. Stillborn pigs appear completely normal but are dead at birth. Risk of stillbirth is greater for piglets born late in the farrowing process, for sows farrowing large litters, and for piglets born >20 min after the last piglet. If piglets fail to be delivered on time then it may indicate that there is a piglet lodged within the birth canal (dystocia). If this occurs then the piglet is removed manually (gloved hand passed through the cervix and into the uterus) by the person monitoring the sow at farrowing. Oxytocin can be administered to sows that fail to deliver piglets on time if there appears to be weak myometrial activity and there is no blockage within the uterus. The placenta are passed within 4 h of the delivery of the last piglet.

Lactation

The increase in prolactin, glucocorticoids, growth hormone, and relaxin during the days prior to parturition stimulates lactogenesis in the pig. Milk let down occurs following oxytocin release when piglets suckle. Piglets nurse as a group when allowed to suckle by the sow. The group nursing ensures that milk from all of the teats is consumed at the same time. Nursing interval during early

lactation is less than 1 h but this interval increases over time. Milk production in the sow increases during the third and fourth weeks of lactation.

Greater than 90% of live born pigs should survive until weaning. A common cause of death before weaning is crushing where the mother accidentally lays on top of a piglet and kills it. Runt piglets (extremely small piglets at birth) may also die before weaning because they are either crushed or fail to compete for milk. Piglets are typically cross-fostered at one to two days after farrowing by moving piglets from a sow with a large litter to a sow with a small litter. Cross-fostering is most-successful for sows farrowing within 1 or 2 days of one another.

Most sows are weaned at approximately 21 days (3 week) of lactation. Sows rarely come into estrus while they are lactating because pulsative LH release is suppressed by suckling. The development of large estrogenic ovarian follicles depends on LH and therefore the ovary has only small follicles when the sow is lactating. After weaning, however, there is a rapid increase in LH pulsatility that initiates the development of preovulatory follicles on the ovary.

Sows are moved out of farrowing crates into breeding and gestation barns immediately after weaning. Most sows will be in estrus within 3–7 days after weaning and are inseminated at that time. The endocrine and follicular changes after weaning are very similar to what was previously described for proestrus. Some sows do not return to estrus after weaning. These sows may be cystic (have large non-ovulatory follicular cysts on the ovary), may have ovulated during lactation (relatively rare) or may be anestrus (fail to develop follicles after weaning). There are no effective treatments for follicular cysts in the sow. Sows that are anestrus can be treated with P.G. 600 to induce estrus and then can be AI.

Sows remain in the herd until they either become subfertile (reduction in litter size or farrowing rate), become injured, or experience skeletal problems generally associated with feet and legs. A sow will typically be productive for 3–4 parities. The production cycle for a sow is approximately 142 days (gestation (114 days)+lactation (21 days)+return to estrus (7 days); assumes 100% efficiency). A sow, therefore, can farrow approximately 2.5 litters per years if she is healthy and highly fertile. If she weans 12 piglets per litter then 30 piglets per sow per year are produced. This level of productivity is achieved by approximately 10% of sows today. A more typical target for swine farms is 25 piglets weaned per sow per year.

Further Reading

- Anderson, L. L. (2000). Pigs. In E. S. E. Hafez, & B. Hafez (Eds.), *Reproduction in Farm Animals* (pp. 182–191). London: Lippincott Williams & Wilkins.
- Geisert, R. D., Whyte, J. J., Meyer, A. E., et al. (2017). Rapid conceptus elongation in the pig: An interleukin 1 beta 2 and estrogen regulated phenomenon. *Molecular Reproduction and Development*, 84(9), 760–774. <https://doi.org/10.1002/mrd.22813>.
- Geisert, R. D., Johnson, G. A., & Burghardt, R. C. (2015). Implantation and establishment of pregnancy in the pig. In regulation of implantation and establishment of pregnancy in mammals: Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy.". In R. D. Geisert, & F. W. Bazer (Eds.), *Advances in Anatomy, Embryology and Cell Biology* (pp. 137–164). Springer.
- Mauget, R. (1982). Seasonality of reproduction in the wild boar. In D. J. A. Cole, & G. R. Foxcroft (Eds.), *Control of Pig Reproduction* (pp. 509–526). London: Butterworth Scientific.
- Perry, J. S. (1981). The mammalian fetal membranes. *Journal of Reproduction and Fertility*, 62, 321–335.
- Senger, P. L. (2012). *Pathways to Pregnancy & Parturition*. Redmon, OR: Current Conceptions, Inc.

Beef Cattle

Niamh Forde, University of Leeds, Leeds, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

Beef cattle breeds are mono-ovulatory and polyestrous in nature and tend to have a defined estrous cycle length of approximately 21 days. Female reproduction begins when a beef heifer begins to cycle on a regular basis and normally coincides with the heifer reaching 50% of its adult weight at approximately 12–15 months of age. The estrous cycle is regulated in a similar manner to other mono-ovulatory species with the preparation of the uterus for successful pregnancy irrespective of whether or not an embryo is present. The embryo undergoes a period of rapid elongation of the trophectoderm cells, and maternal recognition of pregnancy is required in order for the corpus luteum (generated by restructuring of the cells from the ovulated follicle) and progesterone concentrations in circulation to be maintained. Placental formation in beef cattle is cotyledonary in nature and quite superficial with pregnancy lasting between 279–287 days depending on breed and sex of the foetus. Parturition is similar as in dairy cattle, but beef cattle do not experience the post-partum negative energy balance that dairy cows do and have comparative ease to become pregnant again. Specialist breeds of beef cattle are adapted to adverse conditions, e.g., tropical climates, which have led to some interesting reproductive functions. These comparative differences in female beef cattle reproduction will be explored in greater depth in the sections below (see [Table 1](#) for a summary).

Development and Puberty

Puberty is defined as the time at which estrus, accompanied by the ovulation of a dominant follicle, occurs. Reaching puberty, i.e., estrus and ovulation, in beef heifers is dependent on weight gain but occurs on average around 13–15 months of age. The optimum age at which beef cattle should first calf is 24 months of age. In order for this to occur, beef heifers should be inseminated at 15 months of age and should have been cycling prior to this. In general beef cattle hit puberty once they have reached 50% of their mature size (300–325 kg) and so the timing of puberty is dependent on the nutritional plane that the animal is on. Moreover, initiation of ovulation can also be affected by breed of cattle with faster growing breeds such as Charolais reaching puberty at a later time than slower growing breeds such as Angus and Hereford. The presence of a bull can also hasten puberty onset, likely through a pheromone response, though this has yet to be proven conclusively.

The Estrous Cycle and Ovarian Follicle Development

The role of the estrous cycle of beef cattle (typically lasting between 18–24 days in duration) is to ensure a cyclical pattern of ovarian activity that will culminate in the ovulation and successful fertilisation of a high-quality oocyte, allowing development of the embryo in a reproductive tract that has been hormonally primed to drive successful early pregnancy. Within each cycle there are two distinct phases, the luteal phase (14–18 days in duration) and the follicular phase (4–6 days) which, in beef cattle, are predominantly made up of three waves of follicle growth. These waves of follicle growth are initiated when a cohort of follicles is recruited from the ovarian follicle pool, begin to increase in diameter, and become more estrogenic. The fate of the dominant follicle is dependent on the stage of the estrous cycle during which it grows and this is regulated by a series of positive and negative feedback mechanisms acting through the hypothalamus, pituitary and gonad axis (HPG-axis) ([Forde et al., 2011](#)).

Table 1 Comparison of major reproductive events of female cattle compared to different model and domestic species

	<i>Cattle (Bovine)</i>	<i>Sheep (Ovine)</i>	<i>Pig (Porcine)</i>	<i>Mouse (Murine)</i>	<i>Human</i>
Ovulation type	Spontaneous	Seasonal	Continual	Spontaneous	Spontaneous
Ovulation number	Mono-ovulatory	Mono-ovulatory (mainly)	Poly-ovulatory	Poly-ovulatory	Mono-ovulatory
Embryonic genome activation	8–16 cell stage	8–16 cell stage	4–8 cell stage	2-cell stage	4–8 cell stage
Completion of X-Chromosome inactivation	Post-blastocyst	Possibly blastocyst	Post-blastocyst	Blastocyst	Blastocyst
Elongation rate	Rapid	Rapid	Extremely rapid	N/A	N/A
Pregnancy recognition signal	Bovine Interferon Tau	Ovine Interferon Tau	Estrogens	Prolactin	Human chorionic gonadotrophin
Placentation	Cotyledonary	Cotyledonary	Diffuse	Discoid	Discoid
	Synepitheliochorial	Synepitheliochorial	Epitheliochorial	Haemochorial	Haemochorial
Duration of gestation (Days)	279–292	142–152	114	21	280

Gonadotrophin releasing hormone (GnRH), a low molecular weight neuropeptide produced in the hypothalamus, regulates the estrous cycle by controlling the secretion of the gonadotrophins luteinising hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. GnRH is released in a pulsatile manner via the hypophyseal portal blood system to prevent desensitisation of its receptor on the gonadotroph cells of the anterior pituitary. The release of LH and FSH is mediated by the binding of GnRH to its G-protein coupled receptor on the cell surface of the gonadotrophs. This stimulates intracellular calcium release and activation of the mitogen-activated protein kinase (MAPK) signalling pathway culminating in the release of stored LH and/or FSH depending on the stage of the estrous cycle. During the follicular phase the pre-ovulatory GnRH surge leads to high amplitude and frequency of LH pulse release as there are negligible amounts of progesterone in circulation. This allows for the ovulation of the dominant follicle. Following ovulation LH is responsible for rearranging the granulosa and theca cells of the ovulated follicle into the corpus luteum and thus progesterone concentrations in circulation begin to increase and the estrous cycle enters the luteal phase. At all stages during the estrous cycle a transient rise in FSH is responsible for the recruitment of the cohort of follicles from the ovarian pool. These follicles in a given cohort undergo distinct stages of growth with (1) emergence of the cohort of follicles (preceded by increased FSH), (2) selection of the dominant follicle for that cohort, and (3) follicle dominance followed by either atresia (during the luteal phase) or ovulation (during the follicular phase) of the dominant follicle. Each of these stages are characterised by distinct events with emergence of a follicle wave defined as the growth of a cohort of follicles ≥ 5 mm in diameter and coincides with a transient increase in FSH secretion. Selection of one dominant follicle in beef cattle occurs following a decline in FSH concentrations in circulation and all other follicles in that cohort undergo atresia via programmed cell death by apoptosis. During the dominance stage of the wave, the single selected follicle suppresses any remaining estrogen active follicles in that cohort. Whether or not that dominant follicle ovulated is dependent on whether or not it is in the luteal phase (cannot ovulate) or the follicular phase (can ovulate). As beef cattle have predominantly three waves of follicle growth per cycle, the dominant follicle from two out of these three waves of growth will never be destined to ovulate.

Role of the Dominant Follicle in Regulating the Estrus Cycle

Both FSH and LH play a role in the growth and development of ovarian follicles by binding to their respective receptors. FSHR is expressed during the emergence stage of follicle development, allowing the follicles in a cohort to grow. Once the dominant follicle is selected it produces increased quantities of estradiol and inhibin which suppresses FSH release from the anterior pituitary. The dominant follicle acquires LHR which allows it to continue to grow under a low FSH but high LH environment. If this occurs during the luteal phase, i.e., when progesterone concentrations are high, then the dominant follicle cannot ovulate, will regress and undergo cell death. This results in the removal of estradiol and inhibin from circulation, allowing another transient rise in FSH and the emergence of another cohort of follicles.

One of the hallmarks of a healthy follicle is the production of estradiol, which is detectable in the follicular fluid and is responsible for regulation of the estrus cycle as well as the display of estrus behaviour. Most follicles in a cohort at the emergence stage are estrogen active although some studies in beef cattle have shown that there are higher concentrations of estradiol in the dominant follicle prior to detectable differences in the diameter of the dominant follicle. Estradiol production occurs in a two-step manner with the production of androgens by the vascularised theca cells of the follicle and subsequent aromatisation of these androgens to estradiol in the avascular granulosa cells. The binding of LH to its receptor in the theca cells stimulated the conversion of cholesterol to testosterone via 17α -hydroxylase. Following diffusion of testosterone into the granulosa cells it is aromatised to estradiol via P450_{aromatase} (CYP19A) and estradiol is released into the follicular fluid. Not only does estradiol have a local effect on the follicle but is also involved in systemic regulation of the estrous cycle. The production of estradiol by the pre-ovulatory dominant follicle is sufficient to induce a surge of GnRH release from the hypothalamus resulting in an LH surge of sufficient pulse frequency as to stimulate final maturation of the follicle. This increases estradiol concentrations in follicular fluid, resulting in positive feedback to GnRH, LH, and FSH culminating in ovulation and estrous behaviour display. The display of estrus behaviour in beef heifers is between 12–14 h whereas beef cows, kept indoors, tends to display estrus behaviour for less time (8.5 h). As with dairy cattle, the duration and intensity of estrous display are affected by environmental factors including surface type, size of the group and bull presence. But there is no evidence that estrus behaviour follows any distinct diurnal pattern.

Two additional families of genes and proteins can indirectly impact the feedback system and display of estrus behaviour in beef cattle. These are the insulin-like growth factor (IGF) family and the transforming growth factor Beta superfamily of genes, which alters estradiol production by the ovarian follicle. IGF-1 stimulates estradiol production by follicles, but its bioavailability is strictly controlled by the IGF binding proteins. The composition of IGF binding proteins in the follicular fluid of a given follicle can alter the follicle's ability to drive estradiol production and may affect the expression of estrus behaviour. Similarly, activin, follistatin and inhibin can inhibit its production (Roche, 1996). The careful balance between these members of the TGF Beta superfamily can modify estradiol production with consequences for estrous behaviour.

The Corpus Luteum

Unlike dogs and cats, the CL of beef cattle has a finite life-span and requires an inhibition of the luteolytic mechanism to maintain high concentrations of P4 in circulation that will support and maintain pregnancy. Throughout the luteal phase of the estrous cycle,

progesterone secretions from the corpus luteum are sufficient to suppress LH amplitude and pulse frequency as to inhibit ovulation of the dominant follicle. In the non-pregnant state, regression of the corpus luteum is similar to other ruminants and is a result of the actions of endometrial-derived prostaglandin $F_{2\alpha}$ (PGF_{2A}), acting in an extra-uterine manner and stimulating CL regression. The CL produces oxytocin which binds to its cognate receptor on the endometrial epithelium and stimulates production of PGF_{2A} in a positive feedback loop. If there is an appropriately developed conceptus present, producing sufficient quantities of the pregnancy recognition signal Interferon Tau, then oxytocin receptor up-regulation in the endometrial epithelial cells is inhibited and PGF_{2A} production and release are suppressed.

Ovarian Reserve and Oocyte Growth

The ovarian follicle reserve in beef cattle is likely laid down during development with the oocyte housed in a primordial follicle with one layer of granulosa cells surrounding it. The reproductive lifespan of female beef cattle is dependent on the ovarian reserve and different subspecies had different ovarian reserves. For example, the subspecies *Bos taurus indicus* has a significantly larger ovarian reserve as well as numbers of follicles per cohort, compared to their *Bos taurus taurus* counterparts. This phenomenon has been demonstrated through ovarian scanning as well as measurement of anti-mullarian hormone (AMH) in blood, which is a proxy marker for ovarian reserve. The majority of oocytes reside in the ovarian reserve in an immature state and as such go through a 90-day period of growth where they can acquire developmental competency. This process involved both nuclear and cytoplasmic maturation of the oocyte in order for it to acquire sufficient mRNAs and proteins to drive embryo development up to embryonic genome activation (EGA).

Pre-Implantation Embryo Development

If there is successful fertilisation of the oocyte in the oviduct by a sperm, this structure is referred to as the zygote. This consists of a single cell with the diploid number of chromosomes arising from the fusion of the two haploid gametes. This is distinct to the formation of a parthenode which can be artificially induced in beef cattle embryos *in vitro* by chemical activation of the oocyte without fertilisation. The first distinctive morphological stage of embryo development is cleavage formation i.e. mitotic division of the cell (s) within the embryo to produce more cells (known as blastomeres) without an overall increase in the size of the embryo. The time to first cleavage in beef cattle has been used as a marker of developmental potential and during this stage the embryo drives these cell cycle divisions by using maternal-derived and stored transcripts and proteins. This is then followed by EGA, whereby the embryonic genome begins the process of active transcription and translation of genes and proteins to drive embryo development. The process of EGA is species specific but occurs in bovine embryos (both beef and dairy cattle) between the 8-cell and 16-cell stage of development. More recently there has been the identification of genes expressed at the 4-cell stage of development that are proposed to be involved in driving EGA (Li *et al.*, 2013). This discovery was made possible by exploiting the subspecies differences between *Bos taurus* and *Bos taurus indicus* whereby *indicus* sperm was used to fertilise *taurus* oocytes. Taking advantage of single polymorphism differences between these two subspecies and next generation sequencing, the authors could identify what transcripts were generated from the embryo rather than those that were maternally-derived. The authors identified the genes upstream binding transcription factor, RNA polymerase I-like 1 (UBTF1), heterogeneous nuclear ribonucleoprotein A2/B (HNRNPA2B1), Krüppel-like factor 17 (KLF17), and Kelch-like family member 28 (KLHL28) as well as gene ontologies involved in RNA processing and transport as novel genes involved in driving the EGA process in bovine embryos (Graf *et al.*, 2014).

A second key event in early embryo development is the formation of distinct cell lineages from the blastomeres that go on to form components of the embryo proper (inner cell mass) and the outer cell mass that forms the extraembryonic membranes. This is a process that is different in bovine embryos to that which occurs in mice. There are specific genes associated with the trophectoderm cell lineage including H+/K+ transporting non-gastric alpha2 subunit (ATP12A), caudal type homeobox 2 (CDX2), disabled homolog 2 (DAB2), Interferon Tau (IFNT), ATPase keratin 8 (KRT8), and transcription factor AP-2 alpha (TFAP2A). While markers for the epiblast include Nanog homeobox (NANOG), PR/SET domain 14 (PRDM14), POU class 5 homeobox 1 (POU5F1) and teratocarcinoma-derived growth factor 1 (TDGF1), GATA binding protein 6 (GATA6) and goosecoid homeobox (GSC) for the primitive endoderm. It is only when the formation of the morula stage of development (approximately day 4–5) that the distinction in cell lineage begins.

By the blastocyst stage of development, another key event that is still poorly understood in species other than mice, occurs; that is X-chromosome inactivation (XCI). In female embryos, there are two X chromosomes, while in male embryos there is one X and one Y chromosome. The Y chromosome in the male has a number of genes that are required to drive some of the male specific characteristics and include the sex determining region Y (SRY) gene. In female embryos, one of the X chromosomes must be inactivated in order for dosage compensation to occur. This process is regulated by the long non-coding RNA (lncRNA) X-inactive specific transcript (XIST). This lncRNA, along with other lncRNAs and proteins form the X-chromosome inactivation center (XIC) to allow for the inactivation of one of the X-chromosomes. However, unlike the process in mice which seems to be completed by the blastocyst stage of development, XCI in cattle is not complete at the blastocyst stage of development and seems to occur after the lineage specification into inner and outer cell mass but prior to gastrulation occurring.

In addition to EGA and XCI, another important process early embryo development in cattle is the differential methylation of different regions of the genome including areas hosting imprinted genes. Imprinting is a process whereby one allele of either the maternal or the paternal origin is silenced and differential methylation occurs at distinctive sites of the embryonic genome alter gene function. In the mouse model, where this has been most comprehensively studied, this process is stable throughout embryo development, however in cattle, the expression of the machinery and degree of methylation of these imprinted sites is very variable – particularly during the blastocyst stage of development.

One of the clear differences in early pregnancy morphology in beef cattle is the process of post hatching elongation. On approximately Day 8–10 the blastocyst hatches from the zona pellucida and under goes morphological changes from an ovoid conceptus on Day 13, to a tubular and then filamentous conceptus on days 14 and 15 respectively. This entire process of conceptus elongation is driven by secretions from the maternal uterine endometrium (see below) but the rate of conceptus elongation is also dependent on the embryo itself. The evidence for this is from multiple embryo transfer models where by *in vitro* produced bovine embryos are transferred into the same recipient and recovered on Day 14 display variation, even between embryos exposed to the same environment.

Interaction With the Female Tract for Early Pregnancy Success

In beef cattle, there is no strict requirement for the embryo to be exposed to the oviduct environment given it is possible to grow beef cattle embryos *in vitro* up to the blastocyst stage of development. These embryos can be transferred into synchronised beef heifer or cow recipients to result in successful pregnancy outcome. It is true however, that exposure to the oviduct environment, i.e., *in vivo* produced blastocysts, are of higher quality than their *in vitro* grown counterparts. This is likely due to exposure to the secretions from the oviduct epithelial cells which are not necessarily included as components of culture medium for embryos grown *in vitro*. In contrast, elongation of the conceptus must occur in the female tract as attempts to recapitulate this *in vitro* have not been successful. The elongation of the conceptus in cattle is governed by the post-ovulatory rise in concentration of the hormone progesterone. Progesterone in circulation acts indirectly on the embryo, by altering the transcriptome of the endometrium and subsequently the composition of the proteins, amino acids, ions, and other molecules that comprise the uterine luminal fluid. Elevated concentrations of progesterone advance the normal changes that occur throughout the estrous cycle while a delay in the post-ovulatory rise in progesterone causes a delay in the normal temporal changes that occur. What is clear from recent advances in omic technologies and utilizing these to understand early pregnancy events is that there is an optimum range of progesterone concentration in circulation that will drive successful elongation (through changes to the endometrial transcriptome and uterine fluid composition) (Forde and Lonergan, 2012). Data in the literature has shown that blanket progesterone supplementation treatments, either through vaginal pessaries or through stimulation of the endogenous and/or an accessory corpus luteum, do not necessarily result in improved pregnancy rates. Moreover, the changes that are observed in the endometrium/uterus that are modified with different concentrations of progesterone are not specific to external manipulation of progesterone as modifying the size of the ovulated dominant follicle (and subsequently the CL size) results in similar modifications to the endometrium (Fig. 1).

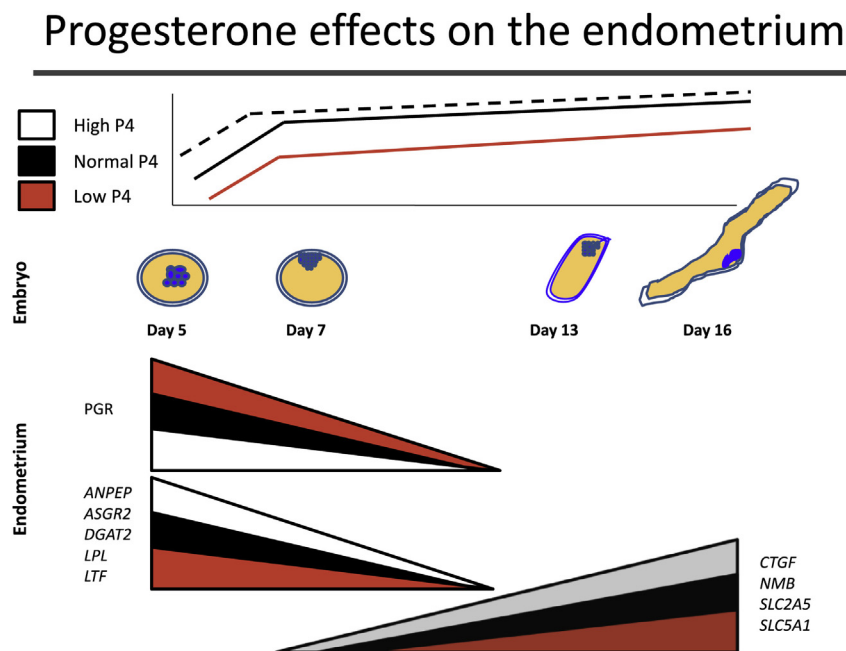


Fig. 1 Schematic diagram of the relationship between progesterone concentrations in circulation and mRNA expression values in the endometrium that are required to be down- and up-regulated in the post-hatching/elongation stage of conceptus development in order to establish uterine receptivity to implantation.

The rate of conceptus trophoblast elongation is correlated with IFNT production. Sufficient quantities of IFNT are required to signal the presence of the conceptus to the mother and maintain a functional CL and thus P4 concentrations in circulation. A significant number of studies have demonstrated there is a transcriptional response of the beef cow endometrium to the conceptus, predominantly on Day 16 (consistent with conceptus transfer data). There is a consistent transcriptomic signature which is amplified as day of pregnancy progresses from Day 15–20 and is predominantly due to the endometrial response to conceptus-derived IFNT. However, there are substantial changes to the endometrial transcriptome that are not a result of IFNT. Indeed, when IFNT was discovered there were a larger number of additional conceptus-derived proteins that were present/*de novo* synthesised by the conceptus but were not identified. More recent advances in proteomic technologies have identified additional conceptus-derived proteins that may modify endometrial function. An additional interesting phenomenon has been the discovery that conceptuses with different developmental competencies (*in vivo*, *in vitro*, and cloned embryos) elicit a different transcriptomic response in the endometrium indicating that the endometrium may modify its uterine receptivity to facilitate implantation to embryos with potentially different pregnancy outcomes.

The endometrium of beef cattle, unlike that of human or mice, does not undergo a decidualization response prior to implantation. What is clear however, is that there is some conservation in terms of gene expression patterns that are required for uterine receptivity to implantation, e.g., downregulation of the nuclear progesterone receptor from the luminal and glandular epithelium, regulation of secreted phosphor protein 1, increased production of IGF binding protein 1 and other proteins (Spencer *et al.*, 2016). This indicates that there are receptive and non-receptive uterine states in the endometrium of beef cattle.

Placental Development and Post-Partum Reproductive Issues

As mentioned above placental formation in cattle is very superficial and cotyledonary in nature. The main interaction sites are the aglandular caruncular regions of the endometrium which fuse with the foetal cotyledons to form the placentomes. The microscopic interactions between the maternal and foetal systems are termed synepitheliochorial in nature as binucleate cells from the foetus migrate and fuse with the epithelial cells of the endometrium to form syncytia which are responsible for the production of placental lactogen (Burton *et al.*, 2006). In beef cattle, 80% of the progesterone produced during pregnancy originates from the corpus luteum with only 20% derived from the placenta, demonstrating that the corpus luteum is required for pregnancy success for the majority of gestation.

Post-partum issues in cattle are distinct from those that occur in dairy cows, as they do not experience the post-partum negative energy balance complications and reproductive issues that dairy cows undergo in the demands for energy to drive milk production. However, post-partum there are some reproductive issues that are specific to beef cattle. Beef cattle are exposed to a transient rise in FSH 3–5 days after calving which results in resumption of recruitment and growth of a cohort of follicles 7–10 days post-partum. Ovulation of the resulting dominant follicles is dependent of LH pulsatile. Resumption of ovulation however, can be inhibited in beef cattle by severe body condition score loss or suckling stimulated inhibition which reduces GnRH release as consequently LH pulse amplitude and frequency.

Recent studies are demonstrating that beef cattle offspring may also be susceptible to postnatal consequences due to changes to the maternal environment. The developmental origins of health and disease (DOHaD) hypothesis, originating as the Barker hypothesis following observations of increased risk of death from cardiovascular disease in humans with low birth weight, has given rise to an entirely new field of study (covered in detail in other chapters in this book) (Pérez-Cerezales *et al.*, 2017). Recent studies looking at this phenomenon in beef cattle have reported some interesting findings. Restriction of dietary intake in the first three months of gestation in beef cattle results in offspring with decreased ovarian reserve and impairment of the cardiovascular system. More recently, culture of embryos from beef cattle donors for two days supplemented with Dickkopf-related protein one for two days had no impact on blastocyst development but did result in lower birth weight (Tribulo *et al.*, 2017). Collectively these data demonstrate that beef cattle can be susceptible to maternal environmental insults that may have transgenerational consequences.

Conclusion

Beef cattle have an interesting reproductive lifespan with some mechanisms that are conserved between closely related species, e.g., the sheep. However, there are some events that are unique to the bovine as a model, as indeed unique to beef cattle, which is an important consideration when trying to extrapolate reproductive processes between different species.

References

- Burton, G., Kaufmann, P., & Huppertz, B. (2006). Anatomy and genesis of the placenta. In J. Neill (Ed.), *Knobil and Neill's Physiology of Reproduction* (third ed.). pp. 189–232. Elsevier.
- Forde, N., Beltman, M. E., Lonergan, P., et al. (2011). Oestrous cycles in *Bos taurus* cattle. *Animal Reproduction Science*, 124, 163–169.

- Forde, N., & Lonergan, P. (2012). Transcriptomic analysis of the bovine endometrium: What is required to establish uterine receptivity to implantation in cattle? *Journal of Reproduction and Development*, 58, 189–195.
- Graf, A., Krebs, S., Zakhartchenko, V., et al. (2014). Fine mapping of genome activation in bovine embryos by RNA sequencing. *Proceedings of the National Academy of Sciences USA*, 111, 4139–4144.
- Li, L., Lu, X., & Dean, J. (2013). The maternal to zygotic transition in mammals. *Molecular Aspects of Medicine*, 34, 919–938.
- Pérez-Cereales, S., Ramos-Ibeas, P., Rizo, D., et al. (2017). Early sex-dependent differences in response to environmental stress. *Reproduction*, 153, 1–13.
- Roche, J. F. (1996). Control and regulation of folliculogenesis – A symposium in perspective. *Reviews of Reproduction*, 1, 19–27.
- Spencer, T. E., Forde, N., & Lonergan, P. (2016). The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *Journal of Dairy Science*, 99, 5941–5950.
- Tribulo, P., Bernal Ballesteros, B. H., Ruiz, A., et al. (2017). Consequences of exposure of embryos produced in vitro in a serum-containing medium to dickkopf-related protein 1 and colony stimulating factor 2 on blastocyst yield, pregnancy rate, and birth weight. *Journal of Animal Science*, 125, 4407–4412.

Sheep

Kathrin A Dunlap, Texas A&M University, College Station, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

ACTH	Adrenocorticotropin hormone	LH	Lutenizing hormone
CA	Corpus albicans	NRC	National Research Council
CH	Corpus hemorrhagicum	oPL	Ovine placental lactogen
CL	Corpus luteum	OXTR	Oxytocin receptor
CO	Corn oil	P4	Progesterone
CST3	Cystatin C	PAGs	Pregnancy associated glycoproteins
CST6	Cystatin 6	PGE2	Prostaglandin E2
CTSL1	Cathepsin L1	PGF2a	Prostaglandin F2 alpha
ECM	Extracellular matrix	PGR	Progesterone receptor
enJSRV	Endogenous Jaagsiekte sheep retrovirus	PSPB	Pregnancy specific protein B
ESR1	Estradiol receptor 1	PTGS2	Prostaglandin G/H synthase and cyclooxygenase 2
FSH	Follicle stimulating hormone	sGE	Superficial glandular epithelia
GE	Glandular epithelia	SLC1A5	Solute carrier family 1 member 5
GnRH	Gonadotropin releasing hormone	SLC2A1	Solute carrier family 2 member 1
GRP	Gastrin releasing peptide	SLC2A5	Solute carrier family 2 member 5
HSD11B	Hydroxysteroid (11-β) dehydrogenase 1	SLC2A12	Solute carrier family 2 member 12
IGFBP1	Insulin-like growth factor binding protein 1	SLC5A1	Solute carrier family 5 member 5
IFNT	Interferon tau	SLC5A11	Solute carrier family 5 member 11
IRF2	Interferon regulatory factor 2	SLC7A	Solute carrier family 7 member
LGALS15	Galectin 15	SPP1	Secreted phosphoprotein1
LE	Luminal epithelia	UGKO	Uterine gland knockout

Glossary

Aglandular Without glands.

Anestrus Period in which female is not cycling. Ewes are anestrus when they are prepubertal ewe lambs, also during the non-breeding season or pregnancy.

Breeding season Period of time during the year in which sheep are capable of breeding. Regulated by light exposure do to photoperiod. Ovine breeding season is during reduced day length (i.e. fall-winter).

Caruncle Aglandular area of tissue lined by single layer of luminal epithelium, located on the uterine endometrium, projecting into the uterine lumen. Forms the maternal component of the placentome.

Conceptus Embryo and extraembryonic membranes.

Corpus luteum Post-ovulatory ovarian structure necessary for progesterone production.

Cotyledon Structure found on the placental membrane that provides the fetal component of the placentome.

Estrous cycle Female reproductive cycle between ovulatory periods. In sheep the estrous cycle is approximately 17 days long.

Estrus Period of female receptivity to mating as part of the estrous cycle.

Ewe Female ovine after at least one parturition.

Ewe lamb Female ovine between birth and first parturition.

Interferon tau Maternal recognition of pregnancy signal in the sheep, produced by the embryo.

Lamb Ovine offspring between birth and one year of age.

Luteolysis Destruction of the functional corpus luteum.

Maternal recognition of pregnancy A biochemical signal (interferon tau in sheep) produced by the conceptus for maintenance of pregnancy.

Oocyte Female gamete.

Ovulation Release of an oocyte from an ovarian follicle.

Parturition Expulsion of the fetus and placental membranes at the end of pregnancy.

Placentome Structure formed via interdigitation of maternal caruncle and fetal cotyledon. Site of high throughput nutrient exchange.

Puberty Time point of chronological and physiological maturity in which the animal is capable of releasing a gamete and becoming pregnant.

Ram Intact male ovine.

Sheep General term for all ages and sexes of ovine.

Synepithelialchorial placentation Minimally invasive type of placentation in which there is formation of a maternal-fetal syncytial layer at the placental-uterine interface.

Introduction

Sheep are the primary source of animal protein and clothing fiber for much of the world, ranking second to beef cattle in total numbers. Globally, they are a significant species as a greater number of individual sheep may be sustained on the same amount of forage as a single cow and sheep are frequently raised in arid climates with extreme variation in nutrient availability. The combination of sustainability on limited forage, minimal management requirements, and the potential protein and fiber production in such conditions makes them a favorable species for utilization in most of the world. As such, increased reproductive performance and efficiency is critically important. This article serves to review general aspects of female sheep reproduction.

Female Reproductive Tract Anatomy

In utero the reproductive tract of the ewe develops, anatomically, to a level that is very similar to its mature state. The reproductive tract is located ventrally to the rectum and is suspended within the body cavity by a structure known as the broad ligament. The broad ligament can be classified into subareas depending upon localization. The mesovarium supports the ovary, mesosalpinx the oviduct and mesometrium the uterus. At pregnancy the mesometrium will help to maintain the placement of the uterus despite the increased size and weight from the developing fetus.

The ovary is home to the female gametes that are at their maximal population just prior to birth. Unlike the ram, which begins a continuous production of gametes following puberty, the ewe is born with a finite supply of oocytes (ranging between 40,000 and 300,000 in total number) that are released at from meiotic arrest at puberty and are then capable of maturation, release and subsequent fertilization. The ewe ovary is an almond shaped structure in which the innermost component is termed the medulla and the external layer the cortex. The cortex is home to the gametes or oocytes. Oocytes are found within structures known as follicles. At birth groups or nests of primordial follicles can be detected throughout the cortex. As the animal matures, so do the follicles. After the female has reached the physiological maturity associated with puberty the follicular population will undergo a series of steps involved in maturation. The primordial follicle will progress through stages of development to become primary, secondary, tertiary and then ovulatory. As the ovulatory follicle is housed in the cortex, at ovulation, the oocyte will be released from the surface of the ovary.

The ovulated oocyte will be collected by the infundibulum, which is the anterior most portion of the oviduct. The infundibulum is lined by small finger like projections known as fimbriae that sweep over the cortex of the ovary in order to collect the oocyte and move it further into the oviduct. The next region of the oviduct encountered is the ampulla. Marked by the presence of cilia, the ampulla is also a secretory structure, which adds to the oviduct milieu that is necessary for successful fertilization. The ampulla transitions to the isthmus, a more muscular segment of the oviduct that at the time of mating will help to facilitate movement of spermatozoa to from the uterus toward the waiting oocyte. In the sheep fertilization takes place within the oviduct and the embryo begins the process of cellular division and development prior to entering the uterus. The sheep uterus has two distinctive horns that come together to form a single uterine body. The area of at which the uterine body separates into the two horns is known as the bifurcation.

The sheep uterus is clearly organized into a series of tissue layers, each serving a specific purpose. The exterior layer of the uterus or perimetrium is the outer most covering of the uterus. The next layers are smooth muscle termed the myometrium. The myometrium is comprised of layers of longitudinal and smooth muscle that are capable of coordinated muscle contraction, such as what is necessary for expulsion of the fetus at birth. Located between the myometrium and the lumen (central canal of the reproductive tract) is the endometrium. The uterine endometrium is made up of the uterine mucosa and submucosal layer, and as such it is a dynamic, secretory tissue that will ultimately provide a place for attachment and development of the embryo and extraembryonic membranes known as the placenta. The mucosa is lined by epithelial cells and the submucosa can be divided into two distinctive regions, the stratum compactum and stratum spongiosum stroma. The stroma of the adult ewe is populated with blood and lymphatic vessels as well as uterine glands.

While the majority of the reproductive anatomy of the ewe does not change after birth, the uterine endometrium undergoes a critical process: development of uterine glands ([Fig. 1](#)). The uterine submucosa or stroma of a mature ewe is densely populated with uterine glands. The secretory products of the uterine glands are necessary for pregnancy as they are the source of histotroph, a milieu of cytokines, lymphokines, growth factors, hormones, amino acids, glucose, fructose, vitamins, and additional substances that is an essential for early embryonic nutrition. However, at birth the endometrium of the ewe lamb lacks uterine glands. Immediately post-partum there is a series of invaginations of the uterine luminal epithelium that will extend as buds. They will continue to grow into the stroma and will undergo subsequent coiling and branching to give rise to the uterine glands. This developmental process is under strict endocrine control and inappropriate exposure to the steroid hormone progesterone during neonatal life can prevent gland formation. Of interest, this phenomenon has been utilized by researchers via the creation of the uterine gland knock out (UGKO) ewe, which has proven an exceptional tool for investigation of the role of uterine glands in pregnancy. The UGKO population is devoid of glands and incapable of maintaining a pregnancy ([Fig. 1](#)).

The surface of the ruminant endometrium is unique in that it contains discreet aglandular regions of tissue known as caruncles ([Fig. 1](#)). The endometrial surface of the sheep can contain upwards of 100 caruncles. In the ewe, the caruncles have a concave shape as they protrude from the surface of the endometrium. The caruncles are lined by luminal epithelium and are vascularized. They are fairly uniformly distributed throughout the uterine body and horns. They can range in diameter and those located closer to the tips of the uterine horns are smaller. During pregnancy, the caruncles will form the maternal component of structures known as placentomes.

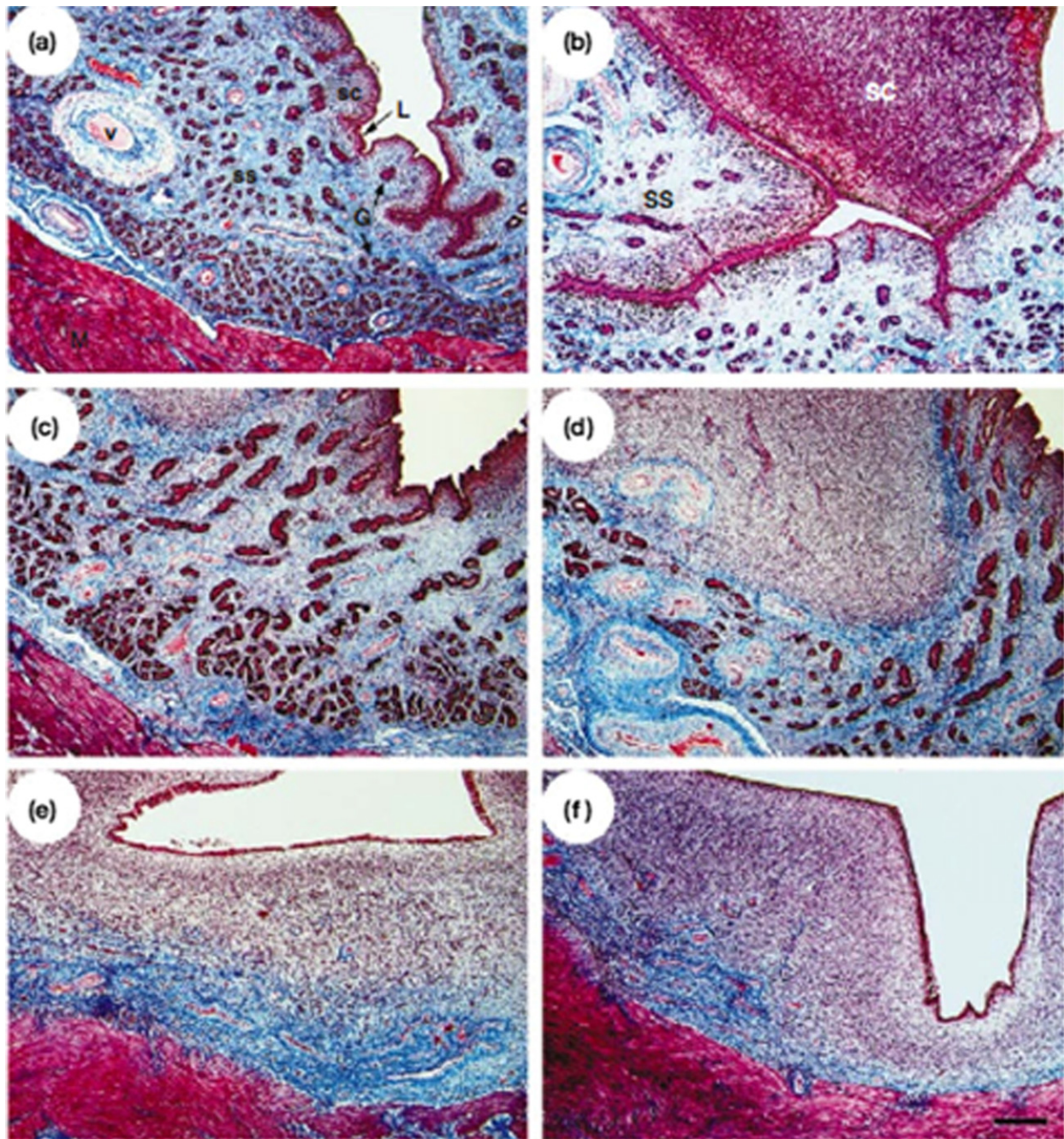


Fig. 1 Representative photomicrographs of the uterus from cyclic (a,b), pregnant (c,d) and uterine gland knockout (UGKO) ewes (e,f) 14 days after mating, using a Masson's trichrome stain. This procedure stains nuclei black, cytoplasm and muscle fibers red and extracellular matrix (ECM) components blue. The left column (a,c) from normal ewes depicts intercaruncular endometrial regions and the right column (b,d) depicts caruncular endometrial area. No distinct caruncular or intercaruncular regions are distinguishable in uteri from UGKO ewes (e,f). L, luminal epithelium; G, glandular epithelium; SC, stratum compactum; SS, stratum spongiosum; M, myometrium; V, blood vessel. Scale bar represents 200 μ M. Reproduced from Gray, C.A., Burghardt, R.C., Johnson, G.A., Bazer, F.W., Spencer, T.E., 2002. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction* 124, 289–300.

Placentomes are the sight of high throughput nutrient exchange between the uterus and developing fetus. They are formed by the interdigitation of maternal caruncular crypts and cotyledonary villi (**Fig. 2**). Cotyledons are located on the fetal chorion (outermost layer of the placenta) known as cotyledons and serve as the fetal component of the placentome. Placentomes are detectable as early as day 40 of pregnancy, becoming fully functional by day 60. The placentomes are the primary point of physical attachment between the uterus and placenta throughout pregnancy. At parturition (birth) the caruncles will separate from the

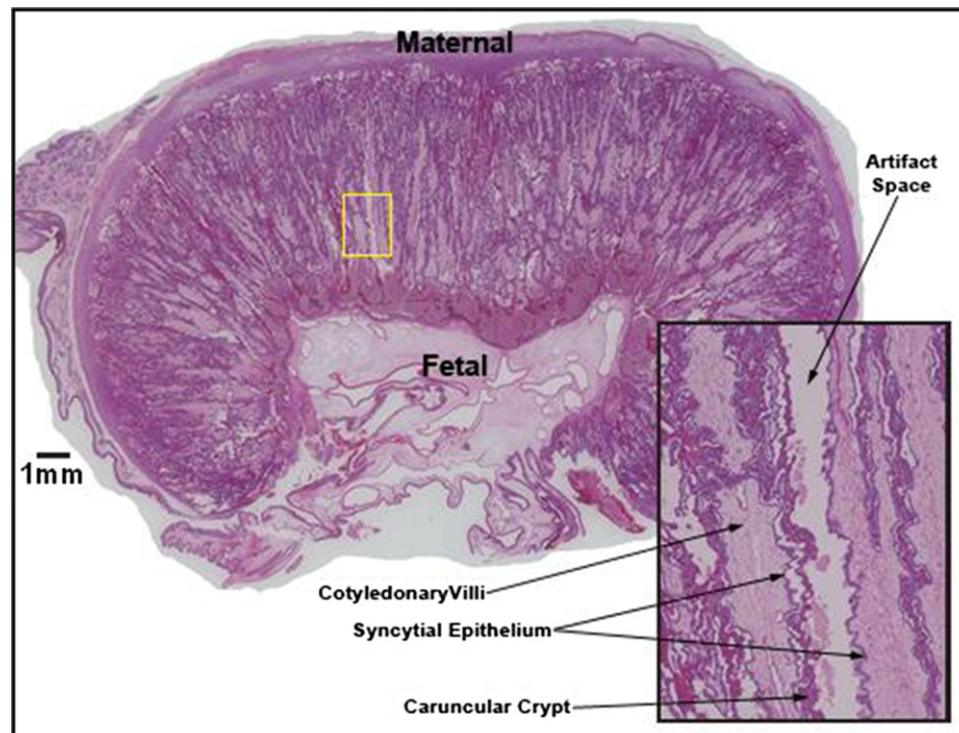


Fig. 2 Representative photomicrograph of a placentome using hematoxylin and eosin stain. The large panel depicts a complete cross section of a typical concave, A type placentome of the sheep. The maternal and fetal labels provide orientation, as maternal is adjacent to the uterine stroma and the fetal is proximal to the uterine lumen. The high magnification inset depicts the interdigitation of the caruncular crypt and cotyledonary villi, which are separated by a layer of syncytial epithelium. Fixation artifact space is also labeled. Scale bar represents 1 mm.

placental membranes and remain attached to the uterine endometrium. In the postpartum interval the caruncles will undergo necessary repair of tissue and vasculature in order to return to their original disposition.

The size and shape of placentomes can vary within and between animals. In sheep the placentomes can be classified by shape ranging from A-D type based upon the relative configuration of the caruncle to cotyledon. In an A type placentome the caruncle will maintain a concave position with the cotyledon being centrally attached. The caruncle will essentially surround the cotyledon, forming a nearly spherical structure with placental membranes exiting the apex of the placentome. Conversely, a D type placentome will be inverted in that the caruncle will have taken on a convex shape and the cotyledon will be surrounding it. This is the type of placentome characteristic of the cow, in that bovine caruncles are convex rather than concave in shape. Total mass of placentomes has been shown to correlate to fetal weight and pre-mating surgical removal of caruncles, which causes an overall reduction in placentome number and mass, will reduce fetal weight and can compromise pregnancy.

Immediately caudal to the uterus is the cervix. This is a fibrous structure that primarily functions as a protective barrier. In the sheep the cervix is identifiable by the series of annular rings that it possesses. These rings (typically 4–7) provide an initial barrier to foreign particles as well as spermatozoa as they create a tortuous pathway for spermatozoa to navigate. The cervix is a secretory organ whose products will vary dependent upon the endocrine cues provided. At the time of mating (i.e., estrus) the cervix will secrete mucins to help facilitate spermatozoa transport. If pregnancy is successful a more solid mucous barrier is formed to help protect the uterus from influx of foreign material. At parturition the cervix will dilate to enable passage of the fetus and placental membranes from the uterus to the vagina.

The vagina of the ewe is the most caudal interior region of the reproductive tract. At mating this is the receiving site for the male's penis and at parturition is part of the fetal birth canal. Resultantly the epithelium must protect against abrasions as well as introduction of foreign materials. The secretory activities of the vagina are also heavily influenced by endocrine control. As example, under influences of high estrogen (i.e., at estrus) the vagina becomes highly secretory, and can lead to discharge visible at the vulva. The vulva is the portion of the female reproductive tract visible from outside the body cavity. While not as prevalent a concern as it is in pigs, vulva structure can influence mating efficiency in sheep. A small or tipped vulva can make penetration by the ram more difficult thus potentially reducing fertilization.

Puberty and the Breeding Season

The chronological age at which sheep typically reach puberty is 5–12 months. Influencing factors include breed, environment, photoperiod and nutritional status. Physiologically, a lamb is considered pubertal when it is capable of producing gamete and

maintaining a pregnancy. Body size is one of the most visible external measures. Ewe lambs must meet a minimum threshold of frame size and body condition prior to initiation of puberty. An additional factor that impacts a lamb's entry into the breeding population is the photoperiod in which they were born.

Sheep are termed to be "short day" breeders, meaning that the decreased light-levels associated with shorter days will positively influence the hormonal control of gamete production in both the male and female. Resultantly most sheep have a breeding season that is inversely correlated with the amount of light they are exposed to. As the typical gestation length of a sheep is 147 days, an ewe that is bred in the fall will lamb in the early spring. Therefore those lambs will first be exposed to increasing photoperiod and will be growing during this time (i.e., the non-breeding season). Their body composition will be suitable so that when they are then exposed to the shorter days of breeding season they are more apt to undergo puberty and successfully mate. Conversely, lambs born in the summer and/or early fall will not have reached a sufficient body mass to be pubertal during their first exposure to decreased day length. Nor will they have experienced the pattern of increasing and then decreasing photoperiods, which are necessary to stimulate puberty in sheep. Resultantly, they will enter puberty at an older age and will not breed until the following short-day time period, thus making them less reproductively efficient than their peers.

The transition from an anestrus or pre-pubertal state to one of cyclicity requires a change in the ewes' endocrine profile. Specifically, within the ewe's brain the action of kisspeptin neurons will trigger an increased frequency of pulsatile release of gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH). The change in endocrine release pattern will support ovarian function, including steroid hormone production and follicular maturation.

Behavioral signs of estrus are the most frequently utilized to determine a female is capable of breeding. However, those signs often accompany the second ovulation occurring during the transition from anestrus to estrus. When ewes enter the period of cyclicity either at puberty or the start of the breeding season, they frequently undergo a "silent ovulation" meaning that an oocyte is released from a dominant follicle without accompanying signs of estrus behavior. The hypothalamus requires progesterone priming in order to facilitate the endocrine cues necessary for such behavior. The corpus luteum (CL) formed from the silent ovulation will produce the progesterone necessary to feedback upon the hypothalamus to trigger initiation of estrus-like behavior in the subsequent cycle. The nuance of neuroendocrine control of cyclicity is one of the many challenges associated with determining the exact point at which a ewe enters puberty and/or the breeding season.

Cyclicity

The ewe typically has an estrous cycle (period of time between ovulations) that is approximately 17 days in length. The cycle is characterized by specific intervals of time that correspond to the presence of specific ovarian structures and endocrine profiles. During the period of estrus a female is receptive to mating. Behaviorally, she typically will stand to be mounted by the male, may mount other females and there may be discharge detectable from her vulva. During this time there is a dominant follicle present on the ovary that is producing quantities of estrogen capable of exerting positive feedback on the hypothalamus. At the end of estrus, estrogen levels will begin to decrease and there will be a surge in LH from the anterior pituitary that will trigger ovulation of the dominant follicle. The post-ovulatory follicle will then be termed a corpus hemorrhagicum (CH). The post estrus time period is metestrus and is the time of transition in which the remaining post-ovulatory follicular theca and granulosa cells give rise to the small and large luteal cells. These cells will comprise the corpus luteum (CL), which is the ovarian structure responsible for progesterone production. The CL is the dominant structure during diestrus and if the ewe were to become pregnant would remain intact throughout gestation. If the ewe does not become pregnant the CL will undergo a process of destruction known as luteolysis following exposure to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) produced by the uterine endometrium. The regressed CL leaves an inactive structure resembling a pale scar, the corpus albicans (CA).

In the absence of an embryo, endometrial $PGF_{2\alpha}$ travels from the uterine vein to the ovarian artery via counter-current exchange and with a series of coordinated frequent, high amplitude pulses is capable of inducing apoptosis of the CL. In the non-pregnant ewe this will occur at the end of diestrus and will result in a decrease in progesterone production. The ewe will then enter proestrus and with it will have increasing levels of estrogen, follicle stimulating hormone (FSH) and LH, as there is a follicular pool that has been preparing for ovulation during the subsequent estrus period.

The development of follicles suitable for ovulation is frequently described as a wave like pattern. In sheep there are typically three to four follicular waves within an estrous cycle (Fig. 3). The last wave in a cycle is the wave that results in ovulation. The proceeding waves do have an increase in FSH and estrogen levels from the follicles that have matured prior to undergoing regression. Manipulation of the follicular waves remains a key area of research interest within all ruminants.

Embryonic Development and Implantation

Early embryonic loss is an important factor in livestock production and resultantly considerable effort has been extended to understand the process of embryo development and subsequent implantation into the uterus in an effort to mitigate such losses. Following successful fertilization the embryo undergoes a series of morphological and developmental changes within the oviduct prior to entering the uterus (Fig. 4). After the initial fusion of the male and female pronuclei to form a single celled zygote the embryo will cleave into two blastomeres. The blastomeres will continue to divide and the embryo will reach the morula stage of

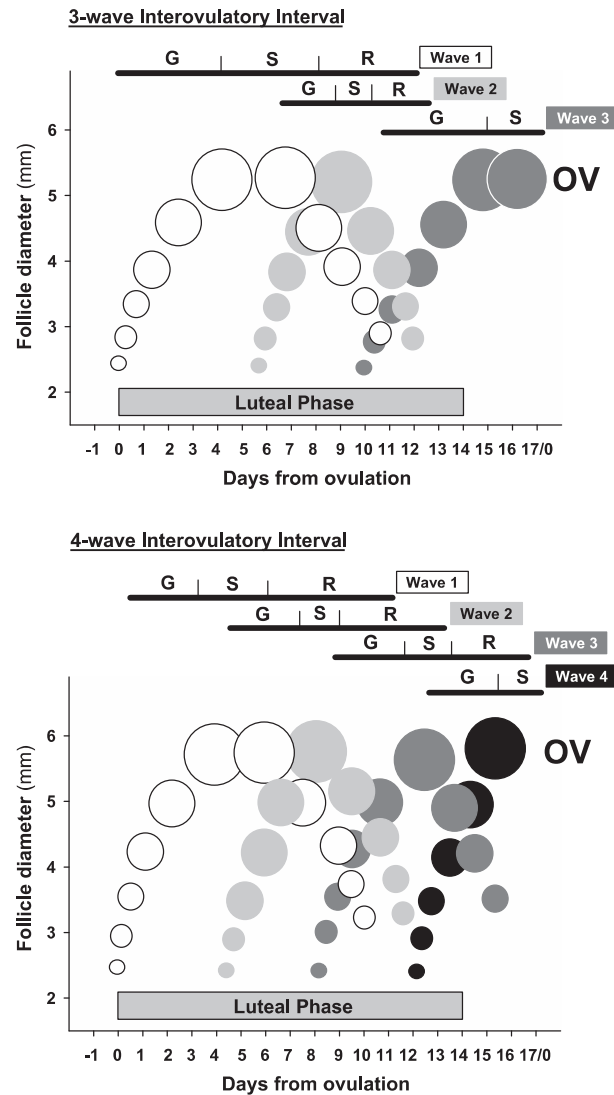


Fig. 3 Schematic illustration of the growth of ovarian antral follicles emerging at 2–3 mm in diameter during the ewes' estrous cycle. The largest follicles that grow to an ostensibly ovulatory diameter of ≥ 5 mm before regression or ovulation emerge in an orderly succession throughout the 17-day interovulatory interval, giving typically 3 or 4 follicular waves. OV=ovulation. The approximate durations of the growing (G), static (S) and regressing (R) phases of the lifespan of the largest follicles of waves are shown in the upper chart area. Reproduced from Bartlewski, P.M., Baby, T.E., Giffin, J.L., 2011. Reproductive cycles in sheep. *Anim. Reprod. Sci.* 124, 259–268.

classification by day 6 of pregnancy. At this point in development, the embryo is moving from the oviduct to the uterine horn and will enter the uterus as a blastocyst. The blastocyst is the embryo after it has undergone the process of compaction, which yields a defined inner cell mass, blastocoel and trophoblast. Upon hatching from the zona pellucida on day 8 of gestation the blastocyst will expand to form a spherical shape and begin the process of maternal recognition of pregnancy. The sheep embryo produces a type 1 interferon known as Interferon tau (IFNT) that serves as the maternal recognition of pregnancy signal and as such prevents production of endometrial $\text{PGF}_{2\alpha}$ and subsequent loss of the CL.

The mononuclear trophoblast cells, which are located on the periphery of the embryo, will secrete IFNT. The actions of IFNT on the uterine endometrium include the downregulation of estrogen receptor alpha, which will prevent stimulation of oxytocin receptor gene transcription. Loss of available oxytocin receptors will preclude oxytocin from stimulating the pulsatile release of $\text{PGF}_{2\alpha}$ capable of lysing the CL. In addition to down-regulation of factors driving luteolysis, binding of IFNT to its endometrial receptors causes upregulation of a series of IFN-stimulated genes necessary for successful implantation (Table 1).

Occurring concomitantly with maternal recognition of pregnancy is a rapid morphological change in the development of the sheep embryo (Fig. 4). After blastocyst formation and hatching, the embryo will undergo a series of changes from gestational days 9–15, that will result in it taking on an ovoid, tubular and ultimately filamentous form (approximately 10–15 cm in length). Successful elongation and implantation is dependent upon multiple factors.

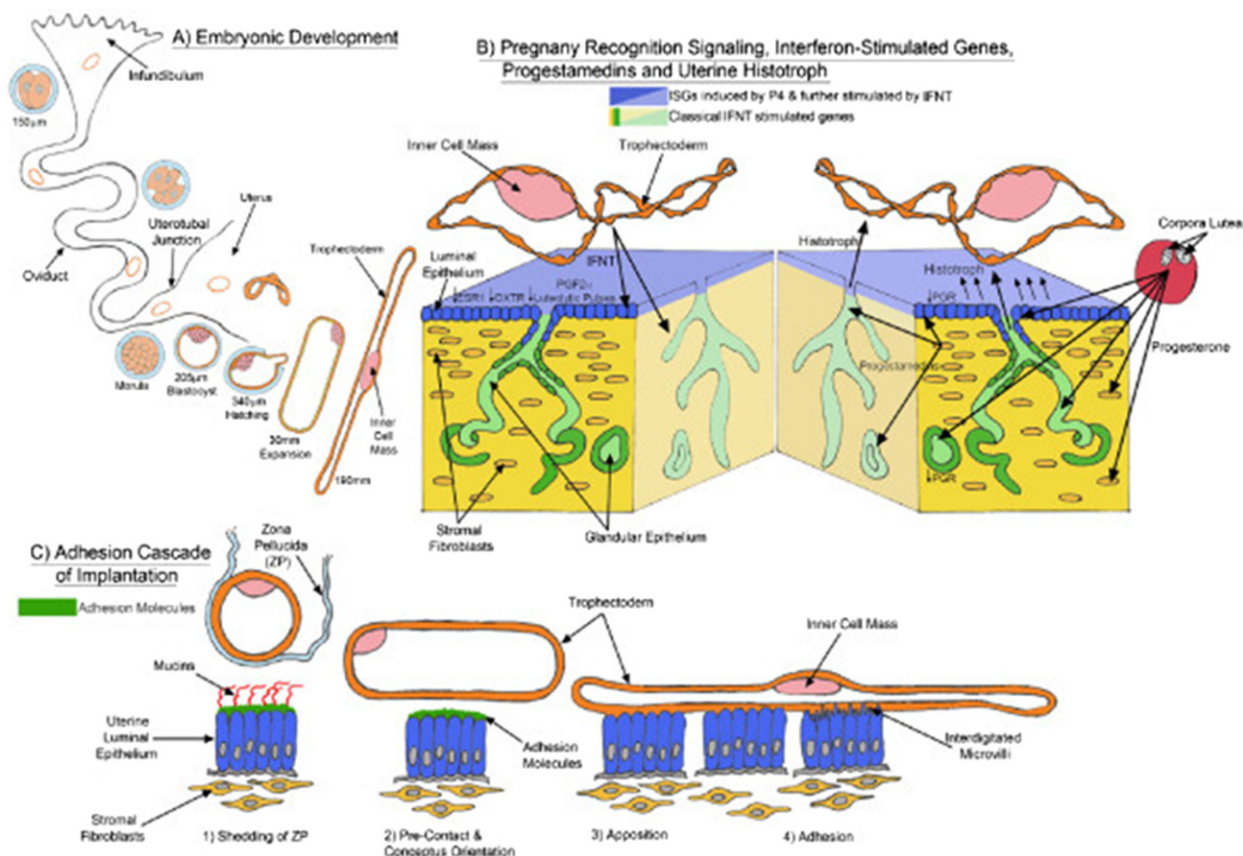


Fig. 4 (A) Oocytes fertilized in the oviduct enter the uterus at the morula stage, hatch from the zona pellucida as spherical blastocysts and then transition to large spherical, tubular and filamentous conceptuses (embryo and its extra-embryonic membranes) with interferon tau (IFNT), the pregnancy recognition signal, being secreted from mononuclear trophoblast cells between Days 10 and 21 of pregnancy. (B) Endometrial epithelia cease expressing receptors for progesterone (PGR) due to autoregulation by progesterone and IFNT silences expression of receptors for estradiol (ESR1) and oxytocin receptors (OXTR) to abrogate development of the mechanism for oxytocin-mediated pulsatile release of prostaglandin $F_{2\alpha}$ (PGF) which would otherwise cause regression of the corpus luteum and cessation of progesterone secretion. The endometrial stromal fibroblasts express PGR and secrete fibroblast growth factor 10 that regulates uterine epithelia cell function. With down-regulation of PGR in uterine epithelia the uterine luminal (LE) and superficial glandular (sGE) epithelia express genes that are either induced by progesterone (P4) or induced by P4 and further stimulated by IFNT. Further, IFNT induces expression of interferon regulatory factor 2 (IRF2) in uterine LE and sGE to silence expression of classical interferon stimulated genes and allow expression of a unique set of genes that promote conceptus growth and development. The endometrial glandular epithelial cells (GE) and stromal fibroblasts do not express IRF2 and, therefore, express classical interferon stimulated proteins. Collectively, molecules secreted by uterine epithelia or transported into the uterine lumen by uterine epithelia form histotroph required for conceptus development. (C) The ovine conceptus undergoes the adhesion cascade for implantation. Reproduced from Bazer, F.W., Song, G., Kim, J., *et al.*, 2012. Uterine biology in pigs and sheep. *J. Anim. Sci. Biotechnol.* 16:3 (1), 23.

Progesterone produced by the CL is necessary for conceptus elongation and implantation. Ovarian progesterone also induces expression of a variety of genes in the uterine endometrium (Table 1). The general classifications include those encoding transport of glucose, transport of amino acids, cell proliferation, migration, attachment, proteases and their inhibitors, and intracellular enzymes. Exposure to increased quantities of exogenous progesterone after ovulation will enhance conceptus elongation (Fig. 5).

Another key factor in conceptus growth and successful establishment of pregnancy in the peri-implantation period is the presence of the envelope protein of the endogenous Jaagsiekte sheep retrovirus (enJSRV). Unique to sheep, enJSRVs have integrated into the genome. Multiple copies of enJSRVs have been detected within the genome of domestic sheep. The enJSRV envelope protein has been deemed essential for appropriate cellular proliferation of the conceptus of the sheep, as its absence results in failed elongation leading to embryonic loss.

Among the hypothesized methods for communication between the developing conceptus and uterine endometrium of the sheep are exosomes or microvesicles. These structures are present in multiple tissues and body fluids including the uterine luminal fluid of the sheep as well as the endometrial epithelium. As they are membrane-covered nanovesicles (30–150 nm) that are capable of carrying genetic material such as mRNAs, they are potentially capable of exerting a great deal of influence on uterine gene expression as well as cellular function such as migration and elongation which is necessary for conceptus development and implantation in the sheep.

Table 1 Effects of ovarian progesterone (P4) and intrauterine infusion of interferon tau (IFNT) on elongation- and implantation-related genes expressed in the endometrial epithelia of the ovine uterus^a

<i>Gene symbol</i>	<i>P4</i>	<i>IFNT</i>
Transport of glucose		
<i>SLC2A1</i>	↑	+
<i>SLC2A5</i>	n.d.	n.e.
<i>SLC2A12</i>	n.d.	+
<i>SLC5A1</i>	↑	+
<i>SLC5A11</i>	↑	+
Transport of AA		
<i>SLC1A5</i>	n.d.	n.d.
<i>SLC7A2</i>	↑	+
Cell proliferation, migration, attachment		
<i>GRP</i>	↑	+
<i>IGFBP1</i>	↑	+
<i>LGALS15</i>	↑	+
<i>SPP1</i>	↑	+
Proteases and their inhibitors		
<i>CTSL1</i>	↑	+
<i>CST3</i>	↑	+
<i>CST6</i>	↑	+
Intracellular enzymes		
<i>HSD11B1</i>	↑	+
<i>PTGS2</i>	↑	+

^aEffect of hormone or factor denoted as induction (↑), stimulation (+), no effect (n.e.), or not determined (n.d.).

Data from Spencer, T.E., Forde, N., Lonergan, P., 2015. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J. Dairy Sci.* 99, 5941–5950.

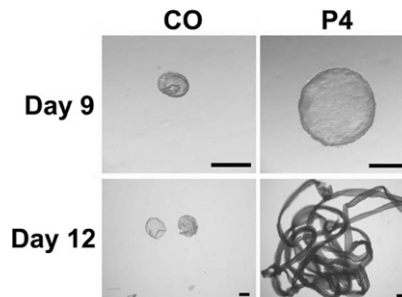


Fig. 5 Effects of early exogenous progesterone treatment on blastocyst morphology on Days 9 and 12 of pregnancy. In study two ewes were treated daily with corn oil (CO) vehicle or progesterone (P4) from 36 h postmating. On Day 9, blastocyst diameter was greater ($P < 0.02$) for ewes receiving P4 ($636 \pm 64 \mu\text{m}$) than CO ($282 \pm 64 \mu\text{m}$). In study 1, ewes were treated daily with CO or P4 from 36 h postmating to D 12. Elongated and filamentous conceptuses were recovered from P4-treated ewes, while only spherical to early tubular blastocysts were recovered from CO-treated ewes. Bar = 400 μm . Modified from Satterfield, M.C., Bazer, F.W., Spencer, T.E., 2006. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol. Reprod.* 75, 289–296.

The fully elongated sheep conceptus will adhere to the endometrial luminal epithelium of the uterine horn adjacent to the CL and begin the process of implantation. This process requires changes in relative uterine gene expression to signal down regulation of anti-adhesion molecules, and upregulation of adhesion molecules, as well upregulation of multiple IFN-stimulated genes. Successful implantation will continue to trigger changes in endometrial gene expression that will maintain production of histotroph, stimulate increased vascularization and angiogenesis within the endometrium and caruncles, as well as facilitate formation of syncytia necessary for successful placentation.

Placentation

Sheep have a synepithelialchorial type of placentation whereby the embryo does not invade into the uterine wall as it does in rodents or primates, but rather extends upon the uterine surface. In sheep the mononuclear trophoblast cells will form binucleate

cells that will interact with the luminal epithelium to form a syncytial layer. The intercaruncular endometrium and placenta maintain contact through a series of focal adhesions. These contact points are not as rigid as those found within the placentomes.

Subsequent to IFNT production by the trophoblast, the placenta continues to produce pregnancy specific proteins such as pregnancy associated glycoproteins (PAGs), pregnancy specific protein B (PSPB) as well as placental lactogen (oPL). The growing placenta is also a source of progesterone production. Interestingly, in the sheep the placenta produces sufficient quantities of progesterone after day 60 of pregnancy to maintain pregnancy without the contribution of the CL.

As pregnancy progresses there is a switch between histotrophic and hematotrophic nutrition in the sheep. In early pregnancy the intercaruncular areas of the pregnant uterus experiences a significant increase in capillary density, further facilitating the transfer of nutrients from the mother to the fetus through histotroph. Later in gestation, 85% of uterine blood flow to the uterus travels through developed placentomes, which are the primary locations for hematotrophic support of the developing fetus. Uterine blood flow increases by approximately three fold in the later half of gestation in the ewe. The initial increase in uterine blood flow is followed by an increase in placental mass, reaching its peak around day 90 of gestation. A secondary increase in flow occurs between days 120–140, coinciding with rapid fetal growth. Fetal and placental weights and uterine blood flow are highly correlative. Factors that alter fetal weight such as genotype, maternal nutrient intake, heat stress, altitude, and fecundity, result in parallel alterations in placental weight and uterine blood flow. Increased blood flow and placental mass help to facilitate the transfer of nutrients necessary to sustain the rapid fetal growth that is characteristic of the third trimester of pregnancy in the sheep.

Parturition

Parturition is an intricate process requiring interaction and endocrine regulation by the ewe and lamb. As pregnancy progresses the endocrine profile of the fetus begins to change. There is a stimulation of the hypothalamic-pituitary-adrenal axis of the fetus. This is responsible for an increased production of adrenocorticotropin hormone (ACTH) and adrenal cortisol, which are necessary for initiation of parturition.

Additionally, late in gestation placental hormone production begins to shift. There is an increase in production of prostaglandin E₂ (PGE₂) that occurs after day 100 of gestation. The PGE₂ can further stimulate the production of fetal ACTH and cortisol as well as initiating matrix breakdown necessary for cervical ripening. As parturition becomes eminent fetal cortisol production continues and there is a decrease in progesterone and corresponding increase in estrogen. Elevated estrogen increases secretions of the cervix and vagina to facilitate lubrications of the reproductive tract for delivery, and also serves to upregulate oxytocin receptors within the uterine myometrium. Binding of oxytocin to its receptors will facilitate the coordinated muscle contraction necessary for expulsion of the fetus and placental membranes.

Parturition can be complicated by the presence of multiple fetuses. Ideally lambs will present for delivery individually, with their head positioned between their forelimbs. Each lamb possesses an individual placenta and membranes must be expelled after parturition. The uterus will begin the process of involution allowing for repair and a return to its non-pregnant state. The udder of the ewe has two teats enabling her to nurse twins effectively. In cases in which there are greater than two lambs per ewe supplementation is frequently necessary. Lactation is beyond the scope of this article.

Influence of Maternal Nutrition on Reproductive Processes

Maternal nutrition impacts ovulation, fertilization, and fetal development. The process of increasing maternal nutritional intake through administration of a high energy feedstuff in the month prior to breeding is known as flushing. In ewes suffering from diminished body condition this process has the potential to increase ovulation and fertilization rates. Maternal nutrition is also critically important during pregnancy. Ewes receiving less than the National Research Council (NRC) recommendations are at risk for compromised pregnancies as maternal nutrition during pregnancy impacts fetal growth. Insufficient nutrient availability can cause restricted fetal growth resulting in lambs that are small for gestational age. Lamb production, the main source of income for sheep flocks worldwide, is dependent on the birth of viable lambs with appropriate birth weight, which is a key determinate of neonatal survival.

The placenta plays a key role in responding to maternal nutritional constraints to facilitate fetal growth. As pregnancy depends upon cross-talk between the maternal and the fetal compartments via the placenta an insufficient supply of nutrients from the mother, leads to compensatory adaptations to enhance size, architecture and function of the placenta allow demands for fetal-placental growth to be met. Placental gene expression influencing nutrient transport is altered in an effort to promote fetal growth as a means to enhance lamb viability.

Maternal metabolic stress resulting in diminished birth weight also translates into poor post-natal performance of lambs. Growth restricted and/or small birth weight lambs demonstrate slower growth rates, decreased feed intake, and either reduced adult carcass merit or reproductive performance when compared to their counterparts from well-fed dams.

Poor maintenance of a ewe's body condition during pregnancy can also lead to post-partum difficulties including decreased lactation and a slower return to cyclicity. While sheep are adaptable to a wide range of forage quality and availability, it is necessary to consider nutritional needs in order to facilitate reproductive success and offspring viability.

Conclusion

The sheep is a valuable commodity in terms of food and fiber production as well as serving as an interesting and useful model for research in reproduction. The understanding of basic sheep reproduction and how it is impacted by factors such as genetics, environment, and developmental programming, will enable development of more optimal management tools that will allow for increased reproductive performance and profitability.

Further Reading

- Belkacemi, L., Nelson, D.M., Desai, M., Ross, M.G., 2010. Maternal undernutrition influences placental-fetal development. *Biol. Reprod.* 83, 325–331.
- Borowicz, P.P., Arnold, D.R., Johnson, M.L., *et al.*, 2007. Placental growth throughout the last two thirds of pregnancy in sheep: Vascular development and angiogenic factor expression. *Biol. Reprod.* 76, 259–267.
- Burns, G., Brooks, K., Wildung, M., *et al.*, 2014. Extracellular vesicles in luminal fluid of the ovine uterus. *PLOS ONE* 10:9 (3), e90913.
- Driancourt, M.A., Gougeon, A., Royere, D., Thibault, C., 1993. Ovarian function. In: Thibault, C., Levasseur, M.C., Hunter, R.H.F. (Eds.), *Reproduction in Mammals and Men*. Paris: Ellipses, pp. 281–305.
- Dun, R., 1955. The cervix of the ewe. Its importance in artificial insemination of sheep. *Aust. Vet. J.* 101–103.
- Dunlap, K.A., Palmarini, M., Varela, M., *et al.*, 2006. Endogenous retroviruses regulate periimplantation placental growth and differentiation. *Proc. Natl. Acad. Sci. USA* 103, 14390–14395.
- Dunlap, K.A., Kwak, H.I., Burghardt, R.C., *et al.*, 2010. The sphingosine 1-phosphate (S1P) signaling pathway is regulated during pregnancy in sheep. *Biol. Reprod.* 82, 876–887.
- Dwyer, C.M., 2008. The welfare of the neonatal lamb. *Small Rumin. Res.* 76, 31–41.
- Gootwine, E., 2004. Placental hormones and fetal-placental development. *Anim. Reprod. Sci.* 82–83, 551–566.
- Hay, W.W., 1991. In vivo measurements of placental transport and metabolism. *Proc. Nutr. Soc.* 50 (2), 355–362.
- Igwebuike, U., 2010. Impact of maternal nutrition on ovine foetoplacental development: A review of the role of insulin-like growth factors. *Anim. Reprod. Sci.* 121, 189–196.
- Louey, S., Cock, M.L., Harding, R., 2005. Long term consequences of low birthweight on postnatal growth, adiposity and brain weight at maturity in sheep. *J. Reprod. Dev.* 51, 59–68.
- Matthews, S.G., Challis, J.R.G., 1996. Regulation of the hypothalamo-pituitary-adrenocortical axis in fetal sheep. *Trends Endocrinol. Metab.* 7, 239–246.
- Mellor, D.J., Mitchell, B., Matheson, I.C., 1977. Reductions in lamb weight caused by pre-mating carunclectomy and mid-pregnancy placental ablation. *J. Comp. Pathol.* 87, 629–633.
- Redmond, J.S., Baez-Sandoval, G.M., Spell, K.M., *et al.*, 2011. Developmental changes in hypothalamic Kiss1 expression during activation of the pulsatile release of luteinizing hormone in maturing ewe lambs. *J. Neuroendocrinol.* 23, 815–822.
- Reynolds, L.P., Borowicz, P.P., Vonnahme, K.A., *et al.*, 2005. Placental angiogenesis in sheep models of compromised pregnancy. *J. Physiol.* 565, 43–58.
- Reynolds, L.P., Redmer, D.A., 1995. Utero-placental vascular development and placental function. *J. Anim. Sci.* 73, 1839–1851.
- Rhind, S.M., Rae, M.T., Brooks, A.N., 2001. Effects of nutrition and environmental factors on the fetal programming of the reproductive axis. *Reproduction* 122, 205–214.
- Satterfield, M.C., Song, G., Kochan, K.J., *et al.*, 2009. Discovery of candidate genes and pathways in the endometrium regulating ovine blastocyst growth and conceptus elongation. *Physiol. Genom.* 39, 85–99.
- Senger, P.L., 2012. *Female anatomy, Pathways to Pregnancy and Parturition*, third ed. Redmond, OR: Current Conceptions, pp. 11–37.
- Sharma, R.K., Blair, H.T., Jenkinson, C.M., *et al.*, 2012. Uterine environment as a regulator of birth weight and body dimensions of newborn lambs. *J. Anim. Sci.* 90, 1338–1348.
- Shelton, M., 1964. Relation of birth weight to death losses and to certain productive characters of fall-born lambs. *J. Anim. Sci.* 23, 355–359.
- Smith, G.M., 1977. Factors affecting birth weight, dystocia and preweaning survival in sheep. *J. Anim. Sci.* 44, 745–753.
- Spencer, T.E., Johnson, G.A., Bazer, F.W., Burghardt, R.C., Palmarini, M., 2007. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. *Reprod. Fertil. Dev.* 19, 65–78.
- Steyn, C., Hawkins, P., Saito, T., *et al.*, 2001. Undernutrition during the first half of gestation increases the predominance of fetal tissue in late-gestation ovine placentomes. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 98, 165–170.
- Vonnahme, K.A., Hess, B.W., Nijland, M.J., Nathanielsz, P.W., Ford, S.P., 2006. Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions. *J. Anim. Sci.* 84, 3451–3459.
- Wu, G., Bazer, F.W., Wallace, J.M., Spencer, T.E., 2006. Board-invited review: Intrauterine growth retardation: Implications for the animal sciences. *J. Anim. Sci.* 84, 2316–2337.

Introduction

The reproductive biology of the mare features a number of enigmatic events that presumably evolved to facilitate the birth, at yearly intervals, of a single precocious neonate able to stand, suckle and run within hours of delivery. Given that gestation lasts 11 months, to produce a foal annually at a time when grass is abundant a mare needs to conceive in late spring to early summer, and within a month of any previous foaling. Equine pregnancy incorporates a number of unusual features, including the selective descent of developing embryos into the uterus, and an unusually long period of intrauterine conceptus migration that is essential for successful ‘maternal recognition of pregnancy’. In fact, the preimplantation period as a whole is unusually long, with the chorioallantois only finally establishing a stable villous attachment to the endometrium at day 40, by when it must support a fetus with precursors of all its major organs. Intriguingly, definitive non-invasive placentation is preceded by endometrial invasion by a specialized band of trophoblast cells to form temporary endocrine structures known as the endometrial cups. Once placental interdigitation has been initiated, to meet the nutritional and respiratory needs of a fetus that will weigh around 10% of maternal body weight at term (e.g., 80 kg in a draft breed), the surface area for exchange is maximized by extensive branching of the chorioallantoic villi to form the ‘microcotyledons’, accompanied by expansion of the chorioallantoic sac to line the entire uterine lumen. For the first 3 months of gestation, pregnancy maintenance depends on ovarian progesterone; thereafter the placenta assumes the (pro)gestagenic support role. Moreover, during mid-gestation the placenta cooperates with the tremendously enlarged fetal gonads to produce large quantities of unique estrogens that promote uterine blood flow and help prepare the uterus for parturition.

Reproductive Seasonality

Mares are long-day seasonally polyoestrus, i.e., in the absence of pregnancy they exhibit repeated estrous cycles during the late spring to early autumn (Hughes *et al.*, 1975) (Table 1). In the winter, the archetypal mare enters a period of deep anestrus characterized by profound ovarian inactivity, i.e., little or no follicle development and no functional corpus luteum. As the days lengthen in the spring, the mare passes into a ‘transitional phase’ characterized by the growth of follicles that fail to mature and ovulate (Nagy *et al.*, 2000). The principle stimulus to the onset of seasonal cyclicity is day-length, mediated primarily by melatonin secreted by the pineal gland exclusively during periods of darkness. Longer days/shorter nights restrict the periods of melatonin secretion, thereby removing a ‘physiological brake’ on hypothalamic secretion of the major stimulator of the reproductive endocrine axis, gonadotrophin releasing hormone (GnRH). Neuroendocrine pathways involving kisspeptin and dopamine-prolactin are also known to be involved in the control of seasonal reproductive cyclicity (Williams *et al.*, 2012) and, while it is not clear exactly how they are stimulated or controlled, they almost certainly feed in at the level of the hypothalamus, facilitating the increased frequency and amplitude of pulsatile GnRH release. GnRH secreted from the hypothalamus enters specific hypothalamic-pituitary portal vessels that transport it to the anterior pituitary, where it stimulates the gonadotrope cells to secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH). While the follicles that develop during the spring transition fail to ovulate, they do produce estrogens that in turn stimulate the mare to display sexual receptivity, either intermittently or for a prolonged period, a phenomenon known as ‘spring estrus’. The spring transition ends, and cyclical period begins, when the pituitary is able to generate an ‘LH surge’ capable of inducing ovulation (Williams *et al.*, 2012).

Table 1 Reproductive cycle parameters in the mare

	Mean	Range	Comments
Natural onset of seasonal cyclicity	Early April	March to May	Depends on breed, body condition, environmental temperature, exposure to other horses
Estrous cycle duration	21 days	17–24 days	Shorter in mid-summer Breed differences
Diestrus (luteal phase)	15 days	13–15 days	
Estrus (Follicular phase)	6 days	3–9 days	Shorter in mid-summer
Size of pre-ovulatory follicle	40 mm	30–60 mm	Consistent within individual mares. Breed differences
Follicle size at deviation	22 mm		Onset of LH-dependence Follicle dominance

While increasing day-length is the primary instigator of the spring transition, there are other contributors, including environmental temperature, body condition or nutritional plane, and pheromonal cues transmitted by close contact with stallions or other, cycling, mares (Nagy *et al.*, 2000). With respect to nutrition, mares in good to excessive body condition may cycle year-round, or show a shorter depth and duration of winter anestrus. A rising plane of nutrition can also hasten the spring transition. From a practical perspective, most horse registries assign an arbitrary birth date of January 1st. Since horses of some breeds (e.g., Thoroughbred racehorses) compete as juveniles (2–3 years old) or are sold via public auctions as foals or yearlings, there is considerable commercial pressure to ensure that foals are born early in the year so that they are more mature than their peers at the time of sale or racing. To aid this process, it is common to stimulate early seasonal cyclicity by exposing mares to ‘artificial long days’ (up to 16 h light) by providing additional lighting at dusk from late December (Nagy *et al.*, 2000).

The Estrous Cycle

The estrous cycle in the mare has a mean duration of 21 days, divided over a luteal or diestrous phase (progesterone dominance) of relatively constant duration (14–15 days) interspersed with follicular or estrous phases (estrogen dominance: period of sexual receptivity) of more variable duration (mean of 5–7 days: Hughes *et al.* (1975), Aurich (2011); Table 1). While recruitment of pre-antral follicles to the growing pool is probably gonadotropin independent, a ‘wave’ of small antral follicles develops FSH responsiveness and is stimulated to develop further by one of the FSH peaks that occur at approximately 10 day intervals. When the largest follicle(s) in a wave reaches 22 mm in diameter, one (occasionally two or more) is ‘selected’ for dominance and switches from FSH to LH dependence, allowing LH to promote further growth and maturation (Donadeu and Pedersen, 2008). The other (subordinate) follicles do not make this transition and instead shrink and become atretic. Indeed, the dominant follicle produces hormones (e.g., estrogens and inhibin) that suppress FSH release, thereby removing trophic support to the subordinate follicles. When growth of the dominant follicle to pre-ovulatory size (35–60 mm; varying between breeds and individuals) coincides with the end of the luteal (progesterone dominated) phase, a positive feedback between follicular estrogens and pituitary LH develops that generates the ‘surge’ in LH release that stimulates final follicle maturation and ovulation. By contrast, if a large follicle develops midway through the luteal phase it will not usually ovulate because circulating progesterone inhibits LH release and prevents the development of an ovulatory surge. In fact, the LH surge in mares is more gradual and prolonged than in other domestic species, only reaching a peak 1–2 days after ovulation, in part because of the long circulating half-life of LH in the mare (Stabenfeldt *et al.*, 1975). However, a relatively modest rise in LH is sufficient to trigger ovulation approximately 40 h later, about 10 h after an acute increase in intra-follicular prostaglandins (E2 and F2 α : Sirois and Doré (1997)) thought to be instrumental to weakening and rupture of the follicle wall. That relatively little LH is required to induce ovulation presumably explains why diestrous (mid-cycle) ovulation is more common in the mare than other species. The anatomy of follicle rupture highlights another peculiarity of the mare, namely that the ovarian cortex in which the follicles are situated is surrounded by both a vascular medulla and a thick tunica albuginea, rather than being located peripherally as in other species (Stabenfeldt *et al.*, 1975). The cortex reaches the surface of the ovary only at the ‘ovulation fossa’, which thanks to the absence of the tough overlying tunica, is the only site at which a follicle can rupture and release its oocyte into the adjacent oviductal infundibulum. When combined with the large size of equine pre-ovulatory follicles, the need to ovulate via the restricted fossa imposes a spatial impediment to ovulation of more than one follicle simultaneously, and undoubtedly contributes to the difficulty in stimulating multiple ovulation in mares.

Following ovulation, the ruptured follicle fills with blood to form a ‘corpus haemorrhagicum’, within which granulosa cells from the follicle wall migrate, and are transformed by LH (‘luteinized’) into luteal cells that organize the blood clot to form a highly vascular, progesterone-secreting corpus luteum (CL), the dominant endocrine organ of diestrus and early pregnancy. In the mare, the post-ovulatory rise in circulating progesterone is rapid, exceeding the 1 ng/mL that suppresses estrous behavior within 24–36 h, and continuing to a peak at around 8 days after ovulation. Luteal progesterone secretion then plateaus until day 13–14 when, in the absence of pregnancy, luteolysis (i.e., functional destruction of the CL) is triggered abruptly. Although the precise endocrine conditions that establish and initiate luteolysis are not known, a period of 8–10 days of progesterone priming enables oxytocin receptors to develop in the endometrium. By day 12–13 of diestrus, the endometrial oxytocin receptor population is sufficient to allow oxytocin, produced either in the hypothalamus and secreted from the posterior pituitary or produced locally within the endometrium, to bind to the endometrial receptors and trigger the release of the uterine luteolysin, PGF2 α . In turn, PGF2 α stimulates further release of oxytocin, thereby completing a positive feedback loop that results in the generation of the large pulses of PGF2 α (de Ruijter-Villani *et al.*, 2015) required to bring about functional demise of the CL and the associated precipitous fall (within 24 h) in circulating progesterone concentrations. For various reasons, e.g., reduced PGF2 α production capacity of degenerate endometrium, spontaneous failure of luteolysis leading to prolonged CL survival (typically 60–90 days) in the absence of pregnancy, is relatively common in the mare (Stabenfeldt *et al.*, 1975).

Mating, Fertilization and Tubal Development of the Embryo

Estrous behavior in the mare (sexual receptivity) is determined primarily by the absence of progesterone (<1 ng/mL), with rising estrogen concentrations serving to enhance the intensity of expression. Although there are significant individual variations, mares

are typically demonstrative in their expression of sexual receptivity. During progesterone dominance (e.g., diestrus or early pregnancy), mares react aggressively to the approach of a stallion (i.e., squeal, bite or kick) making it clear that his attention is unwelcome (Aurich, 2011). In the absence of progesterone, a mare's behavior can vary, or progress, from passive acceptance of a stallion's presence to actively seeking him out and demonstrating her readiness to mate by straddling her hind legs and elevating her tail; more intense displays to a stallion include rhythmic clitoral eversion ('winking') and/or micturition of a thick cloudy pheromone-laden urine that stimulates stallion interest. Even though estrogen concentrations decline from 1 to 2 days prior to ovulation, the mare will continue to display estrus until progesterone concentrations rise above 1 ng/mL, 1–2 days after ovulation. Assuming that a mare is mated naturally or inseminated artificially with freshly collected semen, it is assumed that the spermatozoa enter an oviductal 'sperm reservoir' by binding to oviduct epithelial cells in the distal isthmus of the oviduct (adjacent to the uterotubal junction); these spermatozoa retain their fertilizing capacity for at least 48 h.

LH stimulation of final follicle maturation 'reawakens' the oocyte to resume meiosis such that, at the time of ovulation, the oocyte has reached metaphase of the second meiotic division (MII) and is ready to be fertilized. The ovulated oocyte contained within an investment of supporting cumulus cells is 'caught' by the oviductal fimbriae, even though only a small proportion of the 40–50 mL of fluid evacuated from the follicle enters the oviduct; the cumulus-oocyte complex is then channeled via the infundibulum into the ampulla to await fertilization. At this point, a small number of the spermatozoa stored in the isthmus are activated ('capacitated'), by an as yet undefined signal, to detach from the oviduct epithelium and ascend through the isthmus to the ampulla, where they pass through the 'expanded' cumulus investment and bind to the outer coat of the oocyte (the zona pellucida). A single sperm will penetrate the zona pellucida, enter the perivitelline space and fuse with the oocyte to deliver its haploid packet of chromosomes and trigger fertilization.

After fertilization, the equine embryo remains in the oviduct for an unusually long time, approximately 6 days (Table 2), before descending into the uterus as a compacted morula comprising hundreds of morphologically indistinguishable cells. In fact, developing embryos are selectively transported into the uterus, whereas unfertilized oocytes are retained within the ampulla where they slowly degenerate. The key to this 'selective oviductal transport' is embryonic production of prostaglandins, principally PGE₂, from approximately day 5 after fertilization; PGE₂ relaxes the circular muscle of the oviductal isthmus causing it to dilate and allow the embryo to pass rapidly through and into the uterine lumen (Weber *et al.*, 1995).

Early Uterine Development

Soon after its arrival in the uterus on day 6, the horse embryo displays the first signs of cell lineage segregation, namely the coalescence of inter-cellular vacuoles to form a central cavity (blastocoel) lined by differentiated trophoblast cells, and within which the pluripotent cells aggregate to form the inner cell mass. At this point, the embryo is still surrounded by its original coat, the zona pellucida. Coincident with uterine entry, however, a second embryo coat known as the blastocyst 'capsule' forms within the perivitelline space between the trophoblast and zona pellucida (Table 2). When the embryo 'hatches' from the zona on day 7, as a result of rapid blastocyst expansion aided, presumably, by trophoblastic and/or endometrial proteases, the capsule persists. The capsule is initially composed of trophoblast-derived, mucin-like glycoproteins; however, these

Table 2 Timing of important events during equine pregnancy

Event	Time	Comments
Embryo descent into uterus	Day 6	Unfertilized oocytes remain in oviduct
Blastocyst formation	Day 7	Segregation of trophoblast and inner cell mass
Blastocyst capsule formation	Day 6–7	Embryonic glycoproteins require uterine contribution to coalesce
Zona pellucida loss (hatching)	Day 7	Soon after uterine entry & capsule formation
Yolk sac completed	Day 8	Primitive endoderm lines trophoblast
Conceptus migration phase	Days 6–16	Prostaglandins involved in propulsion Essential for maternal pregnancy recognition
Conceptus fixation	Day 16	Base of a uterine horn Coincident with capsule desialylation
Blastocyst capsule disintegration	Days 21–23	Disappearance completed around day 28
Apposition and adhesion phases of implantation	Days 22–40	Associated with formation and initial expansion of chorioallantois
Endometrial cup formation	Day 35–40	Chorionic girdle cells detach from conceptus and invade endometrium
eCG secretion/secondary CLs	Days 35–140	Varies between pregnancies (and equid genus)
Onset of placentation	Day 40	Stable microvillous attachment of chorion to endometrium
Microcotyledon development	Days 40–90	Secondary and tertiary branches increase surface area
Placental progesterone production	Day 60-term	Primarily 5 α DHP (very little native progesterone) Able to maintain pregnancy soon after day 70
Fetal-placental estrogen production	Day 60-term	Correlated with fetal gonad enlargement Peaks at about day 240
Foaling/parturition	Day 335–345	Enormous range (320–370 days). Depends on breed, parity, fetal sex, time of year

embryonic substrates require an unknown uterine contribution to coalesce into a confluent layer (Stout, 2016). Once formed, the capsule increases progressively in size and mass up to day 18, with endometrial proteins contributing significantly to this growth. While the functions of the capsule are not completely understood, it is essential for intra-uterine survival of the early embryo, and instrumental to maintaining the spherical shape of the rapidly expanding vesicle. The capsule also provides essential mechanical protection to the delicate yolk-sac vesicle during the period of intrauterine migration, and is present at the embryo-maternal interface during a period when the mobile conceptus must signal its presence to ensure 'maternal recognition of pregnancy', and harvest sufficient nutrients from the proteinaceous uterine secretions (histotroph) to support continued growth and development. It has been proposed that the capsule aids these processes by sequestering transport molecules that store or transmit biochemical signals or nutrients between the conceptus and endometrium (Stout, 2016). The capsule has also been proposed to protect against microbial or maternal immunological attack, and there is compelling evidence that it facilitates conceptus migration. In the latter respect, the abundant negatively charged sialic acid residues of the mucin-like capsular glycoproteins are thought to inhibit adhesion to the endometrium. Indeed, the end of the mobile period at day 16 is temporally associated with extensive desialylation of the capsular glycoproteins (Quinn *et al.*, 2007). With its role presumably completed, the capsule decreases in mass from around day 18, and by day 23 has lost its structural integrity and begun to disintegrate (Table 2); disappearance is completed by around day 28 (Allen and Wilsher, 2009).

Conceptus Migration and the Maternal Recognition of Pregnancy

During days 6–16, the equine conceptus vesicle expands rapidly (0.2 mm on day 6; 2 mm on day 10; 20 mm on day 14) but remains spherical and completely unattached to the uterine wall. Instead, the conceptus migrates continuously throughout the uterine lumen, propelled by myometrial contractions stimulated by prostaglandins either secreted by the conceptus or produced locally in the uterine wall in response to other conceptus products (Stout, 2016). Migration has been proposed to serve two major functions; (1) efficient harvesting of nutrients or growth factors from the uterine secretions, and (2) dispersal of the conceptus 'maternal recognition of pregnancy factor' (Table 2). Maternal recognition of pregnancy (MRP) involves the transmission of a biochemical signal(s) from the conceptus to its dam, to ensure that she makes the physiological changes required to support the continued survival and development of that conceptus. Since the most obvious outcome of MRP signaling is prolonged survival of the primary CL, MRP is generally taken to refer specifically to the processes involved in preventing luteal demise, and thereby ensuring ongoing provision of the progesterone required to maintain a qualitatively and quantitatively adequate supply of histotroph. Although the identity of the equine conceptus antiluteolytic factor(s) is not known, studies in which conceptuses failed to prevent luteolysis when restricted to a single uterine horn indicate that conceptus migration is critical to MRP signal transmission (McDowell *et al.*, 1988), just as blastocyst elongation ensures direct exposure of as much endometrium as possible to the MRP signals in pigs and ruminants. In fact, the spherical horse conceptus must deliver its anti-luteolytic signal to, and prevent PGF2 α release from, the entire endometrium rather than just the horn ipsilateral to the CL because the mare lacks the intimate intertwining of uterine vein and utero-ovarian artery that allows direct transfer of PGF2 α from the uterus to the ipsilateral ovary in ruminants; instead endometrial PGF2 α reaches the ovaries via the systemic circulation in the mare. Mechanistically, it appears that the equine conceptus disables luteolysis by a combination of suppressing the expression or binding affinity of endometrial oxytocin and PGF2 α receptors involved in the reciprocal stimulation loop that generates luteolytic PGF2 α pulses, and suppressing endometrial expression of prostaglandin endo-peroxide synthase 2 (PTGS2), an enzyme critical to PGF2 α synthesis (de Ruijter-Villani *et al.*, 2015).

Conceptus Fixation, Adhesion and Preparation for Implantation

After prolonging CL lifespan, the conceptus ceases to migrate and instead becomes lodged ('fixed'), at the base of a uterine horn as a result of its increasing diameter combined with a marked increase in uterine tone. The trophoctoderm and endometrium are still not in direct contact, however, because of the interposed capsule. Nevertheless, after vesicle fixation the capsular glycoproteins begin to undergo structural modifications, including desialylation and degradation of integral proteins (Quinn *et al.*, 2007), followed by attenuation, and culminating in a loss of structural integrity between days 21 and 23. Capsule disintegration allows the apposition and adhesion phases of implantation to begin and, not surprisingly, is associated with extensive upregulation, in both endometrium and trophoctoderm, of genes implicated in the implantation process in other species (Stout, 2016). In the diaspora following fixation and preceding stable placental attachment, it is likely that conceptus secretion of large quantities of estrogens and other vasoactive factors supports increasing nutrient demands by inducing an intense local endometrial hyperaemia and increased uterine blood flow; removal of the capsular barrier should also facilitate nutrient transfer. In addition, soon after fixation the embryo develops a primitive heart, blood cells and intra and extra-embryonic blood vessels yielding a functional circulation that will help deliver yolk-sac imbibed nutrients and oxygen to the developing embryo. The transition to a true placenta begins around day 20 with the development of the allantois as an outgrowth from the embryonic hindgut. By day 22, the allantois has enlarged to abut and fuse with the overlying chorion to form the chorioallantois which, during the coming days and weeks, will develop and vascularize in preparation for definitive placentation (Allen and Wilsher, 2009).

Implantation: Endometrial Cup Formation and Placental Interdigitation

Establishment of a stable placental attachment is preceded by an enigmatic invasive ‘implantation event’ involving a discrete band of specialized trophoctoderm cells that develop at the junction of the expanding allantois and regressing yolk-sac. These invasive trophoctoderm cells proliferate rapidly and pile up to form a distinct white band known as the ‘chorionic girdle’. At approximately day 35, the chorionic girdle cells detach from the conceptus, adhere to the endometrium and begin to invade via the endometrial glands (Table 2); the girdle cells pass through the epithelial basement membrane and into the endometrial stroma where they become binucleate, and cease to divide or invade further (Allen and Wilsheer, 2009). Here, the girdle cells organize into temporary endocrine structures known as the endometrial cups, which secrete large quantities of equine chorionic gonadotrophin (eCG), a hormone with considerable FSH-like activity in non-equid species. In the horse, eCG is almost exclusively LH-like in its effects, presumably to prevent overstimulating and wasting the ovarian follicle reserve during pregnancy, and instead stimulates steroid hormone (primarily progesterone) secretion by the primary CL and induces ovulation or luteinization of any medium to large follicles that develop under the influence of the ongoing waves of pituitary FSH production, resulting in the so-called secondary (ovulated) or accessory (non-ruptured, luteinized) corpora lutea. These additional CLs boost circulating progesterone concentrations, thereby acting as a ‘fail safe’ against loss of progestagenic support during the transition from ovarian to placental production. The endometrial cups are, however, recognized as foreign and trigger an intense cell-mediated and humoral maternal immune response characterized by the appearance of large numbers of lymphocytes and other immune cells around the endometrial cups, and of anti-paternal antibodies in the maternal circulation. This immune reaction eventually leads to ‘rejection’ of the endometrial cups, which are sloughed off the endometrial surface somewhere around day 120–140 of gestation, accompanied by a fall in circulating eCG concentrations.

By day 35, the embryo proper has developed limb buds and precursors of all major organs, to become a fetus. Only 5 days later does the principle implantation event start in earnest with the development of a microvillous attachment between the outermost chorion and underlying endometrial luminal epithelium. This establishment of a stable placental attachment is accompanied by interdigitation of the blunt primary chorioallantoic villi with corresponding up-growths from the endometrium (sulci). As gestation progresses, the surface area of the non-invasive, epitheliochorial interface increases dramatically in two ways; (1) the chorioallantois elongates rapidly such that it extends throughout the gravid horn and uterine body by day 65, and has also lined the whole of the non-gravid horn by day 85; (2) secondary and tertiary branching of the primary villi, matched by the accommodating endometrial sulci, leads to the increasingly complex labyrinthine interface characteristic of the mature microcotyledons (Table 2). Together these developments hugely increase the total area over which nutrients and gases can be exchanged, thereby compensating for the relatively long diffusion pathway inherent to an epitheliochorial placenta. By mid-gestation, microcotyledon formation is essentially complete although, as gestation proceeds, the microcotyledons enlarge and remodel to meet the increasing nutritional demands of the growing fetus (Allen and Wilsheer, 2009). The need to interdigitate with the vast majority of the endometrial surface to provide sufficient placental exchange to support the late gestation fetus, explains why it is extremely rare for a mare to successfully carry twins to term.

Endocrine Support of Pregnancy

Maintenance of early equine pregnancy depends on ovarian progesterone, initially from the primary CL and, following endometrial cup formation, supplemented by secondary CLs. As the fetus and placenta grow, so do their endocrine capacities and, from around day 60, measurable quantities of placental-derived 5α -pregnan-3,20-dione (5α -DHP) and other progestagens appear in the maternal circulation (Ousey, 2012)(Table 2). A parallel increase in uterine blood flow means that increasing quantities of steroid precursors are delivered to the fetal circulation, where they can be metabolized into progestagens before being secreted back into the maternal circulation. Studies involving the ovariectomy of pregnant mares, suggest that placental progestagen production is sufficient to maintain pregnancy from soon after day 70.

Horse conceptuses also secrete large quantities of estrogens from as early as day 8, with proposed functions during early pregnancy including promotion of uterine blood flow and optimizing histotroph quality and production. However, early conceptus estrogens are not measurable in the mare’s circulation and, while maternal estrogen concentrations do rise after day 35, the initial source is the eCG-stimulated primary CL. From about day 80, maternal circulating estrogen concentrations rise again; this time they are derived from the fetal-placental unit and including large quantities of the equine pregnancy specific unsaturated estrogens, equilin, equilenin and their hydroxy-derivatives (Ousey, 2012). This mid-gestation rise in fetal-placental estrogens is based on an enormous enlargement of the fetal gonads which produce the primary estrogen precursor, DHEA, that is aromatized by the placenta to yield the estrogens that enter maternal blood. Fetal-placental estrogens appear to stimulate uterine blood flow, and oppose the quietening effects of progesterone on the myometrial smooth muscles as parturition approaches.

Parturition and Post-Partum Recovery

While the mean length of gestation in the mare is 335–345 days, the exact duration varies tremendously between individuals and is affected by time of year (gestation is shorter for foals born late in the year), maternal parity, breed and sex of the foal (Table 2).

Table 3 Major events at foaling and during post-partum recovery in the mare

Event	Timing	Comments
Endocrine changes associated with preparation for parturition	2–5 days before foaling	Fetal ACTH and cortisol release Falling maternal progestagens, Increased estrogen: progestagen ratio
Foaling	20–45 min	Rapid and forceful
Expulsion of fetal membranes	15–90 min post-foaling	Significant delay endangers mare health
Post-partum estrus	8–12 days after foaling	In 80% of mares
Uterine involution	14–21 days	Recovery of endometrial architecture Contraction of myometrial musculature
Arrival of foal heat embryo in uterus	From day 14 after foaling	Embryo remains 6 days in oviduct Survival compromised by delayed involution
Desired re-establishment of pregnancy	<30 days after foaling	To maintain yearly foal production

That ‘normal’ gestation can range from at least 320–370 days makes it difficult to predict when a mare will foal. This is not helped by the fact that, while the endocrine changes indicating preparation for parturition in other species are also seen in the mare, they are much more rapid, acute and are only detectable in the last few days before equine delivery (Fowden *et al.*, 2008) (Table 3). Nevertheless, the major drive to parturition appears to be an increase in fetal adrenal cortical activity (fetal stress), with the resulting fetal cortisol triggering a rise in the maternal estrogen: progestagen ratio. Increasing estrogen dominance activates various pathways associated with myometrial contractility, including increased uterine oxytocin receptor expression and PGF2 α production, and allows the onset of the uterine contractions characteristic of the first stage of labour (Ousey, 2012). Increasingly coordinated myometrial contractions cause the chorioallantois to bulge into a cervix relaxed by PGE2 of utero-placental origin and the resulting dilation of the cervix triggers release of hypothalamic-pituitary oxytocin, via the Ferguson reflex, to further stimulate uterine myometrial contractions both directly and by provoking further uterine PGF2 α release. Second stage labour (i.e., fetal delivery) begins once the chorioallantois has ruptured, is assisted by powerful abdominal contractions and is normally completed very rapidly, i.e., within about 30 min (Table 3). After delivery of the foal, release of the fetal components of the placenta (i.e., the chorioallantois) during third stage labour is also completed relatively rapidly, normally within 15–90 min of birth, presumably in part because each non-invasive microcotyledon can relatively easily disengage from its endometrial ‘glove’.

Post-partum uterine involution, i.e., discharge of pregnancy and parturition-related debris, remodeling of the endometrium and contraction of the uterus to something approaching its pre-gravid size, is also surprisingly rapid in the mare. Again the non-invasive nature of placentation may contribute to rapid recovery since the endometrium presumably has to undergo a comparatively modest repair and remodeling processes. The post-partum period also features the development of a mature ovarian follicle leading to a ‘post-partum’ estrus at around day 9 after foaling (Table 3). Rapid uterine involution means that mating at this ‘foal heat’ can lead to successful conception, although anything that delays involution will reduce the likelihood of establishing or maintaining pregnancy. Interestingly, post-partum follicle growth appears to be a specific phenomenon related to the endocrine conditions of late gestation; once the resulting CL has regressed, the mare may still return to seasonal anestrus if she foaled early in the year before the days started to lengthen.

References

- Allen, W. R., & Wilsher, S. (2009). A review of implantation and early placentation in the mare. *Placenta*, 30, 1005–1015.
- Aurich, C. (2011). Reproductive cycles of horses. *Animal Reproduction Science*, 124, 220–228.
- de Ruijter-Villani, M., van Tol, H. T., & Stout, T. A. (2015). Effect of pregnancy on endometrial expression of luteolytic pathway components in the mare. *Reproduction Fertility and Development*, 27, 834–845.
- Donadeu, F. X., & Pedersen, H. G. (2008). Follicle development in mares. *Reproduction in Domestic Animals*, 43(suppl. 2), 224–231.
- Fowden, A. L., Forhead, A. J., & Ousey, J. C. (2008). The endocrinology of equine parturition. *Experimental and Clinical Endocrinology and Diabetes*, 116, 393–403.
- Hughes, J. P., Stabenfeldt, G. H., & Evans, J. W. (1975). The oestrous cycle of the mare. *Journal of Reproduction and Fertility Suppl.*, 23, 161–166.
- McDowell, K. J., Sharp, D. C., Grubbaugh, W., Thatcher, W. W., & Wilcox, C. J. (1988). Restricted conceptus mobility results in failure of pregnancy maintenance in mares. *Biology of Reproduction*, 39, 340–348.
- Nagy, P., Guillaume, D., & Daels, P. (2000). Seasonality in mares. *Animal Reproduction Science*, 60–61, 245–262.
- Ousey, J. C. (2012). Endocrinology of pregnancy. In A. O. McKinnon, E. L. Squires, W. E. Vaala, & D. D. Varner (Eds.), *Equine Reproduction* (second ed., pp. 2222–2233). Chichester, West Sussex: Wiley-Blackwell.
- Quinn, B. A., Hayes, M. A., Waelchli, R. O., Kennedy, M. W., & Betteridge, K. J. (2007). Changes in major proteins in the embryonic capsule during immobilization (fixation) of the conceptus in the third week of pregnancy in the mare. *Reproduction*, 134, 161–170.
- Sirois, J., & Doré, M. (1997). The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. *Endocrinology*, 138, 4427–4434.
- Stabenfeldt, G. H., Hughes, J. P., Evans, J. W., & Geschwind, I. I. (1975). Unique aspects of the reproductive cycle of the mare. *Journal of Reproduction and Fertility Suppl.*, 23, 155–160.
- Stout, T. A. (2016). Embryo-maternal communication during the first 4 weeks of equine pregnancy. *Theriogenology*, 86, 349–354.

- Weber, J. A., Woods, G. L., & Lichtenwalner, A. B. (1995). Relaxatory effects of prostaglandin E2 on circular smooth muscle isolated from the equine oviductal isthmus. *Biology of Reproduction Mongraph*, 7, 125–130.
- Williams, G. L., Thorson, J. F., Prezotto, L. D., et al. (2012). Reproductive seasonality in the mare: Neuroendocrine basis and pharmacologic control. *Domestic Animal Endocrinology*, 43, 103–115.

Rodents

Jennifer L Herington, John C Reese, and Bibhash C Paria, Vanderbilt University Medical Center, Light Hall, Nashville, TN, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Research using animal models has played a vital role in virtually every major area of biomedicine, including our understanding of female reproduction. Among animals used in laboratory research, rodents have emerged as the model of choice. There are more than 2000 rodent species with diverse physiology and behavior. The mouse, rat, hamster, gerbil and guinea pig are the most commonly used rodents for laboratory research. Therefore, the genomes of mouse, rat, hamster, guinea pig and human have been sequenced. These genomes are composed of varying numbers of genes packaged in chromosomes. Based on size, the genome of the rat and guinea pig appears the closest to humans (Table 1).

Reproduction (sexual or asexual) is crucial for the purpose of genetic propagation to the next generation. Sexual reproduction includes inbreeding and outbreeding. The benefit of outbreeding males and females of the same species is the increased genetic variability due to exchange of genes. Conversely, inbreeding reduces the genetic diversity by increasing homozygosity leading to high risk of inheriting genetic diseases. Inbred homozygous animal models are preferred in biomedical studies in order to limit variations in experimental parameters. Unfortunately, the usage of a single inbred strain of species likely does not capture the genetic variations observed in outbred species like humans.

Reproductive Maturity and Cyclicity

Puberty

The chromosomal sex of the zygote (fertilized egg) determines the sexual phenotype of the offspring. The time of sexual differentiation varies among species. In long gestational species (human and guinea pig) sex differentiation occurs prenatally, but in short gestational species (mice and rats) it continues into the neonatal period. The reproductive ability of females begins at puberty, which is marked by the onset of the reproductive cycle: the estrous cycle in rodents and menstrual cycle (menarche) in humans. Rats begin cycling immediately after vaginal opening, whereas mice and hamster attain sexual maturity several days or weeks after their vaginal opening. The onset of the human menstrual cycle occurs between the ages of 8–12 (Table 1).

Anatomy of the Female Reproductive Tract

The female reproductive system consists of a pair of ovaries, each attached to fallopian tubes connected to either a single uterine body (human) or two separate uterine horns (bicornuate; rodents). The uterus is connected to a cervix to separate it from the vaginal canal. In rodents, the ovaries are encapsulated with a bursa (a thin membranous structure) to guard it from the peritoneal environment. The bursa is not continuous in humans, making a peritoneal ectopic pregnancy possible. In the majority of rodents, the bicornuate uterus leads into one cervix. However, in hamsters and chinchillas, the bicornuate uterus leads into two cervical canals.

Stages of the Reproductive Estrous Cycle

The estrous cycle length for the majority of commonly used laboratory rodents is shorter (4–5 days) than the less commonly used rodents which exhibit a lengthy cycle (greater than two weeks; Table 1). One factor underlying the extended period of estrous cycle in the guinea pig is formation of a functional corpus luteum (CL) in the absence of mating. This is very similar to humans where luteal phase continues for ~14 days after ovulation.

The estrous cycle of rodents is divided into four stages based on vaginal smear cytology and proportion of vaginal epithelial cells, cornified epithelial cells and leukocytes. In a standard 4-day cycle, the order of stages includes: 1) *proestrus*, the preovulatory day in which there is predominance of nucleated epithelial cells; 2) *estrus*, the day of ovulation and receptive phase for breeding whereby there are irregular-shaped, needle-like and enucleated cornified (or keratinized) squamous epithelial cells; 3) *metestrus*, the day of non-receptivity in which there is a mixture of cornified epithelial and leukocytes; and 4) *diestrus*, another day of non-receptivity whereby there is a predominance of leukocytes (Deb et al., 2006). When determining the stage of estrus, it is important to note that: 1) the regular estrous cycle in mice is not as distinct as rats and hamsters; 2) it is possible that the day of estrus or diestrus lasts for 2 consecutive days, thereby creating a five day cycle; and 3) sometimes a vaginal smear may reflect a transitory period between estrous cycle days.

The major differences between the reproductive cycle of rodents and humans are the longer cycle length (~28–30 days; Table 1) and uterine cyclic changes, including menstruation. In humans, the beginning of the menstrual cycle is marked by the shedding of uterine endometrium (menstruation), which lasts ~3–7 days. Similar to rodents, the menstrual cycle is divided into phases based on ovarian or uterine changes, which are described in detail below. During each reproductive cycle of most rodents and humans,

Table 1 The chromosome number and reproductive parameters of rodents and humans

		<i>Chromosome (#, 2n); Genome (# base pairs)</i>	<i>Sexual maturity</i>	<i>Cycle length (Days)</i>	<i>Ovulation type</i>	<i>Blastocyst Implantation Type</i>	<i>Blastocyst Implantation^b</i>	<i>Gestation length (Days)</i>	<i>Postpartum estrus</i>	<i>Lactation delay</i>
Rodent										
Commonly used in the laboratory	Mouse	40; ~2.6 billion	4–5 weeks	4–5	Spontaneous	Eccentric	Midnight of day 4	20	Yes	Yes
	Rat	42; ~2.7 billion	4–5 weeks	4–5	Spontaneous	Eccentric	Afternoon of Day 5	21–23	Yes	Yes
	Gerbil	44; unknown	9–12 weeks	4–5	Spontaneous	Interstitial	Day 7	23–26	Yes	Yes
	Syrian Hamster	44; ~2.5 billion	4–6 weeks	4	Spontaneous	Eccentric	Afternoon of day 4	16	Yes	Yes
	Guinea pig	64; ~2.7 billion	4–6 weeks	15–18	Spontaneous	Interstitial	Day 7–8	60–72	Yes	Yes
Less-common in the laboratory	Voles	17–64; ^a unknown	35–47 days	14–19	Induced	Eccentric	Evening of Day 4	21	Yes	Yes
	Chinchilla	64; unknown	4–8 months	25–50	Blend of Both	Interstitial	Day 5	110–130	Yes	Yes
Human		46; ~2.9 billion	8–12	28–30	Spontaneous	Interstitial	Day 20–23 of secretory phase	~280	No	Unknown

^aSpecies dependent; males and female have different chromosome numbers; female have chromosome from both sexes.^bCopulatory plug or presence of sperm in vaginal smear = day 1 of pregnancy.

ovulation occurs spontaneously regardless of mating with male. However, in some rodents such as voles, ovulation is induced by vaginal stimulation from copulation with a male (Jemiolo, 1983) (Table 1).

Hormonal Changes During the Estrous Cycle

In mammals, the reproductive cycle is regulated by cyclic variations in the levels of: 1) peptide hormones from the hypothalamus and pituitary gland and 2) steroid hormones from the ovary. The gonadotropin releasing hormone (GnRH) of the hypothalamus triggers the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. FSH is responsible for stimulating ovarian follicular growth and estrogen (E) production by the developing follicle. LH triggers oocyte release from the mature follicle (ovulation) and development of corpus luteum (CL) which secretes more progesterone (P_4) than E (Christensen *et al.*, 2012).

The differences observed in the levels of pituitary and ovarian hormones among rodent species during their estrous cycles are detailed below:

- 1) Mouse: The proestrous phase corresponds to an increase in E levels followed by increased release of FSH and LH into the circulation. During estrus, E levels decline. P_4 levels start to rise during metestrus, peak during diestrus and then decline until estrus (McLean *et al.*, 2012).
- 2) Rats: Unlike the mouse, serum E levels remain elevated during the early part of estrus, before returning to basal levels on the afternoon of estrus. Moreover, serum P_4 levels of this species significantly increase during the transition from proestrus to estrus, following peak levels of FSH and LH, which is different compared to mice.
- 3) Hamster: Unlike the variable 4–5 day cycle length of rats and mice, hamsters exhibit a regular 4 day cycle. Serum FSH and LH levels peak on the afternoon of proestrus prior to ovulation. In hamsters, FSH shows another peak on the morning of the estrus (unlike mouse and rats). E and P_4 levels show an inverse relationship during the cycle. E secretion markedly increases a full day prior to LH release, but P_4 secretion begins only in response to the LH surge.
- 4) Gerbil: Serum FSH and LH levels peak at proestrus and estrus, respectively. Serum E levels peak at proestrus and displayed no significant difference throughout the estrus, metestrus or diestrus stages. Serum P_4 levels remain constant through proestrus, metestrus and diestrus stages, peaking at estrus.
- 5) Guinea pig: Little information is available regarding the changes in E and P_4 levels during the estrous cycle of guinea pigs, largely due to the relatively lengthy 16 day cycle. E levels are elevated during proestrus and beginning of preovulatory estrus, thereby preceding the preovulatory LH peak. There is no preovulatory FSH peak. The peak levels of plasma P_4 are observed on luteal phase of the cycle (day 12) and fall thereafter, reaching extremely low levels on day 1 of the cycle.

As shown in Fig. 1, the major difference in hormonal changes between the estrous and menstrual cycle is the slight secondary increase in E levels during the middle of the post-ovulatory (luteal) phase.

Ovarian Changes During the Cycle

The ovary contains its maximum number (millions) of oocytes at birth, which then deplete in number from puberty to menopause. In each reproductive cycle of humans and rodents, several ovarian follicles containing an oocyte begin to grow or mature. During the follicular maturation period, at least one follicle in humans, and more than one follicle in rodents, becomes dominant and grows faster than others; marking the major difference in ovarian changes between humans and rodents. The stages of follicular development include: primordial → primary → secondary → tertiary (or Graafian) follicles.

Based on follicular changes, the ovarian cycle of rodents and humans is divided into three phases (Schank and McClintock, 1992):

- 1) Preovulatory phase (follicular phase) is defined as the follicular growth and maturation for ovulation. The follicular phase occurs during the proestrus stage of rodents, and uterine proliferative phase of humans.
- 2) Ovulatory phase (ovulation) is the event of oocyte release from the follicle and is defined as estrus/heat/lordosis in which the female exhibits maximum sexual motivation and receptivity.
- 3) Postovulatory phase (luteal phase) occurs when Graafian follicles, in the absence of their oocyte, develop into CL. The CL secretes P_4 during the luteal phase. The luteal phase is analogous to the metestrus and diestrus of rodents, and uterine secretory phase of the human cycle.

During the proestrus stage, follicles grow and mature into Graafian follicles. The estrus stage of the cycle is recognized by the presence of newly formed CL and the absence of Graafian follicles. At metestrus, the luteal cells of the CL begin producing P_4 . The functional demise of the CL (referred to as luteolysis) begins at the end of metestrus and continues to diestrus. Presence of a maximally-sized CL is the best marker of a diestrus ovary. Luteolysis is visible in the diestrus stage and results in decreased P_4 synthesis, which releases inhibition of LH secretion, allowing proestrus to begin. Although the aforementioned ovarian changes occur during the rodent estrous cycle, the specific stage is difficult to discern by ovarian morphology alone, since all stages contain follicles in each phase of development as well as multiple generations of CL. A regressed CL remains in the ovary for 10–15 days in most rodents, except hamsters which show no presence of CL in their next cycle.

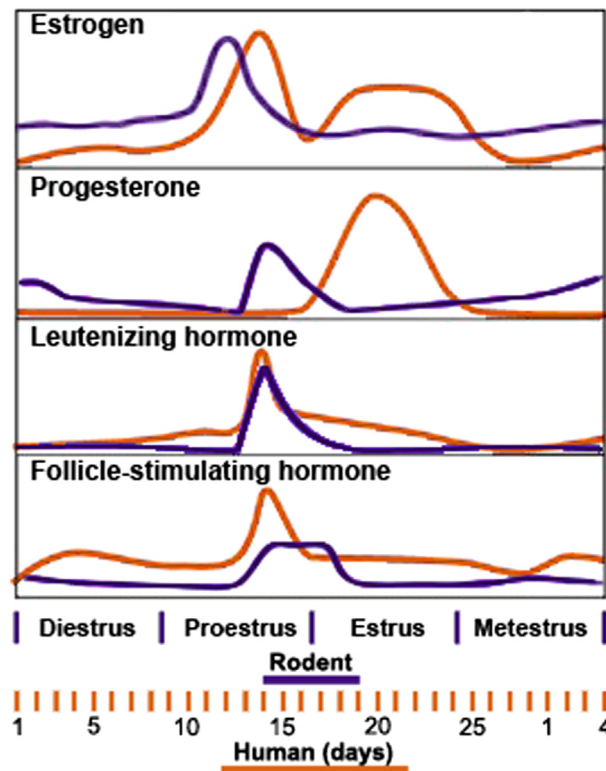


Fig. 1 A schematic drawing of hormonal profiles during female reproductive cycles of rodents and humans.

Uterine Changes During the Cycle

At proestrus, the rodent uterus is somewhat distended due to fluid accumulation. Uterine luminal epithelial (LE) cells become columnar shaped and proliferate. Stromal edema is also observed at proestrus. During the estrus stage, the uterus is fully distended and has maximum secretory activity due to a pronounced increase in the endometrial extracellular fluid. During estrus, proliferation occurs in the stroma but not epithelium. At metestrus, proliferation occurs in luminal and glandular epithelial cells, simultaneous to apoptosis of stromal cells as well as the proliferating LE cells. Finally, during the diestrus stage, the rodent uterus is small and poorly vascularized, with a collapsed lumen.

There are key differences between the uterine changes occurring in the estrous and menstrual cycles. In the human, the cyclic uterus undergoes changes to achieve receptivity and stromal cell decidualization in order to support the possible presence of a blastocyst for implantation. The human uterine cyclic changes are divided into three phases (Mihm *et al.*, 2011):

- 1) Menstrual phase is the event of endometrial shedding occurring the first 4–7 days of the cycle.
- 2) Proliferative phase is defined by uterine endometrial regrowth, lasting ~10 days between the menstrual and secretory phases. After menstruation, ovarian follicular E production stimulates the proliferation of endometrial epithelial, glandular and endothelial cells, thereby increasing the uterine lining thickness.
- 3) Secretory phase is characterized by an increase in endometrial secretory activity corresponding to the ovarian luteal phase, lasting ~14 days after ovulation on day 14. On cycle days 16–20, endometrial glands become more tortuous and dilated, and accumulate glycogen. On cycle days 21 and 22, the endometrial stroma begins becoming edematous. On cycle days 23–25, stromal mitosis is apparent. Predecidual cells appear first around the spiral arterioles and later under the LE. On cycle day 27, there is a marked lymphocytic infiltration and endometrial stroma appears as a solid sheet of well-developed decidual-like cells.

Regulation of the Reproductive Cycle by the Hypothalamic-Pituitary-Ovarian Axis

Transition from the prepubertal period to puberty is marked by increase in GnRH production. Upon the onset of puberty, occurrence of repeated regular cycles is a function of phasic production and secretion of hormones from the hypothalamus, pituitary and ovary. As shown in Fig. 2, hypothalamic GnRH stimulates pituitary production of FSH and LH which then act on the ovary. Under the control of FSH during the late stage of the proestrus in rodents and follicular phase in humans, follicles grow and produce increased levels of E, causing a positive feedback loop for additional FSH and LH production by the anterior pituitary. Under the control of LH during early estrus in rodents and ovulatory phase in humans, ovulation occurs and mature follicles are transformed into CLs. The CLs begin producing more P_4 than E, resulting in an inhibition of the hypothalamic-pituitary-ovarian positive

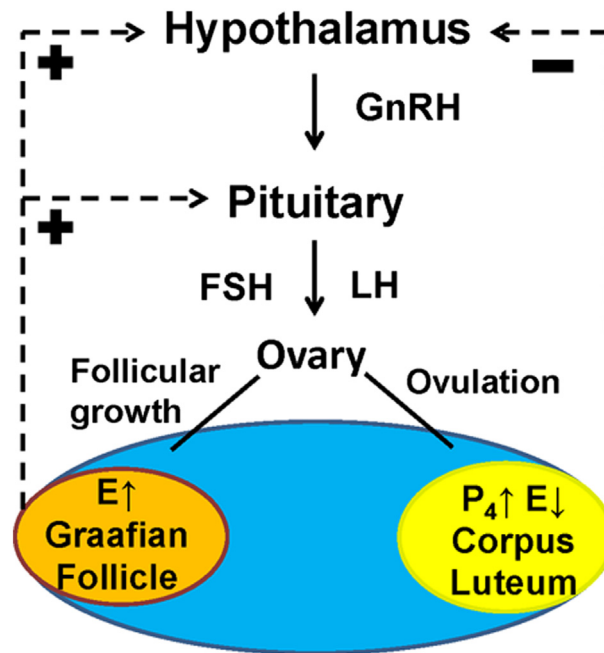


Fig. 2 A schematic view of the hypothalamus, pituitary and ovarian reproductive axis. E, estrogen; FSH, follicle stimulating hormone; LH, luteinizing hormone; GnRH, gonadotropin releasing hormone.

feedback loop. If pregnancy does not occur following ovulation, the CL undergoes luteolysis, resulting in decreased P_4 production, which in turn allows the hypothalamus to produce GnRH to resume the cycle (Mihm *et al.*, 2011).

Current dogma is that the occurrence of repeated regular cycles is a function of phasic ovarian E and P_4 secretion. However, E and P_4 receptor gene ablation studies in mice and rats suggest that while both ovarian E and P_4 functions are required for estrous cyclicity in mice, E but not P_4 function is needed for estrous cyclicity of rats. Perhaps, the maintenance of regular menstrual cycles in humans is also not dependent on functional P_4 , since women that are P_4 – resistant exhibit regular cycles with normal luteal phase duration (Kubota *et al.*, 2016).

Pregnancy Establishment

Identification of Pregnancy

Pregnancy begins when successful mating occurs around the time or day of ovulation, to allow fertilization of the egg. After a successful copulation, components of the male rodent ejaculate coagulate to form a plug occluding the vagina. The semen of humans coagulates to form a loose gel rather than a fibrous plug seen in rodents. The copulatory plug is used as an indicator of successful coitus in most rodents. However, the copulatory plugs do not stay in the vagina for as long of time in rats, gerbils or hamsters, compared to mice. Thus, the best method to ascertain mating in any species is the microscopic identification of sperm in a vaginal saline lavage. The estrous stage of the hamster cycle is also determined by visually inspecting the vaginal discharge.

Fertilization of the oocyte occurs within the fallopian tube. The window of fertilization lasts for a limited time span: 6 days in humans, but only hours in rodents due to the short survival time of sperm and egg. Remarkably in humans, an oocyte can survive for a day and sperm can remain active for up to 5 days.

The earliest that pregnancy can be identified in rodents is during blastocyst attachment to the uterus. At this time, due to an increase in localized vascular permeability, a macromolecular dye can be used to visualize sites of blastocyst attachment at various time points in early rodent pregnancy, as outlined in Table 1. In humans, blood or urine tests for human chorionic gonadotropin (hCG) levels allows a convenient method to identify early pregnancy following copulation, since hCG is increasingly produced by the syncytiotrophoblast after implantation.

Uterine Preparation for Pregnancy Establishment

In the event of fertilization, two processes occur in synchrony: 1) the CL survives and produces P_4 to prepare the uterus for embryo attachment and implantation, and 2) the fertilized egg mitotically divides to reach the blastocyst stage and migrates from the oviduct to the uterus. In general, the uterine preparation period from the day of fertilization to the day of blastocyst attachment is known as the preimplantation period. The preimplantation uterine changes begin on day 2 of pregnancy and continue until

implantation. The preimplantation uterine period is divided into prereceptive and receptive phases. During the prereceptive phase (days 2–4 in mice), the uterine LE is non-adhesive to the blastocyst. The receptive phase allows blastocyst implantation to occur during a limited window of opportunity known as the 'window of implantation'. In pregnant and pseudopregnant mice, the window extends from late day 4 of pregnancy to early day 5. In women, the window of receptivity begins on cycle day 19–20 and lasts for 4–5 days. After the uterine window of implantation, the uterus gradually loses its receptivity and becomes refractory to support blastocyst implantation (Dey *et al.*, 2004).

Steroid Hormones Required for Blastocyst Implantation

The preovulatory E secretion from the growing ovarian follicles induces uterine LE proliferation on day 2 of pregnancy, which is then suppressed by the postovulatory increase in P_4 secretion from the CL. Postovulatory P_4 influences the initiation of uterine stromal cell proliferation, allowing the uterus to acquire a receptive phenotype permissive to implantation. The period of uterine receptivity begins in a P_4 -primed uterus following a small elevation of circulating E levels in mice, rats and gerbils. In these species, the necessity of a small estrogen increase was ascertained by studies showing that removal of preimplantation estrogen elevation experimentally leads to a condition called "delayed implantation" (discussed in detail below). While a small spike of ovarian E secretion prior to blastocyst implantation is crucial for the initiation of implantation in rodents (like mice, rats and gerbils), it is not important for implantation in other rodents (such as guinea pigs and hamsters) (Fig. 3). The role of ovarian E in initiation of implantation in humans remains debatable. Since ovarian E secretion may not be necessary for blastocyst implantation in humans, hamsters or guinea pigs, it is possible that the blastocysts of these species are capable of producing E of which there are a handful of reports (Reese *et al.*, 2008).

Delayed Blastocyst Implantation

Any delay in blastocyst implantation, whether natural or experimental, is referred to as 'delayed implantation'. The growth arrest of the blastocyst prior to implantation can be considered as a quiescent (latent) period. The length of this latent period is dependent on the uterine ability to attain receptivity, and could last 1–2 days or even months depending on the species. Thus, preimplantation blastocyst latency within a prereceptive uterus is a protective phenomenon from an unfavorable uterine environment, and is an embryonic trait conserved across mammalian species. Rodents experience a lactational delay of blastocyst implantation due to a combination of postpartum estrus, mating and lactation. Immediately following parturition, rodents experience a fertile postpartum estrus. However, since rodents are nursing their offspring, the implantation of latent blastocysts occurs after the end of suckling stimulus (Mead, 1993).

Delayed implantation can be experimentally induced in rodents. To do so, mice, rats and gerbils are ovariectomized or hypophysectomized prior to the preimplantation ovarian E secretion in order to prevent the uterus from becoming receptive. Experimentally-induced delayed implantation can be maintained for several weeks with daily P_4 supplementation. However, a single low dosage injection of estradiol-17 (E_2) causes both the uterus and blastocyst to achieve implantation competency resulting in initiation of the implantation process. The experimental delay in implantation cannot be initiated in rodents such as hamsters and guinea pigs, whose implantation is P_4 -dependent (Paria *et al.*, 1993; Reese *et al.*, 2008). Experimentally-induced delayed implantation cannot be tested in humans due to ethical reasons.

There is no clinical data in humans concerning ovulatory status immediately after parturition and lactational delays in implantation. Clinical findings have showed that women undergo postpartum amenorrhea (the absence of menstrual cycle). The length of

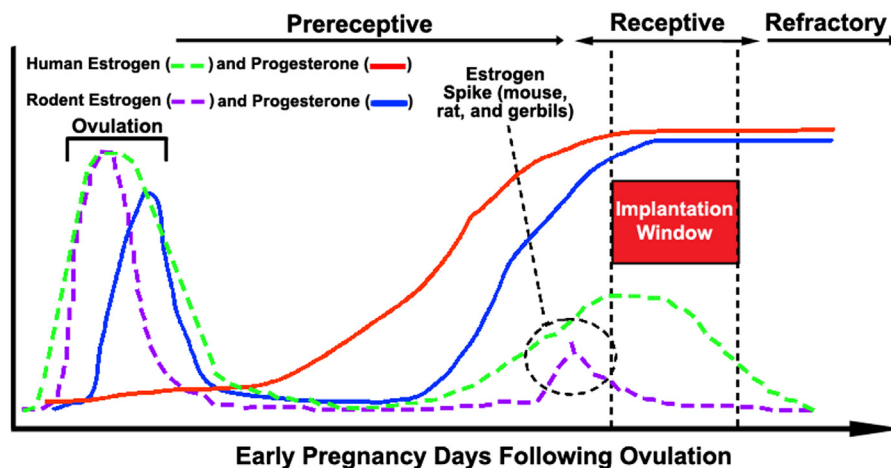


Fig. 3 A schematic drawing of ovarian steroid hormonal profiles during early pregnancy in rodents and humans.

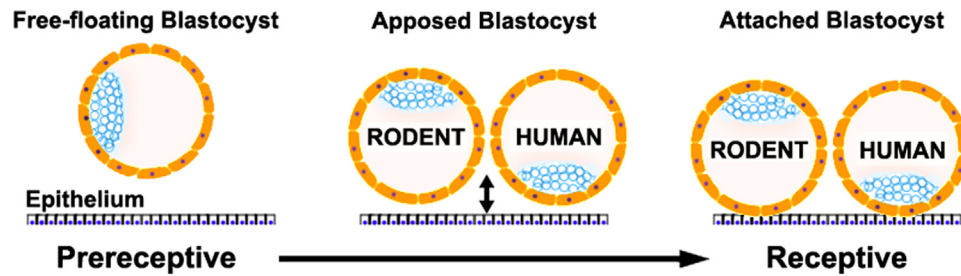


Fig. 4 A schematic depiction of early blastocyst implantation events.

amenorrhea varies depending on: 1) whether the woman is breastfeeding or not, 2) frequency and length of breast feeding, 3) maternal age and parity. Most women will not begin ovulating in the first 4–6 weeks after giving birth (McNeilly, 2001).

Key Events of Implantation and Decidualization

Embryo implantation and stromal cell decidualization are prerequisites for the establishment of pregnancy in the majority of mammals. Implantation involves a physical, molecular and physiological contact between the blastocyst and the uterus. It occurs in three phases: apposition, attachment and invasion. In rodents, the uterine LE enfolds creating small crypt-like structures called implantation chambers. The blastocyst within an implantation chamber gradually orients (apposition) and places itself within proximity to the LE, initiating the process of attachment. The orientation of the blastocyst during implantation differs between humans and rodents. In contrast to humans in which the polar trophoblast (embryonic side) of the blastocyst initiates the process of attachment, this process is initiated by the mural trophoblast (abembryonic side) of the rodent blastocyst (Fig. 4). In most species, blastocyst attachment occurs at the antimesometrial side of the uterus (Carson *et al.*, 2000).

Attachment of the trophoblast ensues immediately after the loss of zona pellucida (a membrane surrounding the blastocyst) except in guinea pigs where the trophoblast penetrates the zona pellucida to attach with the uterine LE. In mice and rats, initial attachment is loose, allowing the blastocyst to be flushed out of the uterus. However, in hamsters, the attachment reaction is extremely tight, making flushing of the blastocyst almost impossible. In rodents, the mural trophoblast cells stop dividing after attachment to the uterus, but continue to endoduplicate to form trophoblast giant cells. The polar trophoblast cells continue to proliferate, migrate towards the mesometrial side of the uterus and form a fully developed ectoplacental cone. In contrast to rodents, the human embryonic trophoblast not only attaches, but continues to proliferate and invade the endometrium (Carson *et al.*, 2000).

Based on the interaction between the blastocyst and uterus, the process of implantation has been classified into the following types (Carson *et al.*, 2000).

- 1) Centric (fusion or non-invasive) implantation occurs when the blastocyst makes extensive surface contact and fuses with the uterine LE without any penetration. Examples include: rabbits, dogs, domestic animals and several marsupials.
- 2) Eccentric (displacement) implantation occurs when the uterine LE forms an invagination to surround the abembryonic trophoblast. The trophoblast makes contact with stromal cells by displacing or shedding the underneath uterine LE. Examples include: rats, mice, hamsters and voles.
- 3) Interstitial (intrusive) implantation takes place when the trophoblast or the entire blastocyst invades through the LE to embed within the endometrial stroma. Examples include: humans, guinea pigs, gerbils and chinchilla.

The blastocyst-uterine attachment reaction is followed by decidualization, only at embryo implantation sites. Decidualization is common among many mammalian species, including rodents and humans. Decidualization occurs due to the proliferation of stromal cells and their transformation into decidual cells, which are polyploid and epithelioid-like. In humans, predecidual cells are observed at focal sites within the endometrium (surrounding the spiral arteries) during the late secretory phase of the cycle, in the presence or absence of the blastocyst. However, in rodents decidualization relies on contact of the blastocyst with the uterus. Therefore, decidual formation begins at the antimesometrial side of the implantation sites. Following initiation of the blastocyst-uterine attachment reaction, an avascular primary decidual zone (PDZ) is formed immediately underneath and lateral to the implanting embryo. Cells of the PDZ are tightly packed and epithelioid in nature. Later, proliferating stromal cells around the PDZ differentiate into decidual cells forming a secondary decidual zone (SDZ). In parallel to the antimesometrial decidual formation, stromal cells of the mesometrial side of the implanting embryo also proliferate and differentiate to the non-polyploid decidual zone (Gao *et al.*, 2015).

Decidualization can be experimentally-induced in pregnant, pseudopregnant or ovariectomized rodents. To do so, the uterus must be hormonally-primed for decidualization by various artificial means, such as an intraluminal oil injection, silk thread placement or surface scratching using a needle. In ovariectomized rodents, P₄ but not E₂, is an absolute requirement for induction of decidualization. The differential hormonal requirement of the uterus for artificial decidualization has been confirmed by the use of progesterone receptor knockout mice in which decidualoma formation is not possible, while artificial decidualization can occur

in P₄-primed ovariectomized estrogen receptor knockout mice (Paria *et al.*, 1999). While a physical stimulus is required to induce artificial-decidualization in rodents, it is unclear why a similar physical stimulus is not required for decidualization in humans.

Concluding Remarks

Reproductive cycle abnormalities, fertility issues and early pregnancy disorders in women are common reproductive health concerns that arise because of ovarian and uterine functional deficiencies. Examining key events and hormonal regulation of ovarian and uterine functions during the menstrual cycle and early pregnancy in human subjects remains problematic due to: 1) ethical and regulatory concerns; 2) inadequate patient health information, and 3) genetic diversity among humans. Therefore, researchers have relied on the use of rodent models; however no rodent model truly recapitulates all aspects of the human reproductive cycle and early pregnancy. Here, we highlighted the similarities and differences that exist among the most commonly used laboratory rodent species, as well as between rodents and humans. Different rodent models may be better suited to study specific steps in the processes involved in human reproductive cycle and early pregnancy. Comparative genomic analysis of humans and laboratory rodents, as well as extension of genetic manipulation techniques from the mouse to other rodent species, will allow generation of meaningful data that can be applied to human reproduction.

References

- Carson, D. D., Bagchi, I., Dey, S. K., et al. (2000). Embryo implantation. *Developmental Biology*, 223, 217–237.
- Christensen, A., Bentley, G. E., Cabrera, R., et al. (2012). Hormonal regulation of female reproduction. *Hormone and Metabolic Research*, 44, 587–591.
- Deb, K., Reese, J., & Paria, B. C. (2006). Methodologies to study implantation in mice. *Methods in Molecular Medicine*, 121, 9–34.
- Dey, S. K., Lim, H., Das, S. K., et al. (2004). Molecular cues to implantation. *Endocrine Review*, 25, 341–373.
- Gao, F., Bian, F., Ma, X., Kalinichenko, V. V., & Das, S. K. (2015). Control of regional decidualization in implantation: Role of FoxM1 downstream of Hoxa10 and cyclin D3. *Scientific Reports*, 5, 1–16.
- Jemiolo, B. (1983). Ovulation and fertilization in the vole, *Pitymys subterraneus*. *Biology of Reproduction*, 28, 523–527.
- Kubota, K., Cui, W., Dhakal, P., et al. (2016). Rethinking progesterone regulation of female reproductive cyclicity. *Proceedings of the National Academy of Sciences USA*, 113, 4212–4217.
- McLean, A. C., Valenzuela, N., Fai, S., & Bennett, S. A. (2012). Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *Journal of Visualized Experiments*, 67, e4389.
- McNeilly, A. S. (2001). Lactational control of reproduction. *Reproduction Fertility and Development*, 13, 583–590.
- Mead, R. A. (1993). Embryonic diapause in vertebrates. *Journal of Experimental Zoology*, 266, 629–641.
- Mihm, M., Gangooly, S., & Muttukrishna, S. (2011). The normal menstrual cycle in women. *Animal Reproduction Science*, 124, 229–236.
- Paria, B. C., Huet-Hudson, Y. M., & Dey, S. K. (1993). Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proceedings of the National Academy of Sciences USA*, 90, 10159–10162.
- Paria, B. C., Tan, J., Lubahn, D. B., Dey, S. K., & Das, S. K. (1999). Uterine decidual response occurs in estrogen receptor- α -deficient mice. *Endocrinology*, 140, 2704–2710.
- Reese, J., Wang, H., Ding, T., & Paria, B. C. (2008). The hamster as a model for embryo implantation: Insights into a multifaceted process. *Seminars in Cell and Developmental Biology*, 19, 194–203.
- Schank, J. C., & McClintock, M. K. (1992). A coupled-oscillator model of ovarian-cycle synchrony among female rats. *Journal of Theoretical Biology*, 157, 317–362.

Selected Comparative Aspects of Canine Female Reproductive Physiology

Mariusz P Kowalewski, University of Zurich, Zurich, Switzerland

© 2018 Elsevier Inc. All rights reserved.

Introduction

Why the Dog?

In recent years, considerable progress has been achieved in understanding reproductive physiology in the domestic dog, in particular regarding female reproduction.

This was driven by the following underlying facts:

1. The dog is one of the most suitable laboratory animal models. Out of over 711 traits/disorders currently (Oct. 2017) listed in the Online Mendelian Inheritance in Animals (OMIA) database for the dog, 413 present potential models for human diseases. Other domestic animals trail with a maximum of roughly 200 possible models, e.g. for cattle and cats, and less for others. Mostly for social reasons, the potential of the dog as a model animal is not fully utilized in research studies.
2. The dog is among the most important laboratory animals in pharmacological and toxicological studies.
3. Social aspects: the domestic dog is among the most important pets and veterinary patients. Thus, social and economic interest in better understanding of canine reproduction is justified. Many clinical issues are still not solved, such as luteal insufficiency and endometrial hyperplasia/pyometra complex, because basic regulatory processes are not yet fully understood in this species. Consequently, several therapeutic approaches are based solely upon clinical experience and empirical evidence, rather than on understanding of underlying molecular biological processes.
4. Due to several species-specific regulatory mechanisms, possibilities for translational research to be adapted from other species to dogs are limited. This underlines the importance of the dog as an interesting model for investigating comparative aspects of reproductive physiology in other mammals, broadening the horizons and even breaking some of the existing dogma.
5. In taxonomically-related endangered species, the domestic dog could be used as a valuable model for studying reproduction and developing assisted reproduction techniques.

Goals

Here, a general overview of reproductive cycles in pregnant and non-pregnant dogs is presented. Species-specific peculiarities and comparative aspects are emphasized. Focusing on endocrine regulatory processes, the most recent developments in understanding canine female reproductive physiology are presented. These include ovarian and luteal function, embryo-maternal oviductal and uterine communication and the feto-maternal utero-placental dialog, during the establishment, maintenance and termination of canine gestation.

Original research papers and reviews published from our and other laboratories were used to gather the information presented herein.

More comprehensive reviews and details regarding source publications can be found in (Concannon, 2009, 2011; Feldman and Nelson, 2004; Hoffmann *et al.*, 2004; Kowalewski, 2014, 2017; Kowalewski *et al.*, 2015; Wright, 2017). In this respect, noteworthy is the pioneering work on canine reproductive physiology, dating from the middle of the 19th century (1845), by the German researcher and at that time professor at the University in Giessen, Theodor Ludwig Wilhelm von Bischoff (Bischoff, 1845). Even though at that time the role of the corpus luteum was not known, and progesterone (P4) was not yet isolated (not until the beginning of the 20th century), von Bischoff, among others, with a great deal of detail, for the first time described the process of preovulatory luteinization in the dog.

What Makes the Dog Special? – Species-Specific Peculiarities of Canine Reproduction at a Glance

The sexual cycle in the domestic dog is exceptionally long compared with other domestic animal species, showing tremendous breed and individual variations with regard to the length of the particular phases of the cycle (Fig. 1). Corpora lutea (CL) are the only major source of circulating steroids in the female dog, both in pregnant and non-pregnant bitches. Because of this, the dog is the only domestic animal species not producing steroids in the placenta, and thus is fully reliant on luteal steroids for successful pregnancy. This also underlines the central role of CL in controlling canine reproduction. Compared with other domestic animal species, canine CL require a longer time to fully attain their maximal steroidogenic capacities (around days 15 to 30 after ovulation). At the time of implantation, the uterus is exposed to relatively high P4 concentrations, apparently much higher than those required for the maintenance of pregnancy. Hysterectomy does not affect ovarian cyclicity in the dog, neither could substantial production of luteolysin (PGF2 α) be confirmed in the CL of pregnant or non-pregnant bitches. The sexually active phases are always separated by an obligatory quiescence (resting) phase, referred to as anestrus. One of the biggest peculiarities is the lack of an antiluteolytic principle in the absence of pregnancy, resulting in a physiological pseudopregnancy. This, along with similar secretion patterns of circulating steroids in pregnant and non-pregnant bitches, precludes P4 as a reliable marker for pregnancy detection in

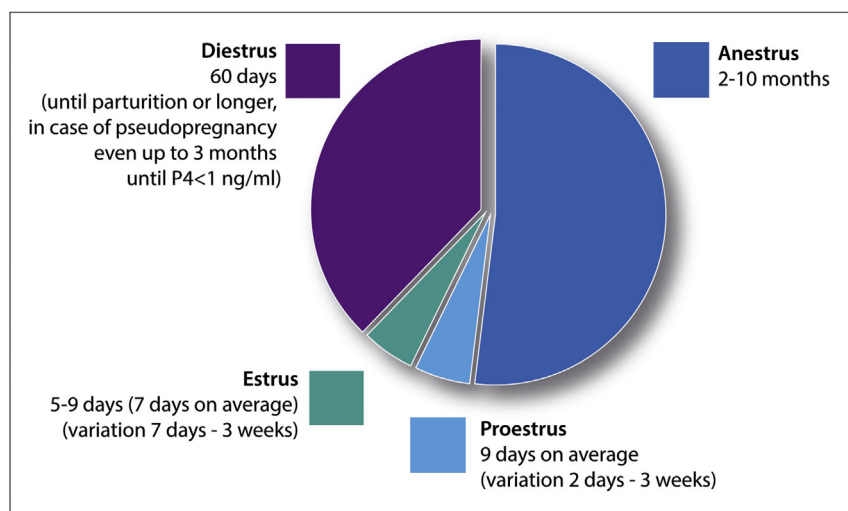


Fig. 1 The canine reproductive cycle.

dogs. Pseudopregnancy can last at least as long as, or even exceed, the time span of normal pregnancy. Consequently, the classical definition of embryo-maternal recognition of pregnancy, understood to be the existence of different strategies evolutionarily established for preventing luteolysis and allowing the CL to persist in order for pregnancy to succeed, also does not apply in the dog. Instead another, more general definition was proposed for the dog as more suitable (Kowalewski *et al.*, 2015). Accordingly, in the canine species the maternal recognition of pregnancy appears to involve morphological and functional interplay between the uterus, the embryo, and the CL as the only provider of P4.

Further, in the domestic dog, as in other *Canidae*, immature oocytes are ovulated at the primary oocyte stage. They require a further 2–3 days of oviductal maturation, only then acquiring the developmental stage of fertile secondary oocytes. This makes the dog an absolute exception among domestic animal species in which the resumption of meiosis and completion of the chromosome reduction division is stimulated by the preovulatory LH surge and takes place before ovulation. In other words, whereas in all other domestic animal species oocytes are ovulated as secondary oocytes, in the dog primary oocytes are ovulated. This also greatly complicates artificial reproduction techniques (ART) applications in dogs because information on the requirements for completion of oocyte meiosis in vitro is sorely lacking, unlike in several other domesticated animal species such as cattle.

Also contrasting with other domestic animal species, including the cat, is the absence of a pregnancy or prepartum/parturition-related increase of estrogens in the dog. The prepartum signaling cascade appears, therefore, also to follow species-specific regulatory mechanisms.

Finally, regarding luteotropic support, although hypophyseal hormones, (predominantly) PRL and LH, are required for luteal maintenance during the second half of the canine luteal phase, luteal regression occurs in spite of increasing availability of both circulating PRL and LH. Although gestational PRL levels are increased when compared with pseudopregnancy, they cannot be used for detection of pregnancy because elevated levels of this hormone can be also observed in clinical condition of overt pseudopregnancy (*Lactatio sine graviditate* or *Lactatio falsa*) and large individual variations are observed. Consequently, so far, from the endocrinological perspective, fetal placental relaxin (RLN) is the only reliable marker of pregnancy in the dog.

Reproductive Cycles During Pregnancy and Pseudopregnancy

Bitches exhibit their first sexual cycle at highly variable ages, depending on the breed, their size and individual predispositions; smaller dogs may reach puberty between 6 to 10 months of age whereas larger breeds may not exhibit their first cycle until the age of 18–24 months.

The domestic dog is an aseasonal (can experience ovarian cycles independently throughout the year) and monoestrous (experience only one estrus or “heat” which is followed by a spontaneous ovulation only once in a breeding season) breeder with an obligatory anestrus in between.

The length of a canine sexual cycle can vary considerably and appears to depend on the breed and genetic predispositions. It can range from 5 up to even 13 months (Concannon, 2011), so in most cases, 2 cycles are observed per year. This does not include the basenji breed, in which the cycle is regulated photoperiodically with the breeding season in autumn in Northern and Southern hemispheres (information on cycles near the equator are missing). The dingo, another representative of the family *Canidae*, is seasonal at both hemispheres, but aseasonal at the equator (Concannon *et al.*, 2009).

The duration of canine pregnancy is approximately 2 months. Anestrus can last for 2–10 months (genetic, individual and breed/size variations all play a role) and ends with increasing GnRH and LH pulsatility (Concannon *et al.*, 2009). Although fertility

decreases with age, sexual senescence in the sense of menopause does not occur in dogs. The optimal breeding age ends at around 6–7 years of age.

Ovarian Cycle

The ovarian cycle in the dog is divided into 4 phases: proestrus, estrus, diestrus (the luteal phase, in some literature frequently designated as metestrus) and anestrus. The length of particular phases is variable (Fig. 1).

It is not known what triggers proestrus and initiation of a new estrous cycle; however, breed, age, environmental factors and genetic background are influencing factors.

Nevertheless, at the end of anestrus GnRH pulses increase, followed by increased LH and FSH pulsatility. The LH pulses appear to be greater than those of FSH (Concannon *et al.*, 2009).

Proestrus and transition to estrus

Proestrus lasts on average 9 days, but can be as short as 2–3 days or as long as 3 weeks. It is characterized by growth of follicles and rising estradiol (E2) concentrations. A bitch becomes attractive to males, however, she does not allow them to mount. Prior to the end of proestrus, E2 starts to decrease and proestrus ends with the LH surge. Estrogens lead to: increased cornification of vaginal epithelium, serosanguinous vaginal discharge caused by predominantly endometrial diapedesis, and anatomically visible edema of the vulva and vagina. The proestrus bleeding typically becomes a more transparent exudate when the cycle enters the stage of estrus. Proestrus and estrus are together referred to as “the heat”.

Anestrus LH levels remain low (<1 ng/mL) with pulse intervals of 4–24 h, occasionally reaching levels between 2–25 ng/mL. Approximately one week before proestrus, LH pulses increase in frequency to every 60–90 min, and average concentrations increase to 3 ng/mL (Concannon *et al.*, 2009). At proestrus, due to negative feedback from estrogens, the pulses and quantity of LH secreted decrease. Its levels increase, however, considerably towards the LH peak, to reach concentrations between 4–40 ng/mL (8–15 ng/mL on average) (Concannon *et al.*, 2009).

As for FSH, it starts to increase at mid- through late anestrus, however without detectable estrogenic activity (the reasons for this remain unclear). A week before proestrus, concomitantly with increasing LH pulsatility, FSH pulse frequencies also rise. Quantitatively however, the FSH increase is less pronounced than that of LH (Feldman and Nelson, 2004).

Estrogen (E2) levels increase with the progression of proestrus, from initial low values of 5–10 pg/mL to the highest preovulatory concentrations of 45–120 pg/mL. At the end of proestrus, E2 levels start to decrease, and the LH peak marking the transition to estrus occurs 1–3 days later. Concomitantly with decreasing E2, P4 levels increase, and both events are needed for the LH surge to occur. By exerting a positive feedback on the hypothalamus and hypophysis, both also lead to enhanced FSH production (Kowalewski, 2017; Concannon *et al.*, 2009; Feldman and Nelson, 2004).

Progesterone: the LH-driven preovulatory luteinization starts during proestrus (Fig. 2(A) and (B)), characterized by increasing P4 even up to 6 days before the LH surge (from basal concentrations of 0.2–0.4 to 0.6–1.0 ng/mL). At the LH surge, P4 rises rapidly, reaching relatively high levels of at least 5 ng/mL (up to 10 ng/mL) at the time of ovulation (which takes place during estrus). The phenomenon of preovulatory luteinization described in the dog by von Bischoff (Bischoff, 1845) is exceptionally strong, and is not observed to this extent in other domestic animal species.

Estrus

Lasts 5–9 days (on average 7 days, but up to 3 weeks, with large variability) (Fig. 2(A) and (B)). Clinically and behaviorally, estrus is characterized by the mounting willingness of a bitch. Endocrinologically, estrus begins 0–1 day from the LH surge. The LH surge is defined as the time when LH for the first time exceeds its basal levels (<200% over the basal values and >50% of the concentrations at the LH peak; (Concannon, 2009, 2011)). The surge is characterized by an initial increase in LH levels over 12–36 h, followed by a 12–24 h decrease, and takes on average 36 h, with a range between 24–72 h (Kowalewski, 2017; Concannon, 2011).

In addition to the initial LH burst, estrus is marked by progressively decreasing E2 concentrations and increasing P4 levels. In particular, following the LH surge, P4 increases strongly towards ovulation. Thus, simplifying, one could say that whereas proestrus is the phase of increasing and high E2 levels, estrus in the bitch is the phase of decreasing E2.

In addition to stimulating the LH surge, the decreasing E2:P4 ratio triggers ovulation (Fig. 2(B)). Ovulation is spontaneous and takes place within 24–72 h after the LH surge (Feldman and Nelson, 2004) and formation of the CL starts immediately thereafter; P4 levels continue to increase, first during the estrus phase and then during diestrus. Furthermore, the decreasing E2/P4 ratio is also responsible for estrus behavior, and bitches typically present “standing heat” with declining E2 levels and with increasing P4 having a positive impact on the length and intensity of estrus behavior (Feldman and Nelson, 2004).

Endocrinologically, estrus ends when E2 levels decrease below 15 pg/mL, and the cycle becomes strongly dominated by P4 (luteal phase) (Feldman and Nelson, 2004).

From the clinical perspective, it has to be emphasized that the estrogen-dependent cornification of vaginal epithelial cells (appearance of anuclear superficial cells or anuclear squames): (i) does not necessarily correlate with the profile of circulating peripheral E2, because (ii) a lag-time of 3–6 days was reported in the response of the vaginal epithelium to circulating estrogens; consequently (iii), the first day when 80–90% superficial keratinized cells are observed can be as soon 6 days before the LH surge, or as late as 4 days afterwards (i.e., at either increasing/maximal, or decreasing E2 concentrations) (Feldman and Nelson, 2004); thus (iv), it is not possible to distinguish late proestrus from estrus based on vaginal cytology; and finally, (v) vaginal cytology, even if

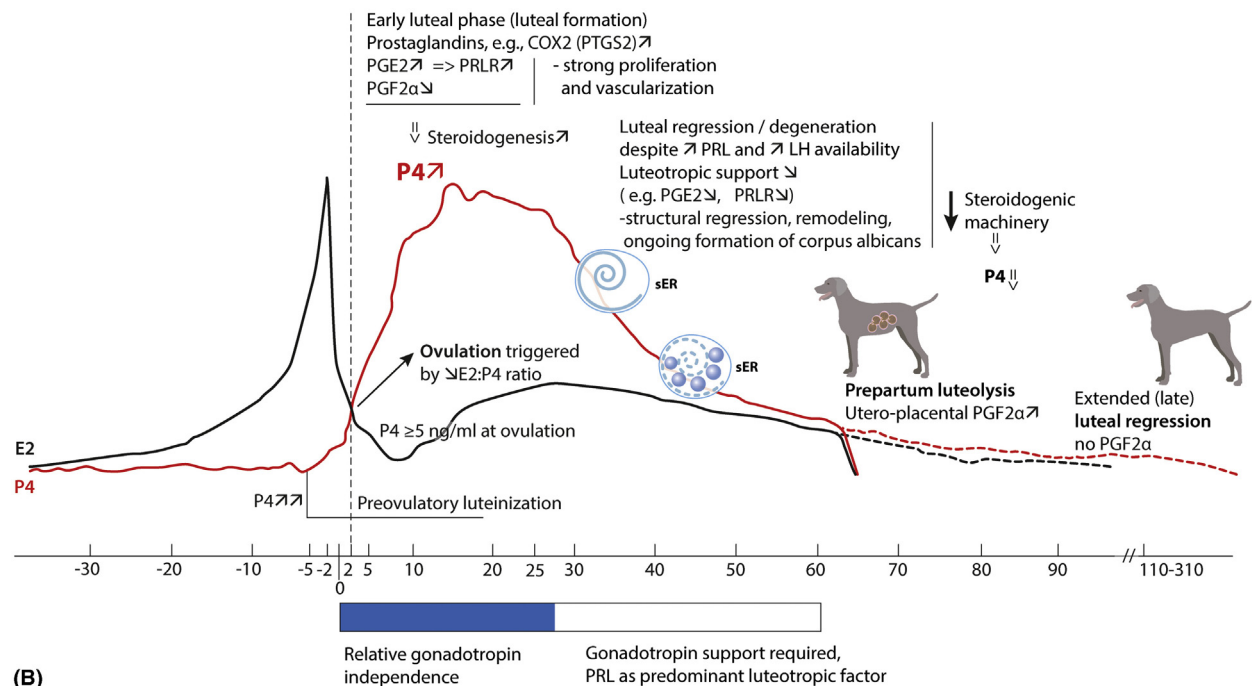
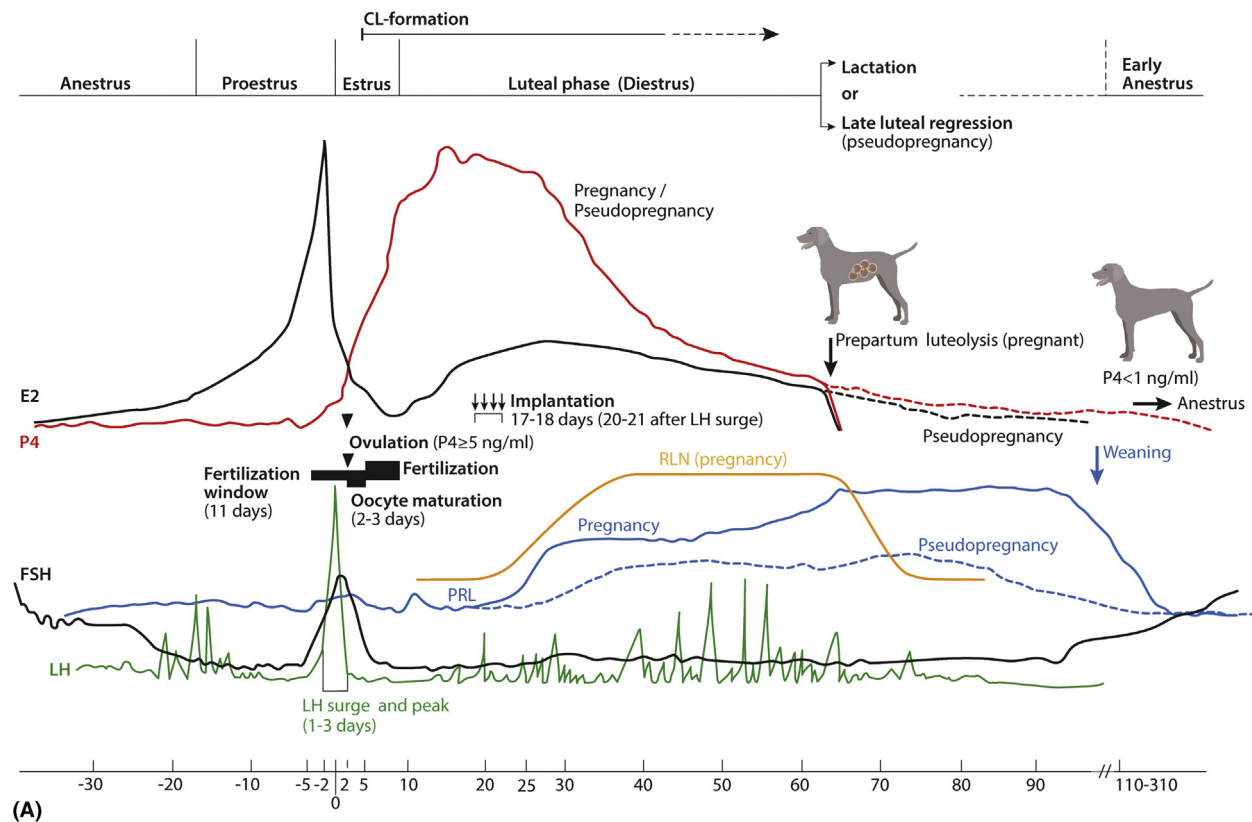


Fig. 2 (A) Schematic representation of changes in estradiol (E2), progesterone (P4), follicle stimulating hormone (FSH), luteinizing hormone (LH), prolactin (PRL), and relaxin (RLN) during pregnant and non-pregnant reproductive cycles in the dog. Placental RLN is the only specific marker of pregnancy. (B) The bottom panel shows a schematic representation of the most important regulatory mechanisms regulating canine luteal function. Timelines are shown in relation to LH peak. COX2 (PTGS2), cyclooxygenase 2; PGE2, prostaglandin E2; PGF2 α , prostaglandin F2 α ; PRLR, PRL-receptor; sER, smooth endoplasmic reticulum (first signs of degeneration within sER can be seen at approximately day 35 after ovulation exhibiting "whorl like" structures, and proceeds further towards fatty degeneration seen around day 45). For both panels detailed explanations are provided in text.

reflects the level of E2-dependent keratinization of the vaginal epithelium, does not allow us to identify the day of the LH peak or ovulation and does not predict the best time for fertilization (Feldman and Nelson, 2004). Even if at “standing heat” up to 100% of superficial cells are cornified, vaginal cytology is not a reliable method for proper timing of a bitch, in particular for artificial insemination.

Diestrus, i.e., luteal phase in pregnant and non-pregnant cycles (Figs. 1 and 2(A) and (B))

Endocrinologically, by definition, diestrus is the phase of the cycle dominated by P4 secretion. Behaviorally, diestrus starts with the end of “standing heat”. Histologically, based on the vaginal epithelial cytology, the onset of diestrus is the day when a dramatic shift from E2 to P4 dominance is observed, i.e., 80–100% of superficial cells appear in estrus vs. the same amount of intermediate or parabasal cells indicating diestrus (Feldman and Nelson, 2004).

From the biological point of view, and for research purposes, based on structural and functional changes observed in ruptured follicles, the time point of ovulation which induces CL formation, is seen as the beginning of diestrus (Kowalewski, 2017).

In non-pregnant dogs, diestrus can last as long as pregnancy (around 60 days) or can extend beyond this time. It ends when peripheral P4 levels decrease below <1 ng/mL and, by definition, the cycle enters the stage of anestrus. This can last even up to 3 months after ovulation. Thus, physiological pseudopregnancy in non-pregnant dogs can exceed by far the length of pregnancy, which is an exception among domestic animal species.

Morphologically, preovulatory luteinization is characterized by strong proliferation and folding in canine preovulatory follicles (Bischoff, 1845). Intense vascularization, morphological and functional changes within the theca interna layers, result in relatively high P4 concentrations at the time of ovulation (see above). Prostaglandins (PGE2 and PGF2 α) appear to be involved in the process of ovulation (Kowalewski, 2017). The local production of endogenous PGF2 α , but not of PGE2, greatly decreases following ovulation (Kowalewski, 2017). Interfering with P4 function at the level of its nuclear receptor (PGR), by application of the antigestagen aglepristone, does not prevent ovulation in dogs (Reynaud *et al.*, 2015).

Following ovulation, further luteinization of theca interna cells and luteinization of the remaining follicular granulosa cells take place, giving rise to the development of a unique population of luteal cells (Kowalewski, 2017). Thus, in contrast to other domestic animal species, in fully developed canine CL, both morphologically and functionally only one population of steroidogenic cells can be identified. Intense vascularization and strong proliferative activity of steroidogenic and non-steroidogenic cellular components take place. The steroidogenic machinery becomes strongly activated and P4 concentrations rise quickly within 15–30 days when CL are fully formed and maximal P4 concentrations of usually 30–35 ng/mL (but even up to 80 ng/mL or higher) are observed. This exposure of the reproductive system to high circulating P4 concentrations is not observed in other domestic animal species. During the early luteal phase, developing CL are, at least to some extent, gonadotropin-independent and prostaglandins, in particular PGE2, appear to be among the most important regulatory factors. Indeed, interfering with prostaglandin synthesis, by administration of a selective cyclooxygenase 2 (COX2/PTGS2) blocker (firocoxib), results in partial suppression of luteal steroidogenic function. More detailed, cumulative descriptions of the underlying regulatory mechanisms can be found in (Kowalewski, 2014, 2017).

Generally, especially following implantation and placentation, numerically but not statistically higher P4 levels are observed in pregnant bitches. The lack of any statistical differences relates most probably to large individual and diurnal variations. Starting at approximately day 25 of the luteal life span, CL become dependent on gonadotropin support, with PRL being the predominant luteotropic factor (Kowalewski, 2017). However, shortly after reaching the highest P4 levels, functional and structural luteal regression starts. Progesterone levels decrease gradually despite increasing availability of hypophyseal PRL and LH.

The slowly ongoing luteal regression is associated with structural and functional changes in steroidogenic cells and remodeling processes within luteal parenchyma. First, around day 35 after ovulation degenerative processes can be seen within smooth endoplasmic reticulum and mitochondria. The appearance of large intracytoplasmic vacuoles further indicates ongoing fatty degeneration (Fig. 2(B)). The steroidogenic machinery slows down, and expression of important luteotropic factors decreases (Fig. 2(B)). Also, the PGE2-driven expression of PRL-receptor (PRLR) diminishes. Whether this is the cause or result of the degenerative process remains unclear.

The similar P4 secretion patterns observed in pregnant and non-pregnant dogs start to diverge just prior to parturition (Fig. 2(A) and (B)), when in pregnant bitches a steep prepartum decrease in P4 levels is observed during prepartum luteolysis, approximately 12–24 h ante partum (prior to any clinical and/or behavioral signs of parturition, like nest building). Concomitantly, circulating levels of PGF2 α of predominantly fetal-placental origin increase, indicating acute luteolytic process (Figs. 3 and 4). In pseudopregnant bitches, the slow luteal regression continues until baseline P4 levels (<1 ng/mL) are achieved at the transition to anestrus. Whereas prepartum luteolysis is associated with massive apoptotic signals, in the CL of non-pregnant bitches apoptotic events are only sporadically observed. As mentioned elsewhere, an intraluteal source of luteolytic PGF2 α can be ruled out in both cases. Interestingly, however, the luteal phase can be terminated by exogenous application of luteolysin, even as early as day 5 of cytologic diestrus, though relatively high dosages and/or repeated treatments are necessary, associated with a high risk of side effects (emesis, diarrhea, panting) (Romagnoli *et al.*, 1991). The constitutive and stable expression of the respective PGF2 α receptor in fully functional canine CL may explain its receptivity to exogenous PGF2 α (Kowalewski *et al.*, 2008). Apparently, therefore, contrasting with the CL of pregnancy, an endogenous source of PGF2 α is missing in pseudopregnant dogs.

The immune system seems to be involved in regulating canine CL function: CD4- and CD8-positive cells, and MHCII-expressing cells, together representing lymphocytes and macrophages, and their immunoactive products (i.e., cytokines) are present in the CL of both pregnant and non-pregnant dogs. As revealed by the transcriptomic approach, the prepartum luteolysis especially appears to be dominated by strong activation of the immune response (Zatta *et al.*, 2017) (Fig. 3).

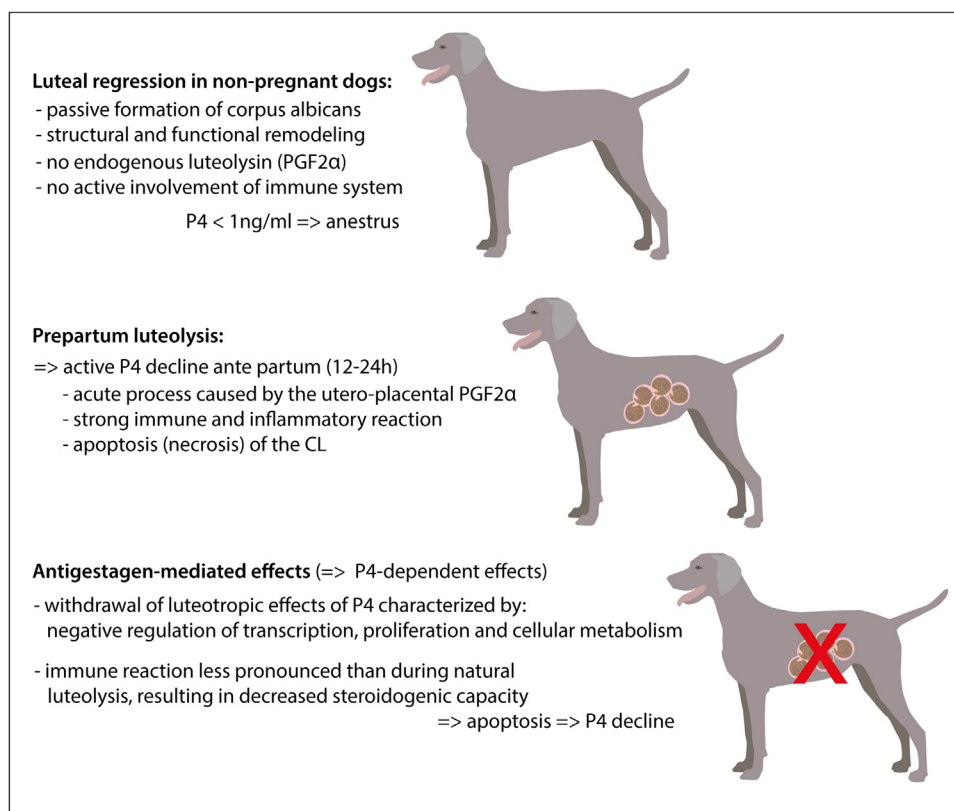


Fig. 3 Summary of regulatory events involved in termination of canine CL function in pregnant and non-pregnant dogs. Progesterone (P4)-mediated effects indicating the nature of luteotropic actions of P4 in the canine CL are presented, as revealed by the use of an antigestagen (aglepristone) at mid-gestation. Modified after Zatta, S., Rehrauer, H., Gram, A., Boos, A., Kowalewski, M.P., 2017. Transcriptome analysis reveals differences in mechanisms regulating cessation of luteal function in pregnant and non-pregnant dogs. *BMC Genomics* 18, 757.

Cumulatively, contrasting with the PGF2 α -driven, acute (immune) process of prepartum luteolysis, the extended luteal regression observed in pseudopregnant dogs appears to be a passive degenerative process of corpus albicans formation without an (acute) involvement of the immune system. It seems to be modulated, however, by several metabolic, paracrine and most probably autocrine factors, but is apparently devoid of any active luteolytic principle as indicated by the lack of apoptosis. Its inherent life span is not affected by hysterectomy.

Progesterone as a luteotropic factor

The functional withdrawal of P4 caused by an antigestagen results in a pre-term luteolysis/abortion. This is associated with induction of the immune system (a weaker response, however than in natural luteolysis), and suppression of cell proliferation and luteal transcriptional activity (Zatta *et al.*, 2017) (Fig. 3).

Estrogens

E2 is entirely of luteal origin; following the preovulatory peak, E2 continues to decrease, at 15 pg/mL diestrus starts, and the lowest values (8–9 pg/mL) are reached shortly thereafter. Luteal formation and rising P4 are associated with a new increase in E2, starting at approximately day 10, both in pregnant and pseudopregnant dogs (Fig. 2(A) and (B)). This increase is significant, and E2 remains present at average values of 15–40 pg/mL throughout diestrus (varying individually, diurnally and depending on the breed). Importantly, pre-ovulatory E2 concentrations are never reached (Kowalewski, 2017). The secretion pattern of E2 roughly follows that of P4. It decreases towards the end of the luteal life span, further indicating its source within the CL. Therefore, as mentioned elsewhere and contrasting with other domestic animals, there is no prepartum increase in E2.

PRL

This hormone is the predominant luteotropic factor in the dog from approximately day 25 (24–28) onwards after ovulation (Fig. 2(A) and (B)). It is mostly of hypophyseal origin. Interfering with its function (e.g., by administration of the dopamine agonist bromocriptine) during this time leads to premature cessation of luteal function in both pregnant and non-pregnant dogs. These effects can be reversed by PRL but not by LH, which is a weaker luteotropic factor than PRL. The role of PRL is in the maintenance of luteal function rather than in active stimulation of P4 production (PRL administration does not increase P4 levels). Its

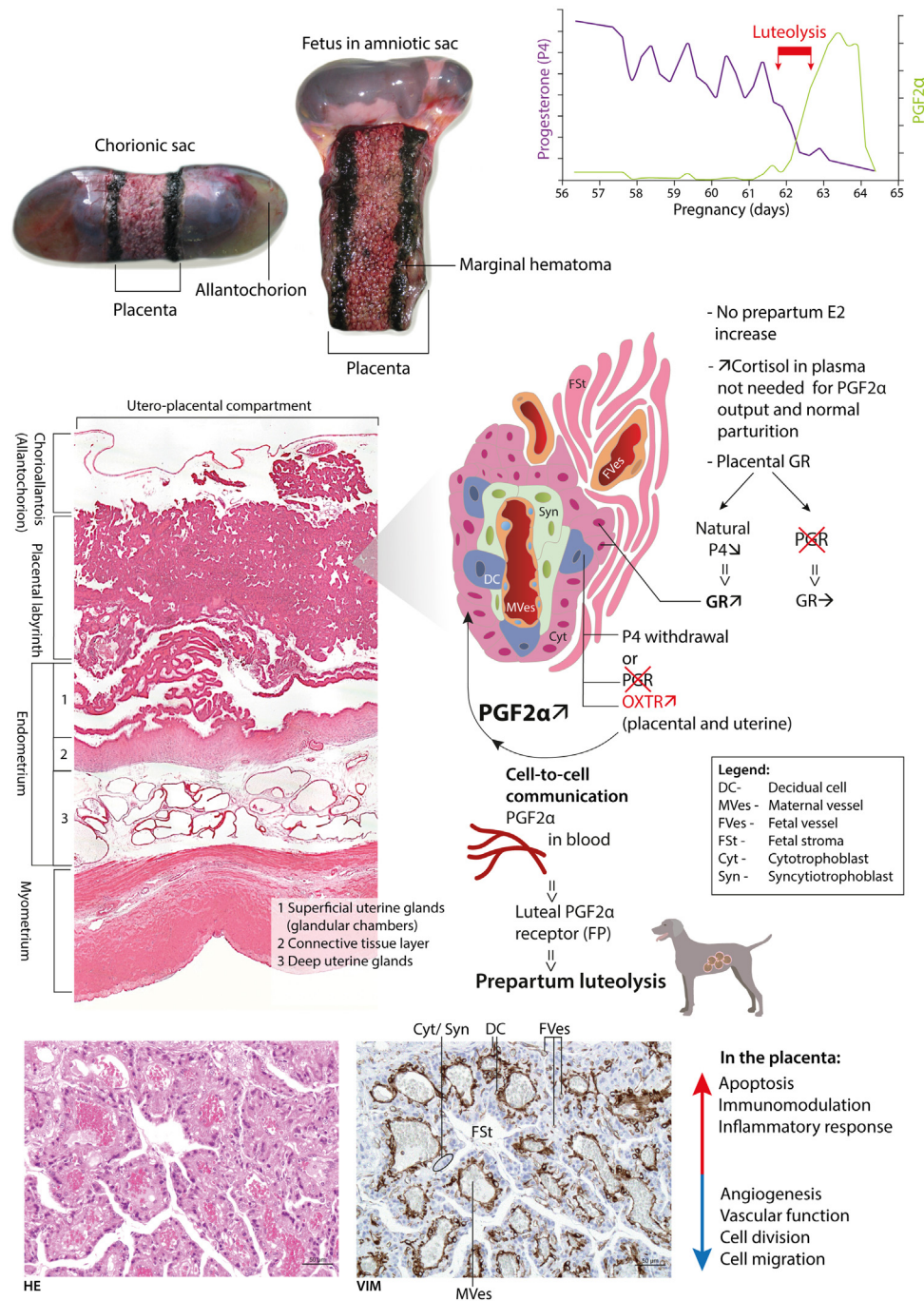


Fig. 4 Proposed model of signals participating in feto-maternal communication at the time of prepartum luteolysis in the dog. The key hormonal events associated with the prepartum luteolysis, characterized by decreasing progesterone (P4) and increasing PGF2α levels, are shown in the upper right panel. Photographs represent: (upper panel, left) following removal from the uterus, conceptus (fetus with fetal membranes) is shown before and after the opening of the chorioallantoic sac at mid-pregnancy. Pictures shared by courtesy of Dr. Christine Reich, Justus-Liebig-University Giessen, Giessen, Germany. The macroscopic appearance of the mature canine girdle placenta (*placenta zonaria*) is shown; (middle panel, left) a histological cross-section through the canine utero-placental compartment (full thickness, uterus with adjacent placenta from the middle part of the girdle without the marginal hematoma), shown at low magnification; (middle panel right) a fragment of the canine placenta is presented schematically, decidual cells are the only cells in the canine placenta expressing the nuclear P4 receptor (PGR) and oxytocin receptor (OXTR). The natural- or antigestagen-mediated withdrawal of P4 leads to activation of prostaglandin synthesis (PGF2α) in the fetal part of the placenta (trophoblast). Antigestagens interfere with the biological function of P4 by blocking the function of its receptor (PGR). GR (NR3C1) = glucocorticoid receptor is localized in fetal trophoblast; its expression increases in normal, but not in antigestagen (aglepristone)-induced parturition; (lower panel) depicted are two consecutive microphotographs of canine placenta stained with hematoxylin and eosin (HE; left picture), or vimentin (VIM, right picture; an intermediate filament protein and marker of mesenchymal type cells, stains positively for fibroblasts, endothelial cells, and maternal decidual cells).

concentrations increase significantly in the second half of pregnancy, presumably driven by gestational placental RLN, and reach maximal values prior to term (≈ 50 ng/mL) (Kowalewski, 2017) (Fig. 2(A)). In pseudopregnant dogs, it increases only slightly during the course of diestrus, with maximal values of around 9 ng/mL observed late in diestrus at the time corresponding to parturition. As mentioned elsewhere, due to highly variable concentrations and the occasionally occurring clinical condition of *Lactatio falsa*, circulating levels of PRL do not serve for pregnancy detection. Progesterone withdrawal appears to increase circulating PRL levels.

RLN

RLN increases strongly following implantation and placentation, at approximately 25–30 days after the LH surge (3–4 weeks of gestation) (Fig. 2(A)). It originates mostly in the fetal cytotrophoblasts and exerts local, i.e., intraplacental and uterine, autocrine and paracrine as well as endocrine effects (Nowak *et al.*, 2017). Direct (auto-/paracrine) and indirect (endocrine) luteotropic roles, e.g., through stimulation of hypophyseal PRL secretion, are proposed (Nowak *et al.*, 2018). The highest concentrations of RLN (4–6 ng/mL) are observed 2–3 weeks before parturition and then decrease at parturition. Only low levels of RLN are observed during lactation (0.5–2 ng/mL) for approximately 4–9 weeks (Steinetz *et al.*, 1987). So far, RLN is the only reliable marker of pregnancy in dogs.

Embryo-Maternal Communication, and Establishment and Maintenance of Canine Pregnancy

Following ovulation, immature primary oocytes require 2–3 days (4–5 days after the LH surge) to gain the secondary oocyte developmental stage and acquire the capacity for fertilization. The process of oocyte maturation is still not fully understood, profoundly limiting the success of assisted reproduction techniques. Apparently, P4 acting through its nuclear receptor PGR plays an important role in the maturation process, and when blocked leads to delayed resumption of meiosis and embryonic development retardation (Reynaud *et al.*, 2015).

The “fertilization window”, i.e., the time period when a bitch can be successfully mated is relatively long in the dog (Kowalewski *et al.*, 2015); fertile mating can take place as early as 5 days before ovulation until even 6 days afterwards (all together 11 days) (Fig. 2(A)). This is due to: (i) the long survival time of spermatozoa in the female genital tract with the uterus acting as the main sperm reservoir, and (ii) prolonged survival of oviductal oocytes (7–8 days, including 2 days needed for maturation) (Concannon, 2009; Bischoff, 1845). Embryonic development and oviductal passage are slow in the dog. It takes up to 7–10 days for embryos to reach the uterotubal junction. At this time, their developmental stage can vary widely, being at the early stage of 16 cells or later at morulae or blastocysts. The uterine migration takes 9–10 days; the last blastocysts can hatch as late as day 19 after the LH surge (i.e., approximately 14 days after fertilization). Apposition starts on day 12–14 after fertilization, and uterine implantation and invasion are synchronized, starting at day 17–18 of embryonic life (days 20–21 after the LH surge) (Fig. 2(A)).

Although there is no anti-luteolytic signal in the dog, early embryo-maternal communication does take place and embryos modulate uterine function, facilitating implantation and preventing their own rejection. This contact is initiated prior to implantation and first involves biochemical and functional, rather than morphological, modifications of the uterus, indicating early decidualization processes (Kowalewski *et al.*, 2015). Modification of matrix assembly and immunomodulatory effects are observed, as indicated by transcriptomic studies (Graubner *et al.*, 2017a; Kowalewski *et al.*, 2015). More detailed studies regarding involvement of the immune system in these processes are presently in progress.

The factors produced by embryos relating to implantation are not yet fully described (Kowalewski *et al.*, 2015). In addition to immune system-derived factors observed in other species, PGE2 appears to play an important role: (i) it is expressed by embryos and exhibits higher levels in hatched than in unhatched blastocysts prior to implantation, (ii) expression of the PGE2 synthesizing enzymes and the respective PGE2 receptor (EP2) is elevated in the early pregnant uterus, and (iii) PGE2 exerts a strong decidualization effect on canine stromal cells (Graubner *et al.*, 2017b).

The presence of attaching conceptuses induces the first morphological changes in the uterus. The subepithelial morpho-functional decidualization of maternal endometrial stromal compartments starts and decidual cells begin to develop (Graubner *et al.*, 2017b). Importantly, and in contrast with humans, the decidualization process in the dog is not driven by circulating P4 (even if it is exceptionally high), but similar to rodents, by the presence of embryos (Graubner *et al.*, 2017b). Following implantation, maternal stromal cells further develop into placental decidual cells and become an integral part of the placenta. These are the only cells of the canine placenta expressing the nuclear P4 receptor (PGR) and oxytocin receptor (OXTR). As shown in ovariectomized bitches, P4 alone is enough for establishment and maintenance of pregnancy in previously estrogenized bitches (Concannon *et al.*, 2001).

Termination of Canine Gestation, and the Prepartum Luteolytic Cascade

The canine placenta is classified as an *endotheliochorial* (possessing strongly invasive trophoblast, *deciduata*) *zonary* placenta, i.e., forming a girdle of chorionic villi around the middle of the chorionic sac (Fig. 4). It develops glandular chambers (greatly enlarged superficial endometrial glands, also called the *spongy zone*). These are 1–2 mm thick and separate the placental labyrinth from the connective tissue layer covering the deep uterine glands. They continue to grow until day 40 of gestation, from then on fully developed utero-placental compartments are established.

The prepartum increase in PGF2 α seems to originate in the fetal part of the placenta (trophoblast), where the entire machinery producing PGF2 α is localized and is concomitantly strongly activated. The underlying mechanisms, at least to some extent, are P4-dependent, because interfering with PGR function in decidual cells by administration of antigestagens also induces synthesis of PGF2 α in neighboring fetal trophoblast cells (Fig. 4). Thus, maternal stroma-derived decidual cells appear to be involved in the underlying P4-dependent feto-maternal cross-talk leading to the prepartum release of PGF2 α . In both situations, i.e., during normal and induced parturition, expression of the OXTR increases in these cells (Fig. 4). Based on our own data, including transcriptomic analyses (unpublished), the functional involvement of the immune system during maintenance and termination of pregnancy is under discussion (Fig. 4).

Cortisol

The increased presence of cortisol in the maternal circulation pre-partum has been described as being of an “erratic nature”, i.e., it is observed occasionally, but is not required for normal parturition. The circulating cortisol levels, however, do not have to fully mirror local concentrations of glucocorticoids. Therefore, local effects cannot be excluded. The increased placental expression of glucocorticoid receptor (GR/NR3C1) is, however, not needed to induce prepartum PGF2 α synthesis in the dog. Being localized in fetal trophoblast, its expression increases significantly in normal but not in induced parturition (Kowalewski, 2017) (Fig. 4). Importantly, clinical application of glucocorticoids (dexamethasone) does not seem to mimic the natural prepartum signaling cascade, since prolonged treatments, on average over 8 days, are needed for pregnancy termination, associated with side effects like polydipsia and polyuria (Wanke *et al.*, 1997).

Closing Remarks

As shown herein, canine reproductive cycles indeed appear to be quite unique among domestic animal species. Driven by the same goal of reproductive success, dogs present different regulatory strategies. Many biological, clinical and breeding questions remain unsolved. Thus, for example, knowledge regarding mechanisms involved in termination of anestrus, the regulation of oocyte maturation, resumption of meiosis and early embryonic development, needs to be improved. So far, in vitro fertilization of in vivo matured oocytes has been reported, but obtaining mature oocytes post-ovulation is tedious and erratic. As for in vitro oocyte maturation, the efficiency is still low. Better understanding of mechanisms involved in the establishment and maintenance of pregnancy could be helpful in understanding the pathogenesis of some frequently occurring uteropathies and could lead to development of better clinical protocols, providing better assistance for patients during maintenance and termination of canine gestation.

Acknowledgements

The work was supported by funds from the Swiss National Science Foundation (SNSF) research grant number 31003A_160251 to MPK. The author would like to thank Jeanne Peter from the Department of Scientific Communication of the Vetsuisse Faculty, University of Zurich, for her help with preparing schematic figures and to Dr. Barry Bavister for careful editing of the manuscript. The author thanks Prof. Iris Reichler and Prof. Alois Boos from the University of Zurich, for critical reading of the manuscript and their helpful comments.

References

- Bischoff, T.L.W., 1845. Entwicklungsgeschichte des Hunde-Eies. (Eng: the development of the canine oocyte.). Braunschweig Druck und Verlag von Friedrich Vieweg und Sohn. Available at: <http://digi.ub.uni-heidelberg.de/diglit/bischoff1845/0005?sid=d04cee3b21867da2b18620756659777e>.
- Concannon, P., Tsutsui, T., & Shille, V. (2001). Embryo development, hormonal requirements and maternal responses during canine pregnancy. *Journal of Reproduction and Fertility, Supplement*, 57, 169–179.
- Concannon, P. W. (2009). Endocrinologic control of normal canine ovarian function. *Reproduction in Domestic Animals*, 44(Suppl. 2), 3–15.
- Concannon, P. W. (2011). Reproductive cycles of the domestic bitch. *Animal Reproduction Science*, 124, 200–210.
- Concannon, P. W., Castracane, V. D., Temple, M., & Montanez, A. (2009). Endocrine control of ovarian function in dogs and other carnivores. *Animal Reproduction*, 6, 172–193.
- Feldman, E. C., & Nelson, R. W. (2004). Ovarian cycle and vaginal cytology. *Canine and Feline Endocrinology and Reproduction* (third ed., pp. 752–774). St. Louis, MO: Saunders (An Imprint of Elsevier).
- Graubner, F. R., Gram, A., Kautz, E., et al. (2017a). Uterine responses to early pre-attachment embryos in the domestic dog and comparisons with other domestic animal species. *Biology of Reproduction*, 97, 197–216.
- Graubner, F. R., Reichler, I. M., Rahman, N. A., et al. (2017b). Decidualization of the canine uterus: From early until late gestational in vivo morphological observations, and functional characterization of immortalized canine uterine stromal cell lines. *Reproduction in Domestic Animals*, 52(Suppl. 2), 137–147.
- Hoffmann, B., Busges, F., Engel, E., Kowalewski, M. P., & Papa, P. (2004). Regulation of corpus luteum-function in the bitch. *Reproduction in Domestic Animals*, 39, 232–240.
- Kowalewski, M. P. (2014). Luteal regression vs. prepartum luteolysis: Regulatory mechanisms governing canine corpus luteum function. *Reproductive Biology*, 14, 89–102.
- Kowalewski, M. P. (2017). Regulation of corpus luteum function in the domestic dog (*Canis familiaris*) and comparative aspects of luteal function in the domestic cat (*Felis catus*). In R. Meidan (Ed.), *The Life Cycle of the Corpus Luteum* (pp. 133–157). Cham: Springer International Publishing.

- Kowalewski, M. P., Gram, A., Kautz, E., & Graubner, F. R. (2015). The dog: Nonconformist, not only in maternal recognition signaling. *Advances in Anatomy, Embryology and Cell Biology*, 216, 215–237.
- Kowalewski, M. P., Mutembei, H. M., & Hoffmann, B. (2008). Canine prostaglandin F2alpha receptor (FP) and prostaglandin F2alpha synthase (PGFS): Molecular cloning and expression in the corpus luteum. *Animal Reproduction Science*, 107, 161–175.
- Nowak, M., Boos, A., Kowalewski, M.P., 2018. Luteal and hypophyseal expression of the canine relaxin (RLN) system during pregnancy: Implications for luteotropic function. *PLOS ONE*.
- Nowak, M., Gram, A., Boos, A., et al. (2017). Functional implications of the utero-placental relaxin (RLN) system in the dog throughout pregnancy and at term. *Reproduction*, 154, 415–431.
- Reynaud, K., Saint-Dizier, M., Tahir, M. Z., et al. (2015). Progesterone plays a critical role in canine oocyte maturation and fertilization. *Biology of Reproduction*, 93, 87.
- Romagnoli, S. E., Cela, M., & Camillo, F. (1991). Use of prostaglandin F2 alpha for early pregnancy termination in the mismated bitch. *Veterinary Clinics of North America: Small Animal Practice*, 21, 487–499.
- Steinetz, B. G., Goldsmith, L. T., & Lust, G. (1987). Plasma relaxin levels in pregnant and lactating dogs. *Biology of Reproduction*, 37, 719–725.
- Wanke, M., Loza, M. E., Monachesi, N., & Concannon, P. (1997). Clinical use of dexamethasone for termination of unwanted pregnancy in dogs. *Journal of Reproduction and Fertility. Supplement*, 51, 233–238.
- Wright, S. J. (2017). Spotlight on reproduction in domestic dogs as a model for human reproduction. In H. Schatten, & G. M. Constantinescu (Eds.), *Animal Models and Human Reproduction: Cell and Molecular Approaches with Reference to Human Reproduction* (pp. 99–109). Ames, IA: Blackwell Publishing.
- Zatta, S., Rehrauer, H., Gram, A., Boos, A., & Kowalewski, M. P. (2017). Transcriptome analysis reveals differences in mechanisms regulating cessation of luteal function in pregnant and non-pregnant dogs. *BMC Genomics*, 18, 757.

Relevant Website

<http://omia.angis.org.au/home/>. — OMIA.

Female Cat Reproduction

Janine L Brown and Pierre Comizzoli, Smithsonian Conservation Biology Institute, Front Royal, VA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The domestic cat is one of the world's most popular companion animals, in addition to playing an important role in biomedical research, contributing to the fields of genetics, fertility, developmental biology, neurophysiology, cancer, immunology, and infectious disease. Being obligate carnivores, cats share the instinct to hunt prey. With the exception of lions, felids are solitary and do not live in socially structured adult groups. They are generally adaptable to a wide range of environments, and are found on every continent except Antarctica. Of the 41 known wild cat species, at least two-thirds are threatened or endangered with extinction in some part of their natural range. Felids are placed into small (<6.5 kg, 45% of species), medium (7–20 kg, 35%) and large (35–135 kg, 20%) body weight categories. The major causes of wild population declines are habitat loss and fragmentation, human-animal conflict, and for some species, poaching for pelts and traditional medicines. Yet, despite a long history in captivity, a significant challenge continues to be determining how to manage these generally secretive animals in environments that allow them to thrive and reproduce. Even in relatively naturalistic enclosures, reproduction in captive felids remains poor, with the exception of some of the *Panthera* species. As reproduction is the essence of species survival, an understanding of how female cats reproduce can contribute to their sustainability and conservation.

Domestic Cat

Reproductive Anatomy

The vulva is located just below the anus and is followed by a 10–30 mm vestibule ending with a narrow ring-like structure (close to the urethra). The straight vagina is long (up to 70 mm) and ends with a short cervix that has a dorso-cranial opening. The small uterine body is partially divided internally by a septum leading to two horns (50–70 mm long) that are suspended dorsally by the mesometrium. After the utero-tubal junction, the long and convoluted oviducts are lateral to the ovaries and terminate into an ovarian bursa formed by the mesosalpinx. The ovaries (10×5 mm) are caudal to the kidneys and attached proximally by the suspensory ligament and dorsally by the mesovaria.

Sexual Maturation and Seasonality

The female reaches puberty at 5–12 months of age, at approximately 75% of adult body weight, with some dependence on season of birth and growth rate (Tsutsui and Stabenfeldt, 1993; Wildt *et al.*, 1998). In general, short-haired breeds reach puberty earlier than long-haired cats. The domestic cat is seasonally polyoestrous under natural photoperiods, but will cycle year-round when housed on a 12–14 h light cycle (Shille *et al.*, 1979). Cats are long-day breeders, so ovarian activity is reduced during winter and resumes after exposure to increasing daylight (February or March through September in the northern hemisphere). Because seasonality is affected by photoperiod, geographic latitude influences degree of seasonality. Melatonin regulates photoperiod-induced seasonality in the cat, with concentrations being highest during the dark phase (Leyva *et al.*, 1989). Females appear to exhibit a graded response to melatonin, with greater follicular suppression occurring as the daily duration of melatonin elevation is increased (Leyva *et al.*, 1989; Graham *et al.*, 2004).

Gametogenesis

Like in many other mammal species, the cat ovarian follicle is composed of an oocyte surrounded by granulosa cells. Morphology changes as the oocyte grows and the surrounding cells differentiate during folliculogenesis. After the migration of primordial germ cells into the gonad, the early stages of gametogenesis begin during the second or third week of fetal development. There is then a large number of primordial follicles located throughout the ovarian cortex, beneath the tunica albuginea (Bristol-Gould and Woodruff, 2006). In addition to follicles, the ovarian cortex contains stroma cells and an extra-cellular matrix that plays a role in the regulation of the early stages of folliculogenesis. The ovarian medulla is mainly comprised of blood vessels dispatched to the rest of the gonad.

Primordial follicles have an oocyte ranging from 20 to 30 μ m in diameter directly surrounded by flat cells. After activation, which is controlled by intra-ovarian factors and not gonadotropins, primordial follicles transition to primary follicles containing oocytes ranging from 30 to 50 μ m in diameter and a single layer of cuboidal granulosa cells. At this stage, the zona pellucida is easily detected, in addition to a basement membrane separating the surrounding layer of granulosa cells from the ovarian stroma (Bristol-Gould and Woodruff, 2006). Primary follicles grow into secondary follicles that vary greatly in size due to multiple layers of granulosa cells, ranging from 100 to 400 μ m, and contain oocytes ranging from 40 to 75 μ m in diameter. At least two, but usually more layers of granulosa cells are apparent and a theca cell layer is assembled on the outer side of the basement membrane. The next stage is the early/small antral follicle that is similar in diameter at the onset of antral formation to secondary follicles. Antral follicle

growth (from 300 to 1000 μm in diameter as follicular fluid accumulates) and cell differentiation are dependent on gonadotropins. While oocytes are approximately 75–90 μm in diameter, these antral follicles are surrounded by two to three layers of thecal cells. Large antral follicles are the ultimate stage that grow to 2–3 mm in diameter and contain intact mural and cumulus granulosa cell layers, many theca cell layers, a large antrum, and an oocyte. Not all ovarian follicles reach that stage, however, as more than 60% will undergo atresia. At the final stage, the oocyte diameter is $\sim 100 \mu\text{m}$ (without the zona pellucida) and the germinal vesicle (nucleus at the Prophase I of the meiosis) is larger than in other species (35–40 μm diameter) (Comizzoli *et al.*, 2011). The oocyte cytoplasm is dark because of the high concentration in lipid droplets. Preovulatory follicles and the enclosed oocytes require an adequate luteinizing hormone (LH) stimulus to trigger oocyte maturation (resumption of the meiosis to the metaphase II stage) and ovulation.

Ovarian Cycle

There are four phases of the domestic cat ovarian cycle: proestrus, estrus, diestrus, and anestrus (or interestrus). Based on regular fluctuations in estrogens, unbred domestic cats have an estrous cycle length of ~ 2 weeks, with estrus lasting 3–7 days.

Proestrus

The proestrus phase lasts for 1–2 days, but the signs are not always obvious. This phase consists of the presence of ovarian follicles (1–2 mm diameter) and synthesis of estradiol-17 β that circulates at high concentrations (Shille *et al.*, 1979). The female demonstrates behaviors, such as vocalizing, rubbing the head and neck against objects, and rolling on the ground. Occasionally, there is interest in males, but it excludes copulation.

Estrus

Females are in estrus for 3–16 days, during which time they display clear mating behaviors: for example, calling, rolling, urinating. Females assume a lordosis posture, head and front legs down, rump held high, during mating. Estrous behavior rapidly follows increased circulating estradiol-17 β associated with advanced follicular development (Shille *et al.*, 1979). Estrus is characterized by permitting male mounting and coitus, and usually vocalization, lordosis, and foot-treading. Behavioral signs are strongest in association with peak estrogen secretion around Day 3 (Tsutsui and Stabenfeldt, 1993). Estradiol-17 β usually remains above 20 pg/mL of serum (mean range, 25–100 pg/mL) during estrus (Fig. 1). Distinct pre-ovulatory follicles (>2 mm diameter; usually one to four follicles/ovary) protrude slightly above the ovarian surface. Neither coitus nor ovulation affects the duration of elevated estradiol-17 β or estrus. Frequent matings are usually needed to stimulate sufficient LH to cause final follicular maturation and ovulation, which occurs 24–48 h post-coitus depending upon the time of mating onset (Fig. 1). The ovulatory LH surge occurs within minutes of copulation, peaks in a few hours and returns to baseline within 4–16 h (Shille *et al.*, 1983; Johnson and Gay, 1981). If ovulation is not induced during estrus, the queen will enter an anovulatory phase, referred to as anestrus or interestrus, and go into proestrus again. If the mated queen does ovulate, diestrus ensues, which can proceed to gestation if conception occurs or to a nonpregnant luteal phase if the mating is infertile.

The cat is typically classified as an induced ovulator; however, spontaneous ovulations occur in many laboratory-maintained females, often frequently, depending upon the individual, housing conditions, proximity to a male, and possibly genetics (Concannon, 1991). Reflex ovulations depend on coitus-induced neuronal stimulation of the medial basal hypothalamus, release of gonadotropin-releasing hormone (GnRH), and a subsequent LH surge from the pituitary (Fig. 1). Cats copulate frequently

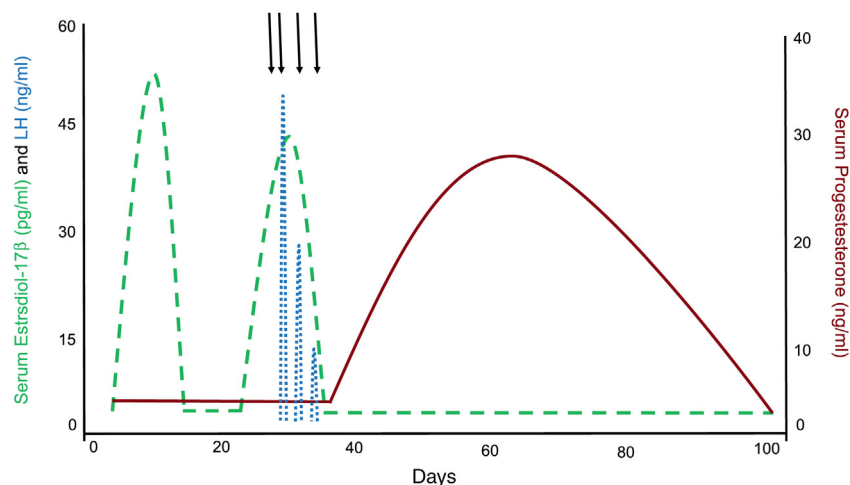


Fig. 1 Schematic diagram of circulating estradiol-17 β (dashed line), LH (dotted line) and progesterone (solid line) concentrations during the estrous cycle and post-ovulation in domestic cats. Arrows designate mating.

during estrus (as often as 36 times within 36 h), and early matings serve more to finalize folliculogenic events (oocyte maturation and release) than to ensure the presence of many sperm. With frequent mating, blood LH surges within 8 h of coital onset and then gradually declines during the next 20–24 h (Fig. 1). Such LH surges are sufficient to cause final follicular maturation, with ovulation then occurring at variable time after first breeding. Queens mated three times/day beginning on Day 1 of estrus require ~48 h to ovulate, whereas cats mated initially on Day 2 of estrus ovulate within 30–36 h. Matings starting on Day 3 or 4 complete ovulation within ~32 h. Pre-ovulatory follicles (2.5–3.5 mm diameter) become vascularized, develop a central hyperemic stigma, and release follicular contents onto the ovarian surface, with a slight inward collapse of the follicular apex. Ovulation is all-or-none because follicle number before mating corresponds to corpora lutea (CL) number after coitus (~1–4 CL/ovary). If mating is limited to one copulation, neither an LH surge nor ovulation usually occurs. This neuroendocrine pathway can become refractory because copulations on consecutive days result in fewer LH surges of lower amplitude (Fig. 1). The last copulations on a given day of unrestricted matings fail to elicit increased LH, and suppression may persist for 48–72 h. However, injecting GnRH can induce a pronounced LH surge, indicating that decreased responsiveness is caused by depleted hypothalamic GnRH or feedback inhibition of GnRH on further hypothalamic GnRH release.

Diestrus

Ovulating queens enter diestrus, a phase in which the queen no longer displays estrual postures and is unresponsive to the male. There is vacuolation (luteinization) of granulosa and theca interna cell cytoplasm in corpora hemorrhagica as early as 64 h after first mating. By 100 h (48–72 h after ovulation), CL develop as raised, reddish-orange structures with polygonal cellular morphology (Fig. 2), each reaching maximal size within 10–16 days. Circulating progesterone increases above baseline (<1 ng/mL) ~76 h after first mating (~40 h after ovulation), increasing to 25–90 ng/mL within 4–22 days. In response to rising progesterone, uterine glandular epithelia show increased cell height and cytoplasmic vacuolation (Fig. 2). Luteal progesterone and LH receptor content are static for the first 100 h and then increase in parallel with peripheral progesterone (Fig. 2). Rapid progesterone increases beginning at 76 h reflects accelerated synthesis within the CL, with LH playing a luteotropic role. Progesterone is elevated for 36–38 days in nonpregnant cats, after which CL gradually regress to form persistent luteal scars. Many females then reinstate estrous cyclicity within 7–14 days. The interval of elevated progesterone during a sterile luteal phase (~36 days) is shorter than that during pregnancy (63–67 days) (Fig. 3). Though often referred to as ‘pseudopregnancy’, the nonpregnant luteal phase in the cat is not associated with maternal behaviors or mammary gland development.

Anestrus

Anestrus (or interestrus) is a phase of reproductive dormancy characterized behaviorally by indifference or active resistance to sexual advances. Mature ovarian follicles undergo atresia, and peripheral estradiol-17 β is at nadir. The normal interestrus phase in cycling, nonovulating females ranges from 7 to 21 days. Seasonal anestrus also is observed in cats exposed to natural photoperiods, with reduced ovarian activity during short days. However, many cats appear to cycle year-round.

Conception, Pregnancy and Parturition

Multiple oocytes are ovulated at the metaphase II stage, are fertilized in the proximal oviduct, and most reach the 2-cell stage by 64 h after first breeding. Blastomeres then cleave every 24 h (Fig. 2). Embryos undergo compaction at the morula stage while passing through the utero-tubal junction and form early blastocysts 124–148 h after first mating. This timing correlates with the decline of circulating estradiol 17 β and rapidly increasing progesterone. This period also corresponds with pronounced morphological changes in the uterine histology (Fig. 2). Cat embryos then migrate between uterine horns to evenly distribute fetuses. After zona lysis, implantation and the beginning of the formation of a zonary endotheliochorial type placenta begins on Day 13–14 post mating.

Temporal progesterone profiles after ovulation and through the demise of the CL vary among individuals, so progesterone concentrations cannot be used as a reliable indicator of pregnancy during the first 36 days after ovulation. Luteal LH receptor and progesterone content increases after 100 h in parallel with the rise in circulating progesterone (Fig. 2; Roth *et al.*, 1995). After ovulation, serum estradiol-17 β periodically fluctuates above basal levels, indicating steroidogenic activity occurs even during gestation. Estrogens increase significantly and steadily during the latter half of gestation, with a distinct surge occurring about a week before parturition (Schmidt *et al.*, 1983). Progesterone is produced primarily by CL during the first 40–45 days of pregnancy. Ovariectomy does not induce abortion, so the placenta has been credited with producing progesterone during the last 3 weeks of pregnancy. During the second half of pregnancy, estradiol-17 β periodically fluctuates above basal levels, indicating that there are continuous waves of follicular development. A distinct estradiol-17 β surge occurs 8 or 9 days before parturition. Queens occasionally mate during pregnancy, with superfetation a possible but infrequent occurrence. Peripheral prolactin increases about Day 35 of pregnancy, plateaus 2 weeks later, is maintained through parturition and the first 4 weeks of lactation, and is at nadir shortly after weaning. Prolactin may be luteotropic in the pregnant cat because administering the anti-prolactin, dopamine antagonist, cabergoline, at 30–40 days reduces progesterone to baseline and causes fetal absorption or abortion (Johnson and Gay, 1981; Jochle and Jochle, 1993). Peripheral relaxin from the fetoplacental unit increases beginning about Day 25 of gestation to help soften pelvic connective tissue, reaches a plateau 5–10 days later and then declines to baseline at parturition (Stewart and Stabenfeldt, 1985). Progesterone declines slowly, beginning ~30 days into gestation, returning to nadir on, or shortly after, the day of parturition. Circulating prostaglandin F $_{2\alpha}$ from the fetoplacental unit and endometrium increases at ~Day 30, plateaus at Day 45, and surges

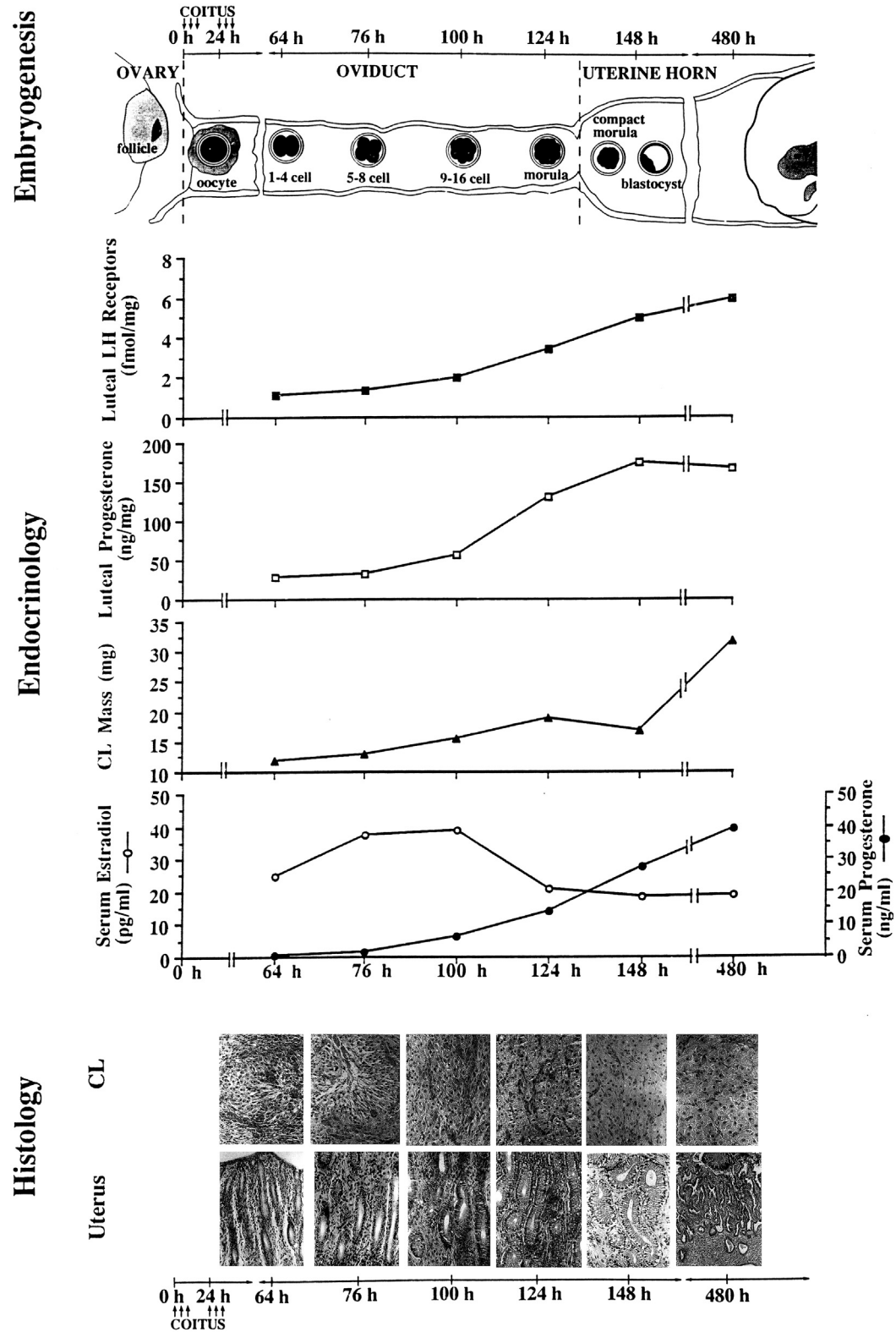


Fig. 2 Composite diagram of *in vivo* embryogenesis, endocrine profiles, and histological traits in natural estrus and mated domestic cats.

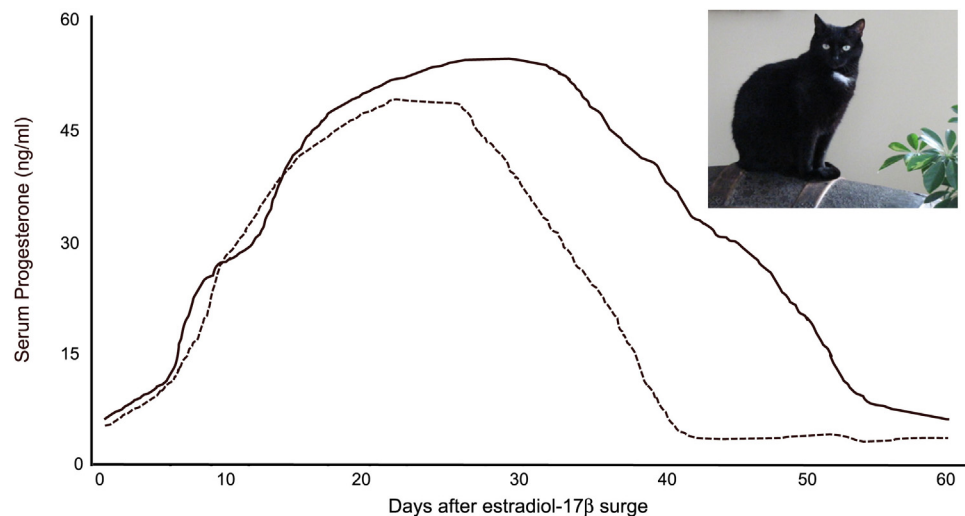


Fig. 3 Serum progesterone profiles during a pregnant (solid line) and nonpregnant (dashed line) luteal phase in the domestic cat.

just at the end of gestation to facilitate parturition (Tsutsui and Stabenfeldt, 1993). This hormone has a direct luteolytic effect and can induce abortion and fetal expulsion if administered after Day 30 of gestation (Versteegen *et al.*, 1993).

Diagnosing pregnancy based on progesterone analysis during the first half of gestation is not effective because luteal phase profiles are similar (Fig. 3). However, because the nonpregnant luteal phase is shorter, it is possible to diagnose pregnancy if progesterone is still elevated after ~40 days post-mating. Relaxin and prolactin measured in serum or plasma also are diagnostic of pregnancy after mid-gestation because neither are secreted during nonpregnant luteal phases. Though most effective using serum or plasma, commercial canine relaxin immunoassays have been used to diagnose pregnancy during late pregnancy in domestic cat using urine (de Haas van Dorsser, 2006, 2007). Recently, an immunoassay for a prostaglandin F_{2α} metabolite in domestic cat urine and feces has been shown to be diagnostic of pregnancy (Dehnhardt *et al.*, 2012). Prostaglandin F_{2α} in circulation is quickly metabolized to 13,14-dihydro-15-keto-PGF_{2α} (PGFM) in the lungs, and has a longer half-life in blood than its original compound. In dogs, PGFM is elevated 2–4 days before birth (Luz *et al.*, 2006), but in domestic cats, it increases several weeks before parturition.

Kittens are weaned after ~8 weeks, thus permitting a female to produce up to three litters per year. Domestic cats have long served as the model species for developing assisted reproductive techniques in wild felids (Howard *et al.*, 1992, 1996).

Non-Invasive Steroid Hormone Monitoring

A series of studies in the 1990s on domestic cats paved the way for development of noninvasive techniques to monitor reproductive hormones in felids. Unlike other taxa, where steroids typically are found in both urine and feces, in cats the main route of steroid excretion is the feces. For example, after injection of radiolabelled estradiol-17β and progesterone into domestic cats, 95% of metabolites were excreted in feces within 1–2 days (Shille *et al.*, 1990; Brown *et al.*, 1994). High performance liquid chromatography, gas chromatography/mass spectrometry and immunoreactivity analyses demonstrated that: (1) fecal estrogens consist primarily of unconjugated and conjugated (3-sulfate, 17α-sulfate and 17β-sulfate) estradiol, with minor amounts of estrone and estriol; and (2) progesterone is metabolized and excreted as mostly polar compounds (>75%), presumably conjugates, and some unconjugated pregnanes. Steroid metabolites can be extracted from feces by boiling, vortexing or shaking in combinations of organic solvents (e.g., ethanol, methanol), usually containing at least 10% water or buffer to assist in recovery of conjugated steroids. A number of variables can affect measures of steroid metabolites: age of sample after defecation, environmental exposure, sample volume and consistency, and sample homogeneity. These effects are not always consistent between species or hormones, necessitating that each assay and fecal handling technique be properly validated using both laboratory and biological tests. As discussed below, noninvasive fecal monitoring has played a key role in our understanding of the wide range in reproductive patterns across felid species.

Wild Felids

General Biology

Information on sexual maturity, estrous cycle characteristics, gestation length, and litter size of the 41 currently recognized nondomestic felids (Kitchener *et al.*, 2017) is summarized in Table 1. Because of their secretive nature, limited information is

Table 1 Life history and reproductive characteristics of Felidae

Common name	Genus/species	Geographic range	Sexual maturity (months)	Estrous cycle (days)	Estrus (days)	Gestation (days)	Litter size (range)	Seasonal breeder
African golden cat	<i>Caracal aurata</i>	West/Central Africa	11	nd	nd	75–81	1–2	nd
African wildcat	<i>Felis lybica</i>	North Africa and Near East	11	nd	5–9	56–70	2–5	yes
Andean mountain cat	<i>Leopardus jacobitus</i>	South America-Andes	nd	nd	nd	nd	1–2	nd
Asian golden cat	<i>Catopuma temmincki</i>	Tropical Asia	12–24	10–21	3–6	80–92	1–3	no
Black-footed cat ^a	<i>Felis nigripes</i>	Southern Africa	12–21	5–29	2–9	63–71	1–2	no
Bobcat ^a	<i>Lynx rufus</i>	North America	10–12	44	5–10	50–70	1–6	yes
Bornean bay cat	<i>Catopuma badia</i>	Borneo	nd	nd	nd	nd	nd	nd
Canadian lynx ^a	<i>Lynx canadensis</i>	North America	10–23	monoestrus	2–5	60–65	2–5	yes
Caracal ^a	<i>Caracal caracal</i>	Sub-Saharan Africa, North Africa and Southwest Asia	14–16	12–16	1–6	78–81	1–3	no
Cheetah ^a	<i>Acinonyx jubatus</i>	Southern and Eastern Africa and Iran	24–36	7–23	2–6	90–98	1–8	no
Chinese mountain cat	<i>Felis bieti</i>	China	nd	nd	nd	nd	2–4	yes
Clouded leopard ^a	<i>Neofelis nebulosa</i>	Tropical Asia	20–30	25–30	3–6	85–93	1–5	yes
Domestic cat ^a	<i>Felis catus</i>	Worldwide	5–12	14–21	3–7	64–67	1–9	variable ^b
Eurasian lynx ^a	<i>Lynx lynx</i>	Eurasia	20–24	monoestrus	2–7	68–72	1–4	yes
European wildcat	<i>Felis silvestris</i>	Europe	10–12	nd	2–8	63–68	1–8	yes
Fishing cat ^a	<i>Prionailurus viverrinus</i>	Tropical Asia	12–15	16–24	4–8	63–70	1–4	no
Flat-headed cat	<i>Prionailurus planiceps</i>	Tropical Asia	nd	nd	nd	56	1–4	no
Geoffroy's cat ^a	<i>Leopardus geoffroyi</i>	South America	12–18	12–20	2–3	72–78	2–3	yes
Iberian lynx ^a	<i>Lynx pardinus</i>	Spain	8–10	monoestrus	2–5	63–65	2–3	yes
Iriomote cat	<i>Prionailurus bengalensis iriomotensis</i>	Japan-Iriomote island	nd	nd	nd	60–70	1–4	yes
Jaguar ^a	<i>Panthera onca</i>	Americas	24–36	30–42	4–12	91–111	1–4	no
Jaguarundi	<i>Puma yagouaroundi</i>	Americas	24–36	nd	3–5	70–75	1–4	no
Jungle cat	<i>Felis chaus</i>	Tropical Asia	11–18	nd	5	63–68	1–6	yes
Kodkod	<i>Leopardus guigna</i>	South America	nd	nd	nd	72–78	1–3	nd
Leopard ^a	<i>Panthera pardus</i>	Africa and Tropical Asia	24–36	7–42	6–9	90–105	1–3	no
Leopard cat ^a	<i>Prionailurus bengalensis</i>	Tropical Asia	8–12	nd	5–9	56–70	1–4	variable ^b
Lion ^a	<i>Panthera leo</i>	Sub-Saharan Africa and India	13–24	8–30	2–9	105–114	1–6	no
Marbled cat	<i>Pardofelis marmorata</i>	Tropical Asia	21–22	nd	nd	66–81	1–4	nd
Margay ^a	<i>Leopardus wiedii</i>	Central and South America	18–24	11–25	2–6	76–84	1–3	no
Ocelot ^a	<i>Leopardus pardalis</i>	Central and South America	18–22	7–31	2–6	79–85	1–3	no
Pallas' cat ^a	<i>Otocolobus manul</i>	Central Asia	10–12	7–21	4–12	66–75	2–5	yes
Pampas cat	<i>Leopardus colocolo</i>	South America	24	nd	nd	80–85	1–3	nd
Puma ^a	<i>Puma concolor</i>	Americas	18–24	21–28	5–8	84–96	1–6	no
Rusty-spotted cat	<i>Prionailurus rubiginosus</i>	India and Sri Lanka	11–13	nd	1–11	65–71	1–3	no
Sand cat ^a	<i>Felis margarita</i>	North Africa, Middle East and Central Asia	9–14	5–28	2–11	59–69	1–5	variable ^b
Serval ^a	<i>Leptailurus serval</i>	Sub-Saharan Africa	18–24	12–20	1–4	67–77	1–5	variable ^b
Snow leopard ^a	<i>Panthera uncia</i>	Central and South Asia	24–36	15–39	2–12	98–104	1–5	yes
Southern tigrina	<i>Leopardus guttulus</i>	South America	nd	nd	nd	nd	nd	nd
Sunda clouded leopard	<i>Neofelis diardi</i>	Indonesia	nd	nd	nd	nd	nd	nd
Sunda leopard cat	<i>Prionailurus javanensis</i>	Southeast Asia	nd	nd	nd	nd	nd	nd
Tiger ^a	<i>Panthera tigris</i>	Tropical Asia	36–48	10–29	3–7	93–112	1–5	no
Tigrina ^a	<i>Leopardus tigrinus</i>	Central and South America	10–12	11–27	2–6	78–86	1–3	nd

^aAt least some hormonal data are available (serum and/or fecal metabolite analyses).^bSeasonality occurs in some populations but not others.Source: Based on Kitchener, A.C., Breitenmoser-Würsten, C., Eizirik, E., *et al.*, 2017. A revised taxonomy of the Felidae. The final report of the cat classification task force of the IUCN/SSC Cat Specialist Group. Cat News Special Issue 11, pp. 1–80.

available on the biology of felids in situ. Most is based on studies of captive individuals. Hormone data are available for about 23 species, mostly through fecal steroid metabolite analyses, although much are based on only one or two animals and for short durations. For some species, however, there is almost no reproductive information: Andean mountain cat, Bornean bay cat, Chinese mountain cat, flat-headed cat, Iriomote cat, kodkod, Southern tigrina, Sunda clouded leopard and Sunda leopard cat.

Reproductive tract and ovarian anatomies described so far are similar to the domestic cat and only vary in size proportionally to the body weight of the species.

Ovarian Cycle and Seasonality

Folliculogenesis is not different from what has been described in the domestic cat. Also, similar to the domestic cat, estrous cycles of wild felids are characterized by periods of high (estrus) and low (interestrus) estrogen concentrations (**Fig. 4**). Based on estrogen analyses, demographic data or behavioral observations, estrous cycle lengths range from less than a week to just over a month across species, with estrus ranging from 1 to 12 days (**Table 1**). Wide variability in reproductive data are due to limited sample collection and/or assessment of only a few individuals/species. Endocrine data are the most accurate for defining reproductive cycle characteristics because of the high prevalence of silent estrus. Most wild felids mate frequently; thus, it has been assumed they too are strictly induced ovulators. However, we now know that felids exhibit a range of ovulatory patterns, from almost exclusively induced to varied combinations of induced and spontaneous (e.g., **Fig. 5**). These differences occur not only across species, but also between individuals within a species (**Brown, 2006, 2011**). Thus, spontaneous increases in progestagens after estrogen surges are nonexistent or rare in species like the tiger, puma, snow leopard, cheetah, tigrina, jaguar, sand cat, lynx and ocelot; occur at least occasionally in lion, leopard, black-footed cat, Pallas' cat, bobcat, and fishing cat; and are quite common in the clouded leopard and margay. In some species, non-mating induced ovulations are more prevalent when females are housed together, whereas in others they also can occur in singletons. Thus, within this taxon ovulatory mechanisms vary, regulated to a greater or lesser degree by species and perhaps individual-specific responses to physical and/or psychosocial stimuli.

Seasonality of reproduction is specified in **Table 1**. At least 13 species exhibit seasonal breeding in the wild and/or captivity, whereas for 15 others, births have been observed in every month of the year. Ten of these species have no data on seasonality of breeding or births. Seasonality of reproduction is controlled in part by photoperiod, similar to that for the domestic cat. For example, clouded leopards exhibit anestrus in the late summer/early fall both in northern latitudes and in Asia, but if housed indoors with exposure to 12 h of continuous artificial light per day, females will cycle year-round (**Brown et al., 1995**). Pallas' cats have the shortest breeding season, with ovarian activity observed only for ~3 months of the year (Jan-Mar) (**Brown et al., 2002**). Sudden transitions to 'long days', as in a zoo lights festival, can stimulate premature follicular steroidogenesis in highly seasonal cat species, and negatively impact reproduction (**Brown et al., 2002**). A few species, like leopard cat, sand cat and serval, exhibit 'variable' seasonal reproduction that is linked to environmental conditions or prey availability rather than being restricted to specific months.

Many felids exhibit periods of follicular inactivity that are not associated with season. Cycles are observed in every month of the year, but not continuously for many individuals (e.g., cheetah, ocelot, fishing cat) (**Brown, 2006, 2011**). There is no common cause of ovarian acyclicity in felids. Suppression of estrous cyclicity in cheetahs (**Brown et al., 1996**) and Canadian

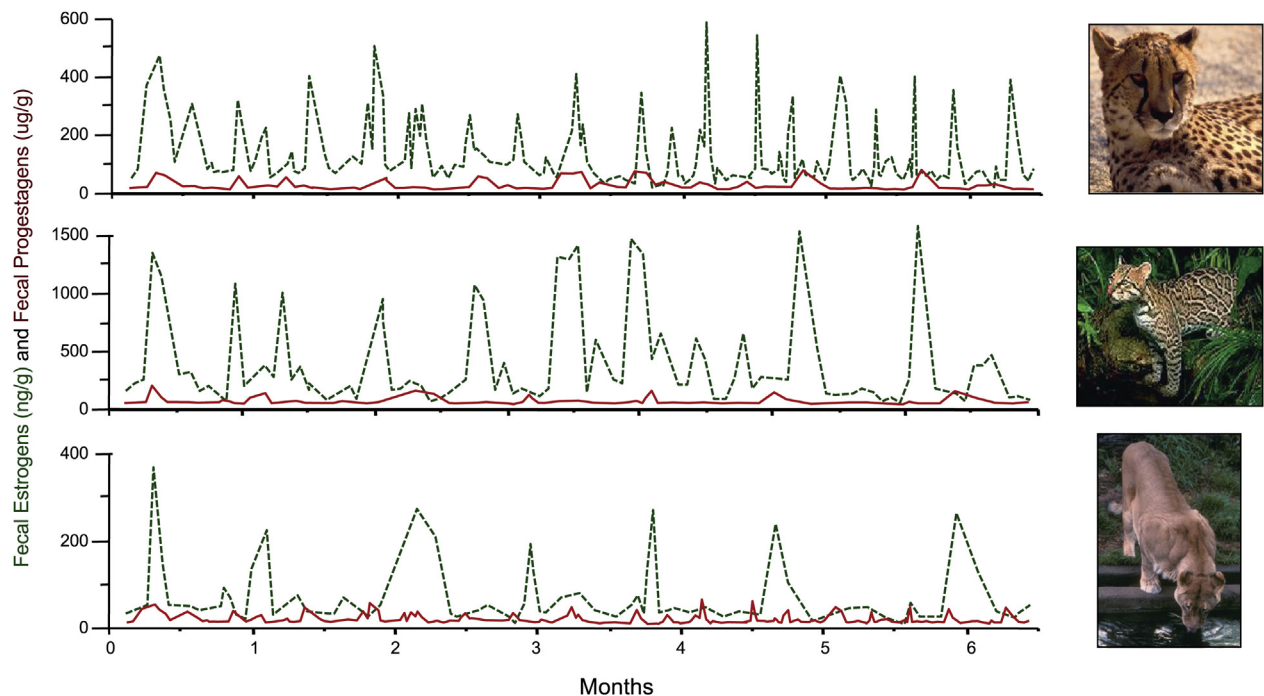


Fig. 4 Comparative profiles of fecal estrogens (dashed lines) and progestagens (solid lines) in the cheetah (top panel), tigrina (middle panel) and lion (bottom panel) showing lack of spontaneous ovulations.

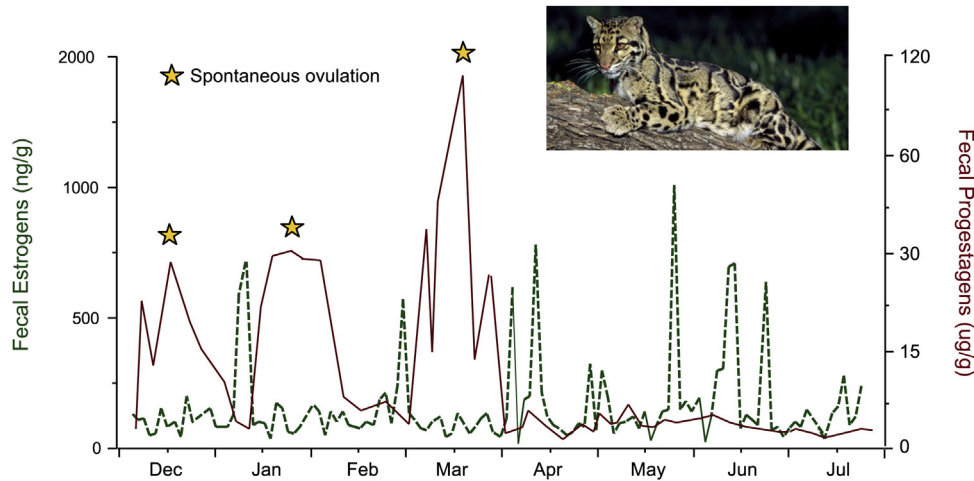


Fig. 5 Concentrations of fecal estrogens (dashed line) and progestagens (solid line) in the clouded leopard during cycles with and without spontaneous ovulations. Non-mating induced ovulations are indicated by the stars.

lynx (Fanson *et al.*, 2010) often occurs when females are housed together, and in cheetahs, separation of pairs results in normal cyclic ovarian activity (Wielebnowski *et al.*, 2002). In ocelots, ovarian inactivity was associated with poor husbandry management, and was ameliorated by addition of enrichment items and hiding places (Moreira *et al.*, 2001). For many species, however, causes of ovarian acyclicity remain elusive.

Compared to other felids, ovarian function in lynx differs in many aspects. For one, estrogen and progestagen immunoassays are not effective in clearly identifying ovarian follicular and luteal function, respectively, despite excretion of native hormone into urine and feces (Fanson *et al.*, 2009; Dehnhard *et al.*, 2008). Clear cyclic estrogen patterns even with daily collections are not evident, nor are progestagens effective at diagnosing pregnancy. Instead, overall estrogen concentrations increase throughout the breeding season, regardless of whether mating or pregnancy ensues. Iberian and Eurasian lynx are strictly seasonal breeders with, normally, a single ovulatory cycle, and thus are the only monovulatory felids (Fanson *et al.*, 2009; Dehnhard *et al.*, 2008). Another peculiarity is the presence of persistent CL and elevated progestagen concentrations, which are maintained for at least 2 years after ovulation, regardless of origin (pregnant, pseudopregnant), possibly to prevent continued follicular activity (Jewgenow *et al.*, 2014). Lynx also are unusual in that there is significant co-excretion of estrogen and progestagen metabolites in feces (up to $r=0.8$), suggesting some estrogen immunoreactivity may, in fact, be of luteal origin (Dehnhard *et al.*, 2008). Finally, continued progestagen production postpartum is observed due to the presence of active CL during the lactational anestrus period (Göritz *et al.*, 2009; Dehnhard *et al.*, 2008; Jewgenow *et al.*, 2014). This activity may act to support lactation, prevent new follicular cycle activity, and thus restrict the breeding season to midwinter. Though not as extensively studied, the Canadian lynx appears to share many of these traits, including persistent CL across cycles (Fanson *et al.*, 2010). The bobcat is the only cat in the lynx genus that is seasonally polyestrous, but they too exhibit CL that persist in subsequent cycles (Jewgenow *et al.*, 2014).

Pregnancy and Post-Partum

Progestagen concentrations during pregnant and nonpregnant luteal phases in nondomestic cats are quantitatively similar, as in the domestic cat (e.g., Fig. 6). In felids where comparative data are available, length of the non-pregnant luteal phase is about one third to one half that of pregnancy (Brown, 2006, 2011). There is an interesting species difference in estrogen excretory patterns throughout gestation, with concentrations increasing after mid gestation in the cheetah, Pallas' cat and fishing cat, similar to the domestic cat, but remain stable throughout pregnancy in the clouded leopard and tiger (Brown, 2011). Relaxin measured in urine using a canine RIA can diagnose pregnancy after mid-gestation in leopards (de Haas van Dorsser, 2006). A simple strip test from the same company (Witness Relaxin[®]) was moderately effective in diagnosing late pregnancy in Iberian lynx urine samples, but only if concentrated by ultrafiltration and brought up in serum (Braun *et al.*, 2009). More recently, PGFM has been found to increase in urine and/or feces in the weeks before parturition in several nondomestic cat species/subspecies (sand cat, African wildcat, Siberian tiger, North Chinese leopard, leopard cat, ocelot, serval, tigrina, caracal, fishing cat, Geoffrey's cat, Iberian and Eurasian lynx, cheetah, puma), thus providing a clear signal differentiating between pregnant and pseudopregnant luteal cycles (Dehnhard *et al.*, 2012, 2014, 2015). By contrast, only moderate increases in PGFM during gestation were found in Indochinese tiger and Persian leopard.

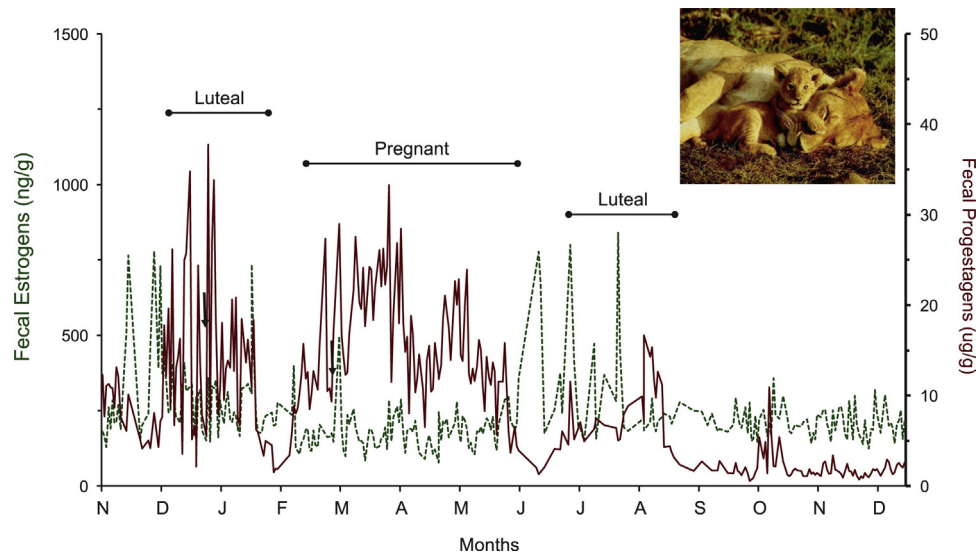


Fig. 6 Concentrations of fecal estrogens (dashed line) and progestagens (solid line) during pregnant and nonpregnant luteal phases in a lion.

Biomedical Science and Conservation

Domestic cat females have been used as animal models in reproductive science for decades, and compared to laboratory rodents, they offer many advantages because of greater similarities in follicular anatomy and gamete biology with women. Fertility preservation approaches developed in the female cat have direct applications to human reproductive medicine (Comizzoli *et al.*, 2011). Of course, the domestic cat also has been a critical model to better understand the reproductive biology of wild counterparts.

Felids express marked variations in reproductive mechanisms among species. Identifying the type of ovulation (induced versus spontaneous) and effect of seasonality on reproduction for each species is particularly important as these two characteristics impact both natural and assisted breeding efforts. The ability to easily and safely assess reproductive status through noninvasive means is vital to our understanding the causes of poor fertility of cats in natural breeding situations and in response to assisted reproductive procedures. In the nearly three decades since fecal steroid techniques were developed for wild felids, over 300 papers have been published that describe gonadal cycle patterns for just over half of the species. Thus, an impressive endocrine database now exists, and hormone monitoring has played a major role in the breeding management of many felid species.

One of the most useful benefits of steroid metabolite monitoring has been assessing causes of poor fertility in response to assisted reproductive procedures, like artificial insemination and embryo transfer, allowing these tools to more reliably contribute to species propagation. A high priority today is developing ovulation induction protocols that result in consistent responses, without ovarian hyperstimulation, to provide an optimal maternal environment for fertilization and embryo development. Along with this effort is the need to control the reproductive cycle, including down-regulating endogenous ovarian activity and synchronizing estrus (Pelican *et al.*, 2006; Swanson, 2006). More reliable tests for diagnosing pregnancy are still needed, preferably ones that are noninvasive and applicable across all species. Developing optimal protocols for breeding cats, by both natural and assisted means, are key to conserving and managing endangered populations.

References

- Braun, B. C., Frank, A., Dehnhard, M., et al. (2009). Pregnancy diagnosis in urine of Iberian lynx (*Lynx pardinus*). *Theriogenology*, 71, 754–761.
- Bristol-Gould, S., & Woodruff, T. K. (2006). Folliculogenesis in the domestic cat (*Felis catus*). *Theriogenology*, 66, 5–13.
- Brown, J. L. (2006). Comparative endocrinology of domestic and nondomestic felids. *Theriogenology*, 66, 25–36.
- Brown, J. L. (2011). Female reproductive cycles of wild felids. *Anim Reprod Sci*, 124, 155–162.
- Brown, J. L., Graham, L. H., Wu, J., Collins, D., & Swanson, W. F. (2002). Reproductive endocrine responses to photoperiod and exogenous gonadotropins in the Pallas' cat (*Otocolobus manul*). *Zoo Biol*, 21, 347–364.
- Brown, J. L., Wildt, D. E., Graham, L. H., et al. (1995). Comparison of natural versus chorionic gonadotropin-induced ovarian responses in the clouded leopard (*Neofelis nebulosa*) assessed by fecal steroids. *Biol Reprod*, 53, 93–102.
- Brown, J. L., Wildt, D. E., Wielebnowski, N., et al. (1996). Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. *J Reprod Fert*, 106, 337–346.
- Brown, J. L., Wasser, S. K., Wildt, D. E., & Graham, L. H. (1994). Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non-invasively in feces. *Biol Reprod*, 51, 776–786.

- Comizzoli, P., Pukazhenthi, B. S., & Wildt, D. E. (2011). The competence of germinal vesicle oocytes is unrelated to nuclear chromatin configuration and strictly depends on cytoplasmic quantity and quality in the cat model. *Hum Reprod*, 26, 2165–2177.
- Concannon, P. W. (1991). Reproduction in the dog and cat. In P. T. Cupps (Ed.), *Reproduction in Domestic Animals* (fourth ed., pp. 517–553). San Diego, CA: Academic Press.
- De Haas van Dorsser, F. J., Lasano, S., & Steinetz, B. G. (2007DeHaas). Pregnancy diagnosis in cats using a rapid, bench-top kit to detect relaxin in urine. *Reprod Dom Anim*, 42, 111–112.
- De Haas van Dorsser, F. J., Swanson, W. F., Lasano, S., & Steinetz, B. G. (2006DeHaas). Development, validation, and amplification of a urinary relaxin radioimmunoassay for the diagnosis and monitoring of pregnancy in felids. *Biol Reprod*, 74, 1090–1095.
- Dehnhard, M., Finkenwirth, C., Crosier, A., et al. (2012). Using PGFM (13,14-dihydro-15-keto-prostaglandin F₂alpha) as a non-invasive pregnancy marker for felids. *Theriogenology*, 77, 1088–1099.
- Dehnhard, M., Kumar, V., Chandrasekhar, M., & Jewgenow, K. (2015). Non-invasive pregnancy diagnosis in big cats using the PGFM (13,14-dihydro-15-keto- PGF₂α) assay. *PLOS ONE*. Available at: <https://doi.org/10.1371/journal.pone.0143958>.
- Dehnhard, M., Naidenko, S., Frank, A., et al. (2008). Non-invasive monitoring of hormones: A tool to improve reproduction in captive breeding of the Eurasian lynx. *Reprod Dom Anim*, 43, 74–82.
- Dehnhard, M., Naidenko, S. V., & Jewgenow, K. (2014). Comparative metabolism of PGFM (13,14-dihydro-15-keto- PGF₂alpha) in feces of felids. *Theriogenology*, 81, 733–743.
- Fanson, K., Wielebnowski, N., & Lucas, J. (2009). Reproductive physiology of Canada lynx (*Lynx canadensis*). In A. Vargas, C. Breitenmoser, & U. Breitenmoser (Eds.), *Iberian Lynx Ex Situ Conservation: An Interdisciplinary Approach* (pp. 390–399). Madrid: Fundación Biodiversidad.
- Fanson, K. V., Wielebnowski, N. C., Shenk, T. M., et al. (2010). Patterns of ovarian and luteal activity in captive and wild Canada lynx (*Lynx canadensis*). *Gen Comp Endocrinol*, 169, 217–224.
- Görztz, F., Dehnhard, M., Hildebrandt, T. B., et al. (2009). Non cat-like ovarian cycle in the Eurasian and the Iberian lynx – Ultrasonographic and endocrinological analysis. *Reprod Dom Anim*, 44(Suppl. 2), 87–91.
- Graham, L. H., Swanson, W. F., DE Wildt, D. E., & Brown, J. L. (2004). Influence of oral melatonin on natural and gonadotrophin-induced ovarian function in the domestic cat. *Theriogenology*, 61, 1061–1076.
- Howard, J. G., Barone, M. A., Donoghue, A. M., & Wildt, D. E. (1992). The effect of pre-ovulatory anaesthesia on ovulation in laparoscopically inseminated domestic cats. *J Reprod Fertil*, 96 (1), 175–186.
- Howard, J. G., Byers, A. P., Brown, J. L., et al. (1996). Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (*Neofelis nebulosa*). *Zoo Biol*, 15 (1), 55–69.
- Jewgenow, K., Painer, J., Amelkina, O., Dehnhard, M., & Goeritz, F. (2014). Lynx reproduction – Long-lasting life cycle of corpora lutea in a feline species. *Reprod Biol*, 14, 83–88.
- Jochle, W., & Jochle, M. (1993). Reproduction in a feral cat population and its control with a prolactin inhibitor, cabergoline. *J Reprod Fertil Suppl*, 47, 419–424.
- Johnson, L. M., & Gay, V. L. (1981). Luteinizing hormone in the cat. I. Tonic secretion. *Endocrinology*, 109, 240–246.
- Kitchener, A.C., Breitenmoser-Würsten, C., Eizirik, E., et al., 2017. A revised taxonomy of the Felidae. The final report of the cat classification task force of the IUCN/SSC Cat Specialist Group. *Cat News Special Issue* 11, pp. 1–80.
- Leyva, H., Madley, T., & Stabenfeldt, G. H. (1989). Effect of melatonin on photoperiod responses, ovarian secretion of estrogen and coital responses in the domestic cat. *J Reprod Fert Suppl*, 39, 135–142.
- Luz, M. R., Bertan, C. M., Binelli, M., & Lopes, M. D. (2006). Plasma concentrations of 13,14-dihydro-15-keto prostaglandin F₂-alpha (PGFM), progesterone and estradiol in pregnant and nonpregnant diestrus cross-bred bitches. *Theriogenology*, 66, 1436–1441.
- Moreira, N., Monteiro-Filho, E. L. A., Moraes, W., et al. (2001). Reproductive steroid hormones and ovarian activity in felids of the *Leopardus* genus. *Zoo Biol*, 20, 103–116.
- Pelican, K. M., Wildt, D. E., Pukazhenthi, B., & Howard, J. G. (2006). Ovarian control for assisted reproduction in the domestic cat and wild felids. *Theriogenology*, 66, 37–48.
- Roth, T. L., Munson, L., Swanson, W. F., & Wildt, D. E. (1995). Histological characteristics of the uterine environment and corpus luteum during early embryogenesis and the relationship to embryonic mortality in the domestic cat. *Biol Reprod*, 54, 1012–1021.
- Schmidt, P. M., Chakraborty, P. K., & Wildt, D. E. (1983). Ovarian activity, circulating hormones and sexual behavior in the cat. II. Relationships during pregnancy, parturition, lactation and the postpartum estrus. *Biol Reprod*, 28, 657–671.
- Shille, V. M., Haggerty, M. A., Shackleton, C., & Lasley, B. L. (1990). Metabolites of estradiol in serum, bile, intestine and feces of the domestic cat (*Felis catus*). *Theriogenology*, 34, 779–794.
- Shille, V. M., Lundstrom, K. E., & Stabenfeldt, G. H. (1979). Follicular function in the domestic cat as determined by estradiol-17β concentrations in plasma: Relation to estrous behavior and cornification of exfoliated vaginal epithelium. *Biol Reprod*, 21, 953–963.
- Shille, V. M., Munro, C., Farmer, S. W., & Papkoff, H. (1983). Ovarian and endocrine responses in the cat after coitus. *Reprod Fertil*, 69, 29–39.
- Stewart, D. R., & Stabenfeldt, G. H. (1985). Relaxin activity in the pregnant cat. *Biol Reprod*, 32, 848–854.
- Swanson, W. F. (2006). Application of assisted reproduction for population management in felids: The potential and reality for conservation of small cats. *Theriogenology*, 66, 49–58.
- Tsutsui, T., & Stabenfeldt, G. H. (1993). Biology of ovarian cycles, pregnancy and pseudopregnancy in the domestic cat. *J Reprod Fertil Suppl*, 47, 29–35.
- Versteegen, J. P., Onclin, K., Silva, L. D. M., & Donnay, I. (1993). Abortion induction in the cat using prostaglandin F_{2α} and a new anti-prolactinic agent, cabergoline. *J Reprod Fert Suppl*, 47, 411–417.
- Wielebnowski, N. C., Ziegler, K., Wildt, D. E., Lukas, J., & Brown, J. L. (2002). Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). *Anim Cons*, 5, 291–301.
- Wildt, D. E., Brown, J. L., & Swanson, W. F. (1998). Reproduction in cats. In E. Knobil, & J. Neill (Eds.), *Encyclopedia of Reproduction*, 1 pp. 497–510). New York: Academic Press.

Reproductive Toxicity of Environmental Contaminants in the Female

Warren G Foster, McMaster University, Hamilton, ON, Canada

Anne-Marie Gannon, Health Canada, Ottawa, ON, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

It has been estimated that there are more than 80,000 chemicals in commercial use and thus assessing risk associated with potential human exposure is a daunting task. Human exposure to chemical contaminants has been widely documented through biomonitoring and epidemiological studies. Among the many pathological and iatrogenic conditions that jeopardize reproductive function, chemical exposures have been associated with adverse health effects on the structure and function of the reproductive tract in females including: altered timing of pubertal onset, disruption of menstrual cycle function (Cooper et al., 2005; Jukic et al., 2015), sub-fertility and impaired response to ovulation induction (Alviggi et al., 2014; Petro et al., 2012; Tanrikut et al., 2014), ovarian cancer (Langseth and Kjaerheim 2004), increased time to achieve pregnancy (De Cock et al., 1994), spontaneous abortion (Arbuckle et al., 2001; Korrick et al., 2001), polycystic ovarian syndrome (PCOS) (Kandaraki et al., 2010; Kandaraki et al., 2011; Rutkowska and Rachon 2014) endometriosis (Cobellis et al., 2003; Kim et al., 2011; Louis et al., 2005; Reddy et al., 2006) and earlier age at menopause (Grindler et al., 2015). While the literature demonstrates numerous examples of potential associations between environmental contaminant exposure and adverse reproductive health outcomes, many others have been unable to show similar associations (Foster et al., 2008; Sadeu et al., 2010; Sifakis et al., 2017), hence links with adverse reproductive health outcomes in females remains equivocal. Although potential effects in the general population remains uncertain, the strongest evidence and potential for modifiable risk is limited to occupational groups (e.g. farmers) or specific populations whose lifestyle, such as sport fishing, presents risks for high exposure to known reproductive toxicants (Mendola et al., 2008). Furthermore, the mechanisms underlying these adverse effects are poorly defined. For many commercial chemicals though, there is an absence of evidence of human exposure, and thus causal associations are difficult to establish. Consequently, improved methods are necessary to assess reproductive health in women for occupational screening programs and epidemiological studies.

Although the literature is generally equivocal on the relationship between exposure to environmental contaminants and adverse reproductive outcomes in humans, animal studies support the above associations. Environmental contaminants inhibit germinal nest breakdown and induce oocyte aneuploidy (Hunt et al., 2003; Susiarjo et al. 2007), produce anomalies of reproductive tract development (Flaws et al., 1997; Gray et al., 1997; Gray and Ostby 1995), disrupt ovarian steroidogenesis (Craig et al., 2011), decrease ovarian follicle counts (Borgeest et al., 2002; Borman et al., 2000; Peretz et al., 2012), and impair fertility. Consequently, animal studies have become indispensable in furnishing the scientific community with experimental evidence central to establishing biological plausibility. In order to enhance the pace for screening chemicals, reduce the numbers of animals required for testing, and to define mechanisms of action (MOA), sensitive screening methods and innovative techniques are required to assess reproductive hazards.

Biomarkers of Female Reproductive Function

In women's health, clinical markers of follicle development, oocyte health, and embryo quality, as well as for the diagnosis of endometriosis, polycystic ovarian syndrome (PCOS), and ovarian cancer are either non-existent or sub-optimal. Assessing reproductive fitness in a population exposed to environmental contaminants remains a challenge owing to the relative paucity of sensitive and specific clinical markers of adverse effects. While menstrual cycle irregularity, difficulty conceiving, and repeated spontaneous abortion are all indicators of reproductive dysfunction, none are rare or unique to chemical exposures. Thus, ability to assess reproductive health in women and determine the impact of environmental contaminants in exposed populations is limited. The search for novel biomarkers of reproductive health and disease therefore continues to be a high priority research need in toxicology and reproductive medicine.

Circulating concentrations of follicle stimulating hormone (FSH) together with antral follicle counts is often used to assess ovarian reserve and detect effects of advanced maternal age. However, these parameters of reproductive fitness are of limited value in women under 40 years of age and the impact of exposure to environmental contaminants on them is poorly defined. Serum anti-Müllerian hormone (AMH) has emerged in the clinical literature as a marker of ovarian reserve. AMH is produced by healthy growing follicles and thus it serves as both a marker of follicle health as well as an indirect marker of follicle reserve (Verma et al., 2016). To wit, circulating AMH concentrations are lower in women who smoke relative to a control population (Plante et al., 2010),

suggesting that it may be a useful biomarker of effect. However, AMH has not been validated for use as a clinical marker of effect as it is unclear how it is influenced by body mass index, comorbidities, physical activity, and diet.

Clinical markers are also lacking for common gynecological disorders such as PCOS and endometriosis. Accordingly, novel biomarkers of effect and diagnostic markers of reproductive disease continue to be an unmet need. Recent studies employing phenomics, genomics, proteomics, and metabolomics are being brought to bear on the issue. Increasingly there is recognition that single markers may not have adequate sensitivity and specificity to be predictive of disease and thus marker panels have also been explored. Of the techniques emerging, research into the exosome and measurement of the concentrations of small non-coding RNA such as microRNA (miRNA) and piwi-interacting RNA (piRNA) have shown promise as potential clinical markers; however, issues of validity, reproducibility, and predictive value remain to be addressed. Hence, experimental animal studies continue to be necessary for identification of potential chemical hazards.

Experimental Evidence and Biological Plausibility

Owing to the potential for serious adverse health effects with potential life-long health consequences following exposure to chemicals, screening tests have been developed to identify potential health hazards. Animal studies are highly valuable in this arena because they allow for the testing of adverse effects using defined concentrations of test chemicals of known purity in genetically similar animals, under stringent conditions and distinct developmental stages in the absence of confounding comorbidities – all conditions that cannot be achieved in human studies.

Regulatory test methods– Regulatory test guidelines have been established by various federal and international agencies in an effort to standardize testing methods across laboratories following the same guideline. Although each test guideline developed by different regulatory bodies differs from its counterpart designed by other regulatory bodies, their aim remains the same. Specifically, these regulatory studies are designed to evaluate test chemical effects on a broad range of endpoints that cover all major reproductive and developmental events in a single screening assay that can be routinely carried out in standard laboratories. Broadly, reproduction can be broken down into six stages, which are typically assessed using a combination of three *in vivo* studies. Stage 1 encompasses pre mating to conception, Stage 2 comprises conception to implantation, Stage 3 covers implantation to the closure of the hard palate (considered the end of major organogenesis), Stage 4 includes the time between the end of major organogenesis to parturition, Stage 5 embodies birth to weaning and Stage 6 comprises weaning to sexual maturity (Parker, 2012). The main regulatory studies designed to assess adverse effects of potential toxicants on the reproductive system across all stages of reproduction are: the fertility and reproductive toxicity study (which allows for the assessment of effects on stages 1 and 2), the prenatal and developmental toxicity study (which covers stages 3 and 4), and the prenatal and postnatal study (which combines stages 3 through 6). For a listing of regulatory test guidelines put out by the various agencies, the reader is directed to Hoberman and Lewis (2012). Reproductive performance measures the steps in the reproductive cycle beginning with mating and includes gestation length that captures the processes of fertilization, implantation, embryogenesis, and fetal development followed by birth, lactation, puberty, and gametogenesis. Key outcome measures assessed are the mean time to mating (precoital interval), litter size, sex ratio, and mating, fertility, gestation, live birth, and survival indices. Developmental landmarks include birth weight, anogenital distance (AGD), eye opening, pinna unfolding, vaginal opening, and soft tissue and skeletal anomalies (e.g. vaginal septum).

All *in vivo* test guidelines, irrespective of their aim, require the measurement of tissue weights for all major organs and histopathological assessment. Test guideline-specific outcome measures include: vaginal cytology; uterine and ovarian histology (including assessment of ovarian follicle counts); circulating serum hormone concentrations (luteinizing hormone (LH), FSH, prolactin (PRL), estradiol (E₂) and progesterone (P₄). In recent years, hormones such as inhibin, activin, and AMH have been added to the list of target hormones; however, although useful for research purposes they have not been validated as biomarkers of adverse effect in a regulatory context.

These regulatory studies are designed to produce robust reproducible data for regulatory risk assessment purposes. Owing to the large volume of data produced and the potential for type I error, it is essential to evaluate each outcome against concurrent controls; however, in the event that control data falls below the normal range for the outcome of interest it may be necessary to consider using historical control data. If comparing with historical controls it is best to use contemporary controls from the same laboratory though this may not always be possible and thus historical controls from the literature may also be employed. In addition, it is important to integrate the data and ensure biological consistency across multiple related outcome measure to ensure that data are statistically significant, biologically relevant, and compound related. Results from regulatory studies help define the no or lowest observable adverse effect level (NOAEL or LOAEL) for the most sensitive adverse outcome measure. Data generated from such studies are useful in identifying potential hazards that, together with human exposure data, contribute to the risk assessment process (Council NR, 1983).

Regulatory studies have proven to be valuable for the identification of reproductive and developmental hazards and possess a number of strengths including: multiple dose groups; robust sample size typically providing adequate statistical power; genetically similar animals that have been shown to be sensitive to reproductive toxicants; an appropriate route of exposure; and integration of multiple physiological processes over specific stages of an organism's lifespan directly related to the stage of reproduction or development being evaluated. These studies are conducted according to internationally harmonized test guidelines in accordance with good laboratory practices (GLP) and standard operating procedures (SOPs), which together

enhance study reliability and generate reproducible data. Although GLP and the use of SOPs enhances study reliability and reproducibility of data, they cannot provide mechanistic insight and cannot provide an assessment of all potential pathophysiological processes that may reveal more sensitive reproductive/developmental outcomes. While regulatory screening methods provide an integrated assessment of reproductive and developmental function, there are a number of inherent weaknesses as well. The fundamental assumption in developmental and reproductive toxicology is the ability of the models employed to predict human response. However, while basic events in development are highly conserved across species, the pharmacokinetics of the maternal metabolism varies greatly resulting both false positives and negatives depending on whether the parent compound or its metabolite causes toxicity. Moreover, these screening assays are time consuming and expensive to carry out. The statistical power necessary to make confident conclusions from the data necessitates large numbers of animals and requires a large testing capacity for the laboratories undertaking these studies as they take several months to conduct due to the length of gestation and the time required following tissue collection to process and evaluate the data obtained. Additionally, most regulatory tests are conducted solely in one strain of a species, which can affect the endpoints displaying adverse effects. Use of the most sensitive strain is recommended, but it is not always immediately apparent which strain fits that condition. Inter-lab variation of data interpretation can alter the assessment of toxicity of a chemical, particularly with respect to examination of fetal morphology. Specifically, evaluation criteria and nomenclature have evolved substantially over time, making the standardization of these criteria difficult particularly in light of the fact that individual laboratories utilize different strains, assess fetuses on different days of gestation and possess large volumes of historical data based on their own criteria (Carney et al., 2012). Moreover, test guidelines often require evidence of a dose-response when assigning importance to an effect; however, in the event where one does not exist, particularly where fetal malformations are concerned, it is incumbent upon the testing laboratory to look at whether or not the reason for a lack of dose-response is due to fetal death, whereby the most affected conceptuses at the highest dose died and thus weren't available for assessment at parturition. Physiological and anatomical differences in the type of placenta utilized by test species and humans create discordance between results in a test species and the plausibility of it occurring in humans. Maternal toxicity can further muddy the waters with respect to interpretation of the data generated in reproductive and developmental toxicity studies, as developmental toxicity often occurs at the same dose at which maternal toxicity occurs, making causal determination difficult. Lastly, no routine test guidelines directly test for changes in mating behavior though it is susceptible to disruption by toxic substances (Ben Slima et al., 2017) and represents a significant concern for human reproduction.

Traditional tests employ a control group plus three dose levels designed to produce a graded response and which encompass a NOAEL, a mid-dose which elicits minimal observable adverse effects and a high dose designed to elicit clinical signs of toxicity (maternal and/or developmental) but not death or suffering. However, the high dose exposure, which is not representative of human exposure of the vast majority of chemicals, can overwhelm the detoxification machinery, leading to shifts in the metabolism of the test compound and altered toxicokinetics. Changes in toxicokinetics may produce false positives, leading risk assessors to erroneously classify compounds as harmful when in fact they are not. Important differences in biochemistry, exposure, and metabolism leave uncertainty and thus experimental animal studies designed to elucidate key mechanisms underlying observed toxic phenomenon are frequently pursued in hypothesis-driven experimental studies. Furthermore, these screening tests are not designed to provide information on target tissue exposure or MOA but may suggest a mode of action.

High throughput screening methods—To address some of these shortcomings, Tox21, a tiered testing paradigm for the 21st century (Thomas et al., 2013), was proposed to provide high throughput solutions to regulatory agencies facing the challenge of assessing the thousands of chemicals currently used in commerce but which are lacking conventional toxicity data by increasing the speed, efficiency and accuracy of toxicity testing. It represents a shift from traditional *in vivo* animal tests to *in silico* modeling, high throughput screening (HTS) of human cells *in vitro*, and toxicogenomics. This tiered testing paradigm is divided into three tiers, designed to address the question of a chemical's toxicity, as well as to elucidate potential MOA, while reducing the numbers of animals required to do so at the same time allowing for limited testing in animals as required to fill data gaps not answered by the early tiers. The testing protocol is still in its early inception and thus requires the generation of a robust database of integrated studies demonstrating the comparable application of these methods with the traditional tests, which are the current gold standard used for risk assessment purposes. Its utility and reliability in predicting MOA using only *in silico* and *in vitro* methods continues to be improved upon; however, in the absence of conventional toxicity tests, these modeling methods can be useful in providing toxicity data for risk assessment.

Mechanistic studies—In reproductive toxicology it is difficult to completely replace animal studies with tissue culture screens owing to issues related to critical windows of exposure (Pryor et al., 2000) and complex interaction of multiple specialized cell types whose physiology remains imperfectly understood. However, advances have been made in several areas that provide insight for screening chemicals and exploring potentially important mechanistic pathways. Mechanistic studies enhance the data generated on a given test chemical by identifying potential effects on pathophysiological processes as well as gene, protein, lipid, and miRNA expression. This in turn provides insight into central mechanistic pathways that could be incorporated into cell-based screening assays or identify more sensitive outcomes to drive regulatory decisions to protect human health. Regulatory studies are already complicated, time consuming and expensive and thus the risk of further complicating these studies must also be considered. Mechanisms of toxic action are explored in specialized culture systems such as granulosa and isolated ovarian follicle culture studies.

Granulosa cell cultures exploit the availability of mural granulosa cells obtained following oocyte retrieval in ovulation induction cycles. While there are obvious benefits of using human primary cells from the developing follicle, these cells are terminally

differentiated and obtained from women who have compromised fertility and thus may not be representative of granulosa cells from healthy fertile women. To obviate these limitations, some investigators have employed rodent antral follicle cultures to assess the effects of target chemicals on follicle growth, ovarian steroidogenesis, and target protein expression (Basavarajappa et al., 2011; Peretz et al., 2010; Peretz et al., 2012). However, these cells are obtained following gonadotrophin stimulation to maximize the number of cells obtained and are limited to the assessment of compound effects on a narrow follicle development stage and in an artificial environment. In an attempt to mitigate these issues and to better understand the impact of test chemicals on the development of follicles, isolated ovarian follicle culture systems have been developed to evaluate the time- and stage-dependent effects of test chemicals on follicle development, ovulation, and fertilization (Cortvrindt et al., 1998). Two-dimensional pre-antral follicle cultures have been used to demonstrate follicle stage- and time-dependent effects of ovarian toxicants (Neal et al., 2007; Sadeu and Foster 2011a, 2011b). Isolated follicle culture methods have been further enhanced to allow for three-dimensional growth to better approximate in vivo conditions (Jin et al., 2010; Kreeger et al., 2005; Xiao et al., 2017b). Recent developments have extended culture techniques to include the reproductive tract on a chip that will enable assessment of test chemical effects on the menstrual cycle in culture (Xiao et al., 2017a).

Challenges and Future Directions

There are many challenges facing reproductive biologists and toxicologists hoping to protect the health of women over the course of their lifetime. While the pressing need for novel clinical markers of disease are well recognized and their potential application in toxicology is appreciated, it is unclear if the search for individual markers or panels of markers will be more productive. Advances in omics biology have been very exciting revealing mechanisms underlying dysregulation of signaling pathways in gynecological diseases. Markers identified in these studies have potential as outcome measures to detect sensitive effects of test compounds. Moreover, the validity of the biomarkers must be demonstrated and their relationship with an adverse health outcome demonstrated. Another challenge will be how to integrate new outcome measures into the risk assessment review process. Moreover, it must be appreciated that there is a profound difference between screening assays for hazard identification and studying mechanisms of action. Results of mechanistic studies are indeed valuable; however, their role and contribution to the risk assessment process requires evaluation. Adverse outcomes must be valid and linked with a potential adverse outcome in women.

References

- Alviggi, C., Guadagni, R., Conforti, A., Coppola, G., Picarelli, S., De, R. P., et al. (2014). Association between intrafollicular concentration of benzene and outcome of controlled ovarian stimulation in ivf/icsi cycles: A pilot study. *Journal of Ovarian Research*, 7, 67.
- Arbuckle, T. E., Lin, Z., & Mery, L. S. (2001). An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. *Environmental Health Perspectives*, 109, 851–857.
- Basavarajappa, M. S., Craig, Z. R., Hernandez-Ochoa, I., Paulose, T., Leslie, T. C., & Flaws, J. A. (2011). Methoxychlor reduces estradiol levels by altering steroidogenesis and metabolism in mouse antral follicles in vitro. *Toxicology and Applied Pharmacology*, 253, 161–169.
- Ben Slima, A., Chtourou, Y., Barkallah, M., Fetoui, H., Boudawara, T., & Gdoura, R. (2017). Endocrine disrupting potential and reproductive dysfunction in male mice exposed to deltamethrin. *Human & Experimental Toxicology*, 36, 218–226.
- Borgeest, C., Symonds, D., Mayer, L. P., Hoyer, P. B., & Flaws, J. A. (2002). Methoxychlor may cause ovarian follicular atresia and proliferation of the ovarian epithelium in the mouse. *Toxicological Sciences*, 68, 473–478.
- Borman, S. M., Christian, P. J., Sipes, I. G., & Hoyer, P. B. (2000). Ovotoxicity in female fischer rats and b6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: Comparison through calculation of an ovotoxic index. *Toxicology and Applied Pharmacology*, 167, 191–198.
- Carney, E. W., Ellis, A. L., Tyl, R. W., Foster, P. M. D., Scialli, A. R., Thompson, K., & Kim, J. K. (2012). Critical evaluation of current developmental toxicity testing strategies - a case of babies and their bathwater. In R. D. Hood (Ed.), *Developmental and reproductive toxicology: A practical approach* (pp. 592–601). New York: Taylor and Francis.
- Cobellis, L., Latini, G., De Felice, C., Razzi, S., Paris, I., Ruggieri, F., et al. (2003). High plasma concentrations of di-(2-ethylhexyl)-phthalate in women with endometriosis. *Human Reproduction*, 18, 1512–1515.
- Cooper, G. S., Klebanoff, M. A., Promislow, J., Brock, J. W., & Longnecker, M. P. (2005). Polychlorinated biphenyls and menstrual cycle characteristics. *Epidemiology*, 16, 191–200.
- Cortvrindt, R. G., Hu, Y., Liu, J., & Smits, J. E. (1998). Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotropin. *Fertility and Sterility*, 70, 1114–1125.
- Council NR. (1983). *Risk assessment in the federal government. Managing the process*. Washington, D.C: National Academy Press.
- Craig, Z. R., Wang, W., & Flaws, J. A. (2011). Endocrine disrupting chemicals in ovarian function: Effects on steroidogenesis, metabolism and nuclear receptor signaling. *Reproduction*, 142(5), 633–646.
- De Cock, J., Westveer, K., Heederik, D., te, V. E., & van Kooij, R. (1994). Time to pregnancy and occupational exposure to pesticides in fruit growers in the Netherlands. *Occupational and Environmental Medicine*, 51, 693–699.
- Flaws, J. A., Sommer, R. J., Silbergeld, E. K., Peterson, R. E., & Hirshfield, A. N. (1997). In utero and lactational exposure to 2, 3,7,8-tetrachlorodibenzo-p-dioxin (tcdd) induces genital dysmorphogenesis in the female rat. *Toxicology and Applied Pharmacology*, 147, 351–362.
- Foster, W. G., Neal, M. S., Han, M. S., & Dominguez, M. M. (2008). Environmental contaminants and human infertility: Hypothesis or cause for concern? *Journal of Toxicology and Environmental Health Part B: Critical Reviews*, 11, 162–176.
- Gray, L. E., Wolf, C., Mann, P., & Ostby, J. S. (1997). In utero exposure to low doses of 2,3,7,8-tetrachlorodibenzo-p-dioxin alters reproductive development of female long evans hooded rat offspring. *Toxicology and Applied Pharmacology*, 146, 237–244.
- Gray, L. E., Jr., & Ostby, J. S. (1995). In utero 2,3,7,8-tetrachlorodibenzo-p-dioxin (tcdd) alters reproductive morphology and function in female rat offspring. *Toxicology and Applied Pharmacology*, 133, 285–294.

- Grindler, N. M., Allsworth, J. E., Macones, G. A., Kannan, K., Roehl, K. A., & Cooper, A. R. (2015). Persistent organic pollutants and early menopause in U.S. women. *PLoS One*, *10*, e0116057.
- Hoberman, A. M., & Lewis, E. M. (2012). Perspectives on the developmental and reproductive toxicity guidelines. In R. D. Hood (Ed.), *Developmental and reproductive toxicology: A practical approach, Part 3rd* (pp. 602–642). New York: John Wiley.
- Hunt, P. A., Koehler, K. E., Susiarjo, M., Hodges, C. A., Ilagan, A., Voigt, R. C., et al. (2003). Bisphenol a exposure causes meiotic aneuploidy in the female mouse. *Current Biology*, *13*, 546–553.
- Jin, S. Y., Lei, L., Shikanov, A., Shea, L. D., & Woodruff, T. K. (2010). A novel two-step strategy for in vitro culture of early-stage ovarian follicles in the mouse. *Fertility and Sterility*, *93*, 2633–2639.
- Jukic, A. M., Calafat, A. M., McConaughy, D. R., Longnecker, M. P., Hoppin, J. A., Weinberg, C. R., et al. (2015). Urinary concentrations of phthalate metabolites and bisphenol a and associations with follicular-phase length, luteal-phase length, fecundability, and early pregnancy loss. *Environ Health Perspect*, *124*(3), 321–328.
- Kandaraki, E., Chatzigeorgiou, A., Livadas, S., Palioura, E., Economou, F., Koutsilieris, M., et al. (2010). Endocrine disruptors and polycystic ovary syndrome (pcos): Elevated serum levels of bisphenol a in women with pcos. *The Journal of Clinical Endocrinology and Metabolism*.
- Kandaraki, E., Chatzigeorgiou, A., Livadas, S., Palioura, E., Economou, F., Koutsilieris, M., et al. (2011). Endocrine disruptors and polycystic ovary syndrome (pcos): Elevated serum levels of bisphenol a in women with pcos. *The Journal of Clinical Endocrinology and Metabolism*, *96*, E480–484.
- Kim, S. H., Chun, S., Jang, J. Y., Chae, H. D., Kim, C. H., & Kang, B. M. (2011). Increased plasma levels of phthalate esters in women with advanced-stage endometriosis: A prospective case-control study. *Fertility and Sterility*, *95*, 357–359.
- Korrick, S. A., Chen, C., Damokosh, A. I., Ni, J., Liu, X., Cho, S. I., et al. (2001). Association of ddt with spontaneous abortion. A case-control study. *Annals of Epidemiology*, *11*, 491–496.
- Kreeger, P. K., Fernandes, N. N., Woodruff, T. K., & Shea, L. D. (2005). Regulation of mouse follicle development by follicle-stimulating hormone in a three-dimensional in vitro culture system is dependent on follicle stage and dose. *Biology of Reproduction*, *73*, 942–950.
- Langseth, H., & Kjaerheim, K. (2004). Ovarian cancer and occupational exposure among pulp and paper employees in norway. *Scandinavian Journal of Work, Environment and Health*, *30*, 356–361.
- Louis, G. M., Weiner, J. M., Whitcomb, B. W., Sperrazza, R., Schisterman, E. F., Lobbell, D. T., et al. (2005). Environmental pcb exposure and risk of endometriosis. *Human Reproduction*, *20*, 279–285.
- Mendola, P., Messer, L. C., & Rappazzo, K. (2008). Science linking environmental contaminant exposures with fertility and reproductive health impacts in the adult female. *Fertility and Sterility*, *89*, e81–e94.
- Neal, M. S., Zhu, J., Holloway, A. C., & Foster, W. G. (2007). Follicle growth is inhibited by benzo-[a]-pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay. *Human Reproduction*, *22*, 961–967.
- Parker, R. M. (2012). Reproductive toxicity testing - methodology. In R. D. Hood (Ed.), *Developmental and reproductive toxicology: A practical approach* (pp. 425–487). New York: CRC Press.
- Peretz, J., Gupta, R. K., Singh, J., Hernandez-Ochoa, I., & Flaws, J. A. (2010). Bisphenol a impairs follicle growth, inhibits steroidogenesis, and down-regulates rate-limiting enzymes in the estradiol biosynthesis pathway. *Toxicol Sci*, *119*(1), 209–217.
- Peretz, J., Craig, Z. R., & Flaws, J. A. (2012). Bisphenol a inhibits follicle growth and induces atresia in cultured mouse antral follicles independently of the genomic estrogenic pathway. *Biol Reprod*, *87*(3), 63.
- Petro, E. M., Leroy, J. L., Covaci, A., Franssen, E., De, N. D., Dirtu, A. C., et al. (2012). Endocrine-disrupting chemicals in human follicular fluid impair in vitro oocyte developmental competence. *Human Reproduction*, *27*, 1025–1033.
- Plante, B. J., Cooper, G. S., Baird, D. D., & Steiner, A. Z. (2010). The impact of smoking on antimüllerian hormone levels in women aged 38 to 50 years. *Menopause*, *17*, 571–576.
- Pryor, J. L., Hughes, C., Foster, W., Hales, B. F., & Robaire, B. (2000). Critical windows of exposure for children's health: The reproductive system in animals and humans. *Environmental Health Perspectives*, *108*(Suppl 3), 491–503.
- Reddy, B. S., Rozati, R., Reddy, B. V., & Raman, N. V. (2006). Association of phthalate esters with endometriosis in Indian women. *BJOG*, *113*, 515–520.
- Rutkowska, A., & Rachon, D. (2014). Bisphenol a (bpa) and its potential role in the pathogenesis of the polycystic ovary syndrome (pcos). *Gynecological Endocrinology*, *30*, 260–265.
- Sadeu, J. C., Hughes, C. L., Agarwal, S., & Foster, W. G. (2010). Alcohol, drugs, caffeine, tobacco, and environmental contaminant exposure: Reproductive health consequences and clinical implications. *Critical Reviews in Toxicology*, *40*, 633–652.
- Sadeu, J. C., & Foster, W. G. (2011a). Cigarette smoke condensate exposure delays follicular development and function in a stage-dependent manner. *Fertility and Sterility*, *95*, 2410–2417.
- Sadeu, J. C., & Foster, W. G. (2011b). Effect of in vitro exposure to benzo[a]pyrene, a component of cigarette smoke, on folliculogenesis, steroidogenesis, and oocyte maturation. *Reproductive Toxicology*, *31*, 402–408.
- Sifakis, S., Androussopoulos, V. P., Tsatsakis, A. M., & Spandidos, D. A. (2017). Human exposure to endocrine disrupting chemicals: Effects on the male and female reproductive systems. *Environmental Toxicology and Pharmacology*, *51*, 56–70.
- Susiarjo, M., Hassold, T. J., Freeman, E., & Hunt, P. A. (2007). Bisphenol a exposure in utero disrupts early oogenesis in the mouse. *PLoS Genetics*, *3*, e5.
- Tanrikut, E., Karaer, A., Celik, O., Celik, E., Otlu, B., Yilmaz, E., et al. (2014). Role of endometrial concentrations of heavy metals (cadmium, lead, mercury and arsenic) in the aetiology of unexplained infertility. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, *179*, 187–190.
- Thomas, R. S., Philbert, M. A., Auerbach, S. S., Wetmore, B. A., Devito, M. J., Cote, I., et al. (2013). Incorporating new technologies into toxicity testing and risk assessment: Moving from 21st century vision to a data-driven framework. *Toxicological sciences: An official journal of the society of Toxicology*, *136*, 4–18.
- Verma, A. K., Rajbhar, S., Mishra, J., Gupta, M., Sharma, M., Deshmukh, G., et al. (2016). Anti-müllerian hormone: A marker of ovarian reserve and its association with polycystic ovarian syndrome. *Journal of Clinical and Diagnostic Research*, *10*, QC10–QC12.
- Xiao, S., Coppeta, J. R., Rogers, H. B., Isenberg, B. C., Zhu, J., Olalekan, S. A., et al. (2017a). A microfluidic culture model of the human reproductive tract and 28-day menstrual cycle. *Nature Communications*, *8*, 14584.
- Xiao, S., Zhang, J., Liu, M., Iwahata, H., Rogers, H. B., & Woodruff, T. K. (2017b). Doxorubicin has dose-dependent toxicity on mouse ovarian follicle development, hormone secretion, and oocyte maturation. *Toxicological sciences: an official journal of the Society of Toxicology*, *157*, 320–329.

Plastic Compounds

Zelieann R Craig, University of Arizona, Tucson, AZ, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blastocyst An early embryonic structure forming a hollow ball of cells which have begun the process of differentiation.

Corpus luteum A hormone-secreting structure that develops in an ovary after ovulation occurs.

Developmental exposure An exposure occurring during the period of fetal development; also known as gestational and in utero exposure.

Embryogenesis The process by which the embryo forms and develops.

Endometriosis A disorder in which tissue that normally lines the uterus grows outside the uterus.

Epidemiological studies Studies that analyze the patterns, causes, and effects of health and disease conditions in defined populations.

Epigenetic regulation A process during which gene expression is controlled through changes in a chromosome without alterations in the DNA sequence.

Estrogenicity The ability of an agent to mimic the effects of estrogen in a cell or tissue.

Estrous cyclicity Process through which the recurring physiologic changes that are induced by reproductive hormones in a female are monitored via vaginal washings.

Follicular atresia The process through which ovarian follicles are lost.

Folliculogenesis The maturation of the ovarian follicle; describes the progression of small primordial follicles into large preovulatory follicles.

Germinal vesicle breakdown Refers to the dissolution of the nucleus of an oocyte that is arrested in prophase of meiosis I in response to hormonal stimulation prior to ovulation.

Gestational age The term used to describe how far along a pregnancy is.

Gestational exposure An exposure occurring during the period of gestation or pregnancy; also known as developmental and in utero exposure.

Gonadal differentiation The process during which an undifferentiated gonad develops into a male or female gonad.

Implantation The stage of pregnancy at which a fertilized egg adheres to the wall of the uterus.

Imprinted gene A gene that is expressed in a parent-of-origin-specific manner.

In vitro fertilization The process of fertilization by manually combining an egg and sperm outside of the body.

Leiomyoma Noncancerous growths in the uterus; also known as fibroids.

Meiotic resumption Process during which a developing oocyte exits the arrested stage and completes meiosis I in preparation for ovulation.

Micro RNAs A small non-coding RNA molecule that functions in RNA silencing and post-transcriptional regulation of gene expression.

Myometrial Related to the middle layer of the uterine wall consisting of uterine smooth muscle cells, and supporting stromal and vascular tissue.

Oocyte-cumulus complexes An oocyte surrounded by specialized granulosa cells released during an ovulation.

Oocyte maturation Process during which oocytes re-entry meiosis just prior to ovulation.

Oocyte retrieval Technique used in in vitro fertilization to remove oocytes from the ovary to achieve fertilization outside the body.

Oogenesis The differentiation of the oocyte into an ovum capable of fertilization and further development into an individual.

Oxidative stress An imbalance between the production of free radicals and the ability to counteract their harmful effects through neutralization by antioxidant.

Preterm birth A birth that occurs before the 37th week of pregnancy.

Primordial follicle recruitment Step of folliculogenesis during which a dormant primordial follicle is activated and begins development towards the primary follicle stage.

Steroidogenesis The process by which steroids are generated from cholesterol.

Trophoblast Is the layer of tissue located on the outside of an embryo that supplies it with nourishment and later forms the placenta.

Zygotes A diploid cell resulting from the fusion of two haploid gametes; a fertilized egg.

Introduction

In the first part of her book on humanity's love story with plastics, Susan Freinkel describes the history of plastics and how they have changed our lives (Freinkel, 2011). Plastics have changed our lives by eliminating the limitations of requiring only natural products to satisfy our everyday needs. Their versatility gave us the ability to create many new products in a cost-effective manner which also translated into better opportunities for those of modest means to acquire previously unattainable products. The name "plastic" comes from a Greek verb that means "to mold of shape" and truly describes this wonderful invention. From the invention of celluloid to more complex contemporary plastics, humans have been in contact with these products for nearly 150 years. Ironically, a key chemical feature of the most versatile plastics allows the compounds forming their backbone to escape the products they are in and enter the environment and, hence, wildlife and humans.

This chapter introduces the reader to current knowledge about plastic compounds and their interactions with the female reproductive system. An emphasis on two widely known plasticizers, bisphenol A and phthalates, is taken to summarize both classical and contemporary studies highlighting the effects of these chemicals. Major reviews of the literature are cited and included as suggested readings in hopes of stimulating the reader to examine these thorough reports in their quest to learn more about these compounds.

Bisphenol A (BPA) is a plasticizer used in a variety of consumer products including polycarbonate plastics, the epoxy resins that line aluminum cans, and thermal receipts. Due to its ability to leach out of products, BPA has been detected in many human tissues including urine, serum, breastmilk, and ovarian follicular fluid. In their thorough review of experimental and human studies published between 2007 and 2013, Peretz et al. (2014) concluded that BPA is a reproductive toxicant based on its ability to disrupt ovarian, uterine, and developmental function. Furthermore, a detailed review of studies published between 2007 and 2016 by Ziv-Gal and Flaws (2016) has summarized current evidence for BPA-induced infertility.

Phthalates are synthetic chemicals used in polyvinyl chloride plastics, beauty and infant products, medical devices and on the enteric coating of some medications. As with BPA, phthalates are not covalently bound to the products in which they are used, thus, they are able to leach out into the environment under a variety of conditions. One challenging aspect of understanding phthalates as endocrine disruptors is that, contrary to BPA, they are a chemical class that includes many related chemicals. The basic structure of a phthalate includes a phthalic acid and alkyl chains of various lengths and functional groups. This diversity of chemical structures allows phthalates to potentially interact with a large variety of tissues. Like BPA, the epidemiological and experimental animal literature examining the relationship between phthalate exposure and adverse female reproductive health outcomes has been systematically reviewed previously by Kay et al. (2013). In their report, Kay et al. (2013) conclude that despite a paucity of data in specific research areas for several phthalates, there is sufficient evidence supporting phthalates as reproductive toxicants. Based on classical studies assessing phthalate toxicity, it was thought that phthalates could only cause reproductive toxicity at high concentrations. Interestingly, newer reports employing environmentally relevant doses of phthalates (i.e. those that resemble human exposures) and evaluating their effects on reproductive processes have highlighted aspects of phthalate toxicity that were not as obvious in high dose classical studies.

In the following sections, the status of our knowledge regarding BPA and phthalates will be summarized with an emphasis on their effects on various aspects of ovarian and uterine function (Fig. 1) as well as developmental processes including embryogenesis, placental, and pregnancy (Fig. 2). In the case of phthalates, most studies have focused on di-2-ethylhexyl phthalate (DEHP), di-n-butyl phthalate (DBP), and butyl benzyl phthalate (BBP) as well as their primary metabolites mono-2-ethylhexyl phthalate (MEHP) and mono-butyl phthalate (MBP; common metabolite for DBP and BBP), respectively. Very few studies have characterized the reproductive toxicity of other phthalates, thus, most of this section will be concerned with DEHP, DBP, and BBP and their metabolites.

Effects of Plastic Compounds on Ovarian Function

The ovary is responsible for oogenesis, folliculogenesis, steroidogenesis and ovulation in the female. Therefore, proper ovarian development and function are absolutely required for female fertility. Naturally, any chemical exposure that causes disruptions in these processes has the potential to cause infertility. The processes of ovarian follicle formation, oocyte meiosis, and steroidogenesis are particularly sensitive to environmental stressors. Ovarian toxicants are agents that cause detrimental effects on the ovary. Due to the diversity of processes that control ovarian development and function, ovarian toxicants can cause adverse effects via a variety of mechanisms. BPA and phthalates have been shown to intervene with processes required for the proper development of the ovary in the womb, the assembly and maturation of ovarian follicles, ovarian hormone production, and the ability of oocytes to mature into fertilizable eggs.

Oogenesis and Folliculogenesis

The processes of oogenesis and folliculogenesis ensure that the female will have a complement of fertilizable eggs to give rise to healthy offspring. Disruptions in these processes increase a female's risk for adverse reproductive outcomes including premature menopause, infertility, and early pregnancy loss to name a few. Many studies have assessed the impact of plasticizer exposures on these processes and found the effects of BPA and phthalates to be detrimental to them. Exposure to BPA in the womb, has been reported to cause defects in the process of oogenesis in mice and macaques. Specifically, developmental exposure to BPA affects the onset of meiosis, the accuracy with which chromosomes pair and recombine during meiosis, and disrupts the expression of key meiosis and mitosis genes in early oocytes and their precursors. Human fetal oocytes exposed in vitro to BPA undergo increased degeneration, have

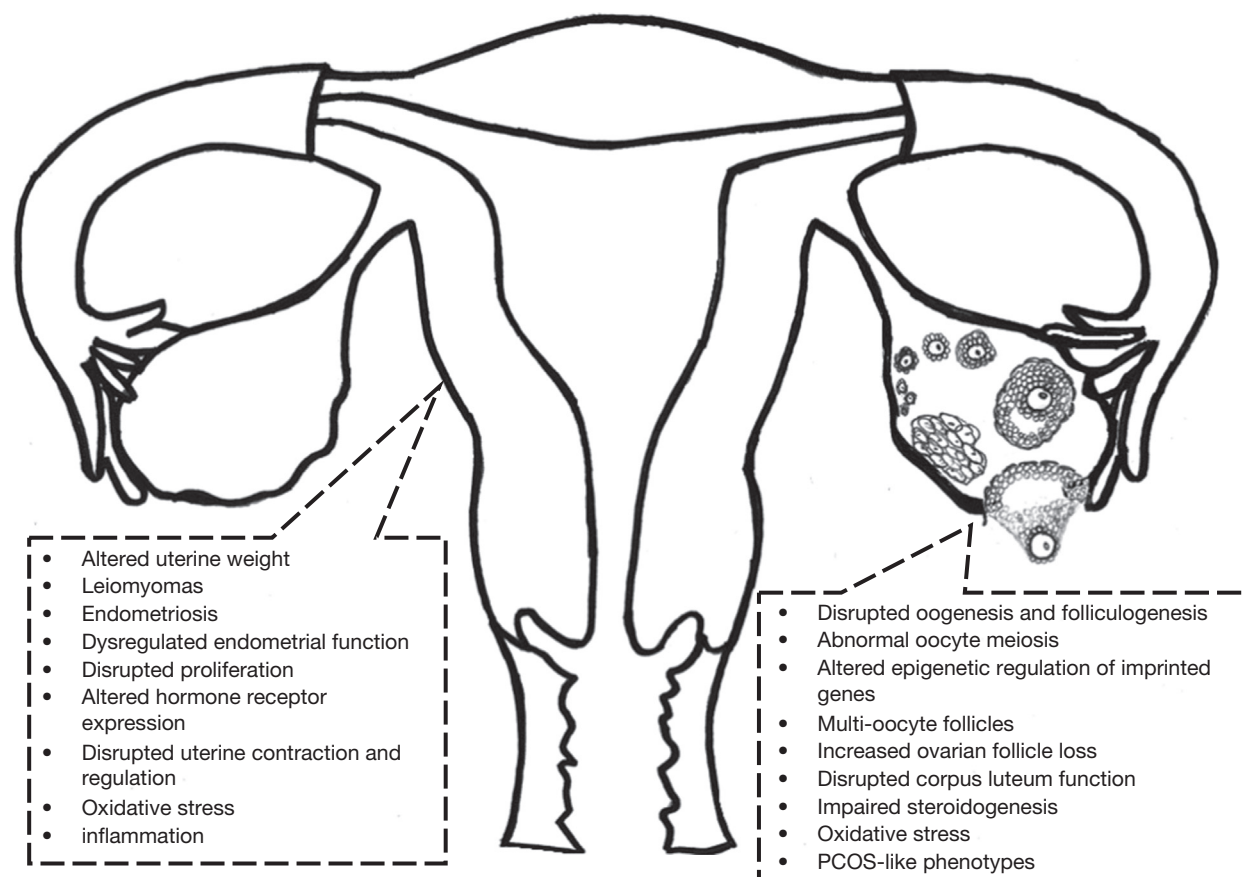


Fig. 1 Aspects of ovarian and uterine function susceptible to plastic exposure. According to human and animal studies various aspects of ovarian and uterine function may be targeted by plastic exposures. Overall, these processes include ovarian oogenesis, folliculogenesis, steroidogenesis, and susceptibility to oxidative stress and PCOS. In the uterus, processes involved include uterine morphology, endometrial function, hormone signaling, uterine function, and oxidative stress.

increased chromosome recombination defects, and show altered gene expression patterns when compared to control treated oocytes. Also, mouse oocytes exposed to BPA *in vitro* show increased errors in the epigenetic regulation of maternally imprinted genes. Following gestational exposure, BPA increased the incidence of multi-oocyte follicles in lambs and macaques. In mice and macaques, various studies suggest that gestational exposure to BPA causes an increase in unenclosed oocytes (not encased within follicles), decreased number of primordial follicles and increased follicular atresia. Several studies in lambs and rats have also provided evidence that BPA alters the rate at which follicles transition through their various stages of development during folliculogenesis. A study in sheep exposed prenatally to BPA revealed that BPA exposure disrupts gonadal differentiation and folliculogenesis by altering the expression of microRNAs required for the regulation of these processes. Interestingly, there seem to be dose-specific effects of BPA exposure. Specifically, low doses of BPA have been shown to accelerate the growth of preantral follicles *in vitro*, while higher doses have been shown to alter cell cycle and apoptosis gene expression and cause atresia in cultured mouse antral follicles.

Phthalates have been shown to cause various disruptions in follicular and oocyte development. Interestingly, phthalates have been shown to disrupt folliculogenesis at various levels of follicle maturation as well as at various ages or developmental stages in rodents. Phthalate exposures have been shown to cause imbalances in the number of unenclosed oocytes, the rate of primordial follicle recruitment, numbers of primary, secondary, and antral follicle numbers. Results indicate that disruptions in phosphatidylinositol 3 kinase (PI3K) signaling pathway, cell cycle/apoptosis dysregulation, and increased oxidative stress are likely mechanisms for phthalate-induced alterations in folliculogenesis. *In vitro* studies have also provided evidence for phthalate effects on follicle survival and function. Specifically, ovarian follicles treated *in vitro* with phthalates show significant growth inhibition, reduced estrogen production, and increased apoptosis. In pregnant mice, phthalates have also been shown to inhibit the function of the post-ovulatory gland known as the corpus luteum.

Steroidogenesis

Ovarian steroidogenesis is the process through which ovarian cells produce hormones for the maintenance of reproductive tissues, regulation of ovarian function and ovulation, and establishment of pregnancy. BPA and phthalate exposures have been associated with disruptions in ovarian steroidogenesis in various human and animal studies. Studies on women undergoing *in vitro*

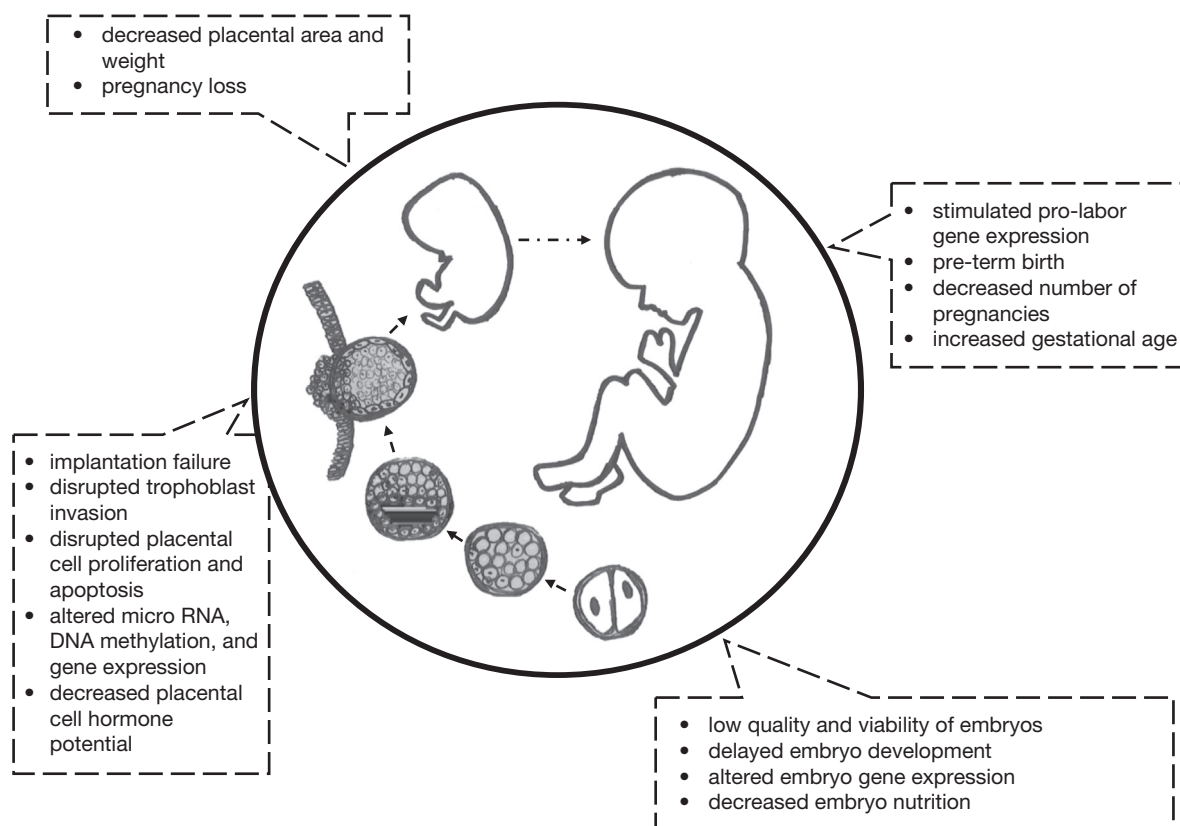


Fig. 2 Aspects of embryo development, placental function, and pregnancy susceptible to plastic exposure. According to human and animal studies various aspects of embryo development, placental function, and pregnancy may be targeted by plastic exposures. In the embryo, overall quality, viability, nutrition, progression through the appropriate stages of development, the ability to implant in the uterus may be disrupted by plastics. In placental cells, plastics seem to affect proliferation, apoptosis, epigenetic regulation, gene expression, and hormone producing potential. Overall in pregnancy, plastic exposures are suspected to increase pro-labor gene expression, induce preterm birth, pregnancy losses, and, in some cases, increase gestational age at birth.

fertilization have provided evidence for associations between BPA exposure and impaired hormone levels. Specifically, the level of BPA exposure in women has been associated with a decrease in peak serum hormone levels (prior to oocyte retrieval), increased testosterone and androstenedione levels, and disrupted expression of the hormone-producing enzyme, aromatase. Studies in laboratory animals have provided evidence for effects of BPA on hormone levels although the direction of these changes has been equivocal. For example, some studies in rodents reported that BPA exposures lead to increased levels of estrogen, its hormone precursors and their receptors, while other studies found no effects on levels of these hormones as well as the enzymes that produce them and the receptors that detect them. Furthermore, *in vitro* studies have demonstrated that the effects of BPA exposure on ovarian steroidogenesis are dependent on the BPA concentration used and the type of ovarian tissue exposed. Overall, production of estrogen and its precursors is mostly thought of as being sensitive to BPA exposure. Finally, there is suspicion that BPA exposure could be associated with polycystic ovary syndrome (PCOS) and/or a PCOS-related phenotype. This is based on studies reporting associations between BPA exposure level and PCOS hallmarks such as increased serum androgen levels and insulin resistance in PCOS patients. Furthermore, rodents experimentally exposed to BPA show a phenotype reminiscent of the hallmarks of PCOS including disruption of estrous cyclicity and accumulation of ovarian cysts and large ovarian antral follicles.

Like BPA, phthalates have been shown to disrupt the process of steroidogenesis in the ovary. Both DEHP and its metabolite MEHP have been shown cause disruptions in ovarian steroidogenesis by decreasing estrogen synthesis through alterations in the expression of enzymes required for its synthesis and inactivation in ovarian tissues and/or cells derived from humans, mice, and rats. Furthermore, in cultured follicles from mice and oocyte-cumulus-complexes from pig, phthalates were shown to cause a precocious increase in the synthesis of the estrogen precursor, progesterone. Finally, a study in mice demonstrated that phthalate exposure increases steroidogenesis inhibiting secretions from the anterior pituitary and causes a decrease in serum progesterone levels, thus, suggesting the involvement of the anterior pituitary in phthalate-induced disruptions in ovarian steroidogenesis.

Oocyte Maturation

Although fewer in number relative to folliculogenesis and steroidogenesis, studies characterizing the effects of BPA exposure on oocyte yield, maturation, and fertilization have provided enough evidence to suspect that BPA exposure could adversely affect

oocyte maturation and function in women. Epidemiological studies in women undergoing in vitro fertilization have demonstrated associations between increased BPA exposure and decreased oocyte quality, decreased oocyte retrieval, and decreased maturation and fertilization in retrieved oocytes. In animal models, the effects of BPA exposure on germinal vesicle breakdown and polar body extrusion are not completely understood since various studies have shown contradicting results or no detrimental effects. In vitro exposures to BPA have been shown to cause defects in spindle formation, congressional failure, chromosome misalignment, and meiotic arrest in mouse and human oocytes. Collectively, phthalates have been shown to disrupt oocyte function by decreasing their viability, causing alterations in heritable DNA methylation patterns, increasing the number of degenerating oocytes and follicles in the fetal rat ovary, and causing dysregulation of pro-steroidogenesis lipid and cholesterol synthesis in cultured human fetal ovaries. Furthermore, phthalates have been reported to cause disruptions in oocyte maturation including the oocyte's progression through the stages of germinal vesicle breakdown, meiotic resumption, and meiotic spindle formation in various species including zebrafish, cows, mares, mice and rats. Additionally, phthalate exposures have been shown to result in oocyte and accompanying cumulus cell apoptosis, increased oxidative stress, inhibited cumulus cell expansion, poor quality embryos, and defects in ovulation and corpus luteum formation and function in various species including livestock, zebrafish, rats, and marmosets.

Effects of Plastic Compounds on Uterine Function

Appropriate uterine function is key to ensure that pregnancy is possible and to avoid reproductive diseases such as leiomyomas and endometriosis. Several studies have linked BPA and phthalate exposures to disruptions in uterine function. In the uterus, work in animal models has shown that BPA exposure leads to disruptions in the morphology of the uterus, function of the endometrium, and receptivity of the uterus for embryo implantation after fertilization. In women, epidemiological studies have reported that women with higher levels of markers of BPA exposure have increased odds of having endometriosis and implantation failure. Studies in prenatally and/or neonatally exposed mice have shown that BPA exposure leads to gross uterine lesions, decreased expression of key regulators of endometrial cell proliferation, and the formation of endometrial-like structures outside the uterus later in life. BPA has also been shown to interfere with apoptosis and hormone-stimulated uterine epithelium proliferation in neonatally exposed mice and with hormone receptor expression in the non-human primate uterus. BPA exposure during early pregnancy, has been shown to inhibit embryo implantation in mice and, through the paternal gamete, decrease implantation, pregnancy maintenance and increase early fetal loss. BPA-treated mice have also been shown to suffer proliferative lesions in their oviducts, which raises concerns regarding potential effects of this plasticizer on processes that depend on proper oviduct function such as fertilization and embryo transport. In vitro, BPA has been shown to decrease the proliferation of human endometrial stromal fibroblasts and endothelial cells, and to disrupt uterine cell contractions, oxytocin and prostaglandin signaling.

Phthalates have also been shown to be associated with uterine pathologies in women and to cause deviations in uterine function in rodents. In women, increased urinary concentration of markers of phthalate exposure have been associated with the presence and severity of endometriosis as well as with the occurrence of leiomyoma or fibroids. Phthalates have been reported to enhance the proliferative activity, exert anti-apoptotic effects, and increase collagen content in myometrial and leiomyoma cells derived from women. In adult rats, phthalate exposure was shown to alter hormone receptor levels in the uterus and to cause structural abnormalities such as decreased luminal diameter, thinning of endometrial layers, and disruption of the glandular epithelium. In pregnant mice, phthalate exposure was shown to compromise endometrial receptivity and impair embryo implantation by altering the expression of hormonal and signaling factors key to these processes. When multiple phthalates were administered together in a mixture with the goal of mimicking human exposure in mice, combined phthalate exposure was associated with increased uterine weight, an observation classically considered as a sign of estrogenicity. Finally, phthalate exposure has been shown to induce oxidative stress and inflammatory responses in primary endometrial cells.

Effects of Plastic Compounds on Embryo, Placenta and Pregnancy

Not much is known about the effects of BPA and phthalate exposures on embryo development and placental function, but the few studies current available support the idea that these plasticizers may impact these aspects of reproduction. As with many aspects of BPA research, this is an active area of study and further studies will likely provide enough data to elucidate the effects of BPA on pregnancy.

Embryonic Development

Defects in the processes of fertilization and early embryonic development can lead to pregnancy loss and developmental defects. In women, urinary concentrations of BPA have been associated with decreased rate of blastocyst formation and an increase in low-quality embryos during in vitro fertilization procedures. In studies using laboratory rodents, BPA exposure has been associated with delayed embryo development and disrupted expression of imprinted genes in embryos and placentas. A few studies have demonstrated the ability of phthalates to interfere with embryonic development. Specifically, phthalates have been shown to impair the formation of viable zygotes in rats, block the formation of two-cell embryos in mice, and cause altered gene expression in bovine blastocysts. In mice, phthalate exposure decreased embryo nutrition, an effect that was exacerbated in embryos with neural tube defects.

Placental Development and Function

Appropriate placental development is key to the survival, nourishment, and metabolic programming of the fetus. Defects in this process could lead to pregnancy loss and developmental and metabolic disease in adulthood. A small group of studies have evaluated the ability of BPA and phthalates to affect placental development and function. Specifically, in vitro studies have revealed that BPA exposure inhibits the rate at which placental cells proliferate and undergo apoptosis, an observation that could have significant implications for the proper development and nourishment of the fetus. These observations have been supported by studies in humans reporting that the placentas of women with greater urinary phthalate levels have altered miRNA, DNA methylation, and gene expression patterns. In placental cells, in vitro phthalate exposures have been shown to decrease aromatase activity, inhibit the process by which trophoblasts invade the endometrium during early placental development, disrupt regulation of placental cell apoptosis, and induce pro-labor gene expression. In mice, phthalate treatment reduced placental weight and area, disrupted vascularization, inhibited proliferation, and activated apoptosis of placental cells.

Pregnancy

In pregnant women, higher levels of BPA in urine have been associated with preterm birth. Interestingly, several studies have evaluated the ability of BPA to adversely affect pregnancy outcomes using animal models but have reported conflicting results. Specifically, some studies have reported defects in the number of pregnancies and pups delivered by BPA-treated animals while others reported observing no such effects. A potential association between phthalate exposure and pregnancy outcomes has been highlighted by studies showing that higher phthalate exposures are associated with increased risk for pregnancy loss and, in surviving pregnancies, with increased gestational age. Finally, phthalates have been shown to increase pregnancy loss and fetal mortality in rats.

Conclusion

In summary, plastics have given us the ability to mimic the usefulness of many natural products in an affordable way thanks to their versatility and low production cost. Ironically, the compounds that give plastics their desirable characteristics allow them to escape their products and enter our environment and bodies. A large body of work continues to accumulate and provide strong evidence for the ability of plastic compounds to interact with the female reproductive system and increase risk for adverse reproductive health outcomes. Although much work is still needed in specific areas and at concentrations that mimic human exposures, there is sufficient evidence to conclude that BPA and phthalates are significant concerns to female fertility. Finally, replacement of these chemicals in our products is currently the best way to manage the risks they impose to human and animal health. Although classical substitutes have fallen under intense scrutiny recently due to potential toxicity, a group of newer chemicals seem to have promising features such as cost effectiveness, low leaching potential, and low toxicity.

References

- Freinkel, S. (2011). *Plastic: A Toxic Love Story*, Houghton Mifflin. New York, USA: Harcourt.
- Kay, V., Chambers, C., & Foster, W. (2013). Reproductive and developmental effects of phthalate diesters in females. *Critical Reviews in Toxicology*, 43, 200–219.
- Peretz, J., Vrooman, L., Ricke, W., Hunt, P., Ehrlich, S., Hauser, R., Padmanabhan, V., Taylor, H., Swan, S., VandeVoort, C., & Flaws, J. (2014). Bisphenol A and reproductive health: Update of experimental and human evidence, 2007–2013. *Environmental Health Perspectives*, 122, 775–786.
- Ziv-Gal, A., & Flaws, J. (2016). Evidence for bisphenol A-induced female infertility: A review (2007–2016). *Fertility and Sterility*, 106, 827–856.

Further Reading

- Cho, Y., Park, S., & Han, M. (2015). Di-(2-ethylhexyl)-phthalate induces oxidative stress in human endometrial stromal cells in vitro. *Molecular and Cellular Endocrinology*, 407, 9–17.
- Craig, Z. R., Hannon, P. R., Wang, W., Ziv-Gal, A., & Flaws, J. A. (2013). Di-n-butyl phthalate disrupts the expression of genes involved in cell cycle and apoptotic pathways in mouse ovarian antral follicles. *Biology of Reproduction*, 88(1), 23.
- Craig, Z. R., Wang, W., & Flaws, J. A. (2011). Endocrine-disrupting chemicals in ovarian function: Effects on steroidogenesis, metabolism and nuclear receptor signaling. *Reproduction (Cambridge, England)*, 142(5), 633–646.
- Guo, M., Lai, L., Zong, T., Lin, Y., Yang, B., Zhang, L., Li, M., & Kuang, H. (2015). Exposure to di(2-ethylhexyl) phthalate inhibits luteal function via dysregulation of CD31 and prostaglandin F2alpha in pregnant mice. *Reproductive Biology and Endocrinology*, 3, 11–13.
- Hannon, P. R., Niermann, S., & Flaws, J. A. (2016). Acute exposure to di(2-Ethylhexyl) phthalate in adulthood causes adverse reproductive outcomes later in life and accelerates reproductive aging in female mice. *Toxicological sciences: an official journal of the Society of Toxicology* (vol. 150(1)), 97–108.
- Hannon, P., & Flaws, J. (2015). The effects of phthalates on the ovary. *Frontiers in Endocrinology*, 6, 1–19.
- Huang, Q., Zhang, H., Chen, Y., Chi, Y., & Dong, S. (2016). The inflammation response to DEHP through PPARgamma in endometrial cells. *Int J Environ Res Public Health* (vol. 13), E318. <https://doi.org/10.3390/ijerph13030318>.
- Kim, J., Kim, S., Oh, Y., Ihm, H., Chae, H., Kim, C., & Kang, B. (2017). In vitro effects of phthalate esters in human myometrial and leiomyoma cells and increased urinary level of phthalate metabolite in women with uterine leiomyoma. *Fertility and Sterility*, 107, 1061–1069.
- Liu, T., Jia, Y., Zhou, L., Wang, Q., Sun, D., Xu, J., Wu, J., Chen, H., Xu, F., & Ye, L. (2016). Effects of di-(2-ethylhexyl) phthalate on the hypothalamus-uterus in pubertal female rats. *International Journal of Environmental Research and Public Health*, 13, E1130.

- Rasmussen, L., Sen, N., Vera, J., Liu, X., & Craig, Z. (2017). effects of in vitro exposure to dibutyl phthalate, mono-butyl phthalate, and acetyl tributyl citrate on ovarian antral follicle growth and viability. *Biol Reprod* (Epub ahead of print) <https://doi.org/10.1095/biolreprod.116.144691>.
- Sant, K., Dolinoy, D., Jilek, J., Shay, B., & Harris, C. (2016). Mono-2-ethylhexyl phthalate (MEHP) alters histiotrophic nutrition pathways and epigenetic processes in the developing conceptus. *The Journal of Nutritional Biochemistry*, 27, 211–218.
- Sen, N., Liu, X., & Craig, Z. (2015). Short term exposure to di-n-butyl phthalate (DBP) disrupts ovarian function in young CD-1 mice. *Reproductive Toxicology*, 53, 15–22.
- Somasundaram, D., Manokaran, K., Selvanesan, B., & Bhaskaran, R. (2017). Impact of di-(2-ethylhexyl) phthalate on the uterus of adult Wistar rats. *Human & Experimental Toxicology*, 36, 565–572.
- Zhou, C., Gao, L., & Flaws, J. (2017). Prenatal exposure to an environmentally relevant phthalate mixture disrupts reproduction in F1 female mice. *Toxicology and Applied Pharmacology*, 318, 49–57.

Metals in Female Reproduction

Sakhila K Banu, Jone A Stanley, and Joe A Arosh, Texas A&M University, College Station, TX, United States
Mariajoseph Michael Aruldas, University of Madras, Chennai, India

© 2018 Elsevier Inc. All rights reserved.

Heavy metals have been used by humans since ancient times. Metals may be divided into four groups: (i) those with greatest toxicological significance that are wide-spread in the human environment, for example, arsenic (As), cadmium (Cd), lead (Pb), mercury (Hg), chromium (Cr), and uranium (U); (ii) essential trace metals, for example, copper (Cu), iron (Fe), manganese (Mn), selenium (Se), and zinc (Zn); (iii) other metals with evident biological or toxicological interest, for example, nickel (Ni) and vanadium (V); and (iv) metals of pharmacological interest, for example, aluminum (Al), gallium (Ga), and lithium (Li) (Banu, 2013). The focus of this article is twofold: (1) examination of the adverse or beneficial effects of commonly used metals, including essential trace metals on female reproductive function, and (2) consideration of the role of essential trace metals such as Cu, Fe, Mn, Se, and Zn on physiological aspects of female reproduction. Discussion related with each metal includes their beneficial and/or the adverse effects on humans. Additional information pertaining to other mammalian species, including primates and experimental animal models, source of exposure, maximum contamination limit (MCL) or minimal risk level (MRL), and half-life are given in **Table 1** for the metals included in this commentary.

Anthropologic use of heavy metals started long before the beginning of industrial revolution with earliest recorded use of heavy metals by humans dating to about 6000 BC (Adebowale, 2004). The *Stone Age* includes an extended prehistoric period that lasted for about 3.4 million years ending between 4500 BC and 2000 BC with the advent of metal usage. The subsequent ages of human history were named for introduction of the usage of metals. The *Copper Age* started around 6000 BC; the *Bronze Age* around 3200 BC, and the *Iron Age* began around 1200 BC. During the copper age, humans used an iron/nickel alloy that naturally occurred in iron/nickel meteorites. The production and usage of heavy metals increased exponentially after the industrial revolution (AD 1750–1850) (Banu, 2013).

Exposure to endocrine disrupting chemical (EDC), including heavy metals can cause adult reproductive failure, endocrine dysfunction, and nutrient homeostasis instability. Since the female mammals have their oocyte number determined by birth or soon after birth, the ovaries are direct targets for environmental insults. Any environmental insult to the ovary has the potential to affect the future generation of the affected individual. The placenta is the vascular interface between the mother and fetus, which helps regulate nutrient, oxygen, and waste exchange. It is evident that pregnancy related conditions, including intrauterine fetal growth restriction (IUGR) and preeclampsia, originate from placental dysfunctions. Placenta is a potential gateway for chemicals and toxicants to cross, resulting in adverse effects within the fetus and restricting fetal growth and development. Therefore, environmental influence on placental origin of adult diseases is a growing field to understand the influence of placenta in determining fetal health.

Arsenic

Arsenic (As) toxicity is a major global health concern due to its wide distribution and adverse health effects. The shallow ground water of the western United States is more contaminated with As than the eastern United States. Arsenic is used as a pesticide and to preserve wood from rot and decay. The oxidized form of As (arsenate) is the principal inorganic As species found in drinking water. Toxicity of arsenal compounds results in the production of free radicals that can cause DNA single-strand breakage. Exposure to As induces oxidative stress in several organs due to significant reduction in antioxidants. Exposure of pregnant women to As resulted in increased rates of abortion, preterm births, and neonatal mortality, as well as decreased birth weight, even at low exposure levels. Maternal exposure to As also decreased thymic size in the children and increased infectious diseases in both mothers and children, as a result of suppression of the immune system. Arsenic concentrations in pregnant women were negatively associated with birth weight, height and chest circumference (Liao et al., 2018). Fetal As concentration was negatively associated with head circumference. Maternal exposure to As during pregnancy increases oxidative stress and inflammation in the placenta by elevating proinflammatory cytokines (IL-1 β , TNF α , and IFN γ). Arsenic also silences gene transcription in umbilical artery and placenta.

Cadmium

Cadmium (Cd) is listed by the US Environmental Protection Agency (EPA) as one of the 126 priority pollutants. Soluble Cd salts accumulate in mammals and result in toxicity to various reproductive organs. Cd exposure is linked to numerous human health problems, including infertility. Cd accumulates in the ovary, uterus and pituitary of humans and alters the histopathology of these endocrine organs.

Cd is one of the EDCs with a long half-life of 20–30 years. An early study reported that Cd levels in the ovary increased linearly between 30 and 65 years of age. Below 30 years, there was no age dependent increase observed. However, over 65 years of age, ovarian Cd levels began to decrease. Interestingly, in smokers, the amount of Cd in the ovaries was elevated compared to

Table 1 Encyclopedia of metals

<i>Metal</i>	<i>MCL, MRL, SMCL, NL or PEL</i>	<i>Sources or uses</i>	<i>Species</i>	<i>Major finding/pathway(s)</i>
Aluminum (Al)	SMCL 0.05 to 0.2 mg/L	Leaching from rock and soil, mining, Al ores, coal-fired power plants and incinerators	Rats	Disrupted ovarian structure; altered metabolism of Fe, Zn and Cu; decreased activities of Na(+)-K(+)-ATPase, Mg(2+)-ATPase and Ca(2+)-ATPase in the ovary
			Mice	Placental atrophy, hemorrhagic uterus, intra uterine fetal growth restriction (IUGR) and increased embryo mortality
			Fish	Decreased protein deposition in eggs, decreased plasma levels of 17 α -hydroxyprogesterone
Arsenic (As)	MCL 0.01 mg/L	Natural erosion from land, discarded mine and mill tailings	Rats	Follicular atresia and thinning of uterine luminal diameter; decreased weight of the ovary and uterus; altered histomorphology of the uterus with degeneration of luminal epithelial cells and endometrial glands
			Mice	Spontaneous abortion with aberrant placental vasculogenesis and placental insufficiency
Boron (B)	NL 1000 μ g/L	Glass manufacturing, coal burning power plants, copper smelters, fertilizer, and pesticide	Rabbit	Increased fetal resorptions or fetal deaths, prenatal mortality; decreased litter size and number of live fetuses per litter; decreased gravid uterine weight, and vaginal bleeding associated with pregnancy loss in mothers
			Rats	Increased fetal resorption, IUGR, lethality of embryos and fetuses, decreased litter size, and fetal malformations
			Mice	Increased fetal resorption, decreased fetal weight, uterine weight, increased prenatal mortality and malformed fetuses
Cadmium (Cd)	MCL 0.005 mg/L	Corrosion of galvanized pipes, erosion of natural deposits; discharge from metal refineries; runoff from waste batteries and paints	Hamster	Ovarian lesions, diffused hemorrhage in all the developing follicles and interstitial cells
			Rats	Stromal hemorrhages and cellular degeneration in oviduct and uterus; ovarian follicular necrosis, persistent diestrus; increased weight and thickness of the uterine epithelium and endometrial stroma
			Mice	Hemorrhagic foci in the ovary and endometrial stroma
			Hen	Ovarian lesions with severe necrosis, and degeneration of ovarian follicles and apoptosis, and increased oxidative stress, decreased antioxidants and decreased steroidogenesis.
			Silk worm	Changes in the oviposition behavior, and reduced fertilization rates
Chromium (Cr)	MCL 0.1 mg/L (USEPA); 0.05 mg/L (WHO)	Industrial processes and manufacturing activities including discharges from steel and pulp mills, leather industries, paint pigments, automobile emissions, chrome plating, and corrosion inhibitors	Rats	Disrupted placental histology; increased cell death and oxidative stress, and decreased antioxidants in the placenta; caused premature ovarian failure, delayed puberty, extended estrus cycle, decreased pregnancy rate and disrupted ovarian steroidogenesis; advanced germ cell nest breakdown during fetal ovarian development, increased apoptosis of the oocytes and granulosa cells, and increased ovarian follicle atresia
			Mice	Decreased implantation and viable fetuses, decreased litter size, and increased fetal resorption; increased endometrial stromal cells apoptosis.
			Fish	Impaired vitellogenesis and decreased vitellogenic oocytes
Cobalt (Co)	MRL 0.03 μ g/m ³	Mining and mineral processing. Enters dust, seawater spray, volcanic eruptions, forest fires; and surface water or rainwater runoff	Rabbits	Decreased body weight
			Rats	Cobalt crosses the placenta, enters maternal blood, fetal blood and amniotic fluid and induced IUGR and fetal malformations.
			Mice	Decreased body weight, increased malformed fetuses, and induced postimplantation loss of the embryos
Copper (Cu)	MCL 1.3 mg/L	Mining, smelting and refining; Cu in wire, sheet metal, water pipes and fossil; and fuel combustion	Mice	Reduced number of healthy primordial, primary, growing, secondary and antral follicles and corpus luteum; induced ovarian lesions; and increased follicle atresia
			Crab	Lower gonadosomatic index

(Continued)

Table 1 Encyclopedia of metals—cont'd

<i>Metal</i>	<i>MCL, MRL, SMCL, NL or PEL</i>	<i>Sources or uses</i>	<i>Species</i>	<i>Major finding/pathway(s)</i>
Gold (Au)	No limit identified	Open pit mining, cyanide heap leaching, gold ore dumps. Gold treatment for rheumatoid arthritis	Human Rats	In utero exposures to gold resulted in stillborn birth, severe malformations of the newborn, decreased estriol levels, hypertension and proteinuria in mother Embryo toxicity at high doses; gold sodium thiomalate decreased fetal weight and caused fetal malformation, increased numbers of eosinophils and inflammation in endometrial and myometrial cells
Lead (Pb)	MCL 0.015 mg/L	Fossil fuels, industrial products, lead smelters, paints, pipes, cables, ceramics, stained glass and batteries	Monkey Rats Mice	Lead suppressed corpora luteal production of progesterone; suppressed the plasma levels of LH, FSH and E ₂ Delayed the timing of puberty, suppressed serum levels of insulin-like growth factor-1, luteinizing hormone, and estradiol; increased thickening of the endometrium, narrowing of uterine lumen, caused damage to endometrial glands and vacuolar degeneration. Decreased primordial follicles, increased atretic antral follicles; maternal exposure to Pb retarded embryonic growth, reduced litter size, induced IUGR and impaired postnatal survival. Progeny of the lead-exposed mothers experienced pubertal delay Decreased number of litters, increased number of fetal death; and decreased number of healthy primary follicles in the ovary.
Lithium (Li)	PEL 0.025 mg/m ³	Present in the earth's crust in 65 ppm; released from <i>mining</i> processes, and highly used to treat bipolar disorder	Rats	Teratogenic and fetotoxic; decreased cell proliferation in preimplantation embryos
Manganese (Mn)	SMCL 0.05 mg/L	Mn is an essential trace element. Found in air, soil, water, and food; disposal of Mn based products, fungicide, seed protectants, antiknock agent in gasoline, and a contrasting agent in nuclear magnetic resonance tomography	Rats Mice Fish	Altered myometrial cell proliferation; advanced puberty and elevated serum levels of LH, FSH, and estradiol Embryo toxicity with late resorptions, postimplantation loss and reduced fetal body weight Reduction in diameter and number of oocytes, formation of large inter follicular space, necrosis and vacuolation in the ovary with oocyte atresia, and decreased gonado-somatic-index
Mercury (Hg)	MCL 0.002 mg/L	Mining, burning coal and waste, natural deposits, industrial waste disposal and volcanic activity; skin lightening creams and dental amalgam, thermometers, fluorescent light bulbs, and blood pressure instruments	Hamster Rats Mice Fish	HgCl ₂ inhibited ovulation rate and increased the number of degenerated oocytes Prolonged estrus cycle, increased ovarian follicular atresia, increased irregular follicles with abnormal oocyte borders, and reduced number of ovarian follicles HgCl ₂ caused damage to the ovary and reduced the number of oocytes retrieved by superovulation; reduced oocyte quality and viability of oocytes, and reduced the rate of IVF Disrupted transcription of HPG-axis genes and impaired reproductive function
Molybdenum (Mo)	PEL 3 mg/m ³	Mining, smelting, coal-fired power plants and coal combustion	Cow Rats Mice	Cessation of milk synthesis. Prolonged estrus cycle, increased fetal resorption, decreased birth weight in pups, and had adverse effects on embryogenesis Ovarian hyperemia, increased oxidative stress, and altered ovarian function; poor oocyte quality and blastocyst quality, and degenerated blastocysts; impaired embryo development
Nickel (Ni)	MCL 0.1 mg/L	Oil and coal combustion, metal refining, sewage sludge and incineration	Rats Fish	Increased fetal mortality and fetal malformations, and IUGR Irregular shaped oocytes, scattered yolk granules in the inter follicular space, abnormal growth of the previtellogenic oocytes

Table 1 Encyclopedia of metals—cont'd

<i>Metal</i>	<i>MCL, MRL, SMCL, NL or PEL</i>	<i>Sources or uses</i>	<i>Species</i>	<i>Major finding/pathway(s)</i>
Platinum (Pt)	PEL 1.0 mg/m ³	Mining, catalytic converters, laboratory equipment, electrical contacts and electrodes, thermometers, dentistry equipment, and jewelry. Pt-containing compounds used for cancer chemotherapy	Rats	Interstitial edema and dilation of blood vessels of the ovary, inflammation of follicular epithelium, theca cells and corpus luteum; primordial follicle degeneration and oocyte degradation
Selenium (Se)	MCL 0.05 mg/L	Weathering of rocks and soils, Se in air from burning coal or oil	Duck	Decrease in the rate of egg hatching; yolk peritonitis in egg, malformed embryo and infertile egg
			Hen	Decreased egg weight and hatchability rate at very high doses 7 and 9 ppm
Strontium (Sr)	MCLG 4.4 mg/L	Ceramic manufacturing and glass products, pyrotechnics, paint pigments, fluorescent lights manufacturing	Human	Increased perinatal mortality rates in the regions of Ukraine and Belarus surrounding the Chernobyl site correlate with the strontium content in pregnant women
			Mice	Reduced oocytes in the fetal ovary, and reduced reproductive capacity.
			Rats	Reduced implantation, increased fetal resorption and embryo mortality, induced developmental defects, and IUGR
Thallium (Tl)	MCL 0.002 mg/L	Coal burning power plants, cement factories, and smelting operations	Human	Prematurity and IUGR
			Rats	Reduced ovarian cell viability and decreased glutathione (GSH), and GSH peroxidase and GSH reductase activities; embryo toxicity and increased fetal death; decreased number of live fetuses
Tin (Sn)	PEL 0.1 mg/m ³	Mining, coal and oil combustion	Rats	Pregnancy failure, preimplantation loss, postimplantation loss, fetal resorptions, and fetal death; embryo toxic and teratogenic, and atrophy of uterine glands
Titanium (Ti)	PEL 15 mg/m ³	Emissions from facilities, chemical and industrial processes such as petrochemicals, pulp and paper	Mice	Increased Graafian follicles and corpus luteum; increase in atretic follicles, intense inflammatory response and ovarian necrosis; reduced number of mating and pregnancy rate; smaller uteri and smaller fetuses, reduced serum concentrations of P ₄ , LH, FSH, and testosterone
Uranium (U)	MCL 30 µg/L.	Wind and water erosion and volcanic eruptions, mining, milling, and processing of uranium	Rats	In utero exposure to uranium decreased pregnancy rate, affected labor, and decreased survival rate of offspring
			Mice	Reduced pregnancy rate; decreased number of litters, decreased weight and diameter of placentas, decreased number of embryos, caused irregular estrus cycle, increased percentage of dysmorphic oocytes; and altered ratio of primary to secondary follicles
			Fish	Decreased number of spawning and the number of eggs per spawn; increased mortality of F1 embryos; decreased circulating concentrations of vitellogenin
Zinc	SMCL 5 mg/L	Mining, purifying of Zn, Pb, and Cd ores, steel production, coal burning, and burning of wastes. Disposal of Zn wastes from metal manufacturing industries and coal ash from electric utilities	Sheep	Induced severe copper deficiency and caused a high incidence of abortions and stillbirths
			Rats	A two-generation study with ZnCl ₂ exposure reduced fertility, viability and litter size, as well as uterus weight in the offspring
			Mice	ZnCl ₂ significantly reduced fertility and litter size, and body weight.
			Fish	Decreased ovarian glycogen, cholesterol, total proteins and total lipid in the ovary

IUGR, intra uterine growth restriction; *IVF*, in vitro fertilization; *MCL*, maximum contaminant level; *MRL*, minimal risk level; *NL*, state notification level; *PEL*, personal exposure limit; *SMCL*, secondary maximum contaminant level.

nonsmokers. In multiparous women (> 3 children), a tendency to decreased Cd ovarian levels was observed. Thus, it was proposed that Cd might be a risk factor for conception and pregnancy in women in their forties. In the human subjects, different groups of individuals have been identified as being more susceptible to Cd exposure (e.g., individuals with a low iron status, mainly women). Therefore, it is important to identify vulnerable groups, especially when setting recommendations for daily intake of Cd. Exposure to Cd during human pregnancy through either occupational exposure or cigarette smoking has been linked to decreased birth

weights and premature birth, with the enhanced levels of placental Cd and decreased progesterone biosynthesis by the placental trophoblast. In the placenta, Cd decreases progesterone synthesis by decreasing cytochrome P450 cholesterol side-chain cleavage enzyme (P450_{scc}) which converts cholesterol to pregnenolone, the metabolic intermediate in the synthesis of most steroid hormones. In addition, Cd inhibits placental low-density lipoprotein receptor (LDL-R) mRNA resulting in decreased precursor of cholesterol from the maternal peripheral circulation. Cd affects steroidogenesis by interfering with the DNA binding zinc (Zn²⁺)-finger motif through the substitution of Cd²⁺ for Zn²⁺. Cd acts as an estrogenic compound by either mimicking or inhibiting the actions of endogenous estrogens. Cd alters human chorionic gonadotropin and zinc transfer in human placenta, and induced ultrastructural changes in the placenta such as syncytiotrophoblastic vesiculations, stromal edema, vacuoles in Hofbauer cells and necrosis.

There is a sexual dimorphism in Cd accumulation, where women have higher internal levels of Cd than men, due to higher gastrointestinal absorption of Cd in women with low iron stores or due to genetic factors. Further, pregnant women appear to accumulate more Cd than nonpregnant women. A recent study in pregnant women from China on qualitative placental proteomic analyses demonstrated that prenatal Cd exposure at an electronic waste disposal area caused significant changes in proteins of mitochondrial respiratory chain (Xu et al., 2016). Cd exposure in pregnancy alters fetal DNA methylation in a marked sex-specific manner. Besides various molecular mechanisms such as genetic and epigenetic changes, increased oxidative stress and lipid peroxidation play key roles in Cd-induced female reproductive toxicity. Cd exposure during pregnancy leads to accumulation in the placenta, disturbed zinc transfer to the fetus, interferes with glucocorticoid balance, and affects the regulation of insulin growth factor-related proteins. It has been shown to inhibit the migration of human placental trophoblast cells. Finally, prenatal exposure to Cd causes changes in the epigenetic machinery, which could possibly lead to adverse outcome of the fetal health.

Chromium

Hexavalent chromium (CrVI) usage is increasing exponentially worldwide; and Cr pollution is a continuous, ongoing global problem. A report from the Environmental Working Group indicates increasing CrVI levels in 35 cities in the United States (Banu, 2013). At least 74 million people in nearly 7000 communities drink tap water polluted with total Cr. Unlike most other divalent metal ions, CrVI is unique, specific, and behaves like an anion due to its structural similarity with anions (such as CrO₄²⁻, HCrO₄⁻, and SO₄²⁻). Due to its anionic nature, CrVI is rapidly transported into cells through anion transporters and reduced to CrIII by various antioxidants such as ascorbate (vitamin C), GSH, cysteine and antioxidant enzymes (Banu, 2013).

Cr disrupts ovarian function in women working in Cr industries who exhibited increased levels of Cr in their blood and urine. Exposed women experienced abnormal menses, infertility or increased postpartum bleeding. Several reports from studies on experimental animals demonstrate adverse effects of CrVI on the ovary. Brief details are described in Table 1. Premature ovarian failure (POF) is a defect of ovarian follicle development and is characterized by primary or secondary amenorrhea, with elevated levels of gonadotropin hormones or by early menopause. *Xpnep2* is a marker gene in POF women, which is located on the X chromosome. Findings from our laboratory using rat as an experimental model indicate that prenatal exposure to CrVI disrupts *Xpnep2* gene during ovarian development, resulting in POF (Banu et al., 2015). However, it is not known whether there is a correlation between CrVI exposures in women and POF. Preterm birth is a leading cause of infant mortality and significant precursor to future morbidity.

Premature abortion and preterm birth are common problems in Cr-exposed women. Every year, about 15 million babies are born prematurely, and more than one in 10 of all babies born around the world are premature. Cr can transfer through the placenta to developing fetuses. A recent birth cohort study evaluated the association between maternal CrVI exposure during pregnancy and the risk of preterm birth among 7290 pregnant women in Hubei province, China. Data indicated an increased risk of preterm birth associated with higher maternal urinary Cr levels, and the associations appeared to be more evident in male infants. Thus, Cr not only causes preterm birth, it is also sexually dimorphic. Recent epidemiological and clinical data indicate that environmental CrVI exposure in women living in Willits, California adversely affects pregnancy outcomes and subsequent health of two generations, resulting in low birth rate, higher risk of pregnancy loss, and spontaneous abortion. Children (F1 offspring) of CrVI exposed women experienced respiratory problems, perinatal jaundice, and increased birth defects. The study was conducted using data released from the hospital records that included newborn records from 29,311-unexposed (control) and 5036-exposed (Willits population), as well as 31,444-unexposed (control) and 5558-exposed pregnant women (Willits population). For the first time, the detrimental reproductive effects of nonoccupational CrVI exposure in women and their infants in the United States was reported (Remy et al., 2017). Another epidemiological study indicated that occupational exposure to Cr during pregnancy caused intrauterine growth retardation (IUGR) and resulted in low birth weight of the newborn children. Increased levels of Cr in the blood and urine of chrome plating workers is associated with elevated levels of free radicals and chromosomal aberrations. The placenta is essential for fetal programming since it determines the growth, development and health of the growing fetuses. Understanding environmental exposure on placental development and its functions helps to identify molecular mechanisms that have both early- and long-term effects on health of the fetus. A recent study from our laboratory on the human term placenta revealed a positive correlation between Cr burden in the placenta and oxidative stress. Moreover, increase in Cr burden decreased antioxidant proteins and elevated apoptotic protein sex-dependently (Banu et al., 2017).

Copper

Copper (Cu) is an essential trace metal. The majority of plasma Cu is transported bound to ceruloplasmin (>75%), whereas the remainder is bound to albumin, transcuprein, and Cu-amino acid complexes. The concentration of free Cu ions in human plasma is

10^{-13} M. Cu bound to these carriers is less tightly bound and is exchangeable. Cu is a component of a number of metalloenzymes, including cytochrome C oxidase, superoxide dismutase, glutathione peroxidase and transferase, tyrosine, dopamine β -hydroxylase, amino oxidase, lysyl oxidase, ceruloplasmin, and enzymes of fatty acid metabolism.

Cu is essential for normal reproduction, however, over exposure can cause various toxic effects. Cu is toxic in its unbound form, and causes redox imbalance due to its highly redox active nature. Prolonged use of Cu intrauterine devices (Cu-IUDs) cause a wide spectrum of adverse side effects on surrounding tissues due to long-term proximity with local tissue. Cu concentrations of uterine washings were 0.7 and 0.9 mg/L for IUD nonusers, while those with T-380A IUDs showed concentrations that varied according to their menstrual cycle (i.e., proliferative phase: 5.9 ± 1.8 ; secretory phase: 10.4 ± 4.9 and menstruation: 12.6 ± 5.4 mg/L) (Arancibia et al., 2003).

Iron

Iron (Fe) is a metallic element that makes up about 5% of the Earth's crust. Water hardness and acidity influence the amount of iron that dissolves during the percolation process in the water cycle. These iron-rich waters recharge surface waters and aquifers that inevitably serve as drinking water sources. Corrosion can often be a source of iron in drinking water. Iron contamination as a result of corroded pipes is a common occurrence in many cities with old water systems.

Increased Fe intake increases fertility levels in women. However, overexposure is detrimental to the reproductive health of the women. A previous study evaluated the effect of iron burden on hypogonadism and diminished ovarian reserve and on infertility in women with thalassemia major (TM). Iron burden in the ovary of these women had an inverse correlation with antimüllerian hormone levels and nontransferrin bound iron. An increase in reactive oxygen species and lower enzymatic antioxidant defense mechanisms have been shown to accelerate follicle aging. High redox activity in the ovarian follicular fluid, increased oxidative injury and low levels of antioxidants were reported in the ovary of a woman with TM.

Overweight and obese women with PCOS have increased serum ferritin levels indicating increased iron stores. In PCOS patients, increased iron stores contribute to insulin resistance and hyperinsulinemia. During follicular atresia and luteal regression, heme oxygenase-1 (HO-1), an enzyme-catalyzing heme degradation, was observed in apoptotic granulosa cells in atretic follicles (Harada et al., 2004) resulting in the release of iron ions from heme structures. Free iron ions, particularly ferrous iron, increase free radical generation resulting in oxidative damage. Increased iron uptake associated with the iron storage disease hemochromatosis is also associated with a higher incidence of ovarian cancer. The frequency of two mutations (C282Y, H63D) in heterozygous carriers of the hemochromatosis gene was assessed in 677 women consisting of 80 controls, 124 benign, 96 with low metastatic potential and 264 women with invasive ovarian tumors. The proportion of women with a single allele of the C282Y mutation was significantly elevated in women with benign tumors and ovarian cancers (8%–9%) compared with the control group (2.5%). This was reflected in a 4.9-fold increase in the risk of developing ovarian cancer. Survival rates for women with high-grade serous ovarian cancer with the heterozygous C282Y mutation were reduced from 39 to 19 months (Gannon et al., 2011). It is evident from the above reports that increased iron burden is deleterious to the ovary, and could predispose women to development of ovarian cancers.

Lead

Lead (Pb) is a ubiquitous environmental and industrial pollutant. Pb concentrations have increased in the atmosphere as a consequence of extensive industrialization and environmental pollution. In human studies, women occupationally exposed to Pb revealed increased incidence of spontaneous abortion, decreased birth weight and minor congenital malformations in their babies, infertility, and changes in ovarian function. When granulosa cells of women (in vitro fertilization/embryo transfer patients) were treated with Pb in vitro, Pb significantly decreased progesterone levels. This study also detected Pb in the follicular fluid of human ovary. This finding proves that Pb is able to enter the ovary and accumulate in the ovarian compartment. In occupationally exposed humans, decreased plasma levels of LH have been found. Pb also increased MMPs and decreased TIMP2 in human placenta with increased Pb burden (Paksy et al., 2001).

Manganese

Millions of kilograms of manganese (Mn) are released into the air and surface waters each year in the United States. Mn may also be released to the environment through the use of MMT (methylcyclopentadienyl manganese tricarbonyl) as a gasoline additive. Human exposure to Mn is widespread. In mammalian cells, Mn causes DNA damage and chromosome aberrations. Large amounts of Mn affect fertility in mammals and are toxic to the embryo and fetus (Colomina et al., 1996). Mn is a component of the mitochondrial enzymes, pyruvate carboxylase, superoxide dismutase (MnSOD), glutamine synthetase, alkaline phosphatase, and arginase. Mn also activates several enzymes. MnSOD, also known as SOD2, is a mitochondrial enzyme with potent antioxidant activity. Mn is an antagonist of iron and can replace Mg^{2+} in certain enzymes. Because of its similar ionic radius Mn can also interfere with the metabolism of calcium. Therefore, it is possible that Mn could induce early menarche in women. However, information is lacking on the direct toxic effects of Mn on the ovary in human. Early studies from Dees' laboratory reported direct effects of Mn on the hypothalamus, follicular development, and steroidogenesis in the ovary (see Table 1). In addition, umbilical cord blood concentration of Mn is directly correlated with intrauterine growth restriction in newborn children compared to newborns appropriate for gestational age (Vigeh et al., 2008).

Mercury

The three most common forms of mercury (Hg) (elemental, inorganic, and methylmercury) can produce adverse health effects at sufficiently high doses. The USEPA has determined that eating Hg-contaminated fish is the primary route of exposure to Hg for most people. Methylmercury is the most toxic form. It affects the immune system, alters genetic and enzyme systems, and damages the nervous system. Methylmercury is particularly damaging to developing embryos, which are 5–10 times more sensitive than adults. Elemental mercury, Hg(0), the form released from broken thermometers, causes tremors, gingivitis, and excitability when vapors are inhaled over a long period of time. Once in surface water, Hg enters a complex cycle in which one form can be converted to another. Hg attached to particles can deposit in sediment where it can diffuse into the water column, get resuspended and buried by other sediments, or be methylated. Methylmercury can enter the food chain or it can be released into the atmosphere by volatilization. Intracellular Hg is rapidly oxidized by cytosolic catalase to mercuric Hg (Hg^{2+}), the reactive species for most Hg compounds. Hg^{2+} can be formed by oxidation of Hg, reduction of mercuric salts, or demethylation of methylmercury. Hg^{2+} is highly reactive and rapidly combines with intracellular ligands such as sulfhydryls, potentially disrupting enzymes and proteins. Epidemiological studies indicate that occupational exposure to Hg in women resulted in abnormal menstrual cycles in terms of bleeding pattern and cycle length (Rodríguez-Villamizar et al., 2015). Dental assistants exposed to Hg vapor experience decreased fertility.

Nickel

The most probable route of nickel (Ni) exposure from hazardous waste sites would be from consumption of contaminated drinking water, inhalation of dust, dermal contact with bath/shower water, soil, or dust, and ingestion of Ni-contaminated soil. Groundwater contamination may occur where the soil has a coarse texture and where acid waste, such as waste from plating industries is discarded. Ni(II) may induce genotoxic effects such as DNA-protein crosslinking, DNA strand breaks, sister chromatid exchange and oxidative DNA base damage, including the mutagenic 8-oxo-29-deoxyguanosine and other lesions in CHO cells (Kasprzak, 1995). Ni^{2+} was found to be cytotoxic at 62.5 μM in primary cultures of granulosa cells from women (23–37 years of age) who undergo in vitro fertilization. Ni^{2+} decreased human chorionic gonadotropin (0.1 IU/mL hCG) or dibutyryl cyclic adenosine monophosphate (1 mM db-cAMP)-induced progesterone production. Women working in a metal refinery in Northwestern Russia were reported to have spontaneous abortions, and the newborns of the Ni-exposed women suffer from congenital musculoskeletal defects, and perinatal mortality (Vaktskjold et al., 2008).

Selenium

The trace element selenium (Se) was discovered in 1817 by the Swedish physician and chemist Berzelius; however, its essentiality in mammals was not discovered until the 1950s. Se is found in highest amounts in organ meats, such as kidney and liver, while some seafood contains nearly as much. Se is initially taken up from the soil and concentrated by plants; it is absorbed in the small intestine and incorporated into proteins. At least 25 selenoproteins including the glutathione peroxidases (GPX), thioredoxin reductases, and deiodinases contain Se. Selenoproteins play an important role in many biological functions, such as antioxidant defense, formation of thyroid hormones, DNA synthesis, fertility, and reproduction.

Se is used in animal husbandry as an essential supplement for successful reproduction. Of particular importance to reproduction and pregnancy are the GPXs, in which Se is an important component. Few clinical studies in patients with unexplained infertility revealed decreased Se in serum and follicular fluid samples. Women with polycystic ovary syndrome (PCOS) had decreased levels of Se relative to the control group, indicating that this element may play a role in the pathogenesis of PCOS related to hyperandrogenism (Coskun et al., 2013). Interestingly, women presenting with unexplained infertility or premature ovarian failure were found to have significantly increased serum levels of the ovarian autoantibody against Se-binding protein-1. Se accumulates in granulosa cell layers of large healthy follicles. Increased Se in these cells is attributed to increase in GPX1. In another study, human cumulus cells derived from cumulus-oocyte complexes collected for both in vitro fertilization and intracytoplasmic sperm injection revealed a positive correlation between GPX1 and successful fertilization and pregnancy (Ceko et al., 2015). This suggests a strong role for GPX1, and Se indirectly, in determining follicle growth, maturation, and dominance in both the cow and in women leading to specific dietary recommendations for Se for improved fertility. Thus, Se plays a significant role in female reproductive function. Because of its contribution to the formation of selenoproteins combined with its antioxidant action, a low Se status in the body results in low resistance to free radical damage. Unfortunately, studies related with Se and female reproduction are very few compared to studies in male species. More studies are needed to fill this gap in understanding the importance of Se in female reproduction.

Titanium

Titanium oxide (TiO_2) nanoparticles are used for protection against UV ray exposure due to their high refractive index. Many sunscreens contain TiO_2 as well as surface coating products which are colorless and reflect and scatter UV rays more efficiently than larger particles. Some types of TiO_2 nanoparticles have been reported to cause toxicity in vitro and in vivo, attributed to the generation of reactive oxygen species (ROS) resulting in apoptosis, micronuclei formation (Rahman et al., 2002), mitochondrial

abnormalities and other forms of cell toxicity. CHO cells treated with nano-TiO₂ rapidly formed extracellular aggregates with dimensions that were generally > 100 nm. Aggregates were readily internalized by CHO cells and appeared in vacuoles taken up by a phagocytotic or endocytotic process. Internalized aggregates were restricted to the cytoplasm and were not localized to any specific organelles or intracellular sites. Exposure to TiO₂ (5 and 200 nm size) increased the production of reactive oxygen species in both short- and long-term exposures (Thomas et al., 2011). Superoxide dismutase quenched the ROS signal produced by nano-TiO₂ of human hepatocytes. There are no studies indicating deleterious effects of Ti on the human ovary. More studies are needed to address the effects of Ti on ovarian development and function.

Zinc

Due to its nature as an essential trace element, oral uptake of small amounts of zinc (Zn) is essential for survival. The recommended dietary allowance (RDA) for zinc is 11 mg/day for men and 8 mg/day for women. Lower Zn intake is recommended for infants (2–3 mg/day) and children (5–9 mg/day) because of their lower average body weights. Oral uptake of Zn leads to absorption throughout the small intestine and subsequent distribution occurs via the serum, where it predominately exists bound to several proteins such as albumin, α -microglobulin, and transferrin. Inhalation of Zn-containing smoke generally originates from industrial processes like galvanization, primarily affecting manufacture workers. Uptake of large doses of supplemental Zn over extended periods is frequently associated with Cu deficiency. This correlation seems to be caused by the competitive absorption relationship of Zn and Cu within enterocytes, mediated by metallothioneins (MT). The expression of MT is upregulated by high dietary Zn content, and MT binds Cu with a higher affinity than Zn. Zn is a component of > 300 enzymes and an even greater number of other proteins, which emphasizes its indispensable role for human health. The cellular homeostasis of Zn is mediated by two protein families; the Zn-importer (Zip; Zrt-Irt-like proteins) family, containing 14 proteins that transport Zn into the cytosol, and the Zn transporter (ZnT) family, comprising 10 proteins transporting Zn out of the cytosol. The same transporter families also regulate the intracellular distribution of Zn into endoplasmic reticulum, mitochondria, and Golgi. In addition, many mammalian cell types also contain membrane-bound vesicular structures, so-called zincosomes. These vesicles sequester high amounts of Zn and release it upon stimulation, for example, with growth factors. Optimal nucleic acid and protein metabolism, as well as cell growth, division, and function require sufficient availability of Zn. Zn acts as a co-factor for at least 200 enzymes, including carbonic anhydrase enzymes and Cu/ZnSOD. It also stabilizes DNA conformation through its involvement in numerous DNA repair enzymes, especially important during early embryogenesis. Through its association with Zn finger proteins, it is involved in the regulation of gene expression and control of apoptosis.

Zn seems to have a pivotal role in reproduction and thus fertility via mechanisms that control genome stability. It is an important co-factor in the folate cycle, involved in the recycling of homocysteine to methionine, both of which have a major influence on methylation reactions through S-adenosyl methionine (SAM). SAM is formed in the oocyte and homocysteine impairs both methionine incorporation and normal methylation reactions. Moreover, human oocytes have a very limited capacity for homocysteine recycling, because the cystathionine β synthase (CBS) pathway is absent and the betaine homocysteine methyltransferase (BHMT) pathway, which is Zn dependent, is poorly expressed. Zn also has an important role in preventing generation of reactive oxygen species (ROS) during the first embryonic cleavage division. ZnSOD, located in the cytoplasm, is found in human oocytes and fallopian tubes and in the environment of the preimplantation embryo. Zn is present throughout the embryo's journey in the oviduct. In vitro, nonphysiologically high Zn concentrations may have negative effects on preimplantation development (Stephenson and Brackett, 1999).

Heavy Metal Toxicity: Mechanism of Action and Possible Intervention

Commonly used metals such as Fe, Cu, Cd, Cr, Hg, Ni, Pb, and As possess the ability to generate free radicals, resulting in depletion of enzyme activities, damage to lipid bilayer and DNA (Stoys and Bagchi, 1995). There are a wide variety of oxygen-, carbon-, sulfur-, and nitrogen-radicals, originating not only from superoxides, hydrogen peroxide, and lipid peroxides but also from chelates of amino acids, peptides, and proteins complexed with the toxic metals. The resulting oxidative stress may affect the levels and functions of redox-sensitive signaling molecules such as AP-1, NF- κ B, and p53, derange the cell signaling and gene expression systems, and/or induce apoptosis. Both AP-1 and NF- κ B are considered stress response transcription factors that govern the expression of a variety of proinflammatory and cytotoxic genes (Hu et al., 2002). The heavy metals have electron-sharing affinities that can result in the formation of covalent attachments mainly between heavy metal and sulfhydryl groups of proteins. Several enzymes in antioxidant defense systems may protect the proper balance between pro-oxidant and antioxidant species but unfortunately, enzymes contain sulfhydryl groups which can bind metal ions at their active sites, and hence, become inactive due to direct binding of metal ions to sulfhydryl groups. GSH is found in mammalian tissues at millimolar concentrations. The oocyte has a GSH concentration of 10 mM which accounts for > 90% of total nonprotein sulfur. Pb is known to deplete GSH levels. Zn can be replaced by Pb which inactivates the function of several Zn-dependent enzymes. The antioxidant enzymes SOD, catalase and GPX are potential targets of Pb. Se is essential for GPX activity, and Pb forms a complex with Se and decreases GPX activity. Pb inhibits heme synthesis. Because catalase is a heme-containing enzyme, Pb decreases its activity. Pb also replaces Cu and Zn, thus, decreasing the activity of Cu/Zn SOD. Therefore, inhibitory effects of metal ions such as Pb and Cu on various

enzymes impair antioxidant defense mechanisms by cells and render them more vulnerable to oxidative stress. An important property of Se is its interaction with other elements including As, Cu, Ni, Co, Cr, Mn, Zn, Cd, Sn, Pb, Hg, Bi, Mo, Ag, Au (Banu, 2013). The sequestration of elements by Se represents an efficient natural detoxification mechanism for some of these elements but also results in the physiological inactivation of Se. Se is essential for cellular antioxidant defense systems to scavenge free radicals. Supplementation of Se or Zn mitigated or inhibited the deleterious effects of Cd in causing follicular atresia in Japanese quail. Therefore, supplementation of an additional dose of Se and/or Zn (other than the dietary level) could help mitigate Cd toxicity, or other metal toxicities that respond to Se or Zn supplementation. Free radicals generated by As lead to the activation of oxidative signaling pathways that result in cell apoptosis. Supplementation of vitamins C and E alter the extent of DNA damage by reducing TNF- α levels and inhibiting activation of the caspase cascade in As-intoxicated animals. A combination of vitamin C and thiamine was effective in reducing Pb levels in blood, liver, and kidney. α -Lipoic Acid (LA) is an endogenous thiol antioxidant, which possesses the potential to quench ROS, regenerate GSH, and chelate metals such as iron, Cu, Hg and Cd. LA is also known to mediate free-radical damage in biological systems (Gorąca and Asłanowicz-Antkowiak, 2009). LA is readily available in the diet, absorbed through the gut, and easily passes through the blood-brain barrier. Exogenous supplementation with LA has been reported to increase unbound LA levels, which can act as a potent antioxidant and reduce oxidative stress both in vitro and in vivo (Bustamante et al., 1998). LA serves as a protective agent against Cd-induced membrane damage and cell dysfunction in hepatocytes. Another hormone with antioxidant properties is melatonin. Melatonin stimulates several antioxidant enzymes including glutathione reductase, GPX and SOD, promotes quick disposal of H₂O₂, enhances the production of enzymes that are involved in the synthesis of GSH, and prevents the reduction of membrane fluidity caused by lipid peroxidation. In this way, melatonin helps in scavenging free radicals. Thus, it could be a possible intervention against metal toxicity, along with other candidates such as Zn, Se and LA.

In conclusion, prenatal exposure to heavy metal EDCs is a growing concern, because such exposures have been shown to be associated with a variety of pregnancy complications as well as developmental deficits and birth defects. One of the major consequences of heavy metal exposure is increased oxidative stress and decreased levels of antioxidants (Fig. 1). Experimental data document various female reproductive dysfunctions due to metal exposure (Table 1). As heavy metals enter the water supply by numerous routes, strategies must be identified and validated to mitigate the adverse effects of metal exposure. Additional research is also needed to delineate the molecular mechanisms underlying heavy metal-induced reproductive and developmental toxicity.

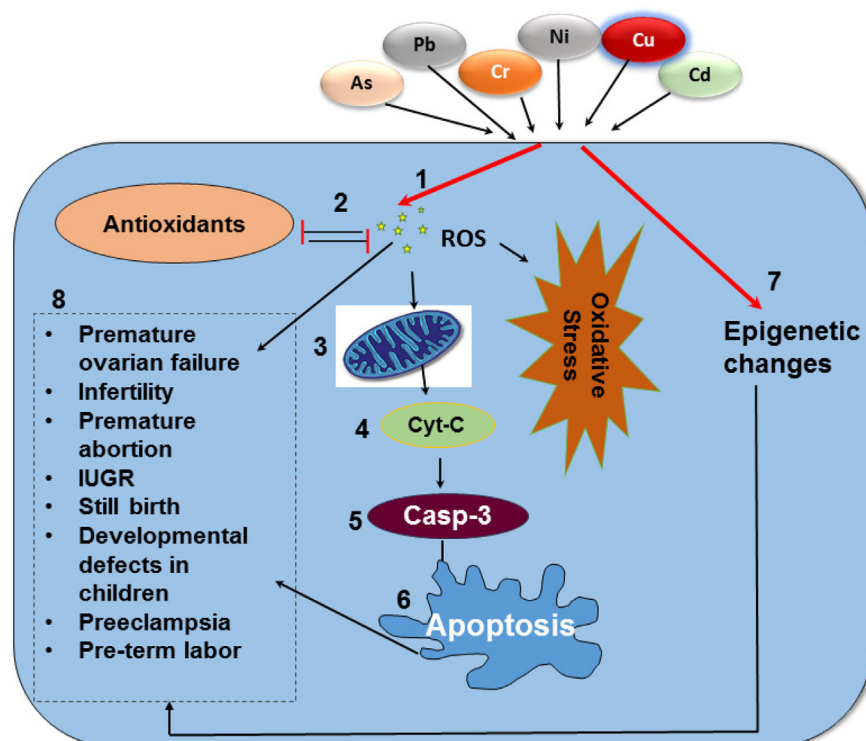


Fig. 1 A schematic diagram of heavy metals-induced female reproductive disorders. (1) Heavy metals enter into a cell by active transport or passive diffusion, which results in the (2) generation of large amount of free radicals or reactive oxygen species (ROS). ROS deplete antioxidants (AOX) and AOX quenches ROS. (3) Increased oxidative stress generated by ROS changes mitochondrial membrane permeabilization. (4) Release of cytochrome *c* from the mitochondria and (5) activation of caspases, lead to (6) apoptosis. (7) Heavy metals also cause epigenetic modifications. (8) All these events culminate in several reproductive and pregnancy disorders in women, as well as intrauterine growth restriction in children. *IUGR*, intra uterine fetal growth restriction. Modified from Banu, S. K. (2013). Heavy metals and the ovary. In: Hoyer, P. B. (ed.) *Ovarian toxicology*. (2nd ed.), pp. 191–228. CRC Press.

Acknowledgement

Most of the data on chromium and the ovary and placenta from the author's laboratory were generated and published with the funding support from the National Institute of Health (NIH)/National Institute of Environmental Health Sciences (NIEHS) grants ES016605-A2 and ES020561-01. The authors acknowledge Dr. Robert C Burghardt, Professor & Associate Dean for Research and Graduate Studies, College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, for his editorial comments.

References

- Adebowale, A. (2004). *Bioremediation of arsenic, chromium, lead, and mercury* (pp. pp. 1–43). Washington, DC: U.S. Environmental Protection Agency.
- Arancibia, V., Peña, C., Allen, H. E., & Lagos, G. (2003). Characterization of copper in uterine fluids of patients who use the copper T-380a intrauterine device. *Clinica Chimica Acta*, 332, 69–78.
- Banu, S. K. (2013). Heavy metals and the ovary. In P. B. Hoyer (Ed.), *Ovarian toxicology* (2nd edn., pp. 191–228). CRC Press.
- Banu, S. K., Stanley, J. A., Sivakumar, K. K., Arosh, J. A., Barhoumi, R., & Burghardt, R. C. (2015). Identifying a novel role for X-prolyl aminopeptidase (Xpnep) 2 in Crvi-induced adverse effects on germ cell Nest breakdown and follicle development in Rats1. *Biology of Reproduction*, 92, 1–18.
- Banu, S. K., Stanley, J. A., Taylor, R. J., Sivakumar, K. K., Arosh, J. A., Zeng, L., Pennathur, S., & Padmanabhan, V. (2017). Sexually dimorphic impact of chromium accumulation on human placental oxidative stress and apoptosis. *Toxicological Sciences*. <https://doi.org/10.1093/toxsci/kfx224>.
- Bustamante, J., Lodge, J. K., Marcocci, L., Tritschler, H. J., Packer, L., & Rihn, B. H. (1998). Lipoic acid in liver metabolism and disease. *Free Radical Biology and Medicine*, 24, 1023–1039.
- Ceko, M. J., Hummitzsch, K., Hatzirodos, N., Bonner, W. M., Aitken, J. B., Russell, D. L., Lane, M., Rodgers, R. J., & Harris, H. H. (2015). X-ray fluorescence imaging and other analyses identify selenium and Gpx1 as important in female reproductive function. *Metallomics*, 7, 71–82.
- Colomina, M. T., Domingo, J. L., Llobet, J. M., & Corbella, J. (1996). Effect of day of exposure on the developmental toxicity of manganese in mice. *Veterinary and Human Toxicology*, 38, 7–9.
- Coskun, A., Arikian, T., Kilinc, M., Arikian, D. C., & Ekerbicer, H. C. (2013). Plasma selenium levels in Turkish women with polycystic ovary syndrome. *European Journal of Obstetrics & Gynecology*, 168, 183–186.
- Gannon, P. O., Medelci, S., Le Page, C., Beaulieu, M., Provencher, D. M., Mes-Masson, A. M., & Santos, M. M. (2011). Impact of hemochromatosis gene (Hfe) mutations on epithelial ovarian cancer risk and prognosis. *International Journal of Cancer*, 128, 2326–2334.
- Gorąca, A., & Aslanowicz-Antkowiak, K. (2009). Prophylaxis with A-Lipoic acid against lipopolysaccharide-induced brain injury in rats. *Archivum Immunologiae et Therapiae Experimentalis*, 57, 141–146.
- Harada, T., Koi, H., Kubota, T., & Aso, T. (2004). Haem oxygenase augments porcine granulosa cell apoptosis in vitro. *Journal of Endocrinological Investigation*, 181, 191–205.
- Hu, Y., Jin, X., & Snow, E. T. (2002). Effect of arsenic on transcription factor Ap-1 and Nf-Kb DNA binding activity and related gene expression. *Toxicological Letters*, 133, 33–45.
- Kasprzak, K. S. (1995). Possible role of oxidative damage in metal-induced carcinogenesis. *Cancer Investigation*, 13, 411–430.
- Liao, K.-W., Chang, C.-H., Tsai, M.-S., Chien, L.-C., Chung, M.-Y., Mao, I. F., Tsai, Y.-A., & Chen, M.-L. (2018). Associations between urinary total arsenic levels, fetal development, and neonatal birth outcomes: A cohort study in Taiwan. *Science of the Total Environment*, 612, 1373–1379.
- Paksy, K., Gati, I., Naray, M., & Rajczy, K. (2001). Lead accumulation in human ovarian follicular fluid, and in vitro effect of lead on progesterone production by cultured human ovarian granulosa cells. *Journal of Toxicology and Environmental Health. Part A*, 62, 359–366.
- Rahman, Q., Lohani, M., Dopp, E., Pemsel, H., Jonas, L., Weiss, D. G., & Schiffmann, D. (2002). Evidence that ultrafine Titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. *Environmental Health Perspectives*, 110, 797–800.
- Remy, L. L., Byers, V., & Clay, T. (2017). Reproductive outcomes after non-occupational exposure to hexavalent chromium, Willits California, 1983–2014. *Environmental Health*, 16, 1–15.
- Rodriguez-Villamizar, L. A., Jaimes, D. C., Manquian-Tejos, A., & Sanchez, L. H. (2015). Human mercury exposure and irregular menstrual cycles in relation to artisanal gold mining in Colombia. *Biomédica*, 35, 38–45.
- Stephenson, J. L., & Brackett, B. G. (1999). Influences of zinc on fertilisation and development of bovine oocytes in vitro. *Zygote*, 7, 195–201.
- Stohs, S. J., & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal-ions. *Free Radical Biology and Medicine*, 18, 321–336.
- Thomas, K. V., Farkas, J., Farnen, E., Christian, P., Langford, K., Wu, Q., & Tollefsen, K. E. (2011). Effects of dispersed aggregates of carbon and titanium dioxide engineered nanoparticles on rainbow trout hepatocytes. *Journal of Toxicology and Environmental Health, Part A*, 74, 466–477.
- Vaktskjold, A., Talykova, L., Chashchin, V., Odland, J. Ø., & Nieboer, E. (2008). Spontaneous abortions among nickel-exposed female refinery workers. *International Journal of Environmental Health Research*, 18, 99–115.
- Vigeh, M., Yokoyama, K., Ramezanzadeh, F., Dahaghin, M., Fakhriazad, E., Seyedaghamiri, Z., & Araki, S. (2008). Blood manganese concentrations and intrauterine growth restriction. *Reproductive Toxicology*, 25, 219–223.
- Xu, L., Ge, J., Huo, X., Zhang, Y., Lau, A. T., & Xu, X. (2016). Differential proteomic expression of human placenta and fetal development following E-waste lead and cadmium exposure in utero. *Science of the Total Environment*, 550, 1163–1170.

Relevant Websites

<http://www.acgih.org>.
<https://www.atsdr.cdc.gov>.
<http://www.blacksmithinstitute.org>.
<https://www.cdc.gov/niosh/index.htm>.
<https://www.epa.gov>.
<http://www.who.int/en/>.

Pesticides

Shanthi Ganesan, Mackenzie J Dickson, and Aileen F Keating, Iowa State University, Ames, IA, United States

© 2018 Elsevier Inc. All rights reserved.

General Female Reproductive Physiology

The mammalian ovary is the female gonad and has two major functions: germ cell and sex steroid hormone production. The ovarian follicular structures are the functional units in which the oocyte (female gamete) is surrounded by granulosa cells, and eventually also by theca cells. Interaction and communication between the granulosa cell layer and oocyte maintains oocyte viability, while 17β -estradiol (E_2) production within the dominant follicles requires interaction between the granulosa and theca cells.

There are a variety of different developmental stage follicles within the ovary, the most immature of which is the primordial follicle. The ovary is endowed with a finite number of primordial follicles at birth, and within the primordial follicle structure, the meiotically-arrested oocyte resides and is surrounded by squamous granulosa cells (Hirshfield, 1991). Upon receiving an activation signal, primordial follicles mature through primary, secondary, and antral follicle stages during which the number of granulosa cells increase, and they become cuboidal in structure. A tertiary follicle is characterized by the presence of a fluid filled sac, or antrum. The antrum is located between the granulosa cell layer and the oocyte and contains follicular fluid. The composition of follicular fluid arises from filtered blood running through the thecal capillaries (Shalgi et al., 1973). Ovulation occurs when a follicle ruptures and the oocyte released. Cumulus granulosa cells may be ovulated along with oocyte. The oocyte remains arrested at prophase I of meiosis until the luteinizing hormone (LH) surge, just prior to ovulation, at which time, the first polar body is extruded and the first round of meiosis is completed. The oocyte is then arrested at metaphase II until fertilization occurs. At this point, the second polar body is extruded and meiosis II is completed. Although follicular growth and development is well characterized, the mechanisms, and signals initiating follicular growth and selection as well as maintenance or regression are still not fully understood. After ovulation, a corpus hemorrhagicum forms due to luteinization of the granulosa and theca cells under the control of LH. This structure next becomes a corpus luteum (CL) which is the site of progesterone (P_4) production. The CL is the primary source of P_4 during the luteal phase of the cycle and is maintained on the ovary if the animal is pregnant and is essential for pregnancy maintenance, inhibits the hypothalamic-pituitary-ovarian axis and is involved in mammary gland preparation for lactation, though in the majority of species, placental takes over from luteal P_4 production as gestation progresses (Fig. 1).

Female Reproductive Toxicity

Ovarian follicles at any developmental stage can be impacted by chemical exposures, resulting in negative effects on oocyte or somatic cell viability, endocrine function, ovulation, oocyte competence, and germline integrity. The reproductive impact of an ovarian chemical exposure largely depends on what follicular development stage is targeted: if primary, secondary or antral follicles are targets and damaged or deleted, temporary perturbations to reproductive function will occur since these follicles can ultimately be replaced by recruitment from the ovarian reserve. However, damage or demise of the primordial

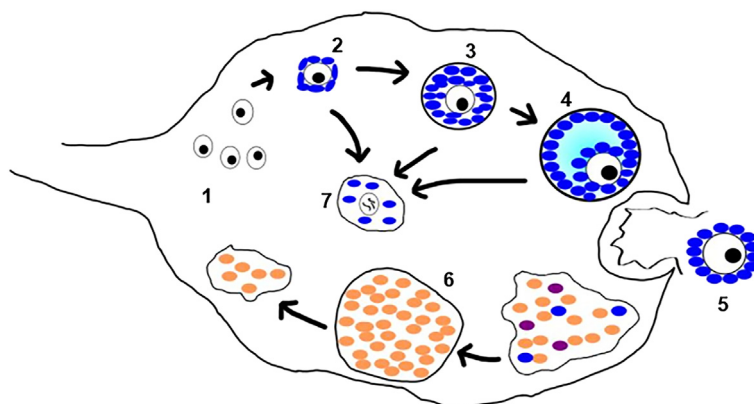


Fig. 1 Summary of ovarian physiology. During follicular development, primordial follicles (1) are recruited to primary follicles (2) when growth is initiated. Secondary follicles (3) are characterized by at least two layers of granulosa cells (shown in blue) surrounded by theca cells (shown in purple). The tertiary follicles (4) contain an antrum filled with follicular fluid. Ultimately the oocyte is ovulated (5) and a corpus luteum forms (6) from luteinized granulosa and theca cells. Nevertheless, the majority of follicles, never reach ovulation and regress via atresia (7). 17β -estradiol is produced within the preovulatory follicle while the corpus luteum produces progesterone.

follicles represent more insidious chemical exposure effects and will lead to permanent infertility and premature ovarian senescence. Since they are irreplaceable, if depleted or hyperactivated into the growing pool, females will eventually undergo ovarian failure, commonly known as menopause in women. The postmenopausal phase of a woman's life places her at greater risk to develop cardiovascular disease, obesity, osteoporosis, Alzheimer's disease, and colorectal and ovarian cancer, thus a longer time frame spent in the postmenopausal physiological state is detrimental for general female health (Hoyer and Keating, 2014).

The level and duration of exposure to an environmental reproductive toxicant may also influence the phenotypic manifestations and effects. Chronic, low dose exposures are difficult to identify since their phenotypic display may go unrecognized for years. Selective damage of small preantral follicles would not initially be observed and raise concern until the end of ovarian function that may occur years later. Further, the developmental age of the female at which exposure occurs should be considered. During childhood and prior to puberty, there are higher numbers of primordial follicles within the ovary, ergo, a chemical exposure during this life stage may not result in the same extent of ovarian damage as might be observed postpubertally. However, damage to oocytes by chemical exposures in utero and/or during childhood years present a significant concern, since these effects would not be detected until later reproductive years at which point the impact could be permanent.

There are additional nonovarian points of action that should also be considered in female reproductive toxicity which will not be further discussed herein in interests of brevity but which include impacts on the uterus, placenta, hypothalamic-pituitary-gonadal axis, hepatic metabolism of steroid hormones and lactation to name a few (Fig. 2).

Examples of Pesticides Documented to Impact Female Reproductive Capacity

Atrazine

Atrazine (ATR) is a herbicide used to kill broadleaf plants in rural and urban environments and is widely used. ATR levels in US drinking water can exceed the drinking water criteria (Barbash et al., 2001) and human exposure has been confirmed by detection of ATR in urine samples (Curwin et al., 2007). Interestingly, when ATR levels were high in Illinois drinking water, as compared to women in Vermont (a low ATR-using state) consumption of >2 cups of unfiltered water daily was associated with increased risk of irregular menstrual periods in women (Cragin et al., 2011). In rodent in vivo studies ATR-induced effects on ovarian function have been mechanistically examined. ATR-exposed rats (100 or 300 mg kg⁻¹; oral gavage daily for 2 weeks) had reduced ovarian and uterine weights, reduced circulating E₂ levels, and lengthened estrous cycles relative to control-treated females (Eldridge et al., 1994). Another study that evaluated a range of ATR exposures (0.75–100 mg kg⁻¹ day⁻¹) and two different routes (oral gavage or dietary consumption) observed altered estrous cyclicity, and attenuated LH surge, and lower numbers of oocytes ovulated, though no impacts on fetus number were noted. Interestingly, dietary ATR exposure did not alter any of

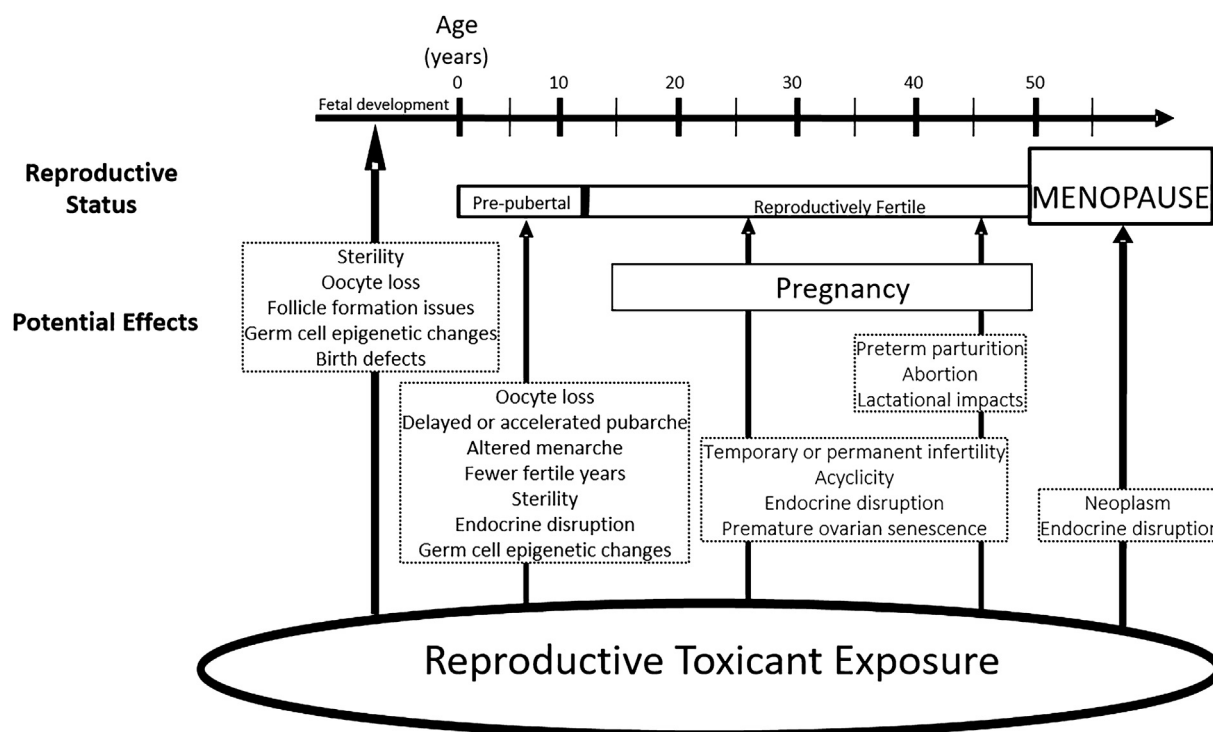


Fig. 2 Summary of potential phenotypic outcomes of ovotoxicant exposures in females across the reproductive lifespan.

the endpoints examined (Foradori et al., 2014). Thus, the route of TAR exposure is worth considering when assessing reproductive endpoints.

A study in rats examining the impact of ATR (75 mg kg⁻¹) exposure or exposure to the ATR metabolites desisopropylatrazine (DIA) and diamino-*s*-chlorotriazine (DACT) at equimolar dosages demonstrated increased both ATR and DIA increased circulating P₄ but this effect was not observed in the DACT-treated females (Fraites et al., 2009). Cultured rat granulosa cell primary cultures had increased E₂, P₄, and cytochrome P450 19a1 (*Cyp19a1*) mRNA abundance (Tinfo et al., 2011). Evidence for adrenal P₄ production due to ATR exposure has been provided in a study where ATR was administered either in a single or multiple doses and in which P₄ was elevated, the LH surge altered and increased *Kiss1* mRNA abundance in the hypothalamus demonstrated. Also this study demonstrated eloquently that timing of the ATR exposure relative to the LH surge was importance in the physiological effects observed (Goldman et al., 2013).

Gestational exposure to ATR is also a concern and administration of ATR at the higher dose (100 mg kg⁻¹ day⁻¹) during gestation days 14–21 in rats reduced female offspring birth weight and delayed vaginal opening (Davis et al., 2011). Embryonic exposure to ATR affected meiotic processes in exposed offspring and induced oxidative stress, impacted DNA double strand break repair, as well as chromosome synapsis and crossover numbers. Coupled with the meiotic effects were a reduction in primordial follicle number and increased numbers of multioocyte follicles in ovaries of adult mice (Gely-Pernot et al., 2017). Epidemiological studies in humans which have examined ATR levels in drinking water have associated ATR exposure with increased risk for preterm birth (Stayner et al., 2017), as well as abdominal defects and gastroschisis in exposed offspring (Brender and Weyer, 2016).

In nonmammalian aquatic and avian species, ATR is strongly implicated as an endocrine disruptor and a reproductive toxicant, and as an example, a study in quail discovered that ATR exposure arrested ovarian and oviductal development and increased levels of circulating E₂, P₄, LH, and PRL while downregulated follicle stimulating hormone (FSH) and testosterone (T) (Qin et al., 2015). In addition, ATR increased the level of ovarian cytochrome P450 11A1 (*Cyp11a1*), *Cyp19a1*, 3β-hydroxysteroid dehydrogenase (*Hsd3b*), and 3β-hydroxysteroid dehydrogenase (*Hsd17b*) but decreased the estrogen receptor beta (*ERβ*) level (Qin et al., 2015).

Taken together, the literature supports that ATR as a reprotoxicant with effects on the reproductive system of mammals at many different points, and the widespread exposure to ATR is of concern.

Glyphosate

With the development of herbicide-tolerant soybeans, corn and cotton in 1996, glyphosate (GLY) use has increased dramatically over the past two decades, now accounting for 50% of total herbicide applied, equating to 155 million pounds per annum with almost 100% of wheat, corn, soybean, cotton, and potato cultivation receiving GLY treatment. Outside of agriculture, GLY is extensively utilized for weed control in home gardens, railways, and roadways, therefore, both urban and rural populations are exposed to GLY. Worryingly, GLY residues are found in food and drinking water and a recent screen of 182 individuals in 18 European Union countries detected GLY presence in 44% of urine samples (Bremen, 2013). Due to widespread use, GLY residues in waterways in the range of 10–15 µg L⁻¹ are reported (Byer et al., 2008; Struger et al., 2008). GLY is water soluble and has a half-life in soil of 2–215 days and in water of 2–91 days (Giesy et al., 2000; Battaglin et al., 2014). In United States glyphosate is detectable in 79% of waterways (Bradley et al., 2017) and there have been many recent publications on the negative effects of GLY exposure on aquatic species.

No effect of GLY on E₂ production using a standardized H295R steroidogenesis assay was observed (Hecker et al., 2011). There is, however, a growing body of scientific literature providing evidence that GLY represents a mammalian health risk. In the ovary, steroid acute regulatory protein (STAR) protein increases cholesterol translocation from the outer to the inner mitochondrial membranes; and represents the rate-limiting step in ovarian E₂ production. GLY exposure reduces STAR protein in male testicular steroidogenic leydig cells (Walsh et al., 2000), thereby potentially reducing sex steroid hormone production. Also, a placental cell line, JEG3, had lowered cell viability and microsomal CYP19a activity due to GLY exposure (Richard et al., 2005), findings that were recapitulated in a human hepatic cell line, HepG2 (Gasnier et al., 2009). Use of an embryonic cell line also found that GLY decreased cell viability and CYP19a activity (Benachour et al., 2007). In addition, cultured bovine granulosa cells had a reduction in both granulosa cell viability and E₂ production due to GLY exposure while these effects were not observed in cultured theca cells at the same concentrations of GLY (Perego et al., 2017).

In vivo experiments investigating the impact of GLY exposure on female reproductive health are limited, however, both ovarian and offspring impacts have been demonstrated. Maternal demise as a consequence of GLY exposure during gestation has been previously reported. A 50% mortality rate occurred in pregnant Wistar rats exposed from gestation day 6–15 to GLY (1000 mg kg⁻¹) (Dallegrave et al., 2003), however, the mechanisms underlying these fatalities were not explored. The Ontario farm family health study correlated GLY exposure during late pregnancy with risk of spontaneous abortion (Arbuckle et al., 2001).

There are, however, also a number of studies where gestation was completed successfully in GLY-exposed mice and rats albeit with accompanying teratogenic effects on the offspring including skeletal (Dallegrave et al., 2003), male reproductive (Dalsenter et al., 2003), hepatic enzymatic (Darwich et al., 2001), behavioral (Gallegos et al., 2016; Cattani et al., 2014), and hepatic peroxidation (Beuret et al., 2005). Neonatal exposure to a glyphosate-based herbicide (2 mg kg⁻¹ day⁻¹) on postnatal days 1, 3, 5, and 7 increased resorption sites in exposed female rats when they were experiencing gestation in adulthood. In addition, there was a decreased in levels of E₂ and P₄ receptors, and mRNAs at the implantation sites (Ingaramo et al., 2016). When adult ovariectomized rats were exposed to GLY (0.5, 5, 50 mg kg⁻¹ day⁻¹) for three consecutive days increased uterine

epithelia cell height, decreased mRNA encoding the ER alpha (ER α) and P₄ receptor (PR) were demonstrated in the luminal epithelium but increased expression in the stromal epithelium (Varayoud et al., 2017). Additionally, postnatal day 1 to postnatal day 7 rat pups exposed to glyphosate in 48 h intervals had altered expression of uterine genes, hyperplasia of the luminal epithelium and increased uterine stromal and myometrium thickness (Guerrero Schimpf et al., 2017).

While a contentious area of research, there is documented scientific data supporting that GLY has female reproductive effects, though whether these effects are observed at exposure levels reflective of human exposure levels remain unclear, thus it is currently difficult to extrapolate risk.

Methoxychlor

Methoxychlor (MXC) is an organochlorine pesticide used in agriculture and was a replacement for dichlorodiphenyltrichloroethane. MXC induces ovarian atrophy and decreases steroidogenesis. Interestingly, MXC also induces growth of the ovarian surface epithelium, contrasting with the pro-death impact on the antral follicles (Symonds et al., 2005), however, oxidative stress-induced DNA damage to these cells is observed (Symonds et al., 2008). Thus, MXC induces ovarian atresia and acts as an endocrine disruptor. Many of the data documented in this section has come from the research group of Dr. Flaws at the University of Illinois, thus if a study is not cited below, it has come from this group.

In a two-generation exposure experiment, MXC was provided to rats via the diet at 0–1500 ppm. At the higher doses, MXC reduced food consumption and body weight, lengthened the estrous cycle, negatively affected fertility parameters, had deleterious effects on implantation, reduced the numbers of implantation sites and offspring, and decreased ovarian weight. Additionally, a uterotropic effect, consistent with an endocrine disrupting effect of MXC, was noted and the female offspring had increased uterine weight (Aoyama et al., 2012). These effects were dose-dependent.

Mice exposed to MXC (64 mg kg⁻¹ day⁻¹) for 20 days had reduced primordial follicle number 30 days postdosing, but this effect was not evident after a longer postdosing timeframe (60 days). Another study has indicated reduced primordial follicle number due to MXC exposure in adult rats that received exposure during gestation, and increased abundance of the insulin-like growth factor receptor was also noted in growing follicles within these ovaries, potentially contributing to altered folliculogenesis (Ozden-Akkaya et al., 2017).

Mechanistic insights into the mode of action of MXC have been gained using both in vivo and in vitro models. MXC binds to the aryl hydrocarbon receptor (AHR) in cultured ovarian antral follicles from mice to induce atresia and inhibit antral follicle growth, and deletion of *Ahr* in mice, partially protected against the ovotoxic effects of MXC exposure. Cultured antral follicles from mice exposed to MXC had a temporal pattern of increased abundance of mRNAs encoding pro- and antiapoptotic genes. Initially, increased Bcl-xl and Bax, as well as increased caspase were noted, which were followed by morphological observation of atresia, supporting that atresia resulted from apoptosis.

ER-mediated pathways may mediate the ovarian toxicity of MXC. Incubation of antral follicles with E₂ protected against both MXC- and HPTE-induced atresia. Further, ovarian atresia was greater in antral follicles from MXC-exposed *Er β* -overexpressing mice compared with their wild type counterparts. In *Er α* -deficient mice, higher levels of atresia due to MXC exposure were noted, likely through altered apoptosis within these follicles.

MXC also has actions via oxidative stress-induced mitochondrial damage. Reduced expression of superoxide dismutase 1 (*Sod1*), glutathione peroxidase (*Gpx*), and catalase (*Cat*) due to MXC exposure were observed, and co-treatment with the antioxidant N-acetylcysteine (NaC) prevented these impacts, supporting that MXC induced oxidative stress as a mode of action during ovarian toxicity. Alteration of cell cycle regulators may contribute to slower growth of antral follicles in mice exposed to MXC. Decreased abundance of proliferating cell nuclear antigen (PCNA), decreased *Ccnd2* and *Cdk4* was observed in the MXC-exposed ovaries. Protection from MXC-induced oxidative stress via co-treatment with NaC reversed this effect of MXC on *Ccnd2* and *Cdk4*. Similar findings were observed in the caprine ovary (Bhardwaj and Saraf, 2017) supporting that oxidative stress contributes to ovotoxicity due to MXC exposure.

MXC undergoes hepatic metabolism to 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) and mono-hydroxy methoxychlor (mono-OH MXC). In addition to the MXC parent compound, mono-OH MXC negatively affects steroidogenesis. Antral follicles from mice cultured in the mono-OH MXC had decreased ovarian sex steroid hormone levels, but pregnenolone supplementation blunted this effect and returned ovarian hormone to the levels of follicles cultured in vehicle control, thus the endocrine disrupting effects of mono-OH MXC may be at an early stage in ovarian steroidogenesis. Pregnenolone supplementation was, however, ineffective at reversing the pro-atretic effects of mono-OH MXC in cultured antral follicles. HPTE exposure of cultured rat granulosa cells negatively affected E₂ and P₄ through interaction of disruption of signaling via FSH signaling (Zachow and Uzumcu, 2006). In addition, HPTE inhibited cyclic AMP (cAMP) production and reduced the abundance of *Er α* and *Er β* in cultured rat granulosa cells (Harvey et al., 2015). Luteal cells had reduced P₄ formation as well as reduced *Cyp11a1* activity (Akgul et al., 2008, 2011), thus MXC can also negatively affect the luteal phase of the estrous cycle and thereby potentially impacts pregnancy maintenance.

There are also ovarian epigenetic effects of MXC exposure as evidenced by epigenetic alterations to pathways that have important roles in ovarian physiology due to gestational exposure to MXC (Zama and Uzumcu, 2013). Epigenetic alterations have been recently identified that are induced by MXC and that correlate with increased rates of renal and ovarian pathology as well as obesity in offspring that were exposed gestationally, and these effects had transgenerational patterns of inheritance (Manikkam et al., 2014).

Thus, MXC alters female reproductive function by dysregulating steroidogenesis, slowing antral follicle growth, inducing atresia and inducing epigenetic impacts due to gestational exposure.

Chlorpyrifos

Chlorpyrifos (CPF) is an organophosphate insecticide, widely used in agricultural and domestic settings. Relative to the previously discussed pesticides, less is known about female reproductive effects of CPF. In terms of ovarian exposure, in adult rats exposed to CPF (5 mg/kg/day) for 21 days orally, 0.03% of CPF was distributed to the ovary (Tanvir et al., 2016). Increased oocyte death as a consequence of CPF exposure in a dose-dependent manner was reported in cultured buffalo oocytes, along with reduced oocyte maturation, oocyte nuclear maturation, meiotic progression, fertilization, cleavage, and blastocyst development rates (Nandi et al., 2011). As a mode of action, oxidative stress has been evidenced in the ovary and brain of exposed rats, which was ameliorated by co-exposure to an antioxidant extract from ginger (Abolaji et al., 2017).

Exposure to CPF-methyl (CPM) (1, 10, and 100 mg kg⁻¹ bw day⁻¹) before mating, during mating, gestation and lactation by oral gavage decreased ovarian weight, decreased fertility, the numbers of implantations and the numbers of offspring and there was a higher number of male pups born to females who received 100 mg/Kg. The offspring were exposed until 13 weeks of age via lactation for 3 weeks and then via oral gavage. F1 pups exposed to CPM 100 mg kg⁻¹ in utero and during lactation via milk had reduced body weights. Circulating E₂, T, as well as thyroid hormone (T3 and T4) were reduced and there was a concomitant increased in thyroid-stimulating hormone (TSH) and cholesterol in F1 female rats (Jeong et al., 2006).

Adult rats exposed to CPF (0.1 and 2.5 mg kg⁻¹ day⁻¹) for 8 weeks had alterations to estrous cyclicity, with longer time spent in metestrous. As is the case for MXC, increased ovarian surface epithelium height was noted and there was increased atresia in follicles of CPF-exposed rats. In addition, the uterus had increased surface epithelium and endometrial gland epithelial heights, increased myometrial thickness and increased luminal epithelium height (Nishi and Hundal, 2013).

Gestational CPF exposure altered brain development, specifically glial and neuronal cell numbers in offspring of dams that received exposure (5 mg kg⁻¹ day⁻¹) during gestation day 13–17 (Chen et al., 2017). Epidemiological studies have indicated an association between CPF exposure prenatally in humans and arm tremor later in childhood, indicating prenatal impacts of CPF on the developing nervous system (Rauh et al., 2015). Additionally, behavioral deficits have been demonstrated in the offspring of rats who received exposure to CPF prior to pregnancy, indicating maternal germline transmission of neurological dysfunction (Grabovska and Salyha, 2015). Indeed, there is now a wealth of information supporting that CPF impacts the developing brain. The placenta may also be a CPF target as evidenced by ex vivo experiments on human placenta in which structural alterations induced by CPF were observed (Ridano et al., 2017).

Thus, although the evidence that CPF exposure negatively impacts the developing brain of offspring, the target point at which these impacts initiate are unknown, nor do we appreciate if these effects begin at the gamete level, thus there is a considerable amount of scientific investigation that is lacking on the reproductive effects of CPF.

Summary

ATR, GLY, MXC, and CPF are abundantly used pesticides for which the information on female reproductive effects are limited, though our knowledge in this research area is gaining. All of these chemicals have been detected in human bodily fluids and the scientific literature supports that exposure to these pesticides have reproductive and endocrine outcomes as well as effects on offspring. Whether they all induce epigenetic alterations to the maternal germline to facilitate transgenerational inheritance is unclear currently. There are additional pesticides that are not addressed in this article, and it should be borne in mind that pesticide exposures typically do not occur in isolation, thus combination exposure experiments are warranted to fully understand the impacts on all aspects of female reproductive physiology.

References

- Abolaji, A. O., Ojo, M., Afolabi, T. T., Arowoogun, M. D., Nwawolor, D., & Farombi, E. O. (2017). Protective properties of 6-gingerol-rich fraction from *Zingiber officinale* (ginger) on chlorpyrifos-induced oxidative damage and inflammation in the brain, ovary and uterus of rats. *Chemico-Biological Interactions*, 270, 15–23.
- Akgul, Y., Derk, R. C., Meighan, T., Rao, K. M., & Muroso, E. P. (2008). The methoxychlor metabolite, Hpte, directly inhibits the catalytic activity of cholesterol side-chain cleavage (P450_{sc}) in cultured rat ovarian cells. *Reproductive Toxicology*, 25, 67–75.
- Akgul, Y., Derk, R. C., Meighan, T., Rao, K. M., & Muroso, E. P. (2011). The methoxychlor metabolite, Hpte, inhibits rat luteal cell progesterone production. *Reproductive Toxicology*, 32, 77–84.
- Aoyama, H., Hojo, H., Takahashi, K. L., Shimizu-Endo, N., Araki, M., Takeuchi-Kashimoto, Y., Saka, M., & Teramoto, S. (2012). Two-generation reproduction toxicity study in rats with methoxychlor. *Congenital Anomalies*, 52, 28–41.
- Arbuckle, T. E., Lin, Z., & Mery, L. S. (2001). An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. *Environmental Health Perspectives*, 109, 851–857.
- Barbash, J. E., Thelin, G. P., Kolpin, D. W., & Gilliom, R. J. (2001). Major herbicides in ground water results from the National Water-Quality Assessment. *Journal of Environmental Quality*, 30, 831–845.
- Battaglin, W. A., Meyer, M. T., Kuivila, K. M., & Dietze, J. E. (2014). Glyphosate and its degradation product AMPA occur frequently and widely in U.S. soils, surface water, groundwater, and precipitation. *Journal of the American Water Resources Association*, 50, 275–290.

- Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., & Seralini, G. E. (2007). Time- and dose-dependent effects of roundup on human embryonic and placental cells. *Archives of Environmental Contamination and Toxicology*, 53, 126–133.
- Beuret, C. J., Zirulnik, F., & Gimenez, M. S. (2005). Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses. *Reproductive Toxicology*, 19(4), 501.
- Bhardwaj, J. K., & Saraf, P. (2017). N-acetyl cysteine-mediated effective attenuation of methoxychlor-induced granulosa cell apoptosis by counteracting reactive oxygen species generation in caprine ovary. *Environmental Toxicology*, 32, 156–166.
- Bradley, P. M., Journey, C. A., Romanok, K. M., Barber, L. B., Buxton, H. T., Foreman, W. T., Furlong, E. T., Glassmeyer, S. T., Hladik, M. L., Iwanowicz, L. R., Jones, D. K., Kolpin, D. W., Kuivila, K. M., Loftin, K. A., Mills, M. A., Meyer, M. T., Orlando, J. L., Reilly, T. J., Smalling, K. L., & Villeneuve, D. L. (2017). Expanded target-chemical analysis reveals extensive mixed-organic-contaminant exposure in U.S. streams. *Environmental Science & Technology*, 51, 4792–4802.
- Bremen, M. L. 2013. *Determination of glyphosate residues in human urine samples from 18 european countries*. Report Glyphosate MLHB-2013-06-06
- Brender, J. D., & Weyer, P. J. (2016). Agricultural compounds in water and birth defects. *Current Environmental Health Reports*, 3, 144–152.
- Byer, J. D., Struger, J., Klawunn, P., Todd, A., & Sverko, E. (2008). Low cost monitoring of glyphosate in surface waters using the ELISA method: An evaluation. *Environmental Science & Technology*, 42, 6052–6057.
- Cattani, D., de Liz Oliveira Cavalli, V. L., Heinz Rieg, C. E., Domingues, J. T., Dal-Cim, T., Tasca, C. I., Mena Barreto Silva, F. R., & Zamoner, A. (2014). Mechanisms underlying the neurotoxicity induced by glyphosate-based herbicide in immature rat hippocampus: Involvement of glutamate excitotoxicity. *Toxicology*, 320, 34–45.
- Chen, X. P., Chao, Y. S., Chen, W. Z., & Dong, J. Y. (2017). Mother gestational exposure to organophosphorus pesticide induces neuron and glia loss in daughter adult brain. *Journal of Environmental Science and Health. Part B*, 52, 77–83.
- Cragin, L. A., Kesner, J. S., Bachand, A. M., Barr, D. B., Meadows, J. W., Krieg, E. F., & Reif, J. S. (2011). Menstrual cycle characteristics and reproductive hormone levels in women exposed to atrazine in drinking water. *Environmental Research*, 111, 1293–1301.
- Curvin, B. D., Hein, M. J., Sanderson, W. T., Striley, C., Heederik, D., Kromhout, H., Reynolds, S. J., & Alavanja, M. C. (2007). Urinary pesticide concentrations among children, mothers and fathers living in farm and non-farm households in Iowa. *The Annals of Occupational Hygiene*, 51, 53–65.
- Dallegre, E., Mantese, F. D., Coelho, R. S., Pereira, J. D., Dalsenter, P. R., & Langeloh, A. (2003). The teratogenic potential of the herbicide glyphosate-roundup in Wistar rats. *Toxicology Letters*, 142, 45–52.
- Dalsenter, P. R., DE Araujo, S. L., de Assis, H. C., Andrade, A. J., & Dallegre, E. (2003). Pre and postnatal exposure to endosulfan in Wistar rats. *Human & Experimental Toxicology*, 22, 171–175.
- Daruch, J., Zirulnik, F., & Gimenez, M. S. (2001). Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. *Environmental Research*, 85, 226–231.
- Davis, L. K., Murr, A. S., Best, D. S., Fraitas, M. J. P., Zorrilla, L. M., Narotsky, M. G., Stoker, T. E., Goldman, J. M., & Cooper, R. L. (2011). The effects of prenatal exposure to atrazine on pubertal and postnatal reproductive indices in the female rat. *Reproductive Toxicology*, 32, 43–51.
- Eldridge, J. C., Fleenor-Heyser, D. G., Extrom, P. C., Wetzel, L. T., Breckenridge, C. B., Gillis, J. H., Luempert, L. G., 3rd, & Stevens, J. T. (1994). Short-term effects of chlorotriazines on estrus in female Sprague-Dawley and Fischer 344 rats. *Journal of Toxicology and Environmental Health*, 43, 155–167.
- Foradori, C. D., Sawhney Coder, P., Tisdell, M., Yi, K. D., Simpkins, J. W., Handa, R. J., & Breckenridge, C. B. (2014). The effect of atrazine administered by gavage or in diet on the LH surge and reproductive performance in intact female Sprague-Dawley and long Evans rats. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, 101, 262–275.
- Fraitas, M. J., Cooper, R. L., Buckalew, A., Jayaraman, S., Mills, L., & Laws, S. C. (2009). Characterization of the hypothalamic-pituitary-adrenal axis response to atrazine and metabolites in the female rat. *Toxicological Sciences*, 112, 88–99.
- Gallegos, C. E., Bartos, M., Bras, C., Gumilar, F., Antonelli, M. C., & Minetti, A. (2016). Exposure to a glyphosate-based herbicide during pregnancy and lactation induces neurobehavioral alterations in rat offspring. *Neurotoxicology*, 53, 20–28.
- Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M. C., & Seralini, G. E. (2009). Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. *Toxicology*, 262, 184–191.
- Gely-Pernot, A., Saci, S., Kernanec, P. Y., Hao, C., Giton, F., Kervarrec, C., Tevosian, S., Mazaud-Guittot, S., & Smagulova, F. (2017). Embryonic exposure to the widely-used herbicide atrazine disrupts meiosis and normal follicle formation in female mice. *Scientific Reports*, 7, 3526.
- Giesy, J. P., Dobson, S., & Solomon, K. R. (2000). Ecotoxicological risk assessment for roundup herbicide. *Reviews of Environmental Contamination and Toxicology*, 167, 35–120.
- Goldman, J. M., Davis, L. K., Murr, A. S., & Cooper, R. L. (2013). Atrazine-induced elevation or attenuation of the LH surge in the ovariectomized, estrogen-primed female rat: Role of adrenal progesterone. *Reproduction*, 146, 305–314.
- Grabovska, S., & Salyha, Y. (2015). ADHD-like behaviour in the offspring of female rats exposed to low chlorpyrifos doses before pregnancy. *Arhiv za Higijenu Rada i Toksikologiju*, 66, 121–127.
- Guerrero Schimpf, M., Milesi, M. M., Ingaramo, P. I., Luque, E. H., & Varayoud, J. (2017). Neonatal exposure to a glyphosate based herbicide alters the development of the rat uterus. *Toxicology*, 376, 2–14.
- Harvey, C. N., Chen, J. C., Bagnell, C. A., & Uzumcu, M. (2015). Methoxychlor and its metabolite HPTE inhibit cAMP production and expression of estrogen receptors alpha and beta in the rat granulosa cell in vitro. *Reproductive Toxicology*, 51, 72–78.
- Hecker, M., Hollert, H., Cooper, R., Vinggaard, A. M., Akahori, Y., Murphy, M., Nellesmann, C., Higley, E., Newsted, J., Laskey, J., Buckalew, A., Grund, S., Maletz, S., Giesy, J., & Timm, G. (2011). The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study. *Environmental Science and Pollution Research International*, 18, 503–515.
- Hirshfield, A. N. (1991). Development of follicles in the mammalian ovary. *International Review of Cytology*, 124, 43–101.
- Hoyer, P. B., & Keating, A. F. (2014). Xenobiotic effects in the ovary: Temporary versus permanent infertility. *Expert Opinion on Drug Metabolism & Toxicology*, 10, 511–523.
- Ingaramo, P. I., Varayoud, J., Milesi, M. M., Schimpf, M. G., Muñoz-de-Toro, M., & Luque, E. H. (2016). Effects of neonatal exposure to a glyphosate-based herbicide on female rat reproduction. *Reproduction*, 152, 403–415.
- Jeong, S. H., Kim, B. Y., Kang, H. G., Ku, H. O., & Cho, J. H. (2006). Effect of chlorpyrifos-methyl on steroid and thyroid hormones in rat F0- and F1-generations. *Toxicology*, 220, 189–202.
- Manikkam, M., Haque, M. M., Guerrero-Bosagna, C., Nilsson, E. E., & Skinner, M. K. (2014). Pesticide methoxychlor promotes the epigenetic transgenerational inheritance of adult-onset disease through the female germline. *PLoS One*, 9, e102091.
- Nandi, S., Gupta, P. S., Roy, S. C., Selvaraju, S., & Ravindra, J. P. (2011). Chlorpyrifos and endosulfan affect buffalo oocyte maturation, fertilization, and embryo development in vitro directly and through cumulus cells. *Environmental Toxicology*, 26, 57–67.
- Nishi, K., & Hundal, S. S. (2013). Chlorpyrifos induced toxicity in reproductive organs of female Wistar rats. *Food and Chemical Toxicology*, 62, 732–738.
- Ozden-Akkaya, O., Altunbas, K., & Yagci, A. (2017). Effects of methoxychlor on IGF-I signaling pathway in rat ovary. *Biotechnic & Histochemistry*, 92, 230–242.
- Perego, M. C., Schutz, L. F., Caloni, F., Cortinovis, C., Albonico, M., & Spicer, L. J. (2017). Evidence for direct effects of glyphosate on ovarian function: Glyphosate influences steroidogenesis and proliferation of bovine granulosa but not theca cells in vitro. *Journal of Applied Toxicology*, 37, 692–698.
- Qin, L., Du, Z.-H., Zhu, S.-Y., Li, X.-N., Li, N., Guo, J.-A., Li, J.-L., & Zhang, Y. (2015). Atrazine triggers developmental abnormality of ovary and oviduct in quails (*Coturnix Coturnix coturnix*) via disruption of hypothalamo-pituitary-ovarian axis. *Environmental Pollution*, 207, 299–307.
- Rauh, V. A., Garcia, W. E., Whyatt, R. M., Horton, M. K., Barr, D. B., & Louis, E. D. (2015). Prenatal exposure to the organophosphate pesticide chlorpyrifos and childhood tremor. *Neurotoxicology*, 51, 80–86.
- Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., & Seralini, G. E. (2005). Differential effects of glyphosate and roundup on human placental cells and aromatase. *Environmental Health Perspectives*, 113, 716–720.

- Ridano, M. E., Racca, A. C., Flores-Martin, J. B., Fretes, R., Bandeira, C. L., Reyna, L., Bevilacqua, E., Genti-Raimondi, S., & Panzetta-Dutari, G. M. (2017). Impact of chlorpyrifos on human villous trophoblasts and chorionic villi. *Toxicology and Applied Pharmacology*, 329, 26–39.
- Shalgi, R., Kraicer, P., Rimon, A., Pinto, M., & Soferman, N. (1973). Proteins of human follicular fluid: The blood-follicle barrier. *Fertility and Sterility*, 24, 429–434.
- Stayner, L. T., Almberg, K., Jones, R., Graber, J., Pedersen, M., & Turyk, M. (2017). Atrazine and nitrate in drinking water and the risk of preterm delivery and low birth weight in four Midwestern states. *Environmental Research*, 152, 294–303.
- Struger, J., Thompson, D., Staznik, B., Martin, P., McDaniel, T., & Marvin, C. (2008). Occurrence of glyphosate in surface waters of southern Ontario. *Bulletin of Environmental Contamination and Toxicology*, 80, 378–384.
- Symonds, D. A., Tomic, D., Miller, K. P., & Flaws, J. A. (2005). Methoxychlor induces proliferation of the mouse ovarian surface epithelium. *Toxicological Sciences*, 83, 355–362.
- Symonds, D. A., Merchenthaler, I., & Flaws, J. A. (2008). Methoxychlor and estradiol induce oxidative stress DNA damage in the mouse ovarian surface epithelium. *Toxicological Sciences*, 105, 182–187.
- Tanvir, E. M., Afroz, R., Chowdhury, M., Gan, S. H., Karim, N., Islam, M. N., & Khalil, M. I. (2016). A model of chlorpyrifos distribution and its biochemical effects on the liver and kidneys of rats. *Human & Experimental Toxicology*, 35, 991–1004.
- Tinfo, N. S., Hotchkiss, M. G., Buckalew, A. R., Zorrilla, L. M., Cooper, R. L., & Laws, S. C. (2011). Understanding the effects of atrazine on steroidogenesis in rat granulosa and H295R adrenal cortical carcinoma cells. *Reproductive Toxicology*, 31, 184–193.
- Varayoud, J., Durando, M., Ramos, J. G., Milesi, M. M., Ingaramo, P. I., Munoz-de-Toro, M., & Luque, E. H. (2017). Effects of a glyphosate-based herbicide on the uterus of adult ovariectomized rats. *Environmental Toxicology*, 32, 1191–1201.
- Walsh, L. P., McCormick, C., Martin, C., & Stocco, D. M. (2000). Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. *Environmental Health Perspectives*, 108, 769–776.
- Zachow, R., & Uzumcu, M. (2006). The methoxychlor metabolite, 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane, inhibits steroidogenesis in rat ovarian granulosa cells in vitro. *Reproductive Toxicology*, 22, 659–665.
- Zama, A. M., & Uzumcu, M. (2013). Targeted genome-wide methylation and gene expression analyses reveal signaling pathways involved in ovarian dysfunction after developmental EDC exposure in rats. *Biology of Reproduction*, 88, 52.

Ovarian Toxicity of Polycyclic Aromatic Hydrocarbons

Ulrike Luderer, University of California, Irvine, Irvine, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

AHR Aryl hydrocarbon receptor
AKT AKT serine–threonine kinase (also called Protein Kinase B)
ANF Alpha-naphthoflavone
BaP Benzo[*a*]pyrene
BAX BCL-2 associated X
BPDE BaP-7,8-diol-9,10-epoxide
CYP Cytochrome P450
DBC Dibenzo[*def,p*]chrysene (also known as dibenzo[*a,l*]pyrene)
DMBA 7,12-Dimethyl-benz[*a*]anthracene
DMBA-DHD DMBA-3,4-diol-1,2-epoxide
EC50 Effective concentration 50%
ED50 Effective dose 50%
FSH Follicle stimulating hormone
GD Gestational day
GSH Glutathione
i.p. Intraperitoneal
LH Luteinizing hormone
3-MC 3-Methylcholanthrene
PAH Polycyclic aromatic hydrocarbon
PI3K Phosphatidylinositol-3-kinase
PND Post-natal day
ROS Reactive oxygen species
Tp53 Tumor protein 53 (also known as p53)

Structure and Metabolism of PAHs

Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more benzene rings with or without hydrocarbon side chains. The structure of BaP, a representative PAH, is shown in [Fig. 1](#). PAHs are formed during incomplete combustion of organic materials, for example, when tobacco, wood or other organic materials are burned, when foods are grilled or smoked, and when fossil fuels are combusted. People are mainly exposed to PAHs by inhalation of polluted air or tobacco smoke and by ingestion of grilled or smoked foods.

Metabolism of BaP as a representative PAH is shown in [Fig. 1](#). PAHs like BaP generally require metabolism to reactive metabolites to exert toxicity. The best studied metabolic activation pathway is the bay region diol epoxide pathway, which begins with oxidation by cytochrome P450 enzymes (CYPs) to arene oxides, which are then hydrolyzed by microsomal epoxide hydrolase to dihydrodiols, which undergo another CYP-catalyzed oxidation to form diol epoxides, such as BaP-7,8-diol-9,10-epoxide (BPDE) ([Xue and Warshawsky, 2005](#)). Radical cations of PAHs are formed via one electron oxidation catalyzed by cytochrome P450 and peroxidases ([Cavalieri and Rogan, 1995](#)). Diol epoxides and radical cations are highly reactive and form DNA adducts, which can result in mutations and carcinogenesis. In the aldo–keto reductase pathway, the dihydrodiol is oxidized by dihydrodiol dehydrogenases (aldo–keto reductases) to a ketol, which spontaneously rearranges to a catechol, which undergoes one electron oxidation to *o*-semiquinone anion radical, producing hydrogen peroxide, and a second one electron oxidation to *o*-quinone, producing superoxide anion radical ([Penning et al., 1996; Penning, 2004](#)). The *o*-quinone can undergo one or two electron reduction, setting up a redox cycle ([Penning et al., 1996; Penning, 2004](#)). The generation of ROS in this pathway results in oxidative DNA damage, which can result in mutations if not repaired ([Xue and Warshawsky, 2005](#)). Damage to DNA and other cellular macromolecules has been implicated in the ovarian toxicity of PAHs, as will be discussed in this chapter. The enzymes required for metabolic activation of PAHs are expressed in the ovary ([Cannady et al., 2002; Shimada et al., 2003; Rajapaksa et al., 2007](#)).

Detoxification of reactive metabolites of PAHs occurs via Phase II biotransformation pathways. Conjugation with GSH catalyzed by glutathione transferases (GSTs) is an important detoxification mechanism for quinone and diol epoxide metabolites, and glucuronidation and sulfation are important detoxification mechanisms for phenol, quinone, and dihydrodiol metabolites

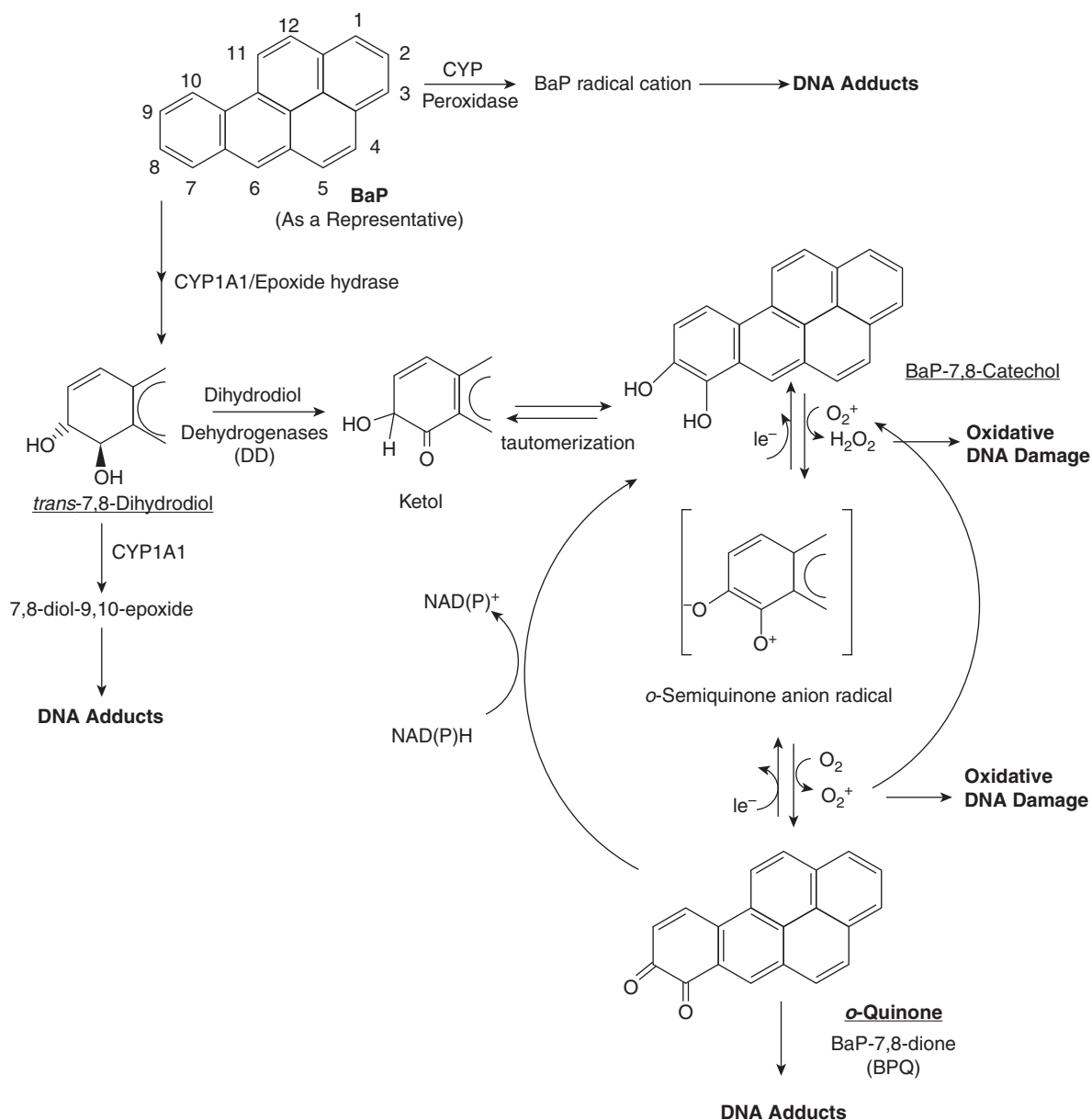


Fig. 1 Metabolic activation of BaP. Key enzymes involved in formation of reactive metabolites that can react with DNA, forming mutagenic adducts include the CYPs CYP1A1, CYP1B1, and CYP1A2, epoxide hydrase, and aldo-keto reductases. The aldo-keto reductase metabolic pathway generates reactive oxygen species, H_2O_2 and $SO^{\bullet-}$.

(Swanson et al., 1986; Romert et al., 1989; ATSDR, 1995; Jernström et al., 1996; Seidel et al., 1998; Fang et al., 2002). Metabolism of PAHs by CYPs to mono-hydroxylated phenols, such as 1-, 3-, 5-, 7-, and 9-hydroxy-BaP, is considered a detoxification pathway, as these compounds do not undergo further transformation to reactive metabolites (Hecht et al., 2006). These mono-hydroxylated metabolites also undergo Phase II biotransformation and are then excreted.

PAHs enhance their own metabolic activation by binding to the ligand activated transcription factor, the aryl hydrocarbon receptor (AHR) (Shimizu et al., 2000; Denison and Nagy, 2003). AHR activation has been implicated in the ovarian toxicity of PAHs, as will also be discussed in this chapter.

Ovarian Effects of PAHs in Peripubertal Animals and Adult Animals

More than 50 years ago, it was discovered that exposure to the PAHs BaP, DMBA, and 3-MC by multiple routes induced ovarian tumors in rodents (Marchant, 1960; Mody, 1960; Biancifiori et al., 1961; Krarup, 1967). More recently, DBC was also found to

be a potent ovarian carcinogen (Buters et al., 2002; Chen et al., 2012). The initial investigators noted that ovarian atrophy, with few or no follicles remaining in the ovaries, preceded tumor formation. Detailed dose-response and time course analyses of follicle destruction by single i.p. doses of PAHs were subsequently conducted in peripubertal mice and rats (Mattison, 1979, 1980; Mattison and Thorgeirsson, 1979; Mattison et al., 1980). A strong species difference in sensitivity to primordial/primary follicle destruction was noted with mice being considerably more sensitive than rats (Mattison, 1979). Among mouse strains tested, C57BL/6 was more sensitive than DBA/2 N (Mattison and Thorgeirsson, 1979). Fifty percent of primordial follicles were destroyed in C57BL/6 mice at doses of 25 mg/kg BaP and 3MC and 1 mg/kg DMBA, while in DBA/2 N mice, 50% of primordial follicles were destroyed at 500 mg/kg BaP and 3MC and 10 mg/kg DMBA (Mattison and Thorgeirsson, 1979). No significant depletion of primordial follicle numbers was observed after dosing with 80 mg/kg of the lower molecular weight PAH pyrene (Mattison and Thorgeirsson, 1979). A more recent study compared the effects of repeated lower daily doses of DMBA, BaP, or 3-MC for 15 days on ovarian follicle numbers in peripubertal B6C3F1 mice and Fisher 344 rats (Borman et al., 2000). Similar to findings with single higher doses, mice were more sensitive than rats (Borman et al., 2000). Again, DMBA was the most potent destroyer of primordial follicles, followed by 3-MC and BaP. Differences were noted among the three PAHs in terms of which follicles were targeted, with BaP and 3-MC targeting only primordial and primary follicles, while DMBA in addition targeted granulosa cells of secondary and antral follicles (Mattison, 1980). With repeated dosing, depletion of primordial follicles results in eventual depletion of follicles at more mature stages of development (Borman et al., 2000).

Intraperitoneal injection of prepubertal mice with 50 mg/kg DMBA induced ovarian expression of the proapoptotic gene *Bax* and increased BAX protein in oocytes of primordial and primary follicles 24 h after injection (Matikainen et al., 2001). Similar increases in BAX protein expression were observed in xenografts of human fetal ovaries implanted under the kidney capsules of immunodeficient mice 24 h after a single i.p. dose of 50 mg/kg DMBA, and 76% of primordial follicles in the human ovarian grafts were dead or dying at 6 days after dosing, showing that human follicles are also sensitive to PAHs (Matikainen et al., 2001). The same group observed that the proapoptotic BCL-2 family gene *Bid* is induced in the ovary of prepubertal mice 24 h after i.p. injection of 50 mg/kg DMBA; however, *Bid*^{-/-} mice were equally sensitive to follicle depletion by DMBA as wild type mice (Pru et al., 2009). In contrast, expression of the tumor suppressor *Tp53* was also induced in the ovaries, and *Tp53*^{-/-} mice were completely resistant to follicle depletion by DMBA (Pru et al., 2009). The p53 protein responds to DNA damage by initiating repair pathways and if the damage is too great, initiating apoptosis (Zhou and Elledge, 2000).

Few studies have examined the effects of PAH exposure on ovarian follicle numbers in older adult rodents. Adult 8-month old mice showed similar (DBA/2N) or somewhat lower (C57BL/6) sensitivity to primordial follicle depletion by a single 80 mg/kg dose of DMBA or 3-MC compared to peripubertal mice (Mattison and Thorgeirsson, 1979). Corpora lutea numbers were dose-dependently decreased at 1 week after a single i.p. dose of BaP in C57BL/6N mice, with 50% depletion at a dose of 1.6 mg/kg (Swartz and Mattison, 1985). Corpora lutea were completely absent 1 week after doses of 50, 100 and 500 mg/kg, and even at 4 weeks, no corpora lutea were present in ovaries of mice dosed with 500 mg/kg (Swartz and Mattison, 1985). These results are consistent with lack of ovulation due to destruction of antral follicles or derangement of hypothalamic-pituitary-ovarian axis signaling. Further evidence that BaP acutely prevents ovulation comes from a study in which 7-week old female F344 rats were exposed by inhalation to BaP doses of 25–100 µg/m³ for 4 h/day, for 14 days, then paired with unexposed males (Archibong et al., 2012). Number of oocytes ovulated and litter size were significantly decreased and estrous cycle length was prolonged in the 100 µg/m³ group only (Archibong et al., 2012).

The above in vivo studies do not provide information about whether the parent compound or a metabolite is the ultimate ovotoxicant or whether metabolism occurs in the ovary or elsewhere. To address these questions, intraovarian injection of BaP and several of its metabolites was performed in peripubertal C57BL/6N, DBA/2N, and B6D2F1 mice; primordial and primary follicle counts were compared in the injected versus contralateral uninjected ovary (Takizawa et al., 1984). BaP and BaP diol were approximately equipotent within mouse strain, with C57BL/6N mice more sensitive than DBA/2N mice. BPDE was much more potent and was equipotent in all three strains. Together these data are consistent with BPDE being the ultimate ovotoxicant and with intraovarian metabolism of BaP to BPDE being most efficient in C57BL/6 mice. It is possible that the greater sensitivity of mice to the ovarian toxicity of PAHs compared to rats may be due to more efficient metabolism in the murine versus the rat ovary, but this has not been tested directly.

Ovarian Effects of PAHs in Neonatal Rodents

The ovarian effects of neonatal exposure to PAHs have been examined in several in vivo studies. Neonatal Swiss mice dosed i.p. from PND 4–10 with 1 mg/kg/day DMBA had about 50% fewer total follicles per section, decreased percentage of primordial follicles and increased percentages of activating (transitional between primordial and primary), primary, and secondary follicles (Sobinoff et al., 2011). Neonatal Swiss mice were dosed i.p. from PND 4–10 with 0, 1.5 or 3 mg/kg BaP and euthanized 24 h after the last dose (Sobinoff et al., 2012). Primordial follicle percentages were significantly and dose-dependently decreased and activating follicles increased by both BaP doses, while primary and secondary follicles were significantly increased and total follicles per section were decreased by about 50% only at the higher dose (Sobinoff et al., 2012). Thus, the sensitivity of neonatal mice to primordial follicle depletion by these PAHs appears to be similar to that of peripubertal mice (Borman et al., 2000).

Effects of PAHs in Cultured Neonatal Ovaries and Follicles

The mechanisms by which PAHs destroy follicles have been examined in follicle and ovary culture models. These studies suggest that the mechanisms differ for primordial compared to growing follicles.

Cultured Neonatal Ovaries

Because isolated primordial follicles do not survive in culture, the cultured neonatal (PND 3–4) ovary model, which contains almost exclusively primordial follicles, is often used to examine the effects of toxicants on primordial follicles. Neonatal rat and mouse ovaries can be cultured for at least 15 days, during which time some primordial follicles are activated into the growing pool, developing into primary and secondary follicles (Devine et al., 2002, 2010). Both DMBA and BaP have been shown to deplete primordial follicles in this culture system.

Consistent with the *in vivo* studies in peripubertal rodents, cultured neonatal mouse ovaries are more sensitive than rat ovaries to destruction by PAHs. Culture of B6C3F1 neonatal mouse ovaries with 12.5–1000 nM DMBA for 15d depleted primordial and primary follicles, with significant decreases at ≥ 12.5 and ≥ 25 nM DMBA, respectively (Rajapaksa et al., 2007). Time-course analyses showed that healthy primordial and primary follicle numbers began to decline after 6 h in culture with 1000 nM, and no healthy primordial or primary follicles remained after 8d of culture (Rajapaksa et al., 2007). Morphological changes consistent with apoptotic death were observed in oocytes and granulosa cells of primordial and primary follicles (Rajapaksa et al., 2007). Culture of F344 neonatal rat ovaries with 12.5–1000 nM DMBA or DMBA-DHD for 15d dose-dependently depleted primordial and primary follicles, but significant decreases occurred at ≥ 75 and ≥ 372 nM DMBA and ≥ 12.5 and ≥ 75 nM DMBA-DHD respectively (Igawa et al., 2009). Time-course analyses showed that healthy primordial and primary follicle numbers began to decline after 2d and 4d in culture with 1000 nM, respectively (Igawa et al., 2009). DMBA treatment increased microsomal epoxide hydrolase protein expression in oocytes of primordial and primary follicles and in both rat and mouse ovaries, and treatment with a microsomal epoxide hydrolase inhibitor was protective against DMBA, but not DMBA-DHD (Rajapaksa et al., 2007; Igawa et al., 2009). The greater potency of DMBA-DHD, as well as the protective effect of the microsomal epoxide hydrolase inhibitor, support earlier conclusions from *in vivo* studies (Takizawa et al., 1984) that PAH diol epoxide metabolites are the ultimate ovotoxicants. To examine effects of DMBA on large primary follicles and secondary follicles, neonatal rat ovaries were treated with 12.5 or 75 nM DMBA for 1 day and analyzed 1, 2, 4, or 8 days later (Madden et al., 2014). Primordial and small primary follicle numbers were unaffected, but large primary follicles and secondary follicles were decreased at 8 days after both doses and secondary follicles were decreased at 8 days after 12.5 nM (Madden et al., 2014).

The molecular mechanisms by which PAHs deplete primordial follicles have been examined in the cultured neonatal ovary. Culture of neonatal Swiss mouse ovaries for 4 days with 50 nM DMBA increased caspase 2 and 3 immunostaining and TUNEL labeling in granulosa cells of primary and secondary follicles, but not primordial follicles, consistent with induction of apoptosis in growing follicles, but not in primordial follicles (Sobinoff et al., 2011). In contrast, DMBA treatment increased AKT phosphorylation in oocytes of primordial follicles and significantly modulated phosphorylation of mTOR and expression of FOXO3A in primordial follicle oocytes, two downstream targets of phosphorylated AKT (Sobinoff et al., 2011). Microarray analysis of whole neonatal ovaries after 4d dosing showed upregulation of immune response signaling and follicular growth and development pathways, including mTOR, integrin-linked kinase, EIF2, and VEGF (Sobinoff et al., 2011). Similar results were reported by the same group for BaP. Microarray analysis identified alterations in expression of genes associated with follicular growth and development, DNA repair and follicular atresia, AHR signaling, and oxidative stress in ovaries cultured for 4 d with 1000 nM BaP (Sobinoff et al., 2012). Immunofluorescence of cultured ovaries showed increased PCNA protein in granulosa and oocyte nuclei of primordial follicles, consistent with activation, and increased activated caspases 2 and 3 and TUNEL in primary and secondary follicles, consistent with apoptosis, of BaP treated ovaries (Sobinoff et al., 2012). Taken together these results suggest that BaP and DMBA deplete follicles in the neonatal ovary by stimulating activation of primordial follicles and initiating apoptosis in primary and secondary follicles. PI3K is a positive upstream regulator of AKT signaling, which increases phosphorylation of AKT by phosphoinositide-dependent kinase 1 (PDK1). Therefore, the conclusions of Sobinoff and coworkers would lead one to predict that PI3K inhibition should protect against follicle depletion by DMBA. However, in cultured neonatal F344 rat ovaries, PI3K inhibitor enhanced depletion of both primordial and primary follicles by 1000 nM DMBA (Keating et al., 2009). One way to reconcile these apparently divergent findings is to posit that DMBA acts downstream of PI3K to activate AKT signaling. Alternatively, upregulation of AKT signaling may be a late effect that occurs after primordial follicles have died.

An earlier study, which compared DMBA and its metabolite DMBA-DHD, reported direct induction of apoptosis of primordial follicles in cultured neonatal ovaries (Matikainen et al., 2001). PND 4 mouse ovaries were cultured for 12, 24, 48, or 72 h with 1 μ M DMBA or 0.01, 0.1, or 1 μ M DMBA-DHD with or without the AHR antagonist, ANF. BAX protein was increased in oocytes of primordial follicles by 24 h, and this increase was blocked by ANF. Histologically apoptotic primordial and primary follicles were evident at 48 and 72 h; 1 μ M DMBA caused 50% and 0.1 μ M DMBA-DHD caused more than 75% depletion of healthy primordial follicles by 72 h, while no healthy primordial follicles were found in ovaries exposed to 1 μ M DMBA-DHD at 72 h (Matikainen et al., 2001). The requirement for AHR activation and BAX induction was further supported by showing that cultured *Ahr*^{−/−} or *Bax*^{−/−} PND4 ovaries were completely resistant to follicle depletion by 0.1 μ M DMBA-DHD. To test whether activation of the *Bax* promoter by DMBA involves AHR, oocytes from PND 12 small secondary follicles were microinjected with a *Bax*-promoter GFP reporter; 68% of wild type oocytes that were exposed to 1 μ M DMBA-DHD demonstrated *Bax* promoter activation, compared to

only 16% of similarly treated *Ahr*^{−/−} oocytes (Matikainen et al., 2001). Mutation of the two AHR response elements in the *Bax* promoter abolished the ability of DMBA-DHD to activate the promoter.

Cultured Growing Follicle

The effects of PAHs on follicular growth and apoptosis have been studied in cultured, isolated secondary and preovulatory mouse and rat follicles. BaP concentration-dependently inhibited FSH-stimulated growth and estradiol production of secondary follicles from PND 22 rats cultured for 5 days, with all concentrations, including the lowest of 1.5 ng/mL (6 nM) significantly inhibiting growth (Neal et al., 2007). The same group reported that BaP also concentration- and time-dependently inhibited in vitro maturation to the antral stage of small secondary follicles from PND 13 mice cultured for up to 12 days with LH and FSH (Sadeu and Foster, 2011). The lowest effective concentration was 45 ng/mL (180 nM). Oocyte nuclear maturation in response to hCG after 12 days of culture was not affected by BaP (Sadeu and Foster, 2011). BaP at 180 nM, but not lower, concentrations upregulated follicular mRNA expression of *Cyp11a1* and *Cyp11b1* at 8 and 12 days of culture, respectively, consistent with AHR activation (Sadeu and Foster, 2013). Expression of the proapoptotic genes *Bax* and *Hsp90ab1* was increased after 12, but not 8, days of culture (Sadeu and Foster, 2013). Levels of BPDE and BaP-7,8-quinone DNA adducts and the oxidative damage markers 8-OH-dG and 8-isoprostane increased in a BaP concentration dependent manner in follicles cultured for 13 days (Yao et al., 2017). No direct markers of apoptosis were examined in the preceding studies. DMBA concentration-dependently induced apoptosis in preovulatory Sprague–Dawley rat follicles cultured in the presence of FSH for up to 48 h; the lowest effective concentration was 1 μM (Tsai-Turton et al., 2007). ROS were increased after 12 h, followed by increased numbers of BAX-positive granulosa and theca cells at 24 and 48 h, and increased numbers of activated caspase 3 and TUNEL-positive granulosa and theca cells at 48 h (Tsai-Turton et al., 2007). Treatment with glutathione ethyl ester, a cell permeable form of GSH, protected against and depletion of GSH with buthionine sulfoximine enhanced the induction of apoptosis by DMBA. Taken together, these studies are consistent with inhibition of follicular growth and maturation and induction of oxidative stress and apoptosis of secondary and antral follicles by PAHs.

Effects of PAHs on Oocyte Quality

Oral administration of 13 mg/kg BaP to peripubertal mice resulted in DNA damage measured by the alkaline comet assay in ovulated oocytes 4 and 6 days after dosing and in cumulus cells at 2, 4, and 6 days; levels had returned to baseline at 15 days (Einaudi et al., 2014). BPDE DNA adducts in cumulus cells were significantly elevated 2, 4, and 6 days after exposure, but not at 15 and 22 days (Einaudi et al., 2014). Oocytes ovulated 4 and 6 days after exposure would have been enclosed in large secondary and small antral follicles at the time of exposure, while oocytes ovulated at 15 and 22 days would have been in primary and primordial follicles at the time of exposure and may have been more likely to have undergone atresia as a result of DNA damage. A study by another group showed that surviving oocytes that had been exposed at the quiescent primordial follicle stage had indicators of decreased oocyte quality. Postnatal mice were dosed i.p. from PND 4–10 with 0, 1.5, or 3 mg/kg BaP and were superovulated 6 week. after the last dose. Oocyte mitochondrial superoxide anion levels and oxidized lipid were dose-dependently increased, while in vitro sperm-egg binding was dose-dependently decreased (Sobinoff et al., 2012).

Effects of PAH Exposure During Prenatal Ovarian Development

Germ Cell Depletion In Vivo

Germ cells in the developing ovary that are not yet enclosed in follicles appear to be even more highly sensitive to destruction by PAHs than follicle-enclosed oocytes. Studies summarized below collectively support that induction of apoptosis of germ cells by PAHs requires AHR activation, which induces metabolism to reactive metabolites and increases expression of *Tp53*, leading to apoptosis initiation via expression of proapoptotic BCL-2 family members *Hrk* and *Bax* and activation of caspases (Fig. 2). Furthermore, Phase II metabolism and ROS detoxification by glutathione are important defenses against germ cell depletion in the developing ovary.

Oral dosing of CD-1 mice with 0, 10, 40, or 160 mg/kg BaP from gestational day 7 to 16, spanning the critical windows of primordial germ cell (PGC) formation, migration to the gonadal ridge, proliferation during migration and in the ovary, and entry into meiosis, resulted in dose-dependently decreased fertility in the F1 female offspring; all females in the 40 and 160 mg/kg groups were infertile (MacKenzie and Angevine, 1981). Very few large follicles or corpora lutea were observed in the ovaries of F1 females exposed to 10 mg/kg/day, while ovaries from the 40 and 160 mg/kg/day groups were absent or very small (MacKenzie and Angevine, 1981). Using the same dosing route and dosing window, F1 offspring of C57BL/6J female mice dosed with 2 mg/kg/day BaP, had 81% fewer primordial follicles and 66% fewer follicles of all stages than control F1 females at 6 week. of age (Luderer et al., 2017).

Depletion of fetal ovarian germ cells by DMBA requires the proapoptotic BCL-2 family protein BAX. Pregnant mice were treated with 1 mg/kg DMBA or vehicle i.p. on gestational day 14.5, and ovaries of female offspring were analyzed on PND4 (Matikainen et al., 2002). Healthy primordial follicle numbers were depleted by more than 80% in wild type mice exposed to DMBA in utero, while primordial follicle numbers were not decreased by DMBA in *Bax*^{−/−} mice (Matikainen et al., 2002).

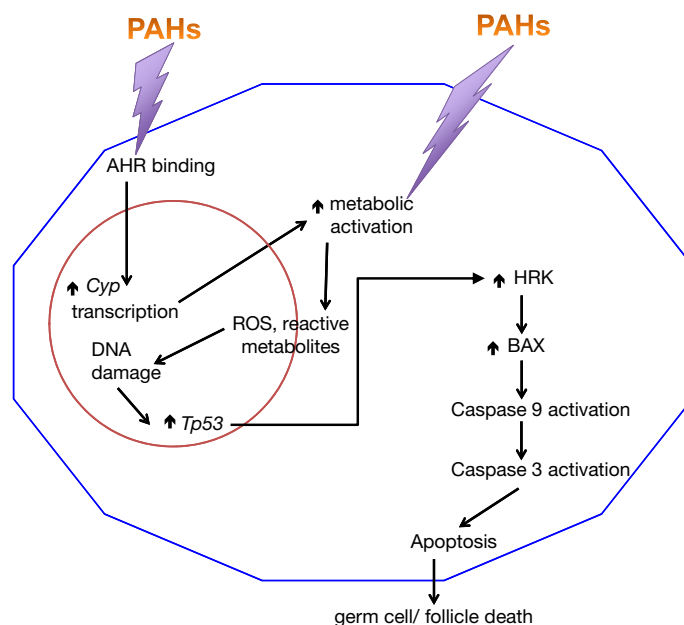


Fig. 2 Model of activation of apoptosis in ovarian germ cells by PAHs. The model is a composite of results from in vivo and in vitro studies of prenatal and postnatal exposure to PAHs or their metabolites. PAHs are metabolically activated via the pathways shown in Fig. 1 in ovarian germ cells (blue polygon). Metabolism is enhanced by PAH binding to the AHR, which activates this transcription factor, causing it to translocate to the nucleus (red circle), where it increases transcription of CYPs that metabolize PAHs, as well as transcription of the proapoptotic gene *Bax*. Reactive metabolites and ROS cause DNA damage which increases *Tp53* expression. TP53 activates apoptosis by increasing proapoptotic BCL-2 family proteins, including HRK and BAX, leading to caspase activation, and ultimately apoptotic cell death of germ cells in the prenatal ovary or primordial follicles in the postnatal ovary.

Consistent with the important role of GSH in Phase II detoxification of PAH metabolites and in reduction of ROS generated as a result of PAH metabolism, embryos deficient in GSH due to deletion of the modifier subunit of the rate limiting enzyme in its synthesis (*Gclm*^{−/−} mice) were more sensitive to germ cell destruction by prenatal exposure to BaP. Oral gavage from GD 7–16 with 0, 2 or 10 mg/kg/d BaP of pregnant *Gclm*^{+/−} mice mated with *Gclm*^{+/−} males dose-dependently decreased fertility and ovarian follicle numbers in their F1 female offspring, with the *Gclm*^{−/−} offspring significantly more affected than their wild type littermates (Lim et al., 2013).

Ovarian follicle numbers were decreased in 3 week old offspring of C57BL/6 mothers treated preconception and/or during lactation with a mixture of 1 mg/kg BaP and 1 mg/kg DMBA in corn oil administered s.c. once a week for 3 week, followed by mating with an untreated male and/or administered on PND3 and then twice more at 1 week intervals (Jurisicova et al., 2007). Primordial and primary follicles were depleted by about 40% after either preconceptional or lactational dosing and by about 70% after treatment during both windows, and this was prevented by cotreatment with the AHR-antagonist resveratrol. Protein and mRNA expression of the BH3-only BCL-2 family member Harikiri (*Hrk*) were upregulated 24 h after culture of neonatal ovaries with 1 μM BaP and DMBA, and resveratrol prevented upregulation of *Hrk*. No significant depletion of follicles was observed in *Ahr*^{−/−} offspring of *Ahr*^{+/−} mothers treated in the same manner during both preconceptional and lactational windows, nor was *Hrk* upregulated. Upregulation of *Bax* by preconceptional and lactational administration of the PAH mixture was absent in *Hrk*^{−/−} offspring. The authors stated that the preconceptional dosing regimen did not result in any exposures during gestation. However, others have shown that PAH metabolites persist in offspring tissues for more than 2 weeks after oral or inhalation dosing (Wu et al., 2003; Archibong et al., 2012; Jules et al., 2012). Therefore, the preconceptional treatment most likely resulted in gestational exposure as well as preconceptional exposure, and one cannot exclude direct effects on the developing germ cells.

Germ Cell Depletion in Cultured Embryonic Ovaries

Cultured E13.5 mouse ovaries with attached mesonephros are an excellent model system in which to study the mechanisms of toxicity of PAHs in the embryonic ovary. E13.5 corresponds to the onset of entry of oogonia into meiosis. These studies support that germ cell destruction by PAHs at this developmental stage is apoptotic, requiring AHR-mediated increased BAX expression and caspase activation.

Culture of E13.5 ovaries with 0.01–1 μM DMBA-DHD for 72 h concentration-dependently depleted germ cells, and germ cell depletion was prevented by the AHR antagonist ANF (Matikainen et al., 2002). DMBA-DHD upregulated BAX protein in germ cells at 24 h, prior to any morphological indicators of apoptosis, and ANF also prevented the increase in BAX. Culture of E13.5 ovaries with 50–1000 ng/mL (0.2–4 μM) BaP, concentration-dependently increased BAX protein levels in germ cells at 6 h, followed at 24 h

by germ cell activation of caspase 3 and caspase 9, and at 48 h by decreased germ cell numbers (Lim et al., 2016). Treatment with a pan caspase inhibitor completely prevented germ cell depletion by BaP.

Culture of human gestational week 8–9 ovary-mesonephros complexes with 1 μ M DMBA-DHD for 7d did not significantly alter the percentage of apoptotic, activated caspase 3 positive germ cells, but decreased the percentage of proliferating, phospho-histone H3-positive germ cells (Anderson et al., 2014). Germ cell numbers were not affected, but the counts were expressed per unit area rather than per ovary, which may have obscured an effect on germ cell number. Oogonia are still proliferating and have not begun to enter meiosis during weeks 8–9 of human pregnancy. This may explain the different results observed in this experiment compared to the studies that used cultured E13.5 mouse ovaries. Alternatively, apoptotic cells may have already been cleared after 7 days of culture, and no earlier time points were examined.

Transplacental Carcinogenesis

As noted above, exposure of adult rodents to PAHs causes ovarian tumors later in life. Prenatal development appears to be an even more sensitive window for ovarian tumorigenesis induction by PAHs. Exposure from GD 7–16 via oral treatment of pregnant *Gclm*+/- mice with BaP dose-dependently depleted ovarian germ cells and induced ovarian tumors in the F1 female offspring, with more severe effects in *Gclm*-/- than *Gclm*+/+ offspring (Lim et al., 2013). All of the 10 mg/kg/d *Gclm*-/- offspring had bilateral tumors, while 50% of the 10 mg/kg/d *Gclm*+/+ offspring had unilateral tumors and 33% had bilateral tumors. In the 2 mg/kg/d exposed groups, no *Gclm*+/+ offspring had tumors, while 11% of *Gclm*-/- females had a unilateral tumor (Lim et al., 2013). No tumors in other organs were noted at these doses of BaP. Two other PAHs, DMBA and DBC have also been found to be transplacental ovarian tumorigens at doses that cause tumors in other organs. A single oral dose of 60 mg/kg DMBA at any of GDs 6, 8, and 10–20 induced malignant ovarian tumors later in life in 30%–80% of exposed offspring, with peak sensitivity on GDs 8–13 (Goerttler et al., 1981). Exposure of pregnant mice to a single oral dose of 15 mg/kg DBC on GD 17 induced ovarian tumors in about 18% of the female offspring, while four doses of 3.75 mg/kg on GD 5, 9, 13, and 17 induced ovarian tumors in about 50% (Shorey et al., 2012). These studies suggest that the window of greatest sensitivity for transplacental ovarian tumorigenesis is the embryonic period prior to entry of germ cells into meiosis.

Conclusions

The PAHs BaP, DMBA, and 3MC are potent ovarian toxicants at all developmental stages—embryonic, neonatal, peripubertal, and adult. They are metabolized to reactive metabolites that destroy germ cells in the developing ovary and primordial follicles postnatally by activation of AHR, induction of BAX- and caspase-dependent apoptosis, resulting in premature ovarian follicle depletion, reproductive senescence, and ovarian tumors (Fig. 2). These PAHs also destroy follicles at later stages of development and acutely prevent ovulation. These data from experimental studies with PAHs are consistent with epidemiological data on the effects of smoking in women, which show that smoking is associated with early menopause, decreased fecundity, and ovarian cancer in women smokers and with decreased fecundity and earlier age at menopause in daughters exposed in utero.

References

- Anderson, R. A., McIlwain, L., Coutts, S. M., Kinnell, H. L., Fowler, P. A., & Childs, A. J. (2014). Activation of the aryl hydrocarbon receptor by a component of cigarette smoke reduces germ cell proliferation in the human fetal ovary. *Molecular Human Reproduction*, 20(1), 42–48.
- Archibong, A. E., Ramesh, A., Inyang, F., Niaz, M. S., Hood, D. B., & Kopsombut, P. (2012). Endocrine disruptive actions of inhaled benzo[a]pyrene on ovarian function and fetal survival in fisher F344 adult rats. *Reproductive Toxicology*, 34, 635–643.
- ATSDR. (1995). *Toxicological profile for polycyclic aromatic hydrocarbons*. Atlanta, GA: US Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry.
- Biancifiori, C., Bonser, G. M., & Caschera, F. (1961). Ovarian and mammary Tumours in intact C3Hb virgin mice following a limited dose of four carcinogenic chemicals. *British Journal of Cancer*, 15, 270–283.
- Borman, S. M., Christian, P. J., Sipes, I. G., & Hoyer, P. B. (2000). Ovotoxicity in female Fischer rats and B6 mice induced by low-dose exposure to three polycyclic aromatic hydrocarbons: Comparison through calculation of an Ovotoxic index. *Toxicology and Applied Pharmacology*, 167, 191–198.
- Buters, J. T. M., Mahadevan, B., Quintanilla-Martinez, L., Gonzalez, F. J., Greim, H., Baird, W. M., & Luch, A. (2002). Cytochrome P450 1B1 determines susceptibility to dibenzo[a,h]pyrene-induced tumor formation. *Chemical Research in Toxicology*, 15, 1127–1135.
- Cannady, E. A., Dyer, C. A., Christian, P. J., Sipes, I. G., & Hoyer, P. B. (2002). Expression and activity of microsomal epoxide hydrolase in follicles isolated from mouse ovaries. *Toxicological Sciences*, 68(1), 24–31.
- Cavallieri, E. L., & Rogan, E. G. (1995). Central role radical cations in metabolic activation of polycyclic aromatic hydrocarbons. *Xenobiotica*, 25(7), 677–688.
- Chen, K.-M., Zhang, S.-M., Aliaga, C., Sun, Y.-W., Cooper, T., Gowdahlall, K., Zhu, J., Amin, S., & El-Bayoumy, K. (2012). Induction of ovarian cancer and DNA adducts by dibenzo[a,h]pyrene in the mouse. *Chemical Research in Toxicology*, 25, 374–380.
- Denison, M. S., & Nagy, S. R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and Endogenous chemicals. *Annual Review of Pharmacology and Toxicology*, 43, 309–334.
- Devine, P. J., Sipes, I. G., Skinner, M. K., & Hoyer, P. B. (2002). Characterization of a rat in vitro ovarian culture system to study the ovarian toxicant 4-vinylcyclohexene diepoxide. *Toxicology and Applied Pharmacology*, 184, 107–115.
- Devine, P. J., Petrillo, S. K., & Cortvriendt, R. (2010). In vitro ovarian model systems to study toxicology. In J. Richburg, & P. Hoyer (Eds.), *Reproductive and endocrine toxicology* (p. 11). Oxford, UK: Elsevier.

- Einaudi, L., Courbiere, B., Tassistro, V., Prevot, C., Sari-Minodier, I., Orsiere, T., & Perrin, J. (2014). In vivo exposure to benzo[a]pyrene induces DNA damage in mouse oocytes and cumulus cells. *Human Reproduction*, 29(3), 548–554.
- Fang, J.-L., Beland, F. A., Doerge, D. R., Wiener, D., Guillemette, C., Marques, M. M., & Lazarus, P. (2002). Characterization of benzo(a)pyrene-*trans*-7,8-dihydrodiol glucuronidation by human tissue microsomes and overexpressed UDP-glucuronosyltransferase enzymes. *Cancer Research*, 62, 1978–1986.
- Goerttler, K., Loehrke, H., Hesse, B., Milz, A., & Schweizer, J. (1981). Diaplacental initiation of NMRI mice with 7,12-dimethylbenz[a]anthracene during gestation days 6–20 and postnatal treatment of the F1-generation with the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate: Tumor incidence in organs other than skin. *Carcinogenesis*, 2(11), 1087–1094.
- Hecht, S. S., Carmella, S. G., Yoder, A., Chen, M., Li, Z.-Z., Le, C., Dayton, R., Jensen, J., & Hatsukami, D. K. (2006). Comparison of polymorphisms in genes involved in polycyclic aromatic hydrocarbon metabolism with urinary phenanthrene metabolite ratios in smokers. *Cancer Epidemiology, Biomarkers & Prevention*, 15(10), 1805–1811.
- Igawa, Y., Keating, A. F., Rajapaksa, K. S., Sipes, I. G., & Hoyer, P. B. (2009). Evaluation of ovotoxicity induced by 7,12-dimethylbenz[a]anthracene and its 3,4-diol metabolite utilizing a rat in vitro ovarian culture system. *Toxicology and Applied Pharmacology*, 234, 361–369.
- Jernström, B., Funk, M., Frank, H., Mannervik, B., & Seidel, A. (1996). Glutathione-S-transferase A1-1-catalysed conjugation of bay and fjord region diol epoxides of polycyclic aromatic hydrocarbons with glutathione. *Carcinogenesis*, 17(7), 1491–1498.
- Jules, G. E., Pratap, S., Ramesh, A., & Hood, D. B. (2012). In Utero exposure to benzo[a]pyrene predisposes offspring to cardiovascular dysfunction in later-life. *Toxicology*, 295, 56–67.
- Juriscova, A., Taniuchi, A., Li, H., Shang, Y., Antenos, M., Detmar, J., Xu, J., Matikainen, T., Hernandez, A. B., Nunez, G., & Casper, R. F. (2007). Maternal exposure to polycyclic aromatic hydrocarbons diminishes murine ovarian reserve via induction of *Harakiri*. *The Journal of Clinical Investigation*, 117(12), 3971–3978.
- Keating, A. F., Mark, C. J., Sen, N., Sipes, I. G., & Hoyer, P. B. (2009). Effect of phosphatidylinositol-3 kinase inhibition on ovotoxicity caused by 4-vinylcyclohexene diepoxide and 7,12-dimethylbenz[a]anthracene in neonatal rat ovaries. *Toxicology and Applied Pharmacology*, 241, 127–134.
- Krarp, T. (1967). 9:10-Dimethyl-1:2-benzanthracene induced ovarian Tumours in mice. *Acta Pathologica et Microbiologica Scandinavica*, 70(2), 241–248.
- Lim, J., Lawson, G. W., Nakamura, B. N., Ortiz, L., Hur, J. A., Kavanagh, T. J., & Luderer, U. (2013). Glutathione-deficient mice have increased sensitivity to transplacental benzo[a]pyrene-induced premature ovarian failure and ovarian tumorigenesis. *Cancer Research*, 73(2), 908–917.
- Lim, J., Kong, W., Lu, M., & Luderer, U. (2016). The mouse fetal ovary has greater sensitivity than the fetal testis to benzo[a]pyrene-induced germ cell death. *Toxicological Sciences*, 152(2), 372–381.
- Luderer, U., Myers, M. B., Banda, M., McKim, K. L., Ortiz, L., & Parsons, B. L. (2017). Ovarian effects of prenatal exposure to benzo[a]pyrene: Roles of embryonic and maternal glutathione status. *Reproductive Toxicology*, 69, 187–195.
- MacKenzie, K. M., & Angevine, D. M. (1981). Infertility in mice exposed in utero to benzo(a)pyrene. *Biology of Reproduction*, 24, 183–191.
- Madden, J. A., Hoyer, P. B., Devine, P. J., & Keating, A. F. (2014). Acute 7,12-dimethylbenz[a]anthracene exposure causes differential concentration-dependent follicle depletion and gene expression in neonatal rat ovaries. *Toxicology and Applied Pharmacology*, 276, 179–187.
- Marchant, J. (1960). The development of ovarian tumors in ovaries grafted from mice pretreated with dimethylbenzanthracene. *British Journal of Cancer*, 14, 514–519.
- Matikainen, T., Perez, G. I., Juriscova, A., Pru, J. K., Schlezinger, J. J., Ryu, H.-Y., Laine, J., Sakai, T., Korsmeyer, S. J., Casper, R. F., Sherr, D. H., & Tilly, J. L. (2001). Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nature Genetics*, 28, 355–360.
- Matikainen, T., Moriyama, T., Morita, Y., Perez, G. I., Korsmeyer, S. J., Sherr, D. H., & Tilly, J. L. (2002). Ligand activation of the fetal aromatic hydrocarbon receptor transcription factor drives Bax-dependent apoptosis in developing fetal ovarian germ cells. *Endocrinology*, 143(2), 615–620.
- Mattison, D. R. (1979). Difference in sensitivity of rat and mouse primordial oocytes to destruction by polycyclic aromatic hydrocarbons. *Chemico-Biological Interactions*, 28, 133–137.
- Mattison, D. R. (1980). Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice. *Toxicology and Applied Pharmacology*, 53, 249–259.
- Mattison, D. R., & Thorgeirsson, S. S. (1979). Ovarian aryl hydrocarbon hydroxylase activity and primordial oocyte toxicity of polycyclic aromatic hydrocarbons in mice. *Cancer Research*, 39, 3471–3475.
- Mattison, D. R., White, N. B., & Nightingale, M. R. (1980). The effect of benzo(a)pyrene on fertility, primordial oocyte number, and ovarian response to pregnant Mare's serum gonadotropin. *Pediatric Pharmacology*, 1, 143–151.
- Mody, J. K. (1960). The action of four carcinogenic hydrocarbons on the ovaries of IF mice and the Histogenesis of induced tumors. *British Journal of Cancer*, 14, 256–266.
- Neal, M. S., Zhu, J., Holloway, A. C., & Foster, W. G. (2007). Follicle growth is inhibited by benzo[a]pyrene, at concentrations representative of human exposure, in an isolated rat follicle culture assay. *Human Reproduction*, 22(4), 961–967.
- Penning, T. M. (2004). Aldo-keto reductases and formation of polycyclic aromatic hydrocarbon *o*-quinones. *Methods in Enzymology*, 378, 31–67.
- Penning, T. M., Ohnishi, S. T., Ohnishi, T., & Harvey, R. G. (1996). Generation of reactive oxygen species during the enzymatic oxidation of polycyclic aromatic hydrocarbon *trans*-dihydrodiols catalyzed by dihydrodiol dehydrogenase. *Chemical Research in Toxicology*, 9(1), 84–92.
- Pru, J. K., Kaneko-Tariu, T., Juriscova, A., Kashiwagi, A., Selesniemi, K., & Tilly, J. C. (2009). Induction of Proapoptotic gene expression and recruitment of p53 herald ovarian follicle loss caused by polycyclic aromatic hydrocarbons. *Reproductive Sciences*, 16(4), 347–356.
- Rajapaksa, K. S., Sipes, I. G., & Hoyer, P. B. (2007). Involvement of microsomal epoxide hydrolase enzyme in ovotoxicity caused by 7,12-dimethylbenz[a]anthracene. *Toxicological Sciences*, 96(2), 327–334.
- Romert, L., Dock, L., Jenssen, D., & Jernström, B. (1989). Effects of glutathione transferase activity on benzo[a]pyrene 7,8-dihydrodiol metabolism and mutagenesis studied in a mammalian cell co-cultivation assay. *Cancer Research*, 49(9), 1701–1707.
- Sadeu, J. C., & Foster, W. G. (2011). Effect of in vitro exposure to benzo[a]pyrene, a component of cigarette smoke, on folliculogenesis, steroidogenesis, and oocyte nuclear maturation. *Reproductive Toxicology*, 31, 402–408.
- Sadeu, J. C., & Foster, W. G. (2013). The cigarette smoke constituent benzo[a]pyrene disrupts metabolic enzyme, and apoptosis pathway member gene expression in ovarian follicles. *Reproductive Toxicology*, 40, 52–59.
- Seidel, A., Friedberg, T., Löllman, B., Schwierzok, A., Funk, M., Frank, H., Holler, R., Oesch, F., & Glatt, H. (1998). Detoxification of optically active bay- and fjord-region polycyclic aromatic hydrocarbon dihydrodiol epoxides by human glutathione transferase P1-1 expressed in Chinese hamster V79 cells. *Carcinogenesis*, 19(11), 1975–1981.
- Shimada, T., Sugie, A., Shindo, M., Nakajima, T., Azuma, E., Hashimoto, M., & Inoue, K. (2003). Tissue-specific induction of cytochromes P450 1A1 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in engineered C57BL/6J mice of *Arylhydrocarbon Receptor* gene. *Toxicology and Applied Pharmacology*, 187, 1–10.
- Shimizu, Y., Nakatsuru, Y., Ichinose, M., Takahashi, Y., Kume, H., Mimura, J., Fujii-Kuriyama, Y., & Ishikawa, T. (2000). Benzo[a]pyrene carcinogenicity is lost in mice lacking the aryl hydrocarbon receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 97(2), 779–782.
- Shorey, L. E., Castro, D. J., Baird, W. M., Siddens, L. K., Löhr, C. V., Matzke, M. M., Waters, K. M., Corley, R. A., & Williams, D. E. (2012). Transplacental carcinogenesis with dibenz[def,p]chrysene (DBC): Timing of maternal exposures determines target tissue response in offspring. *Cancer Letters*, 317, 49–55.
- Sobinoff, A. P., Mahony, M., Nixon, B., Roman, S. D., & McLaughlin, E. A. (2011). Understanding the villain: DMBA-induced preantral ovotoxicity involves selective follicular destruction and primordial follicle activation through PI3K/Akt and mTOR signaling. *Toxicological Sciences*, 123(2), 563–575.
- Sobinoff, A. P., Pye, V., Nixon, B., Roman, S. D., & McLaughlin, E. A. (2012). Jumping the gun: Smoking constituent BaP causes premature primordial follicle activation and impairs oocyte fusibility through oxidative stress. *Toxicology and Applied Pharmacology*, 260, 70–80.
- Swanson, M. S., Haugen, D. A., Reilly, C. A. J., & Stamoudis, V. C. (1986). Protection by uridine diphosphoglucuronic acid and DT-diaphorase against the cytotoxicity of polycyclic aromatic hydrocarbons isolated from a complex coal gasification condensate. *Toxicology and Applied Pharmacology*, 84, 336–345.
- Swartz, W. J., & Mattison, D. R. (1985). Benzo(a)pyrene inhibits ovulation in C57BL/6N mice. *The Anatomical Record*, 212, 268–276.

- Takizawa, K., Yagi, H., Jerina, D. M., & Mattison, D. R. (1984). Murine strain differences in ovotoxicity following intraovarian injection with benzo(a)pyrene, (+)-(7R,8S)-oxide, (-)-(7R,8S)-dihydrodiol, or (+)-(7R,8S)-diol-(9S,10R)-epoxide-2. *Cancer Research*, 44, 2571–2576.
- Tsai-Turton, M., Nakamura, B. N., & Luderer, U. (2007). Induction of apoptosis by 9,10-dimethyl-1,2-benzanthracene (DMBA) in cultured preovulatory rat follicles is preceded by a rise in reactive oxygen species and is prevented by glutathione. *Biology of Reproduction*, 77(3), 442–451.
- Wu, J., Ramesh, A., Nayyar, T., & Hood, D. B. (2003). Assessment of metabolites and AhR and CYP1A1 mRNA expression subsequent to prenatal exposure to inhaled benzo(a)pyrene. *International Journal of Developmental Neuroscience*, 21, 333–346.
- Xue, W., & Warshawsky, D. (2005). Metabolic activation of polycyclic aromatic hydrocarbon and heterocyclic aromatic hydrocarbons and DNA damage: A review. *Toxicology and Applied Pharmacology*, 206, 73–93.
- Yao, C., Foster, W. G., Sadeu, J. C., Siddique, S., Zhu, J., & Feng, Y.-L. (2017). Screening for DNA adducts in ovarian follicles exposed to benzo(a)pyrene and cigarette smoke condensate using liquid chromatography-tandem mass spectrometry. *Science of the Total Environment*, 575, 742–749.
- Zhou, B.-B. S., & Elledge, S. J. (2000). The DNA damage response: Putting checkpoints in perspective. *Nature*, 408, 433–439.

Female Antiestrogens

Radwa Barakat, University of Illinois at Urbana-Champaign, Urbana, IL, United States; and Benha University, Benha, Egypt

Chan Jin Park, University of Illinois at Urbana-Champaign, Urbana, IL, United States

Pablo A Perez, University of Illinois at Urbana-Champaign, Urbana, IL, United States; and National University of Cordoba, Cordoba, Argentina

Karen Chiu and CheMyong Ko, University of Illinois at Urbana-Champaign, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Estrogen Biosynthesis and Function

Estrogens are members of the steroid hormone family that play physiological roles in the development and function of reproductive and nonreproductive organs as well as pathological roles in a number of the pathogenesis such as endometriosis, polycystic ovary syndrome, and breast tumor. Estrogens are synthesized by the cells that express an enzyme, aromatase, that catalyzes the conversion of a C19-steroid precursor (androgen) to estrogen. In females, the primary sites of aromatase expression are the ovarian granulosa cells where theca cells convert cholesterol to pregnenolone, which is then further converted to androstenedione and testosterone by 17,20-lyase and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzymes, respectively. These androgens are then transported to granulosa cells by simple diffusion and converted to estrone, or 17 β -estradiol (estradiol), the primary and most potent estrogen, by aromatase. Estrone can be converted to estradiol by the 17 β -HSD enzyme (Findlay et al., 2010). Secondary sites include adrenal glands, skin, osteoblasts, pancreas, brain, and lymph nodes. In each site of estrogen synthesis, aromatase expression is regulated by a unique promoter, while its biochemical function and estrogenic products are the same in all sites of estrogen synthesis (Kamat et al., 2002). Precursors (androgens) of estrogen synthesis are produced in some estrogen synthetic sites such as gonads, but most extra-gonadal sites use an external source of androgens as precursors as summarized in Fig. 1. In women, during pregnancy and after menopause, the placental syncytiotrophoblast and adipose tissues are considered the primary sites of estrogen production, respectively (Cui et al., 2013).

Estrogens synthesized in the ovary play a key role in folliculogenesis and steroid production within the ovary. When secreted into the systemic circulation, estrogen regulates FSH and LH release and stimulates the growth and differentiation of the uterine epithelium (Stocco, 2008). In the mammary and pituitary glands, estrogens regulate proliferation and differentiation of stromal and epithelial cells and stimulate prolactin secretion, respectively. These estrogenic actions occur during fetal mammary development, adult morpho-physiological changes, and pregnancy-associated mammary gland growth for lactation preparation (Saji et al., 2000).

Extra-gonadally synthesized estrogens play tissue-specific roles. In the adipose tissue, they modulate lipogenesis, increase high-density lipoprotein, and decrease low-density lipoprotein production. Estrogen stimulates bone formation and regulates synthesis of proteins responsible for blood clotting in the liver. Estrogens also play a neuroprotective role in the brain by modulating neuronal metabolism, pain sensitivity, and cognitive behavior. In the adrenal cortex and pancreatic cells, estrogen enhances steroidogenic activity and increases insulin expression. Estrogen synthesized in lymph nodes regulates the maturation, differentiation, and migration of monocytes, macrophages, and dendritic cells (Barakat et al., 2016).

Estrogen Receptors

Estrogens exert their actions in a target cell or tissue mostly through the activation of two estrogen receptors (ER α and ER β) encoded by the *Esr1* and *Esr2* genes, respectively. Both ERs belong to the superfamily of nuclear receptors and act as transcription factors. Estrogens can bind to these ERs with similar affinities but trigger different signaling pathways. In a cell, an estrogen-bound ER travels to the nucleus where it either stimulates or inhibits transcription of its target genes via two distinctive mechanisms. An estrogen response element (ERE)-dependent mechanism involves a direct interaction between an ER and a specific DNA sequence (ERE) of the promoter of the estrogen target gene. In this process, ER α and ER β probably interact with other transcription factors that may confer signaling specificity between the two ERs. ER may regulate gene expression via an ERE-independent pathway. Under this mechanism, an ER influences the expression of a gene by binding not to an ERE but to other transcription factors that directly or indirectly interact with DNA sequences. Stimulating protein-1 (SP-1), activator protein 1 (AP-1), nuclear factor κ B (NF- κ B), and c-Jun are known to interact with ERs (Findlay et al., 2010).

Estrogens are also known to bind to a cohort of cytoplasmic or membrane proteins that are not classical ERs. Binding of estrogens to these proteins activates a subset of different G proteins and/or protein kinase cascades, such as MAPK/ERK, PI3K/AKT, cAMP, PKA, and PKC which in turn phosphorylate substrates, including membrane-based ion channels and secondary messenger systems, leading to rapid cellular responses. This pathway is mainly responsible for the acute and rapid estrogen actions that occur much faster than transcriptional processes (Cui et al., 2013; Findlay et al., 2010).

ER Expression in Female Reproductive Organs and Impact of Losing ER in Mouse Models

A target tissue or organ responds to estrogens differently depending on the types of ERs expressed and the expression levels. In the ovary, ER α is expressed in theca and surface epithelial cells, while ER β is expressed exclusively in granulosa cells (Drummond and Fuller, 2012). This differential distribution suggests that estrogen actions in the theca cells are partly mediated by ER α , and follicle

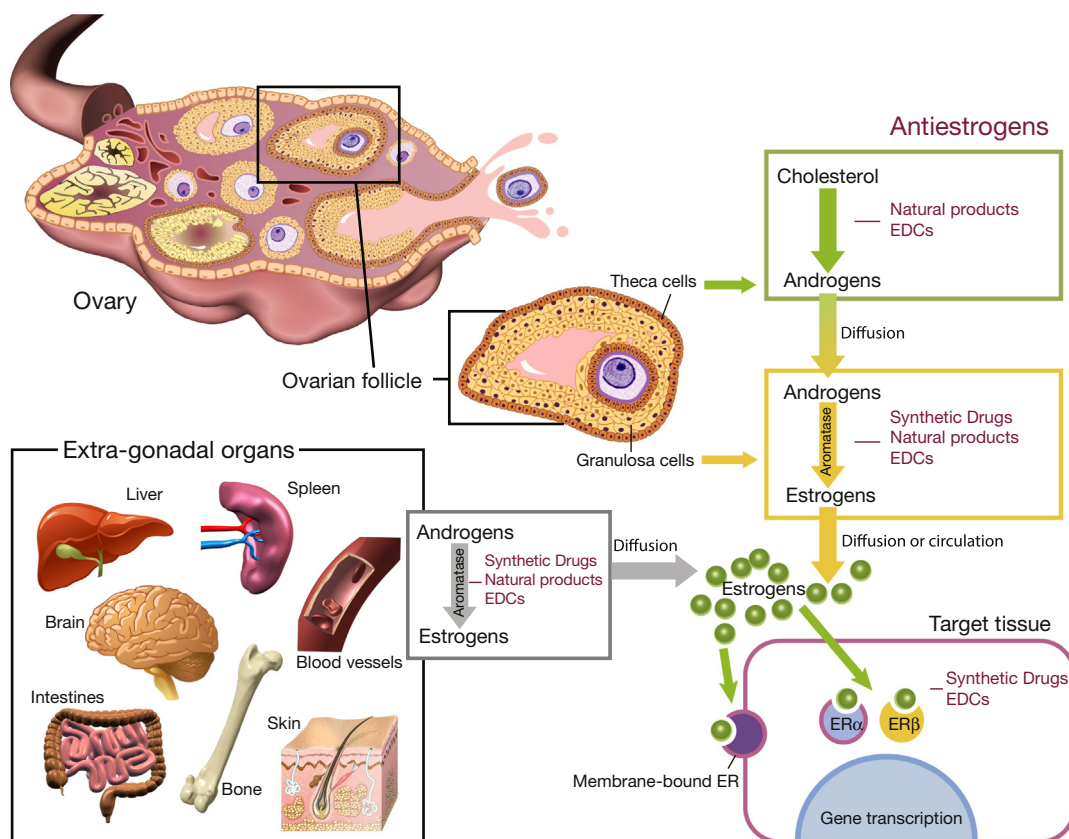


Fig. 1 Sites of estrogen syntheses, mode of estrogen action, and targets of antiestrogens in females. Estrogens are synthesized in the ovary and a variety of extra-gonadal tissues where aromatase is expressed. Aromatase is an enzyme that converts androgens to estrogens. Once synthesized, estrogens reach their target tissues by either diffusion or circulation and exert their actions by binding to estrogen receptors (ERs) that are localized in the cell membrane (membrane-bound ERs) or nucleus (ER α and ER β). Antiestrogens inhibit estrogen production or interfere ER action, disrupting estrogen-regulated pathways or events at cell, tissue, or organ levels. Several synthetic drugs and natural products are used as antiestrogens for the purpose of inhibiting aromatase or ERs. Some endocrine disrupting chemicals (EDCs) act as antiestrogens by decreasing aromatase activity or binding to ERs.

growth and estrogen biosynthesis in granulosa cells are mediated mainly by the activation of ER β . In the uterus and oviduct, ER α is highly expressed in luminal and glandular epithelial cells and in the stroma, while ER β is found only in the endometrial glandular epithelial cells and rarely in the oviduct (Wang et al., 2000).

In the mammary gland, ER α is present in epithelial cells of intralobular acini and interlobular ducts, whereas ER β is expressed in epithelial cells as well as in the luminal and myoepithelial cells. ER α expression in a breast tumor indicates a poor prognosis (Saji et al., 2000).

Estrogens play numerous modulatory roles in the hypothalamus–pituitary–ovary axis. In the hypothalamus, both ERs are expressed in distinct patterns in each hypothalamic nuclei. In the anterior lobe of the pituitary gland, ER α is the predominant receptor, and diverse lines of evidence indicate that ER α is the major mediator of estrogen action in the pituitary. ER β expression is low in the anterior lobe but high in the posterior and intermediate lobes.

Most of the roles that estrogens play were discovered using genetically modified mice that lack either or both subtypes of ERs. The phenotype of ER α -deficient (ER α KO) mice is different from that of ER β KO mice, indicating unique pathways regulated by the two ERs as shown in Table 1. ER α KO female mice are infertile; they exhibit hyperemic ovaries with hemorrhagic follicles and do not ovulate. These ovarian defects are in part a consequence of a direct effect on theca cells and ovary function, but also a result of pituitary dysfunction. Furthermore, ER α KO female mice exhibited a hypoplastic uterus that is incapable of maintaining fetal implantation. Interestingly, mammary glands of ER α KO mice exhibited normal fetal development, but they remain rudimentary, suggesting that ER α expression is necessary for mediating growth stimulation. Previous studies show that global ER β KO females exhibit reduced fertility, mainly explained by the defects in the ovary and a marked increase in the number of premature atretic follicles (Drummond and Fuller, 2012). Nevertheless, a novel ER β KO mouse, which was generated by knock-in of codon-improved Cre recombinase (iCre) gene into the exon of the *Esr2* gene, was completely infertile in the female (Cacioppo et al., 2016). The uterus of ER β KO females is hyper-responsive to estrogens, leading to uterine dysfunction and infertility (Walker and Korach, 2004).

Table 1 Estrogen receptors in female reproductive organs and pathological abnormalities of ER-deficient mice

Organs	Sites of ER expression		Phenotypes of ERKO mice	
	ER α	ER β	ER α KO	ER β KO
Ovary	Theca and interstitial cells and germinal epithelium	Granulosa cells	Anovulation, hemorrhagic follicles	Follicle atresia, defective folliculogenesis
Uterus	Luminal, glandular epithelial cells, and stroma	Glandular epithelial cells	Hypoplastic uterus, no implantation	Hyperplastic, hypersensitive to estrogens
Mammary gland	Epithelial cells of intra-lobular acini and inter-lobular ducts	Epithelial, luminal, and myoepithelial cells	Defective myoepithelial cell layers	Hyper-proliferation of alveolar cells

Antiestrogens and Their Medical Uses

Antiestrogens are agents that either inhibit estrogen production or interfere with estrogen receptor signaling, preventing estrogen from mediating its biological effects in the body. Natural antiestrogens are often used to reduce estrogen production in women with excessive estrogen levels or with an ER-positive tumor. Of note, ER α is considered as one of the most important clinical markers in breast tumor patients and as the most successful molecular target in cancer drug discovery. Antiestrogen therapy has thus become the main approved treatment for ER α -positive breast cancer.

Commercial Antiestrogens

Estrogens stimulate proliferation and metastasis of ER α -positive breast cancer cells, making aromatase and ERs ideal targets for treating breast cancers. Therefore, antiestrogenic drugs are used for ER α -positive (and/or PR-positive), but not for ER α -negative, breast cancers. Antiestrogenic drugs are administered before surgery to reduce tumor size and/or after surgery to suppress the proliferation of remaining cancer cells (Mallick et al., 2016). Selective estrogen receptor modulators (SERM) are used for targeting ERs and aromatase inhibitors for decreasing estrogen synthesis. Table 2 lists antiestrogenic medications, their modes of actions, and possible side effects.

Tamoxifen

Tamoxifen is the most widely used SERM that acts as agonist and antagonist in a tissue-selective manner. Tamoxifen is metabolized to endoxifen, a bioactive form, in the liver by cytochrome p450 enzymes. It exerts an antiestrogenic effect in the breast by inhibiting ER α signaling but a proestrogenic effect by promoting cell proliferation in the uterus, brain, and bone (Robertson, 2004). Tamoxifen is an FDA-approved drug for treatment of all stages of breast cancer. However, because tamoxifen acts as an ER α agonist in the uterus, prolonged exposure to this SERM increases the risk of developing endometrial cancers. Tamoxifen alters the plasma protein profile by increasing sex hormone-binding globulin and antithrombin III, whose expressions are regulated by estrogens. Tamoxifen treatment of breast cancer often continues for 5 years. Use of tamoxifen decreases the bone density as it leads to decreases in markers of bone turnover (serum osteocalcin and urinary N-telopeptide). This prolonged treatment leads to a number of side effects such as sweating, hot flashes, vaginal discharge, vaginal dryness, itching, and depression.

Raloxifene

Raloxifene is an SERM that has an antiestrogenic effect similar to tamoxifen, as it has antiestrogenic effects in breast and proestrogenic effects in the uterus, brain, and bone. This drug is metabolized in the gut, but it does not require a cytochrome enzyme. Moreover, raloxifene has greater affinity for the estrogen receptor than tamoxifen; it has greater estrogenic effects on bone and lesser

Table 2 Antiestrogenic medications, their modes of actions, and associated side effects

Antiestrogens	Drug name	Mode of action	Side effects
Selective estrogen receptor modulators (SERMs)	Tamoxifen, raloxifene, toremifene	ER α antagonist in mammary cells; ER α agonist in the endometrium	Fatigue, hot flashes, vaginal dryness, mood swings, mental impairment, blood clot, increased risk of endometrial cancers
Nonsteroidal aromatase inhibitors	Anastrozole, letrozole	Inhibit aromatase enzyme activity; inhibit estrogen synthesis	Gastrointestinal disturbances, hot flashes, muscle pain, joint stiffness and/or pain, osteoporosis
Steroidal aromatase inhibitors	Exemestane		
Others mechanism of actions	Fulvestrant	Block estrogen receptors and eliminate them temporarily	Hot flashes, night sweats, mild nausea, fatigue, osteoporosis

estrogenic effects on the uterus compared to tamoxifen (Mallick et al., 2016). Raloxifene displays many of the same side effects as tamoxifen when used for an extended period of time.

Toremifene

Toremifene is another SERM that has an action mode similar to tamoxifen. This drug was developed as an alternative to tamoxifen with the hope that it would produce fewer uterine side effects. However, the side effects of toremifene treatment are similar to those of tamoxifen treatment (Robertson, 2004). Toremifene is used less frequently and is approved only to treat metastatic breast cancer.

Anastrozole

As a pure antiestrogen, this drug produces antiestrogenic effects in any estrogen-responsive tissues including breast and uterus. Anastrozole is a nonsteroidal aromatase inhibitor that reversibly binds to the aromatase enzyme and suppresses estrogen synthesis. This drug is used for treating ER-positive breast cancer and is also used as a measure for preventing breast cancer development in women at high risk. Anastrozole is preferred over tamoxifen for treating postmenopausal women with localized breast cancer because of its good safety profile for postmenopausal women as well as younger women. Side effects include hot flashes, altered mood, joint pain, nausea, osteoporosis, and rheumatic disorders.

Letrozole

Letrozole is a nonsteroidal aromatase inhibitor that reversibly binds to aromatase enzyme, and inhibits estrogen synthesis. It is used for treating ER α -positive local or metastatic breast cancer in postmenopausal women. Letrozole is also used to induce ovulation in women who exhibit polycystic ovary syndrome or anovulation. When the enzyme aromatase is inhibited by letrozole, estrogen levels are attenuated. This results in the increased output of FSH from the brain and pituitary gland, which induces the development of a mature follicle in the ovary and ovulation. The most common side effects of letrozole are sweating, hot flashes, joint pain, and fatigue.

Exemestane

Exemestane is used for the adjuvant treatment of postmenopausal women with ER α -positive breast cancer who have received 2 to 3 years of tamoxifen treatment for the completion of 5 consecutive years of hormonal therapy (Mallick et al., 2016). Because this steroidal aromatase inhibitor irreversibly binds to the active site of the aromatase enzymes, preventing them from converting androgens into estrogens, it is contraindicated in premenopausal women. The most common side effects are hot flashes, sweating, insomnia, headache, and joint pain. Nausea and fatigue are observed mainly in patients with advanced breast cancer.

Fulvestrant

Fulvestrant is an estrogen receptor antagonist that competitively binds to the ER α with a much greater affinity than tamoxifen. This drug is not an SERM, as it acts like an antiestrogen throughout the body. In addition to blocking the estrogenic effects of estradiol, fulvestrant has no estrogenic effects on the endometrium. Fulvestrant is used in postmenopausal women with ER-positive breast cancer. Prolonged treatment with fulvestrant causes side effects such as hot flashes, osteoporosis, mild nausea, and fatigue (Robertson, 2004).

Natural Products as Antiestrogens

Many natural molecules present in plants have estrogenic and/or antiestrogenic mimetic activities. Plants produce a wide variety of secondary metabolites, some of which are phytoestrogens that have chemical structures that are similar to estrogens and display estrogenic activity (Liu et al., 2010). Exposure to phytoestrogens occurs primarily through dietary intake. Most of phytoestrogens have antiestrogenic properties that may make them effective therapeutic agents. Therefore, blocking the activity of ERs in the signal transduction pathway is important for reversing the development of estrogen-responsive diseases such as cancer. Furthermore, some phytoestrogens are used as an effective treatment for breast cancer because of their apoptotic properties and antiestrogenic effects. However, phytoestrogen therapy is safe only for postmenopausal women because they have lower estrogen levels in their bodies (Lecomte et al., 2017). This therapy can also be used conjunctively with antiestrogenic drug therapies. Phytoestrogens can also act as endocrine disruptors by affecting estrogen biosynthesis. Isoflavones, flavones, lignans, coumestans, and mycoestrogens are representative phytoestrogens (Table 3).

Isoflavones

Isoflavones are widely consumed phytoestrogens such as genistein, equol, daidzein, biochanin A, and formononetin. They are found in soybeans, red clover, certain fruits, legumes, and nuts. They have a weak inhibitory activity on aromatase enzyme and a strong inhibitory activity on 3 β -HSD and 17 β -HSD (Sifakis et al., 2017). Soybeans are the most recognized sources of phytoestrogens as they are rich in genistein and daidzein contents. Isoflavone supplements that containing predominantly genistein are often used in dietary supplements for reducing hot flashes in postmenopausal women. Intake of isoflavone-rich food or dietary supplements seems to lower the incidence of breast cancer and osteoporosis in postmenopausal women. Moreover, genistein can compete against estrogen and bind to both estrogen receptors with a much higher affinity toward ER β than ER α . It has been

Table 3 Exemplary phytoestrogens, dietary sources, and their mode of action

Groups	Phytoestrogens	Dietary source	Mode of action
Isoflavones	Genistein, equol, daidzein, biochanin A, formononetin	Legumes, soybean, nuts, red clover	Inhibit 3 β -HSD and 17 β -HSD; inhibit aromatase enzyme
Flavones	Apigenin, chrysin, quercetin, kaempferol	Mostly red and yellow fruits and vegetables	Potent inhibitors of 17 β -HSD and aromatase enzyme; inhibit 3 β -HSD
Flavonones	Flavanone, naringenin	Citrus fruits	Potent inhibitors of 17 β -HSD and aromatase enzyme; inhibit 3 β -HSD
Lignans	Enterodiol, enterolactone	Most fruits, cereals, vegetables, flaxseed, sesame seed	Weak inhibitor of aromatase enzyme
Coumestans	Coumestrol	Spinach, bean shoots, clover, Alfalfa sprouts, sunflower seeds	Inhibit 17 β -HSD; inhibit aromatase enzyme; higher binding affinity to estrogen receptors
Mycoestrogens	Zearalenone	Mold on bread and plants	Higher binding affinity to estrogen receptors; inhibit 17 β -HSD

identified as an angiogenesis inhibitor because it inhibits the proliferation and survival of cancer cells. In addition, moderate doses of genistein inhibit cell proliferation on cancers of the prostate, cervix, brain, breast, and colon (Spagnuolo et al., 2015).

Flavones and flavonones

Flavonoids are natural products that are widely distributed in the plant kingdom and categorized according to their molecular structures as flavones, flavanones, catechins, and others. Flavones are a class of flavonoids that includes apigenin, chrysin, quercetin, and kaempferol. Flavones are present mainly in cereals, red and yellow fruits, and vegetables (Liu et al., 2010). Flavones and flavonones appear to be more potent than isoflavones in inhibiting 17 β -HSDs and 3 β -HSD. Compared with isoflavones, flavones are relatively potent inhibitors of aromatase enzyme. Flavones can be used conjunctively with antiestrogenic drug therapies, inhibiting aromatase, which is considered a major strategy in treating breast cancer patients.

Lignans

Lignans are a class of phytoestrogens that contain enterolactone and enterodiol. Enterolactone and enterodiol are formed by bacteria in the intestinal tract from the plant lignans matairesinol and secoisolariciresinol, which exist in various whole-grain cereals (barley, rye, and wheat), seeds, nuts, legumes, and vegetables. They are found mainly in fiber-rich food products such as cereals, vegetables, and fruits. The richest known dietary sources of enterolactone precursors are flaxseed and sesame seed. These lignans exert weak inhibitory actions on aromatase. Enterolactone levels are lower in breast cancer patients than in healthy controls, indicating its potential anticarcinogenic activity.

Coumestans

Coumestrol, a natural organic compound in the class of phytochemicals known as coumestans, is contained in a variety of plants including soybeans, clover, alfalfa sprouts, sunflower seeds, spinach, and legumes. Coumestrol inhibits the enzymatic activity of 17 β -HSD and aromatase (Sifakis et al., 2017). It also exerts antiestrogenic activity 30- to 100-fold higher than that of isoflavones by binding to ER α and ER β . Because of its estrogenic activities, coumestrol could be used as a supplement to hormone therapy and chemotherapy in breast cancer patients. Studies looking at coumestrol in animals found that it delays vaginal opening in rats. Cows that consume a significant amount of alfalfa tend to have low pregnancy rates and higher spontaneous abortion rates, resulting in a low fertility.

Mycoestrogens

Mycoestrogens are produced by fungi that contaminate cereal grains, animal feed sources, corn silage, and hay. Zearalenone is a potent estrogenic mycotoxin produced by some *Fusarium* and *Gibberella* species. Zearalenone is heat-stable and found worldwide in many cereal crops such as maize, barley, oats, wheat, rice, and sorghum (Liu et al., 2010). It is considered to be the most potent phytoestrogen in inhibiting the activity of both the reductive and oxidative activity of 17 β -HSD. Natural exposure to zearalenone in contaminated food has been implicated as a cause of female reproductive changes because of its powerful estrogenic activity. Zearalenone is metabolized by the intestinal tissue into major active metabolites: α - and β -zearalenol. The estrogenic activity of zearalenone and its metabolites is mediated by their binding affinity to estrogen receptors. Girls chronically exposed to zearalenone through the diet suffer from early puberty and several sexual disorders.

Antiestrogens as Endocrine Disrupting Chemicals

Endocrine disrupting chemicals (EDCs) are exogenous substances that can disturb the production, metabolism, transportation, action, and elimination of natural hormones due to their natural hormone-mimicking property (Casals-Casas and Desvergne, 2011; Shanle and Xu, 2011). Most EDCs exert their actions by binding to hormone receptors, including ERs, androgen receptors (AR), thyroid hormone receptors (TRs), and nonsteroidal receptors (membrane-bound receptors). An EDC may interfere with gene expression, enzyme activity, and epigenome modification (Casals-Casas and Desvergne, 2011; Patel et al., 2015; Shanle and Xu, 2011). Exposure to EDCs is frequently associated with reproductive abnormalities, metabolic disorders, cancers, and physiological problems (Casals-Casas and Desvergne, 2011; Patel et al., 2015). Of note, a variety of EDCs directly affect the ovarian function as well as steroidogenesis in females (Patel et al., 2015).

Antiestrogenic EDCs are defined as chemical compounds with an antagonistic effect on ERs or inhibitory activity on aromatase. Broadly, this definition can be extended to chemicals that inhibit the expression/activity of key enzymes that contribute to the synthesis of estradiol (E2) from cholesterol. An antiestrogenic EDC disturbs the expression/activity of aromatase or competes against endogenous estrogens in binding estrogen ERs. Antiestrogenic EDCs are classified according to their modes of actions: (1) inhibitory activity on estrogen synthesis, (2) antagonistic activity on ERs, and (3) other antiestrogenic action. Table 4 lists representative EDCs that are known to exert an antiestrogenic effect in females.

Antiestrogens With Inhibitory Activity on Estrogen Synthesis

Exposure to a variety of EDCs disrupts ovarian estrogen synthesis, thus causing ovarian dysfunction and infertility (Patel et al., 2015). Bisphenol A (BPA), methoxychlor (MXC), phthalates, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are representative antiestrogens acting on estrogen synthesis machinery.

BPA (bisphenol A)

BPA is used in the process of manufacturing polycarbonate plastics and epoxy resins as a plasticizer. Specifically, it is used for packaging foods and beverages, baby bottles, thermal papers (i.e., receipts), and dental sealants. In the liver, BPA is converted to 4-methyl-2,4-bis(4-hydroxyphenyl) pent-1-ene (MBP). MBP has a 1000-fold stronger binding affinity to ERs compared BPA alone. BPA exposure inhibits steroidogenesis in several animal models (Patel et al., 2015). BPA decreased estradiol synthesis in cultured ovarian cells by decreasing aromatase expression. In porcine granulosa cell culture, BPA stimulated estradiol production at a low BPA concentration (0.1 μ M) but inhibited estradiol production at higher BPA concentrations (1 and 10 μ M). In mouse antral follicle cultures, BPA acutely decreased *Cyp11a1* expression, causing a decrease of progesterone, androstenedione, testosterone, and estradiol. Also, BPA significantly decreased serum estradiol levels in rats by decreasing aromatase and *Star* expression in granulosa cells and theca interstitial cells, respectively. In humans, high BPA concentration in plasma or urine is associated with a reduced number of mature/fertilized oocytes, low response to hyperstimulation with human chorionic gonadotrophin (hCG), implantation failure, and infertility (Sifakis et al., 2017).

Table 4 List of antiestrogenic endocrine disruptors, their mode of action, and associated disorders

Chemicals	Bioactive metabolites	Usage	Mode of action	Disorders
Bisphenol A (BPA)	4-Methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene (MBP)	Plasticizers	Inhibition of E2 synthesis; ER α antagonist	Endometriosis, PCOS, implantation failure, infertility
Bisphenol AF (BPAF)	BPAF diglucuronide, BPAF glucuronide (BPAF-G), BPAF glucuronide dehydrated, BPAF sulfate	Plasticizers	ER β antagonist; metabolites have no activity on ERs	Not known
Methoxychlor (MXC)	1,1,1-Trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl) ethane (MOH) 2,2-Bis(<i>p</i> -hydroxyphenyl)-1,1,1-trichloroethane (HPTE)	Pesticides	E2 synthesis Inhibition of E2 synthesis; ER β antagonist; ER β hyper-methylation	Not known Impaired folliculogenesis and implantation failure in rodents
Di-(2-ethylhexyl) phthalate (DEHP)	Mono-(2ethylhexyl) phthalate (MEHP)	Plasticizers	Inhibition of E2 synthesis	Endometriosis, infertility
2,3,7,8-Tetrachlorodibenzo- <i>p</i> -dioxin (TCDD)	2,3,7-Trichloro-8-hydroxydibenzo- <i>p</i> -dioxin (OH-TriCDD); 1,3,7,8-tetrachloro-2-hydroxydibenzo- <i>p</i> -dioxin (OH-TCDD)	Byproducts	Inhibition of E2 synthesis	Endometriosis, endometriosis in TCDD-exposed rhesus monkeys

MXC (methoxychlor)

MXC is a synthetic insecticide developed as an alternative to dichlorodiphenyltrichloroethane (DDT), which was banned by the US Environmental Protection Agency in 2003 because of its acute toxicity, bioaccumulation, and endocrine disrupting properties. In the body, MXC is metabolized to 1,1,1-trichloro-2-(4-hydroxyphenyl)-2-(4-methoxyphenyl) ethane (MOH) and the 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) by cytochrome p450 enzymes. MXC inhibits folliculogenesis, decreases *Esr2* expression, and delays growth of antral follicles in laboratory animals. MXC and its metabolites (MOH and HPTE) decrease estrogen synthesis by inhibiting the expression of steroidogenic enzymes such as *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* mRNA in laboratory animals (Patel et al., 2015; Shanle and Xu, 2011).

Phthalates

Phthalates are synthetic chemicals used as plasticizers for producing a variety of products such as medical tubing, food packaging, and infant toys. Di-(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), butyl benzyl phthalate (BBP), and diethyl phthalate (DEP) are the most prevalent phthalates in consumer products. DEHP exposure inhibits aromatase expression in antral follicles and decreases *Cyp19a1* expression and serum estradiol levels in rats. Mono-(2ethylhexyl) phthalate (MEHP), the bioactive metabolite of DEHP, inhibits aromatase expression in the cultured granulosa cells of mice, rats, and humans (Patel et al., 2015). High urine content of phthalate metabolites is associated with endometriosis incidence (Sifakis et al., 2017).

TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin)

TCDD is produced as a byproduct of solid waste burning, herbicides, pesticides, disinfectants, and paper mills. This lipophilic toxicant has a long environmental half-life and accumulates in the food chain. TCDD is found in animals and humans (in adipose tissue, serum, milk, and ovarian follicular fluid). The International Agency for Research on Cancer has classified TCDD as a carcinogen. TCDD causes reproductive dysfunctions due to its antiestrogenic actions (Patel et al., 2015). In the mouse, the nanomolar range of TCDD exposure decreases the concentration of progesterone, androstenedione, testosterone, and estradiol and inhibits the expression of steroidogenic enzymes including *Hsd17b1* and aromatase in isolated antral follicles.

EDCs on Estrogen Receptors

EDCs such as bisphenols and MXC bind to ERs and modulate ER functions by inducing conformational changes. Even though most antiestrogenic EDCs bind to ER with relatively weak affinity, the binding still induces a significant disruption in ER signaling. In addition to bisphenols and MXC, benzanthrone, disulfiram, 3,3,3',3'-tetramethyl-1,1'-spirobisindane-5, 5',6,6'-tetrol, and atrazine exhibit antiestrogenic properties with low ER α binding affinity (Akahori et al., 2008).

BPAF (bisphenol AF)

Because of the antagonistic activities of BPA against ER α , research on replacing BPA has been actively conducted. BPAF, a BPA derivative in which two methyl groups in BPA are replaced with trifluoromethyl groups is a substitute for BPA, and is manufactured to produce "BPA-free" plastic products. However, BPAF acts as an antagonist for ER β in an in vitro study (Li et al., 2012). Of note, BPAF acts as either ER agonists or antagonists depending on its concentration or tissue type. At present, various alternatives for BPA are being developed and presented, but as in the case of BPAF, their safety is not yet ensured.

MXC

As noted above, MXC decreases estradiol synthesis by inhibiting the expression of steroidogenic enzymes. MXC itself has 10,000 times lower binding affinity than E2 for ERs. A main metabolite of MXC, 2,2-Bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), acts as an agonist for ER α and an antagonist for ER β . In peripubertal female rhesus monkeys, administration of MXC for 12 months induced precocious puberty and shortened the follicular phases. Also, HPTE inhibits follicular development in the rat ovary (Patel et al., 2015; Shanle and Xu, 2011).

Other Antiestrogenic Mechanisms of EDCs

Some EDCs disrupt the endocrine system via a nonclassical nuclear receptor signaling pathway, aryl hydrocarbon receptor (AhR), or epigenetic modification. While studies on EDCs in membrane-associated ER α or G protein-coupled receptors for estrogen (GPR30) are primarily concerned with estrogenic action, other studies showed that MXC exerts an antiestrogenic effect via epigenetic modification (Shanle and Xu, 2011). MXC induces hypermethylation in the *Esr2* gene promoter but not in the *Esr1* gene (Sifakis et al., 2017). Because DNA methylation in the CpG island of the promoter in certain genes generally causes reduction of gene expression, hypermethylation in the *Esr2* promoter by MXC may affect the receptivity for E2 in the ovary, particularly in granulosa cells.

In summary, estrogens play important roles in regulating female reproductive function and embryonic development. Some natural/synthetic antiestrogens are currently used as therapeutics for treating female reproductive disease. The most important use of antiestrogen medication is to treat breast cancer and reduce the risk of cancer remission. Research on targeting estrogen receptors as a potential anticancer drug is still ongoing. Antiestrogenic EDCs disrupt estrogen synthesis and ERs signaling, and normal

estrogen signaling is affected by unwanted exposure to antiestrogenic EDCs. The effects of antiestrogenic therapeutics and EDCs on ovaries and extra-gonadal organs that synthesize estrogen warrant further research because of their potential to drastically improve various reproductive health diseases and cancer.

References

- Akahori, Y., Nakai, M., Yamasaki, K., Takatsuki, M., Shimohigashi, Y., & Ohtaki, M. (2008). Relationship between the results of in vitro receptor binding assay to human estrogen receptor alpha and in vivo uterotrophic assay: Comparative study with 65 selected chemicals. *Toxicology In Vitro*, 22(1), 225–231.
- Barakat, R., Oakley, O., Kim, H., Jin, J., & Ko, C. J. (2016). Extra-gonadal sites of estrogen biosynthesis and function. *BMB Reports*, 49(9), 488–496.
- Cacioppo, J. A., Koo, Y., Lin, P. J., Osmulski, S. A., Ko, C. D., & Ko, C. (2016). Generation of an estrogen receptor beta-iCre knock-in mouse. *Genesis*, 54(1), 38–52.
- Casals-Casas, C., & Desvergne, B. (2011). Endocrine disruptors: From endocrine to metabolic disruption. *Annual Review of Physiology*, 73, 135–162.
- Cui, J., Shen, Y., & Li, R. (2013). Estrogen synthesis and signaling pathways during aging: From periphery to brain. *Trends in Molecular Medicine*, 19(3), 197–209.
- Drummond, A. E., & Fuller, P. J. (2012). Ovarian actions of estrogen receptor-beta: An update. *Seminars in Reproductive Medicine*, 30(1), 32–38.
- Findlay, J. K., Liew, S. H., Simpson, E. R., & Korach, K. S. (2010). Estrogen signaling in the regulation of female reproductive functions. *Handbook of Experimental Pharmacology*, 198, 29–35.
- Kamat, A., Hinshelwood, M. M., Murry, B. A., & Mendelson, C. R. (2002). Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans. *Trends in Endocrinology and Metabolism*, 13(3), 122–128.
- Lecomte, S., Demay, F., Ferrière, F., & Pakdel, F. (2017). Phytochemicals targeting estrogen receptors: Beneficial rather than adverse effects. *International Journal of Molecular Sciences*, 18(7), 1381.
- Li, Y., Burns, K. A., Arao, Y., Luh, C. J., & Korach, K. S. (2012). Differential estrogenic actions of endocrine-disrupting chemicals bisphenol A, bisphenol AF, and zearalenone through estrogen receptor alpha and beta in vitro. *Environmental Health Perspectives*, 120(7), 1029–1035.
- Liu, Z., Kanjo, Y., & Mizutani, S. (2010). A review of phytoestrogens: Their occurrence and fate in the environment. *Water Research*, 44, 567–577.
- Mallick, S., Benson, R., & Julka, P. K. (2016). Breast cancer prevention with anti-estrogens: Review of the current evidence and future directions. *Breast Cancer*, 23(2), 170–177.
- Patel, S., Zhou, C., Rattan, S., & Flaws, J. A. (2015). Effects of endocrine-disrupting chemicals on the ovary. *Biology of Reproduction*, 93(1), 20.
- Robertson, J. (2004). Selective oestrogen receptor modulators/new antioestrogens: A clinical perspective. *Cancer Treatment Reviews*, 30(8), 695–706.
- Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M., & Gustafsson, J. A. (2000). Estrogen receptors alpha and beta in the rodent mammary gland. *Proceedings of the National Academy of Sciences of the United States of America*, 97(1), 337–342.
- Shanle, E. K., & Xu, W. (2011). Endocrine disrupting chemicals targeting estrogen receptor signaling: Identification and mechanisms of action. *Chemical Research in Toxicology*, 24(1), 6–19.
- Sifakis, S., Androutsopoulos, V. P., Tsatsakis, A. M., & Spandidos, D. A. (2017). Human exposure to endocrine disrupting chemicals: Effects on the male and female reproductive systems. *Environmental Toxicology and Pharmacology*, 51, 56–70.
- Spagnuolo, C., Russo, G. L., Orhan, I., Habtemariam, S., et al. (2015). Genistein and cancer: Current status, challenges, and future directions. *Advances in Nutrition*, 6, 408–419.
- Stocco, C. (2008). Aromatase expression in the ovary: Hormonal and molecular regulation. *Steroids*, 73(5), 473–487.
- Walker, V. R., & Korach, K. S. (2004). Estrogen receptor knockout mice as a model for endocrine research. *ILAR Journal*, 45(4), 455–461.
- Wang, H., Eriksson, H., & Sahlin, L. (2000). Estrogen receptors alpha and beta in the female reproductive tract of the rat during the estrous cycle. *Biology of Reproduction*, 63(5), 1331–1340.

Female Antiandrogens

Radwa Barakat, University of Illinois at Urbana–Champaign, Urbana, IL, United States; and Benha University, Benha, Egypt
CheMyong J Ko, University of Illinois at Urbana–Champaign, Urbana, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Androgens in the Female Reproductive System

In females, androgens play important roles in regulating development and functions of reproductive organs as well as in maintaining muscle strength, bone density, protein balance, sexuality, and overall well-being. The main circulating androgens in women are testosterone and dihydrotestosterone (DHT). Their actions are mediated by the androgen receptor (AR), a member of the nuclear receptor superfamily. Antiandrogens are any compounds that inhibit either the production of androgens or their interaction with androgen receptor (Zhou, 2010; Walters, 2015).

In healthy women, ovaries and adrenal glands produce approximately 50% of the body's testosterone, the primary androgen, while the other half is synthesized by peripheral conversion of androgen precursors (androstenedione and DEHA), which are secreted from the adrenal glands and the ovaries (Burger, 2002). Proper androgen production is critical for both reproduction and health in females, as hyper- and hypoandrogenism lead to a variety of disorders and infertility. Hyperandrogenism caused by overproduction of androgens or decreased androgen metabolism is responsible for the most prevalent gynecological disorder, polycystic ovary syndrome (PCOS). Women with PCOS suffer from ovulatory dysfunction, infertility, and metabolic abnormalities (Goodarzi et al., 2011). Because androgens are precursors for estrogen biosynthesis, a shortage of androgens in a woman results in a hypoestrogenic condition. Hypoandrogenism is one of the main causes of osteoporosis in women.

The actions of androgens are mediated by the AR. Therefore, AR expression in a tissue is considered to be an indication that the tissue is a target of androgen action. In the ovary, the AR is expressed in the oocytes, granulosa cells, and theca cells of both preantral and antral follicles (Walters, 2015). Mice deficient in AR (ARKO) have been an essential tool for identifying the roles that androgens play in females. ARKO mice display a markedly reduced fertility accompanied by defective follicular growth, ovulation, and corpora lutea formation, indicating that androgens may play an essential role in the ovary (Cheng et al., 2013). In the uterus, AR is expressed in the glandular and luminal epithelial cells and stromal cells where androgens stimulate cell proliferation. In support, ARKO mice have thinner uteruses than their wild type littermates and also display a diminished uterine hypertrophy in response to superovulation (Cheng et al., 2013). Exposure to hydroxyflutamide, an AR antagonist, leads to suppressed decidualization and delayed initiation of implantation and fetal development in rats. Androgens also play important roles in regulating pregnancy and parturition by preventing uterine contractions during pregnancy (Burger, 2002).

In the breast, androgens suppress mammary gland growth by inhibiting the proliferation of epithelial and stromal cells where AR is expressed. Athletic women who are exposed to an excess androgen level show atrophy in their breast tissue, and dihydrotestosterone (DHT) is often used to suppress breast growth during puberty. Importantly, steroidogenic enzymes involved in androgen biosynthesis are expressed in the mammary tissue at different stages of pregnancy and lactation, indicating a *de novo* androgen synthesis in the developing mammary gland. Androgens also decrease milk production and are used to suppress unwanted lactation. Therefore, an exposure to antiandrogens will influence both development and function of reproductive organs in females.

Antiandrogens and Their Medical Uses

In women, the main androgens are testosterone and DHT, which is more potent than testosterone and is produced from testosterone by an enzyme, 5- α reductase. A variety of pathological conditions such as polycystic ovary, ovarian carcinoma, and adrenal gland carcinoma or hyperplasia lead to hyperandrogenism, a condition in which the blood androgen level is higher than the physiologically normal concentration. Women with hyperandrogenism may suffer from symptoms such as hirsutism, acne, androgenetic alopecia, menstrual abnormality, anovulation, recurrent pregnancy loss, and dyslipidemia, and they often seek medical attention for treating the symptoms (Walters et al., 2016).

Commercial Antiandrogens

Antiandrogen medications are currently used in a variety of androgen-driven pathological conditions. Antiandrogens are classified as steroidal or nonsteroidal. Nonsteroidal antiandrogens are considered to be purer than steroidal antiandrogens because they have higher specificities to their targets than steroidal ones do. Steroidal antiandrogens such as cyproterone acetate and spironolactone not only bind to AR but exhibit cross reactivity to other steroid hormone receptors. The following are antiandrogens widely used in women (Gao and Mahto, 2016).

Flutamide is a nonsteroidal antiandrogen and is used for women with PCOS as a means to control symptoms such as acne, hirsutism, and alopecia. Flutamide acts as a selective AR antagonist, displaying competitive binding affinity against testosterone and DHT.

Table 1 Natural products as antiandrogens

Product names	Sources	Treatments of	Action mechanism
White peony	Ornamental plants (<i>Paeonia lactiflora</i>)	Hirsutism	Inhibits the production of testosterone and promotes the activity of the aromatase
Licorice	Herbaceous plants (<i>Glycyrrhiza glabra</i>)	Hirsutism, hyperlipidemia atherosclerosis	Reduces free testosterone in the blood and block androgen receptors
Red reishi	Mushroom (<i>Ganoderma lucidum</i>)	Hirsutism	Inhibits 5 α reductase
Saw palmetto	Small palm tree (<i>Serenoa repens</i>)	Acne	Inhibits 5 α reductase
Spearmint	Herbaceous plants (<i>Mentha spicata</i>)	Alopecia	Inhibits 5 α reductase
		PCOS	Reduce free testosterone in blood
		Hirsutism	
		Breast cancer	
Black cohosh	Buttercup plants (<i>Actaea racemosa</i>)	Premenopausal symptoms	Inhibit breast cancer cell proliferation

Cyproterone acetate is a steroidal antiandrogen used mainly for treating acne and hirsutism and also for hormone therapy for transgender women. Cyproterone is a potent AR antagonist that competitively binds to AR against testosterone and DHT (Gao and Mahto, 2016). It also has antigonadotropic effects: it reduces the hypophyseal storage and synthesis capacity of gonadotropins in the pituitary, which suppress ovarian function and reduce serum androgen levels.

Spironolactone is a steroidal antiandrogen used for treating acne, hirsutism, and alopecia and as hormone therapy for transgender women. It reduces testosterone level by a direct inhibition of the 17 α -hydroxylase enzyme that is necessary for the biosynthesis of testosterone. Spironolactone also inhibits 5- α reductase, which is the enzyme that converts testosterone to the more potent DHT hormone. It also act as an aldosterone antagonist by inhibiting aldosterone binding to mineralocorticoid receptors in the adrenal glands, thus disrupting the sodium/potassium balance. Therefore, this drug is primarily used to treat fluid build-up due to heart failure, liver scarring, or kidney disease, and in the treatment of high blood pressure (Gao and Mahto, 2016).

Natural Products as Antiandrogens

In recent years, there has been an increasing demand for natural (or plant-derived) antiandrogens for treating hyperandrogenism in women. Natural antiandrogens are used mainly to reduce symptoms associated with polycystic ovarian syndrome (PCOS), hirsutism, and acne (Grant and Ramasamy, 2012). Natural antiandrogens are used mostly to either decrease testosterone synthesis (white peony, licorice, and spearmint) or reduce DHT level by inhibiting 5- α reductase (red reishi, saw palmetto, and *Camellia sinensis*). Table 1 lists some of the most widely used natural antiandrogens and their medical uses.

Antiandrogens as Endocrine Disrupting Chemicals

The endocrine disrupting chemicals (EDCs) are exogenous substances that can alter the normal balance of endocrine system. Some EDCs are from plants or animals, but the vast majority are synthetic chemical compounds that are produced as industrial products and released into the environment. There is considerable concern about the impact of these synthetic compounds on public health, as they are associated with a variety of pandemic health problems in men and women. In particular, exposure to EDCs during the fetal developmental period may have life-long or even transgenerational impacts, even when exposure levels are below those considered to be harmful to adults (Costa et al., 2014).

EDCs mainly interfere with the synthesis, secretion, metabolism, and transport of endogenous hormones through direct binding to specific hormone receptors. An EDC either mimics the action of a hormone or therefore exerts an “agonistic effect,” or it blocks/inhibits the hormone’s action, inducing an “antagonistic effect.” Initially, it was thought that EDCs exert their actions only by binding to nuclear hormone receptors, including estrogen receptors (ERs), AR, and thyroid hormone receptors (TRs) (Costa et al., 2014). However, recent studies show that EDCs also act through nonsteroidal receptors, transcriptional coactivators, and enzymatic pathways that are involved in steroid biosynthesis. They can also modify gene expression by causing epigenetic modifications without inducing mutation.

A number of EDCs exert antiandrogenic activity. Antiandrogenic EDCs can be classified according to their modes of actions. Some EDCs inhibit androgen synthesis, while others bind to AR. Table 2 lists representative EDCs that are known to exert antiandrogenic effect on females, and more details about different pathological effects of antiandrogens are provided in Fig. 1.

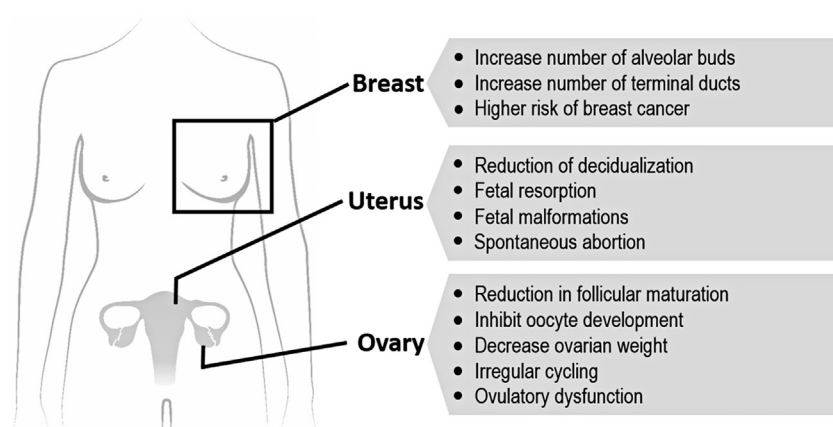
Antiandrogens With Inhibitory Activity on Testosterone Synthesis

DEHP (di-ethylhexyl phthalate)

Phthalates are a family of compounds that are used extensively in medical, automotive, and cosmetics production. There are six different types of phthalates; DEHP (di-ethylhexyl phthalate), BBP (benzyl-butyl phthalate), and DINP (diisononyl phthalate) have potent antiandrogenic properties. DEHP is the most widely used phthalate and is used mainly as a plasticizer in polyvinyl

Table 2 Synthetic chemicals as antiandrogens

<i>EDCs</i>	<i>Types</i>	<i>Active metabolite</i>	<i>Mode of action</i>
DEHP (di-ethylhexyl phthalate)	Plasticizers	MEHP	Inhibit testosterone synthesis
BBP (benzyl butyl phthalate)	Plasticizers	MBP	Inhibit testosterone synthesis
DBP (dibutyl phthalate)	Plasticizers	MBP	Inhibit testosterone synthesis
DDT (dichlorodiphenyltrichloroethane)	Organochlorine pesticide	DDE	AR antagonist
Methoxychlor	Organochlorine pesticide	HPTE	AR antagonist
Vinclozolin	Dicarboximide fungicide	M1, M2	AR antagonist
Procymidone	Dicarboximide fungicide	Not known	AR antagonist
Linuron	Urea-based herbicide	Not known	AR antagonist

**Fig. 1** Pathological effects of exposure to EDC antiandrogens in ovary, uterus and breast.

chloride (PVC) products such as plastic toys or intravenous blood bags. The main active metabolite of DEHP is mono-(2-ethylhexyl) phthalate (MEHP), which is more potent than DEHP itself and responsible for most of pathological alteration coming from DEHP exposure. Prenatal exposure to DEHP leads to reduced ovarian weight and increased uterine weight in female mice. In utero or lactational DEHP exposure alters steroidogenic gene expression in the ovary, decreases estrogen synthesis, reduces ovarian weight, and reduces ovulation. Recent studies have found harmful effects of prenatal exposure to DEHP. Prenatal DEHP exposure caused increased uterine weight and decreased anogenital distance, disrupted estrous cyclicity, and reduced fertility when the subjects were examined at their adulthood. Exposure during fetal development altered follicular recruitment and development, eventually causing premature ovarian failure.

BBP (benzyl-butyl phthalate) and DBP (dibutyl phthalate)

BBP is used mainly to make vinyl tiles, car upholstery, and adhesives, and it is often metabolized into DBP, a monobutyl phthalate (MBP). In rats, in utero exposure to BBP leads to decreased uterine and ovarian weights, decreased blood progesterone levels, and postimplantation embryonic loss. Furthermore, exposure to BBP or MBP increased the risk of postimplantation embryonic loss in rats. Fetal exposure to BBP increased embryonic demise, increased resorptions, decreased the weight of live fetuses, and increased the incidence of skeletal malformations in rats. Uterine weights and progesterone levels decreased after BBP exposure, the lower serum concentrations of progesterone explained the reduction of decidualization in pregnant rats. A study reported an increase in the number of alveolar buds and terminal ducts after in utero exposure to BBP (Harris and Sumpter, 2001).

DBP exposure via the diet from GD15-PND21 induced a trend for delayed onset of vaginal opening in Sprague–Dawley rats. DBP exposure has been shown to decrease maternal weight gain, fetal weight, and food consumption and increase the risk of losing pregnancy by in rats. Another study showed that in utero exposure to doses as low as 20 ppm DBP in the diet induced hypoplasia of the mammary glands.

Antiandrogens With Inhibitory Activity on Androgen Receptor

Vinclozolin

Vinclozolin (VZ) is a dicarboximide fungicide used mainly to treat fruits and vegetables, such as lettuce, grapes and beans. Vinclozolin or its active metabolites (M1, M2) competitively inhibits the binding of androgens to their receptors, which leads to an inhibition of androgen-dependent gene expression in mammals. Embryonic exposure to vinclozolin alters steroid receptor expression and fetal genital tubercle development, causing virilization in female mice (Kelce et al., 1997). Prenatal exposure to

Vinclozolin also reduces the anogenital distance in the female rat. In fish, exposure to 700 µg vinclozolin suppressed oocyte development. Exposure to high concentrations of vinclozolin caused abnormal ovarian pathology in rats, which exhibited an elevated incidence of ovarian lipodosis and interstitial cell hyperplasia. Furthermore, a previous study has showed the transgenerational effect of vinclozolin on DNA methylation of several imprinted genes in a mouse.

Procymidone

Procymidone is another dicarboximide fungicide similar in structure to vinclozolin and widely used to protect fruits from fungal infection. Procymidone has antiandrogenic properties, and it competitively inhibits the binding of androgens to its receptor, thereby preventing androgen function. When administered by gavage to pregnant rats (GD14–to day 3 after birth), the male pups displayed a reduced anogenital distance, nipple retention, hypospadias, and reduced accessory sex gland size. It had no effect on female pups (Ostby et al., 1999).

DDT (dichlorodiphenyltrichloroethane)

DDT is an organochlorine pesticide that has been banned in North America since 1972. The active metabolite of DDT is DDE (di-chlorodiphenyl-ethylene), which acts as androgen receptor antagonist in vivo and in vitro. DDE is ubiquitous and found in various tissues of humans throughout the world. It accumulates and persists in the environment with a half-life of more than 50 years. DDE was also found in ovarian follicular fluids and serum of women. In women undergoing in vitro fertilization, there was a correlation between high levels of DDE in follicular fluid and high levels of failed fertilization. In immature female rats, the administration of DDE resulted in a significant increase in vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 in ovarian tissue, which may lead to ovarian dysfunction. In utero exposure to DDE on GD 14–18 led to reduction of AGD, hypospadias, and retained nipples in male rat offspring. A study of Alaska Native women reported that DDE concentration in serum was related to an increased risk of breast cancer. The largest study performed includes more than 2000 American women and reports that PCB and DDE concentration in serum was related to increased menstrual cycle length and irregularity. A study of Chinese textile workers indicated increased odds for spontaneous abortion with increasing total DDT and DDE serum levels (Kelce et al., 1997).

Methoxychlor

Methoxychlor is a synthetic organochlorine used as an insecticide to protect crops, livestock, and pets against insects. It was intended to replace DDT, but it has been banned because of its acute toxicity, bioaccumulation, and endocrine disruption activity. The main active metabolite of methoxychlor is HPTE (2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane), which acts as an estrogen receptor alpha (ER α) agonist, ER β antagonist, and AR antagonist. Methoxychlor and its metabolized product HPTE are reproductive toxicants that target antral follicles of the mammalian ovary as they inhibit follicular development, reducing the number of antral follicles and increasing the preantral follicles in the rat ovary (Uzumcu et al., 2006). Oral exposure to methoxychlor in female rats for 14 days led to atresia of the ovarian follicles with pyknosis and karyorrhexis of the granulosa cells; this altered folliculogenesis and inhibited the gap junction formation between oocyte and cumulus. Administration of methoxychlor (25 and 50 mg kg⁻¹ day⁻¹) to female rhesus monkeys in the peripubertal period led to shorter follicular stages, which influence the ovarian cyclicity.

Linuron

Linuron is a urea-based herbicide that acts as a weak androgen receptor antagonist in vitro and in vivo and disrupts androgen-dependent male reproductive tract development after gestational exposure (Gray et al., 2001). When administered to pregnant rats (GD14–18; 100 mg kg⁻¹ day⁻¹), the male pups displayed a reduced anogenital distance, retention of areolas, and reduced size of androgen-dependent tissues, including seminal vesicles, ventral prostate, and epididymis.

In conclusion, it is clear that androgens play important roles in the regulation of female reproductive function and have a critical role during embryonic development and sex differentiation. It is possible that some natural antiandrogens could be used as therapeutics for modulating female and male reproductive disease. Antiandrogens have a great impact in female reproductive capacity, while many synthetic chemicals with antiandrogenic activity are still in use. Future research is needed to explore the different possible pathological hazards from these chemicals and how we can minimize the health hazards associated with them and increase the public awareness in this issue.

References

- Burger, H. G. (2002). Androgen production in women. *Fertility and Sterility*, 77, 3–5.
- Cheng, X. B., Jimenez, M., Desai, R., Middleton, L. J., Joseph, S. R., Ning, G., et al. (2013). Characterizing the neuroendocrine and ovarian defects of androgen receptor-knockout female mice. *AJP: Endocrinology and Metabolism*, 305(6), 717–726.
- Costa, E. M. F., Spritzer, P. M., Hohl, A., & Bachega, T. (2014). Effects of endocrine disruptors in the development of the female reproductive tract. *Arquivos Brasileiros de Endocrinologia e Metabologia*, 58(2), 153–161.
- Gao, J., & Mahto, A. (2016). Anti-androgen therapy in female adult acne. *Journal of Dermatology Research and Therapy*, 2(4), 24–26.
- Goodarzi, M. O., Dumesic, D. A., Chazenbalk, G., & Azziz, R. (2011). Polycystic ovary syndrome: Etiology, pathogenesis and diagnosis. *Nature Reviews Endocrinology*, 7(4), 219–231.
- Grant, P., & Ramasamy, S. (2012). An update on plant derived anti-androgens. *International Journal of Endocrinology and Metabolism*, 1010(22).

- Gray, L. E., Ostby, J., Furr, J., Wolf, C. J., Lambright, C., Parks, L., et al. (2001). Effects of environmental antiandrogens on reproductive development in experimental animals. *Human Reproduction Update*, 7(3), 248–264.
- Harris, C. A., & Sumpter, J. P. (2001). The endocrine disrupting potential of phthalates. In *Endocrine disruptors—part I*. Berlin, Heidelberg: Springer.
- Kelce, W. R., Lambright, C. R., Gray, L. E., & Roberts, K. P. (1997). Vinclozolin and p,p'-DDE Alter androgen-dependent gene expression: In vivo confirmation of an androgen receptor-mediated mechanism. *Toxicology and Applied Pharmacology*, 142(1), 192–200.
- Ostby, J., Kelce, W. R., Lambright, C., Wolf, C. J., Mann, P., & Gray, L. E. (1999). The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. *Toxicology and Industrial Health*, 15(1–2), 80–93.
- Uzumcu, M., Kuhn, P. E., Marano, J. E., Armenti, A. E., & Passantino, L. (2006). Early postnatal methoxychlor exposure inhibits folliculogenesis and stimulates anti-Müllerian hormone production in the rat ovary. *The Journal of Endocrinology*, 191(3).
- Walters, K. A. (2015). Role of androgens in normal and pathological ovarian function. *Reproduction*, 149(4), 193–218.
- Walters, K. A., Simanainen, U., & Gibson, D. A. (2016). Androgen action in female reproductive physiology. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 23(3), 291–296.
- Zhou, X. (2010). Roles of androgen receptor in male and female reproduction: Lessons from global and cell-specific androgen receptor knockout (ARKO) mice. *Journal of Andrology*, 31(3), 235–243.

Estrogenic Agonist

Enrique H Luque, Mónica Muñoz-de-Toro, and Jorge G Ramos, National University of the Littoral, Santa Fe, Argentina

© 2018 Elsevier Inc. All rights reserved.

Introduction

Estrogen plays vital roles by regulating many different physiological functions in both male and female organs. Estrogen mediates its action almost entirely through its alpha and beta estrogen receptors (ER α and ER β), which further function as transcription factors. The actions of estrogens are also mediated by membrane-bound proteins such as G protein-coupled estrogen receptor (GPER), inducing a nongenomic cellular effect. Estrogenic agonists are compounds that selectively stimulate estrogen-like actions in target tissues. These compounds have different chemical structures, and may be either natural or synthetic, or can be metabolic products from a compound lacking estrogenic activity (Table 1). These compounds act or function according to the tissue in which they are.

Sources of Estrogenic Agonists

A large number of man-made chemicals are manufactured worldwide and most of them are currently released into the environment. Throughout their lives, both humans and animals are exposed to many of these chemicals, including estrogenic agonists also known as xenoestrogens or environmental estrogens, which can have a positive or negative impact on their health and lifestyle. These compounds can be found in the environment, food, cosmetics, household items, pharmaceutical products, and many other products. Some of them have been tested for hormonal activity and have been shown to have the potential to disrupt the endocrine system of many animal species, including humans. This led to the introduction of the term “endocrine disruption.” In both human and rodent models, the exposure to ubiquitous environmental estrogenic agonists during early life has been shown to disrupt the normal development of target tissues and may cause permanent adverse effects. Environmental agents that mimic or block various reproductive hormones may also have transgenerational effects, and their ubiquity has increased the concern for human and ecological health.

A well-known example of a widespread estrogen agonist is bisphenol A (BPA), one of the highest-volume chemicals produced worldwide. Most people are exposed to it daily by consuming food and beverages into which BPA has leached from polycarbonate containers, including reusable bottles and baby bottles. Although initially considered a weak environmental estrogen, BPA may be similar to 17 β -estradiol (E2) in stimulating cellular responses, especially at low but environmentally relevant doses (nM), as demonstrated by recent studies.

Agrochemical compounds such as endosulfan, glyphosate-based herbicides (GBHs), and atrazine also behave as estrogenic agonists. In adult ovariectomized rats, environmentally relevant doses of endosulfan have been found to mimic the nonuterotrophic E2 actions, strengthening the hypothesis that this pesticide is a widespread estrogen agonist. Regarding glyphosate, which is the

Table 1 Representative examples of synthetic and natural estrogenic agonists

Industrial byproducts	Polychlorinated biphenyls (PCBs) Polybrominated biphenyls (PBBs) Dioxins
Plastics and plasticizers	Bisphenol A (BPA) Phthalates
Pesticides	Methoxychlor Chlorpyrifos Dichlorodiphenyltrichloroethane and metabolites (DDT, DDE) Endosulfan (END) Atrazine (ATZ) Glyphosate-based herbicides (GBH)
Pharmaceutical agents	DES Tamoxifen Gestodene
Detergent and biodegradation products	Octyl-phenol (OP) Nonylphenol (NP)
Phytoestrogens	Genistein Coumestrol Daidzein Resveratrol Naringenin
Mycotoxins	Zearalenone
Metals	Cadmium

active ingredient of several herbicide formulations, uterotrophic assays with a GBH formulation have shown that although GBH does not alter uterine weight, it increases the luminal epithelial cell height and downregulates ER α mRNA in all GBH- and E2-treated groups. Finally, atrazine is also considered an estrogenic agonist due to its ability to induce aromatase activity and thus lead to inappropriate and excess estrogen production.

Pharmacological compounds such as tamoxifen, the first selective estrogen receptor modulator used clinically to treat breast cancer and still widely prescribed in premenopausal women, can act as both an estrogen antagonist and an estrogen agonist. Tamoxifen is an estrogen antagonist in breast and brain tissue but is an estrogen agonist in the uterus and liver. Gestodene, a potent third-generation synthetic progestin, has beneficial effects on bone, due to its biotransformation into metabolites with intrinsic estrogenic activity.

Besides the above-mentioned synthetic compounds, phytoestrogens, zearalenone (ZEN), and cadmium are examples of naturally occurring estrogen agonists. Phytoestrogens are nonsteroidal plant compounds that can behave as weak estrogen mimics or as antiestrogens. These natural plant products are grouped into several classes, including lignans, isoflavones, and coumestans. In vitro studies have shown that phytoestrogens bind both ER α and ER β . ZEN is a mycotoxin with high estrogenic activity in vitro and in vivo, which accumulates in grains mainly at preharvest but also at postharvest under poor storage conditions. Thus, ZEN is a widespread contaminant in grain-based food products. Although ZEN exhibits low acute toxicity, long-term exposure to ZEN may present a health risk due to its high estrogenic activity. Cadmium also has potent estrogen-like activity in vivo. Exposure to cadmium increases uterine wet weight, promotes growth and development of the mammary glands, and induces hormone-regulated genes in ovariectomized animals.

Tissue Targets and Mechanisms of Action of Estrogenic Agonists

Estrogen contributes to the maintenance of body homeostasis by regulating many different physiological functions. Estrogen actions in reproductive and nonreproductive tissues rely on a complex net of nuclear and extranuclear signal transduction pathways triggered by at least two ER subtypes (ER α and ER β), which belong to the nuclear receptor superfamily of ligand-inducible transcription factors. There are two major mechanisms of ER-mediated transcriptional regulation (**Fig. 1**) the so-called classical mechanism, where ERs bind directly to the promoter regions of target genes by canonical sequences like estrogen responsive elements (EREs), and the nonclassical mechanism, which involves ERs regulating gene expression by associating with other transcription factors such as c-Jun and c-Fos, which bind DNA but not by direct ER–DNA binding. Estrogenic agonists bind to ERs and interfere with their signals and intracellular functions. As aforementioned, the chemical nature of these compounds includes a variety of structures; however, a growing mass of evidence has shown that structural similarities of estrogenic agonists correlate with their estrogenic activity when the classical mechanism of action of ERs is involved. By using a 3 $\times$ perfect ERE Luc reporter (GGTCAnnnTGACC) and a pS2 imperfect ERE Luc (GGTCAnnnTGGCC), [Li et al. \(2013\)](#) demonstrated that BPA, fluorinated BPA, and HPTE (an estrogenic metabolite of the pesticide methoxychlor) strongly activate ER α ERE-mediated responses (**Fig. 1**). Other estrogenic agonists like Daidzein, Genistein, Kaempferol, and Coumestrol also induce both ER α and ER β ERE-mediated activity (**Fig. 1**). Estrogenic agonists can also activate the nonclassical ER mechanism in a manner not associated with chemical structure similarity, modulating the activity of other transcription factors such as AP-1 and Sp1. In 1995, [Webb et al.](#) first reported ER activation of the -73 collagenase AP-1 promoter. By using this promoter in different constructions in HeLa cells, these authors found ER α AP-1-mediated activation by Kaempferol, Apigenin, and Coumestrol and significant activation of the ER β AP-1 promoter by Daidzein. These data indicate that estrogen agonists activate the nonclassical ER mechanism in a manner not associated with their chemical structure.

Recent studies indicate that environmental estrogens have the ability not only to disrupt ER signaling, but also to reprogram the epigenome by altering DNA and histone methylation through rapid, nongenomic ER actions. MCF-7 breast cancer cells have been identified as targets for epigenetic reprogramming by estrogenic agonists. These observations, in addition to other published data, support the hypothesis that activation of rapid signaling by environmental estrogens can lead to epigenetic reprogramming and contribute to fertility problems and progression of some diseases.

Biomarkers

The term “biomarker” is used for a wide variety of purposes. Thus, confusion often results when biomarker development, validation, and qualification are discussed. Biomarkers are useful to bio-monitor estrogenic exposure and/or estrogenic actions. The concentrations of estrogen agonists and/or of their metabolites in samples such as urine, serum, tissue, eggs, etc. are classified as exposure biomarkers, whereas morphological, biochemical, and transcriptional (estrogen-regulated gene expression: mRNA and proteins) biomarkers that evidence biological responses are classified as Effect Biomarkers.

Representative useful biomarkers of estrogenic actions (Effect Biomarkers) used in toxicology and ecotoxicology are listed in **Table 2**.

Exposure to Estrogenic Agonists and Toxicological Effects

The developmental origins of health and disease (DOHaD) paradigm posits that, during development, there are sensitive windows in which tissue formation and function can be modified by environmental stressors (such as estrogenic agonists), which can lead to

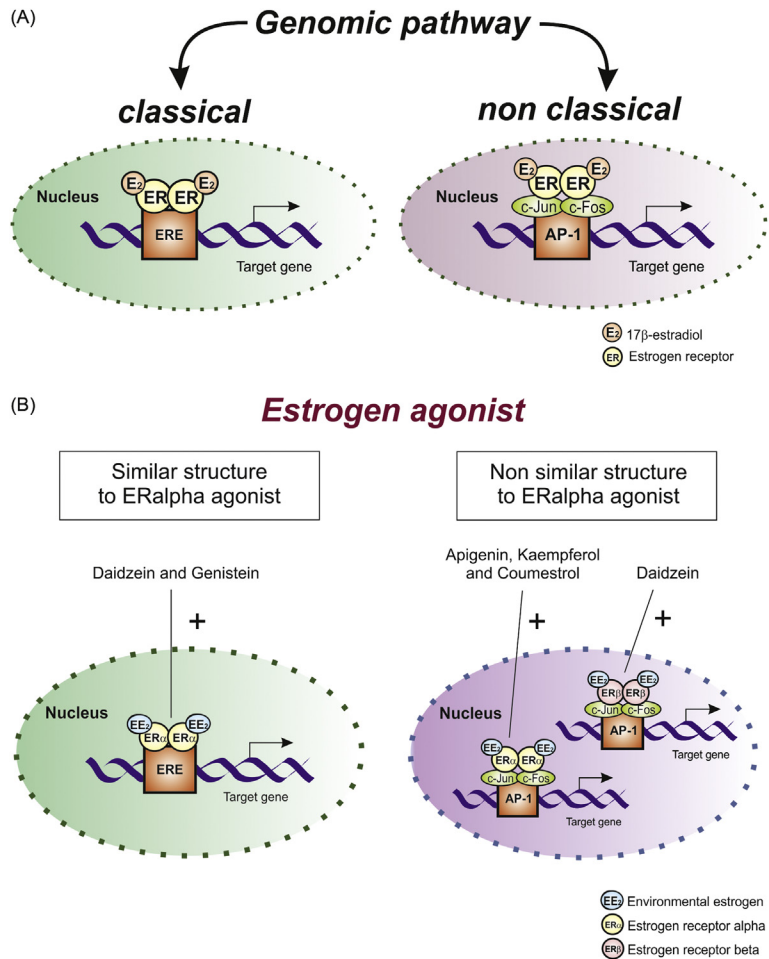


Fig. 1 Panel (A) shows a schematic representation of the classical and nonclassical genomic mechanisms of ER-mediated transcriptional regulations of estrogens. Panel (B) shows the hypothesis that structural similarities of estrogenic agonists correlate with different mechanisms of action, using a plethora of protein intermediates that lead to a selected genomic expression (illustrated by Paola Ingaramo and Gabriela Altamirano).

Table 2 Biomarkers of estrogenic actions

Biomarkers	
Biological responses	Estrogen-sensitive genes
Uterine wet weight	Progesterone receptor (PR)
Onset of puberty	Vitellogenin
Sexual receptivity	Complement component C3
Lordosis response	Ornithine decarboxylase
Ovarian follicular dynamic	Casein
MG growth and differentiation	Whey acidic protein
Osteoblast proliferation, differentiation, and mineralization	Lactoferrin
Reptilian sex determination	

MG, mammary gland.

increased susceptibility to adverse health outcomes across the life course. The principle for this paradigm comes from data published in the 1970s showing that daughters of mothers who took the estrogenic drug diethylstilbestrol (DES) during pregnancy experienced a greater risk of a rare vaginal cancer and other reproductive abnormalities. Since then, many studies on natural or experimental exposures to estrogenic agonists have been performed in animal models, wild animals, and humans. These studies showed that exposure to estrogenic agonists during gestation induces morphological alterations in both the stroma and epithelium of estrogen-sensitive tissues during fetal and/or neonatal life, and it has been postulated that the primary targets of estrogenic agonists are the fetal tissues that express ER. An estrogenic compound would then alter the reciprocal stroma–epithelial interactions that mediate an altered tissue differentiation and/or proliferation. In addition to this direct effect on estrogen target tissues,

estrogenic agonists may result in what has been called developmental estrogenization syndrome, indicating that many estrogenic pathways may be affected, thus resulting in altered puberty, disrupted ovarian function and infertility, endometriosis, uterine fibroids, and premature menopause.

The environmental health sciences have generated considerable attention for the possible “low-dose effects” of estrogenic agonists in mammals. The term “low-dose effects” is defined as biological changes that occur at environmentally relevant exposure levels or at doses that are lower than those reported in the United States Environmental Protection Agency standard toxicity testing guidelines. Moreover, the estrogenicity of the dose effects of estrogenic agonists varies significantly among species, and there are variations between *in vivo* and *in vitro* tests. For example, temperature sex determination in reptiles can be altered by exogenous treatment with an estrogenic substance, thus providing an important model system to determine the estrogenicity of various environmental contaminants (Table 2). However, labeling chemicals as simply estrogenic or antiestrogenic given their action in a single test species is inappropriate, because the potency of the response can also be altered given the species studied. It has been demonstrated that, in reptiles, BPA causes developmental effects similar to those observed after E2 exposure, such as sex reversal and altered gonadal histoarchitecture. The BPA dose needed to evoke an effect comparable to that of E2 in a sex reversal *in vivo* system is 100-fold larger. In contrast, data from *in vitro* studies suggest that BPA is 10,000 less potent than E2. Published results highlight the usefulness of *Caiman latirostris*, a temperature-dependent sex-determined species, as a tool to assess estrogenic activity *in vivo* and as a sentinel to monitor endocrine-disrupting chemicals in aquatic environments. Concern regarding environmental exposure to xenoestrogen and its potential impact on the development and reproduction of wildlife species is raised based on these data and upon publications showing the presence of xenoestrogens in surface waters, sediments, liquid manure samples, and sewage. Another significant issue is that most chemical exposures in the environment occur in complex combinations and mixtures.

In female mammals, estrogen agonists act as endocrine disruptors, negatively affecting several organs and systems. A detailed description of some of these adverse effects can be found below.

Brain and Hypothalamic-Pituitary Axis

Neonatal exposure to BPA alters the hypothalamic circuitry responsible for controlling estrous cyclicity and female sexual behaviors of adult rats. One of the targets of BPA action is the hypothalamic ER α gene. When female rats are neonatally exposed to BPA, this receptor is downregulated in both the medial preoptic and ventromedial nuclei, thus leading to alterations in the estrous cyclicity and inability to induce a normal E2-induced LH surge. Besides, in BPA-exposed rats, ER α protein expression is upregulated in the anteroventral periventricular nucleus and downregulated in the arcuate nucleus. Thus, neonatal BPA exposure permanently disrupts hypothalamic LHRH pre-mRNA processing in nuclei that control estrous cyclicity during adulthood. Interestingly, BPA-exposed females display significantly lower levels of proceptive sexual behavior.

Ovary and Female Reproductive Tract

Prenatal and neonatal exposure to estrogenic agonists may be responsible for disruption of follicular development. Some studies in rats have demonstrated that progesterone inhibits the assembly of primordial follicles, whereas both progesterone and estrogen reduce their initial recruitment. In mice, progesterone and estrogen prevent primordial follicle assembly, but not subsequent development. Recent observations have shown that normal folliculogenesis requires androgen receptor (AR)-mediated androgen action and that cyclin-dependent kinase inhibitor 1B, commonly known as p27kip1 or p27, suppresses primordial follicle endowment and activation and promotes follicle atresia. Exposure to DES or BPA via sc during the first 6 postnatal days disrupts follicle development in rats by reducing the primordial follicle pool by stimulating the initial recruitment, associated with an increased proliferation rate likely mediated by an estrogenic pathway. Neonatal exposure to DES or BPA also affects the ovarian follicular dynamics in lambs, showing a decline in the stock of primordial follicles with stimulation of follicular development. In these species, BPA reduces ovarian weight and increases the number of multioocyte follicles, promoting proliferation of granulosa/theca cells in antral follicles, and increasing both the number of antral atretic follicles and p27 expression. Moreover, in female lambs neonatally exposed to low doses of DES or BPA, the ovarian response to an exogenous gonadotropin stimulus (oFSH) is impaired, showing a low number of small follicles together with a lower number of atretic follicles and no increase in E2 serum levels. These alterations due to neonatal exposure to estrogen agonists may be the pathophysiological basis of the subfertility syndrome in female adulthood.

Oral exposure to BPA during rat gestation affects pup primordial follicle recruitment and increases the number of corpora lutea, indicating a larger number of ovulated oocytes, coupled with higher levels of mRNA expression of 3 β -hydroxysteroid dehydrogenase and serum progesterone. BPA-treated rat pups have lower expression of AR at different stages of the growing follicle population, evidencing an imbalance of AR expression between primordial/primary follicles, with higher mRNA-follicle-stimulating hormone receptor expression. These results add to the growing evidence that folliculogenesis and steroidogenesis are targets of BPA within the ovary.

Uterus and Implantation Process

The uterus is also a target for estrogenic agonists. Successful implantation is the result of complex molecular interactions between the hormonally primed uterus and a mature blastocyst. This carefully synchronized interplay of hormonal signals and feedback

loops is potentially vulnerable to chemicals such as estrogenic agonists that may disrupt endocrine signaling. Morphoregulator genes like members of the *Hox* gene family regulate uterine development and may be associated with endocrine-related processes such as endometrial proliferation and differentiation in the uterus. Neonatal exposure to estrogen agonists could affect the signaling events governed by *Hox* genes, altering the developmental trajectory of the uterus with lasting consequences during the preimplantation period. Neonatal exposure to xenoestrogens alters *Hoxa10* and *Hoxa11* uterine expression shortly after treatment as well as in the adult. Xenoestrogen exposure also affects the steroid-hormone responsiveness of the preimplantation uterus. These molecular alterations could explain, at least in part, the adverse effects of xenoestrogens on uterine implantation. Exposure to estrogenic agonists such as BPA, DES, endosulfan or glyphosate could contribute to the impaired female fertility noted over the past decades.

Perinatal (gestation + lactation) exposure to DES and estrogen replacement therapy induce cystic glands, glands with daughter glands, conglomerate of glands, and a decrease in progesterone receptor (PR) in the subepithelial stroma of the rat uterus. These long-lasting effects of perinatal exposure to DES include the induction of abnormalities in uterine tissues of aged female rats and an altered response of the adult uterus to E2.

Studies on the effects of neonatal exposure to BPA on the uterine response to estrogen replacement therapy in aged rats have shown an increased density of glands with squamous metaplasia and glands with daughter glands. Rats exposed to BPA and then treated with estrogen show lower *Wnt7a* (a member of the *Wnt* gene family) expression in squamous metaplasia. BPA-treated rats also show lower expression of ER α and its 5'-untranslated exons ESR1-O and ESR1-OT in the uterus. Moreover, BPA modifies the expression of uterine ER α coactivator proteins and epigenetic regulatory enzymes. Thus, perinatal estrogenic agonists-exposed rats show different glandular abnormalities associated with deregulated expression of E2-target genes.

As aforementioned, endosulfan behaves as an estrogenic agonists, and neonatal exposure to low doses of endosulfan alters the expression of proteins regulating uterine development and differentiation (i.e., *Hoxa 10* and α -smooth muscle actin expression), namely a dysregulation of steroid receptors. An aberrant ER α uterine expression is associated with a decrease in the DNA methylation status during the preimplantation period. Endosulfan increases the expression of ER α and its transcript variants ER α -OS, ER α -O, ER α -OT, and ER α -E1. Moreover, decreased DNA methylation levels have been detected in some ER α regulatory regions, suggesting an epigenetic upregulation of its transcriptions. ER α overexpression has been associated with an induction of its downstream genes, *MUC-1* and *IGF-1*, suggesting that endosulfan might alter the uterine estrogenic pathway, compromising uterine receptivity. These alterations could account, at least in part, for endosulfan-induced implantation failures.

Neonatal exposure to a GBH affects uterine morphology, proliferation and expression of proteins that regulate uterine organogenetic differentiation in rats. GBH-exposed uteri show morphological changes, characterized by an increase in the incidence of luminal epithelial hyperplasia and an increase in the stromal and myometrial thickness. GBH treatment increases cell proliferation and alters the expression of proteins involved in uterine organogenetic differentiation (ER α , PR, *Hoxa10*, *Wnt7a*). All these changes may alter the functional differentiation of the uterus, affecting female fertility and/or promoting the development of neoplasias. Neonatal exposure to a GBH also alters the reproductive performance and the molecular mechanisms involved in the decidualization process in adult rats. GBH-exposed rats show an increased number of resorption sites, associated with an altered decidualization response, as well as morphological and molecular changes at the implantation sites, associated with decreased expression of ER α and PR, a downregulation of *COUP-TFII* (*Nr2f2*) and *Bmp2* mRNA and increased expression of *HOXA10* and increased cellular proliferation. These alterations in endometrial decidualization might be the mechanism of GBH-induced postimplantation embryo loss.

The female reproductive tract disorders caused by exposure to endocrine disruptors have not been totally clarified. Different mechanisms would be involved in the effects on the uterus during implantation and development depending on the estrogenic agonist compound (BPA, DES, endosulfan, or glyphosate) and the dose administered. BPA may alter overall female reproductive capacity by affecting the morphology and function of the female reproductive system, including the ovary, and may be associated with infertility in women.

Mammary Gland and Lactation

Exposure to estrogens throughout a woman's life, including the period of intrauterine development, is a risk factor for the development of breast cancer. The increased incidence of breast cancer noted during the last years may have been caused, in part, by exposure of women to estrogenic agonists. The mammary glands (MG) of BPA-exposed rodents show differences in the rate of ductal migration into the stroma during initial development and a significant increase in the percentage of ducts, terminal ducts, terminal end buds, and alveolar buds at adult age. The altered cellular proliferation and histoarchitecture, coupled with an increased presence of secretory product within alveoli, resemble those of early pregnancy, suggesting disruption of the hypothalamic-pituitary-ovarian axis and/or misexpression of developmental genes. The altered relationship in cellular proliferation between the epithelium and stroma and the increase in terminal ducts and terminal end buds are striking, because these changes are associated with carcinogenesis in both rodents and humans.

Prenatal exposure to BPA alters the endocrine environment of the MG and its angiogenic process, associated with higher expression of vascular endothelial growth factor and increased vascular areas. The increased angiogenesis and altered steroid hormone signals could explain the higher frequency of preneoplastic lesions later in life. Of special concern is the increased terminal end bud density at puberty and the increased number of terminal ends at adulthood, as these two structures are the sites at which

cancer arises in humans and rodents. Besides affecting the female MG, BPA, and endosulfan modify the development of the male rat MG by inducing low expression of AR.

Xenoestrogen-exposed rats also show changes in milk protein and fatty acid synthesis and secretion during lactation. Modifications in female MG morphology and differentiation due to estrogenic agonists exposure could alter the quality and quantity of milk produced by the exposed individuals. Thus, perinatal exposure to environmentally relevant xenoestrogen doses results in persistent alterations in MG morphogenesis and functional activity.

Organochlorine compounds (OCCs), such as pesticides and polychlorinated biphenyls (PCBs), are persistent lipophilic chemicals, some of which have been identified as endocrine disruptors, mainly with estrogen-like effects. Some areas of Argentina characterized by intensive farming and agricultural activities and industrial development present a high incidence of environmental and dietary exposure to OCCs. Both the frequency of occurrence and levels of OCC residues in breast adipose tissue are high. The diet is a relevant source of OCC exposure, with consumption of animal fat and freshwater fish playing a significant role. Bioaccumulation is evidenced by the significant positive association between organochlorine levels and body mass index and the age of the patient. The amount and quality of OCC residues in adipose tissue adjacent to breast carcinoma affect the biological behavior of the tumor. All the ER α -positive breast carcinomas from postmenopausal women exhibit high proliferation when OCC levels in the surrounding adipose tissue reach high levels, supporting the hypothesis that OCC residues in adipose tissue adjacent to breast carcinoma generate an estrogenic microenvironment that influences the biological behavior of the tumor through ER α activation and ER α -dependent proliferation.

Estrogenic Agonists as Obesogens

The prevalence of obesity, metabolic syndrome, and type-2 diabetes has dramatically increased worldwide over the last few decades. Although genetic predisposition and lifestyle factors like decreased physical activity and energy-dense diet are well-known factors in the pathophysiology of these conditions, accumulating evidence suggests that the increase in estrogenic agonists in the environment also explains a substantial part of the incidence of the mentioned diseases. "Obesogens" are defined as chemicals that promote obesity by increasing the number of fat cells and/or the storage of fat in existing adipocytes. Obesogens can also promote obesity indirectly by shifting the energy balance to favor storage of calories, altering the basal metabolic rate, changing the gut microbiota to promote food storage, and disturbing hormonal control of appetite and satiety. New obesogens, including chemicals that cause or lead to diabetes as well as to altered lipid metabolism and fatty liver, are being identified at an increasing rate.

Epidemiological evidence and in vivo experimental data suggest an association between prenatal exposure to BPA and increased body fat at school age, induction of insulin resistance, and/or disruption of pancreatic beta cell function. Thus, it may be inferred that BPA exposure may be involved in some metabolic disorders associated with obesity.

Perinatal exposure to BPA impairs glucose homeostasis, induces obesity, and increases food intake in adult rodents, altering hypothalamic feeding circuitry. These effects could be mediated by the downregulation of ER α through a reduction of its promoter activity. BPA partially mimics the mechanisms of obesity produced by the feeding with a high-fat diet, and the combination of exposure to BPA and a high-fat diet results in an exacerbation of the individual effects.

Exposure to other estrogen agonists such as dichlorodiphenyldichloroethylene, hexachlorobenzene, or PCBs has been also linked to obesity later in life.

Conclusions

Estrogenic agonists are compounds that selectively stimulate estrogen-like actions in target tissues. These compounds have different chemical structures, and can be natural, synthetic, or metabolic products from a compound lacking estrogenic activity. As representative examples of estrogenic agonists, we can mention industrial byproducts, plastics and plasticizers, pesticides, pharmaceutical agents, detergents and biodegradation products, phytoestrogens, mycotoxins, and metals. All these compounds are present in the environment and it has been demonstrated that domestic animals, wildlife animals, and humans are contaminated with them. It is important to mention that neither animals nor humans are prepared to metabolize and detox these man-made chemicals, a fact that increases their body burden.

In environmental health sciences, the principle for the DOHaD paradigm assumes that there are developmental sensitive windows in which environmental estrogenic agonists can modify tissue differentiation and function, thus leading to increased susceptibility to adverse health outcomes during the individual's life. A limited number of studies in humans have related the exposure to estrogenic agonists to a health outcome. These include some endpoints and a variety of chemicals related to adverse effects on female reproductive organs and fertility. A better understanding of this research area will enable recommendations to prevent prenatal/in utero adverse exposures related to later adverse health outcomes.

References

- Li, Y., Luh, C. J., Burns, K. A., et al. (2013). Endocrine-disrupting chemicals (EDCs): In vitro mechanism of estrogenic activation and differential effects on ER target genes. *Environmental Health Perspectives*, 121, 459–466.
- Webb, P., Lopez, G. N., Unt, R. M., & Kushner, P. J. (1995). Tamoxifen activation of the estrogen receptor/AP-1 pathway: Potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology*, 9, 443–456.

Further Reading

- Andreoli, M., Stoker, C., Rossetti, M., et al. (2015). Withdrawal of dietary phytoestrogens in adult male rats affects hypothalamic regulation of food intake, induces obesity and alters glucose metabolism. *Molecular and Cellular Endocrinology*, 401, 111–119.
- Björnström, L., & Sjöberg, M. (2005). Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. *Molecular Endocrinology*, 19, 833–842.
- Hall, J. M., & McDonnell, D. P. (2005). Coregulators in nuclear estrogen receptor action: From concept to therapeutic targeting. *Molecular Interventions*, 5, 343–357.
- Heindel, J. J., Blumberg, B., Cave, M., et al. (2017). Metabolic disrupting chemicals and metabolic disorders. *Reproductive Toxicology*, 68, 3–33.
- Heindel, J. J., Skalla, L. A., Joubert, B. R., Dilworth, C. H., & Gray, K. A. (2017). Review of developmental origins of health and disease publications in environmental epidemiology. *Reproductive Toxicology*, 68, 34–48.
- Kass, L., Altamirano, G., Bosquiaz, V., Luque, E. H., & Muñoz-de-Toro, M. (2012). Perinatal exposure to xenoestrogens impairs mammary gland differentiation and modifies milk composition in Wistar rats. *Reproductive Toxicology*, 33, 390–400.
- Monje, L., Varayoud, J., Muñoz-de-Toro, M., Luque, E. H., & Ramos, J. (2010). Exposure of neonatal female rats to bisphenol A disrupts hypothalamic LHRH pre-mRNA processing and estrogen receptor alpha expression in nuclei controlling estrous cyclicity. *Reproductive Toxicology*, 30, 625–634.
- Nilsson, E. E., & Skinner, M. K. (2015). Environmentally induced epigenetic transgenerational inheritance of reproductive disease. *Biology of Reproduction*, 93(145), 1–8.
- Rey, F., Ramos, J. G., Stoker, C., et al. (2006). Vitellogenin detection in broad-snouted caimans, *Caiman latirostris* (Crocodylia: Alligatoridae). A tool to assess environmental estrogen exposure in wildlife. *Journal of Comparative Physiology. B*, 176, 243–251.
- Rivera, O., Varayoud, J., Rodriguez, H., et al. (2015). Neonatal exposure to xenoestrogens impairs the ovarian response to gonadotropin treatment in lambs. *Reproduction*, 149, 645–655.
- Stoker, C., Rey, F., Rodriguez, H., et al. (2003). Sex reversal effects on *Caiman latirostris* exposed to environmental relevant doses of the xenoestrogen bisphenol A. *General and Comparative Endocrinology*, 133, 287–296.
- Varayoud, J., Ramos, J., Muñoz-de-Toro, M., & Luque, E. H. (2014). Long-lasting effects of neonatal bisphenol A exposure on the implantation process. In G. Litwack (Ed.), *94. Endocrine disruptors (vitamins and hormones)* (pp. 253–275). Cambridge, MA: Academic Press/Elsevier.
- Vigizzi, L., Ramos, J. G., Kass, L., et al. (2016). A deregulated expression of estrogen-target genes is associated with an altered response to estradiol in aged rats perinatally exposed to bisphenol A. *Molecular and Cellular Endocrinology*, 426, 33–42.
- Ziv-Gal, A., & Flaws, J. A. (2016). Evidence for bisphenol A-induced female infertility: A review. *Fertility and Sterility*, 106, 827–856.

Diethylstilbestrol (DES)

Sarah Bjorkman and Hugh S Taylor, Yale School of Medicine, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Diethylstilbestrol (DES) is a synthetic estrogen analogue prescribed to pregnant women during the mid 1900s with the belief that it helped to reduce the risk of miscarriage and preterm delivery (Smith and Gabbe, 1999). This thought continued despite a large placebo-controlled, double-blind study in 1951 showing that DES had no such positive benefit (Dieckmann et al., 1999). The continued prescription of DES without evidence of its efficacy, combined with a large production base and vast potential patient population, led millions of women and developing infants in the US and around the world to be exposed to DES between 1940 and 1971 (Goodman et al., 2011). This widespread exposure has, unfortunately, not been without consequence. There are now well-established adverse effects of direct, and most notably, in utero exposure to DES, including vaginal and clear cell adenocarcinoma, (Herbst et al., 1971; Hill, 1973) structural and functional reproductive tract anomalies (Bibbo et al., 1977; Kaufman et al., 2000; Senekjian et al., 1988), many pregnancy complications (Kaufman et al., 2000, 1980; Barnes et al., 1980; Goldberg and Falcone, 1999), and even deleterious psychosexual effects (Guterman et al., 1986; Katz et al., 1987).

History, Development, and Initial Use

DES was first synthesized in 1938 by Leon Golberg and Sir Robert Robinson at the University of Oxford's Courtauld Institute of Biochemistry–Dyson Perrins Laboratory (Dodds et al., 1938). Funding for the research leading to the initial synthesis of DES was provided by the UK Medical Research Council, which prohibited the patenting of medications discovered using public funds. This lack of patent restriction subsequently led DES to be produced by over 200 different makers world-wide. This resulted in a rapid distribution and vast area of influence (Meyers, 1983). Between 1940 and 1978, it is estimated that 5–10 million pregnant women, and children born to these women, were exposed to DES in the United States, with millions more exposures across the globe (Giusti et al., 1995).

Developed as an estrogen analogue, DES was initially marketed and utilized for the relief of a wide variety of symptoms related to estrogen-deficient states, with initial FDA approved indications for gonorrheal vaginitis, atrophic vaginitis, menopausal symptoms, and postpartum lactation suppression to prevent breast engorgement (Goodman et al., 2011; Meyers, 1983). In the early 1940s, placental hormones were believed to play a positive role in the continuation of pregnancy, and research at the time suggested beneficial results of using DES as the estrogen of choice in preventing miscarriage (Bamigboye and Morris, 2003). This presumptive data lead to the off-label use of DES for the prevention of miscarriage beginning in the early 1940's, with official FDA approval for this indication beginning in 1947. An inadequately controlled observational study by O. Watkins Smith and her husband in 1948 claimed to further support the use of DES in prevention of early abortion and later-pregnancy complications (Smith and Gabbe, 1999). However, in 1953, W. J. Dieckmann presented his placebo-controlled, double-blind study to the American Gynecological Society showing that DES did not reduce the incidence of abortion, prematurity, or postmaturity. His work also refuted the Smith's findings that DES decreased the rates of toxemias of pregnancy and perinatal mortality (Dieckmann et al., 1999). Despite Dieckmann's work, DES continued to be prescribed to pregnant mothers in the US until 1971.

In 1971, a landmark article by Herbst published in the New England Journal of Medicine reported that in utero exposure to DES was associated with the development of vaginal clear cell adenocarcinoma in adolescent females (Herbst et al., 1971). This finding was followed by a nationwide notification of physicians by the FDA that DES should not be prescribed to pregnant women (Selected Item from the FDA Drug Bulletin—November 1971, 1972). In 1975, further investigation into the scope and severity of the effect from DES exposure was undertaken by the National Cancer Institute. The institute's initiation of the DES-Adenosis Project thus began the illumination of many teratogenic and carcinogenic effects from prenatal exposure to DES (Hoover et al., 2011).

After 1971, DES use continued for medical indications outside of pregnancy. Its use as a post-coital contraceptive slowed in 1975 when the FDA mandated that packaging required the label, "This drug product should not be used as a post-coital contraceptive" (FDA, 1975). Advanced breast cancer in postmenopausal women was treated primarily with DES until the approval of Tamoxifen in 1977 (Ingle et al., 1981). In men, DES was used as the first-line treatment for advanced prostate cancer from 1941 through 1985, until Leuprolide was found to have similar efficacy (The Leuprolide Study Group, 1984). The FDA finally withdrew approval for DES in September of 2000 (FR Doc No: 00-23477, 2000).

Outside of direct human exposure, DES was also used as an additive to livestock feed in the 1960s. Given an incidence of similar unique diagnoses occurring in women who had not been exposed to DES directly or in utero, there is some conjecture that the mothers of young women who developed CCA may have been exposed to DES through dietary sources (Goodman et al., 2011) (Fig. 1).

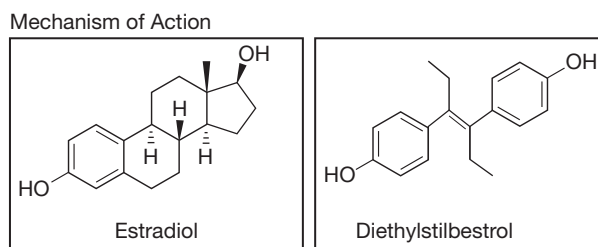


Fig. 1 Chemical structures of Estradiol and Diethylstilbestrol.

Mechanism of Action

As an Estrogen Receptor Agonist

DES is a nonsteroidal open-ring analogue of estradiol, and functions as a full agonist of nuclear estrogen receptors (ERs). ERs are expressed in two separate classes, ER α and ER β . In vivo activation of ERs is accomplished by estrogens, with the most abundant being estradiol (17 β -estradiol) (Dahlman-Wright et al., 2006). In discussing the effects of DES, it is important to note the expression patterns of ER α and ER β . ER α is expressed in the uterine endometrium, ovarian stromal cells, the hypothalamus, and breast cancer cells (Yaghmaie et al., 2005). It is also expressed in the epithelium of the efferent ducts in males (Hess, 2003). ER β is expressed in the kidneys, brain, bone, heart, lungs, intestinal mucosa, prostate, ovarian granulosa cells, and endothelial cells (Babiker et al., 2002).

Molecular dimensions of DES, as determined by x-ray crystallography, are nearly identical to those of estradiol, with notable equal distances between the hydroxyl groups at either end of the molecules (Ravina, 2011). The widespread effect of DES is secondary to the nearly ubiquitous expression of ERs throughout the female and male bodies, and further compounded by DES's elevated binding affinity over that of estradiol. When compared to estradiol, DES has a binding affinity 4.68 times greater at ER α and 2.95 times greater at ER β (Kuiper et al., 1997). Without competition, DES preferentially binds ER β with EC50 of 0.06 nM compared to an EC50 of 0.18 nM required at ER α (Coss et al., 2012).

As a Modulator of Fetal Development

The most notorious effect of DES use occurs not because of its classic action as an estrogen receptor agonist, but rather from its ability to cross the placenta and cause in utero effects on fetal development. DES affects fetal development by acting as an endocrine disruptor (Bromer et al., 2009; Hill et al., 1980; Akbas et al., 2004). The developing müllerian system, which eventually forms the female reproductive tract, is coded for by the homeobox gene orthologs HOXA9, HOXA10, HOXA11, and HOXA13 (Taylor et al., 1997). Normally, HOXA9 develops the oviduct at the rostral end of the human paramesonephric duct, HOXA10 is associated with the rudimentary uterus (uterine anlage), HOXA11 associated with both uterine and cervical anlagen, and HOXA13 is expressed in the caudally developing vagina (Taylor, 2008). In the short-term, use of DES directly stimulates HOXA10, causing hypermethylation of this HOXA10 gene during what is thought to be a critical window of development. This leads to long-term epigenetic change (Taylor, 2008; Titus-Ernstoff et al., 2008). Through teratogenic effect, hypermethylation subsequently functions as a mechanism of change to developmental programming where HOXA10 is expressed in both uterine and cervical segments of the developing paramesonephric duct, and HOXA11 is confined to the cervical anlage (Bromer et al., 2009; Daftary and Taylor, 2006; Sassoon, 1999). Common defects that appear as consequence of these teratogenic effects include structural, functional, and cellular changes throughout the female and male reproductive tracts which are detailed later in this article (Fig. 2).

As a Carcinogen

In addition to teratogenic changes in fetal development, DES has been discovered to play a significant role as a carcinogen. Its effect on HOX and Wnt gene families is believed to be responsible for preventing the stratification of vaginal epithelium and resorption of vaginal glands, leading to vaginal adenosis and loss of the uterotubal junction (Block et al., 2000; Ma, 2009; Laronda et al., 2012). This change is thought to play a role in estrogen's alteration of stromal epithelium leading to carcinogenic change (Block et al., 2000; Ma, 2009; Laronda et al., 2012). During the postmenarcheal adolescent period, hormonal changes trigger vaginal adenosis whereby the glandular epithelium is exposed to the environment. Glandular cells comprising the adenosis are normally protected inside of the uterus and cervix, however after DES exposure are located in the vagina, which is normally protected by a squamous epithelium. The glandular cells of the adenosis are now inappropriately "in harm's way" exposed to carcinogens in the environment and leading to the well-known peak incidence of clear cell adenocarcinoma during the teenage years of women exposed to DES during their fetal development (Guzman and Zambrano, 2007). Exposure to DES during in utero development also increases the risk for other vaginal and cervical carcinogenic change, including cervical intraepithelial neoplasia (Hoover et al., 2011). The lasting carcinogenic effects and epigenetic changes of DES are now known to be heritable in animal models, and are associated with increased rates of malignant and benign uterine and ovarian tumors in the female offspring of females who were exposed to DES during their in utero fetal development (Titus-Ernstoff et al., 2006, 2008; Newbold et al., 1998, 2006; Holliday, 1998; Walker, 1984; Turusov et al., 1992).

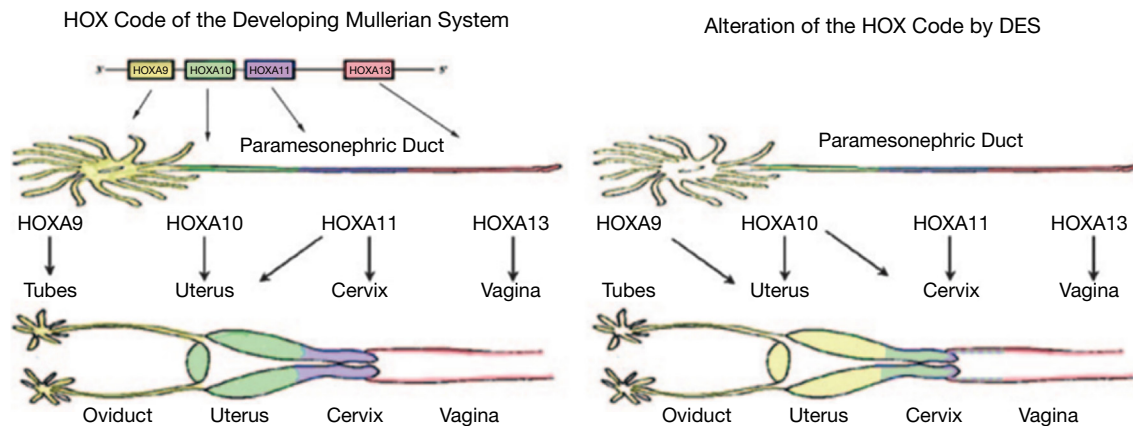


Fig. 2 Schematic representation of the spatial domains of expression of the homeobox gene orthologs HOXA9, HOXA10, HOXA11, and HOXA13 in the developing human paramesonephric duct. In keeping with the property of collinearity, the most 3' ortholog HOXA9 is expressed rostrally in the developing oviduct, whereas the most 5' ortholog HOXA13 is expressed in the developing vagina. The ortholog HOXA10 is associated with the uterine anlage, and HOXA11 is expressed in both the uterine and cervical anlagen. In utero exposure to the endocrine disruptor DES results in a posterior shift in the spatial domains of expression of these HOX orthologs. Consequently, in DES-exposed animals, HOXA9 is expressed in the uterine anlage, HOXA10 is expressed in the caudal uterine and cervical anlagen, and HOXA11 is confined to the developing cervix. The spatial domain of expression of HOXA13 remains unaffected. A shift in the spatial domains of expression of the various HOX genes may be one mechanism for DES-related teratogenicity. As taken from *Endocrine Regulation of Hox Genes* Daftary, G. S., Taylor, H. S. (2006). *Endocrine regulation of HOX genes. Endocrine Reviews*. 27, 331–355.

Deleterious Effects

DES gained its infamy from the unintended negative effects caused to women who were exposed to the medication during pregnancy (DES mothers), and, to a much greater extent, on those who were exposed to DES while developing in utero (DES daughters and DES sons). The total number of these DES mothers, daughters, and sons in the US is estimated between 5 and 10 million persons, with millions more exposed around the world (Giusti et al., 1995). As research continues on the lasting epigenetic changes caused by DES, deleterious effects are also being found in generations removed from direct exposure to DES (DES granddaughters, etc) (Titus-Ernstoff et al., 2008).

Outcomes From Direct Exposure (DES Mothers)

Women directly exposed to DES have been shown to have a moderately increased risk of breast cancer, with a reported relative risk of 1.27–1.4 as compared unexposed controls (Titus-Ernstoff et al., 2001; Laitman, 2002). There is also an increased risk of fatal breast cancers (Calle et al., 1996), and a slight but significant increase in all-cause mortality in women exposed to DES (Titus-Ernstoff et al., 2006). No evidence exists that DES mothers are at a higher risk for any type of cancer other than breast cancer, including endometrial or ovarian cancers (Giusti et al., 1995; Titus-Ernstoff et al., 2001; Troisi et al., 2007).

Outcomes From In Utero Exposure in Women (DES Daughters)

Women exposed to DES while developing in utero (DES daughters) are known to have a significant increased risk of developing various types of cancers, malformations of the female reproductive tract, pregnancy problems, and many other adverse effects.

Clear cell adenocarcinoma and precursors to reproductive tract cancer

The most prominent and first discovered negative effect of DES was the dramatic increase in the risk of clear cell adenocarcinoma (CCA) in adolescent females who had been exposed prenatally (Herbst et al., 1971). This causality was first discovered in the late 1960s after the appearance of a cluster of cases in one institution where the affected young women had prenatal exposure to DES (Herbst and Scully, 1970). Further investigation has shown a 40-fold increase in the risk of CCA of the vagina and cervix in DES daughters (Troisi et al., 2007; Swan, 2000). The incidence of CCA has been reported as 1 per 1000 by age 34 (Melnick et al., 1987), and 1.6 per 1000 by age 39 (Troisi et al., 2007). Notably, the median age of CCA diagnosis in DES daughters is in the late second decade, as compared to the previously rare occurrence of CCA seen almost exclusively in postmenopausal women (Goodman et al., 2011; Herbst and Anderson, 1990). The increased risk of CCA in DES daughters has been associated with several risk factors including maternal history of miscarriage, exposure to DES early in gestation, birth during autumn, and prematurity (Herbst and Anderson, 1990). There is no evidence of an association between pregnancy and adverse tumor characteristics, or worse prognosis (Herbst and Anderson, 1990). DES daughters are at increased risk of developing other carcinogenic changes in the reproductive tract. They are nearly 2.3 times more likely to develop cancer precursor cells (cervical intraepithelial neoplasia Grade II or worse) than nonexposed women (Hoover et al., 2011). Nearly 95% of DES daughters will have abnormal columnar epithelium

of the cervix and/or upper vagina, and 75% exposed prior to the 9th week of gestation will have vaginal adenosis (Barclay, 1979; Herbst et al., 1972).

Breast cancer

Repeated evidence in the US shows that DES daughters are at an increased risk of developing breast cancer (Hatch et al., 1998), with 2% of DES daughters developing breast cancer attributable to their exposure (Hoover et al., 2011). This increased risk is demonstrated in women after 40 years of age, with DES daughters approximately twice as likely to develop breast cancer as unexposed women with similar risk factors after this age (Troisi et al., 2007; Palmer et al., 2002, 2006). The mechanism for an increased risk of breast cancer after DES exposure is unknown, but speculated that changes in breast tissue may be mediated by the proliferative effects of estrogen (Larson et al., 2006). Epigenetic programming of genes that increase risk of breast cancer has also been described (Doherty et al., 2010).

Reproductive tract anomalies and issues with fertility

DES daughters frequently have abnormalities of the reproductive tract that can predispose to problems with reproduction (Kinch, 1982). These abnormalities can include cervical hoods or ridges, small t-shaped uteri, other abnormalities of the uterine cavity, uterine leiomyomas, and abnormalities of the fallopian tubes (Barclay, 1979; Kinch, 1982; Kaufman et al., 1977; DeCherney et al., 1981; Newbold, 2004; Baird and diethylstilbestrol, 2005). Primary cervical abnormalities include hypoplasia and shortened cervical structure (Milhan, 1992). In relation to the structural abnormalities of the uterus and fallopian tubes, DES daughters are more than twice as likely to have primary infertility than nonexposed women (Milhan, 1992; Palmer et al., 2001). A higher percentage of DES daughters than non-exposed women report trying to become pregnant without success for at least 12 months, and are also more likely to never have become pregnant (Palmer et al., 2001).

Complications in pregnancy

In addition to having difficulty becoming pregnant, DES daughters have significantly increased risk of pregnancy complications. Major complications include preterm delivery, spontaneous abortions, second trimester losses, stillbirth, neonatal death, ectopic pregnancy, and preeclampsia (Goodman et al., 2011; Kaufman et al., 2000; Kinch, 1982). Many of these complications are believed to be related to structural abnormalities in the uterus that make it difficult for the uterus to accommodate for a growing fetus (Goodman et al., 2011). In 2011, a paper published in the New England Journal of Medicine reported excess risk of these complications in DES daughters as follows: preterm delivery 35.4%, spontaneous abortions 38.6%, second trimester losses 14.7%, stillbirth 6.3%, neonatal death 7.2%, ectopic pregnancy 11.7%, and preeclampsia 13.7% (Goodman et al., 2011).

Other deleterious effects

DES daughters are reported to have more problematic sexual lives, to seem more depressive, to be more poorly adjusted to life, and to have other negative psychosexual effects. These effects are postulated to be secondary to a psychological vulnerability related to long-term stress from the obstetric and gynecologic problems they face as a result of their DES exposure (Guterman et al., 1986; Katz et al., 1987; Giusti et al., 1995). Additionally, DES daughters are 50% more likely to experience early menopause (menopause prior to age 45) than unexposed women, giving them an incidence rate of nearly 3% (Hoover et al., 2011; Hatch et al., 2006). DES daughters have also been reported to have immune system disorders (NIH DES Research Update, 1999), but there has been no difference found in the rates of rheumatologic disorders such as lupus, rheumatoid arthritis, optic neuritis, and idiopathic thrombocytopenic purpura (Strohsnitter et al., 2010).

Unique medical management

Given the many known adverse effects of in utero exposure to DES, DES daughters should have a complete pelvic examination yearly beginning at menarche or by age 14, with an interval for follow-up determined on an individual basis (Barclay, 1979; Anon, 1983).

Outcomes From In Utero Exposure in Men (DES Sons)

In addition to the major effect of in utero DES exposure on the female reproductive tract, significant consequences have also been found in males who were exposed to this substance during their fetal development. These deleterious effects seen at increased rates in DES sons can be structural, functional, or cellular abnormalities, and include hypospadias, microphallus, retained testes, testicular hypoplasia, cryptorchidism, epididymal cysts, and testicular inflammation (Giusti et al., 1995; Guzman and Zambrano, 2007; Kinch, 1982; NIH DES Research Update, 1999). Relative risks for these abnormalities are as follows: cryptorchidism 1.9%, epididymal cyst 2.5%, and testicular inflammation 2.4%. These relative risks are increased when examining only DES sons who were exposed prior to the 11th week of gestation to 2.9%, 3.5%, and 3.0% respectively (Palmer et al., 2009). Notably, there is no evidence to suggest a statistically significant difference in rates of varicocele, urethral stenosis, benign prostatic hypertrophy, or inflammation of the prostate between DES sons and unexposed men (Palmer et al., 2009). DES sons have been found to not have an increased rate of subfertility or infertility (Wilcox et al., 1995). There is currently no significant evidence to show increased risk of testicular cancer in DES sons, despite suspicion that a statistically significant difference may appear as this population continues to be studied (Strohsnitter et al., 2001). There is also no increase in the incidence of prostate cancer in DES sons to

date (Newbold, 2004). As is the case in their female counterparts, DES sons have been shown to have an increased rate of psychosexual effects (Giusti et al., 1995).

Lasting Epigenetic Change in Future Generations (DES Granddaughters and Grandsons)

Research continues to follow the children of DES daughters, as repeated animal models have shown multigenerational effects of in utero DES exposure (Titus-Ernstoff et al., 2008; Turusov et al., 1992; Newbold et al., 1998). These findings have given concern that similar events may be seen in future human generations. To date, however, limited such evidence has been found (Titus-Ernstoff et al., 2008). Some recent studies have shown that DES granddaughters and DES grandsons may have slightly increased risks of cancer and birth defects, but these have all been limited by a small sample size (Titus-Ernstoff et al., 2008, 2010; Klip et al., 2002). Evidence that DES granddaughters have a higher incidence of later menarche and menstrual irregularities has been found, but also lacked statistical significance given the small number of events (Titus-Ernstoff et al., 2006).

References

- Akbas, G. E., Song, J., & Taylor, H. S. (2004). A HOXA10 estrogen response element (ERE) is differentially regulated by 17 beta-estradiol and diethylstilbestrol (DES). *Journal of Molecular Biology*, 340, 1013–1023.
- Babiker, F. A., De Windt, L. J., van Eickels, M., Grohe, C., Meyer, R., & Doevendans, P. A. (2002). Estrogenic hormone action in the heart: Regulatory network and function. *Cardiovascular Research*, 53, 709–719.
- Baird, D. D., & Newbold, R. (2005). Prenatal diethylstilbestrol (DES) exposure is associated with uterine leiomyoma development. *Reproductive toxicology* (Elmsford, NY), 20, 81–84.
- Bamigboye, A. A., & Morris, J. (2003). Oestrogen supplementation, mainly diethylstilbestrol, for preventing miscarriages and other adverse pregnancy outcomes. *The Cochrane Database of Systematic Reviews*. Cd004353.
- Barclay, D. L. (1979). Congenital diethylstilbestrol-associated vaginal/cervical adenosis (DES babies). *The Journal of the Arkansas Medical Society*, 75, 451–452.
- Barnes, A. B., Colton, T., Gundersen, J., Noller, K. L., Tilley, B. C., Strama, T., et al. (1980). Fertility and Outcome of Pregnancy in Women Exposed *in utero* to Diethylstilbestrol. *New England Journal of Medicine*, 302, 609–613.
- Bibbo, M., Gill, W. B., Azizi, F., Blough, R., Fang, V. S., Rosenfield, R. L., et al. (1977). Follow-up study of male and female offspring of DES-exposed mothers. *Obstetrics and Gynecology*, 49, 1–8.
- Block, K., Kardana, A., Igarashi, P., & Taylor, H. S. (2000). *In utero* diethylstilbestrol (DES) exposure alters Hox gene expression in the developing müllerian system. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 14, 1101–1108.
- Bromer, J. G., Wu, J., Zhou, Y., & Taylor, H. S. (2009). Hypermethylation of homeobox A10 by *in utero* diethylstilbestrol exposure: An epigenetic mechanism for altered developmental programming. *Endocrinology*, 150, 3376–3382.
- Calle, E. E., Mervis, C. A., Thun, M. J., Rodriguez, C., Wingo, P. A., & Heath, C. W., Jr. (1996). Diethylstilbestrol and risk of fatal breast cancer in a prospective cohort of US women. *American Journal of Epidemiology*, 144, 645–652.
- Coss, C. C., Jones, A., Parke, D. N., Narayanan, R., Barrett, C. M., Kearbey, J. D., et al. (2012). Preclinical characterization of a novel diphenyl benzamide selective ERalpha agonist for hormone therapy in prostate cancer. *Endocrinology*, 153, 1070–1081.
- Daftary, G. S., & Taylor, H. S. (2006). Endocrine regulation of HOX genes. *Endocrine Reviews*, 27, 331–355.
- Dahlman-Wright, K., Cavailles, V., Fuqua, S. A., Jordan, V. C., Katzenellenbogen, J. A., Korach, K. S., et al. (2006). International union of pharmacology. LXIV. Estrogen receptors. *Pharmacological Reviews*, 58, 773–781.
- DeCherney, A. H., Cholst, I., & Naftolin, F. (1981). Structure and function of the fallopian tubes following exposure to diethylstilbestrol (DES) during gestation. *Fertility and Sterility*, 36, 741–745.
- Dieckmann, W. J., Davis, M. E., Rynkiewicz, L. M., & Pottinger, R. E. (1999). Does the administration of diethylstilbestrol during pregnancy have therapeutic value? *American Journal of Obstetrics and Gynecology*, 181, 1572–1573.
- Dodds, E. C., Goldberg, L., Lawson, W., & Robinson, R. (1938). OE strogenic activity of certain synthetic compounds. *Nature*, 141, 247–248.
- Doherty, L. F., Bromer, J. G., Zhou, Y., Aldad, T. S., & Taylor, H. S. (2010). *In utero* exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. *Hormones and Cancer*, 1, 146–155.
- FDA. (1975). Certain estrogens for oral use. Notice of withdrawal of approval of new drug applications. *Federal Register*, 40, 5384.
- FR Doc No: 00-23477. Federal Register Online 2000;65:55264–5.
- Giusti, R. M., Iwamoto, K., & Hatch, E. E. (1995). Diethylstilbestrol revisited: A review of the long-term health effects. *Annals of Internal Medicine*, 122, 778–788.
- Goldberg, J. M., & Falcone, T. (1999). Effect of diethylstilbestrol on reproductive function. *Fertility and Sterility*, 72, 1–7.
- Goodman, A., Schorge, J., & Greene, M. F. (2011). The long-term effects of *in utero* exposures—The DES story. *The New England Journal of Medicine*, 364, 2083–2084.
- Gutterman, E. M., Ehrhardt, A. A., Markowitz, J. S., Veridiano, N. P., & Neuwalder, H. F. (1986). Long-term distress subsequent to pregnancy drug administration: Women with *in utero* diethylstilbestrol (DES) exposed daughters. *Journal of Psychosomatic Obstetrics and Gynecology*, 5, 51–63.
- Guzman, C., & Zambrano, E. (2007). Endocrine disruptor compounds and their role in the developmental programming of the reproductive axis. *Revista De Investigacion Clinica; Organo del Hospital de Enfermedades de la Nutricion*, 59, 73–81.
- Hatch, E. E., Palmer, J. R., Titus-Ernstoff, L., Noller, K. L., Kaufman, R. H., Mittendorf, R., et al. (1998). Cancer risk in women exposed to diethylstilbestrol *in utero*. *Journal of the American Medical Association*, 280, 630–634.
- Hatch, E. E., Troisi, R., Wise, L. A., Hyer, M., Palmer, J. R., Titus-Ernstoff, L., et al. (2006). Age at natural menopause in women exposed to diethylstilbestrol *in utero*. *American Journal of Epidemiology*, 164, 682–688.
- Herbst, A. L., & Anderson, D. (1990). Clear cell adenocarcinoma of the vagina and cervix secondary to intrauterine exposure to diethylstilbestrol. *Seminars in Surgical Oncology*, 8(6), 343.
- Herbst, A. L., Kurman, R. J., & Scully, R. E. (1972). Vaginal and cervical abnormalities after exposure to stilbestrol *in utero*. *Obstetrics and Gynecology*, 40, 287–298.
- Herbst, A. L., & Scully, R. E. (1970). Adenocarcinoma of the vagina in adolescence. A report of 7 cases including 6 clear-cell carcinomas (so-called mesonephromas). *Cancer*, 25, 745–757.
- Herbst, A. L., Ulfelder, H., & Poskanzer, D. C. (1971). Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *The New England Journal of Medicine*, 284, 878–881.
- Hess, R. A. (2003). Estrogen in the adult male reproductive tract: A review. *Reproductive Biology and Endocrinology*, 1, 52.
- Hill, E. C. (1973). Clear cell carcinoma of the cervix and vagina in young women. A report of six cases with association of maternal stilbestrol therapy and adenosis of the vagina. *American Journal of Obstetrics and Gynecology*, 116, 470–484.

- Hill, D. E., Slikker, J. W., Helton, E. D., Lipe, G. W., Newport, G. D., Szisak, T. J., et al. (1980). Transplacental pharmacokinetics and metabolism of diethylstilbestrol and 17 β -estradiol in the pregnant rhesus monkey. *The Journal of Clinical Endocrinology & Metabolism*, 50, 811–818.
- Holliday, R. (1998). The possibility of epigenetic transmission of defects induced by teratogens. *Mutation Research*, 422, 203–205.
- Hoover, R. N., Hyer, M., Pfeiffer, R. M., Adam, E., Bond, B., Cheville, A. L., et al. (2011). Adverse health outcomes in women exposed *in utero* to diethylstilbestrol. *The New England Journal of Medicine*, 365, 1304–1314.
- Ingle, J. N., Ahmann, D. L., Green, S. J., Edmonson, J. H., Bisel, H. F., Kvols, L. K., et al. (1981). Randomized clinical trial of diethylstilbestrol versus tamoxifen in postmenopausal women with advanced breast cancer. *The New England Journal of Medicine*, 304, 16–21.
- Katz, D. L., Frankenburg, F. R., Benowitz, L. I., & Gilbert, J. M. (1987). Psychosis and prenatal exposure to diethylstilbestrol. *The Journal of Nervous and Mental Disease*, 175, 306–308.
- Kaufman, R. H., Adam, E., Binder, G. L., & Gerthoffer, E. (1980). Upper genital tract changes and pregnancy outcome in offspring exposed *in utero* to diethylstilbestrol. *American Journal of Obstetrics and Gynecology*, 137, 299–308.
- Kaufman, R. H., Adam, E., Hatch, E. E., Noller, K., Herbst, A. L., Palmer, J. R., et al. (2000). Continued follow-up of pregnancy outcomes in diethylstilbestrol-exposed offspring. *Obstetrics and Gynecology*, 96, 483–489.
- Kaufman, R. H., Binder, G. L., Gray, P. M., Jr., & Adam, E. (1977). Upper genital tract changes associated with exposure *in utero* to diethylstilbestrol. *American Journal of Obstetrics and Gynecology*, 128, 51–59.
- Kinch, R. A. (1982). Diethylstilbestrol in pregnancy: an update. *Canadian Medical Association Journal*, 127, 812–813.
- Klip, H., Verloop, J., van Gool, J. D., Koster, M. E., Burger, C. W., & van Leeuwen, F. E. (2002). Hypospadias in sons of women exposed to diethylstilbestrol *in utero*: A cohort study. *Lancet (London, England)*, 359, 1102–1107.
- Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., et al. (1997). Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology*, 138, 863–870.
- Laitman, C. J. D. E. S. (2002). exposure and the aging woman: mothers and daughters. *Current Women's Health Reports*, 2, 390–393.
- Laronda, M. M., Unno, K., Butler, L. M., & Kurita, T. (2012). The development of cervical and vaginal adenosis as a result of diethylstilbestrol exposure *in utero*. *Differentiation; Research in Biological Diversity*, 84, 252–260.
- Larson, P. S., Ungarelli, R. A., de Las Morenas, A., Cupples, L. A., Rowlings, K., Palmer, J. R., et al. (2006). *In utero* exposure to diethylstilbestrol (DES) does not increase genomic instability in normal or neoplastic breast epithelium. *Cancer*, 107, 2122–2126.
- Ma, L. (2009). Endocrine disruptors in female reproductive tract development and carcinogenesis. *Trends in Endocrinology and Metabolism: TEM*, 20, 357–363.
- Melnick, S., Cole, P., Anderson, D., & Herbst, A. (1987). Rates and risks of diethylstilbestrol-related clear-cell adenocarcinoma of the vagina and cervix. *The New England Journal of Medicine*, 316, 514–516.
- Meyers, R. (1983). *DES: The bitter pill*. New York: Putnam Pub. Group.
- Milhan, D. (1992). DES exposure: Implications for childbearing. *The International journal of childbirth education: the official publication of the International Childbirth Education Association*, 7, 21–28.
- Newbold, R. R. (2004). Lessons learned from perinatal exposure to diethylstilbestrol. *Toxicology and Applied Pharmacology*, 199, 142–150.
- Newbold, R. R., Hanson, R. B., Jefferson, W. N., Bullock, B. C., Haseman, J., & McLachlan, J. A. (1998). Increased tumors but uncompromised fertility in the female descendants of mice exposed developmentally to diethylstilbestrol. *Carcinogenesis*, 19, 1655–1663.
- Newbold, R. R., Padilla-Banks, E., & Jefferson, W. N. (2006). Adverse effects of the model environmental estrogen diethylstilbestrol are transmitted to subsequent generations. *Endocrinology*, 147, S11–7.
- NIH DES Research Update. NIH Publication No 00–4722. 1999.
- Palmer, J. R., Hatch, E. E., Rao, R. S., Kaufman, R. H., Herbst, A. L., Noller, K. L., et al. (2001). Infertility among women exposed prenatally to diethylstilbestrol. *American Journal of Epidemiology*, 154, 316–321.
- Palmer, J. R., Hatch, E. E., Rosenberg, C. L., Hartge, P., Kaufman, R. H., Titus-Ernstoff, L., et al. (2002). Risk of breast cancer in women exposed to diethylstilbestrol *in utero*: Preliminary results (United States). *Cancer Causes and Control: CCC*, 13, 753–758.
- Palmer, J. R., Herbst, A. L., Noller, K. L., Boggs, D. A., Troisi, R., Titus-Ernstoff, L., et al. (2009). Urogenital abnormalities in men exposed to diethylstilbestrol *in utero*: a cohort study. *Environmental Health: A Global Access Science Source*, 8, 37.
- Palmer, J. R., Wise, L. A., Hatch, E. E., Troisi, R., Titus-Ernstoff, L., Strohsnitter, W., et al. (2006). Prenatal diethylstilbestrol exposure and risk of breast cancer. *Cancer Epidemiology, Biomarkers and Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, 15, 1509–1514.
- Anon. (1983). Prenatal diethylstilbestrol (DES) exposure recommendations of the diethylstilbestrol-adenosis (DESAD) project for the identification and management of exposed individuals. *Clinical Pediatrics*, 22, 139–143.
- Ravina, E. (2011). *The evolution of drug discovery: From traditional medicines to modern drugs*. Hoboken, NJ: John Wiley & Sons.
- Sassoon, D. (1999). Wnt genes and endocrine disruption of the female reproductive tract: A genetic approach. *Molecular and Cellular Endocrinology*, 158, 1–5.
- Selected Item from the FDA Drug Bulletin—November 1971. (1972SelectedItemfromtheFDADrugBulletinNovember,). Diethylstilbestrol Contraindicated in Pregnancy. *California Medicine*, 116, 85–86.
- Senekjian, E. K., Potkul, R. K., Frey, K., & Herbst, A. L. (1988). Infertility among daughters either exposed or not exposed to diethylstilbestrol. *American Journal of Obstetrics and Gynecology*, 158, 493–498.
- Smith, O. W., & Gabbe, S. G. (1999). Diethylstilbestrol in the prevention and treatment of complications of pregnancy. *American Journal of Obstetrics and Gynecology*, 181, 1570–1571.
- Strohsnitter, W. C., Noller, K. L., Hoover, R. N., Robboy, S. J., Palmer, J. R., Titus-Ernstoff, L., et al. (2001). Cancer risk in men exposed *in utero* to diethylstilbestrol. *Journal of the National Cancer Institute*, 93, 545–551.
- Strohsnitter, W. C., Noller, K. L., Troisi, R., Robboy, S. J., Hatch, E. E., Titus-Ernstoff, L., et al. (2010). Autoimmune disease incidence among women prenatally exposed to Diethylstilbestrol. *The Journal of Rheumatology*, 37, 2167–2173.
- Swan, S. H. (2000). Intrauterine exposure to diethylstilbestrol: long-term effects in humans. *APMIS (Acta Pathologica, Microbiologica et Immunologica Scandinavica)*, 108, 793–804.
- Taylor, H. S. (2008). Endocrine disruptors affect developmental programming of HOX gene expression. *Fertility and Sterility*, 89, e57–8.
- Taylor, H. S., Vanden Heuvel, G. B., & Igarashi, P. (1997). A conserved Hox axis in the mouse and human female reproductive system: Late establishment and persistent adult expression of the Hoxa cluster genes. *Biology of Reproduction/Biology of reproduction*, 57, 1338–1345.
- The Leuprolide Study Group. (1984TheLeuprolide). Leuprolide versus diethylstilbestrol for metastatic prostate cancer. *The New England Journal of Medicine*, 311, 1281–1286.
- Titus-Ernstoff, L., Hatch, E. E., Hoover, R. N., Palmer, J., Greenberg, E. R., Ricker, W., et al. (2001). Long-term cancer risk in women given diethylstilbestrol (DES) during pregnancy. *British Journal of Cancer*, 84, 126–133.
- Titus-Ernstoff, L., Troisi, R., Hatch, E. E., Hyer, M., Wise, L. A., Palmer, J. R., et al. (2008). Offspring of women exposed *in utero* to diethylstilbestrol (DES): A preliminary report of benign and malignant pathology in the third generation. *Epidemiology (Cambridge, Mass.)*, 19, 251–257.
- Titus-Ernstoff, L., Troisi, R., Hatch, E. E., Palmer, J. R., Hyer, M., Kaufman, R., et al. (2010). Birth defects in the sons and daughters of women who were exposed *in utero* to diethylstilbestrol (DES). *International Journal of Andrology*, 33, 377–384.

- Titus-Ernstoff, L., Troisi, R., Hatch, E. E., Palmer, J. R., Wise, L. A., Ricker, W., et al. (2006). Mortality in women given diethylstilbestrol during pregnancy. *British Journal of Cancer*, 95, 107–111.
- Titus-Ernstoff, L., Troisi, R., Hatch, E. E., Wise, L. A., Palmer, J., Hyer, M., et al. (2006). Menstrual and reproductive characteristics of women whose mothers were exposed *in utero* to diethylstilbestrol (DES). *International Journal of Epidemiology*, 35, 862–868.
- Troisi, R., Hatch, E. E., Titus-Ernstoff, L., Hyer, M., Palmer, J. R., Robboy, S. J., et al. (2007). Cancer risk in women prenatally exposed to diethylstilbestrol. *International Journal of Cancer*, 121, 356–360.
- Turusov, V. S., Trukhanova, L. S., Parfenov Yu, D., & Tomatis, L. (1992). Occurrence of tumours in the descendants of CBA male mice prenatally treated with diethylstilbestrol. *International Journal of Cancer*, 50, 131–135.
- Walker, B. E. (1984). Tumors of female offspring of mice exposed prenatally to diethylstilbestrol. *Journal of the National Cancer Institute*, 73, 133–140.
- Wilcox, A. J., Baird, D. D., Weinberg, C. R., Hornsby, P. P., & Herbst, A. L. (1995). Fertility in Men Exposed Prenatally to Diethylstilbestrol. *The New England Journal of Medicine*, 332, 1411–1416.
- Yaghmaie, F., Saeed, O., Garan, S. A., Freitag, W., Timiras, P. S., & Sternberg, H. (2005). Caloric restriction reduces cell loss and maintains estrogen receptor-alpha immunoreactivity in the pre-optic hypothalamus of female B6D2F1 mice. *Neuro Endocrinology Letters*, 26, 197–203.

Environmentally Induced Epigenetic Transgenerational Inheritance of Female Reproductive Pathology

Eric E Nilsson and Michael K Skinner, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

DNA methylation An epigenetic process in which a methyl group attaches to a cytosine molecule that is next to a guanine on the DNA strand.

Epigenetics Molecular factors and processes around DNA that regulate genome activity independent of DNA sequence, and which are mitotically stable.

Polycystic ovarian syndrome (PCOS) An individual having Oligo/anovulation, hyperandrogenism, or polycystic ovaries on an ultrasound. In humans, PCOS is characterized as an individual having two of the three above conditions.

Primary ovary insufficiency (POI) A condition characterized by marked follicular loss within the ovaries and menopause before the age of 40 in humans.

Transgenerational epigenetic inheritance Germline (sperm or egg) transmission of epigenetic information between generations in the absence of any continued direct exposures or genetic manipulations.

Introduction

Epigenetic mechanisms can mediate changes in an organism's phenotype or gene expression patterns that occur in response to environmental factors. Epigenetic processes include DNA methylation, modifications to histone proteins, expression of non-coding RNA, and changes to chromatin structure. Ancestral exposure to a variety of environmental factors, such as toxicants or abnormal nutrition, has been shown to promote changes in transgenerational gene expression and disease frequency (Skinner, 2014). This concept is known as multigenerational or transgenerational epigenetic inheritance. Transgenerational phenomena are inherited changes that do not involve any form of continued direct environmental factor exposure, while multigenerational inheritance involves direct exposures to the animal, developing embryo, or developing gametes within a developing embryo. Transgenerational epigenetic inheritance requires that epigenetic information or epigenetic changes are present in germ cells (i.e., sperm or eggs), as it is through germ cells that inheritance occurs.

There are some periods of an organism's development that are sensitive to environmental impacts, during which exposure to environmental factors can change the epigenetic landscape around an organism's DNA. In mammals one such sensitive window is during the period of gonadal sex determination, at which time extensive epigenetic modifications are already occurring in the developing germ cells (Messerschmidt et al., 2014). Toxicants administered to a pregnant F0 generation female at this time directly expose the developing pups (F1 generation) to the toxicant, as well as exposing the developing gametes within those pups, which will become the F2 generation. Thus, a transgenerational effect can only be seen in the F3 generation animals (great-grand-offspring) and beyond because those animals would not be directly exposed to any toxicants. In contrast, in the event an adult male or nonpregnant female is exposed, the F0 generation adult and the germ cells that will generate the F1 generation are directly exposed, and examination of the F2 generation (grand-offspring) is required to demonstrate a transgenerational phenomenon (Skinner, 2008, Fig. 1).

Ovarian diseases affect many individuals throughout the world. The two most common types of ovarian diseases are polycystic ovarian syndrome (PCOS) and primary ovarian insufficiency (POI). Polycystic ovarian syndrome affects 12%–21% of women of reproductive age (March et al., 2010). PCOS in humans is characterized by a combination of different ovarian abnormalities including oligo/anovulation, hyperandrogenism, and polycystic ovaries on an ultrasound (Boyle and Teede, 2012). Primary ovary insufficiency is a condition characterized by oocyte loss within the ovaries and menopause before the age of 40 in humans (Laven, 2016).

Transgenerational epigenetic effects caused by different factors, such as environmental pollutants, can be evaluated both in terms of molecular epigenetic changes induced within the cells of descendants, and in characterizing observable phenotypic changes in descendants, such as increases in disease frequencies.

Transgenerational Reproductive Effects

Several environmental toxicants have been studied in model animal species for the potential transgenerational epigenetic effects they may have on female reproduction.

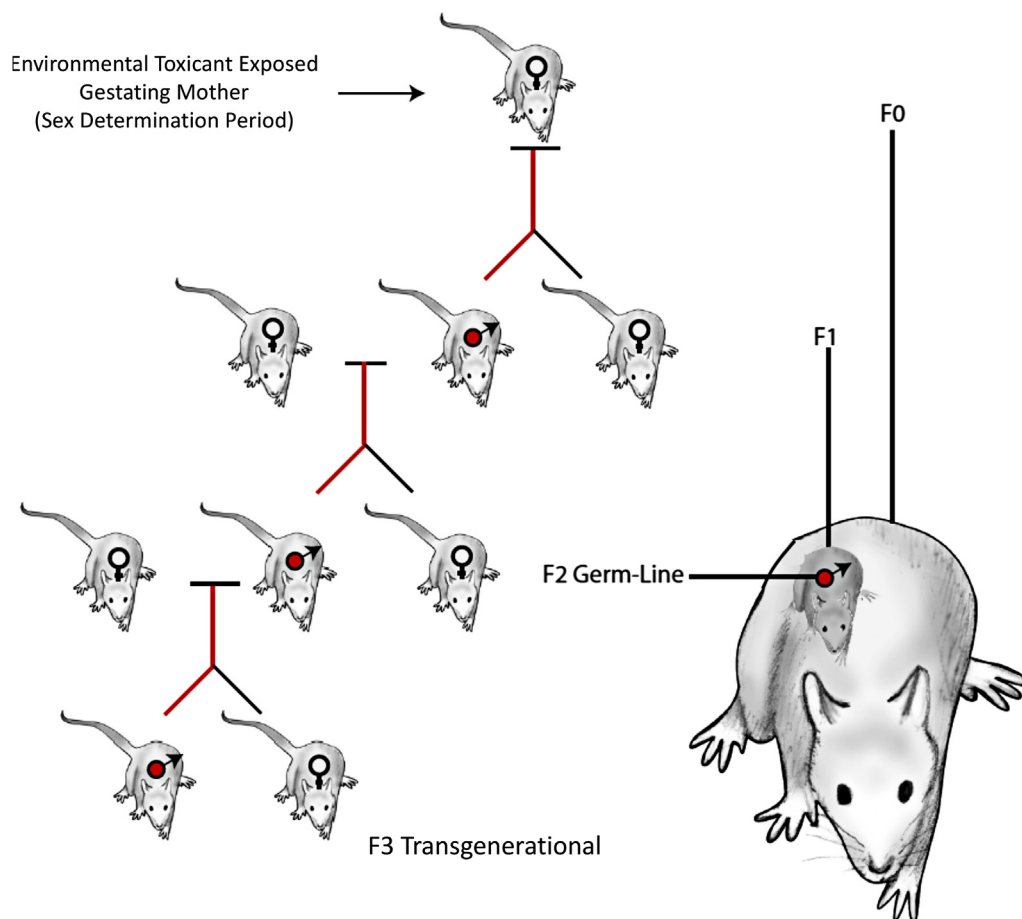


Fig. 1 Rat lineage example showing an F0 generation pregnant animal exposed to a toxicant, and illustrating that the multigenerational F1 and F2 generations were also directly exposed, while the transgenerational F3 generation had no direct exposure. Modified from Skinner, M.K., 2008. What is an epigenetic transgenerational phenotype? F3 or F2. *Reproductive Toxicology*, 25, 2–6.

Vinclozolin is a known antiandrogenic fungicide that is commonly used on fruits and vegetables, specifically wine grapes in the United States (Anway and Skinner, 2006). Following exposure of F0 generation pregnant rats to vinclozolin, the directly exposed F1 generation females at 1 year of age showed a significant increase in small ovarian cysts compared to controls. This resembles PCOS in humans. These F1 generation ovaries also had a significant decrease in primordial and developing follicles, reflecting a reduction in the total number of oocytes in the ovary. Such decreases in the primordial follicle pool size are similar to what is seen in POI in humans and may lead to lower fertility. In the non-exposed F3 generation of vinclozolin lineage animals, the ovaries showed an increased incidence of both small and large ovarian cysts, as well as a significant decrease in oocyte numbers (Nilsson et al., 2012). This F3 generation phenotype indicates epigenetic transgenerational inheritance of ovarian disease. Ancestral exposure to vinclozolin in rats has also resulted in an increase in uterine bleeding events late in pregnancy in the F1–F3 generations (Nilsson et al., 2008). Granulosa cells isolated from antral follicles of the transgenerational F3 generation vinclozolin lineage ovaries showed epigenetic differences compared to controls. Forty-three genes showed changes in DNA methylation in promoter regions, some of which are known to be correlated with ovarian disease (Nilsson et al., 2012).

Permethrin is an insecticide used to treat both animals and crops (Li et al., 2016). DEET (*N,N*-diethyl-*meta*-toluamide) is the most common active ingredient in insect repellents and is commonly applied topically to the skin (Osimitz and Grothaus, 1995). Following exposure of F0 generation gestating female rats to a mixture of permethrin and DEET, the directly exposed F1 generation females showed a decrease in primordial follicles, indicating a lower follicle pool size. The non-exposed F3 generation rats also experienced a decrease in primordial follicle numbers, and an increase in both small and large cyst frequency as compared to F3 non-exposed vehicle control rats (Nilsson et al., 2012) (see Table 1). In addition, the incidence of F3 generation females with an altered age of puberty was increased (Manikkam et al., 2012b). This demonstrates a transgenerational effect.

Dichlorodiphenyltrichloroethane (DDT) is an environmentally persistent insecticide that was heavily used in the United States until its use was banned 1972. Exposure to DDT may occur when consuming meat, fish, or dairy products (CDC, 2016). Following

Table 1 Toxicants having evidence of transgenerational effects on female reproduction

<i>Environmental toxicant</i>	<i>Transgenerational effect</i>	<i>References</i>
Vinclozolin	Ovarian oocyte decrease, cyst increase, granulosa cell gene expression change. Uterine bleeding	Nilsson et al. (2008, 2012)
Pesticides mix	Ovarian oocyte decrease, cyst increase. Altered pubertal age	Nilsson et al. (2012) and Manikkam et al. (2012b)
DDT	Ovarian cyst increase	Skinner et al. (2013)
Methoxychlor	Ovarian cyst increase	Manikkam et al. (2014)
Plastics mix	Ovarian oocyte decrease, cyst increase. Altered pubertal age	Nilsson et al. (2012) and Manikkam et al. (2012b)
DEHP (mice)	Ovarian oocyte decrease, reduced embryo viability	Pocar et al. (2017)
BPA (mice)	Altered ovarian gene expression	Berger et al. (2016)
Dioxin	Ovarian oocyte decrease, cyst increase. Altered pubertal age (rats). Reduced pregnancy rates, increased pre-term birth, endometriosis (mice)	Nilsson et al. (2012) and Brunner-Tran and Osteen (2011)
Jet fuel	Ovarian oocyte decrease, cyst increase. Altered pubertal age	Nilsson et al. (2012) and Tracey et al. (2013)

the treatment of pregnant F0 generation rats with DDT, the transgenerational F3 generation rats had an increase in incidence of small and large cysts compared to controls (Skinner et al., 2013).

Methoxychlor is an insecticide used on agricultural crops and in animal feed (ATSDR, 2002). In rat studies of F0 generation gestating females exposed to methoxychlor, the resulting F3 generation animals showed a transgenerational increase in polycystic ovary incidence (Manikkam et al., 2014).

Bisphenol-A (BPA), dibutyl phthalate (DBP), and di(2-ethylhexyl)phthalate (DEHP) are components of plastics that are environmental toxicants that humans are exposed to on a regular basis (Lorz et al., 2002). After F0 generation pregnant female rats were exposed to a mixture of BPA, DBP, and DEHP, the F1 generation females were found to have fewer oocytes in their ovaries, and an increase in small and large cysts. In the F3 generation, there was a transgenerational decrease in oocytes, an increase in small and large cysts, and an increased incidence of females with an altered age of puberty (Nilsson et al., 2012; Manikkam et al., 2013). In a mouse study investigating BPA alone, after F0 generation pregnant animals were exposed it was found that the F3 generation had ovaries with altered expression of hormone receptor, steroidogenic, apoptotic, and anti-oxidant genes (Berger et al., 2016). In a mouse study investigating DEHP alone, following exposure of the pregnant F0 generation animals, DEHP was found to cause reduced oocyte number and reduced embryo viability over several generations of female mice (Pocar et al., 2017).

Dioxin is an organic pollutant that is a byproduct of chemical manufacturing processes. It is a reproductive toxicant and can alter hormone release, and cause developmental issues or cancer when direct exposure occurs (WHO, 2010). Following the treatment of F0 generation gestating female rats with dioxin, the F1 generation treated lineage ovaries were found to have a significant decrease in oocyte numbers. The F3 generation animals also experienced a significant transgenerational decrease in oocyte pool size, as well as a significant increase in the incidence of small cysts, and a decrease in the age of puberty (Nilsson et al., 2012). In mice exposure of F0 generation pregnant animals to dioxin resulted in a transgenerational reduction in pregnancy rates seen in the F3 and F4 generations, as well as a transgenerational increase in the incidence of pre-term birth, and increased uterine pathologies resembling endometriosis (Brunner-Tran et al., 2016; Bruner-Tran and Osteen, 2011).

JP-8 is a hydrocarbon mixture used as fuel for jet aircraft. It has been used as a carrier for pesticides and is commonly applied for dust control on gravel roads (Witzmann et al., 2003). After pregnant rats were exposed to jet fuel, the F1 generation directly exposed animals experienced a decrease in oocyte numbers, as well as an increase in small ovarian cysts. The F3 generation showed a transgenerational decrease in oocyte numbers, as well as an increase in the incidence of both small and large cysts, and a decrease in the age of puberty (Nilsson et al., 2012; Tracey et al., 2013).

As can be seen above, epigenetic transgenerational inheritance of female reproductive disease susceptibility can be induced by ancestral exposure to several different environmental toxicants Table 1. The question is raised whether different toxicants promote different epigenetic changes that are passed transgenerationally. A study in rats addressed this question by comparing the DNA methylation patterns in the sperm of the F3 generation descendants of F0 generation gestating females that had been exposed to either vinclozolin, jet fuel hydrocarbons, dioxin, a plastics mixture, or a pesticide mixture (Manikkam et al., 2012a). Interestingly, the sites of changes in DNA methylation, known as differentially methylated regions (DMR), formed a unique pattern with each different ancestral toxicant exposure. No transgenerational DMR was common to all the exposures. This shows that there are molecular epigenetic changes that correlate with inherited transgenerational phenotypes seen with each of these toxicant exposures. This also suggests that DNA methylation patterns could be used as biomarkers to detect ancestral exposure to environmental toxicants. In the future, individuals so tested might then be able to predict whether they have an increased susceptibility to the correlated diseases.

Conclusion

Studies in rodents have shown that environmental toxicants can induce epigenetic changes that can be inherited and lead to an increased incidence of reproductive diseases in females. Care must be taken when interpreting these data since the manifestations of rodent ovarian and reproductive diseases are different from those in humans. However, it is interesting to note that the incidence of human ovarian diseases is now as high as 20% (Sirmans and Pate, 2013), and ancestral exposures to environmental toxicants must be considered as one underlying cause.

Exposure to different environmental factors has the potential to alter the epigenome of an individual, even transgenerationally in subsequent non-exposed generations, which induces changes in reproductive disease frequency. Therefore environmental exposures could contribute to the incidence of reproductive abnormalities in the current human population. Epigenetic profiling of individuals may be able to detect biomarkers of ancestral exposure, and so predict their susceptibility to specific diseases.

References

- Anway, M. D., & Skinner, M. K. (2006). Epigenetic transgenerational actions of endocrine disruptors. *Endocrinology*, 147, S43–9.
- ATSDR. (2002). *Toxicological profile for methoxychlor*. U.S. Department of Health and Human Services, Public Health Service, Agency for Toxic Substances and Disease Registry. ATSDR.
- Berger, A., Ziv-Gal, A., Cudiamat, J., Wang, W., Zhou, C., & Flaws, J. A. (2016). The effects of in utero bisphenol A exposure on the ovaries in multiple generations of mice. *Reproductive Toxicology*, 60, 39–52.
- Boyle, J., & Teede, H. J. (2012). Polycystic ovary syndrome—An update. *Australian Family Physician*, 41, 752–756.
- Bruner-Tran, K. L., & Osteen, K. G. (2011). Developmental exposure to TCDD reduces fertility and negatively affects pregnancy outcomes across multiple generations. *Reproductive Toxicology*, 31, 344–350.
- Bruner-Tran, K. L., Duleba, A. J., Taylor, H. S., & Osteen, K. G. (2016). Developmental toxicant exposure is associated with transgenerational adenomyosis in a murine model. *Biology of Reproduction*, 95, 73.
- CDC. (2016). United States Center for Disease Control and Prevention DDT Factsheet. https://www.cdc.gov/biomonitoring/DDT_FactSheet.html (Online) (Accessed September 2017).
- Laven, J. S. (2016). Primary ovarian insufficiency. *Seminars in Reproductive Medicine*, 34, 230–234.
- Li, W., Morgan, M. K., Graham, S. E., & Starr, J. M. (2016). Measurement of pyrethroids and their environmental degradation products in fresh fruits and vegetables using a modification of the quick easy cheap effective rugged safe (QuEChERS) method. *Talanta*, 151, 42–50.
- Lorz, P. M., Towae, F. K., Enke, W., Jäckh, R., & Bhargava, N. (2002). Phthalic acid and derivatives. In *Ullmann's encyclopedia of industrial chemistry*. Weinheim: Wiley-VCH.
- Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, M. M., & Skinner, M. K. (2012a). Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLoS One*, 7, e31901.
- Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. (2012b). Pesticide and insect repellent mixture (permethrin and DEET) induces epigenetic transgenerational inheritance of disease and sperm epimutations. *Reproductive Toxicology*, 34, 708–719.
- Manikkam, M., Tracey, R., Guerrero-Bosagna, C., & Skinner, M. (2013). Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of adult-onset disease and sperm epimutations. *PLoS One*, 8, e55387.
- Manikkam, M., Haque, M. M., Guerrero-Bosagna, C., Nilsson, E., & Skinner, M. K. (2014). Pesticide methoxychlor promotes the epigenetic transgenerational inheritance of adult onset disease through the female germline. *PLoS One*, 9, e102091.
- March, W. A., Moore, V. M., Willson, K. J., Phillips, D. I., Norman, R. J., & Davies, M. J. (2010). The prevalence of polycystic ovary syndrome in a community sample assessed under contrasting diagnostic criteria. *Human Reproduction*, 25, 544–551.
- Messerschmidt, D. M., Knowles, B. B., & Solter, D. (2014). DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes & Development*, 28, 812–828.
- Nilsson, E. E., Anway, M. D., Stanfield, J., & Skinner, M. K. (2008). Transgenerational epigenetic effects of the endocrine disruptor vinclozolin on pregnancies and female adult onset disease. *Reproduction*, 135, 713–721.
- Nilsson, E., Larsen, G., Manikkam, M., Guerrero-Bosagna, C., Savenkova, M., & Skinner, M. (2012). Environmentally induced epigenetic transgenerational inheritance of ovarian disease. *PLoS One*, 7, e36129.
- Osimitz, T. G., & Grothaus, R. H. (1995). The present safety assessment of deet. *Journal of the American Mosquito Control Association*, 11, 274–278.
- Pocar, P., Fiananese, N., Berrini, A., Secchi, C., & Borromeo, V. (2017). Maternal exposure to di(2-ethylhexyl)phthalate (DEHP) promotes the transgenerational inheritance of adult-onset reproductive dysfunctions through the female germline in mice. *Toxicology and Applied Pharmacology*, 322, 113–121.
- Sirmans, S. M., & Pate, K. A. (2013). Epidemiology, diagnosis, and management of polycystic ovary syndrome. *Clinical Epidemiology*, 6, 1–13.
- Skinner, M. K. (2008). What is an epigenetic transgenerational phenotype? F3 or F2. *Reproductive Toxicology*, 25, 2–6.
- Skinner, M. K. (2014). Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. *Molecular and Cellular Endocrinology*, 398, 4–12.
- Skinner, M. K., Manikkam, M., Tracey, R., Guerrero-Bosagna, C., Haque, M. M., & Nilsson, E. (2013). Ancestral dichlorodiphenyltrichloroethane (DDT) exposure promotes epigenetic transgenerational inheritance of obesity. *BMC Medicine*, 11, 228.
- Tracey, R., Manikkam, M., Guerrero-Bosagna, C., & Skinner, M. (2013). Hydrocarbons (jet fuel JP-8) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. *Reproductive Toxicology*, 36, 104–116.
- WHO. 2010. World Health Organization WHO Ten chemicals of major public health concern. http://www.who.int/ipcs/assessment/public_health/chemicals_phc/en/ [Online]. [Accessed 17 May 2015].
- Witzmann, F. A., Bobb, A., Briggs, G. B., Coppage, H. N., Hess, R. A., Li, J., Pedrick, N. M., Ritchie, G. D., Rossi, J., III, & Still, K. R. (2003). Analysis of rat testicular protein expression following 91-day exposure to JP-8 jet fuel vapor. *Proteomics*, 3, 1016–1027.

MAMMARY GLAND

The Mammary Gland: An Overview

Deirdre K Tucker, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States; and National Institute of Environmental Health and Science (NIEHS), National Institute of Health (NIH), Research Triangle Park, NC, United States

Suzanne E Fenton, National Institute of Environmental Health and Science (NIEHS), National Institute of Health (NIH), Research Triangle Park, NC, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Body mass index A measurement of body fat adjusted by height and weight.

Endocrine disruptor Chemicals in the environment that modify some aspect of normal hormone function, causing an adverse change in response.

Epidermal growth factor Hormone responsible for cell proliferation, differentiation, and growth.

Estrogen receptor Hormone receptor that can bind endogenous estrogens and estrogen mimicking compounds.

Growth hormone Somatotropin produced by the pituitary gland that stimulates growth, reproduction during early development and maintains tissue during adulthood.

Hepatocyte growth factor Paracrine growth factor found in epithelial, endothelial, mesenchymal, and multiple other tissues that is involved in cell proliferation and motility.

Mammary stem cells Multipotent cells located in the mammary gland, which are capable of self-renewal and differentiation to produce luminal and myoepithelial cell types during different stages of development.

Matrix metalloproteinase Enzymes that degrade the extracellular matrix during growth and regeneration.

Terminal ductal lobular unit Functional unit of the human breast composed of a duct and several clusters of acini that are grouped together in a lobule.

Terminal end bud Highly proliferative structures located at the end of the ducts that promote growth into the mammary fat pad during puberty.

Thelarche The beginning of secondary breast development; often associated with the beginning of puberty in girls.

Vascular endothelial growth factor Signaling protein responsible for stimulating the growth of new lymph and blood vessels.

Introduction

The mammary gland or breast is an exocrine gland that is responsible for producing milk to nourish offspring. This organ is the defining characteristic of mammals and encompasses several species including primates (humans, macaques, gorillas, etc.), ruminants (cattle, deer, goats, etc.), rodents (mice, rats, rabbits, etc.), and many others. Similar to other glandular structures, the mammary gland is susceptible to developing cancer and remains the most commonly diagnosed cancer among females in the United States and many other industrialized countries ([Breastcancer.org, 2017](#); [World Cancer Research Fund International, 2017](#)). Heritable factors contribute to approximately 15% of breast cancer cases, whereas the remaining cases are associated with risk factors that cannot be altered, such as sex, age, race, age at menarche and menopause, and breast density, as well as factors that may be controlled. These can include lifestyle and environmental factors that include, but are not limited to, parity, physical activity, contraceptives use, and chemicals in the environment that are occupational hazards or unintentional exposures ([American Cancer Society, 2017](#); [Rudel et al., 2011](#)).

In addition to breast cancer, early breast development in young girls (aged 6–8 years), prior to other signs of puberty, has become quite common. Over the past five decades, the age of breast bud development has steadily declined in the United States and in several European countries ([Juul et al., 2006](#); [Herman-Giddens et al., 1997](#); [Lindgren, 1996](#)). Differences in age at breast development by race and ethnicity have been noted in the United States, where breast development occurs much earlier in African American females compared to Hispanics, white non-Hispanics, and Asians; however the overall trend remains that precocious puberty is occurring in all races and ethnicities ([Biro et al., 2013](#); [Wu et al., 2002](#)). Body mass index (BMI) plays a role in breast developmental timing, but recent studies that adjusted for BMI and socioeconomic status found that thelarche is significantly earlier in their populations, indicating that there are other factors strongly influencing this developmental endpoint. Therefore, understanding breast development is essential to understanding how nonheritable factors may modify both pubertal breast timing and breast cancer risk.

Life Stages of Normal Mammary Gland Development

Mammary gland morphogenesis is a unique and intricate process that requires extensive crosstalk between multiple endocrine, paracrine, autocrine, and cellular networks during each stage of development regardless of species (Fig. 1). There are however, fundamental intra- and interspecies similarities and differences that exist.

Rodents

During mid-pregnancy in the rodent, rudimentary mammary placodes arise from the ectoderm. Maternal hormones and crosstalk between the fetal mammary epithelium and estrogen receptor (ER)-rich mesenchyme promote the formation of a primary ductal tree that is present before birth (LaMarca and Rosen, 2007; Saji et al., 2000; Feng et al., 2007; Veltmaat et al., 2003). The maternal hormones estrogen and growth hormone are released and enter the placenta where they are introduced into fetal circulation and signal to receptors located on epithelial and/or stromal cells. Binding of estrogen to the ER- α in the fetal mammary stroma induces hepatocyte growth factor (HGF), which is required for epithelial branching (Cunha et al., 1997). As a result, by embryonic day 12 (E12) a bilayered duct forms, comprised of an inner luminal compartment and an outer monolayer of cap cells that eventually develops into the myoepithelial layer (Fig. 2; Silberstein, 2001). By E13–14, male gland development in some mouse strains begins to digress (Richert et al., 2000). In females, the gland progresses into a bulbous structure that continues to elongate until it penetrates the mammary fat pad (~E17). In contrast, the male mouse gland undergoes complete regression caused by testicular development and increased androgen production and release (Richert et al., 2000). Stalk retraction occurs within 2 days of this process due to the epithelium detaching from the epidermis resulting in limited to no epithelium in the male mouse fat pad and no nipple or areola formation. Male rats, on the other hand, possess epithelium in the mammary fat pads, but like mice, have no nipple or areola. In at least one rat strain, development in both sexes was similar at E15.5 and consisted of epithelium concentrically arranged and subjacent to the epidermis (Filgo et al., 2016). Bud growth remained static up to E17.5, but continued to penetrate the fat pad. By E18.5, female rat mammary buds began to invaginate to prepare for nipple development (E19.5). At the same time, male glands continued to progress into the fat pad, but underwent “bud destruction” and invagination to form a nipple did not occur, again due to endocrine cues from the male testes (Filgo et al., 2016). Prior to birth, mammary sprouts from male and female rats and female mice reach a secondary fat pad precursor where it promotes ductal branching and the formation of a rudimentary ductal tree (Filgo et al., 2016). At the time of birth, regardless of strain or sex, the mammary gland consists of rudimentary epithelial stalks and stroma, consisting of fibroblasts, connective tissue, immune cells, and large adipocytes (Richert et al., 2000). Growth remains consistent with other body size increases until puberty.

The prepubertal rodent gland often consists of a single or double layer of epithelium that surrounds a central lumen. With the onset of puberty, exponential gland growth results in ductal elongation and lateral branching. During early puberty, highly proliferative structures known as terminal end buds (TEBs) appear and are prominent until 6–7 weeks of age in rats and mice. The TEBs are teardrop-shaped structures located at the end of the extending ducts of the gland and contain four to six layers of cuboidal epithelial cells, where cap cells are located on the basal surface situated just below the basal lamina (Russo and Russo, 1996). Cap cells are thought to have pluripotent characteristics and will form the luminal and myoepithelial compartments

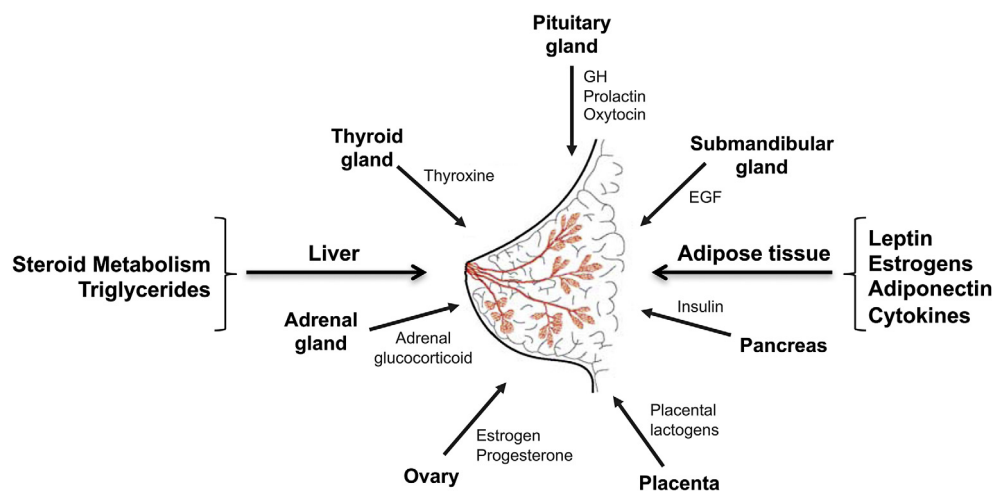


Fig. 1 The growth and function of the mammary gland is affected by numerous endocrine tissues. The many hormones and growth factors that modify development, and transitions between life stages, are produced by endocrine tissues, as well as within the breast. These numerous hormones are targets for endocrine disruption, by environmental chemicals and pharmaceuticals with off-target effects, and when significantly altered can lead to misdirected growth patterns, breast function, and potentially susceptibility to later life disease. GH, growth hormone; EGF, epidermal growth factor. Modified from Macon and Fenton (2013). *Journal of Mammary Gland Biology and Neoplasia* 18(1), 43–61. <https://doi.org/10.1007/s10911-013-9275-7>.

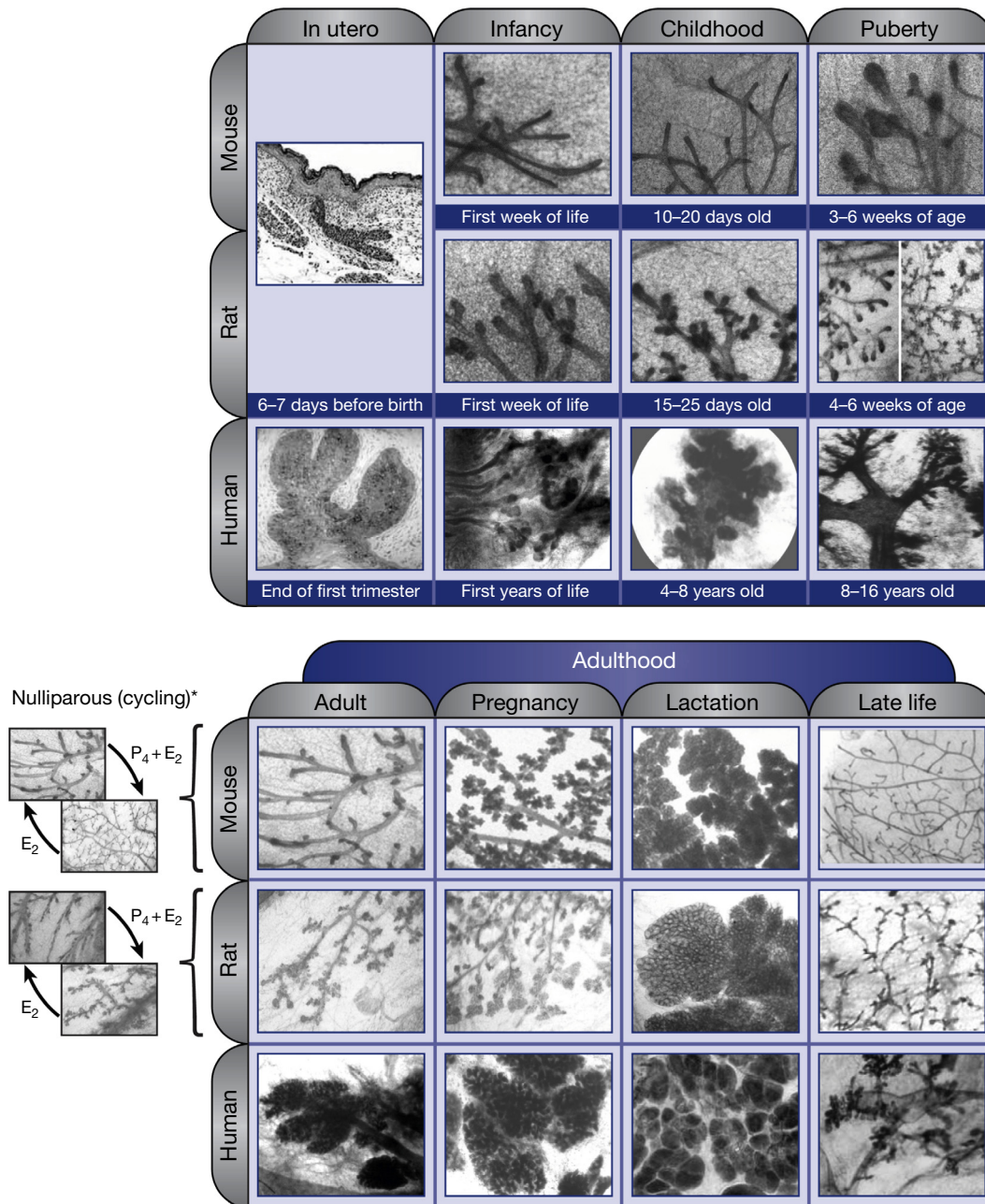


Fig. 2 Structure of the female mammary gland of the mouse, rat, and human during different life stages. In this comparison of the rodent mammary gland and human breast over the life course, mouse and rat tissues are magnified 1.8 times and human tissue is magnified 2.5 times (rat tissues at 4–6 weeks are less magnified than mouse and human tissues at the same age). Evaluation of similar life stages demonstrates the similarities and differences between the rat and mouse mammary glands and the human breast. In childhood, puberty, and adulthood, the mouse demonstrates a more simple ductal morphology (lacks buds and lobule development) than the rat and human. During adult life, all species demonstrate morphological changes in lobule development characteristics reflective of differences in cycle-dependent ovarian hormone levels (E_2 , estradiol; P_4 , progesterone). This figure does not show the cyclic changes in the human breast. Numerous morphological similarities are evident across species during pregnancy and lactation. Regression of the mammary ducts and lobules to a static state is seen late in life in all species. Male tissues are not shown for rats and humans due to the lack of representative data in the literature. Reproduced from IBCERCC (2013). Inter-agency Breast Cancer and the Environment Research Coordinating Committee. Department of Health and Human Services. Breast Cancer and the Environment: Prioritizing Prevention. Available at: https://www.niehs.nih.gov/about/assets/docs/breast_cancer_and_the_environment_prioritizing_prevention_508.pdf (last accessed May 8, 2017).

(Russo and Russo, 1996). Evidence suggests that TEBs are packed with epithelial cells that undergo apoptosis to create the lumen (Humphreys, 1999). As lumen is being created, ductal elongation also takes place as a result of end bud pressure through the stromal compartment (Williams and Daniel, 1983). As new cells are added to the TEB, the extracellular matrix (ECM) begins to constrict which allows for elongation. Matrix metalloproteinase 14 was particularly integral in this process and was elevated in the cells surrounding the TEBs (Szabova et al., 2005). While collagen has been shown to play an essential role in tubulogenesis, it can also bind to the ECM to help create mammary cell shape. The combination of fibroblast growth factor and ECM remodeling by MMP2 and MMP3 causes TEBs to bifurcate, leading to epithelial branching (Silberstein, 2001; Wiseman et al., 2003). ECM remodeling also promotes angiogenesis and increased vascular endothelial growth factor (VEGF) expression during puberty. Adipocytes express an estrogen response element in their VEGF promoter that encourages communication between endothelial cells, epithelial cells, and adipocytes (Liang et al., 2006). Binding of estrogen to ER α in the epithelium also induces amphiregulin (AREG), a natural ligand for the epidermal growth factor receptor, which induces ductal branching (Wiesen et al., 1999). Lateral duct branching may also be inhibited by stromal TGF- β 1 that in turn inhibits the activity of HGF (Soriano et al., 1998). In male Harlan Sprague-Dawley rats, the mammary gland looks identical to the female gland until puberty. Thereafter it begins to form more lobuloalveolar structures with enlarged vacuoles by PND 46, which are completely mature by PND 70 (Filgo et al., 2016). Once lobuloalveolar structures start to form, longitudinal and lateral growth is inhibited resulting in a stunted phenotype compared to age-matched females.

Similar to puberty, pregnancy is governed by many of the same endocrine and paracrine factors. Although gestation length can vary across different strains and species, mammary gland growth during pregnancy and lactation is similar. Early in pregnancy, secondary and tertiary branching is increased to promote alveolar development (Fig. 2). At the same time the majority of adipose tissue is replaced by epithelium surrounded by capillaries (Richert et al., 2000). During late pregnancy, the alveoli fill an extensive portion of the fat pad. Prolactin and progesterone work in concert in preparation for lactation by promoting differentiation of alveoli, the structures responsible for synthesizing and secreting milk. Pathways downstream of progesterone signaling have implicated receptor activator of NF κ B1 and receptor activator of NF κ B1 ligand (RANKL) in alveologenesis. Mammary involution is the final phase of lactation and occurs when milk-producing epithelial cells undergo apoptosis to return the gland to a simple ductal structure. Involution occurs in two distinct phases. In the first phase, apoptosis is triggered and plasmin helps by disrupting the cell-cell interactions with the ECM. During the second phase, remodeling of the basement membrane, collapse of alveoli, and differentiation of adipocytes occurs (Macias and Hinck, 2012), while plasmin works in concert with MMP3 to disrupt the basal lamina and cause premature involution (Simpson et al., 1994). The gland then returns to a resting phase, similar to that seen in the virgin gland.

Humans

Human breast development begins around the 5th week of gestation, when a two to four cell layer “milk streak” or “mammary band” forms from the thickening of the ectoderm (Dabelow, 1957; Hughes, 1949). Involution of the milk streak is involved in condensing the bud below the ectoderm into the mesenchyme where it will become the nipple primordium. The formation of early epithelial ducts appears shortly after week 12 of gestation (Fig. 2). Ultrastructural assessment of the gland in the second trimester fetus shows two distinct epithelial populations, central and basal cells, which share a similar morphology to the basal cells in the epidermis, where glycogen accumulation is greatest. Unlike the mouse where there is one primary duct that extends from the nipple into 5–10 secondary ducts, the human has many lactiferous ducts that will radially extend to form their own separate ductal system with surrounding stroma, separate from neighboring ducts. Between 18 and 21 weeks of pregnancy, fetal epithelial outgrowths begin in the mesenchyme and quickly undergo formation of lumen that become lined with cuboidal cells. During this time the myoepithelium is fully developed. During the third trimester, secondary epithelial buds undergo canalization and the area surrounding the nipple is reduced forming a “mammary pit” (Osin et al., 1998; Tobon and Salazar, 1974). Within the final weeks of gestation, the vasculature surrounding the adipose tissue is increased. Approximately 15–20 lobules composed of lactiferous ducts that connect to the breast surface appear (Javed and Lteif, 2013). The breast epithelium is functional at birth, when hormones from the mother may cause secretory activity in the newborn, but within the first 2 years following birth the gland will begin to involute due to the removal of maternal hormones, resulting in a small ductal tree that is surrounded by fibroblast-enriched mesenchymal tissue (Anbazhagan et al., 1991). At this point, male and female breast morphology is indistinguishable from one another. Both are composed of a ductal tree that extends onto a small indent that is located on the surface of the skin and where breast development can vary between simple blunt-ended tubular structures or well-defined acinar structures (Howard and Gusterson, 2000).

Between birth and puberty, the mammary gland grows in proportion to the body with little to no differences between males and females. Pubertal development in females usually occurs between 8 and 14 years of age (Marshall and Tanner, 1969), but as mentioned earlier, may occur much earlier. Of the few studies that report cellular development from peri-pubertal cadaver tissues, the breast was described as having small bundles of primary and secondary ducts whose distal ends developed into bulbous structures analogous to the rodent TEBs. New branches arise from the main ducts and lateral buds from these structures eventually evolve into alveolar buds that will form clusters around the duct that forms the lobule type 1. This lobule type 1 structure is termed the terminal ductal lobular unit (TDLU) (Russo et al., 1990). TDLUs are reported in some but not all tissues, which may be accounted for by the specimens stage within pubertal development (Fig. 2; Russo and Russo, 2014). TDLU structures are thought to contain an undifferentiated cell population, similar to the TEBs in rodents.

In the clinic, breast development during this period is scored by Tanner staging. Timing of breast development often varies by ethnicity, growth velocity, and levels of exposure to endogenous and exogenous hormones (Javed and Lteif, 2013). During stage I, there is very little glandular tissue and a slight elevation of the papilla (Javed and Lteif, 2013). By Tanner stage II, breast buds begin to form, nipple elevation occurs, and the diameter surrounding the areola widens and this growth is continued into stage III (Tanner, 1962). Tanner stage II (thelarche) is typically when pediatricians diagnose the beginning of puberty. With age advancement, girls can directly enter stage IV, where the nipple and areola enlarge forming a secondary mound on the breast tissue. In some cases, however, girls can transition from stage III to V without ever going into stage IV. During this final stage the breast reaches its adult size and the areola regresses resulting in a loss of the separation of contours. Adult female breasts are composed of 80% fibrous tissue in the form of intra- and extra-lobular connective tissue, where the fat is pushed to the periphery (Fig. 2).

Once breast development is complete, the TDLUs in humans and minor bud development in rodents are hormonally influenced by the menstrual cycle/estrous cycle (Fig. 2). In the virgin postpubertal gland Lobule 1-type (Lob1-type) TDLUs are the predominant structures and are analogous to the TEBs found in rodents. These structures (in both rodents and humans) are the site at which ductal carcinomas arise. Eventually these structures develop to Lob2, which have more lobules per duct (Russo et al., 2005). Transition from Lob2 to Lob3 structures occurs over years with the development of new alveoli, and if pregnancy is not achieved this may not occur. During puberty, male breast tissue does not undergo any additional development with the exception of 4%–69% of males who may develop transient gynecomastia, or increased proliferation of male breast tissue due to estrogen dominance (Lemaine et al., 2013; Braunstein, 1993). This condition tends to correct itself and is only apparent in ~10% of the pubertal male population by age 17 (Ma and Geffner, 2008). This phenomenon can also occur during neonatal development and late adulthood (due to regression of androgen secretion) but the prevalence is greatest during puberty (Javed and Lteif, 2013; Braunstein, 1993; Nuttall, 1979).

During pregnancy, ducts containing the Lob2 and Lob3 eventually develop secretory acinar cells of Lob4 (Russo and Russo, 2014). Following lactation and weaning, glands regress back to predominantly Lob2 structures, while the glands of nulliparous women contain more Lob1. When menopause occurs, all women regardless of childbearing status typically regress back to Lob1 structures.

Mammary Stem and Progenitor Cells

The emergence of improved technologies and differentiating assays has advanced the field of mammary biology and the ability to isolate and characterize mammary stem cells (MaSC) in mice. The defining characteristics of a stem cell are its ability to self-renew and produce multi-lineage cell types. The stem cell portion of the mammary gland is thought to play an essential role in development, differentiation, maintenance, pregnancy, and even carcinogenesis.

Similarities among the human and rodent mammary structure and mammary stem/progenitor populations have shown that there is great conservation of genes that are responsible for these populations (Lim et al., 2010). Gene signatures from flow cytometry sorted FVB/N mice were identified and compared to the gene signatures from flow sorted human breast cell populations, revealing that once normalization for species was accounted for, the cells for both species colocalized into three distinct types (luminal, stromal, and stem or basal cells) (Lim et al., 2010). When they compared transcriptional activity scores from each mouse subtype gene signature to that of the different human cell subtypes it revealed that genes within each subtype were highly conserved across species. Approximately 1200 signature mouse genes for all cell populations corresponded to their human orthologue, with the MaSC population having the highest number of signature genes and best conservation between species. Of these conserved genes, an array of upregulated genes included transcription factors (i.e., p63, *Egr2*, and *Sox11*), cytokeratins (CK5, 14, and 16), and genes associated with the Wnt/ β -catenin and Notch pathways which have been shown to play a role in stem cell maintenance and differentiation (Asselin-Labat et al., 2006; Roarty and Rosen, 2010; Sun et al., 2010). Luminal progenitor and mature luminal cells were observed to have a lower degree of conservation. Luminal progenitor cells expressed *Kit*, *Elf5*, and *Cxcr4*, a gene involved in metastasis of breast cancer, and more mature luminal cells expressed ER α , PR, and the RANKL; qRT-PCR confirmed these findings (Lim et al., 2010). A more recent study detailed the major pathways involved in MaSC maintenance and demonstrated that the Wnt pathway, which is involved in patterning during development, is required (Badders et al., 2009). Signaling of the Wnt pathway leads to stabilization and accumulation of β -catenin which then translocates into the nucleus binding to LEFT/TFC, and results in transcription of various proliferation genes. MaSC increased 6.4-fold when *Wnt1* was overexpressed using a MMTV promoter (Asselin-Labat et al., 2006). Conversely, decreased Notch signaling in the Cbf-1 knockout mouse and p53 $^{-/-}$ mice have resulted in increased MaSC activity, where activity was defined as the transplant repopulating frequency (Bouras et al., 2008; Cicalese et al., 2009). The Δ N63 isoform of the transcription factor p63, which is associated with p53, can induce Wnt signaling and self-renewal capacity, whereas the Δ T-63 isoform is more involved in luminal commitment and the hedgehog pathway (Badders et al., 2009; Li et al., 2008).

The MaSC and Puberty

Several of the genes and signaling pathways identified during the discovery of MaSC are essential during pubertal mammary gland development, a period of extensive epithelial growth (Tiede and Kang, 2011). In the mouse, TEBs are prevalent during this period and contain highly mitotic cells. Within the cap cells of the TEBs resides a pluripotent stem cell population that is capable of self-

renewal and differentiation. Since these structures are increased during puberty it is also thought that the MaSC and luminal populations are increased and genes involved in these processes are also changed to coordinate with the changes in cellular morphology. Exposure to environmental toxicants may alter the MaSC and luminal epithelial cells. Advances in this research may provide details into the initiation, promotion, and progression of cancer by environmental exposure to endocrine disrupting chemicals during early life stages such as puberty.

The MaSC and Pregnancy

Rodents

Emerging evidence has suggested that another type of MaSC may contribute to the alveolar expansion that occurs during pregnancy. Parity-induced mammary epithelial cells (PI-MEC) are thought to originate from cells that have differentiated during pregnancy. Unlike more committed alveolar cells, this population is thought to either de-differentiate or not undergo apoptosis during involution or remodeling after lactation (Wagner et al., 2002). Pregnant double transgenic mice that expressed *Rosa-lacZ* and X-Gal, which identifies β -galactosidase activity in cells, revealed that immediately following parturition most differentiated alveolar cells expressed *Rosa-lacZ*. During involution cells located in close proximity to TDLUs had the greatest X-gal staining and this was also observed in the developing alveoli of subsequent pregnancies. When stained whole tissue from mice undergoing involution was transplanted into the mammary fat pad of virgin mice, ~40% produced outgrowths that were X-gal positive throughout the entire mammary gland. This group also showed that when the PI-MEC population was sorted by flow cytometry that 98% of were CD24+, and a substantial number of them co-expressed CD49f, both markers seen in pubertal stem cell populations. Notably, these cells were also able to form mammospheres, an epithelial cell derived form of the mammary gland that possess stem cell-like properties, in culture, a phenomenon that has been shown to occur with multipotent human cells (Matulka et al., 2007). In other studies, luciferase expressing MaSCs were transplanted into cleared fat pads of 3-week-old mice, followed by induction of pregnancy, and the authors reported that MaSC populations expanded 200-fold followed by an immediate decrease following parturition. Baseline levels were dependent on whether nursing of pups occurred (Tiede et al., 2009). Progenitor cells have also been shown to increase later in pregnancy and during lactation. At GD 18, luminal mammary populations collected from the dam were further sorted by CD61 expression, a marker that can define less differentiated luminal cells from more differentiated cells. A 15-fold decrease in less differentiated luminal cells was reported, compared to an increase in the number of alveolar luminal differentiated cells (Asselin-Labat et al., 2006).

Humans

MaSC identification in the human has not been obtained in the same fashion as rodents due to the ethical issues that surround collection of cells from human subjects. However, human mammary epithelial cells isolated from mammary tissue from healthy individuals undergoing reduction mammoplasties were successfully found to survive, proliferate, and form spheroids in nonadhesive medium while most of the cells experienced cell death (Dontu et al., 2003). These mammospheres contained 8-fold more bi-lineage progenitor cells than freshly cultured human mammary cells. Successive passages produced colonies that contained all three epithelial cell types. Secondary and later spheroid generations were generated from single cells. Secondary cells were found to express $\alpha 6$ integrin, CD10, CK5 with moderate expression of epithelial specific antigen and CK14. Genotyping and transcription confirmation studies identified genes within the Wnt/Frizzled pathways, CXCR4 and transcription factors that are responsible for cell cycle arrest. Further understanding of the roles that MaSC play in development, function, and susceptibility to cancer in the breast is an urgent research need.

Conclusions

The mammary gland undergoes extensive changes and remodeling over the course of an individual's lifetime, and there are numerous similarities of structures and regulatory cues between rodents and humans. With factors such as increased exposure to environmental chemicals that can mimic endogenous hormones, there has been a significant rise in childhood and adult obesity, altered breast developmental timing during puberty, and breast cancer risk. Scientific efforts should continue to focus on how these factors contribute to these risks to increase public awareness, which in turn may lead to modified lifestyle choices and better nutritional and health resources, especially in the most susceptible populations.

References

- American Cancer Society. (2017American). Breast cancer risk and prevention: lifestyle-related breast cancer risk factors. <https://www.cancer.org/cancer/breast-cancer/risk-and-prevention/lifestyle-related-breast-cancer-risk-factors.html> (accessed January 5, 2017).
- Anbazhagan, R., Bartek, J., Monaghan, P., & Gusterson, B. A. (1991). Growth and development of the human infant breast. *American Journal of Anatomy*, 192, 407–417.
- Asselin-Labat, M. L., Shackleton, M., Stingl, J., Vaillant, F., Forrest, N. C., Eaves, C. J., Visvader, J. E., & Lindeman, G. J. (2006). Steroid hormone receptor status of mouse mammary stem cells. *Journal of the National Cancer Institute*, 98, 1011–1014.
- Asselin-Labat, M. L., Sutherland, K. D., Barker, H., Thomas, R., Shackleton, M., Forrest, N. C., Hartley, L., Robb, L., Grosveld, F. G., van der Wees, J., Lindeman, G. J., & Visvader, J. E. (2006). Gata-3 is an essential regulator of mammary gland morphogenesis and luminal cell differentiation. *Nature Cell Biology*, 9, 201–209.

- Badders, N. M., Goel, S., Clark, R. J., Klos, K. S., Kim, S., Bafico, A., Lindvall, C., Williams, B. O., & Alexander, C. M. (2009). The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. *PLoS One*, 4(e6594), 1–14.
- Biro, F. M., Greenspan, L. C., Galvez, M. P., Pinney, S. M., Teitelbaum, S., Windham, G. C., Deardorff, J., Herrick, R. L., Succop, P. A., Hiatt, R. A., Kushi, L. H., & Wolff, M. S. (2013). Onset of breast development in a longitudinal cohort. *Pediatrics*, 132, 1019–1027.
- Bouras, T., Pal, B., Vaillant, F., Harburg, G., Asselin-Labat, M. L., Oakes, S. R., Lindeman, G. J., & Visvader, J. E. (2008). Notch signaling regulates mammary stem cell function and luminal cell-fate commitment. *Cell Stem Cell*, 3, 429–441.
- Braunstein, G. D. (1993). Gynecomastia. *The New England Journal of Medicine*, 328, 490–495.
- Breastcancer.org. (2017). Breastcancer.org. U.S breast cancer statistics. http://www.breastcancer.org/symptoms/understand_bc/statistics (accessed March 15, 2017).
- Cicalese, A., Bonizzi, G., Pasi, C. E., Faretta, M., Ronzoni, S., Giulini, B., Briskin, C., Minucci, S., Di Fiore, P. P., & Pelicci, P. G. (2009). The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell*, 138, 1083–1095.
- Cunha, G. R., Young, P., Hom, Y. K., Cooke, P. S., Taylor, J. A., & Lubahn, D. B. (1997). Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *Journal of Mammary Gland Biology and Neoplasia*, 2, 393–402.
- Dabelow, A. (1957). Die Milchdrüse. In *Hanbuch der Mikroskopischen Anatomie des mensechen. Haut und Sinnes Organs* (pp. 277–485). Berlin/Göttingen/Heidelberg: Springer-Verlag.
- Dontu, G., Abdallah, W. M., Foley, J. M., Jackson, K. W., Clarke, M. F., Kawamura, M. J., & Wicha, M. S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes and Development*, 17, 1253–1270.
- Feng, Y., Manka, D., Wagner, K. U., & Khan, S. A. (2007). Estrogen receptor- α expression in the mammary epithelium is required for ductal and alveolar morphogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 14718–14723.
- Filgo, A. J., Foley, J. F., Puvanesarajah, S., Borde, A. R., Midkiff, B. R., Reed, C. E., Chappell, V. A., Alexander, L. B., Borde, P. R., Troester, M. A., Bouknight, S. A., & Fenton, S. E. (2016). Mammary gland evaluation in juvenile toxicity studies: temporal developmental patterns in the male and female Harlan Sprague-Dawley rat. *Toxicologic Pathology*, 44, 1034–1058.
- Herman-Giddens, M. E., Slora, E. J., Wasserman, R. C., Bourdony, C. J., Bhapkar, M. V., Koch, G. G., & Hasemeier, C. M. (1997). Secondary sexual characteristics and menses in young girls seen in office practice: a study from the Pediatric Research in Office Settings network. *Pediatrics*, 99, 505–512.
- Howard, B. A., & Gusterson, B. A. (2000). Human breast development. *Journal of Mammary Gland Biology and Neoplasia*, 5, 119–137.
- Hughes, E. S. R. (1949). The development of the mammary gland. *Annals of the Royal College of Surgeons of England*, 6, 66–119.
- Humphreys, R. C. (1999). Programmed cell death in the terminal endbud. *Journal of Mammary Gland Biology and Neoplasia*, 4, 213–220.
- Javed, A., & Lteif, A. (2013). Development of the human breast. *Seminars in Plastic Surgery*, 27, 5–12.
- Juul, A., Teilmann, G., Scheike, T., Hertel, N. T., Holm, K., Laursen, E. M., Main, K. M., & Skakkebaek, N. E. (2006). Pubertal development in Danish children: comparison of recent European and US data. *International Journal of Andrology*, 29, 247–255. discussion 286–290.
- LaMarca, H. L., & Rosen, J. M. (2007). Estrogen regulation of mammary gland development and breast cancer: amphiregulin takes center stage. *Breast Cancer Research*, 9, 304.
- Lemaine, V., Cayci, C., Simmons, P. S., & Petty, P. (2013). Gynecomastia in adolescent males. *Seminars in Plastic Surgery*, 27, 56–61.
- Li, N., Singh, S., Cherukuri, P., Li, H., Yuan, Z. Q., Ellisen, L. W., Wang, B. L., Robbins, D., & Drenzo, J. (2008). Reciprocal intraepithelial interactions between TP63 and hedgehog signaling regulate quiescence and activation of progenitor elaboration by mammary stem cells. *Stem Cells*, 26, 1253–1264.
- Liang, Y. Y., Brekken, R. A., & Hyder, S. M. (2006). Vascular endothelial growth factor induces proliferation of breast cancer cells and inhibits the anti-proliferative activity of anti-hormones. *Endocrine-Related Cancer*, 13, 905–919.
- Lim, E., Wu, D., Pal, B., Bouras, T., Asselin-Labat, M. L., Vaillant, F., Yagita, H., Lindeman, G. J., Smyth, G. K., & Visvader, J. E. (2010). Transcriptome analyses of mouse and human mammary cell subpopulations reveal multiple conserved genes and pathways. *Breast Cancer Research*, 12, 1–14.
- Lindgren, G. (1996). Pubertal stages 1980 of Stockholm schoolchildren. *Acta Paediatrica*, 85, 1365–1367.
- Ma, N. S., & Geffner, M. E. (2008). Gynecomastia in prepubertal and pubertal men. *Current Opinion in Pediatrics*, 20, 465–470.
- Macias, H., & Hinck, L. (2012). Mammary gland development. *WIREs Developmental Biology*, 1, 533–557.
- Marshall, W. A., & Tanner, J. M. (1969). Variations in pattern of pubertal changes in girls. *Archives of Disease in Childhood*, 44, 291–303.
- Matulka, L. A., Triplett, A. A., & Wagner, K. U. (2007). Parity-induced mammary epithelial cells are multipotent and express cell surface markers associated with stem cells. *Developmental Biology*, 303, 29–44.
- Nuttall, F. Q. (1979). Gynecomastia as a physical finding in normal men. *Journal of Clinical Endocrinology and Metabolism*, 48, 338–340.
- Osin, P. P., Anbazhagan, R., Bartkova, J., Nathan, B., & Gusterson, B. A. (1998). Breast development gives insights into breast disease. *Histopathology*, 33, 275–283.
- Richert, M. M., Schwertfeger, K. L., Ryder, J. W., & Anderson, S. M. (2000). An atlas of mouse mammary gland development. *Journal of Mammary Gland Biology and Neoplasia*, 5, 227–241.
- Roarty, K., & Rosen, J. M. (2010). Wnt and mammary stem cells: hormones cannot fly wingless. *Current Opinion in Pharmacology*, 10, 643–649.
- Rudel, R. A., Fenton, S. E., Ackerman, J. M., Euling, S. Y., & Makris, S. L. (2011). Environmental exposures and mammary gland development: state of the science, public health implications, and research recommendations. *Environmental Health Perspectives*, 119, 1053–1061.
- Russo, J., Gusterson, B. A., Rogers, A. E., Russo, I. H., Wellings, S. R., & Vanzwieten, M. J. (1990). Biology of disease – comparative-study of human and rat mammary tumorigenesis. *Laboratory Investigation*, 62, 244–278.
- Russo, J., Maillo, D., Hu, Y. F., Balogh, G., Sheriff, F., & Russo, I. H. (2005). Breast differentiation and its implication in cancer prevention. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 11, 931s–936s.
- Russo, I. H., & Russo, J. (1996). Mammary gland neoplasia in long-term rodent studies. *Environmental Health Perspectives*, 104, 938–967.
- Russo, J., & Russo, I. H. (2014). Histological evaluation of the normal breast. In *Techniques and methodological approaches* (pp. 45–73). New York: Springer Science + Business Media.
- Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M., & Gustafsson, J. A. (2000). Estrogen receptors α and β in the rodent mammary gland. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 337–342.
- Silberstein, G. B. (2001). Postnatal mammary gland morphogenesis. *Microscopy Research and Technique*, 52, 155–162.
- Soriano, J. V., Pepper, M. S., Orci, L., & Montesano, R. (1998). Roles of hepatocyte growth factor scatter factor and transforming growth factor- β (1) in mammary gland ductal morphogenesis. *Journal of Mammary Gland Biology and Neoplasia*, 3, 133–150.
- Sun, P., Yuan, Y., Li, A., Li, B., & Dai, X. (2010). Cytokeratin expression during mouse embryonic and early postnatal mammary gland development. *Histochemistry and Cell Biology*, 133, 213–221.
- Sympton, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J., & Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary-gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene-expression. *Journal of Cell Biology*, 125, 681–693.
- Szabova, L., Yamada, S. S., Birkedal-Hansen, H., & Holmbeck, K. (2005). Expression pattern of four membrane-type matrix metalloproteinases in the normal and diseased mouse mammary gland. *Journal of Cellular Physiology*, 205, 123–132.
- Tanner, J. M. (1962). *Growth and adolescence* (2nd edn, pp. 291–303). Springfield, IL: Thomas.
- Tiede, B., & Kang, Y. (2011). From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. *Cell Research*, 21, 245–257.
- Tiede, B. J., Owens, L. A., Li, F., DeCoste, C., & Kang, Y. (2009). A novel mouse model for non-invasive single marker tracking of mammary stem cells in vivo reveals stem cell dynamics throughout pregnancy. *PLoS One*, 4, e8035.

- Tobon, H., & Salazar, H. (1974). Ultrastructure of the human mammary gland. I. Development of the fetal gland throughout gestation. *Journal of Clinical Endocrinology and Metabolism*, 39, 443–456.
- Veltmaat, J. M., Mailleux, A. A., Thiery, J. P., & Bellusci, S. (2003). Mouse embryonic mammaryogenesis as a model for the molecular regulation of pattern formation. *Differentiation*, 71, 1–17.
- Wagner, K., Boulanger, C., Henry, M., Sgaglia, M., Hennighausen, L., & Smith, G. (2002). An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. *Development*, 129, 1377–1386.
- Wiesen, J. F., Young, P., Werb, Z., & Cunha, G. R. (1999). Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development*, 126, 335–344.
- Williams, J. M., & Daniel, C. W. (1983). Mammary ductal elongation – differentiation of myoepithelium and basal lamina during branching morphogenesis. *Developmental Biology*, 97, 274–290.
- Wiseman, B. S., Sternlicht, M. D., Lund, L. R., Alexander, C. M., Mott, J., Bissell, M. J., Soloway, P., Itohara, S., & Werb, Z. (2003). Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *Journal of Cell Biology*, 162, 1123–1133.
- World Cancer Research Fund International. (2017). Breast cancer statistics. <http://www.wcrf.org/int/cancer-facts-figures/data-specific-cancers/breast-cancer-statistics> (accessed March 1, 2017).
- Wu, T., Mendola, P., & Buck, G. M. (2002). Ethnic differences in the presence of secondary sex characteristics and menarche among US girls: the Third National Health and Nutrition Examination Survey, 1988–1994. *Pediatrics*, 110, 752–757.

The Cell Biology of the Lactating Mammary Epithelium

Margaret C Neville, University of Colorado School of Medicine, Aurora, CO, United States
Jenifer Monks, University of Colorado Anschutz Medical Center, Aurora, CO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The cell biology of the mammary epithelium can be most easily visualized in three groups of structures: (1) the cytoskeleton consisting of actin and microfilaments and their interacting membrane molecules that link the cell to its environment via cell–cell and cell–matrix interactions, (2) the cellular structures within which the enzyme reactions catalyze the synthesis and secretion of milk proteins, and (3) the transporters localized to the basolateral and apical plasma membranes as well as to the membranes of intracellular compartments. Set 1 are the structures that anchor the cell in its environment and lead to the formation of an epithelium, one cell or about 20 μm thick, that firmly separates the interstitial environment, providing both nutrients and hormonal signals, from the extracellular environment. Set 2 are the structures that compartmentalize the enzymes that transform nutrient molecules into milk proteins, sugars, and lipids, setting up elaborate secretion mechanisms. The transporters of Set 3 bring nutrient molecules, ions, and trace elements into the cells, regulate their distribution into cellular compartments, and provide a pathway across the apical membrane into the milk space. For a comprehensive review of the current status of the field and the regulation of the development of these structures see the chapter by Anderson and coworkers in Knobil and Neill's *Physiology of Reproduction* (Anderson et al., 2015).

Fine Structure of the Mammary Epithelial Cell

Fig. 1 shows an electron micrograph of a portion of a mammary epithelial cell from a lactating mouse. Lipid droplets and secretory vesicles containing casein micelles are seen toward the apical portion of the cell while a bevy of compartments including the nucleus, mitochondria, Golgi vesicles, the rough endoplasmic reticulum, and lysosomes that carry out the enzymatic work of the cell are found throughout. A section through a myoepithelial cell is seen embedded at the basal surface; these cells with their long processes surrounding the alveoli are stimulated to contract by oxytocin from the hypothalamus/pituitary bringing about ejection of milk from the lumen and out the nipple. The fine fibrils of the basal lamina can be seen at the exterior surface of the basal membrane of the cell. The myoepithelial cells are within this layer. While not apparent in this picture, this is only part of a single cell in a tightly organized layer of epithelial cells that separate the interstitial space that supplies nutrients from the lumen containing the secreted product, milk.

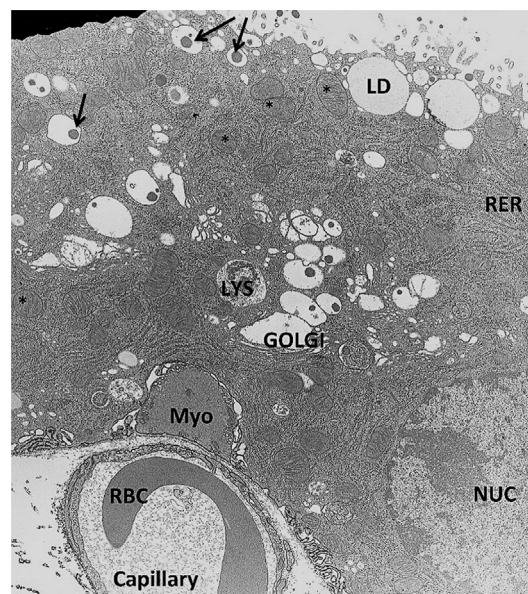


Fig. 1 Electron micrograph of a lactating mammary epithelial cell. Shown are fat droplets (LD) approaching the apical membrane, secretory vesicles containing casein micelles (arrows), the rough endoplasmic reticulum (RER), golgi vesicles (GOLGI), nucleus (NUC), lysosomes (LYS), and mitochondria (*) within the cell. A myoepithelial cell (Myo) is embedded in the basal side of the cell and a capillary containing a red blood cell (RBC) is snuggled up to the basal lamina separating the interstitial space from the epithelial and myoepithelial compartments. A part of the lumen is shown at the upper pole of the cell and the interstitial space occupies the lower right corner of the image.

Structural Elements of the Alveolar Epithelium

The epithelium of all mucosal surfaces is a highly disciplined structure whose basic form is determined both by an internal cytoskeleton and by interactions with neighboring cells and the extracellular matrix. The mammary epithelium is no exception; the cytoskeleton, which defines the intracellular space, is formed by a network of actin microfilaments mostly lining cell borders (represented in *orange* in Fig. 2) as well as keratin intermediate filaments and microtubules composed of α - and β -tubulin; both are represented by the *green wash* in the interior of the cell. These interacting elements give the cells shape and coherence, providing a framework by which both plasma membrane structures and interior organelles are organized. The cytoskeletal elements interact with a variety of transmembrane molecules that link them to neighboring cells or to the basement membrane (Fig. 2). The classic work of Dorothy Pitelka that defined the electron microscopy of these junctions is still relevant 40 years after its publication in 1977 (Pitelka and Hamamoto, 1977). In all cases the extracellular portion of each transmembrane protein specifically interacts with proteins on an adjacent cell or in the extracellular space. Its intracellular portion interacts with an anchoring protein that mediates its attachment to the cytoskeleton. We will first describe the junctions that mediate cell–cell interactions and then the molecules that anchor the cell to the extracellular matrix.

Cell–Cell Interactions

Tight junctions

The tight junction complex that encircles each epithelial cell at its apical pole (Fig. 2) forms a barrier to the flux of molecules across the paracellular pathway between the luminal and intracellular spaces. This barrier is permeable to ions and even to large molecules such as albumin and immunoglobulins during pregnancy, probably allowing synthesized milk components to return to the interstitial compartment. At secretory activation (sometimes termed lactogenesis II) the decrease in plasma progesterone leads to a change in the configuration of the tight junction proteins so that they form a set of ridge-like structures extending across the cell boundary at the apical ends of the cells. These structures completely prevent passage of any molecules across the paracellular pathway, allowing milk components to accumulate in the lumen. Recent studies indicate that the tetraspanin molecules claudin-3 and claudin-8 are localized to the tight junctions of the lactating mammary epithelium (Baumgartner et al., 2017) interacting with the membrane molecule occludin as well as with zonula occludin proteins like ZO-1 which provide a link to the actin cytoskeleton.

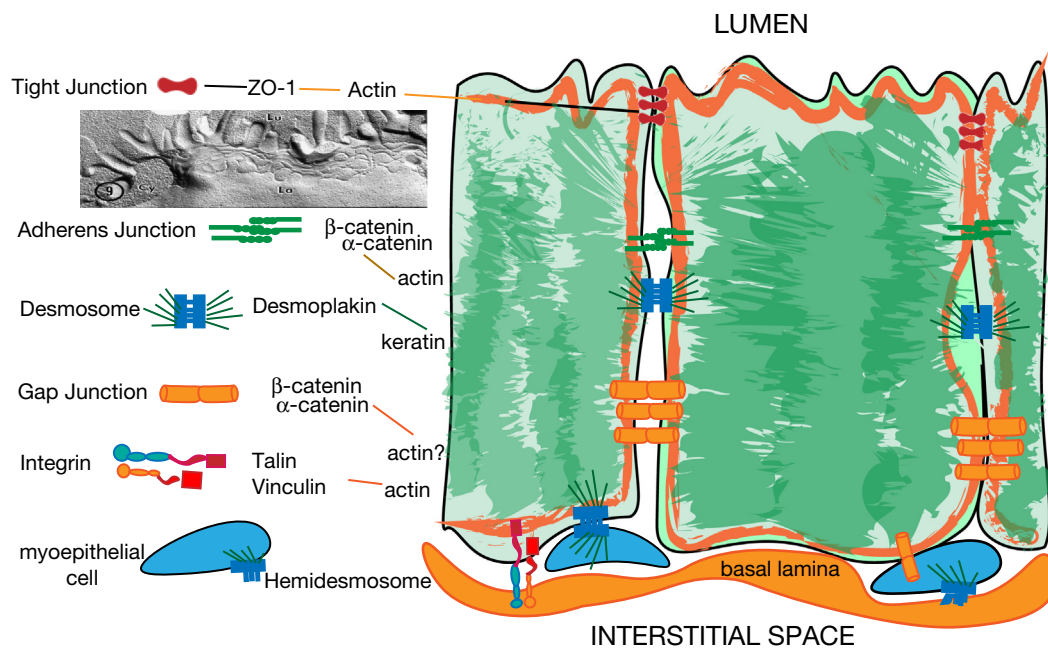


Fig. 2 Cartoon illustrating the cell–cell and cell–matrix interactions of the mammary alveolar cell. The cytoskeleton is composed of actin microfilaments, generally localized to the cell borders (*orange*) and the keratin intermediate filaments and microtubules composed of α - and β -tubulin (both represented as *green*). Cell–cell interacting junctions include the tight junction, the adherens junction, the desmosome, and the gap junctions, all composed of a transmembrane molecule that interacts with a specific submembrane protein, which in turn interacts with the cytoskeleton. Hemidesmosomes mediate interactions between the epithelial cell and the myoepithelial cell as well as between the myoepithelial cell and the basal lamina. Integrins mediate interactions between the epithelial cell and the basal lamina. (See text for additional details.)

Adherens junctions

Cadherin makes up a set of sticky transmembrane fibers that encircle many epithelial cells, providing a strong, calcium dependent linkage across the paracellular pathway that stabilizes the epithelium. Cadherin is bound intracellularly to β -catenin which in turn links cadherin to the actin cytoskeleton. β -Catenin in turn interacts with α -catenin. The linkage between cadherin and catenins regulates a number of signaling pathways including the NF κ B, Wnt, and MAPK pathways thought to give cadherins an important role in the regulation of development.

Desmosomes

Desmosomes are composed of plaques of proteins named desmoglein and desmocollin, members of the cadherin family that interact across the lateral borders between epithelial cells. They are transmembrane proteins whose extracellular domains form calcium dependent adhesions with similar structures on adjacent cells; their intracellular domains interact with desmoplakin, a large dumbbell-shaped protein that also binds keratin, providing a link to the cytoskeleton. Desmosomes form strong cell–cell interactions that contribute to development of the mammary epithelium. They also form connections between the epithelial cells and the basal myoepithelial cells. However, desmosomes linking the epithelial cells are rare in the lactating gland, presumably because they would impair the shape change that must occur in order for the lumen to accommodate the accumulating milk during lactation.

Gap junctions

Direct transfer of molecules up to 1500 Da between cells occurs through gap junctions, channels formed by six connexin molecules that aggregate to form a hemichannel. Alignment of two of these hemichannels between cells allows passage of signaling molecules, ions, vitamins, and other small molecules but not macromolecules. Four types of connexins, Cx26, Cx30, Cx32, and Cx43, are expressed in rodent mammary glands, connexins Cx26 and Cx32 are markedly upregulated in lactation. Cx43 is been shown to be associated with myoepithelial cells. Connexins in the luminal cells of the mammary gland are thought to interact with catenins, giving them a role in the regulation of mammary development.

Cell–Matrix Interactions***Integrin-mediated attachment to the extracellular matrix***

Integrins are transmembrane dimers made up of α and β subunits. The most prevalent integrin in the lactating mammary epithelium is integrin $\alpha 5/\beta 1$. Integrins provide an important mechanism by which the mammary epithelium attaches to the basal lamina, a extracellular matrix layer composed of laminin, collagen IV, nidogens (also known as entactins), the proteoglycan perlecan, fibronectin, and a number of other proteins (Maller et al., 2010). In the mammary gland integrins are thought to bind both to laminin and fibronectin in the basal lamina providing a firm anchor to the extracellular matrix. Intracellularly the integrins bind to an intracellular anchoring protein, most often talin which then interacts with the actin-binding protein vinculin. A kinase, focal adhesion kinase or FAK, is often a part of the complex providing a means by which transcription or cell movement can be regulated through the phosphorylation of signaling molecules such as Src and Rac1 when cell matrix interactions are perturbed.

Other transmembrane mediators

Other membrane molecules through which cells interact with the extracellular matrix are DDR1 (discoidin domain receptor 1), dystroglycan, which binds laminin in the extracellular space and interacts with the Ras signaling pathway intracellularly, and the proteoglycans decorin and syndecans-1 and syndecan-4. Although all have been implicated in mammary development, much less is known about their detailed interactions with both extracellular matrix molecules and intracellular anchoring and signaling proteins.

Cell Biology of Milk Secretion

The four major pathways by which nutrients transported to the lactating mammary cell are turned into components of the mammary secretion are shown in Fig. 3. These pathways are (I) the secretory vesicle pathway, responsible for secreting most milk proteins as well as milk sugars, (II) the lipid secretion pathway responsible for secretion of the milk fat globule (MFG), a specialized structure containing the milk triglyceride, (III) a transcytotic pathway responsible for moving immunoglobulins and other molecules from the interstitial space to the milk lumen, and (IV) a variety of membrane channel and transport proteins responsible for transmembrane movement of nutrients, micronutrients, and the ionic components of milk. During pregnancy the paracellular channel (V) is open allowing molecules of all sizes to move between the interstitial and milk spaces. We will now deal in more detail with each of these pathways.

Pathway I. Secretion of Milk Proteins and Sugars

Pathway I begins in the rough endoplasmic reticulum (Fig. 3, RER) where most milk proteins as well the enzymes responsible for synthesis of lactose and oligosaccharides are created. These molecules are transferred to the Golgi compartment for processing of

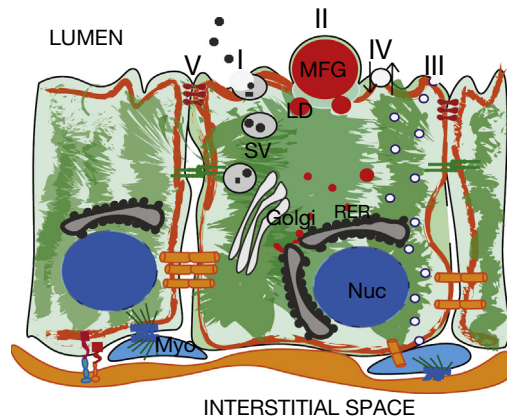


Fig. 3 Cartoon illustrating the major intracellular compartments and their role in milk secretion. The major milk secretion pathways are (I) protein and lactose synthesis secretion by Golgi secretory vesicles (SV). (II) Lipid secretion as the milk fat globule (MFG). Cytoplasmic lipid droplets (LD) are seen approaching and fusing with the MFG. (III) Transcytosis, some proteins are endocytosed from the interstitial compartment at the basal surface and transported across the cell to be secreted into the lumen. (IV) Membrane transport. Transporter molecules are located at both basolateral and apical surfaces and on intracellular membranes (see [Table 1](#)). (V) Paracellular pathway, open in pregnancy, closed in lactation. Note that desmosomes mediating epithelial cell interactions are lacking in the lactating mammary cell.

proteins including phosphorylation and attachment of oligosaccharides in many cases. Synthesis of lactose and oligosaccharides also occurs in this compartment. Secretory vesicles containing the milk proteins and sugars bud off the Golgi compartment, traveling to the apical plasma membrane, fusing and releasing their contents into the lumen.

Milk proteins

The major proteins synthesized and secreted by this endogenous pathway are the caseins and the so-called whey proteins, α -lactalbumin, β -lactoglobulin (in ruminants and pigs), whey acidic protein (rodents and pigs) and lactoferrin. Caseins are a class of phosphoproteins that make up 20% (humans) to 80% (cows) of the proteins in milk. The major isoforms of casein are α - and β -caseins, hydrophobic molecules that are highly phosphorylated so that they form distinct electron dense structures called micelles in the presence of ionic calcium. κ -Casein is a more soluble glycoprotein found at the surface of the casein micelle. Casein can be separated from the rest of the milk components by centrifugation, by precipitating at pH 4.6 or by enzymatic cleavage, usually with rennin, precipitating the casein as curds used in cheese making.

α -Lactalbumin (LALBA), with a molecular structure similar to lysozyme, is an important co-factor in lactose synthesis increasing the affinity of the enzyme galactosyltransferase for glucose up to 1000 times. Lactoferrin is an 80 kDa highly cationic iron-binding glycoprotein that is a member of the innate immune system. The protein has anti-microbial properties, both by direct action on bacteria and by depriving bacteria of needed iron. Lactoferrin also induces secretion of interferons with antimicrobial activity.

β -Lactoglobulin is a lipid-binding protein similar to retinoic acid binding protein. It is present in the milk of most mammals but not in rodent or human milk. Although it is known to bind fatty acids, its function in milk is not known.

Many proteins present at lower concentrations in milk are thought to be synthesized via the secretory pathway including lysozyme, bile salt stimulated lipase, amylase, the B-12 binding protein haptocorrin, a folate binding protein and many others. The role of bile salt stimulated lipase in triglyceride digestion in the infant is well known, but the exact role of most of the other minor milk proteins requires additional investigation.

Lactose and oligosaccharide synthesis

Lactose is synthesized from glucose and UDP-galactose. Cytoplasmic glucose is transported into the Golgi by the transporter GLUT-1. Glucose is also converted to UDP-galactose in the cytoplasm by a series of enzymes and then transported into the Golgi by the UDP-galactose transporter. In the Golgi compartment glucose and UDP-galactose are combined by the complex of α -lactalbumin and β 4-galactosyl transferase into the major milk sugar, lactose. Lactose is present at very high concentration in the milk of most species (180–200 mM in human milk) and its osmotic activity draws water into the Golgi.

The oligosaccharides in human milk are present at concentrations from 5 to 10 mg/L, similar to the concentration of human milk protein. The core structure has lactose at the initiating end extended by lactose or acetyl glucosamine units and decorated with fucosyl or sialyl units. These compounds are thought to be of great importance in human infants, regulating the intestinal microbiome and potentially stimulating intestinal development.

Pathway II: Secretion of Milk Lipid and Milk Extracellular Vesicles

The milk fat globule

The MFG is a distinct structure with a large core of triglyceride and a membrane, the milk fat globule membrane, containing a variety of proteins, the most abundant of which are adipophilin (ADPH) also known as perilipin 2, lactadherin, xanthine dehydrogenase, butyrophilin, and, in some species, fatty acid binding protein 3. The fatty acids used to synthesize triglycerides are either transported from the plasma into the alveolar cells by a fatty acid transporter, usually FATP6, or synthesized as medium chain fatty acids in the mammary gland itself. These fatty acids are transferred to the endoplasmic reticulum where they are combined with acetyl CoA and channeled toward esterification with glycerol by the enzymes AGPAT1 and AGPAT6. Small lipid droplets formed between the two leaves of the endoplasmic reticulum acquire a coat of phospholipid that mediates budding into the cytoplasm where they interact with ADPH. These cytoplasmic lipid droplets (CLD) travel toward the apical membrane, fusing into larger CLD as they reach the membrane where they eventually bud into the alveolar lumen ([Masedunskas et al., 2017](#)).

Milk extracellular vesicles

Small extracellular vesicles (EV) ranging in size from 50 to 200 nm in diameter are secreted in large numbers into milk (3×10^{12} particles/mL). They are surrounded by a double layer membrane and contain the traditional EV proteins CD9, CD63, CD81, Hsc70, MHC-class II, MFGE-8, and many others as well as both RNA and microRNA. In bovine milk these RNA's are about 58% non-coding RNA and 13% microRNA. They are thought to interact with intestinal cells and regulate intestinal development and immunity in the infant.

Pathway III. Transepithelial Protein Transport—Immunoglobulins and Other Molecules

Immunoglobulins

Sophisticated mechanisms exist to assure that important immunoglobulins made in the immune cells of the mother are transported to the milk at times and in quantities necessary for protection of the neonate from pathogens to which the mother is exposed. Plasma cells, the immune cells that make immunoglobulins, home from the intestine to the mammary gland during lactation and secrete IgA into the interstitial medium. The IgA binds to the polymeric IgA receptor at the basal surface of the epithelial cell and is then endocytosed via clathrin-coated vesicles, sorted into endosomes and subsequently secreted at the apical membrane bound to a portion of the receptor called secretory component ([Kraehenbuhl and Hunziker, 1998](#)).

Albumin and other plasma-delivered molecules

Many other molecules present in the plasma of lactating animals are transported into milk via transcytosis including albumin in rodents, prolactin, insulin, and drugs and toxins. However, much more work is necessary before the pathways for transcytosis of proteins and other molecules are properly understood.

Pathway IV. Transport of Water, Ions, and Trace Elements Into Milk

Major pathways

The major transporters by which nutrients, ions, and trace elements are moved across the membrane barriers in the lactating mammary epithelium are listed in [Table 1](#). Specific transporters exist for movement of these molecules across the basolateral and apical plasma membranes as well as for transport into various intracellular compartments including the RER, Golgi, and mitochondria.

Water

Water is the most abundant constituent of milk transferred passively across both the basolateral and apical membranes of the lactating mammary cell in response to an osmotic gradient. The water transporters aquaporins 1, 3, and 5 have been identified in the mammary gland and are likely responsible.

Monovalent ions

Sodium, potassium, chloride concentrations in milk are lower than those in the mammary epithelial cell, where sodium and potassium concentrations are thought to be maintained by the Na^+/K^+ -ATPase on the basolateral membrane. An electroneutral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ transporter may be responsible for the concentration of Cl^- in the mammary cell possibly responsible for the low concentration of milk Cl^- .

Calcium

Calcium is present in milk at concentrations of 10–80 mM mostly bound to casein and citrate. Free calcium ion is present at about 3 mM despite an intracellular free calcium level several orders of magnitude lower ([Cross et al., 2014](#)). Calcium influx across the plasma membrane is mediated by Orai1 channels, so-called store operated channels because they open in response to calcium depletion in the endoplasmic reticulum and Golgi vesicles. Calcium ATPases SERCA2 and SPCA2 transport calcium into the endoplasmic reticulum and Golgi vesicles, respectively, where they interact with calcium binding proteins, particularly

Table 1 Some major transporters in the lactating mammary epithelium

Transported molecule	Major transporter		Location	Major function
	Protein	Gene name		
Glucose	GLUT-1	SLC2A1	Basal membrane, Golgi membrane	Glucose transport across the plasma and Golgi membranes
UDP-galactose	UDP-galactose transporter	SLC35A2	Golgi membrane	UDP-galactose transport into Golgi
Fatty acid	Long chain fatty acid transporter FATP6	SLC27A6	Basal plasma membrane	Long chain fatty acid transport into secretory mammary cells
Neutral amino acids	LAT1	SLC3A2, SLC7A5	Basal plasma membrane	System L neutral amino acid transporter
Neutral amino acids; sodium dependent	SNAT1, SNAT2	SLC38A1, SLC38A2	Basal plasma membrane	System A sodium dependent neutral amino acid transporter
Calcium	Calcium release activated calcium channel protein 1	ORAI1	Basal plasma membrane	Calcium release activated calcium channel; regulated by calcium ATPase SPCA2
Calcium	SERCA2	ATP2A2	Endoplasmic reticulum	Sarco/endoplasmic reticulum Ca ATPase
Calcium	PMCA2	ATP2B2	Apical membrane	Transport of cytosolic calcium into milk
Calcium/magnesium	SPCA2	ATP2C2	Golgi vesicles	Golgi calcium transport; activation of ORAI1
Zinc	ZIP5, ZIP8, ZIP10	SLC39A5, SLC39A8, SLC39A10	Basal membrane	Transport of zinc into the cell
Zinc	ZnT2, ZnT4, ZnT5	SLC30A2, SLC30A4, SLC30A5	Intracellular membranes	Zinc transport into endoplasmic reticulum and Golgi compartments
Zinc	ZIP3	SLC39A3	Apical membrane	Transport of zinc from milk into the cell
Iron	TFR (transferrin receptor)	TFRC	Basolateral membrane	Transports transferrin bound iron from the plasma into endoplasmic vesicles
Iron	DMT1 (also called NRAMP2)	SLC11A2	Apical membrane?	Transport of iron across endosomal membrane and apical membrane
Iron	FPN (ferroportin)	SLC40A1	Plasma membrane	Iron exporter
Copper	CTR1	SLC31A1	Basal membrane	Transport of copper removed from plasma ceruloplasmin into the mammary cell
Water	Aquaporins	AQP1, AQP3, AQP5	Basal and apical membranes and internal membranes	Water equilibration across all membranes of the alveolar cell

casein, and ions such as citrate, bicarbonate, and phosphate. Secretory vesicles transfer this calcium into milk. In addition free calcium is transported from the cytosol into milk via a specific plasma membrane calcium ATPase 2 (PMCA2) expressed in the apical membrane. Magnesium is thought to be transported by many of the mechanisms known to transport calcium, but definitive studies are lacking.

Trace elements

Zinc, iron, and copper are important constituents of milk each of which has its own transport pathways. Two major categories of zinc transporters in the lactating mammary gland ([McCormick et al., 2014](#)) move zinc across membranes: the ZIP transporters move zinc across plasma membranes into the cell and from the cell into the milk and the ZNT2 transporters move zinc into intracellular compartments such as the endoplasmic reticulum. Ferric iron in serum is bound to transferrin which binds to transferrin receptor at the basal surface of the cell and is taken up into endosomes where the low pH leads to iron dissociation. The transferrin receptor recycles to the surface and the iron, reduced to ferrous iron is transported into the cytoplasm by the divalent ion transporter, DMT1. Plasma copper is largely bound to the protein ceruloplasmin, a protein that is synthesized in mammary epithelial cells and is present in milk. Current evidence suggests that interstitial copper ions can be removed from ceruloplasmin with a reductase at which point they become available for transport into the cell by CTR1, the only known membrane transporter for copper. Thus the picture that emerges is that plasma copper is rapidly transferred into the mammary gland by CTR1. Within the gland it must be transferred to the secretory pathway by an unknown mechanism, bound to ceruloplasmin and secreted into milk. However, details of this pathway are lacking.

Manganese, selenium, and cobalt

Manganese levels in human milk are in the ng/mL range but tend to be higher in other animals. Selenium is present at concentrations in the ng/mL range but varies with selenium intake. Cobalt is part of Vitamin B12. However, the mechanisms of transfer from the plasma to the milk are not known.

Pathway V. Paracellular Transport

The tight junctions are open in the gland of the pregnant animal allowing passage of molecules as large as albumin. This pathway is responsible for the high concentrations of such diverse molecules as sodium and albumin in the prepartum mammary secretion as well the transfer of secreted components such as lactose into the plasma, preventing build-up of mammary secretion in the lumen prior to the birth of the offspring.

References

- Anderson, S. M., Maclean, P. S., Mcmanaman, J. L., & Neville, M. C. (2015). Lactation and its hormonal control. In T. M. Plant, & A. J. Zelenik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn.). New York: Elsevier.
- Baumgartner, H. K., Rudolph, M. C., Ramanathan, P., Burns, V., Webb, P., Bitler, B. G., Stein, T., Kobayashi, K., & Neville, M. C. (2017). Developmental expression of claudins in the mammary gland. *Journal of Mammary Gland Biology and Neoplasia*, 22, 141–157.
- Cross, B. M., Breitwieser, G. E., Reinhardt, T. A., & Rao, R. (2014). Cellular calcium dynamics in lactation and breast cancer: from physiology to pathology. *American Journal of Physiology. Cell Physiology*, 306, C515–26.
- Kraehenbuhl, J.-P., & Hunziker, W. (1998). Epithelial transcytosis of immunoglobulins. *Journal of Mammary Gland Biology and Neoplasia*, 3, 289–304.
- Maller, O., Martinson, H., & Schedin, P. (2010). Extracellular matrix composition reveals complex and dynamic stromal–epithelial interactions in the mammary gland. *Journal of Mammary Gland Biology and Neoplasia*, 15, 301–318.
- Masedunskas, A., Chen, Y., Stussman, R., Weigert, R., & Mather, I. H. (2017). Kinetics of milk lipid droplet transport, growth, and secretion revealed by intravital imaging: lipid droplet release is intermittently stimulated by oxytocin. *Molecular Biology of the Cell*, 28, 935–946.
- McCormick, N. H., Hennigar, S. R., Kiselyov, K., & Kelleher, S. L. (2014). The biology of zinc transport in mammary epithelial cells: implications for mammary gland development, lactation, and involution. *Journal of Mammary Gland Biology and Neoplasia*, 19, 59–71.
- Pitelka, D. R., & Hamamoto, S. T. (1977). Form and function in mammary epithelium: the interpretation of ultrastructure. *Journal of Dairy Science*, 60, 643–654.

Mammary Gland Development

Lucia Speroni, Cheryl M Schaeberle, Carlos Sonnenschein, and Ana M Soto, Tufts University School of Medicine, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Overview of the Histology and Anatomy of the Breast

Mammary glands are the defining feature of the class Mammalia. In humans, mammary glands, though present in both females and males, are functional only in the postpartum female. Anatomically, the breasts are round protuberances that contain a parenchyma, namely, the epithelial tree or mammary gland proper, and a stroma, represented by adipose tissue and both loose and dense irregular connective tissue. The glands occupy the subcutaneous layer of the anterior and lateral thoracic wall. Each breast contains 15–20 lobes which in turn consist of many lobules. The gland reaches the exterior through the nipple, a protrusion surrounded by a pigmented areola. The breast parenchyma extends from the level of the second or third rib to the inframammary fold, which is at about the level of the sixth or seventh rib, and from the border of the sternum to the anterior axillary line. The posterior surface of the breast reaches the fasciae of the pectoralis major, serratus anterior, external abdominal oblique, and rectus abdominis muscles (Jesinger, 2014).

The breast ductal system is a branching structure that extends from a lactiferous duct that opens at the nipple and ends distally in structures named the terminal ductal lobular units. Such a ductal system represents a lobe. Rats and mice have a unilobular system. In the rhesus monkey there are three to seven lobes that converge to the nipple. Among women, the number of lobes is variable (Going and Moffat, 2004) and physiologically different within the same breast and overtime (Mills et al., 2016). Ultrasound data suggest the presence of 15–20 ducts converging on the nipple whereas 3D modeling of the nipple from serial sections shows that there is a median of 27 ducts (Going and Moffat, 2004). The number of ducts are greater than the number of openings in the nipple (6–8) suggesting that there could be distinct populations of duct; some produce milk while others derive from skin appendages and do not (Going and Moffat, 2004).

Histologically and functionally, as stated above, the breast is made up of two main tissue types, namely, (1) the parenchyma (epithelium), represented by the epithelial cells, whose function it is to generate milk to nourish the growing newborn, and (2) the stroma which surrounds the glandular epithelium. The epithelium is composed of two layers of cells: a continuous luminal cuboidal cell layer and a basally located discontinuous myoepithelial cell layer. The epithelial duct is in intimate contact with fibrous connective tissue and distant from the adipose tissue (Parmar and Cunha, 2004). The stroma or connective tissue surrounding the epithelium is composed of various cell types (fibroblasts, adipocytes, and immune cells), blood vessels, nerves, and an extracellular fibrous matrix of which the main component is collagen (Howard and Gusterson, 2000; Masso-Welch et al., 2000; Richert et al., 2000). In the mature mammary gland, at the distal end of the ducts, there are lobules composed of sacs or alveoli where milk is produced in response to hormonal cues, these structures are called terminal ductal lobular units and are the sites where breast tumors commonly develop starting as intraductal hyperplasias. These terminal ductal lobular units have been classified as types 1, 2, and 3. Again, ducts and alveolar buds are lined with a bi-layered epithelium composed of luminal epithelial cells and myoepithelial cells residing between the luminal epithelial cells and the basement membrane that separates them from the stroma. Throughout development, reciprocal interactions between the epithelium and the stroma are responsible for the morphogenesis of mammary glands. In the early stages of development the mesenchyme plays an inductive role on the morphogenesis of the epithelium.

Fetal Development

Humans

Fetal breast is particularly vulnerable to carcinogenic insults such as synthetic hormones and endocrine disruptors that cause organizational defects that permanently alter the anatomy and function of the gland (Soto and Sonnenschein, 2010). Most of what is currently known about fetal mammary development in humans resulted from observations of postmortem, difficult-to-obtain specimens. Processes involved in morphogenesis have been mostly inferred from animal studies, mostly carried out in mice. Like in rodents, there is individual variability regarding the timing of developmental events. Breast development is visible by 4 weeks of gestation as paired areas of proliferating epithelial cells in the epidermis of the thoracic region. These areas extend in a line from the axilla to the inguinal region and form two mammary ridges or milk lines. By week 6 of gestation, the mammary ridges have regressed back and only two epithelial masses, the mammary buds, are visible and begin to penetrate into the underlying mesenchyme. The epithelium then becomes surrounded by fibroblast-like cells and collagenous stroma. Until week 9 the placode grows further inward (cone stage) and between weeks 10 and 12, the mammary buds sprout from the placode (budding stage) and become lobular in shape creating an indentation along the epithelial–stromal border. Meanwhile the nipple begins to form in the overlying epidermis and the mesenchyme differentiates into fibroblasts, smooth muscle cells, endothelial cells, and adipocytes. At week 15, the epithelium branches into solid epithelial cords (branching stage) which develop lumen at around week 20. The mammary pit is formed as a depression of the epidermis in the region of the nipple. The ductal epithelium first appears as a bilayer of cuboidal cells. The luminal layer rapidly differentiates into secretory cells and the basal layer into

myoepithelial cells. By 6 months of gestation, a rudimentary ductal system has formed and the ducts are separated by fat islands within a fibrous connective tissue. The secretory epithelium becomes functional near the end of gestation in response to lactogenic hormones (Parmar and Cunha, 2004). The human mammary gland develops similarly in female and male fetuses (Howard and Gusterson, 2000).

Mice

The mouse model has revealed most of what is known about fetal development of the mammary gland. Using tissue recombination techniques, it was observed that the main determinant of morphogenesis is the reciprocal interaction between the mesenchyme and the parenchyma (Howard and Lu, 2014; Sakakura et al., 1976). Mouse fetal mammogenesis starts at about embryonic day 10.5 (E10.5) with the formation of two presumptive milk lines. The mammary placodes become visible between embryonic day (E) 11 and 12 and they then develop into mammary buds by E13, which are elevated knob-like structures. During the next day, they invaginate and thus are no longer observable from the exterior. At this time, several layers of mesenchyme condense surrounding the buds in a concentric fashion and they acquire a "light bulb" shape. In male embryos, testicular development results in testosterone secretion, which acts on the androgen-receptor expressing cells in the mammary mesenchyme. At E13.5 the mesenchyme starts condensing around the bud which then separates from the epidermis hindering further epithelial development (Kratohwil, 1971, 1977). In female mouse embryos, after a 24 h resting phase where practically no cell proliferation is observed, the mammary bud sprouts as it pushes through the mesenchyme penetrating the presumptive fat pad made up of a cluster of preadipocytes. Conversion of preadipocytes to adipocytes starts by E18 attaining a characteristic adipose tissue structure neonatally. At E18, the mammary epithelium consists of a rudimentary ductal tree (Balinsky, 1950; Robinson et al., 1999) (Fig. 1). Apart from the action of testosterone described above, fetal mammary gland development in mice does not require the action of other hormones; null mutation of the estrogen-receptor alpha does not alter fetal development (Dupont et al., 2000). However, fetal mammary morphogenesis can be altered by in utero exposure to hormonally active chemicals such as the xenoestrogen Bisphenol-A (Vandenberg et al., 2007). The fact that fetal DES exposure in rodents and humans leads to increased propensity to breast cancer also indicates that the gland is responsive to these chemicals and that the effects of such exposure could be deleterious (Hoover et al., 2011; Soto and Sonnenschein, 2010). Moreover, nuclear ERs and GPR30 are only expressed in the mesenchyme at this developmental stage, thus reinforcing the idea that the stroma plays a central role on morphogenesis (Wadia et al., 2013). Estrogen receptor alpha appears in the mammary epithelium at the end of the fetal period (E18–19) and is clearly more abundant in the epithelium than in stromal cells after birth and throughout postnatal life (Vandenberg et al., 2007).

Prepubertal and Pubertal Development

Humans

The prepubertal breast consists of a rudimentary tree of simple branched ducts and it grows at the same pace as the general body until the onset of puberty. Breast tissue is similar in prepubertal boys and girls. Pubertal rises in estrogen levels cause breast growth in girls and frequently though transiently in boys (Dimitrakakis and Bondy, 2009). Puberty in girls is marked by initiation of ovulation and establishment of menstrual cycles; also, breasts increase in volume and undergo changes in the stroma and glandular tissues. The stroma increases in the amount of fibrous and adipose tissue which constitutes 80% of the adult nonlactating breast (Parmar and Cunha, 2004). Glandular growth occurs as ducts elongate, branch, and form specialized club-shaped structures at the distal end called terminal end buds (TEB) (Russo and Russo, 2004). New branches will originate from TEBs expanding the epithelial tree and originating small ductules or alveolar buds. Alveolar buds are structures that are morphologically more developed than TEBs yet underdeveloped compared to the acinus, that is, the terminal structure present in the mature nonlactating mammary gland. A cluster of alveolar buds is called a lobule. Lobular formation occurs 1–2 years after the first menstrual cycle and the mammary gland develops gradually throughout adulthood and fully if pregnancy happens. The complexity of the lobules depends on the developmental stage of the breast (Fig. 2). Lobules gradually mature from type 1–2 and 2–3, as they increase in area and number of ductules and decrease in cell number. Once sexual maturity is reached, this initial phase of rapid growth ceases. Epithelial proliferation and morphological changes occur during each menstrual cycle (Söderqvist et al., 1997). Each cycle is regulated by the sequential and combined action of estrogen and progesterone: estrogen peaks at the follicular phase which is followed by a second peak during the luteal phase simultaneously with a peak in progesterone levels (Soules et al., 1989). Breast lobules become more complex and bigger in size during the luteal phase of the cycle. The breast of a nulliparous woman contains predominantly lobules type 1 and occasionally lobules type 2 and rarely type 3 (Russo and Russo, 2004). Lobules in parous women undergo a complete cycle of development to type 4 lobules, the state of maximal development of the adult gland when milk needs to be secreted, namely at the very end of pregnancy and after parturition (Parmar and Cunha, 2004).

Mice

In mice, like in humans, the gland grows isometrically until the onset of puberty. Ovarian activity results in increased prepubertal estradiol serum levels by PND14 (François et al., 2017). Estrogens trigger the peri-pubertal growth of the epithelial ductal tree, which results in the development of terminal end buds, the bulb-shaped invasive structure that guides ductal growth. The main

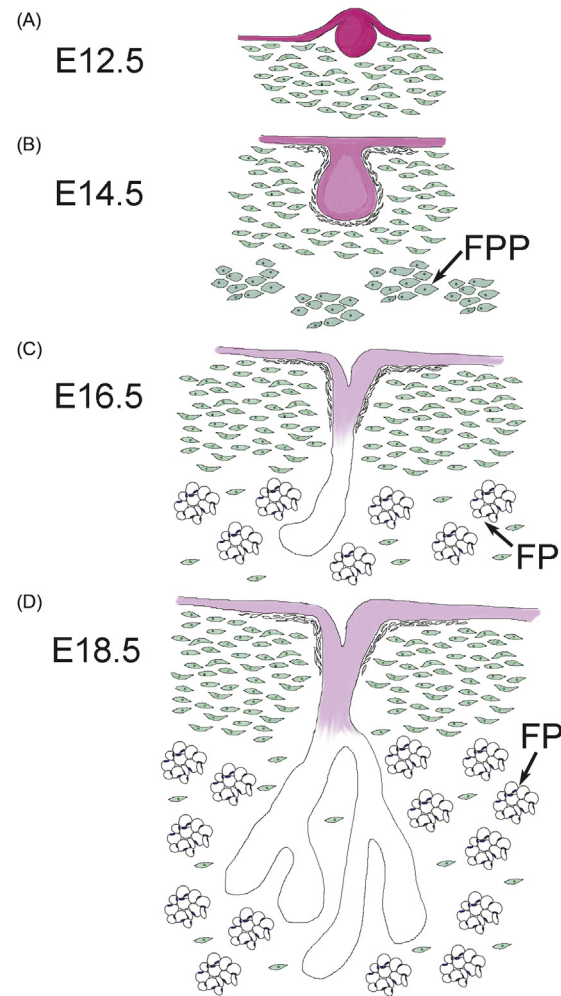


Fig. 1 Schematic of prenatal development of the mouse mammary gland. (A) The epithelial bud is surrounded by dermal mesenchyme (E12.5). (B) The bud invaginates into the surrounding stroma. The fat pad precursor (FPP) starts to differentiate (E14.5). (C) The epithelial compartment elongates, forming the sprout (E16.5). (D) The fat pad (FP) has now differentiated, a prerequisite for ductal growth and branching. The epithelial compartment has now branched forming solid epithelial cords. Lumen formation starts at this point (E18.5). Reprinted with permission from Soto, A.M., Rubin, B.S., Sonnenschein, C. (2007). *Endocrine disruption and the female*. In Gore, A.C. (eds) *Endocrine-disrupting chemicals. Contemporary endocrinology*. New York, NY: Humana Press.

ductal system is produced by this invasive activity, by TEB bifurcation, and by the elongation of the subtended duct. The TEB is characterized by high levels of proliferation producing the cells needed for ductal elongation, and apoptosis which results in patency of the ducts. Once the TEBs reach the edges of the fat pad they regress (Paine and Lewis, 2017).

The mammary gland undergoes developmental changes during each estrus cycle. Depending on the strain, the ductal system of adult, nonpregnant females will exhibit only ducts, which undergo lateral branching, a process regulated by progesterone (Fig. 3). In some strains, alveolar buds may appear during each cycle. These alveolar structures contain a single layer of epithelial cells; they undergo complete maturation during pregnancy, and thus, become fully developed to sustain milk production (Robinson et al., 1995). Lateral buds may elongate to form branches or cleave to form alveolar buds. As branching morphogenesis occurs, the connective tissue within the extracellular matrix is broken down to allow growth of the epithelial structures (Richert et al., 2000).

The mouse mammary stroma is dissimilar to that of the human breast. In the former, a thin layer of loose connective tissue containing sparse fibroblasts surrounds the ductal tree. This stroma is in turn, surrounded by abundant adipose tissue. In humans, instead, ducts are surrounded by loose connective tissue containing abundant fibroblasts and a distinct fibrous stromal component, which is also surrounded by adipose tissue. In mice, the epithelium is in proximity to the adipose tissue, while in humans it is not. In both species, the extracellular matrix (ECM) is composed of the basement membrane separating the epithelium and stroma, and the interstitial matrix filling the spaces among the stromal cells. The ECM is a functionally important structure that plays an important role in ductal morphogenesis. The ECM is a dynamic structure that undergoes remodeling, a process influenced by enzymes secreted by both the epithelial and stromal cells (Howard and Lu, 2014).

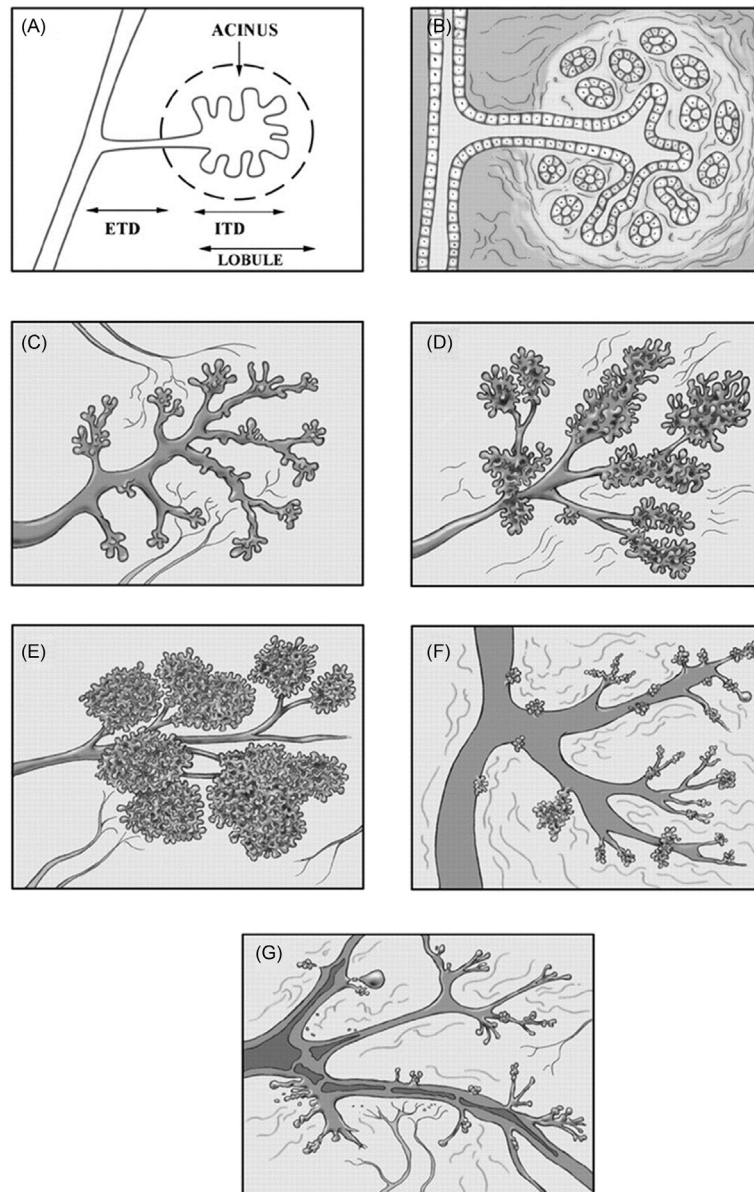


Fig. 2 Drawings depicting the functional development of the human breast, the basic functional unit of which is the terminal ductal-lobular unit (TDLU). (A) Diagram of a TDLU emerging from the extralobular terminal duct (ETD). (B) Diagram depicting the histology of a normal TDLU in the resting nulliparous state, showing the terminal duct and associated acini. (C) Drawing of terminal branching structures from a 15-year-old human breast. An extralobular duct is shown with rudimentary terminal branching structures. True lobules and acini are not yet present. (D) Drawing of TDLUs from a 22-year-old nulliparous human mammary gland having well developed lobules and acini. (E) Drawing of breast structures of a 30-year-old pregnant woman. Note well developed TDLUs. (F) Drawing of breast tissue of a 55-year-old parous menopausal woman. Ducts are dilated, and the TDLUs are atrophied. (G) Drawing of breast tissue of an 80-year-old woman. Marked atrophy of ducts and TDLUs can be seen. Reprinted with permission from Parmar, H. and Cunha, G. R. (2004). Epithelial-stromal interactions in the mouse and human mammary gland in vivo. *Endocrine-Related Cancer*, 11, 437–458.

Pregnancy

Humans

During pregnancy, the breast undergoes changes from a resting to a functional organ in preparation for feeding the newborn. Although the breast is remodeled in every menstrual cycle, it is pregnancy hormones that trigger remodeling to the extent of a fully mature organ. Circulating levels of lactogenic hormones, estrogen, progesterone, and prolactin as well as the metabolic hormones insulin, growth hormone and glucocorticoids orchestrate ductal branching, alveolar morphogenesis, and secretory differentiation (production of lactose). The levels of prolactin increase during pregnancy and induce secretory differentiation by mid-pregnancy.

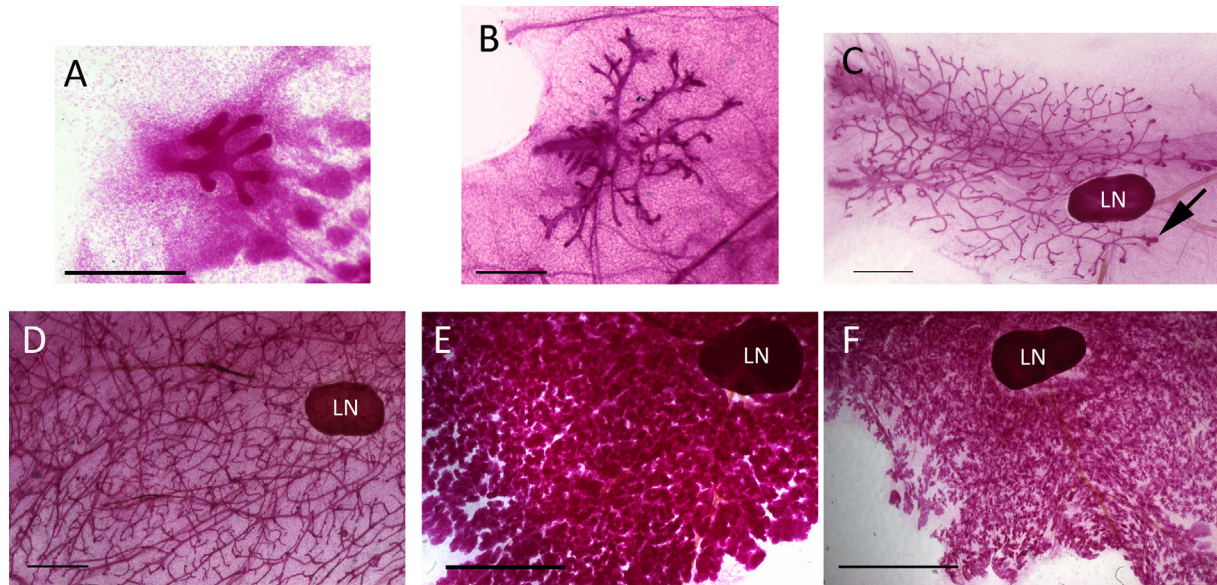


Fig. 3 Development of female mouse mammary glands throughout life. (A) E18, (B) PND5, (C) 1 month (pubertal) (arrow depicts terminal end bud), (D) 7 months, showing extensive lateral branching (E) lactating gland, and (F) involuting gland 4 days postweaning. Scale bar in (A and B) 500 μ m, in (C through F) 2 mm. LN denotes lymph node.

(Pang and Hartmann, 2007). Specimens taken during pregnancy and lactation are usually from operations due to a cancer diagnosis during these developmental stages. In general, they show similarities with those observed in rodents; the main difference is heterogeneity. Some parts of the breast are showing clear changes like those described in rodents; other areas seem to be resting (Howard and Gusterson, 2000).

Mice

During the first half of pregnancy, mammary growth occurs through a massive proliferation of the epithelium which manifests as enhanced ductal branching and one of alveolar bud formation. This is due to estrogen and progesterone secreted by the corpus luteum, which during pregnancy is under prolactin stimulation in rodents. In humans, it is HCG that maintains the corpus luteum. The stromal compartment is reduced, although there is an increase of capillaries surrounding the alveoli. The alveolar buds cleave to form alveoli which are organized in lobules that fill the fat pad at the end of pregnancy (lobulo-alveolar phase). Alveoli have a layer of epithelial luminal cells that produce milk components. These luminal cells are surrounded by myoepithelial cells that abut the basement membrane. By day 18 of pregnancy, the alveolar cells are already producing milk proteins and lipids; and at parturition the mammary gland is fully functional (i.e., a lactating mammary gland). Lactogenesis 1 begins during the second half of pregnancy with the progressive expression of various genes involved in the synthesis of milk components. In the mouse, the morphological manifestation of this phase is the appearance of cytoplasmic lipid droplets in the mammary epithelium while in humans it is the appearance of alpha-lactalbumin in serum. This phase is thought to be regulated by progesterone, prolactin and/or plasma lactogen, and growth hormone. During this period, progesterone suppresses milk secretion (Neville et al., 2002).

Lactation

Humans

The signal for the breast to begin secretory activity is the drop in progesterone levels just after the placenta is delivered. The infant is nurtured by colostrum, a high protein concentrate including lactoferrin and IgA that is secreted right after birth; and then by milk which starts to be secreted 30–40 h postpartum. Ejection of milk from the breast results from the contraction of the myoepithelial cells surrounding the alveoli. Mechanical stimulus such as suckling and breast massages can aid milk production and breastfeeding (Hassiotou and Geddes, 2013; Pang and Hartmann, 2007).

Mice

Around parturition, the mouse mammary gland undergoes further changes that lead to the secretion of colostrum first and then milk. This phase is referred to as lactogenesis 2 and is characterized by a further increase in the number of expressed milk protein genes, and the presence of lipid droplets and casein in the alveolar lumen. Colostrum is characterized by the presence of

immunoglobulins. This phase 2 requires high prolactin levels and a drastic lowering of progesterone levels. After the beginning of suckling there is a final expansion of the alveolar compartment and a further increase of milk protein gene expression. Lactation is maintained by suckling, triggering oxytocin secretion which is needed for the contraction of myoepithelial cells, and prolactin secretion, which acts in the luminal epithelial cells to maintain milk production (Neville et al., 2002) (Fig. 3E).

Postlactational Involution

Humans

When milk removal from the breasts slows down or stops (weaning), the glandular tissue gradually involutes. The number of epithelial cells in the alveoli decreases and adipocytes repopulate the stroma. Experiments in mice have shown that macrophages are crucial in extracellular matrix remodeling to return the gland to a resting state similar though not identical to that before pregnancy occurred (O'Brien et al., 2012). Typically, there are more type 2 and 3 lobules than in the nulliparous breast.

Mice

Cessation of suckling triggers mammary gland involution. During the first hours, histology reveals engorgement of the alveoli with milk. After 2 days, the process becomes irreversible with signs of tissue remodeling and of epithelial cell death by apoptosis peaking at day 4 (Fig. 3F). After the first 2 days, stromal cells secrete matrix metalloproteinases that break down extracellular matrix. Macrophages are prominently observed during tissue remodeling. At this point, alveoli start to decrease in number giving way to clusters of disorganized epithelial alveolar cells. Simultaneously, adipocytes re-appear in the stroma. Ducts surrounded by dense stroma are now visible and dense stroma is visible around the ducts. The stroma also increases around the clusters of collapsing alveoli. By day 6 massive cell death has already occurred, but apoptotic bodies are still detected. Concomitantly, a large increase in macrophage numbers is observed, which return to nulliparous levels when regression is completed (O'Brien et al., 2010). By day 21 of involution the remodeled gland appears quite like the gland of nulliparous animals, although with somewhat more differentiated glandular development (Richert et al., 2000).

Age-Related Lobular Involution

Humans

As a woman ages, breast lobules involute and the number and size of acini per lobule in the breast decreases. The intralobular stroma is initially replaced by collagen followed by a gradual replacement of both glandular tissue and collagen by fatty tissue (Hutson et al., 1985). Age-related involution is different from postlactational involution; in the later, mammary tissues regress without loss of glandular tissue. The rate and extent of the process varies among individuals; it is more pronounced when the levels of estrogen and progesterone produced by the ovaries decrease signaling menopause. Partial involution has been detected in women younger than 40 years of age and ongoing in women older than 70. Factors other than age and menopause such as obstetrical history can influence age-related involution (Milanese et al., 2006).

Rodents

Mice and rats do not undergo a process similar to human menopause. Aging of the hypothalamus–pituitary–ovarian axis has been studied in more detail in rats than in mice. Cessation of estrus cyclicity is followed by a chronic anovulatory state. In various rat strains this status is referred to as persistent estrus that is characterized by persistent vaginal cornification. Contrary to menopause in women, rat ovaries of persistent estrous animals contain follicles in various stages of development. The mammary glands of old female nulliparous and multiparous mice at 16–20 months of age show features of early pregnancy, clearly indicating the presence of mammotropic hormones (Raafat et al., 2012; Rubin, 2000).

Acknowledgments

A special thanks to Nafis Hasan for his critical assistance in preparation of this article. This work was supported by National Institute of Environmental Health Sciences grant ES08314. The content does not necessarily represent the official views of the funding agencies including the National Institute of Environmental Health Sciences or the National Institutes of Health.

References

- Balinsky, B. I. (1950). On the prenatal growth of the mammary gland rudiment in the mouse. *Journal of Anatomy*, 84, 227–235.
- Dimitrakakis, C., & Bondy, C. (2009). Androgens and the breast. *Breast Cancer Research*, 11, 212.
- Dupont, S., Krust, A., Gansmuller, A., Dierich, A., Chambon, P., & Mark, M. (2000). Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development*, 127, 4277–4291.

- François, C. M., Petit, F., Giton, F., Gougeon, A., Ravel, C., Magre, S., Cohen-Tannoudji, J., & Guigon, C. (2017). A novel action of follicle-stimulating hormone in the ovary promotes estradiol production without inducing excessive follicular growth before puberty. *Scientific Reports*, 7, 46222.
- Going, J. J., & Moffat, D. F. (2004). Escaping from flatland: Clinical and biological aspects of human mammary duct anatomy in three dimensions. *Journal of Pathology*, 203, 538–544.
- Hassiotou, F., & Geddes, D. (2013). Anatomy of the human mammary gland: Current status of knowledge. *Clinical Anatomy*, 26, 29–48.
- Hoover, R. N., Hyer, M., Pfeiffer, R. M., Adam, E., Bond, B., Cheville, A. L., Colton, T., Hartge, P., Hatch, E. E., Herbst, A. L., Karlan, B. Y., Kaufman, R., Noller, K. L., Palmer, J. R., Robboy, S. J., Saal, R. C., Strohsnitter, W., Titus-Ernstoff, L., & Troisi, R. (2011). Adverse health outcomes in women exposed in utero to diethylstilbestrol. *The New England Journal of Medicine*, 365, 1304–1314.
- Howard, B. A., & Gusterson, B. A. (2000). Human breast development. *Journal of Mammary Gland Biology and Neoplasia*, 5, 119–137.
- Howard, B. A., & Lu, P. (2014). Stromal regulation of embryonic and postnatal mammary epithelial development and differentiation. *Seminars in Cell & Developmental Biology*, 25–26, 43–51.
- Hutson, S. W., Cowen, P. N., & Bird, C. C. (1985). Morphometric studies of age related changes in normal human breast and their significance for evolution of mammary cancer. *Journal of Clinical Pathology*, 38, 281–287.
- Jesinger, R. A. (2014). Breast anatomy for the interventionalist. *Techniques in Vascular and Interventional Radiology*, 17, 3–9.
- Kratochwil, K. (1971). In vitro analysis of the hormonal basis for the sexual dimorphism in the embryonic development of the mouse mammary gland. *Journal of Embryology & Experimental Morphology*, 25, 141–153.
- Kratochwil, K. (1977). Development and loss of androgen responsiveness in the embryonic rudiment of the mouse mammary gland. *Developmental Biology*, 61, 358–365.
- Masso-Welch, P. A., Darcy, K. M., Stangle-Castor, N. C., & Ip, M. M. (2000). A developmental atlas of rat mammary gland histology. *Journal of Mammary Gland Biology and Neoplasia*, 5, 165–185.
- Milanese, T. R., Hartmann, L. C., Sellers, T. A., Frost, M. H., Vierkant, R. A., Maloney, S. D., Pankratz, V. S., Degnim, A. C., Vachon, C. M., Reynolds, C. A., Thompson, R. A., Melton, L. J., Goode, E. L., & Visscher, D. W. (2006). Age-related lobular involution and risk of breast cancer. *Journal of the National Cancer Institute*, 98, 1600–1607.
- Mills, D., Gomberwalla, A., Gordon, E. J., Tondre, J., Nejad, M., Nguyen, T., Pogoda, J. M., Rao, J., Chatterton, R., Hennig, S., & Love, S. M. (2016). Examination of duct physiology in the human mammary gland. *PLoS One*, 11, e0150653.
- Neville, M. C., McFadden, T. B., & Forsyth, I. (2002). Hormonal regulation of mammary differentiation and milk secretion. *Journal of Mammary Gland Biology and Neoplasia*, 7, 49–66.
- O'Brien, J., Lyons, T., Monks, J., Lucia, M. S., Wilson, R. S., Hines, L., Man, Y. G., Borges, V., & Schedin, P. (2010). Alternatively activated macrophages and collagen remodeling characterize the postpartum involuting mammary gland across species. *American Journal of Pathology*, 176, 1241–1255.
- O'Brien, J., Martinson, H., Durand-Rougely, C., & Schedin, P. (2012). Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. *Development*, 139, 269–275.
- Paine, I. S., & Lewis, M. T. (2017). The terminal end bud: The little engine that could. *Journal of Mammary Gland Biology and Neoplasia*, 22, 93–108.
- Pang, W. W., & Hartmann, P. E. (2007). Initiation of human lactation: Secretory differentiation and secretory activation. *Journal of Mammary Gland Biology and Neoplasia*, 12, 211–221.
- Parmar, H., & Cunha, G. R. (2004). Epithelial–stromal interactions in the mouse and human mammary gland in vivo. *Endocrine-Related Cancer*, 11, 437–458.
- Raafat, A., Strizzi, L., Lashin, K., Ginsburg, E., McCurdy, D., Salomon, D., Smith, G. H., Medina, D., & Callahan, R. (2012). Effects of age and parity on mammary gland lesions and progenitor cells in the FVB/N-RC mice. *PLoS One*, 7, e43624.
- Richert, M. M., Schwertfeger, K. L., Ryder, J. W., & Anderson, S. M. (2000). An atlas of mouse mammary gland development. *Journal of Mammary Gland Biology and Neoplasia*, 5, 227–241.
- Robinson, G. W., McKnight, R. A., Smith, G. H., & Hennighausen, L. (1995). Mammary epithelial cells undergo secretory differentiation in cycling virgins but require pregnancy for the establishment of terminal differentiation. *Development*, 121, 2079–2090.
- Robinson, G. W., Karpf, A. B. C., & Kratochwil, K. (1999). Regulation of mammary gland development by tissue interaction. *Journal of Mammary Gland Biology and Neoplasia*, 4, 9–19.
- Rubin, B. S. (2000). Hypothalamic alterations and reproductive aging in female rats: Evidence of altered luteinizing hormone-releasing hormone neuronal function. *Biology of Reproduction*, 63, 968–976.
- Russo, J., & Russo, I. H. (2004). Development of the human breast. *Maturitas*, 49, 2–15.
- Sakakura, T., Nishizuka, Y., & Dawe, C. J. (1976). Mesenchyme-dependent morphogenesis and epithelium-specific cytodifferentiation in mouse mammary gland. *Science*, 194, 1439–1441.
- Söderqvist, G., Isaksson, E., von Schoultz, B., Carlström, K., Tani, E., & Skoog, L. (1997). Proliferation of breast epithelial cells in healthy women during the menstrual cycle. *American Journal of Obstetrics and Gynecology*, 176, 123–128.
- Soto, A. M., & Sonnenschein, C. (2010). Environmental causes of cancer: Endocrine disruptors as carcinogens. *Nature Reviews. Endocrinology*, 6, 363–370.
- Soules, M. R., McLachlan, R. I., Ek, M., Dahl, K. D., Cohen, N. L., & Bremner, W. J. (1989). Luteal phase deficiency: Characterization of reproductive hormones over the menstrual cycle. *Journal of Clinical Endocrinology and Metabolism*, 69, 804–812.
- Vandenberg, L. N., Maffini, M. V., Wadia, P. R., Sonnenschein, C., Rubin, B. S., & Soto, A. M. (2007). Exposure to environmentally relevant doses of the xenoestrogen bisphenol-A alters development of the fetal mouse mammary gland. *Endocrinology*, 148, 116–127.
- Wadia, P. R., Cabaton, N. J., Borrero, M. D., Rubin, B. S., Sonnenschein, C., Shioda, T., & Soto, A. M. (2013). Low-dose BPA exposure alters the mesenchymal and epithelial transcriptomes of the mouse fetal mammary gland. *PLoS One*, 8, e63902.

The Marvels of Milk and Lactation

Russell C Hovey, University of California, Davis, CA, United States

© 2018 Elsevier Inc. All rights reserved.

The Evolution of Lactation, and the Benefits of Milk

Lactation is the core reproductive strategy shared by all mammals, which includes the Monotremata (the egg-laying monotremes, the echidna and the platypus), the Marsupialia (birthing altricial young and no true placenta, such as the opossum, the koala, the wallaby and the kangaroo), and the Eutherian mammals (placental mammals accounting for 95% of all mammals, including humans and domesticated livestock). In turn, the composition of milk is the lifeblood of the neonate, and has been evolutionarily-tailored for its developmental needs and the environment it is raised in. The formidable question over the ages has been “why did lactation evolve as an advantageous reproductive strategy some 300 million years ago?” The answer likely comes from several angles. First, the ability of a mother to produce nutritious milk almost certainly conferred an advantage to terrestrial mammals through their ability to nourish undeveloped infants in drier climates with longer intervals between feedings, all while capitalizing on her nutrient intake and reserves. Milk also likely provided hydration through the parchment-like shell of eggs while providing antimicrobial protection by way of proteins such as lysozyme and lipocalins. The nutritive profile of milk was also surely a major advantage, providing an optimal balance of energy, amino acids, fatty acids and minerals for the growing neonate, while at the same time providing a continuous source of water. The ability to produce milk may have also allowed for shorter generation intervals and delayed tooth eruption, and the faster evolution of successful species (Ofstedal, 2002).

Mammary Gland Anatomy as Relates to Milk Secretion

The functional anatomy of the mammary glands varies considerably. The monotremes have “mammo-pilo-sebaceous patches,” a primitive form of the gland that distributes its milk to the neonate via their associated hairs. In other mammals, the mammary glands have either teats (a single opening, the galactophore) or nipples (served by two or more galactophores). Hence, mice, rats, cows, goats and sheep have teats, whereas pigs, rabbits/elephants and humans have nipples with 2, 8–11, and 10–15 galactophores, respectively (Table 1). Some species such as horses, goats and cows have their glands arranged into a suspended udder. Gland location also differs appreciably across species. Those bearing litters and large numbers of offspring have numerous glands positioned ventrally (i.e., mice and pigs) whereas in species such as humans or elephants the glands are located thoracically. Generally speaking, the glands are arranged symmetrically. The teats/nipples also serve as a critical barrier to pathogens, both physically through the presence of a sphincter surrounding the streak canal, and chemically via their secretion of keratins to form a “keratin plug.”

The Initiation of Milk Synthesis

Fascinatingly, the mammary epithelium transitions from a proliferative state during pregnancy to a state of terminal differentiation for the synthesis and secretion of milk, which is collectively referred to as “lactogenesis.” During lactogenesis I, the epithelium assumes many of the properties needed for milk synthesis—redistribution and enhanced activity of organelles, an assumption of polarity to allow basal-to-apical secretion, and increased enzymatic activity as well as the ability to form colostrum. Lactogenesis II, also referred to as secretory activation, corresponds to the initiation of milk flow around parturition, coincident with the movement of milk fat droplets into the lumen, the synthesis of lactose and the parallel movement of water, and the closure of inter-epithelial tight junctions. Once lactation initiates, the continued copious secretion of milk is referred to as galactopoiesis.

Table 1 Comparative mammary gland structure and milk composition in various species. Data are weight percentage

Species	Water	Fat	Caseins	Whey proteins	Lactose	No. of gland pairs	No. of galactophores per gland	Gland positions
	%	%	%	%	%			
Cow	87	3.7	2.6	0.6	4.6	2	1	Inguinal
Human	87	3.8	0.4	0.6	7.1	1	10–15	Thoracic
Mare	89	1.9	1.3	1.2	6.2	1	2	Inguinal
Pig	81	6.8	2.8	2.0	5.5	6	2	Thoracic/abdominal/inguinal
Mouse	74	13.1	7.0	2.0	3.0	5	1	Thoracic/abdominal/inguinal
Blue whale	46	39.4	7.2	3.7	0.4	1	1	Inguinal

Data are adapted from Akers, R.M. (2002). *Lactation and the mammary gland*. Ames, Iowa: Iowa State Press, and Davies, D.T., Holt, C. and Christie, W.W. (1983). *The composition of milk*. In: Mepham, T. B. (ed.) *Biochemistry of Lactation* No. 1., p. 71–117. Amsterdam, New York: Elsevier Science.

The progression through lactogenesis reflects a complex interplay between the local and endocrine environments. A key requirement for lactogenesis I is a subtending basement membrane to which epithelial cells attach via integrins; this physical environment also facilitates the formation of 3-dimensional, single-layered alveoli. The matrix is also crucial for the response to the key endocrine signals—insulin, prolactin, and glucocorticoid. While these hormones synergize to facilitate synthesis of a range of proteins unique to milk, it is the parallel elevation in circulating progesterone that acts as a “brake”; the timing of the subsequent switch to lactogenesis II upon the removal of this “brake” as the result of a decline in circulating progesterone varies between species, ranging from a few days before parturition (i.e., cows) to a few days after (i.e., humans).

The continued synthesis of milk absolutely requires consistent endocrine stimulation, although requirements vary with species, genetics, and environment. Serum prolactin levels must remain elevated for lactation to continue. Elegant historical studies using endocrine ablation strategies also revealed crucial roles for thyroid hormone, adrenal corticoids, and insulin for the continued synthesis of milk (Tucker, 1974). In addition, supplemental growth hormone increases milk yield and is often administered to dairy cows as recombinant bovine somatotropin.

The various stages of lactogenesis can also be recapitulated *in vitro* and *in vivo*. Mammary epithelial cells in their stromal microenvironment can undergo lactogenesis *in vitro* in the presence of combinations of hormones in “whole organ culture.” Isolated epithelial cells can form hollowed spheroids when exposed to extracellular matrices, then can initiate lactogenesis I after hormonal stimulation. Perhaps the most striking model of lactogenesis is “induced lactation” in nulliparous females (such as sheep or heifers), or in males such as bulls, and can also be realized in humans. In this model, exogenous ovarian steroids can mimic all the mammatogenic and lactogenic changes seen during a normal gestation; with frequent milking, the resulting yields and composition are similar to that from a pregnancy-induced lactation (Smith and Schanbacher, 1973).

Milk and Its Composition

At the broadest level, milk comprises an array of proteins, lipids and carbohydrates that are dissolved or suspended in water. Removing the fat layer rich in triglycerides packaged into milk fat globules leaves “skim” milk that can be further separated into the aqueous “whey” (containing water, lactose, soluble proteins such as α -lactalbumin, β -lactoglobulin, lactoferrin and lysozyme, and ions including potassium, sodium and chloride) and the precipitable “casein” fraction (containing casein molecules in their colloidal, micellar form in association with Ca, Mg, inorganic phosphate and citrate).

While many of these molecules are synthesized *de novo* by the epithelium, others are present in milk due to their active or passive transfer directly from the maternal circulation. One should also not overlook that milk contains various somatic cells including those from the immune system as well as epithelial cells shed from the gland. Milk can also transmit beneficial microbes from the mother (Williams et al., 2017), and can support the intramammary growth of external pathogens, leading to mastitis.

The composition of milk differs significantly across species, ranging from dilute milks with a water content of ~90% (as in dairy animals and humans), through to more viscous milk produced by marine mammals such as whales, seals and dolphins that contains ~50% water (Table 1). Generally speaking, the fat and protein contents of milk are positively correlated across species; conversely, the ratio of fat and lactose in milks is negatively correlated. Thus, more dilute milks typically have a higher lactose content, reflecting the fact that lactose draws water into milk due to its osmotic potential.

Lactose and Water Content

Lactose is a disaccharide produced by the mammary epithelium that not only serves as a nutritious carbohydrate but also as the major osmole in milk. Mammary epithelial cells can produce lactose through their unique synthesis of α -lactalbumin, a modifier that enables galactosyl transferase to facilitate the linkage of glucose and galactose to form lactose. While the lactose content of milk is relatively static throughout lactation, the concentration of lactose (or other oligosaccharides) in milk differs appreciably across species, creating differences in the water content of milk. As an extreme, Cape fur seals have evolved mutations in the α -lactalbumin gene that precludes their ability to synthesize lactose (Sharp et al., 2008).

Milk Fat

The composition of triglyceride in milk depends on several key variables, the first of which is the nature of circulating precursors. Roughly speaking, half of the fat in milk triglyceride is derived from preformed fatty acids taken up from the circulation which contribute to the C₁₆ and longer fatty acid chains. For monogastrics, the circulating fatty acid profile largely reflects that of the diet, thereby increasing the abundance of unsaturated fatty acids found in their milk. By contrast, the majority of circulating fatty acids in ruminants are saturated due to ruminal biohydrogenation, yielding milk fat having a higher content of saturated fatty acids. The remainder of the fatty acids in milk triglyceride are synthesized *de novo* by the mammary epithelial cells. The precursors that are available vary with species. In monogastrics, epithelial cells convert high levels of circulating glucose into a pool of cytoplasmic acetyl CoA ready for conversion to fatty acid chains. By contrast, ruminants have low levels of free circulating glucose, but a readily-available pool of the volatile fatty acid, acetate, which can be directly converted to acetyl CoA. In both cases, the newly-synthesized acetyl CoA is then shunted through the malonyl CoA pathway, which elongates the fatty acid chain by two carbons with every round of the fatty acid synthetase enzyme complex. Lastly, ruminants can also direct rumen-derived

β -hydroxybutyrate into fatty acid synthesis as an alternative four carbon primer. In all cases these newly-synthesized fatty acids are then incorporated into triglyceride via glycerol esterification prior to their secretion via pinocytosis of milk fat globules (Bauman et al., 2006).

Genetics also dictates the composition of milk fat and impacts the mechanisms by which it is secreted. For example, among *Bos taurus* dairy breeds, Holsteins and Jerseys differ considerably in the fat content of their milk (approximately 3.6% and 5%, respectively), while polymorphisms in key milk fat synthesis genes such as DGAT1 also account for variation within a breed (Buitenhuis et al., 2014). Furthermore, goats produce a higher proportion of medium chain fatty acids (C_6 , C_8 , and C_{10}) in their milk fat than do cows; goats also secrete milk fat globules in a true apocrine fashion that captures additional cytoplasmic cellular material, compared to the merocrine secretion of exclusive fat droplets by cows during pinocytosis. Recent evidence also highlights that a key regulator of milk fat droplet secretion is oxytocin-induced contraction of the alveoli (Masedunskas et al., 2017). Milk fat synthesis is also sensitive to specific metabolites, as occurs during “milk fat depression.” In this situation, such as in ruminants fed a diet high in unsaturated fatty acids, elevated levels of rumen-derived conjugated linoleic acid can suppress de novo fatty acid synthesis, lowering the fat content of milk without any appreciable effect on the output of other components (Jenkins and Harvatine, 2014).

Milk Proteins

Milk contains an abundance of proteins that literally come in many shapes and sizes. The caseins are a collection of proteins encoded by different genes arising from a common ancestor that are uniquely synthesized by the mammary epithelium. In bovine milk they account for approximately 80% of all protein while this is closer to 30% for humans. The caseins form the hydrophobic micelles that represent the colloidal fraction of milk; not only are they of high biological value for the infant due to their optimal amino acid profile, but the calcium and phosphate that help form these micelles are equally-important for the growing infant. A number of genetic variants of the different bovine caseins have also been described and are increasingly being harvested as separate forms of cow’s milk with different processability and purported health benefits.

As outlined above, α -lactalbumin is also a key protein found in milk due to the abundance required for lactose synthesis. In some species such as pigs, cows, sheep and goats the whey protein β -lactoglobulin is abundant and can exist as genetic variant A and B forms. Humans on the other hand do not synthesize β -lactoglobulin. Similarly, the whey acidic protein is produced in the milk of species such as mice, rabbits, pigs, and the tammar wallaby, but not in humans or other domestic livestock.

Beyond the caseins and major whey proteins, numerous other proteins are found in milk that differ according to species as well as stage of lactation. The immunoglobulins found in the first milk, or colostrum, serve a critical role for the delivery of immunity to the neonate via passive immunity, and are present at concentrations exceeding those in the maternal circulation. The nature of colostrum also varies as a function of the differential importance of various immunoglobulins (IgA, IgG, and IgM) across species, and depending on the relative contribution of transplacental transfer of immunity from the mother to the fetus. Other proteins such as serum albumin are found in milk as the result of tight junction leakage, and are much more abundant in colostrum, during mastitis, and during involution. Lastly, the mammary epithelium synthesizes a diverse pool of proteins including antimicrobial proteins and growth factors having many, potentially unresolved, functions that might benefit the neonate, or even the gland itself.

Vascular and Lymphatic Support

Sustained lactation requires the delivery of adequate nutrients via the vasculature, which also delivers endocrine signals and removes metabolic byproducts. Blood flow to the lactating glands increases dramatically coincident with increased maternal cardiac output at parturition. With the onset of lactogenesis II the mammary epithelium also increases its ability to extract water, nutrients and oxygen (Linzell, 1974). The importance of the blood supply for milk production is emphasized by the fact that approximately 500 units of blood must flow through the mammary gland to produce 1 unit of milk (Linzell, 1974). A complementary lymphatic vasculature drains the extracellular space surrounding the secretory epithelial cells toward the lymph nodes spaced within and around the mammary glands. While drainage of lymph from the glands is normally pulsatile and unidirectional, the frequency this pulsatility increases significantly during mastitis. These same lymphatics also represent a major route for migration of metastatic cancer cells from a primary mammary tumor to distal lymph nodes and other organs.

Regulation of Milk Synthesis and Secretion

Mammals use a range of strategies to deliver milk to their offspring, largely reflecting adaptation to their environment. For example, pups of mice are permanently attached to the teat and feed approximately every 20 min, while sows feed their piglets every hour. At the other extreme, some seals such as the Hooded seal have an extremely short lactation (4 days) when they do not forage, whereas other seals such as the fur seal can forage offshore for weeks before returning to suckle their pups, without any negative impact on their milk production. Hibernating bear sows can go for months without any intake of water or food while raising their altricial cubs. Dairy animals such as cows and goats are routinely milked twice daily. These are but a few of many different lactation “strategies” that have evolved, or have been selected for—combined, they emphasize that the mammary glands have enormous potential for plasticity, although we understand very little about how far and wide these strategies are used across mammals.

The evacuation of milk at the time of feeding or milking is promoted by the actions of oxytocin (or mesotocin in marsupials) through a neuro-endocrine mechanism. Tactile stimulation of the nipple/teat and their papillary nerves leads to the rapid activation of hypothalamic neurons to release stored oxytocin, a 9-amino acid peptide, from the posterior pituitary. Circulating oxytocin then acts via receptors to stimulate the contraction of basal myoepithelial cells woven around the alveoli, and longitudinally around the ducts. In species such as cows and goats having a gland cistern, the first milk to be harvested is from this region, while the subsequent milk comes from the alveolar tissue and peripheral ducts in response to oxytocin-induced milk letdown. Sensitivity to oxytocin is often countered by factors such as stress, whereas conversely it can also become a conditioned reflex leading to precocious milk letdown in response to various external stimuli such as certain sights or sounds.

The range of different milk removal intervals highlights how the rate of milk accumulation and the local control of milk secretion are regulated within the gland. After milk is evacuated from each alveolus, the reduced intramammary pressure coincides with a maximal rate of milk synthesis and secretion, then, as intraluminal pressure increases over time, the rate of milk synthesis slows. The extended accumulation of milk past a critical time (considered to be approximately 18 h in dairy animals) initiates the disruption of tight junctions between epithelial cells, leading to the appearance of various milk constituents (such as lactose) in the maternal circulation. The factors governing the local regulation of milk synthesis continues to be a topic of interest and may reflect the actions of molecules such as serotonin or dimerized α -lactalbumin, as well as through mechanisms such as physical stretch.

Part of the yield potential of the mammary glands is also likely regulated at the local level. An example is the response to unilateral, frequent milking of a dairy animal's udder when one half is milked on a regular schedule ($2 \times$ /day) while the other half is milked more frequently (i.e., $4 \times$ /day); within both halves the epithelium is assumed to experience the same endocrine environment (Wall and McFadden, 2012). While $4 \times$ milking increases the overall yield as expected, there is also a sustained increase in output, even once it reverts back to $2 \times$ /day. Along similar lines, the mammary epithelium within a gland can be asynchronous, given that different alveoli, as well as lobes, differ in their expression of genes encoding various milk proteins (Molenaar et al., 1992). A gland can also apparently compensate for disrupted function in other glands by producing more milk. Position also influences milk yield; the rear quarters of a cow's udder produces approximately 50% more milk than the fore glands, while the anterior glands in pigs produce more milk than those that are posterior. Several studies suggest there may be left/right asymmetry in milk yield and/or composition, while emerging evidence also suggests that the composition of a mother's milk might reflect the sex of her offspring.

For many of the most widely-studied species, the composition of the major milk components is relatively constant over the course of lactation, excluding the abundance of immunoglobulins in colostrum. However, these profiles often reflect that the female has a relatively static plane of nutrition or environment. On the other hand, certain wild mammals have evolved unique lactation strategies that alter milk composition with lactation stage. Perhaps the most fascinating of these is that used by macropod marsupials such as the tammar wallaby to feed two young at strikingly-different developmental stages by producing milks of different composition and volume—a strategy referred to as “asynchronous concurrent lactation.” Of their four glands, one can produce small volumes of an “early lactation” milk that is high in complex oligosaccharides and low in protein and lipid for nourishment of a continuously-attached altricial young. At the same time, an adjacent gland produces larger volumes of a “late lactation” milk that is high in monosaccharides, fat, and proteins including specific late-lactation proteins (Brennan et al., 2007).

Maternal Adaptation to Lactation

The volume of milk produced by a gland is ultimately a function of the number of epithelial cells in the gland, and the secretory activity per cell. At the same time, the composition of milk and its volume dictates the sum amount of nutrients required from the mother. All of these variables must be addressed to ensure a successful lactation, and are realized through significant changes that occur before, during or after parturition. During lactation, a female markedly increases her dry matter intake and selection of energy-dense foodstuffs, concurrent with changes in her gastrointestinal system including hepatic hypertrophy, lengthening of the intestine, increased uptake of various dietary nutrients, and increased renal retention of water and salts. The liver also simultaneously increases its gluconeogenic potential, particularly in ruminants, to provide the massive amount of glucose required for lactose synthesis. These changes become necessary because the mammary gland becomes a net sink for a range of nutrients with the onset of lactation. At the same time, the female changes her metabolic partitioning of nutrients; whereas adipose tissue was a store for triglyceride during pregnancy, it becomes a net exporter of lipids with the onset of lactation, having less sensitivity to insulin and greater sensitivity to hormone sensitive lipase. Consequently, the liberated glycerol and non-esterified fatty acids become available to the liver for glucose synthesis via gluconeogenesis in support of lactation, and to the mammary epithelium for the synthesis of milk lipids. This striking shift in nutrient partitioning between organs in support of lactation has been termed “homeorrhexis.” Perhaps some of the most striking examples of this concept are in wild animals such as seals and bears that must rely on their accumulated lipid stores to sustain lactation during extended foraging periods or hibernation. Lactating females often then enter a postpartum period of negative energy balance as the nutrient demands to support lactation exceed their ability to consume and extract nutrients from the diet, and to access maternal stores. Thus, it is not surprising that lactating females, particularly high-producing dairy animals, are prone to metabolic diseases such as ketosis and hypercalcemia immediately postpartum. One should also not overlook the ability of lactation to control the other essential maternal function, that is, reproduction. In some species, such as humans, lactation leads to anovulation and its associated contraceptive benefits, while in species such as the tammar wallaby, lactation halts development of a blastocyst leading to its dormancy in a state of diapause (Brennan et al., 2007).

The Lactation Cycle

The course of a lactation in terms of overall yield is typically described by a lactation “curve” describing milk yield over time. For a typical modern dairy cow, lactation length is defined as 305 days—it is important to note that this period reflects dairy management practices designed to have animals concurrently pregnant and re-initiating lactation approximately every year. Longer lactations can be realized if milk harvesting is continued. The lactation curve of a dairy cow typically reflects a “peak” of milk output around 4–6 weeks, then declines thereafter. By contrast, milk yield in species such as pigs and rodents increases over time such that greatest output is realized at weaning after 3 weeks; by contrast, humans, guinea pigs and rabbits have a “peak” of yield about half way through a typical lactation. These changes are presumed to reflect changes in cell activity as well as a balance between any change in cell number due to cell growth and death.

The composition of milk is also not fixed, and can change throughout a lactation. The elevated level of protein in milk post-partum reflects the presence of immunoglobulins in colostrum. Following peak lactation in dairy animals, when lactose concentrations are highest, a declining milk yield is associated with a reduction in lactose output, leading to concomitant increases in the concentrations of fat and protein. By contrast, as indicated earlier, other species such as the tammar wallaby change their composition dramatically to meet the different developmental states of the offspring. Similarly, hibernating species such as bears also change their milk composition appreciably during lactation, moreso as a function of their using maternal nutrient stores.

The Future for Lactation and Its Physiology

For the past 166 million years, mammals have relied on lactation as a cornerstone mechanism for species survival. With continued predictions for a drier and hotter environment as the result of climate change alongside less water availability and restricted range, the very biology of successful lactation could be under threat. We also continue to learn about the benefits of milk for developing neonates, and the importance of breastfeeding. And, aided by selection technologies, the emerging market for tailored milk products from dairy animals stands to expand, which may well benefit enormously from gene-editing approaches. At the same time, the prospect for producing various biologics in transgenic animals continues worldwide, albeit at different rates in different countries. Regardless, one can surely not say that the future for the study of milk and lactation biology is without many questions!

References

- Bauman, D. E., Mather, I. H., Wall, R. J., & Lock, A. L. (2006). Major advances associated with the biosynthesis of milk. *Journal of Dairy Science*, 89(4), 1235–1243. [https://doi.org/10.3168/jds.S0022-0302\(06\)72192-0](https://doi.org/10.3168/jds.S0022-0302(06)72192-0).
- Brennan, A. J., Sharp, J. A., Lefevre, C., Topcic, D., Auguste, A., Digby, M., & Nicholas, K. R. (2007). The tammar wallaby and fur seal: Models to examine local control of lactation. *Journal of Dairy Science*, 90(Suppl 1), E66–75. <https://doi.org/10.3168/jds.2006-483>.
- Buitenhuis, B., Janss, L. L., Poulsen, N. A., Larsen, L. B., Larsen, M. K., & Sorensen, P. (2014). Genome-wide association and biological pathway analysis for milk-fat composition in Danish Holstein and Danish Jersey cattle. *BMC Genomics*, 15, 1112. <https://doi.org/10.1186/1471-2164-15-1112>.
- Jenkins, T. C., & Harvatine, K. J. (2014). Lipid feeding and milk fat depression. *The Veterinary Clinics of North America. Food Animal Practice*, 30(3), 623–642. <https://doi.org/10.1016/j.cvfa.2014.07.006>.
- Linzell, J. L. (1974). Mammary blood flow and methods of identifying and measuring precursors of milk. In B. L. Larson, & V. R. Smith (Eds.), *Lactation—A comprehensive Treatise No. 1* (pp. 143–225). New York and London: Academic Press.
- Masedunskas, A., Chen, Y., Stussman, R., Weigert, R., & Mather, I. H. (2017). Kinetics of milk lipid droplet transport, growth, and secretion revealed by intravital imaging: Lipid droplet release is intermittently stimulated by oxytocin. *Molecular Biology of the Cell*, 28(7), 935–946. <https://doi.org/10.1091/mbc.E16-11-0776>.
- Molenaar, A. J., Davis, S. R., & Wilkins, R. J. (1992). Expression of alpha-lactalbumin, alpha-S1-casein, and lactoferrin genes is heterogeneous in sheep and cattle mammary tissue. *The Journal of Histochemistry and Cytochemistry*, 40(5), 611–618. <https://doi.org/10.1177/40.5.1374090>.
- Oftedal, O. T. (2002). The mammary gland and its origin during synapsid evolution. *Journal of Mammary Gland Biology and Neoplasia*, 7(3), 225–252.
- Sharp, J. A., Lefevre, C., & Nicholas, K. R. (2008). Lack of functional alpha-lactalbumin prevents involution in Cape fur seals and identifies the protein as an apoptotic milk factor in mammary gland involution. *BMC Biology*, 6, 48. <https://doi.org/10.1186/1741-7007-6-48>.
- Smith, K. L., & Schanbacher, F. L. (1973). Hormone induced lactation in the bovine. I. Lactational performance following injections of 17-estradiol and progesterone. *Journal of Dairy Science*, 56(6), 738–743.
- Tucker, H. A. (1974). General endocrinological control of lactation. In B. L. Larson, & V. R. Smith (Eds.), *Lactation—A comprehensive treatise No. 1* (pp. 277–326). New York and London: Academic Press.
- Wall, E. H., & McFadden, T. B. (2012). Triennial lactation symposium: A local affair: How the mammary gland adapts to changes in milking frequency. *Journal of Animal Science*, 90(5), 1695–1707. <https://doi.org/10.2527/jas.2011-4790>.
- Williams, J. E., Carrothers, J. M., Lackey, K. A., Beatty, N. F., York, M. A., Brooker, S. L., Shafii, B., Price, W. J., Settles, M. L., McGuire, M. A., & McGuire, M. K. (2017). Human milk microbial community structure is relatively stable and related to variations in macronutrient and micronutrient intakes in healthy lactating women. *The Journal of Nutrition*, 147(9), 1739–1748. <https://doi.org/10.3945/jn.117.248864>.

Further Reading

- Akers, R. M. (2002). *Lactation and the mammary gland*. Ames, Iowa: Iowa State Press.
- Davies, D. T., Holt, C., & Christie, W. W. (1983). The composition of milk. In T. B. Mepham (Ed.), *Biochemistry of Lactation No. 1* (pp. 71–117). Amsterdam, New York: Elsevier Science.

Hypothalamic–Pituitary–Mammary Gland (HPM) Axis

Darcie D Seachrist, Elyse Donaubauer, and Ruth A Keri, Case Western Reserve University, Cleveland, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Alveologenesis The formation of alveoli, which are hollow cavities in the body. In the lungs, alveoli are small air sacs, while in the mammary gland, alveoli refer to hollow sacks lined with epithelial cells that can differentiate into milk producing units.

Arborization Branching of the mammary ducts within the mammary fat pad.

GHIH Growth hormone inhibiting hormone, also called somatostatin.

Gonadotropic hormones Hormones produced by the gonadotroph cells in the pituitary.

Hypophysectomy Removal of the pituitary.

Invaginate To fold inside upon itself.

Lobuloalveologenesis In the mammary gland, the formation of groups of alveoli into functional units that produce and drain milk into ducts that empty into the nipple.

Morphogenetic Processes that control organ formation and function.

Ovariectomize To surgically remove the ovaries.

Parturition The act of giving birth; childbirth.

STAT5 Signal transducer and activator of transcription 5, a transcription factor.

Abbreviations

ACTH Adrenocorticotrophic hormone

cAMP Cyclic adenosine monophosphate

CRH Corticotropin releasing hormone

E2 17 β -Estradiol

ER Estrogen receptor

FSH Follicle stimulating hormone

GH Growth hormone

GHR Growth hormone receptor

GHRH Growth hormone releasing hormone

GHRHR Growth hormone releasing hormone receptor

GnRH Gonadotropin releasing hormone

GnRHR Gonadotropin releasing hormone receptor

GPER G protein-coupled estrogen receptor

HP Hypothalamic–pituitary

HPM Hypothalamic, pituitary, mammary gland axis

IGF1 Insulin-like growth factor 1

JAK2 Janus kinase 2

LH Luteinizing hormone

MCR2 Melanocorticotropin receptor

OXTR Oxytocin receptor

OXT Oxytocin

P4 Progesterone

POMC Pro-Opiomelanocortin

PR Progesterone receptor

PRKO Progesterone receptor knockout

PRL Prolactin

PRLR Prolactin receptor

WAP Whey acidic protein

Introduction

The impact of the pituitary on mammary gland function was first discovered in 1928 by Stricker and Grüter when they injected pituitary extracts into ovariectomized rabbits and found that this induced mammary gland development and milk production (Stricker and Grueter, 1928; Riddle, 1940). This discovery was followed by additional analyses of pituitary extracts on the growth of the mammary gland and stimulation of lactation in the 1930s (Nelson, 1935, 1936; Trott et al., 2008). Soon after, several groups identified the pituitary hormone that controls lactation, which we now know as prolactin (Trott et al., 2008). Further supporting the importance of the pituitary in controlling development of the mammary gland, removal of the pituitary (hypophysectomy) abolished lactation in adult guinea pigs (Nelson, 1935). It is now widely accepted that the structure and function of the mammary gland is exquisitely controlled by neuronal and hormonal signaling generated from the hypothalamic/pituitary (HP) axis. Pituitary hormones that directly regulate mammary gland development, such as prolactin, oxytocin and growth hormone, and others that indirectly impinge upon the gland, are necessary for complete morphogenesis and the production and ejection of milk. Neuronal signals from the hypothalamus control synthesis and secretion of these hormones, which in turn relay various signals to the mammary gland. Specifically, prolactin (PRL), growth hormone (GH), and oxytocin (OXT) directly impact the mammary gland by binding to their cognate receptors in the mammary epithelium or mesenchyme. Other pituitary hormones, such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH) regulate mammary gland function indirectly by promoting the synthesis and secretion of the ovarian hormones, estrogen and progesterone (Table 1).

The basic morphological stages of mammary gland development are consistent across mammals and much of what we know about the molecular mechanisms contributing to hormonal regulation of the gland comes from studying developmental processes in genetically engineered mice (Briskin and O'Malley, 2010). Mammary gland development can be divided into three stages: embryonic, pubertal, and pregnancy/lactation (Fig. 7). While the pubertal and pregnancy/lactation stages of development are highly dependent upon the hormonal milieu and input from the HP axis, embryonic mammary morphogenesis is largely independent of systemic hormonal cues. Rather, early formation and growth of the mammary gland depends on paracrine signaling originating in the mesenchyme surrounding the mammary epithelium.

Mammary morphogenesis initiates with the formation of bilateral ectodermal ridges in the ventral skin to form the milk or mammary lines (Macias and Hinck, 2012). These quickly resolve into the mammary placodes, of which there are five on each side of the mouse, by embryonic day 11.5 (E11.5). The placodes then invaginate to form the mammary buds and subsequently primary ductal mammary sprouts. By E18.5 of the mouse, a rudimentary ductal tree has formed that remains morphogenetically quiescent until the onset of puberty. At this stage and those that follow, mammary gland development becomes dependent on hormones synthesized and secreted by the HP axis and its target organs, in particular, the ovary. Hormone-induced elongation and branching of the ductal tree occurs until it extends to the edges of the mammary fat pad. Pregnancy and lactation are considered to be the final stages of mammary development and differentiation. At the onset of pregnancy, tertiary side branching of the ducts and lobuloalveologenesis begins and extends through parturition at which time milk production commences and lactation can occur. When the pups are weaned and lactation ceases, the mammary epithelium undergoes apoptosis and involutes until the gland returns to a near-virgin state both morphologically and molecularly. Remarkably, this process of epithelial expansion, lactational differentiation, and involution can occur repeatedly without exhaustion of the gland and each of these waves of differentiation and remodeling is controlled, in part, by the hormones synthesized and secreted by the hypothalamic/pituitary axis.

Table 1 Impact of pituitary hormones on mammary morphogenesis and lactation

<i>Hormone</i>	<i>Timing</i>	<i>Direct/Indirect</i>	<i>Source</i>	<i>Function</i>
Adrenocorticotrophic hormone (ACTH)	Adult	Indirect	Anterior pituitary	Lobuloalveologenesis, milk production by modulating adrenal production of glucocorticoids
Estrogens (E)	Juvenile	Direct	Ovary	Ductal elongation
Follicle stimulating hormone (FSH)	Juvenile/adult	Indirect	Anterior pituitary	Ductal elongation and side branching by modulating ovarian production of progestins
Glucocorticoids (GC)	Adult	Direct	Adrenal	Lobuloalveologenesis, milk production, suppression of involution
Growth hormone (GH)	Juvenile	Direct/indirect	Anterior pituitary	Elongation of mammary primary ductal sprout and lactation enhancement through IGF1 regulation
Insulin-like growth factor 1 (IGF1)	Juvenile	Direct/indirect	Liver/mammary stroma	
Luteinizing hormone (LH)	Juvenile/adult	Indirect	Anterior pituitary	Elongation of mammary primary ductal sprout
Progestins (P)	Adult	Direct	Ovary	Ductal elongation and side branching by modulating ovarian production of estrogens
Prolactin (PRL)	Pregnancy/lactation	Direct	Anterior pituitary	Tertiary side branching and lobuloalveologenesis
Oxytocin (OXT)	Lactation	Direct	Posterior pituitary	Lobuloalveologenesis, milk production
				Milk ejection and expanded lobuloalveolar development

Luteinizing Hormone and Follicle-Stimulating Hormone

In most cases, ovarian steroids (estrogens and progestins) are essential for mammary morphogenesis, although dietary exposures to conjugated linoleic acids can substitute for estrogens in rodents and possibly other mammals. The production of ovarian steroids relies on pituitary synthesis and secretion of two gonadotropic hormones, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). When the ovaries of prepubertal animals are removed, the mammary glands fail to develop despite a sharp rise in the levels of LH and FSH. Thus, these pituitary hormones have indirect roles on mammary gland development.

Both LH and FSH are peptide hormones that are synthesized and secreted from gonadotroph cells of the anterior pituitary gland (Fig. 1). LH and FSH production and secretion from these cells depends upon the pulsatile release of a 10 amino acid peptide known as Gonadotropin Releasing Hormone (GnRH) from the hypothalamus that then travels through the portal vasculature to the anterior pituitary. Binding of GnRH to its receptors (GnRHR) on gonadotroph cells induces the expression of the genes encoding LH and FSH as well as secretion of the fully functional hormones. In the ovary, LH and FSH bind to their cognate G-protein coupled receptors, LHR and FSHR, and signal through cAMP (cyclic adenosine monophosphate) to coordinate ovarian follicular growth, ovulation, and estrogen and progesterone production. Estrogens, principally 17 β -estradiol (E2), from the ovary promote continued production of LH in a positive feedback loop until ovulation. After ovulation, progestins (primarily progesterone or P4) and estrogens produced by the ovary now act in a negative feedback loop at the hypothalamus and pituitary to inhibit synthesis and secretion of LH and FSH. In addition to sex steroids, LH and FSH induce the production of activin and inhibin, members of the TGF β superfamily of cytokines that also impact mammary gland development.

In addition to ovariectomy/hormone replacement studies, the specific roles of estrogens and progestins within the mammary gland have also been elucidated through examination of mice lacking the receptors for these hormones. Two classes of estrogen receptors (ER) exist: the G protein-coupled receptor, GPER (also known as GPR30) and the nuclear hormone receptors, ER α and ER β , which are each encoded by separate genes, *ESR1* and *ESR2*, respectively. All three of these receptors are expressed in the mammary epithelium and its surrounding stroma. The mammary glands of *ESR1* null mice fail to develop beyond a rudimentary duct, indicating a requirement for estrogen signaling through ER α for ductal elongation. Indeed, both mammary and stromal ER α are necessary for proper ductal outgrowth as demonstrated through transplantation/tissue recombination studies. In contrast, while ER β and GPER can impact mammary epithelial cell proliferation upon ligand binding in vitro, these receptors are not essential for mammary gland growth or development, in vivo.

Progestins signal through binding to the progesterone receptor (PR), a nuclear hormone receptor. Two isoforms of PR exist, PR α and PR β , but unlike the estrogen receptors, PR α and PR β are products of the same gene (*PGR*). Both isoforms of PR are also expressed in the stromal and epithelial compartments of the mammary gland. Mice lacking PR expression (PRKO) display normal

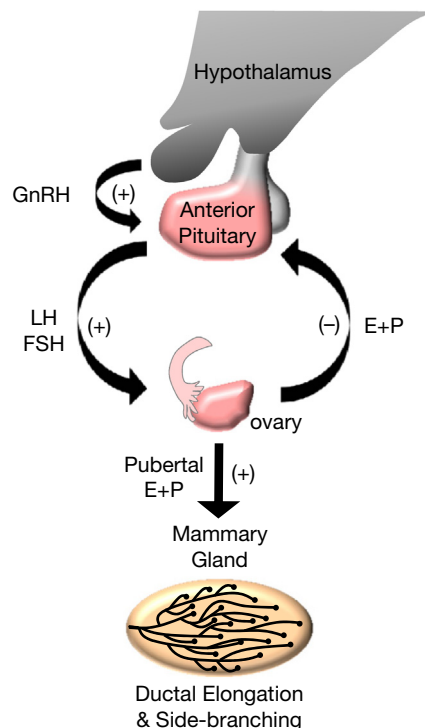


Fig. 1 Hypothalamic/gonadotropin/ovarian/mammary gland axis. The gonadotropins, LH and FSH, are synthesized and secreted from gonadotrophs of the anterior pituitary gland following stimulation by hypothalamic GnRH. LH and FSH act at the ovary to induce production and release of estrogens (E) and progestins (P). These ovarian hormones then stimulate mammary gland ductal elongation and side branching during puberty.

ductal elongation, but are deficient in tertiary side-branching in adulthood and alveologenesis during pregnancy. When PR null epithelial cells are engrafted into wild type mammary stroma, ductal elongation proceeds normally, but the ducts fail to undergo side branching and alveologenesis. Thus, PR is principally necessary for expansion of differentiated structures of the mammary gland. Genetic disruption of PR in the mammary stroma yields phenotypically normal mammary outgrowth, indicating an intrinsic requirement for PR in the mammary epithelium for arborization of the ductal tree and alveologenesis. Notably, progesterone signaling in the mammary gland also primes the gland for responding to prolactin by increasing the expression of prolactin receptors.

Prolactin

As indicated above, prolactin (PRL) was initially identified as a key pituitary regulator of mammary gland function using injections of pituitary extracts into rabbits and analyzing mammary gland responses. Prolactin is a peptide hormone that is produced and secreted by lactotroph cells in the anterior pituitary gland (Fig. 2). Unlike all other anterior pituitary hormones, prolactin does not require a stimulus from the hypothalamus to be synthesized and secreted. Rather, prolactin is chronically negatively regulated by dopamine that is released from neuroendocrine dopaminergic (NEDA) neurons in the hypothalamus. Lactotroph cells have dopamine receptors on their surface, and following dopamine binding, prolactin secretion is reduced. Dopamine secretion from the hypothalamus varies across the light-dark cycle, resulting in regulation of prolactin secretion by the circadian clock. Prolactin also acts as a negative regulator of its own release through a short feedback loop. As prolactin secretion increases, it binds to receptors on hypothalamic neurons, stimulating dopamine production and secretion. In contrast, loss of PRL signaling in mice leads to hyperproliferation of the pituitary lactotroph cells resulting in prolactinomas. Dopamine, and hence prolactin, can also be regulated by gonadal steroids such as estradiol, progesterone, and testosterone. For estradiol, this involves desensitization of NEDA neurons to prolactin, blocking its negative feedback in the hypothalamus and hence, freeing the pituitary from dopamine suppression and inducing prolactin release.

In pregnancy, serum prolactin levels can follow a bimodal pattern wherein this hormone is elevated during early and late gestation/parturition (rodents) or can progressively increase throughout the pregnancy (primates) (Fig. 3). In early pregnancy, elevated prolactin, in conjunction with progesterone, induces extensive proliferation of the mammary epithelium, ductal side branching,

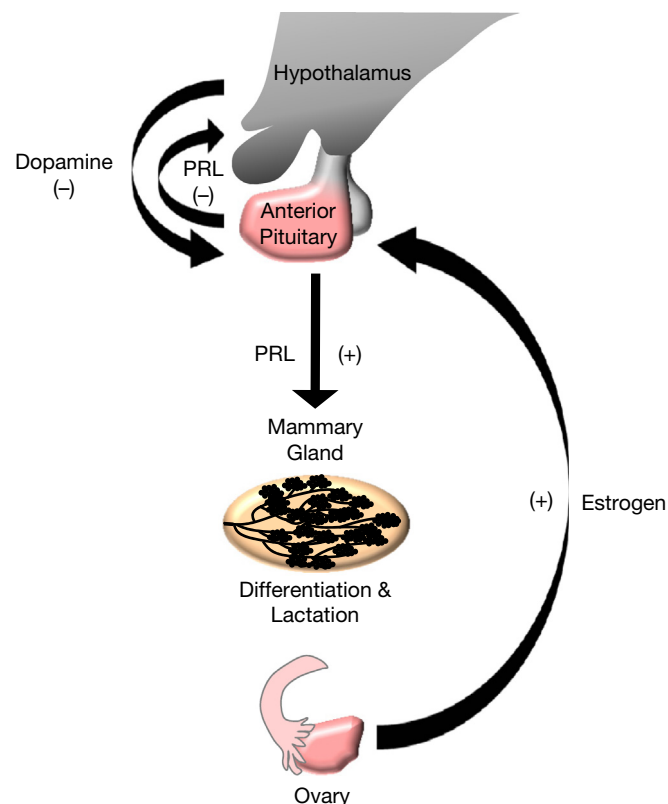


Fig. 2 Hypothalamic/PRL/mammary gland axis. PRL is synthesized and secreted from lactotroph cells of the anterior pituitary gland in response to elevated estrogens during pregnancy. PRL stimulates lactation and differentiation of mammary epithelial cells and is negatively controlled by dopamine that is released by hypothalamic neurons. PRL also feeds back to suppress its own production by inducing dopamine release.

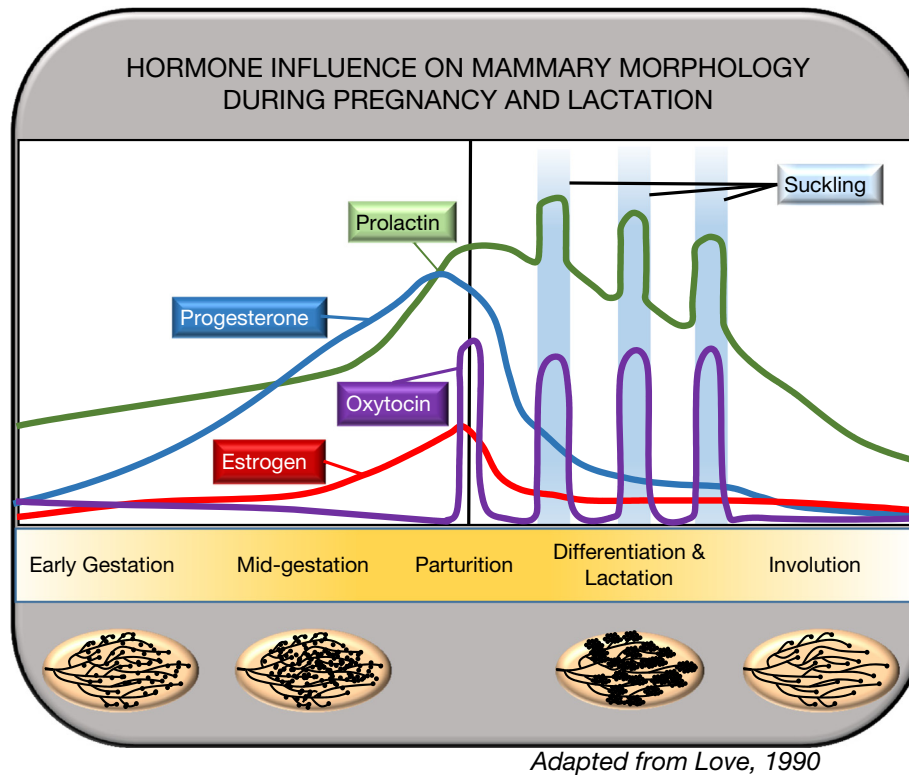


Fig. 3 Hormonal influence on mammary gland morphology during pregnancy, lactation and involution. Serum hormone levels fluctuate greatly during pregnancy impacting mammary gland development and differentiation. In particular, PRL and OXT hormone levels cycle during lactation to stimulate both milk production and ejection during breastfeeding. At weaning, milk-promoting hormones decline and the mammary gland undergoes involution, morphologically returning to a near-virgin state.

and alveologensis. In contrast, when progesterone levels fall in late pregnancy/parturition, the elevated prolactin causes lobuloalveolar differentiation in preparation for milk production.

There are several mechanisms that ensure elevated prolactin signaling during pregnancy. The number of lactotroph cells in the pituitary increases starting in early pregnancy and this is followed by an increase in prolactin secretion. Prolactin production is also regulated by other hormonal and paracrine factors, such as estradiol, progesterone, glucocorticoids, and insulin. Additionally, human placental lactogen, which is structurally similar to human growth hormone and can bind and activate the prolactin receptor, is present at increasing levels as gestation advances. Through these adaptations, increased prolactin signaling is assured as pregnancy progresses in preparation for lactation. Post-partum, prolactin levels are sustained by signals induced by the suckling infant through the nipple, which continues to suppress the short feedback loop and dopamine secretion by the hypothalamus.

In the mammary gland, prolactin binds to its cognate receptor (PRLR) in the mammary epithelia. The prolactin receptor is a single transmembrane cytokine receptor that is associated with two signaling proteins at its c-terminus [Janus Kinase 2 (JAK2) and Signal Transducer and Activator of Transcription 5 (STAT5)]. Binding of prolactin to PRLR induces phosphorylation of JAK and then STAT5. STAT5 then translocates to the nucleus and induces transcription of target genes involved in promotion of the cell cycle (Cyclin D1) and expression of milk proteins such as β -casein, whey acidic protein (WAP), and β -lactoglobulin. Prolactin deficient female mice (*Prl*^{-/-}) are infertile but develop normally branched mammary glands that can be observed in wild type virgin females, however these glands lack terminal or lateral lobules. Rather than forming lobuloalveoli at the ends of ducts, the ducts terminate as blunt or tapered ends. PRL receptor deficient mice are also infertile and have global reproductive abnormalities, yet their mammary morphogenesis is normal until puberty. At this point, the glands maintain terminal end buds but do not form alveoli. Given the reproductive defects, assessing the impact of prolactin and its receptor required transplantation of PRLR glands into wild-type recipient mouse mammary fat pads. This approach revealed that prolactin signaling is not necessary for pregnancy-associated ductal side branching and alveolar bud formation, but is essential for lobuloalveolar development. Notably, heterozygous PRLR null females are fertile, yet they also display incomplete mammary gland development during pregnancy and fail to nurse their first litter, indicating that a threshold level of prolactin production is essential for these processes to occur. Some *Prlr* heterozygous null mice have restored alveologensis and lactational capacity after subsequent pregnancies, depending on the mouse strain, suggesting that repeated low-level hormonal stimulation can overcome this dosage defect.

Notably, genetic disruption of the *Jak2* gene within the mammary epithelium of mice phenocopies the pregnancy-associated defects in branching morphogenesis and lactation observed in *Prlr* null animals, indicating that it is an essential intermediate of

the prolactin receptor in the mammary epithelium. STAT5 is a downstream effector of JAK2 and genetic activation of STAT5 in the mammary gland induces precocious alveolar development in virgin mice, suggesting that it is also important for regulating gland development. However, loss of both STAT5 isoforms, STAT5a and STAT5b, in the mammary gland only recapitulates some of the defects of *Prlr* loss, indicating that while JAK2 is a primary mediator of prolactin signaling, it functions through STAT5a/b and other, as yet unknown, intermediates.

Growth Hormone

During the time that prolactin was identified as a key pituitary hormone that regulated mammary gland development, growth hormone (GH) was also discovered to have a growth-promoting and lactogenic effect, with GH purified from pituitary extracts inducing lactation. This discovery ultimately led to the broad-scale use of growth hormone to increase milk production in dairy cows. GH is a peptide hormone that is produced and secreted by somatotroph cells in the anterior pituitary gland (Fig. 4). The regulation of growth hormone production by somatotrophs is dependent on the balance of two neuropeptides released by the neurosecretory nuclei of the hypothalamus: growth hormone releasing hormone (GHRH) and growth hormone inhibiting hormone (GHIH, also called somatostatin). GHRH stimulates the G protein-coupled receptor, GHRHR, on somatotrophs. This induces cAMP production that then leads to synthesis and secretion of GH. GH is secreted in a pulsatile pattern in response to multiple stimuli, including ovarian estrogens. The inhibitory protein, somatostatin, is produced by numerous organs in addition to the brain and provides a mechanism for suppressing GH release from the pituitary.

Growth hormone binds to its cognate receptor (GHR) in target tissues to elicit its effects. Like PRLR, GHR is a transmembrane cytokine receptor that also signals through JAK and STAT proteins. The specific functions of GH and GHR on the mammary gland were discovered through the use of genetically modified mice. Both GH and GHR deficient mice are smaller than their wildtype littermates and have a stunted and rudimentary mammary ductal tree, indicating that GH signaling is essential in some way for ductal morphogenesis, although a sparse mammary ductal tree can eventually form in GHR deficient animals. The growth hormone receptor (GHR) is detectable in all postnatal stages of mammary gland development, with higher expression in the stroma than in the epithelial compartment, suggesting that the effects of GH could be directly on the mammary epithelium, or indirectly through the surrounding stromal tissue. To distinguish between these possibilities, GHR null mammary epithelium was transplanted into the mammary fat pads of wild type mice. Such transplants were able to undergo normal mammary morphogenesis with complete arborization of the ductal tree. Hence, GH signaling is not required within the mammary epithelium for ductal outgrowth. Further, these studies suggest that GH signaling may act through the mammary stroma or remotely through another tissue that provides another endocrine hormone that controls the mammary gland.

A key mediator of GH action in many tissues is Insulin-like Growth Factor 1 (IGF1). GH stimulates the release of IGF1 from many tissues, including the liver, to generate systemic IGF1. Locally, GH can also stimulate the production of IGF1 from the

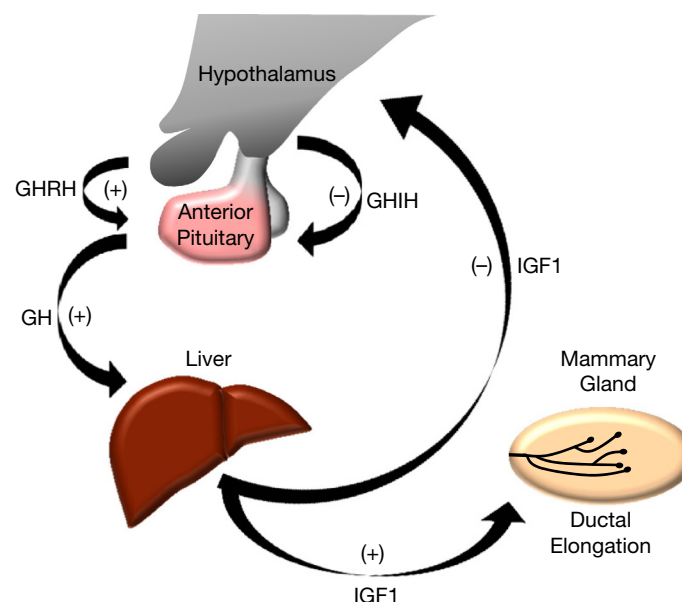


Fig. 4 Hypothalamic/GH/liver/mammary gland axis. GH is synthesized and secreted from the anterior pituitary gland following stimulation by GHRH from the hypothalamus. Circulating GH stimulates synthesis and secretion of IGF1 from the liver, which acts on the mammary gland in conjunction with pubertal estrogens to promote ductal elongation. IGF1 negatively feeds back to the hypothalamus to signal release of GHIH (somatostatin), thus inhibiting GH release.

mammary stroma. GH deficient mice have severely reduced IGF1 levels. In addition, genetic disruption of the *Igf1* gene leads to an identical phenotype in the mammary gland as mice lacking the GHR even though they have normal GH levels. Together, these data suggest that the primary target of GH signaling in the mammary gland is regulation of IGF1 which then controls development of the gland. Whether this impact was conveyed through regulation of IGF1 at the liver or mammary stroma required generation of more sophisticated mouse models that only produced IGF1 from the liver. These mice have completely normal mammary gland development, hence GH action on the mammary gland likely involves stimulation of IGF1 production by the liver that, in turn, systemically stimulates growth of the mammary gland. Notably, IGF1 also negatively feeds back to directly inhibit somatotroph production of GH, as well as stimulates release of somatostatin from the hypothalamus.

Adrenocorticotrophic Hormone

Similar to LH and FSH, adrenocorticotrophic hormone (ACTH) exerts its effects on the mammary gland indirectly, through stimulation of glucocorticoids (GC) generated from the adrenal gland. ACTH was first isolated from pituitary extracts in the early 1930s and shown to stimulate adrenal glucocorticoid production. The impact of the adrenal glands on mammary gland function was discovered when removal of the adrenal glands of lactating mice caused cessation of casein secretion and lactational dysfunction. Continued dissection of the pathway was performed with hormonal deletion and restoration experiments in rabbits and rats. Exogenous ACTH induced lactogenesis in pseudopregnant rabbits and treatment with hydrocortisone induced full mammary differentiation and lactogenesis in adrenalectomized and ovariectomized rats. Thus, a role for ACTH in regulation of mammary epithelial secretion and lactation through modulating glucocorticoids was established.

ACTH is a peptide hormone generated by the cleavage of a large precursor protein, pro-opiomelanocortin (POMC). ACTH is secreted from anterior pituitary corticotrophs upon stimulation by hypothalamic corticotropin-releasing hormone (CRH) (Fig. 5). Circulating ACTH binds to the melanocorticotropin receptor (MCR2), a G protein-coupled receptor in the adrenal cortex, which stimulates a cAMP signaling cascade and production of glucocorticosteroids (glucocorticoids), such as cortisol (corticosterone in mice). Glucocorticoids then signal back to the hypothalamus to reduce CRH and subsequently, ACTH production and secretion in a negative feedback loop.

Glucocorticoids/corticosteroids bind to and activate nuclear hormone receptors (GR) that are expressed in the mammary stroma and epithelium. As with most nuclear hormone receptors, GR acts as a ligand-bound transcription factor, inducing or suppressing gene expression through DNA binding. GR also influences gene expression independently of DNA binding, through

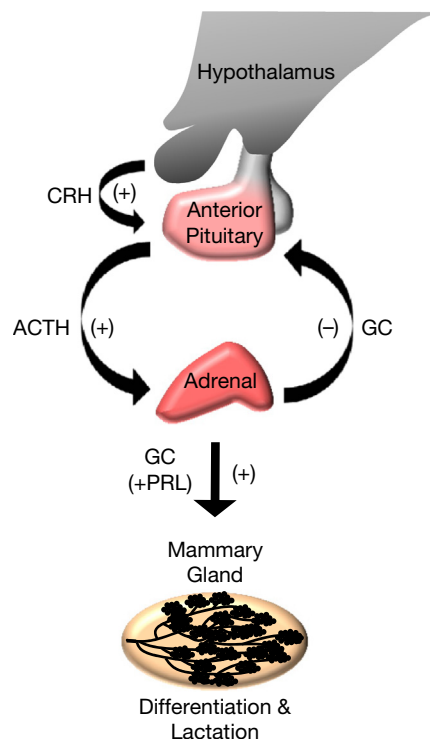


Fig. 5 Hypothalamic/ACTH/adrenal/mammary gland axis. CRH, released from the hypothalamus, promotes synthesis and release of ACTH from corticotrophs of the anterior pituitary gland. ACTH stimulates production of glucocorticoids (GC) from the adrenal cortex, which in conjunction with PRL, promote lactation and differentiation of the mammary epithelium. GC negatively feeds back to the hypothalamus and pituitary to inhibit ACTH expression and release.

protein–protein interactions with factors such as phosphorylated STAT5. Corticosteroid treatment paradigms have shown that these agents inhibit involution of the lactating mammary gland. The impact of GR on mammary gland development has been elucidated using genetically engineered mouse models, yet its specific mechanism is not well defined. Mice lacking GR in all tissues die at birth making them unusable for dissection of GR regulation of post-natal mammary development. Transplantation of GR null mammary epithelial cells into wild-type hosts resulted in impaired ductal development of virgin mice, yet there was no overt impact on mammary epithelial differentiation during pregnancy, lactation, or involution. Using a different mouse model that can only express a mutant form of GR that lacks the ability to bind to DNA, the mice are viable and fertile. Females had a reduction in mammary epithelial cell proliferation, decreased ductal arborization, and a lack of alveolar buds. However, they were fully lactationally competent. Lastly, disruption of the GR gene (*NCR31*) only in the mammary gland during pregnancy leads to decreased lobuloalveologenesis that persists through lactation, yet milk protein production is unaffected and pups can be nursed until weaning. Together, these data indicate that ACTH-stimulated production of glucocorticoids regulates mammary ductal morphogenesis but is dispensable for milk production and lactation.

Glucocorticoids can also synergize with prolactin in mammary epithelial differentiation and milk protein gene expression. Furthermore, prolactin and glucocorticoids have been reported to modulate the levels of one another's receptors, supporting a complex interplay between these two hormones in their actions on the mammary gland.

Oxytocin

Oxytocin (OXT) is a nonapeptide that is essential for milk ejection in response to suckling. OXT was the first peptide hormone to be sequenced in the 1950's by Dr. Du Vigneaud, who was awarded the Nobel Prize for his efforts. OXT is necessary for milk ejection from the lobuloalveolar compartments into the mammary ducts, by inducing contraction of the alveolar myoepithelial cells of the mammary gland. Despite the presence of milk in their mammary glands, OXT-deficient mice fail to nurse their young confirming the necessity of this hormone for milk ejection. OXT is also required for continued proliferation and expansion of lobuloalveoli after birth to accommodate the expanding size of the offspring. Interestingly, while OXT also induces contraction of the uterine wall during childbirth, it is not necessary for parturition.

In contrast to GH, PRL, LH, FSH, and ACTH, which are synthesized and secreted from the anterior pituitary gland, oxytocin is released from the posterior lobe (Fig. 6). Synthesized by the neurosecretory neurons in the hypothalamus, OXT is transported in vesicles down axons to the posterior pituitary gland. Suckling stimulates OXT secretion in a pulsatile manner from the posterior pituitary (Fig. 3). Because mammary tissue responds to oxytocin only at very high concentrations and rapidly becomes desensitized, pulsatile secretion of oxytocin is the most effective for milk release.

OXT exerts its function through binding to the oxytocin receptor (OXTR) in the mammary myoepithelial cells which surround the luminal cells of the ducts and alveoli. The number of OXTR in the mammary myoepithelia increases progressively during pregnancy and remains high during lactation. OXTR deficient dams phenocopy those with loss of the OXT ligand indicating that OXTR is the primary mediator of OXT function. OXTR is a G-protein coupled receptor, which transmits signals across the cell membrane using heterotrimeric G-proteins. OXT binding to the OXTR induces stimulation of phospholipase C and intracellular calcium release, resulting in a contractile force in the myoepithelial cells surrounding the milk-producing luminal cells and causing milk

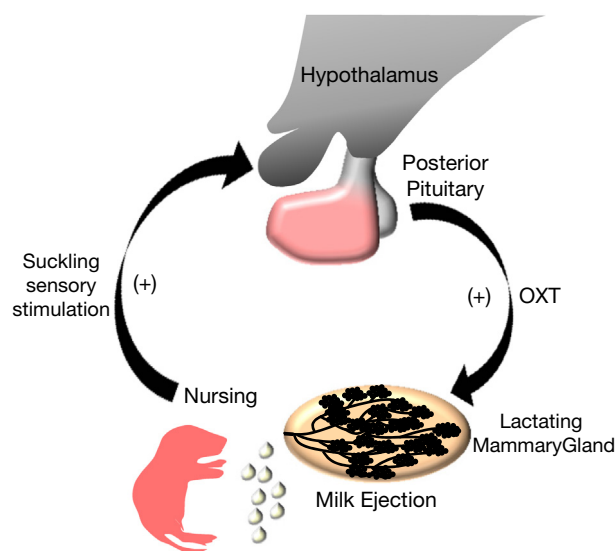


Fig. 6 Hypothalamic/OXT/mammary gland axis. OXT is synthesized in the hypothalamus and released from the posterior pituitary gland in response to suckling. OXT stimulates mammary gland myoepithelial cell contraction to promote milk ejection.

ejection. In the absence of OXT, milk stasis occurs and involution ensues. Hence, OXT is essential for maintaining lactational competency in addition to milk release.

Overall, there has been no single sensory pathway identified that regulates signals from the mammary gland to the oxytocin neurons. Multiple neurotransmitters have been shown to modulate the milk ejection response, with glutamate and noradrenaline transmitting signals from the hypothalamus, and OXT acting on its own neurons to modulate the response. OXT-producing neurons have excitatory glutamate receptors on their cell surface, and injection of a glutamate receptor antagonist into mice blocks suckling-induced OXT release. Activation of sensory nuclei in the medulla is observed following suckling, and these neurons end in the hypothalamus and can act on OXT neurons. Additionally, an increase in noradrenaline in the hypothalamus is observed in response to a suckling stimulus. Hence, it is likely a complex interplay of numerous neurotransmitters that relays the suckling signal to the hypothalamus and pituitary and ultimately stimulates milk ejection.

Putting It All Together: The Integrated Hormonal Milieu

The majority of mammary gland development occurs postnatally at the onset of puberty and is exquisitely dependent on the hormones produced by the hypothalamus and pituitary (Fig. 7). LH/FSH secretion from gonadotrophs stimulates ovarian estrogen production inducing mammary epithelial cell proliferation and elongation of the ducts through the fat pad. Estrogen levels increase dramatically compared to progesterone, augmenting the expression of PR in the mammary gland in a process called “estrogen priming.” Concomitantly, GH secreted from somatotrophs induces systemic IGF1 levels, which then synergize with estrogens to promote extension of the ductal tree with each estrus wave until the ducts have reached the edges of the fat pad. GH also induces

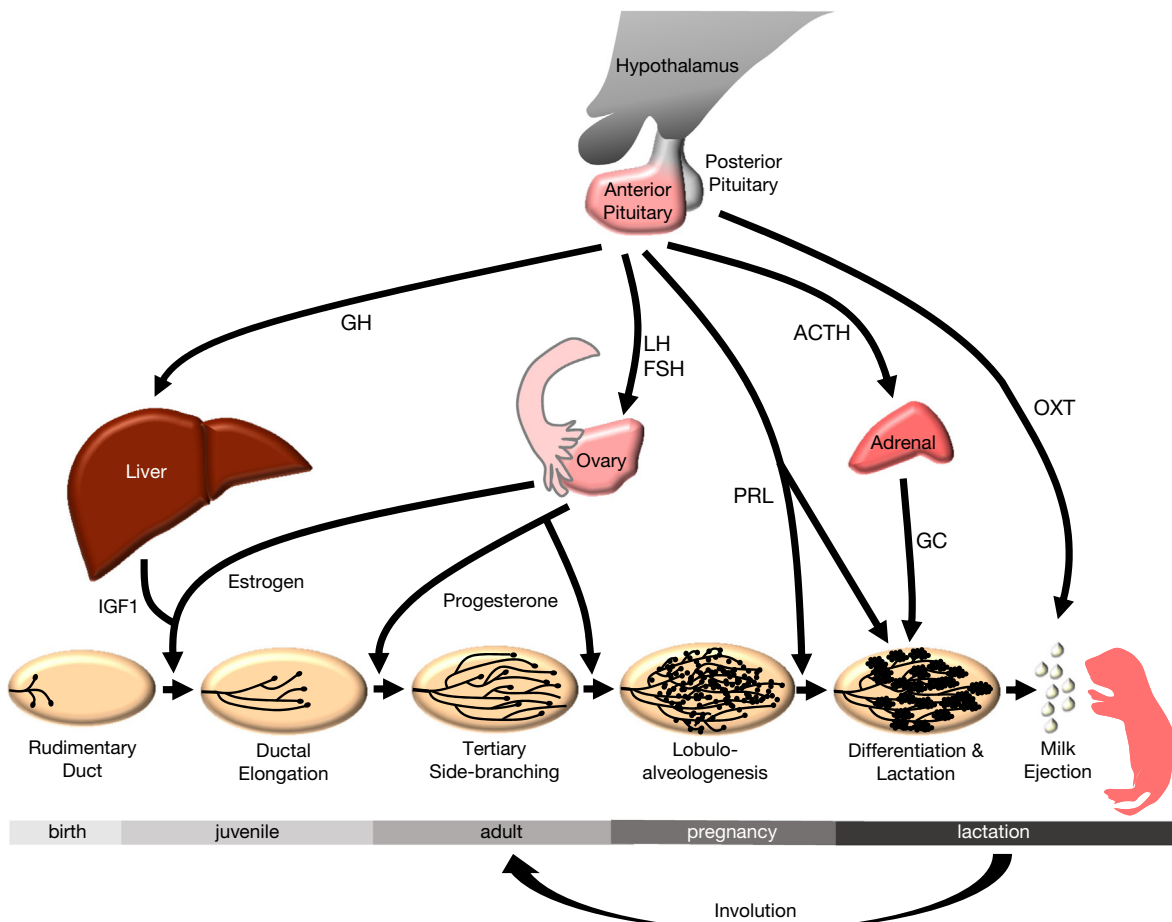


Fig. 7 The hormonal interplay that controls mammary morphogenesis and lactation from birth to post-lactational involution. The hypothalamus controls the production of a diverse set of pituitary hormones that then directly or indirectly stimulate the development, differentiation, and milk release from the mammary gland. Overall, the formation of a fully functional mammary gland involves the complex integration of inputs from several organs including the hypothalamus, pituitary, ovary, liver, and adrenal glands. Termination of suckling ultimately rewires the hormonal circuitry, leading to involution, a process by which unneeded mammary epithelial cells are pruned from the ductal tree. The cyclic process of alveolar expansion and milk production occurs with each pregnancy, providing a dynamic system to ensure offspring viability.

ER expression resulting in a synergistic impact of estrogens and GH (and, indirectly, IGF1) on ductal development. Tertiary side branching of the ductal tree begins in the adult gland and is dependent on LH/FSH-induced ovarian progesterone synthesis and mammary epithelial PR. During pregnancy, progesterone in concert with glucocorticoids and PRL, prepares the gland for lactation. Together, these three hormones promote extensive tertiary side branching and lobuloalveologenesis leading to complete filling of the mammary fat pad with epithelial cells and initiating milk production. Upon parturition, PRL promotes terminal differentiation of the alveoli into milk producing units. OXT, secreted from the posterior pituitary, then induces the contraction of the alveolar myoepithelial cells, stimulating the ejection of milk into the ducts and through the nipple. Suckling promotes OXT release and OXT then stimulates further proliferation and expansion of lobuloalveoli to accommodate increased milk demand. Upon weaning, the mammary gland involutes and returns to a near-virgin state, resetting the gland for pituitary hormone-induced growth and differentiation with subsequent pregnancies.

It should be noted that none of these pituitary-derived hormones act in isolation to promote mammary development and lactation. Instead, they act in concert, often synergistically, to promote postnatal mammary epithelial cell proliferation and differentiation. This system of positive and negative feedback loops from target organs back to the hypothalamus/pituitary uses a cascade of downstream signaling components to finely tune their relative contribution to the hormonal milieu. This culminates in a fully developed mammary gland at lactation that is dependent on the Hypothalamic/Pituitary/Mammary gland axis (Fig. 7).

References

- Briskin, C., & O'Malley, B. (2010). Hormone action in the mammary gland. *Cold Spring Harbor Perspectives in Biology*, 2, a003178.
- Macias, H., & Hinck, L. (2012). Mammary gland development. *Wiley Interdisciplinary Reviews: Developmental Biology*, 1, 533–557.
- Nelson, W. O. (1935). The effect of hypophysectomy upon mammary gland development and function in the Guinea pig. *Proceedings of the Society for Experimental Biology and Medicine*, 33, 222–224.
- Nelson, W. O. (1936). Endocrine control of the mammary gland. *Physiological Reviews*, 16, 488–526.
- Riddle, O. (1940). Lactogenic and mammogenic hormones. *Journal of the American Medical Association*, 115, 2276–2281.
- Stricker, P., & Grueter, R. (1928). Action du lobe antérieur de l'hypophyse sur la montée laiteuse. *Compte Rendu Seance Société Biologie*, 99.
- Trott, J. F., Vonderhaar, B. K., & Hovey, R. C. (2008). Historical perspectives of prolactin and growth hormone as mammogens, lactogens and galactagogues—Agog for the future! *Journal of Mammary Gland Biology and Neoplasia*, 13, 3–11.

Mammary Gland-Endocrinology

Priscilla A Furth, Sara Afridi, Sahar J Allothman, Redha I Azhar, Laxmi Y Gusain, Shaunice M Shreeves, Weisheng Wang, and Diba Zomorrodi, Georgetown University, Washington, DC, United States

© 2018 Elsevier Inc. All rights reserved.

Overview and Introduction of Endocrine Regulation of the Mammary Gland in Health and Disease

The mammary gland, or breast in humans and some other mammals, is the gland that mediates lactation, that is production of milk providing nourishment for offspring. Both males and females can have glandular mammary tissue. Growth and development of the mammary gland is regulated endocrinologically. Both sexes can be born with rudimentary mammary tissue. In females glandular tissue begins significant development with puberty in response to the release of steroid hormones estrogen and progesterone. Glandular development in males also is stimulated by exposure to estrogen, either exogenously or due to disease induced hormonal imbalances. During pregnancy main actors estrogen and progesterone work in concert with cytokine-like molecules including prolactin to develop a milk-producing gland. For decades it has been recognized that these hormones do not act in isolation but are influenced by a multiplicity of factors including other hormones such as glucocorticoids and androgens to execute normal mammary gland development and influence development of disease (Topper and Freeman, 1980). Benign and malignant diseases occur in the mammary gland. Both types of conditions can be pathophysiologically influenced by endocrine hormone stimulation. For example, fibrocystic disease of the breast has been linked to imbalanced production of estrogen and progesterone and breast cancer risk is modified by estrogen exposure, for example duration of estrogen exposure or post-menopausal exposure to exogenous estrogen and progesterone replacement therapy. Mammary gland tissue is composed of mammary epithelium, fat and connective collagenous tissue supported by vasculature that delivers nutrients and hormones and lymphoid system that drains extracellular fluids and mediates local immune response.

Steroid Hormones

Steroid hormones with significant activity in mammary gland tissue are grouped into two classes: sex steroids (estrogens, progestogens and androgens) and corticosteroids (glucocorticoids). Estrogens include estrone (E1) produced from ovary and fat tissue, estradiol (E2) primarily produced by ovarian follicles but also breast tissue, fat, liver, adrenal gland, and neural tissues, estriol (E3) produced primarily by the placenta during pregnancy and estetrol (E4) produced only during pregnancy by the liver (Simpson, 2003). Progesterone (P4) is a progestogen produced by the ovary and adrenal glands in the absence of pregnancy and by the corpus luteum and placenta during pregnancy. In males estrogens are produced in fat, testes and neural tissue and progesterone in testes and adrenal glands. Androgens typically act as inhibitors of normal mammary gland development but under some conditions can act as growth stimulants for some types of breast cancers (Hickey et al., 2012). Androgens are produced in the adrenal gland and testes in men and ovaries in women. In both females and males the glucocorticoid cortisol is produced by the adrenal gland. Cortisol (F) has many roles in normal physiology and development, most prominently in non-mammary tissue. In the mammary gland it generally acts as a relative survival factor for mammary epithelial cells.

Estrogens, progesterone, androgens, and glucocorticoids are carried from their non-mammary sites of production to the mammary gland through the vasculature or diffuse locally from sites of production within the gland (Fig. 1). From either production site they freely diffuse across biomembranes (Oren et al., 2004) and when in the cytosol can bind to their corresponding nuclear hormone receptors, primarily estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) for estrogens, progesterone receptor (PGR) for progesterone, glucocorticoid receptor (Nuclear Receptor Subfamily 3 Group C Member 1 or NRC31) for cortisol and androgen receptor (AR). ESR1 is generally considered (not considering) as growth promoting for mammary epithelial cells and ESR2 as more growth inhibitory (Renoir, 2012). In classical genomic signaling these ligand/receptor bound complexes are translocated to the nucleus where they can act on genomic DNA, either directly through their cognate DNA response elements or indirectly by binding other transcription factors, to mediate alterations in gene transcription (Bojcsuk et al., 2017). Non-genomic actions of steroid hormones can also occur, which, at times, can work together with classical hormone receptor signaling to mediate hormone action (Levin, 2015). Interactions between different steroid hormone receptors may influence their coordinate action (Sikora, 2016). Classical and non-classical hormone signaling produces both transcriptional and protein-protein interactions that influence cell growth, survival and differentiation.

In the mammary gland estrogen is the steroid hormone with the most positive influence on stimulating ductal growth and progesterone acting with estrogen are a critical steroid hormone combination mediating lobuloalveolar differentiation and lactation. Androgens generally act to suppress mammary epithelial cell growth. All four of the steroid hormones estrogen, progesterone, cortisol, and androgens have been shown to influence breast cancer growth. Approximately 70% of breast cancers express ESR1 some of which co-express PGR. AR and GR are also found expressed in specific breast cancer subsets and can be present in the absence of ESR1 and PGR expression. For ESR1 positive breast cancers therapeutic mainstays are anti-estrogen therapies including selective estrogen modulators (SERMs) that interfere with estrogen receptor action and aromatase inhibitors that interrupt production of estrogens.

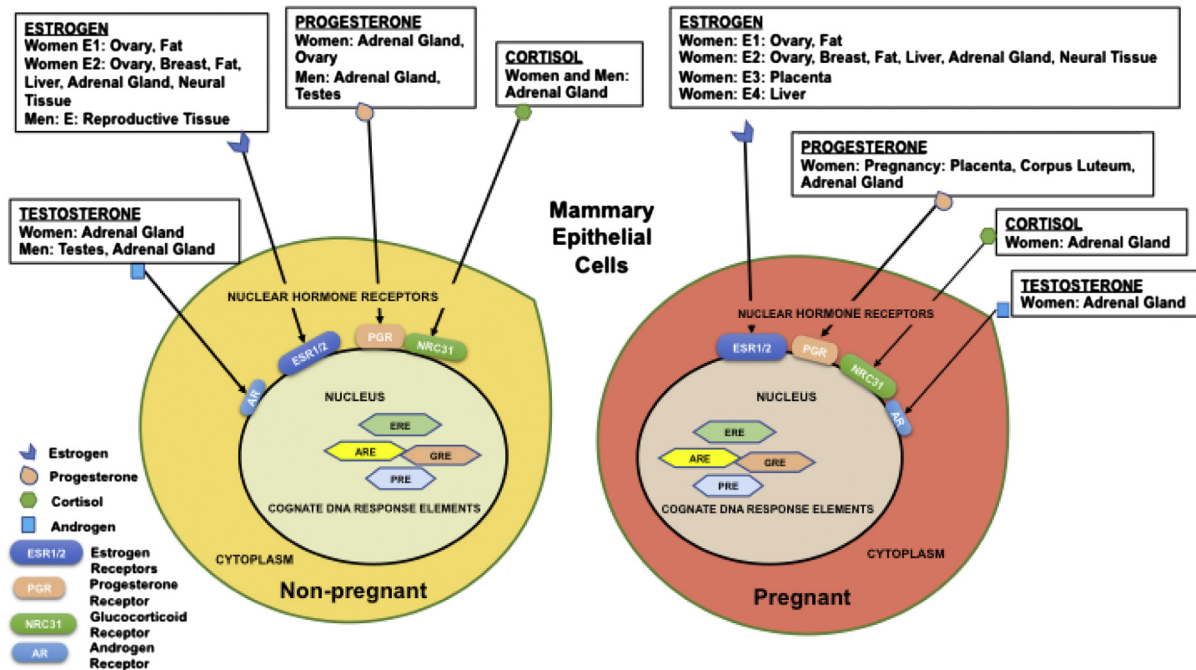


Fig. 1 Diagram illustrating four different steroid hormones (estrogens E1, E2, E3, and E4), progesterone, cortisol and testosterone active in mammary gland development, their sites of production, interacting receptors and DNA cognate response elements in mammary epithelial cells in women and men and in women during pregnancy. Interacting receptors: estrogen receptor alpha/beta (ESR1/2) for estrogens, progesterone receptor (PGR) for progesterone, glucocorticoid receptor (Nuclear Receptor Subfamily 3 Group C Member 1 or NRC31) for cortisol and androgen receptor (AR) for testosterone. DNA cognate response elements: ERE, estrogen response element for ESR1/2; PRE, progesterone response element for PGR; GRE, glucocorticoid response element for NRC31; ARE, androgen response element for AR.

Cytokine Hormones

Type I cytokine prolactin (PRL) is the major cytokine mediating normal development and physiology of the mammary gland. It is produced predominantly by the pituitary gland and travels to the mammary gland through the vasculature to act endocrinologically (Hovey et al., 2002). It can also be produced locally by the mammary gland and mammary epithelial cells can demonstrate auto-crine signaling where it is both produced and acts on the same cell as well as paracrine signaling acting on a neighboring cell. Prolactin signals through binding to the prolactin receptor (PRLR) and is most closely linked to lobuloalveolar development and lactation in mammary gland development (Horseman, 1999). Growth hormone (GH1) is a Type I cytokine that binds to the growth hormone receptor (GHR) (Kleinberg et al., 2009). Placental lactogens are Type I cytokines produced by the placenta during pregnancy that share structural homology with PRL and GH and can also influence pregnancy-related mammary development (Forsyth, 1994). Local production of Type II cytokines interleukins (IL) 4 and 13 by mammary epithelial cells can contribute to mammary epithelial luminal cell differentiation after binding to the interleukin 4 receptor (IL4R), a dimer composed of interleukin 13 receptor alpha 1 (IL-13R α 1) and interleukin 4 receptor alpha (IL-4R α) (Khaled et al., 2007). A feature of both Type I and Type II cytokine action in the mammary gland is signaling through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathway to regulate gene transcription through DNA elements referred to as gamma-activated sites (GAS) that recognize and bind STAT proteins (Fig. 2). STAT5 is the major actor in mammary gland development whereas STAT3 plays a more predominant role in involution (Furth et al., 2011). Different types of breast cancer sub-types demonstrate different patterns of STAT protein activation (Furth, 2014). GH/GHR signaling also contributes to mammary gland development through stimulation of insulin growth factor 1 (IGF1) signaling.

Parathyroid Hormone Related Protein

Parathyroid hormone related protein (PTHrP) is produced by many tissues including the mammary gland, acts predominantly locally and plays a role in embryonic mammary gland development (Hiremath and Wysolmerski, 2013). It acts through the G-protein coupled parathyroid hormone receptor 1 (PTHrP1) to specify the mammary mesenchyme.

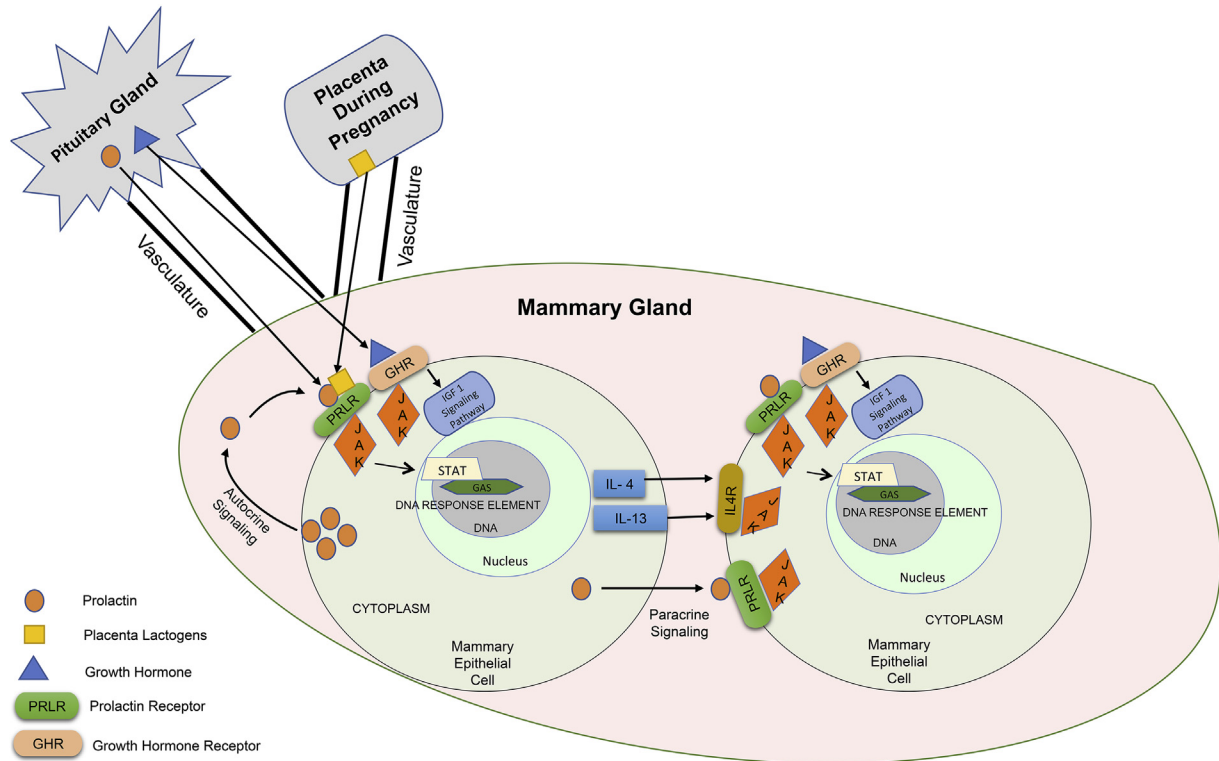


Fig. 2 Diagram illustrating Type I cytokine hormones prolactin, growth hormone and placental lactogens and Type II cytokine hormones interleukins (IL) 4 and 13 active in mammary gland development, interacting receptors, downstream signaling molecules Janus kinase (JAK), signal transducer and activator of transcription (STAT) insulin growth factor 1 (IGF1), and the STAT DNA response element gamma-activated site (GAS). JAK proteins are intracellular proteins that bind to Type I and II cytokine receptors and mediate recruitment and activation of STAT proteins that subsequently translocate to the nucleus of the cell where they can act as transcription factors through binding to GAS elements as well as other transcriptionally active proteins. Prolactin, growth hormone and placental lactogens act as endocrine hormones secreted by the pituitary or placenta and traveling through the vasculature to reach the mammary gland. Prolactin, IL4, and IL13 can exhibit autocrine and/or paracrine signaling. Interacting receptors: prolactin receptor (PRLR) for prolactin and placental lactogens, growth hormone receptor (GHR) for growth hormone, interleukin 4 receptor (IL4R) for IL4 and IL13. DNA response element for STAT proteins: GAS.

Synergy of Endocrine Hormone Action in Normal Mammary Physiology

Given that the normal mammary gland's primary function is to lactate and produce milk, it is essential to appreciate the endocrinological influences and important processes of the female lifespan that influence mammary gland development and lead to lactation. Over the course of her lifetime, a female will undergo birth, puberty, possible pregnancy and lactation, and menopause, all of which are endocrinologically mediated through different patterns of steroid and cytokine hormone stimulation that synergize with downstream growth factors and receptors, to mediate onset and ending and influence mammary gland development and function (Fig. 3). During the embryonic stage of female life, a rudimentary mammary gland develops from ectoderm and mesoderm germ layers influenced by PTHrP action. Neonatal milk production can occur transiently at birth in both sexes, due to high levels of maternal hormones passing through the placenta to stimulate the neonatal mammary tissue. Mammary gland tissue is then generally quiescent until puberty. Release of gonadotropin releasing hormone (GNRH1) from the hypothalamus triggers the pituitary to secrete gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then stimulate the ovary to cyclically produce estrogens and progesterone. Their cyclic production acts on mammary ductal tissue to stimulate development of terminal end buds that then travel through the mammary fat pad to lead to the development of a branched ductal tree in the mammary gland by the end of puberty (Paine and Lewis, 2017). Mammary epithelial cells experience cyclic rounds of growth stimulation and regression with each menstrual cycle dictated by waves of higher and lower levels of estrogens and progesterone. During pregnancy, a surge of estrogen and progesterone from the ovaries, corpus luteum and placenta stimulate the anterior pituitary to increase production of prolactin and this coupled with synthesis of placental lactogens from the placenta and local production of IL-4 and IL-13 results in milk production and secretion into the alveolar lumens. Milk becomes available to the offspring upon suckling of the nipple. Nipple sucking stimulates the pituitary gland to release the hormone oxytocin (OXT) stimulating contractile action that moves the milk from the alveolar lumens through the ducts to the nipple for ingestion by the offspring. When the offspring are weaned from the mother and the milk is no longer removed from the gland, involution is triggered. A series of changes in hormonal stimulation including immediate changes in patterns of STAT5/STAT3 activation occur, which is followed

Mammary Gland Growth, Development, and Regression Over the Reproductive Lifespan of Women

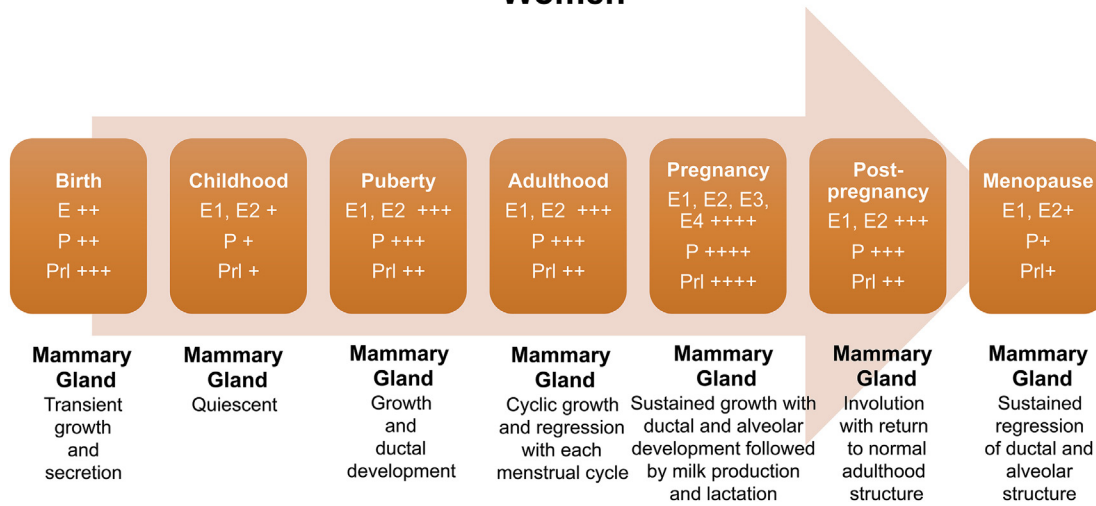


Fig. 3 Chart illustrating how changes in secretion levels of three major hormones governing mammary gland development (estrogen (E), progesterone (P) and prolactin (Prl)) are related to different stages of mammary gland development over the reproductive lifespan of a woman. Numbers of plus signs (+) indicate higher and lower secretion levels.

by resolution of hormone levels to cyclical pre-pregnancy patterns (Li et al., 1997). Menopause is reached when the ovarian follicle reserve is exhausted and cyclical production of estrogens and progesterone no longer occurs (Hale et al., 2013) and the mammary epithelium regresses. In humans breast density decreases as the proportion of fatty as compared to collagenous stromal tissue increases. Significantly, risk of breast cancer development actually increases with age, coincident with menopause and loss of cyclic hormonal stimulation.

Endocrinology of Breast Cancer and Breast Disease

For many decades hormones have been recognized as factors modifying breast cancer risk for women (Horn and Vatten, 2017) with longer estrogen exposure over a woman's lifetime increasing risk and early life pregnancy and longer durations of breast feeding associated with reduced risk. Men develop breast cancer at much lower frequency than women (Fentiman, 2016). Activation of the estrogen signaling pathway can promote development of mammary cancer associated with mutation of the *BRCA1*, DNA repair associated (*BRCA1*) gene (Wang and Di, 2014) (Fig. 4). Patterns of hormonal stimulation can influence cytokine secretion and the immune environment of the breast (Need et al., 2014). Deregulation of the normal cross-talk between ligand and receptors expressed in different tissue compartments within the mammary gland and the development of autocrine activation of signaling pathways can increase risk of breast cancer development (Hynes and Watson, 2010). Estrogens can also directly mediate genotoxicity (Cavalieri and Rogan, 2010). Benign breast diseases including cyclic breast pain and appearance of solid and cystic lesions are largely hormonally mediated (Santen and Mansel, 2005). Abnormally elevated prolactin or thyrotropin levels can lead to milk production (galactorrhea) in non-pregnant or menopausal women.

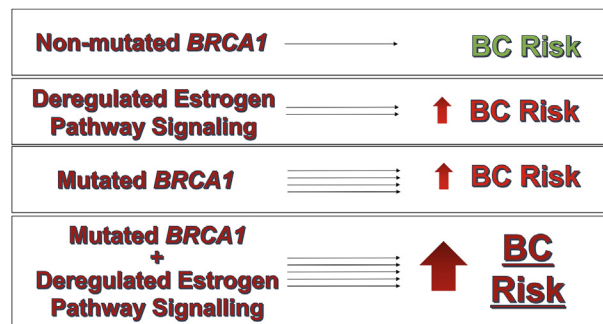


Fig. 4 Table illustrating an example of an interaction between deregulation of a steroid hormone signaling pathway (estrogen) and gene mutation (*BRCA1*, DNA Repair Associated (*BRCA1*)) in increasing risk of breast cancer development. Each of the pathophysiological changes individually increase risk of breast cancer development with the combination posing an even higher risk.

Summary

In summary, the mammary gland is an endocrinological organ that is both the target of exogenous hormonal stimulation and creator of its own endogenous hormonal network. Dominant players include steroid hormones, cytokine Type 1 and 2 hormones and their receptors. Some of the same hormonal networks that regulate normal mammary gland development can function as risk factors for breast cancer development when deregulated. Normal development is mediated by a well-orchestrated organization of hormonal signaling both spatially and temporally. Imbalances in hormonal signaling can increase the risk for benign as well as malignant breast disease.

References

- Bojcsuk, D., Nagy, G., & Balint, B. L. (2017). Inducible super-enhancers are organized based on canonical signal-specific transcription factor binding elements. *Nucleic Acids Research*, 45, 3693–3706.
- Cavalieri, E. L., & Rogan, E. G. (2010). Depurinating estrogen–DNA adducts in the etiology and prevention of breast and other human cancers. *Future Oncology*, 6, 75–91.
- Fentiman, I. S. (2016). Male breast cancer is not congruent with the female disease. *Critical Reviews in Oncology/Hematology*, 101, 119–124.
- Forsyth, I. A. (1994). Comparative aspects of placental lactogens: Structure and function. *Experimental and Clinical Endocrinology*, 102, 244–251.
- Furth, P. A. (2014). STAT signaling in different breast cancer sub-types. *Molecular and Cellular Endocrinology*, 382, 612–615.
- Furth, P. A., Nakles, R. E., Millman, S., Diaz-Cruz, E. S., & Cabrera, M. C. (2011). Signal transducer and activator of transcription 5 as a key signaling pathway in normal mammary gland developmental biology and breast cancer. *Breast Cancer Research*, 13, 220.
- Hale, G. E., Robertson, D. M., & Burger, H. G. (2013). The perimenopausal woman: Endocrinology and management. *The Journal of Steroid Biochemistry and Molecular Biology*, 142, 121–131.
- Hickey, T. E., Robinson, J. L., Carroll, J. S., & Tilley, W. D. (2012). Minireview: The androgen receptor in breast tissues: Growth inhibitor, tumor suppressor, oncogene? *Molecular Endocrinology*, 26, 1252–1267.
- Hiremath, M., & Wysolmerski, J. (2013). Parathyroid hormone-related protein specifies the mammary mesenchyme and regulates embryonic mammary development. *Journal of Mammary Gland Biology and Neoplasia*, 18, 171–177.
- Horn, J., & Vatten, L. J. (2017). Reproductive and hormonal risk factors of breast cancer: A historical perspective. *International Journal of Women's Health*, 9, 265–272.
- Horsman, N. D. (1999). Prolactin and mammary gland development. *Journal of Mammary Gland Biology and Neoplasia*, 4, 79–88.
- Hovey, R. C., Trott, J. F., & Vonderhaar, B. K. (2002). Establishing a framework for the functional mammary gland: From endocrinology to morphology. *Journal of Mammary Gland Biology and Neoplasia*, 7, 17–38.
- Hynes, N. E., & Watson, C. J. (2010). Mammary gland growth factors: Roles in normal development and in cancer. *Cold Spring Harbor Perspectives in Biology*, 2(8), a003186.
- Khaled, W. T., Read, E. K. C., Nicholson, S. E., et al. (2007). The IL-4/IL-13/Stat6 signaling pathway promotes luminal mammary epithelial cell development. *Development*, 134, 2739–2750.
- Kleinberg, D. L., Wood, T. L., Furth, P. A., & Lee, A. V. (2009). Growth hormone and insulin-like growth factor-I in the transition from normal mammary development to preneoplastic mammary lesions. *Endocrine Reviews*, 30, 51–74.
- Levin, E. R. (2015). Extracellular steroid receptors are essential for steroid hormone actions. *Annual Review of Medicine*, 66, 271–280.
- Li, M., Liu, X., Robinson, G., et al. (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proceedings of the National Academy of Sciences of the United States of America*, 94, 3425–3430.
- Need, E. F., Atashgarian, V., Ingman, W. V., & Dasari, P. (2014). Hormonal regulation of the immune microenvironment in the mammary gland. *Journal of Mammary Gland Biology and Neoplasia*, 19, 229–239.
- Oren, I., Fleishman, S. J., Kessel, A., & Ben-Tal, N. (2004). Free diffusion of steroid hormones across biomembranes: A simplex search with implicit solvent model calculations. *Biophysical Journal*, 87, 768–779.
- Paine, I. S., & Lewis, M. T. (2017). The terminal end bud: The little engine that could. *Journal of Mammary Gland Biology and Neoplasia*, 22, 93–108.
- Renoir, J.-M. (2012). Estradiol receptors in breast cancer cells: Associated co-factors as targets for new therapeutic approaches. *Steroids*, 77, 1249–1261.
- Santen, R., & Mansel, R. (2005). Benign breast disorders. *New England Journal of Medicine*, 353, 275–285.
- Sikora, M. J. (2016). Family matters: Collaboration and conflict among the steroid receptors raises a need for group therapy. *Endocrinology*, 157, 4553–4560.
- Simpson, E. R. (2003). Sources of estrogen and their importance. *The Journal of Steroid Biochemistry and Molecular Biology*, 86, 225–230.
- Topper, Y. J., & Freeman, C. S. (1980). Multiple hormone interactions in the developmental biology of the mammary gland. *Physiological Reviews*, 60, 1049–1106.
- Wang, L., & Di, L.-J. (2014). BRCA1 and estrogen/estrogen receptor in breast cancer: Where they interact? *International Journal of Biological Sciences*, 10, 566–575.

Further Reading

- Andres, A. C., & Strange, R. (1999). Apoptosis in the estrous and menstrual cycles. *Journal of Mammary Gland Biology and Neoplasia*, 4, 221–228.
- Amal, J. F., Lenfant, F., Metivier, R., et al. (2017). Membrane and nuclear estrogen receptor α actions: From tissue specificity to medical implications. *Physiological Reviews*, 97, 1045–1087.
- Dall, G. V., & Britt, K. L. (2017). Estrogen effects on the mammary gland in early and late life and breast cancer risk. *Frontiers in Oncology*, 7.
- Diaz-Cruz, E. S., Sugimoto, Y., Gallicano, I. G., et al. (2011). Comparison of increased aromatase versus ER α in the generation of mammary hyperplasia and cancer. *Cancer Research*, 71, 5477–5487.
- DVall, S. A., & Radovick, S. (2009). Endocrinology of female puberty. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 16, 1–4.
- Frech, M. S., Halama, E. D., Tilli, M. T., et al. (2005). Deregulated estrogen receptor α expression in mammary epithelial cells of transgenic mice results in the development of ductal carcinoma in situ. *Cancer Research*, 65, 681–685.
- Hennighausen, L., & Robinson, G. W. (2005). Information networks in the mammary gland. *Nature Reviews Molecular Cell Biology*, 6, 715–725.
- Howlander, N., Noone, A. M., Krapcho, M., Miller, D., Bishop, K., Kosary, C. L., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D. R., Chen, H. S., & Feuer, E. J. (1975–2014). In K. A. Cronin (Ed.), *SEER Cancer Statistics Review*. Bethesda, MD: National Cancer Institute. https://seer.cancer.gov/csr/1975_2014/, based on November 2016 SEER data submission, posted to the SEER web site, April 2017.
- Jones, L. P., Tilli, M. T., Assefnia, S., et al. (2008). Activation of estrogen signaling pathways collaborates with loss of Brca1 to promote development of ER α negative and ER α positive mammary preneoplasia and cancer. *Oncogene*, 27, 794–802.
- Li, W., Xiao, C., Vonderhaar, B. K., et al. (2007). A role of estrogen/ER α signaling in BRCA1-associated tissue-specific tumor formation. *Oncogene*, 26, 7204–7212.

- Ma, H., Ursin, G., Xu, X., et al. (2017). Reproductive factors and the risk of triple-negative breast cancer in white women and African-American women: A pooled analysis. *Breast Cancer Research*, 19, 6.
- Obr, A. E., & Edwards, D. P. (2012). The biology of progesterone receptor in the normal mammary gland and in breast cancer. *Molecular and Cellular Endocrinology*, 357, 4–17.
- Pan, D., Kocherginsky, M., & Conzen, S. D. (2011). Activation of the glucocorticoid receptor is associated with poor prognosis in estrogen receptor-negative breast cancer. *Cancer Research*, 71, 6360–6370.
- White, B. and Porterfield, S. Endocrine and reproductive physiology. 4th edn., pp. 177–197, 210–238. Philadelphia, Pennsylvania: Elsevier Mosby.
- Zheng, Y., & Murphy, L. C. (2016). Regulation of steroid hormone receptors and coregulators during the cell cycle highlights potential novel function in addition to roles as transcription factors. *Nuclear Receptor Signaling*, 14, e001.

Relevant Websites

mheducation, n.d. https://highered.mheducation.com/sites/9834092339/student_view0/chapter46/mechanism_of_steroid_hormone_action.html – Glencoe Online Learning Center. McGraw Hill Education. See: <http://www.glencoe.com/>.

Lactocrine Programming

Frank F Bartol, Auburn University, Auburn, AL, United States

Carol A Bagnell, Rutgers University, New Brunswick, NJ, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Developmental plasticity The idea that tissue/organismal phenotype is not fixed genetically, but is defined dynamically through the course of development by macro- and micro-environmentally defined epigenetic events that affect the developmental program at molecular and cellular levels.

Developmental program The series of molecular, cellular and related biological processes which, together, define events that determine structure and function of tissues, organs, organ systems and organisms.

Developmental trajectory The organizational path defined by a developmental program.

Lactocrine A mechanism by which bioactive factors are communicated from mother to offspring in colostrum/milk as a specific consequence of nursing.

Lactocrine hypothesis The idea that disruption of lactocrine signaling shortly after birth will alter the program and trajectory of development with short-term organizational effects and long-term consequences for health and well-being.

Phenome The totality of genetic, epigenetic and extragenetic (environmental) factors that, collectively, determine organismal phenotype.

Introduction

Lactocrine is a term coined to describe a mechanism by which milk-borne bioactive factors (MbFs) are communicated from mother to offspring in colostrum (first milk) as a specific consequence of nursing (Bartol et al., 2008). Lactocrine transmission of MbFs is a well-recognized mammalian phenomenon (Bagnell et al., 2017; Bartol et al., 2017). Also well-established is the fact that, beyond genetics, maternal effects on organizational events through the course of gestation affect the trajectory of development and, ultimately, offspring phenotype. Such maternal effects do not end at birth, but continue for a period of time postnatally through signals communicated from mother to offspring via a lactocrine mechanism.

A developmental program is defined by the series of molecular, cellular and related biological processes that, collectively, determine phenotype or organismal structure and function. The nature of these events, which occur in time and space, determine developmental trajectory and connect genotype to phenotype. The totality of genetic, epigenetic and environmental factors that determine organismal phenotype constitute the phenome (Houle et al., 2010; Bush et al., 2016). The lactocrine hypothesis for maternal programming of phenotype posits that lactocrine-mediated signaling is a significant part of the phenomic puzzle and, as such, that disruption of lactocrine support will alter the program and trajectory of postnatal development with short-term organizational effects and long-term phenotypic consequences.

Developmental Plasticity

Phenotype is not fixed genetically, but is defined dynamically through the course of development. Consequently, a single genotype can give rise to more than one phenotype. Thus, phenotype is plastic and developing tissues are said to display developmental plasticity. Inherent in this observation is the long-established idea that disruption of organizational events during “critical moments” of development can have lasting consequences (Stockard, 1921). Such “critical moments” (periods of time during which disruption of the normal path or optimal trajectory of development can affect phenotype) vary for different tissues and organs across species and with respect not only to the timing, but also to the nature, magnitude and duration of disruptive events. Therefore, defined at both genetic and epigenetic levels, a program of development can follow optimal or suboptimal trajectories depending upon the environmental and physiological conditions present during critical organizational periods (Kicheva and Briscoe, 2015).

Many tissues, including those of the female reproductive tract and the central nervous system, remain organizationally plastic for a period of time postnatally and are, therefore, subject to developmental disruption with phenotypic consequences (Hanson and Gluckman, 2014; Ismail et al., 2017; Bartol et al., 2017). Neonatal mammals are subject to effects of the environmental conditions into which they are born, including effects of physiologically significant, organizationally important signals communicated in milk.

Milk-Borne Bioactive Factors (MbFs)

Milk is more than food (Hinde and Milligan, 2011). Beyond nutrients, colostrum contains a complex array of bioactive factors (Table 1). Bioactive molecules identified in colostrum and milk include proteins, growth factors and cytokines, native and latent bioactive peptides, oligosaccharides, fatty acid-derived molecules, steroid hormones, and noncoding microRNAs (miRNAs). Peptides and miRNAs can be packaged selectively in and delivered as elements of exosomes (McGough and Vincent, 2016), found in milk and other body fluids. Additionally, lactocrine transmission of information from mother to offspring may occur via maternal somatic cells delivered in milk. Milk-borne immune cells delivered to nursing infants can cross the neonatal intestinal mucosa, enter the bloodstream and accumulate in somatic tissues. Similarly, the presence of stem cells with multilineage potential in breast milk suggests that lactocrine-mediated microchimerism, involving colonization of neonatal tissues by stem cells of maternal origin, could also affect postnatal development and offspring phenotype (Bartol et al., 2017).

Milk can also serve as a conduit for transmission of bioactive factors of environmental origin to nursing young (Bartol and Bagnell, 2012). Both naturally occurring (e.g., phytochemicals) and manmade (e.g., endocrine-disrupting chemicals) environmental factors can be communicated to neonates in milk. Such substances can be introduced into the maternal system via ingestion,

Table 1 Milk-borne Bioactive Factors^a

Category	Species ^b	Bibliographic Sources
Bioactive peptides ^c	Buffalo, cow, goat, human, mouse, pig, sheep	Pandya (2009), Garcia et al. (2013), Clare and Swaisgood (2000), Nagpal et al. (2011), Park and Nam (2015), Jandal (1996), Jensen (1995), Frankshun et al. (2012), Schanbacher et al. (1998), Meisel (1998), Bartol et al. (2017), and Sharp et al. (2014)
Cytokines	Cow, human, mouse	Jensen (1995), Bartol et al. (2017), Collado et al. (2015), Garofalo (2010), and Munoz et al. (1990)
Enzymes	Buffalo, cow, goat, horse, human, pig, sheep	Pandya (2009), Garcia et al. (2013), Jensen, (1995), Hata et al. (2010), Jenness (1980), Kerner et al. (1984), and Reinhardt et al. (2012)
Fatty acid-derived factors	Cow, goat, human, sheep	Garcia et al. (2013), Jandal (1996), and Collado et al. (2015)
Gastrointestinal peptides	Cow, dog, human, pig	Jensen (1995), Grosvenor et al. (1993)
Growth factors	Cow, dog, goat, horse, human, mouse, nonhuman primates, pig, rat, tammar wallaby	Garcia et al. (2013), Jensen (1995), Bartol et al. (2017), Grosvenor et al. (1993), Baxter et al. (1984), Blum and Baumrucker (2008), Connolly and Rose (1988), Donnet-Hughes et al. (2000), Donovan et al. (1991a), Donovan et al. (1991b), Donovan and Odle (1994), Iacopetta et al. (1992), Jin et al. (1991), Malven et al. (1987), Okada et al. (1991), Power et al. (2017), Read et al. (1984), Vega et al. (1991), Collado et al. (2015), Sharp et al. (2014), and Wright (1983)
Hypothalamic hormones	Cow, horse, human, pig, rat, sheep	Jensen (1995) and Grosvenor et al. (1993)
Immunoglobulins	Buffalo, cow, human, pig	Pandya (2009), Jensen (1995), and Blum and Baumrucker (2008)
Lipid-derived factors	Cow, goat, human, sheep	Garcia et al. (2013) and Jandal (1996)
MicroRNA	Cow, goat, human, pig, tammar wallaby	Chen et al. (2014), Hata et al. (2010), Kosaka et al. (2010), Sharp et al. (2014), Modepalli et al. (2014), Gu et al. (2012), Zhou et al. (2012), and Chen et al. (2010)
Nucleosides	Cow, human	Jensen (1995)
Nucleotides	Cow, goat, human, pig, sheep	Jensen (1995)
Oligosaccharides	Buffalo, cow, dog, goat, horse, human, rat, sheep, tammar wallaby	Kunz et al. (2000) and Urashima et al. (2001)
Pituitary hormones	Cow, goat, human, mouse, pig, rat, sheep	Jensen (1995), Grosvenor et al. (1993), Blum and Baumrucker (2008), Donnet-Hughes et al. (2000), and Donovan and Odle (1994)
Prostaglandins	Cow, human, rat	Grosvenor et al. (1993)
Steroid hormones	Cow, goat, human, nonhuman primates, pig	Garcia et al. (2013), Jandal (1996), Jensen (1995), Bartol et al. (2017), Grosvenor et al. (1993), Malekinejad et al. (2006), Hinde et al. (2015), and Farmer and Quesnel (2009)
Thyroid/parathyroid hormones	Cow, goat, human, rat, sheep	Jensen (1995) and Grosvenor et al. (1993)
Other peptides/proteins ^d	Cow, goat, guinea pig, human, horse, mouse, pig, sheep	Garcia et al. (2013), Jandal (1996), Jensen (1995), Grosvenor et al. (1993), Masson and Heremans (1971) and Legrand et al. (2008)

^aBioactive factors identified in mammalian colostrum and/or mature milk.

^bMilk-borne bioactive factors in a given category have been identified in colostrum/milk from one or more of the species listed.

^cBioactive peptides include those bioactive peptides that are encrypted in larger milk proteins.

^dOther peptides/proteins include, Delta Sleep Inducing Peptide, Erythropoietin, Transferrin, Vitamin B12-Binding Protein, Vitamin D-Binding Protein.

inhalation and/or transdermal transport (LaKind et al., 2004). Thus, lactocrine communication can also provide chemical information to nursing offspring related to the nature of environmental conditions to which the lactating dam is exposed. In turn, macro-environmentally modified lactocrine signals can affect the way in which neonates respond and adapt to extrauterine conditions into which they are born (Bartol and Bagnell, 2012).

The Lactocrine Hypothesis, Colostrum and Lactocrine Programming

The lactocrine hypothesis for maternal programming of postnatal development advanced the idea that imposition of a lactocrine-null or lactocrine deficient state from birth will alter the developmental program and trajectory of somatic development with short-term organizational effects and long-term phenotypic consequences (Bartol et al., 2008, 2017). A lactocrine-null condition is defined by the absence of colostrum consumption. This condition can be imposed purposefully or experimentally, by substitution of a milk replacer for colostrum, or by necessity when conditions of disease or drug addiction make consumption of mother's milk inadvisable clinically. A lactocrine deficient condition is imposed upon nursing offspring when the relative availability, abundance and/or quality of colostrum/milk is suboptimal. This can occur naturally in conditions of mastitis, agalactia and/or competition for teat position in litter-bearing species. A unique secretory product of the mammary gland, colostrum forms during late pregnancy as the mammary gland is prepared for lactation. Because colostrum production is transient, and its composition is optimized to support nutritional, immunological and developmental needs of nursing offspring, colostrum intake is a major determinant of neonatal survival and development (Bartol et al., 2017 and references therein).

Asynchronous concurrent lactation (ACL), characteristic of macropodid marsupials, provides an excellent model system for study of lactocrine effects on patterns of offspring development (Bartol et al., 2017 and references therein). In species that display ACL, two pouch young can nurse from adjacent mammary glands that are secreting milk of different composition. Comparisons of developmental characteristics of pouch young nursed normally, on mammary glands functionally synchronous with their age, or asynchronously, on mammary glands functionally ahead of their age, showed that growth and development were influenced markedly by differences in milk composition. Studies of this kind established that maternally regulated milk composition, rate of milk production, timing of milk consumption, and milk intake can affect developmental trajectory and offspring phenotype.

Effects of nursing from birth and the composition of colostrum or milk on patterns of offspring development are also documented for eutherian mammals (Bartol and Bagnell, 2012; Bartol et al., 2017). Information presented in Table 1 documents the fact that colostrum/milk acts as a conduit for lactocrine transmission of a vast array of signaling molecules. From data generated primarily in rodent models, it is clear that neonatal consumption of colostrum affects differentiation and, ultimately, function of anterior pituitary mammotropes, development of other tissues including liver, kidney, spleen and muscle, and maturation of both the immune system and gastrointestinal tract (Bartol and Bagnell, 2012 and references therein). In nursing mice, the array of chemokines in milk affects patterns of hippocampal cell proliferation in the central nervous system with lasting effects on adult spatial memory. Maternal effects on infant behavioral trajectory mediated by milk-borne glucocorticoids were documented in rhesus macaques. Data for dairy calves suggested a role for MbFs in programming of mammary gland development and function. Collectively, observations indicate that maternal effects on postnatal development at multiple levels include functional contributions from colostrum and milk that must be communicated to offspring via a lactocrine mechanism. Generally, available data indicate that lactocrine signaling can have broad ranging effects on patterns of growth and body composition with lasting implications for health (Bartol and Bagnell, 2012). Data for the pig (*Sus scrofa domestica*) provide the most complete picture of lactocrine programming effects, as reflected by studies of the developing female reproductive tract.

Lactocrine Effects on Female Reproductive Tract (FRT) Development

The mammalian uterine wall is incompletely organized at birth (postnatal day = PND 0) (Cooke et al., 2013). In species including the mouse, rat, sheep, and pig, uterine growth and morphogenesis of the uterine wall, including the uterine mucosal lining or endometrium, proceed normally for a period of time neonatally after bilateral ovariectomy at birth. Continued development of the uterine wall after ovariectomy at birth, including differentiation and proliferation of uterine glands, indicates that extraovarian factors support uterine development in the early neonatal period. Data for the pig indicate that one source of such extraovarian support comes from colostrum via a lactocrine mechanism (Bartol et al., 2013, 2017).

In the pig, endometrial glands are absent or rudimentary at birth. Nascent endometrial glandular epithelium (GE) differentiates from luminal epithelium (LE) and begins to proliferate and penetrate uterine stroma (ST) between birth and PND 3. Differentiation of GE from LE and histogenesis of the uterine wall during this period are marked by spatially unique endometrial expression of a number of gene products, including estrogen receptors (ESR1). This period coincides with an optimal window for lactocrine transmission of colostral MbFs to offspring. Protein and other components of colostrum are present at highest concentrations, and are delivered steadily on day 1 of lactation. Gut closure in the pig, the point after which macromolecular components of colostrum may no longer simply diffuse across intestinal epithelium, occurs within approximately 48 h of birth. These relationships are reviewed in detail elsewhere (Bartol et al., 1993, 2013, 2017; Cooke et al., 2013).

Lactocrine signaling from birth affects the porcine uterine developmental program rapidly (Bagnell et al., 2017; Bartol et al., 2017). A single feeding of colostrum at birth is sufficient to support normal levels of uterine and cervical cell proliferation at

12 h postnatal, suggesting that lactocrine effects on FRT development are initiated at first ingestion of colostrum. When colostrum consumption was delayed by milk replacer feeding for 0.5 h from birth, or limited to 12 h from birth, developmental markers including uterine matrix metalloproteinase-9 (MMP9) and tissue inhibitor of metalloproteinase-1, as well as cervical MMP9 expression levels on PND 2 were similar to those observed for nursed gilts that consumed colostrum continuously for 48 h from birth. Since nursing for as little as 1 h from birth is sufficient to establish passive immunity in newborn piglets, observations indicate that lactocrine signals supportive of FRT development are likely to be communicated within 12 h of birth.

Imposition of the lactocrine-null state for 2 days from birth, by feeding milk replacer in lieu of colostrum, affected the neonatal porcine uterine transcriptome profoundly between birth and PND 2 (Bartol et al., 2017). Global RNA sequencing (RNAseq) revealed over 3000 differentially expressed genes associated with uterine development between birth and PND 2 in nursed gilts, whereas over 4500 differentially expressed genes were associated with this period in gilts fed milk replacer from birth. Comparison of uterine gene expression on PND 2 between nursed and replacer-fed gilts revealed over 800 differentially expressed, lactocrine-sensitive genes. Evaluation of small noncoding microRNA (miRNA) expression also revealed unique age and lactocrine effects on uterine miRNA expression between birth and PND 2 in nursed as compared to replacer-fed gilts (George et al., 2017). Integration of uterine RNAseq and small RNAseq data, followed by informatic analyses, predicted lactocrine-sensitive biological processes associated with uterine development during this period to include: cell–cell signaling; cell, tissue and organ development; tissue morphology; and reproductive system development and function.

Beyond effects on the neonatal uterine transcriptome identified in lactocrine-null gilts on PND 2, imposition of the lactocrine-null state for 2 days from birth also altered porcine uterine developmental trajectory as evaluated on PND 14. Compared to nursed controls, endometrial thickness, uterine gland penetration depth, GE and stromal cell proliferation, and ESR1 expression by nascent GE were reduced on PND 14 in lactocrine-null gilts. Thus, experimental disruption of lactocrine support from birth by milk replacer feeding is antiuterotrophic in the porcine neonate.

Consistent with these observations, natural disruption of lactocrine input in nursing piglets, resulting in a lactocrine deficient state, also affects uterine development negatively. Evaluation of uterine wall development on PND 14 in nursed gilts determined to be lactocrine deficient as reflected by low serum immunoglobulin immunocrit (iCrit) on PND 0 (Bartol et al., 2017), revealed effects similar to those observed in lactocrine-null gilts at the same point. Again, endometrial development was generally more advanced on PND 14 in high (optimal colostrum consumption) as compared to low (minimal colostrum consumption) iCrit gilts, indicating an antiuterotrophic effect of lactocrine deficiency from birth.

Long-Term Consequences of Lactocrine Deficiency

The lactocrine hypothesis predicts that disruption of lactocrine signaling will affect the developmental trajectory of lactocrine-sensitive tissues with lasting consequences. This hypothesis was tested retrospectively for the uterus in a study designed to define the relationship between PND 0 iCrit, indicative of the amount of colostrum acquired by piglets on the day of birth, and their fecundity as adults (Bartol et al., 2013). Initial data included observations for 381 gilts for which PND 0 iCrit values were obtained, and live litter sizes were recorded for the same animals as adults. As predicted, low serum iCrit on PND 0, reflecting minimal colostrum consumption (lactocrine deficiency), was associated with reduced lifetime fecundity through four parities (Bartol et al., 2013). This observation was confirmed and extended in a complementary study of the same design involving 799 females (Bartol et al., 2017 and references therein). Results showed that lactocrine deficiency from birth was associated with reduced average preweaning growth rate during lactation, increased age at puberty, and reduced live litter size. The difference in live litter size for adult females that had low as compared to high PND 0 iCrit values was approximately 1.4 piglets per litter, with no effect of parity. These data were interpreted to indicate permanent impairment of reproductive performance in neonatally lactocrine-deficient female pigs (Bartol et al., 2017).

Major reproductive losses in the pig are associated with early embryonic mortality attributable, in part, to endometrial dysfunction during the peri-attachment period of early pregnancy (Bazer et al., 2011). Such losses during early pregnancy could account for differences in live litter size at the end of gestation. To test the hypothesis that permanent impairment of reproductive performance in neonatally lactocrine-deficient adult female pigs is associated with endometrial functionality, uterine tissues were obtained from adult, neonatally low (lactocrine deficient) and high (control) iCrit gilts on pregnancy day (PxD) 13. Effects of lactocrine deficiency at birth on adult, peri-attachment stage endometrial gene expression were determined by RNAseq analysis. Preliminary results indicate hundreds of differential endometrial gene expression events in adults on PxD 13 associated with lactocrine deficiency from birth. Overall, observations for the pig can be interpreted to indicate that disruption of lactocrine signaling for a relatively short period of time from birth: (1) alters the neonatal uterine developmental program with lasting consequences, as reflected by altered endometrial responses to conceptus signals during early pregnancy; (2) reduces uterine capacity for conceptus support; and (3) reduces fecundity, reflected by smaller litters in neonatally lactocrine deficient adults. Collectively, observations support the lactocrine hypothesis for maternal programming of uterine development, endometrial functionality, and the functional capacity of uterine tissues in the pig.

Maternal Lactocrine Continuum

An abundance of data provide compelling support for the idea that maternal influence over development of offspring extends beyond the womb into neonatal life by consequence of nursing and the transmission of lactocrine signals (Bagnell et al., 2017;

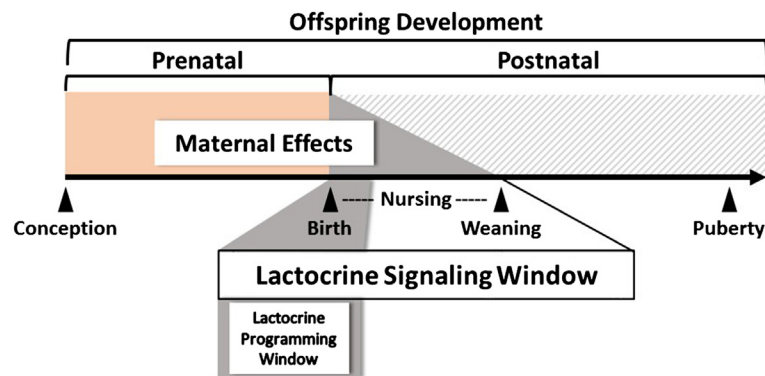


Fig. 1 Developmental programming and lactocrine effects. Maternal effects on offspring development begin at conception and extend through the prenatal period into postnatal life through signals transmitted from mother to nursing young in colostrum (first milk) and mature milk via a lactocrine mechanism. The potential lactocrine signaling window encompasses the period from birth until weaning. The lactocrine programming window, defined as the postnatal period when lactocrine-active factors can affect the organizational programming of somatic cells and tissues with lasting consequences, is likely to be species-specific, and to encompass a shorter period of hours or days from birth. During this period concentrations of potentially lactocrine-active factors in colostrum are highest, and conditions facilitative of delivery of lactocrine-active factors from the neonatal gastrointestinal tract into the circulation are broadly functional. In the pig, lactocrine disruption from birth (PND 0): (i) alters uterine development at cellular and molecular levels by 12 h postnatal; (ii) alters uterine gene expression globally by PND 2; (iii) inhibits endometrial histogenesis by PND 14; (iv) alters adult endometrial gene expression in adults on PxD 13; and (v) reduces functional uterine capacity in adults (See: [Bagnell et al., 2017](#); [Bartol et al., 2017](#)).

[Bartol and Bagnell, 2012](#); [Bartol et al., 2013, 2017](#)). The fact that lactation is a defining characteristic of mammals suggests that lactocrine programming of postnatal development is an evolutionarily conserved, mammalian phenomenon. Data for the pig support the idea of a continuum of events affecting lactocrine signaling and reproductive performance ([Bartol and Bagnell, 2012, 2017](#)). Aspects of this continuum are illustrated in [Fig. 1](#).

During the prenatal period, maternal environmental conditions program fetal and placental development. Factors of maternal and fetoplacental origin define the path of mammary gland development, colostrogenesis and lactogenesis. This determines the potential quantity and quality of colostrum/milk and, therefore, the relative availability and nature of lactocrine signals during the postnatal period. Transmission of lactocrine-active factors from mother to offspring can occur throughout the period from birth to weaning. However, a critical window for lactocrine programming of uterine development is likely open for a short period of time (12–24 h) postnatally ([Fig. 1](#)). For nursing offspring, the relative availability and quality of colostrum determines whether neonates are lactocrine sufficient or lactocrine deficient from birth. This, in turn, affects the uterine developmental program and, ultimately, determines functional uterine capacity in adults. Reduced functional uterine capacity in neonatally lactocrine deficient adults may affect the ability of these animals to support fetoplacental development, thereby affecting mammogenesis, lactogenesis and the nature and quality of lactocrine signals in the next generation. By the same token, lactocrine sufficiency from birth supports optimal postnatal programming of lactocrine-sensitive tissues.

Summary and Conclusions

Nursing and factors delivered to mammalian offspring in colostrum and milk are recognized as essential elements of a continuum of maternal epigenetic contributions to the organizational program that defines developmental trajectory and offspring phenotype. Identification of lactocrine mechanisms regulating postnatal development will aid in understanding the developmental origins of mammalian health and disease. Defining the functional roles and interactions of the immense array of potentially lactocrine-active factors in colostrum and milk ([Table 1](#)) poses a significant challenge that demands to be accepted. Studies are also needed to determine how colostrogenesis, which is programmed maternally during late gestation, affects the nature and quality of lactocrine-active factors found in colostrum. Efforts to identify lactocrine programming mechanisms will contribute to understanding of the postnatal phenome by defining new environmental and epigenetic linkages connecting genotype to phenotype.

Acknowledgments

Authors thank Kathleen K. Gray and Dori J. Miller for assembly of information compiled in [Table 1](#).

References

- Bagnell, C. A., Ho, T. Y., George, A. F., Wiley, A. A., Miller, D. J., & Bartol, F. F. (2017). Maternal lactocrine programming of porcine reproductive tract development. *Molecular Reproduction and Development*, 84, 957–968.
- Bartol, F. F., Wiley, A. A., Spencer, T. E., Vallet, J. L., & Christenson, R. K. (1993). Early uterine development in pigs. *Journal of Reproduction and Fertility Supplement*, 48, 99–116.
- Bartol, F. F., Wiley, A. A., & Bagnell, C. A. (2008). Epigenetic programming of porcine endometrial function and the lactocrine hypothesis. *Reproduction in Domestic Animals*, 43(Suppl. 2), 273–279.
- Bartol, F. F., & Bagnell, C. A. (2012). Lactocrine programming of female reproductive tract development: Environmental connections to the reproductive continuum. *Molecular and Cellular Endocrinology*, 354, 16–21.
- Bartol, F. F., Wiley, A. A., Miller, D. J., Silva, A. J., Roberts, K. E., Davolt, M. L., Chen, J. C., Frankshun, A. L., Camp, M. E., Rahman, K. M., Vallet, J. L., & Bagnell, C. A. (2013). Lactation Biology Symposium: Lactocrine signaling and developmental programming. *Journal of Animal Science*, 91, 696–705.
- Bartol, F. F., Wiley, A. A., George, A. F., Miller, D. J., & Bagnell, C. A. (2017). Physiology and Endocrinology Symposium: Postnatal reproductive development and the lactocrine hypothesis. *Journal of Animal Science*, 95, 2200–2210.
- Baxter, R. C., Zaltsman, Z., & Turtle, J. R. (1984). Immunoreactive somatomedin-C/insulin-like growth factor I and its binding protein in human milk. *Journal of Clinical Endocrinology & Metabolism*, 58, 955–959.
- Bazer, F. W., Spencer, T. E., Johnson, G. A., & Burghardt, R. C. (2011). Uterine receptivity to implantation of blastocysts in mammals. *Frontiers in Bioscience (Schol Ed)*, 3, 745–767.
- Blum, J. W., & Baumrucker, C. R. (2008). Insulin-like growth factors (IGFs), IGF binding proteins, and other endocrine factors in milk: Role in the newborn. *Advances in Experimental Medicine and Biology*, 606, 397–422.
- Bush, W. S., Oetjens, M. T., & Crawford, D. C. (2016). Unravelling the human genome-phenome relationship using phenome-wide association studies. *National Reviews. Genetics*, 17, 129–145.
- Chen, T., Xi, Q. Y., Ye, R. S., Cheng, X., Qi, Q. E., Wang, S. B., Shu, G., Wang, L. N., Zhu, X. T., Jiang, Q. Y., & Zhang, Y. L. (2014). Exploration of microRNAs in porcine milk exosomes. *BMC Genomics*, 15, 100.
- Clare, D. A., & Swaisgood, H. E. (2000). Bioactive milk peptides: A prospectus. *Journal of Dairy Science*, 83, 1187–1195.
- Collado, M. C., Santaella, M., Mira-Pascual, L., Martínez-Arias, E., Khodayar-Pardo, P., Ros, G., & Martínez-Costa, C. (2015). Longitudinal study of cytokine expression, lipid profile and neuronal growth factors in human breast milk from term and preterm deliveries. *Nutrients*, 7, 8577–8591.
- Connolly, J. M., & Rose, D. P. (1988). Epidermal growth factor-like proteins in breast fluid and human milk. *Life Sciences*, 42, 1751–1756.
- Cooke, P. S., Spencer, T. E., Bartol, F. F., & Hayashi, K. (2013). Uterine glands: Development, function and experimental model systems. *Molecular Human Reproduction*, 19, 547–558.
- Donnet-Hughes, A., Duc, N., Serrant, P., Vidal, K., & Schiffrin, E. (2000). Bioactive molecules in milk and their role in health and disease: The role of transforming growth factor-beta. *Immunology & Cell Biology*, 78, 74–79.
- Donovan, S. M., Hintz, R. L., & Rosenfeld, R. G. (1991a). Insulin-like growth factors I and II and their binding proteins in human milk: Effect of heat treatment on IGF and IGF binding protein stability. *Journal of Pediatric Gastroenterology and Nutrition*, 13, 242–253.
- Donovan, S. M., Hintz, R. L., Wilson, D. M., & Rosenfeld, R. G. (1991b). Insulin-like growth factors I and II and their binding proteins in rat milk. *Pediatric Research*, 29, 50–55.
- Donovan, S. M., & Odle, J. (1994). Growth factors in milk as mediators of infant development. *Annual Review of Nutrition*, 14, 147–167.
- Farmer, C., & Quesnel, H. (2009). Nutritional, hormonal, and environmental effects on colostrum in sows. *Journal of Animal Science*, 87, 56–65.
- Frankshun, A. L., Chen, J., Barron, L. A., Ho, T. Y., Miller, D. J., Rahman, K. M., Bartol, F. F., & Bagnell, C. A. (2012). Nursing during the first two days of life is essential for the expression of proteins important for growth and remodeling of the neonatal porcine cervix. *Endocrinology*, 153, 4511–4521.
- Garcia, C., Duan, R. D., Brevaut-Malaty, V., Gire, C., Millet, V., Simeoni, U., Bernard, M., & Armand, M. (2013). Bioactive compounds in human milk and intestinal health and maturity in preterm newborn: An overview. *Cellular and Molecular Biology (Noisy-le-grand)*, 59, 108–131.
- Garofalo, R. (2010). Cytokines in human milk. *The Journal of Pediatrics*, 156, S36–S40.
- George, A. F., Rahman, K. M., Camp, M. E., Prasad, N., Bartol, F. F., & Bagnell, C. A. (2017). Defining age- and lactocrine-sensitive elements of the neonatal porcine uterine microRNA-mRNA interactome. *Biology of Reproduction*, 96, 327–340.
- Grosvenor, C. E., Picciano, M. F., & Baumrucker, C. R. (1993). Hormones and growth factors in milk. *Endocrine Reviews*, 14, 710–728.
- Gu, Y., Li, M., Wang, T., Liang, Y., Zhong, Z., Wang, X., Zhou, Q., Chen, L., Lang, Q., He, Z., Chen, X., Gong, J., Gao, X., Li, X., & Lv, X. (2012). Lactation-related microRNA expression profiles of porcine breast milk exosomes. *PLoS One*, 7, e43691.
- Hanson, M. A., & Gluckman, P. D. (2014). Early developmental conditioning of later health and disease: Physiology or pathophysiology? *Physiological Reviews*, 94, 1027–1076.
- Hata, T., Murakami, K., Nakatani, H., Yamamoto, Y., Matsuda, T., & Aoki, N. (2010). Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. *Biochemical and Biophysical Research Communications*, 396, 528–533.
- Hinde, K., & Milligan, L. A. (2011). Primate milk: Proximate mechanisms and ultimate perspectives. *Evolutionary Anthropology*, 20, 9–23.
- Hinde, K., Skibi, A. L., Foster, A. B., Del Rosso, L., Mendoza, S. P., & Capitano, J. P. (2015). Cortisol in mother's milk across lactation reflects maternal life history and predicts infant temperament. *Behavioral Ecology*, 26, 269–281.
- Houle, D., Govindaraju, D. R., & Omholt, S. (2010). Phenomics: The next challenge. *Nature Reviews Genetics*, 11, 855–866.
- Iacopetta, B. J., Griew, F., Horisberger, M., & Sunahara, G. I. (1992). Epidermal growth factor in human and bovine milk. *Acta Paediatrica*, 81, 287–291.
- Ismail, F. Y., Fatemi, A., & Johnston, M. V. (2017). Cerebral plasticity: Windows of opportunity in the developing brain. *European Journal of Paediatric Neurology*, 21, 23–48.
- Jandal, J. M. (1996). Comparative aspects of goat and sheep milk. *Small Ruminant Research*, 22, 177–185.
- Jenness, R. (1980). Composition and characteristics of goat milk: Review 1968 – 1979. *Journal of Dairy Science*, 63, 1605–1630.
- Jensen RG (1995) Handbook of milk composition (1st edn.) [ebook]. Sandiego: Academic Press.
- Jin, Y., Cox, D. A., Knecht, R., Raschdorf, F., & Cerletti, N. (1991). Separation, purification, and sequence identification of TGF-β1 and TGF-β2 from bovine milk. *Journal of Protein Chemistry*, 10, 565–575.
- Kerner, J., Froseth, J. A., Miller, E. R., & Bieber, L. L. (1984). A study of the acylcarnitine content of sows colostrum, milk and newborn piglet tissues: Demonstration of high amounts of isovalerylcarnitine in colostrum and milk. *Journal of Nutrition*, 114, 854–861.
- Kicheva, A., & Briscoe, J. (2015). Developmental pattern formation in phases. *Trends in Cell Biology*, 25, 579–591.
- Kosaka, N., Izumi, H., Sekine, K., & Ochiya, T. (2010). microRNA as a new immune-regulatory agent in breast milk. *Silence*, 1, 7.
- Kunz, C., Rudloff, S., Baier, W., Klein, N., & Strobel, S. (2000). Oligosaccharides in human milk: Structural, functional, and metabolic aspects. *Annual Review of Nutrition*, 20, 699–722.
- LaKind, J. S., Amina Wilkins, A., & Berlin, C. M., Jr. (2004). Environmental chemicals in human milk: A review of levels, infant exposures and health, and guidance for future research. *Toxicology and Applied Pharmacology*, 198, 184–208.
- Legrand, D., Pierce, A., Ellass, E., Carpentier, M., Mariller, C., & Mazurier, J. (2008). Lactoferrin structure and functions. *Advances in Experimental Medicine and Biology*, 606, 163–194.
- Malekinejad, H., Scherpenisse, P., & Bergwerff, A. A. (2006). Naturally occurring estrogens in processed milk and in raw milk (from gestated cows). *Journal of Agricultural and Food Chemistry*, 54, 9785–9791.

- Malven, P. V., Head, H. H., Collier, R. J., & Buonomo, F. C. (1987). Periparturient changes in secretion and mammary uptake of insulin and in concentrations of insulin and insulin-like growth factors in milk of dairy cows. *Journal of Dairy Science*, 70, 2254–2265.
- Masson, P. L., & Heremans, J. F. (1971). Lactoferrin in milk from different species. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 39, 119–129.
- McGough, I. J., & Vincent, J. P. (2016). Exosomes in developmental signalling. *Development*, 143, 2482–2493.
- Meisel, H. (1998). Overview on milk protein-derived peptides. *International Dairy Journal*, 8, 363–373.
- Modepalli, V., Kumar, A., Hinds, L. A., Sharp, J. A., Nicholas, K. R., & Lefevre, C. (2014). Differential temporal expression of milk miRNA during the lactation cycle of the marsupial tammar wallaby (*Macropus eugenii*). *BMC Genomics*, 15, 1012.
- Munoz, C., Endres, S., van der Meer, J., Schlesinger, L., Arevalo, M., & Dinarello, C. (1990). Interleukin-1 beta in human colostrum. *Journal of Immunology Research*, 141, 505–513.
- Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., Morotta, F., Jain, S., & Yadav, H. (2011). Bioactive peptides derived from milk proteins and their health beneficial potentials: An update. *Food & Function*, 2, 18–27.
- Okada, M., Ohmura, E., Kamiya, Y., Murakami, H., Onoda, N., Tsushima, T., & Shizume, K. (1991). Transforming growth factor (TGF)- α in human milk. *Life Sciences*, 48, 1151–1156.
- Pandya, A. J. (2009). *Bioactive Components in buffalo milk. Bioactive components in milk and dairy products*. Wiley-Blackwell.
- Park, Y. W., & Nam, M. S. (2015). Bioactive peptides in milk and dairy products: A review. *Korean Journal for Food Science of Animal Resources*, 35, 831–840.
- Power, M. L., Schulkin, J., Drought, H., Milligan, L. A., Murtough, K. L., & Bernstein, R. M. (2017). Patterns of milk macronutrients and bioactive molecules across lactation in a western lowland gorilla (*Gorilla gorilla*) and a Sumatran orangutan (*Pongo abelii*). *American Journal of Primatology*, 79, 1–11.
- Read LUP, Francis GL, Wallace JC, Dahlenberg OW, Ballard FJ (1984) Changes in the growth-promoting activity of human milk during lactation. *Pediatric Research* 18, 133–139.
- Reinhardt, T. A., Lippolis, J. D., Nonnecke, B. J., & Sacco, R. E. (2012). Bovine milk exosome proteome. *Journal of Proteomics*, 75, 1486–1492.
- Schanbacher, F. L., Talhouk, R. S., Murray, F. A., Gherman, L. I., & Willett, L. B. (1998). Milk-borne bioactive peptides. *International Dairy Journal*, 8, 393–403.
- Sharp, J. A., Modepalli, V., Enjapoori, A. K., Bisana, S., Abud, H. E., Lefevre, C., & Nicholas, K. R. (2014). Bioactive functions of milk proteins: A comparative genomics approach. *Journal of Mammary Gland Biology and Neoplasia*, 19, 289–302.
- Stockard, C. R. (1921). Developmental rate and structural expression: An experimental study of twins, 'double monsters' and single deformities, and the interaction among embryonic organs during their origin and development. *The American Journal of Anatomy*, 28, 115–277.
- Urashima, T., Saito, T., Nakamura, T., & Messer, M. (2001). Oligosaccharides of milk and colostrum in non-human mammals. *Glycoconjugate Journal*, 18, 357–371.
- Vega, J. R., Gibson, C. A., Skaar, T. C., Hadsell, D. L., & Baumrucker, C. R. (1991). Insulin-like growth factor (IGF)-I and -II and IGF binding proteins in serum and mammary secretions during the dry period and early lactation in dairy cows. *Journal of Animal Science*, 69, 2538–2547.
- Wright, C. E., & Gaul, GE (1983). Nerve growth factor is present in human milk. *Pediatric Research*, 17.
- Zhou, Q., Li, M., Wang, X., Li, Q., Wang, T., Zhu, Q., Zhou, X., Wang, X., Gao, X., & Li, X. (2012). Immune-related microRNAs are abundant in breast milk exosomes. *International Journal of Biological Sciences*, 8, 118–123.

Content and Volume Overview

John R McCarrey, Department of Biology, University of Texas at San Antonio, San Antonio, TX, USA

Wei Yan, Department of Physiology and Cell Biology, University of Nevada, Reno School of Medicine, Reno, NV, USA

© 2018 Elsevier Inc. All rights reserved.

Volume 3 of this encyclopedia covers three critical and sequential topics of reproduction: gametogenesis, fertilization, and development. **Gametogenesis** refers to the production of gametes in each sex. This process is markedly different in males and females, involving spermatogenesis and oogenesis, respectively, leading to the production of very distinct gametes: spermatozoa/sperm and oocytes/eggs. Gametogenesis begins with the specification of primordial germ cells in a similar manner in the early embryo in both sexes, followed by the sexually dimorphic processes of spermatogenesis in the male and oogenesis in the female. Although these processes differ phenotypically, they share common features, including the expression of many genes that are not expressed in any other cell types in the body; for example, meiosis, which is unique to germ cells and leads to the production of haploid gametes, and germ line–specific epigenetic reprogramming that erases most inherited epigenetic marks and resets new epigenetic programming to foster gametogenesis and epigenetic inheritance between generations. Processes that differ between spermatogenesis and oogenesis include the ongoing presence of a gametogenic stem cell—the spermatogonial stem cell (SSC) throughout the reproductive life span—in the male, such that adult human males can produce up to 85 million sperm per day, and the absence of any equivalent ongoing presence of a gametogenic stem cell in the female. Rather, in the female, all of the primordial germ cells do not undergo cell death transition briefly to premeiotic oogonia and then to meiotic oocytes that progress to diplotene of the first meiotic division during the fetal period, and then become quiescent until the time of ovulation of each surviving oocyte. The products of gametogenesis—the spermatozoon in the male and the oocyte/ovum in the female—are dramatically dimorphic in terms of morphology, gene expression, epigenetic programming, ongoing signaling pathways, motility, and several other functions that ultimately contribute to the genesis of functional gametes competent to participate in fertilization.

Fertilization is the process by which the male and female haploid gametes become unified to establish the diploid fertilized egg or zygote. This process follows transport of the paternal genome by the sperm from the male reproductive tract to the female reproductive tract, as well as transport of the maternal genome by the oocyte from the ovary into the Fallopian tubes where fertilization takes place. A complex system normally regulates sperm–egg interactions to ensure proper fertilization of the oocyte by a single sperm while simultaneously blocking the ability of all other sperm to fertilize the egg, thus preventing polyspermy. Fertilization marks the point at which individual gametes cease to exist and a new embryo is generated. This process is associated with significant changes in epigenetic programming associated with both the paternal and maternal genomes, plus recruitment of maternally produced mRNAs and proteins to sustain preimplantation development, and activation of the embryonic genome, such that the embryo can initiate development.

Development is the process by which a fertilized egg or zygote grows into a newborn baby in mammals. This begins with preimplantation development—a process that is largely intrinsically regulated within the early embryo on the basis of maternal factors present in the ooplasm, plus new macromolecules generated by gene expression in the early embryo. The initial fertilized egg divides to yield multiple cells in a structure termed a morula, which then continues to grow by cell division and cellular differentiation, as well as cavitation to form the blastocyst. The blastocyst consists of an outer layer of trophoblast cells that will form the extraembryonic structures such as the chorion and the allantois, plus an inner cell mass that will give rise to the embryo proper and, eventually, to the newborn offspring. The zygote is considered to be a totipotent cell because it possesses the potential to form all cell types, including embryonic and extraembryonic cell types. The trophoblast cells rapidly lose this potential, such that they can only form extraembryonic structures, while the inner cell mass are “pluripotent,” meaning they can produce any cell type in the embryo proper.

At the blastocyst stage the embryo implants into the uterine wall, which marks the transition from preimplantation development to postimplantation development. The postimplantation embryo undergoes organogenesis to form the fetus. In the fetus, tissues and organs continue to grow and differentiate so that they can eventually function independently following birth. Each somatic cell lineage develops on the basis of cells sensing their position within the embryo and activating gene expression patterns that will facilitate development of proper structures or organs in proper places within the body. This is associated with unique epigenetic programming and differential gene expression in each.

In addition to all of the somatic cell lineages, the germ line becomes reestablished as the earliest specific cell type allocated within the embryo proper. This marks the beginning of gametogenesis in the new generation, which will start the gametogenesis–fertilization–development cycle over again.

References

Gametogenesis

Wassarman, P. M. (2013). Gametogenesis. *Current Topics in Developmental Biology*, 102.

Fertilization

Gwatkin, R. (2012). *Fertilization mechanisms in man and mammals*. New York City, NY: Springer Science & Business Media.

Development

Tam, P. P. L., Nelson, W. J., & Rossant, J. (2013). *Mammalian development*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

GAMETOGENESIS

Gametogenesis Overview

John R McCarrey, University of Texas at San Antonio, San Antonio, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Gametogenesis

As the name implies, gametogenesis refers to the process by which the gametes—oocytes or eggs in females and spermatozoa or sperm in males—are generated. The gametes are the final, differentiated product of the germ line in each sex. However, unlike all other differentiated cells present in the adult, the gametes do not represent terminally differentiated cell types because they participate in fertilization to give rise to an entirely new individual. In this regard, it has been suggested that the germ line is essentially immortal. The gametes are also the only haploid cell type found in the adult. This is because the developing germ cells undergo the process of meiosis which includes one round of DNA replication followed by two rounds of cytokinesis or cellular division. Upon fertilization, the union of the two haploid gametes (sperm + egg) restores diploidy in the zygote.

Development of the Germ Line

The germ line is the first individual cell type allocated during development of the embryo proper. In mammals, the first manifestation of the germ line in each sex is the formation of primordial germ cells (PGCs) at about the stage of gastrulation in the embryo. This process is common to germline development in both the male and female and at this stage the PGCs appear identical in each sex. The PGCs arise in the epiblast and subsequently migrate to the site of the developing genital ridges that will eventually give rise to the gonads. As with the PGCs, the early stages of gonadal development are not significantly different in males and females, although subsequent to this stage both the gonads and the germ cells undergo sexually dimorphic development. The developing gonads are pre-programmed to form ovaries in (XX) females, but expression of the Y-linked *Sry* gene in developing Sertoli cells in the testes in (XY) males induces the indifferent, bipotential gonad to develop into a testis instead of an ovary. Once this gonadal sex differentiation is initiated in the somatic cells of the developing gonads in each sex, the previously indifferent PGCs are induced to initiate sex-specific gametogenesis. Thus, PGCs in a developing ovary are induced to enter the oogenic pathway, whereas those in a developing testis are induced to enter the spermatogenic pathway. It is the sexual phenotype of the somatic elements of the developing gonad that directs the initial choice of which sex-specific gametogenic differentiation pathway the PGCs will enter, regardless of the genetic sex of the PGCs. Normally, genotypically female XX PGCs will be present in a phenotypically female developing ovary and will be induced to initiate oogenesis, whereas genotypically male XY PGCs will be present in a phenotypically male developing testis so will be induced to initiate spermatogenesis. However, even when XY PGCs are present in a developing ovary, they will be induced to initiate oogenesis, just as XX PGCs present in a developing testis will be induced to initiate spermatogenesis. Nevertheless, although the sex-specific somatic phenotype of the developing gonad directs the sex-specific initiation of either oogenesis or spermatogenesis, the ability of the germ cells to complete gametogenesis by forming either functional oocytes or sperm is limited by their sex-specific genotype, such that XY germ cells do not normally produce functional oocytes (though this has been reported on occasion) and XX germ cells never produce functional spermatozoa.

The first manifestation of sexually dimorphic gametogenesis is the entry by oogenic cells into prophase of meiosis I during fetal development while at this same stage spermatogenic cells remain mitotic and do not enter meiosis until well after birth in association with puberty. In both males and females, entry of germ cells into meiosis is stimulated by retinoic acid (RA) produced by gonadal somatic cells. This takes place during the fetal stages in both the developing ovary and the developing testis. However, in the fetal testis, there is also production of an inhibitor of RA (cytochrome P450 26B1 encoded by the *Cyp26b1* gene) which effectively blocks the meiosis-inducing signaling function of RA, and so prevents the induction of entry into meiosis by germ cells in the fetal testis. Prior to and at the time of puberty in the postnatal testis, RA is again produced by somatic cells in the pubertal testis in the absence of any simultaneous production of cytochrome P450 26B1, resulting in induction of entry of spermatogonia into meiosis at this time.

In response to production of RA in the fetal ovary, PGCs enter first meiotic prophase and progress to the diplotene stage. But they then suspend further progression through subsequent phases of meiosis and remain meiotically quiescent until stimulated to resume progression through meiosis upon ovulation in the adult. This prolonged suspension of meiotic progression at the stage when homologous chromosomes have paired on the first meiotic metaphase plate is the source of the marked increase in aneuploid offspring of older mothers due to non-disjunction of the paired homologues.

At the same time when female germ cells are entering into meiosis followed by entry into meiotic quiescence, fetal male germ cells remain mitotic, but also enter a state of mitotic quiescence. This quiescence is more transient than that observed in developing oocytes, lasting only until resumption of expansion of the spermatogonial pool in the immature, postnatal testis.

Nevertheless, it is very intriguing to note that both female and male germ cells enter a stage of quiescence during the latter portion of fetal development. This period of quiescence corresponds to a period of genome-wide epigenetic reprogramming unique to the developing germ line. Whether or not proliferative quiescence is necessary to facilitate germ line-specific epigenetic reprogramming is not known.

Postnatal Gametogenesis

Another striking dimorphism in the manner in which gametogenesis takes place in females and males, respectively, is that the cohort of primary oocytes that develops during the fetal period represents the entirety of the lifelong oogenic pool under normal circumstances in the female. It appears that all of the developing female germ cells enter first meiotic prophase during the fetal period, after which they retain the ability to complete the meiotic divisions, but are not able to further propagate their numbers, thereby limiting the oogenic pool to those primary oocytes that are present at birth. Indeed, many of the early primary oocytes undergo cell death, thus further limiting the pool of oocytes available during the reproductive lifespan of the female. Typically, exhaustion of the primary oocyte pool correlates with menopause in female mammals.

In contrast to the developmental dynamics of oogenesis, the dynamics of spermatogenesis are quite distinct. In the fetal testis, male PGCs develop into prospermatogonia that continue to divide mitotically, thus increasing their numbers significantly. After birth, these mitotically expanded prospermatogonia can either give rise directly to progenitor and/or differentiating spermatogonia, or they can enter a cell death pathway, or, in the case of a small subpopulation of the prospermatogonial pool, they can form spermatogonial stem cells (SSCs). SSCs are unique in that they can either self-renew or proceed through the spermatogenic differentiation pathway. The SSCs are the only cell type in the entire spermatogenic lineage capable of undergoing self-renewal. To accomplish both maintenance of the SSC pool and contribution of cells that will continue through the spermatogenic pathway, the SSCs must undergo, directly or indirectly, an asymmetric division whereby a portion (likely half) of the mitotic progeny of SSCs retain SSC identity and function, while the other portion become committed to entering the spermatogenic pathway. The latter progenitor spermatogonia undergo further mitotic expansion before initiating the spermatogenic pathway as differentiating spermatogonia.

Meiosis

Meiosis is a critical, unique feature of gametogenic cell lineages. Meiosis facilitates multiple beneficial outcomes. Meiotic recombination generates unique rearrangements of the paternal and maternal genomes prior to fertilization. In addition, meiosis achieves a reduction division that yields haploid eggs or sperm that can then re-establish diploidy upon the union of a sperm and egg at fertilization. However, the manner in which meiosis is achieved is also dimorphic during female and male gametogenesis. As noted above, during fetal development in females, all PGCs give rise to oogonia that then proceed into first meiotic prophase. From this stage on, there are no premeiotic (mitotic) female germ cells, and there is therefore no subsequent opportunity to expand the oogenic cell pool beyond completion of the two meiotic divisions. As a result, females possess their entire lifetime supply of germ cells when they are born and this pool will then be steadily reduced by ovulation or cell death throughout the remainder of the female's reproductive lifespan. In contrast, the mitotic SSCs persist throughout the reproductive lifespan of the male and, in addition to sustaining the SSC pool by self-renewal, give rise to progenitors and then differentiating spermatogonia that then progress into meiosis during each succeeding wave of spermatogenesis in the testis.

Albeit on the basis of very different developmental schedules, germ cells progress through the same stages of meiosis in both males and females. This includes an initial round of DNA replication, followed by the various stages of first meiotic prophase (preleptotene, leptotene, zygotene, pachytene, and diplotene) and completion of the first meiotic division as primary oocytes or spermatocytes followed by completion of the second meiotic division as secondary oocytes or spermatocytes prior to formation of the haploid products of meiosis—the ovum in the female and the spermatids in the male. These steps occur as distinct stages during spermatogenesis as cells progress from premeiotic spermatogonia to meiotic primary spermatocytes and then secondary spermatocytes to postmeiotic round and then elongating spermatids to spermatozoa. During oogenesis, these steps occur in rapid succession following resumption of first meiotic prophase in the female, and are triggered by ovulation and fertilization.

Postmeiotic Gametogenesis

The completion of the first and second meiotic divisions is very disproportionate in oocytes, yielding the very small first and second polar bodies while leaving the large majority of the ooplasm intact in the ovum. Thus, only one of the four products of female meiosis yields a functional gamete, but that one gamete will contain abundant maternal nutrients and factors that will sustain preimplantation embryogenesis following fertilization. In the male, all four products of meiosis emerge as round spermatids that then undergo the differentiative process of spermiogenesis to progress from small, round haploid cells to elongating spermatids and finally to the highly differentiated spermatozoa, all in the absence of any further cellular division. The resulting testicular spermatozoa are then released from the seminiferous epithelium by the process of spermiation and are then transported through the lumen of the seminiferous tubule to the rete testis which serves as a collection area prior to transport of spermatozoa out of the testis and into the epididymis via the efferent ducts. The spermatozoa then traverse the various sections of the epididymis, including the caput, corpus, and cauda regions before moving into the vas deferens from where they are available for release from the male via ejaculation. Transit of the testicular spermatozoa through the epididymis is critical to the acquisition of motility and fertility, yielding mature spermatozoa competent to participate in fertilization.

The Gametogenic Transcriptomes

Unique patterns of gene expression are required to facilitate the unique gametogenic processes of oogenesis and spermatogenesis. Because the gametes are, in many ways, distinct from any of the somatic cell types, there are numerous oogenesis- or spermatogenesis-specific members of gene families, and/or variants of mRNAs (e.g., splicing variants) or proteins (e.g., post-translational modifications). The transcriptomes associated with oogenesis and spermatogenesis are highly complex and largely unique, rivaled only by the brain in these respects. Coordination of expression of these unique transcriptomes is facilitated by many gametogenesis-specific regulatory factors including oogenesis- or spermatogenesis-specific transcription factors, chromatin remodeling factors, and components of other regulatory networks.

Germline-Specific Epigenetic Reprogramming During Gametogenesis

In addition to undergoing meiosis common to both oogenesis and spermatogenesis, as well as the sex-specific differentiation patterns associated with oogenesis or spermatogenesis to yield the egg (ovum) or spermatozoon (sperm), respectively, the germ cells also undergo a unique process of epigenetic reprogramming coincident with the process of gametogenesis in each sex. As noted above, germ cells are unique in that they undergo extensive differentiation to yield the male and female gametes, but these gametes then participate in fertilization to give rise to a complete new individual in which all cell types will subsequently re-emerge. Thus, distinct regulatory mechanisms are required to (1) direct the gametogenic differentiation processes and (2) prepare the gametogenic genomes to contribute to development of the embryo following fertilization. This is achieved by reprogramming of the epigenome in both the preimplantation embryo and the developing germ lines.

Embryonic reprogramming is achieved by erasing a majority of the epigenetic programming that is inherited via the gametes at the time of fertilization. This is followed by resetting of epigenetic programming that is common to the precursors of both somatic and germline cells and is complete by the time of gastrulation in the developing embryo. Subsequently, all somatic cell lineages retain the majority of this reset epigenetic programming. However, a second round of germline-specific epigenetic programming is initiated in the PGCs in which extant epigenetic programming is once again erased—to an even greater extent than the erasure that occurs in the preimplantation embryo, and this is followed by yet another round of resetting of epigenetic programming that follows distinct patterns in oogenic and spermatogenic cells, respectively. This germline-specific epigenetic reprogramming facilitates the processes of gametogenesis in both sexes, including meiosis and either oogenesis or spermatogenesis, as well as the preparation of the gametogenic genomes to direct subsequent early embryonic development following fertilization.

Maintenance of Enhanced Genetic Integrity During Gametogenesis

A critical function of the germ line that must be maintained during gametogenesis in each sex is the integrity of the gametogenic genomes, such that the genetic information transmitted from one generation to the next is preserved in an optimally pristine state. While a relatively small amount of genetic variation among individuals and between generations is beneficial, most spontaneous or induced genetic variation is not advantageous. Therefore it is not surprising that genetic integrity is maintained more stringently and faithfully in the germ line than in any somatic cell lineage. This is achieved by elevated levels of expression and corresponding activity of multiple cell death and DNA repair pathways. The former are typically induced in response to the occurrence of large scale genetic defects or damage such as polyploidy, aneuploidy, major chromosomal rearrangements or extensive DNA damage genome wide, thus dealing with these defects retrospectively. However, more minor genetic defects such as point mutations which are the most common disease-related genetic defects do not typically induce cell death and can only be minimized by prospectively preventing their occurrence. Enhanced DNA repair activities serve to reduce the initial occurrence of point mutations by increasing the likelihood that initial DNA damage will be corrected prior to becoming stabilized as a heritable point mutation. These elevated levels of cell death and DNA repair activities exemplify a situation in which a long-term evolutionary benefit offsets the evolutionary expense of the elevated investment of additional energy required to achieve the beneficial outcome.

Further Reading

Gametogenesis

Wassarman, P. M. (2013). Gametogenesis. *Current Topics in Developmental Biology*, 102, 243–266.

Germ Cells

Wolstenholme, G. E. W. (Ed.). (2008). *Mammalian germ cells*Ciba Foundation Symposium. Novartis Foundation Symposia. <https://doi.org/10.1002/9780470718841>.

Meiosis

Benavente, R., & Volff, J.-N. (2009). *Meiosis*. Basel, Switzerland: Karger Medical and Scientific Publishers.

Epigenetics

Allis, C., Caparros, M.-L., Jenuwein, T., & Reinberg, D. (Eds.). (2015). *Epigenetics* (2nd edn). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

SPERMATOGENESIS

Meiosis Overview

Paula E Cohen, Cornell University, Ithaca, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction and History of Meiosis

Meiosis is the specialized cell division process that takes a diploid precursor cell and from it generates up to four haploid cells for sexual reproduction in eukaryotes. In mammals, the haploid products of meiosis are referred to as gametes: the spermatozoa of males, and the oocytes (or eggs) of females. Meiosis differs from the mitotic cell division in many respects, most notably in that it is comprised of two divisions preceded by only a single phase of DNA replication. While many other differences exist, much of the generalized mitotic machinery is conserved in meiosis, and this conservation is also apparent across organisms and between sexes.

Meiosis was first observed in sea urchin eggs in 1876 by German biologist, Oscar Hertwig. A decade later, Belgian zoologist, Edouard Van Beneden, described a similar process in the eggs of the roundworm, *Ascaris*. It was not until the 1890s, however, that August Weismann noted the importance of the two cellular division events for generation of haploid gametes for fertilization. The term *meiosis*, derived from the Greek word for “lessening” was coined in 1905 by Farmer and Moore to reflect the halving of the chromosomes. These early observations preceded the rediscovery of Mendel’s work by Hugo de Vries and others in the 1900s, and culminated in 1911 with the discovery by Thomas Hunt Morgan of crossing over, the exchange of genetic information between homologous chromosomes as a result of recombination, a key and essential feature of meiosis.

In order to derive haploid daughter cells following a single round of DNA replication, meiosis consists of two distinct segregation events: meiosis I and meiosis II. Both meiotic divisions consist of prophase, metaphase, anaphase, and telophase (Fig. 1), but whereas the first division is considered equational (maintaining the number of genome copies, or ploidy, of the daughter cells), the second is reductional (halving the ploidy of the daughter cells), and thus very similar to mitosis. During meiosis I, homologous chromosomes, derived from each parent (maternal and paternal), must first find each other and pair, in order to then be segregated equally into two daughter cells. Like mitosis, meiosis II involves separation of sister chromatids, which are connected through their cohesin rings, the protein complexes that maintain sister chromatid interactions through the cell cycle. These sister chromatids are segregated to opposite poles prior to cytokinesis, resulting in the halving of the genome content. The fact that sister chromatids are already paired makes mitosis and meiosis II fairly easy to orchestrate: a simple separation of the chromatids following dissolution of the cohesin ring. For meiosis I, however, the homologous chromosomes are not usually tethered together, and thus, they must undergo a fascinating, and relatively poorly understood process of homology searching, pairing and then physical tethering, in order to allow them to be segregated equally at the first meiotic division. Thus, it is meiosis I, involving homologous chromosome interactions, that represents the unique and defining phase of meiosis.

General Scheme of Meiotic Progression in Eukaryotes

While differences exist across species in the functional regulation of meiosis, the staging and progression of meiotic events follows a generally similar pattern as that of mammals, described here for male mammals, with particular emphasis on what is known in the laboratory mouse. Following a set and species-specific number of mitotic divisions, the spermatogonia of the adult testis are triggered to enter meiosis predominantly through the actions of retinoic acid (RA). RA-responsive genes, including *STRA8*, promote premeiotic S-phase, which doubles the genetic content of the cell. These cells are now known as 2N, because they contain two copies of every chromosome, and 4C, because they contain a total of four sister chromatids (or four copies of the genome). These cells then enter prophase I of meiosis, which is by far the longest stage, lasting up to 10 days in male mice. Prophase I can be further subdivided into five distinct substages (Fig. 2): *Leptonema* (adjective: leptotene), *Zygonema* (adjective: zygotene), *Pachynema* (adjective: pachytene), *Diplonema* (adjective: diplotene), and *Diakinesis*. During these stages, homologous chromosomes, each consisting of paired sister chromatids must first find each other, pair, and then undergo a physical tethering process known as *synapsis*. This tethering is achieved by the accumulation of a set of proteins that comprise a scaffold structure along and between homologous chromosomes that are known collectively as the *synaptonemal complex* (SC). The sequential accumulation of the SC defines the substages of Prophase I and goes hand-in-hand with the second defining feature of this stage: *meiotic homologous recombination* (HR). Meiotic recombination results in the formation of crossovers, which are regions of exchanged chromosome length between the parental chromosomes. The cytological manifestation of crossing over are the *chiasmata*, and these serve to tether homologous chromosomes together until the first meiotic division. SC formation and HR are intricately intertwined events, the latter being initiated first and seeding the formation of the former (see below).

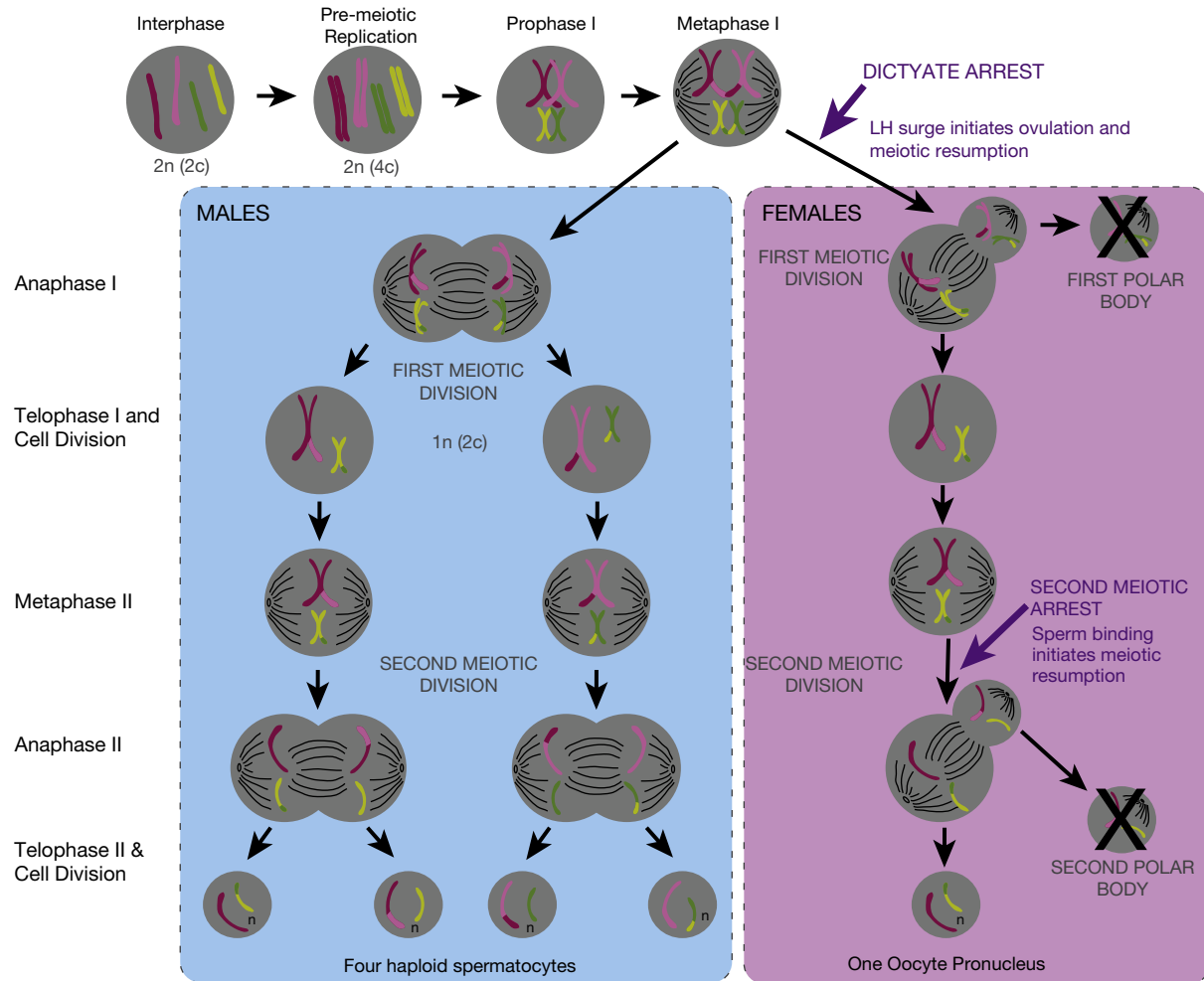


Fig. 1 Overview of mammalian meiosis. A representative cell is shown containing two pairs of homologous chromosomes (red/pink and green/yellow). At interphase, these cells are diploid, containing 2n and 2c content. “n” refers to the number of homologous chromosomes and “c” refers to the number of chromatids and/or the number of copies of each gene locus. After DNA replication, therefore, the cell is still 2n, but is now 4c (four copies of each chromatid, two for each chromosome). After the first meiotic division, when homologous chromosomes have divided, the daughter cells (one secondary spermatocytes or one secondary oocyte) are now 1n, 2c, containing only one homologous chromosome, but two sister chromatids. By the end of the second division, this chromosome content is reduced further to 1n, 1c. Note that only one cell emerges at the end of meiosis in females, the remaining genetic content being expelled in the first and second polar bodies. Modified from Chapter 1, *Physiology of Reproduction*, eds. Knobil and Neil (2013).

The outcome of prophase I is the tethering of homologous chromosomes, firstly through SC-mediated protein:protein interactions and then through HR-mediated DNA:DNA interactions, the latter which much then be appropriately processed and distributed to result in a precisely determined cohort of chiasmata. These chiasmata persist into metaphase I when, in conjunction with the cohesin rings which encircle the sister chromatids across their entire lengths, they allow for accurate tension to be established on the meiosis I spindle. This tension is crucial for ensuring that homologs segregate equally to opposite poles of the cell at anaphase I. Homolog segregation is facilitated by the resolution of chiasmata to form intact and appropriately recombined homologous chromosomes, as well as by the specific release of cohesion along chromosome arms, but not at the centromere. The maintenance of centromere cohesion at the first meiotic division ensures that sister chromatids remain together through until meiosis II.

The segregation of homologous chromosomes at the first meiotic division results in two daughter cells that are each now 2C and N in terms of genomic content, containing one of each chromosome, with each chromosome consisting of two sister chromatids (or two copies of the genome, hence the “equational” division). Each of these cells will undergo a mitosis-style division, culminating in segregation into two daughter cells each (for a total of four daughter cells). These final products, termed *gametes* in mammals, or *meiospores* in fungi/plants, are now 1C (one copy of the genome) and 1N (one chromosome) in total genomic content, the haploid products of a reductional division. In males, these gametes will now enter the postmeiotic differentiation stages of spermatogenesis as newly-formed spermatids.

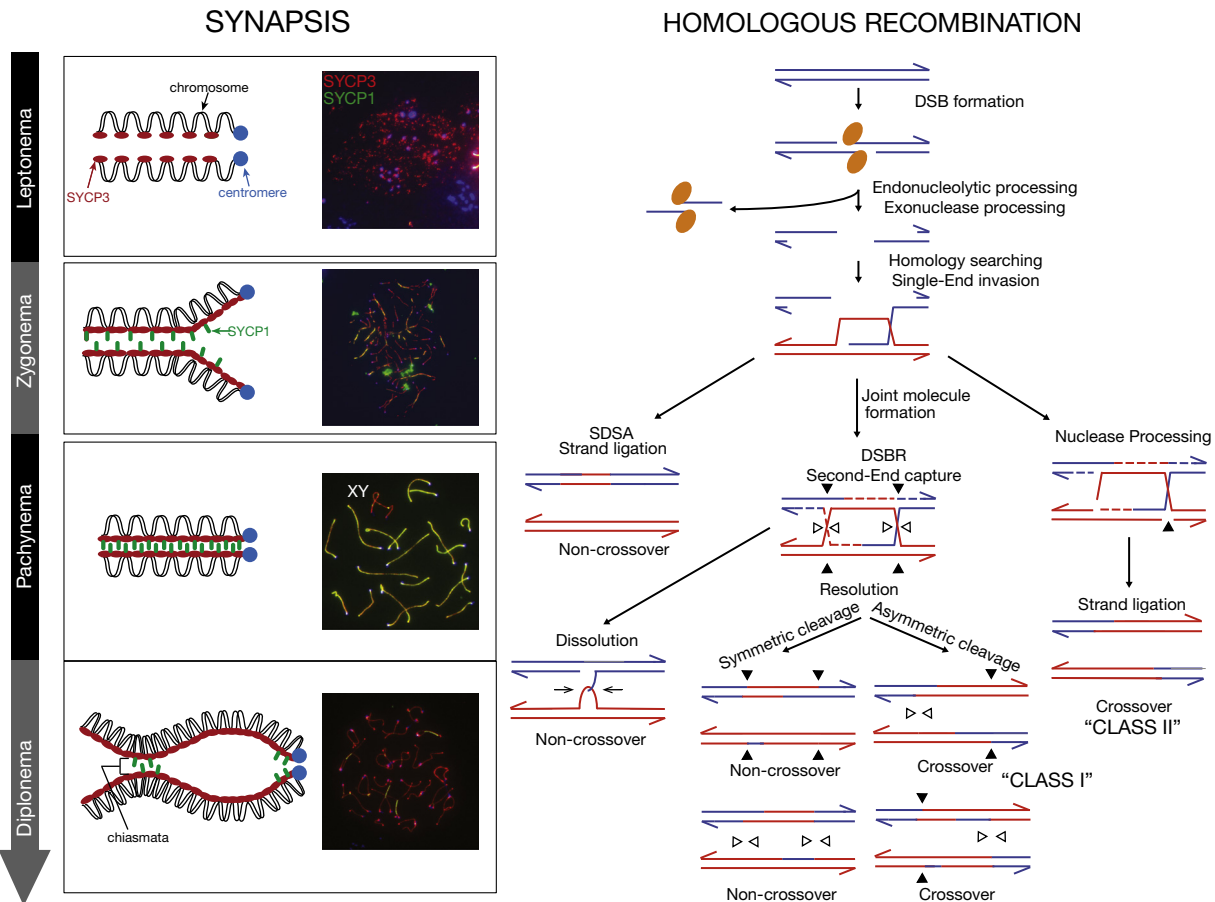


Fig. 2 Overview of prophase I and alignment of the events of synapsis (left) with that of recombination (right). Synapsis is depicted in cartoon form (left) and with representative fluorescence micrographs (right) showing mouse spermatocytes in prophase I. The SC lateral element protein, SYCP3 is shown in red, and the central element protein, SYCP1, in green, at each of the first four stages of prophase I. The last stage, diakinesis, is not shown because SC proteins are no longer present at this time. Note that the staining depicts proteins only; the DNA is distributed throughout the field as loops emanating away from the SC structure. The right panel depicts DSB formation and repair through one of several pathways. At least two pathways give rise to non-crossovers (SDSA and Dissolution), and at least two pathways give rise to crossovers (Class I and Class II) and the approximate timing of the appearance of these products is related to the vertical scale of prophase I stages on the left. Modified from Gray and Cohen, *Annual Review of Genetics* (2016).

Female meiosis follows a similar profile to that of males, but with several exceptions. Most notably, meiosis occurs in a synchronous fashion amongst all oocytes during embryonic development (described later). Additionally, at meiosis I when the primary oocyte has divided, only one set of homologous chromosomes become encapsulated in a secondary oocyte. The remaining chromosomes are discarded in a polar body that degenerates over several hours. At the second meiotic division, when sister chromatids are segregated, again only one set of sister chromatids become encapsulated in the final oocyte, and the remaining genetic material is again expelled as the second polar body. Thus, female meiosis results in the production of only one oocyte.

Regulation of Prophase I Events: Meiotic Homologous Recombination

As described above, Prophase I is the defining feature of meiosis and by far the longest phase. Upon entry into leptonema, homologous chromosomes are long and uncompacted and must first initiate meiotic HR and undergo the intricate and poorly understood process of homology recognition and pairing. The first key event in leptonema, therefore, is the induction of hundreds of DNA double strand break (DSB) across the genome, mediated by the topoisomerase VI subunit A-like protein, SPO11, in conjunction with its recently-identified B-type subunit, TopoVIB-like (TOPOVIBL) (Keeney et al., 1997; Grelon, 2016). Together, SPO11 and TOPOVIBL act with several co-factors to induce DSB formation at hundreds of sites across the genome, with some 250 DSBs formed in mouse meiosis, and over 600 DSBs formed during human meiosis. Of these, only 10% will become the crossovers (CO) that are needed to ensure homologous chromosome interactions, while the remaining DSBs will be processed as noncrossovers (NCO) in which flanking DNA sequence is not exchanged between homologs, and thus will not promote interhomolog connections (Fig. 2).

Importantly, all of the DSBs must be repaired in an accurate and temporally defined manner, both to ensure the correct distribution of COs across the genome, but also to prevent incurring a DNA lesion in the resulting gamete.

Regardless of the final outcome of DSB formation (CO or NCO), DSB events are processed in a similar fashion for the early events of DSB repair (Hunter, 2015). The DSB becomes resected to form 3' overhangs on either side of the DSB and these become stabilized by replication protein A (RPA). Two distinct RecA homologs, RAD51 and meiosis-specific DMC1, then associate with the overhang and these proteins promote strand invasion, also known as *single end invasion*, into closely apposed chromosomes. In doing so, RAD51 and DMC1 promote strand displacement in the opposing chromosome, and thereby mediate a homology search process that culminates with the complete pairing of homologous chromosomes by late zygonema/early pachynema. Thus, regardless of the outcome of the DSB repair process, the induction of many DSBs is thought to be critical for homology searching and recognition.

Following single end invasion, the major repair pathway is thought to result exclusively in NCO events, and may involve up to 90% of the original DSBs. This pathway, known as *synthesis-dependent strand annealing* (SDSA), and involves first extension, and then rejection of the invading DNA strand, followed by reengagement/annealing with the original opposing end of the DSB (McMahill et al., 2007). Following further DNA synthesis to repair all the gaps in the strand caused by the original DSB processing, the resulting repair is now termed an NCO because of the lack of exchange of flanking DNA sequence between DNA strands.

As an alternative to SDSA, following single end invasion, the free end of the single strand is captured at the other side of the DSB, resulting in a D-loop structure that can be processed in multiple ways, depending on the DNA repair pathway that is invoked. In terms of COs, the major pathway is the ZMM pathway (or Class I pathway), named for the proteins that regulate these events in budding yeast (Fig. 2). These include the DNA mismatch repair proteins, MutS homologs-4 and -5 (MSH4 and MSH5), collectively termed MutS γ , which associate with a subset of DSB repair events, driving them towards a CO fate. Importantly in mammals, however, not all of the MutS γ -defined intermediates become COs since the number of MSH4 or MSH5 foci found on the chromosomes is considerably higher than the final tally of COs, suggesting that a cohort of these intermediates is destined to become NCO events (Kneitz et al., 2000). The current understanding is that these CO-destined MutS γ sites are stabilized progressively by proteins that invoke posttranslational modifications. These so-called CO-licensing factors may include the SUMO E3 ligase, RNF212 (Reynolds et al., 2013). Final *crossover designation* is achieved by the accumulation of the DNA mismatch repair heterodimer of MLH1 and MLH3, collectively termed MutL γ , which associates with only those MutS γ sites that will become COs (Lipkin et al., 2002; Edelmann et al., 1996). Crossover designation is orchestrated by other factors, including the cyclin family member, cyclin N-terminal domain containing-1, CNTD1, and by the Ubiquitin E3 ligase, Human Enhancer of Invasion-10, HEI10 (Qiao et al., 2014; Holloway et al., 2014).

As alluded to above, the distribution of COs (and therefore, also of NCOs) must be tightly regulated during prophase I to conform to several rules. Firstly, *crossover assurance* is the process by which an *obligate crossover* is present on every chromosome, ensuring accurate homolog engagement through until the first meiotic division. Secondly, the placement of one CO reduces the likelihood of a second nearby chromosome, in a process called *crossover interference*, ensuring that no one stretch of chromosome receives too many or too few COs. Finally, in a process termed *crossover homeostasis*, the number of DSBs can be reduced many fold at the expense of NCOs, while COs are retained at their optimal level. Thus CO regulation encompasses all of these requirements and must be orchestrated within the complex DSB repair mechanisms that also ensure complete repair of the entire DSB cohort through prophase I.

Regulation of Prophase I Events: Synaptonemal Complex Formation

Concurrent with DSB induction is the onset of synapsis, in which the synaptonemal complex (SC) progressively tethers the two homologous chromosomes together (Cahoon and Hawley, 2016) (Fig. 2). The synaptonemal complex was first visualized by Moses and Fawcett in 1956, and consists of two axial/lateral elements that align each homolog and the central element that resides between the two homologs. Transverse filaments, which have one end anchored in the lateral element and the other in the central element, function to “zip” the two homologs together during early prophase I, in the process that is intricately linked to the homologous recombination events taking place concurrently with synapsis. In mouse and other organisms, DSB induction precedes SC assembly while in *Drosophila melanogaster*, SC assembly initiates in advance of DSB formation (McKim et al., 1998). In the former, it has been postulated that DSB repair creates single stranded overhangs that are required to initiate homolog interactions leading to synapsis, perhaps explaining why so many more DSBs are generated than are actually required for crossing over. Interestingly, though most meiotic species exhibit synapsis (there are exceptions to this rule), and while the overall structure of the SC is conserved, the proteins that comprise the SC vary quite markedly. This suggests that different organisms have evolved distinct mechanisms to create the same structure.

In mice, the initiation of synapsis is dependent on the 250–300 DSBs that form, such that, any reduction in DSB formation leads to delayed SC assembly. The importance of the SC in mouse is demonstrated by the fact that loss of one or more SC proteins, such as the axial element protein SYCP3, or the central element protein, SYCP1, leads to prophase I disruption (Yuan et al., 2000, 2002). Other proteins also participate in this process, including the HORMA-domain containing proteins, HORMAD1 and HORMAD2 (Wojtasz et al., 2009; Shin et al., 2010). These two proteins associate exclusively with unsynapsed axes, and their presence is essential for normal DSB repair, resulting in an inextricable co-dependence between synapsis and homologous recombination (Shin et al., 2010; Fukuda et al., 2010).

The First Meiotic Division

Upon completion of prophase I, the SC has broken down, and crossing over has taken place. Upon entry into metaphase I, the homologous chromosome pairs line up along the mid-plate of the cell, the orientation of maternal/paternal chromosomes toward each pole being random. Homologous chromosomes must attach to the spindle in a bi-orientated manner to ensure that homologous chromosomes separate, while sister chromatids adopt a co-orientation to ensure that they remain together. Thus, unlike mitosis and the second meiotic division (MII), where the kinetochores of the sister chromatids undergo *amphitelic attachment* to the bipolar spindle (attaching to opposing poles of the spindle, thus called *bi-polar* attachment) to ensure equal segregation thereof, during the first meiotic division (MI), the kinetochores of the sister chromatids must attach in a *syntelic* fashion (attaching to the same pole, called *mono-polar attachment*) in order to ensure that they segregate together to the same pole.

At MI, bi-orientation of homologs is achieved by a combination of the presence of cohesin rings along the arms of the chromosomes and at the centromeres, together with the physical exchange of DNA at the CO sites. In this respect, it is not the CO alone that is important, but the overall tension achieved by the interlocked homologs that have exchanged DNA sections but which still retain the original cohesin complexes that exist along each homologous chromosome.

Cohesin removal at metaphase I is distinct from that of mitotic metaphase in the requirement for a specific sequential loss of cohesion along chromosome arms the first meiotic division (MI) followed by loss of centromeric cohesion at the second meiotic division (MII). The loss of cohesins along chromosome arms is necessary to achieve accurate segregation of the newly-recombined homologous chromosomes. However, in order to maintain sister chromatid interactions through until the second meiotic division, it is necessary to facilitate the retention of cohesin complexes at the centromere. Thus, at MI, arm cohesion is lost through two distinct mechanisms. Firstly, a nonenzymatic *Prophase I* pathway involving the Wings Apart-Like protein, WAPL, promotes opening of the cohesin ring at part of a phosphorylation cascade involving NIMA-like kinase-1 (NEK1) and protein phosphatase-1 γ (PPI γ) (Bri  o-Enr  quez et al., 2016). As a second mechanism, the phosphorylation of the kleisin subunit of the cohesin ring, REC8, leads to its subsequent cleavage by the enzyme, Separase, but only after the latter has been released by its negative regulator, securin (Fig. 3). Meanwhile, centromeric REC8 is protected from the actions of Separase by the recruitment of phosphatase 2A (PP2A) to the centromere by Shugoshins 1 and 2 (SGO1/2). PP2A prevents Separase-mediated cleavage of the cohesin ring by dephosphorylation of REC8.

The bi-polar orientation of homologs at MI and of sister chromatids at MII, establishes tension across the entire spindle structure that is critical, not only for accurate segregation, but also as a sensing mechanism to ensure that the conditions are met to facilitate the metaphase-to-anaphase transition. This so-called *spindle assembly checkpoint* (SAC) monitors bi-polar attachment and prevents further progression of the cell cycle until stable kinetochore attachment to the spindle. Once these conditions have been met, release of the SAC allows for the activation of the *anaphase promoting complex/cyclosome* (APC/C) and binding to CDC20 (Fig. 3). APC/C, an ubiquitin E3 ligase, targets several proteins for destruction through polyubiquitylation, including

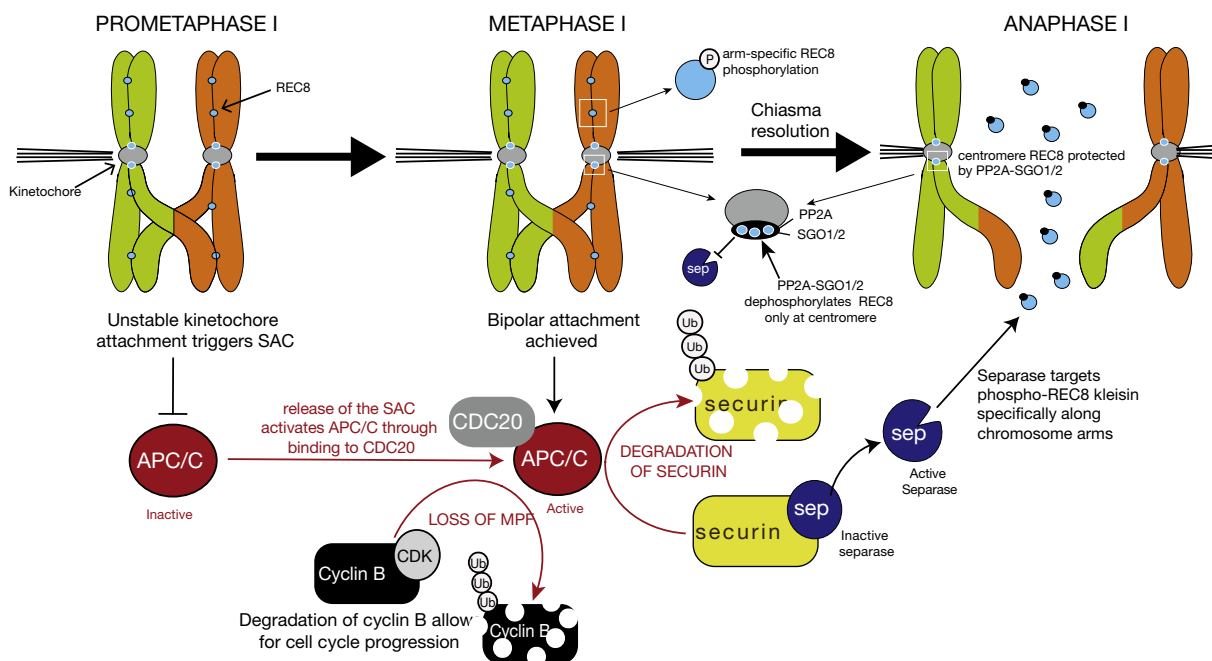


Fig. 3 The spindle assembly checkpoint and progression through the first meiotic division. Cartoon depiction linking SAC activation and monitoring to chromosome segregation at MI. Modified from Chapter 1, *Physiology of Reproduction*, eds. Knobil and Neil (2013).

the Cyclin B component of *maturation promoting factor* (MPF), and Securin. Destruction of Securin, in turn releases the enzyme Separase, allowing the latter to cleave REC8 specifically along the chromosome arms. This elegant system, therefore, allows for the timely destruction of cohesin along chromosome arms only once bi-orientation of homologs, and appropriate spindle tension, is assured. It should be noted that the importance and functionality of the SAC in male MI is unclear currently, while in females, the MI checkpoint is somewhat more permissive, allowing a few unattached kinetochores to evade the SAC arrest (Gui and Homer, 2012).

The Second Meiotic Division

The second meiotic division, or MII, is very similar to the mitotic divisions, involving the amphitelic attachment of sister kinetochores to the spindle. The actions of Separase are no longer restricted at the centromere through mechanisms that remain unclear. In mammals, two Shugoshin proteins exist, SGO1 and SGO2. While SGO2 appears to redistribute prometaphase II in mouse oocytes in a manner that is dependent on microtubule attachment of kinetochores, SGO1 and PP2A persist at the centromere. This retention of PP2A suggests that other mechanism must allow for Separase-mediated REC8 cleavage. Instead, it has been proposed that PP2A is inactivated by other mechanisms during MII, at least in female mammals (Miller et al., 2013).

Female Meiosis is Punctuated by Arrest Periods

While the general scheme of meiosis is similar across sexually reproducing organisms, there are some distinct differences between species and, in mammals, between sexes. In males, meiosis initiates postnatally and, most often, postpubertally, proceeding in waves across the seminiferous tubules of the testis. Self-renewing spermatogonial stem cells proliferate as spermatogonia and then enter meiosis sequentially, with cohorts of spermatogonia embarking on meiosis throughout the life of the individual. In female mammals, meiosis initiates synchronously, or semisynchronously, amongst the entire population of oogonia, such that prophase I is completed during gestational life. Toward the end of prophase I, instead of progressing through to metaphase I, cells enter an arrest period known as *dictyate arrest*. During this arrest phase, homologous chromosome interactions remain intact, manifested by the persistence of the cohesin rings along chromosome arms and the chiasmata. These *bivalent* chromosomes persist through dictyate arrest until after puberty, when cohorts of oocytes are triggered to resume meiosis with each estrus/menstrual cycle. Thus, in species such as humans, chiasmate-intact bivalents must persist for up to several decades before resuming meiosis.

Meiotic resumption in most mammals is triggered by the surge in pituitary-derived luteinizing hormone (LH) that also triggers ovulation (Fig. 4). Under conditions of dictyate arrest, oocyte meiosis remains arrested by the persistent phosphorylation and inactivation of MPF, which consists of cyclin B and its cyclin-dependent kinase partner, CDK1. Phosphorylation of CDK1 by

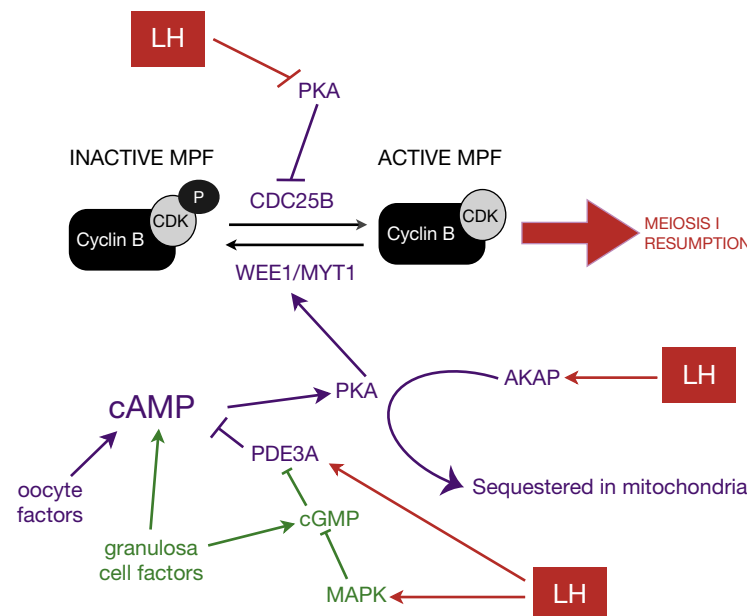


Fig. 4 Maintenance of dictyate arrest and resumption of the first meiotic division. Purple depicts events in the oocyte, and green depicts events in the neighboring granulosa cells. Dictyate arrest is maintained by keep maturation promoting factor (MPF) phosphorylated (by WEE1 kinase). This is achieved through the activity of protein kinase A (PKA) that is activated by high cAMP levels in the oocyte. Orange depicts LH stimulated events at ovulation, all of which culminate in the activation of MPF through dephosphorylation by CDC25B by removing PKA. Modified from Chapter 1, *Physiology of Reproduction*, eds. Knobil and Neil (2013).

WEE1 is promoted by high levels of cyclic AMP (cAMP) in the oocyte which, in turn, activates Protein Kinase A (PKA) to phosphorylate (and activate) WEE1. Simultaneously, PKA suppresses the activity of CDC25B, the phosphatase that dephosphorylates CDK1. cAMP is maintained at high levels through dictyate arrest by multiple mechanisms that are intrinsic to the oocyte, as well as by extrinsic factors from neighboring granulosa cells. At the time of the LH surge, LH reduces cAMP and PKA levels in the oocyte by suppressing granulosa cell-stimulated cAMP-promoting factors, by enhancing the inhibitory effect of Phosphodiesterase 3A (PDE3A) on cAMP, and by sequestering PKA in the mitochondria. All these events serve to reduce WEE1 and CDC25B phosphorylation, resulting in a dephosphorylation of MPF. This newly activated MPF then drives the resumption of meiosis, allowing completion of prophase I and entry into MI. However, as described above, MPF is then rapidly degraded by APC/C following bi-orientation of homologous chromosomes on the microtubular spindle, allowing for the metaphase-to-anaphase transition. Thus, resumption of meiosis I from dictyate arrest is achieved through the stepwise activation of MPF through dephosphorylation, followed by CDC20-mediated APC/C activation and subsequent degradation of MPF, co-incident with Separase-mediated release of chromosome arm cohesion.

Resumption of MI is followed by rapid progression into meiosis II, up until metaphase II, during which time MPF levels reaccumulate, and the oocyte once again arrests in anticipation of fertilization. This second arrest is maintained by *cytostatic factor*, the major components of which are c-Mos and EMI2 (Masui and Markert, 1971; Sagata et al., 1989). EMI2 is an inhibitor of APC/C, thereby preventing a second round of ubiquitylation and degradation of MPF. Thus arrest is maintained until fertilization, when sperm-egg fusion leads to the release into the oocyte cytoplasm of a sperm-derived phospholipase-C isoform, PLC-zeta (PLC ζ ; Fig. 5) (Saunders et al., 2002). PLC ζ induces increases in intracellular calcium that ultimately lead to upregulation and activation of two kinases: calmodulin-dependent protein kinase II (CaMKII) and Polo-like kinase (Plx1). These two kinases act sequentially to phosphorylate EMI2, leading to the proteolytic destruction of CSF and releasing APC/C inhibition. This now allows APC/C to target MPF for destruction, and permits MII resumption.

Human Meiosis is Error Prone

Aneuploidy is the presence of an abnormal number of chromosomes and is highly prevalent in human gametes. Indeed, aneuploidy is the leading genetic cause of spontaneous miscarriage and congenital birth defects in our species. The reported incidence of aneuploidy in human gametes and embryos varies widely, with approximately 4% aneuploidy incidence in stillbirths, 35% aneuploidy incidence in spontaneous miscarriages, and up to 70% aneuploidy incidence in eggs or polar bodies from assisted fertility clinics (Nagaoka et al., 2012). Regardless of the true incidence in humans, two clear facts remain uncontested: that the majority of aneuploidy errors are the result of maternal meiosis, with maternal age being a strong contributing component, and that the rates of aneuploidy in humans is staggeringly high compared to other meiotic species.

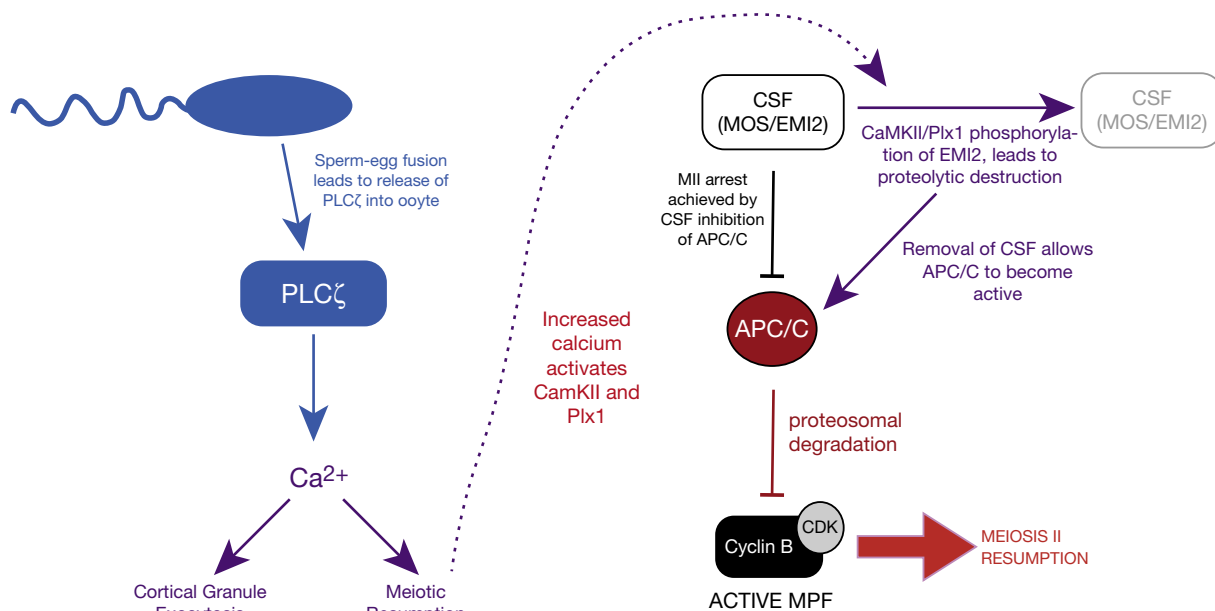


Fig. 5 Resumption of the second meiotic division in mice. The second meiotic arrest is maintained by high levels of cytostatic factor (CSF), consisting of MOS and EMI2. Upon sperm-egg fusion, the release of sperm-derived phospholipase C zeta into the oocyte cytoplasm leads to increases in intracellular calcium, that initiates various events, including meiotic resumption. Increased calcium activates two kinases, CaMKII and Plx1, which together phosphorylate EMI2, thereby leading to the proteolytic destruction of CSF. This releases the inhibition of APC/C by CSF, allowing the former to now degrade MPF (cyclin B-CDK) through ubiquitylation. Loss of MPF leads to meiotic resumption. Modified from Chapter 1, *Physiology of Reproduction*, eds. Knobil and Neil (2013).

The causes of the high incidence of aneuploidy in humans are likely to be varied and complex. That maternal age is a contributing factor underscores the fact that the protracted dictyate arrest observed in women may predispose eggs from older women to errors and/or inappropriate segregation at the first meiotic division. This is supported by observations demonstrating that age-related loss of meiosis-specific cohesin components can lead to increased aneuploidy in mice (Chiang et al., 2010). However, maternal age is certainly not the only cause of aneuploidy in humans, since oocytes from ovaries of human XX fetuses show substantial variation in DSB repair events, and CO processing, as defined by the localization of MLH1 foci during prophase I (Lenzi et al., 2005). Thus DSB repair processing and/or inefficient CO formation are likely to play key roles in ensuring appropriate chromosome segregation at MI. Moreover, it is likely that differences exist between male and female meiosis in the maturation of DSB repair sites to become COs, whereby female meiosis exhibits a more inefficient and error prone CO placement and maturation than observed in male meiosis (Wang et al., 2017). Other sex-specific differences are likely to exist, particularly in the checkpoint mechanisms that sense errors in DSB repair and/or synapsis, and that these differences between the sexes account for different error rates in male versus female meiosis. Indeed, mutant mouse models that exhibit synaptic defects display apoptosis of male spermatocytes, whereas some oocytes can survive these defects, suggesting that errors in prophase I progression can be better tolerated in females than in males.

Whether deficits in meiotic processes during human fetal development arise from genetic errors, in key DSB repair genes for example, or whether they are environmental/toxicological in nature remain to be seen. Almost certainly, genetic factors exist, as evidenced by the numerous polymorphisms in DSB repair genes that associate with infertility. In addition, however, environmental toxins, such as bisphenol A, have also been implicated since these estrogenic chemicals can cross the placenta and have dramatic effects on meiotic events in the fetal ovary (Hunt et al., 2012). More recently, it has emerged that meiotic recombination errors are more prevalent in the sperm of adolescent boys than in older men (Zelazowski et al., 2017) suggesting that some requirement for endocrine maturation of the hypothalamic-pituitary-gonadal axis may be important for supporting proper meiotic progression in mammals.

References

- Brieño-Enríquez, M. A., et al. (2016). Cohesin removal along the chromosome arms during the first meiotic division depends on a NEK1-PP1 γ -WAPL Axis in the mouse. *Cell Reports*, 17, 977–986.
- Cahoon, C. K., & Hawley, R. S. (2016). Regulating the construction and demolition of the synaptonemal complex. *Nature Structural & Molecular Biology*, 23, 369–377.
- Chiang, T., Duncan, F. E., Schindler, K., Schultz, R. M., & Lampson, M. A. (2010). Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes. *Current Biology*, 20, 1522–1528.
- Edelmann, W., et al. (1996). Meiotic pachytene arrest in MLH1-deficient mice. *Cell*, 85, 1125–1134.
- Fukuda, T., Daniel, K., Wojtasz, L., Toth, A., & Höög, C. (2010). A novel mammalian HORMA domain-containing protein, HORMAD1, preferentially associates with unsynapsed meiotic chromosomes. *Experimental Cell Research*, 316, 158–171.
- Grelon, M. (2016). Meiotic recombination mechanisms. *Comptes Rendus Biologies*, 339, 247–251.
- Gui, L., & Homer, H. (2012). Spindle assembly checkpoint signalling is uncoupled from chromosomal position in mouse oocytes. *Development*, 139, 1941–1946.
- Holloway, J. K., Sun, X., Yokoo, R., Villeneuve, A. M., & Cohen, P. E. (2014). Mammalian CNTD1 is critical for meiotic crossover maturation and deselection of excess precrossover sites. *The Journal of Cell Biology*, 205, 633–641.
- Hunt, P. A., et al. (2012). Bisphenol A alters early oogenesis and follicle formation in the fetal ovary of the rhesus monkey. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 17525–17530.
- Hunter, N. (2015). Meiotic recombination: The essence of heredity. *Cold Spring Harbor Perspectives in Biology*, 7.
- Keeney, S., Giroux, C. N., & Kleckner, N. (1997). Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell*, 88, 375–384.
- Kneitz, B., et al. (2000). MutS homolog 4 localization to meiotic chromosomes is required for chromosome pairing during meiosis in male and female mice. *Genes & Development*, 14, 1085–1097.
- Lenzi, M. L., et al. (2005). Extreme heterogeneity in the molecular events leading to the establishment of chiasmata during meiosis I in human oocytes. *American Journal of Human Genetics*, 76, 112–127.
- Lipkin, S. M., et al. (2002). Meiotic arrest and aneuploidy in MLH3-deficient mice. *Nature Genetics*, 31, 385–390.
- Masui, Y., & Markert, C. L. (1971). Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *The Journal of Experimental Zoology*, 177, 129–145.
- McKim, K. S., et al. (1998). Meiotic synapsis in the absence of recombination. *Science*, 279, 876–878.
- McMahill, M. S., Sham, C. W., & Bishop, D. K. (2007). Synthesis-dependent strand annealing in meiosis. *PLoS Biology*, 5, e299.
- Miller, M. P., Amon, A., & Únal, E. (2013). Meiosis I: When chromosomes undergo extreme makeover. *Current Opinion in Cell Biology*, 25(6), 687–696.
- Nagaoka, S. I., Hassold, T. J., & Hunt, P. A. (2012). Human aneuploidy: Mechanisms and new insights into an age-old problem. *Nature Reviews: Genetics*, 13, 493–504.
- Qiao, H., et al. (2014). Antagonistic roles of ubiquitin ligase HEI10 and SUMO ligase RNF212 regulate meiotic recombination. *Nature Genetics*, 46, 194–199.
- Reynolds, A., et al. (2013). RNF212 is a dosage-sensitive regulator of crossing-over during mammalian meiosis. *Nature Genetics*, 45, 269–278.
- Sagata, N., Watanabe, N., Vande Woude, G. F., & Ikawa, Y. (1989). The c-Mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature*, 342, 512–518.
- Saunders, C., et al. (2002). PLC ζ : A sperm-specific trigger of Ca²⁺ oscillations in eggs and embryo development. *Development*, 129(15), 3533–3544.
- Shin, Y. H., et al. (2010). HORMAD1 mutation disrupts synaptonemal complex formation, recombination, and chromosome segregation in mammalian meiosis. *PLoS Genetics*, 6, e1001190.
- Wang, S., et al. (2017). Inefficient crossover maturation underlies elevated aneuploidy in human female meiosis. *Cell*, 168, 977–989.e17.
- Wojtasz, L., et al. (2009). Mouse HORMAD1 and HORMAD2, two conserved meiotic chromosomal proteins, are depleted from synapsed chromosome axes with the help of TRIP13 AAA-ATPase. *PLoS Genetics*, 5, e1000702.
- Yuan, L., et al. (2000). The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. *Molecular Cell*, 5, 73–83.
- Yuan, L., et al. (2002). Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3. *Science*, 296, 1115–1118.
- Zelazowski, M. J., et al. (2017). Age-dependent alterations in meiotic recombination cause chromosome segregation errors in spermatocytes. *Cell*. <https://doi.org/10.1016/j.cell.2017.08.042>.

Spermatogenesis: Overview

Erwin Goldberg, Northwestern University, Evanston, IL, United States

Barry R Zirkin, Johns Hopkins University, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome A Golgi derived organelle that forms over the nucleus to become a membrane-bound vesicle containing dense granules; contains hydrolytic enzymes necessary for the acrosome reaction at fertilization.

Cycle A complete sequential progression of the cellular associations (or stages) that occur in the formation of spermatozoa that occur over time is called a cycle of the seminiferous epithelium.

Seminiferous epithelium Consists of two cell types, a Sertoli cell, and male germ cells at various steps of development.

Sertoli cell The somatic cells of the testis that are essential for testis formation and spermatogenesis. Sertoli cells facilitate the progression of germ cells to spermatozoa via direct contact and by controlling the environment milieu within the seminiferous tubules.

Spermiation A complex process by which spermatozoa are released into the seminiferous tubule lumen after detaching from the Sertoli cell junctional complex.

Stages A stage (numbered with Roman numerals) is represented by a defined association of spermatogonia, spermatocytes, spermatids in a cross section of seminiferous epithelium, at a specific phase in time during spermatogenesis. The acrosomal system is commonly used to identify specific stages in the cycle of the seminiferous epithelium.

Stem cell Spermatogonial stem cells (SSCs) are a subtype of undifferentiated spermatogonia that both self renew or differentiate to undergo the stages of spermatogenesis.

Introduction to Spermatogenesis

The mammalian testis is comprised of two major compartments: the interstitium, within which the testosterone-producing Leydig cells and the testicular vasculature reside, and the seminiferous tubules, which contain the somatic Sertoli cells and the developing germ cells with which the Sertoli cells associate ([Fig. 1](#)). Spermatogenesis begins with diploid spermatogonial stem cells that mitotically divide to become spermatocytes, followed by two meiotic divisions resulting in haploid round spermatids, and then the differentiation of the spermatids during spermiogenesis to form mature spermatozoa ([Fig. 2](#)). The mature spermatozoa then enter the epididymis where they develop into spermatozoa with the ability for forward motility and fertility. Germ cell divisions and differentiation occur in association with the Sertoli cells. The Sertoli cells produce proteins that are required for spermatogenesis and, in turn, are themselves regulated in part by changes in the developing germ cells with which they are associated. The complex interactions between the Sertoli cells and the surrounding germ cells are crucial for spermatogenesis ([Russell et al., 1983](#)).

Sertoli Cells

Sertoli cells extend from the basement membrane of the tubules to the tubule lumen ([Figs. 2 and 3](#); [Russell et al., 1983](#); [Russell and Tallon-Doran, 1983](#)). Spermatogonial cell divisions occur in the basal compartment of the seminiferous tubules, between the tubule basement membrane and the tight junctions between adjacent Sertoli cells. As the germ cells enter meiosis as primary spermatocytes, they move from the basal to the adluminal compartment of the testis, with tight junctions reforming beneath them. Meiosis involves pairing of homologous chromosomes and crossing over/genetic recombination between the pairs. This results in the production of haploid secondary spermatocytes with genetic compositions that differ from one another and from the cells from which they derive. The secondary spermatocytes and cells derived from them (spermatids) express unique surface antigens and thus require protection from the immune system. Sertoli–Sertoli tight junctions form the blood–testis barrier, which helps to protect the meiotic and subsequent developing germ cells from the immune system and from potentially harmful blood-borne chemicals ([Franca et al., 2016](#)).

Phases of Spermatogenesis

Spermatogonial Proliferation

The continuous production of spermatozoa, and thus male fertility, requires the maintenance of a pool of stem spermatogonial cells (SSCs) and the regulated differentiation of a subset of these cells ([Fig. 4](#)) ([de Rooij, 2017](#)). The SSCs are single cells (A_s) that encode

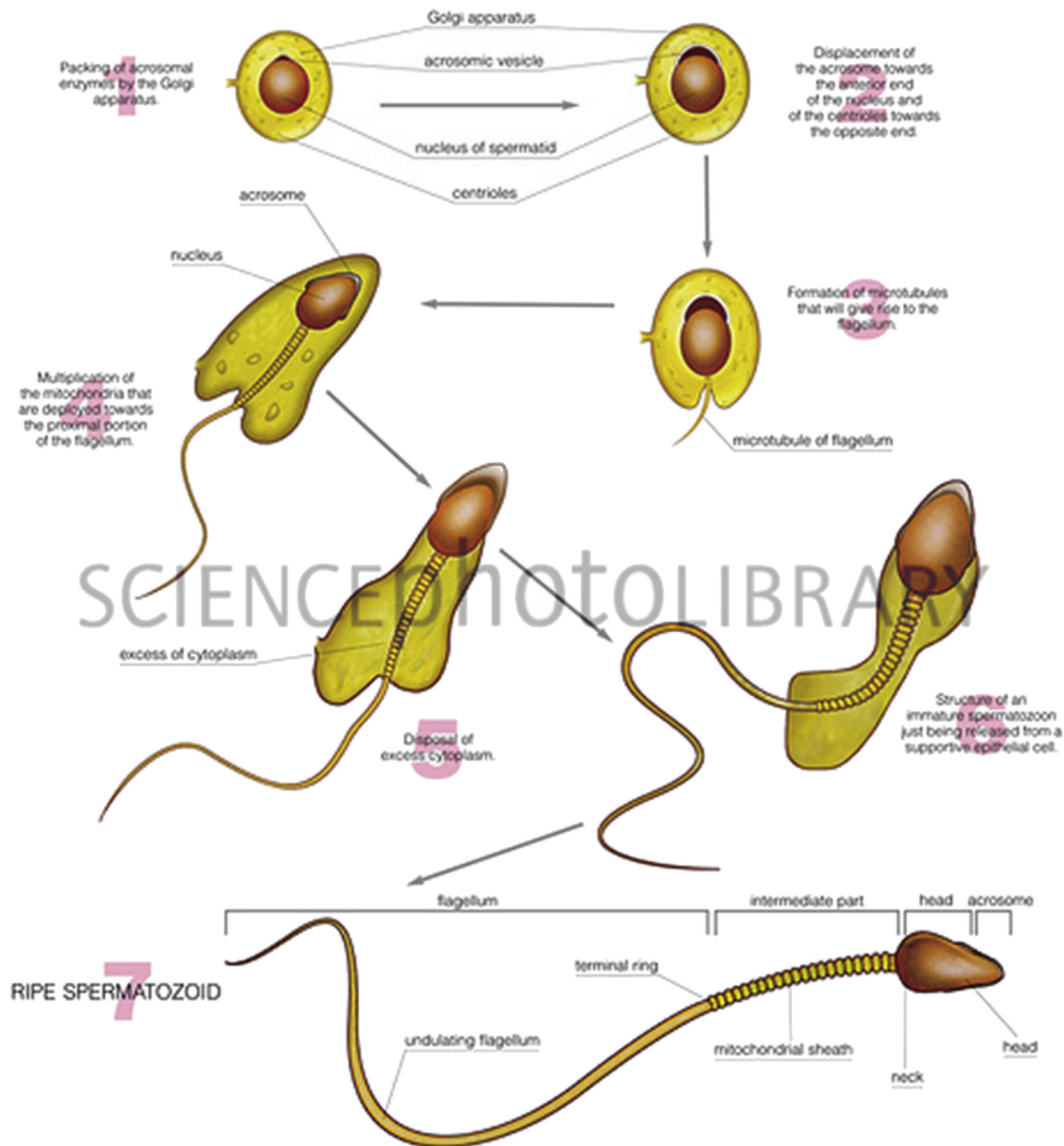


Fig. 1 Depiction of transition steps from round spermatid to elongated spermatozoan.

subunits of the glial cell line-derived neurotrophic factor (GDNF) receptor Ret and GFR α 1, as well as the transcription factor PLZF. The SSCs do not express Kit, which is expressed by more mature, differentiating spermatogonia. SSCs are scarce; in mice, there are approximately 30,000/testis. In culture or after injection into a blastocyst, the stem cells are able to give rise to cells of ectodermal, mesodermal and endodermal lineages, indicating that they are pluripotent. Divisions and subsequent differentiation of the SSCs are regulated at least in part by factors derived from their surroundings (their niche). The niche is created in part by the Sertoli cells which secrete glial cell line-derived neurotrophic factor (GDNF) as well as other growth factors that are involved in regulating SSC function. Analyses of transgenic mice have shown that GDNF is involved in the suppression of SSC differentiation and thus in maintaining populations of SSCs. In contrast, inhibition of GDNF signaling reduces the self-renewing replication of the SSCs, thus allowing their differentiation to form progenitor spermatogonia. The progenitor spermatogonia replicate to give rise to fully

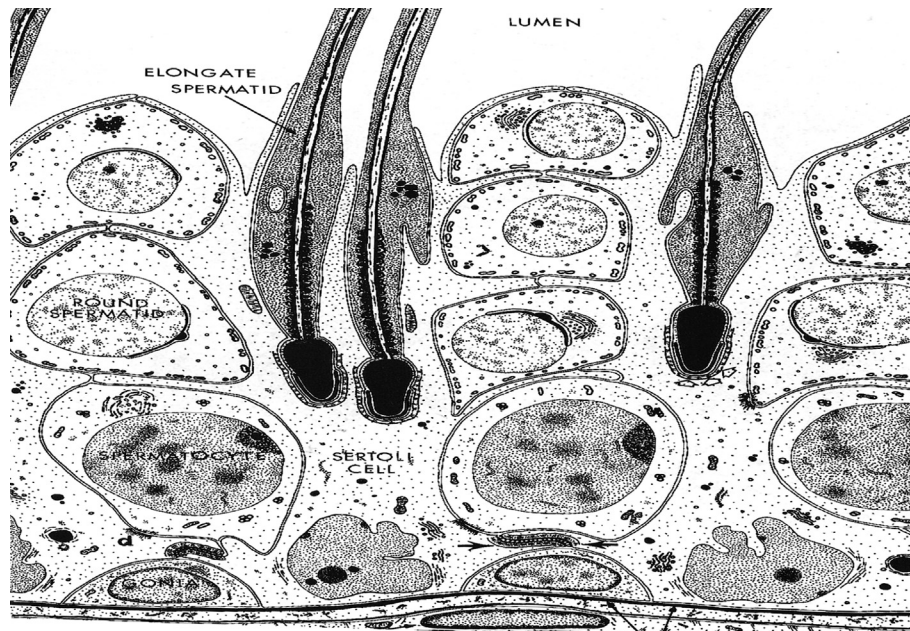


Fig. 2 Formation of Manchette during spermiogenesis.

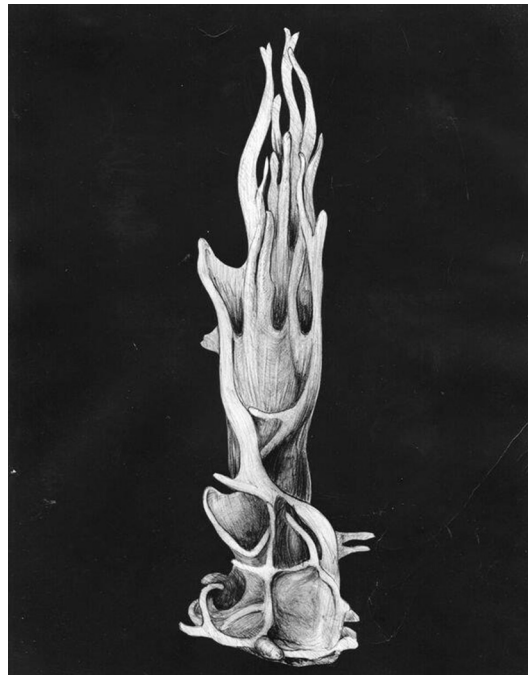


Fig. 3 Drawing of reconstructed rat Sertoli cell. The cell is columnar, with the base below and apex above. Cup-shaped processes of the cell encompass various germ cell types. From Russell, L.D., and Tallon-Doran, M. (1983) Three-dimensional reconstruction of a rat stage V Sertoli cell: 111. A study of specific cellular relationships. *American Journal of Anatomy*. 167, 181–192.

differentiated spermatogonia. After their last division, the spermatogonia enter meiosis as preleptotene spermatocytes. These cells migrate upwards away from the base of the seminiferous tubule and cross the Sertoli–Sertoli junction.

Much of what is understood about SSC function and regulation derives from studies of the mouse. It should be noted, however, that the mouse and human differ in significant ways (Singh et al., 2017). In contrast to the mouse, there are two populations of SSCs in humans and other primates: mitotically active stem cells, referred to as A_{pale} spermatogonia, that sustain spermatogenesis in the normal testis; and reserve stem cells, referred to as A_{dark} spermatogonia, that replicate after loss of substantial numbers of

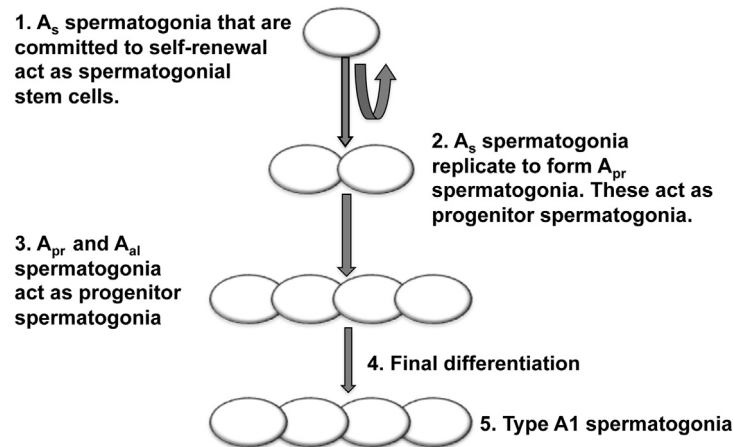


Fig. 4 Diagram of spermatogonial stem cell self-renewal, and stem cell differentiation to form type A1 spermatogonia.

spermatogenic cells. The stem/progenitor cells also differ from their mouse counterparts because they divide only once or twice before differentiating. Despite the limited numbers of replications, the number of sperm produced per gram of human testis is similar in human and mouse, suggesting that the numbers of stem/progenitor cells in the human testis must be far greater than in the mouse. GDNF is considered to play an important *in vivo* role in the regulation of both human and mouse. As in the mouse, human Sertoli cells express GDNF mRNA, and transcripts encoding the subunits of the GDNF receptor GFRA1 and RET are detectable in spermatogonia isolated from both mouse and human testes. Furthermore, GFRA1, the ligand-binding domain of the GDNF receptor, is expressed by human A_{pale} and A_{dark} spermatogonia.

Meiosis

Meiosis results in the production of haploid spermatids (Criswold, 2016). In meiosis, DNA replication occurs during the S-phase of interphase. Immediately following DNA replication, the cells enter meiotic prophase (Meiosis I). During leptotene of meiotic prophase, the chromosomes, each consisting of two sister chromatids, become apparent in the nuclei. During zygotene, homologous chromosomes begin to pair, facilitated by the formation of the synaptonemal complex. At pachytene, homologous chromosomes pair fully and undergo chromosomal breakage and reunion and thus genetic recombination. During diplotene, the synaptonemal complex begins to degrade and homologous chromosomes separate from one another, remaining bound at chiasmata, the regions where crossing-over occurred. In the mammalian female, oocytes develop to this stage before birth and remain in this state until meiosis is resumed in preparation for ovulation. In the male, there is no arrest during meiosis. Rather, the process is continuous. At diakinesis, chromosomes condense and the meiotic spindle begins to form. At metaphase I, homologous chromosomes align on the metaphase plate. At anaphase I, homologous chromosomes, each consisting of a pair of sister chromatids, move to opposite poles. The first meiotic division effectively ends when the chromosomes arrive at the poles at telophase. The daughter cells have the haploid chromosome number, and each chromosome is comprised of a pair of chromatids. Meiosis II is not preceded by DNA synthesis. Chromatids separate into the resulting daughter cells such that the haploid spermatids receive half the DNA amount.

Spermiogenesis

Spermatids, the products of meiosis, mature to become elongated spermatids (aka spermatozoa) during spermiogenesis (O'Donnell, 2015). In part, spermatid maturation involves the elongation of the nucleus and the condensation of chromatin, the latter a consequence of the replacement of histones by arginine- and cysteine-rich protamine during spermatid elongation. Condensation of the nucleus results in transcriptionally inactive, tightly packed chromatin. Spermatid maturation also involves the formation of the acrosomal granule by the Golgi apparatus, and its subsequent elaboration over the head of the nucleus. The acrosome contains hydrolytic enzymes that are required for sperm penetration through egg investments during fertilization. Additionally, the sperm tail forms, and the mitochondria gather at the base of the nucleus, forming the midpiece. Testicular spermatozoa, which lack motility or the ability to penetrate an egg, gain both qualities in the epididymis.

Cycle of the Seminiferous Epithelium

Spermatogenesis is a continuous process which, as described above, involves spermatogonial proliferation and differentiation, meiosis and spermiogenesis (Russell et al., 1983). Spermatozoa are produced at all times, from puberty through old age. In

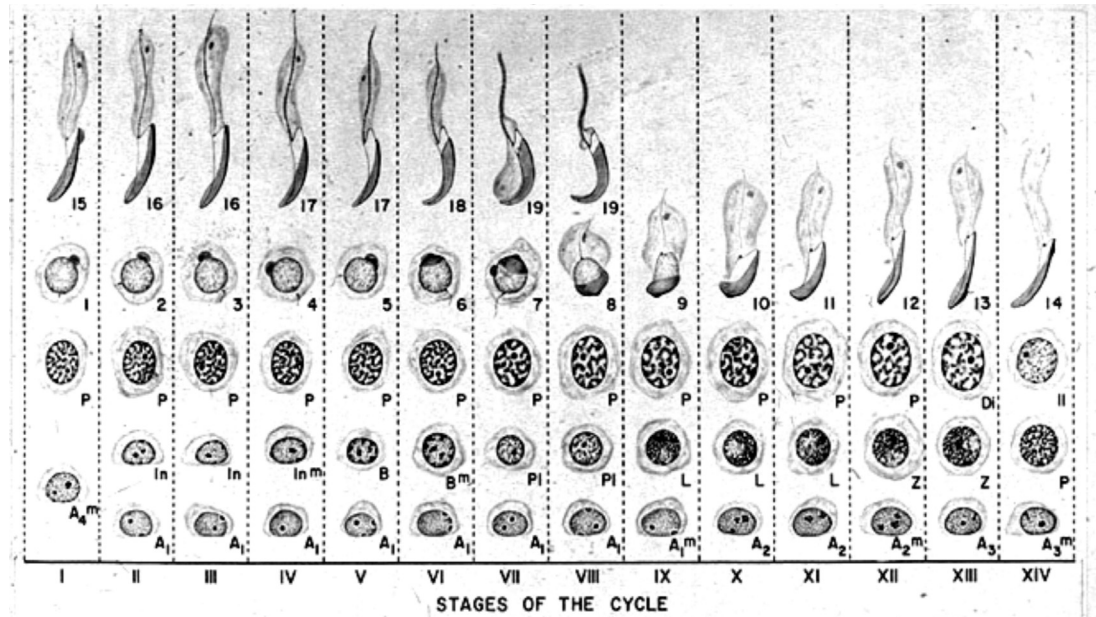


Fig. 5 Stages of the cycle of the seminiferous epithelium in the rat. The vertical columns, designated by Roman numerals, show progressive associations (stages) of spermatogenesis, including spermatogonia, spermatocytes and spermatids. These associations occur over time at any given location of the tubule. Modified from Fig. 4-1, Russell, L.D., Ettlin, R.A., Sinha Hikim, A.P., and Clegg, E.D. (1983) In: *Histological and Histopathological Evaluation of the Testis*. Cache River Press, Clearwater, FL.

any given location along a seminiferous tubule, cohorts of spermatogonia, spermatocytes and spermatids are seen, developing concurrently (Figs. 2 and 5). Spermatogonia are situated along the basement membrane, spermatocytes are closer to the tubule lumen, and spermatids are nearest the lumen. The cells are not arranged at random, but rather, at any location along the tubule, cells are organized into well-defined stages defined by the four to five germ cell cohorts that are present. Any given cross section of seminiferous tubule will contain one or two generations of spermatogonia, one or two generations of spermatocytes, and one or two generations of spermatids. The various generations of germ cells form identifiable morphological stages, defined by specific sets of cell associations that follow each other over time in any given area of seminiferous tubule. Different regions of a given seminiferous tubule will contain different stages of the cycle, the net result of which is the continuous and asynchronous production of sperm. The cellular associations present at any given location change over time as the cells differentiate. The series of cellular associations that ultimately produce spermatozoa from spermatogonia is referred to as the cycle of the seminiferous tubule (Fig. 5). The term “cycle” is used because any specific cellular association that is present at a given time and location will be seen again at a later time at that same tubular location. The cycle continues from puberty throughout the life of the male. The time that is required for spermatozoa to be produced from spermatogonia varies according to the species. In the human, spermatogenesis occurs over the course of about 64 days; and in the rat about 48 days.

Cells do not move laterally along the length of the tubules. However, observation along the length of a tubule reveals that the stages are largely in consecutive order, though there are short reversals of this segmental order, called modulations. The sequential order of stages along the tubules constitutes what is referred to as “the wave of the seminiferous epithelium.”

Hormonal and Paracrine Regulation of Spermatogenesis

A number of factors, both endocrine and paracrine, are known to regulate germ cell survival during spermatogenesis. Testosterone, produced by Leydig cells of the testicular interstitial compartment, is essential for spermatogenesis (Zirkin, 1998). Prolonged reduction in intratesticular testosterone concentrations can result in significant germ cell loss and thus reduced testicular volume (Fig. 6) and ultimately infertility (Wang et al., 2016). Little is known about the cellular and molecular mechanisms that underlie the regulation of spermatogenesis by testosterone. The situation is complicated by the fact that germ cells, whose survival depends on adequate concentrations of testosterone, do not express the androgen receptor. Thus, the germ cell apoptosis that results from decreased intratesticular testosterone concentration is considered to be mediated by the androgen receptor-expressing somatic Sertoli cells. In addition to testosterone, the maintenance of spermatogenesis also may be dependent on follicle-stimulating hormone (FSH). The Sertoli cells contain receptors for both testosterone and FSH. Paracrine factors from Sertoli cells, some of which may be regulated by testosterone, play an integral role in regulating spermatogenesis.



Fig. 6 Effects of prolonged reduction in intratesticular testosterone concentrations on testicular volume. The reduced testicular volume results from germ cell loss.

Summary

The production of male gametes is essential to the survival and perpetuation of species. Cellular and molecular changes during spermatogenesis are exquisitely regulated by both extrinsic and intrinsic factors. As perturbations at any stage of spermatogenesis can result in non-functional gametes or abnormal embryos, the importance of spermatogenesis is apparent.

Bibliography

- França, L. R., Hess, R. A., Dufour, J. M., Hofmann, M. C., & Griswold, M. D. (2016). The Sertoli cell: One hundred fifty years of beauty and plasticity. *Andrology*, 4, 189–212.
- Griswold, M. D. (2016). Spermatogenesis: The commitment to meiosis. *Physiological Reviews*, 96, 1–17.
- O'Donnell, L. (2015). Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis*, 4, e979623.
- de Rooij, D. G. (2017). The nature and dynamics of spermatogonial stem cells. *Development*, 144, 3022–3030.
- Russell, L. D., & Tallon-Doran, M. (1983). Three-dimensional reconstruction of a rat stage V Sertoli cell: 111. A study of specific cellular relationships. *American Journal of Anatomy*, 167, 181–192.
- Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P., & Clegg, E. D. (1983). *Histological and histopathological evaluation of the testis*. Clearwater, FL: Cache River Press.
- Singh, D., Paduch, D. A., Schlegel, P. N., Orwig, K. E., Mielnik, A., Bolyakov, A., & Wright, W. W. (2017). The production of glial cell line-derived neurotrophic factor by human sertoli cells is substantially reduced in Sertoli cell-only testes. *Human Reproduction*, 32, 1108–1117.
- Wang, C., Festin, M. P., & Swerdloff, R. S. (2016). Male hormonal contraception: Where are we now? *Current Obstetrics and Gynecology Reports*, 5, 38–47.
- Zirkin, B. R. (1998). Spermatogenesis: Its regulation by testosterone and FSH. *Seminars in Cell and Developmental Biology*, 9, 417–421.

Prospermatogonia

Massimo De Felici, University of Rome Tor Vergata, Rome, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

In mammals, prospermatogonia, also called gonocytes or prespermatogonia, are transient cells of the male germ cell line between the primordial germ cells (PGCs) and the spermatogonia stem cells (SSCs). PGCs are embryonic cells coming from the extraembryonic mesoderm of the yolk sac wall which colonize the gonadal anlagen to give rise to oogonia in the female and prospermatogonia in the male. SSCs are the stem cells of the postnatal testis derived from prospermatogonia which sustain spermatogenesis during the adult life.

The lifetime of prospermatogonia spans from the embryonic to the early postnatal or pre-pubertal period depending on the species. For example, in rodents, where the most part of studies about these cells have been performed, this period lasts about 2 weeks, whereas in primates it takes several months or some years. The transient nature of prospermatogonia is testified by their morphological and molecular heterogeneity due to the variety and dynamics of the processes occurring in such cells. These characteristics are exacerbated in human by the asynchrony of these processes resulting in the presence of prospermatogonia at different developmental stages at the same time in the same testis cord region. Such heterogeneity led to the proposal of various prospermatogonia subpopulations identified with different terminology.

Only scant information is available about human prospermatogonia, due to the obvious difficulty to obtain these cells. Such information should be, however, of great biological and clinical interest since several developmental processes occur in prospermatogonia crucial for male fertility and probably, if defective, at the origin of certain types of testicular germ cell tumors (TGCTs). In fact, disturbance of testis development in fetal life can result in testicular dysgenesis syndrome (TDS), which includes disorders, such as cryptorchidism, hypospadias, infertility, and TGCTs.

Formation

Prospermatogonia derive from PGCs during the embryonal–fetal period. In the human embryo, between the 2nd and 3rd week, the progenitors of PGCs probably arise in the posterior region of the epiblast, one of the two cell disk (the other being the hypoblast) forming the embryo proper at this time (De Felici, 2016) or in the forming extraembryonic amnios membrane (Sasaki et al., 2016). Shortly before the beginning of gastrulation, the progenitors move in the extraembryonic mesoderm/endoderm of the yolk sac where they possibly become PGCs and from which, around 4th week, PGCs start migration toward the gonadal anlagen (or gonadal ridges) developing in the celomatic wall of the embryo hindgut.

In the developmental testis (5th–6th week), PGCs become progressively enclosed within the testis cords by pre-Sertoli cells; this starts about on 7th week and continue up to the end of first trimester of pregnancy. During this period, PGCs differentiate into prospermatogonia at the center of the cords (for a review, see De Felici, 2016) (Fig. 1).

Heterogeneity

On the basis of morphological and molecular studies human prospermatogonia have been differently classified and named (for reviews, see Culty, 2013; McCarrey, 2013).

According to early studies, Wartenberg et al. (1971) on morphological grounds distinguished three types of human prospermatogonia: primitive phase 1-gonocytes, phase 2-gonocytes, and the more differentiated, fetal spermatogonia. Gondos and Hobel (1971), however, distinguished only two types of fetal germ cells according to their localization within the tubules: isolated and centrally situated gonocytes, and fetal spermatogonia clustering near the basal lamina. Fukuda et al. (1975), again described three types of germ cells within human testis from 9 to 30 weeks *postconception*: the gonocytes, the intermediate cells and the fetal spermatogonia. The gonocytes were described as cells with the highest nucleus–cytoplasmic ratio, a round nucleus with a centrally located, prominent nucleolus, a cytoplasm displaying a well-developed Golgi apparatus, lipid droplets and parallel arrays of short cisternae of the rough endoplasmic reticulum. The intermediate cells were found to extend several cytoplasmic processes and to contain a moderate number of long, branched and/or widened reticulum cisternae. Intermediate cells were often connected to one another by intercellular cytoplasmic bridges. Fetal spermatogonia also displayed cytoplasmic bridges. These latter showed the lowest nucleus–cytoplasmic ratio and a more condensed chromatin. The mitochondria were situated close to the nucleus. Glycogen particles, polyribosomes, and chromatoid bodies (“nuage”) were present in all the three germ cell types. All these morphologically distinct fetal germ cells can be considered prospermatogonia in progressive stages of differentiation.

At molecular level, early prospermatogonia (equivalent to gonocytes) have been then reported to express several genes and surface markers in common to PGCs and typical of pluripotent cells such OCT4, NANOG, c-KIT, AP-2γ, placental alkaline phosphatase (PLAP), and SSEA-1, SSEA-4, M2A. Most of these markers are downregulated during the subsequent migratory phase and differentiation and progressively replaced by others, more germ cell specific, such as VASA (or DDX4), PUM2, DAZL, and MAGE-A4

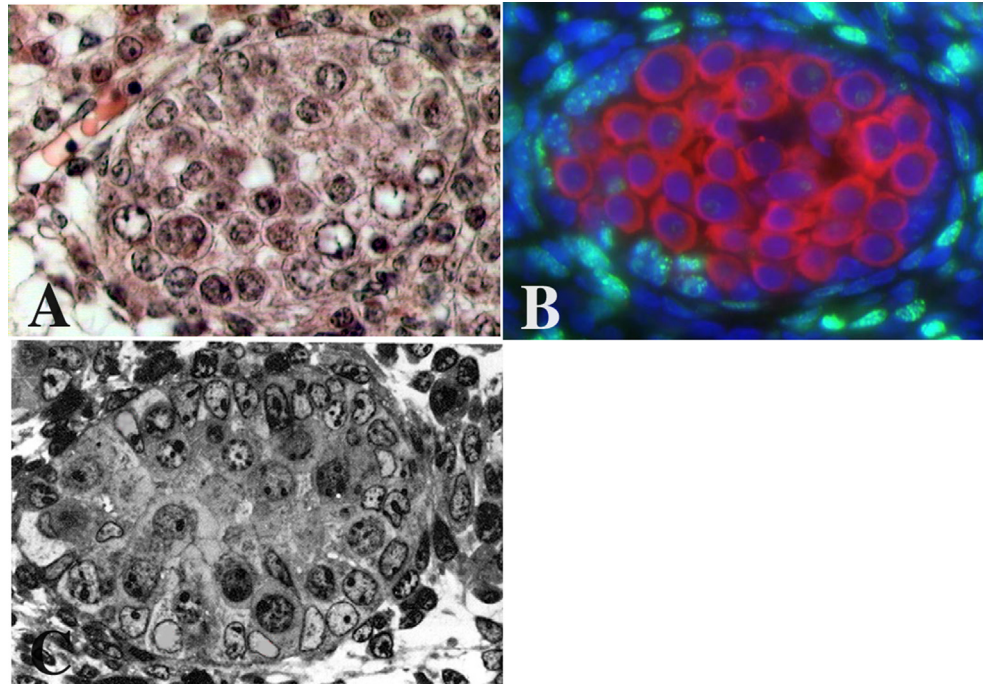


Fig. 1 Mouse prospermatogonia in the center of a testis cords. (A) Paraffin section stained with E&E, (B) immunolocalization in paraffin section of VASA in the cytoplasm of prospermatogonia (*red*) (cell nuclei are stained with the fluorescent *blue* dye DAPI), and (C) semithin toluidine *blue* stained section for transmission electron microscope.

(Gaskell et al., 2004 and references here in). Interestingly, most of the pluripotency and some of the more advanced prospermatogonia developmental stage markers, are expressed at various level in the most part of TGCTs, supporting the hypothesis that this tumors originate from undifferentiated or poor differentiated prospermatogonia (for a review, see Dolci et al., 2015).

Proliferation

Colonizing PGCs and prospermatogonia in the center of the testis cords, undergo active proliferation. The number of germ cells per testis shows an exponential increase between 6 and 9 weeks *postconception* from about 10,647 to 74,626. In third-trimester samples, this number has been estimated to increase to $1.5\text{--}3.8 \times 10^6$, which is consistent with further mitotic activity of prospermatogonia in the second trimester (Cortes, 1990; Mamsen et al., 2011). The rate of increase in germ cell numbers is then reduced and low proliferation is detected in prospermatogonia in the late gestational period, followed by a small increase, between 2.5- and 6-month-old infants. So that, differently than in rodents, human prospermatogonia do not appear to undergo a period of complete proliferative quiescence.

In rodents, several molecules, mostly produced by testis somatic cells, have been reported to induce or inhibit prospermatogonia proliferation in *in vitro* models. For example, retinoic acid (RA) was found to increase the proliferation of fetal rat prospermatogonia. In mouse and/or in rat, PDGF-BB, 17- β -estradiol, and RA were shown to induce neonatal prospermatogonia proliferation whereas activin A, prostaglandin D2, FGF9, androgen, and T3 hormones, were reported to be negative regulators of fetal prospermatogonia proliferation. Moreover, Activin A/Nodal/TGF β signaling appears to inhibit the proliferation both of fetal and postnatal prospermatogonia in the mouse. While stimulation of proliferation seems to occur through classical MAPK and/or PI3K-dependent pathways, SMAD2/3 and p38 act in concert to inhibit mouse prospermatogonia proliferation (for a review, see Manku and Culty, 2015). The final targets of the inhibitors of proliferation is likely to be the activation of genes such as *Nanos2* and *Dnd1*, encoding RNA binding proteins, upregulation of the CDK inhibitors p15, p16, p21, and p27 and of the tumor suppressor RB1 protein and downregulation of some cyclins as for example E1, E2, and D3. Finally, the expression of transcription factors such DMRT1, baso-nuclin2, and FOX1, were reported to be associated to the G0 quiescence of mouse prospermatogonia (for a review, see De Felici and Farini, 2012).

If these factors are involved in the control of human prospermatogonia proliferation is not known. Moreover, since the marked asynchrony of such processes in human, we must assume that the supply of stimulatory or inhibitory proliferation factors to prospermatogonia must be finely regionally regulated.

Migration

The various stages of the prospermatogonia migration from the center to the testicular cord periphery are indicated by changes in morphological features and, in particular, by the extension of cytoplasmic processes toward the basal lamina (Fukuda et al., 1975) (Fig. 2). Reaching such matrix, some prospermatogonia appear to be arranged in pairs and groups connecting by intercellular bridges derived by incomplete cytokinesis (Wartenberg et al., 1971; Gondos and Hobel, 1971).

In contrast to rodents, where prospermatogonia migration starts and ends during the first week after birth, human prospermatogonia commence to migrate around the end of the second trimester of gestation and continue up to 8 months after birth. Despite that this migration coincides with the physiological activation of the hypothalamic-pituitary-gonadal axis, characterized by a surge in serum gonadotropins and testosterone levels at age 1–3 months, data from mice with global inactivation of the androgen receptor, FSH receptor or lacking GNRH, indicate that androgens and gonadotropins are not required for prospermatogonia migration and differentiation.

A current list of factors that have been directly shown to influence prospermatogonia migration in rodents includes c-KIT/stem cell factor, various adhesion molecules, thyroid hormone, PDGF receptor beta, and Notch. In such species, a model has been proposed in which the ADAM–integrin–tetraspanin complex, known to constitute a network of membrane microdomains called the “tetraspanin web,” is involved in the migration of prospermatogonia (for a review, see Culty, 2009).

Programmed Cell Death

Programmed cell death (PCD) affects primarily prospermatogonia at the center of the developing sex tubules. The physiological significance of this process is still not clear. Some researchers have hypothesized that it may cooperate in maintaining an optimal germ cell/Sertoli cell ratio. Others suggest this as a selection mechanism to eliminate chromosomally abnormal cells, eventually appearing because of high proliferation rate.

In the mouse, prospermatogonia death occurs primarily in the middle of the fetal period and again around birth. According to morphological observations and molecular markers, such process occurs with typical apoptotic features. Studies on knockout mice have identified some important players of the process. For example, haploinsufficiency of the zinc-finger transcription factor *Zfp148* has been shown to cause complete loss of prospermatogonia correlated with impaired phosphorylation of p53. Another member of the p53 family, p63, localized in the nucleus of the prospermatogonia, might be also involved in triggering their PCD. The RNA binding protein *Dazl* is, on the contrary, important for prospermatogonia survival since disruption of the gene leads to their massive degeneration. Bcl-2 family members are also positively or negatively involved in prospermatogonia survival. In fact, in Bcl-x hypomorphic mice, severe loss of prospermatogonia occurs and the phenotype is rescued by concomitant disruption of Bax. Interestingly, some authors reported that testis somatic cells play an active role in prospermatogonia PCD. Extrinsic apoptotic pathways are usually initiated by binding to cell surface “death receptors,” including Fas (CD95) and TNF receptor1, by their specific ligands. Interestingly, c-FlipL, a natural inhibitor of Fas-mediated apoptosis, is expressed at high level in mouse prospermatogonia and might have a role in protecting prospermatogonia from Fas-dependent apoptosis (Giampietri et al., 2006).

The second postnatal round of death in mice does not occur with typical apoptosis features so that other mechanisms can be involved.

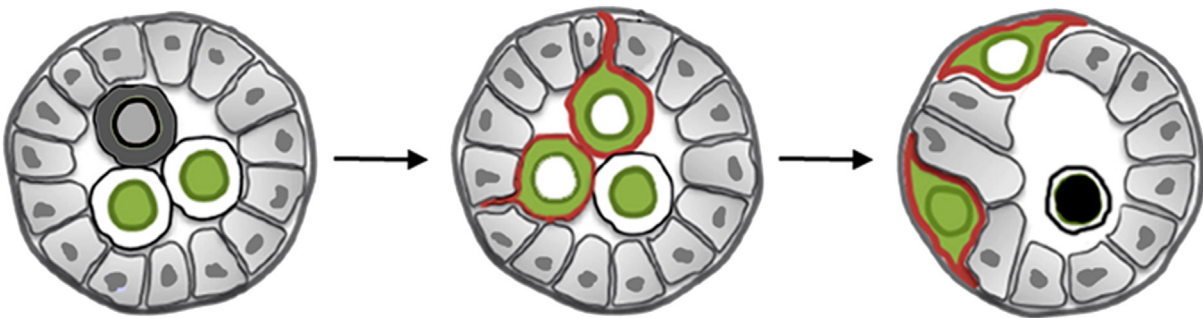


Fig. 2 Diagram of prospermatogonia formation and differentiation within a testis cord. (A) Prospermatogonia (green nucleus and white cytoplasm) arise from primordial germ cells (pale gray nucleus and dark gray cytoplasm); (B) and (C) prospermatogonia migration from the center to the testicular cord periphery is indicated by changes in morphological features (in particular, by the extension of cytoplasmic processes toward the basal lamina), in expression of adhesion proteins and receptors (red plasmalemma), several transcription factors and epigenesis (green nucleus). Moreover, programmed cell death occurs in significant number of prospermatogonia (black nucleus). Cells originated from prospermatogonia laid onto the basal lamina are stem cell spermatogonia.

In human, significant level of apoptosis occurs in fetal testis during the first trimester, both in Sertoli and Leydig cells and prospermatogonia and continues throughout pregnancy. RA could be one of the factors involved since its presence at 10^{-6} M for 4 days in culture increases significantly apoptosis in testis obtained from 6 to 10 weeks.

Epigenesis

Shortly after specification, throughout migration and for a couple of days after gonad colonization mouse PGCs undergo extensive epigenetic reprogramming (changes in gene expression due to mechanisms, mainly DNA methylation/demethylation and histone modification, other than changes in DNA sequence). Such process is a key feature of PGC development and includes wide genome demethylation and erasure of the DNA methylation marks associated with imprinted genes, allowing genome reprogramming of the nascent germ cells and the establishment of sex-specific imprints during gametogenesis. There are also extensive changes in several histone marks. For a review, about of epigenesis in mouse PGC, see [De Felici \(2011\)](#).

In human PGCs, a few observations have been reported with regard to chromatin changes and DNA demethylation. Nevertheless, a timeline and some characteristics of these events are becoming to be available also thanks to the in vitro models of PGC-like formation for stem cell lines in vitro ([Tang et al., 2015](#)). In this regard, immunohistochemical studies and DNA methylome analyses have shown that both female and male fetal germ cells are hypomethylated already at 5th and 6th weeks of gestation when most of them are still migrating or have just arrived in the developing gonads. Like in mouse, consistent with global demethylation, UHRF1 (a protein involved in the maintenance of DNA methylation), was not detectable in proliferating oogonia/prospermatogonia while de novo DNA methyltransferase DNMT3A and DNMT3B were repressed. Also resembling mouse PGCs, despite global hypomethylation, potentially hazardous retrotransposons such as the LINE SINE-VNTR-*Alu* (SVA) and endogenous retrovirus (ERV) elements remain methylated in gonadal human PGCs. Interestingly, other mechanisms, including histone modifications or PIWI-interacting RNAs (piRNAs), have been identified in germ cells to inactivate transposable elements during global demethylation. Remarkably, some loci associated with metabolic and neurological disorders were also resistant to DNA demethylation, revealing potential for transgenerational epigenetic inheritance that may have phenotypic consequences.

The subsequent re-establishment of methylation and correct imprinting are essential for ensuring functional germ cells. In contrast to mouse prospermatogonia, which remain demethylated for only a few days, human prospermatogonia remain hypomethylated for weeks and only show signs of re-methylation in the II trimesters (around 16th week onward). As for chromatin modifications, at around 16th–20th weeks human prospermatogonia were reported to have absence/low levels of repressive H3K9me2, H3K27me3, and H3K9me3 modifications but high levels of permissive H3K9ac and H2A.Z marks. In the mouse, global DNA methylation levels rise from ~10% in 13.5 dpc PGCs to ~50% in 16.5 dpc prospermatogonia, coinciding with redistributing the active marks of H3K4me2/3 and H3K36me3 in prospermatogonia. The elevated expression of DNA methyltransferase 3A/3B/3L (DNMT3s) during these stages suggests the potential contribution of de novo DNA methylation at this stage. The time course of complete re-methylation and paternal imprinting establishment in humans are not known. However, some results allow dating the completion of DNA methylation pattern to occur sometime postnatally, before puberty, while imprinting continues during spermatogonial differentiation and early stages of meiotic prophase I.

These results support the notion that human PGCs undergo a pattern of genome reprogramming similar on the whole to the mouse model but with differences in timing and chromatin changes. The significance and importance of such differences remain, however, to be clarified.

Differentiation

After reaching the periphery of the developing tubules, prospermatogonia differentiate into SSCs which in turn will give rise to differentiating spermatogonia ([Fig. 2](#)). In humans, these processes are gradual and asynchronous, which means that germ cells at different stages of development will be present throughout fetal and neonatal development.

The differentiation step from prospermatogonia to SSCs is particularly important since, as reported above, carcinoma in situ (CIS) cells, the progenitors of the most part of TGCTs, are believed to arise from arrested/dysfunctional prospermatogonia. In this context, it has been reported that in the normal human testis during the perinatal period, those prospermatogonia which remain OCT4 positive are almost exclusively located in the center of the seminiferous cords while increasing intensity of VASA immunostaining coincides with relocation of prospermatogonia to the basal membrane in the second trimester of pregnancy. In older children with disorders of sexual development, who are at increased risk of TGCTs, prospermatogonia expressing OCT4 are reported to be both more numerous and located at the basal lamina.

In the mouse, known genes expressed in SSCs that must presumably be activated during their differentiation from prospermatogonia, include *Gfra1* and *Ret*, encoding the receptor of the growth factor GDNF, and *Plzf* and *Nanos2*, encoding transcription factors likely important for the maintenance of the SSC stemness. Moreover, the integrins $\beta 1$ and $\alpha 6$ and the transcription factor FOXO1 have been described to undergo nuclear-cytoplasm translocation when prospermatogonia differentiate in SSCs. In this latter, FOXO1 will be subsequently relocated into the nucleus. According to some authors, FGF signaling is a master regulator of prospermatogonia to SSC transition and acts upstream of GDNF and RA signaling for the activation of the MEK/ERK and PI3K/Akt pathways.

Several studies in mice suggest that two different populations of prospermatogonia are present in the neonatal testis, in which one subpopulation progress directly into differentiating spermatogonia and complete the first round of postnatal spermatogenesis, whereas a second population transforms into SSCs that than provide the basis for subsequent rounds of spermatogenesis. Whether this two waves of spermatogenesis are conserved in human is currently unknown. According to a widely accepted model for human spermatogenesis in the adult, SSCs correspond to single type-A_{dark} spermatogonia, which can self-renew, while paired type-A_{pale} spermatogonia are differentiating daughter cells connected by an intercellular bridge.

References

- Cortes, D. (1990). Histological versus stereological methods applied at spermatogonia during normal human development. *Scandinavian Journal of Urology and Nephrology*, 24, 11–15.
- Culty, M. (2009). Gonocytes, the forgotten cells of the germ cell lineage. *Birth Defects Research. Part C, Embryo Today*, 87, 1–26.
- Culty, M. (2013). Gonocytes, from the fifties to the present: Is there a reason to change the name? *Biology of Reproduction*, 89, 1–6.
- De Felici, M. (2011). Nuclear reprogramming in mouse primordial germ cells: Epigenetic contribution. *Stem Cells International*, 2011, 15. Article ID 425863.
- De Felici, M. (2016). The formation and migration of primordial germ cells in mouse and man. *Results and Problems in Cell Differentiation*, 58, 23–46.
- De Felici, M., & Farini, D. (2012). The control of cell cycle in mouse primordial germ cells: Old and new players. *Current Pharmaceutical Design*, 18, 233–244.
- Dolci, S., Campolo, F., & De Felici, M. (2015). Gonadal development and germ cell tumors in mouse and humans. *Seminars in Cell & Developmental Biology*, 45, 114–123.
- Fukuda, T., Hedinger, C., & Groscurth, P. (1975). Ultrastructure of developing germ cells in the fetal human testis. *Cell and Tissue Research*, 161, 55–70.
- Gaskell, T. L., Esnal, A., Lynn, L. L., et al. (2004). Immunohistochemical profiling of germ cells within the human fetal testis: Identification of three subpopulations. *Biology of Reproduction*, 71, 2012–2021.
- Giampietri, C., Petrunaro, S., Klinger, F. G., et al. (2006). C-Flip expression and function in fetal mouse gonocytes. *The FASEB Journal*, 20, 124–126.
- Gondos, B., & Hobel, C. J. (1971). Ultrastructure of germ cell development in the human fetal testis. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 119, 1–20.
- Mamsen, L. S., Lutterodt, M. C., Andersen, E. W., et al. (2011). Germ cell numbers in human embryonic and fetal gonads during the first two trimesters of pregnancy: Analysis of six published studies. *Human Reproduction*, 26, 2140–2145.
- Manku, G., & Culty, M. (2015). Mammalian gonocyte and spermatogonia differentiation: Recent advances and remaining challenges. *Reproduction*, 149, 139–157.
- McCarrey, J. R. T. (2013). Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. *Biology of Reproduction*, 89, 1–7.
- Sasaki, K., Nakamura, T., Okamoto, I., et al. (2016). The germ cell fate of Cynomolgus Monkeys is specified in the nascent amnion. *Developmental Cell*, 39(2), 169–185. <https://doi.org/10.1016/j.devcel.2016.09.007>.
- Tang, W. W. C., Dietmann, S., Irie, N. A., et al. (2015). Unique gene regulatory network resets the human Germline Epigenome for development. *Cell*, 161, 1453–1467.
- Wartenberg, H., Holstein, A. F., & Vossmeier, J. (1971). Cytology of the prenatal development of human gonads. II. Electronmicroscopic studies on the cytogenesis of gonocytes and foetal spermatogonia in the testis. *Zeitschrift für Anatomie und Entwicklungsgeschichte*, 134, 165–185.

Spermatogonia

Adetunji Fayomi*, Sherin David*, Chatchanan Doungkamchan*, and Kyle E Orwig, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Introductory Overview

Spermatogenesis is a highly organized process that produces millions of sperm each day in postpubertal mammals (Sharpe, 1994; Gupta et al., 2000; Thayer et al., 2001). This productivity depends on the activity of spermatogonial stem cells (SSCs), which are the adult tissue stem cells in the testes that balance self-renewing divisions with differentiating divisions to maintain the stem cell pool and fuel spermatogenesis, respectively (Clermont and Bustos-Obregon, 1968; Huckins and Oakberg, 1978; Tegelenbosch and de Rooij, 1993; de Rooij and Grootegeod, 1998). When SSCs differentiate, they give rise to progenitor spermatogonia that undergo a species-specific number of transit amplifying mitotic divisions, followed by two meiotic divisions and spermiogenesis (morphological transformation) to produce terminally differentiated sperm (Fig. 1). Spermatogenesis occurs in a cyclic manner along the length of the seminiferous tubules, ensuring continuous sperm production throughout the postpubertal life of mammals. In mice, the cycle of the seminiferous epithelium is divided into 12 distinct stages (I–XII) and each stage is defined by specific associations of spermatogonia, spermatocytes, spermatids and sperm. A segment of seminiferous tubules progresses through all 12 stages every 8.5 days (Russell et al., 1990). SSCs reside in a specialized niche located on the basement membrane of the seminiferous tubules where they are in direct contact with the basement membrane and Sertoli cells, which produce some of the paracrine factors required to regulate self-renewal and differentiation fate decisions. Stem, progenitor and differentiating spermatogonia are all located on the basement membrane of the seminiferous tubules. Differentiating spermatogonia give rise to spermatocytes that initiate meiosis, migrate off the basement membrane and give rise to secondary spermatocytes, spermatids and sperm. The identity of the SSC pool is the subject of active research and debate.

Spermatogonial Development—Clonal Dynamics

Spermatogenesis arises from a population of A_{single} (A_s) spermatogonia located on the basement membrane of seminiferous tubules (Fig. 2A, red) (Huckins, 1971a; Oakberg, 1971; de Rooij, 1973). A_s spermatogonia are rare, comprising 0.03% of all germ cells in the mouse testis; they are evenly distributed along the basement membrane of the seminiferous tubules; have a relatively large nuclear

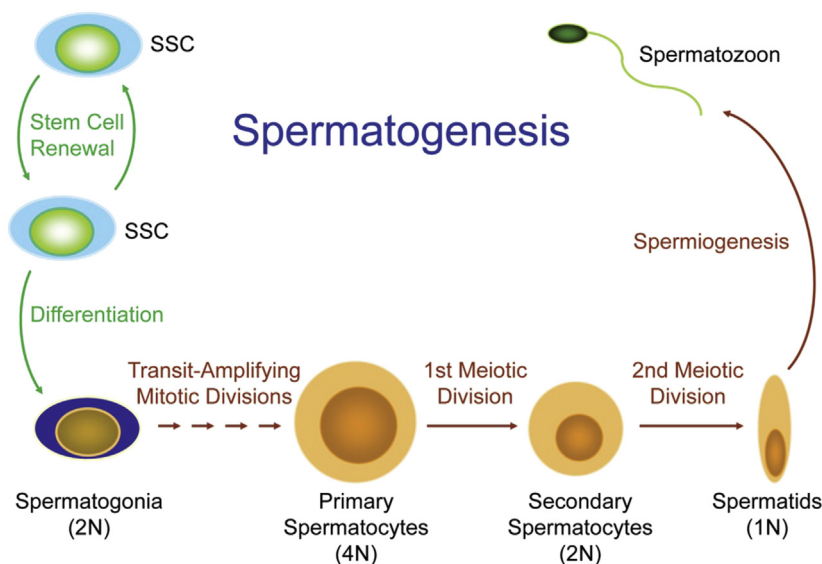


Fig. 1 *Mammalian Spermatogenesis.* The pool of diploid spermatogonial stem cells (SSCs) balance self-renewing and differentiating divisions to maintain the stem cell pool as well as continuous sperm production. Once committed to differentiate, SSCs give rise successively to undifferentiated progenitor spermatogonia and differentiating spermatogonia through a species-specific number of transit amplifying mitotic divisions that can dramatically increase the yield of sperm from a single stem cell. Differentiating spermatogonia give rise to primary spermatocytes, which undergo two meiotic divisions to produce haploid spermatids that undergo spermiogenesis to produce terminally differentiated sperm.

*These authors contributed equally to this work.

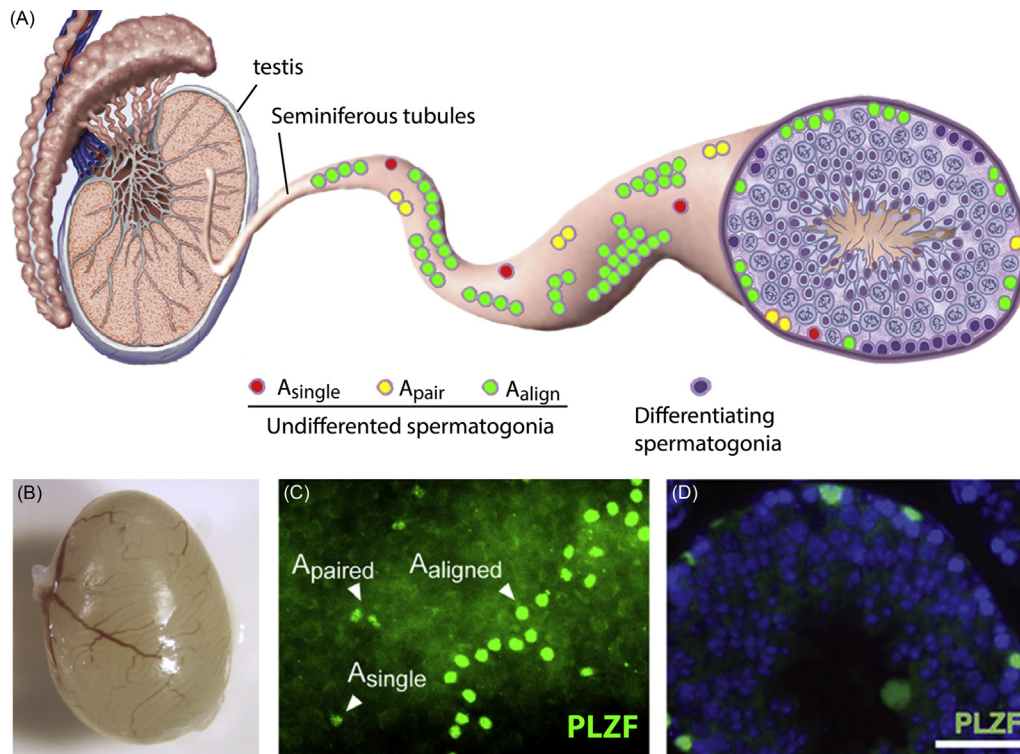


Fig. 2 Testis anatomy *in toto*, whole mount and cross-sectional views. (A) The mammalian testis is comprised of numerous seminiferous tubules where spermatogenesis occurs. Undifferentiated A_{single} (red), A_{paired} (yellow) and A_{aligned} (green) spermatogonia are located on the basement membrane of seminiferous tubules. Undifferentiated spermatogonia expand clonally through transit-amplifying mitotic divisions, where individual cells remain connected via intracytoplasmic bridges, and give rise to differentiating spermatogonia (purple). Differentiating spermatogonia, which are also located on the basement membrane of seminiferous tubules, undergo a species-specific number of transit amplifying divisions (see Fig. 3) before giving rise to primary spermatocytes that migrate off the basement membrane and initiate meiosis. (B) Macroscopic view of whole mouse testis. (C) Whole mount preparation of mouse seminiferous tubules stained with PLZF (ZBTB16), a marker of undifferentiated A_{single} , A_{paired} and A_{aligned} spermatogonia. (D) Cross section of seminiferous tubule stained with PLZF. Note that it is not possible to determine clonal arrangement (A_{single} , A_{paired} , A_{aligned}) in cross sectional views of the seminiferous tubules. Spermatogonial terminology is for rodent but conceptually relevant to spermatogonial development for monkeys and humans (see text).

to cytoplasmic ratio and diffuse chromatin (lacking heterochromatin) (Tegelenbosch and de Rooij, 1993). When A_{s} spermatogonia divide they produce A_{paired} (A_{pr}) spermatogonia (Fig. 2A, yellow) that either complete cytokinesis to produce two new A_{s} spermatogonia (self-renewal) or stay connected via an intracytoplasmic bridge and produce a chain of four A_{aligned} (A_{al4}) spermatogonia at the next division. Subsequent divisions of A_{al4} spermatogonia give rise to A_{al8} and A_{al16} spermatogonia (Fig. 2A, green). A_{s} , A_{pr} and A_{al} are collectively termed, undifferentiated spermatogonia. A_{s} and A_{pr} are equally distributed along the length of the seminiferous tubules, while A_{al} are distributed in a stage-dependent manner with peak numbers in the mid-stages before the appearance of differentiating type A1 spermatogonia (Tegelenbosch and de Rooij, 1993). The transition to differentiating type A1 spermatogonia can occur from clones of A_{s} , A_{pr} or A_{al4} , but most frequently occurs from clones of A_{al8} or A_{al16} (Nakagawa et al., 2010; Griswold and Oatley, 2013; de Rooij and Russell, 2000; de Rooij and Griswold, 2012). Eight successive transit amplifying divisions from A1 spermatogonia in rodents give rise to differentiating spermatogonial types A2, A3, A4, Intermediate and B, followed by primary spermatocytes, secondary spermatocytes and round spermatids. With a total of 10 transit amplifying mitotic divisions followed by two meiotic divisions, a single stem cell that commits to differentiate can theoretically produce 4096 sperm in rodents (Russell et al., 1990) (Fig. 3, Top row).

The Spermatogonial Stem Cell in Rodents

A_{s} , A_{pr} and A_{al} spermatogonia all have an undifferentiated appearance with homogenous, diffuse chromatin. They can be distinguished by clone size in whole mount preparations of seminiferous tubules (Fig. 2A center and C), but not in histological cross section (Fig. 2A right and D). This distinction is important because there is general consensus that A_{s} spermatogonia comprise at least a part of the spermatogonial stem cell pool and that stem cell activity decreases with increasing clone size. In 2011, Oatley and colleagues identified for the first time, a molecular marker (ID4) with expression that was limited to A_{s} spermatogonia on the basement membrane of seminiferous tubules (Oatley et al., 2011). ID4 represents a subpopulation of A_{s} spermatogonia; it is co-expressed with GFRa1 and PLZF, but represents a subpopulation of GFRa1⁺ and PLZF⁺ cells. ID4⁺ cells can be maintained and

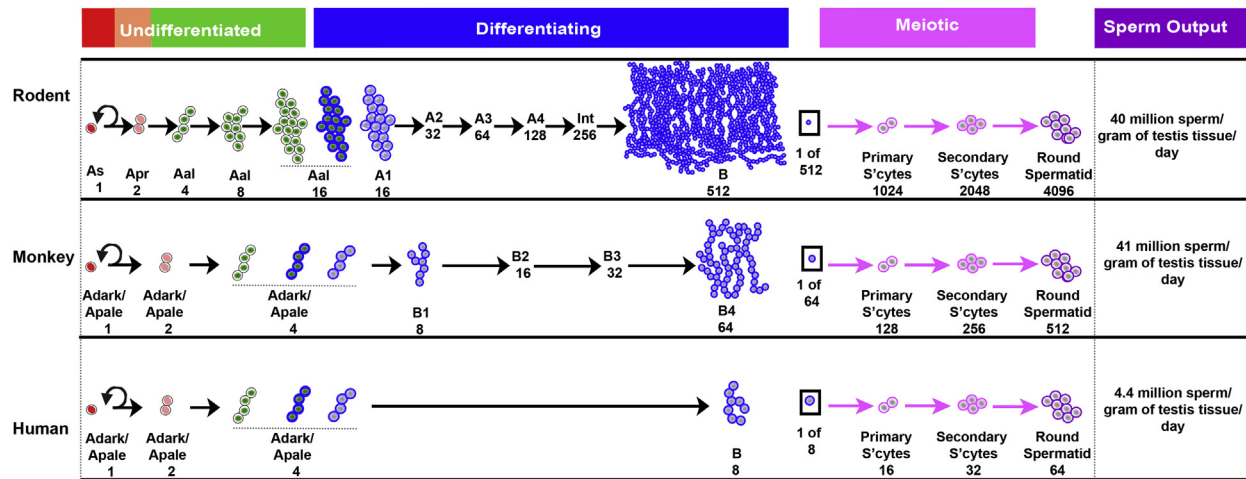


Fig. 3 Spermatogenic lineage development in rodents, monkeys and humans. In the rodent, monkey and human, spermatogonial development begins with a single spermatogonia ($A_{\text{single}}-A_s$ in rodent, $A_{\text{dark}}/A_{\text{pale}}$ in monkey and human)-red nucleated cell, which can divide to either self-renew or remain attached to form a clone of two cells ($A_{\text{paired}}-A_{\text{pr}}$)-orange nucleated cell. Depending on the species, the clone of two cells undergo one or more mitotic divisions as undifferentiated spermatogonia-green nucleated cells. Each mitotic division is represented by a black arrow. In the rodent, A_{aligned} (A_{al}) clones 4, 8 or 16 cells could result from these mitotic divisions before differentiation to type A1 spermatogonia without mitotic division. In monkeys and humans, $A_{\text{dark}}/A_{\text{pale}}$ clones of two or four divide and differentiate to become type B spermatogonia with clone sizes of four or eight. The number of mitotic divisions in differentiating spermatogonia (blue periphery cells) also vary by species. There are six generations of differentiating spermatogonia in the rodent. This includes the A1 (clone size-16), A2 (clone size-32), A3 (clone size-64), A4 (clone size-128), intermediate spermatogonia (Int, clone size-256) and type B spermatogonia (clone size-512). Differentiating type B1 spermatogonia in monkeys and type B spermatogonia in humans correspond to type A1 differentiating spermatogonia in rodents based on nuclear morphology and cKIT expression. There are four generations of differentiating type B spermatogonia in monkeys (B1–B4), but only one generation of differentiating B spermatogonia in humans, which has a significant impact on sperm output (right column). In all three species, the last generation of differentiated spermatogonia (blue cell in the box) undergo a division to enter meiosis (first pink arrow) and the resulting primary spermatocytes (two pink cells) undergo first meiotic division (second pink arrow), which results in the production of secondary spermatocytes (four pink cells). The second meiotic division (third pink arrow) results in postmeiotic round spermatids (eight purple cells). Sperm output per gram of testicular tissue per day from rodents, monkeys and humans is indicated in the last column.

expanded in culture and regenerate spermatogenesis when transplanted into infertile recipients. Relative to $ID4^{\text{bright}}$ cells, transplantable stem cell activity is substantially reduced in $ID4^{\text{dim}}$ and $ID4^-$ cells, but not absent (Oatley et al., 2011; Chan et al., 2014; Helsel et al., 2017). Since 2011, PAX7 (Aloisio et al., 2014), BMI1 (Komai et al., 2014) and EOMES (Braun et al., 2017) have also emerged as markers restricted to A_s spermatogonia on the basement membrane of seminiferous tubules. Each marker is expressed by a sub-population of $GFRa1^{+}$ cells and a subpopulation of A_s spermatogonia. Cells expressing each of these markers produce and maintain colonies of spermatogenesis after transplantation and/or in lineage tracing studies (see Table 1 for list of spermatogonial markers).

It seems reasonable to suppose that the stem cell pool also extends to some A_{pr} spermatogonia because A_s must transit through an A_{pr} state in the process of self-renewal (this concept is nicely reviewed in de Rooij and Griswold, 2012). In fact, self-renewal and differentiation could be precisely balanced if half of A_{pr} spermatogonia completed cytokinesis to produce two new A_s , while the other half produced chains of $A_{\text{al}4}$ at the next division. However, pulse labeling of $GFRa1^{+}$ cells indicated that the balance was skewed more toward the A_{pr} to $A_{\text{al}4}$ transition than the A_{pr} to 2 A_s transition. To reconcile this apparent imbalance, Hara and colleagues provided live video imaging $GFRa1$ -GFP spermatogonia data to suggest that clone fragmentation (e.g., $A_{\text{al}4}$ fragment to $A_{\text{al}3} + A_s$ or $A_{\text{pr}} + 2A_s$ or 4 A_s) was an important contributor to maintenance of the A_s pool (Hara et al., 2014) (Fig. 4). While the fate of the fragmenting clones could not be documented in that study, clones of $A_{\text{al}3}$ have been observed by others and apoptosis rarely occurs in undifferentiated spermatogonia (Suzuki et al., 2009; Gassei and Orwig, 2013). However, one concern with the clone fragmentation model is that it does not account for the contribution of $GFRa1$ negative cells to the pool of A_s (Suzuki et al., 2009; Gassei and Orwig, 2013) or the pool of transplantable stem cells (Grisanti et al., 2009; Garbuzov et al., n.d.).

Spermatogonial Tool Box for Rodents

Spermatogonial stem cells are rare in rodent testes because they are outnumbered by the progenitor and differentiating spermatogonia, spermatocytes and spermatids that they produce; and this challenges efforts to understand the molecular regulation of SSC function. Therefore, to learn the molecular features of SSCs, it was necessary to (1) study testes early in development prior to spermatogonial differentiation (Shima et al., 2004); (2) study testes in which differentiating germ cells were depleted (Orwig et al., 2008); (3) enrich transplantable SSCs by FACS or MACS (Hermann et al., 2015) or (4) modulating growth factors in SSC culture (Oatley et al., 2006). Each of these approaches identified molecules expressed by stem and progenitor spermatogonia and provided

Table 1 Spermatogonial markers

Germ cell marker ^a	Rodents			Nonhuman primates			Humans			Comments	References
	RT-PCR	IHC ICC	Transplant/ Lineage tracing	RT-PCR	IHC ICC	Transplant/lineage tracing	RT-PCR	IHC ICC	Transplant/ lineage tracing		
ID4 ^{Bright}	X	IHC-XS IHC-WM	LT and T				X	IHC-XS		A _s , A _d , A _p	Oatley et al. (2011), Helsel et al. (2017), Chan et al. (2014), Sachs et al. (2014), and Sun et al. (2015)
PAX7		IHC-XS	LT and T							A _s	Aloisio et al. (2014)
BMI1 ^{Bright}		IHC-XS	LT							A _s	Takada et al. (2007), Komai et al. (2014), and Zhang et al. (2008)
EOMES		IHC-XS IHC-WM	LT								Braun et al. (2017)
EXOSC10							X	IHC-XS		A _{dark}	von Kopylow et al. (2012a)
OCT2										A _{dark}	Lim et al. (2011)
FGFR3							X	IHC-XS ICH-WM		A _{dark} , A _{pale}	von Kopylow et al. (2012b) Zheng et al. (2014)
EGR3		IHC-WM								A _s , A _{pr}	Kent Hamra et al. (2004)
NANOS2	X	IHC-XS IHC-WM	LT					IHC-XS		A _s , A _{pr} , A _{dark} , A _{pale} , B-RS	Sada et al. (2009), Suzuki et al. (2007, 2009), Tsuda et al. (2003), and Kusz et al. (2009)
GFRA1		IHC-XS IHC-WM	LT and T		IHC-XS			IHC-XS		A _s -A _{al4} , A _{dark} , A _{pale} , B1, B2	Hermann et al. (2009), Grisanti et al. (2009), Maki et al., 2009, Buageaw et al. (2005), Zohni et al. (2012), He et al. (2010), Meng et al. (2000), Detlin et al. (2003), and Hara et al. (2014)
UTF1	X	IHC-XS					X	IHC-XS		A _s -A _{al4} , A _{dark} , A _{pale} , BM	Kristensen et al. (2008) van Bragt et al. (2008)
AXIN2		IHC-XS IHC-WM	LT							A _s -A _{al}	Takase and Nusse (2016)
ZBTB16		IHC-XS IHC-WM	T		IHC-XS		X	IHC-XS		A _s -A _{al} , A _{dark} , A _{pale} , B1, B2, BM	Hermann et al. (2009), Grisanti et al. (2009), He et al. (2010), Eildermann et al. (2012a); Wu et al., 2009, Buaas et al. (2004), Costoya et al. (2004), Hobbs et al. (2012), and Gassei and Orwig (2013)
SSEA4					IHC-XS	T		IHC-XS	T	Undifferentiated spermatogonia	Maki et al. (2009), Eildermann et al. (2012a); Muller et al. (2008), Izadyar et al. (2011), and Kokkinaki et al. (2011)
ENO2								IHC-XS		Spermatogonia on the BM	Valli et al. (2014)
SALL4		IHC-XS IHC-WM			IHC-XS			IHC-XS		A _s -A _{al} , A _{dark} , A _{pale} , B	Eildermann et al. (2012a,b); Hobbs et al. (2012), and Gassei and Orwig (2013)
FOXO1		IHC-XS IHC-WM								A _s -A _{al}	Goertz et al. (2011)
POU5F1 (OCT4)		IHC-XS IHC-WM	T		IHC-XS					A _s -A _{al} , BM	Mitchell et al. (2008), Pesce et al. (1998), and Ohbo et al. (2003)
POU3F1		IHC-XS		X						Spermatogonia on the BM	Wu et al. (2010)
RET		IHC-XS								A _s -A _{al}	Ballow et al. (2006)
BCLB6		IHC-XS		X						Rare cells on BM. RS in stage 7	Oatley et al. (2007)

(Continued)

Table 1 Spermatogonial markers—cont'd

Germ cell marker ^a	Rodents			Nonhuman primates			Humans			Comments	References
	RT-PCR	IHC ICC	Transplant/ Lineage tracing	RT-PCR	IHC ICC	Transplant/lineage tracing	RT-PCR	IHC ICC	Transplant/ lineage tracing		
LHX1		IHC-XS		X						Rare cells on BM.	Oatley et al. (2011)
ETV5		IHC-XS		X						RS in stage 7	Oatley et al. (2007)
SOX3		IHC-XS								On the BM	Raverot et al. (2005)
GPR125		IHC-XS IHC-WM	T		IHC-XS		X	IHC-XS ICC	T	A _S -A _{al} A _S -A _{al} , rare cells on the BM	He et al. (2010), Eildermann et al. (2012a); Wu et al. (2009), Seandel et al. (2007, 2008), Nickkholgh et al. (2014)
LIN28 (TEX17)		IHC-XS IHC-WM			IHC-XS			IHC-XS		A _S -A _{al} , rare cells on the BM	Aeckerle et al. (2012) Zheng et al. (2009)
UCHL1 (PGP9.5)		IHC-XS			IHC-XS		X	IHC-XS		Spermatogonia, cells on the BM	He et al. (2010), Eildermann et al. (2012a); Wu et al. (2009), and Wang et al. (2006)
DMRT1		IHC-XS IHC-WM					X	IHC-XS		A _S -B	Zhang et al. (2016) Jørgensen et al. (2012)
NGN3		IHC-XS IHC-WM	LT		IHC-XS					A _{pale} -Spc	Hermann et al. (2009), Raverot et al.
SOHLH1	X	IHC-XS IHC-WM			IHC-XS			IHC-XS		A _S -A _{al} , A _{pale} , B1–4, PS	(2005), and Yoshida et al. (2004, 2006)
NANOS3	X	IHC-XS IHC-WM								GFR α 1 [−] spermatogonia	Suzuki et al. (2012), Barrios et al. (2012), Ramaswamy et al. (2014), and Zhang et al. (2015)
SOHLH2	X	IHC-XS IHC-WM						IHC-XS		A _S -A1	Suzuki et al. (2007, 2009) Tsuda et al. (2003), and Lolicato et al. (2008)
CBL							X	IHC-XS		GFR α 1 [−] spermatogonia	Ballow et al. (2006), Suzuki et al. (2012), Barrios et al. (2012), Zhang et al. (2015), and Toyoda et al. (2009)
DSG2							X	IHC-XS		BM	(von Kopylow et al., 2010)
SAGE1								IHC-XS		BM	(von Kopylow et al. (2010)
TRA-1-81					IHC-XS					B	Lim et al. (2011)
MAGEA4					IHC-XS			IHC-XS		Rare cells on the BM	Eildermann et al. (2012a) Muller et al. (2008)
SNAP91							X	IHC-XS		BM, some spc	Zohni et al. (2012), He et al. (2010), Eildermann et al. (2012a) Mitchell et al. (2008), and Wyns et al. (2008)
RBM	X	IHC-XS						IHC-XS		BM, some spc	(von Kopylow et al. (2010)
RAR γ	X	IHC-XS IHC-WM								A _S -A4 A _{dark} , A _{pale} , B-RS	Jarvis et al. (2005), Elliott et al. (1996, 1997), Maymon et al. (2001), and Osterlund et al. (2001)
STRA8	X	IHC-XS IHC-WM					X			A _{al} -A1	Gely-Pernot et al. (2012)
ckIT	X	IHC-XS IHC-WM	T	X	IHC-XS		X	IHC-XS		A _S -RS	Giulii et al. (2002), Oulad-Abdelghani et al. (1996), Antonangeli et al. (2009), and Miyamoto et al. (2002)
										A _{al} -RS A _{pale} , B1–4, PS, BM	Hermann et al. (2009), Maki et al. (2009), He et al. (2010), Gassei and Orwig (2013), Izadyar et al. (2011), Shinohara et al. (2000), Sá et al. (2013), Manova et al. (1990), Yoshinaga et al. (1991), Schrans-Stassen et al. (1999), and Unni et al. (2009)

DAZL	X	IHC-XS			IHC-XS	X	IHC-XS	A _s -Spc	Wu et al. (2009), Hermann et al. (2007), Ruggiu et al. (1997), and Cooke et al. (1996)
VASA		IHC-XS			IHC-XS	X	IHC-XS	A _s -RS	Maki et al. (2009), Eildermann et al. (2012a); Wu et al. (2009), Tanaka et al. (2000), and Castrillon et al. (2000)
<i>Cell-surface^b</i> GFRa1		IHC-XS IHC-WM	LT and T		IHC-XS		IHC-XS	A _s -A _{al4} , A _{dark} , A _{pale} , B1, B2	Hermann et al. (2009), Grisanti et al. (2009), Maki et al. (2009), Buageaw et al. (2005), Zohni et al. (2012), He et al. (2010), Meng et al. (2000), Detlin et al. (2003), and Hara et al. (2014)
cKIT	X	IHC-XS IHC-WM	T	X	IHC-XS	X	IHC-XS	A _{al} -RS A _{pale} , B1–4, PS, BM	Hermann et al. (2009), Maki et al. (2009), He et al. (2010), Gassei and Orwig (2013), Izadyar et al. (2011), Shinohara et al. (2000), Sá et al. (2013), Manova et al. (1990), Yoshinaga et al. (1991), Schrans-Stassen et al. (1999), and Unni et al. (2009)
THY-1 (CD90)			T		T		IHC-XS T	BM	Hermann et al. (2009), Maki et al. (2009), He et al. (2010), Izadyar et al. (2011), Kubota et al. (2003), Conrad et al. (2008), and Ryu et al. (2004)
E-cadherin CDH1, CD324)		IHC-XS IHC-WM	T					A _s -A _{al}	Tokuda et al. (2007) Zhang et al. (2011)
CD9		IHC-XS	T				IHC-XS T	BM	Zohni et al. (2012), Kanatsu-Shinohara et al. (2004, 2011), and Paul et al. (2013)
β1-integrin (CD29)			T			X	IHC-XS	BM	Sá et al. (2013), Shinohara and Avarbock (1999), and Kanatsu-Shinohara et al. (2008)
α6-integrin (CD49f)			T		IHC-XS	X	IHC-XS ICC	BM	Maki et al. (2009), He et al. (2010), Izadyar et al. (2011), Shinohara et al. (2000), Sá et al. (2013), Conrad et al. (2008), Shinohara and Avarbock (1999), and Chen et al. (2009)
EpCAM		IHC-XS	T			X		BM	(Wu et al. (2009), Ryu et al. (2004), Kanatsu-Shinohara et al. (2011), Dovey et al. (2013), and Anderson et al. (1999)
SSEA4					IHC-XS T		IHC-XS T	Undifferentiated spermatogonia	(Maki et al. (2009), Eildermann et al. (2012a); Muller et al. (2008), Izadyar et al. (2011), and Kokkinaki et al. (2011)
GPR125		IHC-XS IHC-WM	T		IHC-XS	X	IHC-XS ICC	A _s -A _{al} , rare cells on the BM	He et al. (2010), Eildermann et al. (2012a); Wu et al. (2009), Seandel et al. (2007, 2008), and Nickkholgh et al. (2014)

X denotes that the technique was used. IHC-XS, immunohistochemistry cross-section; IHC-WM, immunohistochemistry whole-mount; ICC, immunocytochemistry; T, transplantation; LT, Lineage tracing; A_s, A single spermatogonia; A_{pr}, A paired spermatogonia; A_{al}, A aligned spermatogonia; A_d, A dark spermatogonia; A_p, A pale spermatogonia; B, type B spermatogonia; Spc, spermatocytes; RS, round spermatids; PS, pachytene spermatocytes; BM, basement membrane.

^aWe attempted to sort the germ cell markers in the order of their expression from undifferentiated to differentiating spermatogonia.

^bExamined with flow cytometry or FACS experiments. FGFR3 and TRA-1-81 are also cell surface markers but not included here because they have not been studied in the context of flow cytometry or FACS.

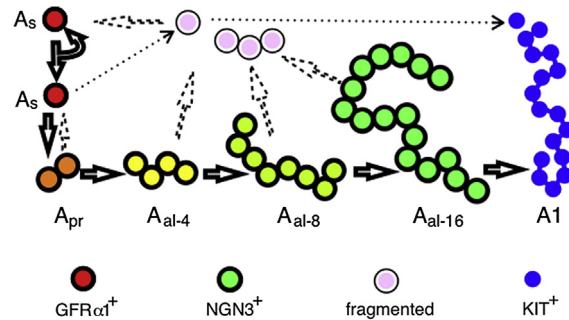


Fig. 4 *Clone fragmentation model.* In the rodent, molecular markers such as GFR α 1, NGN3 and KIT reveal the clone size of spermatogonia during development. GFR α 1 marks the A_{single} (A_s), A_{paired} (A_{pr}) and A_{aligned} (A_{al}) spermatogonia. However, the intensity of GFR α 1 expression diminishes progressively from A_{pr} to larger clones. In contrast, the intensity of NGN3 expression increases from smaller clones of A_{pr} and A_{al4} to larger clone of A_{al16}. The canonical progression for spermatogonial development is for A_s (red cell) to either self-renew through a complete mitotic division or to form A_{pr} (orange cell) through incomplete cytokinesis following mitotic division. A_{pr} then undergo one or more mitotic divisions to give rise to A_{al} clone of 4, 8 and 16 following one, two or three sequential mitotic divisions. A_{al16} then transit to A1 spermatogonia. However, the clone fragmentation model (Hara et al., 2014) opines that A_{al4} spermatogonia can fragment and contribute to replenishment of the pool of A_s spermatogonial stem cells. Larger clones can also fragment, but this occurs with lower frequency than fragmentation of A_{al4}. Figure reproduced with permission from Spradling, A. and Fan, C. M. (2010). Counterfeiting the family jewels. *Cell Stem Cell*, **6**, 405–406.

clues about pathways that regulate SSC self-renewal and differentiation. However, gene expression data generated from these approaches should be interpreted with caution because the cell populations were heterogeneous and approaches 1, 2 and 4 do not represent the steady state condition with homeostatic balance of self-renewal and differentiation. Although SSC culture does not represent steady state spermatogenesis, it has served as a powerful tool to dissect the mechanisms that regulate SSC self-renewal, differentiation and transplantation potential (Oatley et al., 2006; Lee et al., 2007; Yeh et al., 2011; Chapman et al., 2015).

It is tempting to speculate that a pure population of stem cells could be isolated from the adult testis (with steady state spermatogenesis) by sorting cells that express ID4, PAX7, BMI1 or EOMES. However, that approach could provide an incomplete picture because it is not certain that these four molecules mark the same cell population or that they capture the entire population of SSCs. It is possible, and perhaps likely, that the stem cell pool is dynamic and molecularly heterogeneous; reflecting changes that occur (1) at different stages of the cell cycle, (2) during development, (3) through the stages of the seminiferous epithelium or (4) under conditions of stress. A new generation of *single cell sequencing* technologies (Fluidigm, drop-seq and 10X genomics) now allow researchers to study the heterogeneous mammalian testis and SSC pool with a throughput and resolution that was not previously possible (Hermann et al., 2015; Guo et al., 2017). Furthermore, the drop-seq and 10X platforms can process hundreds of thousands of cells in a single run, which means it is possible to study all cell populations in the mammalian testis without the bias of preselection enrichment. These approaches will certainly expand the list of spermatogonial stem cell markers and may identify subpopulations of spermatogonia that were not previously recognized. So, how should researchers validate the expanding list of SSC markers?

The simplest and least stringent approach to validate candidate markers of undifferentiated stem/progenitor spermatogonia is by RT-PCR or immunocytochemical staining of an experimental cell population with known markers of undifferentiated spermatogonia (e.g., ID4, GFR α 1, PLZF). A more stringent approach is to examine the pattern of candidate marker expression in situ by immunohistochemical analyses in histological cross-section. A marker that is restricted to stem and/or progenitor spermatogonia should be expressed by a subpopulation of cells located on the basement membrane of seminiferous tubules (see PLZF for example, Fig. 2D). Co-staining in histological cross-section helps to establish whether the candidate marker is expressed by undifferentiated spermatogonia (e.g., co-stain with PLZF or GFR α 1), differentiating spermatogonia (e.g., co-stain with cKIT) or both, but does not provide any information about clone size. Immunohistochemical analyses in whole mount preparations of seminiferous tubules (see PLZF example in Fig. 2C) provides the same basement membrane localization and co-expression information as histological cross section as well as information about the clonal arrangement of marked cells. Unfortunately, markers that work well in histological cross section do not always work well in whole mount preparations of seminiferous tubules and vice versa. Each marker needs to be tested empirically in each approach with the appropriate negative and positive controls. In rodent, monkey and human testes, the most undifferentiated spermatogonia are arranged in the smallest clones of 1, 2 or 4 cells.

The descriptive methods described above are useful, but cannot demonstrate the biological potential of an experimental cell population to produce and maintain spermatogenesis, which is the hallmark of SSCs. Ralph Brinster and colleagues pioneered the method of SSC transplantation, which demonstrates the potential of an experimental cell population to regenerate spermatogenesis in the testes of infertile recipients (Brinster and Zimmermann, 1994; Brinster and Avarbock, 1994). More than 20 years after the initial reports, SSC transplantation remains the “gold standard” bioassay of spermatogonial stem cells. In recent years lineage tracing has emerged as a powerful tool that demonstrates the in vivo capacity of marker positive cells to produce and sustain spermatogenesis over a long period of time (Nakagawa et al., 2007; Hara et al., 2014; Aloisio et al., 2014; Komai et al., 2014). Lineage tracing results can be a little more difficult to quantify than transplantation, but have the advantage that stem cell potential can be assayed in the steady state condition of the adult testis.

Stem, Progenitor and Differentiating Spermatogonia in Nonhuman Primates and Humans

Nonhuman primate and human testes contain two morphologically distinct types of undifferentiated spermatogonia, classified as A_{dark} and A_{pale} based on differences in nuclear architecture and staining with hematoxylin in histological cross sections (Clermont and Leblond, 1959; Clermont, 1966). Clermont initially proposed that A1 spermatogonia (later renamed A_{dark}) were the SSCs that gave rise to progressively more differentiated A2 (later renamed A_{pale}) and B spermatogonia. However, he later revised this model based on observations that A_{dark} failed to label after a single bolus injection of [^3H]-thymidine. Since A_{dark} did not appear to self-renew under steady state conditions, he proposed that A_{dark} and A_{pale} represented reserve and active stem cells, respectively (Clermont, 1969). In this reserve stem cell model, spermatogenesis is maintained by the “active” A_{pale} SSCs that self-renew to maintain the A_{pale} pool and differentiate to produce spermatogenesis. The “reserve” A_{dark} spermatogonia are quiescent and become active only when A_{pale} are depleted by noxious insult. This notion is supported by observations in nonhuman primates and men indicating that X-irradiation caused a rapid decline in the pool of A_{pale} spermatogonia, which were later replenished by the surviving A_{dark} spermatogonia. Whether A_{dark} are truly quiescent and reserve or slow cycling stem cells that contribute to steady state spermatogenesis is uncertain. Chronic or repetitive administration of a mitotic labeling reagent followed by a period of washout would identify slow cycling stem cells that retain label for an extended period of time, a classic feature of adult tissue stem cells. This is an experiment that has been performed to identify slow cycling, label retaining A_{s} stem cells in rats (Huckins, 1971b) and could be performed in nonhuman primates.

A_{dark} and A_{pale} spermatogonia are present in equal numbers on the basement membrane of the seminiferous tubules and together represent 4% of germ cells in the testis, a frequency that is more than 100-fold greater than rodent A_{s} spermatogonia (0.03%). A_{dark} are evenly distributed along the length of the seminiferous tubules (Similar to rodent A_{s} and A_{pr}), while A_{pale} exhibit a stage-dependent distribution with peak numbers at mid-cycle (similar to rodent A_{al}) that precedes the appearance of differentiating type B1 spermatogonia. A_{dark} and A_{pale} are arranged predominantly as singles, pairs or chains of four (Clermont, 1969), while differentiating B1 spermatogonia appear as clones of 4 or 8, indicating that there are 1–2 transit amplifying divisions in the undifferentiated A_{dark} or A_{pale} spermatogonia before differentiating to B (Ehmcke et al., 2005). In monkeys, four additional transit-amplifying divisions from B1 produce differentiating spermatogonia types B2, B3 and B4, followed by primary spermatocytes. Based on expression of the differentiation marker, cKIT, differentiating type B spermatogonia in nonhuman primates and humans are equivalent to differentiating type A spermatogonia in rodents (Valli et al., 2015). In contrast to rodents, which have a small stem cell pool and many transit amplifying divisions to produce 40×10^6 sperm per gram of testicular parenchyma per day (Fig. 3, top), nonhuman primates achieve a similar sperm output ($41 \times 10^6/\text{g/day}$) with a relatively larger stem cell pool and few transit amplifying divisions (Fig. 3, middle). Sperm output in humans is lower than rodents or monkeys and this can be attributed to the fact that humans have only one generation of differentiating type B spermatogonia (Fig. 3, bottom).

Spermatogonial Tool Box for Nonhuman Primates and Humans

Many markers of undifferentiated and differentiating spermatogonia are conserved from rodents to nonhuman primates to humans (Table 1). While some markers appear species-specific, this could reflect lack of experimentation or antibody availability. Nonetheless, several good markers of undifferentiated, transitioning and differentiating spermatogonia are available and can be used for the methods of RT-PCR, immunocytochemistry (ICC), immunohistochemistry in histological cross-section (IHC-XS) or immunohistochemistry in whole mount preparations of seminiferous tubules (IHC-WM). High throughput single cell sequencing is as accessible for monkey and human studies as it is for mice, assuming the availability of normal tissues (Guo et al., 2017). Monkey to monkey spermatogonial stem cell transplantation is technically feasible and can demonstrate the potential of an experimental cell population to produce and maintain spermatogenesis, long-term (Hermann et al., 2012). However, this approach is not practical or accessible to most researchers as a routine bioassay and impossible for human application. Primate to mouse xenotransplantation has emerged as a valuable surrogate assay of monkey and human SSCs. SSCs from higher primates do not produce complete spermatogenesis in mouse seminiferous tubules. However, they do recapitulate several aspects of spermatogonial function that are not shared with other cell types: (1) they migrate through Sertoli cells to engraft the basement membrane of seminiferous tubules; (2) they proliferate to produce chains and clusters of cells with typical spermatogonial appearance and (3) they persist for long periods of time. One day it may be possible to recapitulate complete spermatogenesis from monkey or human cells in organoid cultures, as previously described for mice (Zhou et al., 2016). However, the mouse results still need to be replicated to establish a robust assay and translation to monkeys and humans will likely require a source of monkey and/or human fetal gonadal cells that are not widely available, if at all.

Concluding Remarks

While different vocabulary is used to describe stem, progenitor and differentiating spermatogonia in rodents, nonhuman primates and humans, many aspects of spermatogenic lineage development are conserved. Undifferentiated spermatogonia, including SSCs, exist as individual cells or small clones of interconnected cells. Transit-amplifying divisions lead to larger clones that are progressively more differentiated. Key differences in spermatogenic lineage development between rodents and higher primates include the

size of the stem cell pool and the number of transit-amplifying divisions that precede meiosis. Many markers of undifferentiated spermatogonia are conserved from mice to monkeys to humans (e.g., GFR α 1, ZBTB16, SALL4). This is true also for transitioning spermatogonia (e.g., SOHLH2, NGN3) and differentiating spermatogonia (e.g., cKIT) (See [Table 1](#) for complete list). It is important to understand how molecular markers of spermatogonia in monkeys and humans correspond with clone size and dark and pale descriptions of nuclear morphology. This knowledge helps to link contemporary studies with the classic literature and identifies parallels between rodents, monkeys and humans. This is particularly important because few modern-day investigators have the experience or patience to conduct classic morphometric studies that were common in the 1950s to 1970s.

Acknowledgements

AF, SD and CD contributed equally to this work with literature review and production of figures and tables. KEO wrote the manuscript. We are grateful to the pioneers cited in this chapter who provided insights using tools of the past that are equally impactful as the tools we use today. We have sprinkled our own experiences throughout this chapter from work that has been supported by the National Institutes of Health (RR18500, AG024992, HD055475, HD008610, HD061289, HD055475, HD075795, HD076412, HD092084), PA Department of Health Tobacco Funds, the US-Israel Binational Science, the Magee-Womens Research Institute and Foundation and the department of Obstetrics, Gynecology and Reproductive Sciences at the University of Pittsburgh School of Medicine.

References

- Aeckerle, N., et al. (2012). The pluripotency factor LIN28 in monkey and human testes: a marker for spermatogonial stem cells? *Molecular Human Reproduction*, 18(10), 477–488.
- Aloisio, G. M., Nakada, Y., Saatcioglu, H. D., Pena, C. G., Baker, M. D., Tarnawa, E. D., Mukherjee, J., Manjunath, H., Bugde, A., Sengupta, A. L., Amatruda, J. F., Cuevas, I., Hamra, F. K., & Castrillon, D. H. (2014). PAX7 expression defines germline stem cells in the adult testis. *The Journal of Clinical Investigation*, 124, 3929–3944.
- Anderson, R., et al. (1999). Expression of the homophilic adhesion molecule, Ep-CAM, in the mammalian germ line. *Journal of Reproduction and Fertility*, 116(2), 379–384.
- Antonangeli, F., et al. (2009). Expression profile of a 400-bp Stra8 promoter region during spermatogenesis. *Microscopy Research and Technique*, 72(11), 816–822.
- Ballow, D. J., et al. (2006). Sohlh2 is a germ cell-specific bHLH transcription factor. *Gene expression patterns : GEP*, 6(8), 1014–1018.
- Barrios, F., et al. (2012). SOHLH1 and SOHLH2 control Kit expression during postnatal male germ cell development. *Journal of Cell Science*, 125(Pt 6), 1455–1464.
- van Bragt, M. P., et al. (2008). GDNF expression of the pluripotency marker UTF1 is restricted to a subpopulation of early A spermatogonia in rat testis. *Reproduction*, 136(1), 33–40.
- Braun, R. E., Sharma, M., Srivastava, A., Fairfield, H. E., & Bergstrom, D. E. (2017). *Identification of slow-cycling long-term Spermatogonial stem cells and their regulation by PLZF*. Washington DC: Society for the Study of Reproduction.
- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11303–11307.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Buaas, F. W., et al. (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nature Genetics*, 36(6), 647–652.
- Buageaw, A., et al. (2005). GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. *Biology of Reproduction*, 73(5), 1011–1016.
- Castrillon, D. H., et al. (2000). The human VASA gene is specifically expressed in the germ cell lineage. *Proceedings of the National Academy of Sciences of the United States of America*, 97(17), 9585–9590.
- Chan, F., Oatley, M. J., Kaucher, A. V., Yang, Q.-E., Bieberich, C. J., Shashikant, C. S., & Oatley, J. M. (2014). Functional and molecular features of the Id4+ germline stem cell population in mouse testes. *Genes & Development*, 28, 1351–1362.
- Chapman, K. M., Medrano, G. A., Chaudhary, J., & Hamra, F. K. (2015). NRG1 and KITL signal downstream of retinoic acid in the germline to support soma-free syncytial growth of differentiating spermatogonia. *Cell Death Discovery*, 1, 15018.
- Chen, B., et al. (2009). Xeno-free culture of human spermatogonial stem cells supported by human embryonic stem cell-derived fibroblast-like cells. *Asian Journal of Andrology*, 11(5), 557–565.
- Clermont, Y. (1966). Renewal of spermatogonia in man. *The American Journal of Anatomy*, 118, 509–524.
- Clermont, Y. (1969). Two classes of spermatogonial stem cells in the monkey (*Cercopithecus Aethiops*). *The American Journal of Anatomy*, 126, 57–71.
- Clermont, Y., & Bustos-Obregon, E. (1968). Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted “in toto”. *The American Journal of Anatomy*, 122, 237–247.
- Clermont, Y., & Leblond, C. P. (1959). Differentiation and renewal of spermatogonia in the monkey, *Macacus rhesus*. *The American Journal of Anatomy*, 104, 237–273.
- Conrad, S., et al. (2008). Generation of pluripotent stem cells from adult human testis. *Nature*, 456(7220), 344–349.
- Cooke, H. J., et al. (1996). A murine homologue of the human DAZ gene is autosomal and expressed only in male and female gonads. *Human Molecular Genetics*, 5(4), 513–516.
- Costoya, J. A., et al. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature Genetics*, 36(6), 653–659.
- Detlin, L., et al. (2003). Morphological characterization of the spermatogonial subtypes in the neonatal mouse testis. *Biology of Reproduction*, 69(5), 1565–1571.
- Dovey, S. L., et al. (2013). Eliminating malignant contamination from therapeutic human spermatogonial stem cells. *The Journal of Clinical Investigation*, 123(4), 1833–1843.
- Ehmcke, J., Luetjens, C. M., & Schlatt, S. (2005). Clonal organization of proliferating spermatogonial stem cells in adult males of two species of non-human primates, *Macaca Mulatta* and *Callithrix Jacchus*. *Biology of Reproduction*, 72, 293–300.
- Eildermann, K., Gromoll, J., & Behr, R. (2012a). Misleading and reliable markers to differentiate between primate testis-derived multipotent stromal cells and spermatogonia in culture. *Human Reproduction*, 27(6), 1754–1767.
- Eildermann, K., et al. (2012b). Developmental Expression of the Pluripotency Factor Sal-Like Protein 4 in the Monkey, Human and Mouse Testis: Restriction to Premeiotic Germ Cells. *Cells, Tissues, Organs*, 196, 206–220.
- Elliott, D. J., et al. (1996). An RBM homologue maps to the mouse Y chromosome and is expressed in germ cells. *Human Molecular Genetics*, 5(7), 869–874.
- Elliott, D. J., et al. (1997). Expression of RBM in the nuclei of human germ cells is dependent on a critical region of the Y chromosome long arm. *Proceedings of the National Academy of Sciences*, 94(8), 3848–3853.
- Garbuzov, A., Pech, M. F., Sukhwani, M., Hasegawa, K., Zhang, R. J., Orwig, K. E. & Artandi, S. E. (February 13, 2018). Purification of GFR α 1+ and GFR α 1- spermatogonial stem cells reveals a niche-dependent mechanism for fate determination. *Stem Cell Reports*.
- Gassei, K., & Orwig, K. E. (2013). SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. *PLoS One*, 8, e53976.
- Gely-Pernot, A., et al. (2012). Spermatogonia differentiation requires retinoic acid receptor gamma. *Endocrinology*, 153(1), 438–449.
- Giulii, G., et al. (2002). Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. *EMBO Reports*, 3(8), 753–759.

- Goertz, M. J., et al. (2011). Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. *The Journal of Clinical Investigation*, 121(9), 3456–3466.
- Grisanti, L., Falcatori, I., Grasso, M., Dovore, L., Fera, S., Muciaccia, B., Fuso, A., Bero, V., Boitani, C., Stefanini, M., & Vicini, E. (2009). Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. *Stem Cells*, 27, 3043–3052.
- Griswold, M. D., & Oatley, J. M. (2013). Concise review: Defining characteristics of mammalian spermatogenic stem cells. *Stem Cells*, 31, 8–11.
- Guo, J., Grow, E. J., Yi, C., Mlcochova, H., Maher, G. J., Lindsog, C., Murphy, P. J., Wike, C. L., Carrell, D. T., Goriely, A., Hotaling, J. M., & Cairns, B. R. (2017). Chromatin and single-cell RNA-Seq profiling reveal dynamic signaling and metabolic transitions during human Spermatogonial stem cell development. *Cell Stem Cell*, 21, 533–546.e6.
- Gupta, G., Maikhuri, J. P., Setty, B. S., & Dhar, J. D. (2000). Seasonal variations in daily sperm production rate of rhesus and bonnet monkeys. *Journal of Medical Primatology*, 29, 411–414.
- Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., Simons, B. D., & Yoshida, S. (2014). Mouse Spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell*, 14, 658–672.
- He, Z., et al. (2010). Isolation, characterization, and culture of human spermatogonia. *Biology of Reproduction*, 82(2), 363–372.
- Helsel, A. R., Yang, Q. E., Oatley, M. J., Lord, T., Sablitzky, F., & Oatley, J. M. (2017). ID4 levels dictate the stem cell state in mouse spermatogonia. *Development*, 144, 624–634.
- Hermann, B. P., et al. (2007). Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. *Stem Cells*, 25(9), 2330–2338.
- Hermann, B. P., et al. (2009). Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. *Human Reproduction*, 24(7), 1704–1716.
- Hermann, B. P., Sukhwani, M., Winkler, F., Pascarella, J. N., Peters, K. A., Sheng, Y., Valli, H., Rodriguez, M., Ezzelarab, M., Dargo, G., Peterson, K., Masterson, K., Ramsey, C., Ward, T., Lienesch, M., Volk, A., Cooper, D. K., Thomson, A. W., Kiss, J. E., Penedo, M. C., Schatten, G. P., Mitalipov, S., & Orwig, K. E. (2012). Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. *Cell Stem Cell*, 11, 715–726.
- Hermann, B. P., Mutaji, K. N., Velte, E. K., Ko, D., Oatley, J. M., Geyer, C. B., & Mccarrey, J. R. (2015). Transcriptional and translational heterogeneity among neonatal mouse spermatogonia. *Biology of Reproduction*, 92, 54.
- Hobbs, R. M., et al. (2012). Functional antagonism between Sall4 and Plzf defines germline progenitors. *Cell Stem Cell*, 10(3), 284–298.
- Huckins, C. (1971a). The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. *The Anatomical Record*, 169, 533–557.
- Huckins, C. (1971b). The spermatogonial stem cell population in adult rats. III. Evidence for a long-cycling population. *Cell and Tissue Kinetics*, 4, 335–349.
- Huckins, C., & Oakberg, E. F. (1978). Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules. I. The normal testes. *The Anatomical Record*, 192, 519–528.
- Izadyar, F., et al. (2011). Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. *Human Reproduction*, 26(6), 1296–1306.
- Jarvis, S., et al. (2005). Molecular markers for the assessment of postnatal male germ cell development in the mouse. *Human Reproduction*, 20(1), 108–116.
- Jørgensen, A., et al. (2012). Analysis of meiosis regulators in human gonads: a sexually dimorphic spatio-temporal expression pattern suggests involvement of DMRT1 in meiotic entry. *MHR: Basic science of reproductive medicine*, 18(11), 523–534.
- Kanatsu-Shinohara, M., Toyokuni, S., & Shinohara, T. (2004). CD9 is a surface marker on mouse and rat male germline stem cells. *Biology of Reproduction*, 70(1), 70–75.
- Kanatsu-Shinohara, M., et al. (2008). Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. *Cell Stem Cell*, 3(5), 533–542.
- Kanatsu-Shinohara, M., et al. (2011). Dynamic Changes in EPCAM Expression during Spermatogonial Stem Cell Differentiation in the Mouse Testis. *PLoS One*, 6(8), e23663.
- Kent Hamra, F., et al. (2004). Defining the spermatogonial stem cell. *Developmental Biology*, 269(2), 393–410.
- Kokkinaki, M., Djourabchi, A., & Golestaneh, N. (2011). Long-term culture of human SSEA-4 positive spermatogonial stem cells (SSCs). *Journal of Stem Cell Research and Therapy*, S2, 003.
- Komai, Y., Tanaka, T., Tokuyama, Y., Yanai, H., Ohe, S., Omachi, T., Atsumi, N., Yoshida, N., Kumano, K., Hisha, H., Matsuda, T., & Ueno, H. (2014). Bmi1 expression in long-term germ stem cells. *Scientific Reports*, 4, 6175.
- von Kopylow, K., et al. (2010). Screening for biomarkers of spermatogonia within the human testis: a whole genome approach. *Human Reproduction*, 25(5), 1104–1112.
- von Kopylow, K., et al. (2012a). Differential marker protein expression specifies rarefaction zone-containing human Adark spermatogonia. *Reproduction*, 143(1), 45–57.
- von Kopylow, K., et al. (2012b). Fibroblast growth factor receptor 3 is highly expressed in rarely dividing human type A spermatogonia. *Histochemistry and Cell Biology*, 138(5), 759–772.
- Kristensen, D. M., et al. (2008). Presumed pluripotency markers UTF-1 and REX-1 are expressed in human adult testes and germ cell neoplasms. *Human Reproduction*, 23(4), 775–782.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2003). Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 100(11), 6487–6492.
- Kusz, K. M., et al. (2009). The highly conserved NANOS2 protein: testis-specific expression and significance for the human male reproduction. *Molecular Human Reproduction*, 15(3), 165–171.
- Lee, J., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Kimura, T., Nakano, T., Ogura, A., & Shinohara, T. (2007). Akt mediates self-renewal division of mouse spermatogonial stem cells. *Development*, 134, 1853–1859.
- Lim, J., et al. (2011). OCT2, SSX and SAGE1 reveal the phenotypic heterogeneity of spermatocytic seminoma reflecting distinct subpopulations of spermatogonia. *The Journal of Pathology*, 224(4), 473–483.
- Lolicato, F., et al. (2008). Potential role of Nanos3 in maintaining the undifferentiated spermatogonia population. *Developmental Biology*, 313(2), 725–738.
- Maki, C. B., et al. (2009). Phenotypic and molecular characterization of spermatogonial stem cells in adult primate testes. *Human Reproduction*, 24(6), 1480–1491.
- Manova, K., et al. (1990). Gonadal Expression of C-Kit Encoded at the W-Locus of the Mouse. *Development*, 110(4), 1057–1069.
- Maymon, B. B., et al. (2001). The contribution of RNA-binding motif (RBM) antibody to the histopathologic evaluation of testicular biopsies from infertile men. *Human Pathology*, 32(1), 36–41.
- Meng, X., et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287(5457), 1489–1493.
- Mitchell, R. T., et al. (2008). Germ cell differentiation in the marmoset (*Callithrix jacchus*) during fetal and neonatal life closely parallels that in the human. *Human Reproduction*, 23(12), 2755–2765.
- Miyamoto, T., et al. (2002). Isolation and expression analysis of the testis-specific gene, STRA8, stimulated by retinoic acid gene 8. *Journal of Assisted Reproduction and Genetics*, 19(11), 531–535.
- Muller, T., et al. (2008). Glycan stem-cell markers are specifically expressed by spermatogonia in the adult non-human primate testis. *Human Reproduction*, 23(10), 2292–2298.
- Nakagawa, T., Nabeshima, Y., & Yoshida, S. (2007). Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Developmental Cell*, 12, 195–206.
- Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R. E., & Yoshida, S. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science*, 328, 62–67.
- Nickkholgh, B., et al. (2014). Enrichment of spermatogonial stem cells from long-term cultured human testicular cells. *Fertility and Sterility*, 102(2), 558–565. e5.
- Oakberg, E. F. (1971). Spermatogonial stem-cell renewal in the mouse. *The Anatomical Record*, 169, 515–531.
- Oatley, J. M., Avarbock, M. R., Telaranta, A. I., Fearon, D. T., & Brinster, R. L. (2006). Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 9524–9529.
- Oatley, J. M., Avarbock, M. R., & Brinster, R. L. (2007). Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. *The Journal of Biological Chemistry*, 282(35), 25842–25851.

- Oatley, M. J., Kaucher, A. V., Racicot, K. E., & Oatley, J. M. (2011). Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. *Biology of Reproduction*, 85, 347–356.
- Ohbo, K., et al. (2003). Identification and characterization of stem cells in prepubertal spermatogenesis in mice. *Developmental Biology*, 258(1), 209–225.
- Orwig, K. E., Ryu, B. Y., Master, S. R., Phillips, B. T., Mack, M., Avarbock, M. R., Chodosh, L., & Brinster, R. L. (2008). Genes involved in post-transcriptional regulation are overrepresented in stem/progenitor spermatogonia of cryptorchid mouse testes. *Stem Cells*, 26, 927–938.
- Osterlund, C., et al. (2001). Specific localization of RBM1a in the nuclei of all cell types except elongated spermatids within seminiferous tubules of the human. *International Journal of Andrology*, 24(5), 272–277.
- Oulad-Abdelghani, M., et al. (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *The Journal of Cell Biology*, 135(2), 469–477.
- Paul, C., Nagano, M., & Robaire, B. (2013). Aging Results in Molecular Changes in an Enriched Population of Undifferentiated Rat Spermatogonia. *Biology of Reproduction*, 89(6), 147, 1–10.
- Pesce, M., et al. (1998). Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. *Mechanisms of Development*, 71(1-2), 89–98.
- Ramaswamy, S., et al. (2014). Spermatogonial SOHLH1 nucleocytoplasmic shuttling associates with initiation of spermatogenesis in the rhesus monkey (*Macaca mulatta*). *Molecular Human Reproduction*, 20(4), 350–357.
- Raverot, G., et al. (2005). Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Developmental Biology*, 283(1), 215–225.
- de Rooij, D. G. (1973). Spermatogonial stem cell renewal in the mouse. I. Normal situation. *Cell and Tissue Kinetics*, 6, 281–287.
- de Rooij, D. G., & Griswold, M. D. (2012). Questions about Spermatogonia posed and answered since 2000. *Journal of Andrology*, 33, 1085–1095.
- de Rooij, D. G., & Grootegoed, J. A. (1998). Spermatogonial stem cells. *Current Opinion in Cell Biology*, 10, 694–701.
- de Rooij, D. G., & Russell, L. D. (2000). All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology*, 21, 776–798.
- Ruggiu, M., et al. (1997). The mouse Dazl gene encodes a cytoplasmic protein essential for gametogenesis. *Nature*, 389(6646), 73–77.
- Russell, L. D., Ettlin, R. A., Sinhaikim, A. P., & Clegg, E. D. (1990). *Histological and histopathological evaluation of the testis*. Clearwater, FL: Cache River Press.
- Ryu, B. Y., et al. (2004). Phenotypic and functional characteristics of spermatogonial stem cells in rats. *Developmental Biology*, 274(1), 158–170.
- Sá, R., et al. (2013). Expression of stem cell markers: OCT4, KIT, ITGA6, and ITGB1 in the male germinal epithelium. *Systems Biology in Reproductive Medicine*, 59(5), 233–243.
- Sachs, C., et al. (2014). Evaluation of candidate spermatogonial markers ID4 and GPR125 in testes of adult human cadaveric organ donors. *Andrology*, 2(4), 607–614.
- Sada, A., et al. (2009). The RNA-Binding Protein NANOS2 Is Required to Maintain Murine Spermatogonial Stem Cells. *Science*, 325(5946), 1394–1398.
- Schrans-Stassen, B. H., et al. (1999). Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. *Endocrinology*, 140(12), 5894–5900.
- Seandel, M., et al. (2007). Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. *Nature*, 449(7160), 346–350.
- Seandel, M., et al. (2008). Niche players: spermatogonial progenitors marked by GPR125. *Cell Cycle*, 7(2), 135–140.
- Sharpe, R. M. (1994). Regulation of spermatogenesis. In E. Knobil, & J. D. Neill (Eds.), *The Physiology of Reproduction*. New York: Raven Press, Ltd.
- Shima, J. E., Mclean, D. J., McCarrey, J. R., & Griswold, M. D. (2004). The murine testicular transcriptome: Characterizing gene expression in the testis during the progression of spermatogenesis. *Biology of Reproduction*, 71, 319–330.
- Shinohara, T., Avarbock, M. R., & Brinster, R. L. (1999). β 1- and α 6-integrin are surface markers on mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 96(10), 5504–5509.
- Shinohara, T., et al. (2000). Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proceedings of the National Academy of Sciences of the United States of America*, 97(15), 8346–8351.
- Sun, F., et al. (2015). Id4 Marks Spermatogonial Stem Cells in the Mouse Testis. *Scientific Reports*, 5, 17594.
- Suzuki, A., Tsuda, M., & Saga, Y. (2007). Functional redundancy among Nanos proteins and a distinct role of Nanos2 during male germ cell development. *Development*, 134(1), 77–83.
- Suzuki, H., Sada, A., Yoshida, S., & Saga, Y. (2009). The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. *Developmental Biology*, 336, 222–231.
- Suzuki, H., et al. (2012). SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. *Developmental Biology*, 361(2), 301–312.
- Takada, Y., et al. (2007). Mammalian Polycomb Scmh1 mediates exclusion of Polycomb complexes from the XY body in the pachytene spermatocytes. *Development*, 134(3), 579–590.
- Takase, H. M., & Nusse, R. (2016). Paracrine Wnt/beta-catenin signaling mediates proliferation of undifferentiated spermatogonia in the adult mouse testis. *Proceedings of the National Academy of Sciences of the United States of America*, 113(11), E1489–97.
- Tanaka, S. S., et al. (2000). The mouse homolog of *Drosophila* Vasa is required for the development of male germ cells. *Genes and Development*, 14(7), 841–853.
- Tegelenbosch, R. A., & de Rooij, D. G. (1993). A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutation Research*, 290, 193–200.
- Thayer, K. A., Ruhlen, R. L., Howdeshell, K. L., Buchanan, D. L., Cooke, P. S., Preziosi, D., Welshons, W. V., Haseman, J., & Vom Saal, F. S. (2001). Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17 α -ethinyl oestradiol. *Human Reproduction*, 16, 988–996.
- Tokuda, M., et al. (2007). CDH1 Is a Specific Marker for Undifferentiated Spermatogonia in Mouse Testes. *Biology of Reproduction*, 76(1), 130–141.
- Toyoda, S., et al. (2009). Sohlh2 affects differentiation of KIT positive oocytes and spermatogonia. *Developmental Biology*, 325(1), 238–248.
- Tsuda, M., et al. (2003). Conserved role of nanos proteins in germ cell development. *Science*, 301(5637), 1239–1241.
- Unni, S. K., et al. (2009). Stage-specific localization and expression of c-kit in the adult human testis. *The Journal of Histochemistry and Cytochemistry*, 57(9), 861–869.
- Valli, H., et al. (2014). Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. *Fertility and Sterility*, 102(2), 566–580.
- Valli, H., Phillips, B. T., Gassei, K., Nagano, M. C., & Orwig, K. E. (2015). Spermatogonial stem cells and spermatogenesis. In T. M. PLANT, & A. J. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th ed). San Diego: Elsevier.
- Wang, Y.-L., et al. (2006). Overexpression of ubiquitin carboxyl-terminal hydrolase L1 arrests spermatogenesis in transgenic mice. *Molecular Reproduction and Development*, 73(1), 40–49.
- Wu, X., et al. (2009). Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. *Proceedings of the National Academy of Sciences of the United States of America*, 106(51), 21672–21677.
- Wu, X., et al. (2010). The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. *Biology of Reproduction*, 82(6), 1103–1111.
- Wyns, C., et al. (2008). Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. *Human Reproduction*, 23(11), 2402–2414.
- Yeh, J. R., Zhang, X., & Nagano, M. C. (2011). Wnt5a is a cell-extrinsic factor that supports self-renewal of mouse spermatogonial stem cells. *Journal of Cell Science*, 124, 2357–2366.
- Yoshida, S., et al. (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Developmental Biology*, 269(2), 447–458.
- Yoshida, S., et al. (2006). The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development*, 133(8), 1495–1505.
- Yoshinaga, K., et al. (1991). Role of C-Kit in Mouse Spermatogenesis - Identification of Spermatogonia as a Specific Site of C-Kit Expression and Function. *Development*, 113(2), 689–699.
- Zhang, S., et al. (2008). Expression localization of Bmi1 in mice testis. *Molecular and Cellular Endocrinology*, 287(1-2), 47–56.
- Zhang, Y., et al. (2011). E-cadherin can be expressed by a small population of rat undifferentiated spermatogonia in vivo and in vitro. *In Vitro Cellular & Developmental Biology. Animal*, 47(8), 593–600.

- Zhang, X., et al. (2015). Immunohistochemical Study of Expression of Sohlh1 and Sohlh2 in Normal Adult Human Tissues. *PLoS One*, 10(9), e0137431.
- Zhang, T., et al. (2016). DMRT1 Is Required for Mouse Spermatogonial Stem Cell Maintenance and Replenishment. *PLoS Genetics*, 12(9), e1006293.
- Zheng, K., et al. (2009). The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. *BMC Developmental Biology*, 9(1), 38.
- Zheng, Y., et al. (2014). Quantitative detection of human spermatogonia for optimization of spermatogonial stem cell culture. *Human Reproduction*, 29(11), 2497–2511.
- Zhou, Q., Wang, M., Yuan, Y., Wang, X., Fu, R., Wan, H., Xie, M., Liu, M., Guo, X., Zheng, Y., Feng, G., Shi, Q., Zhao, X. Y., Sha, J., & Zhou, Q. (2016). Complete meiosis from embryonic stem cell-derived germ cells in vitro. *Cell Stem Cell*, 18, 330–340.
- Zohni, K., et al. (2012). CD9 is expressed on human male germ cells that have a long-term repopulation potential after transplantation into mouse testes. *Biology of Reproduction*, 87(2), 27.

Further Reading

- Spradling, A., & Fan, C. M. (2010). Counterfeiting the family jewels. *Cell Stem Cell*, 6, 405–406.

Spermatocytes

John Schimenti and Priti Singh, Cornell University, Ithaca, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Primary spermatocyte A developing male germ cell derived from differentiation of a type B spermatogonium. It undergoes the major events of meiosis, including recombination and pairing of homologous chromosomes, before undergoing the first meiotic division.

Secondary spermatocyte A transient cell generated from the division of a primary spermatocyte. Each of these progeny cells undergoes an equational (mitotic-like) cell division to generate two haploid spermatids.

Synapsis Following “pairing,” or close alignment of maternal and paternal homologous chromosomes in primary spermatocytes, synapsis refers to the formation of the SC between these chromosome pairs.

Synaptonemal complex (SC) A structurally conserved, zipper-like tripartite proteinaceous structure along the entire length of homologous chromosomes. The SC is a hallmark feature of maturing primary spermatocytes, and serves to physically tether the homologs together at multiple points along their lengths.

Development of Spermatocytes

Spermatogenesis comprises three main phases: (I) mitoses of spermatogonia, (II) meiosis of spermatocytes ([Fig. 1](#)), and (III) spermiogenesis by which round spermatids change their shapes to become elongated spermatids. Here, we will discuss the transition from phase I to phase II.

Following several rounds of cell division, spermatogonia exit from the mitotic cell cycle and enter meiosis as spermatocytes. This complex process of transition from spermatogonium to spermatocyte has two important elements—*first*, commitment to the spermatogenic differentiation program, and *second*, activation of the meiotic program. Spermatogenic differentiation is influenced by the intricate balance between two sets of antagonistic factors—one promoting and/or maintaining the undifferentiated state of spermatogonial stem cells (SSCs), and the other promoting differentiation of spermatogonia. Retinoic acid (RA) signaling and meiotic competency of germ cells are two important components of differentiation. RA promotes meiotic prophase I entry through the induction of several RA-responsive elements (RARE) ([Koubova et al., 2014](#)). RA production in males begins during embryogenesis, however the induction of RA-responsive genes is delayed by the production by gonadal somatic cells of a cytochrome P450 enzyme (CYP26B1) that efficiently degrades RA. After birth, CYP26B1 is downregulated and RA produced by the testicular Sertoli cells acts upon RAREs of spermatogonia, prompting them to differentiate into primary spermatocytes that enter meiosis ([Saba et al., 2014](#)).

During their development, spermatocytes grow in size ([Bellve et al., 1977](#)). Importantly, during these extensive nuclear reorganization and proliferation processes, cohorts of germ cell types (spermatogonia, spermatocytes, and spermatids) undergo incomplete cytokinesis and are retained as syncytia of synchronously differentiating cells conjoined by narrow intercellular cytoplasmic bridges. Mouse primary spermatocytes undergo meiotic prophase I in a syncytium of over 500 cells, and these bridges are maintained through the meiotic divisions and well into the later stages of spermiogenesis. These bridges enable the sharing of cytoplasmic constituents between neighboring cells ([Braun et al., 1989](#)). The exact roles and functions in spermatogenesis are unclear, but the intercellular bridges are thought to coordinate the spermatogenic progression of developing cohorts, and also to enable haploid spermatids to be “phenotypically diploid,” that is, if the male heterozygous for a mutation in a crucial haploid expressed gene such as a protamine, the spermatids bearing the mutant copy are rescued by obtaining mRNA from the genetically WT syncytium-mates ([Greenbaum et al., 2011](#)). Furthermore, knockout of a gene (*Tex14*) that is required for intercellular bridge formation causes arrest of spermatogenesis at the prophase I spermatocyte stage ([Greenbaum et al., 2006](#)). Thus, meiosis in spermatocytes requires some function of this structure, but that function remains unknown.

Another important intercellular interaction is that between germ cells and Sertoli cells. Intimate contact between germ cells and Sertoli cells is initiated when seminiferous cords are formed early in testicular morphogenesis. However, their proliferation/differentiation schedules are distinct after birth. In the mouse, Sertoli cells proliferate in the perinatal period, and their proliferation stops ~15 days after birth. Conversely, male germ cells become mitotically quiescent after populating the fetal gonad. Soon after birth they begin proliferating and migrate to the basal lamina of the seminiferous tubule, after which their proliferation slows they undergo differentiation into spermatogonia. During this pubertal development, Sertoli cells interact and, under androgen regulation, establish specialized cell–cell junctions that are the functional basis for the basal and adluminal compartments of the seminiferous epithelium. As a critical event of early meiotic prophase, germ cells pass through these junctions and exit the basal compartment to establish themselves in the adluminal compartment as primary spermatocytes ([Smith and Braun, 2012](#)).

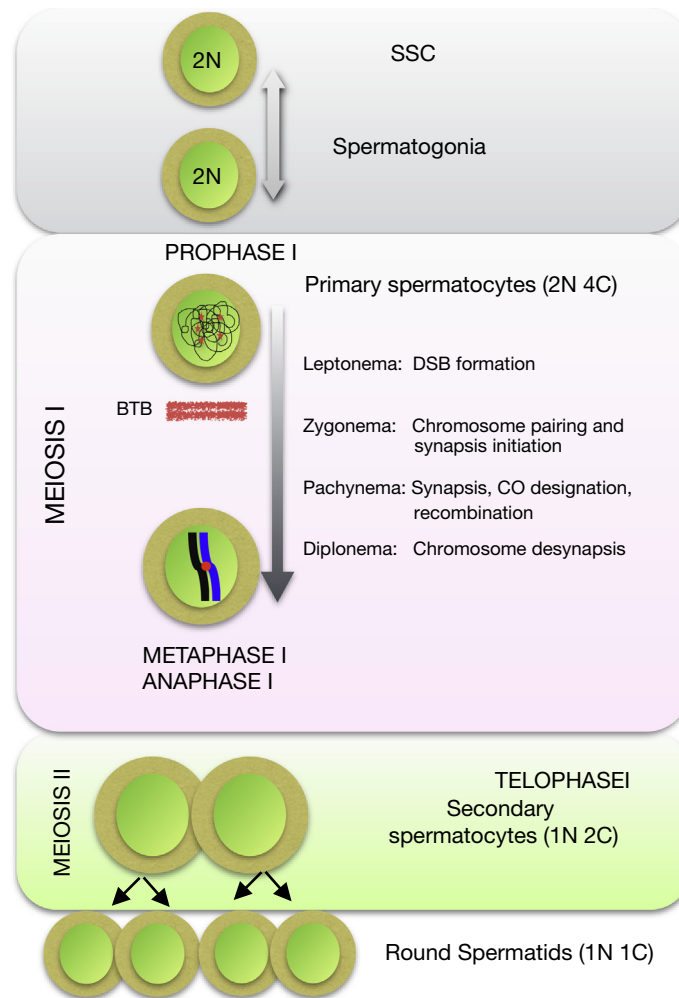


Fig. 1 Male gamete development. The *double ended arrow* in the spermatogonia panel denotes that proliferating “transit-amplifying” spermatogonia may revert to stem cell status. Once a spermatogonium enters meiosis, it is irreversible. SSC, spermatogonial stem cell; BTB, blood testis barrier; N, chromosome number; C, DNA content. The *red dot* in the Meiosis I cell represents a chiasma (crossover).

What Makes Spermatocytes Unique?

Spermatocytes are the only cells in males that undergo meiosis, a specialized cell division process characterized by a single round of DNA replication followed by two rounds of chromosome segregation. These divisions produce four haploid spermatids from a single primary spermatocyte (Fig. 2). The reduction in ploidy is essential for gametogenesis in all sexually reproducing organisms.

Spermatocytes are “born” from a Type B spermatogonium that undergoes a final instance of DNA replication called premeiotic DNA replication. The cells are now called primary spermatocytes, which are formally in “prophase I” of the G2 stage of the meiotic cell cycle. Prophase I lasts several days, during which the defining events of meiosis occur, namely alignment (pairing) and synapsis (intimate attachment) of the homologous chromosomes, and genetic recombination. The process of recombination, namely the subset of recombination events called crossovers, occurs at least once on every chromosome pair, leading to an infinite number of potential chromosome constitutions in each haploid spermatid product. At the end of prophase I, primary spermatocytes undergo the first meiotic division (MI), which is “reductional” (homologous chromosomes segregate to daughter cells, not sister chromatids). The resulting two cells are called secondary spermatocytes, which immediately undergo the second meiotic division (MII), which is “equational” (sister chromatids segregate to daughter cells). The resulting cells (total of four from each original spermatocyte) are haploid round spermatids (Fig. 2). The following sections describe these events in greater detail.

Homologous Chromosome Synapsis in Spermatocytes

The events leading to MI require that chromosomes first pair in a process that is dependent upon homologous recombination. Following homolog recognition and initial alignment, homologous chromosomes then synapse via the assembly of a proteinaceous

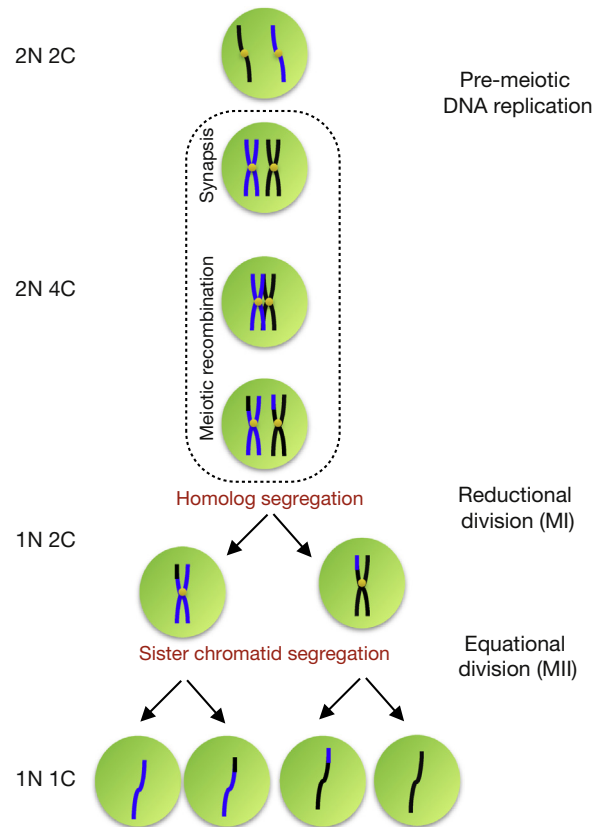


Fig. 2 Reductional and equational meiotic divisions. The *black* and *blue* chromosomes at the top represent homologs, not sister chromatids. N, chromosome number; C, DNA content.

structure called the synaptonemal complex (SC). The process of retaining the homologs together (at a distance of ~ 100 nm), in the context of the SC, is termed synapsis. The SC, which is unique to meiosis, reinforces the process of meiotic recombination. The chromatin of meiotic chromosomes is arranged into a series of loops originating from the SC-defined meiotic chromosome axis. Only a small fraction of the DNA is actually in contact with the SC along its linear length, which spans each autosome in its entirety.

MI is subdivided into four main substages: prophase I, metaphase I, anaphase I, and telophase I. Each stage is identified by the major characteristic events, which allow the dividing cell to progress toward the completion of meiosis. Prophase I is the longest stage, because it involved so many intricate events. Prophase I is subdivided into five substages based on the appearance and dynamics of meiotic chromosomes. The chromosomes can be cytologically defined by the physical state of SCs (**Fig. 3**).

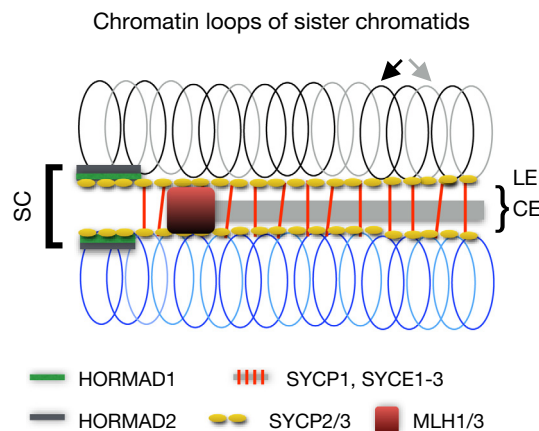


Fig. 3 Structure of synaptonemal complex (SC) during pachynema. SC, synaptonemal complex; LE, lateral element; CE, central element.

Chromosomes begin to condense during *preleptonema*. The SC assembly and homolog pairing follow this during *leptonema* (adjective = leptotene), which is particularly defined by the formation of immature SC fragments composed of two lateral elements (LEs) that begin forming along each chromosome. The LEs mature later into the axial elements (AEs). SYCP2 and SYCP3 are two major components of AE/LEs that create a bipartite structure along the bivalent axes. SYCP2 (but not SYCP3) serves as a linker between AE and the transverse filaments (TFs). The AE/LE coalign with cohesin cores (see below) and are connected by the TF, so that C-terminal end fastened to the LE and the other N-terminal to the central element (CE), which forms in later stages.

In *zygonema*, homologs begin to pair and CEs are deposited between the AE/LEs along the entire chromosome length. SYCP1 in mammals is a component of TFs, which is turn are a major component of the CE. There are many other proteins have been identified that associate transiently and provide stability to SCs. SYCP1 forms unstable homodimers by N-terminal self-associations and further require SC central element proteins 1–3 (SYCE1, SYCE2, and SYCE3) for stabilization and initiation of synapsis. These proteins are not only important for the structure of the SC in forming stable attachments between homologous chromosomes, but the axis proteins SYCP2/3, and also the HORMAD1 and HORMAD2 proteins, which bind only with unsynapsed chromosome regions of AEs are important for regulating recombination such that homologs, rather than sister chromatids, recombine (see below) (Rinaldi et al., 2017; Li et al., 2011; Shin et al., 2013). There are also other proteins that are important for synapsis, such as FKBP6, which are not discussed here (Noguchi et al., 2008).

During *pachynema*, synapsis occurs along the entire length of homologs and SCs are completely formed. This event allows close association between maternal and paternal axes. This is the stage when spermatocytes are fully prepared for recombination (described in sections below) between homologs. The physical connections established during this stage, as a consequence of cross-overs (chiasmata), are crucial for proper disjunction of homologous chromosomes to daughter cells at MI. They maintain physical attachment of homologs at the metaphase I plate. The onset of *diplonema* is described by the disassembly of the SC and homolog desynapsis. The final stage of prophase I is *diakinesis*, which quickly progresses into metaphase I.

Homologous Recombination (HR) in Primary Spermatocytes

HR is a mechanism, present in essentially all forms of DNA-based life, for repairing double stranded breaks (DSBs) in DNA. As indicated above, meiotic recombination is an essential and integral event in spermatocytes. The processes of stable homolog alignment, chromosome synapsis, and accurate MI disjunction are dependent upon HR. Since HR is a DNA repair mechanism, it requires generation of DSBs on the DNA during early prophase I. DSBs are generated by a topoisomerase type II-like protein, SPO11, and the action of this protein is essentially tantamount to meiotic initiation. These DSBs are nonrandomly distributed throughout the genome; they tend to cluster in discrete genomic regions called *hotspots*, and the locations of these hotspots are influenced in humans and mice by the action of the PRDM9 protein (Parvanov et al., 2010; Baudat et al., 2010). DSBs are processed to expose 3' overhanging ends that are bound by several enzymes, including recombinases called RAD51 and DMC1, which are homologs to bacterial RecA. These protein coated filaments conduct a "homology search" that ultimately ends with alignment to the allelic region of the homologous chromosome, invading the double helix of the homologous strand to form recombination intermediates. Ultimately these intermediates are resolved; ~90% of the time as "noncrossovers," and the remainder as crossovers.

Crossovers can be cytologically detected as structures called *chiasmata*. Chiasmata and cohesins along sister chromatid arms hold homologous chromosomes together during recombination and prior to their segregation in anaphase I. These processes occur by the end of prophase I, where *primary spermatocytes* undergo diakinesis and the first meiotic (reductional) division to produce two secondary spermatocytes, which divide equationally to generate the four haploid products (Fig. 2). Each spermatid is unique, the consequence of random combinations of recombination events in Prophase I and random segregation of recombinant homologs at the first meiotic division.

Meiotic Sex Chromosome Inactivation (MSCI) in XY Chromosomes

X and Y chromosomes in eutherian mammals are highly divergent in structure and gene content. In primary spermatocytes, these can synapse only through a small distal region of homology called as pseudoautosomal region (PAR). In pachynema, the paired XY chromosomes become sequestered into a defined heterochromatin-rich subdomain of the nucleus called the "sex body" (alternatively, the "XY body"). In this condensed state, the genes on the X and Y chromosomes are inaccessible to the transcriptional machinery, effectively causing chromosome-wide silencing, termed as "meiotic sex chromosome inactivation" (MSCI) (Handel et al., 1994). MSCI controls the unpaired regions of the X and Y and is critical for completion of meiosis in male mice. Experiments in mice and nematodes have shown that when either the X or Y chromosome is provided with a homologous pairing partner, it can form a synapsed bivalent that remains untouched by MSCI. These results established the sex chromosome *asynapsis* as the principal cause of MSCI. Upon entering pachynema, several proteins including BRCA1, TOPBP1, and ATR coat unsynapsed AEs of the sex chromosomes followed by their further spreading into the X and Y chromatin. The sex body chromatin is modified with specific histone marks associated with MSCI including H3K9me2, ubiquitylated H2A, and the phosphorylated histone variant of H2AX, γ H2AX. Together, these and other proteins enable MSCI (Hoyer-Fender, 2003).

Similar to MSCI, unpaired autosomes are also transcriptionally silenced by a process referred to as meiotic silencing of unpaired chromatin (MSUC) (Turner et al., 2005). Similar phenomena have been described in many organisms including eutherians,

marsupials, chickens, nematodes and fruit flies, and key features are conserved. In mammals, sites of asynapsis are associated with proteins that are components of DNA repair and DNA damage signaling machinery. Asynapsis triggers phosphorylation of many axis proteins, leading accumulation of the same protein and protein modifications that cause MSCI (Turner et al., 2004). These events ultimately trigger heterochromatinization and transcriptional silencing of unsynapsed regions.

Checkpoint and Genetic Quality Control Mechanisms in Spermatocytes

A fundamental charge of gametogenesis is to minimize the mutational load transmitted to offspring. Mutations and genetic aberrations have several forms, including single nucleotide changes, insertions/deletions, aneuploidy, and gross chromosomal rearrangements (GCRs). In vegetatively proliferating cells, errors in DNA replication are responsible for the majority of accumulated mutations. This is understandable given the task of duplicating a diploid genome consisting of 6 billion nucleotides. Whereas most mutations introduced during DNA replication entail single nucleotide changes and small indels associated with low complexity duplicated sequences, the mutagenic events to which meiotic cells are prone are more serious: aneuploidy and GCRs such as translocations and interstitial deletions. Gametic aneuploidy is the major cause of birth defects and spontaneous abortions in humans. Aneuploidy can occur during either MI or MII of meiosis, but the following discussion concentrates on the elaborate challenges to genome integrity in prophase I and the mechanisms to mitigate them.

As discussed earlier, proper chromosome disjunction at MI requires meiotic recombination, which in turn requires genetically-programmed DSBs. In a sense, these DSBs are a double edged sword; while essential for meiosis and fertility, they constitute a dangerous form of damage that can lead to gene disruption and GCRs. Under normal circumstances, the number of SPO11-induced DSBs is regulated so as to be sufficiently high to drive HR-driven chromosome synapsis, but sufficiently low such that all the DSBs can ultimately get repaired. Still, the system is not perfect. Accordingly, spermatocytes have adaptive mechanisms that render them highly sensitive to unrepaired DSBs and the presence of asynapsed chromosomes. The most prominent is the “pachytene checkpoint” that triggers apoptotic death of spermatocytes that fail to repair all DSBs, or which bear one or more asynapsed chromosomes. Mutant mice that are defective for DSB repair or synapsis die at Stage IV of the seminiferous tubule developmental cycle, equivalent to the mid-pachytene stage of meiosis (Barchi et al., 2005).

Experiments with mutant mice have identified MSCI and MSUC as being crucial processes in the pachytene checkpoint that prevent the production of chromosomally abnormal sperm. This is not immediately intuitive, but these mechanisms are essentially tied to conserved DNA damage response (DDR) pathways. Unsynapsed chromosomes or unrepaired DSBs activate canonical DDR components including the checkpoint sensor kinases ATM and ATR. BRCA1 and TOPBP1 act as cofactors promoting ATR activity in response to unsynapsed meiotic chromatin, which in turn phosphorylates histone H2AX. These events lead to meiotic silencing (MSUC for autosomes or MSCI in sex chromosomes). Because the same proteins are involved in sensing asynapsis and unrepaired DSBs, and some are present in limiting quantities, autosomal defects that trigger MSUC can weaken MSCI, causing apoptosis as described earlier. This disruption of MSCI is thought to constitute the major genetic quality control checkpoint in spermatocytes, although more canonical DDR signaling via the ATM signaling pathway is also active (Pacheco et al., 2015).

References

- Barchi, M., Mahadevaiah, S. D., Giacomo, M., Baudat, F., de Rooij, D. G., Burgoyne, P. S., Jasin, M., & Keeney, S. (2005). Surveillance of different recombination defects in mouse spermatocytes yields distinct responses despite elimination at an identical developmental stage. *Molecular and Cellular Biology*, 25, 7203–7215.
- Baudat, F., Buard, J., Grey, C., Fiedel-Alon, A., Ober, C., Przeworski, M., Coop, G., & de Massy, B. (2010). PRDM9 is a major determinant of meiotic recombination hotspots in humans and mice. *Science*, 327, 836–840.
- Bellve, A., Cavicchia, J., Millette, C., O'Brien, D., Bhatnagar, Y., & Dym, M. (1977). Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. *The Journal of Cell Biology*, 74, 68–85.
- Braun, R. E., Behringer, R. R., Peschon, J. J., Brinster, R. L., & Palmiter, R. D. (1989). Genetically haploid spermatids are phenotypically diploid. *Nature*, 337, 373–376.
- Greenbaum, M. P., Yan, W., Wu, M. H., Lin, Y. N., Agno, J. E., Sharma, M., Braun, R. E., Rajkovic, A., & Matzuk, M. M. (2006). TEX14 is essential for intercellular bridges and fertility in male mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 4982–4987.
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3, a005850.
- Handel, M. A., Park, C., & Kot, M. (1994). Genetic control of sex-chromosome inactivation during male meiosis. *Cytogenetics and Cell Genetics*, 66, 83–88.
- Hoyer-Fender, S. (2003). Molecular aspects of XY body formation. *Cytogenetic and Genome Research*, 103, 245–255.
- Koubova, J., Hu, Y. C., Bhattacharyya, T., Soh, Y. Q., Gill, M. E., Goodheart, M. L., Hogarth, C. A., Griswold, M. D., & Page, D. C. (2014). Retinoic acid activates two pathways required for meiosis in mice. *PLoS Genetics*, 10, e1004541.
- Li, X. C., Bolcun-Filas, E., & Schimenti, J. C. (2011). Genetic evidence that synaptonemal complex axial elements govern recombination pathway choice in mice. *Genetics*, 189, 71–82.
- Noguchi, J., Ozawa, M., Nakai, M., Somfai, T., Kikuchi, K., Kaneko, H., & Kunieda, T. (2008). Affected homologous chromosome pairing and phosphorylation of testis specific histone, H2AX, in male meiosis under FKBP6 deficiency. *The Journal of Reproduction and Development*, 54, 203–207.
- Pacheco, S., Marcet-Ortega, M., Lange, J., Jasin, M., Keeney, S., & Roig, I. (2015). The ATM signaling cascade promotes recombination-dependent pachytene arrest in mouse spermatocytes. *PLoS Genetics*, 11, e1005017.
- Parvanov, E. D., Petkov, P. M., & Paigen, K. (2010). *Prdm9* controls activation of mammalian recombination hotspots. *Science*, 327, 835.
- Rinaldi, V. D., Bolcun-Filas, E., Kogo, H., Kurahashi, H., & Schimenti, J. C. (2017). The DNA damage checkpoint eliminates mouse oocytes with chromosome synapsis failure. *Molecular Cell*, 67(1026–1036), e2.
- Saba, R., Wu, Q., & Saga, Y. (2014). CYP26B1 promotes male germ cell differentiation by suppressing STRA8-dependent meiotic and STRA8-independent mitotic pathways. *Developmental Biology*, 389, 173–181.

- Shin, Y. H., McGuire, M. M., & Rajkovic, A. (2013). Mouse HORMAD1 is a meiosis I checkpoint protein that modulates DNA double-strand break repair during female meiosis. *Biology of Reproduction*, 89, 29.
- Smith, B. E., & Braun, R. E. (2012). Germ cell migration across Sertoli cell tight junctions. *Science*, 338, 798–802.
- Turner, J. M., Aprelikova, O., Xu, X., Wang, R., Kim, S., Chandramouli, G. V., Barrett, J. C., Burgoyne, P. S., & Deng, C. X. (2004). BRCA1, histone H2AX phosphorylation, and male meiotic sex chromosome inactivation. *Current Biology*, 14, 2135–2142.
- Turner, J. M., Mahadevaiah, S. K., Fernandez-Capetillo, O., Nussenzweig, A., Xu, X., Deng, C. X., & Burgoyne, P. S. (2005). Silencing of unsynapsed meiotic chromosomes in the mouse. *Nature Genetics*, 37, 41–47.

Spermatids

Barry R Zirkin, Johns Hopkins University, Baltimore, MD, United States
Erwin Goldberg, Northwestern University, Evanston, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome A membrane bound organelle closely applied to the surface of spermatids and spermatozoa. Formed by the Golgi apparatus of early spermatids, it is rich in hydrolytic enzymes and important for fertilization.

Axoneme The central portion of the sperm tail composed of a microtubular array with associated structural proteins.

Fibrous sheath The cytoskeletal structure along the principle piece of the tail. Glycolytic enzymes important for sperm metabolism are tightly bound to the fibrous sheath.

Manchette Appears in elongating spermatids as a sheath extending from the nucleus, surrounding the tail and disappearing during the late steps of spermiogenesis; involved in protein transport to the base of the axoneme.

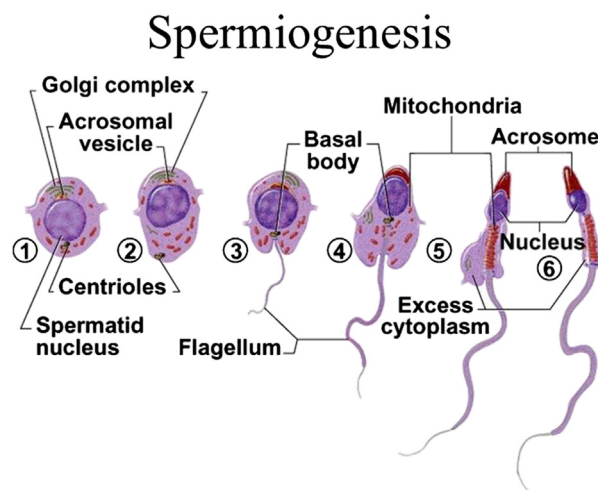
Outer dense fibers Major cytoskeletal elements of the tail of elongated spermatids and spermatozoa. In the middle piece there are nine ODFs, one per axonemal doublet. In the principle piece of the tail only seven ODFs are present.

Introduction

As described in the section “Overview of Spermatogenesis,” building a sperm consists of the formation of spermatogonia from spermatogonial stem cells, mitotic divisions of the spermatogonia to form spermatocytes, two meiotic divisions of the spermatocytes that result in the formation of spermatids, and spermatid differentiation to form spermatozoa. The latter process, spermiogenesis, is the focus of this article.

The round spermatids that result from meiosis exhibit a remarkable transformation during spermiogenesis to become flagellated spermatozoa with extremely compact nuclei. During this process, the round spermatids undergo profound remodeling in sequential “steps” to form elongated spermatids. The steps are distinguished from each other by the specific structural changes that occur in the forming acrosome and the nucleus. In addition to morphological changes, there are molecular events that lead to the formation of the sperm acrosome, mid-piece and flagellum. The morphological and molecular changes that occur during spermiogenesis are critically important in achieving production of functional male gametes.

During the cellular transformation of haploid round spermatids to spermatozoa, major structural changes occur that include formation of the acrosome from the Golgi apparatus, nuclear elongation, chromatin condensation, axoneme formation triggered by the centriolar complex, and assembly of sperm-specific cytoskeletal elements including the mitochondrial sheath surrounding the axoneme. The distinct steps of spermiogenesis identified in man (Oko and Clermont, 1999) are depicted in Fig. 1. Recently,



- Changes that transform spermatids into spermatozoa
 - discarding excess cytoplasm & growing tails

Fig. 1 Depiction of transition steps from round spermatid to elongated spermatozoan. From Oko, R and Clermont, Y 1999. Spermiogenesis. *Encyclopedia of Reproduction*, Vol. 4 Pro-Z, pp. 602–609. Cambridge, MA: Academic Press.

high-resolution light microscopy (HRLM) has enabled improved morphological identification of all germ cells (spermatogonia, spermatocytes, spermatids) in the human testis (Nihi et al., 2017). This approach has led to a highly reliable way to identify the events of spermatogenesis in man. Precise staging of germ cells is important for predicting causes of aberrant spermatogenesis and infertility.

Spermiogenesis

Spermatid development, or spermiogenesis, extends over 22.7 days in rats and 21.6 days in humans. Formation of the acrosome is a key event in spermatid development. The acrosome is a membrane-bound organelle on the surface of the nuclei of spermatids and spermatozoa. Acrosome formation begins in the early spermatid by coalescence of proacrosomic vesicles to a single large vesicle which tightly adheres to the nuclear membrane. The acrosomal vesicle is rich in hydrolytic enzymes that are released during the acrosome reaction, a critical step in fertilization that enables sperm penetration of the zona pellucida of the egg.

DNA Compaction

Morphological remodeling of the spermatid involves nuclear elongation and chromatin condensation, producing the species-specific form that is characteristic of the sperm head. During these processes, there is reorganization of the haploid genome and compaction of DNA. Human sperm chromatin undergoes a complex remodeling in which histones are replaced by protamines during a regulated transition that involves histone modifications (H1t histone is modified or lost) as well as intermediate and temporary replacement of the histones by sperm-specific transition nucleoproteins TP1 and TP2. Chromatin compaction is influenced by TP1 and TP2. These transition proteins are then replaced late in spermiogenesis by arginine and cysteine-rich protamine. Protamine polymorphisms can lead to compaction defects, increased susceptibility to DNA damage, and infertility. The replacement of most histones by protamine 1 (PRM1) and protamine 2 (PRM2) facilitates a high order of chromatin packaging necessary for normal sperm function, and may also be required for DNA silencing and imprinting changes (Carrell et al., 2007).

Epigenetic modifications, characterized by DNA methylation, histone substitutions, and chromatin remodeling, also are important regulators in spermiogenesis. Several genes in the germinal epithelium are regulated through epigenetic mechanisms, and there have been suggestions of an influence of epigenetics on spermatogenesis. Epimutations (often hypermethylation) in several genes (e.g., MTHFR, PAX8, NTF3, SFN, HRAS, JHM2DA, IGF2, H19, RASGRF1, GTL2, PLAG1, D1RAS3, MEST, KCNQ1, LIT1, SNRPN) have been reported to be associated with poor semen parameters or male infertility. Recent reports suggest that epigenetic modifications in mature sperm may also have effects on embryo development (Carrell and Hammoud, 2010).

The Manchette (Acrosome and Tail Formation)

Spermiogenesis also involves the elongation of spermatids and removal of excess cytoplasm, as well as the formation of the acrosome and tail. Protein transport during spermatid elongation is required for the formation of the sperm tail and acrosome, and for shaping of the sperm head. Proteins are transported to the developing sperm head and tail by the manchette, a microtubular structure that surrounds the elongating spermatid head and that is present only during spermatid elongation, and by the axoneme, a microtubule-based cytoskeleton composed of nine pairs of microtubules doublets and two central microtubules that is the core structure of the sperm tail depicted in Fig. 2 from Chen et al. (2016).

In the mouse and human, the manchette is first seen first in mid-spermiogenesis (step 8 in the mouse, and step 5 in the human; see Fig. 1). It forms between the perinuclear ring that surrounds the nucleus and the elongating sperm axoneme. Much is now known about the assembly, disassembly and function of the manchette, and the roles of these processes in spermatid head-shaping and sperm tail formation, including the molecular events that are involved (Lehti and Sironen, 2016). Among the molecular players is SPEM1, a protein expressed only in developing spermatids (Bao et al., 2011). Inactivation of Spem1 in mice has been shown to result in deformed spermatozoa. UBQLN1 has been identified as a SPEM1-interacting partner (Bao et al., 2010). UBQLN1 and SPEM1 have been co-localized to the manchette of elongating spermatids. UBQLN1 functions through binding and directing poly-ubiquitinated proteins to the proteasome for degradation. Consequently, the interactions shown to occur between UBQLN1 and SPEM1 suggest a role for UBQLN1 in regulating protein ubiquitination during spermiogenesis. Ran-binding protein 17 (RANBP17) also was identified as an interacting partner of SPEM1 (Bao et al., 2011). RANBP17 was shown to localize to the XY body of primary spermatocytes, later in spermatogenesis to the nuclei of round spermatids (steps 1–7), and finally, together with SPEM1, to the manchette of elongating spermatids (steps 8–14). As a member of a protein family involved in nucleocytoplasmic transport, RANBP17 has been suggested as possibly playing a role in sex chromosome inactivation during meiosis and/or in intramanchette transport during spermiogenesis. Interactions between RANBP17 and SPEM1 suggest that SPEM1 might function in RANBP17-mediated nucleocytoplasmic transport.

Observations by electron microscopy have revealed rod-like elements linking the manchette microtubules to the nuclear membrane (Russell et al., 1994). The manchette appears to be connected through a protein complex called the LNC complex (linker

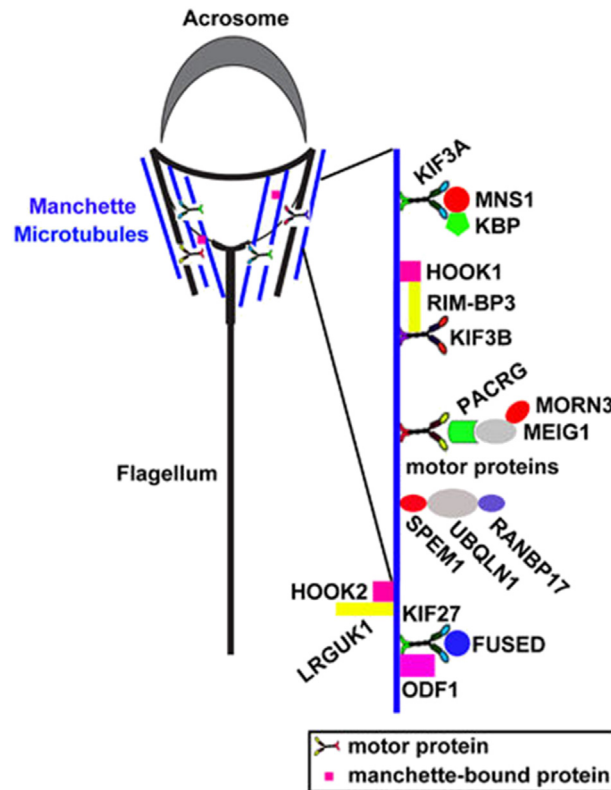


Fig. 2 Formation of Manchette during spermiogenesis. From Chen, S.R. et al. 2016. *Cell Death & Disease* 7(11), e2472.

of nucleoskeleton and cytoskeleton). Two LINC complexes have been identified during spermatid elongation: the SUN3/Nesprin 1 protein complex, which has been shown to connect the manchette to the nucleus; and SUN1/Nesprin 3 which, with SUN3/Nesprin, has been localized to sites at which manchette microtubules contact the nuclear envelope. It has been suggested that the LINC complex may be involved in nuclear shaping (Göb et al., 2010; Calvi et al., 2015).

Successful sperm production requires proteins and protein complexes to be transported to the correct assembly sites during sperm tail formation. However, the exact roles of microtubule and actin-based delivery complexes remain uncertain. Some proteins appear to be transported through the manchette to the base of the developing sperm tail. However, not all sperm-tail proteins have been localized to the manchette, raising the prospect that different, more complex, mechanisms for protein storage and transport may be involved in sperm formation (Lehti and Sironen, 2016). Functional studies will provide a more complete understanding of the mechanisms involved in constructing a fertile sperm, important for developing therapies for male infertility and perhaps for identifying targets for a reversible male contraceptive technology.

Formation of the Tail

Sperm tail formation is an important step in remodeling the spermatid. Early in spermiogenesis, a pair of centrioles migrates toward the nucleus and eventually binds to it (Fig. 1). Formation of the central component, the axoneme, is initiated by one of the pair. The axoneme is comprised of a characteristic “9 + 2” microtubule arrangement, dynein arms, radial spokes, and the nexin-dynein regulatory complex (N-DRC) (Inaba, 2011). As noted above, there is an intimate association of the nucleus and manchette for delivery of essential proteins. It currently is hypothesized that proteins are moved by intramanchette transport to the base of the sperm tail and to the developing sperm tail itself by intraflagellar transport. Outer dense fibers and a fibrous sheath appear shortly after the axoneme completes its growth. The T-complex-associated-testis-expressed 1 (TCTE1) evolutionarily conserved axonemal protein localizes to the nexin-dynein regulatory complex and is testis-enriched in its expression, with its mRNA appearing in early round spermatids and protein localized to the flagellum. TCTE1 is 498 aa in length with a leucine rich repeat domain at the C terminus. Recent studies (Castaneda et al., 2017) revealed that knockout of Tcte1 results in male sterility. Tcte1-null spermatozoa show aberrant motility. Although the axoneme is structurally normal in Tcte1 mutant spermatozoa, Tcte1-null sperm demonstrate a significant decrease of ATP, which is used by dynein motors to generate the bending force of the flagellum. As the authors note, these data provide a link to defining the molecular intricacies required for axoneme function, sperm motility, and male fertility as discussed below.

Sperm Tail Enzymes

While structure and motility are relatively well-defined, the functions of the outer dense fibers and fibrous sheath are less clear. Most of the glycolytic enzymes responsible for meeting sperm energy requirements are tightly bound to the fibrous sheath. These enzymes include a sperm specific form of glyceraldehyde phosphate dehydrogenase (GAPDH-S) and lactate dehydrogenase A (LDHA). The most abundant sperm specific protein, LDHC, is cytosolic and distributed throughout the sperm tail. Many species of spermatozoa, including mouse and human, generate ATP by aerobic glycolysis to satisfy their energy requirements even though the mid-piece houses the mitochondria that in most cells are the efficient energy producers. Both GAPD-S and LDHC have been proven to be required for sperm function by targeted disruption of the cognate genes. When and how these sperm proteins become distributed to the sperm tail is not obvious. For example, the *Ldhc* gene is transcribed and translated prior to and during meiosis, whereas *Gapds* may be transcribed in spermatocytes but not translated until meiosis is completed. Clearly, it would be expected that the transcriptional apparatus is disabled in haploid cells. To date, most of the testicular mRNAs known to be translationally regulated are initially transcribed in postmeiotic cells. There are numerous studies in the last decade showing that mRNA is stored (often referred to as “masked mRNA”) and is translated during spermiogenesis (Xu and Hecht, 2008). Additionally, there are examples of post-transcriptional modifications of gene products during spermiogenesis that are beyond the scope of this article. Generally speaking there are changes in transcription and translation throughout spermatogenesis, including spermiogenesis.

Mitochondria Migration

In elongating rodent and human spermatids, the mitochondria migrate toward the forming tail as the manchette appears (Fig. 1). The mitochondria assemble along outer dense fibers, between the neck and the annulus, and assemble into a spiral structure in what becomes the mid-piece of the sperm tail. The length of the sperm tail is species specific.

Intercellular Bridges

Intercellular bridges connect all the spermatids originating from the same stem cell, a consequence of the incomplete cytokinesis of spermatogonia during mitosis and of spermatocytes during meiosis. Disruption of the intercellular bridges can cause male infertility. The importance of intercellular bridges is clear. Thus, movement of molecules among haploid cells is made possible by intercellular bridges, and this movement is likely to be involved in the sharing of mRNAs among germ cells. This sharing, in turn, is important for sperm production because numerous essential proteins are encoded on sex chromosomes (Kim et al., 2015).

Spermiation

When the remodeling process of the spermatid has been completed, elongated spermatids are ready for release from the germinal epithelium. The action of the steroid hormones estrogen and androgen, which are known to control transcription in various reproductive and nonreproductive tissues, are important for the control of sperm release from the seminiferous epithelium (spermiation). Spermiation is characterized by extensive remodeling of actin filaments and endocytosis. There is evidence suggesting that genes regulated by estrogen and androgen may in some way be involved in actin remodeling and endocytosis.

Summary

This outline of spermiogenesis briefly describes the exquisitely complex structural and molecular intracellular changes required for producing and shaping what is destined to become a functional male gamete. Other chapters will describe the involvement of Sertoli cell–germ cell interactions, and will provide further details about the contribution of Leydig cells in the interstitium. These interactions too are essential for completing the highly-ordered process necessary for sperm production and thus survival of the species.

References

- Bao, J., Zhang, J., Zheng, H., Xu, C., & Yan, W. (2010). UBQLN1 interacts with SPEM1 and participates in spermiogenesis. *Molecular and Cellular Endocrinology*, 327(1–2), 89–97. <https://doi.org/10.1016/j.mce.2010.06.006>.
- Bao, J., Wu, Q., Song, R., Jie, Z., Zheng, H., Xu, C., & Yan, W. (2011). RANBP17 is localized to the XY body of spermatocytes and interacts with SPEM1 on the manchette of elongating spermatids. *Molecular and Cellular Endocrinology*, 333(2), 134–142. <https://doi.org/10.1016/j.mce.2010.12.021>.
- Calvi, A., Wong, A. S., Wright, G., Wong, E. S., Loo, T. H., et al. (2015). SUN4 is essential for nuclear remodeling during mammalian spermiogenesis. *Developmental Biology*, 407(2), 321–330. <https://doi.org/10.1016/j.ydbio.2015.09.010>.
- Carrell, D. T., & Hammoud, S. S. (2010). The human sperm epigenome and its potential role in embryonic development. *Molecular Human Reproduction*, 16(1), 37–47. <https://doi.org/10.1093/molehr/gap090>.

- Carrell, D. T., Emery, B. R., & Hammoud, S. (2007). Altered protamine expression and diminished spermatogenesis: What is the link? *Human Reproduction Update*, 13(3), 313–327.
- Castaneda, J., Huac, R., Miyata, H., Ojib, A., Guoc, Y., et al. (2017). TCTE1 is a conserved component of the dynein regulatory complex and is required for motility and metabolism in mouse spermatozoa. *Proceedings of the National Academy of Sciences of the United States of America* 114(27):E5370–E5378. <https://doi.org/10.1073/pnas.1621279114>
- Chen, S. R., et al. (2016). *Cell Death & Disease*, 7(11), e2472.
- Göb, E., Schmitt, J., Benavente, R., & Alsheimer, M. (2010). Mammalian sperm head formation involves different polarization of two novel LINC complexes. *PLoS One*, 5(8), e12072. <https://doi.org/10.1371/journal.pone.0012072>.
- Inaba, K. (2011). Sperm flagella: Comparative and phylogenetic perspectives of protein components. *Molecular Human Reproduction*, 17(8), 524–538. <https://doi.org/10.1093/molehr/gar034>.
- Kim, H. J., Yoon, J., Matsuura, A., Na, J. H., Lee, W. K., et al. (2015). Structural and biochemical insights into the role of testis-expressed gene 14 (TEX14) in forming the stable intercellular bridges of germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, 112(40), 12372–12377. <https://doi.org/10.1073/pnas.1418606112>.
- Lehti, M. S., & Sironen, A. (2016). Formation and function of the manchette and flagellum during spermatogenesis. *Reproduction*, 151(4), R43–54. <https://doi.org/10.1530/REP-15-0310>.
- Nihi, F., Gomes, M. L. M., Carvalho, F. A. R., Reis, A. B., et al. (2017). Revisiting the human seminiferous epithelium cycle. *Human Reproduction*, 32(6), 1170–1182. <https://doi.org/10.1093/humrep/dex064>.
- Oko, R., & Clermont, Y. (1999). Spermiogenesis. In , vol. 4 *Pro-Z. Encyclopedia of Reproduction* (pp. 602–609). Cambridge, MA: Academic Press.
- Russell, L. D., Ying, L., & Overbeek, P. A. (1994). Insertional mutation that causes acrosomal hypo-development: Its relationship to sperm head shaping. *The Anatomical Record*, 238(4), 437–453.
- Xu, M., & Hecht, N. B. (2008). MSY2 and polypyrimidine tract binding protein 2 stabilize mRNAs in the mammalian testis. *International Journal of Andrology*, 31(5), 457–461. <https://doi.org/10.1111/j.1365-2605.2008.00885.x>.

The First Wave of Spermatogenesis

Ricardo D Moreno, Pontifical Catholic University of Chile, Santiago, Chile

© 2018 Elsevier Inc. All rights reserved.

Introduction

The first wave of spermatogenesis can be defined as every process of division and differentiation undergone by a germinal cell from the spermatogonium (a stem cell), until the production of the first spermatozoa, which signals the beginning of male fertile life. In rodents, this wave, is completed within the first 6 weeks of life and is followed by successive waves, which overlap each other ensuring the continuous production of spermatozoa throughout the male fertile life.

Since the first wave of spermatogenesis is a milestone in the male reproductive life it has certain characteristics of its own that are not shared with the spermatogenesis in the adult and makes it worth to study.

Spermatogenesis in the Adulthood

Mammalian adult testes have two important functions: one is endocrine, since they are the main source of testosterone; the second is the production of male gametes, spermatozoa. The process by which sperm is formed is known as spermatogenesis, and occurs within tubular structures—the seminiferous tubules. These tubules contain two types of cells—somatic cells (named Sertoli), and germ cells (those that will form spermatozoa)—and in the interstitial compartment, a third cell type can be found—the Leydig cell—which is the major source of testosterone. Once the adult stage is reached, spermatogenesis may be continuous, as in humans, but it can also occur only at certain periods of the year, as in Siberian hamsters.

Spermatogenesis in adults can be divided into three phases:

1. In the Proliferative Phase, a group of diploid stem cells (called spermatogonia) which has the ability of self-renewal, that is, to divide constantly, begins the path of spermatogenesis. In order to increase their number, they start to divide and after the sixth cell division, approximately, they enter into meiosis, which marks the next stage of spermatogenesis.
2. The Meiosis is a type of cell division that occurs only in the testes and ovaries and originates four haploid cells. During meiosis, the recombination process ensures that every haploid cell that is produced has a unique combination of genes and thus guarantees the diversity of the progeny. Cells in meiosis are known as spermatocytes, which in turn can be subdivided according to the different substages of meiosis.
3. During the Differentiation Phase or Spermiogenesis, haploid cells (now called spermatids) experience morphological, biochemical, and physiological changes such as modification of the shape of the head, flagellum development and cytoplasm remodeling, hence transitioning into a spermatozoon. According to its morphology and degree of differentiation it is possible to unveil two types of spermatids: round and elongated.

As mentioned above, spermatogenesis is continuous throughout the male fertile life. Given this, in an adult testis we can find all the previously described germ cell types at the same time, which, as we will see, does not occur during the first wave of spermatogenesis since they are sequentially formed as the differentiation progresses are unfold.

Spermatogenesis begins very early in fetal stage (De Felici, 2000). During development, the so-called primordial germ cells or PGCs migrate to the mesonephros, where they meet with somatic cells (the future Sertoli cells) around embryonic day 12.5. When these two types of cells are brought together, they arrange the seminiferous cords, that is, the precursors of the seminiferous tubules without lumen. At this stage, germ cells (called pro-spermatogonia or gonocytes) enter into a proliferative resting phase, nondividing until puberty, where proliferation reinitiates and the first wave of spermatogenesis begins.

The First Wave of Spermatogenesis or the Awakening of the Force

The onset of the first wave of spermatogenesis is marked by the proliferation of prespermatogonia or gonocytes, which begins in prepuberal males (Fig. 1).

At this step, the seminiferous cords are populated only by Sertoli cells and gonocytes are located in the lumen. From a morphological point of view, gonocytes, or prespermatogonia, are cells with a large, round nucleus placed in the center of the seminiferous cords, whereas spermatogonias are elongated cells with prominent nucleoli and situated at the periphery of the cords. The differences regarding the location and shape of these cells are product of important changes in the expression of multiple genes that control cell proliferation and differentiation. In the mouse, this transition from gonocytes to spermatogonia occurs during the first 3 days of life, whereas in the human it takes several weeks or months and occurs during the fetal life (Fig. 1).

Underlying this differentiation process are various types of signals that are received by the gonocytes and eventually determine their differentiation. In the mouse, 1–2 days after birth, gonocytes begin to proliferate and migrate to the periphery of the seminiferous cords. This proliferation is induced by molecules or factors secreted by Sertoli cells such as platelet-derived factor (PDGF-BB), 17- β -estradiol, and retinoic acid (Manku and Culty, 2015). This is one of many examples that we can find where there is a paracrine

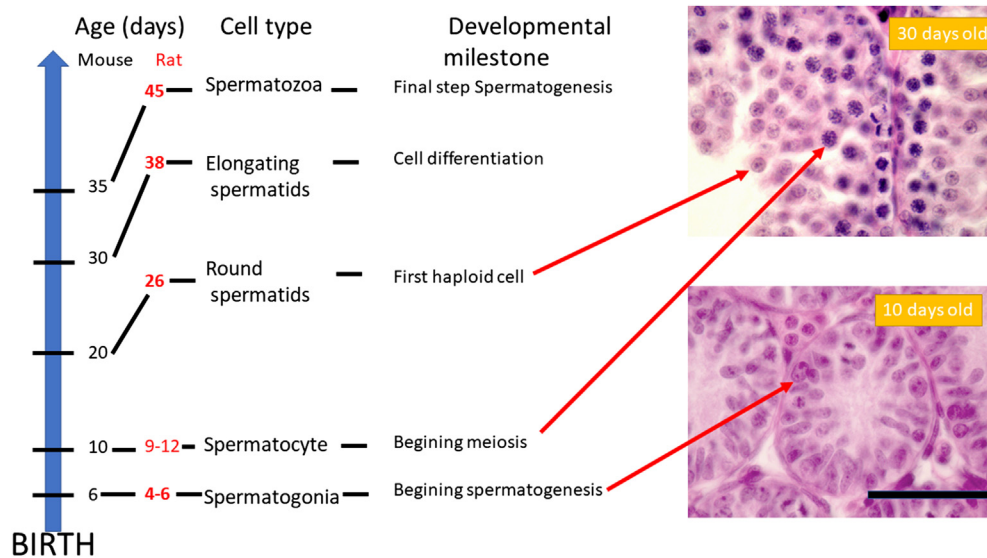


Fig. 1 Comparative timeline of the first wave of spermatogenesis. The first wave of spermatogenesis begins a few days after birth in both mice and rat. The timeline on the left indicates the age at which different types of germ cell differentiations can be detected according to their degree of differentiation. The photos show sections of testicles of 30 and 10-day-old rats where the arrows indicate the cell types that can be found in each of the respective cases. Bar = 50 μ m.

communication between the Sertoli and germ cells. Once spermatogonia arise, they begin to proliferate and accommodate in pairs or rows, one after another, which reflects different subtypes (A1, A2, A3, A4, In and B) each one with a greater commitment degree to differentiation. The spermatogonia subtype-B is the one that effectively enters meiosis. Among the signals involved in this process, the retinoic acid, which is a derivative of vitamin A that is produced by the Sertoli cell, is quite relevant since germ cells contain proteins (receptors) that are capable of binding to it and thus induce the expression of specific genes (Manku and Culty, 2015). It is interesting to note that retinoic acid has also been implicated in the induction of meiosis in females, a process that occurs during fetal life, hence indicating that it is a conserved mechanism between males and females. Spermatocytes in leptotene, which is the first stage of meiosis, initially appear in 10 days-old mice and 9–12 days-old rats and continue their differentiation program and progress through the following stages of meiosis (Fig. 1). Once these cells have completed meiosis they give rise to the haploid or round spermatids (haploid cells), which can be first detected in 20 days-old mice and 26 days-old rats. Then, around the 35th and 45th day in mice and rats, respectively, round spermatids experience spermiogenesis (the final stage of differentiation) resulting in testicular spermatozoa (Figs. 1 and 2). Eventually, these cells detach themselves from the other germ cells and are released into the

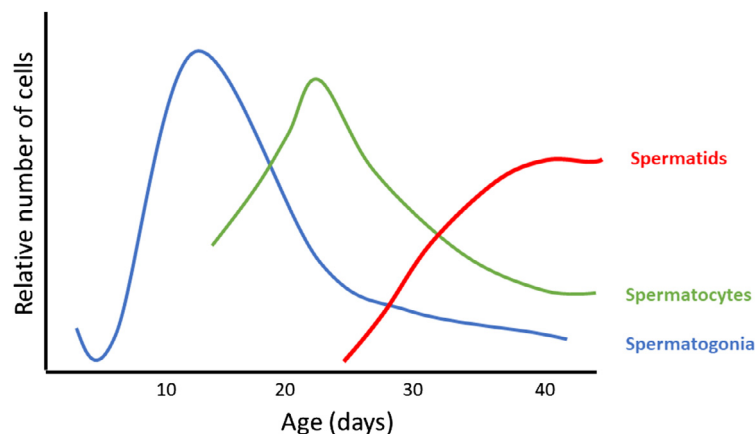


Fig. 2 Relative amount of different germ cell types according to the age of development. The graph shows the relative abundance of spermatogonia (blue line), spermatocytes (green line), and spermatids (round and elongated, red line), according to the age in rat. In the early days of age there is a significant increase in spermatogonia, which decreases around 12 days because they start to enter the meiosis and spermatocytes are able to be observed. Subsequently, these cells complete meiosis and spermatids are produced, becoming the most abundant cell population in the seminiferous tubules. Graph data was adapted from Yang, Z.W., Wreford, N.G. and de Kretser, D.M. (1990). A quantitative study of spermatogenesis in the developing rat testis. *Biology of Reproduction* 43, 629–623.

lumen of the seminiferous tubules, and transported to the epididymis, where they undergo a maturation process becoming ready to be ejaculated. It is important to mention *in vitro* studies have shown that spermatids formed during the first wave of spermatogenesis are functionally active and capable of generating viable embryos, although these are produced at a much lower rate than those from spermatids formed during adult spermatogenesis (Miki et al., 2004). These results have been achieved using assisted reproduction techniques where spermatids are injected into mouse oocytes (since they are immobile), and the embryos produced *in vitro* are subsequently transferred to recipient females.

From a technical point of view, morphology of the biology of the first wave of spermatogenesis due to the sequential apparition of the different germ cell types has been very useful when studying spermatogenesis, since it is possible to take advantage of the sequential apparition of specific germ cell types and design more efficient isolation procedures using testis (mice or rats) of specific ages. That is, if we want to isolate round spermatids, we should use testicles of a 20 days-old specimen, while if we wanted to isolate spermatogonia we should use a 6–8 days-old one.

While the first wave of spermatogenesis progresses, Sertoli cells also suffer alterations, especially in shape and function (Holsberger et al., 2005). A few days after birth, these cells, along with germ cells, begin to proliferate until around the 30th day in rat. The cessation of proliferation of Sertoli cells is associated with its differentiation process. One of the critical events in this process is the generation of the so-called hemato-testicular barrier, which is the product of tight junctions between the Sertoli cells in a way that prevent the entering of the majority of soluble plasma compounds into the seminiferous tubules. These junctions are formed developmentally right after the differentiation of the first spermatocytes into zygotene (the stage that follows leptotene), and in this way isolate all the subsequent germ cells from the rest of the body (Morales et al., 2007). Thus, Sertoli cells provide nutrients and support germ cell differentiation until the spermatozoa are released into the lumen of the seminiferous tubule. Studies in rat indicate that the developing of this barrier begins between the 15th and 16th day and is completed at the 18th day (Fig. 3). On the other hand, the formation of the lumen within the seminiferous cords, which is another parameter of Sertoli cells differentiation, begins to be observed as early as the 10th day in mouse (Russell et al., 1989). The number of seminiferous tubules increases rapidly and at the 20th day almost all the cords have been transformed into seminiferous tubules (Fig. 3). At the same time, the size of the lumen progressively increases during the first wave of spermatogenesis and in mouse it reaches that of the adult at the age of 50 days. (See Fig. 4.)

Even though the general process between the first wave of spermatogenesis and the one observed in the adult are similar, there are some differences between them and it may be related to the multiple developmental changes that are observed during the spermatogenesis. One important difference is that in the former, the type of germ cell that can be found will depend on the age of the specimen, while in the latter, all types of germ cells coexist at the same time. Some authors have reported that the kinetics of the first wave of spermatogenesis in the mouse are similar to the ones in the adult but others have shown that it is about 20% faster than the adult, at least in the mouse (Kluin et al., 1982). This difference in velocity normalizes at 35 days of age, when, in the mouse, the first wave has already ended and spermatogonia begin to produce the following waves of differentiation (spermatogenesis). One of the possible causes of this difference in sperm production velocities may be due to the very origin of spermatogonia, which seems not to be exactly the same as in adults. As we have seen, spermatogonia are produced from the gonocytes, which migrate from the lumen and adhere to the walls of the seminiferous tubules and form the spermatogonial stem cells (SSC). In the adult, these cells express specific markers such as neurogenin-3 and are source of all spermatogonia. However, there are data, in the mouse, showing that the

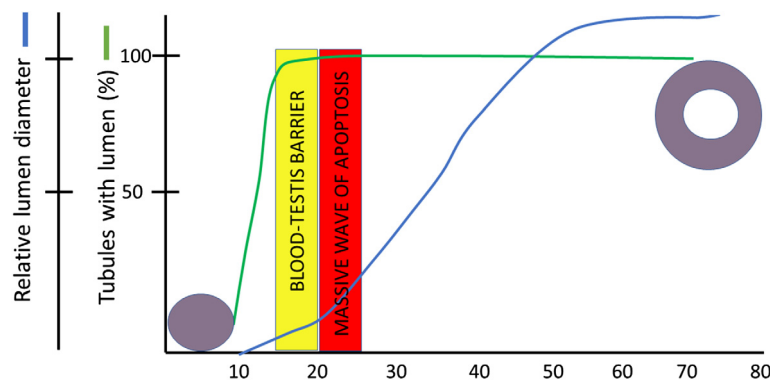


Fig. 3 Relationship between Sertoli cell differentiation and germ cell apoptosis according to the age of development. The graph shows that from 10 days of age some seminiferous cords with lumen can be observed, which is indicative of the beginning of the differentiation of the Sertoli cell and the seminiferous tubule formation. This is a quick process and it is concomitant with the progressive increase in diameter of the lumen that reaches adult levels of around 45 days of age. Another important event in the differentiation of the Sertoli cell is the formation of the blood-testis barrier (yellow bar), which occurs just before the massive wave of apoptosis in the germinal cells (red bar). The data in this chart are adapted from Russell, L.D. (1978). The blood–testis barrier and its formation relative to spermatocyte maturation in the adult rat: A lanthanum tracer study. *The Anatomical Record* **190**, 99–111; Russell, L.D., Bartke, A. and Goh, J.C. (1989). Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion and flow. *The American Journal of Anatomy* **184**, 179–89; Moreno, R.D., Lizama, C., Urzúa, N., Vergara, S.P. and Reyes, J.G. (2006). Caspase activation throughout the first wave of spermatogenesis in the rat. *Cell and Tissue Research* **325**, 533–540.

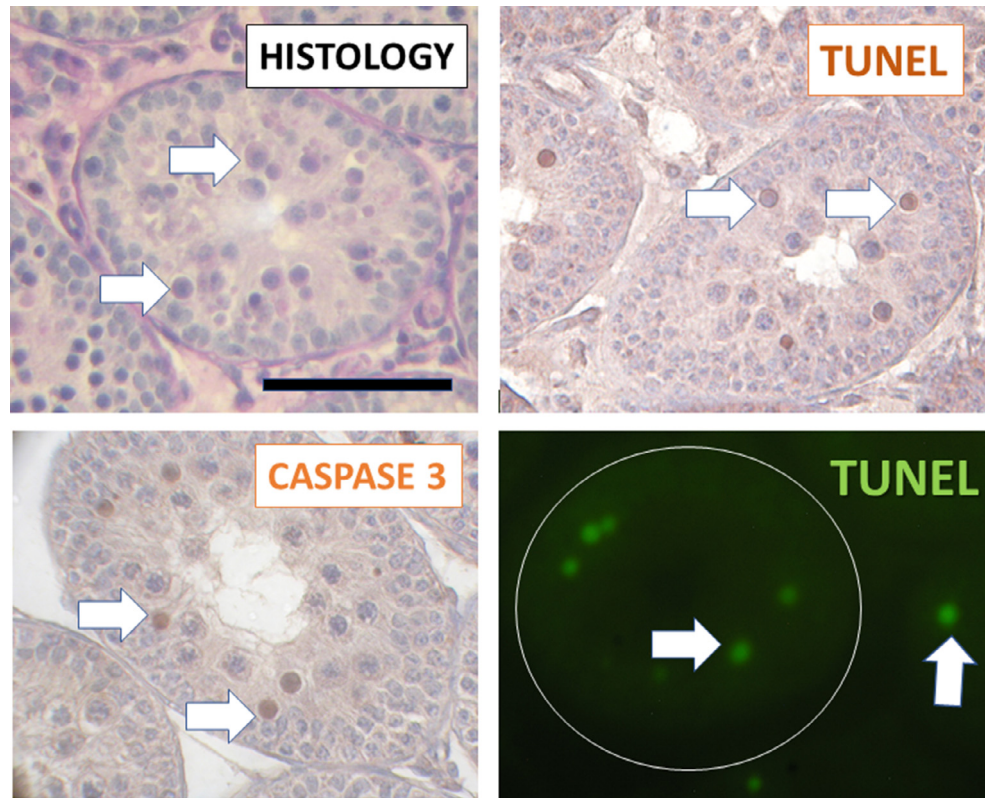


Fig. 4 Different ways of viewing cells in apoptosis. The figure shows four different techniques through which cells can be seen and quantified in apoptosis. In a testicle cut, which can be stained with a combination called PAS-hematoxylin, cells in apoptosis can be osseous (arrows). On the other hand, they can be seen by another technique called TUNEL that detects DNA rupture. This technique can be used with dyes (brown cells) or fluorescence (green cells). Since the tissue cannot be seen in this technique, a circle indicating the seminiferous tubule has been superimposed. During apoptosis caspase-3 is activated which can also be detected using a specific antibody (brown cells, arrows). Black bar = 100 mm.

first wave of spermatogenesis comes from a group of gonocytes and not from the SSC population, suggesting that they do not have exactly the same origin as the spermatogonia that form in the adult, which would explain the aforementioned differences (Yoshida et al., 2006).

Apoptosis in the First Wave of Spermatogenesis

Apoptosis is a type of programmed death in which the cell begins a genetic program to “commit suicide.” This genetic program includes several types of genes, and has been observed in different organisms being fundamental in the development of organs and tissues. In the case of the first wave of spermatogenesis, it has been observed that there is a massive germ cell death in both rat and mouse, occurring in 20–25 days-old rats and 18–20 days-old mice (Moreno et al., 2006). This massive germ cell death during the first wave has not been observed in other species such as human or ruminants, mainly due to the technical limitations and the limited biological material available during this specific developmental window. In the case of rats and mice, most of germ cells that die during this period are spermatocytes in the stage of pachytene, although some authors have also observed that some spermatogonia also enter into apoptosis (Rodriguez et al., 1997). It is interesting that this massive event of cell death occurs immediately after the formation of the hemato-testicular barrier (Fig. 3), suggesting that it is possible that the differentiation process of the Sertoli cell is in some way linked to the death of germ cells. It is interesting that within the whole set of genes known to be involved in apoptosis, there are some of them that are more important or that participate almost exclusively in apoptosis of germ cells. Among these genes is BAX, whose deletion (knockout) stops the first wave of spermatogenesis at the same stage where the apoptosis is observed, that is to say the stage of spermatocytes in pachytene (Knudson et al., 1995). These mice are therefore infertile since they do not produce spermatozoa. Another interesting gene is BCL-W, which has an antiapoptotic function, that is, it prevents apoptosis (Meehan et al., 2001). Genetic studies in which this gene has also been deleted (knockout) interestingly show that the first wave of spermatogenesis proceeds normally, but that adults are infertile due to a massive germ cell apoptosis. This is because during the first wave of spermatogenesis at least two other antiapoptotic genes (*Bcl-2* and *Bcl-XL*) are expressed, mainly in spermatogonia, whereas in the adult stage only *Bcl-w* is detected (Meehan et al., 2001). Thus, by deleting *bcl-w*, its function is compensated by the presence of *Bcl-2* and *Bcl-xl* during the first wave of spermatogenesis, which does not occur in the adult. These

results indicate that the regulation of apoptosis during the first wave of spermatogenesis and in the adult, are different and that this process is important for the production of spermatozoa.

The Hormonal Control of the First Wave of Spermatogenesis

Central to the production of spermatozoa and the progression of spermatogenesis is the hormonal control that is exerted on this process by the endocrine system. The production of testosterone in the male occurs mainly in Leydig cells, which are formed during the fetal stage 12.5 days of gestation in the mouse and between 7 and 14 weeks in the human (Svechnikov et al., 2010; O'Shaughnessy, 2014). Very soon after birth, the hypothalamus–pituitary gland gonad (HPG) axis is activated, which is reflected in an increase in testosterone sustained up to adult levels. That is, the release of testosterone is controlled by the luteinizing hormone (LH) produced by the pituitary, which in turn is regulated by the hormone GnRH released by the hypothalamus. This increase in testosterone production is critical for the onset and development of puberty, but it is also known that, at least in humans, there is a transient increase in testosterone levels in 2–3 months old children, which is called the “mini puberty” (Svechnikov et al., 2010; Li et al., 2015). Several genetic studies have been very valuable to study the contribution of various hormones in the progression of spermatogenesis, and in particular the first wave of spermatogenesis. For example, it is known that the generation of PGCs, their migration, differentiation in prespermatogonia (gonocytes), and formation of seminiferous cords occurs before maturation of the HPG axis, therefore these stages are not under control of this axis. This is also seen in mutant mice that do not produce GnRH (*hpg* mice) or in knockout mice for the receptors of the hormones FSH, LH or testosterone (Singh et al., 1995; O'Shaughnessy, 2014). In these experimental models, it has been observed that the first wave of spermatogenesis can be initiated and germ cells can reach meiosis, although there is a strong decrease in the number of germ cells as early as 5 days of life, compared to wild, suggesting an hormonal control of testosterone during this period. In addition, recent data suggest that, in the mouse, “mini-puberty” appears to coincide with a rise in FSH levels (Li et al., 2017).

Experimental evidence suggesting that both FSH and testosterone act by modulating massive apoptosis during the first wave of spermatogenesis comes from two lines of evidence. The first evidence emerges from experiments in which the exogenous administration of testosterone reduces the first wave of apoptosis (Rodriguez et al., 1997). The second group of evidence rises from the data obtained using KO mice for the FSH receptor and androgen receptor (total or only in Sertoli cells), which show that in these mice there is an exacerbated apoptosis of germ cells around 20–25 days of age relative to controls (O'Shaughnessy, 2014; Shiraishi and Matsuyama, 2017). Together these data indicate that both FSH and androgens, in particular testosterone, protect germ cells from apoptosis.

In addition, from the effects described in apoptosis of germ cells during the first wave of spermatogenesis, data indicate that mice lacking the androgen receptor gene in Sertoli cells are infertile due to the absence of spermatids. It is interesting that this same phenotype is observed in KO mice for the hormone receptor LH, indicating that even from the first wave of spermatogenesis, testosterone is fundamental for survival, and differentiation of haploid cells (spermiogenesis). Several studies have shown that the receptor for testosterone belongs to the family of steroid receptors and is mainly located in the nucleus of Sertoli, Leydig, peritubular (germinal cells surrounding the seminiferous tubules), and germinal cells. However, deletion of the androgen receptor in germ cells or peritubular cells has no effect on the fertility or progression of spermatogenesis of these mice (Wang et al., 2009). This suggests that the main effect of testosterone on spermatogenesis is exerted indirectly through the Sertoli cell.

In addition to testosterone and FSH, there is evidence that other hormones may also exert regulatory function during the first wave of spermatogenesis. The role of estrogen in spermatogenesis has been very controversial, but germ and Sertoli cells express the two isoforms of the estrogen receptor. In addition, the testis expresses the enzyme CYP19A1 (aromatase) which converts testosterone into estradiol (Carreau and Hess, 2010). The transcript for this enzyme can be detected in Sertoli cell and spermatogonia from 10 days-old rat onward. Mice KO for the estrogen receptor (alpha subunit) or overexpression of the *Cyp19A1* gene, are fertile and do not show major alterations in the first wave of spermatogenesis suggesting that this hormone may regulate spermatogenesis but it is dispensable for the normal progression of the first wave. On the other hand, it has been shown that the differentiation of Sertoli cells depend upon thyroid hormones (Holsberger et al., 2005). In fact, inhibition of T3/T4 production (hypothyroidism) in the neonatal rat impairs testicular growth, germ cell maturation, seminiferous tubule formation and other differentiation processes. In addition, it delays and increases the rate of germ cell apoptosis during the first wave of spermatogenesis (Silva et al., 2011). Therefore, the first wave of spermatogenesis is hormonally regulated at multiple levels.

References

- Carreau, S., & Hess, R. A. (2010). Oestrogens and spermatogenesis. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 365, 1517–1535.
- De Felici, M. (2000). Regulation of primordial germ cell development in the mouse. *The International Journal of Developmental Biology*, 44, 575–580.
- Holsberger, D. R., Kiesewetter, S. E., & Cooke, P. S. (2005). Regulation of neonatal Sertoli cell development by thyroid hormone receptor alpha1. *Biology of Reproduction*, 73, 396–403.
- Kluin, P. M., Kramer, M. F., & de Rooij, D. G. (1982). Spermatogenesis in the immature mouse proceeds faster than in the adult. *International Journal of Andrology*, 5, 282–294.
- Knudson, C. M., Tung, K. S., Tourtellotte, W. G., Brown, G. A., & Korsmeyer, S. J. (1995). Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*, 270, 96–99.
- Li, R., Vannitamby, A., Meijer, J., Southwell, B., & Hutson, J. (2015). Postnatal germ cell development during mini-puberty in the mouse does not require androgen receptor: Implications for managing cryptorchidism. *The Journal of Urology*, 193, 1361–1367.

- Li, R., Vannitambay, A., Yue, S. S. K., Handelsman, D., & Hutson, J. (2017). Mouse minipuberty coincides with gonocyte transformation into spermatogonial stem cells: A model for human minipuberty. *Reproduction, Fertility, and Development*. <https://doi.org/10.1071/RD17100>.
- Manku, G., & Culty, M. (2015). Mammalian gonocyte and spermatogonia differentiation: Recent advances and remaining challenges. *Reproduction*, 149, R139–57.
- Meehan, T., Loveland, K. L., de Kretser, D., Cory, S., & Print, C. G. (2001). Developmental regulation of the bcl-2 family during spermatogenesis: Insights into the sterility of bcl-w^{−/−} male mice. *Cell Death and Differentiation*, 8, 225–233.
- Miki, H., Lee, J., Inoue, K., Ogonuki, N., Noguchi, Y., Mochida, K., Kohda, T., Nagashima, H., Ishino, F., & Ogura, A. (2004). Microinsemination with first-wave round spermatids from immature male mice. *The Journal of Reproduction and Development*, 50, 131–137.
- Morales, A., Mohamed, F., & Cavicchia, J. C. (2007). Apoptosis and blood–testis barrier during the first spermatogenic wave in the pubertal rat. *Anatomical Record (Hoboken)*, 290, 206–214.
- Moreno, R. D., Lizama, C., Urzúa, N., Vergara, S. P., & Reyes, J. G. (2006). Caspase activation throughout the first wave of spermatogenesis in the rat. *Cell and Tissue Research*, 325, 533–540.
- O'Shaughnessy, P. J. (2014). Hormonal control of germ cell development and spermatogenesis. *Seminars in Cell & Developmental Biology*, 29, 55–65.
- Rodriguez, I., Ody, C., Araki, K., Garcia, I., & Vassalli, P. (1997). An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. *The EMBO Journal*, 16, 2262–2270.
- Russell, L. D., Bartke, A., & Goh, J. C. (1989). Postnatal development of the Sertoli cell barrier, tubular lumen, and cytoskeleton of Sertoli and myoid cells in the rat, and their relationship to tubular fluid secretion and flow. *The American Journal of Anatomy*, 184, 179–189.
- Shiraishi, K., & Matsuyama, H. (2017). Gonadotropin actions on spermatogenesis and hormonal therapies for spermatogenic disorders. *Endocrine Journal*, 64, 123–131.
- Silva, D., Lizama, C., Tapia, V., & Moreno, R. D. (2011). Propylthiouracil-induced hypothyroidism delays apoptosis during the first wave of spermatogenesis. *Biological Research*, 44, 181–188.
- Singh, J., O'Neill, C., & Handelsman, D. J. (1995). Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. *Endocrinology*, 136, 5311–5321.
- Svechnikov, K., Landreh, L., Weisser, J., Izzo, G., Colón, E., Svechnikova, I., & Söder, O. (2010). Origin, development and regulation of human Leydig cells. *Hormone Research in Paediatrics*, 73, 93–101.
- Wang, R. S., Yeh, S., Tzeng, C. R., & Chang, C. (2009). Androgen receptor roles in spermatogenesis and fertility: Lessons from testicular cell-specific androgen receptor knockout mice. *Endocrine Reviews*, 30, 119–132.
- Yoshida, S., Sueno, M., Nakagawa, T., Ohbo, K., Nagamatsu, G., Suda, T., & Nabeshima, Y. (2006). The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development*, 133, 1495–1505.

Further Reading

- Russell, L. D. (1978). The blood–testis barrier and its formation relative to spermatocyte maturation in the adult rat: A lanthanum tracer study. *The Anatomical Record*, 190, 99–111.
- Sung, W. K., Komatsu, M., & Jagiello, G. M. (1986). The kinetics of the first wave of spermatogenesis in the newborn male mouse gamete. *Research*, 14, 245–254.
- Yang, Z. W., Wreford, N. G., & de Kretser, D. M. (1990). A quantitative study of spermatogenesis in the developing rat testis. *Biology of Reproduction*, 43, 629–635.

Spermatogonial Response to Somatic Cell Interactions

Tessa Lord and Jon M Oatley, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The spermatogonial pool is diverse, however can be divided into two primary populations; the undifferentiated and differentiating pools. The spermatogonial stem cells (SSCs) are the undifferentiated self-renewing reservoir that facilitate continual sperm production from the time of puberty throughout the life of the adult male. Along with the SSCs, progenitor spermatogonia are also considered to be part of the “undifferentiated” population, however, can no longer self-renew and instead undergo transit-amplifying mitotic divisions to achieve clonal expansion. For the most part, it is accepted that progenitor spermatogonia in the undifferentiated pool exist as pairs or chains of up to 16 cells long, interconnected by intercellular bridges (referred to as A_{paired} or A_{aligned} respectively), while the SSCs are considered to be a subset of the A_{single} population (reviewed by Lord and Oatley, 2017). Progenitors also differ functionally from SSCs in that they are responsive to the differentiating signal in the testis; retinoic acid (RA). Thus, following the RA pulse that occurs every 8.6 days across the seminiferous epithelium of the mouse, the majority of progenitors will transition from type A (undifferentiated) to type A1 (differentiating) spermatogonia. This transition is immediately followed by further transit-amplifying mitotic divisions that accompany the formation of A2, A3, A4, intermediate, and finally type B spermatogonia. The type B spermatogonia are those which are poised to enter meiosis as preleptotene spermatocytes; thus beginning the journey to production of haploid male germ cells with the capacity to fertilize an egg.

Fate decisions within the spermatogonial population require tight regulation to ensure maintenance of the germline and optimal fertility. For instance, the decision between maintenance of the SSC population versus transition into a progenitor state; or the decision for progenitors to continue in an undifferentiated state versus commitment to differentiation; must be controlled to prevent exhaustion of the reservoir from which spermatogenesis arises. Extrinsic cues from the surrounding environment are instrumental in ensuring such regulation of these distinct spermatogonial populations. These cues primarily originate from the somatic cells in the testes. Somatic cells that are known to interact with the spermatogonial population exist both within the seminiferous tubules themselves, alongside the spermatogonia; i.e., Sertoli cells, but also exist on the outer of the seminiferous tubules; such as the androgen-producing Leydig cells that reside in the interstitial space, the peritubular myoid cells (PMCs), macrophages, and vascular cells (the orientation of these cells in the testis is diagrammatically represented in Fig. 1). Somatic cells primarily influence spermatogonial populations via the production of growth factors, the majority of which have been characterized to be important for driving self-renewal in SSCs, and maintenance of the undifferentiated population. However, depending on the stage of the seminiferous cycle, the same somatic cells that secrete factors required for maintenance of the SSC population can also facilitate the RA-driven transition of a subset of undifferentiated progenitors into differentiating spermatogonia; depicting the intricate and dichotomous role of these cells in terms of their interaction with the spermatogonial population. In this article we will provide an overview of somatic interaction with the spermatogonial population; examining the origins and actions of growth factors known to stimulate maintenance of the undifferentiated population, and also the production and degradation of RA within the seminiferous tubules to regulate the differentiating transition.

Somatic Cell Interaction With the Undifferentiated Spermatogonial Pool

Perhaps the most extensively studied somatic cell-spermatogonial interaction is the stimulation of SSC maintenance and self-renewal by growth factors produced by Sertoli cells in the SSC niche; particularly Glial cell-line derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2) (Fig. 1). The SSC niche is thought to exist as a microenvironment at the basement membrane of the seminiferous epithelium, on the outer of the blood-testis barrier. This “open niche” is thought to be populated by the entire undifferentiated spermatogonial population, and true understanding of how SSCs and progenitor spermatogonia behave differently in the microenvironment remains to be determined. However, the action of GDNF on the SSC population is particularly well understood, with this growth factor exerting its effects via the GFRA1/RET receptor to stimulate PI3K/AKT signaling (Oatley et al., 2007) and upregulate expression of transcription factors known to be important in SSC maintenance and self-renewal, such as ETS variant 5 (*Etv5*), B-Cell CLL/Lymphoma 6B (*Bcl6b*) (Oatley et al., 2007) and Inhibitor of DNA binding 4 (*Id4*) (Oatley et al., 2011). As such, supplementation of GDNF into culture medium has been shown to directly stimulate self-renewal of SSCs in primary spermatogonial culture (Kubota et al., 2004), and further, although GDNF, GFRA1 and RET null mice do not survive into adulthood (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Enomoto et al., 1998), GDNF^{+/-} mice exhibit severe impairments in proliferation of the spermatogonial population (Meng et al., 2000). Although FGF2 supplementation of culture media also influences SSC proliferation, in the absence of GDNF, FGF2 alone is not sufficient for SSC maintenance (Kubota et al., 2004). Based primarily on the outcomes of in vitro studies, GDNF and FGF2 concentration within the niche is thought to be critically important for regulation of the undifferentiated population in vivo. However, it has become apparent that growth factor production by somatic cells *outside* the niche can also directly influence the undifferentiated pool; as well as indirectly influence spermatogonial populations via interactions with other somatic cells. As the SSC niche has been covered extensively in another

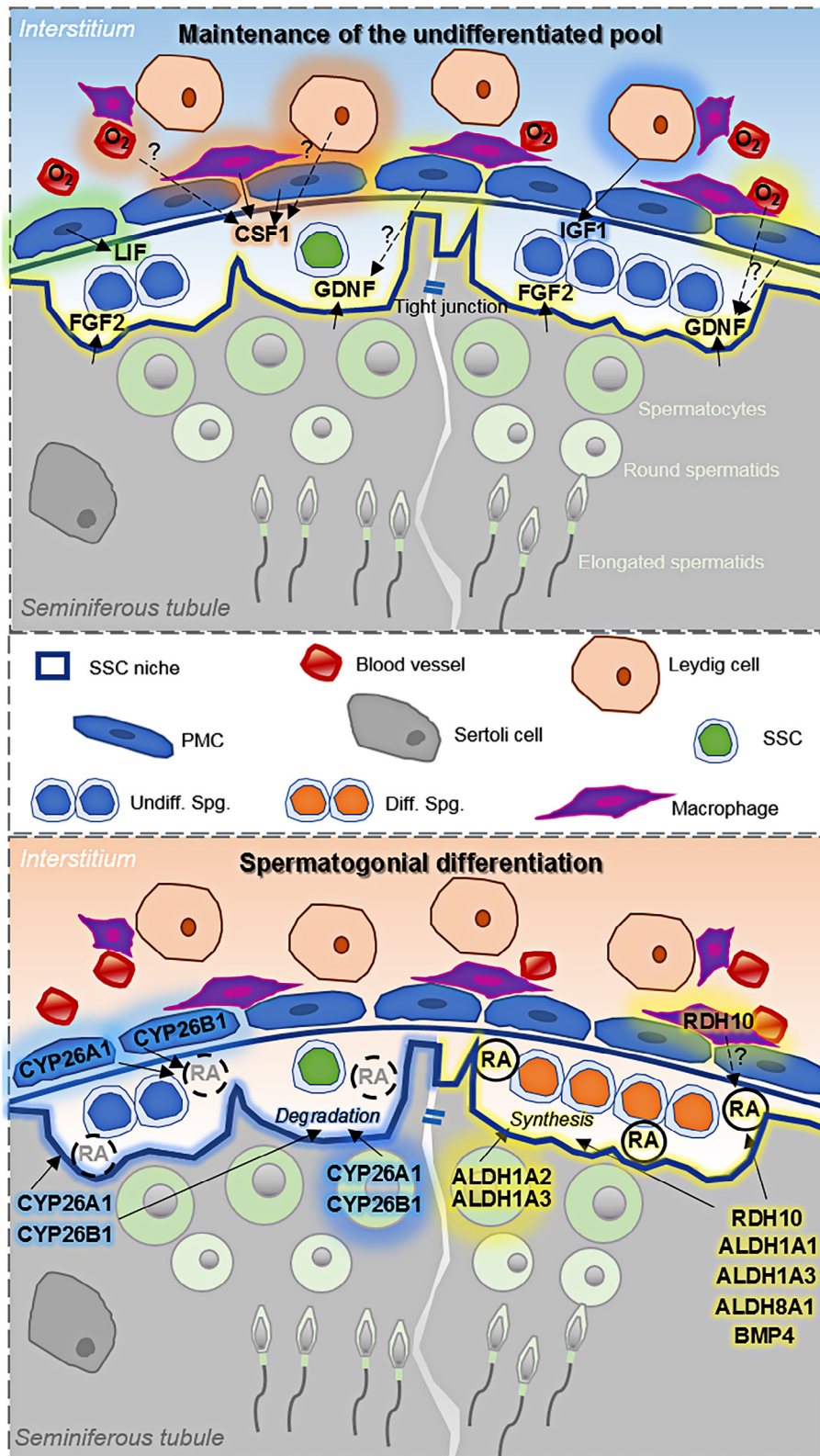


Fig. 1 Somatic cell interactions with spermatogonia. Top: Somatic cells in the testes produce growth factors to drive self-renewal of SSCs and maintenance of the undifferentiated spermatogonia population. These growth factors include GDNF, FGF2, CSF1 and LIF. Middle: Key depicting cell types in diagram. Bottom: Somatic cells regulate differentiation of transit amplifying progenitor spermatogonia by controlling synthesis (RDH, ALDH) and degradation (CYP26) of RA, and by producing growth factors that support RA-driven differentiation (BMP4).

article, here we will primarily explore how somatic cells residing outside the SSC niche influence the dynamics of the undifferentiated pool; including macrophages, PMCs, Leydig cells and vascular cells.

Macrophages are primarily considered to be a phagocytic cell involved in immune defense, however it is becoming apparent that these cells play an alternate role in the testis. Indeed, two populations of testicular macrophages exist, one within the interstitium and the other directly on the surface of the seminiferous tubules (DeFalco et al., 2015). These testicular macrophages are biased toward an M2 phenotype, meaning that they have far less pro-inflammatory properties than bone macrophages, and instead may have an immunosuppressive function (Wang et al., 2017). In assessing macrophage interaction with the spermatogonial population, recent observations by DeFalco et al. (2015) noted that, although not in direct contact with peritubular macrophages, the undifferentiated spermatogonial pool appears to preferentially reside adjacent to these cells. To confirm crosstalk and a direct functional link between macrophages and spermatogonia, targeted ablation of the testicular macrophage population was instigated. Remarkably, following macrophage ablation, 75% of the undifferentiated spermatogonial population (as labeled by ZBTB16 immunostaining) was lost. While the full extent of the macrophage-spermatogonia interaction remains unknown, DeFalco et al. (2015) did identify expression of the growth factor Colony Stimulating Factor 1 (CSF1) by the peritubular macrophage population. Importantly, it has been demonstrated that CSF1 can work alongside GDNF and FGF2 to directly influence SSC maintenance and self-renewal; with CSF1 supplementation to primary cultures of spermatogonia stimulating a threefold enrichment in SSC content over 63 days; as demonstrated by spermatogonial transplantation analyses (Oatley et al., 2009). Indeed, expression of the CSF1 receptor (CSF1R) is significantly upregulated in populations of spermatogonia that are enriched for SSC content using THY1 + selection (Oatley et al., 2009); further suggesting that this growth factor is important for regulating SSC activities in the testes, and that peritubular macrophages may play a role in stimulating self-renewal in the SSC pool (Fig. 1).

The potential origins of CSF1 span beyond the testicular macrophage population, with immunostaining analyses showing that a portion of the PMC population also express this growth factor (Oatley et al., 2009) (Fig. 1). The PMCs are a flattened muscle-like cell that surround the outer of the basal lamina of the seminiferous tubule, and are thought to aid progressive transport of male germ cells through the seminiferous tubule via their contractile activity (Maekawa et al., 1996). In addition to production of CSF1, their ability to potentially influence the spermatogonial population spans to the production of another growth factor; leukemia inhibitory factor (LIF) (Piquet-Pellorce et al., 2000). LIF production in the rodent testis was identified using an RT-PCR approach, and was sourced primarily to the PMC population following purification of each testicular somatic cell type (Piquet-Pellorce et al., 2000). Some evidence exists to suggest that LIF, like CSF1, works alongside GDNF to stimulate SSC proliferation. This evidence comes from in vitro studies in which LIF has been supplemented into rodent spermatogonial cultures both alone (Kubota et al., 2004; Wang et al., 2014), and in conjunction with GDNF (Wang et al., 2014). While both independent research groups reported no significant improvement in SSC content (Kubota et al., 2004) nor proliferation of cultures (Wang et al., 2014) following the additional of LIF alone, Wang et al. reported that a combination of LIF and GDNF significantly improved proliferation in these cultures (Wang et al., 2014). Despite this, the spermatogonial population observed by Wang et al. was heterogeneous; being comprised of both SSCs and progenitors, thus the significance of LIF production by PMCs in the testes in terms of spermatogonial regulation remains undetermined.

In addition to the production of CSF1 and LIF, investigation has also been conducted into PMCs as an alternate source of GDNF itself, contributing to the stimulation of maintenance and self-renewal of the SSC population (Spinnler et al., 2010; Chen et al., 2016) (Fig. 1). In the human testis, PMCs have been shown to express GDNF using immunocytochemistry techniques, and isolated PMCs were demonstrated to release GDNF into surrounding culture media (Spinnler et al., 2010). Further, in the mouse, it has been reported that conditional knockout of GDNF in PMCs results in diminishment of the undifferentiated spermatogonial population, and as a consequence, an age-associated decline in fertility (Chen et al., 2016). Despite this, an independent research group (Chen and Liu, 2016) have pointed out that the Myh-11 Cre utilized by Chen et al. (2016) is not specific for the PMC population, but rather its expression transcends all types of smooth muscle cells (Xin et al., 2002). Thus, although expression of GDNF by PMCs in the testis is evident, further investigation into the relevance of this in regulating activities of the undifferentiated spermatogonial pool is required. As a whole, however, it is apparent that the PMC population has the capacity to synthesize and secrete a number of growth factors that are known to be influential on the undifferentiated spermatogonial pool.

The Leydig cells exist in the interstitial space where their primary function is to synthesize androgens in response to stimulation by luteinizing hormone. Although the Leydig cells are not likely to directly influence the spermatogonia in this manner as spermatogonia do not express the androgen receptor (Sar et al., 1990), it has been demonstrated that androgens can indirectly influence activities in the spermatogonial pool. Specifically, Leydig cells stimulate the production of FGF2 by Sertoli cells (Gonzalez-Herrera et al., 2006); which, as mentioned previously, is critical for working alongside GDNF to stimulate maintenance and self-renewal of the SSC pool (Kubota et al., 2004). Additionally, androgen secretion has been proposed to stimulate GDNF production by PMCs (Chen et al., 2014, 2016). In conjunction with their primary role in androgen synthesis, Leydig cells also secrete a number of factors themselves that are known to influence proliferation of the spermatogonial population; thus, these cells may also have a direct influence on the spermatogonial population. Such factors include Insulin like growth factor 1 (IGF1); which has been shown to significantly improve proliferation of SSCs in cultures of undifferentiated spermatogonia (Kubota et al., 2004), as well as CSF1 (Oatley et al., 2009). Again, these findings suggest that the Leydig cells may play some role in maintaining the undifferentiated spermatogonial pool in the testis.

While this review has thus far considered how growth factors produced by somatic cells can influence the undifferentiated spermatogonia population; when discussing the vascular cells in the testes we also need to consider the influence of oxygen tension.

While other *closed* stem cell niche systems such as the hematopoietic niche are for the most part accepted to maintain hypoxic conditions (reviewed by Eliasson and Jönsson, 2010), it is controversial as to whether this is the case in the germline stem cell niche. A study by Yoshida et al. (2007) reported that “SSCs” preferentially exist in regions of the seminiferous tubule adjacent to vasculature. However, the SSC marker used in this study was Nuerogenin3 (NEUROG3), which has more recently been described to preferentially label the transit amplifying progenitor population (Nakagawa et al., 2010; Kaucher et al., 2012; Hara et al., 2014). Contrastingly, a study by our own research group suggested that SSCs preferentially reside in avascular regions of the testes (Chan et al., 2014), in this case utilizing ID4 as an SSC marker; a transcription factor whose expression has been shown to be highly enriched in the SSC population, and that can be used to isolate a seemingly pure population of SSCs (Chan et al., 2014; Helsel et al., 2017b). In considering the influence of oxygen tension, and thus association with vascular cells, on the spermatogonial population; the beneficial nature of hypoxic conditions on maintenance of the SSC pool has recently been demonstrated using in vitro culture studies (Helsel et al., 2017a). Indeed, when maintained in atmosphere of 21% oxygen and 5% CO₂, SSCs in primary culture have a significantly diminished life span of regenerative capacity when compared to their counterparts cultured in 10% oxygen and 5% CO₂ (Helsel et al., 2017a). Interestingly, in addition to regulation of oxygen tension, the possibility that vascular cells also interact with the undifferentiated population via production of growth factors cannot be discounted. Indeed, DeFalco et al. (2015) have suggested that CSF1 could be released from the perivascular compartment, while Chen and Liu (2016) have suggested, based on immunofluorescence staining, that some GDNF production may occur within the vasculature (Chen et al., 2016). Clearly, the functional relevance of growth factor production within the vascular compartment in terms of regulation of the spermatogonia pool requires further investigation, however, the potential impact of proximity of spermatogonia to the oxygen carrying erythrocytes within these structures has certainly been shown to have the capacity to influence SSC maintenance and functional longevity.

Clearly, the ability for somatic cells to interact with and influence self-renewal of SSCs and maintenance of the undifferentiated pool is highly intricate and spans beyond the direct Sertoli-spermatogonia association that occurs within the niche. However, the signals that stimulate maintenance of the undifferentiated pool must be balanced by signals that drive differentiation of the transit amplifying progenitors; lest the production of mature spermatozoa be halted. In the next section, somatic cell production and degradation of RA; the spermatogonial differentiation-driving signal, will be explored.

Involvement of Somatic Cells in Driving the A to A1 Transition in Spermatogonia

The critical role for the vitamin A metabolite RA in continuation of spermatogenesis has been appreciated since the 1920's (Wolbach and Howe, 1925). More recently, vitamin A- and RA- deficient rodent models have been characterized; depicting a complete block in spermatogenesis at the stage of spermatogonial differentiation that can be rescued by exogenous RA treatment (van Pelt and de Rooij, 1990, 1991; Hogarth et al., 2013). Exogenous RA drives differentiation in transit-amplifying progenitors via interaction with the RA receptors, “RARs” and their heterodimerization partners, the retinoid X receptors (RXRs). Upon the appearance of RA, RAR/RXR heterodimers bind to RA responsive elements (RAREs) in specific genes, modulating their expression (reviewed by Cunningham and Duester, 2015). Two classically upregulated genes that are indicative of the RA-driven differentiating transition in spermatogonia are *Kit* and *Stimulated by retinoic acid 8 (Stra8)* (Pellegrini et al., 2008). As with VA deficient models, knockdown of *KIT* results in cessation of spermatogenesis as a consequence of a block in the differentiating transition (reviewed by Rossi et al., 2000). Contrastingly, knockdown of *STRA8* expression instigates a less severe phenotype, however a disruption of spermatogonial differentiation is still apparent (Endo et al., 2015).

Clearly, it would not be beneficial for RA to be constantly present in the spermatogonial microenvironment, as this could result in exhaustion of the undifferentiated pool. Instead, the seminiferous cycle is such that a pulse of RA occurs in a “wave” along the length of the tubule every 8.6 days, is rapidly degraded, and is bookended by conditions that stimulate rebuilding of the transit-amplifying progenitor population (Hogarth et al., 2015a). The synthesis of RA at stages VI–VIII of the seminiferous cycle (Hogarth et al., 2015a), and the subsequent degradation, is thought to be controlled in part by somatic cells in the testis. The Sertoli cells in particular have been thoroughly investigated, and play an integral role in driving the differentiating transition in spermatogonia via their capacity for RA synthesis and storage (Tong et al., 2013), as well as production of enzymes for RA degradation (Hogarth et al., 2015b).

Sertoli cells possess isoforms of both retinol dehydrogenase (RDH) and aldehyde dehydrogenase (ALDH) enzymes that act to convert dietary Vitamin A into its metabolite retinal, and subsequently convert retinal into biologically active RA, respectively (Tong et al., 2013; Kent et al., 2015). The requirement for these Sertoli expressed enzymes for the A to A1 transition in spermatogonia is highlighted by a series of conditional knockout and inhibitor studies in rodent models. For instance, knockout of *Rdh10* in Sertoli cells results in a significant reduction in the percentage of *KIT* and *STRA8* positive spermatogonia in neonatal mice, while simultaneous loss of *RDH10* expression in Sertoli and germ cells results in a complete block in spermatogonial differentiation (Tong et al., 2013). Similarly, inhibition of ALDH activity using the compound WIN 18,446 mimics the phenotype of a vitamin A deficient mouse during neonatal life (Hogarth et al., 2013), with *ALDH1A1*, *ALDH1A2*, *ALDH1A3* and *ALDH8A1* isoforms being expressed in both somatic (Sertoli and interstitial) and germ cell populations of the testes; their specific localization depending on stage of development (Kent et al., 2015). In combining these data, one can conclude that the somatic cells, particularly the Sertoli cells, undoubtedly play a role in synthesizing RA to drive spermatogonial differentiation during the first wave of spermatogenesis in neonatal life. However, these dynamics shift upon transition into adulthood, at which time RA synthesizing enzymes within later stage germ cells take on a role, and the degree to which Sertoli cells participate in RA synthesis in steady state conditions becomes

uncertain. Indeed, in *Rdh10* conditional knockout mice (Sertoli- and germ cell- specific), fertility is restored at ~10 weeks of age, suggesting that a new source of RA synthesizing enzymes appears at this time (Tong et al., 2013). Similarly, the capacity for ALDH inhibition to enforce a complete block of spermatogenesis is lost in the adult around the time that the first preleptotene spermatocytes appear (Raverdeau et al., 2012).

Importantly, although RA synthesis to drive differentiation of spermatogonia is primarily thought to be coordinated through Sertoli cells and later stage germ cells, it is also possible that other somatic cells in the testes are involved in regulating local RA levels. For instance, DeFalco et al. (2015) identified expression of the RA synthesis enzyme RDH10 in testicular macrophages, and showed that the differentiating population of spermatogonia was dramatically affected when the peritubular macrophage population was ablated. As such, it will be interesting to assess in the future how much of a contribution the macrophage population plays to RA synthesis in the testes.

In conjunction with somatic cell regulation of the differentiating transition in spermatogonia via the synthesis of RA, it is becoming apparent that regulation of this transition is also reliant on rapid degradation of the RA signal by CYP26 enzymes synthesized in the same somatic cell populations. Indeed, when *Cyp26a1* and *Cyp26b1* isoforms were simultaneously knocked down in Sertoli cell populations, precocious and overabundant expression of STRA8 could be identified in the spermatogonial population (Hogarth et al., 2015b), suggesting that rapid degradation of RA may be important to “hold back” a portion of the transit-amplifying progenitor pool from entering the differentiating pathway. Further, when *Cyp26b1* was knocked down in Sertoli and germ cells simultaneously, fertility was significantly impaired, and 96% of seminiferous tubules were found to be completely devoid of germ cells (Hogarth et al., 2015b). CYP26 expression is also evident in PMCs, where it has been postulated to act as a protective barrier for the spermatogonia from circulating retinoids that could stimulate unwanted transition into a differentiating state and loss of the undifferentiated pool (Vernet et al., 2006; Hogarth, 2015).

Finally, the differentiation-driving activities of the somatic cell population in the testis are not limited to RA synthesis and degradation, but also the synthesis of growth factors that act alongside RA to stimulate the A to A1 transition. Specifically, bone morphogenic protein 4 (BMP4) has been shown by northern blot analysis to be synthesized by Sertoli cells in early postnatal life (Pellegrini et al., 2003). Experimental evidence suggests that BMP4 works alongside RA to stimulate differentiation in spermatogonia, as well as DNA synthesis, and the expression of key differentiating markers such as KIT and STRA8 (Pellegrini et al., 2003; Yang et al., 2016). As BMP4 expression begins to decline in Sertoli cells from post-natal day 7, this somatic cell signal is primarily thought to be influential on the spermatogonial population in postnatal life (Pellegrini et al., 2003).

Conclusions

Somatic cell interactions drive a number of different spermatogonial responses in the mammalian testes, depending on the stage of the cycle of the seminiferous epithelium, as well as the subset of the spermatogonial population being examined. In this review, we have detailed how testicular somatic cells, even those that reside on the outer of the seminiferous tubules—thus not in direct contact with spermatogonia, produce a number of growth factors that can stimulate self-renewal of SSCs as well as maintenance of the undifferentiated spermatogonial pool. These growth factors include GDNF produced by Sertoli cells and PMCs; FGF2 produced by Sertoli cells, and CSF1 produced by macrophages, PMCs and potentially Leydig cells. Further, this review has explored how somatic cells; particularly the Sertoli cells, also orchestrate transition to a differentiating state in a subset of transit amplifying progenitors at stages VI to VIII of the seminiferous epithelial cycle by regulating synthesis and degradation of RA. In conclusion, the somatic cells in the testes play an indispensable role in regulating cell fate decisions of spermatogonia, ensuring that the self-renewing SSC reservoir is not lost, and driving spermatogonia through a differentiating pathway at discriminant times to maintain continual sperm production throughout the life of the adult male.

References

- Chan, F., Oatley, M. J., Kaucher, A. V., Yang, Q.-E., Bieberich, C. J., Shashikant, C. S., & Oatley, J. M. (2014). Functional and molecular features of the Id4+ germline stem cell population in mouse testes. *Genes & Development*, 28, 1351–1362.
- Chen, L.-Y., Willis, W. D., & Eddy, E. M. (2016). Targeting the *Gdnf* gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. *Proceedings of the National Academy of Sciences*, 113, 1829–1834.
- Chen, L., Brown, P. R., Willis, W. B., & Eddy, E. M. (2014). Peritubular Myoid cells participate in male mouse Spermatogonial stem cell maintenance. *Endocrinology*, 155, 4964–4974.
- Chen, S.-R., & Liu, Y.-X. (2016). Myh11-Cre is not limited to peritubular myoid cells and interaction between Sertoli and peritubular myoid cells needs investigation. *Proceedings of the National Academy of Sciences of the United States of America*, 113, E2352.
- Cunningham, T. J., & Duester, G. (2015). Mechanisms of retinoic acid signalling and its roles in organ and limb development. *Nature Reviews. Molecular Cell Biology*, 16, 110–123.
- DeFalco, T., Potter, S. J., Williams, A. V., Waller, B., Kan, M. J., & Capel, B. (2015). Macrophages contribute to the Spermatogonial niche in the adult testis. *Cell Reports*, 12, 1107–1119.
- Eliasson, P., & Jönsson, J.-I. (2010). The hematopoietic stem cell niche: Low in oxygen but a nice place to be. *Journal of Cellular Physiology*, 222, 17–22.
- Endo, T., Romer, K. A., Anderson, E. L., Baltus, A. E., de Rooij, D. G., & Page, D. C. (2015). Periodic retinoic acid–STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. *PNAS*, 112, E2347–E2356.
- Enomoto, H., Araki, T., Jackman, A., Heuckeroth, R. O., Snider, W. D., Johnson, E. M., Jr., & Milbrandt, J. (1998). GFR α 1-deficient mice have deficits in the enteric nervous system and kidneys. *Neuron*, 21, 317–324.

- Gonzalez-Herrera, I. G., Prado-Lourenco, L., Pileur, F., Conte, C., Morin, A., Cabon, F., Prats, H., Vagner, S., Bayard, F., Audigier, S., & Prats, A. (2006). Testosterone regulates FGF-2 expression during testis maturation by an IRES-dependent translational mechanism. *The FASEB Journal*, 20, 476–478.
- Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., Simons Benjamin, D., & Yoshida, S. (2014). Mouse Spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell*, 14, 658–672.
- Helsel, A. R., Oatley, M. J., & Oatley, J. M. (2017a). Glycolysis-optimized conditions enhance maintenance of regenerative integrity in mouse Spermatogonial stem cells during long-term culture. *Stem Cell Reports*, 8, 1430–1441.
- Helsel, A. R., Yang, Q., Oatley, M. J., Lord, T., Sablitzky, F., & Oatley, J. M. (2017b). ID4 levels dictate the stem cell state in mouse spermatogonia. *Development*, 144, 624–634.
- Hogarth, C. A. (2015). *Sertoli Cell Biology*. Netherlands: Elsevier.
- Hogarth, C. A., Arnold, S., Kent, T., Mitchell, D., Isoherranen, N., & Griswold, M. D. (2015a). Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. *Biology of Reproduction*, 92(2), 37.
- Hogarth, C. A., Evanoff, R., Mitchell, D., Kent, T., Small, C., Amory, J. K., & Griswold, M. D. (2013). Turning a Spermatogenic wave into a tsunami: Synchronizing murine spermatogenesis using WIN 18,446. *Biology of Reproduction*, 88(40), 41–49.
- Hogarth, C. A., Evans, E., Onken, J., Kent, T., Mitchell, D., Petkovich, M., & Griswold, M. D. (2015b). CYP26 enzymes are necessary within the postnatal seminiferous epithelium for normal murine spermatogenesis. *Biology of Reproduction*, 93, 19.
- Kaucher, A. V., Oatley, M. J., & Oatley, J. M. (2012). NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor Spermatogonia. *Biology of Reproduction*, 86(5), 164.
- Kent, T., Arnold, S. L., Fasnacht, R., Rowsey, R., Mitchell, D., Hogarth, C. A., Isoherranen, N., & Griswold, M. D. (2015). ALDH enzyme expression is independent of the Spermatogenic cycle, and their inhibition causes Misregulation of murine Spermatogenic processes. *Biology of Reproduction*, 94, 11–13. Article 12.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *PNAS*, 101, 16489–16494.
- Lord, T., & Oatley, J. M. (2017). A revised A_{single} model to explain stem cell dynamics in the mouse male germline. *Reproduction*, 154, R55–R64.
- Maekawa, M., Kamimura, K., & Nagano, T. (1996). Peritubular myoid cells in the testis: Their structure and function. *Archives of Histology and Cytology*, 59, 1–13.
- Meng, X., Lindahl, M., Hyvönen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G., Westphal, H., Saarma, M., & Sariola, H. (2000). Regulation of cell fate decision of undifferentiated Spermatogonia by GDNF. *Science*, 287, 1489–1493.
- Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reichardt, L. F., Ryan, A. M., Carver-Moore, K., & Rosenthal, A. (1996). Renal and neuronal abnormalities in mice lacking GDNF. *Nature*, 382, 76–79.
- Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R. E., & Yoshida, S. (2010). Functional hierarchy and reversibility within the murine Spermatogenic stem cell compartment. *Science*, 328, 62–67.
- Oatley, J. M., Avarbock, M. R., & Brinster, R. L. (2007). Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse Spermatogonial stem cells is dependent on Src family kinase signaling. *The Journal of Biological Chemistry*, 282, 25842–25851.
- Oatley, J. M., Oatley, M. J., Avarbock, M. R., Tobias, J. W., & Brinster, R. L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development*, 136, 1191–1199.
- Oatley, M. J., Kaucher, A. V., Racicot, K. E., & Oatley, J. M. (2011). Inhibitor of DNA binding 4 is expressed selectively by single Spermatogonia in the male germline and regulates the self-renewal of Spermatogonial stem cells in mice. *Biology of Reproduction*, 85, 347–356.
- Pellegrini, M., Filippini, D., Gori, M., Barrios, F., Lolicato, F., Grimaldi, P., Rossi, P., Jannini, E. A., Geremia, R., & Dolci, S. (2008). ATRA and KL promote differentiation toward the meiotic program of male germ cells. *Cell Cycle*, 7, 3878–3888.
- Pellegrini, M., Grimaldi, P., Rossi, P., Geremia, R., & Dolci, S. (2003). Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: A potential role of BMP4 in spermatogonia differentiation. *Journal of Cell Science*, 116, 3363–3372.
- Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A.-C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H., & Westphal, H. (1996). Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature*, 382, 73–76.
- Piquet-Pellorce, C., Dorval-Coiffec, I., Pham, M., & Jégou, B. (2000). Leukemia inhibitory factor expression and regulation within the testis. *Endocrinology*, 141, 1136–1141.
- Raverdeau, M., Gely-Pernot, A., Féret, B., Dennefeld, C., Benoit, G., Davidson, I., Chambon, P., Mark, M., & Ghyselinck, N. B. (2012). Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. *PNAS*, 109, 16582–16587.
- Rossi, P., Sette, C., Dolci, S., & Geremia, R. (2000). Role of c-kit in mammalian spermatogenesis. *Journal of Endocrinological Investigation*, 23, 609–615.
- Sar, M., Lubahn, D. B., French, F. S., & Wilson, E. M. (1990). Immunohistochemical localization of the androgen receptor in rat and human tissues*. *Endocrinology*, 127, 3180–3186.
- Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., & Pachnis, V. (1994). Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor ret. *Nature*, 367, 380–383.
- Spinnler, K., Köhn, F. M., Schwarzer, U., & Mayerhofer, A. (2010). Glial cell line-derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man. *Human Reproduction*, 25, 2181–2187.
- Tong, M., Yang, Q., Davis, J. C., & Griswold, M. D. (2013). Retinol dehydrogenase 10 is indispensable for spermatogenesis in juvenile males. *PNAS*, 110, 543–548.
- van Pelt, A. M., & de Rooij, D. G. (1990). Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. *Biology of Reproduction*, 43, 363–367.
- Van Pelt, A. M., & De Rooij, D. G. (1991). Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of Spermatogenic cells. *Endocrinology*, 128, 697–704.
- Vernet, N. G., Dennefeld, C., Cc, R.-E., Oulad-Abdelghani, M., Chambon, P., Ghyselinck, N. B., & Mark, M. (2006). Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology*, 147, 96–110.
- Wang, M., Fijak, M., Hossain, H., Markmann, M., Nüsling, R. M., Lochnit, G., Hartmann, M. F., Wudy, S. A., Zhang, L., Gu, H., Konrad, L., Chakraborty, T., Meinhardt, A., & Bhushan, S. (2017). Characterization of the micro-environment of the testis that shapes the phenotype and function of testicular macrophages. *The Journal of Immunology*, 198, 4327–4340.
- Wang, P., Suo, L., Wang, Y., Shang, H., Li, G., Hu, J., & Li, Q. (2014). Effects of GDNF and LIF on mouse spermatogonial stem cells proliferation in vitro. *Cytotechnology*, 66, 309–316.
- Wolbach, S. B., & Howe, P. R. (1925). Tissue changes following deprivation of fat-soluble vitamin. *The Journal of Experimental Medicine*, 42, 753–777.
- Xin, H.-B., Deng, K.-Y., Rishniw, M., Ji, G., & Kotlikoff, M. I. (2002). Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. *Physiological Genomics*, 10, 211–215.
- Yang, Y., Feng, Y., Feng, X., Liao, S., Wang, X., Gan, H., Wang, L., Lin, X., & Han, C. (2016). BMP4 cooperates with retinoic acid to induce the expression of differentiation markers in cultured mouse Spermatogonia. *Stem Cells International*, 2016, 9536192.
- Yoshida, S., Sukeno, M., & Nabeshima, Y. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science*, 317, 1722–1726.

Differential Expression of Golgi Proteins During Spermatogenesis

Louis Hermo, Regiana L Oliveira, Charles E Smith, and John JM Bergeron, McGill University, Montreal, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

The Golgi apparatus is a cellular organelle that is found in each of the estimated greater than 200 different cell types that make up the human body (Valentine et al., 1994). The Golgi apparatus plays a major role in protein biosynthesis, post-translational modification of endoplasmic reticulum (ER) derived proteins and as a sorting station for proteins directed for the plasma membrane, lysosomes and other destinations. Golgi apparatus structure visualized by standard thin sectioning techniques at the electron microscope (EM) level, that reached its apogee in the latter part of the last century, can identify solely and unambiguously any of the differentiated cell types in a given species (Clermont et al., 1995; Rambourg and Clermont, 1990). Remarkably, its structure is strictly conserved with the number and size of cisternae in Golgi stacks and overall size of the Golgi apparatus under strict control for each of the many differentiated cell types of a given tissue or organ of the human body. This conjecture is illustrated in this work for the Golgi apparatus of male germ cells in the adult rat testis as they undergo differentiation through the process called spermatogenesis.

Part 1: Spermatogenesis: A Discovery Based on the Golgi Apparatus

In order to fully understand the differential distribution of Golgi proteins during germ cell development, one must commence with an understanding of the organization and composition of the germ cell types comprising the many seminiferous tubules of the adult testis (Clermont, 1972). In mammalian species (our focus will be on the rat), these tubules are lined by a unique stratified seminiferous epithelium bordering a centrally located lumen (Fig. 1A–F). The seminiferous epithelium consists of germ cells that are at different phases of their differentiation process, with the end result being the production of sperm (Fig. 1A–F). The latter after being fully formed enter the centrally located lumen, whereby they then gain access to the epididymis, the site of sperm maturation. Germ cells are accompanied by nondividing Sertoli cells, which serve to support and nourish germ cells, but are beyond the topic of the present study (Fig. 1A–F).

On the other hand, germ cells in the seminiferous epithelium of the testis undergo a differentiation process as they evolve from the youngest germ cells called spermatogonia into maturing spermatozoa (Fig. 2). Defined as spermatogenesis, this event occurs over a span of several weeks and is characterized by a period of mitotic, meiotic and postmeiotic events (Fig. 2; Leblond and Clermont, 1952; Clermont, 1972; Steinberger and Steinberger, 1975; Clermont et al., 1993; Hermo et al., 2010a). Spermatogonia undergo several mitotic divisions to self-renew as well as gradually increase their numbers to eventually form spermatocytes. The latter undergo two meiotic divisions thereby reducing the number of chromosomes by half to form haploid spermatids, which subsequently undergo a structural metamorphosis to become spermatozoa (Fig. 2; Dym and Clermont, 1970). Spermatids undergo a series of dramatic structural and functional modifications and, as defined by acrosome development and sperm head shape alterations, result in 19 well defined steps in their differentiation, a process referred to as spermiogenesis, a subdivision of spermatogenesis (Fig. 3; Clermont, 1972; Hermo et al., 2010a; Hess et al., 2015).

A continuously renewing seminiferous epithelium creates a unique situation for the differentiation of germ cells unlike any other in the human body. In any given cross sectional profile of a seminiferous tubule, the different germ cell types develop in groups and form several concentric layers around the tubule. Generally, spermatogonia and early spermatocytes localize to the base of the epithelium, more advanced spermatocytes and early round spermatids occupy the mid region, and late elongating spermatids prior to release appear closest to the lumen (Fig. 1A–F). A distinguishing feature of the seminiferous epithelium is that the composition of germ cells in any given portion or segment along the length of the tubule can be very different from adjacent segments. In fact, only one of 14 unique germ cell associations, defined as stages, occupies a given cross sectional profile of a seminiferous tubule at any given time in the rat testis (Fig. 1A–F).

The key to figuring out the complexity of these cellular associations came with the discovery of the periodic acid Schiff (PAS) technique. In the mid-1950s, detailed light microscope observations elucidated the complexity of the differentiation pathway of male germ cells in the seminiferous epithelium of the testis. The PAS technique, specific to vicinyl hydroxyls and when applied to the testis, revealed staining of the Golgi apparatus and a Golgi derived membrane derived lysosomal structure called the acrosome that was being produced in a specific population of germ cells referred to as early spermatids (Figs. 2 and 3). The reaction due to carbohydrate modifications on glycoproteins was modulated by Golgi-localized glycosyltransferases. In fact it was the Golgi apparatus of spermatids and its role in the formation of the lysosomal acrosome, to be used by sperm at the time of fertilization of the egg that was the defining point in unraveling spermatogenesis. The developing acrosome which grew with time in size while attaching itself to the nucleus of spermatids, along with changes to the shape of the spermatid head (from spherical to elongating) served to identify 14 specific cellular associations that were called stages of the seminiferous epithelium. Examples of several stages can be seen in Fig. 1A–F. The PAS method provided a precise mapping of how germ cells associate with each other in the numerous cross sectional profiles of seminiferous tubules that made up the testis proper (Fig. 2) and has survived the test of time for future

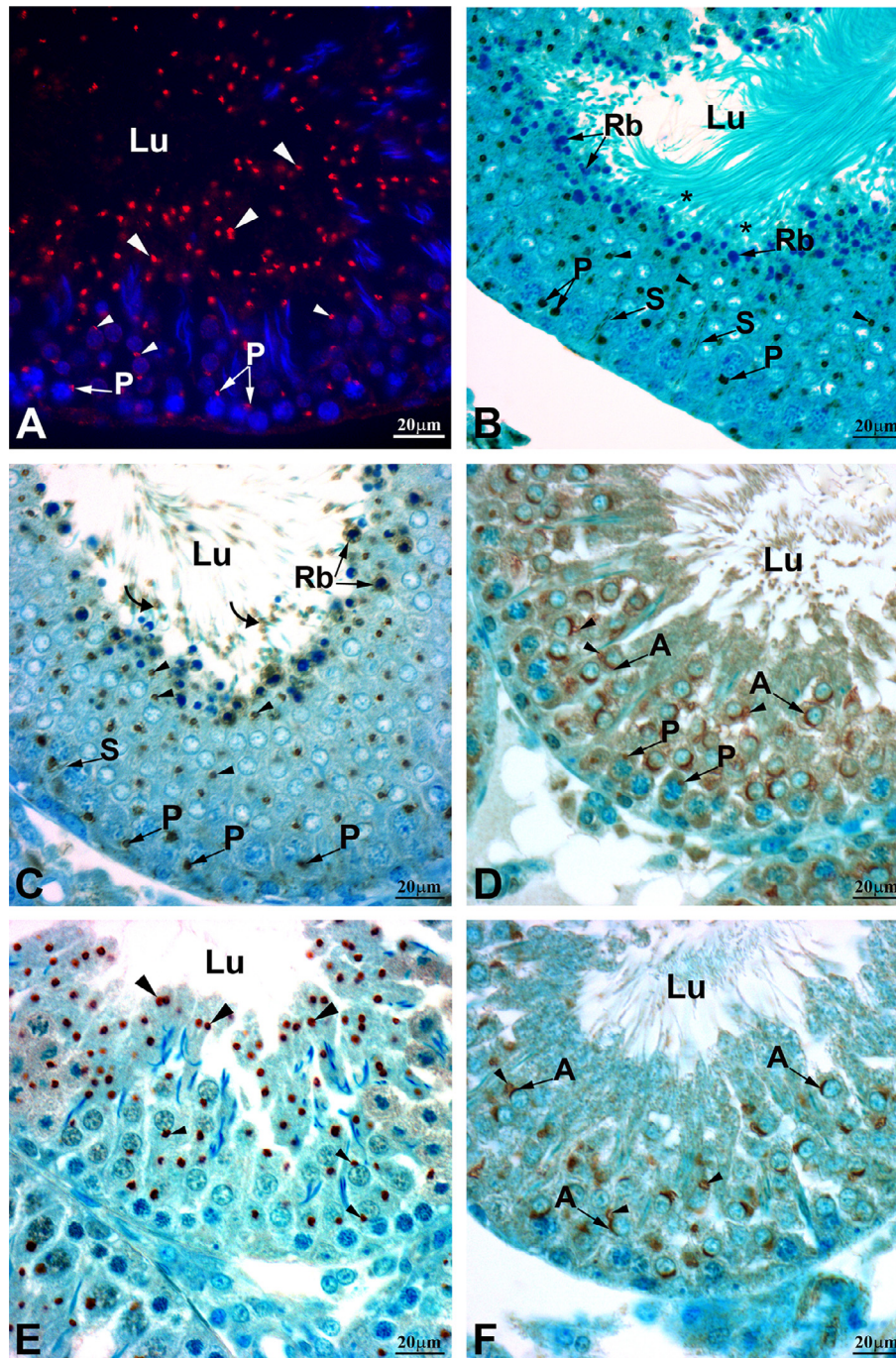
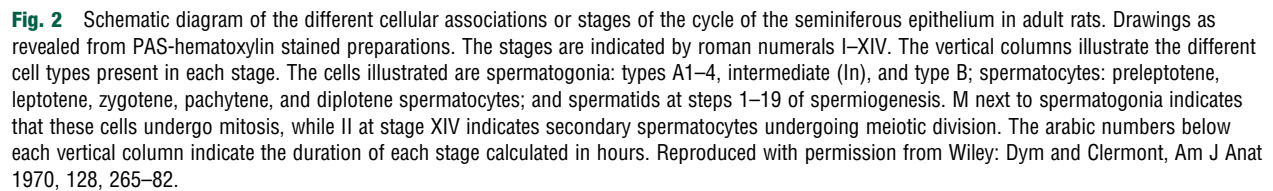


Fig. 1 Cross sectional profiles of seminiferous tubules at different stages of the cycle are shown to enhance understanding of spermatogenesis as discussed in Part 1. Each tubule contains germ cells that are specific to the stage that they occupy as defined by the criteria set down by [Leblond and Clermont \(1952\)](#). The non-dividing Sertoli cells are also shown. Germ cells at one specific stage do not appear in another stage, establishing the precise order of spermatogenesis. These images also serve to localize proteins that were reactive for the Golgi apparatus at the stages indicated in each figure (A–F) as discussed in Part 2. LM-IF (A) of a tubule at stage III–IV and LM-IHC (B) of a tubule at stage VII revealing punctate reactions for the Golgi apparatus of germ cells and Sertoli cells of sections stained for TM9SF3. LM-IHC (C) of a tubular section at stage VIII showing reactive Golgi apparatus of germ and Sertoli cells stained for TMED7/p27. Rbs and the forming Hermes body are also reactive. LM-IHC (D) of a tubular section at stage V–VI revealing reactions over the Golgi apparatus of pachytene spermatocytes and steps 5–6 spermatids and the acrosome stained for GBF1. LM-IHC (E) of a tubular section at stage XIV showing Golgi reactions for germ cells stained for GL54D. LM-IHC (F) of a tubular section at stage V–VI revealing reactions over the Golgi apparatus of steps 5–6 spermatids and the acrosome stained for GPP34 (GOLPH3). Abbreviations: Lumen (Lu), pachytene (P) spermatocytes, cytoplasm of late spermatids (*asterisks*), Sertoli cells (S), seminiferous epithelium (SE), residual bodies (Rb), Golgi apparatus early (*small arrowheads*) and late (*large arrowheads*) spermatids, forming Hermes body (*curved arrows*), acrosome (A).



Setting the Stage for Germ Cell Differentiation and Golgi Protein Expression Profile

Cycling Through the Stages of Germ Cell Development

In addition **Fig. 2** shows how any given germ cell type will appear with time as it differentiates through the 14 stages of the cycle. In some cases these changes are simply a change in size (e.g. pachytene spermatocytes), or more dramatically shape (round nucleus of early spermatids versus elongating nucleus of late spermatids) or addition of newly formed structures (acrosome of early spermatids; tails of spermatids). Therefore, each generation of germ cells depicted in a given column of **Fig. 2** and representing a particular stage e.g. stage I, will advance in its development to form the distinct composition of generation of germ cells of the next stage, i.e. stage II. This continues for germ cells at stage II to stage III and so forth and so on until stage XIV, whereupon

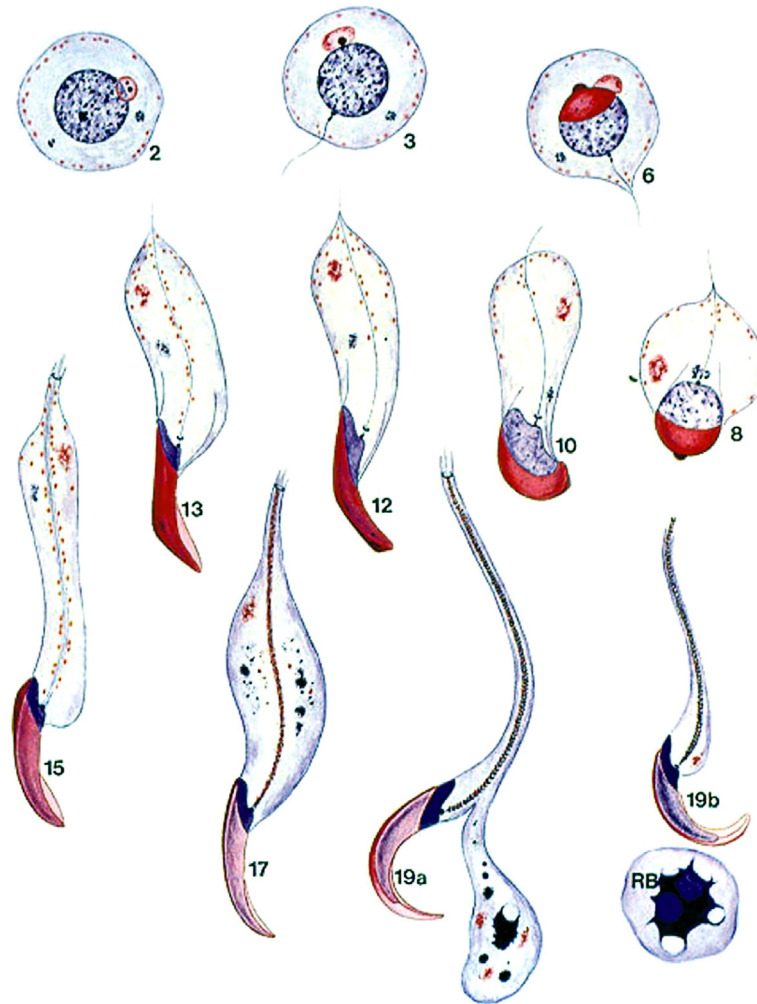


Fig. 3 Schematic representation of the role of the Golgi apparatus in acrosome formation during spermiogenesis as visualized by the PAS method. At step 2 the Golgi apparatus seen as a small *magenta colored bubble* has formed two small dense proacrosomic granules placed next to the nucleus, which at step 3 have fused together to form a single larger dense granule. At step 6 the acrosome has grown in size and covers a sizeable portion of the nucleus. At step 8 the nucleus occupies an eccentric position next to one pole of the nucleus, while the Golgi apparatus becomes spherical in shape and floats freely in the cytoplasm. From steps 10–19 the nucleus becomes gradually compacted, elongated and sickle shaped. The spherical Golgi apparatus is still evident as a *reddish bubble* in the vast cytoplasm of these cells. At step 19a, the cytoplasm has displaced itself along the tail to form the residual body (RB) seen hanging as a sac onto the sperm at the junction of its head and tail. At step 19b, the residual body has completely liberated itself from the sperm, while a small dilated focal area of cytoplasm at the neck region of the tail constitutes the Hermes body (HB) (cytoplasmic droplet). It reveals the presence of a small reddish remnant of the Golgi apparatus, as does the residual body. Reproduced with permission from Wiley: *Andrology*, 2015 Nov; 3 (6):1015–21. Hess RA, Hermo L, Robaire B; Lessons learned in andrology: Yves Clermont, an interview by Lonnie D. Russell.

germ cells of stage I then reappear. The sequential appearance of the different generation of germ cells from stage I to stage XIV and then back to stage I constitutes the so-called cycle of the seminiferous epithelium.

Here is how this works. An important feature of germ cell differentiation is that the different generations of germ cells at a given stage are evolving over time in a given segment of a tubule that they occupy, i.e. germ cells do not move laterally along the tubule as they differentiate. Indeed, if one were able to watch in the living organism the various generations of germ cells defining a distinct stage, e.g. stage I, at a particular segment of the tubule, with time the different germ cell generations would evolve into those characterizing the next stage (stage II), and this would go on and on until stage I would reappear once again in that same segment of the tubule. Hence germ cells advance in differentiation in a given cross sectional profile of the tubule that results in all 14 stages appearing in a particular area of the tubule over time. Leblond and Clermont (1952) defined the advancement of germ cell differentiation over the 14 stages of a given area of the tubule as the “cycle” of the seminiferous epithelium (Fig. 2). Considering that sperm are being released continuously at the end of spermatogenesis, the different generations of germ cells evolve and advance in development, and this continuous production is due to the actively mitotic divisions of the spermatogonial stem cells.

Using a variety of techniques, the duration of each stage of the cycle of the seminiferous epithelium was calculated and shown to be constant but varying in time for different stages (Fig. 2). Some stages lasted for a short period of time, while others lasted longer, i.e. stages IX and X, 7 h; stage VII, 58 h. Adding up the different durations of each stage, the total duration of the cycle was calculated to be 12.9 days in Sprague Dawley rats and that of the complete process of spermatogenesis 51.6 days (Clermont and Harvey, 1965; Clermont, 1972). A comprehensive analysis of the stages of the cycle of the rat and several other species is provided in a complete book on the testis (Russell et al., 1990). A video by W. Vogl and R. Hess depicting the 14 stages of the cycle of the rats in time can be viewed at <https://www.facebook.com/stagescycle/>. In addition, for those that might be interested, the gross characteristics of adult rat seminiferous tubules, first described by Clermont and Huckins (1961), has been elegantly redefined in a 3D reconstruction of their number, length, run and mutual relationships (Nakata et al., 2015, 2017). Knowledge of the stages of spermatogenesis is also useful for analyzing abnormalities in mutant mice undergoing different degrees of germ cell generation (Meistrich and Hess, 2013). An assessment of the functions of several critical genes during spermatogenesis has been examined in mutant mice (Matzuk and Lamb, 2008; Lin and Matzuk, 2014).

Spermiogenesis: A Key Event for Golgi Apparatus Function Serving Sperm Fertilizing Capability

Spermiogenesis is a timely event defining the last phase of germ cell differentiation. It begins after the 2nd meiotic division of spermatocytes with the formation of haploid spermatids and follows them through their metamorphosis into spermatozoa. It is subdivided into distinct steps with 19 being identified in rats; the entire process extends over 22.7 days (Fig. 3). During spermiogenesis, many genes/proteins are expressed, some for the first time, with implications in the diverse dynamic events occurring at this time point of germ cell development. These events include formation of the flagellum, nuclear condensation, and modification of specific organelles, such as the Golgi apparatus and ER. A significant event is formation of the acrosome by the Golgi apparatus of early spermatids leading to the formation of spermatozoa enhanced with the ability to penetrate the protein coats of the egg (Clermont et al., 1993; Hermo et al., 2010a,b; Gao et al., 2016).

The Good, the Bad and the Sticky

The Golgi apparatus (the good) of early spermatids is the key player in the formation of the acrosome during the early steps of spermiogenesis. The acrosome (the bad) is a membrane bound lysosome filled with an arsenal of hydrolytic enzymes that is used at the time of fertilization to break through the coverings of the egg. The nucleus (sticky) provides a platform for tight binding of the acrosome to ensure its presence on the sperm at the time of fertilization. Hence the Golgi apparatus, acrosome and its attachment to the nucleus are critical to fertilization.

The Golgi Apparatus Forms the Acrosome During Steps 1–7 of Spermiogenesis

Steps of spermatid differentiation, as defined by Golgi identification and acrosome formation were discovered through the PAS reaction which alone enabled a distinction in the rat amongst the transformation of early round spermatids (steps 1–8) to late elongated spermatids (steps 9–19) (Leblond and Clermont, 1952; Hermo et al., 2010a). The PAS staining of the Golgi apparatus and the acrosome is found in the original illustration from Dr. Yves Clermont shown here in Fig. 3.

From steps 1–3 spermatids as noted in the LM, small proacrosomic granules made by the hemispherical Golgi apparatus appear adjacent to the spherical nucleus. With continued production these granules fuse with one another creating a larger membrane bound structure called the acrosome (step 4). The latter applies itself to the nuclear surface to which it tightly adheres, and with time and progressive enlargement by contributions from the Golgi apparatus, it spans more and more of the nuclear surface (steps 5 and 6), until its formation is completed at which time it covers almost half of the nuclear circumference (step 7) (Fig. 3). During acrosome formation, the hemispherical Golgi is closely associated with the developing acrosome at one pole of the nucleus.

Stacking of Golgi Cisternae and Tubular Interconnections to Form the Golgi Ribbon: The Dynamics of the Golgi Ribbon in Forming the Acrosome

The structure of the Golgi apparatus of early spermatids at the time of acrosome formation has been described amply by EM analysis (Thorne-Tjomsland et al., 1988; Griffiths et al., 1981; Hermo et al., 2010a,b; Hermo et al., 1980; Susi et al., 1971; Hermo et al., 2018). The Golgi apparatus is a continuous structure with alternating compact stacked and non-compact tubular interconnecting regions (Hermo et al., 1980; Clermont et al., 1994; Thorne-Tjomsland et al., 1988; Fig. 4), with an overall appearance in mammalian cells to that of a ribbon. The term Golgi ribbon was first introduced by Rambourg and Clermont (1990). Its usage has been adopted universally by the scientific community to define the conserved properties of Golgi apparatus structure in all mammalian cell types (Jarvela and Linstedt, 2014; Vinke et al., 2011; Lavieu et al., 2014).

In essence, the stacked compact region of the Golgi ribbon consists of a highly organized assembly of tightly apposed flattened cisternae (Fig. 4). The latter show remarkable structural differences from the *cis* (forming) face to the *trans* (mature) face of the Golgi stack. Proteins synthesized in ER cisternae facing the *cis* face and carried by vesicles fuse with an anastomotic closely knit tubular network (CGN) forming the *cis*-most element of the Golgi ribbon. Beneath and closely opposed to one another are nine flattened cisternae. Dilated tubules extend from the edges of these flattened cisternae and serve to connect adjacent compact regions to each

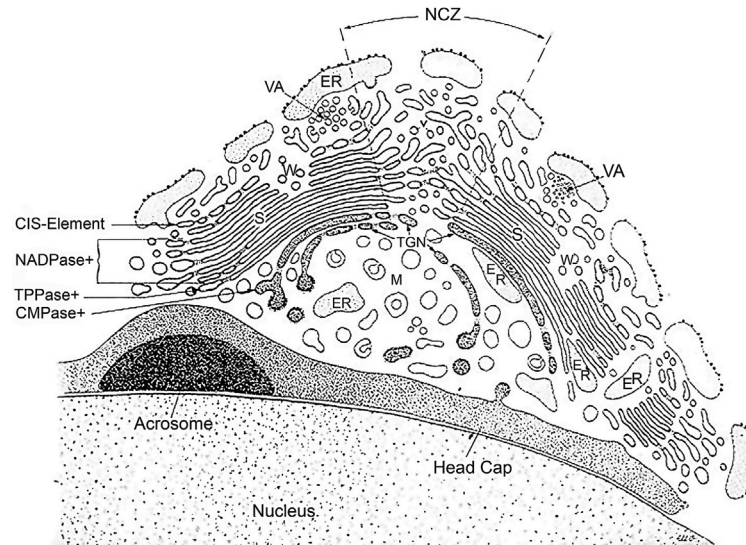


Fig. 4 Schematic representation of the Golgi apparatus of a step 7 spermatid. Represented are stacks of Golgi cisternae (S) forming the compact zone and dilated tubules extending from the cisternal edges serving to join adjacent stacks that form the non-compact zone (NCZ). The Golgi stack consists of an anastomotic tubular network on the *cis* face called the CIS-Element, mid cisternae stained for NADPase, a *trans* cisterna stained for TPPase and 2 CMPase cisternae called the trans Golgi networks. The latter reveal bristle (clathrin) coated buds at their edges and a peeling off configuration. Endoplasmic reticulum (ER) is seen overlying the *cis* element, applied to *trans* cisterna and the TGN, as well as in the NCZ and core or medulla (M) of the Golgi apparatus. Pan shaped spaces of the *cis* cisternae form wells (W). Vesicular aggregates (VA) also appear on the *cis* face of the Golgi apparatus. This material was originally published in Cell and Molecular Biology of the Testis, chapter 14 "Cell Biology of Mammalian Spermatogenesis", Clermont, Y., L. Hermo, and R. Oko. 1993, edited by Claude Desjardins and Larry L. Ewing and has been reproduced by permission of Oxford University Press.

other. These tubules form the so-called non compact regions. Overall the various Golgi stacks and interconnecting non compact regions form a continuum defining the Golgi ribbon. Many small 50–60 nm vesicles and tubules are associated with the Golgi ribbon (Fig. 4).

On the trans-most side of the Golgi ribbon, 1–3 morphologically distinct thickened cisternae characterize the so-called trans Golgi networks (TGNs), which at times reveal a peeling off configuration from the Golgi ribbon (Fig. 4). Vesicular budding showing a bristle or clathrin coat is often seen emanating from their edges. The TGNs are apposed to the developing acrosome, which during its formation applies itself to one pole of the nuclear surface. Continuity between the TGN and acrosome are seen but rarely. The area in between the TGNs and the developing acrosome also contains many vesicular and tubular profiles decorated with bristle coats, some of which fuse with the acrosome (Burgos and Gutierrez, 1986; Mollenhauer and Zebrun, 1960; Susi et al., 1971). EM cytochemical markers indicate that nicotinamide adenine dinucleotide phosphatase (NADPase) localizes to middle cisternae, thiamine pyrophosphatase (TPPase) to the trans most cisternae, and cytidine 5-monophosphatase (CMPase) to the TGNs (Fig. 4; Clermont et al., 1981; Smith et al., 1990; Thorne-Tjomsland et al., 1988). Because the acrosome also shows a cytochemical reaction for CMPase, TPPase, it is suggested that these respective Golgi cisternal elements contribute directly to acrosome formation (Thorne-Tjomsland et al., 1988; Hermo et al., 2010b). As the TGNs reveal a peeling off configuration from the stack and an apparent breakdown of their structure into tubules and vesicles, it has been suggested that the Golgi cisternae turnover as they form the acrosome (Thorne-Tjomsland et al., 1988). But this theory may be an oversimplification of Golgi dynamics as will be discussed in Part 2.

Postacrosome Phase of Spermiogenesis (Steps 8–18): The Golgi Is Functional After Acrosome Formation

Beginning at step 8 and thereafter, the Golgi apparatus becomes spherical and migrates away from the acrosome to lie freely in the cytoplasmic lobe of late spermatids. At later steps of spermiogenesis, it continues to be involved in glycoprotein synthesis destined for the plasma membrane (Batista et al., 2012; Clermont and Tang, 1985; Susi et al., 1971; Clermont et al., 1993). During this time, the acrosome conforms to the changes to the shape of the nucleus, which in the rat takes on its species-specific sickle shaped appearance. At step 15 the Golgi ribbon begins to fragment and become vacuolated (Susi et al., 1971), with individual flattened cisternae of the compact Golgi stack dispersing into the cytoplasm (Oko et al., 1993).

Step 19 Spermatid (The Last Frontier): The Golgi Cisternae Congregate Into the Forming Hermes Body (Cytoplasmic Droplet)

At the last step of spermiogenesis, the flattened Golgi cisternae of the step 19 spermatids congregate into a small pouch of cytoplasm located at the neck region of the flagellum, formerly called the cytoplasmic droplet (Oko et al., 1993), and renamed recently as the

Hermes body (Au et al., 2015a). These authors have suggested a role for the Golgi cisternae and other proteins of the Hermes body in the maturation of sperm as they traverse the epididymal duct. This topic is discussed in a separate section of the Encyclopedia of Reproduction.

Part 2: Defining Golgi Proteins Expressed in Germ Cells During Spermatogenesis

One of the shortcomings about the Golgi ribbon of germ cells is the lack of appropriate markers across the full spectrum of germ cell differentiation. In 2015, a reproducible method to isolate Golgi apparatus selectively from male germ cells of whole adult rat testis homogenates was developed (Au et al., 2015a). By quantitative proteomics, a characterization of over 1000 different proteins in this subcellular fraction was elucidated, with several representing novel Golgi proteins as well as known Golgi proteins.

With monospecific antibodies to 20 different proteins obtained from different sources (Au et al., 2015a) and concluded to be Golgi localized by LM immunohistochemical localization (LM-IHC), a map of their expression profile during spermatogenesis was realized. Here we discuss 14 of these Golgi proteins to illustrate their complex distribution pattern during the process of germ cell differentiation (spermatogenesis) in attempting to define their functional significance to spermatogenesis. The insight gained from the detailed analysis of this data will be summarized by the illustration of spermatogenesis truncated to 30 of the 64 distinct cell types identified. Here germ cells are represented undergoing mitosis, meiosis, acrosome formation (steps 1–7), postacrosome Golgi migration (steps 8–18), and ending at the terminal step of spermiogenesis, step 19 spermatids (Fig. 5). A more detailed analysis of Golgi proteins in germ cells based on the work of Au et al. (2015a) and that of others can be found in Hermo et al. (2018).

Defining Golgi Proteins to Specific Germ Cells During Differentiation Using the Staging Scheme of Leblond and Clermont (1952)

Golgi proteins localized to spermatogonia, spermatocytes and spermatids: TMED7/p27, TMED4/p25 and TM9SF3

The periodic renewal of type A spermatogonia was recognized early in the last century (Regaud, 1901), but the complex process of spermatogonial renewal is only being fully understood 60 years later. In the early 1950s, it was the inspirational work of Clermont and Leblond (1953) that first defined stem cells in the testis and paved the way for all subsequent significant contributions on their renewal (Oatley and Brinster, 2008; Yoshida et al., 2009; Griswold, 2016; Mecklenburg and Hermann, 2016; Boitani et al., 2016; Komeya and Ogawa, 2015).

While there are many descriptions of markers for undifferentiated and differentiated spermatogonia (Herme et al., 2010a), those that demarcate the Golgi apparatus of these cells are lacking. Three of the 20 proteins characterized by Au et al. (2015a), although widely distributed throughout spermatogenesis, clearly labeled the Golgi apparatus of spermatogonia. These were one member of the transmembrane superfamily 9, TM9SF3, and two members of the TMEDp24 (transmembrane emp24 domain) family of Golgi (GOLD domain) integral membrane proteins, TMED4/p25 and TMED7/p27.

In the isolated germ cell Golgi fraction, three members of the TM9SF family of proteins of unknown function were identified (Au et al., 2015a). Localization of one of these, TM9SF3, revealed it to be a Golgi integral membrane protein uncovered for the first time as a Golgi marker not only to germ cells but also with a ubiquitous expression in other cell types, such as Sertoli and Leydig cells, epididymal epithelial cells (Au et al., 2015a) and neurons of the brain (unpublished data). In germ cells, TM9SF3 was expressed in the Golgi apparatus from spermatogonia to step 16 spermatids (Figs. 1A, B and 5). TM9SF3 has been shown to positively regulate cell proliferation and hence may play a role during the many mitotic divisions occurring during spermatogonial renewal of stem cells and more differentiated spermatogonia (Shen et al., 2017).

TMED4/p25 and TMED7/p27 proteins also revealed a widespread distribution commencing in spermatogonia and extending to late spermatids (Au et al., 2015a). However, while TMED4/p25 extended from spermatogonia to step 16 spermatids, TMED7/p27 was maintained in step 19 spermatids and in the forming Hermes body (Figs. 1C and 5). TMED2/p24 was also expressed in germ cells but only at step 19 spermatids (see below).

The TMEDp24 (transmembrane emp24 domain) family of Golgi (GOLD domain) membrane proteins regulate trafficking, cargo selection, and lipid makeup of Golgi derived carriers (Dominguez et al., 1998; Pastor-Cantizano et al., 2016). Ten TMED proteins are subdivided into four subfamilies based on sequence similarity (Strating and Martens, 2009). They are regulated during differentiation and development in many cell types (Jerome-Majewska et al., 2010) and may serve this function in germ cells.

Golgi protein localized exclusively to spermatocytes (MG160)

Meiosis is unique to germ cells and involves one round of DNA replication followed by two consecutive cell divisions enabling diploid cells to form haploid cells. Meiosis serves important cellular activities such as DNA repair, genetic exchange, and synapsis of homologous chromosomes, with many genes implicated in this process (Herme et al., 2010a; Mikolcevic et al., 2016; Griswold et al., 2012).

One of the 14 Golgi proteins analyzed by Au et al. (2015a) was expressed solely in spermatocytes. MG160 (Golgin160/MEA-2/Golga3) is a coiled coil protein and part of the Golgin family (Munro, 2011). It is normally expressed in all somatic cells and is a universal Golgi marker (Bell et al., 2001; Dahan et al., 1994). MG160 has long been known to be a prominent gene product of male germ cells (Kondo and Sutou, 1997). In the testis, MG160 expression demarcates the boundary between spermatogonial renewal and spermiogenesis, i.e. spermatocyte differentiation, with expression exclusive to spermatocytes (Au et al., 2015a; Fig. 5). When the gene is truncated in a transgenic mouse model, exactly as for the MG160 null mutant, spermatocytes are depleted

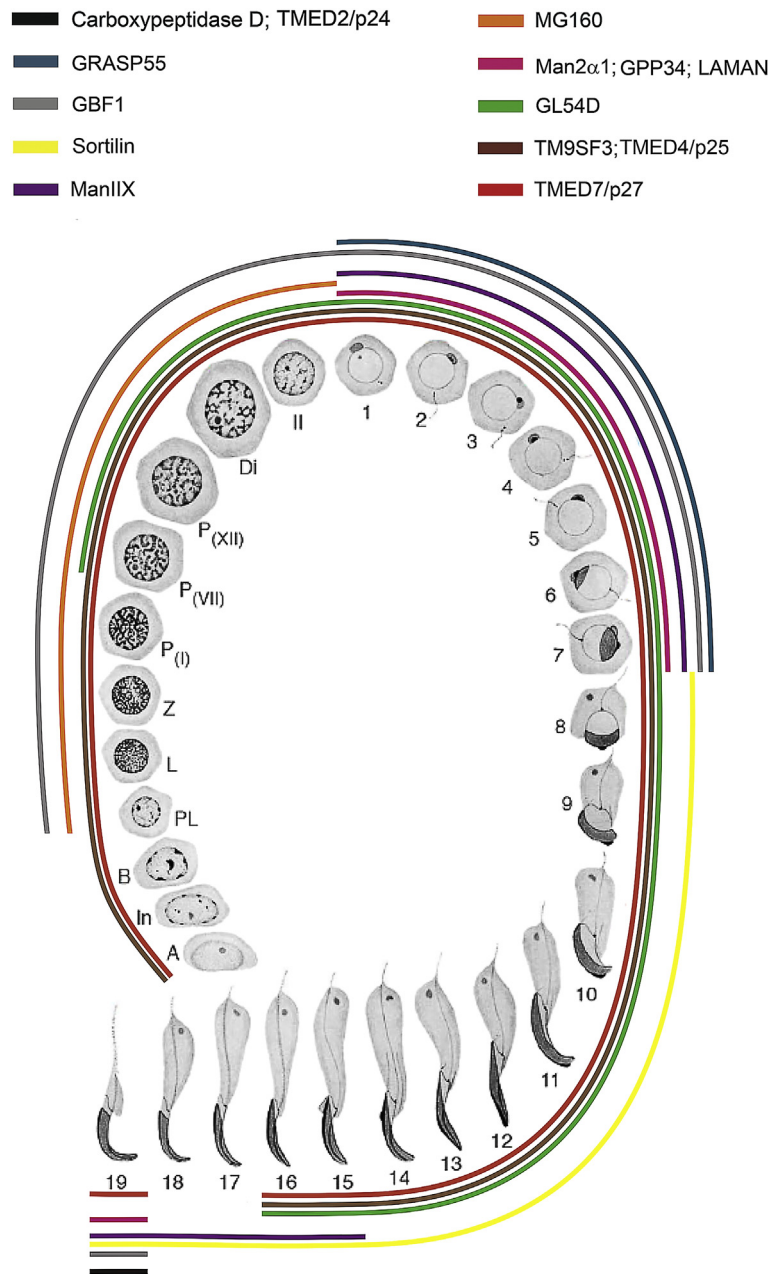


Fig. 5 Thirty representative germ cell types are depicted as they undergo differentiation during spermatogenesis. Illustrated are the mitotic spermatogonia types A, intermediate (In) and B; spermatocytes undergoing meiosis: preleptotene (PL), leptotene (L), zygotene (Z), pachytene (P), diplotene (Di) and secondary spermatocytes (II). Spermatids evolving through 19 steps of spermiogenesis that can be subdivided into acrosome formation (1–7), postacrosome events (8–18) and Hermes body (cytoplasmic droplet) formation (step 19). The expression profile of each Golgi protein in accordance with the different phases of germ cell differentiation is depicted by a specific colored line as deduced by analysis of germ cells at all 14 stages of the cycle from LM-IHC experimentation.

(Bentson et al., 2013). Sperm development is decreased and the resultant few sperm are infertile. MG160 has been implicated in tethering Golgi mini stacks together (Cheung and Pfeffer, 2016), and in this role it may be implicated in the doubling in size of the Golgi apparatus of spermatocytes and its eventual partitioning into four Golgi apparatuses with each one destined for the four resulting haploid spermatids (Suarez-Quian et al., 1991).

Golgi proteins localized to spermatocytes and spermatids (GBF1 and GL54D)

GBF1 is one of the guanine nucleotide exchange factors for ADP-ribosylation factor that regulates vesicular trafficking for active secretion by recycling between *cis*-Golgi and the ER (Wright et al., 2014). GBF1 expression begins at preleptotene spermatocytes

and continues through spermatocyte development with expression extending into early spermatids from steps 1–7 where it localizes to the Golgi apparatus and acrosome (Au et al., 2015a; Figs. 1D and 5). Hence the burst of GBF1 expression in spermatocytes and early spermatids likely reflects their high secretory activity. In fact, forerunners of the proacrosomic granules first appear in zygotene primary spermatocytes and extend into late pachytene spermatocytes (Anakwe and Gerton, 1990; Fawcett, 1975), with acrosome formation advancing through the first half of spermiogenesis (Hermo et al., 2010a,b) when GBF1 is also expressed (Au et al., 2015a).

A study of the isolated testis germ cell Golgi fraction (Au et al., 2015a) revealed that the most abundant Golgi protein was a protein designated from the annotation of UniProtKB/Swiss-Prot, as GLYCOSYLTRANSFERASE 54 DOMAIN-CONTAINING PROTEIN (GL54D), an MGAT4 family member also known as GnT1IP-S (Huang and Stanley, 2010). GL54D was uncovered for the first time at the endogenous protein level by mass spectrometry of the isolated testis Golgi fraction by Au et al. (2015a). GL54D, a type II, N-linked integral membrane terminally glycosylated glycoprotein largely facing the lumina of Golgi cisternae is a Golgi marker like TM9SF3. However, whereas TM9SF3 is ubiquitous to many cells as a universal Golgi marker, GL54D is germ cell-specific revealing a differential expression profile during spermatogenesis. GL54D is first detected in pachytene spermatocytes at stage VII of the cycle with expression extending up to step 16 spermatids (Figs. 1E and 5). Its initial onset of expression coincided exactly with that of the glucose transporter 3 (GLUT-3) protein that decorates the plasma membrane of germ cells and where it may be involved in its transportation to the cell surface (Au et al., 2015a). GLUT-3 was also noted to be the most abundant protein of an isolated Hermes body fraction of sperm, where it was proposed to serve as an ATP energy source for initiation of sperm motility as sperm transited through the epididymal duct (Au et al., 2015b).

Golgi proteins localized to early spermatids for acrosome formation (steps 1–7)

In addition to TMED7/p27, TMED4/p25, TM9SF3, GL54D, and GBF1 all with varied distributions, several additional proteins with Golgi characteristics (GRASP55, GPP34, Man2 α 1, ManIIX and LAMAN) revealed onset of Golgi expression in early spermatids during acrosome formation (Au et al., 2015a).

The acrosome is a lysosomal structure that is formed by the Golgi apparatus during early spermiogenesis (steps 1–7), with an unprecedented specificity for adherence to the nuclear surface of spermatids (Fig. 3). As such it follows the contours and modifications that occur to the shape of the head and nucleus of spermatids during late steps of spermiogenesis (Hermo et al., 2010b). At the time of fertilization, sperm undergo the acrosome reaction, an event unique in the life of a spermatozoon. Acrosomal contents released during fertilization promote sperm penetration through the oocyte's investments, leading to sperm-egg interaction and fusion (Hermo et al., 2010c). Acrosome formation is an intense period of protein synthesis that is marked by the expression of a plethora of proteins localized to the Golgi and developing acrosome (Hermo et al., 2018). In this article, we present only a few of the many Golgi proteins involved in acrosome formation. However, the list of Golgi proteins critical for acrosome formation is impressive and continues to grow as evidenced from numerous studies involving mutant and/or deficient mouse models (see reviews Foster and Gerton, 2016; Niu et al., 2016; Hermo et al., 2018).

GRASP 55 and GPP34

GRASP55 (Golgi reassembly protein) has been implicated in the stacking of Golgi cisternae (Bekier et al., 2017; Zhang and Wang, 2015; Seemann et al., 2000; Shorter et al., 1999). In the testis, its expression first appears after meiosis with Golgi immunoreactivity limited to steps 1–7 spermatids (Au et al., 2015a; Fig. 5) with involvement in acrosome formation, along with interactions with junctional adhesion molecule-C (Cartier-Michaud et al., 2017). However, no localization was noted for the Golgi apparatus of Sertoli or Leydig cells of the testis, all of which reveal stacked Golgi cisternae (Rambourg et al., 1974; Christensen, 1975). Alternative functions to those of cisternae stacking have been proposed for GRASP55 such as unconventional protein secretion and Golgi ribbon formation (Rabouille and Linstedt, 2016). Unlike typical lysosomes, the acrosome has to bind to the nuclear surface of spermatids and this may involve unconventional secretion pathways to accommodate this novel function for the acrosome.

GPP34, now called GOLPH3 (Golgi phosphoprotein 3), the first example of a Golgi resident oncogene protein, has a well-defined role in the regulation of Golgi ribbon structure (Buschman et al., 2015; Bergeron et al., 2017), and this property may also be shared with GRASP55 (Rabouille and Linstedt, 2016). During steps 1–7 spermatids, the Golgi apparatus is crescent in shape with the TGNs exposed to the nuclear surface of early spermatids (Au et al., 2015a). This would allow easy access of the proacrosomic granules to the nuclear surface to which they bind. Defining the shape of the Golgi ribbon may be in part the role of GPP34 and GRASP55, but this would be limited to its expression profile during a defined time point during spermatogenesis, i.e. steps 1–7 spermatids when both are expressed (Au et al., 2015a; Figs. 1F and 5).

Man2 α 1, ManIIX and LAMAN

Man2 α 1 and ManIIX are both well known Golgi mannosidases (Man). Man2 α 1 is a universal Golgi marker also localizing to Sertoli cells (Igdoura et al., 1999; Ramalho-Santos et al., 2001). ManIIX is a male germ cell specific mannosidase partially overlapping with Man2 α 1 in its specificity from steps 1–7 spermatids with both the Golgi and acrosome being reactive (Au et al., 2015a; Fig. 5). These sequence-related proteins likely have related functions in alpha mannosidase trimming of high mannose containing N-linked oligosaccharides during acrosome formation (Moremen, 2002; Herscovics, 1999; Rose, 2012).

LAMAN, also known as Man2B1, a major glycoprotein was also found in the Golgi apparatus and acrosome of steps 1–7 spermatids (Au et al., 2015a; Fig. 5). LAMAN is a ubiquitous lysosomal alpha mannosidase that cleaves alpha mannosidase linkages in glycans (Sun and Wolfe, 2001).

Segregation of Golgi Localized Proteins Exclusive to the Golgi Ribbon Versus Those Expressed in the Golgi Ribbon and Acrosome

During acrosome formation, 10 of the 14 Golgi proteins examined in this study localized to the Golgi apparatus of steps 1–7 spermatids (Au et al., 2015a). Of the 10, 6 i.e., GRASP55, GBF1, ManIIX, GPP34, Man2 α 1, and LAMAN localized to both the Golgi apparatus and developing acrosome (Au et al., 2015a). However, four Golgi proteins, i.e. GL54D, TM9SF3, TMED4/p25 and TMED7/p27, reacted only for the Golgi apparatus with no evidence of reactivity for the acrosome (Au et al., 2015a). Hence a segregation of proteins exists during acrosome formation, whereby some proteins are excluded from the acrosome but are present in the Golgi apparatus. Acrosome formation is often explained by the cisternal maturation model for Golgi biogenesis and traffic (Hermo et al., 2010b; Thorne-Tjomsland et al., 1988), however, the segregation of the four Golgi markers is not consistent with this model. In addition to the cisternal maturation model, other models have been proposed for Golgi traffic (Patterson et al., 2008; Dmitrieff and Sens, 2013; Lavieu et al., 2013; Dancourt et al., 2016; Mani and Thattai, 2016; Glick and Luini, 2011). Based on our observations the spermatid Golgi is well suited to examine the validity of these various models.

Postacrosome Period: Late Bloomers and Their Role in Late Spermatids: SORTILIN and ManIIX

After acrosome formation, the Golgi apparatus becomes spherical and positions itself in the cytoplasmic lobe of late spermatids. Coincident with the movement of the Golgi apparatus into the cytoplasmic lobe at step 8 spermatids, Golgi located immunogenicity of 12 proteins, i.e. GRASP55, GBF1, GPP34, ManIIX, Man2 α 1, and LAMAN was below detection (Au et al., 2015a). By contrast four Golgi proteins, i.e. GL54D, TM9SF3, TMED4/p25 and TMED7/p27 maintained reactivity into late spermiogenesis thus defining the identity of the Golgi apparatus in late spermatids (Au et al., 2015a; Fig. 5).

Two proteins revealed onset of expression at the late steps of spermiogenesis. Surprisingly, Golgi migration away from the acrosome also corresponded precisely with the onset of expression of sortilin in the Golgi apparatus at step 8 which continued till step 19 spermatids (Au et al., 2015a; Fig. 5). Sortilin transports lysosomal enzymes from the Golgi apparatus to MVBs and lysosomes (Braulke and Bonifacino, 2009). During acrosome formation trafficking from the Golgi apparatus to the developing acrosome appears to utilize mannose 6 phosphate receptors, as their RNA transcripts are prominent in early spermatids (O'Brien et al., 1994). However, from steps 8 onwards, the expression of sortilin likely represents its functions in plasma membrane remodeling (Clermont and Tang, 1985).

The Golgi is responsible for glycosylation (Zhang and Wang, 2016) and this occurs during acrosome formation with expression of Man2 α 1 and ManIIX. These sequence-related proteins likely have related functions in alpha mannosidase trimming of high mannose containing N-linked oligosaccharides (Moremen, 2002). The lack of detection of both of these proteins from steps 8–14 suggests a diminution in Golgi mannosidase activity that in turn would be linked to the well known major changes in N-glycosylation of proteins accounting for the high mannose and complex glycoproteins differentially expressed during spermatogenesis (Batista et al., 2012; Huang and Stanley, 2010). However, from steps 15–19, ManIIX expression returns with expression in the forming Hermes body (Au et al., 2015a; Fig. 5) likely heralding a return of complex sugar modification reactions on N-linked glycoproteins, with its expression being essential for spermatogenesis and male fertility (Fukuda and Akama, 2003a,b). In fact, terminal sugar incorporation in the Golgi apparatus has been well documented in late spermatids for glycoprotein synthesis destined for the plasma membrane (Clermont and Tang, 1985).

Exclusive Expression of Several Golgi Proteins During Formation of the Hermes Body at Step 19 Spermatids and Renewed Expression of Other Golgi Proteins Shut Down From Steps 8–18

At the last step of spermiogenesis, a resurgence of protein expression occurs at step 19 spermatids, with reactivity highlighting the forming Hermes body. Reactivity returns for TMED7/p27, which persisted till step 16, as well as GBF1, GPP34, Man2 α 1, LAMAN, which were shut down at step 8 (Au et al., 2015a; Fig. 5). In addition, two additional Golgi proteins became expressed for the first time, i.e. carboxypeptidase D (concentrated in the Golgi apparatus, where it functions by cycling to and from the plasma membrane (Breiner et al., 1998) and TMED2/p24 (Au et al., 2015a; Fig. 5). Although a stacked Golgi ribbon is no longer detectable at step 19, these proteins define the flattened membranous elements that congregate into the forming Hermes body as Golgi elements (Au et al., 2015a).

An important function of step 19 spermatids is in spermatid individualization into spermatozoa by disruption of the syncytium formed by intercellular bridges (Dym and Fawcett, 1971; Fabian and Brill, 2012; Greenbaum et al., 2009). It is attractive to propose the internal membranes (Golgi cisternae and vesicular transport) of the forming Hermes body as the source of new plasma membrane to seal off each individual spermatid resulting in spermatozoa.

The Hermes body of step 19 spermatids is maintained in epididymal sperm where its protein composition has been analyzed in detail by proteomics and localization studies. Au et al. (2015a,b) revealed that the flattened cisternal membranes of the forming Hermes body show marked differences in their Golgi protein composition from the Golgi apparatus of germ cells evolving during spermatogenesis, with some Golgi localized proteins not entering the Hermes body, i.e. GL54D, TMED4/p25, sortilin, TM9SF3 and GRASP55. In this way, the flattened cisternae of the step 19 spermatids appear to have different molecular constituents from the stacked Golgi cisternae of earlier germ cells, and this may be analogous to that postulated for functional individual yeast-flattened cisternae (Papanikou and Glick, 2009). In addition, cisternae of the step 19 spermatids may function independent of a stacked configuration as suggested for Golgi cisternae (Klumperman, 2011) and without the conventional setup of a continuous

Golgi ribbon. Thus, the Hermes body of epididymal sperm formed at step 19 spermatids appears to have evolved a system of unique flattened membranous cisternae unlike the conventional stacked Golgi apparatus to carry out specific functions for sperm maturation (Au et al., 2015a).

Proteins of Unknown Function

In the work of Au et al. (2015a), two abundant proteins of unknown function were identified as new Golgi markers (GL54D and TM9SF3). Moreover, over 200 other proteins of unknown function were uncovered in the isolated testis Golgi fraction, each of which may now be studied by future generations of scientists to elucidate their distribution and functions during germ cell differentiation.

Conclusion

The differential expression pattern of proteins related to the Golgi apparatus during germ cell development has been elucidated by incorporating knowledge of the stages of the cycle first described by Leblond and Clermont (1952). Their varied expression profile at select time points during germ cell differentiation strongly suggests diversified complex regulatory mechanisms that are in play to ensure the precise turning on and off of these proteins of different functions which are related to the diversified differentiation process of germ cells. The endpoint of spermiogenesis resulting in Hermes body formation in step 19 spermatids has also characterized and defined by a unique set of Golgi markers. The differential specific expression of novel and established Golgi markers during spermatogenesis may now help define a new model system to answer the unresolved questions in Golgi apparatus structure and function (Emr et al., 2009).

Acknowledgments

I hope that all aspiring young scientists early in their career would be so privileged as to have such great role models as I (LH) was with Dr. Y. Clermont and Dr. C.P. Leblond. I would like to thank Dr. Clermont my supervisor and mentor who challenged and inspired me over my 45 years at McGill University, as did Dr. Leblond. Their sincere dedication and love of science allowed me and generations of students to find our niche and aspire us to be as great as they were. I would also like to thank Dr. M.F. Lalli who assisted me as a graduate student in learning the ropes of electron microscopy, tissue fixation and preparation and spermatogenesis. This paper is dedicated to Yves Clermont and CP Leblond who elaborated the stem cell renewal theory for male germ cell differentiation. <http://www.mcgill.ca/anatomy/stem-cell-renewal-theory>.

References

- Anakwe, O. O., & Gerton, G. L. (1990). Acrosome biogenesis begins during meiosis: Evidence from the synthesis and distribution of an acrosomal glycoprotein, acrogranin, during guinea pig spermatogenesis. *Biology of Reproduction*, 42, 317–328.
- Au, C. E., Hermo, L., Byrne, E., Smirle, J., Fazel, A., Simon, P. H., Kearney, R. E., Cameron, P. H., Smith, C. E., Vali, H., Fernandez-Rodriguez, J., Ma, K., Nilsson, T., & Bergeron, J. J. (2015a). Expression, sorting, and segregation of Golgi proteins during germ cell differentiation in the testis. *Molecular Biology of the Cell*, 26, 4015–4032.
- Au, C. E., Hermo, L., Byrne, E., Smirle, J., Fazel, A., Kearney, R. E., Smith, C. E., Vali, H., Fernandez-Rodriguez, J., Simon, P. H., Mandato, C., Nilsson, T., & Bergeron, J. J. (2015b). Compartmentalization of membrane trafficking, glucose transport, glycolysis, actin, tubulin and the proteasome in the cytoplasmic droplet/Hermes body of epididymal sperm. *Open Biology*, 5.
- Batista, F., Lu, L., Williams, S. A., & Stanley, P. (2012). Complex N-glycans are essential, but core 1 and 2 mucin O-glycans, O-fucose glycans, and NOTCH1 are dispensable, for mammalian spermatogenesis. *Biology of Reproduction*, 86, 179.
- Bekier, M. E., 2nd, Wang, L., Li, J., Huang, H., Tang, D., Zhang, X., & Wang, Y. (2017). Knockout of the Golgi stacking proteins GRASP55 and GRASP65 impairs Golgi structure and function. *Molecular Biology of the Cell*, 28, 2833–2842.
- Bell, A. W., Ward, M. A., Blackstock, W. P., Freeman, H. N., Choudhary, J. S., Lewis, A. P., Chotai, D., Fazel, A., Gushue, J. N., Paiement, J., Palcy, S., Chevet, E., Lafreniere-Roula, M., Solari, R., Thomas, D. Y., Rowley, A., & Bergeron, J. J. (2001). Proteomics characterization of abundant Golgi membrane proteins. *Journal of Biological Chemistry*, 276, 5152–5165.
- Bentson, L. F., Agbor, V. A., Agbor, L. N., Lopez, A. C., Nfonam, L. E., Bornstein, S. S., Handel, M. A., & Linder, C. C. (2013). New point mutation in Golga3 causes multiple defects in spermatogenesis. *Andrology*, 1, 440–450.
- Bergeron, J. J. M., Au, C. E., Thomas, D. Y., & Hermo, L. (2017). Proteomics identifies golgi phosphoprotein 3 (GOLPH3) with a link between golgi structure, cancer, DNA damage and protection from cell death. *Molecular & Cellular Proteomics*, 16, 2048–2054.
- Boitani, C., Di Persio, S., Esposito, V., & Vicini, E. (2016). Spermatogonial cells: Mouse, monkey and man comparison. *Seminars in Cell and Developmental Biology*, 59, 79–88.
- Braulke, T., & Bonifacio, J. S. (2009). Sorting of lysosomal proteins. *Biochimica et Biophysica Acta*, 1793, 605–614.
- Breiner, K. M., Urban, S., & Schaller, H. (1998). Carboxypeptidase D (gp180), a Golgi-resident protein, functions in the attachment and entry of avian hepatitis B viruses. *Journal of Virology*, 72, 8098–8104.
- Burgos, M. H., & Gutierrez, L. S. (1986). The Golgi complex of the early spermatid in guinea pig. *Anatomical Record*, 216, 139–145.
- Buschman, M. D., Xing, M., & Field, S. J. (2015). The GOLPH3 pathway regulates Golgi shape and function and is activated by DNA damage. *Frontiers in Neuroscience*, 9, 362.
- Cartier-Michaud, A., Bailly, A. L., Betzi, S., Shi, X., Lissitzky, J. C., Zarubica, A., Serge, A., Roche, P., Lugari, A., Hamon, V., Bardin, F., Derviaux, C., Lembo, F., Audebert, S., Marchetto, S., Durand, B., Borg, J. P., Shi, N., Morelli, X., & Aurand-Lions, M. (2017). Genetic, structural, and chemical insights into the dual function of GRASP55 in germ cell Golgi remodeling and JAM-C polarized localization during spermatogenesis. *PLoS Genetics*, 13, e1006803.
- Cheung, P. Y., & Pfeffer, S. R. (2016). Transport vesicle tethering at the trans Golgi network: Coiled coil proteins in action. *Frontiers in Cell and Developmental Biology*, 4, 18.
- Christensen, A. K. (1975). Leydig cells. In D. W. Hamilton, & R. O. Greep (Eds.), *Handbook of physiology*. American Physiological Society: Washington, DC.
- Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiological Reviews*, 52, 198–236.

- Clermont, Y., & Harvey, S. C. (1965). Duration of the cycle of the seminiferous epithelium of normal, hypophysectomized and hypophysectomized-hormone treated albino rats. *Endocrinology*, 76, 80–89.
- Clermont, Y., & Huckins, C. (1961). Microscopic anatomy of the sex cords and seminiferous tubules in growing and adult male albino rats. *Developmental Dynamics*, 108, 79–97.
- Clermont, Y., & Leblond, C. P. (1953). Renewal of spermatogonia in the rat. *American Journal of Anatomy*, 93, 475–501.
- Clermont, Y., & Tang, X. M. (1985). Glycoprotein synthesis in the Golgi apparatus of spermatids during spermiogenesis of the rat. *Anatomical Record*, 213, 33–43.
- Clermont, Y., Lalli, M., & Rambourg, A. (1981). Ultrastructural localization of nicotinamide adenine dinucleotide phosphatase (NADPase), thiamine pyrophosphatase (TPPase), and cytidine monophosphatase (CMPase) in the Golgi apparatus of early spermatids of the rat. *Anatomical Record*, 201, 613–622.
- Clermont, Y., Hermo, L., & Oko, R. (1993). Cell biology of mammalian spermatogenesis. In C. Desjardins, & L. L. Ewing (Eds.), *Cell and molecular biology of the testis*. New York: Oxford University Press.
- Clermont, Y., Rambourg, A., & Hermo, L. (1994). Connections between the various elements of the cis- and mid-compartments of the Golgi apparatus of early rat spermatids. *Anatomical Record*, 240, 469–480.
- Clermont, Y., Rambourg, A., & Hermo, L. (1995). Trans-Golgi network (TGN) of different cell types: Three-dimensional structural characteristics and variability. *Anatomical Record*, 242, 289–301.
- Dahan, S., Ahluwalia, J. P., Wong, L., Posner, B. I., & Bergeron, J. J. (1994). Concentration of intracellular hepatic apolipoprotein E in Golgi apparatus saccular distensions and endosomes. *Journal of Cell Biology*, 127, 1859–1869.
- Dancourt, J., Zheng, H., Bottanelli, F., Allgeyer, E. S., Bewersdorf, J., Graham, M., Liu, X., Rothman, J. E., & Lavie, G. (2016). Small cargoes pass through synthetically glued Golgi stacks. *FEBS Letters*, 590, 1675–1686.
- Dmitrieff, S., & Sens, P. (2013). Transient domain formation in membrane-bound organelles undergoing maturation. *Physical Review. E, Statistical, Nonlinear, and Soft Matter Physics*, 88, 062704.
- Dominguez, M., Dejgaard, K., Fullekrug, J., Dahan, S., Fazel, A., Paccaud, J. P., Thomas, D. Y., Bergeron, J. J., & Nilsson, T. (1998). gp25L/emp24/p24 protein family members of the cis-Golgi network bind both COP I and II coatomer. *Journal of Cell Biology*, 140, 751–765.
- Dym, M., & Clermont, Y. (1970). Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. *American Journal of Anatomy*, 128, 265–282.
- Dym, M., & Fawcett, D. W. (1971). Further observations on the numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in the mammalian testis. *Biology of Reproduction*, 4(2), 195–215.
- Emr, S., Glick, B. S., Linstedt, A. D., Lippincott-Schwartz, J., Luini, A., Malhotra, V., Marsh, B. J., Nakano, A., Pfeffer, S. R., Rabouille, C., Rothman, J. E., Warren, G., & Wieland, F. T. (2009). Journeys through the Golgi—Taking stock in a new era. *Journal of Cell Biology*, 187, 449–453.
- Fabian, L., & Brill, J. A. (2012). Drosophila spermiogenesis: Big things come from little packages. *Spermatogenesis*, 2, 197–212.
- Fawcett, D. W. (1975). Gametogenesis in the male: Prospects for its control. *The Symposium. Society for Developmental Biology*, 25–53.
- Foster, J. A., & Gerton, G. L. (2016). The Acrosomal Matrix. *Advances in Anatomy, Embryology and Cell Biology*, 220, 15–33.
- Fukuda, M. N., & Akama, T. O. (2003a). The in vivo role of alpha-mannosidase IIx and its role in processing of N-glycans in spermatogenesis. *Cellular and Molecular Life Sciences*, 60, 1351–1355.
- Fukuda, M. N., & Akama, T. O. (2003b). The role of N-glycans in spermatogenesis. *Cytogenetic and Genome Research*, 103, 302–306.
- Gao, Y., Xiao, X., Lui, W. Y., Lee, W. M., Mruk, D., & Cheng, C. Y. (2016). Cell polarity proteins and spermatogenesis. *Seminars in Cell and Developmental Biology*, 59, 62–70.
- Glick, B. S., & Luini, A. (2011). Models for Golgi traffic: A critical assessment. *Cold Spring Harbor Perspectives in Biology*, 3, a005215.
- Greenbaum, M. P., Iwamori, N. N., Agno, J. E., & Matzuk, M. M. (2009). Mouse TEX14 is required for embryonic germ cell intercellular bridges but not female fertility. *Biology of Reproduction*, 80, 449–457.
- Griffiths, G., Warren, G., Stuhlfauth, I., & Jockusch, B. M. (1981). The role of clathrin-coated vesicles in acrosome formation. *European Journal of Cell Biology*, 26, 52–60.
- Griswold, M. D. (2016). Spermatogenesis: The commitment to meiosis. *Physiological Reviews*, 96, 1–17.
- Griswold, M. D., Hogarth, C. A., Bowles, J., & Koopman, P. (2012). Initiating meiosis: The case for retinoic acid. *Biology of Reproduction*, 86, 35.
- Hermo, L., Rambourg, A., & Clermont, Y. (1980). Three-dimensional architecture of the cortical region of the Golgi apparatus in rat spermatids. *American Journal of Anatomy*, 157, 357–373.
- Hermo, L., Pelletier, R. M., Cyr, D. G., & Smith, C. E. (2010a). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: Background to spermatogenesis, spermatogonia, and spermatocytes. *Microscopy Research and Technique*, 73, 241–278.
- Hermo, L., Pelletier, R. M., Cyr, D. G., & Smith, C. E. (2010b). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 2: Changes in spermatid organelles associated with development of spermatozoa. *Microscopy Research and Technique*, 73, 279–319.
- Hermo, L., Pelletier, R. M., Cyr, D. G., & Smith, C. E. (2010c). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 3: Developmental changes in spermatid flagellum and cytoplasmic droplet and interaction of sperm with the zona pellucida and egg plasma membrane. *Microscopy Research and Technique*, 73, 320–363.
- Hermo, L., Oliveira, R. L., Smith, C. E., & Bergeron, J. J. M. (2018). Golgi apparatus regulation of differentiation: A case study for male germ cells of the rat testis. In *Spermatogenesis: Biology and clinical implications*. Taylor & Francis.
- Herscovics, A. (1999). Importance of glycosidases in mammalian glycoprotein biosynthesis. *Biochimica et Biophysica Acta*, 1473, 96–107.
- Hess, R. A., Hermo, L., & Robaire, B. (2015). Lessons learned in andrology: Yves Clermont, an interview by Lonnie D. Russell. *Andrology*, 3, 1015–1021.
- Huang, H. H., & Stanley, P. (2010). A testis-specific regulator of complex and hybrid N-glycan synthesis. *Journal of Cell Biology*, 190, 893–910.
- Igdoura, S. A., Herscovics, A., Lal, A., Moremen, K. W., Morales, C. R., & Hermo, L. (1999). Alpha-mannosidases involved in N-glycan processing show cell specificity and distinct subcompartmentalization within the Golgi apparatus of cells in the testis and epididymis. *European Journal of Cell Biology*, 78, 441–452.
- Javela, T., & Linstedt, A. D. (2014). Isoform-specific tethering links the Golgi ribbon to maintain compartmentalization. *Molecular Biology of the Cell*, 25, 133–144.
- Jerome-Majewska, L. A., Achkar, T., Luo, L., Lupu, F., & Lacy, E. (2010). The trafficking protein Tmed2/p24beta(1) is required for morphogenesis of the mouse embryo and placenta. *Developmental Biology*, 341, 154–166.
- Klumpperman, J. (2011). Architecture of the mammalian Golgi. *Cold Spring Harbor Perspectives in Biology*, 3, 1–19.
- Komeya, M., & Ogawa, T. (2015). Spermatogonial stem cells: Progress and prospects. *Asian Journal of Andrology*, 17, 771–775.
- Kondo, M., & Sutou, S. (1997). Cloning and molecular characterization of cDNA encoding a mouse male-enhanced antigen-2 (Mea-2): A putative family of the Golgi autoantigen. *DNA Sequence*, 7, 71–82.
- Lavie, G., Zheng, H., & Rothman, J. E. (2013). Stapled Golgi cisternae remain in place as cargo passes through the stack. *eLife*, 2, e00558.
- Lavie, G., Dunlop, M. H., Lerich, A., Zheng, H., Bottanelli, F., & Rothman, J. E. (2014). The Golgi ribbon structure facilitates anterograde transport of large cargoes. *Molecular Biology of the Cell*, 25, 3028–3036.
- Leblond, C. P., & Clermont, Y. (1952). Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfurous acid technique. *American Journal of Anatomy*, 90, 167–215.
- Lin, Y. N., & Matzuk, M. M. (2014). Genetics of male fertility. *Methods in Molecular Biology*, 1154, 25–37.
- Mani, S., & Thattai, M. (2016). Stacking the odds for Golgi cisternal maturation. *eLife*, 5.
- Matzuk, M. M., & Lamb, D. J. (2008). The biology of infertility: Research advances and clinical challenges. *Nature Medicine*, 14, 1197–1213.
- Mecklenburg, J. M., & Hermann, B. P. (2016). Mechanisms regulating spermatogonial differentiation. *Results and Problems in Cell Differentiation*, 58, 253–287.
- Meistrich, M. L., & Hess, R. A. (2013). Assessment of spermatogenesis through staging of seminiferous tubules. *Methods in Molecular Biology*, 927, 299–307.

- Mikolcevic, P., Isoda, M., Shibuya, H., Del Barco Barrantes, I., Igea, A., Suja, J. A., Shackleton, S., Watanabe, Y., & Nebreda, A. R. (2016). Essential role of the Cdk2 activator RingoA in meiotic telomere tethering to the nuclear envelope. *Nature Communications*, 7, 11084.
- Mollenhauer, H. H., & Zebrun, W. (1960). Permanganate fixation of the Golgi complex and other cytoplasmic structures of mammalian tests. *Journal of Biophysical and Biochemical Cytology*, 8, 761–775.
- Moremen, K. W. (2002). Golgi alpha-mannosidase II deficiency in vertebrate systems: Implications for asparagine-linked oligosaccharide processing in mammals. *Biochimica et Biophysica Acta*, 1573, 225–235.
- Munro, S. (2011). The golgin coiled-coil proteins of the Golgi apparatus. *Cold Spring Harbor Perspectives in Biology*, 3, 1–14.
- Niu, C. M., Guo, J. Q., Ma, H. T., Zheng, Z., & Zheng, Y. (2016). Sperm acrosome formation-associated genes in mice: Advances in studies. *Zhonghua Nan Ke Xue*, 22, 72–76.
- Nakata, H., Wakayama, T., Sonomura, T., Honma, S., Hatta, T., & Iseki, S. (2015). Three-dimensional structure of seminiferous tubules in the adult mouse. *Journal of Anatomy*, 227, 686–694.
- Nakata, H., Sonomura, T., & Iseki, S. (2017). Three-dimensional analysis of seminiferous tubules and spermatogenic waves in mice. *Reproduction*, 154, 569–579.
- Oatley, J. M., & Brinster, R. L. (2008). Regulation of spermatogonial stem cell self-renewal in mammals. *Annual Review of Cell and Developmental Biology*, 24, 263–286.
- O'Brien, D. A., Welch, J. E., Fulcher, K. D., & Eddy, E. M. (1994). Expression of mannose 6-phosphate receptor messenger ribonucleic acids in mouse spermatogenic and Sertoli cells. *Biology of Reproduction*, 50, 429–435.
- Oko, R., Hermo, L., Chan, P. T., Fazel, A., & Bergeron, J. J. (1993). The cytoplasmic droplet of rat epididymal spermatozoa contains saccular elements with Golgi characteristics. *Journal of Cell Biology*, 123, 809–821.
- Papanikou, E., & Glick, B. S. (2009). The yeast Golgi apparatus: Insights and mysteries. *FEBS Letters*, 583, 3746–3751.
- Pastor-Cantizano, N., Montesinos, J. C., Bernat-Silvestre, C., Marcote, M. J., & Aniento, F. (2016). p24 family proteins: Key players in the regulation of trafficking along the secretory pathway. *Protoplasma*, 253, 967–985.
- Patterson, G. H., Hirschberg, K., Polishchuk, R. S., Gerlich, D., Phair, R. D., & Lippincott-Schwartz, J. (2008). Transport through the Golgi apparatus by rapid partitioning within a two-phase membrane system. *Cell*, 133, 1055–1067.
- Rabouille, C., & Linstedt, A. D. (2016). GRASP: A multitasking tether. *Frontiers in Cell and Developmental Biology*, 4, 1.
- Ramalho-Santos, J., Moreno, R. D., Wessel, G. M., Chan, E. K., & Schatten, G. (2001). Membrane trafficking machinery components associated with the mammalian acrosome during spermiogenesis. *Experimental Cell Research*, 267, 45–60.
- Rambourg, A., & Clermont, Y. (1990). Three-dimensional electron microscopy: Structure of the Golgi apparatus. *European Journal of Cell Biology*, 51, 189–200.
- Rambourg, A., Clermont, Y., & Marraud, A. (1974). Three-dimensional structure of the osmium-impregnated Golgi apparatus as seen in the high voltage electron microscope. *American Journal of Anatomy*, 140, 27–45.
- Regaud, C. (1901). Etudes sur la structure des tubes seminiferes et sur la spermatogenese chez les mammiferes. Part 1. *Archives d'Anatomie microscopiques et de Morphologie experimentale*, 4, 101–156.
- Rose, D. R. (2012). Structure, mechanism and inhibition of Golgi alpha-mannosidase II. *Current Opinion in Structural Biology*, 22, 558–562.
- Russell, L. D., Ettlin, R. A., Sinhaikim, A. P., & Clegg, E. D. (1990). *Histological and histopathological evaluation of the testis*. Clear-water, FL: Cache River Press.
- Seemann, J., Jokitalo, E., Pypaert, M., & Warren, G. (2000). Matrix proteins can generate the higher order architecture of the Golgi apparatus. *Nature*, 407, 1022–1026.
- Shen, L., Du, X., Ma, H., & Mei, S. (2017). MiR-1193 suppresses the proliferation and invasion of human T-cell leukemia cells through directly targeting the transmembrane 9 superfamily 3 (TM9SF3). *Oncology Research*, 25(9), 1643–1651.
- Shorter, J., Watson, R., Giannakou, M. E., Clarke, M., Warren, G., & Barr, F. A. (1999). GRASP55, a second mammalian GRASP protein involved in the stacking of Golgi cisternae in a cell-free system. *EMBO Journal*, 18, 4949–4960.
- Smith, C. E., Hermo, L., Fazel, A., Lalli, M. F., & Bergeron, J. J. (1990). Ultrastructural distribution of NADPase within the Golgi apparatus and lysosomes of mammalian cells. *Progress in Histochemistry and Cytochemistry*, 21, 1–120.
- Steinberger, E., & Steinberger, A. (1975). Spermatogenic function of the testis. In R. O. Greep, & E. B. Astwood (Eds.), *Handbook of physiology*. Baltimore, MD: Waverley Press.
- Strating, J. R., & Martens, G. J. (2009). The p24 family and selective transport processes at the ER-Golgi interface. *Biology of the Cell*, 101, 495–509.
- Suarez-Quian, C. A., An, Q., Jelesoff, N., & Dym, M. (1991). The Golgi apparatus of rat pachytene spermatocytes during spermatogenesis. *Anatomical Record*, 229, 16–26.
- Sun, H., & Wolfe, J. H. (2001). Recent progress in lysosomal alpha-mannosidase and its deficiency. *Experimental & Molecular Medicine*, 33, 1–7.
- Susi, F. R., Leblond, C. P., & Clermont, Y. (1971). Changes in the golgi apparatus during spermiogenesis in the rat. *American Journal of Anatomy*, 130, 251–267.
- Thorne-Tjomsland, G., Clermont, Y., & Hermo, L. (1988). Contribution of the Golgi apparatus components to the formation of the acrosomic system and chromatoid body in rat spermatids. *Anatomical Record*, 221, 591–598.
- Valentine, J. W., Collins, A. G., & Meyer, C. P. (1994). Morphological complexity increase in metazoans. *Paleobiology*, 20, 131–142.
- Vinke, F. P., Grieve, A. G., & Rabouille, C. (2011). The multiple facets of the Golgi reassembly stacking proteins. *Biochemical Journal*, 433, 423–433.
- Wright, J., Kahn, R. A., & Sztul, E. (2014). Regulating the large Sec7 ARF guanine nucleotide exchange factors: The when, where and how of activation. *Cellular and Molecular Life Sciences*, 71, 3419–3438.
- Yoshida, S., Hiyoshi, K., Ichinose, T., Takano, H., Oshio, S., Sugawara, I., Takeda, K., & Shibamoto, T. (2009). Effect of nanoparticles on the male reproductive system of mice. *International Journal of Andrology*, 32, 337–342.
- Zhang, X., & Wang, Y. (2015). GRASPs in Golgi structure and function. *Frontiers in Cell and Developmental Biology*, 3, 84.
- Zhang, X., & Wang, Y. (2016). Glycosylation quality control by the Golgi structure. *Journal of Molecular Biology*, 428, 3183–3193.

Further Reading

- Flechon, J. E. (2016). The acrosome of eutherian mammals. *Cell and Tissue Research*, 363, 147–157.
- Taloni, A., Font-Clos, F., Guidetti, L., Milan, S., Ascagni, M., Vasco, C., Pasini, M. E., Gloria, M. R., Ciusani, E., Zapperi, S., & La Porta, C. A. M. (2017). Probing spermiogenesis: A digital strategy for mouse acrosome classification. *Scientific Reports*, 7, 3748.

Inherent Sperm Maturation: A Role for the Hermes Body (Cytoplasmic Droplet) of Sperm

Louis Hermo, Regiana L Oliveira, and Charles E Smith, McGill University, Montreal, QC, Canada
John JM Bergeron, McGill University Health Centre, Montreal, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction: Background on the Formation and Constituents of the Hermes Body (Cytoplasmic Droplet)

The cytoplasmic droplet (CD) of sperm was first identified by Retzius (1909) more than a century ago, and over time it has been a lost soul in quest for an identity. The CD is a feature of epididymal sperm and corresponds to a small dilated bulge enveloping a focal area along the length of the sperm flagellum (Fig. 1). In the testis it is formed at the last step of spermatid development, a process defined as spermiogenesis, which in the rat is composed of 19 steps. At step 19 spermatids, the forming CD appears as a dilated area of the flagellum located at the connecting piece (neck), which anchors the head of the sperm to the flagellum (Fig. 1). These spermatids are ready to be released into the seminiferous tubule lumen whereupon they enter the epididymal duct. Hence the CD forms a distinct compartment of the flagellum with a unique setup of organelles that has eluded generations of scientists as to a functional significance.

During sperm transit through the epididymis, the CD slides along the mid piece of the flagellum, where mitochondria are arranged in a spiral manner, to the annulus, a ring-like structure separating the mid piece from the principal piece of the

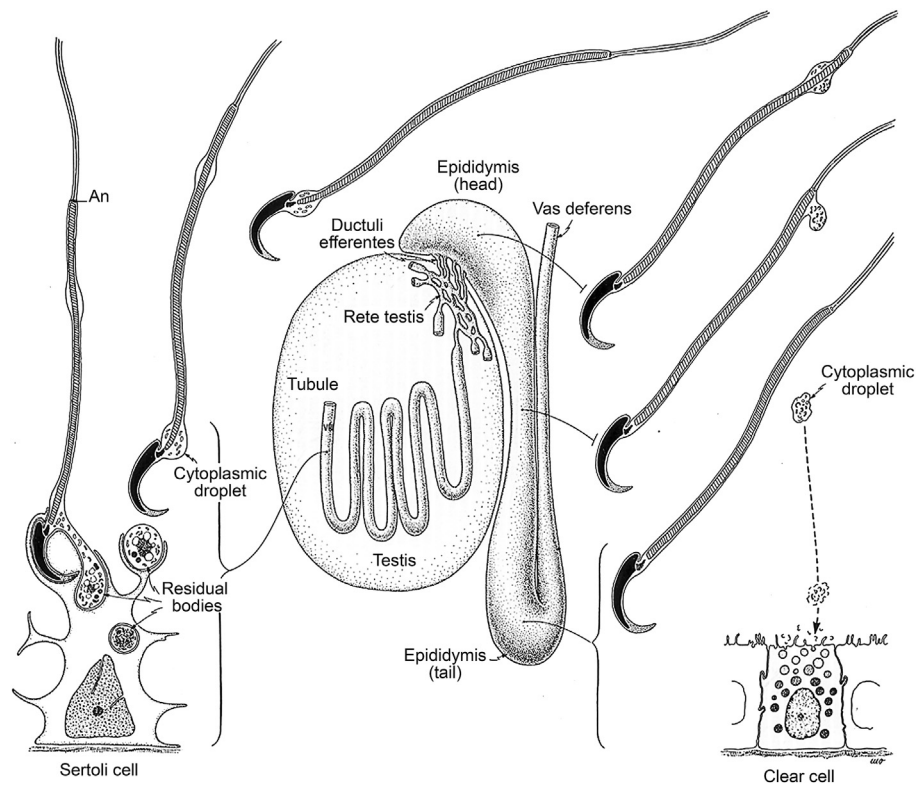


Fig. 1 Schematic representation of the forming cytoplasmic droplet (recently renamed the Hermes body) at step 19 spermatid in the testis and fate of the CD as sperm transit through the epididymis. At step 19 spermatids, the forming cytoplasmic droplet appears as a dilated area of the flagellum located at the connecting piece (neck), which anchors the head of the sperm to the flagellum. The bulk of cytoplasm of the step 19 spermatid forms the residual body, which is phagocytosed and degraded by Sertoli cells of the seminiferous epithelium. The step 19 spermatids are released as spermatozoa from the seminiferous tubule lumen whereupon they traverse the epididymal duct. As sperm enter the initial segment of the epididymis, the cytoplasmic droplet is still located at the neck area of the flagellum. During sperm transit through the caput to the corpus region of the epididymis, the cytoplasmic droplet slides along the mid piece of the flagellum, where mitochondria are arranged in a spiral manner, to the annulus (An), a ring-like structure separating the mid piece from the principal piece of the flagellum. Thereafter the cytoplasmic droplet detaches from the flagellum to be liberated into the epididymal lumen, whereupon it fragments with its contents being taken up by the epithelial clear cells. Diagram drawn by Dr. Yves Clermont. Figure taken from Hermo L, Dworkin J, Oko R. (1988). Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. *The American Journal of Anatomy* **183**, 107–124. Reproduced with permission of Wiley and sons.

flagellum (Hermo et al., 2010) (Fig. 1). In some species, the CD detaches from the flagellum and is liberated into the lumen whereupon it fragments and releases its contents, which are then taken up by the epithelial clear cells of the epididymis (Hermo et al., 1988) (Fig. 1). As sperm traverse the epididymis, they acquire their maturational features, motility and fertility (Orgebin-Crist, 1969), an event coincident with the presence of the CD on sperm suggesting a cause-effect relationship (Hermo et al., 1988, 2010). Moreover, the CD is an evolutionarily conserved structure unique to epididymal spermatozoa where it has been observed in over 30 vertebrate species from fish to primates and humans (Yuan et al., 2013) suggesting a functional role for sperm.

Early biochemical studies identified activities of lysosomal hydrolases and glycolytic enzymes in CDs of several species (Dott and Dingle, 1968; Roberts et al., 1976; Moniem and Glover, 1972). As first revealed with the advent of the electron microscope (EM), the most conspicuous features of the CD were numerous short flattened as well as dilated membranous profiles suggested to be remnants of Golgi and ER cisternae (Bloom and Nicander, 1961; Fawcett and Ito, 1965). Later studies revealed that the numerous short flattened membranous profiles appeared to resemble the Golgi cisternae of the Golgi stack. However, unlike cisternae of the Golgi apparatus, these cisternae did not form a typical stacked configuration and were unreactive for various Golgi cytochemical markers (Hermo et al., 1988) (Fig. 2). The identity of the cisternae awaited more sophisticated techniques.

As will become apparent in the present article, the current data from the literature suggest that sperm themselves through the CD also play a role in their own maturation (Au et al., 2015b; Yuan et al., 2013), a function previously ascribed mainly to the epithelial epididymal cells (Robaire and Hermo, 1988; Cornwall, 2009). By its protein and organellar makeup the CD appears to regulate the initiation of sperm motility, a prerequisite for sperm maturation. In the literature confusion exists about the CD a structure formed by a normal testis, versus excess residual cytoplasm (Aitken et al., 1994) denoting a structure found on abnormally formed sperm liberated from a testis displaying damage to the seminiferous epithelium (review Cooper, 2005). As a consequence of this confusion, the term Hermes body (HB), the winged messenger of the Greek gods, transforming the mortal to the immortal was proposed to identify the true CD (Au et al., 2015b). In the reproductive context, the presence and functions of the HB along the mid piece of sperm during epididymal transit, where sperm mature, represents a transformation of sperm from an immotile unfertile to a motile fertile state.

Sweetening up the Sperm by Glycolysis: A Major Function of the Hermes Body

In order to determine a functional role for the HB of sperm, a method to isolate fractions of isolated HBs of adult rat caput epididymal sperm was developed with proteins characterized by quantitative tandem mass spectrometry (Au et al., 2015b). From the 1511 proteins identified in the isolated HB fraction, a selection of proteins was analyzed with antibodies to reveal by light microscope (LM) immunohistochemical (IHC) and immunofluorescence (IF) their localization to the sperm HB of in situ epididymal tissue sections. Here we will focus on some of the proteins in the life history of the HB from the work of Au et al. (2015b) that appear to serve a role in the maturation process of sperm.

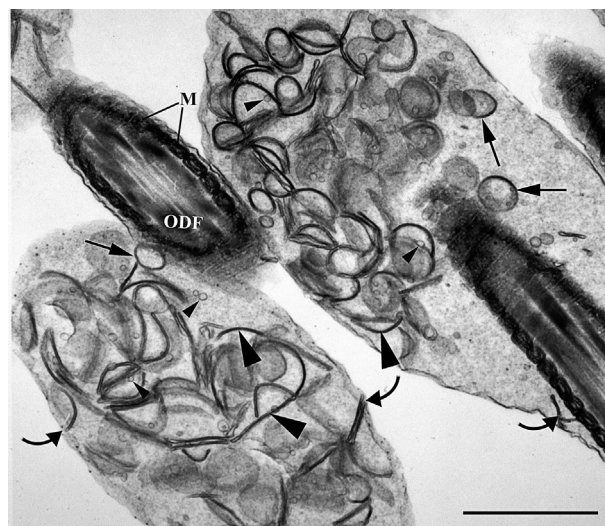


Fig. 2 Electron micrograph of tangential sections through the CD of caput epididymal sperm. Short flattened cisternal elements are abundant (large arrowheads) with some being straight, while others are curved. They congregate together in one pole of the cytoplasm of the CD. Many small vesicles (small arrowheads) are evident some in close proximity to the cisternae. The edges of the cisternae (curved arrows) are often contiguous with one another and the plasma membrane. M, mitochondria; ODF, outer dense fibers. Original Mag 48,750 $\times$.

Out of the 1151 detected proteins, the plasma membrane protein, glucose transporter 3 (GLUT3) (Solute Carrier Family 2 Facilitated Glucose Transporter Member 3) was the most abundant protein of the isolated HB of rat epididymal sperm. This along with the finding that Hexokinase1 was the second most abundant protein provided the first clue as to the function of this elusive structure in providing ATP via glycolysis to sperm. This conjecture was further supported by the characterization of proteins of the entire glycolytic pathway to the isolated HB by proteomics and also by the LM-IHC visualization of GLUT3 (**Fig. 3A**), HEX1 (**Fig. 3B**) and sperm-specific lactate dehydrogenase C to this structure in situ (**Au et al., 2015b**). During epididymal sperm maturation, ATP generation from glycolysis appears to direct the initiation of sperm motility (**Mannowetz et al., 2012; Miki, 2007; Odet et al., 2013**). However, while several investigators localized glycolytic enzymes to the mid piece and/or principal piece of the sperm flagellum, (**Tanii et al., 2007; Eddy et al., 2003; Miki, 2007; Ford, 2006; Nakamura et al., 2010**), the LM-IHC/IF and proteomic observations of **Au et al. (2015b)** emphasized the concentration of glycolytic enzymes and relevance of glycolysis in the HB of epididymal sperm.

Previous proteomics studies on whole sperm (**Baker et al., 2013; Chocu et al., 2012; Chauvin et al., 2012**) uncovered GLUT-3 and other glycolytic metabolic enzymes as prominent. In humans, 436 proteins of the 1056 found in sperm belonged to various metabolic pathways, with glycolytic proteins being the highest expressed (**Baker and Aitken, 2009**). Proteomic analyses on proteins from enriched purified HBs from murine epididymal spermatozoa identified 105 proteins, 71 (68%) of which were enzymes involved in energy metabolism (**Yuan et al., 2013**). ATP generation has long been established as a requirement for sperm motility. The findings of **Au et al. (2015b)** and **Yuan et al. (2013)** that enzymes of the glycolytic pathway are enriched in the HB proposes a function for this structure in the initiation of sperm motility by ATP generation that is a prerequisite for sperm maturation as they transit through the epididymal duct.

Also relevant was the finding of Monocarboxylate Transporters (MCT) in the Hermes body. These utilize proton gradients to move molecules with one carboxylate group such as lactate or pyruvate across the plasma membrane. MCT1 and MCT2 localized to the Hermes body of epididymal sperm (**Figs. 3C and D**).

Internal Flattened Cisternae of the Hermes Body Reveal Golgi Proteins Differing From That of Germ Cells of the Testis

A clue as to the composition and functions of the HB came in 1993, when Oko et al. provided evidence by EM that the flattened internal membranes corresponded to intact Golgi cisternae. Indeed at the last steps of spermiogenesis (steps 15–18), it was noted that flattened Golgi cisternae that constituted the Golgi stack separated from each other and gradually became free floating

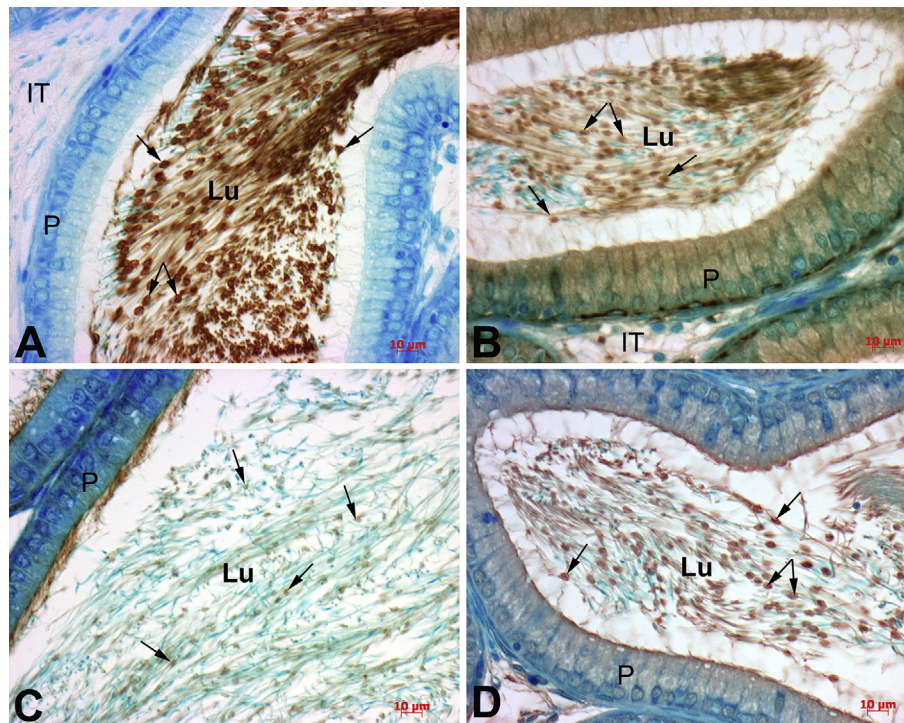


Fig. 3 A–D: LM photomicrographs of sections of the epididymal duct immunostained with antibodies to: GLUT3 (**Fig. 3A**), HEX1 (**Fig. 3B**), MCT1 and MCT2 (**Figs. 3C and D**). The Hermes body of epididymal sperm are reactive (*arrows*) with each appearing as a small dilated area along the flagellum. Lu, lumen; P, principal cells; IT, intertubular space.

in the spermatid cytoplasm (Fig. 4). At the last step of spermiogenesis, i.e., step 19 (stage VIII of the cycle; Leblond and Clermont, 1952), these Golgi elements congregated into the forming HB, as loosely organized unstacked cisternae (Okamoto et al., 1993) (Fig. 4). Confirmation that the HB of epididymal sperm contained Golgi proteins and cisternae was demonstrated by western blots and in situ immunoreactivity for the Golgi markers TGN38, alpha 2, 6 sialyltransferase and beta 1, 4 galactosyl transferase

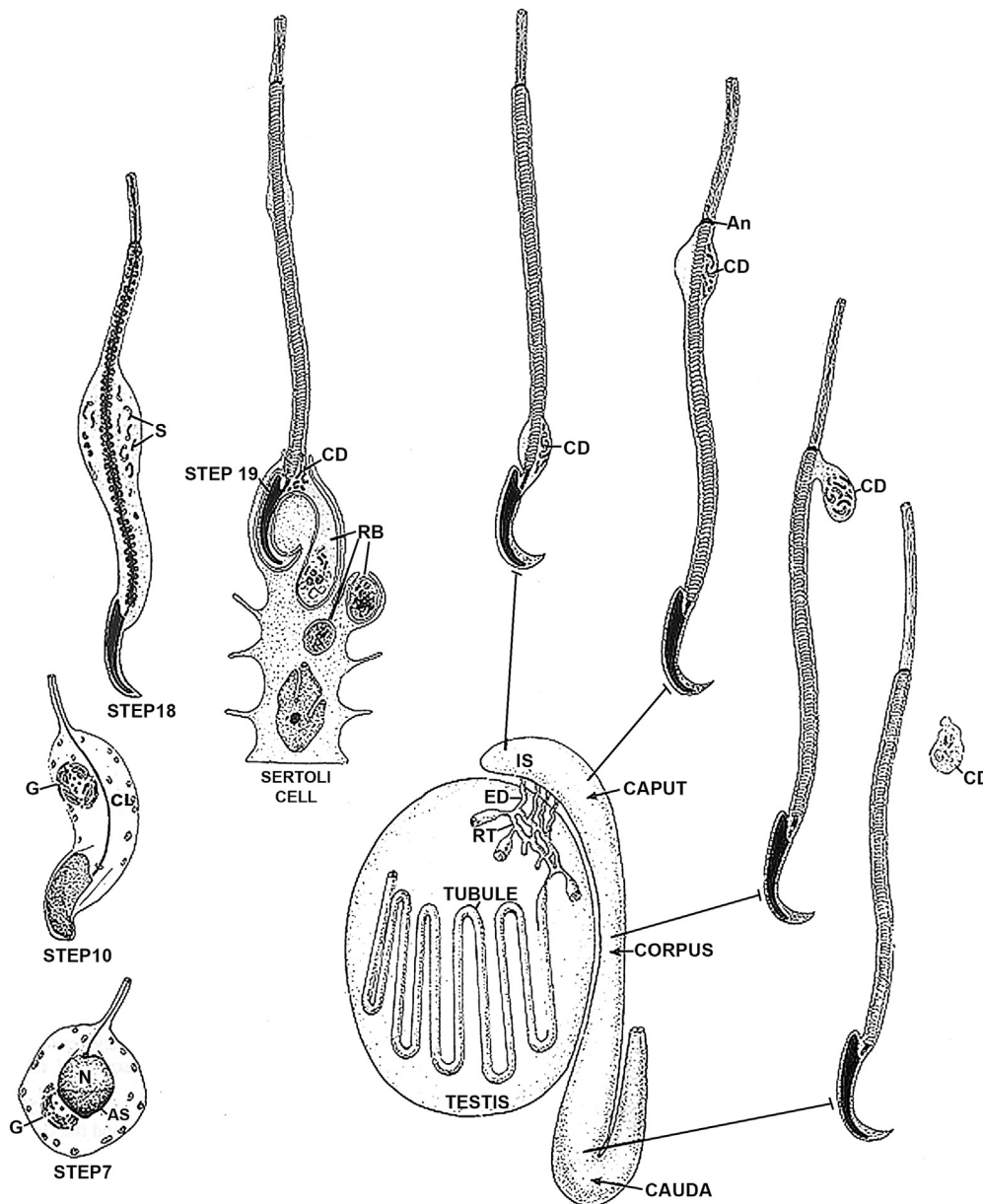
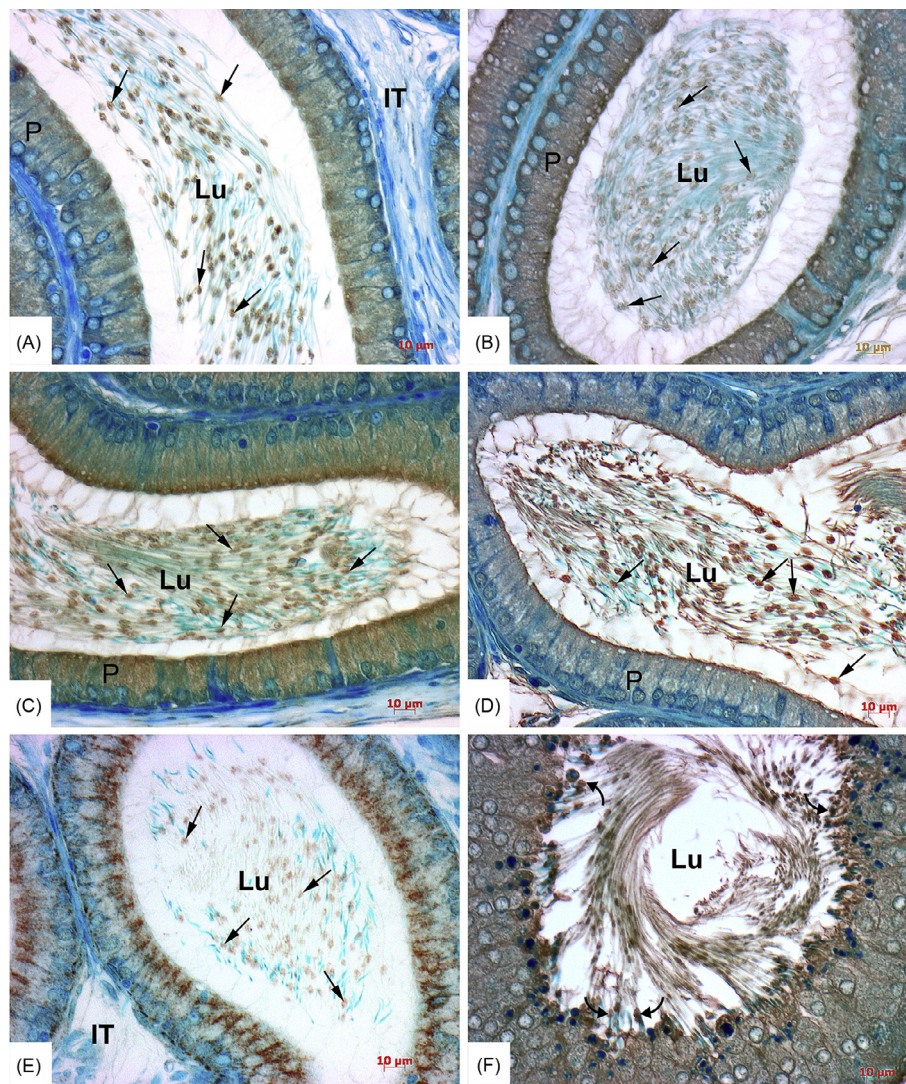


Fig. 4 Schematic representation of the fate of the Golgi apparatus of spermatids and formation of the cytoplasmic droplet (CD), recently renamed the Hermes body, at step 19 of spermiogenesis of the adult rat testis. In early spermatids the Golgi apparatus (G) is hemispherical and forms the acrosomic system (AS) overlying the nucleus (N) as seen at step 7. At step 10 spermatids, the Golgi is spherical and shifts from an acrosomic position to the cytoplasmic lobe (CL). By step 18 the Golgi apparatus has fragmented resulting in dispersal of the individual flattened Golgi cisternae (sacculi, S) into the cytoplasmic lobe. At step 19, the Golgi cisternae enter the forming CD located at the neck area of the flagellum. Other organelles (ER, mitochondria, lipid) enter the residual body of the step 19 spermatids, which is larger in size and destined to be phagocytosed and degraded by the Sertoli cells of the seminiferous epithelium (Morales et al., 1985). After being released from the seminiferous epithelium, the step 19 spermatids (spermatozoa) pass through the efferent ducts (EDs), initial segment (IS), caput, corpus and cauda regions of the epididymis. While the CD is situated at the neck area of the flagellum in the IS, it migrates along the flagellum to the annulus (An) which demarcates the site where the mid piece of the flagellum becomes the principal piece. This occurs by the time spermatozoa enter the caput region of the epididymis. By the time spermatozoa enter the cauda epididymidis, some of the CDs liberate themselves from the sperm flagellum. RT: rete testis; Tubule: seminiferous tubule. Diagram drawn by Dr. Yves Clermont. Figure taken from Okamoto, R., Hermo, L., Chan, P.T., Fazel, A. and Bergeron, J.J. (1993). The cytoplasmic droplet of rat epididymal spermatozoa contains saccular elements with Golgi characteristics. *The Journal of Cell Biology* 123(4), 809–821. Reproduced with permission of The Journal of Cell Biology.

(Oko et al., 1993). By LM-IF, Moreno et al. (2000a, b) localized the Golgi markers giantin, Golgin-97, Golgin95/GM130, Golgin160 and GRASP65 (Moreno et al., 2000a, b) to the HB of isolated epididymal sperm.

In order to determine the validity of this Golgi hypothesis, a method was devised to isolate the Golgi apparatus of germ cells of the testis to elucidate which Golgi proteins were expressed during germ cell differentiation, as well as those that were maintained in Golgi cisternae of the forming HB of step 19 spermatids and the HB of epididymal sperm. By quantitative proteomics a total 1318 were uncovered by Au et al. (2015b). From these 20 were examined by LM-IHC/IF. The data on protein composition of the Golgi cisternae of the HB revealed that many of the proteins localized to the Golgi of germ cells during their differentiation process, such as GL54D, TM9SF3, TMED4/p25, MG160, GRASP55, Sortilin, and UBXD8, were completely absent from the forming HB and that of epididymal sperm (Au et al., 2015a).

However, a host of Golgi proteins of different functions resided in the HB, of which TMED7/p27 was abundant by proteomics and as seen by LM-IHC of in situ HBs of epididymal sperm (Fig. 5A). Other Golgi proteins such GBF1, GPP34, LAMAN, Man2 α 1, MANIX, TMED2/p24 and carboxypeptidase D (Breiner et al., 1998) also resided in the HB as revealed by proteomics and LM-IHC (Au et al., 2015b). Hence even though a Golgi stack is no longer detectable in the HB, these proteins define the flattened membranous elements that congregate therein as Golgi cisternae but differing in protein composition from that of germ cells. Moreover, even the expression profiles of different Golgi proteins expressed by germ cells differed during the various phases of their differentiation process (Au et al., 2015a). Also see review of Hermo et al. (2018).



Figs. 5 A–F: LM photomicrographs of sections of the epididymal duct immunostained with antibodies to: TMED7/p27 (Fig. 5A), and BIP (Fig. 5B); ERP57 (Fig. 5C), annexin 6 (Fig. 5D), Vps13c (Fig. 5E). Hermes bodies of sperm are reactive in the epididymal lumen (arrows). Fig. 5F: LM photomicrograph of a testicular section immunostained with anticarboxypeptidase D antibody illustrating reactive forming Hermes body situated at the neck region of the flagellum (curved arrows) of the step 19 spermatids at stage VIII of the cycle. Lu, lumen; P, principal cells; IT, intertubular space.

In this way, the flattened cisternae of the step 19 spermatids appear to have different molecular constituents from those of a stacked Golgi apparatus and this may be analogous to that postulated for individual yeast-flattened cisternae (Losev et al., 2006; Papanikou and Glick, 2009). In addition, these cisternae may function independent of a stacked configuration as suggested for Golgi cisternae (Klumperman, 2011) and without the conventional setup of a continuous Golgi ribbon. A similar situation exists in circulating platelets where Golgi proteins are present as nonstacked, scattered, separate structures, with selective sets of Golgi enzymes being secreted during platelet activation (Yadav et al., 2017). Thus, the Hermes body of epididymal sperm formed at step 19 spermatids appears to have evolved a system of unique flattened membranous cisternae unlike the conventional stacked Golgi apparatus to carry out specific functions related to sperm maturation (Au et al., 2015b). The specific role(s) of the flattened cisternae of the HB has yet to be resolved thoroughly and awaits the minds of curious young investigators.

Function of the Golgi Cisternae in Relation to the Size of the Hermes Body and Enhancing Glycolysis

A consistent observation by EM of the HB of step 19 spermatids and epididymal sperm was the presence of numerous small 50-60 nm vesicles that associated with the flattened Golgi cisternae (Fig. 2). These appeared to bud off not only at the edges but also from their mid regions. A role for these vesicles comes from EM observations of cryo-fixed in situ HB of epididymal sperm using anti-TMED7/p27 and GLUT3 antibodies. With the former, gold particles localized over the flattened Golgi cisternae of the HB in greater numbers than that over the plasma membrane, while the reverse was the case for GLUT3 (Au et al., 2015b). Thus TMED7/27 Golgi derived vesicles may traffic GLUT3 to the surface of the Hermes body.

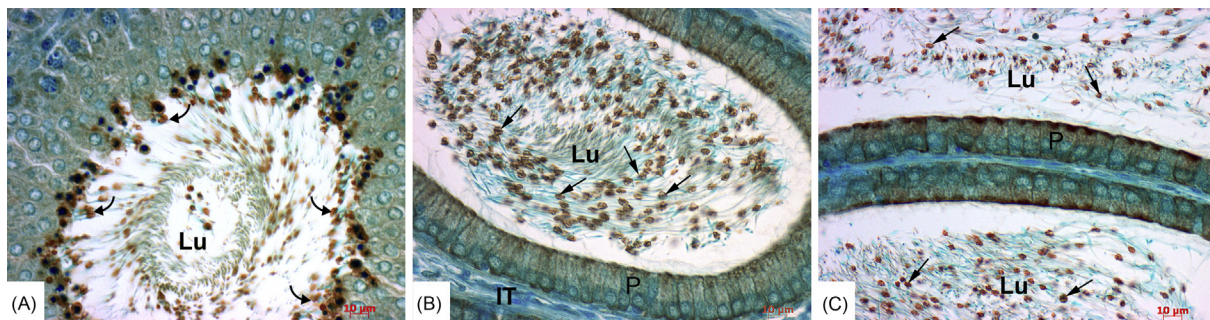
Validity for this hypothesis comes from an LM quantitative analysis of the size of the forming HB at step 19 spermatids versus that seen of the HB of sperm in the initial segment of the epididymis. Hermes bodies of step 19 spermatids appeared to be smaller than those of the Hermes body of epididymal sperm of the initial segment (compare Fig. 6A with B). Also the size of the Hermes body of the latter appeared to be larger in size as compared to those of epididymal sperm of the caput/corpus/cauda epididymidis (compare Fig. 6B with C).

This was confirmed by quantitative analysis of the mean transverse diameter (at its largest axis) of the forming Hermes body of step 19 spermatids with those of epididymal sperm of the initial segment and cauda epididymidis. The mean transverse diameter of atlastin 3 reactive forming Hermes bodies of step 19 spermatids was 2.914 μm with a standard deviation of ± 0.160 . The mean transverse diameter of atlastin 3 reactive Hermes bodies measured in the initial segment of the epididymis was 3.558 μm with a standard deviation of ± 0.348 . An unpaired *t*-test at the 95% confidence interval revealed that the difference was statistically significant (*P*-value < 0.0001). Moreover, the mean transverse diameter of atlastin 3 reactive Hermes bodies measured in the cauda epididymidis was 2.335 μm with a standard deviation of ± 0.202 . An unpaired *t*-test at the 95% confidence interval revealed that the difference was statistically significant (*P*-value < 0.0001) from that of the initial segment. The size of the HB of caput epididymal sperm was also significantly reduced as compared to the initial segment.

Hence a proposed function for the Golgi cisternae may be to traffic Golgi derived vesicles to the cell surface for enhancing the size of the HB. Thus TMED7/p27 may traffic vesicles carrying GLUT-3 to the surface of the HB to serve 2 purposes: to add additional plasma membrane to the cell surface of the HB to increase its size, which in turn would add GLUT3 for purposes of increasing uptake of glucose for increased ATP production for initiation of sperm motility which occurs in the proximal region of the epididymal duct.

Endoplasmic Reticulum (ER), Endosomes, COP Coats, Clathrin, SNAREs, Annexins, AAA + Proteins and GTPases Function in Vesicular Trafficking and Membrane Fusion in the Hermes Body

The proteomic study of Au et al. (2015b) also revealed many ER-related proteins in the HB. ER proteins involved in protein folding included BIP (Fig. 5B) an ER HSP70 family member, also known as glucose-regulated protein 78 or HSPA5, and the glycoprotein



Figs. 6 (A) LM photomicrograph of a testicular section immunostained with anti-atlastin 3 antibody illustrating reactive forming Hermes body of step 19 spermatids at stage VIII of the cycle (*curved arrows*). (B and C) LM of Hermes bodies of epididymal sperm (*arrows*) of the initial segment and caput epididymidis reactive for atlastin 3 antibody. The size of the Hermes body appears to be largest for epididymal sperm of the initial segment. Lu, lumen; P, principal cells; IT, intertubular space.

folding enzyme, ERP57 (Fig. 5C). The glycoprotein folding sensor glucosyl transferase UGGT, the diglucose lectin malectin, and calnexin, the ER lectin chaperone, were also evident.

A striking morphological feature of the HB was the preponderance of flattened cisternae largely unstacked but with an extreme curvature at their edges. The membrane curvature and tubulation proteins, such as atlastin 1, 2, and 3 (Fig. 6B and C) (Hu et al., 2009; Orso et al., 2009) and reticulon 1 and 3 (Chen et al., 2013) were evident in the HB and as such were implicated (Au et al., 2015b).

Protein synthesis proteins via the 68 characterized ribosomal proteins, were characterized by proteomics, as was the abundant ribosome-associated ELONGATION FACTOR 1 ALPHA 1 (EF1A1) along with EF2 (Au et al., 2015b).

Not only were Golgi and ER proteins noted in the HB, but also ER to Golgi, Golgi to ER and endosome to Golgi trafficking proteins (Au et al., 2015b). All seven subunits of COPI coatomer were found in the HB predicting retrograde membrane trafficking (Brandizzi and Barlowe, 2013). Anterograde trafficking COPII coatomer subunits were represented by the GTPase activating protein, sec23, a coat component of COPII vesicles. The coat protein CLATHRIN HEAVY CHAIN (CHC) and the clathrin adaptors AP1 (CLATHRIN ADAPTIN-1) and AP2 (CLATHRIN ADAPTIN-2) were also characterized (Au et al., 2015b).

Proteins suggesting an active role of the HB in membrane fusion were represented by annexins (Fig. 3D), the AAA proteins p97, NSF and its associated alpha SNAP. The endosome to Golgi R SNARE, VAMP 3 (vesicle associated membrane protein 3, also known as cellubrevin), its partner the GTP-binding protein RAB14 (Gonzalez and Scheller, 1999; Junutula et al., 2004), and the Q-SNARE, SYNTAXIN-13 were suggestive of endosomes in this structure and as confirmed by EM observations of large membrane bound vesicular structures in the HB of in situ epididymal sperm (Au et al., 2015b).

The most abundant GTPase was IQGAP known to coordinate actin polymerization and microtubule formation (Watanabe et al., 2004). This may be needed for the known migration of the Hermes body along the mid piece of the flagellum (Cooper, 2011), although a role in signaling (Smith et al., 2015) to coordinate membrane trafficking with glycolysis may also be considered. The most abundant Rab of the GTPase category was Rab14 (Au et al., 2015b), known to be involved in the trafficking and internalization of the GLUT3 paralog, GLUT4, in other cell types (Sadacca et al., 2013).

Taken together, the abundance and localization of these membrane fusion and trafficking proteins with the localization of GLUT3 to the surface and TMED7/p27 to the internal Golgi membranes of the HB is consistent with GLUT3 trafficking to regulate glucose uptake and glycolysis for ATP production and initiation of sperm motility. Yet the full extent of the functions of the proteins of the HB is in dire need of investigation.

Movement of the Hermes Body Along Sperm Flagellum During Epididymal Transit

The HB of epididymal sperm moves in a peristaltic-like manner along the mid piece of the flagellum from the neck to the annulus as sperm move from the caput to the cauda epididymidis (Fig. 1), while the internal organellar membranes rotate in a vortex manner around the axoneme and mitochondrial sheath (Hermo et al., 2010; Cooper, 2011; Sadacca et al., 2013).

Movement of the HB along the mid piece varies between species. Peristaltic motions of the smooth muscle, myoid cells, positioned as several concentric layers at the outer wall of epididymal tubules (Oliveira et al., 2016), has been suggested for some species, as the application of repeated or sustained centrifugal force mimics this function (Cooper, 2005). In the guinea pig, formation of thin filaments cross linking the plasma membrane and outer mitochondrial membranes in the area of the HB appear to function in squeezing it along the midpiece distally to the annulus (Suzuki-Toyota et al., 2010). Such a network of filaments has not been noted in the rat, however, actin and myosin were noted in a proteomic study of the HB of rat epididymal sperm and represent candidates to regulate this movement (Au et al., 2015b). An additional function for the actin based motility system and the characterized myosin motors of the HB would be for active mixing of cytosolic metabolites (Zimmermann et al., 2012), such as ATP for initiating sperm motility.

Role of Hermes Body in Osmolality Regulation

Various studies have pointed to a potential role of the HB in regulating its volume by allowing water entrance/exit and uptake of epididymal osmolytes (Cooper et al., 2004; Fetic et al., 2006). This would allow spermatozoa to adapt to osmolality changes and maintain membrane integrity during their transit through the epididymis and through the female reproductive tract, because when spermatozoa are challenged with low osmolality, the flagellum of epididymal spermatozoa becomes coiled at the position of the HB (Cooper, 2007). As suggested by Cooper (2011), the volume of the Hermes body may be a consequence of the presence of the highly abundant aquaporin water channels localized to this structure (Hermo et al., 2008; Chen et al., 2011). In fact, defective water channels and volume regulation results in swelling and initiation of angulation of the flagellum at the HB leading to a hairpin bend morphology (Yeung et al., 2009). This phenomenon can also be mimicked by blocking cation and anion channels involved in volume regulation (Yeung et al., 2002, 2005; Barfield et al., 2005; Cooper et al., 2004), with sperm failing to reach the oviduct resulting in infertile mice (Yeung et al., 2000; Cooper et al., 2004).

Taken together with the prior localization of the highly abundant water channel aquaporin 7 to the Hermes body in situ (see Fig. 2C of Hermo and Smith, 2011), then the Hermes body represents an expanded aqueous environment segregated from

the rest of the more compacted intracellular environment of the sperm flagellum (Hermo and Smith, 2011). This would enable reversible protein-protein interactions and the ready diffusion of metabolites.

Cholesterol Removal and Internal Membranes of Hermes Body

A striking morphological feature of the Hermes body is the preponderance of flattened cisternae largely unstacked but with an extreme curvature at their edges. The membrane curvature and tubulation proteins, such as atlastins (Fig. 6) and reticulons (Chen et al., 2013), localized to the HB and as such are implicated. A further feature of the flattened cisternae is the close proximity of their curved edges to each other but also with that of the plasma membrane (Oko et al., 1993) (Fig. 2). The latter apposition may be relevant to lipid remodeling of epididymal sperm (Helle et al., 2013; Levine, 2004) especially for cholesterol removal which coincides with sperm transit through the epididymis and which is essential for sperm fertility (Saez et al., 2011).

Fate of the Hermes Body of Epididymal Sperm

The release and fate of the HB during epididymal transit appears to be species dependent. In the rat and monkey, HBs detach from spermatozoa by the time they reach the cauda epididymidis (Xu et al., 2013; Hermo et al., 1988, 2010), but a more recent study suggested that many HBs retain them in the cauda epididymidis (Au et al., 2015b). In the mouse, bull, boar, ram, and goat, the HBs remain on spermatozoa in the epididymis and are not released until the time of ejaculation (Cooper, 2005; Cooper and Yeung, 2003; Xu et al., 2013).

In bovine ampulla vas deferens and seminal vesicle, a haemolytic phospholipid binding protein has been suggested to liberate HBs from sperm (Matousek and Kysilka, 1980; Matousek and Kysilka, 1984), with fructose functioning in this capacity in boars (Harayama et al., 1996; Temple-Smith, 1984).

During sperm maturation in the brushtailed possum, HBs were shed from maturing spermatozoa in the caput region of the epididymis. Disappearance of detached HBs from the lumen was associated with a region of specialized principal cells, which selectively sequestered and phagocytosed them from the epididymal lumen (Temple-Smith, 1984). These observations contrasted the rat where after detaching from the flagellum, the HBs appeared to fragment in the epididymal lumen with their contents taken up by the epithelial clear cells (Fig. 1) (Hermo et al., 1988).

Does the Presence of the Hermes Body Affect Sperm Fertility

Failure to properly form or shed the HB has been associated with male infertility. Male *Alox15* mice lacking the organelle degradation enzyme 15-lipoxygenase (15LOX) displayed sperm HB anomalies (Moore et al., 2010). There were more sperm with attached HBs in the cauda region as compared to wild type mice and presence of isolated, intact mitochondria not noted in wild type HBs. These findings may explain the reduced fertility and smaller litter size of *Alox15* mice.

Spermatid maturation 1 (*Spem1*) encodes a protein exclusively expressed in the cytoplasm of steps 14–16 mouse elongated spermatids. Male mice deficient in *Spem1* were completely infertile because of deformed sperm characterized by aberrant HBs located in atypical positions and deformed flagella (Zheng et al., 2007).

The majority of *Tnp1*-null and *Tnp1-Hils1* double KO spermatozoa were mostly devoid of HBs. *Tnp1* encodes transition protein 1 (TNP1) and *Hils1* encodes a histone variant specifically expressed in elongated spermatids (HILS1). *Tnp1* KO male mice were sub-fertile, while *Tnp1-Hils1* double KO male mice were infertile (Zheng et al., 2007).

There have also been reports that the retention of HBs on sperm in domestic animals is associated with infertility (Amann et al., 2000; Thundathil et al., 2001; Kuster et al., 2004). However, in humans the majority of motile sperm found in the ejaculate bear a HB, which differs in its position from other species, being at the neck region rather than the annulus (Cooper, 2005). Their persistence does not appear to be an abnormal feature of human sperm, as HBs are not deleterious to sperm motility and may be related to physiological volume regulation (Cooper et al., 2004; Fetic et al., 2006). This may reflect their short mid piece obviating droplet migration and flagellar angulation and hampering droplet loss (Cooper, 2011).

A recent case report has shown that live births resulted from two separate IVF cycles in a couple in which ICSI was performed with sperm specifically selected for presence of small cytoplasmic droplets. Cycles using standard sperm selection methods resulted in very poor embryo development and no pregnancies (Bellish et al., 2015).

Selective Protein Loss in the Hermes Body: Proteasomes and Proteins Involved in Degradation

The concentration of ubiquitin, all subunits of the proteasome, the ATPase P97 linked to protein translocation in ERAD to the proteasome, and the highly abundant known germ cell ubiquitin ligase cullin3 (Wang et al., 2006), the stress response sperm ubiquitin

ligase (Genschik et al., 2013; Wang et al., 2006) appeared to explain the selective and progressive loss of 12 of the 43 proteins of the Hermes body as sperm proceeded from the initial segment to the cauda epididymidis (Au et al., 2015b). However, their presence also suggests a role for the abundance of glycolytic enzymes in the Hermes body, i.e., to generate ATP for protein degradation. Proteasome subunits and ubiquitin conjugating enzymes have also been characterized in a proteomic study of whole epididymal sperm (Chauvin et al., 2012). Localization studies of ubiquitin-proteasome pathway and 15-lipoxygenase (15LOX) (Moore et al., 2010) confirmed their presence in the HB in situ for sperm maturation (Hou and Yang, 2013).

The Hermes Body Compartmentalizes Stress Proteins

Intracellular antioxidant proteins are involved in protecting sperm against oxidative stress (Robaire et al., 2006; Hermo et al., 2010; Moore et al., 2010). Peroxiredoxins 1, 3 and 6 noted in sperm (O'flaherty and De Souza, 2011) and which scavenge reactive oxygen species, were abundant in the HB, as well as glutathione peroxidase and transglutaminase (Au et al., 2015b). Glutathione S transferases were noted by proteomics in the isolated HB (Au et al., 2015b), and by LM-IHC in previous studies (Veri et al., 1993; Papp et al., 1995).

Eighteen different heat shock proteins protective against stress were also evident in the HB (Au et al., 2015b). This included the germ cell specific HSP70.2 protein, HSP70.3 and HSP86. The HSP70 ER luminal protein, GRP170 was also abundant. Thus the Hermes body contains proteins that provide protection of whole sperm from oxidative stress (Aitken et al., 2014).

Step 19 Spermatids: Formation of the Hermes Body

While the proteomic data characterized proteins to the HB of epididymal sperm, a striking observation was made when these proteins were analyzed as they evolved during the course of germ cell differentiation. A burst of both non Golgi and Golgi protein expression was prominent exclusively in step 19 spermatids during formation of the HB (Au et al., 2015a, b; Hermo et al., 2018). These included AnnexinA1, AP1 and AP2, malectin, atlastin 2, syntaxin 13, Vps13c (member of the Vps13 family of vacuolar protein sorting-associated proteins), tubulin, peroxiredoxins 1 and 6, and the monocarboxylate transporter MCT2, all of which were also present in the HB as sperm entered the proximal region of the epididymis (e.g., Vps13c, Fig. 3E and MCT2, Fig. 3D). This also conformed with expression selectively to step 19 spermatids for the Golgi proteins TMED2/p24 and carboxypeptidase D (Fig. 3F). This has been also noted for several GSTs that became expressed solely at step 19 (Papp et al., 1995). Thus step 19 spermatids appear as highly active cells for protein expression contrary to dogma. This makes the forming Hermes body a site of major expression of proteins that had not been reported in any earlier studies and which may serve an important role in sperm as they leave the seminiferous tubule lumen and traverse the epididymis (Au et al., 2015b).

Since transcription ceases immediately at the end of acrosome formation by step 8 (Monesi, 1965), then the differential expression of the Golgi and non Golgi proteins after step 8 must necessarily reflect translational regulation as described for many other proteins during late spermiogenesis (Hecht, 1990). Transcriptional regulation of changes in the structure and function of organelles of the secretory pathway is well established in somatic cells (Schroder and Kaufman, 2005). Thus the translation controlled pathway for organelle modification and morphogenesis of germ cells (Chappell et al., 2013) must be prominent at step 19 in the formation and eventual function of the HB as it persists on sperm as they travel through the epididymis.

Proteins of Unknown Function

215 proteins of unknown function were also characterized by proteomics in the isolated Hermes body by Au et al. (2015b). Information on these proteins should extend considerably the role of this micro-compartment in epididymal sperm maturation.

Conclusion

As seen by EM, the forming Hermes body in step 19 and of epididymal sperm concentrates isolated flattened cisternae defined as Golgi elements, as well as dilated irregularly shaped membranous profiles suggestive of being ER and endosomes. Taken together with protein markers, this heterogeneity is indicative that the HB is a macromolecular structure enriched with subsets of endosomal, Golgi, coat and ER proteins for functions in membrane traffic and stress proteins for protection and proteins involved in degradation, as well as cytoskeletal elements for migration and a concentration of cytoplasmic proteins for glycolysis (Fig. 7). A detailed analysis of the expression profiles of germ cell Golgi and non-Golgi proteins and their overlapping distribution profiles can be found in our recent review (Hermo et al., 2018).

The HB is conserved amongst all mammalian species. The current data indicate that sperm themselves through the HB may play a role in their own maturation. Taken together with the proteins of the biosynthetic secretory apparatus characterized by Au et al.

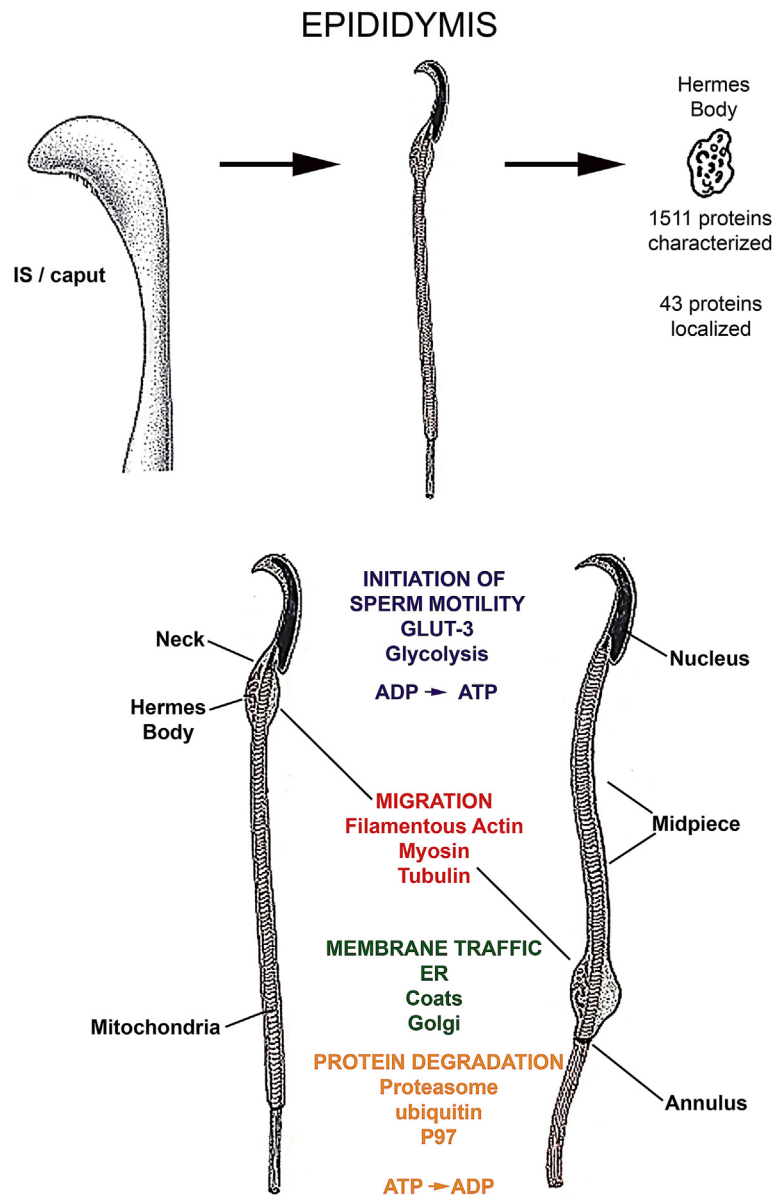


Fig. 7 The initial segment and caput epididymidis of adult rats were dissected free from the testis and surrounding fat. A method was developed to isolate fractions of isolated HBs from epididymal sperm of these regions (Oko et al., 1993). Proteins were characterized by quantitative tandem mass spectrometry (Au et al., 2015b). From the 1511 proteins identified in the isolated HB fraction, 43 proteins were selected and analyzed with antibodies to reveal their localization by light microscope immunohistochemical (IHC) and immunofluorescence (IF) to the HBs of sperm of in situ epididymal tissue sections. The data revealed that the HB is involved in glycolysis, migration along the flagellum, membrane trafficking and protein degradation.

(2015b) and Chauvin et al. (2012) then far from being a cell devoid of a translational machinery, epididymal sperm may be reassessed as in a dynamic state of protein turnover as proposed for all cells (Schoenheimer, 1942).

Acknowledgments

I hope that all aspiring young scientists early in their career would be so privileged as to have such great role models as I (LH) was with Dr. Y. Clermont and Dr. C. P. Leblond. I would like to thank Dr. Clermont my supervisor and mentor who challenged and inspired me over my 45 years at McGill University, as did Dr. Leblond. Their sincere dedication and love of science allowed me and generations of students to find our niche and aspire us to be as great as they were. I would also like to thank Dr. M. F. Lalli who assisted me as a graduate student in learning the ropes of electron microscopy, tissue fixation and preparation and spermatogenesis. This article is dedicated to Yves Clermont and CP Leblond who elaborated the stem cell renewal theory for male germ cell differentiation. <http://www.mcgill.ca/anatomy/stem-cell-renewal-theory>.

References

- Aitken, J., Krausz, C., & Buckingham, D. (1994). Relationships between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. *Molecular Reproduction and Development*, 39, 268–279.
- Aitken, R. J., Smith, T. B., Jobling, M. S., Baker, M. A., & De Iulius, G. N. (2014). Oxidative stress and male reproductive health. *Asian Journal of Andrology*, 16, 31–38.
- Amann, R. P., Seidel, G. E., Jr., & Mortimer, R. G. (2000). Fertilizing potential in vitro of semen from young beef bulls containing a high or low percentage of sperm with a proximal droplet. *Theriogenology*, 54, 1499–1515.
- Au, C. E., Hermo, L., Byrne, E., Smirle, J., Fazel, A., Simon, P. H., Kearney, R. E., Cameron, P. H., Smith, C. E., Vali, H., Fernandez-Rodriguez, J., Ma, K., Nilsson, T., & Bergeron, J. J. (2015a). Expression, sorting, and segregation of Golgi proteins during germ cell differentiation in the testis. *Molecular Biology of the Cell*, 26, 4015–4032.
- Au, C. E., Hermo, L., Byrne, E., Smirle, J., Fazel, A., Kearney, R. E., Smith, C. E., Vali, H., Fernandez-Rodriguez, J., Simon, P. H., Mandato, C., Nilsson, T., & Bergeron, J. J. (2015b). Compartmentalization of membrane trafficking, glucose transport, glycolysis, actin, tubulin and the proteasome in the cytoplasmic droplet/Hermes body of epididymal sperm. *Open Biology*, 5(8).
- Baker, M. A., & Aitken, R. J. (2009). Proteomic insights into spermatozoa: Critiques, comments and concerns. *Expert Review of Proteomics*, 6, 691–705.
- Baker, M. A., Naumovski, N., Hetherington, L., Weinberg, A., Velkov, T., & Aitken, R. J. (2013). Head and flagella subcompartmental proteomic analysis of human spermatozoa. *Proteomics*, 13, 61–74.
- Barfield, J. P., Yeung, C. H., & Cooper, T. G. (2005). The effects of putative K⁺ channel blockers on volume regulation of murine spermatozoa. *Biology of Reproduction*, 72, 1275–1281.
- Bellish, J., McCulloch, D. H., Ahmad, K., & McGovern, P. G. (2015). Successful pregnancy in a couple with severe male factor infertility after selection of sperm with cytoplasmic droplets. *Case Reports in Obstetrics and Gynecology*, 2015, 276931.
- Bloom, G., & Nicander, L. (1961). On the ultra-structure and development of the protoplasmic droplet of spermatozoa. *Zeitschrift für Zellforschung*, 55, 833–844.
- Brandizzi, F., & Barlowe, C. (2013). Organization of the ER-Golgi interface for membrane traffic control. *Nature Reviews. Molecular Cell Biology*, 14, 382–392.
- Breiner, K. M., Urban, S., & Schaller, H. (1998). Carboxypeptidase D (gp180), a Golgi-resident protein, functions in the attachment and entry of avian hepatitis B viruses. *Journal of Virology*, 72, 8098–8104.
- Chappell, V. A., Busada, J. T., Keiper, B. D., & Geyer, C. B. (2013). Translational activation of developmental messenger RNAs during neonatal mouse testis development. *Biology of Reproduction*, 89, 61.
- Chauvin, T., Xie, F., Liu, T., Nicora, C. D., Yang, F., Camp, D. G., 2nd, Smith, R. D., & Roberts, K. P. (2012). A systematic analysis of a deep mouse epididymal sperm proteome. *Biology of Reproduction*, 87, 141.
- Chen, Q., Peng, H., Lei, L., Zhang, Y., Kuang, H., Cao, Y., Shi, Q. X., Ma, T., & Duan, E. (2011). Aquaporin3 is a sperm water channel essential for postcopulatory sperm osmoadaptation and migration. *Cell Research*, 21, 922–933.
- Chen, S., Novick, P., & Ferro-Novick, S. (2013). ER structure and function. *Current Opinion in Cell Biology*, 25, 428–433.
- Chocu, S., Calvel, P., Rolland, A. D., & Pineau, C. (2012). Spermatogenesis in mammals: Proteomic insights. *Systems Biology in Reproductive Medicine*, 58, 179–190.
- Cooper, T. G. (2005). Cytoplasmic droplets: The good, the bad or just confusing? *Human Reproduction*, 20, 9–11.
- Cooper, T. G. (2007). Sperm maturation in the epididymis: A new look at an old problem. *Asian Journal of Andrology*, 9, 533–539.
- Cooper, T. G. (2011). The epididymis, cytoplasmic droplets and male fertility. *Asian Journal of Andrology*, 13, 130–138.
- Cooper, T. G., & Yeung, C. H. (2003). Acquisition of volume regulatory response of sperm upon maturation in the epididymis and the role of the cytoplasmic droplet. *Microscopy Research and Technique*, 61, 28–38.
- Cooper, T. G., Yeung, C. H., Fetic, S., Sobhani, A., & Nieschlag, E. (2004). Cytoplasmic droplets are normal structures of human sperm but are not well preserved by routine procedures for assessing sperm morphology. *Human Reproduction*, 19, 2283–2288.
- Cornwall, G. A. (2009). New insights into epididymal biology and function. *Human Reproduction Update*, 15, 213–227.
- Dott, H. M., & Dingle, J. T. (1968). Distribution of lysosomal enzymes in the spermatozoa and cytoplasmic droplets of bull and ram. *Experimental Cell Research*, 52, 523–540.
- Eddy, E. M., Toshimori, K., & O'Brien, D. A. (2003). Fibrous sheath of mammalian spermatozoa. *Microscopy Research and Technique*, 61, 103–115.
- Fawcett, D. W., & Ito, S. (1965). The fine structure of bat spermatozoa. *The American Journal of Anatomy*, 116, 567–609.
- Fetic, S., Yeung, C. H., Sonntag, B., Nieschlag, E., & Cooper, T. G. (2006). Relationship of cytoplasmic droplets to motility, migration in mucus, and volume regulation of human spermatozoa. *Journal of Andrology*, 27, 294–301.
- Ford, W. C. (2006). Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go round? *Human Reproduction Update*, 12, 269–274.
- Genschik, P., Sumara, I., & Lechner, E. (2013). The emerging family of CULLIN3-RING ubiquitin ligases (CRL3s): Cellular functions and disease implications. *The EMBO Journal*, 32, 2307–2320.
- Gonzalez, L., Jr., & Scheller, R. H. (1999). Regulation of membrane trafficking: Structural insights from a Rab/effector complex. *Cell*, 96, 755–758.
- Harayama, H., Shibukawa, T., Miyake, M., Kannan, Y., & Kato, S. (1996). Fructose stimulates shedding of cytoplasmic droplets from epididymal boar spermatozoa. *Reproduction, Fertility, and Development*, 8, 1039–1043.
- Hecht, N. B. (1990). Regulation of 'haploid expressed genes' in male germ cells. *Journal of Reproduction and Fertility*, 88, 679–693.
- Helle, S. C., Kanfer, G., Kolar, K., Lang, A., Michel, A. H., & Kornmann, B. (2013). Organization and function of membrane contact sites. *Biochimica et Biophysica Acta*, 1833, 2526–2541.
- Hermo, L., & Smith, C. E. (2011). Thirsty business: Cell, region, and membrane specificity of aquaporins in the testis, efferent ducts, and epididymis and factors regulating their expression. *Journal of Andrology*, 32, 565–575.
- Hermo, L., Dworkin, J., & Oko, R. (1988). Role of epithelial clear cells of the rat epididymis in the disposal of the contents of cytoplasmic droplets detached from spermatozoa. *The American Journal of Anatomy*, 183, 107–124.
- Hermo, L., Schellenberg, M., Liu, L. Y., Dayanandan, B., Zhang, T., Mandato, C. A., & Smith, C. E. (2008). Membrane domain specificity in the spatial distribution of aquaporins 5, 7, 9, and 11 in efferent ducts and epididymis of rats. *The Journal of Histochemistry and Cytochemistry*, 56, 1121–1135.
- Hermo, L., Pelletier, R. M., Cyr, D. G., & Smith, C. E. (2010). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 3: Developmental changes in spermatid flagellum and cytoplasmic droplet and interaction of sperm with the zona pellucida and egg plasma membrane. *Microscopy Research and Technique*, 73, 320–363.
- Hermo, L., Oliveira, R. L., Smith, C. E., & Bergeron, J. (2018). Golgi Apparatus Regulation of Differentiation: A case study for male germ cells of the rat testis. In *Spermatogenesis: Biology and Clinical Implications*. Taylor & Francis.
- Hou, C. C., & Yang, W. X. (2013). New insights to the ubiquitin-proteasome pathway (UPP) mechanism during spermatogenesis. *Molecular Biology Reports*, 40, 3213–3230.
- Hu, J., Shibata, Y., Zhu, P. P., Voss, C., Rismanchi, N., Prinz, W. A., Rapoport, T. A., & Blackstone, C. (2009). A class of dynamin-like Gtpases involved in the generation of the tubular ER network. *Cell*, 138, 549–561.
- Junutula, J. R., De Maziere, A. M., Peden, A. A., Ervin, K. E., Advani, R. J., Van Dijk, S. M., Klumperman, J., & Scheller, R. H. (2004). Rab14 is involved in membrane trafficking between the Golgi complex and endosomes. *Molecular Biology of the Cell*, 15, 2218–2229.
- Klumperman, J. (2011). Architecture of the mammalian Golgi. *Cold Spring Harbor Perspectives in Biology*, 3.
- Kuster, C. E., Hess, R. A., & Althouse, G. C. (2004). Immunofluorescence reveals ubiquitination of retained distal cytoplasmic droplets on ejaculated porcine spermatozoa. *Journal of Andrology*, 25, 340–347.

- Leblond, C. P., & Clermont, Y. (1952). Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfurous acid technique. *The American Journal of Anatomy*, 90, 167–215.
- Levine, T. (2004). Short-range intracellular trafficking of small molecules across endoplasmic reticulum junctions. *Trends in Cell Biology*, 14, 483–490.
- Losev, E., Reinke, C. A., Jellen, J., Strongin, D. E., Bevis, B. J., & Glick, B. S. (2006). Golgi maturation visualized in living yeast. *Nature*, 441, 1002–1006.
- Mannowetz, N., Wandernoth, P. M., & Wennemuth, G. (2012). Glucose is a pH-dependent motor for sperm beat frequency during early activation. *PLoS One*, 7, e41030.
- Matousek, J., & Kysilka, C. (1980). The haemolytic factor (phospholipid-binding protein) of the bull reproductive tract—its synthesis and effect on spermatozoal cytoplasm droplets. *Animal Reproduction Science*, 3, 195–205.
- Matousek, J., & Kysilka, C. (1984). The phospholipid-binding protein of the reproductive tract of the bull – Effect on the removal of spermatozoal cytoplasm droplets in other species and influence of antibodies on its reactivity. *Animal Reproduction Science*, 7, 433–440.
- Miki, K. (2007). Energy metabolism and sperm function. *Society of Reproduction and Fertility Supplement*, 65, 309–325.
- Monesi, V. (1965). Differential rate of ribonucleic acid synthesis in the autosomes and sex chromosomes during male meiosis in the mouse. *Chromosoma*, 17, 11–21.
- Moniem, K. A., & Glover, T. D. (1972). Alkaline phosphatase in the cytoplasmic droplet of mammalian spermatozoa. *Journal of Reproduction and Fertility*, 29, 65–69.
- Moore, K., Lovercamp, K., Feng, D., Antelman, J., Sutovsky, M., Manandhar, G., Van Leyen, K., Safranski, T., & Sutovsky, P. (2010). Altered epididymal sperm maturation and cytoplasmic droplet migration in subfertile male Alox15 mice. *Cell and Tissue Research*, 340, 569–581.
- Morales, C., Clermont, Y., & Hermo, L. (1985). Nature and function of endocytosis in Sertoli cells of the rat. *The American Journal of Anatomy*, 173, 203–217.
- Moreno, R. D., Ramalho-Santos, J., Chan, E. K., Wessel, G. M., & Schatten, G. (2000a). The Golgi apparatus segregates from the lysosomal/acrosomal vesicle during rhesus spermiogenesis: Structural alterations. *Developmental Biology*, 219, 334–349.
- Moreno, R. D., Ramalho-Santos, J., Sutovsky, P., Chan, E. K., & Schatten, G. (2000b). Vesicular traffic and golgi apparatus dynamics during mammalian spermatogenesis: Implications for acrosome architecture. *Biology of Reproduction*, 63, 89–98.
- Nakamura, N., Mori, C., & Eddy, E. M. (2010). Molecular complex of three testis-specific isozymes associated with the mouse sperm fibrous sheath: Hexokinase 1, phosphofructokinase M, and glutathione S-transferase mu class 5. *Biology of Reproduction*, 82, 504–515.
- Odet, F., Gabel, S., London, R. E., Goldberg, E., & Eddy, E. M. (2013). Glycolysis and mitochondrial respiration in mouse Ldhc-null sperm. *Biology of Reproduction*, 88, 95.
- O'flaherty, C., & De Souza, A. R. (2011). Hydrogen peroxide modifies human sperm peroxiredoxins in a dose-dependent manner. *Biol. Reprod.*, 84, 238–247.
- Oko, R., Hermo, L., Chan, P. T., Fazel, A., & Bergeron, J. J. (1993). The cytoplasmic droplet of rat epididymal spermatozoa contains saccular elements with Golgi characteristics. *The Journal of Cell Biology*, 123, 809–821.
- Oliveira, R. L., Parent, A., Cyr, D. G., Gregory, M., Mandato, C. A., Smith, C. E., & Hermo, L. (2016). Implications of caveolae in testicular and epididymal myoid cells to sperm motility. *Molecular Reproduction and Development*, 83, 526–540.
- Orgebin-Crist, M. C. (1969). Studies on the function of the epididymis. *Biology of Reproduction*, 1(Suppl 1), 155–175.
- Orso, G., Pendin, D., Liu, S., Toso, J., Moss, T. J., Faust, J. E., Micaroni, M., Egorova, A., Martinuzzi, A., Mcnew, J. A., & Daga, A. (2009). Homotypic fusion of ER membranes requires the dynamin-like GTPase atlastin. *Nature*, 460, 978–983.
- Papanikou, E., & Glick, B. S. (2009). The yeast Golgi apparatus: Insights and mysteries. *FEBS Letters*, 583, 3746–3751.
- Papp, S., Robaire, B., & Hermo, L. (1995). Immunocytochemical localization of the Ya, Yc, Yb1, and Yb2 subunits of glutathione S-transferases in the testis and epididymis of adult rats. *Microscopy Research and Technique*, 30, 1–23.
- Retzius, G. (1909). Die Spermen der Huftiere. *Niol. Untersuch.*, 14, 163–178.
- Robaire B and Hermo L (1988) Efferent ducts, epididymis, and vas deferens: Structure, functions, and their regulation. In: Knobil E and Neill J (eds.) *The Physiology of Reproduction*, pp. 999–1080. New York: Raven Press.
- Robaire, B., Hinton, B. T., & Orgebin-Crist, M. C. (2006). The epididymis. In J. D. Neill (Ed.), *Physiology of Reproduction* (pp. 1071–1148).
- Roberts, M. L., Scouten, W. H., & Nyquist, S. E. (1976). Isolation and characterization of the cytoplasmic droplet in the rat. *Biology of Reproduction*, 14, 421–424.
- Sadacca, L. A., Bruno, J., Wen, J., Xiong, W., & McGraw, T. E. (2013). Specialized sorting of GLUT4 and its recruitment to the cell surface are independently regulated by distinct Rabs. *Molecular Biology of the Cell*, 24, 2544–2557.
- Saez, F., Ouvrier, A., & Drevet, J. R. (2011). Epididymis cholesterol homeostasis and sperm fertilizing ability. *Asian Journal of Andrology*, 13, 11–17.
- Schoenheimer, R. (1942). *The dynamic state of body constituents*. Cambridge, MA: Harvard University Press.
- Schroder, M., & Kaufman, R. J. (2005). ER stress and the unfolded protein response. *Mutation Research*, 569, 29–63.
- Smith, J. M., Hedman, A. C., & Sacks, D. B. (2015). Iqgaps choreograph cellular signaling from the membrane to the nucleus. *Trends in Cell Biology*, 25, 171–184.
- Suzuki-Toyota, F., Ito, C., Maekawa, M., Toyama, Y., & Toshimori, K. (2010). Adhesion between plasma membrane and mitochondria with linking filaments in relation to migration of cytoplasmic droplet during epididymal maturation in guinea pig spermatozoa. *Cell and Tissue Research*, 341, 429–440.
- Tani, I., Yagura, T., Inagaki, N., Nakayama, T., Imaizumi, K., & Yoshinaga, K. (2007). Preferential localization of rat GAPDS on the ribs of fibrous sheath of sperm flagellum and its expression during flagellar formation. *Acta Histochemica et Cytochemica*, 40, 19–26.
- Temple-Smith, P. D. (1984). Phagocytosis of sperm cytoplasmic droplets by a specialized region in the epididymis of the brushtailed possum, *Trichosurus Vulpecula*. *Biology of Reproduction*, 30, 707–720.
- Thundathil, J., Palasz, A. T., Barth, A. D., & Mapletto, R. J. (2001). The use of in vitro fertilization techniques to investigate the fertilizing ability of bovine sperm with proximal cytoplasmic droplets. *Animal Reproduction Science*, 65, 181–192.
- Veri, J. P., Hermo, L., & Robaire, B. (1993). Immunocytochemical localization of the Yf subunit of glutathione S-transferase P shows regional variation in the staining of epithelial cells of the testis, efferent ducts, and epididymis of the male rat. *Journal of Andrology*, 14, 23–44.
- Wang, S., Zheng, H., Esaki, Y., Kelly, F., & Yan, W. (2006). Cullin3 is a Khl10-interacting protein preferentially expressed during late spermiogenesis. *Biology of Reproduction*, 74, 102–108.
- Watanabe, T., Wang, S., Noritake, J., Sato, K., Fukata, M., Takefuji, M., Nakagawa, M., Izumi, N., Akiyama, T., & Kaibuchi, K. (2004). Interaction with IQGAP1 links APC to Rac1, Cdc42, and actin filaments during cell polarization and migration. *Developmental Cell*, 7, 871–883.
- Xu, H., Yuan, S. Q., Zheng, Z. H., & Yan, W. (2013). The cytoplasmic droplet may be indicative of sperm motility and normal spermiogenesis. *Asian Journal of Andrology*, 15, 799–805.
- Yadav, S., Williamson, J. K., Aronova, M. A., Prince, A. A., Pokrovskaya, I. D., Leapman, R. D., & Storrie, B. (2017). Golgi proteins in circulating human platelets are distributed across non-stacked, scattered structures. *Platelets*, 28, 400–408.
- Yeung, C. H., Wagenfeld, A., Nieschlag, E., & Cooper, T. G. (2000). The cause of infertility of male c-ros tyrosine kinase receptor knockout mice. *Biology of Reproduction*, 63, 612–618.
- Yeung, C. H., Anapolski, M., Sipila, P., Wagenfeld, A., Poutanen, M., Huhtaniemi, I., Nieschlag, E., & Cooper, T. G. (2002). Sperm volume regulation: Maturation changes in fertile and infertile transgenic mice and association with kinematics and tail angulation. *Biology of Reproduction*, 67, 269–275.
- Yeung, C. H., Barfield, J. P., & Cooper, T. G. (2005). The role of anion channels and Ca²⁺ in addition to K⁺ channels in the physiological volume regulation of murine spermatozoa. *Molecular Reproduction and Development*, 71, 368–379.
- Yeung, C. H., Callies, C., Rojek, A., Nielsen, S., & Cooper, T. G. (2009). Aquaporin isoforms involved in physiological volume regulation of murine spermatozoa. *Biology of Reproduction*, 80, 350–357.
- Yuan, S., Zheng, H., Zheng, Z., & Yan, W. (2013). Proteomic analyses reveal a role of cytoplasmic droplets as an energy source during epididymal sperm maturation. *PLoS One*, 8, e77466.

- Zheng, H., Stratton, C. J., Morozumi, K., Jin, J., Yanagimachi, R., & Yan, W. (2007). Lack of Spem1 causes aberrant cytoplasm removal, sperm deformation, and male infertility. *Proceedings of the National Academy of Sciences of the United States of America*, *104*, 6852–6857.
- Zimmermann, J., Brunner, C., Enculescu, M., Goegler, M., Ehrlicher, A., Kas, J., & Falcke, M. (2012). Actin filament elasticity and retrograde flow shape the force-velocity relation of motile cells. *Biophysical Journal*, *102*, 287–295.

Further Reading

- Matousek, J., & Kysilka, C. (1984). The phospholipid-binding protein of the reproductive tract of the bull—effect on the removal of spermatozoal cytoplasm droplets in other species and influence of antibodies on its reactivity. *Animal Reproduction Science*, *7*, 433–440.

Chromatin Structure, Composition, and Function During Spermatogenesis

Mark E Gill, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland

Antoine HFM Peters, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland; and University of Basel, Basel, Switzerland

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromatin A complex composed of proteins and nucleic acid that serves to package and regulate the genetic material.

Histone A group of small, highly conserved, basic proteins that package DNA in most eukaryotic cells.

Nucleosome A complex composed of four different histone proteins and associated DNA.

Protamines Spermatogenesis-specific, small, basic proteins, which package DNA in late stage spermatids and mature spermatozoa.

Spermatid A postmeiotic male germ cell.

Spermatocyte A meiotic male germ cell.

Spermatogonium A premeiotic, mitotically dividing male germ cell.

General Introduction to Chromatin Composition and Structure

Germ cells, like somatic cells, package their DNA into chromatin, a complex of proteins and nucleic acids that serves to regulate DNA sequence accessibility and ultimately control processes such as gene transcription, recombination, DNA repair, and genome stability. The primary protein constituents of chromatin are histones, small highly basic DNA packaging proteins. Histone proteins are generally ultra-conserved within eukaryotes, for instance they show no amino acid sequence divergence between mice and humans. The basic unit of chromatin, the nucleosome, consists of two pairs of four different histone proteins around which 146 bp of DNA is wrapped (Cutter and Hayes, 2015). (See Fig. 1).

Histone proteins are extensively posttranslationally modified with a variety of chemical modifications including methylation, phosphorylation, and acetylation (among many others). These modifications control the affinity of the histone proteins for DNA, as well as serving as a signaling platform for interaction with modification-specific binding proteins. These interactors, called chromatin readers, can then recruit additional modifying enzymes or transcriptional machinery to induce changes in gene expression. Modifications of histones occur in a variety of combinations and correlate highly with specific genomic localization and transcriptional activity. For instance tri-methylation of different lysine residues on the N-terminus of histone H3 associate with either

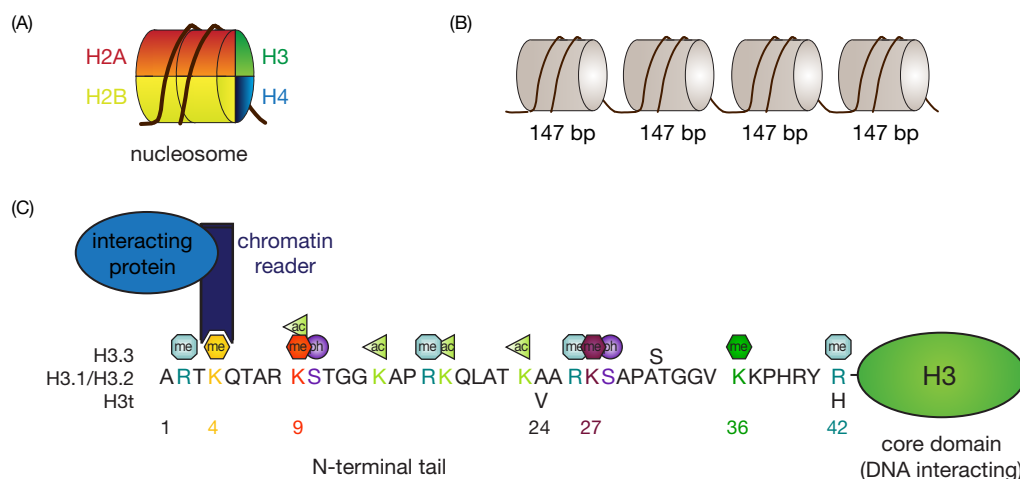


Fig. 1 Chromatin structure. (A) Schematic representation of a nucleosome, the basic unit of chromatin. DNA wraps around the four pairs of histone proteins (H2A, H2B, H3, and H4). (B) Representation of local chromatin structure. 147 bp of DNA is wrapped around each nucleosome with a short stretch of linker DNA between adjacent nucleosomes. (C) Model for the role of posttranslational modifications in chromatin. The N-termini of the different histone proteins are extensively modified. These modifications are recognized by chromatin readers, which in turn recruit interacting proteins to drive changes in transcription and other chromatin-mediated functions. Amino acid variations between N-termini of canonical H3.1/H3.2 and variant H3.3 and H3t proteins are indicated.

transcriptional activity or transcriptional repression, while presence of both modifications is suggested to represent a “poised” state capable of establishing either permanent activation or silencing following differentiation cues (Bernstein et al., 2006). The number of different modification states on different histone amino acid residues has led to the model of a “histone code,” wherein different combinations of histone modifications provide regulatory information on top of genetic sequence-encoded information to establish the gene regulatory landscape of a particular cell type (Jenuwein and Allis, 2001).

While canonical histone proteins are highly conserved, there exist within the mammalian genome several genes encoding variant histone proteins containing a small number of amino acid substitutions (Zink and Hake, 2016). Many of these histone variants are specifically expressed in the testis of both mouse and man. The amino acid differences specific to these variants are thought to function primarily in two different ways. Some substitutions result in changes in residues subject to posttranslational modification, creating new binding sites for chromatin readers. Other substitutions appear to alter the affinity of these variant histones for DNA. Decreases in affinity for DNA by histone variants can result in increases in DNA sequence accessibility, allowing previously repressed regions to become transcriptionally active. Analysis of mouse mutants of several histone variants revealed that these proteins are essential for several different phases of male germ cell development. A particularly important variant histone of the H3 family, known as H3.3, is expressed in all cell types. Unlike canonical histones, which are only synthesized and loaded onto chromatin during DNA synthesis, H3.3 (in addition to some other variants of different histone families) is produced throughout the cell cycle and its loading into chromatin is also not limited to S phase (Szenker et al., 2011). This variant is expressed at high levels throughout all stages of spermatogenesis, and mutations in H3.3-encoding genes have been found to disrupt male fertility in a variety of ways (Tang et al., 2015; Yuen et al., 2014).

Chromatin Composition and Structure in Premeiotic Spermatogonial Germ Cells

In mammals, germ cells are specified from a specific population of multipotent embryonic cells. These cells possess a chromatin configuration fairly similar to that of somatic cells, a state which is thought to remain largely unchanged throughout embryonic development. At birth a population of cells present in the testis will serve as adult spermatogonial stem cells to produce sperm throughout the life of the animal. These cells will first be induced to differentiate at puberty. As in somatic cells, different classes of chromatin modifying enzymes, catalyzing tri-methylation of histone H3 at lysine 4 (H3K4me3) and of histone H3 at lysine 27 (H3K27me3), are required for proper regulation of transcriptional activity or repression in spermatogonial stem cells and their survival (Glaser et al., 2009; Mu et al., 2014).

Spermatogonial differentiation involves a defined number of cell divisions, which serve to amplify the population of male germ cells that can become mature spermatozoa (see Fig. 2). It has been clear for many years that cellular differentiation of premeiotic spermatogonia in both humans and mice correlates with changes in chromatin configuration. Classical staining techniques to visualize chromatin packaging within the nucleus reveal an increase in the amount of compacted DNA within the nuclei of more differentiated spermatogonia. The quantity of these densely stained regions, known as heterochromatin, also increases during somatic differentiation. Heterochromatin is generally associated with regions of the genome showing low transcriptional activity, including many repetitive sequences. These transcriptionally repressed regions harbor modifications to their histones and DNA important for preventing inappropriate transcription. In male germ cells, loss of transcriptional repressive pathways including those mediating tri-methylation of lysine 9 of histone H3 (H3K9me3) or methylation of cytosines within the DNA lead to upregulation of endogenous retroviruses, some of which possess the capacity to move around the genome, potentially leading to deleterious mutations (Zamudio and Bourc’his, 2010). In order to prevent these elements from activating during spermatogenesis, a special small RNA-mediated pathway, known as the piRNA pathway, has evolved to work in concert with chromatin modifications. Together these pathways serve to safeguard the germline from acquisition of large numbers of mutations due to transposon activity.

Induction of differentiation in spermatogonial stem cells is also associated with expression of a variant form of histone H3 known as H3t. This variant, which is found in mammals, is restricted in its expression to testicular germ cells and carries several

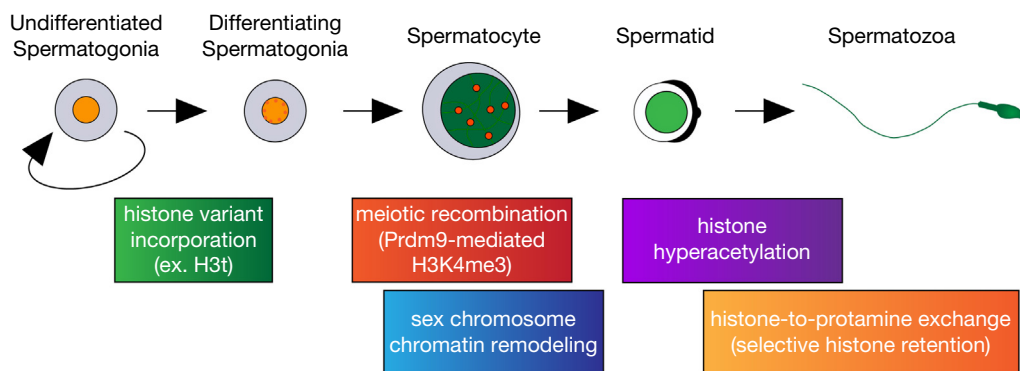


Fig. 2 Major changes in chromatin states occurring during postnatal spermatogenesis. Timing of major events resulting in changes in chromatin structure and state during spermatogenesis are indicated in colored boxes. See text for details of each event.

amino acid substitutions relative to canonical histone H3, some of which are conserved within mammals. Biochemical analysis of the human H3T protein suggest that this variant forms nucleosomes that exhibit decreased affinity for DNA (Tachiwana et al., 2010). In addition human H3T has been found to interact preferentially with certain chromatin associated proteins. Mouse mutants for H3t are viable, but males exhibit infertility (Ueda et al., 2017). However, how the amino acid substitutions and/or regulation of this histone variant result in successful spermatogenesis remains unknown.

Chromatin Composition and Structure in Meiotic Male Germ Cells

Following their mitotic divisions, male germ cells initiate the specialized cell division process known as meiosis. Meiosis serves to randomly distribute homologous chromosomes from different parental origins in haploid descendent cells, called spermatids. This is accomplished by performing DNA replication followed by two subsequent cell divisions. A characteristic of mammalian male meiosis is a protracted period in prophase of the first meiotic division. This extended period occurs following the final DNA replication step in the male germline, meaning that changes to chromatin occurring during meiosis cannot rely upon canonical histone proteins, which normally incorporate during DNA replication. Therefore, increasing quantities of DNA replication independent variant histones, such as H3.3, become deposited into chromatin with time during meiosis.

A key feature of meiosis in all eukaryotes is the process of homologous recombination. During this process programmed double strand breaks in the DNA are formed, which are then repaired using homologous sequences on chromosomes of different parental origin as templates. This process results in the physical linkage of parental homologs together, a process necessary for appropriate homolog segregation during the first meiotic division. The chromatin surrounding meiotic double strand breaks acquires a phosphorylation of an H2A variant known as H2AX. This modification, known as γ H2AX, spreads from the site of the double strand break into the surrounding chromatin serving to recruit essential DNA repair pathway proteins.

Interestingly, the location of the double strand breaks formed during meiosis is not random, but instead occurs preferentially at so-called “hotspots.” These hotspots have been shown to be located at specific DNA sequence motifs that also exhibit important chromatin features. The Prdm9 enzyme, which can catalyze H3K4me3 and H3K36me3, has been found to recognize the sequences of these hotspots and both H3K4me3 and H3K36me3 levels have been found to be increased in such sequences (Baudat et al., 2013). These histone modifications are normally associated with transcription start sites of active genes and gene bodies of active genes respectively. This therefore represents a potential germline specific alteration of the transcription associated histone code found in somatic cells. Whether other histone modifications are also associated with hotspots and what the exact role that such histone modifications play during the process of recombination is still unclear.

During prophase, following DNA double strand break formation autosomes undergo the process of synapsis in which homologous chromosomes are linked together by structural meiotic proteins. Failure of synapsis of autosomal chromosome regions leads to a general phenomenon known as meiotic silencing of unsynapsed chromatin (MSUC), in which genes located in these regions become transcriptionally silenced (Turner, 2015). Such transcriptional silencing is associated with wholesale changes in chromatin composition and modification states, and if not resolved is frequently associated with cell death and male infertility. One feature of these unsynapsed chromosomal regions is the maintenance of γ H2AX on their chromatin, well after other synapsed regions have de-phosphorylated this site following repair of double strand breaks.

One interesting feature of mammalian recombination occurs on the sex chromosomes. Because the X and Y chromosomes in mammals exhibit significant sequence divergence, they lack an effective homolog with which to pair and to be used as a template for DNA repair. This results in their global transcriptional silencing during prophase. To compensate for loss of expression of genes located on the X chromosome, additional paralogous gene copies have evolved on autosomal chromosomes. Similar to the process of MSUC, the transcriptionally silent sex chromosomes display enrichment for γ H2AX throughout much of meiotic prophase (Turner, 2015). In addition, they also undergo a global histone replacement process in which canonical nucleosomes become depleted and replaced with nucleosomes containing H3.3 (van der Heijden et al., 2007). Following this global transition, the new H3.3 containing nucleosomes become specifically enriched for a histone modifications associated with transcriptional silencing. What role the global replacement of nucleosomes on transcriptionally silenced sex chromosomes plays in transcriptional regulation of these cells during and after meiosis remains unknown.

At endogenous retroviral sequences, DNA methylation and di-methylation of H3K9 (H3K9me2) protect against transcriptional activation and acquisition of a chromatin state permissive for meiotic double strand break formation and repair. At pericentromeric heterochromatin regions, H3K9me2 and H3K9me3 as well as associated heterochromatin proteins prevent the underlying major satellite sequences, localized on autosomes and the X chromosome, from becoming engaged in illegitimate interactions between nonhomologous chromosomes, thereby ensuring synapsis of such sequences only at homologous chromosomes and safeguarding male fertility (Peters et al., 2001).

Chromatin Composition and Structure in Postmeiotic Male Germ Cells

Postmeiotic male germ cells, called spermatids, contain a chromatin consisting of a large quantity of variant histone proteins, inherited from their meiotic predecessors. Immediately following meiosis, these cells remain in a relatively standard, transcriptionally active chromatin state. The sex chromosomes in these cells also remain largely in a configuration similar to that of

spermatocytes, enriched globally for histone modifications associated with transcriptional repression. A small number of sex-linked genes, however, become expressed following meiosis and are thought to play important roles in completion of spermatogenesis. Many of these genes are present in multiple copy arrays on the sex chromosomes (Namekawa et al., 2006). How these genes become transcriptionally activated in spermatids and whether their multicopy nature is important for this activation is not clear.

After a relatively extended initial phase, spermatids initiate final changes to their chromatin that enable their function as mature spermatozoa. One of the first dramatic changes to chromatin to occur during spermatid differentiation is a global increase in the level of histone lysine acetylation. Acetylation of lysine residues on histones neutralizes positive charges found in the histones, decreasing their affinity for negatively charged DNA. This process in male germ cells may play three different roles. First, histone acetylation may increase the accessibility of DNA on a global scale. Second, histone acetylation may serve to recruit chromatin reader proteins required for further differentiation of spermatids. Indeed mutation of a testis specific histone acetylation reader protein (Brdt) has been shown to disrupt spermatogenesis (Berkowits and Wolgemuth, 2013). Finally, acetylated histones are targets for a spermatogenic-specific proteasome complex, which can lead to the degradation of histone proteins during spermatid differentiation (Qian et al., 2013). This change in histone lysine acetylation again reflects potential differences in the germline histone code relative to that of somatic cells. In somatic cells increased histone lysine acetylation is associated with increased transcriptional activity, while in spermatids the timing of the increase in acetylation is associated with a global downregulation of transcription. How this downregulation in the presence of high levels of histone lysine acetylation is controlled at the chromatin level is still unknown.

Following the global acetylation of histones in spermatids, a uniquely spermatogenic alteration of chromatin occurs. Histone proteins are removed from chromatin and replaced with other small spermatogenesis-specific basic proteins. While exact sequences for these proteins show divergence throughout metazoans, diverse species of animals use a similar strategy to produce their spermatozoa. In mammals, initially a class of proteins known as transition proteins become incorporated into chromatin, and are then themselves evicted to be replaced by another class of proteins, known as protamines. This dramatic alteration in chromatin composition results in a greatly reduced nuclear volume. It is hypothesized that this decrease in nuclear volume results in an increased capacity for spermatozoa to swim through the female reproductive tract. Further, protamine-packaged DNA has been shown to be more resistant to DNA damaging agents than histone-packaged DNA, suggesting that histone to protamine exchange may also serve a function in protecting the genetic material during its transit to the oocyte.

As previously mentioned, meiotic, and early postmeiotic differentiation during spermatogenesis involves the incorporation of increasing quantities of variant histone proteins. These variants are produced in a DNA replication-independent manner. Interestingly, mutations in several of these variants suggest that possessing variant histone-containing chromatin is essential for the efficient transition from nucleosomal chromatin to protamine-containing chromatin. Mutations in the previously mentioned H3 variant H3.3 has been shown to result in a block in differentiation in early postmeiotic germ cells, just at the onset of histone eviction (Tang et al., 2015). Likewise, a variant of H2B (TH2B, which is expressed exclusively in male and female germ cells) is also required to enable proper nuclear condensation and protamine incorporation in spermatids (Montellier et al., 2013). An H2A variant, H2A.L.2, has also been shown to load onto chromatin prior to transition proteins (Barral et al., 2017). This loading was found to be essential for subsequent transition protein loading onto chromatin. These results suggest that rather than a two-step transition from histone to transition protein to protamine, various substeps involving variant histone incorporation prior to and in parallel with transition protein incorporation may occur in this process. The specific molecular details of the roles of these variants in the removal and subsequent replacement of histones by protamines are a subject of active investigation by many researchers.

Mutations in the protamine genes, in both mouse and human, are associated with male infertility. Indeed in mice mutations in protamine genes have been shown to be haplo-insufficient for male fertility (Cho et al., 2001). Additionally, germ cells from some human patients exhibiting spermatogenic failure are enriched for histone proteins relative to normal functional spermatozoa. Testing of human samples in infertility clinics now routinely involves use of DNA intercalating dyes, which query spermatozoa as to their nuclear compaction. Decreased nuclear compaction, a proxy for increased histone retention, indicates a decreased functional capacity for this material for in vitro fertilization. Whether retention of histone proteins is actually causative for human spermatogenic failure and infertility, however, remains unknown.

Chromatin Composition and Function of Mature Spermatozoa

Early studies of mature spermatozoa from a variety of species suggested the possibility that some histone proteins might be retained in mature sperm. More recent data analyzing several different species suggest indeed that histone to protamine exchange is not fully complete in mammals (Brykczynska et al., 2010). A small percentage of nucleosomes, the number of which appears to be species specific, are retained within the chromatin of mature spermatozoa. These nucleosomes were shown to carry posttranslational modifications found normally within active and repressed gene promoters. Identification of the sequences to which these nucleosomes bind showed an enrichment for regulatory regions of “housekeeping” genes and of genes driving developmental fate processes. These regulatory regions contain high levels of the dinucleotide CpG, in its non-DNA methylated state (Erkek et al., 2013). Between species, the fraction of retained nucleosomes in CpG-rich sequences is inversely correlated with the percentage of total retained nucleosomes in spermatozoa. Species such as mouse, which retain very few nucleosomes, show prominent retention of nucleosomes in CpG-rich regions, while humans, which retain more nucleosomes in spermatozoa, harbor nucleosomes in both CpG-rich regions and other genomic loci. It remains unclear whether the presence of nucleosomes and their associated modifications

in mature spermatozoa play a regulatory role in the establishment of transcription or in the organization of the paternal genome in subsequent generations. Protamine proteins are rapidly removed from paternal DNA after fertilization, but it is not clear whether this is also the fate of sperm-derived histone proteins.

Several experiments have suggested that increased nucleosome content on paternally inherited chromosomes may impair development of embryos. Genetic and chemical approaches that effect the level of a particular chemical modification (known as poly(ADP)-ribosylation) found on many proteins (including histones) were shown to increase nucleosome retention in mouse spermatozoa (Ihara et al., 2014). Embryos fathered by such sperm were then found to develop less successfully than those fathered by unaltered sperm. Whether this failure in development is directly induced by nucleosome retention, and not by some other effect of the method, however, has not been definitely shown.

Nucleosome-containing paternal chromosomes are also present in embryos produced by the assisted reproduction technique known as ROSI (round spermatid injection). This technique, used experimentally in animal models, is also occasionally utilized in human assisted reproductive clinics when a male patient lacks differentiated condensed haploid cells in his testes. Round spermatids from a testis biopsy are then isolated and injected into oocytes in vitro that, following oocyte activation, can develop into embryos. While this technique can give rise to full-term offspring in humans, it is still considered an experimental procedure and is not standard in most infertility clinics. This is because the success rate for ROSI is substantially lower relative to techniques using more differentiated male germ cell types (such as ICSI—intracytoplasmic sperm injection). Whether the decreased rate of success for ROSI relative to these other techniques is caused by the presence of high levels of histone containing chromatin or by some other unknown differences between spermatid and spermatozoa affecting gene regulation, genome stability and/or embryonic fitness remains to be resolved.

References

- Barral, S., Morozumi, Y., Tanaka, H., et al. (2017). Histone variant H2A.L.2 guides transition protein-dependent protamine assembly in male germ cells. *Molecular Cell*, 66(1), 89–101.
- Baudat, F., Imai, Y., & de Massy, B. (2013). Meiotic recombination in mammals: Localization and regulation. *Nature Reviews. Genetics*, 14(11), 794–806.
- Berkowitz, B. D., & Wolgemuth, D. J. (2013). The role of the double bromodomain-containing BET genes during mammalian spermatogenesis. *Current Topics in Developmental Biology*, 102, 293–326.
- Bernstein, B. E., Mikkelsen, T. S., Xie, X., et al. (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell*, 125(2), 315–326.
- Brykczynska, U., Hisano, M., Erkek, S., et al. (2010). Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nature Structural & Molecular Biology*, 17(6), 679–687.
- Cho, C., Willis, W. D., Goulding, E. H., et al. (2001). Haploinsufficiency of protamine-1 or -2 causes infertility in mice. *Nature Genetics*, 28(1), 82–86.
- Cutter, A. R., & Hayes, J. J. (2015). A brief review of nucleosome structure. *FEBS Letters*, 589, 2914–2922.
- Erkek, S., Hisano, M., Liang, C. Y., et al. (2013). Molecular determinants of nucleosome retention at CpG-rich sequences in mouse spermatozoa. *Nature Structural & Molecular Biology*, 20(7), 868–875.
- Glaser, S., Lubitz, S., Loveland, K. L., et al. (2009). The histone 3 lysine 4 methyltransferase, Mll2, is only required briefly in development and spermatogenesis. *Epigenetics & Chromatin*, 2(1), 5. <https://doi.org/10.1186/1756-8935-2-5>.
- van der Heijden, G. W., Derijck, A. A., Posfai, E., et al. (2007). Chromosome-wide nucleosome replacement and H3.3 incorporation during mammalian meiotic sex chromosome inactivation. *Nature Genetics*, 39(2), 251–258.
- Ihara, M., Meyer-Ficca, M. L., Leu, N. A., et al. (2014). Paternal poly (ADP-ribose) metabolism modulates retention of inheritable sperm histones and early embryonic gene expression. *PLoS Genetics*, 10(5), e1004317.
- Jenuwein, T., & Allis, C. D. (2001). Translating the histone code. *Science*, 293(5532), 1074–1080.
- Montellier, E., Boussouar, F., Rousseau, S., et al. (2013). Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes & Development*, 27(15), 1680–1692.
- Mu, W., Starmer, J., Fedoriv, A. M., Yee, D., & Magnuson, T. (2014). Repression of the soma-specific transcriptome by Polycomb-repressive complex 2 promotes male germ cell development. *Genes & Development*, 28(18), 2056–2069.
- Namekawa, S. H., Park, P. J., Zhang, L. F., et al. (2006). Postmeiotic sex chromatin in the male germline of mice. *Current Biology*, 16(7), 660–667.
- Peters, A. H., O'Carroll, D., Scherthan, H., et al. (2001). Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell*, 107(3), 323–337.
- Qian, M. X., Pang, Y., Liu, C. H., et al. (2013). Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. *Cell*, 153(5), 1012–1024.
- Szenker, E., Ray-Gallet, D., & Almouzni, G. (2011). The double face of the histone variant H3.3. *Cell Research*, 21(3), 421–434.
- Tachiwana, H., Kagawa, W., Osakabe, A., et al. (2010). Structural basis of instability of the nucleosome containing a testis-specific histone variant, human H3T. *Proceedings of the National Academy of Sciences of the United States of America*, 107(23), 10454–10459.
- Tang, M. C., Jacobs, S. A., Mattiske, D. M., et al. (2015). Contribution of the two genes encoding histone variant h3.3 to viability and fertility in mice. *PLoS Genetics*, 11(2), e1004964.
- Turner, J. M. (2015). Meiotic silencing in mammals. *Annual Review of Genetics*, 49, 395–412.
- Ueda, J., Harada, A., & Urahama, T. (2017). Testis-specific histone variant H3t gene is essential for entry into spermatogenesis. *Cell Reports*, 18(3), 593–600.
- Yuen, B. T., Bush, K. M., Barrilleaux, B. L., et al. (2014). Histone H3.3 regulates dynamic chromatin states during spermatogenesis. *Development*, 141(18), 3483–3494.
- Zamudio, N., & Bouc'his, D. (2010). Transposable elements in the mammalian germline: A comfortable niche or a deadly trap? *Heredity*, 105, 92–104.
- Zink, L. M., & Hake, S. B. (2016). Histone variants: Nuclear function and disease. *Current Opinion in Genetics & Development*, 37, 82–89.

Regulation of Spermatogenesis by Noncoding RNAs

Wei Yan, University of Nevada, Reno School of Medicine, Reno, NV, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Spermatogenesis refers to the process through which the male gamete, or sperm, are produced. Spermatogenesis occurs inside the seminiferous tubules of the testis, and involves spermatogonial proliferation (mitotic phase) followed by meiotic division of spermatocytes (meiotic phase) and differentiation of round spermatids into elongating, elongated and eventually spermatozoa (haploid phase, also called spermiogenesis) (Hermo et al., 2010). It takes several weeks for a spermatogonial stem cell to go through all three phases and ultimately differentiate into numerous spermatozoa (Hermo et al., 2010). The fully developed testicular spermatozoa are then released from the seminiferous epithelia through a process called spermiation, and the released testicular spermatozoa are further transported to rete testis and then transit through the male reproductive tract for further maturation (Sofikitis et al., 2008). Given such a complex biological process, spermatogenesis requires precise regulation of spatiotemporal expression of numerous genes, which occur at both transcriptional and post-transcriptional levels (Eddy, 1998). Genetic networks that control spermatogenesis have been partially dissected over the past two decades, and thousands of protein-coding genes have been identified to be essential for each of the three phases of spermatogenesis (Matzuk and Lamb, 2008). In addition to protein-coding genes, non-protein coding genes, which are transcribed as noncoding RNAs (ncRNAs), have also been found to play critical roles in the regulation of spermatogenesis (Holt et al., 2016). In addition to long noncoding RNAs (> 200 nt, lncRNAs) (Luk et al., 2014), a large number of small ncRNAs (sncRNAs) have been identified to be abundantly expressed in the testis, including microRNAs (miRNAs) (Ro et al., 2007), piwi-interacting RNAs (piRNAs) (Bortvin, 2013), endogenous small interference RNAs (endo-siRNAs) (Song et al., 2011), mitochondrial genome-encoded small RNAs (mitosRNAs) (Ro et al., 2013), tRNA-derived small RNAs (tsRNAs) (Peng et al., 2012), small nucleolar RNAs (snoRNAs) (Ortoger et al., 2013), etc. These sncRNAs were identified through the next-gen deep sequencing technologies. In general, these sncRNAs act jointly to fine-tune the expression of protein-coding genes at both transcriptional and post-transcriptional levels. Except for miRNAs and piRNAs, the biogenesis and physiological roles of other sncRNAs are largely unknown. Therefore, the discussion below is confined to the roles of lncRNAs, miRNAs, endo-siRNAs, and piRNAs in the regulation of spermatogenesis.

Roles of Testicular ncRNAs in Spermatogenesis

lncRNAs. Using either microarray and deep sequencing technologies, thousands of lncRNAs have been identified in both spermatogenic cells and testicular somatic cell types (Sun et al., 2013; Bao et al., 2013). lncRNAs can be divided into five subtypes based on their proximity to mRNA genes, including sense, antisense, bidirectional, intronic, and intergenic. The majority of lncRNAs are nuclear and low in abundance. lncRNAs have been shown to play critical roles in chromatin remodeling by recruiting chromatin-remodeling complexes to specific loci, which, in turn, mediate epigenetic modifications, for example, XIST, HOTAIR and H19 (Mercer and Mattick, 2013). Some lncRNAs, including promoter-associated RNAs (PARs), enhancer RNAs (eRNAs), and transcripts from ultra-conserved regions (T-UCRs), have also been found to regulate transcription through interacting with transcription factors (Mercer and Mattick, 2013; Lee, 2012). However, these putative functions were mostly established based on in vitro experiments and functions of individual lncRNAs in spermatogenesis remains to be demonstrated.

miRNAs. miRNAs were the first sncRNA species identified to be highly expressed in the testis (Ro et al., 2007). miRNAs are 22 nt in length and miRNA biogenesis starts with transcription of miRNA genes by the same transcriptional machinery used for mRNA synthesis. The miRNA-containing region of the primary miRNAs possesses unique sequences which can fold into a stem-loop/hairpin structure (Voinnet, 2009). DROSHA, a nuclear RNase III enzyme, recognizes the stem-loop and cleaves it out. The hairpin-shaped precursor miRNAs are then shipped out of the nucleus and reach the cytoplasm by exportin 5, where a cytoplasmic RNase III called Dicer dices out the two mature miRNAs from the stem region of the hairpin (Voinnet, 2009). Mature miRNAs then bind RNA-induced silencing complex (RISC) and lead the RISC to their target sites, 3'UTRs of protein coding genes. miRNAs bind to the sites in the 3'UTRs by complementary annealing using the "seed sequence" (the 2nd–7th nt) (Friedman et al., 2009). The net effects of miRNA-RISC binding to the 3'UTRs of protein coding mRNAs include translational inhibition, and stabilization/destabilization of mRNAs depending on the proximity of their binding sites to the stop codon within the 3'UTRs (Voinnet, 2009; Zhang et al., 2017). Hundreds of miRNAs are expressed in the testis and many display cell specificity; for example, many X-linked miRNAs are exclusively expressed in spermatogenic cells (Ro et al., 2007; Song et al., 2009). Ablation of *Drosha* and *Dicer* in either Sertoli cells or spermatogenic cells leads to male infertility due to severely disrupted spermatogenesis, highlighting the necessity of miRNA production in spermatogenesis (Wu et al., 2012; Papaioannou et al., 2009). However, knockout of single miRNAs seldom generates significant disruptions in spermatogenesis. This is most likely due to the fact that one miRNA can target multiple mRNAs and the 3'UTR of a particular mRNA tends to harbor binding sites for multiple miRNAs. This "one-to-multi" relationship constitutes interwoven networks among miRNAs and their targets. Thus, lack of one miRNA may not be able to generate significant effects sufficient to disrupt the function of their target mRNAs. When miRNAs that belong to the same family (i.e., share the same seed sequence) or target genes of the same pathways are inactivated simultaneously, phenotypes in particular developmental or physiological processes often show up, suggesting that miRNAs tend to function in a redundant manner (Wu et al., 2014). This phenomenon

may represent a “fail-safe” mechanism, and it is also consistent with the fine-tuning role of miRNAs in the post-transcriptional regulation of gene expression.

endo-siRNAs. Chemically synthesized small interference RNAs (siRNAs) have been used to knock down mRNAs for over two decades. But endogenous siRNAs were not believed to exist because their production requires double-stranded RNAs (dsRNAs) as substrates, which, in theory, are non-existent in healthy cells due to interferon/OAS/RNase L-mediated interferon response (Lamontagne et al., 2001). However, endo-siRNAs were first discovered in developing oocytes and early embryos, and later in spermatogenic cells (Svoboda and Flemr, 2010). Endo-siRNAs are 22 nt sncRNAs processed from endogenous dsRNAs by cytoplasmic DICER. The endogenous dsRNAs are likely transiently formed by regional annealing between the same or different transcripts (Sontheimer and Carthew, 2005). Dicer recognizes dsRNAs and cleaves them into siRNAs, which can induce rapid and efficient degradation of their host and target transcripts. Comparative studies on the phenotypic differences in *Dicer* and *Drosha* male germ cell-specific knockout mice have demonstrated that endo-siRNAs is indeed critical for normal spermatogenesis (Wu et al., 2012). However, the precise function of individual endo-siRNAs in specific time points and spermatogenic cell types during spermatogenesis remain unknown. Given the potency of siRNA-mediated mRNA decay, these endo-siRNAs are likely act when rapid degradation of certain transcripts becomes necessary during spermatogenesis.

piRNAs. piRNAs were first identified as 24–30 nt sncRNAs that bind PIWI family proteins (Luteijn and Ketting, 2013). In mice, two types of piRNAs are expressed in the testis: one expressed predominantly in spermatocytes and spermatids in adult testes, and the other abundant only in primordial germ cells (PGCs) and pro-spermatogonia in fetal testes (Bortvin, 2013). Fetal testicular piRNAs are mainly processed by the piRNA processing machinery involving MIWI2, MILI, Mael, MitoPLD and other unknown factors using the transcripts from repetitive elements as substrates (Bortvin, 2013). The massive transcriptional activation of retrotransposons, for example, LINE1, is caused by the global reprogramming events, characterized by rapid genome-wide DNA demethylation and re-methylation in PGCs between E13.5 and E15.5 in mice. By producing repetitive sequence-derived piRNAs, transcripts synthesized from the repetitive sequences including retrotransposons, can be degraded through the RNAi pathway; more importantly, these piRNAs are required for re-methylation of repetitive sequences during the establishment of the germline-specific epigenome (Bortvin, 2013). Therefore, these piRNAs appear to be able to lead to methylation of repetitive sequences from which they are derived. However, it remains unknown how piRNAs achieve this at molecular levels. The adult testicular piRNAs are abundantly expressed in meiotic and haploid male germ cells, especially in pachytene spermatocytes and round spermatids. Recent data suggest that these piRNAs are mainly transcribed from several large clusters in the genome and appear to be able to induce degradation of many transcripts expressed in haploid male germ cells, that is, spermatids (Gou et al., 2014, 2015). Given that quick turnover is critical for spermiogenesis, these piRNAs have been assigned a role in inducing massive degradation of haploid transcripts. However, given that the quick turnover of haploid transcripts occurs in an orderly manner at precise time points, how these piRNAs select specific transcripts and the identify of these piRNA targets need to be elucidated in future studies.

References

- Bao, J., Wu, J., Schuster, A. S., Hennig, G. W., & Yan, W. (2013). Expression profiling reveals developmentally regulated lncRNA repertoire in the mouse male germline. *Biology of Reproduction*, 89(5), 107.
- Bortvin, A. (2013). PIWI-interacting RNAs (piRNAs)—A mouse testis perspective. *Biochemistry (Moscow)*, 78(6), 592–602.
- Eddy, E. M. (1998). Regulation of gene expression during spermatogenesis. *Seminars in Cell & Developmental Biology*, 9(4), 451–457.
- Friedman, R. C., Farh, K. K., Burge, C. B., & Bartel, D. P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Research*, 19(1), 92–105.
- Gou, L. T., et al. (2014). Pachytene piRNAs instruct massive mRNA elimination during late spermiogenesis. *Cell Research*, 24(6), 680–700.
- Gou, L. T., et al. (2015). Pachytene piRNAs instruct massive mRNA elimination during late spermiogenesis. *Cell Research*, 25(2), 266.
- Hermo, L., Pelletier, R. M., Cyr, D. G., & Smith, C. E. (2010). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: Background to spermatogenesis, spermatogonia, and spermatocytes. *Microscopy Research and Technique*, 73(4), 241–278.
- Holt, J. E., Stanger, S. J., Nixon, B., & McLaughlin, E. A. (2016). Non-coding RNA in spermatogenesis and epididymal maturation. *Advances in Experimental Medicine and Biology*, 886, 95–120.
- Lamontagne, B., Larose, S., Boulanger, J., & Elela, S. A. (2001). The RNase III family: A conserved structure and expanding functions in eukaryotic dsRNA metabolism. *Current Issues in Molecular Biology*, 3(4), 71–78.
- Lee, J. T. (2012). Epigenetic regulation by long noncoding RNAs. *Science*, 338(6113), 1435–1439.
- Luk, A. C., Chan, W. Y., Rennert, O. M., & Lee, T. L. (2014). Long noncoding RNAs in spermatogenesis: Insights from recent high-throughput transcriptome studies. *Reproduction*, 147(5), R131–141.
- Luteijn, M. J., & Ketting, R. F. (2013). PIWI-interacting RNAs: From generation to transgenerational epigenetics. *Nature Reviews. Genetics*, 14(8), 523–534.
- Matzuk, M. M., & Lamb, D. J. (2008). The biology of infertility: Research advances and clinical challenges. *Nature Medicine*, 14(11), 1197–1213.
- Mercer, T. R., & Mattick, J. S. (2013). Structure and function of long noncoding RNAs in epigenetic regulation. *Nature Structural & Molecular Biology*, 20(3), 300–307.
- Ortogero, N., et al. (2013). Computer-assisted annotation of murine Sertoli cell small RNA transcriptome. *Biology of Reproduction*, 88(1), 3.
- Papaioannou, M. D., et al. (2009). Sertoli cell Dicer is essential for spermatogenesis in mice. *Developmental Biology*, 326(1), 250–259.
- Peng, H., et al. (2012). A novel class of tRNA-derived small RNAs extremely enriched in mature mouse sperm. *Cell Research*, 22(11), 1609–1612.
- Ro, S., Park, C., Sanders, K. M., McCarrey, J. R., & Yan, W. (2007). Cloning and expression profiling of testis-expressed microRNAs. *Developmental Biology*, 311(2), 592–602.
- Ro, S., et al. (2013). The mitochondrial genome encodes abundant small noncoding RNAs. *Cell Research*, 23(6), 759–774.
- Sofikitis, N., et al. (2008). Hormonal regulation of spermatogenesis and spermiogenesis. *The Journal of Steroid Biochemistry and Molecular Biology*, 109(3–5), 323–330.
- Song, R., et al. (2009). Many X-linked microRNAs escape meiotic sex chromosome inactivation. *Nature Genetics*, 41(4), 488–493.
- Song, R., et al. (2011). Male germ cells express abundant endogenous siRNAs. *Proceedings of the National Academy of Sciences of the United States of America*, 108(32), 13159–13164.
- Sontheimer, E. J., & Carthew, R. W. (2005). Silence from within: Endogenous siRNAs and miRNAs. *Cell*, 122(1), 9–12.
- Sun, J., Lin, Y., & Wu, J. (2013). Long non-coding RNA expression profiling of mouse testis during postnatal development. *PLoS One*, 8(10), e75750.

- Svoboda, P., & Flemr, M. (2010). The role of miRNAs and endogenous siRNAs in maternal-to-zygotic reprogramming and the establishment of pluripotency. *EMBO Reports*, 11(8), 590–597.
- Voinnet, O. (2009). Origin, biogenesis, and activity of plant microRNAs. *Cell*, 136(4), 669–687.
- Wu, Q., et al. (2012). The RNase III enzyme DROSHA is essential for microRNA production and spermatogenesis. *The Journal of Biological Chemistry*, 287(30), 25173–25190.
- Wu, J., et al. (2014). Two miRNA clusters, miR-34b/c and miR-449, are essential for normal brain development, motile ciliogenesis, and spermatogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 111(28), E2851–2857.
- Zhang, Y., et al. (2017). MicroRNAs control mRNA fate by compartmentalization based on 3' UTR length in male germ cells. *Genome Biology*, 18(1), 105.

Epigenetic Programming of Paternally Imprinted Genes During Spermatogenesis

Stella K Hur and Marisa S Bartolomei, University of Pennsylvania, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Mammals are diploid, having a set of chromosomes derived from their mothers through oocytes and a set of chromosomes from their fathers through sperm. Most genes in the genome are expressed or repressed from both parental chromosomes (i.e., alleles). However, a small number of genes, termed imprinted genes, deviate from this norm. Genes that undergo the process of genomic imprinting are expressed from a single parental allele, either the maternal or the paternal allele. Genomic imprinting, including the genes that are imprinted and defined regulatory mechanisms, is well-conserved among mammals. The significance of genomic imprinting in mammalian development was formally demonstrated by nuclear transplantation experiments in the 1980s. The experiments revealed that uniparental embryos are not viable—diploid androgenetic embryos derived from two male pronuclei or diploid gynogenetic embryos derived from two female pronuclei failed to develop (McGrath and Solter, 1984; Surani et al., 1984). These results suggested that developmental failure must be due to absence or overexpression of genes that are expressed solely from one of the parental genomes, and thus normal development requires both a maternal and a paternal pronuclear contribution to the zygote. This was the first suggestion that the mammalian genome contained imprinted genes.

Approximately 200 imprinted genes have been identified in mouse, the mammal in which most experimentation has been conducted. Notably, most of the lessons learned about imprinting and imprinted genes are conserved in humans as well as other mammals that have been examined. Imprinted genes are typically located in clusters of 3–12 genes that are spread over a few Mb of DNA, although examples of single imprinted genes have been identified (Barlow and Bartolomei, 2014). The clusters harbor maternally- and paternally-expressed imprinted genes that encode both protein-coding and long noncoding (lnc) RNAs. Each well-studied cluster has a discrete imprinting control region (ICR) that governs imprinted expression and exhibits parent-of-origin-specific epigenetic marks such as DNA methylation and posttranslational histone modifications that are present throughout life (Fig. 1). For an element to be called an ICR, a functional role must be demonstrated. This is typically shown by deleting the ICR which then results in the loss of imprinting of genes in the cluster. Thus, while there are other sequences in and around imprinted genes that contain parental-specific epigenetic modifications, they cannot be claimed as ICRs unless they fulfill the criteria of (1) harboring parental-specific epigenetic marks acquired in the germline, (2) maintaining the marks throughout life, and (3) functioning to maintain imprinted gene expression. In addition to conferring parental-origin of the allele, sequences that constitute the ICRs have other roles, two of which will be briefly described here (Fig. 2). First, for many clusters including the *Igf2r/Airn*, *Cdkn1c/Kcnq1ot1*, and *Snrpn* clusters, ICRs contain the promoter for lncRNAs, which are over 100 kb in length. When transcribed, these parental allele-specific lncRNAs repress the expression of linked protein-coding genes, either by attracting repressive chromatin or transcriptionally interfering at the promoters (Latos et al., 2012). The second function of ICRs is that of an allele-specific CCCTC-binding factor (CTCF)-binding enhancer blocker. This function has been largely elucidated in studies of the *H19/Igf2* imprinted locus (Bartolomei, 2009). At this locus, *H19* and *Igf2* share enhancers and depend on the ICR to regulate which gene accesses these enhancers. On the maternal allele, the unmethylated ICR binds CTCF and blocks access of *Igf2* to enhancers, enabling *H19*

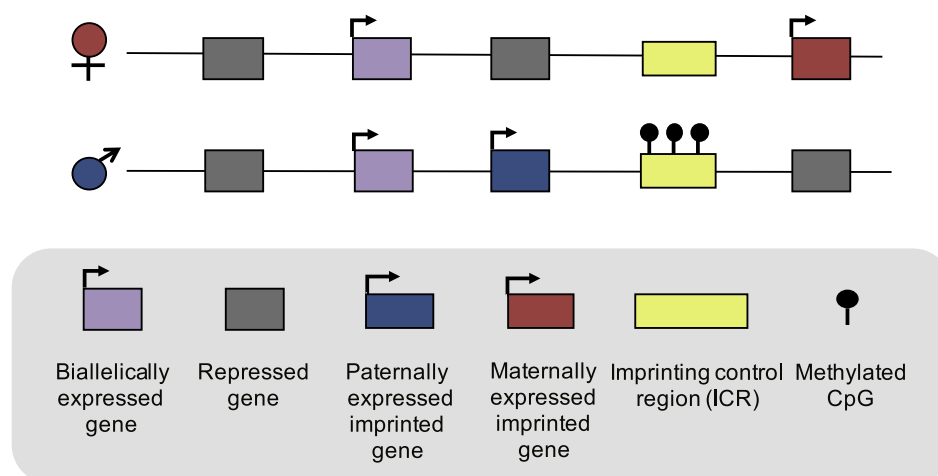


Fig. 1 Majority of the genes in the mammalian genome are expressed/repressed from both parental alleles (purple/gray boxes, respectively). However, imprinted genes (red and blue boxes) are expressed exclusively from either the maternal allele (♀) or the paternal allele (♂). Imprinted genes typically exist in clusters, and a *cis*-acting regulatory element called the imprinting control region (ICR, yellow box) coordinately regulates the expression of the imprinted genes within a cluster. One hallmark characteristic of ICRs is differential modification on each parental allele by various epigenetic modifications, such as DNA methylation at CpG dinucleotides (filled lollipops).

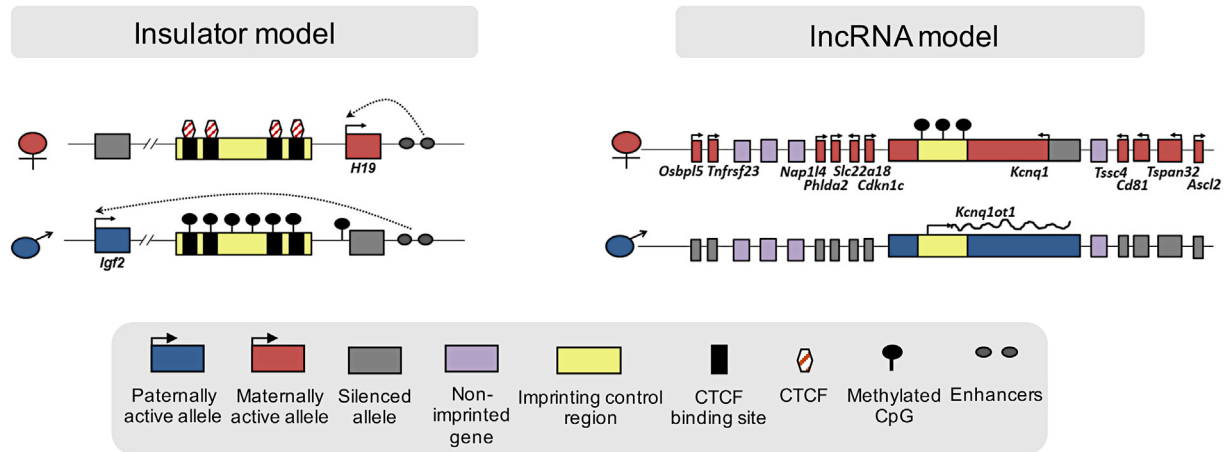


Fig. 2 Two main models for imprinted gene expression have been described (see text for details). In the insulator model, insulator proteins such as CTCF bind to ICR in a DNA-methylation sensitive manner, so an allele-specific insulation is achieved. In the lncRNA model, a lncRNA is transcribed from the unmethylated ICR, and silences genes *in cis* by forming a sense–antisense overlap or by affecting recruitment of transcriptional or epigenetic regulators. As a consequence, on the allele where ICR is methylated and the lncRNA is not expressed, the imprinted genes within the domain are expressed.

expression. In contrast, the ICR and *H19* are methylated on the paternal allele, which blocks CTCF binding at the ICR and enables *Igf2* to engage the shared enhancers.

As mentioned above, the parental-specific epigenetic marks at the ICRs (also referred to as “imprints”) are essential for the appropriate parent-of-origin expression of the linked imprinted genes. Therefore, proper regulation of the ICR is the key to genomic imprinting. Remarkably, ICRs (that are only a few kb in length and bare no obvious distinguishing sequence feature) are specifically modified in the germline when the two parental genomes are separated and can be distinguished. Moreover, ICRs escape the genome-wide epigenetic reprogramming that occurs after fertilization. Such escape is essential for imprinted genes to maintain their parental identity throughout the life of the organism. The focus of this article will be to describe the reprogramming that occurs in the male germline that results in parental-allele-specific marking of ICRs. Although most ICRs are marked in the maternal germline by DNA methylation during oocyte growth, there are a few well studied examples of ICRs that are epigenetically marked in the male germline.

Lifecycle of Epigenetic Programming

Imprinted genes undergo a cycle of erasure and establishment of epigenetic marks during development (Fig. 3). In mammals, the germline is recruited from somatic cells based on location in the postimplantation embryo. These somatic cells start forming the germline by repressing the somatic expression pattern and activating the germline expression pattern (Tang et al., 2016). These cells, which are destined to be reprogrammed and differentiated into mature gametes, have both maternal and paternal imprints inherited from the egg and sperm at fertilization, respectively. Following the initial specification of these so called primordial germ cells (PGCs), the cells divide and migrate along a well-defined pathway, which is accompanied by largescale erasure of the somatic epigenetic signatures of most of the genome. At this time, imprints are also erased, through both active and passive mechanisms (see below). Subsequently, reacquisition of sex-specific imprints at ICRs occurs in the germline to reflect the parental origin of the gamete as described below. ICRs are fully marked with their parental origin in the mature gametes and largely maintain this epigenetic marking despite the global epigenetic reprogramming that occurs after fertilization in the preimplantation embryo.

DNA Methylation as the Epigenetic Mark

The first identified imprinted genes in 1991 enabled investigators to assess how parental alleles were marked so that appropriate allelic expression patterns were achieved during development. The first and most easily testable candidate was DNA methylation, a modification that covalently adds a methyl group to the 5th carbon position in the cytosine residue predominantly in a CpG dinucleotide context. This modification is typically associated with gene repression either through interactions with repressive chromatin machinery or by blocking DNA access of factors that bind directly to the DNA sequence. DNA methylation is established through the action of de novo methyltransferases (DNMT3A and DNMT3B) together with their partner DNMT3L. DNA methylation is maintained during each cell division *in situ* by the action of the maintenance methyltransferase DNMT1 (which recognizes

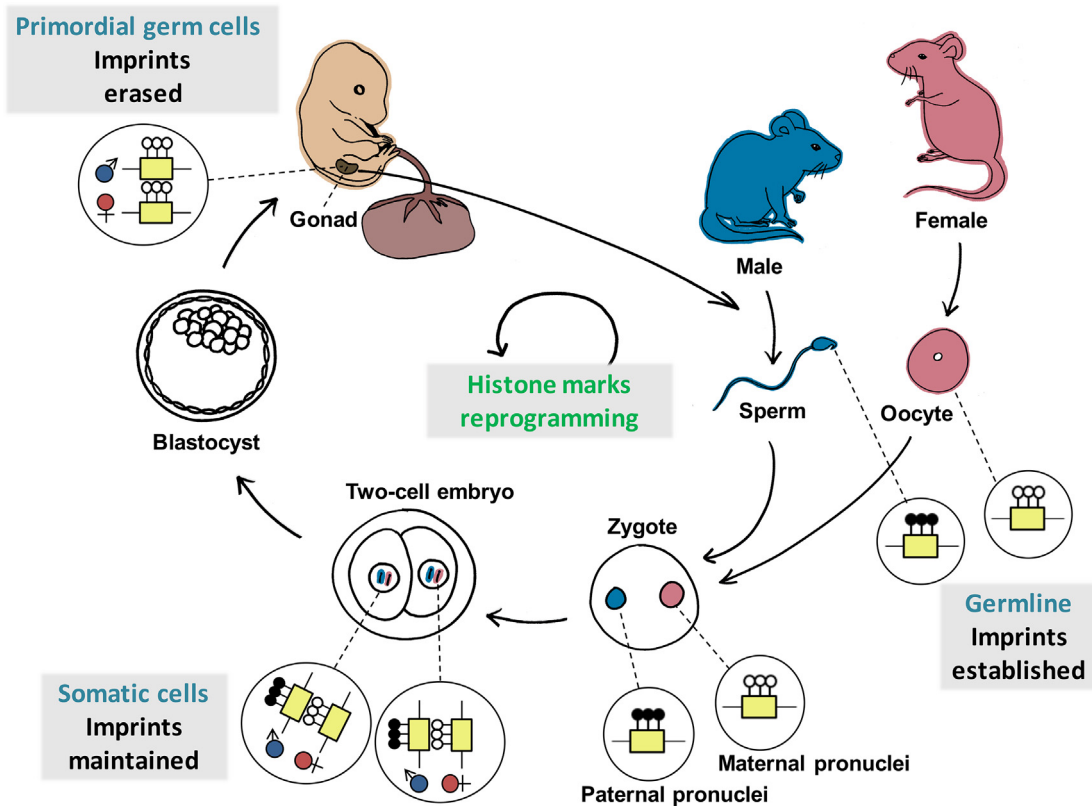


Fig. 3 The differential ICR methylation is established during gametogenesis according to the sex of the germ cells. Depicted here is an example of paternally methylated ICR (yellow box), which is methylated only in sperm (filled lollipops represent methylated CpGs; empty lollipops represent unmethylated CpGs). Following fertilization, the parental-allele specific ICR methylation is stably maintained in the somatic cells. In the PGCs of growing embryos, however, the existing somatic imprinting marks are erased, to allow for reestablishment of a sperm- or oocyte-specific methylation at the ICR. It should also be noted that post-translational histone modification marks at ICRs are reprogrammed during development.

hemi-methylated CpG dyads and methylates the unmethylated CpG) and its functional partner UHRF1 (Li and Zhang, 2014). Thus, ICR methylation patterns that are set up in the germline or early embryo can be stably maintained.

Despite the remarkable stability of DNA methylation, there are times when DNA methylation is necessarily erased. This includes periods of genome reprogramming that occur in the germline and after fertilization or when a regulatory element that drives a specific expression state or nuclear architecture requires activation. DNA methylation can be reversed either through passive or active mechanisms. Reversal occurs passively when functional DNA methylation machinery is lacking. In this case, downregulation or cytoplasmic localization of DNMTs and the cofactor UHRF1 prevents reestablishment of DNA methylation patterns after replication, eventually resulting in dilution of 5-methylcytosine. Alternatively, erasure of DNA methylation can occur actively through the Ten–eleven translocation (TET) family of proteins (TET1, TET2, and TET3). These proteins mediate the iterative oxidation of 5-methylcytosine to 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine (Wu and Zhang, 2017). The oxidized forms of 5-methylcytosine can either be diluted through replication or removed through various DNA repair pathways. Although still not completely resolved, base excision repair of 5-formylcytosine and 5-carboxylcytosine by thymine DNA glycosylase (TDG) has gained the most support in the removal of these oxidized forms of cytosine and replacement with unmodified cytosine.

To summarize, based on the properties above, the methylcytosine modification fulfills the criteria for a parental identity mark or “imprint” because: (1) it can be established in either the sperm or oocyte by de novo DNA methyltransferases, (2) it can be stably propagated at each embryonic cell division by a maintenance DNA methyltransferase, and (3) it can be erased in the germline to reset the imprint in the next generation, either by a passive or active mechanism.

Acquisition of DNA methylation at ICRs in the germline has been well-studied in the last 20 years and will be described in more detail in the next section. Nevertheless, it still remains unclear what precise signals govern acquisition and reprogramming of these allelic imprints. Moreover, until a few years ago, it was thought that the entire genome was demethylated in PGCs at approximately the same time, providing a convenient explanation for how genomes could be reset to a “ground state” in preparation for the PGCs to differentiate into germ cells and acquire sex-specific imprints. It is now known that some DNA sequences maintain DNA methylation during this germline reprogramming. These elements include the repetitive intracisternal A-particle (IAP) elements as well as single copy sequences. The mechanisms of escape from reprogramming and maintenance of DNA methylation at these sequences are not understood. Careful epigenomic profiling of germline cells, including PGCs and immature gametes, additionally

demonstrated that DNA demethylation occurs in two main sequential stages. Initially, a global decrease in 5-methylcytosine occurs through passive demethylation. Subsequently, locus specific demethylation occurs at ICRs and some meiotic genes through both active and passive processes. Here again, it is unknown how these particular sequences are protected from the initial DNA demethylation step. It is known, however, that elimination of the delay in the second step of DNA demethylation, through *Dnmt1* perturbation in a mouse model, results in precocious germline differentiation as well as hypogonadism and infertility (Hargan-Calvopina et al., 2016). That is, enabling the entire genome to be demethylated in a premature and nonsequential manner has detrimental effects on the germline. While one could clearly conclude that premature activation of meiotic genes would impact germ cell maturation, it is also possible that aberrant regulation of imprinted gene expression through dysregulation of ICRs could also prompt defects in the germline.

Epigenetic Marking of ICRs in the Paternal Germline

As stated above, the mammalian germline is derived from somatic cells, which have maternally and paternally marked chromosomes. Thus, it is important that ICRs in the earliest PGCs first be reprogrammed to a “ground state” by removing the somatic parental imprints. Ultimately, the haploid sperm will harbor paternal-specific epigenetic marks regardless of whether the somatic genome was originally maternal or paternal in origin. Likewise, the haploid egg will harbor maternal-specific epigenetic marks regardless of the parental source of the genome. As described above, the ICRs must therefore first be stripped of their DNA methylation and other epigenetic marks. Due to our ability to study DNA methylation on small numbers of cells, DNA methylation reprogramming is best understood in the field. Thus, this section will focus on DNA methylation erasure and reestablishment at ICRs.

As described in the previous section, the DNA methylation of ICRs is erased in PGCs. Through the use of *Tet1* and *Tet2* deficient mice it has been shown that different ICRs have varying degrees of reliance on the process of active DNA demethylation (Dawlaty et al., 2013; Yamaguchi et al., 2013). Both *Tet1*^{-/-} and *Tet1*^{-/-}; *Tet2*^{-/-} mice derived from the relevant heterozygous crosses are viable and fertile, although females are subfertile. Analysis of sperm from both single and double knockout males shows that ICRs that normally exhibit maternal-specific methylation (thus should not be methylated in the male germline) do indeed exhibit some DNA methylation, suggesting that the germ cells were not fully erased and reprogrammed. Curiously, not every ICR is equally affected, suggesting that *Tet1* and *Tet2* deficiency impacts various ICRs differently. For example, *Peg3* and *Peg10* ICRs, which are highly methylated in oocytes and unmethylated in wild-type sperm, exhibit abnormally high DNA methylation in sperm of *Tet* mutant mice. In contrast, the *Snprn* ICR appears unaffected by *Tet* gene deficiency. To determine whether abnormal germ cell DNA methylation patterns could impact imprinting in the next generation, offspring were produced from single (*Tet1*^{-/-}) and double (*Tet1*^{-/-}; *Tet2*^{-/-}) mutant mice mated with wild-type mice (i.e., the gametes were *Tet* null but the offspring were heterozygous). Here again, a subset of offspring exhibited abnormal DNA methylation patterns and abnormal imprinted gene expression patterns, although allelic expression was not examined in these mice. These experiments illustrate a number of points. First, active DNA demethylation is required for appropriate ICR methylation patterns in sperm and normal imprinting in some offspring. Second, some germ cells and offspring exhibit normal DNA methylation patterns at ICRs, suggesting that other mechanisms are involved in resetting imprints, such as passive DNA demethylation. Finally, the difference between the results from single and double mutants is minor, suggesting that TET1 mediates the majority, if not all, of the observed ICR demethylation.

Following DNA demethylation in PGCs, the maternal-specific imprints are acquired in late stage oocytes after birth (Stewart et al., 2016). In mouse oocytes, over 20 maternally-methylated sequences at imprinted loci have been defined (designated as gDMRs or gametic differentially methylated sequences), although not all of these sequences have been demonstrated to function as ICRs. Maternal gDMRs are CpG rich, usually being considered as CpG islands, and located in promoters or genes. Although a discussion of DNA methylation acquisition at maternal ICRs is beyond the scope of this article, it has been postulated that transcription initiated upstream of the ICR is linked to acquisition of ICR methylation in the maternal germline (Chotalia et al., 2009).

In contrast to the large number of maternal gDMRs, only three paternally methylated ICRs that acquire methylation during spermatogenesis have been defined (Stewart et al., 2016). The three paternally methylated ICRs are located at the *Rasgrf1*, *H19/Igf2*, and *Gtl2/Dlk1* loci. In contrast to the gene proximal location of the CpG dense maternally-methylated gDMRs/ICRs, paternally methylated ICRs are intergenic and exhibit the genome average of DNA methylation. In the male germline in the mouse, de novo methylation is initiated at approximately embryonic day 13.5 in prospermatogonia that are arrested in mitosis, and the male DNA methylome is completely established prior to birth. Because male germline undergoes mitotic proliferation after birth and prior to entering meiosis, the paternal-specific ICRs must also undergo DNA methylation maintenance. The question of how methylation is established at the three paternal ICRs is poorly understood. The acquisition of DNA methylation at the *Rasgrf1* ICR is most clearly defined. Early DNA methylation in the male germline occurs at repetitive sequences through a de novo methylation pathway involving testis-specific Piwi-interacting RNAs (piRNAs) and DNMT3B. The piRNAs are derived from repetitive sequences and direct methylation to the corresponding sequences. The retrotransposon in the paternally-methylated *Rasgrf1* ICR triggers the piRNA pathway that targets methylation to the ICR (Watanabe et al., 2011). Because the other two intergenic paternal ICRs (*H19/Igf2* and *Gtl2/Dlk1*) do not harbor transposable elements, they likely use a distinct pathway. It has been suggested that other paternally-methylated sequences may be targeted by low level transcription, as transcription has been observed across relevant sequences prior to acquisition of DNA methylation through DNMT3A (Singh et al., 2013). However, because the male germline is more highly methylated and DNA methylation and transcription occur more promiscuously in the male germline compared

to the female germline, it is not at all clear if transcription is the critical step for the acquisition of DNA methylation imprints. In fact, it may be that methylation is indiscriminately established all over the paternal genome during spermatogenesis and the key to paternal ICRs is maintenance of methylation asymmetry following fertilization (Li et al., 2008).

Although most work has focused on DNA methylation, it is clearly known that the chromatin environment at ICRs must be favorable for de novo DNA methylation. For example, failure to remove histone 3, lysine 4 trimethylation at target sequences is inhibitory to the de novo DNA methyltransferases. This is the likely reason that DNA methylation is acquired on the two parental alleles in the germline at different times at some ICRs (Davis et al., 2000; Lucifero et al., 2004). Thus, the asymmetric establishment of DNA methylation at the maternal and paternal alleles strongly suggests that ICRs also harbor differential chromatin imprints.

Conclusions and Future Prospects

Germline imprinting represents an intriguing paradigm of mammalian epigenetic gene regulation in which parental alleles are differentially recognized in the germline to undergo allele-specific epigenetic modifications. These modifications are remembered throughout the life of the individual, except when preparing for the next generation. The mechanisms governing the DNA methylation marking of ICRs are well-established as are the mechanisms for maintenance of DNA methylation imprints. It is also clear that ICRs undergo passive and active demethylation during reprogramming, although the extent to which the active and passive mechanisms contribute to any given locus requires further study, including analyses in PGCs. Moreover, a role for other epigenetics mechanisms in establishment and maintenance is incompletely understood. Perhaps the least well understood aspect of the acquisition of male-specific imprints is how the sequences at the *H19/Igf2* and *Gtl2/Dlk1* are reproducibly recognized over all of the other sequences in the male germline. Given the powerful tools for analysis and manipulation of the germline in model organisms, it is anticipated these various questions will be addressed in the future.

References

- Barlow DP and Bartolomei MS (2014) Genomic imprinting in mammals. *Cold Spring Harbor Perspectives Biology* 6, pii: a002592.
- Bartolomei, M. S. (2009). Genomic imprinting: Employing and avoiding epigenetic processes. *Genes and Development*, 23, 2124–2133.
- Chotalia, M., Smallwood, S. A., Ruf, N., et al. (2009). Transcription is required for establishment of germline methylation marks at imprinted genes. *Genes and Development*, 23, 105–117.
- Davis, T. L., Yang, G. J., McCarrey, J. R., & Bartolomei, M. S. (2000). The H19 methylation imprint is erased and reestablished differentially on the parental alleles during male germ cell development. *Human Molecular Genetics*, 9, 2885–2894.
- Dawlaty, M. M., Breiling, A., Le, T., et al. (2013). Combined deficiency of Tet1 and Tet2 causes epigenetic abnormalities but is compatible with postnatal development. *Developmental Cell*, 24, 310–323.
- Hargan-Calvopina, J., Taylor, S., Cook, H., et al. (2016). Stage-specific demethylation in primordial germ cells safeguards against precocious differentiation. *Developmental Cell*, 39, 75–86.
- Latos, P. A., Pauler, F. M., Koerner, M. V., et al. (2012). Airn transcriptional overlap, but not its lnc RNA products, induces imprinted Igf2r silencing. *Science*, 338, 1469–1472.
- Li, E., & Zhang, Y. (2014). DNA methylation in mammals. *Cold Spring Harbor Perspectives Biology*, 6, a019133.
- Li, X., Ito, M., Zhou, F., et al. (2008). A maternal-zygotic effect gene, *Zfp57*, maintains both maternal and paternal imprints. *Developmental Cell*, 15, 547–557.
- Lucifero, D., Mann, M. R., Bartolomei, M. S., & Trasler, J. M. (2004). Gene-specific timing and epigenetic memory in oocyte imprinting. *Human Molecular Genetics*, 13, 839–849.
- McGrath, J., & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, 37, 179–183.
- Singh, P., Li, A. X., Tran, D. A., et al. (2013). De novo DNA methylation in the male germ line occurs by default but is excluded at sites of H3K4 methylation. *Cell Reports*, 4, 205–219.
- Stewart, K. R., Veselovska, L., & Kelsey, G. (2016). Establishment and functions of DNA methylation in the germline. *Epigenomics*, 8, 1399–1413.
- Surani, M. A., Barton, S. C., & Norris, M. L. (1984). Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*, 308, 548–550.
- Tang, W. W., Kobayashi, T., Irie, N., Dietmann, S., & Surani, M. A. (2016). Specification and epigenetic programming of the human germ line. *Nature Review Genetics*, 17, 585–600.
- Watanabe, T., Tomizawa, S., Mitsuya, K., et al. (2011). Role for piRNAs and noncoding RNA in de novo DNA methylation of the imprinted mouse *Rasgrf1* locus. *Science*, 332, 848–852.
- Wu, X., & Zhang, Y. (2017). TET-mediated active DNA demethylation: Mechanism, function and beyond. *Nature Review Genetics*, 18, 517–534.
- Yamaguchi, S., Shen, L., Liu, Y., Sandler, D., & Zhang, Y. (2013). Role of Tet1 in erasure of genomic imprinting. *Nature*, 504, 460–464.

Specification of Spermatogonial Stem Cells

John R McCarrey, University of Texas at San Antonio, San Antonio, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Spermatogonial Stem Cells

Spermatogenesis is the process by which spermatozoa, the male gametes, are continually produced in the testis. This process takes place in the seminiferous epithelium. This epithelium consists of a series of multiple levels of cells that progress through the process of spermatogenesis by transitioning from less differentiated cells to more differentiated cells. Because this is an ongoing process that leads to the production of spermatozoa that are then released into the lumen of the seminiferous tubules, the epithelium must be continually replenished with additional spermatogenic cells. This is achieved by virtue of the presence of spermatogonial stem cells (SSCs) at the base of the seminiferous epithelium. These SSCs have the capability to divide asymmetrically (either directly or indirectly) such that they can sustain their own population via self-renewal as well as contributing new cells to the process of spermatogenesis via commitment to differentiation.

SSCs are the only cell type within the spermatogenic lineage capable of self-renewal and asymmetric division. In addition to undergoing self-renewal to sustain the SSC population, these cells can give rise to progenitor spermatogonia that are committed to enter the spermatogenic differentiation pathway. Progenitors then give rise to differentiating spermatogonia that then form spermatocytes, spermatids, and spermatozoa.

Development of Spermatogonial Stem Cells

The initial manifestation of the germ cell lineage in either males or females is the specification of primordial germ cells (PGCs) which are set aside as distinct from all other cell types (collectively known as somatic cells) in the early embryo at about the stage of gastrulation. In mammals, allocation of the PGCs occurs in the epiblast and the resulting PGCs then migrate to the developing genital ridges that will differentiate into either testes (in males) or ovaries (in females). In males, the PGCs give rise to prospermatogonia, which persist throughout the fetal and early postnatal period (in the mouse) before giving rise to spermatogonia. The SSCs represent only a small fraction of the undifferentiated spermatogonia. Other undifferentiated spermatogonia give rise directly to progenitors that, in the mouse, contribute to the unique first wave of spermatogenesis. These spermatogonia do not undergo self-renewal and so this population is exhausted very early during the male reproductive lifespan. Other undifferentiated spermatogonia undergo cell death. The initial SSCs give rise to the second and all subsequent waves of spermatogenesis by undergoing asymmetric divisions that facilitate self-renewal of the SSC population as well as contributing progenitors that continually replenish the seminiferous epithelium and the process of spermatogenesis. However, a key unanswered question is the nature of the mechanism by which a small subset of prospermatogonia give rise to the foundational SSCs while all other prospermatogonia transition into undifferentiated spermatogonia that immediately form progenitors that contribute to the first wave of spermatogenesis or that undergo cell death. The mechanism by which certain prospermatogonia become fated to form SSCs represents the initial specification of SSCs.

Specification of Spermatogonial Stem Cells

There are at least two theories regarding the mechanism responsible for specification of the foundational SSCs. The first theory holds that some sort of selection process operates at the cellular level to determine which prospermatogonia will give rise to SSCs. The nature of such a selection process is not at all well defined. It is not clear what cellular characteristics might form the basis upon which phenotypic distinctions arise among prospermatogonia and facilitate a mechanism that then identifies a specific subset to become SSCs. It is also difficult to imagine that such a mechanism would underly the critically important process of SSC specification in each individual in any sort of consistent manner. The alternate prevailing theory holds that a subset of prospermatogonia becomes predetermined to form the foundational SSCs on the basis of proactively regulated epigenetic programming that ensures that this subset of prospermatogonia and early undifferentiated spermatogonia will develop characteristics optimal to the formation and subsequent function of SSCs. Among these characteristics will be the ability to undergo self-renewal and asymmetric divisions, the ability to give rise to entire colonies of differentiating spermatogenic cells (something that can be directly assessed by a spermatogonial transplantation assay), and maintenance of advantageous characteristics such as enhanced genetic integrity.

Evidence for Predetermination of Spermatogonial Stem Cells

Recent studies appear to support the predetermination theory. Analysis of gene expression at the single cell level has clearly revealed the presence of distinct subpopulations of undifferentiated spermatogonia, and transplantation analyses have shown that one of these subpopulations possesses the majority of the capability to seed spermatogenesis in a recipient testis (= SSC function). Further

studies have shown that this same subpopulation also maintains enhanced genetic integrity relative to the other spermatogonial subtypes, and that these indications of heterogeneity can also be seen in earlier prospermatogonia.

Reproduction is central to the process of evolution, and, in the male, SSCs are critical to the process of reproduction. An attractive feature of the predetermination theory is that it invokes a proactive developmental mechanism that has the capacity to ensure that prospermatogonia bearing optimal characteristics will preferentially give rise to the foundational SSCs that will, in turn, ultimately give rise to the male gametes. This will then maximize the likelihood that optimal characteristics will be transmitted to subsequent generations, all of which will benefit the species.

Summary

The male germ line is unique in that it derives from PGCs that are allocated as germ cells very early during development, and then give rise to prospermatogonia, a subset of which then give rise to the foundational SSCs. Prior to the emergence of SSCs, none of the earlier male germ cell types has the capacity to self-renew, rather they progress via a unidirectional developmental process from PGCs to prospermatogonia and then, in the case of a small subset of prospermatogonia, to SSCs. Following the SSC stage, all subsequent spermatogenic cell types also lack the capacity to self-renew as they also progress through the unidirectional differentiation process of spermatogenesis to form spermatozoa. Thus, the SSCs are truly unique among male germ cells and are the only cells that participate in more than one wave of spermatogenesis. As such, it is critical that they develop and maintain optimal characteristics, and a predetermination mechanism of specification appears to be most consistent with this concept.

Further Reading

Spermatogonia

Oatley, J., & Griswold, M. (Eds.). (2017). *The biology of mammalian spermatogonia*. New York City, NY: Springer-Verlag.

Spermatogonial Stem Cells

Orwig, K., & Hermann, B. (Eds.). (2011). *Male germline stem cells: Developmental and regenerative potential*. New York City, NY: Humana Press.

Predetermination of Mammalian Spermatogonial Stem Cells

McCarrey, J. R. (2017). In J. Oatley, & M. Griswold (Eds.), *Transition of prenatal prospermatogonia to postnatal spermatogonia. The biology of mammalian spermatogonia*. New York City, NY: Springer-Verlag.

Regulation of Spermatogonial Stem Cell Function

Ina Dobrinski, Whitney Alpaugh, and Taylor Goldsmith, University of Calgary, Calgary, AB, Canada

© 2018 Elsevier Inc. All rights reserved.

Glossary

SSC	Spermatogonial stem cell
Rhox10	Reproductive homeobox 10
A _s	A single spermatogonia
A _{pr}	A paired spermatogonia
A _a	A aligned spermatogonia
PI3K	Phosphoinositide 3-kinase
AKT	Protein kinase B (PKB)
MAP2K1	Mitogen-activated protein kinase kinase 1
Etv5	ETS Variant 5
CXCR4	C-X-C chemokine receptor 4
RAR γ	Retinoic acid receptor gamma
mTORC1	Mamalian target of rapamycin complex 1
REDD1	Regulated in development and DNA damage response 1
STAT3	Signal transducer and activator of transcription 3

Spermatogonial Stem Cell Dynamics

The majority of our understanding of SSC biology comes from rodent models; therefore, this chapter will focus on evidence from the mouse model. SSCs arise postnatally from the transient pro-spermatogonia or gonocyte population. Prospermatogonia are the only germ cells present in the neonatal testis cords until expression of the transcription factor reproductive homeobox 10 (*Rhox10*) provides the signal to differentiate and establish the SSC pool (Song et al., 2016). In an adult testes, SSCs are rare and believed to constitute only about 0.03% of all germ cells (Tegelenbosch and De Rooij, 1993). The low proportion of SSCs in the total germ cell population can support spermatogenesis through multiple mitotic amplification divisions. The number of amplification divisions differs between species which greatly influences their spermatogenic output (Hess and Renato de Franca, 2008). In a mouse testes, SSCs are a subset of A_{single} (A_s) spermatogonia, which divide to A_{paired} (A_{pr}) and then A_{aligned} (A_{al}) (4, 6, 8, 16 and rarely 32 cells) spermatogonia. A_{al} cells then differentiate to A1 spermatogonia without division, then divide to form A2, A3, A4, intermediate and type B spermatogonia before entering meiosis as a spermatocyte (De Rooij, 2001). These spermatogonia clones are all linked by intercellular bridges resulting from incomplete cytokinesis. The main hypotheses for the role of these intercellular bridges involve the ability for the cellular clone to share cytoplasmic content and facilitate synchronous cell division in the later spermatogonia clones (A1-type B spermatogonia). This becomes especially important for coordinating meiotic entry (Greenbaum et al., 2011).

It has long been thought that once A_s spermatogonia divide to become A_{pr} spermatogonia, the cells are committed to differentiation. However, more recently, a series of studies demonstrated that early progenitor cells are capable of reverting back to a stem cell phenotype (Barroca et al., 2009; Nakagawa et al., 2010; Hara et al., 2014). Through transplantation experiments, Barroca et al. (2009) found that isolated progenitor cells were able to repopulate a germ-cell-depleted testes albeit at a much lower efficiency than isolated stem cells. Through in vivo imaging studies, Nakagawa et al. (2010) established that, during steady state spermatogenesis in mouse, dividing clones up to A_{al-8} have the ability to fragment from their dividing sister cells, become an A_s spermatogonium and regain stem cell function. The rate of fragmentation was increased considerably during regeneration after toxic insult to the testes. Similar findings were previously reported in *Drosophila melanogaster* (Brawley and Matunis, 2004) which demonstrates that this phenomenon of transient amplifying progenitor cells being capable of de-differentiating is highly conserved.

Spermatogonial Stem Cell Markers

One persistent challenge in the field of SSC biology is the ability to specifically identify and isolate this cell population for study. Currently, the only conclusive way to determine if a population of cells contains stem cells is by transplanting them into a germ cell-depleted recipient animal and examine the recipient testes 2 months post transplantation for donor derived colonization and spermatogenesis. However, the dynamic nature of SSCs described above confounds this as well as other approaches to examine SSCs.

The identification of several differentially expressed proteins have helped to characterize subpopulations of spermatogonia that have increased stem cell potential (Table 1). In Shinohara et al., 1999, Shinohara et al. found that cell populations enriched

Table 1 Summary of factors involved in SSC regulation discussed in this article.

<i>Name</i>	<i>Role in SSC Biology</i>	<i>Evidence</i>
$\beta 1$ -integrin	Positive population enriched in stem cell activity	Transplantation
$\alpha 6$ -integrin	Positive population enriched in stem cell activity	Transplantation
THY-1	Positive population enriched in stem cell activity	Transplantation
ID4	SSC marker	Immunohistochemistry and transplantation
PAX7	SSC marker	Immunohistochemistry, lineage tracing and transplantation
GDNF	Required for SSC self-renewal signaling through GFR $\alpha 1$ /RET	Knock-out studies and in vitro culture system
FGF2	Important for SSC self-renewal	In vitro culture system
CSF1	Important for SSC self-renewal	Microarray studies, immunohistochemistry and in vitro culture system
CXCL12	Required for SSC maintenance signaling through CXCR4	Immunohistochemistry, in vitro culture system and knockout model studies
Retinoic acid	Pulsatile retinoic acid stimulation is required for differentiation and spermatogenesis	Vitamin A deficiency models, immunohistochemistry, knockout model studies and in vitro culture system
PLZF	Undifferentiated spermatogonia marker and required for SSC self-renewal and maintenance	Immunohistochemistry, knock-out studies, transplantation and in vitro culture system
GFR $\alpha 1$ /RET	Undifferentiated spermatogonia marker and required for SSC self-renewal	Immunohistochemistry, in vivo imaging and in vitro culture system
BCL6B	Expression required for SSC self-renewal	Microarray studies, in vitro culture system and knockout model studies
RB1	Required for SSC self-renewal and maintenance of the pool	In vitro culture system and conditional knockout studies
NANOS2	Undifferentiated spermatogonia marker, required for SSC self-renewal and maintenance	Immunohistochemistry, lineage tracing, knockout and overexpression models
Reactive oxygen species	Finite amounts required for SSC self-renewal	In vitro culture system, inhibitor/enhancer studies and transplantation studies
SALL4	Early differentiating spermatogonia marker required for SSC differentiation	Immunohistochemistry, co-immunoprecipitation, knockout studies and ChIP-seq
NGN3	Early differentiating spermatogonia marker required for SSC differentiation	Immunohistochemistry, lineage tracing experiments, transplantation and in vitro culture system
SOX3	Expression required for early spermatogonia differentiation	Immunohistochemistry and knockout model studies

for $\beta 1$ -integrin and $\alpha 6$ -integrin expressing spermatogonia have higher stem cell capacity following transplantation than non-enriched cells. Transplantation experiments using THY-1⁺ (CD90.2) spermatogonia have also established this population to be enriched in stem cell activity (Kubota et al., 2003). Glial cell line-derived neurotrophic factor receptor $\alpha 1$ (GFR $\alpha 1$) is expressed in A_s, A_{pr} and some A_{al} spermatogonia and has become a well established protein marker of the undifferentiated spermatogonia population that contains stem cells (Hofmann et al., 2005).

Recently, two other proteins, inhibitor of DNA binding 4 (ID4) and paired box transcription factor 7 (PAX7), have been introduced to the field as more specific stem cell markers (Oatley et al., 2011; Aloisio et al., 2014). ID4 expression is restricted to a subset of A_s spermatogonia and, upon transplantation of ID4 positive spermatogonia, recipient testes are extensively colonized (Oatley et al., 2011; Chan et al., 2014). PAX7 is expressed in a very rare subpopulation of A_s spermatogonia. Lineage tracing experiments found that a single PAX7 positive spermatogonium can differentiate to germ cells of all spermatogenic stages. Additionally, PAX7 positive spermatogonia are resistant to germ cell ablation by chemo- and radiotherapy and expand in number after these toxic insults (Aloisio et al., 2014). This regenerative cell behavior has been established as a stem cell hallmark in other stem cell systems.

Spermatogonial Stem Cell Niche Regulation

The specific cellular, molecular and structural environment that supports stem cell function is known as its niche. The SSC niche is located at the periphery of the seminiferous tubules along the basement membrane produced by Sertoli and peritubular myoid cells. A schematic representation of the cell associations and factors contributing to the stem cell niche in the testis is presented in Fig. 1. Time-lapse imaging experiments have also revealed that undifferentiated type A spermatogonia are over-represented along sections of the seminiferous tubules adjacent to blood vessels and migrate away from this vascular-associated niche upon differentiation (Yoshida et al., 2007).

Several niche-cell secreted ligands are important for SSC maintenance (Table 1). The best studied ligand, glial cell-line derived neurotrophic factor (GDNF), is secreted by Sertoli as well as peritubular myoid cells under testosterone pulsatile controlled release

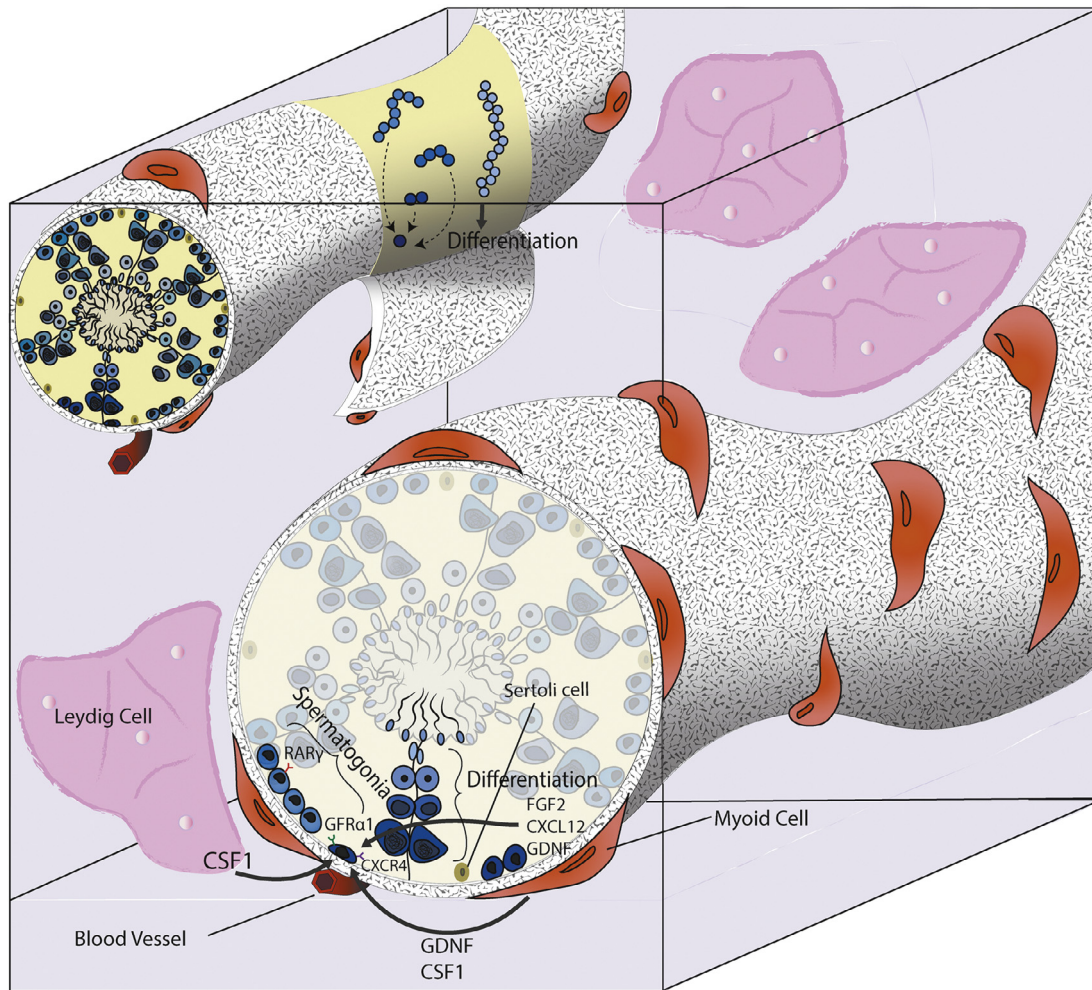


Fig. 1 Schematic illustration of the SSC niche in the testis.

(Meng et al., 2000; Tadokoro et al., 2002; Yomogida et al., 2003; Chen et al., 2016). GDNF signals through the GFR α 1/RET tyrosine kinase receptor dimer located on undifferentiated spermatogonia to activate the PI3K-Akt pathway which promotes proliferation and self-renewal (Braydich-Stolle et al., 2007; Lee et al., 2007; Oatley et al., 2007). Another important factor for SSC self-renewal is fibroblast growth factor 2 (FGF2) which activates MAP2K1 and ultimately up-regulates *Etv5* and *Bcl6b* gene expression (Kubota et al., 2004; Ishii et al., 2012). Mouse spermatogonia in vitro culture systems require these two growth factors, GDNF and FGF2, for expansion of undifferentiated spermatogonia.

A microarray screen revealed that colony stimulating factor 1 receptor (*Csf1r*) is up-regulated on THY1⁺ compared to THY1⁻ testis cells (Oatley et al., 2009). Colony stimulating factor 1 (CSF1) ligand is secreted by both Leydig and peritubular myoid cells and promotes self-renewal of SSCs in vitro. Another ligand-receptor interaction that is important for SSC self-renewal is CXCL12 secreted by Sertoli cells and CXCR4 which is expressed on a subset of undifferentiated spermatogonia (Yang et al., 2013b). Upon inhibition of CXCR4, spermatogonia display increased propensity to differentiate in vitro upon retinoic acid (RA) stimulation (a potent inducer of differentiation) and this leads to disrupted spermatogenesis in vivo.

RA is synthesized from dietary vitamin A and RA signaling from Sertoli and other germ cells is essential for spermatogenesis (Morales and Griswold, 1987; Sugimoto et al., 2012). As GFR α 1 positive undifferentiated spermatogonia begin to differentiate, they start expressing the transcription factor neurogenin-3 (NGN3). NGN3⁺ spermatogonia express retinoic acid receptor gamma (RAR γ) and are therefore responsive to retinoic acid induced differentiation (Ikami et al., 2015).

Transcriptional Regulation of Spermatogonial Stem Cells

Our understanding of the transcriptional regulation of SSC maintenance is constantly evolving and a hot area of research. Several factors that play a key role in SSC self-renewal and differentiation have been identified (Table 1).

Self-Renewal

In 2004, the *luxoid* mutant mouse, which has a mutation in the gene encoding *Plzf*, was observed to have severe spermatogenic defects (Buaas et al., 2004; Costoya et al., 2004). PLZF is expressed in undifferentiated spermatogonia and its absence leads to progressive germ cell loss. More recently, Hobbs et al. (2010) demonstrated that PLZF decreases mammalian target of rapamycin complex 1 (mTORC1) activity, which promotes proliferation and differentiation in multiple stem cell systems, via inducing mTORC1 inhibitor regulated in development and DNA damage response 1 (REDD1). There is evidence that this regulation of mTORC1 by PLZF affects the responsiveness of undifferentiated spermatogonia to GDNF self-renewal signaling. Microarray analysis of established spermatogonia cultures grown in the presence or absence of GDNF identified expression of the transcription repressor B-cell lymphoma member 6 (*Bcl6b*) as being regulated by GDNF signaling and important for SSC self-renewal (Oatley et al., 2006). Further analysis of *Bcl6b* knock-out testes revealed degenerating tubules, devoid of spermatogenesis confirming the role of BCL6B in SSC self-renewal in vivo.

Expression of a tumor suppressor gene that encodes for retinoblastoma protein (RB) has also been shown to be important for SSC maintenance and self-renewal (Hu et al., 2013; Yang et al., 2013a). Conditional knockout studies of *Rb1* in the germline show that first wave spermatogenesis occurs followed by progressive loss of all germ cells due to failure of the GRF α 1⁺ A_s spermatogonia population to self-renew. Lineage tracing experiments demonstrated undifferentiated spermatogonia expressing RNA-binding protein NANOS2 give rise to the entire spermatogenic lineage (Sada et al., 2009). Conditional knockout and over-expression studies in vivo show that NANOS2 is required for SSC self-renewal and long-term maintenance of the pool. In addition to expression of these specific genes, the generation of finite amounts of reactive oxygen species (ROS) within SSCs was shown to be required for self-renewal in vitro (Morimoto et al., 2013). When ROS generation was inhibited in vitro, numbers of spermatogonia in cultures decreased but when ROS production is slightly elevated, SSC doubling time decreases which, in turn, increases SSC cell numbers.

Differentiation

Several transcriptional mechanisms of SSC differentiation have also been identified. Expression of the transcription factor spalt-like 4 (*Sall4*) in undifferentiated spermatogonia promotes differentiation by suppressing PLZF activity (Hobbs et al., 2012; Gassei and Orwig, 2013; Lovelace et al., 2016). PLZF promotes SSC self-renewal, in part, by suppressing gene networks required for differentiation. SALL4 removes this repression by interacting with PLZF to facilitate differentiation. Similar to the antagonist relationship of SALL4 with PLZF, increasing expression of neurogenin 3 (NGN3) in the GRF α 1⁺ undifferentiated spermatogonia population coincides with differentiation (Yoshida et al., 2004; Nakagawa et al., 2010). NGN3 expression was found to be regulated by STAT3 activation and to ultimately induce differentiation in vitro and in vivo following transplantation of *Ngn3* deficient spermatogonia (Kaucher et al., 2012). Sry-related homeobox 3 (SOX3) has also been associated with NGN3 expression and early spermatogonia differentiation (Raverot et al., 2005). Undifferentiated spermatogonia fail to differentiate in *Sox3* knockout testes resulting in tubules containing undifferentiated spermatogonia and Sertoli cells only.

Summary

In summary, regulation of SSC function to achieve a balance between self-renewal and differentiation is essential for male fertility. While several key factors and pathways active in mammalian SSCs have been elucidated, predominantly in the mouse model (summarized in Table 1 and Fig. 1), a more complete picture and translation to other non-rodent species are still under intense investigation. Translating these mouse model findings as well as elucidating novel mechanisms in human SSC regulation are important for increasing our understanding of clinical male infertility. Knowing which pathways are critical for normal SSC maintenance provides potential targets for diagnosis and ultimately treatment of male infertility.

References

- Aloisio, G. M., Nakada, Y., Saatcioglu, H. D., Pena, C. G., Baker, M. D., et al. (2014). PAX7 expression defines germline stem cells in the adult testis. *The Journal of Clinical Investigation*, 124, 3929–3944.
- Barroca, V., Lassalle, B., Coureuil, M., Louis, J. P., Le Page, F., et al. (2009). Mouse differentiating spermatogonia can generate germinal stem cells in vivo. *Nature Cell Biology*, 11, 190–196.
- Brawley, C., & Matunis, E. (2004). Regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo. *Science*, 304, 1331–1334.
- Braydich-Stolle, L., Kostereva, N., Dym, M., & Hofmann, M. C. (2007). Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. *Developmental Biology*, 304, 34–45.
- Buaas, F. W., Kirsh, A. L., Sharma, M., Mclean, D. J., Morris, J. L., et al. (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nature Genetics*, 36, 647–652.
- Chan, F., Oatley, M. J., Kaucher, A. V., Yang, Q. E., Bieberich, C. J., et al. (2014). Functional and molecular features of the Id4⁺ germline stem cell population in mouse testes. *Genes & Development*, 28, 1351–1362.
- Chen, L. Y., Willis, W. D., & Eddy, E. M. (2016). Targeting the Gdnf gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 1829–1834.
- Costoya, J. A., Hobbs, R. M., Barna, M., Cattoretti, G., Manova, K., et al. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature Genetics*, 36, 653–659.
- De Rooij, D. G. (2001). Proliferation and differentiation of spermatogonial stem cells. *Reproduction*, 121, 347–354.
- Gassei, K., & Orwig, K. E. (2013). SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. *PLoS One*, 8, e53976.

- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3, a005850.
- Hara, K., Nakagawa, T., Enomoto, H., Suzuki, M., Yamamoto, M., et al. (2014). Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell*, 14, 658–672.
- Hess, R. A., & Renato de Franca, L. (2008). Spermatogenesis and cycle of the seminiferous epithelium. *Advances in Experimental Medicine and Biology*, 636, 1–15.
- Hobbs, R. M., Seandel, M., Falcior, I., Rafii, S., & Pandolfi, P. P. (2010). Plzf regulates germline progenitor self-renewal by opposing mTORC1. *Cell*, 142, 468–479.
- Hobbs, R. M., Fagoonee, S., Papa, A., Webster, K., Altruda, F., et al. (2012). Functional antagonism between Sall4 and Plzf defines germline progenitors. *Cell Stem Cell*, 10, 284–298.
- Hofmann, M. C., Braydich-Stolle, L., & Dym, M. (2005). Isolation of male germ-line stem cells; influence of GDNF. *Developmental Biology*, 279, 114–124.
- Hu, Y. C., De Rooij, D. G., & Page, D. C. (2013). Tumor suppressor gene Rb is required for self-renewal of spermatogonial stem cells in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 12685–12690.
- Ikami, K., Tokue, M., Sugimoto, R., Noda, C., Kobayashi, S., et al. (2015). Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. *Development*, 142, 1582–1592.
- Ishii, K., Kanatsu-Shinohara, M., Toyokuni, S., & Shinohara, T. (2012). FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. *Development*, 139, 1734–1743.
- Kaucher, A. V., Oatley, M. J., & Oatley, J. M. (2012). NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. *Biology of Reproduction*, 86(164), 1–11.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2003). Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 6487–6492.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 16489–16494.
- Lee, J., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., et al. (2007). Akt mediates self-renewal division of mouse spermatogonial stem cells. *Development*, 134, 1853–1859.
- Lovelace, D. L., Gao, Z., Mutoji, K., Song, Y. C., Ruan, J., et al. (2016). The regulatory repertoire of PLZF and SALL4 in undifferentiated spermatogonia. *Development*, 143, 1893–1906.
- Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., De Rooij, D. G., et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287, 1489–1493.
- Morales, C., & Griswold, M. D. (1987). Retinol-induced stage synchronization in seminiferous tubules of the rat. *Endocrinology*, 121, 432–434.
- Morimoto, H., Iwata, K., Ogonuki, N., Inoue, K., Atsuo, O., et al. (2013). ROS are required for mouse spermatogonial stem cell self-renewal. *Cell Stem Cell*, 12, 774–786.
- Nakagawa, T., Sharma, M., Nabeshima, Y., Braun, R. E., & Yoshida, S. (2010). Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science*, 328, 62–67.
- Oatley, J. M., Avarbock, M. R., Talaranta, A. I., Fearon, D. T., & Brinster, R. L. (2006). Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 9524–9529.
- Oatley, J. M., Avarbock, M. R., & Brinster, R. L. (2007). Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. *The Journal of Biological Chemistry*, 282, 25842–25851.
- Oatley, J. M., Oatley, M. J., Avarbock, M. R., Tobias, J. W., & Brinster, R. L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development*, 136, 1191–1199.
- Oatley, M. J., Kaucher, A. V., Racicot, K. E., & Oatley, J. M. (2011). Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. *Biology of Reproduction*, 85, 347–356.
- Raverot, G., Weiss, J., Park, S. Y., Hurley, L., & Jameson, J. L. (2005). Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Developmental Biology*, 283, 215–225.
- Sada, A., Suzuki, A., Suzuki, H., & Saga, Y. (2009). The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science*, 325, 1394–1398.
- Shinohara, T., Avarbock, M. R., & Brinster, R. L. (1999). beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 5504–5509.
- Song, H. W., Bettegowda, A., Lake, B. B., Zhao, A. H., Skarbreik, D., et al. (2016). The Homeobox transcription factor RHOX10 drives mouse Spermatogonial stem cell establishment. *Cell Reports*, 17, 149–164.
- Sugimoto, R., Nabeshima, Y., & Yoshida, S. (2012). Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. *Mechanisms of Development*, 128, 610–624.
- Tadokoro, Y., Yomogida, K., Ohta, H., Tohda, A., & Nishimune, Y. (2002). Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mechanisms of Development*, 113, 29–39.
- Tegelenbosch, R. A., & De Rooij, D. G. (1993). A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutation Research*, 290, 193–200.
- Yang, Q. E., Gwost, I., Oatley, M. J., & Oatley, J. M. (2013a). Retinoblastoma protein (RB1) controls fate determination in stem cells and progenitors of the mouse male germline. *Biology of Reproduction*, 89, 113.
- Yang, Q. E., Kim, D., Kaucher, A., Oatley, M. J., & Oatley, J. M. (2013b). CXCL12-CXCR4 signaling is required for the maintenance of mouse spermatogonial stem cells. *Journal of Cell Science*, 126, 1009–1020.
- Yomogida, K., Yagura, Y., Tadokoro, Y., & Nishimune, Y. (2003). Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. *Biology of Reproduction*, 69, 1303–1307.
- Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., et al. (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. *Developmental Biology*, 269, 447–458.
- Yoshida, S., Sukeno, M., & Nabeshima, Y. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science*, 317, 1722–1726.

Spermatogonial Transplantation and Spermatogonial Stem Cell Competence

Hiroshi Kubota, Kitasato University, Towada, Aomori, Japan

Ralph L Brinster, University of Pennsylvania, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Spermatogenesis is complex, but well-organized, and is one of the most productive systems in mammalian tissues. Millions to hundreds of millions of spermatozoa are produced from the beginning of puberty in the male testes, and the high productivity continues until old age. Spermatogonial stem cells (SSCs) are responsible for maintaining life-long spermatogenesis and are capable of regenerating complete spermatogenesis when it is damaged by injury.

Since the 1950s using rodents, mammalian SSCs have been studied by means of histological sections, whole mounts of seminiferous tubules, and H^3 -thymidine labeling experiments for cell-kinetics. As a result, putative SSCs are identified as a small subpopulation of type A spermatogonia, namely undifferentiated spermatogonia. Undifferentiated spermatogonia are the initial stage of spermatogenesis and are subdivided into type A_{single} (A_s), A_{paired} (A_{pr}), and A_{aligned} (A_{al}) according to the number of cells connected by intercellular bridges. A_s spermatogonia have long been considered to be SSCs because A_{pr} and A_{al} spermatogonia are derived from A_s spermatogonia, and the interconnected forms are thought to be a well-conserved sign of germ cell differentiation in various species. However, these definitions were not established by the biological activity of A_s spermatogonia. Therefore, it is impossible to know whether A_s spermatogonia are indeed SSCs. Like other stem cells in the body, SSCs must be defined by their biological competence, in which single cells possess the capacity of both self-renewal and differentiation to the terminal cell type characteristic of that tissue. Spermatogonial transplantation is an experimental system that allows evaluating SSC competence in donor cell populations, and the only method to unequivocally identify SSCs based on their competency.

Spermatogonial Transplantation as a Functional Assay for SSCs

Spermatogonial Transplantation Procedure

The spermatogonial transplantation technique was developed to identify SSCs using mice in 1994 (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). To establish the transplantation technique in the initial experiments, donor cells that express a reporter gene were directly injected into the seminiferous tubule of an infertile recipient male. Although three injection methods have been developed (Kubota and Brinster, 2017), microinjection via efferent ducts appeared to be the best for controlling the injection volume, which is important for quantitative analysis of SSCs. The recipient males were originally made infertile by injecting the alkylating chemotherapeutic agent, Busulfan. Alternatively mutant mice with deficiencies of germ cell development, such as W mutant mice (Brinster and Avarbock, 1994), can also be used as recipients to avoid chemotherapeutic treatment of the recipient. After microinjection of testicular cells into the spermatogenesis-impaired seminiferous tubules, a small subpopulation of the donor cells is able to pass through the blood-testis barrier of the Sertoli cells and colonizes the basement membrane of the tubule. The donor cells that reach the basement membrane begin to proliferate laterally, and only after 1 month donor-derived spermatocytes appear in the adluminal compartment of the seminiferous tubule. Spermatozoa can be identified in the lumen of the tubule after 2 months (Nagano et al., 1999). The reconstituted spermatogenesis continues throughout the remaining life of the recipients, and spermatozoa derived from donor cells can fertilize eggs, resulting in production of fertile progeny carrying the donor male haplotype. These characteristics of donor cells following transplantation establish SSC competence of the transplanted cells. Thus, development of the spermatogonial transplantation procedure has made it possible to unequivocally identify SSCs in any donor-cell population based on their biological function.

Quantitative Functional Assay for SSCs

The transplantation procedure can be used as a quantitative assay for retrospective identification of SSCs. When testis cells from a transgenic mouse that expresses a reporter gene, such as β -galactosidase (β -gal) or green fluorescent protein (GFP), are transplanted into infertile recipient testes, donor-derived spermatogenesis can be unequivocally identified by visualizing the reporter proteins. In recipient testes transplanted with β -gal-expressing germ cells, donor-derived spermatogenesis can be identified as blue colonies after staining with the substrate, X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), 2 months following transplantation (Fig. 1). These colonies are each derived from a single donor cell, and the colony number does not change from 1 to 4 months, whereas the length of colonies increases (Nagano et al., 1999). Thus, the number of blue colonies represents the number of SSCs in the donor cell population that can colonize the recipient testis. Only about 10% of SSCs in a donor cell population that is transplanted form colonies; consequently, the number of colonies is believed to represent only one tenth of the true stem cell number (Nagano, 2003).

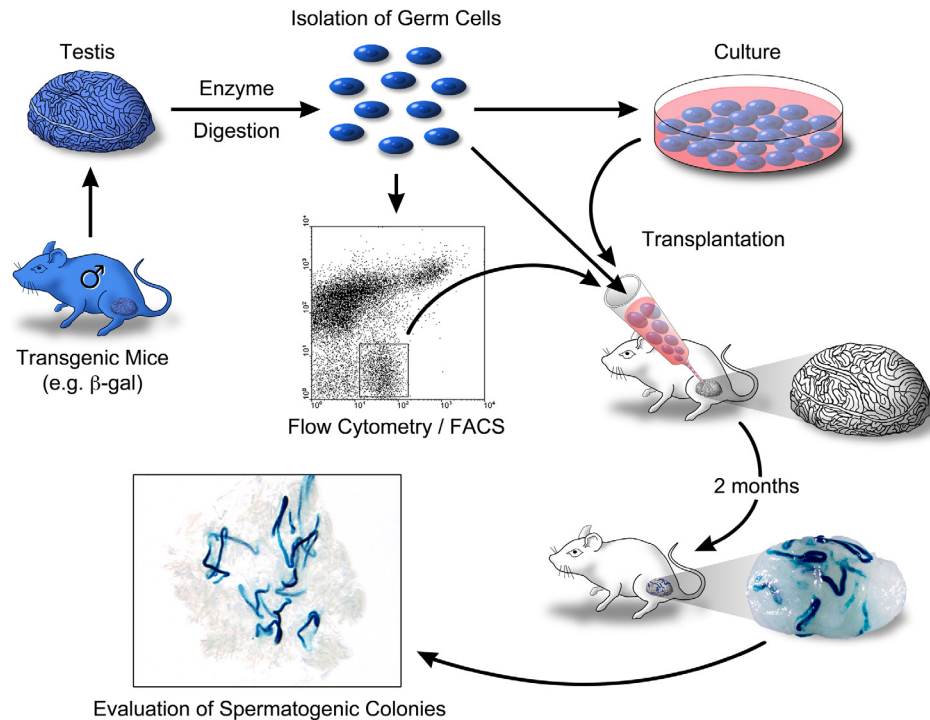


Fig. 1 Outline of quantitative transplantation assay for SSCs. A single cell suspension prepared from testes of transgenic mice expressing a reporter gene (e.g. β -gal) by enzymatic digestion is injected into the seminiferous tubules of an infertile recipient male. Cells from in vitro culture or cells fractionated by FACS or other selection methods can be used for a donor cell population. Two months after transplantation, donor-derived spermatogenesis can be detected in the recipient testis as *blue* stretches. Because each spermatogenic colony is derived from a single SSC, the number of colonies represents the number of SSCs in the donor cell suspension. The length of the colonies demonstrates the degree of SSC expansion. Modified from Nagano, M., Avarbock, M. R. and Brinster, R. L. (1999). Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biology of Reproduction* **60**, 1429–1436; Kubota, H. and Brinster, R. L. (2006). Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature Clinical Practice Endocrinology & Metabolism* **2**, 99–108.

Defining the Identity of SSCs

Estimated SSC Number in a Testis

The number of A_s spermatogonia in the testis is very low. A whole mount analysis of seminiferous tubules indicated that only 0.03% of all germ cells in an adult mouse testis were A_s spermatogonia. Because all A_s spermatogonia may not be SSCs, the number of SSCs would be fewer than that of A_s spermatogonia. Estimation of the SSC number by the spermatogonial transplantation assay indicated that approximately 0.01% of all seminiferous tubule cells are functional SSCs and that the SSC number in an individual testis is as low as 3000 (Nagano, 2003). The rarity of SSCs in the testis makes studies of SSCs very difficult; therefore, defining the identity of SSCs is crucial to clarifying their biological and molecular characteristics.

Cell Surface Characteristics of SSCs

One of the most extensively studied characteristics of SSCs is their cell surface phenotype. Determining the cell surface characteristics of SSCs is important to enrich the rare SSC population, and the following process can be used. Initially, a single cell suspension is prepared from testes and stained with fluorochrome-conjugated antibodies against cell surface molecules. Subsequently, the stained testicular cell population is fractionated based on the expression patterns of the surface molecules, and each fraction of cells is isolated by fluorescence activated cell sorting (FACS). To determine which subpopulation of testis cells includes SSCs, isolated cell populations are subjected to the transplantation assay. Two months after transplantation of each cell fraction, recipient testes are examined to identify donor-derived spermatogenesis (Fig. 1). The number of spermatogenic colonies generated by different cell populations indicates the number of SSCs in that population of cells. By repeating this process, the cell surface phenotype of mouse SSCs has been determined to be $THY1^{lo}$ $ITGA6^{+}$ $KIT^{-/lo}$ $MHC-I^{-}$ (major histocompatibility complex class I) in mice (Kubota et al., 2003). Further characterization by flow cytometry demonstrated that mouse SSCs express several other surface molecules including GFRA1 (GDNF family receptor alpha 1), CDH1, CD9, EPCAM, MCAM, and others (Kubota and Brinster, 2017). These cell surface molecules are mainly categorized as cell adhesion molecules and growth factor receptors, some of which regulate SSC functions. Furthermore, cellular activity, such as efflux pump activity, mitochondrial activity, cell-cycle, and intracellular enzymatic activity, can be used for FACS-based cell fractionation. At present, no cell surface molecule or cellular characteristic that

exclusively identifies SSCs has been identified. Nevertheless, while the concentration of SSCs in adult testes is 1–2 in 10,000 testicular cells, that of $\text{THY1}^{\text{lo}} \text{ITGA6}^+ \text{KIT}^{-/\text{lo}} \text{MHC-I}^-$ cells in adult testes is increased to approximately 1 in 30 (Kubota et al., 2003). The SSC-enriched cell population greatly facilitates further investigation of SSC characteristics at cellular and molecular levels.

Intracellular Characteristics of SSCs

Although flow cytometry of cell surface molecules is a powerful and an effective approach to dissect the SSC phenotype, knowledge about gene expression characteristics of undifferentiated spermatogonia is increased by comparative or comprehensive gene expression analyses between SSCs and differentiating cells. Consequently, many intracellular molecules, such as transcription factors or RNA binding proteins, which are expressed exclusively in undifferentiated spermatogonia or primarily in A_s spermatogonia, have been identified. For determining whether they are SSC-specific genes, transgenic mice in which a reporter gene, such as *GFP*, is inserted downstream of the promoter of a putative SSC-specific gene, have been used. Following identification of reporter gene-expressing cells in the testes of the transgenic mice, the cells are isolated by FACS and subjected to the spermatogonial transplantation assay (Kubota and Brinster, 2017). Again, no SSC specific molecules have been identified, but this approach could allow better SSC enrichment than existing techniques relying on cell surface markers alone. Furthermore, this approach is important to delineate the ordered expression of gene expression during spermatogonial differentiation from SSCs and to determine the molecular signature of the stem cell.

Factors Influencing SSC Competence

Impact of SSC Niche

The surrounding microenvironment of stem cells is called the stem cell niche, which has been shown to influence SSC competence. When SSCs from adults were transplanted into pups and adult recipient testes, the number of spermatogenic colonies in pup testes was approximately 10 times greater than in adult testes (Shinohara et al., 2001; Fig. 2). In addition, colonies generated were four times longer in pup testes compared with those in adults (Fig. 2). These findings indicated that the SSC niche in pups is more accessible and supportive of transplanted SSCs than that in adults. Conversely, aged atrophic testes ($\sim 2+$ years) are less supportive of transplanted SSCs, and the competence of resident SSCs in those testes deteriorates (Zhang et al., 2006). Although some males can maintain spermatogenesis until old age ($\sim 2+$ years), the amount of spermatogenesis begins to decrease after the first year, which results from a decreasing number of SSCs (Zhang et al., 2006; Ryu et al., 2006). By 2 years of age, the SSC number in the atrophic testes is significantly decreased, which is clearly established by the SSC transplantation technique. Furthermore, the SSC niche in atrophic testes of 2-year-old mice appears to be deteriorated because young SSCs rarely colonized and regenerated spermatogenesis in those recipient mice following transplantation (Zhang et al., 2006). Thus, the decrease in SSC competence during aging is largely influenced by the SSC niche. To support this conclusion, serial transplantation of SSCs into young recipient males revealed that the SSC number and their competency did not change up to 32 months, during which SSCs were serially transplanted eight times, every 3 months into young recipients (Ryu et al., 2006). These serial transplantation experiments clearly indicate that the young SSC niche extends the SSC competency beyond the normal life span of the mouse.

Sertoli cells, myoid cells, Leydig cells, and potentially other interstitial cells, as well as spermatogonia make up the SSC niche. In most cases, their exact contribution is unknown; however, the number of Sertoli cells appear to have a major influence on the number of niches available for SSCs. This may result from their production of glial cell-line derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), the primary and secondary critical soluble factors (Kubota et al., 2004b). When the number of Sertoli cells in the recipient testis is increased, donor cell-derived colonies are significantly increased (Oatley et al., 2011).

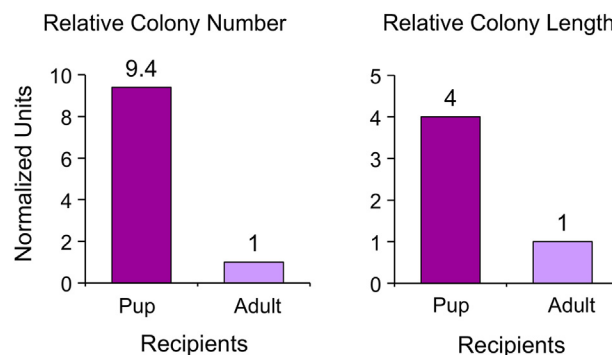


Fig. 2 Developmental effect of the stem cell niche on SSC competence. Donor SSCs from adult testes were transplanted into pup or adult recipient testes. The colony number and length generated in recipient testes were analyzed 2 months after transplantation. The colony number and length in pup testes were 9.4-fold and 4-fold greater than those in adult testes, respectively (Shinohara et al., 2001). This result indicated that the SSC niche in immature testes is more accessible and supportive for transplanted SSCs.

Additionally, a supportive role of insulin-like growth factor 1 (IGF1) and colony stimulating factor 1 (CSF1) on SSC proliferation in culture has been reported (Kubota et al., 2004b; Oatley et al., 2009). Because IGF1 and CSF1 are expressed in Sertoli cells and Leydig/myoid cells, respectively, these factors likely play a role as niche factors for SSCs.

Functional Evaluation of Spermatogenic Defects

Many congenitally infertile mouse lines that are caused by spontaneous or introduced genetic mutations have been established, and spermatogonial transplantation provides a valuable approach to elucidate the mechanisms of these spermatogenic defects. If donor germ cells from an adult spermatogenesis-impaired mutant mouse are able to colonize a wild-type recipient mouse following transplantation and reconstitute donor-derived spermatogenesis, then the SSCs of the mutant infertile mice are competent. We also know in this mutant mouse that the SSC niche is intact, because the SSCs were able to survive until puberty. Therefore, the spermatogenic failure likely results from a defect in the differentiation environment. This can be confirmed by transplantation of wild-type SSCs into the testis of the spermatogenesis-impaired mutant mouse. If the differentiation environment is defective, spermatogenesis would not be reconstituted by wild-type SSCs. On the other hand, if donor germ cells from an adult mutant mouse are unable to reconstitute donor-derived spermatogenesis in a wild-type recipient mouse, the germ cells themselves likely have a defect. Again, this can be confirmed by transplantation of wild-type SSCs. If germ cells of the mutant mice are the causal factor for impaired spermatogenesis, donor wild-type SSCs would reconstitute spermatogenesis in the spermatogenesis-impaired mutant recipients. The congenital mutations of the *Steel* (*Sl*) locus (encoding KIT-ligand, KITL) and the *white-spotting* (*W*) locus (encoding c-kit receptor tyrosine kinase, KIT) are examples of the above situations (Ogawa et al., 2000). Mice with the *Sl^d* mutation (e.g. *Kitl^{Sl}/Kitl^{Sl-d}*) show no expression of the membrane-bound form of KITL on Sertoli cells and have impaired spermatogenesis (Fig. 3B). KIT is expressed

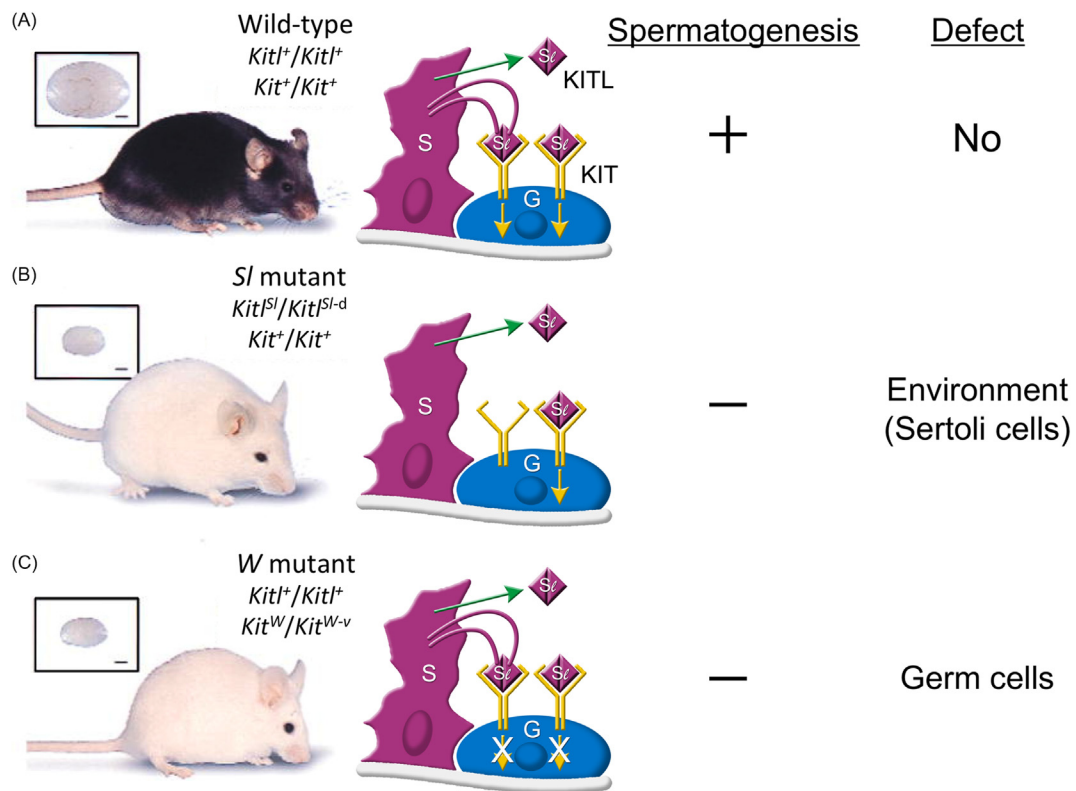


Fig. 3 Functional evaluation of congenital spermatogenic defects by spermatogonial transplantation. (A) In wild-type mice, Sertoli cells (S) express both membrane-bound KITL and soluble KITL. Spermatogonia (G) express KIT, the receptor for KITL, which interacts with both the membrane-bound and soluble forms of KITL. The membrane-bound KITL can stimulate spermatogonia more strongly than soluble KITL. Wild-type mice have no defect of these molecules and show normal spermatogenesis. (B) *Sl* mutant mice (*Kitl^{Sl}/Kitl^{Sl-d}*) have mutations in the *Sl* locus and express soluble KITL only, but not the membrane-bound KITL, by Sertoli cells. No spermatogenesis occurs in *Sl* mutant mice. (C) *W* mutant mice (*Kitl^W/Kitl^{W-v}*) have mutations in the *W* locus, and the mutant cannot transduce the stimulatory signal after binding of KITL. No spermatogenesis occurs in *W* mutant mice. When testis cells from *Sl* mutant mice are transplanted into the seminiferous tubules of *W* mutant mice, they establish spermatogenesis in the recipient *W* mutant mice, because spermatogonia of *Sl* mutant mice display the normal, wild-type KIT on immediate descendants of the SSCs, and the Sertoli cells of the *W* mutant mice express membrane-bound KITL. The recipient *W* mice are fertile and produce progeny with the *Sl* phenotype. Modified from Ogawa, T., Dobrinski, I., Avarbock, M. R. and Brinster, R. L. (2000). Transplantation of male germ line stem cells restores fertility in infertile mice. *Nature Medicine* 6, 29–34.

on differentiating spermatogonia, but its expression is negligible on undifferentiated spermatogonia in wild-type mice (Fig. 3A). Because SSCs from the Sl^d mice could reconstitute spermatogenesis after transplantation into wild-type mice that express both soluble and membrane-bound forms of KITL (Fig. 3A, $Kitl^+/Kitl^+$), it indicates that Sl^d SSCs are normal and the membrane-bound form of KITL is required for spermatogonial differentiation (Shinohara et al., 2000). Conversely, the W mutant mice have a congenital mutation of KIT (e.g. Kit^W/Kit^{W-v}) and show impaired spermatogenesis (Fig. 3C). When SSCs from wild-type mice that express KIT (Fig. 3A, Kit^+/Kit^+) were transplanted into the W mouse testes, complete spermatogenesis could be reconstituted (Brinster and Avarbock, 1994). This result indicated that the stimulatory signal via KIT on spermatogonia is crucial to complete spermatogenesis (Ogawa et al., 2000). Most notably, transplantation of SSCs from the Sl^d mutant mice into the testes of W mutant mice resulted in normal spermatogenesis and produced fertile progeny from the donor Sl^d SSCs (Ogawa et al., 2000). Using the transplantation assay between mutant and wild-type mice, the mechanisms of spermatogenic defects of a number of mutant mice have been investigated, and the functions of mutated genes are clarified. Spermatogonial transplantation is an extremely valuable method to elucidate deficiencies in the competency of SSCs including self-renewal potential and differentiation ability, their niche, or differentiation environments in infertile males.

Spermatogonial Transplantation of Non-Mouse SSCs

Application to Non-Mouse Species

Spermatogonial transplantation has now been applied to rats, goats, cattle, pigs, cats, dogs, and monkeys to study SSCs and determine their competency (Kubota and Brinster, 2017). For rats and hamsters, the procedure is identical to mouse transplantation. However, in non-rodents, the transplantation procedure and recipient preparation have been modified according to the anatomical structure of the testis. In species with a large testis and a centrally located rete testis, donor cells are generally injected using ultrasound-guided pipette placement into the rete testis, which has resulted in long-term donor-derived spermatogenesis. Furthermore, in goats and sheep, progeny have been generated by mating with females, and the donor haplotype was transmitted to subsequent generations (Herrid et al., 2009; Honaramooz et al., 2003). Therefore, spermatogonial transplantation has been shown to be applicable for identification and evaluation of SSCs of many mammalian species and has occasionally been used to assess the colonizing ability of the injected cell population. The possible limitation in studies of non-rodent SSC competency using a donor and recipient of the same species is the availability of transgenic strains, time to reach puberty, and cost for experiments in larger animals.

Xenogeneic Transplantation

An alternative method to assess SSC competence of donor cell populations from larger animals is the use of xenogeneic transplantation of the donor cell population into the testis of an immunocompromised mouse recipient (Dobrynski et al., 1999; Dobrynski et al., 2000). This approach has been used for many large animal species and partially resolves the problems mentioned above. The first xenogeneic transplantation was demonstrated when rat SSCs were transplanted into immunocompromised mouse testes, and complete long-term rat spermatogenesis was reconstituted and generated functional spermatozoa (Clouthier et al., 1996). However, when germ cells from species more phylogenetically divergent from mouse, including rabbit, dog, cat, pig, cattle, horse, monkey, and human, were transplanted into infertile immunocompromised male mice, donor-derived spermatogenesis did not occur, but primitive spermatogonia from all the species examined colonized and proliferated for 1–12 months on the basement membrane of the recipient mouse seminiferous tubules. These cells are thought to be SSCs and undifferentiated spermatogonia. To reconstitute xenogeneic spermatogenesis in mice, species specific differentiation factors will be necessary, and transplantation of testicular somatic cell suspensions including Sertoli cells, in addition to SSCs from xenogeneic species, might reestablish the environment necessary for foreign species spermatogenesis in mice. However, xenogeneic transplantation is a valuable technique to assess the SSC colonizing and self-renewal ability of cell populations from non-rodent species and has been used to judge SSC competency of cell populations from those species.

Spermatogonial Transplantation as a Method to Determine SSC Competency of Cultured Cell Populations

Competency of Mouse SSCs in Culture

Development of culture systems to support self-renewal and expansion of SSCs is enormously valuable, and the spermatogonial transplantation assay has been indispensable to develop culture systems and to determine the SSC competency of any cell population. In 1998, long-term culture of murine SSCs was first demonstrated from an unfractionated population of testicular cells using STO (SIM mouse embryo-derived thioguanine and ouabain resistant) feeders in a serum-supplemented medium, and the SSCs were maintained for about 4 months (Nagano et al., 1998). Transplantation of the cultured cells resulted in donor cell-derived spermatogenesis in the recipient testes, indicating that SSCs existed in the long-term culture. The functional assay unambiguously demonstrated that SSCs could be maintained in culture for several months, although the culture condition was insufficient to support SSC maintenance in number. Subsequently, GDNF, which had been found to be a critical factor for spermatogonial proliferation in vivo (Meng et al., 2000), showed a beneficial effect on SSC maintenance in the culture condition; however, the SSC number rapidly decreased after 1-week in culture (Nagano et al., 2003). The first report of long-term ex vivo expansion of SSCs was from

mice with a DBA/2 genetic background cultured in a proprietary medium containing serum and several growth factors, including GDNF, FGF2, leukemia inhibitory factor (LIF), and epidermal growth factor (EGF) (Kanatsu-Shinohara et al., 2003). Using the enriched culture medium, gonocytes, precursors of SSCs in neonate testes, were cultured on mouse embryonic fibroblast feeder cells. Soon after the cultivation, DBA mouse gonocytes resumed proliferation and formed grape shape aggregates, which continuously proliferated. Because SSCs in adult testes are believed to behave as single cells, which are thought to be A_s spermatogonia, the aggregated cells did not look like typical spermatogonial cells. However, the transplantation assay unequivocally demonstrated that the aggregated cells possess SSC competency, because they generated donor-derived spermatogenic colonies following transplantation. Furthermore, progeny were produced by mating with female mice. The aggregating cells derived from DBA/2 mice were named germline-stem (GS) cells; however, GS cells were not established from other mouse strains such as C57BL/6 or 129/Sv.

Essential Factors for Rodent SSC Self-Renewal

Identification of essential mitogenic factors for self-renewing proliferation of SSCs is crucial to understanding SSC competence, and this can be accomplished by development of a defined SSC culture system and subsequent transplantation of the cultured cells (Kubota et al., 2004a, Kubota et al., 2004b). A system consisting of a serum-free defined medium containing minimum components and STO feeder cells demonstrated that GDNF is the essential growth factor for proliferation of SSCs. In the serum-free culture condition, undifferentiated spermatogonia from mouse testes formed morula-like, tightly packed cellular clumps, which continuously proliferate (Fig. 4A–D). Significant strain variation existed in development of long-term SSC cultures. While SSCs from DBA/2 mice require GDNF only for their continuous proliferation, SSCs from other mouse strains, such as C57BL/6 or 129/Sv require FGF2 and a soluble GDNF receptor GFRA1, which potentiates the GDNF stimulatory signal (Kubota et al., 2004b). The cell surface phenotype of proliferating clump cells was identical to undifferentiated spermatogonia in testes, and the functional assay demonstrated that their SSC competence is also equivalent to freshly isolated undifferentiated spermatogonia from testes (Kubota et al., 2004a), indicating that the clump-forming cells in the serum-free condition are functionally identical to SSC-enriched undifferentiated spermatogonia. Under the culture condition, the clump cells having SSC competence doubled every 5.6 days and continued to proliferate for more than 6 months (Kubota et al., 2004b). The recipient males transplanted with the cultured cells produced fertile progeny carrying the donor SSC haplotype (Fig. 5).

The culture system was improved further by reducing the oxygen concentration (Kubota et al., 2009). A low O₂ atmosphere (10% O₂) compared with an air atmosphere (21% O₂) enhanced self-renewal of SSCs in culture. In particular, SSCs from *W* mouse testes, which contain very few SSCs, could proliferate under the 10% O₂ condition, but not in an air atmosphere.

The simple serum-free culture system was applicable to cultivation of SSCs from rats, and the growth factor requirements for self-renewal of rat SSCs was also determined (Ryu et al., 2005). Like mouse SSCs, GDNF is the key growth factor for rat SSCs, and FGF2 enhanced their proliferation. Under culture condition containing GDNF, FGF2, and STO feeders, rat undifferentiated spermatogonia indefinitely proliferated as clumps (Fig. 4E), and cultured clump cells generated spermatogenic colonies in Busulfan-treated infertile recipients following transplantation. By mating with females, offspring with donor haplotype was successfully produced indicating that rat clump-forming germ cells possess the full competency of SSCs. An important finding arising from the rat experiments is that self-renewal of rat SSCs is dependent on GDNF, suggesting that this growth factor may be essential for SSC self-renewal in other species.

Characteristics and Competency of Rabbit SSCs in Culture

Although rodent SSCs have been investigated extensively using the culture and transplantation systems, knowledge about non-rodent SSCs is limited. The first long-term SSC culture of non-rodent mammals was from rabbit (Kubota et al., 2011). Mice and rats diverged phylogenetically about 11 million years ago, but rabbits diverged from rodents about 60 million year ago, similar to the divergence of large animals and primates from rodents. Thus, cultivation of rabbit germ cells could provide an excellent foundation for development of SSC cultures from other non-rodent species. While STO feeder cells used for rodent SSC cultures could not support clump formation and proliferation of rabbit spermatogonia, C166 feeder cells, a yolk-sac derived endothelial cell line, did support both when GDNF was present in the serum-free medium (Fig. 4F). When these clump-forming cells were transplanted into recipient immunocompromised mice, donor cells colonized and proliferated in the recipient testes for 6 months after transplantation (Kubota et al., 2011). Furthermore, the rabbit donor cells in recipient mouse testes retained their undifferentiated spermatogonial characteristics during this period. These results suggest that the clump-forming cells cultured on C166 feeders contained rabbit SSCs. To unequivocally demonstrate that they are rabbit SSCs, evaluation by transplantation into rabbit recipients is required.

Evaluation of SSC Competency of Domestic Animals and Primates in Culture

Continuous propagation of SSCs derived from large animals and primates, including human, is critical to develop new technologies of reproductive biology and therapeutic intervention for infertility (Kubota and Brinster, 2006). Reconstitution of donor-derived spermatogenesis by cultured SSCs of non-rodent animals followed by transplantation into the same species recipients has shown limited success. Currently, no long-term in vitro expansion of bona fide non-rodent SSCs has been reported. Therefore, the rabbit culture experiments serve as a model of initial assessment of the SSC competency of the cultured cells of non-rodent animals. There

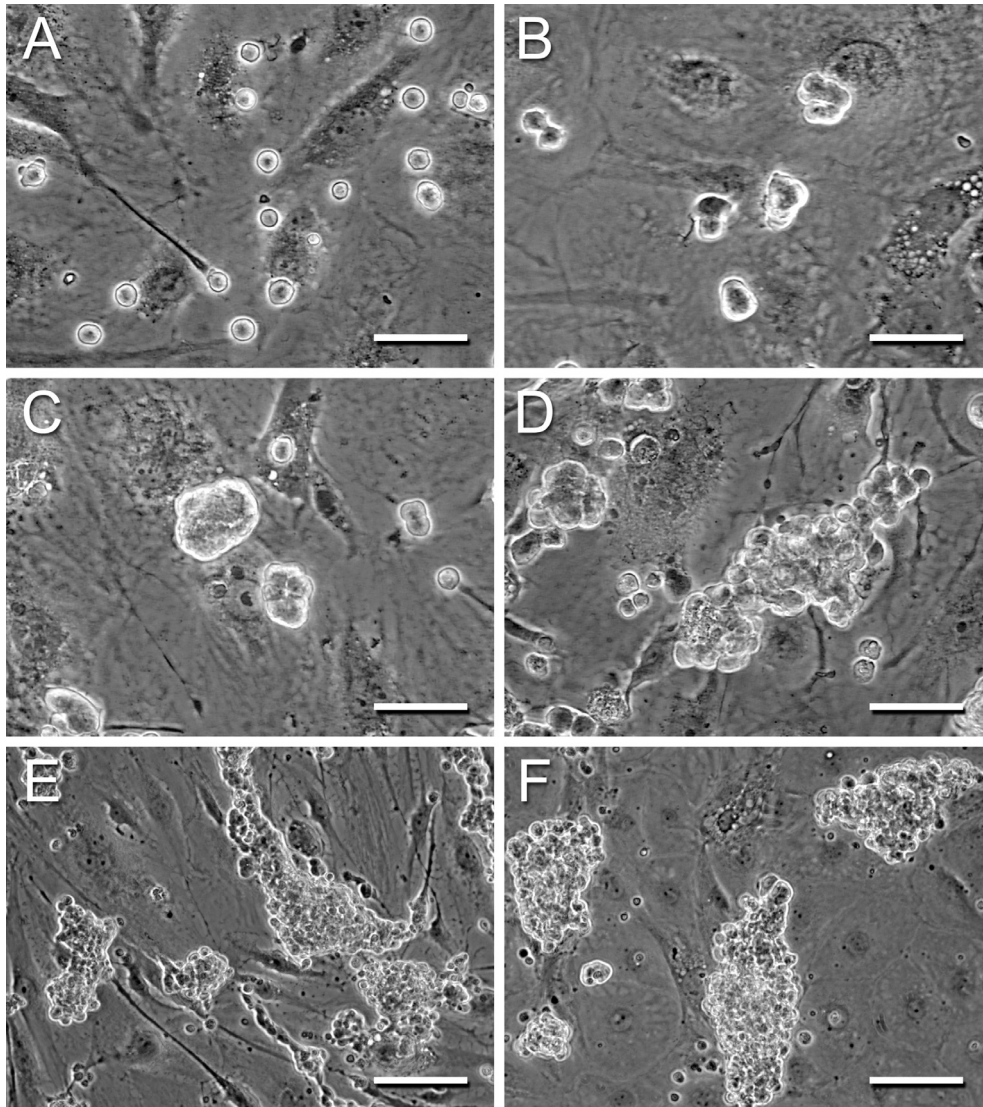


Fig. 4 Long-term in vitro expansion of SSCs. (A–D) Initiation of clump-formation of undifferentiated spermatogonia and their continuous proliferation. Undifferentiated spermatogonia from 129/Sv mice were cultured on STO feeder cells. (A) 5 h, (B) 2 days, (C) 5 days, and (D) 5 months in cultures. The clump-forming germ cells retain SSC competency equivalent to undifferentiated spermatogonia from the testis. (E) Clump-forming germ cells developed from rat undifferentiated spermatogonia on STO feeders. (F) Clump-forming germ cells developed from rabbit undifferentiated spermatogonia on C166 feeder cells. Their SSC competency was confirmed by spermatogonial transplantation into rats (E) or immunocompromised mice (F). Scale bars: (A–D) 50 μ m, (E and F) 100 μ m. Modified from Kubota, H., Avarbock, M. R. and Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16489–16494; Ryu, B. Y., Kubota, H., Avarbock, M. R. and Brinster, R. L. (2005). Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14302–14307; Kubota, H., Wu, X., Good-year, S. M., Avarbock, M. R. and Brinster, R. L. (2011). Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. *The FASEB Journal* **25**, 2604–2614.

are several studies of cultivation of putative SSCs from non-rodent species, which were evaluated by xenogeneic transplantation into immunocompromised mice (Kubota and Brinster, 2017). In most of the studies, donor cells in the recipient testes were characterized by immunohistochemistry or whole mount immuno-staining using SSC markers previously identified in rodents. Unfortunately, some of SSC markers of animals of interest were expressed in non-germ cells of primary and cultured testis cells; therefore, it was not clear that the colonized cells are indeed SSCs, which has resulted in controversy (Kubota and Brinster, 2017). Thus, for non-rodent SSCs the first step is identification of specific cellular or molecular markers of SSCs or undifferentiated spermatogonia in fresh testis samples, which can be used to identify colonized donor-cells in the recipient testes. Although the research on human SSCs is still in its infancy, phenotypic characterization of putative primate SSCs has been partially achieved (Her-mann et al., 2009). The identified molecular markers are valuable to characterize colonized putative primate donor-cells in recipients when potential SSCs in culture are transplanted. Short-term in vitro maintenance of clump cells of putative primate

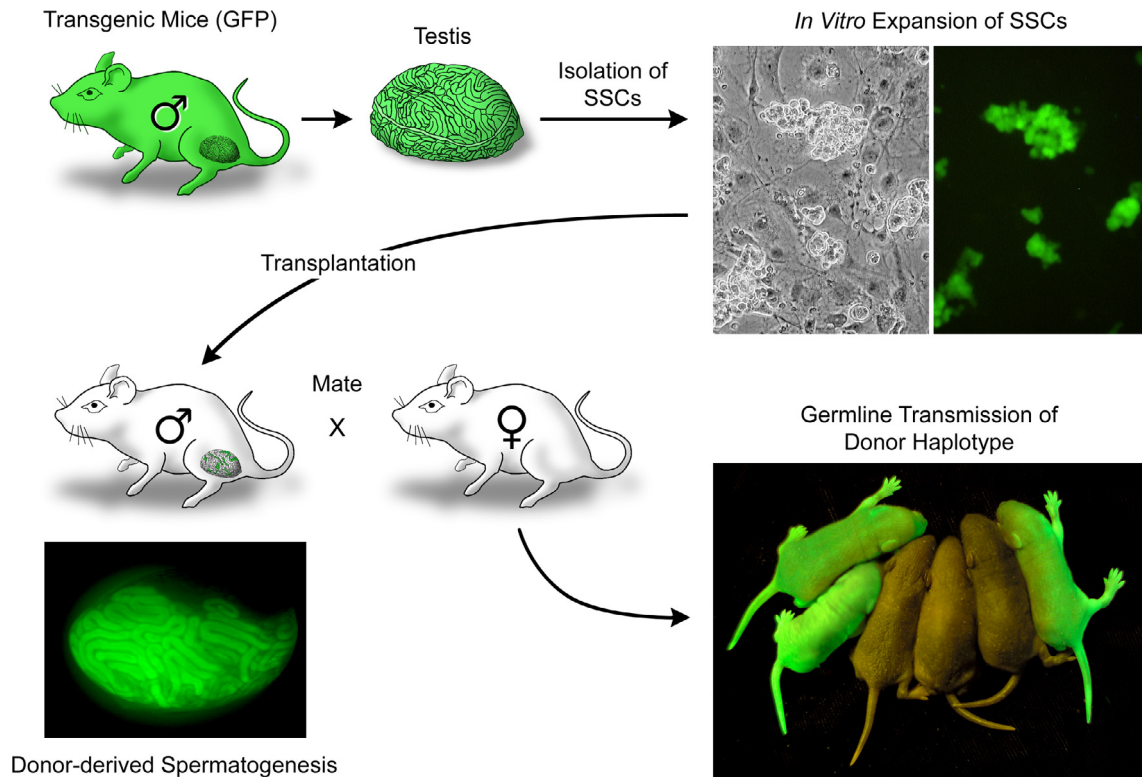


Fig. 5 Schematic diagram of SSC culture and spermatogonial transplantation. A subpopulation enriched for SSCs from testes of transgenic mice expressing a fluorescent reporter gene (e.g. GFP) are cultured on STO feeders in a serum-free medium in the presence of GDNF. After expansion in culture, clump-forming germ cells are transplanted into infertile recipient males. Mating the recipient males to wild-type females results in production of progeny with the donor male haplotype. Genetic modification during cultivation can be employed. Modified from Kubota, H., Avarbock, M. R. and Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 16489–16494.

spermatogonia has been achieved in preliminary results using the rabbit culture system (unpublished data). Xenogeneic transplantation of the clump cells into immunocompromised mice followed by characterization of colonized cells using molecular markers will provide reliable information for primate SSC characteristics. Nevertheless, evaluation of the SSC competency of the germ cell clumps is possible only by a functional transplantation assay; therefore, development of a functional assay to evaluate both the self-renewal and differentiation capacity using the same or phylogenetically-close species is essential for the future studies.

Concluding Remarks

Because stem cells are defined by their biological competence, development of a functional assay is crucial to explore their biology in depth. Since 1994, studies using spermatogonial transplantation have succeeded in clarify the identity of SSCs, function of the SSC niche, and factors influencing SSC competence, which was not possible without the approach. One of the most important achievements of the spermatogonial transplantation method is the development of culture systems that support ex vivo expansion of SSCs. The spermatogonial transplantation method in conjunction with in vitro culture systems maximizes the use of SSCs and allows development of new technology for male germline intervention and therapeutic treatments for male infertility in any mammalian species including humans.

Acknowledgments

We thank James Hayden, RBP, FBCA, for assistance in preparation of figures.

References

- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, *91*, 11303–11307.

- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Clouthier, D. E., Avarbock, M. R., Maika, S. D., Hammer, R. E., & Brinster, R. L. (1996). Rat spermatogenesis in mouse testis. *Nature*, 381, 418–421.
- Dobrynski, I., Avarbock, M. R., & Brinster, R. L. (1999). Transplantation of germ cells from rabbits and dogs into mouse testes. *Biology of Reproduction*, 61, 1331–1339.
- Dobrynski, I., Avarbock, M. R., & Brinster, R. L. (2000). Germ cell transplantation from large domestic animals into mouse testes. *Molecular Reproduction and Development*, 57, 270–279.
- Hermann, B. P., Sukhwani, M., Simorangkir, D. R., Chu, T., Plant, T. M., & Orwig, K. E. (2009). Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. *Human Reproduction*, 24, 1704–1716.
- Herrid, M., Olejnik, J., Jackson, M., Suchowerska, N., Stockwell, S., Davey, R., Hutton, K., Hope, S., & Hill, J. R. (2009). Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. *Biology of Reproduction*, 81, 898–905.
- Honaramooz, A., Behboodi, E., Megee, S. O., Overton, S. A., Galantino-Homer, H., Echelard, Y., & Dobrynski, I. (2003). Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. *Biology of Reproduction*, 69, 1260–1264.
- Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., & Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biology of Reproduction*, 69, 612–616.
- Kubota, H., & Brinster, R. L. (2006). Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature Clinical Practice Endocrinology & Metabolism*, 2, 99–108.
- Kubota, H., & Brinster, R. L. (2017). Transplantation and culture of spermatogonial stem cells. In J. Oatley, & M. Griswold (Eds.), *The biology of mammalian spermatogonia*. New York: Springer-Verlag.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2003). Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 6487–6492.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004a). Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biology of Reproduction*, 71, 722–731.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004b). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 16489–16494.
- Kubota, H., Avarbock, M. R., Schmidt, J. A., & Brinster, R. L. (2009). Spermatogonial stem cells derived from infertile Wv/Wv mice self-renew in vitro and generate progeny following transplantation. *Biology of Reproduction*, 81, 293–301.
- Kubota, H., Wu, X., Goodyear, S. M., Avarbock, M. R., & Brinster, R. L. (2011). Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. *The FASEB Journal*, 25, 2604–2614.
- Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., De Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., Pichel, J. G., Westphal, H., Saarma, M., & Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287, 1489–1493.
- Nagano, M. C. (2003). Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biology of Reproduction*, 69, 701–707.
- Nagano, M., Avarbock, M. R., Leonida, E. B., Brinster, C. J., & Brinster, R. L. (1998). Culture of mouse spermatogonial stem cells. *Tissue & Cell*, 30, 389–397.
- Nagano, M., Avarbock, M. R., & Brinster, R. L. (1999). Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biology of Reproduction*, 60, 1429–1436.
- Nagano, M., Ryu, B. Y., Brinster, C. J., Avarbock, M. R., & Brinster, R. L. (2003). Maintenance of mouse male germ line stem cells in vitro. *Biology of Reproduction*, 68, 2207–2214.
- Oatley, J. M., Oatley, M. J., Avarbock, M. R., Tobias, J. W., & Brinster, R. L. (2009). Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development*, 136, 1191–1199.
- Oatley, M. J., Racicot, K. E., & Oatley, J. M. (2011). Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. *Biology of Reproduction*, 84, 639–645.
- Ogawa, T., Dobrynski, I., Avarbock, M. R., & Brinster, R. L. (2000). Transplantation of male germ line stem cells restores fertility in infertile mice. *Nature Medicine*, 6, 29–34.
- Ryu, B. Y., Kubota, H., Avarbock, M. R., & Brinster, R. L. (2005). Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 14302–14307.
- Ryu, B. Y., Orwig, K. E., Oatley, J. M., Avarbock, M. R., & Brinster, R. L. (2006). Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. *Stem Cells*, 24, 1505–1511.
- Shinohara, T., Avarbock, M. R., & Brinster, R. L. (2000). Functional analysis of spermatogonial stem cells in steel and cryptorchid infertile mouse models. *Developmental Biology*, 220, 401–411.
- Shinohara, T., Orwig, K. E., Avarbock, M. R., & Brinster, R. L. (2001). Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 6186–6191.
- Zhang, X., Ebata, K. T., Robaire, B., & Nagano, M. C. (2006). Aging of male germ line stem cells in mice. *Biology of Reproduction*, 74, 119–124.

Sex Chromosomes and Sex-Linked Genes in Spermatogenesis

Seth D Kasowitz and P Jeremy Wang, University of Pennsylvania, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Sex determination

A founding population of primordial germ cells are designated early in embryonic development. These cells migrate to the gonadal ridge, which are bipotential embryonic structures capable of developing into either male or female reproductive organs. Expression of the Y-linked sex determining gene *Sry* triggers differentiation of the embryonic gonads into testes (Berta et al., 1990). Individuals inheriting a Y chromosome lacking *Sry* are phenotypically female, and conversely those with an XX karyotype possessing a translocated *Sry* gene develop as males. As testes develop, secreted sex hormones—androgens—bind to the X-encoded *androgen receptor*, *Ar*, and drive the continued development of the male reproductive system. Another gene on the X chromosome, the nuclear receptor *Nr0b1* (previously known as *Dax1*), is also needed for development of testis cords and establishment of Sertoli and Leydig somatic cell.

Evolution of Sex Chromosomes

The mammalian X and Y are derived from an ancient pair of autosomes. Their role as sex chromosomes began 160–250 million years ago, following divergence of therians from monotremes, with the initial acquisition of *Sry* as a sex-determining gene. *Sry* is thought to have become a male determining factor resulting from a dominant mutation in the ancestral transcription factor *Sox3* (Graves, 2006). The acquisition of sex-specific genes created regions where the chromosomal pair could no longer recombine. Nonhomologous regions between X and Y impede meiotic pairing and the gene contents of nonrecombining regions decay rapidly over evolutionary time. The extant Y chromosome presents multiple “strata” resulting from consecutive rounds of rearrangement, loss of recombination, and degeneration (Lahn and Page, 1999).

The Y chromosome is much smaller than the X, and most of it composes a nonrecombining male-specific Y region (MSY) (Hughes and Page, 2015). A small stretch of X–Y homology, called the pseudoautosomal region (PAR), still exists. Synapsis and crossover formation at the PARs are critical for male meiosis. The human PARs represent about 5% of the genome yet contain about half the gene content of the Y chromosome. MSY is largely composed of segmental duplications creating palindromic regions, some of which are grouped and designated as azoospermia factor regions (AZF) due to their deletion being the frequent source of spermatogenic failure.

Dynamics of Gene Contents on the Sex Chromosomes

The gene content of the X chromosome is not constant. The X chromosome has an excess of genes expressed in testis, presumably because of its hemizygous status in males. The hemizygous status of X-linked genes in males provides rapid selection for X-linked recessive alleles that benefit males than heterozygous autosomal recessive alleles. Any new recessive allele is immediately selected for when located on the X in males. Even if the allele would be subject to purifying selection in females, sufficient rarity would make homozygosity unlikely, and thus male benefiting traits could become fixed regardless of their effect in females. As the frequency of this allele increases, any deleterious effect can be minimized by modulating the expression of the gene to be testis-specific. This sexual antagonism provides an explanation for spermatogenesis-related genes disproportionately populating the X chromosome (Wang et al., 2001).

Studies in marsupial mammals have provided examples for accumulation of testis genes on the X chromosome. *Nr0b1* encoding nuclear receptor DAX1 has a conserved function in gonadal development across all mammals (eutherians, metatherians, and prototherians), and as noted above is critical for the formation of the testis cord structure. The recruitment of *Nr0b1* to the X occurred only after divergence of placental mammals from marsupials, where it is expressed during gonadal development despite being autosomal (Stickels et al., 2015). This pattern of marsupial autosome to placental X chromosome recruitment for genes expressed in reproductive organs has been observed in several other genes. With recruitment of marsupial autosomal *TGIF* gene to the X chromosome in both human and mouse (*Tgiflx*), its expression also shifts from ubiquitous to an exclusively testis-specific pattern. As another example, *Arx* is autosomal in marsupials but X-linked in therian mammals. Although *Arx* is not testis-specific, it is expressed in germ cells in both marsupials and therians. Therefore, the role of *Nr0b1*, *Tgiflx*, and *Arx* in testis development was established prior to their translocation to the X.

Meiotic Sex Chromosome Inactivation (MSCI)

During the prophase of meiosis I, homologous chromosomes pair, synapse, and form crossovers. At the leptotene stage, homologous chromosomes pair and programmed DNA double strand breaks (DSBs) are generated to initiate meiotic recombination. At the

zygotene stage, homologous chromosomes begin to synapse through the synaptonemal complex. DNA damage response (DDR) proteins are recruited to DSBs and form discrete foci on meiotic chromosomes. DSBs are repaired and resolved at the pachytene stage, resulting in formation of crossovers. These crossovers yield the chiasmata evident in diplotene and diakinesis stages.

Meiosis occurs in both male and female germ cells but exhibits sexual dimorphism. In oocytes, the two X chromosomes pair without issue, as they are entirely homologous. In male meiotic germ cells, the X and Y chromosomes can only synapse at the pseudoautosomal regions, leaving the majority of sex chromosomes unsynapsed. At pachytene, unsynapsed chromatins are transcriptionally silenced. In the context of the X and Y chromosomes, this general surveillance process is referred to as meiotic sex chromatin inactivation (MSCI) (Turner et al., 2005).

MSCI is triggered by phosphorylation of the histone variant H2AX and the rapid recruitment of many DDR proteins. ATR catalyzes phosphorylation of H2AX (Royo et al., 2013). MSCI is marked with silencing histone modifications including H3K9me3 and is associated with incorporation of the histone variant H3.3. MDC1 and BRCA1 recruit BRCA2 and the ubiquitin ligase RNF8, and these in turn interact with a cascading network of DDR proteins (Alavattam et al., 2016). Through interactions with FANCD2, RNF8 links the BRCA and *Fanconi anemia* (FA) repair networks. As BRCA1 signal amplifies across the unsynapsed chromosomes, RNF8 recruits the FA proteins, changing the chromatin structure and initiating chromosome-wide gene silencing. The silencing of the sex chromosomes by MSCI is essential for progression through spermatogenesis. As discussed below, failure to initiate MSCI leads to meiotic failure.

Impact of MSCI on sex chromosome gene contents

As a direct consequence of MSCI, genes on sex chromosomes are enriched for those with expression in spermatogonia and early spermatocytes (Khil et al., 2004; Wang et al., 2001). Meiotic DNA double strand breaks are generated by the enzyme SPO11. In *Spo11* knockout testis, meiosis fails to progress to the pachytene stage of prophase I. Studies of *Spo11* knockout testes confirmed that premeiotically expressed genes are over-represented on the X chromosome.

Silencing of sex-linked genes during meiosis creates a need to compensate for critical X-linked genes. *Pgk1* is an X-linked gene encoding the phosphoglycerate kinase responsible for the conversion of glucose to pyruvate, a fundamental metabolic process. Coincident with *Pgk1* silencing during meiosis, an autosomal gene *Pgk2* is transcribed exclusively in the testis (McCarrey and Thomas, 1987). The *Pgk2* gene lacks introns and thus is a retrotransposed paralogue of *Pgk1*. Retrotransposition occurs when a processed mRNA is reverse-transcribed and reintegrated into the genome. The resulting copies are often nonexpressed pseudogenes, but some can become functional retrogenes. Genes on the X chromosome are parents to a disproportionately large number of retrogenes compared with autosomally derived copies (Emerson et al., 2004). X-derived retrogenes are more likely to be subject to positive selection than being autosomal, and this selection often favors male germline biased expression.

Many additional examples of X-to-autosome retrotransposition and subsequent testis specialization have been reported. TFIID is an essential component of the RNA polymerase holoenzyme. *TAF1* encoding a subunit of TFIID is X-linked. An autosomal retrogene paralogue *TAF1L* in humans is expressed specifically in the testis and is functionally equivalent to *TAF1* in a cell line (Wang and Page, 2002). A recessive mouse mutant called *juvenile spermatogonial depletion* (*jsd*) maps to an autosomal locus where a gene, *Utp14b*, contains a frameshift mutation (Bradley et al., 2004). The UTP14 protein functions in ribosome biogenesis. *Utp14b* is a testis-specific retrogene of the X-linked *Utp14a*. In humans, the X-to-autosome retrotransposition of *UTP14A* appears to have occurred independently. *Rpl10* is X-linked and encodes a ribosomal subunit (Jiang et al., 2017). *Rpl10* is expressed in early germ cells but not in spermatocytes at the pachytene stage and beyond. An autosomal retrogene copy, *Rpl10l*, is expressed specifically in the testis and in the same stage of germ cells where *Rpl10* is silenced. RPL10-deficient cells can be rescued by RPL10L. Further evidence for the functional substitution came from the generation of a mouse model expressing the X-linked *Rpl10* gene under control of the autosomal *Rpl10l* promoter. This transgene partially rescues spermatogenic defects in *Rpl10l*-deficient mice. These examples demonstrate that the X to autosome retrotransposition provides one important evolutionary mechanism to compensate for silencing of X-linked genes due to MSCI.

Multicopy Sex-Linked Genes and MSCI Escape

The gene content of the X chromosome across mammalian species is largely conserved. However, more than 300 genes differ between mouse and human X chromosomes (Mueller et al., 2013). Most of these genes were independently recruited to the X chromosome from autosomes. The majority of these genes are present as multicopy genes in ampliconic regions on the X chromosome. The mouse Y chromosome also acquired multicopy genes (Soh et al., 2014). While many sex-linked genes remain silenced postmeiotically, some sex-linked genes reestablish transcription in postmeiotic round spermatids (Namekawa et al., 2006). In particular, multicopy genes on the X and Y chromosomes escape postmeiotic silencing. These multicopy genes are preferentially expressed in postmeiotic germ cells. The absolute expression level per gene copy is low. Therefore, it has been hypothesized that the amplification of these sex-linked genes is to compensate for the continued silencing effect on sex chromosomes in postmeiotic germ cells (Mueller et al., 2013).

Hox genes are found in multigene clusters throughout the genome. They regulate developmental processes by functioning as *Homeobox* transcription factors. Following duplication, *Hox* genes are often found to acquire specialized expression profiles and

regulatory functions. A cluster of 12 *Hox*-related genes on the mouse X chromosome are referred to as *Rhox* genes owing to their expression in reproductive tissues (MacLean et al., 2005). Some of these *Rhox* genes have been individually inactivated in mouse. Inactivation of *Rhox5* leads to subfertility owing to defects in spermatozoa maturation (MacLean et al., 2005). *Rhox8* is exclusively expressed postnatally in Sertoli cells. Knockdown of *Rhox8* impairs male fertility. Disruption of *Rhox13* has no effect on fertility, although sperm count is reduced. The multicopy gene clusters on the sex chromosomes evolve rapidly and are often divergent between species. In comparison with the mouse *Rhox* cluster, the human *RHOX* locus contains only three gene copies.

Multicopy gene families present a challenge to characterizing both the structure of the genome and determining its physiological functions. The repetitive nature of regions produced by segmental duplication confounds genome assembly, although strategies have been developed to enable sequencing of complex regions on the sex chromosomes (Mueller et al., 2013; Soh et al., 2014). A genetic approach of knocking out individual genes is ineffective for assessing the function of some multicopy gene clusters, because of functional redundancy. One successful method adapts Cre/loxP-mediated genome editing to deleting large regions containing multicopy genes. Humans with a 1.1 Mb deletion on the X chromosome (Xq22.1 deletion syndrome) develop a range of symptoms including epilepsy, mental retardation, and cleft palate. Mice lacking the syntenic region of the X chromosome phenocopied the human deletion syndrome (Zhou et al., 2014). Among the 20 genes deleted between *Nxf2* and *Nxf3* are five near identical genes in the *preferentially expressed antigen in melanoma* (*Pram*) family and several closely related copies of *Gm6215* along with multiple pseudogene copies. Many genes in this region are specifically expressed in testis. Germline-specific deletion of this region results in meiotic defects and male sterility. While a defect in chromosomal synapsis was characterized, a specific gene or gene family within the deleted region is yet to be identified. This study demonstrates that large scale deletions and subdeletions present one method for genetically characterizing the roles of multicopy gene families.

The *Sly* (Sycp3-like Y-linked) gene is present in multicopies in the MSY region of the mouse Y chromosome. As many as 70 of the ~100 copies contain open reading frames, suggesting that they may encode functional proteins. Knocking down *Sly* transcripts with silencing RNAs (siRNA) shows that SLY plays a role in maintaining postmeiotic silencing of the sex chromosomes. By directing heterochromatin formation, SLY helps repress sex-linked spermatid genes, including itself. Therefore, amplification of this gene on the Y may be, in part, a response to its self-silencing effect.

Knocking down *Sly* upregulated, among other genes, the multigene X-linked *Slx* (homologous to *Sly*) family of genes (Cocquet et al., 2012). Where SLY proteins localize to the nucleus and specifically to the sex chromosomes, SLX protein products are cytoplasmic. *Slx* transcripts were knocked down with a similar shRNA-mediated approach as developed for *Sly*. Knockdown of this gene family does not alter sex chromosome silencing, but affects sperm maturation and release into the seminiferous lumen. These X and Y chromosome multigene families are closely related. When SLY proteins are reduced, SLX proteins localize to the nucleus and appear to fill the vacated role of chromatin remodeling (Cocquet et al., 2012). Knocking down *Sly* corrects the gene regulation defects caused by knocking down *Slx*. Mice lacking both *Slx* and *Sly* gene products have better sperm maturation and fertility than knockdown of either *Slx* or *Sly* gene family alone. These studies reveal an intragenomic conflict between the X and Y chromosomes in postmeiotic germ cells.

X-Linked MicroRNA Gene Families

MicroRNAs are 22-nucleotide RNAs processed from longer primary transcripts, and are involved in translation repression and gene silencing. The density of miRNA clusters is significantly higher on the X chromosome than the autosomes. The evolution and expression of miRNAs on the X chromosome share common features with protein-coding genes discussed above. Clusters of many paralogous miRNA copies are found on the X chromosome because of recent rapid divergence. The testis expresses miRNAs from across the genome, however X-linked multimember miRNA families are more highly expressed in spermatocytes and spermatids than autosomal ones. The X-linked testis-expressed microRNA clusters are rapidly evolving, showing a substitution rate 25 times greater than autosomal clusters.

Transcription of X-linked microRNA genes is subject to MSCI (Royo et al., 2015; Song et al., 2009). Some miRNA genes on the X chromosome that have expanded by gene duplication are able to escape transcriptional silencing. Similar to MSCI-escaping protein coding genes, these microRNA genes transcribe primary transcripts through the pachytene stage. However, RNA FISH has shown that X-linked microRNA genes are silenced by MSCI and that the misexpression of these miRNAs in pachytene spermatocytes causes spermatogenic defects (Royo et al., 2015). As observed for protein coding genes, some X-linked miRNA genes have nearly identical copies on autosomes, which may substitute for their loss during MSCI.

Role of Y-Linked Genes in Spermatogenesis

The Y chromosome is dispensable for survival, but is crucial for male sex differentiation and spermatogenesis. *Sry* drives testis development and thus determines the male sex. Other Y-linked genes are not involved in sex determination but are important for spermatogenesis. The identification of Y-linked genes involved in spermatogenesis relied on mutants with naturally occurring segmental deletions of the Y chromosome. Functional requirements of candidate genes identified in the Y chromosome deletion region were tested by transgenic expression in mice with the Y chr deletion. One such study systematically examined genes lost in a sterile mouse model with a deletion on the Y chromosome. One Y-linked gene, *Eif2s3y*, was found to be essential for spermatogenesis (Mazeyrat

Table 1 Mouse sex-linked genes involved in spermatogenesis.

Gene symbol	Knockout phenotype	Chr
<i>Akap4</i>	Shortened sperm flagellum and impaired motility	X
<i>Ar</i>	Meiotic arrest at the pachytene stage	X
<i>Cdk16</i>	Defective sperm morphology and impaired motility	X
<i>Fmr1</i>	Chromosome asynapsis and impaired crossover formation	X
<i>Gcna</i>	Male sterility	X
<i>Nr0b1</i>	Testis cord formation defect; rete testis blockage	X
<i>Nxf2</i>	Reduced spermatogonial proliferation; meiotic arrest	X
<i>Prdx4</i>	Increased spermatogenic cell death due to oxidative stress	X
<i>Rhox5</i>	Increased male germ cell apoptosis; reduced fertility	X
<i>Scml2</i>	Defective spermatogenesis; reduced fertility	X
<i>Slx/Slx1-1</i>	Delayed sperm release; increased spermatid apoptosis	X
<i>Sox3</i>	Impaired differentiation of spermatogonia	X
<i>Taf7l</i>	Reduced male fertility; defects in sperm morphology	X
<i>Tex11</i>	Meiotic failure	X
<i>Tsc22d3</i>	Loss of male germ cells	X
<i>Tsx</i>	Increased apoptosis in spermatocytes	X
<i>Eif2s3y</i>	Defect in spermatogonial proliferation	Y
<i>Sry</i>	Male to female sex reversal	Y
<i>Zfy1/Zfy2</i>	Defects in sperm morphology	Y

et al., 2001). Through in vitro fertilization, only two Y-linked genes, *Sry* and *Eif2s3y*, are found to be required for development of the testis and normal spermatogenesis through the end of meiosis (Yamauchi et al., 2014). However, the mice with only two Y-linked genes (*Sry* and *Eif2s3y*) can produce some round spermatids but do not complete spermiogenesis.

Two Y-linked genes, *Zfy1* and *Zfy2*, are important for efficient MSCI and also function as a checkpoint for sex chromosome silencing during meiosis. Genes on the X and Y chromosomes escape MSCI in mice lacking *Zfy1/Zfy2*. Targeted deletion of Y-linked genes in embryonic stem (ES) cells through homologous recombination was not successful for unknown reasons. Recently, the CRISPR/Cas9 genome-editing approach has been successfully used to delete *Zfy1* and *Zfy2* in mouse (Nakasuji et al., 2017). Disruption of either gene alone has no effect on fertility. However, *Zfy1/2* double knockout mice are male sterile and show defects in sperm morphology, suggesting that these two Y-linked genes function complementarily in spermiogenesis (Table 1).

Role of X-Linked Genes in Spermatogenesis

The X chromosome contains a large number of testis-specific genes. Gene knockout approach has been informative in elucidating their functions (Table 1). Males produce sperm throughout life, because of the presence of spermatogonial stem cells. Spermatogonial stem cells proliferate, self-renew, and sustain continuous spermatogenesis. X-linked genes have been identified with critical roles at each stage of spermatogenesis. The nuclear mRNA export factor *Nxf2* and phosphatidylcholine biosynthesis protein *Pcyt1b* are both X-linked genes affecting spermatogonial proliferation. Defects in proliferation often do not impact the first wave of spermatogenesis in juveniles. Instead germ cells are depleted as spermatogonia enter meiosis without replacing themselves. Therefore, mice lacking either *Pcyt1b* or *Nxf2* progressively lose germ cells with age. In addition, *Nxf2* also regulates meiosis in spermatocytes, depending on the genetic backgrounds.

A number of X-linked genes regulate meiosis including *Fmr1*, *Tex11*, and *Scml2* (Table 1). Meiotic recombination involves formation of programmed DNA double strand breaks and subsequent repair of these breaks. The *Fragile X mental retardation protein* gene, *Fmr1*, regulates protein translation in the cytoplasm of neurons, and is also involved in the DNA damage response to meiotic double strand breaks in gametogenesis (Alpatov et al., 2014). Disruption of *Fmr1* impairs chromosome synapsis and crossover formation. TEX11 interacts with SYCP2, a component of the synaptonemal complex. TEX11 localizes as foci along the synaptonemal complex on meiotic chromosomes. TEX11 promotes chromosomal synapsis and meiotic recombination. *Tex11* knockout male mice are sterile. Some chromosomes in *Tex11*-deficient spermatocytes fail to synapse, leading to meiotic arrest and apoptosis. Additionally, *Tex11*-deficient spermatocytes form fewer crossovers, which leads to the formation of univalent chromosomes and chromosome missegregation. Testes from humans with mutations in the *TEX11* gene also display meiotic arrest (Yang et al., 2015). Germ cell specific polycomb protein, SCML2, is a critical chromatin remodeling factor during spermatogenesis (Hasegawa et al., 2015; Luo et al., 2015). SCML2 is expressed in spermatogonia and spermatocytes. In pachytene spermatocytes, SCML2 localizes to the sex body and recruits a deubiquitinating protein, USP7 to modulate histone ubiquitination in the XY chromatin. It is intriguing that these X-linked genes (*Fmr1*, *Tex11*, and *Scml2*) are presumably subject to MSCI but function in meiosis, suggesting the existence of additional developmental and posttranscriptional mechanisms in coping with MSCI.

Following meiosis, spermiogenesis proceeds with extensive chromatin remodeling, formation of an acrosome, and development of the sperm tail. Targeted deletion of *Akap4*, an X-linked gene expressed in spermatids, does not affect the number of sperm produced (Miki et al., 2002). However, maturation of sperm lacking this structural protein was severely impacted. AKAP4-deficient sperm display short abnormal tails and lack motility. Sperm from mice lacking the X-encoded protein kinase gene, *Cdk16*, also display a range of morphological abnormalities and impaired motility. Both of these X-linked knockout males are infertile. In sum, genetic studies have shown that X-linked genes, particularly testis-specific genes, function throughout spermatogenesis: mitotic proliferation, meiosis, and postmeiotic differentiation.

Sex-Linked Genes and Spermatogenic Failure in Humans

Some sex-linked genes discussed here are implicated in spermatogenic failure in humans. Mutations in *SRY* or the X-linked androgen receptor gene, *AR*, are responsible for cases of sex reversal (Jager et al., 1990; Lubahn et al., 1989). As mentioned in the previous section, mutations in the X-linked meiosis gene *TEX11* leads to meiotic arrest and nonobstructive azoospermia (Yang et al., 2015). Clinical analysis of infertile men presenting sperm with fibrous sheath dysplasia identified mutations in *AKAP4*.

The Y chromosome has multiple azoospermia factor regions (AZF) associated with male infertility: AZFa, AZFb, and AZFc. In the AZFa region, deletions lead to a Sertoli cell only phenotype, due to a complete loss of germ cells. Work in induced pluripotent stem cells (iPSC) suggests this phenotype is due to loss of *DDX3Y*, which is primarily expressed in spermatogonia. Deletion of the AZFb region causes infertility due to spermatocyte arrest. Deletion of the AZFc region, containing the *DAZ* genes, is associated with highly variable testicular defects (Reijo et al., 1995). These human studies demonstrate the important role of sex-linked genes in human sex differentiation and reproductive fecundity.

References

- Alavattam, K. G., Kato, Y., Sin, H.-S., Maezawa, S., Kowalski, I. J., et al. (2016). Elucidation of the Fanconi anemia protein network in meiosis and its function in the regulation of histone modifications. *Cell Reports*, 17, 1141–1157.
- Alpatov, R., Lesch, B. J., Nakamoto-Kinoshita, M., Blanco, A., Chen, S., et al. (2014). A chromatin-dependent role of the fragile X mental retardation protein FMRP in the DNA damage response. *Cell*, 157, 869–881.
- Berta, P., Hawkins, J. R., Sinclair, A. H., Taylor, A., Griffiths, B. L., et al. (1990). Genetic evidence equating *SRY* and the testis-determining factor. *Nature*, 348, 448–450.
- Bradley, J., Baltus, A., Skaletsky, H., Royce-Tolland, M., Dewar, K., et al. (2004). An X-to-autosome retrogene is required for spermatogenesis in mice. *Nature Genetics*, 36, 872–876.
- Cocquet, J., Ellis, P. J. I., Mahadevaiah, S. K., Affara, N. A., Vaiman, D., et al. (2012). A genetic basis for a Postmeiotic X versus Y chromosome Intragenomic conflict in the mouse. *PLoS Genetics*, 8, e1002900.
- Emerson, J. J., Kaessmann, H., Betrán, E., & Long, M. (2004). Extensive gene traffic on the mammalian X chromosome. *Science*, 303, 537–540.
- Graves, J. A. M. (2006). Sex chromosome specialization and degeneration in mammals. *Cell*, 124, 901–914.
- Hasegawa, K., Sin, H.-S., Maezawa, S., Broering, T. J., Kartashov, A. V., et al. (2015). SCML2 establishes the male germline epigenome through regulation of histone H2A ubiquitination. *Developmental Cell*, 32, 574–588.
- Hughes, J. F., & Page, D. C. (2015). The biology and evolution of mammalian Y chromosomes. *Annual Review of Genetics*, 49, 507–527.
- Jager, R. J., Anvret, M., Hall, K., & Scherer, G. (1990). A human XY female with frame shift mutation in the candidate testis-determining gene *SRY*. *Nature (London)*, 348, 452–454.
- Jiang, L., Li, T., Zhang, X., Zhang, B., Yu, C., et al. (2017). RPL10L is required for male meiotic division by compensating for RPL10 during meiotic sex chromosome inactivation in mice. *Current Biology*, 27, 1498–1505.e6.
- Khil, P. P., Smirnova, N. A., Romanienko, P. J., & Camerini-Otero, R. D. (2004). The mouse X chromosome is enriched for sex-biased genes not subject to selection by meiotic sex chromosome inactivation. *Nature Genetics*, 36, 642–646.
- Lahn, B. T., & Page, D. C. (1999). Four evolutionary strata on the human X chromosome. *Science*, 286, 964–967.
- Lubahn, D. B., Brown, T. R., Simental, J. A., Higgs, H. N., Migeon, C. J., et al. (1989). Sequence of the intron/exon junctions of the coding region of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proceedings of the National Academy of Sciences*, 86, 9534–9538.
- Luo, M., Zhou, J., Leu, N. A., Abreu, C. M., Wang, J., et al. (2015). Polycomb protein SCML2 associates with USP7 and counteracts histone H2A ubiquitination in the XY chromatin during male meiosis. *PLoS Genetics*, 11, e1004954.
- MacLean, J. A., Il, Chen, M. A., Wayne, C. M., Bruce, S. R., Rao, M., et al. (2005). Rhox: A new Homeobox gene cluster. *Cell*, 120, 369–382.
- Mazeyrat, S., Saut, N., Grigoriev, V., Mahadevaiah, S. K., Ojarikre, O. A., et al. (2001). A Y-encoded subunit of the translation initiation factor Eif2 is essential for mouse spermatogenesis. *Nature Genetics*, 29, 49–53.
- McCarrey, J. R., & Thomas, K. (1987). Human testis-specific PGK gene lacks introns and possesses characteristics of a processed gene. *Nature*, 326, 501–505.
- Miki, K., Willis, W. D., Brown, P. R., Goulding, E. H., Fulcher, K. D., et al. (2002). Targeted disruption of the *Akap4* gene causes defects in sperm flagellum and motility. *Developmental Biology*, 248, 331–342.
- Mueller, J. L., Skaletsky, H., Brown, L. G., Zaghlul, S., Rock, S., et al. (2013). Independent specialization of the human and mouse X chromosomes for the male germ line. *Nature Genetics*, 45, 1083–1087.
- Nakasuiji, T., Ogonuki, N., Chiba, T., Kato, T., Shiozawa, K., et al. (2017). Complementary critical functions of *Zfy1* and *Zfy2* in mouse spermatogenesis and reproduction. *PLoS Genetics*, 13, e1006578.
- Namekawa, S. H., Park, P. J., Zhang, L.-F., Shima, J. E., McCarrey, J. R., et al. (2006). Postmeiotic sex chromatin in the male germline of mice. *Current Biology*, 16, 660–667.
- Reijo, R., Lee, T. Y., Salo, P., Alagappan, R., Brown, L. G., et al. (1995). Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. *Nature Genetics*, 10, 383–393.
- Royo, H., Prosser, H., Ruzankina, Y., Mahadevaiah, S. K., Cloutier, J. M., et al. (2013). ATR acts stage specifically to regulate multiple aspects of mammalian meiotic silencing. *Genes & Development*, 27, 1484–1494.
- Royo, H., Seitz, H., Ellnati, E., Peters, A. H. F. M., Stadler, M. B., et al. (2015). Silencing of X-linked MicroRNAs by meiotic sex chromosome inactivation. *PLoS Genetics*, 11, e1005461.

- Soh, Y. Q. S., Alföldi, J., Pyntikova, T., Brown, L. G., Graves, T., et al. (2014). Sequencing the mouse Y chromosome reveals convergent Gene Acquisition and amplification on both sex chromosomes. *Cell*, 159, 800–813.
- Song, R., Ro, S., Michaels, J. D., Park, C., McCarrey, J. R., et al. (2009). Many X-linked microRNAs escape meiotic sex chromosome inactivation. *Nature Genetics*, 41, 488–493.
- Stickels, R., Clark, K., Heider, T. N., Mattiske, D. M., Renfree, M. B., et al. (2015). DAX1/NROB1 was expressed during mammalian gonadal development and gametogenesis before it was recruited to the eutherian X chromosome. *Biology of Reproduction*, 92, 22.
- Turner, J. M. A., Mahadevaiah, S. K., Fernandez-Capetillo, O., Nussenzweig, A., Xu, X., et al. (2005). Silencing of unsynapsed meiotic chromosomes in the mouse. *Nature Genetics*, 37, 41–47.
- Wang, P. J., & Page, D. C. (2002). Functional substitution for TAF(II)250 by a retroposed homolog that is expressed in human spermatogenesis. *Human Molecular Genetics*, 11, 2341–2346.
- Wang, P. J., McCarrey, J. R., Yang, F., & Page, D. C. (2001). An abundance of X-linked genes expressed in spermatogonia. *Nature Genetics*, 27, 422–426.
- Yamauchi, Y., Riel, J. M., Stoytcheva, Z., & Ward, M. A. (2014). Two Y genes can replace the entire Y chromosome for assisted reproduction in the mouse. *Science*, 343, 69–72.
- Yang, F., Silber, S., Leu, N. A., Oates, R. D., Marszalek, J. D., et al. (2015). TEX11 is mutated in infertile men with azoospermia and regulates genome-wide recombination rates in mouse. *EMBO Molecular Medicine*, 7, 1198–1210.
- Zhou, J., Goldberg, E. M., Leu, N. A., Zhou, L., Coulter, D. A., et al. (2014). Respiratory failure, cleft palate and epilepsy in the mouse model of human Xq22.1 deletion syndrome. *Human Molecular Genetics*, 23, 3823–3829.

Further Reading

- Graves, J. A. M. (2006). Sex chromosome specialization and degeneration in mammals. *Cell*, 124, 901–914. <https://doi.org/10.1016/j.cell.2006.02.024>.
- Hu, Y.-C., & Namekawa, S. H. (2015). Functional significance of the sex chromosomes during spermatogenesis. *Reproduction*, 149, R265–277. <https://doi.org/10.1530/REP-14-0613>.
- Krausz, C., & Casamonti, E. (2017). Spermatogenic failure and the Y chromosome. *Human Genetics*, 136, 637–655. <https://doi.org/10.1007/s00439-017-1793-8>.
- Turner, J. M. A. (2015). Meiotic silencing in mammals. *Annual Review of Genetics*, 49, 395–412. <https://doi.org/10.1146/annurev-genet-112414-055145>.
- Wang, P. J. (2004). X chromosomes, retrogenes and their role in male reproduction. *Trends in Endocrinology and Metabolism*, 15, 79–83. <https://doi.org/10.1016/j.tem.2004.01.007>.

The Cycle of the Seminiferous Epithelium

Michael D Griswold, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

The formation of spermatozoa via spermatogenesis is a continuous process throughout the reproductive lifetime of most mammals. The end products (sperm) are expelled (spermiation) from the testis and the next generation of sperm begin to develop from spermatogenic stem cells. In order to maintain the continuum of sperm production, the initiation of spermatogenesis and spermiation are coordinated. In most mammals the time required to generate spermatozoa from spermatogenic stem cells is about 30–40 days. The complexities of spermatogenesis are best known in the mouse but appear to be similar in most mammalian species. The testicular germ cells all arise from a population of spermatogenic stem cells that proceed through a well-defined differentiation process. In the mouse, the first pool of cells formed from the spermatogenic stem cells are termed the “progenitor spermatogonia” or “undifferentiated spermatogonia” (Oatley and Brinster, 2006, 2008). The term “undifferentiated spermatogonia” is a misnomer in that these cells are committed to the spermatogenic lineage. This pool acts as a reservoir of undifferentiated spermatogonia (progenitor cells) that replicate and undergo incomplete cytokinesis to form clonal chains of 2, 4, 8, or 16 cells connected by intracellular bridges. The pool of progenitor cells can be maintained for a period of time without undergoing differentiation but when acted on by retinoic acid (RA) the chains of progenitor cells immediately enter an irreversible differentiation pathway (Griswold, 2016). Once acted on by retinoic acid the clonal progenitor cells form differentiating spermatogonia that undergo a series of highly synchronized, controlled and irreversible cell divisions and differentiation steps that result in the formation of preleptotene spermatocytes. The preleptotene cells while maintaining the clonal structure undergo meiosis and spermiogenesis in additional tightly controlled events. The transition of differentiating spermatogonia also termed “A₁ spermatogonia” from progenitor cells as a result of the action of RA can be considered the initial committed step in spermatogenesis that will lead 35 days later to the release of spermatozoa in the mouse.

The key to murine reproduction is the rapid and efficient production of gametes so while the development of A₁ spermatogonia to the point of spermiation takes 35 days, the initiation of the process occurs four times more frequently (every 8.6 days in the mouse). The net result is that at any point along the seminiferous tubule there are multiple developing cell types present. The periodic and frequent initiation of the A₁ transition of spermatogonia results in a buildup of the germ cell component with the more advanced cells being moved towards the tubule lumen. The end result after 35 days are multiple germ cell layers consisting of four distinct differentiating germ cell types with spermiation occurring into the lumen of the tubule and initiation of the next round of spermatogenesis at the basal aspect of the tubule.

The linear development of germ cells over 35 days with the initiation of the A₁ transition occurring every 8.6 days results in the same grouping of germ cells being continuously repeated over time. There are 8.6 days between spermiation/initiation events and in this time each layer of cells undergoes a series of developmental steps that result in recurring groups of morphologically distinct cells found interrelated with each other (cell associations). In Fig. 1 the murine linear germ cell development is shown in more detail with each 8.6 day window shown as a blue rectangle. It is apparent from Fig. 1 that each 8.6 day period results in a series of repeating cellular associations. It is these repeating cellular associations that give rise to the 12 stages of the cycle of the seminiferous epithelium of the mouse shown in the expanded view of an 8.6 day period (Clermont, 1972; Leblond and Clermont, 1952). The number of identifiable stages in the cycle for any given species is determined solely by the morphology of the developing germ cell types. The length of one cycle is determined by the time between the transitions of undifferentiated spermatogonia to differentiating spermatogonia. If the cycle of the seminiferous epithelium from many different species is examined several commonalities can be seen. First, the A₁ transition, the onset of spermatid development and spermiation are linked to the same or closely aligned stages and four rounds of the cycle are required for the development of sperm from the onset of spermatogonial differentiation. This relationship suggests a possible common signaling mechanism for these events.

The description of the cycle thus far has focused on a single point along the seminiferous tubule and how the cell population changes with time. If the transition of undifferentiated to differentiating spermatogonia occurred at the same time in all tubules, spermatozoa would be released only every 8.6 days. This type of spermatogenesis is termed synchronous as opposed to the normal asynchronous timing. The initiation of the cycle as a result of the A₁ transition of spermatogonia is staggered along the tubule in a continuous and progressive manner. The net result is the continuum of stages of the cycle in space and the distance from one stage to a repeat of that stage constitutes the spermatogenic wave (Fig. 2). The spermatogenic cycle (repeating cellular associations with time) and the wave (repeating cellular associations along the tubule) are similar in most mammals but differ somewhat in humans and great apes. When it was shown that a cross section of a seminiferous tubule contained more than one stage of the cycle in great apes and humans, it was proposed that this was the result of a helical orientation of several spirals of spermatogenic waves (Luetjens et al., 2005; Wistuba et al., 2003). The concept of a spiral or helical arrangement of the stages to form a wave has also been challenged (Johnson, 1994).

The key factor in the formation of differentiating spermatogonia or the A₁ transition is retinoic acid. In the absence of retinoic acid the undifferentiated spermatogonia are the most advanced cell type in the testis. The presence of retinoic acid stimulates a rapid and defined progression of undifferentiated to A₁ spermatogonia that move through spermatogenesis to ultimately form

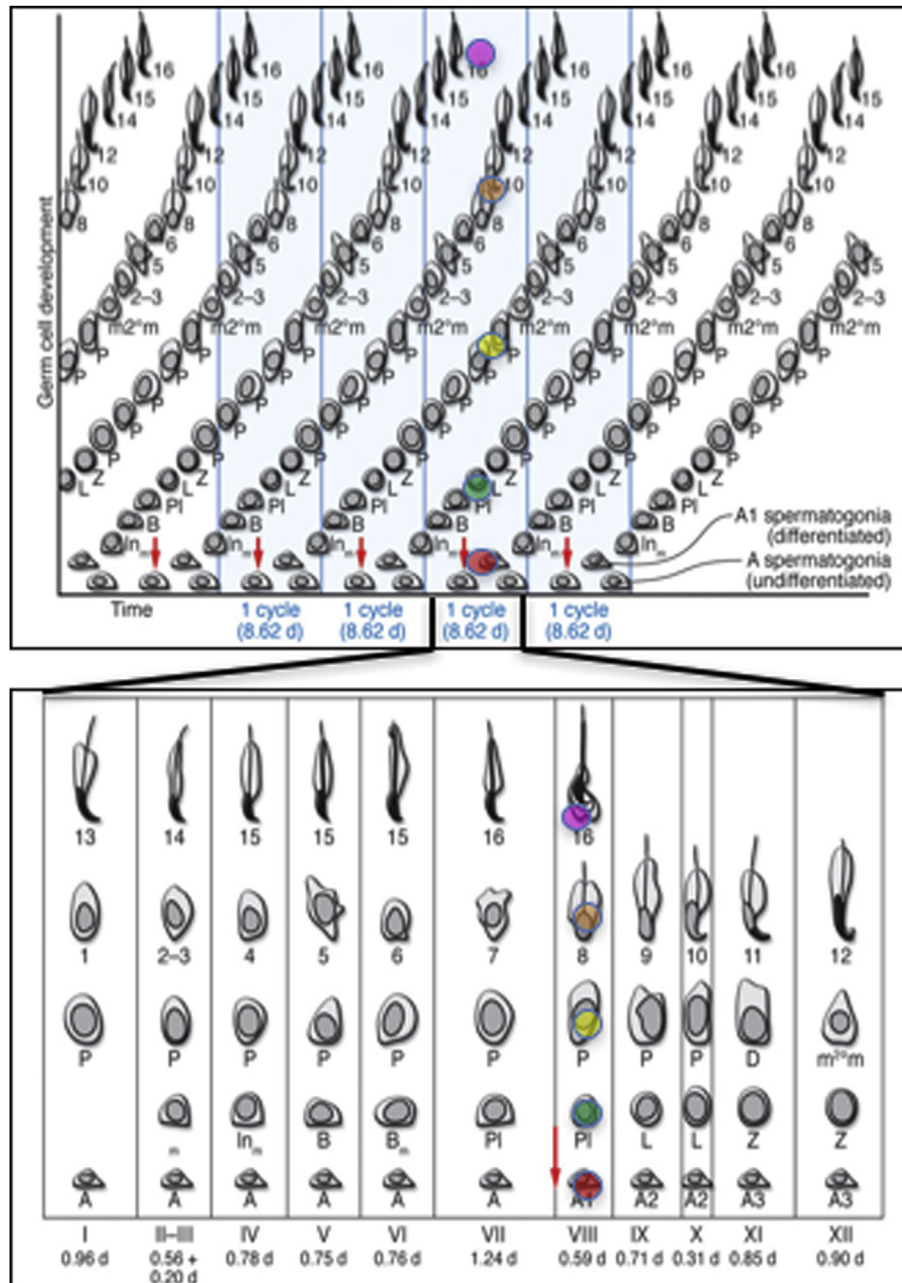


Fig. 1 (Top) Generating the recurring cellular associations that define the cycle of the seminiferous epithelium. The cycle is generated by the precisely timed transition of A spermatogonia into A₁ spermatogonia (red circle). In the mouse this transition occurs every 8.6 days. In addition it takes 8.6 days for the A₁ spermatogonia to become preleptotene spermatocytes and enter meiosis and an additional 8.6 days $\times$ 3 to form elongated spermatids ready for spermiogenesis. The net result is that once fully established the same cell associations or the same group of cell types appears every 8.6 days (designated by blue rectangles). In the 8.6 day period between the transition of A spermatogonia to A₁ spermatogonia there is a continuum of development of each cell type. Red (differentiating A₁ spermatogonia), green (preleptotene spermatocytes), yellow (pachytene spermatocytes), orange (round or elongating spermatids), magenta (elongated spermatids). The red arrows designate the timing of the pulses of retinoic acid that initiate the differentiation. In the bottom of the figure one blue rectangle is expanded to illustrate the cycle as it is normally pictured.

spermatozoa. In the normal murine testis there are pulses of retinoic acid that move progressively along the tubule to both stimulate the initiation of the cycle of the seminiferous epithelium and the release of spermatozoa (spermiogenesis).

The timing of the cycle is dependent on the time required to form spermatozoa and the frequency of retinoic acid pulses and therefore is species specific. However, the function of the cycle is to assure nonsynchronous and continuous spermatogenesis as opposed to synchronous and pulsatile release of sperm.

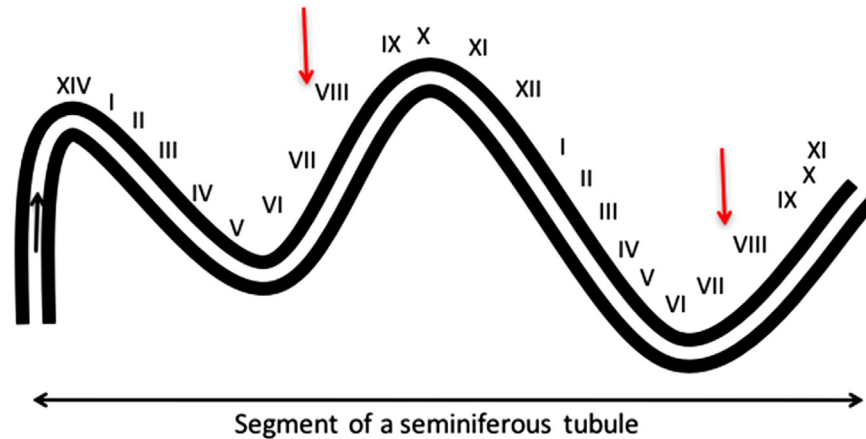


Fig. 2 Depiction of a spermatogenic wave. A section of a seminiferous tubule is shown with the distribution of stages of the cycle shown to be progressively aligned along the tubule. The wave is created by the movement of pulses of retinoic acid (*red arrow*) moving along the tubule.

References

- Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: Seminiferous epithelial cycle and spermatogenesis renewal. *Physiological Reviews*, 52, 198–205.
- Griswold, M. D. (2016). Spermatogenesis: The commitment to meiosis. *Physiological Reviews*, 96, 1–17.
- Johnson, L. (1994). A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial wave along the length of human seminiferous tubules. *Journal of Andrology*, 15, 435–441.
- Leblond, C. P., & Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Annals of the New York Academy of Sciences*, 55, 548–573.
- Luetjens, C. M., Weinbauer, G. F., & Wistuba, J. (2005). Primate spermatogenesis: New insights into comparative testicular organisation, spermatogenic efficiency and endocrine control. *Biological Reviews of the Cambridge Philosophical Society*, 80, 475–488.
- Oatley, J. M., & Brinster, R. L. (2006). Spermatogonial stem cells. *Methods in Enzymology*, 419, 259–282.
- Oatley, J. M., & Brinster, R. L. (2008). Regulation of spermatogonial stem cell self-renewal in mammals. *Annual Review of Cell and Developmental Biology*, 24, 263–286.
- Wistuba, J., Schrod, A., Greve, B., Hodges, J. K., Aslam, H., Weinbauer, G. F., & Luetjens, C. M. (2003). Organization of seminiferous epithelium in primates: Relationship to spermatogenic efficiency, phylogeny, and mating system. *Biology of Reproduction*, 69, 582–591.

Structure of the Spermatozoon

George L Gerton, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA, United States
Melissa L Vadnais, The Scripps Research Institute, La Jolla, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The functions of the sperm are to transport and contribute a haploid copy of the paternal genome to the egg and initiate development of the embryo. To achieve this goal efficiently, the structure of the sperm cell has evolved to be compact and compartmentalized. The spermatozoon can be divided into two major, morphologically distinct compartments, the head and the tail (flagellum) (Fig. 1). The head can be further subdivided into the acrosomal region and the nucleus. The acrosomal region includes the secretory acrosomal vesicle and the plasma membrane overlying the acrosome. This structure is involved in acrosomal exocytosis, zona pellucida binding, and sperm binding/fusion to the plasma membrane of the oocyte. The nucleus contains a copy of the paternal genetic material to be transported to the oocyte. The flagellum is often termed the “powerhouse” or “motor” of the sperm cell. This structure produces energy by glycolysis and oxidative phosphorylation which is used by the motile machinery in the tail to propel the sperm through the female reproductive tract and oocyte investments. The flagellum can be structurally segmented into four distinct, concatenated regions that are termed the connecting piece, the midpiece, the principal piece, and the end piece. The entire sperm cell is encased by the plasma membrane.

Sperm from diverse species may be structured differently and vary considerably in length. For example, in rodent sperm, the centrosomes and the centrioles are missing (Goto et al., 2010). In most other species, a reduced form of the centrosome with a single proximal centriole is present. In rodent sperm, the head is falciform or “hook-shaped.” In many other mammalian species, including ungulates, carnivores, and primates, the heads are spatulate or “oval-shaped.” The human sperm cell is about 60 μm long, and the flagellum is greater than 1 μm in diameter, 55 μm in length, and tapers toward the end piece. Among rodents, the sperm length varies. In the mouse, the sperm is about 120 μm in length, and rat sperm is about 190 μm long. The Chinese hamster has one of the longest mammalian sperm cells at 250 μm . The rabbit and sea urchin produce some of the shortest sperm that are 46 and 50 μm long, respectively (Eddy, 2006). On the other hand, the longest spermatozoon described to date is from the fruit fly, *Drosophila hydei*, at about 23 mm in length (Pitnick and Markow, 1994)! It should also be noted that some sperm, such as the

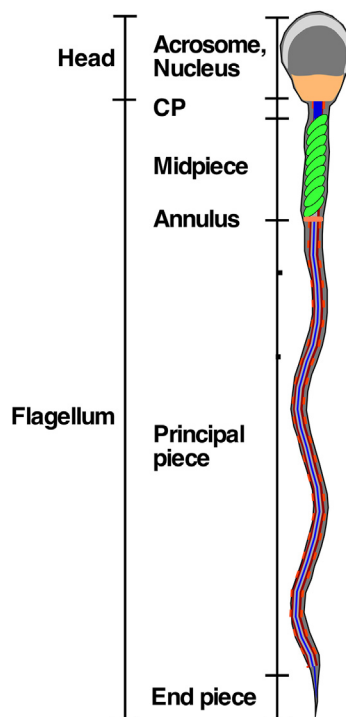


Fig. 1 Generalized picture of a mammalian sperm based on the guinea pig. The head and flagellum are indicated as are the major subdivisions of these organelles. CP: connecting piece. Green structures in the midpiece represent the mitochondria. Blue line running through the tail represents the axoneme and the red and reddish-orange structure in principal piece represent the longitudinal columns and circumferential ribs of the fibrous sheath, respectively. Orange represents the nucleus. Light gray and dark gray represent the apical and principal-equatorial segments of the acrosome, respectively.

amoeboid sperm from the round worm *Caenorhabditis elegans*, lack a flagellum (Nelson et al., 1982). Although sperm cells are generally uniform in size and shape within most species, abnormalities of morphology can exist within individuals, which may lead to subfertility or infertility.

Sperm Head

The mammalian sperm head contains the nucleus, which is capped at its anterior end by a single secretory vesicle called the acrosome (Fig. 2). During the course of spermatogenesis, the nucleus becomes extensively condensed and residual cytoplasm is removed from the cell body, resulting in a tightly compact, mature spermatozoon. In the head, the nucleus is surrounded by moderate amounts of cytoskeletal components and a very small volume of cytoplasm. Cytoskeletal components lie in the narrow space between the acrosome and the nucleus as well as between the acrosome and the plasma membrane.

Nucleus

During spermatogenesis, the chromatin is extensively remodeled. The histones associated with the deoxyribonucleic acid (DNA) are largely replaced by protamines in the sperm nucleus. Protamines are positively-charged, DNA-binding proteins that facilitate the packaging of the sperm chromatin into a compact hydrodynamic nucleus enabling the transport of a haploid copy of the male's genome to the oocyte (Brewer et al., 2002; Dadoune, 2003). The nuclear volume is less than 5% of that of a somatic cell, and the chromatin is extensively condensed to 1/13th that of the oocyte nucleus (Martins and Krawetz, 2007). The nucleus is covered by a nuclear envelope. Except for some species with redundant nuclear envelope material containing nuclear pore complexes at the base of the sperm nucleus, the nuclear pore complexes are removed during spermatogenesis (Ho and Suarez, 2003). The nuclear lamina is a protein meshwork lining the inner surface of the nuclear envelope (Elkhatib et al., 2015). It forms part of the nuclear skeletal network anchoring the chromatin and is made up of lamins, which are members of the intermediate filament family of proteins. The perinuclear theca forms a rigid shell composed of disulfide bond-stabilized structural proteins incorporated with numerous other protein molecules (Korley et al., 1997).

Acrosome

The acrosome is a Golgi-derived, membrane-enclosed vesicle that forms a cap in the proximal segment of the sperm head (Figs. 1 and 2). Morphologically, the acrosome consists of three segments: *apical*, which extends beyond the anterior aspect of the nucleus; *principal*, which overlies the anterior aspect of the nucleus; and *equatorial*, which forms a thinner posterior band around the nucleus.

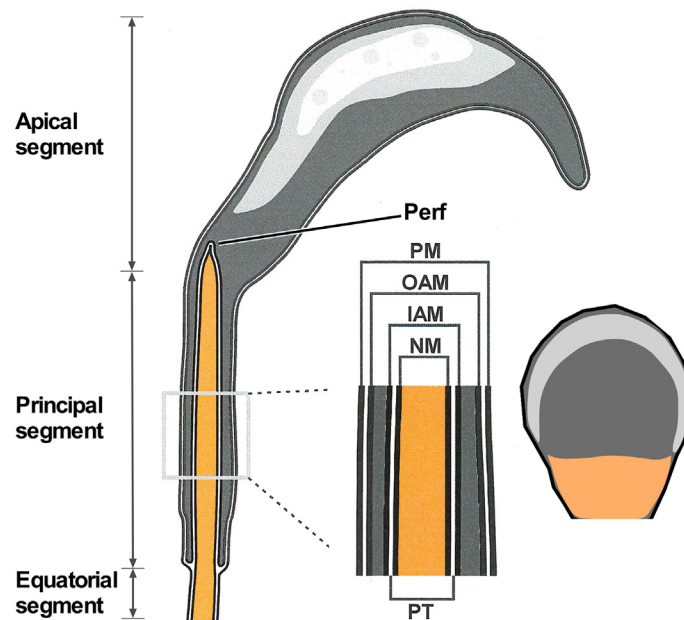


Fig. 2 Diagram of the nucleus and acrosomal regions of the guinea pig sperm. On the *left*, a longitudinal section through the head shows that the acrosome can be delineated into three major zones: apical, principal, and equatorial segments. The central diagram shows the various membrane domains: PM, plasma membrane; OAM, outer acrosomal membrane; IAM inner acrosomal membrane; and NM, nuclear membrane. Also shown is the perinuclear theca (PT) that lies between the inner acrosomal membrane and the nuclear membrane. The nucleus is colored *orange*. The figure on the right shows a dorsal view of the sperm head.

Biochemically, the contents of the acrosome can be considered to be of two general states: freely soluble or insoluble (matrix or membrane-associated). Some of the proteins found in the acrosome are thought to be necessary for sperm binding to or penetration of the oocyte zona pellucida or to be required for the dissolution of the insoluble acrosomal matrix (Gerton, 2002; Yoshinaga and Toshimori, 2003). The membrane enclosing the acrosome can be demarcated into the inner acrosomal membrane, which is laminated to the nuclear envelope, and the outer acrosomal membrane, which is apposed to the inner face of the plasma membrane overlying the acrosome. The distinctions of these two zones are set up during early spermiogenesis when the membrane of the pro-acrosomal vesicle contacts and then flattens against the nuclear envelope, forming the inner acrosomal membrane. As spermiogenesis proceeds, the expanding acrosomal vesicle continues to spread over the apical end of the nucleus as spermatid elongation occurs (Hermo et al., 1980). Some proteins of the acrosomal matrix associate with the inner and/or outer acrosomal membranes; in addition, they may partition within specific zones of the matrix. Like other Golgi-derived vesicular elements, the acrosomal membranes contain glycoproteins like IZUMO1 (the inner acrosomal membrane, oolemma-binding protein (Inoue et al., 2005)) which have specific functions in gamete fusion. During acrosomal exocytosis, the outer acrosomal membrane fuses with the overlying plasma membrane and the newly created hybrid vesicles are shed from the sperm while the inner acrosomal membrane remains intact and becomes a new part of the plasma membrane in that region of the sperm head.

Acrosomal shape and size vary widely between species (Fawcett, 1970). In animals with a spatulate, oval-shaped, sperm head, the acrosome is flattened in the plane of the lateral (x) and anterior-posterior (z) axes of the sperm cell. In contrast, mureoid rodent sperm cells, such as those from mouse, rat, and hamster, have a falciform or hooked-shaped head containing an acrosome that overlies the convex and, to a lesser extent, concave margins of the nucleus; the nuclei of these cells are flattened in the y-axis. Conversely, sperm from caviid rodents such as guinea pigs start out with a spatulate morphology that morphs during epididymal maturation into a cupped shape that resembles the jai alai basket known as a *cesta*; in longitudinal section (yz plane), these sperm appear to have falciform-shaped heads. The acrosomes of guinea pig sperm are quite large and the apical segments can be readily isolated, making these sperm an excellent model for studying the biochemistry of acrosomal dynamics.

Cytoskeletal, Cytoplasmic, and Membrane Distinctions

Various terms have been utilized to describe extra-nuclear and extra-acrosomal regions of the sperm head and the reader is warned that the use of these terms may vary slightly among different investigators. The plasma membrane region that is in close association with the outer acrosomal membrane may be described by the term *periacrosomal* (from the Greek *peri* meaning “about, around”) (Flaherty and Olson, 1988). Cytoskeletal and cytoplasmic regions can be considered as three specific compartments: *subacrosomal* (between the inner acrosomal membrane and the nuclear envelope), *postacrosomal* (between the plasma membrane and posterior nucleus), and *para-acrosomal* (the cytoplasmic/cytoskeletal region adjacent to the outer acrosomal membrane) (Eddy, 2006). The *perinuclear theca* is a sheath that encases the full extent of the sperm nucleus; it can be further subdivided into the postacrosomal perinuclear theca and the subacrosomal perinuclear theca. The *perforatorium* is a specialized structure found in the apical region of the subacrosomal perinuclear theca. Each segment serves a distinct function. For example, the subacrosomal layer of the perinuclear theca functions to anchor the acrosome to the anterior region of the nucleus.

Sperm Flagellum

The biogenesis of the mammalian sperm flagellum is an orderly process that occurs during spermiogenesis, the last phase of spermatogenesis when the haploid spermatids differentiate into spermatozoa. Immediately following the completion of meiosis, each round spermatid elaborates a primitive flagellum, which initially consists of the *axoneme* surrounded by the plasma membrane. Subsequently, the remaining three major flagellar accessory structures, *outer dense fibers*, *mitochondrial sheath*, and *fibrous sheath*, are produced in a temporally-regulated manner. Since the axoneme is the key for flagellar structure, it will be addressed first.

The Axoneme: The Foundation for Flagellar Structure and Function

The axoneme extends from the connecting piece along the full length of the flagellum and generates the propulsive force for sperm cell movement (Figs. 1 and 3). In addition to numerous other proteins, the major components of the axoneme are cytoskeletal tubulins and dynein motor proteins. The axonemal structure is composed of nine circumferentially arranged outer microtubular doublets and two central microtubular singlets (canonical “9 + 2” arrangement of microtubules), composed of dimers of α -tubulin and β -tubulin. Each doublet consists of an “A microtubule” and a “B microtubule.” The B microtubule is configured in a C-shaped and attaches onto the side of the A microtubule. On cross section, two dynein arms extend from the A microtubule toward the B microtubule of the next microtubular doublet. In most mammals, the central pair of microtubules extends longitudinally from the implantation fossa through the connecting piece to the end piece, whereas the other microtubules travel from the remnants of the base of the distal centriole to the end piece. Radial spokes are structures that extend from the base of the A microtubules to the central pair (Fig. 3D) (Inaba, 2011).

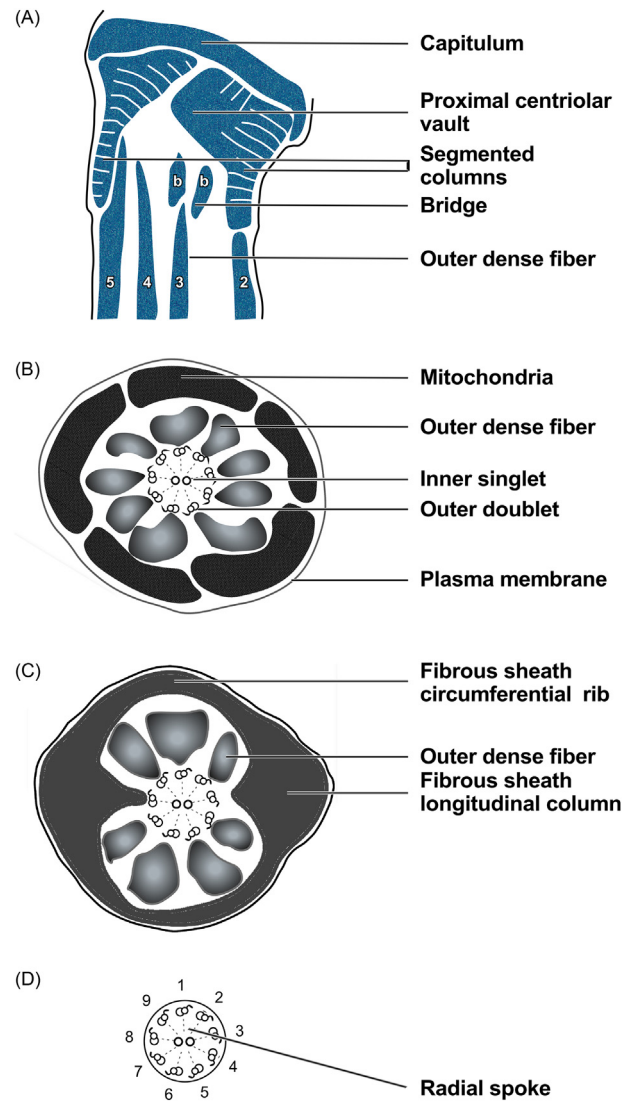


Fig. 3 Diagram of the four major regions of the mammalian sperm flagellum in cross section. (A) Connecting piece (based on the study of Ounjai et al. (2012)). (B) Midpiece. (C) Principal piece. (D) End piece. The numerals in D represent the numbers assigned to the outer doublets of microtubules.

Flagellar Segments and Their Accessory Structures

Connecting piece

The flagellum fastens to the base of the sperm head by the *connecting piece* (Fig. 3A). The *implantation fossa* is a specialized domain of the posterior nucleus that adheres to the proximal face of an electron dense structure of the connecting piece, known as the *basal plate*. Additionally, the *capitulum* initiates at the distal face of the basal plate, conforming to the shape of the implantation fossa. The connecting piece retains some centriolar proteins, which act to stabilize the proximal part of the flagellum (Ounjai et al., 2012; Woolley et al., 2008). In most mammals, except rodents, the capitulum contains the proximal centriole, which is a remnant of the bi-centriolar centrosome found in early haploid spermatogenic cells. The complete degradation of both centrioles during spermiogenesis distinguishes rodents from other species. The centrosome is an amorphous cytoplasmic region approximately 0.5 μm in length and 0.2 μm in diameter that functions as the primary microtubule organizing center and regulates the nucleation and spatial organization of microtubules.

The capitulum is contiguous with the *segmented or striated columns*. At the zone where the connecting piece transitions into the midpiece, there are nine striated or segmented columns, held together by filamentous linkages. The segmented columns extend 1–2 μm posteriorly from the capitulum and connect at their distal ends to the outer dense fibers, cytoskeletal structures that continue into the midpiece and principal piece segments. At the proximal end, the segmented columns fuse with each other to form the centriolar vault, created by the disintegration of the proximal centriole. At the distal end, the segmented columns meld with the outer dense fibers to form the distal centriolar vault, organized by outer dense fibers 3 and 8 that associate with a bar-like structure.

Midpiece

The *midpiece* of the sperm flagellum is distinguished by its relatively greater thickness compared to the more distal principal piece (Fig. 3B). The midpiece is bordered at its anterior aspect by the connecting piece and at its posterior limit by the annulus, a transverse ring of dense material. Mitochondria and the outer dense fibers are key accessory structures of the midpiece.

There are nine outer dense fibers and these are located between the mitochondrial sheath and the axoneme (Fawcett, 1975). They are numbered corresponding to the adjacent outer microtubule axonemal doublets from which they emanate. The outer dense fibers adhere to the connecting piece at the proximal end of the flagellum and, generally, run longitudinally the full length of the midpiece and most of the principal piece. The exceptions to this rule are outer dense fibers 3 and 8, which are replaced by the longitudinal columns of the fibrous sheath in the principal piece. Outer dense fibers 1, 5, 6, and 9 are larger in cross section than fibers 2, 3, 4, 7, and 8. In the proximal region of the midpiece, the outer dense fibers start out thick and then gradually taper in size as they progress toward the flagellar end piece. They terminate in the proximal half of the principal piece in primate sperm but extend to near the end of the principal piece in rodent sperm (Fawcett, 1975). The last to be curtailed are the large outer dense fibers 1, 5, and 6. Electron microscopy has determined that each outer dense fiber is composed of a cortex and medulla. The cortex is a thin layer and seems to be composed of a single lamina of globular particles 6–7 nm in diameter; whereas, the medulla appears electron dense with no obvious substructure (Olson and Sammons, 1980). The cortex appears to be continuous with the satellite fibers that are present in the flagellar matrix at the surface of the outer dense fibers facing the axoneme (Irons and Clermont, 1982).

The predominant characteristic of the midpiece is the sheath of mitochondria wrapped in a spiral pattern around the nine outer dense fibers that form intimate associations with the outer microtubular doublets of the axoneme. In most species, the structure of the mitochondrial sheath is highly uniform. The midpiece length is related to the length of the sperm cell. In most mammals it is between 5 and 12 μm , but in rats and Chinese hamsters, the midpiece is 64 and 100 μm long, respectively (Baccetti, 1982, 1986). Sperm mitochondria are elongated and flattened compared to those found in somatic cells. The number of helices made by the mitochondria and the lengths of the midpieces vary among species (Eddy, 2006). Each sheath consists of approximately 75–100 mitochondria that are degraded by targeted proteolysis once the sperm flagellum has been drawn inside the fertilized ovum; as a consequence, the mitochondrial genome is inherited maternally (Sutovsky et al., 2004). In the midpiece, the mitochondrial sheath adheres to an underlying complex of filaments named the sub-mitochondrial reticulum (Olson and Winfrey, 1990). This complex is a network of ribbons of filamentous material that are laterally interconnected. The sub-mitochondrial reticulum terminates and fuses with the *annulus* at the junction between the midpiece and the principal piece.

Principal piece

Just posterior of the annulus, the principal piece begins and continues for most of the remainder of the flagellar length (Fig. 3C). Mitochondria are not found beyond the annulus although the axoneme and seven of the nine outer dense fibers are continuous with the same structures in the midpiece. A new flagellar accessory structure, the *fibrous sheath*, is the longest section of the sperm flagellum and closely underlies the plasma membrane. The fibrous sheath delineates the morphological boundaries of the principal piece and is characterized by circumferential ribs that bridge two longitudinal columns. The fibrous sheath can be considered as a tapering cylinder. The tips of the ribs thicken as they approach the longitudinal columns. This thickness decreases toward the end of the principal piece. The circumferential ribs end abruptly at the posterior margin of the principal piece where it transitions to the end piece (Fawcett, 1970, 1975). As mentioned above, outer dense fibers 3 and 8 are replaced by these columns in the principal piece. The longitudinal columns are 15–20 nm in diameter and formed by longitudinally positioned, loosely packed filamentous structures. The longitudinal columns run tangentially to and attach to ridges projecting from microtubule doublets 3 and 8 of the axoneme (Fawcett, 1970). In sperm with a falciform shape (mostly rodents species), the dorsal longitudinal column is closest to the microtubule outer doublet 3 while the ventral longitudinal column is closest to the microtubule outer doublet 8 (Fawcett, 1970). The fibrous sheath is an important structure of the sperm flagellum. It functions as a scaffold for glycolytic enzymes and proteins involved in signaling pathways that regulate sperm maturation, motility, capacitation, hyperactivation, and/or acrosomal exocytosis (Krisfalusi et al., 2006).

The plasma membrane of the principal piece differs in its composition compared to the midpiece. For example, components of the CATSPER calcium channel, which is required for male fertility, are restricted to the principal piece and are exquisitely structurally aligned with the longitudinal columns of the fibrous sheath. This, in addition to axoneme-driven motility, the principal piece of the sperm flagellum is significant for both metabolic reasons (compartmentalization of metabolic processes such as glycolysis) and cell signaling (specific protein kinases and ion channels).

End piece

The *end piece* of the sperm flagellum is the most distal segment. It contains no structural elements other than the axoneme (Fig. 3D). The end piece contains the axonemal doublets and begins where the outer dense fibers and fibrous sheath end. The plasma membrane of the end piece is contiguous with that of the principal piece.

Concluding Comments

The function of the spermatozoon is dictated by its structure. The complexity of this cell with its unique composition of proteins and organelles that are assembled in a temporally and spatially organized manner during spermiogenesis provides a challenging subject for study in the quest to understand the basis for male fertility.

References

- Baccetti, B. (1982). The evolution of the sperm tail. *Symposia of the Society for Experimental Biology*, 35, 521–532.
- Baccetti, B. (1986). Evolutionary trends in sperm structure. *Comparative Biochemistry and Physiology. A, Comparative Physiology*, 85(1), 29–36.
- Brewer, L., Corzett, M., & Balhorn, R. (2002). Condensation of DNA by spermatid basic nuclear proteins. *The Journal of Biological Chemistry*, 277(41), 38895–38900.
- Dadoue, J.-P. (2003). Expression of mammalian spermatozoal nucleoproteins. *Microscopy Research and Technique*, 61(1), 56–75.
- Eddy, E. M. (2006). Chapter 1. The Spermatozoon. In E. Knobil (Ed.) (3rd ed.), *vol. 1. Knobil and Neill's physiology of reproduction* (pp. 3–54). Amsterdam, Boston: Elsevier.
- Elkhatib, R., Longepied, G., Paci, M., Achard, V., Grillo, J.-M., Levy, N., Mitchell, M. J., & Metzler-Guillemain, C. (2015). Nuclear envelope remodelling during human spermiogenesis involves somatic B-type lamins and a spermatid-specific B3 lamin isoform. *Molecular Human Reproduction*, 21(3), 225–236.
- Fawcett, D. W. (1970). A comparative view of sperm ultrastructure. *Biology of Reproduction*, 2(suppl_2), 90–127.
- Fawcett, D. W. (1975). The mammalian spermatozoon. *Developmental Biology*, 44, 394–436.
- Flaherty, S. P., & Olson, G. E. (1988). Membrane domains in guinea pig sperm and their role in the membrane fusion events of the acrosome reaction. *Anatomical Record*, 220, 267–280.
- Gerton, G. L. (2002). Function of the sperm acrosome. In D. M. Hardy (Ed.), *Fertilization* (pp. 265–302). San Diego, CA: Academic Press.
- Goto, M., O'Brien, D. A., & Eddy, E. M. (2010). Sperliolin is a novel human and mouse sperm centrosome protein. *Human Reproduction*, 25(8), 1884–1894.
- Hermo, L., Rambourg, A., & Clermont, Y. (1980). Three-dimensional architecture of the cortical region of the Golgi apparatus in rat spermatids. *The American Journal of Anatomy*, 157(4), 357–373.
- Ho, H.-C., & Suarez, S. S. (2003). Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biology of Reproduction*, 68(5), 1590–1596.
- Inaba, K. (2011). Sperm flagella: Comparative and phylogenetic perspectives of protein components. *Molecular Human Reproduction*, 17(8), 524–538.
- Inoue, N., Ikawa, M., Isotani, A., & Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature*, 434(7030), 234–238.
- Irons, M. J., & Clermont, Y. (1982). Formation of the outer dense fibers during spermiogenesis in the rat. *The Anatomical Record*, 202(4), 463–471.
- Korley, R., Pouresmaeili, F., & Oko, R. (1997). Analysis of the protein composition of the mouse sperm perinuclear theca and characterization of its major protein constituent. *Biology of Reproduction*, 57(6), 1426–1432.
- Krisfalusi, M., Miki, K., Magyar, P. L., & O'Brien, D. A. (2006). Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. *Biology of Reproduction*, 75(2), 270–278.
- Martins, R. P., & Krawetz, S. A. (2007). Nuclear organization of the protamine locus. *Society of Reproduction and Fertility Supplement*, 64, 1–12.
- Nelson, G. A., Roberts, T. M., & Ward, S. (1982). *Caenorhabditis elegans* spermatozoan locomotion: Amoeboid movement with almost no actin. *The Journal of Cell Biology*, 92(1), 121–131.
- Olson, G. E., & Sammons, D. W. (1980). Structural chemistry of outer dense fibers of rat sperm. *Biology of Reproduction*, 22(2), 319–332.
- Olson, G. E., & Winfrey, V. P. (1990). Mitochondria-cytoskeleton interactions in the sperm midpiece. *Journal of Structural Biology*, 103(1), 13–22.
- Ounjai, P., Kim, K. D., Lishko, P. V., & Downing, K. H. (2012). Three-dimensional structure of the bovine sperm connecting piece revealed by electron cryotomography. *Biology of Reproduction*, 87(3), 73.
- Pitnick, S., & Markow, T. A. (1994). Large-male advantages associated with costs of sperm production in *Drosophila hydei*, a species with giant sperm. *Proceedings of the National Academy of Sciences of the United States of America*, 91(20), 9277–9281.
- Sutovsky, P., Van Leyen, K., McCauley, T., Day, B. N., & Sutovsky, M. (2004). Degradation of paternal mitochondria after fertilization: Implications for heteroplasmy, assisted reproductive technologies and mtDNA inheritance. *Reproductive Biomedicine Online*, 8(1), 24–33.
- Woolley, D. M., Carter, D. A., & Tilly, G. N. (2008). Compliance in the neck structures of the guinea pig spermatozoon, as indicated by rapid freezing and electron microscopy. *Journal of Anatomy*, 213(3), 336–341.
- Yoshinaga, K., & Toshimori, K. (2003). Organization and modifications of sperm acrosomal molecules during spermatogenesis and epididymal maturation. *Microscopy Research and Technique*, 61(1), 39–45.

Further Reading

- Toshimori, K., & Eddy, E. M. (2015). Chapter 3—The spermatozoon. In *Knobil and Neill's physiology of reproduction* (4th edn., pp. 99–148). San Diego: Academic Press.

Chromatin Structure in Sperm: Composition and Function

W Steven Ward, University of Hawaii at Manoa, Honolulu, HI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromatin DNA packaged by proteins. This is the way DNA molecules, which are much longer than the cell, that fit into the nucleus.

Epigenetics Heritable structures associated with chromatin that are not part of the DNA sequence. The most common forms of epigenetic modifications are DNA methylation and histone posttranslational modifications.

Histone A family of basic proteins that bind to DNA in most cells to form chromatin.

Nucleosomes The basic unit of somatic cell chromatin structure. One nucleosome contains two tetramers of histones each made up of one histone H1, H2A, H2B and H3, and about 200 bp of DNA coiled twice around the histones.

Protamine Very basic proteins that fold DNA into chromatin only in sperm cells.

Spermatozoa The male gamete. It is the final, mature form of the sperm cell that carries the father's DNA to the oocyte.

Somatic cell Any cell that is not a germ cell. The vast majority of cells in the body are somatic.

Introduction: The Complexity of the Problem

The sperm cell has the seemingly impossible task of packaging all the father's genes into a tiny compartment (the sperm nucleus) that must make a voyage that is equivalent to a person walking 1 (mouse) or 10 (human) miles to deliver that genetic information completely and utterly intact. The genes are encoded in 23 strands of DNA (in humans) that if stretched end to end is 1 m long, and is packaged into a tiny volume about 10 millionths of a meter (10 μm) long and half to 1 millionths of a meter (0.5–1.0 μm) wide. This is a difficult problem to visualize, and the best image is one that the distinguished professor at Johns Hopkins, Don Coffey, used to explain it with the now outdated technology of tape cassettes (Fig. 1). Tape cassettes organized long stretches of magnetized tape into a coils that the player could read. The tape had to be organized on the cassette's rollers for it to be functional. In somatic cells—the cells that make up our bodies—with the full complement of 46 chromosomes, 2 m of DNA must fit into a sphere about 10 μm in diameter—much larger than a sperm cell, but still relatively small. In both sperm and somatic cells, the DNA is coiled and



Fig. 1 Tape cassette as model for chromatin. All the tape has been pulled from the cassette and piled below it. The tape cassette is a good model for the folding the long strands of DNA into chromosomes. In both the tape cassette and the chromosome, the information-bearing strands, which are many orders of magnitude longer than the final compact package, must be ordered in a way that the information on the strands is accessible.

packaged by proteins into chromatin—complex units of DNA and protein that coil the long strands of DNA into functional units inside the cell. The major difference between the two is that in somatic cells the DNA must be available for all its normal functions—transcription of the 23,000 protein encoding genes and the million or so non-coding RNA genes, and DNA replication when the cell divides. The sperm cell, on the other hand, shuts down all DNA functions and can further compact the DNA. But, the sperm cell must deliver that DNA in a packaged form that can be easily opened, and, probably contains information about which genes to activate first, and where to start DNA replication. The sperm cell, like the oocyte, has the added burden of delivering a pristine copy of one parent's DNA to the oocyte to form the embryo, and, unlike the oocyte, to take that DNA on a long and hazardous journey protecting the DNA all the way.

There is a great deal we know about chromatin structure in sperm, but even more that we have yet to discover. In this article, we will explore both known aspects of sperm chromatin structure and the questions that remain.

Histone Based Chromatin (Most Cells and a Small Part of the Sperm Chromatin)

To understand sperm chromatin structure, we need a brief reminder about chromatin structure in most other cells. The 2 m of DNA is first coiled into nucleosomes by an octamer of proteins called histones ([Fig. 2B](#)). To coil DNA, the biggest problem is the negative charges that line both strands of the double helix because of the phosphates. These negative charges cause the DNA to repel from itself. The four human core histones are composed of 22%–25% basic amino acids (histidine, lysine and arginine) that have positive charges that can counter the negative charges of the DNA. This allows the histone octamers to wind about 200 bp of DNA twice around it to form nucleosomes, condensing the DNA strand into a “beads on a string”. Nucleosomes are usually depicted as short cylinders, but are, in fact, dimers of two short coils of four histones (H2A, H2B, H3 and H4) each connected together ([Luger et al., 1997](#)). They form a natural path for the DNA to coil twice around them. Note that the DNA is fairly accessible on at least one side, and many proteins are capable of binding to it in sequence-specific manners even in the nucleosome form. Nucleosomes are then compacted further ([Fig. 2C](#)) into what is called heterochromatin, a chromatin form that usually indicates genes that have been shut down. This packaging does not shorten the 2 m of DNA enough to fit inside the nucleus, however, and further packaging is required. To do this, the chromatin is organized into loop domains by a nuclear matrix ([Fig. 2D and E](#)). Thus, in somatic cells, the 2 m of DNA is compacted by histones and the nuclear matrix to fit inside the nucleus, but in a form that is still accessible for its functions.

Protamine Based Sperm Chromatin

During spermatogenesis, the chromatin of the precursor to the sperm cell, round spermatids, transforms its structure markedly from a typical histone-bound structure to a much more compact form. The proteins, called protamines, that form mammalian sperm chromatin are even more highly specialized to bind to DNA than histones. There are two types of protamines in humans called protamine 1 and protamine 2, and in their final form, they are composed of 57% and 67% basic amino acids, respectively. They bind to the DNA in the major groove ([Prieto et al., 1997](#)) essentially completely neutralizing the DNA ([Fig. 2G](#)). In sperm chromatin that is bound by protamines, there is virtually no access to the DNA double helix as there is in even the most compact forms of somatic chromatin (compare [Fig. 2D and I](#)). When all the charges are neutralized (and this can be accomplished either by protamines or divalent metals; [Hud and Downing, 2001](#)), DNA forms into tightly compact toroids ([Fig. 2I](#)). In the sperm cell, these toroids each contain 20,000–50,000 bp of DNA. The DNA was already almost completely inaccessible by its coating of tightly bound protamines ([Fig. 2G](#)), and with the formation of the toroid, most of the DNA is buried deep within the toroid, protected by surrounding DNA/protamine fibers. In this way the DNA is in an almost crystalline state, protected from damage by nucleases and mechanical stresses. One experiment is a particularly vivid illustration of how protected this sperm chromatin is. Even a brief sonication of somatic cells destroys them because the histone bound DNA does not prevent DNA double strand breaks. Mouse sperm, however, can be sonicated and then injected into oocytes to generate live pups ([Tatenio et al., 2000](#)). This demonstrates that all the sperm DNA survived the mechanical stress of the sonication. Protamines are uniquely evolved to compact that DNA about as tightly as it is possible for DNA to be packaged. They provide the protection needed to transport the father's DNA safely to the oocyte for productive fertilization.

In mammalian sperm chromatin, depending on the species, 1%–10% of the DNA remains bound to histones in fully mature sperm indicating that some of the sperm chromatin remains organized into nucleosomes rather than protamine toroids. An obvious question that has been asked is whether these histones are randomly placed or positioned on specific genes. Human sperm chromatin has a relatively large component of histone bound DNA, 10%, and several labs have provided evidence that the histones are positioned in at specific sites along the chromosome but they do not always agree on where these sites are ([Hammoud et al., 2009](#); [Brykczynska et al., 2010](#)). This specificity suggests that sperm DNA is packaged with functional consequences for development. For example, evidence from at least two laboratories suggests that some of the genes required for early development in the embryo are bound by histones in the sperm nucleus, suggesting that they are programmed by chromatin structure to activate shortly after fertilization ([Arpanahi et al., 2009](#); [Hammoud et al., 2009](#)).

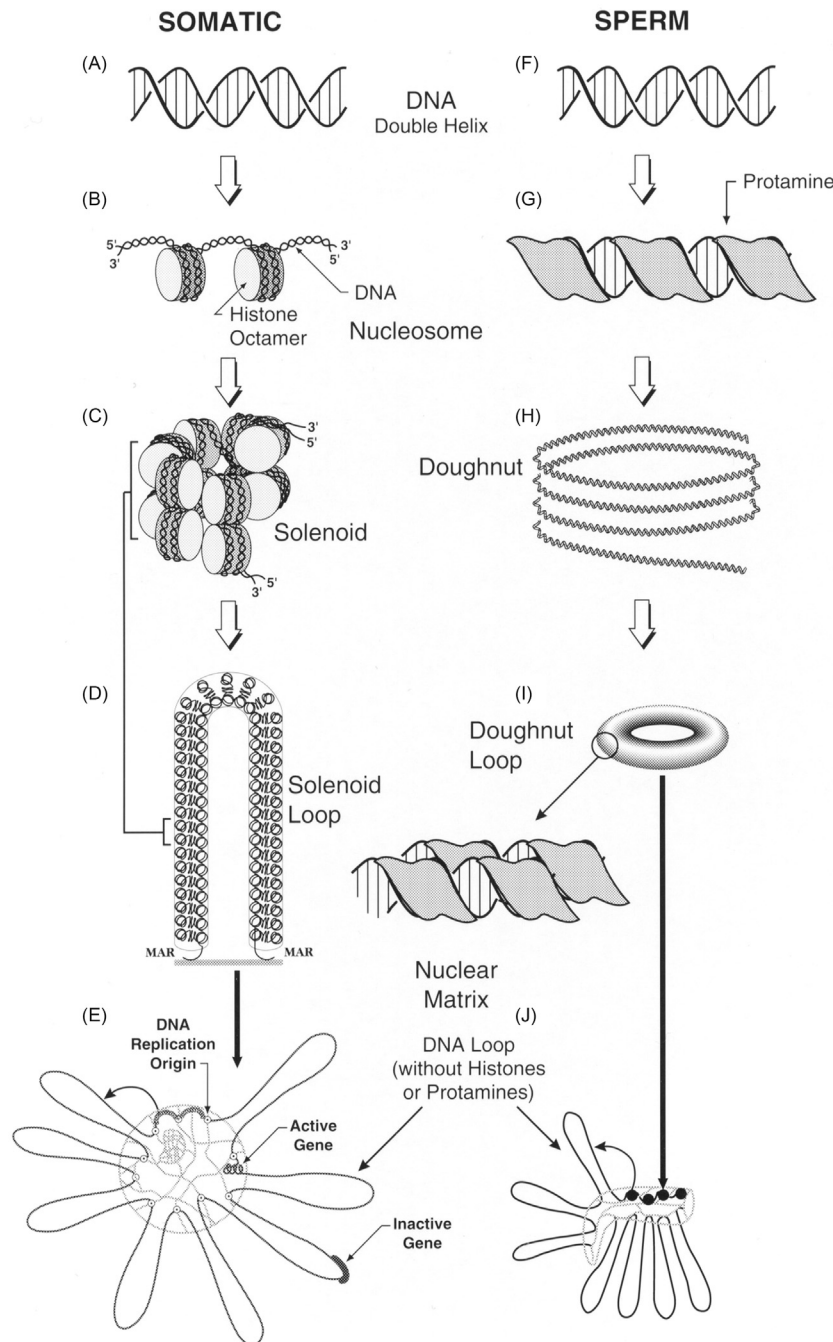


Fig. 2 Comparison of somatic cell and sperm chromatin. (A–C) DNA in most cells is compacted by coiling around histone octamers. Note that the outside of the DNA is exposed, no matter how tightly the DNA is coiled by histones. (D–E) The chromatin is still relatively long, compared to the nucleus, and it is further collapsed by organizing into loops by the nuclear matrix. (F–I) In sperm, most of the DNA (90%–99%, depending on the species) is bound much more tightly by protamines that wrap around the DNA in the major groove. This protamine bound DNA folds into large toroids. (J) Some data suggests that each toroid is a loop domain. This figure is a modified version of a previously published figure in [Ward \(1993\)](#).

Higher Order Packaging in Sperm Chromatin

All the principles discussed above are fairly well established and accepted. But several important questions remain to be answered. One can predict, for example, that the 23 chromosomes are packaged into 30,000–60,000 protamine toroids which immediately begs the question of how these toroids are arranged in chromosome. We now enter the realm of predictive models for sperm chromatin structure that necessarily change as more data are accumulated. **Fig. 3** depicts our most current model for how the DNA is packaged in sperm chromatin based on several different lines of experiments, which are described in greater detail in this review

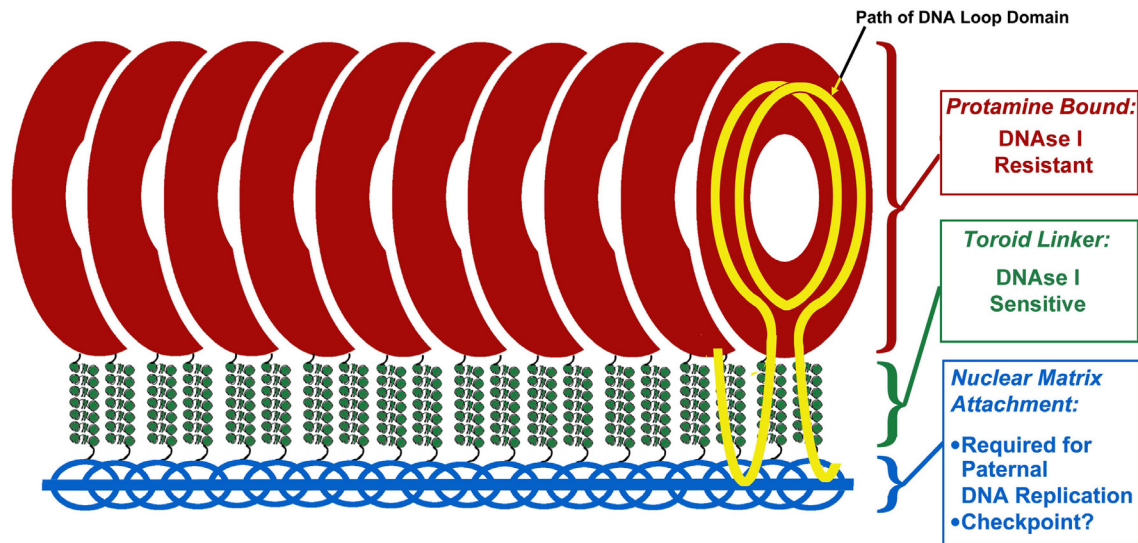


Fig. 3 Model for sperm chromatin structure. Protamine toroids are proposed to be stacked together, with nuclease sensitive linker regions of chromatin that also attach to the nuclear matrix. See text for details. A more complete description of this model can be found in [Ward \(2010\)](#).

([Ward, 2010](#)). There are two experimental results this model attempts to incorporate. First, even though it is packaged by protamines into impenetrable toroids, nuclease digestion of intact sperm nuclei result in the degradation of chromatin to 20–50 kb fragments ([Sotolongo et al., 2003](#)). Second, it is clear from different laboratories (reviewed in [Ward, 2010](#)) that sperm DNA is organized into loop domains similar to somatic cells (compare [Figs. 2E and J](#)). The fragments of DNA that are released by nucleases are within the range of predicted loop sizes, and it appears that each protamine toroid is equivalent to a single loop domain ([Sotolongo et al., 2003](#)). The most efficient way to package the toroids would be to stack them as shown in [Fig. 3](#) like a roll of life-saver candies. At least one laboratory has presented experimental evidence that this is the case ([Mudrak et al., 2009](#)). We have shown that the chromatin between the toroids is nuclease sensitive even though the DNA within the toroids is not ([Sotolongo et al., 2003](#)). Our model predicts that these toroid linker regions contain histone bound DNA, but there is no clear direct experimental evidence for that as of now. Our evidence also suggests that the toroid linker regions are the sites where the DNA loops attach to the sperm nuclear matrix. Beyond the predicted stacking of the toroids, there must also be further coiling to shorten the chromatin fibers to fit into the sperm cell. Any attempt to predict what that structure is would be pure speculation at this point.

We do have some idea, however, how the condensed chromosomes are arranged in the sperm nucleus. The telomeres of each chromosome appear to be attached to each other in at least six different mammalian species, and they are located towards the periphery of the sperm nucleus ([Zalensky et al., 1995, 1997](#)). The centromeres, on the other hand, coalesce together in the center of the nucleus. This results in a model for chromosome packaging in which the chromosome is bent in to a hairpin structure with the telomeres at the periphery and the centromeres at the center.

Functional Sperm Chromatin

Is there a function that the unique structure of sperm chromatin serves beyond protecting the DNA in the transit of the spermatozoon during fertilization? At least two different ideas have been proposed and the model for sperm packaging shown in [Fig. 3](#) suggests a structural focus for both functions.

The first proposed function is that the structure of the sperm chromatin functions in early embryogenesis. In other words, the overall role of the sperm is to not only deliver the intact paternal genome, but to provide instructions to the embryo on how to use that DNA. This is supported by several groups and has multiple levels of action. At least three levels of chromatin structure are thought to participate in embryogenesis and we have proposed a fourth. (i) DNA methylation is the clearest example. It has long been known that in a process called imprinting certain genes are methylated differently in paternal versus maternal DNA and that the correct methylation patterns on both are required for embryonic development ([Bartolomei and Tilghman, 1997](#)). This was the first evidence that something besides the genetic sequence of the DNA was inherited by the embryo from the gametes that was essential. As an extension of this, it is now clear that methylation of other genes may also be important for embryogenesis, even if they are methylated the same way on both gametes (ii) More recently, several laboratories have shown that the few histones that remain on the sperm genome are posttranslationally modified and that these modified histones are associated with specific genes ([Brykczynska et al., 2010](#)). This raises the possibility that these modified histones direct the embryo to focus on specific genes or chromosomal regions during early embryogenesis. (iii) It has recently been shown that sperm contain a host of paternal RNAs, the functions of which are only now being identified. Again, the possibility here is that these RNAs provide instructions to the

embryo on the proper use of the paternal genome. This model has gained considerable support with the recent discoveries of the importance of non-coding RNAs in gene regulation. (iv) Finally, we have proposed that the attachment sites of DNA to the nuclear matrix in the protamine toroid regions also provide information required for embryogenesis and have shown that these attachment sites are required for the replication of the paternal DNA in the embryo shortly after fertilization (Shaman et al., 2007). This suggests that the nuclear matrix attachment sites mark the origins of replication.

The proposed model in Fig. 3 suggests another possible function for sperm chromatin structure. The toroid linker regions are sensitive to nuclease digestion (Sotolongo et al., 2003), and we have shown that mammalian sperm can be induced to degrade its own DNA to loop-sized fragments in a process that we have termed sperm chromatin fragmentation (SCF) (Yamauchi et al., 2007). SCF mimics the DNA degradation of somatic cells in that the DNA is first reversibly fragmented by topoisomerase II, then irreversibly digested by nucleases (Li et al., 1999). SCF appears to occur on the nuclear matrix at the nuclease sensitive toroid linker regions. This suggests that when sperm DNA is compacted during spermiogenesis, the toroid linker regions remain accessible to topoisomerases and nucleases to allow the sperm cell to respond to external stimuli when necessary to initiate an apoptotic-like mechanism that incapacitates the sperm. The ability of spermatozoa to undergo apoptosis makes sense when one considers the millions of individual cells that invade the female uterus for each fertilization attempt. Most of these mobile carriers of foreign (to the female) DNA will not contribute to the production of progeny and must be destroyed. It has been argued that default status of spermatozoa is apoptosis and outside factors in the semen and uterus prevent it (Pujianto et al., 2010). The model for sperm chromatin structure presented in Fig. 3 illustrates how possible nuclease sensitive regions could allow the sperm cell to degrade its own DNA even while protecting 90%–99% of it as compact protamine bound toroids.

Summary

Mammalian sperm chromatin is the most densely packaged DNA known, yet it maintains windows of nuclease sensitivity that may also serve key functional roles in fertility and embryogenesis. Current models offer many hints as to how this highly condensed DNA can remain functional. We still have a long way to go before we understand just how the sperm is able to fold a meter of DNA inside its tiny volume.

References

- Arpanahi, A., Brinkworth, M., Iles, D., Krawetz, S. A., Paradowska, A., Platts, A. E., Said, M., Steger, K., Tedder, P., & Miller, D. (2009). Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. *Genome Res*, 19(8), 1338–1349.
- Bartolomei, M. S., & Tilghman, S. M. (1997). Genomic imprinting in mammals. *Annual Review of Genetics*, 31, 493–525.
- Brykczynska, U., Hisano, M., Erkek, S., Ramos, L., Oakeley, E. J., Roloff, T. C., Beisel, C., Schubeler, D., Stadler, M. B., & Peters, A. H. (2010). Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nature Structural & Molecular Biology*, 17, 679–687.
- Hammoud, S. S., Nix, D. A., Zhang, H., Purwar, J., Carrell, D. T., & Cairns, B. R. (2009). Distinctive chromatin in human sperm packages genes for embryo development. *Nature*, 460, 473–478.
- Hud, N. V., & Downing, K. H. (2001). Cryoelectron microscopy of lambda phage DNA condensates in vitreous ice: The fine structure of DNA toroids. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 14925–14930.
- Li, T. K., Chen, A. Y., Yu, C., Mao, Y., Wang, H., & Liu, L. F. (1999). Activation of topoisomerase II-mediated excision of chromosomal DNA loops during oxidative stress. *Genes & Development*, 13, 1553–1560.
- Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, 389, 251–260.
- Mudrak, O., Chandra, R., Jones, E., Godfrey, E., & Zalensky, A. (2009). Reorganisation of human sperm nuclear architecture during formation of pronuclei in a model system. *Reproduction, Fertility, and Development*, 21, 665–671.
- Prieto, M. C., Maki, A. H., & Balhorn, R. (1997). Analysis of DNA-protamine interactions by optical detection of magnetic resonance. *Biochemistry*, 36, 11944–11951.
- Pujianto, D. A., Curry, B. J., & Aitken, R. J. (2010). Prolactin exerts a prosurvival effect on human spermatozoa via mechanisms that involve the stimulation of Akt phosphorylation and suppression of caspase activation and capacitation. *Endocrinology*, 151, 1269–1279.
- Shaman, J. A., Yamauchi, Y., & Ward, W. S. (2007). The sperm nuclear matrix is required for paternal DNA replication. *Journal of Cellular Biochemistry*, 102, 680–688.
- Sotolongo, B., Lino, E., & Ward, W. S. (2003). Ability of hamster spermatozoa to digest their own DNA. *Biology of Reproduction*, 69, 2029–2035.
- Tateno, H., Kimura, Y., & Yanagimachi, R. (2000). Sonication per se is not as deleterious to sperm chromosomes as previously inferred. *Biology of Reproduction*, 63, 341–346.
- Ward, W. S. (1993). Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. *Biology of Reproduction*, 48, 1193–1201.
- Ward, W. S. (2010). Function of sperm chromatin structural elements in fertilization and development. *Molecular Human Reproduction*, 16, 30–36.
- Yamauchi, Y., Shaman, J. A., & Ward, W. S. (2007). Topoisomerase II mediated breaks in spermatozoa cause the specific degradation of paternal DNA in fertilized oocytes. *Biology of Reproduction*, 76, 666–672.
- Zalensky, A. O., Allen, M. J., Kobayashi, A., Zalenskaya, I. A., Balhorn, R., & Bradbury, E. M. (1995). Well-defined genome architecture in the human sperm nucleus. *Chromosoma*, 103, 577–590.
- Zalensky, A. O., Tomilin, N. V., Zalenskaya, I. A., Teplitz, R. L., & Bradbury, E. M. (1997). Telomere-telomere interactions and candidate telomere binding protein(s) in mammalian sperm cells. *Experimental Cell Research*, 232, 29–41.

In Vitro Spermatogenesis

Yukiko Ishikura and Mitinori Saitou, Kyoto University, Kyoto, Japan; and JST, ERATO, Kyoto, Japan

© 2018 Elsevier Inc. All rights reserved.

Introduction

Male germ-cell development begins with the specification of primordial germ cells (PGCs), the common precursors for the spermatozoa and the oocytes, and culminates in the generation of spermatozoa that function to convey paternally derived genetic as well as epigenetic information to subsequent generations. The entire spermatogenesis process is one of the most complex cellular differentiation processes during mammalian development and physiology. In vitro spermatogenesis is a research discipline that aims at reconstituting the spermatogenesis processes in vitro, most ambitiously by means of pluripotent stem cells (PSCs) such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) as a starting material, and has been one of the most critical challenges in reproductive science (for review see Daley, 2007; Handel et al., 2014; Saitou and Miyauchi, 2016). If successful, it will represent a powerful strategy with which to study salient mechanisms underlying the whole spermatogenesis process, ranging from epigenetic reprogramming/programming to genetic stability to meiotic recombination (Baudat et al., 2013; Handel and Schimenti, 2010; Kong et al., 2012; Saitou et al., 2012), in a precise manner, and the resultant spermatozoa generated in vitro might serve as a critical resource for regenerative biology or medicine (e.g., see Hirota et al., 2017).

Much effort by numerous researchers has been dedicated toward this important objective, and as a result there have been a number of reports showing the induction of haploid spermatid-like cells from PSCs (see relevant references in Daley, 2007; Handel et al., 2014; Saitou and Miyauchi, 2016). However, most such reports have documented inductions with very low efficiency, which do not recapitulate the in vivo processes, and which somehow are not convincingly reproduced (Daley, 2007; Handel et al., 2014; Saitou and Miyauchi, 2016), leaving in vitro spermatogenesis as an unattained and formidable goal. On the other hand, during the last two decades, the methods for culturing rodent and human PSCs have become highly sophisticated (for review see Nichols and Smith, 2009), methods for culturing rodent spermatogonial stem cells (SSCs) have been established (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004), and there have been solid advances in our understanding of the basic mechanisms underlying the entire spermatogenesis process (Griswold, 2016; Hermann et al., 2010; Saitou and Miyauchi, 2016). Accordingly, it has been demonstrated that mouse PSCs are induced into PGC-like cells (PGCLCs), which undergo spermatogenesis upon transplantation into neonatal mice lacking endogenous germ cells, and the resultant spermatozoa contribute to healthy offspring (Hayashi et al., 2011). It is also of note that an improved method for testis organ culture has been developed and has been used to differentiate and mature undifferentiated spermatogonia or a primary cell line bearing the SSC activity into haploid spermatids (Sato et al., 2011a,b). These advances heighten the prospect that the recapitulation of the entire process of spermatogenesis from PSCs may one day be possible. Indeed, remarkably, the entire mouse oogenesis process, the female counterpart of germ-cell development, has been reconstituted in vitro based on the PGC/PGCLC culture system (Hayashi et al., 2012; Hikabe et al., 2016; Morohaku et al., 2016). Here, we review the current state and ongoing progress of in vitro spermatogenesis studies, with an emphasis on those from mouse and human PSCs, and discuss future perspectives.

Male Germ-Cell Development and Spermatogenesis in Mice and Humans

Spermatogenesis begins with the specification of PGCs early during embryonic development (Fig. 1). In mice, a representative model organism for mammalian development, PGCs are induced within the most proximal posterior epiblast cells at around embryonic day (E) 6.0 in response to bone morphogenetic protein 4 (BMP4) and WNT3, and form a cluster of ~40 cells within the extra-embryonic mesoderm at around E7.25 (Ginsburg et al., 1990; Lawson and Hage, 1994; Ohinata et al., 2009; Saitou et al., 2002). The pluripotent epiblast cells bear competence for the germ cell fate from around E5.5 to E6.25 (Ohinata et al., 2009), and the transcription factors (TFs) essential for PGC specification include BLIMP1 (also known as PRDM1), PRDM14 and TFAP2C (also known as AP2γ) (Ohinata et al., 2005; Vincent et al., 2005; Weber et al., 2010; Yamaji et al., 2008). Upon specification, PGCs repress the somatic mesodermal program, re-acquire potential pluripotency, and embark on epigenetic reprogramming (for review see Saitou and Yamaji, 2012). Note that the epigenetic properties of PGCs at specification are similar to those of the epiblast cells and PGCs retain parental imprints inherited from spermatozoa and oocytes (Hajkova et al., 2002; Kagiwada et al., 2013; Seisenberger et al., 2012). PGCs initiate migration after around E7.25, move through the developing hindgut endoderm and mesentery, and colonize the incipient gonad primordium from around E10.5. During migration and colonization of the embryonic gonads, PGCs proliferate substantially (from ~40 cells at E7.25 to ~25,000 cells at E13.5) (Kagiwada et al., 2013; Tam and Snow, 1981), acquire the expression of key translational regulators DAZL (Cooke et al., 1996; Lin et al., 2008) and DDX4 (also known as mouse vasa homolog (mVH)) (Fujiwara et al., 1994; Tanaka et al., 2000), and importantly, undergo epigenetic reprogramming that includes genome-wide DNA demethylation, imprint erasure, and histone modification remodeling, thereby acquiring an epigenetic “blank slate” with equivalent epigenotypes for the paternal and maternal alleles (Hajkova et al., 2002; Popp et al., 2010; Seisenberger et al., 2012; Seki et al., 2005).

It is within the embryonic gonads that PGCs initiate their sexually dimorphic development (Fig. 1). In embryonic ovaries, in response to cues from ovarian somatic cells, from around E13.5, PGCs progress toward the oogenic pathway and enter into the

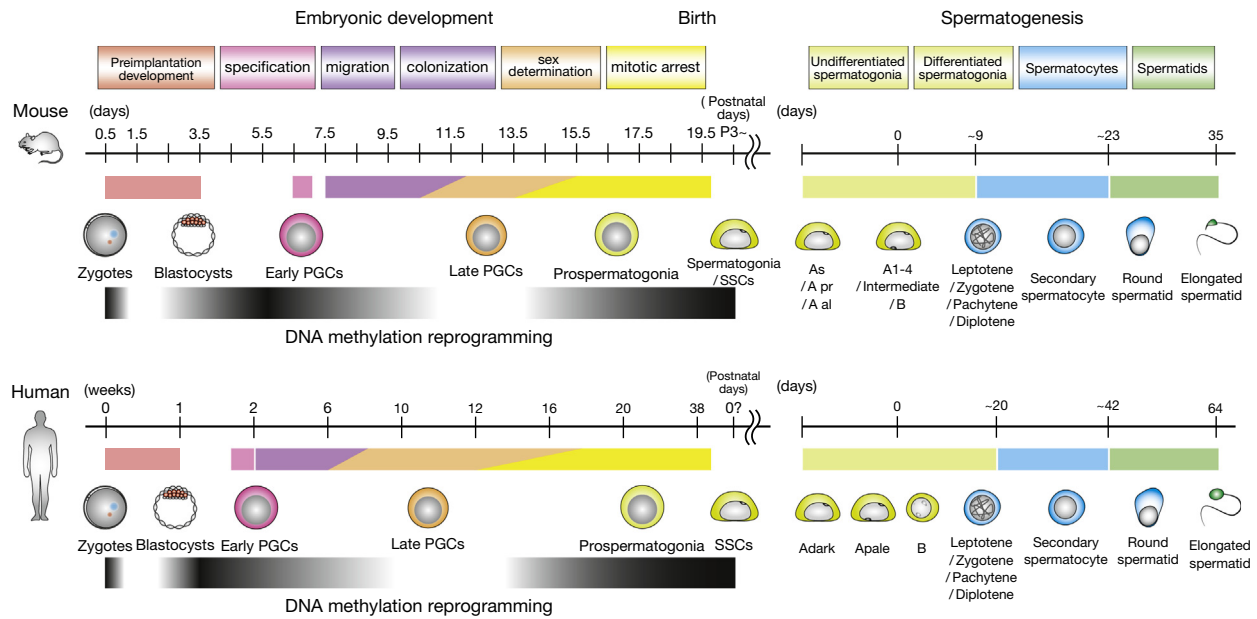


Fig. 1 Male germ-cell development and spermatogenesis in mice and humans. Scheme showing the key cell types and events during male-germ cell development and spermatogenesis in mice (top) and humans (bottom). The color code of the key events is shown on the top. PGCs, primordial germ cells; SSCs, spermatogonial stem cells; As, A single; Apr, A paired; Aal, A aligned.

prophase of meiosis I, heralding the oocyte development (see [Edson et al., 2009](#); [McGee and Hsueh, 2000](#) for further details on oocyte development). In contrast, in embryonic testes, in response to signals from or environments created by embryonic testicular somatic cells, from around E13.5, PGCs cease their cell cycle (mitotic arrest) and differentiate into pro-spermatogonia or gonocytes (for review see [Manku and Culty, 2015](#); [Spiller et al., 2017](#)). The mechanism that drives the differentiation of PGCs into the male pathway remains obscure, but the signaling pathways involving nodal, prostaglandin D2, and fibroblast growth factor 9 (FGF9) appear to play key roles ([Manku and Culty, 2015](#); [Spiller et al., 2017](#)), and the translational regulator NANOS2 integrates the system that represses the female pathway and promotes the male differentiation ([Suzuki and Saga, 2008](#); [Tsuda et al., 2003](#)). Located within the luminal side of the testicular cords (primitive seminiferous tubules) created by embryonic Sertoli cells, the pro-spermatogonia continue to be mitotically arrested until after birth and gradually establish the properties of spermatogonia, including the expression of a key TF, PLZF (also known as ZBTB16), and of receptors for the key mitogen of SSCs, glial-cell-derived neurotrophic factor (GDNF) ([Meng et al., 2000](#)), GFR α 1 and RET ([Griswold, 2016](#); [Hermann et al., 2010](#); [Manku and Culty, 2015](#)). The key event that takes place during this period is the establishment of the androgenetic epigenome including paternal imprints ([Kubo et al., 2015](#); [Seisenberger et al., 2012](#)), which requires de novo DNA methyltransferases (DNMT) 3A and 3L and the machinery for PIWI-interacting RNA (piRNA) biogenesis, such as PIWIL2 (also known as MILI), PIWIL4 (also known as MIWI2), and Tudor domain-containing proteins (TDRDs) (for review see [Chuma and Nakano, 2013](#); [Siomi et al., 2011](#)).

From at around postnatal day (P) 3, the pro-spermatogonia translocate to the basal compartment of the seminiferous tubules, differentiate into spermatogonia by an unknown mechanism, and resume mitotic proliferation ([Griswold, 2016](#); [Hermann et al., 2010](#); [Manku and Culty, 2015](#); [Fig. 1](#)). Some of the spermatogonia in turn proceed directly to spermatogenesis by entering into meiosis, and produce spermatozoa by 4 weeks after birth (first-wave spermatogenesis) ([Bellve et al., 1977](#); [Yoshida et al., 2006](#)). On the other hand, some spermatogonia are recruited into SSCs, which, with their remarkable capacity for self-renewal and spermatogenic differentiation, support the spermatogenesis throughout the life of individual animals ([Griswold, 2016](#); [Hermann et al., 2010](#); [Yoshida, 2016](#)). The identity of SSCs has long been debated: one idea posits that A single (As) spermatogonia or a subpopulation of As spermatogonia are the only bona fide SSCs that can self-renew and support the life-long spermatogenesis, whereas another proposal submits that a collective pool of cells expressing GFR α 1, including As, A paired (Apr), and A aligned (Aal), can function as SSCs (for review see [de Rooij, 2017](#); [Griswold, 2016](#); [Hermann et al., 2010](#); [Yoshida, 2016](#)). The TFs such as PLZF, and signaling molecules such as GDNF, FGFs, and WNTs ensure the self-renewal of SSCs, and the TFs such as SOHLH1/2 and STRA8, and signaling molecules such as retinoic acid (RA) and stem cell factor (SCF) are critical for SSCs to enter into meiosis ([de Rooij, 2017](#); [Griswold, 2016](#); [Hermann et al., 2010](#); [Yoshida, 2016](#)). Upon entry into meiotic divisions, spermatogonia translocate to the luminal compartment of the seminiferous tubules to differentiate into spermatocytes, which upon completion of meiosis, differentiate into spermatids and then into mature spermatozoa to acquire the characteristic sperm morphology with protamine-based compact chromatin (spermiogenesis) (for review see [Kimmins and Sassone-Corsi, 2005](#)). It is critical to note that the luminal compartment of the seminiferous tubules represents a distinct fluid and hormonal milieu established by the tight junctions of the Sertoli cells (blood-testis barrier) ([Russell et al., 1990](#); [Yoshida, 2016](#)). It takes approximately ~35 days for differentiating spermatogonia to form mature spermatozoa, with ~14 days for the completion of meiosis I ([Griswold, 2016](#); [Hermann et al., 2010](#)).

In humans, male germ-cell development and spermatogenesis take a pathway generally similar to that in mice, but require a much longer time, appear to involve substantial developmental heterogeneity/asynchronicity, and exploit molecular mechanisms divergent from those in mice (for review see [Hermann et al., 2010](#); [Manku and Culty, 2015](#); [Saitou and Miyauchi, 2016](#); [Tang et al., 2016](#); [Fig. 1](#)). Human PGCs (hPGCs) are specified presumably at around week 2 (Wk2) of development via an unknown mechanism. Notably, it has been shown that in cynomolgus monkeys, PGCs are specified as early as E11 in the nascent amnion segregated from the epiblast upon implantation ([Sasaki et al., 2016](#)), suggesting that hPGCs are also specified in the amnion. hPGCs undergo migration similarly to mouse PGCs and colonize the anlage of embryonic testes from around Wk5 ([De Felici, 2013](#); [Witschi, 1948](#)). hPGCs continue to proliferate at least until Wk10–12 in embryonic testes, and thereafter gradually enter into mitotic arrest in an asynchronous fashion to differentiate into pro-spermatogonia ([Culty, 2009](#)). The number of hPGCs is around ~100 at Wk3, which increases to around ~1000 at Wk4 and to around ~150,000 at Wk9 ([De Felici, 2013](#); [Mamsen et al., 2011](#)), and during this period, hPGCs undergo the epigenetic reprogramming, acquiring an epigenetic blank slate ([Gkoutela et al., 2015](#); [Guo et al., 2015](#); [Tang et al., 2015](#)).

Some of the pro-spermatogonia translocate to the basal compartment of the seminiferous tubules and differentiate into spermatogonia during the embryonic period ([Culty, 2009](#); [Fig. 1](#)). After birth, human spermatogonia population appears to be maintained in a dynamic balance for more than a decade until the onset of puberty ([Paniagua and Nistal, 1984](#)), after which they undergo spermatogenesis and SSCs continue to provide spermatogenesis throughout the lifetime of the individual. It takes ~64 days for differentiating human spermatogonia to form mature spermatozoa, with ~23 days for the completion of meiosis I ([Griswold, 2016](#); [Hermann et al., 2010](#); [Yoshida, 2016](#)). Obviously, our knowledge of the mechanisms of human germ-cell development and spermatogenesis is limited, apart from the information obtained from classical morphological observations. The recent development of many key technologies, including those that allow massively parallel transcriptome analyses from single cells, is expected to provide valuable insights into the mechanisms of human germ-cell development ([Guo et al., 2015](#); [Li et al., 2017](#)), for which obtaining sufficient experimental materials is often difficult.

Male Germ-Cell Development and Spermatogenesis From Mouse PSCs

Based on the current knowledge of male germ-cell development and spermatogenesis, and on relevant reproductive technologies—most prominently, the SSC transplantation assays ([Brinster and Avarbock, 1994](#); [Brinster and Zimmermann, 1994](#); [Fig. 2](#))—these processes are beginning to be reconstituted step-by-step using mouse PSCs as a starting material ([Saitou and Miyauchi, 2016](#)). As a first step in this process, mouse PSCs were induced by activin A and bFGF into epiblast-like cells (EpiLCs), which were in turn induced essentially by BMP4 into PGC-like cells (PGCLCs) in floating aggregates ([Hayashi et al., 2011](#); [Fig. 3](#)). Gene-expression dynamics during the induction of mPSCs to EpiLCs to PGCLCs recapitulated those during progression from the E4.5 epiblast to E6.0 epiblast to PGC specification, and EpiLCs and PGCLCs induced for 4–6 days (d4/d6 PGCLCs) exhibited gene-expression properties similar to those of the E6.0 epiblast and E9.5 PGCs, respectively ([Hayashi et al., 2011](#)). Remarkably, like PGCs ([Chuma et al., 2005](#)) and those induced from the epiblast *ex vivo* ([Ohinata et al., 2009](#)), when PGCLCs were transplanted into the testes of neonatal mice lacking endogenous germ cells (*W/W^u* mice bearing mutations in *KIT*), they successfully reconstituted spermatogenic colonies and differentiated into spermatozoa, and the resultant spermatozoa robustly contributed to apparently healthy offspring ([Hayashi et al., 2011](#)). These findings represent the first demonstration that mPSCs can differentiate *in vitro* into germline cells with an appropriate function. Subsequently, it was shown that when aggregated with embryonic ovarian somatic cells and transplanted under the ovarian bursa of immune-deficient mice, female PGCLCs are differentiated and matured into oocytes at the germinal vesicle stage, and the resultant oocytes contribute to healthy offspring following *in vitro* maturation (IVM) and fertilization (IVF) ([Hayashi et al., 2012](#)). Thus, mouse PGCLCs function as precursors both for the spermatozoa and the oocytes and are considered as a bona fide *in vitro* counterpart of PGCs.

Accordingly, the system for PGCLC induction has been exploited for the identification of TFs sufficient for conferring the germ-cell fate ([Nakaki et al., 2013](#)), for clarifying a detailed signaling and transcriptional cascade leading to PGC specification ([Aramaki et al., 2013](#)), and for delineating the histone modification remodeling and DNA methylation reprogramming associated with PGC specification ([Kurimoto et al., 2015](#); [Shirane et al., 2016](#); [von Meyenn et al., 2016](#)). Importantly, the latter studies ([Kurimoto et al., 2015](#); [Shirane et al., 2016](#); [von Meyenn et al., 2016](#)), together with other studies on PGCs *in vivo* ([Arand et al., 2015](#); [Kagiwada et al., 2013](#); [Seisenberger et al., 2012](#)), have clarified that genome-wide DNA demethylation in PGC(LC)s occurs in a manner consistent with a replication-coupled passive dilution having differing dilution rates for single-copy sequences (unique regions), distinct regulatory elements, and repeats. This appears to be achieved through the repression of key components of the *de novo* as well as the maintenance DNA methyltransferase machineries (DNMT3A/B and UHRF1) upon PGC(LC) specification by BLIMP1/PRDM14/TFAP2C ([Kagiwada et al., 2013](#); [Kurimoto et al., 2008, 2015](#); [Seisenberger et al., 2012](#); [Seki et al., 2005](#); [Shirane et al., 2016](#)). Consequently, the genome-wide DNA methylation profiles of PGCs/PGCLCs show a diluted configuration of those of epiblast/EpiLCs, and d6 PGCLCs bear ~40% of genome-wide 5mC levels ([Shirane et al., 2016](#)).

A more recent study has attempted an induction of SSCs from PGCLCs *in vitro* ([Ishikura et al., 2016](#); [Fig. 3](#)). d4 PGCLCs were aggregated with somatic cells of E12.5 embryonic testes and were cultured on a Trans-well system under an air-liquid interface culture condition. Under this condition, the aggregates formed an embryonic testis-like structure with reconstituted seminiferous tubules maintaining the expression of GATA4 and SOX9, key TFs for Sertoli cells ([Vidal et al., 2001](#); [Viger et al., 1998](#)). Within these structures (reconstituted testes), PGCLCs survived preferentially inside the seminiferous tubules, increased in number, and

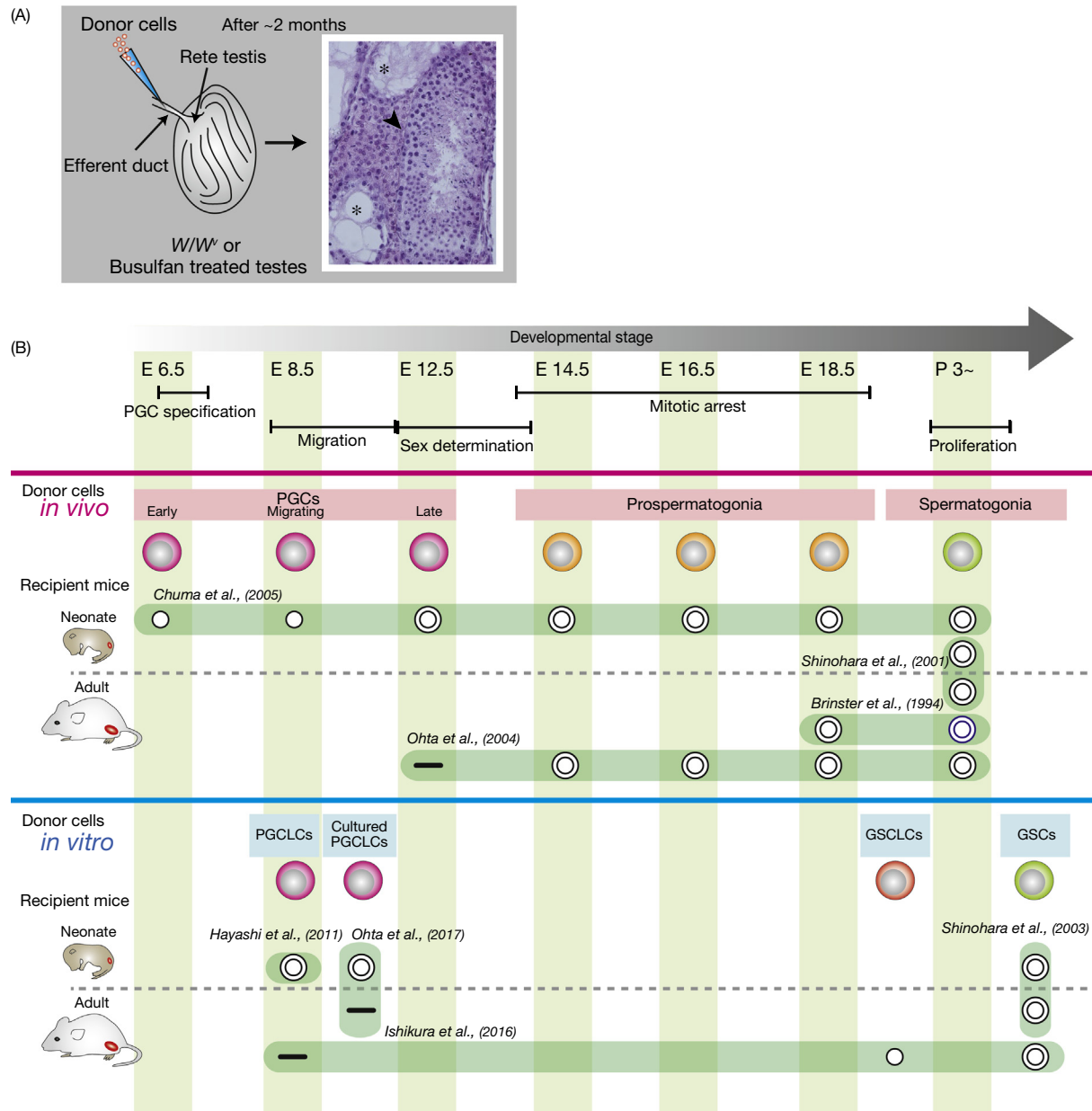


Fig. 2 Germ-cell transplantation assays. (A) Scheme showing the germ-cell transplantation assay. Donor germ cells are transplanted into the rete testis of germ-cell depleted mice (mice treated with busulfan or *W/W^u* mice) and their colonization and spermatogenesis are examined 2–3 months after the transplantation. The photograph on the right shows a section of the transplanted testis with seminiferous tubules showing spermatogenesis (arrowhead) or no spermatogenesis (*: un-colonized tubules). The section was stained by hematoxylin and eosin. (B) Scheme showing donor cell types (top: cells *in vivo*; bottom: cells *in vitro*) and recipients used for germ-cell transplantation assays in mice. ⊙: Colonization and spermatogenesis with high efficiency; ○: colonization and spermatogenesis with low efficiency; –: no colonization. Key references are shown.

up-regulated DDX4, a key marker for gonadal PGCs (Fujiwara et al., 1994; Tanaka et al., 2000), from around 10 days of the culture; in addition, a subset of the DDX4-positive cells up-regulated PLZF, an SSC marker that initiates expression perinatally in pro-spermatogonia (Buas et al., 2004; Costoya et al., 2004), from around 20 days of culture. Thereafter, the cultures of the reconstituted testes were maintained up to ~2 months at 34°C, a permissive temperature for spermatogenic meiosis (Sato et al., 2011a; Steinberger et al., 1964), and a few PGCLC-derived cells became positive for SCP3, a marker for the onset of meiosis (Yuan et al., 2000), but no cells completed meiosis under the reported condition (Ishikura et al., 2016). These findings indicate that PGCLCs differentiate at least up to spermatogonia-like cells in the reconstituted testes *in vitro*, although their differentiation kinetics is protracted compared to that of PGCs *in vivo* (Fig. 3).

It has been well-established that mouse SSCs can be propagated *in vitro* in the presence of cytokines including GDNF as germline stem cells (GSCs) (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). Upon transplantation into either neonatal or adult mouse

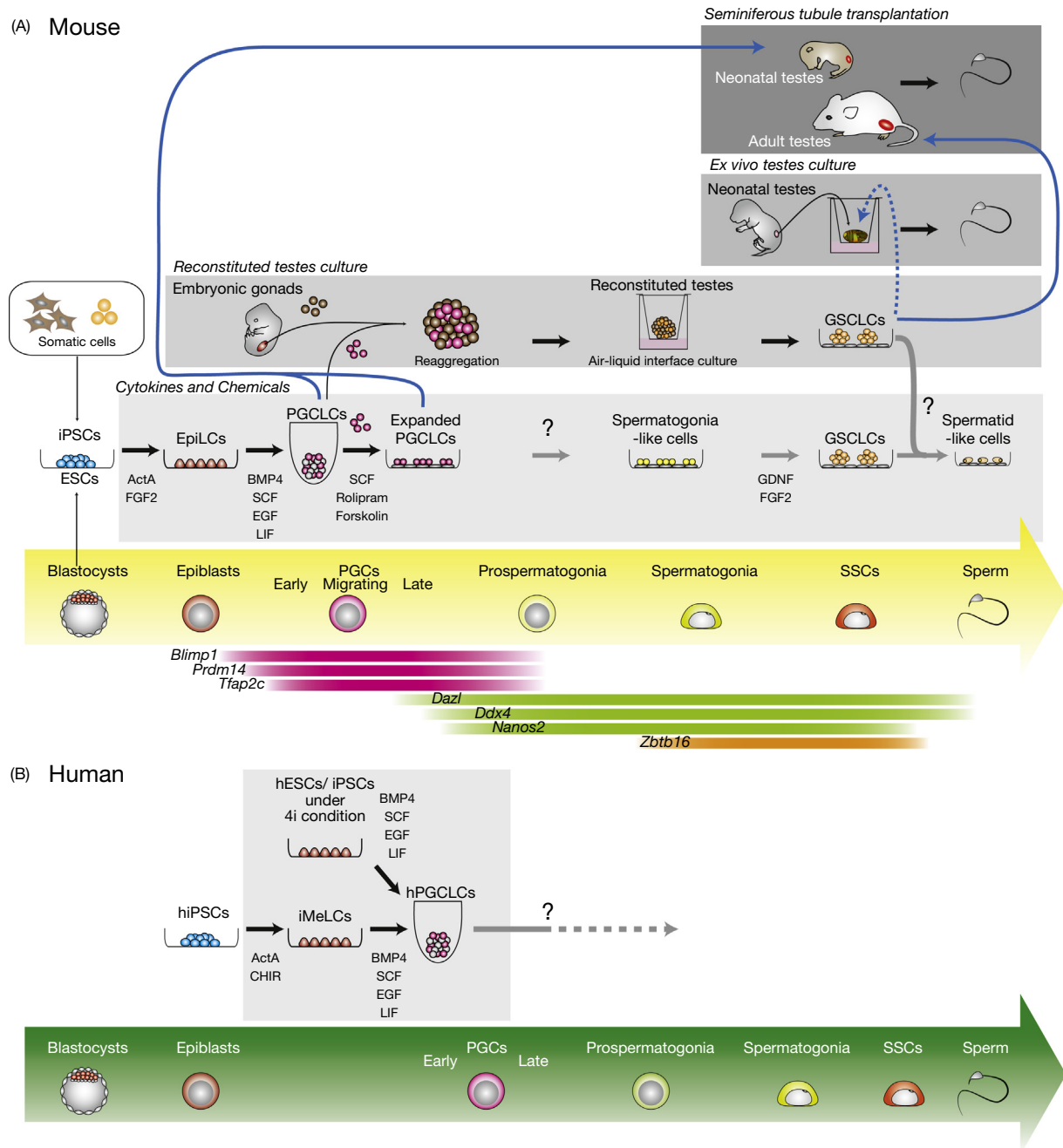


Fig. 3 Reconstitution of male germ-cell development in vitro and ex vivo. (A) Scheme showing the current state of in vitro (focused on the experiments recapitulating the in vivo processes) and ex vivo reconstitution of male germ-cell development and spermatogenesis in mice. Key cell types and gene expression during male germ-cell development are shown at the bottom. Reconstitution pathways involving cytokines and chemicals, reconstituted testes culture, ex vivo testes culture, and transplantation into seminiferous tubules, are shown. **Black bold arrows**, the induction steps reported; **gray bold arrows**, the induction steps necessary to be established; **blue arrows**, transplantation; **blue dotted arrow**, transplantation necessary to be performed. See text for details and key references. (B) Scheme showing in vitro reconstitution of germ-cell development in humans. See text for details and key references.

testes depleted of endogenous germ cells, GSCs colonize the testes to form spermatogenic colonies, and the resultant spermatozoa contribute to healthy offspring (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). It is important to note that while PGCs/PGCLCs colonize and undergo spermatogenesis only in testes of neonatal mice, SSCs/GSCs do so in testes of both neonatal and adult mice, and the male germ cells acquire the capacity to colonize/undergo spermatogenesis in adult testes upon their differentiation into pro-spermatogonia (after ~E14.5) (Chuma et al., 2005; Ohta et al., 2004; Shinohara et al., 2001; Fig. 2). Thus, GSCs are a primary cell line that is enriched with cells bearing the SSC activity, although their cellular heterogeneity remains

to be fully elucidated (Kanatsu-Shinohara and Shinohara, 2013). Accordingly, spermatogonia-like cells derived from PGCLCs in the reconstituted testes were propagated as GSC-like cells (GSCLCs) under the GSC culture condition (Ishikura et al., 2016). GSCLCs exhibited a morphology, proliferation kinetics, surface-marker expression, and expression of key genes highly similar to those of GSCs. Remarkably, unlike PGCLCs, GSCLCs colonized adult testes robustly upon transplantation. However, it was found that only a few GSCLC lines (3 out of 15 examined) underwent spermatogenesis in a fraction of colonized tubules, and a majority of colonized cells halted the spermatogenesis at around the entry into or early stages of the first meiotic prophase. Nonetheless, spermatozoa derived from GSCLCs were retrieved consistently and contributed to healthy offspring. Thus, PGCLCs can be induced into the male pathway to form spermatogonia-like cells in vitro, which can be propagated as a stable cell line with the SSC activity (Fig. 3).

Compared to GSCs, the capacity of GSCLCs for spermatogenesis was found to be limited. Transcriptome and genome-wide DNA methylome analyses revealed that GSCLCs bear global transcription and DNA methylation profiles similar to GSCs and to P7 KIT-negative undifferentiated spermatogonia, but in promoters and non-promoter CpG islands, GSCLCs exhibited 5mC levels consistently higher than such cell types (Ishikura et al., 2016). The hyper-methylated promoters in GSCLCs constituted ~3.0%–5.0% of all promoters and the associated genes exhibited a tendency to be repressed and included key genes for spermatogenesis, such as *Pou5f1*, *Sohlh1*, *Dpep3*, *Spo11*, *Tex19.1*, *Tdrd9*, and *Piwi1*. GSCLCs also exhibited aberrant methylation profiles of some of the differentially methylated regions (DMRs) of maternally imprinted genes. Investigation of the methylation-level dynamics of the hyper-methylated promoters during male germ-cell development in vivo revealed that the hyper-methylation of these promoters can be a combinatorial consequence of an incomplete DNA demethylation during reprogramming and aberrant DNA methylation during androgenetic programming (Ishikura et al., 2016). Thus, an improvement of culture conditions for reconstituted testes to prevent the hyper-methylation of these promoters, e.g., by using an appropriate index for epigenetic reprogramming/androgenetic programming, or separate culture for PGCLCs for the completion of genome-wide DNA demethylation followed by reconstituted testis culture, will lead to an induction of spermatogonia-like cells and GSCLCs with better function.

It was reported that d6 mPGCLCs enter into the prophase of meiosis I during the first 6 days when co-cultured with dissociated testicular cells of neonatal W/W^m mice in the presence of retinoic acid (RA), BMP2/4/7, and activin A, and such cells complete meiosis over the following 8 days when follicle stimulating hormone (FSH), bovine pituitary extract (BPE), and testosterone are provided, resulting in the generation of spermatid-like cells that contribute to healthy offspring (Zhou et al., 2016). This is a surprising result, because the reported procedure skips a period of male germ-cell development necessary for completing the epigenetic reprogramming, acquiring the androgenetic epigenome and differentiating into spermatogonia (more than 2 weeks of development in vivo—i.e., the period from PGCs at around E9.5 (d6 PGCLCs) to spermatogonia initiating the first wave spermatogenesis at around P10), and also because the culture was conducted at 37°C, a non-permissive temperature for spermatogenesis (Sato et al., 2011a; Steinberger et al., 1964). Hence, we consider that the validity of this study, including a more precise analysis of the induced intermediate cell types, warrants re-examination.

On the other hand, it has recently been shown that mouse d4 PGCLCs can be expanded up to ~50 fold on a sub-line of m220 feeders in the presence of SCF, forskolin and rolipram, the latter two of which elevate cAMP levels in PGCLCs (Ohta et al., 2017; Fig. 3). The PGCLCs essentially maintain their transcriptome as migrating PGCs during the expansion culture, but remarkably, consistent with the notion that genome-wide DNA demethylation in PGCs proceeds predominantly through replication-coupled passive dilution, the PGCLCs progressively erase their genome-wide DNA methylation, resulting in genome-wide 5mC levels of ~20% and ~6% at culture day 3 (d4c3) and 7 (d4c7), respectively (Ohta et al., 2017). Importantly, the 5mC distribution patterns in all genomic regions, including single-copy sequences (unique regions), repeats and the DMR of imprinted genes, of d4c3 and d4c7 PGCLCs were essentially identical to those of E10.5 PGCs and E13.5 male/female germ cells, respectively (Ohta et al., 2017). This work demonstrates that the genome-wide DNA demethylation and sexual differentiation of germ cells that occur in embryonic gonads by E13.5 are controlled by distinct genetic mechanisms, providing a potentially ideal starting material for the in vitro sex differentiation of germ cells and the reconstituted testis culture. Accordingly, a recent study has demonstrated that in response to BMP and RA, cultured PGCLCs can be induced into embryonic primary oocytes, and initiate and progress the prophase of meiosis I up to the pachytene stage (Miyauchi et al., 2017). It would be an interesting subject for future research to identify a defined condition—i.e., cytokines or compounds—to induce the male germ-cell fate in cultured PGCLCs without the use of testicular somatic cells.

It is critical to point out that recent years have also seen significant progress in the methodologies for the ex vivo organ culture of mouse testes, allowing the differentiation and maturation of undifferentiated spermatogonia into haploid spermatids with the capacity for contributing to healthy offspring (Sato et al., 2011a; Fig. 3). In these experiments, neonatal mouse testes that contain undifferentiated spermatogonia, but not their differentiated progeny, were isolated and their fragments preserving the organization of seminiferous tubules were placed on an agarose gel and cultured under an air-liquid interface condition at 34°C in the presence of knockout serum replacement (KSR), and the spermatogonia successfully underwent meiosis and formed haploid spermatids. Importantly, this system was extended to differentiate mouse GSCs into spermatids: GSCs were transplanted into neonatal W/W^m mouse testes and their fragments were cultured ex vivo, resulting in the generation of GSC-derived spermatids and healthy offspring (Sato et al., 2011b). More recently, a system to culture testicular fragments on a microfluidics apparatus was developed, allowing a constant, periodical production of spermatids from spermatogonia over more than 6 months (Komeya et al., 2016). Currently, with this ex vivo culture system, spermatids can be generated even from embryonic testes after E14.5, but not from those in earlier stages (Kojima et al., 2016). It would be an interesting line of research to combine the induction of GSCLCs from PSCs and their differentiation in the ex vivo culture system.

Germ-Cell Development From Human PSCs

Based on the system for the in vitro germ-cell development from mouse PSCs, human germ-cell development has begun to be reconstituted from human PSCs (Fig. 3). In one method, hESCs/hiPSCs maintained under a distinct condition with four kinase inhibitors (4i) (Gafni et al., 2013) were induced into human PGCLCs by BMP4 (Irie et al., 2015; Kobayashi et al., 2017), whereas in another, hiPSCs cultured under a conventional condition (Nakagawa et al., 2014) were first induced by activin A and WNT signaling into incipient mesoderm-like cells (iMeLCs) expressing genes such as *T*, *MIXL1*, and *EOMES*, which were in turn induced into hPGCLCs by BMP4 (Sasaki et al., 2015; Yokobayashi et al., 2017). hPGCLCs bear a property similar to human and cynomolgus monkey PGCs just after specification and do not yet up-regulate genes such as *DAZL* and *DDX4* (Irie et al., 2015; Sasaki et al., 2015, 2016). hPSCs cultured with 4i exhibited expression of some mesodermal genes and showed some of the properties of gastrulating cells, and were thus likely to exist in a state similar to iMeLCs (Sasaki et al., 2015). hPSCs cultured under conventional conditions bear a property similar to the post-implantation epiblast of cynomolgus monkeys, a primate model closely related to humans (Nakamura et al., 2016), and the PGCLC induction efficiency among multiple hiPSC lines has been correlated with the expression levels of genes such as *T*, *MIXL1*, and *EOMES* (Yokobayashi et al., 2017).

Importantly, using these in vitro systems, it has been shown that the mechanism for hPGC specification likely involves signaling and transcriptional mechanisms distinct from those in mouse PGC specification (Irie et al., 2015; Kobayashi et al., 2017; Kojima et al., 2017; Sasaki et al., 2015; Yokobayashi et al., 2017). In response to WNT signaling, *EOMES*, but not *T*, activates *SOX17*, and *SOX17* and *TFAP2C* establish the hPGC program, including *BLIMP1* expression, in an interdependent fashion (Kojima et al., 2017). Thus, key TFs themselves as well as their hierarchy of actions are different between human and mouse PGC specification.

At present, in vitro reconstitution of human germ-cell development from hPSCs is in its incipience and should be conducted in a step-by-step manner, based on the concepts and reconstitution procedures established in mice, as well as in part on the knowledge obtained from appropriate primate models, such as the fact that primate PGCs are specified in the amnion (Sasaki et al., 2016). It may also be important to take much longer developmental times for human germ-cell development into consideration. We consider that a key next step would be to explore the methodologies to induce epigenetic reprogramming and sex determination in hPGCLCs. The development of a method to propagate human SSCs in vitro is also a salient challenge.

Perspective

The last decade has seen significant progress in the in vitro reconstitution of germ-cell development in mice. Indeed, the entire female germ-cell development and the male germ-cell development up to the differentiation of spermatogonia/SSCs reflecting the in vivo germ-cell development have been reconstituted using mouse PSCs as starting materials (Hikabe et al., 2016; Ishikura et al., 2016). The key to the reconstitution of the entire spermatogenesis process in vitro is to realize the trans-meiotic differentiation of spermatogonia into spermatids, which has been pursued for many years, but appears to remain elusive, although one report claims to have gone through this process using a relatively simple methodology (Zhou et al., 2016). This goal will perhaps require a better understanding and the reconstitution of the intra-luminal environment of seminiferous tubules generated by the Sertoli cells, within which spermatogenic meiosis progresses. Toward this goal, efforts using GSCs as a starting material would be critical (Wang et al., 2016). At the same time, considering the key roles played by Sertoli cells and other testicular somatic cells in spermatogenesis (Russell et al., 1990), investigation into their development and their induction from PSCs would be a promising field of research. On the other hand, in vitro reconstitution of human germ-cell development and gametogenesis is an emerging challenge with many exciting potential applications (Saitou and Miyauchi, 2016; Tang et al., 2016). Given the much longer times required for human germ-cell development and the ethical and social impacts of the realization of human in vitro gametogenesis, such studies will require much persistent and careful effort (Ishii and Saitou, 2017).

Acknowledgments

We thank the members of our laboratory for their discussion of this study. This work was supported in part by a Grant-in-Aid for Research Activity Start-up from JSPS (17H06802) to Y.I., a Grant-in-Aid for Specially Promoted Research from JSPS (17H06098) to M.S., and by a grant from JST-ERATO (JPMJER1104) to M.S.

References

- Aramaki, S., Hayashi, K., Kurimoto, K., Ohta, H., Yabuta, Y., Iwanari, H., Mochizuki, Y., Hamakubo, T., Kato, Y., Shirahige, K., et al. (2013). A mesodermal factor, *T*, specifies mouse germ cell fate by directly activating germline determinants. *Developmental Cell*, 27, 516–529.
- Arand, J., Wossidlo, M., Lepikhov, K., Peat, J. R., Reik, W., & Walter, J. (2015). Selective impairment of methylation maintenance is the major cause of DNA methylation reprogramming in the early embryo. *Epigenetics & Chromatin*, 8, 1.
- Baudat, F., Imai, Y., & de Massy, B. (2013). Meiotic recombination in mammals: Localization and regulation. *Nature Reviews. Genetics*, 14, 794–806.
- Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnagar, Y. M., & Dym, M. (1977). Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. *Journal of Cell Biology*, 74, 68–85.

- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11303–11307.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Buaas, F. W., Kirsh, A. L., Sharma, M., McLean, D. J., Morris, J. L., Griswold, M. D., de Rooij, D. G., & Braun, R. E. (2004). Plzf is required in adult male germ cells for stem cell self-renewal. *Nature Genetics*, 36, 647–652.
- Chuma, S., & Nakano, T. (2013). piRNA and spermatogenesis in mice. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 368, 20110338.
- Chuma, S., Kanatsu-Shinohara, M., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Hosokawa, M., Nakatsuji, N., Ogura, A., & Shinohara, T. (2005). Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. *Development*, 132, 117–122.
- Cooke, H. J., Lee, M., Kerr, S., & Ruggiu, M. (1996). A murine homologue of the human DAZ gene is autosomal and expressed only in male and female gonads. *Human Molecular Genetics*, 5, 513–516.
- Costoya, J. A., Hobbs, R. M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K. E., Wolgemuth, D. J., & Pandolfi, P. P. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells. *Nature Genetics*, 36, 653–659.
- Culty, M. (2009). Gonocytes, the forgotten cells of the germ cell lineage. *Birth Defects Research. Part C, Embryo Today*, 87, 1–26.
- Daley, G. Q. (2007). Gametes from embryonic stem cells: A cup half empty or half full? *Science*, 316, 409–410.
- De Felici, M. (2013). Origin, migration, and proliferation of human primordial germ cells. In G. Coticchio, D. F. Albertini, & L. De Santis (Eds.), *Oogenesis* (pp. 19–37). London: Springer-Verlag.
- de Rooij, D. G. (2017). The nature and dynamics of spermatogonial stem cells. *Development*, 144, 3022–3030.
- Edson, M. A., Nagaraja, A. K., & Matzuk, M. M. (2009). The mammalian ovary from genesis to revelation. *Endocrine Reviews*, 30, 624–712.
- Fujiwara, Y., Komiya, T., Kawabata, H., Sato, M., Fujimoto, H., Furusawa, M., & Noce, T. (1994). Isolation of a DEAD-family protein gene that encodes a murine homolog of *Drosophila vasa* and its specific expression in germ cell lineage. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 12258–12262.
- Gafni, O., Weinberger, L., Mansour, A. A., Manor, Y. S., Chomsky, E., Ben-Yosef, D., Kalma, Y., Viukov, S., Maza, I., Zviran, A., et al. (2013). Derivation of novel human ground state naive pluripotent stem cells. *Nature*, 504, 282–286.
- Ginsburg, M., Snow, M. H., & McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development*, 110, 521–528.
- Gkoutela, S., Zhang, K. X., Shafiq, T. A., Liao, W. W., Hargan-Calvopina, J., Chen, P. Y., & Clark, A. T. (2015). DNA demethylation dynamics in the human prenatal germline. *Cell*, 161, 1425–1436.
- Griswold, M. D. (2016). Spermatogenesis: The commitment to meiosis. *Physiological Reviews*, 96, 1–17.
- Guo, F., Yan, L., Guo, H., Li, L., Hu, B., Zhao, Y., Yong, J., Hu, Y., Wang, X., Wei, Y., et al. (2015). The transcriptome and DNA methylome landscapes of human primordial germ cells. *Cell*, 161, 1437–1452.
- Hajkova, P., Erhardt, S., Lane, N., Haaf, T., El-Maarri, O., Reik, W., Walter, J., & Surani, M. A. (2002). Epigenetic reprogramming in mouse primordial germ cells. *Mechanisms of Development*, 117, 15–23.
- Handel, M. A., & Schimenti, J. C. (2010). Genetics of mammalian meiosis: Regulation, dynamics and impact on fertility. *Nature Reviews. Genetics*, 11, 124–136.
- Handel, M. A., Eppig, J. J., & Schimenti, J. C. (2014). Applying “gold standards” to in-vitro-derived germ cells. *Cell*, 157, 1257–1261.
- Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., & Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell*, 146, 519–532.
- Hayashi, K., Ogushi, S., Kurimoto, K., Shimamoto, S., Ohta, H., & Saitou, M. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science*, 338, 971–975.
- Hermann, B. P., Sukhwani, M., Hansel, M. C., & Orwig, K. E. (2010). Spermatogonial stem cells in higher primates: Are there differences from those in rodents? *Reproduction*, 139, 479–493.
- Hikabe, O., Hamazaki, N., Nagamatsu, G., Obata, Y., Hirao, Y., Hamada, N., Shimamoto, S., Imamura, T., Nakashima, K., Saitou, M., et al. (2016). Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature*, 539, 299–303.
- Hirota, T., Ohta, H., Powell, B. E., Mahadevaiah, S. K., Ojarikre, O. A., Saitou, M., & Turner, J. M. A. (2017). Fertile offspring from sterile sex chromosome trisomic mice. *Science*, 357, 932–935.
- Irie, N., Weinberger, L., Tang, W. W., Kobayashi, T., Viukov, S., Manor, Y. S., Dietmann, S., Hanna, J. H., & Surani, M. A. (2015). SOX17 is a critical specifier of human primordial germ cell fate. *Cell*, 160, 253–268.
- Ishii, T., & Saitou, M. (2017). Promoting in vitro gametogenesis research with a social understanding. *Trends in Molecular Medicine*, 23, 985–988.
- Ishikura, Y., Yabuta, Y., Ohta, H., Hayashi, K., Nakamura, T., Okamoto, I., Yamamoto, T., Kurimoto, K., Shirane, K., Sasaki, H., et al. (2016). In vitro derivation and propagation of spermatogonial stem cell activity from mouse pluripotent stem cells. *Cell Reports*, 17, 2789–2804.
- Kagiwada, S., Kurimoto, K., Hirota, T., Yamaji, M., & Saitou, M. (2013). Replication-coupled passive DNA demethylation for the erasure of genome imprints in mice. *EMBO Journal*, 32, 340–353.
- Kanatsu-Shinohara, M., & Shinohara, T. (2013). Spermatogonial stem cell self-renewal and development. *Annual Review of Cell and Developmental Biology*, 29, 163–187.
- Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Miki, H., Ogura, A., Toyokuni, S., & Shinohara, T. (2003). Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biology of Reproduction*, 69, 612–616.
- Kimmins, S., & Sassone-Corsi, P. (2005). Chromatin remodelling and epigenetic features of germ cells. *Nature*, 434, 583–589.
- Kobayashi, T., Zhang, H., Tang, W. W. C., Irie, N., Withey, S., Klisch, D., Sybirna, A., Dietmann, S., Contreras, D. A., Webb, R., et al. (2017). Principles of early human development and germ cell program from conserved model systems. *Nature*, 546, 416–420.
- Kojima, K., Sato, T., Naruse, Y., & Ogawa, T. (2016). Spermatogenesis in explanted fetal mouse testis tissues. *Biology of Reproduction*, 95, 63.
- Kojima, Y., Sasaki, K., Yokobayashi, S., Sakai, Y., Nakamura, T., Yabuta, Y., Nakaki, F., Nagaoka, S., Woltjen, K., Hotta, A., et al. (2017). Evolutionarily distinctive transcriptional and signaling programs drive human germ cell lineage specification from pluripotent stem cells. *Cell Stem Cell*, 21, 517–532.
- Komeya, M., Kimura, H., Nakamura, H., Yokonishi, T., Sato, T., Kojima, K., Hayashi, K., Katagiri, K., Yamanaka, H., Sanjo, H., et al. (2016). Long-term ex vivo maintenance of testis tissues producing fertile sperm in a microfluidic device. *Scientific Reports*, 6, 21472.
- Kong, A., Frigge, M. L., Masson, G., Besenbacher, S., Sulem, P., Magnusson, G., Gudjonsson, S. A., Sigurdsson, A., Jonasdottir, A., Jonasdottir, A., et al. (2012). Rate of de novo mutations and the importance of father's age to disease risk. *Nature*, 488, 471–475.
- Kubo, N., Toh, H., Shirane, K., Shirakawa, T., Kobayashi, H., Sato, T., Sone, H., Sato, Y., Tomizawa, S., Tsurusaki, Y., et al. (2015). DNA methylation and gene expression dynamics during spermatogonial stem cell differentiation in the early postnatal mouse testis. *BMC Genomics*, 16, 624.
- Kubota, H., Avarbock, M. R., & Brinster, R. L. (2004). Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 16489–16494.
- Kurimoto, K., Yabuta, Y., Ohinata, Y., Shigeta, M., Yamanaka, K., & Saitou, M. (2008). Complex genome-wide transcription dynamics orchestrated by Blimp1 for the specification of the germ cell lineage in mice. *Genes & Development*, 22, 1617–1635.
- Kurimoto, K., Yabuta, Y., Hayashi, K., Ohta, H., Kiyonari, H., Mitani, T., Moritoki, Y., Kohri, K., Kimura, H., Yamamoto, T., et al. (2015). Quantitative dynamics of chromatin remodeling during germ cell specification from mouse embryonic stem cells. *Cell Stem Cell*, 16, 517–532.
- Lawson, K. A., & Hage, W. J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Foundation Symposium*, 182, 68–84.

- Li, L., Dong, J., Yan, L., Yong, J., Liu, X., Hu, Y., Fan, X., Wu, X., Guo, H., Wang, X., et al. (2017). Single-cell RNA-Seq analysis maps development of human germline cells and gonadal niche interactions. *Cell Stem Cell*, 20, 858–873. e854.
- Lin, Y., Gill, M. E., Koubova, J., & Page, D. C. (2008). Germ cell-intrinsic and -extrinsic factors govern meiotic initiation in mouse embryos. *Science*, 322, 1685–1687.
- Mamsen, L. S., Lutterodt, M. C., Andersen, E. W., Byskov, A. G., & Andersen, C. Y. (2011). Germ cell numbers in human embryonic and fetal gonads during the first two trimesters of pregnancy: Analysis of six published studies. *Human Reproduction*, 26, 2140–2145.
- Manku, G., & Culty, M. (2015). Mammalian gonocyte and spermatogonia differentiation: Recent advances and remaining challenges. *Reproduction*, 149, R139–R157.
- McGee, E. A., & Hsueh, A. J. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews*, 21, 200–214.
- Meng, X., Lindahl, M., Hyvonen, M. E., Parvinen, M., de Rooij, D. G., Hess, M. W., Raatikainen-Ahokas, A., Sainio, K., Rauvala, H., Lakso, M., et al. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, 287, 1489–1493.
- Miyauchi, H., Ohta, H., Nagaoka, S., Nakaki, F., Sasaki, K., Hayashi, K., Yabuta, Y., Nakamura, T., Yamamoto, T., & Saitou, M. (2017). Bone morphogenetic protein and retinoic acid synergistically specify female germ cell fate in mice. *EMBO Journal*, 36, 3100–3119.
- Morohaku, K., Tanimoto, R., Sasaki, K., Kawahara-Miki, R., Kono, T., Hayashi, K., Hirao, Y., & Obata, Y. (2016). Complete in vitro generation of fertile oocytes from mouse primordial germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 9021–9026.
- Nakagawa, M., Taniguchi, Y., Senda, S., Takizawa, N., Ichisaka, T., Asano, K., Morizane, A., Doi, D., Takahashi, J., Nishizawa, M., et al. (2014). A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells. *Scientific Reports*, 4, 3594.
- Nakaki, F., Hayashi, K., Ohta, H., Kurimoto, K., Yabuta, Y., & Saitou, M. (2013). Induction of mouse germ-cell fate by transcription factors in vitro. *Nature*, 501, 222–226.
- Nakamura, T., Okamoto, I., Sasaki, K., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Yamamoto, T., & Saitou, M. (2016). A developmental coordinate of pluripotency among mice, monkeys and humans. *Nature*, 537, 57–62.
- Nichols, J., & Smith, A. (2009). Naïve and primed pluripotent states. *Cell Stem Cell*, 4, 487–492.
- Ohinata, Y., Payer, B., O'Carroll, D., Ancelin, K., Ono, Y., Sano, M., Barton, S. C., Obukhanych, T., Nussenzweig, M., Tarakhovsky, A., et al. (2005). Blimp1 is a critical determinant of the germ cell lineage in mice. *Nature*, 436, 207–213.
- Ohinata, Y., Ohta, H., Shigetani, M., Yamanaka, K., Wakayama, T., & Saitou, M. (2009). A signaling principle for the specification of the germ cell lineage in mice. *Cell*, 137, 571–584.
- Ohta, H., Wakayama, T., & Nishimune, Y. (2004). Commitment of fetal male germ cells to spermatogonial stem cells during mouse embryonic development. *Biology of Reproduction*, 70, 1286–1291.
- Ohta, H., Kurimoto, K., Okamoto, I., Nakamura, T., Yabuta, Y., Miyauchi, H., Yamamoto, T., Okuno, Y., Hagiwara, M., Shirane, K., et al. (2017). In vitro expansion of mouse primordial germ cell-like cells recapitulates an epigenetic blank slate. *EMBO Journal*, 36, 1888–1907.
- Paniagua, R., & Nistal, M. (1984). Morphological and histometric study of human spermatogonia from birth to the onset of puberty. *Journal of Anatomy*, 139(Pt 3), 535–552.
- Popp, C., Dean, W., Feng, S., Cokus, S. J., Andrews, S., Pellegrini, M., Jacobsen, S. E., & Reik, W. (2010). Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. *Nature*, 463, 1101–1105.
- Russell, L. D., Ettlin, R. A., Sinha Hikim, A. P., & Clegg, E. D. (1990). *Histological and histopathological evaluation of the testis*. Clearwater: Cache River Press.
- Saitou, M., & Miyauchi, H. (2016). Gametogenesis from pluripotent stem cells. *Cell Stem Cell*, 18, 721–735.
- Saitou, M., & Yamaji, M. (2012). Primordial germ cells in mice. *Cold Spring Harbor Perspectives in Biology*, 4, a008375.
- Saitou, M., Barton, S. C., & Surani, M. A. (2002). A molecular programme for the specification of germ cell fate in mice. *Nature*, 418, 293–300.
- Saitou, M., Kagiwada, S., & Kurimoto, K. (2012). Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. *Development*, 139, 15–31.
- Sasaki, K., Yokobayashi, S., Nakamura, T., Okamoto, I., Yabuta, Y., Kurimoto, K., Ohta, H., Moritoki, Y., Iwatani, C., Tsuchiya, H., et al. (2015). Robust in vitro induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell*, 17, 178–194.
- Sasaki, K., Nakamura, T., Okamoto, I., Yabuta, Y., Iwatani, C., Tsuchiya, H., Seita, Y., Nakamura, S., Shiraki, N., Takakuwa, T., et al. (2016). The germ cell fate of cynomolgus monkeys is specified in the nascent amnion. *Developmental Cell*, 39, 169–185.
- Sato, T., Katagiri, K., Gohbara, A., Inoue, K., Ogonuki, N., Ogura, A., Kubota, Y., & Ogawa, T. (2011a). In vitro production of functional sperm in cultured neonatal mouse testes. *Nature*, 471, 504–507.
- Sato, T., Katagiri, K., Yokonishi, T., Kubota, Y., Inoue, K., Ogonuki, N., Matoba, S., Ogura, A., & Ogawa, T. (2011b). In vitro production of fertile sperm from murine spermatogonial stem cell lines. *Nature Communications*, 2, 472.
- Seisenberger, S., Andrews, S., Krueger, F., Arand, J., Walter, J., Santos, F., Popp, C., Thienpont, B., Dean, W., & Reik, W. (2012). The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. *Molecular Cell*, 48, 849–862.
- Seki, Y., Hayashi, K., Itoh, K., Mizugaki, M., Saitou, M., & Matsui, Y. (2005). Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. *Developmental Biology*, 278, 440–458.
- Shinohara, T., Orwig, K. E., Avarbock, M. R., & Brinster, R. L. (2001). Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *Proceedings of the National Academy of Sciences of the United States of America*, 98, 6186–6191.
- Shirane, K., Kurimoto, K., Yabuta, Y., Yamaji, M., Satoh, J., Ito, S., Watanabe, A., Hayashi, K., Saitou, M., & Sasaki, H. (2016). Global landscape and regulatory principles of DNA methylation reprogramming for germ cell specification by mouse pluripotent stem cells. *Developmental Cell*, 39, 87–103.
- Siomi, M. C., Sato, K., Pezic, D., & Aravin, A. A. (2011). PIWI-interacting small RNAs: The vanguard of genome defence. *Nature Reviews. Molecular Cell Biology*, 12, 246–258.
- Spiller, C., Koopman, P., & Bowles, J. (2017). Sex determination in the mammalian Germline. *Annual Review of Genetics*, 51, 265–285.
- Steinberger, A., Steinberger, E., & Perloff, W. H. (1964). Mammalian testes in organ culture. *Experimental Cell Research*, 36, 19–27.
- Suzuki, A., & Saga, Y. (2008). Nanos2 suppresses meiosis and promotes male germ cell differentiation. *Genes & Development*, 22, 430–435.
- Tam, P. P., & Snow, M. H. (1981). Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. *Journal of Embryology and Experimental Morphology*, 64, 133–147.
- Tanaka, S. S., Toyooka, Y., Akasu, R., Katoh-Fukui, Y., Nakahara, Y., Suzuki, R., Yokoyama, M., & Noce, T. (2000). The mouse homolog of Drosophila Vasa is required for the development of male germ cells. *Genes & Development*, 14, 841–853.
- Tang, W. W., Dietmann, S., Irie, N., Leitch, H. G., Floros, V. I., Bradshaw, C. R., Hackett, J. A., Chinnery, P. F., & Surani, M. A. (2015). A unique gene regulatory network resets the human germline epigenome for development. *Cell*, 161, 1453–1467.
- Tang, W. W., Kobayashi, T., Irie, N., Dietmann, S., & Surani, M. A. (2016). Specification and epigenetic programming of the human germ line. *Nature Reviews. Genetics*, 17, 585–600.
- Tsuda, M., Sasaoka, Y., Kiso, M., Abe, K., Haraguchi, S., Kobayashi, S., & Saga, Y. (2003). Conserved role of nanos proteins in germ cell development. *Science*, 301, 1239–1241.
- Vidal, V. P., Chaboissier, M. C., de Rooij, D. G., & Schedl, A. (2001). Sox9 induces testis development in XX transgenic mice. *Nature Genetics*, 28, 216–217.
- Viger, R. S., Mertineit, C., Trasler, J. M., & Nemer, M. (1998). Transcription factor GATA-4 is expressed in a sexually dimorphic pattern during mouse gonadal development and is a potent activator of the Mullerian inhibiting substance promoter. *Development*, 125, 2665–2675.
- Vincent, S. D., Dunn, N. R., Sciammas, R., Shapiro-Shalef, M., Davis, M. M., Calame, K., Bikoff, E. K., & Robertson, E. J. (2005). The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. *Development*, 132, 1315–1325.
- von Meyenn, F., Berrens, R. V., Andrews, S., Santos, F., Collier, A. J., Krueger, F., Osomo, R., Dean, W., Rugg-Gunn, P. J., & Reik, W. (2016). Comparative principles of DNA methylation reprogramming during human and mouse in vitro primordial germ cell specification. *Developmental Cell*, 39, 104–115.
- Wang, S., Wang, X., Ma, L., Lin, X., Zhang, D., Li, Z., Wu, Y., Zheng, C., Feng, X., Liao, S., et al. (2016). Retinoic acid is sufficient for the in vitro induction of mouse spermatocytes. *Stem Cell Reports*, 7, 80–94.

- Weber, S., Eckert, D., Nettersheim, D., Gillis, A. J., Schafer, S., Kuckenberg, P., Ehlermann, J., Werling, U., Biermann, K., Looijenga, L. H., et al. (2010). Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. *Biology of Reproduction*, 82, 214–223.
- Witschi, E. (1948). Migration of germ cells of human embryos from the yolk sac to the primitive gonadal folds. *Contributions to Embryology Carnegie Institution*, 209, 67–80.
- Yamaji, M., Seki, Y., Kurimoto, K., Yabuta, Y., Yuasa, M., Shigeta, M., Yamanaka, K., Ohinata, Y., & Saitou, M. (2008). Critical function of Prdm14 for the establishment of the germ cell lineage in mice. *Nature Genetics*, 40, 1016–1022.
- Yokobayashi, S., Okita, K., Nakagawa, M., Nakamura, T., Yabuta, Y., Yamamoto, T., & Saitou, M. (2017). Clonal variation of human induced pluripotent stem cells for induction into the germ cell fate. *Biology of Reproduction*, 96, 1154–1166.
- Yoshida, S. (2016). From cyst to tubule: Innovations in vertebrate spermatogenesis. *Wiley Interdisciplinary Reviews: Developmental Biology*, 5, 119–131.
- Yoshida, S., Sukeno, M., Nakagawa, T., Ohbo, K., Nagamatsu, G., Suda, T., & Nabeshima, Y. (2006). The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. *Development*, 133, 1495–1505.
- Yuan, L., Liu, J. G., Zhao, J., Brundell, E., Daneholt, B., & Hoog, C. (2000). The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility. *Molecular Cell*, 5, 73–83.
- Zhou, Q., Wang, M., Yuan, Y., Wang, X., Fu, R., Wan, H., Xie, M., Liu, M., Guo, X., Zheng, Y., et al. (2016). Complete meiosis from embryonic stem cell-derived germ cells in vitro. *Cell Stem Cell*, 18, 330–340.

OOGENESIS

Growth and Meiotic Maturation of Mammalian Oocytes: An Overview

Hugh J Clarke, McGill University, Montreal, QC, Canada; and McGill University Health Centre, Montreal, QC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

A newborn girl carries up to about a million germ cells, known as oocytes, each housed in a structure termed a primordial follicle (Kerr et al., 2013). The weight of current evidence indicates that female mammals do not generate new oocytes after birth—in contrast to the continuous production of new sperm by sexually mature males—meaning that the endowment of oocytes that a female receives at birth represents her lifetime supply. The generation of oocytes and their prenatal differentiation and assembly into follicles has been recently reviewed and will not be discussed here (Jorgensen, 2013; Pepling, 2012). Postnatal development of the oocyte can be divided into two phases—growth, which is a slow process that requires 3–4 months in humans, and meiotic maturation, a much more rapid process that occurs at the time of ovulation and requires about 36 h in humans (Fig. 1). During

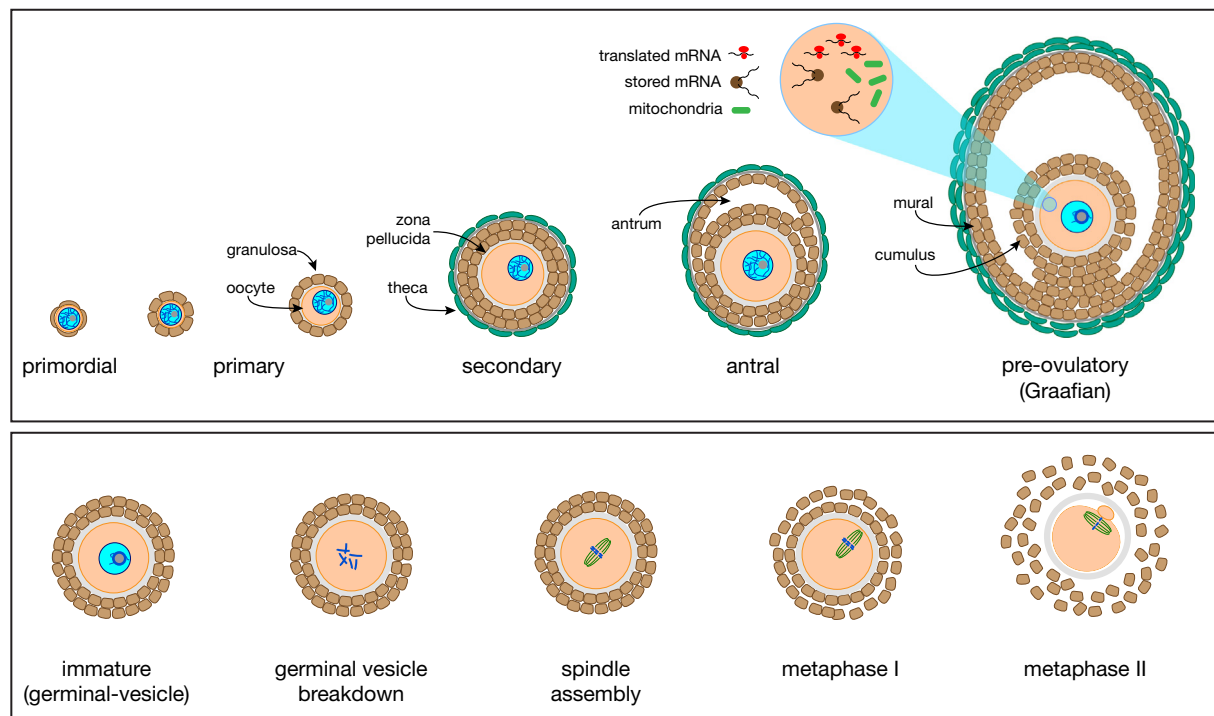


Fig. 1 Growth and meiotic maturation of the oocyte. Upper: Growth of the oocyte and its follicle are coordinated as shown. Entry into growth is marked by a transition of the granulosa cells from a squamous to a cuboidal morphology and an increase in the size of the oocyte. As the oocyte continues to grow, the granulosa cells proliferate, generating multiple layers. The elaboration of the zona pellucida, an extracellular coat assembled from oocyte-secreted glycoproteins, physically separates the oocyte from the bodies of the granulosa cells. At the secondary follicle stage, thecal cells come to lie external to the granulosa cells, from which they are separated by a basement membrane. Near the time that the oocyte reaches full-size, a fluid-filled cavity termed the antrum appears in the follicle, separating the granulosa cells into mural and cumulus subpopulations. The chromatin of fully grown oocytes becomes partially condensed around the nucleolus-like body; this process is termed the NSN-to-SN transition and is associated with transcriptional arrest. Growing oocytes accumulate large reserves of mRNAs, some of which are stored for future translation, and organelles including mitochondria. Lower: During meiotic maturation, the oocyte enters M-phase of the cell cycle. The membrane surrounding the germinal vesicle (nucleus) breaks down, and the chromosomes condense and become aligned on a spindle. Following translocation of the spindle to the oocyte cortex, the first meiotic division occurs such that one set of homologous chromosomes is discarded in the small first polar body. The oocyte advances to metaphase II where it becomes arrested until fertilization triggers completion of the second meiotic division. Numerous cytoplasmic changes that are not shown, including translational activation of previously silent mRNAs, also occur during maturation. As the oocyte matures, the cumulus granulosa cells secrete a gelatinous matrix that pushes them away from the oocyte and from each other, thus terminating physical contact between the two cell types.

growth and maturation of the oocyte, the follicle also undergoes dramatic and coordinated changes, and so these are discussed together below.

Growth

In order to maintain a stock of oocytes throughout reproductive life, the entry of oocytes within primordial follicles into the growth phase must be strictly regulated. We do not know what controls the rate or number of primordial follicles that begin to grow—why one or several primordial follicle may begin to grow whereas their neighbors does not. It is intriguing, however, that when follicles are aggregated together to generate an ovary-like structure, all begin to grow at the same time (Hayashi et al., 2012; Eppig and Wigglesworth, 2000). This suggest that the mechanisms that regulate entry of follicles into the growth phase are embedded in the architecture of the intact ovary.

The first indication that an oocyte within a primordial follicle has begun to grow is an increase in the diameter of the oocyte and a morphological transition of the somatic granulosa cells that surround the oocyte from a squamous to a cuboidal shape (Fig. 2) (Mora et al., 2012; Eppig et al., 2000). Since both cell types manifest changes at approximately the same time, which one is responsible for the initiating signal? Evidence so far points to the granulosa cells (Saaticioglu et al., 2016; Zhang et al., 2014). The granulosa cells are known to produce Kit ligand (KITL) while oocytes express its receptor KIT. Activation of KIT by its ligand in other cell types activates the phosphatidylinositol 3-kinase (PI3K) signaling pathway, which in turn leads to an increase in protein synthesis, and recent studies indicate that an increase in KITL production by the granulosa cells triggers oocyte growth through this mechanism. Through the use of appropriate genetic mutations, however, it is possible to experimentally activate KIT signaling in the oocyte in a ligand-independent manner. In this case, the oocyte begins to grow, but the granulosa cells remain squamous. This result suggests that oocyte growth per se does not trigger the squamous-cuboidal transition of the granulosa cells, favoring the idea that the signal for entry of the primordial follicle into the growth phase originates in the granulosa cells. Nonetheless, the question is not entirely resolved. We still do not know what causes the granulosa cells to increase production of KITL, and it is conceivable that this occurs in response to a signal from the oocyte.

Entry into the growth phase is accompanied by major changes in the pattern of gene expression in the oocyte. Although mechanistic links remain to be established, this genetic reprogramming probably plays a key role in driving oocyte growth. FOXO3 is predominantly nuclear in oocytes in primordial follicles, but translocates to the cytoplasm shortly after initiation of oocyte growth, and genetic deletion of *Foxo3* causes most oocytes in primordial follicles to begin to grow (Castrillon et al., 2003). Similarly, deletion of the oocyte-specific transcription factor *Sohlh2* (Choi et al., 2008) or oocyte-specific deletion of transcription factor *Lhx8* (Ren et al., 2015) also trigger initiation of oocyte growth, apparently independently of signals from the granulosa cells. These changes in transcription factor activity seem likely to represent part of the mechanism that responds to the growth-promoting signals sent by the granulosa cells.

Once oocyte growth has been initiated, it continues in an apparently continuous manner until the oocyte reaches a species-specific size—for example, approximately 120 μm in humans and 75 μm in mouse. This represents an increase of 100- to 200-fold in volume as compared to an oocyte in a primordial follicle. Whether growth requires continuous “activating” signals from the granulosa cells is not clear; however, KITL and KIT continue to be expressed by granulosa cells and growing oocyte, respectively, and KITL can stimulate continued growth of partially grown oocytes in vitro (Kidder and Vanderhyden, 2010). What is certain,

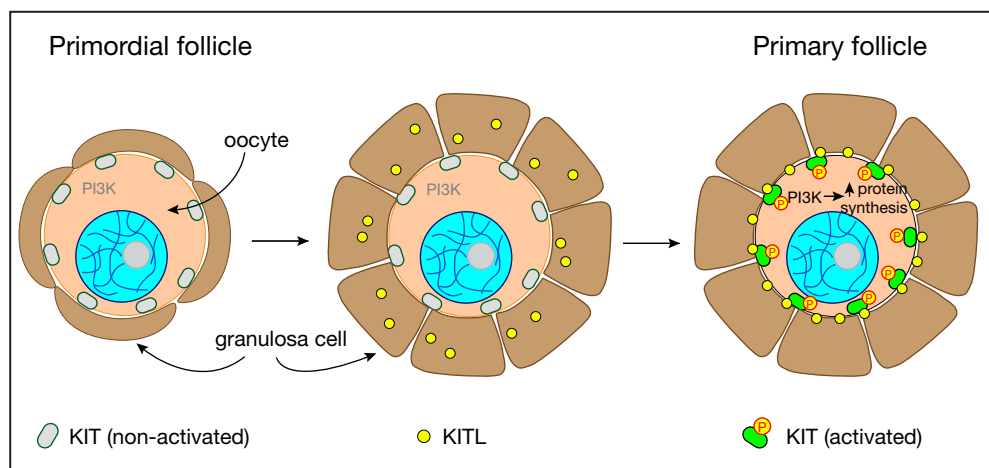


Fig. 2 Initiation of growth. Oocytes in primordial follicles are enclosed by a layer of squamous granulosa cells. Unknown signals induce the granulosa cells to assume a cuboidal morphology and probably also to increase synthesis of KITL. This ligand binds to its receptor, KIT, on the oocyte surface leading to activation of the PI3-kinase signaling pathway. This in turn increases protein synthesis in the oocyte, which begins to grow. Activating PI3-kinase signaling in the oocyte can trigger its growth even when the granulosa cells remain squamous.

however, is that the granulosa cells play an indispensable role. This has been shown in two ways. First, when partially grown oocytes enclosed by granulosa cells are removed from the follicle and placed in culture, they will continue to grow, whereas such oocytes from which the granulosa cells have been removed will not. Second, an extensive network of gap junctions couples the oocyte to adjacent granulosa cells, as well as the granulosa cells to each other. When either oocyte–granulosa cell or granulosa–granulosa cell gap junction coupling is disrupted by mutation of genes encoding their constituent proteins, oocytes fail to grow to full-size and are unable to develop as embryos (El-Hayek and Clarke, 2016; Winterhager and Kidder, 2015). These results demonstrate that the granulosa cells provide essential products to the oocytes via gap junctions and that disrupting granulosa cell interactions impairs their ability to supply these products. It is noteworthy that there is partial, albeit limited, oocyte growth when gap junctional coupling with the granulosa cells is abolished, but no oocyte growth when the granulosa cells are physically separated from the oocyte. This implies that oocyte growth requires an additional contact-dependent interaction with the granulosa cells—possibly the KIT-KIT signaling described above.

Several essential products provided to oocytes by the granulosa cells have been identified (Fig. 3) (Su et al., 2009). Oocytes express only low levels of mRNAs encoding enzymes required to metabolize glucose to pyruvate that acts as a source of ATP, whereas these mRNAs are expressed much higher levels in the granulosa cells. Consistent with these observations, pyruvate produced in the granulosa cells is transferred to the oocyte. Oocytes also take up certain amino acids poorly, which are transferred to them from the granulosa cells via the gap junctions. Finally, cholesterol produced by the granulosa cells is transferred to the oocyte. Granulosa cells also send signals via gap junctions that regulate the initiation of meiotic maturation to the oocyte; this function is discussed in detail below.

Signaling between the oocyte and granulosa cells does not run only in one direction. Several growth factors produced by the oocyte that act on the granulosa cells have been identified (Fig. 3) (Mottershead et al., 2015; Peng et al., 2013; Chang et al., 2016). The best-known are two members of the transforming growth factor β family, GDF (growth-differentiation factor) 9 and BMP (bone morphogenetic protein) 15. Both factors are produced by growing oocytes, although the relative portions and timing of synthesis may vary among species. Both can act as diffusible factors in vitro, although this does not exclude the possibility that, in vivo, they may be tethered to the oocyte surface. In the absence of either or both growth factors, granulosa cells show defects in proliferation and differentiation and they fail to undergo a process termed cumulus layer expansion at the time of oocyte maturation and ovulation. FGF (fibroblastic growth factor) 8 produced by oocytes also influences granulosa cell differentiation and activity. Importantly, in the mouse, production of GDF9 by the oocyte is essential, via its effect on granulosa cell physiology, for normal oocyte development. Thus, the oocyte actively modifies its microenvironment to ensure normal differentiation.

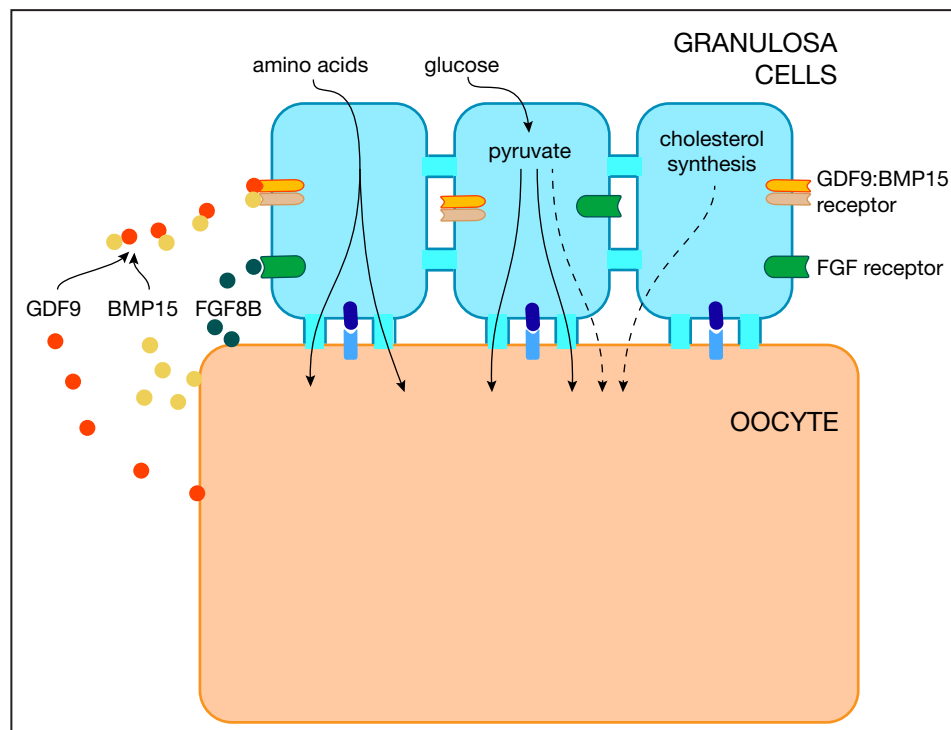


Fig. 3 Communication between the growing oocyte and neighboring granulosa cells. Gap junctions link the granulosa cells to the oocyte and to each other and permit the passage of small molecules. Glucose and certain amino acids are poorly taken up by the oocyte, which relies on the granulosa cells to provide glucose-derived pyruvate and the amino acids. Pyruvate may be transferred to the oocyte through gap junctions or by diffusion. Cholesterol synthesized by the granulosa cells is also transferred to the oocyte. The oocyte secretes several growth factors, including GDF9, BMP15 and FGF8B, that help to regulate granulosa cell differentiation and function.

Apart from the enormous increase in volume, what happens during oocyte growth? Below, I describe several key events, though many equally important changes occur and others certainly remain to be identified. Early during its growth, the oocyte secretes a family of glycoproteins that become assembled into an extracellular matrix termed the zona pellucida (Wassarman and Litscher, 2013). The zona pellucida completely envelopes the growing oocyte, reaching a final thickness of about 15 μm in humans. It serves at least two functions: first, because fertilization triggers modifications to the zona pellucida that prevent sperm penetration through it, it acts as a barrier to polyspermic fertilization; second, it is thought to protect the embryo from becoming trapped within the oviduct, thereby enabling it to reach the uterus where it will implant.

Because the zona pellucida physically separates the oocyte from the adjacent granulosa cells that supply it with essential nutrients and signals via gap junctions, a mechanism is required to enable the two cells to remain in physical contact. This mechanism consists of finger-like extensions of the granulosa cells, termed transzonal projections (TZPs), that traverse the zona pellucida to reach the oocyte (Fig. 4) (Li and Albertini, 2013; Clarke, 2017). The gap junctions connecting the granulosa cells to the oocyte are located where the tips of the TZPs contact the oocyte plasma membrane.

Growing oocytes also produce a large quantity of messenger RNAs. Some mRNAs synthesized by the growing oocyte are translated immediately to provide the proteins that sustain growth, but many others remain silent (Fig. 5) (Kang and Han, 2011; Svoboda et al., 2015; Clarke, 2012; Reyes and Ross, 2016). These stored mRNAs will be translated during either meiotic maturation or after fertilization, which are periods when the oocyte is transcriptionally silent. Although the oocyte may employ multiple strategies to identify whether specific mRNA species should be translated or stored, a well-characterized mechanism relies on a specific U-rich sequence, termed the cytoplasmic polyadenylation element (CPE), that is located in the 3'-untranslated region (3'-utr) within about 200 nt of the polyadenylation signal. The poly(A) tail of mRNAs bearing an appropriately positioned CPE becomes shortened (deadenylated) in growing oocytes and the mRNAs are likely stored in ribonucleoprotein particles in cytoplasm. Thus, unlike the case in many other cell types, deadenylation of an mRNA in oocytes is not coupled to its degradation.

Growing oocytes also generate an enormous number of mitochondria (St John, 2014). Although a wide range of values have been reported and there may be significant differences among species, the number in a fully grown oocyte could be conservatively estimated to be at least 150,000. As in the case of the mRNAs, this huge accumulation reflects the fact that embryos do not generate new mitochondria until after the embryo has implanted into the uterus and begun to grow in size. Other changes that occur during oocyte growth include the synthesis of membrane-bound vesicles, termed cortical granules (Cheeseman et al., 2016), whose contents will modify the zona pellucida following fertilization to prevent polyspermy, the assembly of a cytoplasmic lattice that may act as a storage unit for ribonucleoprotein particles carrying silent mRNAs (Liu et al., 2017), and the methylation of specific cytosine residues in DNA that establishes the female genomic imprinting pattern (Kelsey and Feil, 2013).

As oocytes approach full-size, the chromatin undergoes a morphological change termed the non-surrounded nucleolus (NSN) to surrounded nucleolus (SN) transition and transcriptional activity drops to an essentially undetectable level and the. Although the NSN–SN transition and transcriptional arrest are closely coupled temporally, their mechanistic link remains to be fully elucidated. Nor is the function of the transcriptional arrest in fully grown oocytes well-understood. It is worth noting that oocytes stop growing once they reach a species-characteristic size and it is interesting to speculate whether transcriptional arrest is the cause or the consequence of the growth arrest.

Meiotic Maturation

Near the end of the growth phase, oocytes acquire the ability to undergo meiotic maturation—the final stage of oocyte development before fertilization. During maturation, the oocyte, which has been arrested at late G2 of the cell cycle since entering meiosis during fetal life, enters M-phase, completes the first meiotic division and becomes arrested at metaphase of the second meiotic division (metaphase II). These divisions are discussed in more detail below; first, I will describe how maturation is controlled.

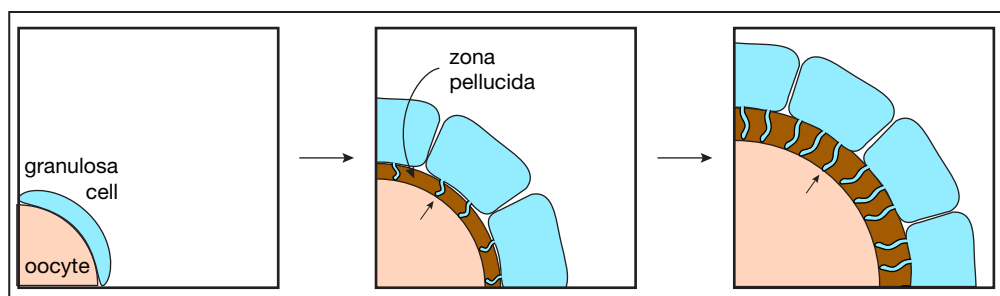


Fig. 4 Specialized structures that enable the oocyte and granulosa cells to maintain contact. The elaboration of the zona pellucida by the growing oocyte physically separates it from the adjacent granulosa cells. Contact between the two cell types is maintained by narrow cytoplasmic fingers, termed transzonal projections (TZPs, arrows), that extend from the granulosa cells through the zona pellucida to the surface of the oocyte. The gap junctions that metabolically couple the two cell types and are essential for oocyte growth are located at the tips of the TZPs. Granulosa cell–oocyte contact via TZPs is maintained throughout oocyte growth but is lost during meiotic maturation.

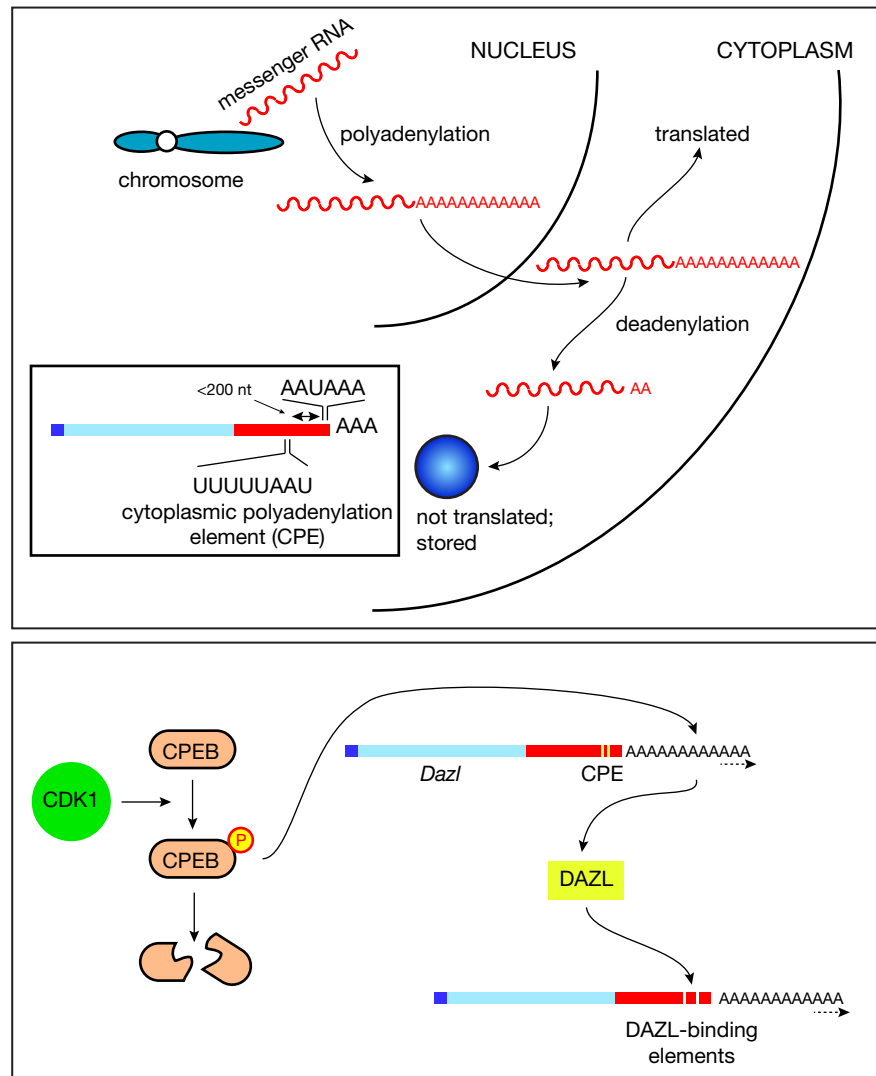


Fig. 5 Regulation of mRNA translation during growth and maturation. Upper: Growing oocytes. Most newly transcribed mRNAs are polyadenylated at the 3'-end in the nucleus. Following export to the cytoplasm, those following the default pathway remain polyadenylated and are translated. However, mRNAs carrying specific sequences in the 3'-untranslated region (3'-utr; red portion of mRNA in box) become deadenylated and translationally silent. The best-characterized sequence is the cytoplasmic polyadenylation element (CPE), typically located within 200 nt (double-headed arrow) of the poly(A) signal (AAUAAA). These silent mRNAs are very stable and thought to be stored in ribonucleoprotein particles. Lower: Maturing oocytes. Activation of CDK1 leads to phosphorylation and subsequent degradation of the CPE-binding protein, CPEB. This triggers elongation of the poly(A) tail (dashed arrow) and translational activation. *Dazl* mRNA is translationally activated by this mechanism, producing DAZL protein. DAZL in turn can bind to sequences in the 3'-utr of other mRNAs, triggering their polyadenylation and translational activation. Such mechanisms enable protein synthesis to be temporally regulated during maturation.

Maturation is initiated physiologically by luteinizing hormone (LH) released by the pituitary gland. Through a series of signaling steps described below, this leads to activation of cyclin-dependent kinase (CDK) 1 within the meiotically immature oocyte (Conti et al., 2012; Jaffe and Egbert, 2017), which has been arrested at late G2 of the cell cycle since entering meiosis during embryonic development, driving it into M-phase. Although fully grown immature oocytes contain CDK1, it is maintained—until the LH release—in an inactive form as follows (Fig. 6). NPPC (natriuretic peptide C) secreted by the mural granulosa cells of the follicle binds to and activates the G-protein coupled membrane receptor, NPR2 (natriuretic peptide receptor 2), on the mural and cumulus granulosa cells, triggering production of the nucleotide, cyclic (c) GMP (Zhang et al., 2010). Via the gap junctions located at the tips of the TZPs, cGMP flows from the granulosa cells into the oocyte, where it inhibits the activity of phosphodiesterase (PDE) 3A. This inhibition allows the concentration of cAMP, which is constitutively synthesized by the oocyte, to remain elevated. cAMP activates protein kinase A, which indirectly maintains inhibitory phosphorylation of specific sites on CDK1.

The preovulatory release of LH and binding to its receptor (LHGCR) located on the mural granulosa cells of the follicle (LHGCR is also present on the thecal cells) causes them to release ligands that bind to the epidermal growth factor receptor (EGFR) located on both the mural and cumulus granulosa cells. Via mechanisms that remain to be fully elucidated, activation of LHGCR and EGFR

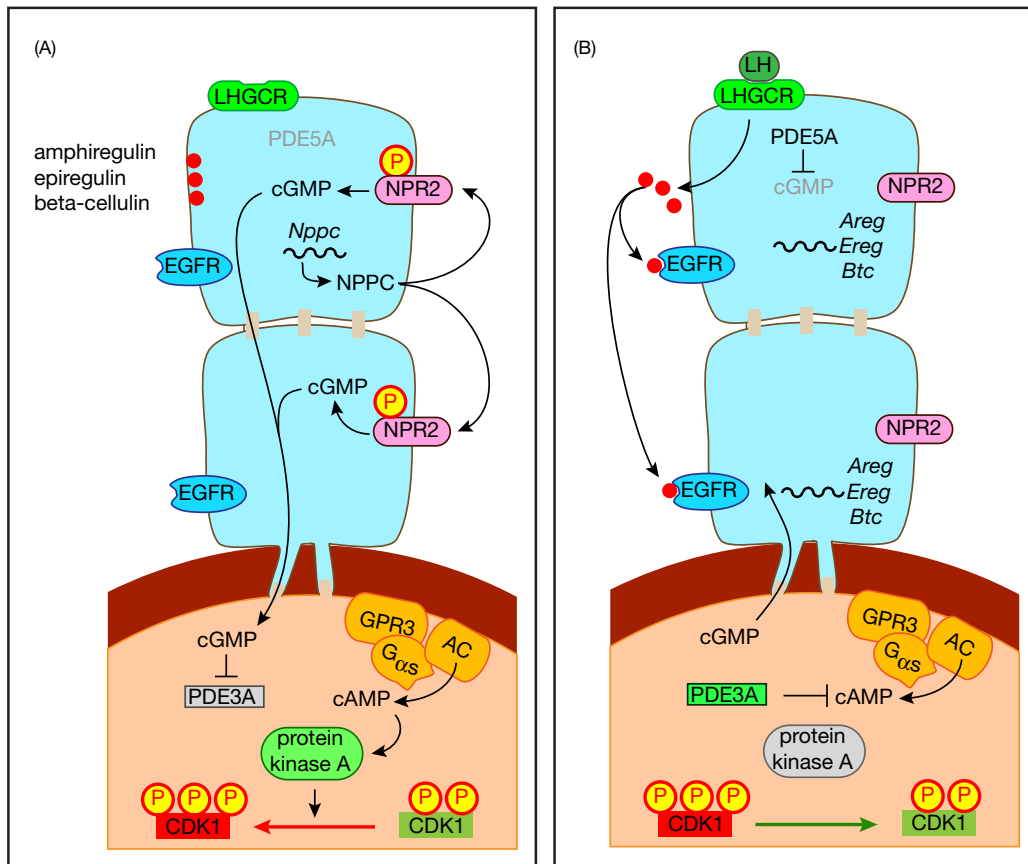


Fig. 6 Regulation of meiotic maturation. A. Before the activation of the LH receptor (LHCGR), the mural granulosa cells produce and release NPPC, which activates its receptor, NPR2, located on both mural and cumulus granulosa cells. Active NPR2 generates cGMP, which diffuses through gap junctions and reaches a concentration in the oocyte that is sufficient to inhibit phosphodiesterase (PDE) 3A. This allows the concentration of cAMP, produced in the oocyte by adenyl cyclase (AC) coupled to G-protein coupled receptor (GPR) 3, to remain high, thereby maintaining CDK1 in a hyperphosphorylated inactive form. B. Binding of LH to LHCGR triggers the release of ligands from the mural granulosa cells that activate EGFR on the mural and cumulus granulosa cells. cGMP levels within the granulosa cells fall due both to inhibition of its synthesis owing to dephosphorylation of NPR2 and (later) reduced production of NPPC and to increased hydrolysis owing to activation of PDE5A in the granulosa cells. The relative contribution of LHCGR-mediated and EGFR-mediated signaling to these events remains to be fully elucidated. As the concentration of cGMP within the granulosa cells falls, it flows out of the oocyte to equalize its concentration throughout the granulosa cell-oocyte compartment. The decreased cGMP in the oocyte permits PDE3A to become active and hydrolyze cAMP, enabling dephosphorylation and activation of CDK1.

turns off production of cGMP by the granulosa cells, leading to a drop in its concentration within the oocyte. This permits the activation of PDE3A and consequently a decrease in intra-oocyte cAMP and activation of CDK1. In mice, no new protein synthesis within the oocyte is required to activate CDK1 and initiate maturation; in other mammals, however, new protein synthesis—possibly of cyclin B1, the regulatory subunit of CDK1—may be required.

The earliest easily identified morphological indicator of maturation is the dissolution of the nuclear envelope, termed germinal vesicle breakdown (GVBD). The chromosomes condense and progressively become aligned in the center of the first meiotic spindle (Holt et al., 2013; Polanski, 2013; Howe and FitzHarris, 2013) (Fig. 7). The poles of oocyte spindles lack centrioles; instead, they are generated by the acentriolar microtubule-organizing centers (aMTOCs) and associated proteins that are opposed to the nucleus of immature oocytes. Alignment of the chromosomes on the spindle is achieved by balanced forces of microtubules, termed K-fibers, that run from the kinetochores of each chromosome to each pole of the spindle (other microtubules run between the poles of the spindle) and thereby pull the chromosomes in opposing directions. A mechanism termed the spindle assembly checkpoint (SAC) ensures that the first meiotic division is not initiated until the chromosomes have become correctly aligned.

Once chromosomal alignment has been achieved, thereby satisfying the SAC, a protein termed CDC20 is released from an inhibited state (Fig. 8). CDC20 activates the APC/C (anaphase-promoting complex/cyclosome), which in turn degrades two key protein targets—cyclin B1 and securin. Degradation of cyclin B1 inactivates CDK1, triggering exit from M-phase of the cell cycle. In parallel, degradation of securin enables activation of separase, a protease. The target of separase are proteins known as cohesins, which hold homologous chromosomes together. Degradation of cohesins located on the arms of the chromosomes enables the homologues to be pulled to opposing poles of the spindle, a process that is achieved by the shortening of the K-fibers. Importantly,

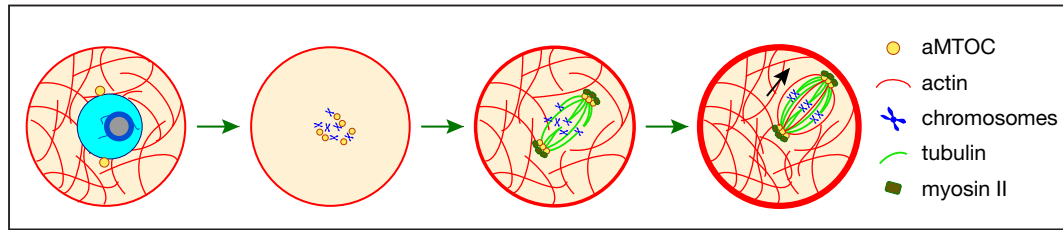


Fig. 7 Translocation of the meiotic spindle to the oocyte cortex to permit asymmetric cytokinesis. The immature oocyte of the mouse contains a cytoplasmic actin mesh as well as cortical actin (red) and a small number of microtubule-organizing centers (yellow) located near the nuclear membrane. These lack centrioles, so are termed acentriolar (aMTOC). At the time of GVBD, the actin meshwork disappears and a process of fragmentation generates several aMTOCs, which associate with the condensing chromosomes. The aMTOCs organize the poles of the spindle, where myosin II (brown) is also present. The spindle contains both long microtubules running between the poles and shorter microtubules (both shown in green), termed K-fibers, running between the poles and chromosomal kinetochores. Balanced tension between K-fibers running in opposite directions helps to align the chromosomes in the middle of the spindle, thus satisfying the spindle assembly checkpoint. Concomitantly, a cytoplasmic actin meshwork reforms, the cortical actin thickens and the spindle begins to drift off-center. Interactions between myosin II and the cortex amplify the initial displacement and thus pull (arrow) the spindle towards the thickened cortex where it becomes anchored in preparation for the first meiotic division and cytokinesis.

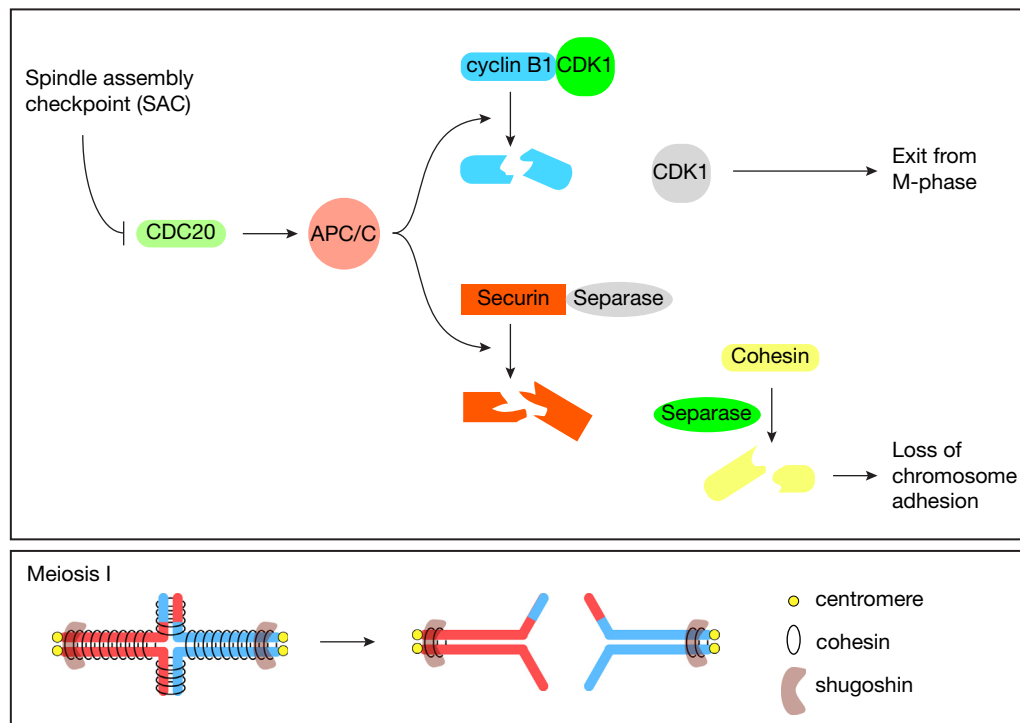


Fig. 8 Initiation of the first meiotic division. Upper: When the spindle-assembly checkpoint is satisfied, CDC20 is released from an inhibited state and is able to activate the anaphase-promoting complex/cyclosome (APC/C). The APC/C degrades cyclin B1, thereby inactivating CDK1 and allowing the oocyte to move from M-phase to anaphase. The APC/c also degrades securin, thereby freeing separase that is able to degrade cohesin. The loss of cohesin enables homologous chromosomes to separate and thus move to opposite poles of the spindle at anaphase. Lower: Maternal (red) and paternal (blue) homologous chromosomes are held together at metaphase I by cohesin (black rings). Degradation of cohesin is necessary for the homologues to separate. The shugoshin protein (brown) located near the centromeres (yellow) locally protects cohesin from degradation at this time, so that sister chromatids remains tightly associated. Degradation of centromeric cohesin is required for the second meiotic division that will occur after fertilization.

in contrast to the cohesins located on the chromosomal arms, the cohesins associated with the centromeric regions of the chromosomes is protected from degradation, via the shugoshin protein. This ensures that sister chromatids remain associated during the first meiotic division. Mis-segregation of chromosomes during the first meiotic division is the most frequent origin of fetal aneuploidy. Following completion of the first meiotic division, the chromosomes retained in the oocyte rapidly become aligned on the second meiotic spindle. The oocyte becomes arrested at this stage, however, and does not complete the second meiotic division until it is activated by fertilization or by parthenogenetic agents. Meiotic arrest at metaphase II is achieved by stabilization of CDK1.

A remarkable feature of meiotic maturation is the extreme asymmetry of the cytokineses associated with the first and second meiotic divisions, which generate one very large cell—the oocyte—and one small cell—the first and second polar bodies, respectively. Thus, in contrast to the meiotic divisions in males, which generate four germ cells, these divisions in females generate only one germ cell. It is necessary to retain a large oocyte because the mitotic cleavage divisions following fertilization will divide it into many small cells, termed blastomeres, before the embryo implants into the uterus and begins to grow. To achieve the asymmetric cytokinesis, the meiotic spindle must be translocated to the cortex (periphery) of the oocyte. This depends on an actin meshwork in the cytoplasm, including an actin cage that encloses the spindle (Chaigne et al., 2017) (Fig. 7). This meshwork enables the caged spindle to be carried to the cortex through a process that depends on myosin II located at the poles of the spindle. It is thought that, owing to a slight off-centering of the nucleus in the immature oocyte, the spindle is initially displaced slightly towards one region of the cortex; subsequently, this displacement is amplified by a positive-reinforcement mechanism as the leading edge of the spindle approaches the cortex. Concomitantly, the actin-rich cortex thickens owing to increased assembly of actin fibers and, due to the exclusion of myosin II, becomes softer. This change in cortical tension, which must be gated within a narrow range and is regulated by CDK1 via activation of MAP (microtubule-associated protein) kinase, is essential for proper migration and positioning of the spindle.

These so-called nuclear events of maturation are accompanied by a diverse array of cytoplasmic changes. These include actin-mediated transport of membrane-bound vesicles termed cortical granules, which play a key role in preventing polyspermic fertilization, from the cytoplasm to the cortex and aggregation of mitochondria around the meiotic spindle. Notably, a subset of previously silent mRNAs become translationally activated (Fig. 5). The most extensively characterized mechanism involves the CPE. Its associated protein, CPEB, becomes phosphorylated early during maturation and is subsequently largely degraded (Clarke, 2012; Kang and Han, 2011; Reyes and Ross, 2016; Chen et al., 2013). Concomitantly, the poly(A) tail of CPE-containing mRNAs lengthens and these mRNAs become translationally activated. Whether phosphorylation or degradation of CPEB enables lengthening, as well as whether it is due to increased activity of poly(A) polymerases or decreased activity of deadenylases remain to be fully established. However, the translational activation of specific CPE-containing mRNAs, such as *Ccnb1* (cyclin B1), are essential for the completion of maturation. In addition to the CPE, other sequences in the 3'-utr also control mRNA translational activity during maturation (Chen et al., 2013). The *Dazl* mRNA contains CPEs and is translationally activated early during maturation. The newly synthesized DAZL protein then binds to specific sequences in the 3'-utr of mRNAs that may not contain a CPE, activating their translation. Such mechanisms enable the translation of different mRNA species may be temporally regulated during maturation.

Once the oocyte has completed meiotic maturation, having reached metaphase II, it becomes developmentally arrested. Now termed an egg, its developmental journey is complete. The egg awaits fertilization, which will release it from developmental arrest, enabling the second meiotic division to be completed and embryogenesis to begin.

Acknowledgments

Supported by grants from the Eunice Kennedy Shriver National Institute of Child Health & Human Development of the National Institutes of Health (R21HD086407), Canadian Institutes of Health Research (CIHR), the Natural Sciences and Engineering Research Council of Canada, and the Research Institute of the McGill University Health Centre (RI-MUHC). Research reported in this publication is solely the responsibility of the author and does not necessarily represent the official views of the National Institutes of Health. We apologize to colleagues whose research could not be cited owing to limitations on the number of references.

References

- Castrillon, D. H., Miao, L., Kolipara, R., Horner, J. W., & Depinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*, 301, 215–218.
- Chaigne, A., Terret, M. E., & Verlhac, M. H. (2017). Asymmetries and symmetries in the mouse oocyte and zygote. *Results and Problems in Cell Differentiation*, 61, 285–299.
- Chang, H.-M., Qiao, J., & Leung, P. C. K. (2016). Oocyte–somatic cell interactions in the human ovary—Novel role of bone morphogenetic proteins and growth differentiation factors. *Human Reproduction Update*, 23, 1–18.
- Cheeseman, L. P., Boulanger, J., Bond, L. M., & Schuh, M. (2016). Two pathways regulate cortical granule translocation to prevent polyspermy in mouse oocytes. *Nature Communications*, 7, 13726.
- Chen, J., Torcia, S., Xie, F., et al. (2013). Somatic cells regulate maternal mRNA translation and developmental competence of mouse oocytes. *Nature Cell Biology*, 15, 1415–1423.
- Choi, Y., Yuan, D., & Rajkovic, A. (2008). Germ cell-specific transcriptional regulator sohlh2 is essential for early mouse folliculogenesis and oocyte-specific gene expression. *Biology of Reproduction*, 79, 1176–1182.
- Clarke, H. J. (2012). Post-transcriptional control of gene expression during mouse oogenesis. *Results and Problems in Cell Differentiation*, 55, 1–21.
- Clarke, H. J. (2017). Control of mammalian oocyte development by interactions with the maternal follicular environment. *Results and Problems in Cell Differentiation*, 63, 17–41.
- Conti, M., Hsieh, M., Musa Zamah, A., & Oh, J. S. (2012). Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Molecular and Cellular Endocrinology*, 356, 65–73.
- El-Hayek, S., & Clarke, H. J. (2016). Control of oocyte growth and development by intercellular communication within the follicular niche. *Results and Problems in Cell Differentiation*, 58, 191–224.
- Eppig, J. J., & Wigglesworth, K. (2000). Development of mouse and rat oocytes in chimeric reaggregated ovaries after interspecific exchange of somatic and germ cell components. *Biology of Reproduction*, 63, 1014–1023.
- Eppig, J. J., Wigglesworth, K., & Hirao, Y. (2000). Metaphase I arrest and spontaneous parthenogenetic activation of strain Lxbx oocytes: Chimeric reaggregated ovaries establish primary lesion in oocytes. *Developmental Biology*, 224, 60–68.

- Hayashi, K., Ogushi, S., Kurimoto, K., et al. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science*, 338, 971–975.
- Holt, J. E., Lane, S. I., & Jones, K. T. (2013). The control of meiotic maturation in mammalian oocytes. *Current Topics in Developmental Biology*, 102, 207–226.
- Howe, K., & FitzHarris, G. (2013). Recent insights into spindle function in mammalian oocytes and early embryos. *Biology of Reproduction*, 89, 71.
- Jaffe, L. A., & Egbert, J. R. (2017). Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. *Annual Review of Physiology*, 79, 237–260.
- Jorgensen, J. S. (2013). Defining the neighborhoods that escort the oocyte through its early life events and into a functional follicle. *Molecular Reproduction and Development*, 80, 960–976.
- Kang, M. K., & Han, S. J. (2011). Post-transcriptional and post-translational regulation during mouse oocyte maturation. *BMB Reports*, 44, 147–157.
- Kelsey, G., & Fell, R. (2013). New insights into establishment and maintenance of DNA methylation imprints in mammals. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 368, 20110336.
- Kerr, J. B., Myers, M., & Anderson, R. A. (2013). The dynamics of the primordial follicle reserve. *Reproduction*, 146, R205–215.
- Kidder, G. M., & Vanderhyden, B. C. (2010). Bidirectional communication between oocytes and follicle cells: Ensuring oocyte developmental competence. *Canadian Journal of Physiology and Pharmacology*, 88, 399–413.
- Li, R., & Albertini, D. F. (2013). The road to maturation: Somatic cell interaction and self-organization of the mammalian oocyte. *Nature Reviews. Molecular Cellular Biology*, 14, 141–152.
- Liu, X., Morency, E., Li, T., et al. (2017). Role for pad16 in securing the mma-msy2 complex to the oocyte cytoplasmic lattices. *Cell Cycle*, 16, 360–366.
- Mora, J. M., Fenwick, M. A., Castle, L., et al. (2012). Characterization and significance of adhesion and junction-related proteins in mouse ovarian follicles. *Biology of Reproduction*, 86, 153.
- Mottershead, D. G., Sugimura, S., Al-Musawi, S. L., et al. (2015). Cumulin, an oocyte-secreted heterodimer of the transforming growth factor-beta family, is a potent activator of granulosa cells and improves oocyte quality. *Journal of Biological Chemistry*, 290, 24007–24020.
- Peng, J., Li, Q., Wigglesworth, K., et al. (2013). Growth differentiation factor 9: Bone morphogenetic protein 15 heterodimers are potent regulators of ovarian functions. *Proceedings of the National Academy of Sciences of the United States of America*, 110, E776–785.
- Pepling, M. E. (2012). Follicular assembly: Mechanisms of action. *Reproduction*, 143, 139–149.
- Polanski, Z. (2013). Spindle assembly checkpoint regulation of chromosome segregation in mammalian oocytes. *Reproduction, Fertility, and Development*, 25, 472–483.
- Ren, Y., Suzuki, H., Jagarlamudi, K., et al. (2015). Lhx8 regulates primordial follicle activation and postnatal folliculogenesis. *BMC Biology*, 13, 39.
- Reyes, J. M., & Ross, P. J. (2016). Cytoplasmic polyadenylation in mammalian oocyte maturation. *Wiley Interdisciplinary Reviews RNA*, 7, 71–89.
- Saatcioglu, H. D., Cuevas, I., & Castrillon, D. H. (2016). Control of oocyte reawakening by kit. *PLoS Genetics*, 12, e1006215.
- St John, J. (2014). The control of mtDNA replication during differentiation and development. *Biochimica et Biophysica Acta*, 1840, 1345–1354.
- Su, Y. Q., Sugiura, K., & Eppig, J. J. (2009). Mouse oocyte control of granulosa cell development and function: Paracrine regulation of cumulus cell metabolism. *Seminars in Reproductive Medicine*, 27, 32–42.
- Svoboda, P., Franke, V., & Schultz, R. M. (2015). Sculpting the transcriptome during the oocyte-to-embryo transition in mouse. *Current Topics in Developmental Biology*, 113, 305–349.
- Wassarman, P. M., & Litscher, E. S. (2013). Biogenesis of the mouse egg's extracellular coat, the zona pellucida. *Current Topics in Developmental Biology*, 102, 243–266.
- Winterhager, E., & Kidder, G. M. (2015). Gap junction connexins in female reproductive organs: Implications for women's reproductive health. *Human Reproduction Update*, 21, 340–352.
- Zhang, H., Risal, S., Gorre, N., et al. (2014). Somatic cells initiate primordial follicle activation and govern the development of dormant oocytes in mice. *Current Biology*, 24, 2501–2508.
- Zhang, M., Su, Y. Q., Sugiura, K., Xia, G., & Eppig, J. J. (2010). Granulosa cell ligand nppc and its receptor npr2 maintain meiotic arrest in mouse oocytes. *Science*, 330, 366–369.

Oocyte Meiotic Arrest

Meijia Zhang, China Agricultural University, Beijing, PR China

© 2018 Elsevier Inc. All rights reserved.

Introduction

The female germ cells, oocytes, arise from the primordial germ cells during development of the fetus. Following a round of DNA replication the cells enter meiosis progress to the dictyate stage of first meiotic prophase (prophase I) at about the time of birth, at which point meiosis arrests and the oocytes display a characteristic large nucleus called the germinal vesicle (GV). The diplotene arrest in oocyte may last for several months or years in follicular microenvironment depending on the mammalian species. These prophase I arrested oocytes are referred to as primordial oocytes and reside in primordial follicles. During follicular growth, the somatic cells divide to form several layers, the oocyte enlarges, and a fluid-filled antrum begins to form. Some follicles at the early antral stage are "recruited" by follicle-stimulating hormone (FSH) from the pituitary to continue growing and develop into preovulatory antral follicles (Graafian follicles, Fig. 1). Antrum formation physically separates the granulosa cells into a mural granulosa cell compartment along the follicle wall and a cumulus cell compartment surrounding the oocyte. The meiotic arrest of oocytes within preantral follicles is caused by inherent factors in the oocyte and correlates with low levels of activity by cell cycle regulatory proteins. Once the growing oocyte reaches its full size and an antral space begins to form, the oocyte acquires the ability to complete meiosis. However, these meiotically competent oocytes maintain meiotic prophase arrest until the preovulatory surge of luteinizing hormone (LH) from the pituitary gland triggers the resumption of meiosis during the estrous or menstrual cycle (Fig. 1). The process by which the oocyte completes the first meiotic division and progresses to metaphase of the second meiotic division is called oocyte maturation. Germinal vesicle breakdown (GVB) is the first change occurring, and is widely used as an experimental criterion for assessing meiotic resumption or oocyte maturation.

In 1935, Pincus and Enzmann demonstrated that rabbit oocytes could resume meiosis spontaneously without hormonal stimulation when they were liberated from antral follicles and cultured under simple nutritionally supportive conditions. Such spontaneous maturation of oocytes cultured either denuded of their cumulus cells or within their cumulus complex is confirmed in all mammalian species examined, including human. Interestingly, the time course of spontaneous maturation is similar to that of hormonal stimulation in vivo. These observations lead to general acceptance of the hypothesis that the somatic cells of the antral follicle provide a signal to the oocyte that keeps it arrested at prophase I. This hypothesis is further corroborated by the studies that co-culture of oocytes with follicular granulosa cells, granulosa cell extract, and follicular fluid inhibits oocyte maturation. The inhibitory action of granulosa cells is effective from small to medium sized follicles, but is significantly lower in follicles yielding mature oocyte. For a long time, many studies focus on identifying the factors participating in the maintenance of meiotic arrest, and lead to partial characterization and purification of a factor, oocyte maturation inhibitor (OMI), from follicular fluids. This inhibitory effect is not species specific; thus human follicular fluids inhibit the maturation of porcine and rat oocytes, bovine follicular fluids inhibit hamster oocytes, and porcine follicular fluids inhibit the maturation of rat oocytes. In 1976, Tsafiri et al. revealed that OMI had a low molecular weight (about 2000 Da), and the activity was lost when it was treated with protease. These results suggest that OMI is a peptide of low molecular weight. The inhibitory effect of OMI requires the mediation of cumulus cells surrounding the oocyte, since OMI inhibits the resumption of meiosis by oocytes cultured within their intact cumulus cells, but has no inhibitory effect on the maturation of denuded oocytes (Tsafiri and Pomerantz, 1986). LH overcomes the inhibitory effect of OMI, supporting that OMI has a physiological role in the regulation of meiosis. Some investigators are unable to demonstrate OMI activity of follicular preparations. These varying findings may be related, at least in part, to the low OMI activity of some batches of follicular fluid

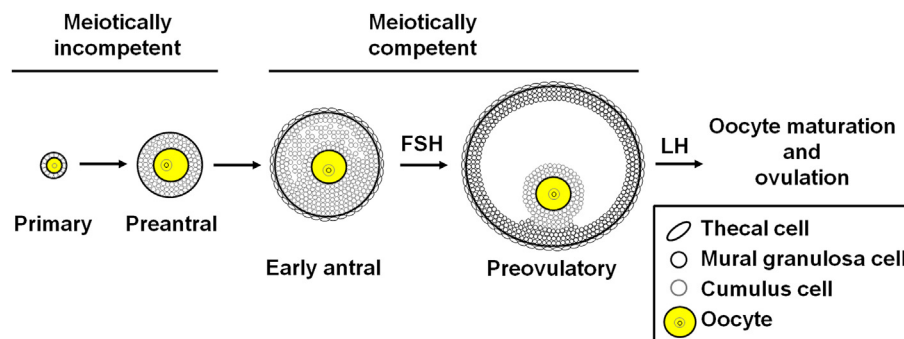


Fig. 1 Ovarian follicle development in mammal. The follicle consists of somatic cells surrounding an oocyte (yellow). Oocytes within the primary and preantral follicles are meiotically incompetent. At the early antral stage, the oocytes acquire meiotic competence, but are held in meiotic arrest by the signaling from somatic cells. These early antral follicles are recruited for further growth by follicle-stimulating hormone (FSH), and become preovulatory follicles. The preovulatory follicle develops luteinizing hormone (LH) receptors on the mural granulosa cells. A surge of LH stimulates the oocyte to resume meiosis (maturation) and ovulation.

and of granulosa cell cultures. Collectively, these results support the notion that the inhibitory influence of the follicle is due to a factor, OMI, produced by granulosa cells. Purification of OMI to homogeneity will allow the assessment of the exact role of OMI in meiotic arrest in mammals.

Cyclic AMP

Cyclic adenosine 3',5'-monophosphate (cAMP) is a key second messenger regulating oocyte maturation. [Cho et al. \(1974\)](#) were the first to propose that spontaneous maturation of mouse oocytes in vitro when released from their follicles can be reversibly blocked by addition of a derivative of cAMP. Further studies indicate that oocyte maturation can be blocked by adenylyl cyclase activators, membrane permeant cAMP analogs or cAMP phosphodiesterase inhibitors, such as hypoxanthine or 3-isobutyl-1-methylxanthine (IBMX). Moreover, oocytes resume meiosis spontaneously in parallel with decreases in cAMP levels. These findings, confirmed in numerous mammalian species, indicate that maintenance of meiotic arrest in meiotically competent oocytes depended on high levels of cAMP within the oocyte.

Intraoocyte cAMP regulates the maturation-promoting factor (MPF) activity through stimulation of cAMP-dependent protein kinase A (PKA). MPF is the protein complex of cyclin-dependent kinase 1 (CDK1) and cyclin B (CyB), and its activity is regulated by the phosphatase cell division cycle 25B (CDC25B) and the kinases Wee1B and myelin transcription factor 1 (MYT1); CDC25B dephosphorylates CDK1, whereas WEE1B, and MYT1 phosphorylate CDK1. High cAMP levels result in the phosphorylation of CDK1 and inactivation of the MPF complex so that the oocyte is arrested at the dictyate stage of prophase I ([Fig. 2](#)).

Cyclic AMP Synthesis

Cyclic AMP could be produced either by the oocyte or by the follicular somatic cells that surround it. A long-standing hypothesis is that cAMP is generated by somatic cells and continuously diffuses into oocytes through heterologous gap junctions between cumulus cells and oocytes. Although the inhibitors of gap junction permeability induce oocyte maturation ([Sela-Abramovich et al., 2006](#)), the permeability of these junctions to cAMP has not been definitively determined. The increasing evidences indicate that the oocyte itself produces sufficient cAMP to maintain meiotic arrest.

It is reported that meiotic arrest could be interrupted by microinjection of either an antibody of stimulatory G protein (Gs) or a dominant negative form of a subunit of Gs into follicle-enclosed oocytes, suggesting that the Gs-adenylyl cyclase mediates production of cAMP within the oocyte. The G-protein-coupled, Gs-linked receptor, GPR3, is expressed in mouse oocyte, and adenylyl cyclase (ADCY) is found at the oolemma. GPR3 or ADCY3-deficient oocytes show precocious resumption of meiosis within the antral follicles ([Mehlmann et al., 2004](#)). GPR3 is also as the predominant receptor signaling for meiotic arrest in human oocytes.

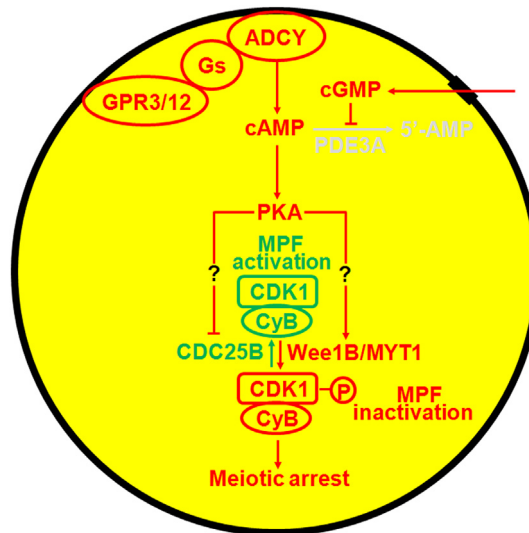


Fig. 2 Oocyte signaling maintaining meiotic arrest at diplotene stage of prophase I in the antral follicle. GPR3/12, activated constitutively by an unknown ligand endogenous to the oocyte, activates Gs, which stimulates ADCY to cause an elevation of cAMP. The activity of the cAMP phosphodiesterase, PDE3A, is kept low by cGMP of somatic cell origin, thus preventing the breakdown of cAMP and maintaining high levels of cAMP within the oocyte. The increased level of cAMP increases PKA activity. The increased PKA activity inactivates CDC25B phosphatase and activates Wee1B/MYT1 kinase that results in phosphorylation of CDK1 and inactivation of MPF (CDK1/CyB complex), and therefore maintains meiotic arrest. *GPR3/12*, G-protein-coupled receptor 3 and 12; *Gs*, guanine nucleotide binding protein, α stimulating; *ADCY*, adenylyl cyclase; *cAMP*, cyclic adenosine 3',5'-monophosphate; *cGMP*, cyclic guanosine 3',5'-monophosphate; *PDE3A*, phosphodiesterase 3A; *PKA*, protein kinase A; *MPF*, maturation promoting factor; *CDK1*, cyclin-dependent kinase 1; *CyB*, cyclin B; *CDC25B*, cell division cycle 25B; *MYT1*, myelin transcription factor 1.

A closely related receptor, GPR12, has been shown to be expressed specifically in rat oocytes, and serves an analogous function in rat oocytes. These receptors exhibit constitutive activity that is independent of granulosa cell influence, but the natural ligand(s) has not been identified. Taken together, the above findings strongly suggest that meiotic arrest is dependent on continuous cAMP signaling through active GPR/Gs/ADCY endogenous to the oocyte (Fig. 2). Although the transfer of cAMP from surrounding cumulus cells to oocytes is possible, it is not sufficient by itself to maintain meiotic arrest.

Cyclic AMP Degradation

Intracellular concentration of cAMP depends on the synthesis by ADCY and the hydrolysis by cyclic nucleotide phosphodiesterases (PDEs). It has been shown that maintenance of meiotic arrest is associated with undetectable cAMP-PDE activity. In 1998, Wiersma et al. reported that injection of PDE3 inhibitors into rats and mice prevented meiotic resumption without affecting ovulation, strongly suggesting an essential role of the PDE3 family in regulating oocyte maturation via cAMP degradation. PDE3A is the major PDE expressed in mouse, rat, monkey, and human oocytes. The selective PDE3A inhibitors such as cilostamide and milrinone maintain oocyte meiotic arrest in several mammalian species, and PDE3A activity increases during gonadotropin-stimulated oocyte maturation. Vaccari et al. (2008) reported that oocytes deficient in PDE3A had the continuous cAMP accumulation and meiotic arrest even after removal from the follicle, indicating that the meiotic arrest is maintained by constitutive cAMP signaling associated with a lack of PDE3A activity. Depleting both of GPR3 and PDE3A genes allows spontaneous meiosis resumption in vivo as depletion of GPR3 alone, suggesting that PDE3A is downstream of GPR3. These results illuminate that intraoocyte cAMP levels are regulated via control of PDE3A-mediated degradation, rather than endogenous synthesis. Inhibition of PDE3A activity is essential for sustaining elevated cAMP levels that maintain meiotic arrest.

Cyclic GMP

In 1980, Hubbard reported that cyclic guanosine 3',5'-monophosphate (cGMP) levels were highest at dioestrus but lowest during oestrus in hamster ovary, suggesting a functional relationship between cGMP levels and meiosis. Many studies show that cGMP plays an important role in the maintenance of meiotic arrest. Cyclic GMP levels decrease during oocyte spontaneous maturation. Exposure of oocytes to the cGMP analog 8-Br-cGMP, or to the cGMP PDE-specific inhibitors maintains meiotic arrest in several mammalian species.

It is known that cGMP shows an inhibitory effect on PDE3A activity via competition with cAMP in the hydrolysis process. PDE3A hydrolyzes both cAMP and cGMP, but the velocity of cAMP hydrolysis is one order of magnitude higher than that for cGMP hydrolysis. Consistent with these results, the microinjection of a cGMP-specific PDE5 into oocytes causes meiotic maturation of wild-type oocytes, but this effect is absent in PDE3A-deficient oocytes. Moreover, cGMP suppresses cAMP hydrolysis in oocyte extracts, suggesting that cGMP maintains meiotic arrest through the inhibition of PDE3A activity. Guanylyl cyclase agonists have inhibitory effects on spontaneous meiotic resumption in cumulus-oocyte complexes (COCs), but not in denuded oocytes, and the inhibition of cGMP production by the somatic cells leads to a decrease in oocyte cGMP and meiotic resumption (Norris et al., 2009), suggesting that the oocyte depends on the somatic cells for its supply of cGMP. Cyclic GMP diffuses into oocytes via heterologous gap junctions to inhibit PDE3A activity (Fig. 2). This inhibition sustains a high concentration of intraoocyte cAMP sufficient to maintain meiotic arrest. Thus, cGMP of somatic cell origin is a critical component required to maintain meiotic arrest.

NPPC

Cyclic GMP can be produced by nitric oxide (NO) and natriuretic peptides. NO, a chemical messenger enzymatically produced by three isoforms of nitric oxide synthases (NOS), activates soluble guanylyl cyclase. NO donor sodium nitroprusside has the inhibitory effect on mouse oocyte spontaneous maturation only in a high concentration, with an increase in the percentage of atypical. However, knockouts of *Nos1* and *Nos2* affect ovulation, and knockout of *Nos3* appears to impair oocyte development. The role of NO appears to be not in the regulation of meiotic arrest.

The natriuretic peptides, including type A (NPPA, also known as ANP), type B (NPPB, also known as BNP), and type C (NPPC, also known as CNP), activate particulate guanylyl cyclase family. In general, NPPA and NPPB activate natriuretic peptide receptor 1 (NPR1, also known as GC-A, NPRA), and NPPC activates receptor NPR2 (also known as GC-B, NPRB). NPPA and NPPB are cardiac hormones, and have an important role in the regulation of cardiovascular homeostasis. It is reported that NPPA slightly inhibits spontaneous meiotic resumption of rat oocytes. However, NPPA and NPPB genes in mouse ovary could not be detected by in situ hybridization, and by specific riboprobes. Moreover, the activity of these ligands could not be detected in inhibiting meiotic resumption of mouse oocytes or in promoting elevated cGMP levels in vitro. All these results indicate that NPPA and NPPB do not seem to be the crucial mechanism of meiotic arrest.

NPPC, on the contrary, is expressed in a wide variety of central and peripheral tissues and acts locally as autocrine and paracrine regulator. NPPC and its cognate receptor NPR2 are reported in rat follicles, and show variation during the estrous cycle. Zhang et al. (2010) found that NPPC of granulosa cell origin acted on the NPR2 receptor in cumulus cells to stimulate cGMP synthesis (Fig. 3). This provides a critical piece to understanding the molecular basis for maintenance of meiotic arrest. NPPC gene is expressed

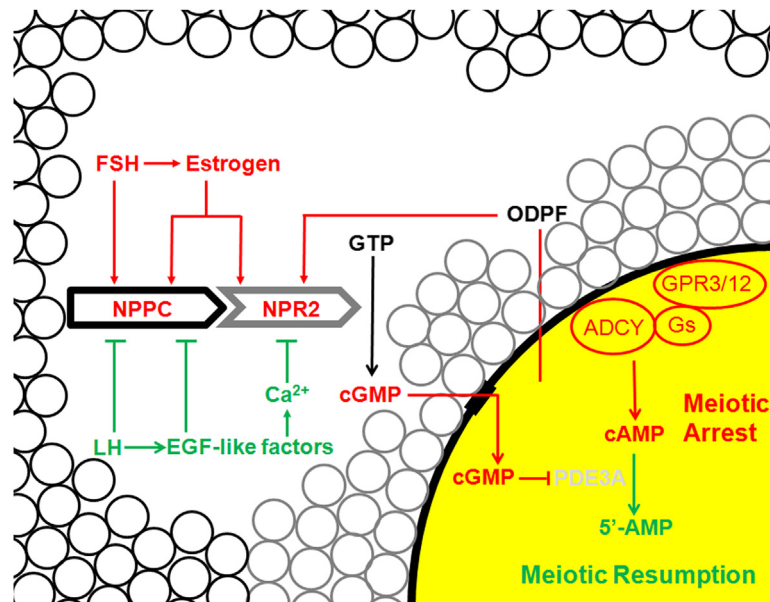


Fig. 3 Somatic cell signaling regulating oocyte meiosis. NPPC produced by mural granulosa cells stimulates the generation of cGMP by cumulus cell NPR2. Cyclic GMP, diffusing into oocyte via gap junctions, maintains high cAMP levels and meiotic arrest by the inhibition of PDE3A activity. ODPF promotes cumulus cell expression of NPR2 to elevate cGMP levels for meiotic arrest. FSH, through estrogen and other signaling, enhances NPPC/NPR2 expression during antral follicular development. LH-induced EGF-like growth factors decrease NPPC content and NPR2 activity, resulting in cGMP decrease and meiotic resumption. *NPPC*, natriuretic peptide type C; *NPR2*, natriuretic peptide receptor 2; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone; *EGF-like factors*, epidermal growth factor-like factors. *cGMP*, cyclic guanosine 3',5'-monophosphate; *cAMP*, cyclic adenosine 3',5'-monophosphate; *GPR3/12*, G-protein-coupled receptor 3 and 12; *Gs*, guanine nucleotide binding protein, α stimulating; *ADCY*, adenylyl cyclase; *ODPF*, oocyte-derived paracrine factors; *PDE3A*, phosphodiesterase 3A.

predominantly in mouse mural granulosa cells (MGCs), which line the inside of the follicular wall, and in contrast, NPR2 gene is most concentrated in the cumulus cells surrounding the oocyte. NPPC prevents spontaneous resumption of cumulus cell-enclosed mouse oocytes in vitro, and increases cumulus cell and oocyte cGMP levels. NPPC do not prevent spontaneous resumption of denuded oocytes, possibly for the lack of NPR2 receptor in oocyte. Importantly, mutations in either the NPPC or NPR2 gene in mice result in failure to maintain meiotic arrest and precocious meiotic resumption in oocytes within antral follicles. Further study shows that both connexin-43 (GJA1) and connexin-37 (GJA4) gap junctions are involved in NPPC-mediated meiotic arrest. Thus, NPPC from follicular MGCs stimulates the generation of cGMP by NPR2 in cumulus cells. Cyclic GMP diffuses into oocyte via heterologous gap junctions and maintains meiotic arrest by inhibiting PDE3A activity and cAMP hydrolysis (Fig. 3). The expression of NPR2 on granulosa cells suggests that cGMP generated in granulosa cells via autocrine role may also contribute to meiotic arrest. In mammals, oocyte maturation must be coordinated precisely with ovulation to produce a developmentally competent egg at the right time for fertilization. Therefore, NPPC/NPR2 prevents precocious meiotic resumption in oocytes, which is critical for maturation and ovulation synchrony and for normal female fertility.

It has long been known that the granulosa cells of follicles produce OMI that keeps oocyte arrested at the dictyate stage of prophase I. Interestingly, granulosa-derived NPPC has the character similar to the OMI: a 22 amino acid residues peptide with molecular weight of 2197.6, action on cumulus cell NPR2, and the identical sequences among mouse, rat, pig, and human. NPPC from human inhibits oocyte maturation of mouse, rat, pig, and cattle. Moreover, LH can overcome the inhibitory effect of NPPC. A better understanding of NPPC on meiotic arrest will allow the assessment of the detailed mechanism(s) in the regulation of meiosis in mammals.

The Regulation of NPPC/NPR2

Oocyte-Derived Paracrine Factors

In general, oocyte-derived paracrine factors (ODPFs) affect cumulus cell differentiation, and granulosa cells have important endocrine functions. NPR2 gene is expressed predominantly in cumulus cells, and its expression could be promoted by ODPFs. The expression of NPR2 gene in cumulus cells decreases when the oocyte is removed, but could be restored when these cumulus cells culture with denuded oocytes. Further studies focus on growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15; also called GDF9B), and fibroblast growth factor 8B (FGF8B), the members of oocyte-secreted transforming growth factor- β (TGF- β) superfamily. Each of these three ODPFs slightly promotes expression of NPR2 gene by cumulus cells, and combinations of three proteins have the same effect as that of oocytes on promoting NPR2 gene expression in isolated cumulus cells

(Fig. 3). Knockout of Smad4, the central component of the canonical TGF- β signaling pathway, reduces NPR2 expression in cumulus cells, and the maintaining oocyte meiotic arrest is weakened, indicating that these ODPFs act on cumulus cells by the activation of SMAD (Sma and Mad-related protein) signaling pathway.

It is reported that the function of the NPPC/NPR2 system depends upon IMPDH activity for the production of cGMP and maintaining meiotic arrest. IMPDH is the rate-limiting enzyme required for the production of guanylyl metabolites and cGMP. Inhibition of IMPDH with mizoribine prevents NPPC/NPR2 from providing the sufficient levels of cGMP required for maintaining oocyte meiotic arrest. Interestingly, ODPFs, particularly GDF9-BMP15 heterodimer, also promote expression of IMPDH. Therefore, ODPFs could promote expression of IMPDH and NPR2 and elevate cGMP levels in cumulus cells for meiotic arrest. It has been long known that the follicular somatic cells are essential in maintaining oocyte meiotic arrest. However, the function of the somatic cells is surprisingly regulated by oocyte-derived paracrine signals. Thus, the maintenance of oocyte meiotic arrest is not unidirectional from the somatic compartment to oocytes but bidirectional, requiring orchestration by oocytes.

FSH

During each estrous cycle, FSH recruits some early antral follicles to continue growing and develop into Graafian follicles. It is reported that the expression of NPPC and NPR2 in rat ovary vary during the estrous cycle with maximal expression at proestrus. The treatment of equine chorionic gonadotropin (eCG), a glycoprotein hormone that possesses primarily FSH activity, stimulates NPPC gene expression in rodent MGCs and increases NPPC content in the ovaries. eCG also increases NPR2 gene expression in both MGCs and cumulus cells. However, FSH cannot stimulate the expression of NPPC and NPR2 genes in MGCs cultured in vitro, suggesting that FSH activity of eCG induces the expression of NPPC and NPR2 indirectly (Fig. 3). The distinct physiological action of FSH is to stimulate follicular growth. Thus, FSH-promoted NPPC and NPR2 insure their ability to maintain oocyte meiotic arrest during antral follicular development. Inappropriate expression of NPPC and NPR2 in the growing follicles may disrupt meiotic arrest and normal follicular development.

FSH increases estrogen levels by the aromatization of testosterone, which partially mediates FSH action. The injection of synthetic estrogen diethylstilbesterol (DES) promotes NPPC and NPR2 genes expression in rat ovary. In vitro, 17 β -estradiol (E2) increases the levels of NPPC and NPR2 genes in cultured mouse MGCs. E2 also stimulates the expression of NPR2 gene by cumulus cells, thereby augmenting NPPC ability to produce cGMP and maintain meiotic arrest of COCs. All these results implicate that the physiological role of estrogens may be involved in maintaining oocyte meiotic arrest via promoting the expression of NPPC and NPR2. However, the deletion of estrogen receptors shows slightly precocious meiotic resumption of oocytes within antral follicles. Thus, other pathways may participate in the regulation of NPPC and NPR2 expression maintaining meiotic arrest.

LH

Fully grown mammalian oocytes within Graafian follicles are held in meiotic arrest by NPPC/NPR2-produced cGMP until LH triggers oocyte maturation and ovulation during the estrous or menstrual cycle. It has been known that LH receptor activation, via a series of incompletely understood steps, ultimately causes a decrease in cAMP within the oocyte. Recent studies provide strong evidence that LH acts by decreasing cGMP synthesis in somatic cells, resulting in the decrease of intraoocyte cGMP levels. The reduction of cGMP levels in oocytes relieves the inhibition of PDE3A, resulting in the decrease of cAMP levels. Further studies show that LH decreases cGMP levels in follicular somatic cells by the reductions of NPPC/NPR2 expression and function (Fig. 3). The activation of LH receptor decreases NPPC gene and/or protein levels in mouse, rat, pig, and human ovaries (Kawamura et al., 2011). LH receptor signaling also decreases NPR2 gene levels in mouse cumulus cells. However, this decrease occurs after meiotic resumption. On the other hand, LH could induce meiotic resumption of oocytes within cultured follicles even in the present NPPC, suggesting that LH may decrease NPR2 guanylyl cyclase activity. Consistent with this, LH treatment rapidly causes NPR2 dephosphorylation in rat granulosa cells without the change of NPR2 protein levels (Egbert et al., 2014). This dephosphorylation results in the inactivation of NPR2 and the rapid reduction in follicle cGMP levels. Thus, LH decreases NPPC content and NPR2 activity, each of which is enough to induce oocyte maturation by decreasing cGMP levels in the somatic cells and then in oocytes. The timing of meiotic resumption and ovulation is so critical that multiple pathways may have evolved into the fail-safe mechanism to ensure normal female fertility.

It is well known that LH promotes the production of epidermal growth factor (EGF)-like growth factors in MGCs, including amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC) in MGCs, which is essential for the regulation of oocyte maturation and ovulation (Park et al., 2004). The activation of EGFR by amphiregulin and EGF decreases NPPC gene levels in cultured granulosa cells. However, the EGFR inhibitor AG1478 partially reverses LH-induced the decrease of NPPC gene, suggesting that EGFR activity is not required for all of the LH-induced NPPC decrease. In COCs culture system that NPPC is used to prevent oocyte spontaneous maturation, EGF could decrease NPR2 activity of cumulus cells via the elevation of intracellular calcium concentrations. Further study shows that EGF-elevated calcium in cumulus cells decreases NPR2 affinity for NPPC and cGMP levels (Fig. 3), resulting in meiotic resumption (Hao et al., 2016). It needs to elucidate whether calcium also reduces NPR2 phosphorylation levels of cumulus cells as reported in a 293T cell line stably expressing NPR2.

It has been long hypothesized that the action of LH either relieves a maturation arresting substance from the somatic cells, or alternatively provides a positive maturation-promoting substance to override the follicular inhibition. Above data is consistent with a model in which LH removes the inhibitory function of NPPC/NPR2 to subsequently trigger oocyte maturation.

References

- Cho, W. K., Stern, S., & Biggers, J. D. (1974). Inhibitory effect of dibutyryl cAMP on mouse oocyte maturation in vitro. *The Journal of Experimental Zoology*, 187, 383–386.
- Egbert, J. R., Shuhaibar, L. C., Edmund, A. B., et al. (2014). Dephosphorylation and inactivation of NPR2 guanylyl cyclase in granulosa cells contributes to the LH-induced decrease in cGMP that causes resumption of meiosis in rat oocytes. *Development*, 141, 3594–3604.
- Hao, X., Wang, Y., Kong, N., et al. (2016). Epidermal growth factor-mobilized intracellular calcium of cumulus cells decreases natriuretic peptide receptor 2 affinity for natriuretic peptide type C and induces oocyte meiotic resumption in the mouse. *Biology of Reproduction*, 95, 45.
- Hubbard, C. J. (1980). Ovarian cAMP and cGMP fluctuations in the hamster during the oestrous cycle. *Journal of Reproduction and Fertility*, 59, 351–355.
- Kawamura, K., Cheng, Y., Kawamura, N., et al. (2011). Pre-ovulatory LH/hCG surge decreases C-type natriuretic peptide secretion by ovarian granulosa cells to promote meiotic resumption of pre-ovulatory oocytes. *Human Reproduction*, 26, 3094–3101.
- Mehlmann, L. M., Saeki, Y., Tanaka, S., et al. (2004). The Gs-linked receptor GPR3 maintains meiotic arrest in mammalian oocytes. *Science*, 306, 1947–1950.
- Norris, R. P., Ratzan, W. J., Freudzon, M., et al. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development*, 136, 1869–1878.
- Park, J. Y., Su, Y. Q., Ariga, M., et al. (2004). EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science*, 303, 682–684.
- Pincus, G., & Enzmann, E. V. (1935). The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs. *The Journal of Experimental Medicine*, 62, 665–675.
- Sela-Abramovich, S., Edry, I., Galiani, D., Nevo, N., & Dekel, N. (2006). Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology*, 147, 2280–2286.
- Tsafir, A., & Pomerantz, S. H. (1986). Oocyte maturation inhibitor. *Clinics in Endocrinology and Metabolism*, 15, 157–170.
- Tsafir, A., Pomerantz, S. H., & Channing, C. P. (1976). Porcine follicular fluid inhibitor of oocyte meiosis: Partial characterization of the inhibitor. *Biology of Reproduction*, 14, 511–516.
- Vaccari, S., Horner, K., Mehlmann, L. M., & Conti, M. (2008). Generation of mouse oocytes defective in cAMP synthesis and degradation: Endogenous cyclic AMP is essential for meiotic arrest. *Developmental Biology*, 316, 124–134.
- Wiersma, A., Hirsch, B., Tsafir, A., et al. (1998). Phosphodiesterase 3 inhibitors suppress oocyte maturation and consequent pregnancy without affecting ovulation and cyclicity in rodents. *The Journal of Clinical Investigation*, 102, 532–537.
- Zhang, M., Su, Y. Q., Sugiura, K., Xia, G., & Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*, 330, 366–369.

Germ Cell Nests and Germline Cysts

Melissa Pepling, Syracuse University, Syracuse, NY, United States

Lei Lei, University of Michigan Medical School, Ann Arbor, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cystoblast Cyst forming cell in the *Drosophila ovariole*.

Cyst fragments Groups of interconnected cells released from a germline cyst by breakage of one or more of its intercellular bridges; unless single, the cells in each cyst fragment will still have the properties of a cyst.

Fusome Cytoplasmic organelle found in insect intercellular bridges thought to be important for synchronizing divisions and specifying the oocyte during cyst formation in *Drosophila*, has not been detected in mammalian cysts.

Germ cell nest Germ cells that clump together morphologically; the interconnected nature of such cells cannot be determined from morphological observation.

Germarium Structure at the anterior end of the ovariole in *Drosophila* where the germline stem cells reside.

Germline cyst A cluster of interconnected germ cells generated by mitotic divisions with incomplete cytokinesis.

Intercellular bridges The arrested cytokinesis furrows that join the cytoplasm of individual germ cells within a cyst.

Ovariole Subdivision of the *Drosophila* ovary where oocytes are produced.

Primordial follicle One primary oocyte surrounded several flattened pregranulosa cells, found in mammals.

Primordial germ cells (PGCs) Precursors of oocytes and sperm found in fetal gonads.

Ring canal *Drosophila* intercellular bridge.

Introduction

Germ cells throughout the animal kingdom develop in structures called germline cysts. These structures share several conserved characteristics and are most well studied in the *Drosophila* ovary. The cells that make up a germline cyst divide synchronously by mitosis from precursor cells, remain connected by intercellular bridges and share cytoplasm. This article will focus on our current understanding of these structures in mammalian fetal ovaries and in particular, the mouse.

Germline Cysts—The *Drosophila* Model System

Drosophila female germline cysts are the most the well-studied and will be described here as a frame work for the discussion of mammalian germ cell cysts. Each *Drosophila* ovary consists of about 16 ovarioles where eggs are produced (for reviews of *Drosophila* oogenesis see [Bastock and St Johnston, 2008](#); [De Cuevas et al., 1997](#); [Hudson and Cooley, 2014](#); [Spradling, 1993](#)). Each ovariole has eggs at various stages of development often referred to as an assembly line of egg production. Germline stem cells are found in the germarium, a structure at the anterior end of the ovariole. There are about two to three stem cells in each germarium and they divide to form a daughter germline stem cell and a cyst forming cell called the cystoblast. The cystoblast then divides exactly four times but cytokinesis is not completed leaving a structure of 16 connected germ cells called a germline cyst. The connections or intercellular bridges are also referred to as ring canals based on their appearance. In each *Drosophila* cyst, a cellular structure named the fusome plays an essential role in orientating centrosomes and thus to generate a branched cyst structure. In each *Drosophila* 16-cell cyst, two germ cells are connected by four intercellular bridges, two cells are connected by three bridges, four cells are connected by two bridges and eight cells are connected by one bridge. The fusome is a cytoplasmic organelle found within the intercellular bridges of the cysts in many insects ([Hirschler, 1955](#)). Under the electron microscope (EM), the fusome is recognized by a distinctive region that is rich in fibrils and endoplasmic reticulum (ER)-like vesicles but excluding mitochondria ([De Cuevas et al., 1997](#)). During oocyte formation in each *Drosophila* cyst, one of the two germ cells with four bridges collects cytoplasm from the remaining 15 cells and differentiates into an oocyte. The remaining 15 germ cells, namely nurse cells die in an apoptotic-like process after donating their cytoplasmic content. Transfer of nurse cell cytoplasm across the ring canals to the oocyte occurs slowly at first and then more quickly in a process called nurse cell dumping.

Conservation of Cysts and Features of Mammalian Cysts

Germline cysts have been found during gametogenesis in many vertebrates and invertebrates. Since the late 19th century, intercellular bridges connecting adjacent germ cells have been observed in a wide range of animals by using EM ([Fawcett et al., 1959](#)).

During the oogenesis of invertebrates and some lower vertebrates, oocyte production in the adult ovary is sustained by germline stem cells or oogonia. Germline cysts can be observed in the adult ovary of these animals, including worms (Gibert et al., 1984; Hirsh et al., 1976; Siekierska, 2003), insects (Gutzeit et al., 1993; Klag and Bilinski, 1993), fishes (Leu and Draper, 2010; Marlow and Mullins, 2008; Nakamura et al., 2010), amphibians (Coggin, 1973; Filosa and Taddei, 1976; Kloc et al., 2004; Ruby et al., 1970), lizards (Filosa and Taddei, 1976), and chicks (Skalko et al., 1972; Ukeshima and Fujimoto, 1991). During mammalian oogenesis, oocyte production is sustained by a pool of primary oocytes that form during fetal ovarian development. Germline cysts can be observed in mammalian fetal ovaries. Animals that have been examined include bats (Lechowska et al., 2012), mice (Ruby et al., 1969), rats (Franchi and Mandl, 1962), golden hamsters (Weakley, 1967), rabbits (Zamboni and Gondos, 1968), pigs (Bielanska-Osuchowska, 2006), bovines (Garverick et al., 2010; Russe, 1983), and humans (Gondos, 1973; Ruby et al., 1970). Germline cysts have been identified in adult testes during spermatogenesis in many invertebrates and vertebrates as well. The animals that have been examined include hydras, *Drosophila*, opossums, pigeons, rats, hamsters, guinea pigs, rabbits, monkeys, and humans (Fawcett et al., 1959).

The cell biology of mammalian germline cysts remains largely unknown but is beginning to be investigated in mice (see Fig. 1). In the mouse embryo, germ cell progenitors, primordial germ cells (PGCs) can be first identified at around embryonic day 5 (E5) (Wear et al., 2016). PGCs initially proliferate with complete cytokinesis while migrating into the gonad. Starting at E10.5, individual PGC proliferate with incomplete cytokinesis to form germline cysts in both fetal ovaries and testes (Lei and Spradling, 2013; Pepling and Spradling, 1998). Unlike the *Drosophila* cyst, in which 16 germ cells remain interconnected in a fixed geometry, mouse cysts become fragmented during and after cyst formation. On average, each PGC produce a 30-cell clone, which comprises about 5 cysts whose size ranging from 2 to about 30 cells. Cysts from a PGC cluster together with cysts derived from other PGCs form nests of germ cells in fetal ovaries (Fig. 2). At around E14.5, when mitosis ceases, germ cells in the cyst enter meiosis synchronously in the mouse fetal ovary (Lei and Spradling, 2013). Mouse cysts continue fragmenting into single cells (but still nesting together) from E14.5 to postnatal day 0 (P0). In the P4 ovary, germ cells become individualized due to nest breakdown (detailed in section five). These individual primary oocytes are enclosed by several pregranulosa cells to form primordial follicles. From E14.5 to P4, approximately 80% of the fetal germ cells undergo apoptosis during a process similar to the “nursing process” observed in *Drosophila* oogenesis (Lei and Spradling, 2016).

During oocyte differentiation in the human fetal ovary, PGCs are observed in the embryo of about 3 weeks, at the number of about 40 (Witschi, 1948). These germ cells continue to proliferate in the fetal ovary and the number of germ cell per ovary peaks at ~3.5 million by 5 months, indicating the end of germline cyst formation in the human fetal ovary (Baker, 1963). Unlike in the mouse fetal ovary, where most germ cells develop at the same stage, such as cyst formation (E10.5–14.5), cyst fragmentation (E14.5–P0), nest breakdown (P0–P4), germ cells of each above stage can be observed in a relatively wide range of time during oocyte differentiation in the human fetal ovary. Germline cysts/intercellular bridges appear to be present between 50 days and 140 days/22 weeks (Hartshorne et al., 2009). Primordial follicles can be observed as early as 15 weeks, and the

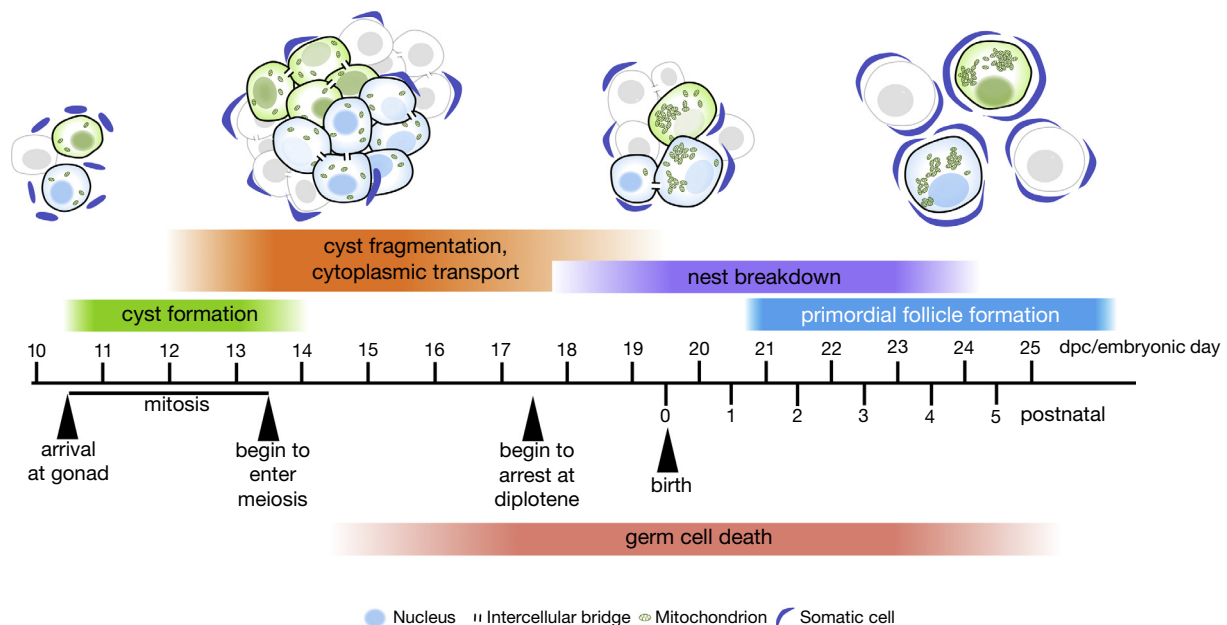


Fig. 1 Germ cell development in the mouse ovary. Primordial germ cells arrive at the genital ridge at approximately E10.5 and germ line cysts form with cysts becoming fragmented during and after cyst formation. Mouse cysts continue to fragment into single cells but remain together in the nests. Starting at E13.5, germ cells enter meiosis and progress through prophase I. Subsequently, oocytes arrest in the diplotene stage beginning at E17.5. After birth, germ cell nests break apart and pregranulosa cells enclose individual oocytes to form primordial follicles.

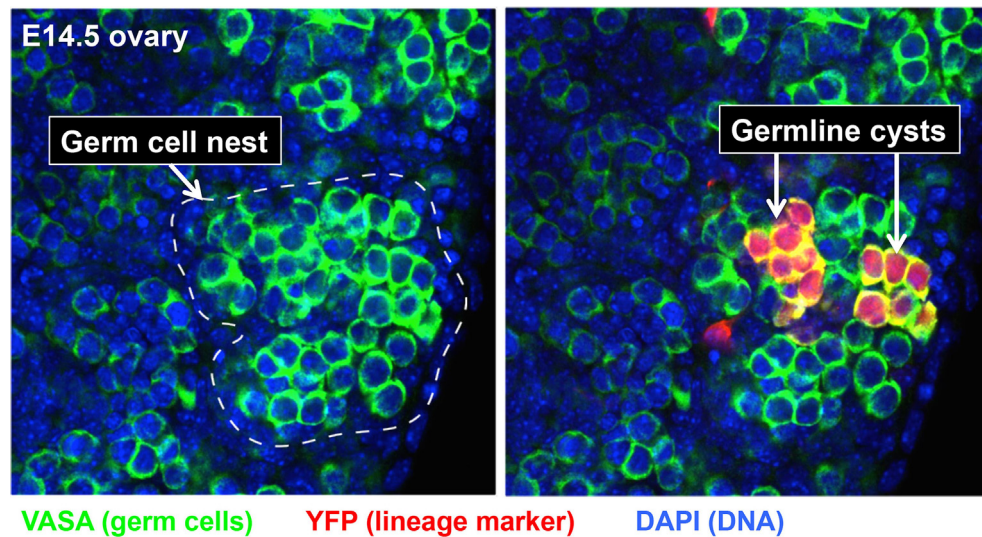


Fig. 2 Germ cell morphology in the E14.5 mouse fetal ovary. VASA-expressing germ cells are clustered together in nests. Two germline cysts (red) are revealed by lineage marker yellow fluorescent protein (YFP) by the single-PGC lineage tracing approach (Lei and Spradling, 2013).

number steadily increases during the second trimester and plateaus in the third trimester with $\sim 350,000$ – $400,000$ /ovary at birth (Kerr et al., 2013). These primordial follicles formed in the fetal ovary, namely the ovarian reserve, serve as the only source for sustaining egg production in the adult ovary.

Cyst Formation, Structure, and Composition

As early as 1880–90s, intercellular bridges were described by several scientists when they studied cell division in limax eggs, arthropod cells, and trout embryos (Fawcett et al., 1959; Carnoy, 1885; Flemming, 1891; Hennequy, 1896; Mark, 1881). The intercellular bridges were initially considered as a transient structure during cytokinesis of mitotic division. In 1955, Burgos and Fawcett recognized intercellular bridges as “a normal occurrence” when studying spermatogenesis in the cat (Burgos and Fawcett, 1955). These early ultrastructural morphological studies lay the foundation for recognizing the mechanism of germline cyst formation through incomplete cytokinesis. By using EM, intercellular bridges were described as “short cylindrical channels bounded by an electron opaque membrane continuous with the plasma membrane of the conjoined cells” (Gondos, 1973). The morphology of intercellular bridges is highly conserved in vertebrates and invertebrates. A typical intercellular bridge is 0.5 – $3\ \mu\text{m}$ in diameter in mammals, and 0.5 – $10\ \mu\text{m}$ in *Drosophila* (Cooley, 1998; Greenbaum et al., 2011). While the importance of the fusome in *Drosophila* cyst formation has been demonstrated, the mechanism of cyst formation in other animals remain largely uncovered. Fusome-like structures have been found in the *Xenopus* ovary and dogfish testis (Kloc et al., 2004; Loppion et al., 2008), but have not been observed within the intercellular bridges in mammalian ovaries or testes (Greenbaum et al., 2011; Pepling and Spradling, 1998).

The structure of mouse cysts during oocyte differentiation has been investigated (Lei and Spradling, 2013; Pepling and Spradling, 1998). Similar to *Drosophila* cysts, mouse cysts are in branched structures. By the time cyst formation is completed in the E14.5 mouse ovary, about 14% of germ cells in cysts are connected by three or four intercellular bridges (Fig. 3). The mechanism underlying branched cyst formation in mice remains unknown. The fact that the fusome structure was not found in mouse cysts suggests that mammals may use a different mechanism to position centrosomes and spindles during cyst formation.

The molecular composition of intercellular bridges has been characterized in *Drosophila* female and male cysts and mouse cysts in adult testes. In *Drosophila* cysts, intercellular bridges are initially composed of proteins involved in contractile ring formation during cytokinesis, including anillin, septins, mitotic kinesin-like protein (MKLP), actin, and myosin II. As the bridges mature, myosin II and actin are lost in the bridges in male cysts. In contrast, actin remains a major component of the mature bridges in female cysts, anillin and septins are no longer present. Two female cyst-specific proteins, hu-li tai shao (Hts) and kelch, are essential for female bridge growth and expansion (Cooley, 1998).

The protein composition of the intercellular bridges in adult mouse testes was revealed by proteomic analyses (Greenbaum et al., 2007). Besides anillin, septin, MKLP, and Rac GTPase-activating protein (RacGAP) that are found in the cytokinesis machinery of somatic cells, germ cell-specific intercellular bridge proteins, including testis-expressed 14 (Tex14) and RNA-binding protein 44 (RBM44) were also identified. Tex14 is a highly conserved protein that is expressed in human testes (Houmard et al., 2009), the loss of Tex14 in mice led to defects in bridge formation and male infertility (Greenbaum et al., 2006).

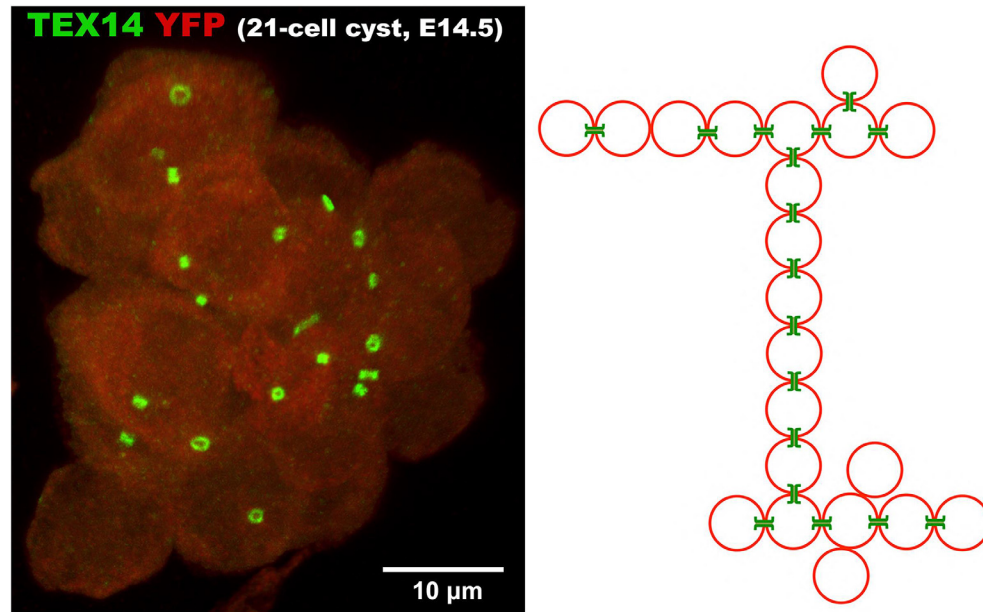


Fig. 3 Left: A lineage-labeled 21-cell cyst (red, lineage marker YFP) in the E14.5 mouse fetal ovary. Intercellular bridges are revealed by immunostaining of a bridge marker Tex14 (green). Structure of the cyst is shown in the diagram on right. Adapted from Lei, L. and Spradling, A. C. (2016). Mouse oocytes differentiate through organelle enrichment from sister cyst germ cells. *Science* **352**, 95–99.

Regulation of Nest Breakdown and Follicle Formation

The regulation of cyst formation and oocyte specification in mammals is not well understood. Recently, however, headway has been made in our understanding of the genetic control of nest breakdown into individual germ cells and primordial follicle formation and can be broken down into several classes of regulatory molecules including steroid hormones, signaling pathways, transcription factors, and cell adhesion molecules (Table 1).

Several hormones have been implicated in the control of nest breakdown and follicle formation including at least two steroids and one polypeptide hormone. The steroid hormone estrogen is thought to be important for maintaining oocytes in nests and thus, must be downregulated for follicle formation to occur. Ovaries from adult female mice treated as neonates with estrogen or estrogenic compounds have more multiple oocyte follicles (MOFs) which are likely oocyte in nests that did not separate and subsequently became enclosed in follicles together (Iguchi et al., 1986; Jefferson et al., 2006; Suzuki et al., 2002). Exposure of neonatal mouse ovaries to estrogen in ovary organ culture reduced the number of single oocytes and increase the number of oocytes still in nests (Chen et al., 2007). In addition, the phytoestrogen genistein and synthetic estrogens diethylstilbestrol, ethinyl estradiol and bisphenol A (BPA) also blocked nest breakdown (Karavan and Pepling, 2012). Further, progesterone also inhibited nest breakdown and with estradiol had an additive effect. In contrast to steroid hormones, follicle stimulating hormone (FSH) was found to promote nest breakdown and primordial follicle formation in both hamsters and mice (Lei et al., 2010; Roy and Albee, 2000).

There is evidence for the involvement of several signaling pathways in the control of nest breakdown and primordial follicle formation. Several members of the transforming growth factor beta (TGFβ) superfamily are expressed in the developing ovary. Mutants for either *bone morphogenetic protein 15* (*Bmp15*) or *growth differentiation factor 9* (*Gdf9*) have more MOFs than normal mice suggesting defects in nest breakdown (Yan et al., 2001). Both gene products are oocyte-secreted TGFβ family members (Elvin et al., 2000). Treatment of ovaries with another TGFβ family member, activin A, promotes follicle formation (Bristol-Gould et al., 2006). In contrast, overexpression of activin antagonist, inhibin B causes an increase in MOFs (Mcmullen et al., 2001). One isoform of Follistatin, another activin antagonist, when deleted causes defects in nest breakdown (Wang et al., 2015; Kimura et al., 2011). *Lunatic fringe* (*Lfng*) mutants are infertile with MOFs (Hahn et al., 2005). LFNG belongs to the fringe protein family which function by regulating notch signaling (Bruckner et al., 2000; Hicks et al., 2000) suggesting that notch signaling may play a role in nest breakdown. Interestingly, In *Drosophila*, *fringe* mutants also have follicles with more than one oocyte (Grammont and Irvine, 2001). In mammals, there are four notch receptors (NOTCH1–4) and five notch ligands (jagged (JAG) 1 and 2; and deltalike (DLL) 1, 3, and 4) (Bray, 2006). Inhibition of notch signaling in cultured ovaries caused a reduction in follicle formation (Trombly et al., 2008). Loss of NOTCH 2 in somatic cells blocks nest breakdown (Xu and Gridley, 2013). In addition, deletion of ADAM10, a secretase that cleaves and activates the Notch receptor also disrupted nest breakdown and primordial follicle formation (Feng et al., 2016). The c-Jun amino-terminal kinase (JNK) signaling cascade plays important roles in many developmental processes including oogenesis. Specifically, JNK is important for nest breakdown and follicle formation as JNK inhibition disrupts these processes (Niu et al., 2016). Ovary organ culture studies have revealed additional factors with potential roles in nest breakdown. Addition of KIT ligand to developing ovaries in organ culture accelerates follicle formation, while a function blocking antibody against the KIT tyrosine

Table 1 Molecules involved in regulating nest breakdown

<i>Gene</i>	<i>Protein/function</i>	<i>Evidence for role in NBD</i>	<i>References</i>
<i>Activin A</i>	TGF β family member	Promotes follicle formation while an activin antagonist causes multiple oocyte follicles	Bristol-Gould et al. (2006) and McMullen et al. (2001)
<i>Adam10</i>	Regulator of Notch signaling	Mutants have disrupted nest breakdown and follicle formation	Feng et al. (2016)
<i>Ahr</i>	Aryl hydrocarbon receptor, basic helix loop helix transcription factor	Follicle formation is accelerated in mutants	Benedict et al. (2000) and Robles et al. (2000)
<i>Bmp15</i>	Bone morphogenetic protein 15, TGF β family member	Mutants have multiple oocyte follicles	Yan et al. (2001)
<i>E-cadherin</i>	Cell adhesion molecule	Inhibition causes premature nest breakdown in hamsters and in mice	Niu et al. (2016) and Wang and Roy (2010)
<i>Figla</i>	Factor in the germ line alpha, folliculogenesis specific basic helix loop helix	Mutants are defective in follicle formation and exhibit perinatal oocyte loss	Soyal et al. (2000)
<i>Fsh</i>	Follicle stimulating hormone, glycoprotein polypeptide hormone	Promotes follicle formation	Lei et al. (2010) and Roy and Albee (2000)
<i>Follistatin</i>	Activin antagonist	Mutants defective in follicle formation	Kimura et al. (2011) and Wang et al. (2015)
<i>Foxl2</i>	Forkhead box L2, winged helix transcription factor	Mutants are defective in follicle formation and exhibit perinatal oocyte loss	Schmidt et al. (2004) and Uda et al. (2004)
<i>Gdf9</i>	Growth differentiation factor 9, TGF β family member	Mutants have multiple oocyte follicles	Yan et al. (2001)
<i>JNK</i>	The c-Jun amino-terminal kinase	Inhibition disrupts nest breakdown and follicle formation	Niu et al. (2016)
<i>Kit signaling</i>	Kit ligand Kit receptor tyrosine kinase	Promotes follicle formation in hamsters and mice	Jones and Pepling (2013) and Wang and Roy (2004)
<i>Lunatic fringe</i>	Regulator of Notch signaling	Mutants have multiple oocyte follicles	Hahn et al. (2005)
<i>N-cadherin</i>	Cell adhesion molecule	Inhibition delays nest breakdown in hamsters	Wang and Roy (2010)
<i>Ngf</i>	Nerve growth factor, neurotrophin signaling	Follicle formation is reduced in mutants	Abir et al. (2005) and Dissen et al. (2001)
<i>Notch ligands</i>	Jagged 1 and 2	Inhibition of Notch signaling reduces follicle formation	Trombly et al. (2008)
<i>Notch receptors</i>	Notch 1 and 2	Inhibition of Notch signaling reduces follicle formation. Notch 2 mutants have defective nest breakdown	Trombly et al. (2008) and Xu and Gridley (2013)
<i>Nobox</i>	Newborn ovary homeobox-encoding gene	Mutants have delayed follicle formation and oocyte loss	Rajkovic et al. (2004)
<i>Ntrk1</i>	NGF receptor, neurotrophin signaling	Follicle formation is reduced in mutants	Kerr et al. (2009)
<i>Ntrk2</i>	Receptor for NT-4 and BDNF, neurotrophin signaling	Follicle formation and germ cell number is reduced in mutants	Kerr et al. (2009) and Spears et al. (2003)
<i>p27</i>	Cyclin-dependent kinase inhibitor 1, downstream of PI3K signaling	Follicle formation is accelerated in mutants	Rajareddy et al. (2007)
<i>Taf4b</i>	Ovary specific subunit of TFIID	Mutants have delayed nest breakdown	Grive et al. (2014)

kinase receptor inhibits follicle formation in both mice and hamsters (Jones and Pepling, 2013; Wang and Roy, 2004). Nerve growth factor (NGF) has also been implicated in primordial follicle formation. Not only do *Ngf* homozygous mutants have reduced numbers of primary and secondary follicles, they also have more oocytes not enclosed in follicles at P7 (Dissen et al., 2001). In addition, mutants of NGF receptors, NTRK1, and NTRK2, have reduced numbers of follicles lending support to the idea that NGFs are important for follicle formation (Kerr et al., 2009; Spears et al., 2003).

Mouse knockouts of several transcription factors have phenotypes suggesting roles in primordial follicle formation. Mutants in the gene encoding the aryl hydrocarbon receptor (AHR), a basic helix loop helix (bHLH) protein, form follicles at a faster rate than normal (Benedict et al., 2000; Robles et al., 2000). Factor in the germline alpha (FIGLA) is another bHLH protein (Liang et al., 1997). *Figla* mutants begin to lose oocytes at birth and oocytes still present are not enclosed in primordial follicles (Soyal et al., 2000). Disruption of *Nobox*, an oocyte specific homeobox gene results in an increased oocyte loss and a delay in nest breakdown (Suzumori et al., 2002; Rajkovic et al., 2004). Mutants in *Foxl2*, a winged-helix forkhead transcription factor, are sterile and germ cell nests are present in adult ovaries (Uda et al., 2004). Finally, mutation of TAF4b, an ovary-specific subunit of the TFIID general transcription complex results in delayed nest breakdown (Grive et al., 2014).

Cell adhesion molecules (CAMs) may be important to keep oocytes together before nest breakdown and also during nest breakdown to promote the association of primary oocytes and pregranulosa cells. Recent work using the hamster model has implicated

two CAMS, N and E-cadherin in follicle formation (Wang and Roy, 2010). These CAMs appear to have opposing roles in nest breakdown and primordial follicle assembly with E-cadherin involved in keeping oocytes together and N-cadherin important for oocyte/pregranulosa cell interactions. More recently, there is evidence from studies in the mouse that E-cadherin is important for maintaining oocytes in nests and/or cysts and must be downregulated for primordial follicle formation to occur (Niu et al., 2016).

Functions of Connected Germ Cells

Intercellular bridges provide a means of exchanging signaling molecules and other cytoplasmic contents among sister germ cells, which is essential for normal oogenesis and spermatogenesis.

Synchronization of Differentiation and Maturation

It has been observed in cysts of *Drosophila*, *Xenopus*, and mammals that germ cells connected by intercellular bridges develop in a synchronized manner. Germ cells in cysts undergo synchronized mitosis and meiosis during oogenesis in mice and rabbits (Lei and Spradling, 2013; Pepling and Spradling, 1998; Ruby et al., 1969; Zamboni and Gondos, 1968). In adult mammalian testes, the connections between genetically distinct haploid spermatids allow these cells to share the cytoplasm and hence develop equally (Braun et al., 1989). The connection in *Drosophila* male cysts facilitate the death of the whole cyst when one germ cell encounters damage in its genome, suggesting that cysts may serve a purpose of protecting genomic integrity during sperm production (Lu and Yamashita, 2017).

Nursing Mechanism During Oocyte Formation

A well-characterized function of female *Drosophila* germline cysts is to facilitate cytoplasmic transport and oocyte formation in the cyst. In the *Drosophila* cyst, asymmetric distribution of the fusome establishes polarity in the cyst that directs cytoplasmic enrichment into the oocyte through microtubule-dependent transport. The similar function of nursing in mammalian female cysts was initially suggested by studies using EM. As a canal that connects two cells, organelles including smooth ER, ribosomes, smooth vesicles, mitochondria, and microtubules can be found within the bridge in mouse, rabbit, and human fetal ovaries (Gondos, 1973; Pepling and Spradling, 1998; Ruby et al., 1969; Zamboni and Gondos, 1968). Through the observation of organelle distribution in individual germline cysts in mouse fetal ovaries, centrosomes, Golgi complexes and mitochondria were found to redistribute extensively within germline cysts during oocyte differentiation, leading to about 20% of the fetal germ cells differentiating into primary oocytes with enriched cytoplasmic content. Similar to *Drosophila*, the germ cells that donate their cytoplasm undergo apoptosis (Lei and Spradling, 2016). However, in the ovaries of primitive insects (grasshopper, cockroaches) and *Xenopus*, no significant germ cell apoptosis in cysts were observed and all germ cells develop into oocytes (Kloc et al., 2004; Pritsch and Buning, 1989).

Other Functions

Besides the above two major functions of cysts in gametogenesis, it has been proposed that cyst may serve a function of: (1) *facilitating the onset of meiosis*, given the fact that the cyst-formation cycle involves a significant alteration in the cell cycle and cyst formation always seems to precede the onset of meiosis; (2) *facilitating organelle biogenesis* through enriching mitochondria; (3) *inhibition of mitosis* to restrict the number of germ cells entering meiosis; (4) *restriction of motility* (Gondos, 1973; Pepling and Spradling, 1998; Pepling and Spradling, 2001).

References

- Abir, R., Fisch, B., Jin, S., Barnet, M., Ben-Haroush, A., Felz, C., Kessler-Icekson, G., Feldberg, D., Nitke, S., & Ao, A. (2005). Presence of NGF and its receptors in ovaries from human fetuses and adults. *Molecular Human Reproduction*, 11, 229–236.
- Baker, T. G. (1963). A quantitative and cytological study of germ cells in human ovaries. *Proceedings of the Royal Society B: Biological Sciences*, 158, 417–433.
- Bastock, R., & St Johnston, D. (2008). *Drosophila* oogenesis. *Current Biology*, 18, R1082–7.
- Benedict, J. C., Lin, T. M., Loeffler, I. K., Peterson, R. E., & Flaws, J. A. (2000). Physiological role of the aryl hydrocarbon receptor in mouse ovary development. *Toxicological Sciences*, 56, 382–388.
- Bielanska-Osuchowska, Z. (2006). Oogenesis in pig ovaries during the prenatal period: Ultrastructure and morphometry. *Reproductive Biology*, 6, 161–193.
- Braun, R. E., Peschon, J. J., Behringer, R. R., Brinster, R. L., & Palmiter, R. D. (1989). Protamine 3'-untranslated sequences regulate temporal translational control and subcellular localization of growth hormone in spermatids of transgenic mice. *Genes & Development*, 3, 793–802.
- Bray, S. J. (2006). Notch signalling: A simple pathway becomes complex. *Nature Reviews. Molecular Cell Biology*, 7, 678–689.
- Bristol-Gould, S. K., Kreeger, P. K., Selkirk, C. G., Kilen, S. M., Cook, R. W., Kipp, J. L., Shea, L. D., Mayo, K. E., & Woodruff, T. K. (2006). Postnatal regulation of germ cells by activin: The establishment of the initial follicle pool. *Developmental Biology*, 298, 132–148.
- Bruckner, K., Perez, L., Clausen, H., & Cohen, S. (2000). Glycosyltransferase activity of fringe modulates Notch-Delta interactions. *Nature*, 406, 411–415.
- Burgos, M. H., & Fawcett, D. W. (1955). Studies on the fine structure of the mammalian testis. I. Differentiation of the spermatids in the cat (*Felis domestica*). *Journal of Biophysical and Biochemical Cytology*, 1, 287–300.
- Carnoy, J. B. (1885). La cytodierèse chez les arthropodes. *La Cellule*, 1, 375.

- Chen, Y., Jefferson, W. N., Newbold, R. R., Padilla-Banks, E., & Pepling, M. E. (2007). Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary in vitro and in vivo. *Endocrinology*, 148, 3580–3590.
- Coggins, L. W. (1973). An ultrastructural and radioautographic study of early oogenesis in the toad *Xenopus laevis*. *Journal of Cell Science*, 12, 71–93.
- Cooley, L. (1998). *Drosophila* ring canal growth requires Src and Tec kinases. *Cell*, 93, 913–915.
- De Cuevas, M., Lilly, M. A., & Spradling, A. C. (1997). Germline cyst formation in *Drosophila*. *Annual Review of Genetics*, 31, 405–428.
- Dissen, G. A., Romero, C., Hirshfield, A. N., & Ojeda, S. R. (2001). Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology*, 142, 2078–2086.
- Elvin, J. A., Yan, C., & Matzuk, M. M. (2000). Oocyte-expressed TGF-beta superfamily members in female fertility. *Molecular and Cellular Endocrinology*, 159, 1–5.
- Fawcett, D. W., Ito, S., & Slautterback, D. (1959). The occurrence of intercellular bridges in groups of cells exhibiting synchronous differentiation. *Journal of Biophysical and Biochemical Cytology*, 5, 453–460.
- Feng, L., Wang, Y., Cai, H., Sun, G., Niu, W., Xin, Q., Tang, X., Zhang, J., Wang, C., Zhang, H., & Xia, G. (2016). ADAM10-Notch signaling governs the recruitment of ovarian pregranulosa cells and controls folliculogenesis in mice. *Journal of Cell Science*, 129, 2202–2212.
- Filosa, S., & Taddei, C. (1976). Intercellular bridges in lizard oogenesis. *Cell Differentiation*, 5, 199–206.
- Flemming, W. (1891). Neue Beiträge zur Kenntniss der Zelle. *Archiv für Mikroskopische Anatomie*, 37, 685.
- Franchi, L. L., & Mandl, A. M. (1962). The ultrastructure of oogonia and oocytes in the foetal and neonatal rat. *Proceedings of the Royal Society of London, Series B: Biological Sciences*, 157.
- Garverick, H. A., Juengel, J. L., Smith, P., Heath, D. A., Burkhart, M. N., Perry, G. A., Smith, M. F., & McNatty, K. P. (2010). Development of the ovary and ontogeny of mRNA and protein for P450 aromatase (arom) and estrogen receptors (ER) alpha and beta during early fetal life in cattle. *Animal Reproduction Science*, 117, 24–33.
- Gibert, M. A., Starck, J., & Beguet, B. (1984). Role of the gonad cytoplasmic core during oogenesis of the nematode *Caenorhabditis elegans*. *Biology of the Cell*, 50, 77–85.
- Gondos, B. (1973). Intercellular bridges and mammalian germ cell differentiation. *Differentiation*, 1, 177–182.
- Grammont, M., & Irvine, K. D. (2001). Fringe and notch specify polar cell fate during *Drosophila* oogenesis. *Development*, 128, 2243–2253.
- Greenbaum, M. P., Yan, W., Wu, M. H., Lin, Y. N., Agno, J. E., Sharma, M., Braun, R. E., Rajkovic, A., & Matzuk, M. M. (2006). TEX14 is essential for intercellular bridges and fertility in male mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 4982–4987.
- Greenbaum, M. P., Ma, L., & Matzuk, M. M. (2007). Conversion of midbodies into germ cell intercellular bridges. *Developmental Biology*, 305, 389–396.
- Greenbaum, M. P., Iwamori, T., Buchold, G. M., & Matzuk, M. M. (2011). Germ cell intercellular bridges. *Cold Spring Harbor Perspectives in Biology*, 3, a005850.
- Grive, K. J., Seymour, K. A., Mehta, R., & Freiman, R. N. (2014). TAF4b promotes mouse primordial follicle assembly and oocyte survival. *Developmental Biology*, 392, 42–51.
- Gutzeit, H. O., Zissler, D., & Fleig, R. (1993). Oogenesis in the honeybee *Apis mellifera*: Cytological observations on the formation and differentiation of previtellogenic ovarian follicles. *Roux's Archives of Developmental Biology*, 202, 181–191.
- Hahn, K. L., Johnson, J., Beres, B. J., Howard, S., & Wilson-Rawls, J. (2005). Lunatic fringe null female mice are infertile due to defects in meiotic maturation. *Development*, 132, 817–828.
- Hartshorne, G. M., Lyraou, S., Hamoda, H., Oloto, E., & Ghafari, F. (2009). Oogenesis and cell death in human prenatal ovaries: What are the criteria for oocyte selection? *Molecular Human Reproduction*, 15, 805–819.
- Henneguy, L. F. (1896). *Leçons sur la Cellule*. Paris: Georges Carré.
- Hicks, C., Johnston, S. H., Disibio, G., Collazo, A., Vogt, T. F., & Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nature Cell Biology*, 2, 515–520.
- Hirschler, J. (1955). On the cooperation of fusomes in the development of egg nursecell complexes in the animal ovary. *Cellule*, 57, 65–87.
- Hirsh, D., Oppenheim, D., & Klass, M. (1976). Development of the reproductive system of *Caenorhabditis elegans*. *Developmental Biology*, 49, 200–219.
- Houmard, B., Small, C., Yang, L., Nalwai-Cecchini, T., Cheng, E., Hassold, T., & Griswold, M. (2009). Global gene expression in the human fetal testis and ovary. *Biology of Reproduction*, 81, 438–443.
- Hudson, A. M., & Cooley, L. (2014). Methods for studying oogenesis. *Methods*, 68, 207–217.
- Iguchi, T., Takasugi, N., Bern, H. A., & Mills, K. T. (1986). Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. *Teratology*, 34, 29–35.
- Jefferson, W., Newbold, R., Padilla-Banks, E., & Pepling, M. (2006). Neonatal genistein treatment alters ovarian differentiation in the mouse: Inhibition of oocyte nest breakdown and increased oocyte survival. *Biology of Reproduction*, 74, 161–168.
- Jones, R. L., & Pepling, M. E. (2013). KIT signaling regulates primordial follicle formation in the neonatal mouse ovary. *Developmental Biology*, 382, 186–197.
- Karavan, J. R., & Pepling, M. E. (2012). Effects of estrogenic compounds on neonatal oocyte development. *Reproductive Toxicology*, 34, 51–56.
- Kerr, B., Garcia-Rudaz, C., Dorfman, M., Paredes, A., & Ojeda, S. R. (2009). NTRK1 and NTRK2 receptors facilitate follicle assembly and early follicular development in the mouse ovary. *Reproduction*, 138, 131–140.
- Kerr, J. B., Myers, M., & Anderson, R. A. (2013). The dynamics of the primordial follicle reserve. *Reproduction*, 146, R205–15.
- Kimura, F., Bonomi, L. M., & Schneyer, A. L. (2011). Follistatin regulates germ cell nest breakdown and primordial follicle formation. *Endocrinology*, 152, 697–706.
- Klag, J., & Bilinski, S. (1993). Oosome formation in two ichneumonid wasps. *Tissue & Cell*, 25, 121–128.
- Kloc, M., Bilinski, S., Dougherty, M. T., Brey, E. M., & Etkin, L. D. (2004). Formation, architecture and polarity of female germline cyst in *Xenopus*. *Developmental Biology*, 266, 43–61.
- Lechowska, A., Bilinski, S. M., Rasweiler, J. J. T., Cretokos, C. J., Behringer, R. R., & Kloc, M. (2012). Early oogenesis in the short-tailed fruit bat *Carollia perspicillata*: Transient germ cell cysts and noncanonical intercellular bridges. *Genesis*, 50, 18–27.
- Lei, L., & Spradling, A. C. (2013). Mouse primordial germ cells produce cysts that partially fragment prior to meiosis. *Development*, 140, 2075–2081.
- Lei, L., & Spradling, A. C. (2016). Mouse oocytes differentiate through organelle enrichment from sister cyst germ cells. *Science*, 352, 95–99.
- Lei, L., Jin, S., Mayo, K. E., & Woodruff, T. K. (2010). The interactions between the stimulatory effect of follicle-stimulating hormone and the inhibitory effect of estrogen on mouse primordial folliculogenesis. *Biology of Reproduction*, 82, 13–22.
- Leu, D. H., & Draper, B. W. (2010). The zwi promoter drives germline-specific gene expression in zebrafish. *Developmental Dynamics*, 239, 2714–2721.
- Liang, L., Soyak, S. M., & Dean, J. (1997). FlGalpha, a germ cell specific transcription factor involved in the coordinate expression of the zona pellucida genes. *Development*, 124, 4939–4947.
- Loppion, G., Crespel, A., Martinez, A. S., Auvray, P., & Sourdaire, P. (2008). Study of the potential spermatogonial stem cell compartment in dogfish testis, *Scyliorhinus canicula* L. *Cell and Tissue Research*, 332, 533–542.
- Lu, K. L., & Yamashita, Y. M. (2017). Germ cell connectivity enhances cell death in response to DNA damage in the *Drosophila* testis. *eLife*, 6.
- Mark, E. L. (1881). Maturation, fecundation and segmentation of *Limax campestris*. *Bulletin of the Museum of Comparative Zoology at Harvard College*, 6.
- Marlow, F. L., & Mullins, M. C. (2008). Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. *Developmental Biology*, 321, 40–50.
- McMullen, M. L., Cho, B. N., Yates, C. J., & Mayo, K. E. (2001). Gonadal pathologies in transgenic mice expressing the rat inhibin alpha-subunit. *Endocrinology*, 142, 5005–5014.
- Nakamura, S., Kobayashi, K., Nishimura, T., Higashijima, S., & Tanaka, M. (2010). Identification of germline stem cells in the ovary of the teleost medaka. *Science*, 328, 1561–1563.
- Niu, W., Wang, Y., Wang, Z., Xin, Q., Wang, Y., Feng, L., Zhao, L., Wen, J., Zhang, H., Wang, C., & Xia, G. (2016). JNK signaling regulates E-cadherin junctions in germline cysts and determines primordial follicle formation in mice. *Development*, 143, 1778–1787.

- Pepling, M. E., & Spradling, A. C. (1998). Female mouse germ cells form synchronously dividing cysts. *Development*, 125, 3323–3328.
- Pepling, M. E., & Spradling, A. C. (2001). The mouse ovary contains germ cell cysts that undergo programmed breakdown to form follicles. *Developmental Biology*, 234, 339–351.
- Pritsch, M., & Buning, J. (1989). Germ cell cluster in the panoistic ovary of Thysanoptera (Insecta). *Zoomorphology*, 108, 309–313.
- Rajareddy, S., Reddy, P., Du, C., Liu, L., Jagarlamudi, K., Tang, W., Shen, Y., Berthet, C., Peng, S. L., Kaldis, P., & Liu, K. (2007). p27kip1 (cyclin-dependent kinase inhibitor 1B) controls ovarian development by suppressing follicle endowment and activation and promoting follicle atresia in mice. *Molecular Endocrinology*, 21, 2189–2202.
- Rajkovic, A., Pangas, S. A., Ballow, D., Suzumori, N., & Matzuk, M. M. (2004). NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science*, 305, 1157–1159.
- Robles, R., Morita, Y., Mann, K. K., Perez, G. I., Yang, S., Matikainen, T., Sherr, D. H., & Tilly, J. L. (2000). The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. *Endocrinology*, 141, 450–453.
- Roy, S. K., & Albee, L. (2000). Requirement for follicle-stimulating hormone action in the formation of primordial follicles during perinatal ovarian development in the hamster. *Endocrinology*, 141, 4449–4456.
- Ruby, J. R., Dyer, R. F., & Skalko, R. J. (1969). The occurrence of intercellular bridges during oogenesis in the mouse. *Journal of Morphology*, 127, 307–313.
- Ruby, J. R., Dyer, R. F., Skalko, R. G., & Volpe, E. P. (1970). Intercellular bridges between germ cells in the developing ovary of the tadpole, *Rana pipiens*. *The Anatomical Record*, 167, 1–9.
- Russe, I. (1983). Oogenesis in cattle and sheep. *Bibliotheca Anatomica*, 24, 77–92.
- Schmidt, D., Ovitt, C. E., Anlag, K., Fehsenfeld, S., Gredsted, L., Treier, A. C., & Treier, M. (2004). The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. *Development*, 131, 933–942.
- Siekierska, E. (2003). The structure of the ovary and oogenesis in the earthworm, *Dendrobaena veneta* (Annelida, Clitellata). *Tissue & Cell*, 35, 252–259.
- Skalko, R. G., Kerrigan, J. M., Ruby, J. R., & Dyer, R. F. (1972). Intercellular bridges between oocytes in the chicken ovary. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 128, 31–41.
- Soyal, S. M., Amleh, A., & Dean, J. (2000). FlGalpa, a germ cell-specific transcription factor required for ovarian follicle formation. *Development*, 127, 4645–4654.
- Spears, N., Molinek, M. D., Robinson, L. L., Fulton, N., Cameron, H., Shimoda, K., Telfer, E. E., Anderson, R. A., & Price, D. J. (2003). The role of neurotrophin receptors in female germ-cell survival in mouse and human. *Development*, 130, 5481–5491.
- Spradling, A. C. (1993). Germline cysts: Communes that work. *Cell*, 72, 649–651.
- Suzuki, A., Sugihara, A., Uchida, K., Sato, T., Ohta, Y., Katsu, Y., Watanabe, H., & Iguchi, T. (2002). Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. *Reproductive Toxicology*, 16, 107–116.
- Suzumori, N., Yan, C., Matzuk, M. M., & Rajkovic, A. (2002). Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. *Mechanisms of Development*, 111, 137–141.
- Trombly, D. J., Woodruff, T. K., & Mayo, K. E. (2008). Suppression of notch signaling in the neonatal mouse ovary decreases primordial follicle formation. *Endocrinology*, 150, 1014–1024.
- Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J. E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., & Pilia, G. (2004). Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Human Molecular Genetics*, 13, 1171–1181.
- Ukeshima, A., & Fujimoto, T. (1991). A fine morphological study of germ cells in asymmetrically developing right and left ovaries of the chick. *The Anatomical Record*, 230, 378–386.
- Wang, J., & Roy, S. K. (2004). Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: Modulation by follicle-stimulating hormone. *Biology of Reproduction*, 70, 577–585.
- Wang, C., & Roy, S. K. (2010). Expression of E-cadherin and N-cadherin in perinatal hamster ovary: Possible involvement in primordial follicle formation and regulation by follicle-stimulating hormone. *Endocrinology*, 151, 2319–2330.
- Wang, Z., Niu, W., Wang, Y., Teng, Z., Wen, J., Xia, G., & Wang, C. (2015). Follistatin288 regulates germ cell cyst breakdown and primordial follicle assembly in the mouse ovary. *PLoS One*, 10, e0129643.
- Weakley, B. S. (1967). Light and electron microscopy of developing germ cells and follicle cells in the ovary of the golden hamster: Twenty-four hours before birth to eight days postpartum. *Journal of Anatomy*, 101, 435–459.
- Wear, H. M., Mcpike, M. J., & Watanabe, K. H. (2016). From primordial germ cells to primordial follicles: A review and visual representation of early ovarian development in mice. *Journal of Ovarian Research*, 9, 36.
- Witschi, E. (1948). Migration of the germ cells of human embryos from the yolk sac to the primitive gonadal folds. *Contributions to Embryology Carnegie Institution*, 32, 67–80.
- Xu, J., & Gridley, T. (2013). Notch2 is required in somatic cells for breakdown of ovarian germ-cell nests and formation of primordial follicles. *BMC Biology*, 11, 13.
- Yan, C., Wang, P., Demayo, J., Demayo, F. J., Elvin, J. A., Carino, C., Prasad, S. V., Skinner, S. S., Dunbar, B. S., Dube, J. L., Celeste, A. J., & Matzuk, M. M. (2001). Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Molecular Endocrinology*, 15, 854–866.
- Zamboni, L., & Gondos, B. (1968). Intercellular bridges and synchronization of germ cell differentiation during oogenesis in the rabbit. *The Journal of Cell Biology*, 36, 276–282.

Oocyte Meiotic Resumption Upon Puberty

Deepak Adhikari and John Carroll, Monash University, Melbourne, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Anaphase promoting complex A complex cellular machinery that marks the target cell proteins for degradation.

Connexins Proteins making gap junctions for the transfer of molecules between cells.

Cyclic adenosine monophosphate A second messenger important in many biological processes. It is derived from ATP by adenylate cyclase enzyme. It plays key role in maintaining the meiotic arrest of oocyte.

Cyclic guanosine monophosphate A second messenger synthesized from GTP by guanylate cyclase enzyme.

Cyclin dependent kinase 1 A serine/threonine kinase that plays key roles in both mitosis and meiosis progression.

Follicle stimulating hormone A hormone synthesized and secreted by anterior pituitary gland. It regulates development, growth, pubertal development, and follicle development in the ovary.

Gap junctions Special intercellular connections between different cell types.

Ligand A substance that binds with its specific receptor and produces a signal.

Luteinizing hormone A hormone synthesized and secreted by anterior pituitary gland. It functions closely with follicle stimulating hormone for testis and ovary development.

Maturation promoting factor A complex of cyclin and cyclin dependent kinase that stimulates cell cycle progression both in mitosis and meiosis.

Phosphodiesterase 3A An enzyme that hydrolyses cyclic adenosine monophosphate.

Phosphorylation A chemical reaction adding a phosphoryl group to its substrates.

Receptor A protein molecule on or inside a cell that selectively receives chemical signal (ligand) from outside a cell.

Introduction

Meiosis is a specialized cell division consisting of two rounds of successive divisions that halves the number of chromosomes in the sperm or oocyte. Oocytes enter meiosis during fetal life but become arrested at diplotene stage of prophase I until puberty. Oocytes arrested at prophase I contain a large nucleus surrounded by nuclear envelope also known as the germinal vesicle (GV). GV breakdown (GVBD) and dissolution of the nuclear membrane is the first morphological sign of oocyte meiotic resumption. A surge of luteinizing hormone (LH) induces meiotic resumption and ovulation of the oocyte. In the follicle, the oocyte is surrounded by somatic cells called granulosa cells. The interaction of oocytes and granulosa cells is an essential aspect of reproductive biology as it is necessary for development of both the oocyte and the follicle (Fig. 1). One of the first known examples of oocyte–granulosa cell interaction provides a graphic example of this interaction; the demonstration that removal of the fully grown oocyte from the follicle into a suitable culture medium allows spontaneous resumption of meiosis completely independent of LH. We now understand the signaling mechanisms responsible for this remarkable ability of the granulosa cell compartment to maintain meiotic arrest and then transduce the LH signal to the oocyte where it triggers meiotic resumption.

Mechanism of Oocyte Meiotic Arrest: A Role for cAMP

The spontaneous resumption of meiosis that occurs when the oocyte is removed from the fully grown antral follicle provides a critical insight into the mechanism of meiotic arrest. It suggests that the somatic cells of the follicle prevent the resumption of meiosis in the oocyte and that the LH surge overcomes this inhibitory action rather than providing an independent positive stimulus. The action of LH is also dictated by the location of its receptors on the theca and mural granulosa cells (McGee and Hsueh, 2000). Thus, LH does not stimulate meiotic resumption by acting directly on the oocyte. Rather, recent evidence shows that LH stimulates meiotic resumption indirectly by disrupting intercellular communication between the somatic cells of the follicle and also between the cumulus cells and the oocyte.

A number of lines of evidence suggest that a high level of cyclic adenosine monophosphate (cAMP) in the oocyte is responsible for maintenance of oocyte meiotic arrest (Fig. 2A). Examples include, the spontaneous meiotic resumption of fully grown oocyte is prevented if oocyte cAMP concentration is maintained by adding its analog dibutyryl cAMP to the culture medium. cAMP is inactivated by an enzyme cAMP phosphodiesterase 3A (PDE3A) and inhibition of phosphodiesterases in oocytes in vitro leads to maintenance of meiotic arrest. In vivo evidence that PDE3A is the key physiological regulator of cAMP in oocytes is provided by mutant mice lacking *Pde3a*; in these mice oocytes are permanently arrested at the GV stage and never resume meiosis due to

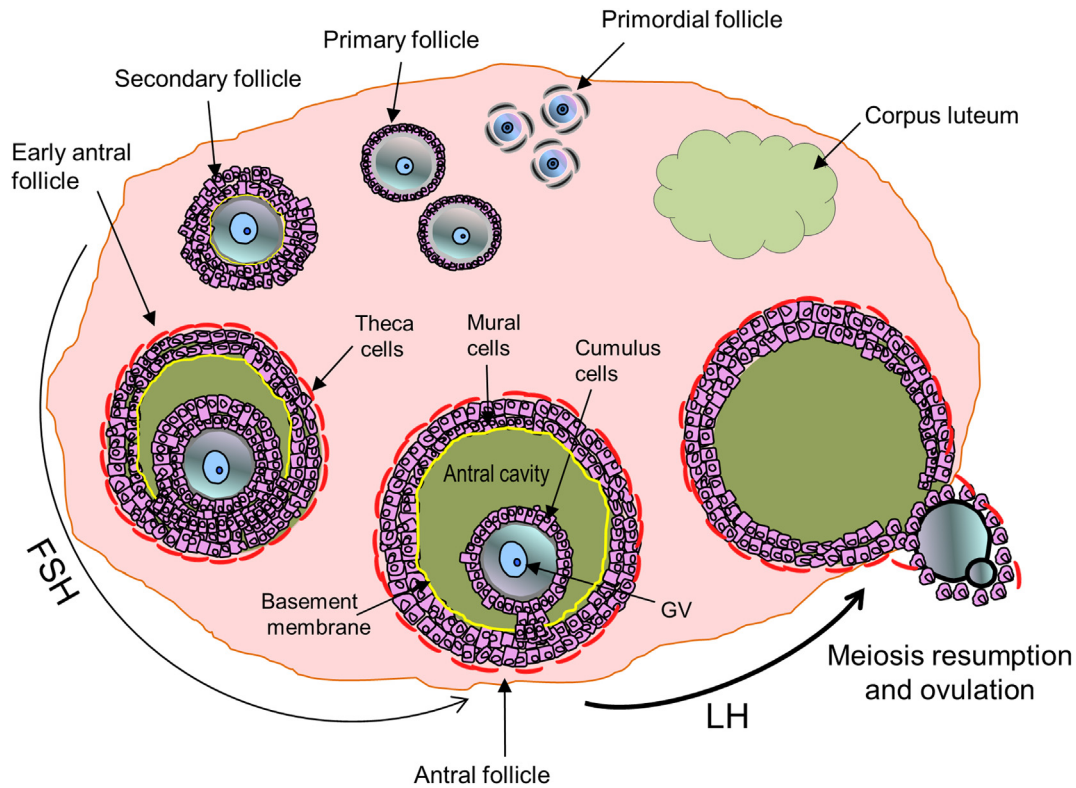


Fig. 1 Schematic representation of mammalian ovary showing the process of follicle development. Primordial follicle contains an oocyte surrounded by flattened pregranulosa cells. It develops through primary, secondary and early antral follicle stages before forming an antral follicle containing a fluid-filled antral cavity. Within the follicle, oocytes remain arrested at prophase I and are characterized by a germinal vesicle (GV). Antral follicle contains mural granulosa cells closer to the basement membrane and cumulus granulosa cells closer to the oocyte. Follicle stimulating hormone (FSH) stimulates the development of antral follicles. A preovulatory surge of luteinizing hormone (LH) at puberty stimulates fully-grown antral follicle leading to oocyte meiotic resumption and ovulation of mature egg for fertilization. The remaining of the antral follicle becomes corpus luteum.

failure in cAMP degradation (Masciarelli et al., 2004). This indicates that regulation of PDE3A activity is a key factor in linking cAMP levels and meiotic maturation in vivo.

Further support for the cAMP hypothesis of meiotic arrest comes from interrupting oocyte cAMP production through genetic ablation of adenylyl cyclase (AC), the enzyme responsible for catalyzing production of cAMP from ATP. Mouse oocytes deficient in AC fail to arrest at GV stage in vivo and undergo spontaneous maturation in the follicle (Horner et al., 2003). The activation of oocyte AC is thought to be mediated by G-protein-coupled receptors (GPCRs) present in the oocyte plasma membrane. The GPCR activates a G-protein, Gs, known to activate AC, leading to cAMP synthesis (Fig. 2A). When the function of Gs is blocked, oocytes cannot synthesize cAMP leading to a spontaneous meiosis resumption within the follicle (Mehlmann et al., 2002; Ledent et al., 2005). Similarly, mutant mouse oocytes lacking an orphan receptor GPCR3 also resume meiosis within the follicles in vivo. Together these experiments tightly couple oocyte cAMP to meiotic resumption and suggest that oocyte cAMP is generated by an orphan GPCR coupled to AC and that this cAMP is elevated in oocytes due to repression of PDE3A.

Somatic Cells Prevent cAMP Degradation in Oocyte

Throughout folliculogenesis, bidirectional communication between oocyte and its surrounding somatic cells is essential for the coordinated development of both oocyte and somatic cells (Fig. 1). A fully grown follicle has two types of granulosa cells: those immediately surrounding the oocyte are called cumulus granulosa cells and those which line the basement membrane are called mural granulosa cells (Fig. 1). The somatic cells in the follicle are extensively connected via gap junctions, which are intercellular membrane channels that allow adjacent cells to share molecules smaller than 1 kDa. Gap junction channels are composed of proteins belonging to the connexin (Cx) family, and Cx43 is the most predominant Cx in the gap junctions between granulosa and cumulus cells while Cx37 is on the oocyte surface, presumably coupling the oocyte to the cumulus cells (Fig. 2). The major question is how this somatic cell–oocyte syncytium functions to maintain meiotic arrest by promoting an elevation of oocyte cAMP.

In a remarkable series of experiments it has been discovered that follicular cells synthesize cyclic guanosine monophosphate (cGMP) which diffuses into the oocyte through Cx37-containing gap-junctions. cGMP is a potent inhibitor of PDE3A thereby

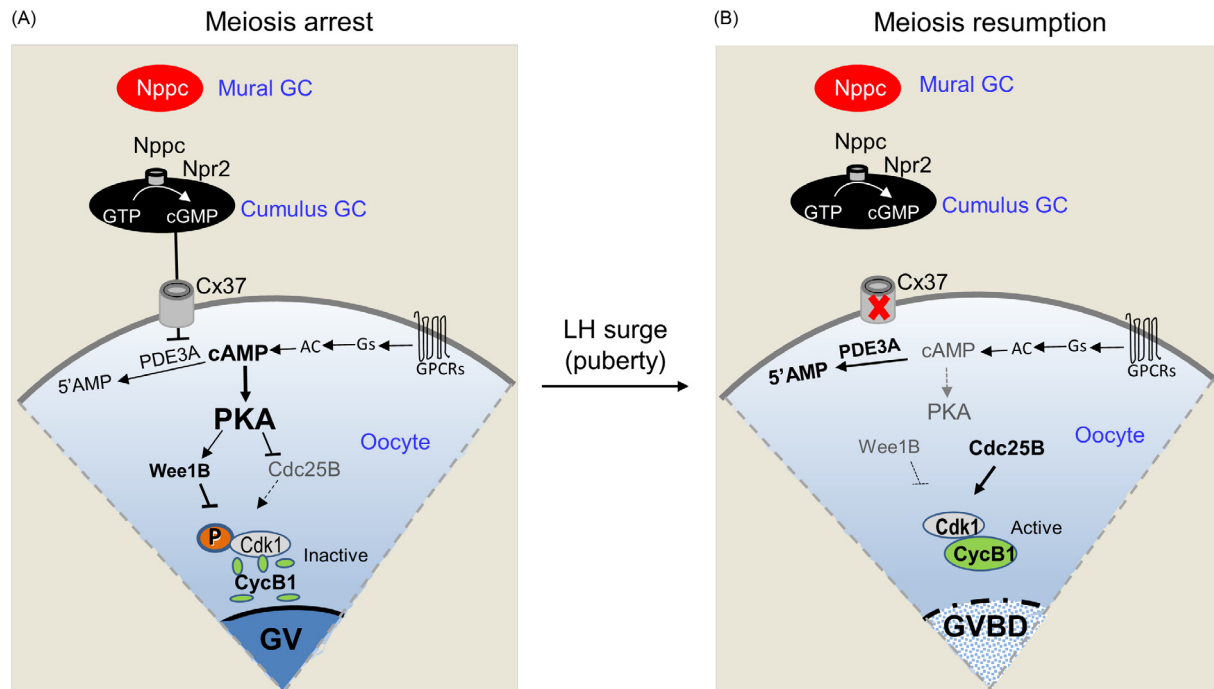


Fig. 2 Model showing the regulation of oocyte meiotic arrest and the resumption of meiosis upon puberty onset. (A) Oocyte meiotic arrest at GV stage requires the synthesis and maintenance of high levels of cAMP, which is produced via the activation of the GPCR/Gs/AC pathway in oocyte. A high cAMP level is maintained in the oocyte by preventing its degradation by inhibiting PDE3A. Mural GCs produce Nppc, which stimulates the generation of cGMP by Npr2 (a guanylyl cyclase) present in cumulus GCs. cGMP enters the oocyte via Cx37 containing gap junctions and prevents PDE3A from hydrolyzing cAMP in the oocyte. cAMP activates PKA that in turn activates the Wee1B kinase and inhibits the Cdc25B phosphatase leading to the inactivation of CDK1. Also shown in the figure is the constantly degraded cyclin B1 (cycB1) that prevents MPF activation in prophase I-arrested oocytes. (B) A preovulatory surge of LH upon puberty onset causes closure of gap junctions throughout the follicle halting the supply of cGMP to the oocyte. This in turn increases cAMP hydrolysis by PDE3A. Low levels of cAMP and PKA can no longer activate Wee1B and inactivate Cdc25B and Cdk1 becomes dephosphorylated and catalytically active. Active Cdk1–cycB1 complex causes the GVBD and meiotic resumption. P—phosphorylation. Modified from Adhikari, D. and Liu, K. (2014). The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. *Molecular and Cellular Endocrinology* **382**, 480–487.

allowing the endogenous oocyte AC activity to drive up cAMP levels (Fig. 2A) (Norris et al., 2009; Vaccari et al., 2009; Conti et al., 2012). These observations further helped to dispel the hypothesis that meiotic arrest is maintained by cAMP diffusing from the somatic compartment and established cGMP as the critical somatic compartment messenger that is essential for maintaining meiotic arrest.

The guanylyl cyclase activity for synthesizing the cGMP from GTP is mediated by natriuretic peptide receptor 2 (Npr2) which is expressed on cumulus cells (Zhang et al., 2010). Furthermore, the stimulation of Npr2 on cumulus cells is mediated by natriuretic peptide precursor type C (Nppc) secreted from mural granulosa cells (Fig. 2A) (Zhang et al., 2010). As such, genetic studies show that oocytes from either *Npr2* or *Nppc*-null mice cannot sustain meiotic arrest in vivo (Zhang et al., 2010).

Taken together, these findings provide a clear understanding of how the oocyte is maintained in meiotic arrest in the follicle but the question remains as to how the LH surge overrides this arrest and initiates the resumption of meiosis in the oocyte.

Puberty and Meiosis Resumption: Overcoming cAMP-Mediated Arrest by LH

Before puberty, the fate of follicles starting to grow is to die by atresia. After puberty, the secretion of pituitary-derived gonadotrophins and the maturation of the hormonal reproductive cycle mark the initiation of ovulatory reproductive cycles. This increase in gonadotropins allows the growth and survival of only a limited number of antral follicles that reach to the preovulatory stage (Fig. 1) (McGee and Hsueh, 2000). In response to a complex interaction of follicle stimulating hormone (FSH), ovarian regulators, and growth factors it is usual that one (in human and monovular species) or several (in polyovular species) follicles grow faster and establish dominance so as to become the preovulatory follicles. In response to the preovulatory LH surge, changes in the follicle compartment lead to the oocyte within the dominant follicle(s) resuming meiosis, and undergoing ovulation as a mature oocyte prepared for fertilization (Fig. 1) (McGee and Hsueh, 2000).

The LH surge is known to promote substantial changes in gene expression in granulosa cells, which leads to a cascade of changes that indirectly promotes oocyte meiotic resumption. A key trigger is that LH causes the breakdown of gap junctions throughout the

follicle thereby halting the supply of cGMP from somatic cells to the oocyte (Fig. 2B). When the cumulus cells stop supplying cGMP to the oocyte, the activity of PDE3A enzyme increases leading to cAMP hydrolysis and the resumption of meiosis (Norris et al., 2009; Adhikari and Liu, 2014; Zhang et al., 2010). The model for LH-mediated oocyte meiosis resumption is presented in Fig. 2B. In support of this model, LH stimulation of rat granulosa cells in vitro can mediate the phosphorylation and decrease in the expression of Cx43 leading to the disruption of the gap-junction communication between the granulosa cells. In order to test whether the closure of gap junctions alone is sufficient for oocyte meiotic resumption, follicle-enclosed oocytes from rat were treated with a chemical known to block the gap junctions. Blocking of gap junctions indeed reduced cAMP concentrations in the oocytes and caused resumption of meiosis in follicle-enclosed oocytes (Sela-Abramovich et al., 2006).

Several LH-induced signaling pathways are responsible for these dramatic changes in the follicle that ultimately lead to meiotic resumption in the oocyte. The most upstream pathway identified is the rapid induction of expression of epidermal growth factor (EGF)-like factors. Such factors include epiregulin, amphiregulin and betacellulin, all of which show a dramatic increase in expression within 3 h of LH stimulation. In vitro, when these EGF-like growth factors are added to the cultures of cumulus–oocyte complex or follicles, they activate EGF receptor (EGFR) on somatic cells and promote oocyte meiotic resumption. These events are mediated via the EGFR activation because they are inhibited by small molecule inhibition of EGFR (Park et al., 2004). In vivo, the physiological roles of EGF-like growth factors in LH-induced oocyte meiosis resumption have been demonstrated in mutant mouse models. LH-induced Cx43 phosphorylation and gap junction closure is impaired in mutant mouse models with inactivating mutations of EGFR (Conti et al., 2012). Thus the evidence indicates that LH-induced EGFR activity stimulates oocyte meiotic resumption by causing the closure of gap junctions and decreasing cGMP production in the follicle.

In addition to EGF-signaling pathways, LH also induces a dramatic increase in activity of the mitogen activated protein kinase (MAPK) pathway. Disruption of MAPK signaling in mouse granulosa cells prevents the LH-induced resumption of oocyte maturation (Fan et al., 2009). In fact, MAPK is a major downstream regulator of the EGF-like growth factors because they can no longer stimulate oocyte meiosis resumption in cumulus–oocyte complex if the cumulus cells are lacking MAPK. The requirement for MAPK appears to be in its ability to interrupt cell-to-cell communication among mural and cumulus cells through phosphorylating connexin 43. Thus, LH-induces a coordinated signaling cascade in the follicle causing decreased cGMP production and a breakdown in gap-junctions that ultimately starves the oocyte of cGMP. The resultant release of PDE3A activity allows a decrease in oocyte cAMP and release of meiotic arrest.

These discoveries of the last decade have unlocked a long-standing question in reproductive biology; that of how the follicle cells and hormonal signals interact in order to coordinate oocyte maturation with ovulation so as to ensure successful fertilization. Concurrently, the question of how cAMP interfaces with the cell cycle machinery that control the biochemical pathways of meiotic arrest and resumption were being uncovered.

Signaling From cAMP to the Cell Cycle Machinery That Drives Meiotic Resumption

During cAMP-mediated meiotic arrest it is essential that the activity of maturation promoting factor (MPF), a complex of cyclin dependent kinase 1 (CDK1) and its regulatory subunit cyclin B1, remains low (Adhikari et al., 2012). Suppression of MPF activity is ensured by inhibitory phosphorylation of CDK1 (Adhikari et al., 2016) and maintenance of a stable level of cyclin B1 through a steady rate of regulated proteolysis (Reis et al., 2006; Marangos et al., 2007). The inhibitory state of CDK1 is maintained by Wee1B/Myt1 kinases and its activation requires dephosphorylation by the phosphatase Cdc25B. The critical nature of this kinase–phosphatase pair is demonstrated in experiments in which they are specifically deleted from growing oocytes. In Wee1b knock out oocytes there is induction of spontaneous resumption of meiosis while Cdc25b knock out oocytes fail to resume meiosis in response to LH.

The effector kinase of cAMP, protein kinase A (PKA) acts as the critical pivot for suppressing CDK1 activity, phosphorylating and activating Wee1B kinase (Han and Conti, 2006) and at the same time providing inhibitory phosphorylation of Cdc25B phosphatase (Pirino et al., 2009). Thus cAMP–PKA activity takes a belt and braces approach to maintain CDK1 inhibition (Fig. 2A) activating the inhibitor and inhibiting the activator.

In response to the LH surge the subsequent decrease in oocyte cAMP drives an increase in Cdc25B activity and a release of Wee1B-mediated inhibition. This allows Cdc25B-mediated removal of inhibitory phosphates on CDK1 and the resultant increase in CDK1 activity drives the resumption of meiosis. Having been released from what may have been many years of arrest at prophase I of meiosis, the oocyte is released from the shackles of cAMP and progresses under translational and posttranslational modifications to proceed through meiosis I to metaphase of meiosis II, where it again arrests, awaiting fertilization to drive the transition from oocyte to embryo.

Conclusion

The generation of eggs or ova involves a specialized type of cell division called meiosis, which occurs in oocyte over a prolonged period of time. Although the oocyte enters meiosis during fetal life, it becomes arrested at the diplotene stage of the first meiotic prophase until puberty. Extracellular stimuli, mainly generated by surrounding granulosa cells control meiosis arrest and resumption of oocyte. In particular, cGMP synthesized in the granulosa cells inhibits the activity of CDK1/Cyclin B1 through maintaining high cAMP levels in oocyte. LH activates several signaling pathways in the granulosa cells that disconnects the oocyte

from its surrounding granulosa cells by closure of gap junctions. Without the supply of cGMP from granulosa cells, the oocyte cannot maintain high levels of cAMP and as a result undergoes meiotic resumption. A number of pathways modified in granulosa cells after LH stimulation have been reported and the list of such molecules is increasing. However, the ovarian follicle, being a complex tissue and technically challenging to study, only yields mechanistic data on how the signaling pathways interact to orchestrate oocyte meiosis after many years of intensive investigation. An improved understanding of how the LH surge drives the resumption of meiosis in oocytes will help us to treat female infertility that results from oocytes that fail to resume meiotic maturation.

References

- Adhikari, D., & Liu, K. (2014). The regulation of maturation promoting factor during prophase I arrest and meiotic entry in mammalian oocytes. *Molecular and Cellular Endocrinology*, 382, 480–487.
- Adhikari, D., Zheng, W., Shen, Y., et al. (2012). Cdk1, but not Cdk2, is the sole Cdk that is essential and sufficient to drive resumption of meiosis in mouse oocytes. *Human Molecular Genetics*, 21, 2476–2484.
- Adhikari, D., Busayavalasa, K., Zhang, J., et al. (2016). Inhibitory phosphorylation of Cdk1 mediates prolonged prophase I arrest in female germ cells and is essential for female reproductive lifespan. *Cell Research*, 26, 1212–1225.
- Conti, M., Hsieh, M., Zamah, A. M., & Oh, J. S. (2012). Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Molecular and Cellular Endocrinology*, 356, 65–73.
- Fan, H. Y., Liu, Z., Shimada, M., et al. (2009). MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science*, 324, 938–941.
- Han, S. J., & Conti, M. (2006). New pathways from PKA to the Cdc2/cyclin B complex in oocytes: Wee1B as a potential PKA substrate. *Cell Cycle*, 5, 227–231.
- Horner, K., Livera, G., Hinckley, M., et al. (2003). Rodent oocytes express an active adenylyl cyclase required for meiotic arrest. *Developmental Biology*, 258, 385–396.
- Ledent, C., Demeestere, I., Blum, D., et al. (2005). Premature ovarian aging in mice deficient for Gpr3. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 8922–8926.
- Marangos, P., Verschuren, E. W., Chen, R., Jackson, P. K., & Carroll, J. (2007). Prophase I arrest and progression to metaphase I in mouse oocytes are controlled by Emi1-dependent regulation of APC(Cdh1). *Journal of Cell Biology*, 176, 65–75.
- Masciarelli, S., Horner, K., Liu, C., et al. (2004). Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. *The Journal of Clinical Investigation*, 114, 196–205.
- McGee, E. A., & Hsueh, A. J. (2000). Initial and cyclic recruitment of ovarian follicles. *Endocrine Reviews*, 21, 200–214.
- Mehlmann, L. M., Jones, T. L., & Jaffe, L. A. (2002). Meiotic arrest in the mouse follicle maintained by a Gs protein in the oocyte. *Science*, 297, 1343–1345.
- Norris, R. P., Ratzan, W. J., Freudzon, M., et al. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development*, 136, 1869–1878.
- Park, J. Y., Su, Y. Q., Ariga, M., et al. (2004). EGF-like growth factors as mediators of LH action in the ovulatory follicle. *Science*, 303, 682–684.
- Pirino, G., Wescott, M. P., & Donovan, P. J. (2009). Protein kinase A regulates resumption of meiosis by phosphorylation of Cdc25B in mammalian oocytes. *Cell Cycle*, 8, 665–670.
- Reis, A., Chang, H. Y., Levasseur, M., & Jones, K. T. (2006). APCcdh1 activity in mouse oocytes prevents entry into the first meiotic division. *Nature Cell Biology*, 8, 539–540.
- Sela-Abramovich, S., Edry, I., Galiani, D., Nevo, N., & Dekel, N. (2006). Disruption of gap junctional communication within the ovarian follicle induces oocyte maturation. *Endocrinology*, 147, 2280–2286.
- Vaccari, S., Weeks, J. L., 2nd, Hsieh, M., Menniti, F. S., & Conti, M. (2009). Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes. *Biology of Reproduction*, 81, 595–604.
- Zhang, M., Su, Y. Q., Sugiura, K., Xia, G., & Eppig, J. J. (2010). Granulosa cell ligand NPPC and its receptor NPR2 maintain meiotic arrest in mouse oocytes. *Science*, 330, 366–369.

Oocyte Maturation During Folliculogenesis

Teresa K Woodruff, Hoi C Lee, Jaewang Lee, and Maxwell Edmonds, Northwestern University, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Fertility in the female is determined by the developmental competence of the oocyte, specifically its ability to undergo growth, meiosis, fertilization, and produce a healthy embryo (Kidder and Vanderhyden, 2010). The mammalian ovarian follicle is composed of a single oocyte, one or more layers of adjacent and surrounding somatic cells (granulosa cells), and a marginal rim of theca cells separated from the granulosa cells by a basal lamina (Fig. 1; Araujo et al., 2014). During folliculogenesis, a number of primordial follicles grow and develop into pre-ovulatory follicles. Preovulatory follicles contain a grown oocyte, capable of maturing into a fertilizable oocyte through both hormonal regulation, stimulation by the gonadotropins follicle stimulating hormone [FSH] and luteinizing hormone [LH], and bi-directional oocyte–somatic cell communication through gap junctions.

Oocyte Maturation From Primordial Through Growing Follicles

The ovary has a number of different stage of follicles which are classified by the number of granulosa cells in the largest cross section of each follicle as proposed by Pedersen and Peters (1968). In mammals, a finite and maximum number of oocytes is present at birth, which decreases throughout the female lifespan. These oocytes are initially arrested in the diplotene stage of prophase I of the meiosis I from fetal life throughout childhood until puberty, when LH surges stimulate the resumption of meiosis. During folliculogenesis, the diameter of the ovarian follicle and oocyte gradually increase across time; as confirmed in the mouse, hamster, pig and human as shown in Fig. 2 (Griffin et al., 2006).

Before primordial follicles initiate their growth and development, they are “activated” by currently unidentified factor(s) from their surrounding environment. These factor(s) might include nutrients, oxygen tension, and other signaling molecules. Activation stimulates for oocyte growth, leading to extremely large amounts of mRNA transcription and protein translation (Liu et al., 2006). At this time, the primordial follicle relies on the bi-directional between oocyte–somatic cell gap junctions, in order to fuel their fast development.

Oocyte Maturation Through Ovulation

The mammalian oocyte is arrested as a diploid cell ($2n$) with $4c$ DNA during prophase I of the first meiotic division, which occurs during the oocyte growth phase. Ovulation is triggered by the LH surge, which in turn is triggered by increasing levels of estradiol secreted by the pre-ovulatory follicle. Before LH surge stimulation, cyclic guanosine 5'-monophosphate (cGMP) travels through gap junctions from the surrounding somatic cells into the oocyte. cGMP is produced by the transmembrane guanylyl cyclase natriuretic peptide receptor (NPR2). The NPR2 is present in all granulosa cells, although is not present in the oocyte. cGMP inhibits the degradation of another cyclic nucleotide called cAMP in the oocyte. This high level of cGMP in the oocyte inhibits the hydrolysis of cyclic adenosine 3', 5'-monophosphate (cAMP) by the phosphodiesterase 3A (PDE3A), and maintains a high level of cAMP, which blocks meiotic progression. The PDE3A, an antagonized enzyme by cGMP, primarily affects cAMP. After the LH surge just before ovulation, the gap junctions between the oocyte and the cumulus cells (immediately adjacent somatic cells) are closed, and cGMP begins to decrease in the oocyte, relieving the inhibition of PDE3A. This causes a decrease in oocyte cAMP, re-enabling the meiosis process (Norris et al., 2009). Metaphase I then initiates, as an orchestrating of the nuclear membrane breaking down (germinal vesicle breaks down) and chromosomes condensing into bivalents and aligning on the meiotic spindle. The homologous chromosomes segregate and one set are extruded in the first polar body, while the other remains in the oocyte. Meiosis is then arrested again until the oocyte has been fertilized (Shuhaibar et al., 2015).

The LH surge leads not only to resumption of meiosis and nuclear maturation, but also to cytoplasmic maturation and the maturation of the zona pellucida, a protective outer layer of the oocyte. This cytoplasmic maturation includes the migration of cortical granules to the outer cortex of the oocyte, a reorganization of the endoplasmic reticulum, an increase in the number of 1,4,5-inositol triphosphate receptors, and an increase in the concentration of calcium ions in the endoplasmic reticulum. LH also stimulates the transformation of the follicular mural cells from estrogen producing cells, into progesterone producing cells. LH also stimulates local prostaglandin production and the enzymatic digestion of the follicular wall, leading to its rupture (ovulation), releasing the cumulus–oocyte complex from the follicular wall. When released from the follicle, the cumulus–oocyte complex stays near the ovarian surface and is picked up by the fimbriae of the fallopian tube (Strömstedt and Byskov, 1999).

Meiosis

Meiosis is the unique cell division that reduces the number of chromosomes in germ cells. It consists of two separate divisions, which end in the production of haploid gametes. The major difference between mitosis and meiosis is the segregation of chromatids.

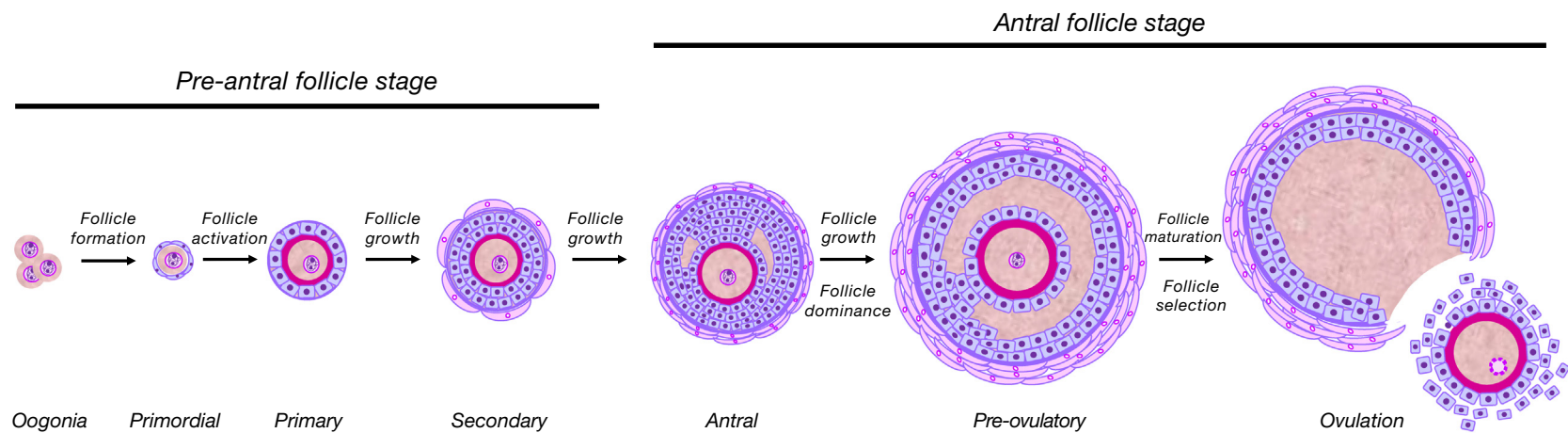


Fig. 1 Schematic sequence of a complete follicular development. Oogonia develop from a primordial germ cell and differentiate into an oocyte in the ovary. All the pre-antral follicles have a primary oocyte which is diploid/46 (2N), containing 4 chromatids arrested in diplotene of prophase I. The pre-ovulatory stage is the last stage of follicle development; these follicles have increased antral fluid and may contain a secondary oocyte.

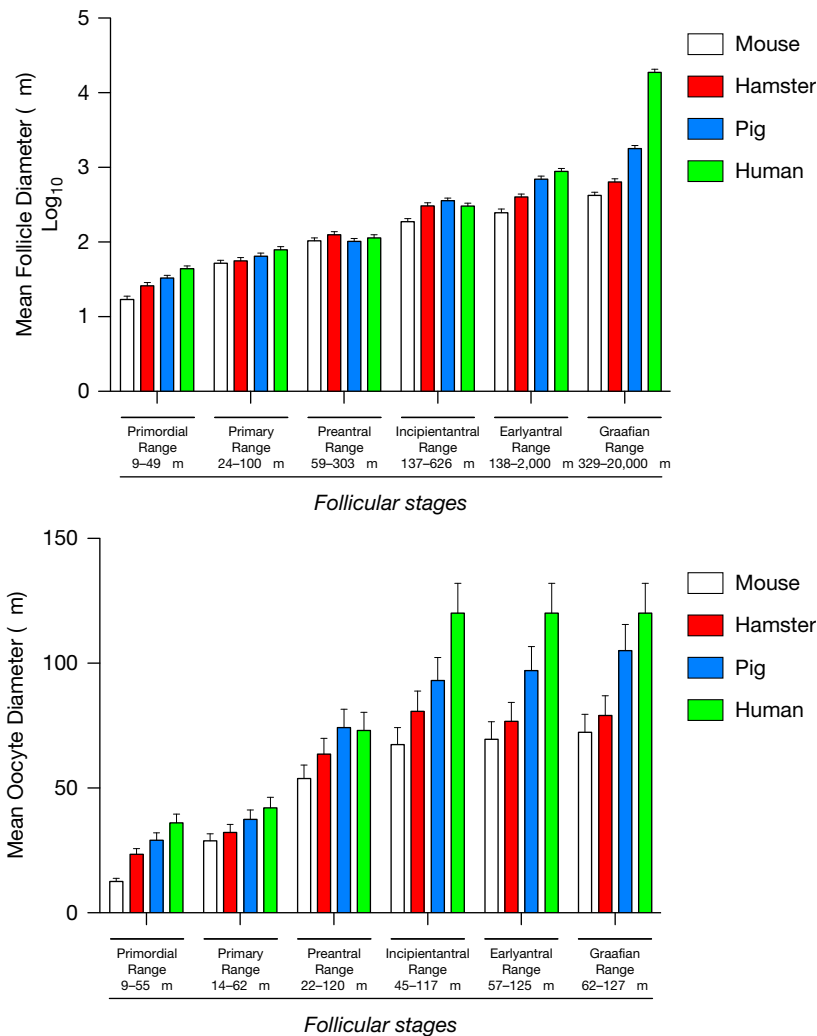


Fig. 2 Follicle and oocyte growth during folliculogenesis in different mammal. Semi-log bar graph of follicle diameter versus follicular stage. * indicates values from previously published data for reference, and therefore do not include error bars. Values are the mean $\pm$ standard error.

Pre-meiotic DNA synthesis produces 4C DNA, with a chromosome number of $2n$, as in normal diploid cells. The first meiotic division, meiosis I, begins with prophase I. In the first meiotic prophase a unique exchange between the maternal and paternal genes takes place. This occurs as homologous chromosomes are separated into daughter cells because the centromere does not divide as in mitotic divisions. The two daughter cells thus contain $1n$ chromosomes and $2c$ DNA and are prepared with different genetic constitutions. The first meiotic prophase consists of four successive stages defined as leptotene, zygotene, pachytene, and diplotene stages. During the leptotene, zygotene, and pachytene stages the homologous chromosomes line up and condense gradually. In the pachytene stage, a synaptonemal complex is produced, allowing for normal segregation of the chromosomes in the first meiotic division, which takes place at the time of ovulation. In the diplotene stage, the chromosomes decondense. Interestingly, the nuclear membrane is present throughout the first meiotic prophase. When the oocyte reaches the end of meiotic I prophase, the meiotic process stops, and the oocyte remains in the diplotene stage until it is either excluded by atresia, undergoes apoptosis, or succeeds in reaching the maturation and resumes meiosis during ovulation. The second meiotic division, meiosis II, is a mitotic division of each of the haploid cells and resembles a normal meiotic division without DNA synthesis; the sister chromatids separate and segregate. As a result, haploid oocytes have $1n$ chromosomes and $1c$ DNA, and all four daughter cells are haploid and genetically different. The second meiotic divisions in oocytes are asymmetric, resulting in the formation of one large oocyte and small polar bodies. Therefore, only one egg cell results from the two meiotic divisions in the female germ line (Strömstedt and Byskov, 1999).

Cumulus Cell Expansion

During the pre-ovulatory period, the cumulus–oocyte complex (COC) expands, preparing for meiotic maturation and enabling developmental capability. Cumulus cell expansion is dependent on extracellular matrix synthesis by cumulus cells. Hyaluronic

acid is the most plentiful component of this extracellular matrix, and synthesized from glucose through the hexosamine biosynthesis pathway. Cumulus expansion occurs during meiotic oocyte maturation. During oocyte maturation, cumulus expansion is thought to influence a variety of essential processes supporting the oocyte's development. After the LH surge, granulosa cells produce EDF-like factors that act on cumulus cells to induce cumulus expansion and oocyte maturation. EGF receptor expressed on cumulus cells up-regulates the ERK1/2-dependent pathway. The signaling pathway plays important roles in oocyte meiotic maturation and cumulus cell expansion because cumulus cells regulate oocyte growth and oocyte maturation (Shimada, 2009).

Acknowledgments

This work was supported by the Center for Reproductive Health After Disease (P50 HD076188) from the National Center for Translational Research in Reproduction and Infertility (NCTRI).

References

- Araujo, V. R., Gastal, M. O., Figueiredo, J. R., et al. (2014). In vitro culture of bovine preantral follicle: A review. *Reproductive Biology and Endocrinology*, 12, 78.
- Griffin, J., Emery, B. R., Huang, I., et al. (2006). Comparative analysis of follicle morphology and oocyte diameter in four mammalian species (mouse, hamster, pig, and human). *Journal of Experimental & Clinical Assisted Reproduction*, 3, 2. <https://doi.org/10.1186/1743-1050-3-2>.
- Kidder, G. M., & Vanderhyden, B. C. (2010). Bidirectional communication between oocytes and follicle cells: Ensuring oocyte developmental competence. *Canadian Journal of Physiology and Pharmacology*, 88, 399–413.
- Liu, K., Rajareddy, S., Liu, L., et al. (2006). Control of mammalian oocyte growth and early follicular development by the oocyte PI3K kinase pathway: New roles for an old timer. *Developmental Biology*, 299, 1–11.
- Norris, R. P., Ratzan, W. J., Freudzon, M., et al. (2009). Cyclic GMP from the surrounding somatic cells regulates cyclic AMP and meiosis in the mouse oocyte. *Development*, 136, 1869–1878.
- Pedersen, T., & Peters, H. (1968). Proposal for a classification of oocytes and follicles in the mouse ovary. *Journal of Reproduction and Fertility*, 17, 555–557.
- Shimada, M. (2009). Cumulus oocyte complex: Cumulus cells regulate oocyte growth and maturation. *Journal of Mammalian Ova Research*, 26(4), 189–194.
- Shuhaibar, L. C., Edbert, J. R., Noris, R. P., et al. (2015). Intercellular signaling via cyclic GMP diffusion through gap junctions restarts meiosis in mouse ovarian follicles. *PNAS*, 112, 5527–5532.
- Strömstedt, M., & Byskov, A. G. (1999). Oocyte, mammalian. In *Encyclopedia of reproduction* (1st edn, 3 pp. 468–480). London, UK: Academic Press.

Structural Aspects of Oocyte Maturation

Lynda K McGinnis, University of Southern California, Los Angeles, CA, United States

Patricia Rodrigues, Lusofona University, Lisbon, Portugal

Darlene Limback, University of Kansas Medical Center, Kansas City, KS, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Actin-related protein 2/3 (Arp2/3) Arp2/3 is a conserved protein complex of actin-related proteins ARP2 and ARP3, which enucleates branching actin filaments.

Asymmetric cell division Asymmetric cell division is a characteristic of female meiotic maturation in which the smallest daughter cell, the polar body, is designed to degenerate, safeguarding all the organelles and molecules within the larger cell—the oocyte.

Cytoplasmic streaming Cytoplasmic streaming is the movement of actin, organelles and the fluidic cytoplasm in a coordinated pattern. In large cells, such as the oocyte it can be the most important means of reorganization and intracellular transportation.

Germinal vesicle breakdown (GVBD) The germinal vesicle (GV) is the name used for the nuclear envelope in an oocyte. At the initiation of meiotic maturation, this nuclear envelope is disassembled, a process similar to the somatic cell nucleus. GVBD is essential, prior to spindle formation and completion of meiosis.

Microtubule organizing centers (MTOCs) MTOCs are the sites of microtubule nucleation and anchoring. In somatic cells, these would be the centrosomes. However, in mammalian oocytes, meiotic spindles lack centrioles and therefore they do not have true centrosomes. The MTOCs are maternally inherited and used by the oocyte until after fertilization, when embryos assemble centrioles either denovo or contributed from the sperm.

Transzonal projections (TZPs) TZPs are long extensions from the cumulus cells that stretch through the zona pellucida and contact the oolema. These projections contain an actin and/or tubulin cytoskeleton and gap junctional pores that allow for direct intracellular communication between the oocyte and its companion cumulus cells. These connections are essential. The cumulus cells provide molecules to both nourish the oocyte and to maintain meiotic arrest. In turn, the oocyte sends signals to the cumulus cells indicating that the oocyte is healthy and maintains the cumulus in a quiet state. The surge in luteinizing hormone (LH) during the estrous cycles causes the gap junctions to close, preventing exchange of molecules from the cumulus and oocyte, thus triggering oocyte maturation.

Zona pellucida (zona) The zona is a thick extracellular matrix that surrounds the oocyte and physically separates the oocyte from its companion cumulus cells. The TZPs extend from the cumulus cells through this matrix to maintain contact and to communicate with the oocyte.

Introduction

The oocyte's size and function make it a unique cell within the animal kingdom. Even though oocytes and spermatozoa (sperm) contribute an equal number of chromosomes to the zygote, the oocyte alone contributes the cytoplasmic stockpile of molecules and organelles needed throughout the long developmental and maturation process of oogenesis (Li and Albertini, 2013; Coticchio et al., 2015). The last step in oocyte maturation is in fact very fast and occurs immediately before ovulation, triggered by the maternal surge in LH (Li and Albertini, 2013; Coticchio et al., 2015; Plancha, 1996).

The oocyte begins its journey as a primordial germ cell migrating from the allantois to the genital ridge, where it differentiates into an oocyte (McLaren, 2003). Oocytes enter meiosis and arrest in diplotene of prophase-I (PI) of meiosis-I (MI), while still interconnected by inter-cytoplasmic bridges and surrounded by somatic cells forming the oocyte clusters of the ovigerous cords. Follicle formation, the acquisition of flattened granulosa cells, occurs after birth (rodents) or during fetal life (human) coinciding with cluster breakdown and major germ cell loss. The primordial follicles stay in a quiescent state throughout reproductive life until the oocyte undergoes nuclear and cytoplasmic maturation to accomplish fertilization (Peters and McNatty, 1980). Growth from primordial to preovulatory follicle is regulated by endocrine and paracrine, as well as intra- and inter-cellular communication. Bidirectional communication between oocyte and the cumulus cells surrounding it is fundamental (Li and Albertini, 2013; Rodrigues et al., 2008) and requires maintenance of intact TZPs extending from the somatic cumulus cells to the oocyte cell surface (oolema) (Albertini et al., 2001) (Fig. 1B, white arrow). The TZPs provide the means for dialogue between somatic and germ cells, maintaining meiotic arrest, while enabling development of the oocyte cytoplasm and its stockpile of molecules and organelles; maternal contributions that will be required for processes of oocyte maturation, fertilization and embryo development.

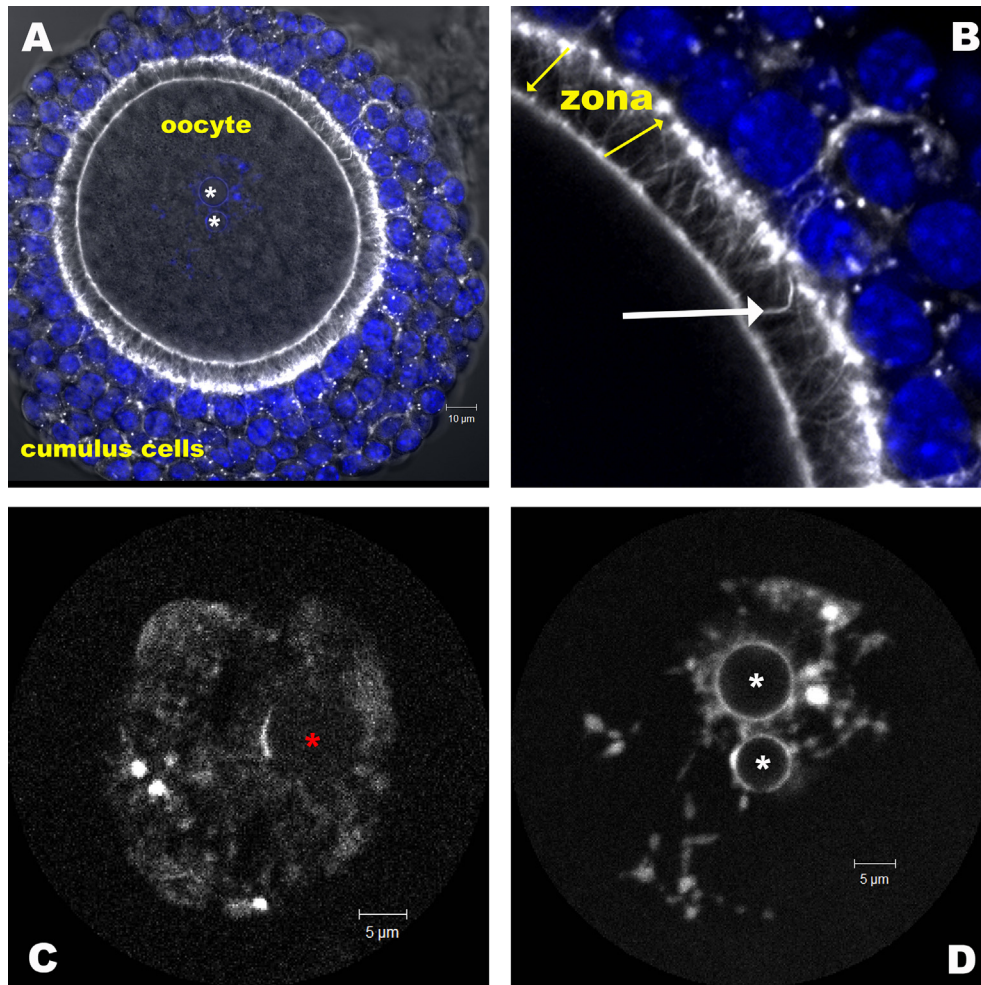


Fig. 1 Transzonal processes (TZPs) and GV maturation. (A and B) An intact cumulus-enclosed GV stage oocyte that has been release from a large antral follicle and stained with fluorescently labeled molecules that identify DNA (blue) and F-actin (white). At this stage prior to the LH-surge, the cumulus cells tightly enclose the oocyte, but are physically separated from it by the zona (B, yellow arrows). To maintain critical communication with the oocyte, the cumulus cells project through the zona to contact the oocyte cell surface. These processes can be visualized by staining for actin, which forms the cytoskeleton of the TZPs (A and B, white). Nuclei are seen as blue in (A and B); in (B), the blue are the nuclei of the cumulus cells. (C and D) Demonstrate two phases of GV maturation, the NSN configuration (C), and the SN configuration (D). The GV can have a single or multiple nucleoli (C, red asterisk; D, white asterisks). The GV in (D) is an enlargement of the nucleus from the oocyte in (A; note white asterisks that identify the nucleoli in both images). Images were taken on a Zeiss LSM Pascal scanning confocal microscope. Each image is 10 μm thick. White bars show scale: (A) 10 μm , (C and D) 5 μm). Actin labeled with phalloidin. DNA labeled with Hoechst 33258.

Oocyte maturation is the stage of meiotic progression from PI to metaphase-II (MII) and first polar body extrusion. This event involves carefully orchestrated and rapid changes in cytoskeletal organization, organelle redistribution, and membrane polarization. In this chapter, we will focus on the structural aspects of oocyte maturation.

Nuclear Maturation

While arrested in PI the oocyte nucleus is called the germinal vesical (GV). It is large and centrally located within the oocyte cytoplasm. It contains a prominent nucleolus with condensed heterochromatin or diffuse euchromatin (Albertini, 2015). The nuclear envelope of the GV has pores, allowing and mediating RNA trafficking during the oocyte growth phase. In human oocytes, chromatin remodeling is linked to meiosis resumption (Combelles et al., 2003; Escrich et al., 2010). Specifically, chromatin is transformed from diffuse (non-surrounded nucleolus, NSN; Fig. 1C, asterisk) to a perinucleolar and heterochromatin state (surrounded nucleolus, SN) (Miyara et al., 2003) (Fig. 1D, asterisk). This transition occurs in the fully-grown oocyte during antral follicle formation and is considered an early sign that the oocyte is ready to begin meiotic maturation. Oocytes that reach an SN configuration have higher developmental potential than NSN oocytes (Luciano et al., 2012). Communication with cumulus cells

is essential for the transition from NSN to SN chromatin configuration, evidenced by the arrest of the oocyte in GV stage if cultured without granulosa/cumulus cells (De La Fuente and Eppig, 2001). This cumulus cell communication likely occurs via gap junctions between the TZPs and oolema, as indicated by the failure of chromatin remodeling in the oocytes of female mice with connexin 37 gene deletion, which is an oocyte specific gap junction protein (Carabatsos et al., 2000).

According to its chromatin configuration, the GV has also been further classified into four stages: GV0 to GV3. Global DNA methylation is associated with the increasing condensation of the DNA and is also implicated in the acquisition of meiotic and developmental competence (Luciano et al., 2012). GV0 chromatin is diffuse and filamentous throughout the nucleus. GV1 is an early stage of chromatin remodeling, where the chromatin appears as a few foci of condensation, which progress to distinct clumps of chromatin condensation in GV2, an intermediate stage. The highest level of chromatin condensation is achieved at GV3, with all chromosomes clustered together, immediately prior to spindle formation. Noteworthy, GV0 oocytes have lower capacity to resume and complete meiosis, while GV1-3 oocytes, regardless of configuration stage, will all mature to MII stage in vitro. However, developmental competence of these oocytes is affected by GV stage: GV2 and GV3 oocytes can develop to blastocyst stage after in vitro fertilization, whereas GV1 have much less developmental potential under the same conditions (Luciano et al., 2012). This progressive chromatin condensation within the intact GV is the earliest stage of oocyte meiotic maturation and is associated with oocyte developmental competence.

The LH surge triggers oocytes to exit from PI arrest, initiate germinal vesical breakdown (GVBD), and begin MI spindle assembly (Fig. 2). Depending on species and external conditions (in vivo versus in vitro), the MI spindle forms in the center of the oocyte. However, the spindle needs to be eccentrically located, just under the oocyte cytoplasmic membrane to ensure that cytokinesis occurs with minimal loss of cytoplasm. One set of chromosomes will be expelled from the oocyte into a small polar body—extremely asymmetrical division (Dalton and Carroll, 2013; Yi et al., 2013).

Formin-2 (Fmn2) is an actin-nucleating protein expressed in oocytes and is required for progression through MI. This has been demonstrated in mice lacking Fmn2 in which, despite normal karyokinesis their oocytes fail to extrude a polar body (Leader et al., 2002). Recently it was suggested that Fmn2 together with Arp2/3 regulates MI spindle relocation (Yi et al., 2013). In brief, the endoplasmic reticulum (ER) vesicles recruit Fmn2 to the periphery of the spindle. This polarized distribution of Fmn2 causes the spindle to drift (Peters and McNatty, 1980; Leader et al., 2002). This drifting leads to Arp2/3 activation in the proximal cortex that triggers cytoplasmic streaming, which pushes cytoplasmic contents away from the cortex while also pushing the spindle towards the cortex (Peters and McNatty, 1980; Leader et al., 2002) (Fig. 2, red arrows). When the metaphase chromosomes move near to the oolema, F-actin and Arp2/3 are activated and a dense layer of actin forms in the cortex directly above the spindle (Longo and Chen, 1985; Yi et al., 2011). In fact, it has been shown that cortical actin polarization adjacent to the spindle is required for maturation (Coticchio et al., 2014). Moreover, disruption of actin in mouse oocytes leads to both larger GV's and enlarged MI spindles (Barrett and Albertini, 2010) underscoring the importance of actin in maintaining proper oocyte structure. An enlarged MI spindle causes a larger polar body (Plancha et al., 2005), leading to reduced cytoplasmic mass in the MII oocyte, which may compromise the early embryo development (Coticchio et al., 2015).

The actin-driven asymmetric position of the meiotic spindle is not the only difference between the spindle of the oocyte and the somatic cell spindle. The mammalian meiotic spindle lacks astral microtubules. Since the oocyte has no centriole-containing centrosome, it has microtubule organizing centers (MTOCs) instead (Li and Albertini, 2013; Maro et al., 1986; Messinger and Albertini, 1991) (Fig. 2, green asterisk). These MTOCs contain γ -tubulin and pericentrin similar to somatic cell centrosomes, and can be seen as foci within MTOCs and at the spindle poles (Li and Albertini, 2013). In the mouse, γ -tubulin has been implicated in oocyte spindle and polar body position and size (Barrett and Albertini, 2007). Reserves of γ -tubulin and its allocation to specific subcellular sites is vital to early embryonic progression, as shown by embryo developmental arrest when maternal γ -tubulin is absent (Yuba-Kubo et al., 2005).

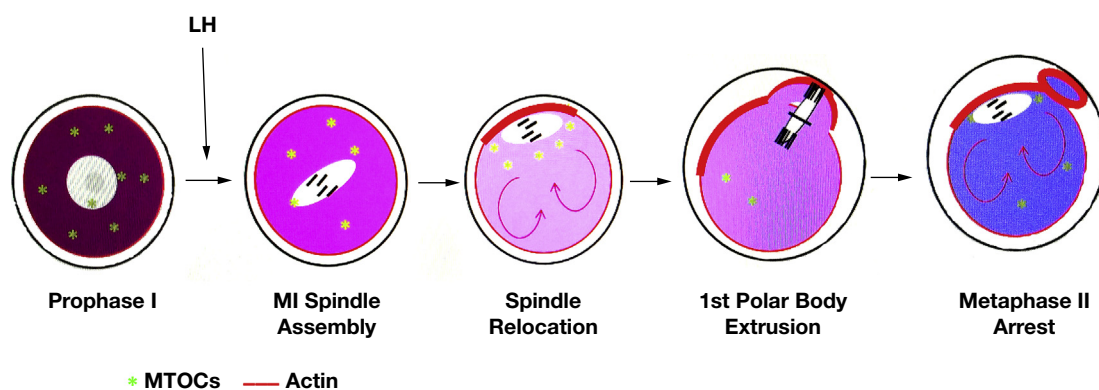


Fig. 2 Oocyte nuclear maturation.

Cytoplasmic Maturation

Cytoplasm, also known as ooplasm, is composed of basic cellular organelles including: endoplasmic reticulum (ER), Golgi apparatus (GA), lysosomes, ribosomes, mitochondria, and annulated lamellae, which are similar to those found in all somatic cells. In addition, cortical granules (CGs) and Balbiani's body are organelles unique to oocytes (Albertini, 2015). Here we will focus on the organelles that are critical for oocyte maturation (Fig. 3).

Mitochondria contribute directly to oocyte metabolic demand providing most of the energy required for oocyte maturation, fertilization and early embryo development. Structurally, the maternal inheritance of mitochondria begins during primordial follicle formation when the mitochondrial founder population is established in the ooplasm of these cells (Combelles et al., 2003). Typically, mitochondria are situated perinuclear and associated with the Balbiani's body in the arrested oocyte (Li and Albertini, 2013). As the oocyte grows, the mitochondrial population amplifies steadily. The mitochondria change in abundance, location and function during maturation, and provide the energy needed for the reorganization of the cytoskeleton and other organelles (Albertini et al., 2001). Mitochondrial-DNA (mtDNA) replication occurs throughout oogenesis, but it stops completely in the fully-grown oocyte; only later, in the developing embryo, is it resumed. The mitochondria are an interesting example of selective migration during maturation which prevents their loss into the extruding polar body. At MI, mitochondria congregate near the MI spindle and migrate with the spindle to the oocyte cortex. But with the initiation of anaphase, the mitochondria segregate towards the spindle pole furthest from the cleaving polar body and thus are retained within the larger oocyte during cytokinesis (Combelles et al., 2003). Mitochondria are also sites of calcium storage, although the primary organelle responsible for cytoplasmic calcium regulation is the endoplasmic reticulum (ER).

The ER stores and releases free Ca^{2+} in the cytoplasm and is required for intracellular calcium oscillations that trigger both meiotic maturation to MII and the completion of meiosis at fertilization (Ducibella and Fissore, 2008; Cheon et al., 2013). ER distribution differs between the GV stage and MII oocytes and is visibly polarized. The GV ooplasm contains ER clusters that are not well defined and are scattered throughout the ooplasm with larger clusters in the interior near to the GV. However, by the MII stage, the ooplasm has developed a reticular network of ER extending over the entire ooplasm with large aggregates at the cortex (reviewed in Coticchio et al., 2015). The ER, by recruiting Fmn2 to the spindle periphery, is also involved in the cytoplasmic streaming of actin and organelles, as seen above (Yuba-Kubo et al., 2005).

The functions of the Golgi apparatus during oocyte maturation is still not well understood, although there is evidence that it is not just a spectator in this process. In somatic cells, the Golgi's primary function is to post-translationally modify, sort, pack, and release proteins and macromolecules for inclusion into other intercellular compartments, insertion into the cell membrane, or for exportation to the extracellular environment. When an inhibitor of membrane trafficking is used during in vitro oocyte maturation (brefeldin A), meiosis is arrested (Moreno et al., 2002). So, even though it is not yet known exactly how, the Golgi's involvement in membrane trafficking appears to be critical for meiotic resumption (Coticchio et al., 2015). Interestingly, a cis-Golgi matrix protein, GM130 is also involved in recruitment of microtubule enucleators such as γ -tubulin (Rivero et al., 2009). Based on this evidence and the fact that some species of oocytes do not have MTOCs, it has been suggested that the Golgi is also involved in spindle formation.

Cortical granules (GCs) are small secretory organelles that function to prevent polyspermy (entry of more than one sperm into the oocyte). They are not found in somatic cells but are present in all mammalian oocytes (Wessel and Wong, 2009). At the GV stage, the CGs are widely distributed throughout the ooplasm, but with the transition to MII stage, CGs aggregate just beneath the oolema (Coticchio et al., 2015). These organelles are vesicles containing cross-linking enzymes, proteases, glycosidases, and structural proteins. They are activated when the first sperm enters the oocyte triggering intracellular Ca^{2+} oscillations that then cause CGs to fuse with the oolema, and to extrude their contents. This exocytosis of CGs, alters the oolema, preventing entry of additional sperm. This cascade is known as the "cortical reaction" and the "block to polyspermy" and is the final act of cortical maturation.

Cortical Maturation

The oocyte cortex is a relatively undefined region that includes the oolema and the cytoplasm directly beneath. "The cortex functions as a boundary zone that gives the cell its shape, a region of selective exchanges and transductions, an active partner for the cytosolic

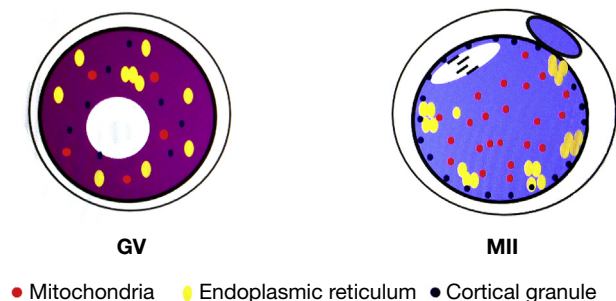


Fig. 3 Oocyte cytoplasmic maturation.

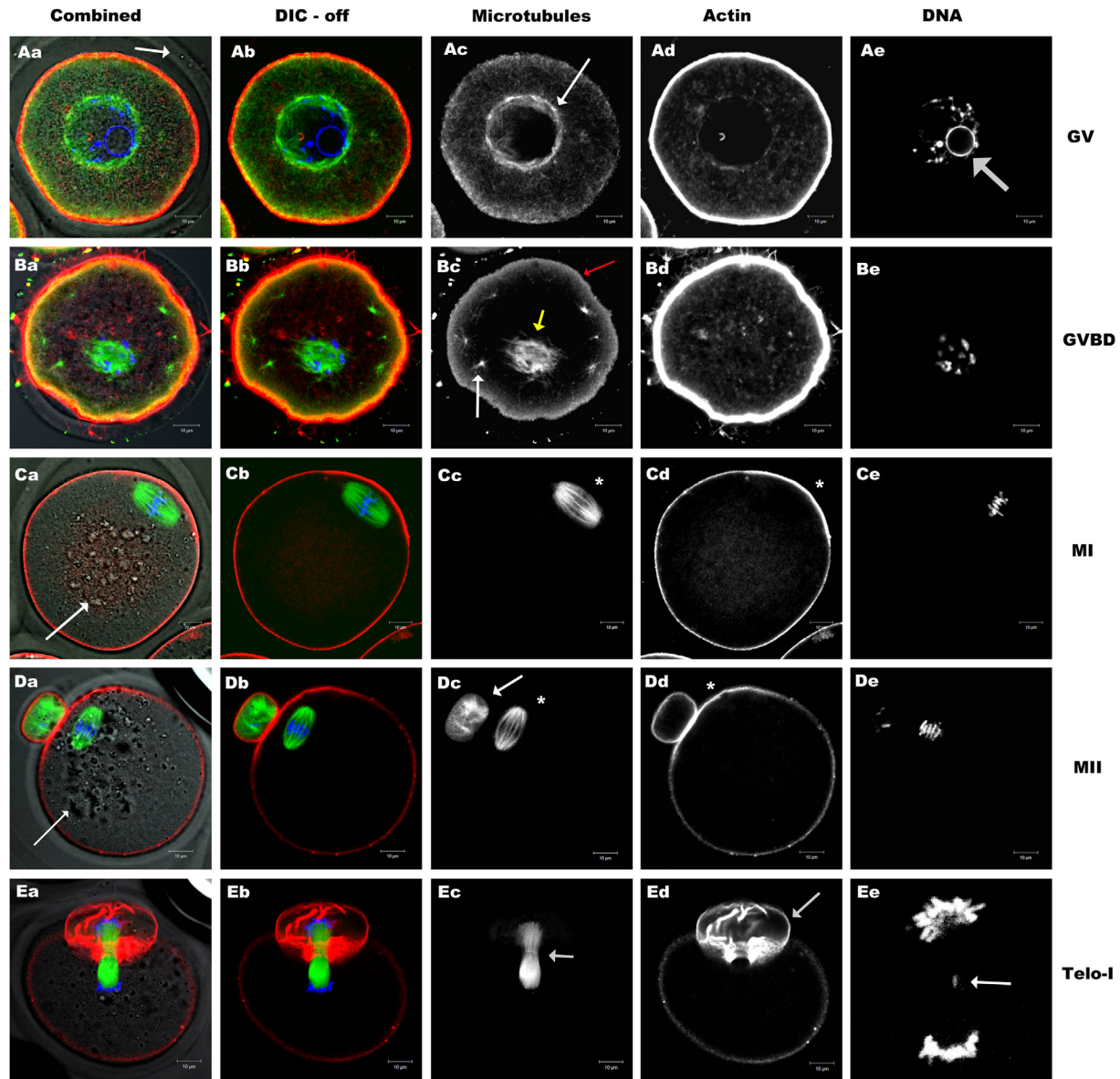


Fig. 4 Structural meiotic maturation. This set of images show the power of immunofluorescence and confocal microscopy. The confocal laser scanning microscope enables visualization of the inner workings of a cell. Here, we have used a mixture of antibodies and dyes labeled with fluorescent molecules to identify multiple structures within each oocyte at different stages of maturation. Female mice were treated with hormones to induce the growth of multiple antral follicles, a procedure similar to those used in clinical assisted reproduction for women. After 2 days of antral follicle growth, oocyte maturation is induced by treating the females with an LH-like hormone. The oocytes are then collected from the ovaries at specific times post-LH, times that are associated with the various stages of oocyte maturation: row A = 0 h GV; row B = 2 h GVBD; row C = 8 h MI; row D = 16 h MII; row E = 16 h meiotic check-point arrested at telophase-I. Column “Combined” is all of the markers over-layered and shown together, including the non-fluorescent view from differential interference contrast (DIC). Column “DIC-off” is the same as “Combined” but only showing the fluorescently labeled structures. The computer software is used to add the colors that we see as *blue* (DNA), *red* (actin), and *green* (tubulin and/or Src-family kinases, SFK). Columns “Microtubules”, “Actin”, and “DNA” are showing the images from single wavelengths, thus the individually labeled structures. We have chosen to show microtubules by identifying two different families of proteins. A and B are labeled with an antibody that identifies activated Src-family kinases. The SFKs are found closely associated with microtubules, so they can also be used to identify the MTOCS (Ac and Bc, *white arrows*), and also shows the polarized location of activated SFKs in the cytoplasm near to the cortex (gradient layer surrounding the oocyte in Bc, *red arrow*). Rows C, D, and E are oocytes labeled with a mixture of two antibodies to identify alpha and beta tubulin, thus only the meiotic spindles are visible (Bc *yellow arrow*, Cc, Dc, Ec). With the addition of DIC, it’s possible to see the dense aggregates of intracellular organelles (Ca, Da *arrows*). Cd and Dd *asterisks* = actin cap over the MI (Cd) and MII (Dd) spindles. Row E—an oocyte that failed to complete MI and was arrested at telophase-I. The confocal microscope demonstrates the cause of this meiotic failure; the chromosomes in Ed have been enlarged to show that the oocyte suffered a lost chromosomes (Ed *arrow*), trapped within the midbody of the telophase spindle, a condition that triggered a meiotic “check point arrest” (Gorbsky, 2015). If this oocyte had completed meiotic maturation with abnormal chromosome segregation, the resulting MII oocytes would have had either an extra or a missing chromosome, a serious and potentially fatal meiotic error called aneuploidy. Images were taken on a Zeiss LSM Pascal scanning confocal microscope. Each image is 10 μm thick. *White bars* show scale: A = 10 μm, C and D = 5 μm). Actin labeled with phalloidin; DNA labeled with Hoechst 33258; activated SFKs labeled with “clone28” antibody; tubulin labeled with anti-tubulin antibody.

cytoskeleton and organelles, and a preferred site for the localization of RNA and protein determinants. It has the properties of a viscoelastic gel and functions as a unit during many cell behaviors" (Sardet et al., 2002). The cortex includes the oolema and the nearby subcortical region including cytoskeleton and cytoplasmic components that are polarized to this region adjacent to the oolema. During oocyte maturation, the cortex undergoes changes that are critical for the completion of meiosis and to enable fertilization. This process includes the movement of organelles and cytoskeleton as mentioned earlier plus changes in the oolema and regionally localized proteins.

Using confocal microscopy, McGinnis and Albertini demonstrated a polarized activation of kinases, including Src- and MAPK-family proteins associated with cell cycle and fertilization (McGinnis and Albertini, 2010). As the oocyte matures, there is a dramatic increase in tyrosine phosphoproteins and Src-family kinases associate with the oolema, while serine/threonine and MAPK-family kinases are activated in the subcortical layer (McGinnis and Albertini, 2010; McGinnis et al., 2011). This reorganization and activation of proteins to the cortex is, like other movements during maturation, preparing the oocyte for sperm entry and a rapid response to fertilization.

Movement of the metaphase spindle to the cortex induces the formation of a thick cortical actin layer immediately overlaying the spindle, often called the "actin cap" (Fig. 4Cd and 4Dd, *asterisk*). This dramatic polarization is accompanied by changes in cortical tension and reorganization of proteins associated with actin dynamics, such as myosin-II and the ezrin/radixin/moesin proteins (Larson et al., 2010). The formation of this dense actin layer is associated with changes in the oolema creating a "microvilli-free zone" directly over the MII spindle. Microvilli are small protrusions of the cell membrane that cover most of the oocyte surface. Sperm normally only enter the oocyte in regions where they contact microvilli, therefore the microvilli-free zone reduces the chance for sperm to enter the egg too close to the MII spindle where sperm are susceptible to extrusion with the polar body (Luo et al., 2009). Cortical maturation is another critical step in oocyte maturation, setting the stage for another meiotic arrest while the oocyte waits for fertilization.

Oocyte maturation is a complex event, carefully orchestrated to match structural elements and molecular signaling cascades. Once maturation is complete, oocyte meiosis arrests in metaphase-II and awaits sperm entry and fertilization. This must occur quickly because mammalian oocytes only maintain optimal fertilizing capacity for a limited time. In the mouse, this window of opportunity is the period between 14 and 18 h after the LH-surge. In humans, the optimum fertilization window is presumed to range from 24 to 36 h post-LH, but the specifics are not well known due to the limitations of this type of research on human oocytes. It is known however, that mammalian oocytes can suffer from postovulatory aging which includes a slow migration of the spindle away from the cortex and an increased susceptibility to abnormal egg activation, apoptosis, and aneuploidy (McGinnis et al., 2014).

Summary

Oocyte structural maturation is a complex event that must occur rapidly and in synchrony involving dramatic movements of intracellular components and polarizing shifts, all with a purpose of preparing the oocyte for fertilization and to support the development of a healthy embryo.

References

- Albertini, D. F. (2015). The mammalian oocyte. In T. Z. A. Plant (Ed.) (4th ed.), vol. 1. *Knobil and Neil's physiology of reproduction* (pp. 59–97). Elsevier.
- Albertini, D. F., Combelles, C. M., Benecchi, E., & Carabatsos, M. J. (2001). Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*, 121, 647–653.
- Barrett, S. L., & Albertini, D. F. (2007). Allocation of gamma-tubulin between oocyte cortex and meiotic spindle influences asymmetric cytokinesis in the mouse oocyte. *Biology of Reproduction*, 76, 949–957.
- Barrett, S. L., & Albertini, D. F. (2010). Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence. *Journal of Assisted Reproduction and Genetics*, 27, 29–39.
- Carabatsos, M. J., Combelles, C. M., Messinger, S. M., & Albertini, D. F. (2000). Sorting and reorganization of centrosomes during oocyte maturation in the mouse. *Microscopy Research and Technique*, 49, 435–444.
- Cheon, B., Lee, H. C., Wakai, T., & Fissore, R. A. (2013). Ca²⁺ influx and the store-operated Ca²⁺ entry pathway undergo regulation during mouse oocyte maturation. *Molecular Biology of the Cell*, 24, 1396–1410.
- Combelles, C. M., Albertini, D. F., & Racowsky, C. (2003). Distinct microtubule and chromatin characteristics of human oocytes after failed in-vivo and in-vitro meiotic maturation. *Human Reproduction*, 18, 2124–2130.
- Coticchio, G., Guglielmo, M. C., Albertini, D. F., Dal Canto, M., Mignini Renzini, M., De Ponti, E., & Fadini, R. (2014). Contributions of the actin cytoskeleton to the emergence of polarity during maturation in human oocytes. *Molecular Human Reproduction*, 20, 200–207.
- Coticchio, G., Dal Canto, M., Mignini Renzini, M., Guglielmo, M. C., Brambillasca, F., Turchi, D., Novara, P. V., & Fadini, R. (2015). Oocyte maturation: Gamete-somatic cells interactions, meiotic resumption, cytoskeletal dynamics and cytoplasmic reorganization. *Human Reproduction Update*, 21, 427–454.
- Dalton, C. M., & Carroll, J. (2013). Biased inheritance of mitochondria during asymmetric cell division in the mouse oocyte. *Journal of Cell Science*, 126, 2955–2964.
- De La Fuente, R., & Eppig, J. J. (2001). Transcriptional activity of the mouse oocyte genome: Companion granulosa cells modulate transcription and chromatin remodeling. *Developmental Biology*, 229, 224–236.
- Ducibella, T., & Fissore, R. (2008). The roles of Ca²⁺, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. *Developmental Biology*, 315, 257.
- Escriv, L., Grau, N., Meseguer, M., Pellicer, A., & Escriva, M. J. (2010). Morphologic indicators predict the stage of chromatin condensation of human germinal vesicle oocytes recovered from stimulated cycles. *Fertility and Sterility*, 93, 2557–2564.
- Gorbsky, G. J. (2015). The spindle checkpoint and chromosome segregation in meiosis. *The FEBS Journal*, 282, 2471–2487.

- Larson, S. M., Lee, H. J., Hung, P. H., Matthews, L. M., Robinson, D. N., & Evans, J. P. (2010). Cortical mechanics and meiosis II completion in mammalian oocytes are mediated by myosin-II and ezrin-radixin-moesin (ERM) proteins. *Molecular Biology of the Cell*, 21, 3182–3192.
- Leader, B., Lim, H., Carabatsos, M. J., Harrington, A., Ecsedy, J., Pellman, D., Maas, R., & Leder, P. (2002). Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. *Nature Cell Biology*, 4, 921–928.
- Li, R., & Albertini, D. F. (2013). The road to maturation: Somatic cell interaction and self-organization of the mammalian oocyte. *Nature Reviews. Molecular Cell Biology*, 14, 141–152.
- Longo, F. J., & Chen, D. Y. (1985). Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. *Developmental Biology*, 107, 382–394.
- Luciano, A. M., Lodde, V., Franciosi, F., Tessaro, I., Corbani, D., & Modena, S. (2012). Large-scale chromatin morpho-functional changes during mammalian oocyte growth and differentiation. *European Journal of Histochemistry*, 56, e37.
- Luo, J., McGinnis, L. K., & Kinsey, W. H. (2009). Fyn kinase activity is required for normal organization and functional polarity of the mouse oocyte cortex. *Molecular Reproduction and Development*, 76, 819–831.
- Maro, B., Johnson, M. H., Webb, M., & Flach, G. (1986). Mechanism of polar body formation in the mouse oocyte: An interaction between the chromosomes, the cytoskeleton and the plasma membrane. *Journal of Embryology and Experimental Morphology*, 92, 11–32.
- McGinnis, L. K., & Albertini, D. F. (2010). Dynamics of protein phosphorylation during meiotic maturation. *Journal of Assisted Reproduction and Genetics*, 27, 169–182.
- McGinnis, L. K., Carroll, D. J., & Kinsey, W. H. (2011). Protein tyrosine kinase signaling during oocyte maturation and fertilization. *Molecular Reproduction and Development*, 78, 831–845.
- McGinnis, L. K., Pelech, S., & Kinsey, W. H. (2014). Post-ovulatory aging of oocytes disrupts kinase signaling pathways and lysosome biogenesis. *Molecular Reproduction and Development*, 81, 928–945.
- McLaren, A. (2003). Primordial germ cells in the mouse. *Developmental Biology*, 262, 1–15.
- Messinger, S. M., & Albertini, D. F. (1991). Centrosome and microtubule dynamics during meiotic progression in the mouse oocyte. *Journal of Cell Science*, 100, 289–298.
- Miyara, F., Migne, C., Dumont-Hassan, M., Le Meur, A., Cohen-Bacrie, P., Aubriot, F. X., Glissant, A., Nathan, C., Douard, S., Stanovici, A., & Debey, P. (2003). Chromatin configuration and transcriptional control in human and mouse oocytes. *Molecular Reproduction and Development*, 64, 458–470.
- Moreno, R. D., Schatten, G., & Ramalho-Santos, J. (2002). Golgi apparatus dynamics during mouse oocyte in vitro maturation: Effect of the membrane trafficking inhibitor brefeldin a. *Biology of Reproduction*, 66, 1259–1266.
- Peters, H., & McNatty, K. P. (1980). *The ovary: A correlation of structure and function in mammals*. Berkeley and Los Angeles: University of California Press.
- Plancha, C. E. (1996). Cytokeratin dynamics during oocyte maturation in the hamster requires reaching of metaphase I. *Differentiation*, 60, 87–98.
- Plancha, C. E., Sanfins, A., Rodrigues, P., & Albertini, D. (2005). Cell polarity during folliculogenesis and oogenesis. *Reproductive Biomedicine Online*, 10, 478–484.
- Rivero, S., Cardenas, J., Bornens, M., & Rios, R. M. (2009). Microtubule nucleation at the cis-side of the Golgi apparatus requires AKAP450 and GM130. *The EMBO Journal*, 28, 1016–1028.
- Rodrigues, P., Limback, D., McGinnis, L. K., Plancha, C. E., & Albertini, D. F. (2008). Oogenesis: Prospects and challenges for the future. *Journal of Cellular Physiology*, 216, 355–365.
- Sardet, C., Prodon, F., Dumollard, R., Chang, P., & Chenevert, J. (2002). Structure and function of the egg cortex from oogenesis through fertilization. *Developmental Biology*, 241, 1–23.
- Wessel, G. M., & Wong, J. L. (2009). Cell surface changes in the egg at fertilization. *Molecular Reproduction and Development*, 76, 942–953.
- Yi, K., Unruh, J. R., Deng, M., Slaughter, B. D., Rubinstein, B., & Li, R. (2011). Dynamic maintenance of asymmetric meiotic spindle position through Arp2/3-complex-driven cytoplasmic streaming in mouse oocytes. *Nature Cell Biology*, 13, 1252–1258.
- Yi, K., Rubinstein, B., Unruh, J. R., Guo, F., Slaughter, B. D., & Li, R. (2013). Sequential actin-based pushing forces drive meiosis I chromosome migration and symmetry breaking in oocytes. *The Journal of Cell Biology*, 200, 567–576.
- Yuba-Kubo, A., Kubo, A., Hata, M., & Tsukita, S. (2005). Gene knockout analysis of two gamma-tubulin isoforms in mice. *Developmental Biology*, 282, 361–373.

Chromatin Modifications During Mammalian Oocyte Growth and Meiotic Maturation

Rabindranath De La Fuente, University of Georgia, Athens, GA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

In mammalian species the process of oocyte growth and maturation ensues from a pool of primordial follicles that become activated shortly after birth. Throughout postnatal development, oocytes are maintained in a protracted meiotic arrest with an intact nucleus at the diplotene stage. Oocyte growth takes place within the ovarian follicle and is regulated by an intimate bidirectional communication with surrounding granulosa cells. The paracrine signals between germ cells and granulosa cells that regulate this process are not clear at present. However, three oocyte intrinsic developmental programs (nuclear maturation, cytoplasmic maturation and epigenetic maturation) are essential for oocyte growth and acquisition of developmental potential. Mouse oocytes first acquire the capacity for nuclear maturation approximately on day 14 of postnatal development following synthesis and accumulation of cell cycle regulatory molecules required to resume and complete the first meiotic division (Fig. 1). Cytoplasmic maturation is acquired during the final stages of oocyte growth and involves a series of metabolic changes that prepare the oocyte for fertilization, pronuclear formation and the first cleavage division before transcriptional activation of the embryonic genome (Fig. 1). Epigenetic maturation involves a series of chromatin modifications that affect the patterns of gene expression without exerting any changes at the level of the DNA sequence. These changes can occur on a genome-wide basis, on specific chromosomal domains such as centromeres and telomeres or may affect chromatin composition and structure at the single gene level thus may directly impact the regulation of global transcription and/or specific gene expression patterns (De La Fuente, 2006).

Epigenetic modifications may induce stable and heritable changes in gene expression through DNA methylation, the induction of histone post-translational modifications such as histone methylation, acetylation, phosphorylation and poly(ADP) ribosylation or the incorporation of histone variants at specific chromosomal domains such as the specialized centromeric histone H3 variant CENP-A or the replication independent H3.3 variant. Epigenetic modifications are required for the control of transcription, nuclear

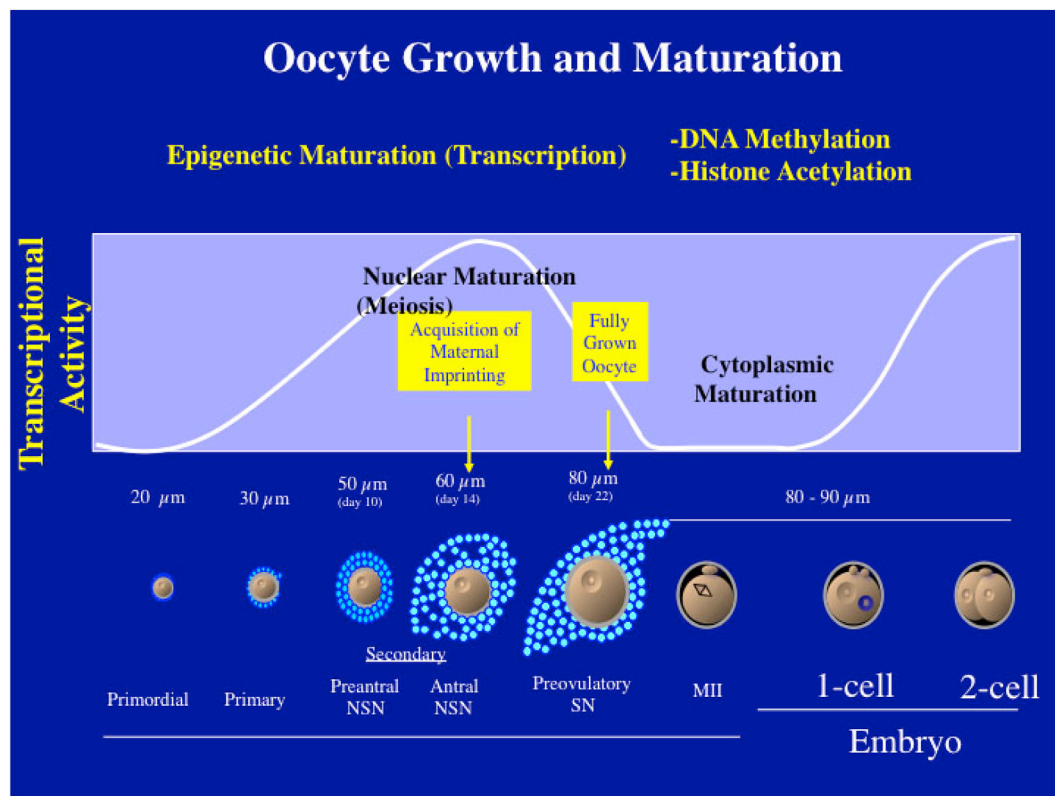


Fig. 1 Epigenetic modifications during mammalian oocyte growth. Functional differentiation of chromatin structure is essential to confer the oocyte with meiotic and developmental potential. During postnasal oocyte growth nuclear maturation, cytoplasmic maturation and epigenetic maturation determine chromosome stability and establishment of maternal imprinting.

architecture, establishment of DNA methylation patterns and maintenance of chromosome stability during meiosis (De La Fuente, 2006).

The mammalian oocyte nucleus or germinal vesicle (GV) is subject to dynamic chromatin modifications at several developmental transitions in preparation for meiosis. At the local level, targeted chromatin modifications at regulatory DNA sequences of specific loci establish oocyte-specific DNA methylation marks, a maternal genomic imprint. Superimposed on these local chromatin changes lays an important nontargeted strategy for the control of global transcription and large-scale chromatin remodeling (De La Fuente, 2006). Large-scale chromatin structure is regulated by multiple and hierarchical global chromatin modifications induced by chromatin remodeling proteins and histone post-translational modifications required for heterochromatin formation and the epigenetic control of centromere function (De La Fuente et al., 2012). Notably, specialized mechanisms are set in place for the control of large-scale chromatin remodeling in the female germ line in order to cope with the unique requirements of meiotic chromosomes (De La Fuente, 2006, 2014; Kimmins and Sassone-Corsi, 2005; Revenkova et al., 2004; Ivanovska and Orr-Weaver, 2006).

The mechanisms regulating the 'meiotic histone code' are only beginning to be unraveled through the generation of transgenic mouse models and the use of genome-wide transcriptome and DNA methylome analyses. These are powerful strategies that will accelerate our understanding of how the meiotic histone code, or changes in large-scale chromatin structure regulate genome function during oogenesis and the maternal inheritance of epigenetic states to the early embryo. This will be critical to gain a mechanistic understanding of the epigenetic origins of chromosome instability, genomic reprogramming and the control of gene expression during the mammalian oocyte to embryo transition.

Establishment of DNA Methylation During Postnatal Oocyte Growth

Establishment of DNA methylation during oogenesis occurs on a locus-by-locus basis and both histone modifications and transcription at regulatory sequences called imprinted control regions (ICRs) or differentially methylated regions (DMRs) located within the 5' flanking regions of imprinted genes dictate the placing and timing of methylation marks. Genomic imprinting results in the establishment of a gamete-specific mark that is strictly required for normal embryonic development, placental differentiation and fetal growth (Hackett and Surani, 2013; Weaver and Bartolomei, 2014). During early postnatal development, maternal-specific DNA methylation patterns begin to be re-established in the growing oocytes within primary follicles. Beginning with day 5 of postnatal development and coincident with the transition from primary to secondary follicles, maternal methylation imprints are established, progressively at different genes, a process that is mostly completed by the germinal vesicle stage before global transcriptional repression takes place in fully-grown preovulatory oocytes (Lucifero et al., 2004; Smallwood et al., 2011).

Establishment of methylation marks at different genes depends on oocyte growth. De novo methylation occurs around the time when the oocyte reaches 50 μ m in diameter and different genes acquire their final methylation status in different oocyte size populations. The time of establishment of DNA methylation marks at specific loci during oogenesis has been described (Lucifero et al., 2004; Smallwood et al., 2011). However, less is known about the primary mechanisms regulating the establishment of de novo DNA methylation at specific imprinted genes during oogenesis. Elegant studies provided the first evidence that the DNA methyltransferase enzyme DNMT3A exhibits de novo methylation activity and that together with its catalytically inactive regulatory cofactor DNMT3L regulate the establishment of DNA methylation at several imprinted loci in mouse oocytes (Kaneda et al., 2004; Hata et al., 2002; Bourc'his et al., 2001). Additional studies indicated that the histone H3 lysine 4 demethylase (KDM1B) is also required for DNA methylation of several but not all maternally imprinted genes during oocyte growth (Ciccone et al., 2009). The precise role of KDM1B in this process remains to be determined. However, it has been suggested that KDM1B may induce demethylation of histone H3 at lysine 4 (H3K4), potentially inducing an open chromatin configuration to recruit the de novo DNA methylation machinery to specific imprinted loci (Ciccone et al., 2009). A different study provided compelling evidence that the KRAB/zinc finger protein Zfp57 is required for methylation of the *Snrpn* locus through a potential interaction with the chromatin binding protein KAP1 (also known as TRIM28), which is known to establish an interaction with the histone methyltransferase SETDB1 and DNMT3A (Li et al., 2008; Messerschmidt et al., 2012; Quenneville et al., 2011).

One of the most intriguing recent discoveries on the mechanisms of oocyte de novo methylation is that transcription across differentially methylated regions (DMR) seems to be required for the establishment and maintenance of maternal imprinting marks during oogenesis. Analysis of DNA methylation patterns in mouse oocytes using the technique of reduced representation bisulfate sequencing (RRBS) indicated that approximately, 1600 CpG islands undergo DNMT3A/Dnmt3L-dependent methylation in mature oocytes. Interestingly, these CpG islands were found to be preferentially located within active transcription units, lacking histone H3 trimethylated at lysine 4 (H3K4me3), a histone mark associated with transcriptional control. Moreover, H3K4me3 is found enriched at unmethylated CpG islands in embryonic stem cells, consistent with the notion that establishment of DNA methylation at imprinted loci during oocyte growth may be linked with active transcription across the differentially methylated regions of specific imprinted genes (Smallwood et al., 2011; Chotalia et al., 2009). The role of transcription in the establishment of DNA methylation marks during oogenesis is not fully understood. However, it has been suggested that it may be required to maintain an open chromatin configuration and allow the recruitment of de novo methylases to regulatory sequences (Chotalia et al., 2009). Changes in transcription may thus coordinate the progressive establishment of maternal methylation marks with oocyte growth. An important observation emerging from genome wide studies is that methylation patterns during oocyte growth are very dynamic and some methylation marks are not established until the final stages of oocyte growth in preovulatory oocytes (Smallwood et al., 2011),

a stage known to be highly susceptible to both hormonal and/or environmental fluctuations (De La Fuente, 2006) and thus with important clinical implications for assisted reproductive technologies.

Chromatin immunoprecipitation (ChIP) studies to generate genome-wide profiles of histone modifications implicated in promoting or inhibiting DNA methylation have recently revealed that histone methylation plays a critical role in the establishment of DNA methylation marks during oogenesis and suggest that the DNA methylation machinery may 'sense' the status of histone H3 modification at specific regulatory sequences (Stewart et al., 2015). Regulatory sequences destined for DNA methylation in both primary and growing oocytes show reduced H3K4me2 and H3K4me3 while permissive H3K36me3 increases specifically at these sites in growing oocytes. Moreover, analysis of KDM1A or KDM1B knockout oocytes indicates that removal of H3K4me3 is required for the establishment of DNA methylation. Thus histone remodeling in the oocyte is critical to direct de novo methylation (Stewart et al., 2015).

Surprisingly, genome wide DNA methylation studies revealed the presence of CpG methylation at a subset of nonimprinted genes in mammalian oocytes (Rutledge et al., 2014). Studies from several independent laboratories indicate that CpG island methylation during oocyte growth is not exclusively associated with genomic imprinting but it determines a significant proportion of the genomic DNA methylation profile inherited by the preimplantation embryo (Smallwood et al., 2011; Rutledge et al., 2014). Notably, amongst the 1600 methylated CpG islands found in the oocyte, some are associated with imprinted genes, some are required to maintain transcriptional repression at a cluster of testis-specific genes and some are required to repress brain-specific genes during embryonic development (Rutledge et al., 2014). These methylated regions are evolutionarily conserved between mouse and human and may be important to regulate phenotypic changes induced by prenatal and postnatal environmental factors that affect DNA methylation (Rutledge et al., 2014). Consistent with these observations, methylome analysis using genome-wide bisulfite sequencing (DNaseq) confirmed the presence of significant DNA methylation at non-CpG islands in the oocyte genome and suggested that methylation in oocytes seems to occur predominantly over gene bodies (Kobayashi et al., 2012; Shirane et al., 2013). This type of genomic non-CpG methylation is also present in pluripotent stem cells and brain (Shirane et al., 2013; Lister et al., 2009). Remarkably, non-CpG methylation comprises up to 25% of all cytosine methylation in embryonic stem cells (Lister et al., 2009). Analysis of genome-wide methylome maps of mouse oocytes indicates that nearly two thirds of all methyl cytosines in the oocyte genome occur in a non-CpG context and is enriched in gene bodies. Importantly, non-CpG methylation accumulates during oocyte growth (Shirane et al., 2013).

The biological significance of non-CpG methylation in the oocyte genome is not clear at present. However, both the recent discovery of different types of DNA methylation during oogenesis, and the mechanistic insight obtained from genome wide sequencing of the oocyte methylome have now shifted the paradigm from initially considering DNA sequence motifs or structural properties as the potential primary mark for the establishment of specific methylation patterns, to that of the presence of critical histone modifications and transcriptional status at the chromatin template that may nucleate the recruitment of the DNMT3A/DNMT3L complex. Exposure of intragenic regions to DNA methyltransferases during transcription as well as other epigenetic marks that induce an open chromatin structure seems critical in this new model. Thus both transcriptional and epigenetic modifications may be required for Dnmt3L mediated intragenic methylation (Weaver and Bartolomei, 2014; Smallwood et al., 2011; Chotalia et al., 2009). Changes in transcription during oocyte growth may thus coordinate the progressive establishment of methylation marks at different DMRs and may be subject to signaling from granulosa cells at different stages of follicular growth and differentiation as well as hormonal or environmental fluctuations.

Chromatin Remodeling and Chromosome Stability During Oocyte Meiosis

Coinciding with the onset of puberty and following stimulation with the gonadotropin hormone LH, fully-grown mammalian oocytes undergo a process of striking large-scale chromatin remodeling in preparation for meiotic division. A growing body of evidence indicates that this is a critical process with far reaching implications for accurate chromosome segregation and maintenance of chromosome stability.

During mammalian oocyte meiosis, two sequential cell divisions taking place without an intervening S-phase decrease the number of chromosomes to a haploid complement. In order to ensure accurate segregation, meiotic chromosomes exhibit unique structural and functional properties to coordinate the initial segregation of homologous chromosomes (meiosis-I) with the subsequent separation of sister chromatids during meiosis-II. Establishment and timely resolution of centromeric cohesion during this process is under stringent molecular regulation and specialized spindle microtubule attachments at meiosis-I or meiosis-II ensure accurate chromosome segregation patterns (Petronczki et al., 2003; Page and Hawley, 2003).

In preparation for meiotic resumption, preovulatory oocytes exhibit a striking large-scale chromatin remodeling process that is critical to regulate nuclear architecture as well as the dynamics of meiotic progression and chromosome segregation. Large-scale chromatin remodeling is critical for the acquisition of both meiotic and developmental competence in mammalian oocytes (De La Fuente, 2006; De La Fuente et al., 2004a; Yang et al., 2009; Baumann et al., 2010). Growing oocytes are characterized by highly decondensed chromatin and exhibit pericentric heterochromatin domains or "chromocenters" distributed throughout the entire germinal vesicle (GV), a configuration termed "non-surrounded nucleolus" (NSN). In the mouse ovary, growing oocytes exhibit the NSN configuration and show high levels of transcriptional activity, required for the synthesis and storage of maternal transcripts essential to support meiotic resumption and the first cleavage divisions until embryonic genome activation takes place in the fertilized embryo. However, during the final stages of oocyte growth, changes in global chromatin structure result in progressive DNA

condensation and the formation of a perinucleolar heterochromatin rim, termed “surrounded nucleolus” (SN) or karyosphere (De La Fuente, 2006). Importantly, transcription run-on assays demonstrate that large-scale chromatin remodeling into the SN configuration is associated with global transcriptional quiescence. This process is critical to confer full developmental potential and to ensure timely progression of meiotic maturation (De La Fuente, 2006). However, the mechanisms involved remain poorly understood.

A unique experimental system designed for the culture of oocyte–granulosa cell complexes obtained from preantral follicles provided the initial evidence suggesting that a developmentally regulated signal(s), presumably of paracrine origin from granulosa cells modulate chromatin remodeling and transcriptional activity in preovulatory oocytes (De La Fuente and Eppig, 2001). Since then independent laboratories have provided additional evidence to support this model (Liu and Aoki, 2002). Studies indicate that a patent gap junctional communication with granulosa cells is required. However, the signals emanating from granulosa cells that might trigger large-scale chromatin remodeling in the oocyte genome remain to be determined (De La Fuente, 2006). Importantly, evidence obtained from pharmacological manipulation of chromatin structure and several transgenic mouse models indicate that large-scale chromatin remodeling and global transcriptional repression are regulated through different pathways (De La Fuente et al., 2004a; Abe et al., 2010; Andreu-Vieyra et al., 2010; Pattabiraman et al., 2015). For example, in oocytes from nucleoplasmin 2 (Npm2) knockout mice chromatin remains in a decondensed, NSN-like configuration following gonadotropin stimulation. However, global transcriptional silencing takes place in a timely manner (De La Fuente et al., 2004a). On the other hand, oocytes lacking the histone H3K4 methyltransferase (MLL2) or the bromo-domain protein (BRWD1) fail to undergo global transcriptional repression despite large-scale chromatin remodeling from the NSN to the SN configuration (Andreu-Vieyra et al., 2010; Pattabiraman et al., 2015). Moreover, loss of function analysis in vaccinia-related kinase (VRK1) knockout oocytes revealed a critical role for this conserved histone H2A kinase in nuclear chromatin remodeling in preparation for meiotic resumption. Depletion of VRK1 causes striking epigenetic changes, such as loss of histone H2A phosphorylation (H2AT119ph), decreased histone H3 (H3K14ac) and histone H4 (H4K5ac) acetylation. Notably, loss of VRK1 in fully-grown oocytes leads to abnormal chromatin remodeling and a reduced proportion of gametes that exhibit the SN chromatin configuration. Thus, the evidence obtained so far indicates that large-scale chromatin remodeling and global transcriptional silencing in mammalian oocytes are concurrent developmental processes under the control of distinct cellular pathways. Notably, chromatin configuration and subcellular position of the germinal vesicle in fully-grown human oocytes has been suggested to provide a noninvasive marker for the gamete’s meiotic and developmental potential. These findings provide additional support indicating that global chromatin remodeling and the resulting epigenetic landscape acquired following large-scale chromatin remodeling play critical roles in the establishment of meiotic and developmental potential in mammalian oocytes of several species (Ivanovska and Orr-Weaver, 2006).

Establishment, maintenance and/or erasure of covalent histone modifications confers structural and functional identity to distinct nuclear and chromosomal domains (Dundr and Misteli, 2001; Dillon and Festenstein, 2002) and is an essential prerequisite for the control of meiotic chromosome segregation. For instance, while a subset of epigenetic marks, such as histone lysine methylation, remains stably associated with specific chromatin domains (i.e., centromeres) of meiotic chromosomes throughout meiosis (Arney et al., 2002; Liu et al., 2004; Ooga et al., 2008; Meglicki et al., 2008; Swain et al., 2007; Wang et al., 2006), other modifications, such as histone acetylation, exhibit highly dynamic patterns during meiotic resumption and chromosome segregation (Adenot et al., 1997; Kim et al., 2003; De La Fuente et al., 2004b; Sarmiento et al., 2004). High levels of acetylation of histone H3 and H4 at most lysine residues are detectable in the nucleus of fully-grown, preovulatory oocytes. However, concomitant with germinal vesicle breakdown, a wave of global histone deacetylation occurs in the oocyte genome (Kim et al., 2003; De La Fuente et al., 2004b). Although, little is known concerning the enzymatic activities that are involved in this process, recent evidence suggests histone deacetylase 2 (HDAC2) as the major enzyme regulating global histone acetylation levels during oocyte development (Abidi et al., 2004).

Timely coordination of developmental transitions in the global epigenetic landscape during meiotic maturation is of outmost importance for the maintenance of chromosome stability in the female gamete. For example, global histone deacetylation during meiotic resumption is required for the localization and function of centromeric proteins such as ATRX (De La Fuente et al., 2004b) as well as the chromosome passenger complex and interference with this process using the HDAC inhibitor trichostatin A (TSA) results in the formation of hyperacetylated, decondensed chromosomes. Exposure to histone deacetylase inhibitors induce the formation of hyper-acetylated chromosomes with highly abnormal meiotic figures including misaligned chromosomes and chromosome lagging in a large proportion of oocytes (De La Fuente et al., 2004a; Yang et al., 2012). TSA exposure leads to severe oocyte aneuploidy and subfertility (Akiyama et al., 2006). However, several HDAC inhibitors (HDACi) have recently entered phase I and in some instances, phase II clinical trials for the treatment of several types of human cancers (Martinet and Bertrand, 2011). Our recent studies indicate that mammalian oocytes exhibit an extreme susceptibility HDACi-induced chromatin changes even at nanomolar concentrations leading to rapid euchromatin decondensation and severe chromosome instability (Yang et al., 2012). Similarly, functional ablation of HDAC2 in oocytes interferes with deacetylation of H4K16ac and significantly increases the proportion of oocytes with chromosome condensation and segregation errors (Ma and Schultz, 2013). Global histone deacetylation in oocytes is critical for centromere function and binding of chromatin remodeling proteins such as ATRX to centromeric domains as hyperacetylation of chromosomes is sufficient to induce loss of centromeric ATRX (De La Fuente et al., 2004b) and interferes with the deposition of the kinetochore-specific histone variant CENP-A (Ma and Schultz, 2013; Wiens and Sorger, 1998).

Notably, reproductive aging is now widely recognized as a major risk factor for oocyte aneuploidy and oocytes obtained from older mice commonly exhibit histone hyper-acetylation at the metaphase-II stage, in particular of lysine 8 and lysine 12 in histone H4 (Akiyama et al., 2006; Suo et al., 2010). A similar correlation between advanced maternal age, defective deacetylation of the

lysine 12 residue in histone H4 and chromosome misalignment has been detected in human oocytes (van den Berg et al., 2011). In addition, reduced global trimethylation of histone H3 (H3K9me3) as well as dimethylation of histone H4 (H4K20me2) and histone H3 (H3K36me2 and H3K79me2) is associated with highly abnormal chromatin configurations, such as condensed, irregular or clumped chromatin at the GV stage in aged mouse oocytes.

Advanced maternal age was initially associated with meiotic spindle defects (Hassold and Hunt, 2001; Hunt and Hassold, 2008; Vialard et al., 2006). Importantly, microarray analyses of oocytes from young and old mice suggest deregulation of the key mechanisms controlling the spindle assembly checkpoint, kinetochore function, a deterioration of cohesion complexes as well as abnormal chromatin remodeling processes during reproductive aging (Revenkova et al., 2004, 2010; Pan et al., 2008; Hodges et al., 2005; Chiang et al., 2010; Lister et al., 2010) that collectively contribute to age-related subfertility and impaired embryonic development. However, recent studies indicate that the epigenetic maturation of centromeric heterochromatin domains may be particularly susceptible to disturbances leading to detrimental effects on oocyte and embryo quality.

Epigenetic Control of Centromere Function and Chromosome Stability

In the mammalian genome, centromeres are specialized chromosomal domains that form the interface between condensed chromosomes and spindle microtubules (Guenatri et al., 2004; Sullivan and Karpen, 2004). Centromeres contain several megabases of repetitive DNA sequences that are essential, albeit not sufficient for its function. Thus, centromere formation is under strict epigenetic control (Dillon and Festenstein, 2002; Karpen and Allshire, 1997). Several epigenetic marks, such as trimethylation of H3K9 (H3K9me3) and H4K20 (H4K20me3), have been implicated in centromere formation by providing specific docking sites for chromatin remodeling proteins such as heterochromatin protein 1 (HP1) and ATRX (Rea et al., 2000; Lachner et al., 2001; Bannister et al., 2001; Kourmouli et al., 2005; Schotta et al., 2004) (De La Fuente et al., 2004b; McDowell et al., 1999).

The chromatin remodeling protein ATRX (α -Thalassemia mental retardation X-linked) is a member of the SWI/SNF2 family of ATP-dependent chromatin remodeling proteins that is found at the centromeres of mouse and human somatic cells (De La Fuente et al., 2004b; McDowell et al., 1999). ATRX plays an essential role at many critical levels of genome organization in human and mouse somatic cells. In mouse oocytes, ATRX localizes to pericentric heterochromatin in GV stage oocytes. Notably, during metaphase-I and metaphase-II of meiosis ATRX acquires a strict centromeric localization. Analysis of a transgenic mouse model carrying an oocyte-conditional deletion of ATRX revealed that loss of maternal ATRX function results in chromosome misalignment at the metaphase-II spindle and severe centromere instability with a high incidence of DNA breaks in zygote stage embryos. Preimplantation embryos deficient for maternal ATRX exhibit rampant aneuploidy and embryo demise resulting in severe subfertility (Baumann et al., 2010; De La Fuente et al., 2004b). At the molecular level, loss of ATRX function interferes with recruitment of the transcriptional regulator DAXX to pericentric heterochromatin in oocytes and embryos suggesting that ATRX regulates the molecular composition of pericentric heterochromatin during the oocyte to embryo transition (Baumann et al., 2010; De La Fuente et al., 2015). *Atrx* transcripts and protein expression are significantly reduced in oocytes of older mice (Pan et al., 2008; Svoboda et al., 2001). Notably, ATRX-deficient oocytes exhibit the same type of chromosome segregation defects (i.e., premature centromere separation and chromosome lagging) detected in aneuploid oocytes obtained from women of advanced reproductive age (Baumann et al., 2010; Vialard et al., 2006). Thus, transgenic mice with an oocyte-conditional deletion of ATRX constitute an important animal model to determine the mechanisms leading to the transmission of aneuploidy during reproductive senescence and to determine the epigenetic origins of chromosome instability during the oocyte to embryo transition.

Recent studies indicate that a striking 70%–90% of human cleavage stage embryos exhibit one or more blastomeres carrying an aneuploid chromosome complement including deletions and/or translocations (Vanneste et al., 2009; Robberecht et al., 2010; Voet et al., 2011). The mechanisms responsible for such high levels of chromosome instability are not known. However, our ATRX model provides novel evidence indicating that centromeric instability is a major mechanism leading to the formation of large-scale chromosomal rearrangements in the cleavage stage embryo (De La Fuente et al., 2015). In addition, our results indicate that maternal inheritance of ATRX contributes to a striking epigenetic asymmetry of maternal and paternal centromeres that is observed during the transition to the first mitosis. Notably, ATRX is essential for transcriptional silencing of major satellite repeats in the maternal genome and required to prevent deleterious centromeric mitotic recombination events in the cleavage stage embryo (De La Fuente et al., 2015). Loss of ATRX during the critical developmental window of epigenetic reprogramming leads to centromeric breaks, sister chromatid exchanges and large-scale chromosomal rearrangements in a striking proportion of cleavage stage embryos (De La Fuente et al., 2015). These findings suggest that ATRX-deficiency is associated with the formation of mitotic recombination breakpoints and the activation of DNA repair pathways providing a unique model to determine the epigenetic origins of cleavage stage chromosome instability (De La Fuente et al., 2015).

We have also recently observed severe chromosome instability during female meiosis in mice lacking BRWD1 (bromo- and WD-containing protein 1) (Pattabiraman et al., 2015). Proteins that contain functional bromodomains play critical roles in a variety of cellular processes, such as chromatin remodeling and transcriptional regulation, through the recognition of acetyl-lysine residues. Loss of BRWD1 function impairs global transcriptional silencing in preovulatory mouse oocytes, as indicated by transcription run-on-assays, while acquisition of the SN chromatin configuration appeared unaffected (Pattabiraman et al., 2015). Further investigation of the protein coding and noncoding transcriptome by micro-array analysis, pathway-specific PCR arrays as well quantitative real-time PCR revealed surprisingly similar expression patterns of protein coding transcripts in mutants and wild-type oocytes. However, loss of BRWD1 is associated with overexpression of mixed lineage leukemia protein 5 (MLL5) as well as long interspersed

nuclear elements (LINE's), indicating that continued run-on expression in mutant oocytes may be attributable to persistent expression of repetitive elements. Most importantly, BRWD1-deficient ova present highly disrupted meiotic spindle configurations and show a severely limited potential to mature to the metaphase-II stage resulting in complete infertility. Prominent defects in axial chromatid condensation and abnormal chromosome-microtubule interactions lead to major defects in chromosome congression and homologous chromosome separation during anaphase-I and result in severe chromatid elongation with loss of structural integrity and chromatin clumping. Surprisingly, chromosomal breaks and meiotic chromosome decondensation in BRWD1 mutant oocytes are not associated with defects in histone deacetylation, monomethylation of H4K20 or loading of the condensin protein Smc4 (Pattabiraman et al., 2015). While the primary epigenetic defect in this model is not known, it is conceivable that large-scale chromatin remodeling processes are disrupted as a consequence of MLL5 and LINE-1 element overexpression leading to severe chromosome instability.

Notably, it is becoming increasingly clear that the establishment of a proper epigenetic landscape and the epigenetic control of transposon expression during oogenesis are essential mechanisms to ensure normal progression of meiotic transitions and the preservation of genome integrity in the female germ line. For example, overexpression of intracisternal A particle (IAP) transposons in MLL2 knockout oocytes is associated with severe meiotic defects and oocyte DNA damage resulting in premature ovarian failure and female sterility (Andreu-Vieyra et al., 2010). Abnormal expression of the mouse transposon (MTs) in DICER knockout oocytes and mutants of the recently discovered oocyte-specific isoform of DICER (DICERo) exhibit severe meiotic defects with lack of proper chromosome alignment to the meiotic spindle in maturing oocytes (Murchison et al., 2007; Flemr et al., 2013). In addition, targeted deletion of MARF1 (meiotic arrest female 1) during oocyte growth results in overexpression of both IAP transposons and LINE-1 elements resulting in widespread DNA damage and meiotic arrest (Su et al., 2012a, 2012b). Finally, experimental overexpression of LINE-1 elements in mouse oocytes results in oocyte aneuploidy and embryonic lethality (Malki et al., 2014). Elucidating the precise epigenetic mechanisms responsible for predisposing female gametes to aneuploidy and CIN is an important first step towards the identification of reliable early detection markers of chromosome stability, oocyte quality and developmental potential.

References

- Abe, K.-i., Inoue, A., Suzuki, M. G., & Aoki, F. (2010). Global gene silencing is caused by the dissociation of RNA polymerase II from DNA in mouse oocytes. *The Journal of Reproduction and Development*, 56, 502–507.
- Abidi, F. E., et al. (2004). Mutation in the 5[prime] alternatively spliced region of the XNP/ATR-X gene causes Chudley-Lowry syndrome. *European Journal of Human Genetics*, 13, 176–183.
- Adenot, P. G., Mercier, Y., Renard, J. P., & Thompson, E. M. (1997). Differential H4 acetylation of paternal and maternal chromatin precedes DNA replication and differential transcriptional activity in pronuclei of 1-cell mouse embryos. *Development*, 124, 4615–4625.
- Akiyama, T., Nagata, M., & Aoki, F. (2006). Inadequate histone deacetylation during oocyte meiosis causes aneuploidy and embryo death in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 7339–7344.
- Andreu-Vieyra, C. V., et al. (2010). MLL2 is required in oocytes for bulk histone 3 lysine 4 Trimethylation and transcriptional silencing. *PLoS Biology*, 8, e1000453.
- Arney, K. L., Bao, S., Bannister, A. J., Kouzarides, T., & Surani, M. A. (2002). Histone methylation defines epigenetic asymmetry in the mouse zygote. *The International Journal of Developmental Biology*, 46, 317–320.
- Bannister, A. J., et al. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, 410, 120–124.
- Baumann, C., Viveiros, M. M., & De La Fuente, R. (2010). Loss of maternal ATRX results in centromere instability and aneuploidy in the mammalian oocyte and pre-implantation embryo. *PLoS Genetics*, 6, e1001137.
- van den Berg, I. M., et al. (2011). Defective deacetylation of histone 4 K12 in human oocytes is associated with advanced maternal age and chromosome misalignment. *Human Reproduction*.
- Bourchis, D., Xu, G. L., Lin, C. S., Bollman, B., & Bestor, T. H. (2001). Dnmt3L and the establishment of maternal genomic imprints. *Science*, 294, 2536–2539.
- Chiang, T., Duncan, F. E., Schindler, K., Schultz, R. M., & Lampson, M. A. (2010). Evidence that weakened centromere cohesion is a leading cause of age-related aneuploidy in oocytes. *Current Biology*, 20, 1522–1528.
- Chotalia, M., et al. (2009). Transcription is required for establishment of germline methylation marks at imprinted genes. *Genes & Development*, 23, 105–117.
- Ciccone, D. N., et al. (2009). KDM1B is a histone H3K4 demethylase required to establish maternal genomic imprints. *Nature*, 461, 415–418.
- De La Fuente, R. (2006). Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. *Developmental Biology*, 292, 1–12.
- De La Fuente, R. (2014). Histone deacetylation: Establishing a meiotic histone code. *Cell Cycle*, 13, 879–880.
- De La Fuente, R., & Eppig, J. J. (2001). Transcriptional activity of the mouse oocyte genome: Companion granulosa cells modulate transcription and chromatin remodeling. *Developmental Biology*, 229, 224–236.
- De La Fuente, R., et al. (2004a). Major chromatin remodeling in the germinal vesicle (GV) of mammalian oocytes is dispensable for global transcriptional silencing but required for centromeric heterochromatin function. *Developmental Biology*, 275, 447–458.
- De La Fuente, R., Viveiros, M., Wigglesworth, K., & Eppig, J. (2004b). ATRX a member of the SNF2 family of helicase/ATPases, is required for chromosome alignment and meiotic spindle organization in metaphase II stage mouse oocytes. *Developmental Biology*, 272, 1–14.
- De La Fuente, R., Baumann, C., & Viveiros, M. (2012). Chromatin structure and ATRX function in mouse oocytes. *Results and Problems in Cell Differentiation*, 55, 45–68.
- De La Fuente, R., Baumann, C., & Viveiros, M. M. (2015). ATRX contributes to epigenetic asymmetry and silencing of major satellite transcripts in the maternal genome of the mouse embryo. *Development*, 142, 1806–1817.
- Dillon, N., & Festenstein, R. (2002). Unravelling heterochromatin: Competition between positive and negative factors regulates accessibility. *Trends in Genetics*, 18, 252–258.
- Dundr, M., & Misteli, T. (2001). Functional Architecture in the cell nucleus. *The Biochemical Journal*, 356, 297–310.
- Flemr, M., et al. (2013). A Retrotransposon-driven Dicer isoform directs endogenous small interfering RNA production in mouse oocytes. *Cell*, 155, 807–816.
- Guenatri, M., Bailly, D., Maisson, C., & Almouzni, G. (2004). Mouse centric and pericentric satellite repeats form distinct functional heterochromatin. *The Journal of Cell Biology*, 166, 493–505.
- Hackett, J. A., & Surani, M. A. (2013). DNA methylation dynamics during the mammalian life cycle. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368.
- Hassold, T., & Hunt, P. (2001). To err (meiotically) is human: The genesis of human aneuploidy. *Nature Reviews Genetics*, 2, 280–291.
- Hata, K., Okano, M., Lei, H., & Li, E. (2002). Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. *Development*, 129, 1983–1993.

- Hodges, C. A., Revenkova, E., Jessberger, R., Hassold, T. J., & Hunt, P. A. (2005). SMC1 β -deficient female mice provide evidence that cohesins are a missing link in age-related nondisjunction. *Nature Genetics*.
- Hunt, P. A., & Hassold, T. J. (2008). Human female meiosis: What makes a good egg go bad? *Trends in Genetics*, 24, 86–93.
- Ivanovska, I., & Orr-Weaver, T. L. (2006). Histone modifications and the chromatin scaffold for meiotic chromosome architecture. *Cell Cycle*, 5, 2064–2071.
- Kaneda, M., et al. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature*, 429, 900–903.
- Karpen, G. H., & Allshire, R. C. (1997). The case for epigenetic effects on centromere identity and function. *Trends in Genetics*, 13, 489–496.
- Kim, J., Liu, H., Tazaki, M., Nagata, M., & Aoki, F. (2003). Changes in histone acetylation during mouse oocyte meiosis. *The Journal of Cell Biology*, 162, 37–46.
- Kimmins, S., & Sassone-Corsi, P. (2005). Chromatin remodelling and epigenetic features of germ cells. *Nature*, 434, 583–589.
- Kobayashi, H., et al. (2012). Contribution of intragenic DNA methylation in mouse Gametic DNA Methylomes to establish oocyte-specific heritable marks. *PLoS Genetics*, 8, e1002440.
- Kourmouli, N., Sun, Y. M., van der Sar, S., Singh, P. B., & Brown, J. P. (2005). Epigenetic regulation of mammalian pericentric heterochromatin in vivo by HP1. *Biochemical and Biophysical Research Communications*, 337, 901–907.
- Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., & Jenuwein, T. (2001). Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, 410, 116–120.
- Li, X., et al. (2008). A maternal-zygotic effect gene, Zfp57, maintains both maternal and paternal imprints. *Developmental Cell*, 15, 547–557.
- Lister, L. M., et al. (2010). Age-related meiotic segregation errors in mammalian oocytes are preceded by depletion of Cohesin and Sgo2. *Current Biology*, 20, 1511–1521.
- Lister, R., et al. (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature*, 462, 315–322.
- Liu, H., & Aoki, F. (2002). Transcriptional activity associated with meiotic competence in fully grown mouse GV oocytes. *Zygote*, 10, 327–332.
- Liu, H., Kim, J., & Aoki, F. (2004). Regulation of histone H3 lysine 9 methylation in oocytes and early pre-implantation embryos. *Development*, 131, 2269–2280.
- Lucifero, D., Mann, M. R., Bartolomei, M. S., & Trasler, J. M. (2004). Gene-specific timing and epigenetic memory in oocyte imprinting. *Human Molecular Genetics*, 13, 839–849.
- Ma, P., & Schultz, R. M. (2013). Histone Deacetylase 2 (HDAC2) regulates chromosome segregation and kinetochore function via H4K16 Deacetylation during oocyte maturation in mouse. *PLoS Genetics*, 9, e1003377.
- Malki, S., van der Heijden, G. W., O'Donnell, K. A., Martin, S. L., & Bortvin, A. (2014). A role for Retrotransposon LINE-1 in fetal oocyte attrition in mice. *Developmental Cell*, 29, 521–533.
- Martinet, N., & Bertrand, P. (2011). Interpreting Clinical assays for histone deacetylase inhibitors. *Cancer Management and Research*, 3, 117–141.
- McDowell, T. L., et al. (1999). Localization of a putative transcriptional regulator (ATRX) at pericentromeric heterochromatin and the short arms of acrocentric chromosomes. *Proceedings of the National Academy of Sciences of the United States of America*, 96, 13983–13988.
- Meglicki, M., Zientarski, M., & Borsuk, E. (2008). Constitutive heterochromatin during mouse oogenesis: The pattern of histone H3 modifications and localization of HP1 α and HP1 β proteins. *Molecular Reproduction and Development*, 75, 414–428.
- Messerschmidt, D. M., et al. (2012). Trim28 is required for epigenetic stability during mouse oocyte to embryo transition. *Science*, 335, 1499–1502.
- Murchison, E. P., et al. (2007). Critical roles for Dicer in the female germline. *Genes & Development*, 21, 682–693.
- Ooga, M., et al. (2008). Changes in H3K79 methylation during preimplantation development in mice. *Biology of Reproduction*, 78, 413–424.
- Page, S., & Hawley, R. (2003). Chromosome choreography: The meiotic ballet. *Science*, 301, 785–789.
- Pan, H., Ma, P., Zhu, W., & Schultz, R. M. (2008). Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Developmental Biology*, 316, 397–407.
- Pattabiraman, S., et al. (2015). Mouse BRWD1 is critical for spermatid postmeiotic transcription and female meiotic chromosome stability. *The Journal of Cell Biology*, 208, 53–69.
- Petronczki, M., Siomos, M., & Nasmyth, K. (2003). Un ménage à quatre: The molecular biology of chromosome segregation in meiosis. *Cell*, 112, 423–440.
- Quenneville, S., et al. (2011). In embryonic stem cells, ZFP57/KAP1 recognize a methylated Hexanucleotide to affect chromatin and DNA methylation of imprinting control regions. *Molecular Cell*, 44, 361–372.
- Rea, S., et al. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature*, 406, 593–599.
- Revenkova, E., et al. (2004). Cohesin SMC1 β is required for meiotic chromosome dynamics, sister chromatid cohesion and DNA recombination. *Nature Cell Biology*, 6, 555–562.
- Revenkova, E., Herrmann, K., Adelfalk, C., & Jessberger, R. (2010). Oocyte Cohesin expression restricted to Predictate stages provides full fertility and prevents aneuploidy. *Current Biology*, 20, 1529–1533.
- Robberecht, C., et al. (2010). Somatic genomic variations in early human prenatal development. *Current Genomics*, 11, 397–401.
- Rutledge, C. E., et al. (2014). Ontogeny, conservation and functional significance of maternally inherited DNA methylation at two classes of non-imprinted genes. *Development*, 141, 1313–1323.
- Sarmiento, O. F., et al. (2004). Dynamic alterations of specific histone modifications during early murine development. *Journal of Cell Science*, 117, 4449–4459.
- Schotta, G., et al. (2004). A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes & Development*, 18, 1251–1262.
- Shirane, K., et al. (2013). Mouse oocyte Methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA Methyltransferases. *PLoS Genetics*, 9, e1003439.
- Smallwood, S. A., et al. (2011). Dynamic CpG island methylation landscape in oocytes and preimplantation embryos. *Nature Genetics*, 43, 811–814.
- Stewart, K. R., et al. (2015). Dynamic changes in histone modifications precede de novo DNA methylation in oocytes. *Genes & Development*.
- Su, Y.-Q., et al. (2012a). MARF1 regulates essential Oogenic processes in mice. *Science*, 335, 1496–1499.
- Su, Y.-Q., Sun, F., Handel, M. A., Schimenti, J. C., & Eppig, J. J. (2012b). Meiosis arrest female 1 (MARF1) has nuage-like function in mammalian oocytes. *Proceedings of the National Academy of Sciences*, 109, 18653–18660.
- Sullivan, B. A., & Karpen, G. H. (2004). Centromeric chromatin exhibits a histone modification pattern that is distinct from both euchromatin and heterochromatin. *Nature Structural & Molecular Biology*, 11, 1076–1083.
- Suo, L., et al. (2010). Changes in acetylation on lysine 12 of histone H4 (acH4K12) of murine oocytes during maternal aging may affect fertilization and subsequent embryo development. *Fertility and Sterility*, 93, 945–951.
- Svoboda, P., Stein, P., & Schultz, R. M. (2001). RNAi in mouse oocytes and preimplantation embryos: Effectiveness of hairpin dsRNA. *Biochemical and Biophysical Research Communications*, 287, 1099–1104.
- Swain, J. E., Ding, J., Brautigan, D. L., Villa-Moruzzi, E., & Smith, G. D. (2007). Proper chromatin condensation and maintenance of histone H3 phosphorylation during mouse oocyte meiosis requires protein phosphatase activity. *Biology of Reproduction*, 76, 628–638.
- Vanneste, E., et al. (2009). Chromosome instability is common in human cleavage-stage embryos. *Nature Medicine*, 15, 577–583.
- Vialard, F., et al. (2006). Evidence of a high proportion of premature unbalanced separation of sister chromatids in the first polar bodies of women of advanced age. *Human Reproduction*, 21, 1172–1178.
- Voet, T., et al. (2011). Breakage–fusion–bridge cycles leading to inv dup del occur in human cleavage stage embryos. *Human Mutation*, 32, 783–793.
- Wang, Q., et al. (2006). Histone phosphorylation and pericentromeric histone modifications in oocyte meiosis. *Cell Cycle*, 5, 1974–1982.
- Weaver, J. R., & Bartolomei, M. S. (2014). Chromatin regulators of genomic imprinting. *Biochimica et Biophysica Acta (BBA)—Gene Regulatory Mechanisms*, 1839, 169–177.
- Wiens, G. R., & Sorger, P. K. (1998). Centromeric chromatin and epigenetic effects in kinetochore assembly. *Cell*, 93, 313–316.
- Yang, F., Baumann, C., & De La Fuente, R. (2009). Persistence of histone H2AX phosphorylation after meiotic chromosome synapsis and abnormal centromere cohesion in poly (ADP-ribose) polymerase (Parp-1) null oocytes. *Developmental Biology*, 331, 326–338.
- Yang, F., Baumann, C., Viveiros, M., & De La Fuente, R. (2012). Histone hyperacetylation during meiosis interferes with large-scale chromatin remodeling, axial chromatid condensation and sister chromatid separation in the mammalian oocyte. *The International Journal of Developmental Biology*, 56, 889–899.

Regulation of Oogenesis by Noncoding RNAs

Yunsheng Li, Malavika K Adur, Benjamin J Hale, and Jason W Ross, Iowa State University, Ames, IA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Atresia The degeneration and death of follicles after they have formed, but before they have fully matured.

Estrous or menstrual cycle A series of cyclical physiological changes occurring in the reproductive organs of a female of the non-primate (estrous) or primate (menstrual) species. The cycle involves intricate endocrine, paracrine and autocrine signals within and between the several tissues in the female including the uterus, ovary, hypothalamus and pituitary glands.

Fertilization The events that occur when a sperm cell penetrates an oocyte and subsequently combine haploid genomes through the process of syngamy to produce a single cell zygote. During normal fertilization, the haploid male and female gametes fuse to produce a diploid zygote. This process can be mimicked in a laboratory culture dish and is known as in vitro fertilization (IVF).

Germinal vesicle breakdown (GVBD) The germinal vesicle refers to the nucleus of an oocyte. When the oocyte resumes meiosis in response to the luteinizing hormone surge that occurs prior to ovulation, the nuclear envelope of the germinal vesicle breaks down in a process known as germinal vesicle break down.

Gonadotrophin hormones These are protein hormones secreted by the pituitary gland, called follicle stimulating hormone (FSH) and luteinizing hormone (LH), that play roles in gamete formation and release.

Luteinization The process by which differentiation of the theca and granulosa cells of the post-ovulatory ovarian follicle results in the formation of a corpus luteum (CL). The result is the production of the steroid hormone progesterone required for maintenance of pregnancy in the event of a successful fertilization.

Non-coding RNA A ribonucleic acid molecule that is not translated into protein, but plays important roles in gene regulation.

Ovulation The process in which an ovarian follicle ruptures and the mature oocyte is released from the ovary into the infundibulum and drawn into the oviduct.

Somatic cell nuclear transfer The process when the diploid nucleus of a somatic cell (found in all organs and tissues within an organism) is used to replace the haploid nucleus of an oocyte to produce a zygote. The organism produced is a clone of the organism that donated the somatic cell.

Steroidogenesis The biochemical process used by cells to produce steroid hormones. The granulosa and theca cells of the developing ovarian follicle produce the steroid hormones, estrogen and progesterone, that are involved in gamete development and pregnancy maintenance, respectively.

Transcription A process in which the genetic information on a strand of DNA is copied into an RNA molecule, by the activity of polymerase enzymes.

Introduction

Oogenesis is an important and complex process in mammals facilitating female gamete development and is regulated by numerous intra- and extra-ovarian factors. The process of oogenesis is dependent on the ovarian follicle, the functional unit of the ovary, and the interaction between the oocyte and the surrounding follicular cells. This cellular association is crucial for the oocyte maturation process as well as for follicle development. The use of knockout models has allowed substantial progress in elucidating the factors regulating oocyte and follicle growth and development, and the signaling pathways involved within these processes. Meanwhile, studies describing the roles of regulatory non-coding RNAs (ncRNA), especially microRNA (miRNA), in oogenesis have recently become an area of increased focus. This article summarizes the various roles of ncRNA in oogenesis, focusing on the better known miRNA regulatory mechanisms, gonadotropin regulation, and oocyte development.

Non-Coding RNA

Non-coding RNA are comprised of two main groups. The long RNA species include long intergenic noncoding RNAs (lincRNAs), long non-coding RNAs (lncRNA), and circular RNAs (cirRNAs). Alternatively, the small RNA species include microRNAs (miRNAs), endogenous small interfering RNAs (*endo*-siRNAs), piwi-interacting RNAs (piwiRNAs), small nucleolar RNA (snoRNA), and transfer RNAs (tRNA). Other non-coding, structural RNAs include ribosomal RNAs (rRNAs) and nuclear RNAs that can often be longer than 200 nucleotides.

Biogenesis of Non-Coding RNA

Small regulatory ncRNA differ in origin, length and Argonaute protein partners through which they elicit their biological function. Both miRNA and *endo*-siRNA are processed from double-stranded precursors that eventually results in 20–22 nucleotide mature small RNA. Alternatively, piRNA are generated from long single-stranded precursors into 26–31 nucleotide functional molecules in a Dicer-independent manner.

MicroRNA can be produced following transcription and processing from their own genes in addition to being processed from the introns or exons of host genes. MicroRNA have two biogenic pathways through which they are produced, canonical and non-canonical. MicroRNA are first transcribed as primary miRNA (pri-miRNA) by RNA polymerase II, and based on sequence complementation within the RNA molecule, secondary RNA structures producing 60–75 nucleotide (nt) hairpins occur within the pri-miRNA. The hairpins are recognized by DGCR8 (DiGeorge syndrome critical region 8), which directs the RNase III enzyme Drosha to cleave the base of the hairpin resulting in a pre-miRNA. Once the pre-miRNA reach the cytosol, they are cleaved by Dicer (an RNase III enzyme) in complex with transactivating response element RNA-binding protein (TRBP), resulting in a functionally mature miRNA. It is the mature miRNA that are capable of being loaded into an RNA induced silencing complex (RISC) and utilized for post-transcriptional gene regulation (PTGR) (Fig. 1).

The biogenesis and mechanism of the piRNA class is less understood than miRNA and *endo*-siRNA. Piwi-interacting RNA are cleaved from long single-stranded RNA precursors in a Dicer independent mechanism. Mature piRNA are approximately 26–31 nt long, and are expressed primarily in germ cells. Long noncoding RNA (lncRNA) are described at their most basic definition as RNA pol II transcribed ncRNA that are greater than 200 nucleotides in length, and they have a role in regulating a diverse range of cellular processes.

Mode of Action of Non-Coding RNA

Following Dicer cleavage, miRNA and *endo*-siRNA interact with the family of Argonaute proteins (AGO1–4) to cause the association of RISC machinery. An Argonaute protein associated with either a miRNA or *endo*-siRNA interacts with target mRNA via

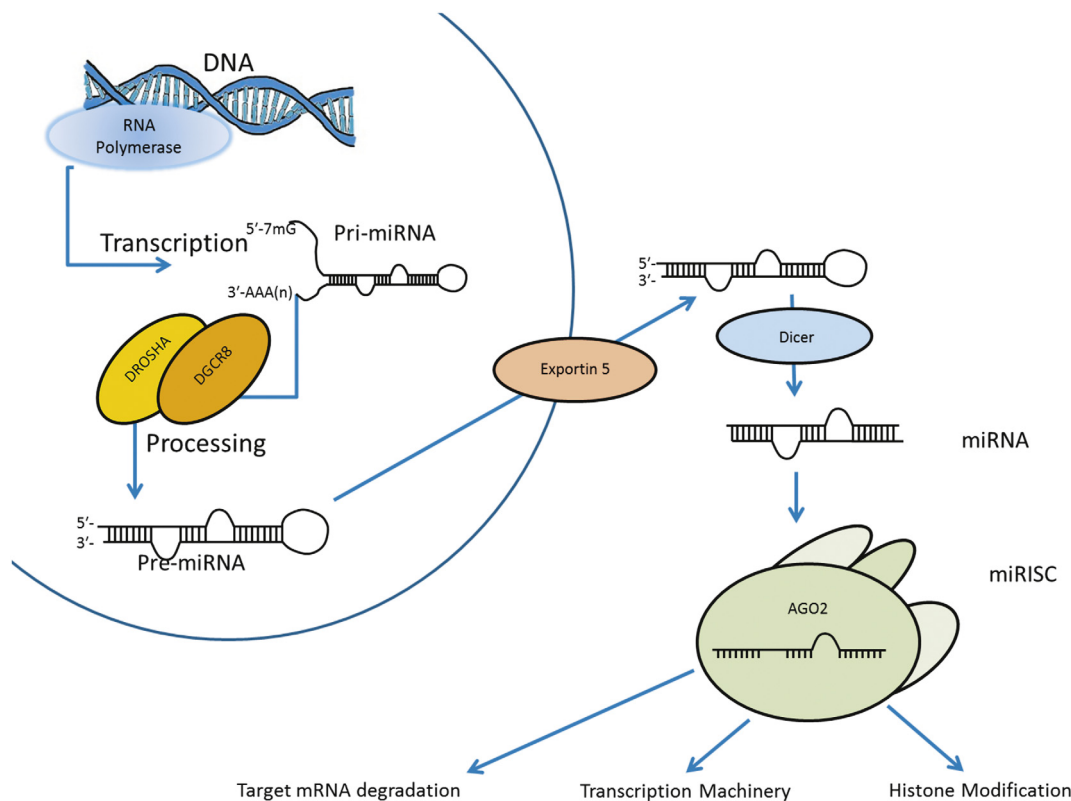


Fig. 1 MicroRNA biogenesis is initiated via the activity of RNA polymerase II resulting in synthesis of a primary miRNA transcript (Pri-miRNA) that is both capped and polyadenylated. The appropriate spatial complementation of specific nucleotides within the transcript results in the formation of hairpin secondary structures that are recognized by the RNA processing complex consisting of DROSHA and DGCR8. The enzymatic activity of this protein complex results in cleavage and removal of the hairpin structure (now considered a pre-miRNA) from the primary transcript. Exportin 5 facilitates the transport of pre-miRNA from the nucleus into the cytoplasm of the cell where it is recognized by Dicer, and the loop is cleaved leaving a short duplex mature miRNA molecule. Upon dissociation, either strand from the duplex miRNA structure can be utilized by the RNA-induced silencing complex to contribute to posttranscriptional gene regulation through impacting mRNA stability and/or translation efficiency in addition to contributing to chromatin modifications to control gene expression. This image is used with permission from Springer Publishing.

complementarity between the small single-stranded RNA molecule and the 3' untranslated region (UTR) of the target mRNA. If complete complementarity exists between the small RNA molecule and target mRNA 3'UTR, then cleavage of the target mRNA can occur. If the complementarity is incomplete the primary mechanism of PTGR occurs through suppression of translation by influencing mRNA interaction with the translational machinery.

PIWI proteins are found to be located in nuclear and/or perinuclear nuage, a structure where piRNA can function to repress genetic elements. The PIWI proteins have an N-terminal PAZ domain followed by the MID (middle) domain, which together recognize and bind the 3' end of piRNA molecules.

Oogenesis

Development of the mammalian oocyte (Fig. 2) begins during fetal life through proliferation of primordial germ cells by mitosis. Mammals are born with a finite number of oocytes that originate from a primordial germ cell pool following migration to the genital ridge during embryonic development. Oogenesis begins with the breakdown of germ cell clusters and the formation of primordial follicles. The oocyte associates with granulosa cells to form primordial follicles that remain arrested in prophase I of meiosis. At puberty, the oocyte in the primordial follicle is triggered to grow under the influence of gonadotropin hormones. It then undergoes numerous molecular and morphological changes in its follicular cell milieu. This process of follicular development is controlled by closely coordinated endocrine and paracrine factors contributed by the developing oocyte and surrounding granulosa and theca cells. In turn, this process is controlled by multiple genes whose expression and interactions are tightly regulated in the oocyte, granulosa and theca cells. Following recruitment and selection, maturing oocytes undergo germinal vesicle breakdown (GVBD) facilitating the progression through meiosis until arrest at metaphase II in most domestic animals. Following GVBD the oocyte is transcriptionally quiescent until after fertilization and the activation of the embryonic genome occurs around the two- to eight-cell stage of development depending upon the species. Distinct sets of transcripts are present in female germ cells, with some maternal mRNAs being translated in the germline to support oocyte formation and maturation, while others are stored in the oocyte and translated during early embryogenesis. Post-transcriptional gene regulation via small ncRNAs has emerged as one of the most important mechanisms to control gene expression in the germline cell and its various developmental stages.

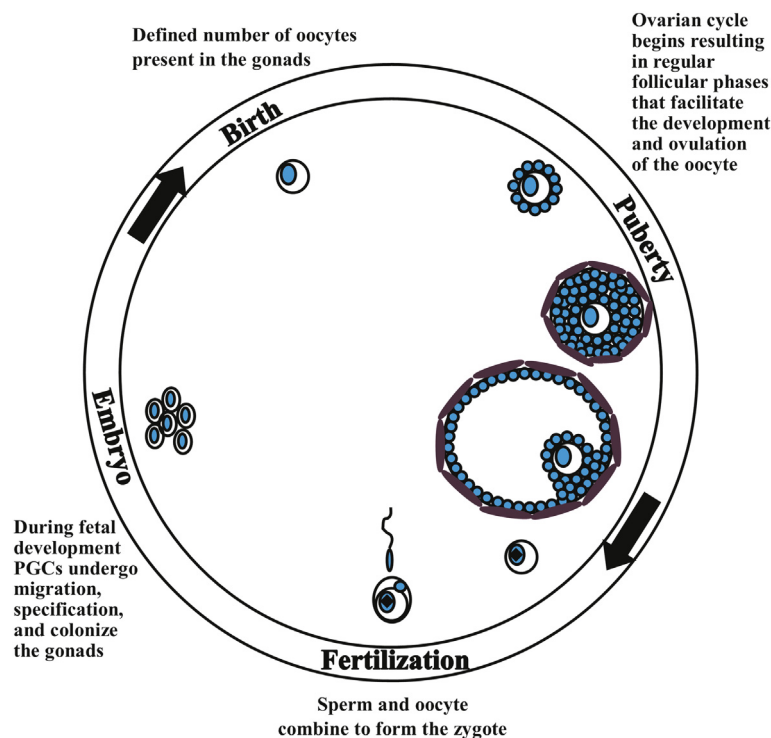


Fig. 2 Oogenesis in mammals begins in the fetal stage. Primordial germ cells colonize the ovary and eventually undergo breakdown of germ cell clusters. The oocyte along with its surrounding granulosa cells forms the primordial follicle, which is arrested in prophase I at birth. At puberty, the active hypothalamus–pituitary–gonadal axis facilitates the development and maturation of follicles and the oocyte within. During the cycle, oocytes within maturing follicles eventually undergo germinal vesicle breakdown and will progress through meiosis until arrested in metaphase II concomitant with ovulation. Meiosis in the oocyte is completed upon fertilization by the sperm. The consequent embryo generates its own set of primordial germ cells that colonize the developing gonads.

Role of Noncoding RNA in Primordial Germ Cell Migration, Development, Specification and Sex Determination

Gametogenesis in mammals begins from primordial germ cells (PGC) that colonize the mesonephros of the developing mammalian embryo and eventually give rise to the testis or ovary. The newborn ovary contains a limited number of oocytes, and with each estrous (domestic animals) or menstrual cycle (primates), a given number of primary oocytes are recruited although only some will undergo maturation and ovulate. The process of gonad formation (in either sex) and oocyte development and maturation is characterized by significant changes in the gene expression profiles. Small regulatory molecules such as miRNA and *endo*-siRNA are present in germ cells and play crucial roles in the regulation of various events in the life of germ cells, including the specification of germ cells, migration and gonad colonization, protection and/or alteration of the genome, and sex-specific differentiation into competent gametes.

Across mammalian species, PGCs migrate to the gonad, begin to associate with somatic cells and commit to a sex-specific fate. MicroRNA clusters which promote cell cycle progression are crucial for primordial germ cell migration, defects of which cause significant reduction in the number of PGCs colonizing the embryonic gonads. Dysregulation of miRNA activity does not specifically affect PGC specification or migration but does affect the ability of PGCs to proliferate and survive during migration, thus resulting in lower number of PGCs colonizing the fetal gonads. Females that fail to compensate for this loss of PGC migration and specification undergo premature ovarian failure and are often sterile. Conversely, somatic cell loss of small RNAs in mice, results in establishment of larger pool of primordial follicles in the neonate ovary, but these mutants are infertile due to premature follicle maturation and loss of oocytes resulting from defects in granulosa cells.

Once PGCs arrive in the gonad, they begin to commit to their sex-specific fate and are highly regulated by their somatic cell niche environment. MiRNA expression profiling of PGCs colonizing the gonad reveals differential expression patterns of miRNAs between the sexes, and hence points towards miRNA regulation of sex specific PGC differentiation. Differential expression of miRNAs was detected when comparing male and female PGCs in mice, sheep and pig. Primordial germ cell specification in mammals involves the silencing of genes associated with somatic cell differentiation and activation of germ cell-specific genes. During early PGC development and specification, miRNAs are highly dynamic in their expression. This is especially important during the period of transcriptional arrest due to higher gene regulation needs.

Role of Noncoding RNA in Pre-pubertal Oogenesis

MiRNAs are involved in the entire process of ovarian follicle development, including follicle growth, atresia and ovulation. A majority of follicles undergo atresia during folliculogenesis. Differentially expressed miRNAs in healthy, early atretic, and progressively atretic follicles signify the roles of miRNAs in regulating follicle development and atresia. In each stage of follicle development, different growth factors contribute to stage-specific functions in different cell types of the follicle. Various studies have shown that miR-21, miR-125b, let-7a and let-7b are the most abundantly expressed miRNAs across the different follicular development stages. Moreover, mouse postnatal ovaries show dynamic changes of miRNA profiles during specific stages indicating their roles at the different phases of ovarian development. Once females reach sexual maturity, oocytes arrested at prophase I of meiosis are recruited in small numbers by gonadotropin hormones (FSH and LH) to produce mature follicles, and are now exposed to a different milieu of miRNAs.

Role of Noncoding RNA in Post-pubertal Oogenesis

The ovarian follicle is the functional unit of the ovary and comprises of the oocyte and its surrounding follicular cells. Maturation of the oocyte begins at puberty and involves maturation of the entire follicular unit, when primordial follicles in their resting stage, are activated to start development. The different developmental stages of the follicle such as primary, secondary, preantral and antral follicles have between one to more than five layers of cuboidal granulosa cells (Fig. 3). The secondary follicle and later stages also have theca cells that form a layer around the oocyte-granulosa complex. At the antral stage, follicles become gonadotropin-dependent and form large Graafian follicles that eventually ovulate under the influence of these gonadotropins.

Effect of gonadotropins on miRNA expression in the ovary

Oogenesis is tightly regulated by pituitary gonadotropins, viz., follicle stimulating hormone (FSH) and luteinizing hormone (LH). Well-synchronized, sequential actions of FSH and LH are critical for follicle growth and pre-ovulatory steroidogenesis, leading to successful ovulation. The cross-talk between ncRNA and gonadotropins has been shown in numerous studies, wherein FSH and LH modulate miRNA expression in ovarian cells. During folliculogenesis, the expression of miR-143, let-7a and miR-15b is negatively regulated by FSH. Studies show FSH changes miRNA expression in in vitro cultured rat granulosa cells and ovarian cell line. The ovulation inducing LH surge increases miR-122 expression in the rat ovary and miR-21, miR-132 and miR-212 in the granulosa cells of the mouse ovary. Though both in vivo and in vitro studies have shown that FSH and LH can affect miRNA expression patterns in ovarian cells, how these gonadotropins modify miRNA expression and how this cross-talk regulates ovarian function still needs investigation.

Role of ncRNAs on oocyte development and maturation

The inability to transcribe mRNA during the final stages of oocyte development and potential for PTGR has led numerous laboratories to pursue the characterization of the ncRNA expressed in the maturing oocyte. The function of miRNA and associated enzymes

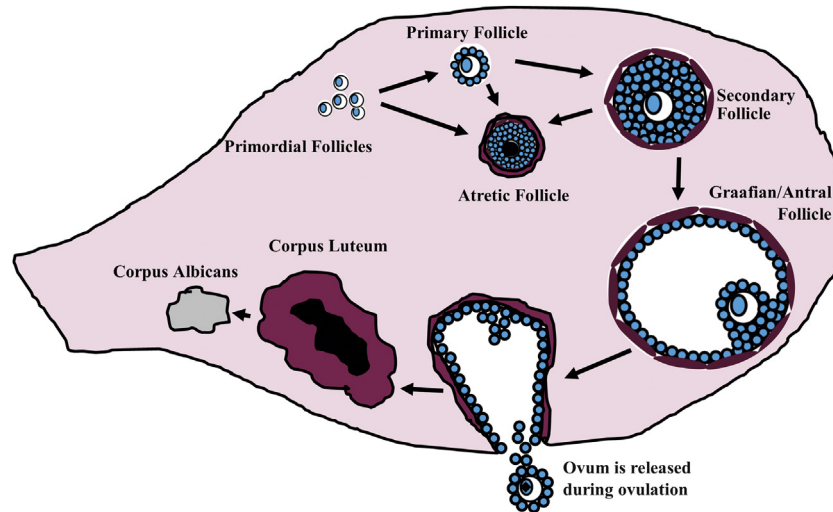


Fig. 3 The ovarian follicle is the functional unit of the ovary. At puberty, under the influence of gonadotropins, the oocyte and its surrounding follicular cells, begin maturation. The primordial follicles in their resting stage are activated to develop into the primary, secondary, preantral and antral follicles. A majority of developing follicles undergo atresia and progress no further. The follicles that successfully continue through the maturation stages, add on granulosa cells that surround the developing oocyte. The later stages also have theca cells that form a layer around the oocyte-granulosa cell unit. The large Graafian follicles eventually ovulate under the influence of pituitary gonadotropins in each reproductive cycle, to form the corpus luteum. This structure enables pregnancy maintenance if the ovulated oocyte is successfully fertilized to form an embryo. In the absence of a viable embryo, the corpus luteum degenerates and the entire process of follicular maturation is repeated in the next reproductive cycle.

within the developing oocyte were determined using oocyte-specific knock out models. Results show loss of *Dicer* has an observable effect on oocytes and both biogenic enzymes are required for healthy oogenesis. Similarly, use of RNA interference knockdown of *Dicer1* in GV stage mouse oocytes significantly decreases both the proportion of oocytes reaching the MII stage and the expression of dicer protein-dependent miRNAs. Using the same knockout system, it was shown that oocyte-specific deletion of *Drosha* demonstrated no discernible phenotypic change. And that *Dgcr8*, a RNA-binding protein critical only for the canonical miRNA biogenesis pathway, is not required for the maturation of mouse oocytes. Collectively, these results suggest that miRNA produced via the canonical pathway in the oocyte are not required for the production of phenotypically normal oocytes in mice.

Endo-siRNA, on the other hand, might have a more critical role in oocyte development and maturation. MicroRNAs and siRNAs silence gene expression post-transcriptionally by guiding an Argonaute protein to their targets. Catalytically inactive knock-in allele of *Ago2* (*Ago2ADH*) in which the catalytic DDH motif is mutated to ADH, inhibiting endonucleolytic cleavage without affecting small RNA binding and disrupts the function of *endo*-siRNAs in oocytes. Oogenesis and hormonal response are normal in *Ago2ADH* oocytes, but meiotic maturation is impaired, with severe defects in spindle formation and chromosome alignment that lead to meiotic catastrophe. In pig oocytes, a microinjection technique to knockdown *DICER* reduces the proportion of oocytes that develop to meiosis II (MII) stages and was significantly lower than oocytes with *DROSHA* and *DGCR8* deficiency.

Role of ncRNA in granulosa cumulus cells

Granulosa cell proliferation and function are essential in follicle development, maturation, and atresia. Several miRNAs are known to regulate granulosa cell proliferation, steroidogenesis and apoptosis. Conditional knockout mouse models help us understand miRNA function in granulosa cell development and oogenesis. Deletion of *Dicer1* results in female sterility and multiple reproductive defects though *Dicer* deficient female mice display normal mating behavior and embryo quality. Comparative analysis of the ovarian tissue from 8-day, 8-week and 8-month old conditional *Dicer1* deleted and wild type mice, showed increased primordial follicle pool endowment, accelerated early follicle recruitment and more degenerate follicles in the *Dicer1* knockout mice. Furthermore, the genes involved in follicle development showed significantly different expression between the *Dicer1* and wild-type mouse ovaries. Female infertility was also the major phenotype observed in a *Dicer1* depletion model and the CL had reduced vascularization. These results together suggest that miRNAs are important regulators of female reproductive tract development and fertility.

Role of ncRNA on regulation of steroid biogenesis

The growth and development of the oocyte is dependent on its bidirectional cross-talk with the surrounding cumulus or granulosa and theca cells. Granulosa and theca cells facilitate steroidogenesis which is critical for successful ovarian folliculogenesis, ovulation, luteogenesis, and oocyte maturation. MicroRNA mediated regulation of steroidogenesis in granulosa cells also potentially affects oocyte maturation. Though no direct studies have implicated a role for ncRNA in theca cells, miRNA function in granulosa cells has been evaluated with different conditional knock out models and in vitro experiments. In an in vitro human granulosa cell culture system treated with miRNA precursors, miRNAs have been shown to enhance or inhibit ovarian progesterone, androgen and estrogen synthesis. Moreover, overexpression of miR-378 decreases estradiol production by regulating aromatase in porcine

granulosa cells, and miR-34a and miR-320 inhibit estradiol release from human granulosa cells and murine ovaries, respectively. In contrast, overexpression of miR-132 promotes estradiol synthesis in mouse granulosa cells via translational repression of Nuclear receptor related 1 (Nurr1). Similarly, overexpression of miR-383 enhances estradiol release from granulosa cells by targeting RBMS1. Recent results show that overexpression of miR-378 in porcine cumulus cells suppress oocyte maturation and embryo developmental potential by inhibiting aromatase expression. Overexpression of miR-224 of cumulus cells leads to the impairment of cell expansion and correlated gene expression, further delaying oocyte maturation and decreasing oocyte developmental potential. Besides the effects on steroidogenesis, miRNA also regulate granulosa cell proliferation and apoptosis.

Ovulation and luteinization

Though the stage-specific expression of miRNAs in the corpus luteum (CL) has been reported, miRNA function in the processes of ovulation and luteal tissue formation, maintenance, rescue or regression is poorly understood. Only few functional studies have been conducted on miRNA in the CL. Microarray analysis of differentially expressed miRNAs in non-regressed and regressed bovine CL shows that miRNA-378 may suppress luteal cell apoptosis by targeting the interferon gamma receptor 1 gene. Moreover, miR-34a expression increases during the follicular to luteal transition in cattle and sheep. Increasing the concentration of miR-34a in in vitro cultured luteal cells results in an increase in progesterone production and decrease in proliferation of steroidogenic cells, which could be due to miR-34a inhibition of both Notch1 and YingYang1 (YY1). *Neat1* is a lncRNA species that has a higher expression level in luteal tissue. *Neat1* knockout mice show CL dysfunction concomitant with reduced progesterone production, thus leading to decreased fertility despite normal ovulation.

Dysregulation of Noncoding RNA Impacting Oogenesis

Abnormal expression of ovarian miRNAs has been associated with numerous reproductive tract disorders. Misregulation of germ cell development at any stage can have deleterious outcomes in oogenesis. MicroRNA have been detected in human and bovine IVF culture media, and differential expression correlated with embryo quality and pregnancy outcomes. MicroRNA expression can also be utilized to explain low cloning efficiency and the high incidence of abnormalities in embryos produced via somatic cell nuclear transfer (SCNT). These biomarker miRNA can also be used to evaluate oocyte competence which is known to decline with ovarian age. Characterization of miRNA impacted by age of the female would be a valuable resource to better understand the biology of aging oocytes and consequent genetic abnormalities. A similar situation is observed in cases of premature ovarian failure (POF), which is characterized by a reduced follicle pool and follicular dysfunction in the ovary manifesting with endocrine and reproductive disorders. The mouse knock-out models for Dicer and Drosha have shown compromised folliculogenesis and defective meiosis, CL insufficiency, POF, and infertility in the adult ovary. Studies have also correlated POF with dysregulated expression of miRNAs in serum and plasma. Corpus luteum insufficiency associated with miRNA dysregulation is also seen in cases of PCOS and obesity. Although the clinical and biochemical signs of PCOS are typically heterogeneous, abnormal folliculogenesis is considered a common characteristic. Aberrant expression of several miRNA has been correlated with the PCOS phenotype, including roles in carbohydrate metabolism, pancreatic beta cell function, insulin resistance and steroid synthesis. These differentially expressed miRNAs are also involved in various signaling pathways that regulate hormone and energy metabolism. This involvement in hormone regulation was shown in a dihydrotestosterone induced rat PCOS model.

It is now also accepted that the miRNAs involved in early embryogenesis share functional similarities in the process of cancer development. These miRNAs can act as tumor suppressors but can also be oncogenes. Multiple studies have identified strong marker miRNA for the different types and stages of ovarian cancer in humans. Furthermore, studies show that tumor cells, derived from effusions of ovarian cancer patients, reflect a distinct miRNA expression signature, associated to metastatic progression. These circulating miRNA have the potential to be specific biomarkers and possibly prognostic aids.

Conclusion

Several studies have revealed preferential expression of miRNAs in the ovary of different species, signifying that they have an important role in ovarian functions. Better understanding of the molecular basis of ncRNA function in oogenesis has provided valuable insight into mammalian reproduction. Oogenesis is a well-coordinated process involving molecular cross-talk between the various somatic cell types and the developing gamete in the follicle. MicroRNA have a definite, synergistic function in the cross-talk between these cell types and the specific regulatory mechanism in the follicle development process. Our increasing understanding of oogenesis, miRNA regulatory networks and the advances in detection, analyses and correlation of miRNA expression specific to ovarian events will help pave the way for newer diagnostic, prognostic and therapeutic strategies for improving female reproductive ability.

Further Reading

Assou, S., Al-edani, T., Haouzi, D., Philippe, N., Lecellier, C. H., Piquemal, D., Commes, T., Ait-Ahmed, O., Dechaud, H., & Hamamah, S. (2013). MicroRNAs: New candidates for the regulation of the human cumulus-oocyte complex. *Human Reproduction*, 28, 3038–3049.

- Banisch, T. U., Goudarzi, M., & Raz, E. (2012). Small RNAs in germ cell development. *Current Topics in Developmental Biology*, 99, 79–113.
- Cook, M. S., & Belloch, R. (2013). Small RNAs in germline development. *Current Topics in Developmental Biology*, 102, 159–205.
- Dallaire, A., & Simard, M. J. (2016). The implication of microRNAs and endo-siRNAs in animal germline and early development. *Developmental Biology*, 416, 18–25.
- Fiedler, S. D., Carletti, M. Z., Hong, X., & Christenson, L. K. (2008). Hormonal regulation of microRNA expression in periovulatory mouse mural granulosa cells. *Biology of Reproduction*, 79, 1030–1037.
- Hale, B. J., Yang, C. X., & Ross, J. W. (2014). Small RNA regulation of reproductive function. *Molecular Reproduction and Development*, 81, 148–159.
- Hale, B. J., Keating, A. F., Yang, C. X., & Ross, J. W. (2015). Small RNAs: Their possible roles in reproductive failure. In , vol. 868. *Male Role in Pregnancy Loss and Embryo Implantation Failure. Advances in Experimental Medicine and Biology* (pp. 49–79). Switzerland: Springer.
- Hossain, M. M., Cao, M., Wang, Q., Kim, J. Y., Schellander, K., Tesfaye, D., & Tsangm, B. K. (2013). Altered expression of miRNAs in a dihydrotestosterone-induced rat PCOS model. *Journal of Ovarian Research*, 6, 36.
- Karakaya, C., Guzeloglu-Kayisli, O., Uyar, A., Kallen, A. N., Babayev, E., Bozkurt, N., Unsal, E., Karabacak, O., & Seli, E. (2015). Poor ovarian response in women undergoing in vitro fertilization is associated with altered microRNA expression in cumulus cells. *Fertility and Sterility*, 103, 6.
- Khan, H. A., Zhao, Y., Wang, L., Li, Q., Du, Y. A., Dan, Y., & Huo, L. J. (2015). Identification of miRNAs during mouse postnatal ovarian development and superovulation. *Journal of Ovarian Research*, 8, 44.
- Lei, L., Jin, S. Y., Gonzalez, G., Behringer, R. R., & Woodruff, T. K. (2010). The regulatory role of Dicer in folliculogenesis in mice. *Molecular and Cellular Endocrinology*, 315, 63–73.
- Li, Y., Fang, Y., Liu, Y., & Yang, X. K. (2015). MicroRNAs in ovarian function and disorders. *Journal of Ovarian Research*, 8, 51.
- Maalouf, S. W., Liu, W. S., & Pate, J. L. (2016a). MicroRNA in ovarian function. *Cell and Tissue Research*, 363, 7–18.
- Maalouf, S. W., Smith, C. L., & Pate, J. L. (2016b). Changes in MicroRNA expression during maturation of the bovine corpus luteum: Regulation of luteal cell proliferation and function by MicroRNA-34a. *Biology of Reproduction*, 94, 71.
- Menon, B., Gulappa, T., & Menon, K. M. (2017). Molecular regulation of LHCG expression by miR-122 during follicle growth in the rat ovary. *Molecular and Cellular Endocrinology*, 15, 81–89.
- Mondou, E., Dufort, I., Gohin, M., Fournier, E., & Sirard, M. A. (2012). Analysis of microRNAs and their precursors in bovine early embryonic development. *Molecular Human Reproduction*, 18, 425–434.
- Murchison, E. P., Stein, P., Xuan, Z., Pan, H., Zhang, M. Q., Schultz, R. M., & Hannon, G. J. (2007). Critical roles for Dicer in the female germline. *Genes & Development*, 21, 682–693.
- Pan, B., Toms, D., Shen, W., & Li, J. (2015). MicroRNA-378 regulates oocyte maturation via the suppression of aromatase in porcine cumulus cells. *American Journal of Physiology. Endocrinology and Metabolism*, 308, 525–534.
- Sanchez, F., & Smitz, J. (2012). Molecular control of oogenesis. *Biochimica et Biophysica Acta*, 1822, 1896–1912.
- Sterin, P., Rozhkov, N. V., Li, F., Cárdenas, F. L., Davydenko, O., Vandivier, L. E., Gregory, B. D., Hannon, G. J., & Schultz, R. M. (2015). Essential role for endogenous siRNAs during meiosis in mouse oocytes. *PLOS Genetics*, 19, e1005013.
- Suh, N., Baehner, L., Moltzahn, F., Melton, C., Shenoy, A., Chen, J., & Belloch, R. (2010). MicroRNA function is globally suppressed in mouse oocytes and early embryos. *Current Biology*, 20, 271–277.
- Yang, C. X., Du, Z. Q., Wright, E. C., Prather, R. S., Rothschild, M. F., & Ross, J. W. (2012a). Small RNA profile of cumulus oocyte complex and early embryos by deep sequencing in the pig. *Biology of Reproduction*, 87, 117.
- Yang, C. X., Wright, E. C., & Ross, J. W. (2012b). Expression of RNA binding proteins DND1 and FXR1 in the porcine ovary and during oocyte maturation and early embryo development. *Molecular Reproduction and Development*, 79, 541–552.
- Yuan, S., Schuster, A., Tang, C., Yu, T., Ortogero, N., Bao, J., Zheng, H., & Yan, W. (2016). Sperm-borne miRNAs and endo-siRNAs are important for fertilization and preimplantation embryonic development. *Development*, 143, 635–647.

Polar Body Extrusion and Ovulation

Eran Gershon, Agricultural Research Organization, Rishon LeZion, Israel
Nava Dekel, Weizmann Institute of Science, Rehovot, Israel

© 2018 Elsevier Inc. All rights reserved.

Introduction

Generation of a haploid nucleus is achieved by the two gametes, oocyte and sperm, during the meiotic cell cycle. Unlike in sperm, meiosis in oocytes is a protracted process, initially arrested at the diplotene stage of the first prophase. Meiotically-arrested oocytes are characterized by diffuse chromosomes surrounded by a nuclear membrane known as germinal vesicle (GV). Reinitiation of meiosis involves dissolution of the nuclear membrane, referred to as germinal vesicle breakdown (GVB), chromosome condensation, formation of the spindle of the first metaphase (MI) and its migration to the oocyte cortex, along with chromosomes segregation. Oocytes complete the first meiotic division by elimination of one set of homologous chromosomes and the formation of the first polar body (PBI) that is immediately followed by their maturation into unfertilized eggs, arrested at the second meiotic metaphase (MII). The ready to be fertilized MII oocytes are released from the ovarian follicle into the oviductal ampulla during ovulation. The meiotic cell cycle in the oocyte is not concluded before fertilization; sperm penetration triggers the segregation of sister chromatids and the extrusion of the second PB (Fig. 1). This chapter summarizes our present knowledge related to the control of meiosis in oocytes with a particular focus on the mechanisms underlying PBI extrusion. Improper execution of the first meiotic division is a major cause for aneuploidy that might result in severe genetic malformations (Hassold and Hunt, 2001).

Regulation of Meiosis in Oocytes

Resumption of meiosis in the oocyte is regulated by intracellular levels of cAMP (Cho et al., 1974; Dekel and Beers, 1978). Unlike most cellular systems, in which the concentrations of cAMP at any given moment represent the balance between its synthesis and degradation, the major contribution of this inhibitory nucleotide in oocytes is uniquely attained by gap junctional-mediated supply from the cumulus/granulosa cells (Dekel et al., 1981). Furthermore, degradation of cAMP, performed by the oocyte specific phosphodiesterase 3A (PDE3A), is predominantly regulated by the transfer of the PDE3A inhibitor, cGMP, from this same somatic compartment of the ovarian follicle (Vaccari et al., 2009). As maintenance of meiotic arrest is heavily dependent on established cell-to-cell communication, it is the closure of gap junctions within the ovarian follicle, mediated by luteinizing hormone (LH), which stimulates the oocyte to resume meiosis (Dekel et al., 1981; Sela-Abramovich et al., 2005; Sherizly et al., 1988; Norris et al., 2008). This response to LH stops the transfer of both cyclic nucleotides, cAMP and cGMP, relieving meiotic arrest.

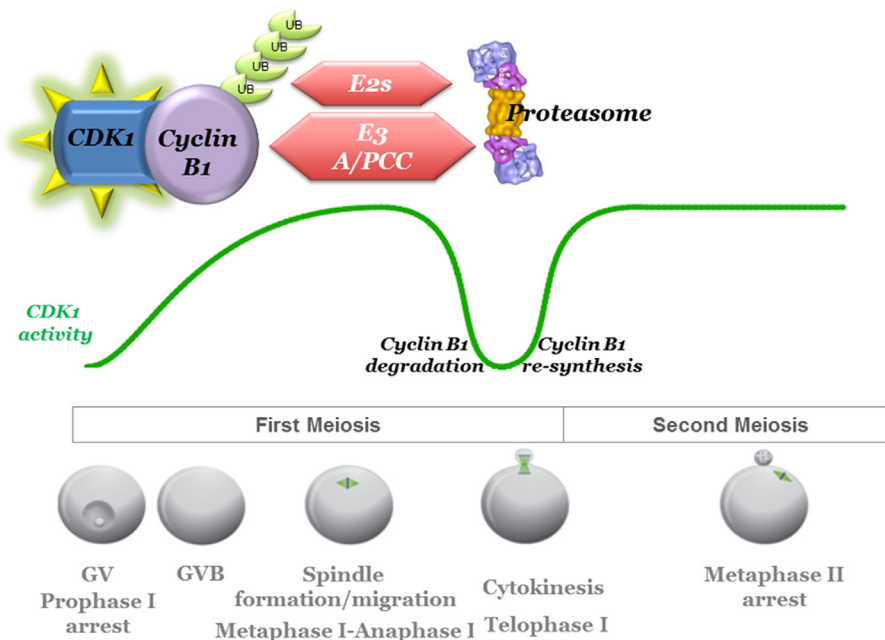


Fig. 1 The master regulator of meiosis in oocytes, CDK1 is activated upon its heterodimerization with cyclin B1. The activity of CDK1 is elevated at reinitiation of meiosis, reaching its maximum at MI. A sharp drop follows this rise in CDK1 activity that occurs due to degradation of cyclin B1 executed by the proteasome and mediated by E3, also known as APC/C. This enzyme decorates cyclin B1 by a polyubiquitin chain, tagging it for degradation.

Cyclin Dependent Kinase (CDK1)

The master regulator of meiosis in oocytes is the cyclin dependent kinase (CDK1), an enzyme that is activated by a series of phosphorylation-dephosphorylation events followed by its heterodimerization with cyclin B1. Activation of CDK1 at the onset of meiosis is inhibited by cAMP (Choi et al., 1991; Fulka et al., 1992). This inhibitory effect of cAMP on CDK1 activation is achieved by the prevention of CDK1 dephosphorylation (Choi et al., 1991; Goren and Dekel, 1994) combined with the inhibition of cyclin B1 synthesis, minimizing its availability (Hampl and Eppig, 1995). Upon the response of the follicle to LH, intraoocyte levels of cAMP drop allowing both, CDK1 dephosphorylation and cyclin B1 accumulation. The resulting activated CDK1 then stimulates reinitiation of the meiotic division (Fig. 1).

Reinitiation of meiosis is characterized by chromosome condensation and the alignment of pairs of homologous chromosomes on the equator of a centrally localized MI spindle. Upon entry into anaphase, characterized by homologous chromosome separation, the MI spindle migrates to the oocyte periphery. The first meiotic division is completed by cytokinesis, and culminates by the formation of PBI. The extrusion of PBI is immediately followed by entry of the oocyte into MII (Fig. 1). Metaphase to anaphase transition and PBI extrusion is absolutely dependent on CDK1 inactivation (Choi et al., 1991; Hampl and Eppig, 1995; Hashimoto and Kishimoto, 1988).

Lowering the activity of CDK1 prior to PBI extrusion is subsequent to degradation of cyclin B1, which is subjected to regulation by the ubiquitin–proteasome pathway. In addition to cyclin B1, this machinery is also responsible for the elimination of securin (Hagting et al., 2002). Proteasomal degradation of securin relieves its inhibitory effect on separase activity allowing the cleavage of the cohesion molecules and resulting in disassociation of pairs of homologous chromosomes (Kudo et al., 2006; Terret et al., 2003; Herbert et al., 2003; Holland and Taylor, 2006).

Degradation of these regulatory proteins, executed by the proteasome–ubiquitin pathway is mediated by particular E2s, such as E2C, E2S and E2D3 (Ben-Eliezer et al., 2015) and the specific E3, also known as APC/C (Jones, 2011). These enzymes decorate protein substrates by a polyubiquitin chain, thus tagging them for degradation (Karabinova et al., 2011; Wickliffe et al., 2009). Upon inhibition of the proteasome and prevention of CDK1 inactivation oocytes arrest at MI and fail to extrude PBI (Terret et al., 2003; Herbert et al., 2003; Ben-Eliezer et al., 2015; Josefsberg et al., 2000; Pomerantz et al., 2012). Interestingly, inactivation of CDK1 along with proteasomal inhibition allowed the extrusion of PBI (Pomerantz et al., 2012). These findings suggest that inactivation of CDK1 is not only necessary but also sufficient for PBI formation (Fig. 2).

Entry into the second meiotic division is dependent on reactivation of CDK1 that is made possible due to another round of cyclin B1 synthesis. The elevated activity of CDK1 keeps the oocytes arrested at metaphase II (Fig. 1).

The MAPK Signaling Pathway

The MAPK pathway represents a sequential phosphorylation events executed by MAPK kinase kinase (MAP3K), MEK, and ERK (Shaul and Seger, 2007). The upstream regulator of MEK in oocytes is MOS (Goldman et al., 1987). It has been shown that activated ERK takes part in the formation of the contractile ring responsible for cytokinesis in pig oocytes (Lee et al., 2000), however, this was not confirmed in mice (Hatch and Capco, 2001). Nevertheless, strong evidence for a role of MAPK pathway in regulating the asymmetric cell division, characteristic to PB formation, has been reported. Activation of the MAPK pathway, taking place in oocytes extruding PBI (Verlhac et al., 1994), affects microtubules dynamics (Verlhac et al., 1996) and is necessary for localization of ERK

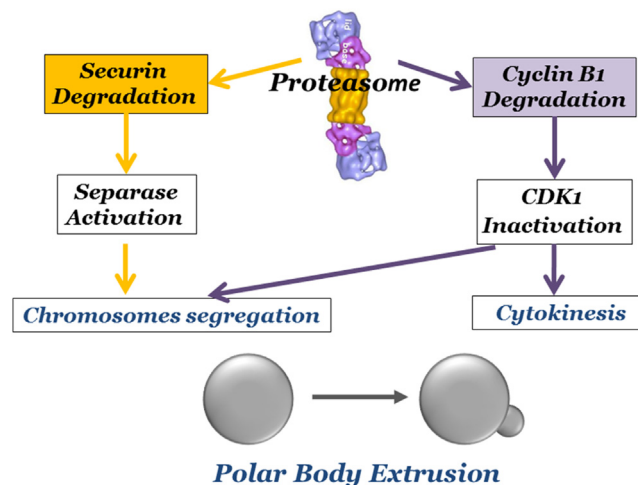


Fig. 2 Extrusion of PBI consists of cytokinesis and chromosome segregation, both subjected to the control of proteasomal degradation. One protein target for the proteasome is cyclin B1, the degradation of which leads to CDK1 inactivation and cytokinesis, whereas degradation of securin brings about chromosome segregation. CDK1 inactivation along with proteasomal inhibition, allowed PBI formation by a mechanism that apparently involves direct activation of separase.

and MEK1/2 on the spindle poles and its migration. Along this line, ablation of the upstream c-mos resulted in inhibition of spindle migration (Verlhac et al., 2000), altered cleavage plane and the production of an abnormally large PBI (Choi et al., 1996). Knocking down GM-130, a *cis*-Golgi protein, colocalizing with phosphorylated MEK at the spindle poles, resulted in the extrusion of a large PBI and elongated spindle (Zhang et al., 2011). In agreement with the role of MAPK pathway in spindle formation, it has been shown that inhibition of MEK1/2 during the first anaphase prevented the contraction of the spindle microtubules and reduced its length (Pomerantz et al., 2012).

Spindle Migration

Extrusion of PBI represents a particular case of asymmetric cell division. This phenomenon is achieved upon localization of the spindle at the cell periphery. Several studies have shown that the central spindle is involved in the positioning of the cleavage furrow (Bonaccorsi et al., 1998; Glotzer, 2005; Rappaport, 1985); therefore, an off-center spindle leads to asymmetric division as occurs during meiosis in oocyte. As mentioned previously reinitiation of the first meiosis is characterized by GVB immediately followed by chromosome condensation (Fig. 1). Concomitantly, a massive organization of microtubules around the chromosomes leads to the formation of a bipolar spindle (Schuh and Ellenberg, 2007). At metaphase of the first round of meiosis, the spindle is centrally localized (Longo and Chen, 1985). It is only later, towards the extrusion of PBI that the spindle migrates to the oocyte cortex (Schuh and Ellenberg, 2008). Once the spindle reached the cortex its axis is arranged at the appropriate orientation to certify the correct separation of the pairs of homologous chromosomes between the oocyte and PBI, establishing the conditions for an asymmetric cell division.

The meiotically-arrested oocyte is covered by a dense population of microvilli. Once the spindle approaches the oocyte periphery these microvilli disassemble (Longo and Chen, 1984; Tremoleda et al., 2001). In addition, actin filaments (F-actin) become denser and accumulate under the oocyte membrane to form the “actin cap” (Schuh and Ellenberg, 2008; Longo and Chen, 1984; Tremoleda et al., 2001). Actin microfilaments are key players in the completion of spindle migration (Longo and Chen, 1985; Maro et al., 1986). The major member of the actin family that regulates the spindle formation is FORMIN2 (Dumont et al., 2007a). FORMIN2 null oocytes fail to position the metaphase spindle during meiosis I and do not form PBI (Leader et al., 2002). A later study showed that FORMIN2 null oocytes failed to extrude PBI also due to mislocalization of myosin II (Dumont et al., 2007a). Myosins are actin-associated motor proteins that surround the F-actin in the cortex. By forming an actomyosin ring, myosins participate in spindle migration (Brunet and Maro, 2005). Myosin 2 was found to concentrate on the spindle poles (Simerly et al., 1998) and myosin 10, the transcripts of which are abundant in mouse oocytes (Evsikov et al., 2006), is necessary for spindle anchoring to the cortex (Weber et al., 2004). Myosin activation occurs by myosin light chain kinase (MLCK, Simerly et al., 1998), which is regulated by MAPK and its upstream c-mos (Brunet and Maro, 2005). The deletion of c-mos prevents the translocation of the spindle to the oocyte cortex, resulting in an altered cleavage plane and subsequent formation of an abnormally large polar body (Choi et al., 1996). The chromosomes themselves also regulate spindle migration and cortical organization, even in the absence of microfilaments (Maro and Verlhac, 2002). This ability of the chromosomes is attributed to RAN, a small GTPase (Dumont et al., 2007b). Upon reinitiation of meiosis, a gradient of GTP-bound RAN (RAN-GTP) is formed around the chromosomes (Zheng, 2004). This gradient is generated due to the localization of its nucleotide exchange factor RCC1 on the chromatin and the diffusion of GTPase activating protein throughout the cytoplasm.

Chromosome Segregation

Unlike the mitotic and the meiotic MII, where sister kinetochores should oppose each other to segregate to different poles, at MI, sister kinetochores must organize and orient side by side to the same pole (Brar and Amon, 2008), requiring sequential loss of cohesion between the chromosomes. The cohesion between the chromosomes is sustained by cohesin, a multi-unit protein, which includes rec8. Chromosome separation at the first anaphase uniquely requires the removal of cohesion, which is, restricted to the chromosome arms, while maintaining the association between sister chromatids (Brar and Amon, 2008). As mentioned previously, the proper chromosome segregation at the end of MI and the extrusion of one set of chromosomes with PBI, is regulated by proteasomal degradation of securin (Hagting et al., 2002). Securin is an inhibitor of separase, an enzyme that cleaves rec8 on chromosome arms, leading to cohesion removal, resolving the chiasmata and allowing homologous chromosome segregation (Kudo et al., 2006; Terret et al., 2003). The cohesion at the centeromeres remains unharmed at MI, guarantying that sister chromatids stay attached until the second metaphase (Terret et al., 2003; Zou et al., 1999).

As mentioned previously, cyclin B1 degradation and the CDK1 inactivation are necessary for PBI extrusion. Inactivation of CDK1 is apparently also critical for chromosomes segregation. The CDK1-cyclin B1 complex binds the phosphorylated separase (Holland and Taylor, 2006; Gorr et al., 2005), thus preventing separation of the homologous chromosomes, blocking PBI extrusion (Gorr et al., 2006). Indeed, expression of non-degradable cyclin B1 in oocytes inhibits chromosome segregation (Herbert et al., 2003). Similarly, pharmacological inactivation of CDK1 along with proteasomal inhibition allowed the extrusion of PBI (Pomerantz et al., 2012).

Protein Degradation: The Ubiquitin–Proteasome Pathway

Oocytes treated with a proteasome inhibitor arrest at MI, exhibiting aborting attempts to extrude PBI (Josefsberg et al., 2000). As mentioned previously, the cellular signal for protein degradation is provided by substrate polyubiquitination. This posttranslational modification is facilitated by a three sequential step-operation executed by a multiple enzyme system as follows. The initial binding of ubiquitin by the ubiquitin-activating enzyme E1 is proceeded by its transfer to a ubiquitin-conjugated enzyme, E2 and completed by its relocation to the substrate via E3, the ubiquitin protein ligase, also known as APC/C (Karabinova et al., 2011; Wickliffe et al., 2009).

The APC/C is a protein complex, the main co-activator of which in meiosis is CDC2 (Peters, 2006). At MI, the APC/C^{CDC20} complex targets securin and cyclin B1 for degradation thus allowing PBI extrusion (Fig. 1) (Reis et al., 2007). The APC/C activity is rigorously regulated during cell division to avoid cell cycle progression prior to the completion of chromosomes condensation and their proper alignment on the spindle equator. This strict regulation is achieved by the spindle assembly checkpoint (SAC), an array of proteins including Mad1, Mad2, Bub1, Bub3, BubR1 and Mps1. This checkpoint ensures that the APC/C activation does not occur before all the kinetochores are properly connected to the spindle. Once the alignment of all chromosomes is accomplished, SAC is released, inhibition of APC/C activity is removed, cyclin B1 and securin are degraded and PBI is extruded (Homer et al., 2005; Leland et al., 2009). Cyclin B1 and securin are the major proteins degraded by the proteasome. However, numerous other meiotic proteins such as Cdc20, Cdc25, Polo-like kinase (PLK-1), ECT2 and C-MOS are subjected to proteasomal degradation (Karabinova et al., 2011).

Ubiquitin, a 76 amino acid polypeptide entails seven lysines, each one of which serves as the acceptor site for the next ubiquitin attachment. The type of ubiquitin signal depends on the lysine residue the chain is assembled on (Hershko and Ciechanover, 1998). Chains linked on lysine48 (K48) signal for degradation whereas linkage on K63 usually signals for DNA repair, inflammation and more (Wickliffe et al., 2009). Mitotic substrates, including APC/C substrates were demonstrated to be tagged for degradation by K11 chains (Jin et al., 2008; Kirkpatrick et al., 2006). Ubiquitin K11 and not K48, was also reported to be the chain topology relevant for PBI extrusion (Pomerantz et al., 2012). This last study further demonstrated that ubiquitin K11 chain mediates not only PBI extrusion but also appropriate chromosome segregation. This finding agrees with previous reports that K11 chains mediate degradation of the APC/C, cyclin B1 and securin (Kirkpatrick et al., 2006).

Unlike the large body of information accumulated regarding the ubiquitination enzymes, very little is known about those enzymes that facilitate, deubiquitinating enzymes (DUBs). Nevertheless, their involvement in regulation of the meiotic cell cycle is suggested by inhibition of carboxy-terminal hydrolyse (UCH)-family DUBs, which resulted in perturbed PBI extrusion and abnormal MII spindle formation; these oocyte could not be fertilized (Mtango et al., 2012a,b).

The family of E2 in human contains around 40 members (Huang et al., 2007), with several of which shown to have roles in mitosis (Jin et al., 2008). Nevertheless, it is only recently that E2s were identified in meiosis. In particular, the E2 family member, E2C and E2S were shown to be essential for spindle formation and their depletion inhibited PBI extrusion. Rescuing this phenotype was achieved upon expressing E2C, but not E2T, suggesting E2C indispensability (Ben-Eliezer et al., 2015). The mechanism by which these two E2s mediate the formation PB1 was suggested by an earlier study (Reddy et al., 2007) demonstrating that their depletion prevented the APC/C binding to its coactivator CDK1 as well as the dissociation of Cdc20 from Mad2 and BubR1. Supporting this notion, high levels of E2C circumvent spindle assembly checkpoint (SAC) inhibition of the APC/C, leading to premature cytokinesis (Ben-Eliezer et al., 2015). E2D3 depletion also led to inhibition of PBI extrusion in oocytes (Ben-Eliezer et al., 2015). This E2 interacts with APC/C and ubiquitinates securin and cyclin B1 (King et al., 1995). It is possible that E2D3 replaces, at least in part E2C in initiating ubiquitin chain formation on those proteins. As mentioned above, the K11 is the major ubiquitin chain in PBI extrusion (Pomerantz et al., 2012). Interestingly, U2C, U2S and U2D3 promote K11 chains on APC/C substrates (Kirkpatrick et al., 2006; Dynek et al., 2010).

Cytokinesis

Cytokinesis is a complex process, which involves five protein clusters essential for the conclusion of the physical split of the oocyte and the PBI (Glotzer, 2005). The initial set of proteins includes the MKLP1-Cyk4 complex (centralspindlin complex) together with the Aurora B-incenp-survivin complex. Depletion of INCENP or inhibition of Aurora kinases B and C activates the APC/C complex before chromosomes have properly arranged on the spindle, arresting the oocytes at MI, thus preventing cytokinesis and PBI extrusion (Sharif et al., 2010). On the other hand, overexpression of Aurora B brings about APC/C inactivation and stabilizes securin; this phenotype is completely suppressed by INCENP depletion (Sharif et al., 2010). The second set of proteins belongs to the RhoA pathway including the ECT2 (RhoGEF) and a RhoGAP. This set of proteins activates the third class of proteins, the myosin II, that are the motor of the contractile ring, its regulators and the F-actin filaments (Jordan and Karess, 1997). Incubation of oocytes in the presence of the microfilament inhibitor cytochalasin D prevents cytokinesis, blocking PBI formation (Verlhac et al., 2000; Kubiak, 1991). The interaction of myosin II with actin and the formation of the actomyosin complex at anaphase are regulated by the fourth class of proteins including the formin2-profilin machinery. This protein complex organizes actin filaments that are essential for the contractile ring assembly (Evangelista et al., 2002). The last set of proteins responsible for the completion of cytokinesis involves the membrane fusion machinery that bridges the space remaining after full ingression of the contractile ring. This contraction of the actomyosin upon PBI extrusion takes place at telophase (Deng et al., 2009) and requires inactivation of CDK1 by

ECT2 (Elbaz et al., 2010). The actomyosin-ring contraction leads to protrusion of the cortex cap overlying the spindle. These events are regulated by the small GTPase, CDC42 and its downstream effector Arp2/3 complex, a major actin regulation complex, both detected in the cortex cap (Leblanc et al., 2011).

Ovulation

Ovulation is a multistep process stimulated by the preovulatory surge of the pituitary LH. The ovulatory response to LH includes oocyte maturation (discussed in details previously in this chapter) concomitant with cumulus expansion and its mucification (Dekel and Kraicer, 1978; Rimón-Dahari et al., 2016), culminating in the disintegration of the follicular wall and the release of a fertilizable oocyte (recently reviewed in Rimón-Dahari et al., 2016). Once the oocyte is released, the residual granulosa and thecal cells undergo reprogramming and differentiate into the corpus luteum (CL) in a process defined as luteinization. The major role of the CL is the production and secretion of progesterone that is crucial for pregnancy establishment and maintenance (Stocco et al., 2007). The complex series of events occurring at ovulation inevitably involves specific ovarian cell types, diverse signaling pathways and temporally controlled expression of specific genes (extensively reviewed in Richards, 2007).

Conclusion

Successful completion of the first meiotic division, characterized by the formation of PBI represents the accomplishment of a complex sequence of events, collectively responsible for proper chromosome segregation. Intensive investigation of the mechanisms, which underlie the fidelity of the separation of the nuclear DNA during meiosis, is of high significance for our understanding of aneuploidy. Aneuploidy and its associated severe genetic malformation, represents a compromised meiotic division in oocytes that is particularly common in women at advanced age. In the western society, in which women tend to postpone motherhood, generation of the relevant knowledge in this regard is of particular importance.

References

- Ben-Eliezer, I., Pomerantz, Y., Galiani, D., Nevo, N., & Dekel, N. (2015). Appropriate expression of Ube2C and Ube2S controls the progression of the first meiotic division. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 29, 4670–4681.
- Bonaccorsi, S., Giansanti, M. G., & Gatti, M. (1998). Spindle self-organization and cytokinesis during male meiosis in asterless mutants of *Drosophila melanogaster*. *Journal of Cell Biology*, 142, 751–761.
- Brar, G. A., & Amon, A. (2008). Emerging roles for centromeres in meiosis I chromosome segregation. *Nature Reviews. Genetics*, 9, 899–910.
- Brunet, S., & Maro, B. (2005). Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: Integrating time and space. *Reproduction*, 130, 801–811.
- Cho, W. K., Stern, S., & Biggers, J. D. (1974). Inhibitory effect of dibutyl cAMP on mouse oocyte maturation in vitro. *The Journal of Experimental Zoology*, 187, 383–386.
- Choi, T., Aoki, F., Mori, M., Yamashita, M., Nagahama, Y., & Kohmoto, K. (1991). Activation of p34cdc2 protein kinase activity in meiotic and mitotic cell cycles in mouse oocytes and embryos. *Development*, 113, 789–795.
- Choi, T., Fukasawa, K., Zhou, R., Tessarollo, L., Borror, K., Resau, J., & Vande Woude, G. F. (1996). The Mos/mitogen-activated protein kinase (MAPK) pathway regulates the size and degradation of the first polar body in maturing mouse oocytes. *Proceedings of the National Academy of Sciences of the United States of America*, 93, 7032–7035.
- Dekel, N., & Beers, W. H. (1978). Rat oocyte maturation in vitro: Relief of cyclic AMP inhibition by gonadotropins. *Proceedings of the National Academy of Sciences of the United States of America*, 75, 4369–4373.
- Dekel, N., & Kraicer, P. F. (1978). Induction in vitro of mucification of rat cumulus oophorus by gonadotropins and adenosine 3',5'-monophosphate. *Endocrinology*, 102, 1797–1802.
- Dekel, N., Lawrence, T. S., Gilula, N. B., & Beers, W. H. (1981). Modulation of cell-to-cell communication in the cumulus-oocyte complex and the regulation of oocyte maturation by LH. *Developmental Biology*, 86, 356–362.
- Deng, M., Gao, J., Suraneni, P., & Li, R. (2009). Kinetochore-independent chromosome poleward movement during anaphase of meiosis II in mouse eggs. *PLoS One*, 4, e5249.
- Dumont, J., Million, K., Sunderland, K., Rassinier, P., Lim, H., Leader, B., & Verlhac, M. H. (2007a). Formin-2 is required for spindle migration and for the late steps of cytokinesis in mouse oocytes. *Developmental Biology*, 301, 254–265.
- Dumont, J., Petri, S., Pellegrin, F., Terret, M. E., Bohnsack, M. T., Rassinier, P., Georget, V., Kalab, P., Gruss, O. J., & Verlhac, M. H. (2007b). A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes. *The Journal of Cell Biology*, 176, 295–305.
- Dynek, J. N., Goncharov, T., Dueber, E. C., Fedorova, A. V., Izrael-Tomasevic, A., Phu, L., Helgason, E., Fairbrother, W. J., Deshayes, K., Kirkpatrick, D. S., & Vucic, D. (2010). c-IAP1 and UbcH5 promote K11-linked polyubiquitination of RIP1 in TNF signalling. *The EMBO Journal*, 29, 4198–4209.
- Elbaz, J., Reizel, Y., Nevo, N., Galiani, D., & Dekel, N. (2010). Epithelial cell transforming protein 2 (ECT2) depletion blocks polar body extrusion and generates mouse oocytes containing two metaphase II spindles. *Endocrinology*, 151, 755–765.
- Evangelista, M., Pruyne, D., Amberg, D. C., Boone, C., & Bretscher, A. (2002). Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. *Nature Cell Biology*, 4, 32–41.
- Evsikov, A. V., Graber, J. H., Brockman, J. M., Hampl, A., Holbrook, A. E., Singh, P., Eppig, J. J., Solter, D., & Knowles, B. B. (2006). Cracking the egg: Molecular dynamics and evolutionary aspects of the transition from the fully grown oocyte to embryo. *Genes & Development*, 20, 2713–2727.
- Fulka, J., Jr., Jung, T., & Moor, R. M. (1992). The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. *Molecular Reproduction and Development*, 32, 378–382.
- Glotzer, M. (2005). The molecular requirements for cytokinesis. *Science*, 307, 1735–1739.
- Goldman, D. S., Kiessling, A. A., Millette, C. F., & Cooper, G. M. (1987). Expression of c-mos RNA in germ cells of male and female mice. *Proceedings of the National Academy of Sciences of the United States of America*, 84, 4509–4513.
- Goren, S., & Dekel, N. (1994). Maintenance of meiotic arrest by a phosphorylated p34cdc2 is independent of cyclic adenosine 3',5'-monophosphate. *Biology of Reproduction*, 51, 956–962.

- Gorr, I. H., Boos, D., & Stemmann, O. (2005). Mutual inhibition of separase and Cdk1 by two-step complex formation. *Molecular Cell*, 19, 135–141.
- Gorr, I. H., Reis, A., Boos, D., Wuhr, M., Madgwick, S., Jones, K. T., & Stemmann, O. (2006). Essential CDK1-inhibitory role for separase during meiosis I in vertebrate oocytes. *Nature Cell Biology*, 8, 1035–1037.
- Hagting, A., Den Elzen, N., Vodermaier, H. C., Waizenegger, I. C., Peters, J. M., & Pines, J. (2002). Human securin proteolysis is controlled by the spindle checkpoint and reveals when the APC/C switches from activation by Cdc20 to Cdh1. *Journal of Cell Biology*, 157, 1125–1137.
- Hampel, A., & Eppig, J. J. (1995). Analysis of the mechanism(s) of metaphase I arrest in maturing mouse oocytes. *Development*, 121, 925–933.
- Hashimoto, N., & Kishimoto, T. (1988). Regulation of meiotic metaphase by a cytoplasmic maturation-promoting factor during mouse oocyte maturation. *Developmental Biology*, 126, 242–252.
- Hassold, T., & Hunt, P. (2001). To err (meiotically) is human: The genesis of human aneuploidy. *Nature Reviews. Genetics*, 2, 280–291.
- Hatch, K. R., & Capco, D. G. (2001). Colocalization of CaM KII and MAP kinase on architectural elements of the mouse egg: Potentiation of MAP kinase activity by CaM KII. *Molecular Reproduction and Development*, 58, 69–77.
- Herbert, M., Levasseur, M., Homer, H., Yallop, K., Murdoch, A., & McDougall, A. (2003). Homologue disjunction in mouse oocytes requires proteolysis of securin and cyclin B1. *Nature Cell Biology*, 5, 1023–1025.
- Hershko, A., & Ciechanover, A. (1998). The ubiquitin system. *Annual Review of Biochemistry*, 67, 425–479.
- Holland, A. J., & Taylor, S. S. (2006). Cyclin-B1-mediated inhibition of excess separase is required for timely chromosome disjunction. *Journal of Cell Science*, 119, 3325–3336.
- Homer, H. A., McDougall, A., Levasseur, M., Murdoch, A. P., & Herbert, M. (2005). Mad2 is required for inhibiting securin and cyclin B degradation following spindle depolymerisation in meiosis I mouse oocytes. *Reproduction*, 130, 829–843.
- Huang, D. T., Hunt, H. W., Zhuang, M., Ohi, M. D., Holton, J. M., & Schulman, B. A. (2007). Basis for a ubiquitin-like protein thioester switch toggling E1-E2 affinity. *Nature*, 445, 394–398.
- Jin, L., Williamson, A., Banerjee, S., Philipp, I., & Rape, M. (2008). Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. *Cell*, 133, 653–665.
- Jones, K. T. (2011). Anaphase-promoting complex control in female mouse meiosis. *Results and Problems in Cell Differentiation*, 53, 343–363.
- Jordan, P., & Karsenti, R. (1997). Myosin light chain-activating phosphorylation sites are required for oogenesis in *Drosophila*. *Journal of Cell Biology*, 139, 1805–1819.
- Josefsberg, L. B., Galiani, D., Dantes, A., Amsterdam, A., & Dekel, N. (2000). The proteasome is involved in the first metaphase-to-anaphase transition of meiosis in rat oocytes. *Biology of Reproduction*, 62, 1270–1277.
- Karabinova, P., Kubelka, M., & Susor, A. (2011). Proteasomal degradation of ubiquitinated proteins in oocyte meiosis and fertilization in mammals. *Cell and Tissue Research*, 346, 1–9.
- King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P., & Kirschner, M. W. (1995). A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell*, 81, 279–288.
- Kirkpatrick, D. S., Hathaway, N. A., Hanna, J., Elsasser, S., Rush, J., Finley, D., King, R. W., & Gygi, S. P. (2006). Quantitative analysis of in vitro ubiquitinated cyclin B1 reveals complex chain topology. *Nature Cell Biology*, 8, 700–710.
- Kubiak, J. Z. (1991). Cell cycle-dependent behavior of microtubules in hybrids of mouse oocytes and blastomeres. *International Journal of Developmental Biology*, 35, 421–427.
- Kudo, N. R., Wassmann, K., Anger, M., Schuh, M., Wirth, K. G., Xu, H., Helmhart, W., Kudo, H., McKay, M., Maro, B., Ellenberg, J., de Boer, P., & Nasmyth, K. (2006). Resolution of chiasmata in oocytes requires separase-mediated proteolysis. *Cell*, 126, 135–146.
- Leader, B., Lim, H., Carabatsos, M. J., Harrington, A., Ecsedy, J., Pellman, D., Maas, R., & Leder, P. (2002). Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. *Nature Cell Biology*, 4, 921–928.
- Leblanc, J., Zhang, X., McKee, D., Wang, Z. B., Li, R., Ma, C., Sun, Q. Y., & Liu, X. J. (2011). The small GTPase Cdc42 promotes membrane protrusion during polar body emission via ARP2-nucleated actin polymerization. *Molecular Human Reproduction*, 17, 305–316.
- Lee, J., Miyano, T., & Moor, R. M. (2000). Localisation of phosphorylated MAP kinase during the transition from meiosis I to meiosis II in pig oocytes. *Zygote*, 8, 119–125.
- Leland, S., Nagarajan, P., Polyzos, A., Thomas, S., Samaan, G., Donnell, R., Marchetti, F., & Venkatachalam, S. (2009). Heterozygosity for a Bub1 mutation causes female-specific germ cell aneuploidy in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 12776–12781.
- Longo, F. J., & Chen, D. Y. (1984). Development of surface polarity in mouse eggs. *Scanning Electron Microscopy*, 703–716.
- Longo, F. J., & Chen, D. Y. (1985). Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. *Developmental Biology*, 107, 382–394.
- Maro, B., & Verlhac, M. H. (2002). Polar body formation: New rules for asymmetric divisions. *Nature Cell Biology*, 4, E281–E283.
- Maro, B., Howlett, S. K., & Houlston, E. (1986). Cytoskeletal dynamics in the mouse egg. *Journal of Cell Science*, (Suppl. 5), 343–359.
- Mtango, N. R., Sutovsky, M., Susor, A., Zhong, Z., Latham, K. E., & Sutovsky, P. (2012a). Essential role of maternal UCHL1 and UCHL3 in fertilization and preimplantation embryo development. *Journal of Cellular Physiology*, 227, 1592–1603.
- Mtango, N. R., Sutovsky, M., Vandevoort, C. A., Latham, K. E., & Sutovsky, P. (2012b). Essential role of ubiquitin C-terminal hydrolases UCHL1 and UCHL3 in mammalian oocyte maturation. *Journal of Cellular Physiology*, 227, 2022–2029.
- Norris, R. P., Freudzon, M., Mehlmann, L. M., Cowan, A. E., Simon, A. M., Paul, D. L., Lampe, P. D., & Jaffe, L. A. (2008). Luteinizing hormone causes MAP kinase-dependent phosphorylation and closure of connexin 43 gap junctions in mouse ovarian follicles: One of two paths to meiotic resumption. *Development*, 135, 3229–3238.
- Peters, J. M. (2006). The anaphase promoting complex/cyclosome: A machine designed to destroy. *Nature Reviews. Molecular Cell Biology*, 7, 644–656.
- Pomerantz, Y., Elbaz, J., Ben-Eliezer, I., Reizel, Y., David, Y., Galiani, D., Nevo, N., Navon, A., & Dekel, N. (2012). From ubiquitin-proteasomal degradation to CDK1 inactivation: Requirements for the first polar body extrusion in mouse oocytes. *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*, 26, 4495–4505.
- Rappaport, R. (1985). Repeated furrow formation from a single mitotic apparatus in cylindrical sand dollar eggs. *Journal of Experimental Zoology*, 234, 167–171.
- Reddy, S. K., Rape, M., Margansky, W. A., & Kirschner, M. W. (2007). Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation. *Nature*, 446, 921–925.
- Reis, A., Madgwick, S., Chang, H. Y., Nabti, I., Levasseur, M., & Jones, K. T. (2007). Prometaphase APCdh1 activity prevents non-disjunction in mammalian oocytes. *Nature Cell Biology*, 9, 1192–1198.
- Richards, J. S. (2007). Genetics of ovulation. *Seminars in Reproductive Medicine*, 25, 235–242.
- Rimon-Dahari, N., Yerushalmi-Heinemann, L., Alyagor, L., & Dekel, N. (2016). Ovarian Folliculogenesis. *Results and Problems in Cell Differentiation*, 58, 167–190.
- Schuh, M., & Ellenberg, J. (2007). Self-organization of MTOCs replaces centrosome function during acentrosomal spindle assembly in live mouse oocytes. *Cell*, 130, 484–498.
- Schuh, M., & Ellenberg, J. (2008). A new model for asymmetric spindle positioning in mouse oocytes. *Current Biology: CB*, 18, 1986–1992.
- Sela-Abramovich, S., Chorev, E., Galiani, D., & Dekel, N. (2005). Mitogen-activated protein kinase mediates luteinizing hormone-induced breakdown of communication and oocyte maturation in rat ovarian follicles. *Endocrinology*, 146, 1236–1244.
- Sharif, B., Na, J., Lykke-Hartmann, K., McLaughlin, S. H., Laue, E., Glover, D. M., & Zernicka-Goetz, M. (2010). The chromosome passenger complex is required for fidelity of chromosome transmission and cytokinesis in meiosis of mouse oocytes. *Journal of Cell Science*, 123, 4292–4300.
- Shaul, Y. D., & Seger, R. (2007). The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica et Biophysica Acta*, 1773, 1213–1226.
- Sherizly, I., Galiani, D., & Dekel, N. (1988). Regulation of oocyte maturation: Communication in the rat cumulus-oocyte complex. *Human Reproduction*, 3, 761–766.
- Simerly, C., Nowak, G., de Lanerolle, P., & Schatten, G. (1998). Differential expression and functions of cortical myosin IIA and IIB isotypes during meiotic maturation, fertilization, and mitosis in mouse oocytes and embryos. *Molecular Biology of the Cell*, 9, 2509–2525.
- Stocco, C., Telleria, C., & Ghibori, G. (2007). The molecular control of corpus luteum formation, function, and regression. *Endocrine Reviews*, 28, 117–149.

- Terret, M. E., Wassmann, K., Waizenegger, I., Maro, B., Peters, J. M., & Verlhac, M. H. (2003). The meiosis I-to-meiosis II transition in mouse oocytes requires separase activity. *Current Biology: CB*, 13, 1797–1802.
- Tremoleda, J. L., Schoevers, E. J., Stout, T. A., Colenbrander, B., & Bevers, M. M. (2001). Organisation of the cytoskeleton during in vitro maturation of horse oocytes. *Molecular Reproduction and Development*, 60, 260–269.
- Vaccari, S., Weeks, J. L., 2nd, Hsieh, M., Menniti, F. S., & Conti, M. (2009). Cyclic GMP signaling is involved in the luteinizing hormone-dependent meiotic maturation of mouse oocytes. *Biology of Reproduction*, 81, 595–604.
- Verlhac, M. H., Kubiak, J. Z., Clarke, H. J., & Maro, B. (1994). Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development*, 120, 1017–1025.
- Verlhac, M. H., Kubiak, J. Z., Weber, M., Geraud, G., Colledge, W. H., Evans, M. J., & Maro, B. (1996). Mos is required for MAP kinase activation and is involved in microtubule organization during meiotic maturation in the mouse. *Development*, 122, 815–822.
- Verlhac, M. H., Lefebvre, C., Kubiak, J. Z., Umbhauer, M., Rassinier, P., Colledge, W., & Maro, B. (2000). Mos activates MAP kinase in mouse oocytes through two opposite pathways. *The EMBO Journal*, 19, 6065–6074.
- Weber, K. L., Sokac, A. M., Berg, J. S., Cheney, R. E., & Bement, W. M. (2004). A microtubule-binding myosin required for nuclear anchoring and spindle assembly. *Nature*, 431, 325–329.
- Wickliffe, K., Williamson, A., Jin, L., & Rape, M. (2009). The multiple layers of ubiquitin-dependent cell cycle control. *Chemical Reviews*, 109, 1537–1548.
- Zhang, C. H., Wang, Z. B., Quan, S., Huang, X., Tong, J. S., Ma, J. Y., Guo, L., Wei, Y. C., Ouyang, Y. C., Hou, Y., Xing, F. Q., & Sun, Q. Y. (2011). GM130, a cis-Golgi protein, regulates meiotic spindle assembly and asymmetric division in mouse oocyte. *Cell Cycle*, 10, 1861–1870.
- Zheng, Y. (2004). G protein control of microtubule assembly. *Annual Review of Cell and Developmental Biology*, 20, 867–894.
- Zou, H., McGarry, T. J., Bernal, T., & Kirschner, M. W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science*, 285, 418–422.

Maternal Programming in Oocytes

Kyeoung-Hwa Kim and Kyung-Ah Lee, CHA University, Bundang-gu, Korea

© 2018 Elsevier Inc. All rights reserved.

Introduction

Mammalian oocytes undergo a unique meiotic cell cycle that produces four daughter cells from one unevenly divided primary oocyte, and in three of these daughter cells, three polar bodies are degenerated, leaving a single large oocyte with most of the cytoplasm containing accumulated maternal RNAs and proteins. Consistent with follicular growth, oocytes in the follicle grow and mature into a large oocyte, and these processes are finely controlled by cell-to-cell communication between follicular cells and oocytes. This cell-to-cell communication is bi-directional and essential for the development and function of oocytes and granulosa cells (Su et al., 2009). Granulosa cells play a nurturing role in supporting oocyte development and a regulatory role in maintaining oocyte meiotic maturation and the global suppression of oocyte transcriptional activity. Additionally, oocytes are the control towers for the proliferation and transition of granulosa cells to cumulus cells in mice (Diaz et al., 2007a, b).

During oocyte growth and development, oocytes produce and store various types of RNAs and proteins in the cytoplasm. The factors stored in the oocyte cytoplasm are required for further processes, such as oocyte maturation, fertilization, and preimplantation embryo development, until new genes are expressed during the oocyte-to-embryo transition that occur after fertilization, namely, the maternal-to-zygote transition (MZT) (De La Fuente, 2006). The MZT is a major developmental transition that starts from fertilization until the stage where embryonic gene expression is concurrently initiated with the degradation of maternal factors from the oocyte stage (Fig. 1). MZT is essential for early embryo development since it organizes embryonic mitotic cell division with zygotic gene activation (ZGA). This unique pattern of dynamics in the transcriptome during the MZT, including the degradation of maternal mRNAs and new transcription of zygotic genes, is the product of maternal programming in oocytes. Both the degradation of maternal mRNAs and new transcription of zygotic genes are prerequisites for the normal development of early stage embryos (De La Fuente and Eppig, 2001; Jukam et al., 2017).

Category of Maternal RNAs

Full-grown mouse oocytes are transcriptionally quiescent, and the loss of the maternal mRNAs initiates when oocytes undergo maturation (Svoboda et al., 2015). This transcriptional quiescence in full-grown oocytes is maintained by the poly(C)-binding protein PCBP1 (Xia et al., 2012). However, while this transition is transcriptionally quiescent, maternal mRNAs are stable with a high turnover rate (Puschendorf et al., 2006). These dormant maternal mRNAs are considered a ready-made-form for use whenever certain developmental necessities arise. Although the functions of many genes have been revealed, the functions of many more genes remain unknown.

Based on studies of the functions of maternal mRNAs using loss-of-function methods, i.e., RNA interference (RNAi), these molecules can be characterized into two groups. The biochemical and molecular changes and the phenotypic changes of the oocytes after RNAi are compared to the in vitro maturation and in vitro fertilization of the control oocytes. One group includes maternal

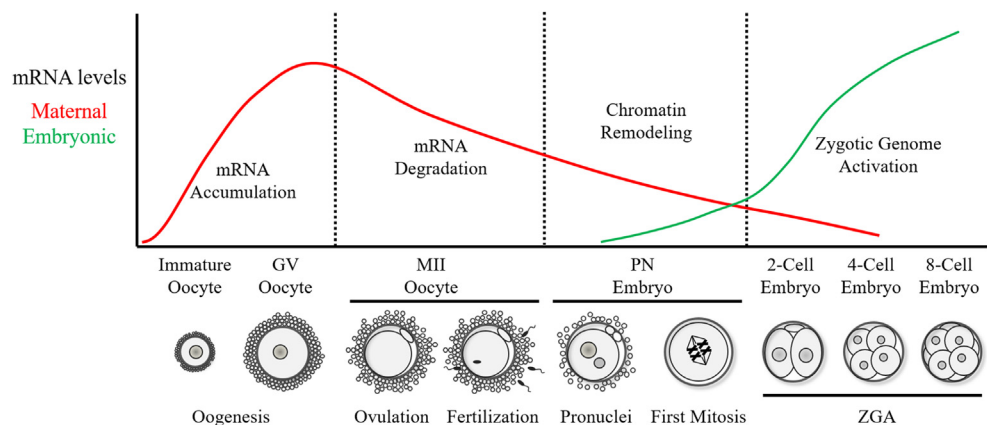


Fig. 1 Maternal-to-zygotic transition. Maternal mRNAs synthesized and stored during oogenesis serve as the maternal contribution supporting oocyte maturation, fertilization, and early embryogenesis. However, accumulated maternal mRNAs undergo general decay after meiotic resumption (red line), followed by ZGA to switch the developmental control from maternal mRNAs to de novo transcripts expressed from the zygotic genome (green line). This transition is referred to as the maternal-to-zygotic transition. Indeed, upon fertilization, the condensed sperm nucleus undergoes chromatin remodeling in which maternally derived histones replace a majority of sperm protamines, resulting in the decondensation of sperm chromatin and the formation of PN.

mRNAs for which the loss-of-function of that specific gene results in the MI-arrest of oocyte maturation (Fig. 2B), while the other group includes maternal mRNAs for which the loss-of-function results in the completion of meiotic maturation but without full competent cytoplasmic maturation (Fig. 2C). When Obox4 was knocked down in GV oocytes, Obox4-RNAi-treated oocytes could not complete nuclear maturation and showed the abnormal organization of spindles and chromosomes and incomplete cytokinesis. The genes affecting RNAi-induced MI arrest are Obox4, Bcl2l10, Txnip, Zap70, etc. (Lee et al., 2013). Interesting phenotypic changes observed after Txnip RNAi included immobilized cytoplasmic streaming of the oocytes (Video B in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64454-9>) (Lee et al., 2013).

The results of the RNAi of other groups of genes showed no changes in progressing nuclear maturation by showing polar body formation after RNAi, but developmental abnormalities were observed after fertilization and beyond. Fertilization without paternal chromosome decondensation and pronucleus (PN) formation was observed in Gas6 RNAi-treated oocytes (Kim et al., 2011). In the case of Sebox RNAi, MII formation and fertilization progressed but no further embryonic development was observed after the 2-cell stage (Kim et al., 2016). According to these phenotypic changes after RNAi, we categorized the genes into two groups and analyzed the second group of genes as new candidates for maternal effect genes (MEGs).

Maternal Effect Genes (MEGs) and Their Functions

As previously explained, the oocytes of vertebrates contain maternal factors, such as mRNAs and proteins, which are accumulated and stored during oogenesis. These maternal factors regulate oocyte growth and maturation, meiosis, fertilization and early embryogenesis, including the transition between meiotic and mitotic cell cycles, the removal of maternal detritus (mRNAs and proteins) and the robust activation of the embryonic genome, the so-called zygotic gene activation (ZGA). Early embryo development is heavily dependent on specific maternal factors of oocytes prior to ZGA, and these specific maternal factors, genes from the maternal genome, are referred to as MEGs. Currently, the lists and functions of MEGs have revealed that these genes are important for the regulation of normal embryo development, as the disruption of the expression of MEGs resulted in defective embryogenesis (Fig. 3).

MEGs were initially described in *Drosophila* 30 years ago, such as dorsal, bicoid and torso, while the first MEG in mammals was described in 2000 using traditional knockout techniques (Kim and Lee, 2014; Tong et al., 2000). Since then, different techniques have been used to identify MEGs. The first MEGs were identified in mice using traditional knockout techniques. However, the global deletion of several MEGs essential for embryogenesis using traditional knockout techniques results in embryonic lethality, preventing the investigation of the function of MEGs in postnatal and adult life, including fertility. The emergence of conditional knockout techniques using a tissue-specific Cre-loxP system has overcome the risks associated with traditional knockout techniques, such as embryonic lethality and complex phenotypes. However, the process to generate a mouse using traditional/conditional knockout or mutant techniques is slow and expensive. Fortunately, the specific knockdown of MEGs in oocytes is a more rapid technique with lower costs, and this technique has been used to identify a growing number of MEGs in mammalian species, such as mice, cows, and pigs (Kim and Lee, 2014). Currently, more than 60 MEGs with pivotal roles in normal embryonic development have been identified in mammals (Condic, 2016).

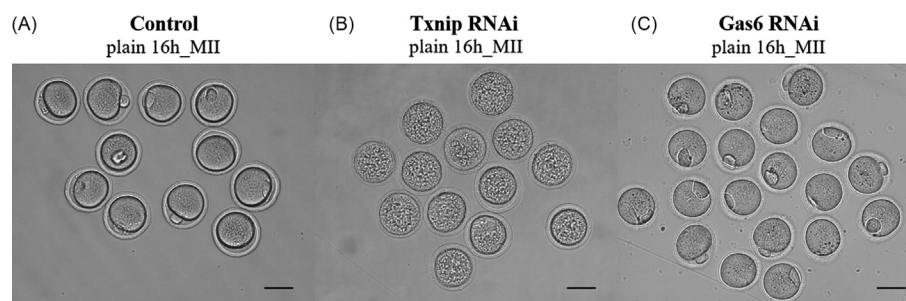


Fig. 2 Meiotic maturation of oocytes after RNAi of the specific genes. Micrographs of control oocytes (A), Txnip RNAi-treated oocytes (B), and Gas6 RNAi-treated oocytes (C) after in vitro culture for 16 h in M16 medium following 8 h of incubation in IBMX-supplemented M16 medium. Bars = 100 μ m. *MI*, metaphase I-arrested oocyte; *MI*, metaphase II oocyte. The video clips (Video A–C) are representative of the data presented in this figure and include a compilation of video microscopy from control oocytes (Video A), Txnip RNAi-treated oocytes (Video B) and Gas6 RNAi-treated oocytes (Video C). Meiotic maturation of oocytes after RNAi of the specific genes. Micrographs of control oocytes (A), Txnip RNAi-treated oocytes (B), and Gas6 RNAi-treated oocytes (C) after in vitro culture for 16 h in M16 medium following 8 h of incubation in IBMX-supplemented M16 medium. Bars = 100 μ m. *MI*, metaphase I-arrested oocyte; *MI*, metaphase II oocyte. The video clips (Video A–C in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64454-9>) are representative of the data presented in this figure and include a compilation of video microscopy from control oocytes (Video A in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64454-9>), Txnip RNAi-treated oocytes (Video B in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64454-9>) and Gas6 RNAi-treated oocytes (Video C in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64454-9>).

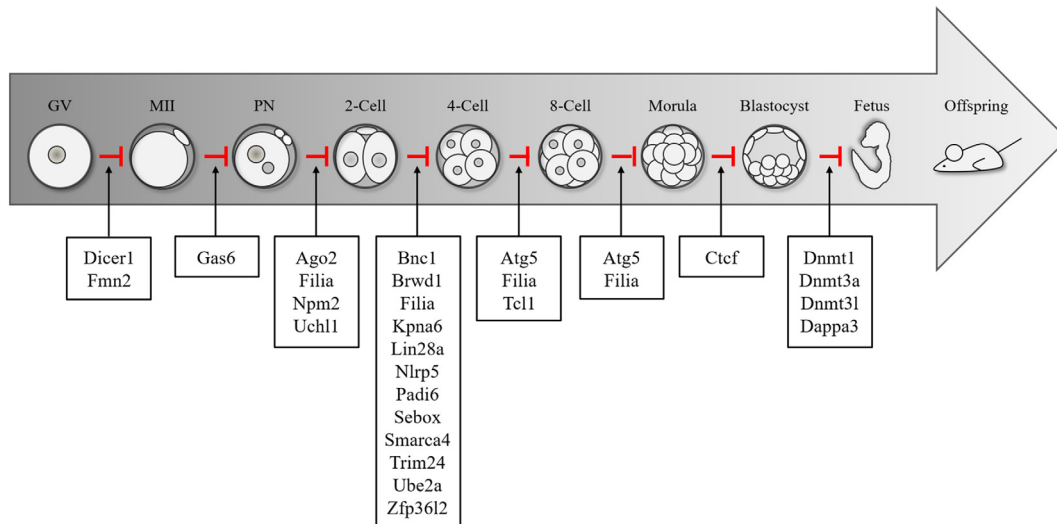


Fig. 3 Mammalian maternal effect genes. Schematic diagram showing arrested embryonic stages after a disruption of appropriate MEGs using various gene interference techniques. *GV*, germinal vesicle oocyte; *MI*, metaphase II oocyte; *PN*, pronucleus stage zygote.

Chromatin Remodeling

The mammalian sperm nucleus contains tightly packed chromatin with protamines. In successful fertilization, by entering the ooplasm, the condensed chromatin in the sperm head undergoes rapid decondensation and becomes repacked with maternally derived histones. This process, known as paternal chromatin remodeling, is induced by specific maternal factors in oocytes. Thus, maternal factors process the male genome and the subsequent formation of the male PN, resulting in syngamy with female PN (Fig. 1). During MZT, the robust activation of embryonic gene expression also requires chromatin remodeling. Brwd1, Gas6, Npm2, Trim24 and Ube2a play essential roles in chromatin remodeling. We identified Gas6 as a MEG using RNAi. Following the microinjection of Gas6 dsRNA into the ooplasm of oocytes, disruption of Gas6 does not affect nuclear oocyte maturation but influences cytoplasmic maturation and chromatin remodeling, resulting in the failure of PN formation (Kim et al., 2011).

Degradation of Maternal mRNAs and Proteins

The MZT is a conserved developmental process during which the stored maternally derived products, i.e., maternal factors, are cleared (Fig. 1; red line), and the embryonic genome is robustly activated (Fig. 1; green line). Thus, the MZT requires both the degradation of maternal products and zygotic activation. In mice, the genome in the growing oocyte is transcriptionally and translationally active, and maternal proteins are stored in the ooplasm of the enlarging oocyte. Hence, maternal products are deposited in the ooplasm during folliculogenesis and oogenesis. In contrast, during meiotic maturation, prior to ovulation, the oocyte genome is translationally quiescent and the polyadenylated mRNAs are cleared. Subsequently, maternal proteins are degraded in early embryo development. However, the degradation of maternal mRNAs and proteins may be necessary for ZGA. Ago2, Atg5, Dicer1, Uchl1 and Zfp3612 are MEGs that play critical roles in maternal factor degradation. Taken together, these MEGs may be involved in autophagy, the ubiquitin-proteasome pathway and the miRNA/siRNA system.

Zygotic Gene Activation (ZGA)

During the MZT, maternal mRNAs and proteins are rapidly erased and new mRNAs and proteins encoded by the embryonic genome are synthesized. The robust activation of the embryonic genome, referred to as ZGA, has been observed in many mammalian embryos, including those of mouse, rat, pig, cow and human, indicating a biological imperative in early embryogenesis. ZGA occurs at the 2-cell stage in mice, the 4-cell stage in pigs, and the 8-cell stage in cows and humans (Fig. 3).

ZGA is essential for the MZT required for subsequent embryonic development, and some MEGs, including Bnc1, Nlrp5, Padi6, Sebox and Smarca4, may be involved in this process. The depletion of these MEGs leads to a reduction of de novo RNA transcription in zygote to 2-cell stage embryos. Upon transcription inhibition during this period, the embryos were primarily arrested at the 2-cell stage, commonly referred to as the 2-cell block. Therefore, ZGA must occur early during mammalian embryo development. In a previous study using RNAi, we identified Sebox and Lin28a as MEGs. Sebox is essential for oocyte cytoplasmic maturation and may prepare the oocyte for embryonic development through orchestrating the expression of other important MEGs and ZGA (Kim et al., 2016). Furthermore, Lin28a is a maternal effect gene expressed in oocyte cytoplasm that does not affect oocyte maturation or fertilization but regulates embryo development, particularly ZGA at the 2-cell stage (Kim et al., 2016).

Genomic Imprinting

Upon fertilization, the oocyte and sperm genomes require epigenetic remodeling and the subsequent formation of a totipotent PN embryo. During this period, the paternal genome is actively demethylated, effectively erasing many spermatogenesis-derived methylation marks. In contrast, the maternal genome is passively demethylated prior to the first mitosis. In addition, the embryonic genome in the blastocyst is demethylated. Maternal factors also maintain DNA imprints during early embryo development. Moreover, dynamic histone modifications, and more specifically lysine methylation, may play important roles in the MZT. Maternal factors play pivotal roles in regulating the epigenetic modifications of the paternal and maternal genomes. Hence, the remodeling of both paternal and maternal chromatin induces the activation or silencing of various genes, ultimately resulting in cellular differentiation and normal offspring. The MEGs Ctf, Dnmt1, Dnmt3a, Dnmt3l and Dapp3 play vital roles in genomic imprinting.

Preimplantation Embryo Development

When the paternal nucleus fuses with the maternal nucleus to generate a zygote, the oocyte transits to embryo development, i.e., from the meiotic cell cycle to the mitotic cell cycle, a process fundamental to successful embryo development. In mice, the molecular components necessary for the reorganization of the division machinery, centrosome re-establishment and changes in spindle properties are produced. The disruption of MEGs in oocytes leads to the inhibition of preimplantation embryo development. Therefore, MEGs are essential for early embryogenesis. The MEGs Filia, Fmn2, Kpna6 and Tcl1 play essential roles in the regulation of the cell cycle and spindle organization.

Conclusion

In summary, oocytes restrain numerous maternal factors for growth and development and sequester resources for fertilization and preimplantation embryo development. Maternal programming is therefore important for normal reproduction and required for the preservation of the species. Studies on the molecular identities and functions, as well as the regulatory pathways for each maternal factor, are important and fascinating issues in reproductive biology.

Supplementary data to this article can be found online at <https://doi.org/10.1016/B978-0-12-801238-3.64454-9>.

References

- Condic, M. L. (2016). The role of maternal-effect genes in mammalian development: Are mammalian embryos really an exception? *Stem Cell Reviews*, 12, 276–284.
- De La Fuente, R. (2006). Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. *Developmental Biology*, 292, 1–12.
- De La Fuente, R., & Eppig, J. J. (2001). Transcriptional activity of the mouse oocyte genome: Companion granulosa cells modulate transcription and chromatin remodeling. *Developmental Biology*, 229, 224–236.
- Diaz, F. J., Wigglesworth, K., & Eppig, J. J. (2007a). Oocytes are required for the preantral granulosa cell to cumulus cell transition in mice. *Developmental Biology*, 305, 300–311.
- Diaz, F. J., Wigglesworth, K., & Eppig, J. J. (2007b). Oocytes determine cumulus cell lineage in mouse ovarian follicles. *Journal of Cell Science*, 120, 1330–1340.
- Jukam, D., Shariati, S. A. M., & Skotheim, J. M. (2017). Zygotic genome activation in vertebrates. *Developmental Cell*, 42, 316–332.
- Kim, K. H., Kim, E. Y., Kim, Y., Kim, E., Lee, H. S., Yoon, S. Y., & Lee, K. A. (2011). Gas6 downregulation impaired cytoplasmic maturation and pronuclear formation independent to the MPF activity. *PLoS One*, 6, e23304.
- Kim, K. H., & Lee, K. A. (2014). Maternal effect genes: Findings and effects on mouse embryo development. *Clinical and Experimental Reproductive Medicine*, 41, 47–61.
- Kim, K. H., Seo, Y. M., Kim, E. Y., Lee, S. Y., Kwon, J., Ko, J. J., & Lee, K. A. (2016). The miR-125 family is an important regulator of the expression and maintenance of maternal effect genes during preimplantational embryo development. *Open Biology*, 6, pii: 160181.
- Lee, S. Y., Lee, H. S., Kim, E. Y., Ko, J. J., Yoon, T. K., Lee, W. S., & Lee, K. A. (2013). Thioredoxin-interacting protein regulates glucose metabolism and affects cytoplasmic streaming in mouse oocytes. *PLoS One*, 8, e70708.
- Puschendorf, M., Stein, P., Oakeley, E. J., Schultz, R. M., Peters, A. H., & Svoboda, P. (2006). Abundant transcripts from retrotransposons are unstable in fully grown mouse oocytes. *Biochemical and Biophysical Research Communications*, 347, 36–43.
- Su, Y. Q., Sugiyama, K., & Eppig, J. J. (2009). Mouse oocyte control of granulosa cell development and function: Paracrine regulation of cumulus cell metabolism. *Seminars in Reproductive Medicine*, 27, 32–42.
- Svoboda, P., Franke, V., & Schultz, R. M. (2015). Sculpting the transcriptome during the oocyte-to-embryo transition in mouse. *Current Topics in Developmental Biology*, 113, 305–349.
- Tong, Z. B., Gold, L., Pfeifer, K. E., Dorward, H., Lee, E., Bondy, C. A., Dean, J., & Nelson, L. M. (2000). Mater, a maternal effect gene required for early embryonic development in mice. *Nature Genetics*, 26, 267–268.
- Xia, M., He, H., Wang, Y., Liu, M., Zhou, Z., Lin, M., Zhou, Z., Huo, R., Zhou, Q., & Sha, J. (2012). PCBP1 is required for maintenance of the transcriptionally silent state in fully grown mouse oocytes. *Cell Cycle*, 11, 2833–2842.

Polar Body Formation After Ovulation and Fertilization

Anne Bourdais, Institute of Genetics and Development of Rennes, Rennes, France

Benoit Dehapiot, Aix Marseille Université, Marseille, France

Guillaume Halet, Institute of Genetics and Development of Rennes, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

In sexual reproduction, male and female gametes unite at fertilization, to generate a genetically unique individual, which combines both parental genomes. Instrumental to the success of embryo development is the haploidization of the parental genomes, so as to restore a diploid chromosome complement after gamete fusion. Meiosis is the process by which haploid gametes (i.e., in which each chromosome is present as a single copy) are generated from diploid parental germ cells (in which each chromosome is present as two copies). In addition, paternal and maternal homologous chromosomes exchange segments of DNA during meiosis, creating genetic diversity. In oocyte meiosis, haploidization is achieved via a succession of two asymmetric divisions, to produce a big egg, and two small daughter cells called polar bodies, in which supernumerary chromosomes are discarded. The fertilized egg will develop into an embryo, while polar bodies will eventually degenerate. In the great majority of mammalian species studied so far, including human, the first meiotic division is achieved shortly before ovulation. Hence, the ovulated egg has emitted the first polar body. Emission of the second polar body, a hallmark of meiosis completion, requires the egg to be fertilized.

In this article, we review the basic principles of polar body formation in the mammalian fertilized egg, and we introduce the concept of cortical polarization, a prerequisite for polar body formation. The results and concepts presented here are based essentially on studies realized with eggs from mammalian species, mostly mouse.

The Need for a Second Meiotic Division

Why does the egg need to execute a second meiotic division before the start of embryo development? The answer lies in the amount of genetic material—that is, the number of chromosomes—in the newly formed embryo (Roeder, 1997; Holt and Jones, 2009). When primordial germ cells, which are diploid cells, invade the embryonic gonads during fetal life, they initiate meiosis after a round of DNA replication (premeiotic S phase), such that every chromosome is made of two identical sister chromatids. Homologous chromosomes of maternal and paternal inheritance are next assembled by pair to form the so-called bivalents, or tetrads, a structure that will facilitate recombination events to create genetic diversity. During the first meiotic division (or meiosis-I), which takes place in the ovarian follicle, the oocyte assembles the meiotic spindle, a bipolar structure made of microtubules, which role is to separate the homologs, one set of which is discarded into the first polar body. This is referred to as the reductional division because the number of chromosomes in the egg is effectively divided by two. Meiosis-I is also referred to as maturation, because the oocyte becomes fertilizable. At the end of meiosis-I, both daughter cells (the egg, and the first polar body) have inherited only one homolog of each pair. Straight away, the chromosomes that have been retained in the egg assemble a new spindle, with chromosomes aligned at its center, primed for the second meiotic division. However, ovulated eggs remain arrested in the so-called metaphase-II stage until fertilization (or until death by apoptosis, if unfertilized). These metaphase-II chromosomes are made of two sister chromatids joined together at their centromeric region. This configuration is similar to chromosome alignment on the mitotic spindle in dividing somatic cells. Since the sperm brings one full set of paternal chromatids, the egg effectively contains three copies of each chromosome after sperm fusion. But this is a transient situation, as fertilization also triggers meiosis resumption in the egg, leading to maternal chromatid segregation (anaphase-II) and the emission of the second polar body in which one set of maternal chromatids is discarded (Fig. 1). Ultimately, the fertilized egg contains one single copy

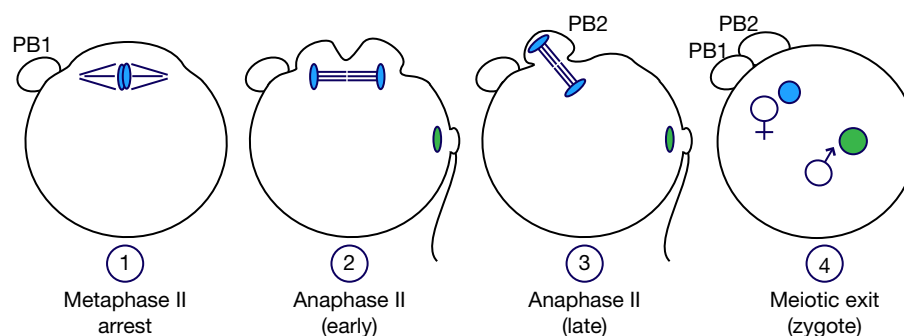


Fig. 1 Emission of the second polar body in the fertilized egg. In most mammalian species, the ovulated egg is blocked at the metaphase-II stage of meiosis (1). Upon fertilization, meiosis resumes and maternal sister chromatids (blue ovals) are segregated by the anaphase-II spindle (2). The fertilizing sperm also brings a full complement of paternal chromatids (green oval). In the mouse, the spindle lies parallel to the egg cortex and must rotate on the side during anaphase-II to achieve polar body formation (3). Next, maternal and paternal chromatids decondense and become enclosed into the maternal and paternal pronuclei (blue and green circles), indicating the end of meiosis (4). PB1: first polar body; PB2: second polar body.

(one chromatid) of each maternal and paternal chromosome, thus restoring a diploid chromosome complement compatible with healthy embryo development. Failure to emit the second polar body would result in triploidy, a condition incompatible with normal embryo development, and a likely cause of spontaneous abortion (Zaragoza et al., 2000).

Polar Bodies: Small Daughter Cells Formed Through Asymmetric Division

The egg contains large amounts of organelles (such as the energy producing mitochondria), proteins, as well as coding and noncoding RNAs, that have accumulated during oocyte growth in the ovary. These maternal stores are vital for successive accomplishment of the first phase of embryo development—the so-called preimplantation development. Therefore, it is important that polar bodies, which inevitably incorporate cytoplasmic content, remain as small as possible, in order for the fertilized egg to retain maximal amount of these precious resources. Emission of the polar body can thus be seen as a paradigm of asymmetric cell division, where daughter cells differ strikingly in size. For instance, the human egg is a large cell with a diameter of about 100 μm , while polar bodies are in the range 15–20 μm . To achieve such asymmetry, the spindle apparatus must localize very close to the egg cortex. During meiosis I, the first meiotic spindle forms in the oocyte center, then migrates towards the cortex while homologous chromosomes are still held together. When the spindle reaches the cortex, homologs separate, and one set is discarded into the first polar body. Next, the metaphase-II spindle forms and remains close to the egg cortex for the duration of the metaphase-II arrest (Fig. 2). The metaphase-II spindle is therefore ideally positioned to produce a second asymmetric division when meiosis II resumes at fertilization. In the mouse egg, the long axis of the metaphase-II spindle lies parallel to the cortex, such that the spindle will have to rotate during anaphase-II in order for the second polar body to protrude (Fig. 1). In human eggs however, as in other mammalian species, the metaphase-II spindle displays a perpendicular orientation relative to the cortex (Coticchio et al., 2013), suggesting that polar body protrusion at fertilization occurs without the need for spindle reorientation.

The Role of Ca^{2+} Signaling in Polar Body Formation at Fertilization

There are fundamental differences in the mechanisms regulating polar body formation in mammalian meiosis-II, versus meiosis-I. This is exemplified in the fact that once oocyte maturation starts, it proceeds autonomously through the successive cell cycle stages, culminating with the emission of the first polar body. In contrast, formation of the second polar body requires the triggering of a signaling cascade that is specifically elicited at fertilization, otherwise the egg remains arrested at metaphase-II. It is well established that the fertilizing sperm triggers a series of Ca^{2+} oscillations (periodic increases in the concentration of Ca^{2+}) in the mammalian egg, with effect to induce anaphase-II, emission of the second polar body, and a number of other events collectively referred to as egg activation (Jones, 2005; Perry and Verlhac, 2008). The trigger for this Ca^{2+} signal is a sperm-specific phospholipase C that is introduced into the egg cytoplasm upon gamete fusion (Swann and Lai, 2016). It is however possible to artificially induce meiosis-II resumption without a fertilizing sperm, using specific culture conditions or molecules that will trigger a rise in Ca^{2+} in the egg. This strategy is sometimes used in the clinic to rescue in vitro fertilized eggs that have failed to activate (Vanden Meerschaut et al., 2014).

Polarization of the Egg's Cortex

Beside the asymmetric positioning of the meiotic spindle, another feature of the ovulated metaphase-II egg is the remarkable remodeling of the cortical area overlying the spindle, a phenomenon referred to as polarization (Fig. 2, left and middle panels).

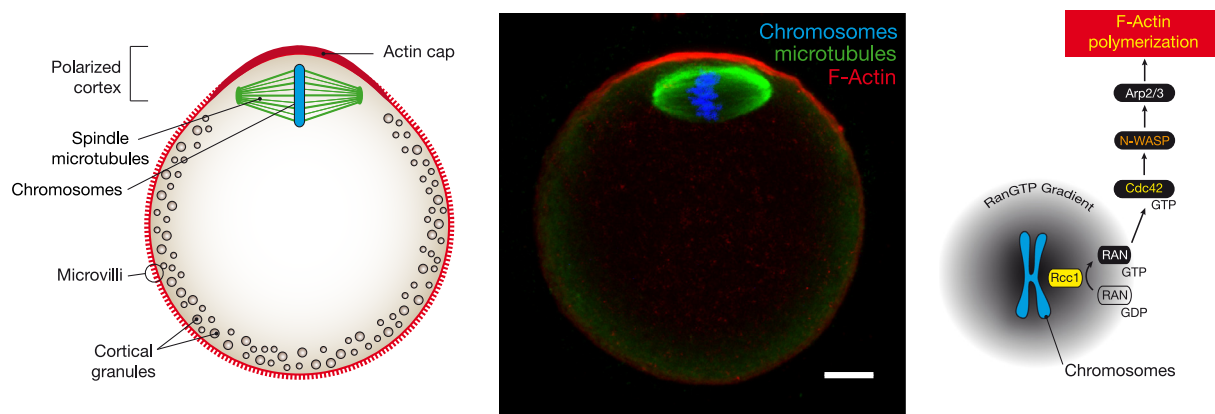


Fig. 2 Egg polarization. Left panel: schematic of a mouse egg arrested at metaphase II, showing some of the typical polarization features. Middle panel: optical slice of a mouse metaphase-II egg obtained by confocal microscopy. The egg was fixed and permeabilized, and stained for metaphase chromosomes (blue), spindle microtubules (green) and actin filaments (F-actin, red). Note the polarized F-actin cap overlying the meiotic spindle. Scale bar is 10 μm . Right panel: the molecular cascade leading to actin polarization in the mouse egg.

This has been extensively described in the mouse egg, where polarization was first detected over 40 years ago, as an exclusion of cortical granules from the spindle area (Nicosia et al., 1977). These granules, which cover the rest of the egg cortex, are exocytosed at fertilization, and their content contributes to establishing a barrier to polyspermy via the modification of the zona pellucida. Simultaneously, it was noticed that microvilli, finger-like membrane processes that are important for sperm binding and fusion, were disassembled in the membrane area overlying the spindle, possibly to prevent sperm fusion in this region (Nicosia et al., 1977). It was later shown that the polarized cortex shows a dramatic enrichment in actin filaments—the so-called actin cap (Longo and Chen, 1985; Maro et al., 1986). Most interestingly, egg polarization is unambiguously associated with chromosomes being in close vicinity to the cortex, no matter the precise location of the chromosomes, while spindle microtubules are not required. This lead to the proposal that maternal chromosomes send a signal to remodel the nearby cortex from a distance, and that this cortical differentiation is not restricted to a predetermined site (Longo and Chen, 1985; Maro et al., 1986). A similar accumulation of actin filaments overlying metaphase chromosomes was reported in other mammalian species (porcine) as well as in amphibians (*Xenopus*) and marine invertebrates (ascidian). The existence of an actin-rich cortex in the vicinity of the spindle has also been suggested in human eggs (Coticchio et al., 2014). Since the first description of the actin cap, other proteins were found to be enriched in the polarized cortex, such as myosin-II and the so-called polarity proteins Par3 and Par6. Reciprocally, some proteins—such as those involved in microvilli formation and sperm binding—have been shown to be selectively excluded from the polarized cortex.

Molecular Insights into the Polarizing Signal Delivered by Chromosomes

While the molecular mechanisms of egg polarization are not fully understood, one particular signaling cascade has been investigated in more details: the formation of the cortical actin cap in the mouse egg (Fig. 2). A major breakthrough was the discovery that the signal emanating from chromosomes to remodel the cortex, is a gradient of activated Ran GTPase (Deng et al., 2007). At the molecular level, cytoplasmic Ran is loaded with GTP (RanGTP, the active form) by its specific guanine nucleotide exchange factor RCC1 which is bound to chromatin, such that egg chromosomes act as a source of freely diffusing RanGTP (Dumont et al., 2007). The intrinsic GTP hydrolyzing activity of Ran (presumably amplified by GTPase-activating proteins) means that RanGTP will turn into inactive RanGDP as it diffuses away from the chromosomes. The net result is a gradient of RanGTP, centered on the maternal chromosomes (Fig. 2). Further studies identified signaling intermediates activated downstream of RanGTP to polarize cortical actin, such as the actin nucleator Arp2/3, the GTPase Cdc42 and the Cdc42 effector N-WASP (Yi et al., 2011; Dehapiot et al., 2013). The signaling cascade as we know it is shown in Fig. 2 (right panel). Accordingly, inhibition of any one of these signaling intermediates, leads to a loss of actin polarity, even though chromosomes are close to the cortex (Deng et al., 2007; Yi et al., 2011; Dehapiot et al., 2013). Importantly, inhibition of this signaling pathway in eggs cultured in vitro, also results in a failure of polar body formation after egg activation (Dehapiot et al., 2013). The Ran/Cdc42/Actin pathway is therefore instrumental to successful polar body emission following egg activation. This pathway seems conserved in meiosis-I, as genetic ablation of Cdc42 in mouse oocytes resulted in a failure to polarize actin and to emit the first polar body, leading to maturation defects and female sterility (Wang et al., 2013). It is likely that additional signaling pathways contribute to achieving egg polarization, possibly also under the control of the RanGTP gradient.

Examination of actin filament distribution in fertilized eggs reveals that the second polar body is enriched in actin and other polarity proteins such as N-WASP, while these proteins are no longer enriched in the egg cortex (Fig. 3). Effectively, the polarized cortical region in the unfertilized egg delineates the contour of the polar body to be formed, and thus polarized proteins end up in the polar body. Because actin filaments can act as force generators, controlling cell shape and deformation, it is likely that the polarized actin cap facilitates membrane protrusion during polar body formation (Dehapiot et al., 2013). In addition, the thick actin layer may provide the polar body with a structural coherence, preventing its collapse during the protrusion process. As mentioned earlier, the mouse egg shows a peculiar configuration whereby the spindle lies parallel to the cortex during anaphase-II (Fig. 1). It was shown that the polarized cortex effectively split in two during anaphase-II, resulting in the formation of two polar body-like protrusions enriched in actin and polarized proteins (Dehapiot et al., 2013). These observations suggest that the molecular cascade elicited by Ran to polarize the cortex is conserved during anaphase-II, and drives the protrusion of the second polar body at fertilization.

New Advances from Live Imaging Studies

A classic approach in egg research utilizes immuno-labeling of proteins of interest, in eggs that have been fixed and permeabilized (as seen in Figs. 2 and 3). Though this technique has generated valuable data regarding the subcellular distribution and relative amount of a number of signaling proteins, it cannot provide conclusive informations on protein dynamics and cytoskeletal remodeling as they occur in the living cell. New achievements in live imaging techniques, associated with the design of dedicated fluorescent probes, have allowed for the monitoring of actin filament dynamics in living eggs. Remarkably, it was shown that actin filaments in the polarized cap flow continuously towards the egg interior, and generates cytoplasmic streaming that contributes to maintaining the cortical positioning of the metaphase-II spindle (Yi et al., 2011). Future studies based on a similar live imaging approach should provide new advances in our understanding of polar body formation after fertilization, and the exact roles of actin filaments.

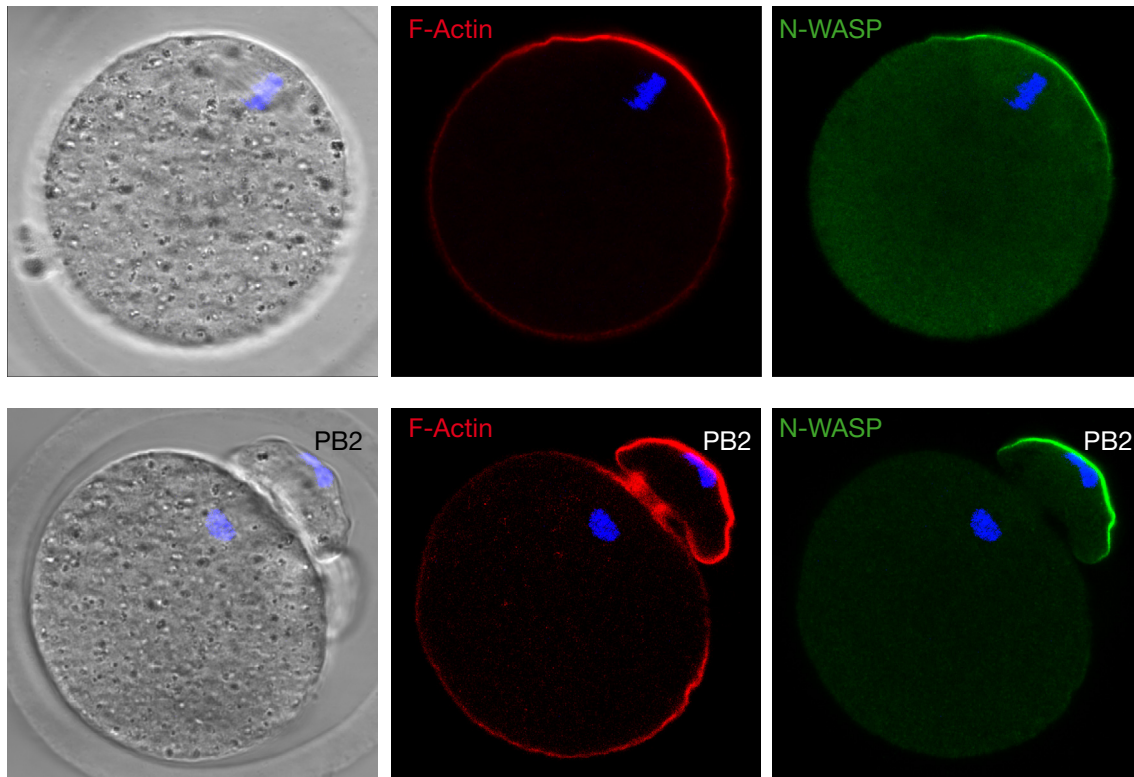


Fig. 3 Polarity features in the second polar body. Images are optical slices of mouse eggs obtained by confocal microscopy. Eggs were fixed and permeabilized before staining. Upper panels: a metaphase-II egg showing the polarized accumulation of actin filaments (F-actin) and N-WASP in the cortex overlying the spindle. Lower panels: a fertilized mouse egg with the second polar body emitted (PB2). Note the accumulation of actin and N-WASP in the cortex of the polar body. Chromosomes are in blue.

References

- Coticchio, G., Guglielmo, M. C., Dal Canto, M., Fadini, R., Mignini Renzini, M., De Ponti, E., Brambillasca, F., & Albertini, D. F. (2013). Mechanistic foundations of the metaphase II spindle of human oocytes matured in vivo and in vitro. *Human Reproduction*, 28, 3271–3282.
- Coticchio, G., Guglielmo, M. C., Albertini, D. F., Dal Canto, M., Mignini Renzini, M., De Ponti, E., & Fadini, R. (2014). Contributions of the actin cytoskeleton to the emergence of polarity during maturation in human oocytes. *Molecular Human Reproduction*, 20, 200–207.
- Dehapiot, B., Carrière, V., Carroll, J., & Halet, G. (2013). Polarized Cdc42 activation promotes polar body protrusion and asymmetric division in mouse oocytes. *Developmental Biology*, 377, 202–212.
- Deng, M., Suraneni, P., Schultz, R. M., & Li, R. (2007). The ran GTPase mediates chromatin signaling to control cortical polarity during polar body extrusion in mouse oocytes. *Developmental Cell*, 12, 301–308.
- Dumont, J., Petri, S., Pellegrin, F., Terret, M. E., Bohnsack, M. T., Rassnir, P., Georget, V., Kalab, P., Gruss, O. J., & Verlhac, M. H. (2007). A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes. *The Journal of Cell Biology*, 176, 295–305.
- Holt, J. E., & Jones, K. T. (2009). Control of homologous chromosome division in the mammalian oocyte. *Molecular Human Reproduction*, 15, 139–147.
- Jones, K. T. (2005). Mammalian egg activation: From Ca^{2+} spiking to cell cycle progression. *Reproduction*, 130, 813–823.
- Longo, F. J., & Chen, D. Y. (1985). Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. *Developmental Biology*, 107, 382–394.
- Maro, B., Johnson, M. H., Webb, M., & Flach, G. (1986). Mechanism of polar body formation in the mouse oocyte: An interaction between the chromosomes, the cytoskeleton and the plasma membrane. *Journal of Embryology and Experimental Morphology*, 92, 11–32.
- Nicosia, S. V., Wolf, D. P., & Inoue, M. (1977). Cortical granule distribution and cell surface characteristics in mouse eggs. *Developmental Biology*, 57, 56–74.
- Perry, A. C. F., & Verlhac, M. H. (2008). Second meiotic arrest and exit in frogs and mice. *EMBO Reports*, 9, 246–251.
- Roeder, G. S. (1997). Meiotic chromosomes: It takes two to tango. *Genes and Development*, 11, 2600–2621.
- Swann, K., & Lai, F. A. (2016). Egg activation at fertilization by a soluble sperm protein. *Physiological Reviews*, 96, 127–149.
- Vanden Meerschaut, F., Nikiforaki, D., Heindryckx, B., & De Sutter, P. (2014). Assisted oocyte activation following ICSI fertilization failure. *Reproductive Biomedicine Online*, 28, 560–571.
- Wang, Z. B., Jiang, Z. Z., Zhang, Q. H., Hu, M. W., Huang, L., Ou, X. H., Guo, L., Ouyang, Y. C., Hou, Y., Brakebusch, C., Schatten, H., & Sun, Q. Y. (2013). Specific deletion of Cdc42 does not affect meiotic spindle organization/migration and homologous chromosome segregation but disrupts polarity establishment and cytokinesis in mouse oocytes. *Molecular Biology of the Cell*, 24, 3832–3841.
- Yi, K., Unruh, J. R., Deng, M., Slaughter, B. D., Rubinstein, B., & Li, R. (2011). Dynamic maintenance of asymmetric meiotic spindle position through Arp2/3-complex-driven cytoplasmic streaming in mouse oocytes. *Nature Cell Biology*, 13, 1252–1258.
- Zaragoza, M. V., Surti, U., Redline, R. W., Millie, E., Chakravarti, A., & Hassold, T. J. (2000). Parental origin and phenotype of triploidy in spontaneous abortions: Predominance of diandry and association with the partial hydatidiform mole. *American Journal of Human Genetics*, 66, 1807–1820.

The Reprogramming Power of the Oocyte

Keith E Latham, Michigan State University, East Lansing, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Somatic cell nuclear transfer (SCNT) was first proposed as a way to assess genetic totipotency of somatic cells within the context of whether genetic material was lost as cells differentiate. Little thought was initially given to the oocyte role in this experiment. Subsequently, the remarkable power of oocytes to reprogram exogenous nuclei was dramatically demonstrated, first in amphibians, and later in mammals, during the production of cloned animals. Cloning has been used for decades thereafter to generate cloned animals, first from embryonic cells, and subsequently from adult somatic cells, with valuable application in basic research, agriculture production, transgenesis and biopharmaceutical production, endangered species preservation, and most recently in the production of novel genetically modified animals capitalizing on advancement in genome editing technologies.

Throughout all this rich history, the nature of ooplasmic factors that support genome reprogramming has remained elusive. Many studies in the cloning field enumerate the many deficiencies and technical barriers to cloning, and report methods for enhancing reprogramming and cloning success by treating donor cells before nuclear transfer or by treating cloned constructs after nuclear transfer. A more limited set of studies addresses ooplasmic factors in cloning, using a combination of genetic, proteomic, and transcriptomic approaches, often coupled to in vitro reprogramming systems to better understand the functions of certain molecules. These lines of study have increased our understanding of oocyte biology, the epigenetic mechanisms that help establish each new life, and how the power of the oocyte might be more efficiently harnessed to achieve desired outcomes. Understanding the nature of oocyte reprogramming capacity will require an appreciation for the unique biological processes that are driven by the ooplasm during normal development, the changes in the cellular abundances and activities of the relevant factors during oocyte maturation and embryo development, and the contributions of these ooplasmic changes to how the overall outcome is influenced by the source of donor cell nuclei as a substrate for ooplasmic actions. Much of this insight has yet to emerge, making this an exciting area of research.

In this article, the reprogramming power of the oocyte is considered both in terms of epigenetic changes that occur during normal development and their contributions to normal events (Fig. 1), and in terms of changes imposed on somatic cell genome following nuclear transfer, to what extent normal events are recapitulated or not recapitulated (Fig. 2), and the consequences for cloned embryo viability. The article also considers reprogramming related to meiotic processes, and reprogramming related to genome function. Both aspects of reprogramming are essential for viability.

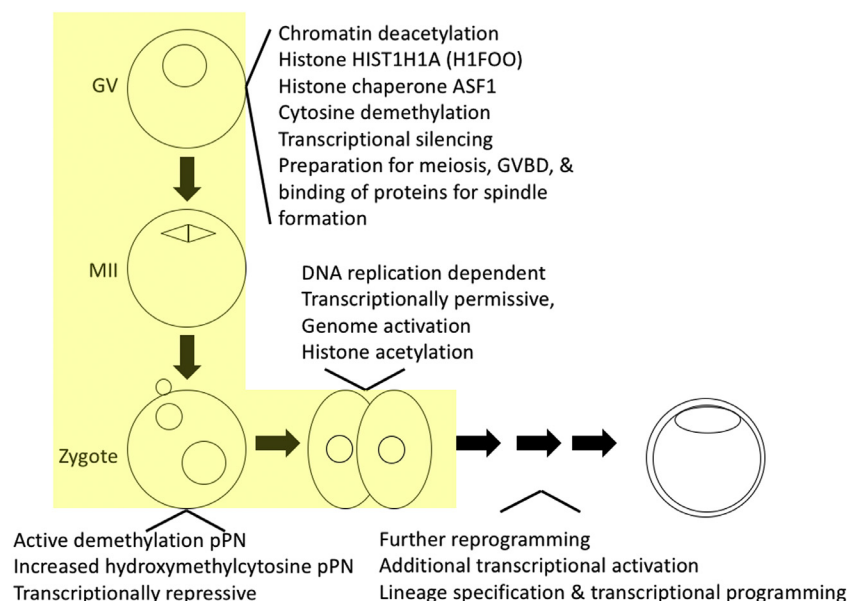


Fig. 1 Summary of major epigenetic changes in mouse oocytes and fertilized embryos. Region in *yellow* highlight indicates the period during which reprogramming events related to meiosis and initial transcriptional programming of the embryonic genome to support development occur. GVBD, germinal vesicle breakdown.

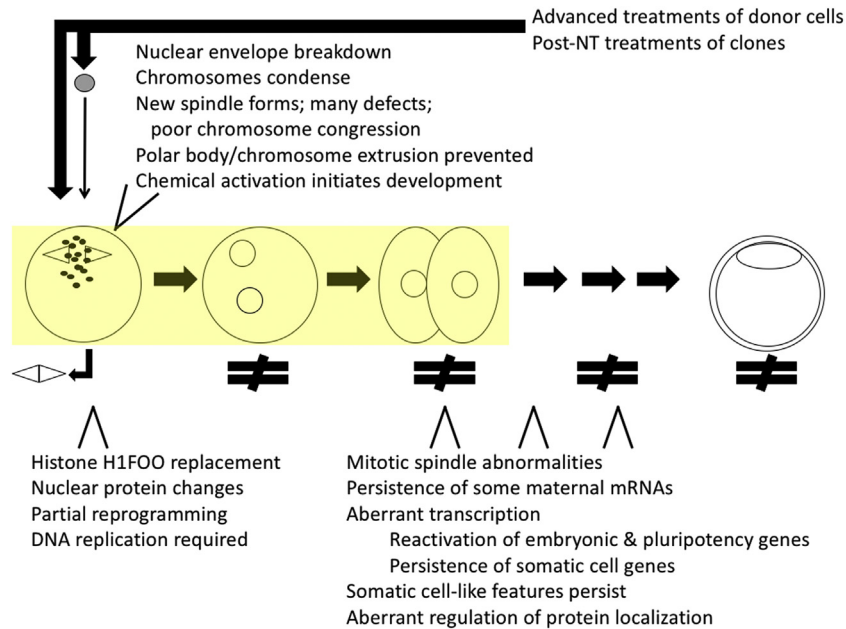


Fig. 2 Summary of major events associated with nuclear transfer in mice. Region in *yellow* highlight corresponds to the highlighted region in **Fig. 1**. As indicated, recapitulation of the events during this period, related to both meiosis and gene transcription, is imperfect, with the result that cloned embryos are markedly dissimilar to normal embryos (as indicated by $\neq$).

Oocyte and Donor Cell Genome Reprogramming Related to Meiosis

The critical cellular organ for meiosis is the meiotic spindle. Chromatin plays a crucial role in spindle formation. Without chromatin spindles can form, but are highly disorganized. Chromatin in which histones H3 and H4 have undergone deacetylation allows binding of RCC1, which in turn initiates the formation of a gradient of *Ran*-GTP emanating from the chromosome and locally directing microtubule polymerization and chromosome attachment (Zierhut and Funabiki, 2015).

After SCNT, donor nuclei lose their nuclear envelopes, undergo chromatin condensation, and direct the formation of new spindles within an hour (Gao et al., 2004). In principle, recapitulating spindle formation following SCNT provides a novel way of studying oocyte meiosis. However, spindles that form after SCNT are defective. They fail to acquire many key proteins in the correct abundances typical of well-formed spindles, and can have abnormal morphologies (Miyara et al., 2006; Han et al., 2010). Chromosomes display gross deficiencies in congression and attachment to the spindle. These defects persist into the initial mitotic cleavage divisions, and cloned embryos display increased incidences of aneuploidy.

Interestingly, spindles that form after transfer of embryonic nuclei (ECNT) are more normal (Miyara et al., 2006), indicating some donor cell-dependent feature of chromatin affects the quality of the spindle that forms. A difference in overall histone H3–H4 acetylation state could account for this. Histone deacetylase complexes are important for maintaining pluripotency and developmental capacity, and for maintaining appropriate patterns of lineage-specific gene expression. These observations indicate that the ability to deacetylate histones may be lost in the oocyte by the mature MII stage, that there may be insufficient opportunity for deacetylation to occur before chromatin condensation, or both. Interestingly, the oocyte variant histone H1FOO assembles onto chromatin within just 5 min of nuclear transfer (Gao et al., 2004), indicating the chromatin remodeling process initiates very rapidly.

The relative timing of histone deacetylation and chromatin condensation may be disrupted with SCNT to mature MII stage ooplasm, in which co-presence of factors that may normally appear and act in a specific temporal sequence during oogenesis precludes the normal required sequence of events. Thus, while spindle formation after SCNT superficially appears to recapitulate oocyte meiotic spindle formation, and while the mature oocyte contains all the factors needed to drive this seeming recapitulation, the key ingredient of temporal specificity of action of some of these factors appears lacking.

Oocyte Reprogramming of Parental Genomes After Fertilization

Following SCNT, there is extensive transcriptional reprogramming of the donor nucleus, so that differentiated genomes cease to express many somatic cell marker genes, and begin expressing pluripotency markers and other genes appropriate to early embryonic stages. The transcriptional reprogramming power of the oocyte reflects the capacity to reprogram gamete genomes during each life cycle. The sperm and egg genomes each directed the formation of highly specialized differentiated cell types. Accordingly, preparing the embryonic genome for embryogenesis requires reprogramming gamete chromatin to an embryonic state. This process

encompasses many global changes in DNA methylation and histone acetylation (**Fig. 1**). The maternal and paternal genomes undergo somewhat different transitions, particularly during the first two cell cycles after fertilization. For example, the paternal genome, but not the maternal genome, undergoes active DNA demethylation during the 1-cell stage within 4 h of fertilization, and then passive demethylation during cleavage. The paternal pronucleus lacks lysine 9 di- and tri-methylated histone H3, but contains histone H3 mono-methylated at lysine 9 and lysine 27, and trimethyl-histone-K27 appears after DNA replication. The maternal pronucleus contains lysine 9 di- and tri-methylated histone H3. With active demethylation of the paternal pronucleus comes increased 5-hydroxymethylcytosine content generated by TET3, and inhibited by DPPA3 in the maternal pronucleus (**Szabo and Pfeifer, 2012**). Reprogramming of the embryonic genome and onset of gene transcription are coupled to DNA replication. Progression through the first S phase after fertilization establishes the ability to perform gene transcription, whereas progression through the second S phase establishes the need for transcriptional enhancers (**Latham, 1999**). Ooplasmic changes accompany these transitions. Early 1-cell stage cytoplasm is transcriptionally repressive and can exert an extended repressive effect on gene expression after nuclear transfer (**Latham, 1999**). Such transitions may contribute to differences in cloning outcome observed by varying the donor and recipient stages. Extensive changes in histone acetylation around the time of transcriptional activation, also dependent on DNA replication, have been described (**Latham, 1999**). The oocyte-expressed SIN3A histone deacetylation complex is required for transcriptional programming of the early embryo. Collectively, these observations demonstrate that the oocyte is endowed with a wide range of factors that modify histone acetylation and methylation and DNA methylation during early cleavage divisions.

Ooplasmic Factors Participating in Exogenous Nuclear Reprogramming

Following nuclear transfer to MII stage oocytes, donor cell nuclear envelopes break down, chromosomes condense, a new spindle forms, and chromosomes try to assemble onto the spindle. Despite the many defects observed, this sequence of events indicates that many of the processes leading to the progression of oocytes to MII stage are recapitulated. As such, it is likely that hundreds of proteins participate in these processes, and thus can be considered as participating in the overall reprogramming process. Thereafter, gene expression must be reprogrammed to an embryonic state; the efficiency of that process affects cloned embryo characteristics and viability (**Fig. 2**). Recent studies provide insight into some of the specific proteins that are likely involved.

Spindle Assembly Factors

The oocyte possesses the necessary factors required for chromatin condensation, and for subsequent spindle assembly. Spindle assembly is linked mechanistically to chromosome condensation. During normal oogenesis, chromosomes undergo histone deacetylation and histone H1 becomes replaced with the oocyte specific variant H1FOO (**De La Fuente, 2014**). Histone deacetylation allows binding of spindle assembly factors such as RCC1, RBBP4, and RBBP7, which allows the formation of a Ran-GTP gradient emanating from the condensed chromosomes, to catalyze microtubule polymerization and spindle assembly (**Zierhut and Funabiki, 2015**). Interestingly, the Ran-GTP gradient is required for formation of the MII spindle, but may be dispensable for forming the MI spindle (**Zierhut and Funabiki, 2015**). Thus, some of the factors that normally participate in progression to first meiotic metaphase may be diminished in abundance from the MII stage oocyte when SCNT is performed, possibly accounting for the many spindle defects observed after SCNT.

Cytoskeleton Components

In a recent genetic screen using recombinant inbred mice to identify genetic loci associated with the ability of SCNT embryos to progress through cleavage, two cytoskeletal scaffold proteins emerged as the strongest genetic candidates for factors affecting early clone development (**Cheng et al., 2013**). These factors may normally control the partitioning of transcription regulators between nuclear and cytoplasmic compartments. This partitioning may be highly regulated to ensure the correct sequence of reprogramming events in the early embryo. However, because the initial chromatin state of the donor genome is very different from gamete genomes following pronucleus formation, the interactions of these cytoskeletal scaffold proteins with factors that modify chromatin may not allow the normal sequence of reprogramming events to occur, leading to partial, or even chaotic, chromatin remodeling. Additional nuclear roles for these proteins have not been examined.

Actin exists in the nucleus, where it may organize nuclear components, regulate chromatin architecture, bind and regulate availability of nuclear transcription factors to participate in transcription, and regulate RNA polymerase II complex function. A major class of chromatin modifying proteins, the SMARC (SWI/SNF-related matrix-associated, actin-dependent regulator of chromatin) family of chromatin regulators, interacts with actin. Studies in *Xenopus* oocytes revealed that the Wave1 protein, which participates in actin filament organization, is involved in reprogramming (**Miyamoto et al., 2013**). Wave1 binds to transcription factors in the nucleus and regulates RNA pol II-dependent transcription. Nuclear actin polymerization regulates expression of the pluripotency factor Oct4/Pou5f1.

Intermediate filaments may also contribute to reprogramming. Vimentin is detected in oocytes and associates with condensing chromatin after SCNT; inhibiting its function negatively affects development of cloned embryos, but not parthenogenetic or fertilized control embryos (**Kong et al., 2014**).

Chromatin, Epigenetic, and Transcriptional Regulators

Oocytes are endowed with a rich supply of specialized histone variants, as well as factors that modify histones. Together, these proteins play significant roles in reprogramming genomes. Oocytes contain a unique form of linker histone H1, denoted H1FOO, which binds chromatin more tightly than somatic H1, and rapidly assembles onto chromatin after SCNT (Gao et al., 2004). Oocyte-expressed histone H3.3 is required for reprogramming, and cannot be substituted by donor cell expressed H3.3 (Wen et al., 2014). Two variants of histone H2, previously denoted as testis specific (TH2A and TH2B) are also expressed in oocytes, and can enhance induced pluripotency in fibroblasts (Huynh et al., 2016). Another oocyte factor, arginine methyltransferase 7 (PRMT7), which methylates H2A, H4, and other chromatin proteins (Miranda et al., 2004), also enhances induced pluripotency (Wang et al., 2016). Histone demethylases are also expressed maternally (Page-Lariviere and Sirard, 2014). Oocytes also contain multiple histone deacetylases, including HDAC1, HDAC2, HDAC3, and other chromatin modifying complexes that interact with HDACs activities, such as SIN3A and nucleoplasmin 2, as well as histone chaperone ASF1, which participate in reprogramming. Additional chromatin regulators are involved in DNA cytosine demethylation, such as DNA methylcytosine dioxygenase TET3, an activity that is inhibited in the maternal genome by the oocyte expressed developmental pluripotency associated protein DPPA3/PGC7/STELLA (Szabo and Pfeifer, 2012), contributing to active demethylation preferentially in the paternal pronucleus. Cullin-ring finger ligase-4, its linker protein DBB1, and substrate adaptor VPRBP/DCAF1 activate TET methylcytosine dioxygenases. Other transcription factors that contribute to oocyte reprogramming function include the transcriptional repressor RE1 silencing transcription factor (REST) (Kong et al., 2016), and oocyte-expressed Gli-like transcription factor GLIS1, which is required for cleavage development and enhances induced pluripotency (Maekawa et al., 2011).

While the oocyte possesses this diverse range of activities, reprogramming with SCNT is limited, and treating either donor cells or SCNT constructs with epigenetic drugs has proven beneficial. These include HDAC inhibitors such as trichostatin A, Scriptaid, valproic acid, oxamflatin, and others. However, excessive treatment with these epigenetic drugs can be detrimental, possibly due to non-specific gene deregulation, loss of imprinting, or loss of other key functional chromatin features. Increased histone acetylation may re-activate some genes contributing to early embryogenesis, but may also inhibit correct chromatin condensation and segregation, as discussed above. Donor cells can also be treated with protamines to enhance cloning (Czernik et al., 2016).

Telomerase and Resetting Replicative Potential

Production of cloned animals, and especially serial cloning through multiple generations or production of clones from aged animals or senescent cells, indicate that the ooplasm has the ability to reset replicative potential, and elongation of telomeres in cloned animals has been reported. This capacity interacts with donor cell characteristics, as both cell type and species affect telomere lengthening.

Mitochondrial and Metabolic Factors

A key component of oocytes is the mitochondrial population, which normally are exclusively matrilineally transmitted. SCNT can create mitochondrial heteroplasmy, although this appears somewhat variable, and subject to complex interactions that differ, as seen by variations in outcomes with different inter-species SCNT donor-recipient combinations. Nuclear reprogramming is an ATP-dependent, energy-intensive process. Metabolic disturbances have been reported in SCNT embryos, and it has been suggested that this negatively affects protein synthesis. The metabolic disturbances may reflect a combination of stress-induced responses and delayed reprogramming affecting embryonic physiology. Mitochondria in the oocyte and early embryo are electron dense with few transverse cristae indicative of reduced ATP-production potential, and become less dense, elongate, and develop transverse cristae during preimplantation development. The initial mitochondrial state may limit ATP production and limit reprogramming capacity. Compatibility between donor nucleus and oocyte mitochondria, and ability of the ooplasm to direct correct transcriptional regulation of mitochondrial genes are likely key factors in defining oocyte reprogramming potential.

Immediate Versus Later Reprogramming Events

Studies of SCNT mouse embryos revealed persistence of somatic cell-like features well into cleavage. Cloned embryos display a preference for somatic cell culture medium over embryo culture medium formulations, a preference for glucose containing media, and a preference for atmospheric oxygen concentration during culture (Latham, 2005). Additionally, cDNA microarray studies revealed hundreds of mis-regulated genes at the two-cell stage, including aberrant expression of transcription factors, which may direct aberrant transcription in a hierarchical manner, accounting for the large number of aberrantly regulated genes (Vassena et al., 2007). At later stages, cloned embryos display aberrant regulation of protein localization, and aberrant gene expression continues (Latham, 2014). Some transcription factors remain associated with the donor genomes after SCNT, and somatic cell-expressed genes continue to be expressed. These observations indicate that reprogramming is slow and likely continues throughout cleavage stages. This likely creates difficulties for providing cloned embryos with optimized culture environments, as their cellular and molecular characteristics would continue to evolve during cleavage development. Treatment of donor cells with epigenetic drugs or chromatin modifying proteins may provide a head start on reprogramming, which could improve outcome.

One study found that inhibiting DNA replication after nuclear transfer inhibited reprogramming and activation of pluripotency markers (Wang et al., 2014). This suggests that, just as DNA replication is required for embryonic genome reprogramming, so too it

contributes to reprogramming during cloning. This is also consistent with persistence of somatic cell characteristics during cleavage, indicating that reprogramming is a slow process that is facilitated by passage through embryonic cell cycles and associated rounds of DNA replication.

Species differences may exist in the pace of reprogramming. One study reported more rapid reprogramming with nuclear transfer to mitotically arrested zygotes in mouse as compared to human (Egli et al., 2011). This difference may reflect different kinetics of regulating ooplasm components following fertilization.

Relationship Between Oocyte Reprogramming Factors and Reprogramming Factors Used to Induce Pluripotency

The study of oocyte reprogramming factors has expanded in recent years through new initiatives to discover factors that can enhance induced pluripotency in cultured somatic cells. For example, oocyte-expressed histone variants, nucleoplasmin, and GLIS1, activation-induced cytidine deaminase, and geminin can enhance cellular reprogramming to make induced pluripotent stem cells (iPSCs) (Huynh et al., 2016; Maekawa et al., 2011). Oocyte expressed PRMT7 can substitute for SOX2 in the iPS protocol (Wang et al., 2016). Transcriptome comparisons between oocytes of different species have led to “candidate oocyte reprogramming factors” that are being tested for use in cellular reprogramming. Oocyte germinal vesicle extracts and oocyte extracts from different species have been considered in cellular reprogramming (Tokmakov et al., 2016). Genes elevated in expression in iPSCs relative to embryonic stem cells have been compared to oocyte transcriptomes for further selection of candidates. Reciprocally, factors used in making iPSCs have been used to facilitate reprogramming with SCNT. The discovery of different combinations of chromatin modifiers and other factors that can be used in combination to convert somatic cells in culture to pluripotency indicates that many pathways to pluripotency may exist, and that extreme overexpression of exogenous factors can overwhelm prior programming by transforming chromatin states at pluripotency-related loci. Discovering which of these pathways also operate during gametogenesis, which do not, and what are the common downstream mechanisms leading to pluripotency should reveal much new knowledge about how gametogenesis generates genomes that can direct the formation of a new individual. In addition, discovering how the oocyte components (cytoplasmic mRNAs, proteins) interact with the two gamete genomes to unlock the gametic programming should prove fascinating.

Conclusions

The oocyte is truly a remarkable cell, being endowed with the ability to vastly restructure chromatin, both in preparation for meiosis, and to generate one of two gamete genomes that must unite to form a new life. The oocyte also contains factors that interact with the sperm-derived genome to ready it for function, as well as factors that maintain and modify epigenetic information related to genomic imprinting, and factors that prepare the embryonic genome for transcription and enable transcription to be properly regulated. No other cell type possesses this capacity. The identification of factors that contribute to reprogramming during cloning by nuclear transfer, combined with identification of factors that enable induced pluripotency in vitro have shed important new light on the nature of ooplasm reprogramming factors. But the factors so far identified are comparatively few in number, and most are well-known for chromatin remodeling and transcriptional control in other contexts. Along with the identification of these factors has come the realization that the oocyte must contain many such factors, and that numerous factors must work in combination to accomplish the overall goal of reprogramming the oocyte genome for meiosis, and reprogramming pronuclei and the embryonic genome during early cleavage to support embryogenesis. Many of the reprogramming factors in the oocyte likely work in complementary pairs, which modify the oocyte and pronuclear genomes, and then reverse those modifications at the appropriate stages. This suggests that the activities of these factors must be coordinated with and regulated by signaling pathways within the oocyte that respond to exogenous stimuli as well as endogenous cues. This further implies that the oocyte will be sensitive to external insults, but that redundancies in functions and partitioning of functions between multiple factors may provide resistance to sustain overall oocyte quality even in the face of adverse environmental conditions. A better understanding of those functional redundancies and partitioning could provide new approaches to enhance oocyte quality for a range of purposes, as well as methods to enhance success of cloning and creation of induced pluripotent stem cells in vitro.

Using oocyte factors to enhance induction of pluripotency, using iPS factor to enhance cloning outcome, or treating donor cells or clone constructs with epigenetic drugs all show some success and some promise. But all these approaches carry risk of incorrect reprogramming, with unintended effects on some target genes potentially negating favorable effects at other genes, as well as risk of disrupting essential imprinting information and other epigenetic information that guides early development and even determines adult phenotype. Additional attention to reprogramming chromatin to a state that can work with oocyte factors to enhance chromosome congression and spindle quality could also be helpful.

References

- Cheng, Y., Gaughan, J., Midic, U., Han, Z., Liang, C. G., Patel, B. G., & Latham, K. E. (2013). Systems genetics implicates cytoskeletal genes in oocyte control of cloned embryo quality. *Genetics*, 193, 877–896.
- Czemik, M., Iuso, D., Toschi, P., Khochbin, S., & Loi, P. (2016). Remodeling somatic nuclei via exogenous expression of protamine 1 to create spermatid-like structures for somatic nuclear transfer. *Nature Protocols*, 11, 2170–2188.

- De La Fuente, R. (2014De). Histone deacetylation: Establishing a meiotic histone code. *Cell Cycle*, 13, 879–880.
- Egli, D., Chen, A. E., Saphier, G., Ichida, J., Fitzgerald, C., Go, K. J., Acevedo, N., Patel, J., Baetscher, M., Kearns, W. G., et al. (2011). Reprogramming within hours following nuclear transfer into mouse but not human zygotes. *Nature Communications*, 2, 488.
- Gao, S., Chung, Y. G., Parseghian, M. H., King, G. J., Adashi, E. Y., & Latham, K. E. (2004). Rapid H1 linker histone transitions following fertilization or somatic cell nuclear transfer: Evidence for a uniform developmental program in mice. *Developmental Biology*, 266, 62–75.
- Han, Z., Liang, C. G., Cheng, Y., Duan, X., Zhong, Z., Potireddy, S., Moncada, C., Merali, S., & Latham, K. E. (2010). Oocyte spindle proteomics analysis leading to rescue of chromosome congression defects in cloned embryos. *Journal of Proteome Research*, 9, 6025–6032.
- Huynh, L. M., Shinagawa, T., & Ishii, S. (2016). Two histone variants TH2A and TH2B enhance human induced pluripotent stem cell generation. *Stem Cells and Development*, 25, 251–258.
- Kong, Q., Xie, B., Li, J., Huan, Y., Huang, T., Wei, R., Lv, J., Liu, S., & Liu, Z. (2014). Identification and characterization of an oocyte factor required for porcine nuclear reprogramming. *The Journal of Biological Chemistry*, 289, 6960–6968.
- Kong, Q. R., Xie, B. T., Zhang, H., Li, J. Y., Huang, T. Q., Wei, R. Y., & Liu, Z. H. (2016). RE1-silencing transcription factor (REST) is required for nuclear reprogramming by inhibiting transforming growth factor beta signaling pathway. *The Journal of Biological Chemistry*, 291, 27334–27342.
- Latham, K. E. (1999). Mechanisms and control of embryonic genome activation in mammalian embryos. *International Review of Cytology*, 193, 71–124.
- Latham, K. E. (2005). Early and delayed aspects of nuclear reprogramming during cloning. *Biology of the Cell*, 97, 119–132.
- Latham, K. E. (2014). Role of aberrant protein modification, assembly, and localization in cloned embryo phenotypes. *Advances in Experimental Medicine and Biology*, 759, 141–158.
- Maekawa, M., Yamaguchi, K., Nakamura, T., Shibukawa, R., Kodanaka, I., Ichisaka, T., Kawamura, Y., Mochizuki, H., Goshima, N., & Yamanaka, S. (2011). Direct reprogramming of somatic cells is promoted by maternal transcription factor Glis1. *Nature*, 474, 225–229.
- Miranda, T. B., Miranda, M., Frankel, A., & Clarke, S. (2004). PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity. *The Journal of Biological Chemistry*, 279, 22902–22907.
- Miyamoto, K., Teperek, M., Yusa, K., Allen, G. E., Bradshaw, C. R., & Gurdon, J. B. (2013). Nuclear Wave1 is required for reprogramming transcription in oocytes and for normal development. *Science*, 341, 1002–1005.
- Miyara, F., Han, Z., Gao, S., Vassena, R., & Latham, K. E. (2006). Non-equivalence of embryonic and somatic cell nuclei affecting spindle composition in clones. *Developmental Biology*, 289, 206–217.
- Page-Lariviere, F., & Sirard, M. A. (2014). Spatiotemporal expression of DNA demethylation enzymes and histone demethylases in bovine embryos. *Cellular Reprogramming*, 16, 40–53.
- Szabo, P. E., & Pfeifer, G. P. (2012). H3K9me2 attracts PGC7 in the zygote to prevent Tet3-mediated oxidation of 5-methylcytosine. *Journal of Molecular Cell Biology*, 4, 427–429.
- Tokmakov, A. A., Iwasaki, T., Sato, K. I., & Kamada, S. (2016). Reprogramming of somatic cells and nuclei by *Xenopus* oocyte and egg extracts. *The International Journal of Developmental Biology*, 60, 289–296.
- Vassena, R., Han, Z., Gao, S., Baldwin, D. A., Schultz, R. M., & Latham, K. E. (2007). Tough beginnings: Alterations in the transcriptome of cloned embryos during the first two cell cycles. *Developmental Biology*, 304, 75–89.
- Wang, B., Pfeiffer, M. J., Schwarzer, C., Arauzo-Bravo, M. J., & Boiani, M. (2014). DNA replication is an integral part of the mouse oocyte's reprogramming machinery. *PLoS One*, 9, e97199.
- Wang, B., Pfeiffer, M. J., Drexler, H. C., Fuellen, G., & Boiani, M. (2016). Proteomic analysis of mouse oocytes identifies PRMT7 as a reprogramming factor that replaces SOX2 in the induction of pluripotent stem cells. *Journal of Proteome Research*, 15, 2407–2421.
- Wen, D., Banaszynski, L. A., Liu, Y., Geng, F., Noh, K. M., Xiang, J., Elemento, O., Rosenwaks, Z., Allis, C. D., & Rafii, S. (2014). Histone variant H3.3 is an essential maternal factor for oocyte reprogramming. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 7325–7330.
- Zierhut, C., & Funabiki, H. (2015). Nucleosome functions in spindle assembly and nuclear envelope formation. *BioEssays*, 37, 1074–1085.

Regulation of the Oocyte Epigenome During Prophase I Arrest

Bruno Marques, Ricardo Matos, and Rui G Martinho, Universidade do Algarve, Faro, Portugal

© 2018 Elsevier Inc. All rights reserved.

Meiosis

Meiosis is a process of cell division where a eukaryotic diploid cell originates one or more haploid cells, being essential for gamete production and maintenance of ploidy after egg fertilization. Meiosis is similarly important for generating genetic diversity through chromosome recombination and segregation. Not surprisingly, meiotic defects often lead to sterility and developmental defects (Ohkura, 2015).

Diploid cells ($2n$) contain two sets of chromosomes, being one set usually from the mother and another set from the father. A homologous chromosome set corresponds to a pair of chromosomes in a diploid cell. After each round of DNA replication during S-phase, a diploid cell contains two copies of each chromosome ($4n$), forming sister chromatids that are held together by the cohesin complex. As a diploid cell enters meiosis, pairs of sister chromatids from the homologous chromosomes are matched together and genetic material is exchanged by crossing over during prophase of meiosis I (prophase I). After synapsis formation and initiation of crossing over during prophase I, the homologous chromosomes are held together by chiasmata during metaphase I and until the onset of anaphase I, when they are resolved and the homologous chromosomes separated. Sister chromatids for each homologous chromosome are subsequently separated in meiosis II in a process similar to mitosis (Ohkura, 2015).

Gametogenesis

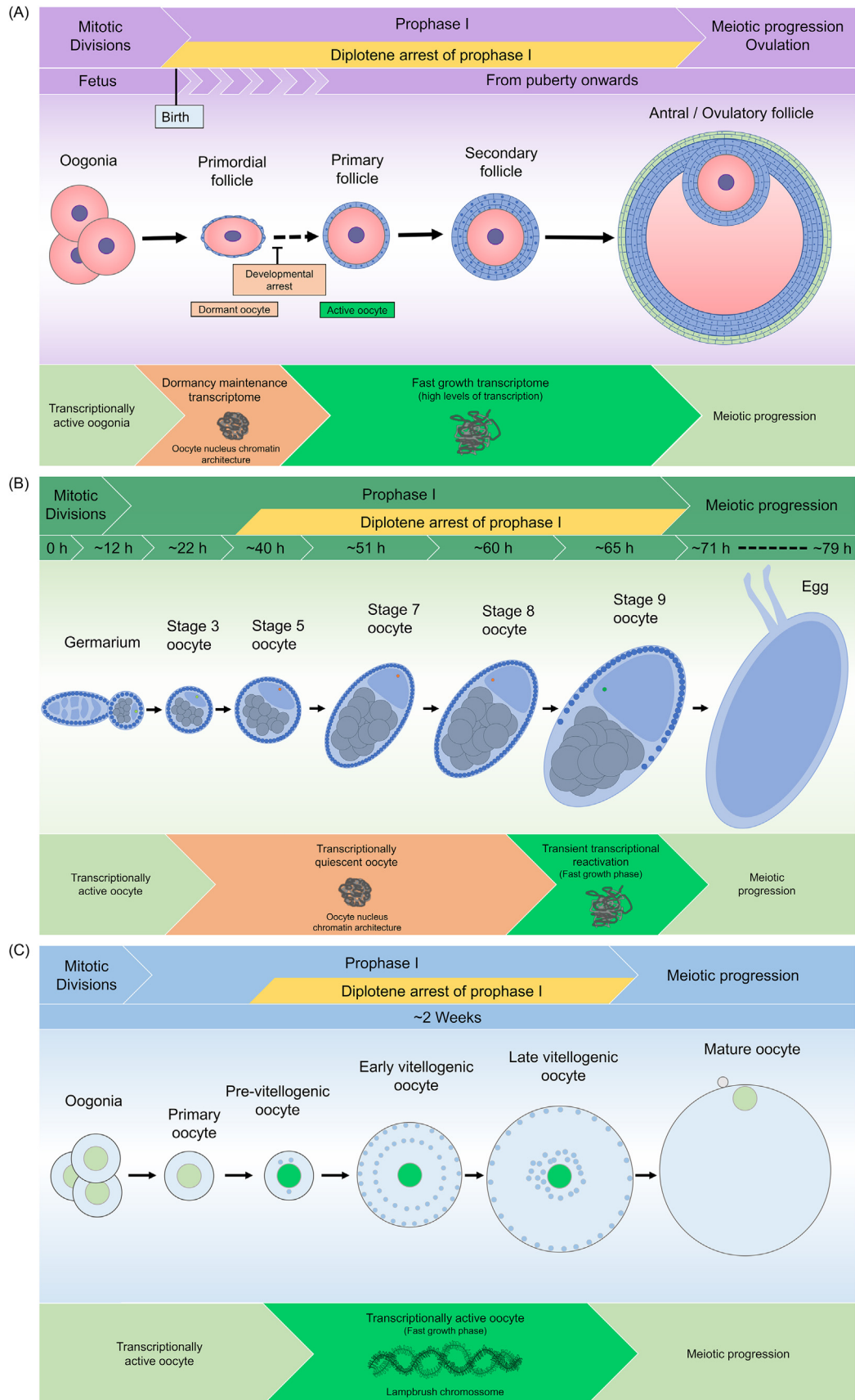
Gametogenesis is the process by which sperm and eggs are produced from the germ cells in the testes and ovaries, respectively. Although males and females have meiosis and differentiation as the central points of gamete formation, they differ in the timing of differentiation in relation to meiosis (Von Stetina and Orr-Weaver, 2011). Whereas spermatogenesis is a continuous process where male germ cells undergo meiosis and then differentiate into the respective spermatozoa (Sharma and Agarwal, 2011), differentiation of female germ cells takes place during meiosis, being its progression frequently arrested during oocyte maturation (Von Stetina and Orr-Weaver, 2011). The meiotic arrest at the diplotene stage of prophase I is a highly conserved feature of female meiosis across all studied metazoans (Fig. 1A–C), being usually coincident with significant oocyte growth and maturation (Arur, 2017; Greenstein, 2005).

Danio Rerio and *Xenopus Laevis* Oogenesis

Oocytes of most animals are large single cells that contain all materials necessary for development of the embryo until the new individual can begin feeding. In the case of non-mammal vertebrates, like zebrafish (*Danio rerio*) or clawed frog (*Xenopus laevis*), their oocytes are remarkably large and rich in storage proteins. In *Danio rerio* the primordial germ cells (PGCs) differentiate into oogonia, which subsequently form the primary oocytes at the beginning of meiosis. The primary oocyte then becomes arrested at the diplotene stage of prophase I (Lubzens et al., 2010). During this arrest there is a massive growth of the oocyte with an accumulation of a large stockpile of storage proteins (vitellogenesis) needed for the development of the embryo after fertilization. Primary oocyte maturation then continues with germinal vesicle breakdown and completion of the first meiotic division. From the first meiotic division results the secondary oocyte and a very small polar body that degenerates. The oocyte maturation process is followed by ovulation, and as in most vertebrates, the secondary oocyte becomes arrested again during the second meiotic division in metaphase (metaphase II) until fertilization (Gilbert, 2000). *Xenopus laevis* oogenesis is similar to *Danio rerio*, as it starts in the embryo roughly 3 weeks after fertilization, as the PGCs begin differentiating to form primary oogonia, being cell growth considerably slow. Meiosis begins after the formation of the secondary oogonia and continues until an arrest at the diplotene stage of prophase I. During this arrest, vitellogenesis takes place and the oocytes grow considerably, with an accumulation of storage proteins. Oocyte meiotic progression then continuous, with chromosome condensation and germinal vesicle breakdown, until it enters meiosis II and becomes arrested again in metaphase II (Rasar and Hammes, 2006; Perry and Verlhac, 2008).

Mammalian Oogenesis

Vertebrate oocyte maturation is remarkably conserved with most differences resulting from adaptations to distinct reproductive strategies. Mammalian oocytes are significantly smaller than oocytes from non-mammal vertebrates as they rely on the mother to provide most of the nutrients required for embryonic development. Mammalian oocytes arrest at diplotene stage of prophase I and subsequently at metaphase II. Most of oocyte growth occurs during diplotene I arrest, where there is a significant change of the primary oocyte transcriptome after reactivation of the dormant primordial follicle and before it progresses into metaphase



I and terminates meiosis I (Ernst et al., 2017). The secondary oocyte then arrests in metaphase II and ovulates. Release of metaphase II arrest occurs after fertilization.

Human oogenesis, starts when the embryonic primordial germ cells begin to differentiate into oogonia between the 6th and 8th weeks of gestation (Pelosi et al., 2015; Kim, 2012). The number of oogonia increases dramatically by mitotic proliferation from approximately 600,000 during the 8th week of gestation up to approximately 7,000,000 by the 5th month. Although the oogonia are formed in large numbers most die by apoptosis, and only the ones that survive will differentiate into an oocyte between the 15th week and 7th month of gestation. Oocyte differentiation results from an asymmetric mitotic division of the oogonium that gives rise to the future primary oocyte and another oogonium. As the primary oocyte enters meiosis, it progresses through meiosis I until it becomes arrested for several years in a dormant state at the diplotene stage of prophase I (Kim, 2012; Pepin et al., 2007; Pan et al., 2012). Granulosa cells surround this primary oocyte to form a structure called primordial follicle and produce various growth factors and cytokines that are crucial for oocyte viability and maturation (Havelock et al., 2004; Ernst et al., 2017). With puberty, and until the onset of menopause, groups of primordial follicles are continuously reactivated for further oocyte maturation. Since primary oocyte maturation takes over a year until ovulation a healthy adult ovary contains a large number of oocytes at distinct stages of maturation. Similar to other non-mammals vertebrates, primary oocyte activation is associated with a burst of gene expression, a significant change of the oocyte transcriptome, and considerable growth and maturation (Pelosi et al., 2015; Ernst et al., 2017).

Mouse oogenesis is largely similar to humans, with the only major differences being the time of each phase. At first, few primordial germ cells can be identified, but they divide mitotically and 12 days *post coitum* the number increases to 2500–6000 cells and by day 16th there are several million germ cells. Similarly to humans, the majority of these cells degenerate, with the rest progressing into the first meiotic division and becoming arrested at diplotene stage of prophase I. Mouse gestation normally lasts 21 days, and similar to humans, the oocytes are arrested in prophase I after birth. Throughout the lifetime of the mouse the dormant primary oocytes are continuously activated but only a small subset of oocytes ovulate, with most degenerating during maturation. The first meiotic division is only completed prior to ovulation so the oocytes are meiotically arrested during growth, and the second meiotic division only occurs after fertilization (Wassarman, 2012).

Drosophila Oogenesis

Drosophila melanogaster oogenesis is divided into 14 developmentally distinct stages, and takes approximately a week to be completed (Bastock and St Johnston, 2008; Lake and Hawley, 2012). Similar to vertebrates, PGCs differentiate within the developing ovary into germ line stem cells (GSCs); which are functionally equivalent to the vertebrate oogonia. *Drosophila* oogenesis starts, again similarly to vertebrates, with an asymmetric division of GSCs that produce an anteriorly localized GSC and a posteriorly localized cystoblast that will ultimately differentiate into a mature oocyte (Bastock and St Johnston, 2008). The cystoblast undergoes four incomplete mitotic divisions to produce a 16 cell-cyst interconnected by actin-based ring canals and a cytoskeleton-rich structure named fusome. Although the two cells that have four intercellular bridges attempt to enter meiosis only one will remain in meiosis and become the oocyte (Bastock and St Johnston, 2008). The other 15 cells will become nurse cells that support oocyte growth and maturation by synthesizing and transporting maternal factors into the oocyte. *Drosophila* oocyte growth and maturation is therefore mostly supported not by the oocyte itself but by the closely associated nurse cells.

Similarly to vertebrates, *Drosophila* oogenesis has a prolonged meiotic arrest at the diplotene stage of prophase I that correlates with a rapid growth of the oocyte. Interestingly, the transcriptionally quiescent diplotene-arrested oocyte nucleus transiently reactivates gene expression just before it progresses into metaphase I (Flora et al., 2017; Navarro Costa et al., 2016). Although potentially important for meiosis and oocyte maturation the signal responsible for such reactivation of gene expression is still poorly understood.

Outstanding Problem: How Is Oocyte Gene Expression Correctly Reactivated After Dormancy?

The meiotic arrest at diplotene stage of prophase I and the significant change of the oocyte transcriptome during reactivation and growth are highly conserved but poorly understood features of oogenesis. How can a dormant human primary oocyte correctly

Fig. 1 The arrest at the diplotene stage of prophase I is a highly conserved feature of female meiosis and is frequently associated with a period of oocyte dormancy followed by a period of rapid growth. (A) In *Homo sapiens* (human), the developing primary oocyte arrests for several years in a dormant state, before reactivation after puberty. Reactivation of the dormant primordial follicle is associated with a dramatic change in the oocyte transcriptome and rapid growth. (B) In *Drosophila melanogaster* (fruit fly), the developing oocyte also arrests at the diplotene stage of prophase I, with its nucleus forming a highly condensed structure, known as the karyosome, and becoming transcriptionally quiescent. Such quiescence does not impair oocyte growth as it mostly relies in the supporting nurse cells. The transcriptionally quiescent oocyte transiently reactivates gene expression before it progresses into metaphase suggesting a role in meiotic progression. (C) In *Danio rerio* (zebrafish), the developing oocyte arrests at the diplotene stage of prophase I. During this meiotic arrest there is a massive growth of the oocyte. The establishment of lampbrush chromosomes structures supports the extremely high levels of gene expression needed for such rapid growth.

reactivate gene expression after decades of dormancy? And to what extent is this reactivation important for female gamete competence and fertility?

As maintenance of female fertility in humans relies in the viability and correct reactivation of the dormant primary oocytes, a better understanding of these processes is likely to be highly relevant not only for fertility but also for the timely onset of menopause.

Our working hypothesis is that the oocyte epigenome plays a crucial role in the correct reactivation of the quiescent primary oocytes and their ability to produce fully competent female gametes.

Meiotic Arrest at the Diplotene Stage of Prophase I

Meiotic prophase I is subdivided into five stages: leptotene, zygotene, pachytene, diplotene, and diakinesis. During the leptotene stage the chromatin arranges into long and thin strands and at zygotene stage the synapsis of homologous chromosomes takes place, facilitated by assembly of central elements of the synaptonemal complex. Pachytene is the stage where homologous recombination, including chromosomal crossover, takes place. Subsequently, the oocyte progresses into the diplotene stage where it frequently enters a prolonged resting phase (Snustad and Simmons, 2015).

The diplotene I arrest is often associated with dramatic rearrangements of the oocyte chromatin architecture. Different species have different strategies to assure the correct maturation of oocyte at this stage (Fig. 1A–C). *Xenopus laevis* and *Danio rerio* chromosomes greatly expand in size and develop symmetric loops forming what is known as lampbrush chromosomes (Fig. 1C). Each loop in these chromosomes represents high levels of transcriptional activity and each loop has a reproducible size and a characteristic pattern. Oocytes of *Xenopus laevis* and *Danio rerio* are much bigger than mammals, so it is crucial that RNA synthesis is enough to sustain oocyte growth. At the end of the diplotene I arrest these lampbrush chromosomes structures disappear, growth is mostly halted, and the oocyte progresses into metaphase I (Appels et al., 2012).

Oocytes from mammals are significantly smaller in size than their vertebrate counterparts, without detectable lampbrush chromosomes, as there is no need to accumulate such large stockpiles of storage proteins to sustain embryonic development after fertilization (Fig. 1A). Before birth, the primary oocytes enter meiotic diplotene I arrest, being surrounded by supporting granulosa cells and forming long-lived quiescent ovarian structures named primordial follicles (Pan et al., 2012). After puberty, activation of the primordial follicles is marked by a change of the supporting granulosa cells from a flat to a cuboid architecture, a major alteration of the oocyte transcriptome, and subsequent resumption of oocyte growth and maturation (Ernst et al., 2017).

Drosophila oocyte arrest at diplotene stage of prophase I is accompanied by condensation of the oocyte chromatin into a structure known as the karyosome and the establishment of transcriptional quiescence (Fig. 1B). Such quiescence does not impair oocyte maturation, as growth is mostly provided by the supporting nurse cells (Bastock and St Johnston, 2008; Lake and Hawley, 2012). Interestingly, and despite the role of the nurse cells, prophase I-arrested oocytes transiently reactivate gene expression approximately 36 hours after karyosome formation, and just before they progress into metaphase I (Navarro-Costa et al., 2016). This transient reactivation of transcription is associated with a global change in oocyte chromatin architecture suggesting a role in meiotic progression.

Epigenetic Regulation

Epigenetics are stable phenotypes that cannot be explained by changes in the DNA sequence. Epigenetic modifications such as DNA methylation and histone modifications play an important role in the regulation of chromatin structure, guaranteeing that gene expression is temporally and spatially appropriate, and that cell-type specific patterns of gene expression are robustly maintained during development (Li et al., 2007). The epigenome of the prophase I-arrested oocyte is surprisingly dynamic, presenting an assortment of euchromatic and heterochromatic markers that vary during oocyte quiescence and reactivation (Navarro-Costa et al., 2016; Kageyama et al., 2007).

Drosophila oocytes show a considerable variation of distinct histone modifications during prophase I-arrest. Although the functional significance of many of these modifications for meiosis and oocyte maturation are still poorly understood, regulation of histone H3 lysine 4 trimethylation (H3K4me3), an euchromatic marker, and histone H3 lysine 27 trimethylation (H3K27me3), a heterochromatic marker, have been reported to be rate-limiting for oocyte chromatin architecture, meiotic completion, and female fertility (Navarro-Costa et al., 2016). During transcriptional quiescence prophase I-arrested oocytes maintain H3K4me3 at low levels compared to H3K27me3, whereas reactivation of oocyte transcription correlates with a significant decrease in the levels of H3K27me3, without any detectable change in the levels of H3K4me3, suggesting that the balance between H3K4me3 and H3K27me3 epigenetically controls oocyte dormancy and reactivation (Navarro-Costa et al., 2016).

Mammalian oocytes also present an assortment of euchromatic and heterochromatic markers, and the levels of H3K4me3 are similarly important for meiotic progression and transcriptional reactivation, as the functional association between H3K4me3 and specific gene expression during meiosis has been established (Kageyama et al., 2007; Shi and Wu, 2009; De La Fuente, 2006). Moreover, and again similarly to *Drosophila*, it has been demonstrated that the levels of H3K4me3 and H3K27me3 are dynamic during oocyte development and maturation, and that the disruption of the balance between them can lead to incorrect meiosis progression (Xu et al., 2017; Pan et al., 2012). Methylation of histone H3 lysine 9 (H3K9) is a heterochromatin epigenetic mark associated with

transcriptional silencing. The levels of H3K9 methylation are dynamic during oocyte maturation (Tachibana et al., 2007), being H3K9 dimethylation regulated by the ESET/SETDB1 complex (Wang et al., 2017). Disruption of H3K9 methylation during prophase I leads to immature gamete formation, being meiosis similarly arrested at pachytene stage in males and females (Tachibana et al., 2007; Wang et al., 2017).

The dynamism of the oocyte epigenome has also been revealed by the patterns of histone acetylation. Fully grown oocytes and growing oocytes have distinct patterns of histone acetylation, being the levels of H3K9, Histone 3 lysine 18 (H3K18), Histone H4 lysine 5 (H4K5), and Histone H4 Lysine 12 (H4K12) acetylation higher in mature oocytes (Kageyama et al., 2007; Pan et al., 2012). Interestingly, the histone acetylation levels are higher in SN (surrounded nucleolus)-type oocytes, where chromosomes are highly condensed around the nucleolus and transcription silenced.

The phosphorylation of histones is also important for meiotic completion and progression, as phosphorylation of H2AThr119 by the *Drosophila* nucleosomal histone kinase-1 (NHK-1) is necessary for acetylation of histones H3 and H4, synaptonemal complex disassembly, and chromosome condensation during karyosome formation (Ivanovska et al., 2005; Lancaster et al., 2010).

Histone Demethylases

Drosophila KDM5 histone demethylase (dKDM5; also known as Lid) localizes to the transcription-start site (TSS) of actively transcribed genes and negatively regulates the levels of H3K4me3 (Lloret-Llinares et al., 2012). dKDM5 also has a demethylase-independent H3K4me3 recognition function that positively regulates gene expression (Li et al., 2010; Liu and Secombe, 2015). Such dual function of dKDM5 potentially explains why the expression of different subsets of genes is positively or negatively regulated after KDM5 depletion.

Drosophila oocytes transiently reactivate gene expression during late prophase I. Female germ-line depletion of dKDM5, leads to a significant increase in the levels of H3K4me3 and precocious transcriptional reactivation of the quiescent prophase I-arrested oocytes (Fig. 2), without any detectable change in the levels of H3K27me3 and histone H3 lysine 9 di-methylation (H3K9me2) (Navarro-Costa et al., 2016). Although the overhaul architecture and growth of the developing egg chamber is not impaired after dKDM5 depletion, its oocyte chromatin architecture is nevertheless highly abnormal, with a defective assembly of the synaptonemal complex, abnormal progression of meiosis I and II, and a significant reduction of oocyte-competence and female fertility (Navarro-Costa et al., 2016; Zhaunova et al., 2016). Importantly, the demethylase activity of KDM5 is required for correct transcriptional reactivation of the oocyte and meiotic progression (Navarro-Costa et al., 2016), suggesting a role of this histone demethylase in the regulation of the oocyte epigenome, and further supporting the hypothesis that correct reactivation of the dormant primary oocyte is epigenetically regulated.

Our working hypothesis is that regardless of distinct reproductive strategies the regulation of oocyte epigenome during prophase I is highly conserved across metazoans. Consistently, depletion of mouse KDM5b leads to secondary female infertility (Zou et al., 2014), whereas deletion of mouse Foxo3 is associated with precocious primary oocyte reactivation and secondary infertility in older females (Castrillon et al., 2003). Interestingly, *Drosophila* KDM5 interacts with Foxo and positively modulates its DNA binding ability (Liu et al., 2014), suggesting a functional crosstalk between these two proteins during oocyte reactivation (Fig. 2). Further confirming the role of distinct histone demethylases in oocytes reactivation and maturation, mouse Sall4 is required for primary oocyte maturation and resumption of meiosis by modulating the expression of KDM5b, KDM6a, and KDM6b that are responsible for maintaining the correct levels of H3K4me3 and H3K27me3 modifications (Xu et al., 2017).

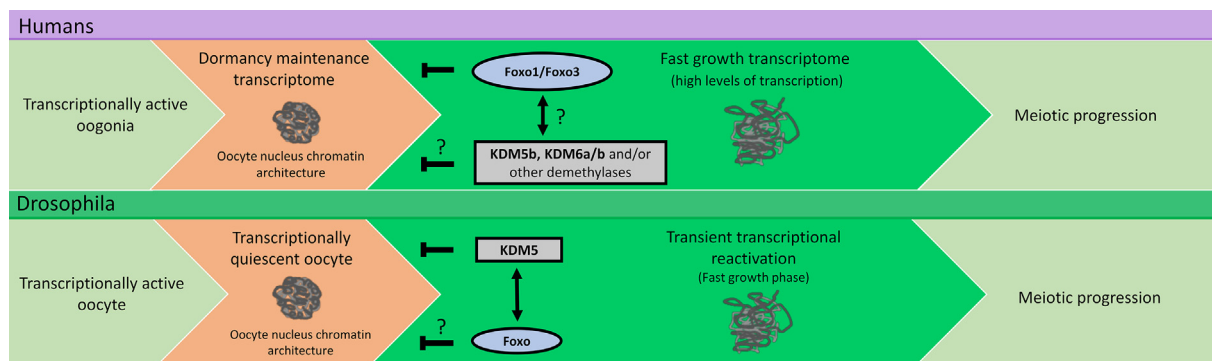


Fig. 2 The epigenetic mechanisms responsible for the correct transcriptional reactivation of the dormant/quiescent primary oocyte are likely to be conserved across metazoans. A functional crosstalk between Foxo transcription factors (e.g., *Drosophila* Foxo, mouse Foxo3, and human Foxo1) and distinct histone demethylases (e.g., *Drosophila* KDM5, and mammalian KDM5b and KDM6a/b) may be regulating oocyte reactivation during the meiotic arrest at the diplotene stage of prophase I.

Final Considerations

Mouse Foxo3 is expressed in the nucleus of the dormant primary oocytes, being responsible for preserving their quiescence state (Castrillon et al., 2003). After puberty, primordial follicle reactivation relies in nutritional and other local factors that stimulate mTOR signaling in granulosa cells of the dormant primordial follicles, leading to the secretion of a ligand that binds the oocytes cell surface receptor Kit (Kim, 2012; Saatcioglu et al., 2016). This binding induces primary oocyte reactivation by promoting PI3K/AKT signaling and subsequent Foxo3 inactivation, as AKT-dependent phosphorylation of Foxo3 leads to its nuclear export and inactivation.

Although the signaling cascades responsible for primordial follicle reactivation are reasonably well established, it is still unclear how an oocyte can correctly reactivate gene expression after long-term dormancy. Reactivation of the primordial follicles after years, if not decades, of dormancy, correlates with a dramatic change in human oocyte transcriptome (Ernst et al., 2017). How is this possible? What are the genetic and epigenetic mechanisms capable of ensuring that the previously dormant oocyte still “remembers” what to do and how to give rise to a fully mature female gamete. We hypothesize that the oocyte epigenome is crucial for the success of such developmental transition, having histone demethylases (like KDM5) a central role in this process.

Acknowledgments

Rui G. Martinho is supported by Portuguese national funding through the following Fundação para a Ciência e a Tecnologia (FCT) grants: PTDC/BEX-BID/0395/2014 and UID/BIM/ 04773/2013 CBMR 1334. Bruno Marques is supported by a FCT PhD studentship PD/BD/128342/2017, within the scope of the ProReGeM PhD program (Ref. PD/00117/2012, CRM:0027030).

References

- Appels, R., Morris, R., Gill, B. S., & May, C. E. (2012). *Chromosome biology*. Berlin: Springer Science & Business Media.
- Arur, S. (2017). Signaling-mediated regulation of meiotic prophase I and transition during oogenesis. *Results and Problems in Cell Differentiation*, 59, 101–123.
- Bastock, R., & St Johnston, D. (2008). *Drosophila* oogenesis. *Current Biology*, 18(23), R1082–7.
- Castrillon, D. H., Miao, L., Kollipara, R., et al. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science*, 301(5630), 215–218.
- De La Fuente, R. (2006De). Chromatin modifications in the germinal vesicle (GV) of mammalian oocytes. *Developmental Biology*, 292(1), 1–12.
- Ernst, E. H., Grondahl, M. L., Grund, S., et al. (2017). Dormancy and activation of human oocytes from primordial and primary follicles: Molecular clues to oocyte regulation. *Human Reproduction*, 32(8), 1684–1700.
- Flora, P., McCarthy, A., Upadhyay, M., & Rangan, P. (2017). Role of chromatin modifications in *Drosophila* Germline stem cell differentiation. *Results and Problems in Cell Differentiation*, 59, 1–30.
- Gilbert, S. F. (2000). *Developmental biology*. Sunderland, MA: Sinauer Associates.
- Greenstein, D. (2005). Control of oocyte meiotic maturation and fertilization. *WormBook*, 1–12. The Online Review of C. Elegans Biology.
- Havelock, J. C., Rainey, W. E., & Carr, B. R. (2004). Ovarian granulosa cell lines. *Molecular and Cellular Endocrinology*, 228(1–2), 67–78.
- Ivanovska, I., Khandan, T., Ito, T., & Orr-Weaver, T. L. (2005). A histone code in meiosis: The histone kinase, NHK-1, is required for proper chromosomal architecture in *Drosophila* oocytes. *Gender and Development*, 19(21), 2571–2582.
- Kageyama, S., Liu, H., Kaneko, N., et al. (2007). Alterations in epigenetic modifications during oocyte growth in mice. *Reproduction*, 133(1), 85–94.
- Kim, J. Y. (2012). Control of ovarian primordial follicle activation. *Clinical and Experimental Reproductive Medicine*, 39(1), 10–14.
- Lake, C. M., & Hawley, R. S. (2012). The molecular control of meiotic chromosomal behavior: Events in early meiotic prophase in *Drosophila* oocytes. *Annual Review of Physiology*, 74, 425–451.
- Lancaster, O. M., Breuer, M., Cullen, C. F., et al. (2010). The meiotic recombination checkpoint suppresses NHK-1 kinase to prevent reorganisation of the oocyte nucleus in *Drosophila*. *PLoS Genetics*, 6(10), e1001179.
- Li, B., Carey, M., & Workman, J. L. (2007). The role of chromatin during transcription. *Cell*, 128(4), 707–719.
- Li, L., Greer, C., Eisenman, R. N., & Secombe, J. (2010). Essential functions of the histone demethylase lid. *PLoS Genetics*, 6(11), e1001221.
- Liu, X., & Secombe, J. (2015). The histone demethylase KDM5 activates gene expression by recognizing chromatin context through its PHD reader motif. *Cell Reports*, 13(10), 2219–2231.
- Liu, X., Greer, C., & Secombe, J. (2014). KDM5 interacts with Foxo to modulate cellular levels of oxidative stress. *PLoS Genetics*, 10(10), e1004676.
- Lloret-Llinares, M., Pérez-Lluch, S., Rossell, D., et al. (2012). dKDM5/LID regulates H3K4me3 dynamics at the transcription-start site (TSS) of actively transcribed developmental genes. *Nucleic Acids Research*, 40(19), 9493–9505.
- Lubzens, E., Young, G., Bobe, J., & Cerda, J. (2010). Oogenesis in teleosts: How eggs are formed. *General and Comparative Endocrinology*, 165(3), 367–389.
- Navarro-Costa, P., McCarthy, A., Prudencio, P., et al. (2016). Early programming of the oocyte epigenome temporally controls late prophase I transcription and chromatin remodelling. *Nature Communications*, 7, 12331.
- Ohkura, H. (2015). Meiosis: An overview of key differences from mitosis. *Cold Spring Harbor Perspectives in Biology*, 7(5), a015859.
- Pan, Z., Zhang, J., Li, Q., et al. (2012). Current advances in epigenetic modification and alteration during mammalian ovarian folliculogenesis. *Journal of Genetics and Genomics*, 39(3), 111–123.
- Pelosi, E., Forabosco, A., & Schlessinger, D. (2015). Genetics of the ovarian reserve. *Frontiers in Genetics*, 6, 308.
- Peplin, D., Vanderhyden, B. C., Picketts, D. J., & Murphy, B. D. (2007). ISWI chromatin remodeling in ovarian somatic and germ cells: Revenge of the NURFs. *Trends in Endocrinology and Metabolism*, 18(5), 215–224.
- Perry, A. C., & Verhac, M. H. (2008). Second meiotic arrest and exit in frogs and mice. *EMBO Reports*, 9(3), 246–251.
- Rasar, M. A., & Hammes, S. R. (2006). The physiology of the *Xenopus laevis* ovary. *Methods in Molecular Biology*, 322, 17–30.
- Saatcioglu, H. D., Cuevas, I., & Castrillon, D. H. (2016). Control of oocyte reawakening by kit. *PLoS Genetics*, 12(8), e1006215.
- Sharma, R., & Agarwal, A. (2011). Spermatogenesis: An overview. In A. Zini, & A. Agarwal (Eds.), *Sperm chromatin: Biological and clinical applications in male infertility and assisted reproduction* (pp. 19–44). New York, NY: Springer New York.
- Shi, L., & Wu, J. (2009). Epigenetic regulation in mammalian preimplantation embryo development. *Reproductive Biology and Endocrinology*, 7, 59.

- Snustad, D. P., & Simmons, M. J. (2015). *Principles of genetics, Binder Ready Version*. New York: John Wiley & Sons.
- Tachibana, M., Nozaki, M., Takeda, N., & Shinkai, Y. (2007). Functional dynamics of H3K9 methylation during meiotic prophase progression. *EMBO Journal*, 26(14), 3346–3359.
- Von Stetina, J. R., & Orr-Weaver, T. L. (2011). Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harbor Perspectives in Biology*, 3(10), a005553.
- Wang, L., Xu, Z., Khawar, M. B., et al. (2017). The histone codes for meiosis. *Reproduction*, 154(3), R65–R79.
- Wassarman, P. (2012). *The mammalian egg coat: Structure and function*. Berlin: Springer Science & Business Media.
- Xu, K., Chen, X., Yang, H., et al. (2017). Maternal Sall4 is indispensable for epigenetic maturation of mouse oocytes. *Journal of Biological Chemistry*, 292(5), 1798–1807.
- Zhaunova, L., Ohkura, H., & Breuer, M. (2016). Kdm5/Lid regulates chromosome architecture in meiotic prophase I independently of its histone demethylase activity. *PLoS Genetics*, 12(8), e1006241.
- Zou, M. R., Cao, J., Liu, Z., et al. (2014). Histone demethylase jumonji AT-rich interactive domain 1B (JARID1B) controls mammary gland development by regulating key developmental and lineage specification genes. *Journal of Biological Chemistry*, 289(25), 17620–17633.

Oogenesis In Vitro

Katsuhiko Hayashi, Kyushu University, Fukuoka, Japan

© 2018 Elsevier Inc. All rights reserved.

The Processes to Be Reconstituted In Vitro: Mice

Oogenesis is a unique sequence of differentiation originated from primordial germ cells (PGCs), the precursor of the germ cell lineage (Fig. 1). PGCs arise from the proximal epiblast in response to bone morphogenetic protein 4 (BMP4) secreted from the adjacent extraembryonic ectoderm and Wnt3 produced autonomously in the epiblast (Lawson et al., 1999; Ohinata et al., 2009). PGCs are located at a base of the allantois soon after the specification, translocate to the definitive endoderm and then start to migrate through the hindgut toward the gonad, which eventually becomes the ovary or testis. Upon entering the gonad, PGCs differentiate in a sex-dependent manner, i.e., they enter meiosis in females to become primary oocytes. In the mid-gestation stage, primary oocytes form a germline cyst, within which they are connected by intercellular bridges. At the perinatal stage, a majority (approximately 70%) of oocytes are eliminated by cell death accompanied with fragmentation of the germline cyst. The remaining oocytes form primordial follicles with the surrounding squamous pregranulosa cells (Fig. 1). The primordial follicles function as a pool of oocytes, ensuring longevity of the reproductive life in the female.

After puberty begins, some of the primordial follicles are periodically activated. The first morphological sign of follicular activation is that squamous granulosa cells surrounding the oocytes become cuboidal; these are called primary follicles. Then the granulosa cells proliferate and form at least two layers of granulosa cells surrounding the growing oocyte; these are called secondary follicles. From primordial follicles to secondary follicles, it appears that folliculogenesis progresses in a gonadotropin-independent manner, based on the evidence that FSH-signaling is dispensable for the formation of secondary follicles. As the oocytes approach full maturation, the follicle structure changes significantly in response to gonadotropins, mainly FSH. The most drastic change is formation of an antrum, by which the granulosa cell population is structurally separated into cumulus and mural granulosa cells. The former population is important for oocyte growth, developmental competence and fertilization, whereas the latter is important for ovulation and estradiol production with theca cells. The fully matured follicles undergo ovulation in response to the LH surge. The oocytes resume meiosis and arrest at meiotic metaphase II (MII), indicating that they are ready for fertilization (Fig. 1).

Reconstitution of PGC Specification In Vitro: Mice

To reconstitute oogenesis in vitro, every differentiation step should be precisely reproduced in culture. Obviously the first step is to reconstitute PGC specification. In the beginning of this century, several groups reported the production of PGC-like cells from mouse embryonic stem cells (ESCs) (Hubner et al., 2003; Toyooka et al., 2003; Geijsen et al., 2004). In principal, these reports showed that embryoid bodies spontaneously, or directionally to some extent, differentiated into PGC-like cells that expressed germ cell-specific markers. However, the results of these reports have not been rigorously compared to PGC specification in vivo, as the molecular mechanisms of the in vivo system were not sufficiently clear at that time. In addition, these reports did not test the functionality—i.e., whether the PGC-like cells are capable of differentiating to fertile eggs and sperm. Therefore, these reports were not sufficient to provide a robust reconstitution of PGCs in culture.

Along with the accumulating evidence showing how PGCs are specified from the epiblast in vivo, reconstitution of PGC specification in vitro has become increasingly possible. Of note, an ex vivo culture condition sufficient for the robust induction of PGCs from the epiblast was determined (Ohinata et al., 2009). This culture system employs a set of growth factors: BMP4, BMP8b, leukemia inhibitory factor (LIF), epidermal growth factor (EGF) and stem cell factor (SCF). Importantly, PGCs derived from the epiblast in this ex vivo culture system gave rise to functional sperm. Using similar culture conditions, PGC-like cells

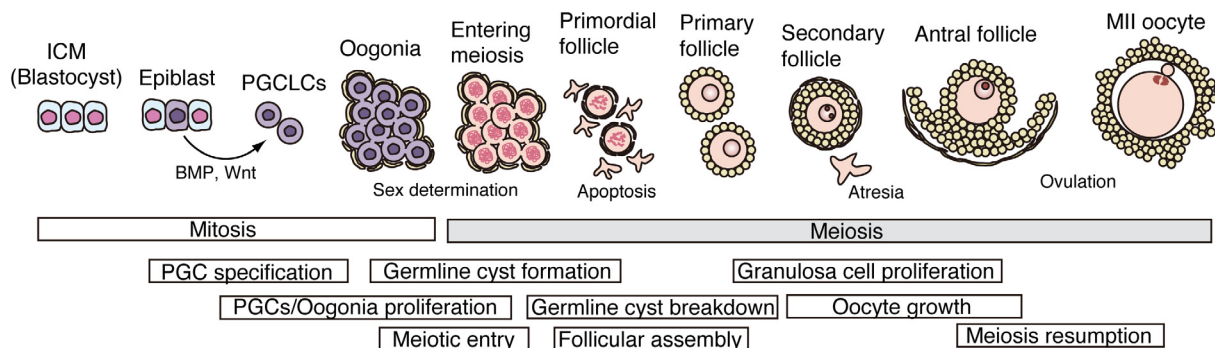


Fig. 1 Schematic diagram of oogenesis to be reconstituted in vitro. Oogenesis is composed of a unique line of differentiation processes. There are several key events (boxed) that can be criteria for evaluation of the in vitro culture system.

were derived from mouse ESCs and induced pluripotent stem cells (iPSCs) (Fig. 2A). In this method, the ability to progress to PGCs in response to BMP4 is conferred to ESCs/iPSCs by culturing with basic fibroblast growth factor (bFGF) and activin A (Hayashi et al., 2011). Gene expression analysis revealed that the transition in vitro was quite similar to that from ICM to the epiblast. The conferred state was essential for PGC differentiation, as ESCs without the treatment could not differentiate into PGC-like cells. The gene expression dynamics during the PGC specification in culture well recapitulates that in vivo. Consistently, epigenetic reprogramming represented by a rapid reduction of di-methylation on histone H3 lysine 9 (H3K9me2) and a gradual increment of tri-methylation of histone H3 lysine 27 (H3K27me3) was observed in the PGC-like cells much as in PGCs in vivo.

Oocyte and Sperm Production From ESC/iPSC-Derived PGC-Like Cells by Transplantation

Whether the PGC-like cells derived from ESCs/iPSCs are capable of differentiating into functional sperm or oocytes is an important criterion to verify the culture system. Recently developed transplantation technologies allow us to test this capability. Transplantation of spermatogenic cells into recipient seminiferous tubules was established by Brinster's group (Brinster and Zimmermann, 1994). In rodents, donor spermatogenic cells give rise to functional mature sperm in the recipient testis. Interestingly, PGCs, which are still an immature type of germ cells, can be used as donor cells in the transplantation: PGCs transplanted into neonatal testes give rise to functional sperm. Consistent with the previous evidence, ESC/iPSC-derived PGC-like cells gave rise to functional sperm after transplantation into the neonatal testes; fertilized eggs with the sperm gave rise to pups (Fig. 2B). The pups appeared to be healthy, as they grew to fertile adult mice without any premature death or developmental delay.

Transplantation technologies for the female germ line are not as sophisticated as those for the male germ line, partially due to the anatomical features of the ovary. In contrast to spermatogenesis, which takes place in the tubular structure of the male reproductive organ, oogenesis proceeds in the follicles, which are structures tightly enclosed by granulosa cells and theca cells. The follicle structure is assembled at the perinatal stage and is not formed de novo after the birth. Therefore, for transplantation of the female germ line, PGCs or oocytes must be transferred with surrounding somatic cells/tissues. Experimentally, transferring either a piece of the ovarian tissue or aggregations composed of PGCs and surrounding somatic cells has been proven technically feasible for oocyte production from donor tissues/cells (Matoba and Ogura, 2011). Accordingly, to produce oocytes from ESC/iPSC-derived PGC-like cells, gonadal somatic cells of fetal ovaries were aggregated with the PGC-like cells and then transplanted into the recipient ovary (Fig. 2B) (Hayashi et al., 2012; Hayashi and Saitou, 2013). At 4 weeks after the transplantation, fully grown oocytes at the germinal

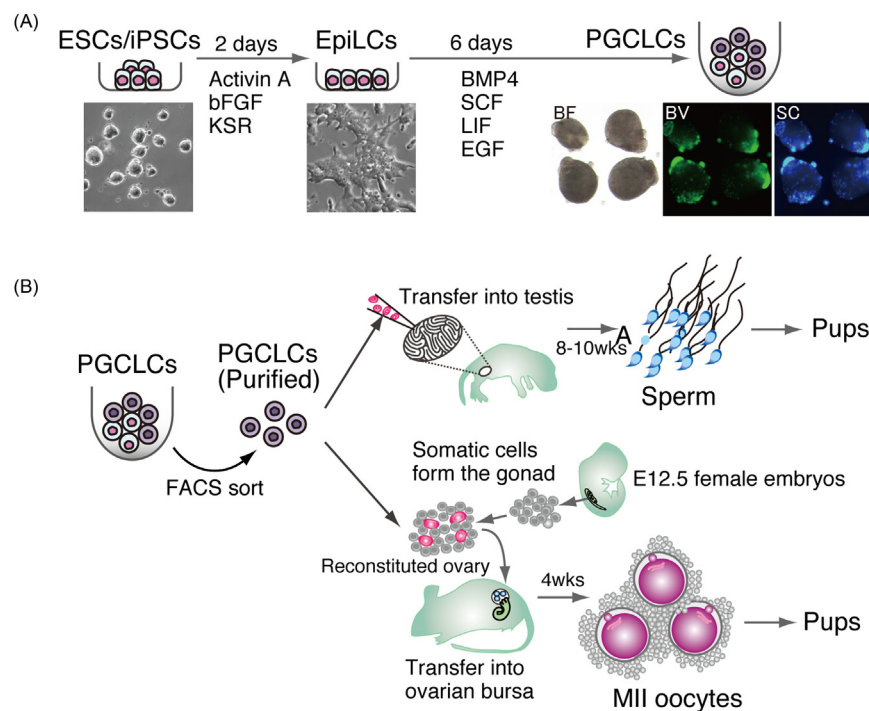


Fig. 2 Reconstitution of PGC specification in vitro. (A) PGCLCs are specified from ES cells (or iPS cells) via epiblast-like cells (EpiLCs). Cells cultured in the cytokines and for the number of days indicated are required for the reconstitution. In the present study, two reporter constructs, Blimp1-mVenus (BV) and stella-ECFP (SC), were used for monitoring PGCLC differentiation. BF: bright field. (B) When PGCLCs are transferred to the testis and ovary, they give rise to functional sperm and MII oocytes, respectively. Sexually matched ES cells (or iPS cells) are required for producing the gametes, e.g., female ES cells for oocyte production. In the case of oocyte production, somatic cells from the E12.5 female gonad are essential for supporting oogenesis in the transplant.

vesicle (GV) stage were collected from the transplanted ovaries. The GV oocytes were capable of resuming meiosis and reached the MII oocytes. At least some of these ES/iPSC-derived MII oocytes were fully potent, as they were fertilized with wild-type sperm and gave rise to pups that grew to fertile adult mice (Hayashi et al., 2012).

Reconstitution of Oogenesis In Vitro: Mice

It has been a long-standing challenge to establish optimal culture conditions under which fully potent eggs can be efficiently matured from immature oocytes, such as those in primordial follicles. In mice, the milestone work in this field was conducted by Obrien and Eppig. In their series of studies, fully functional oocytes were derived from neonatal ovaries in culture (Eppig and O'Brien, 1996). Partly in continuation of their efforts, Morohaku et al. established an organ culture system using fetal ovaries. Notably, the system produced functional egg production from mitotic PGCs in the fetal ovaries, thereby demonstrating the first successful completion of meiosis in a female germ line in culture (Morohaku et al., 2016). These systems provided useful strategies for determining culture conditions that produce fully potent eggs from pluripotent stem cells.

The key advances in the studies described above were mainly (1) the prevention of multiocyte follicle (MOF) formation and (2) the improvement of culture conditions for oocyte growth. In the organ culture of fetal ovaries, MOF, a follicle containing more than two oocytes, is frequently formed, which eventually disturbs oocyte growth in culture. Although the mechanism of MOF formation remains to be clarified, previous studies have suggested that steroid hormones, such as estrogen and progesterone, and their agonistic molecules such as bisphenol A (BPA) and diethylstilbestrol (DES) trigger MOF formation. There is an interesting coincidence between appropriate follicle assembly and reduction of the steroid hormone concentration in newborns upon delivery. Based on these observations, Morohaku et al. tested several estrogen inhibitors and found that ICI182780 was the most effective for preventing fetal gonads from forming MOFs in culture (Morohaku et al., 2016).

There are two ways to make oocytes grow to full-size, namely 2D or 3D culture (Fig. 3). In the 2D culture method, mural granulosa cells with theca cells are removed to directly supply nutrients to cumulus-oocyte complexes (COCs) from the medium. In contrast, the 3D culture method leaves the follicle structure intact like in vivo. Which method is optimal seems to depend on the species used. 2D culture is useful for mice, cows, and pigs, whereas 3D culture is useful for humans and other primates (see below for details). Theoretically, an advantage of 3D culture is that it can encapsulate follicular fluid with COCs, thereby preventing the diffusion of factor(s) in culture. It has been reported that polymer materials, such as polyvinyl pyrrolidone (PVP), improve oocyte growth in the 2D culture system, which may be attributable to prevention of the diffusion and the resulting promotion of the paracrine effect. Morohaku et al. removed the mural follicle wall by treatment with collagenase and used medium containing PVP at 2%, in which oocytes grew to fully grown germinal vesicle (GV) oocytes. The GV oocytes were then transferred to medium for oocyte maturation containing LH and EGF for stimulating maturation accompanied with meiotic resumption. The resultant MII

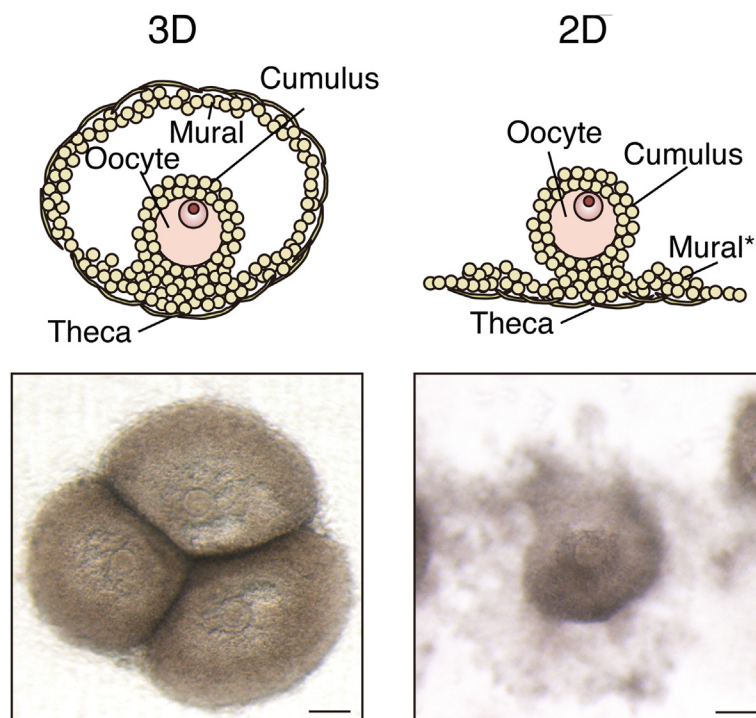


Fig. 3 Two different approaches to follicular growth in vitro. Schematic diagrams of the 3D and 2D culture are shown at the top. Note that the follicle structure is conserved in the 3D culture, whereas a part of the follicle structure is removed in the 2D culture. Representative images of mouse follicles cultured by each method are shown. Scale bars, 100 μ m.

oocytes from the culture were capable of fertilization and full-term development (Morohaku et al., 2016). Although the efficiency of pup production by this approach is not as high as that in the usual in vitro fertilization (IVF) using ovulated oocytes, this study demonstrates, for the first time, that mitotic PGCs can differentiate into functional oocytes in culture.

Generation of Eggs From Pluripotent Stem Cells In Vitro

Building on the advances in the organ culture of fetal ovaries, it has now become possible to generate functional eggs from pluripotent stem cells (Hikabe et al., 2016). This is accomplished as follows. PGCLCs are derived from ES/iPS cells and then aggregated with gonadal somatic cells. The gonadal somatic cells are collected from female embryos at embryonic day E12.5, and then endogenous PGCs are removed from the somatic cells by magnetic beads-conjugated anti-SSEA1 antibody. Aggregation of PGCLCs and gonadal somatic cells is done in a U-bottom 96-well plate, and then the aggregate, called the reconstituted ovary, is transferred onto a culture membrane coated with collagen. The subsequent culture system can be divided into three sections—in vitro differentiation (IVDi), in vitro growth (IVG) and in vitro maturation (IVM)—in which oogenesis proceeds to primary oocytes in the secondary follicle, fully grown GV oocytes and MII oocytes, respectively (Fig. 4). Each section requires culture media and supplements different from those used for organ culture of the fetal ovary. A higher number of oocytes were formed in IVDi culture under the combined condition: alphaMEM (aMEM) for the first 4 days, followed by StemPro34 for 17 days (Hayashi et al., 2017). Consistent with the organ culture of the fetal gonad, addition of ICI182780 to the IVDi medium prevented the formation of MOFs. At the end of the IVDi culture, the individual follicles must be isolated to prevent retardation of the oocyte growth (Fig. 4).

After the isolation of the follicles, as described in the method of the organ culture system, collagenase is also effective to promote the oocyte growth in ES/iPS-derived oocytes. In IVG culture, the granulosa cells surrounding oocytes start to proliferate and eventually lift up the COCs onto the culture membrane, which exhibits a morphology resembling that of antral follicles (Fig. 4). In some cases, recombinant BMP15 and GDF9 can be added to promote the proliferation of granulosa cells. In accordance with the proliferation, oocytes grew in the COC. At 11 days of IVG culture, the COCs are picked up and then transferred to IVM medium that induces resumption of meiosis in the oocytes. During IVM culture, the oocytes extrude the first polar body and become MII oocytes (Fig. 4).

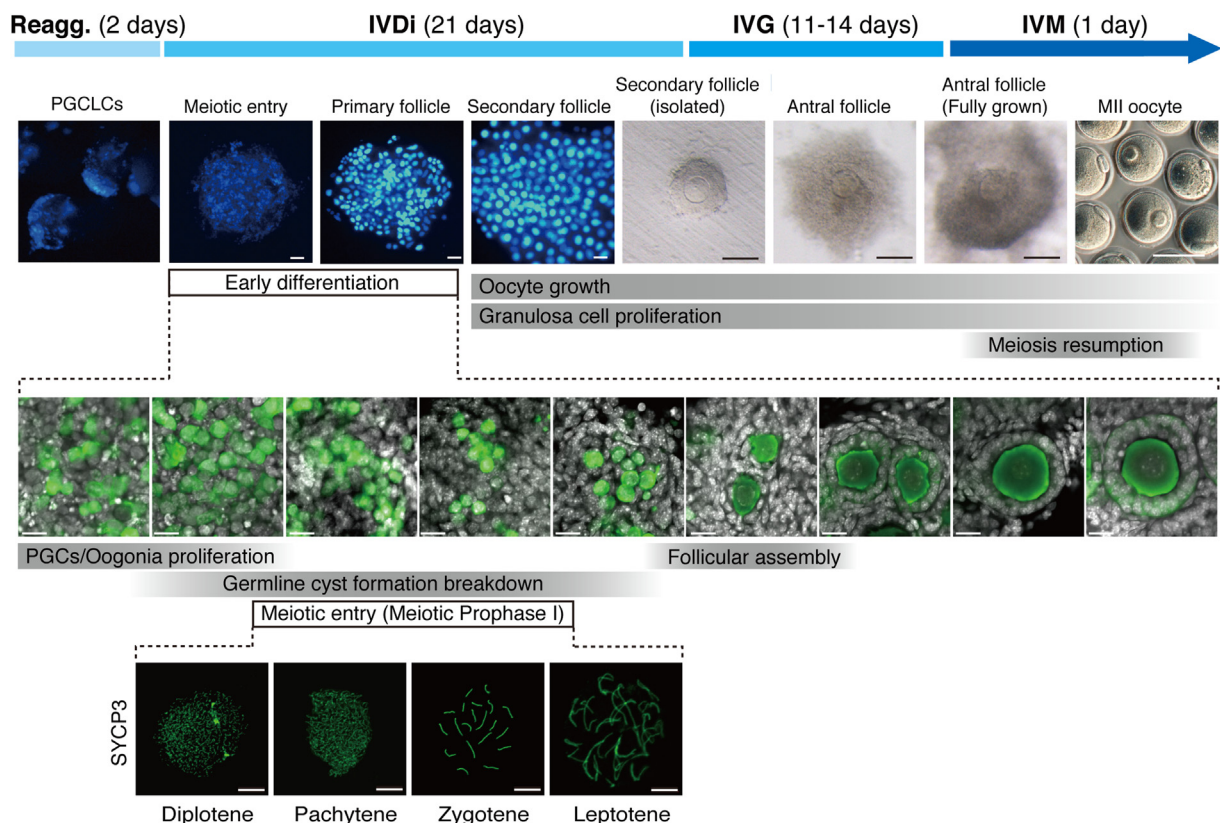


Fig. 4 Reconstitution of oogenesis in vitro. The culture system that reconstitutes the entire process of oogenesis is composed of IVDi, IVG and IVM. PGCLCs aggregated with somatic cells from the E12.5 female gonads are used as a starting material. During culture, the key differentiation processes (boxed) are observed. In the top fluorescence images, oocytes are illuminated by SC. The middle images are from immunofluorescence analyses of SC (green) and DAPI (white). The bottom images show meiotic progression with dynamics of synaptonemal complex protein 3 (SYCP3).

Generation of Pups From ES/iPS-Derived Eggs

ES/iPSC-derived MII oocytes are capable of fertilization with wild-type sperm. Moreover, the fertilized eggs have sufficient potency to give rise to pups. Hikabe et al. demonstrated that, although the developmental rate is low (see below), eggs derived from multiple lines of ESCs, embryonic fibroblast (MEF)-derived iPSCs and tail-tip fibroblast (TTF)-derived iPSCs developed to pups. The pups were apparently healthy, as they grew to adulthood with a frequency comparable to that of wild-type mice and were fertile (Hikabe et al., 2016).

Efficiency of the Oogenesis In Vitro

A reconstituted ovary is composed of 5000 PGCLCs and 50,000 gonadal somatic cells freshly collected from E12.5 female embryos. It is possible to store gonadal somatic cells in liquid nitrogen. In this case, 75,000 frozen-thawed gonadal somatic cells are used for a reconstituted ovary. Hikabe et al. reported that 5000 PGCLCs gave rise to, on average, 237.3 primary oocytes, 55.1 fully grown GV oocytes and 15.9 MII oocytes in IVDi, IVG and IVM culture, respectively. Since 30–50 reconstituted ovaries were produced in one culture experiment, roughly 450–800 MII oocytes were available in the culture. In IVF using wild-type sperm freshly prepared from cauda epididymis, almost half of the MII oocytes were fertilized, and then almost 60% of the fertilized eggs became 2-cell embryos. By transferring to surrogate mothers, 3.5% of the 2-cell embryos became pups. Production of pups from ES cell-derived 2-cell embryos is 20-times less efficient than that from in vivo-derived 2-cell embryos (from IVF using superovulated MII oocytes and wild-type sperm), suggesting that some properties of the oocytes produced in culture were deficient (see Limitations of mouse egg production from ESCs/iPSCs).

Productivity of oocytes from iPSCs is similar to that from ESCs. For example, Hikabe et al. reported that 27.0 MII oocytes on average were obtained from 5000 PGCLCs derived from iPSCs. Despite the comparable productivity, the efficiency of pup production from the iPSC-derived 2-cell embryos was even lower than that from the ESC-derived 2-cell embryos: only 0.3%–0.9% of the iPSC-derived 2-cell embryos transferred gave rise to pups. At least in part, this may be due to the inferior quality of iPSC cells compared to ESCs, which is mainly caused by incomplete reprogramming in the somatic cells.

Limitations of Mouse Egg Production From ESCs/iPSCs

By the in vitro culture system, hundreds of mature oocytes can be obtained from ESCs/iPSCs. However, the developmental potency of the mature oocytes is partially compromised. This is due to frequent asynapsis of the homologous chromosomes, precocious resumption of meiosis and aneuploidy (Hikabe et al., 2016). Almost half of the primary oocytes in the reconstituted ovaries showed asynapsis, which could be identified by phosphorylation of histone H2AX (γ H2AX). This was due not only to the origin of the primary oocytes but also the suboptimal culture conditions, since the asynapsis rate increased even in primary oocytes in the organ culture of fetal ovaries. Further refinement of the basic culture conditions will be needed. The transcriptome analysis revealed that genes expressing immature oocytes, which should be down-regulated in MII oocytes, were still highly expressed in ESC-derived MII oocytes. This indicates that ESC-derived MII oocytes remain immature and precociously resume meiosis. It is generally thought that the follicular environment prevents the oocytes from resuming meiosis until they have sufficiently grown up. This indicates that the 2D culture system fails to reproduce the entire environment of the antral follicles. Although it is difficult to find a clear causal relationship to explain these abnormalities, aneuploidy is slightly increased: aneuploidy is observed in 22.2% of ESC-derived MII oocytes, whereas it is seen in only 2.4% of wild-type oocytes.

Embryonic development seems to also be compromised in ESC-derived embryos. The fertilization rates were not significantly different, but the rate of blastocyst formation of ESC-derived embryos was four times less than that of wild-type embryos. Even after transfer into the uterus there is a gradual loss of embryos, which may be caused by genetic and epigenetic abnormalities. Notably, all of the obtained pups grew up normally without evidence of premature death. This suggests, in a sense, that normal embryos are selected during embryonic development. It is thus essential not only that we refine the culture conditions to increase the success rate of oogenesis, but also that we select in advance competent oocytes/embryos before transferring them to surrogate mothers.

Clear Differences Between In Vivo and In Vitro Oogenesis

Regardless of whether in vitro oogenesis is accomplished in the organ culture system or the reconstituted ovary system, one of the clear differences from in vivo oogenesis will be the formation of primordial follicles, the most immature type of follicles. Histologically, primordial follicles are composed of oocytes surrounded by a monolayer of squamous granulosa cells and located in the cortex of the ovary. They remain strictly dormant (by a mechanism described below) and are sporadically activated in response to environmental cues. The balance between dormancy and activation provides periodical production of oocytes. However, in in vitro oogenesis, almost all oocytes grow up synchronously without resting at the dormant state (Hikabe et al., 2016; Morohaku et al., 2016). Histologically, it is very rare to observe oocytes surrounded by a monolayer of granulosa cells in in vitro oogenesis (Fig. 4). These observations lead to two biologically interesting points. (1) The primordial follicle stage is not essential for functionality of oocytes. (2) In vitro culture is apparently lacking in factors necessary for primordial formation. Since

primordial follicle formation is accomplished by the interaction between oocytes and surrounding somatic cells, not only germ cells but also somatic cells in the culture system must be evaluated more carefully.

Oogenesis In Vitro in Humans

From ethical and clinical points of view, it is less feasible to use human fetal ovaries for developing a culture system that produces oocytes. Rather, it would be more beneficial for patients with infertility to obtain mature oocytes from immature types of oocytes, such as those encapsulated in primordial, secondary and preantral follicles. So far, MII oocytes have not been successfully obtained from primordial follicles. This is partially because the follicles are strictly dormant, since the follicles function as an oocyte storehouse to ensure the longevity of female reproductive life. The stubborn dormancy of the primordial follicles sometimes disturbs in vitro maturation. Therefore, the first step is to trigger follicular activation in the primordial follicles. Based on genetic analysis in mice, several molecules and signaling pathways have been identified. The Foxo3 protein plays a central role in keeping oocytes in the primordial follicles dormant. Disruption of *Foxo3a* causes an acceleration of follicular activation, which results in early exhaustion of the primordial follicles. The activity of the transcription factor is controlled by subcellular localization during follicular activation: Foxo3a is localized in the nuclei of the dormant oocytes, and is exported to the cytoplasm upon follicular activation. The localization is regulated by PI3K-AKT signaling, activation of which eliminates nuclear Foxo3. The signaling is usually antagonized by Pten, a phosphatase for phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in the primordial follicles, but upon activation by an external cue it triggers follicular activation. Based on this background, a research group lead by Hsueh cultured pieces of human ovarian tissues with pharmaceutical reagents, followed by transplantation under the kidney capsule of immunocompromised mice. When a Pten inhibitor (dipotassium bisperoxo(5-hydroxypyridine-2-carboxyl)oxovanadate: bpV) was administered, the number of antral follicles increased up to 27 times compared to that in the controls (Li et al., 2010). In addition, a PI3K-agonistic phospho-peptide (740Y-P) had a synergistic effect on follicular activation. However, the artificially activated follicles often exhibited aberrant growth. Therefore, further refinement will be needed for the initial follicular activation.

Among attempts to mature oocytes in culture, the secondary follicle stage is the most immature stage yet used in the successful production of MII oocytes in primates. The secondary follicles are composed of oocytes surrounded by at least two layers of granulosa cells. In these early structures, the stage is about to switch from a gonadotropin-independent state to a gonadotropin-dependent state. In response to FSH, the follicles vastly increase in size, for example from 150 μ m to 20 mm in diameter, with granulosa cell proliferation and oocyte growth. To reproduce the remarkable morphology, there is a great demand for 3D culture systems that can yield mature oocytes. Among the several methods for reconstituting 3D culture systems, the method using alginate hydrogel currently seems to be the most sophisticated approach for the production of mature oocytes (Xiao et al., 2015). Using this method, MII oocytes were successfully obtained from secondary follicles under a 3D culture system. For the possible ethical implications, it has not yet been evaluated whether the in vitro-matured MII oocytes are capable of fertilization followed by development to term. Instead, culture experiments using nonhuman primates have demonstrated that the in vitro-matured oocytes were fertilized and developed to the morula stage (Xu et al., 2013).

Human Primordial Germ Cells From ES/iPS Cells

One of the ultimate goals in reproductive biology is to reconstitute gametogenesis using pluripotent stem cells in humans. Since a possible strategy has been presented in mice, efforts are underway to adapt the system to humans. Indeed, despite the species difference, it has now become possible to derive human PGCLCs from human ES/iPS cells (Irie et al., 2015; Sasaki et al., 2015). As in the mouse system, BMP and Wnt signaling play key roles in PGC specification in humans. It is interesting that the signaling cascade triggering PGC specification is conserved across the mammalian species. Human PGCLCs largely mimic gene expression and epigenetic modifications in PGCs in vivo. In addition to the ethical implications, however, it is also technically difficult, at the moment, to derive eggs and sperm from human PGCLCs. Since PGCs proceed to gametogenesis in the gonad, signals from gonadal somatic cells, e.g., fetal granulosa cells and fetal Sertoli cells, must be reconstituted in vitro. In the mouse system, the signals are provided by fetal ovarian cells obtained from a number of embryos, which are not available in humans. It is thus necessary to find a way to replace the cells/signals supporting gametogenesis in culture.

References

- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Eppig, J. J., & O'Brien, M. J. (1996). Development in vitro of mouse oocytes from primordial follicles. *Biology of Reproduction*, 54, 197–207.
- Geijsen, N., Horoschak, M., Kim, K., et al. (2004). Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature*, 427, 148–154.
- Hayashi, K., Hikabe, O., Obata, Y., & Hirao, Y. (2017). Reconstitution of mouse oogenesis in a dish from pluripotent stem cells. *Nature Protocols*, 12, 1733–1744.
- Hayashi, K., Ogushi, S., Kurimoto, K., et al. (2012). Offspring from oocytes derived from in vitro primordial germ cell-like cells in mice. *Science*, 338, 971–975.
- Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S., & Saitou, M. (2011). Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. *Cell*, 146, 519–532.
- Hayashi, K., & Saitou, M. (2013). Generation of eggs from mouse embryonic stem cells and induced pluripotent stem cells. *Nature Protocols*, 8, 1513–1524.
- Hikabe, O., Hamazaki, N., Nagamatsu, G., et al. (2016). Reconstitution in vitro of the entire cycle of the mouse female germ line. *Nature*, 539, 299–303.
- Hubner, K., Fuhrmann, G., Christenson, L. K., et al. (2003). Derivation of oocytes from mouse embryonic stem cells. *Science*, 300, 1251–1256.

- Irie, N., Weinberger, L., Tang, W. W., et al. (2015). SOX17 is a critical specifier of human primordial germ cell fate. *Cell*, 160, 253–268.
- Lawson, K. A., Dunn, N. R., Roelen, B. A., et al. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes & Development*, 13, 424–436.
- Li, J., Kawamura, K., Cheng, Y., et al. (2010). Activation of dormant ovarian follicles to generate mature eggs. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 10280–10284.
- Matoba, S., & Ogura, A. (2011). Generation of functional oocytes and spermatids from fetal primordial germ cells after ectopic transplantation in adult mice. *Biology of Reproduction*, 84, 631–638.
- Morohaku, K., Tanimoto, R., Sasaki, K., et al. (2016). Complete in vitro generation of fertile oocytes from mouse primordial germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 9021–9026.
- Ohinata, Y., Ohta, H., Shigeta, M., et al. (2009). A signaling principle for the specification of the germ cell lineage in mice. *Cell*, 137, 571–584.
- Sasaki, K., Yokobayashi, S., Nakamura, T., et al. (2015). Robust in vitro induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell*, 17, 178–194.
- Toyooka, Y., Tsunekawa, N., Akasu, R., & Noce, T. (2003). Embryonic stem cells can form germ cells in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 11457–11462.
- Xiao, S., Zhang, J., Romero, M. M., et al. (2015). In vitro follicle growth supports human oocyte meiotic maturation. *Scientific Reports*, 5, 17323.
- Xu, J., Lawson, M. S., Yeoman, R. R., et al. (2013). Fibrin promotes development and function of macaque primary follicles during encapsulated three-dimensional culture. *Human Reproduction*, 28, 2187–2200.

Further Reading

- McLaughlin, M., Kinnell, H. L., Anderson, R. A., & Telfer, E. E. (2014). Inhibition of phosphatase and tensin homologue (PTEN) in human ovary in vitro results in increased activation of primordial follicles but compromises development of growing follicles. *Molecular Human Reproduction*, 20, 736–744.

SEXUAL DEVELOPMENT

Orgasm

Mihaela Pavličev, Cincinnati Children's Hospital, Cincinnati, OH, United States

Günter P Wagner, Yale University, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Description of the sexual peak, the orgasm, has been dominated by the human experience. Even so, the definitions of what orgasm entails vary broadly, in spite of the general intuitive recognition of the phenomenon. The descriptions range from those focusing on the subjective sensation, the release of neural tension, endocrine effects, or the externally observable changes, such as involuntary muscular contractions (Mah and Binik, 2001). This multitude of descriptions reveals the complexity of the phenomenon, as well as individual variation and highly subjective experience of human orgasm. Orgasm, however, most likely is not a human-specific trait, but much less is known about how common orgasm is across mammalian species. This is at least in part because the current description of orgasm is strongly associated with the characteristics observed and experienced in humans—often too subjective to be expressed without language—thus comparative work on orgasm has been very scarce. For the purposes of this account, we will delineate the orgasm here as a neuro-endocrine event, alluding to the two main physiological signaling systems involved, that is, the nervous and the hormonal systems. We will start by outlining human male and female arousal and orgasm, as the physiology of these is best understood by far. Next, we will summarize what we know about its presence and the possible roles in other mammals, and finally briefly address some of the historical interpretations.

Physiology of Human Orgasm

In males and females the arousal stage precedes the orgasm. Arousal is triggered either by cognitive or tactile stimulation, and begins with the relaxation of smooth muscles lining the blood vessel walls and subsequent rush of blood into genitalia.

In females, the arousal and orgasm have traditionally been described in four stages: excitement, plateau, orgasm and resolution (Masters et al., 1966). Plateau is the stage in which blood flow into the genitalia is increased, leading to vaginal lubrication and vaginal dilation, as well as clitoral swelling. The clitoris is the female anatomical counterpart of the penis and consists of relatively small external visible parts (glans and prepuce or hood) and an appreciable internal portion (paired bulbs and crura with erectile corpora cavernosa; O'Connell et al., 2005; Puppo and Puppo, 2015). The external part of clitoris is located ventro-laterally from vaginal opening, and in particular the glans clitoris—the most sensitive part of clitoris—is positioned at some distance ventral to the vagina. In addition to the involvement of the smooth muscles lining the vessels, the female orgasm manifests itself as involuntary rhythmic muscle contractions of pelvis, uterus and vagina as well as the anus. The majority of women report that stimulation of external clitoral parts, in particular the highly sensitive glans clitoris, is required for the transition from arousal to orgasm (Herbenick et al., 2017; see below for other sensitive regions, such as cervix). The mechanism that underlies the transition from arousal to orgasm is however not understood.

In male arousal the blood rushes into penile *corpus cavernosum*, leading to penile erection. Erection is followed by the ejaculation of sperm from the penis. Physiologically, sperm release consists of two stages; the emission, during which the seminal fluid is collected in the prostatic part of the urethra, and the ejaculation from the penis. We equal here the later stage, the ejaculation, with male orgasm, as the two typically coincide in healthy males. However, the research field is not in complete agreement on this topic (Alexander et al., 1993). Some pathological disorders affect ejaculation and the experience of orgasm independently, which suggests that they are regulated by, to some extent, distinct mechanisms. Arousal, emission and ejaculation are a complex coordinated interplay of several smooth muscles of the ducts epididymis and vas deferens, which connect testes with urethra, further of the seminal vesicle, prostate and several sphincters that likely contribute to accumulating the pressure, with which semen exits penis at ejaculation. As in female orgasm, also male orgasm/ejaculation includes the involuntary action of striated pelvic muscle, the perineal muscles and the anal sphincter. It likely involves more than surpassing a distinct threshold of arousal. Again, the mechanisms that trigger ejaculation/orgasm are currently not understood.

Neurobiology of Human Orgasm

Some of the neurobiological mechanisms involved in mediating arousal and orgasm are known (reviewed in Komisaruk et al., 2006). Genitalia are innervated by the cranial and spinal nerves, integrating the neural connections of autonomous (involuntary) and the somatic nervous systems. Most generally, it is thought that both parts of autonomous neural system are involved in the control of sexual activity: the parasympathetic slow-acting system in arousal, and the sympathetic quick-acting system in actual orgasm. The parasympathetic innervation of genitalia is provided by the cranial *vagus* nerve (Komisaruk et al., 2004) and the sacral pelvic neural fibers, the later associated with pudendal spinal nerve (but see recent suggestion that the pelvic fibers may in fact be

part of the sympathetic system; [Espinosa-Medina et al., 2016](#)). The known sympathetic innervation stems from the hypogastric plexus of the lower abdomen, associated with the lower lumbar spinal nerves. The complex peripheral innervation is also reflected in involvement of multiple brain regions, as shown by the neuroimaging studies of the brain during sexual stimulation. The somatosensory innervation of genitalia in both sexes maps to parts of medial cortex ([Komisaruk et al., 2011](#)), whereas the human orgasm itself has been shown to activate numerous additional brain regions. Mapping female and male orgasm (ejaculation) showed activity in hypothalamic nuclei, cerebellum, preoptic area, bed nucleus of *stria terminalis*, amygdala, hippocampus, lower brainstem, and frontal cortex ([Whipple and Komisaruk, 2006](#); [Georgiadis et al., 2007](#); [Komisaruk and Whipple, 2008](#); [Komisaruk et al., 2011](#); [Huynh et al., 2013](#)). These studies also revealed that to a great extent, male and female orgasm-associated brain circuits overlap. Moreover, many of the same circuits have been also associated with male ejaculation in rodents indicating homologous processes across animal species ([Baum and Everitt, 1992](#)), although differences can be seen when the results are compared to primates ([Michael et al., 1999](#)).

Different neurotransmitter-receptor systems transfer information across synapses in different parts of the vertebrate neural system. Most of the information about the specific neurotransmitters that are active during human sexual arousal and orgasm is derived from the side-effects observed when targeting specific neurotransmitters to treat various conditions, such as for example, depression. The known drug classes that affect arousal and/or orgasm include drugs that act directly on transmitter receptors, such as agonists of serotonin receptors or antagonists of inhibitory noradrenalin receptors. Both these drug types enhance aspects of sexual behavior. Furthermore, the drugs that inhibit degradation of transmitter at the synapse, or inhibit its reuptake (e.g., SSRI drug class), have indicated that serotonin and dopamine modifications significantly modulate sexual reaction and orgasm ([Montejo-Gonzalez et al., 1997](#); [Balon, 2006](#); [Higgins et al., 2010](#)).

Endocrinology of Human Orgasm

Along with the numerous neural circuits of the brain mentioned above, orgasm activates also the hypothalamus. Orgasmic activation of hypothalamus triggers a hormonal response of the pituitary, an endocrine gland functionally connecting the two major signaling systems, the neural and the hormonal system. Pituitary develops partially from neuroectoderm and partially from oral ectoderm. Correspondingly, its function is triggered by the neural signal from the brain, conveyed to pituitary either by the direct neural connections (posterior pituitary), or by the signals secreted from the neurons to the hypothalamic vessel plexus, and transferred into pituitary by the blood (anterior pituitary). Orgasm triggers a surge of pituitary hormone secretion in humans, which is particularly pronounced in females ([Exton et al., 1999, 2001](#)). The strongest hormonal surge is that of prolactin, however also a minor surge of oxytocin has been reported ([Carmichael et al., 1987](#)). The physiological significance of these surges is not clear. As the long-term elevation of prolactin (hyperprolactinemia) is known to inhibit libido, the prolactin surge has been suggested to inhibit promiscuous behavior in females ([Kruger et al., 2002](#)) after coitus. However, the fact that women have more pronounced prolactin surge, and at the same time (in contrast to men) lack refractory period after orgasm, suggests that this effect does not exist and thus such adaptive explanation of the prolactin surge is unlikely ([Bianchi-Demicheli and Ortigue, 2007](#)). An alternative suggestion is that the PRL peak is a remnant of evolutionarily older process that induces ovulation and therefore has had a reproductive function in the evolutionary past (see below). Indeed, prolactin peak occurs at the time of normal ovulation in women ([Vekemans et al., 1977](#); [Braund et al., 1984](#)). Research suggests that PRL may act on ovaries to enhance expression of receptors for luteinizing hormone (LH), the hormone inducing ovulation, and prolong the life span of corpus luteum ([Freeman et al., 2000](#)). Human studies have suggested that women with decreased prolactin levels tend to have shorter luteal phase ([Kauppila et al., 1988](#)). This effect of prolactin is consistent with the role of prolactin in rodent ovarian cycle, which will be discussed further in the section on evolution of orgasm. Mechanistically, the prolactin peak is likely a consequence of brief interruption of dopamine signaling in the hypothalamus, where dopamine constitutively inhibits prolactin secretion, thus dopamine inhibition (for example due to transient increase in serotonin signaling) can account for the prolactin surge ([Bianchi-Demicheli and Ortigue, 2007](#)). The existence of orgasm-associated surge of the second pituitary hormone, oxytocin, has been variably reported ([Kruger et al., 2003](#)) and is possibly a consequence of a different signaling pathway—namely due to projections of the separate neuronal signals from the genitals and breast to the same brain region in the hypothalamus ([Cross and Wakerley, 1977](#)). The reproductive functional significance of oxytocin is likely found in causing the contraction of the myo-epithelial cells in breast milk glands as well as in the reproductive tract, involved in milk ejection in the breast, and propagation of the egg cell in the Fallopian tubes (and fetus during labor) through the reproductive tract. The social function often attributed to oxytocin is that of enhancing partner bonding, in analogy to what is observed in rodents ([Carter, 1992](#); [Witt et al., 1992](#); [Cho et al., 1999](#); [Razzoli et al., 2003](#)). Yet, the orgasm-associated surge reported for humans is not only variably detected, but also small (~1.3-fold change in female orgasm; [Carmichael et al., 1987](#)) relative to the experimental dosages in rodents (up to 10-fold change in experimental settings showing effect on social behavior in rats; [Witt et al., 1992](#)). Given that oxytocin is suggested to reinforce future trust and sexual behavior also in humans, it is not clear whether indeed the weak orgasm-associated increase in oxytocin contributes to these effects, or whether they rather stem from other aspects of intimacy.

Types of Orgasm

Whereas men perceive orgasm relatively uniformly, women report different types of orgasm associated with stimulation of different parts of genitalia ("vaginal", "cervical" vs. "clitoral" orgasm, Grafenberg-spot). The innervation of genitalia described above involves

separate branches supplying different parts of the genitalia, and these all likely respond to stimulation. There is, in addition to spinal nerves, evidence for innervation from cranial *vagus* nerve, which has been suggested to account for cervical orgasm (Sansone et al., 2002; Komisaruk and Sansone, 2003; Komisaruk et al., 2004). Moreover, it is important to realize that the externally visible glans clitoris is only a part of the clitoris, which is much larger and extends internally dorsolateral, parallel to the urethra and also to the ventral vaginal wall (O'Connell et al., 2005; Puppo and Puppo, 2015). It is thus likely that vaginal penetration can achieve indirect stimulation of internal clitoral parts, leading to perception of the ventral vaginal side as a “G-spot”, so called after the German physician Graefenberg (1950), who studied the role of urethra in female orgasm.

Comparative Biology and the Evolutionary Origin of Orgasm

To identify the evolutionary origin of any trait entails recognizing the corresponding (homologous) trait in different species, and subsequently identifying the last common ancestor of those lineages, in which the trait exists. Identifying orgasm in nonprimate species however is difficult. This is because research on orgasm has been primarily conducted in primates, generating a primate-specific definition, which captures lineage-specific characteristics that may be difficult to detect or even be irrelevant in other animals, even though some authors report putative cases of nonprimate orgasm (Pomeroy, 1972; Pfaus et al., 2016). It may be useful to remind the reader that evolution works by a combination of inheritance and modification of traits, and therefore traits that are shared among species, will nevertheless have many species-specific modifications. If these modifications within a certain species (such as human) are considered as a necessary part of the definition of a trait, this precludes the identification of the trait in different species. For this reason, little systematic comparative work has been done to examine the presence, or the role, of orgasm across mammalian species apart from primates. It is very unlikely that the physiological basis of orgasm originated in human, or primate lineage. Rather it is likely that the ancestral trait has acquired modifications in our lineage that make it seem human-specific, and may even have acquired additional roles. It is useful here to consider female and male orgasm separately.

Female Orgasm

The human female orgasm, in contrast to male orgasm, is not necessary to conceive. Indeed, a very high percentage of women do not experience orgasm regularly during reproductive (i.e., penetrative) sex (Hite, 1976; Lloyd, 2005). To explain its existence in humans, various reproductive roles have nevertheless been suggested, by which the presence of female orgasm would positively affect the number of offspring. These roles range from enabling mate choice (Alcock, 1980; Puts et al., 2012), to bonding, or enhancement of sperm transfer (Levin, 2011a, 2011b), to name a few. In spite of efforts, so far no evidence could be found for the current presence of such effects of orgasm on reproductive fitness (Levin, 2011a, 2011b; Zietsch and Santtila, 2013). Here it is important to note that the putative role of female orgasm in humans—whether the female orgasm presently has a (reproductive) role or not—cannot be used as explanation for its origin. What is required is to find and study the trait in other species. This is because the function of a trait often changes during the trait's evolution, and thus evidence is required that the ancestral role was the same as the current role. To answer the question about the origin of female orgasm, it will be necessary to study the presence and evolution of the trait in phylogenetic context.

The most comprehensive discussion of the current hypotheses on evolution of female orgasm has been published by Elizabeth Lloyd. In her 2005 book *The Case of Female Orgasm*, Lloyd (2005) critically reviewed 21 existing theories of origination and maintenance, specifically of female orgasm. Lloyd pointed to the crucial difference between sexes in how closely orgasm is associated with reproductive sex: whereas achieving orgasm in men is effective in reproductive sex, it is highly ineffective in women. Lloyd argued that this makes the explanations based on direct reproductive effects of female orgasm implausible: female orgasm is more efficiently attainable by masturbation or homosexual intercourse, than with reproductive sex. Systematic evaluation of 21 hypotheses based on the evidence that their authors provided, led Lloyd to reject all but the *by-product* hypothesis (Symons, 1979). This hypothesis states that the female orgasm originated and is maintained (in spite of having no function), because the male orgasm is required for reproduction. To understand this hypothesis, it is necessary to know that the male and female genitalia share many of their developmental pathways, that is, the female clitoris is homologous to the male penis. If the underlying genetic program for phallus finds itself in a female individual, the phallus grows into a clitoris, but in a male, it is modified to grow into a penis. As this genetic basis acquires a characteristic that is necessary in one of the sexes, as long as it is not deleterious in another, it is maintained also when it is only required in one sex. Another example for such “vestigial” trait are male nipples. Males do not need them, however the nipples are maintained because males and females have almost identical genomes. Of course some of the adult morphology did become decoupled between males and females: male and female genitalia do look different, penis is larger, and female nipples are as well. But because they are sharing much of the same genetic-developmental program, the hypothesis suggests that the physiology of orgasm simply was not lost in females, because it is crucial when it occurs in males, and does not hurt the female either. The origin and necessity of orgasm in males is thus suggested to drive the origin and maintenance of the orgasm in female. Note that this hypothesis therefore implicitly suggests that female orgasm is as wide spread across species with internal fertilization, as the male orgasm.

Pavlicev and Wagner in 2016 proposed a new hypothesis for the evolutionary origin of female orgasm (Pavlicev and Wagner, 2016; Wagner and Pavlicev, 2017). The authors suggested that human female orgasm is derived from the ancestral neuro-endocrine

signal, which was involved in inducing ovulation during copulation. This mechanism is still maintained in many mammalian species, where ovulation only occurs after an external trigger, often the tactile stimulation during copulation. These so-called induced or reflex ovulators include many carnivores (cats, ferrets, etc.), camels, rabbits and shrews (Bakker and Baum, 2000). In contrast to induced ovulators, humans are spontaneous ovulators, ovulating on an endogenous rhythm, independent of external stimulation. Pavlicev and Wagner noted similarities between the neuroendocrine reflex involved in induced ovulation, and that of the orgasmic neuroendocrine surge and suggested that the signal which triggers pituitary hormones in human female orgasm still carries a signature of the signal that triggers ovulation (via pituitary) in induced ovulators. To support the hypothesis, the authors have shown that induced ovulation is phylogenetically older and thus may have been a predecessor of spontaneous ovulation. As traits lose functions during evolution, their remnants may still remain. Additional evidence is seen in female genital morphology: coincidental with evolution of spontaneous ovulation, also the position of strongly orgasm-triggering glans clitoridis has changed. In species, which require induction by copulation, the glans clitoridis is positioned within copulatory canal where it gets directly stimulated during copulation, whereas in the species that ovulate spontaneously, the clitoris tends to be removed from the copulatory canal. The latter, at the same time, may explain the rarity of female orgasm during reproductive (i.e., penetrative) sex.

An interesting mechanistic insight into the function of copulation-induced reflex may come from laboratory rodents. Mouse is a spontaneous ovulator with very shortened life span of the *corpus luteum* in nonpregnant state. *Corpus luteum* is necessary for implantation, however. The way to prolong the life span of corpus luteum in mice is copulation. If the copulation is sterile, mice still show a long luteal phase in absence of pregnancy (so-called pseudopregnancy). Most interestingly, the mechanism mediating this effect is the induction of high levels of pituitary prolactin. Thus, while mouse ovulation is not copulation induced, the mouse implantation is. This system might be considered as a different derived phenotype from that in primates, based on the same basic mechanisms (copulation-associated prolactin surge acting on ovary).

Male Orgasm

The male orgasm is associated with ejaculation and sperm transfer, and therefore crucial for reproduction. Prompt ejaculation of relatively large amount of sperm thus is generally assumed to be a sufficient explanation for the origin and persistence of orgasm-like impulse in species with internal fertilization. Indeed, erection and ejaculation are widely spread across tetrapods, in particular in amniotes, that is, reptiles and mammals. As mentioned above, some of the neural brain circuits involved in ejaculation are shared at least between rodents and humans, indicating homology of neural regulation.

Historical Cornerstones of Research on Human Orgasm

The irregularity of female orgasm during intercourse has intrigued thinkers since the ancient times. Already Aristotle reflected on the question whether or not female orgasm plays any role in reproduction (reported in Leroi, 2014). Early on, features of female sexuality, including the lack of orgasm during intercourse, has been strongly associated with women's psychological state, and sexual massages were used as treatment for hysteria; *hysteria* standing for a very general description of female mental and behavioral disorders (psychosexual connotation is still reflected in the word *hysterectomy*, meaning uterine removal). This notion was particularly influentially crystalized in the writings of Sigmund Freud, the Viennese founder of psychoanalysis. Freud's work on mental disorders laid great emphasis on the experiences during early development, as well as specifically on sexuality. With respect to female orgasm he proposed a natural developmental succession from infantile clitoral to mature vaginal orgasm (Freud, 1905). The grave consequence of this persuasive concept was the pathologization of the absence of vaginal orgasm during reproductive sex. The lack of vaginal orgasm has become interpreted as a signature of mental immaturity, of women stuck in a childlike sexual state—quite overlooking the fact that the lack of vaginal orgasm is rather the norm than a deviation. One of the early remarkable woman sexologist was Marie Bonaparte (1933). Herself a psychoanalyst, she openly opposed Freud's concept, arguing that glans clitoridis remains the main source of mature female orgasm, and that the lack of orgasm during reproductive sex is due to the lack of clitoral stimulation during penetrative sex, deriving from the placement of the clitoris at distance from the vagina. Remarkably, she collected over 200 individual measurements of the distance between vagina and clitoris in 1930s, and found a correlation between that distance and a decreased rate of copulatory orgasm (a dataset more recently re-evaluated by Wallen and Lloyd, 2011). Best known empirical research on human sexuality and orgasm from the 20th century is undoubtedly that by the zoologist Alfred Kinsey from Indiana University (Kinsey et al., 1948, 1953), followed by the rich work of William Masters and Virginia Johnson, at Washington University in St. Louis (Masters and Johnson, 1960; Masters et al., 1966, 1974, 1993; Kolodny et al., 1979). An important contribution to the body of knowledge on female sexuality, came also from Shere Hite. In a comprehensive review of female sexual experience Hite was the first to document the high percentage of women that rarely or never experience orgasm in penetrative sex—thus considered dysfunctional according to Freud's theory—however are perfectly able to do so in masturbation (Hite, 1976). From the 1970s to 1990s, the evolutionary theories of female orgasm were discussed by a wide range of authors from the ethologist Desmond Morris (1967), the behavioral biologist John Alcock (1980) to the paleontologist Steven J. Gould (1991). With the exception of the work of Symons, Gould and Lloyd, attempts to explain the evolution of orgasm have been predominantly adaptationist, meaning that they aim to explain the origin of female orgasm by its proposed current utility in humans (Wagner

and Pavlicev, 2016, 2017). This debate is synthesized in the influential book by Elisabeth Lloyd (2005). Major contributions to understanding of the physiology of human orgasm were made starting in the 1990s by Barry Komisaruk and collaborators, some of which are documented above.

References

- Alcock, J. (1980). Beyond the sociobiology of sexuality: Predictive hypotheses. *Behavioral and Brain Sciences*, 3(2), 181–182.
- Alexander, C. J., Sipski, M. L., & Findley, T. W. (1993). Sexual activities, desire, and satisfaction in males pre- and post-spinal cord injury. *Archives of Sexual Behavior*, 22(3), 217–228.
- Bakker, J., & Baum, M. J. (2000). Neuroendocrine regulation of GnRH release in induced ovulators. *Frontiers in Neuroendocrinology*, 21(3), 220–262.
- Balon, R. (2006). SSRI-associated sexual dysfunction. *The American Journal of Psychiatry*, 163(9), 1504–1509. quiz 1664.
- Baum, M. J., & Everitt, B. J. (1992). Increased expression of c-Fos in the medial preoptic area after mating in male rats: Role of afferent inputs from the medial amygdala and midbrain central tegmental field. *Neuroscience*, 50(3), 627–646.
- Bianchi-Demicheli, F., & Ortigue, S. (2007). Toward an understanding of the cerebral substrates of woman's orgasm. *Neuropsychologia*, 45(12), 2645–2659.
- Bonaparte, M. (1933). Les deux frigidités de la femme. *Bulletin de la Société de Sexologie*, 5, 161–170.
- Braund, W., Roeger, D. C., & Judd, S. J. (1984). Synchronous secretion of luteinizing hormone and prolactin in the human luteal phase: Neuroendocrine mechanisms. *The Journal of Clinical Endocrinology and Metabolism*, 58(2), 293–297.
- Carmichael, M. S., Humbert, R., Diken, J., Palmisano, G., Greenleaf, W., & Davidson, J. M. (1987). Plasma oxytocin increases in the human sexual response. *The Journal of Clinical Endocrinology and Metabolism*, 64(1), 27–31.
- Carter, C. S. (1992). Oxytocin and sexual behavior. *Neuroscience and Biobehavioral Reviews*, 16, 131–144.
- Cho, M. M., DeVries, A. C., Williams, J. R., & Carter, C. S. (1999). The effects of oxytocin and vasopressin on partner preferences in male and female prairie voles (*Microtus ochrogaster*). *Behavioral Neuroscience*, 113(5), 1071–1079.
- Cross, B. A., & Wakerley, J. B. (1977). The neurohypophysis. *International Review of Physiology*, 16, 1–34.
- Espinosa-Medina, I., Saha, O., Boismoreau, F., Chettouh, Z., Rossi, F., Richardson, W. D., & Brunet, J. F. (2016). The sacral autonomic outflow is sympathetic. *Science*, 354(6314), 893–897.
- Exton, M. S., Bindert, A., Kruger, T., Scheller, F., Hartmann, U., & Schedlowski, M. (1999). Cardiovascular and endocrine alterations after masturbation-induced orgasm in women. *Psychosomatic Medicine*, 61(3), 280–289.
- Exton, M. S., Kruger, T. H. C., Koch, M., Paulson, E., Knapp, W., Hartmann, U., & Schedlowski, M. (2001). Coitus-induced orgasm stimulates prolactin secretion in healthy subjects. *Psychoneuroendocrinology*, 26(3), 287–294.
- Freeman, M. E., Kanyicska, B., Lerant, A., & Nagy, G. (2000). Prolactin: Structure, function, and regulation of secretion. *Physiological Reviews*, 80(4), 1523–1631.
- Freud, S. (1905). *Three essays on the theory of sexuality/Sigmund Freud; with a foreword by Nancy J Chodorow; with an introductory essay by Steven Marcus; translated and edited by James Strachey*. New York: Basic Books.
- Georgiadis, J. R., Reinders, A. A. T. S., Van der Graaf, F. H. C. E., Paans, A. M. J., & Kortekaas, R. (2007). Brain activation during human male ejaculation revisited. *NeuroReport*, 18(6), 553–557.
- Gould, S. J. (1991). *Bully for brontosaurus: Reflections in natural history*. New York: Norton.
- Graefenberg, E. (1950). The role of urethra in female orgasm. *International Journal of Sexology*, 3(3), 145–148.
- Herbernick, D., Fu, T.-C. J., Arter, J., Sanders, S. A., & Dodge, B. (2017). Women's experiences with genital touching, sexual pleasure, and orgasm: Results from a U.S. probability sample of women ages 18 to 94. *Journal of Sex & Marital Therapy*, 1–12.
- Higgins, A., Nash, M., & Lynch, A. M. (2010). Antidepressant-associated sexual dysfunction: Impact, effects, and treatment. *Drug, Healthcare and Patient Safety*, 2, 141–150.
- Hite, S. (1976). *The Hite report: A National Study on female sexuality*. New York: Seven Stories Press.
- Huynh, H. K., Willemsen, A. T. M., & Holstege, G. (2013). Female orgasm but not male ejaculation activates the pituitary. A PET-neuro-imaging study. *NeuroImage*, 76, 178–182.
- Kaupilla, A., Martikainen, H., Puistola, U., Reinila, M., & Ronnberg, L. (1988). Hypoprolactinemia and ovarian function. *Fertility and Sterility*, 49(3), 437–441.
- Kinsey, A. C., Pomeroy, W. B., & Martin, C. E. (1948). *Sexual behavior in the human male*. Philadelphia and London: W.B. Saunders Co.
- Kinsey, A. C., Pomeroy, W. B., Martin, C., & Gebhard, P. (1953). *Sexual behavior in the human female*. Philadelphia: Saunders.
- Kolodny, R. C., Masters, W. H., & Johnson, V. E. (1979). *Textbook of sexual medicine*. Boston: Little, Brown.
- Komisaruk, B., & Whipple, B. (2008). Brain activation during sexual arousal and orgasm in women. *Journal of Sex Research*, 45(2), 94–95.
- Komisaruk, B., Beyer-Flores, C., & Whipple, B. (2006). *The science of orgasm*. Baltimore: The Johns Hopkins University Press.
- Komisaruk, B. R., & Sansone, G. (2003). Neural pathways mediating vaginal function: The vagus nerves and spinal cord oxytocin. *Scandinavian Journal of Psychology*, 44(3), 241–250.
- Komisaruk, B. R., Whipple, B., Crawford, A., Grimes, S., Liu, W. C., Kalnin, A., & Mosier, K. (2004). Brain activation during vaginocervical self-stimulation and orgasm in women with complete spinal cord injury: fMRI evidence of mediation by the vagus nerves. *Brain Research*, 1024(1–2), 77–88.
- Komisaruk, B. R., Wise, N., Frangos, E., Allen, K., & Whipple, B. (2011). Sequential activation of brain regions related to orgasm in women, using fMRI. *The Journal of Sexual Medicine*, 8, 151.
- Kruger, T. H., Haake, P., Hartmann, U., Schedlowski, M., & Exton, M. S. (2002). Orgasm-induced prolactin secretion: Feedback control of sexual drive? *Neuroscience and Biobehavioral Reviews*, 26(1), 31–44.
- Kruger, T. H., Haake, P., Chereath, D., Knapp, W., Janssen, O. E., Exton, M. S., Schedlowski, M., & Hartmann, U. (2003). Specificity of the neuroendocrine response to orgasm during sexual arousal in men. *The Journal of Endocrinology*, 177(1), 57–64.
- Leroi, A. M. (2014). *The lagoon. How Aristotle invented science*. New York: Viking.
- Levin, R. J. (2011a). Can the controversy about the putative role of the human female orgasm in sperm transport be settled with our current physiological knowledge of coitus? *The Journal of Sexual Medicine*, 8(6), 1566–1578.
- Levin, R. J. (2011b). The human female orgasm: A critical evaluation of its proposed reproductive functions. *Sexual and Relationship Therapy*, 26(4), 301–314.
- Lloyd, E. A. (2005). *The case of female orgasm: Bias in the science of evolution*. Cambridge: Harvard University Press.
- Mah, K., & Binik, Y. M. (2001). The nature of human orgasm: A critical review of major trends. *Clinical Psychology Review*, 21(6), 823–856.
- Masters, W. H., & Johnson, V. E. (1960). The human female: Anatomy of sexual response. *Minnesota Medicine*, 43, 31–36.
- Masters, W. H., Johnson, V. E., & Reproductive Biology Research Foundation (US). (1966). *Human sexual response*. Boston: Little, Brown.
- Masters, W. H., Johnson, V. E., & Levin, R. J. (1974). *The pleasure bond: A new look at sexuality and commitment*. Boston: Little.
- Masters, W. H., Johnson, V. E., & Kolodny, R. C. (1993). *Biological foundations of human sexuality*. New York: HarperCollins.
- Michael, R. P., Clancy, A. N., & Zump, D. (1999). Effects of mating on c-Fos expression in the brains of male macaques. *Physiology & Behavior*, 66(4), 591–597.
- Montejo-Gonzalez, A. L., Llorca, G., Izquierdo, J. A., Ledesma, A., Bousono, M., Calcedo, A., Carrasco, J. L., Ciudad, J., Daniel, E., De la Gandara, J., Derecho, J., Franco, M., Gomez, M. J., Macias, J. A., Martin, T., Perez, V., Sanchez, J. M., Sanchez, S., & Vicens, E. (1997). SSRI-induced sexual dysfunction: Fluoxetine, paroxetine, sertraline, and fluvoxamine in a prospective, multicenter, and descriptive clinical study of 344 patients. *Journal of Sex & Marital Therapy*, 23(3), 176–194.

- Morris, D. (1967). *The naked ape: A zoologist's study of the human animal*. New York: McGraw-Hill.
- O'Connell, H. E., Sanjeevan, K. V., & Hutson, J. M. (2005). Anatomy of the clitoris. *The Journal of Urology*, 174(4), 1189–1195.
- Pavlicev, M., & Wagner, G. (2016). The evolutionary origin of female orgasm. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 326(6), 326–337.
- Pfaus, J. G., Scardochio, T., Parada, M., Gerson, C., Quintana, G. R., & Coria-Avila, G. A. (2016). Do rats have orgasms? *Socioaffective Neuroscience and Psychology*, 6, 31883.
- Pomeroy, W. B. (1972). *Dr. Kinsey and the institute of sex research*. New York: Harper & Row.
- Puppo, V., & Puppo, G. (2015). Anatomy of sex: Revision of the new anatomical terms used for the clitoris and the female orgasm by sexologists. *Clinical Anatomy*, 28(3), 293–304.
- Puts, D. A., Dawood, K., & Welling, L. L. M. (2012). Why women have orgasms: An evolutionary analysis. *Archives of Sexual Behavior*, 41(5), 1127–1143.
- Razzoli, M., Cushing, B. S., Carter, C. S., & Valsecchi, P. (2003). Hormonal regulation of agonistic and affiliative behavior in female mongolian gerbils (*Meriones Unguiculatus*). *Hormones and Behavior*, 43(5), 549–553.
- Sansone, G. R., Gerdes, C. A., Steinman, J. L., Winslow, J. T., Ottenweller, J. E., Komisaruk, B. R., & Insel, T. R. (2002). Vaginal stimulation releases oxytocin within the spinal cord in rats. *Neuroendocrinology*, 75(5), 306–315.
- Symons, D. (1979). *The evolution of human sexuality*. Oxford: Oxford University Press.
- Vekemans, M., Delvoe, P., L'Hermite, M., & Robyn, C. (1977). Serum prolactin levels during the menstrual cycle. *The Journal of Clinical Endocrinology and Metabolism*, 44(5), 989–993.
- Wagner, G. P., & Pavlicev, M. (2016). What the evolution of female orgasm teaches us. *Journal of Experimental Zoology Part B-Molecular and Developmental Evolution*, 326(6).
- Wagner, G. P., & Pavlicev, M. (2017). Origin, function, and effects of female orgasm: All three are different. *Journal of Experimental Zoology Part B: Molecular Developmental Evolution*, 328(4), 299–303.
- Wallen, K., & Lloyd, E. A. (2011). Female sexual arousal: Genital anatomy and orgasm in intercourse. *Hormones and Behavior*, 59(5), 780–792.
- Whipple, B., & Komisaruk, B. R. (2006). Where in the brain is a woman's sexual response? Laboratory studies including brain imaging during orgasm. *Journal of Sex Research*, 43(1), 29.
- Witt, D. M., Winslow, J. T., & Insel, T. R. (1992). Enhanced social interactions in rats following chronic, centrally infused oxytocin. *Pharmacology Biochemistry and Behavior*, 43(3), 855–861.
- Zietsch, B. P., & Santtila, P. (2013). No direct relationship between human female orgasm rate and number of offspring. *Animal Behaviour*, 86, 253–255.

Sex Chromosomes

Kim L McIntyre, Shafagh A Waters, and Paul D Waters, University of New South Wales, Sydney, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Allele One of two or more alternative forms of a gene, located at the same position on a particular chromosome.

Autosome Any chromosome apart from a sex chromosome, usually occurring in a pair.

Heterochromatin Condensed chromatin from which gene expression is repressed.

Mutation Change in the DNA sequence, including insertion or deletion.

Pseudoautosomal region (PAR) Region of sex chromosomes that pairs and recombines during meiosis.

Pseudogene A copy of a gene that has lost some of its function.

Recombination Exchange of genetic material between paired chromosomes during meiosis.

Recessive mutation Mutation that impacts function only if occurring on both gene alleles, or in males if occurring on an X- or Y-borne gene.

Sex chromosomes Chromosomes that differ between males and females, defined by the presence of a sex-determining gene on one.

Sex-determining gene Gene located on one sex chromosome that triggers development of the embryonic gonad into either a testis or ovary.

Therian mammal Live-bearing mammals, comprising the eutherian (or placental) mammals and marsupials.

Introduction

In most mammals, sex is determined genetically by an XX female: XY male sex chromosome system. The Y chromosome is small and specialized for functions in male sex and reproduction. It bears a dominant sex-determining gene (called *SRY*) which is expressed in early embryogenesis to trigger a complex regulatory cascade that culminates in testis development. In the absence of the Y chromosome, there is no *SRY* gene and ovary development is initiated.

Live-bearing mammals are divided into two main groups: eutherians and marsupials, collectively called therian mammals. The therian X and Y chromosomes evolved from a normal pair of autosomes by a process of Y chromosome degradation after recombination with the X chromosome was suppressed ([Fig. 1](#)). Consequently, the therian X and Y chromosomes differ from each other in size, structure and gene content ([Fig. 2](#)). All that remains of the once extensive homology between the X and Y chromosomes are a few gene pairs and a small pseudoautosomal region (PAR) that is integral to sex chromosome pairing and segregation at male meiosis. The human PAR contains 24 genes.

X Chromosome: Tailored for Sex

In humans, the X chromosome is 156 Mb and bears 841 protein coding genes and 639 noncoding genes ([Ensembl release 89](#)). The gene density (number of genes per megabase) is about half that of the autosomal average due to an increased density of interspersed repeats.

Enriched for “Balls and Brains” Genes

The genes of the X chromosome have a diverse range of conserved functions in therian mammals, but are enriched (relative to autosomes) for genes associated with sex and reproduction. This sex and reproduction bias was not evident in the gene complement of the ancestral autosome from which the eutherian X chromosome evolved, suggesting that specialization for reproductive functions evolved more recently on the X chromosome ([Julien et al., 2012](#)). It appears that this specialization may have continued after the divergence of the rodent and primate lineages, with the independent emergence in human and mouse of X-borne genes with testis-dominant expression ([Mueller et al., 2013](#)).

The X chromosome is also enriched in genes that function in the brain ([Soumillon et al., 2013](#)). In humans, X-borne genes have higher expression in the brain relative to autosomal genes, and a greater proportion of X-borne genes are expressed in the brain than in other somatic tissues. Intellectual disability associated with X-borne genes is 3.5 times more prevalent than that associated with autosomal genes. Males are predominantly affected by X-borne intellectual disability because they have a single X chromosome, so recessive mutation of an X-borne gene is not protected against. In females, the presence of a nonmutated copy on at least one X chromosome is adequate to protect against disease caused by recessive mutations (reviewed in [Deng et al., 2014](#)).

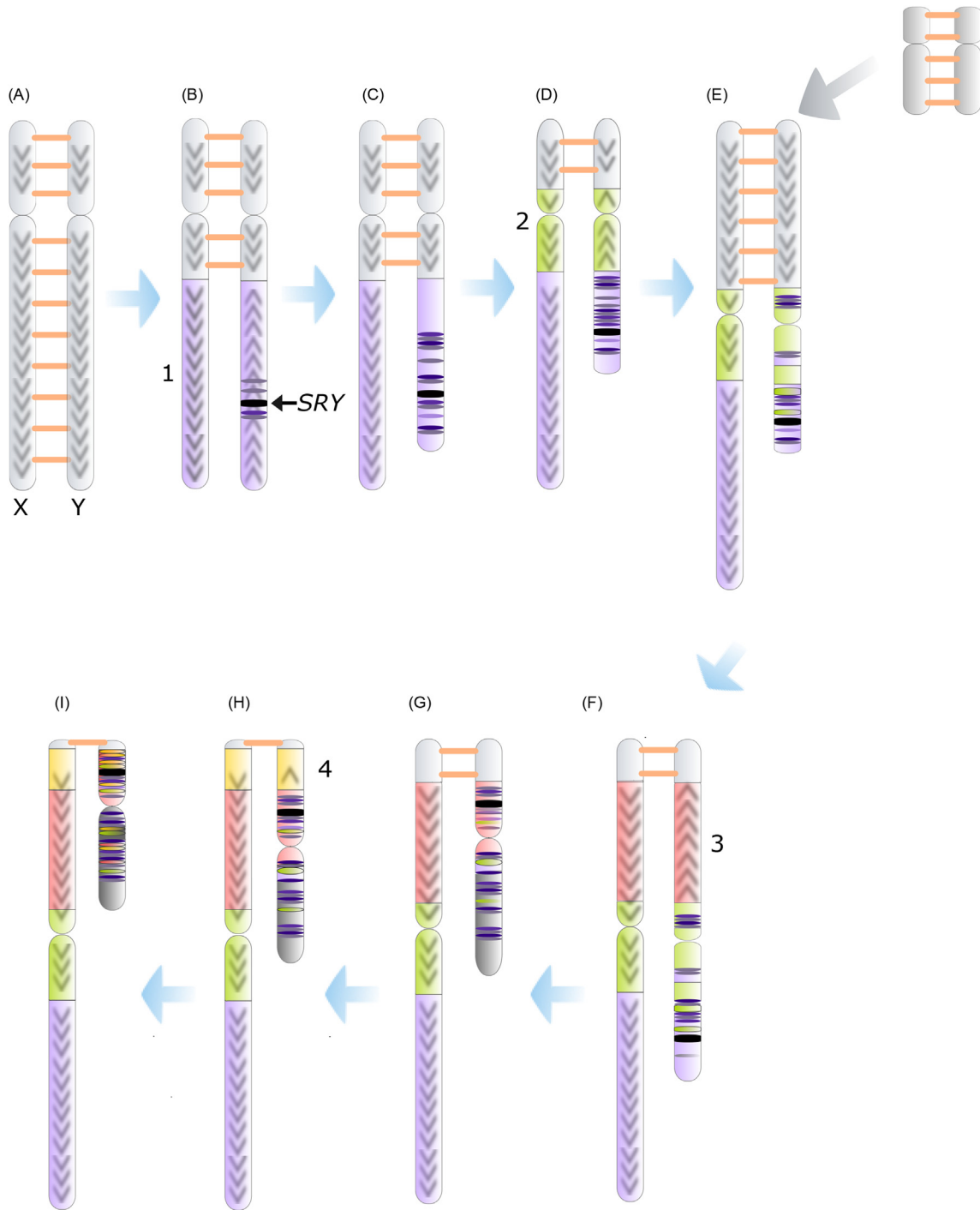


Fig. 1 The generation of evolutionary strata on the human sex chromosomes. Different colors indicate different evolutionary strata, with pale gray representing the PAR. Orange bars represent recombination. Arrows within the chromosomes show inversions on the Y chromosome. (A) The ancestral autosome pair underwent recombination along its length. (B) The sex-determining gene, *SRY* (black), emerged ~180 mya to define the proto-Y chromosome. Spermatogenesis and associated male-benefit genes accumulated near *SRY*, facilitating their transmission only to males. Inversion of the Y chromosome region including *SRY* suppressed recombination, creating evolutionary stratum 1 on the X chromosome (purple). (C) Without recombination with the X chromosome, deleterious mutations accumulated on the Y chromosome leading to loss of gene function, accumulation of heterochromatin and reduction in physical size. (D) A second inversion on the Y chromosome ~117 mya extended the section in which recombination was suppressed between the X and Y chromosomes, creating evolutionary stratum 2 (green). Together, strata 1 and 2 on the X chromosome form the X-conserved region that is common to eutherians and marsupials (Fig. 2). The Y chromosome degenerated further. (E) An autosomal block was fused to the X and Y chromosomes, extending the PAR. This represents the added region of the eutherian sex chromosomes (Fig. 2). (F) A further inversion on the Y chromosome created stratum 3 (pink). (G) The degeneration of the Y chromosome continued. (H) A final inversion on the Y chromosome occurred in the primate lineage ~25–40 mya, leading to the emergence of stratum 4 (yellow) and further reduced the PAR. (I) The contemporary human sex chromosomes. There is possibly a fifth stratum (not shown) (Lahn and Page, 1999; Cortez et al., 2014). Chromosomes not drawn to scale.

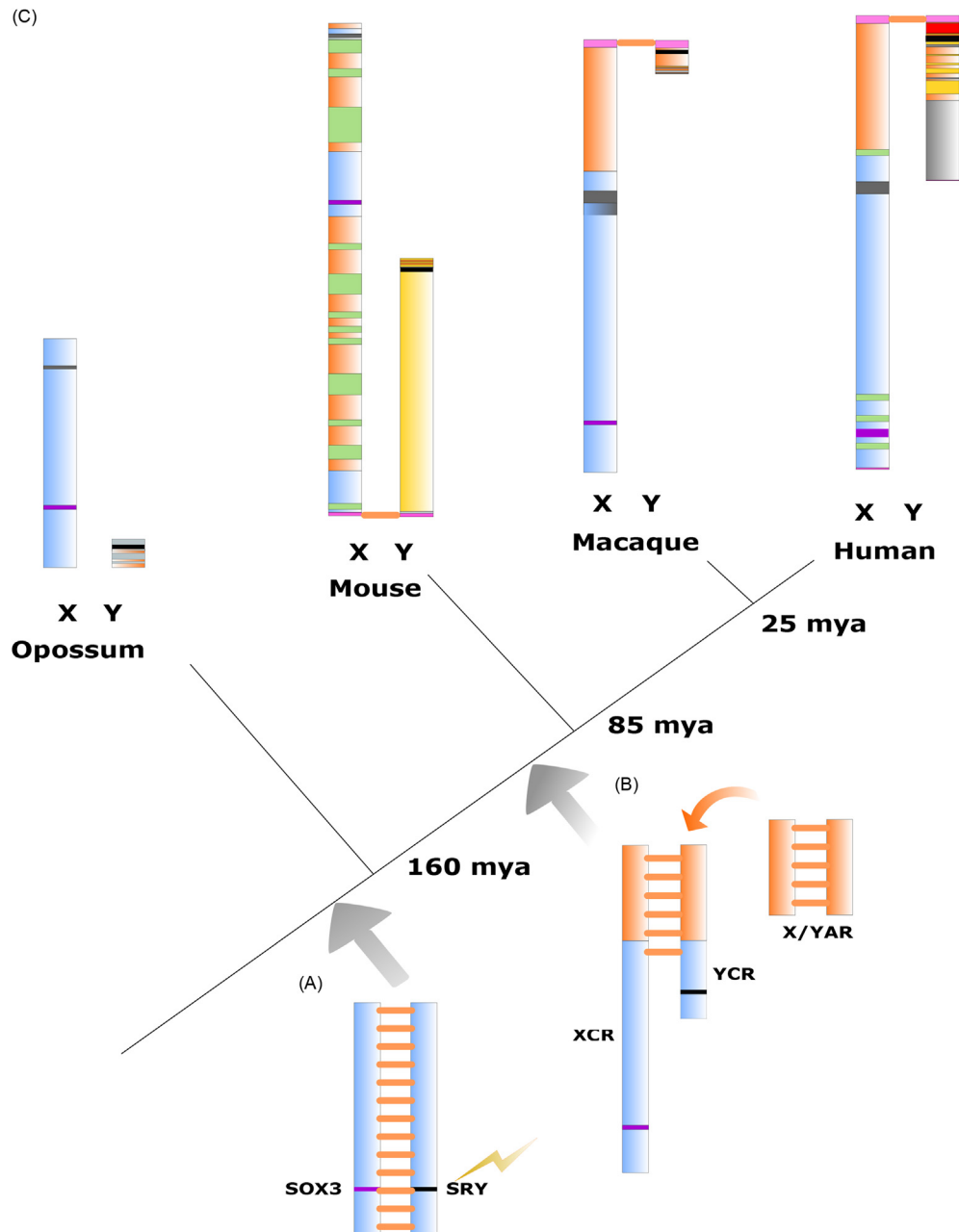


Fig. 2 Sex chromosomes in representative mammals. (A) The mammalian XY chromosomes evolved from a pair of autosomes in a common ancestor of the eutherians and the marsupials when the *SRY* testis-determining gene (black band) evolved from the *SOX3* gene (purple band) to define a new proto-X and -Y chromosome system. The *SOX3* gene is conserved on the eutherian X chromosome. The ancestral section of the XY pair, common to eutherians and marsupials, is called the X- and Y-conserved region (X/YCR) (light blue). After *SRY* emerged, recombination (represented by orange bars) was repressed by a series of inversions on the Y chromosome (Fig. 1). (B) A section of an autosome was fused to the PAR of the sex chromosomes after divergence of the marsupials, forming the X- and Y-added region (X/YAR) (orange). Recombination was also repressed between the XAR and YAR. (C) The contemporary sex chromosomes in opossum, mouse, macaque, and human. The mouse Y chromosome accumulated extensive regions of repeat sequence (yellow) (Soh et al., 2014). The human Y chromosome accumulated repeats (yellow), but also heterochromatin (gray) (Skaletsky et al., 2003). The X chromosome is conserved within primates. The mouse X chromosome has conserved gene content, although the order has been rearranged. Ampliconic repeats have emerged independently on the mouse and human X chromosomes (green) (Mueller et al., 2013). Recombination continues between the X and Y chromosomes within small PARs in eutherian mammals (pink). The marsupial XY pair no longer recombines. Centromeres are dark gray. Divergence dates shown at nodes. Lineage specific chromosome sizes are drawn to scale. Chromosome sections are not drawn to scale.

Many X-borne genes are expressed in both brain and testis, but not in other tissues. This bias may have arisen when X-borne genes with broadly expressed regulatory roles acquired new expression profiles, allowing them to be expressed more specifically in the brain and in association with male sex development. Because males have only one X chromosome, any change in an X-borne gene that is favorable to males is exposed to positive selection, and so more likely to become fixed in the population. It is suggested that the accumulation of “balls and brains” genes on the X chromosome may have been accelerated by sexual selection, where enhanced reproductive success of males results from their attractiveness to females rather than from any intrinsic adaptive fitness (reviewed in [Graves et al., 2002](#)).

X Chromosome Inactivation: Silencing One X in Females

X chromosome inactivation occurs in female therian mammals, where gene expression from one X chromosome is silenced in the somatic cells of females. The silent X chromosome condenses to form a distinct compact body (Barr body) within the nucleus. X chromosome inactivation is controlled by an elaborate molecular mechanism that is not fully understood, although seems to involve both structural and sequence elements of the X chromosome. In eutherians, a noncoding gene (called *XIST*) is expressed from the X chromosome chosen for silencing. The *XIST* RNA then coats the chromosome and recruits an array of protein complexes that mediate silencing. The mechanism underlying the accumulation and spreading of *XIST* RNA along the X chromosome is yet to be resolved, although long interspersed nuclear elements (LINE1s) on the X chromosome may be implicated. LINE1 element density on the human X chromosome is almost twofold that of the autosomes. LINE1 elements are particularly enriched around the *XIST* gene and in regions where silencing is most effective in human and several other eutherian species (excluding mouse). This is consistent with a role for LINE1 elements in localization of *XIST* RNA on the X chromosome in these species, although direct binding appears unlikely (reviewed in [Gendrel and Heard, 2014](#)).

Y Chromosome: Male-Specific Gene Acquisition and Divergence

In contrast to the X chromosome, the Y chromosome is small. In humans, it is the smallest chromosome (57 Mb) and bears 63 protein-coding genes and 109 noncoding genes ([Ensembl release 89](#)). It encodes 27 unique proteins, several of them in multicopy.

Specialized for Males

The Y chromosome is specialized for male reproduction (spermatogenesis). As the chromosome bearing the testis-determining gene (*SRY*), the Y chromosome is transmitted only through males, making it the optimal location for the accumulation of genes that are beneficial to males. Reflecting this particular selective pressure, 11 of the 27 unique proteins encoded on the human Y chromosome are expressed exclusively or predominantly in the testis ([Skaletsky et al., 2003](#)). Given this bias toward testis-specific expression, it is not surprising that the main disorder associated with Y chromosome mutations is impaired spermatogenesis (reviewed in [Hughes and Page, 2015](#)).

The genes on the human Y chromosome that do not have testis-specific expression are broadly expressed in a variety of tissues and are involved in a range of regulatory functions, including nucleic acid binding, transcription, and translation. Most of these broadly expressed Y-borne genes have a functional copy on the X chromosome, consistent with a role that requires two copies of these genes to maintain normal function ([Cortez et al., 2014](#)). It was suggested that subtle differences between the X- and Y-borne versions of regulatory gene pairs could potentially contribute to the differential susceptibility of male and females to a range of complex diseases, including cancer, heart disease and autism. This is a largely unexplored area of enquiry, and one that poses significant experimental challenges, not least of which is disentangling genetic from hormonal effects (reviewed in [Hughes and Page, 2015](#)).

Lineage-Specific Structural Divergence

Unlike the X chromosome, the Y displays considerable difference between species in size, proportion of condensed chromatin (heterochromatin) and proportion of ampliconic (inverted or palindromic) repeats ([Fig. 2](#)). This difference is even evident between closely related groups, such as within the primates ([Li et al., 2013](#)).

Over 50% of the human Y chromosome is heterochromatin. The remainder is comprised of ~45% ampliconic repeats. These ampliconic repeats contain nine protein-coding gene families with copy numbers ranging from 2 to 35 ([Skaletsky et al., 2003](#)). The mouse Y chromosome (92 Mb) is nearly twice the size of the human Y chromosome, and is surprisingly different. It has 183 protein-coding genes, almost no heterochromatin (~3%), and is predominantly (~95%) ampliconic repeats. It contains hundreds of copies of testis-specific genes from three gene families. At least a third of these gene copies are actively transcribed and none have homologs in primates, indicating that they emerged after divergence of the primates and rodents ([Soh et al., 2014](#)).

The extent to which structural features of the human or mouse Y chromosome are characteristic of other therian lineages is largely unknown. Sequencing of Y chromosomes is notoriously difficult, due to a combination of low gene content and highly repetitive sequence. Based on Y sequences that are available, there is evidence of abundant (<30 Mb) ampliconic repeats in Y chromosomes of cat and chimpanzee, but not in rhesus macaque. Similarly, the proportion of heterochromatin in the human Y

chromosome far exceeds that of any other sequenced Y chromosome, most of which (mouse, rhesus, chimpanzee, cat, and marsupials) have virtually none (reviewed in [Li et al., 2013](#)).

Conservation of X Chromosome, Degeneration of Y Chromosome

Gene content and order on the eutherian X chromosome is remarkably conserved, as demonstrated by global conservation of the human, horse, pig, cat, cattle, and elephant chromosomes (reviewed in [Livernois et al., 2012](#)). Even on the rodent X chromosome, where rearrangements have altered gene order, the gene content remains similar to that of other eutherians ([Fig. 2](#)). Compared with the eutherians, marsupials have a somewhat smaller X chromosome: 490 protein coding genes and 213 noncoding genes in the opossum, *Monodelphis domestica* ([Ensembl release 89](#)). The marsupial X chromosome shares homology with the entire long arm and part (the pericentric region) of the short arm of the human X chromosome. The remainder of the eutherian X chromosome is an autosome in marsupials. This autosome was fused to the sex chromosomes in the eutherian ancestor and on the X chromosome is called the X-added region ([Fig. 2](#)).

In contrast to the significant conservation of the X chromosome, on the Y chromosome only 18 genes from the ancestral Y gene complement have been retained in at least one therian representative. On the modern human Y chromosome, only five genes are retained from the ancestral Y chromosome. A further 13 genes (2 of which have degraded to pseudogenes) are retained from the autosome added to the sex chromosomes in the eutherian ancestor ([Cortez et al., 2014](#)).

Evolution of Sex Chromosomes

Recombination Repression Leads to Loss of Genes From the Y

The emergence of the testis-determining gene (*SRY*) on the ancestral Y chromosome provided a region of the genome that was only ever inherited in males. This allowed genes beneficial to spermatogenesis and male development to accumulate in this male-specific region, even if detrimental to females. Repression of recombination between the X and Y chromosomes across this region ensured that the cassette of male-specialized genes remained together. Recombination is critical for the purging of deleterious mutations which, arising on a chromosome, can be replaced by recombination with the paired region of the other chromosome. In the absence of recombination, selection can act only on the entire chromosome rather than individual genes ([Charlesworth, 1991](#)). Consequently, mutations accumulate because they cannot be removed. This is what happens to the Y chromosome: it accumulates deleterious mutations that cannot be removed by recombination, ultimately leading to loss of gene function.

The loss of Y-borne genes is accelerated by the elevated mutation rate of those genes, which results from a higher number of germline cell divisions in male spermatogenesis compared with female oogenesis. Loss of nonfunctional DNA from the Y chromosome has no effect on fitness, and so may result in a reduction in physical size. This is evident in the diminished size of the therian Y chromosome. The therian X chromosome is spared from the fate of the Y chromosome because it undergoes recombination in females.

The process of Y chromosome degeneration slows over time. Y chromosomes at more advanced stages of evolution have very few functional genes and so fewer mutations that are exposed to purifying selection. Accordingly, gene loss from the oldest parts of the human Y chromosome proceeded rapidly at first, and then slowed significantly as only a small number of essential genes were retained ([Cortez et al., 2014](#)).

Loss of Y chromosome genes has not proceeded randomly. Analysis of the rates of nucleotide substitution at nonsynonymous sites (where mutation to the DNA changes the protein) compared with synonymous sites (where mutation to the DNA does not change the protein) indicates that some Y-borne genes were subject to positive selection ([Li et al., 2013](#)). Many of these genes have a partner on the X chromosome that is broadly expressed and has regulatory functions, where transcription from a single gene copy may be insufficient for a normal phenotype. In these cases, conservation of the Y-borne gene partner may have resulted from selection to maintain two gene copies.

Evolutionary Strata

Recombination between the X and Y chromosomes was suppressed by inversion events on the Y chromosome, leaving successive regions of the X chromosome recombinationally isolated from the Y chromosome ([Lahn and Page, 1999](#)) ([Fig. 1](#)). These regions are called evolutionary strata and are defined by the extent of divergence between X and Y gene pairs. Divergence is measured by the rate of accumulation of nucleotide changes that do not affect the protein that is coded for (synonymous substitutions). At least four evolutionary strata are readily identifiable on the human X chromosome. On the Y chromosome, it is more difficult to distinguish evolutionary strata due to extensive rearrangements.

The sex-determining *SRY* gene provided the initial focal point for suppression of recombination between the X and Y chromosomes, and so is part of the first evolutionary stratum. Stratum one arose ~180 mya, before split of the eutherian and marsupial mammals. Stratum two arose in early eutherians, after divergence of the marsupials. At about the same time as the emergence of stratum two, an autosomal block was added to the sex chromosomes, extending the PAR. Suppression of recombination between the X and Y chromosomes within the extended PAR formed stratum three, also in early eutherians. Stratum four emerged only in the primate lineage ([Cortez et al., 2014](#)).

X Chromosome Shaped in Testis

In the testis, expression of protein-coding and noncoding genes is more prolific and complex than in other tissues, particularly during spermatogenesis. This is consistent with a hypothesis that the testis provides an environment that is more conducive than other tissues to gene expression, and to the emergence of new genes over time (Soumillon et al., 2013). Evolution of the X chromosome is particularly impacted by the emergence of new genes because of the evolutionary pressures arising from the presence of only a single X chromosome in males.

The X chromosome is enriched for recessive mutations that confer a male benefit. These recessive mutations are immediately open to positive selection on the single X in males, even if detrimental to females (i.e., sexual antagonism). Any detrimental effects of the new alternative gene form (allele) in females will initially be shielded by normal function of the dominant allele. However, as the new recessive allele occurs more frequently in the population, homozygous females (with two copies of the mutant allele) will emerge and female fitness will be affected. From this point, selection favors restricting expression of the allele to males only. The influence of sexual antagonism is highlighted by the prevalence of genes with testis-biased expression on the X chromosome (Soumillon et al., 2013). This applies particularly to genes that have evolved more recently on the X chromosome in repeat regions, where duplicated genes have redundant functions and so are free to evolve new functions (Zhang et al., 2010).

It was proposed that amplification of some male-specific genes on the X chromosome has been driven by the silencing of X- and Y-borne genes early in male meiosis. This silencing, called meiotic sex chromosome inactivation (MSCI), can silence genes on the X chromosome that have critical function both during and after meiosis. Increased copy number of these genes on the X chromosome can accommodate the silencing effects of MSCI by permitting sufficient gene expression. Consistent with this hypothesis, male-specific genes are enriched in the multicopy gene regions of the human and mouse X chromosomes (reviewed in Deng et al., 2014).

MSCI was also hypothesized to be a driving force that relocates genes required for specific stages of spermatogenesis from the X chromosome to autosomes. These genes can then be expressed unaffected by MSCI. This is supported by the observation that in mouse evolutionarily older spermatogenesis genes tend to be located on autosomes if they are expressed after MSCI initiates silencing, but are enriched on the X chromosome if expressed before MSCI (Soumillon et al., 2013; Zhang et al., 2010).

Sex Chromosome Evolution in Real-Time: Rodents Without a Y

Different sex chromosome systems have arisen many times in diverse vertebrate lineages, and are defined by different sex-determining genes. The genes involved in core testis and ovary developmental pathways are largely conserved across vertebrates, and many sex-determining switches have evolved from a mutation affecting function of one of these genes. For example, *SOX3* is a transcription factor with roles in development of the central nervous system and male gonadogenesis. It has evolved into the sex-determination gene (*SRY*) in therian mammals and independently into a different sex-determining gene in a fish (*Oryzias latipes*). Similarly, the transcription factor *DMRT1* has a conserved role in vertebrate male development, and has evolved into a sex-determining gene independently in chicken (*Gallus gallus*), a fish (*Oryzias latipes*), and a frog (*Xenopus laevis*). This suggests that the conserved gonad development pathway has an inherent plasticity that can accommodate a multitude of different sex-determining triggers (reviewed in Graves, 2013).

The plasticity of sex-determining switches is highlighted in some rodent species where the Y chromosome and *Sry* gene have been lost. Two species of spiny rats lost their Y chromosome and *Sry* gene in a common ancestor. It appears that a mutation in the *Sry* gene weakened its function, likely reducing selection to retain *Sry* and, ultimately, the Y chromosome. A conserved ovary suppressing gene (*Cbx2*) has been identified as the novel candidate sex-determining gene. The presence of additional copies of *Cbx2* in male spiny rats suggests ovarian repression as the new (or emergent) sex determination mechanism (Murata et al., 2012), which will define a new sex chromosome system. Similarly, two species of mole vole have lost their Y chromosomes in independent events. It appears that the role of *Sry* may have been destabilized by mutations in another gene integral to the testis-determination pathway, rendering *Sry* (and consequently the Y chromosome) redundant. An alternative sex-determining gene has not yet been identified in mole voles (Mulugeta et al., 2016), but evolution of a new sex chromosome system must have commenced.

The evolutionary trajectory of sex chromosomes is unique in the genome, and begins with the emergence of a sex-determining gene. The disparities between the human X and Y chromosomes reflect a later stage of the evolutionary process. The end of this process has occurred in some rodents with the loss of their Y chromosome. This heralds the emergence of new mammalian sex chromosome systems, demonstrating the robustness of sex determination in vertebrates despite the array of novel sex determining switches.

References

- Charlesworth, B. (1991). The evolution of sex chromosomes. *Science*, 251, 1030–1033.
- Cortez, D., Marin, R., Toledo-Flores, D., et al. (2014). Origins and functional evolution of Y chromosomes across mammals. *Nature*, 508, 488–493.
- Deng, X., Berletch, J. B., Nguyen, D. K., & Disteche, C. M. (2014). X chromosome regulation: Diverse patterns in development, tissues and disease. *Nature Reviews. Genetics*, 15, 367–378.
- Gendrel, A. V., & Heard, E. (2014). Noncoding RNAs and epigenetic mechanisms during X-chromosome inactivation. *Annual Review of Cell and Developmental Biology*, 30, 561–580.
- Graves, J. A. M. (2013). How to evolve new vertebrate sex determining genes. *Developmental Dynamics*, 242, 354–359.

- Graves, J. A. M., Gecz, J., & Hameister, H. (2002). Evolution of the human X—A smart and sexy chromosome that controls speciation and development. *Cytogenetic and Genome Research*, 99(1), 141–145.
- Hughes, J. F., & Page, D. C. (2015). The biology and evolution of mammalian Y chromosomes. *Annual Review of Genetics*, 49, 507–527.
- Julien, P., Brawand, D., Soumillon, M., et al. (2012). Mechanisms and evolutionary patterns of mammalian and avian dosage compensation. *PLoS Biology*, 10, e1001328.
- Lahn, B. T., & Page, D. C. (1999). Four evolutionary strata on the human X chromosome. *Science*, 286, 964–967.
- Li, G., Davis, B. W., Raudsepp, T., et al. (2013). Comparative analysis of mammalian Y chromosomes illuminates ancestral structure and lineage-specific evolution. *Genome Research*, 23, 1486–1495.
- Livernois, A. M., Graves, J. A. M., & Waters, P. D. (2012). The origin and evolution of vertebrate sex chromosomes and dosage compensation. *Heredity*, 108, 50–58.
- Mueller, J. L., Skaletsky, H., Brown, L. G., et al. (2013). Independent specialization of the human and mouse X chromosomes for the male germ line. *Nature Genetics*, 45, 1083–1087.
- Mulugeta, E., Wassenaar, E., Sleddens-Linkels, E., et al. (2016). Genomes of Ellobius species provide insight into the evolutionary dynamics of mammalian sex chromosomes. *Genome Research*, 26, 1202–1210.
- Murata, C., Yamada, F., Kawauchi, N., et al. (2012). The Y chromosome of the Okinawa spiny rat, *Tokudaia muenninki*, was rescued through fusion with an autosome. *Chromosome Research*, 20, 111–125.
- Skaletsky, H., Kuroda-Kawaguchi, T., Minx, P. J., et al. (2003). The male-specific region of the human Y chromosome is a mosaic of discrete sequence classes. *Nature*, 423, 825–837.
- Soh, Y. Q. S., Alfoldi, J., Pyntikova, T., et al. (2014). Sequencing the mouse Y chromosome reveals convergent gene acquisition and amplification on both sex chromosomes. *Cell*, 159, 800–813.
- Soumillon, M., Necsulea, A., Weier, M., et al. (2013). Cellular source and mechanisms of high transcriptome complexity in the mammalian testis. *Cell Reports*, 3, 2179–2190.
- Zhang, Y. E., Vibrationovski, M. D., Landback, P., et al. (2010). Chromosomal redistribution of male-biased genes in mammalian evolution with two bursts of gene gain on the X chromosome. *PLoS Biology*, 8.

Genetic Mechanisms of Sex Determination

Dagmar Wilhelm and Andrew J Pask, The University of Melbourne, Parkville, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Environmental sex determination (ESD) is believed to be the ancestral state, with genetic sex determination systems evolving later and independently in many lineages. ESD permits skewing of the sex ratio, which can maximize fitness in certain species or under a given set of environmental conditions (Adkins-Regan and Reeve, 2014). Conversely, genetic sex determination is controlled by one or more loci that are located on sex chromosomes and these mechanisms usually result in a stable 1:1 ratio of males-to-females in a population. Species with GSD are not affected by the external environment, which is important for thermo-regulated, viviparous species such as placental mammals, where sex determination and early sexual differentiation occurs in a controlled environment, in utero.

Sex Chromosome Systems

Sex chromosomes have evolved many times independently, but their evolutionary journey is surprisingly similar. Sex chromosomes develop from a pair of autosomes when one of the chromosomes gains a sex-determining locus. To keep the genes with the sex-specific function together, recombination becomes suppressed around this locus. The lack of recombination results in an accumulation of mutations and accelerated degradation of the sex-specific chromosome (for review: Ellegren, 2011; Graves, 2016). Therefore, the size difference between the sex-specific chromosome and its counterpart is an indication of the age of the sex chromosomes.

There are two main sex chromosome systems: XX/XY, in which the male is heterogametic, that is, carries two different sex chromosomes; and ZZ/ZW, where the female is heterogametic. Typical examples are humans with a XX females and XY males, and birds with ZW females and ZZ males (Fig. 1). However, there are variations to this basic model. For example, some sex chromosomes have evolved by translocations and/or fusions, such as the $Z_1Z_1Z_2Z_2$ -male and Z_1Z_2W -female system in the Adélie penguin (Gunski et al., 2017), and the XY_1Y_2 -male and XX-female system in the catfish *Harttia carvalhoi* (Centofante et al., 2006). One of the most complex sex chromosome systems exists in monotremes, such as the Echidna, with a $X_1X_2X_3X_4X_5/Y_1Y_2Y_3Y_4Y_5$ -male and $X_1X_1X_2X_2X_3X_3X_4X_4X_5X_5$ -female system where the sex chromosomes form translocation chains or rings during meiosis (Grutzner et al., 2004; Rens et al., 2004). In addition, some groups have appeared to have lost a sex chromosome. For example, in the vole *Microtus oregoni* females are XO, whereas males are XY (Ohno et al., 1966; Fredga, 1983). In contrast, in two *Ellobius* species, *Ellobius tancrei* and *Ellobius talpinus*, the Y chromosome is lost and both males and females are XX (Just et al., 1995, 2007). In the Japanese spiny rats *Tokudaia osimensis* and *Tokudaia tokunoshimensis*, as well as the mole vole *Ellobius lutescens*, both the Y and the second X chromosome are absent and all animals are XO (Arakawa et al., 2002; Just et al., 1995).

In addition to their differentiation, the gene content of the sex chromosomes also becomes unique over time. The eutherian Y chromosome harbors the sex-determining locus and contains only a handful of other genes, all of which function in either testis development, spermatogenesis or as basal transcriptional regulators (Bellott et al., 2014). The eutherian X chromosome shows an enrichment of genes involved in testis and brain function (Graves et al., 2002). This is due to altered selective pressures placed on sex chromosomes, stemming from their hemizyosity in males. Thus, in males, mutations on the X chromosome that confer a male selective advantage can be rapidly selected for even if they would be recessive in the heterozygous state. The Y chromosome

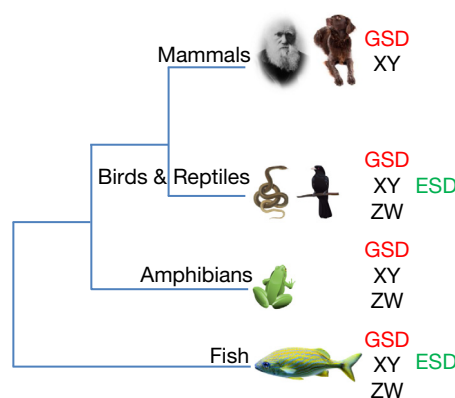


Fig. 1 Sex determination mechanisms in vertebrates. Vertebrate phylogeny showing the various methods of sex determination in each major lineage. Mammals have an exclusively GSD sex determination mechanism involving X and Y chromosome. Birds and reptiles show a broad range of mechanisms including GSD with exclusively ZW sex determination seen in birds but both XY and ZW systems and ESD seen in the reptiles. Amphibians appear to have largely GSD mechanisms with most species having cryptic sex chromosomes. Examples of amphibians with both XY and ZW systems have been described. Finally, fish have a broad array of mechanisms ranging from ESD to GSD including XY, ZW, and polygenic systems. Pictures obtained from <https://pixabay.com/>.

cannot contain any factors required for female function, so the only genes that remain are those that confer a male advantage or that are required to exist in two copies in both males and females (Whitworth and Pask, 2016). Similarly, the W chromosome in birds is enriched for female specific factors, owing to its hemizygoty in females (Moghadam et al., 2012).

Polygenic Systems of Sex Determination

In contrast to the XX/XY and ZZ/ZW system, in a polygenic sex determination system, multiple, independently segregating sex determining loci are present due to additional loci in the genome that can influence gonad development. This can arise through alterations of the sex chromosomes or of one of the autosomes, this can include, for example, the translocation of parts of the Y chromosome to an autosome, creating a so-called “neoY chromosome,” resulting in a multiple sex chromosome system.

Polygenic sex determination mechanisms have been described in a various species of fish, insects, frogs, and even mammals. One of the first species for which a polygenic sex determining system has been identified is platyfish (*Xiphophorus maculatus*), which has a male-determining Y system as well as a female-determining W system. In this system males either are XY or YY, and females either XX, XW, or YW (Vollf and Scharl, 2001). This means the hierarchy of these multiple sex chromosomes can be described as $W > Y$. However, this hierarchy is context dependent. For example, in the Western clawed frog *Xenopus tropicalis*, which also possesses three different sex chromosomes, W, Z, and Y, the hierarchy is $Y > W$. Therefore, males are either YZ, YW, or ZZ, and females are ZW or WW (Roco et al., 2015).

Examples of species in which a modification of a sex chromosome resulted in a polygenic sex determination mechanisms are the wood lemming, *Myopus schisticolor*, several species of the South African field mice (genus *Akodon*), and the African pygmy mouse, *Mus minutoides*. All three have an XY sex determination mechanism, but display both XX and XY females. However, the underlying modifications are different between the different species. In the wood lemming a mutation on the X chromosome, designated X*, most likely a structural rearrangement in the short arm of the X chromosome (Xp), results in the inactivation of the testis-determining factor on the Y chromosome. Therefore, three genotypically different females exist, XX, XX*, and X*Y. X*Y females only produce X*-containing oocytes, hence they give birth to daughters only (XX* and X*Y), resulting in approximately three to four-times more females than males in the population (Winking et al., 1981). Similarly, a chromosomal rearrangement of the X chromosome, however most likely a different one to that in the wood lemming, leading to an X* chromosome has been proposed in the African pygmy mouse. In contrast, in at least six species of *Akodon* the Y chromosome independently acquired a mutation resulting in a Y* and the complete failure to activate the male pathway (Bianchi and Contreras, 1967; Hoekstra and Edwards, 2000). Interestingly, sex reversed XY females in most mammalian species display greatly decreased fertility and fecundity (Marin and Baker, 1998). In contrast, in the species mentioned above, XY females are viable and fully fertile.

An example in which an autosome has undergone modifications is the house fly *Musca domestica*. The genome of the house fly consists of five autosomes and X and Y sex chromosomes. The Y chromosome harbors a male-determining factor M (Y^M). However, this factor can also be encoded on an autosome, A^M , or the X chromosome, X^M (Schmidt et al., 1997). In natural populations, male can carry one to several M factors (Hamm et al., 2014). In populations in which M is only on an autosome or the X chromosome, both males and females are XX (Franco et al., 1982; Hiroyoshi, 1964). The immediate downstream target of M is *Md-tra*, which is located on an autosome and exists in two variants, the wild-type allele *Md-tra* and a dominant allele, *Mda-tra^D*. While *Md-tra* is inhibited by M, *Mda-tra^D* is not and hence functions as a female-determining factor even in the presence of up to 3 M factors (Hediger et al., 1998, 2010).

Sex Determining Genes

While downstream genes in the sex differentiation regulatory cascade are conserved, the master sex determining gene that triggers sexual development shows, similar to the sex determination mechanisms, broad variations. The mode of action of this master sex determining gene can be either dosage sensitive, male- or female-dominant.

In mammals, the gene that initiates sexual development was discovered in 1990. The Y linked sex determining region on Y, or SRY gene was identified from analyses of human XY female and XX male patients (Sinclair et al., 1990). Experiments in mice revealed that it was both necessary and sufficient to drive testis development (Sinclair et al., 1990; Koopman et al., 1991). SRY encodes a transcription factor containing a high mobility group (HMG) DNA-binding domain, that gave the name to a whole family of transcription factor genes, the *Sox* (SRY-related HMG-box) family (Bowles et al., 2000). In contrast to *Sry*, which is only present in mammals, other *Sox* genes are conserved throughout the animal kingdom, including unicellular choanoflagellates (King et al., 2008). However, *Sry* is believed to have evolved from *Sox3* (Fig. 2), which is located on the X chromosome (Foster and Graves, 1994). SRY directly upregulates another HMG box gene, *Sox9* (Sekido and Lovell-Badge, 2008). SOX9 is, like SRY, necessary and sufficient to drive testis development in human and mice (Barrionuevo et al., 2006; Bishop et al., 2000; Chaboissier et al., 2004; Foster et al., 1994). As the only conserved domain between the two factors is the HMG domain, it has been suggested that other SOX proteins could function as male-determining factors. Indeed, ectopic expression of either SOX3 or SOX10 in human and mice result in testis development in XX individuals (Polanco et al., 2010; Sutton et al., 2011).

When *Sry* is not present or not functional, an ovary will form and therefore female development will occur. Hence, the female pathway has been seen as the default pathway. Nevertheless, there also is an active process driving ovarian differentiation, and while in mouse no ovarian counterpart for *Sry* has been identified, an ovarian-determining gene exists, for example in goat. Deletion of the

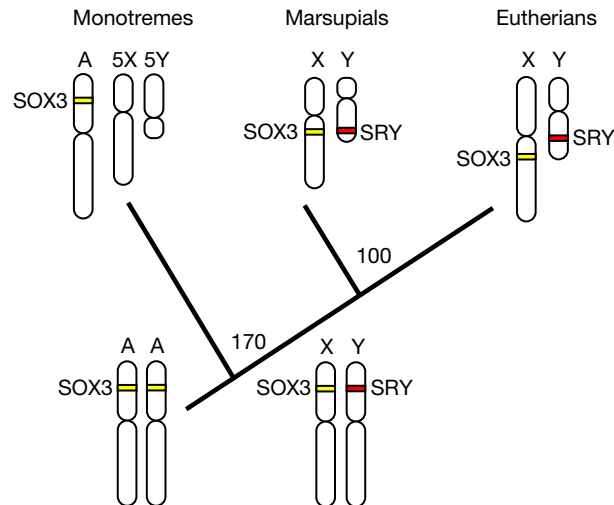


Fig. 2 Mammalian sex chromosome evolution. The sex chromosomes in all species originate from a homomorphic autosomal pair. One gene acquires the ability to determine sex. In mammals, this was the evolution of the *SRY* gene from its X-linked orthologue *SOX3*. Once the sex determination gene has been specified, recombination become restricted around this gene to prevent its cross over onto its chromosome pair. This restricted recombination leads to the accumulation of mutations, deletions and duplications causing the eventual evolution of heteromorphic sex chromosomes. The sex chromosomes also accrue a unique gene content owing to their sex specific distributions. A, Autosome, X, X-chromosome, Y, Y-chromosome. Numbers indicate the time of divergence in millions of years.

gene encoding the forkhead transcription factor *FOXL2* results in testes development instead of ovaries, and therefore female-to-male sex reversal (Boulanger et al., 2014). Similarly, mutation of R-spondin 1 (*RSPO1*) in human can lead to complete female-to-male sex reversal in the absence of *SRY* (Parma et al., 2006). Mice with a null mutation in either *Foxl2* or *Rspo1* “only” display premature ovarian failure and partial sex reversal respectively (Uda et al., 2004; Chassot et al., 2008; Tomizuka et al., 2008), demonstrating clear differences between different mammalian species.

The identification of *SRY* as the male-determining gene in mammals triggered intensive research into the evolution of sex determining genes. It came to a surprise that no other vertebrate has the *SRY* gene. Instead, the first non-mammalian master sex determining gene that was identified in the Japanese rice fish medaka (*Oryzias latipes*) was *Dmy/Dmrt1bY* (DM domain gene on the Y chromosome/doublesex and mab-3 related transcription factor 1b on the Y chromosome) (Matsuda et al., 2002; Nanda et al., 2002). Interestingly, other members of the *Dmrt* gene family were independently recruited as master sex determining genes in other species. These include *Dmrt1* on the Z chromosome in birds, which confers male development using dosage sensitive mechanism (Smith et al., 2009), and *DM-W*, a truncated copy of *Dmrt1* on the W chromosome in the African clawed frog *Xenopus laevis*, which is a female-dominant gene driving ovarian development (Yoshimoto et al., 2008). This relatively widespread distribution of DM genes as master sex determining genes resulted in the suggestions that these could be the equivalent to the mammalian *Sry*. However, further analysis of the *Dmy/Dmrt1bY* in fish uncovered that it is absent in all other fish species studied (Kondo et al., 2003). It became clear that teleost fish not only represent nearly half of all extant vertebrates, but also display one of the widest variety of sex determination mechanisms, including a broad diversity of master sex determining genes. In addition to *Dmy/Dmrt1bY* in medaka, four other promising candidates have been identified to function as the trigger for sex differentiation. These include *amhy* (antiMüllerian hormone on the Y chromosome) in the Patagonian pejerrey *Odontesthes hatcheri*, *amhr2* (antiMüllerian hormone receptor 2) in the pufferfish *Takifugu rubripes*, *gsdf* (gonadal soma-derived growth factor) in *Oryzias latipes*, a species related to medaka, and *sdY* (sexually dimorphic on the Y chromosome) in the rainbow trout *Oncorhynchus mykiss* and most other salmonids (Hattori et al., 2012; Kamiya et al., 2012; Myosho et al., 2012; Yano et al., 2012). Interestingly, none of these four genes encode transcription factors. Instead, two of these genes, *amhy* and *gsdf*, encode for growth factors, one, *Amhr2*, for a receptor, and the last one, *sdY*, for a protein that has homology to interferon regulatory factor 9 (IRF9), involved in SMAD signaling triggered by interferons. This demonstrated that a master sex determining gene does not have to encode a transcription factor to trigger sex differentiation.

Genetic Systems That Can Be Overruled by the Environment

Several groups of reptiles and fish show rapid evolutionary transitions between GSD and ESD mechanisms (Fig. 1; Quinn et al., 2011). Such plasticity in sex determination mechanisms can only occur in species with poorly differentiated sex chromosomes where essential genetic elements have not been lost to one of the sexes. Adding another layer of complexity, some species appear to have both GSD and ESD mechanisms operating concurrently, perhaps representing species at the transition from one mechanism to the other. One such species with both ESD and GSD is the European sea bass (*Dicentrarchus labrax* L.). While genetic mechanisms play a major role in sex determination in this species, elevated temperatures during early development result in masculinization of

fish that would develop into females at standard temperatures (Diaz and Piferrer, 2015). Similarly, in the Australian central bearded dragon lizard (*Pogona vitticeps*), which has a ZZ/ZW GSD system, elevated temperatures during development results in sex reversed genotypic males (ZZ) to phenotypic females (Quinn et al., 2007). Thus, in both cases, temperature can override the gene(s) involved in primary sex determination enabling skewed sex ratios under certain conditions.

Conclusions

From the studies mentioned above, it is clear that the sex determination switch is highly variable across the vertebrates and even between closely related species. In contrast, the underlying mechanisms which then direct the gonadal somatic cells towards either a male or female fate remain highly conserved. This begs the question of *why sex determination mechanisms are so variable*? It is clear that ESD mechanisms can increase fitness for some species where a certain temperature or skewing of the sex ratio leads to increased survivability. But it remains unclear why species with GSD would evolve such a variety of mechanisms and so rapidly transition from one gene driven system to another. This is especially puzzling given that determining sex correctly is arguably the single most important developmental trait in conferring fitness. Thus, we would predict that sex determination genes should be one of the most highly conserved aspects in our genomes.

Defining the mechanisms of sex determination, especially in species where it is rapidly evolving, is going to be critical for our understanding of why sex is so variable. The tractability of next-generation sequencing is likely to have a large impact on our understanding of vertebrate GSD mechanisms over the coming decade. It is now possible to sequence entire genomes from males and females in a population with no discernible sex chromosomes and simply compare the sexes to identify the genes which might be triggering the sex determination cascade. Such approaches can also be coupled with transcriptome sequencing of the developing gonad to identify not only the sex determination switch genes, but also the downstream genes and pathways activated in early sex fate choices across disparate species.

Acknowledgements

Dagmar Wilhelm is supported by research grants from the Australian Research Council (DP150101448, DP170100045). Andrew Pask is supported by Australian Research Council Future Fellowship FT140100964.

References

- Adkins-Regan, E., & Reeve, H. K. (2014). Sexual dimorphism in body size and the origin of sex-determination systems. *The American Naturalist*, 183, 519–536.
- Arakawa, Y., Nishida-Umehara, C., Matsuda, Y., Sutou, S., & Suzuki, H. (2002). X-chromosomal localization of mammalian Y-linked genes in two XO species of the Ryukyu spiny rat. *Cytogenetic and Genome Research*, 99, 303–309.
- Barriouneuo, F., Bagheri-Fam, S., Klattig, J., Kist, R., Taketo, M. M., Englert, C., & Scherer, G. (2006). Homozygous inactivation of Sox9 causes complete XY sex reversal in mice. *Biology of Reproduction*, 74, 195–201.
- Bellott, D. W., Hughes, J. F., Skaletsky, H., Brown, L. G., Pyntikova, T., Cho, T. J., Koutseva, N., Zaghlul, S., Graves, T., Rock, S., Kremitzki, C., Fulton, R. S., Dugan, S., Ding, Y., Morton, D., Khan, Z., Lewis, L., Buhay, C., Wang, Q., Watt, J., Holder, M., Lee, S., Nazareth, L., Alfoldi, J., Rozen, S., Muzny, D. M., Warren, W. C., Gibbs, R. A., Wilson, R. K., & Page, D. C. (2014). Mammalian Y chromosomes retain widely expressed dosage-sensitive regulators. *Nature*, 508, 494–499.
- Bianchi, N. O., & Contreras, J. R. (1967). The chromosomes of the field mouse *Akodon Azarae* (Cricetidae, Rodentia) with special reference to sex chromosome anomalies. *Cytogenetics*, 6, 306–313.
- Bishop, C. E., Whitworth, D. J., Qin, Y., Agoulnik, A. I., Agoulnik, I. U., Harrison, W. R., Behringer, R. R., & Overbeek, P. A. (2000). A transgenic insertion upstream of *Sox9* is associated with dominant XX sex reversal in the mouse. *Nature Genetics*, 26, 490–494.
- Boulanger, L., Pannetier, M., Gall, L., Allais-Bonnet, A., Elzaïat, M., Le Bourhis, D., Daniel, N., Richard, C., Cotinot, C., Ghyselink, N. B., & Pailhoux, E. (2014). FOXL2 is a female sex-determining gene in the goat. *Current Biology*, 24, 404–408.
- Bowles, J., Schepers, G., & Koopman, P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Developmental Biology*, 227, 239–255.
- Centofante, L., Bertollo, L. A., & Moreira-Filho, O. (2006). Cytogenetic characterization and description of an XX/XY1Y2 sex chromosome system in catfish *Harttia Carvalhoi* (Siluriformes, Loricariidae). *Cytogenetic and Genome Research*, 112, 320–324.
- Chaboissier, M. C., Kobayashi, A., Vidal, V. I., Lutzendorf, S., van de Kant, H. J., Wegner, M., de Rooij, D. G., Behringer, R. R., & Schedl, A. (2004). Functional analysis of Sox8 and Sox9 during sex determination in the mouse. *Development*, 131, 1891–1901.
- Chassot, A. A., Ranc, F., Gregoire, E. P., Roepers-Gajadien, H. L., Taketo, M. M., Camerino, G., de Rooij, D. G., Schedl, A., & Chaboissier, M. C. (2008). Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary. *Human Molecular Genetics*, 17, 1264–1277.
- Diaz, N., & Piferrer, F. (2015). Lasting effects of early exposure to temperature on the gonadal transcriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. *BMC Genomics*, 16, 679.
- Ellegren, H. (2011). Sex-chromosome evolution: Recent progress and the influence of male and female heterogamety. *Nature Reviews. Genetics*, 12, 157–166.
- Foster, J. W., & Graves, J. A. (1994). An SRY-related sequence on the marsupial X chromosome: Implications for the evolution of the mammalian testis-determining gene. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 1927–1931.
- Foster, J. W., Dominguez-Steglich, M. A., Guioi, S., Kwok, C., Weller, P. A., Stevanovic, M., Weissbach, J., Mansour, S., Young, I. D., Goodfellow, P. N., et al. (1994). Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. *Nature*, 372, 525–530.
- Franco, M. G., Rubini, P. G., & Vecchi, M. (1982). Sex-determinants and their distribution in various populations of *Musca Domestica* L. of Western Europe. *Genetical Research*, 40, 279–293.
- Fredga, K. (1983). Aberrant sex chromosome mechanisms in mammals. Evolutionary aspects. *Differentiation*, 23(Suppl), S23–30.
- Graves, J. A. (2016). Evolution of vertebrate sex chromosomes and dosage compensation. *Nature Reviews. Genetics*, 17, 33–46.
- Graves, J. A., Geç, J., & Hameister, H. (2002). Evolution of the human X—A smart and sexy chromosome that controls speciation and development. *Cytogenetic and Genome Research*, 99, 141–145.

- Grutzner, F., Rens, W., Tsend-Ayush, E., El-Mogharbel, N., O'Brien, P. C., Jones, R. C., Ferguson-Smith, M. A., & Marshall Graves, J. A. (2004). In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature*, 432, 913–917.
- Gunski, R. J., Caneod, A. D., Del Valle Garner, A., Ledesma, M. A., Coria, N., Montalti, D., & Degrandi, T. M. (2017). Multiple sex chromosome system in penguins (*Pygoscelis*, Spheniscidae). *Comparative Cytoogenetics*, 11, 541–552.
- Hamm, R. L., Meisel, R. P., & Scott, J. G. (2014). The evolving puzzle of autosomal versus Y-linked male determination in *Musca Domestica*. *G3 (Bethesda)*, 5, 371–384.
- Hattori, R. S., Murai, Y., Oura, M., Masuda, S., Majhi, S. K., Sakamoto, T., Ferdinando, J. I., Somoza, G. M., Yokota, M., & Strussmann, C. A. (2012). A Y-linked anti-Müllerian hormone duplication takes over a critical role in sex determination. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 2955–2959.
- Hediger, M., Minet, A. D., Niessen, M., Schmidt, R., Hilfiker-Kleiner, D., Cakir, S., Nothiger, R., & Dubendorfer, A. (1998). The male-determining activity on the Y chromosome of the housefly (*Musca Domestica* L.) consists of separable elements. *Genetics*, 150, 651–661.
- Hediger, M., Henggeler, C., Meier, N., Perez, R., Saccone, G., & Bopp, D. (2010). Molecular characterization of the key switch F provides a basis for understanding the rapid divergence of the sex-determining pathway in the housefly. *Genetics*, 184, 155–170.
- Hirayoshi, T. (1964). Sex-limited inheritance and abnormal sex ratio in strains of the housefly. *Genetics*, 50, 373–385.
- Hoekstra, H. E., & Edwards, S. V. (2000). Multiple origins of XY female mice (genus *Akodon*): Phylogenetic and chromosomal evidence. *Proceedings of the Biological Sciences*, 267, 1825–1831.
- Just, W., Rau, W., Vogul, W., Akhverdian, M., Fredga, K., Graves, J., & Lyapunova, E. (1995). Absence of *Sry* in species of the vole *Ellobius*. *Nature Genetics*, 11, 117–118.
- Just, W., Baumstark, A., Suss, A., Graphodatsky, A., Rens, W., Schafer, N., Bakloushinskaya, I., Hameister, H., & Vogel, W. (2007). *Ellobius Lutescens*: Sex determination and sex chromosome. *Sexual Development*, 1, 211–221.
- Kamiya, T., Kai, W., Tasumi, S., Oka, A., Matsunaga, T., Mizuno, N., Fujita, M., Suetake, H., Suzuki, S., Hosoya, S., Tohari, S., Brenner, S., Miyadai, T., Venkatesh, B., Suzuki, Y., & Kikuchi, K. (2012). A trans-species missense SNP in *Amhr2* is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (fugu). *PLoS Genetics*, 8, e1002798.
- King, N., Westbrook, M. J., Young, S. L., Kuo, A., Abedin, M., Chapman, J., Fairclough, S., Hellsten, U., Isogai, Y., Letunic, I., Marr, M., Pincus, D., Putnam, N., Rokas, A., Wright, K. J., Zuzow, R., Dirks, W., Good, M., Goodstein, D., Lemons, D., Li, W., Lyons, J. B., Morris, A., Nichols, S., Richter, D. J., Salamov, A., Sequencing, J. G., Bork, P., Lim, W. A., Manning, G., Miller, W. T., McGinnis, W., Shapiro, H., Tjian, R., Grigoriev, I. V., & Rokhsar, D. (2008). The genome of the choanoflagellate *Monosiga brevicollis* and the origin of metazoans. *Nature*, 451, 783–788.
- Kondo, M., Nanda, I., Hornung, U., Asakawa, S., Shimizu, N., Mitani, H., Schmid, M., Shima, A., & Scharl, M. (2003). Absence of the candidate male sex-determining gene *dmrt1b(Y)* of medaka from other fish species. *Current Biology*, 13, 416–420.
- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature*, 351, 117–121.
- Marin, I., & Baker, B. S. (1998). The evolutionary dynamics of sex determination. *Science*, 281, 1990–1994.
- Matsuda, M., Nagahama, Y., Shinomiya, A., Sato, T., Matsuda, C., Kobayashi, T., Morrey, C. E., Shibata, N., Asakawa, S., Shimizu, N., Hori, H., Hamaguchi, S., & Sakaizumi, M. (2002). *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*, 417, 559–563.
- Moghadam, H. K., Pointer, M. A., Wright, A. E., Berlin, S., & Mank, J. E. (2012). W chromosome expression responds to female-specific selection. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 8207–8211.
- Myosho, T., Otake, H., Masuyama, H., Matsuda, M., Kuroki, Y., Fujiyama, A., Naruse, K., Hamaguchi, S., & Sakaizumi, M. (2012). Tracing the emergence of a novel sex-determining gene in medaka, *Oryzias luzonensis*. *Genetics*, 191, 163–170.
- Nanda, I., Kondo, M., Hornung, U., Asakawa, S., Winkler, C., Shimizu, A., Shan, Z., Haaf, T., Shimizu, N., Shima, A., Schmid, M., & Scharl, M. (2002). A duplicated copy of *DMRT1* in the sex-determining region of the Y chromosome of the medaka, *Oryzias latipes*. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 11778–11783.
- Ohno, S., Stenius, C., & Christian, L. (1966). The XO as the normal female of the creeping vole (*Microtus oregoni*). In C. D. Darlington, & K. R. Lewis (Eds.), *Chromosome today*. Edinburgh: Oliver and Boyd.
- Parma, P., Radi, O., Vidal, V., Chaboissier, M. C., Dellambra, E., Valentini, S., Guerra, L., Schedl, A., & Camerino, G. (2006). *R-spondin1* is essential in sex determination, skin differentiation and malignancy. *Nature Genetics*, 38, 1304–1309.
- Polanco, J. C., Wilhelm, D., Davidson, T. L., Knight, D., & Koopman, P. (2010). *Sox10* gain-of-function causes XX sex reversal in mice: Implications for human 22q-linked disorders of sex development. *Human Molecular Genetics*, 19, 506–516.
- Quinn, A. E., Georges, A., Sarre, S. D., Guarino, F., Ezaz, T., & Graves, J. A. (2007). Temperature sex reversal implies sex gene dosage in a reptile. *Science*, 316, 411.
- Quinn, A. E., Sarre, S. D., Ezaz, T., Marshall Graves, J. A., & Georges, A. (2011). Evolutionary transitions between mechanisms of sex determination in vertebrates. *Biology Letters*, 7, 443–448.
- Rens, W., Grutzner, F., O'Brien, P. C., Fairclough, H., Graves, J. A., & Ferguson-Smith, M. A. (2004). Resolution and evolution of the duck-billed platypus karyotype with an X1Y1X2Y2X3Y3X4Y4X5Y5 male sex chromosome constitution. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 16257–16261.
- Roco, A. S., Olmstead, A. W., Degitz, S. J., Amano, T., Zimmerman, L. B., & Bullejos, M. (2015). Coexistence of Y, W, and Z sex chromosomes in *Xenopus tropicalis*. *Proceedings of the National Academy of Sciences of the United States of America*, 112, E4752–61.
- Schmidt, R., Hediger, M., Roth, S., Nothiger, R., & Dubendorfer, A. (1997). The Y-chromosomal and autosomal male-determining M factors of *Musca domestica* are equivalent. *Genetics*, 147, 271–280.
- Sekido, R., & Lovell-Badge, R. (2008). Sex determination involves synergistic action of *SRY* and *SF1* on a specific *Sox9* enhancer. *Nature*, 453, 930–934.
- Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A.-M., Lovell-Badge, R., & Goodfellow, P. N. (1990). A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif. *Nature*, 346, 240–244.
- Smith, C. A., Roeszler, K. N., Ohnesorg, T., Cummins, D. M., Farlie, P. G., Doran, T. J., & Sinclair, A. H. (2009). The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. *Nature*, 461, 267–271.
- Sutton, E., Hughes, J., White, S., Sekido, R., Tan, J., Arboleda, V., Rogers, N., Knowler, K., Rowley, L., Eyre, H., Rizzoti, K., Mcaninch, D., Goncalves, J., Slee, J., Turbitt, E., Bruno, D., Bengtsson, H., Harley, V., Vilain, E., Sinclair, A., Lovell-Badge, R., & Thomas, P. (2011). Identification of *SOX3* as an XX male sex reversal gene in mice and humans. *The Journal of Clinical Investigation*, 121, 328–341.
- Tomizuka, K., Horikoshi, K., Kitada, R., Sugawara, Y., Iba, Y., Kojima, A., Yoshitome, A., Yamawaki, K., Amagai, M., Inoue, A., Oshima, T., & Kakitani, M. (2008). *R-spondin1* plays an essential role in ovarian development through positively regulating Wnt-4 signaling. *Human Molecular Genetics*, 17, 1278–1291.
- Uda, M., Ottolenghi, C., Crisponi, L., Garcia, J. E., Deiana, M., Kimber, W., Forabosco, A., Cao, A., Schlessinger, D., & Pilia, G. (2004). *Foxl2* disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Human Molecular Genetics*, 13, 1171–1181.
- Voff, J. N., & Scharl, M. (2001). Variability of genetic sex determination in poeciliid fishes. *Genetica*, 111, 101–110.
- Whitworth, D. J., & Pask, A. J. (2016). The X factor: X chromosome dosage compensation in the evolutionarily divergent monotremes and marsupials. *Seminars in Cell and Developmental Biology*, 56, 117–121.
- Winking, H., Gropp, A., & Fredga, K. (1981). Sex determination and phenotype in wood lemmings with XXY and related karyotypic anomalies. *Human Genetics*, 58, 98–104.
- Yano, A., Guyomard, R., Nicol, B., Jouanno, E., Quillet, E., Klopp, C., Cabau, C., Bouchez, O., Fostier, A., & Guiguen, Y. (2012). An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. *Current Biology*, 22, 1423–1428.
- Yoshimoto, S., Okada, E., Umemoto, H., Tamura, K., Uno, Y., Nishida-Umehara, C., Matsuda, Y., Takamatsu, N., Shiba, T., & Ito, M. (2008). A W-linked DM-domain gene, *DM-W*, participates in primary ovary development in *Xenopus laevis*. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 2469–2474.

Psychological Effects of Sex Differentiation

Matthew S Bramble, Neerja Vashist, and Eric Vilain, Children's National Medical Center, Washington, DC, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Sexual differentiation by definition is the process by which gonadal hormones such as estrogens and androgens influence the development of sex typed characteristics downstream of sexual determination, that is, the development of one's testis or ovaries. During in utero mammalian development, the testes begin secreting testosterone during mid-late gestational weeks. During this active androgen exposure, tissues such as the external genitalia, which are androgen sensitive, become masculinized (Hines, 2003). Both XX and XY genital tissues are typically responsive to androgen, and if stimulated this results in penis formation in male fetuses and clitoral enlargement in female fetuses, a condition discussed in greater detail throughout the review. Gonadal hormones have been shown to act in two distinct ways, either through organizational or activational effects. The organizational effects are permanent nonreversible effects, while the activational effects are shorter-lived nonpermanent effects (Arnold, 2009). This organizational/activational hypothesis was largely developed in the 1950s with the publication by Phoenix et al. (1959), where it was demonstrated that in utero testosterone exposure on genetically female Guinea pigs caused permanent changes to their adult sexual behavior. These changes resulted in the female Guinea pigs taking on more male-typical sexual roles, a behavioral modification that was irreversible. Organizational effects of hormones occur during a distinct window in development called the critical period where the presence of specific hormones can result in long lasting effects. In rodents this critical period is during late gestation or shortly after birth (Arnold, 2009). The exact critical period in human development is less exact, but is believed to be during high levels of early testosterone secretions, between gestational weeks 8–24 and up to 6 months after birth (Hines, 2003). During in utero development some of the organizational effects caused by testosterone on the body include but are not limited to, phallus growth, internal reproductive organ development and the overall proper formation of the external genitalia (Hines, 2003). In addition to the physical effects of hormonal organization, there are also numerous effects on the central nervous system that are thought to be organized by the exposure to gonadal hormones, namely testosterone, during the critical period of human development. Testosterone exposure on the mammalian brain generates sex differences in anatomic structure of various regions such as the bed nucleus of the striatum terminalis (BNST) along with altering neural cell densities in additional brain regions by affecting the rate of cell death. In addition to anatomic changes in the brain as a result of testosterone exposure, it has also been shown that androgens can alter gene expression, DNA methylation and other epigenetic signatures in various regions of the central nervous system (CNS) (McCarthy, 2010; Ngun et al., 2011). Some of these anatomical, genetic and epigenetic sex differences between the male and female brain, which are triggered by androgen stimulation likely affect downstream behaviors and other cognitive measures. Here we will review some of the more heavily studied aspects of the psychological effects of sexual differentiation such as sexual orientation, toy play preferences and other cognitive abilities that seem to be heavily influenced by gonadal hormone exposure during early human development.

Infant and Adolescent Toy Play Preferences

For many, it is taken for granted that boys play with boys toys and girls play with girls toys, however, the causes of these outcomes has been heavily investigated. Studies have demonstrated that clear sex differences exist between infants and adolescent children when it comes to toy selection. On average, when given the choice, boys spend significantly more time playing with male-typed toys such as wheeled vehicles, trains and planes than female-typed toys, with the reverse being true for girls. While there are social components and influences to toy selection as the child develops, what is most striking is the strong link between this behavior and early prenatal androgen exposures.

While it would be unethical to conduct a hormonal manipulation studies on humans, a condition known as congenital adrenal hyperplasia or CAH provides a natural model to study the effects of elevated prenatal androgen exposure on XX and XY genetic backgrounds. Although CAH affects both males and females, sex differences have been most extensively studied in CAH females. CAH results from a mutation in key enzymes required for proper cortisol synthesis, as a result of these mutations, precursors to cortisol are shuttled into the androgen synthesis pathway within the adrenal glands. Due to the overproduction of androgen by the adrenal glands in XX CAH girls, they are born with a wide array of phenotypes. These outcomes range from the simple virilizing (SV) form, causing a more subtle masculinization of the genitals in addition to androgen exposure on the brain during development. The more severe and life-threatening form is the salt wasting (SW) type of CAH, often resulting in a more pronounced masculinization of the genitals along with heavy androgen exposure on the brain. As we will discuss further, some of the reviewed psychological measures often correlate strongly with the type of CAH an individual has, meaning it can also correlate to the level of androgen exposure during development, as SW CAH typically is exposed to more androgen than those with SV CAH while in the womb. Individuals with what is known as nonclassical CAH (NC), experience late onset symptoms of the condition and are not typically exposed to excessive levels of testosterone during developmental periods (Bramble et al., 2017).

The vast majority of studies that have assessed androgen exposure during development and toy choice preferences have largely drawn conclusions from studies involving individuals with CAH. It has been shown that girls with CAH, on average choose

masculine boy's toys when given the chance at a higher frequency than control non-CAH girls. Additionally, girls with CAH tend to play with typical girls toys less than the control girls in most studies (Alexander and Hines, 2002; Hines, 2003; Pasterski et al., 2005). By designing studies focused on individuals with CAH has provided strong evidence indicating that prenatal androgen exposure can indeed influence individual's toy choice. Critics of this work would argue that these observed outcomes are more socially influenced than based on prenatal androgen exposures; interestingly, studies using infants have also demonstrated an androgen effect on toy/object preference. When 12, 18 and 24 month olds were assessed, it was still shown that a sex difference existed between girls and boys in regards to the types of toys and objects they were most visually focused to (Jadva et al., 2010). Using young children to demonstrate sex differences in toy choice preference only strengthens the notion that androgens in part, establish some of these differences adding to the biological component instead of a purely social aspect (Fig.1).

To further support the theory that androgens influence toy choice preference, several studies have been conducted in nonhuman primates to determine if sex differences are still detectable in regards to toy choice selection. A study conducted in 2002 using vervet monkeys found strikingly similar outcomes to those observed in human children. It was shown that male monkeys gravitated towards male-typical toys and female monkeys spent more time with the female-typical toy selections (Hines and Alexander, 2008). This was the first type of study using nonhuman primates which investigated innate factors, that is, testosterone exposure, and the subsequent downstream choice in sex typed toy selection. Similar outcomes were also observed in later studies using rhesus monkeys, where it was shown that male monkeys displayed stronger preferences for wheeled toys over plush toys, which were considered the more feminine choice by this particular group of researchers (Hassett et al., 2008).

Generally speaking, the studies that have assessed toy play preference between male and female children for the most part all detect a sex-difference in regards to the types of selections that each gender are drawn to. While toy play preferences can and do have strong social influences, such as parent encouragement or discouragement when a child plays with an object that would be considered cross-gendered, there appear to be other biological factors which contribute as well. Despite a social link, child toy preferences are not simply influenced by gender socialization alone, but as discussed also influenced by prenatal androgen exposures. Studies which have measured testosterone levels in children often make strong correlations between those levels and the outcomes

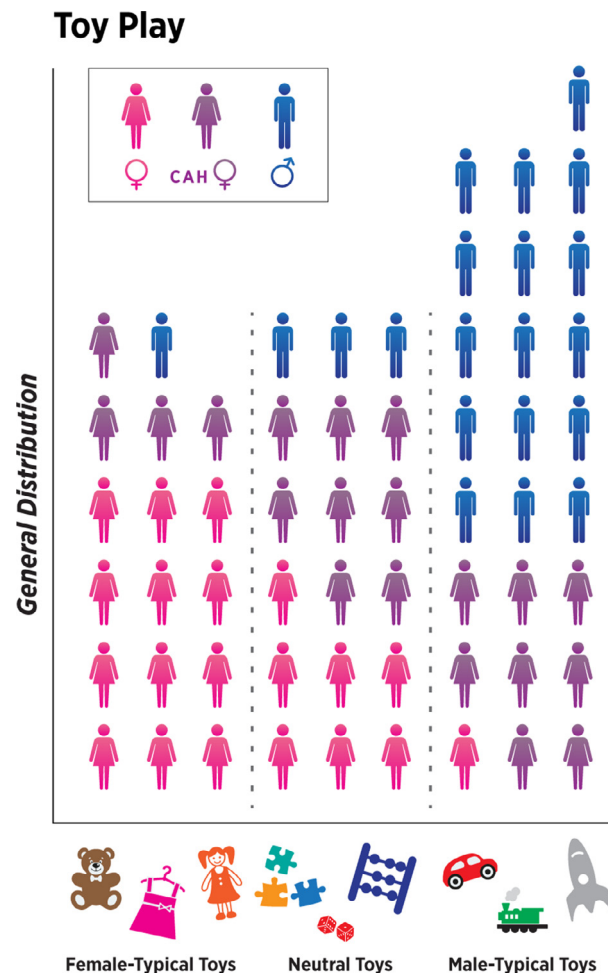


Fig. 1 General distribution of toy choice preferences observed for adolescent girls with CAH versus control-female and control-male populations.

in toy selection. Additionally, works focused on children with CAH and nonhuman primates provide some of the strongest data indicating that androgen exposure is indeed a factor that contributes to these observed sex-differences. Collectively, toy play preferences in children are clearly influenced by early prenatal androgen exposure on the brain, highlighting one of the better documented examples of some of the psychological effects that occur during mammalian sexual differentiation.

Cognitive Differences Influenced by Sexual Differentiation

When focusing on specific cognitive differences, there are many studies that have identified small to moderate sex-differences that exist between males and females of all ages. It has been confidently shown for the most part that males show superiority when tasked with finding solutions involving spatial ability. More specifically, when it comes to 3D mental rotation ability, males tend to outperform females; thereby generating the larger sex-difference identified within the category of spatial ability tests. Other small to moderate cognitive differences which males have shown an advantage include but are not limited to, geographic ability, targeting, mechanical ability/knowledge and navigation tasks. Females on the other hand show superiority in cognitive skills such as memory and tasks involving language and verbal fluency. Some controversy in the field exists when it comes to the cause and size of these identified sex differences (Voyer et al., 1995). It has also been shown that these cognitive abilities can be improved over time with practice such as targeting and geographical awareness. However, some of these sex-differences such as spatial ability are able to be detected at early postnatal time points. Like toy play, it appears that in utero factors such as androgen exposure contribute to establishing such differences between the sexes, with the strongest evidence again coming from studies involving individuals with CAH.

When assessing spatial abilities between girls with CAH versus their unaffected sisters, numerous studies have identified significant differences between the siblings. Girls with CAH on average outperform their unaffected sisters in measures such as mechanical knowledge, geography and 3D mental rotation abilities (Fig. 2A). Interestingly, when assessing males with CAH compared to unaffected brothers, an enhancement in spatial ability was not identified but rather a reduction (Fig. 2A) (Berenbaum et al., 2012; Hines et al., 2003). Some researchers have interpreted these unusual findings in males as possibly being influenced by other factors with the CAH condition and not androgens directly, as males often have more medical difficulties when affected with CAH. But these outcomes could also indicate that it is not just the exposure of androgen on the brain that is important for establishing sex-differences, but also the spatial, temporal and dosage of hormone during development. This enhancement of spatial abilities in girls with CAH is also likely influenced by their early interests such as toy play or their increased interests in male-typical hobbies

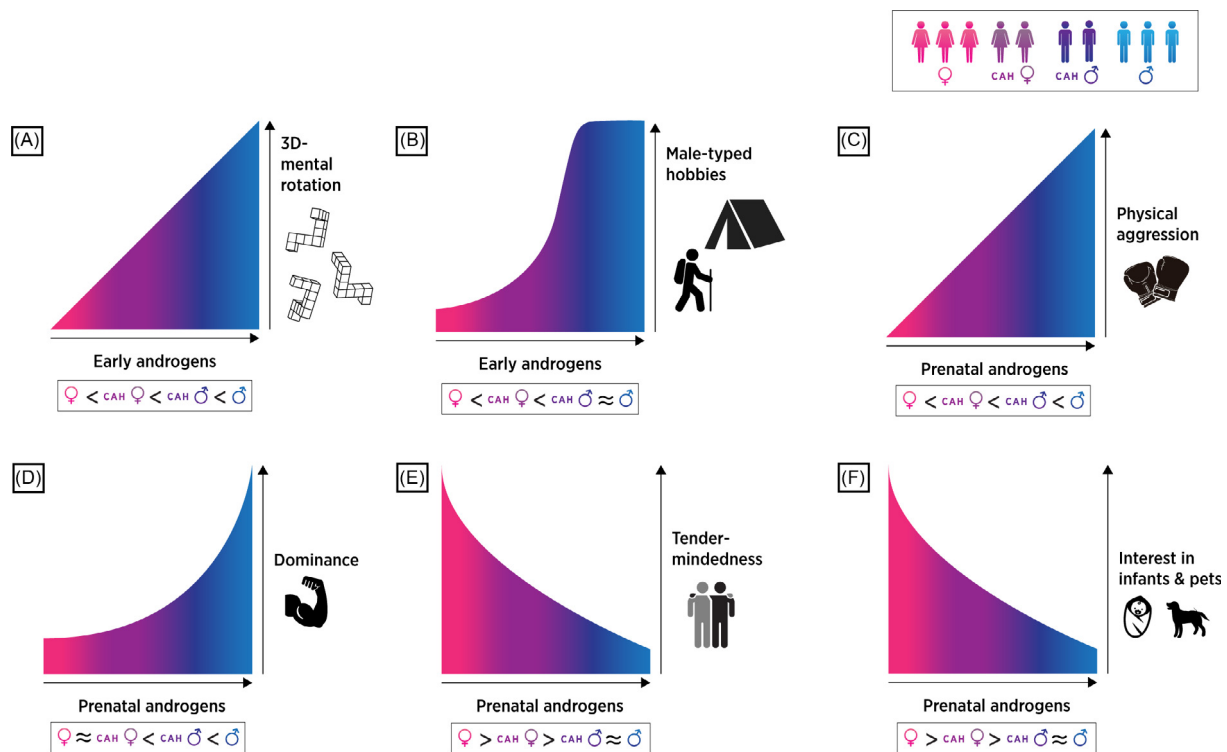


Fig. 2 The general trends observed for the influence on various cognitive and personality traits in control-females, control-males, CAH females and CAH males. (A) Aggression, (B) dominance, (C) tender-mindedness, (D) interest in infants and pets, (E) male-typed hobbies and (F) 3D mental rotation ability.

(Fig. 2B) and activities like rough and tumble play; increasing the chance for improving on these male typical cognitive strengths (Mathews et al., 2009).

The evidence generated from research that has focused on males and females with CAH provide some of the strongest support for the role of androgens on generating spatial capacity and awareness within the human brain. While spatial abilities show one of the larger detectable differences, others have also detected variances between CAH girls and siblings on other personality and cognitive traits. It seems that androgens not only increase spatial ability, but can also increase physical aggression (Fig. 2C) and dominance (Fig. 2D) in girls with CAH versus appropriate control populations; and reducing such measures in CAH males (Mathews et al., 2009; Pasterski et al., 2007). Other smaller differences that have been identified also include, but are not limited to concluding that females with CAH show less interest in female typical traits such as being more tender-minded (Fig. 2E) and having curiosity in babies and pets (Fig. 2F). The effect of androgens on CAH males appears to be either reduction of male typical behavior or in some cases nonexistent. However many sex differences studied in CAH females suggest that prenatal androgens masculinize their behavior. As with toy play, there are social factors that add to increasing such sex-differences in cognitive ability. However, from the comprehensive studies conducted on children with CAH, it appears clear that androgens exposure during in utero development has an organizational effect which at least sets the stage for the establishment of these identified cognitive differences between the sexes. There have been studies with nonhuman mammals also linking prenatal or neonatal levels of gonadal hormones, especially testosterone, on behaviors that exhibit cognitive sex differences. Collectively, these studies strengthen the notion that hormones are important influencers of cognitive traits in humans as well as other mammals.

The Role of Androgen Exposure on Influencing Sexual Orientation

In regards to sex-differences between males and females, sexual orientation shows one of the largest measurable differences, as most males sexually prefer females, and most females sexually prefer males. This sex difference has been reported to have a d value or effect size equal to 6.0–7.0 (Meyer-Bahlburg et al., 2008). For general comparison, the effect size observed for differences in toy play preferences and reported cognitive differences typically fall below a d value of 2.0. The Kinsey scale is often used to quantitatively measure sexual orientation. On this six-point scale, individuals that fall within 0–1 are considered exclusively heterosexual and a score of 5–6 is defined as predominately/exclusively homosexual. While the vast majority of the population is heterosexual, it appears that females display a more variable and fluid sexual orientation pattern, whereas males typically are exclusively heterosexual or homosexual, with a smaller percentage falling into the bisexual range (Fig. 3A). The exact causes of nonheterosexual behavior have yet to be fully elucidated in humans. However, significant genetic, epigenetic and social/psychological works have drawn strong biological conclusions, indicating that sexual orientation is an inborn phenomenon and not a result of socialization (Bailey et al., 2016). As we will discuss further, there are many variable aspects to consider when measuring human sexual orientation, as societal, religious and familial components can affect the display of this human trait. As earlier mentioned, studies conducted on rodents have shown that gonadal hormonal manipulation during the critical period of development can alter downstream sexual behaviors. Specifically, if female rat fetuses are exposed to exogenous testosterone during prenatal development, when stimulated in adulthood with proper hormones, they display a masculinized/defeminized pattern of copulation. These results indicate in part, that androgen exposure on the female brain can alter sexual behavior and possible orientation in humans. Here we will discuss the evidence supporting the role of androgen exposure in affecting female sexual orientation, where again the largest supportive data has been generated from studying women with CAH.

Given that the expression of one's sexual behavior can be constrained by factors independent of biological predisposition, measuring sexual fantasies such as thoughts during masturbation and sexual night or day dreams (total imagery), tend to be more reliable indicators of sexual orientation. When assessing total imagery in women with CAH it has been shown that nonheterosexual outcomes are detected at higher rates depending on the severity of the condition. So by this, individuals with the salt wasting (SW) type of CAH show the most nonheterosexual total imagery outcomes, followed by those with the simple virilizing (SV) type and lastly by women with the nonclassical (NC) category of CAH (Fig. 3B). Studies have also assessed the level of cross-gender fantasies in women with CAH during intercourse or masturbation, as rates of gender dysphoria have been observed at higher rates in CAH women than seen in the general population. These measures have shown that those with the SW form show the highest levels of this cross-gender imagery during these acts, however this measure does not correlate with severity of disease as was observed for total imagery fantasies (Fig. 3C) (Hines, 2011; Meyer-Bahlburg et al., 2008).

When Kinsey score assessments are given to women with CAH, similar trends are observed; individuals having the SW CAH report higher scores (nonheterosexual). These scores decrease as disease severity decreases; all three types of CAH report higher Kinsey scores than control females (Fig. 3D). Using Kinsey score measures it has also been shown that women with the most severe form of the disease report higher scores on assessments measuring various behaviors such as masturbation erotica, masturbation fantasies and attraction (Fig. 3E). However, despite having higher nonheterosexual scores than control population on multiple measures, this does not always necessarily translate to actual sexual partners. The vast majority of individuals with CAH are heterosexual, however, there is a significant increase in the actual number of same sex partners that women with SW CAH have. Women with both SV and NC forms of CAH also display higher rates of having same sex partners when compared to appropriate control females (Fig. 3F) (Meyer-Bahlburg et al., 2008).

Studies conducted on individuals with CAH have strengthened the notion that prenatal androgen exposure can cause increases in masculinized sexual behavior, specifically on a XX genetic background. As mentioned, most women affected with this condition



Fig. 3 General trends and distributions observed within the general population and between individuals with varying types of CAH, including nonclassical, simple virilizing and those with the salt wasting form. (A) General male and female distribution of Kinsey scores observed in general population samples. (B) General observations for total imagery in individuals with CAH. (C) General observations for cross gender imagery in individuals with CAH. (D) Overall reported Kinsey scores for the various types of CAH. (E) A detailed breakdown of Kinsey scores as they pertain to various measures of sexual imagery. (F) The level of nonheterosexual sexual partners that women with CAH report.

tend to not display nonheterosexual behaviors or fantasies; however, the majority of studies conducted on such questions continually find significant differences when compared to control females. As with other androgenic influences on the brain, some argue that the results of increased nonheterosexual behavior in women with CAH are more from a result of social components over biological explanations (Jordan-Young, 2012). While there may be measures that have social components involved, the majority of the scientific community that have assessed sexual behavior in cases of CAH more frequently than not indicate that prenatal androgen exposure does in fact alter sexual behaviors and fantasies in women.

Concluding Remarks

Since the early 1900s researchers have been expanding the understanding of how gonadal hormones masculinize the internal and external reproductive organs of mammals. In 1907, Frank Lillie determined that a freemartin or an infertile female cow with masculinized genitals was a result of sharing a womb with a male twin fetus (Capel and Coveney, 2004). He was successful in determining that a substance secreted by the male twin fetus was the cause for this masculinization, that substance was testosterone. Years later, a highly skilled French surgeon named Alfred Jost was able to expand on the understanding of how testicular secretions were able to alter reproductive organs. Not only did he show that testosterone caused genital masculinization, but he also identified a secondary hormone that was secreted by the testes which is now known as anti-Müllerian hormone or AMH (Jost, 1948). These key findings really opened the field of understanding sexual-differentiation of the mammalian body, specifically the hormonal effects on genital formation. However, with the 1959 publication by Phoenix et al. it became clear that gonadal hormones not only affected the body, but sexual differentiation could also affect the brain and most interestingly, sexual behavior as well (Phoenix et al., 1959).

After the establishment that gonadal hormones act via activational or organizational pathways, downstream rodent studies sought to identify the details and changes that were occurring within the mammalian brain during and post-testosterone exposure. A large number of studies have shown that organizational effects of testosterone on the brain are robust and affect anatomic structures, neural circuitry, sexual behaviors and other cognitive abilities. Despite extensive research on hormonal organization of the brain, the exact molecular mechanisms of this process largely remain elusive. More recent literature is now suggesting that the enduring effects of gonadal hormonal exposure on the brain are modulated in part by chromosomal sex but even more so by epigenetic mechanisms, such as histone modifications and DNA methylation. In fact, a recent publication determined that by altering the DNA methylation machinery and levels of methylation in specific regions of the brain, the downstream behavior of rodents was able to be modified, even post in utero organization (Nugent et al., 2015). Our research group also recently uncovered that sex-differences exist in the early cells of the developing brain, neural stem cells, and that these cells display different responses when exposed to testosterone (Bramble et al., 2016). Collectively, over the past decade significant studies have identified the power of sexual-differentiation on the brain, however much work is still required to fully elucidate the mechanism behind this interesting developmental phenomenon.

While rodent studies are interesting and have provided the foundation for our understanding of sexual differentiation of the rodent brain, a far more limited understanding of sexual differentiation of the human brain still exists. It is through studies of individuals with disorders of sex development (DSD), most specifically CAH that researchers have been able to better understand the downstream psychological effects of sexual-differentiation (Bramble et al., 2017). As reviewed here, it is apparent that gonadal hormone exposure during in utero human development can alter preferences for specific objects, sexual behavior and other cognitive differences.

It has been shown by studying individuals with CAH and nonhuman primates that androgen exposures on the developing brain influence the downstream preference for toy choice. While socialization can affect this outcome, as we have discussed, these differences begin to appear as early as 12 months, indicating that these effects are inborn and largely influenced by testosterone. The studies conducted with CAH men and women have also expanded the understanding of some of the roots for establishing cognitive differences between males and females. These findings for the most part, indicate that testosterone enhances specific male-typical cognitive strengths when female fetuses are exposed to high levels of testosterone during development. In addition to androgen exposure setting the stage for toy preference and specific cognitive abilities, it also becomes apparent from studies in CAH women that it also affects sexual behavior and may increase the rates of nonheterosexual orientation in females.

In summary, from the extensive rodent studies that have been conducted and some of the human research reviewed here, it is very convincing that testosterone exposure on the brain can elicit significant outcomes and largely contribute to the establishment of psychological effects of sexual differentiation.

References

- Alexander, G. M., & Hines, M. (2002). Sex differences in response to children's toys in nonhuman primates (*Cercopithecus aethiops sabaeus*). *Evolution and Human Behavior*, 23(6), 467–479.
- Arnold, A. P. (2009). The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. *Hormones and Behavior*, 55(5), 570–578.
- Bailey, J. M., Vasey, P. L., Diamond, L. M., Breedlove, S. M., Vilain, E., & Epprecht, M. (2016). Sexual orientation, controversy, and science. *Psychological Science in the Public Interest*, 17(2), 45–101.

- Berenbaum, S. A., Bryk, K. L. K., & Beltz, A. M. (2012). Early androgen effects on spatial and mechanical abilities: Evidence from congenital adrenal hyperplasia. *Behavioral Neuroscience*, 126(1), 86–96.
- Bramble, M. S., Roach, L., Lipson, A., Vashist, N., Eskin, A., Ngun, T., Gosschalk, J. E., Klein, S., Barseghyan, H., Arboleda, V. A., & Vilain, E. (2016). Sex-specific effects of testosterone on the sexually dimorphic Transcriptome and Epigenome of embryonic neural stem/progenitor cells. *Scientific Reports*, 6, 36916.
- Bramble, M. S., Lipson, A., Vashist, N., & Vilain, E. (2017). Effects of chromosomal sex and hormonal influences on shaping sex differences in brain and behavior: Lessons from cases of disorders of sex development. *Journal of Neuroscience Research*, 95(1–2), 65–74.
- Capel, B., & Coveney, D. (2004). Frank Lillie's freemartin: Illuminating the pathway to 21st century reproductive endocrinology. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, 301(11), 853–856.
- Hassett, J. M., Siebert, E. R., & Wallen, K. (2008). Sex differences in rhesus monkey toy preferences parallel those of children. *Hormones and Behavior*, 54(3), 359–364.
- Hines, M. (2003). Sex steroids and human behavior: Prenatal androgen exposure and sex-typical play behavior in children. *Annals of the New York Academy of Sciences*, 1007, 272–282.
- Hines, M. (2011). Prenatal endocrine influences on sexual orientation and on sexually differentiated childhood behavior. *Frontiers in Neuroendocrinology*, 32(2), 170–182.
- Hines, M., & Alexander, G. M. (2008). Monkeys, girls, boys and toys: A confirmation letter regarding "sex differences in toy preferences: Striking parallels between monkeys and humans". *Hormones and Behavior*, 54(3), 478–479. author reply 480–1.
- Hines, M., Fane, B. A., Pasterski, V. L., Mathews, G. A., Conway, G. S., & Brook, C. (2003). Spatial abilities following prenatal androgen abnormality: Targeting and mental rotations performance in individuals with congenital adrenal hyperplasia. *Psychoneuroendocrinology*, 28(8), 1010–1026.
- Jadva, V., Hines, M., & Golombok, S. (2010). Infants' preferences for toys, colors, and shapes: Sex differences and similarities. *Archives of Sexual Behavior*, 39(6), 1261–1273.
- Jordan-Young, R. M. (2012). Hormones, context, and "brain gender": A review of evidence from congenital adrenal hyperplasia. *Social Science & Medicine*, 74(11), 1738–1744.
- Jost, A. (1948). *Recherches sur la différenciation sexuelle de l'embryon de lapin*. Translated from French. Paris: Masson et Cie.
- Mathews, G. A., Fane, B. A., Conway, G. S., Brook, C. G. D., & Hines, M. (2009). Personality and congenital adrenal hyperplasia: Possible effects of prenatal androgen exposure. *Hormones and Behavior*, 55(2), 285–291.
- McCarthy, M. M. (2010). How it's made: Organisational effects of hormones on the developing brain. *Journal of Neuroendocrinology*, 22(7), 736–742.
- Meyer-Bahlburg, H. F. L., Dolezal, C., Baker, S. W., & New, M. I. (2008). Sexual orientation in women with classical or non-classical congenital adrenal hyperplasia as a function of degree of prenatal androgen excess. *Archives of Sexual Behavior*, 37(1), 85–99.
- Ngun, T. C., Ghahramani, N., Sanchez, F. J., Bocklandt, S., & Vilain, E. (2011). The genetics of sex differences in brain and behavior. *Frontiers in Neuroendocrinology*, 32(2), 227–246.
- Nugent, B. M., Wright, C. L., Shetty, A. C., Hodes, G. E., Lenz, K. M., Mahurkar, A., Russo, S. J., Devine, S. E., & McCarthy, M. M. (2015). Brain feminization requires active repression of masculinization via DNA methylation. *Nature Neuroscience*, 18(5), 690–697.
- Pasterski, V., Hindmarsh, P., Geffner, M., Brook, C., Brain, C., & Hines, M. (2007). Increased aggression and activity level in 3- to 11-year-old girls with congenital adrenal hyperplasia (CAH). *Hormones and Behavior*, 52(3), 368–374.
- Pasterski, V. L., Geffner, M. E., Brain, C., Hindmarsh, P., Brook, C., & Hines, M. (2005). Prenatal hormones and postnatal socialization by parents as determinants of male-typical toy play in girls with congenital adrenal hyperplasia. *Child Development*, 76(1), 264–278.
- Phoenix, C. H., Goy, R. W., Gerall, A. A., & Young, W. C. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology*, 65, 369–382.
- Voyer, D., Voyer, S., & Bryden, M. P. (1995). Magnitude of sex differences in spatial abilities: A meta-analysis and consideration of critical variables. *Psychological Bulletin*, 117(2), 250–270.

Sex Ratio at Birth

Victor Grech, University of Malta Medical School, Msida, Malta
William H James, University College London, London, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

The human sex ratio (males:females) varies depending on when it is measured. The primary sex ratio is that at fertilization. The secondary sex ratio is that at birth. The tertiary sex ratio is that of the sexually mature members of any particular population and the quaternary ratio is that of the surviving populace at post-reproductive age. This article will deal with the secondary sex ratio, which will henceforth be referred to as M/T (male divided by total births). In humans, male live births slightly exceed female live births, such that this ratio approximates 0.515 (515 male:485 female births) (James, 2015).

The causes for the observed variations of M/T may be classified as “ultimate” or “proximate.” Ultimate causes are those hypothesized to be adaptive that is, to maximize reproductive success, and to therefore have arisen via evolution and natural selection. Proximate causes are those closest to an event and therefore immediately responsible for the observed results. Proximate causes may serve ultimate causes but not all observed proximate causes are adaptive. A veritable plethora of influences have been shown to affect M/T, and this article will provide an outline. Where events are mentioned or reported, this implies that such findings are statistically significant at or below the 5% level (i.e. $P \leq 0.05$).

Historical Notes

The theory and study of M/T has an old and interesting history. In ancient times, it was widely believed that an infant's gender was determined by the degree of heat that a man's ejaculate was exposed to during insemination, adhering to the classical notion of the four elements that comprise nature: earth, air, fire, and water. Laterality was also deemed important by some of the ancient philosophers in that as the male was thought to be superior to the female, so was the right side deemed superior to the left. Hence, by conflating the two theories, it was concluded that the right side/testicle is hotter and associated with the engenderment of males, and the cooler left side was associated with the engenderment of females. Theories continued to multiply with little actual science until the scholarly, post-Enlightenment world of the 18th century began to be intrigued by the regularity and occasional variations in M/T. This investigators included physico-theologians such as John Arbuthnot and Johann Peter Süssmilch, mathematicians such as Nicolas de Condorcet, Pierre-Simon Laplace and Denis Poisson, agronomists such as Charles Gilbert de Morel-Vindé and Charles Girou de Buzareingues, physiologists such as Johann Daniel Hofacker and organizers of statistical observations such as Joseph Fourier and Adolphe Quetelet (Brian and Jaisson, 2007).

The accurate collection of birth data from London allowed John Graunt (1620–74) to publish the first ever descriptive analysis of M/T data. John Arbuthnot (1667–1735) extended this dataset to publish the first ever statistical test based on the symmetric binomial distribution, demonstrating that the male excess was statistically significant, that is, highly unlikely to be due to chance alone (Brian and Jaisson, 2007). Charles Darwin (1809–82) was initially intrigued by the almost precisely equal allocation of male and female births, but later decided “that the whole problem is so intricate that it is safer to leave its solution for the future” (Darwin, 1874).

The explanation for the almost equal sex ratio pertains to the theory of games, in that were male births less common than female, a male would have better mating prospects and would sire more offspring. Thus, parents genetically disposed to produce males would have more offspring and this tendency would spread within the community, increasing male births, such that this advantage disappears when an M/F of 0.5 is reached. The converse would apply were there a dearth of females. This is known as Fisher's principle (Brian and Jaisson, 2007).

Stress: Hormonal Theory of Termination (HTT)

Since the human biological sex does not alter post-conceptually, M/T depends on two factors: the primary sex ratio and its alteration by sex-selective embryonic and/or fetal death (miscarriage and/or stillbirth). Stress decreases M/T and this was strikingly demonstrated following the 9/11/2001 attacks on the United States (US) by Ralph Catalano and colleagues who showed that the terrorist attacks were associated with a decrease in M/T 4 months later (Catalano et al., 2006). The phenomenon was shown to be due to an excess of male fetal loss in pregnancy (Bruckner et al., 2010). The same group has shown other analogous situations that reduced M/T, and these include mass layoffs and the Great East Japan Earthquake in 2011 (as reviewed in James and Grech, 2017).

Ambient temperature is a simple stressor to test and it has been shown that a 1°C increase in annual temperature (as a consequence of a milder winter) predicts one more male than expected for every thousand females born in a given year. Furthermore, males from cold-stressed cohorts who have experienced cold weather in utero culling have, on average, longer life expectancies. This has been calculated as an average decrease in male life-span by 14 days per 1°C increase from one year to the next among those who survived to one year of age (Catalano et al., 2008).

Others have identified a wide spectrum of human stressors associated with subsequent low M/T and these include the sovereignty referendums in Canada, parliamentary elections in Malta, the 2007 recession in the U.S., and maternal occupational stress.

Even relatively mundane events may transiently lower M/T, and these included the death of Princess Diana in 1997 in the UK. Lastly, pregnant Muslim women who observe the fast of Ramadan in early pregnancy have a low M/T (as reviewed in [James and Grech, 2017](#)). A common factor is that with all such acute events, the dip witnessed is transient, occurring approximately 4–5 months after the exogenous stressor ([Catalano et al., 2006](#)).

Such dips are also very sharp and dramatic. To put them in context, the perinatal mortality rate (number of stillbirths and deaths in the first week of life/1000 live births) is universally considered a major marker in the assessment of the quality of health care delivery. This is expected to be under 7/1000 for developed countries. The transient M/T dips observed match and sometimes even double or triple this value, making this not only an important Public Health issue but also a potential and inexpensive metric to objectively assess the effects of population stressors ([Grech, 2015d](#)).

The proximate cause for these effects is the Hormonal Theory of Termination (HTT) by William H. James which can be summarized by the proposal that M/T is positively correlated with R , where R is a function of the form

$$R = \frac{(T + E)}{(G + P)}$$

wherein T , E , G , and P are sex-standardized levels of testosterone, estrogen, gonadotrophins and progesterone of both parents at conception ([James, 2004](#)). Indeed, a major cause of miscarriage is known to be a high level of stress-induced maternal adrenal androgens ([James, 2015](#)). Indirect support for HTT comes from the Baron-Cohen hypothesis which proposes autism as a form of extreme maleness which may be induced by high maternal androgen levels in a condition that is far commoner in males than in females ([Baron-Cohen, 2002](#); [James, 2012](#)).

Relevance to Trivers-Willard Hypothesis (TWH)

The HTT is further strengthened by the fact that all such stress-related variation would be in conformity with the Trivers-Willard hypothesis (TWH). This maintains that evolution should have favored parents with the ability to influence M/F according to conditions surrounding conception and during pregnancy. This is because in polygynous species, a robust son who is conceived under favorable environmental conditions has greater reproductive opportunities than an equivalent daughter who is constrained by pregnancy and lactation. Contrariwise, under adverse conditions, a male fetus (which requires greater resources in order to be carried to term) will be less likely to survive pregnancy. If he does, such a frail male may not survive to reproductive age, and if so, would compete poorly with more robust males for mating privileges. However, a frail female is likelier to survive and reproduce. Hence, under unfavorable conditions, the parental passage of genes is favored if fewer males are produced through the culling of weaker males ([Trivers and Willard, 1973](#)).

Thus, HTT with maternal-stress-induced spontaneous abortion of small, frail male fetuses is a proximate cause, serving the ultimate TWH cause dictated by evolution.

Socioeconomic Status

Many studies have shown that different races have different M/T. This may be due to innate physiological differences or to other factors, which may be environmental or iatrogenic. In the US, M/T was Asian or Pacific Islander > White > American Indian or Alaska Native > Black or African American. M/T declines in association with surrogates of socioeconomic status, presumably due to higher stress levels. The lower baseline M/T of Indian or Alaska Native and Black and African American is equivalent to a constant loss of 3.5–4/1000 male births when compared to White M/T. Race is the most significant variable associated with wealth inequality in this country and may be partially responsible ([Grech, 2017a](#)). The high Asian M/T will be discussed later.

Radiation Elevates M/T

Radiation is the only known toxin which elevates M/T by destroying female in excess of male fetuses, for reasons unknown. This has been shown to occur in proximity to nuclear reactors ([Scherb and Voigt, 2007](#)), and after the meltdown of the Windscale (Sellafield) and Chernobyl reactors in 1957 and 1986 respectively ([Grech, 2014a,b](#)) and in association with the nuclear bomb tests leading up to the Partial Nuclear Test Ban Treaty in 1963 ([Grech, 2015a](#)). Indeed, it has been shown M/T increases 0.0036 (over the approximately 0.515 baseline value) when the normal average background radiation dose rate of 0.1141 $\mu\text{Sv/h}$ is doubled to 0.2282 $\mu\text{Sv/h}$ ([Scherb and Voigt, 2007](#)).

Time of Conception Within the Menstrual Cycle (TWC)

TWC states that the regression of sex ratio (M/T: proportion male) on time of conception within the human menstrual cycle is U-shaped. This is relevant as increased coital activity increases the likelihood of the odds of conception early in the menstrual cycle, thereby skewing the odds toward males, elevating M/T. Statistically significant evidence for TWC comes from a meta-analysis of

births (James, 2008). Indirect evidence also abounds, including the finding that during the Great Wars, M/T rose in belligerent countries. In times of war, an adult sex ratio imbalance prevails, with more males being away from their homes, and soldiers were granted short periods of leave in order to return home. This has been claimed to result in sexual excesses, “actions [that] were viewed as understandable responses to the Frauenuberschuss,” the excess supply of women (Moeller, 1993). Such increased coital activity may have caused the M/T elevations.

This would also explain other reports of high M/T 9 months after occasions of public elation such as the reported high M/T in South Africa 9 months after the 2010 World Cup there and the reported high M/T in the UK after the birth of Prince William in 1982. High coital rates might also explain the reported high fertility and M/T in Hong Kong in the auspicious Dragon years of 1976, 1988, and 2000. Lastly, the powerful decline in coital rates with duration of marriage would persuasively explain the decline in M/T of first births with the duration of time between marriage and birth (James and Grech, 2017).

Temporal and Geographical M/T Variation

M/T may exhibit seasonal variation, typically peaking as summer approaches. This was clearly demonstrated for example in the US with a significant peak in June that exhibited a degree of racial variation (Grech and Borg, 2015).

An overall decreasing trend in M/T is present in Europe and North America. This is offset by a rise in M/T in Asia that will be discussed later (Grech, 2017).

A latitude gradient in M/T is evident, with more males being born in southern, warmer latitudes in Europe. The converse occurs in North America, with more males born in northern latitudes (Grech, 2017).

Gendercide and Faminelect

Gendercide and faminelect (the deliberate infliction of lower standards of care of female neonates, infants and children, simply because of their gender) is rampant, especially in Asia, and has resulted in stark demographic sex imbalances in many countries. This paradigm is impelled by social, cultural, political, and economic factors which are predominantly caused by the tendency for patrilineal inheritance in patriarchal societies, coupled with a reliance on male children to provide economic support indefinitely. For this reason, due to the patriarchal nature of most societies, male offspring preference is far commoner than female preference.

In Asia, this is reinforced by the Confucian tradition that is characterized by strong son preference and female subordination. Gender-selective abortion favors males and this is the likeliest explanation for the high M/F noted in Asian or Pacific Islander births in the US (Grech, 2017a).

This is further reinforced by a US birth order analysis by race which showed that M/T decreased with increasing sibling order for all races except for Asian or Pacific Islander births, where M/T rose progressively to 3rd order births then fell. The implication is that group may be systematically implementing gendercide. Thus, the potential effects of increasing maternal age and/or decreasing coital rates with time to decrease M/T are not only excluded in this race, but go contrary to the findings of this particular study insofar as Asian or Pacific Islander births also had the oldest mean maternal ages for all birth orders (Grech, 2017b).

While the lower baseline M/T of Indian or Alaska Native and Black and African American is equivalent to a constant reduction of 3.5–4/1000 male births when compared to White M/T, the Asian or Pacific Islander strategy is equivalent to a constant loss of 3.5 females fetuses per 1000 live births when compared to Whites (Grech, 2017b).

The likelihood that gendercide is routinely used in these cultures is reinforced by the observation that in Asian countries where education levels rise, M/T drops to values that closely approach those of other races. This was famously observed in South Korea where M/T had peaked at 0.538 in 1990, but dropped to 0.513 by 2013 due to education campaigns, the first Asian country to reverse this trend (Chung and Gupta, 2007).

It is estimated that the number of missing females has reached 126 million in 2010, and this is expected to peak at 150 million in 2035. The annual number of newly missing females is expected to exceed 3 million every year until 2050 (Bongaarts and Guilmoto, 2015).

Faminelect also kills. This may commence antenatally and continue antenatally. For example, in some cultures, when it is known that the fetus is female, failure to attend antenatal clinics and take the tetanus vaccine leads to excess female neonatal mortality. In these cultures, female children may have poorer vaccine uptake and poorer hospital attendances for acute illnesses, with increased morbidity and mortality. Indeed in South and East Asia, there is higher female under-5 mortality compared to males, in stark contrast to the rest of the world (Grech, 2015c).

Women in such countries may experience tremendous social pressure to produce sons, becoming repeatedly pregnant until they do so, since failure may incur violence, rejection or even death (Grech, 2015c).

Globally

A United Nations dataset divided regions into more developed, less developed countries, and least developed countries (as defined by the UN General Assembly). A temporal M/T review showed that in more developed countries, M/T was initially stable at 0.53 up

to 1979, then fell to 0.525. The reason/s for this decline are uncertain but may be due to the trend for delayed pregnancies in educated women with increasing maternal age and possibly decreased coital rates, as intimated above.

In less developed countries (and this group includes Asia), M/T was initially stable at 0.53, then rose after 1984 (presumably after the introduction of ultrasonography and access to this as well as other advanced reproductive techniques as an antenatal sex determining strategy) to 0.545 with a rise in male births and a fall in female births. This further confirms widespread femicide.

The least developed countries exhibited a stable and lowest M/T of 0.52 and this may be due to chronic stress (Grech, 2015b).

Conclusion

While acute events may transiently decrease M/T when related to stress, or chronically decrease M/T in the presence of chronic stress, M/T may also occasionally witness a sharp rise in relation to events that lead to elation and presumably to increased coital activity.

However, the effects of all these pale into insignificance when compared to two man-made effects: radiation exposure, and gendercide and femineglect. These influences have drastically increased M/T, wreaking demographic havoc. The latter influences have most taken effect in Asia, resulting in extensive demographic masculinisation. The large number of missing women has led to speculation that these regions will experience increased levels of antisocial behavior and outright violence. Such individuals are likely to be drawn toward military organizations, potentially threatening global security, particularly since this imbalance is occurring in a global political hotspot (Grech, 2015c).

There are few positive outcomes in these scenarios and these are a reduction in the number of unwanted children and a self-limiting situation since any gender imbalance will lead to a reduction in birth rates. In addition, as the number of girls decreases, their social status should increase due to their rarity, leading to a decline in son preference. Furthermore if, due to scarcity, a significant proportion of men marry older women, there should be fewer single, lonely individuals in old age since women have a greater life expectancy than men. And finally, education may simply eventually obliterate offspring sex bias (Grech, 2015c).

Acknowledgments

WHJ is indebted to Professor Sir David Spiegelhalter FRS (Laboratory of Statistics, University of Cambridge) and to Professor Robert Trivers (Rutgers University) for their generous assessments of his hypothesizing on sex ratio at birth. He is also grateful to Professor Ralph Catalano (Department of Public Health, University of California at Berkeley) and to Professor Andrew Pomiankowski (University College London) for encouragement and advice.

References

- Baron-Cohen, S. (2002). The extreme male brain theory of autism. *Trends in cognitive sciences*, 6(6), 248–254.
- Bongaarts, J., & Guilmo, C. Z. (2015). How many more missing women? Excess female mortality and prenatal sex selection, 1970–2050. *Population and Development Review*, 41(2), 241–269.
- Brian, E., & Jaisson, M. (2007). *The descent of human sex ratio at birth: A dialogue between mathematics, biology and sociology*. Dordrecht: Springer.
- Bruckner, T. A., Catalano, R., & Ahern, J. (2010). Male fetal loss in the U.S. following the terrorist attacks of September 11, 2001. *BMC Public Health*, 10, 273. <https://doi.org/10.1186/1471-2458-10-273>.
- Catalano, R., Bruckner, T., Marks, A. R., & Eskenazi, B. (2006). Exogenous shocks to the human sex ratio: The case of September 11, 2001 in New York City. *Human Reproduction*, 21(12), 3127–3131.
- Catalano, R., Bruckner, T., & Smith, K. R. (2008). Ambient temperature predicts sex ratios and male longevity. *Proceedings of the National Academy of Sciences of the United States of America*, 105(6), 2244–2247.
- Chung, W., & Gupta, M. D. (2007). The decline of son preference in South Korea: The roles of development and public policy. *Population and Development Review*, 33(4), 757–783.
- Darwin, C. (1874). *The descent of man and selection in relation to sex*. London: J. Murray.
- Grech, V. (2014a). Births and male: Female birth ratio in Scandinavia and the United Kingdom after the Windscale fire of October 1957. *The International Journal of Risk & Safety in Medicine*, 26(1), 45–53.
- Grech, V. (2014b). The Chernobyl accident, the male to female ratio at birth and birth rates. *Acta Medica (Hradec Kralove)/Universitas Carolina, Facultas Medica Hradec Kralove*, 57(2), 62–67.
- Grech, V. (2015a). Atomic bomb testing and its effects on global male to female ratios at birth. *International Journal of Risk and Safety in Medicine*, 27(1), 35–44.
- Grech, V. (2015b). Evidence of economic deprivation and female foeticide in a United Nations global births by gender data set. *Early Human Development*, 91(12), 855–858.
- Grech, V. (2015c). Gendercide and femineglect. *Early Human Development*, 91(12), 851–854.
- Grech, V. (2015d). Terrorist attacks and the male-to-female ratio at birth: The Troubles in Northern Ireland, the Rodney King riots, and the Breivik and Sandy Hook shootings. *Early Human Development*, 91(12), 837–840.
- Grech, V. (2017a). Evidence of socio-economic stress and female foeticide in racial disparities in the gender ratio at birth in the United States (1995–2014). *Early human development*, 106–107, 63–65.
- Grech, V. (2017b). Further evidence of male offspring preference for certain subgroups in the United States (2007–2015). *Early Human Development*, 110, 9–12.
- Grech, V. (2017). Latitude gradients and secular trends in sex ratios at birth: Europe and North America and a global overview. *West Indian Medical Journal*. <https://doi.org/10.7727/wimj.2017.093>.
- Grech, V., & Borg, T. (2015). Seasonal variation by race in the male to female ratio at birth in the United States. *West Indian Medical Journal*. <https://doi.org/10.7727/wimj.2015.279>.
- James, W. H. (2004). Further evidence that mammalian sex ratios at birth are partially controlled by parental hormone levels around the time of conception. *Human Reproduction*, 19(6), 1250–1256.

- James, W. H. (2008). The variations of human sex ratio at birth with time of conception within the cycle, coital rate around the time of conception, duration of time taken to achieve conception, and duration of gestation: A synthesis. *Journal of Theoretical Biology*, 255(2), 199–204.
- James, W. H. (2012). A potential explanation of some established major risk factors for autism. *Developmental Medicine & Child Neurology*, 54(4), 301–305.
- James, W. H. (2015). Proximate causes of the variation of the human sex ratio at birth. *Early Human Development*, 91(12), 795–799.
- James, W. H., & Grech, V. (2017). A review of the established and suspected causes of variations in human sex ratio at birth. *Early Human Development*, 109, 50–56.
- Moeller, R. G. (1993). *Protecting motherhood: Women and the family in the politics of postwar West Germany*. Berkeley: University of California Press.
- Scherb, H., & Voigt, K. (2007). Trends in the human sex odds at birth in Europe and the Chernobyl Nuclear Power Plant accident. *Reproductive Toxicology (Elmsford, N.Y.)*, 23(4), 593–599.
- Trivers, R. L., & Willard, D. E. (1973). Natural selection of parental ability to vary the sex ratio of offspring. *Science (New York, N.Y.)*, 179(4068), 90–92.

Sexual Attractants

Barnaby JW Dixon, The University of Queensland, Brisbane, QLD, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

Sexual selection occurs when individuals compete to fertilize the gametes of the opposite sex and operates at precopulatory, copulatory, and postcopulatory levels (Kokko et al., 2003). At the precopulatory level, mate choice between members of the opposite sex (i.e., intersexual selection) and same-sex competition (i.e., intrasexual selection) are the two principle mechanisms of sexual selection. Intersexual selection can shape the evolution of striking ornaments that enhance sexual attractiveness, while intrasexual selection can result in greater access to mates via resource acquisition or directly from winning fights (Kokko et al., 2003). This entry takes a comparative approach and discusses how these mechanisms have imposed on the evolution of visually conspicuous secondary sexual traits in humans.

Evolutionary research into human mate choice proposes that preferences were shaped during human ancestry to priorities morphology reflecting health, competitive ability and parental investment (reviewed in Grammer et al., 2003). A study among 37 diverse cultures revealed that women preferred a partner older than themselves with high earning potential, whereas men stated stronger preferences for youthfulness and physical attractiveness (reviewed in Grammer et al., 2003). Similar findings were reported among the Hadza hunter-gathers of Tanzania, where having a good character, foraging ability and physical attractiveness were the most nominated traits in women and men's ideal partners (reviewed in Dixon, 2012). Sex differences in body composition, skeletal structure, and stature are among several potential targets of intersexual and intrasexual selection during the course of human evolution. The purpose of this brief review is to highlight how sexual selection may have acted on some of these characters in women and men.

Sexual Attractants in Women

Unlike the nonhuman primates, where body mass is markedly sexually dimorphic, in humans sex differences in body composition are more pronounced than overall body size (Dixon, 2012). Women have more body fat than men, which is deposited around the hips, buttocks, and thighs (Singh et al., 2010). Body composition and shape can be measured using the waist-to-hip ratio (WHR), which takes the distance around the waist and divides it by the distance around the hips and buttocks. Premenopausal women from western populations typically have lower WHRs than men ranging from 0.67 to 0.8, while men's WHRs range from 0.85 to 0.95 (Singh et al., 2010). In women, a WHR of around 0.7 and a more "hourglass" body shape is associated with levels of body fat required for the onset of menarche, maintaining regular ovulatory cycles, and hormones associated with fertility (Singh et al., 2010). Fat in the thighs and buttocks is mobilized during pregnancy to support fetal growth, particularly during the third trimester for neural development (Singh et al., 2010). WHRs also become less hourglass with successive pregnancies and as women approach menopause (Singh et al., 2010).

Men's preferences for low WHRs in women may reflect sexual selection for mates who are younger with potentially higher fertility (reviewed in Brooks et al., 2015). Initial research, which was limited to western populations, supported the hypothesis that a low WHR of 0.7 was most attractive to men (reviewed in Brooks et al., 2015). However, subsequent cross-cultural research among small-scale subsistence societies revealed men preferred more masculine WHRs of 0.8–0.9 in women (reviewed in Brooks et al., 2015). Part of this variation was due to not accounting for body mass index (BMI) in the stimulus employed in many of these studies, as men from small-scale societies tended to prefer more corpulent physiques in women (Brooks et al., 2015). Subsequent cross-cultural research that controlled for BMI reported that men prefer low WHRs across a diverse range of traditional small-scale and industrialized societies (Singh et al., 2010). In the context of long-term reproductive processes, a healthy body fat distribution and body weight in women may provide men with cues of youth and fertility that guides their mate choice decisions.

In female nonhuman primates, breast enlargement only occurs during pregnancy and lactation due to milk production (Dixon, 2012). In women, breast development begins during adolescence and continues into young adulthood due to the deposition of stromal and adipose tissue around the mammary glands (reviewed in Dixon et al., 2015). Breast size is unrelated to the ability to produce or store milk. Instead, women with larger breasts have higher levels of estradiol and hence higher potential fertility (Jasienska et al., 2004) and breast fat provides energy reserves that supports lactation (reviewed in Dixon et al., 2015). Like body shape, breast morphology changes following pregnancies and over the lifespan, potentially providing a visual cue of women's sexual maturity, fertility, and maternal investment (reviewed in Dixon et al., 2015). Indeed, men rate images of larger breasts as looking sexually mature, reproductively healthy, and more nurturing as potential mothers (Dixon et al., 2015). However, men's preferences for breast size vary cross-culturally, so that among remote communities in the Highlands of Papua New Guinea, Samoa, Cameroon, and Namibia, men tend to prefer larger breasts than men from Western industrialized cultures like New Zealand and the Czech Republic (Dixon et al., 2011; Havlíček et al., 2017). While the cause for these cross-cultural differences are unknown, they may reflect preferences for greater fat reserves under conditions of nutritional scarcity.

Hormones associated with fertility also influence women's facial attractiveness to men. Female-typical "feminine" facial traits, such as large eyes, a round forehead and small chin are associated with higher levels of estradiol (reviewed in Grammer et al., 2003).

Cross-cultural research among 28 countries revealed that men prefer feminine facial features over more masculinized features (Markinkowska et al., 2014). Higher estradiol is also associated with subtle aspects of women's skin complexion that enhance attractiveness to men (reviewed in Smith et al., 2012). One study also found that facial femininity was positively correlated with estradiol and women's desire to invest as mothers (Smith et al., 2012). Taken together, hormones influence women's facial and bodily attractiveness to men in ways that may reflect sexual selection shaping traits associated with youth, fertility, and maternal investment.

Sexual Attractants in Men

Androgens underpin the expression of male-typical secondary sexual traits in men. During adolescent development, testosterone drives sexual dimorphism in height, musculature, facial shape (i.e., a defined jawline, deeply set eyes, a pronounced brow ridge and a robust midface, beardedness) and body hair. Owing to their reliance on androgens as men enter reproductive maturity, when competition to attract mates is pronounced, male secondary sexual characters are suggested to have been shaped by sexual selection via intersexual and intrasexual selection (Puts, 2010; Dixon, 2012).

In comparison to nonhuman primates, sexual size dimorphism in humans is not pronounced and men are only slightly larger than women (Dixon, 2012). However, muscularity is markedly sexually dimorphic, with men having 61% more muscle than women (Puts, 2010). Men's musculature is distributed around the shoulders and upper back, giving the body an overall v-shaped composition (Dixon et al., 2014). Muscularity is associated with physical strength, aggressiveness and competitive ability, health and disease resistance (Puts, 2010). Women judge a more muscular physique in men as most sexually attractive across cultures (Dixon, 2012) and muscularity is associated with men's mating success (Puts, 2010), suggesting that mate preferences for muscularity are associated with actual mate choices. Likewise, men are typically taller than women and male height is positively associated with men's reproductive success and formidability in male-male competition (Stulp and Barrett, 2016). One study employing multivariate approaches to measuring women's preferences for men's physique reported that muscularity accounted for 80% of the variation in attractiveness judgments and height explained a further 10% (Mautz et al., 2013). Thus, muscularity and height appear to play a strong role in both intersexual mate choice and intrasex competition.

Male primates express a wide diversity of visually conspicuous ornaments, badges of status and weaponry. Some of the most iconic examples include fleshy traits like "cheek flanges" in the male orangutan (*Pongo* spp.), the enlarged nose in male proboscis monkeys (*Nasalis larvatus*), and the large size of the upper-lip in male golden snub-nosed monkeys (*Rhinopithecus roxellana*). Other examples include colorful characters like the red triangle on the chests of male geladas (*Theropithecus gelada*) and the violet fattened rump of alpha male mandrills (*Mandrillus sphinx*). Finally, male primates have conspicuous hairy appendages like the silvery capes of hair that cover the bodies of male hamadryas baboons (*Papio hamadryas*) and facial hair in orangu-tans (*Pongo pygmaeus*; Fig. 1). In the absence of a preserved fossil record of cutaneous characters among the direct ancestors to modern humans, comparative research across the anthropoids offers insights into the selective pressures shaping sexually dimorphic traits (Dixon, 2012).

If these ornaments were sexually selected by female choice, male-male competition, or a combination of the two mechanisms, one would predict that their expression would vary due to the prevailing mating systems (Dixon, 2012). Quantitative research including all male ornamentation, which included all hairy, fleshy, and colorful adornments, reported that primates with polygynous mating systems scored the highest for secondary sexual traits, followed by species with monogamous mating systems, with species with multimale multifemale mating systems scoring the lowest (Dixon, 2012). In these analyses, men were included first as a monogamous species and again as a polygynous species. Both those analyses revealed that polygynous primates scored highest for ornamentation (Dixon, 2012). A follow-up study reported a positive phylogenetic correlation across 154 primate species (including men) between ornamental dimorphism and social group size (Grueter et al., 2015). Ornamental dimorphism also differed according to social organizations, being greatest in males from species with multilevel social systems compared to species with less complex social systems (Grueter et al., 2015). As humans live in large social groups within complex multilevel social systems, men's degree of ornamental sexual dimorphism was similar to males from species of nonhuman primates with large social groups and multilevel social systems.

A large body of research has tested whether intersexual or intrasexual selection is the mechanism that best explains the persistence of men's secondary sexual traits (Dixon, 2012). Results of experiments testing whether masculine facial shape, beardedness, and body hair augment male attractiveness to women are mixed. In some cases, women's preferences are stronger for less masculine than more masculine looking men, while in others preferences are equivocal (reviewed in Dixon et al., 2017). In a similar vein, the majority of studies into women's preferences for male chest and trunk hair have revealed that hairless stimuli are judged as more attractive than hirsute stimuli (Dixon and Rantala, 2016). Studies quantifying women's preferences for men's beards are also mixed, so that beards are rated most attractive in some studies while clean-shaven faces were most attractive in others (reviewed in Dixon et al., 2017). Thus, masculine facial shape, beardedness, and body hair do not consistently enhance men's attractiveness to women.

Rather than augmenting attractiveness, men's secondary sexual traits may play a stronger role in intrasexual communication of dominance and social rank. Indeed, ornamentation in male primates may serve as badges of status and dominance rank that function during male-male agonistic interactions (Dixon, 2012). For instance, in male western gorillas (*Gorilla gorilla*) muscularity, body size, gray body hair and cranial adipose crests are incorporated into male-male displays (Dixon, 2012). Adipose crest size in males also positively predicts the number of females in one-male units and offspring survival (reviewed in Grueter et al., 2015). In geladas, the redness of the skin patch on the chest is brighter among males with larger harems, whereas bachelor and

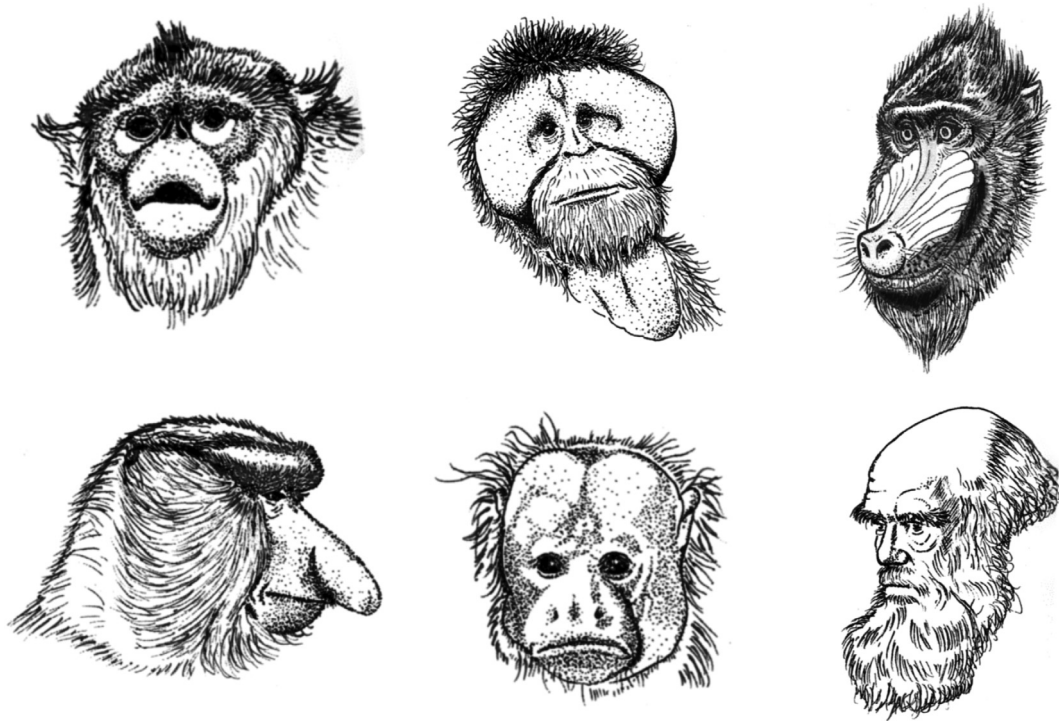


Fig. 1 Examples of visually conspicuous secondary sexual traits in male anthropoids. Portraits in the upper row from left to right show Golden snub-nosed monkey (*Rhinopithecus roxellana*); Bornean orangutan (*Pongo pygmaeus*); Mandrill (*Mandrillus sphinx*). Portraits on the bottom row from left to right show Proboscis monkey (*Nasalis larvatus*); Red uakari (*Cacajao calvus*); Man (*Homo sapiens*). The drawings are used with permission from Alan F. Dixson.

secondary follower males have less pronounced coloration in their skin patches (Dixson, 2012). Red facial coloration in mandrills is most pronounced in dominant males and higher social rank (Dixson, 2015). However, mandrills also have strikingly large canines, which are employed during fights with other males often leading to severe injuries (Fig. 2; Dixson, 2015). Given that male–male competition occurs in almost all species of nonhuman primates and fights are costly, the rapid transmission of rank and status between males could serve to curtail the probability of an agonistic encounter leading to a costly fight.

This process may explain how sexual selection has shaped men's masculine secondary sexual traits. Thus, facial masculinity is associated with upper body strength, muscularity and fighting ability in men (Puts, 2010) and plays a strong role in intrasexual

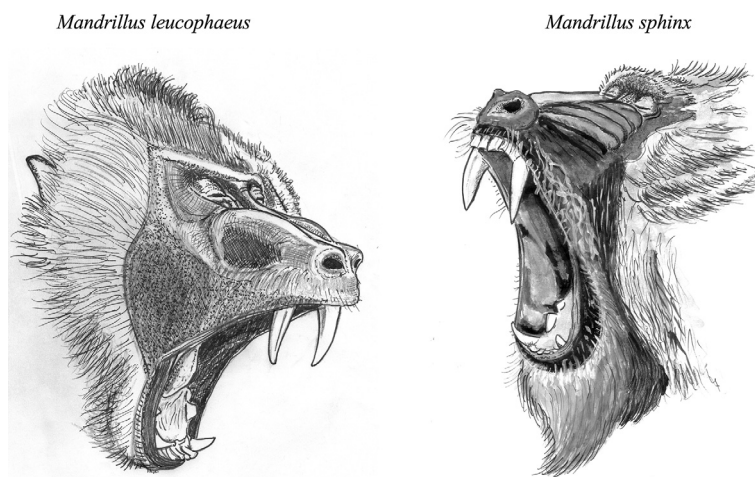


Fig. 2 The portrait on the left is a male drill (*Mandrillus leucophaeus*) yawning and showing the greatly enlarged upper canines along with several secondary sexual traits including black facial skin and the enlarged paranasal swellings. The portrait on the right is a male mandrill (*Mandrillus sphinx*), also yawning and showing enlarged canines as in the drill. Note the skin covering the paranasal swellings consists of a series of bright blue ridges, whereas the central nasal strip and tip of the nose is red and the beard is yellow. These drawings are used with permission from Alan F. Dixson.

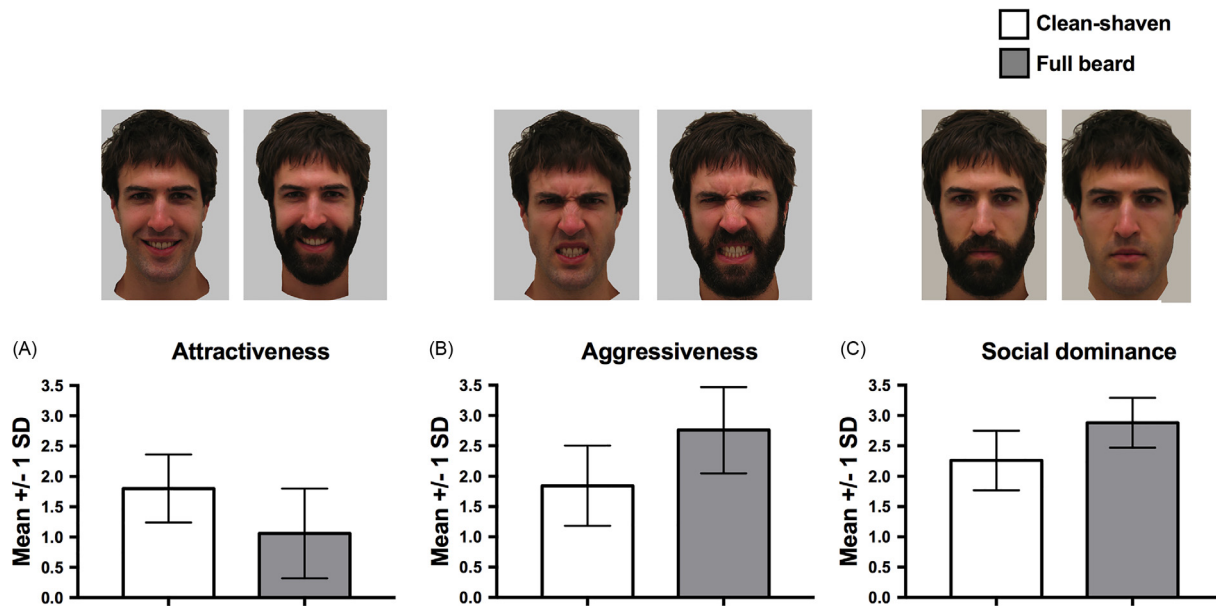


Fig. 3 Ratings of sexual attractiveness by 129 women for clean-shaven and bearded faces posing smiles (A); ratings of aggressiveness by 111 men for clean-shaven and bearded faces posing angry expressions (B); ratings of social dominance by 52 men for clean-shaven and bearded faces posing neutral expressions (C). Data are reproduced from Dixon B. J. and Vasey, P. L. (2012). Beards augment perceptions of men's aggressiveness, dominance and age, but not attractiveness. *Behavioral Ecology*, **23**, 481–490, and the stimuli are owned by Barnaby Dixon.

signaling of dominance and threat (Puts, 2010). Beards also consistently augment ratings of dominance in faces with neutral expressions and angry facial expressions (Dixon and Vasey, 2012; Fig. 3). While facial hair may not directly improve fighting ability, beards likely perform a role in enhancing threatening displays by amplifying masculine facial traits, such as the apparent size of the jaw, the overall length of the face and the effectiveness of threatening facial displays. Fig. 3 shows results of a study among men and women from New Zealand in which men judged angry faces with full beards as more aggressive than when clean-shaven. The same study also showed that men judged bearded faces as looking more dominant than clean-shaven faces, while women rated clean-shaven faces as most attractive (Dixon and Vasey, 2012; Fig. 3). This suggests that like many species of nonhuman primates, masculine facial traits play a role in communicating intrasexual threat.

Evolutionary biologists have long appreciated that the strength and direction of sexual selection by female choice varies with ecological factors (Kokko et al., 2003). Under ecological conditions wherein male–male competition can result in tangible direct benefits, such as protection and resources, females may have higher preferences for males displaying cues of intrasexual formidability (Wong and Candolin, 2005). Interestingly, women's preferences for facial masculinity are stronger in countries with lower healthcare, higher pathogen loads, greater income inequality and higher urban development (reviewed in Dixon et al., 2017). With regards facial hair, full beards are more common under economic conditions where disparity in incomes are greater, in cities with the largest populations, and in countries where women report the strongest preferences for beards (Dixon et al., 2017). These studies suggest that masculine traits and possibly men's decisions to remove or embellish a highly masculine signal through grooming vary due to social, ecological, and economic conditions in ways that are predicted by evolutionary theory.

Conclusion

Studies in human mate choice that employ evolutionary theories to uncover the targets of sexual selection have grown considerably in the past 25 years. Women's body fat distribution and secondary sexual traits may reflect youth, health, and fertility that explain their attractiveness to men. Men's secondary sexual traits appear to play a stronger role in enhancing male dominance to other males rather than aesthetical sexual attractiveness. Women's preferences for masculine traits appear to be stronger under environmental and economic conditions where survival is compromised, resources scarcer and their reproductive success may be enhanced by selecting a partner who can provide tangible benefits that aid their survival and that of their offspring.

Acknowledgments

This work was funded by a University of Queensland Post-Doctoral research fellowship to BJWD. I am also very grateful to Alan Dixon for the use of his drawings.

References

- Brooks, R. C., Jordan, A. L., Shelly, J., & Dixon, B. J. (2015). The multivariate evolution of female body shape in an artificial digital ecosystem. *Evolution and Human Behavior*, 36, 351–358.
- Dixon, A. F. (2012). *Primate sexuality: Comparative studies of the prosimians, monkeys, apes, and humans*. Oxford: Oxford University Press.
- Dixon, A. F. (2015). *The mandrill: A case of extreme sexual selection*. Cambridge: Cambridge University Press.
- Dixon, B. J., Duncan, M., & Dixon, A. F. (2015). The role of breast size and areolar pigmentation in perceptions of women's sexual attractiveness, reproductive health, sexual maturity, maternal nurturing abilities, and age. *Archives of Sexual Behavior*, 44, 1685–1695.
- Dixon, B. J., Grimshaw, G. M., Ormsby, D. K., & Dixon, A. F. (2014). Eye-tracking women's preferences for men's somatotypes. *Evolution and Human Behavior*, 35(2), 73–79.
- Dixon, B. J., & Rantala, M. J. (2016). The role of facial and body hair distribution in women's judgments of men's sexual attractiveness. *Archives of Sexual Behavior*, 45, 877–889.
- Dixon, B. J., Rantala, M. J., Melo, E. F., & Brooks, R. C. (2017). Beards and the big city: Displays of masculinity may be amplified under crowded conditions. *Evolution and Human Behavior*, 38, 259–264.
- Dixon, B. J., & Vasey, P. L. (2012). Beards augment perceptions of men's aggressiveness, dominance and age, but not attractiveness. *Behavioral Ecology*, 23, 481–490.
- Dixon, B. J., Vasey, P. L., Sagata, K., Sibanda, N., Linklater, W. L., & Dixon, A. F. (2011). Men's preferences for women's breast morphology in New Zealand, Samoa, and Papua New Guinea. *Archives of Sexual Behavior*, 40, 1271–1279.
- Grammer, K., Fink, B., Möller, A. P., & Thornhill, R. (2003). Darwinian aesthetics: Sexual selection and the biology of beauty. *Biological Reviews*, 78, 385–407.
- Grueter, C. C., Isler, K., & Dixon, B. J. (2015). Are badges of status adaptive in large complex primate groups? *Evolution and Human Behavior*, 36, 398–406.
- Havlíček, J., Třebický, V., Valentová, J. V., Kleisner, K., Akoko, R. M., Fialová, J., et al. (2017). Men's preferences for women's breast size and shape in four cultures. *Evolution and Human Behavior*, 38(2), 217–226.
- Jasińska, G., Ziolkiewicz, A., Ellison, P. T., Lipson, S. F., & Thune, I. (2004). Large breasts and narrow waists indicate high reproductive potential in women. *Proceedings of the Royal Society B: Biological Sciences*, 271, 1213–1217.
- Kokko, H., Brooks, R., Jennions, M. D., & Morley, J. (2003). The evolution of mate choice and mating biases. *Proceedings of the Royal Society of London B: Biological Sciences*, 270, 653–664.
- Marcinkowska, U. M., Kozlov, M. V., Cai, H., Contreras-Garduño, J., Dixon, B. J., & Oana, G. A. (2014). Cross-cultural variation in men's preference for sexual dimorphism in women's faces. *Biology Letters*, 10(4), 20130850.
- Mautz, B. S., Wong, B. B., Peters, R. A., & Jennions, M. D. (2013). Penis size interacts with body shape and height to influence male attractiveness. *Proceedings of the National Academy of Sciences*, 110, 6925–6930.
- Puts, D. A. (2010). Beauty and the beast: Mechanisms of sexual selection in humans. *Evolution and Human Behavior*, 31, 157–175.
- Singh, D., Dixon, B. J., Jessop, T. S., Morgan, B., & Dixon, A. F. (2010). Cross-cultural consensus for waist-hip ratio and women's attractiveness. *Evolution and Human Behavior*, 31(3), 176–181.
- Smith, M. J. L., Deady, D. K., Moore, F. R., Jones, B. C., Cornwell, R. E., Stirrat, M., & Perrett, D. I. (2012). Maternal tendencies in women are associated with estrogen levels and facial femininity. *Hormones and Behavior*, 61(1), 12–16.
- Stulp, G., & Barrett, L. (2016). Evolutionary perspectives on human height variation. *Biological Reviews*, 91, 206–234.
- Wong, B. B., & Candolin, U. (2005). How is female mate choice affected by male competition? *Biological Reviews*, 80(4), 559–571.

Sexual Imprinting

Hans-Joachim Bischof, Bielefeld University, Bielefeld, Germany

© 2018 Elsevier Inc. All rights reserved.

Introduction

The phenomenon was already known for centuries, but it was [Lorenz, 1936](#) who coined the term imprinting (Prägung) based on his observations of young, artificially bred greylag geese. According to his observations, newly hatched geese immediately follow the first moving object they see (which in nature would be the mother or the siblings) and develop a strong attachment to it, even if, in case of an experiment, it is the animal caretaker who is retrieving the birds from the breeder. If the hatchlings thereafter are asked to choose between that object and another, they decide to follow that first seen object, a choice that persists as long as following behavior can be observed. Lorenz presumed that the attachment to the first object cannot be reversed after it has been established and thus named the underlying learning process “Nachlaufprägung,” named filial imprinting in the English literature. He further observed that the learned preference later in life also affected the mate choice of the birds: the sexually mature geese that were imprinted on the caretaker directed their courtship behavior to human beings instead of the mother. Lorenz called this phenomenon “sexual imprinting” because he presumed the preference for the “wrong” object would persist lifelong.

On the basis of his observations, Lorenz concluded that imprinting associates characteristics of an object with an innate behavior (in the case of sexual imprinting: the traits of the target for the innate courtship behavior). He listed four criteria that characterize imprinting: (1) Imprinting can take place only during a restricted period in the individual's life, the sensitive period; (2) it is irreversible, meaning that a learned preference cannot be forgotten; (3) only species specific, not individual characteristics are learned; (4) learning may be completed at a time where the appropriate behavior is not yet performed, for example, sexual imprinting occurs long before the birds are sexually mature.

These claims were, as usual at that time, based on anecdotal evidence and thus quite speculative. They initiated, however, intense discussion. A lot of research showed that Lorenz' intuition was generally good, but some of his claims had to be revised or even rejected. Over the last six or seven decades, many detailed studies of sexual imprinting contributed to a more complete picture of this important aspect of early development.

The Characteristics and Context of Sexual Imprinting

Early experience contributes to mate choice not only in geese, but also in many other birds, it was also shown for mammals, for several fish species, and even in invertebrates ([Verzijden et al., 2012](#)). For nonhuman primates, there is not much experimental evidence, but for humans, there is an impressive amount of literature dealing with the role of sexual imprinting for mate choice (see below). Such demonstration across the entire animal kingdom including humans already indicates that this type of learning is a universal phenomenon that plays an important role in the development of living beings.

However, the vast majority of studies on the characteristics and context of sexual imprinting have been performed in birds, and most of the avian studies used the Zebra finch (*Taeniopygia guttata castanotis*) as experimental animal which now can be seen as the standard model for sexual imprinting. [Immelmann \(1972\)](#) was the first to study sexual imprinting in detail with Zebra finches as model. He cross-fostered Zebra finches with another finch species, the Bengalese finch (*Lonchura striata*). Adult Zebra finch males, which were raised by Bengalese finches, preferred females of the foster species against those of their own species in a choice test. This indicated that the birds had learned the visual traits of their prospective mate while they were reared by the foster parents and excludes the existence of an innate template of their own species. By varying the duration of the cross fostering period, he found out that the sensitive period for sexual imprinting starts at the time of eye opening (about 6 days after hatch) and lasts up to about 30 days of age, most birds were imprinted from day 12 to day 14. He also showed that the cross-fostered males, if later housed together with a female of the own species, courted this female and even in some cases reared offspring. This result could on first sight indicate that imprinting is not as stable as Lorenz assumed. However, it turned out that the same cross-fostered males, when asked to choose, continued to prefer females of the foster species. Thus, sexual imprinting obviously established a stable preference for the foster species, but it was just a preference that determined the choice of the birds in most cases but could, depending on the actual circumstances, also be ignored (Review: [Bischof, 1994](#); [Ten Cate and Vos, 1999](#)).

By his experiments, Immelmann demonstrated that zebra finches learn the object for sexual behavior, and he presented experimental evidence supporting the first two of Lorenz' criteria: learning is restricted to a sensitive period, and the acquired preference is very stable. He also provided evidence for the notion of exclusive learning of species specific traits of that potential sexual partner (which would indicate that imprinting may be strongly restricted by genetic constraints), but later research indicated that it could not be excluded that individual traits also played a role, and imprinting was even possible on artificially added traits like colored rings or artificial crests (e.g., [Witte and Caspers, 2006](#), see also below).

These basic features were accepted by most researchers, but there was some dispute over other details. It was discussed, for example, whether both, males and females, acquire knowledge about a possible mate by early learning. For ducks, it was shown that only males learned the traits of their mates by imprinting, while females appeared to have an innate template of the appropriate

male mate. According to later experiments, however, females are also imprinted, but on traits of the father in contrast to the males that learned traits of the mother.

The statement of Lorenz that imprinting occurs at a time where the appropriate behavior is not developed was also discussed. Obviously, it is not true for filial imprinting where the following reaction is part of the process by which the bird learns the traits of the object to follow. For sexual imprinting, it appeared not to be logic to assume that young birds before reaching sexual maturity could learn about the object for their courtship behavior that was developing much later. Indeed, experiments demonstrated that the whole process of learning about a prospective sexual partner was more complicated. Young birds which were reared by Bengalese finches and then isolated until reaching sexual maturity, changed their preference to Zebra finches when the first female bird they were able to court was a Zebra finch female, while in the case of first courtship to a Bengalese finch, the preference for the Bengalese finches was preserved. The preference shown after the “first courtship” then could not be changed again. On the basis of these results, [Bischof \(1994\)](#) proposed a model which described imprinting as a two stage process: In an early acquisition period which resembled the sensitive period described by earlier work of Immelmann, the young birds acquire a searching image for a prospective sexual partner, and the “first courtship” towards an object which resembled the previously acquired searching image, after reaching adolescence was seen as a stabilization process which fixes this preference. In a later version, this model was modified in that the acquisition period could also be interpreted as some sort of filial imprinting where the Zebra finches are learning about social partners like parents, siblings, and other birds of the flock; the first courtship of these birds is then directed to members of that group, and thus the chance to imprint sexually on the own species is greatly enhanced ([Fig. 1](#)).

This interpretation, which I shall adopt here, is contradictory to Lorenz’ claim that learning of the mate’s traits occurs before sexual behavior is developed. Lorenz wanted to stress that imprinting might be a process different from associative learning because there was no obvious reinforcement; as shown above, however, later research mainly favored the associative learning idea instead of some process like exposure learning where the object traits are learned just by exposing it to the young animal. A good overview is provided by [Bolhuis \(1991\)](#).

Immelmann presumed that there was an “own species bias” because his experimental males imprinted more readily to the own species than to a foster species. This claim was quite strongly criticized because this could be seen as a genetically fixed bias, an idea which was not very popular in times of a behavioristically dominated learning research. However, such biases were found frequently also in later experiments, and with the acceptance that genetics and environmental both affect the development of brain structure and behavior, the own species bias in sexual imprinting became more and more accepted.

Most of the original experiments to define the bodily traits learned by sexual imprinting were done exclusively with visual cues. However, there is no doubt that others like acoustic or olfactory traits also contribute to the overall mental image of the sexual partner that is established by the imprinting process. Acoustic cues that have been shown to be stored in a two-stage process, also contribute to mate choice ([Bischof, 1997](#)). Likewise, recent research highlights a prominent role of olfactory cues, which are also stored in the course of a sensitive period, for individual recognition and thus also for mate choice ([Caspers et al., 2013](#)).

Neurobiological Correlates of Sexual Imprinting

[Bischof \(1983\)](#) recognized the strong similarity of the behavioral phenomena characterizing sexual imprinting with the development of binocularity of the visual system of cats and monkeys, one of the best investigated models of developmental plasticity. The two main criteria of sexual imprinting, a sensitive period and a remarkable stability of storage of the obtained information, also apply for the development of the wiring of the visual system, and there are other similarities even in details. It is probably more than pure speculation to assume that the physiological processes, which were described for the development of sensory systems, are also the basis for behavioral processes like sexual imprinting, and research on developmental brain plasticity could therefore guide physiological research on imprinting.

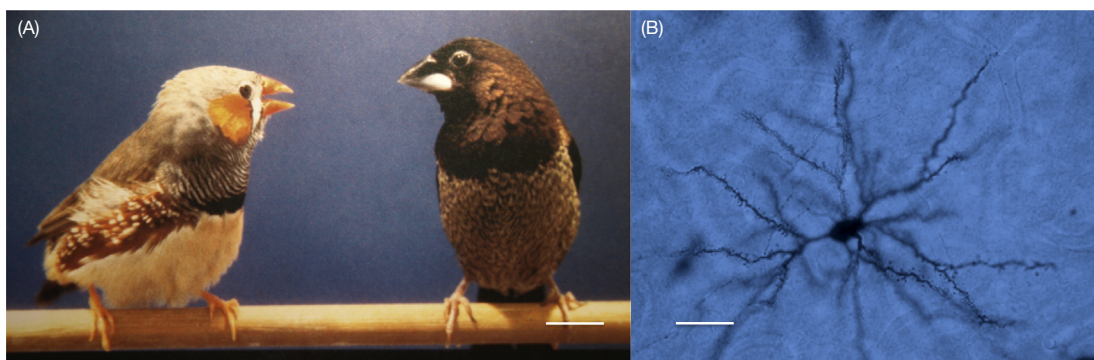


Fig. 1 (A) Male zebra finch (left) that was imprinted on a Bengalese finch and thus courting a Bengalese finch female. (B) A neuron found within one of the imprinting relevant forebrain areas. Dendritic spines are the tiny protrusions along the dendrites, which emanate from the cell body. Spine density (see [Fig. 3](#)) is measured along the length of the distal part of the dendrites.

Because the stabilization of the earlier acquired preference by the “first courtship” appeared to be the most important step in the imprinting process, Bischof and coworkers (review: Bischof, 2007) designed experiments to learn about the physiological basis of the stabilization process of sexual imprinting. By isolating the young birds from the (foster) parents after reaching independence and then exposing them to a female of the own or the foster species for a given period of time, it was possible to exactly adjust the time point at which the stabilization process occurred (see Figs. 2 and 3). Just 1 h exposure to a female, eliciting directed courtship behavior for the first time in the life of the young male, was sufficient to stabilize the previously acquired preference. Details of the physiological correlates of sexual imprinting could then be evaluated by comparing the brain status before and after confrontation with a female.

Among the brain areas that were active during the first exposure to a female, two higher order brain areas showed alterations, which strongly suggested that these areas were involved in the imprinting process. The exposure lead to a very rapid reduction of dendritic spines within the lateral nidopallium, an area which receives visual input from the avian tectofugal visual projection, and the medial nidopallium which is seen as to belong to the acoustic system and shows such reduction also in experiments on song learning. The reduction was permanent, subsequent isolation had no further effect on spine density. This irreversibility of the spine reduction indicated that it could represent the physiological basis of the also irreversible imprinting process: other areas activated by the first courtship event showed another pattern of change: the spine density increased with exposure to a female (much slower

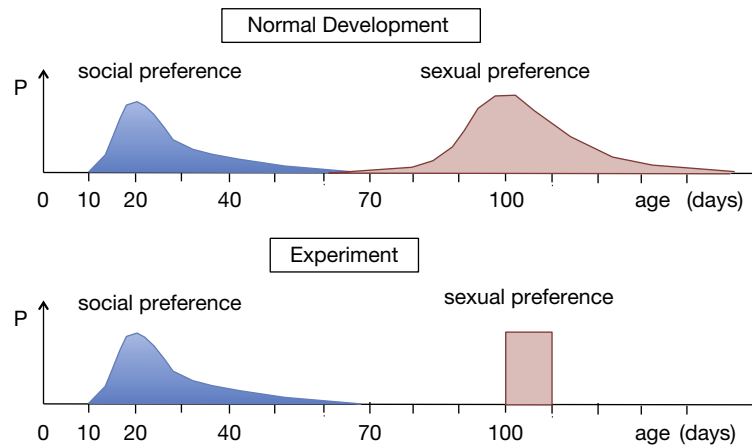


Fig. 2 Sensitive periods in the zebra finch during normal development (upper panel) and experimentally altered (lower panel). P, probability of the occurrence of imprinting (arbitrary units). Upper panel: A social preference is installed around day 20 (blue shaded area), a sexual preference around day 100 (red shaded area). Lower Panel: By isolation, sexual imprinting can be restricted to a defined period of time (see also Fig. 3).

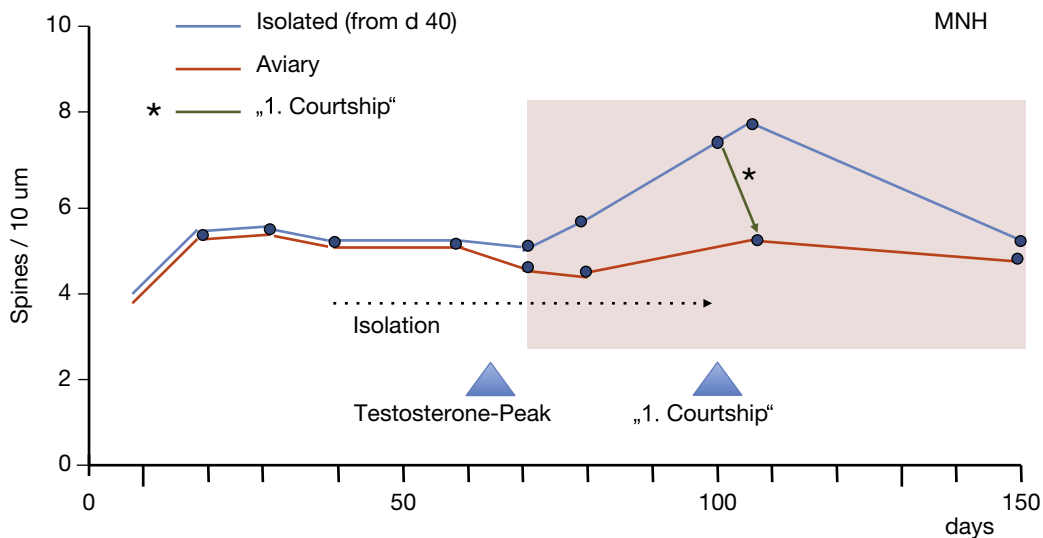


Fig. 3 Changes of dendritic spine density within an imprinting relevant brain area (“MNH”) during development. If the bird is growing up with other birds (“aviary”), the spine density, which indicates the number of connections between neurons, does not show strong variability. If the bird is isolated from day 40 to day 100, the spine density increased from day 70 on up to day 110 and then decreases until day 150 to the “aviary” level. It has been shown that during this time span sexual imprinting is possible (red square). If during this time the bird is exposed to a female, the spine density is dropping very quickly (“1. courtship”). Thereafter, no change of the installed preference is possible.

compared with the “imprinting areas”) and decreased to the value measured before the exposure when the birds were isolated thereafter. The experiment was interpreted as to show that by the stabilization process of sexual imprinting the previously redundant network within the two areas is shaped to a final less (non?) redundant state.

Other experiments demonstrated that isolation before the “first courtship” leads to an increase of spine density within the imprinting relevant areas higher than those observed in not isolated control birds. It was this excess of spines which was reduced when the birds were exposed to a female. This was interpreted as to show that imprinting under normal conditions may work in multiple steps so that the permanent generation of new spines was counteracted by an elimination of spines. It was also shown that the stabilization process of sexual imprinting is also restricted to a sensitive period, which begins at day 70 and ends at day 150. The onset of the sensitive period is characterized by a strong testosterone peak, and chemical castration prevents consolidation and spine density increase. The sensitive period may then be terminated by an increase of GABAergic inhibition. Alternatively, or in addition, it might be possible that, as has been shown for the development of the visual cortex and also for song learning, another variant of early learning which is very similar to sexual and filial imprinting, the development of the perineuronal net, a component of the extracellular matrix which serves to stabilize synaptic contacts, may have an influence on the closing of the sensitive period. Another, as yet not pursued path of investigation is the idea of epigenetic changes of the genome, which are induced under involvement of IEG’s and stress hormones. IEG expression and corticosterone enhancement are also involved in the stabilization period of imprinting, but as yet no evidence is available that would link epigenetic changes to the regulation of the sensitive period. If such changes were involved, it would be interesting to know whether these could be transferred to the next generation. If the effects of sexual imprinting were heritable, this would have immense consequences for our present view of the role of sexual imprinting for evolution.

The results on the neurobiological correlates of sexual imprinting strongly support the view presented above: there are impressive similarities between the physiological basis of brain development, for example that of binocular interaction within the visual cortex, and those of sexual imprinting. Within the visual system, experience from the external world is necessary to fine-tune a genetically prewired processing system. The same could be said from sexual imprinting and the physiological structures underlying it: a given behavioral system like that guiding mate choice may be prestructured to some degree, but it needs fine tuning by external information.

The idea that there may be a prestructured neuronal system, which leads to a predisposition, has also been confirmed in filial imprinting where research demonstrated for example perceptual biases from single traits like colors or preferences for animate objects. It has also been shown that such perceptual biases might be quite universal because they can be demonstrated in chicks as well as in humans (Di Giorgio et al., 2017). However, conceptual paths chosen in physiological research on filial imprinting were very different from those chosen by scientists working on sexual imprinting. Thus, although there is quite strong support for the notion that filial and sexual imprinting as like as song learning are very similar learning paradigms, nobody as yet has tried to reconcile the different concepts and to develop a unified theory of the physiological basis of early learning.

Evolutionary Aspects of Sexual Imprinting

Sexual selection and mate choice are of major importance for evolutionary theories since Darwin (1871). He proposed that the choosing sex (in most cases the female) is able to assess the reproductive investment and/or the reproductive potential of the candidate mate and chooses the best one on the basis of some phenotypic trait. Lots of theoretical essays thereafter refined aspects of Darwin’s ideas (Review: Jones and Ratterman, 2009), and besides the main assumption that mate choice is based on inherited preferences for certain traits, the role of sexual imprinting was also found to have strong impact on sexual selection. It can either enhance or reduce the effects of natural selection. Mathematical models, for example, suggest that learning preferably the parental traits (parental imprinting) can stabilize sexual selection on a specific phenotype, but also cause runaway sexual selection depending on the environmental conditions.

Sexual imprinting may also facilitate speciation by promotion of imprinting to species specific traits, or by the maintenance of sexual isolation between sister species when no postzygotic barrier exists, as shown in sticklebacks. Taken together, sexual imprinting may be one of the most important factors for speciation, even if one considers the many other influences on reproductive isolation. But it may not be possible at present to devise a common theory; instead one has to examine the influence of early learning on sexual selection on a case by case basis. (Review: Verzijden et al., 2012).

Sexual Imprinting in Humans

Psychologists traditionally saw human sexual preferences as to be genetically predetermined adaptations that serve to select the highest quality mate from a range of candidates. However, there are also attempts to find out whether the traits that allow judging the quality of a potential mate, may be learned instead of being inherited. In this context, sexual imprinting has been considered as the learning process that importantly affects mate choice in humans. Comparable with studies in birds, relations between parental traits and sexual or partner preferences have been shown in humans. Most of these studies demonstrated a relationship between partner preferences and traits of the opposite sex parents, similar to the findings in birds. Another important parallel between

animal and human studies was the demonstration of negative imprinting (the so-called Westermarck effect), by which an aversion instead of a preference for the imprinted trait is developed (review: Aronsson, 2011).

Aronsson also put forward the idea that the existence of sexual perversion like fetishism may be an effect of sexual imprinting on sexual preferences. She argues that the sexual preference for fetishes like underwear, socks, etc., may be comparable with sexual imprinting on artificial adornments as it has been shown in birds (e.g., Witte and Caspers, 2006). She also shows convincingly that sexual preferences in humans based on parental traits of parents can also include preferences for accessories like spectacles or behavioral traits like smoking.

There were also negative voices concerning the evidence proving sexual imprinting in humans. Rantala and Marcinkowska (2011) reviewed the current literature, criticized the concept at all and claimed that, due to inadequate methods, the effect of either positive or negative sexual imprinting on mate choice in humans is often more marginal than suggested by the original papers. Although this critique may be justified in some cases, there is enough robust evidence supporting the involvement of sexual imprinting in mate choice. That such an involvement is difficult to detect may probably be due to the fact that development and mate choice in humans is a phenomenon with multiple causes. Also, it has already been shown for Zebra finches that an acquired preference for a given trait or combination of traits does not mean that the birds are condemned to find exactly the ideal mate for courtship and reproduction; Immelmann demonstrated that a cross fostered Zebra finch male, if the preferred foster species is not available, also courts to females of the own species and eventually also raises offspring with one of these females. However, if later on given a choice between females of the foster and the own species, respectively, it will prefer the foster female and abandon the previous mate. If one assumes that human mate choice may be dependent on even more influences apart from imprinting (e.g., social, environmental, and economic ones) than that of Zebra finches, it is plausible to assume that the effects of sexual imprinting may be more strongly obscured and more difficult to prove.

References

- Aronsson, H. (2011). Sexual imprinting and fetishism: An evolutionary hypothesis. In P. R. Adriaens, & A. De Block (Eds.), *Maladapting minds: Philosophy, psychiatry, and evolutionary theory* (pp. 65–90). Oxford: Oxford University Press.
- Bischof, H.-J. (1983). Imprinting and cortical plasticity—A comparative review. *Neuroscience and Biobehavioral Reviews*, 7(2), 213–225.
- Bischof, H.-J. (1994). Sexual imprinting as a two stage process. In J. A. B. Hogan, & J. J. Bolhuis (Eds.), *Causal Mechanisms of Behavioural Development* (pp. 82–87). Cambridge: Cambridge University Press.
- Bischof, H. J. (1997). Song learning, filial imprinting, and sexual imprinting: Three variations of a common theme? *Biomedical Research-Tokyo*, 18, 133–146.
- Bischof, H. J. (2007). Behavioral and neuronal aspects of developmental sensitive periods. *Neuroreport*, 18(5), 461–465. <https://doi.org/10.1097/WNR.0b013e328014204e>.
- Bolhuis, J. J. (1991). Mechanisms of avian imprinting: A review. *Biological reviews of the Cambridge Philosophical Society*, 66(4), 303–345.
- Caspers, B. A., Hoffman, J. I., Kohlmeier, P., Krüger, O., & Krause, E. T. (2013). Olfactory imprinting as a mechanism for nest odor recognition in zebra finches. *Animal Behaviour*, 86, 85–90.
- Darwin, C. R. (1871). *The descent of man, and selection in relation to sex*. London: John Murray.
- Di Giorgio, E., Loveland, J. L., Mayer, U., Rosa-Salva, O., Versace, E., & Vallortigara, G. (2017). Filial responses as predisposed and learned preferences: Early attachment in chicks and babies. *Behavioural Brain Research*, 325(Pt B), 90–104. <https://doi.org/10.1016/j.bbr.2016.09.018>.
- Immelmann, K. (1972). In *The influence of early experience upon the development of social behaviour in estrildid finches* Proceedings of the 15th International Ornithological Congress The Hague 1970 (pp. 316–338). Leiden: E.J.Brill.
- Jones, A. G., & Ratterman, N. L. (2009). Mate choice and sexual selection: What have we learned since Darwin? *Proceedings of the National Academy of Sciences of the United States of America*, 106(Suppl 1), 10001–10008. <https://doi.org/10.1073/pnas.0901129106>.
- Lorenz, K. (1936). Der Kumpan in der Umwelt des Vogels. *Journal für Ornithologie*, 83, 137–213, 289–413. Translation into English: Lorenz, K. (1970). *Studies in animal and human behavior* (Vol. 1). Cambridge, Mass.: Harvard University Press.
- Rantala, M. J., & Marcinkowska, U. M. (2011). The role of sexual imprinting and the Westermarck effect in mate choice in humans. *Behavioral Ecology and Sociobiology*, 65(5), 859–873. <https://doi.org/10.1007/s00265-011-1145-y>.
- Ten Cate, C., & Vos, D.R. (1999). Sexual imprinting and evolutionary processes in birds: A reassessment. In P.J.B. Slater, J. S. Rosenblatt, C. T. Snowden, & T. J. Roper (Eds.), *Advances in the study of behavior*, Vol. 28, pp. 1–31. San Diego: Elsevier Academic Press Inc.
- Verzijden, M. N., Ten Cate, C., Servedio, M. R., Kozak, G. M., Boughman, J. W., & Svensson, E. I. (2012). The impact of learning on sexual selection and speciation. *Trends in Ecology & Evolution*, 27(9), 511–519.
- Witte, K., & Caspers, B. (2006). Sexual imprinting on a novel blue ornament in zebra finches. *Behaviour*, 143, 969–991. <https://doi.org/10.1163/156853906778623626>.

Further Reading

- Aronsson, H. (2011). On sexual imprinting in humans ©Hanna Aronsson, Stockholm 2011. 978-91-7447-308-7.
- Bischof, H. J. (2003). Neural mechanisms of sexual imprinting. *Animal Biology*, 53(2), 89–112. <https://doi.org/10.1163/157075603769700313>.
- Bolhuis, J. J., & Gahr, M. (2006). Neural mechanisms of birdsong memory. *Nature Reviews: Neuroscience*, 7(5), 347–357.
- Laland, K. N. (1994). On the evolutionary consequences of sexual imprinting. *Evolution*, 48(2), 477–489. <https://doi.org/10.2307/2410106>.
- Lichtman, J. W., & Colman, H. (2000). Synapse elimination and indelible memory. *Neuron*, 25(2), 269–278.
- McCabe, B. J. (2013). Imprinting. *Wiley Interdisciplinary Reviews of Cognitive Sciences*, 4(4), 375–390. <https://doi.org/10.1002/wcs.1231>.
- Reul, J. M. (2014). Making memories of stressful events: A journey along epigenetic, gene transcription, and signaling pathways. *Frontiers in Psychiatry*, 5. <https://doi.org/10.3389/fpsy.2014.00005>.
- Skinner, M. K. (2014). Environment, epigenetics and reproduction. *Molecular and Cellular Endocrinology*, 398(1–2), 1–3. <https://doi.org/10.1016/j.mce.2014.07.017>.

FERTILIZATION

Sperm Capacitation

Brett Nixon and Elizabeth G Bromfield, The University of Newcastle, Callaghan, NSW, Australia; and Hunter Medical Research Institute, New Lambton Heights, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

cAMP Cyclic adenosine monophosphate
 HCO_3^- Bicarbonate ion
 $[\text{Ca}^{2+}]_i$ Intracellular calcium concentration
PEBP1 Phosphatidylethanolamine binding protein 1
 pH_i Intracellular pH
PKA protein kinase A
SACY Soluble adenylyl cyclase, ADCY10
ROS Reactive oxygen species
ZP Zona pellucida

Introduction

Sperm capacitation represents the culmination of a spermatozoon's journey to functional maturity that begins within the testes (spermatogenesis) and continues as the cells are conveyed through the male (epididymal maturation) and female (capacitation) reproductive tracts. Since the coining of the term (Austin, 1952) and original description of capacitation by Chang (1951) and Austin (1951) more than half a century ago, significant advances have been made into our molecular understanding of its foundations (Aitken and Nixon, 2013; Gervasi and Visconti, 2016). This has led to an appreciation that capacitation encompasses a series of sophisticated physiological changes, designed to deliver a highly selected subpopulation of spermatozoa to the oocyte, which are capable of effectively engaging in the process of fertilization. Much of this knowledge is grounded in the ability to recapitulate capacitation in vitro using simple chemically defined media, the use of pharmacological interventions, and/or the analysis of loss of function transgenic mouse models. Here, we discuss how this information has transformed our understanding of the timing, mechanistic basis and biological significance of capacitation.

Timing of In Vivo Capacitation

Upon ejaculation, millions of spermatozoa are deposited into the female reproductive tract and tasked with the purpose of locating and fertilizing an ovum. Notwithstanding the level of specialization that the spermatozoa have achieved in preparation for this task, they remain incapable of recognizing the oocyte or engaging in the complex cascade of cell–cell interactions that culminate in syngamy. Indeed, the pioneering work of Austin and Chang independently established that spermatozoa must spend a finite period of residence in the female reproductive tract before acquiring the ability to fertilize an ovum (Austin, 1951; Chang, 1951). Such findings led to the coining of the term capacitation and its original definition by Austin (1952), to include those physiological changes undergone by sperm while in the female reproductive tract that render them competent to penetrate the egg. In the ensuing decades since these seminal discoveries, the necessity for capacitation has held true across the spermatozoa of all studied eutherian mammals. Further, there is emerging evidence supporting the need for analogous, capacitation-like changes for the activation of spermatozoa during their ascent of the female reproductive system in phylogenetically distinct vertebrate species such as the prototheria (Nixon et al., 2016b) and reptiles (Nixon et al., 2016a).

In terms of the timing of capacitation, the original definition proposed by Austin broadly encompasses all events from the moment of insemination into the female reproductive tract through to sperm penetration of the oocyte vestments (Austin, 1952). Notably however, the rate at which sperm cells capacitate is carefully regulated by the changing environment of the female reproductive tract, such that they are delivered to the surface of the oocyte in a fully primed state, ready for fertilization. A key element of this strategy, and one that increases the likelihood of synchrony with ovulation, is the formation of a transient postinsemination sperm reservoir (Fig. 1). Thus, upon reaching the caudal isthmus and uterotubal junction region of the oviduct, spermatozoa establish intimate contact with the ciliated epithelium and thereafter enter a quiescent state (Suarez, 2016). This

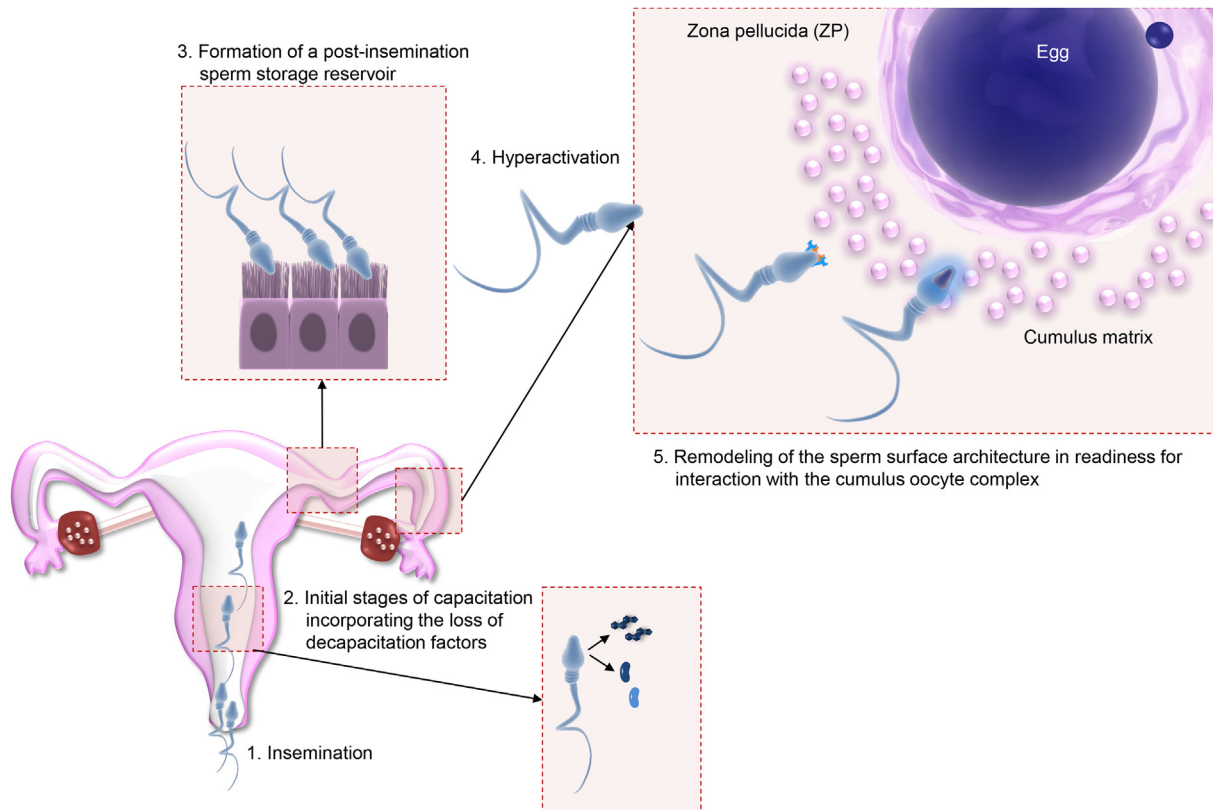


Fig. 1 Phases of *in vivo* sperm capacitation. (1) At the moment of insemination spermatozoa enter the female reproductive tract in a noncapacitated state. (2) The capacitation cascade encompasses a hierarchy of biochemical and biophysical changes that are initiated as spermatozoa disperse from the seminal fluids and begin their ascent of the uterine cavity. These early phases of capacitation are characterized by the loss of decapacitation factors and sterols from the sperm surface. (3) Upon reaching the isthmic region of the Fallopian tubes, spermatozoa bind to the epithelial cells and in so doing, establish a transient storage reservoir in readiness for ovulation. (4) Upon receipt of an appropriate stimulus coincident with ovulation, spermatozoa experience a rapid change in their biochemistry characterized by an increase in reactive oxygen species (ROS) generation, intracellular cyclic adenosine monophosphate and calcium levels, and tyrosine phosphorylation. In response to these signals, spermatozoa begin to express hyperactivated motility, facilitating their release from the oviductal epithelium and migration toward the site of fertilization. (5) These signals also elicit dynamic remodeling of the sperm surface architecture, priming these cells for interaction with the cumulus oocyte complex, the induction of acrosomal exocytosis, and ultimately fertilization of an ovum.

phenomenon is mediated by complex signaling pathways that facilitate cross-talk between the opposing cell types and effectively suppresses sperm activity. In this functionally repressed state, the spermatozoa of most mammals are able to maintained for between 2 and 5 days. Notable exceptions include some marsupials and bats, in which the duration of fertile sperm storage can be extended to between 15 days and several months, respectively (Holt, 2011). The dispersal of the sperm reservoir appears to be driven by the receipt of appropriate extracellular signal(s) coincident with ovulation, whereupon the spermatozoa are able to migrate rapidly toward the oocyte in a state of readiness for fertilization (Suarez, 2016). By the time the spermatozoa have reached the surface of the oocyte within the vicinity of the ampullary-isthmic junction (Fig. 1), they have experienced a functional transformation, including: (i) a transition to hyperactivated motility, (ii) remodeling of their surface architecture to facilitate the expression of receptors for the cumulus oocyte complex and (iii) the activation of the molecular machinery necessary for initiation of acrosomal exocytosis (Aitken and Nixon, 2013).

Mechanistic Basis of Capacitation

Capacitation-Associated Changes in Sperm Membrane Architecture

Much of our current understanding of the molecular basis of sperm capacitation is attributed to the ability to recapitulate this process with either ejaculated or cauda epididymal spermatozoa outside of the female reproductive tract. This *in vitro* approach necessitates the use of chemically defined medium comprising electrolytes (Na^+ , K^+ , Cl^- , HCO_3^- , Mg^{2+} , Ca^{2+} , PO_4^{3-}), energy substrates (glucose, pyruvate, and lactate), and a cholesterol sink; innovations that were first documented by Yanagimachi and Chang (1963). Once this practical milestone had been achieved, one of the first changes documented in capacitating mammalian spermatozoa was that of a substantial depletion of cholesterol from the plasma membrane (Davis et al., 1979). It has since been shown that sterols, such as cholesterol and desmosterol, are transferred from the sperm surface to acceptor molecules such as serum

albumin, high-density lipoproteins and apolipoproteins, thus promoting extensive membrane destabilization and the repositioning of membrane raft microdomains. Accordingly, cholesterol is able to exert a powerful decapacitation effect and, it is for this reason, that albumin features as an important constituent of *in vitro* capacitation media. Notably, the epididymis and male accessory organs also secrete additional proteinaceous decapacitation factors, such as phosphatidylethanolamine binding protein 1 (PEBP1), which bind spermatozoa and exert dual influence over their membrane stability and the modulation of intracellular signaling pathways (Aitken and Nixon, 2013). Acting in tandem with cholesterol, these decapacitation factors are thereby able to suppress the attainment of a capacitated state (Figs. 1 and 2). It follows that spermatozoa from PEBP1-deficient mice experience precocious capacitation and reduced reproductive success.

Notably, cholesterol depletion does not appear to occur uniformly across the entire sperm surface. Rather, there appears to be preferential cholesterol loss from lipid disordered, as opposed to lipid ordered, subdomains. The latter are discriminated on the basis of an enrichment of cholesterol, sphingomyelin, gangliosides, and phospholipids with saturated long-chain acyl groups; and are commonly referred to as either lipid or membrane raft microdomains. There is some evidence that selective sterol transfer from the sperm plasma membrane may be mediated by active cholesterol transporters, such as those of the ATP-binding cassette transporter superfamily. However, this process also appears to be augmented by capacitation-dependent oxidation of sterols. The increased hydrophilicity of the oxysterol products so formed promotes their transfer to albumin acceptors (Gadella and Boerke, 2016). This redox-regulated process is dependent on the presence of bicarbonate and reactive oxygen species (ROS) generation, such that antioxidant supplementation inhibits oxysterol formation and disrupts the capacitation of the spermatozoa. However, in addition to promoting oxysterol formation, both ROS and bicarbonate have been implicated in the modulation of a variety of alternative aspects of the capacitation cascade.

Notwithstanding the importance of sterol depletion in terms of promoting plasma membrane remodeling, once in the female genital tract spermatozoa do not experience pronounced structural changes until they undergo the acrosome reaction. Such observations fueled early speculation that capacitation must be primarily driven by chemical signals that sperm encounter within

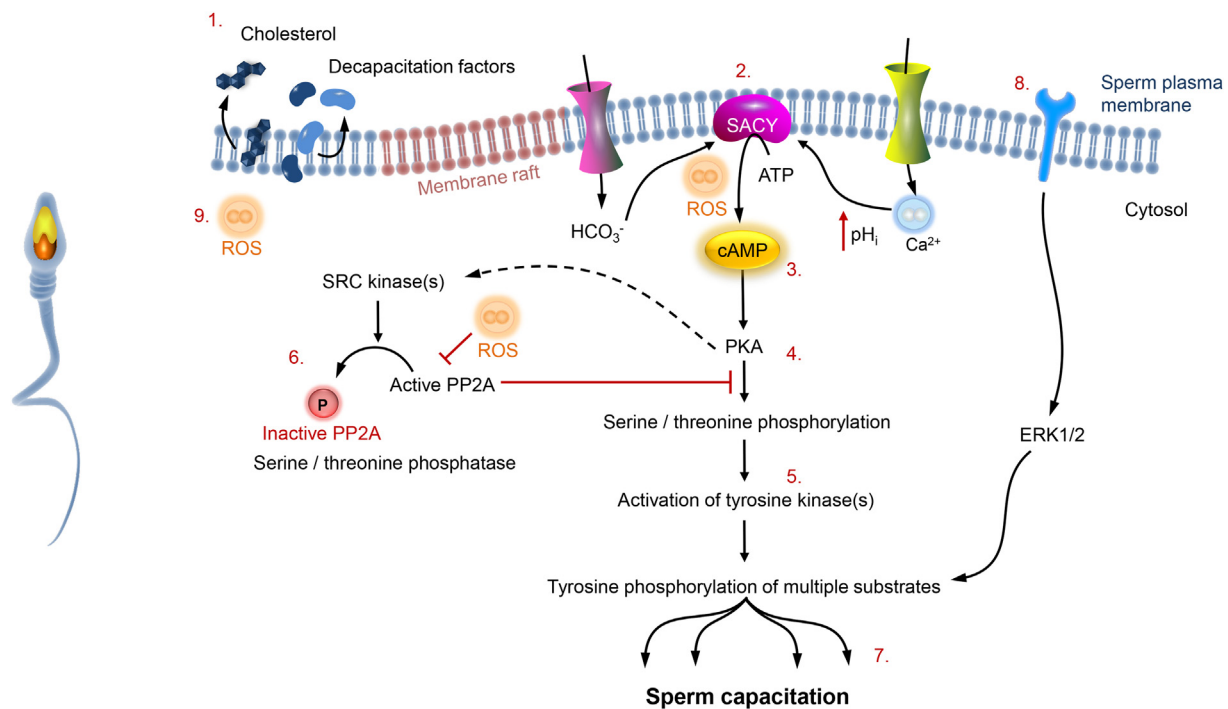


Fig. 2 Model of the capacitation-associated intracellular signaling cascades. (1) The capacitation of mammalian spermatozoa is initiated upon removal of cholesterol and proteinaceous decapacitation factors from the plasma membrane. (2) The dissociation of such factors destabilizes the membrane, promotes sequestration of membrane raft microdomains and is permissive of Ca^{2+} and HCO_3^- influx through appropriate channels including CATSPER and $\text{Na}^+/\text{HCO}_3^-$ cotransporters, respectively. The resultant increase in intracellular calcium and HCO_3^- has been linked to alkalinization of the cell cytosol ($\uparrow \text{pH}_i$). (3) Acting in tandem, these factors stimulate the activity of soluble adenylyl cyclase (SACY), thereby elevating intracellular cAMP concentration. (4) Spermatozoa respond to high cAMP by activating PKA leading to the downstream serine/threonine phosphorylation of multiple substrates. (5) This results in the reciprocal activation of tyrosine kinase(s) and (6) inactivation of the serine/threonine phosphatase(s) (e.g., PP2A). (7) These combined events release spermatozoa from the suppressive mechanisms that normally keep PKA-dependent tyrosine phosphorylation under inhibitory control and thus promote a dramatic increase in the tyrosine phosphorylation of multiple sperm proteins; a pivotal form of posttranslational modification that underpins the functional transformation of spermatozoa and ultimately the attainment of a capacitated state. In addition to the canonical cAMP/PKA signaling pathway, there is a wealth of compelling experimental evidence supporting ancillary roles for (8) alternative signaling cascades such as the extracellular signal regulated kinase (ERKs) module of the mitogen-activated protein (MAP) kinase family and (9) various reactive oxygen species (e.g., in oxysterol formation/efflux, SACY activation, PP2A inactivation).

this environment. Support for this notion now rests with the recognition that spermatozoa are indeed exposed to a variety of extrinsic ligands that control the fate of the cell. Some of these interactions are designed to act early in the capacitation process to promote cell survival (e.g., prolactin) and to impede premature entry of the spermatozoa into capacitation (e.g., decapacitation factors) (Aitken and Nixon, 2013). The dissociation of such factors from the sperm surface makes way for alternative late-acting factors that prepare the cell for fertilization. Among these factors, bicarbonate and calcium ions appear to hold a central role in potentiating capacitation-associated cell signaling (Fig. 2).

Bicarbonate uptake into the maturing sperm cell is facilitated by $\text{Na}^+/\text{HCO}_3^-$ cotransport (Visconti et al., 2011). Thereafter it elicits a suite of interrelated responses, ranging from rapid compositional changes in phospholipid distribution and a concomitant increase in sperm membrane fluidity, activation of soluble adenylyl cyclase and induction of a cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) signaling cascade, intracellular alkalinization, and hyperpolarization of the plasma membrane (Fig. 2). The aforementioned destabilizing effects of bicarbonate, acting synergistically with cholesterol efflux, have been attributed to stimulation of phospholipid scramblase and/or phospholipase enzymes (Gadella and Boerke, 2016). Such enzymatic activity leads to a dramatic reduction in the degree of phospholipid asymmetry, a property that renders the membrane more fusogenic, compatible with the downstream events of acrosomal exocytosis and oocyte plasma membrane (oolemma) fusion. These changes in membrane architecture are also conducive to the lateral redistribution, and concentration, of membrane rafts within the apical region of the sperm head. Such a position is compatible with a role in the mediation of oocyte recognition; a function that resonates with the sequestering of a multitude of receptors implicated in zona pellucida (ZP) adhesion into membrane rafts during capacitation. This also accords with the demonstration that detergent resistant membranes isolated from mouse and human spermatozoa are able to selectively adhere to the ZP of homologous oocytes (Nixon and Aitken, 2009).

An additional hallmark of capacitation is the hyperpolarization of the sperm plasma membrane. Analogous to most somatic cells, spermatozoa are able to maintain an intracellular ion concentration that differs from that of the extracellular environment and in so doing establish a membrane potential. As capacitation proceeds however, spermatozoa experience a hyperpolarization of their plasma membrane, a situation that arises from the closing of Na^+ channels, opening of a K^+ channel, or a combination of these events (Visconti et al., 2011). Such hyperpolarization has been postulated to de-repress Ca^{2+} voltage gated channels and thus facilitate the transient elevation of intracellular calcium needed to support the acrosome reaction. Accordingly, male mice lacking the sperm-specific K^+ channel, SLO3, produce spermatozoa that are refractory to capacitation-associated membrane hyperpolarization and the induction of acrosomal exocytosis, even in the presence of calcium ionophore (Visconti et al., 2011). As an additional tier of regulation, there is evidence that SLO3 activity is influenced by intracellular alkalinization and cAMP, thus reinforcing the importance of bicarbonate-mediated signaling events during capacitation.

Capacitation-Associated Intracellular Signaling

In addition to changes occurring at the plasma membrane, the elevated levels of bicarbonate that sperm encounter within the female reproductive tract also evoke a complex network of intracellular signaling responses (Fig. 2). The widely accepted model of capacitation-associated signaling depicts a sequential process whereby HCO_3^- and Ca^{2+} are positioned as primary drivers of a hierarchical signaling cascade involving increased production of the cAMP second messenger, activation of PKA, and tyrosine phosphorylation of a multitude of sperm proteins (Fig. 2). While this is a well-supported model, it is important to consider that these events are temporally separated into: (i) a subset of biochemical changes (i.e., cAMP generation and PKA activation) that reach maximum levels within as little as a minute following exposure to elevated HCO_3^- concentrations and (ii) a suite of downstream physiological changes (e.g., tyrosine phosphorylation, hyperactivated motility, and plasma membrane remodeling in preparation for acrosomal exocytosis and oocyte interaction) that proceed over a protracted timeframe of up to several hours (Gervasi and Visconti, 2016). Such differences in the activation kinetics of these interdependent correlates of capacitation suggest the necessity for cross-talk between multiple signaling pathways and the compartmentalization of the different processes needed for fertilization.

Canonical cAMP/PKA Signaling

The major regulatory roles assigned to bicarbonate during the early phases of capacitation occur via its direct activation of the atypical soluble adenylyl cyclase, ADCY10 (SACY) (Fig. 2). Accordingly, male mice lacking SACY express an infertility phenotype associated with pronounced sperm motility defects, which cannot be resolved through the application of exogenous cAMP analogues alone. Similarly, pharmacological inhibition strategies have confirmed SACY is required for sperm protein tyrosine phosphorylation and fertility but interestingly, not for hyperactivation or the acrosome reaction (Gervasi and Visconti, 2016). The regulation of these latter events may, instead, rest with classical membrane-bound adenylyl cyclases in a bicarbonate-independent manner. Nevertheless, SACY activation and a concomitant downregulation of phosphodiesterase activity, leads to an impressive increase in intracellular cAMP levels. This, in turn, elicits a rapid upregulation of PKA; a promiscuous serine-threonine protein kinase that acts as a key intermediary in the capacitation cascade (Fig. 2). Indeed, the ablation of the PKA catalytic subunit (PKA-C α 2) leads to a male sterility phenotype associated with defects in sperm capacitation, which mirror those observed in SACY knockout mice (i.e., the spermatozoa from both mouse models are refractory to protein tyrosine phosphorylation when held under capacitation-inducing conditions) (Gervasi and Visconti, 2016). This pivotal role of cAMP/PKA signaling also underpins the ability to accelerate the onset of capacitation in vitro via supplementation of incubation media with phosphodiesterase inhibitors and cAMP analogues.

Tyrosine Phosphorylation

PKA mediated phosphorylation affects the reciprocal activation of tyrosine kinase(s) (PTKs) and deactivation of protein phosphatases (Fig. 2). This results in precocious increases in protein tyrosine phosphorylation, a universal hallmark of capacitation documented in the spermatozoa of all studied eutherian mammals (Aitken and Nixon, 2013). Despite this, the primary kinases responsible for triggering tyrosine phosphorylation remain the matter of some debate. Recent experiments utilizing immunological approaches and small molecule inhibitors have highlighted putative roles for members of the SRC family tyrosine kinases (i.e., cSRC, ABL, FYN) as well as those of the focal adhesion kinase (FAK) family (i.e., FAK, PYK2) (Gervasi and Visconti, 2016). However, the use of transgenic models has challenged the involvement of such enzymes as direct effectors in the canonical capacitation signaling pathway. Rather, on the basis of such studies it is now held that SRC kinases act to phosphorylate, and thereby downregulate the activity of a serine/threonine phosphatase, likely PP2A (Gervasi and Visconti, 2016). This, in turn, releases spermatozoa from the suppressive mechanisms that normally keep PKA-dependent tyrosine phosphorylation under inhibitory control. In terms of alternative tyrosine kinases, several lines of evidence now support a primary role of FER (fer (fps/fes related) tyrosine kinase). Indeed, spermatozoa from mice bearing a kinase-inactivating mutation in *Fer* are refractory to capacitation-associated increases in tyrosine phosphorylation and present with defects in *in vitro* fertilization. Unexpectedly however, these mice retain their fertility, thus challenging the current paradigm that capacitation-associated tyrosine phosphorylation is indispensable for mammalian fertilization (Gervasi and Visconti, 2016).

The functional significance of tyrosine phosphorylation may be resolved by determining the identity of the cohort of sperm proteins that are subjected to this form of modification during capacitation. Although this goal has yet to be fully realized, it is evident that the majority of phosphotyrosine substrates reside within the principal piece of the sperm flagellum. In human spermatozoa for instance, the most prominent targets for capacitation-associated tyrosine phosphorylation are A-kinase anchoring protein (AKAP) 4 (formerly AKAP82) and its precursor pro-AKAP4, both of which represent key structural elements of the fibrous sheath (Bailey, 2010). Owing to the ability of AKAPs to sequester PKA, these findings lend support to the existence of intimate cross-talk between PKA and protein tyrosine kinase pathways. They also raise the prospect that tyrosine phosphorylation may regulate hyperactivation. This notion is congruous with the functions assigned to the fibrous sheath in terms of regulating the degree of flexibility and shape of the flagellar beat during sperm motility, as well as serving as a scaffold for several signaling and/or metabolic pathways. Similarly, Ca^{2+} -binding tyrosine-phosphorylation-regulated protein (CABYR), an ancillary protein associated with the development of the fibrous sheath and regulation of progressive motility, also undergoes tyrosine phosphorylation during capacitation (Bailey, 2010).

Capacitation is also associated with additional foci of tyrosine phosphorylation within the sperm head, and these substrates may be equally, if not more, significant to the attainment of functional competence than those of the flagellum. For instance, the capacitation of boar spermatozoa is correlated with a marked increase in the tyrosine phosphorylation of head-surface proteins; with the major substrate being ACRBP, a (pro)acrosin binding protein that possesses affinity for the zona pellucida (ZP) (Bailey, 2010). A similar situation has also been documented in mouse spermatozoa whereby capacitation is correlated with the appearance of phosphotyrosine residues on the exterior leaflet of the membrane overlying the anterior region of the sperm head. The subset of spermatozoa bearing this hallmark of capacitation correspond to those with the ability to adhere to the ZP, suggesting that tyrosine phosphorylation plays an intermediary role in remodeling the sperm surface to enable ZP recognition. Accordingly, the dominant phosphotyrosine substrates in this domain have been identified as molecular chaperones (namely, HSP90B1 and HSPD1), proteins that are hypothesized to orchestrate the assembly and/or presentation of ZP recognition complex(es) on the sperm surface (Dun et al., 2012).

Work conducted in human spermatozoa suggest that these cells may be primed for oocyte interactions via similar capacitation-associated mechanisms, although in our species this pivotal role appears to rest with HSPA2, an alternative, testis-enriched molecular chaperone. Like HSPD1 and HSP90B1 in the mouse, human sperm HSPA2 is also subject to capacitation-associated tyrosine phosphorylation and is capable of interacting with a number of client proteins (e.g., ARSA, SPAM1, ACE1, PDIA6) that possess affinity for the cumulus-oocyte complex (Nixon et al., 2017). Another notable target for tyrosine phosphorylation within the head of capacitating human spermatozoa is valosin-containing protein (VCP). VCP is a homologue of the SNARE-interacting protein, NSF, which regulates membrane fusion and exocytosis. Thus, the tyrosine phosphorylation of VCP, coupled with its translocation from the connecting piece to the anterior head, could contribute to modulation of acrosomal responsiveness. Alternatively, as is the case for HSPA2, VCP may act to chaperone membrane fusion machinery to their site of action during plasma-acrosomal membrane fusion (Bailey, 2010).

While by no means exhaustive, this brief survey of experimentally validated substrates for capacitation-associated tyrosine phosphorylation does support the importance of this form of posttranslational modification in the priming of spermatozoa for fertilization. Moreover, it suggests that the pervasive impact of tyrosine phosphorylation extends across functional parameters as diverse as motility, surface remodeling, acrosomal exocytosis, and ultimately oolemmal fusion. It is therefore perhaps not surprising that capacitation may encompass alternative tyrosine phosphorylation pathways. Indeed, there is evidence that the extracellular signal regulated kinase (ERK) module of the mitogen-activated protein (MAP) kinase family, may exert regulatory control over certain aspects of capacitation. The fact that key elements of the ERK signaling pathway localize within the peri-acrosomal region of the sperm head suggests that this pathway influences the surface modifications that occur within this domain as capacitation proceeds. This agrees with the demonstration that inhibition of ERK signaling reduces the ability of mouse spermatozoa to engage

in ZP interactions. While there is also some evidence for cross-talk between the cAMP/PKA and ERK pathways during capacitation, the precise nature of this interaction is not well understood (Bailey, 2010).

Intracellular Ca^{2+} and pH

Acting in conjunction with bicarbonate, capacitating spermatozoa experience dramatic changes in intracellular levels of both Ca^{2+} and H^+ ions (Fig. 2). The accompanying increase in $[\text{Ca}^{2+}]_i$ and alkalization of the cell cytosol have been attributed primary messenger status in terms of directly supporting sperm motility and the acrosome reaction (Visconti et al., 2011). They are however, also of fundamental importance in promoting capacitation associated signaling. Indeed, loss- and gain-of-function approaches have established that capacitation is Ca^{2+} -dependent, as are the initiation and maintenance of hyperactivated motility and the acrosome reaction. While informative, this scenario does present some problems owing to the difficulty in differentiating the indirect effects of $[\text{Ca}^{2+}]_i/\text{pH}_i$ during capacitation from that of their direct role in motility and the acrosome reaction. In accounting for the regulation of these two opposing phenomena, it has been postulated that differing mechanisms of calcium entry into the sperm may be particularly relevant (Bailey, 2010). In this context, spermatozoa are known to harbor numerous transporters that could serve as principle candidates responsible for manipulation of Ca^{2+} influx (e.g., CAV and CATSPER channels, $\text{Na}^+/\text{Ca}^{2+}$ exchangers, Ca^{2+} ATPases). Alternatively, they also possess several proteins implicated in Ca^{2+} release from intracellular stores (e.g., IP3 receptors, ryanodine receptors, two-pore channel proteins, and SERCA/SPM ATPases). Notably, the activity of sperm calcium channels such as the voltage dependent calcium channel (VDCC) and the CATSPER family are strongly potentiated by intracellular alkalization, thus accounting for the intimate relationship between $[\text{Ca}^{2+}]_i$ and pH_i in modulating capacitation-associated signaling (Visconti et al., 2011).

Redox Regulation of Capacitation

While the involvement of ROS in sperm capacitation is well established, the identity of the free radical species and the regulatory forces they impose on capacitation has remained the subject of some controversy (Aitken et al., 2015). This situation reflects the existence of compelling evidence implicating key roles for hydrogen peroxide, superoxide anion, and the peroxynitrite anion. In reality, the rapid interconversion of these various reactive species means it is likely that several different redox entities could be involved in various aspects of the capacitation process (Aitken et al., 2015). By way of illustration, the global elevation of protein tyrosine phosphorylation levels that accompany capacitation are intimately tied to the suppression of tyrosine phosphatase activity. This family of enzymes possess a crucial cysteine residue located within their active site that must be held in a reduced state for the expression of phosphatase activity. Potent oxidants generated during sperm capacitation such as hydrogen peroxide and peroxynitrite are both capable of oxidizing this cysteine residue and thus inactivating tyrosine phosphatase activity. Alternatively, superoxide can directly modulate the activity of soluble adenylyl cyclase, thus contributing to increased intracellular levels of cAMP, which drives downstream tyrosine kinase activity. Hydrogen peroxide may also indirectly influence adenylyl cyclase activity thereby creating a self-perpetuating cascade involving ROS generation, adenylyl cyclase activation and tyrosine phosphorylation (Fig. 2).

Additional evidence affirming the role of ROS in the molecular mechanisms regulating sperm capacitation comes from analysis of the biological effects elicited by ROS-specific scavengers. For instance, the elevation of tyrosine phosphorylation during human sperm capacitation can be suppressed via the addition of exogenous catalase to scavenge the hydrogen peroxide generated by these cells. Similarly, membrane permeant ROS scavengers such as 2-mercaptoethanol have been reported to have a profound inhibitory impact on tyrosine phosphorylation. The converse is also true, whereby the direct addition of hydrogen peroxide to mammalian spermatozoa can elicit the tyrosine phosphorylation events associated with capacitation as well as trigger the acrosome reaction (Aitken et al., 2015).

These collective data highlight the phosphatases and kinases regulating tyrosine phosphorylation as being major targets for redox regulation of capacitation. Despite this knowledge, the source of the free radicals that contribute to capacitation remains to be fully resolved, with the involvement of various enzymatic and nonenzymatic biochemical pathways currently under consideration. Irrespective of the source, the ROS that drives capacitation presents spermatozoa with a unique challenge. Indeed, owing to their highly specialized architecture, spermatozoa are inherently vulnerable to oxidative stress. This has prompted the hypothesis that sperm capacitation and the entry of these cells into the intrinsic apoptotic cascade are a continuum, with the ROS that drive tyrosine phosphorylation, cAMP production and cholesterol efflux from the plasma membrane ultimately inducing a state of apoptosis (Aitken et al., 2015). It is for this reason that capacitated spermatozoa rapidly succumb to cell senescence if, upon reaching the site of fertilization, they fail to encounter an oocyte.

Summary

The spermatozoa of all mammalian species acquire the ability to fertilize an ovum during their capacitation within the female reproductive tract. This functional transformation proceeds through a sophisticated hierarchy of biochemical and biophysical changes, the control of which rests with both intrinsic and extrinsic factors. The culmination of capacitation is the generation of

a population of highly specialized spermatozoa that are primed to undergo the acrosome reaction and possess the correct surface architecture to interact with the cumulus oocyte complex and thereby initiate fertilization.

References

- Aitken, R. J., Baker, M. A., & Nixon, B. (2015). Are sperm capacitation and apoptosis the opposite ends of a continuum driven by oxidative stress? *Asian Journal of Andrology*, 17, 633–639.
- Aitken, R. J., & Nixon, B. (2013). Sperm capacitation: A distant landscape glimpsed but unexplored. *Molecular Human Reproduction*, 19, 785–793.
- Austin, C. R. (1951). Observations on the penetration of the sperm in the mammalian egg. *Australian Journal of Scientific Research. Ser. B*, 4, 581–596.
- Austin, C. R. (1952). The capacitation of the mammalian sperm. *Nature*, 170, 326.
- Bailey, J. L. (2010). Factors regulating sperm capacitation. *Systems Biology in Reproductive Medicine*, 56, 334–348.
- Chang, M. C. (1951). Fertilizing capacity of spermatozoa deposited into the fallopian tubes. *Nature*, 168, 697–698.
- Davis, B. K., Byrne, R., & Hungund, B. (1979). Studies on the mechanism of capacitation. II. Evidence for lipid transfer between plasma membrane of rat sperm and serum albumin during capacitation in vitro. *Biochimica et Biophysica Acta*, 558, 257–266.
- Dun, M. D., Aitken, R. J., & Nixon, B. (2012). The role of molecular chaperones in spermatogenesis and the post-testicular maturation of mammalian spermatozoa. *Human Reproduction Update*, 18, 420–435.
- Gadella, B. M., & Boerke, A. (2016). An update on post-ejaculatory remodeling of the sperm surface before mammalian fertilization. *Theriogenology*, 85, 113–124.
- Gervasi, M. G., & Visconti, P. E. (2016). Chang's meaning of capacitation: A molecular perspective. *Molecular Reproduction and Development*, 83, 860–874.
- Holt, W. V. (2011). Mechanisms of sperm storage in the female reproductive tract: An interspecies comparison. *Reproduction in Domestic Animals*, 46(Suppl 2), 68–74.
- Nixon, B., & Aitken, R. J. (2009). The biological significance of detergent-resistant membranes in spermatozoa. *Journal of Reproductive Immunology*, 83, 8–13.
- Nixon, B., Anderson, A. L., Smith, N. D., McLeod, R., & Johnston, S. D. (2016a). The Australian saltwater crocodile (*Crocodylus Porosus*) provides evidence that the capacitation of spermatozoa may extend beyond the mammalian lineage. *Proceedings of the Biological Sciences*, 283.
- Nixon, B., Bromfield, E. G., Cui, J., & De Iuliis, G. N. (2017). Heat shock protein A2 (HSPA2): Regulatory roles in germ cell development and sperm function. *Advances in Anatomy, Embryology, and Cell Biology*, 222, 67–93.
- Nixon, B., Ecroyd, H., Dacheux, J. L., Dacheux, F., Labas, V., Johnston, S. D., & Jones, R. C. (2016b). Formation and dissociation of sperm bundles in monotremes. *Biology of Reproduction*, 95, 91.
- Suarez, S. S. (2016). Mammalian sperm interactions with the female reproductive tract. *Cell and Tissue Research*, 363, 185–194.
- Visconti, P. E., Krapf, D., De La Vega-Beltran, J. L., Acevedo, J. J., & Darszon, A. (2011). Ion channels, phosphorylation and mammalian sperm capacitation. *Asian Journal of Andrology*, 13, 395–405.
- Yanagimachi, R., & Chang, M. C. (1963). Fertilization of hamster eggs in vitro. *Nature*, 200, 281–282.

Mechanisms Involved in Mammalian Gamete Interaction

Patricia S Cuasnicú, Débora J Cohen, Vanina G Da Ros, and Mariana Weigel Muñoz, Institute of Biology and Experimental Medicine, Buenos Aires, Argentina

© 2018 Elsevier Inc. All rights reserved.

Introduction

Mammalian fertilization is a complex process involving a series of coordinated steps. Differently from the situation in most invertebrates and lower vertebrates in which testicular spermatozoa have full fertilizing ability, the highly differentiated mammalian sperm leaving the testis are immotile and do not have the ability to fertilize an egg. To become competent, mammalian sperm need first to undergo a maturation process while passing through the epididymis that confers them progressive motility and the ability to interact with the egg. Mature sperm leaving the epididymis, however, still do not have the immediate capacity to fertilize an egg requiring to undergo another process known as capacitation that occurs while they are ascending through the female tract (Yanagimachi, 1994). After maturation and capacitation, sperm become able to reach, recognize and fertilize the eutherian egg which is ovulated surrounded by two vestments: the cumulus oophorus, a mass of granulosa cells embedded in a matrix of hyaluronic acid, and a glycoprotein coat known as zona pellucida (ZP) (Fig. 1A).

Whereas in all mammals (i.e., monotremes, marsupials, and eutherians), fertilization requires the previous occurrence of the acrosome reaction (AR), an exocytotic event in the head, in eutheria, fertilization also requires the development of a special vigorous and intermittent sperm motility known as hyperactivation. In addition, whereas in mammals the AR involves the release of the acrosomal enzymes as a result of the fusion between the plasma membrane and the underlying acrosomal membrane, in eutheria, it has another important consequence such as the acquisition of fusogenicity of the plasma membrane covering the equatorial segment (Fig. 1B).

Fertilization in eutherian mammals occurs in the ampulla of the oviduct and involves the following successive steps (a) penetration of the cumulus oophorus, (b) binding of sperm to the ZP, (c) penetration of the ZP and, finally, (d) fusion of sperm with the egg plasma membrane (Fig. 2). Each of these steps has different requirements regarding the AR and hyperactivation which are summarized in Fig. 3.

This article will describe the different strategies and molecular mechanisms involved in each of the stages of the fertilization process focusing on eutherian mammals and mainly in rodent models.

Penetration of the Cumulus Oophorus

One of the unique characteristics of most eutherian mammals is the presence of the cumulus oophorus surrounding the oocytes at the time of the ovulation (Bedford, 2014). Thus, penetration of sperm through this cumulus mass becomes the first step towards a successful fertilization. Although not essential, it is widely accepted that the presence of the cumulus oophorus is beneficial for fertilization and reduces individual variations in male fertility. The cumulus layers increase the apparent size of the oocyte and, therefore, the chances that a spermatozoon reaches the oocyte within the ampulla (Bedford, 2014). Evidence also indicates that the radial distribution of the cumulus cells allows the formation of a gradient of chemoattractant molecules secreted by these cells (i.e., progesterone, CRISP1, natriuretic peptides) that guides hyperactivated sperm towards the oocyte (Armon and Eisenbach, 2011). The cumulus may also facilitate fertilization by prolonging the fertile life of the eggs, by preventing spermatozoa to swim away, by filtering those spermatozoa that are unable to interact with the egg, and by enhancing sperm capacitation and AR (Yanagimachi, 1994). In this sense, whereas it is clear that only capacitated sperm can penetrate the cumulus mass, controversial evidence exists regarding the acrosomal status of sperm penetrating the cumulus layers. As described in more detail below, an early model

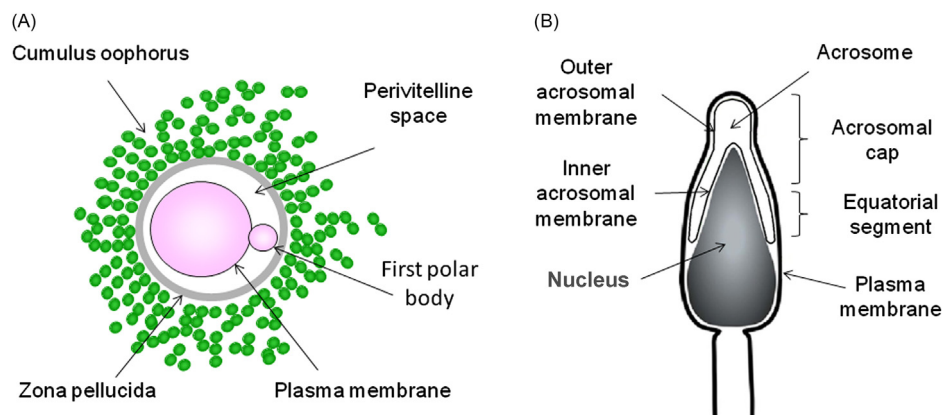


Fig. 1 Diagrams illustrating the general structure of eutherian gametes. Sagittal sections of (A) the ovulated egg and (B) the sperm head.

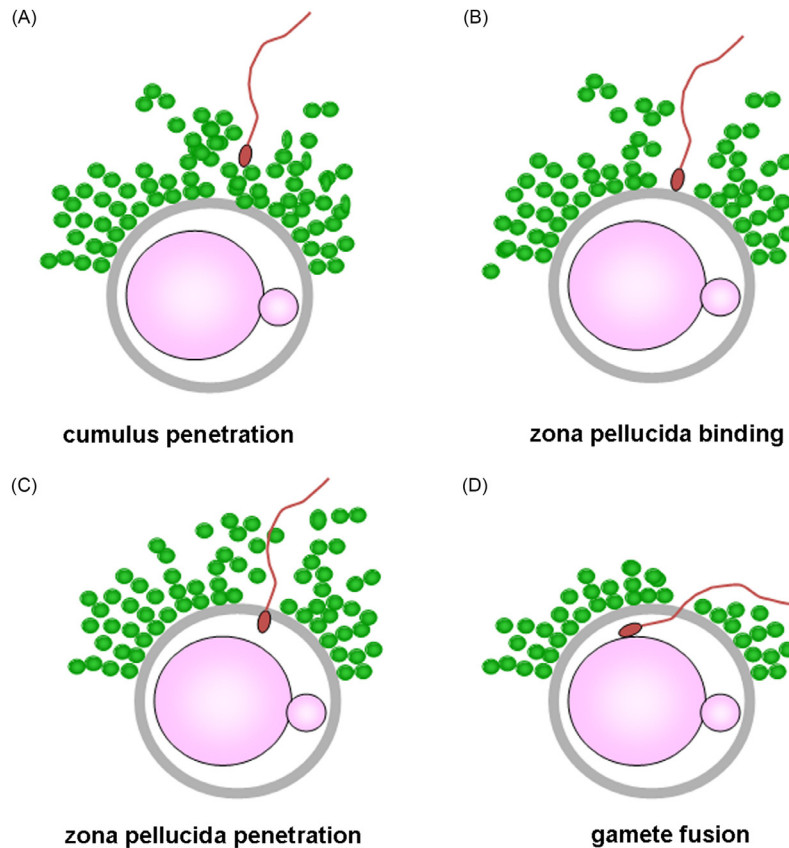


Fig. 2 Successive stages of gamete interaction during mammalian fertilization. (A) Sperm contact and penetrate the cumulus oophorus; (B) sperm binds to the zona pellucida; (C) sperm penetrates de zona pellucida reaching the perivitelline space; (D) sperm fuses with the plasma membrane of the egg.

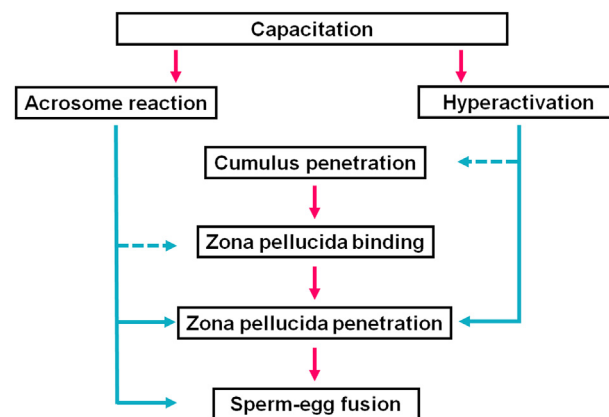


Fig. 3 Requirements of AR and hyperactivation for each stage of gamete interaction. *Solid and dashed lines* refer to an absolute or potential requirement, respectively.

proposed that acrosome intact sperm pass through the cumulus layer and undergo the AR at the ZP level (Florman and Fissore, 2015). However, more recent studies show that the majority of sperm have already completed their AR before entering the cumulus oophorus, reaching the ZP without acrosome (Jin et al., 2011; Hino et al., 2016; Florman and Fissore, 2015). Recent observations by video-recording of sperm within the oviduct show that reacted sperm do not spend much time in the cumulus layers but quickly reach the ZP and swim away from a fertilized egg to enter the cumulus of a nearby unfertilized egg (Hino et al., 2016), supporting a role for the ZP but not for the cumulus oophorus in the prevention of polyspermy.

The molecular mechanisms leading to the sperm entry into the cumulus and subsequent penetration are still poorly understood. It has been proposed that mouse sperm hyaluronidases SPAM1 (formerly known as PH-20) and HYAL5 were both involved in

penetration through the cumulus matrix in acrosome-intact and acrosome-reacted sperm. However, studies using sperm lacking each of these proteins showed that, in spite of a reduced ability to disperse the cumulus mass in SPAM1 knockout mice, the males were fertile (Kimura et al., 2009). The generation of HYAL5/SPAM1 double-knockout mice will provide important information on the relevance of sperm hyaluronidases for the cumulus penetration.

It has also been postulated that sperm hyperactivation may be necessary for cumulus penetration. Evidence favoring this possibility comes from early studies showing that hyperactivated hamster or mouse sperm penetrate more efficiently than fresh sperm, a viscoelastic medium which resembles that from the viscoelastic cumulus matrix (Suarez and Dai, 1992). In addition, it has been reported that sperm lacking testicular CRISP2 have impaired ability to hyperactivate and to penetrate cumulus–oocyte complex in vitro (Brukman et al., 2016). However, further studies are required to clarify the need of hyperactivation for cumulus penetration.

Sperm Binding to the ZP

As mentioned above, besides the cumulus oophorus, the eutherian mammalian egg is ovulated surrounded by the ZP, a thick and elastic coat that plays important roles before and after fertilization. The ZP protects eggs and embryos from physical damage and from sticking to the oviductal walls, contributes to create the specialized microenvironment of the perivitelline space, participates in the mechanisms of prevention of polyspermy and is essential for the development of the preimplantation embryo.

After having penetrated the cumulus oophorus, sperm first bind to and then penetrate the ZP. Sperm–ZP binding has been extensively studied, especially since the biochemical characterization of the protein composition of the ZP in the 80's. Several reports, mainly from Wassarman's laboratory, revealed that the ZP is basically composed by three or four proteins depending on the species that is, ZP1, ZP2, ZP3, and ZP4, significantly contributing to the elucidation of the molecular mechanisms involved in sperm–ZP interaction. As clearly summarized by Florman and Fissore (2015), it was generally believed that the occurrence of the AR which is essential for subsequent steps (i.e., ZP-penetration and gamete fusion) is triggered upon the binding of sperm to the ZP. Moreover, ZP3 was postulated as the molecule involved in both binding of intact sperm to the ZP (primary binding) and induction of the AR, while ZP2 was proposed to participate in the binding of sperm to the ZP after the AR (secondary binding). The use of genetic modified animals for ZP genes, however, opened new insights on the molecular mechanisms involved in gamete recognition showing the critical roles played by the whole ZP structure and the state of ZP2 (cleaved or uncleaved) in sperm–ZP binding (Avella et al., 2013). In addition, as stated above, recent observations using life imaging revealed that most fertilizing sperm undergo the AR before reaching the ZP (Jin et al., 2011), supporting that sperm–ZP interaction is not critical for triggering the AR as previously proposed.

Contrary to the few ZP molecules involved in sperm–ZP binding, numerous sperm proteins have been proposed to be involved in this stage of the fertilization process (i.e. proacrosin, zonadhesin, P56, galactosyltransferase, SED1, etc.) (Okabe, 2014). The use of knockout mice for these molecules, however, questioned their roles in fertilization revealing that most of them were dispensable for fertility. Knockout mice for other molecules proposed to be involved in sperm–ZP binding such as calmegin, Ace (angiotensin-converting enzyme) and members of the ADAM family (ADAM1a, ADAM2, and ADAM3) exhibit sperm with impaired ability to bind to the ZP and either lack or have an aberrant distribution of ADAM3, a sperm surface protein that seems to emerge as a key molecule for gamete interaction (Okabe, 2014). Interestingly, these knockout mice are infertile due to an unexpected sperm inability to migrate into the oviduct, suggesting that oviduct migration and sperm–ZP binding might share a common mechanism. Therefore, evidence seems to support the idea that sperm–ZP interaction is not a simple ligand-receptor event as originally proposed but rather a complex process involving different molecules and recognition mechanisms. Finally, the finding that sperm that are unable to bind to the ZP under in vitro conditions are capable of fertilizing eggs when inseminated in the oviduct led some investigators to reconsider the need of a sperm–ZP binding step for fertilization (Okabe, 2014).

Penetration of the ZP

Sperm penetration through the ZP is the most species-specific stage of the fertilization process. It is a relatively rapid event in which sperm penetrates the ZP at a 45 degree angle. In all mammals, the occurrence of the AR is an absolute requirement for this stage. However, whereas in sub-theria (monotremes and marsupials) ZP-penetration involves a rapid degradation of the coat by the enzymes released from acrosome-reacted spermatozoa, in eutherian mammals, this stage seems to be mediated by mechanical rather than enzymatic mechanisms. Several observations support this idea. The fertilizing sperm passing through the ZP moves the tail vigorously while its narrow head seems to physically cut the zona, it leaves a very thin and curved slit in the zona which completely differs from the enzymatic halo observed in the ZP of other mammals, and inhibitors of acrosomal enzymes proposed to be involved in ZP penetration (i.e., acrosin) impair the previous stage of sperm–ZP binding (Yanagimachi, 1994; Bedford, 2014).

Strong evidence indicates that sperm need to be hyperactivated in order to gain the strength to penetrate the ZP. Hyperactivation development is mediated by the activation of CatSper, the principal sperm calcium channel present in mammals (Ren et al., 2001). Male mice lacking CatSper are infertile because their sperm do not hyperactivate and cannot penetrate the ZP (Carlson et al., 2003), supporting the key role of hyperactivation for successful penetration of the ZP. Thus, although not completely excluded from participating in ZP penetration, acrosomal enzymes in eutheria may be more relevant for other events of the fertilization process such as sperm binding to the ZP or development of the fusogenic properties of the equatorial segment during the AR.

The need of physical thrust for ZP penetration may be associated to the thick and robust ZP of eutherian eggs (Bedford, 2014). Interestingly, accompanying this special nature of the ZP, eutherian sperm also exhibit special features such as a rigid and flat head which allows it to oscillate in the plane during ZP penetration and a stable inner acrosomal membrane (Fig. 1B) which is the one that directly bears the physical effort inherent to ZP penetration. As a consequence of its stability, the inner acrosomal membrane do not exhibit the fusogenic properties required for subsequent gamete fusion which, in eutheria, are preserved on the plasma membrane over the equatorial segment.

Sperm–Egg Fusion

After penetration of the egg vestments, the acrosome-reacted sperm quickly traverses the perivitelline space, a special microenvironment that favors gamete fusion. Evidence shows that gamete fusion in mammals involves a first step in which the sperm binds to the egg plasma membrane followed by a second step of gamete membrane fusion. In this regard, differently from sub-therian mammals in which sperm fuses with the egg through the inner acrosomal membrane, the eutherian sperm fuses with the egg through the equatorial segment of the sperm head (Bedford, 2014). In fact, the AR is an absolute requirement for gamete fusion because only after the occurrence of this event the equatorial segment becomes able to fuse with the egg plasma membrane (Yanagimachi, 1994; Bedford, 2014). The acrosome-reacted sperm fuse with the microvilli present on the entire egg surface except on the area overlying the meiotic spindle, a region through which gamete fusion rarely occurs. In contrast to the absolute need for the AR, evidence indicates that hyperactivation is not required for gamete fusion but that a specific flagellum beat mode is needed for inducing this process (Ravaux et al., 2016). After an abrupt reduction of the sperm tail movement, indicative that gamete fusion has occurred, all the sperm is gradually incorporated into the egg cytoplasm. While the posterior head and tail are incorporated through a membrane fusion process, the anterior region of the head is internalized by a process similar to phagocytosis (Yanagimachi, 1994).

Although gamete fusion is a key step of fertilization, little is known about the molecular mechanisms involved in this process. Proteins involved in gamete fusion were initially identified using specific antibodies to inhibit *in vitro* fertilization. Subsequent gene-disruption experiments revealed that many of these proteins participate in events that precede gamete fusion or that seem to be inessential (Okabe, 2014). Knockout mice for members of the ADAM protein family (i.e., fertilin) postulated as mediators of the fusion process were infertile due to an unexpected phenotype not related to gamete fusion: failure of sperm to migrate to the oviduct *in vivo* and to bind to the ZP *in vitro* (Okabe, 2014). *In vitro* fertilization studies carried out in the presence of antibodies or proteins revealed the involvement of CRISP1 and CRISP2 in gamete fusion through their interaction with complementary sites localized in the egg surface. Although mice lacking these proteins are fertile, their sperm exhibit an impaired fusion ability supporting their relevance for gamete fusion (Da Ros et al., 2008; Brukman et al., 2016). The only sperm protein that proved to be essential for gamete fusion so far is IZUMO, a testis specific member of the immunoglobulin (Ig) superfamily of proteins. Interestingly, IZUMO knockout males are infertile because of an impaired sperm ability to fuse with the egg plasma membrane (Inoue et al., 2005). The tetraspanin CD9 has been identified as an egg plasma membrane protein essential for gamete fusion in mammals (Miyado et al., 2000) and, more recently, the molecule folate receptor 4 (FOLR4), named JUNO, was found to be the egg receptor for sperm IZUMO (Bianchi et al., 2014). IZUMO–JUNO interaction is very weak, and is proposed to mediate the initial adhesion step between gametes but not their fusion. The correct interaction between JUNO–IZUMO molecules would be strengthened by CD9 which has been also proposed as organizer of the microdomains involved in membrane fusion. Whereas these observations provide information on the ligand–receptor interactions required for gamete fusion, the identity of the molecules directly involved in sperm–egg membrane fusion process itself (fusogens) still remain unknown.

Following gamete fusion, the egg initiates a series of morphological and biochemical events known as “activation” that involves resumption of meiosis and block of polyspermy, and leads to cell division/differentiation and the formation of a new individual.

References

- Armon, L., & Eisenbach, M. (2011). Behavioral mechanism during human sperm chemotaxis: Involvement of hyperactivation. *PLoS One*, 6, e28359.
- Avella, M. A., Xiong, B., & Dean, J. (2013). The molecular basis of gamete recognition in mice and humans. *Molecular Human Reproduction*, (5), 279–289.
- Bedford, J. M. (2014). Singular features of fertilization and their impact on the male reproductive system in eutherian mammals. *Reproduction*, 147, R43–52.
- Bianchi, E., Doe, B., Goulding, D., & Wright, G. J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature*, 508, 483–487.
- Brukman, N. G., Miyata, H., Torres, P., et al. (2016). Fertilization defects in sperm from cysteine-rich secretory protein 2 (Crisp2) knockout mice: Implications for fertility disorders. *Molecular Human Reproduction*, 22, 240–251.
- Carlson, A. E., Westenbroek, R. E., Quill, T., et al. (2003). CatSper1 required for evoked Ca²⁺ entry and control of flagellar function in sperm. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 14864–14868.
- Da Ros, V. G., Maldera, J. A., Willis, W. D., et al. (2008). Impaired sperm fertilizing ability in mice lacking cysteine-rich secretory protein 1 (CRISP1). *Developmental Biology*, 320, 12–18.
- Florman, H. M., & Fissore, R. A. (2015). Fertilization in mammals. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction* (4th edn., pp. 149–196). New York: Plenum Press.
- Hino, T., Muro, Y., Tamura-Nakano, M., et al. (2016). The behavior and Acrosomal status of mouse spermatozoa *In Vitro*, and within the oviduct during fertilization after natural mating. *Biology of Reproduction*, 95, 50.
- Inoue, N., Ikawa, M., Isotani, A., & Okabe, M. (2005). The immunoglobulin superfamily protein Izumo is required for sperm to fuse with eggs. *Nature*, 434, 234–238.
- Jin, M., Fujiwara, E., Kakiuchi, Y., et al. (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 4892–4896.

- Kimura, M., Kim, E., Kang, W., et al. (2009). Functional roles of mouse sperm hyaluronidases, HYAL5 and SPAM1, in fertilization. *Biology of Reproduction*, 81, 939–947.
- Miyado, K., Yamada, G., Yamada, S., et al. (2000). Requirement of CD9 on the egg plasma membrane for fertilization. *Science*, 287, 321–324.
- Okabe, M. (2014). Mechanism of fertilization: A modern view. *Experimental Animals*, 63, 357–365.
- Ravaux, B., Garroum, N., Perez, E., Willaime, H., & Gourier, C. (2016). A specific flagellum beating mode for inducing fusion in mammalian fertilization and kinetics of sperm internalization. *Scientific Reports*, 6, 31886.
- Ren, D., Navarro, B., Perez, G., et al. (2001). A sperm ion channel required for sperm motility and male fertility. *Nature*, 413, 603–609.
- Suarez, S. S., & Dai, X. (1992). Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. *Biology of Reproduction*, 46, 686–691.
- Yanagimachi, R. (1994). Mammalian fertilization. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction* (2nd edn., pp. 189–317). New York: Raven Press.

Sperm Acrosome Reaction

Haim Breitbart and Ortal Shabtay, Bar-Ilan University, Ramat-Gan, Israel

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome A secretory vesicle covering the anterior portion of the sperm head underneath the plasma membrane, which consists of a matrix enfolded within a continuous two-layer membrane system; the inner acrosomal membrane and the outer acrosomal membrane.

Acrosome reaction An exocytotic event that is an essential prerequisite to successful fertilization, and occurs in capacitated sperm following a trigger located in the surroundings of the egg. Exocytosis involves the fusion and vesiculation of the plasma membrane overlying the acrosome and the outer acrosomal membrane, so that acrosome contents are released.

Spontaneous acrosome reaction (sAR) Release of acrosome contents under some conditions, in the absence of a trigger, lead to an impairment of this well-regulated exocytotic event and a lowered incidence of a successful fertilization.

Zona pellucida An oocyte-specific extracellular matrix that surrounds the egg and is responsible for specific-specific binding of sperm and induction of the acrosome reaction of bound sperm; the components of the zona pellucida are glycoproteins.

Introduction

The acrosome is a secretory vesicle covering the anterior portion of the sperm head underneath the plasma membrane (PM). This structure is a derivative of the Golgi apparatus, and consists of a matrix enfolded within a continuous two-layer membrane system; the outer acrosomal membrane (OAM) adjoining the sperm PM and the inner acrosomal membrane located near the nuclear envelope. Although the size and shape of the acrosome are different among various species, its basic structure is the same in all eutherian mammals. Sperm must have properly formed acrosomes to be functional in the fertilization process; men or mice carrying mutations affecting the formation or function of the sperm acrosome are infertile or display severe subfertility. In mammals, the acrosome reaction (AR) involves the fusion of the OAM with the overlying PM, thus creating mixed membrane vesicles which later on spread over. AR was studied using intact cells, permeabilized cells and a cell-free system (CFS), in which the membrane fusion event is isolated from other stages of the signaling cascades. In this CFS of sperm AR, PM, and OAM are extracted and the fusion between them was monitored. It was found that both membrane species are primed to fuse during capacitation. The requirement for alkaline pH (7.4) and high Ca^{2+} concentration (about 20 μM) for successful fusion in the CFS mimics those of AR in intact cells. It was discovered that during capacitation, the acrosome undergoes swelling resulting in changes in distance between the OAM and the PM, and that the probable contact between these two membranes could establish the docking or formation of fusion pores.

The head of the acrosome-reacted sperm is turned into an arrowhead-shaped structure allowing the mechanical penetration into the egg. The AR is a well-regulated exocytotic event, which is an absolute prerequisite for successful fertilization. Physiological AR can occur only in sperm that undergo some biochemical transformations in the female reproductive tract, collectively called capacitation.

Functional Significance and Site of the Acrosome Reaction

In many animals the eggs are surrounded by glycoprotein coats, through which the sperm cell must pass in order to reach the egg PM (oolemma). In nonmammalian species the acrosomal content, released after the AR, dissolve the coat to produce a hole through which the spermatozoon can burrow into the egg. In mammals the egg contains a glycoprotein envelope called the zona pellucida (ZP). In the metaphase II-arrested egg, the ZP is further surrounded by the cumulus oophorus, which consists of cumulus cells and their matrix. In many species, sperm that undergo premature AR generally display reduced rate of fertility, due to a failure to penetrate the cumulus oophorus and interact with the egg ZP. It is generally accepted, that only acrosome-intact sperm can bind to the ZP resulting in the occurrence of the AR. Although the ZP of mouse egg and many other species has the ability to induce the AR, it has been demonstrated that at least in the mouse, most fertilizing spermatozoa were acrosome-reacted before reaching the ZP. It was reported that fertilizing mouse sperm begin their AR in the upper isthmus of the oviduct. During the period of fertilization within the oviduct, acrosome-reacted sperm were seen throughout the isthmus, but with higher incidence in the upper than in the mid- and lower segment of the isthmus. Moreover, these authors showed that very few spermatozoa were present in the ampulla, and almost all were acrosome reacted. All fertilizing mouse sperm within the oviduct begin to react before ascending from the isthmus to the ampulla. Others have claimed that cumulus cell mass may be the site of acrosomal exocytosis, a concept supported by recent studies showing that syndecan-1 is present on cumulus cells and possesses the ability to induce exocytosis in vivo. Thus, the site of the AR in vivo should be reconsidered, and the concept regarding the ZP as the only site of physiological AR should be reevaluated. The acrosome-reacted spermatozoon that penetrated the egg-ZP, has the ability to interact and fuse with the egg-PM leading to the zona reaction, a process which prevents polyspermy. A critical protein for sperm-egg fusion is IZUMO1, which is

localized on the acrosomal membrane. As a consequence of acrosomal exocytosis, this protein relocates to the surface of the equatorial region, and sperm become fusion-competent. Thus, the AR has at least dual functions: it renders spermatozoa capable of penetrating through the ZP, and fusing with the egg PM.

Acrosome Reaction Triggers

The AR depends upon an increase of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and PH. This can be artificially established using Ca^{2+} -ionophore A23187, which is a $\text{Ca}^{2+}/2\text{H}^+$ exchanger that triggers the AR when added to capacitated sperm. The mechanism within the spermatozoon, that regulates $[\text{Ca}^{2+}]_i$ for the AR, involves the PM and the acrosome which is a Ca^{2+} storage. Extracellular concentration of Ca^{2+} is in the mM range, and intracellular concentration is in the nM range. The sperm PM contains various Ca^{2+} -channels that mediate Ca^{2+} influx, as well as Ca^{2+} -pump (Ca^{2+} -ATPase) and $3\text{Na}^+/\text{Ca}^{2+}$ exchanger, two systems for Ca^{2+} exclusion from the cell.

The physiologically relevant trigger of the AR that has received the greatest experimental attention is the ZP. Isolated ZP can bind only to acrosome-intact sperm, and can cause the AR. Sperm that contain bound ZP, cannot fertilize the egg, suggesting that the ZP is the physiological ligand that triggers the AR. These initial experiments were carried out in the mouse, and were later on performed in other species including human. Binding of sperm to ZP activates a poorly selective cation channel leading to membrane depolarization and Gi-protein-dependent increase in intracellular PH followed by opening of a voltage sensitive Ca^{2+} channel leading to an increase in $[\text{Ca}^{2+}]_i$.

Spermatozoa also contain several receptors, like P_2 -purinoceptor (activated by extracellular ATP), epidermal-growth-factor-receptor (EGFR), G-protein-coupled-receptors (GPCRs) like lysophosphatidic acid receptor (LPA-R), angiotensin II receptor (AngII-R), and Zn^{2+} -R, progesterone-activated CatSper (a sperm specific Ca^{2+} -channel) and more, all of which can cause an increase in intracellular Ca^{2+} concentrations and trigger the AR. The ligands of these receptors are found in the female reproductive tract at concentrations that can trigger the AR. As mentioned above, for many years the predominant concept was that true AR occurs only and immediately after binding to the ZP, and so this list of triggers were considered to cause false AR. The finding that mouse sperm which undergone AR before reaching the egg are fertile, suggests that other physiological triggers besides ZP might cause true AR if it occurs in the surroundings of the egg. If AR occurs far from the egg, before reaching the isthmus, such sperm is unable to reach the egg in the oviduct and would not be able to fertilize it. We can think of this as false AR, which might be a part of a selection process. It is known that out of 20 million sperm that were transferred to the female, only 10–100 capacitated sperm arrive to the fertilization site. Thus, the AR may also be considered as a selection process designed to ensure the delivery of the genetically selected sperm to the fertilization site.

Signaling Mechanisms in the Acrosome Reaction

During sperm capacitation actin monomers are polymerized into F-actin polymers, and prior to the AR the actin polymers should be dispersed to allow the contact between the OAM and the overlying PM. The increase in intracellular Ca^{2+} levels prior to the AR activates phospholipase C (PLC) to hydrolyse PIP_2 leading to the release of PIP_2 -bound tyrosine-phosphorylated-gelsolin, which undergoes dephosphorylation/activation by Ca^{2+} . Gelsolin is an actin-severing-protein, which causes fast F-actin depolymerisation prior to the AR. All AR triggers, including ZP, cause an increase in Ca^{2+} influx resulting in an increase in intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$). ZP triggers a transient elevation of $[\text{Ca}^{2+}]_i$, which is carried out by a low voltage-activated Ca^{2+} channel, a member of the Ca_v3 family. The sustained Ca^{2+} response occurring after ZP binding to sperm is conducted by TRPC Ca^{2+} -channel. ZP activation of TRPC channels leads also to activation of PI3K to produce PIP_3 , which acts as docking sites for Akt (PKB) and $\text{PKC}\zeta$, two kinases that mediate downstream stages of the AR.

The increase in $[\text{Ca}^{2+}]_i$ described above is necessary, but not enough, to induce the AR. EPAC-dependent Ca^{2+} release from the acrosome should occur in order to achieve the AR. The cyclic adenosine monophosphate (cAMP)-dependent EPAC is an exchange factor, which activates the small GTPases Rap1 and Rab3A by replacing GDP by GTP. EPAC activates $\text{PLC}\epsilon$, which is required for the AR downstream to Rap1 and upstream to intraacrosomal Ca^{2+} mobilization. In turn, $\text{PLC}\epsilon$ hydrolyses PIP_2 to IP_3 , and diacylglycerol (DAG). IP_3 activates IP_3 -receptor, localized in the OAM, to release Ca^{2+} from the acrosome and DAG activates PKC leading to the AR. EPAC can also activate the SNARE protein, known to participate in the membranes fusion in the AR process. When the PM and the OAM are tethered, SNARE forms a trans complex that brings the two bilayers in close proximity.

The list of proteins that participate in the AR also include Rab3A, α -SNAP, NSF, synaptotagmin, calmodulin, dynamin, and complexin.

The AR also depends on signaling molecules like MUPP1, a membrane raft-associated molecular organizer. MUPP1 controls the initial tethering and docking of the acrosomal vesicle, whereas the SNARE protein syntaxin-2 mediates the final step of acrosomal fusion. Inhibition of the Ca^{2+} -ATPase (Ca^{2+} -pump), localized in the OAM and transports Ca^{2+} into the acrosome, causes reduction in intraacrosomal Ca^{2+} concentration resulting in significant increase in cytosolic $[\text{Ca}^{2+}]_i$ due to the opening of the PM store-operated- Ca^{2+} -channel leading to AR.

Progesterone, secreted from the cumulus cells, activates the sperm specific Ca^{2+} -channel CatSper in the sperm tail, and stimulates indirectly the AR. Mouse sperm treated with ZP exhibited a CatSper-dependent Ca^{2+} influx that progressed from the tail to the head leading to the AR.

Sperm EGFR is partially activated during incubation under capacitated conditions. Further activation of the EGFR by EGF or by isolated ZP at the end of the capacitation enhances intracellular Ca^{2+} concentration leading to F-actin breakdown and allows the AR to take place. Under in vivo conditions, the EGFR can be directly activated by its known ligand EGF, which is present in the female reproductive tract, and indirectly by the cascade PKA-Src-EGFR. Moreover, the AR can be induced by GPCRs via the transactivation

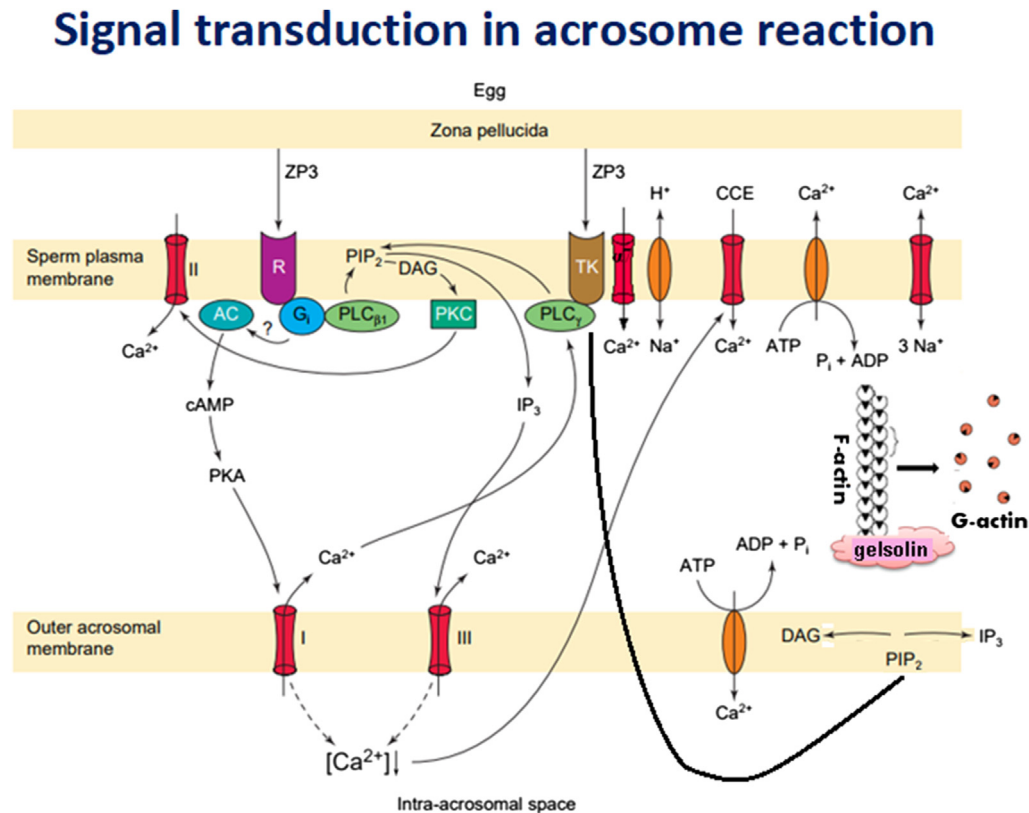
of the EGFR by a signaling pathway involving PKA, SRC family tyrosine kinase and metalloproteinase, and the EGFR downstream effectors PI3K, PLC, and PKC. Regarding the role of the heterotrimeric G-proteins in the AR, it was shown that ZP-dependent intracellular alkalization does not occur when G-protein stimulation is inhibited. Under physiological conditions, sperm PKA is indirectly activated mainly by bicarbonate, which activates the soluble adenylyl cyclase (sAC) to produce cAMP the activator of PKA. GPCR activators like angiotensin II or lysophosphatidic acid are physiological components present in the female reproductive tract.

We suggested that the $\alpha 7$ -nicotinic-acetylcholine receptor ($\alpha 7$ nAChR) might be a sperm receptor activated by ZP to induce EGFR-mediated AR. We found that isolated ZP or $\alpha 7$ agonists induced the AR in sperm from WT but not $\alpha 7$ -null spermatozoa, and the induced AR was inhibited by $\alpha 7$ or EGFR antagonists. Moreover, $\alpha 7$ -null sperm showed very little binding to the egg, and microfluidic affinity in vitro assay clearly showed that $\alpha 7$ nAChR, as well as EGFR, interacted with ZP3. Induction of EGFR activation and the AR by $\alpha 7$ agonist was inhibited by a Src family kinase (SFK) inhibitor. In conclusion, activation of $\alpha 7$ by ZP leads to SFK-dependent EGFR activation, Ca^{2+} influx, and the acrosome reaction.

The most characterized mouse sperm protein that possesses specific binding for ZP3 is the protein sp56, however this protein presents in the acrosomal matrix, not in the sperm PM. A more recent study reveals that ZP3R/sp56 is not essential for mouse fertilization. Moreover, mouse sperm begin to undergo AR in the upper isthmus of the oviduct, thus the acrosomal content including sp56 is spread out from the sperm far away from the egg ZP. Interestingly, the group of Yanagimachi shows that only a few sperm cells migrate from the isthmus to the ampulla during the progression of fertilization suggesting that it is quite difficult for acrosome-reacted sperm to migrate from the isthmus to the ampulla.

Another candidate that has been implicated as a receptor for ZP3 in mouse sperm is a specific form of β -galactosyltransferase (GalTase). It has been postulated that GalTase mediates sperm-ZP binding by interacting with ZP3 oligosaccharide residues, leading to activation of the heterotrimeric Gi-protein. However, targeted mutation of the GalTase gene yields fertile null males, yet their sperm bind less ZP3 than WT and are unable to undergo a ZP3-induced AR. Thus, it was concluded that GalTase is not absolutely critical for fertility.

In conclusion, although several research groups suggested a number of sperm proteins as candidates that mediate sperm-ZP binding, it is not clear yet which sperm protein is indeed necessary for this binding. Moreover, the fact that mouse sperm, which undergo AR before interacting with the egg, are fertile raises the question if indeed we will find a sperm ZP-receptor protein that is absolutely critical for fertilization (Fig. 1).



Intermediate Stages and Spontaneous Acrosome Reaction

Although AR is a precise well-regulated exocytotic process, spontaneous AR (sAR) can occur under some conditions. Sperm samples with high proportion of sAR result in poor success of fertilization. Therefore, sperm contain mechanisms that protect them from undergoing sAR. Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) protects mouse sperm from undergoing sAR by interacting with MUPP1. Inhibition of CaMKII in bovine sperm during the incubation under capacitation conditions strongly induces sAR. We showed that actin polymerization occurs during mammalian sperm capacitation, and the formed F-actin should be dispersed prior to the AR. The process of actin polymerization is mediated by two distinct pathways: the PLD-pathway and the CaMKII-pathway. Actin polymerization during sperm capacitation protects the sperm from undergoing sAR. Thus, activation of PLD and/or CaMKII during sperm capacitation is essential for preventing the occurrence of sAR.

Many studies show that sperm representing intermediate stages of fully and partially acrosome reacted cells. Four basic patterns were discovered in mouse and guinea pig sperm, and six in human sperm. It was also shown that few morphological and biochemical steps of hybrid vesicle formation occur prior to complete the AR. It is concluded that sAR is not a synchronous process.

Further Reading

- Ackermann, F., Zittramski, N., Borth, H., et al. (2009). CaMKII α interacts with multi PDZ domain protein MUPP1 in spermatozoa and prevents spontaneous acrosomal exocytosis. *Journal of Cell Science*, 122, 4547–4557.
- Bleil, J. D., & Wassarman, P. M. (1983). Sperm–egg interactions in the mouse: Sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Developmental Biology*, 95, 317–324.
- Branham, M. T., Bustos, M. A., De Blas, G. A., et al. (2009). Epac activates the small G proteins Rap1 and Rab3A to achieve exocytosis. *The Journal of Biological Chemistry*, 284, 24825–24839.
- Breitbart, H., & Etkovitz, N. (2011). Role and regulation of EGFR in actin remodelling in sperm capacitation and the acrosome reaction. *Asian Journal of Andrology*, 13, 106–110.
- Brenner, E., Rubinstein, S., Cohen, G., et al. (2003). Remodelling of the actin cytoskeleton during mammalian sperm capacitation and acrosome reaction. *Biology of Reproduction*, 68, 837–845.
- Buffone, M. G., Hirohashi, N., & Gerton, G. L. (2014). Unresolved questions concerning mammalian sperm acrosomal exocytosis. *Biology of Reproduction*, 90, 112.
- Cherr, G. N., Lambert, H., Meizel, S., & Katz, D. F. (1986). *In vitro* studies of the golden hamster sperm acrosome reaction: Completion on the zona pellucida and induction by homologous soluble zonae pellucidae. *Developmental Biology*, 114, 119–131.
- Crozet, N., & Dumont, M. (1984). The site of the acrosome reaction during *in vivo* penetration of the sheep oocyte. *Gamete Research*, 10, 97–105.
- Dam, A. H., Feenstra, I., Westphal, J. R., et al. (2007). Globozoospermia revisited. *Human Reproduction Update*, 13, 63–75.
- Etkovitz, N., Tirosh, Y., Chazan, R., et al. (2009). Bovine sperm acrosome reaction induced by G-protein-coupled receptor agonists is mediated by epidermal growth factor receptor transactivation. *Developmental Biology*, 334, 447–457.
- Finkelstein, M., Etkovitz, N., & Breitbart, H. (2010). Role and regulation of sperm gelsolin prior to fertilization. *The Journal of Biological Chemistry*, 285, 39702–39709.
- Fisher, H. M., Brewis, I. A., Barratt, C. L., Cooke, I. D., & Moore, H. D. (1998). Phosphoinositide 3-kinase is involved in the induction of human sperm acrosome reaction downstream of tyrosine phosphorylation. *Molecular Human Reproduction*, 4, 849–855.
- Florman, H. M., & Storey, B. T. (1982). Mouse gamete interactions: The zona pellucida is the site of the acrosome reaction leading to fertilization *in vitro*. *Developmental Biology*, 91, 121–130.
- Florman, H. M., Jungnickel, M. K., & Sutton, K. A. (2008). Regulating the acrosome reaction. *The International Journal of Developmental Biology*, 52, 503–510.
- Hino, T., Muro, Y., Tamura-Nakano, M., et al. (2016). The behaviour and acrosomal status of mouse spermatozoa *in vitro*, and within the oviduct during fertilization after natural mating. *Biology of Reproduction*, 95, 50.
- Hutt, D. M., Baltz, J. M., & Ngsee, J. K. (2005). Synaptotagmin VI and VIII and syntaxin 2 are essential for the mouse sperm acrosome reaction. *The Journal of Biological Chemistry*, 280, 20197–20203.
- Ickowicz, D., Finkelstein, M., & Breitbart, H. (2012). Mechanism of sperm capacitation and the acrosome reaction: Role of protein kinases. *Asian Journal of Andrology*, 14, 816–821.
- Jaldety, Y., Glick, Y., Orr-Urtreger, A., et al. (2012). Sperm epidermal growth factor receptor (EGFR) mediates $\alpha 7$ acetylcholine receptor (AChR) activation to promote fertilization. *The Journal of Biological Chemistry*, 287, 22328–22340.
- Jin, M., Fujiwara, E., Kakiuchi, Y., et al. (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during *in vitro* fertilization. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 4892–4896.
- Joshi, C. S., Khan, S. A., & Khole, V. V. (2014). Regulation of acrosome reaction by Liprin $\alpha 3$, LAR and its ligands in mouse spermatozoa. *Andrology*, 2, 165–174.
- Jungnickel, M. K., Sutton, K. A., Wang, Y., & Florman, H. M. (2007). Phosphoinositide-dependent pathways in mouse sperm are regulated by egg ZP3 and drive the acrosome reaction. *Developmental Biology*, 304, 116–126.
- Kang-Decker, N., Mantchev, G. T., Juneja, S. C., McNiven, M. A., & van Deursen, J. M. (2001). Lack of acrosome formation in Hrb-deficient mice. *Science*, 294, 1531–1533.
- La Spina, F. A., Molina, C. P., Romarowski, A., et al. (2016). For most mouse sperm, the site where acrosomal exocytosis occurs *in vivo* is the upper isthmus of the oviduct. *Developmental Biology*, 15, 172–182.
- Lee, M. A., & Storey, B. T. (1985). Evidence for plasma membrane impermeability to small ions in acrosome-intact mouse spermatozoa bound to mouse zonae pellucidae, using an aminoacridine fluorescent pH probe: Time course of the zona-induced acrosome reaction monitored by both chlortetracycline and pH probe fluorescence. *Biology of Reproduction*, 33, 235–246.
- Lin, Y. N., Roy, A., Yan, W., Burns, K. H., & Matzuk, M. M. (2007). Loss of zona pellucida binding proteins in the acrosomal matrix disrupts acrosome biogenesis and sperm morphogenesis. *Molecular and Cellular Biology*, 27, 6794–6805.
- Lopez, C. I., Belmonte, S. A., De Blas, G. A., & Mayorga, L. S. (2007). Membrane-permeant Rab3A triggers acrosomal exocytosis in living human sperm. *FASEB Journal*, 21, 4121–4130.
- Lu, Q., & Shur, B. D. (1997). Sperm from beta 1,4-galactosyltransferase-null mice are refractory to ZP3-induced acrosome reactions and penetrate the zona pellucida poorly. *Development*, 124, 4121–4131.
- Lucchesi, O., Ruete, M. C., Bustos, M. A., et al. (2016). The signalling module cAMP/Epac/Rap1/PLC ϵ /IP3 mobilizes acrosomal calcium during sperm exocytosis. *Biochimica et Biophysica Acta*, 1863, 544–561.
- Michaut, M., De Blas, G., Tomes, C. N., et al. (2001). Synaptotagmin VI participates in the acrosome reaction of human spermatozoa. *Developmental Biology*, 235, 521–529.
- Muro, Y., Hasuwa, H., Isotani, A., et al. (2016). Behaviour of mouse spermatozoa in the female reproductive tract from soon after mating to the beginning of fertilization. *Biology of Reproduction*, 94, 80.

- Reid, A. T., Lord, T., Stanger, S. J., et al. (2012). Dynamin regulates specific membrane fusion events necessary for acrosomal exocytosis in mouse spermatozoa. *The Journal of Biological Chemistry*, 287, 37659–37672.
- Rodríguez, F., Bustos, M. A., Zanetti, M. N., et al. (2011). A-SNAP prevents docking of the acrosome during sperm exocytosis because it sequesters monomeric syntaxin. *PLoS One*, 6, 21925.
- Roggero, C. M., De Blas, G. A., Dai, H., et al. (2007). Complexin/syntaxin interplay controls acrosomal exocytosis. *The Journal of Biological Chemistry*, 282, 26335–26343.
- Ruete, M. C., Lucchesi, O., Bustos, M. A., & Tomes, C. N. (2014). Epac, Rap and Rab3 act in concert to mobilize calcium from sperm's acrosome during exocytosis. *Cell Communication and Signalling*, 12, 43.
- Saling, P. M., & Storey, B. T. (1979). Mouse gamete interaction during fertilization *in vitro*: Chlorotetracycline as a fluorescent probe for the mouse acrosome reaction. *The Journal of Cell Biology*, 83, 544–555.
- Saling, P. M., Sowinski, J., & Storey, B. T. (1979). An ultrastructural study of epididymal mouse spermatozoa binding to zona pellucidae *in vitro*: Sequential relationship to the acrosome reaction. *The Journal of Experimental Zoology*, 209, 229–238.
- Satouh, Y., Inoue, N., Ikawa, M., & Okabe, M. (2012). Visualization of the moment of mouse sperm–egg fusion and dynamic localization of IZUMO1. *Journal of Cell Science*, 125, 4985–4990.
- Shabtay, O., & Breitbart, H. (2016). CaMKII prevents spontaneous acrosomal exocytosis in sperm through induction of actin polymerization. *Developmental Biology*, 415, 64–74.
- Sosnik, J., Miranda, P. V., Spiridonov, N. A., et al. (2009). Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. *Journal of Cell Science*, 122, 2741–2749.
- Sosnik, J., Buffone, M., & Visconti, P. E. (2010). Analysis of CAPZA3 localization reveals temporally discrete events during the acrosome reaction. *Journal of Cellular Physiology*, 224, 575–580.
- Sotomayor, R. E., & Handel, M. A. (1986). Failure of acrosome assembly in a male sterile mouse mutant. *Biology of Reproduction*, 34, 171–182.
- Spungin, B., & Breitbart, H. (1996). Calcium mobilization and influx during sperm exocytosis. *Journal of Cell Science*, 109, 1947–1955.
- Spungin, B., Levinshal, T., Rubinstein, S., & Breitbart, H. (1992). Fusion of isolated sperm plasma and outer acrosomal membranes: A cell free system for the study of the acrosomal reaction. *FEBS Letters*, 311, 155–160.
- Spungin, B., Margalit, I., & Breitbart, H. (1995). Sperm exocytosis reconstructed in a cell-free system: Evidence for the involvement of phospholipase C and actin filaments in membrane fusion. *Journal of Cell Science*, 108, 2525–2535.
- Stock, C. E., & Fraser, L. R. (1987). The acrosome reaction in human sperm from men of proven fertility. *Human Reproduction (Oxford, England)*, 2, 109–119.
- Tollner, T. L., Yudin, A. I., Cherr, G. N., & Overstreet, J. W. (2003). Real-time observations of individual macaque sperm undergoing tight binding and the acrosome reaction on the zona pellucida. *Biology of Reproduction*, 68, 664–672.
- Tomes, C. N., De Blas, G. A., Michaut, M. A., et al. (2005). Alpha-SNAP and NSF are required in a priming step during the human sperm acrosome reaction. *Molecular Human Reproduction*, 11, 43–51.
- Uto, N., Yoshimatsu, N., Lopata, A., & Yanagimachi, R. (1988). Zona-induced acrosome reaction of hamster spermatozoa. *The Journal of Experimental Zoology*, 248, 113–120.
- Wassarman, P. M. (1988). Zona pellucida glycoproteins. *Annual Review of Biochemistry*, 57, 415–442.
- Wassarman, P. M. (1995). Towards molecular mechanisms for gamete adhesion and fusion during mammalian fertilization. *Current Opinion in Cell Biology*, 7, 658–664.
- Yunes, R., Tomes, C., Michaut, M., et al. (2002). Rab3A and calmodulin regulate acrosomal exocytosis by mechanisms that do not require a direct interaction. *FEBS Letters*, 525, 126–130.
- Zanetti, N., & Mayorga, L. S. (2009). Acrosomal swelling and membrane docking are required for hybrid vesicle formation during the human sperm acrosome reaction. *Biology of Reproduction*, 81, 396–405.
- Zhao, L., Burkin, H. R., Shi, X., et al. (2007). Complexin I is required for mammalian sperm acrosomal exocytosis. *Developmental Biology*, 309, 236–244.

The Zona Pellucida

Paul M Wassarman and Eveline S Litscher, Icahn School of Medicine at Mount Sinai, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

AI	Acrosome-intact
AR	Acrosome-reacted
CFCS	Consensus furin cleavage-site
CTS	C-terminal subdomain
ECM	Extracellular matrix
EHP	External hydrophobic patch
IHP	Internal hydrophobic patch
mRNA	Messenger-RNA
NTS	N-terminal subdomain
SCS	Sperm combining-site
SS	Signal sequence
TMD	Transmembrane domain
VE	Vitelline envelope
ZP	Zona pellucida
ZPD	Zona pellucida domain

Outline

This article describes the nature, synthesis, secretion, assembly, structure, and function of the unique proteins that make up the mammalian egg's extracellular coat, the zona pellucida (ZP). Mention also is made of other vertebrate egg-coat proteins, location and expression of ZP genes, and evolution of ZP proteins.

The Mammalian Egg's ZP

The ovum or unfertilized egg is the link between one generation and the next. It is the process of oogenesis in females that is responsible for production of unfertilized eggs (Albertini, 2015). During oogenesis primordial germ cells generated in the fetus become mitotic oogonia, enter meiosis and become oocytes, achieve full-growth in the ovary, undergo meiotic maturation, are ovulated, and become unfertilized eggs.

All mammalian eggs and preimplantation embryos are completely surrounded by an extracellular matrix (ECM) called the zona pellucida (ZP) (Fig. 1) (Litscher and Wassarman, 2015). The thickness of the ZP varies from < 1 to > 20 μm depending on the species (Table 1) and plays vital roles in vivo during oogenesis, fertilization, and preimplantation development in mammals. For example, the presence of a ZP is required in vivo for normal production of Graafian follicles by the ovary, species-restricted fertilization of ovulated eggs by sperm, and maintenance of intact cleavage-stage embryos in the reproductive tract. Removal of the ZP from eggs results in direct exposure of the egg's plasma membrane to sperm and permits sperm from heterologous species to fertilize eggs (Table 1).

The ZP appears during oogenesis as an extremely thin ECM around oocytes that have begun to grow and increases in thickness as oocytes increase in size (Table 1). For example, mouse oocytes grow > 300-fold in volume, from about 12 to 80 μm in diameter, over 2–3 weeks and at the same time the ZP thickens to about 6.5 μm in width. The ZP consists of long, crosslinked fibrils that make up an elastic, relatively porous ECM that is penetrable by large macromolecules such as antibodies and even by small viruses. The innermost fibrils are oriented radially to the oocyte's plasma membrane and are closely packed, whereas the outermost fibrils are oriented tangentially and are more loosely packed. Following fertilization of ovulated eggs, preimplantation embryos at the expanded-blastocyst stage hatch from their ZP using protease(s) and then implant in the uterus.

ZP Proteins

The mammalian ZP is composed of either 3 (e.g., mouse and cow eggs) or 4 (e.g., rat and human eggs) glycosylated proteins, called ZP1–4, depending on the species (Wassarman, 2008; Litscher and Wassarman, 2015). ZP2 and ZP3 are monomers and ZP1 and ZP4

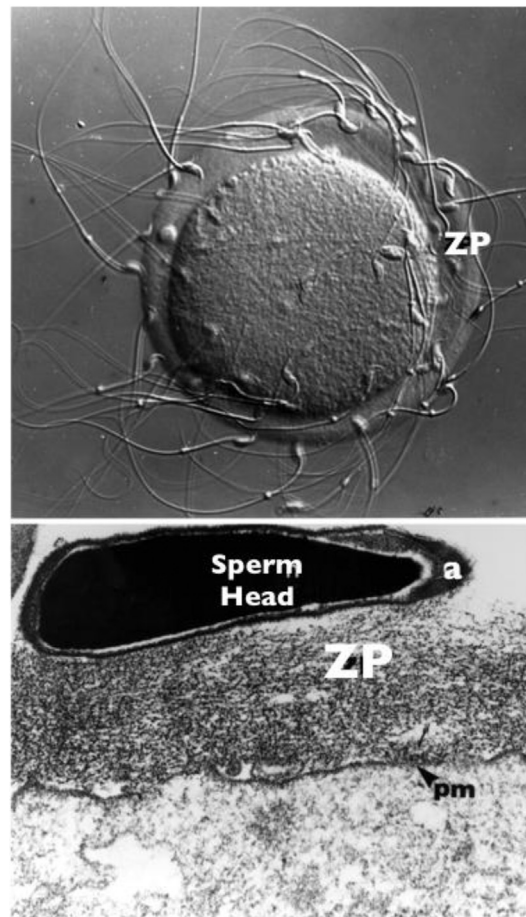


Fig. 1 Light and electron microscopic images of mouse sperm bound to the ZP of unfertilized eggs. Shown are Nomarski differential interference contrast (*top*) and transmission electron microscopic (*bottom*) images. In the bottom panel is a thin section of a sperm head with an intact acrosome (a) bound to the fibrillar ZP of an unfertilized egg. ZP, Zona pellucida; pm, plasma membrane.

are dimers of identical polypeptides connected by intermolecular disulfides. It has been suggested that ZP proteins have properties similar to those of amyloids that self-aggregate and form cross- β sheet fibrillar structures.

All ZP proteins are synthesized as precursor polypeptides that are processed into the mature polypeptides present in the ZP (Table 2). ZP1 and ZP4 are related to one another and have a 45 amino acid sequence, called a P or trefoil domain, that has 6 cysteine residues linked as 3 intramolecular disulfides. ZP2 and ZP3 assemble via non-covalent bonds into long fibrils that are crosslinked by ZP1 and/or ZP4 into a porous ECM. ZP fibrils exhibit a structural repeat due to the presence of a ZP2–ZP3 dimer every 14–15 nm.

Both ZP2 and ZP3 must be present during oogenesis to assemble a ZP. Female mice that are heterozygous-nulls for ZP3 (ZP3^{+/-}) assemble a ZP around oocytes/eggs that is about one-half its normal width, but the mutant mice are as fertile as wild-type mice. On the other hand, female mice that are homozygous-nulls for either ZP2 (ZP2^{-/-}) or ZP3 (ZP3^{-/-}) fail to assemble a ZP around oocytes/eggs and are infertile. Female mice that are homozygous-nulls for ZP1 (ZP1^{-/-}) assemble an extremely porous ZP around oocytes/eggs and exhibit significantly reduced fertility compared to wild-type mice.

Table 1 Approximate dimensions of mammalian eggs and their zona pellucida

Mammal	Egg-diameter (μm)	ZP-width (μm)	Ratio
Platypus	4000	1	4000:1
Possum	230	6	38:1
Mouse	80	6	13:1
Rabbit	125	15	8:1
Cow	120	16	7:1
Pig	130	16	8:1
Baboon	—	13	—
Human	120	22	5:1

Table 2 Size of zona pellucida polypeptides^a

<i>Mammal</i>	<i>ZP1</i>	<i>ZP2</i>	<i>ZP3</i>	<i>ZP4</i>
Platypus	(404) ^b	(700) ^b	403	(593) ^b
Possum	—	712	422	527
Mouse	623	713	424	— ^c
Rat	617	695	424	545
Hamster	616	708	422	543
Rabbit	627	727	419	540
Cow	— ^d	713	421	534
Pig	— ^d	716	421	536
Dog	— ^d	715	426	531
Monkey	640	745	424	539
Chimp	638	745	424	540
Human	638	745	424	540

^aNumber of amino acid residues per polypeptide (for references see [Litscher and Wassarman, 2015](#)).

^bOnly incomplete N- and C-terminal sequences are available.

^cZP4 is a pseudogene in mice.

^dZp1 is a pseudogene in cows and dogs; ZP1 has not been found in pigs.

Location and Expression of ZP Genes

ZP proteins are encoded by single-copy genes located on different chromosomes ([Wassarman, 2008](#); [Litscher and Wassarman, 2015](#)). In mice ZP1–ZP3 are located on chromosomes 19, 7, and 5, respectively. In humans ZP1–ZP4 are located on chromosomes 11, 16, 7, and 1, respectively. ZP genes are expressed coordinately and exclusively by growing oocytes making it female-specific gene expression. The number of copies of ZP messenger-RNA (mRNA) per oocyte or egg increases from undetectable levels (<1000 copies) in non-growing oocytes, to hundreds-of-thousands of copies in mid-stage growing and fully-grown oocytes, to almost undetectable levels (<1000 copies) in oocytes that undergo meiotic maturation and become unfertilized eggs. ZP mRNA also is undetectable in cleavage-stage embryos. During oocyte growth in mice the absolute rate of protein synthesis increases about 40-fold overall, with ZP synthesis representing about 5% of the total.

Conservation of ZP Proteins

ZP2 and ZP3 from different mammals, from monotremes and marsupials to placental mammals, are well conserved, exhibiting about 65%–99% identity. ZP1 and ZP4 exhibit only about 40% identity ([Litscher and Wassarman, 2014, 2015](#)). It has been suggested that proteins with sequence identities of 40% or more perform the same function and those with identities of 25%–40% perform similar functions. ZP proteins have several regions in common (“**Secretion and Assembly of ZP Proteins**” section), such as a signal sequence (SS), ZP domain (ZPD), external and internal hydrophobic patches (EHP, IHP), consensus furin cleavage-site (CFCS), and transmembrane domain (TMD), suggesting they may be derived from a common ancestral gene, perhaps the ZP3 gene, >500 million years ago. This gene duplicated several times during evolution and gave rise to 5 genes in amphibians, 6 genes in birds, and 3–4 genes in mammals. Many fish have 2–4 ZP genes, whereas some fish (e.g., zebrafish) have multiple copies or tandem repeats of ZP1 and ZP3 genes.

Other Vertebrate Egg Coats

It is relevant to note that fish, frog, and bird eggs also have an ECM surrounding them, called the vitelline envelope (VE), that consists of proteins highly related to ZP1–ZP4 ([Litscher and Wassarman, 2014, 2015](#)). The frog and bird egg VE consists of 4–6 proteins, called ZP1–ZP4, ZPd, and ZPax, and for many fish eggs the VE consists of 2–4 proteins, called ZP1, ZP3, ZPax, and variants of ZP1 and ZP3. ZPax is found in a few mammals, but is always present as a pseudo-gene. In fish and birds VE proteins are synthesized by both the ovary and liver, whereas in frogs, as in mammals, they are synthesized only by the ovary.

The ZP Domain

Primary structures of ZP proteins from mammalian eggs have been determined over the past three decades. A hallmark feature of all ZP proteins is an approximately 260 amino acid sequence, containing 8 conserved cysteine residues present as 4 intramolecular disulfides, that is called the ZPD ([Jovine et al., 2005](#); [Litscher and Wassarman, 2015](#)). The ZPD arose >600 million years ago and is found in hundreds of proteins in a variety of animal species, from invertebrates to vertebrates, and in a wide variety of tissues

and organs. These proteins include secreted proteins that function as receptors or mechanical transducers between adjacent cells, as intermediators during differentiation, morphogenesis, and signaling, or as proteins of ECMs other than egg-coats.

The ZPD is a bipartite structure consisting of an amino (N)-terminal subdomain (NTS) and a carboxy (C)-terminal subdomain (CTS) separated by a short protease-sensitive region. The two subdomains are always found in the sequential order NTS → CTS and never CTS → NTS, although the NTS, but not the CTS, is found in some proteins in the absence of a CTS (e.g., Oosp1, Plac1, and papillote). Proteins possessing a ZPD form polymers and isolated ZPDs on their own can polymerize into fibrils, with the NTS, not the CTS, responsible for polymerization. Divergent copies of the NTS are found in single or multiple copies in the amino-terminal regions of ZP1, ZP2, ZP4, and ZPax. Mutation of ZPDs, especially the NTS, can lead to severe pathologies, such as deafness, cancer, and possibly infertility.

Three-Dimensional Structure of ZP Proteins

The three-dimensional structure of the ZPD has been determined by X-ray crystallographic analysis of the (i) NTS of mouse ZP3 at 2.3 Å resolution, (ii) full-length chicken ZP3 at 2.0 Å resolution, (iii) protease resistant core (amino acid residues 295–610) of human uromodulin, a ZPD protein, at 3.2 Å resolution, and (iv) CTS (amino acid residues 463–664) of mouse ZP2 at 2.25 Å resolution. These analyses revealed that both the NTS and CTS adopt an immunoglobulin (Ig)-like fold consisting of an antiparallel sandwich of 2 β-sheets made up of 8 strands of polypeptide (strands A, B, D, and E and C, E', F, G; ≈ 50% of amino acid residues are in β-strands) that enclose a hydrophobic core, with 2 buried disulfides that clamp both sides of the sandwich (Monné and Jovine, 2011). The NTS of mouse ZP3 has approximate dimensions of 52 × 29 × 28 Å. In ZP3 crystals, two ZP3 molecules are arranged as homodimers in antiparallel orientation to form an asymmetric structure. The two ZPDs are bonded by electrostatic interactions between the NTS and CTS of opposing molecules; that is, NTS1:CTS2 and NTS2:CTS1. On the other hand, there are no NTS1:NTS2 or CTS1:CTS2 contacts within the homodimer. However, certain structural features make the ZPD distinct from other known Ig-like domains, such that it is a new Ig superfamily subtype structure. The NTS and CTS of uromodulin are similar to those of ZP3, having the same fold, disulfide connectivities, and a conserved tyrosine residue in the NTS; mutation of this residue in tectorin, a ZPD protein, is associated with hearing loss.

Secretion and Assembly of ZP Proteins

Certain sequence elements involved in secretion and/or assembly of nascent ZP proteins are located near their amino- or carboxy-terminus (Wassarman and Litscher, 2013) (Fig. 2). (i) One sequence element is an SS located at the amino-terminus that targets nascent ZP proteins to the secretory pathway where the SS is removed. The SS is removed during transit of nascent protein from the oocyte's endoplasmic reticulum to the Golgi. (ii) A second sequence element is an EHP located near the carboxy-terminus that interacts with an IHP located within the ZPD. The EHP–IHP interaction locks the ZPD in a conformation that is incompatible with polymerization, thereby preventing ZP protein assembly into fibrils within growing oocytes. Such coupling between proteolytic processing and polymerization is conserved by many or all ZPD proteins. (iii) A third sequence element is a TMD located

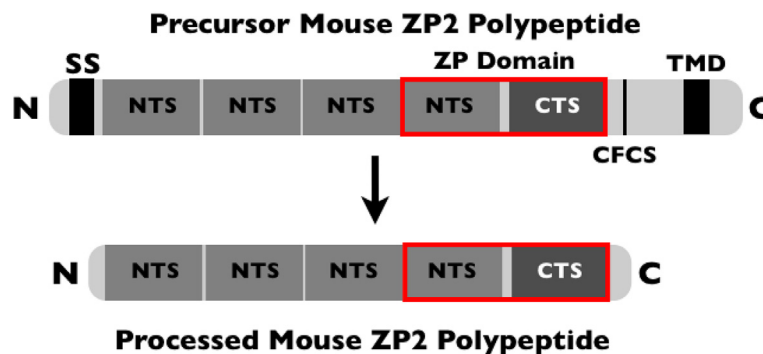


Fig. 2 Diagram of the precursor (*top*) and processed (*bottom*) mouse ZP2 polypeptides. ZP2 serves as an example of the four ZP polypeptides, ZP1–4, found in the ZP of eggs from different mammals. Precursor and processed ZP2 polypeptides consists of 713 and 599 amino acids, respectively. Illustrated at the top are the signal-sequence (SS), ZP domain (ZPD) consisting of an N-terminal and C-terminal subdomain (NTS and CTS, respectively; indicated by a red border), consensus furin cleavage-site (CFCS), and transmembrane domain (TMD). Also shown are the three extra copies of the N-terminal subdomain (NTS) of the ZPD located at the N-terminus of the ZP2 polypeptide [note: ZP3 polypeptide does not have extra copies of the NTS at its N-terminus]. After proteolytic processing at the CFCS the mature polypeptide (*bottom*), lacking the SS, CFCS, TMD, and short C-terminal sequence is incorporated into the ZP. ZP1 and ZP4 also have a trefoil domain, consisting of about 45 amino acids with 3 intramolecular disulfides, N-terminal to the ZPD. N, amino-terminus; C, carboxy-terminus. For additional information see Litscher and Wassarman, 2015.

at the carboxy-terminus that anchors nascent ZP proteins in secretory vesicles and plasma membrane. (iv) A fourth sequence element is a CFCS that is cleaved by a pro-protein convertase so that secretion of nascent ZP proteins takes place.

Within growing oocytes nascent ZP proteins are localized to secretory vesicles that originate from the trans-Golgi and these vesicles fuse with the oocyte's plasma membrane. The oocyte's Golgi is an important site of processing of secretory proteins and it expands dramatically in growing oocytes from flattened stacked lamellae in non-growing oocytes to swollen stacked lamellae associated with numerous vacuoles in fully-grown oocytes. Cleavage of ZP proteins at their CFCS takes place at the oocyte's plasma membrane and results in release of the proteins into the extracellular space. The processed, mature proteins then assemble on the innermost surface of the thickening ZP.

ZP Proteins and Fertilization

Fertilization of mammalian eggs occurs shortly after they are released or ovulated from the ovary into the reproductive tract. There is reason to believe that the ZP plays a role in species-restricted fertilization in mammals in much the same way as the VE restricts interspecies fertilization in some non-mammalian animals. Sperm bind to the ZP of unfertilized eggs and both ZP2 and ZP3 act as sperm receptors (Bleil and Wassarman, 1980; Avella et al. 2013; Florman and Fissore, 2014; Bianchi and Wright, 2016).

Mammalian sperm have an organelle derived from Golgi, called the acrosome, overlying their head (acrosome-intact/AI-sperm) (Fléchon 2016). To penetrate the ZP sperm must undergo the acrosome reaction (AR), a form of cellular exocytosis. This involves fusion of plasma membrane with outer acrosomal membrane and results in exposure of inner acrosomal membrane and its associated enzymes (acrosome-reacted/AR-sperm). Bound sperm are able to penetrate the ZP due to the action of one or more of these enzymes and to forward thrust of the sperm due to flagellar hyperactivity.

AI-sperm bind to ZP3 and AR-sperm bind to ZP2 of unfertilized eggs (Bleil and Wassarman, 1986; Avella et al., 2014; Wassarman and Litscher, 2016). Whereas binding of AR-sperm to ZP2 is due only to its polypeptide, binding of AI-sperm to ZP3 is due to its polypeptide and carbohydrate; the latter binding leads to induction of the AR. The region of ZP3 to which sperm bind is called the sperm combining-site (SCS) and it is a site of positive Darwinian selection perhaps having to do with species-restricted fertilization. It is possible that selective pressure drives the mutations in the SCS and thereby promotes divergence and evolution of ZP3. Following fertilization of eggs sperm can no longer bind to either ZP2 or ZP3 thereby contributing to prevention of polyspermy.

References

- Albertini, D. F. (2015). *The mammalian oocyte. Physiology of Reproduction* (4th edn., vol. 1, pp. 59–97). San Diego, CA: Academic Press.
- Avella, M. A., Xiong, B., & Dean, J. (2013). The molecular basis of gamete recognition in mice and humans. *Molecular Human Reproduction*, 19, 279–289.
- Avella, M. A., Baibakov, B., & Dean, J. (2014). A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *The Journal of Cell Biology*, 205, 801–809.
- Bianchi, E., & Wright, G. J. (2016). Sperm meets egg: The genetics of mammalian fertilization. *Annual Review of Genetics*, 50, 93–111.
- Bleil, J. D., & Wassarman, P. M. (1980). Mammalian sperm–egg interaction: Identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. *Cell*, 20, 873–882.
- Bleil, J. D., & Wassarman, P. M. (1986). Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. *The Journal of Cell Biology*, 102, 1363–1371.
- Fléchon, J. E. (2016). The acrosome of eutherian mammals. *Cell and Tissue Research*, 363, 147–157.
- Florman, H. M., & Fissore, R. A. (2014). *Fertilization in mammals. Physiology of reproduction* (4th edn., Vol. 1, pp. 149–196). San Diego, CA: Academic Press.
- Jovine, L., Darie, C. C., Litscher, E. S., & Wassarman, P. M. (2005). Zona pellucida domain proteins. *Annual Review of Biochemistry*, 74, 83–114.
- Litscher, E. S., & Wassarman, P. M. (2014). Evolution, structure, and synthesis of vertebrate egg-coat proteins. *Trends in Developmental Biology*, 8, 65–76.
- Litscher, E. S., & Wassarman, P. M. (2015). *A guide to zona pellucida domain proteins* (p. 208). Hoboken, NJ: John Wiley and Sons.
- Monné, M., & Jovine, L. (2011). A structural view of egg coat architecture and function in fertilization. *Biology of Reproduction*, 85, 661–669.
- Wassarman, P. M. (2008). Zona pellucida glycoproteins. *Journal of Biological Chemistry*, 283, 24285–24289.
- Wassarman, P. M., & Litscher, E. S. (2013). Biogenesis of the mouse egg's extracellular coat, the zona pellucida. *Current Topics in Developmental Biology*, 102, 243–266.
- Wassarman, P. M., & Litscher, E. S. (2016). A bespoke coat for eggs: Getting ready for fertilization. *Current Topics in Developmental Biology*, 117, 539–552.

The Zona Pellucida Facilitates Fertilization, Blocks Polyspermy and Protects Pre-Implantation Embryos

Keizo Tokuhiko and Jurrien Dean, National Institutes of Health, Bethesda, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome exocytosis The outer membrane of the acrosome, a subcellular organelle in the sperm anterior, fuses with the overlying plasma membrane to release its contents.

Cumulus oophorous Matrix rich in hyaluronic acid interspersed with cumulus cells that surrounds ovulated eggs in the oviduct.

Capacitation Sperm develop the capacity for acrosome exocytosis and hyperactive motility in preparation of fertilization.

Preimplantation development Following fertilization, the 1-cell (1C) zygote undergoes 3 cell division after which it compacts to form the morula (8-12C) and develops into a blastocyst with a fluid filled blastocoel which implants on the wall of the uterus.

Uterotubal junction The narrow passage through which sperm pass from the uterus to the oviduct.

Introduction

At birth, mammals have their complete complement of female germ cells which are arrested at the diplotene stage of the prophase of the first meiotic division. This arrested state, known as the dictyate, persists until just prior to ovulation and, depending on the oocyte, lasts weeks in mice and years in humans. Perinatally, granulosa cells cull out individual oocytes from a cluster to form primordial follicles in which the central germ cell is surrounded by flattened granulosa cells. From this resting pool of germ cells, cohorts, the size of which vary among mammals, are selected to enter a growth phase during which the zona pellucida proteins are secreted to form an extracellular matrix. Zona proteins are no longer produced in transcriptionally inert, fully grown oocytes which precludes further accretion of the zona matrix. During subsequent meiotic maturation and cumulus expansion in ovulatory (Graafian) follicles, a perivitelline space develops between the inner aspect of the zona pellucida and the egg plasma membrane.

Although tens of million sperm are deposited in the lower female reproductive tract at the time of coitus, relatively few (<10/egg) encounter ovulated eggs in the ampulla of the oviduct. Sperm initially ascend the female reproductive tract with linear, progressive motility characterized by low-amplitude, symmetric flagellar beating. Following penetration of the uterotubal junction, sperm accumulate in storage reservoirs in the oviduct isthmus by binding with their heads to the epithelial lining. Sperm undergo capacitation, an incompletely understood process, and the flagellar beat of sperm becomes asymmetric and develops high amplitude which facilitates release from the epithelium of the oviduct and assists in penetration of the investments that surround ovulated eggs. First, the hyperactive sperm encounters the viscoelastic hyaluronan of the cumulus oophorous in the ampulla of the oviduct. After penetrating the cumulus matrix, the sperm bind and penetrate the zona pellucida surrounding ovulated eggs. At some point during passage of the female reproductive tract, sperm undergo acrosome exocytosis and only acrosome-reacted sperm can fuse with the egg.

After fertilization, waves of calcium activate the egg which results in an effective block to polyspermy. There is an immediate block at the egg plasma membrane and additional sperm cannot fuse with the egg. This block requires gamete fusion and does not occur after fertilization by intracytoplasmic sperm injection. The calcium waves in the egg prompt cortical granule exocytosis which provides a potent block to sperm penetration through the zona pellucida matrix by mechanisms yet to be determined. Most importantly, cortical granules release lytic enzymes which modify the zona matrix to prevent sperm binding and provide a definitive block to polyspermy (i.e., if sperm do not bind to the zona surface, they cannot penetrate the zona matrix nor fuse with the egg plasma membrane).

The zona pellucida also facilitates the passage of the embryo through the oviduct. The preimplantation embryo divides within the confines of the zona pellucida during cleavage-stage development and formation of the morula. As the blastocyst forms, the zona pellucida thins and eventually the embryo hatches from the zona matrix leaving a ghost matrix behind. Whether this escape is purely mechanical or is assisted by enzymatic digestion remains unclear. Thus, the zona pellucida plays critical roles in fertilization, the postfertilization block to polyspermy and protecting the embryo prior to implantation.

In this article, we will review the biogenesis of the zona pellucida and its function during fertilization and preimplantation development using research focused on mouse and human model systems.

Biogenesis of the Zona Pellucida

Among mammals, the zona pellucida is composed of three or four homologous glycoproteins with varying designations that we have simplified to ZP1, ZP2, ZP3, and ZP4 based on mouse and human nomenclature. The human zona pellucida is encoded by four zona genes, but mouse *Zp4* is a pseudogene and the mouse zona pellucida contains only ZP1, ZP2, and ZP3 proteins. Each zona protein has a signal peptide at its N-terminus that directs it into the lumen of the endoplasmic reticulum and a transmembrane domain near its C-terminus that tethers it to the lipid bilayer during passage through the endomembrane system (Fig. 1A).

Each zona protein contains a ~260 amino acid bi-partite (N- and C-domains) zona module near the C-terminus. Based on conserved disulfide bond formation, two isoforms of the domain have been defined. One isoform, specific for ZP3 has a longer (44–45 amino acid) extension C-terminal to the zona module and the other isoform, specific for the remaining zona proteins has a shorter (0–5 amino acid) extension. It appears that at least one of each isoform is required to form a zona matrix. The structure of the N- and C-portion domains as well as the complete zona module of the chicken homologue of ZP3 have been solved by X-ray crystallography. Based on these results, a model has been proposed in which proteolysis at the maturation cleavage site (MCS) uncovers a hydrophobic patch on the C-ZP domain which promotes polymerization of the zona matrix in the extracellular space (Han et al., 2010).

Initially, the zona matrix is observed as discrete electron-dense clumps by electron microscopy that fill in during oocyte growth such that the fully-grown mouse (80 μm diameter) and human (120 μm diameter) eggs have 7 μm and 15 μm thick zonae pellucidae, respectively (Fig. 1B and C). By high resolution confocal and scanning electron microscopy, the zona pellucida appears as a porous sponge through which sperm must penetrate to fertilize the encased egg. Using mouse genetics, individual zona genes have been ablated. Mice lacking ZP1 form a zona pellucida that is thinner than normal and egg-free zona ghosts are observed in the oviduct with a concomitant decrease in fecundity. A more severe phenotype is observed in the absence of ZP2 in which a very thin zona matrix is observed during oocyte growth, but no zona pellucida is present surrounding eggs in preovulatory follicles. In the absence of ZP3, no matrix is formed at all and the zona-free eggs lacking either ZP2 or ZP3 are quickly resorbed into the epithelial lining of the oviduct after ovulation rendering female mice sterile (Fig. 2A). A frameshift that resulted in a truncated isoform of human ZP1 in a consanguineous family caused primary infertility in four sisters was reported as a potential dominant negative mutation that prevents formation of the zona pellucida matrix by premature aggregation of zona proteins in oocytes (Huang et al., 2014).

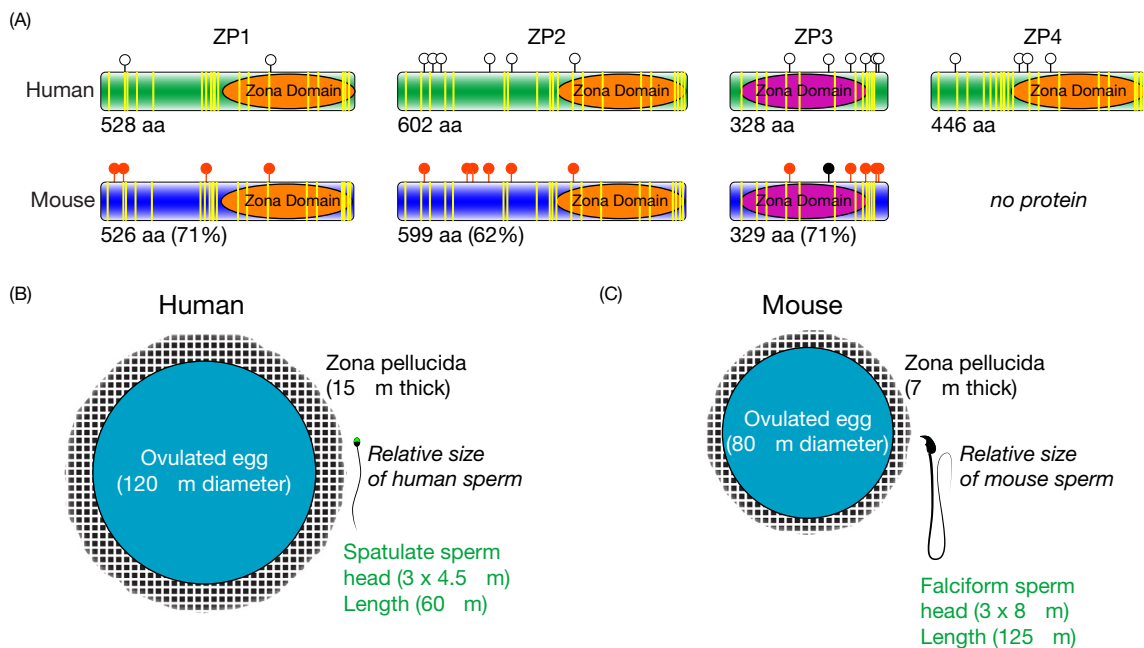


Fig. 1 Human and mouse zona pellucida. (A) Single copy genes encode human (ZP1, ZP2, ZP3, ZP4) and mouse (ZP1, ZP2, ZP3) zona proteins. After processing and secretion, the mature proteins are 62%–71% identical between the two species and the absence of mouse ZP4 reflects a pseudogene. Each protein has a bi-partite zona module for polymerization. Yellow lines, cysteine residues. Circles, potential *N*-glycan attachment sites: red, glycan present; black, glycan absent; open, not determined. The number of amino acids is below each mature zona protein. (B) Schematic representations of human zona pellucida surrounding ovulated egg with human sperm at right. (C) Same as (B), but for mouse.

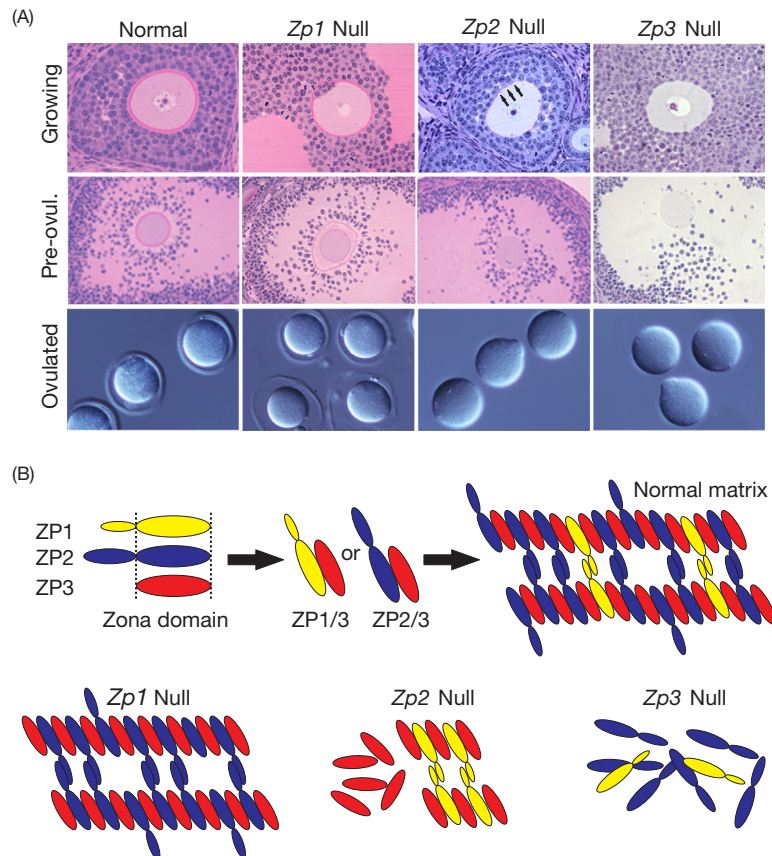


Fig. 2 Genetic ablation of mouse *Zp1*, *Zp2*, and *Zp3*. (A) Ovarian histology of growing (*top*) and preovulatory (*middle*) oocytes from wildtype, *Zp1*^{Null}, *Zp2*^{Null}, and *Zp3*^{Null} mice fixed in glutaraldehyde, embed in methacrylate, and stained with periodic acid Schiff reagent. *Arrows*, thin zona pellucida. Differential interference contrast (DIC) images of unfixed ovulated eggs from each genotype (*bottom*). (B) Hypothetical model of the insoluble zona pellucida (*top*) based on the mutant mouse lines in (A) in which ZP2/3 (*bottom, left*) and ZP1/3 (*bottom, middle*) can form zona matrices, but ZP1/2 (*bottom, right*) cannot. Although less abundant, ZP1 forms disulfide-bonded dimers thought to strengthen the zona pellucida.

Fertilization

Zona Pellucida Ligand

Early models of gamete recognition emphasized the potential role of O-glycans associated with ZP3 as the primary ligand in the zona pellucida for a sperm surface receptor with β -galactosyl transferase a proposed candidate (Fig. 3A). However, biochemical analyses did not detect the proposed O-glycans on mouse ZP3 (Boja et al., 2003) and mutation of the putative attachment sites did not affect fertility (Gahlay et al., 2010). In addition, mice remained fertile after conditional ablation of the gene encoding T-synthase involved in forming implicated core 1 and core 2 O-glycan in zona pellucida (Williams et al., 2007). Because of its abundance in human zona pellucida, sialyl-Lewis^x antigen was proposed as a zona ligand (Pang et al., 2011), but human sperm bind and penetrate “humanized” zonae pellucidae in the absence of the sugar (Avella et al., 2014). The identity of putative sperm surface receptor for the zona ligand remains elusive and genetic ablation of genes encoding candidate proteins does not cause infertility. Models of gamete guidance based on chemo- or thermotaxis have been proposed but mechanistic molecular details remain to be determined, although the CatSper channel has been implicated in rheotaxis (Fig. 3B).

More recently, using genetically modified mice in gain-of- and loss-of-function assays, the N-terminus of ZP2 has been proposed as the zona ligand for sperm binding (Fig. 3C). Capacitated human sperm bind to human, but not mouse, zonae pellucidae. In transgenic mice, individual human ZP1, ZP2, ZP3, and ZP4 proteins were expressed in the absence of the cognate mouse protein. Human sperm bound and penetrated a zona pellucida containing human ZP2 (huZP2), but not huZP1, huZP3 or huZP4 (Baibakov et al., 2012). Mouse sperm did not bind to a zona matrix lacking moZP2 and these mice were infertile in vivo. Using chimeric human/mouse ZP2 proteins expressed in mice lacking endogenous ZP2 as well as ZP2 peptide beads, the sperm binding domain was further defined as moZP2^{51–149} (Avella et al., 2014) and neither N- nor O-glycans in this region are required for sperm-egg recognition and fertility. ZP2 peptide-beads can decoy sperm and act as effective, long-term, reversible contraceptives (Avella et al., 2016).

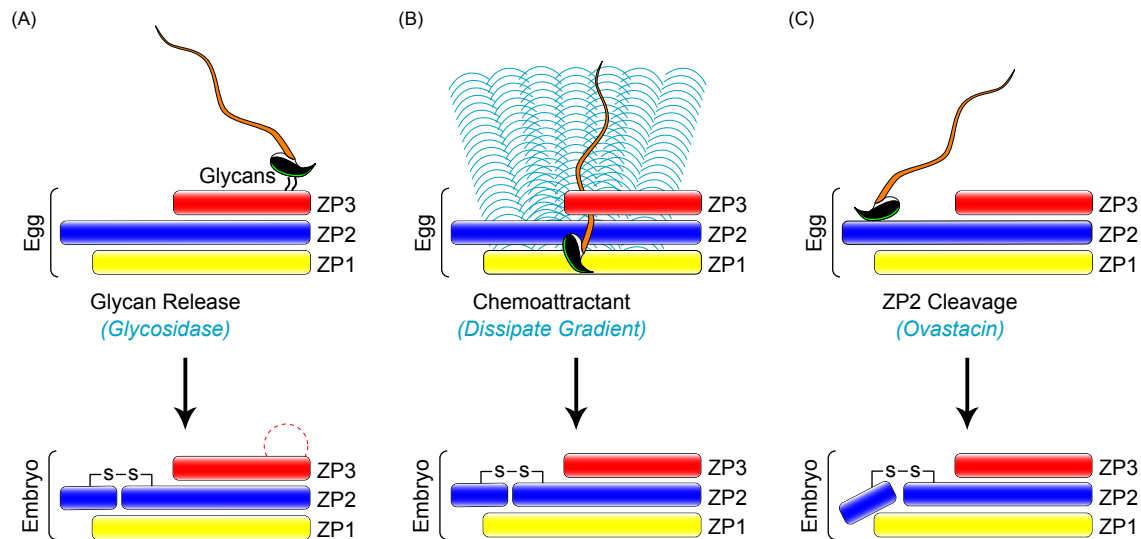


Fig. 3 Models of gamete recognition on the surface of the zona pellucida. (A) In the glycan-release model, *O*-glycans attached to ZP3 act as zona ligands for sperm surface receptors. After fertilization, putative cortical granule glycosidase(s) remove the glycan to prevent sperm binding. (B) In the chemoattractant model, either chemo-, thermo- or rheo-attractants guide sperm to ovulated eggs and dissipation of the gradients after fertilization prevent polyspermy. (C) In the ZP2 cleavage model, sperm bind to the N-terminus of ZP2 which is cleaved after fertilization by ovastacin released by cortical granules to prevent sperm binding. Only the ovastacin–ZP2 cleavage model has been substantiated by mouse genetics.

Sperm Receptor

Although candidate cognate sperm receptors for the zona ligand have been proposed, their genetic ablation does not cause male infertility in the absence of disrupted spermatogenesis. A clue to the putative receptor's identity may lie in the acrosome status when sperm bind to the surface of the zona pellucida. The acrosome is a Golgi derived, subcellular organelle underlying the anterior surface of the sperm that undergoes exocytosis during fertilization. There is general agreement that acrosome-intact sperm ascend into the oviduct to fertilize ovulated eggs in the ampulla and only acrosome-reacted sperm are observed in the perivitelline space between the egg plasma membrane and the inner aspect of the zona pellucida. However, the precise location and trigger for acrosome exocytosis have been difficult to pin down and the functions of the released proteins are incompletely understood.

Early investigators proposed that acrosome exocytosis occurred before sperm encountered ovulated eggs in the ampulla of the oviduct. Recent observations in transgenic mice in which acrosome-reacted sperm penetrate the zona matrix and fertilize cumulus-intact eggs support this hypothesis as does recent *in vitro* and *in vivo* imaging (Hino et al., 2016). An alternative model in which exocytosis is induced by ZP3 at the surface of the zona pellucida has become less compelling with the observation that sperm remain acrosome-intact after binding to the zona matrix *in vitro*. A third model proposes that the fertilizing sperm undergoes acrosome exocytosis as it begins to enter the zona matrix which could account for the presence of acrosome ghosts observed on the surface of the zona matrix. Further investigations using transgenic mice, high resolution imaging and complementary *in vitro* and *in vivo* studies should provide more definitive answers in the future.

Where acrosome exocytosis occurs will help direct the search for the elusive sperm surface receptor that interacts with the zona matrix. If acrosome exocytosis occurs prior to encountering the zona pellucida, the sperm receptor for the zona ligand is likely to reside within the acrosome matrix or on the plasma membrane that remains after exocytosis. A caveat to this line of reasoning is that some acrosome proteins dramatically change their locations after exocytosis. If acrosome exocytosis occurs on the surface or during the initial penetration of the zona matrix, then a search of the intact sperm plasma membrane is more likely to be rewarding. The acrosome has a well-defined matrix that is auto-processed during exocytosis with release of some components (e.g., acrosin, a serine protease) and the retention of others (i.e., neuronal pentraxin 2).

Penetration Through the Zona Pellucida

The ability of sperm to penetrate the zona pellucida has been ascribed to the motility of hyper-activated sperm and to lytic enzymes released by acrosome exocytosis. In support of the first model, sperm specific calcineurin (PPP3CC) deficient spermatozoa with abnormal sperm motility caused by an inflexible midpiece are unable to penetrate the zona pellucida (Miyata et al., 2015). In the second model, sperm binding to ZP3 induces acrosome exocytosis which releases acrosin to establish a penetration slit for passage of sperm through the zona matrix. However, genetic ablation of *acrosin* does not prevent male fertility (Baba et al., 1994), ZP3 is no longer a compelling candidate for gamete recognition (Gahlay et al., 2010) and current literature supports

acrosome exocytosis occurring prior to sperm binding to the zona pellucida. Alternatively, the forceful propulsion of hyper-activated sperm may be sufficient to penetrate the zona pellucida and release of acrosome contents may modify the sperm surface to facilitate fusion with the egg plasma membrane at fertilization or have some other function.

Block to Polyspermy

Monospermic fertilization is critically important for successful onset of development and potent blocks to polyspermy are rapidly invoked following gamete fusion. There is a rapid block at the plasma membrane. Unlike other species, this block does not involve postfertilization changes in membrane voltage in mammals. Rather, Juno, a sperm receptor on the egg plasma membrane interacts with Izumo1 on the surface of sperm and both are essential for fertilization (Bianchi et al., 2014). After sperm egg fusion, Juno is reported to be released from plasma membrane of eggs which would prevent additional sperm from fusing to already fertilized eggs. Fertilization by intracellular sperm injection (ICSI) activates the egg and induces cortical granule exocytosis, but does not trigger a membrane block to sperm binding or fusion. Gamete binding and fusion are necessary for this block which suggests either a direct or indirect sperm contribution.

The zona pellucida also plays an important role in the postfertilization block to polyspermy. After gamete fusion and egg activation, peripherally located cortical granules (0.2–0.6 μm in diameter) approach and fuse with the egg plasma membrane to release their contents into the extracellular space. A pioneer component of the cortical granules is ovastacin, a ~ 47 kD zinc metalloendoprotease that cleaves ZP2 (Burkart et al., 2012). Using deletion mutations in vitro and in vivo, a 7-amino acid motif has been identified that is required to localize ovastacin in the peripheral cortical granules (Xiong et al., 2017). Fetuin B, a ~ 43 kD protease inhibitor produced in the liver and present in the circulation and follicular fluid, inhibits low levels of constitutively secreted ovastacin (Dietzel et al., 2013). However, the amount of ovastacin released during cortical granule exocytosis is thought to overwhelm this fragile defense and modify the extracellular zona pellucida.

Following fertilization, ovastacin cleaves mature mouse ZP2^{35–634} at ¹⁶⁶LA[↓]DE¹⁶⁹ near its N-terminus. Although the two fragments remain di-sulfide bonded, sperm no longer bind to the zona pellucida matrix which provides a definitive block to polyspermy. Thus, embryos derived from female mice either lacking ovastacin or possessing a mutant form of ZP2 that cannot be cleaved, support postfertilization sperm binding to the zona pellucida surrounding early embryos (Gahlay et al., 2010; Burkart et al., 2012). On the other hand, isoforms of ovastacin lacking a genetically defined localization motif are not present in peripheral cortical granules and the resultant premature cleavage of ZP2 profoundly decreases female fertility (Xiong et al., 2017).

Protection of the Early Embryo

There is no obvious biological imperative for order-specific gamete recognition among mammals that fertilize internally. There are many behavioral, environment and physiological barriers to cross-species mating and, although some (e.g., human) gametes are fastidious, others (e.g., rodents) are not. However, the zona pellucida is necessary to prevent resorption of ovulated eggs into the oviduct epithelium. Mice lacking ZP2 or ZP3 do not form a zona pellucida surrounding ovulated eggs which are quickly resorbed into the epithelial lining of the oviduct. Likewise, if the zona pellucida is biochemically removed from 1-cell to 8-cell embryos, they are quickly resorbed and only zona-free embryos that reach the morula stage or beyond have a reasonable chance of survival during preimplantation development.

As the preimplantation embryo divides, individual cells become smaller and the zona pellucida accommodates the cleavage-stage embryo. However, beginning at the early blastocyst stage, the zona pellucida begins to thin and, as the blastocoel fills with fluid, the mature blastocyst escapes from the confines of the zona pellucida to implant on the uterine wall. Non-mammalian vertebrates secrete enzymes from the astacin family of metalloproteases that have been implicated in hatching from the vitelline envelope. A trypsin-like activity and two serine proteases, Prss28 and Prss29 have been investigated as hatching enzymes in mice. However, their roles have not been definitively defined and escape from the confines of the zona pellucida may be mechanical as the late blastocyst outgrows the constraints of the zona pellucida or reflect sequential contractions observed in the hatching blastocyst (Niimura, 2003).

Conclusions

The mammalian zona pellucida is multifunctional in mediating gamete recognition, providing a postfertilization block to polyspermy and facilitating passage of the preimplantation embryo through the oviduct. The three or four zona protein traffic through the endomembrane system as independent proteins and, only after secretion, form the higher order, insoluble extracellular zona pellucida. Despite intense investigations in multiple species, the structure and function of individual zona pellucida proteins remain incompletely understood. Still needed is a detailed understanding of the supramolecular organization of the zona matrix. It is anticipated that improved imaging technologies and structural studies will help resolve the relationship and stoichiometry among the individual proteins within the zona pellucida.

More sophisticated understanding of the molecular basis of gamete recognition would be facilitated by identification of the long-elusive cognate sperm binding protein(s). The difficulty of its identification brings into question the certitude of its existence. Taking advantage of taxon-specific gamete recognition of mice and humans, candidates could be tested in transgenic mice expressing human ligands. Once the three-dimensional structure of the zona pellucida is known, investigations can be undertaken to determine the postfertilization changes that prevent sperm binding. This could involve biochemical, structural, and genetic studies that would determine the molecular basis of the postfertilization block to zona penetration. Lastly, it would be considerable interest to determine the molecular basis by which the blastocyst hatches from the confines of the zona pellucida at the time of implantation. These investigations into the molecular biology of the zona pellucida and its role in fertilization and early development should readily translate to medicine and provide human patients with better reproductive choices.

Acknowledgements

This research was supported by the Intramural Research Program of the NIH, The National Institutes of Diabetes, Digestive and Kidney Diseases (NIDDK).

References

- Avella, M. A., Baibakov, B., & Dean, J. (2014). A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *The Journal of Cell Biology*, 205, 801–809.
- Avella, M. A., Baibakov, B., Jimenez-Movilla, M., Sadusky, A. B., & Dean, J. (2016). ZP2 peptide-beads select human sperm in vitro, decoy mouse sperm in vivo and provide reversible contraception. *Science Translational Medicine*, 8, 336ra60.
- Baba, T., Azuma, S., Kashiwabara, S., & Toyoda, Y. (1994). Sperm from mice carrying a targeted mutation of the acrosin gene can penetrate the oocyte zona pellucida and effect fertilization. *The Journal of Biological Chemistry*, 269, 31845–31849.
- Baibakov, B., Boggs, N. A., Yauger, B., Baibakov, G., & Dean, J. (2012). Human sperm bind to the N-terminal domain of ZP2 in humanized zonae pellucidae in transgenic mice. *The Journal of Cell Biology*, 197, 897–905.
- Bianchi, E., Doe, B., Goulding, D., & Wright, G. J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature*, 508, 483–487.
- Boja, E. S., Hoodbhoy, T., Fales, H. M., & Dean, J. (2003). Structural characterization of native mouse zona pellucida proteins using mass spectrometry. *The Journal of Biological Chemistry*, 278, 34189–34202.
- Burkart, A. D., Xiong, B., Baibakov, B., Jimenez-Movilla, M., & Dean, J. (2012). Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *The Journal of Cell Biology*, 197, 37–44.
- Dietzel, E., Wessling, J., Floehr, J., Schafer, C., Ensslen, S., Denecke, B., Rosing, B., Neulen, J., Veitinger, T., Spehr, M., Tropartz, T., Tolba, R., Renne, T., Egert, A., Schorle, H., Gottenbusch, Y., Hildebrand, A., Yiallouris, I., Stocker, W., Weiskirchen, R., & Jahnke-Dechent, W. (2013). Fetuin-B, a liver-derived plasma protein is essential for fertilization. *Developmental Cell*, 25, 106–112.
- Gahlay, G., Gauthier, L., Baibakov, B., Epifano, O., & Dean, J. (2010). Gamete recognition in mice depends on the cleavage status of an egg's zona pellucida protein. *Science*, 329, 216–219.
- Han, L., Monne, M., Okumura, H., Schwend, T., Cherry, A. L., Flot, D., Matsuda, T., & Jovine, L. (2010). Insights into egg coat assembly and egg-sperm interaction from the X-ray structure of full-length ZP3. *Cell*, 143, 404–415.
- Hino, T., Muro, Y., Tamura-Nakano, M., Okabe, M., Tateno, H., & Yanagimachi, R. (2016). The behavior and acrosomal status of mouse spermatozoa in vitro, and within the oviduct during fertilization after natural mating. *Biology of Reproduction*, 95, 50.
- Huang, H. L., Lv, C., Zhao, Y. C., Li, W., He, X. M., Li, P., Sha, A. G., Tian, X., Papasian, C. J., Deng, H. W., Lu, G. X., & Xiao, H. M. (2014). Mutant ZP1 in familial infertility. *The New England Journal of Medicine*, 370, 1220–1226.
- Miyata, H., Satouh, Y., Mashiko, D., Muto, M., Nozawa, K., Shiba, K., Fujihara, Y., Isotani, A., Inaba, K., & Ikawa, M. (2015). Sperm calcineurin inhibition prevents mouse fertility with implications for male contraceptive. *Science*, 350, 442–445.
- Nimura, S. (2003). Time-lapse videomicrographic analyses of contractions in mouse blastocysts. *The Journal of Reproduction and Development*, 49, 413–423.
- Pang, P. C., Chiu, P. C., Lee, C. L., Chang, L. Y., Panico, M., Morris, H. R., Haslam, S. M., Khoo, K. H., Clark, G. F., Yeung, W. S., & Dell, A. (2011). Human sperm binding is mediated by the sialyl-Lewis(X) oligosaccharide on the zona pellucida. *Science*, 333, 1761–1764.
- Williams, S. A., Xia, L., Cummings, R. D., McEver, R. P., & Stanley, P. (2007). Fertilization in the mouse does not require terminal galactose or *N*-acetylglucosamine on the zona pellucida glycans. *Journal of Cell Science*, 120, 1341–1349.
- Xiong, B., Zhao, Y., Beall, S., Sadusky, A. B., & Dean, J. (2017). A unique egg cortical granule localization motif is required for ovastacin sequestration to prevent premature ZP2 cleavage and ensure female fertility in mice. *PLoS Genetics*, 13, E1006580.

Fertilization in the Oviduct

Janice P Evans, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

The process of fertilization unites the two gametes to create and initiate development of the embryo. However, even before the sperm and egg meet and come together to become one combined cell, there are a noteworthy number of preceding, preparatory events that must occur for each of the gametes. Just as a student has to take precalculus before calculus, or take basic chemistry before organic chemistry, the gametes in both the male and female have to undergo prerequisite steps in order for fertilization itself to be successful. This article will cover these necessary prerequisite steps for the gametes, and then will also address the merger of the gametes and the early steps that initiate embryonic development. This also will highlight how fertilization *in vivo* (i.e., in the oviduct) differs from fertilization *in vitro*. In addition to this overview, there is more detailed information on these events in other chapters of this volume.

The Oocyte's Preparations for Fertilization

Each oocyte develops in its own individual compartment in the ovary, known as the ovarian follicle (or simply "follicle" for short). Oocytes progress through meiosis in a very staggered fashion. Mammalian female gametes initiate meiosis in the fetal ovary, doing DNA replication and progressing through the stages of prophase I (leptotene, zygotene, and pachytene), ultimately to arrive and arrest at what will be an extended version of diplotene phase; this is called the dictyate stage, also known as the resting stage of meiosis. With this as the starting point, there are two things that must change with the oocyte in order for fertilization to occur: (1) the oocyte must leave the ovarian follicle and enter the oviduct (also known as the fallopian tube; specifically the ampulla region of the oviduct, the site where fertilization normally occurs); and (2) the oocyte must progress from this prophase I arrest through meiosis I to a second arrest at metaphase of meiosis II. (One exception to this are dogs and foxes, with oocytes that arrest in meiosis and get fertilized at metaphase I of meiosis.)

These two crucial changes of the oocyte are coordinated by actions downstream from luteinizing hormone (LH), the gonadotropin that triggers ovulation. LH binding to the LH receptor on granulosa cells surrounding the outer wall of the pre-ovulatory follicle (known as mural granulosa cells) induces production of a variety of molecules within the follicle, with one of the most significant being the small signaling lipid Prostaglandin E₂ (Duffy, 2015; Kim and Duffy, 2016). Through paracrine effects on somatic cells within the follicle, PGE₂ facilitates follicle rupture, allowing the oocyte to leave the ovarian follicle and transverse to the nearby ampulla of the oviduct. The oocyte is released from the ovarian follicle surrounded by its associated cumulus cells, as a *cumulus–oocyte complex* (COC).

The actions of LH also induce a cessation of the signaling that maintains prophase I arrest. During early stages of oogenesis and folliculogenesis, oocytes initially are not competent to exit from prophase I arrest. Oocytes develop this meiotic competence during later stages of oocyte growth and follicle development, and once oocytes achieve meiotic competence, signaling within the ovarian follicle keeps the oocytes arrested at prophase I (Jaffe and Egbert, 2017). This arrest of meiotically competent oocytes in preovulatory follicles is mediated by a protein called natriuretic peptide precursor C (NPPC, also known as C-type natriuretic peptide). NPPC is produced by the mural granulosa cells, then acts in a paracrine fashion, binding to its receptor on cumulus cells (the NPPC receptor is known as natriuretic peptide receptor 2, or NPR2). NPR2 has enzymatic activity as a guanylate cyclase that produces the secondary messenger molecule cyclic GMP (cGMP). cGMP is transferred from the cumulus cells to the oocyte through gap junctions, and cGMP in the oocyte inhibits the activity of an enzyme called phosphodiesterase 3A (PDE3A). PDE3A is an enzyme that degrades the secondary messenger molecule cyclic AMP (cAMP), and inhibiting this enzyme and keeping cAMP levels in the oocyte high is crucial for maintain prophase I arrest in meiotically competent oocytes. cAMP in the oocyte acts through the cAMP kinase protein kinase A (PKA), which phosphorylates several cell cycle regulatory proteins that mediate maintenance of the oocyte's prophase I arrest (Jaffe and Egbert, 2017).

When it is time for ovulation and thus for the oocyte to exit from this prophase I arrest, the actions of LH reverse this entire process. This is achieved by several events within the follicle. Two components of this are LH causing both a decrease in NPR2 guanylate cyclase activity in cumulus cells and an increase in degradation of cGMP. These two actions decrease cGMP concentrations in cumulus cells, and in turn reducing the amount of cGMP in the oocyte (Jaffe and Egbert, 2017). In addition, LH binding to the LH receptor on mural granulosa cells leads to a new set of paracrine signals within the follicle, through the release of epiregulin and amphiregulin from the granulosa cells. These two proteins are ligands for epidermal growth factor receptors (EGFRs) present on the cumulus cells surrounding the oocyte. The binding of epiregulin and/or amphiregulin to cumulus cell EGFRs also appears to contribute to the decrease in cGMP (Jaffe and Egbert, 2017). An additional observed effect of LH in the preovulatory follicle is closure of gap junctions connecting closure of gap junctions connecting the cumulus cells to the oocytes, in turn terminating the transfer of cGMP to the oocytes (Jaffe and Egbert, 2017). The end result of all this is decreasing the amount of cGMP in the oocyte, which allows PDE3A to become active and to degrade cAMP. The decline in cAMP activity in the oocyte allows the oocyte to resume meiosis. The oocyte progresses from prophase I into meiosis I, and through cytokinesis (polar body emission), separating the homologous chromosomes. The sister chromatids remain together, and align as the meiotic spindle of metaphase II forms. The

ovulated oocyte in the oviduct is arrested at metaphase II, awaiting the signal from the sperm to complete meiosis (to be addressed below). The terms “metaphase II egg,” “MII egg,” or simply “egg” are sometimes used to describe the oocyte at this stage of meiosis.

The Sperm's Preparations for Fertilization

One obvious change that has to happen with the sperm is in location—the sperm has to be deposited in the female reproductive tract. But even before this, there are other crucial changes that the sperm has to undergo. Sperm complete meiosis during spermatogenesis in the seminiferous tubule of the testis (thus differing from how the female gamete undergoes meiosis). However, even though sperm isolated from the testis are haploid, these haploid sperm in the testis are unable to fertilize an egg. (The exception to this is when a sperm is injected into the egg cytoplasm through an assisted reproductive technology [ART] method known as intracytoplasmic sperm injection [ICSI].)

Two key steps of the sperm's preparations for fertilization occur while the sperm is still in the male, after the sperm has left the testes. The first of these is transit through the epididymis, with the sperm undergoing a process known as *epididymal maturation*. A crucial change that occurs during epididymal maturation is sperm become competent for progressive motility. Changes in sperm during epididymal maturation are thought to be mediated by several events, including sperm interactions with the epididymal epithelium, changes in the sperm surface proteome through acquisition of secreted proteins from the epididymal epithelium, and sperm acquisition of small vesicles released from the epididymal cells (exosomes or epididymosomes) (Gervasi and Visconti, 2017).

The second prerequisite step for the sperm occurring in the male is *mixture of the sperm with the semen*. Seminal plasma components are secreted by the epididymis and accessory glands of the male reproductive tract, the seminal vesicle, prostate, and bulbourethral gland (McGraw et al., 2015). In humans, the components of the semen are important for sperm to survive in the vagina and/or uterus (McGraw et al., 2015). The human vaginal environment is acidic, maintained in large part by the production of lactic acid by bacteria called lactobacilli. The acidic environment is beneficial for preventing certain vaginal infections, as the low pH will kill many types of microbes. However, the acidic environment will also kill sperm. Therefore, the buffering components of seminal fluid are essential to allow the sperm to survive deposition in the vagina. In some animal species that are induced ovulators (e.g., rabbits, camels), seminal plasma contains factors that induce ovulation (Adams and Ratto, 2013). There also are intriguing data that suggest that seminal plasma can affect the health of resulting offspring, based on experimental studies in mice comparing reproductive outcomes from males with and without seminal vesicles (Bromfield et al., 2014; McGraw et al., 2015).

After ejaculation, sperm then make their way through the female tract. The location of sperm deposition in the female tract varies between species (vagina, cervix, or uterus) (Gervasi and Visconti, 2016; McGraw et al., 2015). In humans, sperm travel from the vagina, through the cervix, into the uterus, and to region of the oviduct adjacent to the uterus, known as the utero-tubal junction (UTJ). It should be noted that the human cervix is hormonally primed during follicular phase of the menstrual cycle, such that the cervical mucus around the time ovulation is thin and watery, thus facilitating sperm passage through the cervix into the uterus. Data from a variety of studies, most notably of mouse knockout models, suggest that some process of sperm selection occurs in the female tract; the sperm from several specific knockouts fail to reach the UTJ, indicating that several sperm proteins have direct or indirect roles in successful transit to this part of the female tract (Gervasi and Visconti, 2016; Suarez, 2016).

It is in the female reproductive tract that sperm undergo their next important prerequisite step, *capacitation*, which is broadly defined as the process by which sperm acquire the capacity to fertilize. Capacitation in vivo is thought to be an extended, continuous process, beginning with ejaculation and continuing as sperm reside in the female tract. In the human, the peri-ovulatory cervical mucus may be part of the stimulus for the initiation of capacitation, with the uterine and oviductal environments continuing to support capacitation (De Jonge, 2017). Since the phenomenon of capacitation was first discovered, it has since been determined that capacitation can occur in vitro in certain chemically defined medium (Gervasi and Visconti, 2016). These discoveries and technological developments were part of what paved the way to making in vitro fertilization possible. Capacitation in vivo is mediated by a sequential series of cellular signaling events occurring in sperm as they travel up the female tract, central among them being signaling mediated by the second messenger molecule cAMP (Gervasi and Visconti, 2016). This signaling produces several biochemical changes in the sperm, which combine to culminate in capacitated sperm.

What is different about sperm that have undergone capacitation? The classic definition of capacitation means that the sperm now has the capacity to fertilize an egg, but there are specific changes observed as well, particularly with the later stages of capacitation. One of these is *hyperactivation of motility*. This is a distinct pattern of motility from the progressive motility acquired during epididymal sperm maturation (noted above), with hyperactivated motility characterized by high amplitude, asymmetric beating of the sperm tail (Suarez, 2008a). A key molecular player in hyperactivated motility is the multimeric ion channel known as Catsper, so named for its function as a cation/calcium channel on sperm. Catsper-mediated influx of calcium into the sperm tail drives hyperactivated motility. In human sperm, the activity of Catsper is stimulated by the steroid hormone progesterone, which is present in follicular fluid that is released with the cumulus–oocyte complex upon ovulation (Miller et al., 2015).

Another event associated with the later stages of capacitation is the exocytosis of a large vesicle on the head of the sperm, the *acrosome*. (Note: *Acrosome exocytosis* is also referred to as the *acrosome reaction*.) A long-time model for acrosome exocytosis is that the sperm binding the egg's coat, the zona pellucida (ZP), is the stimulus for acrosome exocytosis. Although there still is strong evidence for sperm interaction with the ZP, including in in vivo contexts (Avella et al., 2014, 2016), there is ongoing evaluation of the biology of acrosome exocytosis, including consideration of acrosome exocytosis in vivo being an event associated with

capacitation of sperm in the female tract (Gervasi and Visconti, 2016; Hirohashi, 2016). This is based on data from a variety of experimental studies (Inoue et al., 2011; Jin et al., 2011; La Spina et al., 2016), with the cautionary note that much of this is based on studies of mouse fertilization, and several aspects of sperm biology differ between species (De Jonge, 2017; Kaupp and Strunker, 2017). Setting aside the question of the physiological location of acrosome exocytosis in vivo, it should be emphasized that acrosome exocytosis is an important prerequisite step for fertilization. Acrosome exocytosis induces changes in the sperm surface, including remodeling and exposure of new surfaces of the sperm that render the sperm capable of binding and fusing with the oocyte plasma membrane (Cuasnicu et al., 2016; Hirohashi, 2016).

A related aspect of sperm residence in the female tract is the formation of *oviductal sperm reservoirs*, specifically in a region of the oviduct called the isthmus (Suarez, 2016). In most species, the establishment of this oviductal sperm reservoir is mediated by sperm binding to the epithelial cells that line the oviduct. The formation of oviductal sperm reservoirs is facilitated by a preceding step with the sperm, the mixture of sperm with semen. Proteins in the seminal plasma help to mediate sperm interactions with the oviductal epithelium (McGraw et al., 2015).

The formation of these oviductal sperm reservoirs is thought to contribute to reproductive success in several ways, including maintaining sperm viability and fertility, regulating capacitation and hyperactivation of motility, and helping to prevent polyspermic fertilization (fertilization of the egg by more than one sperm). Release of sperm from interactions with oviductal epithelial cells appears to be associated with later stages of capacitation and tied with signals with ovulation, although this is not fully understood (Gervasi and Visconti, 2016). Acquisition of hyperactivated motility appears to facilitate detachment of sperm from the oviductal epithelium (Suarez, 2016).

One practical implication of this in human reproductive biology is that these oviductal sperm reservoirs contribute to sperm being able to live in the female tract for several days (and in some species, sperm can live in the female tract for weeks and even months) (Holt, 2011; Suarez, 2008b). Sperm storage thus affects the fertile window that needs to be considered for fertility awareness-based methods of contraception. For example, if a woman has unprotected intercourse on a Monday and then ovulates 5 days later on Friday, this ovulated egg could potentially be fertilized by sperm residing in these reservoirs in her reproductive tract. Fertility awareness-based methods of contraception thus require that a woman abstain from intercourse or use a contraceptive (methods such as condoms, diaphragm, or spermicidal foams or creams) during the time frame in which sperm could survive in the female tract long enough to fertilize an egg that could be ovulated days later.

The Gametes in the Ampulla of the Oviduct

Following the release of sperm from the oviductal sperm reservoirs in the isthmus of the oviduct, sperm then make their way to the ampulla of the oviduct, the site of fertilization. Sperm first make contact with the oocyte's outermost coat, the cumulus layer (also called the cumulus oophorus). This layer contains cumulus cells, the somatic cells that surrounded the oocyte in the ovarian follicle, embedded in an extracellular matrix of hyaluronic acid. Hyperactivated motility facilitates penetration of this extracellular matrix, and there also is a possible role of a sperm-associated hyaluronidase activity, although this has not been conclusively demonstrated (Chang and Suarez, 2010; Hirohashi, 2016; Suarez, 2008a). The next extracellular coat that the sperm makes contact with is the zona pellucida (ZP). As noted above, there are ongoing questions about a sperm has to have an intact acrosome to interact with the ZP and whether the ZP serves as an in vivo agonist to trigger acrosome exocytosis (Buffone et al., 2014), but there are data that provide evidence of sperm–ZP binding (e.g., Avella et al., 2014, 2016). Hyperactivated motility plays a crucial role in sperm passage through the ZP to get the sperm to the space between the ZP and the oocyte plasma membrane, known as the perivitelline space.

Once in the perivitelline space, the sperm binds and fuses with the oocyte plasma membrane with the sperm protein known as IZUMO1 and the oocyte protein called Juno being essential for this process of the individual gametes merging to become the one-cell embryo (Yeste et al., 2017). Obviously one important part of the sperm that is delivered to the oocyte is the paternal DNA. In some species, sperm-provided centrioles may help mediate organization of the mitotic spindle for the first embryonic division (Clift and Schuh, 2013). However, even before embryonic mitosis is going to occur, the oocyte must exit from its arrest in metaphase of meiosis II, where it has been arrested since ovulation (see above). In addition, the oocyte has processes to prevent fertilization by additional sperm, known as blocks to polyspermy. These events are collectively known as *oocyte activation* (or *egg activation*), and are triggered by fertilization by the sperm.

Sperm–oocyte fusion results in cytoplasmic continuity between these two cells, allowing delivery of sperm components into the egg cytoplasm. In one of the earliest steps of this process, the sperm brings the factor that triggers the oocyte's completion of meiosis and initiation of embryonic development—the sperm-specific phospholipase C, PLC ζ , which plays a key role in (Clift and Schuh, 2013; Yeste et al., 2017). PLC ζ , like other phospholipase Cs, hydrolyzes the lipid phosphatidylinositol 4,5-bisphosphate (PIP $_2$) to produce two cleavage products, inositol 1,4,5-trisphosphate (IP $_3$) and diacylglycerol (DAG). IP $_3$ binds to the IP $_3$ receptor on the endoplasmic reticulum, which is an intracellular store for calcium ions (Ca $^{2+}$). The IP $_3$ receptor, with IP $_3$ bound to it, functions as a Ca $^{2+}$ channel, allowing Ca $^{2+}$ to be released from the endoplasmic reticulum to the oocyte cytoplasm. Once in the oocyte cytoplasm, calcium ions serve as the major driver of the oocyte-to-embryo transition, activating calcium/calmodulin-dependent kinase II γ (CaMKII γ) (Clift and Schuh, 2013). This kinase phosphorylates downstream substrates that lead to exit from metaphase II arrest, completion of meiosis, and progression to embryonic mitosis.

In addition to cell cycle resumption, another event of oocyte activation is the establishment of blocks to polyspermy. As noted above, IP₃ receptor-mediated release of calcium ions from the endoplasmic reticulum results in increased cytosolic calcium concentration. This calcium triggers exocytosis of vesicles known as cortical granules. A protease called ovastacin is released from the cortical granules, and this protease cleaves the ZP component ZP2, resulting in a form of the ZP that does not support sperm binding (Burkart et al., 2012). Calcium also appears to mediate the conversion of the oocyte plasma membrane to a form that is less supportive of sperm interaction (Gardner et al., 2007).

Conclusion

The events summarized here can be considered the culminating events of sexual reproduction, the process by which the gametes from two genetically distinct individuals come together to create new, genetically unique offspring. Gaining improved understanding of these central events of reproduction will advance our understanding of how reproduction can go awry, and result in subfertility or infertility. For example, a subset of men seen in infertility clinics have sperm with normal motility and morphology, and that can fuse with eggs, but the eggs fail to undergo egg activation. Research studies of these patients have revealed that sperm from some of these men lack functional PLC ζ (Kashir et al., 2011; Yoon et al., 2008). On the flip side, knowledge of how reproduction works will provide insights into ways to impair reproductive processes, and thus open the door to future methods of contraception. Several of the biological events noted in this article have been proposed to be potential targets for contraception. These include perturbation of ovulation by interfering with production of Prostaglandin E2 (Duffy, 2015), impairing the sperm's acquisition of hyperactivated motility with steroid-like molecules that act in an antagonistic fashion to progesterone (Mannowetz et al., 2017), and placing beads coated with ZP-like peptides in the uterus can serve as a "decoy" for sperm, causing sperm to fail to travel from the uterus to the oviduct (Avella et al., 2016). Research in this and other areas of reproductive biology is poised to produce discoveries that will lead significant advances for human reproductive health.

References

- Adams, G. P., & Ratto, M. H. (2013). Ovulation-inducing factor in seminal plasma: A review. *Animal Reproduction Science*, 136, 148–156.
- Avella, M. A., Baibakov, B., & Dean, J. (2014). A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *The Journal of Cell Biology*, 205, 801–809.
- Avella, M. A., Baibakov, B. A., Jimenez-Movilla, M., Sadusky, A. B., & Dean, J. (2016). ZP2 peptide beads select human sperm in vitro, decoy mouse sperm in vivo, and provide reversible contraception. *Science Translational Medicine*, 8, 336ra360.
- Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J., & Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America*.
- Buffone, M. G., Hirohashi, N., & Gerton, G. L. (2014). Unresolved questions concerning mammalian sperm acrosomal exocytosis. *Biology of Reproduction*, 90, 112.
- Burkart, A. D., Xiong, B., Baibakov, B., Jimenez-Movilla, M., & Dean, J. (2012). Ovastacin, a cortical granule protease, cleaves ZP2 in the zona pellucida to prevent polyspermy. *The Journal of Cell Biology*, 197, 37–44.
- Chang, H., & Suarez, S. S. (2010). Rethinking the relationship between hyperactivation and chemotaxis in mammalian sperm. *Biology of Reproduction*, 83, 507–513.
- Clift, D., & Schuh, M. (2013). Restarting life: Fertilization and the transition from meiosis to mitosis. *Nature Reviews. Molecular Cell Biology*, 14, 549–562.
- Cuasnicu, P. S., Da Ros, V. G., Weigel Munoz, M., & Cohen, D. J. (2016). Acrosome reaction as a preparation for gamete fusion. *Advances in Anatomy, Embryology, and Cell Biology*, 220, 159–172.
- De Jonge, C. (2017). Biological basis for human capacitation-revisited. *Human Reproduction Update*, 23, 289–299.
- Duffy, D. (2015). Novel contraceptive targets to inhibit ovulation: The prostaglandin E2 pathway. *Human Reproduction Update*, 21, 652–670.
- Gardner, A. J., Williams, C. J., & Evans, J. P. (2007). Establishment of the mammalian membrane block to polyspermy: Evidence for calcium-dependent and -independent regulation. *Reproduction*, 133, 383–393.
- Gervasi, M. G., & Visconti, P. E. (2016). Chang's meaning of capacitation: A molecular perspective. *Molecular Reproduction and Development*, 83, 860–874.
- Gervasi, M. G., & Visconti, P. E. (2017). Molecular changes and signaling events occurring in spermatozoa during epididymal maturation. *Andrology*, 5, 204–218.
- Hirohashi, N. (2016). Site of mammalian sperm acrosome reaction. *Advances in Anatomy, Embryology, and Cell Biology*, 220, 145–158.
- Holt, W. V. (2011). Mechanisms of sperm storage in the female reproductive tract: An interspecies comparison. *Reproduction in Domestic Animals = Zuchthygiene*, 46(Suppl 2), 68–74.
- Inoue, N., Satouh, Y., Ikawa, M., Okabe, M., & Yanagimachi, R. (2011). Acrosome-reacted mouse sperm recovered from the perivitelline space can fertilize other eggs. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 20008–20011.
- Jaffe, L. A., & Egbert, J. R. (2017). Regulation of mammalian oocyte meiosis by intercellular communication within the ovarian follicle. *Annual Review of Physiology*, 79, 237–260.
- Jin, M., Fujiwara, E., Kakiuchi, Y., Okabe, M., Satouh, Y., Baba, S. A., Chiba, K., & Hirohashi, N. (2011). Most fertilizing mouse spermatozoa begin their acrosome reaction before contact with the zona pellucida during in vitro fertilization. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 4892–4896.
- Kashir, J., Konstantinidis, M., Jones, C., Lemmon, B., Chang Lee, H., Hamer, R., Heindryckx, B., Deane, C. M., DeSutter, P., Fissore, R. A., Parrington, J., Wells, D., & Coward, K. (2011). A maternally inherited autosomal point mutation in human phospholipase C zeta (PLC ζ) leads to male infertility. *Human Reproduction*, 27, 222–231.
- Kaupp, U. B., & Strunker, T. (2017). Signaling in sperm: More different than similar. *Trends in Cell Biology*, 27, 101–109.
- Kim, S. O., & Duffy, D. M. (2016). Mapping PTGERS to the ovulatory follicle: Regional responses to the ovulatory PGE2 signal. *Biology of Reproduction*, 95, 33.
- La Spina, F. A., Puga Molina, L. C., Romarowski, A., Vitale, A. M., Falzone, T. L., Krapf, D., Hirohashi, N., & Buffone, M. G. (2016). Mouse sperm begin to undergo acrosomal exocytosis in the upper isthmus of the oviduct. *Developmental Biology*, 411, 172–182.
- Mannowetz, N., Miller, M. R., & Lishko, P. V. (2017). Regulation of the sperm calcium channel CatSper by endogenous steroids and plant triterpenoids. *Proceedings of the National Academy of Sciences of the United States of America*, 114, 5743–5748.
- McGraw, L. A., Suarez, S. S., & Wolfner, M. F. (2015). On a matter of seminal importance. *BioEssays*, 37, 142–147.
- Miller, M. R., Mansell, S. A., Meyers, S. A., & Lishko, P. V. (2015). Flagellar ion channels of sperm: Similarities and differences between species. *Cell Calcium*, 58, 105–113.
- Suarez, S. S. (2008a). Control of hyperactivation in sperm. *Human Reproduction Update*, 14, 647–657.
- Suarez, S. S. (2008b). Regulation of sperm storage and movement in the mammalian oviduct. *The International Journal of Developmental Biology*, 52, 455–462.

- Suarez, S. S. (2016). Mammalian sperm interactions with the female reproductive tract. *Cell and Tissue Research*, 363, 185–194.
- Yeste, M., Jones, C., Amdani, S. N., & Coward, K. (2017). Oocyte activation and fertilisation: Crucial contributors from the sperm and oocyte. *Results and Problems in Cell Differentiation*, 59, 213–239.
- Yoon, S. Y., Jellerette, T., Salicioni, A. M., Lee, H. C., Yoo, M. S., Coward, K., Parrington, J., Grow, D., Cibelli, J. B., Visconti, P. E., Mager, J., & Fissore, R. A. (2008). Human sperm devoid of PLC ζ 1 fail to induce Ca²⁺ release and are unable to initiate the first step of embryo development. *The Journal of Clinical Investigation*, 118, 3671–3681.

Decondensation of Sperm Chromatin

Marina Romanato, Lucrecia Piñeiro de Calvo, and Juan Carlos Calvo, Instituto de Biología y Medicina Experimental-CONICET, Buenos Aires, Argentina

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromatin Complex of DNA and proteins that forms chromosomes within the nucleus of eukaryotic cells. DNA is condensed and wrapped around nuclear proteins (histones) to fit inside the nucleus.

Chromatin condensation Denser packaging of DNA in the spermatozoon, as a consequence of replacement of histones by protamines. In humans and other primates, 10%–15% of the sperm's genome remains packaged by histones thought to bind genes that are essential for early embryonic development.

Chromatin decondensation Process involving reduction of disulfide bridges of protamines, by glutathione and removal of protamines by a negatively-charged acceptor, thus allowing the formation of a histone-packed chromatin (nucleosome).

Glutathione (GSH) Important antioxidant in plants, animals, fungi, and some bacteria and archaea. Glutathione can prevent damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals.

Glycosaminoglycans Long unbranched polysaccharides formed by a repeating disaccharide unit, consisting of an amino sugar (*N*-acetylglucosamine or *N*-acetylgalactosamine) along with a uronic acid (glucuronic acid or iduronic acid) or galactose (except for keratan).

Heparin and Heparan sulfate Structurally similar glycosaminoglycans composed of two basic saccharides: *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine as the amino sugar and either D-glucuronic acid or L-iduronic acid as the uronic acid. These basic components are further modified by epimerization, sulfation, and deacetylation. Heparin is only produced by mast cells. It functions as an anticoagulant. Heparan sulfate is made by almost all cell types and has anticoagulant activity, but it is much lower than that of heparin. Heparan sulfate further varies from heparin in the degree of modification of the sugar residues.

Protamines Small, arginine-rich, nuclear proteins that replace histones late in the haploid phase of spermatogenesis; essential for sperm head condensation and DNA stabilization.

Spermatogenesis The process of male gamete production including formation of a diploid spermatocyte from a spermatogonium, meiotic division of the spermatocyte, and transformation of the four resulting haploid spermatids into spermatozoa.

Spermiogenesis Final stage of spermatogenesis, with differentiation of spermatids into mature, motile spermatozoa. Traditionally divided into: the Golgi phase, the cap phase, formation of tail, and the maturation stage.

Synergism An interaction between two or more drugs that causes the total effect of the drugs to be greater than the sum of the individual effects of each drug.

Syngamy The fusion of gametes resulting in the formation of a zygote, which develops into a new organism.

General Considerations

Evolution has devised a means to provide mammalian offspring with the best genetic parental traits, by separating a male and a female sex and, then, combining both genetic materials into a new cell, the zygote, which will develop into a genetically unique new organism. But this revolutionary way to select the best genetic material by choosing the most appropriate partner for the sexual encounter, has its caveats: fertilization occurs in a different organism than that where male gametes were formed.

The journey from the male gonads where spermatogenesis has taken place into the female body to reach the oocyte inside the Fallopian tube, where it resides after being ovulated from the ovarian follicle, has its risks and presents severe challenges to the tiny male germ cell.

The spermatozoon is a unique complex cell that originates from a spermatogonia in the seminiferous tubules of the testis and through spermatogenesis and spermiogenesis arises as a potentially highly motile cell, with a stack of mitochondria providing energy to its flagellum and keeping the genetic material inside a highly condensed nuclear chromatin. Although it is now known that sperm function does not end with the delivery of genetic material inside the oocyte, this is still considered to be its primary goal.

Reaching its destination is not an easy task for the spermatozoon. Although highly protected from the immune system inside the testis and mostly throughout its journey out of the male body, this protection ends when reaching the female counterpart. Physical and chemical barriers: rheological properties of vaginal mucus, pH changes, cells from the female immune system, a long distance to cover until it reaches the oocyte, are deadly threats for this cell for which keeping the genetic material as intact as possible is a must.

Chromatin Condensation

In any somatic cell, DNA is found packed as chromatin by its binding to positively charged proteins, histones, forming nucleosomes which provide not only spatial organization of the chromosomes but also a fine regulation of the dynamics of genetic information: chromosome duplication, gene transcription, genetic recombination, among other processes. However, this nucleosome organization does not provide the protection that sperm DNA requires to reach the fertilization site undamaged. Reactive oxygen species from male and female cells, as well as possible physical rupture from collisions along its journey are among the probable causes of DNA damage.

Sperm chromatin, in different species, is in a highly condensed state due to the removal of histones and their replacement by other positively charged, and smaller, proteins: protamines.

These proteins are arginine-rich, small proteins (50–110 amino acids) that contain multiple cysteine residues that are oxidized to form disulfide bridges both, intra and inter chain, linking protamines together. There are two protamines, P1 and P2. Spermatozoa from all mammalian species contain P1, while P2 is present in mice, hamsters, stallions, some primates and humans.

Protamine P1 is typically a 49–50 amino acids long protein, with a central arginine-rich domain (mainly responsible for binding to DNA) along with two flanking peptides containing cysteine residues. Protamine P1 and P2 seem to derive from a common precursor, but P2 has some characteristics that differ from P1, such as containing histidine residues. In humans, two processed forms of protamine P2 (P2 and P3) which differ in three amino terminal residues are often also found bound to DNA. After processing, approximately 40% of the amino terminus has been removed from P2. This final, processed form of P2, remains slightly larger than P1 (63 amino acids). Another difference between protamine P1 and P2 is that P2 is capable of binding zinc through coordination with its histidine and cysteine residues, forming zinc-fingers which participate in binding to DNA.

Spermatogenesis in humans is a continuous process, very precisely regulated and controlled, which results in the production of spermatozoa containing half the number of chromosomes (haploid) and potentially capable of fertilizing the oocyte (spermatozoa will achieve full capacity after maturation in the epididymis and capacitation in the female reproductive tract). The process of spermatogenesis can be divided into different phases: mitotic cell division with amplification of genetic material, a meiotic phase with reduction to half the number of chromosomes and a final phase known as spermiogenesis. Through these phases, haploidization takes place, as well as reorganization of genetic information within the germ cell. In the spermiogenesis phase, transcription stops as an integral part of germ cell differentiation, and chromatin remodeling takes place. The strong reorganization of chromatin involves gradual replacement of histones by protamines, through binding of testis-specific histones, then of transition proteins and, finally, as nucleosomes are removed, of protamines, generating a highly condensed sperm chromatin in the nucleus. However, the exact process of chromatin remodeling in sperm cells is still not fully comprehended.

In comparison to histone binding to DNA in the nucleosome, each protamine P1 binds to 10–11 bp of DNA, while protamine P2 binds to a larger segment of around 15 bp. This binding, through protamine positive charges, neutralizes the negative charge along the phosphodiester backbone of DNA, thus allowing a more compact packing of adjacent DNA molecules. Apparently, P1 wraps around the DNA helix in the major groove, with one protamine molecule per turn of DNA helix. Combination of hydrogen and electrostatic bonds of the guanidinium portion of the arginine molecule in protamine, provides an anchoring site for protamines and phosphate groups in both DNA strands. This gives rise to a toroidal and more densely packed chromatin. Considering that about 15% of histones remain in sperm chromatin, and the differential binding of protamine P1 and P2 to different stretches of DNA, it can be speculated that sperm chromatin will be packed in a non-homogeneous fashion which could be relevant when decondensation occurs.

Chromatin Decondensation

Decondensation is the first visible change that takes place inside the oocyte, once the spermatozoon has entered the ooplasm after binding to the oolema. The highly-packed, transcriptionally-inactive sperm chromatin needs to be unraveled and converted into a nucleosome-based chromatin, leading to the formation of the male pronucleus, before syngamy can occur.

In mammalian species, including humans, this chromatin decondensation seems to occur in two different stages: first the reduction of disulfide bonds in protamines and, then, replacement of these reduced protamines by oocyte histones. While there is no doubt on the role of oocyte reduced glutathione (GSH) as the reducing agent for protamine disulfide bonds, the nature of the protamine acceptor (the molecule that will compete with DNA for protamine binding and, in so doing allow for histone replacement and nucleosome formation) is still unknown. In fish, amphibians and *Drosophila melanogaster*, nucleoplamin (a pentameric acidic protein, considered to be the first identified molecular chaperone) has been shown to be the protamine acceptor and, thus, responsible for sperm nuclear decondensation. These findings have not been extended to humans and, for a while no evidence of the equivalent molecule was found.

It was shown that heparin (Hep) (a highly negatively charged sulfated glycosaminoglycan, best known for its anticoagulant properties), together with GSH could decondense human sperm chromatin in vitro, and proposed as a putative decondensing agent in vivo.

Since the only cell capable of synthesizing heparin in vivo is the mast cell, and no heparin has been found in the oocyte–cumulus complex, this was highly unlikely. However, evidence of the presence of other glycosaminoglycans (GAGs) in the complex, particularly heparan sulfate, structurally similar to heparin and with similar functions (other than anticoagulant) in different biological systems, led us to investigate the possible role of heparan sulfate (HS) as nuclear-decondensing agent in vivo.

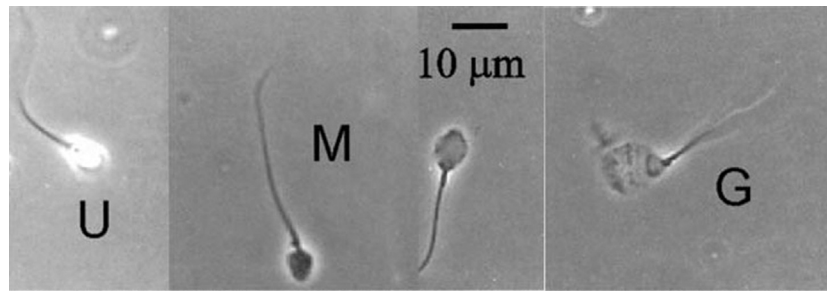


Fig. 1 Nuclear decondensation of human spermatozoa, as visualized under phase contrast. U = unchanged, M = moderately decondensed, G = grossly decondensed. Original magnification $400\times$ (bar = $10\ \mu\text{m}$).

Using a decondensing mixture composed of GSH and different GAGs (heparin, heparan sulfate, dermatan sulfate, hyaluronic acid, or chondroitin sulfate) we determined that heparin and heparan sulfate had the highest (and similar) decondensing ability. Chondroitin sulfate and hyaluronic acid showed no activity at all and dermatan sulfate could decondense to a similar or lower degree than HS/Hep. The percentage of chromatin decondensation was determined by microscopic observation of sperm, using phase contrast light microscopy, according to the aspect of the sperm head: U (unchanged), M (moderately decondensed), or G (grossly decondensed) (Fig. 1).

Decondensation of human sperm chromatin occurs with freshly crushed murine oocytes in the presence of GSH, and is abolished in the presence of heparinase (enzyme that breaks heparan sulfate down to disaccharides). This is a clear indication of the presence and involvement of this GAG in sperm chromatin decondensation. Moreover, the presence of this glycosaminoglycan in the ooplasm was confirmed by immunocytochemistry using a specific antibody, in murine, bovine and human oocytes at various stages of maturation.

Contrarily to what has been usually accepted, the decondensation process seems to be a synergistic cooperation between GSH and heparan sulfate/heparin rather than a two-step mechanism where first the disulfide bonds of protamines are reduced by GSH and, then, the acceptor molecule (heparan sulfate) removes protamines from their association to DNA. The capacity of P2 to bind zinc ions explains the decondensation that occurs in vitro when EDTA (chelate forming agent that complexes divalent cations) is used in combination with heparin/heparan sulfate. The free thiol groups in cysteines, resulting from zinc removal, trigger the auto-reduction of protamine disulfide bonds, thus allowing the GAG to remove these proteins. So far, no transition proteins have been involved in this decondensation process.

When dermatan sulfate is used as decondensing agent instead of heparan sulfate, maximal decondensation achieved is lower, and in rodents, but not in humans, a synergistic effect is found when both GAGs are used together. These differences are an indication of a chromatin with different accessibility to both molecules. Heparan sulfate/heparin are highly-sulfated glycosaminoglycans whose molecules form a spiral-like helix, with their negative charges distributed in a fashion that resembles the DNA molecule with its negative phosphate groups pointing towards the periphery of the helix. On the other hand, dermatan sulfate is less sulfated, with a less ordered distribution of negative charges which creates a more homogeneous negative surface and, thus, quite different from a DNA-like distribution. These differences between both GAGs should influence the way each one interacts with sperm chromatin and could have its counterpart in decondensation. One could speculate that Hep/HS would provide a more DNA-like interaction and, thus, a better competition for protamines; while interaction with DS could be directed towards the remaining nucleosome-like, histone-rich, portions of sperm chromatin.

Chromatin Decondensation and Fertility

It is now well established that changes in protamine content or P1/P2 ratio result in diminished fertility both in humans and in rodents. Other than chromatin compaction/organization no function has been attributed to protamines in the sperm nucleus. It is likely that a lower or higher protamine content or altered protamine ratio will result in a differentially condensed chromatin and, as such, more prone to attack by reactive oxygen species and potential DNA damage. The possibility of analyzing sperm decondensation in vitro with two different GAGs (heparan sulfate and dermatan sulfate) allows for differential screening of possible alterations in chromatin condensation in semen samples from patients attending fertility clinics. Such studies could be relevant in those cases where the sperm nucleus remains condensed inside the oocyte following in vitro fertilization.

Further Reading

- Balhorn, R. (2007). The protamine family of sperm nuclear proteins. *Genome Biology*, 8, 227–235.
- Canel, N. G., Bevacqua, R. J., Hiriart, M. I., Rabelo, N. C., de Almeida Camargo, L. S., Romanato, M., de Calvo, L. P., & Salamone, D. F. (2017). Sperm pretreatment with heparin and L-glutathione, sex-sorting, and double cryopreservation to improve intracytoplasmic sperm injection in bovine. *Theriogenology*, 93, 62–70.
- Julianelli, V., Farrando, B., Alvarez Sedó, C., Calvo, L., Romanato, M., & Calvo, J. C. (2012). Heparin enhances protamine disulfide bond reduction during in vitro decondensation of human spermatozoa. *Human Reproduction*, 27, 1930–1938.
- Kierszenbaum, A. L., & Tres, L. L. (2016). *Histology and cell biology: An introduction to pathology* (4th edition.). Philadelphia: Elsevier.

- Rathke, C., Baarends, W. M., Awe, S., & Renkawitz-Pohl, R. (2014). Chromatin dynamics during spermiogenesis. *Biochimica et Biophysica Acta*, 1839, 155–168.
- Romanato, M., Cameo, M. S., Bertolesi, G., Baldini, C., Calvo, J. C., & Calvo, L. (2003). Heparan sulphate: A putative decondensing agent for human spermatozoa in vivo. *Human Reproduction*, 18, 1868–1873.
- Romanato, M., Julianelli, V., Zappi, M., Calvo, L., & Calvo, J. C. (2008). The presence of heparan sulfate in the mammalian oocyte provides a clue to human sperm nuclear decondensation in vivo. *Human Reproduction*, 23, 1145–1150.
- Romanato, M., Regueira, E., Cameo, M. S., Baldini, C., Calvo, L., & Calvo, J. C. (2005). Further evidence on the role of heparan sulfate as protamine acceptor during the decondensation of human spermatozoa. *Human Reproduction*, 20, 2784–2789.
- Sanchez, M. C., Sedó, C. A., Julianelli, V. L., Romanato, M., Calvo, L., Calvo, J. C., & Fontana, V. A. (2013). Dermatan sulfate synergizes with heparin in murine sperm chromatin decondensation. *Systems Biology in Reproductive Medicine*, 59, 82–90.
- Zini, A., & Agarwal, A. (Eds.). (2011). *Sperm chromatin. Biological and clinical applications in male infertility and assisted reproduction*. New York: Springer.

Polyspermy

Brian Dale, Centre for Assisted Fertilization, Naples, Italy

© 2018 Elsevier Inc. All rights reserved.

What Is Polyspermy

Animals may be classified into two major groups according to the kinetics of sperm–oocyte interaction. In the most commonly studied group, which includes sea urchins and mammals, only one spermatozoon normally enters the oocyte. If more than one spermatozoon does enter the oocyte then the multiple nuclei interact together leading to anomalous division—a term called pathological polyspermy. In other animals e.g., ctenophores, insects, elasmobranchs, some amphibians, reptiles and birds, it is normal for several spermatozoa to enter the oocyte cytoplasm. This is called physiological polyspermy. However, here only one male nucleus will interact with the female nucleus while the others degenerate. In a small minority of animals (about 0.1%) the problem of polyspermy does not exist since fertilization is parthenogenetic and development proceeds without the participation of the spermatozoon.

Our knowledge on fertilization has been obtained mainly from using models, such as the sea urchin and the mammal, that lend themselves to experimentation in the laboratory. Gametes from these animals are easily harvested and fertilization and early development are easy to follow *in vitro*. The text book images of oocytes in the laboratory deprived of their extracellular coats with thousands of spermatozoa attached to their surface are familiar and these led to the idea that a surface mechanism existed that allowed the entry of one spermatozoon while blocking the entry of supernumerary spermatozoa. In particular, two activation events, an electrical depolarization and the cortical reaction were suggested to be mechanisms that had evolved to prevent the entry of multiple sperm. The term polyspermy prevention was born and quickly adopted into the scientific vocabulary and this mystic mechanism of sperm exclusion extrapolated to many other animal groups. However, under natural conditions, contrary to what many think, the number of spermatozoa at the site of fertilization is extremely low, compared to the numbers generated. The sperm: oocyte ratio is regulated first by dilution, in externally fertilizing species, and the female reproductive tract in those with internal fertilization, followed by a bottleneck created by the oocytes extracellular coats, where only the fertilizing spermatozoon encounters and responds to the correct sequence of signals and enters the oocyte. Other spermatozoa are halted or slowed down by defective or sub-optimal signaling. Since under natural conditions, the final sperm–oocyte ratios approach unity it would appear that selective pressures have favored the achievement of monospermy, rather than the evolution of polyspermy preventing mechanisms.

Laboratory Experiments and the Sea Urchin Model

Marine invertebrate oocytes have been the model of choice for centuries owing to their abundance and amenability to laboratory conditions and manipulations, and the experimental animal par excellence is of course the sea urchin, favored by scientists of note such as Hertwig, Boveri and Herbst. Sea urchins are a member of the echinoderm family and are found globally. Animals are collected by divers often without taking into account their breeding patterns or sexual maturity. Gametes are obtained, either by injecting a concentrated salt solution into their body cavity or by manual dissection. The oocytes are surrounded by a jelly layer, that is indiscriminantly removed (by acid treatment) and these large cells are often attached electrostatically to slides to flatten them out, both for imaging or electrophysiological recording. Delage over 100 years ago showed that meiotic and cytoplasmic maturity in sea urchin oocytes is asynchronous leading to heterogeneous populations of oocytes. Although oocytes are effectively arrested at a specific point in the cell cycle, waiting for the activation signal from the spermatozoon to commence development, this metabolic block is not permanent and oocytes *in vitro* will activate spontaneously although at a slower rate than those activated by the spermatozoon. This is called aging and leads to the oocyte having an altered receptivity to spermatozoa, a condition that can be mimicked by treating oocytes with a variety of physical and chemical agents such as heat shock, nicotine and drugs that affect the actin cytoskeleton.

Images of sea urchin oocytes immersed in myriads of spermatozoa in a laboratory setting triggered the concept that fertilization, at least in these animals, was a haphazard “free for all,” analogous to a first order chemical reaction and that the first spermatozoon that successfully interacted with the egg triggered membrane mechanisms that blocked the entry of other spermatozoa. Today we are much more aware that scientific experiments need to be carried out under natural conditions and, in particular, to avoid manipulating oocytes and altering the characteristics of the cells before we start an experiment (Fig. 1).

The Kinetic Experiments of Lord Rothschild

Lord Rothschild and colleagues in the 1940–50s studied sperm–oocyte interaction in sea urchins at various concentrations and conditions and came up with the idea that the fertilizing spermatozoon induced a fast, yet partial, change in the oocyte surface that preceded the cortical reaction and that reduced sperm receptivity by 1/20th. Lord Rothschild made the assumption that a suspension of spermatozoa was analogous to an assembly of gas molecules, and calculated the number of sperm–oocyte collisions at various sperm concentrations. By mixing oocytes and sperm at known densities for varying periods of time and, by treating the

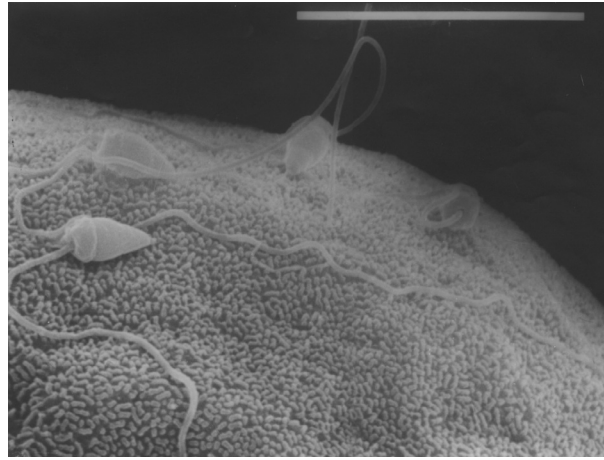


Fig. 1 The surface of a sea urchin oocyte. Are all the attached spermatozoa capable of entering the oocyte?

fertilization reaction as a first order chemical reaction, Rothschild found that the fraction of monospermic oocytes increased in time according to the relationship:

$$M(t) = 1 - e^{-\alpha t} \text{ below sperm densities of } 3 \times 10^6 \text{ per mL}$$

The relationship is similar for polyspermic oocyte at densities between 7×10^7 and 3×10^8 per mL giving a rate of appearance of polyspermic oocytes as α^1 . He argued that since α^1 (the refertilization rate) was found to be much less than α (the monospermic rate), a rapidly acting partial block reduced the probability of successful reactions after the first had occurred.

Although an exercise to be studied by all students of biology, the Rothschild Hypothesis has fundamental flaws: First, fertilization is not a first order chemical reaction and spermatozoa are not analogous to gas molecules. Second, it does not consider the possibility that there are a limited number of sperm entry sites on the oocyte surface. Lastly, it assumes that all spermatozoa are equal in their capacity to penetrate the cell. This is incorrect. Only competent spermatozoa that encounter and respond to the correct sequence of triggering events as they progress through the oocyte investments are successful.

Oocytes Are Programmed for Rapid Change

The oocyte and the spermatozoon are designed to trigger physiological changes in each other. In order to proceed through the layers of extracellular coats that surround the oocyte, the spermatozoon encounters a series of consecutive signals that induce changes in its physiology and are a pre-requisite for further progression. If one observes sperm–oocyte interaction in echinoderms or mammals with their coats intact it can be seen that many sperm are halted partially through their voyage. Once the spermatozoon has reached the oocyte plasma membrane, and probably following fusion of the two cells, this small cell (less than 500,000 smaller than the oocyte) triggers the quiescent oocyte into metabolic activity that eventually leads to meiotic resumption and formation of the zygote.

The first signs of oocyte activation are changes in the ion permeability of the plasma membrane and the entry and subsequent release of intracellular calcium. While the former maybe localized to the site of sperm entry, the latter starts at the entry site and spreads in an autocatalytic wave to the antipode. Simultaneously, and probably in conjunction, there is a massive re-organization of the cell cortex involving cytoskeletal elements and a cascade of cell cycle kinases. The length and density of microvilli, location of cortical granules, and the priming of the intracellular Ca^{2+} release mechanism are events in oocytes that are tightly controlled by the actin cortical cytoskeleton. In the starfish oocyte, the acrosome reaction can be seen to be induced at the outer layer of the jelly and a long tubule then grows from the tip of the spermatozoon to perforate the jelly and the vitelline coat to fuse with the oocyte plasma membrane. The successful spermatozoon needs to encounter a series of consecutive signals that induces changes in its physiology and are a pre-requisite for progression before triggering a localized sub-cortical polymerization of actin, beneath the fertilization cone, that is then involved in the movement of the spermatozoon into the oocyte. This successful sperm anchor site is regulated by the underlying actin cytoskeleton and, in any case, must be pre-determined.

At activation, the oocyte changes its role from a quiescent cell programmed to interact with its male counterpart, to a dynamic zygote that must be kick-started to progress through early development. The early embryo must be protected and isolated from its environment. Protection is afforded by the cortical reaction. Just below the surface of the sea urchin oocyte lie 20,000 granules each measuring about $1 \mu\text{m}$ in diameter containing a cocktail of enzymes and macromolecules. They release their contents into the perivitelline space, following interaction with the fertilizing spermatozoon, by fusing to the oocyte plasma membrane. Fusion starts at the site of sperm entry and then traverses the oocyte in a wave to the antipode taking about 30 s to complete. This leads to a net increase in the total surface area of the plasma membrane, which can be observed as a transient increase in length of microvilli.

The cortical reaction has evolved to change the receptive outer investment of the oocyte into a hardened protective layer to protect the developing embryo in the early stages of embryogenesis and to provide a microclimate for the early division cycles and morphometric movements.

Activation events are propagative, pre-determined and progress at a rate independent of sperm concentration. Thousands of biochemical and physiological pathways are triggered in a spatial and temporal pattern that does not lend itself to traditional molecular biological studies, such as depletion or over-expression of any particular component. These processes are not specific to gametes, but are biochemical and physiological processes common to all cells, however with different kinetics.

Studying Sperm–Oocyte Kinetics Using Electrophysiology

The experiments of Lord Rothschild were the first indirect attempt to measure the receptivity of the oocyte to spermatozoa. However, it was not until the 1970s that electrophysiological recording gave a precise picture of these kinetics. Sea urchin oocytes are large and transparent and easy to impale with microelectrodes to record electrical changes across the oocyte plasma membrane. Many spermatozoa attach to the oocyte, before one, the fertilizing spermatozoon, distinguishes itself by gyrating around its point of attachment. Approximately 3 s later, a small electrical step depolarization occurs, with no change in the morphology of the oocyte surface or sperm behavior, until a further 9 s later when a larger bell-shaped depolarization starts. The successful spermatozoon then stiffens, stops gyrating, and the cortical reaction is initiated at this point. A protuberance of the oocyte cortex (the fertilization cone) is formed and the sperm head slowly disappears into this cone, while the cortical reaction spreads across the oocyte to the antipode. The cortical reaction leads to the elevation of the fertilization membrane and is completed during the repolarizing phase of the fertilization potential. Unsuccessful spermatozoa, that is, those that do not enter the oocyte, attach to the oocyte surface, but do not generate either electrical step depolarizations or fertilization cones, and continue gyrating around their point of attachment for many seconds until their energy resources are depleted and fall limp to the oocyte surface. Thus progression of spermatozoa through the extracellular oocyte coats relies on them encountering and responding in sequence to oocyte borne signals. The successful spermatozoon, which may have pre-determined characteristics, completes this sequential pathway, the supernumerary spermatozoa fail at various stages falling to the wayside. In either case, the oocyte and its topographical organization selects the most fit spermatozoon. In physiological polyspermic species, such as the ctenophores, a parallel spermatozoon selection process by the oocyte occurs, but within the oocyte cytoplasm. In *Beroë* for example, which has a very clear large oocyte, several spermatozoa enter anywhere over the oocyte surface and remain immobile under the surface. The female pronucleus then starts to migrate around the oocyte surface progressively encountering several sperm pronuclei until it decides to fuse with the successful sperm pronucleus. It is not clear how this selection process is regulated however here, as with pathological polyspermy, the fertilizing spermatozoon distinguishes itself from supernumerary spermatozoa.

Fertilization Under Natural Conditions

Gametes vary enormously in form, function and numbers produced and accordingly there are many ways in which sperm–oocyte interactions are regulated.

Animals With Low Sperm Numbers

Contrary to popular belief not all animals produce large sperm: oocyte ratios. For example, in some insects and nematode worms sperm utilization is very efficient. In *Drosophila*, there is a 1:1 ratio between the progeny produced and the number of sperm in the seminal receptacles. In the nematode worm, *Caenorhabditis elegans*, every spermatozoon fertilizes an oocyte, however not all oocytes are fertilized because in fact oocytes are produced in excess. In about 0.1% of all animal species, reproduction is achieved without the participation of spermatozoa and this phenomenon is called parthenogenesis. There are many variations in the precise strategy of parthenogenesis. In aphids, for example, parthenogenetic generations alternate with those generated by fertilization and in bees an egg may be parthenogenetic or fertilized. The creation of female parthenogenetic offspring is widespread among insects such as some Phasmida, Diptera and Lepidoptera while in the crustacea, the best known example of parthenogenesis is the brine shrimp *Artemia salina*. Nematodes, rotifers, snails and flatworms also include a few parthenogenetic species, whereas in the vertebrates the most common form of parthenogenesis and female only species are found in the lizards.

Animals With High Sperm Numbers

In animals with high sperm numbers bottlenecks exist to reduce the number actually reaching the oocyte.

Reduction by elimination

Mammals are pathologically polyspermic and the sperm: oocyte ratio at origin can be as high as $10^9:1$. If we examine sperm size and number in relation to body mass in these animals it appears that evolutionary responses have favored sperm number rather than sperm size with increasing body size. Essentially, larger animals such as elephants, produce more sperm per ejaculate (corrected for

body mass) than a small mammal such as a mouse. Despite these large sperm numbers in the mammals, behavioral adaptations are required to ensure fertilization. Mating must be synchronized and the sperm need to be deposited in the female tract. In humans the spermatozoa are deposited in the vagina, while in mice they are deposited directly into the uterus. In both cases the vast majority of spermatozoa are rapidly eliminated from the tract. Only a minute fraction successfully migrate to the site of fertilization. The major barrier for sperm ascent in mice is the utero-tubal junction, with spermatozoa being progressively released from the lower part of the oviductal isthmus at ovulation. The first barrier to sperm ascent in humans, is the highly folded mucus filled cervix, where sperm are retained and released over a period of several days. Contractile activity of the uterine wall aids sperm ascension to the lower isthmus where they are sequestered until ovulation. Finally, migration from the isthmus to the ampullae appears to be due to sperm motility and contractile activity of the oviduct. In conclusion, in the mammals the number of sperm that reach the site of fertilization is regulated by the female tract. In the few *in vivo* studies where spermatozoa have been counted *in situ*, a few hundred spermatozoa were found in sheep ampullae and only five were found in man, while in rodents the sperm: oocyte ratios at the site of fertilization are usually unity or below.

In birds, which are physiologically polyspermic, hundreds of millions of sperm are inseminated, but only a few hundred reach the ovum, the vast majority are ejected by the female tract early after copulation. In the Zebra Finch and Domestic Fowl the female tract regulates the number of sperm reaching the site of fertilization and it has been suggested that although one or few spermatozoa are sufficient to activate the oocyte, the presence of several supernumerary spermatozoa in the cytoplasm of the oocyte is a pre-requisite for embryogenesis.

Dilution bottleneck

In marine animals, whether or not fertilization occurs at all depends on environmental cues, behavioral adaptations and chemotaxis. Sperm concentration in the sea urchin testis is similar to that in mammals, however many more oocytes are produced than in mammals, consequently the problem faced by these animals is making sure there are enough spermatozoa to satisfy all the oocytes spawned. In sea urchins, the sperm: oocyte ratio at source is $10^4:1$. When dealing with sea urchin gametes in the laboratory a sperm-oocyte ratio that leads to 100% fertilization at time 0, with minimum rates of polyspermy is often selected (10^6 per mL), however data collected from natural spawnings show a much lower fertilization rate and great variability in the proportion of oocytes fertilized in nature. Fertilization success in nature depends on the spawning behavior of the organisms, population size, current velocity, oocyte size, sperm swimming capacity and many other factors. The consensus from field studies is that fertilization success in free spawning benthic organisms can be $< 1\%$ and in any case is highly variable, ranging from 1% to 95%. Thus, in the environment, sperm-oocyte collisions are rare, sperm concentration may be extremely low (below 10^4 per mL) and the availability of sperm may affect female reproductive success. If, under natural conditions, sperm-oocyte collisions are indeed low, perhaps we should re-consider some of our ideas on the evolution of sexual dimorphism.

The egg coat bottlenecks

In all animals the oocyte is surrounded by complex extracellular layers or coats, which may be removed *in vitro* often without inhibiting fertilization. This is often erroneously interpreted as showing the inutility of the extracellular coats and is purely a laboratory artifact. In nature, passage through these coats is a pre-requisite for normal fertilization, oocyte activation and subsequent paternal nuclear de-condensation. Sea urchin oocytes are surrounded by a jelly layer composed of high molecular weight fucose-sulfate-rich glycoconjugates. At spawning, sea urchin sperm are exposed to the high pH of the seawater which leads to the activation of a Na^+/H^+ exchanger and a dynein ATPase causing an increase in motility. Factors released from the jelly layer, the sperm activating peptides (SAPs) further stimulate sperm motility and respiration. The majority of spermatozoa are unable to penetrate the jelly layer and remain immobilized at various depths within the jelly. Those that pass through must arrive in a physiological condition that both promotes binding and subsequently penetration of the vitelline membrane. Thus, in animals with external fertilization, sperm-oocyte ratios are extremely low and need to be enhanced by chemotactic mechanisms even to ensure a minimum of oocytes are fertilized. The outer jelly layer on the one hand attracts and traps spermatozoa, however incompetent spermatozoa (whether it be due to the sperm or the oocyte) are prevented from progressing towards the oocyte.

We have seen that also in mammals, few spermatozoa reach the oocyte under natural conditions, of those that do, some are not able to progress through the outer oocyte coating, the cumulus oophorus. A second extracellular coat, the zona pellucida, that impedes sperm progression even further is composed of several glycoproteins (ZP) that differ between species. The zona pellucida serves to modulate sperm binding and to protect the embryo during early development, however we know little about its topographical constitution and if indeed sperm entry is piloted to a specific site.

The most obvious morphological oocyte bottleneck is found in insects, squid and some teleosts. Here the oocytes lack cortical granules, the extracellular coat, the chorion, is impenetrable, and the spermatozoa lack an acrosome. In these species the spermatozoa enter the oocyte through a pre-formed entry site, the micropyle, where, in fish at least, a sperm attractant, a glyco-protein, has been identified responsible for guiding the spermatozoa to this area. In the anuran, *Discoglossus pictus*, oocytes are highly polarized with the animal pole marking the position of the meiotic plate and organelles distributed in a gradient towards the vegetal pole. Here the spermatozoa may only enter through a restricted depression at the animal pole, called the dimple, where the fine structural organization is different to the rest of the oocyte surface. In tunicates, the spermatozoon enters the oocyte at a preferential site at the vegetal pole. Polarized sperm entry coincides with polarized oocyte activation events. The first event being the release of calcium from intracellular stores which traverses the oocyte from the point of sperm entry to the antipode in a wave. For example, in jellyfish and the anuran *Xenopus laevis*, where the sperm enters the animal pole of the oocyte, the calcium wave starts at the animal pole

and traverses the oocyte to the antipode, while in ascidian and nemertean oocytes, the wave initiates at the vegetal pole, the site of sperm entry. In teleosts, where the spermatozoa are forced to enter at the animal pole through the micropyle, the calcium wave starts at the animal pole. In mammals and echinoderms the calcium wave is also initiated at the point of sperm entry however it is not known whether in these groups there are also preferential sperm entry sites. Finally, the last barrier for sperm interaction is the plasma membrane but to date there is still little information on its structure.

Sperm–Oocyte Encounters Are Infrequent in Nature

In the laboratory, we are used to seeing images of oocytes inundated with hoards of spermatozoa and this triggered the concept that fertilization was a haphazard “free for all,” analogous to a first order chemical reaction and that the first spermatozoon that successfully interacted with the egg triggered membrane mechanisms that blocked the entry of other spermatozoa. This is laboratory artifact. Activation events, triggered by the successful spermatozoon, occur at a rate independent of sperm concentration and serve to change the metabolism and morphology of the oocyte from a dormant cell to a dynamic zygote that needs to be protected from the environment throughout early development.

In nature sperm–oocyte ratios are very low. In some species they approach unity at source, while in others, that produce high numbers of sperm, massive loss by elimination, dilution and extracellular coat bottlenecks drastically reduce the final concentration at the site of fertilization. As spermatozoa progress through the extracellular layers, those encountering or responding to defective signaling fall by the wayside, until one, the successful spermatozoon, reaches its goal. It appears that selective pressures have favored the achievement of monospermy, rather than the prevention of polyspermy.

Further Reading

- Dale, B. (2016). Achieving monospermy or preventing polyspermy. *Research and Reports in Biology*, 7, 47–57.
- Dale, B., & DeFelice, L. J. (2011). Polyspermy prevention: facts and artifacts. *Journal of Assisted Reproduction and Genetics*, 28, 199–207.
- Dale, B., & Monroy, A. (1981). How is polyspermy prevented? *Gamete Research*, 4, 151–169.
- Hemmings, N., & Birkhead, T. (2015). Polyspermy in birds: sperm numbers and embryo survival. *Proceedings of the Royal Society B*, 282, 1682–1688.
- Jaffe, L. A. (1976). Fast block to polyspermy in sea urchin eggs is electrically mediated. *Nature*, 261, 68–71.
- Levitan, D., & Peterson, C. (1995). Sperm limitation in the sea. *Trends in Ecology & Evolution*, 10, 228–231.
- Lupold, S. A., & Fitzpatrick, J. (2015). Sperm number trumps sperm size in mammalian ejaculate evolution. *Proceedings of the Royal Society B: Biological Sciences*, 282, 2122–2130.
- Puppo, A., Chun, J. T., Gragnaniello, G., Garante, E., & Santella, L. (2008). Alteration of the cortical actin cytoskeleton deregulates Ca^{2+} signalling monospermic fertilization and sperm entry. *PLoS One*, 3(10), 3588.
- Rothschild, L. (1954). Polyspermy. *Quarterly Review of Biology*, 29, 332–342.
- Santella, L., Vasilev, F., & Chung, J. T. (2012). Fertilization in echinoderms. *Biochemical and Biophysical Research Communications*, 425, 588–594.
- Snook, R., Hosken, D., & Karr, T. (2011). The biology and evolution of polyspermy: insights from cellular and functional studies of sperm and centrosomal behaviour in the fertilized egg. *Reproduction*, 142, 779–792.
- Yanagimachi, R. (1994). Mammalian fertilization. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction* (2nd edn.). Raven Press: New York.

EMBRYOGENESIS

Cleavage and Cleavage Patterns in Embryogenesis

Heide Schatten, University of Missouri, Columbia, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction and Overview: Cell Division and Cleavages During Pre-Implantation Embryo Development

In early embryo development, a series of cell divisions take place that partition a large single cell, the fertilized oocyte, to become a complex multicellular organism, the mammalian embryo. The fertilized oocyte (zygote) surrounded by a thick external layer, the zona pellucida, is called conceptus. The zygote contains the genetic material from both male and female gametes which amounts to 23 chromosomes each. These combined 46 chromosomes undergo changes during a cell cycle before mitotic divisions occur resulting in the formation of the embryo with two and more cells. Early embryo development is distinguished by different stages which is based on the number of cells and the embryo's location in the female reproductive system, as shown in Fig. 1.

As research on human embryos is limited and largely restricted much of our knowledge on early embryo development and cleavage patterns comes from animal models such as the mouse, the pig, and the bovine systems. The mouse has been utilized for most developmental studies, as it is relatively easy to use for experimental purposes including the amenability for a wide variety of experimental manipulations but it is also different in some aspects from other mammalian systems that need to be taken into consideration when comparisons are made with human embryology. The porcine system has gained increased attention because of its use for biomedical purposes and as a model for human diseases. In this article data are presented that have been obtained from the mouse, the human, and also the pig.

In all mammalian (rodent and non-rodent) systems, after fertilization of the oocyte that takes place in the ampulla of either one of the two oviducts (Fallopian tubes) the fertilized oocyte (zygote) divides into two cells (two-cell stage) followed by five to six mitotic cell divisions. The individual cells resulting from these early cell divisions (cleavages) are called blastomeres. The zygotes of mammalian species undergo well-defined cell cycles but only very limited growth which results in smaller cells after each cleavage division while maintaining the same overall size as the zygote. A typical cell cycle in the early embryo takes between 10 and 12 h. At these early stages the term cleavage is primarily used to describe the division of cells through which the number of cells is increased without increasing the mass which is different from cell division cycles in somatic cells, as in early embryo cells cleavage divisions occur without increasing the volume of the conceptus. The individual blastomere cells resulting from cleavage divisions together

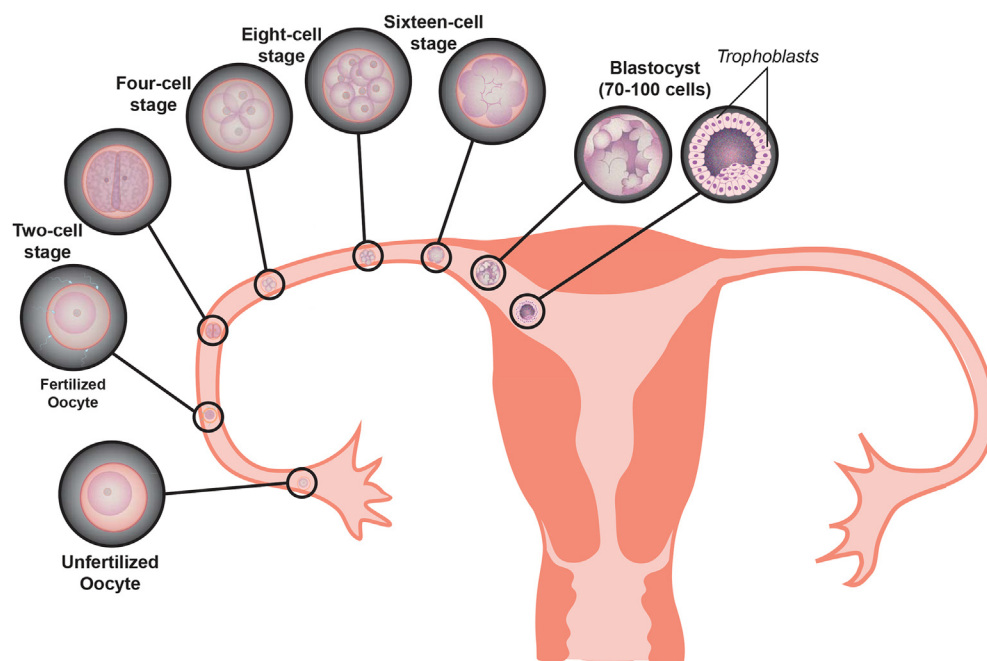


Fig. 1 Fertilization and pre-implantation development takes place at specific locations within the female reproductive system in approximately a time span of 1 week, as described in the text of this article.

form the compact early mammalian embryo, the morula. From the 8- to 16-cell stage flattening of the blastomere cells takes place, a process during which tight connections are formed between the individual blastomeres. This process is called compaction. The process of compaction is primarily mediated by the expression of E-cadherin, a Ca^{++} -dependent adhesion molecule which is essential for the compaction process. When E-cadherin is inhibited compaction does not take place. During the compaction stage from the 8- to 16-cell stage, deposition of extracellular matrix (ECM) occurs which also plays a role in signaling to the embryo cells.

Compaction results in two cellular lineages, the trophoblast which forms the fetal components of the placenta, the chorion, and the inner cell mass which will form the embryo proper. The trophoblast is important for the attachment of the embryo to the uterine lining (endometrium) before implantation can occur. The inner cells will form the inner cell mass (ICM) that will be re-organized into two layers: the epithelial epiblast that will form the embryo and amniotic membrane, and the hypoblast that plays a role in the orientation of the embryonic axes. Synchronous timing of pre-implantation embryo development and the mother's uterus is important for successful implantation, as embryo loss can occur if either one of these events is defective. Compaction of the pre-implantation embryo is a critical event, as fates of the individual blastomeres begin to change at this time to result in differences in individual blastomeres. The differences of individual blastomeres from each other are prompted by specific signals that are communicated to the different blastomeres. Specific signaling depends on several factors including the location of blastomeres within the pre-implantation embryo. These specific signals guide each blastomere's fate which now results in committed cells to pursue their developmental fates. Differentiation of cells includes their status from totipotency (the ability to undergo various cell fates) to pluripotency that allows development only into specific cells in the body. These pluripotent cells will then differentiate to commit to their fate throughout a lifespan.

At about 3 days after fertilization the 16-cell conceptus reaches the uterus. The conceptus continues to divide and in the uterus forms a ball of about 100 cells, called the blastocyst which starts to secrete fluid to form the blastocoel cavity. Blastocyst formation starts at the 16- to 32-cell stage, the morula stage. At this stage tight junctions are developed in the outer cells which do not allow fluid to pass. The outer cells now secrete fluid through a mechanism that involves action by a $\text{Na}^+ - \text{K}^+$ ATPase to pump sodium from the external environment into the embryo which is important for forming the blastocoel cavity. The accumulating fluid fills the blastocoel or blastocyst cavity. By the 64-cell stage (3.5 days) the embryo cells have differentiated and outer trophoblast (TE) cells and the inner cell mass (ICM) cells are clearly distinguished. At this stage the TE consists of about 45 cells while the ICM consists of about 15 cells. Until this time the embryo is still enveloped in the zona pellucida. Hatching from the zona pellucida occurs on day 5, a process during which the blastocyst is released from the zona pellucida and ready for implantation.

In this article, the early stages of development (pre-implantation stages) are discussed while other chapters in this Encyclopedia will detail the stages of implantation and post-implantation development resulting in the birth of a healthy offspring.

The stages of early embryo development are displayed in [Fig. 1](#) and summarized from the first cleavage stages to blastocyst formation as follows. Cleavage follows a partition pattern with no growth until the morula stage.

1-cell stage—fertilized oocyte (zygote)—*day 1*.

2-cell stage—developing embryo at ca 30 h (ca. 1.5 days) post insemination.

4-cell stage—developing embryo at ca 40 h post insemination (ca. 2 days).

8-cell stage—developing embryo at ca 2.5 days post insemination.

16-cell stage—morula; developing embryo at ca 3 days post insemination.

32-cell stage—*morula*, divisions and differentiation takes place in equal-sized blastomere cells of the developing embryo at ca 3 days post insemination.

58-cell stage—early blastocyst developing embryo at ca 4 days post insemination.

107-cell stage—late blastocyst developing embryo at ca 4.5 days post insemination.

The early blastocyst at ca 4.5 days post insemination displays formation of ICM, trophoblast, blastocoel. During blastulation hundreds of cells are produced resulting in the trophoblast that gives rise to the chorion and placenta; the ICM gives rise to the organism, the embryo, while the blastocoel provides nutrients ([Figs. 2 and 3](#)).

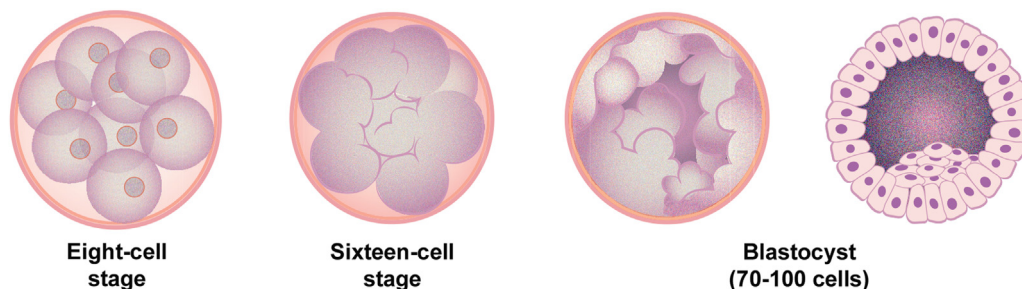


Fig. 2 From the 8-cell stage (left) to the blastocyst stages (right) numerous cleavages take place resulting in a well-defined blastocyst with TE and ICM cells.

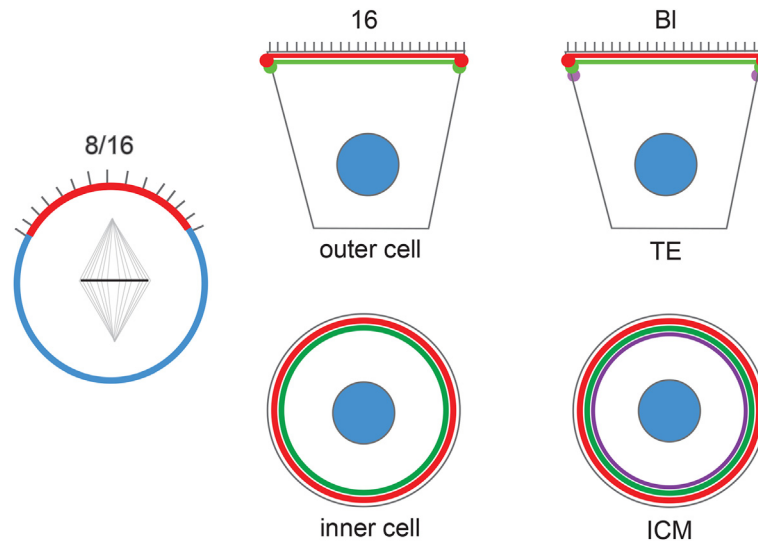


Fig. 3 Shown here are cell divisions during compaction from the 8- to 16-cell stages (8/16) when outer cells (TE cells) and inner cells (ICM cells) are established. *BI*, blastocyst; *TE*, trophectoderm; *ICM*, inner cell mass. Modified from Vinot S, Le T, Ohno S, Pawson T, Maro B, Sophie Louvet-Vallée S (2005). Asymmetric distribution of PAR proteins in the mouse embryo begins at the 8-cell stage during compaction. *Developmental Biology*. 282, 307–319.

Section 2: The Process of Cell Divisions and Establishment of Symmetric and Asymmetric Cleavage Division Patterns

The mechanisms of cleavage divisions are overall similar to cell divisions in other cells in which two major cytoskeletal elements play a role, which are microtubules and microfilaments. Microtubules form the mitotic apparatus (mitotic spindle) whose main function is to separate chromosomes to the newly dividing cells (Fig. 5). These microtubules are organized from centrosomes (microtubule organizing centers; MTOCs) at the two opposite mitotic poles. Two classes of microtubules exist in the mitotic spindle that are important for spindle functions and either connect the two poles (polar microtubules) or connect the polar centrosome from one half of the spindle to the kinetochores of chromosomes of the same half (kinetochore microtubules) (Fig. 5). The mechanisms for embryonic cleavage divisions differ to some extent in rodent and non-rodent mammalian embryo systems. In blastomeres of non-rodent early mammalian embryos centrosomes contain centrioles (microtubule-based tubular structures; see Fig. 5) that are brought into the non-rodent oocyte by sperm at fertilization while blastomeres of rodent early mammalian embryos do not contain centrioles; the sperm in rodent species does not contribute a centriole to the fertilized oocyte. It has been shown that centrioles are not even tolerated by the early rodent embryo and are destroyed if experimentally introduced into the rodent oocyte. In contrast to non-rodent mammalian species rodents display different mechanisms for meiotic and mitotic spindle formation (reviewed in Schatten and Sun, 2011a,b, 2017) up to the early blastocyst stages when centrioles are formed de novo in rodents to participate in subsequent cell divisions. *Microfilaments* located underneath the cell surface play a major role during blastomere cell shape changes and during cytokinesis at the end of cell division when a contractile ring forms between cells to separate the dividing blastomere cells.

In the mouse, the first cleavage occurs at about 16–20 h after fertilization, and the following cleavages each take roughly 12 h. The cell cycle length varies not only among different embryos but even among blastomeres within the same embryo.

The first cell divisions (cleavages) in the mammalian embryo are symmetric while later cell divisions are asymmetric, resulting in distinctly different cells after cytokinesis is completed (Fig. 5 right panel). Unequal cleavages in the mammalian embryo typically occur after asymmetric positioning of the mitotic spindle (Fig. 5, right panel from Marikawa and Alarcón, 2009).

The specific cleavage patterns of embryo cells are only known to some extent. In general, cleavage patterns of blastomeres of 8-cell stage embryos and of external blastomeres of 16-cell stage embryos can be categorized into two groups: symmetric and asymmetric. Symmetric cleavage is defined as the plane of cell division being parallel to the apical–basal axis which results in two external blastomeres; asymmetric cleavage is defined as the plane of cell division being perpendicular to the apical–basal axis, resulting in one external and one internal blastomere (Fig. 5, right panel).

An apical–basal polarity occurs in all eight blastomeres. The number of external and internal blastomeres at the 16-cell stage varies among embryos and amounts to an average of 6–7 internal and 9–10 external blastomeres. The fifth cleavages generate more external and internal cells, and 12–13 internal and 19–20 external blastomeres are present at the 32-cell stage embryo.

The mechanisms and timing of regulation for compaction is not yet completely understood but based on the available data it is likely not controlled by the number of cell divisions, as the inhibition of blastomere division or DNA replication does not delay the process of compaction.

To gather conclusive data studies of fourth cleavages in developing embryos using time-lapse video approaches have revealed that the planes of cell divisions can occur in various different positions relative to the apical–basal axis. It suggests that the orientations of cleavage planes can vary and are strictly following a parallel or perpendicular orientation to the apical–basal axis. Such flexibility in patterning is also observed during the fourth and fifth cleavages. As is known for mitotic cells, a mitotic blastomere cell first becomes spherical and divides into two round blastomeres, which then flatten and incorporate smoothly into the whole embryo. These studies have shown that TE and ICM cells are not strictly determined by cleavage orientations and can alter between symmetric and asymmetric patterns at the fourth and fifth cleavages. At this time it is not clear whether cleavage orientations are regulated by molecular factors such as fate determinants, for example *Cdx2*, that have been studied extensively in the mouse system. Different technologies such as targeted knockout/knockdown of specific gene products combined with high-resolution live cell imaging will be needed to analyze in more detail the timing and requirements for accurate cell divisions leading to implantation-compatible pre-implantation embryos.

As mentioned above, because research on humans is limited and to a large extent restricted much research has been performed on animal models such as the mouse, porcine and bovine systems to understand the mechanisms involved in early cleavages and embryo development. However, animal systems are different in various aspects, including the occurrence of mitotic aneuploidies (chromosomal imbalances; imbalances in chromosome numbers) that are more frequent in the human and are major causes for implantation failures and embryo loss. Studies in animal models have allowed experimental manipulations in order to obtain more informed data for cleavage divisions and embryo development. Much of the experimental research for the past decades has been performed in the mouse. For example, cell fate determination of individual cells has been extensively studied in the mouse, as the mouse system is highly amenable to experimental manipulations. In the mouse embryo and perhaps all other mammalian species, successive asymmetries develop during embryo development. While the issue is not yet completely resolved experimental studies including micromanipulation techniques, blastomere isolation, aggregation, and transplantation, have resulted in concluding that the fates of blastomeres are not determined until the fifth cleavages. During the last decade more data have become available to show that individually dissociated blastomeres of 16- or 32-cell stage embryos become re-aggregated as a single mass with both types of aggregates being capable of forming expanded blastocysts with the correct associations and expressions. These experiments have shown that the location of blastomeres (i.e., external or internal) at around the 32-cell stage is tightly linked to the commitment towards TE or ICM lineage.

While the mouse has allowed us to gain fundamental knowledge and critical data for mammalian embryo development the porcine and bovine systems are being explored increasingly, as these systems in several aspects are important for biomedical research applications for humans and because several cell and molecular processes are similar to those in human reproduction. For example, in humans but not as frequently in other mammalian reproductive systems, meiotic and mitotic error rates are high compared with other mammalian species. Mitotic and cleavage errors resulting in abnormalities are manifested in aneuploidies which are a major cause of implantation failure and embryo loss (Niakan et al., 2012). It has been reported that aneuploidies may occur in one or more blastomeres in more than 80% of human pre-implantation embryos in which abnormal cleavage divisions have been observed. Time-lapse imaging of live human embryos (Wong et al., 2010; Fig. 4) revealed that timing of the early cleavage divisions is especially important for proper embryo development and implantation to avoid embryo loss (reviewed in Niakan et al., 2012). This knowledge is particularly important for in vitro fertilization (IVF) and other assisted reproductive technologies (ARTs) in which adequate in vitro culture of embryos is an important criterion for achieving successful pregnancies and birth of a healthy baby.

In vitro fertilization (IVF) is increasingly being performed to help infertile couples to conceive a child. In this procedure oocytes are removed from the ovary and fertilized with sperm outside the female reproductive tract. The fertilized egg or early embryo is then

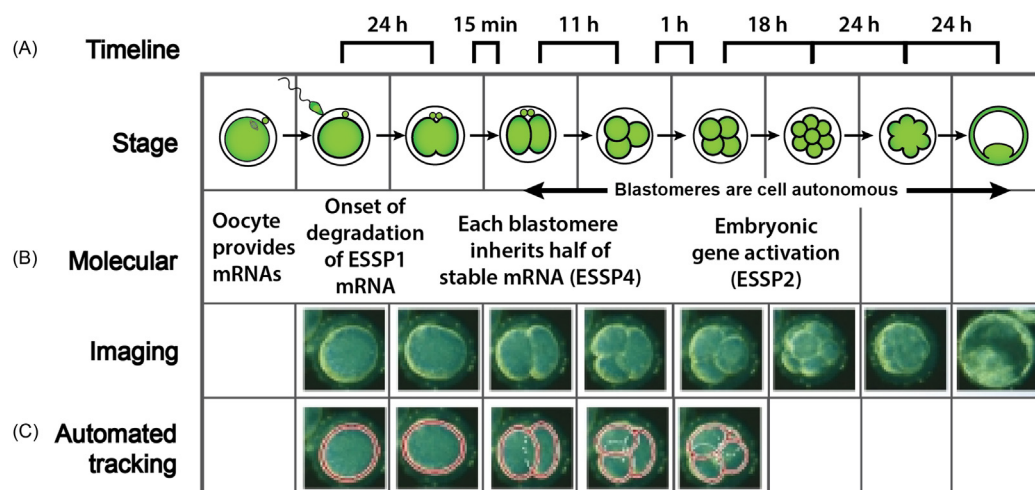


Fig. 4 Time-lapse imaging of live human embryos shows cleavage divisions up to the blastocyst stages.

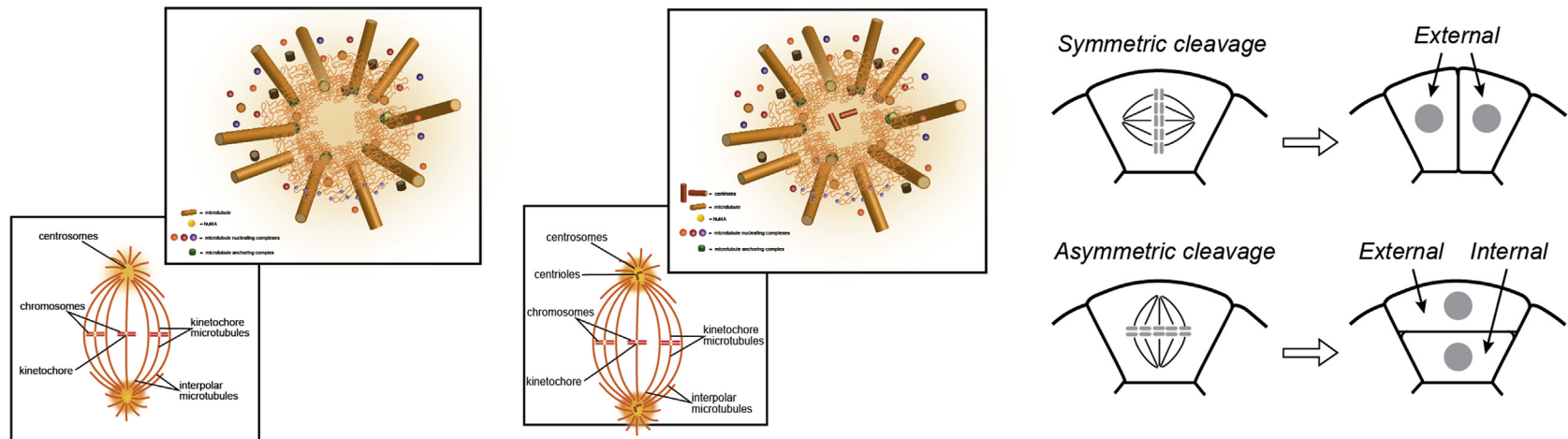


Fig. 5 Mitotic apparatus of first embryonic cell division in rodent (left) and non-rodent (center) species. Rodent systems are different from non-rodents in that a centriole is absent from the center of the centrosome complex while it is present in non-rodent species (for review see Schatten and Sun, 2011a,b, 2017). During early cleavage divisions symmetric and asymmetric cleavages can take place (right).

returned to the woman's uterus where embryo development will take place as known for in vivo-fertilized eggs. This procedure is frequently applied for various reasons, for example when fallopian tubes are damaged or blocked, when hormonal imbalance is experienced or when the woman has endometriosis.

IVF has been used successfully since 1978, when the first child conceived by this procedure was born. Since then thousands of couples have chosen IVF or other ART technologies and methods for improving ART have been developed, recognizing the importance of timing for cleavage as mentioned above.

Embryo selection is among the critical aspects of predicting successful embryo development, implantation, pregnancy, and birth of a healthy baby when IVF is performed.

As aneuploidy is among the highly probable causes for implantation failure, for IVF, time-lapse monitoring primarily uses grading of cleavage-stage embryos to assess the degree of embryo fragmentation, number of nuclei and size, and number and symmetry of blastomeres in the embryo. In humans, high-quality cleavage-stage embryos (day 2 and 3) display four cells on day 2 and eight cells on day 3. On day four, a high-quality morula has developed, with the fourth round of cleavage divisions and compaction. On day 5, the high-quality blastocyst is fully expanded and ICM and TE cells can easily be distinguished. Mitotic aneuploidies, while observed in all developmental stages, are more common during the first three mitotic divisions.

Mitotic aneuploidies may not only contribute to implantation failure but to fetal complications if implantation has taken place, such as intrauterine growth delay, congenital malformations, mental retardation, and uniparental disomy.

Future research using new technologies and methodologies such as single-cell analysis is expected to provide new information on lineage segregation and molecular mechanisms underlying cell and developmental key events. In addition, microscopy and new imaging modalities will contribute valuable data on live and fixed cell samples to enhance our knowledge of cell and molecular events underlying embryo development.

References

- Marikawa, Y., & Alarcón, V. B. (2009). Establishment of trophectoderm and inner cell mass lineages in the mouse embryo. *Molecular Reproduction and Development*, 76(11), 1019–1032. <https://doi.org/10.1002/mrd.21057>.
- Niakan, K. K., Han, J., Pedersen, R. A., Simon, C., & Reijo Pera, R. A. (2012). Human pre-implantation embryo development. *Development*, 139(5), 829–841.
- Schatten, H., & Sun, Q. Y. (2011a). Centrosome dynamics during meiotic spindle formation in oocyte maturation. *Molecular Reproduction and Development*, 78, 757–768.
- Schatten, H., & Sun, Q. Y. (2011b). New insights into the role of centrosomes in mammalian fertilization and implications for ART. *Reproduction*, 142, 793–801.
- Schatten, H., & Sun, Q. Y. (2017). Cytoskeletal functions, defects, and dysfunctions affecting human fertilization and embryo development. Chapter 10. In H. Schatten, & G. M. Constantinescu (Eds.), *Animal models and human reproduction* (1st edn, pp. 355–380). New York: John Wiley & Sons, Inc.
- Wong, C., Loewke, K., Bossert, N., Behr, B., DeJonge, C., Baer, T., & Reijo Pera, R. R. (2010). Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nature Biotechnology*, 28, 1115–1121. 20890283.

Further Reading

- Chazaud, C., & Yamanaka, Y. (2016). Lineage specification in the mouse preimplantation embryo. *Development*, 143, 1063–1074. <https://doi.org/10.1242/dev.128314>.
- Chen, A. E., Egli, D., Niakan, K., Deng, J., Akutsu, H., Yamaki, M., Cowan, C., Fitz-Gerald, C., Zhang, K., Melton, D. A., et al. (2009). Optimal timing of inner cell mass isolation increases the efficiency of human embryonic stem cell derivation and allows generation of sibling cell lines. *Cell Stem Cell*, 4, 103–106 (PMCID: PMC3335201) 19200798.
- Kirkegaard, K., Dyrland, T. F., & Ingerslev, H. J. (2017). Clinical application of methods to select in vitro fertilized embryos. Chapter 7. In H. Schatten, & G. M. Constantinescu (Eds.), *Animal models and human reproduction* (1st edn, pp. 267–312). New York: John Wiley & Sons, Inc.
- Mantikou, E., Wong, K. M., Repping, S., & Mastenbroek, S. (2012). Molecular origin of mitotic aneuploidies in preimplantation embryos. *Biochimica et Biophysica Acta*, 1822, 1921–1930.
- Mordhorst, B. R., & Prather, R. S. (2017). Pig models of reproduction. Chapter 9. In H. Schatten, & G. M. Constantinescu (Eds.), *Animal models and human reproduction* (1st edn). New York: John Wiley & Sons, Inc.
- Oestrup, O., Hall, V., Petkov, S. G., Wolf, X. A., Hyldig, S., & Hyttel, P. (2009). From zygote to implantation: morphological and molecular dynamics during embryo development in the pig. *Reproduction in Domestic Animals*, 44(Suppl. 3), 39–49. <https://doi.org/10.1111/j.1439-0531.2009.01482.x>.
- Petkov, S., Freude, K., Mashayekhi, K., Hyttel, P., & Hall, V. (2017). Towards development of pluripotent porcine stem cells by road-mapping early embryonic development. Chapter 19. In H. Schatten, & G. M. Constantinescu (Eds.), *Animal models and human reproduction* (1st edn). New York: John Wiley & Sons, Inc.
- Schatten, H., & Sun, Q. Y. (2015a). Centrosome and microtubule functions and dysfunctions in meiosis: Implications for age-related infertility and developmental disorders. *Reproduction, Fertility and Development*, 27(6), 934–943. <https://doi.org/10.1071/RD14493>.
- Schatten, H., & Sun, Q.-Y. (2015b). Centrosome-microtubule interactions in health, disease, and disorders. In H. Schatten (Ed.), *The cytoskeleton in health and disease*. New York: Springer Science and Business Media.
- Vinot, S., Le, T., Ohno, S., Pawson, T., Maro, B., & Sophie Louvet-Vallée, S. (2005). Asymmetric distribution of PAR proteins in the mouse embryo begins at the 8-cell stage during compaction. *Developmental Biology*, 282, 307–319.

Zygotic Genome Activation: The Dawn of Independence

Melissa Harrison, University of Wisconsin-Madison School of Medicine and Public Health, Madison, WI, United States
Katharine Schulz, University of Wisconsin-Madison, Madison, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromatin The filamentous material that makes up the eukaryotic chromosome consisting of DNA, histones, and other packaging proteins.

Cytokinesis The physical separation of two daughter cells following mitosis or meiosis.

Haploid Having a single set of chromosomes. Note: Most animals have *diploid* somatic cells (with two paired sets of chromosomes), but produce haploid gametes.

Intron A portion of a gene that is transcribed into RNA but is removed before the remaining *exons* are translated into protein.

Mitosis Part of the cell cycle in which replicated chromosomes are separated to form two new nuclei.

Nucleosome The basic structural unit of chromatin, consisting of a DNA duplex wound around a core of eight histone proteins.

Pioneer factor A specialized transcription factor that establishes or maintains open chromatin at its binding sites; the first factor to engage a region of repressive chromatin.

Pluripotent Capable of giving rise to many of the cell types that make up the body of an organism. Note: As cells undergo *differentiation*, they become restricted in which cell types they can produce.

Transcription The first step of gene expression in which the genetic information encoded in DNA is transferred to RNA.

Introduction: Life Starts Under Maternal Control

In nearly all animals, the embryo relies exclusively on maternal gene products during the first hours following fertilization. At this time, the newly formed zygotic genome remains transcriptionally silent (Newport and Kirschner, 1982b). All the proteins and RNAs needed for this phase of development are produced in the mother and packaged into the egg (Tadros and Lipshitz, 2009). Ultimately, control of development is passed to the zygotic genome through a hand-off known as the maternal-to-zygotic transition (MZT). The MZT encompasses two coordinated processes: the clearance of maternally supplied products and zygotic genome activation (ZGA) (Tadros and Lipshitz, 2009).

Activation of the zygotic genome is ultimately required for survival as maternal products are only capable of driving development for a limited time. To become an adult, an organism must produce an array of diverse cell types and this, in turn, requires the precise expression of select genes. Gene expression is rapidly adjusted to accommodate developmental transitions and to respond to changes in the environment. Thus, as development continues, the prepackaged supply of maternal products must be replaced by proteins and RNAs produced from the zygotic genome to allow the embryo to be responsive to these signals.

In this article, we provide a brief description of the processes that shape the activation of the zygotic genome. In “**Part I: Silence Buys Time**” we discuss the developmental requirements for a period of transcriptional quiescence, and in “**Part 2: Orchestrating the Transfer of Power**” we discuss the mechanisms that enable genome activation.

Part I: Silence Buys Time

The newly assembled zygotic genome must be reprogrammed to allow for embryonic development. The period of transcriptional silence that follows fertilization enables this reprogramming to be carried out. This delay ensures that developmental genes are prepared for expression and provides time for the repeated duplication of the genome, allowing the embryo to increase its production capacity.

Time for Reprogramming

At fertilization, the egg and sperm each contribute one haploid set of chromosomes, which combine to form the new zygotic genome. These chromosomes have been repurposed; they come from two highly specialized cells in which they served to produce a specific set of gene products. To be of use to the embryo, the genome needs to be reprogrammed: the gamete-specific expression program must be erased and the genes needed for early development must be prepared for expression.

Much of our understanding of genome reprogramming stems from efforts to manipulate cell identity. Roughly half a century ago, John Gurdon generated living tadpoles from the nucleus of a fully-differentiated, adult frog cell using a technique now known

as somatic cell nuclear transfer (Gurdon, 1962). In this method, a somatic nucleus is transplanted into an enucleated egg. Cytoplasmic components of the egg reprogram the transplanted genome to allow it to give rise to a new organism. Thus, Gurdon's achievement revealed that all the factors required to revert the somatic nucleus to a pluripotent state come packaged within the egg.

Building on this work, in 2006, Takahashi and Yamanaka generated the first induced pluripotent stem cells (iPSCs). By forcing differentiated cells to express a defined set of transcription factors they converted them to a pluripotent state, much like that of an embryonic stem cell (Takahashi et al., 2006). Since this seminal discovery, many attempts have been made to improve the efficiency of this process in hopes of developing new treatments for disease. Importantly, this work has highlighted the central role that transcription factors and chromatin regulation play in reshaping cell identity.

The reprogramming that takes place in the embryo during the MZT mirrors this in vitro reprogramming. In both cases, the previous cell identity must be cleared to return the genome to a pluripotent state. This flexible chromatin state is what allows iPSCs to be differentiated into numerous cell types and makes them a promising therapeutic tool. Likewise, pluripotency grants the embryo the capacity to give rise to all the diverse cells that make up the adult.

Time for Amplification

The MZT is a highly conserved process. It occurs in nearly all animals and even in some plants. Nonetheless, much of what we know about this transition has been gleaned from a few model species including the fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), frog (*Xenopus laevis*), and mouse (*Mus musculus*) (Tadros and Lipshitz, 2009). While many features of the MZT are shared across species, the timing of this transition differs substantially, both in terms of hours post-fertilization and in terms of cell cycle number (Fig. 1).

Many animals, including frogs and zebrafish, undergo a series of rapid, synchronized cellular divisions after fertilization. These divisions generate a ball of cells, called a blastula, which occupies the same volume as the egg (Tadros and Lipshitz, 2009). Flies undergo a similar process but without cytokinesis. Their nuclei are replicated in a common cytoplasm until the end of the MZT, at which time they are engulfed by cell membranes to form individual cells (Tadros and Lipshitz, 2009).

The mammalian MZT follows a different paradigm. In mice, the first cell cycle takes about a day. For comparison, the first replication cycle takes a mere nine minutes in flies. In the three hours it takes the fly embryo to complete 13 nuclear divisions and undergo ZGA, the mouse embryo is only an eighth of the way through its first cell division (Tadros and Lipshitz, 2009). These different developmental strategies can be traced to the environment of the developing embryo (Yuan et al., 2016). In the protected environment of the mammalian uterus, an embryo has plenty of time to carefully execute a day-long cell cycle. Whereas, if an externally developing embryo is to survive after being abandoned in a hostile environment, it must develop quickly.

Though it may be slow to replicate, the mammalian genome requires little amplification prior to activation. While the fly genome undergoes 13 pre-ZGA replications, the mouse genome is ready after only one (Tadros and Lipshitz, 2009). Again, this difference stems from the unique environments of the developing zygote. Unlike the tiny, placenta-fed mammalian embryo, an externally developing embryo must be loaded with all the nutrients the animal will need prior to hatching (Yuan et al., 2016). A huge cell is required to accommodate this supply. Yet, like any cell, the embryo begins life with only a single genome. Within its bulky cytoplasm, the transcriptional output of one genome is not enough to produce the rapid changes in protein and RNA levels required for development. Thus, in egg-laying species, extensive amplification is needed for the genome to reach sufficient production power.

Part 2: Orchestrating the Transfer of Power

The MZT is not accomplished through a sudden coup but through a gradual relinquishing of maternal control. Accordingly, ZGA is also a progressive process. While embryos experience a major burst of transcription near the end of the MZT, a small subset of genes is expressed much earlier (Harrison and Eisen, 2015). The products of these early genes help prepare the embryo for ZGA by clearing maternal products, modifying the chromatin, and serving as transcriptional activators (Lee et al., 2014). Thus, gene activation is precisely regulated such that each gene is turned on at a particular time.

While the exact sequence of molecular events that leads to ZGA remains unclear, several mechanisms that regulate its onset have been elucidated. To initiate transcription, a permissive chromatin environment is established, transcriptional activators outcompete maternal repressors, and the cell cycle slows.

Prime the Chromatin

In eukaryotes, the expansive genome is compressed to fit it inside the nucleus. DNA is wrapped around histone octamers to form nucleosomes, and linker histones bundle nucleosomes together to provide further compaction. Often, this packaging creates a physical barrier that prevents the transcriptional machinery from accessing the DNA, silencing the expression of underlying genes. In this way, chromatin packing is an important regulator of gene expression.

The cell uses several mechanisms to adjust chromatin accessibility. Nucleosomes are shifted along the DNA or ejected by remodeling complexes. Within the nucleosome, histone variants are swapped in for the standard set and chemical modifications are added to histone tails. These changes can alter how tightly a nucleosome interacts with DNA or serve as a signal that blocks or promotes the

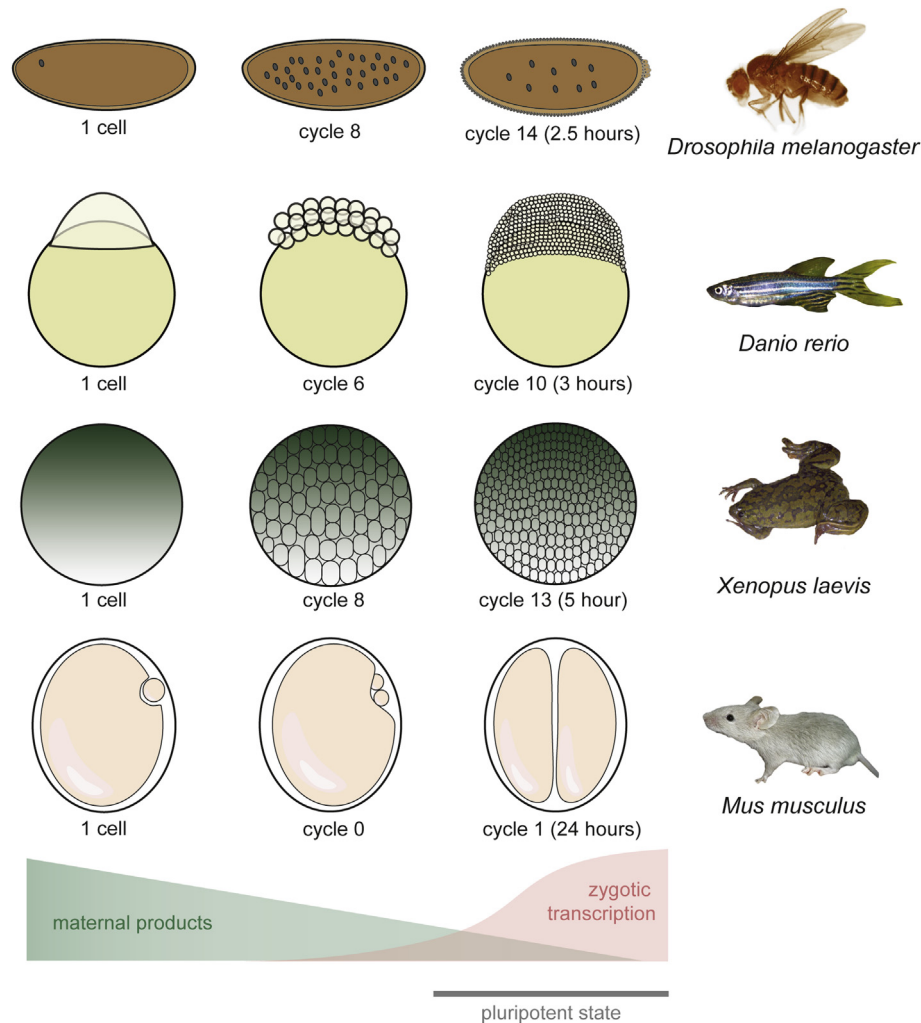


Fig. 1 Zygotic genome activation is shared amongst animals, but the timing of activation differs. Early embryonic development is summarized for four model species, indicated on the right. The “1 cell” stage, an intermediate stage, and the stage at which major onset of ZGA occurs are illustrated. The later stages are labeled with the cleavage cycle number, and the absolute time of ZGA (in hours post fertilization) is included in parenthesis. The timing of clearance of maternal products and activation of zygotic transcription are represented by curves below, and the period of pluripotency is marked with a bar.

recruitment of chromatin regulators. During the first hours of development, a variety of such mechanisms are used to shape the chromatin, ensuring that only the right genomic regions are accessible for activation.

Given the abundance of histones in the early embryo, one might imagine that the DNA would be saturated, contributing to global transcriptional repression. Yet, across species, early chromatin appears to be relatively open (Pálffy et al., 2017). Growing evidence suggests that this chromatin exists in a “naïve” state, analogous to a blank slate. Notably, early chromatin is devoid of histone modifications that typically accompany gene expression (Harrison and Eisen, 2015).

During the MZT, reprogramming erases the gamete cell identity and produces a permissive state that serves as the starting point for genome activation. Right before the major onset of transcription, the genome’s three-dimensional organization becomes more defined (Flyamer et al., 2017; Hug et al., 2017). At the same time, embryonic histone variants are replaced by somatic versions, histone modifications become prevalent, and nucleosomes are repositioned near critical genes (Harrison and Eisen, 2015; Pálffy et al., 2017; Fig. 2A). Together, these changes make the chromatin transcriptionally competent and give activators access to the correct genomic sites.

Overcome Maternal Repression

In the 1980’s Newport and Kirschner proposed that the cell cycles of the early embryo served as a developmental timer (Newport and Kirschner, 1982a,b). As the genome is duplicated within the unchanging cytoplasmic volume of the embryo, the exponentially increasing DNA could titrate a maternally supplied repressor. According to this model, once the DNA levels reach a certain threshold

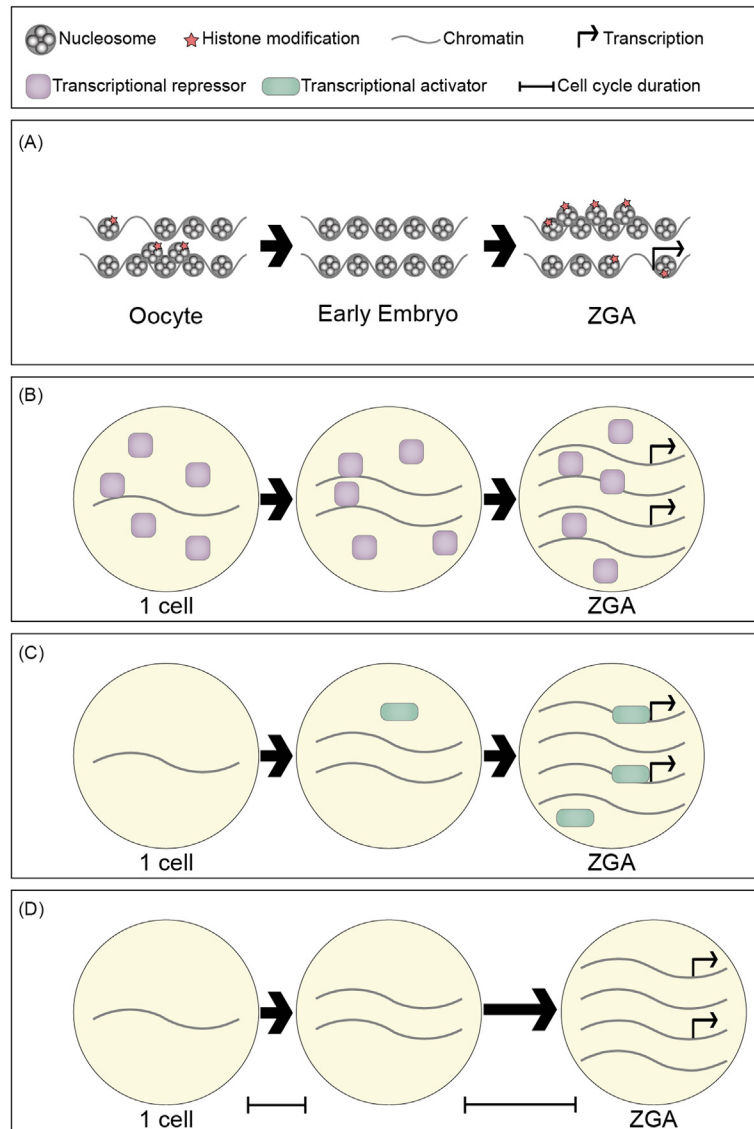


Fig. 2 Multiple complementary mechanisms regulate the onset of zygotic transcription. (A) After fertilization, the chromosomes are reprogrammed to a more naïve state. As the genome is activated, the chromatin is reorganized into defined regions, nucleosomes are repositioned, and histones are post-translationally modified at specific sites. (B) A maternally supplied repressor blocks transcription in the early embryo. As the genome undergoes replication, the exponentially increasing DNA titrates away this repressor, allowing transcription to initiate. Evidence suggests that histones may function as such a repressor. (C) A key transcriptional activator is limiting in the early embryo. Accumulation of this activator enables transcription of target genes. (D) The rapid, early cell cycles interrupt transcription. Cell cycle slowing allows time for completion of transcription.

there is no longer a sufficient quantity of the maternal repressor to silence the genome, allowing for transcriptional activation (Fig. 2B). In support of this model, altering the DNA content of the embryo shifted the timing of ZGA in a number of species (Pálffy et al., 2017).

Naturally, this model hinges on the existence of a titratable repressor. For years, efforts to identify this hypothetical repressor were largely unsuccessful. The few candidates that were identified could only account for the repression of a handful of genes (Tadros and Lipshitz, 2009). However, recent data supports a model in which histones serve a global repressor function (Pálffy et al., 2017). Notably, manipulating histone levels in zebrafish embryos affects the timing of ZGA (Joseph et al., 2017). Histones are maternally deposited in great excess but are progressively titrated by the exponentially increasing DNA content. Fittingly, in zebrafish, a drop in the nuclear histone concentration coincides with the ZGA (Joseph et al., 2017). Histones seem intuitively well suited for global repression; they bind ubiquitously across the genome and prevent gene expression by blocking the access of the transcriptional machinery. However, the density of histones on DNA changes very little in the developmental stages leading up to ZGA. Instead, it is the excess of non-DNA-bound histones that appears to serve as a ZGA timer. In this model, the unbound histones compete with transcriptional activators for binding to DNA. As relative histone levels decrease, activators are able to

successfully compete for DNA binding, and the balance is shifted toward transcriptional activation. Thus, excess histones may serve as a buffer against premature transcription while the genome is reprogrammed.

Accumulate Activators

In theory, the accumulation of a transcriptional activator could also serve as a ZGA timer (Fig. 2C). The classic example of a rate-limiting activator is TATA binding protein (TBP). TBP is a general transcription factor that helps position the transcriptional apparatus over the start of a gene. In the early frog embryo, TBP is present as mRNA, but it is not translated at detectable levels until right before ZGA. The timely increase in the levels of this protein coincides with widespread activation of transcription (Lee et al., 2014).

In addition to general factors, gene-specific transcription factors are also required to ensure that only the correct genes are activated during ZGA. These factors bind to specific DNA sequences, and thus, help to direct the transcriptional machinery to the correct locations. A handful of global genome-activating transcription factors have been identified (Zelda in flies (Harrison and Eisen, 2015), Pou5f3 in zebrafish (Onichtchouk and Driever, 2016), and DUX/DUX4 in mammals (Iturbide and Torres-Padilla, 2017)). These genome activators share a number of features. They bind early in development and promote the activation of hundreds of genes during the major onset of transcription. Their binding is required for the expression of the earliest zygotic genes, and notably, they all appear to function as “pioneer factors.” Pioneer factors are a specialized class of transcription factors that are characterized by their ability to bind to regions of repressive chromatin. Following binding, these factors promote chromatin accessibility, allowing other factors to access the local DNA (Zaret and Carroll, 2011).

Still, the mechanism by which these early transcriptional activators reprogram the genome remains unclear. These factors are maternally deposited and massively upregulated in the hours leading up to ZGA. Thus, they have access to the genome while the chromatin environment is being remodeled and may exploit this nascent state to access their binding sites. Furthermore, recent work in zebrafish suggests that genome activators may use “strength in numbers” to take on the nucleosome (Joseph et al., 2017). As histone levels decrease, the rising levels of transcriptional activators may allow them to outcompete histones for access to DNA. By occupying their binding sites, these activators could shape local nucleosome positioning to favor gene expression.

Allow Time for Transcription

In many species, the early cell cycles occur with unrivaled speed (Tadros and Lipshitz, 2009). Because nascent transcripts are aborted as the chromosomes condense during mitosis, these short cycles leave only a brief window of time for transcription. This constraint is reflected by the fact that early expressed genes tend to be short and lack introns (Lee et al., 2014).

Initially, the embryo undergoes a stripped-down version of the cell cycle. The cell switches between replicating its genome and mitotically dividing, skipping the G1 and G2 gap phases entirely. But over the course of the MZT, the cell cycle slows and a G2 is introduced at the end of this transition (Tadros and Lipshitz, 2009). Thus, it was proposed that cell cycle slowing gradually permits gene expression, culminating in a major burst during the first G2 pause (Fig. 2D).

This model is supported by data from frog embryos, in which experimentally arresting the cell cycle resulted in premature transcription (Lee et al., 2014). But, at least in flies, the relationship between transcription and the cell cycle is likely bidirectional. While arresting the cell cycle can allow for precocious transcription, zygotic transcription is also required to produce proteins that slow the cycle. Transcription may also generate DNA replication stress that contributes to the slowing (Blythe and Wieschaus, 2017). Regardless, the abbreviated replication cycles of the early embryo place limits on transcription that are released as the cycle slows (Yuan et al., 2016).

Conclusion: A Peaceful Handoff Requires Coordination

For development to occur, the genome must produce the correct products, in the right amounts, at specific times. To be prepared to perform this essential function, the genome remains transcriptionally silent during the first stages of development while it undergoes remodeling and rapid replication. The egg is loaded with all the factors necessary for the initial steps of embryogenesis to allow development to proceed during this reprogramming. These maternal products allow the animal to rapidly progress through this vulnerable stage of life. Thus, the MZT has evolved to accommodate the specific needs of the fertilized egg. It allows a giant cell to amplify its inchoate genome without a costly delay in development.

To move the embryo from maternal to zygotic control, the transcriptome is overhauled; maternal transcripts are progressively degraded while the zygotic genome becomes transcriptionally active. These changes occur with precise dynamics such that each transcript is present in the correct amount throughout this transition. The embryo uses a diverse set of mechanisms to execute this complicated handoff. These complementary mechanisms feed back on themselves to reinforce the shift toward zygotic control. As the exponentially increasing DNA overwhelms the supply of maternal repressors and the cell cycle slows, the first zygotic genes are expressed. Some of these early expressed zygotic factors promote the degradation of maternal products, reinforcing the maternal decline and allowing for further zygotic transcription.

Simultaneously, the chromatin of the fertilized egg is reprogrammed to enable the generation of an entirely new organism. This dramatic reorganization of the embryonic genome is similar to the *in vitro* reprogramming attained through either somatic cell

nuclear transfer or the induction of specific transcription factors. In some cases, the same factors function across these contexts. Understanding reprogramming in the context of the developing embryo, where it is accomplished with unprecedented efficiency, will offer important insights not only into a shared and essential aspect of animal development, but may also suggest strategies to increase reprogramming efficiency in culture.

References

- Blythe, S. A., & Wieschaus, E. F. (2017). Zygotic genome activation triggers the DNA replication checkpoint at the midblastula transition. *Cell*, 160(6), 1169–1181.
- Flyamer, I. M., Gassler, J., Imakaev, M., Brandão, H. B., Ulianov, S. V., et al. (2017). Single-nucleus Hi-C reveals unique chromatin reorganization at oocyte-to-zygote transition. *Nature*, 544(7648), 110–114.
- Gurdon, J. B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *Development*, 10, 622–640.
- Harrison, M. M., & Eisen, M. B. (2015). Transcriptional activation of the zygotic genome in *Drosophila*. *Current Topics in Developmental Biology*, 113, 85–112.
- Hug, C. B., Grimaldi, A. G., Kruse, K., & Vaquerizas, J. M. (2017). Chromatin architecture emerges during zygotic genome activation independent of transcription. *Cell*, 169(2), 216–228. e19.
- Iturbide, A., & Torres-Padilla, M.-E. (2017). Starting embryonic transcription for the first time. *Nature Genetics*, 49(6), 820–821.
- Joseph, S. R., Palfy, M., Hilbert, L., Kumar, M., Karschau, J., et al. (2017). Competition between histone and transcription factor binding regulates the onset of transcription in zebrafish embryos. *eLife*, 6.
- Lee, M. T., Bonneau, A. R., & Giraldez, A. J. (2014). Zygotic genome activation during the maternal-to-zygotic transition. *Annual Review of Cell and Developmental Biology*, 30, 581–613.
- Newport, J., & Kirschner, M. (1982a). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell*, 30(3), 675–686.
- Newport, J., & Kirschner, M. (1982b). A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell*, 30(3), 687–696.
- Onichtchouk, D., & Driever, W. (2016). Zygotic genome activators, developmental timing, and pluripotency. *Current Topics in Developmental Biology*, 116, 273–297.
- Pálffy, M., Joseph, S. R., & Vastenhouw, N. L. (2017). The timing of zygotic genome activation. *Current Opinion in Genetics & Development*, 43, 53–60.
- Tadros, W., & Lipshitz, H. D. (2009). The maternal-to-zygotic transition: a play in two acts. *Development*, 136(18), 3033–3042.
- Takahashi, K., Yamanaka, S., Randle, D. H., Kamiyo, T., Cleveland, J. L., et al. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126(4), 663–676.
- Yuan, K., Seller, C. A., Shermoen, A. W., & O'Farrell, P. H. (2016). Timing the *Drosophila* mid-blastula transition: a cell cycle-centered view. *Trends in Genetics*, 32(8), 496–507.
- Zaret, K. S., & Carroll, J. S. (2011). Pioneer transcription factors: establishing competence for gene expression. *Genes & Development*, 25(21), 2227–2241.

Trophectoderm Development

Vernadeth B Alarcon and Yusuke Marikawa, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blastocyst A mammalian embryo at the final stage of development that is competent to implant in the uterus.

Morphologically, it appears as a hollow sphere consisting of two tissue types or cell lineages, the trophectoderm and inner cell mass. The trophectoderm is responsible for implantation and placentation, whereas the inner cell mass gives rise to the fetal body and the extraembryonic tissues, amniotic sac and yolk sac.

Cavitation The process that forms a fluid-filled space in the blastocyst. It is mediated by the trophectoderm, which regulates the directional transport of fluid from the external environment into the developing blastocyst.

Conceptus The products of the fertilized egg, namely the embryonic and extra-embryonic tissues, which form the fetal body and tissues that do not become part of the body (e.g., placenta, amniotic sac), respectively. It is distinct from the term “embryo,” which refers to tissue that forms the body. The blastocyst can also be called a conceptus.

Epithelium A type of tissue which covers or lines the surface of tissues or organs. It functions as a selective barrier, regulating the movement of molecules between the internal and external environments of a tissue or organ. Epithelial cells are characterized as having apical-basal polarity, and are attached to each other by complexes of proteins, including the tight junctions. A simple epithelium, such as the trophectoderm, consists of a single layer of flat-appearing cells owing to being stretched by the expanding blastocyst cavity.

Preimplantation The period of development between the fertilization of the egg and implantation in the uterus. It occurs in the oviduct (fallopian tube) and ends when the conceptus arrives in the uterus.

Introduction

The trophectoderm (TE; from the Greek “trophe,” “ektos” and “derma,” meaning to feed, outside and skin, respectively) is a simple epithelium that makes up the outer wall of the blastocyst (from the Greek “blastos” and “kystis,” meaning bud or sprout and bladder) (Fig. 1A). It is the first tissue to differentiate during preimplantation development, and it is unique to a distinct group

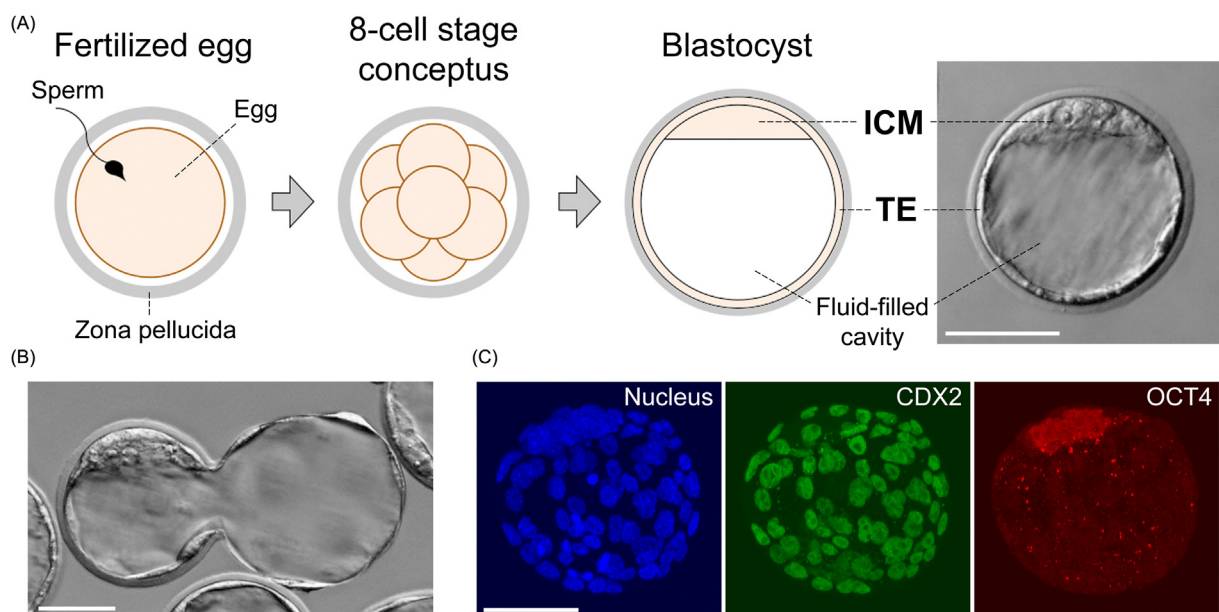


Fig. 1 Preimplantation development. (A) Schematic representations from fertilization to blastocyst formation. Trophectoderm (TE) differentiates as the outer layer that surrounds the inner cell mass (ICM) and blastocyst cavity. A photograph of a mouse blastocyst at around 3 days after fertilization is shown on the right. (B) A photograph of a blastocyst that is hatching out of the zona pellucida is shown. (C) Immunofluorescent visualization of the cell type-specific transcription factors (CDX2 for TE and OCT4 for ICM) in a blastocyst at around 4 days after fertilization. Scale bars = 50 μm.

of mammals called the eutherians, also known as the placental mammals (Eakin and Behringer, 2004). Some examples are human, mouse, dog, and cattle. The hallmark feature of TE is its adaptation for invasion and implantation in the uterus, after which it contributes to the formation of the placenta. The viviparous mode of reproduction of eutherians is made possible by the placenta, which functions in the nutritive and respiratory exchanges between mother and fetus. However, prior to implantation, the TE plays a different role. It transports fluid from the environment into the blastocyst, causing it to increase in diameter. Since the blastocyst is encased by a glycoprotein layer called the zona pellucida, it must hatch from its confinement before the TE can interact with the uterus. It has been observed that when human and mouse blastocysts are cultured outside the maternal body in a Petri dish in a laboratory setting, their expansion due to fluid accumulation appears to promote the tearing of the zona pellucida (Fig. 1B). The failure of hatching leads to the inability of the blastocyst to implant and the failure to produce pregnancy.

In this article, we will examine TE development based on studies conducted primarily in the mouse. It is a popular experimental system because it is amenable to investigations of mechanisms at the cellular and molecular level and is a useful proxy for human since the two species undergo similar morphological changes during the preimplantation period. We will first provide a morphological overview of the emergence of the TE epithelium in the developing blastocyst. We will then discuss the HIPPO signaling pathway and its regulation by cell polarity as the molecular basis of how cells are segregated to differentiate into the TE cell type. Lastly, the current knowledge of TE development is applied to examples of clinical issues that are significant to human reproduction and fertility.

Morphogenesis of the Trophectoderm Epithelium

Preimplantation development prepares the conceptus for attachment to the endometrium, the inner lining of the uterus. The process takes place in the oviduct (fallopian tube), and it begins with the egg. At approximately 60 μm in diameter, the egg is so tiny that it can only be seen with the aid of a microscope. Yet it is one of the biggest cells in the adult body. The sperm must penetrate the zona pellucida to fertilize the egg and produce the conceptus (Fig. 1A). Consisting of only one cell at the start, the conceptus cleaves or divides to increase cell number without growth, so that daughter cells are eventually reduced to the size of adult cells. At approximately 2 days after fertilization, the conceptus appears as a ball of cells or morula (from the Latin “morus,” meaning mulberry), consisting of eight cells. It is from this stage onward that the TE epithelium is gradually constructed. The morula has a bumpy surface that alters dramatically during the process of compaction. Cells change from spherical to wedge shape owing to increases in adhesion between neighboring cells, causing the bumpy surface to smoothen (Fig. 2). This marks the establishment for the first time of the apical-basal polarity in cells. It is the specialization of the plasma membrane into distinct domains that face the external environment (apical) and neighboring (basal) cells. By the 16-cell stage, the morula arranges into an outside layer of cells surrounding a central cluster of cells (Fig. 3). The spatially segregated cells are the progenitors of the first two tissue types in the blastocyst, the TE and inner cell mass (ICM), the latter of which will give rise to the fetal body. The outside cells retain polarity (Fig. 3) and assemble intercellular complexes of tight junction proteins between the apical and basal domains, creating a boundary that segregates the specific functions of the two domains. At 3 days after fertilization or approximately 32-cell stage, a fluid-filled

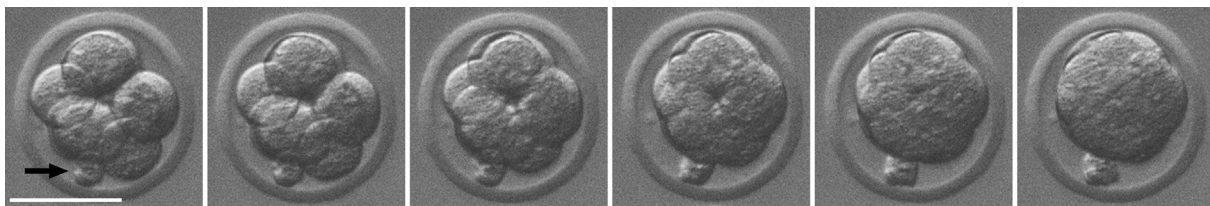


Fig. 2 Compaction. A series of snapshot images that span the period of 3 h at the 8-cell or morula stage, highlighting cell shape changes during the process of compaction. An arrow points to the second polar body, a remnant of oocyte meiosis. Scale bar = 50 μm .

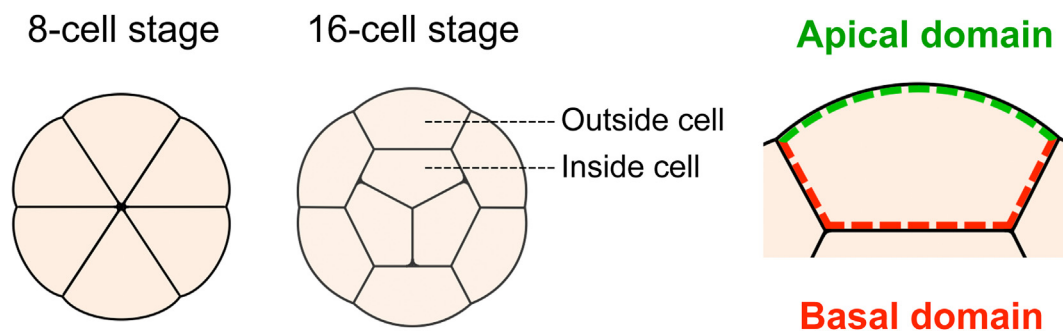


Fig. 3 Establishment of apical-basal cell polarity. Outside and inside cells are generated by the 16-cell stage, as progenitors of TE and ICM, respectively. Distinct apical and basal membrane domains are retained in outside cells.

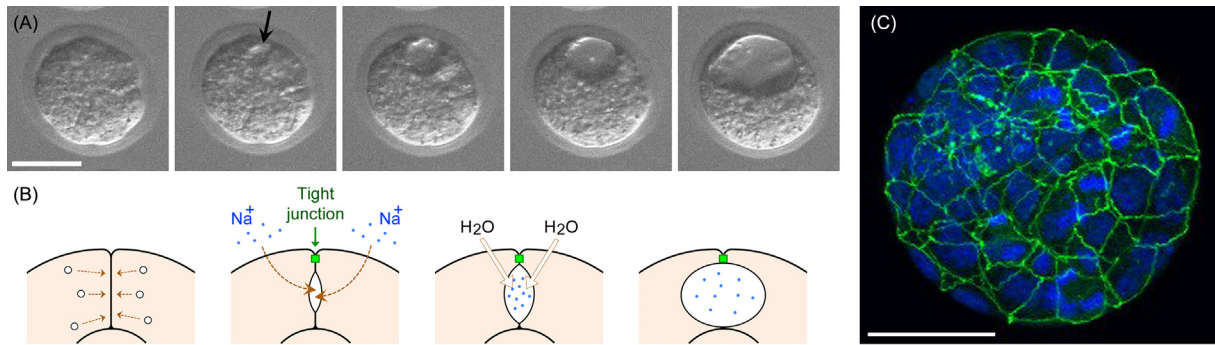


Fig. 4 Formation of the blastocyst cavity. (A) A series of snapshot images that span the period of 3 h around the 16- to 32-cell stages, highlighting the emergence and expansion of the blastocyst cavity. An *arrow* points to a microlumen. (B) Schematic representations, depicting a sequence of cellular and molecular events that lead to cavity formation. (C) Immunofluorescent visualization of Z01 protein (*green*), a key component of tight junction, in the blastocyst. Nuclei (*blue*) are also stained. Scale bars = 50 μ m.

cavity forms inside the morula, transforming it into the blastocyst. The outside cells have matured into the TE epithelium, functioning as a selective barrier between the internal milieu of the blastocyst and the external environment. The blastocyst appears as a hollow, ovoid structure, consisting of a single layer of flat TE cells surrounding the aggregate of ICM cells in one side and the fluid-filled cavity in the other side (Fig. 1A). TE cells adjacent to the ICM and adjacent to the cavity are designated as the polar TE and mural TE, respectively. At 4 days after fertilization, the expanding blastocyst arrives in the uterus, hatches, and is ready for implantation. A time-lapse movie of mouse development, showing progression from the 2-cell stage to the blastocyst stage (Kono et al., 2014), can be viewed online in the PubMed database (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4404313>, see under Supplementary Material, Movie 1: control): images of the conceptuses were captured every 15 minutes over a period of 3 days of culture in a Petri dish.

The blastocyst cavity is essential for hatching and for promoting interactions between the TE epithelium and the endometrium by increasing the surface area of the conceptus. The key to generating the cavity is the TE cell polarity that enables directional transport of fluid, and it involves several steps (Fig. 4A and B). First, the basal domain serves as the target site for the exocytosis of membrane vesicles that generate microlumens (small spaces) between the outside cells. Second, distinct ion pumps are differentially distributed in the apical and basal domains that enable the directional transport of sodium ions from the external environment into the microlumens. As sodium ions accumulate, osmotic pressure elevates, drawing in water and expanding the microlumens, which coalesce to create a single fluid-filled cavity. Finally, to retain fluid in the cavity, an impermeable seal is created by the tight junctions between the TE cells (Fig. 4B and C). The seal also enables blastocyst expansion during hatching, and the increase in surface area enhances contact with the endometrium. Establishment of the apical-basal cell polarity is essential for proper morphogenesis of TE, as the experimental disturbance of cell polarity in the mouse conceptus causes the abnormal distribution of tight junction components and the absence of an impermeable seal (Alarcon, 2010). Consequently, blastocyst cavity formation fails, and the conceptus appears as a morula.

Molecular Mechanisms of Trophectoderm Development

Both the TE and ICM have identical genetic composition, since they originate from the same fertilized egg. However, which genes are transcriptionally activated or repressed are profoundly different between them. Genes that are specifically activated in the TE are responsible for various functions unique to TE, such as blastocyst cavity formation, implantation, and trophoblast development. In contrast, ICM expresses genes that are involved in the maintenance of pluripotency, i.e., the developmental capability that generates various tissues to make the fetal body. Hundreds of genes are specifically expressed in the TE by the time the blastocyst cavity is expanded. Some of the TE-specific genes start their expression at the 16- to 32-cell stages before cavitation occurs, and they play critical roles in the development of functional TE. An example is *Cdx2* gene, which encodes a transcription factor, i.e., DNA-binding protein that activates or represses transcriptions of other genes (Fig. 1C). When the fertilized egg is experimentally created from sperm and oocyte that completely lack the *Cdx2* gene, the resulting morula initially forms the blastocyst cavity but fails to maintain it due to impaired epithelial tight junctions (Strumpf et al., 2005). Moreover, the *Cdx2*-deficient conceptus has reduced expressions of other TE-specific genes (e.g., *Eomes*) and also abnormal ectopic expressions of ICM-specific genes (e.g., *Oct4* and *Nanog*) in the outside cells. *Cdx2* is one of the key regulators of TE development that coordinate differential gene expressions, specifically by up-regulating transcription of other TE genes while repressing transcription of ICM genes.

One of the fundamental questions in elucidating the mechanisms of TE formation is how differential gene expressions between the TE and ICM are achieved. Although the details of such mechanisms are still under investigations, a critical role is played by HIPPO signaling (Cockburn et al., 2013; Hirate et al., 2013). HIPPO signaling is an intracellular signal transduction machinery, consisting of over a dozen of interacting protein components, which regulate transcription of genes that control proliferation and differentiation in various cell types (Fig. 5A). When HIPPO signaling is turned “on,” LATS (kinase protein), in association with NF2 (membrane-associated protein) and AMOT (scaffold protein), phosphorylates YAP (transcriptional co-activator).

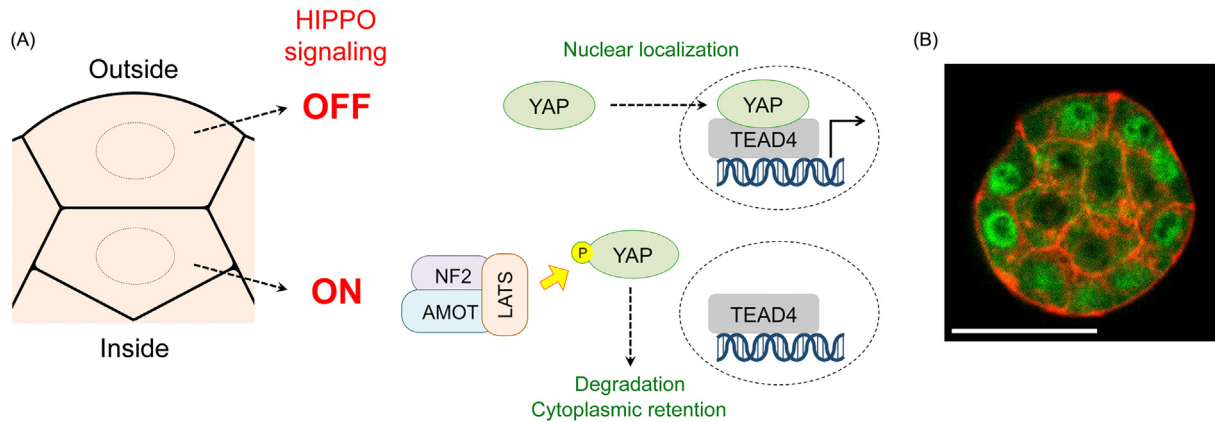


Fig. 5 HIPPO signaling and cell type-specific gene expression. (A) Schematic representations of HIPPO signaling that is differentially regulated between outside and inside cells. Nuclear localization of YAP leads to transcriptional activation of TE-specific genes. (B) Immunofluorescent visualization of YAP (green) and actin filament (red; highlighting cell boundaries) in a cross section of a morula at the 16- to 32-cell stage. Scale bar = 50 μ m.

Phosphorylated YAP is degraded or retained in the cytoplasm, and therefore is functionally inactive. In contrast, when HIPPO signaling is turned “off,” YAP remains unphosphorylated and translocates into the nucleus to associate with TEAD (DNA-binding protein), and together they function as a transcriptional activator. In the mouse embryo before the formation of the blastocyst cavity, HIPPO signaling is turned on in inside cells, but is turned off in outside cells (Fig. 5A and B). As a result, YAP accumulates in the nucleus only in outside cells, and forms a complex with TEAD to activate transcription of TE-specific genes, such as *Cdx2*. Experimental inactivation of HIPPO signaling components causes abnormal expression patterns of TE- and ICM-specific genes. For example, when YAP or TEAD is deficient, expressions of TE-specific genes are abolished and ICM-specific genes are activated in outside cells (Nishioka et al., 2008, 2009). On the other hand, loss of NF2, LATS, or AMOT results in ectopic expression of TE-specific genes in inside cells (Nishioka et al., 2009; Cockburn et al., 2013; Hirate et al., 2013). Therefore, the differential regulation of HIPPO signaling between inside and outside cells is the key to attain cell type-specific gene expression patterns.

The differential activity of HIPPO signaling in relation to cell position is regulated by the apical-basal cell polarity. Whereas inside cells are completely surrounded by neighboring cells, outside cells have the surface that is exposed to the external environment, which allows establishment of the apical-basal polarity. The exposed surface becomes the apical membrane domain, whereas the area adjacent to the neighboring cells becomes the basal membrane domain (Fig. 3). Each membrane domain is populated with unique sets of protein components in a manner similar to the apical-basal polarity found in most epithelial cell types. The components localized to the apical domain are aPKC, PAR3, PAR6, and CDC42, whereas the basal domain components include PAR1, LGL, and SCRIB. aPKC and PAR1 are kinases that modulate the activities of proteins through phosphorylation, whereas the others act as scaffolds to tether proteins to a specific membrane domain. Localization and activity of these polarity proteins are crucial to turn off HIPPO signaling in outside cells (Fig. 5A). For example, when the apical-basal polarity is impaired by experimental disruption of the apical domain (e.g., removal of PAR6) or the basal domain (e.g., removal of PAR1), YAP does not translocate into the nucleus and TE-specific genes are not expressed (Alarcon, 2010; Hirate et al., 2013; Korotkevich et al., 2017). Although the molecular connection between the apical-basal polarity and HIPPO signaling has not yet been fully delineated, a potential mechanism involves localized distribution of AMOT. In outside cells, the apical membrane domain sequesters and possibly inactivates AMOT, leading to the turning off of HIPPO signaling (Hirate et al., 2013). Further investigations are underway to identify other key mechanisms by which the apical-basal polarity influences HIPPO signaling.

Clinical Relevance of the Trophectoderm

The preceding sections are largely based on studies using the mouse as a eutherian model. However, the functional significance and embryological origin of TE are likely to be similar in human. The following section focuses on three specific topics that involve TE and bear clinical significance for human reproduction and fertility.

Early Pregnancy Loss

In human, early pregnancy loss is a common condition characterized by spontaneous abortion or miscarriage of the conceptus before 12 weeks of gestation (Macklon et al., 2002; Larsen et al., 2013). A number of mechanisms have been proposed to cause the miscarriage. One possibility is the abnormal development of the two tissues of the blastocyst, which would result in miscarriage near the time of implantation. For instance, TE development is compromised, and it differentiates into inferior trophoblasts. Normally, healthy trophoblasts secrete the hormone called human chorionic gonadotropin (hCG), which promotes a chain of

molecular events that are essential for the maintenance of the endometrium during the establishment of pregnancy. Since hCG is produced only by the trophoblasts and is excreted in the maternal blood and urine, it is the molecule detected by pregnancy tests. When trophoblasts are defective and fail to produce sufficient hCG, the endometrium degenerates and is shed from the uterus along with the conceptus, thereby terminating the pregnancy. The requirement for proper interactions between trophoblasts and endometrium may serve as a natural screen to select for good quality conceptus. However, TE may also be damaged by exposures to developmental toxicants, resulting in pregnancy loss.

Hydatidiform Mole

In human, the abnormal behaviors of TE are linked to pregnancy-related disorders collectively referred to as gestational trophoblastic disease. The most common type is hydatidiform mole (HM; “hydatid” is from the Greek “hydatidos,” meaning watery vesicle), also known as molar pregnancy or mole. It is a non-viable pregnancy owing to an abnormal blastocyst that overexpresses paternal-derived (i.e., sperm-borne) genes and whose TE gives rise to aberrant placental overgrowth. The ICM may give rise to little or no fetal tissues. According to the United States National Cancer Institute, females in the extreme ends of reproductive age, namely adolescents and perimenopausal women, are at increased risk for HM, affecting 1–3 out of 1000 pregnancies in developed countries.

The placental overgrowth of HM is usually benign, though in rare cases it becomes malignant. Such tumors are unique since they arise from the TE of the conceptus and not from tissue of the maternal body. Patients present with an abnormally high level of hCG secreted by the molar trophoblasts compared to the trophoblasts of a normal pregnancy. Most women can now be cured because of effective anti-neoplastic medications, such as methotrexate, and can have a normal pregnancy after experiencing a molar pregnancy.

HM is classified as complete (CHM) or partial (PHM) based on pathology and genetics (Seckl et al., 2010). CHM is a pregnancy without an embryo and only placental tissues form. The trophoblastic villi of the placenta tend to accumulate fluid and swell, appearing as bunches of grapes. CHM forms from the TE of an unusual blastocyst, the product of an egg that lost its nucleus and is fertilized by two sperm or one sperm whose genome duplicated. While the resulting karyotype is numerically normal (diploid), it is exclusively paternal in origin, and is 46, XX or 46, XY. 46, YY has not been observed since a conceptus with such genetic makeup probably perishes. In rare cases, CHM originates from a conceptus that is biparental but with an abnormal maternal genome that functions like the paternal genome.

PHM, on the other hand, consists of placental overgrowth with patches of swollen trophoblastic villi and some fetal tissues. The blastocyst, in this case, is the product of an apparently normal egg that is fertilized by two sperm. The karyotype is triploid with two sets of paternal-derived genomes in addition to the one set of maternal-derived genome, and is 69, XXX or 69, XXY or 69, XYY.

The occurrence of HM demonstrates that parental genomes are not functionally equivalent owing to the process called imprinting. While paternal-derived and maternal-derived genomes contain the same genes, a parent puts its imprint or biochemical mark on certain genes according to the sex of the parent. The imprint serves to regulate gene expressions, by activating (turning on) or inactivating (turning off) them. Genes required for early placental development are turned on in the paternal-derived genome, and are overexpressed in HM due to the two sets of paternal genomes. In contrast, genes required for early embryonic development are turned on in the maternal-derived genome, and are either absent or underexpressed in HM. The observations with HM are supported by experiments with mouse embryos that were manipulated to contain exclusively paternal genomes or exclusively maternal genomes (McGrath and Solter, 1984).

Trophoctoderm Biopsy

TE biopsy is the sampling of TE cells from the blastocyst produced by in vitro fertilization (IVF) to analyze for the presence of genetic abnormalities (e.g., chromosome defect and single gene defect) or to determine sex (Hyde and Schust, 2015). The procedure is used to aid in the selection of blastocysts for transfer into the uterus of patients undergoing assisted reproductive services.

According to the Centers for Disease Control and Prevention, many people experience problems of fertility in the United States. Such problems are the inability to conceive after 1 year of sexual intercourse with the same partner without the use of contraceptives or the inability to sustain a pregnancy to live birth. Factors that contribute to them originate from either the female or male, or both. Fertility problems are not necessarily symptomatic of sterility, and assisted reproductive technologies (ART) may enable affected individuals to achieve conception and the successful birth of a baby. It is estimated that almost 2% of all babies born annually in the United States are conceived using ART.

IVF is the most commonly used ART. Eggs and sperm are retrieved from the patients, and are combined in a Petri dish. This allows fertilization to take place, and the resulting conceptus is transferred into the uterus for implantation. However, IVF often yields several conceptuses, and patients may decide for them to undergo preimplantation genetic diagnosis (PGD) or screening (PGS) to select only the desired conceptus for uterine transfer. TE biopsy has become a common method for collecting a small number of TE cells for PGD/PGS. During the biopsy, a portion of the mural TE, i.e., the TE covering the blastocyst cavity is aspirated into a pipette, a glass tube with a narrow bore, and is separated from the blastocyst with the aid of a laser device. The collected TE cells are then processed for genetic analyses (e.g., chromosome numbers and structures, and DNA sequences). Although the TE does not become part of the fetal body, it is a suitable proxy for testing since its genetic composition is the same as the ICM.

Due to the hole created in the TE epithelium by the biopsy, the fluid in the cavity leaks out and the roof of the cavity collapses. The biopsied blastocyst is cryopreserved to await the results of the genetic analyses. If a blastocyst is selected for transfer, it is thawed

during which the wound in the TE epithelium closes, and the cavity re-expands. Since the blastocyst consists of more than 100 cells, it is resilient to the removal of a few TE cells and is capable of continuing development to produce a baby.

References

- Alarcon, V. B. (2010). Cell polarity regulator PARD6B is essential for trophectoderm formation in the preimplantation mouse embryo. *Biology of Reproduction*, 83, 347–358.
- Cockburn, K., Biechele, S., Garner, J., & Rossant, J. (2013). The Hippo pathway member NF2 is required for inner cell mass specification. *Current Biology*, 23, 1195–1201.
- Eakin, G. S., & Behringer, R. R. (2004). Diversity of germ layer and axis formation among mammals. *Seminars in Cell and Developmental Biology*, 15, 619–629.
- Hirate, Y., Hirahara, S., Inoue, K., et al. (2013). Polarity-dependent distribution of angiominin localizes Hippo signaling in preimplantation embryos. *Current Biology*, 23, 1181–1194.
- Hyde, K. J., & Schust, D. J. (2015). Genetic considerations in recurrent pregnancy loss. *Cold Spring Harbor Perspectives in Medicine*, 5, a023119.
- Kono, K., Tamashiro, D. A., & Alarcon, V. B. (2014). Inhibition of RHO-ROCK signaling enhances ICM and suppresses TE characteristics through activation of Hippo signaling in the mouse blastocyst. *Developmental Biology*, 394, 142–155.
- Korotkevich, E., Niwayama, R., Courtois, A., et al. (2017). The apical domain is required and sufficient for the first lineage segregation in the mouse embryo. *Developmental Cell*, 40, 235–247.
- Larsen, E. C., Christiansen, O. B., Kolte, A. M., & Macklon, N. (2013). New insights into mechanisms behind miscarriage. *BMC Medicine*, 11, 154.
- Macklon, N. S., Geraedts, J. P., & Fauser, B. C. (2002). Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. *Human Reproduction Update*, 8, 333–343.
- McGrath, J., & Solter, D. (1984). Completion of mouse embryogenesis requires both the maternal and paternal genomes. *Cell*, 37, 179–183.
- Nishioka, N., Yamamoto, S., Kiyonari, H., et al. (2008). Tead4 is required for specification of trophectoderm in pre-implantation mouse embryos. *Mechanisms of Development*, 125, 270–283.
- Nishioka, N., Inoue, K., Adachi, K., et al. (2009). The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. *Developmental Cell*, 16, 398–410.
- Seckl, M. J., Sebire, N. J., & Berkowitz, R. S. (2010). Gestational trophoblastic disease. *Lancet*, 376, 717–729.
- Strumpf, D., Mao, C., Yamanaka, Y., et al. (2005). Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. *Development*, 132, 2093–2102.

Inner Cell Mass Development

Björn Oback and Zachariah McLean, Reproduction, AgResearch, Ruakura Research Centre, Hamilton, New Zealand; and University of Auckland, Auckland, New Zealand

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blastocoel Fluid-filled cavity that forms between ICM and trophoblast.

Blastocyst Mammalian embryo containing a blastocoel.

Blastomere Cell within a cleavage-stage embryo.

Chimera Single organism comprising cells from different zygotes.

Cleavage Cell division in the absence of growth.

Compaction Morphological change of a mammalian embryo from a loose cluster of spherical cells into a tightly packed mass.

Conceptus Product of conception, comprising an embryo and its extraembryonic membranes.

Cytoskeleton Cytoplasmic protein filaments (e.g. actin, tubulin) that shape an eukaryotic cell and direct its movement.

Determination Follows specification. Irreversible process whereby a cell commits to a particular developmental fate if left undisturbed in its environment. Non-determined cells will change their fate if placed in a novel ectopic context but determined (or committed) cells will not.

Differentiation Irreversible changes gene and protein expression to execute specific cellular functions associated with commitment or determination.

Epiblast Pluripotent derivative of the pluriblast or inner cell mass (ICM), giving rise to all embryonic lineages and germ cells.

Epithelium Sheet of polarized cells forming the outer surface of a structure or lining a cavity.

Eutheria Together with prototheria (or monotremes) and metatheria (or marsupials), eutheria (or placentals) form the class of mammals.

Hypoblast Endoderm derivative of the pluriblast or inner cell mass (ICM), giving rise to extraembryonic layers of the yolk sac.

Inner cell mass (ICM) Clump of cells to one side of the blastocyst, entirely enveloped by the trophoblast. In eutherians, the pluriblast is referred to as ICM before segregating into hypoblast and epiblast.

Morula A solid ball of blastomeres contained within the zona pellucida and resembling a mulberry (Latin: morus), preceding the blastocyst stage.

Placenta Transient extraembryonic organ for nutritional supply during pregnancy in mammals.

Pluriblast A group of cells that segregates into hypoblast and pluripotent epiblast, referred to as inner cell mass (ICM) in eutherians.

Pluripotency Ability of a cell to form all embryonic lineages and germ cells.

Specification Reversible process of restricting developmental potential of a cell to a certain fate. It precedes determination and differentiation.

Totipotency Ability of a cell to form a viable animal on its own.

Trophectoderm (TE) Trophoblast epithelium enclosing the ICM in eutherian blastocysts, giving rise to extraembryonic parts of the placenta.

Trophoblast Extraembryonic ectoderm adapted for trophic functions, i.e. taking up nutrients via the placenta, in mammals.

Zona pellucida A proteinaceous extracellular coat that surrounds the developing embryo from the oocyte to the late blastocyst stage.

Zygote A diploid cell formed when two haploid gametes fuse.

Abbreviations

Amot Angiomotin

Carm1 Coactivator-associated arginine methyltransferase 1

Cdx2 Caudal type homeobox 2

Dnmt3b DNA (cytosine-5-)-methyltransferase 3B

Dnmt3l DNA (cytosine-5-)-methyltransferase 3-like

E-cadherin Epithelial cadherin

Fgf4 Fibroblast growth factor 4

Fgfr2 Fibroblast growth factor receptor 2

Gata4 GATA binding protein 4

Gata6 GATA binding protein 6
H3R26me2 Histone 3 arginine 26 di-methylation
ICM Inner cell mass
Lats1/2 Large tumor suppressor 1/2
Mst1/2 Mammalian Ste20-like kinases 1/2
Nf2 Neurofibromin 2
Pdgfr α Platelet derived growth factor receptor alpha
Prdm14 PR domain containing 14
Oct4 POU domain, class 5, transcription factor 1
Sox2 SRY (sex determining region Y)-box 2
Sox21 SRY (sex determining region Y)-box 21
Taz Tafazzin
TE Trophectoderm
Tead4 TEA domain family member 4
Yap Yes-associated protein 1

Mammalian Preimplantation Development

The natural mammalian life cycle begins with fertilization when two haploid gametes fuse to form one diploid zygote. This single cell can give rise to a viable organism on its own, a property referred to as totipotency. The zygote undergoes a series of DNA replications and cleavage divisions, partitioning its cytoplasm into progressively smaller nucleated daughter cells, called blastomeres, without increasing total embryo volume (Fig. 1; Aiken et al., 2004). As the number of cells in the embryo increases from one to two to four and so on, their average nucleo-cytoplasmic ratio increases exponentially (Aiken et al., 2004). After a few cleavage divisions, the blastomeres compact and form a tight morula. This structure is unique to some placental mammals and comprises an outer layer and an internalized cell mass. The outer cells become the first fully functional epithelium of the conceptus, channeling water and ions inside to form a small fluid-filled cavity, the blastocoel. Cavitation marks the appearance of an early blastocyst, a hollow ball of cells with an outside single cell trophoblast layer, often called trophectoderm (TE), enclosing a small inner cell mass (ICM) to one side of the expanding blastocoel. This first lineage decision defines the polarity axis of the blastocyst, with an embryonic pole, marked by the ICM, and an opposite abembryonic pole. As development proceeds, the trophoblast gives rise to fetal placenta, while the ICM segregates into the hypoblast (or primitive endoderm), fated to form the extraembryonic endoderm layers of the yolk sac, and the pluripotent epiblast, which develops into all intraembryonic lineages, including germ cells,

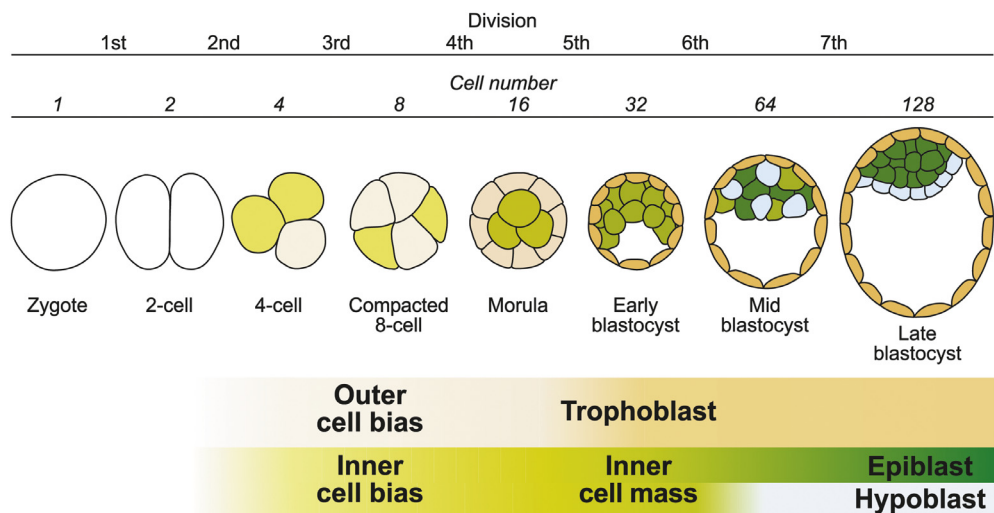


Fig. 1 Overview of mouse preimplantation development. Schematic cross-sections of embryos undergoing cell division, morphological change and lineage segregation from the zygote to the blastocyst stage. Blastomere heterogeneity appears at the 4-cell stage, with the first lineage decision separating inner (pale green) and outer cells (pale orange) in the morula. The ICM (green) appears transiently within the expanding TE (orange) before differentiating into epiblast (dark green) and hypoblast (pale blue) lineages.

and extraembryonic yolk sac mesoderm. The blastocyst then hatches from its surrounding extracellular coat, the zona pellucida, and is ready to attach to the uterus, ending the period of preimplantation embryogenesis.

Principles of Development: Preformation vs Epigenesis

In principle, emergence of the ICM can be explained by two abstract concepts known as preformation (or mosaic) and epigenesis (or regulative) modes of development (Solter, 2016). Pure mosaic development would require the precise localization and heterogeneous distribution of fate-guiding determinants in the zygote, whose presence or absence in parts of the developing embryo will then exactly predict their respective future fate. Many non-mammalian model species (e.g. *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis*) largely follow this invariant pattern. By contrast, pure regulative development would require a homogeneous zygote whose development is solely governed by responding to external factors, such as diffusible intercellular signals, positional information and cell–cell interactions. However, there is probably no organism that exclusively follows one or the other of the two stereotypical modes. Consequently, the distinction between these contradictory models has been somewhat softened by distinguishing “stochastic” from “biased” modes of development. The former argues that early embryonic cells are equipotent and differentiate as a consequence of random processes, whereas the latter poses some heterogeneity between blastomeres resulting in early developmental bias. In reality, mammalian development likely follows a mixed mode, encompassing both deterministic prepatterning and probabilistic regulative elements, creating developmental bias from gradual amplification of small, random heterogeneities.

The Mouse Embryo as a Model for ICM Development

Mammalian embryology is dominated by the study of one species, *Mus musculus*. This small eutherian (placental) animal with its rapid life-cycle and large litter size has supplied the foundation of our understanding of mammalian reproductive physiology and development. The following summary will proceed chronologically through murine ICM development, focusing on cellular and molecular changes between blastomeres that guide fate decisions. It is based on several recent reviews that chronicle the events in greater detail (Leung et al., 2016; Graham and Zernicka-Goetz, 2016; Chazaud and Yamanaka, 2016).

Precompaction Blastomeres and Early Bias in ICM Fate

Early mouse embryos can respond flexibly to compensate for a variety of experimental perturbations. For example, dissociating embryos at the 2-cell and 4- or 8-cell stage results in morphologically identical single blastomeres, some of which can develop into entire animals and contribute to all tissues in chimeras, respectively. Since in any given experiment not every blastomere shows such remarkable developmental plasticity, these observations are compatible with both stochastic and biased views of development (Solter, 2016). Conclusive evidence that the two sister blastomeres of a 2-cell embryo inherit significantly different amounts of proven fate determinants, such as RNA transcripts, has so far been elusive.

By contrast, specific transcriptional and epigenetic heterogeneities have been identified at the 4-cell stage (Fig. 2). The first molecular difference between individual blastomeres relates to the epigenetic modifier *Prdm14* whose mRNA and protein expression is transiently restricted to only two blastomeres at the late 4-cell stage. Its abundance correlates with histone (H) 3

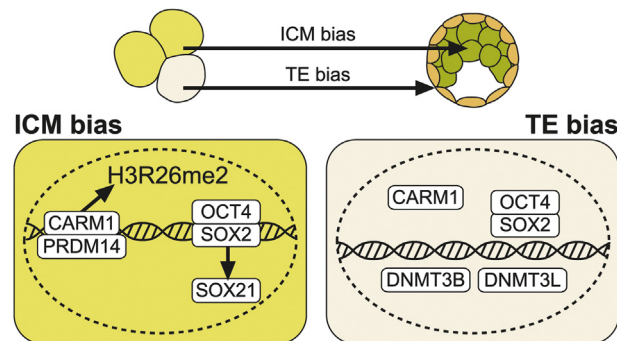


Fig. 2 Molecular heterogeneities bias ICM specification in mouse 4-cell embryo. Increased PRDM14 levels in two blastomeres start off a cascade that promotes the ICM lineage. PRDM14 interacts directly with CARM1 to increase H3R26me2. This correlates with increased DNA-binding of OCT4/SOX2, upregulating SOX21 and biasing blastomeres towards ICM. This hypothetical model possibly also involves *Prdm14* suppressing *Dnmt3b* and *Dnmt3l* expression. By contrast, lack of PRDM14 and CARM1 activity in another blastomere results in low H3R26me2. Along with decreased OCT4/SOX2 DNA-binding and increased *Dnmt3b/Dnmt3l* expression, this biases towards a TE fate.

arginine (R) 26 di-methylation levels (me2), which are also heterogeneous at this stage, predisposing low H3R26me2 blastomeres towards developing into TE derivatives. H3R26me2, in turn, is catalyzed by CARM1, a direct interaction partner of PRDM14. In ectopic overexpression studies, both *Prdm14* and *Carm1* can increase H3R26me2 levels and promote ICM fate. Expression of *Prdm14* is mutually exclusive with that of de novo DNA methyltransferases *Dnmt3b* and *Dnmt3l* in 4-cell embryos. This inverse correlation of expressing epigenetic modifiers is carried through to mid-blastocysts, where *Prdm14* and *Dnmt3b/Dnmt3l* transcripts become transiently ICM- and TE-enriched, respectively. This is consistent with *Prdm14* directly suppressing *Dnmt3b* and *Dnmt3l* expression. Furthermore, CARM1 activity and H3R26me2 levels are positively correlated with abundance of the transcription factor *Sox21*. *Sox21* is a downstream target of *Oct4* and *Sox2*, ICM-specific master regulators of pluripotency, that both antagonize *Cdx2*, a master regulator of TE formation. The findings tie in with heterogeneous DNA-binding dynamics of OCT4 and SOX2 in 4-cells, whereby those blastomeres that have long-lived DNA interactions, indicative of increased OCT4/SOX2-binding and transcriptional activity, are biased towards ICM (White et al., 2016). The same blastomeres that retain stable SOX2-DNA binding also display higher CARM1 and H3R26me2 levels (White et al., 2016).

Together, these findings suggest that individual blastomeres within a subset of 4-cell embryos already display molecular identifiers of the future ICM and exhibit a skewed contribution towards this lineage. However, it remains to be shown that prepatterned variability in the endogenous levels *Prdm14*, OCT4/SOX2-binding and *Sox21* is sufficient to cause a bias towards ICM fate in subsequent cell divisions. It is also unknown how such variability is mechanistically established in the first place.

Compacting Morula: Changes in Cell Shape, Polarity and Position

The 8-cell stage embryo is a symmetrical ball of relatively spherical cells, all morphologically indistinguishable from each other. This appearance drastically changes with the key morphogenetic event that takes place next: compaction. During compaction, all eight blastomeres elongate, flatten and adhere tightly together, eliminating intercellular spaces and minimizing the surface area of the now densely packed embryo. This change in embryo shape is so evident that it can be readily visualized with a standard stereomicroscope. At the same time, each blastomere polarizes into a conventional epithelial structure, containing an outside apical domain and an inside basolateral domain. The apical membrane is typically enriched in surface microvilli and a network of underlying cytoskeletal elements, including actin, myosin and α -tubulin, as well as secretory organelles. The basolateral surface accumulates various cell-cell contacts, including gap, adherens and later tight junctions. These changes in cell shape and polarity critically rely on E-cadherin, a highly conserved calcium-dependent transmembrane adhesion molecule. Without any E-CADHERIN to increase cell-cell contact and initiate compaction, cells continue to divide but only create partially polarized, rounded cells that are loosely attached to one another. Even though they do not form a functional TE epithelium, these aggregates express high levels of the TE specifier *Cdx2*. Individual cells still generate an apical membrane domain, which elevates *Cdx2* expression by suppressing Hippo signaling (see below). Moreover, *Cdx2* mRNA is normally apically anchored. Its dislocation leads to inside cells ectopically expressing this fate-guiding factor, which compromises ICM development. At the same time, low levels of the ICM specifier *Oct4* and absence of spatially segregated ICM cells indicate that E-cadherin normally creates a permissive context for future ICM development. This demonstrates how closely cell polarization is linked to guiding formation of the prospective ICM vs TE lineages.

Importantly, the first acquisition of polarity coincides with changes in cell position that predict cell fate. It results in blastomeres that can divide in different orientations with respect to their apicobasal axis: either symmetric (with the plane of division parallel to the axis of polarity), giving rise to two daughter cells that both remain outside and inherit parts of the apical domain; or asymmetric (with the plane of division perpendicular to the axis of polarity), giving rise to one polar outside daughter cell that retains the complete apical domain and one apolar inside cell. Classic views, embodied in the so-called “inside-outside” (Tarkowski and Wroblewska, 1967) and complementary “polarization” (Johnson and Ziomek, 1981) hypotheses assumed that symmetric divisions produce only future TE (outside) cells, while asymmetric divisions will also produce one future ICM (inside) daughter cell. These orientated cell divisions had been inferred on theoretical grounds and analysis of fixed specimen. Recent advances in live imaging have enabled quantitative tracking of changes in cell shape and position that are central to ICM formation, leading to a revision of the traditional models (Samarage et al., 2015). Asymmetric cell divisions were rare events and internalization occurred when the first outside cell lost its apical domain by shrinkage of the exterior surface, usually at the 12-cell stage. This “apical constriction” mechanism is commonly used during animal development and tissue morphogenesis. It relies on mechanical forces mediated by E-cadherin adhesion and produced by contractile actomyosin tension from apical the cell surface (Samarage et al., 2015). Thus anisotropies in cortical tension, not oriented cell division, may first spatially segregate cells within the mouse embryo. Important questions that remain to be answered are when the asymmetries in cortical tension between cells arise, how they affect polarized fate guides (e.g. *Cdx2*) and to what extent they originate from predetermined or random heterogeneities in biomechanical properties of neighboring cells.

Post-Compacting Morula and ICM Specification From Inner Cells

At the 16-cell stage, the mouse embryo contains on average three inside cells (Samarage et al., 2015), which are smaller and have larger nucleo-cytoplasmic ratios than the outside cells (Aiken et al., 2004). This spatial segregation creates two discrete niches, exposing inner and outer cells to different microenvironments and cell-cell contacts. Once these subpopulations of inside vs

outside cells have been firmly established in the morula, their different cell positions instruct differences in cell signaling, transcriptional activity and ICM vs TE specification.

The main pathway known to coordinate these activities is Hippo signaling, named after its key signaling component, the protein kinase Hippo (MST1/2 in mammals). This evolutionary conserved kinase cascade regulates cell number by modulating cell proliferation, cell death, and cell differentiation. In flies, deletion of any Hippo pathway component leads to “hippopotamus-like” tissue overgrowth but mutant mice show more complicated phenotypes. In the context of the early mouse during embryogenesis, Hippo signals can integrate changes in cell shape and position through sensing both mechanical inputs, such as actomyosin rearrangements, and polarity information. When Hippo signaling is active in inner cells, transcriptional co-activators YAP and TAZ remains cytoplasmic, leading to the expression of ICM genes, such as *Oct4*, *Sox2* and *Nanog* (Fig. 3). In the absence of Hippo signals in outer cells, YAP/TAZ transcriptionally co-activate TE master regulator *Tea4* and its downstream target *Cdx2*. Thus quantitative CDX2 protein expression can be used as an accurate readout of Hippo signaling activity and the process of ICM/TE specification (Posfai et al., 2017).

Activation of Hippo signaling depends on basolateral cell–cell junctions and the absence of an apical membrane domain, which are conditions only found in internalized cells. How does lack of an apical domain activate Hippo signaling in inner cells while presence of an apical domain suppresses it in outer cells? The answer lies in sequestration of key members of the pathway by proteins that regulate adhesion and polarity. AMOT and NF2 are required to activate Hippo kinases LATS1/2 and switch on Hippo signaling. AMOT and LATS1/2 kinases are both inactive as long as they are apically localized, preventing activation of the Hippo pathway in outer cells. In inner cells, which lack an apical domain, AMOT localizes to adherens junctions, interacting with E-cadherin via NF2 and binding LATS1/2. LATS1/2 then phosphorylates and activates AMOT at the adherens junctions, in turn phosphorylating YAP/TAZ. Phosphorylated YAP/TAZ is excluded from the nucleus and degraded. Consequently, YAP/TAZ cannot

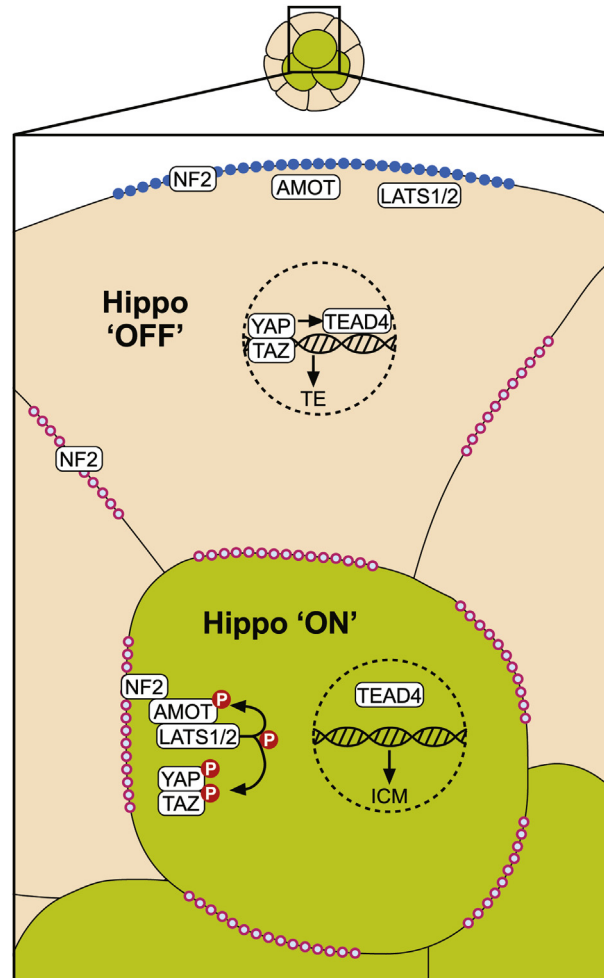


Fig. 3 Hippo signals coordinate cell position, polarity and lineage specification in mouse morula. Inner and outer cell lineage specification is mediated by Hippo signaling. In polarized outer cells, Hippo signaling is “turned off” by the apical membrane (blue) sequestering AMOT and LATS1/2, allowing YAP/TAZ co-activation of TEAD4. In inner cells without an apical membrane, LATS1/2 is free to phosphorylate AMOT, “turning on” Hippo signaling by allowing association with NF2 on basolateral membranes (pink). Phosphorylation excludes YAP/TAZ from the nucleus and allows transcription of ICM genes.

co-activate TEAD4, resulting in failure to induce the TE program via *Cdx2* expression. In this way, inner cells switch on Hippo signals to inactivate YAP/TAZ and allow expression of ICM genes. Without any NF2, the Hippo pathway cannot be activated and such embryos fail to form an ICM. Regardless of their position, inner cells then revert to expressing TE genes instead. In summary, cell position and polarity are mutually reinforcing properties. Hippo signals consolidate differences in cell polarity and position through transcription factor activation, driving the first lineage bifurcation by converting inside cells into ICM and outside cells into TE.

The relationship between cell polarity and cell fate has been further probed by sequentially separating blastomeres after each cell division from the 2-cell to the 32-cell stage, thereby completely inhibiting cell–cell contact and positioning (Lorthongpanich et al., 2012). Each singled out cell adopts a unique gene expression pattern, representing a random mixture of ICM- and TE-specific genes, but overall leaning towards a TE-like profile. Isolated cells also preferentially contribute to TE in morula aggregation experiments. This is consistent with early observations that isolated 8-cell blastomeres give rise to functional TE but do not form ICM-derivatives. On their own, these blastomeres depolarize and consequently activate Hippo/YAP signaling. However, such blastomeres still become biased towards TE, despite the loss of apicobasal polarity and active Hippo signals. Thus lack of an apical domain and Hippo signals are insufficient to entirely control the first lineage decision and induce ICM commitment. Collectively, these data suggest that cell–cell interactions and positional information provide additional cues that are important for correct cell fate specification.

After the 16-cell stage, inner cells expand in numbers while retaining their internal position. At the same time, new inner cells continue to be added by apical constriction and asymmetric divisions of outer cells (Samarage et al., 2015).

Blastocyst Formation and ICM Lineage Segregation

By the 32-cell stage, the nucleo-cytoplasmic ratio of the outer cells has stabilized, marking the end of cleavage division for this subpopulation (Aiken et al., 2004). There is a clear distinction between the apolar inside cells and the outside epithelial monolayer and these divergent compartments herald the formation of the first two lineages: ICM and TE. For cells expressing CDX2, cell fate is basically sealed at this stage, and they are unable to give rise to the ICM. ICM cells, on the other hand, delay their commitment to the next two lineages by another cell cycle, up until some point between the 32- and 64-cell stages (Posfai et al., 2017).

Mouse blastocyst formation starts around the 32-cell stage when the outer morula cells form a fully functional epithelium. The combined presence of tight junctions, sodium and water channels results in the accumulation of liquid within the embryo and formation of the blastocoel cavity. Once cavitation has begun and the ICM appears at the embryonic pole of the cavity, the embryo is referred to as an early blastocyst. At this stage, individual ICM cells are still undergoing cleavage and are not distinguishable by volume or nucleo-cytoplasmic ratio (Aiken et al., 2004). They remain developmentally flexible and can engender all embryonic and extraembryonic lineages. For example, isolated early ICM cells are able to redevelop into miniature blastocysts and contribute to TE after morula complementation. This ability is lost when the ICM number exceeds 16–19 cells, concomitant with end of cleavage divisions and start of the second lineage bifurcation into inner epiblast and outer hypoblast. Each ICM cell becomes irreversibly committed to either epiblast or hypoblast within a single cell cycle (Posfai et al., 2017), coinciding with the end of cleavage divisions and initiation of cell growth, replicative proliferation and stable nucleo-cytoplasmic ratios (Aiken et al., 2004). Perhaps it is no coincidence that ICM cells make their next cell fate choice at the same time. In contrast to the earlier ICM/TE specification, this second lineage decision does not appear to be primarily position-dependent but rather relies on cell–cell signaling (Fig. 4). Early ICM cells co-express epiblast markers, such as SOX2 and NANOG and early hypoblast markers, such as GATA6 and PDGFR α , making the transcriptomes of individual ICM cells at the 32-cell stage indistinguishable. These opposing lineage specifiers are in

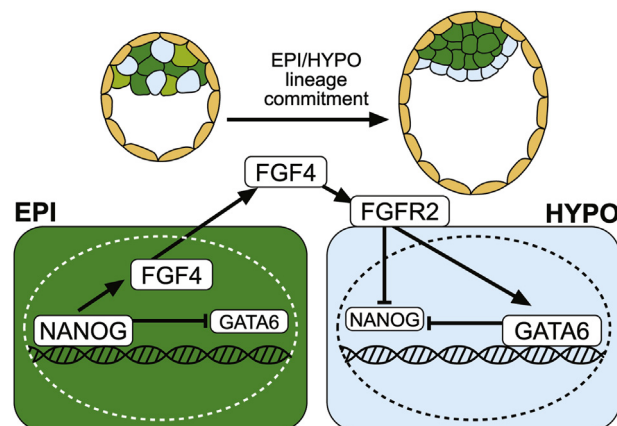


Fig. 4 ICM differentiates into epiblast and hypoblast in mouse blastocyst. In epiblast (EPI) progenitors (green), NANOG drives expression of *Fgf4* while also repressing *Gata6*. Secreted FGF4 then signals through FGFR2 on the surface of hypoblast (HYPO) progenitors (light blue) to activate *Gata6* and repress *Nanog*.

a delicate balance that will gradually tip towards complete lineage segregation within the next few hours. In mouse, this process is mainly driven by FGF4, a secreted factor that is autocrinally produced in nascent epiblast cells. While epiblast progenitors are temporarily unresponsive to the FGF ligand they secrete, nearby hypoblast progenitors express the cognate FGF receptor 2 (FGFR2) and respond by upregulating GATA6 and downregulating NANOG expression (Lanner and Rossant, 2010). Blastocysts carrying a genetic deletion of *Fgf4* or its downstream signaling components do not maintain early hypoblast-specific transcription factors and consequently lack hypoblast (Lanner and Rossant, 2010). Conversely, administering exogenous FGF4 to early embryos sustains expression of hypoblast markers and inhibits epiblast-specific NANOG. As a result, the entire ICM forms hypoblast in a dose-dependent manner (Lanner and Rossant, 2010). Taken together, continuous FGF4-FGFR2 signaling drives stable epiblast/hypoblast segregation and regulates the cell number ratio of these two tissues in the mouse embryo. By the 64-cell or mid-blastocyst stage, segregation of the two lineages is consolidated with the top three inversely correlated gene pairs within the ICM being (i) *Fgf4*, *Nanog* and *Sox2* for the epiblast and (ii) *Fgfr2*, *Gata6* and *Gata4* for the hypoblast, respectively. *Nanog* expression, in particular, is tightly correlated with the nascent epiblast and mutually exclusive with the hypoblast marker *Gata6*. Initially, these markers are expressed in a mutually exclusive “salt and pepper”-like distribution throughout the ICM, before sorting into their correct positions through a combination of cell migration and selective apoptosis.

It is still controversial which developmental mode best explains how binary heterogeneities first arise during ICM differentiation. In the stochastic model, random fluctuations in gene expression, followed by positive feedback and reciprocal repression, are sufficient to explain lineage divergence. Alternatively, cell division history may bias cell fate, with cells being internalized during the 4th (8–16 cell) vs 5th (16–32 cell) and 6th (32–64 cell) cleavages becoming predominantly epiblast vs hypoblast, respectively. The strength of this bias was correlated with relative levels of FGF-producing and -responding internal cells having a dose-dependent effect on ICM composition. Sequential waves of internalization could provide a mechanism for specifying three different lineages (tropho-, epi- and hypoblast) from only two different positional states (in-, outside). However, this internalization time-dependent bias does not preclude stochastic differences. As embryogenesis is a highly regulative and dynamic process, any molecular lineage biases between early vs late entering cells may be subtle and readily overridden by exogenous factors, such as modulation of FGF signaling.

By the late blastocyst stage, two groups of inner cells are morphologically distinguishable: the epithelial hypoblast forms a monolayer facing the blastocoel, covering the surface of an apolar pre-implantation epiblast cluster. Once these two lineages are irreversibly committed, the foundations to all embryonic and extraembryonic lineages are laid and the embryo starts to attach to the uterine endometrium. At that point, the transitory ICM compartment, which in mouse only lasts for just over a day (3.25–4.5) and a couple of cell cycles, has ceased to exist.

Whilst there is compelling evidence that differentiated ICM cells can be permanently captured in vitro, either as hypoblast-derived extraembryonic endoderm or as epiblast-derived naïve pluripotent embryonic stem (ES) cells, this has not yet been achieved with early ICM cells. Compared to epiblast and ES cells, which can self-renew in vivo and in vitro, respectively, it may be more challenging to in vitro culture non-committed, cleavage-stage ICM blastomeres, which do not normally self-renew in vivo (Boroviak and Nichols, 2014).

Evolutionary Context of ICM Development

Mus musculus only represents 0.02% of the existing 5488 mammalian species. In addition to placental eutherians, the class of mammals comprises prototheria (monotremes), which share many developmental features with reptiles and birds, and live-bearing metatheria (marsupials). All three groups develop a functional placenta, but marsupials and placentals rely more heavily than monotremes on this transient extraembryonic organ for nutritional supply during pregnancy. The placenta ultimately derives from the trophoblast, while all other tissues derive from the pluriblast, only referred to as ICM when it is entirely enveloped by the trophoblast, as in the case of eutherians. Thus, strictly speaking, the ICM is an invention of placental mammals. Whilst there are broad similarities between mice and the remaining 99.98% of extant mammals, some critical features of murine preimplantation development are not representative for the phylogenetic group as a whole (Fig. 5), as discussed in more detailed reviews (Sheng, 2015; Frankenberg et al., 2016).

Non-Rodent Eutherians

The chronological sequence of early development is similar between rodents (mouse, rat) and a large number of taxonomically diverse placental orders, such as primates (human, marmoset, macaque), artiodactyls (cow, sheep, deer, pig) and carnivores (cat, dog). This includes key morphogenetic events, such as compaction, inside-out morulation, cavitation, ICM formation and hypoblast-epiblast segregation. However, the timing of cleavage, compaction, blastulation and concomitant lineage specification differs, with the interval between zygote and blastocyst formation taking about 1 week in some species (e.g. cow, pig) compared to 4 days in mouse.

Early embryogenesis in afrotherians, the first group to split from the placental tree, is quite different. Members of this clade, such as elephant shrews or tenrecs, clearly diverge from the rodent prototype. Both species start cavitation at the 4-cell stage, preceding blastomere internalization that would normally occur during morula formation. Skipping the morula stage, a precocious trophoblast-like vesicle with no discernible ICM forms as early as the 16-cell stage. Even though this “blastocyst” resembles

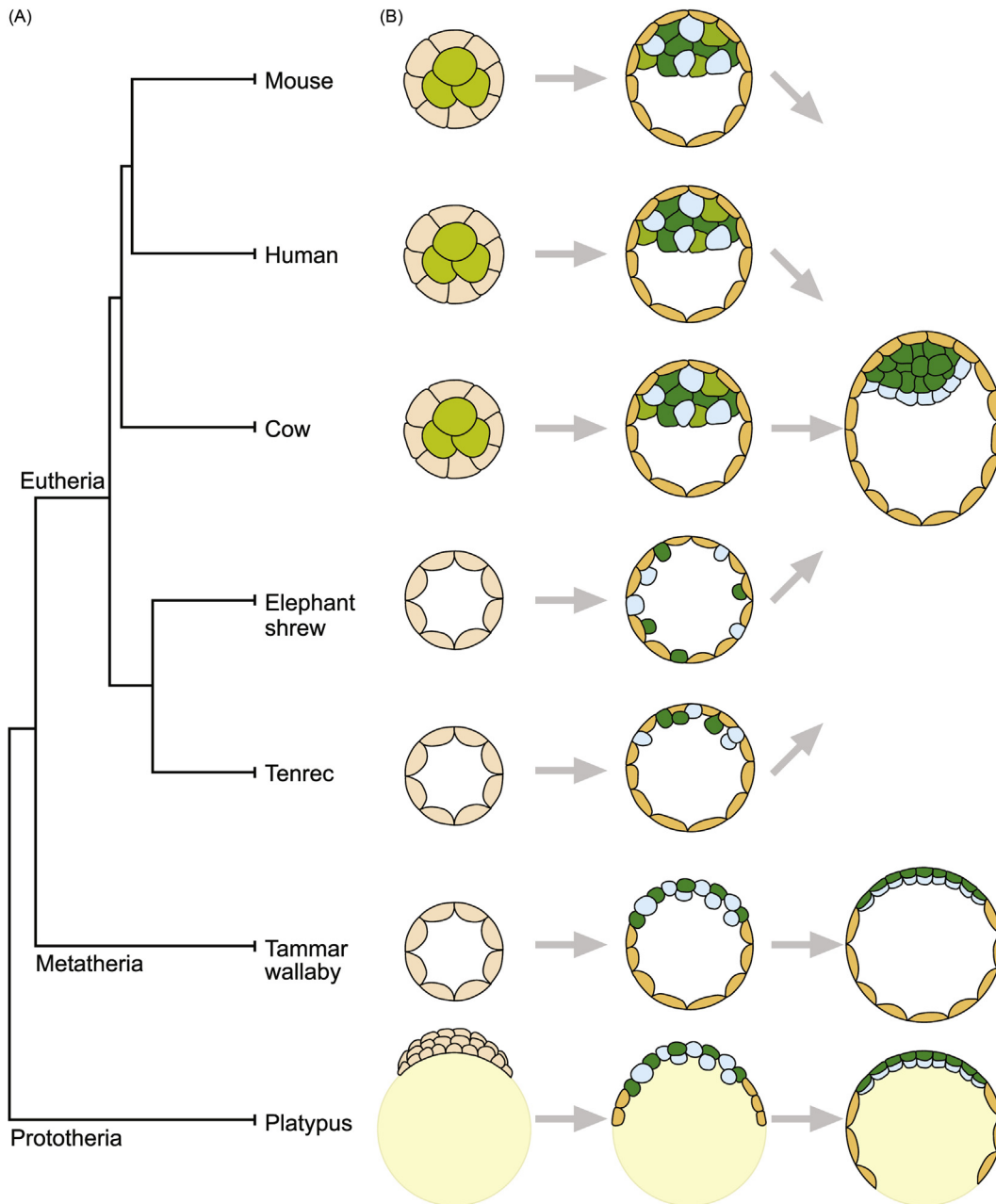


Fig. 5 Comparative mammalian ICM development. (A) Mammalian phylogenetic tree of representative eutherian, prototherian and metatherian species (www.timetree.org). (B) Early embryonic development before apparent ICM vs TE lineage segregation (left column), during formation of a discernible ICM or ICM-equivalent (center column), and after ICM differentiation (right column). Prototherian embryos contain yolk (*pale yellow*) instead of a blastocoel. Embryos are not drawn to scale.

a unilaminar trophoblast epithelium, its cells do not appear to be restricted to the trophoblast fate and will give rise to both epiblast and hypoblast precursors. In the tenrec, epiblast and hypoblast precursors preferentially delaminate from one pole of the trophoblast monolayer, while in the elephant shrew delamination appears to occur randomly. In both cases, the internalized precursors then aggregate and segregate into a similar late ICM structure as in the mouse, with the epiblast mass being sandwiched between the basal sides of polar trophoblast and hypoblast epithelia.

Marsupials

Marsupials are a sister group of placental mammals and their embryo development has been studied in over a dozen species. They all form a trophoblast-like blastocyst, in which individual epithelial cells surround a central blastocoel but lack an ICM. Like in the

tenrec, the precursors of epiblast and hypoblast are restricted to one pole of this unilaminar structure, forming the pluriblast as part of the continuous trophoblast epithelium. Orthologues for key fate specifiers, such as OCT4, SOX2, NANOG, YAP and CDX2 are not differentially expressed, suggesting that these factors are not involved in trophoblast-pluriblast lineage segregation. By the late unilaminar stage, blastocyst cells become molecularly and morphologically heterogeneous with juxtacoelic pluriblast cells adopting a columnar epithelial shape and expressing nuclear-localized NANOG, while GATA6-expressing hypoblast cells delaminate from the pluriblast region. These internalized hypoblast cells migrate to cover both the epiblast, i.e. the non-delaminating remainder of the pluriblast, and the trophoblast, forming a bilaminar structure with three well-defined cell lineages.

Monotremes

Extant monotremes (echidnas and platypuses) represent an early branch of mammalian evolution and they retain ancestral features of reptilian embryogenesis. Knowledge about their early development is limited to just a handful of classical papers and recent interpretations. Monotreme oocytes are an order of magnitude larger in diameter than those of other mammals and contain a large amount of yolk. Cleavage divisions do not divide the embryo completely and result in an embryonic-abembryonic axis that corresponds to the future dorsoventral axis of the embryo. A single cell epithelium eventually covers the entire yolk, the topological equivalent of the blastocoel, forming a blastocyst-like structure. The peripheral monolayer becomes trophoblast, while cells located in the central area of this unilaminar “blastocyst” are specified as future epiblast and hypoblast. These central cells may either still be bipotential precursors or already specified, equivalent to the salt-and-pepper pattern of the mid-ICM or marsupial pluriblast. Similar to marsupials, hypoblast cells then delaminate from the central area and spread on the epiblast and trophoblast surface, forming a bilaminar structure.

Acknowledgments

We thank Pauline Hunt for figure artwork. This work was funded by AgResearch, the University of Auckland and a Callaghan Innovation (LICX1501) PhD stipend to ZM.

References

- Aiken, C. E., Swoboda, P. P., Skepper, J. N., & Johnson, M. H. (2004). The direct measurement of embryogenic volume and nucleo-cytoplasmic ratio during mouse pre-implantation development. *Reproduction*, 128, 527–535.
- Boroviak, T., & Nichols, J. (2014). The birth of embryonic pluripotency. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 369, (1657).
- Chazaud, C., & Yamanaka, Y. (2016). Lineage specification in the mouse preimplantation embryo. *Development*, 143, 1063–1074.
- Frankenberg, S. R., de Barros, F. R., Rossant, J., & Renfree, M. B. (2016). The mammalian blastocyst. *Wiley Interdisciplinary Reviews: Developmental Biology*, 5, 210–232.
- Graham, S. J., & Zernicka-Goetz, M. (2016). The acquisition of cell fate in mouse development: How do cells first become heterogeneous? *Current Topics in Developmental Biology*, 117, 671–695.
- Johnson, M. H., & Ziomek, C. A. (1981). The foundation of two distinct cell lineages within the mouse morula. *Cell*, 24, 71–80.
- Lanner, F., & Rossant, J. (2010). The role of FGF/Erk signaling in pluripotent cells. *Development*, 137, 3351–3360.
- Leung, C. Y., Zhu, M., & Zernicka-Goetz, M. (2016). Polarity in cell-fate acquisition in the early mouse embryo. *Current Topics in Developmental Biology*, 120, 203–234.
- Lorthongpanich, C., Doris, T. P., Limviphuvadh, V., Knowles, B. B., & Solter, D. (2012). Developmental fate and lineage commitment of singled mouse blastomeres. *Development*, 139, 3722–3731.
- Posfai, E., Petropoulos, S., de Barros, F. R. O., Schell, J. P., Jurisica, I., Sandberg, R., Lanner, F., & Rossant, J. (2017). Position- and Hippo signaling-dependent plasticity during lineage segregation in the early mouse embryo. *eLife*, 6, e22906.
- Samarage, C. R., White, M. D., Alvarez, Y. D., Fierro-Gonzalez, J. C., Henon, Y., Jesudason, E. C., Bissiere, S., Fouras, A., & Plachta, N. (2015). Cortical tension allocates the first inner cells of the mammalian embryo. *Developmental Cell*, 34, 435–447.
- Sheng, G. (2015). Epiblast morphogenesis before gastrulation. *Developmental Biology*, 401, 17–24.
- Solter, D. (2016). Preformation versus epigenesis in early mammalian development. *Current Topics in Developmental Biology*, 117, 377–391.
- Tarkowski, A. K., & Wroblewska, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *Journal of Embryology and Experimental Morphology*, 18, 155–180.
- White, M. D., Angiolini, J. F., Alvarez, Y. D., Kaur, G., Zhao, Z. W., Mocskos, E., Bruno, L., Bissiere, S., Levi, V., & Plachta, N. (2016). Long-lived binding of Sox2 to DNA predicts cell fate in the four-cell mouse embryo. *Cell*, 165, 75–87.

Epiblast Development

Bernard AJ Roelen, Utrecht University, Utrecht, The Netherlands

© 2018 Elsevier Inc. All rights reserved.

Formation of the Mammalian Blastocyst

After fertilization of a mammalian oocyte, the formed totipotent zygote starts a series of cleavage divisions. During the first divisions the blastomeres remain totipotent, but this potential gradually decreases upon further development with the timing of developmental restriction dependent on the animal species. The first lineage segregation that occurs is the formation of outer and inner cells around the morula stage. The outer cells form an epithelial sheet of trophectoderm that is important for implantation into the uterus, the fetal maternal interaction and formation of the non-maternal components of the placenta. Pump-like proteins within this epithelium facilitate the formation of a fluid filled cavity, called blastocoel, transforming the embryo into a blastocyst. At this stage the embryo consists of two cell types, an outer trophectoderm layer and an inner cluster of cells referred to as inner cell mass (ICM) facing the blastocoelic cavity. The POU transcription factor OCT3/4 is expressed at high levels in the ICM and is critical for the formation of the pluripotent cell population in the early embryos, while the trophectoderm cells express relatively high levels of the transcription factor CDX2. OCT3/4 and CDX2 not only function as transcription factors, but also as repressors of each other's gene transcription. It is the ICM that will give rise to the epiblast.

Second Lineage Segregation

After the first lineage segregation a second cell fate specification event occurs that segregates the ICM into epiblast, also called primitive ectoderm, and hypoblast, also called primitive endoderm. In the mouse embryo the primitive endoderm after implantation differentiates further to visceral endoderm. The epiblast consists of pluripotent cells that will form the fetus while the primitive endoderm, an amniote specific cell type, although originating from the ICM will predominantly give rise to extra-embryonic tissues such as the yolk sac that provides nutrients to the embryo. During gastrulation most of the visceral endoderm disperses as the epiblast-derived definitive gut endoderm intercalates (Kwon et al., 2008). The primitive endoderm is not only important for its nutritional role as yolk sac, but it also plays an essential role in the organization of the epiblast and therefore the establishment of the body plan.

The transcription factors NANOG and GATA6 control the second lineage segregation of the ICM. Initially, from the 8-cell stage onwards, both transcription factors are present in blastomeric nuclei. Soon after formation of the ICM however, NANOG and GATA6 display a mutually exclusive "salt and pepper"-like expression pattern, with some ICM cells expressing predominantly NANOG while other ICM cells predominantly express GATA6 (Chazaud et al., 2006). This distribution of NANOG and GATA6 appears to be stochastic in a position-independent manner. NANOG-expressing cells will sort out to become the pluripotent epiblast, while GATA6 expressing cells will form an epithelial layer of primitive endoderm overlying the NANOG-positive epiblast and facing the blastocoel. Indeed mice with a genetic deletion for *Gata6* initially do form primitive endoderm but this fails to be specified. After segregation the cells also become morphologically distinct. In mice, the dose of GATA6 in the early ICM regulates the specification of primitive endoderm as demonstrated by a reduction of primitive endoderm cells in *Gata6* heterozygous embryos and defective primitive endoderm in null mutants of *Gata6*. Conversely, mouse embryos genetically deficient for *Nanog* fail to form pluripotent epiblast cells and only produce primitive endoderm cells, besides TE (Mitsui et al., 2003). GATA6 and NANOG repress each other's transcription, and GATA6 seems to be working as an on/off switch since NANOG levels do not increase in the absence of GATA6 (Schrode et al., 2014). After initial expression, the decision for exclusive *Nanog* or *Gata6* expression is subsequently driven by fibroblast growth factor (FGF) and mitogen-activated protein (MAP) kinase signaling. Pre-implantation mouse embryos treated with an inhibitor of FGF receptor activity or an inhibitor of MAP kinase signaling blocked primitive endoderm formation, with all ICM cells expressing NANOG. When embryos were on the other hand cultured in the presence of FGF4, all ICM cells became GATA6-positive (Yamanaka et al., 2010). Exposure of *Gata6* null mutants to exogenous FGF4 could not restore the specification of primitive endoderm, indicating that GATA6 is required for primitive endoderm specification by FGF signaling (Schrode et al., 2014).

In other mammals such as cattle and humans, NANOG and GATA6/GATA4 are initially also expressed in the precursors of epiblast and primitive endoderm in a similar "salt-and-pepper" expression pattern, and later in the epiblast and primitive endoderm respectively after segregation. The signals that regulate NANOG and GATA6 expression are different however. The formation of human GATA6-positive primitive endoderm is for instance not dependent on MAP kinase signaling (Kuijk et al., 2012). In bovine embryos, inhibition of MAP kinase signaling only partially inhibited primitive endoderm formation indicating that although FGF/MAP kinase signaling inhibits NANOG expression, other signals directly stimulate GATA6 expression.

Exactly when the cells are irreversibly committed to either epiblast or primitive endoderm is not known. ICM cells of mouse embryos exhibited plasticity in their developmental fate even after they had established exclusive NANOG or GATA6 expression (Yamanaka et al., 2010). Isolated cells from the epiblast of day 4.5 post-coitum mouse embryos contribute to epiblast or derivatives when injected into the blastocoelic cavity of a host blastocyst, and primitive endoderm cells will only give rise to extra-embryonic endoderm, indicating that at this time epiblast and primitive endoderm are already committed to their lineages. Experiments with

bovine embryos revealed that cells are remarkably flexible and can change fate from becoming NANOG-expressing to GATA6-expressing and vice versa at least until the late blastocyst stage, at day 8 of in vitro culture (Kuijk et al., 2012).

X-Chromosome Inactivation

Sex determination in mammals, with exception of that in monotremes, is determined by the sex chromosomes with homozygous XX in females and heterozygous XY in males. The Y chromosome is much smaller and contains fewer genes than the X chromosome, which could lead to an overexpression of X-linked genes in females. A solution for this phenomenon is a type of dosage compensation by inactivation of one of the X chromosomes in female cells. Central in X-chromosome inactivation is the long noncoding RNA *Xist* that is expressed from the X-chromosome that is to be silenced. *Xist* accumulates on the X-chromosome from which it is expressed and recruits remodeling complexes that repress transcription from this chromosome.

In female cleavage stage mouse embryos, imprinted inactivation of specifically the paternal X-chromosome occurs. At the blastocyst stage, X chromosome reactivation takes place such that in the inner cell mass both X-chromosomes are active in female embryos. Upon formation of the epiblast, X-chromosome inactivation is reestablished but this time in a stochastic fashion in the epiblast and derivatives thereof. In the mouse conceptus, imprinted paternal X chromosome inactivation is maintained in the trophoctoderm and primitive endoderm.

The pattern of X-chromosome inactivation in different mammalian species seems to vary in the cleavage stages, but in the epiblast of most studied species random X-chromosome inactivation takes place (Dupont and Gribnau, 2013). Differences between species are mostly prominent in the lineages that are not derived from the epiblast in particular the trophoctoderm and primitive endoderm. It has been suggested that in mice, rats and cattle the X chromosome inactivation is imprinted in the extraembryonic lineages, while in primates, including human, rabbit and equine species, inactivation of the X chromosome in non-epiblast derived tissues is also random (Dupont and Gribnau, 2013).

Organization of the Epiblast

Before the formation of the definitive fetal germ layers during gastrulation, the human embryo already has implanted into the uterine tissue, making it less accessible for research, and therefore limited information is available regarding this species. In that respect embryos from species such as cattle or horse are more accessible, but high breeding and housing costs together with limited genetic and biochemical tools are disadvantages of these species. Most of the data available on epiblast differentiation therefore have come from studies with mice. In addition, genetic manipulation of pluripotent embryonic stem cells combined with chimaera formation has enabled the generation of gene knock-out and knock-in mice which has substantiated enormously to our current understanding of gene function and pattern formation in the mouse.

The epiblast of mouse embryos forms a radially symmetric cup of epithelial cells, with the extraembryonic ectoderm as an inverted cylinder on top and surrounded on the outside by a single-cell layered visceral endoderm, a derivative of the primitive endoderm (Fig. 1A). The epiblast of most mammals including humans is formed as a flat epithelial sheet of cells, similar to the epiblast of chicken embryos, with the primitive endoderm adjacent/underneath, hence the alternative name hypoblast (Fig. 1B). Up until this stage the epiblast is still radially symmetric, but for induction of the definitive germ layers the establishment of anterior-posterior polarity is essential.

The first sign of anterior-posterior polarity of the conceptus has been detected in the primitive/visceral endoderm of mouse embryos. This axial information in the endoderm is subsequently translated to the epiblast. The endoderm furthest away from the extraembryonic region, the distal visceral endoderm (DVE), derives from a few cells expressing both *Gata6* and *Lefty1* in the late blastocyst. The position of these cells most likely indicates the future mid-anterior side of the conceptus (Takaoka and Hamada, 2012). These cells and progenitors move proximally towards the proximal anterior part of the egg cylinder, triggering global movement of the visceral endoderm. Distal visceral endoderm cells will subsequently lose *Lefty1* expression and migrate laterally-distally away from the proximal anterior region. Simultaneously, prospective anterior visceral endoderm (AVE) cells are newly induced at the distal part of the egg cylinder and migrate towards the anterior side. The function of the DVE is not well known, while the role of AVE in patterning the epiblast has been well established.

The proximal epiblast starts to express proteins such as NODAL and WNT3a. Initially, NODAL is expressed throughout the entire epiblast. The AVE cells on the other hand secrete the NODAL inhibitors LEFTY1 and CER1 and the Wnt inhibitor DKK1, and since Wnt and NODAL are not repressed in the posterior part an anteroposterior axis is established in the epiblast (Perea-Gomez et al., 2002). WNT3A and NODAL in the proximal-posterior part of the epiblast induce expression of the T-box transcription factor BRACHYURY and delamination of epiblast cells through the primitive streak to form the definitive germ layers.

Non-Rodent Species

Compared to early cup-shaped embryos of mice and rats, embryos of other species, including the human, have a sheet-like appearance, much more resembling a peri-gastrulating chick embryo. In the mouse embryo the part of the trophoctoderm that

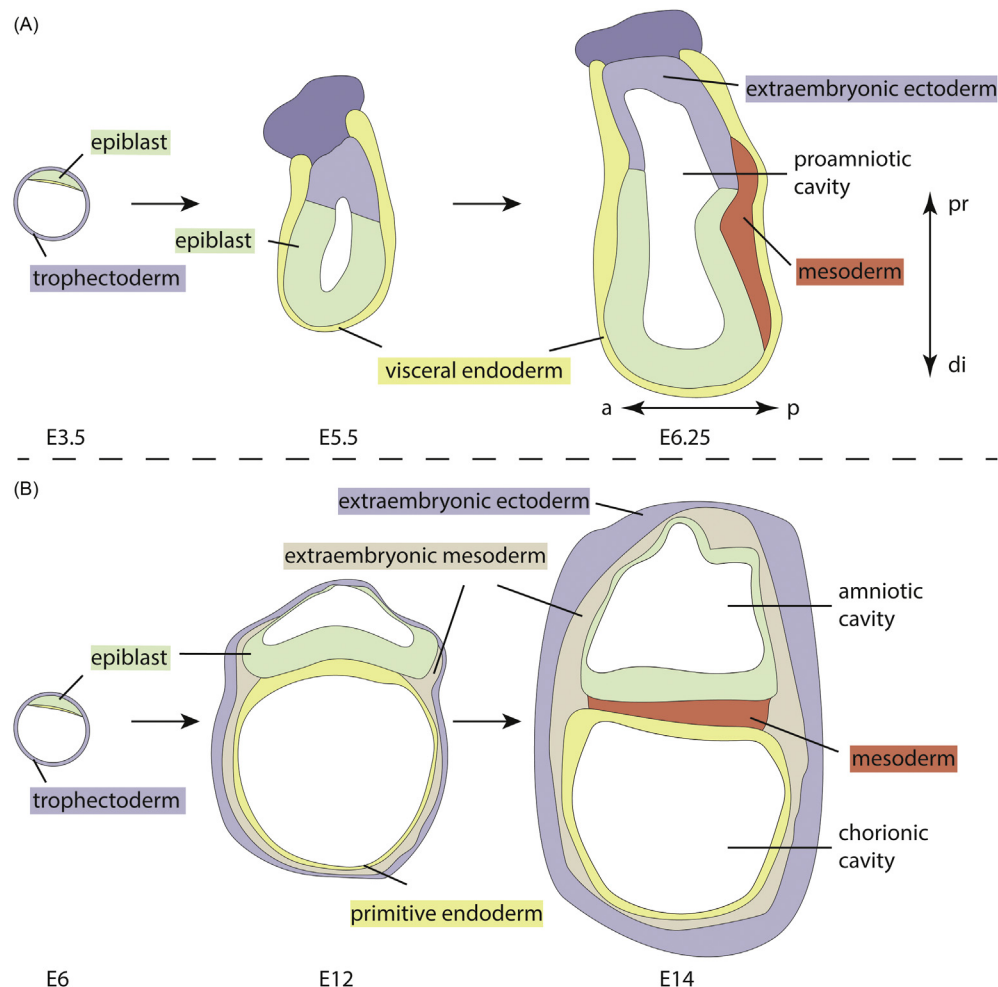


Fig. 1 Schematic representation of lineage specification in developing mouse (A) and human (B) embryos. E refers to embryonic day; proximal (pr)–distal (di) and anterior (a)–posterior (p) axes are indicated. In the human embryo the anterior–posterior axis is perpendicular to the image.

is in contact with the inner cell mass, the polar trophectoderm, grows out to become the extra-embryonic ectoderm. In various species however, including rabbits, ungulates, cats and dogs, the trophectoderm overlying the epiblast is referred to as Rauber's layer and will degenerate. After disappearance of the Rauber's layer, the epiblast is directly exposed to the uterine milieu and connects laterally with the remaining mural trophoblast to form a continuous sheet. In other animals that form a sheet-like epiblast, such as primates, the polar trophectoderm establishes the connection with the uterine epithelium for implantation.

Most information from the epiblast of non-rodent species comes from ungulates such as the pig and cattle. At the pre-implantation state the sheet-like epiblast of ungulates forms a pseudostratified epithelium with tight junctions, desmosomes and a clear basement membrane separating it from the hypoblast (Hall et al., 2010). Ungulate embryos do not implant immediately upon arrival in the uterus and hatching from the zona pellucida; instead their trophectoderm expands dramatically changing the morphology of the conceptus from spherical to ovoid to tubular to filamentous (Fig. 2).

In embryos with a flat epiblast, extra-embryonic mesoderm cells have been identified that later contribute to chorion. The origin of this type of mesoderm has been debated since mesenchymal cells have been identified relatively early in development. It has been suggested that the extra-embryonic mesoderm cells delaminate from the early primitive streak, similar to what has been observed in the mouse. On the other hand, data from monkey embryos suggest that the extra-embryonic mesoderm cells differentiate before gastrulation from the reticulated primitive endoderm.

Gastrulation

The pluripotent epiblast is the source of most if not all fetal and adult lineages, both somatic and germline. In addition, extra-embryonic mesoderm is derived from the epiblast, as well as the ectodermal part of the amnion. Although these extra-embryonic tissues do not contribute to the fetus, they are crucial for survival of the fetus by providing protection and nutrition. Gastrulation

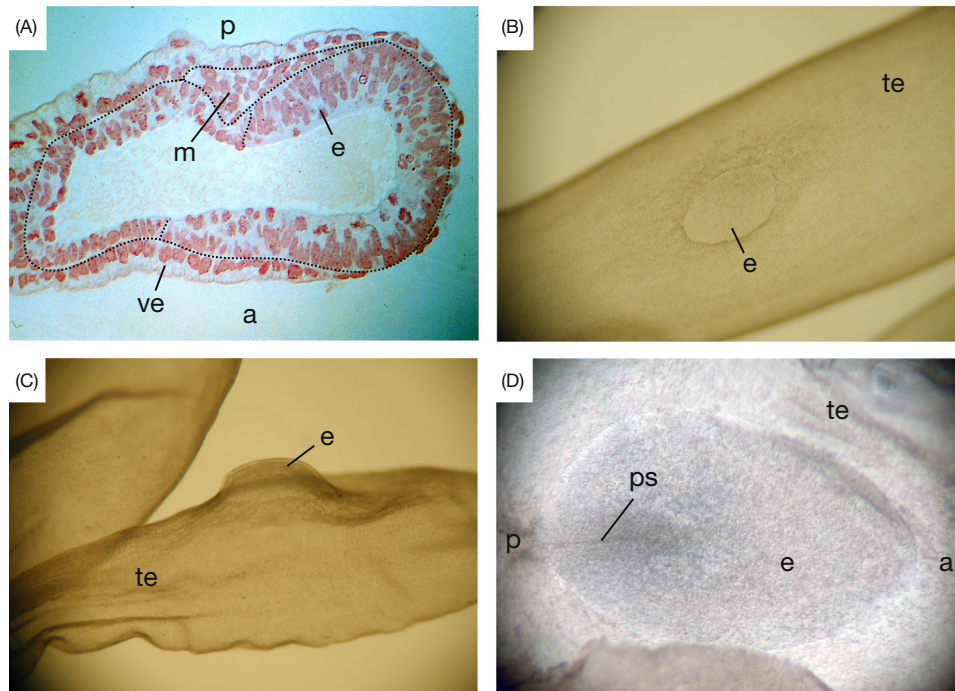


Fig. 2 Microphotographs of (A) a sectioned cup-shaped E6.5 mouse embryo; (B) E10 porcine embryo, dorsal view; (C) E10 porcine embryo lateral view (D) E14 horse embryo. *a*, anterior; *e*, epiblast; *m*, mesoderm; *p*, posterior; *ps*, primitive streak; *te*, trophectoderm; *ve*, visceral endoderm.

is a decisive moment in development as it transforms the epiblast from a single layer of similar cells into a multilayered embryo of which the different layers have made unidirectional choices. The process of gastrulation commences with coordinated growth of the epiblast and movement of epiblast cells towards the primitive streak followed by differentiation and ingression of mesendodermal cells. The internalized cells subsequently converge to the midline and extend along the antero-posterior axis upon undergoing an epithelial to mesenchymal transition and form the mesoderm and the definitive endoderm. The descendants of epiblast cells that do not pass through the primitive streak will constitute the ectodermal lineages (Lawson et al., 1991; Fig. 2).

Proteins belonging to the fibroblast growth factor (FGF) family have been identified as key regulators of the epithelial to mesenchymal transition during gastrulation. Already before a primitive streak is visually present in the mouse embryo, *Fgf8* is expressed in the presumptive dorsal part of the proximal epiblast. Mouse embryos genetically deficient for *Fgf8* besides a lack of FGF8 fail to express FGF4. As a result, mesodermal cells are formed but they fail to migrate away from the streak and instead accumulate at the proximal posterior side of the embryo. A similar clustering of cells at the posterior streak has been observed in embryos deficient for FGF receptor 1 indicating that indeed FGF signaling is crucial for partitioning of the germ layers at the primitive streak. FGF signaling induces the expression of the zinc finger transcription factor SNAIL. Cells that express high levels of SNAIL ingress through the streak while cells that express high levels of the X-linked *Sox3* gene maintain their position in the epiblast. SNAIL and SOX3 repress each other's transcription, thereby ensuring proper subdivision of the epiblast into definitive germ layers (Acloque et al., 2011). Cells of the epiblast have an epithelial character with apical-basal polarity and formation of a basal membrane. Ingression of cells through the primitive streak requires that these break the basal membrane and undergo an epithelial to mesenchymal transition. The formed mesenchymal cells lose cell polarity and cell-cell adhesion but gain migratory and invasive properties. This transition is independent of the differentiation to mesoderm or endoderm, as it has been demonstrated that mouse embryos genetically deficient of *Snail* still form mesoderm but the cells retain epithelial characteristics.

How do cells change from an epithelial sheet, the epiblast, to migratory mesenchymal cells? One important route for this transition is loss of the adhesion protein E-cadherin, a protein that is important for maintenance of adhesion junctions and epithelial tissue. By functioning as a transcriptional repressor, SNAIL can bind to the promoter of the gene coding for E-cadherin leading to a downregulation of E-cadherin expression. The epiblast cells lose their epithelial character and gain the mesenchymal phenotype required for gastrulation.

Fate Map

The developmental capacity of the rodent epiblast, after implantation and the formation of an egg cylinder and a pro-amniotic cavity, has been studied by grafting epiblasts under the kidney capsule of adult animals and analysis of the resulting teratomas. The explanted epiblast cells underwent an epithelial to mesenchymal transition as observed in cells migrating through the primitive

streak and could give rise to descendants of all three germ layers. Experiments by which epiblast cells were meticulously labeled, fragmented and subsequently grafted to other embryos indicated that although the cells are pluripotent, their progeny follows a determined developmental fate based on their position in the epiblast. Labeling individual epiblast cells of pre-streak and early primitive streak mouse embryos with horseradish peroxidase followed by *in vitro* culture revealed that epiblast cells differentiate and migrate in a reproducible and coordinate way. This enabled the construction of a detailed fate map of presumptive embryonic and extra-embryonic germ layers (Lawson et al., 1991). Cells of the lateral epiblast will primarily form embryonic mesoderm while cells from the proximal region will predominantly form extra-embryonic mesoderm and primordial germ cells. Definitive endoderm originates from a posterior region around the (future) streak.

Clonal analysis further revealed that the primordial germ cells also originate from but are not yet lineage restricted in the epiblast. Cells in the proximal epiblast are induced by BMP signals from the extra-embryonic ectoderm to form precursors of the primordial germ cells and allocation takes place during gastrulation in the mesoderm.

Clonal analysis also demonstrated that extensive cell mixing of epiblast cells occurred by morphogenetic movements and that descendants of single cells can contribute to different germ layers (Lawson et al., 1991). The observation that precursor cells for different lineages are present at specific regions of the embryo does not necessarily mean that these cells are irreversibly allocated or restricted in their developmental capacity. Indeed heterotopic explant experiments with pre- and early streak embryos demonstrated that it is the position of the cells, not the intrinsic characteristics of the cells that determines their fate (Tam and Zhou, 1996).

Epiblast Cell Flexibility

It has been demonstrated that, at least in the mouse, epiblast cells can transmigrate into the (primitive) visceral endoderm layer already before gastrulation. This is remarkable, since at this stage the epiblast and primitive endoderm are separated by a basement membrane. Exactly how cell change fate and migrate through the basement membrane is unknown (Hiramatsu et al., 2013). Whether epiblast transmigration to the visceral endoderm layer occurs specifically at the distal tip of the mouse egg cylinder remains unclear. Alternatively, it has become clear that in the mouse, primitive endoderm cells are not all displaced by differentiating epiblast cells that ingress through the primitive streak (Kwon et al., 2008). Rather than displacing and pushing the visceral endoderm cells proximally, the ingressing epiblast cells mix extensively with the visceral endoderm cells. As a result the gut tube, particularly the hindgut, is constituted by both visceral endoderm and epiblast-derived cells. Whether the flexibility of epiblast and primitive endoderm is unique to mouse embryos remains to be established.

Human Epiblast

Understanding human epiblast development beyond the blastocyst stage has been hampered by the invasive implantation into the uterus. Particularly the formation of the epiblast and the events associated with gastrulation has been a black box for the most part. The development of *in vitro* implantation models has allowed the *in vitro* transition from pre/to postimplantation development, although primitive streak formation and gastrulation has not been studied in these models due to ethical and legal restrictions (Shahbazi et al., 2016). Indeed *in vitro* culture of human embryos beyond 14 days or the beginning of primitive streak formation is not allowed in many countries. Questions that remain unresolved are the origin of the extraembryonic mesoderm, origin of the primordial germ cells and the flexibility of epiblast cells to contribute to hypoblast cells prior to primitive streak formation.

Levels of Pluripotency

The epiblast cells of preimplantation embryos, in the mouse between days 3.5 and 4.5 post-coitum, are by definition pluripotent since these cells can give rise to all somatic cell lineages and the germ cells. With the development of the embryo the pluripotency levels gradually diminish, which can be observed for instance by the chromatin state of the cells and the gene expression profile. Shortly after implantation, the pluripotency levels of mouse epiblast cells subside and after gastrulation and accompanying terminal germ layer choice the cells have lost their pluripotent character. In developing embryos, the pluripotency state is very transient as the function of the epiblast is to form a fetus. The pluripotency levels of pre- and post-implantation epiblast cells has been “captured” by *in vitro* culture, and under the right conditions the cells can maintain their pluripotency levels as embryonic stem (ES) cells and epiblast stem cells (EpiSCs) respectively. The pluripotency level of preimplantation epiblast and ES cells is considered to be unrestricted and is therefore referred to as “naïve” while that of post-implantation epiblast and EpiSCs are already “primed” for lineage commitment. Interestingly, human ES cells share more characteristics with mouse EpiSCs and are therefore also considered to be at a primed state of pluripotency. Indeed naïve ES cells form tightly adhering dome-shaped aggregates reminiscent of the blastocyst stage epiblast, while EpiSCs form flat colonies similar to the epithelialized post-implantation epiblast. Naïve pluripotent cells are characterized by high levels of OCT4, NANOG and KLF. Other indications of an open chromatin state and active transcription are large regions of hypomethylated DNA, permissive H3K4 trimethylation and in female cells two active X-chromosomes. Conversely primed epiblast cells, including EpiSCs, also express high levels of OCT4 and NANOG, but low levels of KLF4, and start to express

BRACHYURY, SOX17 and GATA4/6. In addition the EpiSCs display higher DNA methylation levels, and repressive H3K27 trimethylation. In contrast to the naïve state, in female primed epiblast cells one of the X-chromosomes is inactivated. ES and EpiSCs have revealed that alternate enhancer usage dictates the pluripotency character of these cells and it can be assumed that similar differences in enhancer activity states are present in pre- and post-implantation epiblast cells.

During development, the naïve and primed pluripotency states of the epiblast are very transient. However, when the environmental conditions are suboptimal for pregnancy, embryos can arrest in development before implantation, a state known as diapause or delayed implantation. It is thought that in this diapause state the naïve pluripotent epiblast cells self-renew, very much similar to the behavior of ES cells.

Conclusion

The epiblast is the pluripotent primary lineage that will form the definitive germ layers in a complex process of differentiation and morphogenetic movements called gastrulation. After gastrulation the developmental capacity of the differentiating cells is restricted to the residing germ layer. Most of what we know about epiblast development comes from mouse data. However, the mouse epiblast is uniquely cup-shaped, opposed to the planar morphology of the epiblast of most mammalian species. Studies with pluripotent stem cells and in vitro cultured embryos may in the future contribute to our knowledge of the epiblast.

Acknowledgments

Susana Chuva de Sousa Lopes is thanked for critically reading the manuscript. Apologies to colleagues whose work could not be mentioned or cited due to space limitations.

References

- Acloque, H., Ocana, O. H., Matheu, A., Rizzoti, K., Wise, C., et al. (2011). Reciprocal repression between Sox3 and snail transcription factors defines embryonic territories at gastrulation. *Developmental Cell*, 21, 546–558.
- Chazaud, C., Yamanaka, Y., Pawson, T., & Rossant, J. (2006). Early lineage segregation between epiblast and primitive endoderm in mouse blastocysts through the Grb2-MAPK pathway. *Developmental Cell*, 10, 615–624.
- Dupont, C., & Gribnau, J. (2013). Different flavors of X-chromosome inactivation in mammals. *Current Opinion in Cell Biology*, 25, 314–321.
- Hall, V. J., Jacobsen, J. V., Rasmussen, M. A., & Hyttel, P. (2010). Ultrastructural and molecular distinctions between the porcine inner cell mass and epiblast reveal unique pluripotent cell states. *Developmental Dynamics*, 239, 2911–2920.
- Hiramatsu, R., Matsuoka, T., Kimura-Yoshida, C., Han, S. W., Mochida, K., et al. (2013). External mechanical cues trigger the establishment of the anterior-posterior axis in early mouse embryos. *Developmental Cell*, 27, 131–144.
- Kuijk, E. W., van Tol, L. T., Van de Velde, H., Wubbolts, R., Welling, M., et al. (2012). The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos. *Development*, 139, 871–882.
- Kwon, G. S., Viotti, M., & Hadjantonakis, A. K. (2008). The endoderm of the mouse embryo arises by dynamic widespread intercalation of embryonic and extraembryonic lineages. *Developmental Cell*, 15, 509–520.
- Lawson, K. A., Meneses, J. J., & Pedersen, R. A. (1991). Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development*, 113, 891–911.
- Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., et al. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell*, 113, 631–642.
- Perea-Gomez, A., Vella, F. D., Shawlot, W., Oulad-Abdelghani, M., Chazaud, C., et al. (2002). Nodal antagonists in the anterior visceral endoderm prevent the formation of multiple primitive streaks. *Developmental Cell*, 3, 745–756.
- Schrode, N., Saiz, N., Di Talia, S., & Hadjantonakis, A. K. (2014). GATA6 levels modulate primitive endoderm cell fate choice and timing in the mouse blastocyst. *Developmental Cell*, 29, 454–467.
- Shahbazi, M. N., Jedrusik, A., Vuoristo, S., Recher, G., Hupalowska, A., et al. (2016). Self-organization of the human embryo in the absence of maternal tissues. *Nature Cell Biology*, 18, 700–708.
- Takaoka, K., & Hamada, H. (2012). Cell fate decisions and axis determination in the early mouse embryo. *Development*, 139, 3–14.
- Tam, P. P., & Zhou, S. X. (1996). The allocation of epiblast cells to ectodermal and germ-line lineages is influenced by the position of the cells in the gastrulating mouse embryo. *Developmental Biology*, 178, 124–132.
- Yamanaka, Y., Lanner, F., & Rossant, J. (2010). FGF signal-dependent segregation of primitive endoderm and epiblast in the mouse blastocyst. *Development*, 137, 715–724.

Chromatin Structure: Composition and Function During Spermiogenesis

Saadi Khochbin, Université Grenoble Alpes, Grenoble, France

W Steven Ward, University of Hawaii at Manoa, Honolulu, HI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromatin DNA packaged by proteins. This is the way DNA molecules, which are much longer than the cell, that fit into the nucleus.

Histone A family of basic proteins that bind to DNA in eukaryotic cells to form chromatin.

Nucleosome The basic unit of somatic cell chromatin structure. One nucleosome contains a tetramer of H3 and H4 histones and two dimers of H2A and H2B histones, and about 147 bp of DNA coiled 1.7 time around the histone octamer.

Protamine Very basic proteins that fold DNA into chromatin only in sperm cells.

Spermatids Precursor haploid cells that differentiate into spermatozoa.

Spermatozoa The male gamete. It is the final, mature form of the sperm cell that carries the father's DNA to the oocyte.

Spermiogenesis The process of the differentiation of spermatids to spermatozoa.

Introduction

During spermiogenesis, the haploid round spermatid differentiates into a markedly different spermatozoon, with a very long tail and a much more condensed nucleus. During this process, the paternal genome has two functions. It must produce the proteins necessary to complete the changes as the chromatin shuts down all transcription, and it must repackage itself into a much more condensed form. As discussed later, the first task is accomplished by the production of several mRNAs that are stored and not translated until they are needed. The second task, however, is much more complicated, and will take up the majority of this article.

Chromatin Changes During Spermiogenesis

Delayed Translation

The round spermatid is the first haploid cell produced during spermatogenesis. This is the cell that has the correct number of chromosomes (half of the normal) to fertilize an egg initiating the process of embryogenesis. Its chromatin is, as far as we can tell, relatively normal, bound by histones into nucleosomes with DNA that is accessible to transcription factors (Fig. 1A). During spermiogenesis, the histones are gradually replaced with protamines (see below), greatly reducing the access to the DNA by other proteins to almost zero (Fig. 1C). However, there are proteins that are required to complete the differentiation of the round spermatid to the spermatozoon after transcription has shut down. To solve this problem, the round spermatid produces several mRNAs that will be used later in spermiogenesis, and sequesters them with the translational repressor, Y-box protein (YBX2) (Kleene, 2016, Yang et al., 2005). At later stages during spermiogenesis, the YBX2 repression is removed, and the mRNAs are translated into the proteins that are required.

Crystallizing the Sperm Genome

During the later stages of spermiogenesis, the chromatin is condensed into an almost crystalline like state (Fig. 1C). This is to protect the paternal genome during its transit to the egg at fertilization. To understand the complexity of how the sperm chromatin is condensed during this process, it is helpful to consider a few of the topological problems that must be overcome. In the round spermatid, about 1 m of DNA, divided into 23 different chromosomes (in the human) is coiled by histone octamers into roughly 15 million nucleosomes, in which the DNA is wrapped twice around a core of histones (Fig. 1A). These very long, but very thin strands of DNA are coiled into a nucleus that is about 10 μm in diameter. To repackage this DNA in sperm, most of these 15 million nucleosomes must be unwound, then bound to another group of proteins called protamines that form large toroids with much less coiling (Fig. 1). All this must be done sequentially and in situ—the DNA cannot be unwound completely or it would no longer fit into the nucleus. Thus, the condensation of DNA during spermiogenesis is a highly coordinated event that requires precise timing and spatial organization. Many years ago two proteins, called transition proteins (TP) were identified that were important to help the sperm make this crucial change in DNA packaging. Both are small, basic proteins that are only synthesized during late spermiogenesis. Until recently (see below) it was not known exactly how the TPs functioned to assist in the replacement of histones by protamines (PRMs).

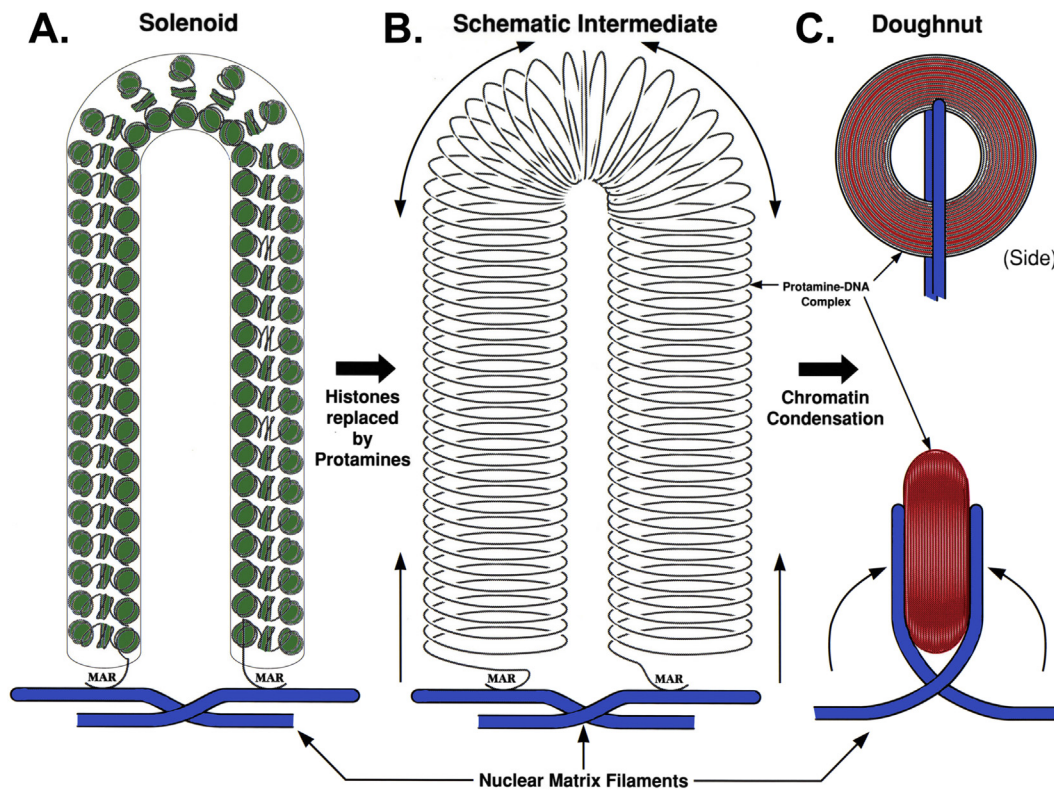


Fig. 1 Schematic representation of how sperm packaging changes during spermiogenesis. (A) Packaging of DNA by nucleosomes into a solenoid-loop. This represents roughly 50,000 bp of DNA. (B) The DNA if all the histones were removed, and the supercoils relaxed by a factor of 6. (C) The same DNA organized into a protamine toroid. This figure is based on a similar in figure (Sotolongo et al., 2003)

Reorganizing the Sperm Chromatin

Spermatid Chromatin at the Time of Histone-to-Protamine Transition: Open Questions

The original model for the function of TPs was that they would completely replace the histones, and then would themselves in turn be replaced by protamines in two sequential complete reorganizations of the future sperm DNA. However, the breakthrough observation by Meistrich laboratory that histones could be replaced in the total absence of TPs (Shirley et al., 2004, Zhao et al., 2004b, Zhao et al., 2004a) prompted the investigators to rethink the function of TPs in histone removal and to question the functional interplay between histones, TPs and PRMs (Barral et al., 2017). Indeed, Meistrich laboratory data suggested that TPs are only necessary for the processing of Pre-PRM2 and the PRM-dependent final tight packaging of the male genome (Shirley et al., 2004, Zhao et al., 2004a). The disappearance of histones under these conditions clearly indicates that PRMs are sufficient to replace histones and hence raised the question of the precise function and utility of TPs in the process of histone removal. Additionally, large-scale histone hyperacetylation and synthesis of specific histone variants are known to accompany histone removal and the subsequent structural transitions. These events are also expected to play a role in driving the genome reorganizations that take place in spermatids (Govin et al., 2004, 2007; Goudarzi et al., 2014).

The full picture of the mechanisms underlying histone-to-protamine transition should therefore integrate a functional interplay between histones, histone variants, histone hyperacetylation, TPs and PRMs. The burning question is therefore how these events act together and how their action is coordinated to drive the genome scale nucleohistone-to-nucleoprotamine transition.

Histone Variant-Directed Histone-to-PRM Transition

In an attempt to identify histone variant candidates involved in a specific organization of chromatin that could facilitate histone eviction, a proteomic approach was set up for an unbiased identification of histones in fractionated spermatogenic cells, which led to the discovery of a series of new histone variants of the H2A and H2B types (Govin et al., 2007). A detailed analysis of the expression of these histone variants pointed to a specific member that accumulated during late spermatogenesis at the same time and in the same cell types as TPs (Govin et al., 2007, Barral et al., 2017). This histone variant of the H2A type, named H2A.L2 (Talbert et al., 2012, El Kennani et al., 2017), belongs to a specific category of histone H2As that are shorter than the canonical histone H2As and lack a region present at the C-terminal part of most of H2A members (Syed et al., 2009, Bao et al., 2004). This region has a critical structural role within a nucleosome as well as in the structure of chromatin fiber. In canonical

H2As, the C-terminal region bears a docking domain that mediates the interaction of H2A with H3–H4 dimers and is essential for the stabilization of the H2A–H2B dimer in the nucleosome (Luger et al., 1997). Additionally, H2A.L.2 also presents significant alterations in an acidic patch, normally present in canonical H2As, which plays an important role in stabilizing nucleosomes–nucleosome interactions (Zhou et al., 2007, Soboleva et al., 2011). Altogether these structural characteristics indicate that nucleosomes bearing H2A.L.2 should present specific features that drastically differentiate these nucleosomes from canonical ones.

In vitro reconstitution assays using purified histones revealed that H2A.L.2-containing nucleosomes indeed possess particular properties that strongly support a specific role for this histone in “preparing” the nucleosomes for histone replacement. H2A.L.2 nucleosomes present flexible DNA ends due to the release of nucleosomal DNA at the entry and exit of nucleosomes and hence, in contrast to canonical nucleosomes, protects only around 120–130 base-pair of DNA against micrococcal nuclease (MNase) digestion (Barral et al., 2017). H2A.L.2’s major function could therefore be related to its ability to create a relaxed chromatin fiber with “open” nucleosomes.

Indeed, our knowledge of another histone variant that also “opens” the nucleosome, the centromeric H3 variant, CENP-A, helps to better understand the function of H2A.L.2. CENP-A’s ability to “open” the nucleosome and release flexible DNA ends is required to load chromatin with the inner kinetochore plate proteins (Roulland et al., 2016). Interestingly, the incorporation of H2A.L.2 into a nucleosome has almost the same structural consequences as the incorporation of CENP-A, and similarly provides a nucleosomal platform for the loading of non-histone proteins. In vitro reconstitution experiments show that, in fact, H2A.L.2 nucleosomes, in contrast to canonical nucleosomes, could load TPs.

A series of in vivo experiments strongly supported the conclusions of these in vitro assays. Indeed, in the absence of H2A.L.2, defective TP incorporation to chromatin was observed and large amounts of TP2-Pre-PRM2 could be immunopurified from elongating spermatids nuclear extracts. These observations indicated first that, in the absence of H2A.L.2, nucleosomes resist invasion by TPs and second that the non-incorporated accumulating TPs trap the incoming Pre-PRM2 and hinders its further processing. From these observations it could be concluded that H2A.L.2 incorporation into nucleosome relaxes chromatin and individually opens all the nucleosomes that become exposed to TPs. The loaded TPs in turn buffer and guide the assembly of PRMs (Fig. 2).

In the absence of H2A.L.2, as observed in the absence of TPs, PRMs can displace histones but the Pre-PRM2 processing and the final compaction of the genome are defective. These observations suggest that TPs’ primary function is not to displace histones but to guide the action of PRMs. These data also invite us to revisit the common belief that histones are first replaced by TPs and that PRMs then replace TPs. In fact, the in vitro reconstitution assays show that while, H2A.L.2 nucleosomes could load TPs, no DNA

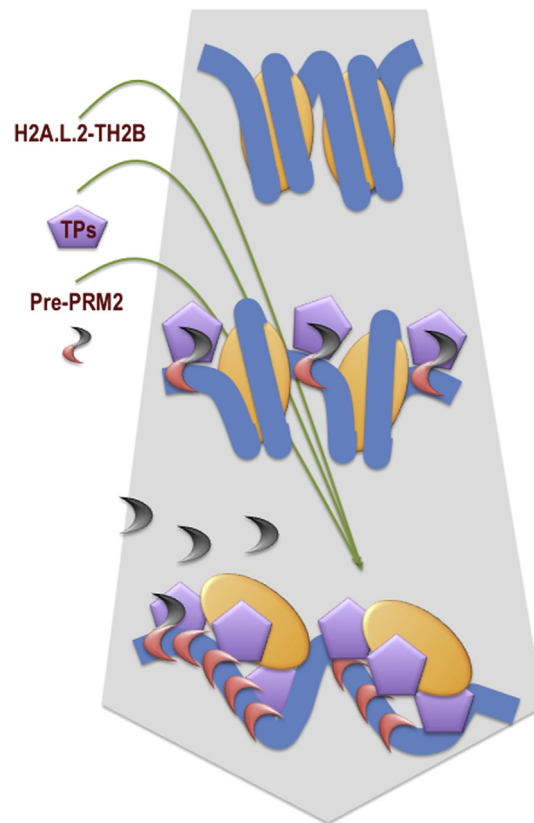


Fig. 2 A new model for the role of histone variants and transition proteins in replacing histones with protamines. See text for details.

release was observed following the incubation of either canonical or H2A.L.2 nucleosomes with increasing amounts of TPs (Barral et al., 2017).

It is of note that, at the time of H2A.L.2 synthesis, the major chromatin H2B species is TH2B (Montellier et al., 2013), which preferentially dimerizes with H2A.L.2 (Govin et al., 2007). It is also known that, compared to the canonical H2Bs, TH2B itself could contribute to some extent to the generation of less stable nucleosomes (Shinagawa et al., 2014, Montellier et al., 2013). In the light of these investigations altogether a new vision of histone-to-protamine transition should be considered, where nucleosomes are first prepared for histone eviction by incorporation of a H2A histone variant which relaxes the chromatin fiber and opens nucleosomes hence mediating the loading of non-histone TPs, whose major role is to buffer the action of the PRMs which are the true displacers of histones (Fig. 2).

Histone Hyperacetylation and Histone Removal

Since the early 1970s different investigators reported the occurrence of histone hyperacetylation in spermatogenic cells from various species, more specifically those undergoing a histone-to-protamine transition during their spermatogenesis (Govin et al., 2004). A detailed study of the timing of histone acetylation during mouse and human spermatogenesis highlighted the occurrence of histone hyperacetylation precisely in the elongating spermatids associated with histone removal (Hazzouri et al., 2000, Faure et al., 2003).

Based on the hypothesis that histone acetylation could signal histone removal through a bromodomain containing protein, the bromodomain testis-specific protein Brdt was characterized as a potentially promising factor, capable of reorganizing hyperacetylated chromatin (Pivot-Pajot et al., 2003). Brdt is a protein containing two tandem bromodomains, uniquely expressed in testis, from spermatocytes all through spermatogenesis (Gaucher et al., 2012). Structural and in vivo studies showed that Brdt's first bromodomain (BD1) specifically recognizes hyperacetylated H4 (Sasaki et al., 2009, Moriniere et al., 2009). Using a mouse model expressing a Brdt deleted of its first bromodomain, the involvement of BD1 in the removal of histones could be demonstrated (Gaucher et al., 2012). Finally, a recent investigation showed that Brdt BD1 is unable to recognize butyrylated histone H4 tails and that, according to the proposed role of Brdt in removing acetylated histones, histone H4 butyrylated at K5 and K8 escapes the wave of acetylation-dependent histone removal (Goudarzi et al., 2016). Despite these accumulating data on the role of Brdt, specifically of its BD1 in histone removal, additional investigations are required to define how Brdt's interaction with hyperacetylated histone H4 mediates histones removal.

Additional recent reports also support a molecular link between histone acetylation and histone removal by proposing a role for the histone acetyltransferases MOF (Lu et al., 2010) and TIP60 (Dong et al., 2017) in histone hyperacetylation and removal, and the action of the H2A ubiquitin ligase RNF8 is histone ubiquitination and degradation (Lu et al., 2010, Gou et al., 2017). The most direct evidence for a role of histone hyperacetylation in their degradation in elongating spermatids comes from the suggested ability of the testis-specific proteasome associated with PA200 to directly bind and degrade acetylated histones in spermatogenic cells (Qian et al., 2013). However, some of these data are controversial, since the role of RNF8 in histone degradation could be questioned. Indeed no effect on histone-to-protamine replacement was observed in RNF8 KO spermatogenic cells (Sin et al., 2012) and, in PA200 KO spermatids, histones are finally removed, although probably with a delay (Qian et al., 2013).

The challenge is now to precisely identify the mechanisms linking histone hyperacetylation to histone eviction, and to discover the molecular link between these histone acetylation-dependent and H2A.L.2-dependent mechanisms.

Conclusion

Recent data has shed much light on the complex mechanisms by which spermatids condense their DNA into the highly compact chromatin of mature spermatozoa. The new model suggests that specific histone variants first open the nucleosomes and transition proteins bring the pre-protamines to the nucleosomes to replace the histones. This coordinated process allows for the orderly transition of the entire paternal genome to a compact, protected state so that the DNA is protected during the transit in fertilization.

References

- Bao, Y., Konesky, K., Park, Y. J., Rosu, S., Dyer, P. N., Rangasamy, D., Tremethick, D. J., Laybourn, P. J., & Luger, K. (2004). Nucleosomes containing the histone variant H2A.Bbd organize only 118 base pairs of DNA. *The EMBO Journal*, 23, 3314–3324.
- Barral, S., Morozumi, Y., Tanaka, H., Montellier, E., Govin, J., De Dieuleveult, M., Charbonnier, G., Coute, Y., Puthier, D., Buchou, T., Boussouar, F., Urahama, T., Fenaille, F., Curtet, S., Hery, P., Fernandez-Nunez, N., Shiota, H., Gerard, M., Rousseaux, S., Kurumizaka, H., & Khochbin, S. (2017). Histone variant H2A.L.2 guides transition protein-dependent protamine assembly in male germ cells. *Mol Cell*, 66, 89–101.e8.
- Dong, Y., Isono, K. I., Ohbo, K., Endo, T. A., Ohara, O., Maekawa, M., Toyama, Y., Ito, C., Toshimori, K., Helin, K., Ogonuki, N., Inoue, K., Ogura, A., Yamagata, K., Kitabayashi, I., & Koseki, H. (2017). EPC1/TIP60-mediated histone acetylation facilitates spermiogenesis in mice. *Mol Cell Biol*, 37(19). pii: e00082-17.
- El Kennani, S., Adrait, A., Shaytan, A. K., Khochbin, S., Bruley, C., Panchenko, A. R., Landsman, D., Pflieger, D., & Govin, J. (2017). MS_HistoneDB, a manually curated resource for proteomic analysis of human and mouse histones. *Epigenetics & Chromatin*, 10, 2.
- Faure, A. K., Pivot-Pajot, C., Kerjean, A., Hazzouri, M., Pelletier, R., Peoc'h, M., Sele, B., Khochbin, S., & Rousseaux, S. (2003). Misregulation of histone acetylation in Sertoli cell-only syndrome and testicular cancer. *Molecular Human Reproduction*, 9, 757–763.

- Gaucher, J., Boussouar, F., Montellier, E., Curtet, S., Buchou, T., Bertrand, S., Hery, P., Jounier, S., Depaux, A., Vitte, A. L., Guardiola, P., Pernet, K., Debernardi, A., Lopez, F., Holota, H., Imbert, J., Wolgemuth, D. J., Gerard, M., Rousseaux, S., & Khochbin, S. (2012). Bromodomain-dependent stage-specific male genome programming by Brdt. *The EMBO Journal*, *31*, 3809–3820.
- Gou, L. T., Kang, J. Y., Dai, P., Wang, X., Li, F., Zhao, S., Zhang, M., Hua, M. M., Lu, Y., Zhu, Y., Li, Z., Chen, H., Wu, L. G., Li, D., Fu, X. D., Li, J., Shi, H. J., & Liu, M. F. (2017). Ubiquitination-deficient mutations in human Piwi cause male infertility by impairing histone-to-protamine exchange during spermiogenesis. *Cell*, *169*, 1090–1104.e13.
- Goudarzi, A., Shiota, H., Rousseaux, S., & Khochbin, S. (2014). Genome-scale acetylation-dependent histone eviction during spermatogenesis. *Journal of Molecular Biology*, *426*, 3342–3349.
- Goudarzi, A., Zhang, D., Huang, H., Barral, S., Kwon, O. K., Qi, S., Tang, Z., Buchou, T., Vitte, A. L., He, T., Cheng, Z., Montellier, E., Gaucher, J., Curtet, S., Debernardi, A., Charbonnier, G., Puthier, D., Petosa, C., Panne, D., Rousseaux, S., Roeder, R. G., Zhao, Y., & Khochbin, S. (2016). Dynamic competing histone H4 K5K8 acetylation and Butyrylation are hallmarks of highly active gene promoters. *Molecular Cell*, *62*, 169–180.
- Govin, J., Caron, C., Lestrat, C., Rousseaux, S., & Khochbin, S. (2004). The role of histones in chromatin remodelling during mammalian spermiogenesis. *European Journal of Biochemistry*, *271*, 3459–3469.
- Govin, J., Escoffier, E., Rousseaux, S., Kuhn, L., Ferro, M., Thevenon, J., Catena, R., Davidson, I., Garin, J., Khochbin, S., & Caron, C. (2007). Pericentric heterochromatin reprogramming by new histone variants during mouse spermiogenesis. *The Journal of Cell Biology*, *176*, 283–294.
- Hazzouri, M., Pivot-Pajot, C., Faure, A. K., Usson, Y., Pelletier, R., Sele, B., Khochbin, S., & Rousseaux, S. (2000). Regulated hyperacetylation of core histones during mouse spermatogenesis: Involvement of histone deacetylases. *European Journal of Cell Biology*, *79*, 950–960.
- Kleene, K. C. (2016). Position-dependent interactions of Y-box protein 2 (YBX2) with mRNA enable mRNA storage in round spermatids by repressing mRNA translation and blocking translation-dependent mRNA decay. *Molecular Reproduction and Development*, *83*, 190–207.
- Lu, L. Y., Wu, J., Ye, L., Gavrilina, G. B., Saunders, T. L., & Yu, X. (2010). RNFB-dependent histone modifications regulate nucleosome removal during spermatogenesis. *Developmental Cell*, *18*, 371–384.
- Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, *389*, 251–260.
- Montellier, E., Boussouar, F., Rousseaux, S., Zhang, K., Buchou, T., Fenaille, F., Shiota, H., Debernardi, A., Hery, P., Curtet, S., Jamshidikia, M., Barral, S., Holota, H., Bergon, A., Lopez, F., Guardiola, P., Pernet, K., Imbert, J., Petosa, C., Tan, M., Zhao, Y., Gerard, M., & Khochbin, S. (2013). Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes & Development*, *27*, 1680–1692.
- Moriniere, J., Rousseaux, S., Steuerwald, U., Soler-Lopez, M., Curtet, S., Vitte, A. L., Govin, J., Gaucher, J., Sadoul, K., Hart, D. J., Krijgsvelde, J., Khochbin, S., Muller, C. W., & Petosa, C. (2009). Cooperative binding of two acetylation marks on a histone tail by a single bromodomain. *Nature*, *461*, 664–668.
- Pivot-Pajot, C., Caron, C., Govin, J., Vion, A., Rousseaux, S., & Khochbin, S. (2003). Acetylation-dependent chromatin reorganization by BRDT, a testis-specific bromodomain-containing protein. *Molecular and Cellular Biology*, *23*, 5354–5365.
- Qian, M. X., Pang, Y., Liu, C. H., Haratake, K., Du, B. Y., Ji, D. Y., Wang, G. F., Zhu, Q. Q., Song, W., Yu, Y., Zhang, X. X., Huang, H. T., Miao, S., Chen, L. B., Zhang, Z. H., Liang, Y. N., Liu, S., Cha, H., Yang, D., Zhai, Y., Komatsu, T., Tsuruta, F., Li, H., Cao, C., Li, W., Li, G. H., Cheng, Y., Chiba, T., Wang, L., Goldberg, A. L., Shen, Y., & Qiu, X. B. (2013). Acetylation-mediated proteasomal degradation of core histones during DNA repair and spermatogenesis. *Cell*, *153*, 1012–1024.
- Roulland, Y., Ouarrhni, K., Naidenov, M., Ramos, L., Shuaib, M., Syed, S. H., Lone, I. N., Boopathi, R., Fontaine, E., Papai, G., Tachiwana, H., Gautier, T., Skoufias, D., Padmanabhan, K., Bednar, J., Kurumizaka, H., Schultz, P., Angelov, D., Hamiche, A., & Dimitrov, S. (2016). The flexible ends of CENP-A nucleosome are required for mitotic fidelity. *Molecular Cell*, *63*, 674–685.
- Sasaki, K., Ito, T., Nishino, N., Khochbin, S., & Yoshida, M. (2009). Real-time imaging of histone H4 hyperacetylation in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 16257–16262.
- Shinagawa, T., Takagi, T., Tsukamoto, D., Tomaru, C., Huynh, L. M., Sivaraman, P., Kumarevel, T., Inoue, K., Nakato, R., Katou, Y., Sado, T., Takahashi, S., Ogura, A., Shirahige, K., & Ishii, S. (2014). Histone variants enriched in oocytes enhance reprogramming to induced pluripotent stem cells. *Cell Stem Cell*, *14*, 217–227.
- Shirley, C. R., Hayashi, S., Mounsey, S., Yanagimachi, R., & Meistrich, M. L. (2004). Abnormalities and reduced reproductive potential of sperm from Tnp1- and Tnp2-null double mutant mice. *Biology of Reproduction*, *71*, 1220–1229.
- Sin, H. S., Barski, A., Zhang, F., Kartashov, A. V., Nussenzweig, A., Chen, J., Andreassen, P. R., & Namekawa, S. H. (2012). RNFB regulates active epigenetic modifications and escape gene activation from inactive sex chromosomes in post-meiotic spermatids. *Genes & Development*, *26*, 2737–2748.
- Soboleva, T. A., Nekrasov, M., Pahwa, A., Williams, R., Huttley, G. A., & Tremethick, D. J. (2011). A unique H2A histone variant occupies the transcriptional start site of active genes. *Nature Structural & Molecular Biology*, *19*, 25–30.
- Sotolongo, B., Lino, E., & Ward, W. S. (2003). Ability of hamster spermatozoa to digest their own DNA. *Biology of Reproduction*, *69*, 2029–2035.
- Syed, S. H., Boulard, M., Shukla, M. S., Gautier, T., Travers, A., Bednar, J., Faivre-Moskalenko, C., Dimitrov, S., & Angelov, D. (2009). The incorporation of the novel histone variant H2AL2 confers unusual structural and functional properties of the nucleosome. *Nucleic Acids Research*, *37*, 4684–4695.
- Talbert, P. B., Ahmad, K., Almouzni, G., Ausio, J., Berger, F., Bhalla, P. L., Bonner, W. M., Cande, W., Chadwick, B. P., Chan, S. W., Cross, G. A., Cui, L., Dimitrov, S. I., Doenecke, D., Eirin-Lopez, J. M., Gorovsky, M. A., Hake, S. B., Hamkalo, B. A., Holec, S., Jacobsen, S. E., Kamieniarz, K., Khochbin, S., Ladurner, A. G., Landsman, D., Latham, J. A., Loppin, B., Malik, H. S., Marzluff, W. F., Pehrson, J. R., Postberg, J., Schneider, R., Singh, M. B., Smith, M. M., Thompson, E., Torres-Padilla, M. E., Tremethick, D. J., Turner, B. M., Waterborg, J. H., Wollmann, H., Yelagandula, R., Zhu, B., & Henikoff, S. (2012). A unified phylogeny-based nomenclature for histone variants. *Epigenetics & Chromatin*, *5*, 7.
- Yang, J., Medvedev, S., Reddi, P. P., Schultz, R. M., & Hecht, N. B. (2005). The DNA/RNA-binding protein MSY2 marks specific transcripts for cytoplasmic storage in mouse male germ cells. *Proceedings of the National Academy of Sciences of the United States of America*, *102*, 1513–1518.
- Zhao, M., Shirley, C. R., Hayashi, S., Marcon, L., Mohapatra, B., Suganuma, R., Behringer, R. R., Boissonneault, G., Yanagimachi, R., & Meistrich, M. L. (2004a). Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis*, *38*, 200–213.
- Zhao, M., Shirley, C. R., Mounsey, S., & Meistrich, M. L. (2004b). Nucleoprotein transitions during spermiogenesis in mice with transition nuclear protein Tnp1 and Tnp2 mutations. *Biology of Reproduction*, *71*, 1016–1025.
- Zhou, J., Fan, J. Y., Rangasamy, D., & Tremethick, D. J. (2007). The nucleosome surface regulates chromatin compaction and couples it with transcriptional repression. *Nature Structural & Molecular Biology*, *14*, 1070–1076.

X-Chromosome Inactivation

Tatsuro Nakajima, Kyushu University, Fukuoka, Japan
Takashi Sado, Kindai University, Nara, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Embryonic stem cells They are derived from the inner cell mass cells of the blastocyst with pluripotency to give rise to tissues of all three germ layers when induced to differentiate.

Embryonic tissue It is derived from the epiblast that contributes to all three germ layers of the embryo giving rise to all the tissues of the fetus.

Extraembryonic tissue It is derived from the trophoctoderm and primitive endoderm of the blastocyst, giving rise to the placenta and part of the extraembryonic membranes surrounding the fetus.

Heterochromatinization It is a process to form heterochromatin, which is originally identified as an electron-dense entity in the nucleus. It is highly condensed and transcriptionally inactive chromatin. An inactive X chromosome is referred to as facultative heterochromatin, which can take euchromatic as well as heterochromatic state depending on the developmental stage and cell-type.

Histone tail An N-terminal amino acid stretch of core histones, which protrudes from a nucleosome and is subjected to various chemical modifications at the specific residues. Such modifications have various impacts on transcriptional state through changes in the chromatin structure.

Yeast artificial chromosome (YAC) It is a functional artificial chromosome containing a centromere sequence, telomere sequences, and a replication origin, which allow it to replicate autonomously and propagate from one cell to its daughter cells, in combination with a couple of selectable markers. A YAC can take a long DNA fragment such as partially digested mouse genomic DNA up to 1 Mb and allows to generate an unlimited number of copies of YAC containing a particular long DNA sequence by introduction into yeast cells.

Introduction

Female mammals have two X chromosomes (XX) and males have one X and one Y chromosome (XY). An X chromosome carries more than 1000 genes including those essential for embryo development, while a Y chromosome contains tens of genes required for only male determination and development. One can expect that such a big difference in the number of X-linked genes between the sexes, if many of them are fully expressed, would result in huge imbalance. To avoid such chaotic situation, female mammals have evolved a mechanism to inactivate one of the two X chromosomes during early development ([Lyon, 1961](#)). It is called X-chromosome inactivation (X-inactivation). Once X-inactivation occurs, the inactivated state of the chromosome is stably transmitted to daughter cells through successive cell divisions. Therefore, this phenomenon has been drawing much attention of researchers as one of epigenetic gene regulations for more than half a century. This article will focus on the X-inactivation mainly in mice, which have been most extensively studied as a model animal and made a significant contribution to our knowledge about this unique epigenetic mechanism.

X-Inactivation in Mice

A single fertilized egg of mammals forms two different types of tissues, embryonic and extraembryonic tissues. The former gives rise to all the tissues of the embryo proper, whereas the latter contributes to the remaining extraembryonic components, such as the yolk sac and placenta. Interestingly, the X-inactivation pattern is different between these two tissues in mice and most probably other rodents ([Fig. 1](#)).

Imprinted X-Inactivation

Each of the X chromosomes derived from an egg and sperm is initially active in the zygote, but one of them becomes transcriptionally inactivated at around the 4- to 8-cell stages in favor of the paternal X chromosome ([Okamoto et al., 2004](#)). This is, therefore, called imprinted X-inactivation. Following cleavage, a fertilized egg develops to a blastocyst, which consists of the trophoctoderm forming the outer layer of the embryo and undifferentiated inner cell mass (ICM) cells inside. A part of the ICM cells subsequently differentiates into the primitive endoderm at around the time of implantation. The trophoctoderm and primitive endoderm will contribute to the extraembryonic tissues, in which the paternal X chromosome inactivated early on stays repressed. It should be noted, however, that there is little evidence of imprinted X-inactivation in the extraembryonic tissues of eutherian mammals except

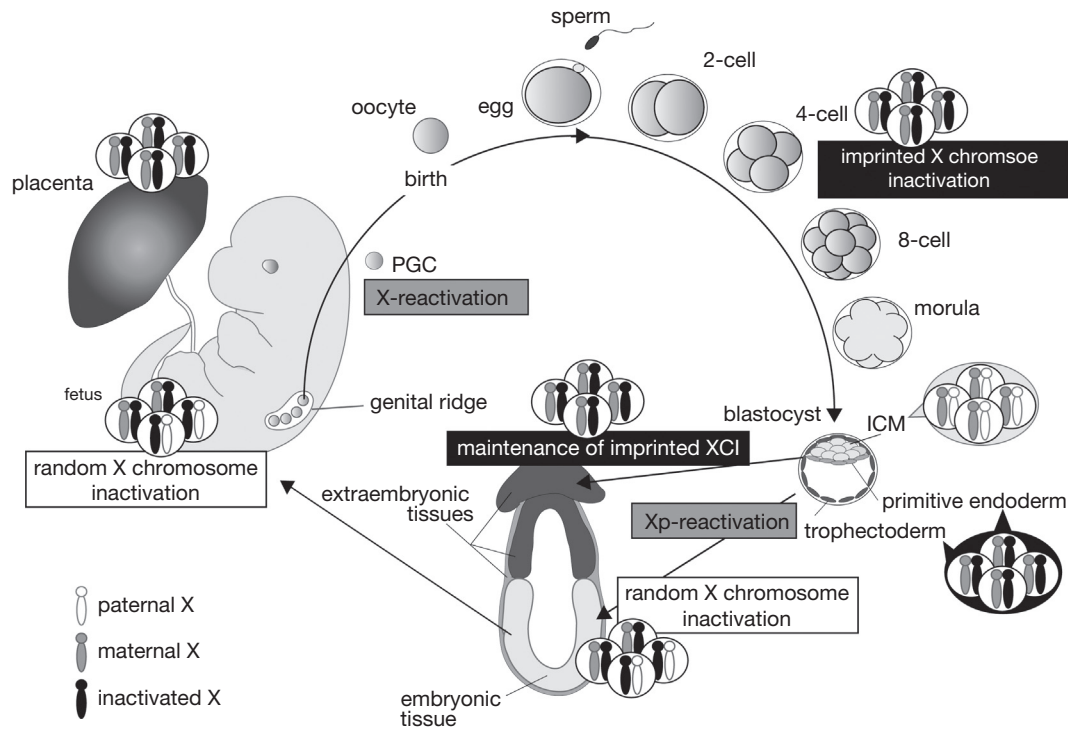


Fig. 1 Changes in the activity of X chromosomes during mouse development. Modified from SadoT: Imaging property.

for rodents. Interestingly, marsupials, which diverged 130 million years ago from the ancestor of therian, show imprinted X-inactivation in not only the extraembryonic but also the embryonic tissue.

Random X-Inactivation

Paternal X chromosome inactivated at the early preimplantation stage transiently restores its transcriptional activity in those cells that have contributed to the ICM cells (Mak et al., 2004). This reactivation is considered to be a part of reprogramming, which cancels the developmental program of the extraembryonic tissues and allows to initiate another developmental program for the embryonic tissue that differentiates into various types of cells of the fetus including germ cells. The ICM cells subsequently form epiblast soon after implantation, which is still undifferentiated pluripotent cell population, and either the maternal or paternal X chromosome undergoes inactivation in a random fashion as cells differentiate (Monk and Harper, 1979). This is referred to as random X-inactivation. It is considered that X-inactivation completes by gastrulation and the inactive state of the X chromosome once established is stably maintained throughout embryogenesis and the life of individual after birth. Mouse embryonic stem (ES) cells, which are derived from the ICM cells and retain pluripotency to differentiate into every cell type of the fetus, have been used for the studies of random X-inactivation as they recapitulate essentially the whole process of random X-inactivation in culture. While female ES cells retains two active X chromosomes in undifferentiated state, one of them undergoes inactivation in a random fashion upon induction of differentiation.

X-Reactivation

It is known that there are two distinct phases when the inactivated X chromosome becomes reactivated during embryonic development. As mentioned earlier, reactivation occurs in the ICM cells of the preimplantation blastocyst for epigenetic reprogramming. Another reactivation of the inactive X chromosome occurs in the primordial germ cells (PGCs), which are the progenitors of future oocytes (Monk and McLaren, 1981). PGCs emerge in a small subset of the somatic cell population of the postimplantation embryo by receiving extrinsic signals from the surrounding somatic cells at around the time of gastrulation. These signals trigger expression of PGC-specific genes. This process also requires reprogramming of the epigenetic state of the somatic cell-lineage to the germ cell-lineage. The X chromosome that has been inactivated early on appears to gradually reactivate in PGCs during their journey from the site they emerge to the genital ridges, the future ovaries. The reactivated X chromosome stays active during the rest of oogenesis until the maternal X chromosome is chosen during random X-inactivation in the embryonic tissues of female offspring.

X-reactivation also occurs in somatic cell nuclear transfer (SCNT) embryos (Eggan et al., 2000). The inactive X chromosome in the donor somatic cell becomes reactivated when exposed to the egg cytoplasm. Similar reactivation is also observed when induced

pluripotent stem (iPS) cells are generated following transduction of the Yamanaka four factors (Oct4, Sox2, Klf4, and c-Myc) in female mouse somatic cells (Maherali et al., 2007). Although the molecular mechanisms of X-reactivation in SCNT embryos and iPS cells as well as in the ICM cells and PGCs in vivo are largely obscure, given the fact that the inactive X chromosome always becomes reactivated when the genome is reprogrammed, it is likely that X chromosome reactivation is one of the most important processes in the genome reprogramming.

XIST/Xist

The X-inactivation center (Xic) has been identified on an X chromosome by classic cytogenetic analysis as a region essential for X-inactivation to occur in cis (Rastan, 1983). In the early 90's, X inactive-specific transcript (*XIST*) RNA was serendipitously found in human as a 17-kb long noncoding RNA exclusively expressed from an inactive X chromosome (Brown et al., 1991). Subsequent cloning of a mouse homolog, *Xist*, accelerated the analysis of the molecular mechanisms of X-inactivation (Borsani et al., 1991; Brockdorff et al., 1991). *Xist* RNA, although, transcribed by RNA polymerase II, and subjected to splicing and polyadenylation as is the case of common protein-coding RNA, it escapes nuclear export and stays in the nucleus to accumulate on the inactive X chromosome it originates from. Extensive studies using mouse ES cells revealed that while the both *Xist* alleles are expressed at a very low basal level in undifferentiated ES cells, one of them is upregulated on the X chromosome to be inactivated upon differentiation and its transcripts spread along the chromosome in cis. It has been suggested that *Xist* RNA thus accumulated on the X chromosome recruits proteins involved in gene silencing and induces chromosome-wide heterochromatinization (Fig. 2). Targeted disruption of *Xist* in ES cells and mice clearly demonstrated that *Xist* is essential for X-inactivation to occur in cis, and therefore, the X chromosome deficient for *Xist* never undergoes inactivation (Penny et al., 1996; Marahrens et al., 1997). It has been shown that yeast artificial chromosome (YAC) containing the *Xist* gene and surrounding region integrated to an autosome as a transgene apparently acts as Xic. *Xist* RNA expressed at the ectopic locus spreads along the chromosome and induces silencing of, at least, some autosomal genes. These results suggest that *Xist* is responsible for the most part of the function of Xic. Comparison of human and mouse *XIST/Xist* RNA sequences revealed the presence of some highly conserved repeated sequences, termed the A-E repeats. Another repeat, termed the F-repeat, was also found as a conserved repeat among mouse subspecies (Fig. 3). Of these conserved repeats, the A-repeat, which is predicted to form a stem-loop structure, has been shown to be indispensable for the proper silencing function of *Xist* RNA in differentiating ES cells and mice (Wutz et al., 2002; Sakata et al., 2017). In addition, since the mutated *Xist* allele lacking the genomic sequence corresponding to the A-repeat fails to be upregulated, it is likely that the A-repeat sequence is also essential as a cis-element for transcriptional regulation of *Xist* (Hoki et al., 2009).

It is known that an antisense RNA is transcribed at the *Xist* locus, which is referred to as *Tsix* (Lee et al., 1999) (Fig. 3). While *Tsix* and *Xist* are expressed on both X chromosomes in undifferentiated female ES cells, *Tsix* becomes downregulated on one X chromosome and then, in turn, *Xist* comes to be upregulated on the same X chromosome and coats it to induce X-inactivation. In contrast, *Tsix* expression on the other X chromosome persists for a while and then both *Tsix* and *Xist* becomes downregulated to leave the X chromosome being active. Therefore, *Tsix* is a negative regulator of *Xist*, which prevents upregulation of *Xist* at the onset of X-inactivation (Sado et al., 2001). Available evidence suggests that *Tsix* is involved in the establishment of the repressive epigenetic modifications at around the *Xist* promoter.

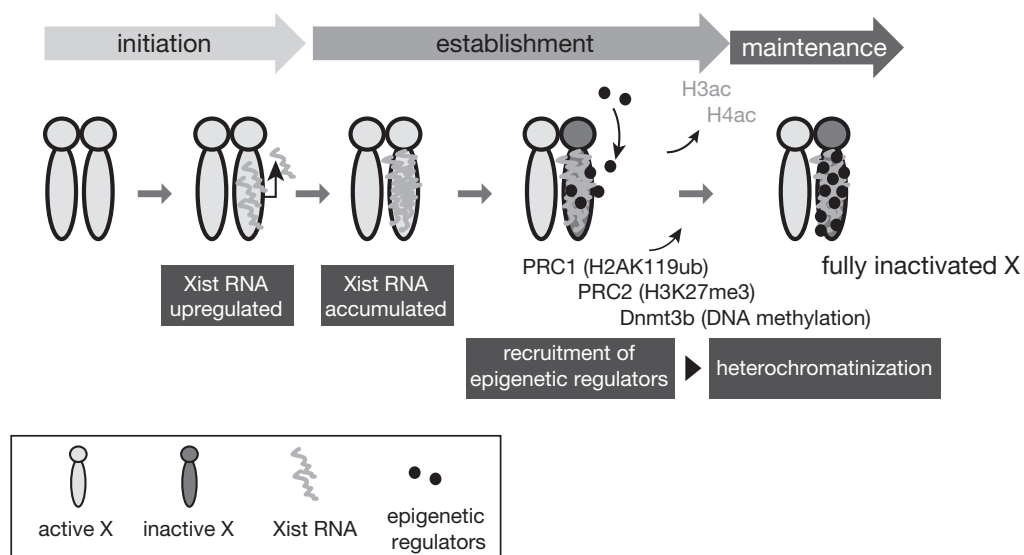
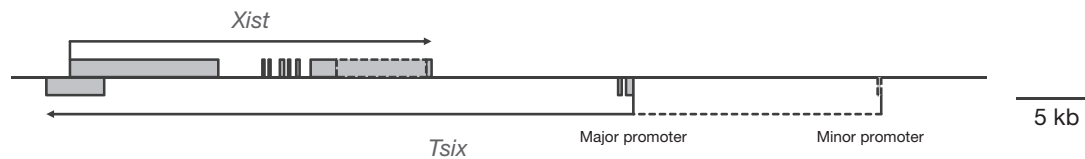


Fig. 2 The process of X chromosome inactivation.

The mouse *Xist* gene and antisense *Tsix* gene



Processing of *Xist* RNA and conserved repeats

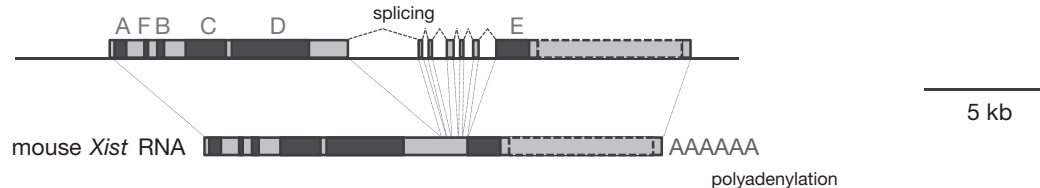


Fig. 3 *Xist* and the antisense regulator, *Tsix*.

Epigenetic Modifications

Epigenetic regulation is a mechanism that controls gene expression via DNA methylation and histone modifications such as acetylation, methylation, and ubiquitination at the N-terminus region, so called histone tail. The inactive X chromosome is characterized by various repressive epigenetic modifications (Fig. 2). Substantial part of DNA methylation occurs at CpG (cytosine-phosphodiester bond-guanine) dinucleotide sequences in the mammalian genome. Classically, it is well known that CpG islands, which are enriched with CpG dinucleotide sequences and often found in the regulatory region of genomic DNA such as the promoters and enhancers, are highly methylated on the inactive X chromosome as compared to those on the active X chromosome. DNA methylation is, however, thought to play a role in the maintenance of X-inactivated state but is dispensable for the initiation of X-inactivation. It has been suggested that de novo DNA methylation of the inactive X chromosome is mediated by de novo DNA methyltransferase Dnmt3b.

While the inactive X chromosome is devoid of histone modifications for active chromatin such as dimethylation of histone H3 at lysine 4 (H3K4me2) as well as acetylation of histone H3 and H4, it is enriched with trimethylation of histone H3 at lysine 27 (H3K27me3), di- and trimethylation of histone H3 at lysine 9 (H3K9me2/3), monomethylation of histone H4 at lysine 20 (H4K20me1), and monoubiquitination of histone H2A at lysine 119 (H2AK119ub1). It has been considered that *Xist* RNA accumulated on the X chromosome recruits polycomb repressive complex 2 (PRC2) whose catalytic core, Ezh2 histone methyltransferase, in combination with Suz12 and Eed mediates H3K27me3. Subsequently, another polycomb repressive complex, PRC1, recognizes H3K27me3 and its E3 ubiquitin ligase activity mediated by Ring1a/b facilitates H2AK119ub1. More recent study using genetics, however, demonstrated that H2AK119ub1 occurs on the X chromosome in the absence of H3K27me3 but not the other way around, therefore, no H3K27me3 on X without H2AK119ub1. It is still unknown, however, how H2AK119ub1 recruits PRC2 on the inactive X chromosome and exerts its effect on heterochromatinization and X-inactivation. Further studies are necessary for elucidating how histone modification patterns are established on the inactive X chromosome and how *Xist* RNA is involved in these processes.

Evolutionary Aspects of X-Inactivation

As mentioned earlier, X-inactivation is also observed in marsupials, but in contrast to eutherians, the paternal X chromosome is always inactivated in every somatic cell like that in the extraembryonic tissues of the mouse. Other differences between eutherian and marsupials include the stability of X-inactivated state and the levels of DNA methylation. It is known in marsupials that spontaneous reactivation of the inactive X chromosome often occurs and differential methylation of the CpG islands between an active and an inactive X chromosome is not prominent. The lack of DNA methylation may partly account for less stable X-inactivation in marsupials. Furthermore, the *Xist* gene is not found in the marsupial genomes. Comparison of the synteny at the Xic region among many different species suggests that the eutherians *Xist* gene has evolved from a protein-coding gene, *LnX3*, which is present up to marsupials. This indicates that marsupials inactivate an X chromosome without *Xist*. Afterwards, a novel noncoding RNA acting like *Xist* RNA was found in marsupials. It is exclusively expressed from and coats the inactive paternal X chromosome in female opossum, and therefore, named *Rsx* (RNA on the silent X) RNA (Grant et al., 2012). *Rsx* RNA is as long as 27-kb in length and subjected to processing such as polyadenylation and splicing like *Xist* RNA. *Rsx* RNA expressed on an autosome in mouse ES cells coats the chromosome, to which the transgene has been integrated, and induces silencing of, at least, three genes nearby the integration site of the transgene. The many characteristic features of *Xist* RNA are shared by *Rsx* RNA, but there is no sequence homology

between them. It is most likely that eutherians and marsupials have evolved noncoding *Xist* and *Rsx* RNA, respectively, as a result of convergent evolution to compensate for the dosage difference of X-linked gene between the sexes by X-inactivation.

References

- Borsani, G., Tonlorenzi, R., Simmler, M. C., Dandolo, L., Arnaud, D., Capra, V., Grompe, M., Pizzuti, A., Muzny, D., Lawrence, C., Willard, H. F., Avner, P., & Ballabio, A. (1991). Characterization of a murine gene expressed from the inactive X chromosome. *Nature*, *351*, 325–329.
- Brockdorff, N., Ashworth, A., Kay, G. F., Cooper, P., Smith, S., McCabe, V. M., Norris, D. P., Penny, G. D., Patel, D., & Rastan, S. (1991). Conservation of position and exclusive expression of mouse *Xist* from the inactive X chromosome. *Nature*, *351*, 329–331.
- Brown, C. J., Ballabio, A., Rupert, J. L., Lafreniere, R. G., Grompe, M., Tonlorenzi, R., & Willard, H. F. (1991). A gene from the region of the human X inactivation centre is expressed exclusively from the inactive X chromosome. *Nature*, *349*, 38–44.
- Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W., 3rd, Yanagimachi, R., & Jaenisch, R. (2000). X-chromosome inactivation in cloned mouse embryos. *Science*, *290*, 1578–1581.
- Grant, J., Mahadevaiah, S. K., Khil, P., Sangrithi, M. N., Royo, H., Duckworth, J., Mccarrey, J. R., Vandeberg, J. L., Renfree, M. B., Taylor, W., Elgar, G., Camerini-Otero, R. D., Gilchrist, M. J., & Turner, J. M. (2012). *Rsx* is a metatherian RNA with *Xist*-like properties in X-chromosome inactivation. *Nature*, *487*, 254–258.
- Hoki, Y., Kimura, N., Kanbayashi, M., Amakawa, Y., Ohhata, T., Sasaki, H., & Sado, T. (2009). A proximal conserved repeat in the *Xist* gene is essential as a genomic element for X-inactivation in mouse. *Development*, *136*, 139–146.
- Lee, J. T., Davidow, L. S., & Warshawsky, D. (1999). *Tsix*, a gene antisense to *Xist* at the X-inactivation centre. *Nature Genetics*, *21*, 400–404.
- Lyon, M. F. (1961). Gene action in the X-chromosome of the mouse (*Mus musculus* L.). *Nature*, *190*, 372–373.
- Maherali, N., Sridharan, R., Xie, W., Utikal, J., Eminli, S., Arnold, K., Stadtfeld, M., Yachechko, R., Tchieu, J., Jaenisch, R., Plath, K., & Hochedlinger, K. (2007). Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell*, *1*, 55–70.
- Mak, W., Nesterova, T. B., de Napoles, M., Appanah, R., Yamanaka, S., Otte, A. P., & Brockdorff, N. (2004). Reactivation of the paternal X chromosome in early mouse embryos. *Science*, *303*, 666–669.
- Marahrens, Y., Panning, B., Dausman, J., Strauss, W., & Jaenisch, R. (1997). *Xist*-deficient mice are defective in dosage compensation but not spermatogenesis. *Genes & Development*, *11*, 156–166.
- Monk, M., & Harper, M. I. (1979). Sequential X chromosome inactivation coupled with cellular differentiation in early mouse embryos. *Nature*, *281*, 311–313.
- Monk, M., & McLaren, A. (1981). X-chromosome activity in foetal germ cells of the mouse. *Journal of Embryology and Experimental Morphology*, *63*, 75–84.
- Okamoto, I., Otte, A. P., Allis, C. D., Reinberg, D., & Heard, E. (2004). Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science*, *303*, 644–649.
- Penny, G. D., Kay, G. F., Sheardown, S. A., Rastan, S., & Brockdorff, N. (1996). Requirement for *Xist* in X chromosome inactivation. *Nature*, *379*, 131–137.
- Rastan, S. (1983). Non-random X-chromosome inactivation in mouse X-autosome translocation embryos—location of the inactivation centre. *Journal of Embryology and Experimental Morphology*, *78*, 1–22.
- Sado, T., Wang, Z., Sasaki, H., & Li, E. (2001). Regulation of imprinted X-chromosome inactivation in mice by *Tsix*. *Development*, *128*, 1275–1286.
- Sakata, Y., Nagao, K., Hoki, Y., Sasaki, H., Obuse, C., & Sado, T. (2017). Defects in dosage compensation impact global gene regulation in the mouse trophoblast. *Development*, *144*, 2784–2797.
- Wutz, A., Rasmussen, T. P., & Jaenisch, R. (2002). Chromosomal silencing and localization are mediated by different domains of *Xist* RNA. *Nature Genetics*, *30*, 167–174.

Embryo Transport

Sierra L Olsen, Shuai Li, and Wipawee Winuthayanon, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Axoneme The central strand of a cilium or flagellum. It is composed of an array of microtubules; typically in nine pairs around two single central ones.

Active stroke The propulsive movement of cilia beating, it involves the entire cilia. Different from basal stroke, which starts from the basal root and propagates like a wave throughout cilia.

Apical membrane The plasma membrane faces to the lumen in a polarized cell.

Basal foot Is an extension of cytoplasmic process from the microtubule-organizing center at the base of the cilia. It determines the direction of cilia beating.

Cilium A hair like organelle that extends outside of the cell.

Ectopic pregnancy A medical condition when an embryo is implanted outside of the uterine cavity. The most common site for this to occur is inside the Fallopian tube.

Estrogen A major sex steroid hormone produced primarily by ovaries. It is responsible for reproductive development.

Progesterone A major sex steroid hormone produced primarily by ovaries. It is responsible for inducing physiological changes to maintain a successful pregnancy.

Prostaglandins Small lipids that are found in most animal tissues and play many physiological roles.

Transudation Passage of the fluid through cell membrane as a result of osmotic pressure.

Transgenic mice Genetically engineered mice used to study genetic functions.

Introduction

The oviduct (or the Fallopian tube in humans) is a trumpet-like structure, that connects the ovary and the uterus, and is the sole site of embryo transport. Embryo transport is the process by which an embryo travels from the site of fertilization, in the ampulla of the oviduct, to the implantation site in the uterus. In humans, embryo transport is difficult to study because it occurs during the first four days of pregnancy before women even know they are pregnant. A large majority of the information known about embryo transport in humans is inferred from studies in animals. Historically, embryo transport is thought to be a passive process, in which the oviduct's role is little more than a path for the embryo to follow. Over the years it has become more and more apparent that embryo transport is much more complex than initially assumed.

It is proposed that a variety of factors can affect embryo transport, including oviductal muscle contractions, ciliary activities, and fluid secretions. All of these mechanisms work together to increase, decrease, or halt the embryos movement, ensuring the delivery of the embryo into the uterus at precisely the right time for successful implantation. Variations in any of these processes can lead to the embryo arriving in the uterus at the wrong time, resulting in an unsuccessful pregnancy.

The amount of time the embryo spends in the oviduct varies between species. For example, human embryos spend approximately 80 h in the Fallopian tube (Croxatto, 2002). In rodents, the embryo travels swiftly through the ampulla to the isthmus where migration slows, and the embryo stays in the isthmus for about 3 days. Pig embryos spend 1–3 days (Oxenreider and Day, 1965), cow embryos 2–4 days (Crisman et al., 1980), sheep embryos 2–3 days, and horse embryos spend 5–6 days in the oviduct (Betteridge et al., 1982). For other species, refer to a comprehensive review by Croxatto (2002). The mechanisms for moving embryos into the uterus are considered to be a combination of oviduct ciliary activity, oviductal fluid flow, and waves of muscle contraction and relaxation all mainly orchestrated by the sex steroid hormones, estrogen (E_2) and progesterone (P_4) (reviewed in Li and Winuthayanon, 2017).

Factors Affecting Embryo Transport

Role of Sex Steroid Hormones During Embryo Transport

E_2 and P_4 play an essential role in regulating many physiological functions of our body. E_2 and P_4 initiate the signaling transduction pathway through their receptors, altering downstream gene expression, and modulating cellular behavior to change the speed of embryo transport. Specifically, three main factors regulating embryo transport are controlled by E_2 and P_4 : (1) muscle contraction, (2) the beat frequency of ciliated cells, and (3) the fluid volume in the tubal lumen. E_2 induces these factors to accelerate the embryo transport. In most circumstances, P_4 opposes E_2 action by suppressing E_2 signaling pathways; hence balancing E_2 activity (Fig. 1). Later sections will explain detailed mechanisms based on the three components of embryo transport.

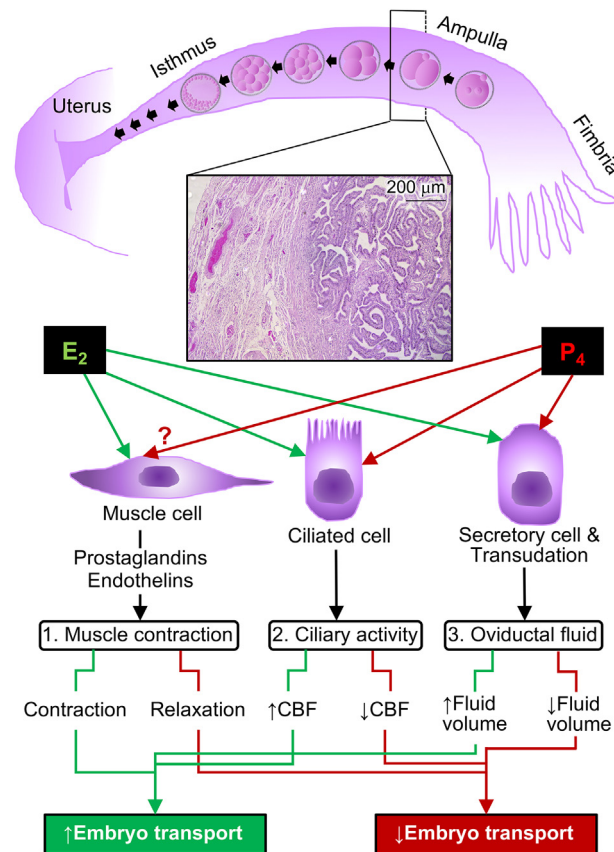


Fig. 1 An illustration of the action of E_2 and P_4 on embryo transport. E_2 increases muscle contractility, CBF, and oviductal fluid volume while P_4 opposes E_2 's action. The pathways regulating muscle relaxation through P_4 still remain unclear. Human Fallopian tube obtained from Dr. Ken-Ichi Takemaru (Stony Brook University, School of Medicine) with institutional ethics approval.

Muscle contractions

Fertilization occurs in the ampullary region of the oviduct. Two smooth muscle layers surround the ampulla and isthmus: an inner circular and the outer longitudinal layers. The contractility of these smooth muscle layers oscillates over the course of the ovarian cycle. In many species, female sex steroid hormones regulate muscle contraction in the oviduct, but there is still speculation as to whether oviductal muscle contractility plays a role in embryo transport. For example, inhibition of muscle activity in the ampullary region of rats and rabbits via a β -adrenergic agonist does not affect egg transport (Halbert et al., 1976, 1989).

As for hormonal regulation of muscle contractility, E_2 increases muscle contraction while P_4 suppresses it (Lindblom et al., 1980; Helm et al., 1982). When circulating level of E_2 peaks, luteinizing hormone (LH) is released. In pigs, E_2 promotes the expression of LH receptor on the smooth muscle and induces oviductal muscle relaxation (Gawronska et al., 1999). In addition, E_2 increases the expression of prostanoid receptors (EP2, EP4, and FP) in the bovine oviductal smooth muscle (Huang et al., 2015). When activated by prostaglandins (PGs), EP2 and FP have a contractile effect while EP4 has a relaxing effect. In humans, PGE_2 and $PGF_{2\alpha}$ are present in the circular and longitudinal smooth muscle of the Fallopian tube (Lindblom et al., 1978). Treatment with PGE_2 and $PGF_{2\alpha}$ induces an increase in the muscle contraction of the human Fallopian tube (Wanggren et al., 2006, 2008). In summary, it appears that the action of E_2 on oviductal smooth muscle contraction is due to the signaling activated by PGs.

P_4 signaling suppresses E_2 -induced oviductal smooth muscle contraction, but the exact mechanism remains unclear. In cow and mouse oviduct, progesterone receptor (PGR) is present in both epithelial and smooth muscle cells (Valle et al., 2007; Ismail et al., 2002). Contraction of smooth muscle in the colon is inhibited through P_4 -mediated increased intracellular cAMP level (Cheng et al., 2010), therefore, it is possible that a similar mechanism of smooth muscle contraction may apply in the oviduct.

Non-hormonal signaling pathways in the oviduct influencing muscle contraction include endothelin, prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX2), hydrogen sulfate, and cannabinoid receptor signaling pathways.

Endothelin is a short amino-acid peptide that directly affects embryo transport by increasing muscle contractility (Shihoya et al., 2016; Kobayashi et al., 2016). In rats and cows, endothelin receptor A (ET_A) is expressed in the isthmus region of the oviduct epithelial cells (Rosselli et al., 1994; Al-Alem et al., 2007). Its role in the ovary and oviduct is reviewed by Bridges et al. (2011).

E_2 is also shown to increase expression of COX2, an enzyme responsible for catalysis of prostaglandin precursor proteins. In rats, treatment with E_2 stimulates the secretion of COX2 into the lumen of the oviduct (Perez Martinez et al., 2006). In cows, COX2 is expressed at the highest level during the follicular and postovulatory phases. This intermediate enzyme indirectly regulates tubal muscle contraction impacting embryo transport (Oda et al., 2006).

Hydrogen sulfate (H_2S) is a significant vasodilator and smooth muscle relaxant in mammals. In humans, H_2S relaxes the spontaneous muscle contraction of the oviduct. Dysregulation of H_2S leads to a delay in embryo transport and development (Ning et al., 2014).

Cannabinoid signaling is also involved in muscle contractility and is proposed as a pathway that prevents prolonged embryo retention in the oviduct. Genetic knockout mice, as well as chemical inhibition, of the cannabinoid receptors resulted in unsuccessful implantation due to an embryo transport defect (Wang et al., 2004).

In addition to facilitating the embryo transport, muscle contraction at junctions of the oviduct can also detain underdeveloped embryos. Human embryos spend majority of their time in the ampulla region of the Fallopian tube and retain at the ampullary-isthmic junction (AIJ) region for couple days, followed by a quick progression through the isthmus (Croatto, 2002).

The last stop for the embryo before entering the uterus is the utero-tubal junction (UTJ). Inflammation or anatomical defects of this junction caused by a mutation in the *Dicer* gene can lead to a transport failure (Nagaraja et al., 2008; Gonzalez and Behringer, 2009). However, in rabbits, removal of the AIJ and the UTJ did not change implantation rate (Eddy et al., 1977; Perez et al., 1981), indicating that embryo transport is not affected by the presence or absence of these two junctions in this species. Removal of a portion of the isthmus also has no effect on embryo transport (Land et al., 1987; McComb et al., 1981). Together, these studies suggest that the presence of a sphincter in the AIJ and isthmic region of the oviduct are not required for normal embryo transport, at least in rabbits.

Ciliary activity

The oviduct epithelium is populated by ciliated cells and nonciliated secretory cells. The majority of the ciliated cells are in the infundibulum and ampulla regions of the oviduct. These cells have hair-like projections known as cilia, extending out of the apical membrane of the cell towards the lumen of the oviduct. Cilia contain axonemes that enable ciliary beating (Fig. 2A). The axoneme is made up of a bundle of microtubules wherein two singlet microtubules are encircled by nine doublets (Fig. 2B). Attached to one of the tubules in a doublet is a protein called dynein, which drives ciliary movement by reaching to a tubule in the adjacent doublet (Raedt et al., 2015). Ciliary beating serves to sweep mucus and other debris off the epithelium of many organs including the respiratory tract and the oviduct (Lodish, 2016). Motile cilia of the oviductal epithelium hydrolyze ATP to create a microtubule movement, generating beat, producing a current, which helps to move the embryo towards the uterus. Changes in the characteristics of the cilia such as orientation and ciliary beat frequency (CBF) can drastically alter oviductal fluid flow, and thus affect embryo transport.

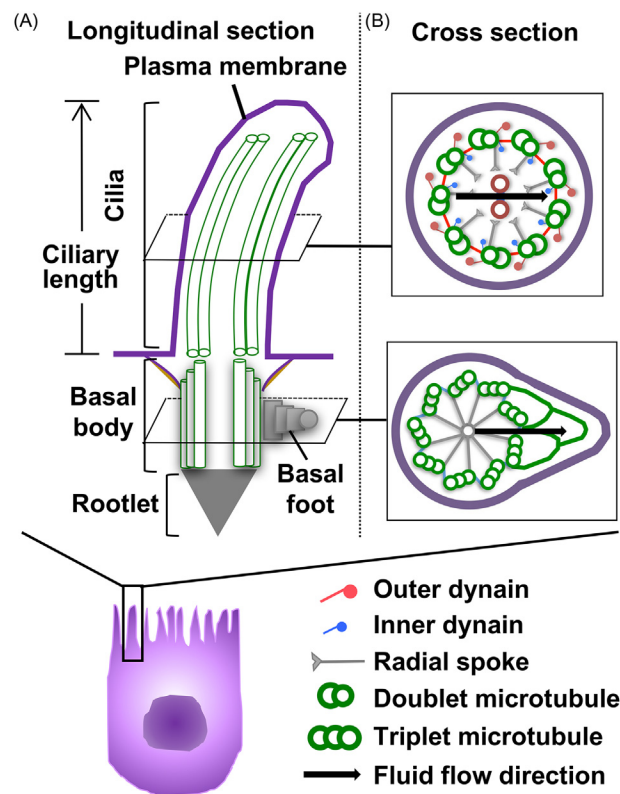


Fig. 2 An illustration of cilia structure. (A) A longitudinal section of the cilia. (B) Cross-sections of the cilia. Arrows in (B) showing the direction of the active stroke of cilia. This direction is organized by the direction of the basal foot and the orientation of axoneme.

Ciliary orientation

The direction of oviductal fluid flow is from the ovary towards the uterus in most studied mammals, with the exception of the pig, wherein the flow in the isthmus is from ovary to uterus (Gaddum-Rosse and Blandau, 1976). The direction of fluid flow is coupled with the orientation of the cilia as seen by the change of mucociliary flow in response to surgical reversal of a segment of ciliated tissue. The cilia are oriented in the direction that the basal foots are pointed to (Fig. 2B). The basal foot designates the direction of active stroke of the ciliary beating. Schatz and colleagues showed that the mucus transport relies on the synchronous alignment of each cilium in relation to its neighbor (Schatz et al., 2013). In the rabbit, surgical reversal of ampulla delayed embryo transport whereas reversal of isthmus did not, suggesting that the direction of cilia beat modulates embryo transport (Nagasaka, 1984; Land et al., 1987; Eddy et al., 1978). A recent study shows that *Celsr1* (Cadherin EGF LAG Seven-Pass G-Type Receptor 1) gene is required for oviduct cilia orientation as a loss of *Celsr* expression leads to abnormal cilia polarity and random directions of beating (Shi et al., 2014). These findings indicate that the orientation of the ciliary beat governs the direction of the fluid flow.

Ciliary beat frequency

Ciliary beat frequency (CBF) is an indicator of mucociliary function. The CBF in the oviduct remains relatively constant, whilst adjusting in response to changes in fluid viscosity and content. The ability to prevent the collapse of mucociliary transport under fluctuating viscosities is called autoregulation. When challenged with viscous loads, cilia auto-regulate by slowing CBF to a frequency that can be maintained. The ability to auto-regulate is a result of a member of the transient receptor potential (TRP) family, TRPV-4, present in the cilia (Andrade et al., 2005). Calcium has been shown to increase CBF in the airway and oviductal epithelium (Lorenzo et al., 2008; Fernandes et al., 2008; Teilmann et al., 2005). Additionally, activation of TRPV-4 results in an influx of calcium into the cell, hence increasing the CBF. The effect of calcium on increasing CBF reaches the maximum at 350 nM concentration in vitro (Lansley and Sanderson, 1999).

Besides viscosity, CBF is also regulated by sex steroid hormones. P_4 represses CBF, while E_2 opposes the action of P_4 (Mahmood et al., 1998). Classical and membrane progesterone receptors (PGRs and mPGRs) are expressed in human and mouse oviduct (Teilmann et al., 2006; Nutu et al., 2009). The rapid reduction of CBF in response to P_4 is mediated through PGRs (Bylander et al., 2013; Bylander et al., 2010). Ciliated cell function can also be modulated by E_2 signal as CBF is reduced when estrogen receptor α (ESR1) is absent from mouse oviductal epithelial cells (Li et al., 2017). PGE_2 also increases CBF in rabbit and hamster oviduct by modulating calcium influx (Hermoso et al., 2001; Verdugo, 1980). In vitro studies have shown that $PGF_{2\alpha}$ activates its receptor, resulting in the release of intracellular calcium. Increased intracellular calcium levels alternate electrical membrane potential, and subsequently increase CBF (Villalon et al., 1989). Additionally, an inflammatory cytokine, interleukin-6 (IL-6), at high concentration decreases the CBF in human Fallopian tubes (Papathanasiou et al., 2008). These findings suggest that the CBF of the oviductal ciliated cells can be directly regulated by P_4 , E_2 , and other factors such as PGs and IL-6.

Oviductal fluid

Oviducts are filled with tubal fluid that not only nourishes and protects the pre-implantation embryo, it also aids in embryo transport to the implantation site. The rate of fluid flow is determined by variations in fluid volume as well as protein content and concentration. These variations are a result of excretions from the secretory cells of the oviductal epithelium, as well as selective diffusion of plasma from the adjacent artery, called "transudation" (Fig. 1) (Leese, 1988).

The volume of oviductal fluid varies between species and between each stage of estrous cycle within a species. In mammals, oviductal fluid production is highest at estrus and lowest at diestrus (Leese et al., 2001). Studies in many different species have shown that oviductal fluid volume increases in response to E_2 and decreases in response to P_4 . It is likely that E_2 and P_4 affect fluid volume by altering the gene expression level of aquaporins (AQPs) on the oviductal epithelium (Branes et al., 2005). AQPs allow water to be transported across the plasma membrane, thus, changes in their expression alter water availability in the oviduct. Expression of AQP1, AQP5, AQP8, and AQP9 have been detected in the oviduct of several species, including humans, pigs, rats, and mice (Huang et al., 2006; Skowronski et al., 2011; Branes et al., 2005). In pigs, AQP1, AQP5, and AQP9 protein expression fluctuates in response to the estrous cycle, reaching their peak expression when E_2 level is elevated. Similarly in rats, expression of AQP9 in the oviductal epithelium is highest at proestrus and estrus. AQPs expression levels coincide with a high oviductal fluid volume in the oviduct, which may explain their role in water transport to the oviductal lumen during proestrus and estrus. Taken together, these findings implicate that steroid hormones, E_2 and P_4 , are the underlying regulators of fluid volume fluctuation in the oviduct in part by altering water transport channels.

Oviductal proteins

While E_2 and P_4 work together to control oviductal fluid volume, they also regulate protein secretion from the secretory cells of the oviductal epithelium. These secreted proteins, either directly or indirectly facilitate embryo transport.

Among all oviductal proteins, mucins and uteroglobins maintain the process of embryo transport by preventing embryo implantation in the oviduct. This implantation of the embryo outside the uterus is one of the pregnancy complications called ectopic pregnancy. In the oviduct, mucins are large glycoproteins that act to lubricate the oviductal lumen with antimicrobial properties (Lagow et al., 1999). Mucin 1, in particular, is a large cell surface protein that inhibits cellular adhesion (Al-Azemi et al., 2009). It acts as a barrier between preimplantation embryos and the oviduct, preventing premature implantation (Aplin, 1999). Another molecule that directly impacts embryo transport is uteroglobin, which is a small protein expressed in the human Fallopian tube. Women with pelvic inflammatory disease (PID) have been associated with reduced levels of uteroglobin, specifically

at the ectopic implantation site in the Fallopian tube (Quintar et al., 2008). These findings suggest that mucins and uteroglobin may be necessary to prevent implantation of the embryo within the Fallopian tube.

Common Risk Factors Impair Embryo Transport

Some environmental and genetic factors can impair embryo transport. Studies have shown cigarette smoking slows muscle contraction and decreases CBF, causing a reduction in embryo transport. These physiological changes impair oocyte pick-up in the oviduct and lead to reduced fertility (Riveles et al., 2007; Knoll et al., 1995; Gieseke and Talbot, 2005; Knoll and Talbot, 1998). In vitro fertilization (IVF) patients, cigarette smoking also induces a much higher rate of miscarriage and ectopic pregnancy (Waylen et al., 2009). Other factors that also affect fertility are environmental contaminants, especially endocrine disruptors such as bisphenol A and S (BPA and BPS), and their chemical derivatives. These chemicals are common in our daily lives and act as weak ligands for ESRs. In the female reproductive system, BPA and BPS affect the functions of the oviducts, uterus, and ovaries, potentially inducing fertility defect (Ziv-Gal and Flaws, 2016). Treatment of BPA to pregnant mice causes embryo retention in the oviduct (Xiao et al., 2011). The pesticide, methoxychlor (MXC), is another type of endocrine disruptor that accelerates embryo transport in the oviduct and causes pre-implantation embryonic loss (Cummings and Perreault, 1990). These works indicate that environmental toxicants disrupt the physiological function of the oviduct and lead to impaired embryo transport.

Genetic disorders such as primary ciliary dyskinesia (PCD) also reduces fertility. PCD is a condition where the physical structure of the cilia dynein arms is distorted, impairing ciliary beating (Zariwala et al., 2007). Women with PCD have a higher risk for ectopic pregnancy and subfertility (Halbert et al., 1997). Genetic mutations of dynein axonemal light chain 1 (*DNAL1*) and dynein axonemal heavy chain 5 (*DNAH5*) in humans cause a defect of the outer dynein arm of the cilia (Zariwala et al., 2007). Structural abnormalities in the cilia lead to dysfunctional ciliary activity, prolonging embryo transport and resulting in ectopic pregnancy (McComb et al., 1986). In summary, mutations of genes involved in ciliary function and exposure to environmental toxicants can lead to ciliary damage, contributes to ectopic pregnancy and reduced fertility.

Conclusion

The oviduct is responsible for gamete and embryo transport. Its role is pivotal for precise timing of implantation. To achieve successful embryo transport, the oviduct utilizes three mechanisms: muscle contraction, ciliary beating, and oviductal fluid. These mechanisms are largely regulated by sex hormones, E_2 and P_4 , in which E_2 increases the overall speed of transport and P_4 decreases transport speed. Embryo retention and prolonged embryo transport can lead to ectopic pregnancy, which can be life-threatening to the mothers. Early exit of the embryos from Fallopian tubes could also lead to failed embryo implantation.

References

- Al-Alem, L., Bridges, P. J., Su, W., et al. (2007). Endothelin-2 induces oviductal contraction via endothelin receptor subtype A in rats. *Journal of Endocrinology*, 193, 383–391.
- Al-Azemi, M., Refaat, B., Aplin, J., & Ledger, W. (2009). The expression of MUC1 in human fallopian tube during the menstrual cycle and in ectopic pregnancy. *Human Reproduction*, 24, 2582–2587.
- Andrade, Y. N., Fernandes, J., Vazquez, E., et al. (2005). TRPV4 channel is involved in the coupling of fluid viscosity changes to epithelial ciliary activity. *Journal of Cell Biology*, 168, 869–874.
- Aplin, J. D. (1999). MUC-1 glycosylation in endometrium: Possible roles of the apical glycocalyx at implantation. *Human Reproduction*, 14(Suppl. 2), 17–25.
- Betteridge, K. J., Eaglesome, M. D., Mitchell, D., et al. (1982). Development of horse embryos up to twenty two days after ovulation: Observations on fresh specimens. *Journal of Anatomy*, 135, 191–209.
- Branes, M. C., Morales, B., Rios, M., & Villalon, M. J. (2005). Regulation of the immunoexpression of aquaporin 9 by ovarian hormones in the rat oviductal epithelium. *American Journal of Physiology. Cell Physiology*, 288, C1048–57.
- Bridges, P. J., Cho, J., & Ko, C. (2011). Endothelins in regulating ovarian and oviductal function. *Frontiers in Bioscience (Scholar Edition)*, 3, 145–155.
- Bylander, A., Lind, K., Goksor, M., et al. (2013). The classical progesterone receptor mediates the rapid reduction of fallopian tube ciliary beat frequency by progesterone. *Reproductive Biology and Endocrinology*, 11, 33.
- Bylander, A., Nutu, M., Wellander, R., et al. (2010). Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube. *Reproductive Biology and Endocrinology*, 8, 48.
- Cheng, L., Biancani, P., & Behar, J. (2010). Progesterone receptor A mediates VIP inhibition of contraction. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, 298, G433–9.
- Crisman, R. O., McDonald, L. E., & Wallace, C. E. (1980). Oviduct (uterine tube) transport of ova in the cow. *American Journal of Veterinary Research*, 41, 645–647.
- Croxatto, H. B. (2002). Physiology of gamete and embryo transport through the fallopian tube. *Reproductive Biomedicine Online*, 4, 160–169.
- Cummings, A. M., & Perreault, S. D. (1990). Methoxychlor accelerates embryo transport through the rat reproductive tract. *Toxicology and Applied Pharmacology*, 102, 110–116.
- Eddy, C. A., Antonini, R., Jr., & Pauerstein, C. J. (1977). Fertility following microsurgical removal of the ampullary-isthmic junction in rabbits. *Fertility and Sterility*, 28, 1090–1093.
- Eddy, C. A., Flores, J. J., Archer, D. R., & Pauerstein, C. J. (1978). The role of cilia in fertility: An evaluation by selective microsurgical modification of the rabbit oviduct. *American Journal of Obstetrics and Gynecology*, 132, 814–821.
- Fernandes, J., Lorenzo, I. M., Andrade, Y. N., et al. (2008). IP3 sensitizes TRPV4 channel to the mechano- and osmotransducing messenger 5'-6'-epoxyeicosatrienoic acid. *Journal of Cell Biology*, 181, 143–155.
- Gaddum-Rosse, P., & Blandau, R. J. (1976). Comparative observations on ciliary currents in mammalian oviducts. *Biology of Reproduction*, 14, 605–609.
- Gawronska, B., Paukku, T., Huhtaniemi, I., et al. (1999). Oestrogen-dependent expression of LH/hCG receptors in pig fallopian tube and their role in relaxation of the oviduct. *Journal of Reproduction and Fertility*, 115, 293–301.
- Gieseke, C., & Talbot, P. (2005). Cigarette smoke inhibits hamster oocyte pickup by increasing adhesion between the oocyte cumulus complex and oviductal cilia. *Biology of Reproduction*, 73, 443–451.

- Gonzalez, G., & Behringer, R. R. (2009). Dicer is required for female reproductive tract development and fertility in the mouse. *Molecular Reproduction and Development*, 76, 678–688.
- Halbert, S. A., Becker, D. R., & Szal, S. E. (1989). Ovum transport in the rat oviductal ampulla in the absence of muscle contractility. *Biology of Reproduction*, 40, 1131–1136.
- Halbert, S. A., Patton, D. L., Zarutskie, P. W., & Soules, M. R. (1997). Function and structure of cilia in the fallopian tube of an infertile woman with Kartagener's syndrome. *Human Reproduction*, 12, 55–58.
- Halbert, S. A., Tam, P. Y., & Blandau, R. J. (1976). Egg transport in the rabbit oviduct: The roles of cilia and muscle. *Science*, 191, 1052–1053.
- Helm, G., Owman, C., Sjöberg, N. O., & Walles, B. (1982). Motor activity of the human fallopian tube in vitro in relation to plasma concentration of oestradiol and progesterone, and the influence of noradrenaline. *Journal of Reproduction and Fertility*, 64, 233–242.
- Hermoso, M., Barrera, N., Morales, B., et al. (2001). Platelet activating factor increases ciliary activity in the hamster oviduct through epithelial production of prostaglandin E2. *Pflügers Archiv*, 442, 336–345.
- Huang, H. F., He, R. H., Sun, C. C., et al. (2006). Function of aquaporins in female and male reproductive systems. *Human Reproduction Update*, 12, 785–795.
- Huang, N., Liu, B., Dong, Z., et al. (2015). Prostanoid receptors EP2, EP4, and FP are regulated by estradiol in bovine oviductal smooth muscle. *Prostaglandins & Other Lipid Mediators*, 121, 170–175.
- Ismail, P. M., Li, J., Demayo, F. J., et al. (2002). A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. *Molecular Endocrinology*, 16, 2475–2489.
- Knoll, M., Shaoulian, R., Magers, T., & Talbot, P. (1995). Ciliary beat frequency of hamster oviducts is decreased in vitro by exposure to solutions of mainstream and sidestream cigarette smoke. *Biology of Reproduction*, 53, 29–37.
- Knoll, M., & Talbot, P. (1998). Cigarette smoke inhibits oocyte cumulus complex pick-up by the oviduct in vitro independent of ciliary beat frequency. *Reproductive Toxicology*, 12, 57–68.
- Kobayashi, Y., Yoshimoto, Y., Yamamoto, Y., et al. (2016). Roles of EDNs in regulating oviductal NO synthesis and smooth muscle motility in cows. *Reproduction*, 151, 615–622.
- Lagow, E., Desouza, M. M., & Carson, D. D. (1999). Mammalian reproductive tract mucins. *Human Reproduction Update*, 5, 280–292.
- Land, J. A., Evers, J. L., Boeckx, W. D., & Brosens, I. A. (1987). Ovum transport after microsurgical anastomosis of the rabbit oviduct. *Journal of Reproductive Medicine*, 32, 103–106.
- Lansley, A. B., & Sanderson, M. J. (1999). Regulation of airway ciliary activity by Ca^{2+} : Simultaneous measurement of beat frequency and intracellular Ca^{2+} . *Biophysical Journal*, 77, 629–638.
- Leese, H. J. (1988). The formation and function of oviduct fluid. *Journal of Reproduction and Fertility*, 82, 843–856.
- Leese, H. J., Tay, J. I., Reischl, J., & Downing, S. J. (2001). Formation of fallopian tubal fluid: Role of a neglected epithelium. *Reproduction*, 121, 339–346.
- Li, S., O'Neill, S. R., Zhang, Y., et al. (2017). Estrogen receptor alpha is required for oviductal transport of embryos. *FASEB Journal*, 31, 1595–1607.
- Li, S., & Winuthayanon, W. (2017). Oviduct: Roles in fertilization and early embryo development. *Journal of Endocrinology*, 232, R1–R26.
- Lindblom, B., Hamberger, L., & Ljung, B. (1980). Contractile patterns of isolated oviductal smooth muscle under different hormonal conditions. *Fertility and Sterility*, 33, 283–287.
- Lindblom, B., Hamberger, L., & Wikqvist, N. (1978). Differentiated contractile effects of prostaglandins E and F on the isolated circular and longitudinal smooth muscle of the human oviduct. *Fertility and Sterility*, 30, 553–559.
- Lodish, H. F. (2016). *Molecular cell biology*. New York: W.H. Freeman-Macmillan Learning.
- Lorenzo, I. M., Liedtke, W., Sanderson, M. J., & Valverde, M. A. (2008). TRPV4 channel participates in receptor-operated calcium entry and ciliary beat frequency regulation in mouse airway epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 12611–12616.
- Mahmood, T., Saridogan, E., Smutna, S., et al. (1998). The effect of ovarian steroids on epithelial ciliary beat frequency in the human fallopian tube. *Human Reproduction*, 13, 2991–2994.
- McComb, P., Langley, L., Villalon, M., & Verdugo, P. (1986). The oviductal cilia and Kartagener's syndrome. *Fertility and Sterility*, 46, 412–416.
- McComb, P. F., Newman, H., & Halbert, S. A. (1981). Reproduction in rabbits after excision of the oviductal isthmus, ampullary-isthmic junction, and uteroisthmic junction. *Fertility and Sterility*, 36, 669–677.
- Nagaraja, A. K., Andreu-Vieyra, C., Franco, H. L., et al. (2008). Deletion of dicer in somatic cells of the female reproductive tract causes sterility. *Molecular Endocrinology*, 22, 2336–2352.
- Nagasaka, T. (1984). Effect of microsurgical ampullary segmental reversal on fertilization in the rabbit. *Nihon Sanka Fujinka Gakkai Zasshi*, 36, 2155–2160.
- Ning, N., Zhu, J., Du, Y., et al. (2014). Dysregulation of hydrogen sulphide metabolism impairs oviductal transport of embryos. *Nature Communications*, 5, 4107.
- Nutu, M., Weijdegard, B., Thomas, P., et al. (2009). Distribution and hormonal regulation of membrane progesterone receptors beta and gamma in ciliated epithelial cells of mouse and human fallopian tubes. *Reproductive Biology and Endocrinology*, 7, 89.
- Odau, S., Gabler, C., Holder, C., & Einspanier, R. (2006). Differential expression of cyclooxygenase 1 and cyclooxygenase 2 in the bovine oviduct. *Journal of Endocrinology*, 191, 263–274.
- Oxenreider, S. L., & Day, B. N. (1965). Transport and cleavage of ova in swine. *Journal of Animal Science*, 24, 413–417.
- Papathanasiou, A., Djahanbakhch, O., Saridogan, E., & Lyons, R. A. (2008). The effect of interleukin-6 on ciliary beat frequency in the human fallopian tube. *Fertility and Sterility*, 90, 391–394.
- Perez, L., Rajkumar, K., & Eddy, C. A. (1981). Fertility and ovum transport after microsurgical removal of the uterotubal junction in rabbits. *Fertility and Sterility*, 36, 803–807.
- Perez Martinez, S., Hermoso, M., Farina, M., et al. (2006). 17-Beta-estradiol upregulates COX-2 in the rat oviduct. *Prostaglandins & Other Lipid Mediators*, 80, 155–164.
- Quintar, A. A., Mukdsi, J. H., Del Valle Bonaterza, M., et al. (2008). Increased expression of uteroglobin associated with tubal inflammation and ectopic pregnancy. *Fertility and Sterility*, 89, 1613–1617.
- Raidt, J., Werner, C., Menchen, T., et al. (2015). Ciliary function and motor protein composition of human fallopian tubes. *Human Reproduction*, 30, 2871–2880.
- Riveles, K., Tran, V., Roza, R., et al. (2007). Smoke from traditional commercial, harm reduction and research brand cigarettes impairs oviductal functioning in hamsters (*Mesocricetus auratus*) in vitro. *Human Reproduction*, 22, 346–355.
- Rosselli, M., Imthurn, B., Macas, E., & Keller, P. J. (1994). Endothelin production by bovine oviduct epithelial cells. *Journal of Reproduction and Fertility*, 101, 27–30.
- Schatz, G., Schneiter, M., Ricka, J., et al. (2013). Ciliary beating plane and wave propagation in the bovine oviduct. *Cells, Tissues, Organs*, 198, 457–469.
- Shi, D., Komatsu, K., Hirao, M., et al. (2014). Celsr1 is required for the generation of polarity at multiple levels of the mouse oviduct. *Development*, 141, 4558–4568.
- Shihoya, W., Nishizawa, T., Okuta, A., et al. (2016). Activation mechanism of endothelin ETB receptor by endothelin-1. *Nature*, 537, 363–368.
- Skowronski, M. T., Skowronska, A., & Nielsen, S. (2011). Fluctuation of aquaporin 1, 5, and 9 expression in the pig oviduct during the estrous cycle and early pregnancy. *Journal of Histochemistry and Cytochemistry*, 59, 419–427.
- Teilmann, S. C., Byskov, A. G., Nogueira, P. A., et al. (2005). Localization of transient receptor potential ion channels in primary and motile cilia of the female murine reproductive organs. *Molecular Reproduction and Development*, 71, 444–452.
- Teilmann, S. C., Clement, C. A., Thorup, J., et al. (2006). Expression and localization of the progesterone receptor in mouse and human reproductive organs. *Journal of Endocrinology*, 191, 525–535.
- Valle, G. R., Cassali, G. D., Nogueira, J. C., et al. (2007). Nuclear estrogen and progesterone receptors in the oviduct of heifers under natural and superovulated estrous cycles. *Animal Reproduction Science*, 101, 28–37.
- Verdugo, P. (1980). Ca^{2+} -dependent hormonal stimulation of ciliary activity. *Nature*, 283, 764–765.
- Villalon, M., Hinds, T. R., & Verdugo, P. (1989). Stimulus-response coupling in mammalian ciliated cells. Demonstration of two mechanisms of control for cytosolic $[\text{Ca}^{2+}]$. *Biophysical Journal*, 56, 1255–1258.

- Wang, H., Guo, Y., Wang, D., et al. (2004). Aberrant cannabinoid signaling impairs oviductal transport of embryos. *Nature Medicine*, 10, 1074–1080.
- Wanggren, K., Lalitkumar, P. G., Stavreus-Evers, A., et al. (2006). Prostaglandin E2 and F2alpha receptors in the human fallopian tube before and after mifepristone treatment. *Molecular Human Reproduction*, 12, 577–585.
- Wanggren, K., Stavreus-Evers, A., Olsson, C., et al. (2008). Regulation of muscular contractions in the human fallopian tube through prostaglandins and progestagens. *Human Reproduction*, 23, 2359–2368.
- Waylen, A. L., Metwally, M., Jones, G. L., et al. (2009). Effects of cigarette smoking upon clinical outcomes of assisted reproduction: A meta-analysis. *Human Reproduction Update*, 15, 31–44.
- Xiao, S., Diao, H., Smith, M. A., et al. (2011). Preimplantation exposure to bisphenol a (BPA) affects embryo transport, preimplantation embryo development, and uterine receptivity in mice. *Reproductive Toxicology*, 32, 434–441.
- Zariwala, M. A., Knowles, M. R., & Omeran, H. (2007). Genetic defects in ciliary structure and function. *Annual Review of Physiology*, 69, 423–450.
- Ziv-Gal, A., & Flaws, J. A. (2016). Evidence for bisphenol A-induced female infertility: A review (2007–2016). *Fertility and Sterility*, 106, 827–856.

Further Reading

Detailed review in embryo transport in mammals

Croxatto, H. B. (2002). Physiology of gamete and embryo transport through the fallopian tube. *Reproductive Biomedicine Online*, 4, 160–169.

Detailed review in the roles of oviducts during fertilization and embryo development

Coy, P., & Yanagimachi, R. (2015). The common and species-specific roles of oviductal proteins in mammalian fertilization and embryo development. *Bioscience*, 65, 973–984.

Li, S., & Winuthayanon, W. (2017). Oviduct: Roles in fertilization and early embryo development. *Journal of Endocrinology*, 232, R1–R26.

Detailed review in ciliated cells in human Fallopian tubes

Lyons, R. A., Saridogan, E., & Djahanbakhch, O. (2006). The reproductive significance of human fallopian tube cilia. *Human Reproduction Update*, 12, 363–372.

Detailed review in ectopic pregnancy

Shaw, J. L., Dey, S. K., Critchley, H. O., & Horne, A. W. (2010b). Current knowledge of the aetiology of human tubal ectopic pregnancy. *Human Reproduction Update*, 16, 432–444.

Detailed review in smoking and IVF outcome

Waylen, A. L., Metwally, M., Jones, G. L., Wilkinson, A. J., & Ledger, W. L. (2009). Effects of cigarette smoking upon clinical outcomes of assisted reproduction: A meta-analysis. *Human Reproduction Update*, 15(1), 31–44.

Origin and Development of Primordial Germ Cells

Massimo De Felici, University of Rome Tor Vergata, Rome, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

The establishment of germline is a process that has always fascinated the scientists. In mammals, the first observations about the putative precursors of the sperm and oocytes, termed primordial germ cells (PGCs), in the wall of the yolk sac of human embryo around 3rd week of gestation, go back to little more than one hundred years ago. From then, the route of mammalian PGCs toward the gonadal primordia inside the embryo proper, has been traced and some of developmental processes of these cells ending with their differentiation into oogonia or prospermatogonia within the developing gonads described. However, only from the eighties years onwards, thanks to development of methods for isolation, precise identification and in vitro culture of mouse PGCs together with the characterization of natural mutations and the use of transgenic animals, important information about the molecular mechanisms underlying these processes has been obtained. More recently, the use of in vitro models for human PGC-like cell specification is providing basic insights into such processes also in the human embryo.

Specification and Determination of Primordial Germ Cells

In the mouse embryo, putative PGCs can be first localized as a small population of about 40 cells positive for alkaline phosphatase at the base of the allantois in the extraembryonic mesoderm at around 7.25 *days post coitum* (dpc) (Ginsburg et al., 1990). Such cells were subsequently shown to originate from a few (about six) of the most proximal epiblast cells, immediately adjacent to the extraembryonic ectoderm (Lawson and Hage, 1994).

From these early observations, several in vivo and in vitro studies, mainly performed in the mouse, extensively reviewed by De Felici (2013, 2016), focused on local molecules able to induce germ cell specification (cell commitment toward a definite fate that can be reversed or transformed to another fate) and subsequently their determination (irreversible commitment toward a definite fate). These showed that prior to and at the beginning of gastrulation, growth factors known as bone morphogenic proteins (BMPs) secreted from the extraembryonic ectoderm (BMP4, BMP8b) and visceral endoderm (BMP2), ultimately converge on proximal epiblast cells to promote PGC specification. Signaling by the wntless and int 3 (WNT3) protein originating from the posterior visceral endoderm and epiblast itself, conferred a receptive environment for proximal epiblast cells for BMP4 response. In fact, the PGC precursors appear to be initially driven toward a somatic mesodermal fate by WNT3, to regain subsequently a latent pluripotency typical of PGCs. The transcription factor B lymphocyte-induced maturation protein 1 (BLIMP1, also known as PR domain zinc finger protein 1, PRDM1), is downstream BMP4 induction, and marks the origin of the mouse PGC precursors around 6.25 dpc. Shortly after, the expression of PRDM14, another transcription factor of the same family, further contributes to PGC specification. The RNA-binding protein LIN28 appears to play a role as an upstream regulator of the BLIMP1 transcript translation whereas one more transcription factor, the activating enhancer binding protein 2γ (AP-2γ), is probably downstream BLIMP1. Sal-like protein 4 (SALL4), cooperates with BLIMP1 in suppressing the somatic cell program (in particular, the expression of *Hox* genes), while PRDM14 is likely involved in reactivating pluripotency in the nascent PGCs (i.e., the expression of sex determining region Y-box 2, *Sox2*, gene).

The PGC precursors increase in number and move toward the posterior region of the embryo. During this phase, other proteins involved in germline specification begin to be expressed. Among these *DPPA3* (developmental pluripotency associated protein 3, also known as Stella or PGC7), involved in epigenetic reprogramming, and the hypoxia-inducible factor-2α (HIF-2α), presumably contribute to regulate the expression of *Pou5f1* (also known as *Oct4*) gene in PGCs. The precise roles of *Oct4* and other key pluripotency genes such as *Sox2*, and *Nanog*, expressed at high level by the specified PGCs, remains to be clarified. Finally, mouse PGC determination is thought to occur in the extraembryonic mesoderm at the base of the allantois. This process is associated to high tissue nonspecific alkaline phosphatase (TNAP) activity, the expression of the tyrosine kinase receptor Kit and of the RNA-binding proteins, Nanos3, T-cell restricted intracellular antigen-1 related (TIAR), and dead-end protein homolog 1 (DND1). While the function of TNAP is unknown, all other proteins play a role in the survival and/or proliferation of the PGC during the subsequent migration and colonization of the gonads (for a review, see De Felici and Farini, 2012).

Fuss (1911, 1912) and Felix (1911) were probably the first to describe the extragonadal location of PGCs in human embryos. The place where PGCs were identified around the end of the 3rd week of gestation, is the same as in the mouse: the wall of the yolk sac at the angle with the allantois. The putative PGCs were distinguished by their large size, spherical shape, a prominent nucleolus, and the presence of abundant glycogen granules in the cytoplasm. Subsequently, histochemical methods for periodic acid-Schiff (PAS)-positive materials and alkaline phosphatase activity were applied to identify human PGCs (McKay et al., 1953). Only recently, the first gene and protein expression data of human PGCs at different stages of development were produced (Kobayashi et al., 2017 and references herein). Noteworthy, these studies show that gene expression patterns in early postmigrating human 7th–9th week PGCs are similar to mouse PGCs of comparable stages. Some important differences have been, however, described. In particular, while the *Sox2* gene is essential for mouse PGC development, its human counterpart is not expressed in PGCs which instead express *SOX17*. Moreover, human PGCs do not express significant level of PRDM14, while they express Kruppel-like factor

4 (*KLF4*), a transcription factor involved in pluripotency maintenance not expressed in mouse PGCs. Recently using the *Cynomolgus macaque*, PGCs were first identified in the amnios wall of the periimplantation embryo at 11 days of conception (Sasaki et al., 2016).

To compensate for the lack of information about the molecular mechanisms of PGC formation in the human embryo, in vitro culture systems based on the possibility to induce embryonic stem (ES) cells derived or induced pluripotent stem (iPS) cells to develop into specific cell lineages including the germline are now available (for a review, see De Felici, 2013). Using such models, it was shown that human ES and iPS cells possess a certain propensity to spontaneously differentiate into human PGC-like cells that can be increased with the addition to the culture medium of BMPs, the same growth factors governing the formation of mouse PGCs. Small changes in stem cell culture conditions or coculture onto human fetal gonad stromal cell, or mouse embryonic fibroblast monolayers in the presence of basic fibroblast growth factor (bFGF), have been also reported to favor the formation of hPGCLC. In addition, silencing the *DAZ* family and *NANOS3* genes in human ES cells resulted in a marked reduction in their capability to give rise to PGCLCs. Recently, defined and stepwise robust differentiation systems for inducing premigratory hPGCLC from iPS cells were reported (Sasaki et al., 2015).

Considering the available information and the different organization of the mouse and human embryos, a hypothetical model for the human PGC formation can be drawn (Fig. 1).

PGC Migration

Mouse PGCs are described to initiate active migration just after specification. Time-lapse photography documented that OCT4-GFP/AP positive cells actively migrated into the allantois/posterior embryo. Before PGCs begin this first step of their journey, however, they express factors associated with cell to cell adhesion such as interferon-inducing transmembrane proteins (IFITM) and E-cadherin. Shortly after PGCs arrive within the hindgut likely along morphogenetic movements, they are likely to receive signals that first promote random motility, and then induce directional migration toward the gonadal ridges. E-cadherin expression disappears in PGCs located in the hindgut, whereas hindgut epithelium expresses high levels of this adhesion protein. Together with decreased E-cadherin expression, receptor tyrosine kinase activity via Kit/KL interactions probably is a factor promoting PGC motility (Fig. 2). Besides Kit/KL activity, another receptor tyrosine kinase/ligand pair, the orphan receptor 2 (ROR2)/WNT5A, was found to favor polarization and elongation of PGCs; disruption of either components caused PGCs to round up and cease migration.

The final part of the mouse PGC journey occurs between 10.5 and 11.5 dpc and requires their migration from the hindgut through the dorsal mesentery to the gonadal ridges. In the absence of TGF β signaling (ALK5 deficiency), PGC migration out of the hindgut was promoted, likely because of the reduced deposition of collagen type I surrounding the gut. In fact, migratory PGCs adhere strongly to collagen; therefore, reduced collagen type I along the gut may result in reduced adhesion, facilitating migration into the dorsal mesentery and gonadal ridges. In this migratory stage, chemokine signals, likely provide attractants and homing mechanisms. In particular, stromal cell-derived factor 1 (SDF1), from neighboring mesenchyme and the gonadal ridges, interacting

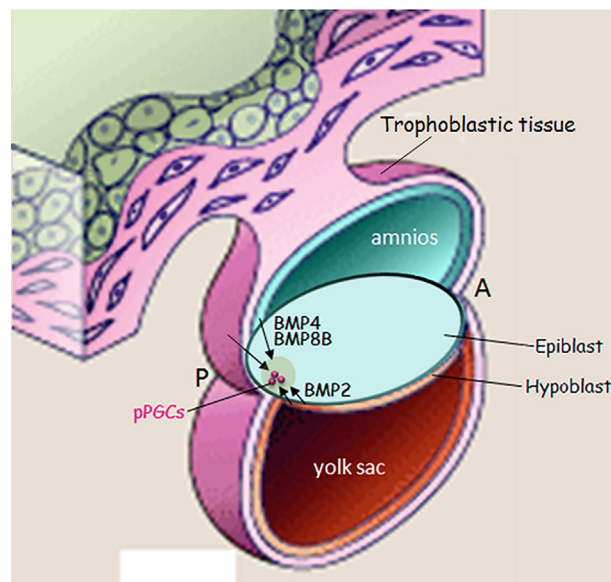


Fig. 1 After implantation and before gastrulation, around the end of the 2th week of gestation, the human embryo consists of a flat disk with two cell layers, epiblast, and hypoblast. It seems plausible that at this stage, the precursors of PGCs (pPGCs) are set aside within the posterior region of the epiblast following the action of BMP signals coming from trophoblastic tissue (including the developing syncytio- and cytotrophoblast) immediately surrounding the periphery of the embryonic disk (or perhaps from the amnioblasts lining the amnios and located above the epiblast) and the hypoblast. A, anterior; P, posterior.

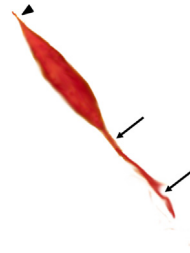


Fig. 2 A mouse PGCs cultured in the presence of a gradient of KL stained for TNAP show a motile phenotype with a lamellipodium/filopodium at the leading edge (arrows) and uropodium at the trailing edge indicated (arrowhead).

with PGCs via its G-protein coupled receptor, CXCR4 (chemokine C-X-C motif receptor 4), and KL, stimulating Kit, are candidate for such activities.

Other studies reported that cell–cell and cell–extracellular matrix (ECM) interactions are also critical for this last stage of PGC migration and gonad colonization. It was hypothesized that such interactions are required to maintain PGCs on the narrow path toward the gonad in the context of diffuse attractant signals. Interactions between PGCs and the ECM glycoproteins such as integrin $\beta 1$, laminin, fibronectin, and collagen IV, dependent on the stage of PGC migration, have been reported. In this regard, it is worthy to mention that integrin $\beta 1$ -deficient PGCs failed to colonize the gonads (for a review, see [De Felici et al., 2005](#)).

PGCs emigrating from the hindgut, were observed to extend long cellular processes that interacted with each other and their surroundings. Subsequent analysis revealed that E-cadherin expression was re-established once PGCs left the hindgut, and was concentrated with β -catenin at sites of PGC–PGC contacts. Moreover, treatment with E-cadherin-blocking antibodies prevented PGCs from directional migration in both in vitro and in vivo experiments, suggesting its role in mediating cell–cell interactions and promoting migration to the gonads.

Surprisingly, gonadal ridges are probably dispensable for the directional migration of PGCs, but required for their precise positioning at the final step of journey. In fact, in *Wt1* mutated mice in which gonadal ridges do not form, most of the PGCs reached the mesenchyme under the coelomic epithelium and no ectopic PGCs were noted; however, the precise positioning of PGCs was disrupted. Similarly, in null mutants for *Gata4*, gonadal ridges never form, however, the PGCs migrate and settle under the coelomic epithelium. Based on morphological observations, some authors claimed that the movement of PGCs from the allantois/yolk sac wall to the hindgut and then the gonadal ridges, might be explained by a simple passive translocation through first an enclosure in the endoderm forming the wall of the hindgut and then a transport by the proliferative expansion of this tissue into the very near gonadal ridges.

Active and passive translocation are applicable also for the journey of human PGCs towards the gonadal ridges. In the human embryo, the superior part of the yolk sac wall containing the PGCs, becomes incorporated in the developing gut during the lateral folding at 4th week of gestation, suggesting that the PGC translocation into the gut may be passive. The gonadal ridges are recognized by a bilateral thickening of the coelomic epithelium near the developing root of the gut mesentery in the posterior embryonic body cavity around 5th week. PGCs present in the endoderm of the gut epithelium were seen from the 5th week onwards. Thereafter, they leave the epithelium and invade the surrounding mesenchyme moving through the gut mesentery toward the gonadal ridges.

Electron microscopic studies describe human PGCs in vivo as having an irregular appearance and possessing pseudopodia during their journey toward the gonadal ridges. These features can be interpreted as a manifestation of active migration. An interpretation confirmed by several in vitro observations reporting that human PGCs show several characteristics of motile cells and can move actively both on cellular and ECM substrates. Such capability is probably used by PGCs to cover the distance of approximately 50 μ m separating the pre-aortic region from the gonadal ridges. Similarly, to mouse PGCs, migratory human PGCs appear to be surrounded by ECM components in which glycosaminoglycans have been histochemically detected. PGCs seem also interacting with the mesenchymal cells through different type of junctions, such as desmosomes, gap junctions, and focal contacts. [Møllgård et al. \(2010\)](#) observed that human PGCs preferentially ascended from the mesentery of the hindgut to the gonadal anlage by migration along autonomic nerve fibers close to the Schwann cells and proposed that these nerve fibers and/or Schwann cells may release chemoattractants supporting PGC migration. In humans, primary sites of ectopic germ cells are within the CNS but they are also seen in the pelvic region, the mediastinum and thorax.

No specific attractants have been identified for human PGCs although they have Kit and are surrounded by cells expressing KL and possess the CXCR4 receptor. The expression of transcripts for member of the olfactory receptor gene family in human PGCs from 10-week-old embryos makes this class of receptor additional candidates for PGC attractants.

During migration, human PGCs contain a large PAS-positive cytoplasmic store of glycogen and several lipid droplets. Following their arrival in the gonadal ridges, the glycogen content is diminished. Round mitochondria with a pale matrix and small tubular vesicular cristae were observed near the nucleus. They significantly increase in number during PGC migration and settlement in the gonadal ridges. Migratory human PGCs have less than 10 mitochondria, while 100 mitochondria are present in ovarian PGCs and 200 in oogonia. These observations suggest that PGCs might prevalently employ an anaerobic metabolism during migration and undergo a transition in their energy metabolism after reaching the gonadal ridges.

An extensive review of mouse and human PGC migration can be found in [De Felici \(2013, 2016\)](#).

Epigenetic Reprogramming

Epigenetics consists of stable heritable changes in gene expression not due to DNA sequence modifications. A unique characteristic of germline is the extensive epigenetics accompanying PGC development. Such process involves primarily DNA wide demethylation and changes of histone marks. This constitutes the so-called epigenome reprogramming or resetting that restores full germline potency for the transmission of genetic information to the next generations (for a review, see [De Felici, 2011](#)).

Immediately following specification, a marked reduction in heterochromatic histone H3K9me2 followed by an increase in repressive H3K27me3 and H2A/H4R3me2s occur in mouse PGC precursors. Such early changes are favored by a period of mitotic arrest and transcriptional quiescence occurring in such cells between 8.5 and 9.5 dpc ([Seki et al., 2007](#)).

Although DNA demethylation begins during this early phase, the bulk of DNA demethylation occurs in PGCs after gonad colonization (between 11.5 and 13.5 dpc). This is accompanied by changes in chromatin organization and loss of numerous histone modifications brought about by genome-wide histone replacement. This second wave of reprogramming includes complete X reactivation in female and the erasure of imprinting (a process by which certain genes are expressed in a parent-of-origin-specific manner), allowing the establishment of sex-specific imprints during the successive gametogenesis.

Evidence exists that DNA demethylation in mouse PGCs employs both a passive (DNA replication-dependent) and active mechanisms. A decrease in the protein level of the de novo methyltransferases DNMT3a and DNMT3b accompanies the PGC postspecification and gonad colonization periods while the maintenance methyltransferase DNMT1 is present but inactive. Active demethylation may involve the DNA repair pathway such as activation-induced cytidine deaminase (AID) and thymine DNA glycosylase (TDG) enzymes, ten-eleven translocation (TET) enzymes and poly (ADP-ribose) polymerase (PARP) activities.

In human PGCs, a few observations have been reported about DNA demethylation and chromatin changes. A timeline and some characteristics of these events are becoming, however, to be available also thanks to the in vitro models of PGC-like formation for stem cells described above (for a review, see [Tang et al., 2016](#)). Immunohistochemical studies and DNA methylome analyses of global 5 mC level showed that both female and male fetal germ cells are hypomethylated already at 5th–7th weeks of gestation when most of them are still migrating or have just arrived in the gonadal ridges. Concomitantly, PGCs undergo chromatin reorganization, X reactivation, and imprinting erasure. Like in mouse, consistent with global demethylation, UHRF1, a factor necessary for DNMT1 activity, was not detectable in proliferating PGCs/oogonia while de novo DNA methyltransferase DNMT3a and DNMT3b were repressed; as note TET1 and TET2 appeared enriched during this period. In human PGCs, demethylation of imprint controlled regions (ICRs) is completed, except for the insulin growth factor2 receptor (*IGF2R*) and the progression elevated gene-10 (*PEG10*) genes, before 7th week when most of PGCs are still migrating towards or are recently arrived into the gonadal ridges, indicating early epigenetic dynamics. Resembling mouse PGCs, despite global hypomethylation, potentially hazardous retrotransposons such as the SINE-VNTR-ALU (SVA) and endogenous retrovirus (ERV) elements remain methylated in human oogonia. Interestingly, in contrast to mouse prospermatogonia, which remain demethylated for only a few days, human prospermatogonia remain hypomethylated for weeks and only showed signs of re-methylation in the II trimesters (around 16th week onward).

As for histone marks, in human PGCs, H3K9me2 levels were persistently lower compared to soma, but it remained detectable in the nuclei. Although at week 4th human PGCs showed 2.5-fold enrichment of H3K27me3, the signal diminished gradually to half of that of soma by week 9th. H3K27me3 foci at the inactivated X chromosome were observed in soma but not in week 7th female PGCs, indicating reactivation of X. At around 16th–20th weeks male germ cells demonstrate absence/low levels of repressive H3K9me2, H3K27me3, and H3K9me3 modifications but high levels of permissive H3K9ac and H2A.Z marks. Remarkably, PGCLCs produced in vitro from human stem cells showed ongoing removal of parental imprinting, erasure of global DNA methylation and histone modifications like their in vivo PGC counterparts.

PGC Differentiation

After arrival in the gonadal ridges, PGCs differentiate into oogonia and prospermatogonia within the developing ovaries and testes, respectively. Both oogonia and prospermatogonia undergo a phase of active proliferation. In oogonia this phase is followed by a mitosis to meiosis switch from which the primary oocytes arise whereas prospermatogonia do not enter meiosis but undergo mitotic arrest into G0.

In the mouse, the transition of PGCs to oogonia or prospermatogonia occurs in a couple of days and is associated to the loss of the motile phenotype, the formation of germ cysts (or nests) due to incomplete cytodieresis, a second wave of epigenetic changes (see above) and the expression of germ cell specific genes such as *Ddx4* (or *Vasa*) and *Dazl*. During this time, no relevant morphological differences between oogonia and prospermatogonia are detectable although their transcriptome begins to diverge significantly in function of the irreversible commitment to oogenesis or spermatogenesis ([Sakashita et al., 2015](#); [Lei and Spradling, 2013](#)).

The sexual differentiation of PGCs is not cell autonomous, meaning that it is not determined by their own sex chromosome constitution. Instead, it is determined by the sexual phenotype of their somatic environment. In the mouse, testis determination is initiated between 10.5 and 11.5 dpc, by expression of the Y-chromosome gene, *SRY* (sex determining region Y chromosome), within the somatic cells, mainly the pre-Sertoli cells, whereas ovary differentiation is guided by the expression in the somatic compartment of at least two genes, namely *RSPO1* and *WNT4* (for a review, see [Ewen and Koopman, 2010](#)).

Since most of the studies focused on PGC sex differentiation into oogonia and prospermatogonia have been performed in the mouse where this process is rapidly followed to entering into meiosis and mitotic arrest, respectively, these two events have been

considered strictly associated to the commitment of PGCs to oogenesis or spermatogenesis. Actually, the decision between male and female germ cell fates is marked by environmental signals that control the expression of two master genes: *Stra8* (stimulated by retinoic acid gene 8), which is required for the initiation of meiosis in females, and *Nanos2*, which blocks *Stra8* expression in males and thereby prevents meiosis. One important environmental factor known to play a role in female-specific development is retinoic acid (RA), which activates *Stra8* in female germ cells (for a review, see Bowles and Koopman, 2007), however, the direct target/s of STRA8 are unknown (Tedesco et al., 2009). In the male, germ cells are protected from exposure to RA by Cyp26b1, an RA-metabolizing enzyme of the cytochrome P450 family that is produced by the Sertoli cells and the Leydig cells. The degradation of RA is likely necessary for arresting the prospermatogonia proliferation and at the same time to suppress entering meiosis (Trautmann et al., 2008). Despite the importance of RA inhibition in males, multiple lines of evidence indicate that additional secreted factors also play crucial roles in determining male germ cell differentiation. One candidate that has been proposed is the secreted male-specific Fgf9. This growth factor signaling maintains the expression of pluripotency-related genes, and actively suppresses entry into meiosis by activating *Nanos2* expression (Barrios et al., 2010) via the transient activation of the Cripto/Nodal pathway (Spiller et al., 2012). A role of prostaglandin D₂ (PGD₂), in this process has been also proposed. Both somatic- and germ cell-produced PGD₂, acting in both a paracrine and an autocrine manner, play a role in the regulation of male fetal germ cell differentiation (Moniot et al., 2014). In addition, the transmembrane protein SDMG1 (sexually dimorphic, expressed in male gonads 1, also known as TMEM184a), associated with endosomes and specifically secreted by Sertoli cells, may be involved in such repressive signaling (Best et al., 2008).

A recent report indicated, however, that the beginning of meiosis and germ cell commitment into oogenesis are dissociable events. In fact, in the mouse *Stra8*-deficient ovarian germ cells grow and differentiate into oocyte-like cells that can be ovulated and fertilized (Dokshin et al., 2013). The drivers of oocyte differentiation are unknown, although the transcription factors SOHL1 and 2 can be involved (Shin et al., 2017). Likewise signaling mechanisms responsible for initiating male commitment of the germ cells may be different from the molecular signals/pathways initiating mitotic arrest and meiosis inhibition. For example, in human, mitotic arrest of germ cells occurs considerably later than somatic sex determination and sex cord formation (and probably germ cell sex determination) and prospermatogonia apparently retain proliferative activity until late gestational period; during this period, accumulating numbers of germ cells appear to exit the cell cycle in a relatively unsynchronised manner.

Human PGCs arrived into the gonadal ridges are rapidly surrounded by somatic cells. The differentiation of PGCs into oogonia occurs during the 9th week of gestation. Oogonia tend to form clusters of dividing cells joined by rims of cytoplasm, termed intercellular bridges, originated by incomplete division of the cell body during cytodieresis. The mitotic proliferation of oogonia lasts several weeks and overlaps the period of their entry into meiosis (10th–11th weeks). Actually, until the 5th month of fetal life, mitotic oogonia and primary oocytes in different stages of meiosis coexist. In the male, PGCs enclosed within seminiferous sex cords become prospermatogonia. Testis differentiation is marked by the expression of sex determining region Y (SRY) and SRY-box 9 (SOX9) genes at 5–6 weeks. Thereafter, sex cords, formed from Sertoli cells and prospermatogonia, become increasingly evident within the testis from the 7th week during late embryonic life. During the first trimester, prospermatogonia are mitotically active and continue to express markers typical of pluripotent cells and PGCs such as OCT4, SSEA1, DDX4 (Vasa), Nanog, and KIT. During the second trimester, most but not all prospermatogonia progressively lose mitotic activity together with the pluripotency and PGC markers.

In the human fetal ovary, the first signs of meiosis initiation are apparent in oogonia around 10 weeks postfertilization. At this stage, meiosis initiation appears strikingly asynchronous, as while more and more germ cells initiate meiosis some oogonia go on proliferating until at least 16 weeks. Interestingly, after 12th week, oogonia seem to segregate into two populations of KIT/OCT4 located at the periphery and VASA positive cells toward the center of the ovary. The latter showing the formation of the synaptonemal complex indicating meiotic entry. Contrary to the antero-posterior wave of meiosis initiation reported in fetal mouse ovaries, this radial gradient in human ovaries suggests the existence of species differentiation in the initiation signals, with local somatic cell interaction operating in human versus diffusible cues in mice. Like in the mouse, RA is a possible intraovarian factor involved in meiosis initiation in the human ovary, although it remains, however, to be determined how the extensive asynchrony of the beginning and progression of this process is regulated (Le Bouffant et al., 2010).

References

- Barrios, F., Filippini, D., Pellegrini, M., et al. (2010). Opposing effects of retinoic acid and FGF9 on *Nanos2* expression and meiotic entry of mouse germ cells. *Journal of Cell Science*, 123, 871–880.
- Best, D., Sahlender, D. A., Walther, N., et al. (2008). Sdmg1 is a conserved transmembrane protein associated with germ cell sex determination and germline-soma interactions in mice. *Development*, 135, 1415–1425.
- Bowles, J., & Koopman, P. (2007). Retinoic acid, meiosis and germ cell fate in mammals. *Development*, 134, 3401–3411.
- De Felici, M. (2011). Nuclear reprogramming in mouse primordial germ cells: Epigenetic contribution. *Stem Cells International*. <https://doi.org/10.4061/2011/425863>.
- De Felici, M. (2013). Origin, migration, and proliferation of human primordial germ cells. In G. Coticchio, et al. (Eds.), *Oogenesis* (pp. 19–37). London: Springer-Verlag.
- De Felici, M. (2016). The formation and migration of primordial germ cells in mouse and man. *Results and Problems in Cell Differentiation*, 58, 23–46.
- De Felici, M., & Farini, D. (2012). The control of cell cycle in mouse primordial germ cells: Old and new players. *Current Pharmaceutical Design*, 18, 233–244.
- De Felici, M., Scaldaferrri, M. L., & Farini, D. (2005). Adhesion molecules for mouse primordial germ cells. *Frontiers in Bioscience*, 10, 542–551.
- Dokshin, G. A., Baltus, A. E., Eppig, J. J., & Page, D. C. (2013). Oocyte differentiation is genetically dissociable from meiosis in mice. *Nature Genetics*, 45, 877–883.
- Ewen, K. A., & Koopman, P. (2010). Mouse germ cell development: From specification to sex determination. *Molecular and Cellular Endocrinology*, 323, 76–93.
- Felix, W. (1911). Die Entwicklung der Harn- und Geschlechtsorgane. In F. Keibel, & F. P. Mall (Eds.), vol. 2. *Handbuch der Entwicklungsgeschichte des Menschen* (pp. 732–795). Leipzig: Hirzel.

- Fuss, A. (1911). Über extraregionäre Geschlechtszellen bei einem menschlichen Embryo von 4 Wochen. *Anat Am*, 39, 407–409.
- Fuss, A. (1912). Über die Geschlechtszellen des Menschen und der Säugetiere. *Anat Entw Mech*, 81, 1–23.
- Ginsburg, M., Snow, M. H., & McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation. *Development*, 110, 521–528.
- Kobayashi, T., Zhang, H., Tang, W. W. C., et al. (2017). Principles of early human development and germ cell program from conserved model systems. *Nature*, 546, 416–420.
- Lawson, K. A., & Hage, W. J. (1994). Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Foundation Symposium*, 182, 68–84.
- Le Bouffant, R., Guerquin, M. J., Duquenne, C., et al. (2010). Meiosis initiation in the human ovary requires intrinsic retinoic acid synthesis. *Human Reproduction*, 25, 2579–2590.
- Lei, L., & Spradling, A. C. (2013). Mouse primordial germ cells produce cysts that partially fragment prior to meiosis. *Development*, 140, 2075–2081.
- McKay, D. G., Hertig, A. T., et al. (1953). Histochemical observations on the germ cells of human embryos. *The Anatomical Record*, 17, 201–219.
- Møllgård, H. K., Jespersen, A., Lutterodt, M. C., et al. (2010). Human primordial germ cells migrate along nerve fibers and Schwann cells from the dorsal hind gut mesentery to the gonadal ridge. *Molecular Human Reproduction*, 16, 621–631.
- Moniot, B., Ujjan, S., Champagne, J., et al. (2014). Prostaglandin D2 acts through the Dp2 receptor to influence male germ cell differentiation in the foetal mouse testis. *Development*, 141, 3561–3571.
- Sakashita, A., Kawabata, Y., & Jincho, Y.t.a. (2015). Sex specification and heterogeneity of primordial germ cells in mice. *PLoS One*, 10, e0144836.
- Sasaki, K., Yokobayashi, S., Nakamura, T., et al. (2015). Robust in vitro induction of human germ cell fate from pluripotent stem cells. *Cell Stem Cell*, 17(2), 178–194. <https://doi.org/10.1016/j.stem.2015.06.014>.
- Sasaki, K., Nakamura, T., Okamoto, I., et al. (2016). The germ cell fate of cynomolgus monkeys is specified in the nascent amnion. *Developmental Cell*, 39, 169–185.
- Seki, Y., Yamaji, M., Yabuta, Y., et al. (2007). Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. *Development*, 134, 2627–2638.
- Shin, Y. H., Ren, Y., & Suzuki, H. (2017). Transcription factors SOHLH1 and SOHLH2 coordinate oocyte differentiation without affecting meiosis I. *The Journal of Clinical Investigation*, 127, 2106–2117.
- Spiller, C. M., Feng, C. W., Jackson, A., et al. (2012). Endogenous nodal signaling regulates germ cell potency during mammalian testis development. *Development*, 139, 4123–4132.
- Tang, W. W., Kobayashi, T., Irie, N., et al. (2016). Specification and epigenetic programming of the human germ line. *Nature Reviews. Genetics*, 17, 585–600.
- Tedesco, M., La Sala, G., Barbagallo, F., et al. (2009). STRA8 shuttles between nucleus and cytoplasm and displays transcriptional activity. *The Journal of Biological Chemistry*, 284, 35781–35793.
- Trautmann, E., Guerquin, M. J., Duquenne, C., et al. (2008). Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes. *Cell Cycle*, 7, 656–664.

FETAL DEVELOPMENT

Formation and Growth of the Fetus

Alison J Forhead, University of Cambridge, Cambridge, United Kingdom; and Oxford Brookes University, Oxford, United Kingdom

Abigail L Fowden, University of Cambridge, Cambridge, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

The conceptus develops from a single fertilized egg into a complex multicellular organism by processes of cell proliferation, growth, and differentiation within the uterus. After organogenesis, the major period of body growth occurs in fetal life, during the second and third trimesters of human pregnancy. Fetal growth follows an orderly and coordinated increase in body weight and length, which is coupled with a proportionate increase in organ weights and their functional development. Normal fetal growth and maturation is essential for both the survival of the offspring at birth and for longer term health in adult life.

Measurement of Fetal Growth

Fetal growth can be assessed by a range of clinical and experimental methods. End-point measurements, such as body weight, length, and adiposity at the time of delivery, are simple indicators of intrauterine growth. It is also possible to measure growth rate while the fetus is developing within the uterus. Ultrasound scanning can be used to make a variety of measurements of growth at different gestational ages. In clinical practice, the most common biometric measurements include biparietal diameter (diameter of a cross-section of the head or skull), femur bone length and abdominal circumference, and these values are combined to estimate the body weight of the fetus.

Using ultrasound data from many individuals, a series of growth charts can be generated to estimate the percentile and the growth trajectory of the fetus. These growth charts have recently been updated by the World Health Organization using data obtained in a multinational study. Longitudinal measurements were made from 14 to 40 weeks in singleton fetuses, where the pregnant women were recruited from middle to high income backgrounds to ensure good nutritional status ([Kiserud et al., 2017; Fig. 1](#)). Fetal growth charts can be used to diagnose abnormalities in intrauterine growth, particularly if fetal growth deviates from its previous trajectory. Estimated fetal body weight less than the 10th centile or greater than the 90th centile indicates a fetus that is small- or large-for-gestational age (SGA and LGA), respectively.

Experimentally, fetal growth has been monitored in large animal models, such as sheep, using both end-point and continuous intrauterine measurements. In the sheep fetus, indwelling growth devices measure the crown-rump length and abdominal circumference over a period of late gestation and incremental growth rates have been demonstrated in well-fed ewes with stable maternal plasma glucose concentrations ([Mellor and Matheson, 1979; Fig. 2](#)). Collectively, the ultrasound and experimental techniques have shown, in many species including the human, that fetal growth is greatest in absolute terms in the later stages of gestation. Close to term, however, the growth rate of the fetus slows as the developing tissues switch from cell proliferation to differentiation and functional maturation, in preparation for birth.

Control of Fetal Growth

The growth of the fetus is driven primarily by the genetic potential of the individual inherited at conception. Intrauterine growth and development, however, can be modified by a variety of factors including physiological, nutritional, environmental, and clinical conditions with consequences for size at birth. Many of these factors interact with each other to generate a wider range of birth weights in the human and animal population than predicted by genotype alone. These factors can be divided into those that originate in (1) the mother, (2) the placenta, and (3) the fetus itself ([Fig. 3](#)).

Maternal Factors

Maternal size

The size of the mother influences the growth rate of the fetus. This may be due to (i) the inherited genetic potential for fetal growth, as paternal height also correlates with birth weight, (ii) the size of the uterus and/or (iii) the body composition and energy reserves of the mother before and during pregnancy. The relative contributions of the genetic and uterine environment in determining fetal growth, however, are difficult to separate. In human populations, both maternal height and body mass index, and paternal height, correlate with offspring birth weight; further analysis shows that neonatal adiposity and head circumference are predicted by maternal height and body mass index, while limb lengths are more strongly associated with paternal height ([Pomeroy et al., 2015](#)). In assisted pregnancies where egg donation was used, the birth weight of the infant correlated with the weight and height

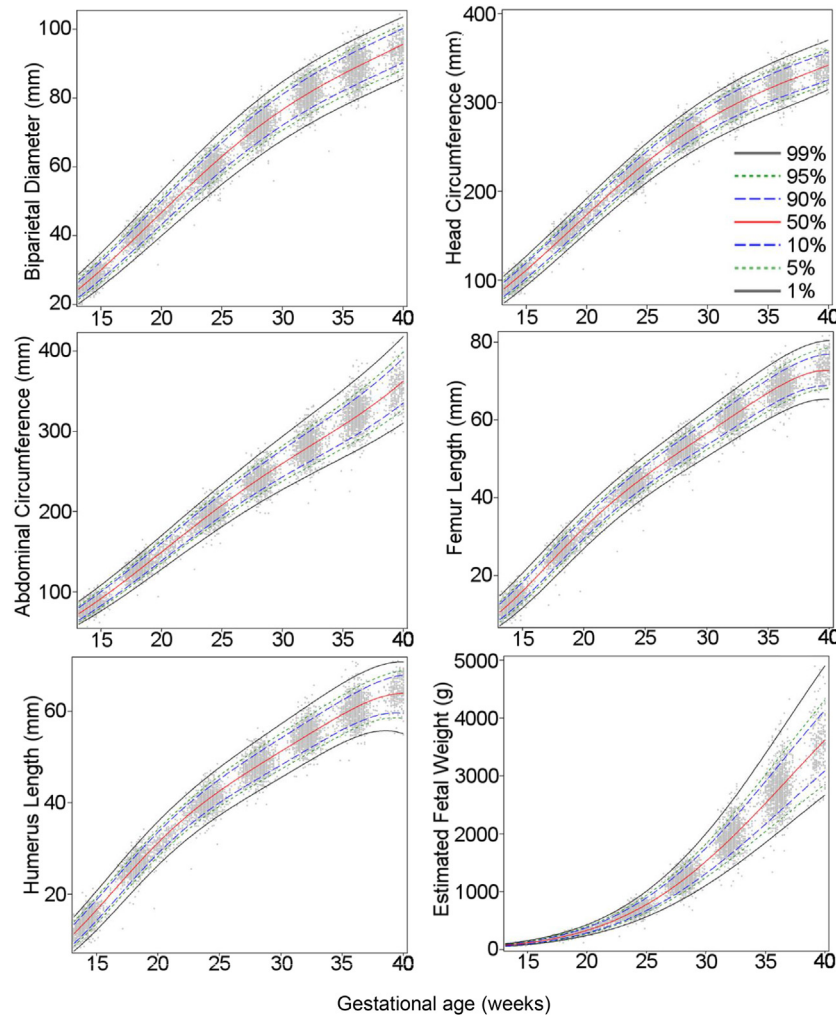


Fig. 1 Growth charts and percentiles for biparietal (*outer–inner*) diameter, head circumference, abdominal circumference, femur length, humerus length, and estimated body weight in human fetuses from 14 to 40 weeks of gestation. Taken from Kiserud, T., Piaggio, G., Carroli, G., Widmer, M., Carvalho, J., Neerup Jensen, L., Giordano, D., Cecatti, J.G., Abdel Aleem, H., Talegawkar, S.A., Benachi, A., Diemert, A., Tshetu Kitoto, A., Thinkhamrop, J., Lumbiganon, P., Tabor, A., Kriplani, A., Gonzalez Perez, R., Hecher, K., Hanson, M.A., Gülmezoglu, A.M. and Platt, L.D. (2017). Correction: The World Health Organization fetal growth charts: A multinational longitudinal study of ultrasound biometric measurements and estimated fetal weight. *PLoS Medicine* **14**(3), e1002284.

of the recipient, but not with measurements from the biological donor mother (Brooks et al., 1995). This suggests that the intra-uterine environment may have a greater role in mediating the effect of maternal size on offspring birth weight than the genetic influence of the mother.

The larger the uterus, the more space is available for growth of both the placenta and fetus and equally, a small uterus can limit the ability of the placenta and fetus to grow to their genetic potential. The effect of uterine size on fetal growth was demonstrated in classical experiments by Walton and Hammond (1938). They developed the technique of artificial insemination to cross breed Shire horses and Shetland ponies. When a Shetland pony mare was inseminated by a Shire horse stallion, the foal produced was three times smaller than the foal conceived by a Shire horse mother and Shetland pony father (Walton and Hammond, 1938; Fig. 4). These studies showed that the size of the uterus can determine the size of the newborn foal, such that the birth weight of the offspring is a function of maternal body weight, around 8% in these experiments. Furthermore, the differences in foal size and body composition at birth persisted into later life.

Maternal nutrition

Maternal nutrition is a key regulator of fetal growth as the mother provides the macro- and micronutrients that both the fetus and placenta require to grow. Several experimental and epidemiological studies have shown that under-nutrition of the mother impairs fetal growth (Sferruzzi-Perri et al., 2013).

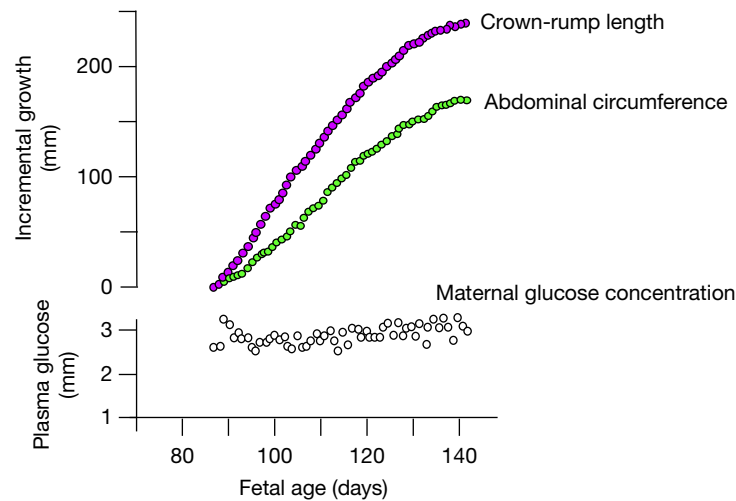


Fig. 2 Growth rates for crown-rump length and abdominal circumference using indwelling growth measuring devices in a sheep fetus during late gestation (term ~145 days). Redrawn from Mellor, D.J. and Matheson, I.C. (1979). Daily changes in the curved crown rump length of individual sheep foetuses during the last 60 days of pregnancy and the effects of different levels of maternal nutrition. *Quarterly Journal of Experimental Physiology* **64**, 119–131.

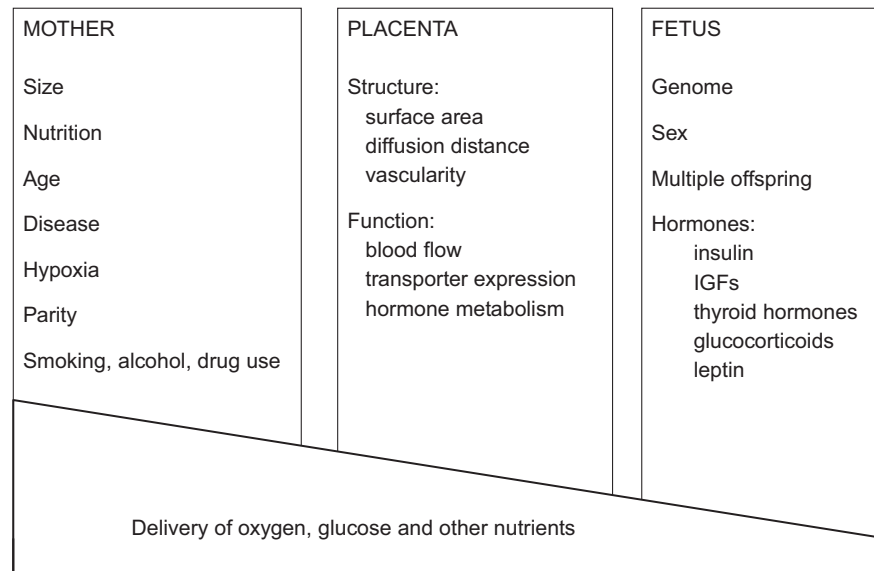


Fig. 3 Factors that influence the growth of the fetus.

Using growth-measuring devices in sheep fetuses, the growth rates of the axial skeleton and abdominal circumference were monitored before, during and after a 10-day period of maternal under-nutrition (Mellor and Murray, 1982; Fig. 5). Maternal plasma glucose levels fell when the mother was under-nourished and the growth rates measured in the fetus were suppressed. When the mother was re-fed to normal levels, maternal plasma glucose concentration returned to normal and the growth of the fetus was restored to the rate observed before the period of under-nutrition.

The Dutch Winter Hunger study is a unique opportunity to examine the effects of maternal under-nutrition on fetal growth in the human population. During World War II, parts of occupied Netherlands were severely rationed by the German army such that, for the last 6 months of the war, the food intake of the people of Western Netherlands, which included Amsterdam, Rotterdam, and The Hague, was reduced to <1000 calories per day. Pregnant women in the third trimester during this time did not show normal weight gain and their babies were around 200–300 g lighter in birth weight (Smith, 1947; Lumey, 1998; Table 1).

Other maternal factors

Fetal growth is also affected by a variety of interacting maternal factors. Parity influences growth of the fetus, whereby first-born offspring are usually smaller than subsequent siblings, in part, as a consequence of changes in uterine size. Extreme ends of maternal

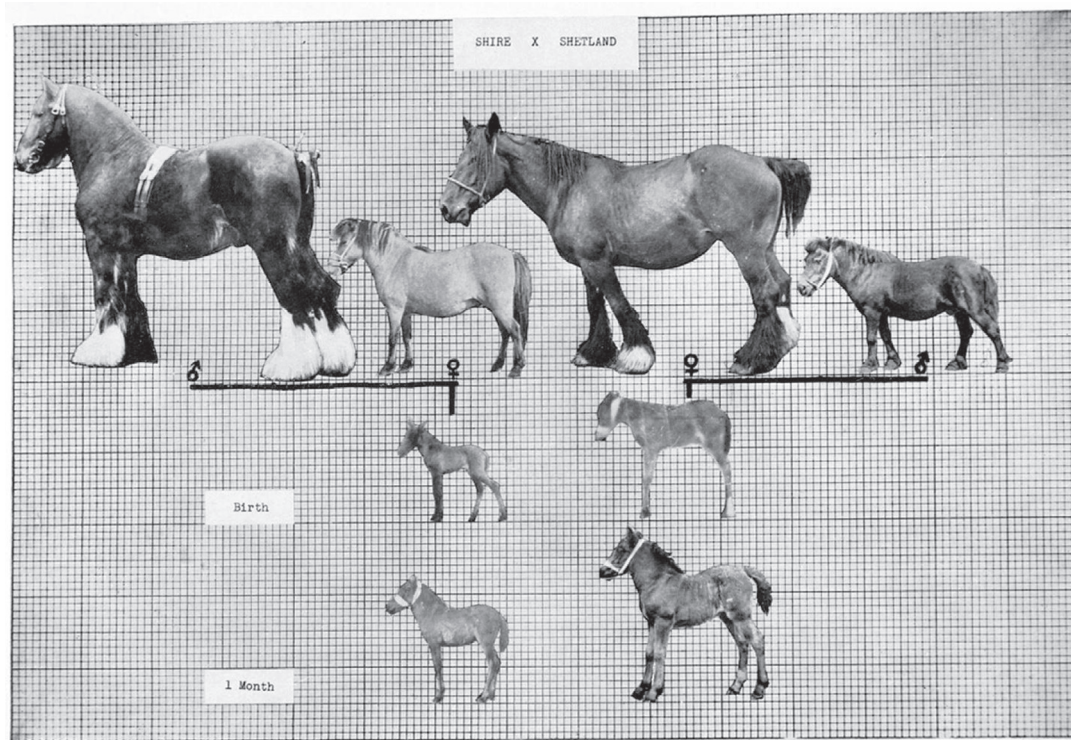


Fig. 4 Parents and offspring of reciprocal Shetland–Shire crosses to show consequences of maternal size for fetal growth. Taken from Walton, A. and Hammond, J. (1938). The maternal effects on growth and conformation in Shire horse–Shetland pony crosses. *Proceedings of the Royal Society London B* **125**, 311–335.

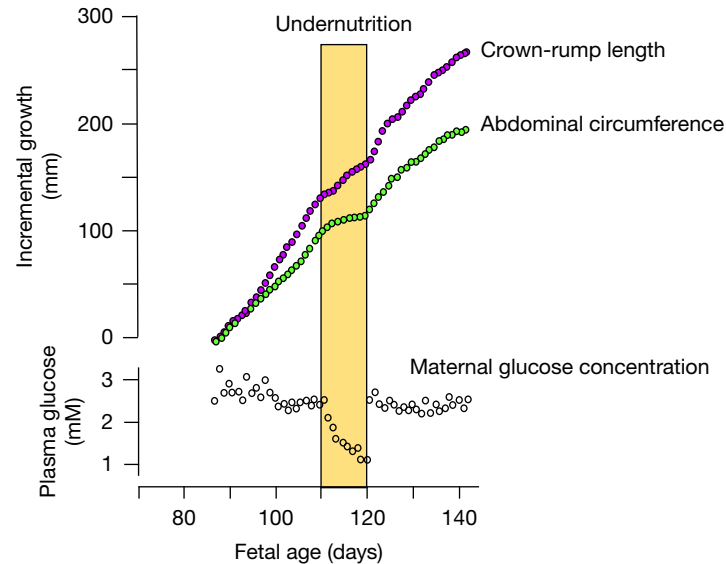


Fig. 5 Effect of maternal nutrition on growth rate of crown-rump length and abdominal circumference in a sheep fetus during late gestation. Redrawn from Mellor, D.J. and Murray, L. (1982). Effects of rate of increase in fetal girth of refeeding ewes after short periods of severe undernutrition during late gestation. *Research in Veterinary Science* **32**, 377–382.

age have been linked to impaired fetal growth, with both teenage and older (>35 years) mothers more likely to give birth to SGA offspring (Restrepo-Méndez et al., 2015). Low birth weight associated with young maternal age may be a result of suboptimal partitioning of resources between the fetus and mother that is still growing herself. In older mothers, preexisting disease may impair placental and fetal growth. These observations, however, are confounded by other factors linked with young and older pregnant women, such as socioeconomic class and nutritional status.

Table 1 Mean placental and birth weights, and placental efficiency, in the Dutch Hunger Winter study

	Control	Trimester of famine exposure		
		1st	2nd	3rd
Placental weight (g)	636	647	611	547
Birth weight (g)	3379	3361	3435	3130
Placental efficiency (g/g)	5.31	5.19	5.62	5.72

Data taken from Lumey, L.H. (1998). Compensatory placental growth after restricted maternal nutrition in early pregnancy. *Placenta* 19, 105–111.

Low birth weight offspring are commonly associated with maternal smoking, chronic stress, alcohol, and drug use, lower socioeconomic class and medical conditions such as preeclampsia, malaria, chronic hypertension, and renal disease (Kramer, 1987). Maternal diabetes and obesity lead to increased incidences of both SGA and LGA offspring. Habitation in the chronic hypoxic environment at high altitude lowers birth weight by approximately 100 g for every 1000 m ascent in altitude, especially in nonnative populations (Moore, 2003).

Placental Factors

The placenta is the interface between the mother and fetus, and ultimately regulates the transfer of nutrients and oxygen from the maternal to fetal circulation. Therefore, the size, structural development and functional transport capacity of the placenta are key factors that affect fetal growth. In many animal species, including humans, the size and growth of the fetus correlates with the size of the placenta, although it is important to consider the common genetic make-up of the fetus and the fetal-derived placental tissues.

After implantation, the early development of the placenta is essential for normal growth of the embryo and fetus. In some species, such as the sheep, the placenta grows at a rapid rate in early gestation and plateaus in size by mid-gestation, while in human pregnancy, the placenta continues to grow at a steady rate throughout gestation. During late gestation, however, the rise in fetal body weight occurs at a faster rate than the change in placental weight which means that the microstructural and functional properties of the placenta, and the transfer of nutrients, are increased to support fetal growth.

The exchange of nutrients, respiratory gasses, water, and waste products between the mother and fetus is governed by the structural development of the placenta. According to Fick's law of diffusion, the transfer rate of freely-permeable substances such as oxygen is dependent on the placental surface area, thickness of the diffusion pathway and the concentration gradient between the maternal and fetal circulations. The concentration gradient is maintained by adequate maternal oxygenation and nutrient status, and uteroplacental blood flow. For nutrients transferred by facilitated diffusion, such as glucose, the placental expression of transporter proteins contributes to the rate of transfer. Specific transporters are also essential for active transfer processes which use energy to transport nutrients, such as amino acids needed for protein synthesis, from mother to fetus against a concentration gradient.

Several experimental models demonstrate the importance of placental size in determining fetal growth. First, the consequences of decreasing placental size from the outset of the pregnancy have been examined in sheep. A number of the implantation sites in the uterus, the caruncles, were surgically removed before mating; these are the sites where the placental cotyledons form during pregnancy. Removal of some of these caruncles led to a reduction in the number of placental cotyledons and subsequently impaired the growth of the fetus (Robinson et al., 1994; Fig. 6). Many of the remaining placental sites were overgrown in an attempt to compensate for the reduction in number, but placental weight overall was still lower which caused poor fetal growth. Placental restriction can also be induced experimentally in rats by ligation of the uteroplacental blood vessels which impairs placental blood flow and leads to significant growth retardation of the offspring.

Data from the Dutch Hunger Winter study also demonstrates the effects of maternal under-nutrition on placental and thereby fetal growth (Lumey, 1998; Table 1). These effects depended on the stage of gestation when the famine occurred. For women in the first trimester of pregnancy during the famine, there was an increase in the size of the placenta at birth and the fetuses were born a normal size; the early growth of the placenta was stimulated by maternal under-nutrition and this was able to support normal fetal growth. In the second trimester, placental size was reduced at birth, but offspring birth weight was normal and overall placental efficiency was significantly improved (the ratio of fetal weight to placental weight, as grams of fetus produced for each gram of placenta). This indicates that the placenta increased its functional capacity and effectiveness in nutrient delivery to maintain fetal growth despite being of a smaller size. Finally, when women were exposed to the famine in the third trimester, birth weight was lower and this was related to a profound reduction in placental weight; although the placenta improved its efficiency, the offspring were born smaller than normal.

The placenta may also contribute to the control of growth in utero by the production and metabolism of hormones that have consequences for maternal and fetal physiology. Placental hormones influence maternal physiology and metabolism in a way that promotes nutrient delivery to the fetus. Placental lactogen induces a degree of insulin resistance in the mother such that less glucose

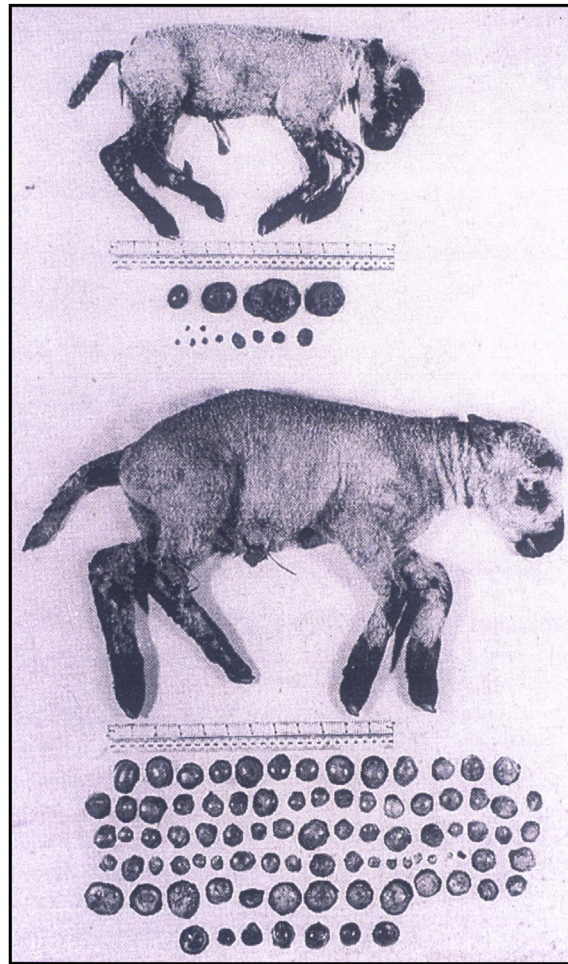


Fig. 6 Effect of reduction in placental size by carunclectomy on fetal growth in sheep. Both fetuses and their placentomes were measured at 127–128 days of gestation (term ~145 days): the upper fetus was taken from a ewe where uterine caruncles were removed before pregnancy, while the lower fetus was taken from a control ewe. Taken from Robinson, J.S., Owens, J.A. and Owens, P.C. (1994). Fetal growth and fetal growth retardation, In Thornburn, G.D. and Harding, R. (eds.) *Textbook of fetal physiology*, pp. 83–94, Oxford: Oxford University Press.

is taken up by maternal tissues and more is readily available for transfer to the fetus. In addition, the pregnancy steroid hormones, progesterone and estrogen, stimulate a range of maternal adaptations in cardiovascular, respiratory and metabolic systems, as well as regulating the contractile activity of the uterine muscles.

The placenta is also the site of metabolism of maternal hormones that can influence fetal growth and development. For instance, the transfer of maternal glucocorticoids to the fetus is limited by the placental enzyme 11β -hydroxysteroid dehydrogenase. This enzyme converts glucocorticoids to biologically-inactive metabolites and thereby protects the fetus from the growth-retarding effects of glucocorticoids from the mother. The placenta also acts as a partial barrier to the transfer of thyroid hormones; placental expression of the type III deiodinase enzyme metabolizes thyroid hormones to inactive forms and regulates circulating thyroid hormone levels in both the placental and fetal circulations.

Fetal Factors

Genetics of the fetus

The genetic makeup or genome of the offspring (which also contributes to the genome of the placenta) influences fetal growth and size at birth. In human populations with diverse backgrounds, there is variation in birth weights between different ethnic groups. For instance, a study in the USA examined the birth weights of infants born 2009–12 from 14 defined races (Morisaki et al., 2017). Compared to non-Hispanic white infants, Japanese babies were on average 289 g lighter and Samoan babies were 126 g heavier. Approximately 60% of the variation in birth weight between ethnic groups, however, was accounted for by differences in the incidence of prematurity, maternal height, body mass index, and gestational weight gain. After adjustment for maternal and socioeconomic factors, black infants were the smallest and American Indians were the largest at delivery.

Several of the genes that are important for placental and fetal growth are imprinted genes (genes that are preferentially expressed from either the maternal or paternal copy). Paternally-expressed genes, such as insulin-like growth factor-II (IGFII), promote fetoplacental growth, whereas maternally-expressed genes, such as the IGF type 2 receptor, tend to restrict growth. The conflict theory proposes that the imprinted genes inherited from the father act to maximize growth and survival of the offspring, while those inherited from the mother limit growth of the fetus, in order to conserve resources for the mother and any future offspring. However, the maternal and fetal genomes also have to interact cooperatively during pregnancy to ensure delivery of a viable infant at term.

There are consequences of genetic defects in the fetus for its ability to grow normally. Genetic defects can affect overall body growth or growth of specific tissues to influence birth size. These defects may be due to single gene mutations such as osteogenesis imperfecta, a brittle bone disease where the individual cannot synthesize collagen normally which has consequences for development of the skeletomuscular system. Alternatively, the defect may involve whole chromosomes; for example, Down's syndrome is due to an extra chromosome 21 which reduces birth weight by 240–300 g, as well as leading to facial and other abnormalities in development. Growth defects can also arise from changes in the expression of single genes without any alterations in chromosome number or DNA sequence. For instance, over-expression of the *IGF2* gene on chromosome 11 due to changes in DNA methylation and imprinting status in Beckwith Wiedemann syndrome typically leads to overgrowth of specific organs and larger than normal birth weight.

The sex of the fetus influences the pattern of growth before birth and newborn weight: female fetuses are smaller than male fetuses at the same gestational age and at birth. Multiple offspring in a pregnancy are also smaller than singleton fetuses in human and other species. Growth of individual fetuses may be compromised by the number of fetuses in a pregnancy by competition for resources and uterine space for placental development.

Hormones of the fetus

There are a variety of hormones produced by the fetus that can influence its body growth and organ development, including insulin, IGFs, thyroid hormones, glucocorticoids, and leptin (Fowden and Forhead, 2009, 2013; Sferruzzi-Perri et al., 2013). Experimental and clinical studies have demonstrated that changes in the levels of these hormones in utero have consequences for the growth of the fetus and placenta (Table 2). In human infants at term, birth weight correlates positively with umbilical cord blood concentrations of insulin, IGF1, thyroxine, and leptin. Hormones act as signals of the intrauterine environment to both fetal and placental tissues, and serve to match the growth and development of the fetus with the levels of oxygen and nutrients available. They have actions on cell proliferation, growth, differentiation, metabolism, and function in a wide range of fetal tissues, and can influence placental growth and transport capacity.

Insulin promotes growth of the fetus, especially fat mass. In fetal tissues, it stimulates uptake of glucose and amino acids, and therefore glucose utilization and protein synthesis, which encourages further transplacental movement of glucose to the fetus down the maternal-fetal concentration gradient. Insulin is responsible for overgrowth of babies born to mothers with poorly controlled diabetes mellitus. The high circulating levels of glucose in the mother transfer to the fetus and stimulate the fetal pancreas to produce insulin. In turn, the high blood levels of insulin in the fetus stimulate overgrowth and fat deposition. Insulin-like growth factors, IGF1 and IGFII, interact with insulin to promote growth of specific tissues such as liver, bones, and adipose tissue, while growth hormone has little effect on fetal body weight and is more important for postnatal growth.

Thyroid hormones are important regulators of fetal growth and the development of the skeletomuscular system, pancreas, brain, and skin. The actions of the thyroid hormones are mediated, in part, by changes in rates of oxygen consumption and oxidative metabolism, and therefore, the energy available for growth. Leptin may also have a role in the control of growth and development of specific fetal tissues, including the pancreas, brain and adipose tissue.

Glucocorticoids, such as cortisol, impair fetal growth in suboptimal intrauterine conditions, such as hypoxia and under-nutrition. These hormones are key physiological signals to suppress general body growth and to divert energy resources to important organs during stressful situations. In addition, glucocorticoids induce a switch in cell processes from proliferation to differentiation in several fetal tissues, which contributes to the normal decline in fetal growth rate near term. Over this period, glucocorticoids promote maturation of a variety of fetal organs in preparation for birth.

Importance of Normal Fetal Growth

The control of fetal growth has important consequences for the short term and longer life-time health of the individual. Neonatal mortality and morbidity is closely related to birth weight, with higher rates associated with the extreme range of birth weight (Basso et al., 2006; Fig. 7). A study in the USA examined the rates of neonatal death, within 28 days of birth, in relation to birth weight in singleton infants born between 1995 and 2000. When all live births were considered, the overall neonatal mortality rate was 38 per 10,000 births with particularly high rates in the smallest, often preterm, infants. The mortality rate was reduced to 8 per 10,000 live births when data from term infants only were assessed, although the relationship between mortality rate and birth weight was maintained (Fig. 7). A number of confounding factors may contribute to the increased risk of death in smaller babies: for example, maternal smoking causes intrauterine growth restriction and increases neonatal mortality by 30% (Basso et al., 2006). Overgrown babies, often born to diabetic mothers, are also at greater risk of neonatal death, in part as a result of obstructed and prolonged labor, and postnatal complications caused by neonatal hypoglycemia and respiratory distress syndrome.

Table 2 Effects of manipulation of hormones on fetal and placental growth

Hormone	Manipulation	Procedure	Species	Weight (% control)		Specific tissue effects
				Fetus	Placenta	
Insulin	Deficiency	Diabetogenic drug treatment	Sheep, monkey	80–85	—	Bone
		Fetal pancreatectomy	Sheep	70	100–110	Liver, spleen, brain
		Insulin gene deletion	Mouse	80	—	Pancreas
		Insulin receptor gene deletion	Mouse	90	100	Liver
		Pancreatic agenesis or insulin receptor defect	Human	50–80	—	
	Overexposure	Fetal insulin treatment	Sheep	100–120	100	Fat
			Monkey	135	—	Fat
IGFI	Deficiency	Maternal diabetes	Human	130	—	Fat
		Igf1 gene deletion	Mouse	60	100	Bone, skeletal muscle
		Igf1 gene defect	Human	50	80	Brain, auditory system
		Igf1r gene deletion	Mouse	45	100	Bone, skin, respiratory muscles
		Igf1r gene defect	Human	50–80	—	Bone, brain
	Overexposure	Fetal IGFI treatment	Sheep	100	100	Liver, lungs, heart, kidney, adrenal gland
IGFII	Deficiency	Igf2 gene deletion	Mouse	60	55	Bone, skeletal muscles
	Overexposure	Igf2 gene expression	Mouse	136	125	Pancreas
		Igf2r gene deletion	Mouse	140	140	Liver, skeletal muscle
		Beckwith–Wiedemann syndrome	Human	150	—	Liver, skeletal muscle
Glucocorticoids	Deficiency	Fetal adrenalectomy	Sheep	115	100	Liver, lungs, gut
	Overexposure	Maternal glucocorticoid treatment	Sheep, monkey, mouse	80–90	65–93	Brain, liver, lungs, heart
			Guinea-pig	84	—	Brain, liver
			Human	85	—	Lungs, gut
		Fetal glucocorticoid treatment	Sheep	80–100	100	Liver, lungs, gut
		Inhibition of placental 11 β -HSD2	Rat	88	—	
Thyroid hormones	Deficiency	11 β -HSD2 gene deletion	Mouse	84	90	
		Deletion of thyroid hormone receptor genes (TR α 1 and β)	Mouse	90	—	
		Fetal thyroidectomy	Sheep	70–80	100	Bone, skeletal muscle, liver, kidneys, heart, fat, pancreas, brain, nervous system
	Overexposure	Fetal thyroxine treatment	Sheep	100	—	Liver, heart
Leptin	Deficiency	Leptin gene deletion	Mouse	100	—	Brain
	Overexposure	Leptin receptor deletion	Mouse	106	100	
		Fetal leptin treatment	Sheep	100	100	Liver, fat, adrenal gland

Adapted from Fowden, A.L. and Forhead, A.J. (2009). Endocrine regulation of feto-placental growth. *Hormone Research* **72**, 257–265, and Sferruzzi-Perri, A.N., Vaughan, O.R., Forhead, A.J. and Fowden, A.L. (2013). Hormonal and nutritional drivers of intrauterine growth. *Current Opinion in Clinical Nutrition and Metabolic Care* **16**, 298–309.

Size at birth is also an important determinant of health in later life. The epidemiologist, Professor David Barker, and his colleagues at the University of Southampton examined the medical records of around 16,000 men and women born in Hertfordshire, UK between 1911 and 1930. Midwives at the time recorded birth weights and body proportions, and the mortality and morbidity statistics of these individuals were traced 50–70 years later. These studies identified an inverse relationship between birth weight and the incidence of death from cardiovascular disease (Barker, 1997; Fig. 8). For both sexes, the standardized mortality ratio for cardiovascular disease, which included coronary heart disease and stroke, was greatest in the individuals who were smallest at birth. This association between birth weight and adult mortality was continuous across the normal range of birth weights and was also independent of other known risk factors for cardiovascular disease; multiple, premature or severely growth-retarded babies were not included in the data analysis. Other risk factors such as smoking, alcohol intake, adult obesity, and social class were, however, additive to the effects of birth weight.

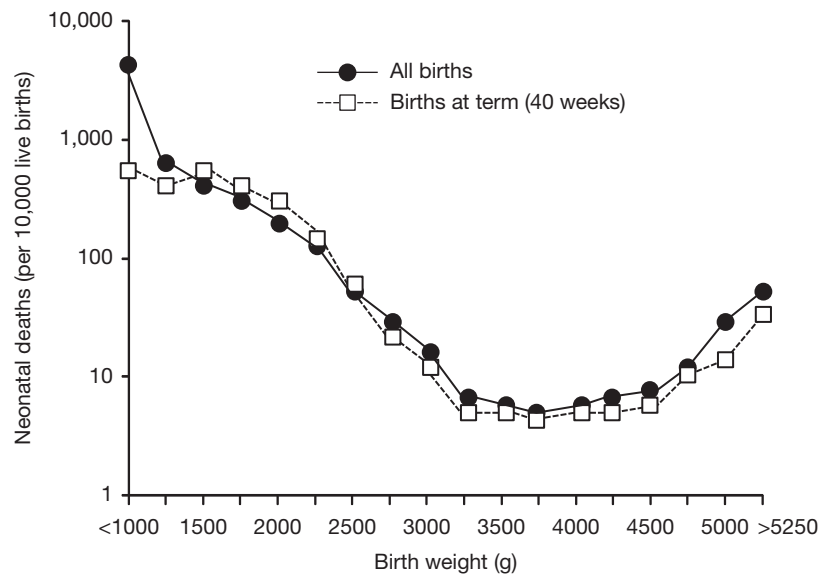


Fig. 7 Relationship between birth weight and neonatal mortality in singleton live births, United States, 1995–2000. All births (dotted line) and births at 40 weeks of gestation (solid line) by last menstrual period. Redrawn from Basso, O., Wilcox, A.J. and Weinberg, C.R. (2006). Birth weight and mortality: Causality or confounding? *American Journal of Epidemiology* **164**, 303–311.

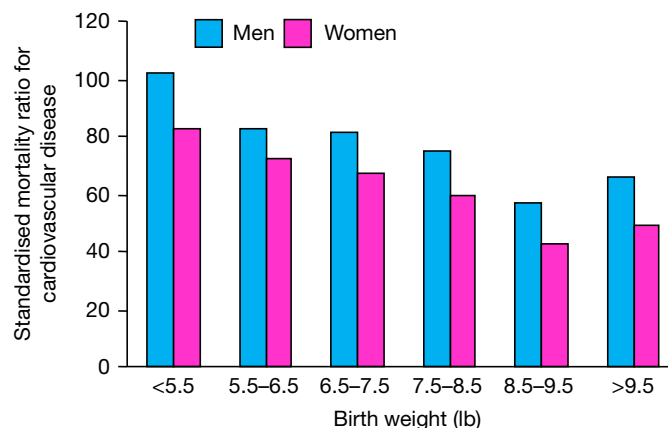


Fig. 8 Relationship between birth weight and risk of death from cardiovascular disease in men and women aged < 65 years. Redrawn from Barker, D.J. (1997). *Mothers, babies and disease in later life*. London: BMJ Publishing Group.

A number of conditions that predispose to cardiovascular disease have also been associated with low birth weight. Individuals that were small at birth have a higher incidence of hypertension, hyperlipidemia, glucose intolerance, and insulin resistance in adulthood. Further epidemiological studies have demonstrated that the relationship is reverse-J-shaped where large babies born to diabetic mothers are also at greater risk of cardiovascular disease and type II diabetes. Therefore, the intrauterine environment has an important role in programming adult pathophysiology, a phenomenon known as the developmental origins of adult disease (Gluckman and Hanson, 2006). Research is on-going to understand the mechanisms by which a suboptimal intrauterine environment, associated with abnormal patterns of fetal growth, leads to long-term consequences for adult health.

References

- Barker, D. J. (1997). *Mothers, babies and disease in later life*. London: BMJ Publishing Group.
- Basso, O., Wilcox, A. J., & Weinberg, C. R. (2006). Birth weight and mortality: Causality or confounding? *American Journal of Epidemiology*, *164*, 303–311.
- Brooks, A. A., Johnson, M. R., Steer, P. J., Pawson, M. E., & Abdalla, H. I. (1995). Birth weight: Nature or nurture? *Early Human Development*, *42*, 29–35.
- Fowden, A. L., & Forhead, A. J. (2009). Endocrine regulation of feto-placental growth. *Hormone Research*, *72*, 257–265.

- Fowden, A. L., & Forhead, A. J. (2013). Endocrine interactions in the control of fetal growth. In J. Bhatia, Z. A. Bhutta, & S. C. Kalham (Eds.), *Nestle nutrition institute workshop series: 74 vols. Maternal and child nutrition: The first 1,000 days* (pp. 93–104). Basel: Karger AG.
- Gluckman, P., & Hanson, M. (2006). *Developmental origins of health and disease*. Cambridge: Cambridge University Press.
- Kiserud, T., Piaggio, G., Carroli, G., Widmer, M., Carvalho, J., Neerup Jensen, L., Giordano, D., Cecatti, J. G., Abdel Aleem, H., Talegawkar, S. A., Benachi, A., Diemert, A., Tshetu Kitoto, A., Thinkhamrop, J., Lumbiganon, P., Tabor, A., Kriplani, A., Gonzalez Perez, R., Hecher, K., Hanson, M. A., Gülmezoglu, A. M., & Platt, L. D. (2017). Correction: The World Health Organization fetal growth charts: A multinational longitudinal study of ultrasound biometric measurements and estimated fetal weight. *PLoS Medicine*, 14(3), e1002284.
- Kramer, M. S. (1987). Determinants of low birth weight: Methodological assessment and meta-analysis. *Bulletin of the World Health Organisation*, 65, 663–737.
- Lumey, L. H. (1998). Compensatory placental growth after restricted maternal nutrition in early pregnancy. *Placenta*, 19, 105–111.
- Mellor, D. J., & Matheson, I. C. (1979). Daily changes in the curved crown rump length of individual sheep fetuses during the last 60 days of pregnancy and the effects of different levels of maternal nutrition. *Quarterly Journal of Experimental Physiology*, 64, 119–131.
- Mellor, D. J., & Murray, L. (1982). Effects of rate of increase in fetal girth of refeeding ewes after short periods of severe undernutrition during late gestation. *Research in Veterinary Science*, 32, 377–382.
- Moore, L. G. (2003). Fetal growth restriction and maternal oxygen transport during high altitude pregnancy. *High Altitude Medicine and Biology*, 4, 141–156.
- Morisaki, N., Kawachi, I., Oken, E., & Fujiwara, T. (2017). Social and anthropometric factors explaining racial/ethnic differences in birth weight in the United States. *Scientific Reports*, 7, 46657.
- Pomeroy, E., Wells, J. C., Cole, T. J., O'Callaghan, M., & Stock, J. T. (2015). Relationships of maternal and paternal anthropometry with neonatal body size, proportions and adiposity in an Australian cohort. *American Journal of Physical Anthropology*, 156, 625–636.
- Restrepo-Méndez, M. C., Lawlor, D. A., Horta, B. L., Matijasevich, A., Santos, I. S., Menezes, A. M., Barros, F. C., & Victora, C. G. (2015). The association of maternal age with birthweight and gestational age: A cross-cohort comparison. *Paediatric and Perinatal Epidemiology*, 29, 31–40.
- Robinson, J. S., Owens, J. A., & Owens, P. C. (1994). Fetal growth and fetal growth retardation. In G. D. Thornburn, & R. Harding (Eds.), *Textbook of fetal physiology* (pp. 83–94). Oxford: Oxford University Press.
- Sferruzzi-Perri, A. N., Vaughan, O. R., Forhead, A. J., & Fowden, A. L. (2013). Hormonal and nutritional drivers of intrauterine growth. *Current Opinion in Clinical Nutrition and Metabolic Care*, 16, 298–309.
- Smith, C. A. (1947). The effect of wartime starvation in Holland upon pregnancy and its product. *American Journal of Obstetrics and Gynecology*, 53, 599–608.
- Walton, A., & Hammond, J. (1938). The maternal effects on growth and conformation in Shire horse-Shetland pony crosses. *Proceedings of the Royal Society of London B*, 125, 311–335.

Amniotic Fluid

Ron Beloosesky, Rambam Medical Center at the Technion Faculty of Medicine, Haifa, Israel
Michael G Ross, Harbor-UCLA Medical Center, Torrance, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Amniotic fluid (AF) is essential for normal human fetal growth and development. The AF that surrounds the fetus serves as protection from mechanical trauma, and its anti-bacterial properties aid in maintaining a sterile intrauterine environment. Adequate AF volume (AFV) is necessary for normal development of both the lungs and the limbs. AF includes fetal cells and metabolic by-products, which allows access for fetal diagnosis more often than any other gestational tissue. The first amniocentesis was first performed in 1956 for fetal diagnosis (i.e., sex determination) ([Fuchs and Riis, 1956](#)).

Volume of AF

In the first trimester, the amniotic cavity is surrounded by the fluid-filled exocoelomic cavity and does not contact the placenta or decidua ([Calvo et al., 2002](#)). At this early gestational period the function of the AF is uncertain, as exchange of molecules between mother and fetus involves the exocoelomic fluid. As pregnancy progress the exocoelomic cavity progressively obliterates, and by the end of the first trimester the amniotic cavity is the only significant deposit of extra-fetal fluid. Measurement of AF volumes in the first half of pregnancy demonstrate logarithmical volume increases of AF ([Smith, 1971](#)).

The earliest estimations of AF volume were done during the latter two thirds of human pregnancies through the use of dye dilution techniques ([Geirsson et al., 1984](#)). Findings from these original quantitative findings have been supported by semiquantitative measurements of AF volume performed with ultrasound ([Geirsson et al., 1984](#); [Rolo et al., 2010](#); [Brace et al., 1989](#)). Both of these methods demonstrate a progressive increase in AF volume between 10 and 30 weeks of gestation. AF volume increases from less than 10 mL at 8 weeks ([Smith, 1971](#)) to 630 mL at 22 weeks and reaches 770 mL at 28 weeks' gestation ([Brace et al., 1989](#)), after which the increase slows, AF volume remains unchanged until 36–38 weeks. AF volume decreases sharply as pregnancy proceeds past the due date, averaging 515 mL at 41 weeks. Subsequently, there is a 33% decline in AF volume per week ([Gadd, 1966](#); [Beischer et al., 1969](#); [Queenan et al., 1968](#)), in accordance with the increased incidence of oligohydramnios in post-term gestations. The complex mechanisms that induce these AF volume alterations throughout gestation are not fully elucidated, though are likely attributable to changes in fluid production and resorption (see below).

In pathologic states, the volume of AF may be dramatically altered. Reduced fluid (oligohydramnios) may be near zero while excessive AF volume (polyhydramnios) may total many liters. The normal processes for production and resorption of AF may be affected by fetal anatomic abnormalities such as renal agenesis or esophageal atresia, leading to abnormal AF volumes. Acute or transient changes such as fetal anemia or maternal dehydration may alter AF production/resorption and therefore AF volume. AF volume abnormalities may also occur without apparent cause, though clinically recognized abnormalities of AF volume may be associated with poorer perinatal outcomes ([Chamberlain et al., 1984](#); [Gumus et al., 2007](#); [Volante et al., 2004](#); [Locatelli et al., 2004a](#)).

Production and Composition of AF

In the first trimester AF is isotonic with maternal or fetal plasma ([Campbell et al., 1992](#)) but contains minimal protein. AF demonstrates an extremely low oxygen tension and an increased concentration of sugar alcohols, the product of anaerobic metabolism ([Jauniaux et al., 2005](#)). In the second half of pregnancy, the urine produced by the human fetus is the major component of AF, causing the AF composition to diverge from that of serum. AF osmolality decreases with advancing gestation by 20–30 mOsm/kg to levels approximately 85%–90% of maternal serum osmolality ([Gillibrand, 1969](#)). While, AF urea, creatinine, and uric acid increase, the AF concentrations of urinary byproducts are two to three times higher than in fetal plasma ([Gillibrand, 1969](#)).

The early AF is thought to arise as a transudate of plasma, either from mother across the uterine decidua or the placenta surface or from the fetus through nonkeratinized fetal skin or both; however, the actual mechanism is unknown ([Faber et al., 1973](#)). AF production and resorption have been studied extensively in the second half of pregnancy, primarily in the sheep model. Findings suggest that the entire volume of AF turns over on a daily basis ([Gitlin et al., 1972](#)), representing a highly dynamic system. AF volume is influenced by a complex interplay of absorptive and productive mechanisms ([Seeds, 1980](#)), which act to maintain a physiologically normal volume, suggesting that they may be regulated to normalize AF volume in pathologic conditions.

There appear to be two major sources for both AF production and clearance near term: Production: fetal urine and fetal lung liquid. Clearance: fetal swallowing and intramembranous pathway. Smaller contributors may arise from flow across fetal skin, secretions from the fetal oral-nasal cavities, transudation across the umbilical cord and clearance by transmembranous pathway.

Intramembranous flow refers to water flow from the amniotic cavity to fetal vasculature, whereas transmembranous flow refers to water and solute exchange between AF and maternal blood across the decidua and myometrium ([Brace, 1997](#)). Transmembranous water and solute fluxes are immeasurably small, although these exchanges were once considered major contributors to AFV and composition. Both the intramembranous and transmembranous pathways permit flow of water and solutes in opposite

directions (i.e., osmotic flow of water and diffusion of solutes), while the other pathways only allow flow of water and solutes in the same direction (i.e., bulk flow) (Brace, 1997). Except for fetal metabolic water production, AF ultimately derives from water flow across the placenta from mother to fetus. Thus, alterations in maternal hydration can result in changes in AFV, presumably due to changes in transplacental water flow (Hanson et al., 1997; Ross et al., 1996).

Daily fetal urine excretion and fetal swallowing are the best-described processes for AF production and clearance. However, there remain major differences in the magnitude of current estimates of human fetal urine production (Wladimiroff and Campbell, 1974; Rabinowitz et al., 1989; Hedriana and Moore, 1994). We feel the best estimates of daily amniotic volume flows in the near term fetus are (Brace and Ross, 1998)

- Fetal urine production—800–1200 mL/day
- Fetal lung liquid secretion—170 mL/day
- Fetal swallowing—500–1000 mL/day
- Intramembranous flow—200–400 mL/day
- Oral-nasal secretions—25 mL/day
- Transmembranous flow—10 mL/day

These values are experimentally derived, in many cases using animal models.

Polyhydramnios

Polyhydramnios (also known as hydramnios) refers to an excessive volume of AF. Polyhydramnios has been associated with an increased risk of adverse pregnancy outcomes, including fetal anomalies, preterm birth and placental abruption (Golan et al., 1994; Many et al., 1995; Smith et al., 1992). Polyhydramnios should be suspected clinically when uterine size is large for gestational age. The diagnosis is made prenatally by ultrasound examination. The increase in AFV (AFV) in most cases is asymptomatic. However, the gravida may experience shortness of breath, uterine contractions or irritability, and abdominal discomfort when uterine distention is severe.

In a general obstetric population the incidence of polyhydramnios ranges from 1% to 2% (Hill et al., 1987; Dashe et al., 2002; Thompson et al., 1998; Biggio et al., 1999; Pri-Paz et al., 2012). Reported rates are influenced by variations in diagnostic criteria, subjective vs. quantitative assessments, the population studied (low or high risk) and the gestational age (preterm, term, or postterm) at examination. In one series of 93,332 singleton pregnancies, polyhydramnios was diagnosed during antepartum sonography in 708 pregnancies (0.7%); mild, moderate, and severe disease occurred in 66%, 22%, and 12% of cases, respectively (Dashe et al., 2002).

Etiology

Relatively minor decrease in fetal swallowing or increase in daily fetal urine production can result in a marked increase in AFV (Harding et al., 1984; Pritchard, 1965, 1966). Fetal structural anomalies commonly associated with polyhydramnios are those that interfere with fetal swallowing and/or absorption of fluid (Ben-Chetrit et al., 1990; Stoll et al., 1991). Decreased swallowing may be due to a primary gastrointestinal obstruction (e.g., duodenal, esophageal, or intestinal atresia), neuromuscular disorders (e.g., anencephaly), or to secondary obstruction of the gastrointestinal tract (e.g., massive unilateral dysplastic kidneys).

Fetal anomalies (often associated with an underlying genetic abnormality or syndrome) are the most common cause of severe polyhydramnios, while factors such as maternal diabetes are more often associated with milder cases. In one series of 272 singleton pregnancies with polyhydramnios, approximately 40% were considered idiopathic, one-third were associated with a congenital anomaly and one-quarter were associated with maternal diabetes (Abele et al., 2012). In up to 25% of cases considered idiopathic prenatally, an abnormality is diagnosed postdelivery (Abele et al., 2012; Dorleijn et al., 2009; Touboul et al., 2007, 2009). Neuromuscular disorders, Bartter syndrome, fetal infection and anemia, account for some of these cases and should be considered in the differential diagnosis. In a retrospective observational study of 294 singleton pregnancies with polyhydramnios only two patients tested positive for parvovirus infection and only one for toxoplasmosis infection (Pasquini et al., 2016). Among these patients, 72% were diagnosed with idiopathic polyhydramnios, 13% with maternal diabetes, 5% with fetal obstructive gastrointestinal lesions, 0.3% with Rhesus isoimmunization, and 1% with chromosomal abnormalities or genetic syndromes.

When occurring with fetal growth restriction, polyhydramnios is suggestive of trisomy 18 or 21.

Increased urine production may occur in high fetal cardiac output states (e.g., fetal anemia due to alloimmunization, fetomaternal hemorrhage, alpha-thalassemia, parvovirus infection, glucose-6-phosphatase deficiency) or, rarely, from entities such as fetal Bartter syndrome (Sieck and Ohlsson, 1984).

In monochorionic multiple gestation, polyhydramnios/oligohydramnios sequence is diagnostic of twin–twin transfusion syndrome (TTTS).

The mechanism for polyhydramnios in cases of maternal diabetes is unclear. Fetal polyuria due to hyperglycemia is one likely etiology, and is supported by the observation that polyhydramnios is often associated with high maternal glycated hemoglobin (A1C) levels and fetal macrosomia (Vink et al., 2006; Idris et al., 2010).

Clinical Significance

Many idiopathic cases resolve spontaneously, especially if mild (Golan et al., 1994; Odibo et al., 2016). However, polyhydramnios has been associated with an increased risk of several adverse outcomes in addition to the poor outcomes related to the associated morphologic abnormalities (Ross and Brace, 2001; Karahanoglu et al., 2016; Wiegand et al., 2016)

- Preterm labor, premature rupture of membranes (PROM), preterm delivery
- Maternal respiratory compromise
- Macrosomia
- Fetal malposition
- Abruptio upon rupture of membranes
- Umbilical cord prolapse
- Postpartum uterine atony

Fetal death risk in nonanomalous pregnancies affected by polyhydramnios is increased compared with normal AF pregnancies at each gestational age, with the greatest increase at term (adjusted odds ratio 5.5, 95% CI 4.1–7.6) (Pilliod et al., 2015). Mortality and morbidity increases with increasing severity of polyhydramnios in some, but not all, studies. The combination of polyhydramnios and small for gestational age fetus has a particularly poor prognosis due to the high prevalence of fetal structural and chromosomal abnormalities in this setting (Sickler et al., 1997). Neonatal outcomes associated with idiopathic polyhydramnios include an increased incidence of low 5-min Apgar score, newborn resuscitation, transient tachypnea of the newborn, ventilator requirement, admission to neonatal intensive care unit, hypoglycemia, jaundice and structural anomalies.

Diagnosis

Polyhydramnios diagnosis is based upon sonographic visualization of increased AFV. A 2014 consensus panel at a fetal imaging workshop suggested the following thresholds for polyhydramnios (Reddy et al., 2014).

- Single deepest pocket ≥ 8 cm
- AF index (AFI) ≥ 24 cm

Qualitative impression of mild, moderate, or severe polyhydramnios should be followed by a quantitative measurement, such as the AFI or single deepest pocket (Table 1). Quantitative approaches are standardized and provide a measurement that can be compared over serial examinations, even if sensitivity and positive predictive value (PPV) are low.

Invasive measurement of AFV is not used clinically. If an invasive method such as dye dilution is performed, polyhydramnios is defined as AFV more than two standard deviations above normal for the gestational age. At 34 weeks of gestation, this would be an AFV > 2200 mL (Brace, 1995). There are few large studies comparing the various methods of AF assessment in women with polyhydramnios against a gold standard (i.e., dye dilution) (Magann et al., 2000; Barnhard et al., 1995). One such study compared AFI 25 cm to a dye standard for diagnosis of polyhydramnios, and sensitivity, specificity, and PPVs and negative predictive values (NPVs) were 30%, 98%, 57%, and 93%, respectively (Chauhan et al., 1997).

Sonographic evaluation should be performed for fetal anomalies or fetal hydrops. The likelihood of identifying the etiology of polyhydramnios correlates with severity of fluid accumulation. In one series, an etiology was determined in only 17% of mild polyhydramnios, but in 91% of those with moderate or severe AF accumulation (Hill et al., 1987). In another study, the frequency of an anomalous infant with mild, moderate, and severe polyhydramnios was 8%, 12%, and 31%, respectively (Dashe et al., 2002). Eighty percent of anomalous infants with polyhydramnios were detected prenatally. The prevalence of aneuploidy in anomalous fetuses with polyhydramnios was reported to be 10% (Dashe et al., 2002). However, studies using microarray for genetic analysis have reported nearly 10% of fetuses with AF index (AFI) > 25 cm had genetic abnormalities while demonstrating a normal detailed fetal ultrasound examination (Allaf et al., 2015; Lamouroux et al., 2016; Boito et al., 2016). The abnormalities included trisomy 18 (10 cases), trisomy 21 (10 cases, sex chromosome aneuploidies (8 cases), tetrasomy 12 p (2 cases), microdeletions/microduplications (15 cases), and uniparental disomy 14 (1 case).

Congenital infection may be associated with fetal abnormalities (e.g., hydrops, growth restriction, cerebral ventriculomegaly, hepatosplenomegaly, intracranial and intraabdominal calcifications, ascites, and hyperechogenic fetal bowel). In the absence of maternal signs and symptoms or fetal findings, congenital infection is an unlikely cause of isolated polyhydramnios (Pasquini et al., 2016; Fayyaz and Rafi, 2012).

Table 1 Criteria for mild, moderate, and severe polyhydramnios

	<i>Mild</i>	<i>Moderate</i>	<i>Severe</i>
Single deepest pocket	8–11.9 cm	12–15.9 cm	≥ 16 cm
Amniotic fluid index	25–30 cm	30.1–35 cm	> 35 cm

Oligohydramnios

Oligohydramnios (reduced AFV) is usually diagnosed by ultrasound examination and may be described qualitatively (e.g., normal, reduced) or quantitatively (e.g., $AFI \leq 5$). Oligohydramnios may inhibit normal fetal movement and growth and can lead to umbilical cord compression, fetal deformation and death. The incidence of oligohydramnios is influenced by variations in diagnostic criteria, the gestational age at the time of the ultrasound examination (preterm, term, or postterm) and the population studied (low or high risk). In one large study of 3050 uncomplicated pregnancies with singleton non-anomalous fetuses between 40 and 41 6/7 weeks of gestation, oligohydramnios (defined as $AFI \leq 5$ cm) was observed in 11% (Locatelli et al., 2004b). The incidence is high in laboring women, largely due to rupture of fetal membranes during or just before labor (Rutherford et al., 1987; Ahn et al., 1989; Mercer et al., 1984).

Etiology

The etiologies of oligohydramnios vary according to the trimester in which they are diagnosed and the severity. The majority of women with oligohydramnios or borderline/low normal AFV have no identifiable cause. Conditions commonly associated with oligohydramnios are listed in Table 2. Homeostatic mechanisms, such as intramembranous absorption (transfer of AF across the amnion into the fetal circulation) serve to maintain AFV. These mechanisms are more successful in limiting excess fluid volume than in preventing reduced fluid volume. As an example, only two-thirds of fetuses with duodenal or proximal jejunal atresia and half of fetuses with esophageal atresia, develop polyhydramnios (Underwood et al., 2005) whereas renal agenesis invariably results in oligohydramnios.

The etiology of oligohydramnios in the first trimester is often unclear. Reduced AF prior to 10 weeks of gestation is rare because gestational sac fluid is primarily derived from the fetal surface of the placenta, transamniotic flow from the maternal compartment, and secretions from the surface of the body of the embryo. Criteria suggested for determining reduced AF at this gestational age have included a difference between mean gestational sac size and crown-rump length of less than 5 mm or a mean gestational sac diameter/crown-rump length ratio outside the normal range for gestational age (Bromley et al., 1991; Tadmor et al., 1994).

In the second trimester fetal urine begins to enter the amniotic sac and the fetus begins to swallow AF. Therefore, disorders related to the fetal renal/urinary system play a prominent role in the etiology of oligohydramnios (Table 3). Placental and maternal factors, as well as rupture of the fetal membranes, are also common causes of oligohydramnios in the second trimester.

The etiologies of mid-trimester oligohydramnios were illustrated in a series of 128 fetuses first noted to have severe oligohydramnios/anhydramnios at 13–24 weeks of gestation (Shipp et al., 1996a). The following etiologies were observed: fetal anomaly (51%), preterm premature rupture of membranes (PPROM) (34%), placental abruption (7%), fetal growth restriction (5%), and unknown (4%). Among the 65 anomalous fetuses, six were aneuploid. An elevated maternal serum alpha fetoprotein (MSAFP) concentration has also been linked to second trimester oligohydramnios, with or without an anomalous fetus. This combination carries an extremely poor prognosis: preterm delivery, fetal growth restriction, fetal death, neonatal death (Dyer et al., 1987). Oligohydramnios associated with an elevated MSAFP level may be caused by fetal membrane or placental damage, with leakage of AF or fetal blood into the maternal circulation (Los et al., 1992).

Oligohydramnios following amniocentesis appears to have a better prognosis. The membranes often “reseal” with reaccumulation of AF and normal pregnancy outcome.

Oligohydramnios first diagnosed in the third trimester is often associated with premature rupture of membranes or with uteroplacental insufficiency. Fetal growth restriction is frequently accompanied to oligohydramnios related to uteroplacental

Table 2 Causes of oligohydramnios

<i>Maternal</i>
Medical or obstetrical conditions associated with uteroplacental insufficiency (e.g., preeclampsia, chronic hypertension, collagen vascular disease, nephropathy, thrombophilia)
Medications (e.g., angiotensin converting enzyme inhibitors, prostaglandin synthetase inhibitors, trastuzumab)
<i>Placental</i>
Abruption
Twin to twin transfusion (i.e., twin polyhydramnios–oligohydramnios sequence)
Placental thrombosis or infarction
<i>Fetal</i>
Chromosomal abnormalities
Congenital abnormalities, especially those associated with impaired urine production
Growth restriction
Demise
Postterm pregnancy
Ruptured fetal membranes
<i>Idiopathic</i>

Table 3 Type and frequency of congenital anomalies associated with oligohydramnios in a literature review

Type	Number of cases (percent)
Renal	94 (65)
Multiple	17 (12)
Aneuploidy	12 (8)
Central nervous system	7 (5)
Skeletal system	5 (4)
Cardiovascular system	4 (3)
Other	6 (4)
Total	145

Adapted from Hill, LM. Clin Obstet Gynecol. 1997; 40:314.

insufficiency. Postterm AFV normally decreases often resulting in oligohydramnios, while many cases of third trimester oligohydramnios are idiopathic. There may be an association between oligohydramnios and pregnancy during the summer season, likely related to suboptimal maternal hydration in hot weather (Feldman et al., 2009). Mechanisms of isolated oligohydramnios also may include alterations in the expression of water pores (aquaporin 1, aquaporin 3) in fetal membranes and placenta (Zhu et al., 2009).

Oligohydramnios may be first suspected because the uterine size is less than expected for gestational age. Clinical diagnosis is based on the finding of decreased AF on ultrasound examination, with either subjective or objective ultrasound criteria. Although use of objective criterion is generally preferable (AF index ≤ 5 cm; single deepest pocket < 2 cm), subjective suspicion of AFV by experienced examiners has similar sensitivity for diagnosing reduced AFV.

AFI 5th %ile averages approximately 7 cm throughout gestation, an AFI ≤ 5 cm is greater than two standard deviations below the mean value (Magann et al., 1997; Moore and Cayle, 1990). For diagnosis of oligohydramnios in a multiple gestation, single deepest vertical pocket is utilized (< 2 cm).

Methods of Increasing AFV

No treatment of oligohydramnios has been proven to be effective long-term. Although, short-term improvement of AFV is possible and may be used under certain circumstances, such as when a fetal anatomic survey is needed.

Amnioinfusion can increase AFV temporarily. Transabdominal amnioinfusion of approximately 200 mL of saline under ultrasound guidance can provide better visualization of fetal anatomy.

Maternal hydration: In isolated oligohydramnios with no indication for delivery oral hydration with one to two liters of water can increase AFV transiently. Maternal hydration is easier and safer than intravenous fluid administration or amnioinfusion. The mechanism of hydration is likely via reduction of maternal plasma osmolality and sodium concentration, resulting in osmotically driven maternal to fetal water flux. A recent review found that maternal hydration was most effective in pregnancies with isolated oligohydramnios and that hypotonic solutions were more effective than isotonic fluids (Phelan et al., 1987).

Prognosis and Management

The prognosis of oligohydramnios depends on the severity, etiology, gestational age at onset, and duration of oligohydramnios. In the first trimester, oligohydramnios is an ominous finding, associated with a high incidence of spontaneous abortion. In the second trimester, pregnancies with borderline/low normal AFV generally have a good prognosis (Mercer et al., 1984) while severe oligohydramnios often ends in fetal or neonatal demise (Gizzo et al., 2015). In a series of 128 fetuses first noted to have oligohydramnios at 13–24 weeks of gestation, survival occurred in 20% of fetuses with PPROM, abruption, and idiopathic cases, 1.5% in fetuses with congenital anomalies, and none in fetuses with fetal growth restriction (Gizzo et al., 2015). Preterm delivery, spontaneous or indicated, occurs in $> 50\%$ of cases (Mercer et al., 1984; Dyer et al., 1987).

In the third trimester, some (Rutherford et al., 1987) but not all (Shipp et al., 1996b) studies have demonstrated an inverse relationship between AFV and the incidence of adverse pregnancy outcome. Adverse outcomes are related to umbilical cord compression, uteroplacental insufficiency, and meconium aspiration.

Idiopathic isolated oligohydramnios at term appears to have a better prognosis. In a retrospective cohort study (Ashwal et al., 2014), no increased risk for a composite adverse outcome were demonstrated when pregnancies complicated by thrombophilia, diabetes, hypertension, fetal growth restriction, or chromosomal/structural abnormalities were excluded.

Duration of oligohydramnios is also a prognostic factor. Idiopathic oligohydramnios diagnosed at an earlier gestational age are at risk for adverse perinatal outcomes compared with those presenting later in gestation (Shipp et al., 1996a). Given the potential for adverse outcome, patients are often delivered if at or near term, while preterm pregnancies may be hospitalized for frequent assessments or managed as outpatients (Clark et al., 1989; Carroll and Bruner, 2000).

References

- Abele, H., Starz, S., Hoopmann, M., et al. (2012). Idiopathic polyhydramnios and postnatal abnormalities. *Fetal Diagnosis and Therapy*, 32, 251.
- Sarno AP Jr, Ahn MO, Brar HS, et al. Intrapartum Doppler velocimetry, AFV, and fetal heart rate as predictors of subsequent fetal distress. I. An initial report. *American Journal of Obstetrics and Gynecology* 1989; 161:1508
- Allaf, B., Dreux, S., Schmitz, T., et al. (2015). AF biochemistry in isolated polyhydramnios: A series of 464 cases. *Prenatal Diagnosis*, 35, 133.
- Ashwal, E., Hirsch, L., Melamed, N., et al. (2014). The association between isolated oligohydramnios at term and pregnancy outcome. *Archives of Gynecology and Obstetrics*, 290, 875.
- Barnhard, Y., Bar-Hava, I., & Divon, M. Y. (1995). Is polyhydramnios in an ultrasonographically normal fetus an indication for genetic evaluation? *American Journal of Obstetrics and Gynecology*, 173, 1523.
- Beischer, N. A., Brown, J. B., & Townsend, L. (1969). Studies in prolonged pregnancy: 3. Amniocentesis in prolonged pregnancy. *American Journal of Obstetrics and Gynecology*, 103(4), 496–503.
- Ben-Chetrit, A., Hochner-Celnikier, D., Ron, M., & Yagel, S. (1990). Hydramnios in the third trimester of pregnancy: A change in the distribution of accompanying fetal anomalies as a result of early ultrasonographic prenatal diagnosis. *American Journal of Obstetrics and Gynecology*, 162, 1344.
- Biggio, J. R., Jr., Wenstrom, K. D., Dubard, M. B., & Cliver, S. P. (1999). Hydramnios prediction of adverse perinatal outcome. *Obstetrics and Gynecology*, 94, 773.
- Boito, S., Crovetto, F., Ischia, B., et al. (2016). Prenatal ultrasound factors and genetic disorders in pregnancies complicated by polyhydramnios. *Prenatal Diagnosis*, 36, 726.
- Brace, R. A. (1995). Progress toward understanding the regulation of AFV: Water and solute fluxes in and through the fetal membranes. *Placenta*, 16(1).
- Brace, R. A. (1997). Physiology of AFV regulation. *Clinical Obstetrics and Gynecology*, 40(2), 280.
- Brace, R. A., & Ross, M. G. (1998). AFV regulation. In R. A. Brace, M. A. Hanson, & C. H. Rodeck (Eds.), *Body fluids and kidney function: vol. 4. Fetus and neonate* (p. 88). Cambridge, MA: Cambridge University Press.
- Brace, R. A., Wolf, E. J., & Normal, A. F. V. (1989). Changes throughout pregnancy. *American Journal of Obstetrics and Gynecology*, 161(2), 382–388.
- Bromley, B., Harlow, B. L., Laboda, L. A., & Benacerraf, B. R. (1991). Small sac size in the first trimester: A predictor of poor fetal outcome. *Radiology*, 178, 375.
- Calvo, R. M., Jauniaux, E., Gulbis, B., et al. (2002). Fetal tissues are exposed to biologically relevant free thyroxine concentrations during early phases of development. *The Journal of Clinical Endocrinology and Metabolism*, 87(4), 1768–1777.
- Campbell, J., Wathen, N., Macintosh, M., et al. (1992). Biochemical composition of AF and extraembryonic coelomic fluid in the first trimester of pregnancy. *British Journal of Obstetrics and Gynaecology*, 99(7), 563–565.
- Carroll, B. C., & Bruner, J. P. (2000). Umbilical artery Doppler velocimetry in pregnancies complicated by oligohydramnios. *The Journal of Reproductive Medicine*, 45, 562.
- Chamberlain, P. F., Manning, F. A., Morrison, I., et al. (1984). Ultrasound evaluation of AFV: II. The relationship of increased AFV to perinatal outcome. *American Journal of Obstetrics and Gynecology*, 150(3), 250–254.
- Chauhan, S. P., Magann, E. F., Morrison, J. C., et al. (1997). Ultrasonographic assessment of AF does not reflect actual AFV. *American Journal of Obstetrics and Gynecology*, 177, 291.
- Clark, S. L., Sabey, P., & Jolley, K. (1989). Nonstress testing with acoustic stimulation and AFV assessment: 5973 tests without unexpected fetal death. *American Journal of Obstetrics and Gynecology*, 160, 694.
- Dashe, J. S., McIntire, D. D., Ramus, R. M., et al. (2002). Hydramnios: Anomaly prevalence and sonographic detection. *Obstetrics and Gynecology*, 100, 134.
- Dorleijn, D. M., Cohen-Overbeek, T. E., Groenendaal, F., et al. (2009). Idiopathic polyhydramnios and postnatal findings. *The Journal of Maternal-Fetal & Neonatal Medicine*, 22, 315.
- Dyer, S. N., Burton, B. K., & Nelson, L. H. (1987). Elevated maternal serum alpha-fetoprotein levels and oligohydramnios: Poor prognosis for pregnancy outcome. *American Journal of Obstetrics and Gynecology*, 157, 336.
- Faber, J. J., Gault, C. F., Green, T. J., et al. (1973). Chloride and the generation of AF in the early embryo. *The Journal of Experimental Zoology*, 183(3), 343–352.
- Fayyaz, H., & Rafi, J. (2012). Torch screening in polyhydramnios: An observational study. *The Journal of Maternal-Fetal & Neonatal Medicine*, 25, 1069.
- Feldman, I., Friger, M., Wiznitzer, A., et al. (2009). Is oligohydramnios more common during the summer season? *Archives of Gynecology and Obstetrics*, 280, 3.
- Fuchs, F., & Riis, P. (1956). Antenatal sex determination. *Nature*, 177(4503), 330.
- Gadd, R. L. (1966). The volume of the liquor amnii in normal and abnormal pregnancies. *The Journal of Obstetrics and Gynaecology of the British Commonwealth*, 73(1), 11–22.
- Geirsson, R. T., Patel, N. B., & Christie, A. D. (1984). In-vivo accuracy of ultrasound measurements of intrauterine volume in pregnancy. *British Journal of Obstetrics and Gynaecology*, 91(1), 37–40.
- Gillibrand, P. N. (1969). Changes in the electrolytes, urea and osmolality of the AF with advancing pregnancy. *The Journal of Obstetrics and Gynaecology of the British Commonwealth*, 76(10), 898–905.
- Gitlin, D., Kumate, J., Morales, C., et al. (1972). The turnover of AF protein in the human conceptus. *American Journal of Obstetrics and Gynecology*, 113(5), 632–645.
- Gizzo, S., Noventa, M., Vitagliano, A., et al. (2015). An update on maternal hydration strategies for AF improvement in isolated oligohydramnios and normohydramnios: Evidence from a systematic review of literature and meta-analysis. *PLoS One*, 10, e0144334.
- Golan, A., Wolman, I., Sagi, J., et al. (1994). Persistence of polyhydramnios during pregnancy—Its significance and correlation with maternal and fetal complications. *Gynecologic and Obstetric Investigation*, 37, 18.
- Gumus, I. I., Koktener, A., & Turhan, N. O. (2007). Perinatal outcomes of pregnancies with borderline AF index. *Archives of Gynecology and Obstetrics*, 276(1), 17–19.
- Hanson, R. S., Powrie, R. O., & Larson, L. (1997). Diabetes insipidus in pregnancy: A treatable cause of oligohydramnios. *Obstetrics and Gynecology*, 89, 816.
- Harding, R., Bocking, A. D., Sigger, J. N., & Wickham, P. J. (1984). Composition and volume of fluid swallowed by fetal sheep. *Quarterly Journal of Experimental Physiology*, 69, 487.
- Hedriana, H. L., & Moore, T. R. (1994). Accuracy limits of ultrasonographic estimation of human fetal urinary flow rate. *American Journal of Obstetrics and Gynecology*, 171, 98.
- Hill, L. M., Breckle, R., Thomas, M. L., & Fries, J. K. (1987). Polyhydramnios: Ultrasonically detected prevalence and neonatal outcome. *Obstetrics and Gynecology*, 69, 21.
- Idris, N., Wong, S. F., Thomae, M., et al. (2010). Influence of polyhydramnios on perinatal outcome in pregestational diabetic pregnancies. *Ultrasound in Obstetrics & Gynecology*, 36, 338.
- Jauniaux, E., Hempstock, J., Teng, C., et al. (2005). Polyol concentrations in the fluid compartments of the human conceptus during the first trimester of pregnancy: Maintenance of redox potential in a low oxygen environment. *The Journal of Clinical Endocrinology and Metabolism*, 90(2), 1171–1175.
- Karahanoglu, E., Ozdemirci, S., Esinler, D., et al. (2016). Intrapartum, postpartum characteristics and early neonatal outcomes of idiopathic polyhydramnios. *Journal of Obstetrics and Gynaecology*, 36(6), 710–714.
- Lamoureaux, A., Mousty, E., Prodhomme, O., et al. (2016). Absent or hypoplastic thymus: A marker for 22q11.2 microdeletion syndrome in case of polyhydramnios. *Journal of Gynecology Obstetrics and Human Reproduction*, 45, 388.
- Locatelli, A., Vergani, P., Toso, L., et al. (2004a). Perinatal outcome associated with oligohydramnios in uncomplicated term pregnancies. *Archives of Gynecology and Obstetrics*, 269(2), 130–133.
- Locatelli, A., Zagarella, A., Toso, L., et al. (2004b). Serial assessment of AF index in uncomplicated term pregnancies: Prognostic value of AF reduction. *The Journal of Maternal-Fetal & Neonatal Medicine*, 15, 233.
- Los, F. J., Beekhuis, J. R., Marrink, J., et al. (1992). Origin of raised maternal serum alpha-fetoprotein levels in second-trimester oligohydramnios. *Prenatal Diagnosis*, 12, 39.

- Magann, E. F., Perry, K. G., Jr., Chauhan, S. P., et al. (1997). The accuracy of ultrasound evaluation of AFV in singleton pregnancies: The effect of operator experience and ultrasound interpretative technique. *Journal of Clinical Ultrasound*, 25, 249.
- Magann, E. F., Chauhan, S. P., Barrilleaux, P. S., et al. (2000). AF index and single deepest pocket: Weak indicators of abnormal amniotic volumes. *Obstetrics and Gynecology*, 96, 737.
- Many, A., Hill, L. M., Lazebnik, N., & Martin, J. G. (1995). The association between polyhydramnios and preterm delivery. *Obstetrics and Gynecology*, 86, 389.
- Mercer, L. J., Brown, L. G., Petres, R. E., & Messer, R. H. A. (1984). Survey of pregnancies complicated by decreased AF. *American Journal of Obstetrics and Gynecology*, 149, 355.
- Moore, T. R., & Cayle, J. E. (1990). The AF index in normal human pregnancy. *American Journal of Obstetrics and Gynecology*, 162, 1168.
- Odiibo, I. N., Newville, T. M., Ounpraseuth, S. T., et al. (2016). Idiopathic polyhydramnios: Persistence across gestation and impact on pregnancy outcomes. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 199, 175.
- Pasquini, L., Seravalli, V., Sisti, G., et al. (2016). Prevalence of a positive TORCH and parvovirus B19 screening in pregnancies complicated by polyhydramnios. *Prenatal Diagnosis*, 36, 290.
- Phelan, J. P., Ahn, M. O., Smith, C. V., et al. (1987). AF index measurements during pregnancy. *The Journal of Reproductive Medicine*, 32, 601.
- Pilliod, R. A., Page, J. M., Burwick, R. M., et al. (2015). The risk of fetal death in nonanomalous pregnancies affected by polyhydramnios. *American Journal of Obstetrics and Gynecology*, 213, 410.e1.
- Pri-Paz, S., Khalek, N., Fuchs, K. M., Simpson, L. L., & Maximal, A. F. (2012). Index as a prognostic factor in pregnancies complicated by polyhydramnios. *Ultrasound in Obstetrics & Gynecology*, 39, 648.
- Pritchard, J. A. (1965). Deglutition by normal and anencephalic fetuses. *Obstetrics and Gynecology*, 25, 289.
- Pritchard, J. A. (1966). Fetal swallowing and AFV. *Obstetrics and Gynecology*, 28, 606.
- Queenan, J. T., Von Gal, H. V., & Kubarch, S. F. (1968). Amniography for clinical evaluation of erythroblastosis fetalis. *American Journal of Obstetrics and Gynecology*, 102(2), 264–274.
- Rabinowitz, R., Peters, M. T., Vyas, S., et al. (1989). Measurement of fetal urine production in normal pregnancy by real-time ultrasonography. *American Journal of Obstetrics and Gynecology*, 161, 1264.
- Reddy, U. M., Abuhamad, A. Z., Levine, D., et al. (2014). Fetal imaging: Executive summary of a joint Eunice Kennedy Shriver National Institute of Child Health and Human Development, Society for Maternal-Fetal Medicine, American Institute of Ultrasound in Medicine, American College of Obstetricians and Gynecologists, American College of Radiology, Society for Pediatric Radiology, and Society of Radiologists in ultrasound fetal imaging workshop. *Obstetrics and Gynecology*, 123, 1070.
- Rolo, L. C., Nardoza, L. M., Araujo, J. E., et al. (2010). Nomogram of AFV at 7 to 10+6 weeks of pregnancy by three-dimensional ultrasonography using the rotational method (VOCAL). *Archives of Gynecology and Obstetrics*, 281(2), 235–240.
- Ross, M. G., & Brace, R. A. (2001). National Institute of child health and development workshop participants. National Institute of child health and development conference summary: AF biology—basic and clinical aspects. *The Journal of Maternal-Fetal Medicine*, 10, 2.
- Ross, M. G., Nijland, M. J., & Kullama, L. K. (1996). 1-Deamino-[8-D-arginine] vasopressin-induced maternal plasma hypoosmolality increases ovine AFV. *American Journal of Obstetrics and Gynecology*, 174, 1118.
- Rutherford, S. E., Phelan, J. P., Smith, C. V., & Jacobs, N. (1987). The four-quadrant assessment of AFV: An adjunct to antepartum fetal heart rate testing. *Obstetrics and Gynecology*, 70, 353.
- Seeds, A. E. (1980). Current concepts of AF dynamics. *American Journal of Obstetrics and Gynecology*, 138(5), 575–586.
- Shipp, T. D., Bromley, B., Pauker, S., et al. (1996a). Outcome of singleton pregnancies with severe oligohydramnios in the second and third trimesters. *Ultrasound in Obstetrics & Gynecology*, 7, 108.
- Shipp, T. D., Bromley, B., Pauker, S., et al. (1996b). Outcome of singleton pregnancies with severe oligohydramnios in the second and third trimesters. *Ultrasound in Obstetrics & Gynecology*, 7, 108.
- Sickler, G. K., Nyberg, D. A., Sohaey, R., & Luthy, D. A. (1997). Polyhydramnios and fetal intrauterine growth restriction: Ominous combination. *Journal of Ultrasound in Medicine*, 16, 609.
- Sieck, U. V., & Ohlsson, A. (1984). Fetal polyuria and hydramnios associated with Bartter's syndrome. *Obstetrics and Gynecology*, 63, 22S.
- Smith, D. L. (1971). AFV: A measurement of the AF present in 72 pregnancies during the first half of pregnancy. *American Journal of Obstetrics and Gynecology*, 110(2), 166–172.
- Smith, C. V., Plambeck, R. D., Rayburn, W. F., & Albaugh, K. J. (1992). Relation of mild idiopathic polyhydramnios to perinatal outcome. *Obstetrics and Gynecology*, 79, 387.
- Stoll, C. G., Alembik, Y., & Dott, B. (1991). Study of 156 cases of polyhydramnios and congenital malformations in a series of 118,265 consecutive births. *American Journal of Obstetrics and Gynecology*, 165, 586.
- Tadmor, O. P., Achiron, R., Rabinowiz, R., et al. (1994). Predicting first-trimester spontaneous abortion. Ratio of mean sac diameter to crown-rump length compared to embryonic. *Journal of Reproductive Medicine*, 39(6), 459–462.
- Thompson, O., Brown, R., Gunnarson, G., & Harrington, K. (1998). Prevalence of polyhydramnios in the third trimester in a population screened by first and second trimester ultrasonography. *Journal of Perinatal Medicine*, 26, 371.
- Touboul, C., Boileau, P., Picone, O., et al. (2007). Outcome of children born out of pregnancies complicated by unexplained polyhydramnios. *BJOG*, 114, 489.
- Touboul, C., Picone, O., Levailant, J. M., et al. (2009). Clinical application of fetal urine production rate in unexplained polyhydramnios. *Ultrasound in Obstetrics & Gynecology*, 34, 521.
- Underwood, M. A., Gilbert, W. M., & Sherman, M. P. (2005). AF Not just fetal urine anymore. *Journal of Perinatology*, 25, 341.
- Vink, J. Y., Poggi, S. H., Ghidini, A., & Spong, C. Y. (2006). AF index and birth weight: Is there a relationship in diabetics with poor glycemic control? *American Journal of Obstetrics and Gynecology*, 195, 848.
- Volante, E., Gramellini, D., Moretti, S., et al. (2004). Alteration of the AF and neonatal outcome. *Acta Biomedica*, 75(Suppl 1), 71–75.
- Wiegand, S. L., Beamon, C. J., Chescheir, N. C., & Stamilio, D. (2016). Idiopathic polyhydramnios: Severity and perinatal morbidity. *American Journal of Perinatology*, 33, 658.
- Wladimiroff, J. W., & Campbell, S. (1974). Fetal urine-production rates in normal and complicated pregnancy. *Lancet*, 1, 151.
- Zhu, X. Q., Jiang, S. S., Zhu, X. J., et al. (2009). Expression of aquaporin 1 and aquaporin 3 in fetal membranes and placenta in human term pregnancies with oligohydramnios. *Placenta*, 30, 670.

Characteristics, Properties, and Functionality of Fetal Membranes: An Overlooked Area in the Field of Parturition

Laura Martin*, São Paulo State University (UNESP), São Paulo, Brazil; and The University of Texas Medical Branch at Galveston, Galveston, TX, United States

Lauren Richardson* and Ramkumar Menon, The University of Texas Medical Branch at Galveston, Galveston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Fetal–Placental–Fetal Membrane Development

Human fertilization begins when a male gamete or sperm fuses with the female gamete or oocyte, generating a unique, and diploid chromosome aggregation called the zygote. The zygote undergoes mitotic divisions to increase the number of embryonic cells and the newly formed morula migrates into the uterus and begins to fill with fluid to form a blastocyst. The telomere (DNA structure that protects chromosomal integrity by forming guanine-rich caps) length, a measure of the biologic age of a cell, and telomerase (an enzyme that maintains telomere length) have also been shown to peak at the blastocyst stage, possibly setting the biologic age of all fetal-derived cells. The blastocyst divides into two parts: an outer layer, the trophoblast, which will form the embryonic part of the placenta, and a group of centrally located blastomeres, the embryoblast, which will develop into an embryo (Watson, 1992).

Blastocyst implantation in the uterine endometrium coincides with the development of a small space in the embryoblast that will form the amniotic cavity. Morphologic changes in the embryoblast result in the formation of the embryonic disc, a bilaminar plate formed by two layers: the epiblast, which constitutes the floor of the amniotic cavity, and the hypoblast, which together with the exocoelomic membrane forms the primitive yolk sac. Isolated cavities or gaps that occur in the syncytiotrophoblast are filled with a mixture of maternal blood and cell debris. Oxygen and maternal blood nutrients present in these gaps become available for embryo development (Fig. 1A). The placenta is made up of fetal-derived tissues through which the fetus derives its nutritional support, maintains its immunological tolerance, and receives and produces regulatory hormones (e.g., progesterone, estrogen,

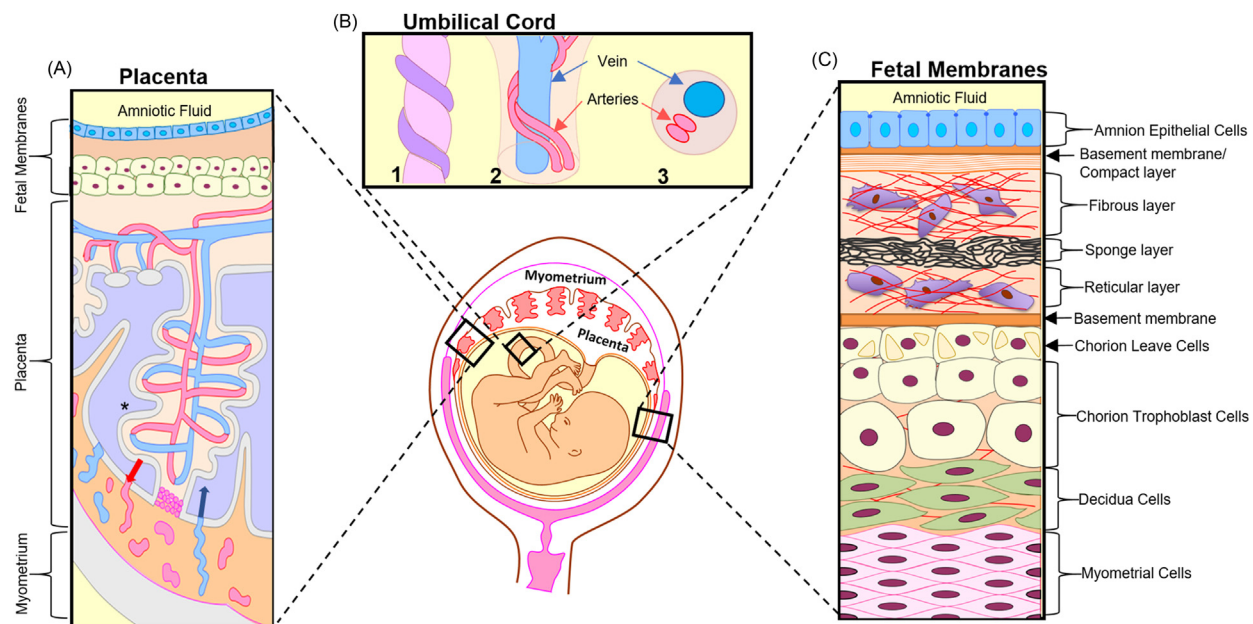


Fig. 1 Graphical representation of in utero organs representation of the entire intrauterine cavity containing the (from outside-in): maternal cavity, uterus/myometrium, placenta, fetal membranes, amniotic fluid, and fetus. (A) Represents the placenta, site of nutrients, and oxygen exchange for the growing fetus. The placenta is attached to the maternal side by the myometrium and the fetal side through the fetal membranes. The *black asterisk* identifies tertiary chorionic villi, while the *red* and *blue* arrows represent arteries and veins respectively. (B) Represents the umbilical cord in its external view (1), internal view (2), and cross-section (3). (C) The description starts from the innermost layer (amnion) and ends at the uterus. Amnion epithelial cells (*blue*) are connected the first layer of the ECM called the basement membrane (*orange*)/compact layer (*orange strips*). The fibroblast (*top red*), spongy (*black*), and reticular layers (*bottom red*) follow, containing stromal cells (*purple*). The chorion (*beige*) is connected to the ECM through a pseudo-basement membrane (*orange*). The chorion is made up of two types of cells: (1) chorion leave cells that contain vacuoles (*dark yellow circles*) and (2) the chorion trophoblast cells. The chorion interfaces with the decidua (*green*), connecting the fetal to the maternal compartments of the uterus.

*These authors contributed equally to this work.

corticotropin-releasing hormone (CRH), human chorionic gonadotropin (hCG), etc.) (Magon and Kumar, 2012) that are vital for pregnancy maintenance. Blood vessels present in the placenta allow the entry and exit of maternal blood, establishing the uteroplacental circulation. Blood circulation is connected to the growing fetus through the umbilical cord which is comprised of two arteries and a vein surrounded by connective tissue (Wharton's jelly) and covered by epithelial cells (Fig. 1B) to help remove CO₂ and other metabolic wastes. Connected to the fetal side of the placenta are the fetal membranes (amniochorion), which surround and line the intrauterine cavity (Fig. 1C). Fully formed amniochorion comprises amnion and chorion connected by an extracellular matrix region. The exact development process of amnion and chorion and their demarcation period into amniochorionic membranes as a unit are still unclear. The development of amnion and chorion begins with embryogenesis, although they do not participate directly in the formation of the embryo or fetus. Like the fetus, early growth of the amnion and chorion layers is rapid and independent of each other. The formation of amniochorion as a unit structure is completed between the 13th and 15th weeks of gestation (Fox, 1997). The growth and development of amniochorion correlate with fetal growth with a longevity of 40 weeks (term gestation period). The telomere length, as mentioned above, of these fetal-derived intrauterine structures has been shown to decrease from ~18 kb (22 weeks gestation) to ~7 kb (41 weeks gestation) during a 19-week period, suggesting (Menon et al., 2012) that all the components of the intrauterine cavity biologically age throughout pregnancy.

Amniochorion Membrane Organization

The amniochorion membrane (fetal or placental membrane) is one of the vital compartments of intrauterine cavity, performing mechanical, immune, and endocrine functions throughout gestation. The avascular fetal membranes are made up of a cuboidal amnion epithelium, a multilayered extracellular matrix (ECM) containing mesenchymal cells, and the chorion that connects to the decidua and myometrium (Fig. 1C), where the latter is maternal tissues. The fetal-derived components of the fetal membranes contain two primary layers: the amnion membrane (AM), consisting of amnion epithelial cells through half of the spongy layer, and the chorion membrane, which contains the other half of the spongy layer through the chorionic trophoblast cells. Both components include mesenchymal stromal cells (Bryant-Greenwood, 1998). By week 15, the amnion and chorion layers are fully formed and fuse to act as the barrier of the uterine cavity.

Amnion Membrane Components

Amnion Membrane

The AM is made up of an amnion epithelium, supported by basement membrane made of Type IV collagen. Beneath the basement membranes are stromal cells embedded in a collagen-rich ECM (compact, fibroblast, and half of the spongy layer). This section of the fetal membranes bears the most tensile strain and mechanistically keeps the whole fetal membrane intact as the fetus grows and develops. The AM is a source of several biologically active factors involved in tissue regeneration and remodeling, as seen in Table 1 (Litwiniuk and Grzela, 2014). Growth factors promote proliferation, migration, differentiation, and re-epithelialization during gestation. Specifically, epidermal and keratinocyte growth factor stimulates the proliferation and migration of epithelial cells, playing an important role in tissue remodeling (Litwiniuk and Grzela, 2014). In the same way, transforming growth factor beta (TGF- β) regulates cell proliferation, migration, and differentiation, as well as extracellular matrix remodeling. Moreover, TGF- β inhibits ECM degradation by increasing collagen production, suppressing the expression of metalloproteinases and stimulating their tissue inhibitors (Ogawa et al., 2004). It is critical that these processes remain in homeostasis throughout gestation to accommodate the changes associated with pregnancy, specifically fetal growth and increase in volume.

Amnion Epithelium

The amnion epithelial layer, bathed in amniotic fluid, is a single layer of cuboidal cells held together by neighboring cells gap junctions. Stress and stretch exerted on this layer make them undergo constant turnover as epithelial cells shed due to either apoptosis or senescence as the membranes age. Amnion cells secrete collagen Types IV and glycoproteins to form the basement membrane on their basal side. This creates a junction and allows communication between the amnion and ECM.

Amnion epithelial cells express a variety of surface markers that include pluripotent stem cells, mesenchymal, hematopoietic, and epithelial markers (Table 2). Current research from our lab shows that amnion epithelial cells and amnion mesenchymal cells co-express both epithelial and mesenchymal intermediate filaments (Cytokeratin-18 and Vimentin), highlighting the intermediate state (metastate) of these cells (Fig. 2), regardless of gestational age. This is indicative of constant turnover of these cells and remodeling of membranes. Regulated inflammatory activity by amnion epithelial cells, with the expression of antiinflammatory proteins like TGF- β , IL-10, IL-1 receptor-agonist and tissue inhibitors of matrix collagen degrading enzymes metalloproteinases (García-Castro et al., 2015), along with pro-inflammatory mediators, promote tissue remodeling throughout gestation.

Extracellular Matrix

In general, the AM ECM is comprised of many layers each with a unique composition of collagens and elastin. Collagen is a major component of the fetal membranes ECM. Elastin and microfibrils give the membranes their viscoelastic properties and allow them

Table 1 Secreted factors of amnion membranes

<i>Tissue type</i>	<i>Categories</i>	<i>Specific name</i>	<i>Abbreviation</i>
Amnion membrane	Tissue regeneration and remodeling	Epidermal growth factor	EFG
		Vascular endothelial growth factor	VEGF
		Keratinocyte growth factor	KGF
		Fibroblast growth factor	FGF
		Hepatocyte growth factor	HGF
		Platelet-derived growth factor	PDGF
		Transforming growth factor alpha and beta	TGF- α and TGF- β
	Antiscarring/fibrotic	Angiotensin	
		Interleukin 8	IL-8
		Transforming growth factor beta	TGF- β
		Hepatocyte growth factor	HGF
	Antibacterial	Interleukin 10	IL-10
		Defensins	
	Antimicrobial	Cathelicidin	
		Elafin	SKALP
	Antiinflammatory	Secretory leucocyte proteinase inhibitor	SLPI
		Interleukin 10	IL-10
		Interleukin-1 receptor antagonist	IL-1RA
		Prostaglandin E2	PGE2

Table 2 Characteristics of amnion epithelial cells

<i>Name of product</i>	<i>Preservation methods</i>	<i>Useful properties</i>
Grafix	Cryopreserved placental allografts	Increase wound closure, shorten healing time, lead to fewer adverse events, and less wound related infection
NEOX Wound Allograft	Cryopreserved placental allografts and umbilical cord	Promote complete epithelialization and speed up wound recovery time
EpiFix	Dehydrated fetal membranes	Contribute to complete wound closure uses less graft material, and delivers a lower median cost to treatment providers
AmnioExcel	Dehydrated fetal membranes	Significant increase in wound closure and a more rapid recovery

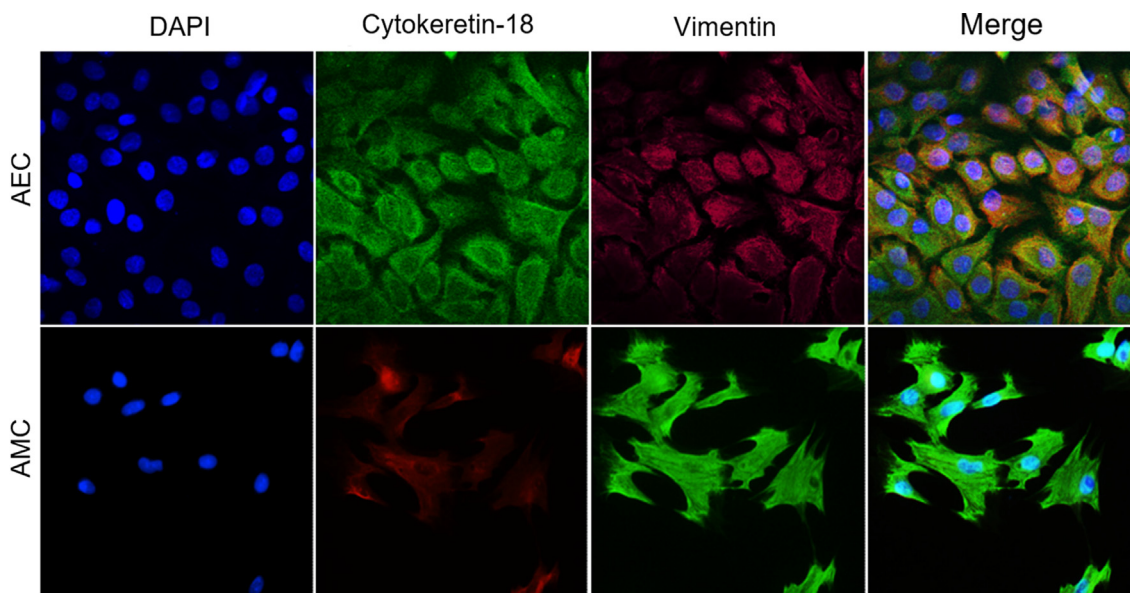


Fig. 2 Amnion epithelial cells and amnion mesenchymal cells co-express epithelial and mesenchymal factors. Confocal immunofluorescence images of amnion epithelial cells (AEC) and amnion mesenchymal cells (AMC) showing the co-expression of cytokeratin-18 and vimentin. In the AECs, cytokeratin-18 is *green* and vimentin is *pink*, while in the AMCs cytokeratin-18 is *red* and vimentin is *green*.

to withstand the stretch and strain associated with fetal growth. Formed by precursor proteins called tropoelastins, elastin forms elastic fibers outside of cells. The cross-linkage of these fibers forms microfibrils, which are abundant in the stromal layers of the AM. Fibronectin, laminins, proteoglycans, hyaluronan, and other types of collagen also play a significant structural and functional role in the development and remodeling of fetal membrane ECM.

Underneath the basement membrane of the amnion epithelium lies the second ECM layer of the AM, the compact layer. This layer is made up of Type 1 and 3 collagens which account for the high tensile strength of AM. Stromal mesenchymal cells secreting Type 1 and 3 collagens create the compact layer and fibroblast layer, forming a fibrous skeleton maintaining amnion integrity (Strauss, 2012). The fibroblast layer connects to the spongy layer made up of proteoglycans, glycoproteins (Strauss, 2012), and Type 3 collagen, producing a spongy meshwork. The reticular layer, made up of fibrillar bundles of collagen, is attached to the spongy layer on its apical side and the chorion pseudo-basement membrane, made of Type 4 collagen, on its basal side.

Amnion Stromal Cells

The amnion ECM layers are filled with amniotic mesenchymal stromal cells, fibroblasts, and small amounts of tissue macrophages (Uchide et al., 2012). These cells can interact with each other, produce inflammatory mediators including enzymes to degrade collagen matrix, and communicate with cellular layers to help with the fetal membrane remodeling. Amniotic mesenchymal cells are characterized morphologically by a small cell body with a round nucleus with a prominent nucleolus. They are long and thin and express various stem cell, mesenchymal, and immune-privileged markers (Huo et al., 2010) (Table 3). Fibroblasts in the amnion ECM are very versatile cells that produce biochemical mediators like growth factors, cytokines, and proteases, and are involved in tissue repair and regeneration (Table 3) (Costa-Almeida et al., 2017). These cells share several characteristics with mesenchymal stem cells (Table 3). Amnion mesenchymal cells and fibroblasts are responsible for making and redistributing ECM components that are critical for cellular and structural support of the fetal membranes throughout gestation.

Chorion Membrane Components

Chorion Membrane

As discussed before, it is thought that the amnion layer is a structural and mechanical barrier of the fetus, whereas the chorion layers prevent amnion degradation and protect the fetus from the maternal immune system. The formation of chorion starts by day 14, when mesenchymal cells, derived from the embryonic disc, spread underneath the inner surface of the cytotrophoblast layer of the implanted blastocyst and form the chorionic sac. The chorion consists of two layers: an outer formed by the trophoblast, and an inner formed by the somatic mesoderm. The trophoblast is made up of an internal layer of cubical cells, named cytotrophoblast, and an external layer of richly nucleated protoplasm devoid of cell boundaries, the syncytiotrophoblast.

Table 3 Characteristics of stromal cells

<i>Tissue type</i>	<i>Categories</i>	<i>Specific name</i>	<i>Abbreviation</i>
Amnion epithelial cells	Pluripotency markers	Sex determining region Y- box 2	SOX2
		Octamer-binding transcription factor 4	4-Oct
	Stem cell markers	NANOG	
		Stage-specific embryonic antigen 3	SSEA-3
		Stage-specific embryonic antigen 4	SSEA-4
		TRA1-60	
	Mesenchymal and hematopoietic markers	TRA1-81	
		Endoglin	CD105
		Thymocyte differentiation antigen 1	Thy-1 or CD90
		Neprilysin	CD10
		Aminopeptidase	CD13
		CD17	
		CD44	
		Integrin beta-1	CD29
		Human leukocyte antigen A	HLA-A
		Human leukocyte antigen B	HLA-B
		Human leukocyte antigen C	HLA-C
	Epithelial markers	N-cadherin	CDH2
		Vimentin	VIM
		E-cadherin	CDH1
		Cytokeritin-18	CK-18
		Cytokeritin-19	CK-19
		Cytokeritin-7	CK-7

The chorion is comprised of multiple layers of cytotrophoblast cells and a stroma that fuses with the amnion in the mature fetal membrane. These cells contain stem cell markers, cluster of differentiation (CD) CD34 and CD45, reinforcing the hematopoietic potential of the chorion. The chorionic membrane also contains more growth factor and more cytokines compared with amniotic membrane. The chorion shows higher levels of adiponectin, fibroblast growth factor, endocrine gland-derived vascular endothelial growth factor, hepatocyte growth factor, insulin-like growth factor-1, platelet-derived growth factor-AA and -BB, tissue inhibitor of metalloproteinase (TIMP)-2 and -4, while the amnion has higher levels of galectin-7, TGF- β 1, and interleukin-1F5 (McQuilling et al., 2017). Besides that, the chorion presents a stronger antibacterial effect than the amnion (Zare-bidaki et al., 2017). These studies reinforce the maternal role of the placenta in protecting the fetus against possible infections.

Summary

From the point of conception, highly unique, pluripotent stem cells that surround the fetus forming the AMs are vital to fetal survival. The amniochorion membranes are composed of multiple cellular and stromal layers containing epithelial and mesenchymal stem cells. These cells are used in a variety of fields, forming organs in vitro, and improving wound healing in clinics. By understanding where these cells come from and what their uses are in other areas, we can get a better idea of how they will act in vivo.

Membrane Properties That Maintain Pregnancy

Factors Maintaining Mechanical Integrity

Recently, it has been shown that progesterone, a pro-gestation hormone constant throughout pregnancy, inhibits signals such as tumor necrosis factor- α , thrombin, TGF- β , or granulocyte-macrophage colony-stimulating factor, which could weaken fetal membrane integrity. Contrary to the use of genomic receptors in maternal uterine tissues, progesterone uses the progesterone receptor membrane component (Feng et al., 2016) (PGRMC) to exert its function in the membranes. This unique ligand-receptor-mediated mechanism highlights progesterone's critical role in ECM homeostasis throughout gestation. The precise mechanism of progesterone- PGRMC mediated signaling is still not fully elucidated. However, this ligand receptor complex exerts a multitude of antiinflammatory properties in in vitro experiments. A decrease in PGRMC during term or preterm labor might contribute to the "functional withdrawal" of progesterone action and shift the balance to a state of inflammation (Zare-bidaki et al., 2017). This is partly facilitated by a constitutive expression of prostaglandin dehydrogenase (PGDH), an enzyme capable of degrading PGs, during gestation to minimize uterotonic functions and keep the uterus quiescent. A reduced functional role of progesterone and reduced expression of PGDH (which will increase bioavailable prostaglandin) may indicate a central role by human fetal membranes at the time of labor, at term or preterm.

Low levels of oxidative stress, especially during membrane remodeling, can help stimulate the small levels of inflammation needed to rebuild and modify the fetal membranes. AM also protects against large amounts of oxidative stress and inflammation through the production of superoxide dismutase, catalase, and expressing antiinflammatory peroxisome proliferator-activated receptor gamma. These factors prevent free radicals from causing DNA, lipid, and protein damage or cause inflammatory mediator activation. Low levels of prostaglandins also localize near sites of injury or infection to increase the levels of inflammation in order to stimulate tissue remodeling.

Role of Microfractures in Membrane Remodeling

Forming a watertight tight seal for the retention of amniotic fluid to protect the inner layers of the fetal membranes is another job of the amnion. Damage to this seal can cause a breakdown of homeostasis, and lead to catastrophic deterioration of the membranes. Temporary loss of cells and matrix collagens is inevitable during growth and remodeling; however, the impact is minimized by remodeling machinery. We have identified sites where such remodeling may occur during gestation, and these sites of fetal membrane remodeling are collectively called microfractures. Microfractures are not necessarily a fracture, but a term that is defined by amnion epithelial cell puckering, basement membrane degradation, collagen matrix degradation, and the development of tunnels with migratory cells. These sites are remodeled by cells and nascent collagen during normal gestation, but under pathological conditions, the persistence of microfractures and a lack of resealing could function as channels for leakage of the amniotic fluid (Fig. 3) (Richardson et al., 2017). We have noticed that the number and morphometric features (height, width, and depth) of microfractures are high in membranes from preterm premature rupture of the membranes (pPROM) and preterm birth compared to preterm births with intact membranes. This suggests that microfractures may serve as a clinical and biomarker for pPROM.

Membranes Contribution to Parturition

Cellular and Biological Regulators of Parturition

The fetal membranes are composed of tissues that are fetal in origin. At the maternal-fetal junction, fetal membranes adhere to the decidualized endometrium and form an immuno-mechanical interface. As mentioned in the opening paragraphs, fetal

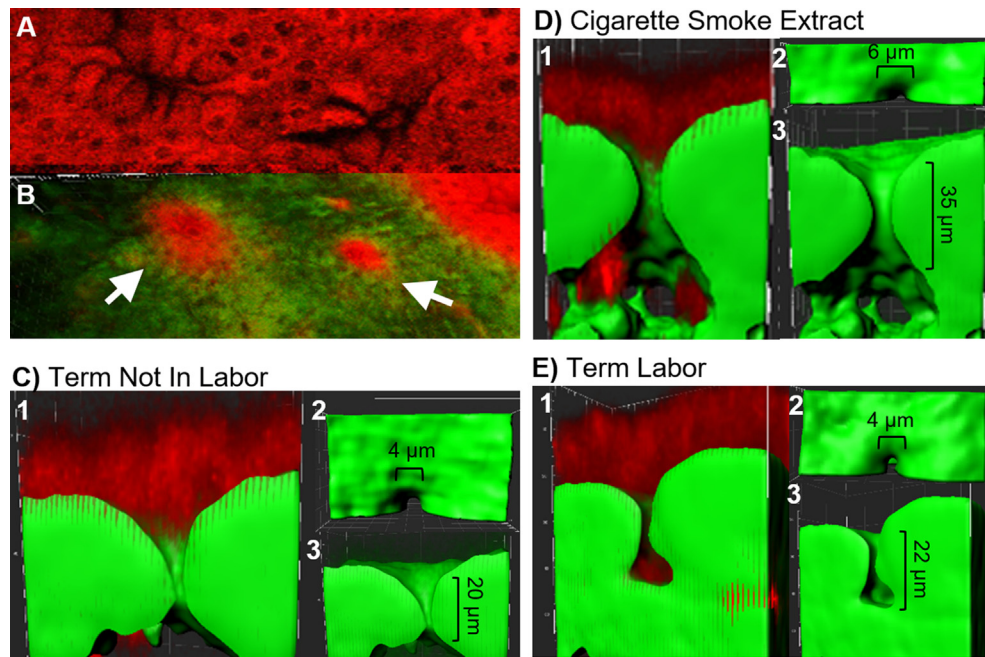


Fig. 3 Characteristics of microfractures. (A) Multiphoton autofluorescence microscopy (MPAM) images of amnion epithelial cell puckering above a microfracture. (B) MPAM and second harmonic generation microscopy image of deteriorated basement membrane (*white arrows*) under areas of altered amnion morphology (*puckering*). (C) Term not in labor (TNIL) microfracture processed with IMARIS demonstrating the average depth and width. (D) Term not in labor (TNIL) membrane exposed to cigarette smoke extract (CSE), an oxidative stress inducer in vitro, shows microfracture that demonstrates significantly deeper and wider microfractures similar to those seen in term labor. (E) Term labor microfracture processed with IMARIS, demonstrating deeper microfractures than TNIL.

membranes undergo a telomere-dependent aging coinciding with fetal growth and maturation. We have noticed that telomerase activity decreases in fetal membranes by the late 1st trimester, and it is minimal to negligible in the 2nd and 3rd trimesters. This suggests that fetal membranes are programmed to age naturally and progressively in utero. Aging is mechanistically mediated by molecular and cellular senescence. As membranes reach their longevity at term, aged membranes can initiate signaling of labor and delivery. Fetal membranes play a critical role in coordinating labor with the help of fetal signals of organ maturation. These fetal signals include endothelin, various growth factors, and chemokines, all of which are capable of increasing inflammation and oxidative stress in the uterine tissues, thus accelerating the membrane aging process (Menon, 2016).

Role of Senescence at Term

Senescence is a mechanism of tissue aging that can contribute to molecular, biochemical, and morphological level changes in the membranes. Amnion epithelial cells treated with cigarette smoke extract (CSE), a well-known oxidative stress inducer, leads to DNA damage and stress signaler p38 mitogen-activated protein kinase activation (MAPK). p38MAPK activation has been shown to cause senescence in fetal membranes as well as producing senescence-associated secretory phenotypes (SASPs), a unique group of inflammatory mediators comprising cytokines, chemokines, and various pro-inflammatory markers. SASPs create an environment of sterile inflammation in the fetal membranes. Besides, senescent fetal membranes also cause the release of damage-associated molecular patterns (DAMPs) from aged and oxidatively damaged tissues (Menon et al., 2016). SASPs and DAMPs are paracrine signalers released from fetal membranes and are capable of signaling parturition by propagating their inflammatory functional signals to the maternal side. We postulate that this signal propagation occurs in two different ways: diffusion through (1) microfractures, or (2) exosomes released from fetal membrane cells. Microfractures are higher in number and deeper at term (in vivo). Experimentally, we have shown that labor in in vitro conditions that mimic term labor also cause the increased appearance of microfractures with larger and deeper features. This suggests their relevance in the propagation of parturition signals. Exosomes are 30–150 nm size microvesicles that are capable of carrying contents from the cell of their origin. We have reported that exosomes from oxidatively stressed fetal membrane cells carry active forms of p38MAPK, SASPs, and DAMPs (Sheller et al., 2016). Thus, fetal membranes at term undergo senescence, they deteriorate and produce powerful pro-inflammatory signals that are sent as signals through various channels to convey to the maternal side the readiness of labor and delivery of the fetus.

(parturition). The primary purpose of these signals is to enhance the inflammatory load on the maternal side, a condition that is considered as the effector of labor and delivery.

Mechanical Contributions of Membranes at Term

Throughout gestation, the fetal membranes, layers of collagen extracellular matrix ($\sim 80 \mu\text{m}$) and cells, are responsible for upholding a protective barrier and watertight seal for the fetus and amniotic fluid. Membranes can withhold tensile and mechanical strain due to the elasticity of the AM and the variety of collagen and elastin that comprise the ECM. At term, a combination of programmed biochemical changes associated with senescence and physical stretch of membranes can lead to collagenolysis, leading to tissue damage (Moore et al., 2006). Excessive stretching and mechanical derangements result in the production of cytokines, prostaglandins, and collagenases, a phenomenon that contributes to the sensitization of the myometrium and the onset of uterine contractions (Waldorf et al., 2015). Stretching of membranes can also cause activation of p38MAPK, accelerating senescence and the release of DAMPs, contributing to mechanical changes. Endocrine factors like progesterone have been shown to reduce the weakness of fetal membranes and thus support pregnancy, whereas tumor necrosis factor- α and granulocyte macrophage colony stimulating factor, pro-labor factors prominent during infection or senescence, have been shown to induce fetal membrane weakening.

Membranes Contribution to Preterm Parturition

Diseases Contributed by Membranes

Preterm birth is defined as birth before the 37th week of complete gestation. Preterm labor and delivery can be medically induced or spontaneous. Spontaneous preterm birth can be subdivided into two categories: preceded by preterm labor with intact membranes, or preceded by pPROM. Among spontaneous preterm births, the incidence of preterm full-term is about 60%, and that of pPROM is 30%–40% (Menon, 2008; Beck et al., 2010). The etiologies, pathways, and mechanisms of spontaneous preterm birth and pPROM are still unclear; however, oxidative stress and inflammation arising from a multitude of risk exposures contribute to these syndromes. As our current understanding of the mechanistic events leading to these conditions is unclear, we have postulated novel mechanisms based on our findings on fetal membrane senescence.

Role of Premature Senescence

Fetal membrane senescence and senescence-associated sterile inflammation at term are normal physiologic responses arising due to the longevity of fetal membranes. This longevity coincides with fetal growth and development. However, with premature activation of senescence in response to various preterm birth risk factors, it may become a pathological factor. Similarities between senescence mechanism-associated factors in term labor and pPROM suggest fetal membranes' contribution to both these conditions. Both term and pPROM demonstrate heightened fetal membrane oxidative stress, oxidative stress-induced cellular, and organelle damage (nucleus, mitochondria, endoplasmic reticulum), stress-associated p38MAPK, senescence and SASP. In vitro studies showed that many of these events could be minimized or terminated by p38MAPK inhibitor, confirming a role for this mechanistic process (Dutta et al., 2015). Thus, fetal membrane senescence prematurely at preterm contributes to pathways of preterm birth.

Membrane Matrix Lysis in pPROM

The rupture of fetal membranes is a process involving collagen degradation. Collagen is a major structural component of the membranes, providing the strength to withstand physical stretch. Matrix metalloproteinases (MMPs) are the only enzymes that act to degrade collagen and play a major role in tissue remodeling. MMP types 2 and 9 degrade Type IV collagen, which is found in the basement membrane (Vadillo-Ortega et al., 1995). The activities of MMPs are regulated mainly by specific TIMPs, and a balance between activators and inhibitors controls the activity of MMPs. Reactive oxygen species, inflammation, and infection have been associated with an imbalance in MMP/TIMP activity, causing increased collagenolysis in the amnion cavity, which is directly related to a decrease in the tensile strength of fetal membranes. MMP-9 are increased in the amniotic fluid of women with intrauterine infection. MMP-2 are increased in membranes exposed to LPS in vitro.

Collagenolysis is mediated by various risk factors inducing inflammation (chorioamnionitis) and oxidative stress. These risk factors can cause increased senescence, apoptosis (programmed cell death), and necrotic types of changes in fetal membranes to activate a collagenolytic process predisposing membranes to rupture (Menon and Richardson, 2017).

Membrane Inflammation, Immune Disruption, and Infection in PTB

Intraamniotic inflammation is a major factor associated with preterm birth and pPROM. The fetal membranes can produce inflammation—sterile and/or infectious—that can arise from a multitude of pathologies and contribute to adverse events during pregnancy.

As described before, sterile inflammation is a feature of senescence-associated aging, and this is characterized mostly by the presence of inflammatory biomarkers, growth factors, and matrix-degrading enzymes. Granulocyte-macrophage colony-stimulating factor is increased in the intrauterine cavity in response to oxidative stress-induced fetal cell senescence. This, an inflammatory marker, can reduce fetal membrane structural strength and compromise fetal membrane integrity, causing the rupture (Murtha et al., 2015). On the other hand, the innate immune system plays a key role in regulating the parturition pathway. The host inflammatory response to microbial colonization and/or infection can determine the pregnancy outcome too. Special sentries of the innate immunity, named pattern recognition receptors (PRRs), recognize structurally stable microbial elements, termed pathogen-associated molecular patterns (PAMPs) (Kawasaki and Kawai, 2014). In addition to microbial ligands, PRRs have been shown to respond to endogenous molecules released by tissue or immune cells in response to injury or inflammation, referred to as DAMPs (Piccinini and Midwood, 2010).

Activation of toll-like receptors by specific PAMPs and DAMPs leads to a downstream cascade orchestrated by the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which results in the production of effector molecules, such as pro-inflammatory cytokines, chemokines, prostaglandins, proteases and other enzymes which can also cause the release of uterotonins to promote preterm parturition.

Another pathway that plays an important role in the processes of human labor and delivery is the activation of AP-1. Differently than the NF- κ B pathway, AP-1 signalization happens through activation of mitogen-activated protein kinases (MAPK). MAPK families play an important role in complex cellular programs like proliferation, differentiation, development, transformation, and apoptosis. At least three MAPK families have been characterized: extracellular signal-regulated kinase (ERK), Jun kinase (JNK/SAPK) and p38 MAPK. MAPK/AP-1 proteins are modulated by growth factors, cytokines, stress, and bacterial infections and play a part in the regulation of both pro- and antiinflammatory cytokines and in the metalloproteinase-9 activity in human fetal membranes (Lappas et al., 2011).

Maternal Factors That Contribute to PTB

There are many socioeconomic, behavioral, and health risks that affect preterm birth rates. Socioeconomic factors include maternal age, race or ethnicity, and socioeconomic and marital status have been shown to affect the timing of parturition. Maternal behaviors can also contribute to this. Women who smoke or take illegal drugs have a higher rate of preterm birth, specifically with ruptured membranes, while women who exercise and maintain a healthy diet have a lower risk. All these factors can contribute to fetal and fetal membrane homeostasis disruption, contributing to preterm birth pathways.

In summary, fetal membranes play a major role in human pregnancy maintenance and their natural aging can contribute to the inflammatory overload associated with labor and delivery.

Beyond Pregnancy: Beneficial Contributions of Fetal Membranes in Organ Regeneration and Tissue Remodeling

Since the early 19th century, physicians and researchers alike have been using either AM or intact fetal membranes for wound repair, as cell-based wound dressings can be beneficial in many settings. Clinical situations presenting in different fields such as diseases of the ocular surface, diabetic wound healing, surgical dehiscence, burns, deep wounds, and leg ulcers all use advanced cell-based wound dressings to induce healing of the epithelial layer (Litwiniuk and Grzela, 2014) (Fig. 4). There are many different forms of commercially available fetal membranes that are both cryopreserved and dehydrated which are useful in various clinical situations due to the abundance of different stimulating factors (Table 4). In obstetrics, the usefulness of fetal membrane ECM is restricted to filling gaps in the membrane to avoid premature rupture of membranes arising from fetal surgery. This lack of application is partly due to the poor understanding of the properties of amnion epithelial cells and other cells in their extracellular matrix.

Due to their epiblast origin, which is the source of all of the germ layers, the amniotic epithelial cells have a wide differentiation capacity. Recent studies have shown that these cells can differentiate into neurogenic epithelial (ectoderm), hepatic (endoderm), adipogenic, myogenic, osteogenic, and cardiac (mesoderm) lineages (Fig. 5A). These studies highlight the usefulness and ease with which amnion cells can act as pluripotent stem cells. The use of these cells in vitro is vital for researchers who are normally faced with other options involving ethical, legal, and religious concerns with the use of embryonic stem cells and the difficulties involved in obtaining stem cells from adult tissue.

Due to these characteristics, amnion mesenchymal cells and fibroblasts with potent stem cell characteristics are often used in wound healing, repair, and differentiation. In the same way as amnion epithelial cells, various studies have reported differentiation of amnion mesenchymal cells into ectoderm (neural), endoderm (pancreatic), hepatogenic, mesoderm (adipogenic) cartilage, osteogenic, and cardiac and trophoblastic lineages (Fig. 5B). The ability of these cells to differentiate into this variety of cell types and lineages under specific tissue/cell culture environments speaks volumes about its pluripotent nature; however, the ability of these cells to transition due to stimulation in vivo has not been addressed.

Many of the uses of AM in the field of wound healing mentioned above also use fetal membrane ECM devoid of amnion cells as a scaffold to promote wound healing. Removal of the amnion epithelial allows the healing tissues' own epithelial cells to colonize the ECM while receiving the beneficial growth factors and cytokines released from the amnion stromal cells.

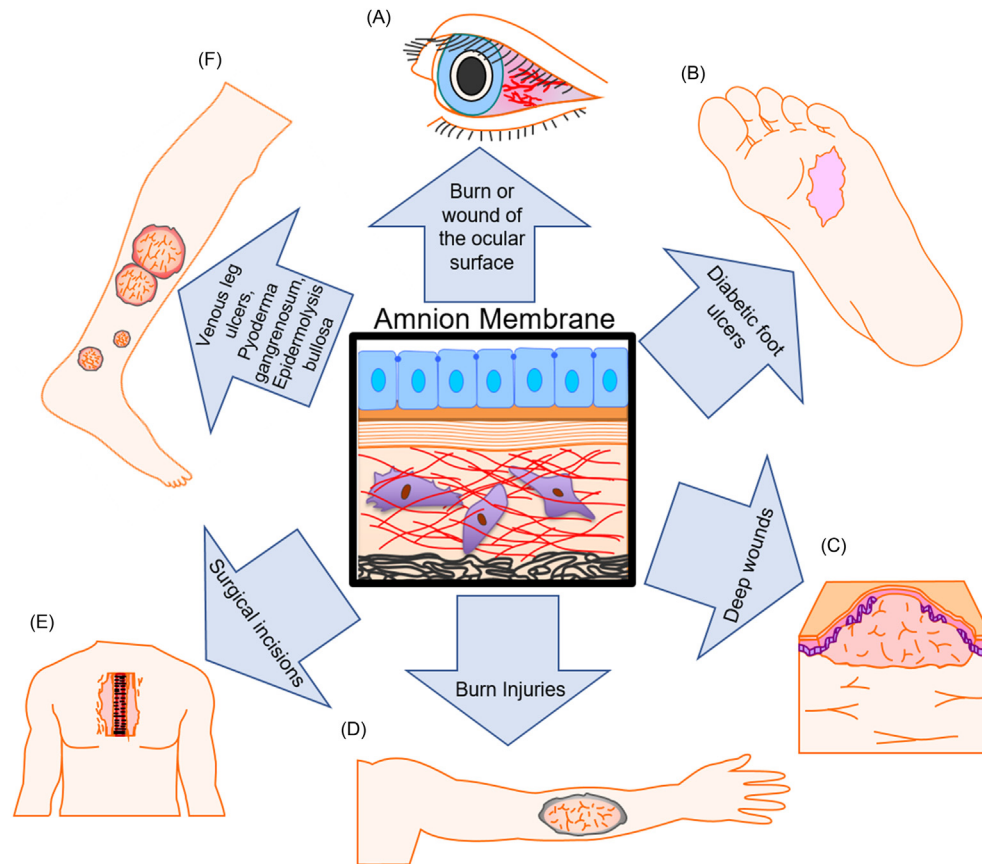


Fig. 4 Amnion membrane uses in wound healing. (A) In the ophthalmology field, amnion membranes are used to treat conjunctivitis scarring, acute burns, and persistent cornea defects. This cell-based dressing was chosen because of its transparent nature and its Type IV collagen basement membrane that promotes the expansion, migration, and invasion of epithelial progenitor cells. (B) In the endocrinology field, amnion membranes are used for diabetic foot ulcers as well as foot and ankle injuries such as chronic wounds, fasciitis, and tendonitis. Amnion membranes are used in these fields because they promote faster ulcer closure and help the healing of chronic wounds due to secreted factors. (C) and (D) In dermatology, amnion membranes are used for burns and deep wounds because they reduce pain, infection, increase mobility, and promote re-epithelization. (E) In the field of surgery, amnion membranes are used to help seal surgical incisions and close dehiscence due to their ability to promote closer healing of hard-to-heal wounds. (F) Venous leg ulcers, pyoderma gangrenosum, and epidermolysis bullosa are all treated with amnion membranes because they provide granulation in tissue, reduce pain, allow for proper epithelialization, and decrease the ulcer surface area and secretion of the fibrinous slough.

Table 4 Types of amnion membranes used clinically

Tissue type	Categories	Specific name	Abbreviation
Amnion mesenchymal cells	Pluripotency markers	Octamer-binding transcription factor 4	4-Oct
		Hepatocyte nuclear factor	HNF-3 β
	Immune privileged markers	NANOG	
		Neuroectodermal stem cell marker	Nestin
Both	Mesenchymal markers	Human leukocyte antigen A	HLA-A
		Human leukocyte antigen B	HLA-B
		Human leukocyte antigen C	HLA-C
		Endoglin	CD105
	Fibroblast markers	CD166/ALCAM	
		Cluster of differentiation 90	CD90
		CD44	
		Integrin beta-1	CD29
		ecto-5'-nucleotidase	CD73/NT5E
		CD9	
Fibroblast stromal cells	Fibroblast markers	Vimentin	VIM
		N-cadherin	CDH2
		Fibroblast specific protein-1	FSP-1/S100A4
		Melanoma cell adhesion molecule	CD146/MCAM

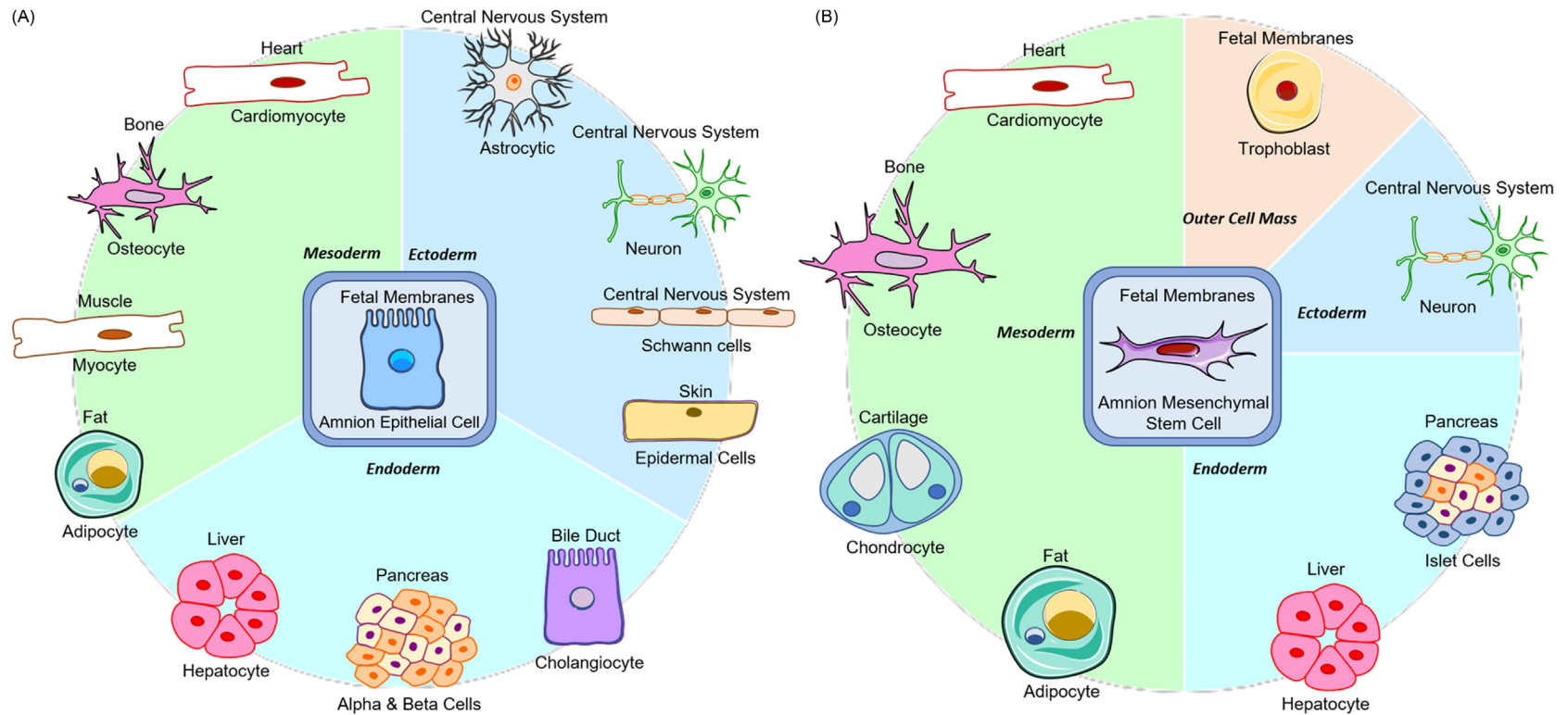


Fig. 5 Pluripotent lineages of amnion epithelial cells and amnion mesenchymal stem cells. (A) Amnion epithelial cells and (B) amnion mesenchymal stem cells can differentiate into cells that derive from the ectoderm, endoderm, mesoderm, and the outer cell mass. Each cell type graphically represented contains the *organ* or system name above and the name of the cell type below.

Summary

Membranes play a key role in the labor cascade by undergoing tissue- and cellular-specific changes that lead to the propagation of labor-initiating signals at term and preterm. As the fetal–maternal interface, if studied properly, the fetal membranes could answer many of the field's questions regarding term labor initiation, inflammation, infection, and diseases which lead to preterm birth. To better understand the mechanism behind the labor cascade, all aspects of fetal membrane origin, as well as cellular characteristics, need to be taken into account. Thus, providing a global understanding of the role of fetal membranes in parturition potentiates the usefulness of membranes during pregnancy, and in the prevention of adverse pregnancies.

Human fetal membranes (placental membranes, amniochorionic membranes) are the innermost lining of the intrauterine cavity. Membranes are fetal tissue in origin, start developing when fetal growth begins and becomes a fully formed structure by 15th week of pregnancy. Fetal membranes are constituted by amnion and chorion connected by collagen rich extracellular matrix forming the innermost layers of the intraamniotic cavity. Extracellular matrix made of fibrous proteins embedded in a polysaccharide gel and various collagen types provide the architectural and structural framework of the fetal membranes respectively. Amnion is constantly bathed in amniotic fluid, signifying its importance as a primary responder to changes in the amniotic cavity. Chorion is in close proximity to maternal decidua and maintains the immune tolerance at the maternal–fetal interface. Membranes perform multiple functions during pregnancy and act as the first line of defense to protect the fetus from mechanical and microbial insults. Membranes express over 400 functionally relevant biomarkers involved in membrane remodeling to maintain homeostasis during pregnancy. Hostile intrauterine environment can force homeostatic imbalance leading to its dysfunction that can contribute to adverse outcomes. Fetal membranes growth and functions are correlated very well with fetal growth; however, membranes are structurally and functionally independent from placenta. Recently, we have reported multiple evidence suggesting that fetal membranes undergo a telomere dependent aging process and membrane's longevity matches that of fetal growth and development. Histologic and molecular evidences suggest that intrauterine redox status helps the membrane to remodel during gestation whereas imbalance in redox status experienced at term accelerates fetal membrane senescence. Membrane senescence is mediated by stress responding kinase p38 mitogen activated kinase. Senescence associated fetal membrane inflammation increases overall uterine inflammatory load prompting parturition associated changes throughout uterine cavity. Thus, fetal membranes not only help to maintain pregnancy but also assist in delivery. Fetal membranes and cells of the fetal membranes are not dead tissues up on delivery. They are rich in pluripotent stem cells. Intact membranes or isolated membrane stem cells are used extensively for various medical applications. This book chapter highlights various functions of human fetal membranes that include, structure, function, biomarkers, mechanisms of membrane aging, its role in parturition and usefulness of fetal membranes and its stem cells postpartum.

Acknowledgments

This book chapter is made possible by funding support from NIH/NICHD (1R01HD084532-01A1) to R Menon.

References

- Beck, S., et al. (2010). The worldwide incidence of preterm birth: A systematic review of maternal mortality and morbidity. *Bulletin of the World Health Organization*, 88(1), 31–38. <https://doi.org/10.2471/BLT.08.062554>.
- Bryant-Greenwood, G. D. (1998). The extracellular matrix of the human fetal membranes: Structure and function. *Placenta*, 19(1), 1–11. [https://doi.org/10.1016/S0143-4004\(98\)90092-3](https://doi.org/10.1016/S0143-4004(98)90092-3).
- Costa-Almeida, R., Soares, R., & Granja, P. L. (2017). Fibroblasts as maestros orchestrating tissue regeneration. *Journal of Tissue Engineering and Regenerative Medicine*, 6125. <https://doi.org/10.1002/term.2405> (Epub ahead of print).
- Dutta, E. H., et al. (2015). Oxidative stress damage-associated molecular signaling pathways differentiate spontaneous preterm birth and preterm premature rupture of the membranes. *Molecular Human Reproduction*, 22(2). <https://doi.org/10.1093/molehr/gav074>.
- Feng, L., et al. (2016). The role of progesterone and a novel progesterone receptor, progesterone receptor membrane component 1, in the inflammatory response of fetal membranes to ureaplasma parvum infection. *PLoS One*, 11(12), 1–20. <https://doi.org/10.1371/journal.pone.0168102>.
- Fox, H. (1997). Aging of the placenta. *Archives of Disease in Childhood—Fetal and Neonatal Edition*, 77(3), F171–F175. <https://doi.org/10.1136/fn.77.3.F171>.
- García-Castro, I. L., et al. (2015). Markers of pluripotency in human amniotic epithelial cells and their differentiation to progenitor of cortical neurons. *PLoS One*, 10(12), 1–18. <https://doi.org/10.1371/journal.pone.0146082>.
- Huo, S., Shi, P., & Pang, X. (2010). Culture and identification of human amniotic mesenchymal stem cells. *Chinese Medical Sciences Journal*, 25(4), 211–214. [https://doi.org/10.1016/S1001-9294\(11\)60004-7](https://doi.org/10.1016/S1001-9294(11)60004-7).
- Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. *Frontiers in Immunology*, 5, 461. <https://doi.org/10.3389/fimmu.2014.00461>.
- Lappas, M., et al. (2011). MAPK and AP-1 proteins are increased in term pre-labour fetal membranes overlying the cervix: Regulation of enzymes involved in the degradation of fetal membranes. *Placenta*, 32(12), 1016–1025. <https://doi.org/10.1016/j.placenta.2011.09.011>.
- Litwiniuk, M., & Grzela, T. (2014). Amniotic membrane: New concepts for an old dressing. *Wound Repair and Regeneration*, 22(4), 451–456. <https://doi.org/10.1111/wrr.12188>.
- Magon, N., & Kumar, P. (2012). Hormones in pregnancy. *Nigerian Medical Journal*, 53(4), 179. <https://doi.org/10.4103/0300-1652.107549>.
- McQuilling, J. P., et al. (2017). Proteomic comparison of amnion and chorion and evaluation of the effects of processing on placental membranes. *Wounds*, 29(6), E38–E42. <http://www.ncbi.nlm.nih.gov/pubmed/28682294>.
- Menon, R. (2008). Spontaneous preterm birth, a clinical dilemma: Etiologic, pathophysiologic and genetic heterogeneities and racial disparity. *Acta Obstetrica et Gynecologica Scandinavica*, 87(6), 590–600. <https://doi.org/10.1080/00016340802005126>.
- Menon, R. (2016). Human fetal membranes at term: Dead tissue or signifiers of parturition? *Placenta*, 44, 1–5. <https://doi.org/10.1016/j.placenta.2016.05.013>.
- Menon, R., & Richardson, L. S. (2017). Seminars in perinatology preterm prelabor rupture of the membranes: A disease of the fetal membranes. *Seminars in Perinatology*, 1–11. <https://doi.org/10.1053/j.semperi.2017.07.012>.

- Menon, R., et al. (2012). Short fetal leukocyte telomere length and preterm prelabor rupture of the membranes. *PLoS One*, 7(2). <https://doi.org/10.1371/journal.pone.0031136>.
- Menon, R., et al. (2016). Placental membrane aging and HMGB1 signaling associated with human parturition. *Aging*, 8(2), 216–230.
- Moore, R. M., et al. (2006). The physiology of fetal membrane rupture: Insight gained from the determination of physical properties. *Placenta*, 27(11–12), 1037–1051. <https://doi.org/10.1016/j.placenta.2006.01.002>.
- Murtha, A. P., et al. (2015). Oxidative stress damage-associated molecular signaling pathways differentiate spontaneous preterm birth and preterm premature rupture of the membranes. *American Journal of Obstetrics and Gynecology*, 22(4), 447–448. <https://doi.org/10.1093/molehr/gav074>.
- Ogawa, K., et al. (2004). Suppression of matrix metalloproteinase-9 transcription by transforming growth factor- β is mediated by a nuclear factor- κ B site. *Biochemical Journal*, 381, 413–422. <https://doi.org/10.1042/BJ20040058>.
- Piccinini, A. M., & Midwood, K. S. (2010). DAMPening inflammation by modulating TLR signalling. *Mediators of Inflammation*, 2010, 1–21. <https://doi.org/10.1155/2010/672395>.
- Richardson, L. S., et al. (2017). Discovery and characterization of human amniochorionic membrane microfractures. *The American Journal of Pathology*, 187(12), 2821–2830. <https://doi.org/10.1016/j.ajpath.2017.08.019>.
- Sheller, S., et al. (2016). Amnion-epithelial-cell-derived exosomes demonstrate physiologic state of cell under oxidative stress. *PLoS One*, 11(6), e0157614. <https://doi.org/10.1371/journal.pone.0157614>.
- Strauss, J. F. (2012). Extracellular matrix dynamics and fetal membrane rupture. *Reproductive Sciences*, 20(2), 140–153. <https://doi.org/10.1177/1933719111424454>.
- Uchide, N., et al. (2012). Possible roles of proinflammatory and chemoattractive cytokines produced by human fetal membrane cells in the pathology of adverse pregnancy outcomes associated with influenza virus infection. *Mediators of Inflammation*, 2012, 270670. <https://doi.org/10.1155/2012/270670>.
- Vadillo-Ortega, F., et al. (1995). 92-kd type IV collagenase (matrix metalloproteinase-9) activity in human amniochorion increases with labor. *The American Journal of Pathology*, 146(1), 148–156.
- Waldorf, K. M. A., et al. (2015). Uterine overdistention induces preterm labor mediated by inflammation: Observations in pregnant women and nonhuman primates. *American Journal of Obstetrics and Gynecology*, 213(6), 830.e1–830.e19. <https://doi.org/10.1016/j.ajog.2015.08.028>.
- Watson, A. J. (1992). The cell biology of blastocyst development. *Molecular Reproduction and Development*, 33(4), 492–504. <https://doi.org/10.1002/mrd.1080330417>.
- Zare-bidaki, M., et al. (2017). Antimicrobial properties of amniotic and chorionic membranes: A comparative study of two human fetal sacs. *Journal of Reproduction & Infertility*, 18(2), 218–224.

The Human Fetal Adrenal Gland—A Review of Its Function and Development

Lucy X Chen and Bruce R Carr, University of Texas Southwestern Medical Center, Dallas, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

CYP 11B2 Aldosterone synthase.

CYP17 17 α -Hydroxylase/17,20-lyase (CYP17).

CYP21 21-Hydroxylase.

Dax-1 Dosage sensitive sex reversal adrenal hypoplasia congenital critical region on the X chromosome.

DHEA Dehydroepiandrosterone.

DHEAS DHEA sulfate.

DZ Definitive zone.

EGR1 Early growth response 1.

FZ Fetal zone.

HFA Human fetal adrenal.

HSD3B2 3 β -Hydroxysteroid dehydrogenase type 2.

NGFIB Nerve growth factor IB.

SULT2A1 DHEA sulfotransferase.

TZ Transition zone.

Introduction

Of all the endocrine glands in the human fetus, the adrenal has perhaps aroused the greatest interest. The human fetal adrenal (HFA) glands secrete very large quantities of steroid hormones; indeed it can be computed that the adrenals of some human fetuses near term secrete 100–200 mg steroids daily, most of which is in the form of dehydroepiandrosterone sulfate (DHEAS). The rate of steroid secretion by the fetal adrenals may be five times that of the adrenal of adults at rest (Siiteri and MacDonald, 1966; Easterling et al., 1966b). The human fetal adrenal has steroidogenic activities that may play a pivotal role in the normal development of the fetus and in the regulation of events leading to parturition.

During human pregnancy the rate of estrogen production increases strikingly. In fact, the production rate of estriol in near-term pregnant woman is 1000-fold greater than that in nonpregnant woman (Brown, 1956). The primary site of estrogen production is the placenta and it relies on the fetal adrenal gland to provide the essential C₁₉ steroid (DHEA/DHEAS) as substrates for placental estrogen (Pritchard and MacDonald, 1980). This was previously attributed to the fact that the human placenta lacks the steroid metabolizing enzyme 17 α -hydroxylase/17,20-lyase (CYP17) to convert progesterone or pregnenolone to DHEA. However, Escobar et al. (2011) have recently demonstrated the presence of CYP17 protein expression and activity in human trophoblasts in vitro, suggesting a larger contribution by the placenta in providing androgenic precursors than previously thought. The regulation of CYP17 in the placenta is still unclear. About 80% of maternal estriol is derived ultimately from fetal adrenal DHEA through the increased activity of placental aromatase. In addition, cortisol is also secreted by the adrenal gland during fetal life but, as will be discussed, at rates considerably less than that of DHEA.

Historical Background

The extremely large relative size of the human fetal adrenals has been recognized for almost 200 years (Lanman, 1953). The uniqueness of the HFA gland was first reported 100 years ago by four separate groups of investigators (Starkel and Wegrzynowski, 1910; Thomas, 1911; Kern, 1911; Elliott and Armour, 1911). They described the rapid growth of the HFA gland during fetal life and the existence of primarily two functionally and histologically distinct zones called the fetal zone (FZ) and the definitive zone (DZ) also known as the adult zone. Furthermore, this group of investigators demonstrated clearly the rapid involution of the former during the first few weeks after birth Fig. 1. The transient fetal zone is not present in most mammals and is unique to humans and a few nonhuman primates. This dramatic decrease in the HFA size after birth, up to 50% (Lanman, 1953), is largely attributed to atrophy of the FZ through apoptosis (Spencer et al., 1999).

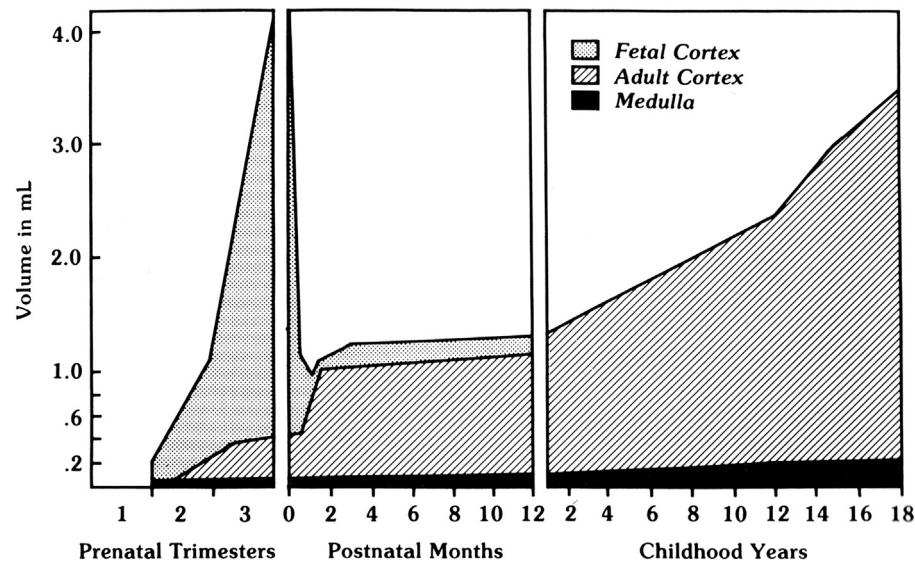


Fig. 1 Size of the adrenal gland and its component parts during fetal life, during infancy, and during childhood. Adapted from Bethune, J. E., *The adrenal Cortex: A scope monograph*, Upjohn, 1974.

Embryology and Histology

The cells comprising the adrenal cortex arise from coelomic epithelium. The cells of the fetal zone can be identified as early as the seventh week of gestation (Keene, 1927; Uotila, 1940). Thereafter, the adrenals of the human fetus continue to grow, and the most rapid rate of growth is detected during the last 6 weeks of gestation (Spector, 1956). At 28 weeks of gestation, the size of the adrenal gland may be equal to the size of the kidney, and the ratio of the weight of the adrenal to total body weight at term is 15–20 times that of the adrenals of the adult (Carr and Simpson, 1981b).

As mentioned above, the HFA is comprised of three histologically distinct zones; the fetal zone, the transitional zone, and the definitive zone also known as the neocortex. The fetal zone accounts for the bulk (80%–90%) of the HFA. It is located centrally and contains large eosinophilic cells with pale staining nuclei. It is the primary site of growth and steroidogenesis. The definitive zone, or neocortex, surrounds the fetal zone and comprises a narrow band of tightly packed cells that has a small quantity of cytoplasm and dark staining nuclei. Fig. 2. It is thought to give rise to the postnatal adrenal glomerulosa. In the second trimester, the transition zone (TZ) develops between the fetal zone and the definitive zone. Cells in the TZ have the capability to synthesize cortisol and is thought to give rise to the postnatal zona fasciculata.

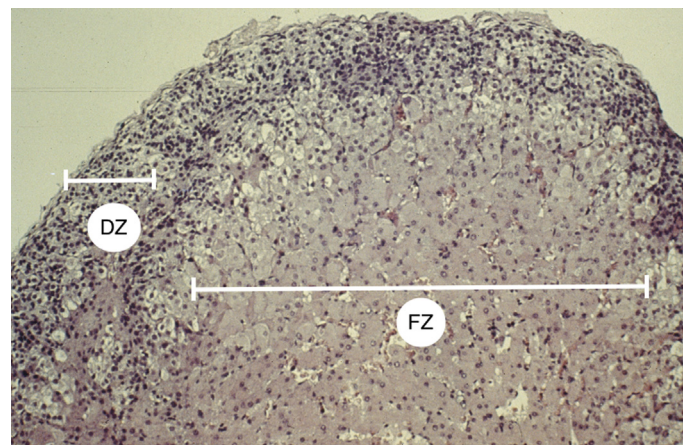


Fig. 2 Photomicrograph of histological section of the adrenal gland obtained from a 14-week-old abortus. The FZ accounts for the bulk (80%–90%) of the HFA and is the primary site of growth and steroidogenesis. The DZ surrounds the FZ and is thought to give rise to the adult adrenal glomerulosa. Original magnification, $\times 33$. FZ, fetal zone; DZ, definitive zone.

Function of the HFA

The primary function of the HFA is to provide steroids, i.e., DHEA and DHEAS, as precursors for the placenta to use in estrogen production. The zones of the HFA are functionally distinct in its steroidogenic roles (Suzuki et al., 2000; Mesiano et al., 1993; Mesiano and Jaffe, 1997). In addition to DHEAS, the fetal zone produces large amounts of other sulfated delta-5 steroids, including pregnenolone sulfate and 17 α -hydroxypregnenolone sulfate. The definitive zone expresses the enzymes needed for production of aldosterone but does not express 17 α -hydroxylase or DHEA—sulfotransferase (SULT2A1). During the second half of gestation the transitional zone expresses both 17 α -hydroxylase and 3 β -hydroxysteroid dehydrogenase type II (HSD3B2), allowing production of fetal cortisol. HSD3B2 is the enzyme needed for the conversion of delta-5 (pregnenolone) to delta-4 steroids (17 α -hydroxypregesterone). During most of gestation, the fetal adrenal lacks HSD3B2, preventing cortisol and aldosterone synthesis and directing steroid production toward DHEAS production. High expression of SULT2A1 in the fetal zone accounts for sulfonation of most of the delta-5 steroids produced, including DHEA and pregnenolone (Suzuki et al., 2000; Mesiano et al., 1993). Near birth, cells of the definitive zone, which express only HSD3B, acquire cytochrome p450 aldosterone synthase (CYP11B2) and begin to secrete mineralocorticoids such as aldosterone. These observations point to the regulation of steroidogenic enzymes being a critical determinant in modulating the biosynthetic capacity and the steroid products of the fetal adrenal gland.

It has been demonstrated that the levels of DHEA in cord plasma increase during pregnancy (Parker and Carr, 1981). After birth, plasma DHEA levels fall precipitously and remain low through childhood until adrenarche, when the levels again rise once more (Parker and Odell, 1980). In contrast, during fetal life cortisol is secreted in smaller quantities as determined on the basis of its concentration in cord plasma (Chang et al., 1976; Barnhart et al., 1980; Campbell and Murphy, 1977) and as determined in vitro studies of adrenal tissue during the first few days of culture (Huhtaniemi, 1977; Simpson et al., 1979; Carr et al., 1980; Branchaud et al., 1978). Although cortisol levels may increase slightly between early and late gestation (Pearson Murphy and Diez D'aux, 1972), there is little evidence to suggest changes in cord plasma cortisol concentration after 25 weeks until term, in contrast to DHEAS levels (Parker and Carr, 1981; Hauth et al., 1978). However, this cannot be construed to mean that the rate of cortisol secretion does not increase as a function of gestational age. As the fetus grows and matures, it is probably that fetal metabolic clearance of plasma cortisol increases. Indeed, the levels of cortisol and cortisol sulfate in amniotic fluid increases as pregnancy advances (Belisle and Tulchinsky, 1980). A significant amount of cortisol in plasma (25%–50%) is derived from maternal sources (Beitins et al., 1973). However, maternal cortisol is converted extensively to cortisone by the placenta during transfer to the fetus (Beitins et al., 1973; Murphy et al., 1974). It is believed that the low secretory rate of cortisol relative to that of DHEAS by the HFA is due to a relative deficiency or reduced activity of HSD3B2 in the fetal zone cells (Goldman et al., 1966; Cavallero and Magrini, 1967). It has been proposed that estrogens or other factors produced by the placenta are responsible for the inhibition of HSD3B2 (Voutilainen and Kahri, 1980).

Lipoproteins as a Source of Cholesterol for Fetal Adrenal Steroidogenesis

As has already been discussed, the adrenal glands of the near-term human fetus secrete large quantities of steroid hormones, 100–200 mg/day. This is a 5–10 times the rate of steroid secretion by the adrenal glands of adults at rest. An important question that arises is the origin of the precursor utilized by the fetal adrenals for steroid hormone biosynthesis.

Cholesterol serves as the principal precursor for steroid biosynthesis in the HFA. LDL binds to a population of receptors present in high concentrations on the cell membranes. Adrenocorticotrophic hormone (ACTH) causes an increase in the number of LDL binding sites, thus optimizing the uptake of LDL cholesterol for steroidogenesis. Fetal adrenals could obtain cholesterol for steroidogenesis from two sources: (1) de novo synthesis from C₂-units and (2) lipoproteins in the fetal plasma. The HFA is capable of de novo synthesis of delta 5-3 β -hydroxysteroid sulfate from C₁₄ acetate, however, based on the large capacity of the fetal adrenals for steroid biosynthesis, de novo synthesis can only account for 30% of daily steroid secretion (Parker and Schimmer, 1997). Thus the remainder of the cholesterol utilized by the HFA gland for steroid hormone biosynthesis is likely obtained from LDL in the fetal circulation. The placenta utilizes large quantities of maternal LDL-cholesterol that is taken up by the placenta and degraded. Because of the rapid rate of utilization of LDL by the HFA, fetal plasma levels of LDL are low and activity of the HFA is a primary determinant of these levels. Thus, in the case of anencephaly, in which the activity of the adrenal is very low, plasma levels of LDL are 2–3 times higher than in normal fetuses, whereas plasma HDL levels are unchanged (Carr and Simpson, 1981b). In addition, in the normal neonate plasma LDL levels rise rapidly after birth, and this event is coincident with the involution of the fetal zone of the adrenal. The fetal liver is likely to be the major source ultimately of the LDL-cholesterol utilized by the HFA. Consequently, factors that regulate cholesterol and lipoprotein synthesis in the fetal liver may, in turn, affect the steroidogenic activity of the HFA through regulation of the supply of cholesterol precursor. Thus, if trophic factors for the HFA other than ACTH exist, an important site of their action might be the fetal liver, rather than a direct action to influence the rate of synthesis of steroids by the fetal adrenal.

Gene Expression Differences in HFA

The HFA and adult adrenal gland have differences in structure and function that, until recently, was not well defined. It was hypothesized that gene expression likely played a critical role in defining their distinct steroidogenic and structural phenotypes. Recent

advances in molecular biotechnology, particularly with cDNA microarrays, has allowed for a more global understanding of gene expression in the HFA and adult adrenal gland.

Using cDNA microarrays, [Rainey et al. \(2001\)](#) contrasted human fetal (15–20 weeks) and adult adrenal gland gene expression profiles and discovered 69 transcripts that have greater than 2.5-fold differences in gene expression. Insulin-like growth factor—II (IGF-II) showed the most striking difference in expression with a 25-fold greater levels in the HFA than in the adult adrenal. In addition, the IGF-II transcript is one of the 20 transcripts consistently shown to have the highest signal intensities in the HFA. The enhanced HFA expression of IGF-II transcripts (second only to the liver) is well documented ([Han et al., 1988](#)). Not unexpectedly, the expression of 24-dehydrocholesterol reductase (DHCR24), an enzyme involved in cholesterol biosynthesis, was expressed at four times higher levels in the HFA than adult adrenal, again emphasizing the importance of de novo cholesterol biosynthesis in HFA steroid production.

Differences in gene expression profiles can result from numerous factors including alterations in key transcription factors that influence specific gene targets. [Rainey et al. \(2002\)](#) identified two transcription factor transcripts, early growth response-1 (EGR1) and nerve growth factor IB (NGFIB) that exhibited at least a 5-fold higher expression than seen in the HFA. EGR1 is a rapid response gene that controls the transcription of numerous response genes including phenylethanolamine *N*-methyltransferase gene in the adrenal medulla ([Ebert et al., 1994](#)). Its elevated expression in the adult adrenal over the HFA may be due to the presence of a fully developed medulla in the former. NGFIB is an important regulator of transcription of several steroidogenic enzymes including CYP21, HSD3B2 and CYP11B2 ([Bassett et al., 2004a,b](#); [Wilson et al., 1993](#); [Martin and Tremblay, 2005](#)). Both microarray and northern analysis have demonstrated a much higher expression of NGFIB in adult adrenal cortex under the control of ACTH ([Davis and Lau, 1994](#)). Multiple studies using transfection into adrenocortical cells have shown that NGFIB is a potent enhancer of HSD3B2 transcription ([Goto et al., 2006](#); [Bassett et al., 2004b](#)).

An extensive body of evidence in identifying steroidogenic factor 1 (SF-1) and dosage-sensitive sex reversal adrenal hypoplasia congenital critical region on the X chromosome (Dax-1), two nuclear transcription factors, as essential for the development and differentiation of the adrenal cortex ([Parker and Schimmer, 1997](#); [Morohashi, 1999](#); [Bakke et al., 2001](#); [Lalli and Sassone-Corsi, 2003](#); [Lyer and McCabe, 2004](#)). SF1 is detected in the HFA from its earliest stages of development and is expressed in all zones of the human adult adrenal cortex. SF-1 regulates the transcription of a diverse array of genes involved in steroidogenesis, reproduction, and sexual differentiation ([Parker and Schimmer, 1997](#); [Parker et al., 2002](#)). Human patients with SF-1 mutations have primary adrenal insufficiency and sex reversal ([Beuschlein et al., 2002](#); [Achermann et al., 1999](#); [Hammer et al., 2005](#)). Dax-1, another orphan nuclear receptor, is known to be responsible for human X-linked congenital adrenal hypoplasia, which is characterized by adrenal insufficiency in infancy and hypoplasia of the HFA with an absence of DZ. Much of its role in adrenal development was revealed through the study of Dax-1 mutations in humans. Dax-1 is also expressed in the HFA in its earliest stages of development and its pattern of expression overlaps significantly with SF-1, suggesting a coregulatory role in steroidogenesis and adrenal gland development ([Beuschlein et al., 2002](#)).

Considerable progress has been made in the last several decades in defining the genes that regulate steroidogenesis and adrenal cortical development, however, most aspects of this process remain poorly understood.

Regulation of HFA Growth

Numerous in vitro and in vivo studies have demonstrated that ACTH is the major trophic hormone that regulates the growth and stimulation of steroid secretion by the HFA. The adrenal glands of human anencephalic fetus lacks the striking increase in the size of the HFA achieved by fetus of normal gestation. The smaller size of the adrenal glands of the anencephalic fetus is thought to be due to atrophy or involution of the fetal zone which comprises 80% of the gland in normal fetuses near term [Fig. 3](#). Adrenal trophy is believed to be due to the low levels of ACTH in plasma of anencephalic fetuses ([Easterling et al., 1966a](#); [Allen et al., 1974](#)). Additionally, maternal glucocorticosteroid therapy leads to suppression of the fetal adrenal steroidogenesis presumably by suppression of fetal ACTH secretion ([Ballard et al., 1980](#)). ACTH is known to promote hypertrophy and production of DHEAS and cortisol by HFA in vitro ([Rainey et al., 2004](#)). Taken together these data indicate that ACTH is needed for fetal adrenal DHEA production. However, the massive amount of fetal steroidogenesis and hypertrophy of the gland as pregnancy progresses cannot be explained by ACTH alone as its level does not rise significantly during the last trimester of gestation ([Branchaud et al., 1978](#)). Therefore, it is hypothesized that other factors, such as placenta derived factors, are responsible for the growth and differentiation of HFA in the later half of pregnancy. This is further supported by the observation that the HFA undergoes rapid involution immediately after birth when placenta derived factors are no longer available.

There is now evidence that placental corticotropin releasing hormone (CRH) may play an integral role in regulating the increase in fetal adrenal steroidogenesis during the last weeks of gestation. CRH is a 41-amino acid peptide that was first identified in the hypothalamus but is also produced by the human placental, fetal membranes, and decidua. CRH controls the ACTH release by the anterior pituitary and thus cortisol and DHEAS secretion by the HFA. Unlike hypothalamic CRH, which is under the negative feedback control of glucocorticoids, cortisol stimulates placental CRH production ([Robinson et al., 1988](#); [Jones et al., 1989](#); [Karalis and Majzoub, 1995](#)). Increase in placental CRH drives HFA cortisol production and vice versa, creating a potent positive feed back endocrine loop between fetal adrenals and the placental that is only terminated when the placenta and fetus separates at the time of delivery.

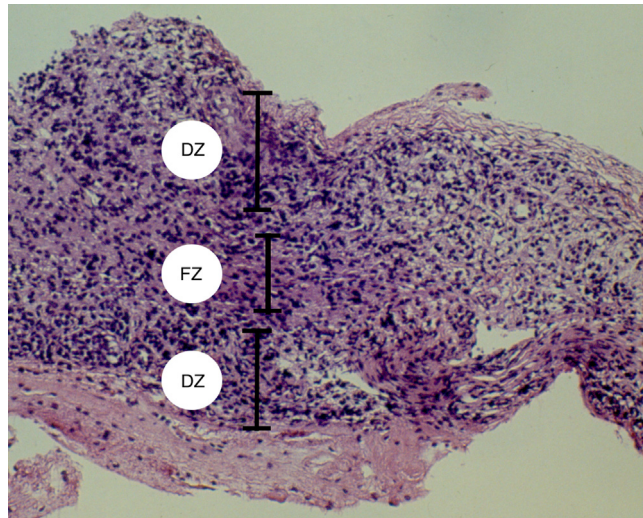


Fig. 3 Photomicrograph of a histological section from the adrenal gland obtained from a 36-week-old anencephalic newborn. The smaller size of the adrenal glands of the anencephalic fetus is thought to be due to absence or involution of the FZ. FZ, fetalzone; DZ, definitive zone.

Maternal plasma CRH levels are decreased in the first trimester and increase from midgestation to term. In the last 12 weeks of gestation, CRH plasma levels increase considerably, peak during labor, and decrease precipitously after delivery (Goland et al., 1986; Stalla et al., 1989). Umbilical cord blood levels and amniotic fluid levels of CRH are increased similarly in late gestation (Lockwood et al., 1996). Fetal CRH levels are lower than those in maternal circulation (50 vs. $1000 \times 10^{-9} \text{ mol/m}^3$) but are substantial compared with levels in men and nonpregnant women. The ability of CRH to directly stimulate human fetal adrenal to produce cortisol and DHEA is well documented (Lanman, 1953; Spencer et al., 1999; Keene, 1927). Sirianni et al. (2005) have also demonstrated that CRH induces ACTH-R expression and cortisol production in HFA in vitro. In primates, CRH is the only trophic hormone to have a specific serum-binding protein (CRH-BP) that binds CRH in the fetal and maternal compartments to tightly regulate the bioactivity of placental CRH (Parker and Carr, 1981). CRH-BP levels decrease in later half of pregnancy, whereas CRH levels increase in maternal plasma and in amniotic fluid. Changes in CRH-BP levels may explain the increase in steroidogenesis in HFA despite relatively constant levels of circulating ACTH in the later half of pregnancy.

Placental CRH may be a key regulator of the fetal-placental stress response machinery as well as in initiation of parturition in humans. In pregnancies in which the fetus can be considered to be stressed as a result of various complications, such as intrauterine growth restriction or preeclampsia, the concentrations of CRH in fetal plasma, amniotic fluid, and in maternal plasma are increased over those seen in a normal gestation (Barnhart et al., 1980; Campbell and Murphy, 1977; Huhtaniemi, 1977). In women who delivered preterm, there was a more rapid rate of increase in CRH levels from the 2nd trimester. Moreover, the biologic impact of increased CRH levels is likely to be amplified in such instances as a result of subnormal levels of CRH-BP (Carr et al., 1980; Branchaud et al., 1978). Such increases in placental CRH production during normal gestation and the excessive secretion of placental CRH in complicated pregnancies may play a role in the normal gestational increases in fetal adrenal cortisol synthesis (Murphy et al., 1974) and the supranormal levels of cortisol in umbilical cord blood that occurs in stressed infants (Barnhart et al., 1980; Hauth et al., 1978; Belisle and Tulchinsky, 1980).

HFA and Parturition

The origin for the signals that control the onset of human labor involves complex biochemical dialog between the mother, fetus, and placenta and is a subject of debate even today. In humans, there are several potential contributions of the fetal adrenal to the timing of parturition. The initiation of fetal adrenal cortisol, which occurs late in gestation, may act in the feed-forward cascade that involves placental CRH as mentioned previously. Some investigators proposed that the increasing level of CRH at the end of gestation reflects a “fetal/placental clock” (i.e., that the human placenta and fetus, through endocrine events, determine the timing of parturition at the end of the normal gestational term) (Carr and Simpson, 1981a,b). The increasing levels of fetal adrenal production of DHEA-S (and its conversion to estrogen) stimulates the production of contraction-associated proteins within the myometrium, resulting in coordinated myometrial activity and onset of labor. This hypothesis is supported by the observation that women who have steroid sulfatase deficiency, which blocks placental use of sulfated precursors, have prolonged pregnancies (Rainey et al., 2001). CRH also stimulates fetal adrenal glands to produce large amount of cortisol, which has numerous effects on preparing fetal organ systems (by fetal lung maturation) and maternal organ systems (by promoting expression of placental genes such as oxytocin and prostaglandins) for labor.

Summary

The human fetal adrenal glands undergo profound physiologic changes during gestation that are unparalleled in any other human endocrine organ. Their steroid products are important for fetal organ maturation and for the correct timing of parturition. Together with the placenta and the maternal adrenal, they form a unique maternal/fetal endocrine unit/system. The development and function of the human fetal adrenal is distinct from those in other species. Their role in steroidogenesis and parturition remains incompletely understood despite more than a century of investigation. However, advances in biotechnology and study of patients with adrenal development disorders have helped to elucidate some of the factors involved in steroidogenic activity regulation and well as factors involved in HFA development and function. The human fetal adrenal is a fascinating organ unique to humans—its biophysiology, regulation, and development continues to remain a fertile area of research.

References

- Achermann, J. C., Ito, M., Ito, M., Hindmarsh, P. C., & Jameson, J. L. (1999). A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. *Nature Genetics*, 22, 125–126.
- Allen, J. P., Greer, M. A., Mogilva, R., Castro, A., & Fisher, D. A. (1974). Endocrine function in an anencephalic infant. *The Journal of Clinical Endocrinology & Metabolism*, 38, 94–98.
- Bakke, M., Zhao, L., Hanley, N. A., & Parker, K. L. (2001). SF-1: A critical mediator of steroidogenesis. *Molecular and Cellular Endocrinology*, 171, 5–7.
- Ballard, P. L., Gluckman, P. D., Liggins, G. C., Kaplan, S. L., & Grumbach, M. M. (1980). Steroid and growth hormone levels in premature infants after prenatal betamethasone therapy to prevent respiratory distress syndrome. *Pediatric Research*, 14, 122–127.
- Barnhart, B. J., Carlson, C. V., & Reynolds, J. W. (1980). Adrenal cortical function in the postmature fetus and newborn infant. *Pediatric Research*, 14, 1367–1369.
- Bassett, M. H., Suzuki, T., Sasano, H., de Vries, C. J., Jimenez, P. T., Carr, B. R., & Rainey, W. E. (2004a). The orphan nuclear receptor NGFI-B regulates transcription of 3 β -hydroxysteroid dehydrogenase. Implications for the control of adrenal functional zonation. *The Journal of Biological Chemistry*, 279(37), 622–630.
- Bassett, M. H., White, P. C., & Rainey, W. E. (2004b). A role for the NGFI-B family in adrenal zonation and adrenocortical disease. *Endocrine Research*, 30, 567–574.
- Beltins, I. Z., Bayard, F., Ances, I. G., Kowarski, A., & Migeon, C. J. (1973). The metabolic clearance rate, blood production, interconversion and transplacental passage of cortisol and cortisone in pregnancy near term. *Pediatric Research*, 7, 509–519.
- Belisle, S., & Tulchinsky, D. (1980). Amniotic fluid hormones. *Maternal-fetal endocrinology*, 169–195.
- Beuschlein, F., Keegan, C. E., Bavers, D. L., Mutch, C., Hutz, J. E., Shah, S., Ulrich-Lai, Y. M., Engeland, W. C., Jeffs, B., Jameson, J. L., & Hammer, G. D. (2002). SF-1, DAX-1, and acd: Molecular determinants of adrenocortical growth and steroidogenesis. *Endocrine Research*, 28, 597–607.
- Branchaud, C. T., Goodyer, C. G., Hall, C. S., Arato, J. S., Silman, R. E., & Giroud, C. J. (1978). Steroidogenic activity of hACTH and related peptides on the human neocortex and fetal adrenal cortex in organ culture. *Steroids*, 31, 557–572.
- Brown, J. B. (1956). Urinary excretion of oestrogens during pregnancy, lactation, and the re-establishment of menstruation. *Lancet*, 270, 704–707.
- Campbell, A. L., & Murphy, B. E. (1977). The maternal-fetal cortisol gradient during pregnancy and at delivery. *The Journal of Clinical Endocrinology and Metabolism*, 45, 435–440.
- Carr, B. R., Parker, C. R., Jr., Milewich, L., Porter, J. C., Macdonald, P. C., & Simpson, E. R. (1980). Steroid secretion by ACTH-stimulated human fetal adrenal tissue during the first week in organ culture. *Steroids*, 36, 563–574.
- Carr, B. R., & Simpson, E. R. (1981a). De novo synthesis of cholesterol by the human fetal adrenal gland. *Endocrinology*, 108, 2154–2162.
- Carr, B. R., & Simpson, E. R. (1981b). Lipoprotein utilization and cholesterol synthesis by the human fetal adrenal gland. *Endocrine Reviews*, 2, 306–326.
- Cavallero, C., & Magrini, U. (1967). In *Histochemical studies on 3 β -hydroxysteroid dehydrogenase and other enzymes in the steroid-secreting structures of human fetus*, Second International Congress on Hormonal Steroids, International Congress Ser (pp. 667–674).
- Chang, R. J., Buster, J. E., Blakely, J. L., Okada, D. M., Hobel, C. J., Abraham, G. E., & Marshall, J. R. (1976). Simultaneous comparison of delta 5-3 β -hydroxysteroid levels in the fetoplacental circulation of normal pregnancy in labor and not in labor. *The Journal of Clinical Endocrinology and Metabolism*, 42, 744–751.
- Davis, I. J., & Lau, L. F. (1994). Endocrine and neurogenic regulation of the orphan nuclear receptors Nur77 and Nur-1 in the adrenal glands. *Molecular and Cellular Biology*, 14, 3469–3483.
- Easterling, W., Simmer, H., Dignam, W., Frankland, M., & Naftolin, F. (1966a). Dehydroepiandrosterone sulfate, 16 α -hydroxydehydroepiandrosterone, and 16 α -hydroxydehydroepiandrosterone sulfate in maternal and fetal blood of pregnancies with anencephalic and normal fetuses. *Steroids*, 8, 157–178.
- Easterling, W. E., Jr., Simmer, H. H., Dignam, W. J., Frankland, M. V., & Naftolin, F. (1966b). Neutral C19-steroids and steroid sulfates in human pregnancy. II. Dehydroepiandrosterone sulfate, 16-alpha-hydroxydehydroepiandrosterone, and 16-alpha-hydroxydehydroepiandrosterone sulfate in maternal and fetal blood of pregnancies with anencephalic and normal fetuses. *Steroids*, 8, 157–178.
- Ebert, S. N., Balt, S. L., Hunter, J. P., Gashler, A., Sukhatme, V., & Wong, D. L. (1994). Egr-1 activation of rat adrenal phenylethanolamine N-methyltransferase gene. *The Journal of Biological Chemistry*, 269, 20,885–98.
- Elliott, T. R., & Armour, R. G. (1911). The development of the cortex in the human suprarenal gland and its condition in hemicephaly. *The Journal of Pathology and Bacteriology*, 15, 481–488.
- Escobar, J. C., Patel, S. S., Beshay, V. E., Suzuki, T., & Carr, B. R. (2011). The human placenta expresses CYP17 and generates androgens de novo. *The Journal of Clinical Endocrinology and Metabolism*, 96, 1385–1392.
- Goland, R. S., Wardlaw, S. L., Stark, R. I., Brown, J. R., Brown, L. S., & Frantz, A. G. (1986). High levels of corticotropin-releasing hormone immunoactivity in maternal and fetal plasma during pregnancy. *The Journal of Clinical Endocrinology & Metabolism*, 63, 1199–1203.
- Goldman, A. S., Yakovac, W. C., & Bongiovanni, A. M. (1966). Development of activity of 3 beta-hydroxysteroid dehydrogenase in human fetal tissues and in two anencephalic newborns. *The Journal of Clinical Endocrinology and Metabolism*, 26, 14–22.
- Goto, M., Piper Hanley, K., Marcos, J., Wood, P. J., Wright, S., Postle, A. D., Cameron, I. T., Mason, J. I., Wilson, D. I., & Hanley, N. A. (2006). In humans, early cortisol biosynthesis provides a mechanism to safeguard female sexual development. *The Journal of Clinical Investigation*, 116, 953–960.
- Hammer, G. D., Parker, K. L., & Schimmer, B. P. (2005). Minireview: Transcriptional regulation of adrenocortical development. *Endocrinology*, 146, 1018–1024.
- Han, V. K., Lund, P. K., Lee, D. C., & D'ercle, J. (1988). Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. *The Journal of Clinical Endocrinology & Metabolism*, 66, 422–429.
- Hauth, J. C., Parker, C. R., Jr., Macdonald, P. C., Porter, J. C., & Johnston, J. M. (1978). A role of fetal prolactin in lung maturation. *Obstetrics and Gynecology*, 51, 81–88.
- Huhtaniemi, I. (1977). Studies on steroidogenesis and its regulation in human fetal adrenal and testis. *Journal of Steroid Biochemistry*, 8, 491–497.
- Jones, S., Brooks, A., & Challis, J. (1989). Steroids modulate corticotropin-releasing hormone production in human fetal membranes and placenta. *The Journal of Clinical Endocrinology & Metabolism*, 68, 825–830.
- Karalis, K., & Majzoub, J. A. (1995). Regulation of Placental Corticotropin-Releasing Hormone by Steroids. *Annals of the New York Academy of Sciences*, 771, 551–555.

- Keene, M. F. (1927). Observations on the Development of the Human Suprarenal Gland. *Journal of Anatomy*, 61, 302–324.
- Kern, H. (1911). Ueber den Umbau der Nebenniere im extra-uterinen Leben. *Deutsche Medizinische Wochenschrift*, 37, 971.
- Lalli, E., & Sassone-Corsi, P. (2003). DAX-1, an unusual orphan receptor at the crossroads of steroidogenic function and sexual differentiation. *Molecular Endocrinology*, 17, 1445–1453.
- Lanman, J. T. (1953). The fetal zone of the adrenal gland: Its developmental course, comparative anatomy, and possible physiologic functions. *Medicine (Baltimore)*, 32, 389–430.
- Lockwood, C. J., Radunovic, N., Nastic, D., Petkovic, S., Aigner, S., & Berkowitz, G. S. (1996). Corticotropin-releasing hormone and related pituitary-adrenal axis hormones in fetal and maternal blood during the second half of pregnancy. *Journal of Perinatal Medicine-Official Journal of the WAPM*, 24, 243–251.
- Lyer, A. K., & McCabe, E. R. (2004). Molecular mechanisms of DAX1 action. *Molecular Genetics and Metabolism*, 83, 60–73.
- Martin, L. J., & Tremblay, J. J. (2005). The human 3beta-hydroxysteroid dehydrogenase/Delta5-Delta4 isomerase type 2 promoter is a novel target for the immediate early orphan nuclear receptor Nur77 in steroidogenic cells. *Endocrinology*, 146, 861–869.
- Mesiano, S., Coulter, C. L., & Jaffe, R. B. (1993). Localization of cytochrome P450 cholesterol side-chain cleavage, cytochrome P450 17 alpha-hydroxylase/17, 20-lyase, and 3 beta-hydroxysteroid dehydrogenase isomerase steroidogenic enzymes in human and rhesus monkey fetal adrenal glands: Reappraisal of functional zonation. *The Journal of Clinical Endocrinology and Metabolism*, 77, 1184–1189.
- Mesiano, S., & Jaffe, R. B. (1997). Developmental and functional biology of the primate fetal adrenal cortex. *Endocrine Reviews*, 18, 378–403.
- Morohashi, K. (1999). gonadal and extragonadal functions of AD4BP/SF-1: developmental aspects. *Trends in Endocrinology and Metabolism*, 10, 169–173.
- Murphy, B. E., Clark, S. J., Donald, I. R., Pinsky, M., & Vedady, D. (1974). Conversion of maternal cortisol to cortisone during placental transfer to the human fetus. *American Journal of Obstetrics and Gynecology*, 118, 538–541.
- Parker, C. R., Jr., & Carr, B. R. (1981). In *The influence of maternal and fetal factors on the fetal plasma concentrations of cortisol (F) and dehydroepiandrosterone sulfate (DS) Program of the 28th Annual Meeting of the Society for Gynecologic Investigation March 1981*, (pp. 18–21).
- Parker, K. L., Rice, D. A., Lala, D. S., Ikeda, Y., Luo, X., Wong, M., Bakke, M., Zhao, L., Frigeri, C., Hanley, N. A., Stallings, N., & Schimmer, B. P. (2002). Steroidogenic factor 1: an essential mediator of endocrine development. *Recent Progress in Hormone Research*, 57, 19–36.
- Parker, K. L., & Schimmer, B. P. (1997). Steroidogenic factor 1: a key determinant of endocrine development and function. *Endocrine Reviews*, 18, 361–377.
- Parker, L. N., & Odell, W. D. (1980). Control of adrenal androgen secretion. *Endocrine Reviews*, 1, 392–410.
- Pearson Murphy, B. E., & Diez D'aux, R. C. (1972). Steroid Levels in the Human Fetus: Cortisol and Cortisone. *The Journal of Clinical Endocrinology & Metabolism*, 35, 678–683.
- Pritchard, J. A., & MacDonald, P. C. (1980). *Williams Obstetrics*. New York: Appleton-Century-Crofts.
- Rainey, W., Carr, B., Wang, Z., & Parker, C. (2001). Gene profiling of human fetal and adult adrenals. *Journal of Endocrinology*, 171, 209–215.
- Rainey, W. E., Cr, P., Jr., Rehman, K., & Carr, B. R. (2002). The adrenal genetic puzzle: how do the fetal and adult pieces differ? *Endocrine Research*, 28, 611–622.
- Rainey, W. E., Rehman, K. S., & Carr, B. R. (2004). In *The human fetal adrenal: making adrenal androgens for placental estrogens Seminars in reproductive medicine* (pp. 327–336). New York, NY: Thieme Medical Publishers, Inc.
- Robinson, B. G., Emanuel, R. L., Frim, D. M., & Majzoub, J. A. (1988). Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta. *Proceedings of the National Academy of Sciences of the United States of America*, 85, 5244–5248.
- Siiteri, P. K., & Macdonald, P. C. (1966). Placental estrogen biosynthesis during human pregnancy. *The Journal of Clinical Endocrinology and Metabolism*, 26, 751–761.
- Simpson, E. R., Carr, B. R., Parker, C. R., Jr., Milewich, L., Porter, J. C., & Macdonald, P. C. (1979). The role of serum lipoproteins in steroidogenesis by the human fetal adrenal cortex. *The Journal of Clinical Endocrinology and Metabolism*, 49, 146–148.
- Sirianni, R., Rehman, K. S., Carr, B. R., Parker, C. R., Jr., & Rainey, W. E. (2005). Corticotropin-releasing hormone directly stimulates cortisol and the cortisol biosynthetic pathway in human fetal adrenal cells. *The Journal of Clinical Endocrinology & Metabolism*, 90, 279–285.
- Spector, W. S. (Ed.). (1956). *Handbook of Biological Data*. Philadelphia: W.B. Saunders Co.
- Spencer, S. J., Mesiano, S., Lee, J. Y., & Jaffe, R. B. (1999). Proliferation and apoptosis in the human adrenal cortex during the fetal and perinatal periods: implications for growth and remodeling. *The Journal of Clinical Endocrinology and Metabolism*, 84, 1110–1115.
- Stalla, G., Bost, H., Stalk, J., Kaliebe, T., Dörr, H., Pfeiffer, D., Werder, K. V., & Müller, O. (1989). Human corticotropin-releasing hormone during pregnancy. *Gynecological Endocrinology*, 3, 1–10.
- Starkel, S., & Wegrzynowski, L. (1910). Beitrag zur Histologie der Nebennieren bei Feten und Kindern. *Archiv für Anatomie und Physiologie*, 214, 214–235.
- Suzuki, T., Sasano, H., Takeyama, J., Kaneko, C., Freije, W. A., Carr, B. R., & Rainey, W. E. (2000). Developmental changes in steroidogenic enzymes in human postnatal adrenal cortex: immunohistochemical studies. *Clinical Endocrinology*, 53, 739–747.
- Thomas, E. (1911). Ueber die Nebenniere des Kindes und ihre Veranderungen bei Infektionskrankheiten. *Beitraege zur Pathologie*, 50, 283–316.
- Uotila, U. U. (1940). The early embryological development of the fetal and permanent adrenal cortex in man. *The Anatomical Record*, 76, 183–203.
- Voutilainen, R., & Kahri, A. (1980). Placental origin of the suppression of 3β-hydroxysteroid dehydrogenase in the fetal zone cells of human fetal adrenals. *Journal of Steroid Biochemistry*, 13, 39–43.
- Wilson, T. E., Mouw, A. R., Weaver, C. A., Milbrandt, J., & Parker, K. L. (1993). The orphan nuclear receptor NGFI-B regulates expression of the gene encoding steroid 21-hydroxylase. *Molecular and Cellular Biology*, 13, 861–868.

Fetal Endocrinology/Hormones

Gerald J Pepe, Eastern Virginia Medical School, Norfolk, VA, United States

Eugene D Albrecht, University of Maryland School of Medicine, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Fetal Hypothalamus–Pituitary

The fetal hypothalamus develops very early in gestation and is comprised of distinct functional nuclei that produce a diverse set of small proteins termed neuropeptides and the biogenic amine dopamine which coordinate multiple aspects of neural/neuroendocrine function. Under the coordinated expression of a growing number of ligands and transcription factors, including Sim 1, Brn-2, Otp, Gsh-1, sonic hedgehog, bone morphogenetic proteins and thyroid hormone, the hypothalamic nuclei that control pituitary-endocrine gland function are identifiable by the 6th week of gestation (Bakos et al., 2016; Morales-Delgado et al., 2014). As shown in Fig. 1, cell bodies of magnocellular neurons in the paraventricular (PVN) and supraoptic (SON) nuclei produce arginine vasopressin (AVP) also known as antidiuretic hormone (ADH) and oxytocin (OXT), respectively, which are axonally transported to and stored/released from the posterior pituitary. Cell bodies of parvocellular neurons in the PVN and anterior PVN (aPVN) produce thyrotropin releasing hormone (TRH), corticotropin releasing hormone (CRH), and growth hormone inhibitory hormone/somatostatin (SS) and those in the arcuate nucleus (ARN) produce growth hormone releasing hormone (GHRH), gonadotropin releasing hormone (GnRH) and the prolactin inhibitory hormone, dopamine. The axons of all cell bodies traverse the median eminence (ME) and neuropeptides are released into the pituitary portal vasculature and secreted into the anterior pituitary gland. In addition to production of these factors, other neurons in cell bodies of the PVN, SO, and ARN as well as those in the ventromedial nucleus (VMN) produce an array of neuropeptides, for example, urocortins, Substance P, enkephalins, Neuropeptide Y, and agouti-related peptide, that impact metabolic as well as reproductive function. Elegant studies in mice (Morales-Delgado et al., 2014) support the more current prosomeric model of hypothalamic development and have identified the rostrocaudal and dorsoventral regions of the hypothalamus in which the CRH, TRH, GHRH, and SS neurons originate, defined the spatiotemporal mRNA expression of these peptidergic factors during fetal development and confirmed that mRNA expression is initiated as early as embryonic day 9. In contrast to neural origin of all other hypothalamic peptidergic neurons, GnRH neurons originate from epithelial progenitor cells of the olfactory placode and GnRH mRNA in the ARN is expressed by gestation week 10. Hypothalamic expression of AVP and OXT is thought to occur relatively late in gestation and based on studies in sheep AVP/OXT neurons are functional and respond to physiologic challenge. While ADH decreases fluid production by and increases fluid reabsorption in the fetal lung following delivery, the specific role(s) of fetal OXT remain to be elucidated.

The program that orchestrates development of the hypothalamus is intricately linked to that guiding development of the pituitary gland which is composed of two anatomically and functionally distinct entities, the posterior pituitary and the anterior pituitary (Kaiser and Ho, 2016). The anterior pituitary which is fully developed by week 3 of gestation, is derived from Rathke's pouch, an ectodermal invagination anterior to the roof of the mouth. In addition, as Rathke's pouch proliferates toward the 3rd ventricle, cells fuse with the diverticulum and the latter along with neural ectoderm associated with development of the 3rd ventricle gives rise to the posterior pituitary. The anterior pituitary vasculature is evident by week 7 and a fully functional system is in place by

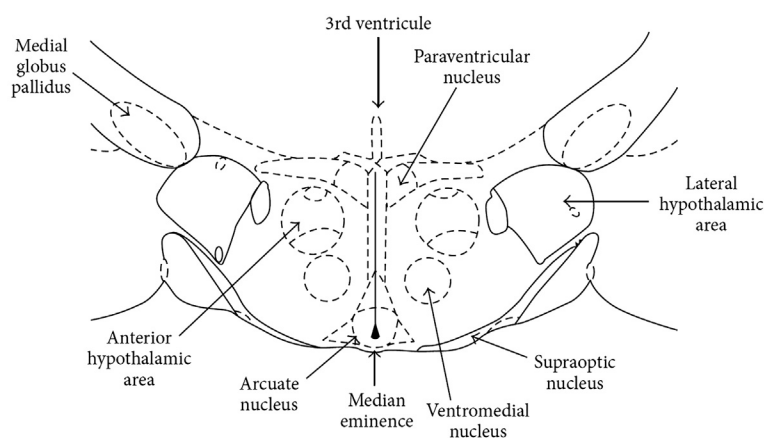


Fig. 1 Overview of the major hypothalamic nuclei producing neuropeptides. Paraventricular and supraoptic nuclei contain neurons producing corticotrophin-releasing hormone, urocortins, thyrotropin releasing hormone, oxytocin, and vasopressin. Ventromedial nucleus neurons produce Substance P, enkephalins, and Neuropeptide Y. Arcuate nucleus neurons express agouti-related peptide, Neuropeptide Y, proopiomelanocortin, melanocyte-stimulating hormones, adrenocorticotrophic hormone-stimulating hormone, and endorphins. Reproduced with permission from Bakos, J., Zatkova, M., Bacova, Z., Ostatnikova, D. (2016) The role of hypothalamic neuropeptides in neurogenesis and neuritogenesis. *Neural Plasticity*, 2016, 3276383, 10 pages.

midgestation. The major cell types of the anterior pituitary, which include corticotrophs, somatotrophs, gonadotrophs, lactotrophs, and thyrotrophs, are derived from totipotent stem cells in a highly coordinated and spatiotemporally controlled process which is completed by week 12 of gestation. The elucidation of the multiple factors that control pituitary cell lineage are based on studies in rodent/murine models and corroborated by pathologic/histologic observations in human subjects (Zhu et al., 2007). Early development requires expression of Rtx and Ptx and subsequent expression of LIM homeodomains by cells of Rathke's pouch as well as FGF8, BMP4 and sonic hedgehog and Pit1 by oral ectoderm and subsequently all pituitary cell types (Fig. 2).

Corticotropes are identifiable by week 6 and produce the peptide hormone ACTH which is a proteolytic product of proopiome-lanocortin (POMC). ACTH protein is detectable by week 7 of gestation and important for fetal adrenal cortical development and production of the steroid hormone dehydroepiandrosterone (DHEA) and its sulfate DHEAS, processes which are curtailed in fetuses with anencephaly/no pituitary. The melanocyte stimulating hormone (MSH) α MSH is a product of the posttranslational processing of precursor ACTH in humans and nonhuman primates and a product of melanotrophs that comprise the intermediate lobe of the anterior pituitary in other species for example, rodents. Fetal serum ACTH levels increase in late gestation in sheep and play a role in onset of parturition. Whether ACTH levels exhibit a similar pattern and function in the primate is equivocal and as discussed below likely reflects the complex interplay between maternal cortisol and impact of placental cortisol metabolism on the fetal-pituitary

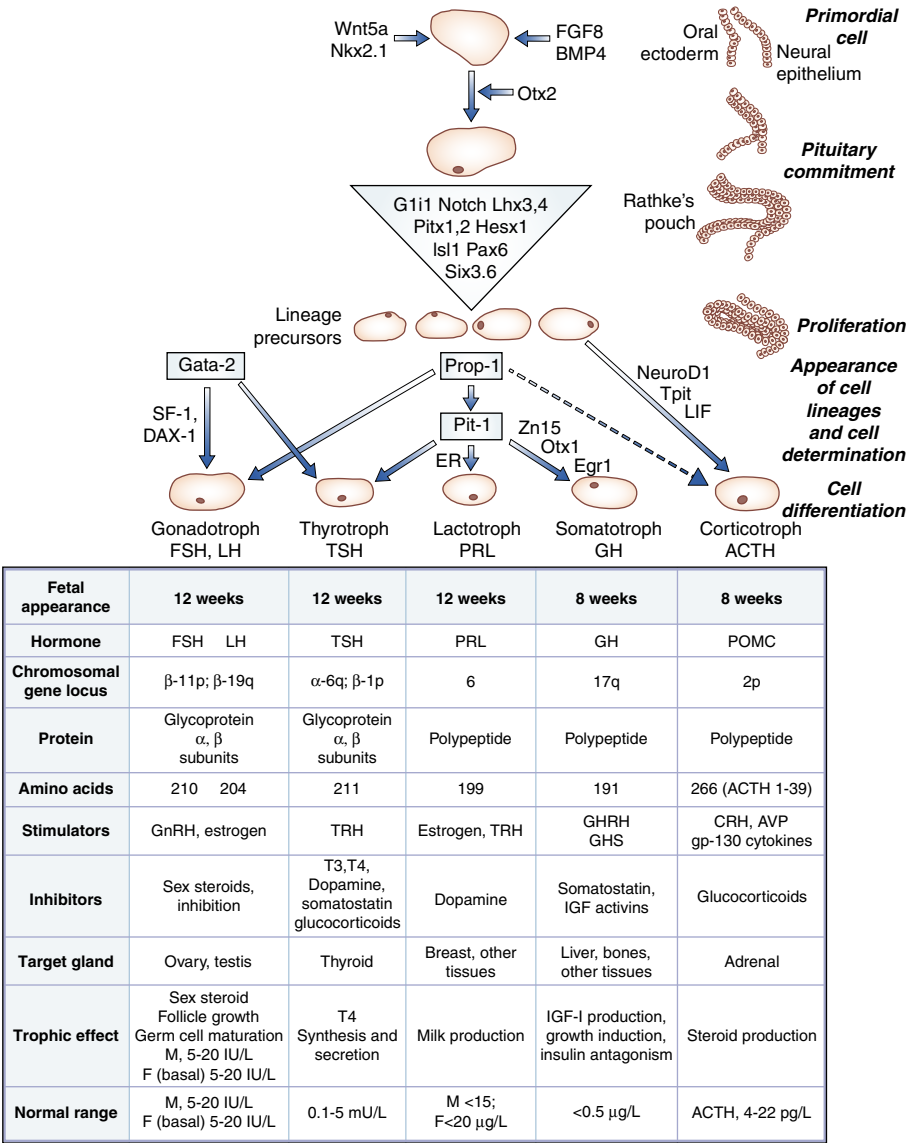


Fig. 2 Model for development of the human anterior pituitary gland and cell lineage determination by a cascade of transcription factors. ACTH, adrenocorticotrophic hormone; ER, estrogen receptor; FSH, follicle-stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PRL, prolactin; TSH, thyrotrophin/thyroid stimulating hormone. Reproduced with permission from Kaiser, U. and Ho, K.K.Y. (2016) Pituitary physiology and diagnostic evaluation. In: Melmed, S., Polonsky, K.S., Larsen, P.R. Kronenberg, H.M., (eds.) *Williams Textbook of Endocrinology* (13th edn.), pp. 176–231. Philadelphia, PA: Elsevier.

adrenal axis as well as the impact of estrogen on zone specific regulation of fetal adrenal ACTH receptor expression and targeting to the cell membrane. Somatotrophs are evident by week 7 and produce the protein hormone growth hormone (GH), which is identifiable by week 8 and acts primarily by enhancing production of insulin like growth factor (IGF)-I. Fetal GH levels increase between weeks 15–25 of gestation and decline precipitously thereafter, presumably reflecting increased response of somatotropes to hypothalamic SS. Because fetuses with idiopathic GH deficiencies exhibit near normal growth, the role of fetal GH appears primarily on intermediary metabolism and thus levels are influenced by supply of maternal nutrients. Lactotroph expression of the protein hormone prolactin is initiated around week 16 of gestation and fetal serum levels steadily increase thereafter and are maximal at term. The increase in fetal prolactin is primarily regulated by fetal estradiol either directly and/or via suppression of hypothalamic dopamine expression which when elevated for example, by bromocriptine, reduces fetal prolactin levels. Prolactin is thought to be important for fetal water and electrolyte balance and thus amniotic fluid osmolality and in concert with cortisol and thyroxine induces maturation of the fetal lung in sheep. The gonadotrophs and thyrotrophs produce the heterodimeric glycoprotein hormones LH, FSH, and TSH, respectively, which have a common α -subunit and a specific β subunit which are detectable by week 12 of gestation. LH and FSH levels increase by midgestation and exhibit sexual dimorphism with levels higher in females than in males due to feedback inhibition of gonadotrophs by elevated fetal testicular secretion of testosterone. However, in both males and females, fetal LH and FSH levels subsequently decline after midgestation due to negative feedback of increasing levels of estradiol on the fetal hypothalamus–pituitary (Dattani and Gevers, 2016). Thus, as discussed below, maturation of the fetal ovary and testes during the second half of gestation appears to be gonadotropin independent. Serum TSH levels are detectable by week 10, increase late in gestation and at term and it appears that TSH functions to regulate the fetal thyroid gland.

Fetal Endocrine Glands

Cellular Development and Secretions of the Adrenal Cortex and Medulla

The primate fetal adrenal is comprised of chromaffin cells and three distinct zones of cortical cells, which arise from intermediate mesoderm known as the adrenogonadal primordium (AGP) which also gives rise to the gonads. AGP development requires expression of Wilms tumor suppressor gene 1 (WT1) and its target SF1 and deletion of either gene results in agenesis of both the gonads and adrenal gland (Del Valle et al., 2017). Stem/progenitor cells also populate the subcapsular region of the fetal adrenal and during the course of gestation these cells migrate centripetally and thereby underpin the unique zone-specific and spatio-temporal pattern of fetal adrenal cortical growth that occurs in primates (Ishimoto and Jaffe, 2011). The primordial adrenal is evident by week 4 and upon activation of stem/progenitor cells, cortical cells undergo rapid growth resulting in a very large fetal zone (FZ) and two smaller zones, the definitive zone (DZ) in the outermost regions of which the progenitor cells reside and the transitional zone (TZ). The FZ synthesizes the androgens DHEA and DHEAS the levels of which steadily increase throughout human pregnancy and are essential for placental estrogen production (Albrecht and Pepe, 2014). Expression of enzymes catalyzing DHEA/S synthesis is evident at 9–12 weeks gestation and earlier in nonhuman primates. Based on these findings and that fetal adrenal growth is compromised in anencephalic fetuses, it appears that the pituitary–adrenal axis is operative very early in gestation. In contrast, cells of the DZ and TZ do not grow or produce aldosterone or cortisol, respectively, until late in gestation and yet these cells like the FZ express ACTH receptor and are ACTH dependent. Therefore, as discussed below additional factors play a significant role in coordinating fetal pituitary–adrenal axis function as well as control FZ growth (Albrecht and Pepe, 2014). The latter are of physiologic relevance as the timely ontogenesis of fetal adrenal cortisol production is important for maturation of fetal organ systems, for example, lung, and in sheep for onset of parturition. It remains to be determined whether aldosterone synthesis is sufficient to impact fetal homeostasis.

The chromaffin cells of the fetal adrenal are derived from the sympathetic nervous system. Neural cells migrate into the adrenal cortex early in gestation and ultimately coalesce to form the medulla, which exhibits the enzymatic capacity to produce the catecholamines epinephrine and norepinephrine as well as dopamine by midgestation. Production of medullary catecholamines is thought to be essential for coordination of fetal cardiovascular, respiratory and metabolic functions critical for intrauterine survival for example, response to stressors including hypoxia and transition to an extrauterine environment. Interestingly, the marked increase in serum epinephrine levels at birth is reduced in neonates with fetal adrenal 21-hydroxylase deficiency and thus lower TZ production of cortisol indicating that intraadrenal cortisol, is required for maturation of the fetal adrenal medulla.

Cellular Development and Secretions of the Thyroid

The thyroid originates from two lateral anlagen in the brachial pouches and from the median anlage which is an outpouching of the floor of the pharynx and the tip of the foramen caecum at the base of the tongue. As the median anlage descends through the neck, it fuses with the lateral anlagen and gives rise to follicular cells which acquire capacity to accumulate iodine obtained by placental transport of maternal stores and form the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) by week 11. The lateral anlage differentiates into the parafollicular “C” cells that synthesize the hormone calcitonin important for calcium homeostasis. The spatio-temporal expression of the genes/transcription factors controlling human fetal thyroid development have been identified (Polak, 2014; Dattani and Gevers, 2016). It is well established that small amounts of maternal thyroid hormones cross the placenta to control development of the fetus. The pattern of the fetal serum levels of the prohormone T_4 , the active hormone T_3 , the inactive hormone reverse T_3 (rT_3) and TSH in the human fetus are shown in Fig. 3. Although pituitary TSH is not essential for growth there

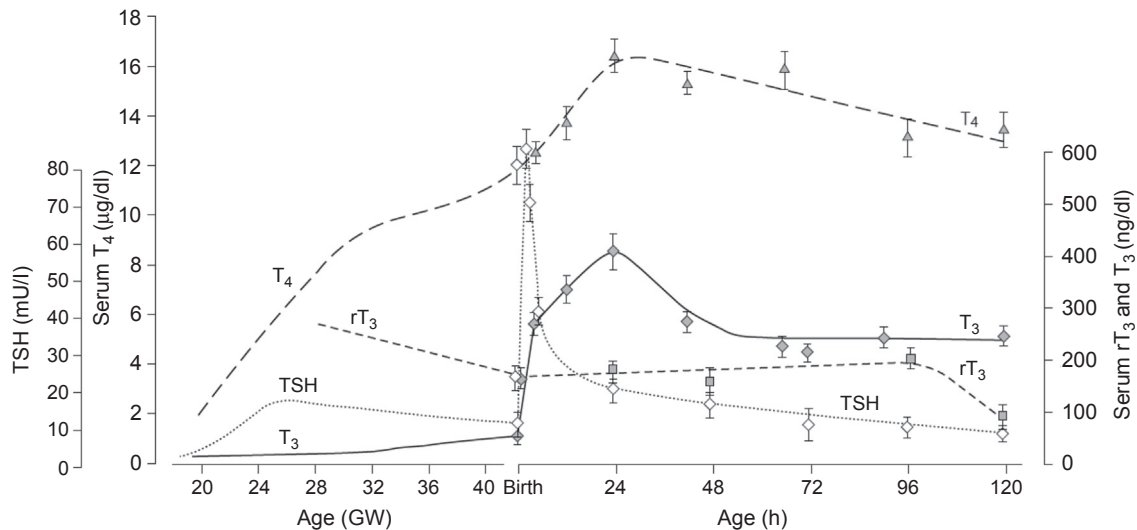


Fig. 3 Representation of the normal serum levels of total T_3 (triiodotyrosine), total T_4 (thyroxine), TSH (thyroid stimulating hormone), and reverse T_3 (rT_3) in the human fetus during weeks 20–40 of gestation (GW), at birth and at 24–120 h of postnatal life. Reproduced with permission from Polak, M. (2014). Human fetal thyroid function. *Endocrine Development* **26**, 17–25.

appears to be increased sensitivity of the fetal thyroid to TSH with advancing gestation since the levels of T_4 , the majority of which is bound to thyroxine-binding globulin synthesized by the fetal liver, continue to rise despite a slight decrease in TSH secretion. The tissue specific development of the deiodinase enzymes types I and II that convert T_4 to T_3 also play an important role in the responsivity of tissue to thyroid hormones. The placenta expresses high levels of deiodinase type III which converts T_4 to rT_3 and explains why fetal levels of rT_3 exceed those of T_3 . Although fetal tissues express receptors for T_3 , human infants born without a thyroid show no signs of hypothyroidism at birth and thus the transport of maternal hormone appears sufficient. However, athyroid infants will develop neural deficits/cretinism if not treated with T_4 . Thus, while the role of the fetal thyroid in human fetal development remains unclear, it is well established that the marked increase in TSH at birth that leads to a rapid and sustained increase in T_4 and T_3 in the perinatal period (Fig. 3) is critical for maturation of the CNS in the first few weeks of extrauterine life.

Cellular Development and Secretions of the Gonads

The gonads are comprised of germ cells which are derived from the yolk sac wall and somatic cells of the genital ridge that forms from the primitive mesonephros. (Del Valle et al., 2017). By week six, the primitive gonad is comprised of gonadal cords continuous with the surface epithelium and a dense internal cellular mass termed the gonadal blastema. At this point the gonad is structurally identical in both genetic male (XY) and female (XX) fetuses and termed the indifferent gonad, which is flanked by the primordia of the Wolffian and Müllerian ducts and the primordia of the external genitalia. Formation of the testis begins at week 7 and results from expression of the SRY gene on the Y chromosome and the genes SOX9, GATA4, and DAX1 (Fig. 4; Dattani and Gevers, 2016). Primitive cords lose their connection with the surface epithelium and Sertoli cells as well as spermatogonia begin to develop, while undifferentiated interstitium form Leydig cells which exhibit the enzymatic capacity to produce testosterone as early as week 8. Upon secretion of placental chorionic gonadotrophin (hCG) into the fetal circulation, the fetal Leydig cells initiate synthesis of testosterone which enhances transformation of the Wolffian duct into epididymis and vas deferens, while the Sertoli cells produce the glycoprotein hormone antimüllerian hormone (AMH) that causes involution of the Müllerian duct (Dattani and Gevers, 2016). Secretion of fetal testicular testosterone into the blood peaks at week 16–18 and declines thereafter in parallel with the decline in placental hCG synthesis (Albrecht and Pepe, 2014). Uptake by and conversion of testosterone to 5α dihydrotestosterone by primordia of the external genitalia leads to formation of prostate, phallus, and penile urethra. Although testicular growth and development continue throughout the second half of gestation, maturation appears to be coordinated by placental estrogen and not fetal pituitary gonadotrophin as discussed below.

In the absence of SRY and presence of a second X chromosome, the indifferent gonad will initiate formation of an ovary. The gonadal blastema differentiates into interstitium and medullary cords containing germ cells now termed oogonia. The genes that initiate ovarian formation remain to be elucidated although there is evidence for a role of Wnt4, DAX1, and SF1. The cells of the developing fetal ovary do not synthesize androgens or estrogens and are not responsive to hCG in vivo or in vitro. Thus, in the absence of any or little testosterone or AMH, the Wolffian duct involutes and the Müllerian duct differentiates into a uterus and oviduct and the primordia of external genitalia develop female. However, like the fetal testis, development of the fetal ovary even very early in gestation appears to be gonadotrophin independent and regulated by placental estrogen as discussed below (Pepe et al., 2006).

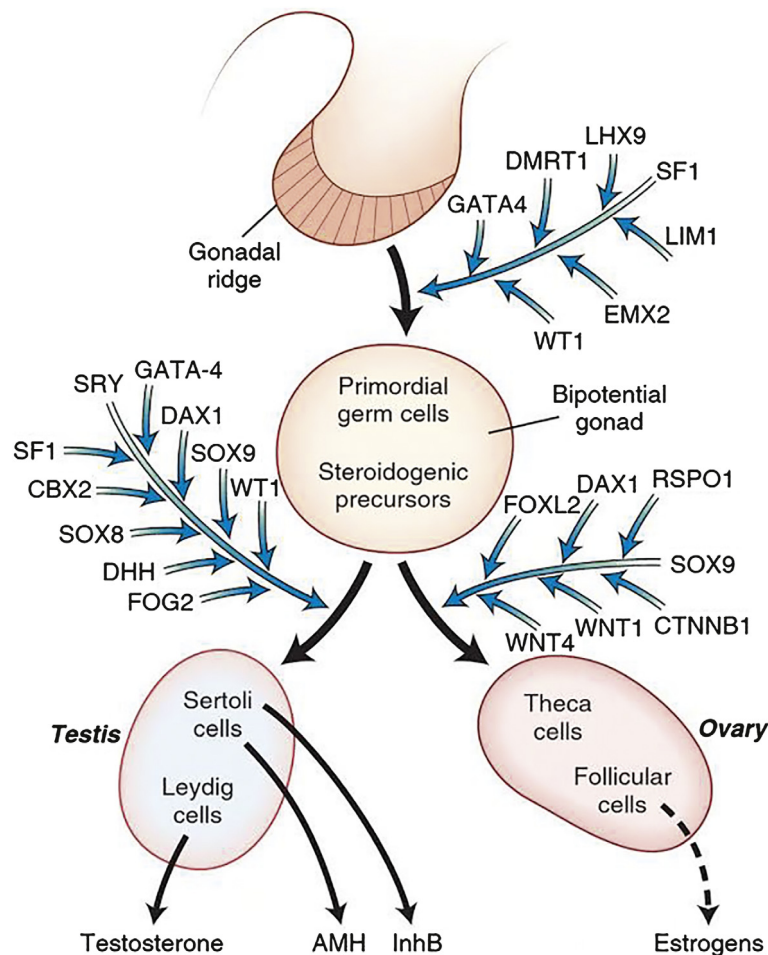


Fig. 4 Summary of the molecular and cellular events of gonadal differentiation. Reproduced with permission from Dattani, M.T. and Gevers, E.F. (2016). Endocrinology of fetal development. In: Melmed, S., Polonsky, K.S., Larsen, P.R. Kronenberg, H.M. (eds.) *Williams Textbook of Endocrinology* (13th edn.), pp. 849–892. Philadelphia, PA: Elsevier.

Cellular Development and Secretions of the Parathyroid

The 4 parathyroid glands originate from the 3rd and 4th pharyngeal pouches and development occurs in synchrony with that of the thyroid and appears to require expression of *HOX15* and several other genes also linked to thyroid formation (Kovacs, 2014). By week 6, the parathyroids become localized to the upper and lower poles of the thyroid and by week 10 are capable of producing parathyroid hormone (PTH). However, throughout most of gestation, fetal serum PTH levels are very low and serum calcium levels are markedly elevated which suppresses PTH production (Kovacs, 2014). Fetal hypercalcemia results from transport of large amounts (up to 150–300 mg/kg/day) of calcium to the fetus, which along with maternal derived phosphorous and magnesium is essential for fetal bone/tissue development and mineral homeostasis. Although fetal serum levels of PTH related peptides (PTHrp) primarily of placental origin are elevated throughout gestation, these peptides do not appear to play a major role in the fetus. Moreover, fetal serum levels of 1, 25 dihydroxyvitamin D₃ (calcitriol) that controls calcium uptake by the GI tract are also very low due to the low levels of PTH and consequently low levels of the renal 1 α -hydroxylase enzyme controlling conversion of precursor 25-hydroxyvitamin D₃ of placental origin to calcitriol. Thus, in the fetus the major hormone controlling fetal bone formation/mineralization is calcitonin produced by the parafollicular “C” cells of the thyroid in response to elevated levels of calcium and consequently fetal serum levels of calcitonin are increased throughout development.

Cellular Development and Secretions of the Endocrine Pancreas

The pancreas is derived from protrusions of the primitive gut epithelium (Edlund, 2002; Dattani and Gevers, 2016). The exocrine pancreas essential for production of enzymes and bicarbonate for digestion is developed by week 4 and the endocrine pancreas containing islets comprised of α and β cells that produce glucagon and insulin, respectively, as well as cells that synthesize somatostatin and pancreatic polypeptide, are identifiable by week 8. The development of α cells appears to peak at midgestation,

whereas β cells continue to proliferate throughout the second half of gestation. The genes controlling pancreatic formation include *IDX1*, *ISL1*, *PAX4*, and *PAX6* which when deleted leads to pancreatic agenesis, islet cell agenesis, and β or α cell agenesis/hypogenesis, respectively. The fetal pancreatic levels of insulin and glucagon are relatively high and increase between mid and late gestation. However, the infusion of glucose or arginine does not stimulate fetal insulin release and thus the capacity of the fetal β cell to release insulin and fetal serum levels of insulin are relatively low throughout most of gestation. In contrast, fetal glucagon levels are relatively high since α cell glucagon release is not responsive to glucose, that is, not suppressed by hyperglycemia. Thus, while fetal pancreatic α and β cells develop and synthesize glucagon and insulin, respectively throughout development, they are not responsive to feedback by glucose. Thus, it is generally thought that in the fetus insulin and glucagon may play a less important role in the regulation of the metabolism of substrate/glucose which is obtained by placental transfer of maternal stores by facilitated diffusion. The constant supply of maternal glucose also precludes a need for gluconeogenesis by fetal tissues and glucagon receptor expression in fetal liver is relatively low. Apparently, a complex interplay of factors for example, IGFs, adipokines produced by the fetus and placenta control the metabolic processes important for fetal growth (Albrecht and Pepe, 2014). However, by late gestation fetal insulin markedly increases glucose uptake and lipogenesis. The latter plus the onset of development of gluconeogenesis and need for glucagon to maintain blood glucose levels immediately after birth and the responsiveness of pancreatic α and β cells to glucose are rapidly established in late gestation/postnatally. It is important to note however, that in pregnancies complicated by maternal gestational diabetes and thus chronic fetal hyperglycemia, fetuses have elevated levels of insulin and exhibit β cell hyperplasia.

Hepatic and Adipose Endocrine Function

It is well established that IGF I and IGF binding protein (IGFBP)-3 produced by the fetal liver and IGF-I expressed in and acting in a paracrine manner in fetal tissues, for example, brain and bone, play a significant role in fetal growth (Dattani and Gevers, 2016). Thus, fetal growth is impaired in mice with mutations in or deletions of IGF-I or its receptor and fetal blood IGF-I levels are correlated with neonatal birth weight/size. Fetal IGF-I levels are modulated by nutrient availability and thus are reduced in nutritionally deprived animals. Administration of IGF-I to normal or growth restricted fetal sheep and guinea pigs increased or restored growth, apparently by upregulating placental nutrient transporters via activation of mammalian target of rapamycin (mTOR) (Albrecht and Pepe, 2014). In addition to the IGF system, the hormones adiponectin and leptin and proinflammatory adipokines for example, resistin, produced by adipose tissue also play a role in fetal growth (Martos-Moreno et al., 2009). Thus, compared to normal full term fetuses, umbilical cord levels of resistin are higher and levels of leptin, adiponectin, IGF-I and IGFBP-3 lower in infants born premature and small for gestational age. Thus, the increase in fetal serum levels of leptin and adiponectin with advancing pregnancy appears to reflect the increase in acquisition of adipose tissue by the fetus. However, our understanding of the factors regulating adipose tissue accumulation and adipokine production remains incomplete.

Role of Placental Estrogen on Fetal Organ System Development

Modulation of Placental Metabolism of Maternal Cortisol and Integration of the Fetal Hypothalamic–Pituitary–Adrenocortical Axis

As indicated earlier, the primate fetal hypothalamus and pituitary can synthesize CRH and ACTH, respectively, very early in gestation, whereas the transitional zone cells of the fetal adrenal cortex do not develop the capacity to produce cortisol until much later. Thus, the fetal pituitary–adrenal axis seems “out of phase” and only becomes functionally integrated near term. Studies have now shown that estrogen, by regulating the expression and cellular localization of the placental 11β -hydroxysteroid dehydrogenase (11β -HSD)-1 and -2 enzymes controlling the interconversion of cortisol–cortisone, dictates the level of biologically active maternal cortisol that arrives in the fetal circulation and via this mechanism plays a critical role in the timely activation of fetal pituitary ACTH dependent maturation of fetal adrenal cortisol synthesis (Albrecht and Pepe, 1999). As shown in Fig. 5, in the presence of relatively low levels of estrogen in early to midgestation, placental 11β -HSD-controlled metabolism favors the formation of cortisol, which is secreted into the fetal circulation and suppresses fetal pituitary ACTH production, thereby preventing ACTH dependent fetal adrenal cortisol production. With advancing gestation and increased estrogen production, there is increased placental 11β -HSD regulated conversion of maternal cortisol to cortisone which would decrease the amount of maternal cortisol arriving in the fetus. The latter results in disinhibition of and thus production of fetal pituitary ACTH and onset of cortisol synthesis by the fetal adrenal gland. In support of this hypothesis, the expression of fetal pituitary ACTH and its precursor pro-opiomelanocortin mRNA and expression of key steroidogenic enzymes in and the ontogenesis of de novo cortisol production by the baboon fetal adrenal were greater at term than at midgestation and increased prematurely at midgestation by treatment with estrogen which induced placental oxidation of cortisol to cortisone. Placental 11β -HSD-dependent regulation of the fetal pituitary–adrenal axis is also likely operative during human pregnancy as women with hypoadrenalism can maintain pregnancy without exogenous corticosteroid treatment, ostensibly because of premature activation of the fetal pituitary–adrenal axis in the absence of placental transport of maternal cortisol to the fetus.

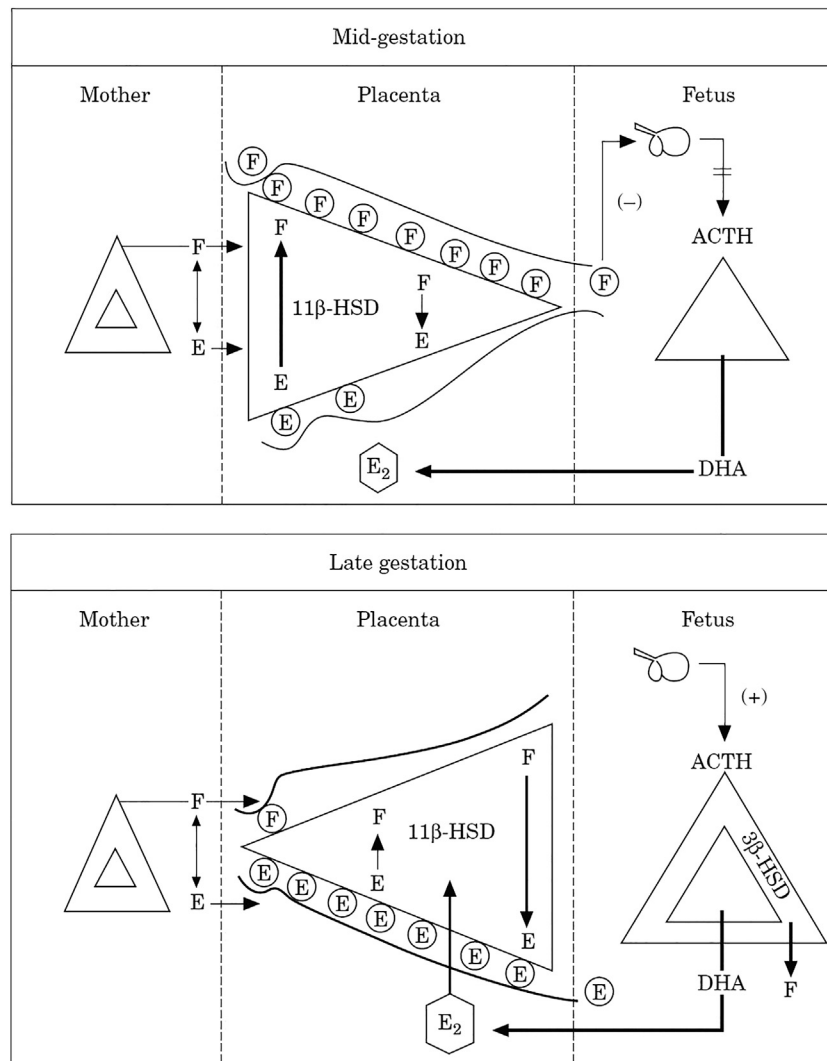


Fig. 5 The role of estrogen (E_2) on placental 11β -HSD-catalyzed metabolism of cortisol (F) and cortisone (E) and regulation of the fetal pituitary-adrenocortical axis at mid- and late gestation in the baboon. 3β -HSD, Δ^3 - 3β -hydroxysteroid dehydrogenase. From Albrecht, E.D. and Pepe, G.J. (1999). Central integrative role of oestrogen in modulating the communication between the placenta and fetus that results in primate fetal-placental development *Placenta* **20**, 129–139.

Development of the Pool of Healthy Ovarian Oocytes/Follicles and Testicular Maturation Essential for Adult Reproductive Function

In primates, the formation of the pool of ovarian primordial follicles available for reproductive function in adulthood occurs in utero. Thus, following differentiation of the indifferent gonad into an ovary there is marked mitotic proliferation of somatic (pregranulosa) and germ (oogonia) cells early in gestation. As germ cells initiate meiosis but arrest in prophase 1 and are now termed oocytes, they make contact with the pregranulosa cells and form a germ cell nest in which primordial follicles are subsequently formed and released into the ovarian cortex by breakdown of the germ cell nest. The oocytes of these newly formed primordial follicles then develop microvilli which contact the single layer of surrounding granulosa cells from which they obtain nutrients to support their survival for up to 50 years (Pepe et al., 2006). Studies have shown that the human and baboon fetal ovary express $ER\alpha$ and $ER\beta$ and that fetal ovarian development is critically dependent upon the increase in estrogen production that occurs with advancing gestation. Thus, in fetal baboons deprived of estrogen in the second half of gestation, primordial follicle numbers were decreased by 50%, whereas germ cell nest breakdown was curtailed and thus the number of germ cell nests increased. Moreover, several of the primordial follicles that did form in ovaries of estrogen-deprived fetuses appeared unhealthy and contained oocytes with a marked depletion in microvilli. Additional studies showed that the onset of puberty was delayed and that serum estradiol and gonadotrophin levels in subsequent menstrual cycles altered in offspring of estrogen-deprived animals, indicating that estrogen programs mechanisms in fetal ovarian development critical for adult reproductive function (Pepe et al., 2006). The importance of estrogen in programming primate fetal ovarian development is heightened by studies showing that polymorphisms of the $ER\alpha$ gene in women are associated with premature ovarian failure and that reproductive function was disrupted in women born very premature and thus not exposed to the increasing levels of estrogen of advancing pregnancy.

Estrogen receptors are also expressed in the primate fetal testis and estrogen also appears to be essential for development of the pool of spermatogonial germ cells and Sertoli cells. Thus, in male fetuses deprived of estrogen during the second half of gestation, the number of reserve spermatogonia stem cells and the development of undifferentiated type A spermatogonia was reduced and the morphology of the seminiferous tubules disrupted. It is likely but remains to be shown whether these perturbations in the germ cell population and the integrity of the seminiferous cords in the estrogen-deprived animals impair spermatogenesis and fertility in adulthood. However, male mice with targeted disruption of the P450 aromatase gene that converts androgens to estrogen exhibit a decline in sperm number in association with infertility and ER α knock-out mice exhibit altered morphology of the testis, efferent ductules, and epididymis as well as reduced sperm motility and infertility (Albrecht and Pepe, 2014).

Control of Fetal Adrenal Fetal Cortical Zone Growth and Androgen Production

In addition to regulating the timely expression of fetal pituitary ACTH via control of placental cortisol metabolism, estrogen via interaction with ER α/β in the fetal adrenal DZ elicits direct effects on the fetal adrenal important for zone-specific development and function of the FZ. Thus, in fetal baboons deprived of estrogen in the second half of gestation, expression of the cell cycle regulator cyclin D1 was increased in the DZ in which progenitor cells essential for fetal adrenal DZ growth reside and the mass/volume of and production of DHAS by the FZ at term were markedly increased, whereas the volumes and steroidogenic function of the DZ and TZ were unaltered (Babischkin et al., 2016). Further study showed that estrogen acts by controlling the interaction of the ACTH receptor (termed the melanocortin 2 receptor; MC2R) with its MC2R accessory protein (MRAP) in the DZ critical for ACTH to elicit its effects. Thus, as shown in Fig. 6, in the first half of pregnancy, the relatively low levels of fetal estrogen promote interaction of MC2R with MRAP leading to movement of MC2R to the cell surface in the DZ, which permits binding of ACTH to MC2R and activation of the MC2R signal transduction pathway. The latter causes increased expression of cyclin D1, progenitor cell proliferation and migration to the FZ and thus increased growth and DHAS production for placental estrogen (E_2) formation. The progressive increase in estrogen levels with advancing gestation restrains MC2R/MRAP interaction and ACTH binding to MC2R, thereby controlling the extent of progenitor cell proliferation/migration and FZ growth and DHAS production to maintain levels of estrogen within a physiologic range.

In summary, the fetal endocrine glands develop very early in gestation and exhibit the potential to produce their respective neuropeptide, protein and steroid hormones by as early as week 6–12 of gestation. Fetal hormone production is critical to the maintenance of fetal homeostasis and growth and integrated with and coordinated in part by maternal and placental hormoneogenesis and placental transport of maternal nutrients. Hormones produced by the fetal endocrine glands are also important for the maturation of fetal organ systems important for neonatal survival in the perinatal period and development and function in adulthood.

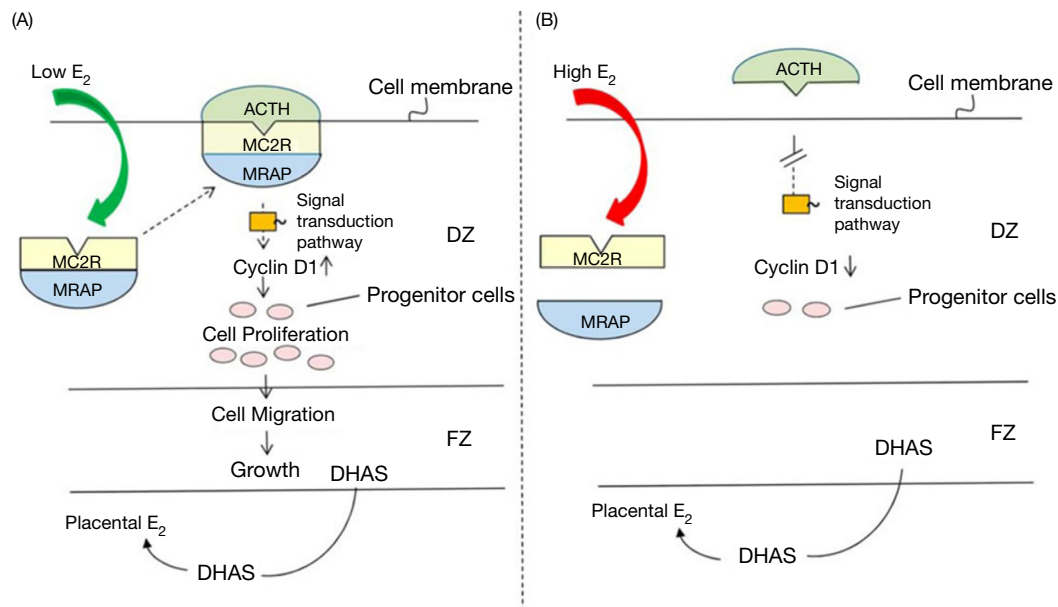


Fig. 6 Proposed role of estrogen (E_2) in regulating the interaction of ACTH receptor (Melanocortin 2 Receptor; MC2R) with the Melanocortin Receptor Accessory Protein (MRAP) in the definitive zone (DZ) important for ACTH dependent growth and production of dehydroepiandrosterone sulfate (DHAS) by the fetal zone (FZ) of the primate fetal adrenal gland important for placental estrogen (E_2) production. Reproduced with permission from Babischkin, J.S., Aberdeen, G.W., Pepe, G.J. and Albrecht, E.D. (2016) Estrogen suppresses interaction of melanocortin 2 receptor and its accessory protein in the primate fetal adrenal cortex *Endocrinology* **157**, 4588–4601.

Acknowledgements

Portions of the material presented in this article were funded by the National Institutes of Health Research Grants R01 HD93070 and R01 DK93950. The authors gratefully acknowledge the assistance of Ms. Sandra Huband with the computer word processing preparation of this article.

References

- Albrecht, E. D., & Pepe, G. J. (1999). Central integrative role of oestrogen in modulating the communication between the placenta and fetus that results in primate fetal-placental development. *Placenta*, 20, 129–139.
- Albrecht, E. D., & Pepe, G. J. (2014). Placental endocrine function and hormone action. In T. Plant, & A. Zeleznik (Eds.), *Knobil and Neill's physiology of reproduction* (4th edn., pp. 1783–1834). New York: Academic Press.
- Babischkin, J. S., Aberdeen, G. W., Pepe, G. J., & Albrecht, E. D. (2016). Estrogen suppresses interaction of melanocortin 2 receptor and its accessory protein in the primate fetal adrenal cortex. *Endocrinology*, 157, 4588–4601.
- Bakos, J., Zatkova, M., Bacova, Z., & Ostatnikova, D. (2016). The role of hypothalamic neuropeptides in neurogenesis and neuritogenesis. *Neural Plasticity*, 2016, 3276383, 10 pages.
- Dattani, M. T., & Gevers, E. F. (2016). Endocrinology of fetal development. In S. Melmed, K. S. Polonsky, P. R. Larsen, & H. M. Kronenberg (Eds.), *Williams textbook of endocrinology* (13th edn., pp. 849–892). Philadelphia, PA: Elsevier.
- Del Valle, I., Buonocore, F., Duncan, A. J., Lin, L., Barengo, M., Parnaik, R., Shah, S., Hubank, M., Gerrelli, D., & Achermann, J. C. (2017). A genomic atlas of human adrenal and gonad development. *Wellcome Open Research*, 2, 25.
- Edlund, H. (2002). Pancreatic organogenesis—Developmental mechanisms and implications for therapy. *Nature Reviews. Genetics*, 7, 524–532.
- Ishimoto, H., & Jaffe, R. B. (2011). Development and function of the human fetal adrenal cortex: A key component in the fetoplacental unit. *Endocrine Reviews*, 32, 317–355.
- Kaiser, U., & Ho, K. K. Y. (2016). Pituitary physiology and diagnostic evaluation. In S. Melmed, K. S. Polonsky, P. R. Larsen, & H. M. Kronenberg (Eds.), *Williams textbook of endocrinology* (13th edn., pp. 176–231). Philadelphia, PA: Elsevier.
- Kovacs, C. S. (2014). Bone development and mineral homeostasis in the fetus and neonate: Roles of the calciotropic and phosphotropic hormones. *Physiological Reviews*, 94, 1143–1218.
- Martos-Moreno, G. A., Barrios, V., Saenz de Pipaón, M., Pozo, J., Dorronsoro, I., Martínez-Biarge, M., Quero, J., & Argente, J. (2009). Influence of prematurity and growth restriction on the adipokine profile, IGF1, and ghrelin levels in cord blood: Relationship with glucose metabolism. *European Journal of Endocrinology*, 161, 381–389.
- Morales-Delgado, N., Castro-Robles, B., Ferran, J. L., Martínez-de-la-Torre, M., Puellas, L., & Diaz, C. (2014). Regionalized differentiation of CRH, TRH, and GHRH peptidergic neurons in the mouse hypothalamus. *Brain Structure & Function*, 219, 1083–1111.
- Pepe, G. J., Billiar, R. B., & Albrecht, E. D. (2006). Regulation of baboon fetal ovarian folliculogenesis by estrogen. *Molecular and Cellular Endocrinology*, 247, 41–46.
- Polak, M. (2014). Human fetal thyroid function. *Endocrine Development*, 26, 17–25.
- Zhu, X., Gleiberman, A. S., & Rosenfeld, M. G. (2007). Molecular physiology of pituitary development: Signaling and transcriptional networks. *Physiological Reviews*, 87, 933–963.

Fetal Lung Development and Function

Colby L Day, University of Florida Jacksonville, Jacksonville, FL, United States

John E Baatz and Rita M Ryan, Medical University of South Carolina, Charleston, SC, United States

© 2018 Elsevier Inc. All rights reserved.

Stages of Lung Development

The development of the respiratory tract begins early in gestation (as early as 3 weeks after conception, or 3 weeks of gestation) and extends until long after birth, with general agreement that development of new alveoli continues until at least 2 years of age, but with much contention that it can extend even longer into childhood and adolescence (Burri, 2006; Fraga and Guttentag, 2012). In this discussion, we will outline the development of the respiratory tract in chronologic order, noting the gestational age of the fetus at each crucial stage. For the purposes of this discussion, the upper respiratory tract will be defined as the airway extending from the nose and mouth to the vocal cords. Alternatively, the lower respiratory tract will be defined as the airway extending below the vocal cords to the alveolar-capillary interface in the lungs. "Alveolar" refers to the alveoli, or the numerous tiny air sacs where gas exchange occurs. It is important to note, however, that the upper and lower respiratory tracts, while they serve distinct functions and have distinct origins and compositions, exist as a *continuum*. During fetal and early postnatal life, the development of the respiratory tract is divided into five stages: embryonic, pseudoglandular, canalicular, saccular, and alveolar. However, these are not entirely distinct phases and exhibit a fair amount of overlap and variation.

Embryonic Stage (3–7 Weeks of Gestation)

The first sign of the burgeoning respiratory tract is the appearance of a groove and the budding of a saccular structure from the ventral (anterior) wall of the innermost layer of cells in the developing foregut (foregut endoderm) (Fig. 1). This primitive lung bud grows anteriorly and downward (ventrocaudally) in parallel with the developing foregut which will eventually become the upper gastrointestinal tract (esophagus). Both the lung and the esophagus are "foregut" structures (Kimura and Deutsch, 2007). The development of the lung bud and its growth in parallel with the future esophagus is coordinated by a complex milieu of transcription factors, i.e., factors that regulate the expression of specific genes and signaling pathways (Shojaie and Post, 2017; Fraga and Guttentag, 2012).

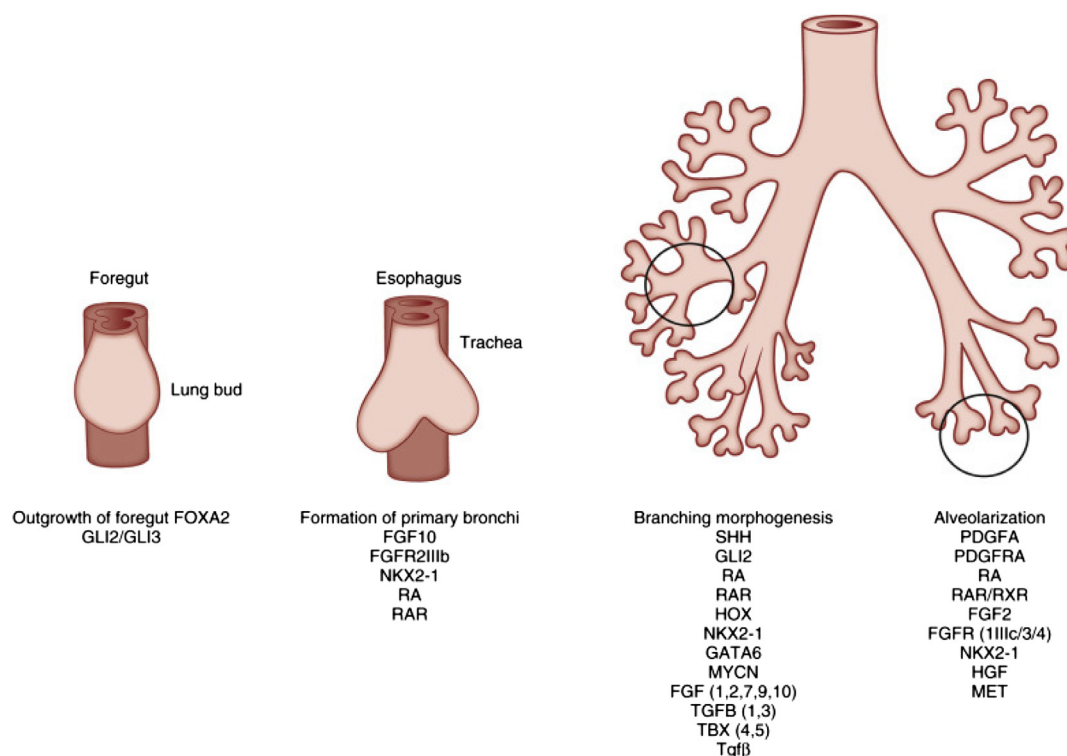


Fig. 1 Regulation of early lung development. Taken from Fig. 64–1 Transcription and growth factors known to regulate lung development from fetal and neonatal physiology, 5th edn. Polin, R., Abman, S., Rowitch, D., Benitz, W., 2017.

As we know, the mature respiratory system consists of two sides: a right lung, comprised of three lobes, and a left lung, comprised of two lobes, which are connected to the trachea and eventually the upper respiratory tract by right and left primary bronchi. This final lung structure is achieved via sequential, transcription factor- and growth factor-mediated branching of the primitive lung bud, along with complex interactions with the surrounding mesenchyme (mesodermal tissue that later develops into connective, lymphatic, circulatory, and skeletal tissues) to promote growth of distal epithelial cells only (Fraga and Guttentag, 2012; Warburton, 2017). This process is initiated at approximately 4 weeks of gestation when the developing lung bud is directed to divide into a right and a left branch, ultimately leading to the development of the right and left bronchi. Subsequent secondary branching results in the formation of three bronchial buds on the right and two bronchial buds on the left. During this stage, the early trachea and bronchial buds are lined by an epithelium of undifferentiated, closely packed cells in a layered array. This epithelium will eventually differentiate into cell types with varying functions including ciliated cells responsible for mucus clearance, secretory cells, basal cells responsible for the repair of injured epithelium, and submucosal glands aiding with immune function, among others (Fraga and Guttentag, 2012). These lung buds also initially lack cartilaginous support but cartilage formation begins at around 4 weeks gestation and proceeds caudally to be complete in the first few postnatal months. By 6–7 weeks gestation, smooth muscle expansion and neural innervation have begun, allowing fetal airway contraction.

During the embryonic stage of development, segmentation of the airway buds is accompanied by the development of the pulmonary (lung) blood vessels, or, vasculature. The mesenchyme surrounding the bronchial buds differentiates into an early vascular bed (vasculogenesis) which eventually connects both with the pulmonary arteries (originating from the 6th pair of aortic arches) and with the pulmonary veins (originating from the left atrium). During fetal life, the majority (90%) of blood flow is directed away from the pulmonary vasculature by the ductus arteriosus, connecting the pulmonary artery with the aorta, completely bypassing the lungs since the developing fetus does not rely on the lungs in utero for gas exchange; this function is provided by the placenta during fetal life (Fig. 2).

Pseudoglandular Stage (5–17 Weeks of Gestation)

The pseudoglandular stage of lung development is characterized by a gland-like appearance where further branching morphogenesis occurs, with the end result of formation of the primitive bronchial tree. Through the complex interactions between growth factors and mesenchyme initiated in the embryonic stage, the respiratory tubules continue to branch into more distal (terminal, or farther away from the center) bronchioles. By the 17th week of gestation, the distal airways consist of acinar tubules and buds (formations that resemble grape clusters) surrounded by mesenchyme (Wert, 2017). These distal airway formations will eventually become the mature adult pulmonary acini (singular: acinus) which represent the most distal ends of the airway, where gas exchange will occur at the alveolar-capillary interface (Fig. 3). While the actual size of the airways will increase throughout gestation and postnatal growth, branching of the large airways is completed in the pseudoglandular stage of fetal

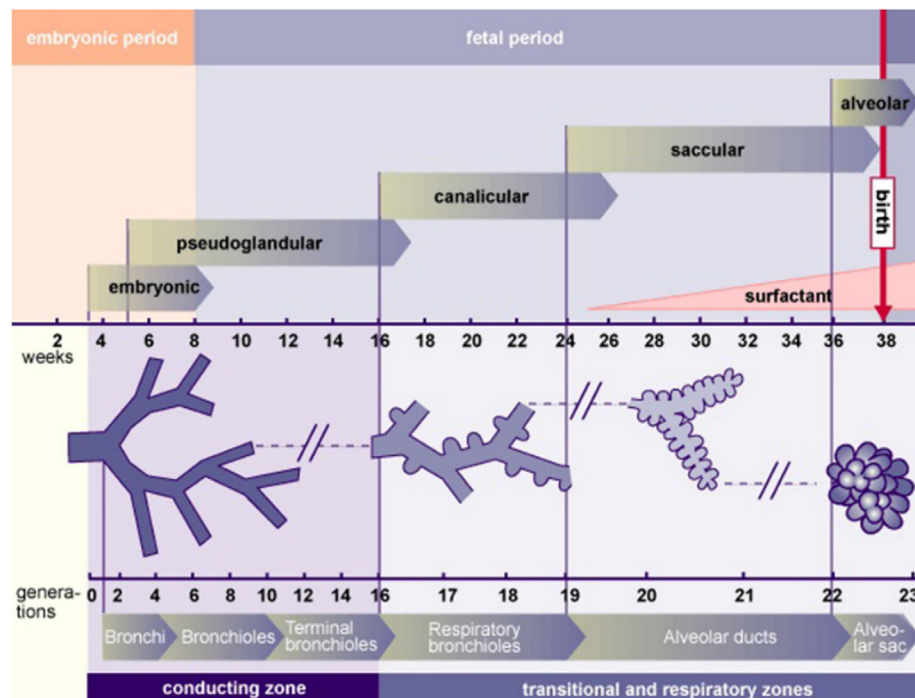


Fig. 2 Stages of lung development. From http://www.embryology.ch/images/rrespiratory/01phasen/r11_ueberblick_airway.gif.

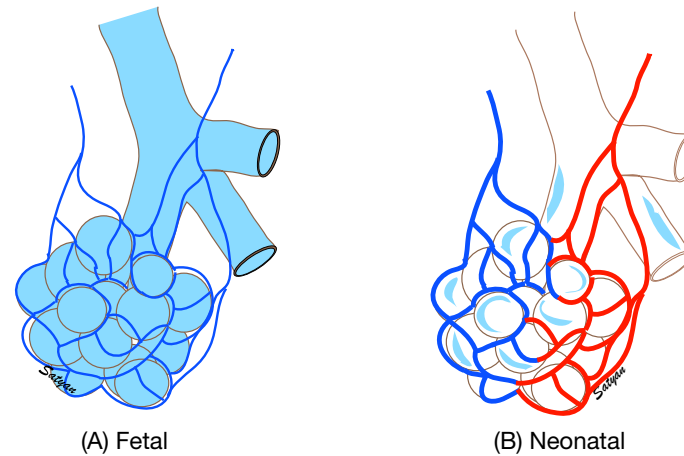


Fig. 3 The acinus. Copyright Satyan Lakshminrusimha (used with permission).

development and no additional branching occurs. The expansion of cartilaginous support, smooth muscle, and neural innervation that began during the embryonic stage continues during the pseudoglandular stage. As the pulmonary vasculature continues to develop alongside the branching airways, the lymphatic system also originates, potentially budding from cardinal veins (Wert, 2017). The lymphatic vessels are very similar to blood vessels but transport lymph, composed of body fluid and white blood cells, instead of blood. The lymphatic vessels are critical later to clearing the lung fluid from the alveolar air spaces at the time of birth. Finally, the separation of the thoracic and the abdominal cavities occurs during the pseudoglandular stage and is a crucial step in development. This process of separation results from partitioning of the two compartments by the pleuroperitoneal fold. As the fetus grows, this fold is pushed down and back and eventually becomes immobilized at the midthoracic region anteriorly (toward the front) and at the lower thoracic region posteriorly (toward the back). Cells destined to become diaphragmatic muscle cells (myoblasts) and cells destined to form the phrenic nerves that stimulate the diaphragm (the large muscle that is used for breathing by lung expansion) migrate to the pleuroperitoneal fold to create the developing diaphragm and begin the process of neural innervation.

Canalicular Stage (16–26 Weeks of Gestation)

With the completion of the large airway branching in the pseudoglandular stage, the canalicular stage is characterized by the development of the rudimentary alveolar-capillary interface or gas exchange unit. A crucial element of the alveolar-capillary exchange is proximity of the air-containing alveolus with the blood-containing capillary, permitting easy diffusion of oxygen and carbon dioxide back and forth (Fig. 3). As a result, during the canalicular stage, development focuses on this ultimate goal of efficient gas exchange through expansion of the capillary bed and thinning of the intervening mesenchymal layer. Simultaneously, the rudimentary pulmonary acini are expanding exponentially yielding a surface area for gas exchange. As mentioned above, the acinus (plural: acini) derives from Latin and refers to formations that resemble grape clusters, with respiratory bronchioles as the stems and alveoli as the grapes (Fig. 3) (Wert, 2017). The canalicular stage is also characterized by significant changes in cellular differentiation of cuboidal epithelial cells into alveolar type I and type II epithelial cells in the alveoli. Differentiation of alveolar type II cells into flattened alveolar epithelial type I cells along the alveolar-capillary membrane results in the thin gas-permeable wall of the alveoli. The cube-like alveolar type II cells begin to have large stores of glycogen which will be used in the production of surfactant, a lipid and protein-rich substance that reduces surface tension so the air sacs stay expanded. As surfactant is produced by alveolar type II cells, it is packaged in multilayered lamellar bodies, ready to be secreted and coat the walls of the alveoli upon the first breath (Fraga and Guttentag, 2012). If this developmental stage progresses normally and without disruption, by its conclusion the fetus has epithelial cells capable of producing fetal lung fluid and the alveolar-capillary exchange system has been developed enough to function; however, actual surfactant production and total surface area for gas exchange in the fetus at this stage are still often not adequate to be compatible with appropriate lung function if preterm birth occurs.

Saccular Stage (24–38 Weeks of Gestation)

The saccular stage of fetal lung development is essentially a refinement in utilization of the basic structures developed during the previous stages. During this time of fetal lung development, there is further expansion of the acini with even further thinning of the mesenchyme, and therefore a further reduction in the distance required for diffusion of oxygen and carbon dioxide; the mean thickness of alveolar-capillary membrane at birth is $0.6\ \mu\text{m}$ (one millionth of a meter) (Wert, 2017). Overall surface area for gas exchange is increased through extension of the terminal saccules and alveolar ducts. There is also continued differentiation of cell types with the alveolar epithelial type II cells become increasingly efficient at surfactant production (including

producing surfactant-associated proteins A, B, C, D) and with the alveolar epithelial type I cells continuing to flatten and multiply to cover additional surface area and come into closer contact with the capillary vascular bed. In the mature lung, although the type I and type II cells are nearly equal in number, the much larger type I cells will cover >90% of the alveolar surface (Wert, 2017).

Alveolar Stage (36 Weeks of Gestation and Thereafter)

The final stage of lung development starts at around the time of delivery of term infants and continues well into childhood. At birth, lungs will have approximately 150 million alveoli, with an astonishing 4m² surface area available for gas exchange (approximately the size of a ping pong table!) (Ochs et al., 2004). During this time, the alveoli mature, creating even more surface area for gas exchange at the alveolar-capillary membrane. To further increase the gas exchange surface there is also development of secondary alveolar septa (or secondary crests) which begin as ridges forming from the primary septa and which are thinner and longer than the more primitive primary septa. This creation of ridges or secondary crests from the primary septa appears to be influenced at least partly by crosslinking of elastin and collagen already deposited in the primary septal structure (Delacourt and Hadchouel, 2017). Additionally, while a double capillary network was initially developed surrounding each primitive alveolar septa, this network is reorganized into a single capillary bed associated with each mature alveolus. There is massive cellular proliferation during this stage, with an increase in both alveolar type I and type II epithelial cells, as well as endothelial cells.

Fetal Lung Expansion and Fetal Lung Fluid

Achievement of the complex development of the fetal respiratory system and the parallel development of the respiratory circulatory system detailed above in brief is not a guarantee of success for a newborn. Although structurally the systems may be sound and prepared for function, a newborn must also successfully transition from intrauterine life characterized by lungs filled with fetal lung fluid to extrauterine life characterized by lungs filled with air. During gestation, the fetus is supplied with oxygen-rich blood by the maternal circulation and placenta, thereby allowing most of the fetal circulation to be diverted away from the lungs via the ductus arteriosus. However, birth signifies not only a sudden introduction into the extrauterine world but also a severing of the connection to the placenta, thus creating a critical need for diversion of neonatal blood to the lungs as well as the capacity of the lungs to fill with air and begin the process of gas exchange at the alveolar capillary membrane. This has to occur immediately after birth to ensure survival (Table 1).

During fetal life, the lungs are filled with fetal lung fluid, distinct in composition from amniotic fluid. In fact, much of the mechanics of fetal lung expansion serve to reduce the amount of amniotic fluid in the lungs to as great an extent as possible, as it can be damaging to developing fetal lungs. Fetal lung fluid is actively secreted into the fetal lung air spaces by transcellular chloride transport, whereas clearance prior to birth (ideally) is mediated by active sodium absorption across the lung epithelium, described in greater detail below.

Table 1 Clinical lung abnormalities in newborns

Stage of lung development	Upper respiratory system	Lower respiratory system	Vasculature or muscle components
Embryonic state (3–7 weeks gestation)	1. Lung agenesis 2. Tracheoesophageal fistula (TEF)/esophageal atresia 3. Tracheal agenesis 4. Bronchogenic cysts 5. Intralobar sequestration 6. CPAM		
Pseudoglandular stage (5–17 weeks)	1. Extralobar sequestration 2. CPAM	1. Congenital acinar dysplasia	1. Congenital diaphragmatic hernia
Canalicular stage (16–26 weeks)		1. Surfactant deficiency (RDS) 2. Severe Bronchopulmonary Dysplasia (BPD) 3. Pulmonary hypoplasia (if low amniotic fluid levels)	1. Congenital alveolar dysplasia 2. Alveolar capillary dysplasia 3. Pulmonary hypertension
Saccular stage (24–38 weeks)		1. RDS 2. BPD 3. Transient tachypnea of the newborn (TTN)	1. Congenital alveolar dysplasia 2. Alveolar capillary dysplasia 3. Persistent pulmonary hypertension of the newborn (PPHN)
Alveolar stage (36 weeks and after)		1. TTN	1. PPHN

The distention of fetal lungs with fetal lung fluid is crucial to the appropriate development of the lungs and the volume of fetal lung fluid present is dependent on multiple factors including secretion of fetal lung fluid, fetal breathing movements, fetal position, fetal transpulmonary pressure, and amniotic fluid volume among some. Fetal breathing movements, reliant upon the fetal diaphragm, occur in conjunction with movement in the fetal laryngeal adductor muscles (which close off the airway). During periods of fetal apnea, these adductors actually function to protect the fetus against loss of fetal lung fluid by creating upper airway obstruction and a net efflux of fetal lung fluid that is less than the rate of fluid production whereas during periods of fetal breathing with diaphragmatic excursion there is a concomitant dilation of the glottis allowing a net efflux of fetal lung fluid (Wallace et al., 2017).

As you may expect from basic physics, the ability of fetal lungs to expand with fetal lung fluid volume is also dependent upon the physical environment in the uterus. If there are conditions causing decreased space for the fetus to fit, the fetus will experience a greater degree of flexion and therefore have less space for fetal lung expansion with fetal lung fluid. Conditions that can cause this include oligohydramnios (decreased amniotic fluid for a variety of reasons) and multiple gestation pregnancies.

While expansion with fetal lung fluid is crucial for lung development, at the time of birth, the fetal lung fluid must be cleared rapidly to allow for air entry and the initiation of gas exchange at the alveolar level. It was originally thought that fetal lung fluid clearance was primarily mediated by a squeeze mechanism induced by a neonate's passage through the birth canal. This was postulated as the reason that infants born by cesarean section delivery are at higher risk for delays in transition related to retained fetal lung fluid (transient tachypnea of the newborn or TTN). However, subsequent studies (both in animal models and by observation of human infants) demonstrated that while infants born by cesarean section without precedent labor do indeed have this elevated risk, those infants born by cesarean section following the initiation of labor have a similar reduction in fetal lung fluid as infants born by vaginal delivery (Carlton, 2017; Bland et al., 1979). Ineffective clearance of this fetal lung fluid can result in TTN, which is a temporary clinical respiratory problem stemming from retained fetal lung fluid and its impact on respiratory effort/function. For a summary of TTN and lung fluid clearance at birth see these reviews by Gugliani et al., 2008, 2009.

Surfactant

In addition to proper progression through the fetal developmental stages and appropriate expansion of the fetal lungs throughout gestation, the neonate must be able to generate appropriate surface tension to keep the alveoli expanded even between active breaths. Without this surface tension, distal airways will collapse between active inhalations leading to atelectasis (collapse of part of the lung) and respiratory distress. This surface tension is in large part achieved through surfactant, a phospholipid managed by alveolar epithelial type II cells. This management by type II cells involves not only production from precursors found in the circulation but also storage and recycling of surfactant to maintain a constant presence in the respiratory system. Surfactant is primarily composed of phospholipids, with dipalmitoyl phosphatidylcholine (DPPC) the most abundant. There are several types of surfactant proteins (SP) including SP-A, SP-B, SP-C, and SP-D and they have varying functions. SP-B and SP-C are the surfactant proteins that are physically associated with the phospholipids of lamellar bodies secreted into the airways. SP-B has the primary function of intercalating with surfactant phospholipid to reduce surface tension in the alveoli so that the alveoli remain open and do not collapse during the respiratory cycle (Whitsett and Baatz, 1992). SP-C appears to have a primary role in efficiently packaging the lipids into the multilayered lamellar bodies. Low amount of surfactant in the air spaces as a result of premature birth can lead to respiratory distress syndrome (RDS), another term for surfactant deficiency. On the other hand, genetic (hereditary) SP-B deficiency is most often lethal, whereas hereditary SP-C deficiency most often leads to nonlethal interstitial lung disease (i.e., progressive scarring of lung tissue, or fibrosis) (Nogee, 2004). Both SP-A and SP-D have significant roles in host innate immunity, important in helping the lung to fight off infections (McCormack and Whitsett, 2002).

Pulmonary Vasculature

The fetal development of pulmonary vasculature occurs alongside the development of the fetal lungs. Additionally, the diversion of the majority of fetal blood flow away from the lungs has been described. To review, oxygenation of fetal blood occurs in the placenta and this oxygenated blood is then returned to the fetus via the umbilical vein. The majority then bypasses the fetal liver via the ductus venosus, travels to the inferior vena cava and into the right atrium of the heart. At that point, rather than travel to the lungs, 90% of the blood is shunted across the foramen ovale to the left atrium to continue on to the left ventricle, or is shunted across the ductus arteriosus and out the aorta to supply blood to the body (Baldwin and Dees, 2012). Despite lower levels of oxygen in the fetal circulation, the fetus receives adequate oxygenation due to the increased concentration of fetal hemoglobin and its higher affinity for oxygen. Once birth occurs and the umbilical cord is cut, the newborn infant is dependent on gas exchange via the lung and the blood circulation can no longer bypass the lung. This is enabled by a drop in the resistance of the blood vessels in the lung, now inviting blood into the lung, to pick up oxygen and deliver carbon dioxide for disposal.

When studying the transition from fetal to neonatal life, there must be a discussion regarding the change in pulmonary vascular resistance that is required for successful transition. During gestation, there is high vascular tone maintained in the fetus due to the

action of vasoconstrictors such as endothelin-1 and platelet activating factor as well as low expression of vasodilators such as endothelium-derived nitric oxide and prostaglandin I₂. After birth, infants are exposed to room air (21% FiO₂) which is a significantly increased oxygen exposure than what infants experience in utero. This sudden change in environment from one of relative hypoxia (fetal environment) to one of relative hyperoxia (neonatal environment), as well as the initiation of respiratory ventilation, prompt a reversal which favors vasodilators over vasoconstrictors. The physical shear stress of the increase in blood flow suddenly traveling to the lungs also contributes to increasing vasodilation. There is a rapid decrease in pulmonary vascular resistance at birth but this decrease also continues over the first few months of postnatal life, before reaching normal adult levels others (Steinhorn and Abman, 2012; Gao and Raj, 2017). Additionally, ductus arteriosus closure occurs naturally in the first week of life as part of normal development, eliminating this as a shunt.

This transition becomes even more obvious in the setting of persistent pulmonary hypertension of the newborn (PPHN; previously known as persistent fetal circulation) in which the normal switch to a state favoring vasodilation does not occur at the time of birth. This can be an idiopathic disorder but more commonly is a result of chronic fetal hypoxia, pulmonary hypoplasia, sepsis, pneumonia, meconium aspiration syndrome, or RDS, among many other causes. The underlying etiology of the PPHN has a definite impact on the outcome and the therapies utilized to treat this disorder but PPHN is universally a disorder with high morbidity and mortality regardless of etiology.

Abnormalities in Development: Clinical Associations

Abnormalities that occur early in development most often affect the structural integrity of the initial developing large airways in the respiratory tract. Actual disruption in the development of lung buds from the primitive foregut can lead to pulmonary agenesis (absence of the lung altogether), with unilateral agenesis more common than bilateral agenesis. If the critical process of separation between the respiratory and the digestive tract does not occur properly in the first 6 weeks of gestation, this can result in the formation of a tracheoesophageal fistula (TEF), whereby there remains an abnormal connection between the two systems. There are several variations of this disorder but the most common is a blind pouch esophageal atresia proximally with a distal esophagus originating from the trachea. Tracheoesophageal fistulas can also be associated with multi-system congenital anomalies such as occur in the VATER/VACTERL association (acronym describing characteristics of the association: vertebral, anal, cardiac, tracheoesophageal, renal, and limb anomalies). Tracheal agenesis, while rare, can also occur in this stage with the trachea ending in a blind pouch and the main stem bronchi instead originating from the esophagus. TEF and tracheal agenesis are characterized by abnormalities in the separation of the respiratory tract from the primitive foregut. Similarly, additional budding from the primitive foregut can result in bronchogenic cysts, often in the mediastinal area. These are often fluid- or mucus-filled and do not usually communicate with the more distal airways. Rarely, abnormal budding from the ventral foregut can lead to the development of areas of abnormal pulmonary tissue, which can either be in connection with the lung (intralobar bronchopulmonary sequestration) or external to the lung (extralobar bronchopulmonary sequestration). Whether intralobar or extralobar sequestration develops is dependent on the timing of the abnormal budding from the foregut: intralobar sequestration occurs earlier, before lung pleura is formed, whereas extralobar sequestration occurs later leading to the development of its separate anatomy and separate pleura. Obstruction of portions of the larger airways can also result in congenital lobar overinflation through air trapping or in congenital pulmonary airway malformations (CPAMs) through abnormal branching morphogenesis (Wert, 2017).

In addition to disorders resulting from improper structural development of the large airways, there can be disorders in the development of the more distal airways, usually resulting in interstitial lung disease and pulmonary vasculature abnormalities such as pulmonary hypertension. These interstitial disorders include *congenital acinar dysplasia* which is a severe disorder characterized by deficiency in normal mature alveoli. This disorder likely occurs during the pseudoglandular stage of development. Growth arrest in the distal airways at later stages of gestation (such as the canalicular and saccular stages) may result in another severe disorder called *congenital alveolar dysplasia* in which distal airspaces are not properly approximated to the pulmonary vascular bed leading to dysfunctional gas exchange. In this disorder, there is sufficient development of the pulmonary vasculature; however it is not in close enough proximity to the distal airways to facilitate gas exchange. In comparison, *alveolar capillary dysplasia* is a disorder in which there are both insufficient pulmonary capillaries as well as poor proximity of the capillaries that do exist to the distal airways, compromising functional gas exchange. The pulmonary veins may also be located in an abnormal placement near the pulmonary arteries (misalignment of pulmonary veins) (Wert, 2017).

Developmental accidents involving other systems can also affect respiratory development and function. For instance, an error in the closure of the pleuroperitoneal fold in the pseudoglandular stage can result in an abnormal persistence of communication between the thoracic and abdominal cavities, allowing abdominal contents such as the stomach, bowel, and liver to herniate into the chest cavity (*congenital diaphragmatic hernia*). This space occupying mass of gastrointestinal structures results in compression of the developing lung and subsequent *pulmonary hypoplasia* with associated abnormal pulmonary vasculature development. Clinically, these infants have varying levels of respiratory failure and pulmonary hypertension depending on the severity and timing during gestation of the lesion, ranging from mild respiratory compromise to death. Pulmonary hypoplasia may also result from abnormal renal development, chronic amniotic fluid leakage, or upper airway obstruction. Each of these conditions result in decreased amniotic fluid and inadequate fetal lung fluid, which are crucial for lung development.

Finally, premature birth results in a growth disruption postnatally in both respiratory development and pulmonary vasculature development, with severity largely dependent upon the degree of prematurity. Infants born during the saccular stage of

development commonly have inadequate surfactant, resulting in surfactant deficiency at birth, called respiratory distress syndrome, or RDS. In addition, this lung developmental growth disruption affects many premature infants and can ultimately result in the chronic lung disease of prematurity called *bronchopulmonary dysplasia*.

References

- Baldwin, H. S., & Dees, E. (2012). Embryology and physiology of the cardiovascular system. In C. A. Gleason, & S. U. Devaskar (Eds.), *Avery's diseases of the newborn* (9th edn.). Philadelphia, PA: Elsevier. chapter 50.
- Bland, R. D., Bressack, M. A., & McMillan, D. D. (1979). Labor decreases the lung water content of newborn rabbits. *American Journal of Obstetrics and Gynecology*, 135(3), 364–367.
- Burri, P. (2006). Structural aspects of postnatal lung development—Alveolar formation and growth. *Biology of the Neonate*, 89, 313–322.
- Carlton, D. P. (2017). Regulation of liquid secretion and absorption by the fetal and neonatal lung. In R. Polin, S. Abman, D. Rowitch, W. Benitz, & W. W. Fox (Eds.), *Fetal and neonatal physiology* (5th edn.). Philadelphia, PA: Elsevier. chapter 65.
- Delacourt, C., & Hadchouel, A. (2017). Regulation of alveolarization. In R. Polin, S. Abman, D. Rowitch, W. Benitz, & W. W. Fox (Eds.), *Fetal and neonatal physiology* (5th edn.). Philadelphia, PA: Elsevier. chapter 62.
- Fraga, M. V., & Guttentag, S. (2012). Lung development. In C. A. Gleason, & S. U. Devaskar (Eds.), *Avery's diseases of the newborn* (9th edn.). Philadelphia, PA: Elsevier. Chapter 42.
- Gao, Y., & Raj, J. U. (2017). Regulation of pulmonary circulation. In R. Polin, S. Abman, D. Rowitch, W. Benitz, & W. W. Fox (Eds.), *Fetal and neonatal physiology* (5th edn.). Philadelphia, PA: Elsevier. Chapter 78.
- Guglani, L., Lakshminrusimha, S., & Ryan, R. M. (2008). Transient tachypnea of the newborn. *Pediatrics in Review*, 29, e59–e65.
- Guglani, L., Ryan, R. M., & Lakshminrusimha, S. (2009). Risk factors and management of transient tachypnea of the newborn. *Pediatric Health*, 3(3), 251–260.
- Kimura, J., & Deutsch, G. H. (2007). Key mechanisms of early lung development. *Pediatric and Developmental Pathology*, 10, 335–347.
- McCormack, F. X., & Whitsett, J. A. (2002). The pulmonary collectins, SP-A and SP-D, orchestrate innate immunity in the lung. *Journal of Clinical Investigation*, 109, 707–712.
- Nogee, L. M. (2004). Alterations in SP-B and SP-C expression in neonatal lung disease. *Annual Review of Physiology*, 66, 601–623.
- Ochs, M., Nyengaard, J. R., Jung, A., Knudsen, L., Voigt, M., Wahlers, T., Richter, J., & Gundersen, H. J. G. (2004). The number of alveoli in the human lung. *American Journal of Respiratory and Critical Care Medicine*, 169, 120–124.
- Shojaie, S., & Post, M. (2017). Molecular mechanisms of lung development and lung branching morphogenesis. In R. Polin, S. Abman, D. Rowitch, W. Benitz, & W. W. Fox (Eds.), *Fetal and neonatal physiology* (5th edn.). Philadelphia, PA: Elsevier. Chapter 64.
- Steinhorn, R. H., & Abman, S. H. (2012). Persistent pulmonary hypertension. In C. A. Gleason, & S. U. Devaskar (Eds.), *Avery's diseases of the newborn* (9th edn.). Philadelphia, PA: Elsevier. Chapter 52.
- Wallace, M., Hooper, S. B., & Harding, R. (2017). Physiologic mechanisms of normal and altered lung growth before and after birth. In R. Polin, S. Abman, D. Rowitch, W. Benitz, & W. W. Fox (Eds.), *Fetal and neonatal physiology* (5th edn.). Philadelphia, PA: Elsevier. Chapter 63.
- Warburton, D. (2017). Overview of lung development in the newborn human. *Neonatology*, 111, 398–401.
- Wert, S. (2017). Normal and abnormal structural development of the lung. In R. Polin, S. Abman, D. Rowitch, W. Benitz, & W. W. Fox (Eds.), *Fetal and neonatal physiology* (5th edn.). Philadelphia, PA: Elsevier. Chapter 61.
- Whitsett, J. A., & Baatz, J. E. (1992). Hydrophobic surfactant proteins SP-B and SP-C: Molecular biology, structure, and function. In B. Robertson, L. M. G. van Golde, & J. J. Batenburg (Eds.), *Pulmonary surfactant: From molecular biology to clinical practice*. Amsterdam: Elsevier.

Fetal Gastrointestinal Tract Development and Function

Norbert Chauvet, Sandrine Faure, and Pascal de Santa Barbara, University of Montpellier, Montpellier, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

The vertebrate gastrointestinal (GI) tract is a vital and specialized organ system, located behind the body wall and characterized by its exceptional length. It is formed as a closed primitive tube that is regionalized during development along the anterior–posterior (AP) axis into various organs such as pharynx, esophagus, stomach (foregut), small intestine (midgut), and large intestine (hindgut) and specialized along the radial axis into concentric layers of morphological and functionally distinct cell types. These processes of differentiation according to different axes that are established during development and maintained throughout life are essential for the digestive tract to ensure its basic function, namely food digestion, absorption of nutrients and water, and elimination of feces (de Santa Barbara et al., 2002; Faure and de Santa Barbara, 2011).

Structural Anatomy and Function of the GI Tract

In adults, each part of the GI tract ensures different and complementary function (Fig. 1A). The stomach secretes acid and enzymes necessary for food digestion and possesses a hypertrophic muscular structure involved in the mechanical digestion of food. Conversely, the small intestine and colon have a thin muscular layer necessary for the transit and elimination of feces. Other functions ensured by the small intestine and the colon are the chemical digestion, absorption of nutrients and water, and the immune defense. Histologically, the adult GI tract is composed of four functional layers (mucosa, submucosa, muscularis propria, and adventitia or serosa). The mucosa is the innermost layer, in contact with the gastrointestinal lumen that harbor morphological features specific to each part of the GI tract; it is composed of epithelial cells with a supporting layer of connective tissue (the lamina propria) and a thin smooth muscle layer (the muscularis mucosae). Underneath the mucosa lays the submucosa, a sheet of loose connective tissue involved in its support. This is followed by the muscularis propria that is involved in the mechanical breakage of food intake, especially in the stomach, and is responsible for its transit along the AP axis by contracting in a phasic manner under the regulation of the autonomous enteric nervous system (ENS). Moreover, the GI tract is surrounded by the adventitia or serosa (depending on its AP position) to prevent frictions between the GI tract and other tissues/organs.

Development of the Fetal GI Tract

Origin and Patterning Along the Anterior–Posterior Axis (AP) During Early Development

The GI tract is a remarkably complex and specialized organ system derived from a primitive and undifferentiated tube that results from the association of the endoderm with the splanchnic mesenchyme at the early embryonic period. During gastrulation, the definitive endoderm ventrally invaginates to first give rise to the anterior intestinal portal (AIP) and secondly the caudal intestinal

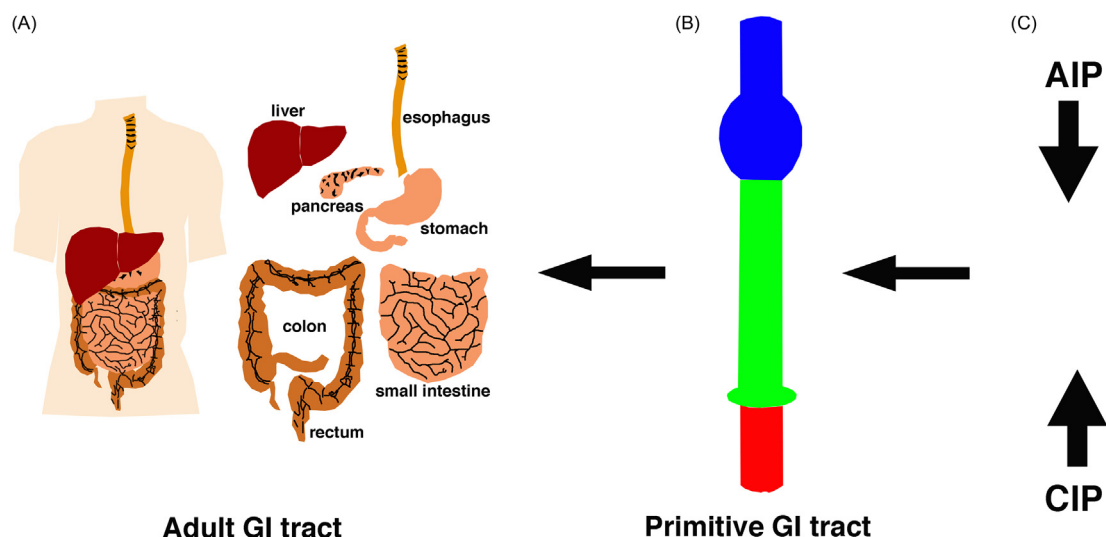


Fig. 1 From AIP/CIP, to primitive to final GI tract. (A) Location of the adult GI tract into the body wall and its final organization into esophagus, stomach, small intestine, colon, and rectum following AP axis. (B) Formation of the primitive GI tube that harbors early AP regionalization divided into foregut, midgut, and hindgut. (C) Arrows show the anterior intestinal portal (AIP) and caudal intestinal portal (CIP) at the opposite poles of the embryo.

portal (CIP) ends of the embryo (Fig. 1C). These two structures elongate toward and fuse to form a straight and uniform primitive GI tube (Fig. 1B) (Roberts, 2000). The human primitive gut is formed at week 4 of gestation and is commonly divided in three embryological structures along the AP axis: foregut, midgut, and hindgut. The foregut will give rise to the pharynx, esophagus, and the stomach; the midgut will develop into the small intestine; and the hindgut will form the colon and the rectum. The foregut is the common embryonic organ that will give rise to two independent systems, the lung and gut. The respiratory system originates from the formation of an endodermal diverticulum in the ventral wall of the foregut, whereas the esophagus forms from the foregut dorsal wall (Faure and de Santa Barbara, 2011). The stomach also arises from the foregut and quickly starts to increase in size compare to the other parts of the primitive gut. The dorsal wall grows faster than the ventral wall, conducting to the stomach curvature. The primitive gut is localized in the coelomic cavity within the body wall and is connected to the dorsal mesentery. At week 6 of gestation, the length of the ileum rapidly increases and brings out part of the intestine. Between week 8 and 10, the intestinal loop rotates around the mesenteric artery and moves back into the coelomic cavity within the body wall. Finally, at week 11, the cecum moves downward, attracting the proximal part of the posterior intestine and the intestine reaches its adult final position. These regional morphological and functional differences are maintained throughout life and are essential for normal GI function.

Colonization of the Fetal GI Tract by the Enteric Nervous System (ENS)

Concomitant with the AP patterning, the GI tract is colonized by the enteric nervous system (ENS), the intrinsic innervation of the GI tract. The ENS is a complex intrinsic network of neurons and glial cells that controls gut motility. In human, it contains around 500 million neurons grouped in different subtypes (around 20 in humans) that differ in neurotransmitter expression, morphology, electrophysiology, and function (Gershon et al., 1993). All of the neurons and glial cells of the ENS are derived from the neural crest, a small population of cells that originate from the dorsal part of the embryonic neural tube (Fig. 2A) and migrate through a ventral route to colonize the developing GI tract (Fig. 2B). Chick and mouse models have contributed to our understanding of the origin and migration of the ENS. ENS precursors arise principally from two distinct regions. In the chick embryo, the vagal-derived progenitors enter the foregut mesenchyme on embryonic day 3 (E3) and begin an anterior to posterior wave of migration to populate the entire GI tract by E8.5 and establish its innervation (Le Douarin and Teillet, 1973). Additional crest-derived cells from the sacral region of the neural tube contribute to the postumbilical ENS progenitors that migrate in the opposite direction through the distal hindgut (Burns and Le Douarin, 1998; Wang et al., 2011) (Fig. 3A). In addition, when colonizing the stomach and the intestine, the migration front of ENS cells is located in the outermost layer of the mesenchyme, adjacent to the serosal epithelium and will migrate inwards to set up plexuses (Fig. 3B) (Burns and Le Douarin, 1998). In contrast, in the colorectum area, ENS cells initially colonize the submucosal layer before migrating outwards to organize the myenteric plexus (Fig. 3B) (Burns and Le Douarin, 1998). To form the human ENS network, precursors must migrate away from the esophagus at week 4 of gestation and spread uniformly throughout the entire bowel, proliferate and undergo sequential lineage restriction to reach the colon at week 7 of gestation before differentiating into many distinct subtypes of interconnected neurons and glia that become organized into enteric ganglia that comprise the myenteric (Auerbach') and submucosa (Meissner') plexi located between the muscle layers of the gut wall (Grundty and Schemann, 2005; Furness, 2006). Contribution of a sacral neural crest pool to the ENS in humans remains unknown. The end result of all this developmental process is the formation of an integrated neuronal network, which controls visceral smooth muscle cell contractions (or peristalsis) and GI motility to ensure food transit.

Late Development: Differentiation of Fetal Into Functional GI Tract

The differentiation of intestinal mesoderm into intestinal smooth muscle occurs also along the radial axis and is conserved in all vertebrates as indicated by the expression of alpha-smooth muscle actin (alpha-SMA) as marker (Fig. 4) (Le Guen et al., 2015; Shyer et al., 2013). At week 7 of gestation, the primitive gut is a simple tube with a stratified undifferentiated endodermal layer that is surrounded by an undifferentiated and uniform mesenchymal layer, in which few alpha-SMA positive cells are present at the periphery of the mesenchyme. In addition, interstitial cells of cajal (ICCs) emerged from the developing gut mesenchyme at

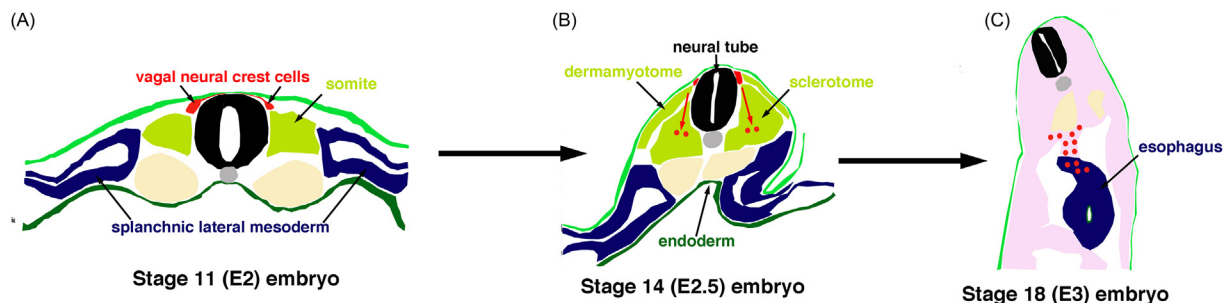


Fig. 2 Origin of the enteric neural crest cell derived cells. (A) After the closure of the neural tube in the trunk of the chick embryo, the vagal neural crest cells (NCCs) emigrate through the neural tube at Stage 11 (E2). (B) Vagal NCCs migrate to the anterior portion of the sclerotome at Stage 14 (E2.5). (C) Vagal NCCs enter into the GI tract at Stage 18 (E3) through invading the anterior foregut mesenchyme.

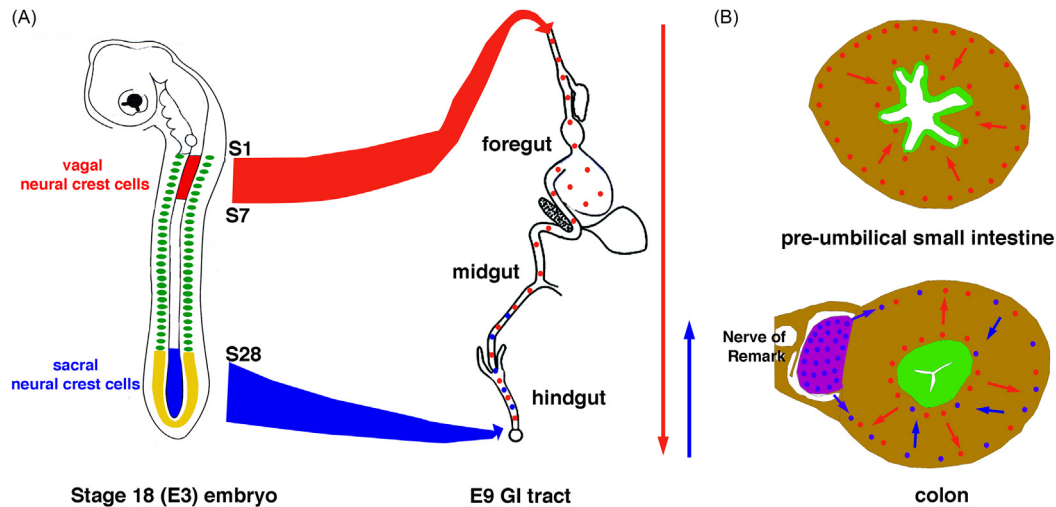


Fig. 3 Colonization of the GI tract by the autonomous ENS from vagal and sacral neural crest origins. (A) Vagal neural crest cells (NCC) adjacent to somites 1–7 colonize the whole GI tract in an anterior-to-posterior direction during days E3–9. Sacral NCC caudal to somite 28 migrate in a - posterior-to-anterior direction to form first the nerve of Remark adjacent to the gut wall during days E4–6 and enter in the gut after E10. (B) Within the preumbilical small intestine, vagal NCCs initially migrate within the outer mesenchymal region, then migrate inwards to colonize the submucosal plexus region. Within the colorectum, vagal NCCs initially migrate within the submucosal region, then migrate outwards to colonize the myenteric plexus region. In addition, after their migration and stop through the Nerve of Remark, within the hindgut, sacral NCC colonize the myenteric plexus region, then migrate inward, to colonize the submucosal plexus.

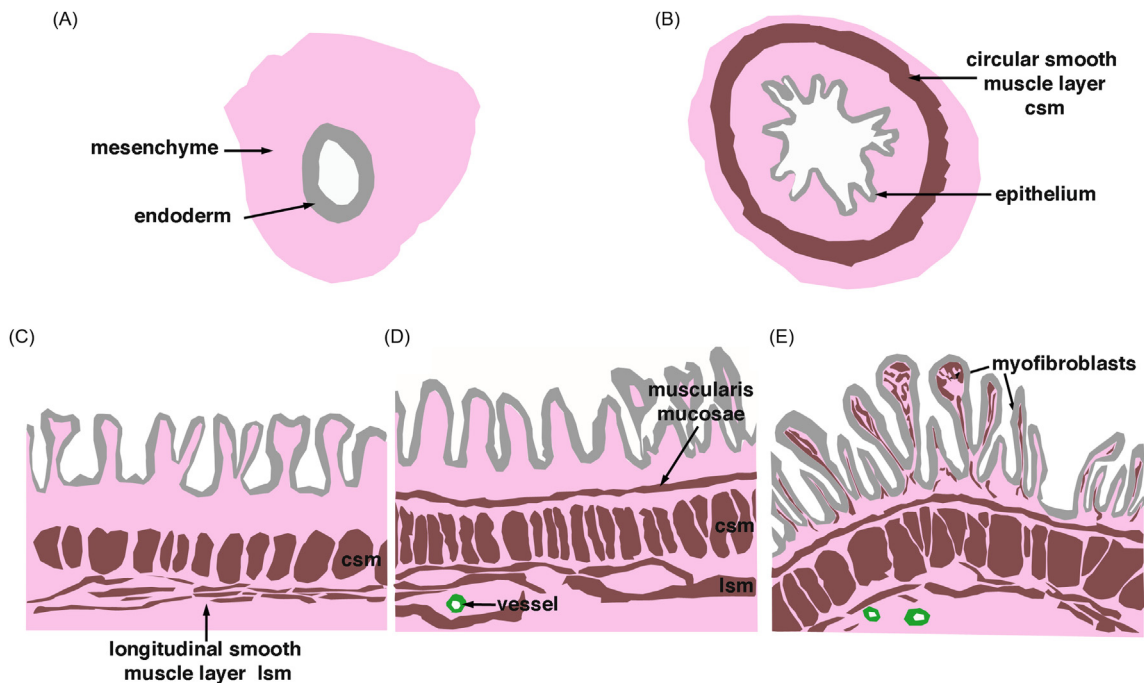


Fig. 4 Development and differentiation of the intestinal SMCs. Smooth muscle cell differentiation in the small intestine of chicken embryos. Smooth muscle cells (SMC) were detected by immunostaining with an antibody against the SMC-specific marker alpha-SMA (brown color), before their transformation into cartoons. (A) Nonorganized and undifferentiated mesenchymal cells in the small intestine of a 6-day-old (E6) chick embryo. During early embryonic development, the visceral endoderm is uniform with stratified layers of cells. (B) alpha-SMA-positive cells in the circular smooth muscle layer of the small intestine of an E13 chick embryo. The ongoing intestinal epithelial cytodifferentiation leads to the formation of an epithelial monolayer. (C) alpha-SMA-positive cells in the longitudinal and circular smooth muscle layers of the small intestine of an E15 chick embryo. (D) alpha-SMA-positive cells in the longitudinal, circular, and submucosal smooth muscle layers in the small intestine of an E16 chick embryo. (E) alpha-SMA-positive cells (myofibroblasts) are detected also in the lamina propria of the small intestine in an E18 chick embryo. (C–E) Intestinal epithelial cytodifferentiation is marked by mesodermal growth into the lumen and villi formation, characterized by specific long and thin villi in the small intestine. *csm*, circular smooth muscle; *lsm*, longitudinal smooth muscle.

week 9 to surround and closely oppose the myenteric ganglia by week 11 to participate to the electrical pacemaker activity of the gut (Wallace and Burns, 2005). The stratified, undifferentiated endodermal cells undergo a columnar transformation accompanied by mesodermal outgrowth. This process results in the development of villi (starting at about week 9 of gestation) along a cranial to caudal axis. A differentiating external circular muscle layer is also observed. The anterior–posterior axis influences the morphologic and epithelial cell differentiation along the radial axis as well.

Hence, in late fetal life, the small intestine epithelium is characterized by long and thin villi, whereas the colon epithelium shows wide and flat villi (Fig. 5) (de Santa Barbara et al., 2003). Villi are separated by a proliferating intervillus epithelium. As the gut develops the intervillus epithelium is reshaped downwards to form crypts. At around week 12–14, primitive crypts begin to appear and at week 13, both circular and longitudinal muscularis can be observed. At week 16, the muscularis mucosa appears and is fully organized by gestational week 18. By week 20, the intestine displays well-developed villi and crypts supported by a specialized connective tissue (the lamina propria), where some alpha-SMA positive cells (myofibroblasts) are observed. The crypt-villous unit allows for a great increase in the surface area for absorption. The small intestine conserves its villus-crypt units throughout life. Conversely, in the adult colon of many species (including humans, but not chickens), embryonic villi will disappear. Transient formation of villi is observed in the embryonic proximal part of the colon, but in humans these villi are flattened before birth and thus the adult human colon is characterized by a relatively flat epithelium regularly separated by crypts. The formation of these crypt-villous structures and epithelial cell differentiation rely on reciprocal signaling between endoderm and mesoderm that occurs during early development period.

Reciprocal Interaction During the Fetal GI Tract Development

In most vertebrates the intestinal endoderm shows a uniform morphology (undifferentiated and stratified cuboidal cells) until mid-gestation when epithelial–mesenchymal interactions direct endodermal differentiation. Signals provided by the mesoderm/smooth muscle will first regulate AP regionalization during endoderm patterning/differentiation, but also will secondarily participate to the epithelium shaping and villi formation. The mature gut epithelia conserve their morphologic and functional pattern specificities throughout adult life.

Mesenchyme–Epithelial Interaction in AP Patterning

After AIP and CIP fusion, the GI endoderm is uniform along its AP axis. However, as differentiation takes place, morphological differences appear, leading to the formation of region-specific epithelium types. These mechanisms involve reciprocal (bi-directional) signals between epithelial cells and the adjacent mesoderm that are required for normal homeostasis. Many studies have demonstrated the importance of the interactions between endoderm and mesoderm during early GI tract development. In vitro experiments have demonstrated that the primitive foregut endoderm needs to be co-cultured with heterologous splanchnic mesenchyme to allow the correct differentiation along the AP axis, while somitic or cephalic mesoderm and limb buds are relatively inefficient. This finding supports the idea that the actors governing the regionalization of digestive epithelia are specifically found in the splanchnic mesenchyme. Many studies reported evidence that in the gut, the mesoderm dictates the ultimate epithelial pattern. For instance, in chicken embryos, early muscular stomach (gizzard) endoderm co-cultured with glandular stomach (proventriculus)

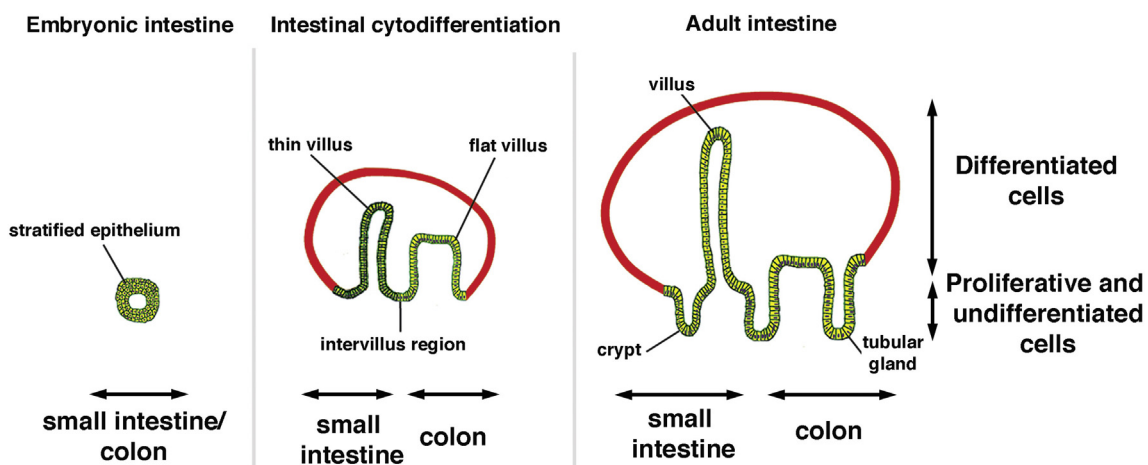


Fig. 5 From development to differentiation of the intestinal epithelium. During early embryonic development, the visceral endoderm appears as a uniform, stratified cell layer. Intestinal epithelial differentiation occurs during fetal development and is marked by mesodermal growth toward the lumen and formation of villi that are separated by proliferative intervillus epithelium. Anterior–posterior axis morphological differences appear: long and thin villi in the small intestine and transitory wide, flat villi in the colon. The intervillus epithelium of the small intestine is reshaped downwards to form crypts. In humans, the final architecture of the small intestine is reached before birth and is characterized by the crypt-villous unit. The colonic villi disappear at the time of birth and the mature colon epithelium present tubular glands (crypts). Modified from de Santa Barbara et al. (2003). Development and differentiation of the intestinal epithelium. *Cellular and Molecular Life Sciences* 60(7), 1322–1332.

mesoderm differentiates into proventricular epithelium (Kedinger et al., 1998). However, there is one exception to the endoderm morphological/cytological plasticity upon exposure to regional-specific mesoderm. Indeed, co-culture of midgut epithelium with heterologous mesenchymal tissues does not influence its specification, as indicated by the retained expression of midgut epithelial digestive enzymes (Kedinger et al., 1998). This difference in the ability of midgut and foregut endoderm to undergo complete heterologous differentiation demonstrates that the mechanisms involved in the gut epithelium cyto-differentiation are different along the AP axis, with midgut endoderm displaying cell-autonomous features.

Reciprocal Interaction Between Enteric Nervous System and Smooth Muscle/Mesenchyme

Interaction of ENS cells with mesenchymal/smooth muscle layer are necessary for their correct migration and differentiation (Burns and Le Douarin, 1998). Moreover, coordinated smooth muscle differentiation and ENS migration are required for normal GI development and patterning. The specific deletion of ENS cells into the stomach impairs smooth muscle development and induces trans-differentiation of both stomach mesenchymal and epithelial layers into a mixed stomach–intestine structure (Faure et al., 2015), supporting a multiple tissular interaction requirement for correct stomach development and differentiation. If the differentiation of the smooth muscle cells of the stomach require the presence of a crucial number of ENS cells, early intestinal smooth muscle differentiation is required for patterning the developing ENS (Graham et al., 2017), demonstrating the difference of mesenchymal/ENS interaction process along the developing GI tract.

Reciprocal Interaction During Smooth Muscle Cell Differentiation and Epithelial Organization

As above commented, during the embryonic period the digestive mesenchyme differentiates into radial axis forming distinct concentric layers around the epithelium: the lamina propria, muscularis mucosae, submucosa, and the smooth muscle layers. Endodermal-derived signals are responsible for the patterning across the radial axis of the gut (Sukegawa et al., 2000). In addition, when skin fibroblasts or somitic mesoderm are co-cultured with gut endoderm, they differentiate into smooth muscle rather than fibroblasts or skeletal muscle through the induction of the expression of smooth muscle marker, such as alpha-SMA (for review, Le Guen et al., 2015). Moreover, epithelial morphogenesis and villus formation of the gut is intimately linked to the sequential differentiation of the three smooth muscle layers, which produce a series of different mechanical forces. Treatment of the intestinal tube with inhibitors of smooth muscle differentiation prevents these progressive villus patterning. Thus, the compressive forces from the smooth muscle layers drive the epithelium to buckle and fold to finally form villus structures (Shyer et al., 2013). Indeed the formation of villi generates gradients of morphogen signaling that restrict the stem cells within the crypt to the base of each villus (Shyer et al., 2015).

Conclusion

As described, the complex and functional pediatric GI tract originates from the lateral splanchnic mesoderm that contributes to the intestine mesoderm and overlays the endoderm to form the primitive intestine. During their development, differentiation and morphogenesis, these structures are colonized by neural crest-derived cells that will form the ENS to participate to the network essential for the peristalsis. Moreover, during development the digestive mesenchyme is also colonized by other cell types such as endothelial and lymphatic cells (Hatch and Mukouyama, 2015). In addition, serosal mesothelial cells also colonize the entire primitive gut and a subset of these cells undergoes epithelial–mesenchymal transition to differentiate into smooth muscle cells that surround the primitive vessels formed by endothelial cells (Wilm et al., 2005; Kim et al., 2007). Intestinal vascular and lymphatic networks serve as efficient nutrient and lipid absorption.

In the neonatal and adult GI tract, multicellular interactions are essential for the development but also for the maintenance of the regionalization and homeostasis of the GI tract. Disruption of these interactions is also associated with bowel malformation or dysfunction, such as tracheo-esophageal atresia (TE), infantile hypertrophic pyloric stenosis, or anal atresia (Faure and de Santa Barbara, 2011; de Santa Barbara et al., 2002).

Summary

This article describes the development and the multitissular interaction mechanisms that drive the differentiation of the fetal gastrointestinal (GI) tract to ensure correct morphogenesis and its basic function. We pass through the anatomy and function of the adult GI tract before focusing on the development of the primitive and fetal GI tract. We describe the early patterning and the late differentiation of the fetal GI tract, its colonization by the enteric nervous system and the reciprocal multilayer interactions to ensure early patterning, late differentiation, and epithelial organization. Finally, we conclude by showing the importance of these events to achieve the normal and functional digestive system.

References

- Burns, A. J., & Le Douarin, N. M. (1998). The sacral neural crest contributes neurons and glia to the post-umbilical gut: Spatiotemporal analysis of the development of the enteric nervous system. *Development*, 125(21), 4335–4347.
- Faure, S., & de Santa Barbara, P. (2011). Molecular embryology of the foregut. *Journal of Pediatric Gastroenterology and Nutrition*, 52(Suppl 1), S2–3. <https://doi.org/10.1097/MPG.0b013e3182105a1a>.

- Faure, S., McKey, J., Sagnol, S., & de Santa Barbara, P. (2015). Enteric neural crest cells regulate vertebrate stomach patterning and differentiation. *Development*, 142(2), 331–342. <https://doi.org/10.1242/dev.118422>.
- Furness, J. B. (2006). The organization of the autonomic nervous system: Peripheral connections. *Autonomic Neuroscience*, 130(1–2), 1–5.
- Gershon, M. D., Chalazonitis, A., & Rothman, T. P. (1993). From neural crest to bowel: Development of the enteric nervous system. *Journal of Neurobiology*, 24(2), 199–214.
- Graham, H. K., Maina, I., Goldstein, A. M., & Nagy, N. (2017). Intestinal smooth muscle is required for patterning the enteric nervous system. *Journal of Anatomy*, 230(4), 567–574. <https://doi.org/10.1111/joa.12583>.
- Grundy, D., & Schemann, M. (2005). Enteric nervous system. *Current Opinion in Gastroenterology*, 21(2), 176–182.
- Hatch, J., & Mukoyama, Y. S. (2015). Spatiotemporal mapping of vascularization and innervation in the fetal murine intestine. *Developmental Dynamics*, 244(1), 56–68. <https://doi.org/10.1002/dvdy.24178>.
- Kedinger, M., Duluc, I., Fritsch, C., Lorentz, O., Plateroti, M., & Freund, J. N. (1998). Intestinal epithelial–mesenchymal cell interactions. *Annals of the New York Academy of Sciences*, 859, 1–17.
- Kim, K. E., Sung, H. K., & Koh, G. Y. (2007). Lymphatic development in mouse small intestine. *Developmental Dynamics*, 236(7), 2020–2025.
- Le Douarin, N. M., & Teillet, M. A. (1973). The migration of neural crest cells to the wall of the digestive tract in avian embryo. *Journal of Embryology and Experimental Morphology*, 30(1), 31–48.
- Le Guen, L., Marchal, S., Faure, S., & de Santa Barbara, P. (2015). Mesenchymal–epithelial interactions during digestive tract development and epithelial stem cell regeneration. *Cellular and Molecular Life Sciences*, 72(20), 3883–3896. <https://doi.org/10.1007/s00018-015-1975-2>.
- Roberts, D. J. (2000). Molecular mechanisms of development of the gastrointestinal tract. *Developmental Dynamics*, 219(2), 109–120.
- de Santa Barbara, P., van den Brink, G. R., & Roberts, D. J. (2002de). Molecular etiology of gut malformations and diseases. *American Journal of Medical Genetics*, 115(4), 221–230.
- de Santa Barbara, P., van den Brink, G. R., & Roberts, D. J. (2003de). Development and differentiation of the intestinal epithelium. *Cellular and Molecular Life Sciences*, 60(7), 1322–1332.
- Shyer, A. E., Tallinen, T., Nerurkar, N. L., Wei, Z., Gil, E. S., Kaplan, D. L., Tabin, C. J., & Mahadevan, L. (2013). Villification: How the gut gets its villi. *Science*, 342(6155), 212–218. <https://doi.org/10.1126/science.1238842>.
- Shyer, A. E., Huycke, T. R., Lee, C., Mahadevan, L., & Tabin, C. J. (2015). Bending gradients: How the intestinal stem cell gets its home. *Cell*, 161(3), 569–580. <https://doi.org/10.1016/j.cell.2015.03.041>.
- Sukegawa, A., Narita, T., Kameda, T., Saitoh, K., Nohno, T., Iba, H., Yasugi, S., & Fukuda, K. (2000). The concentric structure of the developing gut is regulated by Sonic hedgehog derived from endodermal epithelium. *Development*, 127(9), 1971–1980.
- Wallace, A. S., & Burns, A. J. (2005). Development of the enteric nervous system, smooth muscle and interstitial cells of cajal in the human gastrointestinal tract. *Cell and Tissue Research*, 319(3), 367–382.
- Wang, X., Chan, A. K., Sham, M. H., Burns, A. J., & Chan, W. Y. (2011). Analysis of the sacral neural crest cell contribution to the hindgut enteric nervous system in the mouse embryo. *Gastroenterology*, 141(3), 992–1002.e1–6. <https://doi.org/10.1053/j.gastro.2011.06.002>.
- Wilm, B., Ipenberg, A., Hastie, N. D., Burch, J. B., & Bader, D. M. (2005). The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. *Development*, 132(23), 5317–5328.

Germ Cell Sex Differentiation

Kellie S Agrimson, University of Minnesota, Minneapolis, MN, United States

Cathryn A Hogarth, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Overview

The germ cells of both sexes arise from the primordial germ cells (PGCs) that are specified in the mouse epiblast at E6.25 and migrate to the embryonic gonad by E10.5. Germ cell sex is determined by the somatic environment of the gonad which is made up of either Sertoli (testis) or granulosa (ovary) cells, based on the presence or absence of SRY expression, respectively. While germ cell sex determination occurs in response to the somatic environment, germ cell sex differentiation depends on the levels of retinoic acid (RA) present in the gonad. PGCs in the fetal ovary are triggered to become oogonia and enter meiosis, arresting at the diplotene stage of meiosis I just prior to birth. Male PGCs continue to proliferate both prior to and after birth in the testis, separated by a period of cell cycle arrest, and enter meiosis asynchronously from puberty onward (**Fig. 1**). This article will discuss the field's current understanding of mouse germ cell sex differentiation and the role of RA during germ cell meiotic entry. To understand how RA plays a role in germ cell differentiation, first the intricacies to the RA signaling pathway must be explored.

How Is Retinoic Acid Metabolized in the Gonads?

Transport and Storage

Vitamin A, accumulated through the diet, is transported through the circulatory system in the form of retinol. Retinol binding proteins mediate retinol delivery to target organ systems in the body and once retinol reaches the cell, it is either stored as retinyl ester or metabolized into RA, the active metabolite of vitamin A (**Fig. 2**).

Synthesis

A two-step oxidative process converts retinol to RA. The first reversible step forms retinal from retinol via the action of the retinol dehydrogenases (RDH) or short chain dehydrogenases (DHRS), while the irreversible second step occurs when aldehyde dehydrogenases (ALDH) oxidize retinal into RA (**Fig. 2**). Depending on the tissue, there are varying isoforms of the oxidizing enzymes with fluctuating levels of activity necessary for the production of RA. Therefore, the expression patterns of the isoforms may differ within a single tissue which allows for RA levels in an organ system to be tightly controlled. The RDH and ALDH enzymes have been most well studied within the testis, while very little is known about their role in female germ cell development.

Degradation

RA has two fates in a cell, one of which is being catabolized into inactive metabolites by the cytochrome p450 family 26 (CYP26) enzymes, including three isozymes: CYP26A1, CYP26B1, and CYP26C1 (**Fig. 2**). RA degradation in a cell occurs in order to block or stop the expression of RA-responsive genes or to protect a neighboring cell from exposure to this signal. CYP26A1 and CYP26B1 are both expressed within the testis and are essential for the normal progression of spermatogenesis (**Hogarth et al., 2015**). Additionally, the current published data suggest that germ and Sertoli cells are protected from external RA signals by the peritubular myoid cells that surround the seminiferous epithelium based on the localization of *Cyp26* mRNA (**Vernet et al., 2006b; Wu et al., 2008**). As a result, RA levels within the seminiferous epithelium are likely regulated by cross-talk between germ and Sertoli cells.

Signaling

The other fate of RA is to control gene expression by RA receptor (RAR)/retinoid X receptor (RXR) mediated signaling. RA bound to a heterodimer of RAR/RXR can interact with RA response elements (RAREs) within gene regulatory regions (**Bastien and Rochette-Egly, 2004**). Recruitment of coactivators or corepressors control gene activity. In the absence of RA, RAR/RXR bound to RAREs recruit histone deacetylase complexes (HDAC) to repress gene expression. Once RA is bound, the HDAC is released and the RA/RAR/RXR bound complex will recruit histone acetylases or methyltransferases to open chromatin and activate gene expression. Both RAR and RXR families have three different isoforms including α , β , and γ . To date very little is known about the specificity and function of the RAR/RXR receptors in the ovary. However, Sertoli cell expression of RAR α and RXR β and germ cell expression of RAR γ are known to be required for proper spermatogenesis (**Gely-Pernot et al., 2012; Vernet et al., 2006a, 2008**).

How Are Male and Female Germ Cell Paths to Meiotic Entry Different?

Female germ cell entry into meiosis occurs embryonically in response to RA, while male entry occurs after birth in the mouse. RA synthesis occurs in the mesonephros of the developing urogenital ridge and then, in an anterior to posterior direction, moves into

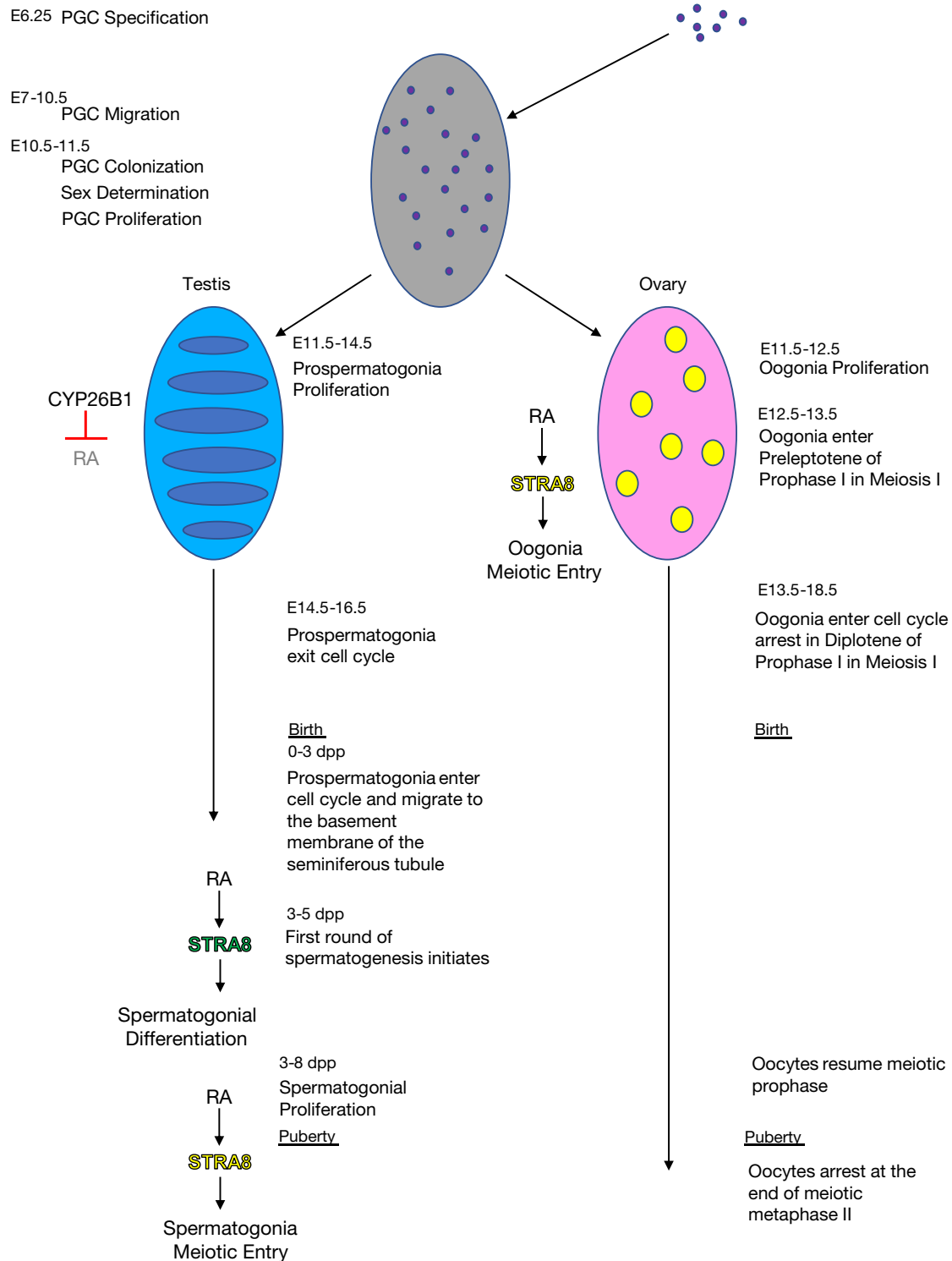


Fig. 1 Timeline summarizing the period of mouse PGC specification through sex determination and germ cell sex differentiation. By E11.5 sex is determined and germ cell fates diverge based on whether the surrounding gonad is male or female. The Sertoli cells express CYP26B1, which degrades any RA present in the testis and protects the male prospermatogonia from entering meiosis prematurely. In contrast, the ovary stops expressing CYP26B1 by E12.5, allowing oogonia to respond to active RA signaling. These oogonia express STRA8 and enter meiosis prior to birth. Male germ cells do not enter meiosis until after birth following spermatogonial differentiation in response to RA and several rounds of proliferation. Spermatogonia enter meiosis as preleptotene spermatocytes in response to STRA8 expression induced by RA signaling.

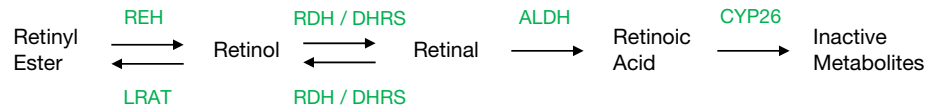


Fig. 2 Network of RA metabolizing enzymes and their role in RA synthesis and degradation. Retinol, transported through the body in the circulatory system, can either be stored as retinyl esters or converted to retinal, both of which are reversible reactions. Lecithin retinol acyltransferase (LRAT) converts retinol into retinyl ester while retinyl ester hydrolase (REH) mobilizes retinyl ester into retinol. Retinol is reversibly converted into retinal by the retinol dehydrogenases (RDH) or short chain dehydrogenases (DHRS). Retinal is irreversibly oxidized into RA by the aldehyde dehydrogenases (ALDH). RA can then signal within cells through the RAR/RXR mediated pathway or be degraded into inactive metabolites by the cytochrome *p*450 family 26 (CYP26) enzymes.

the ovary or testis via the mesonephros ducts at approximately E11.5. However, by this time the Sertoli cells have surrounded the germ cells within the testis cords and express CYP26B1, protecting the male germ cells from the RA signal. CYP26B1 acts as the meiotic inhibitory factor and degrades RA in the fetal testis. In contrast CYP26B1 expression is lost in the ovary by E12.5 and RA from the mesonephros induces female germ cells to express Stimulated by RA 8 (STRA8) and enter into meiosis (Fig. 3). The remaining sections of this article will examine what is known about female and male meiotic entry based on data collected from transgenic mouse gene knockout models and manipulations of RA levels in the gonads.

STRA8

STRA8 is currently described as the “gatekeeper” to meiosis. The regulatory region of *Stra8* includes two RAREs that are targeted directly by RA and STRA8 expression can be detected in as little as 2 h following RA exposure (Evans et al., 2014; Giuili et al.,

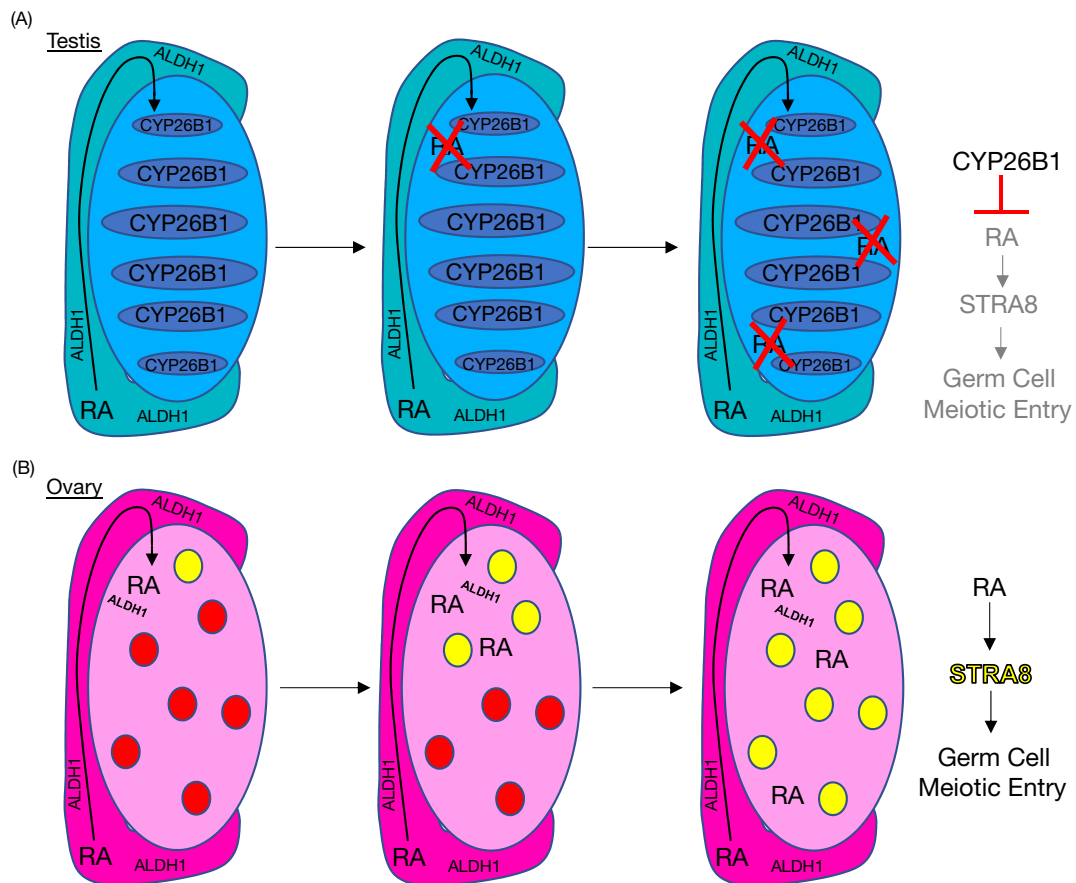


Fig. 3 Hypothesized roles for RA metabolic enzymes in the fetal testis and ovary. (A) CYP26B1, present in the Sertoli cells of the testis cords, degrades RA that is synthesized in the mesonephros by ALDH enzymes and diffuses into the testis in an anterior—posterior direction. RA degradation is shown in this model by the red X marks in the testis. (B) CYP26 is not expressed in the fetal ovary after E12.5, therefore allowing oogonia to turn on STRA8 and enter meiosis. This occurs in an anterior—posterior direction, shown by oogonia transitioning from red to yellow in this model. Additionally, some ALDH expression has been recently reported to be present in the fetal ovary, although likely only highly expressed when having to compensate for loss of other ALDH isoforms.

2002; Oulad-Abdelghani et al., 1996). *Stra8* deficiency results in infertility as meiosis is unable to initiate (Baltus et al., 2006). In the absence of *Stra8*, premeiotic DNA replication, meiotic chromosome condensation, cohesion, synapsis and recombination do not occur in female fetal germ cells (Baltus et al., 2006). Although the exact function of STRA8 is unknown, global deletion of *Stra8* resulted in misregulation of REC8, SYCP3, and H2AFX, all of which are meiotic markers. Ovary size was significantly reduced as were oocyte and follicle numbers (Dokshin et al., 2013).

How Does Deletion or Inhibition of the ALDH1A Synthesizing Enzymes Affect Germ Cell Differentiation?

Until recently, there was controversy over whether or not RA drives female germ cell meiotic entry. Past work investigated female meiotic entry in *Aldh1a2*- and *Aldh1a2/Aldh1a3*-deficient mice to examine the necessity of RA (Kumar et al., 2011). Pregnant mice carrying these ALDH deficient embryos were supplemented with RA from E6.75 to E9.25 to prevent embryonic lethality, as *Aldh1a2/Aldh1a3*-deficiency is embryonic lethal because these embryos do not synthesize enough RA to complete early embryogenesis. This brief supplementation allowed Kumar et al. (2011) to examine the effects from deficiencies following sex determination. RA has an extremely short half-life and the supplemental RA given to the pregnant females was cleared within 12–24 h (Mic et al., 2002). Surprisingly, *Stra8* expression was detected in oogonia deficient in *Aldh1a2/Aldh1a3* and additionally *Sycp3* and H2AFX were expressed in oogonia deficient in *Aldh1a2* alone (Kumar et al., 2011). The idea that the ovary may express a synthesizing enzyme was dismissed due to results collected from *RARE-hsplacZ* reporter transgene mouse model bred to the ALDH mutant mice (Rossant et al., 1991). This mouse model allows for visualization of active RA signaling within individual cells. Kumar et al. (2011) found that the urogenital ridge, not the ovary, expressed β -galactosidase. Although this transgene has been known to lose activity (Sakai and Drager, 2010) and it is possible that the breeding of this reporter line into the different genetic background of the ALDH mutant could have disrupted the expression of the transgene. Based on these results, Kumar et al. (2011) concluded that meiotic initiation can take place in the ovary without RA. However, more recently Bowles et al. (2016) found that *Aldh1a1* is expressed and produces RA in the developing ovary and that *Aldh1a1* expression is sensitive to RA levels in the developing gonads. This would suggest that in the previously discussed *Aldh1a2/Aldh1a3* knockout mouse model the amount of ALDH1A1 available to stimulate germ cell meiosis by synthesizing RA might have been enhanced (Bowles et al., 2016). As a result, it is likely that RA is required for female oogonia to enter meiosis.

RA deficiency can be achieved via either chemical inhibition of ALDH activity using the compound WIN 18,446, an irreversible inhibitor of ALDH1A2 and a reversible inhibitor of ALDH1A1 and ALDH1A3 (Arnold et al., 2015a, b), or by restricting vitamin A levels in the diet and thereby making the mice vitamin A deficient (VAD) and blocking the substrate necessary for RA synthesis. Oogonia from VAD female rat embryos exhibited a lack of *Stra8* expression and never initiated meiosis (Li and Clagett-Dame, 2009) suggesting the necessary role of RA in meiosis. Additionally, treatment of female urogenital ridges with WIN 18,446 resulted in blocked *Stra8* expression in oogonia (Hogarth et al., 2011). Taken together, these data suggest that meiosis does not initiate in RA deficient ovaries (Fig. 4).

How Does Deletion or Inhibition of the CYP26 Degrading Enzymes Affect Germ Cell Differentiation?

Examination of fetal *Cyp26b1* null testes in culture with RARE reporter cells revealed threefold higher RA levels when compared to control littermates (MacLean et al., 2007). Additionally, a significant reduction in germ cell number due to apoptosis and testis size at birth occurred (MacLean et al., 2007). The remaining germ cells left in *Cyp26b1* mutant testes had entered meiosis and some progressed as far as the pachytene stage of prophase I (MacLean et al., 2007).

More convincingly, Sertoli cell-specific deletion of *Cyp26b1* using the *Amh*-Cre revealed that germ cells in G0 reentered the cell cycle and initiated meiosis (Li et al., 2009). These studies support RA as the trigger for meiotic entry in the fetal gonad and provide

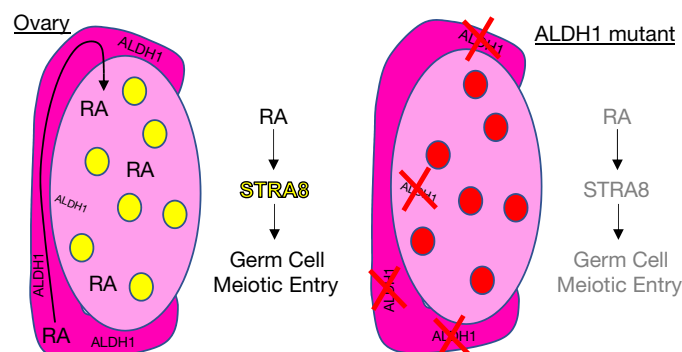


Fig. 4 Hypothesized model displaying loss of ALDH activity in the female urogenital ridge either by gene deletion or protein inhibition. In fetal ovaries that are not exposed to RA, oogonia do not express STRA8 or enter meiosis shown by red oogonia in the mutant compared to yellow differentiating oogonia in the control ovary. Red X marks in the mutant show loss of ALDH activity in the female urogenital ridge.

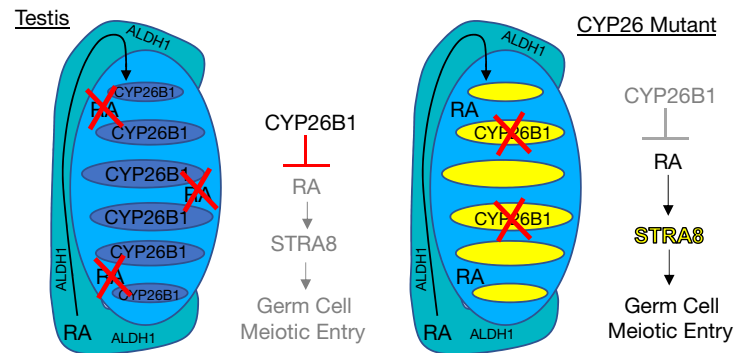


Fig. 5 Hypothesized model representing loss of CYP26 activity in the male urogenital ridge either by gene deletion or protein inhibition. In fetal testes that lose CYP26B1 activity, the Sertoli cells are unable to protect prospermatogonia from prematurely entering meiosis displayed by the mutant testis cords that have germ cells expressing STRA8 shown by *yellow* cords compared to *blue* cords in the control testis. Loss of CYP26B1 activity is represented by the *red X* marks in the testis.

evidence to suggest that the Sertoli cell population acts as a protective barrier for male prospermatogonia (Fig. 5). Interestingly dual knockout of *Cyp26b1*/*Stra8* male embryos rescues this phenotype (Saba et al., 2014). When STRA8 is unable to be expressed, prospermatogonia fail to enter meiosis (Saba et al., 2014). This observation further supports the notion that STRA8, in response to RA, is required for meiotic prophase initiation in both sexes.

Additional evidence to suggest that the CYP26 enzymes are crucial to RA degradation and preventing male germ cells from entering meiosis in the fetal testis was collected in studies using two chemical inhibitors. Ketoconazole, nonspecific cytochrome *p*450 inhibitor, and talarozole (R115866), CYP26-specific inhibitor, were used in separate studies to treat mouse male urogenital ridges in ex vivo organ cultures (Bowles et al., 2006; Koubova et al., 2006; Piprek et al., 2013). In both cases prospermatogonia precociously expressed *Stra8* and other markers of meiotic initiation.

How Does Altering Ra Signaling Affect Germ Cell Differentiation?

RA was first implicated in playing a role in female meiotic entry when rat female ovaries were exposed to exogenous RA and initiated premature meiosis (Livera et al., 2000). Additionally, treatment with a RAR α agonist drove meiotic initiation in female oögonia suggesting that stimulation of the RA signaling pathway also can drive germ cells into meiosis (Livera et al., 2000). Although male prospermatogonia did not respond to physiologically relevant levels of exogenous RA, likely due to normal CYP26B1 activity (Kumar et al., 2011).

Female urogenital ridges that were treated in culture with two different RAR panantagonists, BMS-204493 and AGN193109, did not express *Stra8* and other key meiotic markers to the same level as controls (Bowles et al., 2006; Koubova et al., 2006). This suggests that disrupting the RA signaling machinery alters oögonia meiotic initiation and further supports a role of RA in female germ cell differentiation. Further genetic studies are needed to more clearly understand the role of each isoform of RAR and RXR during meiotic initiation in the female germ cell. However, the redundancy between receptors may pose problematic when trying to tease out specific receptor functions.

Conclusions

Taken together, these data would suggest that RA signaling and STRA8 expression are essential for the normal progression of female germ cell meiotic entry, while fetal male testes protect their germ cells via degradation of RA by the expression of CYP26 enzymes within Sertoli cells. In this article, evidence was provided to demonstrate how female and male germ cells differentiate in response to endogenous RA levels. Further, manipulating RA levels in both male and female fetal gonads affected the normal progression of germ cell differentiation. Altering RA synthesis via ALDH1A enzymes prevented the expression of meiotic markers in the fetal ovary, while disrupting RA degradation through the CYP26 enzymes induced germ cell meiotic entry in male fetal testes. Further work will need to be performed to definitively pinpoint RA as the meiotic inducing factor, and these studies must include measuring RA concentrations without using a transgenic reporter and genetically breeding the triple *Aldh1a* knockout mouse.

References

- Arnold, S. L., Kent, T., Hogarth, C. A., Griswold, M. D., Amory, J. K., & Isoherranen, N. (2015a). Pharmacological inhibition of ALDH1A in mice decreases all-trans retinoic acid concentrations in a tissue specific manner. *Biochemical Pharmacology*, 95, 177–192. <https://doi.org/10.1016/j.bcp.2015.03.001>.

- Arnold, S. L., Kent, T., Hogarth, C. A., Schlatt, S., Prasad, B., Haenisch, M., Walsh, T., Muller, C. H., Griswold, M. D., Amory, J. K., & Isoherranen, N. (2015b). Importance of ALDH1A enzymes in determining human testicular retinoic acid concentrations. *Journal of Lipid Research*, 56, 342–357. <https://doi.org/10.1194/jlr.M054718>.
- Baltus, A. E., Menke, D. B., Hu, Y. C., Goodheart, M. L., Carpenter, A. E., de Rooij, D. G., & Page, D. C. (2006). In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. *Nature Genetics*, 38, 1430–1434. <https://doi.org/10.1038/ng1919>.
- Bastien, J., & Rochette-Egly, C. (2004). Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene*, 328, 1–16. <https://doi.org/10.1016/j.gene.2003.12.005>.
- Bowles, J., Knight, D., Smith, C., Wilhelm, D., Richman, J., Mamiya, S., Yashiro, K., Chawengsaksophak, K., Wilson, M. J., Rossant, J., Hamada, H., & Koopman, P. (2006). Retinoid signaling determines germ cell fate in mice. *Science*, 312, 596–600. <https://doi.org/10.1126/science.1125691>.
- Bowles, J., Feng, C. W., Miles, K., Ineson, J., Spiller, C., & Koopman, P. (2016). ALDH1A1 provides a source of meiosis-inducing retinoic acid in mouse fetal ovaries. *Nature Communications*, 7, 10845. <https://doi.org/10.1038/ncomms10845>.
- Dokshin, G. A., Baltus, A. E., Eppig, J. J., & Page, D. C. (2013). Oocyte differentiation is genetically dissociable from meiosis in mice. *Nature Genetics*, 45, 877–883. <https://doi.org/10.1038/ng.2672>.
- Evans, E., Hogarth, C., Mitchell, D., & Griswold, M. (2014). Riding the spermatogenic wave: Profiling gene expression within neonatal germ and sertoli cells during a synchronized initial wave of spermatogenesis in mice. *Biology of Reproduction*, 90, 108. <https://doi.org/10.1095/biolreprod.114.118034>.
- Gely-Pernot, A., Raverdeau, M., Celebi, C., Dennefeld, C., Feret, B., Klopstein, M., Yoshida, S., Ghyselinck, N. B., & Mark, M. (2012). Spermatogonia differentiation requires retinoic acid receptor gamma. *Endocrinology*, 153, 438–449. <https://doi.org/10.1210/en.2011-1102>.
- Giulii, G., Tomljenovic, A., Labrecque, N., Oulad-Abdelghani, M., Rassoulzadegan, M., & Cuzin, F. (2002). Murine spermatogonial stem cells: Targeted transgene expression and purification in an active state. *EMBO Reports*, 3, 753–759. <https://doi.org/10.1093/embo-reports/kvf149>.
- Hogarth, C. A., Evanoff, R., Snyder, E., Kent, T., Mitchell, D., Small, C., Amory, J. K., & Griswold, M. D. (2011). Suppression of Stra8 expression in the mouse gonad by WIN 18,446. *Biology of Reproduction*, 84, 957–965. <https://doi.org/10.1095/biolreprod.110.088575>.
- Hogarth, C. A., Evans, E., Onken, J., Kent, T., Mitchell, D., Petkovich, M., & Griswold, M. D. (2015). CYP26 enzymes are necessary within the postnatal seminiferous epithelium for normal murine spermatogenesis. *Biology of Reproduction*, 93, 19. <https://doi.org/10.1095/biolreprod.115.129718>.
- Koubova, J., Menke, D. B., Zhou, Q., Capel, B., Griswold, M. D., & Page, D. C. (2006). Retinoic acid regulates sex-specific timing of meiotic initiation in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 2474–2479.
- Kumar, S., Chatzi, C., Brade, T., Cunningham, T. J., Zhao, X., & Duester, G. (2011). Sex-specific timing of meiotic initiation is regulated by Cyp26b1 independent of retinoic acid signaling. *Nature Communications*, 2, 151. ncomms1136 <https://doi.org/10.1038/ncomms1136>.
- Li, H., & Clagett-Dame, M. (2009). Vitamin A deficiency blocks the initiation of meiosis of germ cells in the developing rat ovary in vivo. *Biology of Reproduction*, 81, 996–1001. <https://doi.org/10.1095/biolreprod.109.078808>.
- Li, H., MacLean, G., Cameron, D., Clagett-Dame, M., & Petkovich, M. (2009). Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. *PLoS One*, 4, e7501. <https://doi.org/10.1371/journal.pone.0007501>.
- Livera, G., Rouiller-Fabre, V., Valla, J., & Habert, R. (2000). Effects of retinoids on the meiosis in the fetal rat ovary in culture. *Molecular and Cellular Endocrinology*, 165, 225–231.
- MacLean, G., Li, H., Metzger, D., Chambon, P., & Petkovich, M. (2007). Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. *Endocrinology*, 148, 4560–4567. <https://doi.org/10.1210/en.2007-0492>.
- Mic, F. A., Haselbeck, R. J., Cuenca, A. E., & Duester, G. (2002). Novel retinoic acid generating activities in the neural tube and heart identified by conditional rescue of Raldh2 null mutant mice. *Development*, 129, 2271–2282.
- Oulad-Abdelghani, M., Bouillet, P., Decimo, D., Gansmuller, A., Heyberger, S., Dolle, P., Bronner, S., Lutz, Y., & Chambon, P. (1996). Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. *The Journal of Cell Biology*, 135, 469–477.
- Piprek, R. P., Pecio, A., Laskowska-Kaszub, K., Kloc, M., Kubiak, J. Z., & Szymura, J. M. (2013). Retinoic acid homeostasis regulates meiotic entry in developing anuran gonads and in Bidder's organ through Raldh2 and Cyp26b1 proteins. *Mechanisms of Development*, 130, 613–627. <https://doi.org/10.1016/j.mod.2013.09.001>.
- Rossant, J., Zirngibl, R., Cado, D., Shago, M., & Giguere, V. (1991). Expression of a retinoic acid response element-hsplaZ transgene defines specific domains of transcriptional activity during mouse embryogenesis. *Genes & Development*, 5, 1333–1344.
- Saba, R., Wu, Q., & Saga, Y. (2014). CYP26B1 promotes male germ cell differentiation by suppressing STRA8-dependent meiotic and STRA8-independent mitotic pathways. *Developmental Biology*, 389, 173–181. <https://doi.org/10.1016/j.ydbio.2014.02.013>.
- Sakai, Y., & Drager, U. C. (2010). Detection of retinoic acid catabolism with reporter systems and by in situ hybridization for CYP26 enzymes. *Methods in Molecular Biology*, 652, 277–294. https://doi.org/10.1007/978-1-60327-325-1_16.
- Vernet, N., Dennefeld, C., Guillou, F., Chambon, P., Ghyselinck, N. B., & Mark, M. (2006a). Prepubertal testis development relies on retinoic acid but not retinoid receptors in Sertoli cells. *The EMBO Journal*, 25, 5816–5825, 7601447 [pii] <https://doi.org/10.1038/sj.emboj.7601447>.
- Vernet, N., Dennefeld, C., Rochette-Egly, C., Oulad-Abdelghani, M., Chambon, P., Ghyselinck, N. B., & Mark, M. (2006b). Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. *Endocrinology*, 147, 96–110. en.2005-0953 [pii] <https://doi.org/10.1210/en.2005-0953>.
- Vernet, N., Dennefeld, C., Klopstein, M., Ruiz, A., Bok, D., Ghyselinck, N. B., & Mark, M. (2008). Retinoid X receptor beta (RXRB) expression in Sertoli cells controls cholesterol homeostasis and spermiogenesis. *Reproduction*, 136, 619–626. <https://doi.org/10.1530/REP-08-0235>.
- Wu, J. W., Wang, R. Y., Guo, Q. S., & Xu, C. (2008). Expression of the retinoic acid-metabolizing enzymes RALDH2 and CYP26b1 during mouse postnatal testis development. *Asian Journal of Andrology*, 10, 569–576. <https://doi.org/10.1111/j.1745-7262.2008.00408.x>.

GERMLINE EPIGENETIC INHERITANCE

Genetic Vs. Epigenetic Inheritance Overview

John R McCarrey, University of Texas at San Antonio, San Antonio, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Inheritance

Inheritance refers to the transmission of traits from one generation to the next or from one parent cell to two daughter cells. In a broad sense of the word, inheritance can refer to the persistence of any characteristic that is observable in parents or parent cells that is also observed in offspring or in daughter cells, respectively. Typically, however, inherited characteristics result from the transmission of specific information that can be encoded at the molecular level and transmitted as such from one generation to the next or from one cell to the next. There are two general types of information encoded at the molecular level. One involves information encoded at the level of the base sequence associated with nucleotides in double-stranded DNA, and this is referred to as *genetic inheritance*. The other involves information encoded as chemical modifications of DNA or chromatin, and this is referred to as *epigenetic inheritance*. Inheritance of either genetic or epigenetic information requires propagation of the relevant base sequence or pattern of DNA or chromatin modifications, respectively, throughout the process of DNA replication. Genetic information is typically quite stable, becoming changed only as a result of mutations, or by the physical rearrangements that can occur as a result of meiotic recombination. Epigenetic information is often less stable. The epigenome is different in every cell type and undergoes changes—typically referred to as reprogramming—during each generation. By definition, epigenetic information is reversible, and so is more labile than genetic information. Nevertheless, both genetic and epigenetic information are encoded by molecular mechanisms, and each can be transmitted from generation to generation or from parent cell to daughter cell on the basis of specific molecular mechanisms responsible for the transmission of the respective forms of encoded information.

Genetic Inheritance

Classically, the heritable transmission of traits is dependent upon genes, and in most species, genes are maintained as sequences of double-stranded deoxyribonucleic acid (DNA). This is the basis for *genetic inheritance*. Each strand of DNA is a polymer (chain) of nucleotides that each include one of four different bases—thymine (T), guanine (G), cytosine (C), or adenine (A). Each nucleotide in one strand of the DNA double helix is paired with a complementary nucleotide in the other strand of the double helix. This pairing follows base pairing rules, such that adenine is always paired with thymine (= an “A–T base pair”), and cytosine is always paired with guanine (= a “C–G base pair”). Genetic inheritance refers to the propagation of DNA sequence from one generation to the next or from a parent cell to a daughter cell.

Before cells divide by mitosis, they must first replicate their DNA. This replication process faithfully transmits identical copies of the double-stranded DNA sequence found in the parent cell to each of two daughter cells. DNA replication is a semiconservative process, in that the two strands of the DNA double helix found in the parent cell separate and each acts as a template for synthesis of a new, complementary strand. Because of the base pairing rules, the specific base associated with each nucleotide added to the new DNA strand is dictated by the complementary base present in the template (old) DNA strand. Thus, if a nucleotide with a C is present in the old DNA strand, it will template the addition of a nucleotide with a G during synthesis of the new strand. Similarly, a G or a T or an A in the old/template strand will dictate the addition of a complementary C, A, or T base in the newly synthesized strand. In conjunction with proofreading functions associated with DNA replication, as well as DNA repair mechanisms that function in response to various types of damage incurred in the base sequence in DNA, the DNA sequence is maintained as a relatively stable entity throughout all phases of the cell cycle and is faithfully propagated from generation to generation or parent cell to daughter cell. Genetic inheritance is responsible for the conservation of genetic information among all cell types in the body, as well as that within a pedigree, or among individuals within a particular species or even among related species. As such, genetic information provides the “hard wired” information that forms the essence of each species and of each individual within a species.

Epigenetic Inheritance

Epigenetic inheritance represents the transmission of information that *regulates* the function of the genetic information (i.e., of the genome). All cells within a multicellular individual typically inherit the same genetic information/genome/DNA sequence, yet different cell types become differentiated from one another on the basis of differential expression of the genetic information common to all cell types. Further, each different cell type normally emerges in the correct position within the body and adopts

the correct function(s) associated with the tissue and/or organ in which it is found. This requires that the development of each specific cell type, tissue or organ is highly regulated in a specified and coordinated manner so that all cells within any tissue or organ will function either identically or in a coordinated manner, and this process is regulated by epigenetic information. This is achieved by regulating gene expression. Epigenetic mechanisms regulate the structure of chromatin, and/or they regulate the affinity of genes for specific types of regulatory factors.

Typically, a gene, or at least the promoter plus transcriptional start site of a gene, need to be associated with relatively decondensed (open) chromatin structure to facilitate the process of transcription. Initiation of transcription requires the association of a transcription complex containing RNA polymerase plus associated factors with the gene promoter which will induce a transient, localized denaturation (separating) of the double helix so that one of the two DNA strands can serve as a template for transcription. The requisite transcription complex plus associated factors needed to initiate transcription of a particular gene can be present in the nucleus of a cell but if they are unable to associate with/bind to the promoter of that gene, no initiation of transcription will occur. Thus, regulation of chromatin structure can regulate gene expression, and epigenetic regulators can control the status of chromatin structure.

Epigenetic information occurs in the form of covalent modifications of DNA and/or chromatin. Examples include methylation of DNA. Specifically, in mammals, cytosines present in CpG dinucleotides (= a C-bearing nucleotide followed by a G-bearing nucleotide side by side in a 5' to 3' orientation of a single DNA strand can become methylated at the 5 carbon position = 5-methyl cytosine). Alternatively, but not mutually exclusively, proteins associated with DNA—particularly the core histones that make up nucleosomes that associate with DNA (H2A, H2B, H3, or H4) can undergo modifications including methylation, acetylation, phosphorylation, and/or other post-translational modifications at specific amino acid residues, and this will influence the affinity of the interactions between DNA and the nucleosomes or the interactions between neighboring nucleosomes and this, in turn, will affect overall chromatin structure and gene expression. Specific molecular mechanisms regulate the faithful propagation of these epigenetic modifications such that the associated effects on gene expression are transmitted from a parent cell to its daughter cells. Thus, patterns of DNA methylation are propagated by maintenance methylation. Because a 5'-CpG-3' dinucleotide on one DNA strand will be paired with a complementary 5'-CpG-3' dinucleotide on the other DNA strand, a fully methylated DNA site will include methylation of the cytosines on both strands. When this fully methylated site undergoes semi-conservative DNA replication, each existing, methylated strand will template synthesis of a new complementary strand that is initially unmethylated. However, this "hemimethylated" site is then recognized by the maintenance DNA methyltransferase which adds a methyl group to the unmethylated C such that the site is returned to a fully methylated state. If the same sequence is completely unmethylated when it undergoes DNA replication, no hemimethylated state will be achieved and the unmethylated site will be propagated as such. Thus, while the base sequence of a gene is transmitted via genetic inheritance, the epigenetic modifications associated with that gene are transmitted via epigenetic inheritance. Although not as thoroughly understood, existing evidence suggests that a similar mechanism contributes to propagation of histone modification patterns throughout the process of DNA replication.

Summary

Genetic inheritance refers to the transmission of DNA sequence information, whereas epigenetic inheritance refers to the transmission of patterns of modifications to either DNA or chromatin. These two mechanisms operate simultaneously. As a result, the same set of genetic information can be propagated to all cells in an individual, yet that information can be expressed in different ways in each cell type. Both types of inheritance are necessary to facilitate the normal function of cells, tissues, and organs.

Further Reading

Genetic Inheritance

Moalem, S. (2014). *Inheritance*. New York City, NY: Grand Central Publishing.

Epigenetic Inheritance

Carey, N. (2013). *The epigenetics revolution*. New York City, NY: Columbia University Press.

Epigenetic Transgenerational Inheritance

Michael K Skinner and Eric E Nilsson, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Epigenetics Molecular factors and processes around DNA that regulate genome activity independent of DNA sequence, and which are mitotically or meiotically stable.

Epimutation An altered epigenetic mark at a specific chromosomal site, often in response to exposure to an environmental factor.

Transgenerational epigenetic inheritance Germline (sperm or egg) transmission of epigenetic information between generations in the absence of any continued direct exposures or genetic manipulations.

It is self-evident that the environment has a strong effect on the biology of an organism. Changes in the environment or exposure to environmental toxicants can result in changes in gene expression and development of pathologies or phenotypic variation. Epigenetic factors are the molecular mechanisms an organism uses to respond to environmental changes with changes in gene expression. Although more traditional definitions exist (Skinner, 2011; Skinner et al., 2010), a modern mechanistic definition of epigenetics is:

Molecular factors/processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable.

The term epigenetics was coined by Dr. Conrad Waddington, University of Edinburgh, in the 1940s to describe gene-environment observations that could not be explained with classic genetics (Waddington, 1942). In the 1970s DNA methylation was the first epigenetic molecular mark to be characterized. With DNA methylation a small (methyl) chemical group is attached to DNA, primarily at the cytosine base in animals (Holliday and Pugh, 1975; Singer et al., 1979). In the 1990s the histone proteins that DNA is wrapped around were also found to be chemically modified to alter gene expression (Loidl, 1994; Davie, 1998). In the 2000's non-coding RNA molecules were identified that can act as epigenetic factors (Kornfeld and Bruning, 2014). The coiling, looping and general structure of DNA, termed chromatin structure, is also an epigenetic factor (Yaniv, 2014). Additional DNA cytosine modifications have also been described (Liu et al., 2016). More recently RNA methylation has been shown to alter gene expression and cellular function. The currently known epigenetic molecular processes are DNA cytosine modifications, histone modifications, functional non-coding RNA, RNA methylation and chromatin structure. All these epigenetic processes are important and have distinct roles in the regulation of how genes are expressed in the genome, independent of DNA sequence. New epigenetic marks and processes will also likely be identified in the future.

The vast majority of environmental factors and toxicants do not have the ability to alter DNA sequence or promote genetic mutations (McCarrey, 2012). In contrast, the environment can dramatically influence epigenetic processes to alter gene expression and development. Therefore, epigenetics provides a molecular mechanism for the environment to directly alter the biology of an organism (Jirtle and Skinner, 2007). An altered epigenetic mark at a specific chromosomal site in response to an environmental factor is termed an "Epimutation" (Skinner et al., 2010). Therefore, DNA sequence changes are genetic mutations, while environmentally altered epigenetic sites that influence genome activity are epimutations.

Epigenetic changes can be inherited across generations, and can result in changes in gene expression and phenotype in subsequent generations, even after exposure to the environmental changes or factors has ceased. "Epigenetic Transgenerational Inheritance" is defined as (Skinner et al., 2010; Skinner, 2014):

Germline (sperm or egg) transmission of epigenetic information between generations in the absence of any continued direct exposures or genetic manipulations.

An early study on the toxicant actions of the agricultural fungicide vinclozolin illustrates the epigenetic transgenerational inheritance phenomenon (Anway et al., 2005). F0 generation pregnant rats were exposed to vinclozolin at the time of fetal sex determination and the direct effects on the F1 generation adults were identified. The F1 generation adult males developed a testis abnormality. When the F1 generation offspring was bred to generate the F2 generation (grand-offspring) the F2 generation adult males were found to have the same testis defects as the F1 generation. When the animals were bred to the transgenerational F3 and F4 generations the adult testis defect continued in over 90% of all male progeny (Anway et al., 2005). As the animals aged, both males and females developed disease in a variety of organs (Anway et al., 2006). The frequency of the abnormality did not decline at each generation, but stayed high suggesting a non-Mendelian phenomenon not following classic genetic processes.

Table 1 Environmentally induced epigenetic transgenerational inheritance

<i>Environmental toxicants</i>	
Vinclozolin (agricultural fungicide)	Permethrin and DEET (insect repellant)
Methoxychlor (agricultural pesticide)	DDT (pesticide)
Dioxin/TCDD (industrial contaminant)	Tributyltin (industrial toxicant and biocide)
Plastic compounds (BPA and phthalates)	Hydrocarbons (jet fuel)
<i>Other types exposures</i>	
Nutrition (high fat or caloric restriction)	Smoking and alcohol
Temperature and drought (plant health and flowering)	Stress (behavioral)

When the male vinclozolin lineage animal was outcrossed to a wildtype female the transgenerational phenotype was maintained at the same frequency, but a reverse outcross of a vinclozolin lineage female to a wildtype male resulted in loss of the phenotype (Anway et al., 2005). Therefore, a transgenerational phenomenon was observed that was transmitted through the male germline (sperm). Later experiments with other toxicant exposures (Table 1) have shown transmission through the female germline predominantly (Manikkam et al., 2014), such that the transgenerational phenotype is transmitted in a parent of origin allelic manner, similar to imprinted genes.

To understand transgenerational phenomena it is essential to distinguish between direct exposure effects versus germline (sperm or egg) mediated transgenerational events. When a gestating female is exposed, that F0 generation female, the F1 generation fetus and the germ cells (sperm or eggs) that are inside the fetus and that will produce the F2 generation, are also directly exposed, Fig. 1. Therefore any effects seen in the F0, F1 and F2 generations may be due to direct exposure toxicity as well as to environmentally induced epigenetic changes. Examination of the F3 generation (great grand-offspring) is minimally needed to assess transgenerational phenomenon, since direct exposure effects are not involved (Skinner, 2008), Fig. 1. In contrast, in the event an adult male or non-pregnant female is exposed to an environmental factor, then the F0 generation adult and the germ cells that will

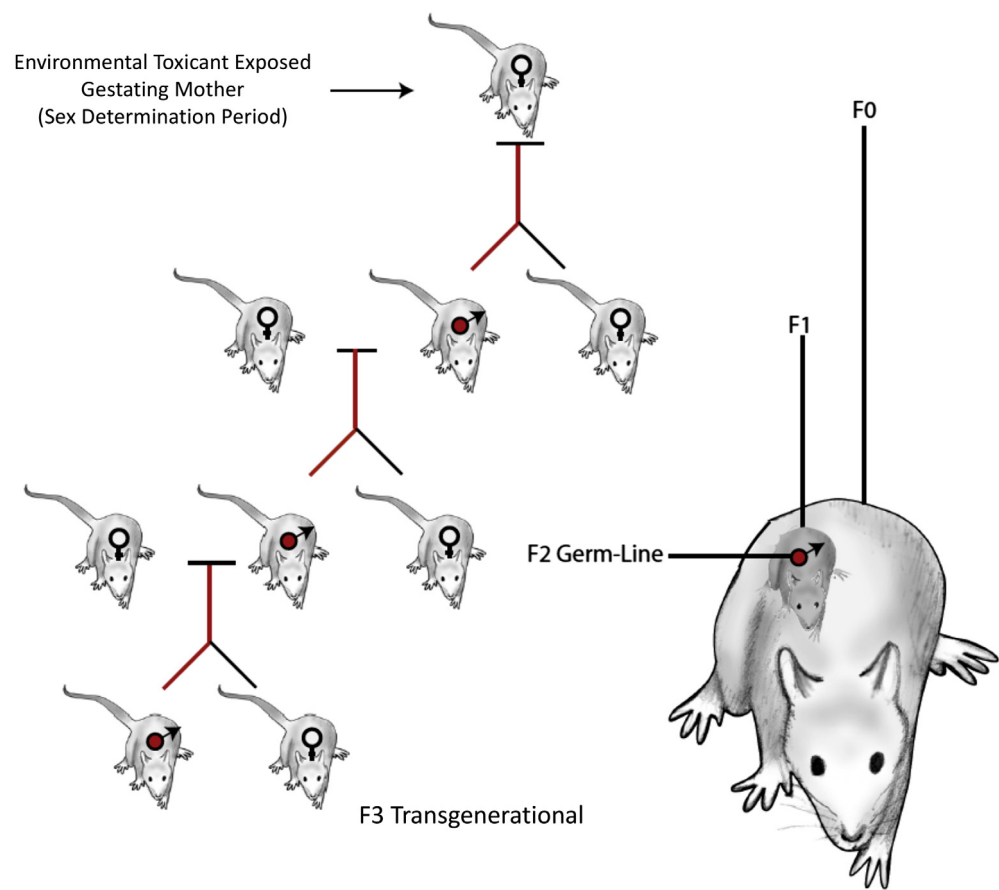


Fig. 1 Environmental factor exposure example showing an F0 generation pregnant animal exposed to a toxicant, and illustrating that the multigenerational F1 and F2 generations were also directly exposed, while the transgenerational F3 generation had no direct exposure. Adapted from Skinner, M. K. 2008. What is an epigenetic transgenerational phenotype? F3 or F2. *Reproductive Toxicology*, 25, 2–6.

generate the F1 generation are directly exposed, and examination of the non-exposed F2 generation (grand-offspring) is required to demonstrate a transgenerational phenomenon. When multiple generations are directly exposed, this is referred to as a multigenerational exposure, which has been shown to have effects with a variety of exposures (Skinner et al., 2010). The ability to transmit information from one generation to the next requires the sperm or egg so transgenerational events are germ cell mediated (Fig. 1).

Consideration of environmentally induced epigenetic transgenerational inheritance is anticipated to have a significant role in all areas of biology. Knowledge of environmental epigenetics and epigenetic inheritance will help to better understand how environmental factors and toxicant exposures can influence our health and disease across generations. In addition, epigenetic transgenerational inheritance will have a critical role in evolution (Skinner, 2015).

References

- Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308, 1466–1469.
- Anway, M. D., Leathers, C., & Skinner, M. K. (2006). Endocrine disruptor vinclozolin induced epigenetic transgenerational adult-onset disease. *Endocrinology*, 147, 5515–5523.
- Davie, J. R. (1998). Covalent modifications of histones: Expression from chromatin templates. *Current Opinion in Genetics & Development*, 8, 173–178.
- Holliday, R., & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science*, 187, 226–232.
- Jirtle, R. L., & Skinner, M. K. (2007). Environmental epigenomics and disease susceptibility. *Nature Reviews. Genetics*, 8, 253–262.
- Kornfeld, J. W., & Bruning, J. C. (2014). Regulation of metabolism by long, non-coding RNAs. *Frontiers in Genetics*, 5, 57.
- Liu, M. Y., Denizio, J. E., Schutsky, E. K., & Kohli, R. M. (2016). The expanding scope and impact of epigenetic cytosine modifications. *Current Opinion in Chemical Biology*, 33, 67–73.
- Loidl, P. (1994). Histone acetylation: Facts and questions. *Chromosoma*, 103, 441–449.
- Manikkam, M., Haque, M. M., Guerrero-Bosagna, C., Nilsson, E., & Skinner, M. K. (2014). Pesticide methoxychlor promotes the epigenetic transgenerational inheritance of adult onset disease through the female germline. *PLoS One*, 9, e102091.
- Mccarrey, J. R. (2012). The epigenome as a target for heritable environmental disruptions of cellular function. *Molecular and Cellular Endocrinology*, 354, 9–15.
- Singer, J., Roberts-Ems, J., & Riggs, A. D. (1979). Methylation of mouse liver DNA studied by means of the restriction enzymes msp I and hpa II. *Science*, 203, 1019–1021.
- Skinner, M. K. (2008). What is an epigenetic transgenerational phenotype? F3 or F2. *Reproductive Toxicology*, 25, 2–6.
- Skinner, M. K. (2011). Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics*, 6, 838–842.
- Skinner, M. K. (2014). Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. *Molecular and Cellular Endocrinology*, 398, 4–12.
- Skinner, M. K. (2015). Environmental epigenetics and a unified theory of the molecular aspects of evolution: A neo-Lamarckian concept that facilitates neo-Darwinian evolution. *Genome Biology and Evolution*, 7, 1296–1302.
- Skinner, M. K., Manikkam, M., & Guerrero-Bosagna, C. (2010). Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends in Endocrinology and Metabolism*, 21, 214–222.
- Waddington, C. H. (1942). The epigenotype. *Endeavour*, 1, 18–20.
- Yaniv, M. (2014). Chromatin remodeling: From transcription to cancer. *Cancer Genetics*, 207, 352–357.

Mechanisms of Epigenetic Transgenerational Inheritance

Michael K Skinner and Eric E Nilsson, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Epigenetics Molecular factors and processes around DNA that regulate genome activity independent of DNA sequence, and which are mitotically stable.

Transgenerational epigenetic inheritance Germline (sperm or egg) transmission of epigenetic information between generations in the absence of any continued direct exposures or genetic manipulations.

Epigenetic processes are some of the main ways that organisms respond to their environment with changes in gene expression. In addition, it is primarily by epigenetic mechanisms that one cell type is able to differentiate and change to develop into another cell type. Epigenetics is defined as: “Molecular factors/processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable” (Skinner, 2011). There are a variety of epigenetic factors that act around the DNA in a cell to regulate gene expression. In the 1970s DNA methylation was the first epigenetic molecular mark to be characterized. With DNA methylation a small (methyl) chemical group is attached to DNA, primarily at the cytosine base in animals (Holliday and Pugh, 1975; Singer et al., 1979) to produce 5-methylcytosine (5mC). Other chemical modifications of cytosine bases in DNA have since been described. The TET family of enzymes can successively oxidize 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Kriaucionis and Tahiliani, 2014). In broad terms, the presence of 5mC often represses DNA transcription, while 5hmC is permissive to transcription (An et al., 2017; Mellen et al., 2017). The functions of the other epigenetic modifications to cytosine are under investigation. N(6)-methyladenine is an epigenetic modification to the adenine base of DNA that was once thought to only be present in prokaryotic organisms, but has now been described in mammalian embryonic stem cells (Wu et al., 2016).

The histone proteins that DNA is wrapped around create the nucleosome and can be chemically modified to alter gene expression. There are many different histone post-translational modifications including lysine acetylation, lysine and arginine methylation, arginine citrullination, lysine ubiquitination, lysine sumoylation, ADP-ribosylation, proline isomerization, and serine/threonine/tyrosine phosphorylation (Rothbart and Strahl, 2014). These modifications can change chromatin structure or recruit transcriptional cofactors to DNA in order to regulate gene expression.

Non-coding RNA molecules can act as epigenetic factors (Kornfeld and Bruning, 2014). These are small RNA molecules that do not code for a protein, but rather function as RNA to regulate gene expression. The non-coding RNA molecules that act as epigenetic factors are not DNA sequence dependent, so the majority do not depend on having a nucleotide sequence that is complementary to a specific DNA or RNA region in order to function. Long non-coding RNAs (lncRNAs) (Wei et al., 2017) and transfer RNA-derived small RNAs (tsRNAs) (Chen et al., 2016a) are examples of RNA classes that are present in sperm and can act as epigenetic factors that affect subsequent generations (Chen et al., 2016b).

The coiling, looping and general structure of DNA, termed chromatin structure, is also an epigenetic factor (Yaniv, 2014). The three-dimensional structure of DNA can make certain regions of the genome accessible to transcription machinery, or bring enhancer regions near to gene promoters, and so affect gene expression. Therefore, epigenetic molecular processes include DNA methylation, histone modifications, non-coding RNAs, RNA methylation and chromatin structure.

Two well-studied examples of epigenetic processes are X-chromosome inactivation and imprinted genes (Lee and Bartolomei, 2013; Henckel et al., 2012). Female mammals have two X-chromosomes and one must be inactivated for normal biological function. This has been shown to involve DNA methylation, histone modifications and long non-coding RNA (Sado and Ferguson-Smith, 2005). Imprinted genes are a small set of genes that are expressed from either the mother's (maternal) or father's (paternal) contributed DNA (allele), but not both. Imprinting involves DNA methylation, histone modifications, chromatin structure and non-coding RNA to control this parent of origin gene expression (Massah et al., 2015). These examples show how epigenetics and genetics cooperate to control genome activity and normal biology.

Altered epigenetic marks at specific DNA sites in response to exposure to an environmental factor are termed “epimutations” (Skinner et al., 2010). Thus, DNA sequence changes are genetic mutations, while environmentally altered epigenetic sites that influence genome activity are epimutations.

Epigenetic information can be transmitted to subsequent generations, and can change gene expression and an organism's phenotype even in transgenerational generations that were themselves never exposed to an inducing factor like an environmental toxicant. The ability to transmit epigenetic information from one generation to the next requires that epigenetic factors be present in sperm or eggs. If the sperm or egg cells are transmitting epigenetic information transgenerationally then epimutations should be detectable in these germ cells. Analysis of the F3 generation (great-grand-offspring) sperm from a line of rats in which the F0 generation pregnant females were treated with the environmental toxicant vinclozolin were found to have sperm epimutations consisting of altered DNA methylation, compared to controls (Guerrero-Bosagna et al., 2010). Interestingly, a variety of different environmental toxicants,

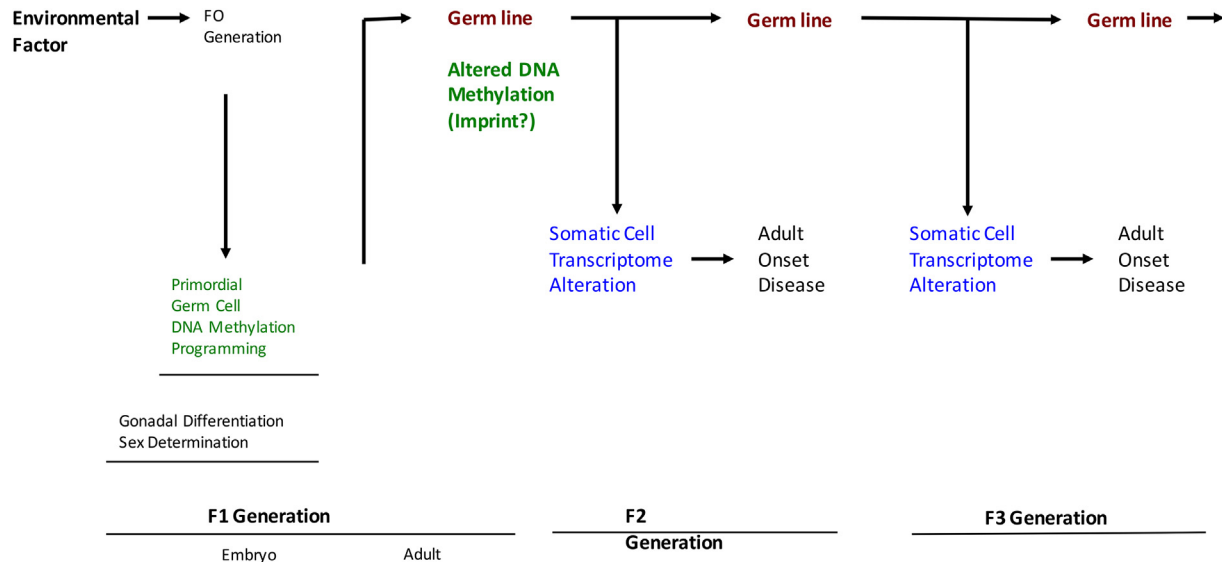


Fig. 1 Role of germline in epigenetic transgenerational inheritance. Summary of environmentally induced epigenetic reprogramming of primordial germ cells that leads to the germline transmission of epimutations, resulting in all somatic cells having altered gene expression. This can result in changes in phenotype or increased disease susceptibility in the F1, F2 or transgenerational F3 generation. Modified from Skinner, M. K., Manikkam, M. & Guerrero-Bosagna, C. 2010. Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends in Endocrinology and Metabolism*, 21, 214–22.

each shown to promote transgenerational disease, were each found to promote a unique signature or pattern of epimutations in their F3 generation sperm (Manikkam et al., 2012).

The example of the processes involved in environmentally induced epigenetic transgenerational inheritance is presented in Fig. 1. The exposure of an F0 generation gestating female to an environmental factor at the critical window of gonadal (testis or ovary) sex determination in the F1 generation fetus modifies the epigenetic programming of the germ cell that the F1 generation adult animal will transmit to the F2 generation. All cell types and tissues derived from cells carrying epimutations will have an altered epigenome and potentially have altered gene expression. For example, isolated transgenerational cells such as Sertoli cells from the testis or granulosa cells from the ovary have been shown to have a transgenerational alteration in gene expression and epigenetics (Guerrero-Bosagna et al., 2013; Nilsson et al., 2012). Tissues sensitive to an altered gene expression profile will have a different phenotype, and may develop disease or abnormalities as the individual ages. For example, the testis and ovary develop pathologies that are associated with the somatic cell epigenetic alterations (Guerrero-Bosagna et al., 2013; Nilsson et al., 2012). An adult F2 generation individual will then transmit germ cell epimutations to the transgenerational F3 generation, and the same mechanism and process can occur in all subsequent generations, Fig. 1.

Environmental exposures can promote non-genetic inheritance through epigenetic transgenerational inheritance mechanisms to promote disease and altered phenotypes in subsequent generations. The potential role of this mechanism in our understanding of disease etiology needs to be considered. For example, environmental exposures in past generations could contribute to the incidence of abnormalities and disease in the current human population. Epigenetic transgenerational inheritance will also have critical roles in other biological processes such as evolutionary biology (Skinner, 2015).

References

- An, J., Rao, A., & Ko, M. (2017). TET family dioxygenases and DNA demethylation in stem cells and cancers. *Experimental & Molecular Medicine*, 49, e323.
- Chen, Q., Yan, M., Cao, Z., Li, X., Zhang, Y., Shi, J., Feng, G. H., Peng, H., Zhang, X., Zhang, Y., Qian, J., Duan, E., Zhai, Q., & Zhou, Q. (2016a). Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science*, 351, 397–400.
- Chen, Q., Yan, W., & Duan, E. (2016b). Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. *Nature Reviews. Genetics*, 17, 733–743.
- Guerrero-Bosagna, C., Settles, M., Lucker, B., & Skinner, M. (2010). Epigenetic transgenerational actions of vinclozolin on promoter regions of the sperm epigenome. *PLoS One*, 5, e13100.
- Guerrero-Bosagna, C., Savenkova, M., Haque, M. M., Sadler-Riggelman, I., & Skinner, M. K. (2013). Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: Molecular etiology of male infertility. *PLoS One*, 8, e59922.
- Henckel, A., Chebli, K., Kota, S. K., Arnaud, P., & Feil, R. (2012). Transcription and histone methylation changes correlate with imprint acquisition in male germ cells. *The EMBO Journal*, 31, 606–615.
- Holliday, R., & Pugh, J. E. (1975). DNA modification mechanisms and gene activity during development. *Science*, 187, 226–232.
- Kornfeld, J. W., & Bruning, J. C. (2014). Regulation of metabolism by long, non-coding RNAs. *Frontiers in Genetics*, 5, 57.
- Kriaucionis, S., & Tahiliani, M. (2014). Expanding the epigenetic landscape: Novel modifications of cytosine in genomic DNA. *Cold Spring Harbor Perspectives in Biology*, 6, a018630.

- Lee, J. T., & Bartolomei, M. S. (2013). X-inactivation, imprinting, and long noncoding RNAs in health and disease. *Cell*, 152, 1308–1323.
- Manikkam, M., Guerrero-Bosagna, C., Tracey, R., Haque, M. M., & Skinner, M. K. (2012). Transgenerational actions of environmental compounds on reproductive disease and identification of epigenetic biomarkers of ancestral exposures. *PLoS One*, 7, e31901.
- Massah, S., Beischlag, T. V., & Prefontaine, G. G. (2015). Epigenetic events regulating monoallelic gene expression. *Critical Reviews in Biochemistry and Molecular Biology*, 50, 337–358.
- Mellen, M., Ayata, P., & Heintz, N. (2017). 5-Hydroxymethylcytosine accumulation in postmitotic neurons results in functional demethylation of expressed genes. *Proceedings of the National Academy of Sciences of the United States of America*, 114, E7812–E7821.
- Nilsson, E., Larsen, G., Manikkam, M., Guerrero-Bosagna, C., Savenkova, M., & Skinner, M. (2012). Environmentally induced epigenetic transgenerational inheritance of ovarian disease. *PLoS One*, 7, e36129.
- Rothbart, S. B., & Strahl, B. D. (2014). Interpreting the language of histone and DNA modifications. *Biochimica et Biophysica Acta*, 1839, 627–643.
- Sado, T., & Ferguson-Smith, A. C. (2005). Imprinted X inactivation and reprogramming in the preimplantation mouse embryo. *Human Molecular Genetics*, 14, R59–64. Spec No 1.
- Singer, J., Roberts-Ems, J., & Riggs, A. D. (1979). Methylation of mouse liver DNA studied by means of the restriction enzymes msp I and hpa II. *Science*, 203, 1019–1021.
- Skinner, M. K. (2011). Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics*, 6, 838–842.
- Skinner, M. K. (2015). Environmental epigenetics and a unified theory of the molecular aspects of evolution: A neo-Lamarckian concept that facilitates neo-Darwinian evolution. *Genome Biology and Evolution*, 7, 1296–1302.
- Skinner, M. K., Manikkam, M., & Guerrero-Bosagna, C. (2010). Epigenetic transgenerational actions of environmental factors in disease etiology. *Trends in Endocrinology and Metabolism*, 21, 214–222.
- Wei, J. W., Huang, K., Yang, C., & Kang, C. S. (2017). Non-coding RNAs as regulators in epigenetics (review). *Oncology Reports*, 37, 3–9.
- Wu, T. P., Wang, T., Seetin, M. G., Lai, Y., Zhu, S., Lin, K., Liu, Y., Byrum, S. D., Mackintosh, S. G., Zhong, M., Tackett, A., Wang, G., Hon, L. S., Fang, G., Swenberg, J. A., & Xiao, A. Z. (2016). DNA methylation on N(6)-adenine in mammalian embryonic stem cells. *Nature*, 532, 329–333.
- Yaniv, M. (2014). Chromatin remodeling: From transcription to cancer. *Cancer Genetics*, 207, 352–357.

Epigenetic Transgenerational Inheritance Across Species

Michael K Skinner and Eric E Nilsson, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Epigenetics Molecular factors and processes around DNA that regulate genome activity independent of DNA sequence, and which are mitotically stable.

Transgenerational epigenetic inheritance Germline (sperm or egg) transmission of epigenetic information between generations in the absence of any continued direct exposures or genetic manipulations.

Introduction

Epigenetics is the molecular factors and processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable (Skinner, 2011). Epigenetic mechanisms are crucial for normal biology and are some of the primary drivers of cell differentiation that produce all the different cell and tissue types in an organism (Klironomos et al., 2013; Skinner et al., 2014b). In addition, epigenetic activity is a normal mechanism by which organisms respond to alterations in the environment by changing gene expression (Aguilera et al., 2010; Gomes and Pelosi, 2013). Epigenetic processes include DNA methylation, modifications to histone proteins, expression of non-coding RNA, and changes to chromatin structure.

It is possible for epigenetic changes and epigenetic responses to environmental factors to be inherited across generations (Anway et al., 2005; Song et al., 2013). Ancestral exposure to environmental factors or toxicants has been shown to promote changes in gene expression and disease frequency (Skinner, 2014). Transgenerational epigenetic inheritance can occur if epigenetic alterations and changes in an organism's phenotype are inherited through the germ line in generations that were themselves never exposed to an inducing factor like an environmental toxicant. Transgenerational epigenetic inheritance requires that epigenetic information or epigenetic changes are present in germ cells (i.e., sperm or eggs), as it is through germ cells that inheritance occurs. This chapter reviews the diversity of organisms and species for which epigenetic transgenerational inheritance has been shown to occur (Table 1).

Overview

Epigenetic transgenerational inheritance phenomena have been shown in many plant species. For example, cold temperature and drought can promote epigenetic transgenerational inheritance of flowering and growth characteristics. These processes involve heritable changes in DNA methylation (Song et al., 2013; Quadrana and Colot, 2016).

In the nematode worm *C. elegans* epigenetic transgenerational inheritance has been shown to occur (Vaiserman et al., 2017). For example, increased longevity that is associated with the histone modification H3K4me3 methylation can be transgenerationally inherited for up to three generations (Greer et al., 2011). Similarly, specific changes in gene expression after an ancestor is exposed to heat shock can be epigenetically inherited for as many as 14 generations (Klosin et al., 2017).

The model insect species *Drosophila melanogaster* has been used in a number of studies investigating epigenetic transgenerational inheritance (Grentzinger et al., 2012). Interestingly, one of the first experiments to establish the potential for epigenetic transgenerational inheritance was performed by Conrad Waddington, who coined the term "epigenetic" (Waddington, 1942, 1953). In these studies it was found that a heat shock induced wing structure changes that persisted for more than seven generations. In more recent examples, it has been found that a high-sugar maternal fly diet can alter the larval body composition for the next two generations (Buescher et al., 2013). Manipulations of the protein levels in the diet of fruit flies can affect longevity and reproduction for three subsequent generations, and is associated with histone modifications (Xia and de Belle, 2016; Xia et al., 2016).

Other arthropod species have also demonstrated epigenetic transgenerational inheritance. The brine shrimp *Artemia* is a genus of crustacean species that have shown the inheritance of an acquired immune resistance (Norouzitallab et al., 2014). In these studies, *Artemia* were exposed to a bacterial pathogen that did not kill them, and to which they developed resistance. Three generations later the *Artemia* were still more resistant to that bacteria. This was associated with changes in DNA methylation and histone acetylation. In *Daphnia magna*, another crustacean species, it was shown that exposure to the toxicant 5-azacytidine resulted in decreased body length and reduced levels of DNA methylation in non-exposed subsequent generations (Vandegheuchte et al., 2010).

Several species of fish have shown epigenetic transgenerational inheritance. Zebrafish exposed to the environmental toxicants benzo(a)pyrene (Knecht et al., 2017), methylmercury (Carvan et al., 2017) or dioxin (Baker et al., 2014) have shown in their grand-offspring behavioral changes, visual defects, increased body mass, skeletal abnormalities and/or decreased fertility, some of which are associated with changes in DNA methylation. Medaka exposed to the endocrine disruptors bisphenol A (BPA) or ethinylestradiol produced grand-offspring and great-grand-offspring with reduced fertility (Bhandari et al., 2015). The pipefish

Table 1 Epigenetic transgenerational inheritance across species

Species	Exposure	Transgenerational phenotype	Generation	References
Plant species	Cold, drought	Change in flowering, growth	F2 +	Quadrana and Colot (2016) and Song et al. (2013)
<i>C. elegans</i> (worm)	Heat shock, altered chromatin modifiers	Longevity, gene expression	F3, F14	Greer et al. (2011) and Klosin et al. (2017)
<i>D. melanogaster</i> (fly)	Heat shock, dietary protein and sugar	Wing changes, body composition, longevity	F2, F3, F7	Waddington (1953), Buescher et al. (2013), and Xia and de Belle (2016)
<i>Artemia</i> (brine shrimp)	Bacteria	Bacterial resistance	F3	Norouzitallab et al. (2014)
<i>Daphnia magna</i>	5-Azacytidine	Decreased body length	F2	Vandeghehuchte et al. (2010)
Zebrafish	Benzo(a)pyrene, methylmercury, dioxin	Visual defects, increased body mass, skeletal abnormalities, decreased fertility	F2	Knecht et al. (2017), Carvan et al. (2017), and Baker et al. (2014)
Medaka (fish)	Bisphenol A (BPA), 17 α -ethinylestradiol	Reduced fertility	F2, F3	Bhandari et al. (2015)
Pipefish	Bacteria	Increased immune gene expression	F2	Beemelmanns and Roth (2017)
Quail	Genistein	Change in age of first egg laid	F3	Leroux et al. (2017)
Duck	Methionine-deficient diet	Altered weight gain	F2	Brun et al. (2015)
Rat	Vinclozolin, calorie restriction	Increased disease rate, behavior changes, body mass	F2, F3, F4	Anway et al. (2005), Skinner et al. (2011), Gillette et al. (2014), and Araminaite et al. (2014)
Mouse	Cage enrichment, vinclozolin, diabetes	Behavior change, increased disease rate, infertility	F2, F3	Yeshurun et al. (2017), Guerrero-Bosagna et al. (2012), and Pavlinkova et al. (2017)
Guinea pig	Ethanol	Reduced fertility	F4	Stockard and Papanicolaou (1918)
Pig	Methyl donor diet	Decreased backfat	F2	Braunschweig et al. (2012)
Human	Calorie restriction	Increased diabetes, mortality	F2	Veenendaal et al. (2013) and Bygren et al. (2001)

Syngnathus typhle has shown transgenerational inheritance of increased expression of immune response genes after grand-parental exposure to heat-killed bacteria, also associated with changes in DNA methylation or histone modifications (Beemelmanns and Roth, 2017).

Some bird species have shown evidence of epigenetic transgenerational inheritance. In a study with quail, eggs were injected with the environmental estrogen genistein (Leroux et al., 2017). In the great-grand offspring the age at which the first egg was laid was significantly greater in genistein-lineage quail compared to the control lineage. Ancestral diet has been shown have a transgenerational effect in ducks (Brun et al., 2015). Ducks fed a methionine-deficient diet produced grand-offspring with altered weight gain and changes in metabolic parameters.

In mammals most studies of epigenetic transgenerational inheritance have occurred in rodents (Skinner et al., 2011). Examples of epigenetic transgenerational inheritance in rats include increased rates of reproductive abnormalities in great-grand offspring after exposure of pregnant rats to the environmental toxicant vinclozolin (Anway et al., 2005) and transgenerational inheritance of altered behaviors (Gillette et al., 2014), both associated with altered DNA methylation. Alterations in longevity and body weight have been seen in grand-offspring after non-pregnant grandmothers were fed a calorie restricted diet (Araminaite et al., 2014).

In mice, males who experience an enriched living environment had grand-offspring with changes in behavior and neuro-endocrine parameters (Yeshurun et al., 2017). Other examples of epigenetic transgenerational inheritance in mice include transgenerational increases in adult onset disease and changes in DNA methylation after ancestral exposure to vinclozolin (Guerrero-Bosagna et al., 2012), and male diabetes resulting in increased rates of infertility in grand-offspring (Pavlinkova et al., 2017).

Interestingly, a study in Guinea pigs from 1918 demonstrated transgenerational inheritance of decreased fertility and increased mortality for four generations after ancestral exposure to ethanol vapor, although this was not attributed to epigenetic inheritance at the time (Stockard and Papanicolaou, 1918).

The domestic pig is another mammal that has shown evidence of epigenetic transgenerational inheritance. Male pigs fed a diet high in methylating micronutrients had grand-offspring with a lower level of backfat (Braunschweig et al., 2012). This change in carcass fat percentage was accompanied by differences in DNA methylation levels of selected genes in liver and muscle.

Evidence of epigenetic transgenerational inheritance in humans comes from retrospective studies (Veenendaal et al., 2013). For example, the Overkalix region of northern Sweden experienced periods of famine in the early 1900s, and the descendants of those people exposed to famine conditions as children 9–12 years of age were investigated (Bygren et al., 2001). It was found that men whose grandfathers were exposed to famine had an increased risk of mortality due to diabetes, and similarly women whose grandmothers were exposed had increased risk.

Conclusions

Epigenetic transgenerational inheritance has been demonstrated to occur across a wide variety of eukaryotic phyla and species, including plants, nematode worms, insects, crustaceans, quail, ducks, rats, mice, Guinea pigs, domestic pigs, and humans. This suggests that epigenetic transgenerational inheritance is a broadly conserved phenomenon, and likely has some adaptive value for each species.

The question that then arises is why can environmental insults sometimes result in transgenerational inheritance of increased disease susceptibility, which is a maladaptive response? One possible answer may be explained by the predictive adaptive response hypothesis (Skinner et al., 2014a; Bateson et al., 2014). In this hypothesis an environmental stressor like famine may epigenetically promote an adaptive (thrifty) phenotype in progeny. If the current environment of those progeny has more than adequate nutrients, diseases like diabetes and obesity are promoted. Another possibility is that an environmental insult, such as exposure to a toxicant, may interfere with the normal molecular epigenetic machinery and result in stochastic and/or directed epigenetic changes that could be considered epimutations. If these epimutations occur in germ cells, then that can lead to transgenerational inheritance of a wider range of phenotypes in the progeny. Some of those phenotypes may be poorly adapted and develop disease. This would explain an increase in disease susceptibility in organisms whose ancestors were exposed to environmental insults. However, the increased phenotypic variation may also result in some individuals who are better adapted to an altered environment, facilitating natural selection (Skinner, 2015).

References

- Aguilera, O., Fernandez, A. F., Munoz, A., & Fraga, M. F. (2010). Epigenetics and environment: A complex relationship. *Journal of Applied Physiology* (1985), 109, 243–251.
- Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308, 1466–1469.
- Araminaite, V., Zalgeviene, V., Simkunaite-Rizgeliene, R., Stukas, R., Kaminskas, A., & Tutkuviene, J. (2014). Maternal caloric restriction prior to pregnancy increases the body weight of the second-generation male offspring and shortens their longevity in rats. *The Tohoku Journal of Experimental Medicine*, 234, 41–50.
- Baker, T. R., Peterson, R. E., & Heideman, W. (2014). Using Zebrafish as a model system for studying the transgenerational effects of dioxin. *Toxicological Sciences*, 138, 403–411.
- Bateson, P., Gluckman, P., & Hanson, M. (2014). The biology of developmental plasticity and the predictive adaptive response hypothesis. *The Journal of Physiology*, 592, 2357–2368.
- Beemelmans, A., & Roth, O. (2017). Grandparental immune priming in the pipefish *Syngnathus typhle*. *BMC Evolutionary Biology*, 17, 44.
- Bhandari, R. K., Vom Saal, F. S., & Tillitt, D. E. (2015). Transgenerational effects from early developmental exposures to bisphenol A or 17alpha-ethinylestradiol in medaka, *Oryzias latipes*. *Scientific Reports*, 5, 9303.
- Braunschweig, M., Jagannathan, V., Gutzwiller, A., & Bee, G. (2012). Investigations on transgenerational epigenetic response down the male line in F2 pigs. *PLoS One*, 7, e30583.
- Brun, J. M., Bernadet, M. D., Cornuez, A., Leroux, S., Bodin, L., Basso, B., Davail, S., Jaglin, M., Lessire, M., Martin, X., Sellier, N., Morisson, M., & Pitel, F. (2015). Influence of grand-mother diet on offspring performances through the male line in Muscovy duck. *BMC Genetics*, 16, 145.
- Buescher, J. L., Musselman, L. P., Wilson, C. A., Lang, T., Keleher, M., Baranski, T. J., & Duncan, J. G. (2013). Evidence for transgenerational metabolic programming in *Drosophila*. *Disease Models & Mechanisms*, 6, 1123–1132.
- Bygren, L. O., Kaati, G., & Edvinsson, S. (2001). Longevity determined by paternal ancestors' nutrition during their slow growth period. *Acta Biotheoretica*, 49, 53–59.
- Carvan, M. J. I., Kalluvila, T. A., Klingler, R. H., Larson, J. K., Pickens, M., Mora-Zamorano, F. X., Connaughton, V. P., Sadler-Riggelman, I., Beck, D., & Skinner, M. K. (2017). Mercury-induced epigenetic transgenerational inheritance of abnormal neurobehavior is correlated with sperm epimutations in zebrafish. *PLoS One*, 12, e0176155.
- Gillette, R., Miller-Crews, I., Nilsson, E. E., Skinner, M. K., Gore, A. C., & Crews, D. (2014). Sexually dimorphic effects of ancestral exposure to vinclozolin on stress reactivity in rats. *Endocrinology*, 155, 3853–3866.
- Gomes, M. V., & Pelosi, G. G. (2013). Epigenetic vulnerability and the environmental influence on health. *Experimental Biology and Medicine* (Maywood, N.J.), 238, 859–865.
- Greer, E. L., Maures, T. J., Ucar, D., Hauswirth, A. G., Mancini, E., Lim, J. P., Benayoun, B. A., Shi, Y., & Brunet, A. (2011). Transgenerational epigenetic inheritance of longevity in *Caenorhabditis elegans*. *Nature*, 479, 365–371.
- Grentzinger, T., Armenise, C., Brun, C., Mugat, B., Serrano, V., Pelisson, A., & Chambeyron, S. (2012). piRNA-mediated transgenerational inheritance of an acquired trait. *Genome Research*, 22, 1877–1888.
- Guerrero-Bosagna, C., Covert, T., Haque, M. M., Settles, M., Nilsson, E. E., Anway, M. D., & Skinner, M. K. (2012). Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. *Reproductive Toxicology*, 34, 694–707.
- Klironomos, F. D., Berg, J., & Collins, S. (2013). How epigenetic mutations can affect genetic evolution: Model and mechanism. *BioEssays*, 35, 571–578.
- Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., & Lehner, B. (2017). Transgenerational transmission of environmental information in *C. elegans*. *Science*, 356, 320–323.
- Knecht, A. L., Truong, L., Marvel, S. W., Reif, D. M., Garcia, A., Lu, C., Simonich, M. T., Teeguarden, J. G., & Tanguay, R. L. (2017). Transgenerational inheritance of neuro-behavioral and physiological deficits from developmental exposure to benzo[a]pyrene in zebrafish. *Toxicology and Applied Pharmacology*, 329, 148–157.
- Leroux, S., Gourichon, D., Leterrier, C., Labruno, Y., Coustham, V., Riviere, S., Zerjal, T., Coville, J. L., Morisson, M., Minvielle, F., & Pitel, F. (2017). Embryonic environment and transgenerational effects in quail. *Genetics, Selection, Evolution*, 49, 14.
- Norouzitallab, P., Baruah, K., Vandegehuchte, M., Van Stappen, G., Catania, F., Vanden Bussche, J., Vanhaecke, L., Sorgeloos, P., & Bossier, P. (2014). Environmental heat stress induces epigenetic transgenerational inheritance of robustness in parthenogenetic *Artemia* model. *The FASEB Journal*, 28, 3552–3563.
- Pavlinkova, G., Margaryan, H., Zatecka, E., Valaskova, E., Elzeinova, F., Kubatova, A., Bohuslavova, R., & Peknicova, J. (2017). Transgenerational inheritance of susceptibility to diabetes-induced male subfertility. *Scientific Reports*, 7, 4940.
- Quadrana, L., & Colot, V. (2016). Plant transgenerational epigenetics. *Annual Review of Genetics*, 50, 467–491.
- Skinner, M. K. (2011). Environmental epigenetic transgenerational inheritance and somatic epigenetic mitotic stability. *Epigenetics*, 6, 838–842.
- Skinner, M. K. (2014). Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. *Molecular and Cellular Endocrinology*, 398, 4–12.
- Skinner, M. K. (2015). Environmental epigenetics and a unified theory of the molecular aspects of evolution: A neo-Lamarckian concept that facilitates neo-Darwinian evolution. *Genome Biology and Evolution*, 7, 1296–1302.
- Skinner, M. K., Manikkam, M., & Guerrero-Bosagna, C. (2011). Epigenetic transgenerational actions of endocrine disruptors. *Reproductive Toxicology*, 31, 337–343.
- Skinner, M. K., Guerrero-Bosagna, C., Haque, M. M., Nilsson, E. E., Koop, J. A. H., Knutie, S. A., & Clayton, D. H. (2014a). Epigenetics and the evolution of Darwin's Finches. *Genome Biology and Evolution*, 6, 1972–1989.

- Skinner, M. K., Savenkova, M., Zhang, B., & Crews, D. (2014b). Gene bionetworks involved in epigenetic transgenerational inheritance of altered mate preference: environmental epigenetics and evolutionary biology. *Bmc Genomics*, *16*, 337.
- Song, J., Irwin, J., & Dean, C. (2013). Remembering the prolonged cold of winter. *Current Biology*, *23*, R807–11.
- Stockard, C. R., & Papanicolaou, G. N. (1918). Further studies on the modification of the germ-cells in mammals: The effect of alcohol on treated Guinea pigs and their descendants. *Journal of Experimental Zoology*, *26*, 119–226.
- Vaiserman, A. M., Koliada, A. K., & Jirtle, R. L. (2017). Non-genomic transmission of longevity between generations: Potential mechanisms and evidence across species. *Epigenetics & Chromatin*, *10*, 38.
- Vandegehuchte, M. B., Lemiere, F., Vanhaecke, L., Vanden Berghe, W., & Janssen, C. R. (2010). Direct and transgenerational impact on *Daphnia magna* of chemicals with a known effect on DNA methylation. *Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology*, *151*, 278–285.
- Veenendaal, M. V., Painter, R. C., De Rooij, S. R., Bossuyt, P. M., van der Post, J. A., Gluckman, P. D., Hanson, M. A., & Roseboom, T. J. (2013). Transgenerational effects of prenatal exposure to the 1944-45 Dutch famine. *BJOG*, *120*, 548–553.
- Waddington, C. H. (1942). Canalisation of development and the inheritance of acquired characters. *Nature*, *150*, 563–565.
- Waddington, C. H. (1953). Gene assimilation of an acquired character. *Evolution*, *1560*, 118–126.
- Xia, B., & de Belle, J. S. (2016). Transgenerational programming of longevity and reproduction by post-eclosion dietary manipulation in *Drosophila*. *Aging (Albany NY)*, *8*, 1115–1134.
- Xia, B., Gerstin, E., Schones, D. E., Huang, W., & Steven de Belle, J. (2016). Transgenerational programming of longevity through E(z)-mediated histone H3K27 trimethylation in *drosophila*. *Aging (Albany NY)*, *8*, 2988–3008.
- Yeshurun, S., Short, A. K., Bredy, T. W., Pang, T. Y., & Hannan, A. J. (2017). Paternal environmental enrichment transgenerationally alters affective behavioral and neuroendocrine phenotypes. *Psychoneuroendocrinology*, *77*, 225–235.

ENVIRONMENTAL IMPACTS ON GAMETOGENESIS & EMBRYOGENESIS

Environmental Impact on Gametogenesis and Embryogenesis: An Overview

Calivarathan Latchoumycandane, Central University of Kashmir, Srinagar, India

Pranitha Jenardhanan and Premendu P Mathur, Pondicherry University, Pondicherry, India

© 2018 Elsevier Inc. All rights reserved.

Glossary

Embryogenesis The process by which the **embryo** forms and develops inside the mother's womb.

Endocrine disruptors Man-made or natural chemicals that can interfere with the endocrine systems and disrupt the normal functions. These disruptions can cause cancerous tumors, birth defects, and other reproductive, and developmental disorders.

Environmental contaminants Substances when accidentally or deliberately introduced into the environment, may have the potential to harm people, wildlife, and plants.

Gametogenesis The development and production of the male and female germ cells required to form a new individual.

Oogenesis The differentiation of the ovum into a cell competent to further development when fertilized. It is developed from the primary oocyte by maturation.

Spermatogenesis The process in which an animal produces spermatozoa from spermatogonial stem cells by way of mitosis and meiosis.

Environmental Impact on Reproduction

Environment has great impact on human reproduction especially during gametogenesis and embryogenesis. Gametogenesis starts from the formation of gametes in the testes or ovaries, followed by embryogenesis, the development of embryo inside the mother's womb. Environmental factors such as life style and occupational exposures to contaminants can cause adverse effects on gametogenesis and embryogenesis. The interaction between early stage of development and the environment has extensively been studied and risks posed by changing lifestyle and the environmental contaminants have been proven to significantly impact our health, either directly by exposing people during early stage of development, or indirectly by disrupting quality of spermatocytes in male or oocytes in female. Changes in the environment due to industrialization have great impact on decreasing sperm quality, which is closely associated to low fertility in females, possibly due to combination of low fertilization rates, and increase embryonic mortality. For instance, observation of adverse trends in male reproductive health together with declining sperm count in Denmark and other countries led to the hypothesis that environmental contaminants having endocrine disrupting properties are harmful to reproductive health. In support of the above studies, a cohort analysis of nearly 45,000 pairs of twins from Sweden, Denmark, and Finland concluded that the environment rather than genetics played a critical role in sporadic cancers of the prostate, breast, and female reproductive system. The endocrine system controls the release of hormones which keeps the body functions normal. However, some environmental or man-made contaminants can impede the endocrine functions by mimicking or blocking hormone response that can disrupt the normal processes of development and reproduction, leading to adverse conditions. A large number of chemicals, mainly pesticides, and industrial wastes possessing estrogenic and/or antiandrogenic properties, are implicated as environmental contaminants. Prolonged exposure to small amounts of environmental contaminants leads to accumulation of considerable burden in animal and human tissues. The US Environmental Protection Agency (EPA) has defined endocrine-disrupting compound as "an exogenous agent that interferes with synthesis, secretion, transport, metabolism, binding action or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process."

Endocrine disruptors are highly heterogeneous molecules, some of them are synthetic chemicals used as industrial solvents/lubricants and their byproducts such as plastics, plasticizers, pesticides, fungicides, and pharmaceutical agents. Susceptibility of individuals to environmental contaminants depends on genetic polymorphisms because each person has unique exposure to a variety of known as well as unknown endocrine disruptors. Individual differences in the metabolism and body composition create considerable variability in the half-life, persistence, degradation in body fluids, and tissues. Initially estrogenic endocrine disruptors received much attention, but soon after antiandrogenic and thyroid hormone disrupting compounds have come into focus due to their adverse effects on reproduction. The mechanisms of action of endocrine-disrupting chemicals were not well understood and were originally proposed to exert their effect primarily through nuclear hormone receptor but recent studies show that the mechanisms are much broader than were originally documented.

Gametogenesis and Embryogenesis of Reproductive Organs

The male and female reproductive systems are under the control of hypothalamic-pituitary-gonadal axis. They interact to fulfill the main roles, in case of female, synthesizing sex hormones, development of female gametes, transport of gametes to a site where sperm can fertilize them, and provide a favorable environment for the development and delivery of the fetus. In the early stage of embryonic development, the two sexes are indistinguishable and a genetic program involving *SRY* gene in the Y chromosome determines the gonadal sex. In the presence of Y chromosome the *SRY* gene directs the gonad to become testis whereas in the absence develops into ovary. Sertoli cells in the testis produce anti-Müllerian hormone (AMH) that induces involution of the paramesonephric duct, also called Müllerian duct. In the absence of AMH it develops into oviducts, uterus and the upper part of vagina. Leydig cells produce testosterone that stimulates fetal mesonephric ducts, also called Wolffian ducts, to develop into epididymides, ejaculatory duct and seminal vesicles. Therefore, hormones play very important role in sex differentiation during early embryonic stage; any adverse effect by environmental contaminants possessing hormonal properties directly interfere the normal development of male or female reproduction.

The testis and ovary act as endocrine glands as well as reproductive organs. They play important role in the production of androgen and spermatozoa in the case of male, and estrogen and egg in the case of female. Spermatogenesis is the formation of the male gamete or spermatozoa and is dependent on the integrity of the seminiferous tubules, Sertoli cells, and endocrine regulation. In response to LH, Leydig cells produce testosterone, which along with FSH binds to Sertoli cell receptors to regulate spermatogenesis. The proper functioning of each organ in the reproductive system is essential to the operation of these complex processes. In animals and humans, there is a potential lag between exposure to factors from change in life-style or environmental contaminants, and manifestation of reproductive disorders because infertility cannot be assessed until the exposed individual attains reproductive age. Adverse effects on male or female reproduction by endocrine disruptors include feminization, demasculinization, reduced fertility, hatchability, viability of the offspring, impaired hormone secretion or activity, and altered sexual behavior.

Lifestyle and Gametogenesis

Lifestyle and morbidity associated with lifestyle due to habitual use of alcohol, tobacco, social or entertainment drugs, improper diet, obesity, and lack of exercise often negatively correlate with reproductive potential. Obesity not only affect the individual reproductive gametes but also impairs metabolic and reproductive health of the offspring suggesting that paternal health cues are transmitted to the next generation via the sperm. Obesity reduces inhibin B, which downregulates FSH biosynthesis and secretion, and thereby decreases spermatogenesis. High fat diet and obesity decrease the number of Sertoli cells and alter testosterone and estradiol ratio. Dietary soy which contains phytoestrogen has deleterious effects on male fertility by mimicking the action of estrogen. In case of female, obesity is associated with the alteration in ooplasm of egg that affects fertilization and implantation. Alcohol consumption can severely affect male fertility by decreasing sperm production and Sertoli cell numbers. Habitual smoking affects all stages of sperm production, sperm count, sperm concentration, sperm morphology, libido, and sometimes causes erectile dysfunction. Nicotine is one the components of tobacco, which causes damage to the DNA of spermatozoa, Leydig cell apoptosis, and hampers androgen production. Nicotine also induces Sertoli cell vacuolization, decreases the number of differential germ cell population and degenerative changes in seminiferous tubules. In case of female, smoking drastically impairs the functions of granulosa cells, hampers oocyte meiosis, depletes primordial follicles, decreases steroidogenesis, and increases the chances of aneuploidy.

Environmental Contaminants and Gametogenesis

Gametogenesis occurs in the testes or ovaries, which have been reported as one of the major target organs for environmental contaminants. The effects of maternal or paternal exposure to adverse environmental factors can be projected to the future offspring, resulting in poor semen quality in the male offspring years later. Paternal exposure in conjunction with the exposure of the adult offspring can amplify the effects, while adverse environmental effects during adulthood are believed to be reversible, damage done before and up to puberty is considered to be irreparable. The environmental contaminants that affect the gametogenesis are grouped into several functional classes viz., estrogen analogues, dioxins, phthalates, polychlorinated biphenyls (PCBs), brominated flame-retardants, and heavy metals. A large number of environmental contaminants possessing endocrine disrupting properties are known to induce abnormalities during gametogenesis both in wildlife and humans. Exposure of female fetuses to androgen leads to the formation of masculine characters whereas exposure of male fetuses to antiandrogens results in under-masculinization or feminization. Furthermore, the development of male reproductive system is under the control of multiple hormones, male fetuses are more susceptible to endocrine disruption than females. A wide range of mechanisms of action are described for several environmental contaminants that affect gametogenesis including agonist of estrogen receptor (genistein, diethylstilbesterol, and bisphenol A), antagonist of androgen receptor (vinclozolin, linuron, procymidone, phthalates, and *p,p'*-DEE), and agonist of aryl hydrocarbon receptor (dioxins, polychlorinated biphenyls, polycyclic aromatic hydrocarbon, and polychlorinated dibenzofurans).

Environmental Contaminants and Spermatogenesis

Even though infertility is considered as an adult problem, several factors including lifestyle and environmental contaminants impact on fertility that have their origin much earlier in life, especially during fetal development. The number of sperm produced per day or daily sperm production is dependent on the numbers of Sertoli cells within the testes. The number of Sertoli cells in the testes depends on the proliferation of the cells during fetal and neonatal periods and any factor that alters FSH or estrogen levels during fetal, neonatal or peri-pubertal life directly affects the sperm count and testicular size in adulthood. The mean sperm counts in men have been shown to decline over the past 60 years and it has been hypothesized that environmental exposure to synthetic estrogenic chemicals and related endocrine active compounds may be responsible for a global decrease in sperm counts and decreased male reproductive capacity. Several organochlorine compounds such as DDT and its metabolites, hexachlorohexane, PCBs, polychlorinated dibenzofurans (PCDFs), and polychlorinated dibenzo-*p*-dioxins (PCDDs), due to their high lipophilicity preferentially bio-accumulate in the adipose tissue. The presence of DDT and its metabolites, HCH, PCBs, and PCDDs has been reported in the fluids of human reproductive tract, such as seminal fluid, cervical mucus, and follicular fluid.

Many chemicals affect spermatogenesis indirectly through their effect on Sertoli cells, [e.g., dibromochloropropane (DBCP), monoethylhexyl phthalate (MEHP)] rather than directly on the germ cells. Hexane, an environmental toxicant and its metabolite 2,5-hexanedione (2,5-HD), cause testicular atrophy, and 2,5-HD induces toxicity by altering Sertoli cell's microtubules by pyrole-dependent cross-linking. The cross-linking of cytoskeletal elements leads to altered protein trafficking in the Sertoli cell. Consequently there is altered paracrine support between Sertoli cells and germ cells. 2-Methoxyethanol (2-ME), an industrial solvent, is toxic to both the male and female reproductive system. In order to attain testicular toxicity 2-ME must be metabolized to 2-methoxyacetic acid (2-MAA) by alcohol and aldehyde dehydrogenases. All stages of spermatocyte development and some stages of spermatid development are affected by 2-ME, which more selectively destroy early- and late-stage pachytene primary spermatocytes. Sertoli cells also are the prime target for 2-ME. The mechanism of testicular toxicity of 2-ME induced germ cell death is by reducing the available purine bases thus causing decreased RNA synthesis in spermatocytes. Alternatively 2-ME induces germ cell death by interfering with signal transduction pathways within either Sertoli cells or germ cells, causing a disruption of cell-to-cell communication. When 2-ME is applied dermally it can cause a decline in epididymal sperm and testicular spermatid counts in rats. Cadmium, one of the heavy metals, is known to affect testicular function by altering the actin filaments of tight junctions of Sertoli cells. Blood levels of cadmium have been reported higher in men from industrialized areas and in current smokers. There is a strong correlation between cadmium levels and number of immotile spermatozoa, and teratozoospermia index.

Esters of *o*-phthalic acid or phthalate esters (PAE) are extensively used as plasticizers in medical devices and other consumer products. Due to their leaching property, they can enter into body from the environment. There are several different PAEs, diethyl hexyl phthalate (DEHP), and its metabolite monoethylhexyl phthalate (MEHP) cause early sloughing of spermatids and spermatocytes and severe vacuolization of Sertoli cell cytoplasm by altering the membrane. DEHP is not only a reproductive toxicant in the male (e.g., rodents) but it can significantly suppress estradiol production from preovulatory follicle granulosa cell. Tri-*o*-cresyl phosphate (TOCP), an industrial chemical used as a plasticizer in lacquers and varnishes, decreases epididymal sperm motility, and density. TOCP interferes with spermatogenic processes and sperm motility directly and not via an androgenic mechanism or decreased vitamin E. Vinclozolin, a fungicide that undergoes biotransformation and its metabolites can effectively act as antagonists to the androgen receptor and disrupts sexual differentiation of male rats. Yet another fungicide benomyl causes a reduction in the number of spermatocytes and produces multinucleated germ cells. Exposure to methoxychlor in rodents during gestation and lactation resulted in slightly smaller testes and epididymides and in lowered sperm counts in male offspring than in controls. Chlordane, another persistent organic pollutant, disturbs spermatogenesis and causes dose-related damage to the testes of mice. The organochlorine insecticides endosulfan and lindane alter the weights of the testis and accessory sex organs in male rats and methoxychlor decrease weights of the accessory sex organs, epididymal sperm counts, and motility by inducing oxidative stress.

PCBs make up a group of synthetic organic chemicals containing about 200 individual compounds, some PCBs bind to estrogen receptor (ER) and consequently exert their toxic effects through estrogenic activity. Some PCBs induce reproductive toxicity through the production of highly toxic free radicals. Rats exposed to mixtures of PCB demonstrated decreased antioxidant enzymes in the testes. The timing of exposure is an important factor that strongly influences the ultimate reproductive effect of exposure to endocrine disruptors. Dioxins act via aryl hydrocarbon receptor and interfere with nuclear receptor, causing genital malformations. The male reproductive system gets adversely affected by the exposure to TCDD which affects germ cells in the testes and reduces Leydig cell volume. Interestingly, prepubertal exposure of men to TCDD has an inverse association between serum concentration of TCDD and semen quality, whereas the men exposed at ages 10–17 years had a positive association with semen quality suggesting that timing of exposure have an impact upon the response to dioxin. The effects have been summarized in [Table 1](#).

Environmental Contaminants and Oogenesis

Environmental contaminants can adversely affect ovary, uterus, vagina, anterior pituitary, and/or steroid production that lead to reproductive disorders such as early puberty, infertility, abnormal cyclicity, premature ovarian failure/menopause, endometriosis, fibroids, and adverse pregnancy outcomes. One of the most common environmental contaminants bisphenol A (BPA) has been shown to increase the incidence of multiocyte follicles, altered fetal ovarian genes responsible for steroidogenesis and expression of microRNA that mediate gonadal differentiation and folliculogenesis in sheep. BPA also interfere with ovarian development by

Table 1 Effect of environmental contaminants on reproduction

S. no.	Environmental contaminants	Source	Effect on reproduction
1	Phthalate (di(<i>n</i>) butylphthalate, di(iso) butylphthalate, benzyl butyl phthalate, di(2-ethylhexyl)phthalate, diphenyl phthalate, diisononyl phthalate, dicyclohexyl phthalate	Plastics, cosmetics, cleaning products, medical devices, toys, and many other everyday products	Reduce anogenital distance, hypospadias and undescended testes in immature male, reduce male fertility, fetal toxicity, and malformation of male organs
2	Bisphenol A (BPA)	Adhesive and sealants, plastic bottles, food packaging, plastic toys, and sporting equipment	Germ cell apoptosis, reduction of sperm count, arrest spermatogenesis at meiosis, affects apical, basal ectoplasmic specialization, disrupt homologous recombination
3	Alkylphenol, nonylphenol, and octylphenol	Use to protect polymers and stabilizer in plastic food packaging industries	Reduce testosterone biosynthesis, reduce testicular size, male fertility, sperm count, and quality
4	Heavy metals (cadmium, lead, arsenic, mercury)	Anthropogenic activities, volcanic eruption, soil erosion, etc.	Increase testicular toxicity, low sperm count, motility, and density Fetal toxicity and malformation of male reproductive organs
5	Volatile organic compounds (toluene, benzene, xylene, etc.)	Paints, varnishes, waxes, cosmetics, degreasing, and hobby products	Testicular toxicity, low sperm count and motility, reduce male fertility, reduce anogenital distance, hypospadias
6	Triclosan	A broad spectrum antimicrobial agent, is widely used in personal care, household as well as health care products, including toothpastes, antibacterial soaps, shampoos, deodorants, cosmetics, kitchen utensils, toys, bedding, and clothes	Alters sperm morphology
7	Polychlorinated biphenyls and its congeners	A wide variety of applications including plasticizers, rubber, resins, carbonless copy paper, adhesives, paints, and inks	Disrupts Sertoli cell function, reduce sperm cell viability and count Alters ovarian steroidogenesis
8	Dioxins	By-products of industrial processes, smelting, chlorine bleaching of paper pulp and the manufacturing of some herbicides and pesticides, volcanic eruptions, and forest fires	Sex hormone disruptors, testicular dysfunctions, low sperm count, and sperm abnormalities. Germ cell apoptosis, decreased sperm count
9	Diesel fuel exhaust	Combustion of diesel fuel	Suppress testicular functions in rats. Prenatal exposure in animals leads to endocrine disruption after birth
10	DTT and its byproduct DDE, methoxychlor, lindane	Organochlorine pesticides	Reduce synthesis of testosterone, induce germ cell apoptosis, functional defects in sperm production and maturation

affecting the onset of meiosis in the fetal ovary in macaques, in CD-1 mice as well as in cultured human fetal oocytes. BPA affects the postnatal ovary by accelerating follicle transition, decreasing primordial and increasing primary follicle numbers in lambs and rats. Prenatal exposure to high doses of BPA increases cystic follicles, depleted corpora lutea, and reduced antral follicle numbers in rats. Bisphenol A exposure also adversely affects the postnatal ovary in vitro by inhibiting the growth of cultured mouse antral follicles, upregulating the expression of cell cycle regulators, and pro- and antiatretic factors thereby causing atresia. The effects of BPA on the ovary also depend on which other chemicals the ovary is exposed to. Interestingly, triclosan, an antibacterial and antifungal agent found in some consumer products, including toothpaste, soaps, detergents, toys, and surgical cleaning treatment interact with BPA thereby magnifying its presence in reproductive tissues in mice.

The increased expressions of liver X receptor α and sterol regulatory element binding protein members in the fetal human ovary are reported following DHEP exposure. Exposure to MEHP decreases the number of viable oocytes in fetal mouse ovaries by dysregulating the expression of Cu-Zn superoxide dismutase and mitochondrial respiratory chain protein such as Nd1. DEHP also impaired the assembly of primordial follicle in cultured newborn mouse ovaries. Methoxychlor increases the expression of a number of genes associated with cell death and primordial follicle activation in mice and lipid peroxidation in neonatal mouse ovaries. One of the major metabolites of methoxychlor, 1,1,1-trichloro-2,2-bis(4-hydroxyphenyl)ethane (HPTE), also inhibits follicular growth and induces atresia in antral follicles from adult mouse ovaries. In addition expression of genes regulating signal transduction, transport, cell cycle, adhesion, differentiation, motility growth, apoptosis, development, and metabolism also get altered.

Environmental Contaminants on Embryogenesis

Embryotoxicity is any morphological or functional alteration caused by chemical or physical agents that interferes with normal growth, homeostasis, development, and differentiation of fetus. Abnormal sex hormone exposure during critical periods of development has been postulated for pathological mechanism for embryotoxicity or teratogenicity. Several environmental chemicals possessing antiandrogenic properties, when administered in utero, affect the normal development of gonads and accessory sex organs in animal models. The endocrine disruptors having antiandrogenic properties are vinclozolin, procymidone, linuron, *p,p'*-DDE (1,1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane) that act as androgen receptor antagonists. Endocrine disruptors possessing antiandrogenic properties such as phthalate esters reduce androgen synthesis. Most chemicals in commercial use have not been checked for possible developmental toxicity to fetus, infants, and children. Even after gestation damage may still occur and toxins can pass on to infants via breastfeeding. One of the historical examples of endocrine disruptors is diethylstilbestrol (DES) affecting female reproductive system. In utero exposure of females to DES is associated with the induction of vaginal carcinomas after puberty. DES exposure causes a characteristic T-shaped uterus, abnormal oviduct anatomy and function, and abnormal cervical anatomy. These effects are believed to occur through estrogen receptor α and abnormal regulation of *Hox* genes. DES administration to humans induces an increase in the incidence of cryptorchidism. Interestingly DES and other potent estrogens are capable of reducing androgen levels and expression of the androgen receptor proteins in rats. It raises a controversy that genital tract abnormalities got arisen from in utero exposure of potent estrogen were due to lowered androgen levels and/or action of estrogen.

In rats, organochlorine environmental contaminants such as kepone and methoxychlor cause masculinization of the exposed female rats, altering ovulation and exhibit male sexual behavior. In humans, estrogenic environmental contaminants can alter natural hormonal cycles and associate with breast cancer induction. Some environmental endocrine disruptors may function as promoters or inducers of carcinogenesis and DDT is one of such examples. Persistent environmental contaminants have been found to correlate with breast cancer incidence. Delayed testicular descent, reproductive dysfunction and sexual impotence have been reported in males occupationally exposed to synthetic estrogens. The widespread effects have been summarized in Table 2.

In rodents, TCDD is both embryotoxic and teratogenic. TCDD has avidity for the estrogen receptor and other receptors (e.g., Ah receptor). Intrauterine exposure to dioxins has been associated with subtle developmental changes in the fetus. In Wistar rats, exposure to TCDD during pregnancy delayed the development of the ovaries in the offspring and reduced the expression of

Table 2 Effect of environmental contaminants on embryogenesis

S. no.	Environmental toxicants	Source	Potential impact on embryogenesis
1	Mercury	Eating contaminated fish or shellfish, breathing vapors from spills or incinerated materials, skin contact with mercury at the workplace	Brain damage, mental retardation, poor coordination, blindness, seizures, nervous, and digestive system problems and renal damage
2	Lead	Paint containing lead, contaminated soil, water, and dust	Low birth weight and preterm birth and neurodevelopmental effects
3	Arsenic	Contaminated food, water, or air; occupational exposure, inhaling sawdust or smoke from arsenic-treated wood; exposure to high-levels of arsenic in soil	Congenital malformations
4	Sulfur dioxide and particulate matter	From polluted air	Increased risk of low birth weight
5	Polychlorinated biphenyls (PCBs)	Drinking water, breathing air or eating foods, or exposure to soil that are contaminated, via older electrical equipment or transformers	Lower birth weight, decreased motor skills, and depressed immune system
6	DDT	Exposure via agricultural work and accidental exposure	Increased risk of miscarriage, preterm birth and small for gestational age
7	Bisphenol-A (BPA)	Polycarbonate plastics, food, consumer products, and packaging	Birth defects and neurodevelopmental disorders
8	Pesticides	Food residues, agricultural settings, in-home use	Impaired cognitive, neurodevelopment and impaired fetal growth
9	Organic solvents	Industrial workplaces, numerous consumer products including plastics, dyes, detergents, food containers, carpeting and cleaning products, nail salons	Fetal loss and miscarriage
10	Phthalates	Plastics, cosmetics, cleaning products, medical devices, toys and many other everyday products	Reduced anogenital distance, hypospadias and undescended testes in immature male, reduced male fertility, fetal toxicity and malformation of male organs, birth defects, shortened gestational age and impaired neurodevelopment

steroidogenic acute regulatory (StAR) protein in fetal ovaries. 2-ME is also embryotoxic and teratogenic in several species. Several plant estrogens bind weakly to estrogen receptors α and β , and are able to induce weak estrogenic and antiestrogenic actions in mammalian tissues.

Further Reading

- Carlsen, E., Giwercman, A., Keiding, N., & Skakkebaek, N. E. (1992). Evidence for decreasing quality of semen during past 50 years. *BMJ*, 305, 609–613.
- Chitra, K. C., Latchoumycandane, C., & Mathur, P. P. (1999). Chronic effect of endosulfan on the testicular functions of rat. *Asian Journal of Andrology*, 1, 203–206.
- Colborn, T., vom Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives*, 101, 378–384.
- De Franciscis, P., Ianniello, R., Labriola, D., Ambrosio, D., Vagnetti, P., Mainini, G., et al. (2015). Environmental pollution due to cadmium: Measure of semen quality as a marker of exposure and correlation with reproductive potential. *Clinical and Experimental Obstetrics & Gynecology*, 42, 767–770.
- Gray, T. J., & Beamand, J. A. (1984). Effect of some phthalate esters and other testicular toxins on primary cultures of testicular cells. *Food and Chemical Toxicology*, 22, 123–131.
- Gray, L. E., Jr., Ostby, J., Ferrell, J., Rehnberg, G., Linder, R., Cooper, R., et al. (1989). A dose-response analysis of methoxychlor-induced alterations of reproductive development and function in the rat. *Fundamental and Applied Toxicology*, 12, 92–108.
- Jenardhanan, P., Panneerselvam, M., & Mathur, P. P. (2016). Effect of environmental contaminants on spermatogenesis. *Seminars in Cell & Developmental Biology*, 59, 126–140.
- Latchoumycandane, C., Chitra, K. C., & Mathur, P. P. (2003). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces oxidative stress in the epididymis and epididymal sperm of adult rats. *Archives of Toxicology*, 77, 280–284.
- Lichtenstein, P., Holm, N. V., Verkasalo, P. K., Iliadou, A., Kaprio, J., Koskenvuo, M., et al. (2000). Environmental and heritable factors in the causation of cancer—Analyses of cohorts of twins from Sweden, Denmark, and Finland. *The New England Journal of Medicine*, 343, 78–85.
- Safe, S. H. (2000). Endocrine disruptors and human health—Is there a problem? An update. *Environmental Health Perspectives*, 108, 487–493.
- Sharpe, R. M. (1993). Declining sperm counts in men—Is there an endocrine cause? *The Journal of Endocrinology*, 136, 357–360.
- Sharpe, R. M. (2000). Environment, lifestyle and male infertility. *Baillière's Best Practice & Research. Clinical Endocrinology & Metabolism*, 14, 489–503.
- Sharpe, R. M., & Skakkebaek, N. E. (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet*, 341, 1392–1395.
- Sujatha, R., Chitra, K. C., Latchoumycandane, C., & Mathur, P. P. (2001). Effect of lindane on testicular antioxidant system and steroidogenic enzymes in adult rats. *Asian Journal of Andrology*, 3, 135–138.
- Toppari, J., Larsen, J. C., Christiansen, P., Giwercman, A., Grandjean, P., Guillelte, L. J., Jr., et al. (1996). Male reproductive health and environmental xenoestrogens. *Environmental Health Perspectives*, 104, 741–803.

Environmental Effects on Developing Germ Cells

Francesca Pacchierotti and Eugenia Cordelli, ENEA, CR Casaccia, Roma, Italy
Antonella Russo, University of Padova, Padova, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

In mammals, germ cell development starts during fetal life and ends up with mature gametes formation after puberty, along the whole reproductive lifespan of individuals. Although a common pattern can be recognized between male and female germ cell development, major differences are present, such as the prenatal occurrence of premeiotic and meiotic phases in oogenesis, the lengthy aging of immature, resting oocytes before ovulation, or the profound postmeiotic differentiation in spermatogenesis.

Gametogenesis is finely regulated at molecular, cellular, tissue, and organism levels; however, external physical and chemical agents can perturb the process, with adverse consequences on fertility and heredity. The environmental impact on germ cells largely depends upon the time of exposure and the developmental phases affected. Chronic exposures may be especially critical for resting oocytes, in which their effect can add up to progressive deterioration of structures and functions with time.

In addition to direct effects on germ cells themselves, environmental exposure may affect germ cells by targeting their supporting somatic niche or the endocrine hypothalamic–pituitary–gonadal axis. The picture is complex since, for instance, the somatic component of testicular and ovarian tissues creates also a structural and functional physiological barrier protecting the maturing gametes from interactions with exogenous molecules.

The ultimate effects on germ cells can be death, with a transient or irreversible decline in gamete production, or genetic and epigenetic alterations with potentially heritable consequences (**Fig. 1**). Additionally, germ cell tumors arising from totipotent primordial germ cells, and especially testicular tumors, have been associated to prenatal environmental exposures to endocrine disrupting chemicals (EDCs). EDCs include a variety of natural and man-made chemicals that may interfere with the body endocrine system, many of which have a long environmental persistence. These compounds are found in daily products, such as plastic food bottles, food cans, detergents, flame retardants, toys, cosmetics, and pesticides. EDCs are a chemical class of high concern for human reproduction, gametogenesis and fertility, which are all hormonally regulated processes.

Nowadays, the attention is moving from the characterization of specific physical and chemical agent hazards to a broader perspective, which aims at understanding the impact on germ cells of complex exposures, diet, and lifestyle habits included. Tobacco smoking is the most potent and widespread environmental toxicant able to induce the full spectrum of adverse effects on germ cells (**Beal et al., 2017**). In the near future, next generation sequencing capacities applied to pedigree genomic analyses will allow to identify the first unquestionable human germ cell mutagens among the most likely candidates, ionizing radiation, cancer chemotherapy, cigarette smoking, and air pollution (**Yauk et al., 2013**) (**Table 1**).

Also, the increasing knowledge about the molecular pathways underlying cell reaction to exogenous stimuli helps to assess the environmental impact on developing germ cells in terms of adverse outcome pathways (AOP), from molecular initiating events to ultimate health effects. This approach is being developed by the Organization for Economic Co-operation and Development (OECD) within its Chemical Testing Program.

Toxic Effects

In Western countries, between 1973 and 2011, the mean sperm count declined, on average, 1.4% per year with an overall decline of 52.4% (**Levine et al., 2017**). The etiology of this decrease is unclear. Sperm count has been plausibly associated with environmental and lifestyle influences, both prenatally and in adult life. In particular, endocrine disruption from chemical exposures (e.g., phthalates) or maternal smoking during critical windows of male reproductive development may play a role in prenatal life, while exposure to pesticides may play a role in adult life (**Fig. 1**). Adverse effects have been documented in many animal studies, in which treatment can be controlled and doses are generally higher than those encountered by humans. Results of human epidemiological surveys, where exposure occurs at low levels for long times, exposure levels are difficult to quantify, and many confounders may affect the study outcome, are less conclusive. Nevertheless, cumulative effects of various low-dose human exposures should not be overlooked.

The risk of environmentally induced cell death is particularly critical for oocytes, of which there is a nonrenewable, finite pool. A reduction of the oocyte number may lead to a shortened reproductive lifespan. Premature ovarian insufficiency (POI) is a medical diagnosis that affects about 1% of women before the age of 40. In more than 75% of cases the cause is undetermined. Environmental factors are suspected of contributing to the onset of POI (**Vabre et al., 2017**).

Studies in rodents have demonstrated that prenatal exposure to mild analgesics, such as paracetamol or indomethacin, may reduce ovarian follicle reserves (a warning sign of POI), possibly by inhibiting primordial germ cell proliferation or interfering with oocyte meiotic progression. Similar effects have been also shown for industrial chemicals like bisphenol A (BPA) and di(2-ethylhexyl) phthalate (DEHP). DEHP is the most widely used plasticizer as it produces a functional polymer with excellent characteristics for industrial and medical applications such as inertness, flexibility, transparency, and high resistance to heat and chemicals. However, DEHP is non-covalently bound to products and may therefore leach out after prolonged use, with potential human exposure through inhalation,

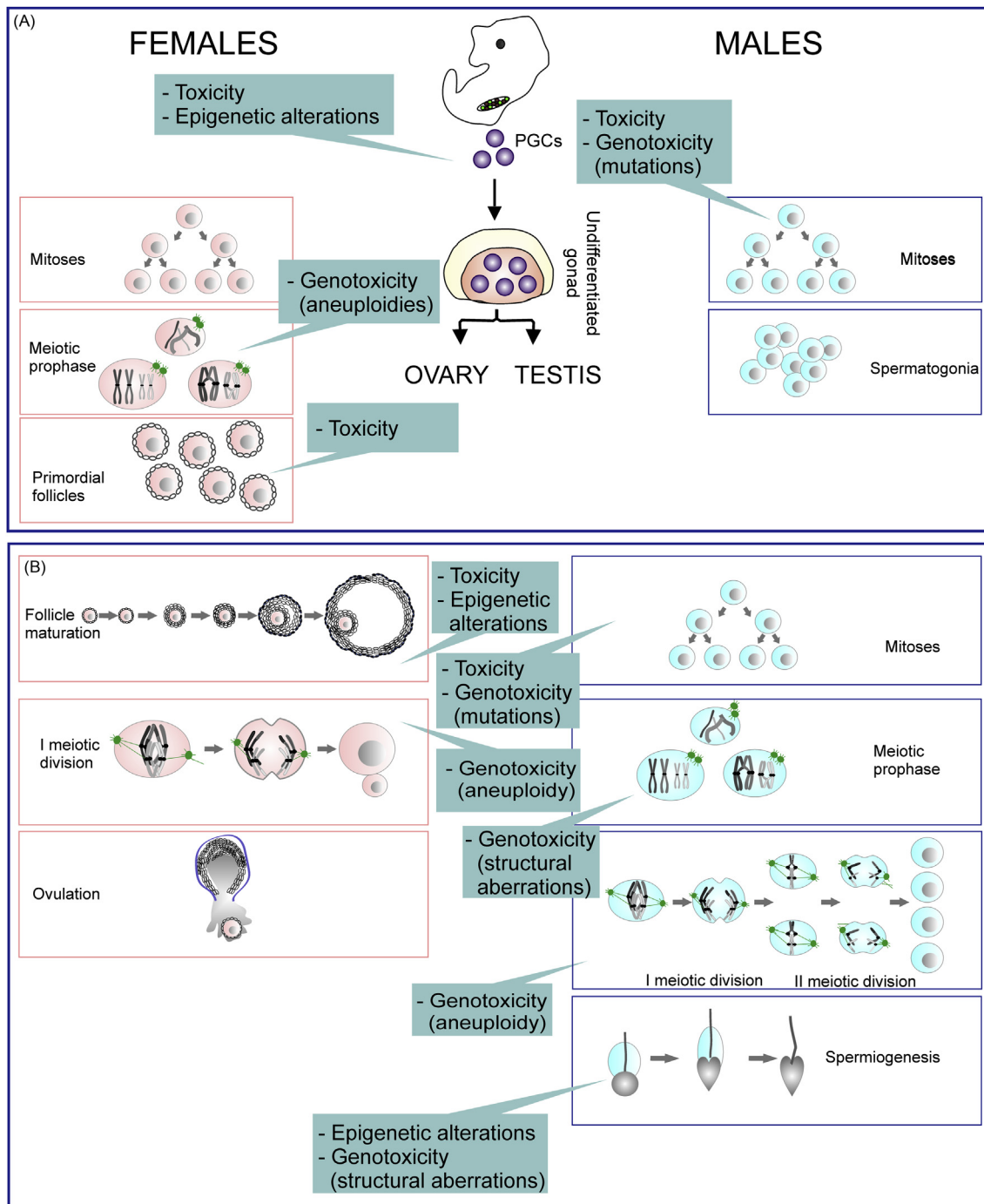


Fig. 1 A schematic representation of main stages of male and female germ cell development in mammals, with indication of prevalent mechanisms of environmental effects and their stage specificity. (A) Prenatal development. (B) Postpubertal development.

ingestion or dermal contact. The mechanisms underlying ovarian toxicity of these chemicals are still unclear and the extent to which their endocrine disrupting properties may contribute needs to be better elucidated. At a molecular level, markers of cell damage (oxidative stress and apoptotic cell death pathway) in follicular cells and oocytes have been reported. DEHP seems to affect oocytes also at an epigenetic level, with alterations of gene expression observed after *in vitro* or *in vivo* exposure of neonatal ovaries, due to DNA methylation changes of imprinted genes and genes critical for oogenesis.

In addition to direct effects on developing germ cells, DEHP and BPA *in utero* exposures have adverse effects on folliculogenesis, during both follicle assembly and recruitment phases. Since the quality and quantity of fertilizable oocytes are dependent on finely tuned paracrine signaling between the oocyte and the surrounding follicular cells, impairment of folliculogenesis will negatively

Table 1 Available evidence, in humans and animal models, for mutagenic potential of human relevant exposures to chemical or physical agents (all of them proven carcinogens in humans)

Agent	Humans		Animal models	
	Somatic cells	Germ cells	Somatic cells	Germ cells
Ionizing radiation	+	Not conclusive ^a	+	+
Smoking	+	Not conclusive ^{a,b}	+	+
Chemotherapeutic drugs	+	Not conclusive ^a	+	+
Air pollutants	+	Not conclusive ^a	+	+

^aYauk, C. L., Lucas Argueso, J., Auerbach, S.S., et al. (2013). Harnessing genomics to identify environmental determinants of heritable disease. *Mutation Research* **752**, 6–9.

^bBeal, M. A., Yauk, C. L., Marchetti, F. (2017). From sperm to offspring: Assessing the heritable genetic consequences of paternal smoking and potential public health impacts. *Mutation Research* **773**, 26–50.

affect germ cell development and female fertility. Although most of the evidence comes from studies with laboratory rodents and few epidemiological surveys are available so far, experimentally determined no-effect levels and human exposure concentrations suggest that safety margins are smaller than recommended, at least for the most highly exposed women (Johansson et al., 2017).

Data in rodents and humans support an effect of prenatal and adult smoking exposure on follicle stock depletion, likely caused by oxidative stress and apoptosis. Among the over 4000 chemical substances contained in cigarette smoke, polycyclic aromatic hydrocarbons are the most likely culprit, as they bind the aryl hydrocarbon receptor on the surface of granulosa cells and through this mechanism may activate pro-apoptotic genes and cause follicular atresia.

The germ cell killing effect of ionizing radiation is well ascertained in laboratory species. After the increasing success of anticancer therapies in children and adolescents, it is important to assess the risk of temporary or permanent sterility in radiotherapy cancer survivors. Fortunately, oocytes enclosed in primordial follicles and slowly cycling/quiescent spermatogonial stem cells, which represent the majority of target cells in women and the only cells with a regenerative potential in men, are relatively radioresistant with respect to the subsequent differentiation stages, growing follicles and committed proliferating spermatogonia; thus in many cases, fertility reduction is only a temporary effect. Notably, different variables influence the risk in women and men, which confirms the role of reproduction physiology in modulating environmental risks. For instance, in women, sterilizing doses decrease with age, as the remaining population of primordial follicles falls, while in men the length of the infertile period appears to increase with radiation dose, as expected on the basis of the survival curves for spermatogonial stem cells.

Genetic Effects

In the 1940s the scientific community started investigating how the genetic information transmitted by gametes could be affected by human exposure conditions. After the end of the Second World War, Hiroshima e Nagasaki populations were monitored for years to understand the transgenerational consequences of irradiation exposure of germ cells. Furthermore, the fast postwar industrial growth lead to the introduction in the environment of an exponential number of new chemical agents, to increasing pollution in several areas of the World, and to novel forms of occupational exposure conditions.

Cell and molecular events associated to the introduction of detrimental genetic changes affecting reproduction may be as complex and specific as the development of male and female gametes is differently organized (Fig. 1). At the same time, the molecular mechanisms dealing with DNA damage surveillance and repair are highly specific and reflect the different cell and chromosome organization during and between male and female gametogenesis process.

Paternal and maternal genomes within fertilized oocytes could be considered the last target of potential environmental effects on developing germ cells. However, this stage is short and is more interesting for mechanistic studies on DNA damage repair than for the assessment of environmental genetic risks. Thus, postfertilization exposures will not be addressed.

Experimental evidence for the action of mutagens in germ cells has been accumulated mainly using male rodents, while databases for female germ cells are poor, because of the intrinsic difficulty to perform analyses on this limited cell compartment. Data are sufficient to have a panel of model agents useful to predict, when necessary, the reasonable profile of action of unknown agents before testing them (Wyrobek et al., 2007) (Table 2).

Unlike the evidence about a toxic impact of environmental, occupational and lifestyle factors on human germ cells, very few studies have so far documented an increase of de-novo mutations due to human environmental exposure. Even for ionizing radiation no conclusive evidence has been obtained in the atomic bomb survivors' offspring and in other cohorts of radiation-exposed people, in spite of very well documented effects in mouse germ cells (Table 1). Beyond the obvious difficulty to set-up appropriate, parent-children, epidemiological or case-control studies, major limitations in genome-wide analytical methods have until now hampered the detection of small increases of genetic changes, at a few, not-necessarily coding, sequences. Genome sequencing, already successfully applied in a proof-of-principle study on the characterization and quantitation of germline mutations in the mouse induced by ethylnitrosourea (the most powerful chemical mutagen in male germ cells), holds promise to overcome these limits.

Table 2 Response of rodent germ cells to genotoxic agents, as assessed by exposing different cell stages and by applying postfertilization assays of clastogenesis, mutagenesis, aneuploidy (dominant lethal assay, heritable translocation assay, specific locus test, cytogenetic analysis of zygotes)

	Prevalent mechanism of action for the observed effects	Male germ cells						Female germ cells
Agent		Stem spermatogonia	Differentiating spermatogonia	Spermatocytes	Early spermatids	Late spermatids	Sperm	Oocytes
Therapeutic agents								
Bleomycin	Clastogenic	—	—	—	—	—	—	+++
Cisplatin	Clastogenic	—	—	—	—	—	—	+++
Colchicine	Aneugenic	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+++
Cyclophosphamide	Clastogenic	—	—	—	++	++	++	+++
Ethyl nitrosourea	Mutagenic	+++	—	—	++	++	++	—
Ionizing radiation	Clastogenic	+	+	+++	+++	++	++	+
Melphalan	Clastogenic	+	+	+	+++	+	+	+++
Mytomicin C	Clastogenic	+	+	+	++	+	—	—
Environmental agents								
Acrylamide	Clastogenic	+	+	—	+	++	+++	—
Benzo(a)pyrene	Clastogenic	—	—	—	—	—	++	—
1,3 Butadiene	Clastogenic	—	—	—	—	+	+	—
Diepoxybutane	Clastogenic	—	—	—	—	+	+	++
Ethylene Oxide	Clastogenic	—	—	—	—	++	++	—
Glycidamide	Clastogenic	—	—	—	—	+++	++	—
Griseofulvin	Aneugenic	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	+++

This table lists a selection of agents representative of different mechanisms of action. + = low response; ++ = mild response; +++ = high response; — = no response; n.a. = not assessed. Note that testing in female germ cells may not be as exhaustive as in male germ cells due to technical limitations.

Adapted from: Wyrobek, A. J., Mulvihill, J. J., Wassom, J. S., et al. (2007). Assessing human germ-cell mutagenesis in the Postgenome Era: A celebration of the legacy of William Lawson (Bill) Russell. *Environmental and Molecular Mutagenesis* 48, 71–95.

Genetic changes can occur both at the gene and at the chromosome level, depending on the mechanism of action and the exposure condition of each environmental factor. For example, gene mutations arise when exogenous compounds reaching the germ cells are able to produce chemical modifications of the DNA sequence. These changes (not all of them) may cause pathological gene variants, which can manifest their detrimental effects at different developmental stages of humans, from early fetal life to elderly. In general, gene mutations induced during gametogenesis do not impact on the gamete before fertilization. Thus these gene changes are not selected and are transmitted to the progeny of exposed individuals. According to *de novo* mutation detection by next generation sequencing, the spontaneous germline mutation rate is substantially lower than the somatic mutation rate and both rates seem to be higher in mice than in humans. The germ cell stage at the highest risk of environmentally induced gene mutations is the mitotically expanding spermatogonial compartment, because many mutagens act by introducing covalently bound adducts to the bases, which, if unrepaired before DNA replication, may induce mispairing and consequent base changes. Benzo(a)pyrene is an ubiquitous environmental pollutant and an ascertained human carcinogen that causes both somatic and male germline mutations. Notable differences in mutation spectra between somatic and germ cells have been reported pointing to male germ cell-specific DNA-repair mechanisms. Benzo(a)pyrene induces mutations in sperm not only after treatment of adult mice, but also after prenatal exposure of primordial germ cells. Also female germ cells may be target of benzo(a)pyrene genotoxicity, as shown by induction of DNA strand breaks in mouse oocytes and their surrounding cumulus cells.

Changes at the chromosome level may be due to a structural damage: several exogenous agents are capable to introduce break-ages into the double helix, or to produce lesions that indirectly cause the formation of such a break. Collectively, these chemicals are defined clastogens. Broken chromosomes are highly reactive and must be sealed, even though imperfectly, to avoid cell death; when more chromosomes are damaged at the same time (e.g., after exposure to high doses of physical or chemical agents) they can rearrange mutually. These events lead to gross genetic changes: loss of large stretches of DNA, duplications or spatial reorganization of the genetic information. Furthermore, at meiosis chromosomes must pair with their homologues. Unbalanced conditions due to structural aberrations may lead to defective chromosome segregation. On the whole, chromosome damage introduced during germ cell development may be associated, with different extents, to prefertilization or early postfertilization selection. Postmeiotic stages (spermatids and spermatozoa) are especially prone to the formation of chromosome breaks and rearrangements, as a consequence of both endogenous (e.g., oxidative DNA damage) and exogenous stressors (Table 2). A compound that has shown a marked clastogenic effect on postmeiotic male germ cells in the mouse is acrylamide. Acrylamide is used worldwide in various scientific and industrial processes, such as water treatment, in cosmetics, and for gel electrophoresis. It is also found in carbohydrate rich foods that have been cooked at high temperatures, and in tobacco smoke. Genotoxic effects on mouse germ cells have been demonstrated also after low-dose chronic treatment relevant to human exposure levels. It is the transformation of acrylamide to its highly DNA-reactive metabolite glycidamide by cytochrome P450 enzyme CYP2E1 that is mainly responsible for the reproductive toxicity of the

compound; CYP2E1 inhibition by chemicals such as resveratrol seems to ameliorate acrylamide effects on male germ cells. Further to genotoxic damage, acrylamide induces also cytotoxic effects in male and female mouse germ cells, with the latter showing impacts on developmental competence, cytoskeleton integrity, reactive oxygen species levels, apoptosis induction, epigenetic modifications and litter size. The potential exposure of large populations to acrylamide at low intake levels through food and smoking has raised concerns regarding its potential health effects, and ad-hoc investigations about possible adverse reproductive effects are certainly warranted.

A second class of well-known chromosome changes affects their number and not their integrity. In mammalian cells there are several structures involved in successful regulation of chromosome segregation, so that the number of genotoxins which can increase numerical changes is large and it includes those acting on tubulin dynamics, centrosome assembly, centromere function. Collectively, these genotoxins are defined aneugens. Again the differences between male and female gamete maturation processes add a further level of complexity; for example minimum effective doses in male and female gametogenesis can differ widely (Pacchierotti et al., 2007). It must be also considered that while gene mutations and/or chromosome structural changes can be virtually introduced along the whole gametogenesis (although stage specific response are described as a function of the mechanism of action of the mutagen and the damage repair capacity), numerical changes in maturing gametes can arise specifically during the two meiotic divisions. Colchicine, a well-known spindle poison, is the paradigm of aneugenic agents acting in male and female gametogenesis (Marchetti et al., 2016) (Table 2). Numerous data support that chromosome segregation checkpoint control in mammalian primary oocytes is more permissive than in primary spermatocytes, making female germ cells specifically sensitive to endogenous and exogenous sources of aneuploidy induction. Bisphenol A has been alleged to induce aneuploidy in mouse oocytes after prenatal or adult exposure, but the underlying adverse outcome pathway has not yet been elucidated (Pacchierotti and Eichenlaub-Ritter, 2011).

Down syndrome, which occurs in children with an extranumerary copy of chromosome 21, originates from missegregation of this chromosome pair of homologues. This condition has a typical maternal origin and it is further influenced by age. A well-described case of Down syndrome increase in an exposed human population has been reported after an event of water pollution caused by the insecticide trichlorfon in Hungary (Czeizel et al., 1993).

Epigenetic Effects

Epigenetic modifications consist in heritable changes in gene function without modification of DNA sequence. The epigenome is substantially plastic and responds to environmental changes with modifications that can be transmitted to daughter cells and across generations. This plasticity is functional for adaptation to a changing environment but it also makes epigenome potentially subjected to dysregulation and susceptible to the induction of epimutations.

Epigenetic modifications comprise three main mechanisms, DNA methylation, chromatin structure modifications and noncoding RNA (ncRNA). All these epigenetic mechanisms have important roles in the development of germ cells from primordial germ cell (PGC) differentiation and migration through meiotic process and maturation of functional gametes. Epigenetic dysregulation of these processes may have detrimental effect on gametogenesis and lead to reproductive dysfunction in adults. Moreover, epigenetic alterations can be retained along germ cell maturation and increasing evidence indicates that they can be transmitted across generations with potential effects on offspring health.

Experimental evidence on different animal models has shown that several toxicant exposures, occurring during prenatal or adult life can affect the germ cell epigenome. A sensitive window to the harmful effects of environmental toxicants is the differentiation of PGCs during prenatal life (Fig. 1). During this period, PGCs colonize the embryonic gonads and start their differentiation into a male or female pathway. A key epigenetic event associated to this process is the genome wide demethylation. At the same time, other epigenetic changes occur, such as modulation of histone modifications; furthermore, a correct balance in the expression of ncRNAs seems to be necessary for a correct PGC development.

The first demonstration that DNA methylation links maternal diet during pregnancy with offspring phenotype was obtained using a mouse model carrying a gene controlled by a methylation-sensitive regulatory region. In this model, treatment of pregnant mothers with BPA induced hypomethylation of the offspring gene with phenotype consequences. These effects could be counteracted by maternal dietary supplementation with methyl donors like folic acid or genistein. These observations raised the question whether prenatal exposures to chemicals that are not direct inhibitors or donors of DNA methylation/reaction can reprogram DNA methylation in PGCs and in the offspring in a physiologically relevant manner.

EDCs are the mostly investigated chemicals in relation to possible effects on PGCs that could be transmitted across multiple generations (Xin et al., 2015). Among them, vinclozolin, a fungicide with antiandrogenic properties, BPA and phthalates. Evidence of transgenerational effects has indeed been obtained, but the picture of the whole pathway, leading from initiating epigenetic changes in PGCs to ultimate reproductive impairment in the following generations, is still incomplete, with few studies, until now, walking the whole pathway step by step. Nevertheless, changes of DNA methylation status and miRNA expression in PGCs have been reported after exposure to vinclozolin. The window of exposure appears to be a critical factor in the manifestation of transgenerational phenotypes. Moreover, the extent to which the epigenetic changes drive the observed phenotypes across generations remains to be determined.

During adult male life, extensive epigenetic reprogramming for the production of functional sperm for fertilization is required during spermatogenesis. Emerging data suggest that the epigenome during this process is susceptible to environmentally induced

alterations. Exposure of adult male mice to particulate air pollution has been shown to increase global methylation of spermatogonia, which persists through spermatogenesis and remains elevated in spermatozoa. Different experimental studies have shown that exposure to toxicants such as acrylamide or EDCs can modify the gain and loss of methylation in sequences involved in genetic imprinting during spermatogenesis. In humans, various epidemiological studies have documented that chemical exposure, mainly cigarette smoking, are linked with alterations of sperm epigenome (Wu et al., 2015).

The mammalian sperm epigenome is suspected to be a mediator of transgenerational transmission of paternal environmental exposures. By now, an extensive literature exists reporting results of experimental studies aiming to relate paternal complex environmental exposure, lifestyle or nutrition conditions with sperm epigenome modifications and offspring health. For example, nutritional manipulation in fetal or adult rodent males, such as over/under-nutrition, prediabetic conditions or folate deficiency, induces differential methylation in sperm and metabolic disorders or birth defects in offspring (Schagdarsurengin and Steger, 2016).

Mature spermatozoa hold a series of ncRNAs that can mediate transgenerational effects. For example, mice subjected to early life traumatic stress exhibited, further to behavioral and metabolic changes, alterations in the abundance of several spermatozoal small ncRNAs, compared to an unstressed control. Progeny generated from injection of sperm RNA from the stressed fathers into embryos, exhibited similar behavior and metabolic patterns as the stressed fathers. This experimental evidence supports the concept that, at least in mice, sperm RNAs can transmit the response of an early life stress to the progeny. The sperm RNA content can be also modulated by dietary exposures (Chen et al., 2016). It appears that sperm may acquire ncRNAs either from their progenitor germ cells or from extracellular sources during epididymal maturation. Interestingly, sncRNAs were also found in human seminal exosomes.

During oogenesis, there are widespread transcriptional changes and de novo DNA methylation, allowing the oocyte to obtain fertilization and embryogenesis competency. A number of posttranslational histone modifications help direct de novo methylation events in the oocyte. All known classes of short ncRNAs are present in mouse oocytes; however, gene-knockout studies have provided evidence that only some of them are required for oocyte development. Several epigenetic changes have been shown in oocytes as a function of female aging, including lower genome-wide DNA methylation, specific histone acetylation and methylation changes, follicular fluid microRNA changes.

In spite of the increasing knowledge about the epigenetic determinants of oocyte quality and of the evidence about maternal effects on epigenetic transgenerational inheritance in human cohorts, so far only a few sparse studies addressed directly the question of possible chemical effects on mammalian oocyte epigenetic setting. An obvious technical difficulty resides in the small number of oocytes collectable from any experimental group. The focus has been mainly on the methylation status of imprinted genes, with specific changes observed, for instance, after prenatal exposure of mice to DEHP, or neonatal exposure of mice to BPA. Imprinted gene methylation changes in mouse oocytes were also associated to a diabetic condition or to a high-fat diet adult exposure. In addition, small but significant histone methylation changes have been reported after exposure of oocytes to BPA or acrylamide. Conversely, the possible role of oocyte-borne sncRNAs in environmentally induced transgenerational phenomena is so far unknown and warrants future investigations.

An open question remains about the relevance of experimental studies for human exposure, especially in view of the low-level (but multicomponent), chronic exposures mostly encountered by human populations. Sorting out an epigenetic impact of environment on human germ cells with potential heritable consequences is not easy task, because of the need to control for genetic and cultural confounders. However, notably, a few human observational studies to date suggest that transgenerational effects exist that cannot easily be attributed to cultural and/or genetic inheritance (Pembrey et al. 2014).

References

- Beal, M. A., Yauk, C. L., & Marchetti, F. (2017). From sperm to offspring: Assessing the heritable genetic consequences of paternal smoking and potential public health impacts. *Mutation Research*, 773, 26–50.
- Chen, Q., Yan, W., & Duan, E. (2016). Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. *Nature Reviews Genetics*, 17, 733–743.
- Czeizel, A. E., Elek, C., Gundy, S., et al. (1993). Environmental trichloron and cluster of congenital abnormalities. *Lancet*, 341, 539–542.
- Johansson, H. K. L., Svingen, T., Fowler, P. A., Vinggaard, A. M., & Boberg, J. (2017). Environmental influences on ovarian dysgenesis—Developmental windows sensitive to chemical exposures. *Nature Reviews Endocrinology*, 13, 400–414.
- Levine, H., Jorgensen, N., Martino-Andrade, A., et al. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*. <https://doi.org/10.1093/humupd/dmx022>.
- Marchetti, F., Massarotti, A., Yauk, C. L., Pacchierotti, F., & Russo, A. (2016). The adverse outcome pathway (AOP) for chemical binding to tubulin in oocytes leading to aneuploid offspring. *Environmental and Molecular Mutagenesis*, 57, 87–113.
- Pacchierotti, F., Adler, I.-D., Eichenlaub-Ritter, U., & Mailhes, J. B. (2007). Gender effects on the incidence of aneuploidy in mammalian germ cells. *Environmental Research*, 104, 46–69.
- Pacchierotti, F., & Eichenlaub-Ritter, U. (2011). Environmental hazard in the aetiology of somatic and germ cell aneuploidy. *Cytogenetic and Genome Research*, 133, 254–268.
- Pembrey, M., Saffery, R., Bygren, L. O., & Network in Epigenetic Epidemiology. (2014). Human transgenerational responses to early-life experience: Potential impact on development, health and biomedical research. *Journal of Medical Genetics*, 51, 563–572.
- Schagdarsurengin, U., & Steger, K. (2016). Epigenetics in male reproduction: Effect of paternal diet on sperm quality and offspring health. *Nature Reviews Urology*, 13, 584–595.
- Vabre, P., Gatimel, N., Moreau, J., et al. (2017). Environmental pollutants, a possible etiology for premature ovarian insufficiency: A narrative review of animal and human data. *Environmental Health*, 16, 37. <https://doi.org/10.1186/s12940-017-0242-4>.
- Wu, H., Hauser, R., Krawetz, S. A., & Pilsner, J. R. (2015). Environmental susceptibility of the sperm epigenome during windows of male germ cell development. *Current Environmental Health Reports*, 2, 356–366.

- Wyrobek, A. J., Mulvihill, J. J., Wassom, J. S., et al. (2007). Assessing human germ-cell mutagenesis in the Postgenome era: A celebration of the legacy of William Lawson (bill) Russell. *Environmental and Molecular Mutagenesis*, 48, 71–95.
- Xin, F., Susiarjo, M., & Bartolomei, M. S. (2015). Multigenerational and transgenerational effects of endocrine disrupting chemicals: A role for altered epigenetic regulation? *Seminars in Cell and Developmental Biology*, 43, 66–75.
- Yauk, C. L., Lucas Argueso, J., Auerbach, S. S., et al. (2013). Harnessing genomics to identify environmental determinants of heritable disease. *Mutation Research*, 752, 6–9.

Further Reading

<https://www.epa.gov>—EPA Endocrine Disruption.

<http://www.oecd.org>—OECD Chemical Testing Program.

Environmental Effects Impacting Preimplantation Development

Tom P Fleming, University of Southampton, Southampton, United Kingdom

Miguel A Velazquez, Newcastle University, Newcastle upon Tyne, United Kingdom

Adam J Watkins, University of Nottingham, Nottingham, United Kingdom

Judith J Eckert, University of Southampton, Southampton, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

The preimplantation embryo, from the time of fertilization up until blastocyst formation before implantation into the uterine endometrium, undergoes an extensive series of cellular and molecular changes over a period of some 4–6 days that are critical for its future development. These changes include: activation of the new embryonic genome to replace the parental gametic genomes; resumption of cell cycling within the zygote from a period of arrest awaiting fertilization; and initiation of morphogenesis leading to the establishment of distinct true embryonic (future fetal) and extra-embryonic (future placental) cell lineages by implantation. Preimplantation morphogenesis results in a blastocyst, a spherical cyst of ~100 μm diameter, comprising (i) an outer layer of differentiated trophoblast (TE) epithelial cells, the progenitor of the placenta; (ii) a blastocoelic cavity generated by TE transport activity, and (iii) an internal cluster of relatively undifferentiated cells, the inner cell mass (ICM), eccentrically placed on the basal surface of the TE (**Fig. 1**). In the late blastocyst stage, the ICM segregates into the epiblast, the progenitor of the entire fetus, and the newly-differentiated primitive endoderm epithelial layer abutting the blastocoel which gives rise to the visceral endoderm yolk sac placenta.

This busy schedule of cellular and molecular activity underlying blastocyst morphogenesis unfolds in a relatively autonomous manner, broadly independent of environmental factors since it can occur in a simple culture solution if an energy substrate and protein source is present. This complex and coordinated intrinsic developmental program involves the generation of radial asymmetry within the embryo, the formation of the distinct cell types, and a series of important cell–cell interactions that collectively guide cell fate and differentiation, coupled with temporal regulation of gene expression (**Bedzhov et al., 2014**). The switch in states from parental gametes (sperm, egg) to being an embryo includes epigenetic reorganization of the new embryonic genome to ensure the different cell types formed throughout development express appropriate genes. Epigenetic reorganization in the early embryo comprises global changes in DNA methylation and histone modifications that collectively regulate the pattern of gene expression (**Messerschmidt et al., 2014**).

During the preimplantation period, the intrinsic developmental program can, however, be influenced by external environmental conditions such as the availability and type of nutrients, a wide range of growth factors, hormones, metabolites, and cytokines present within the mother's reproductive tract, or the chemical composition of culture medium in assisted conception treatment. Early embryos are equipped with an array of membrane receptors and transporters that allow for sensing of environmental conditions such as insulin, amino acids, and glucose. These factors are mainly but not exclusively maternal in origin, may derive from remote sites of synthesis delivered through the vasculature or generated locally such as from uterine endometrial glands. Their release is usually under the control of ovarian steroid hormones. Alternatively, factors may be embryo-derived, acting in an autocrine fashion on the developmental program. The paternal seminal plasma also contains factors that at least indirectly can modulate the developmental program (discussed later).

These environmental factors are primarily embryotrophic, acting as positive regulators on embryo morphogenesis and physiology, stimulating cell cycling, and blastocyst development and attendant cell biological processes (**Hardy and Spanos, 2002; Robertson et al., 2015**). For example, in mice, insulin and the insulin-like growth factors (IGFs) promote mitogenesis, blastocyst formation and embryo cell number particularly in the ICM, glucose uptake, protein synthesis and cellular endocytosis. The cytokine colony stimulating factor 2 (CSF2) acts similarly as a positive regulator of preimplantation development and proliferation in a range of species leading to increased cell number and formation of blastocysts. Transforming growth factor alpha (TGF α) also stimulates development and blastocyst expansion including suppression of apoptosis. In contrast, tumor necrosis factor alpha (TNF α) slows development and increases apoptosis. The phospholipid, embryo-derived platelet activating factor (ePAF), is produced and released by the embryo after fertilization, binds extracellular albumin and acts in an autocrine way to stimulate embryo metabolism and developmental rate. ePAF production by embryos in culture medium and its autocrine activity acts to improve group culture of embryos over singleton culture. Chemokines are also critical in the process of immune tolerance of the mother toward the paternal antigens present in the embryo, coordinating maternal-embryo cross-talk to permit endometrial invasion of the TE derivative cells to occur at implantation—a dialogue that can prevent miscarriage (**Robertson et al., 2015**). Conversely, embryo development outside of the natural maternal environment and lacking ready availability of these stimulatory factors as during *in vitro* culture can reduce developmental progression. In addition, there is increased risk of production of reactive oxygen species (ROS) damage if environmental oxygen level is atmospheric, above that present *in vivo*.

One of the most exciting discoveries in reproductive biology in recent years is that environmental conditions may not be restricted to influencing the preimplantation developmental program in just the short-term but may impose changes in the embryo that persist throughout gestation and into postnatal life (**Fig. 2**). Such examples of longer-term environmental changes mediated during early embryogenesis may alter the growth trajectory during fetal development, disturb metabolic homeostasis, and associate with health and disease risk into adulthood. At one level, this interaction between embryo and environment can provide *plasticity* to

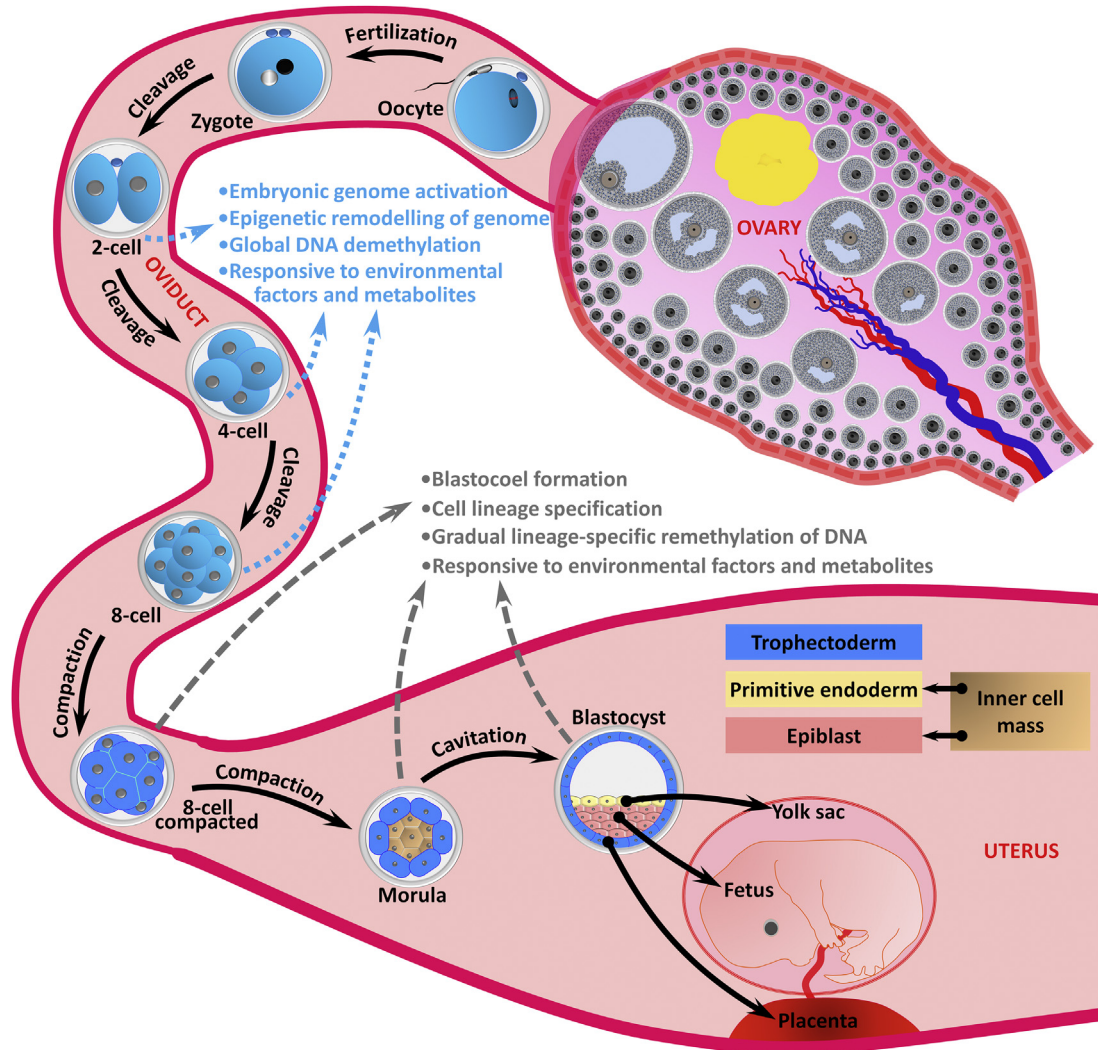


Fig. 1 Developmental milestones during mammalian preimplantation embryo development. The early embryo undergoes cellular and molecular events that are critical for a successful pregnancy. The preimplantation embryo is highly responsive to the environmental milieu present in the reproductive tract. Reproductive tract is representative of rodent and ruminant species.

the developmental program, allowing the early embryo to respond to prevailing environmental conditions and to optimize development accordingly to support survival of offspring. Alternatively, the environmental cues may perturb the developmental program such that changes represent deficiencies in cellular function.

These longer-term environmental effects on the preimplantation embryo are evident *in vivo* in response to the quality and type of maternal nutrition and her health and physiology at the time of conception. They are also apparent in response to *in vitro* culture conditions as occurs during assisted conception treatment. Such enduring effects are commonly known as *periconceptional developmental programming* and associate with a wider phenomenon now known as the “*Developmental Origins of Health and Disease*” (DOHaD). This concept proposes that poor conditions *in utero* mediated through maternal undernutrition and adverse health can disturb fetal development and be the forerunner of disease risk in later life, notably cardiovascular, and metabolic conditions (Barker and Thornburg, 2013). Classic epidemiological evidence in support of the DOHaD concept comes from the findings in several human population studies that low birth weight is a major predictor of hypertension and metabolic syndrome occurring in later adulthood. Below, we consider different examples and associated mechanisms by which environmental exposure to early embryos impose lifetime effects on health.

Effects of Maternal Nutrition and Metabolism on Preimplantation Embryos

Maternal Undernutrition

There is growing evidence that maternal undernutrition affects the development of the early embryo and with long-term consequences. For example, in a human famine dataset, generated following the 5-month Dutch Hunger Winter during WW2, those

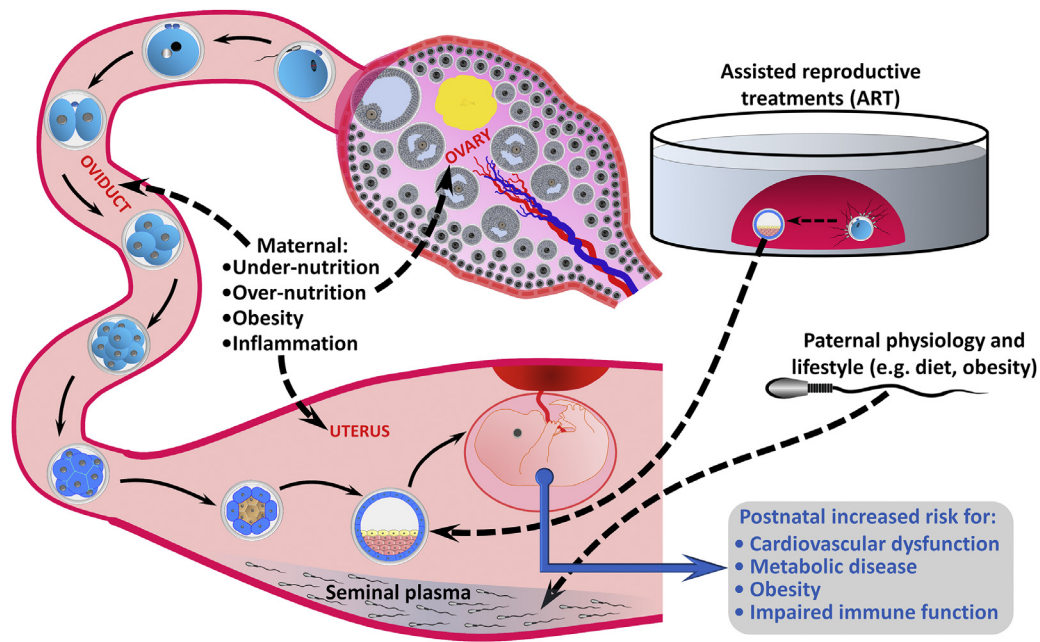


Fig. 2 Long-term effects of the preimplantation environment. Maternal factors can alter the microenvironment of the ovary and the reproductive tract which in turn can impact the early embryo. Paternal factors can affect the preimplantation embryo via maternal tract alterations exerted by seminal plasma. Exsitu development of the preimplantation embryo during assisted reproduction can compromise its viability. All these environmental influences can result in the expression of adverse phenotypes during postnatal life.

people experiencing the famine in utero during *early* gestation, compared with those exposed during *late* pregnancy, developed more severe cardiovascular, metabolic, and neurological deficits in adulthood (Roseboom et al., 2011). In another context, in The Gambia, where seasonal variation in food availability and consumption can also lead to chronic disease in later life, poor nutrition experienced by mothers around conception may have a significant influence. In both of these human examples, poor maternal nutrition affects the pattern of epigenetic reorganization of the genome during embryogenesis and likely contributes to the longevity of the consequences. In the Dutch Famine, gene pathways regulating growth and metabolism become differentially methylated in those experiencing famine during their early development and such changes persist into adulthood. In the Gambian study, the quality of maternal nutrition at conception alters the DNA methylation epigenome, a condition still evident in late childhood (Dominguez-Salas et al., 2014).

Animal models permit more focused studies to identify the mechanisms and consequences of poor maternal nutrition on the early embryo. This is best exemplified in a rodent model in which a maternal low protein diet is provided during only the preimplantation period with normal diet at other times and postnatally (known as Emb-LPD treatment). This treatment caused postnatal offspring to exhibit abnormal growth and adiposity with increased risk of cardiovascular and metabolic disease, especially in females (Watkins et al., 2008). How does this long-term phenotype emerge following this short duration of maternal undernutrition around the time of conception? Emb-LPD has been shown to reduce insulin and amino acid levels in the maternal circulation and this leads to changes in the amino acid composition present in the uterine fluid where embryos reside prior to implantation. Insulin and the branched-chain amino acids (BCAAs) leucine, isoleucine and valine are used by cells as biosensors to set the growth rate through the mTORC1 signal pathway, a master growth regulator integrating nutrient availability and growth. In Emb-LPD blastocysts, mTORC1 signaling is significantly reduced under these low insulin and BCAA conditions indicating this pathway is utilized to sense nutrient availability for growth (Eckert et al., 2012).

This preimplantation nutrient-sensing mechanism subsequently regulates the growth trajectory for fetal development by manipulating the rate of ribosome biogenesis. Thus, if nutrient availability remains low, ribosomal RNA expression is suppressed in embryonic/fetal somatic tissues to restrict growth. However, if nutrient availability increases (as occurs postblastocyst stage in Emb-LPD treatment), ribosome biogenesis is increased beyond that of controls. This capacity for preimplantation experience to manipulate ribosome biogenesis occurs not only during pregnancy but also throughout the lifetime and represents an opportunistic response to “catch up” growth as conditions improve (Denisenko et al., 2016). This coordination between nutrient levels and ribosome biogenesis is regulated epigenetically, with methylation on the ribosomal DNA genes being increased to suppress RNA polymerase 1 binding and rRNA expression in conditions of low nutrients, and vice versa in high nutrients. Along with mTORC1, evidence indicates nutrient sensing to manipulate ribosome biogenesis is achieved through the ribosomal protein, Rrm3 that associates with the mTORC1 complex (Denisenko et al., 2016).

The mouse Emb-LPD model demonstrates a second mechanism activated in the preimplantation period to protect growth under conditions of poor maternal nutrition. Here, the extraembryonic (placental) cell lineages respond to poor diet by increasing the

efficiency of maternal nutrient delivery during pregnancy. Thus, Emb-LPD blastocyst trophectoderm increases proliferation and the rate of cellular endocytosis, thereby capturing more maternal nutrients. The endocytosis response is comprehensive and involves increased expression of endocytosis membrane receptor (megalin), increased uptake of protein ligand and increased collective numbers of lysosomes to digest maternal nutrients. This compensatory mechanism is induced by embryo sensing of reduced environmental insulin and BCAA levels and is regulated by the RhoA GTPase molecular switch which modulates the actin cytoskeleton to promote endocytosis (Sun et al., 2014). A similar stimulation of endocytosis occurs with increased proliferation within the primitive endoderm lineage, here associating with epigenetic modification through histone deacetylation of the Gata6 transcription factor promoter. Collectively, these early extra-embryonic adaptations to poor diet persist beyond the preimplantation period and promote nutrient delivery via the main chorioallantoic placenta and yolk sac placenta throughout gestation (Watkins et al., 2008).

The Emb-LPD model therefore demonstrates distinct embryonic and extra-embryonic compensatory mechanisms whereby a low nutrient preimplantation environment activates developmental plasticity to facilitate survival of the conceptus. These responses, whilst promoting fetal growth, do associate with later cardiovascular disease since they are suited for a nutrient-deprived environment and so are maladaptive for the nutrient-rich conditions experienced after the dietary treatment leading to increased mass, adiposity, and chronic disease. Related studies in sheep whereby below-maintenance feeding of ewes during only the periconceptional period have shown a similar legacy with an altered growth trajectory and adverse cardiovascular and metabolic health in later postnatal life. The importance of altered epigenetic programming has also been clearly demonstrated by periconceptional restriction in sheep B vitamin micronutrients that, through 1-carbon metabolism, provide the methyl donors required for DNA methylation. This treatment significantly alters the offspring epigenome and occurs in association with cardiometabolic abnormalities (Sinclair et al., 2007).

Maternal Obesity and Overnutrition

The global epidemic of overnutrition and obesity is known to affect reproduction by reducing fertility and disturbing fetal metabolism such that children of obese mothers often become obese themselves. This vicious cycle can be traced from the typical high BMI and elevated systemic glucose and insulin levels in mothers driving excess fetal growth and adiposity, often coupled with allergic and atopic disturbance, leading to overweight and cardiometabolic disease risk in offspring.

Focused human and animal model studies have shown that oocyte maturation and the early embryo can be particularly sensitive to maternal obesity and overnutrition. Ovarian follicular fluid becomes richer in metabolites such as insulin, lactate, and fatty acids in overweight mothers and these may subsequently accumulate within oocytes and persist in early embryos where they cause metabolic disturbance (Robker et al., 2009). Firstly, mitochondrial activity is affected with abnormal morphology, cristae structure and membrane potential leading to increased mitochondrial biogenesis to compensate (Lane et al., 2015). Second, the rich maternal metabolite environment causes oxidative stress in oocytes and embryos with the elevated lipid content leading to activation of endoplasmic reticulum stress where protein misfolding occurs during biogenesis, associating with metabolic disorders. These effects lead to reduced embryo development rates and potential. Moreover, transfer of animal embryos from obese mothers, or after in vitro culture in rich nutrients, to lean controls can lead to fetal overgrowth, malformations and glucose-insulin resistance, mimicking the intergenerational conditions found in humans despite the absence of maternal gestational obesity. Thus, environmental exposure to overnutrition around the time of conception and preimplantation development is sufficient to compromise next generation health (Lane et al., 2015).

Effects of In Vitro Assisted Conception Treatments on Preimplantation Embryos

The remarkable success of assisted reproductive treatments (ART) to overcome human infertility is well recognized with several million children born worldwide. Given the influences described above on maternal nutritional effects on the early embryo and their legacy over the lifetime, it is therefore pertinent to evaluate the safety of current techniques used in clinical embryology. Such analysis is complex given the inherent poor fertility of parents involved which may be directly affecting embryo potential, the diversity in patient demographics that may also be influential, and the wide range and changes in procedures used in ART over the years complicating any causal relationships that may be identified. Animal models of ART treatments have therefore been invaluable where many of these variables can be controlled.

Despite these caveats, when considering human ART outcomes after single embryo transfer, several long-term deficiencies compared with natural pregnancies have been identified. These include increased incidence of perinatal complications such as congenital abnormalities, low birth weight, and increased mortality. The effect of embryo culture medium is the most widely studied aspect of human ART postnatal outcomes. Whilst the chemical composition of commercial culture media used are not disclosed, it is apparent, particularly from a randomized control trial, that different culture media impose significant differences in birth weight and this distinction is maintained through early childhood (Kleijkers et al., 2016).

The health of ART children and adults has been followed up in epidemiological studies. These indicate cardiovascular abnormalities are increased in ART offspring including raised blood pressure, altered blood flow rates and vessel thickness, heart shape and size which can in some cases be traced back to defects occurring during the pregnancy period. ART children also exhibit metabolic risks including increased glucose-insulin resistance, increased plasma lipids, and raised adiposity (Sunde et al., 2016). The use

of mouse models with more rigorous experimental design has confirmed an increased risk of cardiometabolic long-term effects such as hypertension and insulin resistance in adult offspring.

The basis for ART environmental effects on embryo potential appear similar to those found from in vivo nutritional studies with evidence of epigenetic disturbance as an early mechanism. ART children have increased risk of rare disorders in imprinted genes which regulate gestational growth. These involve abnormal DNA methylation and such effects can be identified in human embryos. Animal models also show embryo culture causes loss of the normal mono-allelic expression of imprinted genes in embryos and subsequent placental and fetal tissues after transfer. Epigenetic disturbance of imprinted genes also appears causal in “large offspring syndrome,” a condition identified following domestic animal embryo culture in serum-containing medium before transfer to recipients and the occurrence of abnormal excess perinatal weight.

Embryo culture may initiate compromised development because of the absence of natural maternal factors that may occupy the maternal reproductive tract in vivo (see [Introduction](#)). Thus, the cytokine CSF2, shown to have a stimulatory effect on preimplantation survival and morphogenesis, also provides protection for adverse long-term developmental programming when included as a supplement in embryo culture before transfer in mice and sheep. Inclusion of CSF2 to mouse culture medium before transfer protected against abnormal placental structure and fetal growth which occurred in its absence ([Robertson et al., 2015](#)).

Other Models of Environmental Impact on Preimplantation Development

Environmental conditions inducing early embryo programming appear to be broad. For example, maternal systemic inflammation and sickness by endotoxin treatment around the time of conception, inducing a transient pro-inflammatory environment, altered blastocyst cell numbers in mice but, unlike the nutritional models discussed above, had no clear effects on offspring cardiometabolic health. In contrast, the maternal inflammatory condition induced altered programming of adult offspring innate immunity and responsiveness to administered endotoxin such that the immune response was blunted ([Williams et al., 2011](#)). This suggests the preimplantation inflammatory environment had programmed increased tolerance in offspring, perhaps a compensatory mechanism suited for a predicted pathogen-rich environment.

In other studies, a paternal influence on embryo environmental programming has been identified mediated by the composition of seminal plasma. The plasma has traditionally been viewed as a transient nutritious and stabilizing environment for sperm during mating but recent research has demonstrated that it acts within the maternal tract to stimulate both the mother’s immunological response to maintain pregnancy and, either directly or indirectly, the embryo itself, affecting placental phenotype and postnatal offspring metabolism and cardiovascular health ([Bromfield et al., 2014](#)). This paternal environmental influence via seminal plasma on embryo developmental programming adds to the growing body of knowledge that paternal physiology and lifestyle (diet, BMI, obesity) can affect not only his fertility but the development and health of his offspring ([Lane et al., 2015](#); [Watkins et al., 2017](#)).

Conclusion

We have seen that the environment of the early embryo has critical roles in developmental potential, not only facilitating early morphogenesis but also modifying placental, fetal, and postnatal phenotype with important health implications. This argues for exceptional care to be taken in protecting that environment from adverse conditions, a time when the pregnancy will not be known. For example, there is a case for preconception preparation for pregnancy for both partners in terms of weight, physiological state and wellbeing ([Barker et al., 2013](#)). ART practice needs to recognize this phenomenon both in patient condition and also the safety of embryo culture and manipulation conditions. The same is true for agricultural applications using reproductive treatments. We are still in our infancy in terms of understanding the mechanisms by which embryo environment can have such a profound effect across the lifespan and so more research is warranted.

Acknowledgments

The authors are grateful for financial support in their laboratories for research associated with this article, including from the BBSRC (BB/1001840/1, BB/F007450/1), the EU (FP7-CP-FP EpiHealth, 278418), the Rosetrees Trust (M518), and the Gerald Kerkut Trust to TPF; BBSRC (BB/R003556/1), an Aston Research Centre for Healthy Aging fellowship and the Society for Reproduction and Fertility to Adam J. Watkins.

References

- Barker, D. J., & Thornburg, K. L. (2013). The obstetric origins of health for a lifetime. *Clinical Obstetrics and Gynecology*, 56, 511–519.
- Barker, D., Barker, M., Fleming, T., & Lampl, M. (2013). Developmental biology: Support mothers to secure future public health. *Nature*, 504, 209–211.
- Bedzhov, I., Graham, S. J., Leung, C. Y., & Zernicka-Goetz, M. (2014). Developmental plasticity, cell fate specification and morphogenesis in the early mouse embryo. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 369.
- Bromfield, J. J., Schjenken, J. E., Chin, P. Y., Care, A. S., Jasper, M. J., & Robertson, S. A. (2014). Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 2200–2205.

- Denisenko, O., Lucas, E. S., Sun, C., Watkins, A. J., Mar, D., Bomsztyk, K., & Fleming, T. P. (2016). Regulation of ribosomal RNA expression across the lifespan is fine-tuned by maternal diet before implantation. *Biochimica et Biophysica Acta*, 1859, 906–913.
- Dominguez-Salas, P., Moore, S. E., Baker, M. S., Bergen, A. W., Cox, S. E., Dyer, R. A., Fulford, A. J., Guan, Y., Laritsky, E., Silver, M. J., Swan, G. E., Zeisel, S. H., Innis, S. M., Waterland, R. A., Prentice, A. M., & Hennig, B. J. (2014). Maternal nutrition at conception modulates DNA methylation of human metastable epialleles. *Nature Communications*, 5, 3746.
- Eckert, J. J., Porter, R., Watkins, A. J., Burt, E., Brooks, S., Leese, H. J., Humpherson, P. G., Cameron, I. T., & Fleming, T. P. (2012). Metabolic induction and early responses of mouse blastocyst developmental programming following maternal low protein diet affecting life-long health. *PLoS One*, 7, e52791.
- Hardy, K., & Spanos, S. (2002). Growth factor expression and function in the human and mouse preimplantation embryo. *The Journal of Endocrinology*, 172, 221–236.
- Kleijkers, S. H., Mantikou, E., Slappendel, E., Consten, D., van Echten-Arends, J., Wetzels, A. M., van Wely, M., Smits, L. J., van Montfoort, A. P., Repping, S., Dumoulin, J. C., & Mastenbroek, S. (2016). Influence of embryo culture medium (G5 and HTF) on pregnancy and perinatal outcome after IVF: A multicenter RCT. *Human Reproduction*, 31, 2219–2230.
- Lane, M., Zander-Fox, D. L., Robker, R. L., & McPherson, N. O. (2015). Peri-conception parental obesity, reproductive health, and transgenerational impacts. *Trends in Endocrinology and Metabolism*, 26, 84–90.
- Messerschmidt, D. M., Knowles, B. B., & Solter, D. (2014). DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes & Development*, 28, 812–828.
- Robertson, S. A., Chin, P. Y., Schjenken, J. E., & Thompson, J. G. (2015). Female tract cytokines and developmental programming in embryos. *Advances in Experimental Medicine and Biology*, 843, 173–213.
- Robker, R. L., Akison, L. K., Bennett, B. D., Thrupp, P. N., Chura, L. R., Russell, D. L., Lane, M., & Norman, R. J. (2009). Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. *The Journal of Clinical Endocrinology and Metabolism*, 94, 1533–1540.
- Roseboom, T. J., Painter, R. C., van Abeelen, A. F., Veenendaal, M. V., & de Rooij, S. R. (2011). Hungry in the womb: What are the consequences? Lessons from the Dutch famine. *Maturitas*, 70, 141–145.
- Sinclair, K. D., Allegrucci, C., Singh, R., Gardner, D. S., Sebastian, S., Bispham, J., Thurston, A., Huntley, J. F., Rees, W. D., Maloney, C. A., Lea, R. G., Craigon, J., McEvoy, T. G., & Young, L. E. (2007). DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. *Proceedings of the National Academy of Sciences of the United States of America*, 104, 19351–19356.
- Sun, C., Velazquez, M. A., Marfy-Smith, S., Sheth, B., Cox, A., Johnston, D. A., Smyth, N., & Fleming, T. P. (2014). Mouse early extra-embryonic lineages activate compensatory endocytosis in response to poor maternal nutrition. *Development*, 141, 1140–1150.
- Sunde, A., Brison, D., Dumoulin, J., Harper, J., Lundin, K., Magli, M. C., Van den Abbeel, E., & Veiga, A. (2016). Time to take human embryo culture seriously. *Human Reproduction*, 31, 2174–2182.
- Watkins, A. J., Ursell, E., Panton, R., Papenbrock, T., Hollis, L., Cunningham, C., Wilkins, A., Perry, V. H., Sheth, B., Kwong, W. Y., Eckert, J. J., Wild, A. E., Hanson, M. A., Osmond, C., & Fleming, T. P. (2008). Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. *Biology of Reproduction*, 78, 299–306.
- Watkins, A. J., Sirovica, S., Stokes, B., Isaacs, M., Addison, O., & Martin, R. A. (2017). Paternal low protein diet programs preimplantation embryo gene expression, fetal growth and skeletal development in mice. *Biochimica et Biophysica Acta*.
- Williams, C. L., Teeling, J. L., Perry, V. H., & Fleming, T. P. (2011). Mouse maternal systemic inflammation at the zygote stage causes blunted cytokine responsiveness in lipopolysaccharide-challenged adult offspring. *BMC Biology*, 9, 49.

Content and Volume Overview

Carlos Simón, Department of Obstetrics and Gynecology, Valencia University, Valencia, Spain

Craig Niederberger, Department of Urology, University of Illinois at Chicago, College of Medicine, Chicago, Illinois, USA

© 2018 Elsevier Inc. All rights reserved.

Reproductive medicine has evolved rapidly over the last decades, and evaluation and treatment of the female and male have transformed apace. Reproductive medicine in the female in Volume 4 starts with the evaluation of the female in the infertile couple. The diagnostic work-up includes the clinical aspects and the investigation of reproductive endocrinology, reproductive genetics, reproductive tract microbiota, coagulation, as well as the visuals such as imaging and reproductive endoscopy. Then, the most prevalent medical conditions that affect reproductive medicine are considered ranging from endocrine disorders to congenital, infectious, or benign tumors. Oncofertility is the next section that deals with the reproductive treatment and expectations in women suffering malignant tumors of the reproductive tract. Then, medical and surgical treatments of the indicated medical conditions are presented to the reader. Finally, the psychological aspects of the female reproductive medicine as well as legal and ethical aspects are considered.

Reproductive medicine in the male in Volume 4 begins with the evaluation of the male with presumed reproductive dysfunction and includes topics ranging from interrogating the biological system to the organs that are directly involved in reproduction. The volume continues with the effects of the environment on male reproductive potential and proceeds to a thorough treatment of the genetic basis for reproductive diagnosis and therapy in the male. The succeeding sections in the volume present medical conditions that affect reproductive potential and then focuses on therapy, first medical and then surgical. Finally, critical issues in mental health, ethics, and the law are addressed, and the male portion of the volume concludes with benign and malignant diseases affecting male fertility.

EVALUATION FEMALE FERTILITY

Clinical Evaluation of the Subfertile Female

Gemma Castellón*, IVI, Barcelona, Spain

Nuria Muñiz*, IVI, Lleida, Spain

Carlos Simón, IGENOMIX, Valencia, Spain; and University of Valencia/INCLIVA, Valencia, Spain

Joaquín Calaf-Alsina, Autònoma University of Barcelona, Barcelona, Spain

© 2018 Elsevier Inc. All rights reserved.

In fertile couples, the monthly gestation rate is between 20% and 25%, reaching a rate of 84% in the first year of sexual relations. These rates are affected by several prognostic factors, the most important being the age of the woman ([Remohí et al., 2017](#)). Fertility in women is reduced by half at age 35, and declines more quickly after 40 years. In men, the decline begins at age 35, and decreases by 23% per year ([Remohí et al., 2017](#)). An estimated 15%–30% of couples will have fertility problems ([Gelbaya et al., 2014](#)).

Infertility is the inability of a couple to conceive after 1 year of unprotected sex. To enable such couples to produce offspring, a basic study of sterility is recommended in both partners after this time period. Yet, for some couples an earlier infertility study can be beneficial. Earlier initiation of the workup is recommended for women over 35; recurrent pregnancy loss; female antecedents of pelvic surgery or pelvic infectious disease; women with irregular menstrual cycles, amenorrhea, or oligomenorrhea; couples with genetic diseases; or a suspicion of male subfertility (history of surgery or urogenital pathology, history of sexually transmitted diseases) ([Remohí et al., 2017](#)). It is important to differentiate primary infertility (couples without previous offspring) from secondary infertility (couples that have managed to conceive in the past) since secondary infertility typically has a better reproductive prognosis ([Remohí et al., 2017](#)). We define recurrent pregnancy loss as the occurrence of two or more failed pregnancies ([ASRM, 2008a](#)).

Basic Study of the Infertile Woman

It is advisable to study both partners simultaneously, but in this article we will focus on the basic study of infertility in women.

Clinical History and Basic Exploration

The following information should be collected in the first visit with the patient to identify symptoms that explain the infertility or assess the availability of inheritable and/or prognostic factors to guide the selection of additional tests ([ASRM, 2015b](#))

- *Personal history*: Age, allergies, age of menarche, age of onset of development of secondary sexual characteristics, menstrual pattern, history of contraceptive methods, history of pathologies, previous surgeries, history of sexually transmitted diseases, hormone-dependent diseases (thyroid diseases, hirsutism), autoimmune diseases, symptoms related to endometriosis (dyschezia, dysmenorrhea, intercourse pain) and consumption or exposure to toxic drugs.
- *Family history*: Early menopause, genetic or chromosomal diseases, and reproductive problems.
- *Reproductive history*: Length of infertility or recurrent pregnancy loss, primary or secondary infertility, history of reproductive treatments, history of previous pregnancies, and coital frequency.
- *Physical examination*: Body mass index (BMI), blood pressure, secondary sexual characteristics, vaginal and cervical examination, and phenotypic characteristics.
- *Transvaginal ultrasound*: Uterine, annexial, and pelvic assessment.
- Karyotyping.

Principal Causes of Infertility in Women and Additional Tests

The study of infertility should be oriented to be as cost-efficient as possible while avoiding a delay in diagnosis and treatment. In addition, to enable selection of appropriate diagnostic tests, the following should be considered: patient preferences, reproductive age, length of infertility, primary or secondary infertility, clinical history, and physical examination findings ([Table 1](#)).

*The authors contributed equally.

Table 1 Principal causes of infertility in women and appropriate diagnostic tests

<i>Principal causes of infertility in women</i>	<i>Diagnostic tests</i>
Ovulatory function and ovarian reserve	• Clinical signs of abnormal menstrual cycle. Vaginal-Ultrasound. Plasma progesterone levels (between 21st and 22nd day of cycle), urinary luteinizing hormone (LH) near the middle of the menstrual cycle. • Basal FSH, anti-Müllerian hormone (AMH), antral follicle count (AFC)
Assessment of tubal factor	• Hysterosalpingography (HSG), hysterosonosalingoscopy (HSSG), anti-<i>Chlamydia trachomatis</i> antibodies, laparoscopy (LPS)
Study of the cervical canal and uterine cavity	• Three-dimensional ultrasound, hysteroscopy (HSC)

Evaluation of Ovulatory Function

Ovulatory dysfunction is present in 15% of couples, but affects up to 40% of sterile couples. The most frequent clinical signs associated with ovulatory dysfunction are amenorrhea and oligomenorrhea. According to the World Health Organization (WHO), ovulatory dysfunction can be classified into seven groups (Table 2). Ovulatory dysfunction can also result from endocrine disorders (Cushing's syndrome, obesity, congenital adrenal hyperplasia, etc.) or certain medications (neuroleptics, antidepressants, antihypertensives, etc.).

Approximately 97% of women who have regular menstrual cycles (between 26 and 35 days) ovulate spontaneously. In these patients it is generally not necessary to perform diagnostic tests to confirm ovulation. A normal menstrual cycle has a peak of LH hormone between days 12 and 14 (considering day 1 as the day when menstruation starts) and the dominant follicle is ovulated 2–36 h later. The corpus luteum develops and begins to produce progesterone, the hormone needed for endometrial preparation for implantation. Ovulation is typically assessed by assaying plasma progesterone levels in the middle of the luteal phase or urinary LH near the middle of the menstrual cycle, or by transvaginal ultrasonographic visualization of the corpus luteum in the ovary. Plasma progesterone, determined between days 21 and 22 of the cycle, suggests ovulation has occurred if the levels are above 10 pg/mL. In contrast, LH levels typically peak in the urine between 24 and 36 h before ovulation. Importantly, these tests imply ovulation has occurred in the tested cycle but cannot be extrapolated to consecutive cycles (ASRM, 2015b).

Evaluation of the Ovarian Reserve

The ovarian reserve reflects the functional capacity of the ovary, that is, the number of follicles available at a given time (Remohí et al., 2017). As a woman ages, the quantity and quality of the gametes decreases and, consequently, so does the reproductive prognosis (Remohí et al., 2017). Ovarian aging usually results in objective endocrine and ultrasound changes.

Among endocrine markers, the most commonly tested are the basal level of follicle stimulating hormone (FSH) and the level of anti-Müllerian hormone (AMH), both of which are involved in folliculogenesis (ASRM, 2015a).

Basal FSH basal

Levels of FSH between the 2nd and 4th days of the cycle are used to assess ovarian reserve (Remohí et al., 2017). Levels > 10–12 IU/L are indicative of low reserve; however, instances in which normal levels of FSH occur along with estradiol levels above 60 pg/mL are also indicative of low reserve (Remohí et al., 2017). FSH levels are not stable over time, but they tend to present significant inter- and intra-cycle oscillations (Bungum et al., 2011).

Table 2 Classification of amenorrhea according to the WHO, 1970

<i>Category</i>	<i>Origin of the problem</i>	<i>Diseases</i>
GROUP I	Hypothalamus–pituitary axis failure	Anorexia nervosa, Kallman syndrome
GROUP II	Hypothalamus–pituitary axis dysfunction	Polycystic ovary syndrome (PCOS), hypothyroidism
GROUP III	Ovarian failure	Early ovarian failure
GROUP IV	Genital tract malformations or alterations	Rokitansky-Küster-Hauser syndrome, Asherman syndrome
GROUP V	Prolactinomas	Prolactin pituitary tumor
GROUP VI	Functional hyperprolactinemia	Maternal lactation
GROUP VII	Hypothalamus–pituitary tumors, non-prolactin secreting	Pituitary adenomas

The measurement of FSH has low sensitivity (10%–30%) for the diagnosis of low ovarian response (ASRM, 2015a). Indeed, it is common to detect normal FSH levels in patients who have low AMH levels and/or low antral follicle count (AFC). This finding reflects the fact that an increase in FSH levels is not evident until the ovarian reserve is markedly compromised; in contrast, AMH and AFC tend to decrease in earlier stages of ovarian reserve impairment. A prospective study that determined the live birth rate (LBR) in young subfertile patients (mean age 35 years) assessed correlations with elevated FSH and AMH levels, concluded that reproductive prognosis was directly related to AMH levels (> 1 ng/dL) but not FSH levels (> 12 IU/L). Despite elevated FSH levels in some patients, 67% achieved a pregnancy, of which 37% were spontaneous (Yarde et al., 2013). Thus, we conclude that measuring FSH offers a less reliable assessment of ovarian reserve in comparison with other markers, and its predictive capacity for pregnancy prognosis is low.

Anti-Müllerian hormone

AMH is produced and secreted into the bloodstream by the granulosa cells of the preantral and small antral follicles (4–8 mm) and it is responsible for the control and development of the ovarian follicles (Remohí et al., 2017). Currently, two analytical techniques are used to assess AMH levels: Elecsys® assay and Access® AMH Assay. A recent study compared the results of AMH levels obtained using each technique. The Access® technique produced AMH values that were 5%–15% higher than those measured by the Elecsys® technique, especially in patients with high AMH (Iliodromiti et al., 2017).

AMH concentration in blood has been related to the ovarian response to exogenous stimulation. When Elecsys® is used we consider levels between 0.7 and 1.3 ng/mL as predictive of poor response; levels above 3.7–4.6 ng/mL are indicative of hyperresponsiveness (Remohí et al., 2017) while with Access® the values are categorized as low (< 5 pmol/L), normal (5–15 pmol/L) and high (> 15 pmol/L).

There is no universal consensus on the pre-analytical conditions for AMH reference values. The first reports on AMH indicated that its levels were more stable than the levels of FSH, and that it could be measured at any time during the menstrual cycle. Yet, studies emerged to show that AMH levels change under certain circumstances, such as oral contraceptive use or administration of GnRH analogues (Bungum et al., 2016). Further, AMH levels can oscillate over phases of the menstrual cycle and between different cycles. A prospective study indicated that AMH levels increase in the late follicular phase (4–5 days before ovulation), and more markedly so after age 30 (Lambert-Messerlian et al., 2015).

Circadian variations have also been observed, with very low levels detected between 4:00 and 6:00 in the morning relative to the levels at 8:00 in the morning (Bungum et al., 2011). In a retrospective study that evaluated inter-cycle changes in AMH levels in the same individual, a variability of 20% was observed, highlighting the intra-individual variation, as opposed to the well-known variability linked to the analytical technique. The study also noted that 29% of cases had changes in their classification of ovarian reserve because of AMH variation (Bungum et al., 2016). These findings underscore the need for caution when giving reproductive counseling that considers only AMH levels.

The relationship of AMH values with reproductive prognosis is also controversial. A recent study found no statistically significant differences between the pregnancy rate in women without problems of fertility and their AMH levels (regardless of age) (Steiner et al., 2017). In contrast, another study observed that the reproductive prognosis in different age groups improves with AMH levels (Tarasconi et al., 2017). However, the same study found an inverse correlation between AMH levels and rates of spontaneous abortion. The authors hypothesized that low levels of AMH are a consequence of deficient production by defective follicles, giving rise to oocytes of poor quality. Thus, it is unclear whether AMH levels can be used to predict reproductive success (Steiner et al., 2017).

Antral follicle count

Although AFC is not the only ultrasound marker used to assess ovarian reserve, it has the best clinical predictive value (Remohí et al., 2017). Using a transvaginal two-dimensional (2D) ultrasound, usually in the early follicular phase (3rd to 5th day of the cycle), all follicles between 2 and 10 mm are counted in both ovaries. An AFC lower than 6–7 follicles is predictive for low ovarian response but does not predict reproductive prognosis. Three-dimensional ultrasound and volume calculations offer less inter-observer variability but do not improve clinical outcomes (Remohí et al., 2017). Further, the ovarian volume can be evaluated; values < 3 mL are related to low responses, while volumes > 6 –8 mL are associated with a hyperresponse (Remohí et al., 2017). Ovarian vascular flow, and specifically its absence, has also been described as a predictor of low response (Remohí et al., 2017).

In a prospective study comparing ovarian reserve markers, AMH and AFC were able to predict low ovarian response with high accuracy and independently of each other (Jayaprakasan et al., 2010). However, a recent randomized study concluded that AMH is not better than AFC for predicting ovarian response. In one study group, the dose of recombinant FSH (rFSH) was determined by the levels of AMH; this group contained more cases of low response (< 5 ovules obtained) than the group where the rFSH dose was calculated according to the AFC ($p < 0.001$) (Magnusson et al., 2017).

Assessment of Tubal Factor

The fallopian tubes are critical to the uptake and transport of gametes and embryo, sperm capacitation and fertilization process. These processes require an intact anatomical and functional structure. Tubal damage can be usually caused by an infection, endometriosis, or previous pelvic surgery, and tubal factor is a contributor in 14% of cases of sterility. Several tests are available for assessing the tubal factor.

Hysterosalpingography and hysterosonoscopy

HSG involves injecting radiopaque contrast through the cervix and following its passage through the tubes by radiography (Remohí et al., 2017). Compared to laparoscopy, HSG is simple, cost-effective, has minimal complications and does not require anesthesia. This technique has a sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 88%, 87%, 71%, and 94%, respectively (Luciano et al., 2013).

HSSG has a basis similar to HSG—retrograde injection of a liquid through the cervix and assessment of its passage through the tubes—but uses ultrasound rather than radiology. The first reports on HSSG highlighted limitations regarding the type of medium used (air with saline solution), which hindered the examination of the tubes and required that the technique only be performed by expert hands. The advent of new ecovisible media (gel or foam of hydroxyethylcellulose and glycerol) has increased the sensitivity and specificity of HSSG (75%–96% vs. 67%–100%, respectively) (Luciano et al., 2013). Further, the application of 4D technology to HSSG allows a 3D assessment in real or delayed time of the morphology of the uterine cavity and tubes and the presence of pelvic adhesions, thereby facilitating clinical interpretation. With the incorporation of this technology, the sensitivity, specificity, PPV, and NPV of the test are 93.8%, 92.2%, 90.5% and 82.1%, respectively (Wang et al., 2017).

Both scans have a risk of infection around 1%, which can be prevented with the administration orally of 1 g of azithromycin 12 h before the test. Although both techniques can be uncomfortable for the patient, HSG is usually more painful due to the peritoneal irritation produced by the contrast medium, and sometimes, the patient should require subsequent analgesic treatment.

Anti-*Chlamydia trachomatis* antibodies

The Bacterium *Chlamydia trachomatis* (CT) is mandatory intracellular and responsible for the majority of pelvic inflammatory diseases of bacterial origin worldwide. The overall prevalence in Europe is about 30% and the most new cases were diagnosed in patients between 15 and 49 years of age (WHO, 2016). CT usually produces an asymptomatic or subclinical infection, that if left untreated can lead to severe lesions in the tubal anatomy and physiology. Currently there are five methods for the confirmation of an active CT infection (Remohí et al., 2017), but the NAAT Test (nucleic acid amplification test) is considered by the WHO (2016), as the best diagnosis test because it is the most sensitive and specific than the others (93% and 99%, respectively). It is characterized by the rapid sequencing of DNA (by PCR) of the *Omp1* gene, responsible for the expression of the transmembrane antigen protein of *Chlamydia* (MOMP) (Chen et al., 2017a). This can be applied to samples from different locations (cervix, urine, vagina, urethra) and with good results (Remohí et al., 2017). The measurement of serum concentrations of anti-*Chlamydia* antibodies (IgG or IgA), is other test that is used to confirm an active CT infections, but in comparison with the NAAT test, its have a similar sensitivity, ($S = 95\%$); but its specificity is lower ($E = 43\%$ – 81%) (Sahi et al., 2017). On the other hand, we must emphasize that seroconversion of IgG and IgA by a CT infection usually lasts for years, even if the patient has been treated with an antibiotic and no longer has the infection (Taheri Beni et al., 2012). Therefore, its determination will help us to identify an active CT infection, but it will also inform us if the patient has been previously infected, arriving to provide a prognostic value on the current tubal status.

In a non-randomized retrospective study (Den Hartog et al., 2008), serological results were compared with the results of the HSG. In seropositive cases the rate of pathological HSG was 53%–60%, in comparison to 14% among seronegative patients.

Another study (Geisler et al., 2012) evaluated the S and E of a new diagnostic test (elementary body-based enzyme-linked immunosorbent assay, EB-ELISA) compared to other available tests (OmpA: major outer membrane protein peptide-based *C. trachomatis*-IgG and IgA ELISA and Medac Assay). Among antibodies most frequently positive after *Chlamydia* infection, the IgG subtypes IgG1 and IgG3 were the most predominant and stable in time; for IgA, only IgA1 showed a significant increase with respect to its other subtype (IgA2). The levels of these subtypes (IgG1, IgG3, and IgA1) were compared in patients with confirmed *Chlamydia* infection by nucleic acid amplification test (NAAT) at 0 and 6 months. In the study patients (NAAT-positive), 100% of the patients were positive for IgG1 and IgG3, whereas only 74% of patients were positive for IgA1. After 6 months the serological study was repeated; 100% of patients seropositive initially for IgG1 and IgG3 continued to be positive, while only 61% of the patients remained positive for IgA1. Thus, IgG1 and 3 are more stable over time than IgA (IgA1) and should be the preferred markers.

Importantly, patients seropositive for IgG1 or IgG3 for *Chlamydia* were shown to have a pregnancy rate lower than the control group (Steiner et al., 2015). In particular, a positive IgG3 test was associated with a decrease in the pregnancy rate from 38% to 26% ($p < 0.001$) and an increase in the risk of ectopic pregnancy from 4% to 11% ($p = 0.02$). However, despite these results, anti-*Chlamydia trachomatis* antibody testing has a limited clinical application and can be considered in questionable HSG, or as a screening for tubal injury.

Laparoscopy (LPS)

LPS is considered the “gold standard” in the diagnosis and treatment of tubal pathologies. This approach allows direct visualization of tubal morphology and can detect the presence of adhesions that may affect tubal flow. Incorporating the retrograde instillation of methylene blue (chromoperturbation technique) enables assessment of tubal permeability. However, LPS should not be considered as the first-line diagnostic approach, except in cases with suspicion of a pelvic (endometriosis) or tubal pathology (hydrosalpinx or sactosalpinx) that can be treated after diagnosis. Indeed, LPS is a costly, invasive technique that requires anesthesia and has a complication rate of 2%–3% (Gelbaya et al., 2014). Thus, its recommendation should be considered in light of other options.

Falloscopy, salpingoscopy, and fertiloscopy

The techniques of choice for tubal study only offer information on the permeability of the fallopian tubes, and do not guarantee that the inside of the tube is competent (Remohí et al., 2017). Falloscopy (transcervical tubal catheterization) consists of the

introduction of an endoscope through the tubal ostium, to assess its interior and repair the proximal blockage of the tubes to achieve pregnancy spontaneously or facilitate IUI. This is an invasive test that carries complications such as tubal perforation, and performing this test does not promote a pregnancy rate higher than that for IVF. In a recent meta-analysis, the rate of pregnancy after falloscopy was about 27% over the succeeding 6 months, but after that time the pregnancy rate decreased (De Silva et al., 2017). In contrast, the gestation rate with an IVF cycle would be around 35%.

Salpingoscopy (laparoscopic tubal catheterization) encompasses endoscopic study of the distal portion of the tube. It is a costly and invasive technique that requires performing a laparoscopy. In a retrospective study that compared the gestation rate with programmed intercourse or intrauterine insemination (IUI) according to the degree of tubal injury assessed by salpingoscopy, gestation rate was inversely related to the degree of injury in the tubal lumen ($p < 0.05$). In addition, the degree of injury was directly related to the presence of antibodies against *Chlamydia* (Nakagawa et al., 2010).

Fertiloscopy is similar to salpingoscopy but uses vaginal hydrolaparoscopy rather than abdominal laparoscopy. It is based on the creation of a hydroperitoneum through the vaginal cul-de-sac and introduces an optic to see the structures. This procedure does not require general anesthesia and can be performed on an outpatient basis. It has a complication rate of 3%, of which less than 1% are due to intestinal perforation (Wang et al., 2017).

Study of the Cervical Canal and Uterine Cavity

The uterine factor is usually present in 10% of cases of sterility. Myomas, endometrial polyps, and Müllerian malformations are some of the more common disorders that we observe in infertile women or those with recurrent pregnancy loss.

3D ultrasound

The introduction of 3D ultrasound into gynecological consultations has yielded improvement in the diagnosis of Müllerian malformation (Grimbizis et al., 2013) of the uterine cavity. With 3D echo we are able to visualize the coronal uterine plane, which aids the diagnosis of these malformations. The gold standard for diagnosis is magnetic resonance imaging (MRI), but 3D ultrasound presents high-quality images with a sensitivity, specificity, PPV, and NPV of 83%, 100%, 89%, 100%, respectively, comparable to MRI (Saravolos et al., 2016a).

3D ultrasound also enables calculations of endometrial volume, assessment of uterine vascularization, or assessment of the severity of adenomyosis (penetration and invasion by the glands and endometrial stroma within the myometrium). While attempts have been made to establish a relationship between endometrial volume and the pregnancy rate by embryo transfer, the results are contradictory. Some reports suggest that volumes less than 2 mL or greater than 8 mL are related to worse pregnancy outcomes, but other studies do not confirm these results. In fact, when the endometrial thickness is greater than 6.5 mm in the proliferative phase, the endometrial volume measured through virtual organ computer-aided analysis (VOCAL) does not show differences in the rate of evolutionary gestation in oocyte donation cycles, although those patients with very reduced endometrial volume seem to have worse reproductive results (Labarta et al., 2017). Studies of uterine vascularization (assessed by 3D ultrasound) in relation to pregnancy rate are inconclusive (Chen et al., 2017b). In the case of adenomyosis, there is increasing evidence of its negative effect during embryo implantation and perinatal outcomes (Puente et al., 2016).

Hysteroscopy (HSC)

HSC is the technique of choice for the study and treatment of pathologies or alterations of the uterine cavity, due to its high sensitivity and specificity. These alterations are related to worse reproductive prognosis. In a prospective study, women with Müllerian malformations (diagnosed by 3D ultrasound), with the exception of the arcuate uterus, had higher rates of abortion than patients with normal ultrasound (49.3% vs. 12.7%, respectively ($p = 0.05$)). In contrast, no differences were observed in pregnancy rates (Jayaprakasan et al., 2011). In a prospective study where the gestation rate was compared between a group with hysteroscopic myomectomy versus a conservative behavior, the gestation rate increased in the myomectomy group from 27% to 43% ($p < 0.05$) (Casini et al., 2006). In a Cochrane review, the observed improvement in the gestation rate was not statistically significant, although in the case of hysteroscopic endometrial polypectomy, was observed a statistically significant increase in the gestation rate ($p < 0.0001$) (Bosteels et al., 2013). Hysteroscopic treatment of intrauterine synechiae has also been associated to a significant decrease in the rate of abortion, implantation failure and ectopic pregnancy, but increase in the rate of ongoing gestation (Cholkeri-Singh and Sasaki, 2015).

An HSC would be indicated before embryo transfer in the case of diagnosing a refractory endometrium or insufficient thickness by ultrasound. Endometrial thickness should be evaluated by ultrasound before embryo transfer since an endometrium < 7 mm is associated with worse gestational outcomes (Garcia-Velasco et al., 2016). Among the causes of this alteration would be uterine surgery (dilation and curettage, myomectomies, etc.), as one of the main causes for the formation of endometrial adhesions, with Asherman's syndrome being the most serious form. Radiotherapy, Müllerian malformations, or endometritis can also cause insufficient endometrial growth (Garcia-Velasco et al., 2016). Endometritis is characterized by the presence of an endometrial inflammation that can be caused by an infection (mostly), the presence of a foreign body, radiotherapy, or the presence of adenomyosis. It is usually expressed as focal or diffuse hyperemia at the endometrial level, as visualized by HSC (Garcia-Velasco et al., 2016). In some studies, endometritis is associated with repeated miscarriages, implantation failures, and poor perinatal outcomes, but in others, no relationship has been observed (Puente et al., 2016).

More controversial is the systematic performance of an HSC before an IVF cycle. Clinical guidelines published by the National Institute for Health and Care Excellence (NICE) and the American Society for Reproductive Medicine (ASRM) (NICE, 2013; ASRM, 2015b) as well as recently published prospective and randomized studies (El-Toukhy et al., 2016; Smit et al., 2016), conclude that the performance of HSC before an IVF cycle in patients with normal ultrasound does not improve the LBR.

Final Considerations

The present review has critically appraised the current approaches to evaluating factors involved in female fertility. The selection of diagnostic techniques must be guided by clinical wisdom. As in any clinical work-up process, the number and order of the examinations should also adapt to the patient's profile and preferences.

The initial interview enables the clinician to identify some "clues" orienting to a priority evaluation of a given factor. Age above 35 makes a thorough evaluation of ovarian reserve mandatory, even with a regular menstrual pattern. Similarly, the pre-existence of any event related to peritoneal health requires tubal evaluation beyond patency tests. Yet, the evaluation strategy should also adapt to the potential treatments that the couple will need and accept. The findings obtained in a "first round" of examinations will also guide the aspects to be evaluated in depth to enable the best chances of reproductive success.

References

- ASRM. (2008a). Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss. Practice. *Fertility and Sterility*, 90, S60.
- ASRM. (2015a). Committee of the American Society for Reproductive Medicine. Testing and interpreting measures of ovarian reserve. *Fertility and Sterility*, 103, e9–e17.
- ASRM. (2015b). Practice Committee of the American Society for Reproductive Medicine. Diagnostic evaluation of the infertile female: A committee opinion. *Fertility and Sterility*, 103(6), e44–50.
- Bosteels, J., Kasius, J., Weyers, S., et al. (2013). Hysteroscopy for treating subfertility associated with suspected major uterine cavity abnormalities. *Cochrane Database of Systematic Reviews*, 1, CD009461.
- Bungum, L., Jacobsson, A.-K., Rose, F., et al. (2011). Circadian variation in concentration of anti-müllerian hormone in regularly menstruating females: Relation to age, gonadotrophin and sex steroid levels. *Human Reproduction*, 26(3), 678–684.
- Bungum, L., Franssohn, F., Bungum, M., et al. (2016). Quantifying the intraindividual variation of antimüllerian hormone in the ovarian cycle. *Fertility and Sterility*, 106(5), 0015–0282.
- Casini, M. L., Rossi, F., Agostini, R., et al. (2006). Effects of the position of fibroids on fertility. *Gynecological Endocrinology*, 22, 106–109.
- Chen, Y., Chen, J., Yang, L., et al. (2017a). Distribution of *Chlamydia trachomatis* genotypes in infective diseases of the female lower genital tract. *Medical Science Monitor*, 23, 4477–4481.
- Chen, X., Saravelos, S. H., Liu, Y., et al. (2017b). Correlation between three-dimensional power doppler and morphometric measurement of endometrial vascularity at the time of embryo implantation in women with unexplained recurrent miscarriage. *Journal of Molecular Histology*, 48(3), 235–242.
- Cholkeri-Singh, A., & Sasaki, K. J. (2015). Hysteroscopy for infertile women: A review. *Journal of Minimally Invasive Gynecology*, 22(3), 353–362.
- De Silva, P. M., Chu, J. J., Gallos, I. D., et al. (2017). Fallopian tube catheterization in the treatment of proximal tubal obstruction: A systematic review and meta-analysis. *Human Reproduction*, 32(4), 836–852.
- Den Hartog, J. E., Lardenoije, C. M. J. G., Severens, J. L., et al. (2008). Screening strategies for tubal factor subfertility. *Human Reproduction*, 23(8), 1840–1848.
- El-Toukhy, T., Campo, R., Khalaf, Y., et al. (2016). Hysteroscopy in recurrent in-vitro fertilisation failure (TROPHY): A multicentre, randomised controlled trial. *Lancet*, 387(10038), 2614–2621. Epub.
- García-Velasco, J. A., Acevedo, B., Alvarez, C., et al. (2016). Strategies to manage refractory endometrium: State of the art. *Reproductive BioMedicine Online*, 32(5), 474–489. <https://doi.org/10.1016/j.rbmo.02.001>.
- Geisler, W. M., Morrison, S. G., Doemland, M. I., et al. (2012). Immunoglobulin-specific responses to chlamydia elementary bodies in individuals with and at risk for genital chlamydial infection. *The Journal of Infectious Diseases*, 206, 1836–1843.
- Gelbaya, T. A., Potdar, N., Yadava, B., et al. (2014). Definition and epidemiology of unexplained infertility. *Obstetrical and Gynecological Survey*, 69(2), 109–115.
- Grimbizis, G. F., Gordts, S., Di Spiezio, S. A., et al. (2013). The ESHRE/ESGE consensus on the classification of female genital tract congenital anomalies. *Human Reproduction*, 28(8), 2032–2044.
- Iliodromiti, S., Salje, B., Dewailly, D., et al. (2017). Non-equivalence of anti-Müllerian hormone automated assays—Clinical implications for use as a companion diagnostic for individualised gonadotrophin dosing. *Human Reproduction*, 1–6.
- Jayaprakasan, K., Campbell, B., Hopkisson, J., Johnson, I., & Raine-Fenning, N. (2010). A prospective, comparative analysis of Antimüllerian hormone, inhibin-B, and three dimensional ultrasound determinants of ovarian reserve in the prediction of poor response to controlled ovarian stimulation. *Fertility and Sterility*, 93, 855–864.
- Jayaprakasan, K., Chan, Y. Y., Sur, S., et al. (2011). Prevalence of uterine anomalies and their impact on early pregnancy in women conceiving after assisted reproduction treatment. *Ultrasound in Obstetrics & Gynecology*, 37(6), 727–732. Epub.
- Labarta, E., Mariani, G., Holtmann, N., et al. (2017). Low serum progesterone on the day of embryo transfer is associated with a diminished ongoing pregnancy rate in oocyte donation cycles after artificial endometrial preparation: A prospective study. *Human Reproduction*, 13, 1–6.
- Lambert-Messerlian, G., Plante, B., Eklund, E., et al. (2015). Levels of antimüllerian hormone in serum during the normal menstrual cycle. *Fertility and Sterility*, 105, 208–213.
- Luciano, D. E., Exacoustos, C., & Luciano, A. A. (2013). *The Journal of Minimally Invasive Gynecology*, 21(6), 994–998, 2330.
- Magnusson, A., Nilsson, L., Olerod, G., et al. (2017). The addition of anti-müllerian hormone in an algorithm for individualized hormone dosage did not improve the prediction of ovarian response—a randomized, controlled trial. *Human Reproduction*, 32(4), 811–819.
- Nakagawa, K., Inoue, M., Nishi, Y., et al. (2010). A new evaluation score that uses salpingoscopy to reflect fallopian tube function in infertile women. *Fertility and Sterility*, 94, 2753–2757.
- NICE. (2013). *Assessment and treatment for people with fertility problems. NICE Clinical Guideline*. London: NICE.
- Puente, J. M., Fabris, A., Patel, J., et al. (2016). Adenomyosis in infertile women: Prevalence and the role of 3D ultrasound as a marker of severity of the disease. *Reproductive Biology and Endocrinology*, 14, 60.
- Remohí, J., Bellver, J., Ferrando, M., Requena, A., & Pellicer, A. (2017). *Manual práctico de esterilidad y reproducción* (5ª edición). Madrid, Spain: Editorial médica Panamericana.
- Sahi, S.-V., Rogozinska, E., Sobhy, S., & Khan, K. S. (2017). Accuracy of tests used to detect infection with *Chlamydia trachomatis* in asymptomatic pregnant women: A systematic review. *Wolters Kluwer Health*, 29, 375–382.

- Saravelos, S. H., Jayaprakasan, K., Ojha, K., & Li, T.-C. (2016a). Assessment of the uterus with three dimensional ultrasound in women undergoing ART. *Human Reproduction Update*, 1–23.
- Smit, J. G., Kasius, J. C., Eijkemans, M. J., et al. (2016). Hysteroscopy before in-vitro fertilisation (inSIGHT): A multicentre, randomised controlled trial. *Lancet*, 387(10038), 2622–2629. Epub.
- Steiner, A. Z., Diamond, M. P., Richard, S., et al. (2015). Chlamydia trachomatis immunoglobulin G3 seropositivity is a predictor of reproductive outcomes in infertile women with patent fallopian tubes. *Fertility and Sterility*, 104, 1522–1526.
- Steiner, A. Z., Pritchard, D., Stanczyk, F. Z., et al. (2017). Association between biomarkers of ovarian reserve and infertility among older women of reproductive age. *Journal of the American Medical Association*, 318(14), 1367–1376.
- Taheri Beni, B., Jenab, A., Roghanian, R., et al. (2012). Genotyping of endocervical chlamydia trachomatis strains and detection of serological markers of acute and chronic inflammation in their host. *International Journal of Fertility & Sterility*, 6(2), 101–106.
- Tarasconi, B., Tadros, T., Ayoubi, J. M., et al. (2017). Serum antimüllerian hormone levels are independently related to miscarriage rates after in vitro fertilization–embryo transfer. *Fertility and Sterility*, 108(3), 5018–5524.
- Wang, W., Zhou, Q., Gong, Y., et al. (2017). Assessment of fallopian tube fimbria patency with 4 dimensional hysterosalpingo-contrast sonography in infertile women. *Journal of Ultrasound in Medicine*, 00(00–00), 0278–4297.
- World Health Organization. (2016). *Guidelines for the treatment of Chlamydia trachomatis*. Geneva: WHO.
- Yarde, F., Voorhuis, M., Dolleman, M., et al. (2013). Antimüllerian hormone as predictor of reproductive outcome in subfertile women with elevated basal follicle-stimulating hormone levels: a follow-up study. *Fertility and Sterility*, 100(3), 831–838.

Further Reading

- ASRM. (2008b). Practice Committee of the American Society for reproductive. Optimizing natural fertility. *Fertility and Sterility*, 90(Suppl), 3.
- Broekmans, F. J., de Ziegler, D., Howles, C. M., et al. (2010). The antral follicle count: Practical recommendations for better standardization. *Fertility and Sterility*, 94(3), 1044–1051.
- Roberts, J. E., Spandorfer, S., Fasouliotis, S. J., et al. (2005). Taking a basal follicle-stimulating hormone history is essential before initiating in vitro fertilization. *Fertility and Sterility*, 83, 37–41.
- Saravelos, S. H., Kong, G. W. S., Chung, J. P. W., et al. (2016b). A prospective randomized controlled trial of 3D versus 2D ultrasound-guided embryo transfer in women undergoing ART treatment. *Human Reproduction*, 31, 1–6.
- Zhang, R., Xu, A., Wang, Q., et al. (2017). Fertiloscopy improves in vitro fertilization for women with repeated implantation failure. *Journal of Gynecology Obstetrics and Human Reproduction*, 43, 743–746, 07.001.

Imaging in Female Reproductive Medicine

Francisco R Baixauli and Diana B Farinos, University of Valencia, Valencia, Spain

Irene Zoffaroli, Hospital Clínico Universitario Valencia, Valencia, Spain

Belen G Gongora, University of Valencia, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

Female reproductive medicine is undoubtedly one of the fields of medicine that has experienced a greater progression in recent years. Therefore, having a precise control of the changes that take place in the female reproductive system in a physiological way or in the course of techniques of assisted reproduction has become an authentic necessity. Nowadays, different diagnostic techniques in the study of female reproductive medicine are available, such as magnetic resonance, tomographic imaging, laparoscopy, hysteroscopy and ultrasound. The anatomical study of the female's internal genital organs by ultrasound has become a standard practice today. Ultrasound allows us to study both the normal anatomy and its pathology, in an innocuous, cheap and well tolerated way. Therefore, reproductive medicine and ultrasound are intimately linked, allowing us, for example, to obtain very realistic images of fetal intrauterine development.

In this article we will show images that undoubtedly illustrate the above mentioned, allowing the reader to understand the numerous applications of ultrasound as well as other diagnostic imaging techniques in reproductive medicine.

Normal Menstrual Cycle

Female menstrual cycle lasts 28 days with ovulation typically occurring on day 14. After ovulation the corpus luteum develops in the ovary and produces progesterone, this environment allows the endometrium to enter in a secretory phase and to become receptive to the implantation of an embryo. This stage is known as "implantation window" (phase in which the endometrium is receptive to nest a gestation).

Transvaginal ultrasound provides a precise approach of uterine and ovarian size and morphology scrutiny, as well as follicular and endometrial development during the menstrual cycle. Pelvic ultrasounds are performed using both the transabdominal and transvaginal approaches. However, transvaginal ultrasound customarily provides more accurate images of female pelvic organs, since the ultrasound probe lies nearer to these organs. Transvaginal ultrasound also easily depicts changes in the morphological appearance of both the ovary and endometrium due to periodic fluctuations in female hormones secretion (FSH and LH).

In the first days of the cycle, FSH levels are elevated in order to incite follicle development. During this cycle period all the follicles in the ovary measure less than 10 mm and are known as antral follicles. On the other hand, the immediate postmenstrual uterine endometrium is thin. With cycle progression, a dominant follicle is selected to be ovulated while the others undergo atresia. The dominant follicle has a progressive growth, reaching a diameter of 18–27 at the moment of ovulation. Under the influence of increasing estradiol levels, endometrial proliferation appears, this is depicted as a thickening of the endometrium (the so-called trilaminar endometrium).

After ovulation the follicle is transformed in corpus luteum, characterized by a rich vascularization. The post-operative endometrium, under the stimulus of progesterone secreted by corpus luteum, is represented by the loss of the trilaminar appearance and the development of a uniform band (Fig. 1).

Gynecological Pathologies

The different imaging techniques employed in the study of gynecological disease allow us to obtain accurate images of numerous benign and malignant pathologies. Next we will perform a vision of the most relevant pathologies in the different parts of the female anatomy.

- *The uterus.* Uterine fibroid or myoma is the most common benign tumor of the female genital tract. The prevalence of myomas peaks during the fourth decade. The symptomatology of this uterine tumor is related both to its size and location. It appears as a solid myometrium-dependent tumor. Ultrasounds give us the opportunity to diagnose, classify (intramural, subserosal and submucosal) and control their evolution (tumor growth) in an easy way. Magnetic resonance imaging is a precise method to define the size, number and location of the myomas in selected complex cases (Fig. 2).

Congenital anomalies of the female tract usually involve the uterus and the cervix. The diagnosis and precise classification of these congenital uterine anomalies is clinically relevant because of the high associated risk of infertility, endometriosis, and miscarriage. Imaging plays an essential role in its identification and treatment planning. Currently, magnetic resonance and 3D-ultrasound imaging are the preferred means of evaluation due to its ability to simultaneously assess the uterine fundus and cavity. Therefore, by assessing the internal and external contours, 3D-ultrasound and magnetic resonance are able to discriminate between different congenital uterine anomalies (Fig. 3).

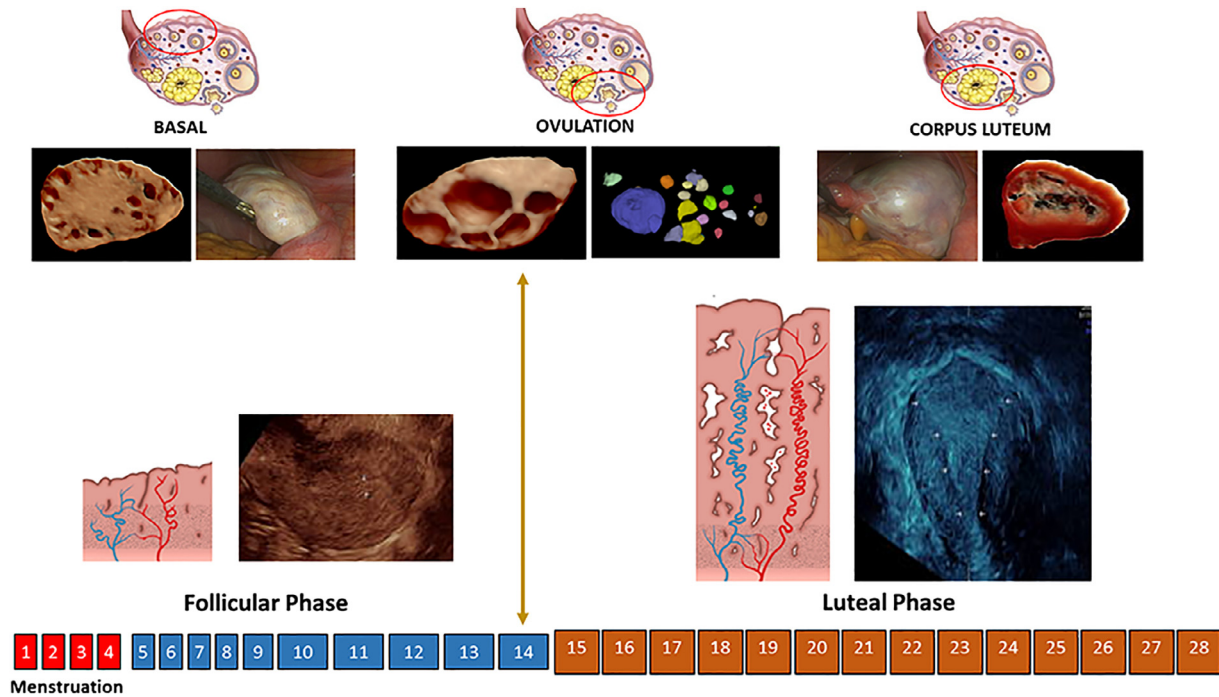


Fig. 1 Menstrual cycle control. Evolutionary control of the menstrual cycle by ultrasound.

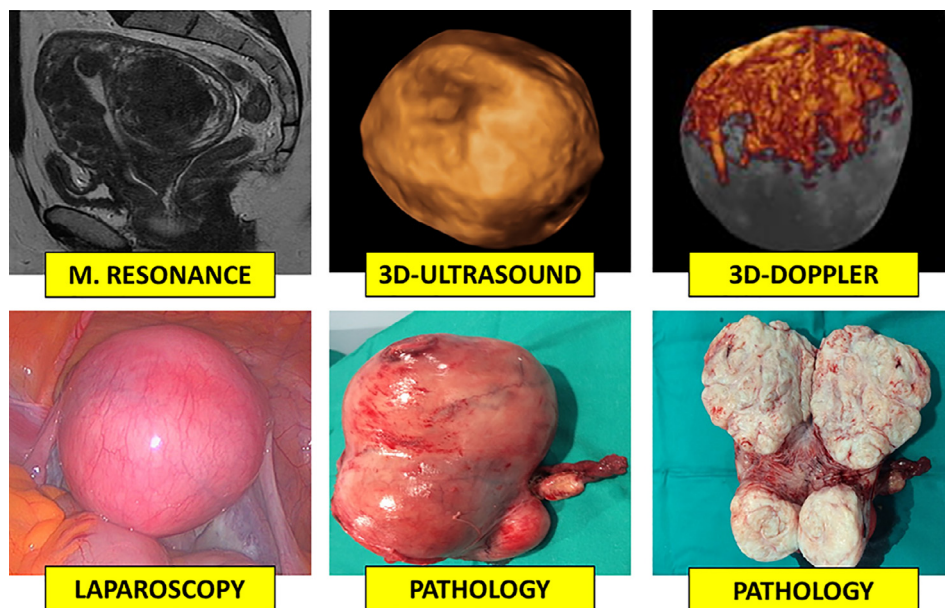


Fig. 2 Myoma or uterine fibroid. The myoma or uterine fibroid is the most frequent gynecological tumor, its assessment with different imaging techniques is of great relevance for its diagnosis and evolutionary control.

- *The endometrium.* Its ultrasonographic appearance as well as its thickness will depend on whether the patient is at a reproductive age or at a post-menopausal condition. The endometrium appears thin just after the menstruation and in menopause. However, it will become progressively thicker as the menstrual cycle progresses (the proliferative phase) and especially after ovulation (the secretory phase). 3D-ultrasound permits to analyze the endometrial volume, a precise parameter linked to endometrial receptivity in assisted reproductive treatments (Fig. 4).

A common benign endometrial pathology, causing abnormal uterine bleeding, is the endometrial polyp that appears as a nodular protrusion on the endometrial surface. The diagnosis of suspicion is firstly established with transvaginal ultrasound, and later confirmed by hysteroscopy (a fine telescope with a light and camera at the end). In addition, hysteroscopy allows its resection using scissors or an electric scalpel giving the opportunity of a posterior histological evaluation (Fig. 5).

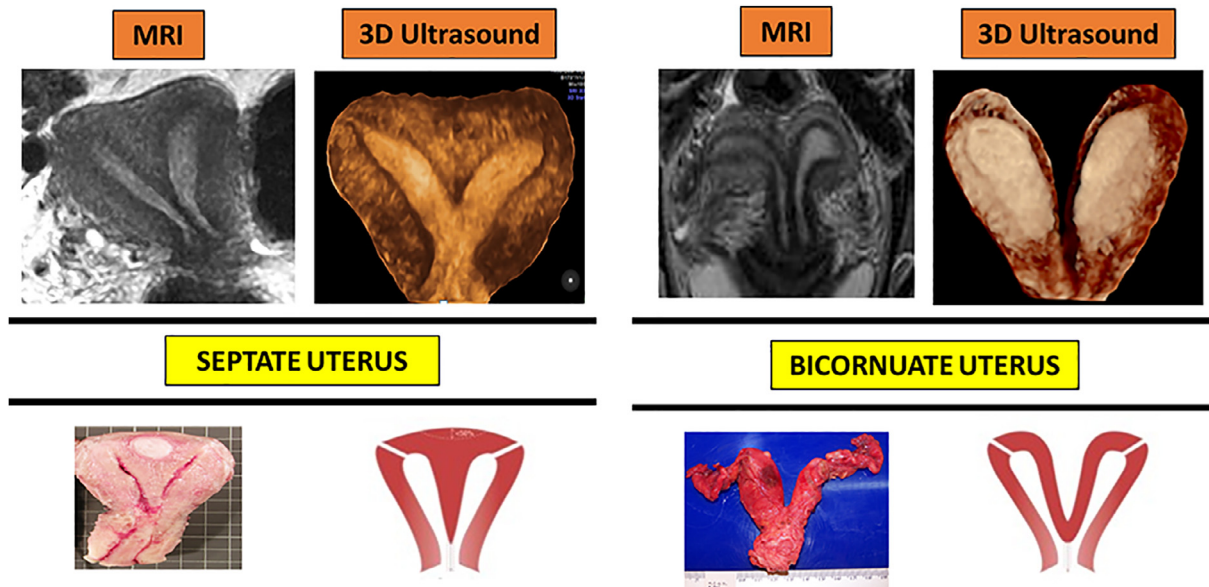


Fig. 3 Congenital uterine malformations. The incorporation of three-dimensional ultrasound and resonance in the diagnosis of congenital uterine malformations has made it possible to differentiate precisely the septal and bicornuate uterus.

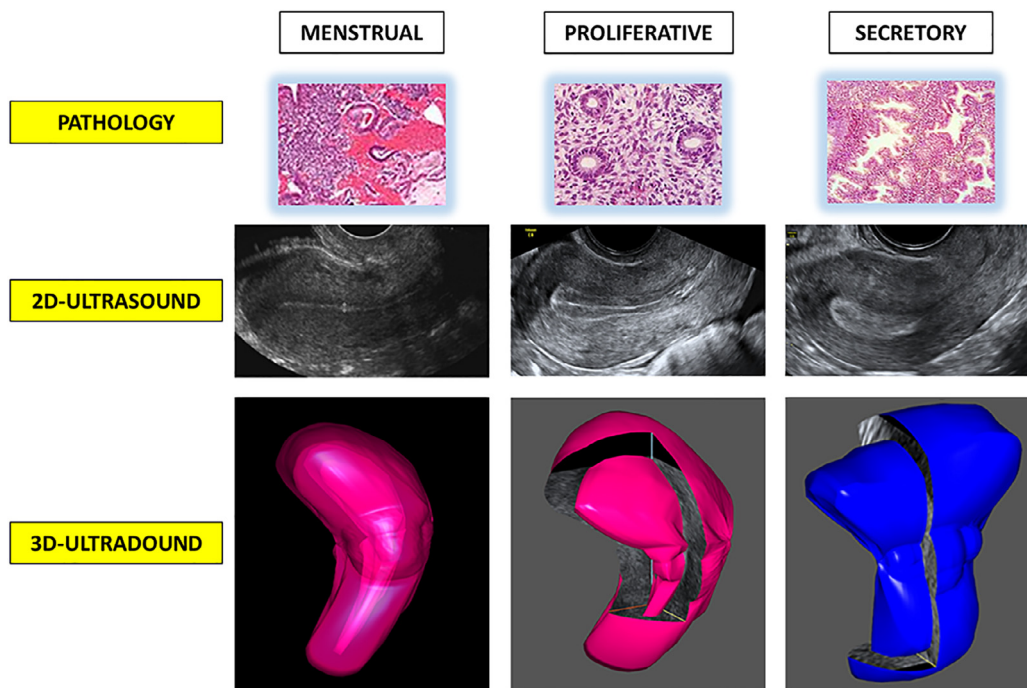


Fig. 4 Menstrual cycle ultrasound. Contribution of 2D and 3D ultrasound to the study of endometrial development during the menstrual cycle.

- *The cervix.* Nowadays ultrasonographic imaging of the uterine cervix is an essential part of preterm delivery risk assessment by measuring the cervical length. The shortening of the cervical length and the presence of the prolapsed membranes inside are signs of premature labor (**Fig. 6**).
- *The fallopian tube.* Hydrosalpinges are fallopian tubes that are blocked at their distal termination and distended with fluid. On the other hand, salpingitis (the acute infection of fallopian tubes) is known as a cause of tubal damage and of later hydrosalpinges. Development. These tubal pathologies are directly related with infertility. Using transvaginal ultrasound or magnetic resonance, the appearance of hydrosalpinx is that of a cystic adnexal mass (**Fig. 7**).

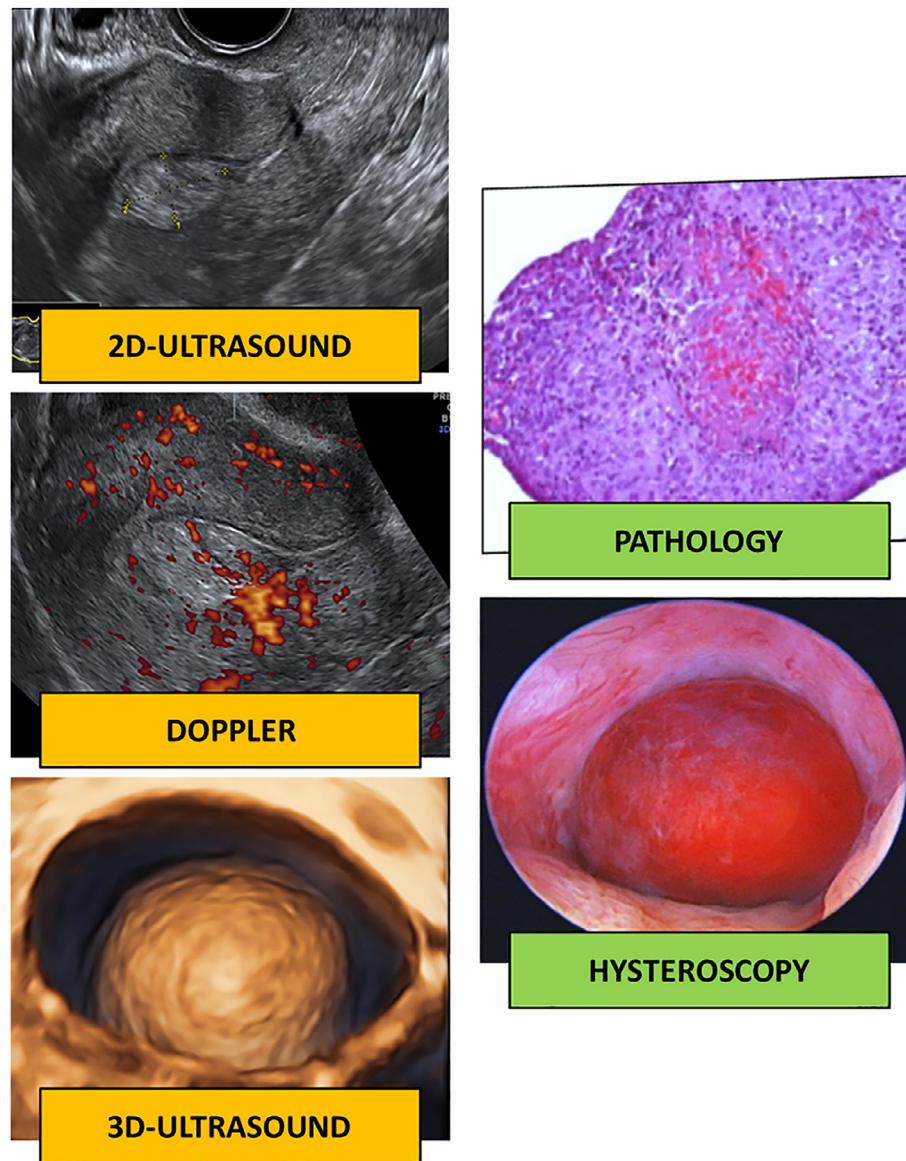


Fig. 5 The Endometrial polyp is one of the most frequent gynaecological pathologies. It's diagnostic assessment is possible thanks to ultrasound and hysteroscopy.

- *The ovaries.* Ovaries modify its aspect throughout the menstrual cycle. Antral follicles are small (about 2–9 mm in diameter) and we can count them with ultrasound during the early proliferative phase. The amount of these follicles along with female age and anti-Mullerian Hormone (AMH) determination (a protein hormone produced by the granulosa cells within the ovary) are by far the best tools that we have for assessing ovarian reserve. Ovarian reserve testing can tell us quite a lot about the remaining quantity of eggs a woman has. Moreover, it informs us about the probable response to ovarian stimulating drugs. This count has been facilitated with the introduction of three-dimensional ultrasound, since it give us the possibility to count the antral follicles in an automatic way (Fig. 8).

Gynecological pathology affecting the ovaries includes endometriosis (growth of endometrial tissue outside the uterus, especially in the pelvic cavity as in the ovaries), and ovarian tumors (both benign and malignant). Endometrioma (ovarian endometriotic cyst) is a common finding in the infertile patient. The typical ultrasonographic appearance is an unilocular cyst with internal homogeneous low-level echogenicity. 3D-ultrasound represents an opportunity to precise the diagnose of ovarian endometriomas due to its ability to perform multiple sections of this ovarian mass (Fig. 9).

Polycystic ovary is one of the most common endocrine disorders impairing females fertility. Major features of this condition include menstrual dysfunction, anovulation and signs of hyperandrogenism. Transvaginal ultrasound is considered the gold

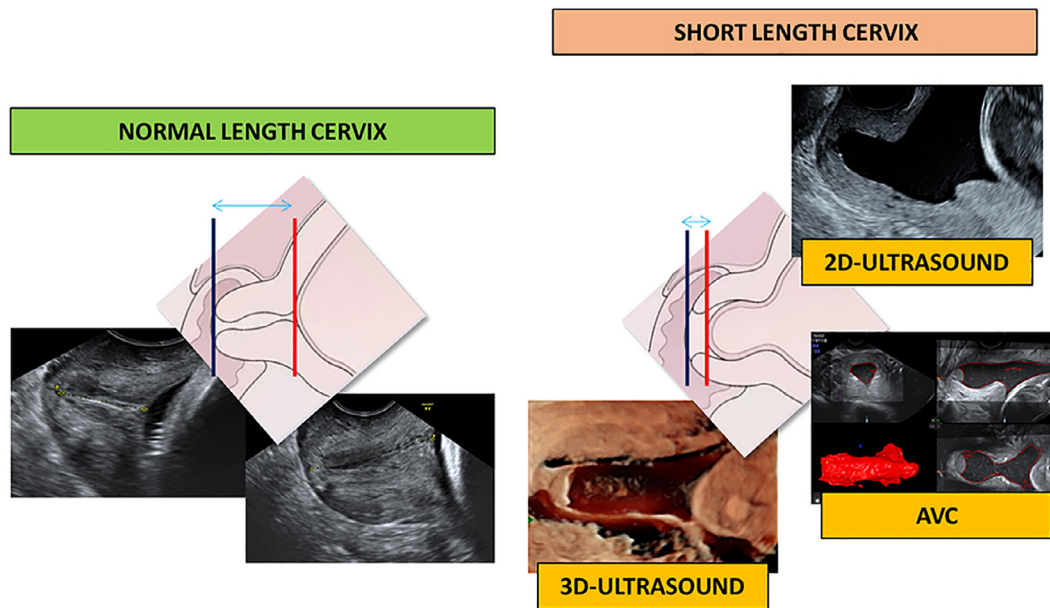


Fig. 6 The measurement of the uterine cervix plays an important role in the diagnosis of the threat of premature labor.

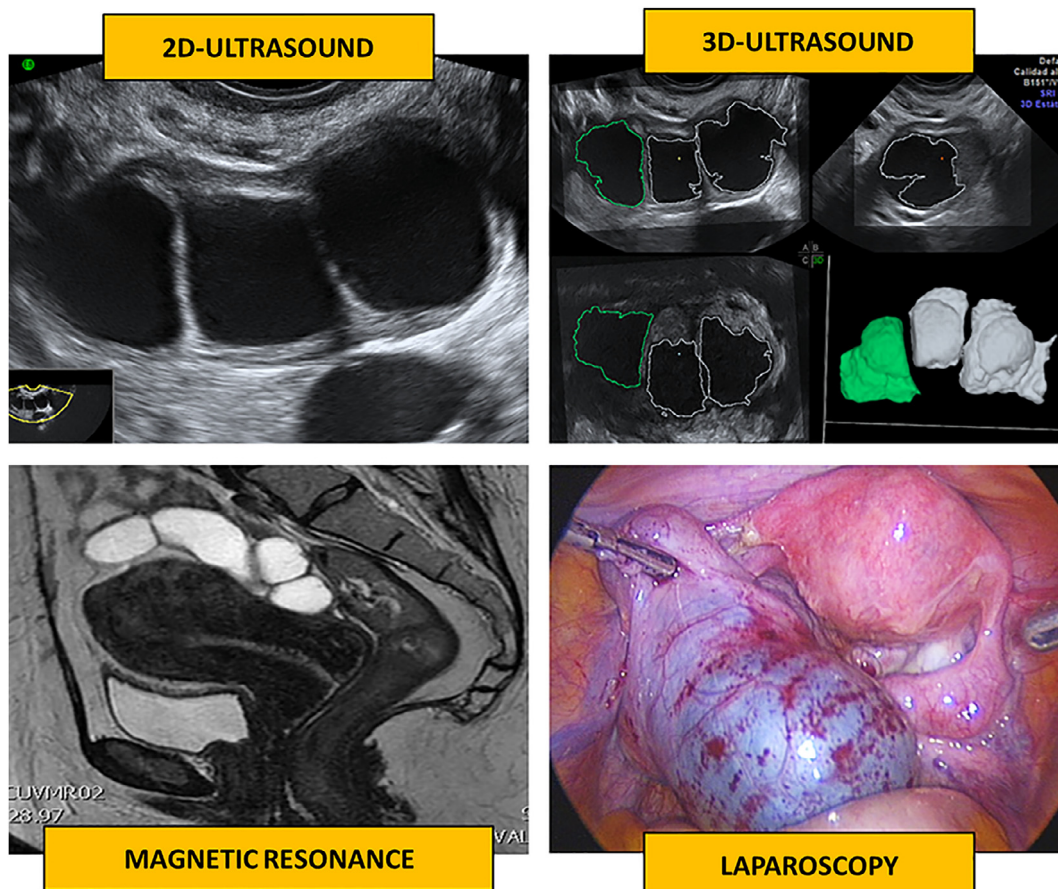


Fig. 7 Pathology fallopian tubes. The pathology of the fallopian tubes determines the fertility of women. The diagnosis of a hydrosalpinx is possible thanks to the different imaging techniques available today.

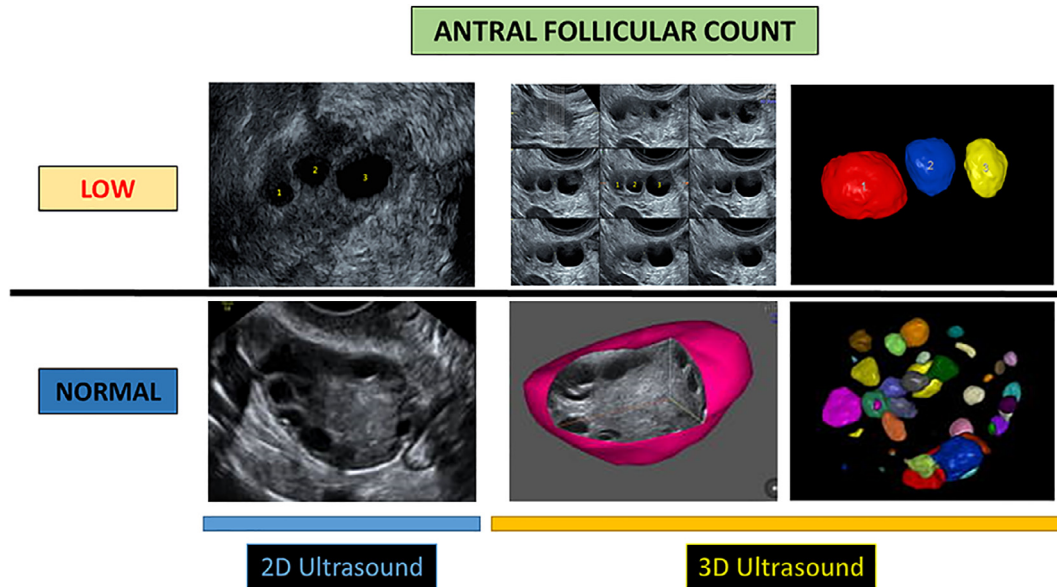


Fig. 8 Evaluation of the reserve of antral follicles using 2D and 3D ultrasound.

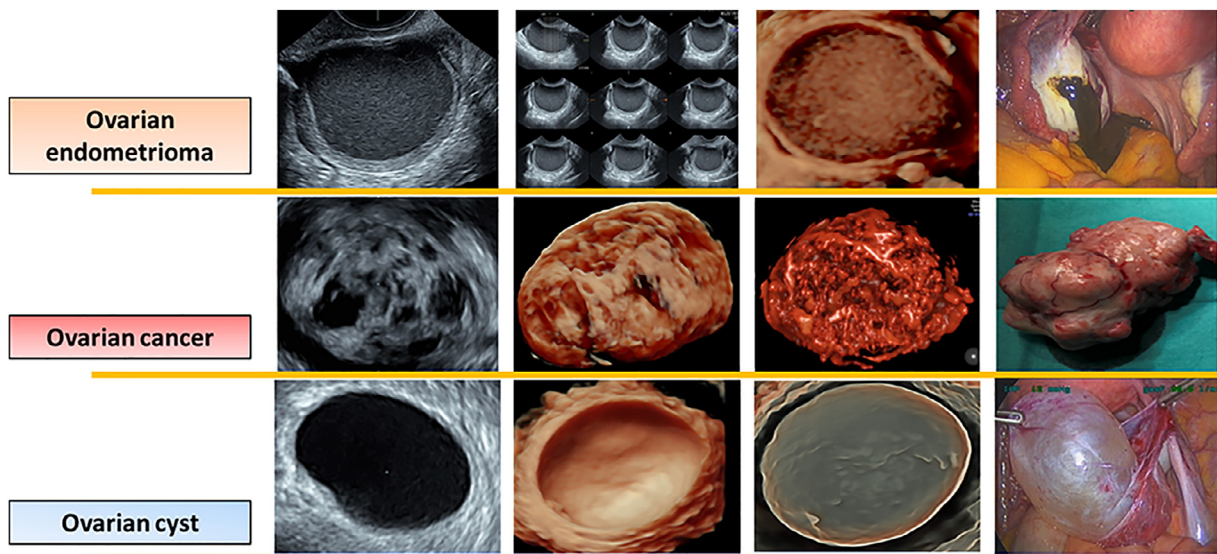


Fig. 9 Benign and malignant ovarian pathology. Evaluation by ultrasound and laparoscopy.

standard in the diagnosis of this disease. Classical features (presence of > 12 follicles per ovary and ovarian volume greater than 10 cm^3) can affect either one or both ovaries. 3D-ultrasound represents a major advance in the diagnosis of polycystic ovary as it provides a clear vision of the rich follicular endowment characteristic of this endocrine pathology (**Fig. 10**).

Reproductive Medicine

Assisted reproductive techniques are generally employed for infertility treatments. These techniques include in vitro handling of both human oocytes and sperm. Furthermore, once fertilized the ovule, it allows the control of early embryonic development with the objective of achieving pregnancy and live birth. In order to enhance the results of these procedures, recruitment of multiple follicles is habitually required and this is reached by controlled ovarian stimulation with hormones (FSH and LH). Transvaginal ultrasound is the diagnostic technique used to control ovulation induction within the cycles of assisted reproduction. It allows us to handle follicular and endometrial development, deciding the precise moment to trigger ovulation and thereafter to transfer the embryos under ultrasound guidance.

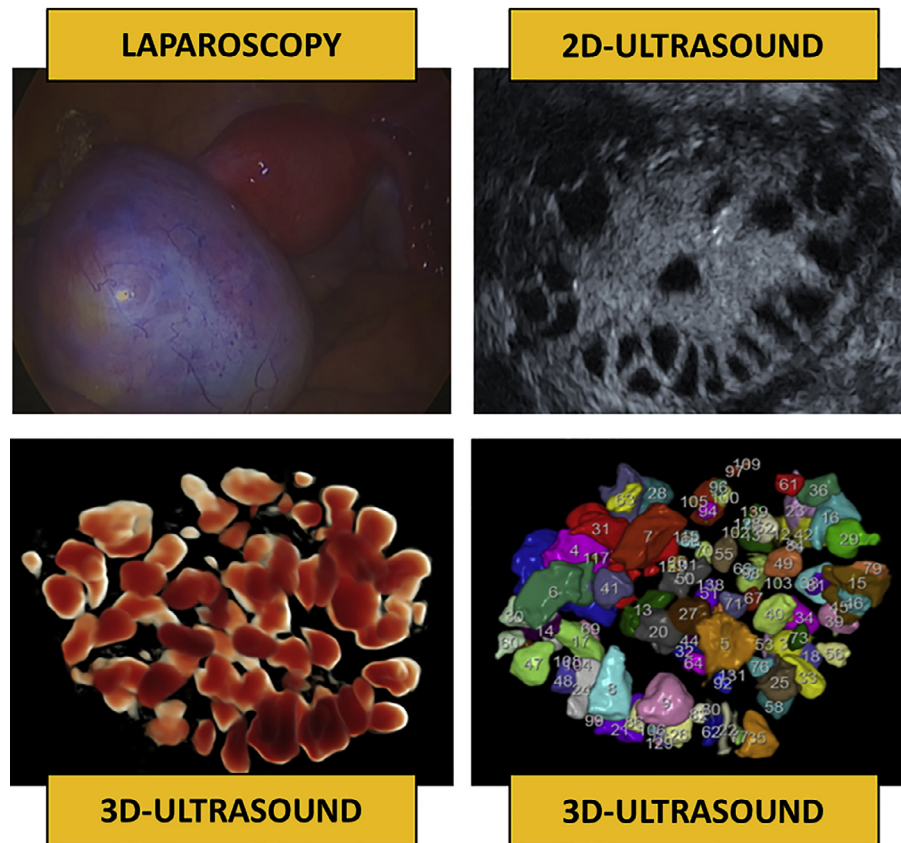


Fig. 10 The polycystic ovary is the most frequent cause of chronic anovulation. This ovary is characterized by its large volume and large reserve of antral follicles.

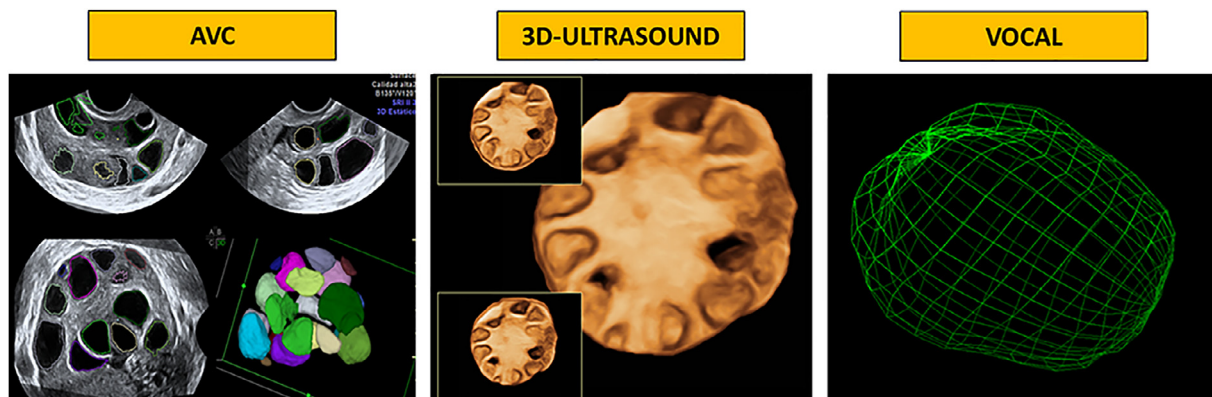


Fig. 11 Control of ovulation induction by ultrasound. We can assess the number of follicles, their size and the ovarian volume.

Ovarian hyperstimulation syndrome is an iatrogenic complication of assisted reproduction technology. The syndrome is characterized by cystic enlargement of the ovaries and the presence of fluid shift from the intravascular to the third space due to increased capillary permeability. Its occurrence is dependent on the administration of human chorionic gonadotrophin (hCG). Since it is a potentially dangerous pathology, its prevention and evolutionary control relies in an appropriate diagnostic ultrasound (**Fig. 11**).

Early Pregnancy Scan

Undoubtedly, the most fascinating application of imaging techniques in reproductive medicine is the vision of the beginning of life. A pregnancy can be viewed as early as 5 weeks from the first day of last menstrual period using transvaginal ultrasound. Since that moment, a dizzying career begins in embryonic development, that has a precise chronology during the first 12 weeks (**Fig. 12**).

EARLY EMBRYONIC DEVELOPMENT

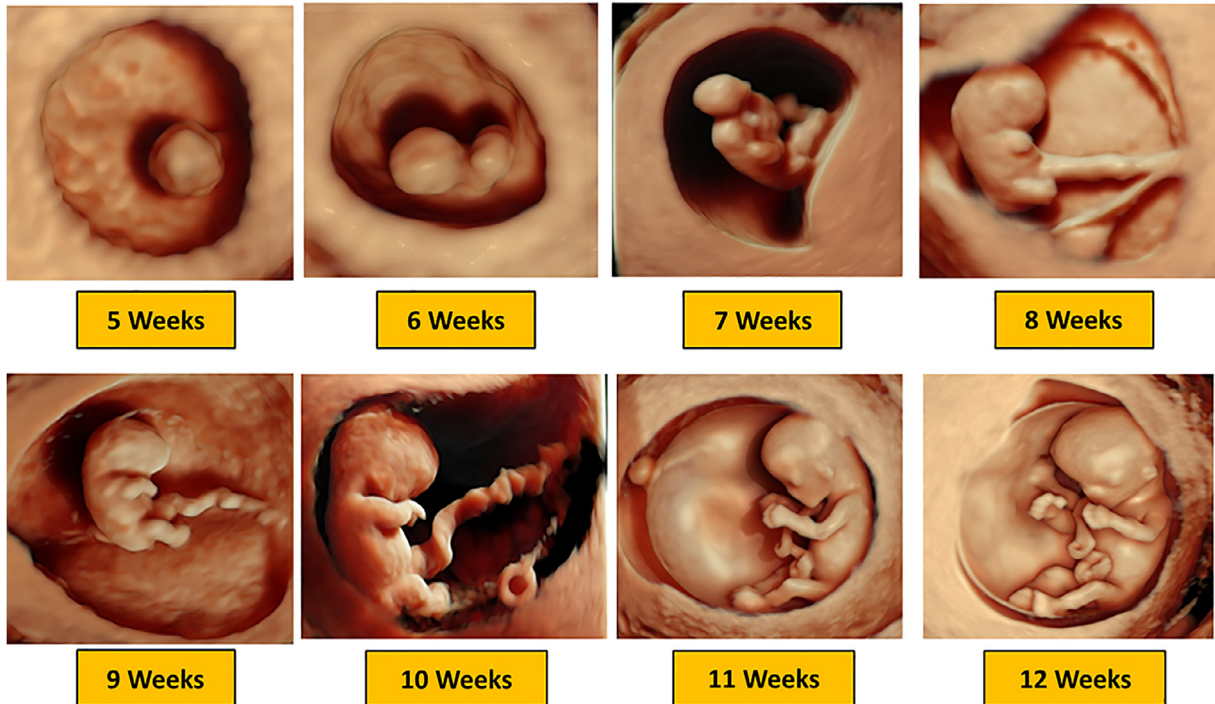


Fig. 12 Embryonic development. Early embryonic development. Its visualization thanks to the three-dimensional ultrasound is of great help.

Subsequently, the vision of fetal development is crucial to ensure its correct development, being the ultrasounds the main diagnostic tool in this field of medicine (Fig. 13).

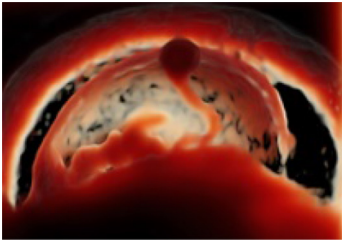
The 3D ultrasound development has led a great improvement in the vision of the early stages of embryonic growth. Nowadays we have multiple 3D ultrasound modalities that allow a detailed view of embryonic and fetal development (Table 1).

Highlights in this cast of 3D modes is the HD-live mode that give us the opportunity to modify the light bump in order to get a better view of the gestation. The TUI (tomographic ultrasound imaging) mode permits to obtain multiple planes of the structures studied. The AVC (automatic volume calculation) mode permits us to perform an automatic count of antral follicles. Moreover, the



Fig. 13 Late fetal development. Its control by means of ultrasounds allows to monitor the correct fetal development.

Table 1 Chronology of embryonic development during the first 12 weeks of gestation

5 Weeks	We visualize the gestational sac, which grows at a rate of 1.15/mm per day, with the yolk sac and embryo growing at a rate of 1 mm per day. In these early stages we can see the omphalomesenteric duct that connects the yolk sac with the umbilical cord.	
6 Weeks	This week begins a rapid development of the embryo, clearly differentiating a cephalic and caudal pole. The amnion membrane covers the entire embryo and the yolk sac is located extra-amniotic in the extraembryonic coelom.	
7 Weeks	The embryo continues its rapid growth. In this week the limbs differ. Also, the embryo initiates the achievement of small movements between the cephalic pole and the caudal pole. The space between the chorion and the amnion called extra-embryonic coelom persists.	
8 Weeks	During this week we can distinguish with great precision the fetal head. The embryo now realizes movements more and more complex. In the limbs, the hands can be visualized. At the umbilical level a physiological herniation occurs.	
9 Weeks	In this week the embryo already measures 25 mm. As characteristic features of this week highlight the visualization of the stomach and urinary bladder. This is the result of the onset of swallowing and renal function with urine production.	
10 Weeks	In this week the embryo measures 35 mm and we can distinguish in the face the eye orbits, the nose and the mouth. The physiological herniation begins to reverse, establishing the base of the umbilical cord at fetal level.	
11 Weeks	In this week the embryo measures 45 mm and we are able to differentiate the fingers and toes. The coiling development within the umbilical cord (one new spiral coil and one extra-centimeter in length for every gestational week approximately) appears to be obvious.	

(Continued)

Table 1 Chronology of embryonic development during the first 12 weeks of gestation—cont'd

12 Weeks	In this week the embryo measures 55 mm. and we can distinguish in the face the eye orbits, the nose and the mouth. The physiological herniation begins to reverse, establishing the base of the umbilical cord at fetal level.	
----------	--	---

advent of radiance or silhouette mode as a new tool in ultrasound diagnosis is intended to assist by generating additional realistic image visualization and a better distinction among different tissues (**Fig. 14**).

Embryo and Fetal Pathology

Prenatal diagnosis techniques allow an early detection of fetal pathology by studying the thickness of the nuchal translucency, the presence or absence of the nasal bone, and the ductus venosus. Nuchal translucency measurement between 11 and 14 gestation's week by ultrasonographic screening is considered a marker for aneuploidies. The presence of a thickened nuchal translucency, even if the karyotype result is normal, can be associated with structural abnormalities (congenital heart diseases) (**Fig. 15**).

In other cases, the ultrasound study permits us to confirm fetal pathologies such as anencephaly (absence of a portion of the brain and skull) or omphalocele (abdominal wall defect in which the intestines and occasionally other organs remain outside of the abdomen) (**Fig. 16**).

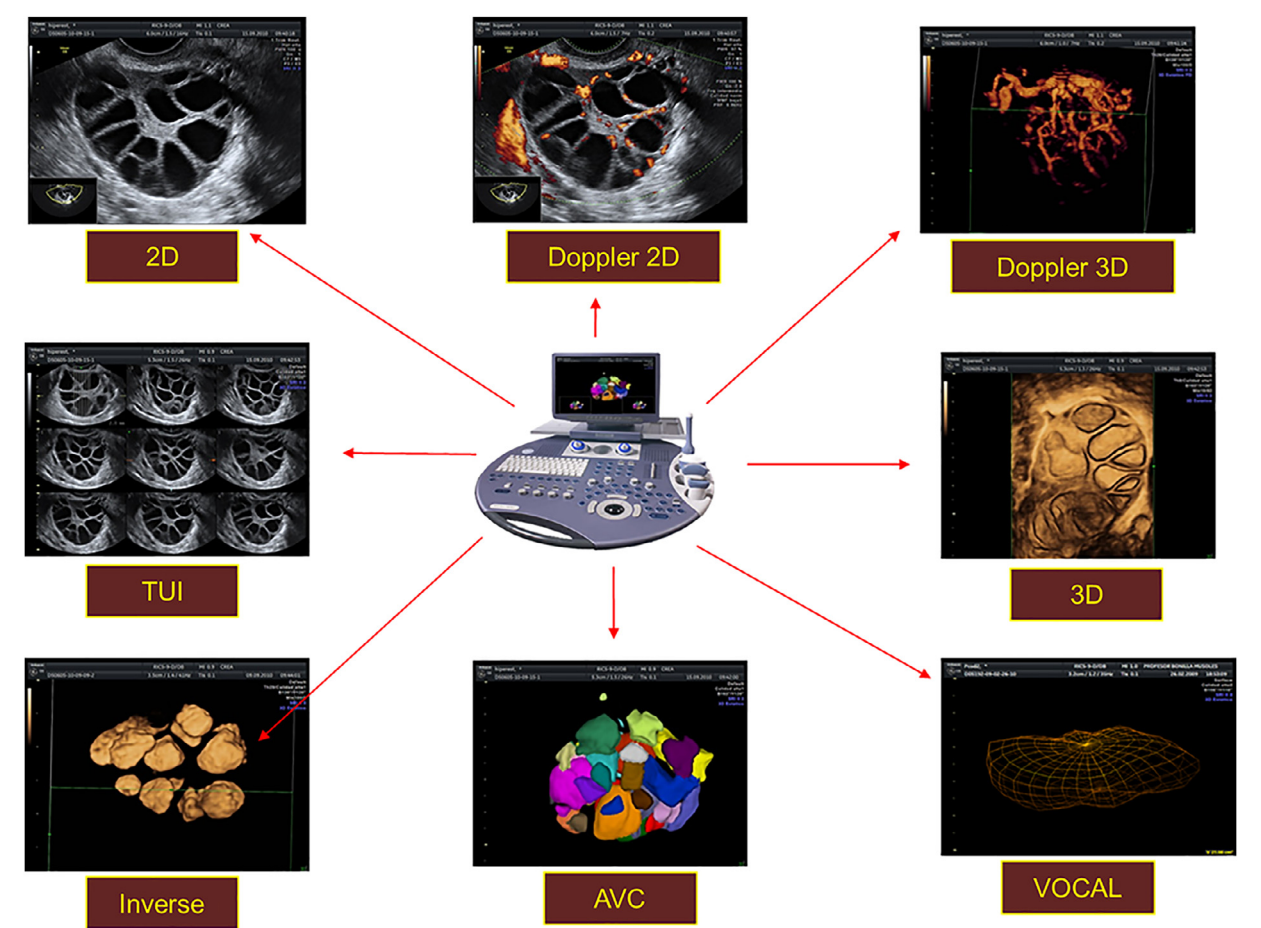


Fig. 14 Different three-dimensional echographic modes. These allow us to assess the morphology, volumetry and vascularization of the structures studied.

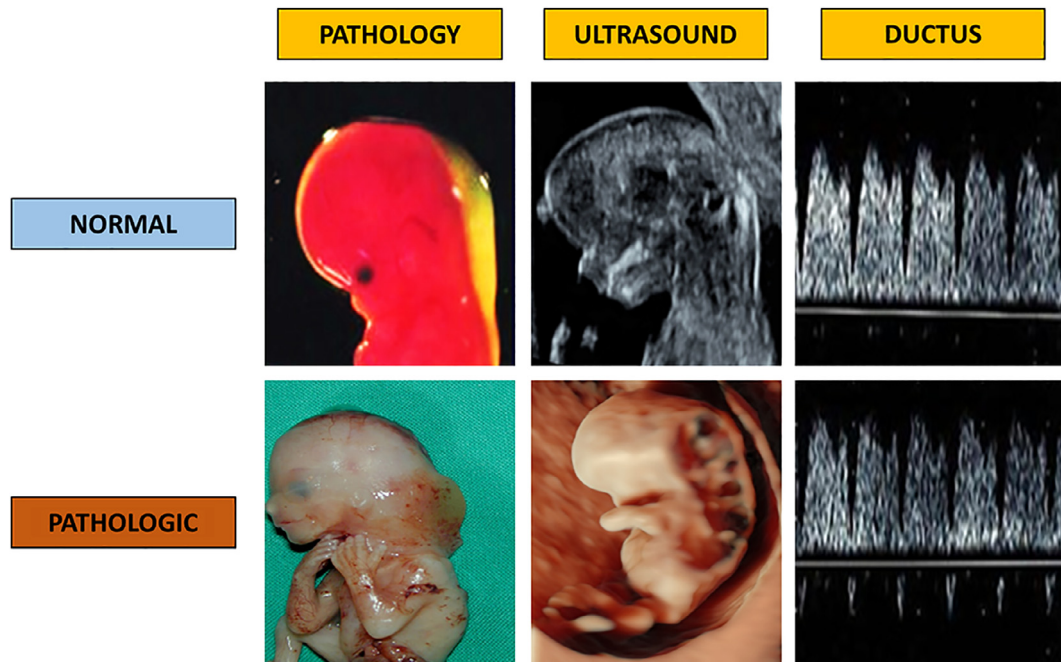


Fig. 15 Prenatal diagnosis. The use of ultrasound in prenatal diagnosis allows us to screen the fetal pathology at week 12.

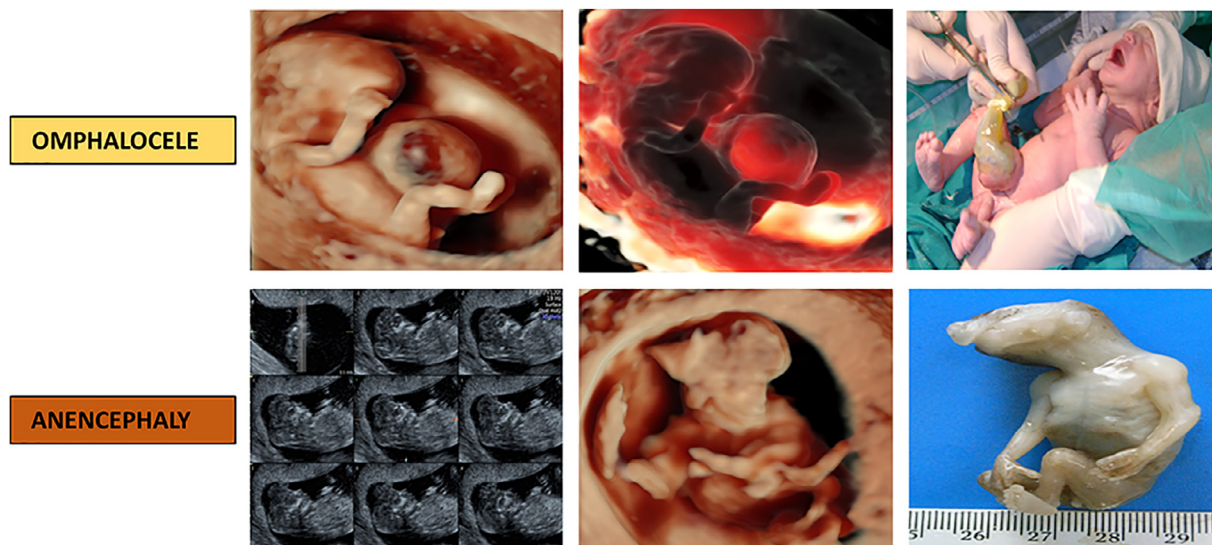


Fig. 16 Diagnosis of malformative fetal pathology by ultrasound.

Ectopic pregnancy (implantation of gestation outside the endometrial cavity) remains the most important cause of first trimester maternal death. Traditionally, laparoscopy has been the gold standard for its diagnosis. The advent of high-resolution transvaginal scan provides an earlier diagnosis, well before surgery becomes necessary. Early diagnosis by transvaginal ultrasound is therefore potentially lifesaving and can reduce surgical morbidity by allowing elective surgery or even non-surgical conservative treatment options.

The diagnosis of ectopic pregnancy combining the ultrasonographic studies and the determination of the levels of hCG is vital for its early diagnosis. In view of the suspicion of a complicated ectopic gestation (bleeding inside the abdominal cavity), we must perform a laparoscopy that will confirm the diagnosis and accomplish an appropriate treatment (removing the ectopic gestation) (Fig. 17).

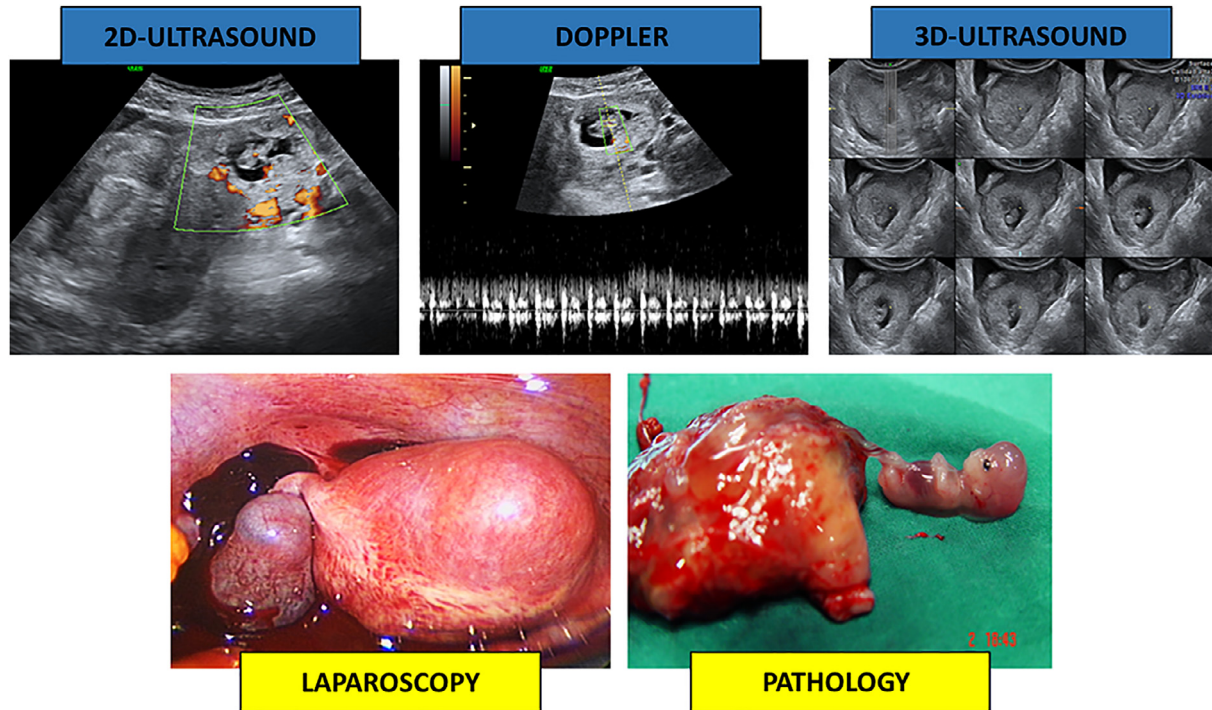


Fig. 17 Ectopic pregnancy is today the leading cause of maternal mortality during pregnancy. Its early diagnosis is mandatory to avoid complications as serious as the death of a pregnant woman.

Further Reading

- Bonilla-Musoles, F., Raga, F., Osborne, N. G., et al. (2013). Multimodality 3-dimensional volumetric ultrasound in obstetrics and gynecology with an emphasis in HDlive technique. *Ultrasound Quarterly*, 29, 189–201.
- Grigore, M., & Mareş, A. (2013). The role of HDlive technology in improving the quality of obstetrical images. *Medical Ultrasonography*, 15, 209–214.
- Kurjak, A., & Chervenak, F. (2017). *Donald School textbook of ultrasound in obstetrics and gynecology* (4th edn.). London: Jaypee Brothers Medical Publishers.
- Merz, E. (2015). 25 Years of 3D ultrasound in prenatal diagnosis (1989–2014). *Ultraschall in der Medizin*, 36, 3–8.
- Merz, E., & Abramowicz, J. S. (2012). 3D/4D ultrasound in prenatal diagnosis: is it time for routine use? *Clinical Obstetrics and Gynecology*, 55, 336–351.
- Pooh, R. K. (2016). Sonoembryology by 3D HDlive silhouette ultrasound—What is added by the “see-through fashion”? *Journal of Perinatal Medicine*, 44, 139–148.
- Pooh, R. K., & Kurjak, A. (2015). Novel application of three-dimensional HDlive imaging in prenatal diagnosis from the first trimester. *Journal of Perinatal Medicine*, 43, 147–158.
- Raga, F., Castillo, J. C., Bonilla, F., & Bonilla-Musoles, F. (2013). HDlive ultrasound images in assisted reproduction treatment. *Reproductive Biomedicine Online*, 26, 269–271.

Chromosomal Abnormalities and Their Reproductive Impact

Valeria Romanelli, GENETYX Molecular Biology Laboratory, Marostica, Italy

Danilo Cimadomo, GENETYX Molecular Biology Laboratory, Marostica, Italy; and GENERA Centers for Reproductive Medicine, CLINICA VALLE GIULIA, Roma, Italy

Maurizio Poli, REPROOMICS, Amsterdam, Netherlands

Cristina Patassini and Laura Girardi, GENETYX Molecular Biology Laboratory, Marostica, Italy

Laura Rienzi and Filippo Maria Ubaldi, GENERA Centers for Reproductive Medicine, CLINICA VALLE GIULIA, Roma, Italy

Antonio Capalbo, GENETYX Molecular Biology Laboratory, Marostica, Italy; and GENERA Centers for Reproductive Medicine, CLINICA VALLE GIULIA, Roma, Italy

© 2018 Elsevier Inc. All rights reserved.

Glossary

Aneuploidy Any deviation from an euploid chromosomal constitution due to the presence of either fewer or more chromosomes.

Chromosomal mosaicism Presence within the same embryo of cells with different karyotypes due to an error in chromosome segregation occurring during the mitotic divisions postfertilization. Both aneuploid mosaicism (different aneuploid cell lines) or euploid/aneuploid mosaicism (mix of euploid and aneuploid cell lines) are possible.

Euploidy The presence of a normal karyotype within a cell. In humans, a correct karyotype is $2n$ (46 chromosomes), where n is the haploid number of chromosomes.

Full chromosome aneuploidy Chromosomal imbalance that involves the whole sequence of a chromosome. Full chromosome aneuploidy can be either a monosomy or a trisomy.

Ploidy Number of sets of chromosomes within a cell. Haploid ($1n$), diploid ($2n$), triploid ($3n$), tetraploid ($4n$), etc.

Preimplantation genetic testing for aneuploidies (PGT-A) Diagnostic methodology that allows comprehensive testing for the presence of aneuploidies using molecular techniques in a biopsy sample retrieved from a human embryo.

Prenatal diagnosis (PND) Diagnostic methodology that allows comprehensive testing for the presence of aneuploidies and/or monogenic disorders using molecular or cytogenetic techniques in a fetus-derived specimen collected via amniocentesis or chronic villi sampling.

Segmental (or partial) aneuploidy Chromosomal imbalance that involves only a portion of a chromosome. Segmental aneuploidies include deletions or duplications.

Structural aneuploidy Karyotypic imbalance that involves the rearrangement of large regions of a chromosome and that occurs within it or between other chromosomes (i.e., inversions, translocations, either robertsonian, which involve acrocentric chromosomes, or reciprocal, which involve any other chromosome).

Targeted amplification DNA amplification strategy directed to specific DNA sequences of interest in the genome.

Technical artifact Chromosomal or genetic imbalance observed in a scientific investigation that is not naturally present, but occurs because of the preparative or investigative procedure (i.e., preferential amplification, allele drop-in, allele drop-out, etc.).

Whole genome amplification (WGA) Random DNA amplification strategy aimed at increasing the starting DNA template, typically from a single or few cells. By amplifying 40%–60% of the whole genome, this strategy allows the generation of a quantity of DNA sufficient to perform downstream molecular analyses.

Preface

In the preimplantation and prenatal periods, chromosomal abnormalities include a wide range of genomic imbalances, from polyploidy, whole chromosome and large structural aneuploidies, down to submicroscopic deletions and duplications. Whilst the impact of some karyotypic abnormalities on embryo and fetal development and associated reproductive outcomes are well-defined, the downstream consequences of many other smaller imbalances are still largely unknown.

This article is structured as an overview of the most prevalent chromosomal abnormalities and their impact on human reproduction. Furthermore, we will focus on preimplantation genetic testing for aneuploidies (PGT-A) as the most recent valuable tool to avoid the transfer of aneuploid embryos during IVF treatments, and review novel findings from basic research that reveal future possibilities for minimizing the occurrence of chromosome segregation errors.

Main Classes of Chromosomal Imbalances in Humans

Whole Chromosome Aneuploidies

Whole chromosome aneuploidies are defined as monosomies and trisomies for entire chromosomes, and undoubtedly represent the single most important cause for implantation failure and miscarriage (> 50% of sporadic first trimester pregnancy losses) in humans (Hassold and Hunt, 2001). Also, they have been extensively investigated due to their clear association with chromosomal syndromes and intellectual disability (Hassold and Hunt, 2001). The overall incidence of chromosomally abnormal pregnancies is highly dependent on female age and it is reported to be as high as 4% in stillbirths and about 0.3% in newborns (Hassold and Hunt, 2001) (Fig. 1). In fact, as women age from menarche to menopause, their oocytes become increasingly susceptible to chromosome segregation errors during the meiotic process. Extensive analysis of the main autosomal trisomies in clinical pregnancies revealed that > 90% had a meiotic origin. Most of the aneuploidies in embryos are due to chromosomal segregation errors occurring in the oocyte, with > 75% due to errors in meiosis I and < 25% due to errors in meiosis II, whilst current estimates suggest that a minority (1%–2%) of the spermatozoa are chromosomally impaired (Hassold and Hunt, 2001). Similarly, at the blastocyst stage, most of the

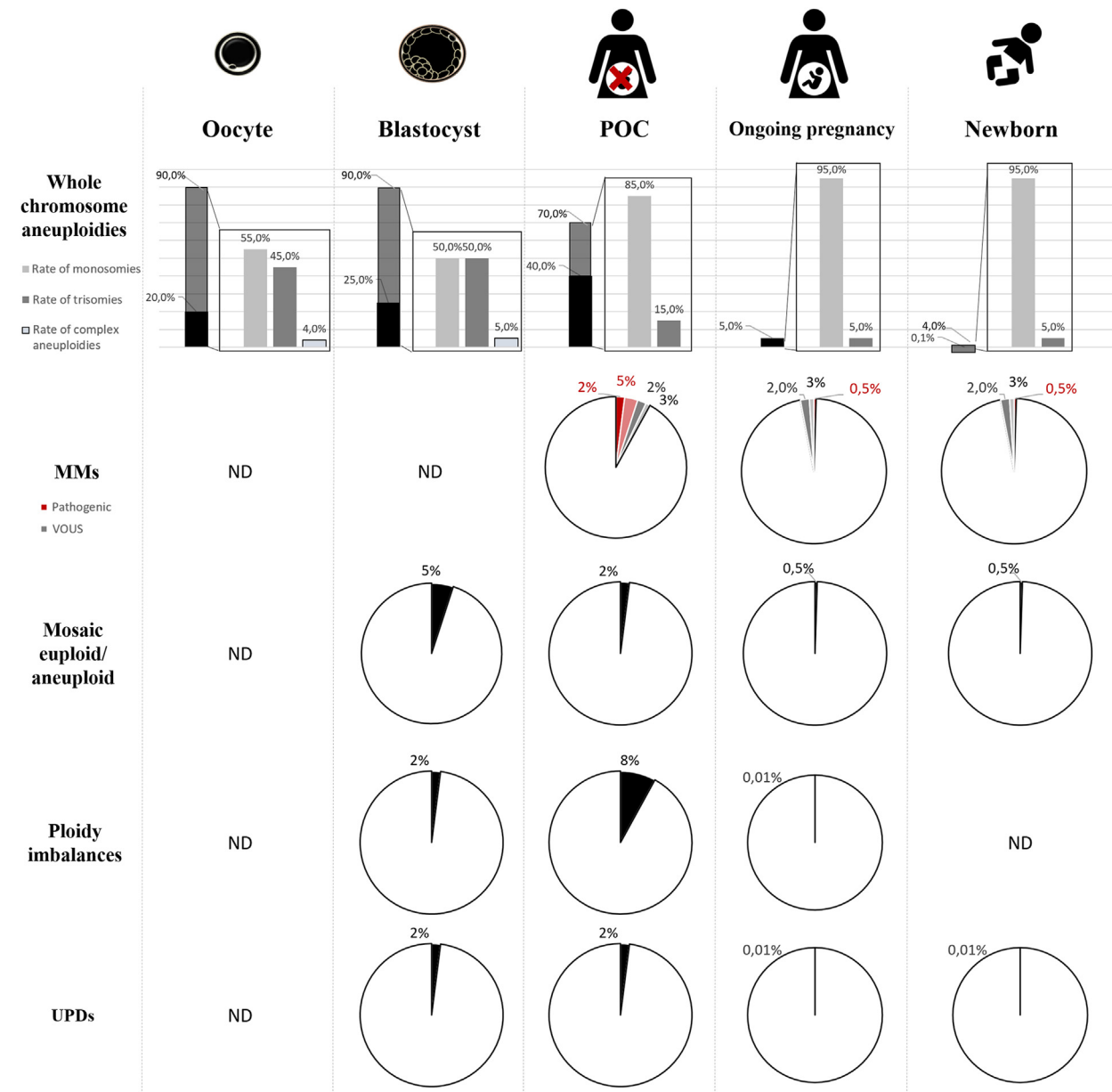


Fig. 1 Incidence of whole chromosome aneuploidies, microdeletions and microduplications (MMs), euploid/aneuploid mosaicism, ploidy imbalance and UniParental Disomy (UPD) across pre-/postimplantation developmental stages: oocyte, embryo, Product of Conception (POC), ongoing pregnancy and newborn. ND, Not detected/detectable; VOUS, Variants of unknown significance. Ranges are shown as full color (=minimum value) and faint color (=maximum value).

aneuploidies were classified as meiotic. The age-related processes that lead to the exponential increase in aneuploid conceptions are increasingly understood and novel insights into the molecular mechanisms of chromosome segregation during female meiosis are being explored (Ottolini et al., 2015). Because there are no therapies available to counteract the age-related increase of aneuploidies, diagnostic programs worldwide, either on preimplantation embryos during IVF cycles or in the prenatal period (prenatal diagnosis, PND), were established to detect the presence of an aneuploid conception, especially for women of advanced reproductive age. At present, the only interventions available to minimize the occurrence of an aneuploid pregnancy are: (i) fertility preservation through oocyte vitrification in young women, or (ii) PGT-A at the blastocyst stage during IVF treatments. Indeed, PGT-A is indicated for those couples with recurrent pregnancy loss (RPL), advanced maternal age (AMA), or recurrent implantation failure (RIF) in previous IVF cycles, to improve the embryo selection process by preventing the transfer of an aneuploidy embryo and reduce the chance of implantation failure and miscarriage (Dahdouh et al., 2015; Chen et al., 2015).

When assessed in embryos at different preimplantation developmental stages, monosomies and trisomies are observed at equivalent rates (Capalbo et al., 2014). On the contrary, population studies of newborns and miscarried products of conception, suggest that all monosomies, except for those involving the X chromosome, are incompatible with late embryonic development and early fetal life. However, although some autosomal trisomies and sex chromosome abnormalities are permissive of implantation, most of them result in developmental arrest within the first 12 weeks of gestation (Fig. 1).

Nearly 40% of human preimplantation embryos arrest around day 3–4, when they reach 8-cell/morula stage and compaction normally occurs. A portion of these embryos contains fragmented cells, suggestive of error-prone cleavage divisions associated with DNA damage. Some data suggest that aneuploidies and associated haploinsufficiency of dosage-sensitive genes are involved in preimplantation embryo arrest (Vega et al., 2014). However, clear evidence of how these mechanisms may cause mitotic arrest in preimplantation embryos is still missing, and other datasets are in favor of an opposite view. Specifically, when human blastocysts are assessed in PGT-A cycles, all range and spectra of aneuploidies are detected, including complex aneuploidies (Figs. 1 and 2). Thus, meiotic aneuploidies do not appear to be selected against during preimplantation development and do not cause major differences neither in the morphological quality nor in the morphokinetic behavior of developing human blastocysts (Capalbo et al., 2014; Rienzi et al., 2015). Notably, although aneuploidy rate in IVF blastocysts produced by women older than 44 reaches > 90%, around 30% of embryos produced by young women are aneuploid (Franasiak et al., 2014) (Figs. 1 and 2). Moreover, blastocysts show a chromosome-specific susceptibility to chromosome segregation errors associated with increased maternal age (Fig. 2). Specifically, larger chromosomes show an overall lower risk of errors and do not show a significant increase in their risk of aneuploidy with advanced female age. In general, the smaller the chromosomes (for instance chromosomes 21 and 22), the higher their susceptibility to the maternal age effect. On the other hand, sex chromosome showed a very low aneuploidy rate (2.3% of all the aneuploidies detected; Fig. 2), and their risk appears to be independent from female age as these abnormalities are generally related to defective segregation during spermatogenesis. Whole chromosome aneuploidies are very well understood and their impact in reproduction has been well defined in the last decades of genetic investigations. Diagnostic programs either at the preimplantation or prenatal stage can be effectively performed and accurate genetic counseling can be provided to couples when an abnormality is detected.

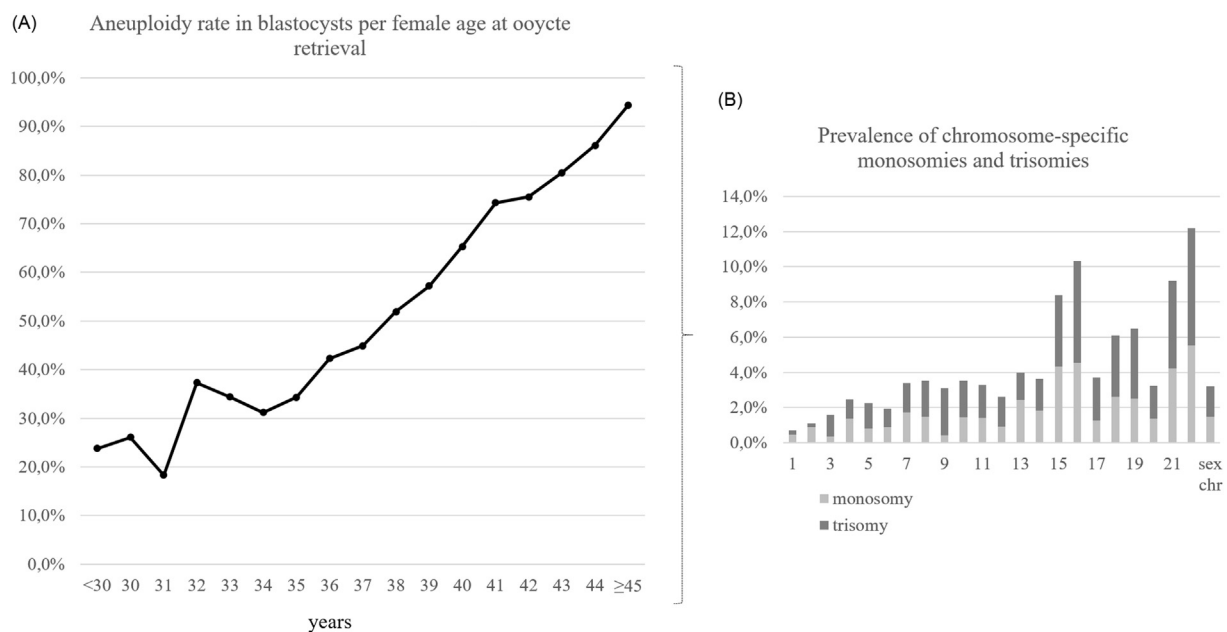


Fig. 2 Incidence of whole-chromosome aneuploidies in human blastocysts per woman age at egg retrieval (A), and prevalence of chromosome-specific monosomies and trisomies among all the aneuploidies detected (B). The figure reports the data produced in >5000 blastocysts tested at GENETYX (years 2013–2016) through a qPCR-based 24-chromosome aneuploidy testing technology (described by Treff et al., 2012).

Chromosomal Mosaicism

Whole chromosome aneuploidies are defined as constitutive when the error occurs along gametogenesis because of defective segregations during meiosis, with the ensuing embryo showing the extra/missing chromosome/sequence. Nonetheless, postzygotic errors in chromosome segregation may also occur and cause aneuploidies. Aberrant mitotic segregations occurring during initial cleavage divisions result in chromosomal mosaicism, a phenomenon that involves the presence of cell lines with different karyotypes within the same embryo. Detection frequency of euploid/aneuploid mosaic blastocysts differs across IVF centers and diagnostic laboratories and represent the main biological source of a potential diagnostic inconsistency in preimplantation/prenatal genetics. For this reason, the incidence and prevalence of chromosomal mosaicism in human blastocysts is a topic at the center of current investigation and debate. The most recent data suggest that about 5% of human blastocysts may show this constitution (Fig. 1), as collectively reported by at least four studies (reviewed in Capalbo et al., 2016) that analyzed the inner cell mass (ICM) and 2–3 fragments of trophoctoderm (TE) from > 100 disaggregated blastocysts donated for research. Due to the absence of appropriate checkpoints at early cleavage stages, it is likely that human embryos do not experience strong negative selection of cells bearing aneuploidy, chromosome breakage or segmental aberrations (Kiessling, 2010). Consequently, these abnormal cells are included in the developing embryo, causing genetic mosaicism to persist beyond early developmental stages without affecting cellular differentiation in a ploidy-dependent fashion. Moreover, in murine mosaic embryo models no evidence has been found for preferential allocation of aneuploid cells to embryonic or extra-embryonic lineages (Bolton et al., 2016). In agreement with these findings, it has not been found any sign for preferential allocation of abnormal cells towards one or the other cell lineage in mosaic human blastocysts (Capalbo et al., 2013). Indeed, abnormal cells in euploid/aneuploid mosaic embryos are evenly distributed across different blastocyst sections, including ICM only. Lastly, the “self-correction” model suggesting that abnormal chromosomes are fixed to a normal disomic configuration by a second mitotic error event correcting the original one is not supported by recent findings showing very low incidence of uniparental disomy (UPD) at the blastocysts stage (Gueye et al., 2014) (Fig. 1). In a PND study involving > 5300 fetuses, the rate of mosaicism in humans did not exceed 1.32% in either spontaneous or IVF-derived pregnancies as reported by Huang and colleagues (Huang et al., 2009). Such rate is in line with the 5% reported at the blastocysts stage. In the same study, the authors also outlined that true fetal mosaicism (not only confined to the placenta) has an even lower prevalence: 0.3%–0.44% (Fig. 1). These data are sufficient to define the prevalence of mitotic errors in human reproduction as relatively limited.

Whilst meiotic aneuploidies are uniformly present in all cells and can be accurately detected and managed in clinical diagnostic programs (Hassold and Hunt, 2001), the embryonic fate and the clinical consequences of mosaic aneuploidies may depend on several variables. These include which chromosome is involved in the aneuploidy, when the error occurred during preimplantation development, what proportion of the embryo is aneuploid, and where the abnormal cells are located within the embryo (Capalbo and Rienzi, 2017). Therefore, the clinical implication of a mosaic aneuploidy can be described as unique for each event and difficult to decipher in the absence of well-defined genotype/phenotype associations. Issues related to segregation and spatial allocations of aneuploid cells in a mosaic preimplantation embryo have recently raised concerns about the applicability and the effectiveness of PGT-A programs. It is widely accepted that, for blastocyst stage embryos, 5–10 randomly selected TE cells can be analyzed to infer the chromosomal configuration of the ICM. Indeed, the diagnosis of genetic imbalances in preimplantation embryos by taking a small TE biopsy does not represent a limitation for uniform aneuploidies. However, when dealing with mosaic euploid/aneuploid embryos, the biopsied TE cells might not be representative of the actual chromosomal constitution of the ICM, potentially causing misdiagnosis of the embryonic karyotype (Capalbo and Rienzi, 2017). Moreover, inherent technical limitations of comprehensive chromosome testing (CCT) technologies for assessment of mosaicism in a multicellular TE biopsy, persist. Presently, the identification of a condition compatible with mosaicism is based on bioinformatics tools, which, in the absence of specific diagnostic approaches, may result in the misinterpretation of an intermediate chromosome copy number falling in between the disomy and the aneuploidy thresholds (intermediate log 2 ratio or copy number value). None of the CCT methods has clinically standardized criteria to unequivocally distinguish between technical variation and genuine mosaicism (Capalbo et al., 2016). Indeed, the fluctuations of copy number profiles can be explained by a multitude of different technical and biological factors (reviewed in Capalbo and Rienzi, 2017). Both functional and prospective nonselection studies aimed at modeling the clinical fate and the reproductive potential of human embryos with an intermediate copy number profile are lacking. Therefore, until a more comprehensive diagnostic strategy is developed, mosaicism should be recognized as a biological limitation for PGT-A programs, as it is also for PND. Indeed, it is still reasonable to avoid reporting mosaicism in PGT-A cycles until its impact on clinical treatment is clarified. Alternatively, genetic reports could include the option of “analysis showing patterns consistent with mosaicism”, in which case patients should undergo extensive genetic counseling prior to transferring the embryo.

Structural and Segmental Chromosome Aneuploidies

Constitutive structural chromosome abnormalities

The most common structural chromosome abnormalities are balanced reciprocal translocations with an incidence in PND of 1 out of 560 fetuses, and balanced Robertsonian translocations and inversions with an incidence of 1 out of 1100–1200 fetuses. Instead, the incidence of unbalanced structural abnormalities is even lower (Franssen et al., 2005). However, a history of recurrent miscarriages in young women suggests an increased risk of structural chromosome abnormality in one of the partners (Franssen et al., 2005). To this regard, the incidence of balanced structural chromosome abnormalities is 0.7% in the general population and increases to 2.2% after one miscarriage, 4.8% after two miscarriages and to 5.2% after three miscarriages (De Braekeleer and Dao, 1990).

Structural chromosomal abnormalities in one of the partners may result in the production of chromosomally unbalanced gametes that cause meiotic impairment and, in general, infertility. Implantation failure, early or late miscarriage, or an ongoing pregnancy resulting in physical and/or mental disabilities in the child are the range of possible consequences underlying the production of an unbalanced embryos. Yet, recurrent miscarriage is more common when the abnormality is present in the maternal karyotype rather than in paternal one. This is due to sex-specific differences in the meiotic silencing checkpoint, which normally eliminates chromosomes that are not perfectly paired in spermatogenesis, thus the meiotic silencing checkpoint is not as stringent in mammalian oocytes compared to sperm. Moreover, structural impairments would commonly result in low fertility, especially in males, as highlighted in cytogenetic studies of gametes from patients with balanced structural chromosome abnormalities (Kurahashi et al., 2012).

Once a structural chromosome abnormality has been revealed in one of the partners, either prenatal chromosomal analysis or preimplantation genetic testing for chromosome structural imbalances during an IVF treatment can be performed to identify normal (balanced) and abnormal (imbalanced) conceptuses.

De novo segmental chromosome aneuploidies

Segmental aneuploidies, such as chromosome deletions and duplications, are major contributors to genomic variability. Although some of them do not affect cellular physiology, a subset of them is pathogenic. Their origins are equally distributed between maternal and paternal chromosomes and female age does not appear to affect their incidence.

Partial aneuploidies (a class of copy number variations, CNVs) can be either inherited or arise because of a de novo event. The former possibility is rather infrequent and occurs in <1% of miscarriages; more often, they originate as an error in the male or female meiosis, apparently at similar rates (Ottolini et al., 2016). The estimated prevalence of chromosomal deletions in the neonatal population ranges from 0.5–2 per 10,000 births (Vega et al., 2014). A recent study reported that $\approx$ 5% of all chromosome abnormalities are deletions, including microdeletions, giving a prevalence of 1.99 per 10,000 births. Duplications are even less common, showing a prevalence of 0.7 per 10,000 births and representing $\approx$ 2% of all the chromosome abnormalities identified (Wellesley et al., 2012). Although CNVs are less frequent than full chromosome aneuploidies, their impact on embryo development may be equally dramatic if they alter the copy number state of putative dosage-sensitive genes.

Among CNVs types, large deletions and duplications are rare events, while chromosomal microdeletions and microduplications (MMs), which involve sequences generally smaller than 5 Mb, make up the most significant fraction of subchromosomal CNVs and a class of them have been clearly defined as pathogenic. These types of recurrent CNVs are classified as genomic disorders and their underlying molecular mechanisms and phenotypes are the best-characterized imbalances in the genome. In the last decade, > 200 recurrent MM syndromes with developmental delay and intellectual disability have been identified. In large prenatal investigations, pathogenic MMs, have been reported with an incidence of about 0.5% in pregnancies and without any identified female age effect (Wapner et al., 2012) (Fig. 1).

As novel technologies have progressively improved the detection of smaller CNVs across the genome, many studies over the last decade suggested a growing spectrum of size and phenotypic effects of CNVs, ranging from adaptive traits to full diseases, as well as embryonic lethality.

While their prevalence in pregnancies and newborns is relatively low, their estimates and pathogenicity in preimplantation embryos are poorly defined. This limited knowledge is a consequence of the paucity of DNA retrieved from the few trophoctoderm cells composing a blastocyst biopsy. Specifically, a whole genome amplification (WGA)-based DNA amplification step is required, which may impact the reliability of the diagnosis. Indeed, current technologies do not cover the genome evenly but just a portion of it (40%–60%), thus limiting genome resolution and MMs detection (Cawad et al., 2016), because of occasional introduction of amplification bias (Capalbo et al., 2015). Moreover, it is noteworthy that a repository database of CNVs and their developmental outcomes for preimplantation embryos is still lacking. The absence of such knowledge makes it complex to give a clinical interpretation of the reproductive impact of the CNVs detected in the preimplantation period.

Preimplantation Genetic Testing for Aneuploidies

The reproductive competence of an embryo is unavoidably dependent on its intrinsic capability to implant. This potential cannot be improved with any of the IVF clinical strategies used, but possibly only predicted through embryo selection tools that, when applied, should not impact the embryo itself. To date, PGT-A has proven to be the only valuable IVF approach to limit the impact of aneuploidies on human reproduction. Indeed, by identifying aneuploid embryos in a cohort produced by a couple during an IVF cycle, and by allowing to transfer euploid embryos, PGT-A does improve the efficiency of ART (increased implantation rate per transfer, reduced miscarriage rate and time-to-pregnancy, etc.), possibly with no impact on its efficacy (i.e., the number of babies born per cycle). Based on the current knowledge, the best PGT-A strategy involves comprehensive chromosome testing (by array single nucleotide polymorphisms, array comparative genomic hybridization, quantitative polymerase chain reaction and, more recently, next generation sequencing) of a trophoctoderm biopsy retrieved at the blastocyst stage. This is a strategy that (i) guarantees the most reliable information about embryonic karyotype (including male-derived aneuploidies and clinically-relevant mitotic errors); (ii) does not impact embryonic intrinsic developmental potential as shown in a paired randomized controlled trial (RCT) (Scott et al., 2013; Cimadomo et al., 2016); (iii) is cost-effective, since only developmentally competent embryos, that developed to the blastocysts stage, are analyzed; (iv) shows high positive ($\geq$ 50%, independently from female age at oocyte retrieval) and

negative ($\geq 96\%$) clinical predictive values as reported in a prospective nonselection RCT (Scott et al., 2012); and (v) is clinically effective, as shown in all the RCTs and observational studies recently reviewed in two meta-analyses (Dahdouh et al., 2015; Chen et al., 2015).

Novel Basic Research Findings

Despite PGT-A being an efficient diagnostic tool, it does not modify the genetic status of the oocytes/blastocysts generated during an IVF cycle. When a woman no longer produces chromosomally normal oocytes/embryos, PGT-A is ineffective in terms of IVF outcomes. Indeed, basic research avantgardes in this field aim at identifying novel strategies to operate onto oocytes/embryos, and to eventually reduce their aneuploidy risk and improve their reproductive competence.

First, unraveling what other factors (beyond woman aging) cause aneuploidies may provide the community with lifestyle guidelines to minimize the incidence of miscarriage, and try to extend female reproductive lifespan. Furthermore, beyond precautionary measures, we may also attempt to target cellular processes involved in aberrant chromosome segregation, and prevent (or rectify) their occurrence. For instance, Wu and colleagues are administering salubrinal to obese mice to counteract metabolic stress in the endoplasmic reticulum triggered by the diet, thus restoring the oocyte maturation potential both in vivo and in vitro (Wu et al., 2015).

Second, the complete characterization of the meiotic machinery, of its individual components function and of upstream regulators may reveal key gene/protein targets whose function must be preserved to guarantee a correct meiosis (e.g., DNA damage response genes). With this knowledge, it will be possible to design novel IVF protocols where immature oocytes are collected before meiotic completion and let them progress through the meiosis process in vitro with the support of stabilizing agents and molecules.

Third, chromosome therapy is a fascinating perspective to perform functional correction on living cells. To date, two main strategies have been tested in human/animal models and published: the *XIST* (*X-inactive specific transcript*)-driven heterochromatization of chromosome 21 to conduct dosage compensation in induced pluripotent stem cells from a Down syndrome individual (Jiang et al., 2013); the *ZSCAN4* (*zinc finger and SCAN domain containing 4*) mRNA-mediated correction of trisomy 18 and 21 to a correct chromosome number in human cell cultures and murine embryonic stem cells (Amano et al., 2015).

Last, mRNAs or inhibitors may be adopted to sustain in vitro maturation or embryo development by preserving genetic integrity.

Although these are only putative desirable perspectives at present, future technologies may be able to counteract the natural mechanism of reproductive aging and ultimately increase the cumulative live birth rate per started cycle in IVF.

Conclusion

Human reproduction is highly dependent upon a correct chromosomal constitution, which is especially reliant on a correct female meiosis. Indeed, maternal age and embryo reproductive competence are closely connected. The possibility of developmental progression in presence of meiotic errors throughout, but mainly beyond, the preimplantation period is limited to few trisomies (e.g., 13, 18, and 21), some segmental and sex chromosomes aneuploidies, especially in presence of low-grade chromosomal mosaicism. Nevertheless, the understanding of human preimplantation development is limited due to ethical and legal restrictions on embryo research and scarcity of material, and currently the only valuable clinical tool to limit the incidence of aneuploidies in humans is PGT-A. Additionally, studies in humans are mainly descriptive and lack functional evidence. Most of the information on embryo development is obtained from animal models and embryonic stem cell (ESC) cultures; hence any conclusion or generalization should be extrapolated with caution. Nonetheless, novel imaging and genetic technologies now provide the tools to further investigate these mechanisms with more accuracy and precision. It is therefore expected that upcoming studies combining genetic, embryology and developmental biology data will be able to answer some of these fundamental biological questions in the near future.

References

- Amano, T., Jeffries, E., Amano, M., Ko, A. C., Yu, H., & Ko, M. S. (2015). Correction of down syndrome and Edwards syndrome aneuploidies in human cell cultures. *DNA Research*, 22, 331–342.
- Bolton, H., Graham, S. J., Van Der Aa, N., Kumar, P., Theunis, K., Fernandez Gallardo, E., Voet, T., & Zernicka-Goetz, M. (2016). Mouse model of chromosome mosaicism reveals lineage-specific depletion of aneuploid cells and normal developmental potential. *Nature Communications*, 7, 11165.
- Capalbo, A., & Rienzi, L. (2017). Mosaicism between trophectoderm and inner cell mass. *Fertility and Sterility*, 107, 1098–1106.
- Capalbo, A., Wright, G., Elliott, T., Ubaldi, F. M., Rienzi, L., & Nagy, Z. P. (2013). FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. *Human Reproduction*, 28, 2298–2307.
- Capalbo, A., Rienzi, L., Cimaglio, D., Maggiulli, R., Elliott, T., Wright, G., Nagy, Z. P., & Ubaldi, F. M. (2014). Correlation between standard blastocyst morphology, euploidy and implantation: An observational study in two centers involving 956 screened blastocysts. *Human Reproduction*, 29, 1173–1181.
- Capalbo, A., Treff, N. R., Cimaglio, D., Tao, X., Upham, K., Ubaldi, F. M., Rienzi, L., & Scott, R. T., Jr. (2015). Comparison of array comparative genomic hybridization and quantitative real-time PCR-based aneuploidy screening of blastocyst biopsies. *European Journal of Human Genetics*, 23, 901–906.
- Capalbo, A., Ubaldi, F. M., Rienzi, L., Scott, R., & Treff, N. (2016). Detecting mosaicism in trophectoderm biopsies: Current challenges and future possibilities. *Human Reproduction*.

- Chen, M., Wei, S., Hu, J., & Quan, S. (2015). Can comprehensive chromosome screening technology improve IVF/ICSI outcomes? A meta-analysis. *PLoS ONE*, *10*, e0140779.
- Cimadomo, D., Capalbo, A., Ubaldi, F. M., Scarica, C., Palagiano, A., Canipari, R., & Rienzi, L. (2016). The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. *BioMed Research International*, *2016*, 7193075.
- Dahdouh, E. M., Balayla, J., & Garcia-Velasco, J. A. (2015). Comprehensive chromosome screening improves embryo selection: A meta-analysis. *Fertility and Sterility*, *104*, 1503–1512.
- De Braekeleer, M., & Dao, T. N. (1990). Cytogenetic studies in couples experiencing repeated pregnancy losses. *Human Reproduction*, *5*, 519–528.
- Franasiak, J. M., Forman, E. J., Hong, K. H., Werner, M. D., Upham, K. M., Treff, N. R., & Scott, R. T., Jr. (2014). The nature of aneuploidy with increasing age of the female partner: A review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertility and Sterility*, *101*, 656–663. e1.
- Franssen, M. T., Korevaar, J. C., Leschot, N. J., Bossuyt, P. M., Knegt, A. C., Gerssen-Schoorl, K. B., Wouters, C. H., Hansson, K. B., Hochstenbach, R., Madan, K., Van Der Veen, F., & Goddijn, M. (2005). Selective chromosome analysis in couples with two or more miscarriages: Case-control study. *BMJ*, *331*, 137–141.
- Gawad, C., Koh, W., & Quake, S. R. (2016). Single-cell genome sequencing: Current state of the science. *Nature Reviews. Genetics*, *17*, 175–188.
- Gueye, N. A., Devkota, B., Taylor, D., Pfundt, R., Scott, R. T., Jr., & Treff, N. R. (2014). Uniparental disomy in the human blastocyst is exceedingly rare. *Fertility and Sterility*, *101*, 232–236.
- Hassold, T., & Hunt, P. (2001). To err (meiotically) is human: The genesis of human aneuploidy. *Nature Reviews. Genetics*, *2*, 280–291.
- Huang, A., Adusumalli, J., Patel, S., Liem, J., Williams, J., 3rd, & Pisarska, M. D. (2009). Prevalence of chromosomal mosaicism in pregnancies from couples with infertility. *Fertility and Sterility*, *91*, 2355–2360.
- Jiang, J., Jing, Y., Cost, G. J., Chiang, J. C., Kolpa, H. J., Cotton, A. M., Carone, D. M., Carone, B. R., Shivak, D. A., Guschin, D. Y., Pearl, J. R., Rebar, E. J., Byron, M., Gregory, P. D., Brown, C. J., Urnov, F. D., Hall, L. L., & Lawrence, J. B. (2013). Translating dosage compensation to trisomy 21. *Nature*, *500*, 296–300.
- Kiessling, A. A. (2010). Timing is everything in the human embryo. *Nature Biotechnology*, *28*, 1025–1026.
- Kurahashi, H., Tsutsumi, M., Nishiyama, S., Kogo, H., Inagaki, H., & Ohye, T. (2012). Molecular basis of maternal age-related increase in oocyte aneuploidy. *Congenital Anomalies (Kyoto)*, *52*, 8–15.
- Ottoloni, C. S., Newnham, L. J., Capalbo, A., Natesan, S. A., Joshi, H. A., Cimadomo, D., Griffin, D. K., Sage, K., Summers, M. C., Thornhill, A. R., Housworth, E., Herbert, A. D., Rienzi, L., Ubaldi, F. M., Handyside, A. H., & Hoffmann, E. R. (2015). Genome-wide maps of recombination and chromosome segregation in human oocytes and embryos show selection for maternal recombination rates. *Nature Genetics*, *47*, 727–735.
- Ottoloni, C. S., Capalbo, A., Newnham, L., Cimadomo, D., Natesan, S. A., Hoffmann, E. R., Ubaldi, F. M., Rienzi, L., & Handyside, A. H. (2016). Generation of meiomaps of genome-wide recombination and chromosome segregation in human oocytes. *Nature Protocols*, *11*, 1229–1243.
- Rienzi, L., Capalbo, A., Stoppa, M., Romano, S., Maggiulli, R., Albricci, L., Scarica, C., Farcomeni, A., Vajta, G., & Ubaldi, F. M. (2015). No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: A longitudinal cohort study. *Reproductive Biomedicine Online*, *30*, 57–66.
- Scott, R. T., Jr., Ferry, K., Su, J., Tao, X., Scott, K., & Treff, N. R. (2012). Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: A prospective, blinded, nonselection study. *Fertility and Sterility*, *97*, 870–875.
- Scott, R. T., Jr., Upham, K. M., Forman, E. J., Zhao, T., & Treff, N. R. (2013). Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertility and Sterility*, *100*, 624–630.
- Treff, N. R., Tao, X., Ferry, K. M., Su, J., Taylor, D., & Scott, R. T., Jr. (2012). Development and validation of an accurate quantitative real-time polymerase chain reaction-based assay for human blastocyst comprehensive chromosomal aneuploidy screening. *Fertility and Sterility*, *97*, 819–824.
- Vega, M., Breborowicz, A., Moshier, E. L., McGovern, P. G., & Keltz, M. D. (2014). Blastulation rates decline in a linear fashion from euploid to aneuploid embryos with single versus multiple chromosomal errors. *Fertility and Sterility*, *102*, 394–398.
- Wapner, R. J., Martin, C. L., Levy, B., Ballif, B. C., Eng, C. M., Zachary, J. M., Savage, M., Platt, L. D., Saltzman, D., Grobman, W. A., Klugman, S., Scholl, T., Simpson, J. L., Mccall, K., Aggarwal, V. S., Bunke, B., Nahum, O., Patel, A., Lamb, A. N., Thom, E. A., Beaudet, A. L., Ledbetter, D. H., Shaffer, L. G., & Jackson, L. (2012). Chromosomal microarray versus karyotyping for prenatal diagnosis. *The New England Journal of Medicine*, *367*, 2175–2184.
- Wellesley, D., Dolk, H., Boyd, P. A., Greenlees, R., Haesler, M., Nelen, V., Game, E., Khoshnood, B., Doray, B., Rissmann, A., Mullaney, C., Calzolari, E., Bakker, M., Salvador, J., Addor, M. C., Draper, E., Rankin, J., & Tucker, D. (2012). Rare chromosome abnormalities, prevalence and prenatal diagnosis rates from population-based congenital anomaly registers in Europe. *European Journal of Human Genetics*, *20*, 521–526.
- Wu, L. L., Russell, D. L., Wong, S. L., Chen, M., Tsai, T. S., ST John, J. C., Norman, R. J., Febbraio, M. A., Carroll, J., & Robker, R. L. (2015). Mitochondrial dysfunction in oocytes of obese mothers: Transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors. *Development*, *142*, 681–691.

Tuboperitoneal Diagnosis

Guillermo Marconi, Instituto Valenciano de Infertilidad (IVI-RAM), Buenos Aires, Argentina

© 2018 Elsevier Inc. All rights reserved.

Glossary

Colpomicrohysteroscope An endoscopic visualization instrument, which unlike other optics, has a magnification capability of up to 40 ×.

Reproductive unit The ovaries, fallopian tubes, and uterus constitute a group of organs that work together to perform a specific function: reproduction.

Abbreviations

ATPF Altered tuboperitoneal factor

HSG Hysterosalpingography

PID Pelvic inflammatory disease

To facilitate understanding, we will define “reproductive unit” as the group of sexual organs inside the female pelvis, each of which performs a specific and irreplaceable function:

- The ovary produces oocytes.
- The fallopian tube transports both gametes, is where fertilization takes place, nourishes the early embryo, and delivers the embryo to the uterus.
- The uterus is the place where the embryo develops until it is ready to be delivered.

This reproductive unit is in close proximity to a number of abdominal-pelvic organs, the closest of which are the bowel and the bladder, and it is also associated with the peritoneal serosa.

The extraperitoneal location of the ovary does not protect it from pathological events occurring in the pelvic peritoneum and that affect the proper functioning of the gonad.

The fallopian tube is not a mere tube that transports cells, but is rather an active organ that plays a crucial role in the reproductive process.

Different pathogens can easily reach the peritoneal cavity and damage the reproduction unit, the abdominal organs, and the peritoneum.

The tuboperitoneal factor is the relationship among the ovaries, fallopian tubes and uterus, and between these reproductive organs and the non-reproductive abdominal organs and the peritoneum.

The tuboperitoneal factor can be affected by several events:

- Adnexal infections (salpingitis, adnexitis, etc.)
- Pelvic infections (pelvic inflammatory disease, PID)
- Degenerative processes, such as endometriosis
- Mechanical factors associated with contraceptive procedures (tube ligation) or with inadequate surgical procedures that damage the surface of the peritoneum or integrity of the organs, especially the adnexae

Tubal factor pathology can present with different manifestations:

- (a) *Adhesions*: Adhesions are pathological bonds that form between organs. They can vary in structure, thickness, and size, and can be vascular or avascular, thick or filmy, and involve only a part or the entire surface of the ovaries and tubes, isolating them or binding them together, to other pelvic organs, and/or to the peritoneum. These adhesions result in altered tuboperitoneal factor (ATPF) (Figs. 1–3).

The causes of ATPF include pelvic inflammatory disease (PID), endometriosis, malformations, and pelvic surgery sequelae affecting the uterus, adnexae, and/or organs in close proximity to the genital system as a result of iatrogenic surgical procedures (appendectomy).

- (b) *Damaged tubal patency caused by obstruction*: Tubal disease can be partial (obstruction) or total (occlusion), and can involve the proximal, medial, or distal portion of the tube.

Proximal tubal obstruction is the blockage of the oviduct at the level of the cornua of the uterus, and is rarely partial (Fig. 4).

Medial tubal occlusion affects the isthmic portion of the fallopian tube and results from mechanical factors, such as voluntary tubal ligation and iatrogenic surgical procedures, as well as infectious degenerative diseases (Fig. 5).

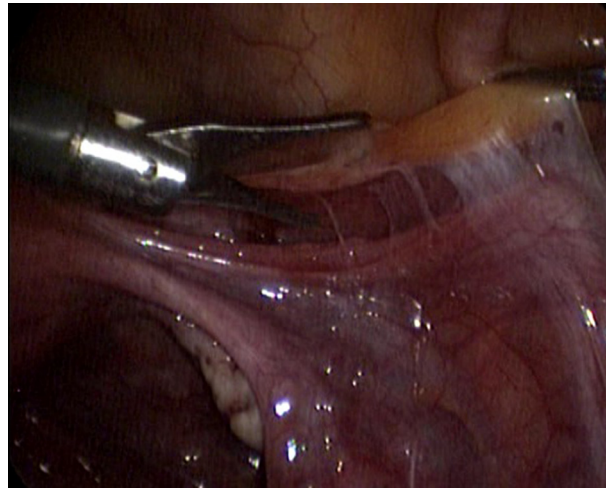


Fig. 1 Filmy adhesions from the ovary to the peritoneum.

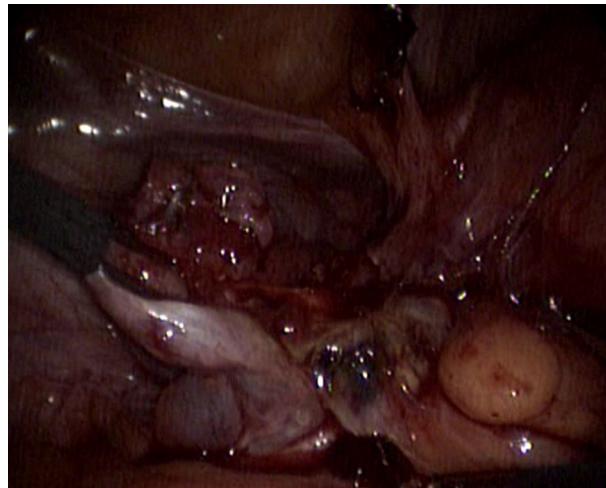


Fig. 2 Dense adhesions between the ovary, the tube, bowel, and uterus.

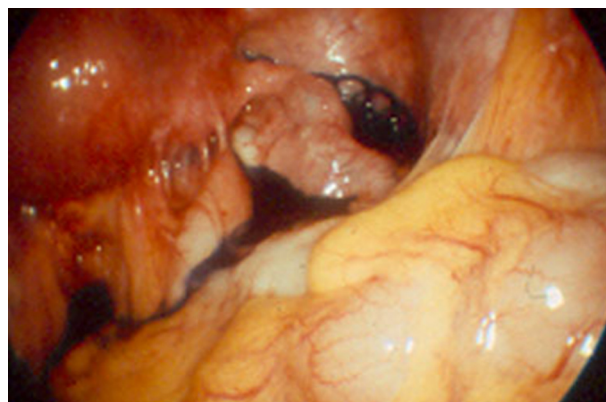


Fig. 3 Severe affected tubo-peritoneal factor.



Fig. 4 Hysterosalpingography. Proximal tubal obstruction. The dye has not spilled into the tubes.

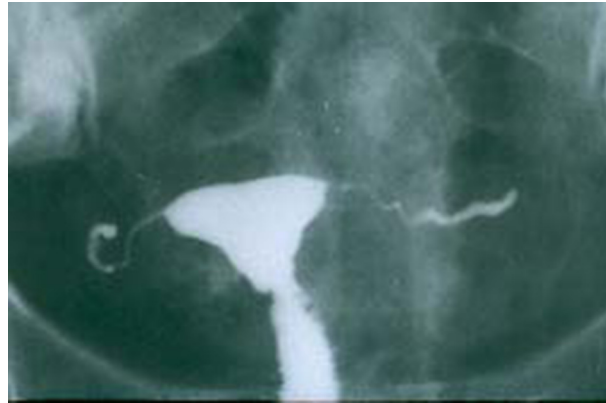


Fig. 5 Medial tubal obstruction.

Distal tubal obstruction involves the fimbrial end of the tube, and is caused by damage to the tubal serosa. It can present as:
Phimosis: (a) prefimbrial: prefimbrial phimosis is a ring-like fibrous narrowing at the ampullary-fimbrial junction; (b) post-fimbrial: postfimbrial phimosis is an adherence that covers all the fimbriae, distorting their typical finger-like appearance. Patency is maintained in both prefimbrial and post fimbrial phimosis.

Fimbrial conglutination: The distal end of the fallopian tube is completely occluded, but with no distention of the lumen.

Hydrosalpinx: It is the most severe form of tubal damage. The tube is distally occluded and dilated. The integrity of the salpingeal epithelium is severely affected (**Figs. 6 and 7**).

(c) *Both pathologies combined*. This is the most frequent case. Tubal damage is usually accompanied by peritoneal adhesions.



Fig. 6 Hysterosalpingography. Bilateral hydrosalpinx.

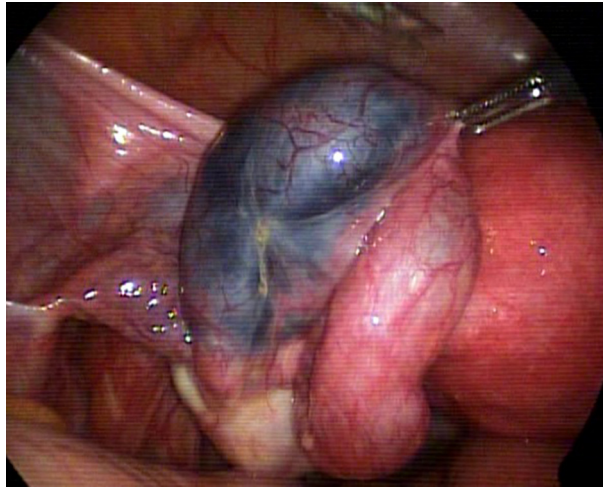


Fig. 7 Laparoscopic view of hydrosalpinx of the left tube.

Diagnostic Methods

Tuboperitoneal factor evaluation means making sure all the pelvic structures are in the right place, in proper relationship to one another, and capable of functioning.

Assessment of tuboperitoneal factor can be performed using hysterosalpingography, hysterosonography, laparoscopy, and salpingoscopy.

Hysterosalpingography (HSG) is a radiographic study that has been used for almost 100 years. Nevertheless, it has not lost validity and is widely used. The method consists of instilling a water-soluble iodinated solution into the uterus through the cervix, using a special cannula. The solution must be injected slowly and progressively to avoid masking surface lesions of the uterus cavity and causing pain to the patient. The procedure is performed while using fluoroscopy with intermittent still images for documentation.

The obtained images allow visualizing the shape of the cervix, isthmus, uterus cavity, tube patency, and the passage of the contrast dye into the abdominal cavity (Fig. 8).

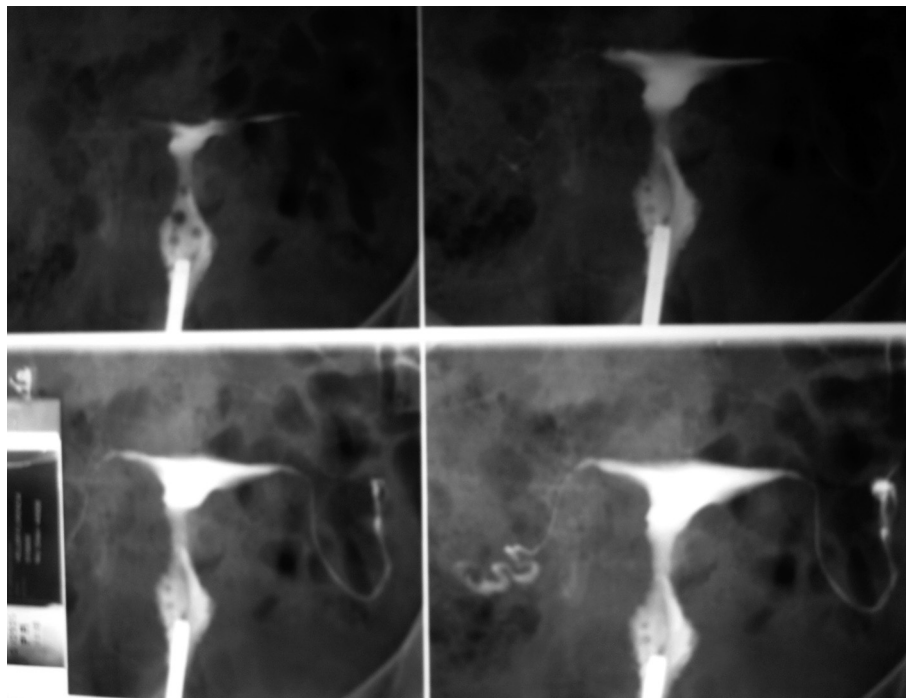


Fig. 8 Normal hysterosalpingography. Note progressive filling of the uterus, both tubes, and passage of the dye into the abdominal cavity.

This method is not useful for diagnosing peritoneal adhesions except for very severe cases involving the distal end of the tube, in which case the images will show peritoneal sacs filled with the contrast dye. These images are often confused with hydrosalpinx (Figs. 6 and 9).

Hysterosonography is a useful method that can be employed in the office setting. It merely involves instilling saline solution through the cervix using a cannula similar to that used for HSG, and then observing the passage of the solution in the abdominal cavity using the transvaginal ultrasound transducer. It is very useful to examine the uterus. Its high resolution not only allows examining the contour of the uterus but also the structure of the uterine wall, thus providing a complete evaluation of the organ. Conversely, it is not recommended for observation of the tubes; the resolution quality of the images of the contour of the salpinx walls and the resolution of the details of the tubal folds is inferior to that of HSG. This method only provides information on the passage of liquid in the pelvic cavity, and is therefore not considered the method of choice by most physicians.

Laparoscopy is the choice method for diagnosing pelvic adhesions; the latter cannot be detected by ultrasound or by radiographic studies. Although it is a surgical procedure, which must be performed under general anesthesia in an operating room, the advent of small caliber laparoscopes, 5 mm in diameter, allows carrying out the procedure on an outpatient basis. It poses the additional advantage of being therapeutic, since pathology can be treated upon detection without affecting the post-operative course of the patient (Figs. 10 and 11).

Salpingoscopy is the micro endoscopic examination of the tubal mucosa. It is performed as follows: (Marconi et al., 1992) during laparoscopy, methylene blue diluted in 10% saline solution is injected through the cervix to stain damaged mucosa cells. Saline solution is instilled immediately after in order to rinse the stain and dilate the tube, and thus facilitate endoscopic visualization. Examination is performed using a colpomicrohysteroscope without an irrigation sheath, inserted in the abdominal cavity through the second laparoscopic trocar, to then canalize the tubal ostium. An advantage of colpomicrohysteroscopy over other types of optics is that the magnification device it employs allows microscopically examining details of the tubal mucosa. The parameters typically analyzed by salpingoscopy include: (a) the appearance of the endosalpingeal folds (Figs. 12–14) (b) the vessels, (c) the presence of adhesions in the tubal lumen (Fig. 15), and (d) the presence of methylene-blue stained cell nuclei in the endosalpinx (Fig. 16). The presence of adhesions in the tubal mucosal folds or blocking the lumen, in addition to the finding of methylene-blue

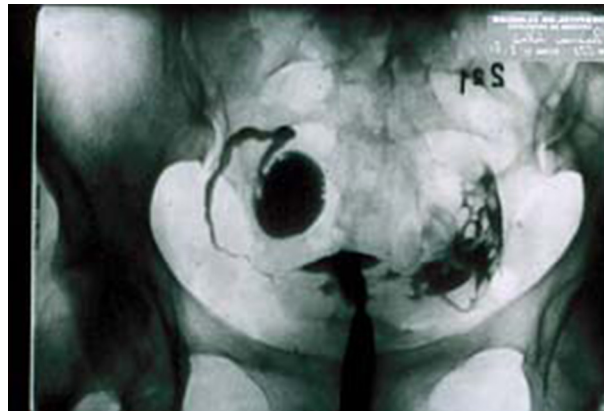


Fig. 9 Hysterosalpingography. What appears to be a hydrosalpinx is in fact a sac of peritoneal adhesions filled with the contrast dye.

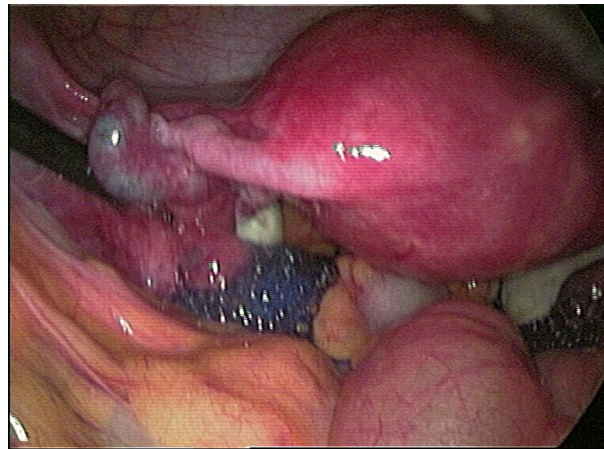


Fig. 10 Normal flow of methylene contrast through *left tube*.

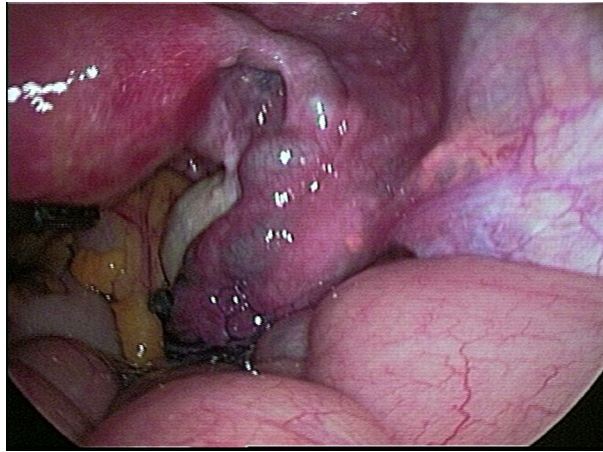


Fig. 11 Normal flow of methylene contrast through *right tube*.

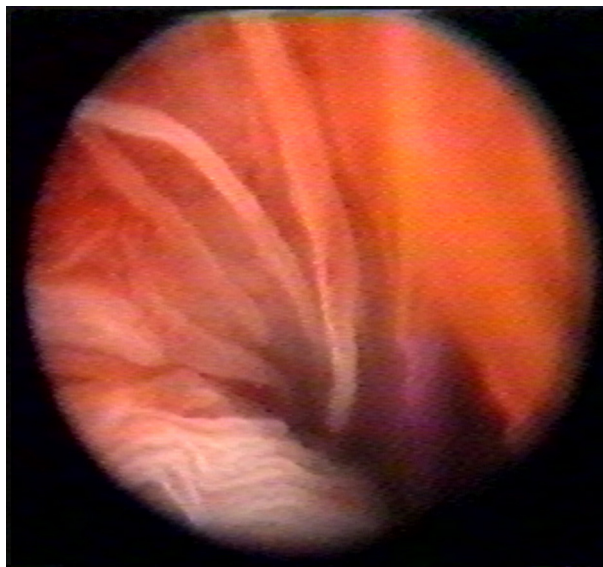


Fig. 12 Salpingoscopy. Normal appearance of the tubal mucosa. The tubal folds can be seen clearly.

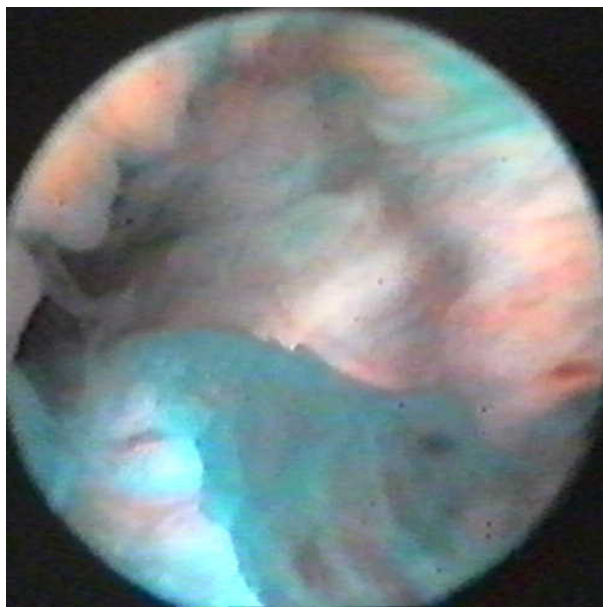


Fig. 13 Salpingoscopy. Damaged tubal mucosa.

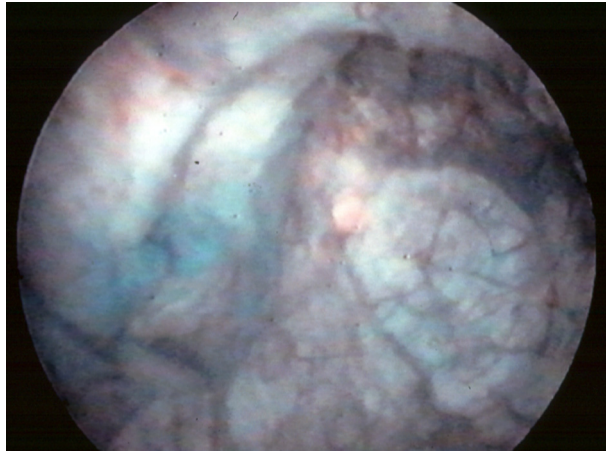


Fig. 14 Severe tubal damage; note the absence of mucosal folds.

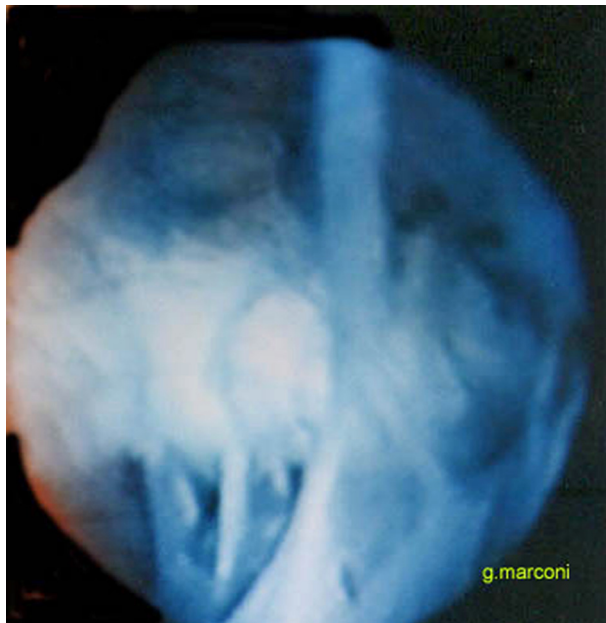


Fig. 15 The tubal lumen is blocked by adhesions.

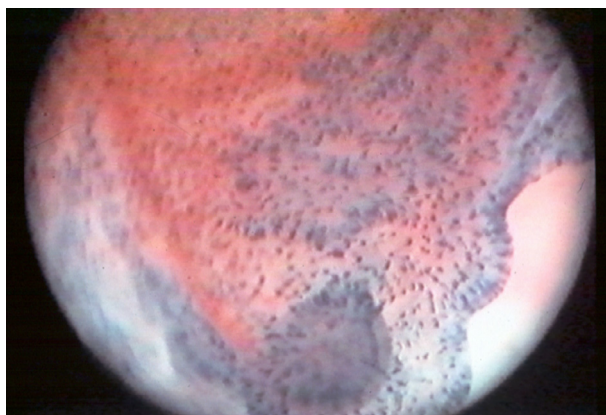


Fig. 16 Salpingeal mucosal folds showing a great number of stained cell nuclei.

stained cell nuclei are indicators of bad prognosis and irreversible damage seriously affecting tube recovery (Video 1 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64728-1>). A number of authors have proposed salpingoscopy as a routine procedure to assess tubal factor (Marconi et al., 1992; De Bruyne et al., 1997; De Bruyne et al., 1989; Marana et al., 1995; Heylen et al., 1995; Shapiro et al., 1988).

Conclusion

As stated at the beginning of this chapter, the key to treatment success is precise and accurate diagnosis.

The diagnostic battery for diagnosis of tuboperitoneal factor includes hysterosalpingography, hysterosonography, laparoscopy, and salpingoscopy. Clearly, it is not mandatory to perform all these tests to rule out tubal pathology. Nevertheless, it can be stated that, like hysterosonography, hysterosalpingography is the method of choice to confirm fallopian tube patency and uterine cavity integrity. The latter can also be confirmed by hysterosonography. Aside from very rare exceptions, without laparoscopy it would be impossible to confirm that the patient has no adnexal-peritoneal adhesions. Salpingoscopy is the real gold standard to evaluate the integrity of the tubal mucosa.

References

- De Bruyne, F., Puttemans, P., Boeckx, W., & Brosens, I. (1989). The clinical value of salpingoscopy in tubal infertility. *Fertility and Sterility*, 51, 339–340.
- De Bruyne, F., Jürgens, H., & Reinhart, W. (1997). The prognostic value of the salpingoscopy. *Human Reproduction*, 12, 266–271.
- Heylen, S. M., Brosens, I. A., & Puttemans, P. J. (1995). Clinical value and cumulative pregnancy rates following rigid salpingoscopy during laparoscopy for infertility. *Human Reproduction*, 10, 2913–2916.
- Marana, R., Rizzi, M., & Muzzi, L. (1995). Correlation between the American fertility Society classification of adnexal adhesion and distal tubal occlusion, salpingoscopy, and reproductive outcome in tubal surgery. *Fertility and Sterility*, 64, 924–929.
- Marconi, G., Augé, L., Sojo, E., Young, E., & Quintana, R. (1992). Salpingoscopy: Systematic use in diagnostic laparoscopy. *Fertility and Sterility*, 57, 742–746.
- Shapiro, B., Diamond, M., & De Charney, A. (1988). Salpingoscopy: An adjunctive technique for evaluation of the fallopian tube. *Fertility and Sterility*, 49, 1076–1079.

Microbiota and Pathogen Screening in the Female Reproductive Tract

Inmaculada Moreno*, Igenomix, Valencia, Spain; and Stanford University, Stanford, CA, United States

Garcia-Graulolanda García-Grau*, Igenomix Foundation, Valencia, Spain; and Valencia University, Valencia, Spain

Carlos Simón, University of Valencia/INCLIVA, Valencia, Spain; Igenomix, Valencia, Spain; Stanford University, CA, United States; and Baylor College of Medicine, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

16S rRNA Is the component of the 30S small subunit of a prokaryotic ribosome. The 16S rRNA gene sequencing is used for phylogenetic studies, as its presence is highly conserved among bacteria, but its sequence has diverged across evolution becoming species-specific.

Biofilm An assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric matrix. Biofilms can be formed by a single bacterium, but are often made up of several species of bacteria, as well as fungi, algae, and protozoa.

Community state types (CST) Profile that defines the total bacterial community of a given body site based on the relative abundances of each bacteria.

Dysbiosis Is defined as a shift in the physiologic microbiota resulting in an imbalance between commensal and pathogenic bacteria. It is speculated that changes in microbial composition contributing to the initiation and/or persistence of many diseases.

Endometrial receptivity analysis (ERA) Is a personalized genetic test, developed and patented in 2009 by IGENOMIX, to diagnose the endometrial receptivity state in the window of implantation.

Human microbiome project (HMP) Initiative launched by the National Institutes of Health (USA) with the goal of identifying and characterizing the microorganisms that are found in association with the human body. This project aimed to test how changes in the human microbiome are associated with human health or disease.

Metagenomics Use of DNA sequencing techniques to study DNA extracted directly from environmental samples, which gives the whole profile of the microbial diversity within the sample, avoiding limitations of traditional microbiological culture. In addition to the information about taxonomic diversity, metagenomics can provide insights into the physiology of the organisms present in the environment.

Microbiome The totality of microorganisms and their collective genetic material present in or on the human body.

Next-generation-sequencing (NGS) Modern technologies that allow the sequencing of DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, revolutionizing the study of genomics and molecular biology.

Operational taxonomic unit (OTU) Is a term used to classify groups of closely related individuals. This concept is generally used to refer to clusters of organisms, grouped by DNA sequence similarity of a specific taxonomic marker gene. OTUs are the most commonly used units of microbial diversity, especially when analyzing 16S rRNA gene.

Abbreviations

16S rRNA 16S ribosomal RNA

ART Assisted reproductive techniques

BV Bacterial vaginosis

CE Chronic endometritis

CSTs Community states types

EF Endometrial fluid

ERA Endometrial receptivity analysis

ET Embryo transfer

HIV Human immunodeficiency virus

HMP Human microbiome project

IVF In vitro fertilization

LD *Lactobacillus* dominated microbiome

NIH National institutes of health

NLD Non-*Lactobacillus* dominated microbiome

OTU Operational taxonomic unit

*Both authors contributed equally to this work.

RIF Repeated implantation failure
RM Recurrent miscarriages
UGT Upper genital tract
WGS Whole genome sequencing

The Human Microbiome

The concept of the human microbiome was suggested in 2001 by Joshua Lederberg who defined it as “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space” (NIH HMP Working Group, 2009). This community includes microorganisms as eukaryotes, archaea, bacteria and viruses, wherein bacteria are the most numerous, and better studied members of the human microbiome.

These microbes are not generally harmful for us; in fact, when these commensal bacteria are in balance, they play fundamental roles in human health. For example, they produce some essential vitamins that the human body is not able to produce on its own, break down our food to obtain nutrients, support the function of the immune system and even produce helpful anti-inflammatory compounds which interfere with other disease-causing microbes. In contrast, it has been seen that changes in the microbiome composition or an imbalance of the microbial communities are correlated with numerous pathologies such as asthma, atopic dermatitis, periodontal disease, obesity, cancer, Crohn’s disease and other gut alterations (Dietert and Dietert, 2015).

Most published medical literature has focused on the microbiome involved in pathogenesis, while only few publications have focused on the physiologic role that the microbiome plays. The importance of this physiologic role was evidenced in 2007 by the Human Microbiome Project (HMP), led by the National Institutes of Health (NIH), that characterized the microbiome of different body sites in healthy volunteers in order to understand the synergistic interactions between the microbiome and its host. This project provided novel and valuable data through the comprehensive analysis of global communities, and raised the possibility of improving human health through monitoring or manipulating the human microbiome (NIH HMP Working Group, 2009).

Characterization of the Human Microbiome

Much of our knowledge about the human microbiota comes from culture-based approaches. Nevertheless, many organisms cannot be identified with this technique. Depending on the body site, it has been estimated that between 20% and 60% of the human microbiome is uncultivable (NIH HMP Working Group, 2009), which results in underestimation of the ecosystem diversity as well as failing to identify microorganisms associated with a disease. For this reason, although the culture data are still informative and useful, must be reinterpreted within the new technological scenario.

Uncultivable species are now detectable thanks to advances in genome sequencing. Metagenomic sequencing techniques have enabled researchers to look deeper into the microbiome and have revealed an enormous amount of information that was not previously accessible. Most published studies use the metagenomic sequencing technique of the 16S rRNA bacterial gene. This gene contains nine hypervariable regions (V1 to V9) placed among highly conserved sequences. These variable regions serve as a molecular fingerprint since they are species-specific, and the comparison of their sequences with annotated bacterial genomes allows for accurate and rapid assignment of the resulting operational taxonomic units (OTUs) with their microbial taxa. Although the microbial world was revolutionized by the analysis of the 16S rRNA gene, new techniques of whole genome sequencing (WGS) have provided another level of depth in bacterial identification as it has facilitated the study of the functional genes expressed by the microorganisms (Mor et al., 2015).

Another aspect that has helped the detection of uncultivable bacteria is the publication of known sequences, which have allowed a taxonomic classification based on DNA sequences of species. The Human Microbiome Project (“HMP,” www.hmpdacc.org) was launched in the United States by the National Institutes of Health to investigate and characterize the human microbiome in 250 healthy volunteers at multiple body sites (oral cavity, skin, respiratory, gastrointestinal and urogenital tract) (NIH HMP Working Group, 2009). A combination of 16S rRNA gene sequencing, metagenomic whole-genome sequencing (WGS), and bioinformatics were used to identify the composition of microorganisms in a particular sample and elucidate the functional complexity of the microbial community. This project focused on the establishment of standards for metagenomic studies and provided a quality database that served as the basis for future research on the human microbiome.

Microbiome of the Reproductive Tract

Bacteria that colonize the genital tract represent 9% of the total human microbiome (Sirota et al., 2014). It has been found that the microbiome of the genital tract is involved in the physiology and pathophysiology of reproduction, affecting from gametogenesis to fertilization, embryo migration and implantation. For example, alterations in this microbiome have been related with pregnancy loss and poor obstetric outcomes during gestation and delivery (Franasiak and Scott, 2015a).

Thanks to sequencing data of the gene coding for the 16S rRNA subunit, the microbiome of the male and female reproductive tract has begun to be characterized in order to understand the potential role in reproductive competence.

The female reproductive tract has long been known to have an active microbiome. Although the greatest focus has been on the vaginal microbiota, there are also studies that demonstrate that the remainder of the female reproductive tract is not sterile. Several studies to date have shown that there is a small but active microbiome in the uterine cavity. In this regard, a recent study has characterized the endometrial microbiome of reproductive age women and has demonstrated the existence of an endometrial microbiota that can differ from that on the vagina (Moreno et al., 2016). Some studies performed in different patient's populations have also found bacteria in the fallopian tubes of women without obvious tubal infectious pathology and there are additional studies that have shown that the ovaries present an indigenous microbiome, being the Firmicutes phylum and the *Lactobacillus* genus the most abundant microorganisms found throughout the female reproductive tract (Franasiak and Scott, 2015b; Miles et al., 2017) (Fig. 1). Moreover, other investigations have revealed that other organs involved in reproduction, such as the placenta, are colonized with their own unique microbiome (Aagaard et al., 2014).

The male reproductive tract has also been seen to have an active microbiome, even in men without evidence of acute or chronic inflammation of their reproductive tract. However, it is still unknown if an altered microbiome may harm the spermatozoa, or alternatively, if differences in the seminal content might create a milieu where different types of bacteria can survive (Franasiak and Scott, 2015b).

On the other hand, it is becoming evident that the microbiome is not only a simple accumulation of floating bacteria on the surface of a human tissue. In many cases, three-dimensional structures called biofilms are formed, which might inhibit immune detection and reduce the effectiveness of antimicrobial treatment. Biofilms are usually present in the vagina, but commonly extend into the endometrial cavity and even up on the fallopian tubes (Franasiak and Scott, 2015b). Although no definitive conclusions have been established regarding the role of biofilms in the reproductive tract, they could have an important impact on reproductive function. In this sense, their study may provide essential information to guide the development of new therapeutic interventions that could improve IVF procedures.

Vaginal Microbiome

In the past, most of the studies that characterized the microbiome of the reproductive tract analyzed vaginal samples, due to the previous belief that the uterine cavity was sterile. The HMP focused the study of the urogenital tract microbiome on the analysis of vaginal samples collected from three specific sites (introitus, midpoint and posterior fornix) obtained from 113 female subjects. The results of this study showed that vaginal tract has the lowest alpha (within-sample) and beta (between-samples) diversity values, compared to other body sites tested, meaning that this is a very stable microbiota (Franasiak and Scott, 2015b).

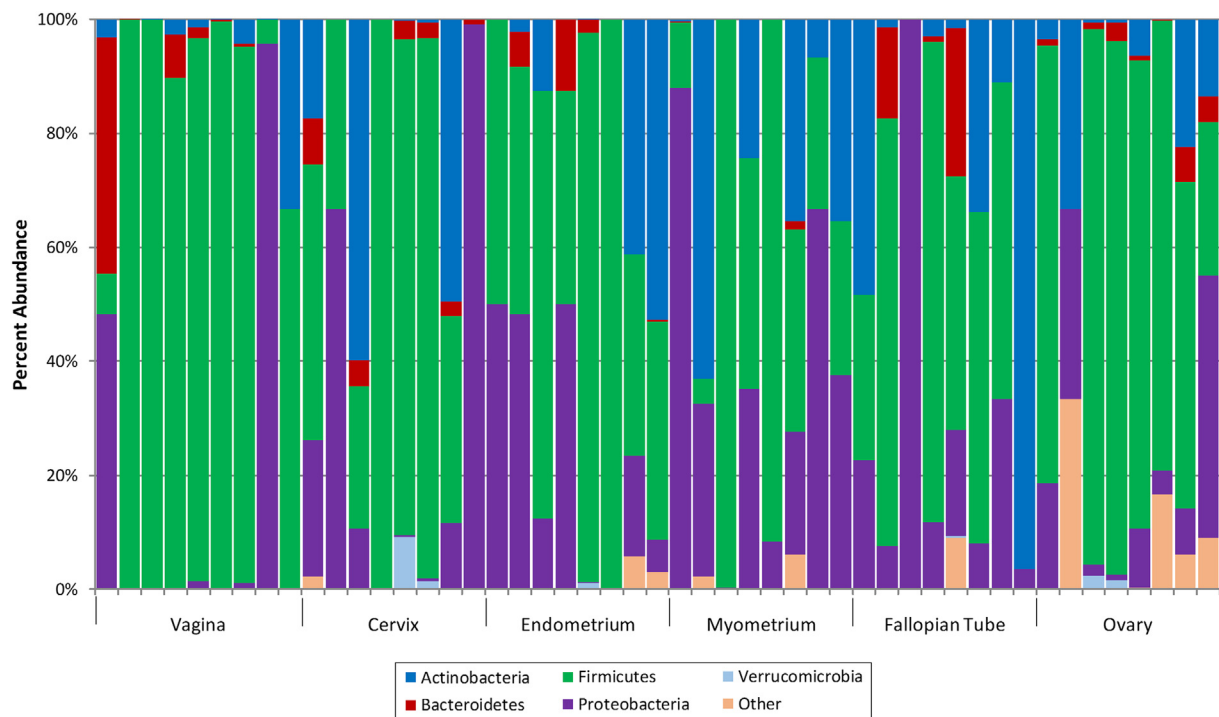


Fig. 1 Distribution of phyla by body site. Samples from each patient are grouped by individual body site. Each column represents the percent abundance of the indicated bacterial phyla present at each designated body site for an individual patient. Data for the five most common phyla are presented; all remaining phyla were grouped and classified as other. Reprinted from Miles, S. M., Hardy, B. L., and Merrell, D.S. (2017). Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oophorectomy. *Fertility and Sterility* 107(3), 813–820, Copyright 2017, with permission from Elsevier.

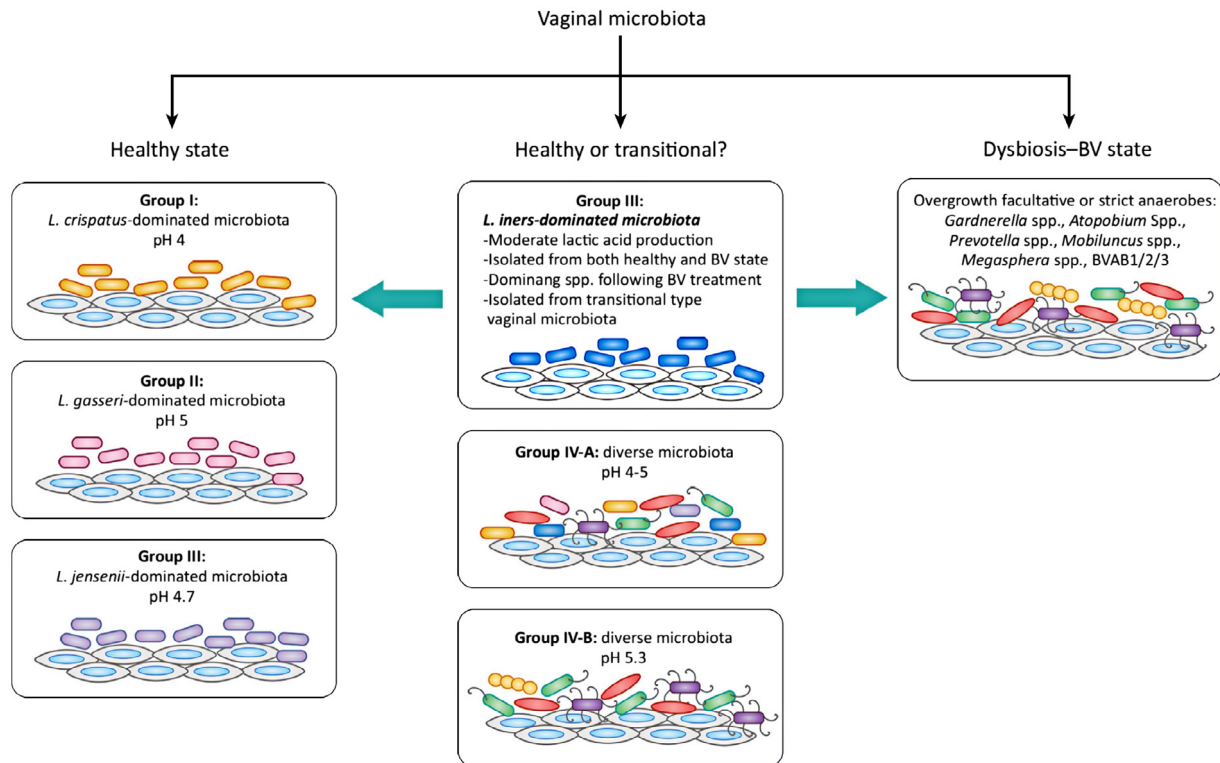


Fig. 2 Composition of vaginal microbiota in reproductive age women. The vaginal microbiota in healthy adult women can be divided into five community groups (CSTs). Group I, II, III, and V are dominated by *Lactobacillus* species belonging to *L. crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii* respectively, while group IV can be separated into two subgroups—IV-A and IV-B. Community group IVA is characterized by the presence of a mixed population of *L. crispatus* or *L. iners* with strict anaerobes, while IVB has a higher proportion of anaerobes members. Reprinted from Petrova, M. I., Reid, G., Vaneechoutte, M., and Lebeer, S. (2017). *Lactobacillus iners*: Friend or foe? *Trends in microbiology*, **25**(3), 182–191, Copyright 2017, with permission from Elsevier.

It has been widely reported that the vaginal microbiota of healthy women is usually dominated by *Lactobacilli*, which represents between 90% and 95% of the total bacteria in the reproductive tract (Sirota et al., 2014). These bacteria play an important role in maintaining the vaginal health of women because they produce lactic acid, which lowers the vaginal pH, and some of them produce hydrogen peroxide (H_2O_2), bacteriocins and other antimicrobial substances to inhibit the growth of pathogenic organisms that might cause infections in the vagina (Sirota et al., 2014).

In a study carried out by Ravel et al. (2011), the vaginal microbiome of 396 women was analyzed using molecular techniques and subsequently classified into five community states types (CSTs) depending on their bacterial composition. More than 70% of women presented vaginal microbiota dominated by *L. crispatus*, *L. gasseri*, *L. iners* and *L. jensenii*, corresponding to CST–I, –II, –III and –V, respectively. Whereas a smaller proportion of women diagnosed of bacterial vaginosis (BV) exhibited a CST-IV vaginal microbiome, characterized by lower percentage of *Lactobacillus* spp. and higher levels of anaerobic bacteria such as *Aerococcus*, *Atopobium*, *Dialister*, *Gardnerella*, *Megaphaera*, *Prevotella* and *Sneathia*, among other genera (Fig. 2).

The vaginal microbiome may be influenced by factors including age, hygiene habits, sexual activity, pregnancy and exogenous hormones (Sirota et al., 2014). In addition, it has been observed that the vaginal microbiota differs among human races and geographical locations; Caucasian and Asian populations present a higher prevalence of *Lactobacillus* dominated microbiome in comparison with Hispanic and African American women who present a higher prevalence of a CST-IV microbiota (Ravel et al., 2011). Apart from the ethnicity, the vaginal microbiome may also be affected by hormonal changes over the course of the menstrual cycle. The intervals of high variability occur during menses, whereas the more stable intervals coincide with the higher levels of estradiol and progesterone during the late follicular and midluteal phases (Gajer et al., 2012).

Bacterial vaginosis

In healthy women, *Lactobacillus* are usually the dominant bacterial species in the vagina, but changes in the bacterial community composition (such as the growth of strict anaerobes and Gram-negative bacteria) may favor an imbalance in the physiological conditions of the urogenital tract, leading to dysbiosis or a pathological state. As a result, a common disorder called bacterial vaginosis (BV) may occur.

BV is the most common vaginal syndrome of reproductive-age women, whose prevalence in the U.S. has been estimated to be 29.2% in the last decade (Moreno and Simon, 2017). This condition is characterized by an excessive vaginal discharge, a malodor

due to amines, an elevated vaginal pH (>4.5) and the presence of “clue” cells (squamous epithelial cells covered with adherent bacteria). BV is typically diagnosed using either the Amsel criteria, based on the aforementioned symptoms, or Nugent scoring method, which examines bacterial composition based on the morphotypes and the proportion of Gram-stained bacteria (Smith and Ravel, 2017).

Dysbiosis of vaginal microbiome in reproductive medicine

In contrast to the HMP, which investigated normal healthy volunteers, several studies have looked at the link between vaginal microbiome and infertility. Specifically, BV has been associated with increased risk of acquiring human immunodeficiency virus (HIV) and other sexually transmitted infections. Moreover, obstetric complications such as spontaneous abortion and preterm delivery have been associated to a disbalanced vaginal microbiota with reduced abundance of *Lactobacilli* (Smith and Ravel, 2017).

Using culture-based approaches, a study carried out by Selman and collaborators showed that vaginal-cervical microbial contamination at the time of embryo transfer (ET) is associated with significantly decreased pregnancy rates (Selman et al., 2007). Bacteria such as Enterobacteriaceae and *Staphylococcus*, isolated from the ET catheter, were associated with poor reproductive outcomes. Another study using sequencing-based technology also suggested that the vaginal microbiome on the day of embryo transfer affects pregnancy outcomes. Specifically, they found that low diversity indexes, with *Lactobacillus* dominant microbiota, have better pregnancy outcomes (Hyman et al., 2012). Similarly, a recent meta-analysis showed that BV is associated with a significantly increased risk of preclinical pregnancy loss; while it is not associated with decreased conception rates after IVF treatment (van Oostrum et al., 2013).

Endometrial Microbiome

Historically, the upper genital tract (UGT) had been considered a sterile site. However, endometrial cultures obtained from patients subjected to hysterectomy for benign conditions suggested the presence of microorganisms in the uterus of approximately one-quarter of the women examined (reviewed in Mor et al., 2015). Furthermore, the use of advanced molecular biology techniques has enhanced our understanding of the UGT microbiome.

A recent study by Mitchell et al. (2015) has provided additional evidence that the upper genital tract in asymptomatic women is not a sterile environment. In their study, 95% of endometrial samples analyzed, tested by qPCR for a set of 12 bacterial genes, were positive for at least one species of bacteria. However, the amount of bacterial DNA detected in the upper genital tract was consistently and significantly lower than in their paired vaginal samples, while significant changes were observed in the bacterial species detected in both samples.

Consistent with this work, a recent study performed using sequencing of the 16S rRNA gene has examined the vaginal and endometrial microbiome of asymptomatic and fertile women and has detected bacterial DNA in the endometrial samples of all subjects. Interestingly, about 20% of the women analyzed had different bacterial communities in the uterine cavity and the vagina, showing that the bacteria detected in the endometrium are not a carry-over from the vagina, demonstrating that, although very similar, both microbiomes are not always identical in each woman (Fig. 3). A total of 166 different OTUs were identified in the endometrial fluid samples analyzed from 22 fertile women. The most represented genus was *Lactobacillus* (71.7% of identified bacteria); while *Gardnerella* (12.6%), *Bifidobacterium* (3.7%), *Streptococcus* (3.2%), and *Prevotella* (0.866%) were the other most common genera in endometrial samples (Moreno et al., 2016).

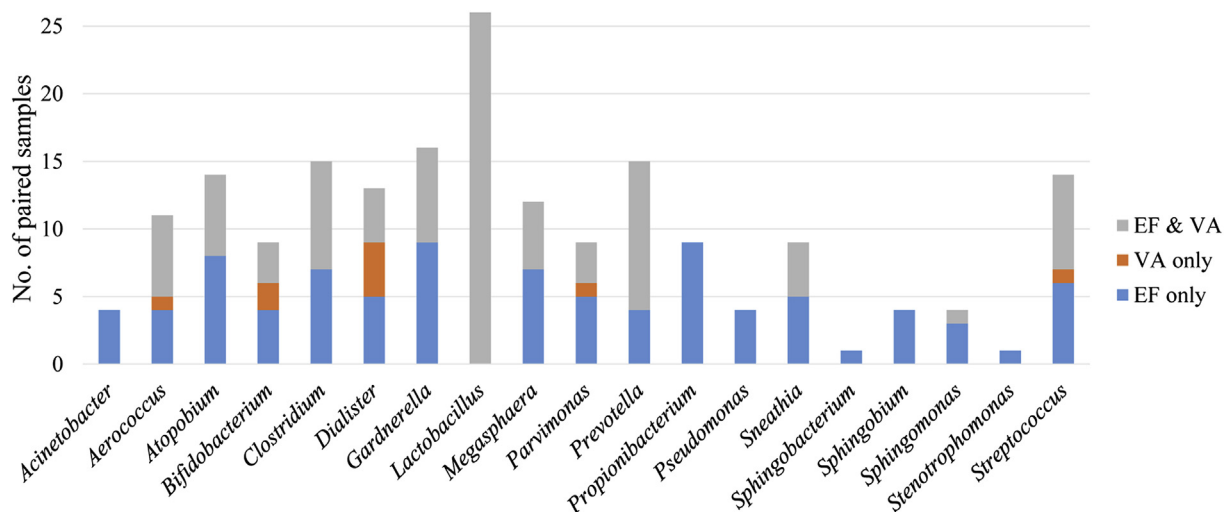


Fig. 3 Number of paired samples with detection of most abundant genera in vaginal aspirate (VA), endometrial fluid (EF) alone, and in both VA and EF. Reprinted from Moreno, I., Codoñer, F.M., Vilella, F. et al., Evidence that the endometrial microbiota has an effect on implantation success or failure. *American Journal of Obstetrics and Gynecology*, **215**(6), 684–703, Copyright 2016, with permission from Elsevier.

The differences observed between the upper and lower genital tract could be explained by the cervix, which serves as a barrier for the ascending bacteria from the vagina to the upper genital tract, and/or by the immune response that is exerted in the cervix to avoid colonization with microbial pathogens. Moreover, these differences could be influenced by the microbial ability to evade the immune response, or even because some microbes may remain in the upper genital tract after disappearing from the vagina and/or have better growth in the upper genital tract (Mitchell et al., 2015).

Uterine microbiome in gynecological diseases and obstetrical complications

Recently the uterine microbiome has also been related to several gynecological conditions. The results from different studies propose bacterial contamination of the endometrium as a potential new factor in the establishment of endometriosis, a disease characterized by the existence of endometrial epithelial and stromal tissue outside the uterine cavity. Lately, there are also studies that show an enrichment of several taxa in the reproductive tract of patients with endometrial hyperplasia and cancer compared to those with benign conditions, pointing to the infectious/inflammatory role of bacteria in the onset of endometrial cancer (reviewed in Moreno and Franasiak, 2017).

On the other hand, the microbiome has also been linked with diseases that affect pregnancy. For example, the chorioamnionitis, an obstetrical complication caused by the inflammation of the fetal membranes, has been linked to polymicrobial infections of microorganisms, which may ascend from the vagina and colonize the intrauterine cavity. In addition, a possible contribution of bacterial infections in the placenta of patients with preeclampsia, an obstetrical condition characterized by high blood pressure, has recently been determined (reviewed in Moreno and Franasiak, 2017).

Endometrial dysbiosis in reproductive medicine

The description of the endometrial microbiota from IVF patients has been attempted using classical microbiological culture. Different studies have linked the isolation of endometrial pathogens such as Enterobacteriaceae, *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli* and Gram-negative bacteria from the catheter tip at the time of embryo transfer, with significantly reduced implantation and pregnancy rates (reviewed in Moreno and Simon, 2017).

Subsequently, the endometrial microbiome of infertile patients and its functional impact on reproductive outcome have been assessed using 16S rRNA gene sequencing. A recent study has reported the endometrial microbiome at the time of ET in 33 IVF patients. The most represented bacteria found in the overall samples belong to the *Lactobacillus* and *Flavobacterium* genera. However, when the endometrial microbiome of patients with ongoing versus nonongoing pregnancies were compared, none of the OTUs found was able to significantly discriminate between these two groups (Fransiak et al., 2016).

In the most recent study, carried out by Moreno and colleagues (Moreno et al., 2016), the impact of the endometrial microbiome in reproductive function was analyzed. Endometrial fluid (EF) samples were obtained from 35 infertile patients with repeated implantation failure (RIF) presenting receptive endometrium analyzed with the Endometrial Receptivity Analysis (ERA) diagnosis tool ("ERA," www.igenomix.com). The results of this study showed that endometrial microbiota profile can be classified according to the structure and relative abundance of the bacterial OTUs identified in EF samples as *Lactobacillus* dominated microbiome (LD) ($\geq 90\%$) or non-*Lactobacillus* dominated microbiome (NLD) ($< 90\%$), as the percentage of *Lactobacillus*, served as a significant variable to predict the reproductive success (Fig. 4). The presence of a NLD microbiota significantly correlated with decreases in implantation (60.7% vs. 23.1%), pregnancy (70.6% vs. 33.3%), ongoing pregnancy (58.8% vs. 13.3%), and live birth (58.8% vs. 6.7%) rates in comparison to subjects with LD endometrial microbiota.

Chronic endometritis and reproductive function

The best example of pathology caused by an altered endometrial microbiota is chronic endometritis (CE). CE is a persistent inflammation of the endometrial lining caused by the infection of the uterine cavity mainly by bacterial pathogens. The presence of certain microorganisms such as *Streptococcus*, *E. coli*, *Enterococcus faecalis*, *Chlamydia*, and mycoplasma species as *Mycoplasma genitalium* and *Ureaplasma urealyticum* (Kitaya et al., 2016) have been related to the onset of CE in a high percentage of cases.

CE is usually asymptomatic which makes it difficult to estimate their prevalence, calculated for the general population between 0.8% and 19%. In infertile patients the prevalence has been estimated in 0.2%–46%, depending on the population type and the diagnostic criteria used on each study (Peymani and DeCherney, 2016). CE has been diagnosed in 12.9% of patients with miscarriages of unknown etiology and in 30.3% of patients with RIF (Kitaya et al., 2016). There are also studies that determine a higher prevalence of CE, reaching 60% in women with unexplained RM and about 66% of women with RIF (reviewed in Moreno et al., 2016). Matteo and colleagues reported that CE might reduce endometrial receptivity and cause infertility owing to an abnormal pattern of lymphocyte subsets causing an aberrant endometrial microenvironment in CE patients (Matteo et al., 2009).

The standard diagnosis of CE relies on histology, which is based on the identification of plasma cells in the endometrial stroma, sometimes supplemented by immunohistochemical quantification of syndecan-1 (CD138) positive cells. Also, the use of hysteroscopy has been proposed as a diagnostic method for CE by evaluating the presence of micropolyps, stromal edema, or hyperemia. Compared to histological findings, hysteroscopy has a diagnostic accuracy of 93.4% (reviewed in Kitaya et al., 2016), however, none of these methods is able to identify the bacterial pathogen causing such inflammation. The last method of CE diagnosis is the culture of infectious agents from the endometrial cavity, which identifies pathogens associated with the disease, providing information for a targeted therapy. In this case, the infectious agents are isolated upon microbial culture in about 73% of women histologically diagnosed of CE (reviewed in Moreno et al., 2016).

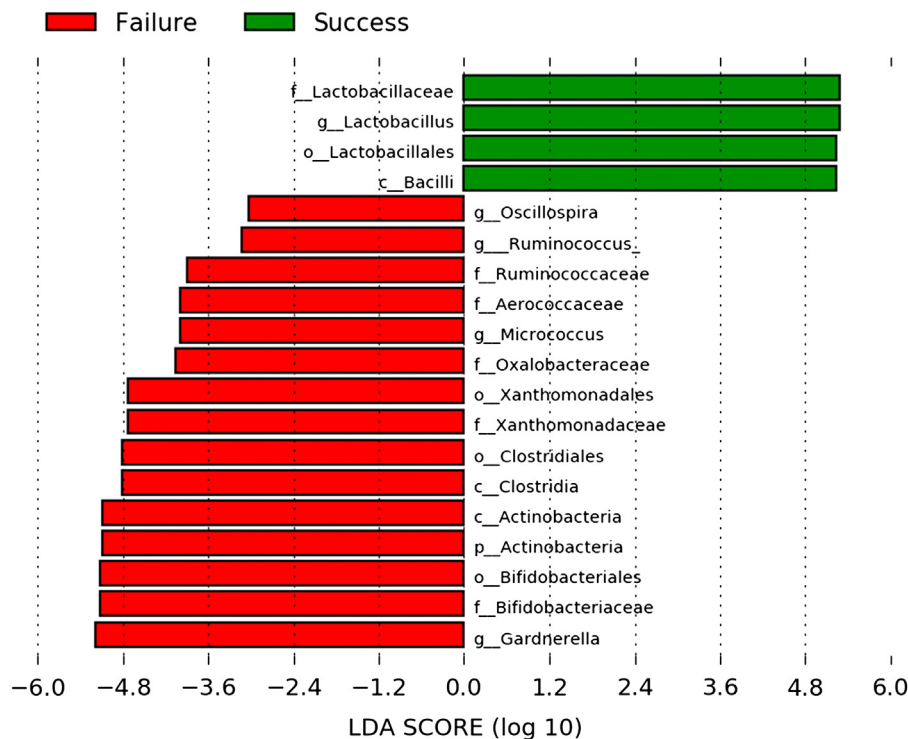


Fig. 4 *Lactobacillus* abundance is a significant variable to predict pregnancy success in IVF patients. Linear discriminant analysis (LDA) showing mean values of the most abundant operational taxonomic units (OTUs) in the receptive subjects grouped by their reproductive success. From data in Moreno, I., Codoñer, F.M., Vilella, F. et al., (2016). Evidence that the endometrial microbiota has an effect on implantation success or failure. *American Journal of Obstetrics and Gynecology*. **215**(6), 684–703.

In case there is no information available on microbiological culture or causative agents, some authors recommend the administration of broad-spectrum antibiotics, such as doxycycline, as the treatment of choice for CE. While treatments based on the antibiogram are critical to select the most effective treatment. This has been seen in recent clinical studies showing an improvement in clinical pregnancy and live birth rates in RIF's patients with CE treated with antibiogram-based antibiotic treatment (reviewed in [Peymani and DeCherney, 2016](#)).

Antimicrobials in Assisted Reproductive Techniques

Although the association between the reproductive tract microbiome and the reproductive outcome remains relatively poorly understood, there is a long history with the use of prophylactic antibiotics during assisted reproductive techniques (ART). Since many years, it has been suggested that microbial contamination during ART could negatively affect the outcomes in this cycle. For this reason, during ART procedures, antibiotics have been proposed as a way to decrease infections of the uterine cavity and thus increase pregnancy rates (reviewed in [Peymani and DeCherney, 2016](#)).

Despite this widespread practice, there are relatively few data supporting or rejecting the usefulness of antibiotics. A recent review analyzing existing data on the effect of antibiotics on reproductive outcomes showed that the administration of antibiotics prior to ET significantly reduced upper genital tract microbial contamination but this was not translated into better pregnancy rates, which were 36% in patients who received antibiotics and 35.5% in those who did not receive them ([Kroon et al., 2012](#)). One possible explanation for the lack of a clear benefit of antimicrobial use prior to ET is that broad-spectrum prophylactic antibiotics may interfere with both dysbiotic bacteria and healthy microbiome, procuring an altered endometrial environment for the ready-to-implant embryo.

For this reason, other ways to restore the physiological reproductive tract microbiome in case of dysbiosis must be explored. In this context, probiotics have been used to increase bacteria with beneficial profiles rather than directly eliminate pathogenic bacteria, since these probiotics present properties antagonistic to pathogens but complementary to host immunity. In fact, these organisms have been associated with conception, reducing the risk of infection, as well as potentially lowering the risk of a number of pregnancy complications ([Reid et al., 2013](#)).

The regeneration of the normal microbiota with the use of probiotics has recently been proposed as a potential therapy for many gynecological and obstetric diseases, either as complementary or as first choice treatment ([Álvarez et al., 2015](#); [Griffin, 2015](#); [Nader-Macías and Juárez Tomás, 2015](#)) (Table 1). In fact, they could be used after antibiotic therapy, with the aim of

Table 1 Possible beneficial effects of the use of probiotics in gynecological and obstetrical conditions

<i>Gynecological and obstetrical conditions</i>	<i>Suggested probiotic</i>	<i>Purpose</i>
Bacterial vaginosis	<i>L. rhamnosus</i> , <i>L. reuteri</i> , <i>L. acidophilus</i> , <i>L. gasseri</i> , <i>L. crispatus</i> , <i>L. fermentum</i> , <i>L. casei</i> , <i>L. bifidus</i> , <i>L. plantarum</i> , etc.	Treatment and prevention
Vulvovaginal candidiasis	<i>L. rhamnosus</i> , <i>L. reuteri</i> , <i>L. fermentum</i> and <i>L. acidophilus</i>	Treatment and prevention
Recurrent urinary tract infections	<i>L. crispatus</i>	Prevention
Preeclampsia	<i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>B. Lactis</i>	Prevention
Preterm birth	<i>L. acidophilus</i> , <i>L. rhamnosus</i> , <i>B. Lactis</i>	Prevention

L., *Lactobacillus*; *B.*, *Bifidobacterium*.

re-colonizing the mucosa before the pathogens can grow again. Specifically, probiotic supplementation with vaginal *Lactobacillus* has been investigated as a way to treat vaginal infections such as BV with efficient results (Recine et al., 2016). Although more functional metagenomic data are needed to characterize the physiological state of the uterine cavity leading to successful pregnancies, this approach could positively affect the outcomes of ART in the future.

Concluding Remarks and Future Perspectives

Knowledge regarding the interactions between the microbiome and the female reproductive tract is rapidly growing thanks to the emerging metagenomic techniques. The progress in the molecular analysis of bacteria has helped to overcome technical obstacles that historically prevented the detection of some bacteria by culture. In addition, these modern technologies allow an analysis that extends beyond phylogenetic descriptions and makes attempts to study the physiology and ecology of the microbiome.

A deeper understanding of normal physiology of the female reproductive tract, the study of biofilms and the identification of different dysbiotic states, could help to characterize the role of the microbiome in reproductive outcomes. All the efforts are now focused on the identification of a microbial signature responsible for reproductive failure or success. The attribution of negative IVF outcome to a specific isolated bacterium is no longer the best option, since to draw conclusions the entire microbial community needs to be addressed. Moreover, rather than trying to eliminate pathogens, nowadays the use of probiotics to restore the beneficial microbiota has been widely suggested. The use of probiotics in the clinical management of infertile patients promises to be a treatment that could improve pregnancy outcomes and prevent obstetrical complications associated with bacterial pathogens, improving thus the clinical care.

Summarizing, the process of implantation is complex and multifactorial, since the reproductive success is not only defined by endometrial histology and gene expression. The ongoing revolution in technology, multiple “-omics”, and multidimensional data analysis has opened the window to a greater level of study. It is time to further investigate the female reproductive tract microbiome and expand research to its virome, fungome, epigenome, and metabolome to increase our 360 degree understanding and to develop targeted therapies. Consequently, the presence of certain microbiome profiles should be considered as an additional risk factor for implantation failure and pregnancy loss, which highlights the importance of using microbiological assessment and probiotic treatments to improve and personalize IVF procedures.

References

- Aagaard, K., Ma, J., Antony, K. M., et al. (2014). The placenta harbors a unique microbiome. *Science Translational Medicine*, 6(237), 237ra65.
- Álvarez, G., Suárez, E., Rodríguez, J. M., & Pérez-Moreno, J. (2015). La microbiota en la mujer; aplicaciones clínicas de los probióticos. *Nutrición Hospitalaria*, 32(Supl 1), 56–61.
- Dietert, R. R., & Dietert, J. M. (2015). The Microbiome and Sustainable Healthcare. *Healthcare (Basel, Switzerland)*, 3(1), 100–129. <https://doi.org/10.3390/healthcare3010100>.
- Franasiak, J. M., & Scott, R. T. (2015a). Introduction: Microbiome in human reproduction. *Fertility and Sterility*, 104(6), 1341–1343.
- Franasiak, J. M., & Scott, R. T. (2015b). Reproductive tract microbiome in assisted reproductive technologies. *Fertility and Sterility*, 104(6), 1364–1371.
- Franasiak, J. M., Werner, M. D., Juneau, C. R., et al. (2016). Endometrial microbiome at the time of embryo transfer: next-generation sequencing of the 16S ribosomal subunit. *Journal of Assisted Reproduction and Genetics*, 33(1), 129–136.
- Gajer, P., Brotman, R. M., Bai, G., et al. (2012). Temporal dynamics of the human vaginal microbiota. *Science Translational Medicine*, 4(132), 132ra52.
- Griffin, C. (2015). Probiotics in obstetrics and gynaecology. *Australian and New Zealand journal of obstetrics and gynaecology*, 55(3), 201–209.
- Hyman, R. W., Herndon, C. N., Jiang, H., et al. (2012). The dynamics of the vaginal microbiome during infertility therapy with in vitro fertilization-embryo transfer. *Journal of Assisted Reproduction and Genetics*, 29(2), 105–115.
- Kitaya, K., Matsubayashi, H., Yamaguchi, K., et al. (2016). Chronic endometritis: Potential cause of infertility and obstetric and neonatal complications. *American Journal of Reproductive Immunology*, 75(1), 13–22.
- Kroon, B., Hart, R. J., Wong, B. M., Ford, E., & Yazdani, A. (2012). Antibiotics prior to embryo transfer in ART. *The Cochrane Database of Systematic Reviews*, 3, CD008995.
- Matteo, M., Cicinelli, E., Greco, P., et al. (2009). Abnormal pattern of lymphocyte subpopulations in the endometrium of infertile women with chronic endometritis. *American Journal of Reproductive Immunology*, 61(5), 322–329.

- Miles, S. M., Hardy, B. L., & Merrell, D. S. (2017). Investigation of the microbiota of the reproductive tract in women undergoing a total hysterectomy and bilateral salpingo-oophorectomy. *Fertility and Sterility*, 107(3), 813–820.e1. <https://doi.org/10.1016/j.fertnstert.2016.11.028>.
- Mitchell, C. M., Haick, A., Nkwopara, E., et al. (2015). Colonization of the upper genital tract by vaginal bacterial species in nonpregnant women. *American Journal of Obstetrics and Gynecology*, 212(5), 611.e1–9.
- Mor, A., Driggers, P. H., & Segars, J. H. (2015). Molecular characterization of the human microbiome from a reproductive perspective. *Fertility and Sterility*, 104(6), 1344–1350.
- Moreno, I., & Franasiak, J. M. (2017). Endometrial microbiota—New player in town. *Fertility and Sterility*. <https://doi.org/10.1016/j.fertnstert.2017.05.034>.
- Moreno, I., & Simon, C. (2017). Microbiological diagnosis: The human endometrial microbiome—Endometritis. In C. Simon, & L. C. Giudice (Eds.), *The endometrial factor: A reproductive precision medicine approach* (pp. 65–77). Boca Raton: CRC Press.
- Moreno, I., Codoñer, F. M., Vilella, F., et al. (2016). Evidence that the endometrial microbiota has an effect on implantation success or failure. *American Journal of Obstetrics and Gynecology*, 215(6), 684–703.
- Nader-Macias, M. E., & Juárez Tomás, M. S. (2015). Profiles and technological requirements of urogenital probiotics. *Advanced drug delivery reviews*, 92, 84–104.
- NIH HMP Working Group. (2009). The NIH Human Microbiome Project. *Genome Research*, 19(12), 2317–2323.
- van Oostrum, N., De Sutter, P., Meys, J., & Verstraelen, H. (2013). Risks associated with bacterial vaginosis in infertility patients: A systematic review and meta-analysis. *Human Reproduction*, 28(7), 1809–1815.
- Peymani R and DeCherney A (2016) Microbiome, Infection and Inflammation in infertility. Prof. A Darwish (Ed.), InTech, DOI: <https://doi.org/10.5772/63090>. Available from: <https://www.intechopen.com/books/genital-infections-and-infertility/microbiome-infection-and-inflammation-in-infertility>.
- Ravel, J., Gajer, P., Abdo, Z., et al. (2011). Vaginal microbiome of reproductive-age women. *Proceedings of the National Academy of Sciences*, 108(Suppl 1), 4680–4687.
- Recine, N., Palma, E., Domenici, L., et al. (2016). Restoring vaginal microbiota: biological control of bacterial vaginosis. A prospective case–control study using *Lactobacillus rhamnosus* BMX 54 as adjuvant treatment against bacterial vaginosis. *Archives of Gynecology and Obstetrics*, 293(1), 101–107.
- Reid, J. N., Bisanz, J. E., Monachese, M., Burton, J. P., & Reid, G. (2013). The rationale for probiotics improving reproductive health and pregnancy outcome. *American Journal of Reproductive Immunology*, 69(6), 558–566.
- Selman, H., Mariani, M., Barnocchi, N., et al. (2007). Examination of bacterial contamination at the time of embryo transfer, and its impact on the IVF/pregnancy outcome. *Journal of Assisted Reproduction and Genetics*, 24(9), 395–399.
- Sirota, I., Zarek, S. M., & Segars, J. H. (2014). Potential influence of the microbiome on infertility and assisted reproductive technology. *Seminars in Reproductive Medicine*, 32(1), 35–42.
- Smith, S. B., & Ravel, J. (2017). The vaginal microbiota, host defence and reproductive physiology. *Journal of physiology*, 595(2), 451–463.

Relevant Websites

<http://www.igenomix.com/tests/endometrial-receptivity-test-era/>—ERA.
www.hmpdacc.org—HMP.

Further Reading

Petrova, M. I., Reid, G., Vaneechoutte, M., & Lebeer, S. (2017). *Lactobacillus iners*: Friend or Foe? *Trends in microbiology*, 25(3), 182–191.

Coagulation Screening

Amparo Santamaría, University Hospital Vall d'Hebron and University Dexeus, Barcelona, Spain
Maria Cerdá Sabater, University Hospital Vall d'Hebron, Barcelona, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chronic myeloproliferative syndromes Are characterized by a clonal evolution associated with a significantly variable course, which may be complicated by thrombocythemia, (secondary) myelofibrosis, and finally acceleration (unstable phase) that merges into blastic crisis. Include as main subtypes polycythemia vera (PV), chronic idiopathic myelofibrosis (IMF), and essential thrombocythaemia (ET).

Factor inhibitor Inhibitors are a serious medical problem that can occur when a person with hemophilia has an immune response to treatment with clotting factor concentrates.

Hemophilia A Also called factor VIII (FVIII) deficiency or classic hemophilia, is a genetic disorder caused by missing or defective factor VIII, a clotting protein. Although it is passed down from parents to children, about 1/3 of cases are caused by a spontaneous mutation, a change in a gene.

Infertility Is defined as difficulty getting pregnant.

Miscarriage Is defined as a spontaneous abortion.

Primary immune thrombocytopenia (ITP) Is an acquired immune-mediated disorder characterized by isolated thrombocytopenia, defined as a peripheral blood platelet count less than 100,000 in the absence of any obvious initiating or underlying cause.

Thrombocytosis Is a disorder characterized by abnormally high levels of platelets in the blood, more than 450,000.

Von Willebrand disease Is a genetic disorder caused by missing or defective von Willebrand factor (VWF), a clotting protein. VWF binds factor VIII, a key clotting protein, and platelets in blood vessel walls, which help form a platelet plug during the clotting process.

Abbreviations

ACL Anticardiolipin antibodies

APCR Activated protein C resistance

APL Antiphospholipid antibodies

APLS Antiphospholipid syndrome

aPTT Activated partial thromboplastin time

cMPS Chronic myeloproliferative syndrome

ITP Primary immune thrombocytopenia

IVF In vitro fertilization

LA Lupus anticoagulant

LMWH Low molecular weight heparin

LVF Leiden V factor

OAPLS Obstetric antiphospholipid syndrome

PTM Prothrombin G20210A mutation

VWD Von Willebrand disease

vWF Von Willebrand factor

β2GP Anti-β2-glycoprotein 1 antibodies

Basis of Coagulation

The haemostatic system consists of blood vessels, platelets, and the plasma coagulation system including the fibrinolytic factors and their inhibitors. When a blood vessel is injured, three mechanisms operate locally at the site of injury to control bleeding: vessel wall contraction, platelet adhesion and aggregation (platelet plug formation), and plasmatic coagulation to form a fibrin clot. All three mechanisms are essential for normal hemostasis. Abnormal bleeding usually results from defects in one or more of these three mechanisms. For a better understanding of the pathogenesis of pathological bleeding, it is customary to divide hemostasis into two stages (i.e., primary and secondary hemostasis). Primary hemostasis is the term used for the instantaneous plug formation

upon injury of the vessel wall, which is achieved by vasoconstriction, platelet adhesion, and aggregation. The fibrin formation is not required for hemostasis at this stage.

Screening and Diagnosis of Coagulation Disorders in Women

After clinical assessment and family history, the clinician often has a general overview concerning the cause of a patient's bleeding or thrombosis. Routine screening tests include a complete blood cell count, platelet count, and evaluation of a peripheral blood sample, a prothrombin time, and an activated partial thromboplastin time. Thrombocytopenia may be due to an Immune Thrombocytopenic Purpura (ITP), and thrombocytosis to a chronic myeloproliferative syndrome. Again, the patient's history, physical findings, and evaluation of a well-prepared peripheral blood smear will be helpful in determining the cause of the patient's thrombocytopenia or essential thrombocythaemia (Lusher, 1996). (See Table 1)

An isolated prolongation of the activated partial thromboplastin time may result from low levels of factors VIII, IX, or XI. A slightly prolonged activated partial thromboplastin time (aPTT) and a moderate decrease in factor VIII may reflect von Willebrand disease (VWD) or the "carrier" state for hemophilia A. In women a greatly prolonged aPTT and very low levels of factor VIII (<3%) most often result from an acquired factor VIII inhibitor (autoantibody against factor VIII) or from severe (type III) of VWD. Also, a prolonged Prothrombin Time (TP) might be observed in other rare bleeding disorders, such as Factor VIIc or Factor X deficiency.

The thrombophilia study includes the following determinations: antithrombin (AT), plasma protein C (PC), antigenic and free protein S (PS), due to functional PS is no longer required as it leads to false diagnoses, functional fibrinogen, antiphospholipid antibodies (APL): lupus anticoagulant (LA), anticardiolipin antibodies (ACL), anti-β2-glycoprotein 1 antibodies (β2GP1), homocysteine, activated protein C resistance (APCR) and Factor VIII. The genetic study includes: Leiden V factor (LVF), prothrombin G20210A mutation (PTM) and ABO Genetic Group.

In the high-risk genetic study to increase the chances of diagnosing patients with hereditary thrombophilia, could be included: F12 Mutation, AT Cambridge Mutation (A384S mutation in the SERPINC1 gene), R67X mutation in the SERPINA10 gene, fibrinogen mutation. If the RPCA is normal, is recommended to perform LVF, if the RPCA is positive but FVL negative, perform factor V Cambridge and Hon Kong mutations (see Table 1).

Bleeding Disorders: Impact on Fertility

Prevalence and Consequences

Women suspected of having a bleeding disorder (or who are hemophilia carriers) should undergo diagnostic testing before becoming pregnant to ensure appropriate preconception counseling and pregnancy management. Bleeding disorders also are more prevalent in women with menorrhagia, and prevalence ranges from 10% to 100%, depending on the specific bleeding disorder. During pregnancy, women with bleeding disorders may have an increased risk of bleeding complications. At delivery, affected women are more likely to experience postpartum hemorrhage, particularly delayed or secondary postpartum hemorrhage.

Women can be affected by several different bleeding disorders such as VWD; platelet defects; Hemophilia A, B or C; and other more rare clotting deficiencies, as well as disorders involving the fibrinolytic system (Kadir and James, 2009).

The prevalence of bleeding disorders in females is unknown. However, VWD, the most common bleeding disorder in humans, may affect between 1% and 2% of the general population. Among those women seeking medical care for menorrhagia, the incidence of VWD or another bleeding disorder may be as high as 20%.

Von Willebrand Disease

VWD is the most common inherited bleeding disorder, and it affects men and women equally. VWD follows an autosomal dominant inheritance pattern. In VWD the defect is in vWF. The affected individual may have abnormal levels of structurally and functionally normal vWF (this is called "classic" or type I VWD) or may produce vWF that is structurally and functionally abnormal (VWD type II). Individuals who inherit a gene for VWD from both parents have severe (type III) VWD, will have extremely low levels (<3%) of vWF and factor VIII and will have a very prolonged bleeding time. In most populations type I disease is the most common form, whereas type III is the least commonly encountered form. It should be noted that levels of vWF can be

Table 1 Screening and diagnosis of coagulation disorders in women

Coagulation study	Result	Possible hemostatic disorders
Platelets count	Thrombocytopenia Thrombocytosis	ITP, vWD cMPS
Prolonged aPTT	LA FVIIIc, FXIc; FIXc, FIIc, FXc, vWF	Autoimmune disease Rare bleeding disorders such as Haemophilia, or vWD
Prolonged TP	FIIc, FVc, FVIIc, FXC	Rare bleeding disorders

influenced by the patient's blood type (persons who have blood type AB have 60%–70% higher levels than persons who have blood type O) and can be elevated during pregnancy, stress, and hyperthyroidism. The two major functions of vWF are to serve as a “bridge” between platelets and injury sites in blood vessel walls and to protect circulating factor VIII from rapid proteolytic degradation. Thus, if a patient has either too little or functionally abnormal vWF, the bleeding time will be prolonged and factor VIII will be decreased (because it is not being protected by vWF). It should be determined which type of von VWD a particular patient has because treatment depends on the type. Multimeric analysis of vWF can be done, and this will allow visualization of the multimeric structure of vWF. In type I disease all bands are present, whereas in the type II variants: 2A and 2B no high-molecular-weight multimers are seen.

Symptomatic Carriers of Hemophilia

Women who are genetic carriers of hemophilia A or B have one gene affected by hemophilia and one that is unaffected. Factor VIII or IX levels that are approximately half of normal are not unusual among hemophilia carriers. Women are found to be hemophilia carriers after giving birth to a son with hemophilia and having genetic testing. A woman whose father has hemophilia is considered an “obligate carrier”, since the only X chromosome that can be given by the father is affected with the disease.

Factor VIII or IX levels in female carriers have been reported to range from 4% to 80%, with levels of 40%–60% being common. Female carriers who have lower than normal factor levels may be considered to be persons with mild hemophilia ([Kupferminc et al., 1999](#)).

Women With Hemophilia

Although rare, cases of severe Hemophilia A and B are found in women. These cases usually fall into one of two categories. Either the woman inherited a hemophilia gene from both her father with hemophilia and her mother who is a carrier, or she is a genotypic carrier who has experienced extreme lyonization of the hemophilia gene, causing the woman's normal gene for the production of Factor VIII or Factor IX to be “turned off.” Women who have moderate to severe hemophilia will have bleeding similar to that of males with the same degree of severity of hemophilia, with the added feature of gynecologic and obstetric problems that make clinical management much more challenging.

Rare Bleeding Disorders in Women

Factor XI deficiency is a very rare bleeding disorder, also called Hemophilia C, it affects approximately 1 in 100,000 people in the United States. Factor XI deficiency follows an autosomal dominant inheritance pattern, meaning that males and females are equally affected. Very little is known about Factor XI deficiency. Prolonged bleeding may occur with surgeries, dental extractions and injury, similar to the bleeding that occurs with VWD. Other rare factor deficiencies may be found in women, like factor V, factor VII, factor X and factor XIII deficiencies, all may produce excessive bleeding. Afibrinogenemia, also called factor I deficiency, may produce severe bleeding symptoms. Dysfibrinogenemia is a disorder in which fibrinogen is found in lower quantity as well as an abnormal quality, leading to prolonged bleeding. The factor XIII deficit causes recurrent abortions and should be discarded.

Obstetric Complications in Women With Bleeding Disorders

Women with bleeding disorders should be able to conceive and carry a pregnancy to term and experience a safe delivery. Managing the pregnancy of a woman with a bleeding disorder requires a multidisciplinary team that will consider the risk of bleeding for the mother and baby. Multiple methods exist for the definitive prenatal diagnosis of hemophilia. Unfortunately these methods are all invasive and may place the woman at risk for excessive bleeding. Amniocentesis, chorionic villus sampling, and cordocentesis must all be undertaken with awareness of the risk of bleeding and/or miscarriage. Conditions such as VWD and platelet dysfunctions are not easily diagnosed before the birth of the infant. Fetal sex determination can be useful in the management of pregnancies at risk for hemophilia, for example, the discovery that a fetus is female may be reassuring to the parents who will know that invasive prenatal testing is not required. When a male child is expected, the delivery can be planned to avoid instrumental deliveries and trauma to the infant during the birth process. Preimplantation genetic diagnosis is a high-tech technique that allows embryos created using in vitro fertilization (IVF) to be tested to determine which are affected by hemophilia. The unaffected embryos are then implanted, avoiding the difficult decision of whether to interrupt a pregnancy because of hemophilia. There have been reports that some women who are carriers of hemophilia have used this method of reproduction to be sure that they will have a child who is not affected with the condition. IVF is an expensive therapy, so the costs of the procedures need to be considered when planning its application. Since normal pregnancy is accompanied by increased levels of several clotting factors and a decrease in fibrinolytic activity, pregnancy is considered a hypercoagulable state.

These women will require treatment to prevent bleeding and to maintain the pregnancy. Infertility is defined as difficulty getting pregnant. Many women with bleeding disorders report having trouble getting pregnant. There is little research to report the incidence or the cause of infertility in women with bleeding disorders. In addition, many women with bleeding disorders report that they have experienced miscarriage. It is unknown whether this can be attributed to their bleeding disorder. It is known that

women with bleeding disorders who have miscarriages are at risk for serious bleeding due to the precipitous drop in factor VIII and vWF levels that occur soon after the loss of a pregnancy. Good hematologic care is advised during and after miscarriage

Primary Immune Thrombocytopenia and Pregnancy

The prevalence of ITP during pregnancy has been reported to be 1 in 1000 to 10,000. In affected patients, despite the potential haemostatic challenges of pregnancy—a time when clotting factors are elevated—platelet counts drop by approximately 10%. During pregnancy, therefore, women with ITP are vulnerable to exacerbation or relapse.

Although the concept is counterintuitive, ITP is a hypercoagulable state. Patients with ITP have approximately a 1.6-fold increased risk of venous thromboembolism (VTE) and a 1.4-fold increased risk of arterial thrombosis. Especially if other risk factors are present, women with ITP may benefit from some form of thromboprophylaxis at delivery, such as use of pneumatic compression devices.

Thrombophilia and Reproduction

What Is Thrombophilia?

Thrombophilia is the tendency to thrombosis. Thrombosis is a complex multifactorial disease, in which multiple interactions between genetic and environmental factors contribute to the development of the disease. Therefore, thrombophilia may be hereditary or acquired, for example, there are thrombophilic genetic mutations (LVF) and other thrombophilic factors or phenotypes (gene-environment interaction) that may be hereditary (like factor VIII) or acquired (APL). There are different phenotypes that have been related to thrombosis, such as APCR, homocysteine, etc. We currently know more than 15 genetic factors and phenotypes associated with venous thrombosis. The association of haemostatic phenotypes (such as RPCA) and risk of thrombosis has been studied, and later search for genetic polymorphisms associated with these risk phenotypes (Factor V Cambridge or factor V Hong Kong, etc.). But nowadays, only 40%–50% of the causes of venous thrombosis are known ([Robertson et al., 2006](#); [Souto et al., 2000](#)).

Thrombophilia and Venous Thromboembolism

Pregnancy is a prothrombotic state per se, it is estimated that the risk of VTE in a pregnant woman during gestation and the puerperium is five times higher than that of a woman who is not pregnant. In addition, presenting thrombophilia, as we will see below, is an added risk factor for thrombosis or repeated miscarriages (RM). Thrombophilia has been linked to infertility, and placental thrombosis with RM.

In the case of fertility treatments, the risk of thrombosis is multiplied. The risk is 10 times higher in women who undergo IVF than women who have a natural pregnancy, and if there is ovarian hyperstimulation syndrome, the risk increases up to 100 times ([Robertson et al., 2006](#); [Bovill et al., 1999](#); [Rosendaal, 1999](#)).

Thrombophilia and Placenta Mediated Complications

Thrombophilia has been proposed as a factor related to gestational complications or placenta mediated complications (PMC). The TREATS study showed that women with thrombophilia were more at risk of developing PMC ([Robertson et al., 2006](#)). The hypothesis is that thrombophilia is a situation that predisposes to a state of hypercoagulability and a tendency to thrombosis, and this could be implicated in gestational complications related to vascular dysfunction, as these complications are due in part to the presence of placental thrombosis. In addition, many factors of coagulation are involved in the correct formation of the placenta such as AT or ANXA.

What prothrombotic factors have been described at present and what evidence is there currently of the association with infertility?

We summarize below the main markers of thrombophilia and the current evidence on their implication in PMC (see [Table 1](#)).

Activated protein C resistance and Leiden V factor

Activated protein C is the most potent endogenous anticoagulant. In 1993, APCR was described as a mechanism responsible for familial thrombophilia. The APCR is an intermediate phenotype associated with the risk of presenting venous thrombosis characterized by a low anticoagulant activity of the protein C system. One year later, at the University of Leiden (The Netherlands), the cause of most cases of hereditary APCR was identified. Although the LVF mutation has been identified as the main cause of this plasma alteration, still 10%–20% of cases of APCR are not carriers of the LVF mutation, indicating the presence of other mutations that cause the same phenotype. Among them, FV Cambridge and FV Hong Kong are genetic variants affecting the amino acid Arg306, which also alter the activation of the PC system. A normal APCR does not exclude the presence of LVF, and if there is an elevated APCR with a normal LVF, it is important to perform a Hong Kong VF, or Cambridge VF. However, there are no studies showing association between these mutations and PMC ([Rai et al., 2002](#)) ([Table 2](#)).

Table 2 Thrombophilia screening

<i>Plasmatic phenotypes</i>
Functional antithrombin
Plasma protein C
Antigenic and free protein S
Functional fibrinogen
APL: LA, ACL, and β 2GP1 antibodies
Homocysteine
ARPC
Factor VIIIc.
Disorders of the fibrinolytic system: dysfibrinogenemia, dysplasminogenemia, hypoplasminogenemia
<i>Genetic test</i>
LVF
Prothrombin mutation
ABO genetic group
If ARPC is positive but LVF is negative, ask for V Cambridge and Hon Kong factors
<i>Other genetic biomarkers under investigation</i>
• F12 mutation
• AT Cambridge (Mutation A384S in <i>SERPINC1</i> gene)
• R67X mutation in <i>SERPINA10</i> gen
• Fibrinogen mutation
• Haplotype ANXA M2

Prothrombin mutation 20210A

Prothrombin is a coagulation factor, precursor of thrombin that activates the coagulation factors V and VIII and converts fibrinogen to fibrin. A mutation in the prothrombin gene, located on chromosome 11, was identified in 1996 in patients with familial thrombophilia, and is associated with a 3 to 5-fold increased risk of thrombosis. The presence of this mutation is associated with a 2 to 3-fold risk of presenting PMC although some studies have not described this association.

Moderate hyperhomocysteinemia

Moderate hyperhomocysteinemia is a risk factor for VTE. The results on whether the thermolabile mutant form of MTHFR is associated with an increased risk of VTE have shown that it is not a cause of thrombosis and therefore should no longer be performed (Robertson et al.). There are studies that demonstrate the association of moderate hyperhomocysteinemia and PMC, in these cases, it is very important to rule out the presence of vitamin B12 or folic acid deficiency, since the treatment of these can resolve the problem. Studies seem to correlate hyperhomocysteinemia with PMC.

Antithrombin deficiency

Antithrombin is an endogenous anticoagulant that blocks the biological activity of thrombin and activated factors IX, X, XI, and XII. Hereditary deficit is not very prevalent, but up to 50% may develop thrombosis. Women with AT deficiency have an increased risk of PMC in addition to having a high risk of thrombosis during pregnancy.

Protein C and Protein S deficiencies

Protein C and protein S deficiencies are inherited in an autosomal dominant manner. Hereditary deficiencies were associated with a VTE risk of 2.5% and 1.3%, respectively. The TREATS study demonstrated association with the presence of PMC.

C46T mutation in the F12 gene and factor XII levels

The C46T mutation of F12 is a risk factor for venous or arterial thrombosis. Factor XII is essential at the onset of the blood coagulation cascade. The concentration of this protein in plasma, or what is the same, this intermediate phenotype, has a significant positive genetic correlation with thromboembolic disease. And it is known that the C46T mutation in the F12 gene is a risk factor and correlates with factor XII levels. In addition, carriers in homozygous T allele have a 5-fold increased risk of thromboembolic events than noncarriers. However, association studies between mutation and factor XIIc levels are inconclusive today.

ABO blood group and factor VIII increase

It has been known since the XX century that carriers of the blood group other than O (No-O) have a 2- to 4-fold higher risk of thrombotic events.

This relationship has been established through the association between the ABO group and factor VIIIc levels, on the one hand, and vWF levels, on the other hand. The non-O blood group presents a 2- to 4-fold higher risk of thrombosis. In addition, the blood group is associated with factor VIIIc levels. And within the genetic blood group, it is known that being a carrier of the allele A1

implies more risk of thrombosis. Finally, in the non-O group, the levels of factor VIIIc has been associated with the onset of thrombosis and could also be associated with RM, although as with previous factors further studies are needed to corroborate these findings.

Haplotype ANXA M2

Annexin-A5 (ANXA5) is an anticoagulant protein that has been postulated as a cause of thrombosis, and is also expressed in the placenta. Its action on the placenta may be affected by APL against it, and the presence of anti-ANXA5 antibodies has been associated with the development of abortions. In addition, a haplotype has been described by the ANXA5 M2 that is associated with a reduction in expression in this protein, and may increase the risk of thrombosis, and it has also been studied that its decrease in the placenta causes inadequate placentation, and therefore is associated with abortions. Genetic transmission may be paternal or maternal. Currently, it is being studied whether it could be a biomarker for the use of personalized antithrombotic therapy.

Other thrombotic risk factors

Other factors of coagulation or fibrinolytic system may be involved in the predisposition to thrombosis. Dysfibrinogenemia, dysplasminogenemia, hypoplasminogenemia, are exceptional causes of familial VTE. And these in turn are associated with PMC problems. From the genetic point of view, there are many polymorphisms in different candidate genes (most of which encode the aforementioned proteins) that are being subjected to multiple association studies to determine their involvement in VTE. Regarding the association with PMC, in the majority of studies the association between FVL, PT20210A and gestational losses has been studied, but in the rest of hereditary thrombophilias there are studies that have shown association and others that have not. In addition, studies are currently under way with the ANXA M2 haplotype to determine if this would be the biomarker that would establish, in the presence or not of thrombophilia, or other factors involved in miscarriage, the need for antithrombotic treatment (Fishe et al., 2016).

Acquired Thrombophilia and Placenta Mediated Complications

Interest in the association between thrombophilia and recurrent abortions arose once antiphospholipid syndrome (APLS), considered being an acquired thrombophilia, was found to be a significant and treatable cause of recurrent miscarriage. Therefore, obstetric antiphospholipid syndrome (OAPLS) is the most well-known and studied acquired cause of thrombophilia. APLS is one of the known causes of gestational loss. The most common obstetric manifestation of APLS is recurrent gestational loss, 10%–15% of women with this manifestation are diagnosed with APLS. Patients with APLS have an 80% risk of having an abortion. Fetal death is associated more powerfully than the earliest losses. All women with recurrent first-trimester abortions should be asked for APL.

The APLS is a systemic autoimmune disease mediated by antiphospholipid antibodies (APL)—lupus anticoagulant (LA), anticardiolipin antibodies (ACL) and anti- β 2-glycoprotein 1 (β 2GP1) antibodies—with clinical, diagnostic criteria—analytical, well defined. APL can be detected in 1%–5% of healthy women. The prevalence of these antibodies increases to 15% in women with recurrent miscarriages in the first trimester. A, APLS should be suspected in all women with systemic lupus or other connective diseases who wish to become pregnant and women with a personal history of: recurrent miscarriages; retarded intrauterine growth; fetal death; hypertension, severe preeclampsia or eclampsia, before 34 weeks in particular; *abruptio placentae*; preterm delivery before the 34th week of gestation; analytical alterations such as hemolysis, elevation of liver enzymes or thrombopenia, is suggestive of HELLP syndrome (Danza et al., 2012; Miyakis et al., 2006).

Malignant Hematological and Fertility

Among the causes to be ruled out, it is important to know myeloproliferative syndromes such as essential thrombocythaemia associated with the presence of the JAK-2 mutation or Polycythemia Vera that can be treated with thrombocytosis and JAK-2 positive.

Myeloproliferative Diseases

Myeloproliferative syndromes (cMPS) are associated with an increased incidence of haemorrhagic and thromboembolic complications as well as progression to myelofibrosis and acute myeloid leukemia. Although cMPS have a peak incidence in the sixth and seventh decade of life, they can occur in women of childbearing age (traditionally 15–45 years). Essential thrombocythaemia (ET) is the most commonly reported cMPS in younger women with an increased prevalence in the third decade. Thrombosis is the main cause of morbidity and mortality: blood rheology, possibly relating to leukocyte adhesion, platelet activation and platelet-leukocyte aggregates, is believed to have a leading role in the development of these complications.

The management of cMPS in pregnancy is complicated. A number of studies have shown that women with ET are at higher risk of developing pregnancy complications. Key challenges include the increased risk of maternal hemorrhage, thrombosis and pregnancy loss (Randi et al., 2014). The principal factors influencing pregnancy outcome include an adequate placental development and the maintenance of a normal uterine blood flow.

Thrombosis Risk Factors in Essential Thrombocythaemia

ET during pregnancy is associated with thrombosis of the placental vessels and can lead to multiple infarcts and secondary placental insufficiency. All this causes a risk of miscarriages in the first trimester of pregnancy of between 25% and 40% (it varies depending on the type of study), an increase compared to the general population, which is around 15%. Other complications during pregnancy are placental abruption, preeclampsia, stillbirth or intrauterine fetal death (Alimam et al., 2016).

The relevance of JAK2V617F mutation as a thrombotic risk factor is still debated, as well as the role of platelet and white blood cell counts. There is controversy as to whether the presence of the JAK2V617F mutation favors thrombotic events. Some studies reported a higher incidence of miscarriages in JAK2V617F positive patients with ET than in JAK2-negative cases (Passamonti et al., 2007), while the Mayo Clinical study demonstrate similar rate of pregnancy loss between JAK2 mutated and nonmutated patients. There is no direct correlation between platelet count and the presence of complications during pregnancy.

References

- Alimam, S., Bewley, S., Chappell, L. C., Knight, M., Seed, P., Gray, G., Harrison, C., & Robinson, S. (2016). Pregnancy outcomes in myeloproliferative neoplasms: UK prospective cohort study. *British Journal of Haematology*, 175(1), 31–36. <https://doi.org/10.1111/bjh.14289>.
- Bovill, E. G., Hasstedt, S. J., Leppert, M. F., & Long, G. L. (1999). Hereditary thrombophilia as a model for multigenic disease. *Thrombosis and Haemostasis*, 82, 662–666.
- Danza, A., Ruiz-Irastorza, G., & Khamashta, M. (2012). Antiphospholipid syndrome in obstetrics. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 26(1), 65–76.
- Fishel, S., Baker, D., Elson, J., Ragunath, M., Atkinson, G., Shaker, A., Omar, A., Kazem, R., Beccles, A., & Greer, I. A. (2016). Precision medicine in assisted conception: A multicenter observational treatment cohort study of the annexin A5 M2 haplotype as a biomarker for antithrombotic treatment to improve pregnancy outcome. *EBioMedicine*, 10, 298–304.
- Kadir, R., James A. (2009). Reproductive health in women with bleeding disorders, *World Federation of Hemophilia. Treatment of Hemophilia*, No 48.
- Kupferminc, M. J., Eldor, A., Steinman, N., Many, A., Bar-Am, A., Jaffa, A., Fair, G., & Lessing, J. B. (1999). Increased frequency of genetic thrombophilia in women with complications of pregnancy. *The New England Journal of Medicine*, 340, 9–13.
- Lusher, J. M. (1996). Screening and diagnosis of coagulation disorders. *American Journal of Obstetrics and Gynecology*, 175(3 Pt 2), 778–783.
- Miyakis, S., Lockshin, M. D., Atsumi, T., Branch, D. W., Brey, R. L., Cervera, R., et al. (2006). International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *Journal of Thrombosis and Haemostasis*, 4(2), 295–306.
- Passamonti, F., Randi, M. L., Rumi, E., Pungolino, E., Elena, C., Pietra, D., et al. (2007). Increased risk of pregnancy complications in patients with essential thrombocythemia carrying the JAK2 (617V>F) mutation. *Blood*, 110(2), 485.
- Rai, R., Backos, M., E Igaddal, S., Shlebak, A., & Regan, L. (2002). Factor V Leiden and recurrent miscarriage—prospective outcome of untreated pregnancies. *Human Reproduction*, 17, 442–445.
- Randi, M. L., Bertozzi, I., Rumi, E., Elena, C., Finazzi, G., Vianelli, N., Polverelli, N., Ruggeri, M., Vannucchi, A. M., Antonioli, E., Lussana, F., Tieghi, A., Iurlo, A., Elli, E., Ruella, M., Fabris, F., Cazzola, M., & Barbui, T. (2014). Pregnancy complications predict thrombotic events in young women with essential thrombocythemia. *American Journal of Hematology*, 89(3), 306–309. <https://doi.org/10.1002/ajh.23635>.
- Robertson, L., Wu, O., Langhorne, P., Twaddle, S., Clark, P., Lowe, G. D., et al. (2006). Thrombosis: Risk and economic assessment of thrombophilia screening (TREATS) study. Thrombophilia in pregnancy: A systematic review. *British Journal of Haematology*, 132(2), 171–196.
- Rosendaal, F. R. (1999). Venous thrombosis: A multicausal disease. *Lancet*, 353, 1167–1173.
- Souto, J. C., Almasy, L., Borrell, M., Blanco-Vaca, F., Mateo, J., Soria, J. M., et al. (2000). Genetic susceptibility to thrombosis and its relationship to physiological risk factors: The GAIT Study. *American Journal of Human Genetics*, 67, 1452–1459.

Further Reading

- Tersigni, C., Marana, R., Santamaria, A., Castellani, R., Scambia, G., & Simone, N. D. (2012). In vitro evidences of heparin's effects on embryo implantation and trophoblast development. *Reproductive Sciences*, 19(5), 454–462.

Surgical Endoscopic Diagnosis of Infertility

Camran Nezhat, Camran Nezhat Institute and Center for Special Minimally Invasive and Robotic Surgery, Palo Alto, CA, United States; Stanford University Medical Center, Palo Alto, CA, United States; and University of California, San Francisco, San Francisco, CA, United States

Daniel Copeland, Camran Nezhat Institute and Center for Special Minimally Invasive and Robotic Surgery, Palo Alto, CA, United States; and University of California, San Francisco, San Francisco, CA, United States

Megan K Burns, Camran Nezhat Institute and Center for Special Minimally Invasive and Robotic Surgery, Palo Alto, CA, United States; and Stanford University Medical Center, Palo Alto, CA, United States

Stacy Young, Camran Nezhat Institute and Center for Special Minimally Invasive and Robotic Surgery, Palo Alto, CA, United States; and Stanford University Medical Center, Palo Alto, CA, United States

Azadeh Nezhat, Camran Nezhat Institute and Center for Special Minimally Invasive and Robotic Surgery, Palo Alto, CA, United States; Stanford University Medical Center, Palo Alto, CA, United States; and University of California, San Francisco, San Francisco, CA, United States

© 2018 Elsevier Inc. All rights reserved.

History of Surgical Evaluation of Infertility

Up until the 1970s, most operations required large incisions for the surgeons to be able to view the pelvis and have room to operate. These large incisions were often painful to recover from, took weeks to heal, and put the patient at more risk of complications. Subjecting infertile but otherwise healthy women to the risks of “open” surgery and the large incisions it requires would have exposed them to greater risk than potential benefit and would violate the principle of “First, do no harm.” Today, gynecologic surgeons can choose from a variety of endoscopic instruments to safely visualize and operate within the abdomen, pelvis, uterus, bladder, and rectum with relatively little downtime for the patient. Surgery can now be performed through small surgical incisions and using a variety of telescopic cameras (videolaparoscopy), and videolaparoscopic instruments. Early instruments were simple tubes through which a surgeon could peer into the abdomen or vagina and were known as endoscopes. They allowed for some diagnostic procedures but were unsuitable for anything but the most rudimentary operative procedures (Nezhat et al., 2013).

Dr. Camran Nezhat’s invention and development of video-assisted endoscopy in the 1970s and early 1980s that transformed the landscape of surgery as we know it today. These instruments use high-resolution video cameras to record, magnify, and display the operative view on monitors to the entire surgical team. Having once been relegated to uncomfortable positions in order to peer through the endoscope, surgeons could now operate in relative comfort and ease and begin performing advanced procedures. In the United States, the vast majority of pelvic surgery is performed videolaparoscopically due to its superior visualization, low morbidity, and increasing surgeon competency (Stovall et al., 2006). Videolaparoscopy is the standard of care for minor gynecologic procedures and is the preferred approach for a sizable number of more complex procedures.

In the course of an infertility workup, imaging studies such as ultrasound and hysterosalpingogram are performed in favor of an exploratory surgery known as diagnostic videolaparoscopy and never be surgically evaluated for endometriosis. Recent data suggests that videolaparoscopic resection of endometriosis can drastically improve pregnancy rates in women with infertility refractory to IVF (Littman et al., 2005; Burney and Nezhat, 2008).

Standard and Specific Pre-Operative Workup

Infertile couples are asked to provide thorough medical, reproductive, and menstrual histories and undergo physical exams. Samples of blood, urine, and semen are analyzed to screen for medical and genetic conditions that could impact a couple’s fertility. Laboratory and imaging studies are further detailed in the medical and radiologic work up chapters. When these studies are unable to establish the cause for infertility, or in cases where the physical exam or imaging studies indicate surgically treatable conditions, surgical evaluation and treatment is offered. In order to reduce the risk of multiple surgeries and optimize a patient’s chances at conception, patients are often consented for simultaneous diagnostic and operative procedures (Nezhat et al., 2013).

Intraoperative Layout

Once the patient has undergone a pre-operative assessment, is medically cleared, and informed consent has been obtained, preparations for surgery begin. Careful consideration must be given to patient positioning during surgery to avoid falls, nerve injuries sustained from being kept in the same position for long periods of time, and inadvertent injury while the patient is asleep.

In gynecology, the patient is typically positioned in dorsal lithotomy (lying on their back with legs separated and positioned in stirrups). In videolaparoscopy, a right-handed surgeon stands to the left of the patient, with their assistant directly across, and sometimes an additional assistant for uterine manipulation. A monitor may be placed by the patient’s legs or by the assistant.

In hysteroscopy, the patient is placed in lithotomy position and the surgeon is between the patient's legs with a screen within easy view (Nezhat et al., 2013). Ultimately, patient safety is tantamount to operating room procedures and variations from these conventions occur to make appropriate accommodations to care.

Hysteroscopic Evaluation

Hysteroscope

Hysteroscopy allows a gynecologic surgeon to visualize and operate upon the inside of the uterus. It is regarded as the gold standard against which other uterine imaging modalities are compared. It requires a hysteroscope, which can pass through the cervix and into the endometrial cavity. The hysteroscope has a channel which allows for fluid under pressure to be instilled into and drained from the endometrial cavity, which allows the uterus to distend. Light shines into the cavity of the uterus through fiberoptic cables and a video camera is connected to back of the hysteroscope and relays high-definition video to a nearby monitor. Thin and flexible hysteroscopes are < 4 mm in diameter and are used primarily for simple outpatient office diagnostic procedures. Thicker, rigid hysteroscopes are often 5 mm in diameter and have an extra channel for the introduction of thin surgical instruments including scissors and graspers. When connected to an energy source, these instruments allow for a gynecologic surgeon to cut, cauterize, and resect or remove tissue from the inside of the uterus and therefore hysteroscopes with this function are termed resectoscopes (Nezhat et al., 2013).

Office Versus Operating Room

Many diagnostic hysteroscopic procedures and operative procedures can now be performed in the outpatient clinical setting. These in-office hysteroscopy procedures may be performed with mild to moderate sedation, with administration of a non-steroidal anti-inflammatory with or without an oral benzodiazepine, or with a simple paracervical block (injection of numbing medication near the cervix). Many patients and providers prefer the office hysteroscopies as they are lower cost, do not require general anesthetic, and do not require use of an operating room in a surgery center or hospital. The vast majority of hysteroscopies, however, still take place in operating rooms and may be incorporated into other planned gynecologic surgeries. Pre-operative ultrasound is often used to guide the surgeon towards anticipating what pathology they may encounter, and preoperative MRI is an option to help define any anomalies of the uterus and specify fibroid characteristics and location. Saline infusion sonogram and hysterosalpingogram are also useful aides to determine whether simple polyps or more complex endometrial pathology is suspected. Such imaging can help a physician predict whether the hysteroscopy will need to take place in the operating room or whether an office procedure would be sufficient (Nezhat et al., 2013).

Timing

Hysteroscopy requires good visualization of the inside of the uterus. Menstrual blood and prolific endometrial tissue can obscure this view, so the gynecologist will time the procedure during the early to mid-follicular phase of the patient's menstrual cycle; just after menses is completed. This also serves to reduce the possibility of disturbing a very early pregnancy. Alternatively, the gynecologist may start the patient on oral contraceptives to thin the endometrial lining (Nezhat et al., 2013).

Cervical Dilation

In order to safely pass the hysteroscope through the cervix and into uterus, surgeons first dilate the muscular cervix. This process can start the night before with the placement of misoprostol tablets into the vagina. Misoprostol is a synthetic prostaglandin medication that softens or "ripens" the cervix in preparation for the procedure. Then the surgeon passes a series of lubricated mechanical dilators through the cervix, gradually increasing in size until the hysteroscope can be introduced.

Procedure With Diagnostics

Once the surgeon has introduced the hysteroscope, and distended the uterus with sterile fluid, they survey the endometrium. By using a rotatable 30 degree angled scope, the surgeon can focus upon any surface within the uterus, as well as the tubal ostia.

When performing hysteroscopy for evaluation of infertility, the anatomic structures within the uterus must be carefully assessed. As many as 25%–35% of patients presenting with infertility are found to have abnormalities in the uterus or fallopian tubes (Abrao et al., 2013). A variety of endometrial pathologies may negatively impact fertility, including endometrial polyps, fibroids, intrauterine cavity adhesions, and Müllerian abnormalities such as a uterine septum. The surgical correction of intrauterine abnormalities, including polypectomy, septoplasty, and submucosal fibroid resection, are associated with improved pregnancy rates.

A tremendous benefit of hysteroscopy is the ability to diagnose and treat many forms of uterine pathology simultaneously. Grasping forceps can facilitate targeted removal of endometrial polyps and scissors can be used to resect intrauterine adhesions and septi. Submucosal fibroids can be shaved down with unipolar loop electrode or technologies such as the Myosure®. Biopsies can be obtained for any suspicious lesions or growths and subsequently sent to pathology for analysis (Nezhat et al., 2013; Perez-Medina et al., 2005).

Endometrial polyps represent the most common intracavitary finding in infertile patients, with the reported incidence of polyps ranging from 10% to 32% (Shalev et al., 2000; Hinckley and Milki, 2004). Most commonly found near the uterine fundus and cornua, polyps are benign irregularly shaped outgrowths that can range in size from millimeters to lesions that completely fill the entire uterine cavity. As some other gynecologic pathologies can have a polyp-like appearance, such as endometrial overgrowth (termed hyperplasia) or malignancies, it is imperative to send removed polyps for pathological analysis. Resection of endometrial polyps can improve fertility outcomes, with infertile women having nearly twice the likelihood of achieving pregnancy following a polypectomy (Perez-Medina et al., 2005).

Leiomyomata, or uterine fibroids, are the most common benign smooth muscle tumors of the uterus with a prevalence of 30%–50% in the population, with some women genetically predisposed to developing fibroids (Stein and Ascher-Walsh, 2009). Their impact upon fertility is dependent on their size, location and number. Their size can range from sub-centimeter to massive growths large enough to fill a woman's entire abdomen. Fibroids are categorized based on their location within the uterus. Pedunculated fibroids are attached only by a narrow stalk to the surface of the uterus. The subserosal variety typically bulge out on the outer surface of the uterus. Neither subserosal nor pedunculated fibroids are meaningfully associated with infertility or heavy menstrual bleeding as they do not impact the endometrial cavity. Intramural fibroids masses within the muscular wall of the uterus. Submucosal fibroids are located just under the surface of the endometrium and are thought to decrease fertility by occupying space within the uterine cavity, leaving fewer areas for implantation of an embryo. In addition, implantation at the site of a fibroid may lead to miscarriage due to decreased vascular flow or abnormal placentation affecting the future pregnancy. They can cause heavy bleeding during menses, and women can become severely anemic. During hysteroscopy, submucosal fibroids appear as rounded masses that bulge into the endometrial cavity. Using a variety of cutting, cauterizing, and suction instruments, submucosal fibroids can be removed at time of hysteroscopy (Nezhat et al., 2013). For subserosal and pedunculated fibroids, see the "Videolaparoscopy" section.

With a prevalence of 2%–3%, *uterine septi* represent the most common congenital anomaly of the uterus. They can range from a thin strip of pale fibrous tissue at the fundus of the uterus to a full-length wall of tissue separating left and right side of the uterus into two entirely separate compartments. Uterine septation is associated with miscarriage in up to 79% of pregnancies, but when detected and resected by hysteroscope, this miscarriage rate decreases to 14% (Hinckley and Milki, 2004). Depending upon its size and thickness, a uterine septation can typically be removed with hysteroscopic scissors, laser, or a unipolar loop electrode.

Hysteroscopically, intrauterine adhesions or *synechiae* are found in up to 21% of women with recurrent pregnancy loss, and in 16% of infertile women prior to starting IVF (Homer et al., 2000). They appear as filmy, white, or devascularized tissue stretching between the endometrium across the uterine cavity. Their presence can prevent the normal fluid distention of the uterus during hysteroscopy, so the uterine cavity can appear collapsed or distorted. Also known as Asherman's syndrome, intrauterine adhesions typically form as a result of mechanical or infectious damage to the endometrium. Most commonly, adhesions form secondary to dilation and curettage (D&C) performed after complications arising from missed or incomplete miscarriage, therapeutic abortion, or retained placenta in the postpartum period. Women with multiple D&Cs or a history of other uterine surgeries such as cesarean section and myomectomy are also at risk of Asherman's syndrome. The incidence of intrauterine adhesions can be as high as 40% following the curettage of retained products of conception; whereas hysteroscopic resection of multiple submucosal uterine fibroids has been associated with a 45.5% incidence of intrauterine adhesions. Following surgical resection of intrauterine adhesions, pregnancy and live birth rates vary between 32% and 76% (Nezhat et al., 2013; March, 2011).

Videolaparoscopic Evaluation

Videolaparoscopy

Videolaparoscopy is a minimally invasive surgical procedure whereby a surgeon can visualize and operate upon abdominal and pelvic organs through several 3–10 mm incisions. Small plastic or metal ports are introduced through the incisions, into the abdomen, and the abdomen is filled with carbon dioxide gas to permit room for the laparoscope and instruments. The laparoscope makes use of fiber optic lighting, a zero degree or angled lens, and a video camera to relay high-definition video from inside the abdomen to a monitor outside of the body. Most surgeries that used to require laparotomies can now be performed videolaparoscopically by expert gynecologists and minimally invasive surgeons. Videolaparoscopic techniques have become the gold-standard for many abdominal and pelvic surgeries (Nezhat et al., 2013).

In contrast to hysteroscopy which can be performed within an office setting, videolaparoscopic procedures almost exclusively require a patient to be under general anesthesia and take place in an operating room. Diagnostic videolaparoscopic procedures make use of at least a single abdominal incision and port, typically through the umbilicus, to thoroughly evaluate the abdominal and pelvic organs. Diagnostic videolaparoscopic procedures can be easily converted to operative videolaparoscopic procedures by the addition of extra-abdominal ports for the passage of videolaparoscopic instruments. These long and thin instruments allow for a surgeon to operate within the abdomen and pelvis.

When performing a diagnostic videolaparoscopic evaluation for infertility, it is customary to attach a uterine manipulator inside the uterus which enables a surgical assistant to move the uterus and helps the surgeon visualize and operate on the entire pelvis and all sides of the uterus. If a manipulator is used that has a channel for instilling fluid, chromoperturbation with dilute methylene blue dye can also be performed during the videolaparoscopic evaluation (Nezhat et al., 2013).

Once abdominal access has been obtained, diagnostic videolaparoscopy begins with careful examination of the bowel, small intestine, and adjacent structures to ensure no damage occurred as a result of the initial port placement, given over 50% of all videolaparoscopic injuries occur at the time of abdominal entry (Vilos et al., 2009). Prior to focusing attention on the pelvis, it is important to perform a thorough evaluation of the abdomen for any pathologic adhesions or disease, which may suggest a history of pelvic inflammatory disease (PID) or the presence of endometriosis. The association between infertility and a history of PID has long been known, as those with a history of PID have a greater likelihood of tubal disease (Westrom et al., 1992).

Endometriosis

Endometriosis is characterized by the ectopic or abnormal growth of endometrial glands and tissue outside the uterus. Endometriosis can affect any organ within the abdomen and pelvis, but most commonly affects the posterior cul de sac, the lining of the uterus, the lining of the parietal peritoneum, and the ovaries (Agarwal and Subramanian, 2010). Endometrial implants are responsive to hormones of the menstrual cycle and may cause pain and discomfort, and bleeding may result in inflammation, scar tissue, and adhesions formation (Nezhat et al., 2013).

There are several mechanisms by which endometriosis can lead to infertility, including inflammatory changes to free fluid within the pelvis directly impacting egg quality and sperm function, damage to the ovaries by way of endometrioma formation, adhesion and scar tissue formation on the tubes and ovaries which impact their ciliary function, and direct infiltration and damage to the fallopian tubes. Endometriosis has also been shown to impact the endometrium within the uterus, making it less hospitable for embryo implantation, and to release prostaglandins and cytokines that impair sperm motility (Oral et al., 1996).

Endometriosis has various appearances during videolaparoscopic evaluation, appearing as strands or adhesions between organs, dense white fibrous scar tissue, and even as clear fluid filled droplets or vesicles. Lesions can appear colored as black, dark blue, dark red, light red, clear, and white. "Typical" lesions are darker, as the iron from menstrual blood is absorbed in surrounding cells and concentrated. Lesions, especially the clear blister variant, commonly have new and enlarged blood vessels radiating from it, called red flame lesions. Endometriosis can form growths of tissue that can appear as small polyps or deeply infiltrating lesions that penetrate into the organs within the pelvis. The deeply infiltrating lesions appear as dense, fibrous, growths that may be felt when palpated with videolaparoscopic instruments. Scarring and adhesions from endometriosis can be so severe that organs can become entirely fused together. A common example of this is when the posterior aspect of the uterus becomes scarred down onto the sigmoid colon and rectum, completely obliterating the space typically found there, called the posterior cul-de-sac (Nezhat et al., 2013).

Uterine Evaluation

When evaluating the uterus, surgeons examine the size, shape, surface, and contours looking for evidence of asymmetry or pathology. A uterus will change in size over the course of a woman's life. After puberty and before carrying a pregnancy, a healthy uterus is on average 7 cm long, 4 cm across, and 3 cm in depth. During pregnancy, a normal uterus can expand up to 40 cm or more. After a pregnancy, during which the uterus expands to accommodate the growing fetus, the uterus shrinks back down to a slightly larger size of 9 cm long, 5 cm across, and 4 cm in depth (Esmaelzadeh et al., 2004). During videolaparoscopy, an assistant adjusts the uterine manipulator to lift and position the uterus to optimize the surgeon's view. The surface of the uterus is covered by a thin layer of visceral peritoneum which appears smooth and light pink. Patches of red inflammation, white or yellow fibrosis, adhesions, and endometriosis can be readily seen upon the surface of the uterus and help guide a surgeon towards a diagnosis.

If present, two varieties of *uterine fibroids* are visible during videolaparoscopy: subserosal and pedunculated. The subserosal variety may bulge out on the outer surface of the uterus. Pedunculated fibroids are attached only by a narrow stalk to the surface of the uterus. Neither subserosal nor pedunculated fibroids are meaningfully associated with infertility or heavy menstrual bleeding as they do not impact the endometrial cavity.

Adenomyosis is a disease in which the endometrial tissue of the uterus is found to have improperly invaded the muscular layer (myometrium) of the uterus. This invasive endometrial tissue causes the myometrium to expand and negatively impacts the muscle's ability to contract. Adenomyosis is associated with pain, heavy menstrual bleeding and infertility. Definitive diagnosis of adenomyosis requires microscopic evaluation of the myometrium following a uterine biopsy or hysterectomy. That said, non-invasive pre-operative imaging with MRI and transvaginal ultrasound can detect adenomyosis with a sensitivity of 0.79 and 0.74, and specificity of 0.91 and 0.85, respectively (Maheshwari et al., 2012). Furthermore, experienced surgeons may detect evidence of adenomyosis by direct visualization and manipulation of the uterus, such as: being abnormally enlarged, with a flushed, asymmetric, soft, boggy, or sagging appearance.

Cesarean scar defect, also known as niche or *isthmocoele*, is an increasingly common uterine defect at the site of hysterotomy on the anterior wall of the uterus. Improper healing of the cesarean incision leads to thinning of the anterior uterine wall, creating an indentation and fluid-filled pouch. Fibrotic tissue at the niche acts as a valve, leading to painful accumulation of blood within the niche, which can then cause delayed menstruation and lead to abnormal bleeding, pelvic pain, vaginal discharge, and infertility. The defect is most easily seen on ultrasound after the menstrual cycle, when the endometrial lining is very thin and blood has accumulated in the defect. Experienced surgeons may repair a niche hysteroscopically for patients who have completed childbearing, or videolaparoscopically for patients desiring fertility (Nezhat, 2016).

Albeit unlikely to have escaped detection by physical exam or pre-operative imaging, *congenital anomalies* of the uterus can be readily seen during videolaparoscopy. During the development of a female fetus, two independent Müllerian (paramesonephric) ducts fuse within the pelvis of the fetus to form the uterus, fallopian tubes, cervix, and the most inner part of the vagina. If there is a disruption in this process and the Müllerian ducts do not fuse appropriately, a range of anatomic anomalies result, including a septate uterus, bicornuate uterus, unicornuate uterus, or a didelphic uterus (Nezhat et al., 2013).

Ovarian Evaluation

The videolaparoscopic evaluation of infertility proceeds with *inspection of the ovaries*. The ovaries are responsible for maintaining the supply of female sex hormones (estrogen and progesterone) and using them to regulate the menstrual cycle as well as to develop and release an egg each month, a process known as ovulation. Certain forms of hormonal supplements or contraceptives work in part by suppressing the function of the ovaries to prevent ovulation. Hormonal contraceptives can therefore mimic regular menstrual cycles, in the absence of any ovulation. In women who are not receiving these prescribed hormones, a regular menstrual cycle each month serves as an external indication that ovulation is occurring successfully each month.

Ovulatory dysfunction can be identified in reproductive-aged-women who experience infertility with irregular, sporadic, or absent periods. Most forms of ovulatory dysfunction can be diagnosed by obtaining a patient's menstrual history, a few blood tests, ultrasound, and a physical exam.

Healthy ovaries vary widely in appearance and function between women, but also throughout the course of a woman's life as she passes through puberty, each menstrual cycle, and menopause. As eggs develop in the ovaries, they form small pockets of fluid called follicular cysts. Women are born with 1–2 million eggs which decrease over time. By the time she enters puberty, she will have approximately 25% of the eggs she was born with. As a woman's quality and quantity of eggs begins to diminish as she approaches menopause, the ovaries get progressively smaller. *Premature ovarian failure* occurs when a woman's ovaries cease functioning and she undergoes menopause prior to age 40. When viewed videolaparoscopically, the ovaries of a woman with premature ovarian failure will appear atrophied and similar to ovaries of a post-menopausal woman.

Polycystic ovary syndrome or PCOS, is another form of ovulatory dysfunction seen during videolaparoscopy as enlarged, distended ovaries, with a notably bumpy surface filled with small follicular cysts. The cutoff volume threshold between normal and enlarged ovaries is 10 mL or ≥ 12 follicles per ovary (Goodman et al., 2015a,b). In addition, PCOS is associated with impaired glucose metabolism and high level of androgens that can cause excessive body hair and acne. In these patients, abnormal endocrine interactions result in over-production of ovarian functional cysts, with excessive estrogen production and irregular or absent ovulation.

Endometriomas, also known as chocolate cysts, are a common complication of endometriosis as the endometrial tissue invades the ovary and repeatedly bleeds into the ovary, forming a blood-filled cyst. They are called chocolate cysts because, when ruptured, the old blood within the cyst looks like chocolate syrup or pudding. Endometriomas impact a patient's fertility through several mechanisms including: inflammatory damage to the ovaries, pressure or a mass effect within the ovaries, damage to ovarian reserve secondary to surgical treatment of the cysts, and formation of adhesions and scar tissue negatively impacting the ovaries' ability to release an egg (Nezhat et al., 1992).

Fallopian Tube Evaluation

The final component of the videolaparoscopic evaluation for infertility is to assess the *fallopian tubes* for possible "tubal-factor infertility." At their distal end, the tubes have fern-like projections known as fimbriae that collect the egg from one of the ovaries each month. Within the tubes, there are ciliated cells that actively sweep the ovum down into the endometrial cavity of the uterus. Sperm, in contrast, propel themselves up from the vagina, through the cervix, through the endometrial cavity of the uterus, and up through the fallopian tubes. The fallopian tubes, therefore, acts as the meeting place between the ovum and sperm, and is where fertilization take place. For these reasons, open and functional fallopian tubes are critical for a patient's fertility. To test whether the tubes are open, the surgeons perform chromopertubation. *Chromopertubation* is a procedure whereby a dye is instilled through the uterine manipulator, into the uterus, and out through the fallopian tubes in order to assess whether the tubes are open and unobstructed. If the dye is not seen, this can be an indication that there is a physical blockage from fertilization to take place. Following operative treatment of any blockages or pathology within the tube, chromopertubation is repeated to ensure that the fallopian tube had been adequately repaired (Nezhat et al., 2013).

There are several pathologic processes that can negatively impact the structure and function of the fallopian tubes. When this happens, it can lead to infertility directly or because of ensuing ectopic pregnancies. *Ectopic pregnancy*, or the implantation of an embryo at a site other than the endometrial cavity, occurs at a rate of 19.7 cases per 1000 in North America, and it is the leading cause of maternal mortality in the first trimester (Tenore, 2000). Ectopic pregnancies occur 98% of the time within a patient's fallopian tube. While an ectopic pregnancy can happen to anyone, it is more common in patients who have already experienced some form of damage to their tubes, such as pelvic inflammatory disease. There are multiple treatment options for tubal ectopic pregnancies: medical treatment with methotrexate, surgical removal of the affected fallopian tube and ectopic pregnancy together (salpingectomy), or tube-preserving surgical procedures that attempt to remove just the ectopic pregnancy from a tube with the hope of leaving a tube that will remain functional (salpingostomy, salpingotomy, segmental resection with anastomosis, and fimbrial milking) (Song et al., 2016).

Infertile patients with a history of an ectopic pregnancy require consideration both to the cause and consequence of the ectopic pregnancy. The surgeon must assess whether there is visible evidence of damage to the tubes or the surrounding tissue: for example, adhesions or scar tissue from PID, or deeply infiltrating endometriosis (DIE), which can be defined as implants have invaded > 5 mm through the tissue and are associated with severe symptoms (Koninckx et al., 1994). When assessing the outcome of an ectopic, it is not uncommon for patients, especially those with limited health literacy or from developing nations, to not know precisely which operation they received and whether they still have both their tubes. The surgeon will assess which if any of the patient's tubes remain, as well as their structural integrity. In cases where the patient underwent a tube-preserving procedure, special care will be given to determine whether the procedure was potentially successful, or whether scar tissue and strictures have occluded the tube.

When strictures or obstructions form in the fallopian tube, it may result in the tube becoming distended over time with a clear sterile fluid, known as *hydrosalpinx* and may affect one or both fallopian tubes. An affected tube can distend in size up to several centimeters and appear sausage-shaped on videolaparoscopy. Such tubes are inherently blocked and do not permit the transfer of sperm or eggs. When occurring on both tubes, this would result in infertility. In addition, hydrosalpinx decreases the success of in vitro fertilization, with afflicted patients suffering from decreased rates of embryo implantation, pregnancy, and live deliveries, while having increased rates of early pregnancy loss and preterm birth (Camus et al., 1999; Kawwass et al., 2013). Therefore, the affected tube is often surgically removed.

Pelvic inflammatory disease (PID) describes a group of inflammatory gynecological conditions most commonly caused by an infection. As the fallopian tubes serve as a conduit between the endometrial cavity of the uterus and open to the pelvic peritoneum, untreated infections may span from the endometrial cavity of the uterus (endometritis) through the fallopian tubes (salpingitis), to involve the ovaries (oophoritis), the pelvic peritoneum (peritonitis), and even reach as high the liver (perihepatitis). PID can be a life-threatening condition and requires expeditious and broad antibiotic treatment. Unfortunately, relatively mild infections can be asymptomatic and untreated before the infection becomes severe. Upon healing, widespread scarring and adhesions form in the afflicted areas due to the tissue damage caused by the infection, as well as the immune system's response. These scars and adhesions can often be seen during videolaparoscopy and represent a significant source of long-term suffering and chronic pelvic pain. PID related scarring within the fallopian tubes can lead to tubal-factor infertility, hydrosalpinx, and ectopic pregnancies. Risk factors for PID include: age at coitarche, prior history of STI, multiple sexual partners, and barriers to care (Simms et al., 2006; Kreisel et al., 2017).

Cystoscopy and Proctoscopy

Cystoscopy is performed in a similar manner to hysteroscopy, with the cystoscope being passed through the urethra and into the bladder which is subsequently distended with fluid. The cystoscope's fiber-optic lighting and video camera then relay high-definition video of the bladder lumen with its two ureteral orifices to a nearby monitor. The path of the ureters from the kidney to the bladder within the female pelvis puts them at risk of being inadvertently injured when operating in that region. Furthermore, treatment of adhesions between the bladder and the uterus or treatment of lesions near the bladder risk inadvertent perforation of the bladder by the surgical instruments or sutures. Visualization of an intact bladder lumen with strong jets of urine draining into the bladder from each of the two ureteral orifices reassures the surgeon that no such injury had occurred (Nezhat et al., 2013). In addition, cystoscopy affords the surgeons an opportunity to concurrently evaluate the bladder for conditions such as endometriosis of the bladder and interstitial cystitis.

Proctoscopy is performed by passing a lubricated proctoscope through the patient's anus and into their rectum. The proctoscope has a channel and a small balloon of air capable of distending the rectum with air. The surgeon can then visualize directly through a lens on the proctoscope the inside of the patient's rectum and evaluate for any signs of bleeding, puncture, sutures, or injury of the rectum. Simultaneously, another surgeon will be using the laparoscope to examine the exterior surface of the rectum within the pelvis. By covering the rectum within a shallow pool of saline, the videolaparoscopic surgeon can perform a "bubble test" to check for air leaking from the rectum, indicating an injury or incomplete closure. In addition to evaluating for iatrogenic injury, inclusion of the proctoscopy allows for the surgeon to evaluate the patient for endometriosis of the bowel (Nezhat et al., 2013).

References

- Abrao, M. S., Muzii, L., & Marana, R. (2013). Anatomical causes of female infertility and their management. *International Journal of Gynaecology and Obstetrics*, 123(Suppl 2), S18–24.
- Agarwal, N., & Subramanian, A. (2010). Endometriosis—Morphology, clinical presentations and molecular pathology. *Journal of Laboratory Physicians*, 2(1), 1–9.
- Burney, R. O., & Nezhat, C. R. (2008). Infertility treatment: The viability of the laparoscopic view. *Fertility and Sterility*, 89(2), 461–464.
- Camus, E., et al. (1999). Pregnancy rates after in vitro fertilization in cases of tubal infertility with and without hydrosalpinx: A meta-analysis of published comparative studies. *Human Reproduction*, 14(5), 1243–1249.
- Esmaelzadeh, S., Rezaei, N., & HajiAhmadi, M. (2004). Normal uterine size in women of reproductive age in northern Islamic Republic of Iran. *Eastern Mediterranean Health Journal*, 10(3), 437–441.
- Goodman, N. F., et al. (2015a). American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and Pcos Society Disease State Clinical Review. Guide to the best practices in the evaluation and treatment of polycystic ovary syndrome—Part 2. *Endocrine Practice*, 21(12), 1415–1426.
- Goodman, N. F., et al. (2015b). American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and Pcos Society Disease State Clinical Review. Guide to the best practices in the evaluation and treatment of polycystic ovary syndrome-part 1. *Endocrine Practice*, 21(11), 1291–1300.

- Hinckley, M. D., & Milki, A. A. (2004). 1000 office-based hysteroscopies prior to in vitro fertilization: Feasibility and findings. *Journal of the Society of Laparoendoscopic Surgeons*, 8(2), 103–107.
- Homer, H. A., Li, T. C., & Cooke, I. D. (2000). The septate uterus: A review of management and reproductive outcome. *Fertility and Sterility*, 73(1), 1–14.
- Kawwass, J. F., et al. (2013). Tubal factor infertility and perinatal risk after assisted reproductive technology. *Obstetrics and Gynecology*, 121(6), 1263–1271.
- Koninckx, P. R., et al. (1994). Deeply infiltrating endometriosis is a disease whereas mild endometriosis could be considered a non-disease. *Annals of the New York Academy of Sciences*, 734, 333–341.
- Kreisel, K., et al. (2017). Prevalence of pelvic inflammatory disease in sexually experienced women of reproductive age—United States, 2013–2014. *MMWR. Morbidity and Mortality Weekly Report*, 66(3), 80–83.
- Littman, E., et al. (2005). Role of laparoscopic treatment of endometriosis in patients with failed in vitro fertilization cycles. *Fertility and Sterility*, 84(6), 1574–1578.
- Maheshwari, A., et al. (2012). Adenomyosis and subfertility: A systematic review of prevalence, diagnosis, treatment and fertility outcomes. *Human Reproduction Update*, 18(4), 374–392.
- March, C. M. (2011). Asherman's syndrome. *Seminars in Reproductive Medicine*, 29(2), 83–94.
- Nezhat, C., Grace, L., Solimannjad, R., Razavi, G. M., & Nezhat, A. (2016). Cesarean scar defect: What is it and how should it be treated? *OBG Management*, 28(4), 32–53.
- Nezhat, F., et al. (1992). Clinical and histologic classification of endometriomas. Implications for a mechanism of pathogenesis. *The Journal of Reproductive Medicine*, 37(9), 771–776.
- Nezhat, C., Buescher, E., Paka, C., Nezhat, C., & Nezhat, F. (2013). Nezhat's Video-Assisted and Robotic-Assisted Laparoscopy and Hysteroscopy. In C. Nezhat, F. Nezhat, & C. Nezhat (Eds.), *Video-assisted laparoscopic treatment of endometriosis* (4th edn.). United States of America: Cambridge University Press.
- Oral, E., et al. (1996). Peritoneal fluid from women with moderate or severe endometriosis inhibits sperm motility: The role of seminal fluid components. *Fertility and Sterility*, 66(5), 787–792.
- Perez-Medina, T., et al. (2005). Endometrial polyps and their implication in the pregnancy rates of patients undergoing intrauterine insemination: A prospective, randomized study. *Human Reproduction*, 20(6), 1632–1635.
- Shalev, J., et al. (2000). Predictive value of transvaginal sonography performed before routine diagnostic hysteroscopy for evaluation of infertility. *Fertility and Sterility*, 73(2), 412–417.
- Simms, I., et al. (2006). Risk factors associated with pelvic inflammatory disease. *Sexually Transmitted Infections*, 82(6), 452–457.
- Song, T., et al. (2016). Laparoscopic tube-preserving surgical procedures for ectopic tubal pregnancy. *Obstetrics and Gynecology Science*, 59(6), 512–518.
- Stein, K., & Ascher-Walsh, C. (2009). A comprehensive approach to the treatment of uterine leiomyomata. *Mount Sinai Journal of Medicine*, 76(6), 546–556.
- Stovall, D. W., Fernandez, A. S., & Cohen, S. A. (2006). Laparoscopy training in United States obstetric and gynecology residency programs. *Journal of the Society of Laparoendoscopic Surgeons*, 10(1), 11–15.
- Tenore, J. L. (2000). Ectopic pregnancy. *American Family Physician*, 61(4), 1080–1088.
- Vilos, G. A., et al. (2009). Three simple steps during closed laparoscopic entry may minimize major injuries. *Surgical Endoscopy*, 23(4), 758–764.
- Westrom, L., et al. (1992). Pelvic inflammatory disease and fertility. A cohort study of 1,844 women with laparoscopically verified disease and 657 control women with normal laparoscopic results. *Sexually Transmitted Diseases*, 19(4), 185–192.

MEDICAL CONDITIONS

Polycystic Ovary Syndrome

Daria Lizneva, Augusta University, Augusta, GA, United States

Ihor Atabiekov, Professional Pathology Hospital, Krasnodar, Russia

Ricardo Azziz, The State University of New York (SUNY) System Administration, Albany, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Androgens Refer to hormones (a type of steroid) that are found in males in much higher concentration than in women and are responsible for many of the male-like features (muscle growth, bears and other male-like hair growth, libido, etc.).

Congenital adrenal hyperplasia Refers to a genetic disorder that affects the ability of the adrenal to produce cortisol and other substances normally.

Polycystic ovary syndrome Is a common genetic trait that presents generally with evidence of hyperandrogenism, ovulatory dysfunction and polycystic ovaries.

Ovulatory dysfunction Refers to abnormal, irregular, infrequent or absent ovulation.

Polycystic ovaries Refer to ovaries that morphologically are larger than usual and contain a larger number of small arrested follicles (cysts) within the ovarian cortex (surface).

Hyperandrogenism Refers to the signs and symptoms of excess male hormone effect in women, including hirsutism, acne, scalp hair loss and high levels of androgens in the blood.

Hirsutism Is a cutaneous sign of hyperandrogenism and defined as excessive male-pattern terminal hair-growth.

Infertility Is a failure of the couple to conceive after one year of regular sexual life.

Abbreviations

17OHP 17-Hydroxyprogesterone

AMH Anti-Müllerian hormone

DHT Dihydrotestosterone

DVT Deep vein thrombosis

FSH Follicle-stimulating hormone

GnRH Gonadotropin-releasing hormone

HA Hyperandrogenism

IGF1 Insulin-like growth factor 1

IGT Impaired glucose tolerance

IVF In vitro fertilization

IVM In vitro maturation

LH Luteinizing hormone

LOD Laparoscopic ovarian drilling

mFG Modified Ferriman–Gallwey scale

OA Oligo/anovulation

OCPs Oral contraceptive pills

OHSS Ovarian hyperstimulation syndrome

PCOM Polycystic ovarian morphology

PCOS Polycystic ovary syndrome

QOL Quality of life

SHBG Sex hormone binding globulin

Introduction

Polycystic ovary syndrome (PCOS) is a disorder affecting 5%–20% of reproductive age women worldwide, depending on diagnostic criteria used (Lizneva et al., 2016a). The main PCOS features include clinical and/or biochemical hyperandrogenism (i.e., hirsutism, acne, androgenic alopecia, hyperandrogenemia), ovulatory dysfunction and polycystic ovarian morphology (PCOM) (i.e., an excessive number of preantral follicles in the ovarian cortex).

The phenotype of PCOS varies across a woman’s lifespan. The clinical presentation of PCOS often starts early in life, and may be associated with premature pubarche in children, early signs of hyperandrogenism (acne, hirsutism) and long-lasting menstrual dysfunction in adolescents. While hyperandrogenic features usually improve in peri-postmenopausal women, the prevalence of metabolic and cardiovascular disorders increases sharply with age, although paradoxically may not increase further than expected for age and body mass after menopause.

The clinical manifestations of PCOS go beyond reproductive signs and symptoms. While polycystic ovaries, hyperandrogenism, and ovulatory dysfunction are considered key features, the majority of affected women also have various degrees of metabolic dysfunction (insulin resistance and secondary hyperinsulinemia). Additional health issues related to PCOS include an increased risk of type 2 diabetes mellitus (T2DM), several pregnancy-related complications (gestational diabetes, meconium aspiration syndrome, preeclampsia), endometrial cancer, and possibly increased risks of cerebrovascular and cardiovascular events, and deep venous thrombosis (Zore et al., 2017).

PCOS Criteria and Phenotypes

PCOS is a syndrome of as yet unknown etiology, although it appears to be a complex genetic trait. Three sets of diagnostic criteria have been proposed to diagnose PCOS (Table 1) (Lizneva et al., 2016a). Currently, there is the general agreement that to be diagnosed with PCOS affected patients should have at least two of three primary features, including chronic anovulation, PCOM, and hyperandrogenism (i.e. the Rotterdam 2003 criteria). Moreover, the 2012 NIH Consensus conference supported the use of Rotterdam criteria, with the caveat that the phenotype(s) of PCOS would also be recorded: Phenotype A—clinical and/or biochemical hyperandrogenism (HA) and oligo-/anovulation (OA) and polycystic ovarian morphology (PCOM) at ultrasound; Phenotype B—HA with OA only; Phenotype C—HA with PCOM only; and Phenotype D—OA with PCOM (Fig. 1).

Table 1 Contemporary diagnostic criteria for PCOS.

NIH (1990)^a Requires both of the following features: 1. Oligo/anovulation (OA) 2. Hyperandrogenism (HA), clinical and/or biochemical
Rotterdam (2003)^a Requires at least two of the following three features: 1. OA 2. HA, clinical and/or biochemical 3. Polycystic ovarian morphology (PCOM)
Androgen Excess & PCOS Society (2006)^a Requires both of the following features: 1. HA, clinical and/or biochemical 2. Ovarian dysfunction, OA and/or PCOM
NIH Consensus (2012)^a Requires at least two of the following three features: 1. OA 2. HA, clinical and/or biochemical 3. PCOM
AND Identification of the specific PCOS phenotypes included: • Phenotype A: HA + OA + PCOM • Phenotype B: HA + OA • Phenotype C: HA + PCOM • Phenotype D: OA + PCOM

Abbreviations: NIH is the US National Institutes of Health; Rotterdam is the 2003 European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine criteria.
^aAll criteria require the exclusion of related or mimicking disorders including, but not limited to, androgen-secreting neoplasms, iatrogenic hyperandrogenism, congenital adrenal hyperplasia, thyroid dysfunction and hyperprolactinemia.

2003 Rotterdam criteria				
2006 AE-PCOS criteria				
1990 US NIH criteria				
	Phenotype A	Phenotype B	Phenotype C	Phenotype D
Hirsutism and/or hyperandrogenemia	+	+	+	-
Anovulatory cycles	+	+	-	+
Polycystic ovarian morphology	+	-	+	+

Fig. 1 PCOS diagnostic criteria and phenotypes. There are four contemporary diagnostic criteria for PCOS. There are also four principal phenotypes of PCOS which differ in their combinations of clinical features, including hyperandrogenism (presence of hirsutism and/or hyperandrogenemia), ovulatory dysfunction, and polycystic ovarian morphology. Phenotypes A and B are most often seen in the clinical setting, because they are the most clinically evident.

PCOS Prevalence

Up to 20% of women of reproductive age have PCOS worldwide ([Lizneva et al., 2016a](#)), although the exact prevalence depends on the diagnostic criteria used: 12%–20% according to the Rotterdam 2003 criteria; 10%–15% when the AE-PCOS 2006 criteria are applied; and 5%–10% when the prevalence is estimated using the NIH 1990 criteria. The higher prevalence of PCOS observed with the Rotterdam and AE-PCOS criteria are related to the inclusion of a greater number of phenotypes of PCOS in these criteria, compared to the NIH 1990 criteria.

PCOS Genetics

PCOS appears to be a complex genetic trait ([Mykhalchenko et al., 2017](#)). Twin studies have demonstrated a high level of heritability (~70%), consistent with the strong influence of genetic factors. The recent genome-wide association studies (GWAS) in several Caucasian and Asian cohorts ([Azziz, 2016](#)) have reported at least 16 robust loci associated with PCOS. Several of these loci are proximate to genes with significant roles regulating gonadotropin secretion and action, ovarian folliculogenesis, insulin secretion and action, weight and energy regulation, and androgen biosynthesis and action.

Mechanisms/Pathophysiology

Potential pathophysiologic mechanisms include primary hypothalamic-pituitary, ovarian, adrenal, and metabolic dysfunctions ([Azziz et al., 2016](#)).

Hypothalamic-Pituitary Dysfunction

Hypothalamically-secreted gonadotropin-releasing hormone (GnRH) is responsible for the secretion of gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]) by the pituitary. In PCOS patients, GnRH appears to be released with increased frequency and amplitude, possibly in part due to hypothalamic resistance to circulating progesterone and/or hypothalamic kisspeptin dysregulation, which leads to increased LH pulse amplitudes, and increased circulating LH and LH/FSH values (more often observed in lean, rather than obese, women with PCOS). This hypothalamic-pituitary dysfunction, together with ovarian resistance to FSH possibly due in part to excess Anti-Müllerian Hormone (AMH) production by the ovary, further increases androgen secretion by the theca cells of ovarian follicles in PCOS. As a result, follicular development is arrested, cycles are anovulatory, and theca cells over-secrete androgens ([Fig. 2](#)).

Ovarian Dysfunction

Much of the ovarian abnormality observed in PCOS may relate to hypothalamic-pituitary dysfunction (see above), it is also possible that intrinsic defects in ovarian function may account for the functional and morphologic gonadal defects seen in the disorder. As

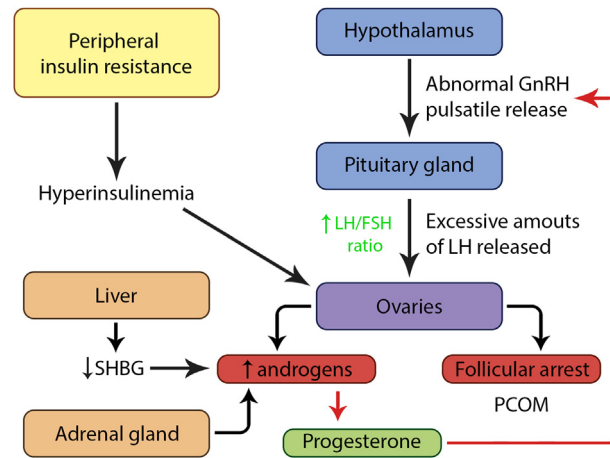


Fig. 2 Scheme of PCOS pathophysiology. Abnormal pulsation of gonadotropin-releasing hormone (GnRH) from hypothalamus, possibly secondary to a reduction in the inhibitory effect of progesterone on GnRH release, leads to increased release of luteinizing hormone (LH) by the pituitary. Although the levels of follicle-stimulating hormone (FSH) are usually normal in patients with PCOS, there may be ovarian follicular resistance to FSH, possibly secondary to increased ovarian concentrations of Anti-Müllerian Hormone (AMH), among other factors. LH hyperproduction, in the face of relative resistance to FSH, leads to excessive secretion of androgens by the ovarian theca, follicular maturation arrest, oligo/anovulation, and the increased accumulation of a greater number of antral follicles in the ovaries, resulting in the characteristic polycystic ovarian morphology (PCOM) and androgen hyperproduction. Adrenocortical dysfunction may contribute modestly to circulating hyperandrogenemia, and metabolic dysfunction results in hyperinsulinemia, which in turn acts on the ovaries to further stimulate androgen secretion by the ovarian theca. *PCOM*, polycystic ovarian morphology; *SHBG*, sex hormone-binding globulin. *Red arrows* indicate inhibitory effect.

noted, anovulation in PCOS is the result of abnormal interactions between LH, FSH, AMH, insulin (see below), insulin-like growth factor 1 (IGF1), and many other substances.

However, and as suggested by the results of recent GWAS, there also may be defects inherent to ovarian follicular function. Dominant follicle maturation occurs irregularly due to relative FSH deficiency and ovarian resistance to FSH, possibly caused by increased levels of AMH. Increased LH stimulates excess androgen production by the theca cells, which in turn promotes excessive primordial follicle accumulation. These arrested primordial follicles individually and as a cohort produce high amounts of AMH, which inhibits aromatase activity, and may also result in FSH resistance, in granulosa cells necessary for further follicular development and maturation (Fig. 3). Thus, even normal levels of FSH in anovulatory PCOS may not be sufficient to overcome the inhibitory effects of AMH.

Increased androgen production in the theca of ovarian follicles in PCOS may also be secondary to the dysregulation of several steroidogenic genes. For example, a recent study observed that *DENND1A* is overexpressed in the theca cells of PCOS, encoding for overactive steroidogenesis in these ovaries (McAllister et al., 2014). Finally, intrinsic defects in insulin action in the ovary may also result in abnormal androgen production. In general, these mechanisms result in ovarian hyperandrogenism, ovulatory dysfunction, and circulating hyperandrogenemia and hyperestrogenemia (secondary to the peripheral conversion of androgens to estrogens).

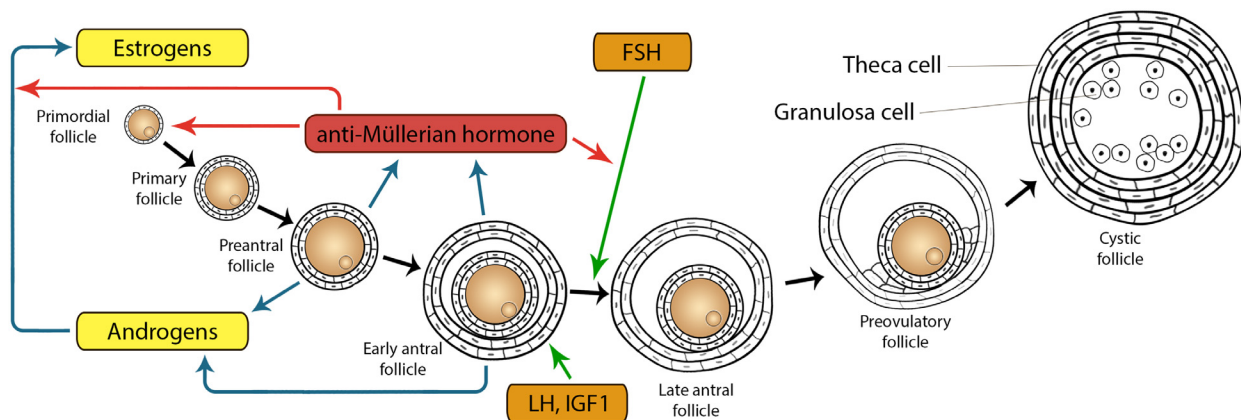


Fig. 3 Ovarian follicular arrest in PCOS. In PCOS ovarian follicular maturation is generally arrested at the early antral stage. *Green lines* indicate stimulation, *red lines* indicate inhibition, and *blue lines* indicate synthesis.

Adrenal Dysfunction

Approximately one-third of patients with PCOS demonstrate evidence of excess adrenal androgen production, principally reflected in increased circulating levels of dehydroepiandrosterone (DHEA) sulfate (DHEAS) ([Goodarzi et al., 2015](#)). These patients demonstrate increased responsivity, but not increased sensitivity, of adrenal androgens to adrenocortical releasing hormone (ACTH) stimulation. However, they do not demonstrate a change in ACTH sensitivity to corticotrophic releasing hormone (CRH) stimulation. Steroidogenically PCOS subjects demonstrate enhanced Δ^5 17–20 lyase activity, reflecting apparent intrinsic cytochrome P450c17 α (CYP17) dysfunction. Overall, however, adrenocortical excess plays a limited role in the pathophysiology of PCOS due to the relative weakness of androstenedione and DHEA, the principal androgens secreted by the adrenal cortex.

Metabolic Dysfunction

Patients with PCOS demonstrate reduced sensitivity to the glucose and lipid, but possibly not the anabolic, effects of insulin. In compensation, patients with PCOS secrete increased levels of insulin, resulting in hyperinsulinemia ([Diamanti-Kandarakis & Dunaif, 2012](#)). Hyperinsulinemia in PCOS can also be exacerbated by decreased hepatic insulin extraction. Excess insulin in PCOS stimulates increased androgen production by the ovarian theca cells and decreased androgen binding in the circulation by inhibiting the hepatic production of sex hormone-binding globulin (SHBG).

We should note that in normal states decreases in insulin sensitivity result in increases in insulin secretion, a constant hyperbolic relationship which reflects the ability of pancreatic islet beta cells to compensate for the insulin resistance. This relationship between insulin secretion and insulin resistance is expressed by the Disposition Index (DI). However, in PCOS despite the prevalent hyperinsulinemia, the insulin secretory response for the degree of insulin resistance is inadequate, resulting in a decreased DI compared with age and body mass index (BMI)-matched controls. Overall, these data indicate that despite the level of hyperinsulinemia observed in PCOS, these patients actually demonstrate relative beta-cell insufficiency.

The molecular pathophysiology of the insulin resistance in PCOS differs from that observed in T2DM or obesity. The molecular defects in insulin action observed vary somewhat according to the tissues studies, although in general there are few defects observed in the mitogenic, as opposed to the glucose transport, insulin signaling cascade. Defects in insulin action have been observed in various tissues including skin fibroblasts, ovary, muscle and myocytes, and adipose tissue and adipocytes ([Diamanti-Kandarakis & Dunaif, 2012](#); [Corbould et al., 2006](#); [Chen et al., 2013](#)). In adipocytes of PCOS inflammatory cytokines (e.g. TNF- α) appear to have a stronger inhibitory effect on insulin-mediated glucose transport and adiponectin (an insulin-sensitizing adipokine) secretion, compared to controls ([Chazenbalk et al., 2010](#)). Further, adipocytes in PCOS demonstrate impaired Glucose Transporter 4 (GLUT4) expression (10).

Clinical Signs

Dermatologic Abnormalities

Hirsutism is observed in at least two-third of White and Black women with PCOS. Hirsutism is characterized by the presence of terminal (thick, coarse and pigmented) hairs in androgen-dependent areas, such as the upper lip, neck and chin, upper and lower abdomen, upper and lower back, buttocks and thighs, and upper arms ([Azziz et al., 2016](#); [Lizneva et al., 2016b](#)). The development of hirsutism in PCOS occurs gradually, which allows it to be clinically differentiated from hirsutism associated with androgen-secreting neoplasms, where the onset of hair growth is usually rapid and more severe, and others signs of virilization are present (e.g. clitoromegaly).

Additionally, 15%–25% of patients with PCOS demonstrate acne. While acne is considered to be a common finding in adolescence, if acne is severe, resistant to treatment, persists after puberty, or exacerbates in the mid-20s, evaluation for hyperandrogenism may be indicated. Finally, some women with PCOS and other hyperandrogenic disorders demonstrate androgenic alopecia (a diffuse thinning of the hair in the sagittal area of the scalp) and in more severe cases demonstrate frank male-like pattern balding.

Menstrual Abnormalities

The vast majority of patients with PCOS demonstrate some degree of menstrual dysfunction ([Azziz et al., 2009](#)) reflecting the underlying ovulatory dysfunction, including oligomenorrhea, amenorrhea and occasionally polymenorrhea. However, some patients may also present with apparent eumenorrhea. In one study, 40% of subjects with hirsutism and apparent eumenorrhea demonstrated underlying ovulatory dysfunction.

Polycystic Ovaries

At least 80% of PCOS subjects demonstrate PCOM, characterized by increased ovarian volume (i.e. generally > 10 mL) and/or increased antral follicle count (AFC, i.e. generally > 12 to > 20 follicles 2–9 mm in diameter). PCOM is usually detected by ultrasonography, and as the technology has improved so has the ability to detect and differentiate small follicles, suggesting the need to increase the cut-off value for AFC for diagnosing PCOM ([Dewailly et al., 2014](#)).

Associated Morbidities

Associated morbidities include increased risks for subfertility, obstetrical complications, obesity, metabolic dysfunction, vascular complications, malignancies, and mood disorders and psychosexual dysfunction ([Zore et al., 2017](#)).

Subfertility

Women with PCOS have impaired fertility primarily due to oligo-anovulation. Infertility is 15 times prevalent in PCOS patients, regardless of BMI, compared to controls.

Obstetrical Complications

Pregnancies in PCOS patients have a significantly higher rate of complications including pre-eclampsia, very preterm delivery (< 32 weeks of gestation), gestational diabetes, fetal macrosomia, and signs of fetal distress (e.g. meconium aspiration syndrome, low Apgar scores) ([Roos et al., 2011](#); [Wang et al., 2013](#)).

Overweightness and Obesity

Recent clinical studies demonstrated a higher prevalence of obesity in PCOS women referred for clinical care, whereas the BMI distribution of PCOS women detected in screening studies in the general population are not much different than that observed in non-affected women in the same population ([Lizneva et al., 2016c](#)). Interestingly, there are no significant differences in the prevalence of PCOS in different countries, despite significant variations in mean population BMI and obesity incidence ([Lizneva et al., 2016a](#)). No association between variants in genes known to be associated with obesity and PCOS has been found ([Cai et al., 2014](#)). These data further support the notion that the relationship of obesity and PCOS is tenuous at best.

Metabolic Dysfunction and Glucose Intolerance

Hyperinsulinemia (both basal and glucose-stimulated) is observed in the majority of PCOS patients. Premenopausal women with PCOS have higher odds for impaired glucose tolerance (IGT), T2DM, and the metabolic syndrome than age- and BMI-matched controls. Approximately 2% of PCOS women progress from normoglycemic status to T2DM yearly.

Cardiovascular and Cerebrovascular Complications

The prevalence of cardiovascular markers of disease is higher in women with PCOS. These include coronary and aortic calcification, aortic stenosis (based on coronary angiography) and a thicker intimal layer of the carotid wall compared with controls. However, while the risk of cerebrovascular complications appears to be increased in PCOS, it is less clear whether the risk of cardiovascular events is actually increased in the disorder. In addition, the odds of deep vein thrombosis (DVT) are at least 1.5 fold higher in untreated women with PCOS vs. BMI-matched controls, and even higher (2-fold greater) in PCOS patients taking oral contraceptive pills (OCPs).

Malignancies

Taking into account the coexistence of anovulation, hyperestrogenemia, and hyperinsulinemia in PCOS, the risk of endometrial cancer is higher in women with PCOS (approximately 2.7 fold greater). The risk of ovarian cancer may also be increased, although data is mixed. No significant increase in the risk of breast cancer in PCOS patients compared with controls has been observed ([Zore et al., 2017](#); [Azziz et al., 2016](#)).

Mood Disorders and Psychosexual Dysfunction

Recent studies report a higher incidence and severity of mood disorders (depression, anxiety) and psycho-sexual dysfunction, in women with PCOS compared to non-PCOS patients. The increased risk is independent of BMI and PCOS phenotype, but correlated with the degree of insulin resistance ([Cinar et al., 2011](#)).

Diagnosis

All criteria used today include assessments for hyperandrogenism, ovulatory dysfunction and ovarian morphology ([Table 1](#)). The evaluation of PCOS patients should include assessment for hyperandrogenism (hirsutism scoring, evaluation for acne and alopecia, monitoring of circulating androgen levels), ovulatory function (detailed menstrual history and luteal progesterone level testing in eumenorrheic hirsute women), and the evaluation of ovarian morphology for PCOM by ultrasonography, preferably transvaginal.

Hirsutism is assessed using a visual scoring method. A score of >6 on a modified Ferriman–Gallwey (mFG) scale defines the presence of hirsutism, although larger populational studies suggest that mFG values as low as 3 may be abnormal. Girls with severe or persistent acne have an increased probability of having PCOS. Biochemically, the measurement of free testosterone is superior to total testosterone alone for the detection of hyperandrogenism. Measurement of testosterone is innately difficult and most commercial or platform assays are insensitive in the female range; consequently the optimum method for assessing total testosterone, also used to calculate free testosterone, is mass spectrometry.

Chronic anovulation is assumed in individuals with oligo-amenorrhea, i.e. prolonged (>35 – 45 days) menstrual cycle or <8 to 10 cycles per year. In such cases, no further testing for anovulation is required. Alternatively, a history of apparent ‘eumenorrhea’ in a patient with other signs of hyperandrogenism (e.g. hirsutism) suggests the need for further assessment of ovulatory status. A mid-luteal progesterone (days 22 to 24 of the cycle) can be used to confirm ovulation.

According to the Rotterdam criteria, PCOM is determined by the presence of 12 or more antral follicles (i.e. measuring 2 to 9 mm in diameter) in the ovary and/or an increase in ovarian volume (>10 mL). However, because more contemporary ultrasound probes have a much greater sensitivity, threshold counts for antral follicles may be as high as 25 (Dewailly et al., 2014). In the absence of accurate ultrasonography AMH values >4.5 ng/mL may be used as a substitute for PCOM, although this remains to be confirmed (Dewailly et al., 2014).

All PCOS criteria used today (Table 1) require the exclusion of mimicking/similar disorders, including but not limited to thyroid disorders and hyperprolactinemia for oligo-anovulation, and androgen-secreting tumors, congenital adrenal hyperplasia, and iatrogenic causes for hyperandrogenism. For instance, nonclassic 21-hydroxylase (CYP21A) deficiency may have the same clinical presentation as PCOS (hyperandrogenism, anovulation, and PCOM), and only a circulating 17-hydroxyprogesterone (17-OHP) can be used to discriminate between the disorders (Pall et al., 2010). Post-stimulation 17-OHP values >10 ng/mL confirm the diagnosis, whereas 17-OHP levels between 2 and 10 ng/mL require an acute ACTH stimulation test for diagnosis (Lizneva et al., 2016b).

Once the diagnosis of PCOS is established, patients should be assessed for glucose intolerance, dyslipidemia, and mood disorders. An oral glucose tolerance test (oGTT) is the preferred method for detecting glucose intolerance or T2DM, and should be repeated every 2 to 3 years, depending on BMI and family history of T2DM, and yearly in women with impaired glucose tolerance as the rates of progression from normal to IGT and T2DM have been reported to be between 5% and 15% within 3 years (Salley et al., 2007).

Every newly diagnosed PCOS patient should also be evaluated for cardiovascular disease and metabolic syndrome risk factors (BMI, age, smoking, family history, lipid profile, liver function tests, high blood pressure, etc.). Further assessment should be considered on an individual basis.

Finally, in PCOS women with a long-standing of anovulation and no endometrial protection, endometrial hyperplasia/cancer should be excluded by ultrasonography and endometrial sampling.

Screening for PCOS in Childhood and Adolescence

Daughters and sisters of women affected by PCOS have a higher chance of developing the disorder (Mykhalchenko et al., 2017); therefore, early screening should be recommended for all first degree relatives of affected patients. Initial treatment with the insulin-sensitizer metformin (see below) may reduce progression to PCOS in girls, at least those with low birthweight and precocious pubarche.

Acne, hyperinsulinemia, and anovulatory cycles are often seen during the pubertal transition. However, irregular menstruation might require medical attention if it persists more than three years after menarche, generally after age 15 years old. In addition, the threshold for defining oligomenorrhea is somewhat higher during in adolescence (i.e. a menstrual cycle length >40 days is considered abnormal) compared to adult women (i.e. cycles >35 days in length are considered abnormal).

The current recommended approach to detecting PCOS in adolescent girls at risk for the disorder is to wait for 2–3 years after menarche before starting vigorous evaluations, unless signs and symptoms mandate an evaluation sooner, and to not finalize the diagnosis unless the clinical picture is unambiguous (Witchel et al., 2015). Early confirmation of the diagnosis helps to reduce the long-term sequelae of hirsutism, acne and alopecia and may reduce the probability of later complications, such as T2DM and endometrial cancer.

Management

An individualized approach should be used for every patient with PCOS as this disorder is heterogeneous, complex, multifactorial and life-long (Azziz et al., 2016; Lizneva et al., 2016b).

Management of PCOS-Associated Obesity

Lifestyle modification

First-line treatment in obese or overweight patients with PCOS is lifestyle modification, which alone may significantly improve ovulatory, mood, fertility and metabolic dysfunction. Even a minimal reduction ($>5\%$) of body weight in a PCOS woman with

increased BMI significantly improves metabolic dysfunction and ovulation. Weight loss achieved by a high-protein diet is well tolerated and improves satiety, but there is still no specific diet recommended for PCOS women that has been studied long-term. Physical activity, even high intensive, usually has a limited effect on weight loss in PCOS patients. However, if performed for at least 30 min per day five times per week, physical exercise may improve insulin resistance and reduce cardiovascular risk.

Medical therapy

Medical treatment is available for weight reduction. Few of these medications have been studied in PCOS. Orlistat, which inhibits lipase in the intestine, has a positive effect on weight reduction in women with PCOS.

Bariatric surgery

Bariatric surgery is another option for obese women with PCOS, reserved for those individuals with morbid obesity (BMI > 40 kg/m²) and for women with BMI > 35 kg/m² who have other health issues. It is highly effective as it improves not only body weight, but also signs of PCOS, by 46% to 7%, with a mean weight loss of about 60% (Skubleny et al., 2016).

Management of Metabolic Dysfunction and Dyslipidemia

Insulin sensitizers

In patients with significant insulin resistance/hyperinsulinemia and/or at increased risk for glucose intolerance (e.g. demonstrating elevated levels of insulin and/or glucose intolerance during an oGTT, a strong family history of glucose intolerance, acanthosis nigricans, etc.) improvement in insulin sensitivity should be considered, either through weight loss (see above) or the use of insulin sensitizers. If lifestyle modification is not sufficient in an obese patient with PCOS, or the patient is not obese (considering that between 40% and 60% of PCOS women may not be obese) additional medical measures should be considered for long-term improvement in insulin sensitivity. Metformin (a biguanide) is commonly used to manage glucose intolerance and T2DM, as it suppresses hepatic glucose synthesis and improves insulin sensitivity peripherally. In PCOS it also reduces insulin-dependent androgen synthesis, and improves menstrual function and fertility. When combined with lifestyle modification, it facilitates weight loss in obese women with PCOS, although metformin alone has no significant impact on BMI in obese women and does not improve fasting glucose and lipid levels.

Thiazolidinediones have a stronger effect on insulin resistance and fasting insulin levels, but are not as efficient in normalizing excess weight and triglyceride levels. Because of possible serious side effects, they are not routinely recommended for use in PCOS, although may be reserved for those patients with serious and severe degrees of insulin resistance.

Inositol isomers (di-*chiro*-inositol and/or myo-inositol) have insulin-mimetic properties because they take act as secondary messengers in the insulin signaling cascade, which explains their effectiveness in reducing post-prandial glucose levels. They may also significantly improve insulin resistance and menstrual regularity in PCOS, although di-*chiro*-inositol alone was not found to be as effective as expected. A combination of the two isomers may be more effective. The exact dosing of these substances is unclear, and their benefit in PCOS management remains to be investigated.

Acarbose suppresses α -glucosidase and is known to improve lipid levels in PCOS patients, but the effect on BMI in these subjects is unclear.

Statins

Statins (HMG-CoA reductase inhibitors) are effective in normalizing dyslipidemia in PCOS, reducing the circulating levels of total cholesterol and triglycerides. The beneficial effect of statins on lipids in PCOS may be further augmented if used together with metformin, but this combination lessens the beneficial effect of metformin on insulin resistance. The safety of statins in pregnancy is unclear, and these drugs should be discontinued before conception.

Management of Hyperandrogenic Signs and Symptoms

Combination hormonal contraceptives

Combination OCPs are first-line treatment for menstrual irregularity in women with PCOS who are not seeking pregnancy. They inhibit ovarian androgen production by suppressing gonadotropin secretion, thus improving hirsutism and acne, and normalize menstrual cyclicity. Further, the estrogenic component of the OCP increases hepatic production of SHBG. This effect is not seen with transdermal contraceptives. The progestin component of OCPs may also directly suppress the synthesis of ovarian androgens. OCPs also protect women with PCOS against their higher risks of endometrial hyperplasia and cancer (see below). Prior to administering OCPs patients should be screened for contraindications to their usage. On occasion, reduction in androgen production can be achieved with continuous progestogens or rarely long-acting GnRH analogues.

Antiandrogens

Direct androgen receptor antagonists in general use in PCOS patients include spironolactone (SPA), flutamide (FLUT), and the progestogen, cyproterone acetate (CPA). SPA is the most commonly used androgen inhibitor in the United States, while CPA and FLUT are more widely used elsewhere. SPA is effective in the management of hirsutism (dosage is 25–100 mg bid). As it is also a diuretic, acting as an aldosterone inhibitor, patients may demonstrate polyuria, hypotension and even syncope. Rarely

will patients develop hyperkalemia and serum potassium levels may be monitored at regular intervals. Other side effects include dyspepsia, excess sensitivity to sun, and atopic reactions. FLUT (used in doses of 50–100 mg/day) has less side-effects than SPA, although may result in greenish urine. More importantly, and although quite rare, PCOS and other patients have been reported to suffer from sudden fulminant hepatic failure and death while on FLUT therapy. Thus FLUT is not recommended as first line therapy for PCOS-associated hyperandrogenism and patients taking FLUT should have their liver function tests monitored before and at regular intervals during treatment. CPA is a progestogen and is frequently included in a combination OCP in doses of 2 mg/day. However, this dosage provides limited antiandrogenic effect, and supplemental CPA in doses of 10–30 mg/day should be considered in patients with significant clinical hyperandrogenism.

5 α -reductase inhibitors (e.g. finasteride) antagonize the effect of androgens by inhibiting the conversion of testosterone to its more potent metabolite, dihydrotestosterone (DHT). It is used in a daily dose of 5 mg and has little side-effects.

All antiandrogens are considered potentially teratogenic, particularly to a male fetus, where underdevelopment of the genitalia may occur, and may also be associated with a slight decrease in libido and muscle strength. Combining antiandrogens with OCPs provides contraceptive protection against pregnancy and potentiates their respective effect on hyperandrogenism. In general, improvements in androgen-associated acne may be evident within 2–3 months and improvements in hirsutism within 6 months of treatment, while improvements in androgenic alopecia are much less consistent and will take longer.

Eflornithine hydrochloride

A topical eflornithine hydrochloride solution irreversibly suppresses ornithine decarboxylase in hair follicles and can be used for the local treatment of excessive facial hair.

Cosmetic treatments

Inhibition of androgen synthesis and their peripheral action helps to prevent progression of skin changes in PCOS (hirsutism, acne, androgenic alopecia), and may ameliorate them over time. However, the addition of cosmetic measures should be considered to both accelerate the process and treat signs that do not regress with medical therapy alone. Hirsutism may be treated temporarily by shaving, depilation, or waxing. More permanent destruction of the offending hair follicles may be achieved with laser therapy and, more effectively, electrolysis. Acne can be managed with various medications including retinoids and antibacterials. Topical minoxidil and hair transplantation can be used for androgenic alopecia.

Endometrial Protection

Chronic anovulation and hyperestrogenism place women with PCOS at greater risk for endometrial hyperplasia and cancer ([Zore et al., 2017](#)), and dysfunctional uterine bleeding, which in turn can result in iron deficiency anemia. To prevent these complications a combination hormonal contraceptive or cyclic progestogen should be used.

Treatment for Subfertility

Not all patients with PCOS are ‘infertile’ as PCOS patients do ovulation, albeit at a third of the rate of normal subjects. Further, satisfaction with ‘fertility’ depends on the age of the patient, how long she has had unprotected intercourse and her reproductive aspirations. When managing the subfertility of PCOS patients it is important to strive to achieve monofollicular ovulation in this group of patients to reduce the inherent risks of multiple births, and taking into account their increased risk of pregnancy-associated complications, including pre-eclampsia, gestational diabetes, preterm labor, and fetal distress. Prior to planned conception, women with PCOS should be counseled about the risks and success rates of future pregnancy, and encourage lifestyle modification. Screening for coexisting conditions should also be performed, including glucose intolerance and insulin resistance.

Monofollicular ovulation is commonly achieved by the use of clomiphene citrate, which competes with estrogen for binding in the hypothalamus, thus increasing production of gonadotropins. More recently, the aromatase inhibitor letrozole has proven superior to clomiphene, since it has a better probability of live births (40%–50%) and is more effective in obese PCOS women. Multiple pregnancies are observed in up to 8% of patents when these drugs are used for ovulation induction. Metformin has a modest beneficial effect on fertility but can increase the effectiveness of clomiphene citrate in some cases ([Legro, 2016](#)). Overall, patients should undergo 5 or 6 cycles of ovulation induction with these medications, assuming the patient is ovulatory, before pursuing other fertility treatments.

Gonadotropins, either an LH and FSH combination, or FSH alone, are considered second-line treatment. Administration and response should be monitored carefully to minimize the risk of multiple pregnancies (<5%) and ovarian hyperstimulation syndrome (OHSS). A low dose regimen is usually prescribed. Gonadotropin therapy is more effective in achieving pregnancy than clomiphene or letrozole, but has greater associated risks and costs.

Surgical approaches have also been used to improve ovulatory function and possibly decrease ovarian androgen production, in patients who fail clomiphene and/or letrozole. The original procedure was to perform an ovarian wedge resection (OWR), where a wedge of tissue is removed from the ovary. This has the effect of decreasing the amount of stromal-thecal tissue and reducing intrinsic androgen production with a significant positive effect on hyperandrogenism and ovulation, at least temporarily. Patients may develop adhesions and diminished ovarian reserve as long-term complications of OWR. Although OWR today can be performed laparoscopically and with preservation of a fair amount of the ovarian cortex, the preferred surgical method of treating

PCOS currently is laparoscopic ovarian drilling (LOD). The criteria for using LOD for ovulation induction is clomiphene resistance, increased LH, no possibility or contraindication to using gonadotropins, and the presence of other indications for laparoscopy. LOD is better tolerated and results in fewer adhesions and less risk of premature ovarian failure than OWR.

In vitro fertilization (IVF) is considered third line treatment for PCOS-associated infertility. The pregnancy rates with IVF are similar in patients with PCOS vs. those with other causes of infertility. In countries where multiple embryo transfers are allowed, the risk of multiple pregnancies may reach 30%. When single embryo transfers are performed, the risk of multiple pregnancies is significantly lower compared to ovulation induction with gonadotropins.

To minimize the risks of multiple pregnancies and OHSS in PCOS, various modifications to IVF have been introduced. In vitro maturation (IVM) is one example, as is cryopreservation of embryos and transfer at a later date, after the ovarian stimulation has receded. Recent studies have indicated that frozen-embryo transfers in PCOS women are associated with a higher probability of a live birth. Alternatively, pregnancy rates with IVM in PCOS is generally inferior to those achieved with IVF (Siristatidis et al., 2013).

Improvement in Quality of Life (QOL)

Quality of life (QOL) is a complex concept including many components (mental, physical, emotional), all of which are compromised in PCOS women. Obesity, dermatologic symptoms (hirsutism, acne, androgenic alopecia), irregular menses, subfertility, and psycho-sexual dysfunction all negatively affect QOL in these patients. The PCOSQ questionnaire is PCOS-specific and includes five domains of questions that are able to assess QOL (Guyatt et al., 2004). Women with PCOS consistently report a reduction in QOL scores vs. controls, as well as poorer body image.

A recent randomized trial indicated that the QOL of women with PCOS was improved (i.e. better PCOSQ scores) in response to lifestyle modifications in combination with OCPs. However, when metformin was added to their treatment, no further QOL improvement occurred. While OCPs improve dermatologic symptoms and menstrual abnormalities, they do not appear to affect mood disorders. Assessment and management of mood disorders in PCOS women should follow standard practice, and should not be ignored just because the patient may have PCOS.

References

- Azziz, R. (2016). Year in Review: PCOS 2015-New insights into the genetics of the polycystic ovary syndrome (PCOS). *Nature Reviews. Endocrinology*, 12, 74–75. Erratum in *Nature Reviews. Endocrinology*, 12, 183 (2016).
- Azziz, R., Carmina, E., Dewailly, D., et al. (2009). The androgen excess and PCOS society criteria for the polycystic ovary syndrome: The complete task force report. *Fertility and Sterility*, 91, 456–488.
- Azziz, R., Carmina, E., Chen, Z. J., et al. (2016). The polycystic ovary syndrome. *Nature Reviews. Disease Primers*, 2, 16057.
- Cai, X., Liu, C., & Mou, S. (2014). Association between fat mass- and obesity-associated (FTO) gene polymorphism and polycystic ovary syndrome: A meta-analysis. *PLoS One*, 9, e86972.
- Chazenbalk, G., Trivax, B. S., Yildiz, B. O., et al. (2010). Regulation of adiponectin secretion by adipocytes in the polycystic ovary syndrome: Role of tumor necrosis factor- α . *The Journal of Clinical Endocrinology and Metabolism*, 95, 935–942.
- Chen, Y.-H., Heneidi, S., Lee, J.-M., Stepp, D. W., Layman, L., Gamboa, G. M., Chazenbalk, G., & Azziz, R. (2013). Increased miRNA-93 inhibits GLUT4 in polycystic ovary syndrome-derived adipocytes. *Diabetes*, 62, 2278–2286.
- Cinar, N., Kizilarlanoglu, M. C., Harmanci, A., et al. (2011). Depression, anxiety and cardiometabolic risk in polycystic ovary syndrome. *Human Reproduction*, 26, 3339–3345.
- Corbould, A., Zhao, H., Mirzoeva, S., Aird, F., & Dunaif, A. (2006). Enhanced mitogenic signaling in skeletal muscle of women with polycystic ovary syndrome. *Diabetes*, 55, 751–759.
- Dewailly, D., Lujan, M. E., Carmina, E., et al. (2014). Definition and significance of polycystic ovarian morphology: A task force report from the androgen excess and polycystic ovary syndrome society. *Human Reproduction Update*, 20, 334–352.
- Diamanti-Kandarakis, E., & Dunaif, A. (2012). Insulin resistance and the polycystic ovary syndrome revisited: An update on mechanisms and implications. *Endocrine Reviews*, 33, 981–1030.
- Goodarzi, M. A., Carmina, E., & Azziz, R. (2015). DHEA, DHEAS, and PCOS. *The Journal of Steroid Biochemistry and Molecular Biology*, 145C, 213–225.
- Guyatt, G., Weaver, B., Cronin, L., Dooley, J. A., & Azziz, R. (2004). Health-related quality of life in women with polycystic ovary syndrome, a self-administered questionnaire, was validated. *Journal of Clinical Epidemiology*, 57, 1279–1287.
- Legro, R. S. (2016). Ovulation induction in polycystic ovary syndrome: Current options. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 37, 152–159.
- Lizneva, D., Suturina, L., Walker, W., Brakta, S., Gavrilova-Jordan, L., & Azziz, R. (2016a). Criteria, prevalence and phenotypes of polycystic ovarian syndrome. *Fertility and Sterility*, 106, 6–15.
- Lizneva, D., Gavrilova-Jordan, L., Walker, W., & Azziz, R. (2016b). Androgen excess: Investigations and management. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 37, 98–118.
- Lizneva, D., Kirubakaran, R., Mykhalchenko, K., Suturina, L., Galina, C., & Azziz, R. (2016c). Phenotypes and body mass in women with PCOS identified in clinical vs. unselected populations: Systematic review and meta-analysis. *Fertility and Sterility*, 106, 1510–1520.
- McAllister, J. M., Modi, B., Miller, B. A., et al. (2014). Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype. *Proceedings of the National Academy of Sciences of the United States of America*, 111, E1519–27.
- Mykhalchenko, K., Lizneva, D. L., Trofimova, T., Walker, W., Suturina, L., Diamond, M. P., & Azziz, R. (2017). Genetics of the polycystic ovary syndrome. *Expert Review of Molecular Diagnostics*, 17(7), 723–733.
- Pall, M., Azziz, R., Beires, J., & Pignatelli, D. (2010). The phenotype of hirsute women: A comparison of polycystic ovarian syndrome and 21-hydroxylase deficient non-classic adrenal hyperplasia. *Fertility and Sterility*, 94, 684–689.
- Roos, N., Kieler, H., Sahlin, L., Ekman-Ordeberg, G., Falconer, H., & Stephansson, O. (2011). Risk of adverse pregnancy outcomes in women with polycystic ovary syndrome: Population based cohort study. *BMJ*, 343, d6309.

- Salley, K. E., Wickham, E. P., Cheang, K. I., Essah, P. A., Karjane, N. W., & Nestler, J. E. (2007). Glucose intolerance in polycystic ovary syndrome—a position statement of the androgen excess society. *The Journal of Clinical Endocrinology and Metabolism*, 92, 4546–4556.
- Siristatidis, C. S., Vrachnis, N., Creatsa, M., Maheshwari, A., & Bhattacharya, S. (2013). In vitro maturation in subfertile women with polycystic ovarian syndrome undergoing assisted reproduction. *Cochrane Database of Systematic Reviews*, 2013, CD006606.
- Skubleny, D., Switzer, N. J., Gill, R. S., et al. (2016). The impact of bariatric surgery on polycystic ovary syndrome: A systematic review and meta-analysis. *Obesity Surgery*, 26, 169–176.
- Wang, Y., Zhao, X., Zhao, H., et al. (2013). Risks for gestational diabetes mellitus and pregnancy-induced hypertension are increased in lean women with polycystic ovary syndrome. *BioMed Research International*, 2013(182582).
- Witchel, S. F., Oberfield, S., Rosenfield, R. L., et al. (2015). The diagnosis of polycystic ovary syndrome during adolescence. *Hormone Research in Paediatrics*, 83, 376–389.
- Zore, T., Joshi, N. V., Lizneva, D., & Azziz, R. (June 28, 2017). PCOS: Long term health consequences. *Seminars in Reproductive Medicine*.

Further Reading

- Dumesic, D. A., & Lobo, R. A. (2013). Cancer risk and PCOS. *Steroids*, 78, 782–785.

Relevant Websites

- www.ae-society.org – The Androgen Excess and Polycystic Ovary Syndrome Society is an international organization dedicated to promoting the generation and dissemination of knowledge related to all aspects of Androgen Excess Disorders.
- www.PCOSChallenge.org – PCOS Challenge is the largest support and advocacy organization for girls and women with PCOS worldwide. It provides support for women with PCOS through the PCOS Challenge nonprofit organization website; PCOS Challenge Expert Series; PCOS Challenge Online Support Network; PCOS Challenge television show; PCOS Challenge radio show; local offline support groups; and multiple 16-week fitness, nutrition, and mental wellness challenges.

Hyperandrogenemia

Neil Chappell and Amy Schutt, Texas Children's Hospital, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Hyperandrogenemia (HA) describes the condition of a patient with increased production of a group of steroid hormones known as androgens, from the Greek prefix "andro" meaning "man." While these hormones play a central role in male physiology, they are also present to a lesser degree in females, with the most predominant androgens including testosterone, dihydrotestosterone (DHT), androstenedione, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEA-S). In women, androgens are synthesized by two endocrine organs, the ovaries and the adrenal glands, with some androgens also produced peripherally in the skin, subcutaneous tissues, and liver. The ovaries and adrenal glands both synthesize testosterone, androstenedione and DHT, while the adrenal glands are responsible for all DHEA-S production. The overall incidence of HA in women is approximately 5%–10%, with polycystic ovarian syndrome (PCOS) accounting for approximately 80% of cases. In general, HA may also be caused by exogenous medications, endogenous neoplasms, or via the nonneoplastic overproduction of androgens. Clinical signs of HA include acne, abnormal hair growth (hirsutism), and male pattern baldness (alopecia) (Azziz et al., 2004; Fritz and Speroff, 2011; Lizneva et al., 2016). Other signs of HA may also be present, including irregular menstrual cycles, virilization (deepening voice, enlarging clitoris, breast atrophy), or insulin resistance.

The steroid biosynthesis pathway and the enzymes that produce these hormones are the same in both men and women (Fig. 1, steroid hormone biosynthesis). However, there are distinct gender and tissue specific differences in the amount and activity of each of these enzymes in the body, and this balance regulates the predominant hormones produced at any one location at any given time. There are additional changes in the steroid biosynthesis pathway during pregnancy, both within the fetal and placental compartments, that can affect exposure to androgens. A complete understanding of the etiology of hyperandrogenemia in a patient requires a full appreciation of this pathway, and thus warrants further discussion in this article.

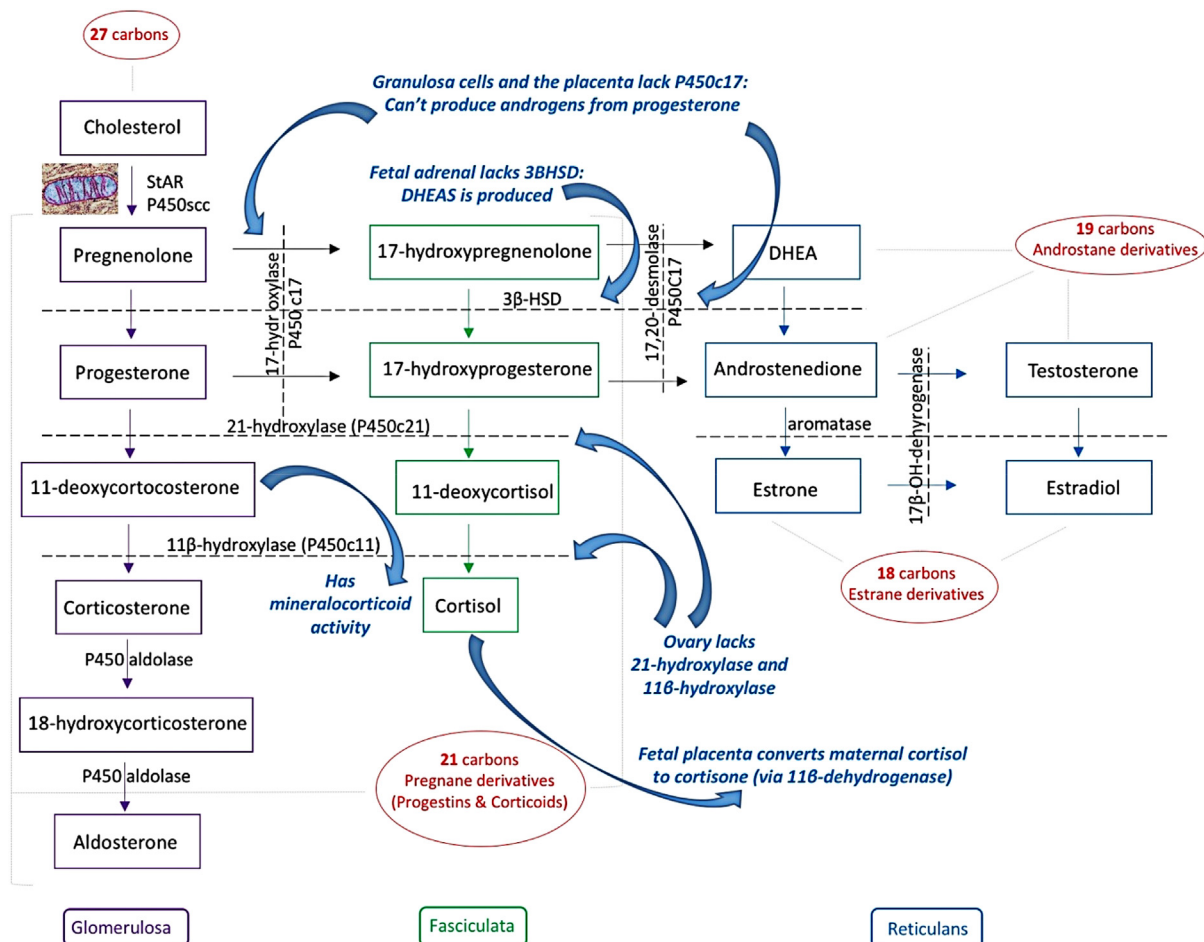


Fig. 1 Steroid hormone biosynthesis.

All steroid hormones that are synthesized via the steroid biosynthesis pathway are formed from the precursor cholesterol, which is transported into the cell by the enzyme StAR (steroid acute regulatory protein). Once inside the cell, cholesterol is cleaved by the side chain cleavage enzyme p450_{scc} into pregnenolone. Pregnenolone is then shuttled to one of several end products, depending on the tissue type and environmental cues from the body: aldosterone, cortisol, testosterone, or estradiol. Additionally, all of the intermediary hormone products formed between pregnenolone and the final end products also have endocrinologic profiles and functions that must not be forgotten. Of note, the adrenal gland has a special enzyme known as a sulfurase, which allows it to convert some DHEA into DHEA-S, making this hormone a unique marker of adrenal production.

The adrenal cortex has three layers—the zona glomerulosa, the zona fasciculata, and the zona reticularis—where aldosterone, cortisol, and androgens are produced, respectively. In the cortex of the adrenal gland, there is significant expression of 21 hydroxylase and 11 beta hydroxylase, to allow for the production of hormones such as aldosterone and cortisol along with a smaller amount of DHEA (Fritz and Speroff, 2011). The ovary, however, has more 17,20-desmolase and 17-beta-hydroxydehydrogenase activity which shunts the steroid pathway toward more androstenedione and estradiol production. The overarching concept in steroid hormone production lies in the regulation of the expression and activity of these steroid producing enzymes which allows for the preferential shunting of steroids to different end hormone products, depending on the tissue site and the body's needs at a given time.

Admittedly, the ovary is perhaps more complicated, and this added complexity is important to understand and appreciate the pathology of ovarian HA. One of the main functions of the ovary is to produce estradiol, the main form of estrogen in the female endocrine system. This is produced in two major steps. First, a specific cell type known as the theca cell produces androstenedione, which is then shuttled to another cell type called the granulosa cell. There, it undergoes a final change by the enzyme aromatase to produce the end product estradiol. This “two cell theory” is a foundational concept when considering ovarian physiology, and will be discussed in detail in the following sections.

With an understanding of steroid hormone production and an appreciation for the vast diversity of action and expression among organ sites, it is also germane to discuss the manner in which production of these hormones is stimulated. Both the ovary and the adrenal gland receive signals from the brain, specifically the pituitary gland, which is in turn regulated by the hypothalamus just superior to the pituitary gland and connected by a fenestrated collection of capillaries known as the hypophyseal portal system. This delicate vascular network allows rapid and frequent communication from the hypothalamus to the pituitary, resulting in tightly regulated control of downstream endocrine organs. The hypothalamic-pituitary connections to the ovary and the adrenal gland are known as the hypothalamic-pituitary-ovarian axis (HPO) and the hypothalamic-pituitary-adrenal axis (HPA), respectively. These axes constitute important endocrine feedback systems that allow for the homeostatic regulation of hormone production. Indeed, the hypothalamic-pituitary stimuli are sensitive to steroid products from the adrenal gland and the ovary, decreasing upstream signals from the brain in response to increasing levels of hormones in the blood through a process known as “negative feedback.”

Specifically, the HPA axis consists of the hypothalamic release of corticotropin-releasing hormone (CRH) to trigger release of adrenocorticotrophic releasing hormone (ACTH) from the pituitary, resulting in the adrenal production of steroid hormones, which then feed back to the hypothalamus and pituitary to downregulate both CRH and ACTH. For the HPO axis, due to the two cell theory, it is slightly more complicated. First, the hypothalamus releases gonadotropin releasing hormone (GnRH) with a specific amplitude and frequency that occurs in part due to stimulation of the GnRH neuron by kisspeptin/GPR54 signaling. GnRH release results in the pituitary secretion of gonadotropins—luteinizing hormone (LH) and follicle stimulating hormone (FSH). The amplitude and frequency of the GnRH signal dictates the ratio of LH to FSH, and this ultimately drives the delicate balance of ovarian steroidogenesis. This in turn controls ovarian recruitment and maturation of an egg (folliculogenesis), ovulation, and the overall cyclicity of the menstrual cycle. Any aberration in kisspeptin, GnRH, LH, and FSH signaling, or the process by which the ovary forms steroid hormones can result in abnormal levels of androgenic precursors, and can subsequently result in pathologic conditions such as HA.

Finally, to fully appreciate the mechanisms responsible for the causes of HA, one must also recognize that each steroid hormone will have different effects, as each will have varying degrees of androgenic actions. Simply put, the amount and type of hormone that is overproduced in a given patient will dictate the severity of the symptoms. For example, DHT has two to three times greater affinity for the androgen receptor compared to testosterone, and additionally has a longer half-life than testosterone, and therefore DHT is a more potent androgen between the two. Similarly, testosterone is a more potent androgen than DHEA. Thus, a patient that is overproducing DHT will likely have a more severe form of HA than a patient that has a slightly elevated DHEA level.

The purpose of this article is to address the various causes of HA in the female population. In order to address this topic in an organized manner, we have divided up the differential diagnosis by age, specifically addressing the population prior to onset of puberty, after puberty, and after menopause.

Medical Causes of Hyperandrogenemia

Prepubertal

Girls that have yet to undergo puberty represent a population in which the ovary is comparatively quiescent, and since this site of androgen production is underactive, overt HA is rare (Catteau-Jonard et al., 2012). Nevertheless, it is becoming increasingly more common to see HA in this cohort due to pathology such as PCOS. As this particular disease is more appropriately covered in the postpubertal section, discussions on the differential diagnosis of HA in prepubertal females will be limited to physiologic HA, congenital adrenal hyperplasia (CAH), and tumors.

Physiologic hyperandrogenism of puberty

As a female enters puberty, at least in part due to direct stimulation from kisspeptin/GPR54 signaling in the brain, the hypothalamic-pituitary axis awakens by initiating small pulses of GnRH from the hypothalamus, which preferentially results in small levels of LH pulses as well. Therefore, there is a higher preponderance of LH to FSH during the initial few months of pubertal awakening which results in more theca cell stimulation and more ovarian androgen production in the predominant form of androstenedione. This is known as physiologic hyperandrogenism of puberty and is historically transient (Catteau-Jonard et al., 2012). FSH production will soon follow these LH pulses as the HPO axis matures and GnRH pulsatility becomes more physiologically consistent with a postpubertal pattern. In turn, estradiol production increases, ultimately resulting in the establishment of a consistent negative feedback cycle on the hypothalamus and pituitary and therefore the establishment of regular, cyclic menses.

Congenital adrenal hyperplasia

Congenital adrenal hyperplasia is a condition that encompasses several enzymatic defects in the steroid pathway from various mutations, usually autosomal recessive in inheritance. Collectively, these conditions are rare, and the resulting phenotype depends both on which enzyme is affected as well as the amount of impairment that the mutation conveys (Fritz and Speroff, 2011). In essence, the enzyme defect results in decreased production of either cortisol, aldosterone, or both from the adrenal gland and the low levels of these hormones results in a compensatory increase in ACTH from the pituitary (due to decreased negative feedback), which then drives more adrenal steroid production. As this does not fix the inherent enzymatic defect, this results in increased shuttling of upstream steroid precursors to more androgenic products, resulting in HA.

The majority of the defects are recognized early in life, and are treated with supplementation of any steroid products that are rendered deficient. The exception is also the most common of these conditions, known as nonclassical CAH, which involves a subtype of mutations in a particular enzyme in the steroid pathway called 21-hydroxylase. Nonclassical CAH presents later in life, around the time of puberty as ovarian steroidogenesis increases with increasing gonadotropin expression in the HPO axis. A hallmark of nonclassical CAH is the significant increase in the steroid immediately upstream of 21-hydroxylase, known as 17-hydroxyprogesterone (17-OHP). Diagnosis of nonclassical CAH from a mutation in 21-hydroxylase is suspected if a morning 17-OHP, preferably in the follicular phase, is $> 200 \text{ ng mL}^{-1}$. Table 1 provides a summary of the enzymatic defects that can cause CAH along with their subtypes and clinical manifestations.

Table 1 Congenital adrenal hyperplasia: summary of the different enzymatic defects seen in congenital adrenal hyperplasia, with details regarding subtypes and their clinical manifestations

<i>Congenital adrenal hyperplasia</i>	<i>Facts</i>	<i>Forms/types</i>	<i>Manifestations</i>
21 hydroxylase deficiency	• 90% of all cases • Autosomal recessive • Chromosome 6	Severe form	• Cortisol and aldosterone deficient • Ambiguous genitalia
		Classical form	• Compensatory ACTH enough to provide sufficient cortisol and aldosterone activity • Ambiguous genitalia
		Nonclassical	• Mildest, not apparent until puberty • Most common: 1/100–1000 Caucasians
11 beta hydroxylase	• 5%–8% of all cases • Autosomal recessive • Chromosome 8	Severe salt wasting	• Cortisol and aldosterone deficient • Ambiguous genitalia • Increased 11-deoxycortisone results in hypertension
		Simple virilizing	• Less severe, compensatory ACTH allows production of cortisol and aldosterone • Ambiguous genitalia • May also have hypertension
		Late onset	• May present later with sexual precocity, hirsutism, and irregular menses
3-beta-hydroxysteroid dehydrogenase	• Two different genes for this enzyme result in two different locations in body sites • All result in mild virilization due to elevated DHEA	Type 1	• Enzyme defects in the placenta and peripheral organs such as skin and breast
		Type 2—salt wasting	• Enzyme defects in more central endocrine organs such as adrenal and ovary • Significant mutation causing severe enzyme defect
		Type 2—nonsalt wasting	• Enzyme defects in more central endocrine organs such as adrenal and ovary • Less significant mutation resulting in mild enzyme defects

Tumor

While a rare cause of HA in the prepubertal population, a diagnosis of malignancy must not be overlooked. Often, these will present with rapid onset and progression, noting a short history of symptoms that also tend to be more severe. Ovarian and adrenal tumors may also result in gonadotropin-independent precocious pubertal development. In the event of suspicion for tumor, imaging studies are recommended of both the ovaries and the adrenal glands. Androgen producing ovarian tumors are usually sex cord stromal in origin and include Sertoli-Leydig cell, Granulosa cell, or hilus cell tumors. Recommended serum hormone markers include testosterone, human chorionic gonadotropin (hCG), DHEA-S, and 17-OHP. Adrenal tumors are classically carcinoma by histology, though may rarely be adenomas.

Other

Other causes of HA in this population are also seen in the postpubertal population and are therefore more appropriately reviewed in the following section. These include idiopathic hirsutism, hyperprolactinemia, Cushing syndrome, and drug ingestion.

Postpubertal

Unlike the prepubertal and postmenopausal populations, the hypothalamic-pituitary axes in both the ovaries and the adrenal glands are fully functional and active during the postpubertal phase. Therein, HA is more commonly seen in this population than in the other extremes of age. Without question, the most common cause of HA in the world is PCOS, though other causes must be evaluated in this cohort as well and warrant discussion in this article.

Polycystic ovary syndrome (PCOS)

The lion's share of HA seen in women that have undergone puberty, but are not yet menopausal, is PCOS ([Azziz et al., 2004](#)). This is the cause of irregular menstrual cycles and HA in 5%–10% of women worldwide, affecting over 100 million women. The complex nature of this multifactorial and diverse disease is impossible to condense into one article; thus, the main focus here will be the HA component of the disease.

PCOS patients manifest HA in approximately 60%–80% of cases, largely from the ovarian overproduction of androgens ([Azziz et al., 2004](#)). As introduced above, this stems in part from an abnormal regulation of the HPO axis whereby the amplitude and frequency of gonadotropin releasing hormone (GnRH) is altered, and thus the delicate balance of LH and FSH expression is also affected, favoring release of LH over FSH and thus increasing the LH:FSH ratio ([Catteau-Jonard and Dewailly, 2013](#)). As seen in the physiologic hyperandrogenism of puberty, any time that LH is significantly increased relative to FSH expression, there is predominance of androgenic precursors in the theca cell of the ovary that cannot be adequately shuttled to estradiol in the granulosa cell. This backup in production of androgenic precursors results in release of these androgens into the bloodstream, making them available for peripheral and systemic effects.

Moreover, PCOS patients commonly manifest a resistance to insulin signaling, augmented in several ways by the concurrent HA. This insulin resistance has several effects. First, it decreases the production of sex hormone binding globulin (SHBG) in the liver, which decreases the binding of androgens to this transport protein, allowing more of the total circulating androgen to be free to exert hormonal effects. Second, insulin can also act in the ovary directly, as a “co-gonadotropin,” to augment the effects of LH in androgen production. Interestingly, insulin action in the ovary via the insulin receptor uniquely favors a certain phosphorylation cascade that leads to downstream inhibition of aromatase, exacerbating the backup of androgen precursors (discussed in more detail below). Finally, it can also bind the insulin like growth factor 1 (IGF-1) receptor, which is present on theca and granulosa cells of the ovary, further modulating steroid hormone production. Therefore, not only does insulin increase free androgens in the serum by decreasing production of its natural “buffer” (SHBG), it also drives production of more androgens directly ([Fritz and Speroff, 2011](#); [Catteau-Jonard and Dewailly, 2013](#)).

Obesity, insulin resistance, and the metabolic syndrome

Many patients with PCOS are obese and insulin resistant, or even have frank metabolic syndrome. Moreover, patients that are obese or insulin resistant are more likely to have PCOS. Thus, the overlap among these conditions is great, in large part due to the effects of insulin on the ovary mentioned above. Insulin acts through several receptors to affect ovarian steroidogenesis, and in fact leads to inhibition of aromatase and stimulation of 17,20-lyase and 17-hydroxylase activity, which results in shuttling the steroid pathway toward production of androgens ([Magoffin, 2006](#); [Yang et al., 2015](#)). In addition to direct steroidogenic actions, insulin also affects the ovary by manipulating the responsiveness to LH and FSH. It does so by preferentially phosphorylating the insulin receptor at serine sites as opposed to tyrosine sites, which alters the function of the receptor to a more proliferative state, increasing FSH and LH receptors on the ovarian granulosa and theca cells, respectively. This augments the responsiveness of the ovary to stimulation from the HPO axis, further increasing steroidogenic signaling while simultaneously inhibiting aromatase, inevitably leading to a buildup of androgens and a deficiency of estradiol ([Fritz and Speroff, 2011](#)). Finally, as mentioned above, there is also a decrease in SHBG production in patients with elevated insulin, and therefore more free androgen in the blood to exert effects.

There is significant overlap with obesity and hyperinsulinemia with PCOS, and sometimes it is clinically difficult to distinguish which condition preceded the other. It is important to note that several syndromes exist where patients have significantly altered insulin sensitivity due to intrinsic receptor defects in the insulin receptor from mutations or autoimmune disease or through some type of abnormal adipose distribution (known as lipodystrophy). The more severe of these syndromes, known as

hyperandrogenic insulin resistant acanthosis nigricans, or HAIR-AN syndrome, bears strong resemblance to PCOS, though again, exhibits extremes of both insulin resistance and HA.

Idiopathic hirsutism

Patients presenting with complaints of clinical HA may not demonstrate elevated serum levels of androgens, nor manifest any other appreciable signs or symptoms other than hirsutism. These patients are classified as having idiopathic hirsutism, which is thought to stem from overactive peripheral enzyme activity in the hair follicles (Azziz et al., 2000; Carmina, 1998). Testosterone may be converted to dihydrotestosterone (DHT), a more potent androgen, by the enzyme 5-alpha reductase, which is present in the hair follicle, and responsible for signaling local hair growth. A small proportion of patients, thought to be approximately 5%–7% of the population presenting with complaints of hirsutism, are thought to have increased activity of this enzyme at the hair follicle (Azziz et al., 2004). Treatment can be difficult as therapies take weeks to months for optimal results, and involves local downregulation of this enzyme, antiandrogens, depilatory agents, or manual removal via laser, shaving, or plucking.

Hyperprolactinemia

Prolactin is yet another hormone released from the anterior pituitary, with one unique caveat. Most hormones are released when a stimulatory signal from the hypothalamus signals their release; however, prolactin is constitutively released unless the release is inhibited by the hypothalamic signal via dopamine. Elevated levels of prolactin can result in increased production of adrenal androgens, specifically DHEA-S, perhaps through direct stimulation on the adrenal gland as prolactin receptors have been identified in the adrenal gland (Fritz and Speroff, 2011). While patients with hyperprolactinemia rarely present with complaints of HA, and indeed, it is rarely the cause of HA, it remains an important consideration in the differential diagnosis.

Cushing syndrome

Cushing syndrome involves elevated levels of cortisol from one of several sources: ingestion of exogenous steroids with cortisol activity, elevated ACTH from a pituitary tumor, elevated cortisol production from an adrenal tumor, or ectopic production of ACTH from another cancer. The latter is a phenomenon known as paraneoplastic syndrome, and classically presents with a particular subtype of lung cancer. Cushing patients not only manifest HA symptoms such as hirsutism, but also several other well-known signs including skin stretching resulting in striae, increasing central obesity, fatigue, muscle weakness, and fat deposition along the back of the neck (termed a “buffalo hump”). Therefore, patients who present with a conglomeration of symptoms such as these described warrant further workup for elevated levels of cortisol. As the overall incidence of this disease is rare (< 1% of HA patients ultimately have this diagnosis) (Fritz and Speroff, 2011), the risk of a false positive test is significant. If initial testing is positive, exogenous sources should be ruled out prior to proceeding to any further testing to localize the source of the overproduction of cortisol (Goodman et al., 2001). The various options of screening for the disease as well as testing strategies to localize the source are beyond the scope of this article.

Tumor

While small benign lesions known as adenomas are not uncommonly found in the pituitary, tumors in the brain that result in overproduction of androgens are quite rare. These would stem from an overgrowth of the pituitary cells known as gonadotrophs that are responsible for the secretion of both LH and FSH. However, adenomas can also grow so large as to affect the hypophyseal portal system, altering the communication between hypothalamic GnRH and the pituitary, resulting in abnormal secretion of some or all of the pituitary end products.

Elevated androgens from tumors in either the ovary or the adrenal gland are rare as well, particularly in this patient population. Suspicion should be high, however, as it is a diagnosis that must not be missed. Again, patients with a malignancy will typically present with more rapidly progressive and more severe symptoms. In these patients, it is prudent to consider imaging the patient's ovaries and adrenal glands to identify a tumor source (Azziz et al., 2004; Carmina et al., 2006; Waggoner et al., 1999).

Iatrogenic

One of the more common causes of HA in any population, and perhaps one of the more commonly overlooked, is iatrogenic HA (Azziz et al., 2004). This usually occurs from the exogenous supplementation of medications that have androgenic side effects, whether by prescription or illicitly. This practice is common in athletes and body builders taking steroids or growth hormone derivatives that result in symptoms consistent with HA. Other prescription drugs that are known for their androgenic actions include DHEA, cortisol, or other steroids such as prednisone and hydrocortisone. Interestingly, Minoxidil, phenytoin, and cyclosporine can result in hypertrichosis, which is a darkening and thickening of hair follicles that can mimic hirsutism, though by a different mechanism of action.

Other

There are also reported cases of extremely rare causes of HA, usually resulting from mutations in enzymes causing deficiencies in the steroid pathway. One such is p450 oxidoreductase deficiency, whereby a mutation on this gene on chromosome 7 affects the ability to provide a specific group of enzymes in the p450 class with the necessary electron donors to be in their active forms. As it happens, this class of enzymes includes some of the hormones in the steroid pathway. Aromatase deficiency is another rare disorder which results in a decreased ability to turn the theca cell precursor androstenedione into estradiol in the granulosa cell, resulting in

a buildup of androgens. Also, defects in glucose metabolism resulting in hyperinsulinemia can cause HA, as previously discussed. Several mutations in the insulin receptor have been reported to cause severe hyperinsulinemia and subsequent HA, for example.

Postmenopausal

The postmenopausal period is one in which the ovary again becomes more quiescent and therefore, is similar to the prepubertal population when HA is overall rare. Interestingly, as the ovary ages, and the number of follicles dwindles, the ratio of theca cells to granulosa cells increases and accordingly, there is a slight increase in androgen production over estrogen. Symptoms from this mild elevation in physiologic androgens are mild, if present at all. The main causes of pathologic HA, similar to the prepubertal cohort, in this patient population include pathologies of overproduction and tumors.

Obesity, insulin resistance, PCOS

As seen in the postpubertal population, patients that experience increased stimulation of the theca cell through various mechanisms will manifest elevated levels of androgens. This is also the case after menopause, whereby patients have higher productivity in these cells producing androstenedione and DHEA, which otherwise would be turned into estradiol if not for the lack of functional granulosa cells in the postmenopausal ovary (Markopoulos et al., 2011). The transiently elevated androgens seen in early menopause discussed above can be further exacerbated by insulin resistance, obesity, or a history of PCOS that can all contribute to further stimulation of this pathway to exacerbate the production of androgens, and result in more impressive HA for these patients. As the ovary continues to atrophy later in life, these symptoms may be ameliorated, though in some patients with enough peripheral stimulation from adiposity and hyperinsulinemia, the androgen production may persist for years.

Ovarian hyperthecosis

Though the exact pathology in this category of HA is not known, ovarian hyperthecosis is characterized by similar symptomatology as PCOS or the metabolic syndrome discussed above. These patients are distinguished, however, by a larger production of androgens driven by an increased gonadotropin secretion from the pituitary (Fritz and Speroff, 2011; Markopoulos et al., 2015). They tend to have isolated high testosterone that is accompanied by high levels of LH and FSH. Ultrasound studies show ovaries larger than expected in menopause, and biopsies of this ovarian tissue have revealed pockets of active cords of cells producing these steroids. With higher levels of androgens, patients will be at higher risk for insulin resistance, obesity, metabolic disease, and, long term, for diabetes and heart disease.

Tumor

Adrenal tumors are rare in this population, though if present are either carcinoma or adenoma. As in the other populations, carcinomas are significantly more likely than adenomas, though still rare overall. Suspicion for adrenal origin is usually reserved for patients that have elevated DHEA-S levels $> 700 \text{ mcg dL}^{-1}$ (Fritz and Speroff, 2011; Waggoner et al., 1999).

Ovarian tumors, while still overall rare, are more common in the postmenopausal population comparatively, with approximately 22,000 cases of ovarian cancer diagnosed in the United States annually. Nonetheless, ovarian malignancies that are capable of producing androgens only comprise a small proportion of this number, making up $< 5\%$ of all ovarian tumors. Again, they tend to be sex cord stromal and bimodal in their presentation, meaning they are more common in the very young or very old populations. As HA is rare at this age and the prognosis of a patient with any malignancy is improved with earlier detection, clinical suspicion must always be high to rule out an ovarian tumor. A serum total testosterone level $> 150 \text{ ng dL}^{-1}$ is suggestive of an androgen producing tumor. Moreover, a tumor is suspected if the onset of HA symptoms such as virilization is over a short period of time, indicating rapid production that would not be consistent with simple theca cell stimulation as in PCOS.

Iatrogenic

Finally, it is worth mentioning iatrogenic HA in this population as well. Many postmenopausal patients will take some form of hormonal replacement therapy for menopausal symptoms at some point, whether over the counter or prescribed. Various treatments available for consumption have levels of androgenic activity and therefore, a thorough review of a patient's medications is prudent prior to other diagnostic modalities.

Conclusion

In conclusion, HA is seen in patients along all ages, and while metabolic disturbances such as PCOS or glucose intolerance are the most common pathologies involved, it is important to bear in mind all the potential causes. All patients presenting with symptoms of HA should be thoroughly evaluated and concern for malignancy should be ruled out, particularly in cases where the onset of symptoms is rapid or severe. It is also important to consider the relationship between HA and insulin resistance, noting that long-standing HA may alter physiology, increasing or exacerbating risk for chronic medical conditions such as diabetes and hypertension; thus, control over HA and appropriate screening and counseling are strongly recommended.

References

- Azziz, R., Carmina, E., & Sawaya, M. E. (2000). Idiopathic hirsutism. *Endocrine Reviews*, 21(4), 347–362.
- Azziz, R., et al. (2004). Androgen excess in women: Experience with over 1000 consecutive patients. *The Journal of Clinical Endocrinology and Metabolism*, 89(2), 453–462.
- Carmina, E. (1998). Prevalence of idiopathic hirsutism. *European Journal of Endocrinology*, 139(4), 421–423.
- Carmina, E., et al. (2006). Extensive clinical experience: relative prevalence of different androgen excess disorders in 950 women referred because of clinical hyperandrogenism. *The Journal of Clinical Endocrinology and Metabolism*, 91(1), 2–6.
- Catteau-Jonard, S., & Dewailly, D. (2013). Pathophysiology of polycystic ovary syndrome: The role of hyperandrogenism. *Frontiers of Hormone Research*, 40, 22–27.
- Catteau-Jonard, S., et al. (2012). Hyperandrogenism in adolescent girls. *Endocrine Development*, 22, 181–193.
- Fritz, M. A., & Speroff, L. (2011). *Clinical gynecologic endocrinology and infertility* (8th edn., p. 1439). Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.
- Goodman, N. F., et al. (2001). American Association of Clinical Endocrinologists medical guidelines for the clinical practice for the diagnosis and treatment of hyperandrogenic disorders. *Endocrine Practice*, 7(2), 120–134.
- Lizneva, D., et al. (2016). Androgen excess: Investigations and management. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 37, 98–118.
- Magoffin, D. A. (2006). Ovarian enzyme activities in women with polycystic ovary syndrome. *Fertility and Sterility*, 86(Suppl. 1), S9–S11.
- Markopoulos, M. C., et al. (2011). Hyperandrogenism in women with polycystic ovary syndrome persists after menopause. *The Journal of Clinical Endocrinology and Metabolism*, 96(3), 623–631.
- Markopoulos, M. C., et al. (2015). Hyperandrogenism after menopause. *European Journal of Endocrinology*, 172(2), R79–91.
- Waggoner, W., Boots, L. R., & Azziz, R. (1999). Total testosterone and DHEAS levels as predictors of androgen-secreting neoplasms: A populational study. *Gynecological Endocrinology*, 13(6), 394–400.
- Yang, F., et al. (2015). Follicular hyperandrogenism downregulates aromatase in luteinized granulosa cells in polycystic ovary syndrome women. *Reproduction*, 150(4), 289–296.

Thyroid Function and Reproduction

Dina M Abdelsamad, Ain Shams University, Cairo, Egypt

Raymond W Ke and William H Kutteh, Fertility Associates of Memphis, Memphis, TN, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Our endocrine system is a highly sophisticated hierarchical system that dictates efficiency as well as dynamic control of different processes in our body such as metabolism, growth, and sexual and emotional development. The thyroid gland is unique among the endocrine organs as it maintains a large store of hormone. The thyroid gland is a brown-red highly vascular gland located anteriorly in the lower neck extending from the fifth cervical vertebra down to the first thoracic vertebra. It weighs approximately 25 g but can be slightly heavier in females and can change in size during menstruation and pregnancy. The estimation of the size of the thyroid gland can be easily achieved noninvasively by clinical examination (Strandberg, 2016).

The thyroid gland is wrapped in an outer false and an inner true capsule, which separates the gland dividing its lobes into lobules. Cystic follicles are the main structural units of the gland and are lined by single epithelial layer surrounding a colloid filled cavity. The principal (follicular) cells produce thyroglobulin (Tg), a dimeric protein used by the gland as a precursor to thyroid hormones. In addition to the principal follicular cells, parafollicular cells (clear cells) secrete calcitonin hormone which is involved in the calcium homeostasis process. An extensively dense network of fenestrated capillaries, lymphatic vessels, and sympathetic nerves oversees the follicles whose size changes according to their activity and hence the abundance of colloid material (Strandberg, 2016).

Synthesis and Regulation of the Thyroid Hormone

Thyroid hormones are produced when the tyrosine residues of thyroglobulin are combined with iodine and the protein is cleaved by action of the enzyme thyroid peroxidase (TPO). Thyroxine (T₄) is the main hormone secreted by the thyroid gland with a longer half-life, though biologically less active, than triiodothyronine (T₃) which is primarily produced outside of the thyroid gland. The thyroid gland secretes 80 µg thyroid hormone daily and is highly dependent on the availability of iodine (Hedden et al., 2015).

More than 99.5% of the circulating thyroid hormones is carried on plasma proteins, mainly thyroxine binding globulin (TBG), transthyretin (TTR), and albumin. The hypothalamic pituitary thyroid axis regulates thyroid hormone homeostasis in the body. Thyroid-stimulating hormone (TSH) secreted from the anterior lobe of the pituitary gland regulates secretion of thyroid hormone. Thyroid-releasing hormone (TRH), produced from the periventricular nucleus of the hypothalamus, regulates secretion of the TSH. This axis is regulated by T₃ and T₄ action on anterior pituitary and hypothalamus using feedback mechanism (Yamada and Mori, 2008).

After conception, TBG and total T₄ concentrations levels are detected in plasma and increase starting from week 7 of gestation and peak by week 16. These concentrations then remain high until delivery. In the first trimester, maternal human chorionic gonadotropin (hCG) directly stimulates the TSH receptor, increasing thyroid hormone production and serum TSH concentration falls. Therefore, during pregnancy, women have lower serum TSH concentrations than before pregnancy, and a TSH below the nonpregnant lower limit of 0.4 mU L⁻¹ is observed in as many as 15% of healthy women during the first trimester of pregnancy. The fraction of women with a suppressed TSH falls to about 10% in the second trimester, and 5% in the third trimester (Poppe et al., 2007; Alexander et al., 2017).

Epidemiology and Classification of Thyroid Disorders

Thyroid disorder classification is based on the levels of thyroid hormone in blood. Euthyroidism, hyperthyroidism, and hypothyroidism reflect the clinical states of normal, excessive, or defective levels of thyroid hormones. In areas of the world with dietary iodine deficiency, an enlargement of the thyroid termed goiter is common. On the contrary, in iodine-replete areas autoimmune etiology is the cause for thyroid disorders, either hypothyroidism including Hashimoto's thyroiditis to hyperthyroidism including thyrotoxicosis caused by Graves' disease (Vanderpump, 2011).

Primary hypothyroidism has a lifetime prevalence as high as 9% for women and 2% for men. The causes can be congenital or iatrogenic but more frequently are autoimmune in origin. The most common cause of hypothyroidism associated with antithyroid autoantibodies is six times more common in females than males. Hashimoto's thyroiditis is more common in middle age women and associated with very high titers of thyroid peroxidase (TPO) antibodies. Postpartum thyroiditis is usually self-limiting following pregnancy but can be permanent if antibodies are detected. Secondary hypothyroidism is due to hypopituitarism from pituitary adenoma, pituitary ablative therapy, or pituitary destruction.

Subclinical hypothyroidism also called mild thyroid failure, is diagnosed when peripheral thyroid hormone levels are within normal reference laboratory range but serum thyroid-stimulating hormone (TSH) levels are mildly elevated (Fatourechi, 2009). The worldwide prevalence of subclinical hypothyroidism ranges from 1% to 10%.

On the other hand, hyperthyroidism affects 2%–5% of females at some time during their life and occurs five times more commonly in women than men. Primary hyperthyroidism is associated with thyrotoxicosis when tissues are exposed to high levels of thyroid hormones. The most common causes of hyperthyroidism are Grave's disease, toxic multinodular goiter, and solitary toxic nodules; the less common causes are the infectious causes, gestational thyrotoxicosis or due to drugs (Kumar and Clark, 2017).

Antithyroid antibodies can be detected in the serum of individuals who are hypothyroid, hyper thyroid, or euthyroid. The presence of autoimmune thyroid antibodies without thyroid dysfunction can be considered a precursor of an upcoming thyroid dysfunction (Monaco, 2003). These antibodies can be destructive such as those directed against thyroglobulin (Tg Ab) or thyroid peroxidase antibodies (TPO Ab). Other antibodies such as those directed against the TSH receptor (TSHR Ab) mostly stimulate, but occasionally block, TSH receptor. TSHR Ab are highly specific for Grave's disease (Kumar and Clark, 2017). TPO Ab are present in 20% of the normal population but only 10%–20% will develop overt hypothyroidism. The IgG TPO AB are able to cross the placenta but do not appear to cause any fetal thyroid dysfunction (Alexander et al., 2017).

Screening for Thyroid Disorders

Serum thyrotropin (TSH) shows a log-linear relationship to free thyroxine. The assay of TSH is commonly accepted as the most sensitive test to detect minor degrees of primary thyroid hormone deficiency. TSH assays are standardized and offer a cost effective method for screening for thyroid disorders (Blumenthal and Eastman, 2017). TSH level elevation in the presence of normal free thyroxine serum levels is defined as subclinical hypothyroidism. Subclinical hypothyroidism is a proven risk factor for the development of overt thyroid dysfunction and for other clinical, mainly cardiovascular, cognitive, and psychiatric disorders. Historically, the range of normal for TSH has been set at approximately 0.4–4.5 mIU L⁻¹. Large population studies have suggested a lower TSH cut-off with an upper limit of 2.5 mIU L⁻¹ in women of reproductive age (Brabant et al., 2006). A comparison of the guidelines from the American Society for Reproductive Medicine, the American College of Obstetricians and Gynecologists, The Endocrine Society, and the Royal College of Obstetricians and Gynaecologists concerning testing and treatment of thyroid disease are listed in Table 1.

It is noteworthy that pregnancy may confound the interpretation of the normal TSH range. In 2007, The Endocrine Society and the American Thyroid Association recommended that the upper limit of the TSH reference range be lowered to <2.5 mIU L⁻¹ in the first trimester and <3 mIU L⁻¹ in the second and third trimesters of pregnancy (Stagnaro-Green et al., 2011). In accordance with these findings, in 2012 The Endocrine Society guidelines recommended treating pregnant women or women planning a pregnancy when serum TSH exceeds 2.5 mIU L⁻¹ in the first trimester and 3 mIU L⁻¹ in the second and third trimesters (DeGroot et al., 2012).

The Effect of Thyroid Hormone on Female Reproductive Physiology

Disorders of thyroid function have a negative impact on female reproduction. Both hypothyroidism and hyperthyroidism have been associated with menstrual disturbances and ovulatory dysfunction. These disturbances sometimes antedate the clinical diagnosis of thyroid dysfunction (DeGroot et al., 2012). Since human oocytes have receptors for thyroid hormone (López Navarro et al., 2016), thyroid hormone disturbances can affect oocyte maturation and ovulation through the change in prolactin levels, alteration of GnRH pulsatile secretion leading to a delayed response of LH and inadequate corpus luteum function (Poppe et al., 2007; Jefferys et al., 2015).

Both increased and decreased levels of thyroid hormone may cause menstrual disturbances, mainly hypomenorrhea and polymenorrhea in hyperthyroidism and oligomenorrhea in hypothyroidism. Hyperthyroidism causes increased circulating sex hormone binding globulin (SHBG) in serum resulting in estrogen levels that may be doubled or tripled. In hyperthyroid women the mean LH levels in both the follicular and luteal phases of the menstrual cycle are significantly higher compared to normal women. In hypothyroid women these physiological changes are skewed to the opposite end of the curve though GnRH levels remain within normal (Krassas et al., 2010).

Table 1 Endocrine Societies guidelines for TSH and antithyroid antibody testing

Guideline recommendation	ASRM	ACOG	Endocrine Society	Royal College
Routine TSH testing	No	No	No	No
Routine TPO/TG antibodies	No	No	No	Not discussed
Treatment if TSH > 2.5	No, if TSH > 4.0	No	Yes	No
If TSH > 2.5 and TPO Ab/TG Ab positive	Yes, if clinically indicated	No	Yes	Not discussed

ASRM, Subclinical hypothyroidism in the infertile female population: a guideline 2015.

ACOG, ACOG Practice Bulletin Number 148: Thyroid disease in pregnancy, April 2015.

Endocrine Society: Management of Thyroid Dysfunction during Pregnancy and Postpartum: An Endocrine Society Clinical Practice Guideline 2012

(DeGroot et al., 2012).

Royal College, produced by RCOG Library staff following the clinical query protocol.

Autoimmune thyroid disease (AITD) is the most common cause of hypothyroidism in women in the reproductive age group 20–40 years. Overt hypothyroidism is associated with a broad spectrum of reproductive disorders, ranging from abnormal sexual development, menstrual irregularities to infertility (Poppe et al., 2007).

Thyroid Disorders and Infertility

Infertility is the failure to conceive after 1 year of unprotected intercourse and is found to affect about 10%–15% of couples in reproductive age (Puscheck and Scott, 2016). In women presenting with fertility issues 2.3% are hyperthyroid, 2%–4% have overt hypothyroidism (Jefferys et al., 2015). In women with ovulatory disorders and irregular menstruation 9%–11% were found to have subclinical hypothyroidism (Lincoln et al., 1999; Poppe, et al., 2007). Among women presenting with infertility, TSH levels were highest among women with ovulatory dysfunction and irregular menstruation (Lincoln et al., 1999).

The prevalence of autoimmune thyroid disease (AITD) is 5–10 times more in women than in men and also higher in women suffering from infertility when compared to an age-matched parous group of women (Ke, 2014). AITD is generally defined as the presence of reactivity to self-thyroid antigens, which are expressed as distinctive inflammatory or antireceptor autoimmune diseases (Iddah and Macharia, 2013). Antithyroid antibodies are also detectable in the ovarian follicles of women with thyroid autoimmunity. The presence of antithyroid antibodies in women with PCOS has been reported to decrease the response of ovaries to clomiphene citrates (Alexander et al., 2017).

The adverse effect of hypothyroidism on fertility can be through the alteration of the estrogen metabolism peripherally, increasing the level of prolactin, which in turn causes negative feedback inhibition on the GnRH level, and the decrease in the levels of SHBG (Hoffman et al., 2016). Most of evidence suggests that IVF outcomes do not differ between women with serum TSH $< 2.5 \text{ mU L}^{-1}$ and those with very mild TSH elevations, defined as a TSH between 2.5 and 4.0 mU L^{-1} . A proposed clinical algorithm for the evaluation of reproductive aged women based on TSH results is presented in Fig. 1.

Despite the physiological evidence that thyroid gland dysfunction influences fertility, AITD alone do not affect embryo implantation (Poppe et al., 2003). The presence of antithyroid antibodies did not affect implantation rates in over 700 euthyroid patients who went through IVF (Kutteh et al., 1999). A recent study found no link between preconception subclinical hypothyroidism and time to pregnancy (Plowden et al., 2016). The presence of AITD did not affect implantation rates in women with a normal thyroid function who went through IUI. Unfortunately, follow-up of patients showed that the patients with AITD have significantly elevated risk of pregnancy loss in the first trimester even if they became euthyroid before conception.

Thyroid Disorders and Pregnancy Loss

Early pregnancy loss is a frustrating and heart-wrenching experience for both the patient and the physician. Almost 25% of women will experience at least one miscarriage, while 2%–4% of couples will suffer recurrent pregnancy loss (Kutteh, 2015). In addition to infertility, miscarriage, preeclampsia, and abruption placenta have been associated with thyroid dysfunction. Fetal complications

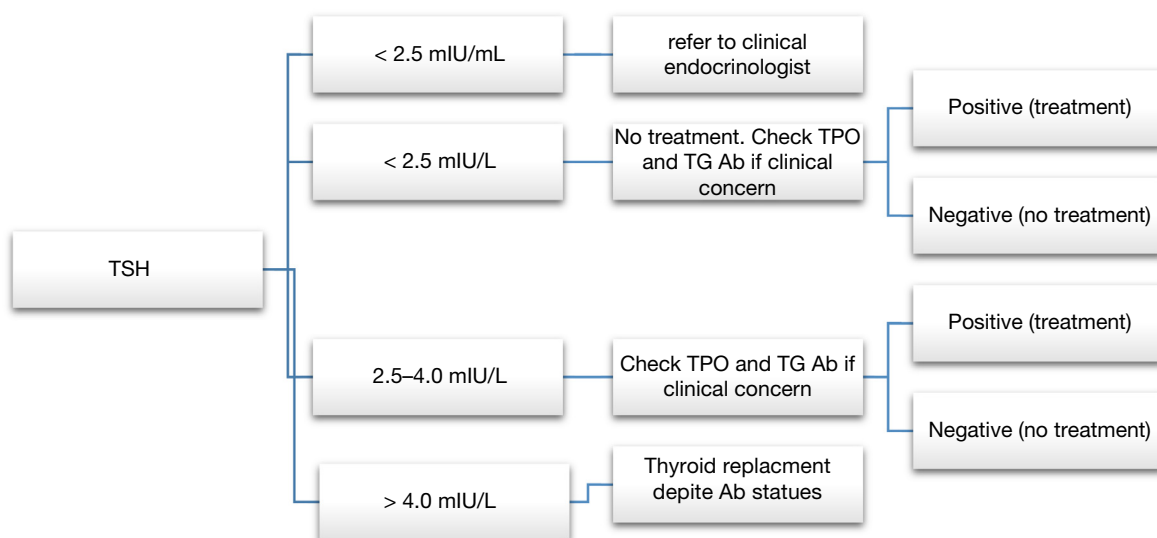


Fig. 1 Proposed clinical algorithm based on TSH results for assessment of reproductive aged women. *TSH*, thyroid stimulating hormone; *TPO Ab*, thyroid peroxidase antibody; *TG Ab*, thyroglobulin antibody.

including preterm birth, low birth weight, impaired neurocognitive development, and fetal death have also been correlated with disorders of thyroid function. A Dutch study of nearly 2500 pregnant women demonstrated that as TSH values rise there is an increased risk for fetal loss (Benhadi et al., 2009).

Among pregnant women, autoimmune thyroid disease has a prevalence of 5%–20% depending on the population studied (Glinioer, 2009). Women demonstrating antithyroid antibodies (ATA) during pregnancy have shown that despite the expected decrease in antibody titers during gestation, thyroid function gradually deteriorated toward hypothyroidism in a significant fraction of such women (Glinioer et al., 1994). The most commonly targeted thyroid antigens are thyroid peroxidase (TPO) and thyroglobulin (Tg), both integral to the production of T4 and T3. The reported prevalence of anti-TPO and anti-Tg antibodies in asymptomatic women is 14.6% and 13.8% respectively (Hollowell et al., 2002).

Stagnaro-Green (1990) first reported an association of ATA with pregnancy loss observing a doubling of the spontaneous miscarriage rate (17.0 vs. 8.4%; $P = 0.001$) in women with a TSH between 2.5 and 5.0 mIU L⁻¹ who were ATA-positive compared with an ATA-negative cohort. A pooled analysis of 21 studies (13 cohort and 8 case control) demonstrated an odds ratio of 2.55 [CI 1.42–4.57; $P = 0.002$] for recurrent losses in women with ATA (Chen and Hu, 2011). Similarly, a recent meta analysis showed a positive association of ATA with spontaneous pregnancy loss in both cohort [OR 3.9 CI: 2.48–6.12; $P < 0.001$] and case-control [OR 1.8; 95% CI 1.25–2.6; $P = 0.002$] studies (Thangaratinam et al., 2012). Based on these studies, a clinical algorithm for the testing of reproductive aged women based on the results of TSH testing along with ATA testing is proposed in Fig. 1.

The precise pathogenic link between ATA and pregnancy loss is unknown and the association does not imply a causal relationship. Autoimmune thyroid disease may impair thyroid reserve to such an extent that the gland is unable to respond to the challenge of pregnancy. ATA may be associated with inappropriate low levels of thyroid hormones for the given gestational period, despite apparent biological euthyroidism. Alternatively, autoimmune thyroid disease could act by delaying the occurrence of conception because of its known association with infertility. Similarly, women with autoimmune thyroid disease are generally older than healthy controls and increased age is an independent risk factor for miscarriage. Finally, ATA may merely be a marker for a generalized immune imbalance that may independently promote pregnancy loss (Glinioer, 2009; Kaprara and Krassas, 2008).

Regardless of the causal relationship, there is evidence that thyroid hormone replacement with levothyroxine in pregnant women with hypothyroidism (TSH > 2.5 mIU L⁻¹), will improve pregnancy outcome (Abalovich et al., 2002; Bussen et al., 2000; Negro et al., 2006). Furthermore, a Cochrane study suggested a significant reduction in preterm birth and a trend toward reduced miscarriage when levothyroxine was used in euthyroid pregnant women with ATA (Reid et al., 2013). Given that thyroid autoimmunity may be present for many years before the onset of hypothyroidism, it seems reasonable to test RPL patients for ATA (both anti-TPO and anti-Tg), preferably immediately before conception. For those with positive antibodies and a TSH > 2.5 mIU L⁻¹, levothyroxine therapy tailored to the TSH level is indicated (Negro et al., 2006). Close monitoring, particularly in the first trimester, is important because of rising thyroid demands with dosage often requiring titration upward during pregnancy (Mandel et al., 1991). There are no clear recommendations in ATA-positive pregnancies with a normal TSH (TSH < 2.5 mIU L⁻¹) but common sense dictates that at the very least, close follow-up during pregnancy is warranted.

References

- Abalovich, M., Gutierrez, S., Alcaraz, G., et al. (2002). Overt and subclinical hypothyroidism complicating pregnancy. *Thyroid*, 12, 63–68.
- Alexander, E. K., Pearce, E. N., Brent, G. A., et al. (2017). Guidelines of the American thyroid association for the diagnosis and management of thyroid disease during pregnancy and the postpartum. *Thyroid*, 27, 315–389. <https://doi.org/10.1089/thy.2016.0457>.
- Benhadi, N., Wiersinga, W., Reitsma, J., Vrijkotte, T., & Bonsel, G. (2009). Higher maternal TSH levels in pregnancy are associated with increased risk for miscarriage, fetal or neonatal death. *European Journal of Endocrinology*, 160, 985–991.
- Blumenthal, N. J., & Eastman, C. J. (2017). Beneficial effects on pregnancy outcomes of thyroid hormone replacement for subclinical hypothyroidism. *Journal of Thyroid Research*, 4601365. <https://doi.org/10.1155/2017/4601365>. Epub 2017 Feb 14.
- Brabant, G., BeckPecoz, P., Jarzab, B., Laurberg, P. et al. (2006) Is there a need to redefine the upper normal limit of TSH? *European Journal of Endocrinology*, 154, 633–637.
- Chen, L., & Hu, R. (2011). Thyroid autoimmunity and miscarriage: a meta-analysis. *Clinical Endocrinology*, 74, 513–519.
- DeGroot, L., Abalovich, M., Alexander, E. K., et al. (2012). Management of thyroid dysfunction during pregnancy and post partum: an Endocrine Society Clinical Practice Guideline. *The Journal of Clinical Endocrinology & Metabolism*, 97, 2543–2565.
- Fatourechi, V. (2009). Subclinical hypothyroidism: An update for primary care physicians. *Mayo Clinic Proceedings*, 84(1), 65–71.
- Glinioer, D. (2009). Thyroidal and immune adaptation to pregnancy: focus on maternal hypo- and hyperthyroidism. In J. Lazarus, V. Pirags, & S. Butz (Eds.), 2009. *The thyroid and reproduction* (pp. 36–58). New York: Thieme.
- Glinioer, D., Riahi, M., Grun, J. P., & Kinthaert, J. (1994). Risk of subclinical hypothyroidism in pregnant women with asymptomatic autoimmune thyroid disorders. *Journal of Clinical Endocrinology and Metabolism*, 79, 197–204.
- Heddwen, B., Barrett, K. E., Boitano, S., & Barman, S. M. (2015). *Ganong's review of medical physiology* (pp. 339–341) New York, NY: Lange-McGraw Hill
- Hoffman, B., Schorge, J., Bradshaw, K., et al. (2016). *Williams gynecology* (3rd edn). New York, NY: McGraw-Hill. Section 2, Chapter 15.
- Hollowell, J., Staehling, N., Flanders, W., et al. (2002). Serum TSH, T4, and Thyroid Antibodies in the United States Population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). *The Journal of Clinical Endocrinology & Metabolism*, 87, 489–499.
- Iddah, M., & Macharia, B. (2013). Autoimmune thyroid disorders. *ISRN Endocrinology*, 2013, 509764.
- Jefferys, A., Vanderpump, M., & Yasmin, E. (2015). Thyroid dysfunction and reproductive health. *Obstet Gynaecology*, 17, 39–45.
- Kaprara, A., & Krassas, G. (2008). Thyroid autoimmunity and miscarriage. *Hormones*, 7, 294–302.
- Ke, R. (2014). Endocrine basis for recurrent pregnancy loss. *Obstetrics and Gynecology Clinics of North America*, 41, 103–112.
- Krassas, G., Poppe, K., & Glinioer, D. (2010). Thyroid function and human reproductive health. *Endocrine Reviews*, 31(5), 702–755.
- Kumar, P., & Clark, M. (2017). *Kumar & Clark's clinical medicine* (9th edn, pp. 2957–2958). Edinburgh: Elsevier. Section 3, Chapter 26.
- Kutteh, W. H. (2015). Novel strategies for the management of recurrent pregnancy loss. *Seminars in Reproductive Medicine*, 33, 161–168.

- Kutteh, W.H., Yetman, D.L., Carr, A.C., Beck, L.A. & Scott, R.T. Jr (1999) Increased prevalence of antithyroid antibodies identified in women with recurrent pregnancy loss but not in women undergoing assisted reproduction. *Fertility and Sterility*, 71, 843–848.
- Lincoln, S. R., Ke, R. W., & Kutteh, W. H. (1999). Screening for hypothyroidism in infertile women. *Journal of Reproductive Medicine*, 44, 455–457.
- López Navarro, E., Ortega, F., Francisco-Busquets, E., et al. (2016). Thyroid hormone receptors are differentially expressed in granulosa and cervical cells of infertile women. *Thyroid*, 26(3), 466–473.
- Mandel, S., Larsen, P., Seely, E., & Brent, G. (1991). Increased need for thyroxine during pregnancy in women with primary hypothyroidism. *International Journal of Gynecology & Obstetrics*, 34, 390–391.
- Monaco, F. (2003). Classification of thyroid diseases: Suggestions for a revision. *The Journal of Clinical Endocrinology & Metabolism*, 88(4), 1428–1432.
- Negro, R., Formoso, G., Mangieri, T., et al. (2006). Levothyroxine treatment in euthyroid pregnant women with autoimmune thyroid disease: Effects on obstetrical complications. *Journal of Clinical Endocrinology and Metabolism*, 91, 2587–2591.
- Plowden, T. C., Schisterman, E. F., Sjaarda, L. A., et al. (2016). Subclinical hypothyroidism and thyroid autoimmunity are not associated with fecundity, pregnancy loss, or live birth. *The Journal of Clinical Endocrinology and Metabolism*, 101(6), 2358–2365. <https://doi.org/10.1210/jc.2016-1049>. Epub 2016 Mar 29.
- Poppe, K., Glinoe, D., Tournaye, H., et al. (2003). Assisted reproduction and thyroid autoimmunity: An unfortunate combination? *Journal of Clinical Endocrinology and Metabolism*, 88, 4149–4152.
- Poppe, K., Velkeniers, B., & Glinoe, D. (2007). Thyroid disease and female reproduction. *Clinical Endocrinology*, 66, 309–321.
- Puscheck E; R. Scott. (2016). Infertility: Practice essentials, overview, etiology of infertility [WWW Document], <http://emedicine.medscape.com/article/274143-overview#a2>
- Reid, S. M., Middleton, P., Cossich, M. C., Crowther, C. A., & Bain, E. (2013). Interventions for clinical and subclinical hypothyroidism pre-pregnancy and during pregnancy. *Cochrane Database of Systematic Reviews*. <https://doi.org/10.1002/14651858.cd007752.pub3>.
- Stagnaro-Green, A. (1990). Detection of at-risk pregnancy by means of highly sensitive assays for thyroid autoantibodies. *The Journal of the American Medical Association*, 264(11), 1422–1425.
- Stagnaro-Green, A., Abalovich, M., & Alexander, E. (2011). Guidelines of the American Thyroid Association for the diagnosis and management of thyroid disease during pregnancy and postpartum. *Thyroid*, 21, 1081–1125.
- Standing, S. (2016). *Gray's anatomy: The anatomical basis of clinical practice* (pp. 470–471). Philadelphia: Elsevier Limited. Section 4, Chapter 29.
- Thangaratnam, S., Tan, A., Knox, E., et al. (2012). Association between thyroid autoantibodies and miscarriage and preterm birth. *Obstetric Anesthesia Digest*, 32(2), 87–88.
- Vanderpump, M. (2011). The epidemiology of thyroid disease. *British Medical Bulletin*, 99(1), 39–51.
- Yamada, M., & Mori, M. (2008). Mechanisms related to the pathophysiology and management of central hypothyroidism. *Nature Clinical Practice Endocrinology & Metabolism*, 4, 683–694.

Further Reading

- American College of Obstetricians and Gynaecologists (2015) *ACOG Practice Bulletin Number 148: Thyroid disease in pregnancy*, April 2015
- Practice Committee of the American Society for Reproductive Medicine. (2015) Practice Committee of the American Society for Reproductive Medicine. Subclinical hypothyroidism in the infertile female population: A guideline. *Fertility and Sterility*, 104(3), 545–553.
- Royal College of Obstetricians and Gynaecologists. (2017). Hypothyroidism in pregnancy (query bank). [online] Available at: <https://www.rcog.org.uk/en/guidelines-research-services/guidelines/hypothyroidism-in-pregnancy-query-bank/> [Accessed 8 Sep. 2017].

Premature Ovarian Insufficiency

Lisa Webber, St Mary's Hospital, London, United Kingdom

Stephen Franks, Hammersmith Hospital, London, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Amenorrhea Absence of periods.

Anti-Mullerian hormone (AMH) Ovarian hormone produced by small antral follicles.

Dyspareunia Pain on intercourse.

Iatrogenic Caused by medical or surgical treatment.

Idiopathic Of unknown cause.

Karyotype Chromosome complement.

Oligomenorrhea Infrequent periods.

Osteoporosis Condition of weakened bone at risk of fracture.

Ovarian reserve Term used to indicate the reproductive potential of the ovary; often used as well to indicate the relative number of eggs remaining in the ovary.

Introduction

Premature ovarian insufficiency (POI) affects 1% of women. It is defined as loss of ovarian activity under the age of 40 years and is characterized by infrequent or absent periods (oligomenorrhea or amenorrhea respectively) and elevated follicle stimulating hormone (FSH). Although it is irreversible, those affected may have episodes of spontaneous ovarian activity, when ovulatory cycles may resume and may even be regular for a limited time. It is for this reason that classifying it as a *premature menopause* or *ovarian failure* is inaccurate. In addition, and importantly, affected women find both these terms unacceptable. Primary ovarian insufficiency is an alternative and accurate name for the condition, indicating that it arises within the ovary, but should not be confused with primary or secondary amenorrhea, as the condition can present with either ([Table 1](#)).

POI is a cause of infertility but natural pregnancies do occur in a small number of women with POI following spontaneous ovulation. Apart from infertility, the consequences of POI are believed to be secondary to low estrogen. Vasomotor symptoms (i.e., hot flashes and night sweats) and osteoporosis can be treated effectively with hormone replacement. However, affected women have a reduced life expectancy, mainly due to cardiovascular disease but also osteoporosis, and data supporting the role of hormone replacement in reversing these complications are, as yet, lacking.

POI can be spontaneous or a consequence of medical or surgical treatments for benign or malignant disease (iatrogenic) in approximately equal numbers ([MacLaran and Panay, 2011](#)). An increasing number of women are living with POI after surviving cancer, although fertility-sparing treatments are now being used when feasible. The major cause of spontaneous POI is unknown, or idiopathic. Genetic causes account for the minority of cases and in an even smaller minority the cause is autoimmune.

Clinical Features

POI presents in females under the age of 40 years with amenorrhea of at least 4–6 months duration, in the presence of elevated FSH levels on two or more occasions a minimum of 4 weeks apart. Amenorrhea may be primary (when there has never been a menstrual period) or secondary (cessation any time after menarche, the first menstrual period). POI presenting with primary amenorrhea may be associated with delayed or arrested puberty and is more likely to be associated with an underlying chromosomal or genetic condition.

The diagnostic cut off level for FSH has not been formally validated, although many biochemistry laboratories will quote > 30 mIU/mL as being “menopausal.” FSH levels tend to be higher in idiopathic POI compared to autoimmune causes ([La Marca](#)

Table 1 Proportion of women experiencing menopause by age

Average age	10% by	1% by	0.1% by	0.01% by
51 years	45 years	40 years	30 years	20 years

The average age of menopause in the United Kingdom is 51 years. The table shows the proportion of women who have undergone menopause by a given age ([POI Guideline Development Group, 2015](#)).

et al., 2009). A review of the literature found levels of 20, 30, and 50 mIU/mL have been used and the ESHRE Guideline on the management of POI proposed a cut off level of 25 mIU/mL to ensure inclusion of those with autoimmune POI (POI Guideline Development Group, 2015).

It is important not to confuse POI with low ovarian reserve, the difference being that women with low ovarian reserve are still having menstrual cycles even though FSH levels may be above the 25 mIU/mL cut off. These conditions are on the same spectrum, however, and some women with low ovarian reserve will progress to POI.

One of the features of POI is that ovarian function can fluctuate, especially within the early years of the condition presenting. In some women, this will result in the resumption of menstrual cycles after a prolonged episode of amenorrhea. Periods may be sporadic but in some can become regular and ovulatory for variable lengths of time.

Women with POI may experience hot flashes and night sweats, the classic symptoms of low estrogen. However, young women and adolescent girls with POI often do not, suggesting that these symptoms represent an estrogen withdrawal effect rather than absolute levels of low estrogen. Genito-urinary symptoms are common throughout the age range, especially dyspareunia in sexually active women. These symptoms can be distressing, although are often not disclosed by affected women unless prompted. Sexuality is often affected and the causes are likely to be multifactorial. The associated infertility of POI may affect how a woman (or her partner) view sexual relations. The underlying diagnosis may have a psychological impact, for example, fear of passing on a genetic cause should pregnancy occur. There may also be physical consequences in iatrogenic POI such as a shortened or stenosed vagina after surgery or radiotherapy.

Osteoporosis is a consequence of POI irrespective of the cause, although there are no data to indicate whether the risk of fracture is increased. Cardiovascular morbidity and mortality are also increased and this is the main reason for the reduced life expectancy seen in POI.

Women with surgical POI may experience a decline in memory in the short-term. The evidence is conflicting as to whether there is a longer-term risk of dementia and Parkinson's disease in this group (Vearncombe and Pachana, 2009). With the exception of women with Turner syndrome, there are no published data on cognitive function in women with spontaneous POI who are not taking estrogen replacement.

Etiology

POI results when the resting pool of primordial follicles is effectively depleted. In most cases this is due to a reduction in primordial follicle number from an unknown cause, but POI can result from, for example, FSH receptor mutations, meaning that a normal number of primordial follicles are present, but follicle development is arrested (Table 2).

Spontaneous POI

It is not known whether women with idiopathic POI are born with a reduced number of primordial follicles or whether they undergo an increased rate of follicle loss (atresia), resulting in early follicle depletion. However, the commonest genetic causes are X chromosome related.

Chromosomal

Two functioning X chromosomes are required to form a primordial follicle in the fetal ovary. Females with Turner syndrome (TS) are 45XO, lacking one X chromosome. They invariably develop POI early and affected girls often require puberty induction, although sometimes puberty will commence but then arrest. Females who are TS mosaics have cells that are 46XX as well as cells that are 45XO. Girls who are TS mosaics may have normal puberty and regular cycles even into adulthood. However, most will present with POI in adolescence or soon after. Women with Turner syndrome, including mosaics, carry particular risks during pregnancy and this is discussed further in the section on Fertility and Pregnancy.

Genetic

It is estimated that 7% of spontaneous POI is due to carriage of a premutation of the fragile X mental retardation 1 gene, FMR-1, which lies on the X chromosome (Schwartz et al., 1994). Fragile X syndrome is a cause of autism, presenting with developmental problems and cognitive impairment. Boys are more severely affected than girls. The mutation is an increase in the number of trinucleotide (CGG) repeats, a high number of repeats (>200) being associated with the syndrome. A lower number of repeats

Table 2 Causes of premature ovarian insufficiency

<i>Spontaneous POI</i>	<i>Iatrogenic POI</i>
Idiopathic	Surgical
Genetic	Chemotherapy
Chromosomal	Radiotherapy
Autoimmune	

(55–200) is known as a premutation, but is unstable and can increase to the full mutation due to instability during meiosis in females (but not males). Female carriers of the premutation do not have any increased risk of cognitive impairment but have an increased risk of developing POI of 13%–26%, although carriers of the full mutation have no increased risk of POI (Bennett et al., 2010).

Maternal carriage of the premutation may have implications for the offspring, should spontaneous pregnancy occur and the full mutation be present. There may also be implications for family members who carry the premutation and so be at risk of POI or of having an affected child. The Genetics Committee of the Society of Obstetricians and Gynecologists of Canada (2008) therefore recommended routine testing for women with spontaneous POI, pretest counseling being a prerequisite.

A number of autosomal gene mutations have been implicated in the etiology of POI. Some of these are associated with recognized clinical syndromes, for example, type 1 blepharophimosis epicanthus ptosis syndrome (BEPS) and galactosemia. However, most mutations have been identified as a result of investigation of affected families (where the mutation(s) may be limited to that particular pedigree), screening of candidate genes (i.e., looking for mutations of genes known to be involved in follicle development or ovarian function), and, more recently, genome-wide association study (Perry et al., 2013). There is also interest in copy number variations (CNVs) which are large areas of DNA that vary in number between individuals and have regulatory roles in gene expression. This raises the possibility that some of the long-term consequences of spontaneous POI could be due to altered expression of genes rather than the consequences of low estrogen.

Autoimmune

There appears to be an association between autoimmune disorders and POI and autoimmune markers are identified in approximately 25% of women with spontaneous POI (Conway et al., 1996). It is disputed whether the relationship is causative (La Marca et al., 2010) and the clinical usefulness/cost effectiveness of screening for coexisting autoimmune disorders is contentious in the absence of suggestive signs or symptoms.

Histological evidence of ovarian inflammation (oophoritis) has been described in POI, although exclusively in the presence of steroid cell autoantibodies detectable in peripheral blood (Hoek et al., 1997). Selective destruction of theca cells is seen, which is the ovarian component binding steroid cell autoantibodies in ovarian biopsies (La Marca et al., 2010). Estrogen levels are low due to loss of theca cells, and FSH and inhibin B are raised, but anti-Müllerian Hormone (AMH) may be relatively normal until such time as follicle numbers decline significantly, usually becoming undetectable within 5 years (Falorni et al., 2012).

Adrenocortical antibodies (ACA) or 21-hydroxylase autoantibodies (21OH-Abs) are the most sensitive autoantibodies to measure as SCA can be identified in over 90% of cryostatic sections of ovaries taken from women who were positive for either of these. The ESHRE Guideline on Management of POI recommends considering screening for either of these autoantibodies when the cause of POI is unknown or if an autoimmune disorder is suspected in order to detect latent Addison's disease. However, only one group has reported the prevalence of these antibodies in POI (4%–5%; Falorni et al., 2012) and a small case series identified progression to impaired adrenal function within 5 years in three out of four women with POI who were positive for ACA (Betterle et al., 1997). Women with autoimmune Addison's disease, invariably positive for ACA/21OH-Abs, and a component of the autoimmune polyendocrine syndromes—APS-1 and APS-2, which also feature POI—should be counseled about and screened for POI.

It is possible that autoantibodies may exist to other components of ovarian follicles, destruction or blockade of which could cause POI. Case reports have been published of autoantibodies against the LH receptor, FSH receptor and zona pellucida (Kelkar et al., 2005), but the clinical application of testing for these is not supported outside of a research program.

Thyroid autoimmunity and dysfunction may be more prevalent in women with POI compared to the normal population (Welt, 2008), where thyroid peroxidase antibodies (TPO-Ab) are found in 12%–15%. Untreated hypothyroidism affects quality of life and, when present in pregnancy, fetal neurocognitive development. The impact of subclinical hypothyroidism on pregnancy outcome is more contentious but is increasingly treated in the context of assisted reproduction. ESHRE consequently recommended testing for thyroid antibodies in women with spontaneous POI (POI Guideline Development Group, 2015).

There is little evidence to support routine testing for other autoimmune conditions in women with karyotypically normal spontaneous POI. However, the possibility of coexisting autoimmune conditions should be considered at routine clinical review. The exception to this approach may be for women with Turner syndrome, who have an increased risk of celiac and inflammatory bowel disease compared to women with karyotypically normal spontaneous POI and to the normal population (Bakalov et al., 2012).

Iatrogenic POI

Bilateral oophorectomy is an obvious cause of POI but ovarian surgery, especially if bilateral or repeated and particularly if for bilateral ovarian endometriosis can terminally compromise ovarian function (Coccia et al., 2011). However, any extensive pelvic surgery can precipitate POI, thought to be due to disturbances of collateral blood supplies.

Age-standardized cancer rates for men and women are increasing, whereas mortality rates are falling in most countries according to WHO figures. This means that more individuals are surviving cancer but living with the consequences of treatment(s), one of which, for women, can be POI. Chemotherapy and pelvic radiotherapy are both gonadotoxic, although dose and age at delivery affect the impact of either. Different chemotherapeutic agents and combinations of agents have differing effects on the ovary. Alkylating agents, for example, busulphan, chlorambucil and cyclophosphamide, carry the highest risk of POI. Others such as vincristine and

5-fluorouracil have a low risk. Concerns about future fertility are significant for women of reproductive age with cancer ([Tschudin and Bitzer, 2009](#)) and the use of fertility preserving techniques may be appropriate for some.

Bone marrow and stem cell transplantation, which require conditioning chemotherapy (the former requires conditioning total body irradiation as well), are increasingly being used for benign conditions such as sickle cell anemia, thalassemia major and, recently, multiple sclerosis. POI may result from treatment and the risks may be increased depending on the condition being treated. For example, females with sickle cell anemia may have preexisting compromised ovarian reserve due to the effects of sickle cell on ovarian blood supply.

Treatments

The only treatment for POI is to replace the estrogen that is no longer being produced by the ovaries. It is not possible to re-populate the ovary with follicles, as these are only produced during fetal life. The primary aim of estrogen replacement is to treat or prevent the consequences of low estrogen. The only proven benefits are for symptoms of low estrogen (hot flashes, night sweats, genitourinary symptoms). Estrogen replacement can also treat or prevent osteoporosis. There is no evidence currently that it prevents bone fractures due to osteoporosis in women with POI (although this is the case in normally menopausal women), but it is biologically plausible that it does. Estrogen replacement may reduce cardiovascular risk factors in women with POI. However, it is not known if it reduces the cardiovascular morbidity or mortality or has any effect on life expectancy. The decline in memory reported in women following oophorectomy can be treated or prevented by estrogen replacement. Women with spontaneous POI taking estrogen replacement have no difference in cognitive function compared to normal controls, although only one paper has addressed this issue ([Ross et al., 2004](#)): however, it is not known if untreated women show any impairment.

As in postmenopausal women, women with POI and an intact uterus should take a progestogen to protect the endometrium from the effect of unopposed estrogen, which could lead to endometrial cancer. Those without a uterus only need to take estrogen and do not require a progestogen. Estrogen and progesterone can be delivered orally or transdermally, and a progestogen can be delivered locally as an intrauterine system as an alternative. Regimens for combined hormone replacement therapy (HRT) can be cyclical (where a progestogen is taken intermittently, usually resulting in a withdrawal bleed), or continuous, to avoid a vaginal bleed. Most young women with POI will require a cyclical combined regimen (unless fitted with a progestogen-releasing intrauterine system), as troublesome vaginal spotting often occurs with a continuous combined replacement due to endometrial instability ([Webber et al., 2017](#)).

Women with POI should be encouraged to adopt healthy lifestyle choices: for example, maintaining a healthy weight and not smoking. This advice applies to the general population, but it may be particularly important for women with POI, given their predisposition to cardiovascular disease and the possibility of cognitive impairment.

Fertility and Pregnancy

Natural pregnancy may occur in up to 5% of women with POI (excluding those after bilateral oophorectomy; [Bidet et al., 2011](#)). There are no proven fertility treatments for women with POI, apart from egg donation, which has an excellent live birth rate, just under 30% per embryo transfer (UK national average in 2015, HFEA). Maintaining the health of the uterus with HRT may be beneficial for future egg donation, and may have a positive effect on the chance of implantation should natural conception occur. It is important that women with POI understand that HRT does not prevent resumption of ovarian activity and, if they are sexually active but want to avoid pregnancy, then contraception should be used.

Spontaneous pregnancies in women with idiopathic POI or after most forms of chemotherapy have no increased risks compared to the normal population. Egg donation pregnancies in those with surgical POI or spontaneous, karyotypically normal POI also appear to have no additional risks in pregnancy compared to any woman who is the recipient of egg donation. The exception is women with autoimmune POI if they have undiagnosed latent Addison's disease, which could be uncovered by pregnancy.

Previous exposure to most chemotherapeutic agents is associated with little risk in a later pregnancy ([Scottish Intercollegiate Guidelines Network \(2013\)](#)). However, anthracyclines and high dose cyclophosphamide can be associated with cardiomyopathy, as can radiotherapy to the heart (e.g., scatter from irradiation to the breast in the treatment of breast cancer, or to treat mediastinal lymphoma). This might be at a subclinical level but could become clinically significant with the physiological stress of pregnancy. Pelvic irradiation often leaves the uterus unresponsive to hormone preparation and implantation will often not occur. If pregnancy does result, there are increased risks of miscarriage (early and late), and disorders of poor placentation (e.g., preeclampsia, stillbirth, and low birth weight) as well as postpartum hemorrhage.

Both natural and egg donation pregnancies are very high risk in women with Turner syndrome (including in those who are a mosaic) due to congenital cardiac anomalies and the risk of aortic dissection, which can be fatal. The estimated risk of a major obstetric complication is 10% and the maternal mortality rate is 3.5% ([Hadnott et al., 2011](#)). Women with TS who desire pregnancy should be referred to a cardiologist specializing in congenital cardiac disease for pre-pregnancy assessment and ongoing care during pregnancy.

Prevention

The risk of iatrogenic POI can be reduced by implementing fertility-sparing treatments when possible. This includes surgical techniques and ovary-sparing surgery, the use of chemotherapeutic agents that are less gonadotoxic and ovarian transposition to remove the ovaries from radiation fields. There are no known options for reducing the risk of spontaneous POI, although maintaining

a healthy lifestyle may be of benefit. Preimplantation genetic screening can be employed for those carrying a mutation known to cause POI to avoid its transmission to the offspring.

References

- Bakalov, V. K., Gutin, L., Cheng, C. M., et al. (2012). Autoimmune disorders in women with Turner syndrome and women with karyotypically normal primary ovarian insufficiency. *Journal of Autoimmunology*, 38, 315–321.
- Bennett, C. E., Conway, G. S., Macpherson, J. N., et al. (2010). Intermediate sized CGG repeats are not a common cause of idiopathic premature ovarian failure. *Human Reproduction*, 25, 1335–1338.
- Betterle, C., Volpato, M., Rees Smith, B., et al. (1997). Adrenal cortex and steroid 21-hydroxylase autoantibodies in adult patients with organ-specific autoimmune diseases: Markers of low progression to clinical Addison's disease. *Journal of Clinical Endocrinology and Metabolism*, 82, 932–938.
- Bidet, M., Bachelot, A., Bissauge, E., et al. (2011). Resumption of ovarian function and pregnancies in 358 patients with premature ovarian failure. *Journal of Clinical Endocrinology and Metabolism*, 96, 3864–3872.
- Coccia, M. E., Rizzello, F., Mariani, G., et al. (2011). Ovarian surgery for bilateral endometriomas influences age at menopause. *Human Reproduction*, 26, 3000–3007.
- Conway, G. S., Kaltsas, G., Patel, A., et al. (1996). Characterization of idiopathic premature ovarian failure. *Fertility and Sterility*, 65, 337–341.
- Falorni, A., Brozzetti, A., Aglietti, M. C., et al. (2012). Progressive decline of residual follicle pool after clinical diagnosis of autoimmune ovarian insufficiency. *Clinical Endocrinology*, 77, 453–458.
- Hadnott, T. N., Gould, H. N., Gharib, A. M., et al. (2011). Outcomes of spontaneous and assisted pregnancies in Turner syndrome: The U.S. National Institutes of Health experience. *Fertility and Sterility*, 95, 2251–2256.
- Hoek, A., Schoemaker, J., & Drexhage, H. A. (1997). Premature ovarian failure and ovarian autoimmunity. *Endocrine Reviews*, 18, 107–134.
- Kelkar, R. L., Meherji, P. K., Kadam, S. S., et al. (2005). Circulating auto-antibodies against the zona pellucida and thyroid microsomal antigen in women with premature ovarian failure. *Journal of Reproductive Immunology*, 66, 53–67.
- La Marca, A., Marzotti, S., Brozzetti, A., et al. (2009). Primary ovarian insufficiency due to steroidogenic cell autoimmunity is associated with a preserved pool of functioning follicles. *Journal of Clinical Endocrinology and Metabolism*, 94, 3816–3823.
- La Marca, A., Brozzetti, A., Sighinolfi, G., et al. (2010). Primary ovarian insufficiency: Autoimmune causes. *Current Opinions in Obstetrics and Gynecology*, 22, 277–282.
- MacLaran, K., & Panay, N. (2011). Premature ovarian failure. *Journal of Family Planning and Reproductive Health Care*, 37, 35–42.
- Perry, J. R., Corre, T., Esko, T., et al. (2013). A genome-wide association study of early menopause and the combined impact of identified variants. *Human Molecular Genetics*, 22, 1465–1472.
- POI Guideline Development Group (2015) Management of women with premature ovarian insufficiency: Guideline of the European Society of Human Reproduction and Embryology. <http://www.eshre.eu/Guidelines-andlegal/Guidelines>
- Ross, J. L., Stefanatos, G. A., Kushner, H., et al. (2004). The effect of genetic differences and ovarian failure: Intact cognitive function in adult women with premature ovarian failure versus turner syndrome. *Journal of Clinical Endocrinology and Metabolism*, 89, 1817–1822.
- Schwartz, C. E., Dean, J., Howard-Peebles, P. N., et al. (1994). Obstetrical and gynecological complications in fragile X carriers: A multicenter study. *American Journal of Medical Genetics*, 51, 400–402.
- Scottish Intercollegiate Guidelines Network. (2013). *Long term follow up of survivors of childhood cancer*. SIGN publication no 132 2013. Available from <http://www.sign.ac.uk>.
- Tschudin, S., & Bitzer, J. (2009). Psychological aspects of fertility preservation in men and women affected by cancer and other life-threatening diseases. *Human Reproduction Update*, 15, 587–597.
- Vearncombe, K. J., & Pachana, N. A. (2009). Is cognitive functioning detrimentally affected after early, induced menopause? *Menopause*, 16, 188–198.
- Webber, L., Anderson, R., Davies, M., et al. (2017). *Human Reproduction Open*, 1–11.
- Welt, C. K. (2008). Primary ovarian insufficiency: A more accurate term for premature ovarian failure. *Clinical Endocrinology*, 68, 499–509.

Further Reading

NICE Guideline (NG23) Menopause: Diagnosis and management.

Relevant Website

www.eshre.eu/guidelines. —European Society of Human Reproduction and Embryology.

Obesity and Reproduction

Tristan SE Hardy, Women's and Children's Hospital, Adelaide, Australia; and University of New South Wales, Sydney, Australia
Sunita MC De Sousa, Royal Adelaide Hospital, Adelaide, Australia; Centre for Cancer Biology (an SA Pathology and University of South Australia alliance), Adelaide, Australia; and University of Adelaide, Adelaide, Australia
Robert J Norman, University of Adelaide, Adelaide, Australia; Fertility SA, Adelaide, Australia; Royal Adelaide Hospital, Adelaide, Australia; and NHMRC Centre for Research Excellence in Polycystic Ovary Syndrome, Adelaide, Australia

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

BMI Body-mass index
ICSI Intracytoplasmic sperm injection
IVF In vitro fertilization
PCOS Polycystic ovary syndrome
PGS Preimplantation genetic screening
WHO World Health Organization
WHR Waist-hip ratio

Introduction

The World Health Organization (WHO) defines obesity as the presence of excess body weight, classified into categories according to body mass index (BMI) ([World Health Organization, 2000](#)). The prevalence of overweight and obesity ($\text{BMI} > 25 \text{ kg/m}^2$ and $\text{BMI} > 30 \text{ kg/m}^2$, respectively) has increased globally in recent decades, with many developed nations reporting up to 60% of individuals classified as overweight or obese in the 20–39 year age bracket ([Ogden et al., 2006](#)). Although the health effects of excess adipocyte deposition are potentially better correlated with measures of central adiposity such as waist-hip ratio (WHR), public awareness of the concept of BMI and the ongoing use of pre-pregnancy or pregnancy booking BMI in the majority of studies in this area has led to the use of BMI as a standard measure of excess weight ([Mitchell and Shaw, 2015](#)). Similarly, the Institute of Medicine guidelines on gestational weight gain ([Institute of Medicine National Research Council Committee to Reexamine IOM Pregnancy Weight Guidelines, 2009](#)) have been extensively validated and remain a useful resource for clinicians providing advice regarding appropriate weight gain based on BMI category at the commencement of pregnancy.

Regardless of the measure used, it is vital that the clinical sequelae of obesity are detected in patients with obesity in the reproductive age group. Although cardiometabolic health concerns have traditionally been the focus of health prevention activities in this area, the spectrum of adverse effects on reproductive outcomes in both men and women is increasingly recognized as an area of major concern. This chapter focuses on the impact of obesity across the reproductive lifespan, from puberty to fertility, pregnancy, and menopause ([Fig. 1](#)). The trans-generational health effects of the current obesity crisis must also be considered, in recognition of the fact that preconception and pregnancy-related obesity may increase the risk of childhood and adult obesity in future generations ([Mullins et al., 2016](#)).

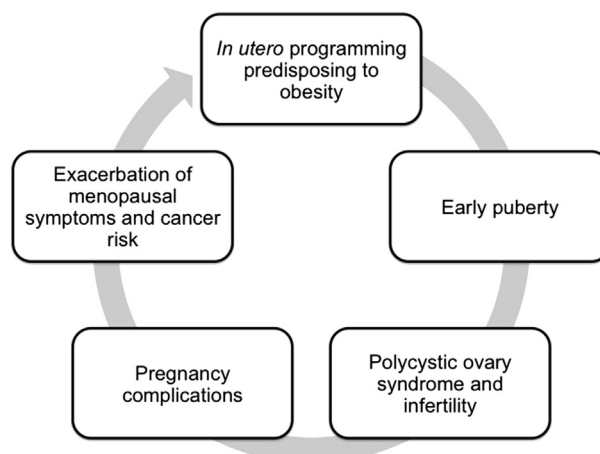


Fig. 1 Consequences of obesity across the reproductive lifespan and trans-generational effects via in utero exposure to maternal obesity.

Obesity and Puberty

The 'critical weight' hypothesis states that an upper threshold of body weight must be attained before menses can commence (Frisch and Revelle, 1970). The presumed teleological basis is that sexual maturation should only occur when an individual is of an adequate weight and nutritional status to bear offspring (Wagner et al., 2012). The corollary is that excess weight may cause advancement of puberty. The majority of data indicate that obesity is associated with earlier menarche (Al-Awadhi et al., 2013; Freedman et al., 2002; Kaplowitz et al., 2001; Lee et al., 2007; Zhai et al., 2015), although some data show that the age of menarche may be unaffected (Demerath et al., 2004) or even delayed by obesity (Kleber et al., 2011). Obesity prevalence is typically higher among children with premature pubarche (de Ferran et al., 2011; l'Allemand et al., 2002), but one study showed delayed pubarche in obese children (Kleber et al., 2011). The relationship between obesity and puberty is more robust for breast development, with multiple studies showing that thelarche occurs earlier in obese compared to normal-weight girls (Heger et al., 2008; Kaplowitz et al., 2001; Lee et al., 2007; Zhai et al., 2015). Body fat percentage and BMI are greater in girls in whom the first sign of puberty is thelarche compared to girls who present with pubarche (Biro et al., 2003). Genetic factors may account for discrepancies between pubertal processes and between studies (Wagner et al., 2012). The rate rather than the final amount of weight gain may influence pubertal onset as rapid weight gain in infancy is associated with early adrenarche (Dunger et al., 2006) and early menarche (Ong et al., 2009). The duration of obesity appears contributory with puberty occurring at an earlier age in proportion to earlier age of weight gain (Jasik and Lustig, 2008).

Adipose tissue is one of the key sites of aromatase with increased adiposity leading to increased aromatase activity and thus increased conversion of testosterone to estradiol. Increased adiposity directly reduces SHBG, leading to further increases in free sex steroids (Jasik and Lustig, 2008). Obesity is also associated with reduced hepatic inactivation of estrogen (Schneider et al., 1983). The mechanism of earlier puberty in obese girls thus appears to be increased circulating sex steroids, which stimulate the GnRH pulse generator in the hypothalamus, leading to gonadotroph stimulation in the pituitary and downstream gonadal steroidogenesis (Wagner et al., 2012). Estrogen excess leads to increased growth hormone and insulin-like growth factor 1 which contribute to insulin resistance and hyperinsulinemia and in turn lead to increased adiposity, driving the cycle of obesity and sex hormone production in females (Jasik and Lustig, 2008).

Similarly to adults, the prevalence of overweight and obesity is increasing in childhood (Ogden et al., 2006), and there has been a concomitant advancement of the age of puberty onset suggesting that these two phenomena are linked. An epidemiological study of 156,835 children over a 39-year period found that increased BMI at age seven in both boys and girls predicted earlier age at puberty (Aksglaede et al., 2009). Downstream effects of obesity-related advancements in puberty include reduced final height (De Leonibus et al., 2014), psychological effects of early sexual maturation, and negative consequences, such as increased breast cancer risk, associated with increased lifetime estrogen exposure (Jasik and Lustig, 2008; Wagner et al., 2012).

Female Obesity and Fertility

It is clear that obesity has a negative effect on reproductive fitness, although the relative contribution of ovarian, endometrial and hypothalamic-pituitary factors is contested (Norman et al., 2008). Obesity is associated with increased time to achieve pregnancy, including in patients with regular menstrual cycles (Gesink Law et al., 2007), and greater risk of first trimester miscarriage and recurrent miscarriage (OR 1.2 and 3.5 for obese patients, respectively) (Lashen et al., 2004). Evidence from oocyte donor cohorts has been used to discriminate between ovarian and endometrial factors. Bellver et al. (2007) examined 2656 first ovum donation cycles where the average BMI of the oocyte donor was within the normal range and demonstrated reduced ongoing pregnancy rate per cycle in overweight and obese recipients compared to normal-weight recipients (38.3% vs. 45.5%). Others have examined the BMI of oocyte donors and demonstrated a reduction in clinical pregnancy rates as donor BMI quartile increased, suggesting an effect of obesity on the oocyte as well as the endometrium (Cardozo et al., 2016).

The impact of obesity on oocyte quality is subtle, as there does not appear to be an association between BMI and the rate of aneuploidy in IVF/ICSI cycles performed with preimplantation genetic screening (PGS) (Goldman et al., 2015). This suggests that the increased risk of miscarriage in obese patients does not result from an increased production of aneuploid embryos. This was confirmed in a study which demonstrated miscarriage rates after single euploid embryo transfer in to be lowest in normal-weight women (14.2%), intermediate in overweight women (29.1%) and highest in obese women (41.9%) (Tremellen et al., 2017). Morphokinetic studies of embryo development have similarly not demonstrated a significant difference in cleavage patterns as a result of maternal obesity (Bellver et al., 2013). Follicular fluid from women with obesity may have biochemical changes indicative of metabolic alteration (increased follicular insulin, lactate, triglycerides) and inflammation (C-reactive protein) (Robker et al., 2009). Subtle mitochondrial dysfunction as a result of lipotoxicity may be the cause of diminished oocyte competence (Wu et al., 2015). Another possibility is impaired oocyte-sperm interaction, with some evidence of the negative effects of BMI on fertility being reduced or absent when ICSI is employed (Wang et al., 2016).

Obesity significantly impacts upon IVF cycle treatments and outcomes. Analysis of 239,127 IVF cycles demonstrated decreased implantation rate from 29.5% to 20.3% (normal BMI vs. highest BMI), progressively decreased clinical pregnancy rate to OR of 0.75 in the highest BMI category, and reductions in live birth rate (31% in normal BMI vs. 21% in highest BMI category) (Provost et al., 2016). Obese women were also more likely to have diminished gonadotrophin response, requiring higher doses of FSH and obtaining significantly lower numbers of mature oocytes despite greater utilization of gonadotrophins (Zander-Fox et al., 2012).

Disturbance of the hypothalamic–pituitary–ovarian axis may occur with increasing weight, and anovulatory dysfunction may occur in the context of obesity with or without PCOS. The overall proportion of women who are overweight or obese with PCOS differs according to the population and subpopulation studied, ranging from 14% to 75% (Joham et al., 2016). Conversely, there is a high proportion of reproductive dysfunction in patients presenting with obesity, with 36% of patients submitted to bariatric surgery having a diagnosis of PCOS (Escobar-Morreale et al., 2017). Although not all women with PCOS are overweight or obese, the overlap between the conditions suggests perturbation of shared metabolic pathways, and the term “obesity-associated gonadal dysfunction” has been applied to describe this patient group with PCOS or secondary hypogonadism as a result of excess adiposity (Escobar-Morreale et al., 2014).

Mechanistic theories of the relationship between PCOS and obesity have focused on insulin resistance and the effect of androgens on fat deposition patterns. Patients with androgen excess may have a tendency towards abdominal fat deposition, which aggravates androgen excess and leads to a vicious cycle resulting in the phenotype of PCOS in susceptible individuals (Escobar-Morreale and San Millan, 2007). Alternatively, obesity may be a source of diagnostic confusion due to prolongation of the follicular phase of the menstrual cycle in women with obesity (Dumesic et al., 2015). This is an important clinical distinction as treatment differs between the anovulatory patient with obesity and PCOS and the ovulatory patient with obesity. However, weight loss should be advocated in both patient groups as a primary treatment goal; lifestyle measures should be considered first-line treatments as weight loss of 5%–10% may significantly improve fertility outcomes (Clark et al., 1995). More profound weight loss as achieved with bariatric surgery leads to resolution of PCOS in up to 96% of patients (Escobar-Morreale et al., 2017), and spontaneous pregnancy in 58% of previously infertile women (Milone et al., 2016).

Male Obesity and Fertility

There has been a broad decline in semen parameters over the last 50 years in developed countries and obesity has been cited as a possible contributor (Du Plessis et al., 2010). Epidemiological studies have shown increased odds ratios for infertility in overweight (1.20, 95% CI 1.04–1.38) and obese (1.36, 95% CI 1.13–1.63) men after correction for confounders, including female BMI (Nguyen et al., 2007). Male and female obesity has synergistic effects on fertility in affected couples, which is particularly concerning as spouses of obese individuals are more likely to be obese compared to spouses of normal-weight individuals (Ramlaui-Hansen et al., 2007). A Danish study of 64,167 pregnant women found that subfertility was more frequent when both partners were overweight (1.41, 95% CI: 1.28–1.56) or obese (2.74, 95% CI: 2.27–3.30) compared to normal-weight controls (Ramlaui-Hansen et al., 2007).

The subfertility observed in obese men is largely a consequence of androgen deficiency secondary to increased aromatase resulting in estrogen excess and hypothalamic–pituitary suppression and hyperinsulinemia-associated SHBG reduction. Other mechanisms of obesity-related infertility in men include direct effects on sperm, altered sexual behavior, erectile dysfunction, local heat generation by excess scrotal fat, and oxidative stress which can lead to DNA damage in sperm (Du Plessis et al., 2010). Weight loss via dietary interventions or bariatric surgery improves gonadal and erectile function (Stokes et al., 2015), although the effect is not as robust as in obese anovulatory women (Clark et al., 1995).

Obesity and Pregnancy

Obesity has been documented to affect virtually all antenatal, intrapartum and postnatal complications, with each individual risk increasing with maternal BMI (Grivell et al., 2016). In many nations, the majority of women entering pregnancy are now overweight or obese (Dodd and Thangaratinam, 2016), and there is pressure on health services to optimize perinatal morbidity and mortality outcomes despite increasing maternal weight.

The risk of congenital anomalies is greater in children of overweight or obese mothers, and may be compounded by poor diet quality (Carmichael et al., 2016). The importance of regular folate supplementation in the preconception period should be stressed to all patients, including those with excess body weight. There may be an additional risk of congenital abnormalities in children born to women with a diagnosis of PCOS (Doherty et al., 2015). Detection of morphological abnormalities may be impaired in the fetus of the obese patient and ultrasound evaluation in a tertiary center is recommended.

Medical complications of pregnancy including gestational hypertension, pre-eclampsia, gestational diabetes and thromboembolic disorders, have all been strongly associated with overweight and obesity. In addition to pregnancy-related medical complications, women may present with pre-existing medical complications of obesity which may be compounded by pregnancy (Grivell et al., 2016).

Obesity is a risk factor for preterm birth, and a recent meta-analysis found that preterm delivery was increased in obese patients, possibly as a result of greater burden of medical complications during pregnancy (Faucher et al., 2016). There may also be an association between lower socioeconomic status, obesity and preterm birth. Stillbirth and neonatal death are both strongly linked to pre-pregnancy BMI, with an odds ratio for infant death of 1.42 (95% CI 1.24–1.63) for women in the obese BMI range compared to women in the normal BMI range prior to pregnancy (Meehan et al., 2014). The risk of neonatal death increases with increasing BMI, and appears to be independent of the demographic and medical risk factors which may confound these results (Declercq et al., 2016).

Induction of labor, instrumental delivery, shoulder dystocia and Caesarean section are all more common in overweight or obese women. There is some evidence to suggest that a policy of elective induction of labor at 37 weeks and 39 weeks gestation in patients with a pre-pregnancy BMI $> 30 \text{ kg/m}^2$ may decrease the risk of Caesarean delivery and fetal macrosomia without increasing the risk of other adverse outcomes (Lee et al., 2016). High birthweight (> 4.0 or 4.5 kg , or $> 90\text{th}$ centile using growth charts) is more common in neonates born to overweight or obese mothers and is associated with a greater risk of early infant and childhood obesity. Lifestyle interventions to reduce gestational weight gain failed to mitigate this negative impact on neonatal body composition in a randomized controlled trial (Dodd et al., 2016).

Given the limited effect of pregnancy-based interventions (Muktabhant et al., 2015), it seems reasonable to focus efforts on the pre-pregnancy period and weight loss strategies should be at the forefront of any interaction with a patient before, during or after pregnancy (Dodd and Thangaratinam, 2016; Dodd et al., 2016). A small increase in BMI between pregnancies may lead to a significant increase in obstetric complications including stillbirth in subsequent pregnancies (Cnattingius and Villamor, 2016). The inter-pregnancy interval is emerging as a key treatment opportunity to reduce weight gain and optimize health prior to subsequent pregnancies.

Obesity and Menopause

Obesity prevalence is highest in the years following menopause. Data from 11,247 Australians showed that waist circumference in women was greatest in the 64–74 years age bracket whilst the prevalence of BMI $> 30 \text{ kg/m}^2$ was greatest in women aged 55–74 years (Teede et al., 2010). Menopause is often considered to be a critical time of weight gain with higher BMI seen in perimenopausal and postmenopausal women compared to premenopausal women, independent of age effects (Pasquali et al., 1994). Sex steroid deficiency appears to mediate the observed anthropometric changes during menopause as a placebo-controlled, cross-over study of combined estrogen–progesterone hormone replacement therapy (HRT) found that HRT increased lean body mass, and decreased total fat mass and abdominal fat (Sorensen et al., 2001). A Swedish prospective study of 1462 women starting in the 1960s found that menopause was not associated with an increase in total body weight, but postmenopausal status was associated with increased waist circumference and reduced waist–hip ratio and estrogen replacement postponed these changes (Bjorkelund et al., 1996). Overall, menopause is associated with a typical weight gain of 2–2.5 kg over 3 years which is similar to age-matched premenopausal women; however, abdominal adiposity appears to rise specifically at the menopausal transition (Polotsky and Polotsky, 2010; Wing et al., 1991). Regardless of the precise prevalence and causality of obesity at the time of menopause, obese women face greater health risks compared to normal-weight postmenopausal women.

Obesity may exacerbate menopausal symptoms. Though the aromatization of androgens to estrogens in obese women would suggest that obese perimenopausal women are less prone to hot flushes, increased total and subcutaneous abdominal adiposity are in fact associated with higher odds of hot flushes, which may relate to heat insulation by adipose tissue (Thurston et al., 2008). In addition, obesity in postmenopausal women is associated with a 2.2-fold increase in the rate of vaginal discharge and a 3.6-fold increase in the rate of vaginal irritation/itching (Pastore et al., 2004). Obesity compounds the risk of urinary incontinence in postmenopausal women. The association is particularly strong with stress and mixed urinary incontinence but it extends to urge incontinence and overactive bladder syndrome (Hunskaar, 2008). The Program to Reduce Incontinence by Diet and Exercise (PRIDE) study found that intensive lifestyle intervention with a mean weight loss of 8% over 6 months in overweight or obese women more than halved the number of stress incontinence episodes, and there was a trend towards reduced episodes of urge incontinence (Subak et al., 2009).

Obesity may have more sinister health effects in postmenopausal women. Data from the Women's Health Initiative showed that increased BMI is associated with a 1.76 hazard ratio of endometrial cancer when comparing postmenopausal women in the highest and lowest BMI categories (Reeves et al., 2011). Breast cancer risk among postmenopausal women also increases in proportion to BMI. These cancer risks appear to be largely mediated by increased estrogen exposure, particularly free estradiol which is a function of obesity-related reductions in SHBG (Key et al., 2003). Moreover, obese women with endometrial or breast cancer suffer higher all-cause and cancer-related mortality compared to their normal-weight counterparts (Chia et al., 2007; Protani et al., 2010).

Age-dependent weight gain at the time of menopause is associated with increases in blood pressure, total cholesterol, low-density lipoprotein cholesterol, triglycerides and fasting insulin compared to normal-weight women (Wing et al., 1991). In addition, BMI, waist circumference and waist–hip ratio are independently associated with coronary heart disease in both premenopausal and postmenopausal women (Rexrode et al., 1998), but the absolute risk of coronary heart disease is greatest in obese postmenopausal women due to the compounding effects of obesity and age. Menopause may exert an independent effect on cardiovascular risk due to sex hormone changes including estrogen deficiency, however data are conflicting. Estrogen replacement in women undergoing spontaneous, age-appropriate menopause does not afford any cardiovascular protection and may in fact increase the risk of cardiovascular disease (Carr, 2003).

Obesity was originally thought to lower osteoporosis risk because of aromatase-derived estrogen production, SHBG reduction leading to increased free estradiol, positive effects of leptin on bone including enhanced production of osteoblasts, and insulin-mediated IGF1 production which stimulates osteoblast proliferation (Albala et al., 1996; Migliaccio et al., 2011). This was borne out empirically with obese postmenopausal women shown to have significantly higher BMD at both the hip and spine in one study (Albala et al., 1996), but other evidence has been conflicting. Studies defining obesity as increased BMI or body weight tend to find beneficial effects on bone density and fracture risk, whilst studies defining obesity as increased body fat percentage or central

adiposity typically show that obesity predisposes to osteoporosis (Migliaccio et al., 2011). The Global Longitudinal Study of Osteoporosis in Women (GLOW) of 44,534 postmenopausal women found that overall fracture prevalence was similar between obese and non-obese women but obese women were at significantly greater risk of incident ankle and upper leg fractures and a significantly smaller risk of wrist fracture (Compston et al., 2011). Dietary factors may modulate the interaction between weight and bone status. In a 12-month randomized controlled trial of participants with BMI between 23.5 and 29.9 kg/m² including 30 postmenopausal women, weight loss by dietary intervention but not by exercise was associated with significant BMD decreases at the hip and spine compared to controls (Villareal et al., 2006). Regardless of any potential benefit of weight on bone health, the negative consequences of obesity across the reproductive span of women dominate and weight loss should be encouraged in obese women.

Conclusions

The rates of overweight and obesity have now reached epidemic proportions (Mitchell and Shaw, 2015), and are having an effect on health in manifold ways across the reproductive lifespan. Whilst the adverse effects of obesity on reproduction have been well documented, there is still a large body of conflicting evidence with regards to mechanisms and outcomes, including outcomes following different weight loss program options. Nevertheless, it is clear that an effective approach to achieve a normal pre-pregnancy weight will be essential to ensure optimal reproductive and pregnancy outcomes, and limit the trans-generational effects of overweight and obesity.

References

- Aksglaede, L., Juul, A., Olsen, L. W., & Sorensen, T. I. (2009). Age at puberty and the emerging obesity epidemic. *PLoS One*, 4, e8450.
- Al-Awadhi, N., Al-Kandari, N., Al-Hasan, T., et al. (2013). Age at menarche and its relationship to body mass index among adolescent girls in Kuwait. *BMC Public Health*, 13, 29.
- Albala, C., Yanez, M., Devoto, E., et al. (1996). Obesity as a protective factor for postmenopausal osteoporosis. *International Journal of Obesity and Related Metabolic Disorders*, 20, 1027–1032.
- Belver, J., Melo, M. A., Bosch, E., et al. (2007). Obesity and poor reproductive outcome: the potential role of the endometrium. *Fertility and Sterility*, 88, 446–451.
- Belver, J., Mifsud, A., Grau, N., Privitera, L., & Meseguer, M. (2013). Similar morphokinetic patterns in embryos derived from obese and normoweight infertile women: a time-lapse study. *Human Reproduction*, 28, 794–800.
- Biro, F. M., Lucky, A. W., Simbartl, L. A., et al. (2003). Pubertal maturation in girls and the relationship to anthropometric changes: pathways through puberty. *The Journal of Pediatrics*, 142, 643–646.
- Bjorkelund, C., Lissner, L., Andersson, S., Lapidus, L., & Bengtsson, C. (1996). Reproductive history in relation to relative weight and fat distribution. *International Journal of Obesity and Related Metabolic Disorders*, 20, 213–219.
- Cardozo, E. R., Karmon, A. E., Gold, J., Petrozza, J. C., & Styer, A. K. (2016). Reproductive outcomes in oocyte donation cycles are associated with donor BMI. *Human Reproduction*, 31, 385–392.
- Carmichael, S. L., Yang, W., Gilboa, S., et al. (2016). Elevated body mass index and decreased diet quality among women and risk of birth defects in their offspring. *Birth Defects Research. Part A, Clinical and Molecular Teratology*, 106, 164–171.
- Carr, M. C. (2003). The emergence of the metabolic syndrome with menopause. *The Journal of Clinical Endocrinology and Metabolism*, 88, 2404–2411.
- Chia, V. M., Newcomb, P. A., Trentham-Dietz, A., & Hampton, J. M. (2007). Obesity, diabetes, and other factors in relation to survival after endometrial cancer diagnosis. *International Journal of Gynecological Cancer*, 17, 441–446.
- Clark, A. M., Ledger, W., Galletly, C., et al. (1995). Weight loss results in significant improvement in pregnancy and ovulation rates in anovulatory obese women. *Human Reproduction*, 10, 2705–2712.
- Chattingius, S., & Villamor, E. (2016). Weight change between successive pregnancies and risks of stillbirth and infant mortality: a nationwide cohort study. *Lancet*, 387, 558–565.
- Compston, J. E., Watts, N. B., Chapurlat, R., et al. (2011). Obesity is Not Protective Against Fracture in Postmenopausal Women: GLOW. *The American Journal of Medicine*, 124, 1043–1050.
- de Ferran, K., Paiva, I. A., Garcia Ldos, S., Gama Mde, P., & Guimaraes, M. M. (2011). Isolated premature pubarche: report of anthropometric and metabolic profile of a Brazilian cohort of girls. *Hormone Research in Paediatrics*, 75, 367–373.
- De Leonibus, C., Marcovecchio, M. L., Chiavaroli, V., et al. (2014). Timing of puberty and physical growth in obese children: a longitudinal study in boys and girls. *Pediatric Obesity*, 9, 292–299.
- Declercq, E., MacDorman, M., Cabral, H., & Stotland, N. (2016). Prepregnancy Body Mass Index and Infant Mortality in 38 U.S. States, 2012–2013. *Obstetrics and Gynecology*, 127, 279–287.
- Demerath, E. W., Li, J., Sun, S. S., et al. (2004). Fifty-year trends in serial body mass index during adolescence in girls: the Fels Longitudinal Study. *The American Journal of Clinical Nutrition*, 80, 441–446.
- Dodd, J., & Thangaratinam, S. (2016). Researchers' position statement on tackling obesity in pregnancy: the International Weight Management in Pregnancy (i-WIP) collaboration pleads for public health intervention. *BJOG*, 123, 163–164.
- Dodd, J. M., Deussen, A. R., Mohamad, I., et al. (2016). The effect of antenatal lifestyle advice for women who are overweight or obese on secondary measures of neonatal body composition: the LIMIT randomised trial. *BJOG*, 123, 244–253.
- Doherty, D. A., Newnham, J. P., Bower, C., & Hart, R. (2015). Implications of polycystic ovary syndrome for pregnancy and for the health of offspring. *Obstetrics and Gynecology*, 125, 1397–1406.
- Du Plessis, S. S., Cabler, S., McAlister, D. A., Sabanegh, E., & Agarwal, A. (2010). The effect of obesity on sperm disorders and male infertility. *Nature Reviews. Urology*, 7, 153–161.
- Dumesic, D. A., Oberfield, S. E., Stener-Victorin, E., et al. (2015). Scientific Statement on the Diagnostic Criteria, Epidemiology, Pathophysiology, and Molecular Genetics of Polycystic Ovary Syndrome. *Endocrine Reviews*, 36, 487–525.
- Dunger, D. B., Ahmed, M. L., & Ong, K. K. (2006). Early and late weight gain and the timing of puberty. *Molecular and Cellular Endocrinology*, 254–255, 140–145.
- Escobar-Morreale, H. F., Alvarez-Blasco, F., Botella-Carretero, J. I., & Luque-Ramirez, M. (2014). The striking similarities in the metabolic associations of female androgen excess and male androgen deficiency. *Human Reproduction*, 29, 2083–2091.
- Escobar-Morreale, H. F., & San Millan, J. L. (2007). Abdominal adiposity and the polycystic ovary syndrome. *Trends in Endocrinology and Metabolism*, 18, 266–272.

- Escobar-Morreale, H. F., Santacruz, E., Luque-Ramirez, M., & Botella Carretero, J. I. (2017). Prevalence of 'obesity-associated gonadal dysfunction' in severely obese men and women and its resolution after bariatric surgery: a systematic review and meta-analysis. *Human Reproduction Update*, 23, 390–408.
- Faucher, M. A., Hastings-Tolsma, M., Song, J. J., Willoughby, D. S., & Bader, S. G. (2016). Gestational weight gain and preterm birth in obese women: a systematic review and meta-analysis. *BJOG*, 123, 199–206.
- Freedman, D. S., Khan, L. K., Serdula, M. K., et al. (2002). Relation of age at menarche to race, time period, and anthropometric dimensions: the Bogalusa Heart Study. *Pediatrics*, 110, e43.
- Frisch, R. E., & Revelle, R. (1970). Height and weight at menarche and a hypothesis of critical body weights and adolescent events. *Science*, 169, 397–399.
- Gesink Law, D. C., Maclehorse, R. F., & Longnecker, M. P. (2007). Obesity and time to pregnancy. *Human Reproduction*, 22, 414–420.
- Goldman, K. N., Hodes-Wertz, B., McCulloh, D. H., Flom, J. D., & Grifo, J. A. (2015). Association of body mass index with embryonic aneuploidy. *Fertility and Sterility*, 103, 744–748.
- Grivell, R. M., O'Brien, C. M., & Dodd, J. M. (2016). Managing Obesity in Pregnancy: A Change in Focus from Harm Minimization to Prevention. *Seminars in Reproductive Medicine*, 34, e38–e46.
- Heger, S., Korner, A., Meigen, C., et al. (2008). Impact of weight status on the onset and parameters of puberty: analysis of three representative cohorts from central Europe. *Journal of Pediatric Endocrinology & Metabolism*, 21, 865–877.
- Hunskaar, S. (2008). A systematic review of overweight and obesity as risk factors and targets for clinical intervention for urinary incontinence in women. *Neurology and Urodynamics*, 27, 749–757.
- Institute of Medicine National Research Council Committee to Reexamine IOM Pregnancy Weight Guidelines. (2009) Institute of Medicine National Research Council Committee to Reexamine IOM Pregnancy. The National Academies Collection: Reports funded by National Institutes of Health. In K. M. Rasmussen, & A. L. Yaktine (Eds.), *Weight Gain During Pregnancy: Reexamining the Guidelines*. Washington (DC): National Academies Press (US) National Academy of Sciences.
- Jasik, C. B., & Lustig, R. H. (2008). Adolescent obesity and puberty: the "perfect storm". *Annals of the New York Academy of Sciences*, 1135, 265–279.
- Joham, A. E., Palomba, S., & Hart, R. (2016). Polycystic Ovary Syndrome, Obesity, and Pregnancy. *Seminars in Reproductive Medicine*, 34, 93–101.
- Kaplowitz, P. B., Slora, E. J., Wasserman, R. C., Pedlow, S. E., & Herman-Giddens, M. E. (2001). Earlier onset of puberty in girls: relation to increased body mass index and race. *Pediatrics*, 108, 347–353.
- Key, T. J., Appleby, P. N., Reeves, G. K., et al. (2003). Body mass index, serum sex hormones, and breast cancer risk in postmenopausal women. *Journal of the National Cancer Institute*, 95, 1218–1226.
- Kleber, M., Schwarz, A., & Reinehr, T. (2011). Obesity in children and adolescents: relationship to growth, pubarche, menarche, and voice break. *Journal of Pediatric Endocrinology & Metabolism*, 24, 125–130.
- l'Allemand, D., Schmidt, S., Rousson, V., et al. (2002). Associations between body mass, leptin, IGF-I and circulating adrenal androgens in children with obesity and premature adrenarche. *European Journal of Endocrinology*, 146, 537–543.
- Lashen, H., Fear, K., & Sturdee, D. W. (2004). Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study. *Human Reproduction*, 19, 1644–1646.
- Lee, J. M., Appugliese, D., Kaciroti, N., et al. (2007). Weight status in young girls and the onset of puberty. *Pediatrics*, 119, e624–e630.
- Lee, V. R., Darney, B. G., Snowden, J. M., et al. (2016). Term elective induction of labour and perinatal outcomes in obese women: retrospective cohort study. *BJOG*, 123, 271–278.
- Meehan, S., Beck, C. R., Mair-Jenkins, J., Leonardi-Bee, J., & Puleston, R. (2014). Maternal obesity and infant mortality: a meta-analysis. *Pediatrics*, 133, 863–871.
- Migliaccio, S., Greco, E. A., Fornari, R., Donini, L. M., & Lenzi, A. (2011). Is obesity in women protective against osteoporosis? *Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy*, 4, 273–282.
- Milone, M., De Placido, G., Musella, M., et al. (2016). Incidence of Successful Pregnancy After Weight Loss Interventions in Infertile Women: a Systematic Review and Meta-Analysis of the Literature. *Obesity Surgery*, 26, 443–451.
- Mitchell, S., & Shaw, D. (2015). The worldwide epidemic of female obesity. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 29, 289–299.
- Muktabhant, B., Lawrie, T. A., Lumbiganon, P., & Laopaiboon, M. (2015). Diet or exercise, or both, for preventing excessive weight gain in pregnancy. *The Cochrane Database of Systematic Reviews*, Cd007145.
- Mullins, E., Murphy, O., & Davies, S. C. (2016). Pre-conception public health to address maternal obesity. *BJOG*, 123, 159–160.
- Nguyen, R. H., Wilcox, A. J., Skjaerven, R., & Baird, D. D. (2007). Men's body mass index and infertility. *Human Reproduction*, 22, 2488–2493.
- Norman, R. J., Chura, L. R., & Robker, R. L. (2008). Effects of obesity on assisted reproductive technology outcomes. *Fertility and Sterility*, 89, 1611–1612.
- Ogden, C. L., Carroll, M. D., Curtin, L. R., et al. (2006). Prevalence of overweight and obesity in the United States, 1999–2004. *Journal of the American Medical Association*, 295, 1549–1555.
- Ong, K. K., Emmett, P., Northstone, K., et al. (2009). Infancy weight gain predicts childhood body fat and age at menarche in girls. *The Journal of Clinical Endocrinology and Metabolism*, 94, 1527–1532.
- Pasquali, R., Casimirri, F., Labate, A. M., et al. (1994). Body weight, fat distribution and the menopausal status in women. The VMH Collaborative Group. *International Journal of Obesity and Related Metabolic Disorders*, 18, 614–621.
- Pastore, L. M., Carter, R. A., Hulka, B. S., & Wells, E. (2004). Self-reported urogenital symptoms in postmenopausal women: Women's Health Initiative. *Maturitas*, 49, 292–303.
- Polotsky, H. N., & Polotsky, A. J. (2010). Metabolic implications of menopause. *Seminars in Reproductive Medicine*, 28, 426–434.
- Protani, M., Coory, M., & Martin, J. H. (2010). Effect of obesity on survival of women with breast cancer: systematic review and meta-analysis. *Breast Cancer Research and Treatment*, 123, 627–635.
- Provost, M. P., Acharya, K. S., Acharya, C. R., et al. (2016). Pregnancy outcomes decline with increasing body mass index: analysis of 239,127 fresh autologous in vitro fertilization cycles from the 2008–2010. Society for Assisted Reproductive Technology registry. *Fertility and Sterility*, 105, 663–669.
- Ramlau-Hansen, C. H., Thulstrup, A. M., Nohr, E. A., et al. (2007). Subfecundity in overweight and obese couples. *Human Reproduction*, 22, 1634–1637.
- Reeves, K. W., Carter, G. C., Rodabough, R. J., et al. (2011). Obesity in relation to endometrial cancer risk and disease characteristics in the Women's Health Initiative. *Gynecologic Oncology*, 121, 376–382.
- Rexrode, K. M., Carey, V. J., Hennekens, C. H., et al. (1998). Abdominal adiposity and coronary heart disease in women. *JAMA*, 280, 1843–1848.
- Robker, R. L., Akison, L. K., Bennett, B. D., et al. (2009). Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. *The Journal of Clinical Endocrinology and Metabolism*, 94, 1533–1540.
- Schneider, J., Bradlow, H. L., Strain, G., et al. (1983). Effects of obesity on estradiol metabolism: decreased formation of nonuterotropic metabolites. *The Journal of Clinical Endocrinology and Metabolism*, 56, 973–978.
- Sorensen, M. B., Rosenfalck, A. M., Hojgaard, L., & Ottesen, B. (2001). Obesity and sarcopenia after menopause are reversed by sex hormone replacement therapy. *Obesity Research*, 9, 622–626.
- Stokes, V. J., Anderson, R. A., & George, J. T. (2015). How does obesity affect fertility in men - and what are the treatment options? *Clinical Endocrinology*, 82, 633–638.
- Subak, L. L., Wing, R., West, D. S., et al. (2009). Weight Loss to Treat Urinary Incontinence in Overweight and Obese Women. *The New England Journal of Medicine*, 360, 481–490.
- Teede, H. J., Lombard, C., & Deeks, A. A. (2010). Obesity, metabolic complications and the menopause: an opportunity for prevention. *Climacteric*, 13, 203–209.
- Thurston, R. C., Sowers, M. R., Sutton-Tyrrell, K., et al. (2008). Abdominal adiposity and hot flashes among midlife women. *Menopause*, 15, 429–434.

- Tremellen, K., Pearce, K., & Zander-Fox, D. (2017). Increased miscarriage of euploid pregnancies in obese women undergoing cryopreserved embryo transfer. *Reproductive BioMedicine Online*, 34, 90–97.
- Villareal, D. T., Fontana, L., Weiss, E. P., et al. (2006). Bone mineral density response to caloric restriction-induced weight loss or exercise-induced weight loss: a randomized controlled trial. *Archives of Internal Medicine*, 166, 2502–2510.
- Wagner, I. V., Sabin, M. A., Pfaffle, R. W., et al. (2012). Effects of obesity on human sexual development. *Nature Reviews. Endocrinology*, 8, 246–254.
- Wang, X., Hao, J., Zhang, F., et al. (2016). Effects of female and male body mass indices on the treatment outcomes and neonatal birth weights associated with in vitro fertilization/ intracytoplasmic sperm injection treatment in China. *Fertility and Sterility*, 106, 460–466.
- Wing, R. R., Matthews, K. A., Kuller, L. H., Meilahn, E. N., & Plantinga, P. L. (1991). Weight gain at the time of menopause. *Archives of Internal Medicine*, 151, 97–102.
- World Health Organization. (2000World). Obesity: preventing and managing the global epidemic. Report of a WHO consultation. In *World Health Organization Technical Report Series* (2001/03/10 ed., vol. 894, pp. i–xii) 1–253.
- Wu, L. L., Russell, D. L., Wong, S. L., et al. (2015). Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors. *Development*, 142, 681–691.
- Zander-Fox, D. L., Henshaw, R., Hamilton, H., & Lane, M. (2012). Does obesity really matter? The impact of BMI on embryo quality and pregnancy outcomes after IVF in women aged ≤ 38 years. *The Australian & New Zealand Journal of Obstetrics & Gynaecology*, 52, 270–276.
- Zhai, L., Liu, J., Zhao, J., et al. (2015). Association of Obesity with Onset of Puberty and Sex Hormones in Chinese Girls: A 4-Year Longitudinal Study. *PLoS One*, 10, e0134656.

Menstrual Disorders: The Need for Agreement on Terminologies and Classification of Causes of Abnormal Uterine Bleeding (AUB)

Hilary OD Critchley, The University of Edinburgh, Edinburgh, United Kingdom

Malcolm G Munro, David Geffen School of Medicine at UCLA, Los Angeles, CA, United States

Ian S Fraser, University of New South Wales, Sydney, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction, Impact, and History of Menstrual Terminologies and Definitions

Menstruation is a fundamental physiological process but for some individuals, and in many cultures and societies is viewed negatively and remains a taboo topic.

The adverse impact of menstruation and menstrual disorders has increased, a circumstance that in large part is due to the increased frequency of menstruation in the developed world. This is a consequence of earlier menarche, an increased access to contraception, and a reduction in parity and associated lactational amenorrhoea. In industrialized nations, women may expect to menstruate perhaps 400 times over their reproductive lifespan compared with as few as 40 times centuries ago, or even now in less industrialized societies. As a result, symptoms related to menstrual function—abnormal uterine bleeding (AUB)—are an increasing area of unmet clinical need for women in the reproductive years.

Heavy menstrual bleeding (HMB) is a common AUB symptom and frequently a debilitating one, affecting as many as a quarter of women of reproductive age (NICE, 2007; Shapley et al., 2004). The symptom of HMB has a significant negative impact on quality of life and imposes a huge socio-economic burden for women affected, their families and healthcare systems, and negatively impacts their productive participation in the workforce and society at large (Bitzer et al., 2015; Frick et al., 2009; Prentice, 1999). A study conducted in the USA reported financial losses of more than \$2000 per patient per annum (year 2009 costs) due to work absence and expenses related to home management (Frick et al., 2009).

Steady depletion of a woman's iron stores is a frequent consequence of HMB, with resulting iron deficiency and, frequently, iron deficiency anemia (IDA) with associated fatigue, and other symptoms that negatively impact health related quality of life (Percy et al., 2017). Leiomyomas (fibroids) are also often associated with the complaint of HMB and their presence may contribute to issues surrounding fertility and pregnancy outcome. Those with large or multiple leiomyomas may experience symptoms related to pressure on surrounding structures such as bowel, vagina, or the urinary bladder. The estimated annual direct costs (surgery, hospital admissions, outpatient visits, and medications) associated with leiomyomas has been reported as between \$4.1 and 9.4 billion (Cardozo et al., 2012). The estimated cost for lost work-hours in the US was estimated to be between \$1.55 and 17.2 billion annually (Cardozo et al., 2012). When costs related to obstetric outcomes attributed to leiomyoma presence were included, the estimated annualized economic impact rose to \$5.9–34.4 billion (Cardozo et al., 2012).

Therapy for the spectrum of causes of AUB in the reproductive years is frequently challenging. Some, such as endometrial polyps and submucous leiomyomas, are best treated with targeted surgical therapy, while others, such as ovulatory disorders, are often successfully managed with hormonal therapy. However, many current hormonal treatments have side effects that result in up to 60% of women seeking treatment with fertility-ending surgical procedures (RCOG, 2014). Consequently, there continues to be an unmet need for acceptable, effective, fertility-preserving medical treatments for HMB, and only delineation of endometrial physiology (Maybin and Critchley, 2015) and the various aberrations that contribute to AUB in the reproductive years will enable future development of personalized medical therapies for affected women.

One of the issues facing clinicians, students, and both basic and clinical investigators has been the lack of a clearly defined set of criteria for describing both normal and abnormal uterine bleeding (AUB) in nonpregnant women of reproductive age. Over the past 100 years or so, >20 descriptions have been employed in clinical practice to describe abnormally heavy and frequent uterine bleeding (Woolcock et al., 2008). These terminologies include, but are not limited to: Menorrhagia; hypermenorrhea; dysfunctional uterine bleeding (DUB); metrorrhagia; meno-metrorrhagia; and polymenorrhea. The word “menorrhagia” (to “burst forth each month”), was initially used in English medical texts by William Cullen, a Professor at the University of Edinburgh in the late 1700s (Woolcock et al., 2008), but the term was never precisely defined.

The term “Dysfunctional uterine bleeding” or DUB has traditionally been used to describe excessive bleeding (heavy, frequent or prolonged) of uterine origin and deemed not to be due to complications of pregnancy, or detectable pelvic pathology or systemic disease. Further, in some circles, “DUB” was subclassified as either ovulatory or anovulatory. This subclassification has implicated either true endometrial dysfunction or disturbance of the hypothalamic-pituitary-ovarian (HPO) axis and thus typically enabled a diagnosis of exclusion. However, as with the symptom of HMB, DUB has been subjected to often subtle but different definitions in the literature (Woolcock et al., 2008). Consequently, the term “DUB” has been interchangeably considered either a symptom, a sign and/or a diagnosis (Fraser et al., 2007a,b).

Hence great confusion has existed over terminologies relating to menstrual bleeding complaints with consequential impacts on patient care (Woolcock et al., 2008). These confusing older terminologies should ideally become obsolete over time.

Why Revised Terminologies and Clear Definitions Assist in Clinical Management, Counseling, and Sharing of Research Findings

The previous assembly of poor definitions surrounding the term AUB, use of confusing terminologies and lack of classification, led to a global initiative to (i) refine language used to describe uterine bleeding complaints, and (ii) to develop a logical classification system of the underlying causes. This process resulted in the development of two systems under the aegis of the Federation Internationale de Gynécologie et Obstétrique, (FIGO). One is a set of terminologies and definitions of normal and AUB in the reproductive years; the second, a classification of potential causes of AUB known by the acronym PALM-COEIN ([Munro et al., 2011](#)).

First was a distinction between chronic AUB in the reproductive years and acute presentations of uterine bleeding. Chronic abnormal uterine bleeding (AUB) was defined as “bleeding from the uterine corpus that is abnormal in frequency, regularity, duration and/or volume that has been present for the majority of the last 6 months” ([Munro et al., 2011](#)). Acute AUB is defined as, “an episode of bleeding in a woman of reproductive age, who is not pregnant, that is of sufficient quantity to require immediate intervention to prevent further blood loss” ([Munro et al., 2011](#)).

Implicit to the successful and effective use of these two FIGO systems is a co-ordinated approach to securing a comprehensive menstrual history, utilizing simple laboratory investigations and performing appropriate imaging techniques. As momentum gathers, this harmonized approach should facilitate clinical care and enhance phenotyping of patients. As a consequence, a more accurate clinical diagnosis and a resulting menu of optimal treatment approaches should be secured. This suggests that most researchers in the field will now be able to compare and contrast their own patient profiles more precisely with those of other researchers than previously ([Woolcock et al., 2008](#)).

Some remain confused about the relationship of the initialism of HMB to AUB. Indeed, HMB represents “excessive menstrual blood loss leading to interference with the physical, emotional, social, and material quality of life of a woman” ([NICE, 2007](#)). Hence, HMB is just one of a range of symptoms which are included under the “umbrella” of AUB symptoms. Reserved for a research setting is a quantitative definition of HMB of blood loss in excess of 80 mL per cycle ([Warner et al., 2004](#)) a volume of blood loss that carries the risk of iron deficiency and anemia because it exceeds the recommended daily iron intake ([Hallberg et al., 1966](#)).

Consequently, and critical to the accurate classification of causes of AUB in the reproductive years, is a detailed menstrual history designed to identify the features of the abnormal bleeding—frequency, regularity, duration, volume, and intermenstrual bleeding—that themselves provide clues to the potential contributors to the clinical problem. With this clinical foundation, appropriate laboratory and uterine imaging techniques are performed to classify the bleeding caused or contributed to by one or more of the PALM-COEIN categories.

Much has been achieved since the initial publication of System 1, the new definitions and descriptions for uterine bleeding in 2007 ([Fraser et al., 2007a,b](#)), and System 2, the PALM-COEIN classification in 2011 ([Munro et al., 2011](#)), to disseminate and share this initiative. FIGO has endorsed and encouraged use of these two systems—new terminologies and definitions ([Fraser et al., 2016](#); [Munro et al., 2011](#)) and the detailed classification of causes: PALM-COEIN ([Madhra et al., 2014](#); [Munro, 2017](#); [Munro et al., 2011](#)). Rational use of the two FIGO systems generally leads to a more focused and precise approach to active management of the presenting symptoms.

The challenge of standardizing assessment tools for measuring outcomes of AUB complaint and management is the current focus of a piece of work being undertaken through the CROWN Initiative (Core Outcomes in Women’s and Newborn Health) where the aim is to address the widespread variation in reporting of women’s health outcomes ([CROWN, 2016](#)).

The Two FIGO Systems

Context

The two FIGO systems that have defined nomenclature and symptoms of abnormal uterine bleeding (AUB) in the reproductive years (System 1), and the PALM-COEIN classification of causes of AUB (System 2), are designed with an objective to facilitate research, education, and provision of optimal clinical care for women with AUB ([Fraser et al., 2016](#); [Munro, 2017](#); [Munro et al., 2011](#)). The development of these two systems has been the output of a globally (six continents represented) collaborative effort involving experts in clinical, translational and bench research and supported by representation from medical societies, journals and regulatory bodies ([Fraser et al., 2016](#); [Munro, 2017](#); [Munro et al., 2011](#)).

Any abnormalities in volume of menstrual blood loss, frequency, regularity and duration were defined as outwith accepted 5th to 95th percentiles by the FIGO Menstrual Disorders Group ([Munro et al., 2011](#)). Further to these definitions of normality, underlying etiologies of AUB should be categorized in a structured manner and aided by the mnemonic PALM-COEIN ([Munro et al., 2011](#)). The acronym “PALM” embodies the structural causes, including polyp, adenomyosis, leiomyoma, and malignancy (and hyperplasia). The second acronym, “COEIN,” includes the nonstructural causes such as coagulopathies, ovulatory dysfunction, endometrial causes, and iatrogenic as well as the not otherwise classified category which includes etiologies such as Caesarean scar defects and vascular malformations.

System 1—Symptoms

Women with AUB in the reproductive years present with one or more of the symptoms defined in FIGO System 1 (Table 1). These include abnormalities in frequency, regularity, duration and volume. The symptom of HMB generally comprises some combination of heavy rate of flow, including significant “gushes” and large “clots” sometimes also lasting a prolonged period. Bleeding (or spotting) between regular menses is called intermenstrual bleeding; when random, a focal lesion is suggested, when cyclical, a hormonal etiology is inferred.

System 2—Classification of Potential Causes of AUB (PALM-COEIN)

The classification categories are designed to facilitate future development of subclassification systems. Importantly it is recognized that any woman may have one or a spectrum of entities that may cause or contribute to AUB (Fig. 1) (Fraser et al., 2016). It is also recognized that definable entities, such as polyps, adenomyosis, and leiomyomas (fibroids), are often asymptomatic and thus may not in fact contribute to the presenting symptom of AUB (Fraser et al., 2016; Munro, 2017; Munro et al., 2011).

The PALM-COEIN classification starts with polyps (AUB-P), which are categorized as either being present or absent (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). Presence of polyps may be ascertained by one or a combination of contrast hysterosonography, typified by the technique of saline infusion sonography (SIS) and hysteroscopic evaluation with or without polypectomy and associated histopathology (Fraser et al., 2016; Munro, 2017; Munro et al., 2011).

Adenomyosis (AUB-A): The criteria for diagnosing adenomyosis have previously relied upon histopathological evaluation of the depth of “endometrial” tissue beneath the endometrial-myometrial interface from the uterus following hysterectomy (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). Currently, imaging with MRI facilitates diagnosis of adenomyosis but this mode of imaging is not accessible to many clinicians globally. Thus, ultrasound criteria for adenomyosis comprise the minimum requirements for assigning an individual the diagnosis of AUB-A (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). A subclassification system for adenomyosis, applicable for AUB-A and other adenomyotic manifestations is currently under development.

Leiomyomas (AUB-L): The primary FIGO classification system reflects only the presence or absence of one or more leiomyomas (fibroids), independent of location, number, and size of leiomyoma (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). These features are ascertained by ultrasound examination in first instance and supported with MRI if available and indicated. In the secondary FIGO classification system, the clinician determines leiomyomas that involve the endometrial cavity, such as submucous (SM) from others (O), as SM lesions are most likely to contribute to AUB (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). This distinction should use either contrast hysterosonography or hysteroscopy to identify and characterize the type of leiomyoma. A tertiary system is discussed below.

Malignancy and premalignant conditions (AUB-M): Should endometrial sampling of women in the reproductive years with the complaint of AUB identify a hyperplastic or malignant process, then the individual is categorized as AUB-M (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). Further subclassification should employ the appropriate WHO or FIGO system gynecological cancer classification systems.

Coagulopathy (systemic disorders of hemostasis; AUB-C): The term coagulopathy embraces the spectrum of systemic disorders of hemostasis, for example, von Willebrand Disease. A coagulopathy may be identifiable in approximately 13% of women with HMB (Fraser et al., 2016; Kadir et al., 1998; Munro et al., 2011). Up to 90% of women with these abnormalities may be identified through taking a careful structured history (Kouides et al., 2005; Munro et al., 2011). Confirmation of the presence of a coagulopathy requires additional testing and includes, for example, measurements of von Willebrand and Ristocetin cofactor, along with other assays of coagulation function (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). It remains unclear how often such

Table 1 FIGO systems for nomenclature and classification of AUB

<i>Clinical dimensions of menstruation and menstrual cycle</i>	<i>Descriptive terms</i>	<i>Normal limits (5th to 95th percentiles)</i>
Frequency of menses (d)	Frequent	<24
	Normal	24–38
	Infrequent	>38
Regularity of menses, cycle-to-cycle	Absent	–
	Regular	Variation $\pm$ 2–20 days
	Irregular	Variation >20 days
Duration of flow (d)	Prolonged	>8.0
	Normal	4.5–8.0
	Shortened	<4.5
Volume of monthly blood loss (mL)	Heavy	>80
	Normal	5–80
	Light	<5

From Fraser, I.S., Critchley, H.O., Munro, M.G., Broder, M. (2007). *Human Reproduction* 22, 635–643; Fraser, I.S., Critchley, H.O., Munro, M.G., Broder, M. (2007). *Fertility and Sterility* 87, 466–476. With permission to reproduce.

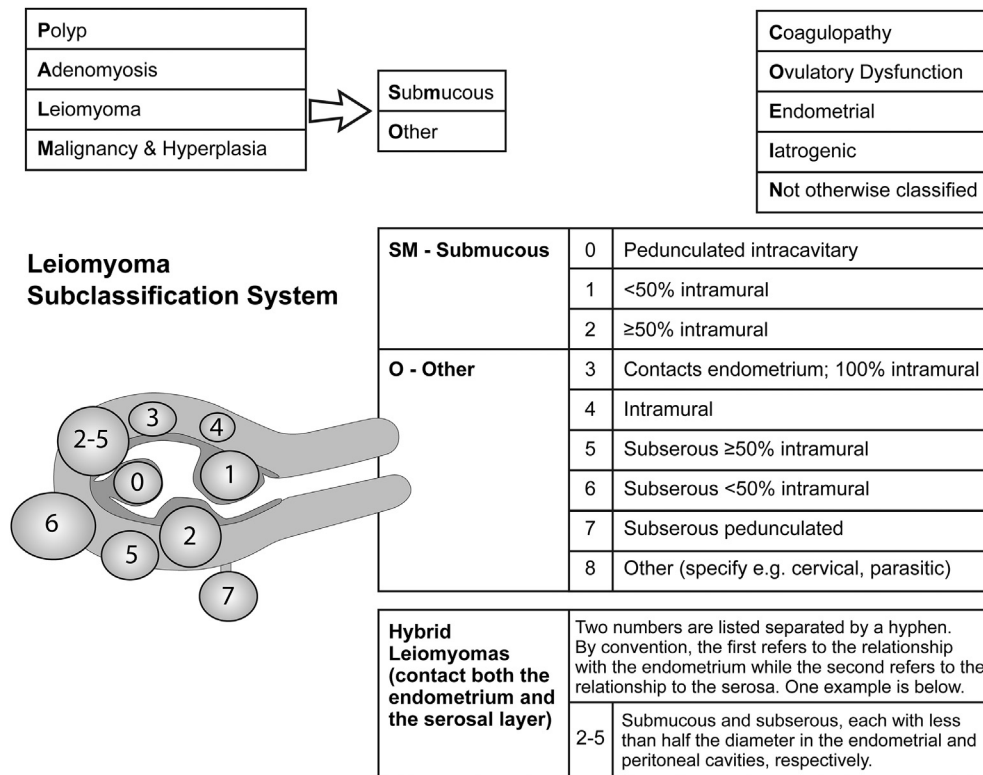


Fig. 1 The PALM-COEIN classification system, including leiomyoma subclassification. Adapted with permission from Munro M.G., Critchley H.O., Broder M.S., Fraser I.S. (2011). *International Journal of Gynecology and Obstetrics* **113**, 3–13.

abnormalities may cause or contribute to AUB, and how often they are asymptomatic or only minimally symptomatic biochemical abnormalities (Fraser et al., 2016; Munro, 2017; Munro et al., 2011).

Ovulatory disorders (AUB-O): Ovulatory dysfunction generally presents as a combination of unpredictable bleeding and variable menstrual blood flow, which is often very heavy (HMB; (Fraser et al., 2016; Munro, 2017; Munro et al., 2011)). Commonly, the woman is either not ovulating, or experiences infrequent ovulation. This pattern of ovulation is common during adolescence and in the latter reproductive years, as the menopause approaches. She may experience “luteal out-of-phase” (LOOP) events (Fraser et al., 2016; Hale et al., 2009; Munro et al., 2011). AUB-O is based on 5th to 95th percentiles of population data and the woman should report cycle lengths in the previous 6 months that vary by at least 20 days (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). It is recognized that such a wide range of normal cycle length reflects the fact that many women experience a short or long cycle in a given year, and that a more reflective range (shortest to longest) of 7–9 days more accurately represents the normal range.

Endometrial causes (AUB-E): If AUB is reported in the presence of predictable and cyclical menstruation (suggestive of normal ovulation) and whether the woman describes HMB or IMB, if other definable causes are absent, then the patient is classified as having AUB-E (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). The cause of AUB-E in a given patient is likely one or a combination of primary endometrial disorders. HMB may be associated with perturbation of mechanisms regulating local endometrial hemostasis (Critchley and Maybin, 2011; Fraser et al., 2016; Maybin and Critchley, 2015; Munro et al., 2011). Currently there are no specific tests available for specific diagnosis of a primary endometrial disorder. A diagnosis of AUB-E is assigned to women with normal ovulatory function and by exclusion of other identifiable abnormalities (such as SM leiomyomas) that are likely to contribute to the abnormal bleeding (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). At present, localized endometrial infections (e.g., chlamydia, TB) would also be included in this category.

Iatrogenic causes (AUB-I): Women are described as having AUB-I if unscheduled endometrial bleeding is reported with use of gonadal steroids (e.g., exogenous oestrogens, progestogens, androgens), or gonadal steroid-related therapies (e.g., GnRH agonists, aromatase inhibitors, SERMs, SPRMS) (Fraser et al., 2016; Munro et al., 2011; Percy et al., 2017; Shapley et al., 2004). AUB considered secondary to administration of anticoagulants, such as warfarin or heparin, or systemic therapies that contribute to disorders of ovulation, for example, medications that interfere with dopamine pathways are now included in this category (Fraser et al., 2016; Munro et al., 2011; Percy et al., 2017; Shapley et al., 2004).

AUB that is “not otherwise classified” (AUB-N) includes entities that may or may not contribute to or cause AUB (Fraser et al., 2016; Munro, 2017; Munro et al., 2011). Such entities, for example, include vascular malformations, the uterine isthmocoele, and other entities that are either uncommonly encountered or that are often poorly defined. Together these entities (or future entities) are positioned in this category AUB-N (Fraser et al., 2016; Munro, 2017; Munro et al., 2011).

It is essential that the FIGO terminology and classification publications (Munro et al., 2011) remain living documents that provide an interactive opportunity for international dialogue and debate. These documents will undergo regular (likely triennial) review during each triennial FIGO World Congress when there is the platform for global discussion concerning clinical utility and recommendations.

Subclassifications of Underlying Causes—Reality and Potential; (Improved Research and Management)

It is recognized that these broad classification categories may, and indeed should, be examined in detail, as there may exist ways to stratify the specific disorder in a way that further facilitates research or therapy. This hypothesis would suggest that

Example of PALM-COEIN Worksheet for Chronic AUB Evaluation

PALM-COEIN Category	Not Assessed	Absent	Present	Diagnostic Method	☒	Date	Comment
Polyps	☐	☐	☐	Vaginal Ultrasound			
				Vaginal Ultrasound with Contrast			
				Hysteroscopy			
				Hysterosalpingogram			
				MRI			
				Histopathology			
Adenomyosis	☐	☐	☐	Vaginal Ultrasound			
				Hysteroscopy			
				MRI			
				Histopathology			
Leiomyoma	☐	☐	☐	Vaginal Ultrasound			
				Vaginal Ultrasound with Contrast			
				Hysteroscopy			
				Hysterosalpingogram			
				Laparoscopy			
				MRI			
				Histopathology			
Malignancy & Hyperplasia	☐	☐	☐	Endometrial Biopsy			
Coagulopathy	☐	☐	☐	Laboratory			
Ovulatory Disorders	☐	☐	☐	History			
				Basal Body Temperature			
				Serum Progesterone			
				Other			
Endometrial Disorders	☐	☐	☐	History			
Iatrogenic	☐	☐	☐	History			
Not Otherwise Classified	☐	☐	☐	Vaginal Ultrasound			
				Vaginal Ultrasound with Contrast			
				Hysteroscopy			
				Hysterosalpingogram			
				Laparoscopy			
				MRI			
				Biopsy			

Fig. 2 Example of PALM-COEIN worksheet for chronic AUB evaluation. Used with permission from Malcolm G. Munro.

“subclassification” systems have utility in informing investigators, clinicians, educators, and patients regarding the potential role of a clinical symptom or diagnosis. Indeed, one such system exists, the FIGO subclassification system for leiomyomas, an approach that was based on a system for submucous myomas originally conceived by (Wamsteker et al., 1993) (Fig. 1). This system allows for the identification of submucous leiomyomas (Types 0, 1, 2, and 3), and distinguishes them from the “O” group (Types 4–8) not known to contribute to AUB, infertility or recurrent pregnancy loss.

Although the leiomyoma subclassification system is the only one currently supported by FIGO, it seems obvious that such an approach is needed for other entities that comprise System 2, the classification of causes of AUB in the reproductive years. For example, we don’t know the impact on uterine bleeding of single versus multiple polyps, or large versus small polyps. Adenomyosis may manifest in a spectrum of symptoms or findings, ranging from a small poorly defined region in the myometrium, to diffuse thickening of the junctional zone. Other possibilities exist as well—there may be different versions of ovulatory disorders and the endometrium may be the source of a spectrum of molecular or infective anomalies that result in symptoms of AUB. The design of a subclassification system for adenomyosis is currently under development; others should follow.

How These FIGO Systems Can Be Used to Assist in Teaching

Any current presentation on FIGO menstrual systems at an international conference raises a number of spontaneous comments and discussion about the effective use of the systems in a range of teaching forums. It seems clear that both systems provide a clear structure where terminologies and definitions assist focus on the likely causes of AUB, resulting in greater clarity in determining required investigations and improved prioritization from the range of potential therapies. This process is often assisted by the use of a standard stylized form in the format of a matrix, logically listing the key symptoms and causes (Fig. 2). The initial focus should be on clarity in defining symptoms, leading to simple categorization of the most likely underlying causes. Such a simple and clear structure did not exist prior to publication of the FIGO PALM-COEIN system (Munro et al., 2011).

Acknowledgments

We wish to thank Sheila Milne for assistance with manuscript preparation and Ronnie Grant for help with illustrations.

References

- Bitzer, J., Heikinheimo, O., Nelson, A. L., Calaf-Alsina, J., & Fraser, I. S. (2015). Medical management of heavy menstrual bleeding: a comprehensive review of the literature. *Obstetrical and Gynecological Survey*, 70, 115–130.
- Cardozo, E. R., Clark, A. D., Banks, N. K., et al. (2012). The estimated annual cost of uterine leiomyomata in the United States. *American Journal of Obstetrics and Gynecology*, 206(211), e211–219.
- Critchley, H. O., & Maybin, J. A. (2011). Molecular and cellular causes of abnormal uterine bleeding of endometrial origin. *Seminars in Reproductive Medicine*, 29, 400–409.
- CROWN. (2016). Initiative (Core outcomes in Women’s and newborn health). <http://www.crown-initiative.org/2016/>.
- Fraser, I. S., Critchley, H. O., Munro, M. G., & Broder, M. (2007a). Can we achieve international agreement on terminologies and definitions used to describe abnormalities of menstrual bleeding? *Human Reproduction*, 22, 635–643.
- Fraser, I. S., Critchley, H. O., Munro, M. G., & Broder, M. (2007b). A process designed to lead to international agreement on terminologies and definitions used to describe abnormalities of menstrual bleeding. *Fertility and Sterility*, 87, 466–476.
- Fraser, I. S., Munro, M. G. and Critchley, H. O. (2016). *Abnormal uterine bleeding in reproductive-age women: Terminology and PALM-COEIN etiology classification*. UpToDate [Online] Available from: https://www.uptodate.com/contents/abnormal-uterine-bleeding-in-reproductive-age-women-terminology-and-palm-coein-etiology-classification?source=search_result&search=abnormal%20uterine%20bleeding%20in%20reproductive-age%20women&selectedTitle=1~150.
- Frick, K. D., Clark, M. A., Steinwachs, D. M., et al. (2009). Financial and quality-of-life burden of dysfunctional uterine bleeding among women agreeing to obtain surgical treatment. *Women’s Health Issues*, 19, 70–78.
- Hale, G. E., Hughes, C. L., Burger, H. G., Robertson, D. M., & Fraser, I. S. (2009). Atypical estradiol secretion and ovulation patterns caused by luteal out-of-phase (LOOP) events underlying irregular ovulatory menstrual cycles in the menopausal transition. *Menopause*, 16, 50–59.
- Hallberg, L., Hogdahl, A. M., Nilsson, L., & Rybo, G. (1966). Menstrual blood loss—a population study. Variation at different ages and attempts to define normality. *Acta Obstetrica et Gynecologica Scandinavica*, 45, 320–351.
- Kadir, R. A., Economides, D. L., Sabin, C. A., Owens, D., & Lee, C. A. (1998). Frequency of inherited bleeding disorders in women with menorrhagia. *Lancet*, 351, 485–489.
- Kouides, P. A., Conard, J., Peyvandi, F., Lukes, A., & Kadir, R. (2005). Hemostasis and menstruation: appropriate investigation for underlying disorders of hemostasis in women with excessive menstrual bleeding. *Fertility and Sterility*, 84, 1345–1351.
- Madhra, M., Fraser, I. S., Munro, M. G., & Critchley, H. O. (2014). Abnormal uterine bleeding: advantages of formal classification to patients, clinicians and researchers. *Acta Obstetrica et Gynecologica Scandinavica*, 93, 619–625.
- Maybin, J. A., & Critchley, H. O. (2015). Menstrual physiology: implications for endometrial pathology and beyond. *Human Reproduction Update*, 21, 748–761.
- Munro, M. G. (2017). Practical aspects of the two FIGO systems for management of abnormal uterine bleeding in the reproductive years. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 40, 3–22.
- Munro, M. G., Critchley, H. O., Broder, M. S., & Fraser, I. S. (2011). FIGO classification system (PALM-COEIN) for causes of abnormal uterine bleeding in nongravid women of reproductive age. *International Journal of Gynaecology and Obstetrics*, 113, 3–13.
- NICE. (2007). *Clinical Guideline 44, Heavy menstrual bleeding*. National Institute for Health and Clinical Excellence (NICE).
- Percy, L., Mansour, D., & Fraser, I. (2017). Iron deficiency and iron deficiency anaemia in women. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 40, 55–67.
- Prentice, A. (1999). Health care implications of dysfunctional uterine bleeding. *Baillière’s Best Practice & Research. Clinical Obstetrics & Gynaecology*, 13, 181–188.
- RCOG. (2014). *National Heavy Menstrual Bleeding Audit - Final Report*. London: RCOG. Available from: https://www.rcog.org.uk/globalassets/documents/guidelines/research-audit/national_hmb_audit_final_report_july_2014.pdf.

- Shapley, M., Jordan, K., & Croft, P. R. (2004). An epidemiological survey of symptoms of menstrual loss in the community. *British Journal of General Practice*, 54, 359–363.
- Wamsteker, K., Emanuel, M. H., & de Kruif, J. H. (1993). Transcervical hysteroscopic resection of submucous fibroids for abnormal uterine bleeding: results regarding the degree of intramural extension. *Obstetrics and Gynecology*, 82, 736–740.
- Warner, P. E., Critchley, H. O., Lumsden, M. A., et al. (2004). Menorrhagia II: is the 80-mL blood loss criterion useful in management of complaint of menorrhagia? *American Journal of Obstetrics and Gynecology*, 190, 1224–1229.
- Woolcock, J. G., Critchley, H. O., Munro, M. G., Broder, M. S., & Fraser, I. S. (2008). Review of the confusion in current and historical terminology and definitions for disturbances of menstrual bleeding. *Fertility and Sterility*, 90, 2269–2280.

Leiomyomas

Hoda ElKafas*, Augusta University, Augusta, GA, United States; and National Organization for Drug Control and Research (NODCAR), Giza, Egypt

Mohamed Ali*, Augusta University, Augusta, GA, United States; and Ain Shams University, Cairo, Egypt

Ayman Al-Hendy, Augusta University, Augusta, GA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Uterine fibroids (UFs) are the most common benign tumor which threaten 70%–80% of women during their reproductive age. They are three times more prevalent among African American women than in Caucasian women, with more aggressive and earlier symptoms. The incidence of UFs is influenced by many factors including age, race, body mass index, and family history. In the United States, the annual economic burden of these tumors is estimated to be 34.4 billion dollars ([Cardozo et al., 2012](#)).

Uterine fibroid has many scientific alternative names such as leiomyoma, leiomyomata, myoma or even descriptive one like fireballs. They appear on the muscular wall of the uterus (myometrium layer) with firm spherical growth of different size ranges, from apple seeds to basketball size. Sometimes they can fill the whole uterus cavity and look like pregnancy ([Sue and Sarah, 2009](#)).

Uterine fibroid tissues are nourished and sustained by the uterine arteries and estrogen hormone effect respectively, which explains why fibroids shrink after menopause ([Borahay et al., 2015](#)).

Fibroids may impair fertility through altering the structure of the uterus, making it unsmooth and curvy with subsequent bleeding ([Pritts et al., 2009](#)).

Types of Uterine Fibroids

Uterine fibroids can be classified based on their location to:

1. *Submucosal*: where tumor is protruding through the uterine cavity. Thus, changing the uterus' capability to regulate the menstrual bleeding and leads to heavy and prolonged periods. Conversely, they typically don't cause pain or extra bleeding in between periods. They can also lead to infertility or miscarriages early in a pregnancy.
2. *Intramural*: Tumor is located within the myometrium and typically doesn't lead to heavy bleeding. However, they can grow to the point that they can lead to chronic pressure with subsequent bulk symptoms as frequent urination, difficulty with bowel function, lower back pain, general aching in the pelvis, and pain during sex.
3. *Subserosal*: where tumor is in the outer border of the myometrium.
4. Pedunculated fibroids where the tumor is attached to the outer border of the uterus by a narrow pedicle containing blood vessels ([Pritts et al., 2009](#)) ([Fig. 1](#)).

Why Women Get Fibroids?

There is no single cause of fibroids, yet there are many internal and external factors that can lead to local hormonal imbalance that creates a growth favoring environment ([Fig. 2](#)).

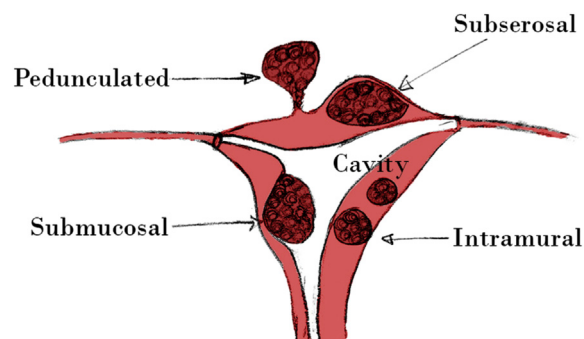


Fig. 1 Types of uterine fibroids.

*These authors contributed equally to this work.

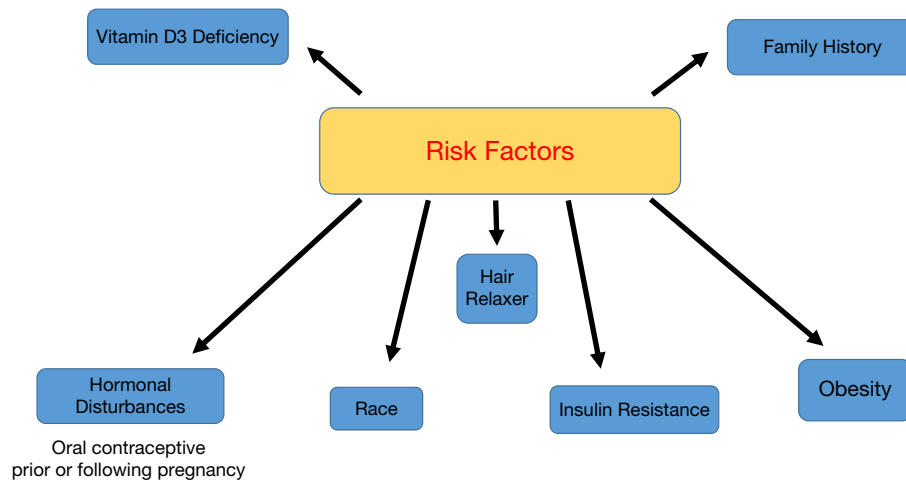


Fig. 2 Risk factors for uterine fibroids.

Hormonal Effects

Fibroid growth is hormonal dependent (both estrogen and progesterone), so women in conditions of high estrogen level exposure are more susceptible to fibroid development; such as in early pregnancy or perimenopause and also hormonal contraceptive use (Borahay et al., 2015).

Genetic Factor

Family history can be a risk factor, daughters of women with UFs history are about three times more likely to develop UFs than the average (Okolo, 2008).

Ethnicity

As previously mentioned, African American women are three to nine times more likely to have UFs than Caucasian women, furthermore, they appear earlier, larger and greater in number (Huyck et al., 2008).

Vitamin D Deficiency

African Americans in particular are at higher risk for vitamin D deficiency (10 times than Caucasians). Black pigmentation in African Americans reduces the absorption of ultraviolet rays from the sun which is crucial for vitamin D biosynthesis. Moreover, decreased milk consumption due to their lactose intolerance diminishes serum levels of vitamin D. Of note, vitamin D may be obtained from dietary sources such as fatty fish, fish oils, fortified foods, and vitamin supplements; but, the main source of vitamin D is still sunlight exposure (Ross et al., 2011). There are factors that affect cutaneous absorption of vitamin D such as the use of sunblock, levels of sunlight exposure (e.g., season, latitude, and time of day), and most importantly skin pigmentation (Brakta et al., 2015).

Obesity

Many studies reported the association between obesity and increased fibroid risk; an association that may be mediated by elevated bioavailable estrogen and/or testosterone associated with obesity (Ciebiera et al., 2016).

Insulin Resistance

Hyperinsulinemia induced by insulin resistance might have a role in the development of uterine fibroids probably by enhancing ovarian hormone secretion or directly promoting myometrial smooth muscle cell proliferation (Wise et al., 2005).

Perimenopause

Women who approach menopause are at the greatest risk for development of uterine fibroids due to exposure to high levels of estrogen (Machado et al., 2013).

Hair Relaxer

Women who used hair relaxers were 1.17 times more likely to have uterine fibroids than those who did not. Uterine fibroids were theorized that endocrine-disrupting chemicals in hair relaxers could enhance a woman's threat to develop uterine fibroids. Hair

Symptoms of uterine fibroids
Painful mass
Heavy bleeding
Low incidence of pregnancy
Severe back pain
Pain during intercourse
Anemia

Fig. 3 Symptoms of uterine fibroids.

relaxer's have endocrine-disrupting phthalates, which often appear on a label as "fragrance" or "perfume." All types of relaxers are known to cause burns and injuries on the scalp, and such injury eases the entry of the chemicals into the body. Phthalates, a group of hormonally-active compounds, can be absorbed through the skin or via inhalation. Phthalates have an estrogenic effect on cells which promote estrogen-dependent fibroid tumors (Wise et al., 2012).

Symptoms of Uterine Fibroids

Generally fibroids do not cause symptoms to all women. Only one in four women with fibroids are symptomatic. Many women ignore symptoms and their lives are miserable. The symptoms depend on the location and size of the fibroid (Pritts et al., 2009) and include (Fig. 3):

1. Heavy painful prolonged menstrual bleeding or even bleeding between the periods which lead to iron deficiency anemia.
2. Firm painful mass located near the middle of the pelvis, which gives a full feeling in the lower abdomen.
3. Severe back pain.
4. Bowel and bladder dysfunction.
5. Reproductive problems such as infertility, multiple miscarriage, or early labor.
6. Pain during intercourse.

Treatment of Uterine Fibroids

The management of uterine fibroids differs significantly based on the woman's age, symptoms, preferences, and her reproductive plans (Doherty et al., 2014). The main goal of medical management is to improve the patient's quality of life by reducing UFs symptoms with minimum adverse effects. Pharmacological treatment may be chosen over a surgical method, especially in patients who would like to avoid risks inherent to surgery or who desire their uterine preservation for future pregnancy or even feminine reasons. However, the side effects accompanied by prolonged treatment with some medications limit the duration of their use. Moreover, a rebound increase in the size of the leiomyomas can be observed after discontinuing some hormonal medical therapy (Doherty et al., 2014) (Fig. 4).

Treatment of Uterine fibroids	
Surgical	I. Hysterectomy II. Myomectomy
Radiological	I. Uterine Artery Embolization II. Magnetic resonance guided focused ultrasound
Pharmacological	I. GnRH agonists II. SPRMs III. Vitamin D supplement IV. Green tea Extract

Fig. 4 Treatment of uterine fibroids.

Pharmacological Treatments

Non-hormonal treatment

COMT inhibitor

Catechol-O-methyl transferase (COMT) is an enzyme that catalyzes local conversion of hydroxylated estrogen metabolites which has antiestrogenic effect to the more estrogenic methylated forms. Fibroids are known to have high levels of COMT and thus are able to promote the growth stimulating effect of estradiol. COMT inhibitor will result in increase 2-hydroxyestradiol level which might help in tumor shrinkage ([Hassan et al., 2011](#)).

Green tea extract

Green tea is a natural product, commonly used by women for multiple purposes. Epigallocatechin gallate (EGCG) is one of the green tea components with an antioxidant, antiinflammatory and antiproliferative effects so might decrease fibroid symptoms ([Doherty et al., 2014](#)).

Vitamin D₃

Studies have demonstrated a lower mean 25-hydroxyvitamin D [25(OH) D₃] concentration in African American females than in Caucasian females aged 20–40 years. Vitamin D is a potent antitumor agent that inhibits leiomyoma cell proliferation and decreases the size of uterine leiomyomas both in vitro and in vivo ([Brakta et al., 2015](#)).

Hormonal medication

Levonorgestrel intrauterine system

The levonorgestrel intrauterine system (LNG-IUS) for example, Mirena[®] has been widely accepted as an effective treatment of heavy menstrual bleeding by reducing menstrual blood loss, increasing hemoglobin, and relieving symptoms by promoting endometrial atrophy ([Machado et al., 2013](#)).

Gonadotropin-releasing hormone analogs (GnRH)

GnRH analogs were approved by the Food and Drug Administration for preoperative controlling of uterine fibroids. GnRH analog treatment induces a menopausal state with low estrogen levels that may result in intolerable postmenopausal like side effects and bone loss. These side effects could be minimized by adding low dose estrogen and progestin. GnRH analog treatment is therefore limited to a maximum of 6 months ([Doherty et al., 2014](#)). GnRH antagonist as cetrorelix was proposed as treatment for uterine fibroid with the advantage of bypassing the initial flare effect accompanied with the agonists, but being expensive with daily dosage requirement limits its use. Recently, Oral GnRH antagonists as Elagolix and Relugolix are being investigated now with promising results.

Selective progesterone receptor modulators

It has been established that progesterone, acting through its progesterone receptors, enhances the proliferative activity of UFs. New compounds that modulate progesterone receptor activity, collectively termed selective progesterone-receptor modulators (SPRMs), could be useful in the treatment of fibroids. Several SPRMs have been investigated including Telapristone, Asoprisnil and most importantly Ulipristal acetate (UPA). The latter one showed promising effects such as rapid amenorrhea induction, shrinkage of tumor, and anemia improvement, currently UPA can be used for both short term preoperative treatment, with superior efficacy as compared to GnRH agonist leuprolide acetate, or even for intermittent long term therapy up to 1 year ([Donnez et al., 2016](#)).

Selective estrogen-receptor modulators

Estrogens are known to stimulate fibroid growth. Antiestrogens, like tamoxifen or raloxifene that block estrogen action, had the prospects for therapeutic activity against fibroids but clinical trials weren't promising and now not considered as possible anti-UF medications ([Borahay et al., 2015](#)).

Aromatase inhibitors (AIs)

AIs are antiestrogens members that block the synthesis of estrogen by blocking the conversion of androgens to estrogen. Two types of AIs are available:

- A. Irreversible steroidal inhibitors, for example, Exemestane, which forms a permanent bond with the aromatase enzyme complex
- B. Nonsteroidal inhibitors, for example, anastrozole and letrozole, which inhibit the enzyme by reversible competition.

In premenopausal women, AIs have been reported to be well tolerated and reduce the symptoms and size of uterine fibroids. General side effects are hypoestrogenic symptoms, such as hot flashes and osteoporosis (bone mineral density loss) ([Doherty et al., 2014](#)).

Non-Surgical Interventions Treatment

Uterine artery embolization (UAE)

UAE is a minimally invasive treatment for uterine fibroids. UAE is a technique where an interventional radiologist block off the arteries that feed the fibroids and results in fibroid shrinkage. UAE presents some advantages over surgical procedures for the

treatment of uterine fibroids such as a shorter hospital stay, lower risk of major complications, and shorter recovery time, yet possibility of fibroid recurrence is still a drawback (Gupta et al., 2014).

Surgical Treatments of Fibroids

Surgical management of uterine fibroid may be required in women with severe pressure symptoms or unresponsiveness to other therapies (medical, UAE) (Stewart et al., 2016). The size and location of the tumor besides the female desire for future fertility both have roles in the choice of the surgical method. Surgical treatment can be either hysterectomy or myomectomy.

Myomectomy

Myomectomy is the surgical removal of tumor from the uterus. Myomectomy therapy has been well established and confirmed to be safe and effective in the uterine-conserving treatment of uterine fibroids. Myomectomy is an alternative to hysterectomy for women who wish to retain their uterus. However, there is a higher risk of bleeding and longer operative time than with hysterectomy and might affect future fertility as well (Jayakrishnan et al., 2013). Fibroids have a 15% recurrence rate especially for patients with multiple UFs and those in the third decade of life. Ten percent of women undergoing a myomectomy will ultimately need hysterectomy within 5–10 years (Radosa et al., 2014).

Hysterectomy

In women who are satisfied with their childbearing, hysterectomy is the choice of a permanent solution for symptomatic fibroids. Hysterectomy is the surgical removal of the uterus, so it remains the definitive surgical intervention for uterine fibroids (Stewart et al., 2016).

Bright Future for Uterine Fibroid Treatments

Many kinds of research and clinical studies are currently being conducted to find more cost effective and efficacious pharmacological alternatives for the treatment of uterine fibroids.

References

- Borahay, M. A., Al-Hendy, A., Kilic, G. S., & Boehning, D. (2015). Signaling pathways in leiomyoma: Understanding pathobiology and implications for therapy. *Molecular Medicine*, 21, 242–256.
- Brakta, S., Diamond, J. S., Al-Hendy, A., Diamond, M. P., & Halder, S. K. (2015). Role of vitamin D in uterine fibroid biology. *Fertility and Sterility*, 104, 698–706.
- Cardozo, E. R., Clark, A. D., Banks, N. K., et al. (2012). The estimated annual cost of uterine leiomyomata in the United States. *American Journal of Obstetrics and Gynecology*, 206(211), e1–9.
- Ciebiara, M., Wlodarczyk, M., Slabuzewska-Jozwiak, A., Nowicka, G., & Jakiel, G. (2016). Influence of vitamin D and transforming growth factor beta3 serum concentrations, obesity, and family history on the risk for uterine fibroids. *Fertility and Sterility*, 106, 1787–1792.
- Doherty, L., Mutlu, L., Sinclair, D., & Taylor, H. (2014). Uterine fibroids: Clinical manifestations and contemporary management. *Reproductive Sciences*, 21, 1067–1092.
- Donnez, J., Donnez, O., Courtot, G. E., & Dolmans, M. M. (2016). The place of selective progesterone receptor modulators in myoma therapy. *Minerva Ginecologica*, 68, 313–320.
- Gupta, J. K., Sinha, A., Lumsden, M., & Hickey, M. (2014). Uterine artery embolization for symptomatic uterine fibroids. *The Cochrane Library*.
- Hassan, M. H., Fouad, H., Bahashwan, S., & Al-Hendy, A. (2011). Towards nonsurgical therapy for uterine fibroids: Catechol-O-methyl transferase inhibitor shrinks uterine fibroid lesions in the Eker rat model. *Human Reproduction*, 26, 3008–3018.
- Huyck, K. L., Panhuysen, C. I., Cuenco, K. T., et al. (2008). The impact of race as a risk factor for symptom severity and age at diagnosis of uterine leiomyomata among affected sisters. *American Journal of Obstetrics and Gynecology*, 198(168), e1–9.
- Jayakrishnan, K., Menon, V., & Nambiar, D. (2013). Submucous fibroids and infertility: Effect of hysteroscopic myomectomy and factors influencing outcome. *Journal of Human Reproductive Sciences*, 6, 35–39.
- Machado, R. B., De Souza, I. M., Beltrame, A., et al. (2013). The levonorgestrel-releasing intrauterine system: Its effect on the number of hysterectomies performed in perimenopausal women with uterine fibroids. *Gynecological Endocrinology*, 29(5), 492.
- Okolo, S. (2008). Incidence, aetiology and epidemiology of uterine fibroids. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 22, 571–588.
- Pritts, E. A., Parker, W. H., & Olive, D. L. (2009). Fibroids and infertility: An updated systematic review of the evidence. *Fertility and Sterility*, 91, 1215–1223.
- Radosa, M. P., Owsianowski, Z., Mothes, A., et al. (2014). Long-term risk of fibroid recurrence after laparoscopic myomectomy. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 180, 35–39.
- Ross, A. C., Taylor, C. L., Yaktine, A. L., & DEL Valle, H. B. (2011). *Institute of Medicine (US) committee to review dietary reference intakes for vitamin D and calcium. Dietary reference intakes for calcium and vitamin D*. Washington, DC: National Academies Press.
- Stewart, E. A., Laughlin-Tommaso, S. K., Catherino, W. H., et al. (2016). Uterine fibroids. *Nature Reviews Disease Primers*, 2, 16043.
- Sue, W., & Sarah, S. B. (2009). Radiological appearances of uterine fibroids. *Indian Journal of Radiology and Imaging*, 19, 222–231.
- Wise, L. A., Palmer, J. R., Spiegelman, D., et al. (2005). Influence of body size and body fat distribution on risk of uterine leiomyomata in U.S. black women. *Epidemiology*, 16, 346–354.
- Wise, L. A., Palmer, J. R., Reich, D., Cozier, Y. C., & Rosenberg, L. (2012). Hair relaxer use and risk of uterine leiomyomata in African-American women. *American Journal of Epidemiology*, 175, 432–440.

Adenomyosis

Aaron Budden and Jason Abbott, School of Women's and Children's Health UNSW, Sydney, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Extirpative Complete removal or eradication of an organ or tissue.

Multiparous Two or more pregnancies resulting in birth by any means, where the child is >400 g or >20 weeks gestation.

Parity The number of children delivered by a woman by any means where the child is >400 g or >20 weeks gestation.

Adenomyosis is a structural uterine pathology specifically denoted in the FIGO PALM-COIEN classification system for abnormal uterine bleeding (AUB-A). The principle symptoms of the pathology are pelvic pain and heavy menstrual bleeding (HMB) and confirmation of adenomyosis requires histological diagnosis. The prevalence of adenomyosis either in the general community or a subgroup of women with these symptoms has therefore been difficult to accurately assess. As accuracy and availability of medical imaging modalities including transvaginal ultrasound (TVUS) and magnetic resonance imaging (MRI) have become more widely available, there has been an increase in image-based diagnosis with high correlation to histological disease when performed by experts. The debate regarding the accuracy of these imaging techniques, which modality to use, and the classification of disease continues to be important factors in this increasingly recognized uterine pathology.

Epidemiology and Etiology

Diagnosis of adenomyosis may only be truly made after invasive extirpative surgery and complete sectioning, or a positive myometrial biopsy with histological demonstration of endometrium or endometrium-like structures within the myometrium and associated smooth muscle hypertrophy or hyperplasia. Given the difficulty in noninvasive diagnosis, the prevalence of adenomyosis is variably reported from as few as only a few percent of women through to 70%, although many studies suggest a 20%–35% prevalence (Vercellini et al., 1995) when post-hysterectomy specimens are examined histologically.

Increasing parity has been commonly associated with an increased risk of adenomyosis and multiparous women who undergo caesarean delivery are reported to be up to five times more likely to develop adenomyosis, although this association is variably reported within these studies (Vercellini et al., 1995). It is hypothesized that breach of the endometrial junction leads to glandular elements growing within the myometrium, which accounts for the increased association with multiparity as well as women who have received curettage at uterine surgery, termination of pregnancy, and caesarean delivery (Parazzini et al., 1997), although these factors are again variably reported.

Endometriosis is commonly noted in the presence of adenomyosis both during imaging and at the time of surgery. As there are similar pathogenic pathways for both diseases, it has been hypothesized that these diseases may occur on a continuum, although current data to support this is lacking. Other risk factors identified include advancing age and tamoxifen use whilst smoking may be protective (Parazzini et al., 1997).

There are a number of pathophysiological theories thought to contribute to adenomyosis, however as for endometriosis, there are likely multiple factors involved including:

1. Infolding of the endometrium with direct penetration into the myometrium
2. Damage to the endometrial–myometrial interface by pregnancy, myometrial incision, or development of leiomyomas
3. Increasing age with cellular damage and repair and
4. Vascular spread

Recent cell biology studies have demonstrated a genetic role in developing adenomyosis. From a well conducted case-control study, down regulation of DNA methyltransferase 3 alpha (DNMT3A) was reported to be significantly decreased in women with adenomyosis compared to women with eutopic endometrium (Zou et al., 2017) and may be responsible for ectopic endometrial cell proliferation and invasion. From separate work, researchers investigated RhoA and ROCK-I messenger RNA and protein expression, which is responsible for various cellular functions including cell migration and proliferation and normally has cyclical expression in concert with menstrual cycles. In women with adenomyosis, the authors reported higher levels of RhoA and ROCK-I messenger RNA and protein expression throughout the menstrual cycle with loss of normal upregulation and downregulation of protein expression (Wang et al., 2016).

Whilst there are common threads in the aetiological theories of adenomyosis with endometriosis and other benign diseases of the reproductive tract there, the complex epigenetic, hormonal, and reproductive factors are not yet fully defined and continues to be an area of research and progress in this field.

Diagnosis

Whilst histology is considered the ultimate manner by which the diagnosis of adenomyosis may be made, even the variations in the pathological definitions thwart a clear determination of what is 'disease'. The presence of glandular and stromal tissue within the myometrium is generally agreed upon, however, the depth at which this occurs from eutopic endometrium in the absence of a basement membrane is more problematic. Specific pathological problems include angle of specimen sectioning, specimen preparation, and the natural irregularity of the endometrial–myometrial border. In addition, the requirement for endometrium to be connected directly to the basalis is fiercely debated since isolated cystic lesions deep in the myometrium, separate to the endometrium, are well recorded. These difficulties have led to a number of reported definitions which are based on boundary disruption, depth of penetration, and distribution within the uterus.

It should be clear from the aforementioned problems, that these histological definitions require the uterus to be removed—a fact that is not acceptable for women who desire current or future fertility. Less invasive methods assessing histology through specific biopsy tools are reported however they each have potential specimen collection and diagnostic issues. For example, given that a biopsy may only sample part of the uterine muscle, this is problematic for accurate histological diagnosis. A small prospective study compared TVUS alone versus TVUS guided needle biopsy prior to performing a hysterectomy and reported adenomyosis in 29/102 (28%) of those women (Vercellini et al., 1998). The sensitivity for TVUS was 83% and just 45% for biopsy with a specificity of 67% and 96% respectively. In a large cross-sectional study involving 292 women examining the use of hysteroscopic biopsy (Dakhly et al., 2016), diagnosis by inspection of the endometrial cavity had a sensitivity of 41% and the endomyometrial biopsy had a sensitivity of 54% compared to the TVUS with a sensitivity of 84%. Specificity of these instruments was 47%, 54%, and 60% respectively.

The diagnostic capabilities of magnetic resonance imaging (MRI) have evolved substantially and are now able to differentiate three distinct layers of the functional zone between the endometrium and myometrium. Thickening of the junctional zone to > 12 mm is a reported surrogate diagnostic marker of adenomyosis on MRI or 3D ultrasound, however the normal physiological change that occurs in the zone with the menstrual cycle has limited its interpretation and variations in both this thickness and this finding in isolation as truly representative of disease continued to be debated.

Ultrasound and MRI

With an increasing desire for uterine retention and improvement in imaging techniques, noninvasive diagnosis of adenomyosis has received greater attention and study. The Morphological Uterus Sonographic Assessment (MUSA) Group reported a consensus statement describing the terms, definitions and measurements that may be used in the sonographic description of uterine diseases including adenomyosis (Van den Bosch et al., 2015). The authors state that ultrasound of the myometrium may be performed using a transabdominal (TAS) or transvaginal (TVS) approach though the resolution of TVS is preferred as it allows for detailed assessment of the myometrium. When assessing the myometrium, the authors recommend measuring the uterine corpus separate to the entire length of the uterus (corpus and cervix) and the fundal length (fundal serosal surface to the fundal tip of the endometrial cavity) and endometrial cavity (fundal tip of the endometrial cavity to the internal os) should also be measured separately. Serosal contour should be reported as either regular or lobulated, the myometrial walls reported as symmetrical or asymmetrical, and the overall echogenicity as homogenous or heterogeneous. If a lesion is visualized it should be described as well-defined or ill-defined and its location specific as well as the ratio of the thickness of the lesion to the uterine wall thickness measured on the same image. Finally the presence or absence of cysts should be reported and where present the number, maximum diameter, and appearance of cyst fluid included.

When evaluating the use of imaging techniques in the diagnosis of adenomyosis, the changes in imaging technology and techniques must be considered. Older studies reported poor diagnostic accuracy of adenomyosis at sonography however with changes in 2D transvaginal ultrasound technology, diagnostic accuracy has improved and more recent studies conducted using expert sonologists and well-defined criteria report very high accuracy (Luciano et al., 2013; Exacoustos et al., 2011). In a prospective study involving 54 symptomatic women scheduled for hysterectomy, 3D ultrasound mapping was undertaken prior to taking full thickness serosa to endometrium biopsies from areas suspected of adenomyosis (Luciano et al., 2013). This resulted in a sensitivity of 92% and a specificity of 83% when compared to histology after hysterectomy. Importantly from this study, women who had previous endometrial ablation or were receiving medical treatment had substantial reductions in diagnostic accuracy reflecting the changes in the endometrial myometrial interface that may occur in these circumstances.

In another small prospective evaluation of women, 2D and 3D TVUS was performed prior to hysterectomy and histological diagnosis (Exacoustos et al., 2011). The presence of myometrial cysts was the most specific 2D TVUS feature and a heterogeneous myometrium was the most sensitive. For 2D TVUS and 3D TVUS, respectively, the sensitivity was 75% and 91%, the specificity was 90% and 88%. It must be recognized that these data occurred in studies with strict methodology but it demonstrates what the best possible accuracy is in the noninvasive diagnosis of adenomyosis.

The increased access to MRI and its relatively less dependence on operator experience has led to considerable investigation in its diagnostic capabilities of adenomyosis. MRI has been compared to ultrasound in a number of studies, again with changes in the diagnostic accuracy over time of both modalities. From a meta-analysis published in 2010, 2312 women from 23 studies examining ultrasound and MRI concluded that both techniques have a high accuracy for the noninvasive diagnosis of

adenomyosis, although MRI was determined to more often result in the correct diagnosis ([Champaneria et al., 2010](#)). The pooled sensitivity of ultrasound from this assessment was 72% and specificity 81% compared to MRI with a sensitivity of 77% and specificity of 89%.

With improvement in imaging technology and more reliable interpretation of findings from images, data support a high accuracy for the noninvasive diagnosis of adenomyosis with either ultrasonography or MRI. Whilst MRI may be more accessible than previously, it continues to be both more expensive and less available than transvaginal ultrasound and this may be driving factors in the choice of imaging for women with suspected adenomyosis.

Symptoms

A woman's clinical presentation is the usual starting point for a differential diagnosis list and therefore remains an essential part of the clinical process. However, the reliance of the clinical symptoms usually associated with the presentation of suspected adenomyosis is neither sensitive nor specific. Broadly speaking, abnormal uterine bleeding (AUB), pelvic pain (dysmenorrhoea and noncyclical pelvic pain), and infertility are three main symptoms that women with adenomyosis may present with to their care provider.

Heavy menstrual bleeding (HMB) does not appear to be highly predictive for all forms of adenomyosis, although increasing severity of disease may increase the likelihood of this symptom. Likewise, dysmenorrhoea and other noncyclical pelvic pain present in a variety of gynecological conditions and there is no evidence about pain quality or site that differentiates it from other conditions such as endometriosis or leiomyomas.

Adenomyosis is increasingly being diagnosed during investigations of the subfertile or infertile couple. MRI studies in women with adenomyosis have demonstrated uterotubal disturbance and substantial dysperistalsis when diffuse forms of the disease occur. Increased symptoms such as dysmenorrhoea may represent worsening of these disorganized contractions of the reproductive musculature and may contribute to poorer sperm transportation for spontaneous pregnancy and increased implantation failure for advanced reproductive technology (ART) cycles ([Vercellini et al., 2014](#)).

Biomarkers

Despite general advancement in the field of cellular biology and the use of specific biomarkers for diagnostics, studies of potential biomarkers for adenomyosis have been limited. Many of the biomarkers being studied are linked to etiology and are nonspecific, leading to issues in terms of differentiation from both gynecological disease and other systemic disease.

One of the more promising biomarkers for diagnosis of adenomyosis is MALDI-TOF-MS which has been associated with both adenomyosis and endometriosis but unfortunately does not differentiate between these diseases ([Long et al., 2013](#)). Another promising biomarker is Moesin, a protein overexpressed in adenomyosis and whilst it is still being developed there is a potential for determine the extent of disease within the myometrium ([Ohara et al., 2014](#)).

Given the current issues with utility of biomarkers for the diagnosis of adenomyosis, none are currently in use clinically and should only be examined in the framework of appropriate clinical research studies.

Classification

Most classification systems for adenomyosis to date have relied on histopathological assessment of the uterus with or without imaging criteria. In part due to an increased desire to diagnose women without performing extirpative surgery, there has been a call for a standardized classification system to aid in diagnosis and management. Previous reviews, studies and consensus meetings have proposed several systems to improve clinical and research descriptions although not as yet has been universally adopted ([Grimbizis et al., 2014](#)). One of the problems of these classification systems is the primary focus on histological findings, specifically relating to depth and/or severity of disease. One small study has reported a classification based on MRI appearance however of the 163 women included only 40 underwent hysterectomy for complete histological comparison ([Kishi et al., 2012](#)). The main classification systems are presented in [Table 1](#).

Treatment

Women suffering from AUB and dysmenorrhoea have commonly been managed with extirpative surgery with adenomyosis diagnosed retrospectively based on histopathological findings. As previously discussed, this method of treatment is not ideal to every woman and available management strategies will depend on specific symptoms, for example dysmenorrhoea or noncyclical pelvic pain, AUB or infertility as well as the desire for current or future fertility. As both the understanding of the disease and diagnosis has improved, the available treatment options has improved and includes medical treatments, surgical options, radiologic or sonographic interventions or combinations of these choices.

Table 1 Classification systems for adenomyosis

Depth	Severity	Other histopathology	MRI
Siegler and Camilien (1994) ^a Grade 1: inner 1/3rd Grade 2: 2/3rd of myometrium Grade 3: entire myometrium	Siegler and Camilien (1994) ^a <i>Mild:</i> 1–3 islets on low powered field (LPF) <i>Moderate:</i> 4–9 islets <i>Severe:</i> > 10 islets Hulka et al. (2002) <i>Mild:</i> microscopic foci present or only inner 1/3 involved <i>Focal:</i> focal adenomyosis <i>Severe</i>	Grimbizis et al. (2014) <i>Diffuse:</i> extensive disease with endometrial mucosa scattered throughout uterine musculature <i>Focal:</i> Hypertrophic and distorted endometrium within the myometrium <i>Polypoid:</i> circumscribed masses of glands and stroma that is predominantly smooth muscle <i>Other:</i> endocervical type and retroperitoneal forms	Kishi et al. (2012) <i>Type I:</i> adenomyosis affects only the inner layer of the myometrium and not other areas <i>Type II:</i> adenomyosis affects only the outer layer of the myometrium <i>Type III:</i> isolated adenomyomas without a relationship to other structural components <i>Type IV:</i> any other variation that does not satisfy these variations
Sammour et al. (2002) 1. < 25% 2. 25–50% 3. 51–75% 4. > 75% of myometrial thickness <i>Penetration ratio:</i> depth of penetration to myometrial thickness	Vercellini et al. (2006) ^b <i>Grade 1:</i> 1–3 islets at LPF <i>Grade 2:</i> 4–10 islets <i>Grade 3:</i> > 10 islets	Vercellini et al. (2006) ^b <i>Configuration of lesion:</i> 1. Focal 2. Nodular Diffuse	
Vercellini et al. (2006) ^b <i>Mild:</i> < 1/3 deep <i>Moderate:</i> 1–2/3rd deep <i>Severe:</i> > 2/3rd			

^aSystem includes both depth and severity.^bSystem include depth, severity, and configuration (other).

Medical Treatment

Medical options are often first line treatments and are of particular benefit where women desire uterine conservation to allow for pregnancy to occur. Options include:

1. Nonhormonal medical to control symptoms of AUB and pain
2. Oral hormonal control such as a combined oral contraceptive pill, oral progestins, GnRHa, danazol, and aromatase inhibitors
3. Intrauterine progestin

In women who complain of symptoms of heavy menstrual bleeding (HMB) are commonly offered options that include nonsteroidal anti-inflammatories, antifibrinolytic agents, and/or the combined oral contraceptive pill. Whilst these agents may be successful to reduce symptoms of HMB in some forms of AUB, there few data to support adenomyosis (AUB-A) specific outcomes for these options. In comparison, pharmacological agents used to suppressed endometrial growth including GnRHa, danazol, oral progestins and aromatase inhibitors are reported to be efficacious for the treatment of adenomyosis (Fawzy and Mesbah, 2015). These agents have also been shown to be efficacious in women with adenomyosis and infertility, although numbers of women involved in high quality studies are few and comparative data are lacking at this time. When considering these agents for HMB or infertility, the benefit of such medication versus the potential side effects must be evaluated carefully as the side effects may be equally unacceptable to the woman compared to the systems experienced by adenomyosis.

The use of the Levonogestrel intrauterine device (LNG-IUD) has received the most attention in studies examining the efficacy of medication to reduce symptoms of AUB and pain in women with adenomyosis. In a recent randomized controlled trial (RCT) 75 women who received either the LNG-IUD or hysterectomy (Ozdegirmenci et al., 2011) were assessed for changes in quality of life (QOL) over 1 year. The authors reported LNG-IUD improved hemoglobin levels of the first 6 months compared to hysterectomy and had a superior effect in the psychological and social life domains of the QOL scores. This has supported previous cohort studies which demonstrated improvement in dysmenorrhoea and high rates of satisfaction.

Surgical Treatment

Surgical management for adenomyosis may be:

1. “Definitive” or “curative” where hysterectomy is performed. Whilst hysterectomy resolves adenomyosis and its associated symptoms of HMB and pain, it is a major surgery and is not appropriate in women wanting future fertility or to maintain their uterus.
2. Conservative excisional surgery where the intent is to maintain the uterus in an intact and functional capacity with the aim for future fertility.
3. Conservative ablative surgery where there is no intent of fertility (and pregnancy would then be contra-indicated).

One of the difficulties commonly faced when considering surgical options is understanding the depth and extent of disease and what type of surgery could reasonably give symptomatic benefit. When considering conservative excisional surgery it is extremely important to perform reliable imaging studies to facilitate informed discussion with the patient regarding outcomes and expectations in addition to accurate preoperative planning. Fertility sparing surgery may be accomplished by:

- Hysteroscopic endomyometrial resection
- Local hysteroscopic resection
- Local laparoscopic resection
- Laparoscopic or ultrasound directed radiofrequency ablation

Hysteroscopic endomyometrial resection requires a high degree of skill and excellent preoperative imaging. Where clinical settings offer such expertise this procedure is reported to be successful with one prospective study reporting good outcomes with superficial adenomyosis. Unfortunately, in women with deep disease, the procedure had much higher failure rates to achieve improvement in symptoms. A recent meta-analysis of 1049 women which evaluated women undergoing hysteroscopic resection, in those where complete surgical removal of disease was reported there was a reduction of dysmenorrhea of 82%, HMB reduction of 69%, and a postoperative pregnancy rate of 60%. Where only partial excision was possible, these results are all reduced at 82%, 50%, and 47% respectively (Grimbizis et al., 2014). A particular challenge with these procedures is intraoperative heavy bleeding leading to increased operating time and decreased resection rates. As such, adjuvant procedures such as temporary uterine artery occlusion or preoperative GnRHa have been suggested to facilitate the procedure however there is currently insufficient evidence to support these approaches.

Similar to hysteroscopic resection, a laparoscopic approach aims to resect local disease whilst retaining a functional uterine corpus but likewise requires a high degree of skill. Most recently a retrospective study of 179 women who underwent laparoscopic uterine artery occlusions and resection of adenomyosis (Liu et al., 2014) reported uterine volume reduction of 58% at 3 years and a significant improvement in QOL scores. This is consistent with older and smaller studies and suggests there is some benefit to this approach.

Radiologic or Sonographic Interventions as Treatment

In alignment with the development of treatment delivery using interventional radiology in a wide range of diseases, there is increased interest in these procedures to deliver a nonsurgical option for women with adenomyosis. Evidence on techniques remain largely retrospective, with few prospective trials and two randomized controlled trials that report on specific adjuvant treatments to the principal intervention rather than comparing between techniques. Currently these techniques are too diverse to compare directly and known studies are summarized in Table 2.

Table 2 Sonographic and interventional radiological techniques for treating adenomyosis

<i>Authors and year</i>	<i>Methodology</i>	<i>Outcome(s)</i>
Kim et al. (2011)	Retrospective cohort study 35 women with symptomatic adenomyosis treated with magnetic resonance guided focused ultrasound (MRfFUS)	Significant improvement in QOL scores at 6 months
Liang et al. (2012)	Retrospective cohort 17 women with symptomatic adenomyosis treated by UAE	Reduction in dysmenorrhoea in 75% of women at 6 months Resolution of pain in 56% Mean reduction in uterine volume of 50%
Smeets et al. (2012)	Retrospective cohort 40 women with symptomatic adenomyosis treated by UAE	Significant improvement in QOL in 72% at 5 years Hysterectomy in 18% for ongoing symptoms
Long et al. (2015)	Prospective study 47 women with symptomatic adenomyosis and uterine volumes greater than 200 cm ³ treated by USgHIFU	Significant reduction in uterine volume Significant reduction in dysmenorrhoea at 12 months and improvement in QOL
Chen et al. (2015)	Retrospective cohort 2549 women with symptomatic adenomyosis treated by USgHIFU	95% of women had successful treatment Mean volume ablation rate 73% 10% adverse outcome (majority were minor-abdominal pain, vaginal discharge)
Ferrari et al. (2016)	Prospective trial 18 women with symptomatic adenomyosis treated by MRgFUS — 7 women with diffuse disease — 11 women with focal disease	There was a reduction in uterine volume for most women 83% of women had a junctional zone thickness of < 12 mm at 1 year post-treatment
Hai et al. (2017)	Prospective trial 87 women with symptomatic adenomyosis treated by TVUS guided transcervical radiofrequency ablation	Mean volume reduction 40.8% at 6 months, 41.2% at 12 months. Dysmenorrhoea statistically declined 2 women with intrauterine adhesions

Infertility and Reproduction

The impact of adenomyosis on female infertility and reproduction is becoming increasingly appreciated. The symptomatic management of women with AUB and pain who wish to conserve fertility has been outlined above however there is evidence to suggest the disease may impact on spontaneous pregnancy and ART efficacy as well as possible complication of pregnancy.

The possible association of adenomyosis to infertility continues to be debated. Whilst transvaginal Imaging studies have shown an increased spontaneous miscarriage rate in women with diffusely enlarged uterus but without distinct uterine masses this it did not affect the pregnancy rate. In contrast, a current meta-analysis review of women with adenomyosis undergoing IVF demonstrated a lower clinical pregnancy rate in women with adenomyosis (40.5% vs. 49.8%) (Vercellini et al., 2014) though there was significant heterogeneity between studies. This review also reported on higher miscarriage rate among women with adenomyosis (32% vs. 14%). Subsequent to this systematic review, studies continue to report on clinical pregnancy rates in women with adenomyosis undergoing IVF however the most important outcome, live birth rate, still remains under reported.

In women who are able to conceive there are several studies that report increased complications of pregnancy in women with adenomyosis. From a large case-control study of 2138 women, those diagnosed with adenomyosis were more likely to have premature delivery (OR 1.84) or premature rupture of membranes (OR 1.98) (Juang et al., 2007).

References

- Champaneria, R., Abedin, P., Daniels, J., Balogun, M., & Khan, K. S. (2010). Ultrasound scan and magnetic resonance imaging for the diagnosis of adenomyosis: Systematic review comparing test accuracy. *Acta Obstetrica et Gynecologica Scandinavica*, 89(11), 1374–1384.
- Chen, J., Chen, W., Zhang, L., et al. (2015). Safety of ultrasound-guided ultrasound ablation for uterine fibroids and adenomyosis: A review of 9988 cases. *Ultrasonics Sonochemistry*, 27, 671–676.
- Dakhly, D. M., Abdel Moety, G. A., Saber, W., et al. (2016). Accuracy of hysteroscopic endomyometrial biopsy in diagnosis of adenomyosis. *Journal of Minimally Invasive Gynaecology*, 23(3), 364–371.
- Exacoustos, C., Brienza, L., & Di Giovanni, A. (2011). Adenomyosis: Threedimensional sonographic findings of the junctional zone and correlation with histology. *Ultrasound in Obstetrics & Gynecology*, 37, 471–479.
- Fawzy, M., & Mesbah, Y. (2015). Comparison of dienogest versus triptorelin acetate in premenopausal women with adenomyosis: A prospective clinical trial. *Archives of Gynecology and Obstetrics*, 292(6), 1267–1271.
- Ferrari, F., Arrigoni, F., Miccoli, A., et al. (2016). Effectiveness of Magnetic Resonance-guided Focused Ultrasound Surgery (MRgFUS) in the uterine adenomyosis treatment: Technical approach and MRI evaluation. *Radiologia Medica*, 121(2), 153–161.
- Grimbizis, G. F., Mikos, T., & Tarlatzis, B. (2014). Uterus-sparing operative treatment for adenomyosis. *Fertility and Sterility*, 101(2), 472–487.
- Hai, N., Hou, Q., Ding, X., et al. (2017). Ultrasound-guided transcervical radiofrequency ablation for symptomatic uterine adenomyosis. *British Journal of Radiology*, 90(1069).
- Hulka, C. A., Hall, D. A., McCarthy, K., & Simeone, J. (2002). Sonographic findings in patients with adenomyosis: Can sonography assist in predicting extent of disease? *AJR. American Journal of Roentgenology*, 179(2), 379–383.
- Juang, C. M., Chou, P., Yen, M. S., et al. (2007). Adenomyosis and risk of preterm delivery. *BJOG: An International Journal of Obstetrics and Gynaecology*, 114(2), 165–169.
- Kim, K. A., Yoon, S. W., Lee, C., et al. (2011). Short-term results of magnetic resonance imaging-guided focused ultrasound surgery for patients with adenomyosis: Symptomatic relief and pain reduction. *Fertility & Sterility*, 95(3), 1152–1155.
- Kishi, Y., Suginami, H., Kuramori, R., et al. (2012). Four subtypes of adenomyosis assessed by magnetic resonance imaging and their specification. *American Journal of Obstetrics Gynecology*, 207(2), 114.e111–114.e117.
- Liang, E., Brown, B., Kirsop, R., Stewart, P., & Stuart, A. (2012). Efficacy of uterine artery embolisation for treatment of symptomatic fibroids and adenomyosis—An interim report on an Australian experience. *The Australian & New Zealand Journal of Obstetrics & Gynaecology*, 52(2), 106–112.
- Liu, M., Cheng, Z., Dai, H., Qu, X., & Kang, L. (2014). Long-term efficacy and quality of life associated with laparoscopic bilateral uterine artery occlusion plus partial resection of symptomatic adenomyosis. *European Journal of Obstetrics, Gynecology, & Reproductive Biology*, 176, 20–24.
- Long, X., Jiang, P., Zhou, L., & Zhang, W. (2013). Evaluation of novel serum biomarkers and the proteomic differences of endometriosis and adenomyosis using MALDI-TOF-MS. *Archives of Gynecology and Obstetrics*, 288(1), 201–205.
- Long, L., Chen, J., Xiong, Y., et al. (2015). Efficacy of high-intensity focused ultrasound ablation for adenomyosis therapy and sexual life quality. *International Journal Of Clinical And Experimental Medicine*, 8(7), 11701–11707.
- Luciano, D. E., Exacoustos, C., Albrecht, L., et al. (2013). Three-dimensional ultrasound in diagnosis of adenomyosis: Histologic correlation with ultrasound targeted biopsies of the uterus. *Journal of Minimally Invasive Gynecology*, 20(6), 803–810.
- Ohara, R., Michikami, H., Nakamura, Y., et al. (2014). Moesin overexpression is a unique biomarker of adenomyosis. *Pathology International*, 64(3), 115–122.
- Ozdegirmenci, O., Kayikcioglu, F., Akgul, M. A., et al. (2011). Comparison of levonorgestrel intrauterine system versus hysterectomy on efficacy and quality of life in patients with adenomyosis. *Fertility & Sterility*, 95(2), 497–502.
- Parazzini, F., Vercellini, P., & Panazza, S. (1997). Risk factors for adenomyosis. *Human Reproduction*, 12(6), 1275–1279.
- Sammour, A., Pirwany, I., Usabutun, A., Arseneau, J., & Tulandi, T. (2002). Correlations between extent and spread of adenomyosis and clinical symptoms. *Gynecologic & Obstetric Investigation*, 54(4), 213–216.
- Siegler, A. M., & Camilien, L. (1994). Adenomyosis. *The Journal of Reproductive Medicine*, 39(11), 841–853.
- Smeets, A. J., Nijenhuis, R. J., & Boekkool, P. F. (2012). Long-term follow-up of uterine artery embolization for symptomatic adenomyosis. *Cardiovascular & Interventional Radiology*, 35(4), 815–819.
- Van den Bosch, T., Dueholm, M., Leone, F. P., et al. (2015). Terms, definitions and measurements to describe sonographic features of myometrium and uterine masses: A consensus opinion from the Morphological Uterus Sonographic Assessment (MUSA) group. *Ultrasound in Obstetrics & Gynecology*, 46(3), 284–298.
- Vercellini, P., Cortesi, I., De Giorgi, O., Merlo, D., Carinelli, S. G., & Crosignani, P. G. I. (1998). Transvaginal ultrasonography versus uterine needle biopsy in the diagnosis of diffuse adenomyosis. *Human Reproduction*, 13(10), 2884–2887.
- Vercellini, P., Parazzini, F., & Oldani, S. (1995). Adenomyosis at hysterectomy: A study on frequency distribution and patient characteristics. *Human Reproduction*, 10(5), 1160–1162.
- Vercellini, P., Vigano, P., Somigliana, E., et al. (2006). Adenomyosis: Epidemiological factors. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 20(4), 465–477.
- Vercellini, P., Consonni, D., Dridi, D., et al. (2014). Uterine adenomyosis and in vitro fertilization outcome: A systematic review and meta-analysis. *Human Reproduction*, 29(5), 964–977.

- Wang, S., Duan, H., Zhang, Y., & Sun, F. Q. (2016). Abnormal activation of RHO/ROCK-I signalling in junctional zone smooth muscle cells of patients with adenomyosis. *Reproductive Sciences*, 23(3), 333–341.
- Zou, Y., Liu, F. Y., Wang, L. Q., et al. (2017). Downregulation of DNA Methyltransferase 3 alpha promotes cell proliferation of ectopic endometrial stromal cells in adenomyosis. *Gene*, 604, 41–47.

Relevant Website

<https://www.endometriosisaustralia.org/single-post/2016/11/08/Adenomyosis-%E2%80%93-sister-to-endometriosis-or-distant-cousin> – Endometriosis Australia.

Congenital Uterine Abnormalities

Pedro Aci n and Maribel Aci n, Miguel Hern ndez University of Elche, Alicante, Spain; and Institute of Gynecology P.A.A, Alicante, Spain

  2018 Elsevier Inc. All rights reserved.

The congenital anomalies affecting the development and morphology of the fallopian tubes, uterus, vagina and vulva, with or without associated ovarian, urinary, skeletal or other organs anomalies, are included within the broad spectrum of female genital tract malformations. The majority of them affect the uterus (fused M llerian ducts) and are therefore reported as uterine or M llerian malformations, which have an influence on the reproductive processes. But we should not forget that many anomalies affecting the M ller ducts may have their origin in a mesonephric (Wolff ducts) or gubernaculum anomaly, being then, in the first case, associated to other genital and extragenital malformations. In fact, the mesonephric anomalies are more complex and present a difficult diagnosis and treatment (Ac n and Ac n, 2016a).

Uterine malformations have been reported to occur in 3%–4% of women overall, in 4% of infertile women and in 15% of those who have experienced recurrent miscarriage (Ac n, 1997; Raga et al., 1997). Other studies that have evaluated the prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure (Saravolos et al., 2008) observed higher percentages: 6.7% in the general population, 7.3% in the infertile population, and 16.7% in the recurrent miscarriage patients. Therefore, common uterine anomalies (M llerian) are frequent and important because of their effects on fertility. Mesonephric anomalies, certain obstructive M llerian malformations and other malformative combinations are particularly important because they cause several clinical symptoms, especially gynecological in young women, and impact the patient's quality of life, in addition to creating fertility problems. Such complex malformations of the female genital tract are not that common (17.3% of all female genitourinary malformations).

Etiology

The etiology of most uterine malformations is generally believed to be multifactorial. In general there is no evident cause or association. The few existing studies have identified a degree of risk for siblings that is similar to most other multifactorial disorders (Jacquinet et al., 2016). The karyotype is generally normal but sometimes there are mosaicisms or other anomalies that do not seem related to the malformation; some familial cases seem to be linked to a recessive autosomal gene and others to a dominant autosomal one, but to date, only a few genes (HNF1B, WNT4, WNT7A, HOXA13) have garnered strong evidence of causality, mainly in syndromic presentations; and the sequencing of candidate genes in series of individuals with isolated uterine abnormalities has been able to suggest an association for several genes, but confirmation of a strong causative effect is still lacking for the majority of them (Jacquinet et al., 2016). And the environmental causes can only explain some cases. Apart from the diethylstilbestrol exposure-related (DES) syndrome, other teratogenic drugs, malnutrition, metabolic alterations, viral infections and placental anomalies have been implied. Numerous reports of discordant phenotypes in monozygotic twins (e.g., Mayer–Rokitansky–Kuster–Hauser (MRKH) syndrome vs. normal uterus) argue against strong heritability and suggest that post-zygotic somatic mutations or non-genetic mechanisms such as epigenetic or microenvironmental factors underlie the occurrence of congenital uterine malformations (Milsom et al., 2015; Rall et al., 2015; Jacquinet et al., 2016). However, whereas these mechanisms may explain sporadic cases, reports of familial cases do support the existence of a predisposing genetic background that may be stronger in some families compared to others (Jacquinet et al., 2016). According to Hammoud et al. (2008), approximately 10% of cases of M llerian anomalies seem to be attributable to a familial association. They conclude that M llerian anomalies have a strong familial aggregation and follow a polygenic and multifactorial inheritance.

Nevertheless, the familial association is difficult to research. Uterine ultrasounds have not usually been performed, or not performed in extended family members with asymptomatic or subtle anomalies. But also, although in many families recurrences tend to recapitulate the same type of uterine malformation, recurrences with variable types of uterine maldevelopment have also been reported in several families suggesting that similar genetic background could still predispose to different types of uterine malformation with variable penetrance and expressivity (Jacquinet et al., 2016).

Thus, it seems then that genital malformations are influenced by multifactorial, polygenic and familiar mechanisms that together can create a favorable environment for the anomaly, but in most cases there is no evident cause or association. However, the embryological development of the genital tract and the chain of anatomical events leading to the production of the malformation are better known, and therefore, we must know the embryology for a better understanding of the pathogenesis.

Embryology

Fig. 1 shows a scheme of female genito-urinary embryology. Briefly, a longitudinal invagination of the coelomic epithelium is formed on the outer side of the urogenital ridge and originates the paramesonephric or M llerian duct. This duct opens into the celomic cavity at the top and descends in parallel and externally to the mesonephric duct. Then, both M ller's ducts cross ventrally the mesonephric ducts, growing in the caudomedial direction until fusing into a Y-shaped middle structure that forms the uterine

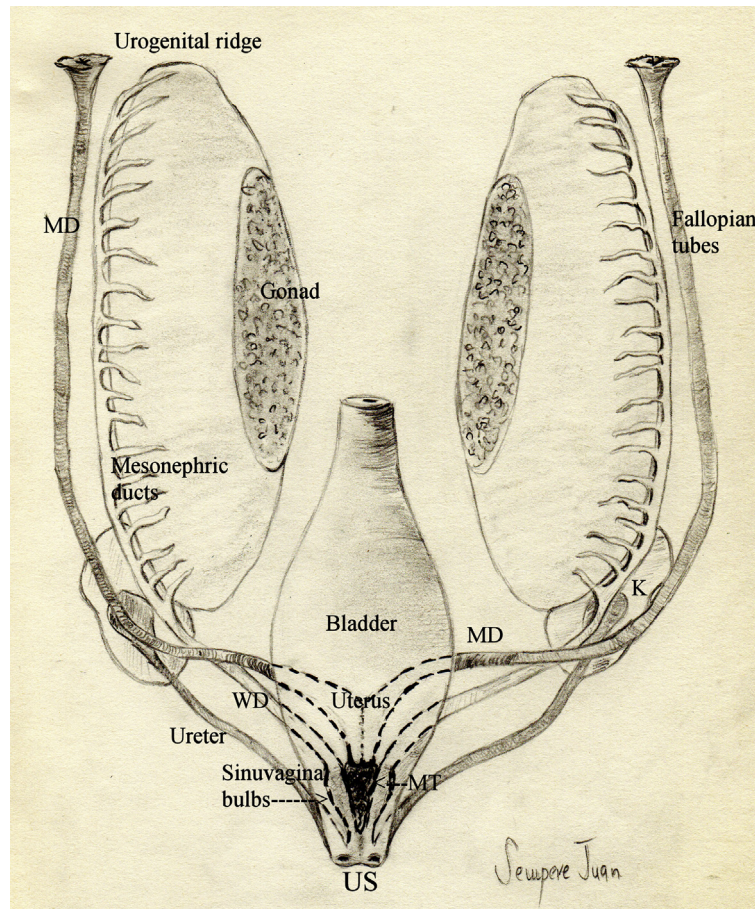


Fig. 1 Embryology of the female genito-urinary tract (frontal view). The formation of the uterine primordium and the opening of the mesonephric ducts into the urogenital sinus are shown. Müllerian tubercle can be seen between both Wolffian ducts and the ureteral buds sprouting from the opening of the Wolffian duct into the urogenital sinus. MD, Müllerian ducts; WD, wolffian ducts; K, kidney; MT, Müllerian tubercle; US, urogenital sinus.

primordium. Thus, it is well known that the uterus is formed from the fused caudal portions of the Müller ducts. However, nowadays we also know that: (1) the appropriate development, fusion and reabsorption of the separating wall between both Müller ducts is induced by the Wolffian ducts placed at both sides and which act as guide elements. (2) The fused Müller ducts form the uterus until the external cervical os, and the inducing mesonephric ducts regress cranially though they enlarge caudally from the level of cervical os, form the sinuvaginal bulbs, incorporate the Müller tubercle's cells and give rise to the vaginal plate whose cavitation is covered by Müllerian cells with a cuboidal or paramesonephric epithelium. Then, by metaplastic induction or by epidermization from the sinus, the vagina is covered by a flat, squamous, stratified epithelium. (3) Since the ureteral bud sprouts from the opening of the wolffian duct into the urogenital sinus, the absence or distal injury of one of these ducts will give rise to a renal agenesis and blind or ipsilateral athretic hemivagina and a uterine anomaly (fusion or reabsorption defect) due to a failure in the inducing function of the injured mesonephric duct. (4) In the absence of formation and caudal growth of the urogenital wedge, there is persistent urogenital sinus and then the opening of the vagina into the sinus can be seen as a vesicovaginal fistula just underneath and between both urethral orifices (Martínez-Escoriza et al., 2011). (5) The female gubernaculum is likely formed by muscle fibers that are not of a mesonephric or paramesonephric origin and their attachment to the Müllerian ducts allows or induces the fusion and adequate development of the uterus. Thus, dysfunction of the female gubernaculum probably results in female genital tract malformations (Acién et al., 2011).

The interaction and correspondence between the analyzed embryonic structures and its normal or abnormal adult derivatives can be seen in Acién et al. (2010c).

Classification

Based on the Müllerian development processes, we should consider the classification of the uterine or Müllerian malformations as shown in Table 1. This classification system for uterine or Müllerian malformations corresponds to the traditional classifications

Table 1 Classification of the uterine or Müllerian malformations

-
1. Anomalies caused by total or partial agenesis:
 - (a) Of both Müllerian ducts: Mayer–Rokitansky–Kuster–Hauser (MRKH) or Rokitansky syndrome, or
 - (b) Müllerian agenesis of just one side: unicornuate uterus
 2. Anomalies caused by total or partial absence of fusion:
 - (a) Didelphys uterus and
 - (b) Bicornuate uterus–bicornis-bicollis and bicornis-unicollis uteri
 3. Anomalies caused by total or partial absence of reabsorption of the septum between the Müllerian ducts:
 - (a) Septate and subseptate uterus
 - (b) Arcuate uterus (minimum subseptate or bicornuate uterus?)
 4. Anomalies caused by a lack of later development:
 - (a) Hypoplastic uterus, T-shaped, and
 - (b) Diethylstilbestrol exposure [DES] syndrome, DES-related uterus
-

(Jarcho, 1946), the widely used [American Fertility Society AFS/ASRM classification \(1988\)](#) or that included in Netter's Atlas (1979). The [AFS/ASRM \(1988\)](#) considered seven basic groups that are analyzed basically from the point of view of the Müllerian development and their relationship to fertility: (I) agenesis and hypoplasias; (II) unicornuate uteri; (III) didelphic uteri; (IV) bicornuate uteri; (V) septate uteri; (VI) arcuate uteri; and (VII) anomalies related to DES syndrome. The additional findings referred to the vagina, cervix, fallopian tubes, ovaries and urinary system must be pointed out apart.

The latest ESHRE/ESGE classification system “uterus-cervix-vagina, UCV” ([Grimbizis et al., 2013](#)) is also based on those concepts (Müllerian development processes), with anatomy providing the basis for the systematic categorization of anomalies. The main types of uterine anomaly are: U0, normal uterus; U1, dysmorphic uterus (T-shaped, infantilis, others); U2, septate uterus (partial, complete); U3, bicorporeal uterus (partial, complete and bicorporeal septate); U4, hemi-uterus (with or without rudimentary cavity, communicating or not horn); U5, aplastic (with or without rudimentary cavity); and U6, unclassified malformations.

However, these classification schemes essentially refer to only Müllerian anomalies or the anatomic visual appearance and do not explain or suggest the actual origin of female genitourinary tract malformations nor their appropriate therapeutic correction. We, based on the embryology presented before, proposed an embryological-clinical classification that was revised and updated in [Acien and Acien \(2011\)](#) including six groups of female genitourinary malformations (see [Table 2](#)). In latter works ([Acien and Acien \(2016a,b\)](#)) we have analyzed the correspondence between the embryological-clinical classification, the [AFS/ASRM \(1988\)](#) and the recent ESHRE/ESGE ([Grimbizis et al., 2013](#)). As expected, the embryological and clinical classification correlates better between vaginal anomaly, uterine anomaly, and ipsilateral renal agenesis or renal dysplasia with or without ectopic ureter, suggesting the origin and possible clinical presentation and thus leading the diagnostic imaging and therapeutical approach, especially in complex malformations.

Nevertheless, for the analysis in this chapter of the clinical aspects of congenital uterine abnormalities, associated or not to mesonephric or extragenital anomalies, we will follow the classification based on the Müllerian development processes presented in [Table 1](#). In [Fig. 2](#) the uterine or Müllerian malformations are graphically exposed.

Previous Considerations

(1) We consider the arcuate uterus as a minor form of the bicornuate uterus, although in the AFS classification it is rather a kind of subseptate uterus and sometimes it can be so; in the ESHRE/ESGE classification it is not considered; (2) any depression in the

Table 2 Embryological-clinical classification for female genito-urinary malformations

-
1. Unilateral genito-urinary agenesis or hypoplasia. They are the cases of unicornuate uterus with contralateral renal agenesis due to agenesis or hypoplasia of an entire urogenital ridge.
 2. Uterine duplicity (bicornuate or didelphys uterus) with a blind hemivagina (or unilateral cervico-vaginal atresia) and ipsilateral renal agenesis. It includes the Herlyn–Werner and Wunderlich syndromes and there can also be cases of partial resorption of the intervaginal septum.
 3. Isolated or common uterine or utero-vaginal anomalies. They include the anomalies in the Müllerian development processes also included in the classification of the [American Fertility Society \(1988\)](#) without other associated anomalies; and also the transverse vaginal septum.
 4. Accessory uterine masses (ACUMs) with an otherwise normal uterus, and other possible gubernaculum dysfunctions.
 5. Anomalies of the urogenital sinus. As imperforated hymen, vesico-vaginal fistulas, persistent urogenital sinus, cloacal anomalies, and other external gastrointestinal or urinary anomalies.
 6. Malformative combinations
-

Revised and updated from Acien, P. (1992); Acien, P., Acien, M. and Sánchez-Ferrer, M. (2004) and Acien, P. and Acien, M. (2011). The history of female genital tract malformation classifications and proposal of an updated system. *Human Reproduction Update* **17**, 693–705.

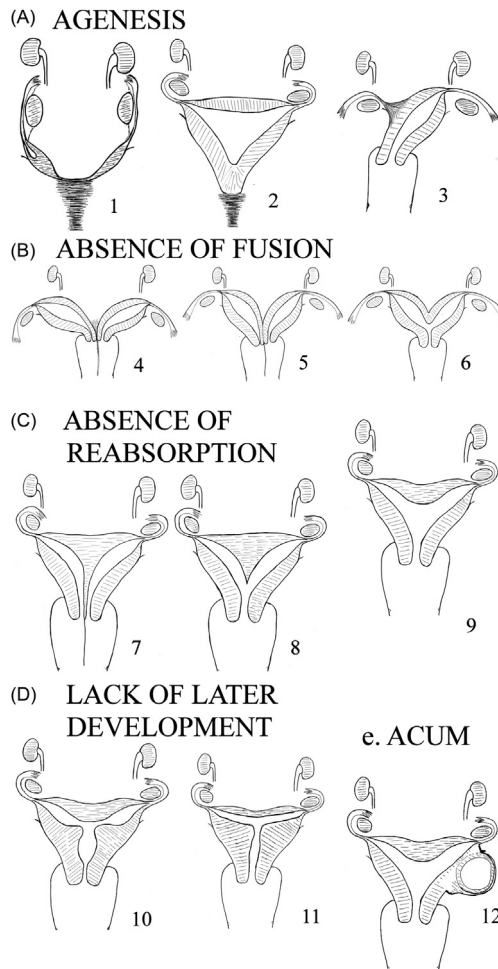


Fig. 2 Graphical representation of uterine or Müllerian malformations. (1) Rokitansky syndrome. (2) Cervico-vaginal agenesis. (3) Unicornuate uterus. (4) Didelphys uterus. (5) Bicornis-bicollis uterus. (6) Bicornis-unicollis uterus. (7) Septate uterus. (8) Subseptate uterus. (9) Arcuate uterus. (10) DES-related uterus. (11) T-shaped hypoplastic uterus. (12) ACUM.

uterine bottom, on its outer surface, makes the malformation then to be included as a bicornuate uterus and not septate, since in this case the external shape of the uterus is supposed to be normal and uniform (this is basically the problem when cataloguing uterine malformations in different publications); (3) many cases are transitional (fusion and reabsorption partial failure). The cataloguing of these cases is sometimes difficult because the upper part is bicornuate (fusion defect, practically didelphys) and is clearly septate (reabsorption defect) lower down. The ESHRE/ESGE classification includes these cases as bicorporal septate uterus. And the other way around, we can also find a septate-bicervical uterus (Acien et al., 2009); (4) the uterus is didelphic when there are two hemiuteri completely detached, at least up to the cervixes, where they may be touching each other. But, as already mentioned, there are also cases with double uterine body and isthmic portion, but with simple exocervix and vagina. They are anatomically and clinically didelphic uteri; (5) we agree with Muller and Musset's bidirectional theory (1967) on the reabsorption of the uterine septum, since when there are communicating uteri, they are generally communicating at an isthmic level and besides, this is always the weakest part of the septum. The explanation to this is in the different portions of the Müller ducts which give rise to different uterine portions and, specifically, to the convergence in the internal cervical os of the Müller ducts with distal divergence up to the external cervical os (Sánchez-Ferrer et al., 2006); (6) the septate or double vagina is associated to a didelphys and bicornis-bicollis uterus, but also to a septate uterus; and the vagina can be septate even in the presence of a normal uterus or of non-septate (Acien et al., 2009).

Clinical Presentation and Diagnosis

Agenesis

1. The combined uterovaginal agenesis is the most common type of agenesis (bilateral Müllerian agenesis) and it corresponds with the MRKH or Rokitansky Syndrome (Oppelt et al., 2012). This is an isolated Müllerian anomaly affecting both the Müllerian tubercle and ducts. Patients report primary amenorrhea. Transrectal ultrasound (TRU), computerized axial tomography (CT) or

the magnetic resonance (MR) demonstrate uterus absence with normal ovaries and two solid rudimentary horns. Some of these rudimentary horns may occasionally present a small functioning endometrial cavity, giving rise to retrograde menstruation, endometriosis and cyclical pain (Acíén and Acíén, 2016a). Occasionally, the cavitated rudimentary horn might be well developed, being possible its implantation in a previously formed neovagina (Grimbizis et al., 2015; Acíén and Acíén, 2016a).

In cases of agenesis or hypoplasia of a urogenital ridge, there will be absence of kidney, ureter, ovary, fallopian tube, hemiuterus, and hemivagina (not detectable) on one side. On the other side there might be a unicornuate uterus, but if there is also contralateral Müllerian agenesis, the diagnosis will be Rokitansky syndrome with unilateral renal agenesis (Acíén et al., 2010b) or atypical Rokitansky. This condition is sometimes associated with skeletal and/or auditory anomalies (Acíén and Acíén, 2010). Apart from the primary amenorrhea, the clinical presentation of the Rokitansky syndrome involves the difficulty for sexual intercourse and infertility, with normal sexual development, the absence of dysmenorrhea and the absence of a vagina under gynecologic examination. Laparoscopy generally reveals a fibrous tract or two solid rudimentary horns and normal ovaries with the distal portion of the tubes.

2. *Cervico-vaginal agenesis and segmentary atresias.* There might be isolated Müllerian anomalies affecting the Müllerian tubercle presenting as complete vaginal (or cervicovaginal) agenesis or atresia; and also segmentary atresias affecting one of the Müllerian ducts (see unicornuate uterus), or the Müllerian tubercle, as in cases of transverse vaginal septum.

Complete vaginal or cervicovaginal agenesis or atresia with a functional uterus is usually a complex malformation in which the external genitals and tubes appear normal. The uterus may be normal or may present with fusion or resorption defects and the cervix may be present, absent or hypoplastic. The clinical presentation involves primary amenorrhea and cyclic pain in post pubertal women. TRU and particularly MR allow a clear diagnosis that includes a normal corpus uteri with endometrium and cervicovaginal atresia. The ovaries are normal, although they might present endometriosis due to retrograde menstruation. Laparotomy with atretic cervix resection and implantation of the uterine corpus in a previously formed neovagina is recommended, having achieved normal menstruations and spontaneous term pregnancy (Acíén et al., 2008).

The vaginal segmentary atresia and transverse vaginal septum correspond to a transverse constriction or septum that is perforated or imperforated. There may be no symptoms until puberty, when the hematocolpos forms and causes episodes of pelvic pain and primary amenorrhea similar to those observed with vaginal atresia. The examination, abdominal or TRU and specially MR allow the diagnosis and help on the surgical evacuation of the hematocolpos. Uterus, fallopian tubes and ovaries are usually normal.

3. *Unicornuate uterus* comes in several variations, based on the degree of development and absence of communication to the contralateral side (see Fig. 3). It can be easily diagnosed with hysterosalpingography (HSG), but attention must be given for the possibility of a didelphys uterus with unilateral canalization and contrast injection. Nowadays, it is a better tool the 3D-US and MR of special interest in the detection of a cavitated non-communicated uterine horn which can also be observed with transvaginal ultrasound (TVU) (or with TRU). It must be remembered that in all isolated Müllerian anomalies both kidneys will be present, but some cases of unicornuate uterus might have a cavitated and non-communicating uterine horn with external bicornuate uterine morphology. The inclusion of these cases as bicornuate or unicornuate uterus is discussed. Functionally, these uteri are unicornuate, but it is important to adequately identify the anomaly to determine whether there is renal agenesis and to decide if surgical excision is required. Patients may experience dysmenorrhea and present endometriosis and ovarian endometriomas in the non-communicating horn side, but sometimes, there are no symptoms or adnexal pathology. Sometimes, the severe symptoms appear after bilateral tubal sterilization following several uneventful pregnancies. Tubal blockage of the side with the cavitated and non-communicating horn led to unilateral haematometra with severe dysmenorrhea. Other times, in the cavitated rudimentary horns (communicated or not), there can be gestation with a possible uterine rupture, usually at the end of the second trimester.

In cases of segmentary atresia of one of the Müllerian ducts with a detached hemiuterus, the HSG will be that of a unicornuate uterus but ultrasound and MR show a didelphys uterus in the superior uterine segment with a unilateral cavitated and non-communicating uterine horn. The cervix is simple. There will generally be dysmenorrhea and endometriosis due to retrograde menstruation from the cavitated uterine horn, but there can also be tubal segmentary atresia and in such cases there will be hematometra without endometriosis. In any case, a differential diagnosis must be determined, mainly with cases of complete unilateral vaginal or cervicovaginal agenesis without communicating uteri.

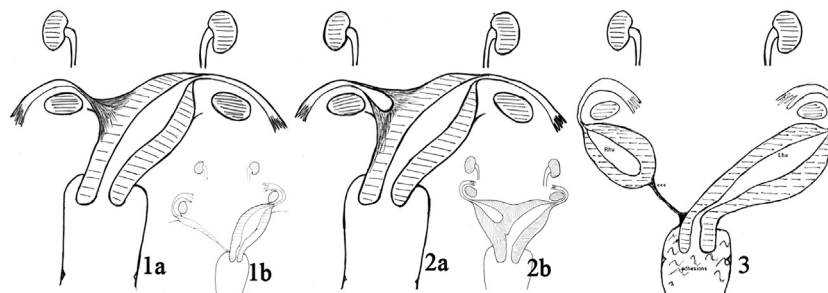


Fig. 3 Graphical representation of the unicornuate uterus subtypes. (1a) With non cavitated rudimentary horn. (1b) Unilateral Rokitansky. (2a and 2b). Cavitated and non-communicated uterine horn. (3) Segmentary atresia of one of the Müllerian ducts with a detached hemiuterus.

The reproductive results of the unicornuate uterus are worse than those in women with normal uterus (abortions: 20% vs. 8%; preterm deliveries: 20% vs. 4%; living children older than 7 days: 70% vs. 89%) but better than in other types of malformations (Acíén, 1993; Acíén et al., 2014). The probability of spontaneous miscarriage during first and second trimester was increased (RR 2.10, 95% CI 1.12–3.94), as well as low birth weight (<2500 g) incidence (RR 3.54, 95% CI 2.22–5.54) compared to controls, in Venetis et al. (2014) meta-analysis.

Fusion defects (didelphys and bicornuate uteri)

1. *Didelphys uterus* presents two completely detached hemiuteri (like two unicornuate uteri) with two cervixes and a double vagina. The new ESHRE/ESGE classification system (2013) has assimilated the didelphys to bicornuate uterus including it as a complete bicorporeal uterus (U3/C2/V2). For the diagnostic imaging, the considerations made on the resorption of the septum and the bidirectional hypothesis of Musset and Müller (Muller et al., 1967) have to be taken into account, and cases with didelphic uterine corpus and simple (normal or septate) cervix and vagina can be found. Apart from the fertility related problems (obstetric mainly), the only symptom might be dyspareunia due to the vaginal septum, but it usually does not present. The probability of pregnancy in natural cycles was not significant different from the controls (RR 0.94, 95% CI 0.83–1.07) (Venetis et al., 2014). And the reproductive results are similar to the unicornuate uterus in our material: 72% of living children (Acíén, 1993), or 91% if there is associated ipsilateral renal agenesis (Acíén et al., 2014); much better than the 40% published by Raga et al. (1997). The miscarriage risk during first and second trimester did not differ from controls but it did the birth weight and intrauterine growth restriction rate in Venetis et al. (2014) meta-analysis.

2. *Bicornuate uterus*: it corresponds to the complete (bicornis-bicollis uterus) and partial (bicornis-unicollis uterus) in the AFS/ASRM classification (1988) or partial, complete, and bicorporeal septate uterus in the new ESHRE/ESGE classification (2013). Some cases can have a cavitated non-communicating horn and their inclusion as bicornuate/septate or unicornuate uterus is discussed. Currently, sonohysterography, 3D-US, and specially the MR may provide the differential diagnosis with the septate uterus without the need of laparoscopy. In a pelvic MR, significant fundal cleft (> 1 cm) indicates no fusion of the upper-mid uterine horns (Graupera et al., 2015). However, if this distance measures < 1 cm, then a septate uterus would be present. In the ESHRE/ESGE classification system (2013), class U3 (bicorporeal uterus) is defined by an external indentation of > 50% of the uterine wall thickness, whereas in the complete bicorporeal uterus (U3b), the width of the fundal indentation at the midline is > 150% of the uterine wall thickness.

The reproductive results in the bicornuate uteri group are worse in cases of bicornis-unicollis uterus (40% of living children older than 7 days) than of bicornis-bicollis (71%) in our material (Acíén, 1993), and in latter analysis: 45% and 65% respectively (Acíén et al., 2014). Also the miscarriage rate is worse in the bicornuate-unicollis (45%) than in the bicornis-bicollis (23.5%). In the Venetis et al. (2014) meta-analysis, there were no statistically significant differences in the probability of pregnancy in natural cycles (RR 0.95) nor under assisted reproductive techniques cycles (RR 1.73) in the bicornuate uterus; but it was higher the first trimester miscarriage rate (RR 2.32), the second trimester (RR 2.90), premature delivery < 28 weeks (RR 5.37), malpresentation (RR 4.65), very low neonatal birth weight (RR 2.78), intrauterine growth restriction (RR 2.80) and perinatal mortality (RR 3.32, 95% CI 1.61–6.86).

3. The fusion Müllerian defects associated to distal mesonephric anomalies (unilateral renal agenesis and ipsilateral blind or atretic hemivagina syndrome) are the most complex malformations; they include uterine duplicity (didelphys, bicornuate or less commonly, septate uterus), renal agenesis (or dysplasia with or without ectopic ureter), and any of the following subtypes (see Fig. 4): (a) large hematocolpos in a blind hemivagina; (b) “Gartner’s duct pseudocyst” in the anterolateral wall of the permeable vagina; (c) partial reabsorption of the intervaginal septum; or (d) complete unilateral vaginal or cervicovaginal agenesis (Acíén and Acíén, 2016a). The last cases can have communication between both hemiuteri and will present as a bicornuate-unicollis uterus. In other cases, there is no communication between the hemiuteri and these cases reflect unilateral hematometra and endometriosis caused by retrograde menstruation on the side of the absent vagina and kidney. In the latter, differential diagnosis must be done with Müllerian segmentary atresias. 2D and 3D-US, pyelography, and MR can help in the diagnosis and treatment includes a hemi-hysterectomy. Reproductive performance was significantly better for a given type of uterine malformation if it was associated with unilateral renal agenesis (URA) (Acíén et al., 2014).

Resorption Defects (Septate, Subseptate) and Arcuate Uterus

1. *Septate uterus*. Corresponds to the complete (septate uterus) and partial (subseptate uterus) in the AFS/ASRM classification (1988) or complete (U2b) and partial (U2a) septate uterus in the new ESHRE/ESGE classification (2013). The diagnosis is equally suggested by TVU and 3D-US, or by HSG. Currently, sonohysterography, 3D-US, CT, and specially MR can provide the appropriate differential diagnosis (Graupera et al., 2015). Imaging description for septate uterus in the AFS/ASRM classification (class V) is convex, flat or minimally indented (< 1 cm) fundal external contour with indentation of the myometrium/septum into the uterine cavity (> 1 cm). In the ESHRE classification (2013), class U2 (septate uterus) is considered by an internal indentation > 50% of the uterine wall thickness and external contour straight or with indentation < 50%. The clinical presentation are reproductive losses, preterm deliveries, breech presentation and eventually hemi-hematometra if there is a non-communicating cavitated uterine horn (Robert’s uterus) (Acíén and Acíén, 2016a).

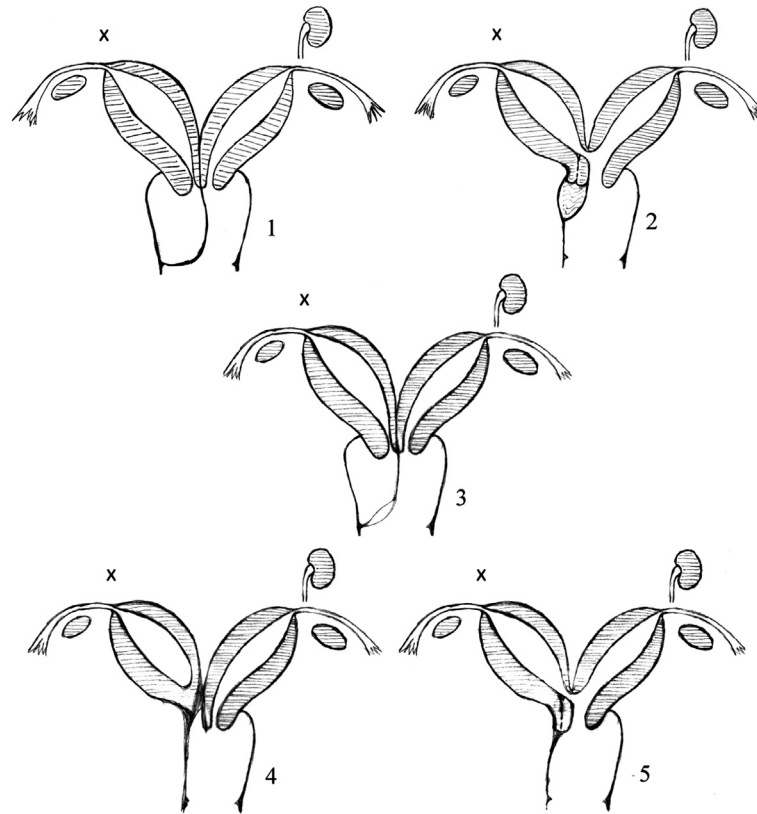


Fig. 4 Subtypes of unilateral renal agenesis and ipsilateral blind or atretic hemivagina syndrome: (1) with large hematocolpos in a blind hemivagina. (2) With “Gartner’s duct pseudocyst” in the anterolateral wall of the permeable vagina. (3) With partial reabsorption of the intervaginal septum. (4) With complete unilateral vaginal or cervicovaginal agenesis and no communication between hemiuteri. (5) Idem with communicating uteri.

The chances for achieving a pregnancy during natural cycles are significantly decreased in women with septated uterus compared with normal controls (RR 0.86, 95% CI 0.77–0.96), but there are no statistically significant differences in women undergoing assisted reproductive techniques cycles (Venetis et al., 2014). The reproductive results in our material (Acien et al., 2014) were similar between the septate and subseptate uterus but with a 40% of miscarriage rate and 55%–51% living children. In the Venetis et al. (2014) meta-analysis, women with septated uterus had a higher rate of first-trimester spontaneous abortion (RR 2.65, 95% CI 1.39–5.06), of second-trimester spontaneous abortion (RR 2.95, 95% CI 1.51–5.77), of malpresentation at delivery (RR 4.35, 95% CI 2.52–7.50), of growth restriction (RR 2.54, 95% CI 1.04–6.23), placental abruption (RR 4.37, 95% CI 1.12–17.08) and perinatal mortality (RR 2.43, 95% CI 1.10–5.36) compared to controls. After hysteroscopic resection of the septum, the probability of achievement of pregnancy was not significantly different (RR 1.14, 95% CI 0.79–1.65) nor the preterm delivery rate reduction (RR 0.66, 95% CI 0.29–1.49), but it did have a significant reduction the spontaneous (combined first-trimester and second-trimester) abortion rate (RR 0.37, 95%; CI 0.25–0.55).

2. *Arcuate uterus* is a minor form of bicornuate uterus, although in the AFS/ASRM classification (1988) it is rather a kind of subseptate uterus. It has not been included in the new ESHRE/ESGE classification system (2013).

The chances of achieving a pregnancy in natural cycles and under assisted reproductive techniques cycles has not been significantly different in women with arcuate uterus on Venetis et al. (2014) meta-analysis. However, it was significantly higher the spontaneous second trimester miscarriage risk (RR 1.98, 95% CI 1.06–3.69), placental abruption (RR 6.60, 95% CI 2.35–18.53) and malpresentation at delivery (RR 2.53, 95% CI 1.45–4.43).

Lack of Posterior Development (Hypoplastic Uterus, T-Shaped, and Diethylstilbestrol Exposure [DES] Syndrome)

Anomalies related to DES syndrome include hypoplastic, tricavitated, and T-shaped hypoplastic uteri with an extremely small uterine cavity, cornual constrictions, and bulbous dilatation of the lower segment. In the new ESHRE/ESGE classification (2013), these anomalies are included as dysmorphic uterus (class U1). From the clinical point of view, most cases are asymptomatic, though it can be associated with menstrual irregularities (hypo or oligomenorrhea), infertility and recurrent pregnancy losses or implantation failures in an IVF programme. Cervical ridges, folds or ectropion with a “glans” aspect, pseudopolyps or cervix hypoplasia may be observed. The diagnosis is made by HSG. Here, the ultrasound is less useful (though it may be suspected with it) and the laparoscopy usually shows normal internal genitalia. The hysteroscopic metroplasty may enhance the fertility and the reproductive performance.

Gubernaculum Dysfunctions

These cases are typified by accessory and cavitated uterine masses (ACUMs) with an otherwise normal uterus. HSG will show normal endometrial cavity and 3D-US and especially MR allow the right diagnosis. Although not properly being a uterine malformation that affects the endometrial cavity, it might provoke relevant symptoms due to menstrual retention in the ACUM. It represents a variant of Müllerian anomaly that is generally located at the level of the insertion of the round ligament and is possibly related to a dysfunction of the female gubernaculum (Acien and Acien, 2011; Acien et al., 2012). Other uterine malformations (e.g., didelphys uterus, Rokitansky syndrome) may also be due to gubernaculum dysfunctions (Acien et al., 2011) affecting the Müller duct development.

The criteria used to diagnose ACUM include the following: (i) an isolated accessory cavitated mass; (ii) a normal uterus (endometrial lumen), fallopian tubes, and ovaries; (iii) a surgical case with an excised mass and a pathological examination; (iv) an accessory cavity lined by endometrial epithelium with glands and stroma; (v) chocolate brown-colored fluid content; and (vi) no adenomyosis (if the uterus has been removed), although there could be small foci of adenomyosis in the myometrium adjacent to the accessory cavity (Acien et al., 2010a, 2012).

References

- Acien, P. (1993). Reproductive performance of women with uterine malformations. *Human Reproduction*, 8, 22–126.
- Acien, P. (1997). Incidence of Müllerian defects in fertile and infertile women. *Human Reproduction*, 12, 1372–1376.
- Acien, P., & Acien, M. (2010). Unilateral renal agenesis and female genital tract pathologies. *Acta Obstetrica et Gynecologica Scandinavica*, 89, 1424–1431.
- Acien, P., & Acien, M. (2011). The history of female genital tract malformation classifications and proposal of an updated system. *Human Reproduction Update*, 17, 693–705.
- Acien, P., & Acien, M. (2016a). The presentation and management of complex female genital malformations. *Human Reproduction Update*, 22, 48–69.
- Acien, P., & Acien, M. (2016b). Diagnostic imaging and cataloguing of female genital malformations. *Insights Imaging*, 7, 713–726.
- Acien, P., Acien, M., Quereda, F., & Santoyo, T. (2008). Cervicovaginal agenesis: Spontaneous gestation at term after previous reimplantation of the uterine corpus in neovagina. *Human Reproduction*, 23, 548–553.
- Acien, P., Acien, M., & Sánchez-Ferrer, M. L. (2009). Müllerian anomalies “without a classification”: From the didelphys-unicollis uterus to the bicervical uterus with or without septate vagina. *Fertility and Sterility*, 91, 2369–2375.
- Acien, P., Acien, M., Fernandez, F., Mayol, M. J., & Aranda, I. (2010a). The cavitated accessory uterine mass: A Müllerian anomaly in women with an otherwise normal uterus. *Obstetrics and Gynecology*, 116, 1101–1109.
- Acien, P., Galán, F., Manchón, I., et al. (2010b). hereditary renal adysplasia, pulmonary hypoplasia and Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome. A case report. *Orphanet Journal of Rare Diseases*, 5, 6.
- Acien, P., Acien, M., & Romero-Maroto, J. (2010c). Blind hemibladder, ectopic ureterocele, or Gartner’s duct cyst in a woman with Müllerian malformation and supposed unilateral renal agenesis: A case report. *International Urogynecology Journal*, 21, 365–369.
- Acien, P., Sanchez del Campo, F., Mayol, M. J., & Acien, M. (2011). The female gubernaculum: Role in the embryology and development of the genital tract and in the possible genesis of malformations. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 159, 426–432.
- Acien, P., Battaller, A., Fernandez, F., et al. (2012). New cases of accessory and cavitated uterine masses (ACUM): A significant cause of severe dysmenorrhea and recurrent pelvic pain in young women. *Human Reproduction*, 27, 683–694.
- Acien, P., Acien, M., Mazaira, N., & Quesada-Rico, J. A. (2014). Reproductive outcome in uterine malformations with or without an associated unilateral renal agenesis. *Journal of Reproductive Medicine*, 59, 69–75.
- American Fertility Society (AFS/ASRM). (1988 American Fertility Society (AFS/ASRM)). The American Fertility Society classifications of adnexal adhesions, distal tubal occlusion, tubal occlusion secondary to tubal ligation, tubal pregnancies, Müllerian anomalies and intrauterine adhesions. *Fertility and Sterility*, 49, 944–955.
- Graupera, B., Pascual, M. A., Hereter, L., et al. (2015). Accuracy of three-dimensional ultrasound compared with magnetic resonance imaging in diagnosis of Müllerian duct anomalies using ESHRE-ESGE consensus on the classification of congenital anomalies of the female genital tract. *Ultrasound in Obstetrics and Gynecology*, 46, 616–622.
- Grimbizis, G. F., Gorkts, S., Spiezo Sardo, A., et al. (2013). The ESHRE/ESGE consensus on the classification of female genital tract congenital anomalies. *Human Reproduction*, 28, 2032–2044.
- Grimbizis, G. F., Mikos, T., Papanikolaou, A., Theodoridis, T., & Tarlatzis, B. C. (2015). Successful isthmo-neovagina anastomosis after Davydov’s colpoptosis in Mayer–Rokitansky–Küster–Hauser syndrome patients with a functional rudimentary uterine horn. *Journal of Minimally Invasive Gynecology*, 22, 142–150.
- Hammoud, A. O., Gibson, M., Peterson, C. M., et al. (2008). Quantification of the familial contribution to Müllerian anomalies. *Obstetrics and Gynecology*, 111, 378–384.
- Jacquinet, A., Millar, D., & Lehman, A. (2016). Etiologies of uterine malformations. *American Journal of Medical Genetics. Part A*, 170A, 2141–2172.
- Jarcho, J. (1946). Malformations of the uterus. *American Journal of Surgery*, 714, 106–166.
- Martínez-Escoriza, J. C., Lobato, J. J., Lorda, E., et al. (2011). Congenital vesicovaginal fistula with menouria: An anomaly of the urogenital sinus. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 159, 472–475.
- Milsom, S. R., Ogilvie, C. M., Jefferies, C., & Cree, L. (2015). Discordant Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome in identical twins. A case report and implications for reproduction in MRKH women. *Gynecological Endocrinology*, 31, 1–4.
- Muller, P., Dellenbach, P., & Gillet, J. Y. (1967). Malformations of the female genital tract and those of the upper urinary tract which may be associated with them: Morphological and physiopathological study. *Semaine des Hôpitaux: Informations*, 43, 912–918.
- Oppelt, P. G., Lermann, J., Strick, R., et al. (2012). Malformations in a cohort of 284 women with Mayer–Rokitansky–Küster–Hauser syndrome (MRKH). *Reproductive Biology and Endocrinology*, 10, 57–64.
- Raga, F., Bauset, C., Remohi, J., et al. (1997). Reproductive impact of congenital Müllerian anomalies. *Human Reproduction*, 12, 2277–2281.
- Rall, K., Eisenbeis, S., Barresi, G., et al. (2015). Mayer–Rokitansky–Küster–Hauser syndrome discordance in monozygotic twins: Matrix metalloproteinase 14, low-density lipoprotein receptor-related protein 10, extracellular matrix, and neoangiogenesis genes identified as candidate genes in a tissue-specific mosaicism. *Fertility and Sterility*, 103, 494–502.e3.
- Sánchez-Ferrer, M. L., Acien, M., Sánchez del Campo, F., et al. (2006). Experimental contributions to the study of the embryology of the vagina. *Human Reproduction*, 21, 1623–1628.
- Saravelos, S. H., Cocksedg, K. A., & Li, T. C. (2008). Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: A critical appraisal. *Human Reproduction Update*, 14, 415–429.
- Venetis, C. A., Papadopoulos, S. P., Campo, R., et al. (2014). Clinical implications of congenital uterine anomalies: A meta-analysis of comparative studies. *Reproductive Biomedicine Online*, 29, 665–683.

ONCOFERTILITY

Cervical Cancer

Melissa Schwartz and Linus Chuang, Icahn School of Medicine at Mount Sinai, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cervical transformation zone An anatomic area of the cervix that contains the transition from ectocervical squamous epithelium to endocervical glandular epithelium.

Colposcopy Procedure used to evaluate the cervix by magnification in order to identify abnormal cells and vascular changes.

Human papillomavirus (HPV) A double-stranded DNA virus with over 40 different types that can infect the genital area and can cause warts as well as cervical cancer. It is the most common sexually transmitted infection in the United States.

Papanicolaou (Pap) test A screening test for cervical cancer in which a smear of exfoliated cells from the cervix are examined under a microscope for abnormal cells.

Parametria Connective tissue that extends laterally from the supracervical portion of the uterus between the broad ligament.

Introduction

Cervical cancer is the third most common gynecologic cancer and cause of gynecologic cancer-related death in the United States ([Berek and Hacker, 2015](#)). The Papanicolaou (Pap) test from screening has decreased the incidence of and mortality from cervical cancer as preinvasive disease and early stage cervical cancers are now more readily detected. Human papillomavirus (HPV) is integral to the development of cervical cancer and is detected in 99.7% of cases. There are two major histologic types of cervical cancer, squamous cell carcinoma and adenocarcinoma, and each has associated precursor lesions. In advanced stages, cervical cancer is a terminal disease.

Epidemiology

Globally, there were over 528,000 new cases and 266,000 deaths due to cervical cancer in 2012. On the other hand, in the United States, cervical cancer is relatively uncommon with approximately 13,000 new cases and 4100 deaths are due to cervical cancer annually ([Berek and Hacker, 2015](#)). These disparate statistics are due to a lack of access to screening and HPV vaccination in developing countries.

The lifetime risk of developing cervical cancer in the United States is estimated to be 0.6%. Cervical cancer incidence and mortality are higher among non-Hispanic black and Hispanic women as opposed to white and Asian women. The mean age of diagnosis 48 years, with very few women diagnosed after age 85 years.

Risk Factors

It has been well-established that persistent infection with HPV is the main cause of cervical cancer ([Cannistra and Niloff, 1996](#); [Schiffman et al., 2007](#)). Two high-risk HPV types, 16 and 18, are responsible for approximately 70% of cervical cancer ([Schiffman et al., 2007](#)). HPV infection is extremely prevalent with a lifetime risk of 80%. However, most of these infections clear spontaneously in immunocompetent individuals within 6–12 months. Risk factors for cervical cancer are predominantly associated with either acquiring the HPV infection or not having the appropriate immune response to fight off HPV infection.

HPV is known to be a sexually transmitted infection. Therefore, cervical cancer has been associated with early onset of sexual activity, increased number of lifetime sexual partners, and having a high-risk sexual partner ([Cannistra and Niloff, 1996](#); [Schiffman et al., 2007](#)). A high-risk partner is someone who has had multiple sexual partners or a known HPV infection. A history of sexually transmitted infections is also a risk factor for cervical cancer. Increasing parity (≥ 3 full term births) and early age at first birth (< 20 years) are also associated with increased risk of cervical cancer.

Human immunodeficiency virus (HIV) causes immunosuppression and a compromised immune response to HPV resulting in persistent infection and increased risk for cervical precancerous lesions to transform into cancer ([Cannistra and Niloff, 1996](#)). Cigarette smoking increases the risk for squamous cell cancer of the cervix, but not adenocarcinoma. Lastly, non-white race and low socioeconomic status have been associated with increased risk of cervical cancer.

Pathogenesis

Cervical cancer is an HPV-associated malignancy. Although genital tract infection with HPV is common, only a small percentage of women develop cervical cancer. Approximately 15 types of HPV have been identified as oncogenic with HPV 16 and 18 being the most common (Schiffman et al., 2007). Most HPV infections are transient and infection with HPV alone does not cause cervical cancer. In order for cervical cancer to develop, an oncogenic HPV strain must first infect the metaplastic epithelium of at the cervical transformation zone. Persistent HPV infection then permits tumorigenesis. If HPV is not cleared, infected epithelial cells can then transform and invade through the basement membrane to become invasive carcinoma. Cervical cancer spreads by direct extension or lymphatic or hematogenous dissemination.

Signs and Symptoms

The most common presenting symptom for patients with cervical cancer is painless vaginal bleeding. This bleeding can be postcoital or postmenopausal. Rarely, patients present with life-threatening hemorrhage requiring immediate intervention. For some women, the presenting symptom is vaginal discharge that can be malodorous or watery. In advanced stages of disease, women may complain of lower back pain, unilateral leg pain or leg edema. These symptoms may indicate that the tumor has invaded the pelvic sidewall. Patients may also have urinary symptoms or recurrent urinary tract infections caused by tumor compression of the bladder or ureters. For many women, especially those with early stage disease, there may be no presenting symptoms, with the diagnosis of cervical cancer made during screening with the use of a Pap test or incidentally during routine pelvic examination.

Diagnosis

Cervical cancer diagnosis is based on histologic evaluation of a cervical biopsy. Any women with symptoms suggestive of cervical cancer should undergo a complete pelvic examination. A speculum examination should also be performed to allow for visualization of the cervix. Any grossly visible cervical lesion should be biopsied. Symptomatic women without a visible cervical lesion should undergo colposcopy and directed biopsy. An adequate colposcopy requires that the entire squamocolumnar junction and any lesions be completely visualized (Cannistra and Niloff, 1996).

Bimanual examination should be performed for palpation and estimation of size of cervical tumor size. The uterus and adnexa should also be assessed. The size and shape of the uterus as well as any adnexal masses should be noted and characterized. A rectovaginal exam should also be done in order to better assess for vaginal, parametrial and pelvic sidewall involvement. The rectum should be palpated for any masses or extension of tumor. Other physical exam findings that are important include palpable groin or supraclavicular lymph nodes.

If a cervical biopsy shows cancer, certain tests can be ordered to assess for spread of disease. Oftentimes these additional tests are only done for large tumors. Additional tests include cystoscopy and proctoscopy. Cystoscopy entails inserted a lens and light through the urethra and into the bladder to assess for cancer in these areas. Proctoscopy allows for visual inspection of the rectum to assess for cancer spread to this area.

Cervical cancer is a clinically staged disease as opposed to surgical staging which is used for other gynecologic malignancies. The International Federation of Gynecology and Obstetrics (FIGO) (Mutch, 2009) utilizes clinical staging, which is largely based on physical examination, given that the majority of women with cervical cancer live in low-resource countries (Table 1). Surgical

Table 1 2009 FIGO staging for cervical cancer

Stage	Description
0	Carcinoma in situ (preinvasive)
I	Invasive carcinoma confined to the cervix
IA1	Stromal invasion ≤ 3.0 mm in depth and ≤ 7.0 mm in horizontal spread
IA2	Stromal invasion > 3.0 mm and ≤ 5.0 mm in depth and ≤ 7.0 mm in horizontal spread
IB1	Clinically visible lesion ≤ 4.0 cm in greatest dimension
IB2	Clinically visible lesion > 4.0 cm in greatest dimension
II	Invasive carcinoma that invades beyond cervix but not to pelvic sidewall or lower 1/3 of vagina
IIA1	Tumor without parametrial involvement. Clinically visible lesion ≤ 4.0 cm in greatest dimension
IIA2	Tumor without parametrial involvement. Clinically visible lesion > 4.0 cm in greatest dimension
IIB	Tumor with parametrial involvement
IIIA	Tumor extends to lower 1/3 of vagina, no extension to pelvic sidewall
IIIB	Tumor extends to pelvic sidewall and/or causes hydronephrosis or nonfunctional kidney
IVA	Tumor invades mucosa of bladder or rectum
IVB	Tumor extends beyond true pelvis

Mutch, D.G. (2009). The new FIGO staging system for cancers of the vulva, cervix, endometrium and sarcomas. *Gynecologic Oncology* 115, 325–328.

staging and advanced imaging modalities may not be available or feasible worldwide. Furthermore, locally advanced disease (i.e., tumor size, vaginal involvement, parametrial involvement) may be better assessed clinically and avoids surgery in women who are not candidates for surgical treatment. Staging procedures include a complete physical exam, cervical biopsy, cystoscopy and proctoscopy, and intravenous pyelogram (IVP) and plain chest radiograph. An IVP is used to evaluate for urinary tract obstruction. A chest X-ray may be done to assess for cancer spread to the lungs.

In resource-rich countries, such as the United States, imaging studies may be performed in order to augment clinical findings and remains controversial. A computed tomography (CT) scan may be done to look for lymphatic spread and in many centers replaces IVP for assessment of urinary tract obstruction. A magnetic resonance imaging (MRI) study may be performed to look for soft tissue involvement. Lastly, a positron emission tomography (PET) scan, which is a nuclear medicine functional imaging technique, may be done to look for increased metabolic activity of rapidly growing tumors in the body.

Management

The management of cervical cancer is dependent on the stage of disease ([Cannistra and Niloff, 1996](#)). Treatment may involve surgery, radiotherapy, chemotherapy, or a combination of therapies. In the United States, 70% of patients with invasive cervical cancer are diagnosed at a stage limited to the cervix, and therefore potential operative candidates. As cervical cancer generally spreads by direct extension, the extent of surgery for localized disease becomes more radical.

Stage IA1 cervical cancer is usually treated with a simple hysterectomy ([Cannistra and Niloff, 1996](#)). Alternatively, if fertility-sparing treatment is desired, then a cold knife cone can be performed. However, if lymphovascular invasion is identified, then a radical hysterectomy should be considered ([Berek and Hacker, 2015](#)). Removal of the ovaries is not necessary as cervical cancer very rarely metastasizes to the ovaries. Stages IA2, IB1, and IIA1 disease can be treated with either radical hysterectomy and pelvic lymph node dissection or primary radiotherapy with concurrent chemotherapy ([Cannistra and Niloff, 1996](#)). Cure rates for radical surgery or radiotherapy with chemotherapy are similar. For stages IB2, and IIA2–IVA, the standard of care is to treat with combination radiotherapy with chemotherapy. Lastly, patients with IVB should be treated with chemotherapy for disseminated disease. In certain advanced stage cases, palliative radiation can be considered.

A number of patients treated surgically will need adjuvant treatment for intermediate or high-risk features ([Berek and Hacker, 2015](#)). The National Cancer Institute supports treatment with the fewest number of interventions. Therefore, if there is a high probability that adjuvant therapy will be needed after surgery, then surgery should not be performed. Intermediate risk features include presence of lymphovascular space invasion (LVSI), deep cervical stromal invasion, and large tumor size. In cases with intermediate-risk disease, adjuvant radiation should be given to decrease risk of recurrence. Women are considered high risk disease if any of the following features are present: positive surgical margins, positive pelvic lymph nodes, or microscopic involvement of the parametrium. Adjuvant treatment for high-risk disease is a chemoradiation.

Chemotherapy has a limited role in the treatment of cervical cancer as most patients are treated with surgery or radiation and have a good response. When chemotherapy is used it is generally given concurrently with radiation. The most commonly used agent is cisplatin as a radiosensitizer. Rarely is chemotherapy used for first-line treatment. The one exception is in cases of metastatic cervical cancer in which combination chemotherapy plus bevacizumab, an angiogenesis inhibitor, is the choice of treatment.

Recurrent disease is managed based on location of recurrence ([Cannistra and Niloff, 1996](#)). If recurrence is local, treatment can be directed, such as surgical resection or radiotherapy. For distant recurrence, chemotherapy can be offered. The role of neoadjuvant chemotherapy for cervical cancer continues to be explored. This mode of treatment has particular implications in low-income countries in which the majority of patients lack access to radiotherapy and present with advanced disease.

Survival

Mortality from cervical cancer is dependent on stage. Five years after diagnosis, survival is very good for early stage disease, but is dismal for advanced disease ([Berek and Hacker, 2015](#)). For treated stage IA (microscopic) disease, nearly 100% of patients are alive after 5 years. Conversely, survival rates for stage IV disease over the same time period range from 5% to 20%. In the United States, the overall survival rate of all patients diagnosed with cervical cancer is approximately 70%. This statistic is largely due to the fact that most cervical cancers in the United States are diagnosed early. Early diagnoses and prevention of cervical cancer is attributed to the Pap test and the HPV vaccine.

Prevention

Routine screening with the Pap test, also known as the Pap smear, was first adopted in the United States in the 1950s. Since the 1970s, the incidence and mortality from cervical cancer has decreased. The Pap test is a cytology-based test and is performed during a speculum exam ([Schiffman and Solomon, 2013](#)). Cells are collected from the cervix using a plastic spatula or soft brush and smeared on a slide for examination under a microscope. The cells are scrutinized for abnormalities. The Pap test is 65% sensitive

and 90% specific for detected abnormal cervical cells. As we have learned more about what causes cervical cancer, molecular testing for HPV has now become a part of screening. HPV testing is >90% sensitive and >90% specific for cervical dysplasia.

The optimal screening method (Pap test, HPV testing, or both) and continues to be debated. The frequency of screening also remains controversial. Current national guidelines recommend that cervical cancer screening be initiated at age 21 years and continued until 65 (Berek and Hacker, 2015). Screening should occur every 3–5 years. It should be noted that positive screening tests generally need to be followed up with a diagnostic test or repeat screening testing needs to be performed at a shorter screening interval. Confirmatory diagnostic testing typically entails a colposcopy with directed cervical biopsies for histopathologic diagnosis.

Another component to prevention of cervical cancer is HPV vaccination. Being vaccinated for HPV reduces the risk of cervical precancers and cancers by >92% by protecting against HPV 16 and 18. HPV vaccination is recommended as a part of routine vaccinations between the ages of 9 and 26 years old.

References

- Berek, J. S., & Hacker, N. K. (2015). *Berek & Hacker's Gynecologic Oncology* (6th edn.). Philadelphia: Wolters Kluwer. Print.
- Cannistra, S. A., & Niloff, H. M. (1996). Cancer of the Uterine Cervix. *The New England Journal of Medicine*, 334, 1030–1038.
- Mutch, D. G. (2009). The new FIGO staging system for cancers of the vulva, cervix, endometrium and sarcomas. *Gynecologic Oncology*, 115, 325–328.
- Schiffman, M., & Solomon, D. (2013). Cervical-cancer screening with human papillomavirus and cytologic cotesting. *The New England Journal of Medicine*, 369, 2324–2331.
- Schiffman, M., Castle, P. E., Jeronimo, J., Rodriguez, A. C., & Wacholder, S. (2007). Human papillomavirus and cervical cancer. *Lancet*, 370(9590), 890.

Vulvar Cancer

Vivek Arora and Neville F Hacker, Royal Hospital for Women, Randwick, NSW, Australia; and University of New South Wales, Randwick, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Colposcope Medical instrument to examine cervix and vagina under magnification.

Dystrophy Abnormal growth of skin on the vulva.

Eczematoid Eczema like skin condition.

Histology Microscopic tissue study.

Immunodeficiency Deficiency in immune response.

Infiltrative Penetrating into the deeper tissues.

Intraepithelial Remaining within the skin.

Lymphadenectomy Removal of lymph nodes.

Lymphedema Persistent tissue swelling from lymphatic fluid after removal of lymph nodes.

Lymphocyst Collection of lymph fluid after removal of lymph nodes.

Malignancy Cancerous growth.

Neoplasia New uncontrolled growth of cells.

Premalignant Precancerous stage.

Introduction

Vulvar cancer is a rare condition, accounting for approximately 5% of all gynecological cancers and < 1% of all cancers diagnosed in women. Typically, vulvar cancer has been considered a disease of old age but increasingly is being diagnosed in younger women. Although arising from an external site, vulvar cancer is often diagnosed at a late stage due to delay in seeking medical advice and delayed diagnosis.

Histologically, squamous cell carcinomas account for the vast majority (85%–90%) of vulvar cancers, with melanomas, basal cell carcinomas, Bartholin's gland carcinomas, invasive Paget' disease and adenocarcinomas arising from the skin appendages accounting for the rest.

The last century has witnessed vast improvements in the management of vulvar cancer, with a significant improvement in 5-year survival. Improved surgical techniques have also improved overall quality of life following treatment ([Hacker and Eifel, 2015](#)).

Risk Factors for Squamous Cell Carcinoma

The risk factors for developing squamous cell vulvar cancer include:

- Smoking
- Vulvar dystrophies such as lichen sclerosus
- Human papilloma virus (HPV) infection
- Vulvar intraepithelial neoplasia (VIN), a premalignant condition of the vulvar skin
- Immunodeficiency syndromes and immunodeficiency states such as post organ transplantation
- Prior history of cervical cancer, which reflects the common association with HPV infection

Vulvar intraepithelial neoplasia (VIN) is an important precursor lesion for vulvar cancer. The International Society for the Study of Vulvovaginal Disease (ISSVD) classifies VIN into two types: the "usual type VIN," which is related to HPV infection and the "differentiated VIN" which is unrelated to HPV and is often diagnosed in association with the diagnosis of lichen sclerosus ([Sideri et al., 2004](#)).

Similarly, two etiologic types of vulvar cancer are recognized. The first type, often diagnosed in younger women, is related to HPV infection and smoking, and is commonly associated with usual type VIN. The second type is diagnosed more commonly in elderly women, is often associated with vulvar dystrophy, most commonly lichen sclerosus, is sometimes associated with differentiated VIN, and is unrelated to HPV infection.

Histologic Types of Vulvar Cancer

Squamous cell carcinoma (SCC) is, by far, the predominant histologic type comprising over 90% of all vulvar cancers. This chapter will concentrate on the management of squamous cell carcinomas.

Melanoma is the second most common type of vulvar cancer, and represents about 5%–10% of lesions. They occur predominantly in post-menopausal white women, most frequently on the labia minora and clitoris (**Fig. 1**). There are no well-defined risk factors that may predispose to the development of vulvar melanomas. Most melanomas are asymptomatic, and any pigmented lesion on the vulva should be biopsied, unless it is known to have been present and unchanged for a long time.

Basal cell carcinomas (BCC) comprise approximately 2% of all vulvar malignancies. Like BCCs elsewhere in the body, they progress locally, but rarely spread to lymph nodes.

Extramammary Paget's disease accounts for <1% of vulvar malignancies. It mainly presents as intraepithelial malignancy, but about 20% are associated with an underlying adenocarcinoma. It predominantly affects post-menopausal white women, and usually presents with vulvar itching and soreness. Clinically, the lesion has an eczematoid appearance, and usually begins on the hair-bearing areas of the vulva. Occasionally, the vulvar disease is secondary to a cancer of the bowel or cancer arising in the bladder, particularly if it is located around the anus or urethra. Even after wide surgical excision, the intraepithelial Paget's disease frequently recurs, because microscopic disease usually extends well beyond the macroscopic lesion (**Fig. 2**).

Bartholin's gland cancer is the least common of the epithelial cancers arising in the vulva. They are usually adenocarcinomas, but may be squamous cell carcinomas if the tumor arises from the duct. These tumors are solid and deeply infiltrative and often diagnosed late due to being misinterpreted as a Bartholin's gland abscess. Not uncommonly, a woman presents with what is thought to be a recurrent Bartholin's gland abscess, but this should raise a suspicion of an underlying malignancy. Bartholin's gland abscess is rare in a postmenopausal woman and should always be regarded as suspicious for malignancy.

Sarcomas, most commonly leiomyosarcomas, are connective tissue tumors that are known to arise as primary tumors in the vulva on rare occasions.



Fig. 1 Melanoma of left labium majus.



Fig. 2 Paget's disease of the vulva.

Clinical Presentation

The signs and symptoms of all histologic types are similar. Most women present with a vulvar lesion, ulcer or a mass. In 5% of cases, the origin of the disease is multifocal.

Vulvar itching and irritation, usually due to VIN or lichen sclerosis, have often been present for an extended period leading up to the diagnosis. Vulvar bleeding, discharge, painful urination, or lump in the groin are late symptoms, and often herald advanced stage disease at presentation. Symptoms of vulvar cancer can often mimic other benign conditions of the skin such as genital herpes, dermatoses, lichen sclerosis, fungal infections and benign genital warts (condyloma acuminatum).

Most cases of vulvar cancer develop on the labia majora. However, the disease can start in any part of the vulva including the clitoris, posterior fourchette and labia minora.

Diagnosis and Investigation

The initial assessment of vulvar cancer includes a thorough history of the presenting symptoms, their duration, an assessment of risk factors and physical examination. Diagnosis depends on biopsy of the lesion and histological review.

Physical examination may reveal an obvious mass or lesion that should be biopsied under a local anesthesia in most cases, but may require an examination under anesthesia if the area is too painful to allow biopsy in the clinic. In the case of small lesions or multifocal disease, application of acetic acid and examination using a colposcope may assist in mapping the extent of disease. More than one biopsy may be required if multifocal disease is suspected. Colposcopy should also be performed on the cervix and vagina, to exclude other foci of in situ or invasive squamous cell carcinoma, as many of these lesions are caused by the human papilloma virus.

The assessment of the vulvar tumor involves assessment of its fixity to underlying structures and its relationship to the midline. Tumors lying within 1–2 cm of the midline are more likely to involve lymph nodes bilaterally due to cross drainage of the lymphatics. The physical examination should include an assessment of any involvement of the vagina, anus or urethra, and the inguino-femoral lymph nodes should be palpated to rule out clinical involvement.

A workup to rule out metastatic disease is recommended even in the absence of obviously involved inguino-femoral lymph nodes. A chest X-ray and a CT scan of the pelvis and abdomen are reasonable for patients with early vulvar cancer. For patients with advanced disease, a positron emission tomography (PET) scan may be used to establish the extent of any metastatic disease. Pelvic lymph node involvement and or distant metastatic disease in other organs may have an impact on treatment decisions. Fine needle aspiration cytology (FNAC) may be performed on sites of apparent metastatic disease to confirm the diagnosis prior to definitive management.

Routes of Spread

Vulvar cancer spreads by

- Direct extension to involve adjoining structures such as the vagina, urethra and anus.
- Lymphatic spread to the lymph nodes of the groin or inguino-femoral nodes. The disease can spread to pelvic lymph nodes in up to 20% of patients who are diagnosed with metastatic disease in inguino-femoral lymph nodes. Involvement of lymph nodes can occur early and the risk increases as the size of the primary tumor and the depth of invasion increases—ranging from 7% for cancers measuring up to 1 cm in diameter to over 30% for cancers measuring over 3 cm ([Gonzalez Bosquet et al., 2007](#)). Overall, about 30% of women presenting with vulvar cancer will be diagnosed with lymph node involvement ([Hacker and Eifel, 2015](#)).
- Hematogenous spread via blood stream to distant sites such as the liver, and lungs. This is rare and a late phenomenon, usually noted only in women with involvement of multiple lymph nodes.

Staging

International Federation of Gynecology and Obstetrics (FIGO) adopted a surgical staging system for vulvar cancer in 1988, which was modified in 2009 ([Table 1](#)).

Principles of Treatment

Treatment of vulvar cancer involves treatment of the primary lesion and an assessment of the risk of involvement of the inguino-femoral lymph nodes. Wherever possible, primary surgery is the preferred modality of treatment, allowing for surgical staging and tailoring of adjuvant therapy if necessary.

Vulvar cancer was typically diagnosed at an advanced stage in the early part of the 20th century, and coupled with poorly developed surgical techniques; the survival rates were in the range of 20%–25%. Patients were treated by resection of the primary lesion

Table 1 FIGO surgical staging for vulvar cancer 2009

FIGO stage	Clinical/pathologic findings
Stage I	Tumor confined to vulva, no evidence of nodal disease
IA	Lesions measuring < 2 cm in size and < 1 mm deep
IB	Lesions measuring > 2 cm in size OR > 1 mm deep
Stage II	Tumor of any size with extension to adjacent perineal structures (lower 1/3 urethra, lower 1/3 vagina, anus) with negative nodes
Stage III	Tumor of any size with or without extension to adjacent perineal structures with positive inguino-femoral lymph nodes
IIIA	(i) 1–2 lymph nodes with metastases ≤ 5 mm
	(ii) 1 lymph node with metastasis ≥ 5 mm
IIIB	(i) ≥ 3 lymph nodes with metastases ≤ 5 mm
	(ii) ≥ 2 lymph nodes with metastases ≥ 5 mm
IIIC	Positive nodes with extra-capsular spread
Stage IV	Tumor invades upper two-third of regional structures like urethra and vagina OR distant metastases
IVA	(i) Tumor invades upper two-third of regional structures like urethra and vagina
	(ii) Fixed or ulcerated inguino-femoral lymph nodes
IVB	Any distant metastases including pelvic lymph nodes

Adapted from FIGO Committee on Gynecologic Oncology (2009). Revised FIGO staging for carcinoma of the vulva, cervix and endometrium. *International Journal of Gynecology and Obstetrics* **105**, 103–104.

without adequate resection of the groin and pelvic lymph nodes (Hacker and Eifel, 2015). Taussig in United States and Way from Great Britain pioneered radical surgery that dramatically improved the survival rates by threefold to 60%–70% (Taussig, 1940; Way, 1960). Their radical surgery for vulvar cancer involved radical vulvectomy with an en-bloc removal of the inguino-femoral lymph nodes (Fig. 3). Although vastly improving cure rates, the procedure was associated with severe post-operative morbidity including groin wound breakdown and infection, deep venous thrombosis and prolonged hospitalization. This procedure was often combined with pelvic exenterative procedures to remove disease involving the anus and the urethra.

There has been a paradigm shift in the management of vulvar cancer since the 1980s. These changes have consisted mainly of a more conservative resection of the primary tumor (radical local excision rather than radical vulvectomy), the use of separate incisions rather than an en bloc approach for the groin dissection, the use of unilateral rather than bilateral groin dissection for tumors which are not within 1–2 cm of the midline, and the appropriate integration of radiation therapy into the treatment protocol (Fig. 4). These modifications have been shown to significantly decrease post-operative without compromising survival (Hacker and Eifel, 2015; Taussig, 1940).

Anatomically, a radical vulvectomy involves the removal of the entire vulva, along with the subcutaneous tissues down to the periosteum of the pubic bone anteriorly and the deep urogenital fascia on the posterior and lateral aspects of the vulva. Radical vulvectomy is still occasionally required for very large lesions or for multifocal disease. It is associated with a high incidence of altered sexual function, body image issues and long-term psychological morbidity (Fig. 5A and B) (Andersen and Hacker, 1983).

Modified radical vulvectomy or radical local excision is now performed for unifocal lesions, with the intention of preserving the appearance and function of the vulva. This approach also allows for preservation of the clitoris in women diagnosed with posterior or lateral lesions. Removal of clitoris, whilst frequently unavoidable in anterior vulvar cancer, carries a particularly significant psychosexual morbidity.

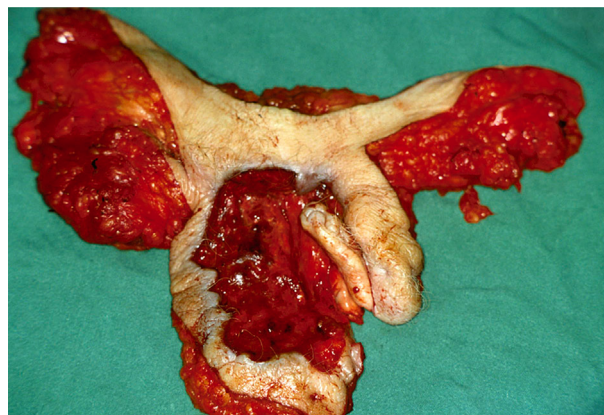
**Fig. 3** En-bloc radical vulvectomy and bilateral inguino-femoral lymphadenectomy for a large squamous cell carcinoma of the vulva.



Fig. 4 Radical local excision and unilateral groin dissection for a left posterior vulvar cancer >1 cm from the midline.

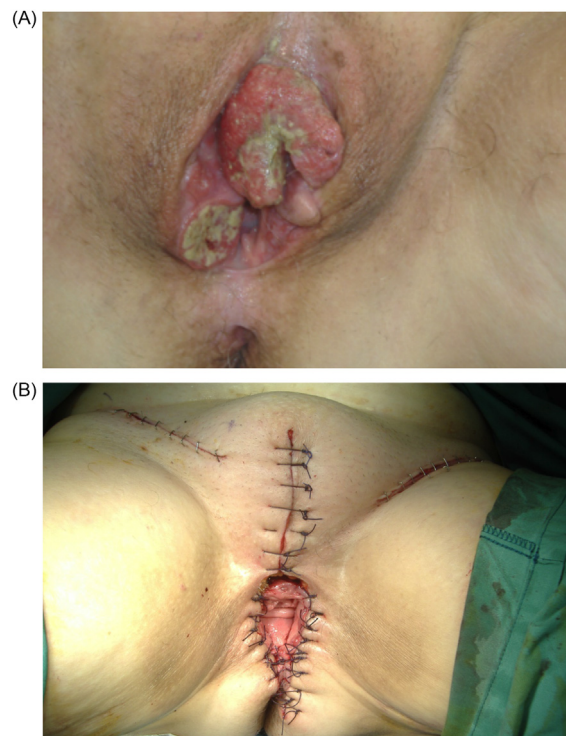


Fig. 5 (A) Large vulvar cancer. (B) Radical vulvectomy and bilateral inguino-femoral lymphadenectomy through separate groin incisions for multifocal squamous cell carcinoma.

Our experience at the Royal Hospital for Women in Sydney would suggest that modified radical vulvectomy can be performed in over 90% of women with disease confined to the vulva, with an overall survival of about 95% at 5 years if the lymph nodes are negative for metastatic disease. The main reasons for performing radical vulvectomy were evidence of multifocal disease or widespread preinvasive disease in addition to the primary cancer ([Tantipalakorn et al., 2009](#)).

Regardless of whether a radical or modified radical vulvectomy is required, a margin of 1–2 cm of normal skin around the visible disease is necessary to avoid local recurrence. Because tissue contracts by about 20% once it is fixed in formalin, a macroscopic surgical margin of 10 mm equates to a histologic margin of 8 mm. If the histologic margin is <5 mm, further surgical resection is indicated. If this is not feasible, because of proximity to the anus or clitoris, for example, adjuvant radiation may be given.

Except in very early stage vulvar cancer (stage IA), patients need to undergo removal of the lymph nodes from one or both groins. Adjuvant radiation to the groins and pelvis may be required, depending on the status of the nodes.

Occasionally, the sequence of treatment may be reversed if there is a large cancer, which would necessitate radical vulvectomy combined with some type of exenterative procedure in order to resect the disease surgically. Primary radiotherapy may be used to shrink the tumor and hopefully avoid the need for urinary or bowel diversion.

Treatment of Early Stage Disease

Early stage vulvar cancer may be defined as (i) the primary tumor confined to the vulva or involving the distal urethra and/or vagina, and amenable to surgical resection without the need for exenterative surgery, and (ii) no clinically involved inguino-femoral lymph nodes.

Malignant vulvar lesions < 2 cm

For vulvar cancers where the maximal dimension of the lesion is < 2 cm and the preoperative biopsy shows a depth of invasion < 1 mm, radical local excision with surgical margins of at least 1 cm should be performed. The entire cancer should then be serially sectioned and examined histologically. If no foci with invasion deeper than 1 mm are found, no groin dissection is required. At least a unilateral inguino-femoral lymph node dissection is required if the final pathology shows the depth of invasion to be > 1 mm.

For tumors < 2 cm in maximal dimension, the risk of lymph node involvement increases with increasing depth of invasion. The risk is < 1% with invasion < 1 mm, increasing to approximately 34% with depth of invasion > 5 mm ([Hacker and Eifel, 2015](#)).

Performing the surgery in two stages with apparent stage IA disease spares a significant proportion of women from the risks and morbidity associated with inguino-femoral lymph node dissection.

Malignant vulvar lesions > 2 cm with or without involvement of adjacent structures

Women presenting with apparent stage IB and II disease (with involvement of the lower vagina or urethra) should be offered radical local excision, or radical vulvectomy if there is multifocal disease. Disease involving the distal urethra can be managed surgically, because at least 1 cm of terminal urethra can be resected without causing urinary incontinence.

Inguino-femoral lymph node dissection should be performed at the time of primary surgery. It is critical to appropriately manage the regional lymph nodes once the diagnosis of vulvar cancer has been confirmed, because recurrent disease in an undissected groin is associated with a very high mortality rate.

Unilateral groin dissection is appropriate for most tumors, but bilateral dissection is required for tumors that involve midline structures or encroach to within 1–2 cm of the midline.

Inguino-femoral lymph node dissection involves an incision just above and parallel to the groin crease. The major vessels of the lower limb, namely the femoral artery, femoral vein and the great saphenous vein, must be identified. The inguinal lymph nodes are situated in the femoral triangle between the superficial fascia and the fascia lata. The femoral nodes, usually only 1–3 in number, are located medial to the femoral vein under the cribriform fascia.

Treatment of Positive Inguinal-femoral Lymph Nodes

If one microscopic lymph node metastasis is found at inguino-femoral lymphadenectomy, no further treatment is required and the patient should have a very good prognosis. If two or more positive nodes are found, or if there is any extracapsular spread histologically, the patient should receive adjuvant groin and pelvic radiation. Earlier practice was to perform a pelvic lymphadenectomy when positive groin nodes were present, but a randomized trial conducted by the Gynecologic Oncology Group showed that these patients had better survival and fewer groin recurrences if treated with bilateral groin and pelvic radiation ([Homesley et al., 1986](#)).

Treatment of Advanced Vulvar Cancer

Advanced vulvar cancer includes disease that is associated with (i) extension of the cancer to the anus, or more proximal vagina and/or urethra, and/or (ii) clinically enlarged groin lymph nodes.

Treatment of Locally Advanced Vulvar Cancer

Vulvar cancer continues to be diagnosed in an advanced stage in a significant proportion of cases. The symptoms, such as itching, have usually been ignored for a prolonged period of time by the patient, and often treated with creams without physical examination by the physician.

Primary surgical management of advanced vulvar cancer would require radical vulvectomy and en bloc resection of one or more pelvic organs, such as the urethra and bladder, vagina and/or anus and rectum. A surgical approach would inevitably necessitate diversion of urine via an ileal conduit and/or fecal diversion via an end colostomy.

In recent years, primary treatment with radiotherapy has been used with great success to shrink or completely treat the vulvar cancer, and avoid the need for a stoma. The radiotherapy may completely eliminate the primary tumor in some cases, but in the majority of cases, it shrinks the tumor and allows a more limited vulvar resection, thereby preserving normal bowel and bladder function ([Moore et al., 2012](#)).

Primary radiotherapy leads to devascularization of the tissues, leading to poor wound healing. Hence, if a large vulvar resection is required as part of the primary treatment or for a local recurrence, there is often a need for plastic surgery. A myocutaneous graft is usually required from the abdomen, buttocks or thigh to close the resultant defect, and to bring a new blood supply into the area to aid in the healing process.

Treatment of Clinically Enlarged Groin Nodes

If patients present with palpably enlarged groin nodes, it is desirable to resect the enlarged nodes and obtain a frozen section to confirm that they contain metastatic disease. If the nodal enlargement is shown to be due to inflammatory changes only, complete inguino-femoral lymphadenectomy should be performed.

Much more commonly, the frozen section will confirm the presence of metastatic disease. In such patients, complete inguino-femoral lymphadenectomy can be avoided, because post-operative radiation will be required to the groins and pelvis, and this will sterilize any microscopic disease that may be present in the un-dissected lymph nodes (**Fig. 6**). Avoiding complete groin dissection in this circumstance minimizes the immediate post-operative risk of developing a lymphocyst or wound infection, so radiation can begin without any significant delay. It also decreases the risk of the later development of lower limb lymphedema ([Hyde et al., 2007](#)).

Rarely, a patient presents with groin nodes that are fixed to underlying structures, such as muscles or blood vessels, or even ulcerating through the skin. Radiotherapy, with or without chemotherapy, should be given, and if significant shrinkage occurs, it may be possible to resect the tumor mass. Erosion of a major femoral blood vessel and fatal hemorrhage is a rare complication.

Sentinel Lymph Node Detection

Sentinel node detection for vulvar cancer has been increasingly used over the past decade. The concept was initially introduced in 1977 for the management of men with penile cancer, and has subsequently been routinely adopted in the management of patients with breast cancer ([Cabanias, 1977](#)).

The hypothesis is that lymphatic drainage from a tumor occurs in an orderly fashion, and if the sentinel node (or nodes) is negative, all other nodes will be negative, so complete groin dissection can be avoided, thereby decreasing the risk of lower limb lymphedema.

The procedure involves the intraoperative intradermal injection of a tracer, isosulfan blue dye, around the tumor, preferably in conjunction with a radioactive colloid (technetium), which is injected several hours earlier. The blue dye tracer is visible to the naked eye, and a blue node can be identified in most cases (**Fig. 7**). The detection rate is increased by the use of the radiotracer, but a gamma probe is required intraoperatively to detect the radioactive node. If the sentinel node is histologically negative on routine hematoxylin and eosin staining, ultrastaging involves taking serial sections from the lymph node and performing immunoperoxidase staining for cytokeratin.

The main concern about sentinel node detection for vulvar cancer is the false negative rate, which will be at least 5%–10% in most centers. The reported false negative rates have ranged from 3% in the setting of the Groningen multicenter study in the Netherlands to 27% in a single center study from Poland ([Van der Zee et al., 2008](#); [Radziszewski et al., 2010](#)). Vulvar cancer is uncommon, and sentinel node detection is only appropriate for tumors up to 3–4 cm diameter, so individual experience with the technique will be limited. Recurrence in an un-dissected groin carries a high mortality and most patients, when properly informed, are not prepared to take this risk ([Farrell et al., 2014](#)). Hence, sentinel node dissection should only be performed for patients with vulvar cancer after properly informed consent.

Indications for Radiotherapy

Radiotherapy may be used for patients with vulvar cancer as primary treatment or in an adjuvant setting.

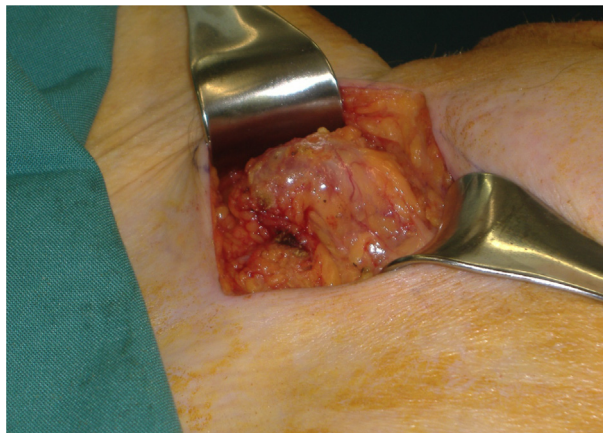


Fig. 6 Resection of a large positive lymph node from the left groin.



Fig. 7 Groin sentinel node detection using Patent Blue Dye.

Primary Radiotherapy

Radiotherapy may be offered as the primary treatment in patients where (i) the medical comorbidities preclude a surgical approach, (ii) there is locally advanced disease, and a surgical approach would necessitate a urinary stoma or colostomy, (iii) there are fixed and/or ulcerated inguino-femoral lymph nodes.

Occasionally particularly in younger patients, a small carcinoma involving the clitoris may be treated with radiation in order to preserve the clitoris.

Adjuvant Radiotherapy

Adjunct radiation is indicated in the following circumstances:

- To prevent local recurrence if the surgical margins around the primary tumor are close (<5 mm) or positive, and further resection cannot be undertaken (e.g., because of proximity to the anus or urethra).
- If more than one micro metastasis is identified after full inguino-femoral lymph node dissection. In this situation both groins and the pelvis should receive adjuvant radiation therapy.

Role of Plastic Surgery in Management of Vulvar Cancer

In most cases it is possible to achieve primary closure after surgical resection of a vulvar cancer. However, primary closure may not be possible if the cancer is extending onto the thigh or buttocks (**Fig. 8A–C**). Resection of recurrent cancers, particularly if there has been prior vulvar radiation, may also require some type of myocutaneous graft or pedicle flap, because radiation causes poor tissue vascularity and progressive fibrosis. It is important to have a plastic surgeon in the multidisciplinary team, because the disease is uncommon and the range of surgical procedures required is considerable.

Complications of Treatment for Vulvar Cancer

The major immediate post-operative morbidity relates to the groin dissection. Wound infection and breakdown have become much less common since the introduction of the separate incision approach, but lymphocyst formation occurs in about 40% of cases. Breakdown of the vulvar wound is relatively common, particularly for posterior incisions, the healing of which is hindered by sitting, but closure by secondary intention is always very effective ([Hacker and Eifel, 2015](#)).

Late complications include lower limb lymphedema from the groin dissection, which occurs in up to 60% of cases, and psychosexual distress from the vulvar surgery. It is important to provide ongoing support for both. As part of the multidisciplinary team management of vulvar cancer, early referral to a physiotherapist should be offered for lymphedema advice and management, and referral to a clinical psycho-oncologist should be offered for psychosexual counseling ([Hacker and Eifel, 2015](#)).

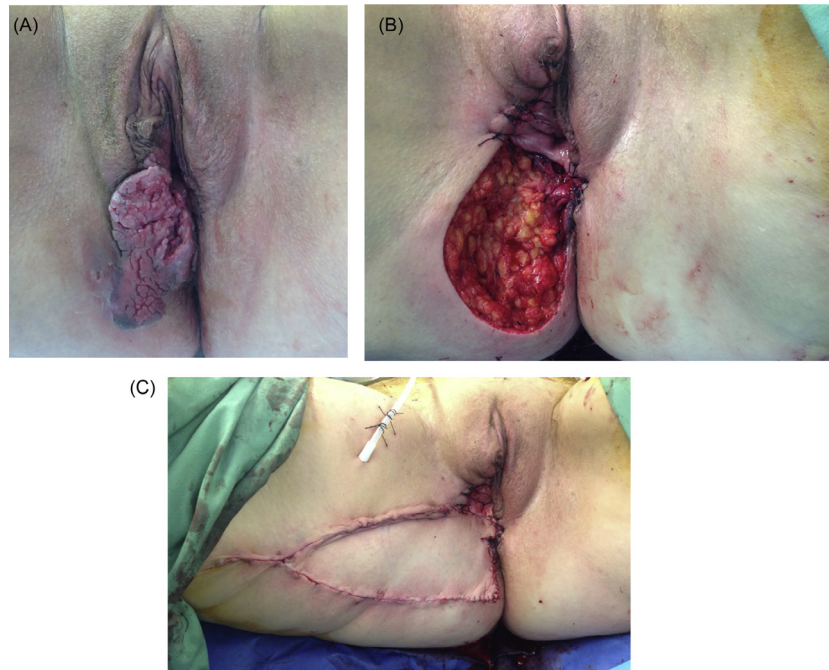


Fig. 8 (A) Large posterior vulvar cancer. (B) Radical local excision of a large posterior squamous cell carcinoma extending on to the right buttocks. (C) Large VY flap used to close the defect.

Conclusion

Vulvar cancer is best managed in the setting of a multidisciplinary team that includes a gynecological oncologist, radiation oncologist, clinical nurse specialist, clinical psychologist, a nursing team specializing in post-operative care following vulvar surgery, physiotherapist and a wound care practitioner. This is a very uncommon malignancy that requires life-long follow up because of its significant tendency to recur even many years later. In addition, the late side effects require ongoing care in a multidisciplinary team setting.

References

- Andersen, B. L., & Hacker, N. F. (1983). Psychosexual adjustment after vulvar surgery. *Obstetrics and Gynecology*, 62, 457–462.
- Cabanas, R. M. (1977). An approach for the treatment of penile carcinoma. *Cancer*, 39, 456–466.
- Farrell, R., Genski, V., & Hacker, N. F. (2014). Quality of life after complete lymphadenectomy for vulvar cancer: Do women prefer sentinel node biopsy. *International Journal of Gynecological Cancer*, 24, 813–819.
- Gonzalez Bosquet, J., Magrina, J. F., Magtibay, P. M., et al. (2007). Patterns of inguinal groin metastases in squamous cell carcinoma of the vulva. *Gynecologic Oncology*, 105, 742–746.
- Hacker, N. F., & Eifel, P. J. (2015). Vulvar cancer. In J. S. Berek, & N. F. Hacker (Eds.), *Gynecologic oncology* (6th edn). Philadelphia: Wolters Kluwer.
- Homesley, H. D., Bundy, B. N., Sedlis, A., et al. (1986). Radiation therapy versus pelvic node resection for carcinoma of the vulva with positive groin nodes. *Obstetrics and Gynecology*, 68, 733–740.
- Hyde, S. E., Valmadre, S., Hacker, N. F., et al. (2007). Squamous cell carcinoma of the vulva with bulky positive groin nodes—Nodal debulking versus full groin node dissection prior to radiation therapy. *International Journal of Gynecological Cancer*, 17, 154–158.
- Moore, D. H., Ali, S., Koh, W. J., et al. (2012). A phase II trial of radiation therapy and weekly cisplatin chemotherapy for the treatment of locally advanced squamous cell carcinoma of the vulva. A gynecologic oncology group study. *Gynecologic Oncology*, 124, 529–533.
- Radziszewski, J., Kowalewska, M., Jedrzejczak, T., et al. (2010). The accuracy of the sentinel node concept in early stage squamous cell vulvar carcinoma. *Gynecologic Oncology*, 116, 473–477.
- Sideri, M., Jones, R. W., Wilkinson, E. J., et al. (2004). Squamous vulvar intraepithelial neoplasia: 2004 modified terminology, ISSVD Vulvar Oncology Subcommittee. *The Journal of Reproductive Medicine*, 50, 807–810.
- Tantipalakorn, C., Robertson, G., Marsden, D. E., et al. (2009). Outcome and patterns of recurrence for International Federation of Gynecology and Obstetrics (FIGO) stages I and II squamous cell vulvar cancer. *Obstetrics and Gynecology*, 113, 895–901.
- Taussig, F. (1940). Cancer of the vulva: An analysis of 155 cases. *American Journal of Obstetrics and Gynecology*, 40, 764–770.
- Van der Zee, A. G., Oonk, M. H., De Hullu, J. A., et al. (2008). Sentinel node dissection is safe in the treatment of early-stage vulvar cancer. *Journal of Clinical Oncology*, 26, 884–889.
- Way, S. (1960). Carcinoma of the vulva. *American Journal of Obstetrics and Gynecology*, 79, 692–697.

Further Reading

- Gonzalez Bosquet, J., Magrina, J. F., Gaffey, T. A., et al. (2005). Long term survival and disease recurrence in patients with primary squamous cell carcinoma of the vulva. *Gynecologic Oncology*, 97, 828–833.

TREATMENT: SURGICAL

Current Techniques and Tools in the Surgical Treatment in Reproductive Medicine

Guillermo Marconi, Instituto Valenciano de Infertilidad (IVI-RMA), Buenos Aires, Argentina

© 2018 Elsevier Inc. All rights reserved.

Introduction

Selection

Surgery demands a strict selection of the patient and her pathology in order to be able to get acceptable results. If a comparative analysis is made between the requirements of in vitro fertilization and surgery the following can be analyzed:

1. *Age*: This factor is preponderant in both treatments. It can be discussed if a young patient, less than 30 years old, should be operated or not, but it would be unquestionably contraindicated for a 35 year old patient, because if the surgery treatment fails, after an 18 month wait her fertility will have decreased drastically due to age, and when she resorts to high complexity assisted fertilization techniques the results would be poor compared to the expectations of her initial age.
2. *Etiology of the illness*: This concept is fundamental in surgery; the recovery prognosis for tubal damage due to a compression adhesive process caused by endometriosis, where the endosalpinx is intact is not the same as the same obstruction caused by a bacterial endosalpingitis where the epithelium of the tube is severely affected. In the first instance the permeability is damaged, in the second the permeability and the functionality are damaged; this is of no concern for in vitro fertilization.
3. *Extent of the illness*: It's obvious that better surgical results are expected in minimal adherence processes, for example, delicate small, thin adherences without vascularization than in a total blockage of the pelvis due to firm, thick adherences with major vascular compromise; once again, this is indifferent for in vitro fertilization.
4. *Related sterility factors*: A profile of a low responsive female and/or an altered masculine factor, Assisted Reproductive Techniques are indicated; surgery is discarded.

Another determining factor in the use of surgery or high complexity techniques is the socio-economical environment.

Not acknowledging that in spite of living in a modern world, full of technology, the socio-economical situation of a patient or of a country can determine a therapeutical attitude is assistential blindness. In cases of personal or demographic poverty where there are no adequate means for the use of the latest technologies, surgery would be indicated even in those cases of poor prognosis.

Surgery can be approached in two ways: laparotomy, which implies going into the abdomen through a wound in the abdominal wall, or laparoscopy which employs endoscopy elements introduced in the abdomen through small incisions. The surgical technique to be used is microsurgery, which has nothing to do with the way that has been chosen.

Halfway through the 1960s microsurgery imposed fundamental concepts and principles which changed the surgical management of the female pelvis. It emphasize the need to treat the pelvic cavity and the related organs with a meticulous, purified surgical technique, with rigid guidelines, independently of the patient being sterile or not. If these recommendations are not followed, surgeons will be potentially responsible for causing severe damages that would make women sterile.

Simultaneously, the conservative factor in surgery was built up. A cycle of total ablations was over, to begin the era of surgeries where the motto was to keep the organs and not to stubbornly remove them surgically without a reason; only the ablation of the pathology should be performed. [Gomel \(1997\)](#) states: microsurgery got hold more than as a surgical technique as a "surgical philosophy."

Instrumental

This philosophical and tactical change in pelvic surgery drive to the manufacture of highly delicate instruments in order to be adequate for these new principles.

The use of magnification devices is essential in laparotomy: at least a magnifying glass; ideally, an operating microscope. This allows us to see with great augmentation structures that would be unseen with an unaided eye, minimal details of sections and super delicate sutures that would be impossible to observe with an unaided eye.

Small scissors, pliers, coagulators, similar to those employed in ophthalmology surgery, some of which have been adapted in length to pelvic surgery.

Irrigation and constant flush is mandatory, the drying of pelvic organs and blood clots are the main cause of post-surgery adhesences.

The use of non-absorbable sutures of extremely fine diameter, 3/0, 4/0, or 5/0, with atraumatic needles is an essential requirement for these procedures.

Table 1 Comparing delivery rate, ectopic pregnancy rate and CPR between laparotomy and laparoscopy surgery

		Laparotomy (59)	Laparoscopy (22)
Delivery rate (DR)		21(36%)	7(32%)
Ectopic pregnancy rate		3(5%)	2(9%)
CPR	12 months	36%	57%
	24 months	40%	57%

DR, delivery rate; CPR, cumulative pregnancy rate.

Laparoscopic surgery comes into the scene and its massive and definite consolidation at the end of the 1980s, although some articles were published between the end of the 1970s and beginning of the 1980s (Gomel, 1975; Mettler et al., 1979).

The difference between both methods is the way of getting into the abdomen, the magnification devices, and the type of surgical instruments employed; the rest of the principles of microsurgery are kept intact. Then, the election of the technique would rely on the criteria of each surgeon to evaluate which of the two techniques he feels more skilled with.

The present preference has, with absolute majority, steered towards the use of laparoscopy. Anyway, it should be pointed out that the percentage of pregnancies and the accumulative pregnancy index aren't different between both techniques.

This is stated in a publication by Saravelos et al. (1995) of the Sheffield group, where no difference is found between patients treated with both techniques (Table 1).

Around 10 years ago the laparoscopy increased its surgery methodology precision with the incorporation of robotics.

The robot allowed for a very peculiar control: the surgeon stays inside a computerized console and from there, through the use of joysticks, moves the surgical instruments with unsurpassable precision. Regretfully, the robot is terribly expensive and doesn't seem to pose any major advantages regarding reproductive purposes.

The hysteroscope together with the resectoscope are incorporated to the surgery reproductive therapeutic arsenal. Any damage that affect the uterine cavity are treated with them.

Surgical Techniques

The surgical techniques can be divided as follows:

1. Adhesiolysis or removal of adhesions.
2. Restitution of permeability of the Fallopian tubes.
3. Surgical removal of tumor-like pathologies.
4. Correction of malformations of the reproductive system.
5. With endocrinological purposes.

Adhesiolysis or Removal of Adhesions

Adhesiolysis or removal of adhesions is the surgical action that tends to free the genital-pelvic organs of the adhesions fixing them.

The proceedings which are used are: divulsion, that is, to detach, break and separate the adhered structures with dragging or separation blunt maneuvers with the tips of an instrument—a scissor or tweezers.

Section: The slack adherences are cut and, when possible, surgically removed with scissors, monopole electrodes or laser; the selection of the instruments employed doesn't seem to have any consequence regarding pregnancy and ectopic pregnancy.

When the adhesions are thick the direct cut with scissors is risky; it could involuntarily cause severe injuries in the compromised organs—bladder, bowel—with severe post-surgery consequences; it is in these instances where the experience and the skill of the surgeon prevail (Video 1 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

The pregnancy percentage in adhesiolysis varies between 25% and 60%, with a 7% (Bruhat, 1983; Donnez, 1989; Favez, 1983; Gomel, 1983; Serour, 1989) ectopic pregnancy index (Table 2).

In the instance of uterine synechias, —adherences, which alters the uterus cavity—an operating hysteroscope, is employed, where a scissor or a mono or bipolar microelectrode is introduced through the operating channel to cut them. A resectoscope can be employed for this procedure, but its larger diameter is a drawback, implying the need to dilate the uterine cervix, which is not always an easy procedure.

Restitution of the Fallopian Tubes Permeability

The aim is to reestablish the integrity of the tubal lumen that for some reason has been totally or partially occluded.

The following are surgical techniques depending on which segment of the tube is affected:

Table 2 Pregnancy rate in adhesiolysis among different instrument used

<i>Author</i>	<i>Technique</i>	<i>No.</i>	<i>% Pregnancy</i>
Gomel (1983)	Scissors	92	62
Fayez (1983)	Scissors	50	56
Donnez (1989)	CO ₂ laser	186	58
Serour (1989)	Scissors-electrocoagulation	25	12
Bruhat (1983)	Scissors-electrocoagulation	93	52

Results in adhesiolysis.

Termino-terminal anastomosis

This technique is applied when the tubal lumen is obliterated at interstitial or medial level.

The goal is to join the two ends of the tube once the obstructed area has been removed.

When the obstruction is cornual (interstitial) the anastomosis can be performed between the isthmic portion of the tube and the uterine cornu. When it is medial, between the two healthy isthmic portion and among isthmic portion and the ampulla when obstruction is in the ampular-isthmic junction; therefore, there are three types of anastomosis: (a) isthmic-cornual, (b) termino-terminal, (c) isthmic-ampular.

Once the obstruction is localized, the occluded area is cut off, then the tubal lumen is canalized with a very thin plastic catheter that joins the distal and proximal tubal segments; this will serve as a guide for the suture stitches for the anastomosis.

The hemostasis has to be performed very carefully to avoid damaging the blood vessels running below the tube that irrigate the organ and carry information to the uterus and the ovary about the reproductive events.

Afterwards, suture stitches of non-absorbable material are made, 5/0 width, in the four cardinal points: mesenteric, internal, external, and superior.

Finally, the tutorial catheter is removed.

This procedure can be performed through laparoscopy and if robotic surgery is employed it's insurmountable.

The results achieved with this technique after 18 months are quite diverse and depend on the etiology which originate the obstruction. Obliteration caused by salpingitis or endosalpingiosis, pregnancies don't surpass 8% or 10%, with a percentage of ectopic pregnancies above 30%; on the other hand, if the goal was to reverse a tubal ligature, the results are between 50% and 80%, depending exclusively on the technique used to perform the ligature, with an ectopic pregnancy percentage between 2% and 10%.

Salpingostomy

It is meant to restore the permeability of the fimbrial or distal end of the tube which is obliterated by adhesions or fibrosis. As previously mentioned, the obstruction can be partial or total.

When it is partial and pre-fimbrial, the objective is to remove the ring which chokes the ampulo-fimbrial joint through dilatation; and if it were post-fimbrial, the adherence tissue fusing the fimbrias is surgically removed employing micro scissors. This procedure is known as fimbrioplasty.

For laparoscopic surgery a pair of atraumatic tweezers are employed; one of them secures the border of the tubal ostium in such a way that one of its ends is introduced inside the lumen of the tube, and the other is introduced in the serous; the second tweezers is introduced closed inside the ampullar cavity; once inside it is withdrawn open, breaking in this way the phimotic ring. Or it is introduced on the opposite end in the same way and both tweezers are removed in opposite directions tearing the phimotic ring.

When the ostium is totally obstructed—conglutination or hydrosalpinx—the tube has to be filled with methylene blue to cause the distention of the obstructed end; in this way a whitish irradiated image is observed, which corresponds to the cicatrization caused by the occlusive process. The opening is performed using a pair of micro scissors, following the path of the white scars (Video 2 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

The goal is to perform three cuts in the conglutinated peritoneum as to form three petals exposing the fimbrias.

Once the opening is performed and in order to avoid a relapse of the occlusion, three nonabsorbable suture stitches (4/0 wide) can be made in each one of the petals. Another quite useful procedure to achieve this goal is the use of a bipolar coagulator with very thin tips (2 mm), to coagulate the peritoneum of each petal; this proceeding achieves the desired peritoneal retraction and eversion.

The fimbrioplasty and the correction of the conglutination achieve approximately 60% of pregnancies with a 6% rate of ectopic pregnancies. On the other hand, in Salpingostomy by hydrosalpinx the pregnancy chances doesn't exceed 30% and a fourth of them are ectopic, both within a time lapse which oscillates between 12 and 18 months after surgery (Bruhat, 1983).

Surgical Removal of Tumor Formations

Uterine myomas, endometrial polyps and ovarian cysts can be co participant for affecting a woman's fertility.

Myomas

Independently from the classic indications for myoma surgery—volume, unexpected growth, neighboring organ compression and complications (painful and/or hemorrhagic)—in reproductive medicine it should be operated when the relationship between the fibroid and uterine cavity is altered.

Interstitial myoma shall be removed when its size is larger than 5 cm or when they compromise the uterine cavity: whether they are in contact with the endometrium or invading the space of the organ in the cavity in different proportions and shapes.

Interstitial myomas

The myomas which occupy the uterine wall can be surgically removed by laparotomy or by laparoscopy.

The surgical techniques of both ways to deal with this are similar: incision of the uterine serous and the myometrium to reach the location of the tumor, being sure you reach the cleavage area to, then, with bold maneuvers divide the fibroid and remove it (Video 3 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

If the surgery were laparoscopic, the division is performed with any blunt instrument and the traction of the myoma is performed with a strong tweezer; if it were laparotomic this is performed with the surgeon's finger (Video 4 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

It is advised to thoroughly coagulate the whole area where the myoma was located with bipolar energy.

The closing of the wound shall be performed in several levels, at least two. The reason for this is to avoid leaving free space in the area where the myoma was located. The ideal suture material is long term absorbent monofilament: PDS II (Polidioxanone) 4/0 in the layer next to the endometrial cavity, or to close the uterine cavity if it was opened during the surgical procedure, and 3/0 for the rest of the wall, including the uterine serous.

The PDS II suture has the property of minimizing the variability of retention in the tension strength and in the speed of absorption, besides rendering a longer support of the wound during the healing process (Video 5 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

At times, when these requirements are not met or not followed, the closing of the wound is made defectively, just at a superficial level, the serosa with only a couple of millimeters of myometrium. The consequence of this is that the walls of the wound don't close correctly, leaving open spaces which are filled with blood, fostering the formation of hematomas which will later be replaced by fibrous tissue; this causes the loss of elasticity of the surrounding myometrium, putting at risk the integrity of the uterine wall when a future pregnancy occurs, and exposing the patient to an uterine rip.

If there's an instance where robotic surgery stands out it is precisely the perfection with which the suture is performed; it can be stated as perfect.

When operating by laparoscopy several techniques and instruments have been designed to remove the myoma or myomas from the abdominal cavity.

- (a) *Rear colpotomy*: Implies the extraction of the myomas through an incision performed in the back of the rear sack of the vagina. It has to be of considerable size according to the dimensions of the fibroid and has the inconvenience of facilitating the escape of the gas used to make the pneumoperitoneum with the corresponding loss of vision due to the collapse of the abdominal cavity.
- (b) *Pfannenstiel micro incision*: A small size suprapubic transversal laparotomy. The criticism to this technique is that a small laparotomy has to be performed to remove the material obtained by laparoscopy, then, why not perform a laparotomy from the very beginning?
- (c) *Morcellation*: It implies the reduction of the fibroid's volume in a mechanical way. An instrument that performs circular cuts—a morcellator—is employed, which is manually or motor controlled and forms tissue cylinders with a 15–20 mm diameter, which are extracted through the surgical trocars.

It's a dangerous instrument because it has a sharp edge and if not used carefully the damage it can cause to the pelvic or viscera vessels is very serious. Furthermore, since the cuts are not perfect small rests of the myoma usually detach and are scattered through the pelvis and the abdomen; their complete removal is usually difficult to do and this minuscule remains can cause parasite myoma formations in different parts of the abdomen (Video 6 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

Myomas that compromise the uterine cavity

The submucous myomas are surgically removed with the use of hysteroscope or resectoscope.

The resectoscope is an endoscopic instrument, which has a cutting system consisting of an incandescent loop fed with mono or bipolar electric power. It performs slice cuts in the tumor-like formation until a complete removal is achieved. The uterine cavity is expanded with a glycine solution when monopole power is used or a physiological solution when bipolar power is used. It's very useful if the surgeon is assisted by ultrasound scan to evaluate the limits of the cut and reduce the risk of a uterine perforation (Video 7 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

Endometrial polyps

Its removal is similar to the one described for submucous myomas. The small polyps can be removed with the operating hysteroscope with the use of scissors and cauterization.

Ovarian cysts

The technique won't be analyzed for each specific case according to its etiology; it will be analyzed considering what's common among all of them.

It's worth pointing out that cystectomy is a field of laparoscopy, with or without robotics. The vision to permit this systematic isn't achieved through laparotomy because the eye of the surgeon is too far away from the pathology; while in laparoscopy, thanks to the optics, the observation is quite close and magnified; this allows to perform focused maneuvers that don't affect the integrity of the ovary.

Cystectomy demands some requirements to avoid irreversibly damaging the gonad.

- (A) *Decapsulate within the layer.* It's fundamental to locate the space separating the cyst from the ovarian parenchyma; once found, both surfaces are separated through traction and counter traction maneuvers performed with pressing endoscopic tweezers. The traction has to be performed quite close to the area where the parts separate. Traction performed too far away produces tearing of the ovary or of the cyst wall to be removed.
- (B) *Dragging of ovarian tissue.* The consequences of not observing the previous procedure will be the dragging of the gonadal tissue with the resulting damage to the organ and increased bleeding.
- (C) *Increased hemorrhage.* During the wall cyst removal, small blood vessels are damaged producing a focalized loss of blood; they should be locally coagulated with bi-polar tweezers with thin tips as soon as the hemorrhage appears; but the increase of bleeding due to an incorrect dragging of the ovarian tissue is notably more abundant; there's not a specific bleeding vessel, the bleeding is in the surface, the whole area bleeds. This phenomenon causes an excessive and abusive use of electric coagulation.
- (D) *Excessive coagulation.* Stubbornness and obstinacy to coagulate the whole bleeding surface to the point of causing a real calcination of the gonad. This excess leads, without fail, to:
- (E) *Irreversible damage of the ovarian function* (Video 8).
Irreversible damage of the ovarian function (Video 8 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

Surgery of Genital Malformations

The uterine malformation which has an indisputable surgical treatment is the uterine septum and its correction is endoscopic with the use of the operating hysteroscope or resectoscope.

If the former is used, just as it was described in the polyps' paragraph, scissors or mono or bipolar micro electrodes are introduced through its operating channel, to section the fibrous septum which divides the cavity.

The use of the resectoscope is somewhat more troublesome due to its larger diameter and the need to perform a cervical dilatation. As previously mentioned, the cutting loop with a curved profile or ending in an L shape, powered with mono or bipolar energy, are ideal to use for the section of the uterine septum (Video 9 in the online version at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>).

Ultrasound scan assistance is very useful, permitting to control the limit of the section.

With Endocrinological Purposes

It could be said that the use of surgery with endocrinological therapeutic purposes has been left absolutely set aside; historically, it was left just for the treatment of the ovarian polycystosis.

Three are the described techniques to be employed: (1) Cuneiform resection of the cortical of both ovaries, whether by laparotomy or laparoscopy. It consists in surgically removing a longitudinal wedge from both ovaries, running in depth from the ovarian cortex to the marrow of the gonad. Generally, the consequences are perianxial adhesions. (2) Multiple removals of the ovarian cortex with the use of biopsy tweezers through laparoscopy, and (3) Ovarian drilling, consisting of 4–10 perforations of the ovarian cortex through laparoscopy, with a monopole or bipolar needle with laser power, with a depth of no more than 5 mm and an electric equipment with a cutting power between 30 and 40 watts, in the case of monopole power.

In the present days it can be said that they have fallen out of fashion; exceptionally, the ovarian drilling is used by some surgeons in some exceptional instances.

Conclusion

To be able to round up a conclusion in a subject as ample as reproduction surgery is almost impossible: its indications are multiple, the surgical techniques are individual for each pathology and the instruments are many; nevertheless, it could be rounded up in four basic concepts; in order for surgery to be useful in sterile treatment it has to have: (a) an appropriate selection of the patient, (b) surgeons with excellent surgical training, (c) complete and state of the art surgery instruments, (d) a proven surgical technique. Supplementary data to this article can be found online at <https://doi.org/10.1016/B978-0-12-801238-3.64753-0>.

References

- Bruhat, M. A. (1983). Laparoscopy procedures to promote fertility: ovariolysis and salpingolysis. Results of 93 selected cases. *Acta Europaea Fertilitatis*, 14, 113–115.
- Donnez, J. (1989). CO₂ Laser laparoscopic surgery. Adhesiolysis, salpingostomy, laser uterine nerve ablation and tubal pregnancy. *Baillière's Clinical Obstetrics and Gynaecology*, 3, 525–543.
- Fayez, J. A. (1983). An assesment of the role of operative laparocopy in tuboplasty. *Fertility and Sterility*, 30, 476–479.
- Gomel, V. (1975). Laparoscopic tubal surgery in infertility. *Obstetrics and Gynecology*, 46, 47.
- Gomel, V. (1983). Salpingo-ovariolysis by laparoscopy in infertility. *Fertility and Sterility*, 28, 607–611.
- Gomel, V. (1997). *Te Linde's Operative Gynecology* (8th ed). Philadelphia: Lippincott-Raven Publishers.
- Mettler, L., Giesel, H., & Semm, K. (1979). Treatment of female infertility due to tubal obstruction by operative laparoscopy. *Fertility and Sterility*, 32, 384.
- Saravelos, H., Li, T.-C., & Cook, I. (1995). An analysis of outcome of microsurgical and laparoscopic adhesiolysis for infertility. *Human Reproduction*, 10, 2887.
- Serour, G. I. (1989). Laparoscopic adhesiolysis for infertile patients with pelvic disease. *International Journal of Gynecology & Obstetrics*, 30, 249–252.

Further Reading

- Schlaff, W. D. (1990). Neosalpingostomy for distal obstruction: prognosis factors and impact of surgical techniques. *Fertility and Sterility*, 54, 984–990.

Surgical Treatment of Asherman's Syndrome

Keith Isaacson, Harvard Medical School, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Intrauterine adhesions were first described in the late 19th century but the term Asherman's syndrome was not coined until 1948 by the Israeli gynecologist Joseph Asherman. He described 29 women who had amenorrhea and had scarring in the lower uterine segment as a result of endometrial trauma. Today we use the term Asherman's syndrome to describe the presence of intrauterine adhesions. The etiology of the formation of intrauterine adhesions is not well understood. Following trauma to the endometrium by surgery, curettage or infection, a reduction in vascular growth factors or involvement of adhesion promoting cytokines may be involved (Conforti et al., 2013). Clearly, the immediate postpregnant state of the uterus places a patient at the highest risk of forming intrauterine adhesions and like most other unknowns, genetics may play a role in the uterus ability to heal with and without scar tissue.

In studies that examine the incidence or prevalence of Asherman's syndrome, we are typically missing the denominator. We know that out of 1000 women with documented Asherman's syndrome, that over half will have had a miscarriage curettage, one quarter with a postpartum curettage with the remainder having a history of cesarean section, infection such as TB, laparoscopic and hysteroscopic myomectomy, uterine artery embolization, and rarely an IUD insertion. The likelihood of developing Ashermans after a first trimester D&C has been reported to be 15%–20% (Gilman et al., 2016; Hooker et al., 2014, 2016). In patients suffering from severe Ashermans, 70% had postpartum instrumentation (Hooker et al., 2016).

As a general rule, the goal of any of the above procedures is to minimize mechanical or thermal trauma to the normal endometrium and not to disrupt the endometrial basalis layer between the functional endometrium and the myometrium. In theory, the deeper the trauma in the uterus the more likely it is to develop intrauterine scar. Moreover, it is appropriate surgical technique not to operate on pathology located on opposing walls of the uterine cavity. Since the cavity is a potential space, this would allow for two traumatic surfaces to adhere to one another. At the end of the day, however, there are no data proving that any curettage technique places a patient at greater or lesser risk for forming post procedural adhesions.

Signs and Symptoms of Asherman's Syndrome

Patients with intrauterine adhesions may be asymptomatic or suffer from severe cyclic pain and amenorrhea. The symptoms are mostly related to the amount and location of the intrauterine scar tissue. While there are several published adhesions scoring classifications, these are not related to the signs and symptoms of the condition. As a general rule, assuming there is no obstruction of menstrual effluent from the scar tissue and the patient is ovulatory, the lesser the amount of flow, the greater the amount of scar tissue. The exception occurs when there is obstruction of flow by lower uterine sear or corneal scar, the resulting hematometria will cause cyclic pain and cramping. In patients who have had an endometrial ablation, this is referred to as posttubal ablation syndrome. The complete canal obstruction at the internal os of the cervix most often occurs after a first trimester curettage.

Other than pain, hypomenorrhea or amenorrhea, intrauterine adhesions can present with recurrent miscarriage or infertility. If an embryo attempts to implant on scar tissue, there is not enough vascularity to support the placental growth. If, however, the embryo implants adjacent to scar tissue, it is likely the scar will not affect the pregnancy.

Candidates for Surgical Therapy

Before we discuss the surgical technique to correct Asherman's syndrome, it is important to identify candidates for therapy. Firstly, the patient should be symptomatic from the syndrome. That is there should be a history of cyclic pain, recurrent miscarriage, abnormal pregnancy outcome related to intrauterine scar or infertility. If a patient has scar within the uterus, light to absent menses, no pain, and does not desire future fertility, there is no need to correct the anatomy. Likewise, the patient should be aware of the likelihood of success given the type of Asherman's and the extent of the disease. It is well recognized that those patients that have more than 70% of the uterine cavity involved in scar tissue, the likelihood of a successful pregnancy is less than 30% (Chen et al., 2017). Another group of patients to consider are those who have an open cavity but hypo or amenorrhea due to the replacement of the endometrium by atrophic scar tissue. The prognosis for these patients who desire fertility, is extremely poor and they should be counseled to consider a gestational carrier or adoption.

Surgical Technique

As with most fertility sparing surgery, the goal is to restore normal anatomy while minimizing damage to surrounding normal tissue. In the case of Asherman's syndrome, the additional goal is to minimize the recurrence of the intrauterine scar tissue. The objective of the surgery is to identify the cervical canal, lower uterine segment, uterine fundus, both cornua and both fallopian tube ostia.

Pre surgical planning is extremely important prior to adhesiolysis. First a bimanual pelvic exam should be performed to determine the uterine and cervical position, anterior or posterior. Second, a transvaginal ultrasound is performed to assess the endometrial echo and thickness. In addition, a saline sonography or a 3-D ultrasound will give the surgeon a good idea of how much of the cavity is involved in scar. When the lower uterine segment is completely obliterated with associated amenorrhea, it is helpful to perform the adhesiolysis with transabdominal ultrasound guidance and a full urinary bladder. This will reduce the risk of creating a false passage, uterine perforation, and missing the uterine cavity entirely.

In order to correct the uterine anatomy while minimizing damage to normal tissue, the smallest, most effective instruments should be used. If possible, instruments that create heat (monopolar or bipolar) should be avoided. As well, there is no need to use mechanical tissue shavers to cut scar tissue and open the cavity. When 5Fr cold semi-rigid "micro" instruments are used, there is minimal risk to damage normal tissue and if a perforation does occur, there is minimal risk to external viscera.

Lysis of intrauterine adhesions should be done hysteroscopically. There is little value in performing this procedure with laparoscopic guidance (Deans and Abbott, 2010).

The laparoscope can demonstrate that a perforation has occurred and not that one is about to occur. As stated, a perforation with a 5 Fr instrument is unlikely to have clinical significance so the risk of laparoscopy is not worth the very small benefit.

Since the instruments of choice are 5 Fr semi rigid blunt tipped or sharp tipped scissors, the outer diameter of the continuous flow dual channel hysteroscope can be between 4.5 and 5.5 mm depending on the manufacturer. The best image is through a rod-lens telescope with a 12 degree foreoblique angle. Some surgeons prefer a 25 or 30 degree lens but keep in mind the instrument exits the hysteroscope at 0 degrees. Therefore the 12 degree lens allows visualization of the distal tip at a greater distance.

Transabdominal ultrasound guidance can be helpful whenever the hysteroscopic encounters complete obliteration of the cervical/endometrial canal at any level. Though the ultrasound only shows two dimensions, it does reduce the incidence of creating a false passage into the myometrium (Myers and Hurst, 2012).

There is a small amount of controversy as to whether RF energy should be used to lyse intrauterine adhesions. At the end of the day there are not enough data to support one technique over another but there are two advantages to using cold instruments to consider. First is the relatively high rate of perforation with Asherman's patients (3%–4%) (Hanstede et al., 2015). Should perforation occur with energy, a laparoscopy is needed to ensure there is no damage to the internal viscera. This is not necessary when a perforation occurs with a 5 Fr cold semi-rigid scissor. Second, it has been reported that pregnancy rates for Asherman's patients are higher when nonenergy devices are used to lyse adhesions (March, 2011).

Whether the procedure is done in the office setting with no anesthesia or the operating theater under local, regional, IV or general anesthesia is irrelevant to the outcome. The one exception is the patient feedback when there are severe adhesions. When the surgeon cuts the adhesions, there is no pain. When the surgeon cuts the myometrium, there is pain and the patient feedback can alert the surgeon that he or she is creating a false passage.

My preference is to perform hysteroscopic adhesiolysis in the office setting using the vaginoscopic technique, no speculum, no tenaculum, and no local anesthesia. It is our experience that approximately 75% of patients tolerate this well. Twenty five percent request that the procedure be performed with local or IV anesthesia. It is recommended that the patient take 400–600 mg of Ibuprofen 1 h prior to the procedure.

In addition to the hysteroscope, and the 5 Fr instruments, the room in which the adhesiolysis is being performed should have a camera, monitor, light source, and a digital capture device. A second monitor for patient viewing is helpful and provides a distraction. Fluid needs to be instilled through the hysteroscope to distend the vagina, cervix, and uterine cavity. The fluid of choice is slightly heated normal saline. The distention flow and pressure can be controlled by an inexpensive fluid pump designed for office use or by gravity or pressure bags. The distention pressure should be the minimum amount that provides adequate flow and distention. Cysto tubing helps to maintain a good flow rate and the fluid should be captured and measured as it leaves the patient or hysteroscope to ensure that fluid overload will not occur. As a rule, a young healthy patient should be able to absorb up to 2 L of saline without clinical consequence. This amount of fluid intravasation rarely occurs during the adhesiolysis procedure but large venous sinuses can be encountered.

The goal of the adhesiolysis is the restoration of the normal cavity. The adhesions can be lysed or removed. When a thick band is encountered, I will try to resect the adhesion at the base and attached wall with the microscissors and not just lyse it. There are no data that resection of an adhesion gives better outcomes than lysis. Restoration of the normal cavity can only be assured if both fallopian tube ostia are visualized. Since the opening of the tube is only 1.2 mm in diameter, this is often difficult when there are corneal adhesions and obstruction.

There are two types of Asherman's fibrosis. The first and most common are the adhesive bands that can be lysed as described above. The second type of Asherman's scar is that which replaces the normal endometrium to the depth of the myometrium including the basalis. There is no successful surgical correction of the latter type of Asherman's and it carries a very poor prognosis when over ¾ of the endometrium has been scarred. These patients should be counseled on gestational carrier opportunities or adoption.

Recurrence of Adhesions

For surgeons that treat a high volume of patients with Asherman's syndrome, it is generally accepted that the initial treatment of the intrauterine adhesions is not technically challenging. What is difficult is preventing and treating the recurrence of the scar tissue that

occurs within the first 6–8 weeks after surgery. Several adjuvants have been and are currently used in effort to minimize recurrence of the intrauterine scar tissue.

- Estradiol
- Intrauterine devices (IUD)
- Intrauterine balloon
- Second look hysteroscopy
- Antiadhesive gels and barriers
- Stem-cell therapy

While there is no standard dose of estrogen for patients who have undergone hysteroscopic adhesiolysis, a recent review suggest that there is benefit to patients desiring future fertility (March, 2011). Our practice is to prescribe 4 mg of oral estradiol daily for 30 days with a dose of 10 mg of medroxyprogesterone acetate on days 25–30 to induce a withdrawal bleed.

Physical barriers such as nonhormonal and not copper containing IUDs, Foley balloons and intrauterine balloons thought to be effective in reducing postoperative adhesion recurrence though there has never been a randomized clinical trial to demonstrate their value. Most reports describe using these in barriers for 7–10 days postsurgery (Di Spiezio Sardo et al., 2016).

Since over 90% of our adhesiolysis procedures are done in the office using a vaginoscopic technique without anesthesia, we prefer the second-look hysteroscopy method to prevent recurrent adhesions. The second procedure is done 2 weeks after the first and the third procedure is done after the withdrawal bleed at 6 weeks. The advantage is not leaving a foreign body within the cavity and being able to lyse recurrent thin adhesions. Since patients have to have their barrier removed in the office, the second look approach only requires one extra visit and no visits in the operating room. The outcomes reported are at least as effective as the barrier approach though no head to head studies have been performed (Robinson et al., 2008).

Outcomes

Spontaneous conception rates after treatment with adhesiolysis are inversely correlated with the extent of adhesive disease (Evans-Hoeker and Young, 2014). For patients with severe IUA, only 25% were able to spontaneously achieve pregnancy, compared to 53% of those with moderate disease (Xiao et al., 2014). One large retrospective study focusing on patients with moderate to severe IUA treated with adhesiolysis, IUD placement, balloon catheter insertion, antiadhesion gel, and oral estrogen therapy together were reported to have pregnancy rates of 66% and live birth rates of 64% (Xiao et al., 2014). A large retrospective study confirms overall conception rates are reported as high as 48% and birth rates less than 80%, with mean time to conception as 8.9 ± 3 months following hysteroscopic adhesiolysis (Chen et al., 2017).

Patients with Asherman's are at increased risk for pregnancy complications. There is a reported 19% combined rate of spontaneous abortion, missed abortion or stillbirth, as well as preterm delivery amongst patients with moderate to severe disease (Xiao et al., 2014). In another study, Asherman's patients after hysteroscopic adhesiolysis were reported to have a 9% incidence of miscarriage (Chen et al., 2017). This is somewhat lower than the baseline incidence of miscarriage of 20%, and is likely underrepresentative of the true incidence. Other outcomes are equally variably reported in the literature, ranging between reporting a 14% risk of abnormal placentation (e.g., accreta) (Fernandez et al., 2006) and 8% risk of postpartum hemorrhage (Chen et al., 2017), to a 63% combined rate of abnormal placentation including increta, placenta previa, and postpartum hemorrhage all together (Xiao et al., 2014). It is difficult to ascertain from the data what the actual risks are, however it is clear that moderate to severe IUA disease has a negative impact on obstetrical outcomes.

Over the past 20 years, many patients have undergone endometrial ablation to treat their heavy menstrual bleeding and subsequently had a desire to conceive. Whether the endometrial cavity is treated with heat or cold to destroy the functional endometrium, there is ultimately severe Asherman's in the treated areas. When these patients are treated, a small percentage with achieve pregnancy. The pregnancy is very high risk with perinatal mortality of 13%, prematurity over 40%, and 25% with abnormal placentation (Hare and Olah, 2005).

In conclusion, intrauterine adhesive disease once thought to be a rare phenomenon, is now known to be relatively common in patients undergoing uterine surgery and endometrial curettage in the postpregnant state. Advances have been made in the minimally invasive approach to treating Asherman's Syndrome whereas most patients can be successfully treated in an outpatient office setting without the need for regional or general anesthesia. Our frustration continues in our poor success with preventing recurrent adhesions. There is great hope for novel stem cell and regenerative therapy to recreate a functional endometrium with a normal intracavitary volume.

References

- Chen, L., Zhang, H., Wang, Q., Xie, F., Gao, S., Song, Y., et al. (2017). Reproductive outcomes in patients with intrauterine adhesions following hysteroscopic adhesiolysis: Experience from the largest Women's Hospital in China. *Journal of Minimally Invasive Gynecology*, 24(2), 299–304.
- Conforti, A., Alviggi Mollo, A., De Placido, G., & Magos, A. (2013). The management of asherman syndrome: A review of the literature. *Reproductive Biology and Endocrinology*, 11, 118.
- Deans, R., & Abbott, J. (2010). Review of intrauterine adhesions. *Journal of Minimally Invasive Gynecology*, 17, 555–569.

- Di Spiezo Sardo, A., Calagna, G., Scognamiglio, M., O'Donovan, P., Campo, R., & De Wilde, R. L. (2016). Prevention of intrauterine post-surgical adhesions in hysteroscopy. A systematic review. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 203, 182–192.
- Evans-Hoeker, E. A., & Young, S. L. (2014). Endometrial receptivity and intrauterine adhesive disease. *Seminars in Reproductive Medicine*, 32(5), 392–401.
- Fernandez, H., Al-Najjar, F., Chauveaud-Lambling, A., Frydman, R., & Gervaise, A. (2006). Fertility after treatment of Asherman's syndrome stage 3 and 4. *Journal of Minimally Invasive Gynecology*, 13(5), 398–402.
- Gilman, A. R., Dewar, K. M., Rhone, S. A., & Fluker, M. R. (2016). Intrauterine adhesions following miscarriage: Look and learn. *Journal of Obstetrics and Gynaecology Canada*, 38(5), 453–457.
- Hanstede, M. M. F., van der Meij, E., Goedemans, L., & Emanuel, M. H. (2015). Results of centralized Asherman surgery, 2003–2013. *Fertility and Sterility*, 104, 1561–1568.
- Hare, A. A., & Olah, K. S. (2005). Pregnancy following endometrial ablation: A review article. *Journal of Obstetrics and Gynaecology*, 25(2), 108–114.
- Hooker, A. B., Lemmers, M., Thirkow, A. L., Heymans, M. W., Opmeer, B. C., Brölmann, H. A. M., et al. (2014). Systematic review and meta-analysis of intrauterine adhesions after miscarriage: Prevalence, risk factors and long-term reproductive outcome. *Human Reproduction Update*, 20(2), 262–278.
- Hooker, A., Fraenk, D., Brölmann, H., & Huime, J. (2016). Prevalence of intrauterine adhesions after termination of pregnancy: A systematic review. *The European Journal of Contraception & Reproductive Health Care*, 21(4), 329–335.
- March, C. M. (2011). Management of Asherman's syndrome. *Reproductive Biomedicine Online*, 23, 63–76.
- Myers, E. M., & Hurst, B. S. (2012). Comprehensive management of severe Asherman syndrome and amenorrhea. *Fertility and Sterility*, 97(1), 160–164.
- Robinson, J. K., Colimon, L. M. S., & Isaacson, K. B. (2008). Postoperative adhesiolysis therapy for intrauterine adhesions (Asherman's syndrome). *Fertility and Sterility*, 90(2), 409–414.
- Xiao, S., Wan, Y., Xue, M., Zeng, X., Xiao, F., Xu, D., et al. (2014). Etiology, treatment, and reproductive prognosis of women with moderate-to-severe intrauterine adhesions. *International Journal of Gynecology & Obstetrics*, 125(2), 121–124.

Surgical Sex Reassignment

Karel Claes, Salvatore D'Arpa, Piet Hoebeke, and Stan Monstrey, University Hospital Ghent, Gent, Belgium

© 2018 Elsevier Inc. All rights reserved.

Glossary

Abdominoplasty Plastic surgery of the abdomen in which fat and skin are removed and muscles are sometimes tightened, usually for cosmetic purposes.

BRCA mutation Mutation in either of the BRCA1 and BRCA2 genes, which are tumor suppressor genes. Harmful mutations in these genes may produce a hereditary breast–ovarian cancer syndrome in affected persons.

Chondrolaryngoplasty Surgical procedure in which the thyroid cartilage is reduced in size by shaving down the cartilage through an incision in the throat, generally to aid those who are uncomfortable with the girth of their Adam's apple.

Pedicled flap An amount of tissue that is transferred, usually including skin and subcutaneous tissue, only partially removed from one part of the body so that it retains its own blood supply during transfer to another site.

Gender dysphoria (GD) Discomfort or distress that is caused by a discrepancy between a person's gender identity and that person's sex assigned at birth (and the associated gender role and/or primary and secondary sex characteristics).

Lipofilling Plastic surgery procedure in which tissue defects are filled with autografted fat tissue obtained through liposuction.

Mastopexy Surgical procedure in which the breasts are lifted or reshaped.

Mayer–Rokitansky–Küster–Hauser (MRKH) syndrome Disorder that occurs in females and mainly affects the reproductive system. This condition causes the vagina and uterus to be underdeveloped or absent.

Orchiectomy Surgical procedure in which one or both testicles are removed.

Sex reassignment surgery (SRS) Also known as gender reassignment surgery (GRS), genital reconstruction surgery or gender confirmation surgery is the surgical procedure (or procedures) by which a gender dysphoric person's physical appearance and function of their existing sexual characteristics are altered to resemble that of their identified gender.

Symmastia Medial aspect of both breasts are bridged across the midline at the sternum because the pouches for breast implants were cut too close to the midline.

Standards of Care (SOC) for the health of transsexual, transgender, and gender-nonconforming people The overall goal of the SOC, provided by the World Professional Association for Transgender Health (WPATH), is to provide clinical guidance for health professionals to assist transsexual, transgender, and gender-nonconforming people with safe and effective pathways to achieving lasting personal comfort with their gendered selves, in order to maximize their overall health, psychological well-being, and self-fulfillment.

Transsexual A person having a strong desire to assume the physical characteristics and gender role of the opposite sex. This definition is still used for those patients who match the criteria for GD and are good candidates for SRS.

Transwoman A person who was born male but whose gender identity is female.

World Professional Association for Transgender Health (WPATH) International, multidisciplinary, professional association whose mission is to promote evidence based care, education, research, advocacy, public policy, and respect in transsexual and transgender health.

History of Transwomen Surgery

The development of modern gender reassignment surgery began in the twentieth century when, following rapid advances in the fields of endocrinology and plastic surgery after World War II, comprehensive medical and surgical treatments for transsexualism started to become available. In 1952, the Danish plastic surgeon Paul Fogh-Andersen initiated the modern era of sex reassignment surgery by using penile skin as a full thickness graft to line the neovagina of Christine Jørgenson ([Fogh-Andersen, 1956](#)). In the United States, things began to change during the late 1950s, when several hundred transsexuals came under the care of Dr. Harry Benjamin, a compassionate endocrinologist who was the first physician to elucidate the nature of gender dysphoria ([Benjamin, 1964](#)).

For the majority of transsexuals seeking surgery, the 'Clinique du Parc' in Casablanca was the place of last resort. There, Dr. Georges Burou (1910–1987), a French gynecologist, invented and applied the anteriorly pedicled penile skin flap inversion technique in 1956 ([Hage et al., 2007](#)).

In 1966, surgeons at the Johns Hopkins Medical Center began performing a limited number of Male-to-Female (MtF) sex reassignment surgeries (SRS) at its new gender identity clinic. Shortly thereafter, hospitals at Stanford, Chicago and Colorado followed suit ([Laub and Fisk, 1974](#); [Pandya and Stuteville, 1973](#)).

The surgeons at Johns Hopkins initially used partial thickness skin grafts to line the neovaginal cavity, but later used penile skin, applied as a full thickness skin graft, much as the technique employed in Copenhagen. The *posteriorly* pedicled penile skin flap

inversion technique for vaginoplasty — a variation of Burou’s method — was introduced by Edgerton and Bull at Johns Hopkins between 1968 and 1970 (Edgerton and Bull, 1970). Beginning in 1967, the Chicago group used the *anteriorly* pedicled penile skin flap to line the vagina, and scrotal skin to form the labia (Pandya and Stuteville, 1973).

In Europe, beginning in the 1970s, the University Hospital of the Free University of Amsterdam became the leading center for medical and surgical treatment of patients with severe gender dysphoria. By the end of the 20th century many centers of excellence employed a multidisciplinary approach for transsexual individuals, providing state of the art treatment, including surgical therapy, for both transwomen and transmen. With the recent increase in number of individuals seeking treatment for gender dysphoria, the quantity and the quality of the multidisciplinary teams has substantially increased worldwide with the largest number of vaginoplasties probably still being performed in Thailand.

Criteria for Male-to-Female Gender Reassignment Surgery

Standards of Care for Surgery

The 7th version of Standards of Care (SOC) of the World Professional Association of Transgender Health (WPATH) offers flexible guidelines for the treatment of people experiencing gender dysphoria and put forth the following criteria for surgical treatments (Coleman et al., 2012):

- For breast augmentation with implants or lipofilling (one referral):
 - Persistent, well documented gender dysphoria
 - Capacity to make a fully informed decision and to give consent for treatment
 - Age of majority in a given country
 - If significant medical or mental health concerns are present, they must be well controlled

Although not an explicit criterion, it is recommended that transwomen undergo feminizing hormone therapy (minimum 12 months) prior to breast augmentation surgery. The purpose is to maximize breast growth in order to obtain better surgical (aesthetic) results.

- For genital surgery, two referrals are recommended and specifically for orchiectomy the following criterion is added to the list above:
 - Twelve continuous months of hormone therapy as appropriate to the patient’s gender goals (unless hormones are not clinically indicated for the individual). The aim of hormone therapy prior to gonadectomy is primarily to introduce a period of reversible testosterone suppression, before a patient undergoes irreversible surgical intervention.
- For vaginoplasty one additional criterion is recommended:
 - Twelve continuous months of living in a gender role that is congruent with their gender identity (formerly called ‘real-life experience’).

Typically, the patient is advised to stop all hormonal therapy at least 2 weeks prior to any surgical procedure. Estrogens, in particular, can cause an increased risk of thromboembolic complications.

Facial Feminization Surgery and Voice Surgery

Facial Feminization Surgery

In most human interactions, the face communicates vital social information. First impressions are largely based upon a person’s facial features and expressions. A face reflects aspects of one’s personality and emotions, and serves as a vehicle to convey verbal and nonverbal communication. The head and face are commonly considered to be the location of the ‘self’. In initial contacts, it is most often the face that provides the clues about the person, including gender. For the transsexual patient, nothing is more important than to appear externally congruent with the internal and emotional self. While many patients undergo surgery to feminize the face, there is a paucity of literature on these procedures (Hage et al., 1997).

Forehead

Spiegel investigated the determinants of female gender and discovered a strong association between facial femininity and attractiveness more specifically attributed to the upper third of the face and the interplay of the glabellar prominence of the forehead, along with the eyebrow shape and position and the hairline shape and position. Based on differences in anthropological measurements, the size of the frontal sinus (in the mid-lower forehead above the eyes and nose) and the general contour of the orbits and forehead, three different ways of modifying the forehead shape have been developed. These vary from simple bony contouring to the more complex procedure, wherein the anterior wall of the frontal sinus is placed into a more posterior position, or in combination with filling of the concavity superior to the frontal bossing with bone cement. Orbital rim contouring may be helpful to make the orbit look bigger, and is completed at the same time. If indicated, a bilateral brow-lift can be simultaneously performed. The female brow is not positioned at the level of the supraorbital rim but just a little above. The female eyebrow has an arch shape with the highest point at one-third laterally in contrast with the male brow that is straight. Scalp

advancement can reduce the distance from the brows to the hairline, which is on average 5 cm for women, in comparison to 7 cm for men.

Cheeks

The cheekbones are more prominent in women and malar augmentation makes the face more heart-shaped. Cheek augmentation is therefore often effective in feminizing the face. Usually this is done with an implant or with soft tissue filling, but certain bone cuts (zygoma osteotomies) and bone segment repositioning may be useful in some cases. The bilateral partial removal of the buccal fat pads of Bichat at the same time can help accentuating the cheekbones by creating a sub-malar hollowing.

Nose

The nose is a feature that varies tremendously from individual to individual, and may be quite masculine appearing. The noses of men are usually larger than those of women and naso-labial and naso-glabellar angles are sharper. Contour changing alone greatly feminizes the basic nasal appearance. In a reduction rhinoplasty mostly dorsum and tip will have to be made smaller and the tip needs to be lifted and refined. The volume of the nostrils can be reduced by alar base excision. Correction of the frontal bossing and tip lifting make the naso-glabellar angle and naso-labial angle more obtuse and more feminine.

Chin

While the male chin is generally wide and vertically high, the female chin tends to be more pointed, narrow, and vertically shorter. The chin can be modified in numerous ways to improve facial appearance and feminize the lower face. This may only require a small implant or little shaving, but it is often necessary to perform extensive bone cuts, with removal of excess of bone and repositioning of the chin to make the chin shorter and narrower.

Angle of the mandible

Males tend to have a more obvious mandible angle, with thicker muscles and resulting fullness, than females. The female tends to have a smaller muscle with a more gradual curve or even a straight line along the lateral border from the posterior border of the mandible to the chin. A lower jaw contouring procedure is completed under general anesthesia through incisions in the mouth. The masseter muscle is reduced on its internal surface, i.e., the portion adjacent to the bone. The bone is then significantly reduced laterally, softening the bony angle. When there is a significant mandibular angle present, the angle is osteotomized to give this area a more rounded and smoother appearance. Botulin toxin can also be used to decrease the volume of the masseter muscle.

Upper lip

Lip lift is regularly used in facial feminization. Its aim is to shorten the upper lip in the area between the alar bases of the nose making the maxillary tooth crowns more visible. Also techniques making the upper lip more voluminous can help in making the face look more feminine. This can be done by fillers, but the use of dermis grafts, harvested from the coronal flap, has few complications.

Orthognatic surgery

Using the Le Fort 1 and bilateral sagittal ramus osteotomy a combination of feminizing effects can be obtained. A clockwise rotation of the bimaxillary complex with posterior impaction of the maxilla gives more volume to the midface making the face look rounder. It also makes the gonial angles more obtuse, makes the chin less pronounced and gives more show to the maxillary tooth crowns.

Hair transplantation

Hair transplantation, or scalp shifting, is very helpful in transwomen who have considerable balding. There are many ways to approximate a female hair pattern. Major procedures like scalp flap or tissue expansion may be required in some cases but, more often, plugs or micro grafts are employed. Scalp reductions are useful in certain individuals. For those with extensive baldness, there is no choice but to use a hairpiece.

Chondrolaryngoplasty and Voice Surgery

The secondary sex characteristics of the larynx with its vocal function remain a major obstacle to achieving gender congruency for many. Estrogen does not have any substantial or lasting influence on voice pitch, and speech therapy alone often does not produce satisfactory results.

Various authors have described operative techniques to raise voice pitch. Basically, this can be achieved by reducing the vibrating vocal cord length (shortening the vocal cords) or increasing the vocal cord tension. The most frequently used technique is the one described by Isshiki (1989). In this technique, the cricoid cartilage is approximated to the lower edge of the thyroid cartilage, causing an increase in vocal cord tension. The cricoid and thyroid cartilages are then fixed to each other with sutures. Nowadays, endoscopic surgery directly on the vocal cords is often performed.

A reduction of the prominent thyroid cartilage can be performed in the same procedure. During this intervention, the thyroid cartilage is exposed, perichondrial flaps are raised, and a resection of the upper limit of the thyroid cartilage is performed. According

to literature, surgical outcomes of voice pitch raising are very good, and the cosmetic benefit of the reduction of the Adam's apple contributes greatly to the patient's psychological wellbeing (Wolfort et al., 1990).

Breast Augmentation

For most transwomen, breast augmentation (or breast "reconstruction") greatly increases subjective feelings of femininity. In a prospective, non-comparative, cohort study it has been shown that the gains in breast satisfaction, psychosocial well-being, and sexual well-being after transwomen undergoing breast augmentation are statistically significant and clinically meaningful to the patient short after surgery as well as in the long term (Weigert et al., 2013). The augmentation mammoplasty may be performed at the same time as genital surgery, or as a preliminary or subsequent procedure.

Breast Development after Hormonal Therapy

While some breast formation occurs after hormonal therapy, for many, it is insufficient. Unfortunately there are no studies looking in detail on what is the optimum delay after start of hormonal treatment. The current version of the SOC does not explicitly indicate a minimum period of hormone therapy that must be completed before breast surgery may be performed. However, most surgeons recommend a 12 month period of feminizing hormone therapy prior to breast augmentation surgery in order to maximize breast growth and obtain better surgical (aesthetic) results.

Surgical Techniques

Since breast prostheses are implanted in transsexuals with "young adolescent" breast development, the patient should be informed that the complex feminine form and age-related changes of the breast cannot be imitated by using symmetric hemispherical prostheses. Therefore, the result of an augmentation mammoplasty in a transwomen with minimal hormone-induced mammogenesis may be poor (Kanhai et al., 1999a, b).

Other anatomical differences, which should be taken into consideration in transwomen, are (1) the wider male chest; (2) a stronger pectoral fascia and a more developed pectoralis muscle; and (3) a smaller dimension of nipple and areola. Usually a larger volume of breast prostheses is chosen by transwomen than that chosen for breast augmentation by a female patient, but even with a larger prosthesis, it is often impossible to avoid an abnormally wide cleavage between the breasts. The nipple areola should always overlie the prosthesis centrally and a very medial position of these prostheses could result in a divergent nipple position with an unacceptable breast appearance (Laub and Fisk, 1974) (Figs. 1 and 2).

In our personal experience we have used lipofilling as an adjunct to make the prosthesis less visible and palpable, as well as to narrow the wide cleavage between the breasts. In transwomen who already have some breast volume due to hormone treatment, lipofilling can be a good option to provide a moderate augmentation of the breast, therefore avoiding the need for an implant (Fig. 3).

Despite some sexual differences in chest wall and mammary anatomy, the implantation of mammary prostheses is not essentially different from breast augmentation in a female patient, except that, usually, larger prostheses are used. The same choices apply as to the kind of prostheses, the position of the pocket, the surgical approach, etc. Patient and surgeon can choose between a silicone gel-filled prosthesis and a saline filled prosthesis. In most cases, a textured prosthesis is chosen to reduce the chance of capsular contraction. When a more cohesive gel-filled prosthesis is chosen, it can be a round prosthesis or a so-called anatomical prosthesis, resulting in additional filling of prominence in the lower part of breast.



Fig. 1 Breast augmentation preoperative view.



Fig. 2 Breast augmentation postoperative view.

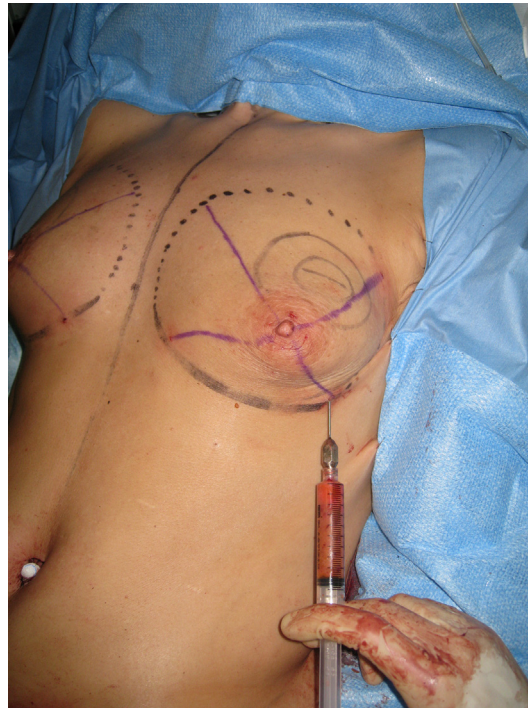


Fig. 3 Lipofilling was used for breast augmentation.

The incision can be made axillary, inframammary, or even periareolar, although the latter is less popular in transwomen because of the smaller size of the areola. If an inframammary incision is used, it should be positioned lower than the preoperative inframammary fold, as the distance between the inferior areolar margin and the inframammary fold will expand after augmentation mammoplasty, likely due to the recruitment of the inframammary or even abdominal skin ([Kanhai et al., 1999a](#)).

The pocket for the prosthesis can be created behind the glandular tissue or behind the pectoralis muscle. Some authors ([Kanhai et al., 1999a](#)) recommend implanting the prostheses in a subglandular position. This is especially indicated in patients who have more subcutaneous and glandular tissue to start with (Tanner Stage IV or V). The surgical procedure is easier to perform and less painful. Many surgeons, however, prefer to put the prostheses in a retropectoral position. In this case, the lower portion (as well as part of the medial origin) of the pectoralis muscle should be detached from the thoracic cage ([Monstrey et al., 2001](#)). In the retropectoral position, the prosthesis is covered with more soft tissue (important in case of thin patients), and a lower risk of capsular contraction has also been reported.

Complications

[Kanhai et al. \(1999b\)](#) reported the main (but rarely occurring) complications after breast augmentation: hematoma, symmastia, capsular contracture, a decreased sensation in the nipple and/or part of the breast, leakage of the prostheses (more obvious in

saline-filled prostheses than in cohesive silicone gel-filled prostheses), and malposition of the prostheses. Although it is very rare in these patients, mastopexy can be the treatment of choice to correct (substantial) mammary ptosis, but usually an augmentation is sufficient to fill out the (slightly) ptotic breasts.

Postoperative follow-up is mandatory in all patients undergoing breast augmentation. Gooren et al. performed a cohort study documenting the occurrence of breast cancer in 2307 transgender persons (Gooren et al., 2013). His study suggested that cross-sex hormone administration does not increase the risk of breast cancer development in transwomen. Breast carcinoma incidences were comparable to male breast cancers, and thus lower than in the female population. However, the historical use of cross-sex hormones may have been too short for malignancies to develop. Therefore good screening and follow up is imperative. Moreover, since breast exams are also very well accepted by transwomen, transgender persons should be encouraged to participate in relevant cancer screening protocols, which for breast cancer screening are the same as for cisgender women (Weyers et al., 2010).

Routine preoperative investigation of family history is imperative. Screening for genetic predisposition (such as BRCA mutations) should be considered in patients with multiple breast and/or ovarian cancers within their family (often diagnosed at an early age); two or more primary breast and/or ovarian cancers in a single family member and/or cases of male breast cancer within their family. Psychological counseling about bilateral prophylactic mastectomy and consecutively primary reconstruction with either autologous tissue (such as a deep inferior epigastric perforator (DIEP) flap) or prosthesis should be offered in patients with a genetic predisposition of breast cancer (Colebunders et al., 2014).

Orchiectomy

An orchiectomy can be performed simultaneously with a vaginoplasty or as a first stage procedure prior to the vaginal reconstruction. If an orchiectomy is performed as a preliminary procedure, it will not compromise the possibility of a vaginoplasty at a later stage, as the testicles can be accessed via a short 5 cm incision at the raphe leaving very little scar tissue. Androgen-blocking treatment can usually be stopped or reduced after the orchiectomy.

Reproductive Options

Attention should be given to reproductive possibilities and restrictions after orchiectomy. A majority of transwomen has been reported to desire to have children (Wierckx et al., 2012). Transwomen should be given the option of sperm preservation in sperm banks, prior to initiating hormones.

Vaginoplasty

As affirmed by Karim et al. (1996), the goal of genital reassignment surgery in male-to-female transsexuals is to create a perineo-genital complex as feminine in appearance and function as possible and free of poorly healed areas, scars, and neuromas. The urethra should be shortened in such a way that the direction of the urinary stream is downward in the sitting position and it should be free of stenosis or fistulas. The neovagina should, ideally, be lined with moist, elastic, and hairless epithelium. Its depth should be at least 10 cm and its diameter 30 mm. The sensation should be sufficient to provide satisfactory erogenous stimulus during sexual intercourse.

The major steps in a vaginoplasty are an orchiectomy (if not previously performed), amputation of the penis, creation of the neovaginal cavity, the lining of this cavity, reconstruction of a urethral meatus and, finally, construction of the labia and clitoris.

In order to obtain a functional and aesthetic outcome, most authors advise to construct a wide and deep vaginal cavity, to perform a near-complete resection of the corpus spongiosum, an eversion of the urethral mucosa and the construction of clitoris/clitoral prepuce, labia minora/majora. Several refinements of vaginoplasty (mostly using the inverted penile-scrotal skin flap technique in combination with skin grafts) have been described (Karim et al., 1995; Selvaggi et al., 2005). These include (1) preoperative epilation of the area at the base of the penis and posterior scrotum; (2) preoperative bowel preparation; (3) the use of a drape with a rectal condom; (4) blunt dissection of the vaginal cavity; and (5) the use of double silicone prosthesis to create and maintain a vaginal cavity of adequate dimension.

Vaginal Lining

Methods for lining the neovagina can be classified into six categories: (1) non-genital skin grafts; (2) penile or scrotal skin grafts; (3) penile-scrotal skin flaps; (4) non-genital skin flaps; (5) pedicled intestinal transplants and (6) recent developments.

Nongenital skin grafts

Laub and Fisk reported the first series of transwomen in whom a split skin graft pulled over a plastic mold was applied in a one-stage vaginoplasty (Laub and Fisk, 1974). The use of a full-thickness skin graft, harvested from the region of a (mini) abdominoplasty, has been described by Hage and Karim (Hage and Karim, 1998). Because of disadvantages like the tendency of the skin graft to shrink

(postoperative daily dilation is required), suboptimal sensation, and the absence of any natural lubrication (McGregor, 1989) it is not used as single reconstructive option.

Penile or scrotal skin as graft

Fogh-Andersen was the first to report the use of a full-thickness skin graft harvested from the penile skin to line the neovagina (Fogh-Andersen, 1956). However the use of penile skin as a graft for vaginoplasty is rarely, if ever, performed as it is usually preserved to be used as a (pedicled) penile flap undergoing less contraction postoperatively.

Penile–scrotal skin flaps

This is the technique of choice. As previously discussed, Burou has been credited with inventing the anteriorly pedicled penile skin flap inversion technique, and it became (and still is) the technique of choice for vaginoplasty in transwomen. However, in 1957, Gillies was the first to report on the use of penile skin as a pedicled flap for vaginoplasty (Gillies and Millard, 1957). Several modifications of this technique were subsequently described. These can be classified into the following three groups (Bouman, 1988):

1. The inverted penile skin is used solely on an abdominal pedicle as an inside-out skin tube (Gillies and Millard, 1957; Eicher, 1989); this penile skin flap can be augmented with a small triangular scrotal skin flap to “break” the circular introitus (Bouman, 1988; Karim et al., 1995). Variations of this technique are used by most surgeons who perform sex reassignment surgery in transwomen today and this technique is therefore considered “the gold standard in vaginoplasty” (Figs. 4, 5, and 6).
2. The pedicled penile skin tube can also be split open to form a rectangular flap which is augmented by a rectangular, posteriorly pedicled scrotal skin flap comparable in size (Jones et al., 1968).
3. The inverted penile skin tube may be applied based on an inferior pedicle (Edgerton and Bull, 1970). Perovic et al. described a modification of the technique wherein a long, vascularized urethral flap is harvested and embedded in the penile skin tube flap (Perovic et al., 2000). They also described the suspension of the neovagina to the sacrospinous ligament to prevent vaginal prolapse, a complication that is rarely, if ever, experienced by others.

There are several advantages to penile skin flaps. They demonstrate fewer tendencies to contract (McGregor, 1989). In the event of inadvertent damage to the rectum, correction is easier, as it is immediately covered with vascularized tissue (Karim et al., 1995). Local innervation is provided and the flap is virtually hairless. Although flaps have much less tendency to contract than grafts, patients are still required to use a dilator postoperatively for 6 months. The disadvantages of penile skin flaps are that a limited amount of penile skin may be available (van Noort and Nicolai, 1993), and that this technique might result in widening of the anterior commissure, which can leave the clitoris more exposed (Karim et al., 1995; Bouman, 1988).

Combining an abdominally pedicled penile skin flap with a wide posteriorly based scrotal skin flap produces an ideal anatomically located introitus and favorable dimensions of the neovagina (van Noort and Nicolai, 1993). However, this technique will introduce hair-bearing scrotal skin within the posterior lining of the vagina if the epilation has not been performed prior to surgery (Figs. 7 and 8).

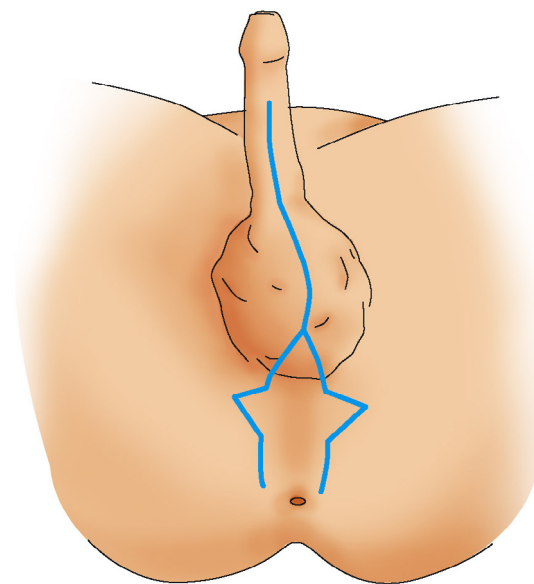


Fig. 4 Inverted penile skin flap technique with dorsal scrotal flap.

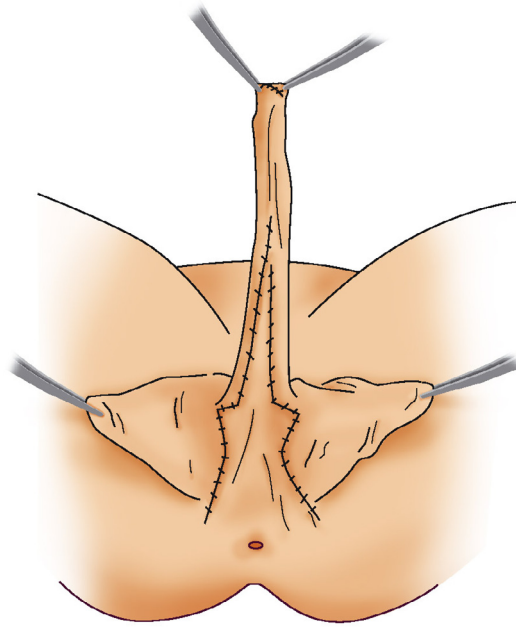


Fig. 5 Inverted penile skin flap technique after resection of all erectile tissue.

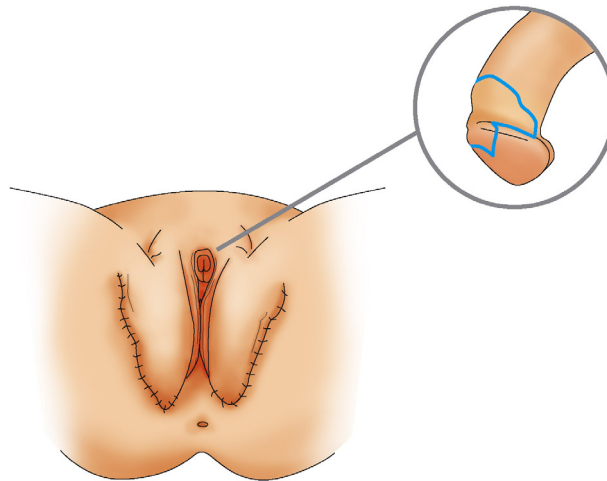


Fig. 6 Inverted penile skin flap technique.

Nongenital skin flaps

In 1980, Cairns and De Villiers reported the use of a medial thigh flap for vaginoplasties in four patients who had previously undergone penile skin inversion vaginoplasty with inadequate results (Cairns and de Villiers, 1980). Huang used two inguino-pudendal flaps, sutured to one another in the midline and then to the penile flap, forming a single large flap (Huang, 1995). The use of distant free flaps for vaginoplasties in transsexuals has never been reported.

The advantages of using non-genital flaps are that they have less risk of contraction and a reduced period of postoperative dilation. The disadvantages are donor site morbidity and scarring, technical complexity (in some cases the flaps are unreliable) (Wee and Joseph, 1989), and their tendency to be bulky as compared to genital skin flaps. This added bulk can decrease the functional dimensions of the neovagina, which is particularly disadvantageous in transwomen, as the male pubic arch is narrower than the female (Karim et al., 1996). Again, these flaps have no natural source of lubrication. Non-genital skin flaps should be reserved as a choice of last resort, after all other options have been exhausted.

Pedicled intestinal transfer

Intestinal vaginoplasty is a good alternative technique in cases where insufficient skin is available. A lack of penile and scrotal skin is often present in young transwomen who started hormonal therapy at an early age. Transwomen who had a previous



Fig. 7 Vaginoplasty: Early post-operative result (2 weeks) with still swollen smaller and greater labia.



Fig. 8 Vaginoplasty—result after 3 months.

failed vaginal reconstruction with skin flaps and/or grafts can also benefit from this procedure (Kim et al., 2003). A pedicled intestinal transfer can also be used to elongate the vagina in transwomen requiring more depth after a previous vaginal reconstruction.

Both small as well as large intestinal segments have been used for vaginal reconstruction (Bouman et al., 2014). For vaginoplasty with a large intestinal segment, the sigmoid is most commonly the first choice. In case the small intestines are used, the ileum is mainly chosen. Harvest of the bowel can be performed through a median or Pfannenstiel laparotomy,

a laparoscopy-assisted-laparotomy or totally laparoscopic. Laparoscopic harvest of bowel has been described by Wedler in 2004 and has been more widely used since (Wedler et al., 2004). Zhang et al. showed that laparoscopic-assisted vaginoplasty using pedicled ileum or sigmoid segment are both effective in reconstructing a vagina (Zhang et al., 2014).

The advantages of using a recto-sigmoid transplant are not only the length it provides, but also the texture and appearance which is more similar to vaginal lining and of course its natural lubrication (Dalton, 1981; Kim et al., 2003). Although the natural lubrication with the production of mucus is usually regarded as an advantage, it may lead to excessive discharge (Hage et al., 1997), especially when ileum is used, compared to colon.

Neovaginal length after an intestinal transfer usually is significantly longer compared to other vaginoplasty techniques. However, if the intestinal segment is taken too long, it can lead to stasis and dehydration of mucus in the deepest portion of the vagina. An intestinal transfer itself has little or no tendency to shrink but dilation remains important to avoid introital stenosis, which is the most common reported complication of intestinal vaginoplasty (Bouman et al., 2014).

Bouman et al. performed a review of the literature focusing on clinical outcomes of intestinal vaginoplasty including 21 studies (Bouman et al., 2014). Prevalence and severity of procedure-related complications were low. Diversion colitis (inflammation that occurs in the bypassed colonic tissue related to diversion of the fecal stream), was very rare. Severe complications were incidental and included necrosis of the intestinal segment, necrotizing fasciitis, bilateral lower extremity compartment syndrome, an intraluminal abscess and intestinal obstruction.

Recent developments

- Autologous buccal mucosa. In 2003 Lin et al. were the first to describe vaginoplasty with autologous buccal mucosa as graft material in eight cases of vaginal agenesis, such as Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS) (Lin et al., 2003). Later, in 2014 Li et al. modified this technique by using micro grafts of the buccal mucosa, minimizing donor site morbidity and enlarging the possible surface area to be grafted (Li et al., 2014). Dessy et al. reduced donor site morbidity even further by taking a 2 cm² full thickness oral biopsy and culturing it into fully differentiated mucosal tissue (Dessy et al., 2014).
- Acellular dermal matrix. To date there are no reports using acellular dermal matrix as vaginal lining in transwomen. However its use has been described for vaginal reconstruction in patients with Mayer-Rokitansky-Küster-Hauser syndrome. Ding et al. reported successful use of acellular porcine small intestinal submucosa graft in MRKHS patients (Ding et al., 2015). In future it might become a choice for transsexual patients who have previously undergone penectomy and orchiectomy. Main drawback of using acellular dermal matrix, however, is the high cost.

Clitoro-labioplasty (Vulvoplasty)

In order to achieve the physiologic and aesthetic equivalent of female external genitalia it is imperative to create labia majora and minora, a clitoris and a clitoral hood. The creation of the labia majora is dependent on the use of either a penile flap or a graft, and the amount of scrotal skin remaining after resection. Secondary corrections may be needed and a common secondary correction is symmetrization of the labia majora and sometimes a commisuroplasty with recreation of the anterior commissure covering the neoclitoris (Selvaggi et al., 2005).

Little has been written specifically about the labia minora. Perovic et al. described using the base of the penile skin to form the labia minora, which are then sutured to the deepithelialized area of the neoclitoris. Thus, the neoclitoris is hooded with labia minora (Perovic et al., 2000). We have used penile foreskin (in continuity with the glans flap for clitoral reconstruction – see below) in order to construct the labia minora and the clitoral hood.

The first person to describe the construction of a neoclitoris was Brown, in 1976. He reported the creation of a functional clitoris using the reduced glans, which remained attached to its dorsal penile neurovascular pedicle (Brown, 1976, 1978). At present, most surgeons performing a clitoroplasty in transsexual patients use the dorsal portion of the glans penis in a horseshoe or W-pattern with the dorsal neurovascular pedicle (Selvaggi et al., 2005; Karim et al., 1995). Dissection of the pedicle can be performed in a plane just above the tunica albuginea of the corpora cavernosa, or in a plane just posterior to this tunica. The latter is a more rapid dissection, but may result in a bulky pedicle and thus an elevated mons pubis.

The clitoral hood and the labia minora can be constructed using a thin inner layer of penile foreskin, which is harvested in continuity with the glans flap. This is the only manner in which the very delicate features of the anterior commissure of the vulva, where the clitoris is located and where the labia minora start from the clitoral hood, can be reconstructed in a natural way (Figs. 9 and 10).

The neoclitoris has the ability to swell and cause a climax on erogenous stimulation. In order to achieve swelling of the labia minora as well, we have designed an extended clitoroplasty in which two lateral extensions of the glans penis are placed in the base of the labia minora (unpublished data). Anatomically, this extended neoclitoris mimics better the female clitoral apparatus. The extensions simulate the female corpora cavernosa; cylindrical organs, made of cavernous erectile tissue who are the “hidden” part of the clitoris (Puppo, 2011).

Selvaggi et al. (2005) described the use of the penile urethra to construct the region between the urethral opening and the neoclitoris. The urethra is incised longitudinally along its ventral aspect, folded open, and sutured just inferior to the neoclitoris. This produces a natural appearance, in both color and texture (Fig. 11).

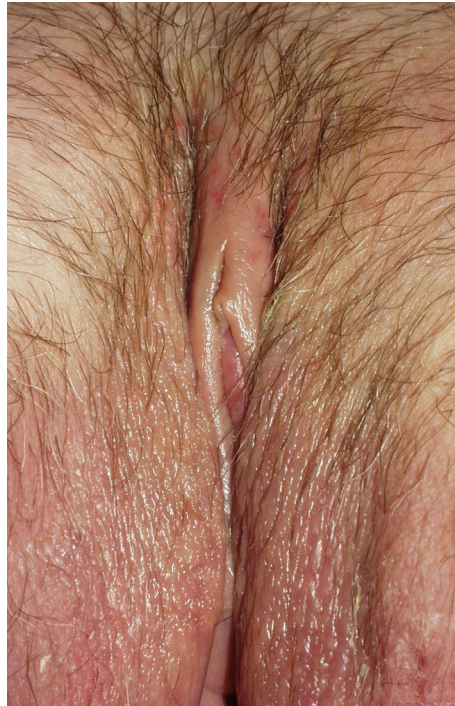


Fig. 9 Vaginoplasty—result after 3 months with a detail of the clitoral hood.

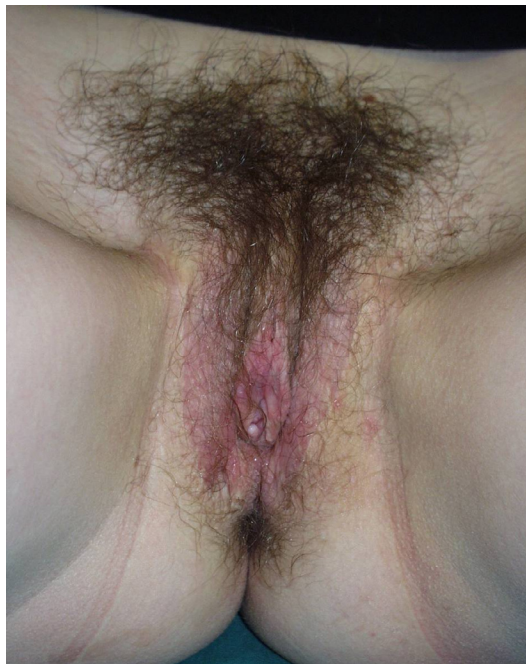


Fig. 10 Long-term result vagino-clitoroplasty.

Postoperative Care

When an anteriorly pedicled penile skin flap technique has been used in combination with a posterior scrotal flap and skin grafts, the patient usually remains in bed for 4–5 days, with the dilator firmly in place. Fractionized Heparin is provided subcutaneously.

After 5 days, the dilator is removed, and the patient is allowed to ambulate. The neovaginal cavity is cleansed daily with an iso-betadine solution and the patients starts to dilate according to a strict protocol. The following day, the urethral catheter is removed and spontaneous voiding is started.



Fig. 11 Long-term result vagino-clitoroplasty: labia minora and clitoris.

The patient is usually discharged on the seventh or eighth postoperative day, and is instructed in the care of the neovagina. This consists of progressive removal of the dilator and irrigation of the vaginal cavity with isobetadine. The period of time that the dilator remains out is gradually increased over the following 3–6 months. Later, if the patient is having regular sexual intercourse, no further dilation is required. Otherwise, routine dilation should continue once or twice per week, especially if a full-thickness skin graft has been used in addition to the pedicled penile skin flap.

Complications

Although the surgical techniques for vaginoplasty have evolved and improved significantly, the patients have to be informed that both medical and surgical treatments are rarely perfect. Revisional surgery is sometimes required to optimize aesthetic results.

Early (but rare) postoperative complications include bleeding (usually resolved by applying some pressure), lower extremity compartment syndrome, infection or impaired wound healing. One specific complication related to the vaginoplasty procedure is the development of a recto-vaginal fistula, as the vaginal cavity needs to be created between the prostate urethra anteriorly and the rectum posteriorly. The rectum has a rather thin wall, and care must be taken to avoid a perforation. If a rectal injury occurs during the dissection of the neovagina, a double layer closure should be performed. This usually heals without problem since a preoperative bowel preparation is routinely performed.

Possible late complications include stenosis of the new urethral meatus, which is rare with the eversion flap of the urethral mucosa.

Life-long vaginal hygiene and dilatation is recommended, especially in operated transwomen undergoing limited or no penetrative sex. Most patients are able to reach an orgasm after the operation (clitoral in most cases), and despite the fact that they often experience some moistening of the vulva during sexual excitation most transwomen require lubrication for sexual intercourse. Until the first uterine transplantation in a transwoman, pregnancy is of course not possible.

References

- Benjamin, H. (1964). Nature and Management of Transsexualism, with a report on thirty-one operated cases. *Western Journal of Surgery, Obstetrics, and Gynecology*, 72, 105–111.
- Bouman, F. G. (1988). Sex reassignment surgery in male to female transsexuals. *Annals of Plastic Surgery*, 21, 526–531.
- Bouman, M. B., van Zeijl, M. C., Buncamper, M. E., Meijerink, W. J., van Bodegraven, A. A., & Mullender, M. G. (2014). Intestinal vaginoplasty revisited: A review of surgical techniques, complications, and sexual function. *The Journal of Sexual Medicine*, 11, 1835–1847.
- Brown, J. (1976). Creation of a functional clitoris and aesthetically pleasing Introitus in sex conversion. In D. Marchac, & J. T. Hueston (Eds.), *Transactions of the Sixth International Congress of Plastic and Reconstructive Surgery* (pp. 654–655). Paris: Masson.
- Brown, J. (1978). A single-stage operative technique for castration, vaginal construction, and Perineoplasty in transsexuals. discussion *Archives of Sexual Behavior*, 4, 313–323.
- Cairns, T. S., & de Villiers, W. (1980). Vaginoplasty. *South African Medical Journal*, 57, 50–55.

- Colebunders, B., T'Sjoen, G., Weyers, S., & Monstrey, S. (2014). Hormonal and surgical treatment in transwomen with BRCA1 mutations: A controversial topic. *The Journal of Sexual Medicine*, 11, 2496–2499.
- Coleman, E., et al. (2012). Standards of care for the health of transsexual, transgender, and gender-nonconforming people, Version 7. *International Journal of Transgenderism*, 13(4).
- Dalton, J. R. (1981). Use of sigmoid colon in sex reassignment operations. *Urology*, 17, 223–227.
- Dessy, L. A., Mazzocchi, M., Corrias, F., Ceccarelli, S., Marchese, C., & Scuderi, N. (2014). The use of cultured autologous oral epithelial cells for vaginoplasty in male-to-female transsexuals: A feasibility, safety, and advantageousness clinical pilot study. *Plastic and Reconstructive Surgery*, 133, 158–161.
- Ding, J. X., Chen, L. M., Zhang, X. Y., Zhang, Y., & Hua, K. Q. (2015). Sexual and functional outcomes of vaginoplasty using acellular porcine small intestinal submucosa graft or laparoscopic peritoneal vaginoplasty: A comparative study. *Human Reproduction*, 30, 581–589.
- Eicher, W. (1989). The inverted penis skin technique in male-to-female transsexuals. In W. Eicher, F. Kubli, & V. Herms (Eds.), *Plastic surgery in the sexually handicapped* (pp. 91–112). Berlin: Springer-Verlag.
- Edgerton, M. T., & Bull, J. (1970). Surgical construction of the vagina and labia in male transsexuals. *Plastic and Reconstructive Surgery*, 46, 529–539.
- Fogh-Andersen, P. (1956). Transvestism and transsexualism; surgical treatment in a case of auto-castration. *Acta Medicinæ Legalis et Socialis (Liege)*, 9, 33–40.
- Gillies, H., & Millard, R. D., Jr. (1957). Genitalia. In *The principles of art of plastic surgery* (pp. 368–388). London: Butterworth.
- Gooren, L. J., van Trotsenburg, M. A., Giltay, E. J., & van Diest, P. J. (2013). Breast cancer development in transsexual subjects receiving cross-sex hormone treatment. *The Journal of Sexual Medicine*, 10, 3129–3134.
- Hage, J. J., & Karim, R. B. (1998). Abdominoplastic secondary full-thickness skin graft vaginoplasty for male-to-female transsexuals. *Plastic and Reconstructive Surgery*, 101, 1512–1515.
- Hage, J. J., Becking, A. G., de Graaf, F. H., & Tuinzing, D. B. (1997). Gender-confirming facial surgery: Considerations on the masculinity and femininity of faces. *Plastic and Reconstructive Surgery*, 99, 1799–1807.
- Hage, J. J., Karim, R. B., & Laub, D. R., Sr. (2007). On the origin of pedicled skin inversion vaginoplasty: Life and work of Dr Georges Burou of Casablanca. *Annals of Plastic Surgery*, 59, 723–729.
- Huang, T. T. (1995). Twenty years of experience in managing gender dysphoric patients: I. Surgical management of male transsexuals. *Plastic and Reconstructive Surgery*, 96, 921–930. discussion 31–4.
- Isshiki, N. (1989). *Phonosurgery – theory and practice*. Berlin, Heidelberg, New York: Springer.
- Jones, H. W., Jr., Schirmer, H. K., & Hoopes, J. E. (1968). A sex conversion operation for males with transsexualism. *American Journal of Obstetrics and Gynecology*, 100, 101–109.
- Kanhai, R. C., Hage, J. J., Asscheman, H., & Mulder, J. W. (1999a). Augmentation mammoplasty in male-to-female transsexuals. *Plastic and Reconstructive Surgery*, 104, 542–549. discussion 50–1.
- Kanhai, R. C., Hage, J. J., Karim, R. B., & Mulder, J. W. (1999b). Exceptional presenting conditions and outcome of augmentation mammoplasty in male-to-female transsexuals. *Annals of Plastic Surgery*, 43, 476–483.
- Karim, R. B., Hage, J. J., Bouman, F. G., de Ruyter, R., & van Kesteren, P. J. (1995). Refinements of pre-, intra-, and postoperative care to prevent complications of vaginoplasty in male transsexuals. *Annals of Plastic Surgery*, 35, 279–284.
- Karim, R. B., Hage, J. J., & Mulder, J. W. (1996). Neovaginoplasty in male transsexuals: Review of surgical techniques and recommendations regarding eligibility. *Annals of Plastic Surgery*, 37, 669–675.
- Kim, S. K., Park, J. H., Lee, K. C., Park, J. M., Kim, J. T., & Kim, M. C. (2003). Long-term results in patients after rectosigmoid vaginoplasty. *Plastic and Reconstructive Surgery*, 112(1), 143–151.
- Laub, D. R., & Fisk, N. (1974). A rehabilitation program for gender dysphoria syndrome by surgical sex change. *Plastic and Reconstructive Surgery*, 53, 388–403.
- Li, F. Y., Xu, Y. S., Zhou, C. D., Zhou, Y., Li, S. K., & Li, Q. (2014). Long-term outcomes of vaginoplasty with autologous buccal micromucosa. *Obstetrics and Gynecology*, 123, 951–956.
- Lin, W. C., Chang, C. Y., Shen, Y. Y., & Tsai, H. D. (2003). Use of autologous buccal mucosa for vaginoplasty: A study of eight cases. *Human Reproduction*, 18, 604–607.
- McGregor, I. A. (1989). *Fundamental techniques of plastic surgery and their surgical applications* (8th edn, pp. 39–63). Edinburgh: Churchill Livingstone.
- Monstrey, S., Hoebeke, P., Dhont, M., De Cuyper, G., Rubens, R., Moerman, M., Hamdi, M., Van Landuyt, K., & Blondeel, P. (2001). Surgical therapy in transsexual patients: A multi-disciplinary approach. *Acta Chirurgica Belgica*, 101, 200–209.
- Pandya, N. J., & Stuteville, O. H. (1973). A one-stage technique for constructing female external genitalia in male transsexuals. *British Journal of Plastic Surgery*, 26, 277–282.
- Perovic, S. V., Stanojevic, D. S., & Djordjevic, M. L. (2000). Vaginoplasty in male transsexuals using penile skin and a urethral flap. *BJU International*, 86, 843–850.
- Puppo, V. (2011). Embryology and anatomy of the vulva: The female orgasm and women's sexual health. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 154, 3–8.
- Selvaggi, G., Ceulemans, P., De Cuyper, G., VanLanduyt, K., Blondeel, P., Hamdi, M., Bowman, C., & Monstrey, S. (2005). Gender identity disorder: General overview and surgical treatment for vaginoplasty in male-to-female transsexuals. *Plastic and Reconstructive Surgery*, 116, 135e–145e.
- van Noort, D. E., & Nicolai, J. P. (1993). Comparison of two methods of vagina construction in transsexuals. *Plastic and Reconstructive Surgery*, 91, 1308–1315.
- Wedler, V., Meuli-Simmen, C., Guggenheim, M., Schneller-Gustafsson, M., & Kunzi, W. (2004). Laparoscopic technique for secondary vaginoplasty in male to female transsexuals using a modified vascularized pedicled sigmoid. *Gynecologic and Obstetric Investigation*, 57, 181–185.
- Wee, J. T., & Joseph, V. T. (1989). A new technique of vaginal reconstruction using neurovascular pudendal-thigh flaps: A preliminary report. *Plastic and Reconstructive Surgery*, 83, 701–709.
- Weigert, R., Frison, E., Sessieq, Q., Al Mutairi, K., & Casoli, V. (2013). Patient satisfaction with breasts and psychosocial, sexual, and physical well-being after breast augmentation in male-to-female transsexuals. *Plastic and Reconstructive Surgery*, 132, 1421–1429.
- Weyers, S., Villeirs, G., Vanherreweghe, E., Verstraelen, H., Monstrey, S., Van den Broecke, R., & Gerris, J. (2010). Mammography and breast sonography in transsexual women. *European Journal of Radiology*, 74, 508–513.
- Wierckx, K., Stuyver, I., Weyers, S., Hamada, A., Agarwal, A., De Sutter, P., & T'Sjoen, G. (2012). Sperm freezing in transsexual women. *Archives of Sexual Behavior*, 41, 1069–1071.
- Wolfort, F. G., Dejerine, E. S., Ramos, D. J., & Parry, R. G. (1990). Chondrolaryngoplasty for appearance. *Plastic and Reconstructive Surgery*, 86, 464–469. discussion 70.
- Zhang, D., Zhang, J., Wang, H., Li, B., Zhu, X., Wang, L., & Wu, J. (2014). Comparative study on laparoscopic vaginoplasty using pedicled ileal and sigmoid colon segment transfer. *Zhonghua Fu Chan Ke Za Zhi*, 49, 172–175.

Further Reading

Ettner, R., Monstrey, S., & Coleman, E. (2016). *Principles of transgender medicine and surgery* (2nd edn). New York: Routledge, Taylor & Francis Group.

MENTAL HEALTH, ETHICS AND THE LAW

Psychological Aspects of Female Reproductive Medicine

Pilar Dolz del Castellar Pareja and Mar Nohales-Córcoles, IVI-RMA IVI, Valencia, Spain

Patricia Díaz-Gimeno, IVI-RMA IVI Foundation, Valencia University, Valencia University, INCLIVA, Valencia, Spain; INCLIVA, Valencia, Spain; and Biopolo La Fe, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Assisted reproductive technology (ART) All treatments or procedures that include the in vitro handling of both human oocytes and sperm or of embryos for the purpose of establishing a pregnancy. This includes, but is not limited to, in vitro fertilization and embryo transfer, gamete intrafallopian transfer, zygote intrafallopian transfer, tubal embryo transfer, gamete and embryo cryopreservation, oocyte and embryo donation, and gestational surrogacy. ART does not include assisted insemination (artificial insemination) using sperm from either a woman's partner or a sperm donor.

Elective embryo transfer The transfer of one or more embryos, selected from a larger cohort of available embryos.

Embryo The product of the division of the zygote to the end of the embryonic stage, 8 weeks after fertilization. (This definition does not include either parthenotes—generated through parthenogenesis—nor products of somatic cell nuclear transfer.)

Embryo donation The transfer of an embryo resulting from gametes (spermatozoa and oocytes) that did not originate from the recipient and her partner.

Implantation rate The number of gestational sacs observed divided by the number of embryos transferred.

In vitro fertilization (IVF) An ART procedure that involves extracorporeal fertilization.

Infertility (clinical definition) Disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.

Initiated cycle An ART cycle in which the woman receives specific medication for ovarian stimulation, or monitoring in the case of natural cycles, with the intention to treat, irrespective of whether or not follicular aspiration is attempted.

Ovulation induction (OI) Pharmacologic treatment of women with anovulation or oligo-ovulation with the intention of inducing normal ovulatory cycles.

Abbreviations

ACTH Adrenocorticotropin hormone

CRH Corticotropin releasing hormone

GnRH Gonadotropin-releasing hormone

LH Luteinizing hormone

Psychological Intervention in a Reproduction Treatment

According to the World Health Organization, infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild et al., 2009). Nearly two decades after the inclusion of psychology in the multidisciplinary teams of AR centers, it is a known fact that in general, the difficulties to conceive may have profound psychological effects and are often accompanied by an evolutionary crisis. This crisis is the reflection of internal conflicts in which stress (referred as distress), anxiety, excessive sadness and depression are manifested (Guerra, 2002).

The search for solutions to getting pregnant usually leads to emotional suffering, which would be more or less intense due to multiple personal and situational factors. Currently we know that between 25% and 65% of patients undergoing an AR treatment display clinical symptoms of stress, anxiety and negative emotions such as hopelessness, low self-esteem or guilt (Guerra, 2002; Guerra et al., 1998).

Accepting the infertility diagnosis can create a vital crisis in both the diagnosed person and in her partner. It is an emotional imbalance produced by a problem for which almost no one is prepared for. Occasionally this suffering is carried in secret, which in turn produces social isolation. This diagnosis implies entering a medical world that they do not know of, with lots of clinical tests,

medications and even surgical interventions that, although not compromising life, it will never be the same. All this combined with the uncertainty of success or failure of the treatment makes one of the most important projects of human beings “stagger” (Hill et al., 2016).

Over the years, the relationship between psychological aspects and infertility has become more and more evident. In some cases, patients can become more anxious, increasing sexual dysfunction (Bracewell-Milnes et al., 2016; Cousineau and Domar, 2007). For years, distress has been recognized among the scientific community as a risk factor associated with infertility because of its more than likely negative influence on reproductive success, probably decreasing the pregnancy rates. This is possibly due to both the sustained stress that people suffer in their daily lives for work or family reasons and the stress that is generated when feeling infertile and becoming an AR patient.

For all of the above reasons, nowadays there is a consensus regarding the comprehensive care of assisted reproduction patients, including the emotional and psychological support from the outset to reduce the levels of stress, anxiety and depression among patients undergoing treatment (Blyth, 2012; Peterson et al., 2012). It has been highlighted that women trying to get pregnant and couples facing IVF treatments often have depression rates similar to women who have cancer (Domar et al., 1993).

The psychologist in the field of AR, and indeed in all areas, is a process facilitator who helps patients, making their relationship with reality more positive. At the beginning of AR treatments, we will find couples with affectionation in different areas. The psychologists can identify problems at the cognitive level, when the ability to face problems and the problem solving skills usually used are overwhelming. Also frequent are the affections at the psychological level, reflecting feelings of denial, confusion, fear, sadness, emotional flattening, disbelief, guilt, excitability and restlessness follow the initial state of shock. In addition, it is common to find a general stress reaction caused by affections at the physiological level (Gameiro et al., 2015).

On the other hand, the medical treatment itself and its vicissitudes are often the source of emotional alterations. If any medical treatment is a source of stress for the patient, the assisted reproduction treatment is especially so. In fact, we can summarize this affectionation in a high percentage of the patients in AR treatment as an adaptive disorder which brings with it anxiety, distress, sadness, and depression.

The approach of patients by the psychology unit follows a protocol within the competencies of the psychologist in an AR center (Guerra, 2002). In our center, we follow a program implemented with the aim of integrating patient-centered attention into the daily activities of the medical team and, at the same time, making use of counseling and psychological intervention to meet any exceptional patient need.

This developed program consists of several actuation areas. The psychological unit offers the possibility of having an initial interview and psychological advice to all those persons or couples who desire it: advice to all couples or single female recipients; advice to all couples after pregnancy (especially multiple pregnancies); council to all embryo reductions and curettages; advice to patients who have to complete their treatment; treatment by cognitive-behavioral intervention of diagnosed disorders or symptomatology; derivation to the psychiatrist in the case of serious pathology consideration and therapeutic groups, for the prevention of possible emotional difficulties through information and teaching strategies to face problems, trying to stimulate psychological support among patients.

For a good psychological intervention, in all the steps of the infertility consulting is very important the training of the professionals that will have contact with the patients (as receptionists, secretaries, nurses, embryologists, and physicians). This training consists of patient care, crisis management, group dynamics, communication skills, and “burn-out” syndrome.

All this training has to be supervised by the expert in emotional support, providing skills in human resources, as support in the personnel selection process, resources and job optimization, group invigoration and emotional support to the team. These tools can be enforced by training courses for mental health professionals.

Finally, there has to be a feedback to evaluate this attention, through a systematic evaluation of the patients satisfaction regarding emotional support by the team, through questionnaires of perceived satisfaction.

The second area is referred to the psychological intervention itself. A systematic evaluation of possible risk populations within the group of patients by the development of screening tools (with a high cut-off point) has to be performed, for the detection of possible pathology. In the first visits or patients that start a new cycle and were not evaluated previously, nurses will give a test to the couple and the results will be evaluated by the psychologist. Couples and single women who choose donated gametes or embryos, will also be provided with advice for “reproduction with a Third Party”. Special psychological interviews will be performed to “reproductively older women” (women older than 40 years old) and gamete and embryo donors. Any patient that requests her evaluation will have it.

This psychological help is focus in facilitate the expression of emotions, identify the causes of emotional difficulty, educate about infertility, being sure that they have the necessary information in order to make decisions about the treatments to which they will undergo, and minimizing distress and equip patients with coping strategies for better adherence to treatments.

To achieve these goals, apart from this patient-centered care and psychological intervention, patients have a psychological support program based on “Brief Behavioral Cognitive Therapy”, in which patients are evaluated and trained in emotional self-control techniques and coping strategies. Some of the evaluation tools used are described above (Table 1).

Psychological Factors Associated to IVF Treatments

Since the development of the in vitro fertilization (IVF) procedure in 1978, there has been growing interest in the emotional experiences of IVF patients. Patients frequently report infertility and its treatment as one of the most stressful experiences of their lives.

Table 1 Evaluation tools

<i>Evaluation tool name</i>	<i>Objective</i>	<i>Characteristics</i>
Fertility quality of life tool (FertiQoL)	To evaluate the influence of infertility problems in different areas of life, such as the impact on self-esteem, emotions, general health, family, the couple, social relationships, work life, future life projects and the emotional impact of assisted reproduction treatments	Self-reported questionnaire, completed by the patients. (Boivin et al., 2011b)
The State–Trait Anxiety Inventory (STAI)	Measure of trait and state anxiety. Used to diagnose anxiety and to distinguish it from depressive syndromes	Self-reported questionnaire (Spielberger et al., 1983)
The Infertility Self-Efficacy Scale (ISES)	To measure the perception of self-efficacy in people facing infertility treatments	(Cousineau et al., 2006)
The Beck Anxiety Inventory (BAI)	To measure the severity of anxiety in children and adults	Multiple-choice self-report inventory (Beck et al., 1988)
State–Trait Anger Expression Inventory 2 (STAXI-2)	To assess state anger, trait anger, and anger expression and to measure the way these components contribute to medical conditions	(Forgays et al., 1998)

Among the undesirable effects of stress on health, there exists evidence that it adversely affects reproductive function (Rockliff et al., 2014).

Currently, lots of psychological factors have been associated to the IVF treatment success and could be used to assist in the identification of prospective IVF patients at high risk of psychological distress (Rockliff et al., 2014). After the meta-analysis of Rockliff and colleagues, neuroticism and the use of escapist coping strategies were linked with increased distress by multiple studies. Neuroticism, also known inversely as “emotional stability”, refers to the tendency to experience negative emotions. Neuroticism personality may experience primarily one specific negative feeling such as anxiety, anger, or depression. Social support was associated with low distress levels across several studies. A few other psychosocial variables, including self-criticism, dependency, situation appraisals and attachment style, appear to be associated with distress but have only been explored by one or two studies at most (Rockliff et al., 2014).

Classically, stress is defined as any physical or psychological stimulus that disturbs individual homeostasis. It can be understood as a condition in which the individual is alerted by an unfavorable situation. However, the concept of stress is more complex, so its interpretation requires some important considerations. First, for a situation to be considered stressful for an individual, that person must perceive it as an alarming or unusual experience. However, this first requirement may also be a component of pleasurable experiences. It is known as eustress, or simply stress, to “the good” stress, to that stress that helps us to be aware of ourselves, to leave our comfort zone and to run certain risks that are not dangerous, but help us to improve our personal development and grow (Witt et al., 2009). On the other hand, it can be defined as distress the “unpleasant” stress. It is a stress that causes an excess of effort in relation to the load or threat. It is always accompanied by a physiological disorder. It is understood as a set of harmful stimuli that weaken, subtracts confidence in oneself and subtracts strength to act. This second condition refers to the individual considering the circumstance in a hostile manner and, therefore, trying to modify or avoid that state. Distress represents the effects of anything that seriously threatens homeostasis. Stress-related outcomes also vary according to personal and environmental factors. Personal risk factors for the development of depression, anxiety, or posttraumatic stress disorder (PTSD) after a serious life event, disaster, or trauma include prior psychiatric history, neuroticism, female gender, and other sociodemographic variables. There is also some evidence that the relationship between personality and environmental adversity may be bidirectional. Levels of neuroticism, emotionality, and reactivity correlate with poor interpersonal relationships as well as “event proneness.” Protective factors that have been identified include, but are not limited to, coping, resources (e.g., social support, self-esteem, optimism), and finding meaning (Schneiderman et al., 2005).

The other component to take into consideration is the ability of the individual to control the stress situation. In this way, subjects able to maintain control during adverse experiences, manage to attenuate the detrimental consequences of stress to a greater extent than individuals who fail to react favorably to the same stimulus. The magnitude of the stress response with its consequent physiological effects will be greatly affected by the individual’s perception and ability to control the situation. The stress response involves physiological and psychological processes that are normally activated in new or adverse situations. Therefore, this response is an advantage for the healthy organism, which allows to adapt and overcome the unfavorable conditions.

Some previous studies in humans have concluded that emotional distress caused by fertility problems or other life events co-occurring with treatment will not compromise the chance of becoming pregnant (Boivin et al., 2011a). The controversy between studies has been related to the criteria to classify patients as distressed or controls, the design of the study, or small sample size (Smeenk et al., 2001).

In 2002, we performed one of the first projects that relate high scores on stress and anxiety with poor results of assisted reproduction treatments (Dolz and Garcia, 2002). This study aimed to verify if there is relation between counting or not on psychological support and a positive result of the assisted reproduction treatment (in terms of pregnancy). Two groups of assisted reproduction patients were compared. The first group was submitted to psychological support (35 patients) and the second one was not (25

patients). As follow-up criteria were considered the number of dropouts of the assisted reproduction treatment and the percentage of pregnancies in both groups. The psychological support program has a variable duration but on average consists of eight sessions given over 2–3 months. The program was based on four fundamental pillars. The first one was training in self-control techniques as breathing, relaxation, stopping negative thoughts, rejection and cognitive reconstruction of ideas and irrational beliefs. The second one was the induction of a positive emotional state (self-reinforcement, assertiveness, positive visualizations). The third one was training in prevention techniques and response control, reinforcing and scheduling critical stages along the treatment, and the fourth was to provide general information about the treatment, description of the steps to follow, desensitization about phobias or possible parallel problems that may arise.

The results obtained in this study were very interesting. In the group that followed the psychological support program, we obtained 54% of pregnancies, with a percentage of abandonment of the ART of 5.7%. However, in the group that did not follow the program, 28% pregnancies and live births were obtained with an abandonment rate of the ART 37.5% (Dolz and Garcia, 2002). As the results reflected, we considered that the patients that received psychological support during the ART cope better with the penalties and show less treatment abandon, due probably a higher quality of life and a better emotional situation. They are livelier to try it more times and therefore they have more probabilities to achieve their goal.

Physiological Mechanism Between Psychology and Infertility

The physiological mechanisms related to stress and reproduction are thought to include centrally controlled GnRH pulse inhibition as well as autonomic nervous system activation leading to alterations in ovarian and uterine function (Rockliff et al., 2014).

Stress hormones are produced by the sympathetic nervous system (SNS) and hypothalamic-pituitary adrenocortical axis. The SNS stimulates the adrenal medulla to produce catecholamines (e.g., epinephrine). In parallel, the paraventricular nucleus of the hypothalamus produces corticotropin releasing hormone (CRH), which in turn stimulates the pituitary to produce adrenocorticotropin (ACTH). Adrenocorticotropin then stimulates the adrenal cortex to secrete cortisol. Together, catecholamines and cortisol increase available sources of energy by promoting lipolysis and the conversion of glycogen into glucose (e.g., blood sugar).

The short term stress response can become maladaptive if it is repeatedly or continuously activated. Chronic SNS stimulation elevated basal levels of stress hormones associated with chronic stress also suppress immunity by directly affecting cytokine profiles. Cytokines are communicatory molecules produced primarily by immune cells involved in inflammatory reactions (Schneiderman et al., 2005).

It is well documented that the long-term stress can lead to serious depression, anxiety, eating disorders, irrational fears, stomach ulcers, irritable bowel syndrome, chronic migraines, blood pressure issues, heart problems, osteoporosis, hair loss, and hormonal imbalances and sexual dysfunction. Long-term untreated stress can lead to serious illness. Stress hormones alters the immune system and other tissues and systems as cardiovascular, digestive, musculoskeletal, reproductive and others as skin, hair, asthma attacks, dry mouth, and mouth ulcers.

Sensory stimuli are processed in the central nervous system through two pathways. The first is a direct and rapid pathway, whose reflex and stereotyped responses are carried out in the spinal cord and brain stem (neurovegetative responses to stress) or in the hypothalamus (neurohumoral responses). In the second route, sensory information is directed through multiple relays and projections to specific areas of the cerebral cortex, such as the primary visual area (area 17 of Brodmann), the auditory area (area 41) or the somatosensory area (areas 3, 1 and 2). These areas are responsible, through their connections with regions such as the hippocampus, the amygdala and the septal area, to modulate information on body activation and behavioral planning. It is precisely this evaluative process that gives the adaptive and plastic character to the response to stress.

Exposure to severe stress situations or for long periods of time can adversely affect the body by compromising the health of the individual and is what we call distress at the present work.

The molecular relationship between stress and reproductive physiology is obvious in the steroid hormones synthesis in adrenal cortex (Fig. 1). During the stress effect, glucocorticoids (cortisol) and mineralcorticoids (aldosterone) are also formed from progesterone. This fact is important because glucocorticoids production could alter progesterone, estrogen and androgens production as they derive from their metabolism. Likewise, key functions in embryo implantation as immune system, cytokines and energetic metabolism are compromised in stress response.

At the ovarian physiology level, it has been demonstrated in sheep that stress increases CRH, increasing ACTH and therefore increasing cortisol. On the other hand, it decreases the GnRH and LH pulse amplitude, delaying the LH peak, decreasing estradiol levels and, although follicle growing can be observed, maturation and ovulation is impaired, as they depend on quicker pulses. In case of lower stress and quicker pulses, granulosa cells integrity is affected, altering their connexion with the oocyte. In this case, progesterone receptors do not seem to be involved. Also if the LH pulse is inadequate, ovulation cannot occur and the follicle can persist provoking a cystic ovarian syndrome (Dobson and Smith, 2000).

The complex relationship between psychological factors and fertility stress begins to become more and more recognized in the scientific literature as a factor that on some occasions can negatively influence the reproductive capacity of human beings. Probably for what we know it can become a determining factor as a mediator in the case of at least 5% of sterility problems of unknown origin. However, more detailed molecular information of what is happening in ovary and endometrial function in humans still remains unclear. Due to all these reasons, systematic attempts have been made to determine the emotional difficulties experienced

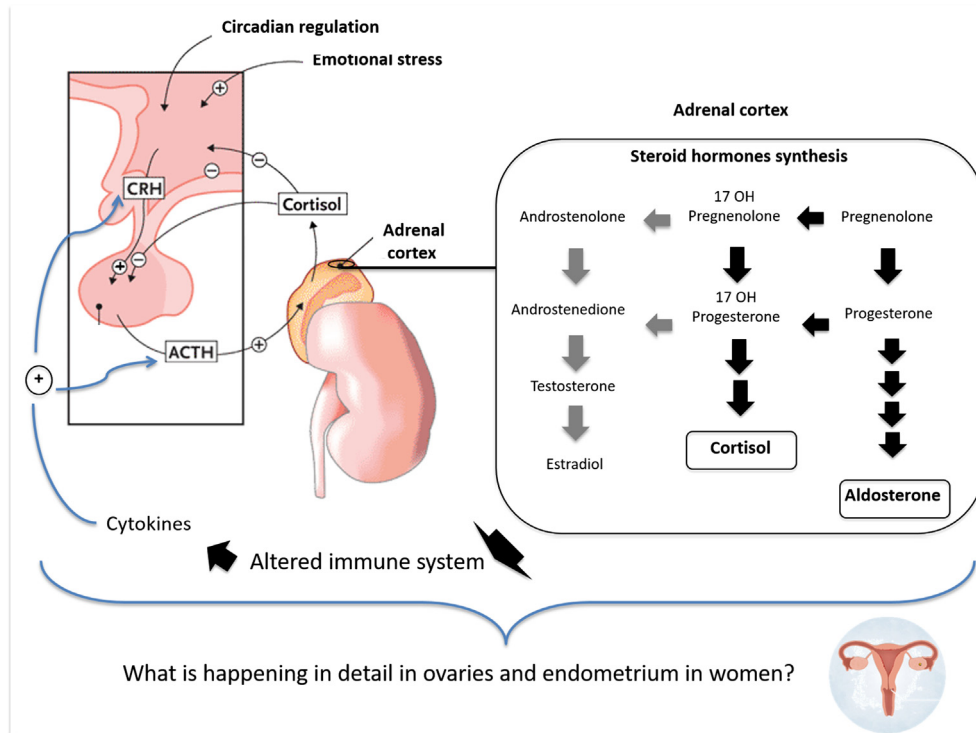


Fig. 1 Molecular relationship between long-term stress effect and reproductive physiology. The hypothalamus hipofisiary axis is activated in long-term stress response. CRH activates ACTH that increases the cortisol and aldosterone production and secretion in adrenal cortex. Immune systems is one of the affected systems by glucocorticoids production that increases cytokines that activates CRH and ACTH.

by sterile couples. The understanding of these difficulties and their therapeutic approach, by the mental health professional, becomes pressing in the clinical context from a perspective of comprehensive vision of the care required by the patients.

In this same line, some studies show that higher levels of stress suffered by the ART patient are correlated with higher abandon rates (Freeman et al., 1987). However, research works that relate high levels of stress (distress) to infertility are still inconclusive. On one hand, some studies show a clear relationship between distress, anxiety and depression with infertility (Borghi et al., 2017; Karaca et al., 2016) but, on the other hand, some works conclude with no evidence of that relationship. Although we must add that there are more of those who conclude that there is a relationship of negative psychological aspects with implantation failures and ultimately with infertility.

Two studies, one performed in Europe and the other in United States, found association between stress and fertility. Buck Louis et al. found that stress significantly reduced the probability of conception each day during the fertile window, possibly exerting its effect through the sympathetic medullar pathway (Louis et al., 2011). In a prospective cohort study Lynch and colleagues found that preconception stress increased the risk of infertility. Data suggest that stress and reproduction are interrelated; however, the directionality of that association is unclear (Lynch et al., 2014). Both studies demonstrated a prospective association between salivary stress biomarkers and longer time to pregnancy, and Lynch et al., the first in the world to observe an association with infertility. In contrast, they found no association between salivary cortisol and fecundability as Buck Louis et al. in 2011 in European population (Louis et al., 2011). After these two studies the evidence to associate stress with infertility have been found due to measured alpha amylase to classify patients as distressed.

Systems Medicine and Psycho-Cognitive Aspects

The systems medicine approach is an information-based discipline focused on a holistic understanding of complex biomedical systems. This is based on the application of information technology to increasingly understand sets of clinical, molecular, cellular, phenotypic, demographic, and psycho-cognitive patient data (Fig. 2) (Hood and Friend, 2011). Nowadays, the systems medicine called P4 medicine (predictive, personalized, preventive and participatory) models to treat patients has been transformed into P5, including as fifth P the "Psycho-cognitive" aspects to be considered in order to empower the patient, increase his/her quality of life and transform him/her from a passive recipient into an active decision-maker. The fifth P analyzes the behavioral component on the

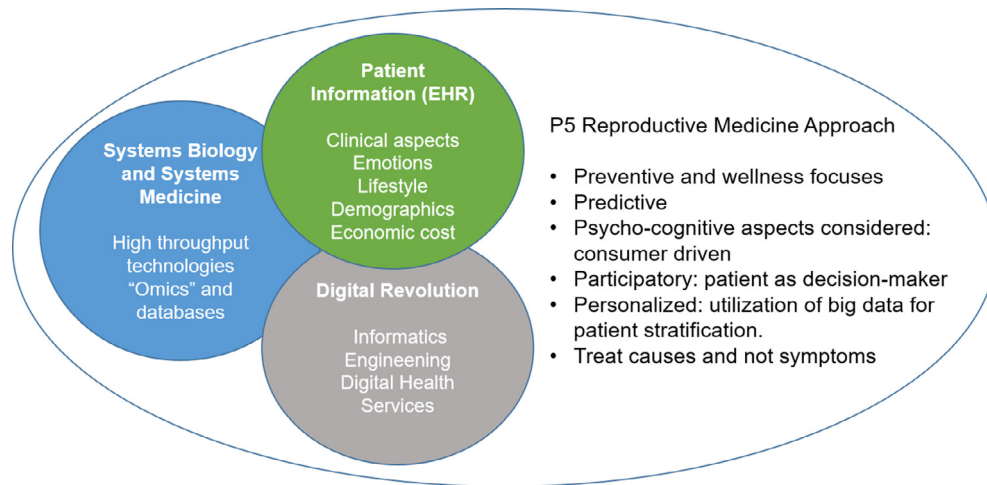


Fig. 2 P5 Systems Medicine Ecosystem. A schematic representation of the main actors involved in Systems P5 Medicine. EHR, Electronic Health Record.

way individuals act to prevent, cope and react to illnesses, how they decide between different therapeutic options and interact with clinicians in the treatment process (Gorini and Pravettoni, 2011).

All this patient information from a P5 medicine approach is a new way to perform medical practices and to develop smart health strategies providing personalized, safe and cost effective treatments (Flores et al., 2013).

This P5 medicine approach is an excellent opportunity to correlate genomic information with personality traits and mental health to understand the physiological effects in reproductive physiology.

In the last decade the controversy related to the effect of psycho-cognitive aspects and its main role in infertility has been solved, demonstrating that is a very important factor for physiology homeostasis and also health in reproduction. P5 medicine from a systems genomics approach is able to integrate all the patient information in a complex and multifactorial system that will allow us to understand in the next decade the detailed molecular relationship with reproductive physiology and the way to stratify patients in order to improve the infertility results and the patient experience in the infertility treatment.

References

- Beck, A. T., Epstein, N., Brown, G., & Steer, R. A. (1988). An inventory for measuring clinical anxiety: Psychometric properties. *Journal of Consulting and Clinical Psychology*, 56, 893–897.
- Blyth, E. (2012). Guidelines for infertility counselling in different countries: Is there an emerging trend? *Human Reproduction*, 27, 2046–2057.
- Boivin, J., Griffiths, E., & Venetis, C. A. (2011a). Emotional distress in infertile women and failure of assisted reproductive technologies: Meta-analysis of prospective psychosocial studies. *BMJ*, 342, d223.
- Boivin, J., Takefman, J., & Braverman, A. (2011b). The fertility quality of life (FertiQoL) tool: Development and general psychometric properties. *Human Reproduction*, 26, 2084–2091.
- Borghii, L., Leone, D., Vegni, E., Galiano, V., Lepadatu, C., Sulpizio, P., & Garzia, E. (2017). Psychological distress, anger and quality of life in polycystic ovary syndrome: Associations with biochemical, phenotypical and socio-demographic factors. *Journal of Psychosomatic Obstetrics and Gynaecology*, 1–10.
- Bracewell-Milnes, T., Saso, S., Bora, S., Ismail, A. M., Al-Memar, M., Hamed, A. H., Abdalla, H., & Thum, M. Y. (2016). Investigating psychosocial attitudes, motivations and experiences of oocyte donors, recipients and egg sharers: A systematic review. *Human Reproduction Update*, 22, 450–465.
- Cousineau, T. M., & Domar, A. D. (2007). Psychological impact of infertility. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 21, 293–308.
- Cousineau, T. M., Green, T. C., Corsini, E. A., Barnard, T., Seibring, A. R., & Domar, A. D. (2006). Development and validation of the infertility self-efficacy scale. *Fertility and Sterility*, 85, 1684–1696.
- Dobson, H., & Smith, R. F. (2000). What is stress, and how does it affect reproduction? *Animal Reproduction Science*, 60–61, 743–752.
- Dolz, P., & Garcia, A. (2002). Incidencia positiva de los Programas de Apoyo Psicológicos en los tratamientos de reproducción asistida. *Revista Iberoamericana de Fertilidad y Reproducción Humana*, 24, 233.
- Domar, A. D., Zuttermeister, P. C., & Friedman, R. (1993). The psychological impact of infertility: A comparison with patients with other medical conditions. *Journal of Psychosomatic Obstetrics and Gynaecology*, 14(Suppl), 45–52.
- Flores, M., Glusman, G., Brogaard, K., Price, N. D., & Hood, L. (2013). P4 medicine: How systems medicine will transform the healthcare sector and society. *Personalized Medicine*, 10, 565–576.
- Forgays, D. K., Spielberger, C. D., Ottaway, S. A., & Forgays, D. G. (1998). Factor structure of the State–Trait Anger Expression Inventory for middle-aged men and women. *Assessment*, 5, 141–155.
- Freeman, E. W., Rickels, K., Tausig, J., Boxer, A., Mastrianni, L., Jr., & Tureck, R. W. (1987). Emotional and psychosocial factors in follow-up of women after IVF-ET treatment. A pilot investigation. *Acta Obstetrica et Gynecologica Scandinavica*, 66, 517–521.
- Gameiro, S., Boivin, J., Dancet, E., de Klerk, C., Emery, M., Lewis-Jones, C., Thorn, P., Van den Broeck, U., Venetis, C., Verhaak, C. M., et al. (2015). ESHRE guideline: Routine psychosocial care in infertility and medically assisted reproduction—a guide for fertility staff. *Human Reproduction*, 30, 2476–2485.
- Gorini, A., & Pravettoni, G. (2011). P5 medicine: A plus for a personalized approach to oncology. *Nature Reviews. Clinical Oncology*, 8, 444. c1.
- Guerra, D. (2002). *Como afrontar la infertilidad*. Barcelona: Planeta.

- Guerra, D., Llobera, A., Veiga, A., & Barri, P. N. (1998). Psychiatric morbidity in couples attending a fertility service. *Human Reproduction*, 13, 1733–1736.
- Hill, B., McPhie, S., Fuller-Tyszkiewicz, M., Gillman, M. W., & Skouteris, H. (2016). Psychological health and lifestyle management preconception and in pregnancy. *Seminars in Reproductive Medicine*, 34, 121–128.
- Hood, L., & Friend, S. H. (2011). Predictive, personalized, preventive, participatory (P4) cancer medicine. *Nature Reviews. Clinical Oncology*, 8, 184–187.
- Karaca, N., Karabulut, A., Ozkan, S., Aktun, H., Oregul, F., Yilmaz, R., Ates, S., & Batmaz, G. (2016). Effect of IVF failure on quality of life and emotional status in infertile couples. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 206, 158–163.
- Louis, G. M., Lum, K. J., Sundaram, R., Chen, Z., Kim, S., Lynch, C. D., Schisterman, E. F., & Pyper, C. (2011). Stress reduces conception probabilities across the fertile window: Evidence in support of relaxation. *Fertility and Sterility*, 95, 2184–2189.
- Lynch, C. D., Sundaram, R., Maisog, J. M., Sweeney, A. M., & Buck Louis, G. M. (2014). Preconception stress increases the risk of infertility: Results from a couple-based prospective cohort study—The LIFE study. *Human Reproduction*, 29, 1067–1075.
- Peterson, B., Boivin, J., Norre, J., Smith, C., Thorn, P., & Wischmann, T. (2012). An introduction to infertility counseling: A guide for mental health and medical professionals. *Journal of Assisted Reproduction and Genetics*, 29, 243–248.
- Rockliff, H. E., Lightman, S. L., Rhidian, E., Buchanan, H., Gordon, U., & Vedhara, K. (2014). A systematic review of psychosocial factors associated with emotional adjustment in in vitro fertilization patients. *Human Reproduction Update*, 20, 594–613.
- Schneiderman, N., Ironson, G., & Siegel, S. D. (2005). Stress and health: Psychological, behavioral, and biological determinants. *Annual Review of Clinical Psychology*, 1, 607–628.
- Smeenk, J. M., Verhaak, C. M., Eugster, A., van Minnen, A., Zielhuis, G. A., & Braat, D. D. (2001). The effect of anxiety and depression on the outcome of in-vitro fertilization. *Human Reproduction*, 16, 1420–1423.
- Spielberger, C., Gorsuch, R., Lushene, R., & Jacobs, G. (1983). *Manual for the State-Trait Anxiety Inventory*. Madrid: Tea Ediciones.
- Witt, W. P., Kahn, R., Fortuna, L., Winickoff, J., Kuhlthau, K., Pirraglia, P. A., & Ferris, T. (2009). Psychological distress as a barrier to preventive healthcare among U.S. women. *The Journal of Primary Prevention*, 30, 531–547.
- Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., Vanderpoel, S., International Committee for Monitoring Assisted Reproductive Technology, & World Health Organization. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009. *Fertility and Sterility*, 92, 1520–1524.

Further Reading

- Moreno, A., & Guerra, D. (2009). Procesos emocionales en pacientes sometidos a Técnicas de Reproducción Asistida. In *Guías de evaluación, consejo, apoyo e intervención psicológica en Reproducción Asistida*. Revista Iberoamericana de Fertilidad y Reproducción Humana.

Legal and Ethical Aspects of Female Reproduction

Heather E Ross, Ross & Zuckerman, LLP, Northbrook, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Legal and Ethical Aspects of Female Reproductive Medicine

The practice of assisted reproductive technology ("ART") carries with it significant legal and ethical challenges, particularly in the area of third party reproduction. Because the physician's role will include treating both his/her patient (the intended parent(s)) and the "third party patient" (the gamete provider or surrogate), the physician should be aware of the potential for conflicting desires about medical treatment (i.e., an intended parent may desire a triplet pregnancy, while the surrogate may prefer to reduce). In addition, unlike other areas of medicine, the purpose of reproductive technology is to create a child, raising issues of disclosure and the rights of donor conceived offspring.

The laws in this area continue to evolve, and in many places remain unsettled or are based on strict compliance with the relevant statute or court practice. Not only do laws vary depending on the type of fertility treatment provided, but also by country, province, state and even counties or districts within a state. Prior to engaging in medical treatment the parties should consider how parentage to the child will be established, and how to ensure the gamete provider or surrogate is protected from financial and social responsibility.

Ethical Considerations

Medical Autonomy

One of the most interesting ethical dilemmas for physicians and lawyers involved in surrogacy arrangements is how to preserve the gestational surrogate's right to medical autonomy, while addressing the intended parent's desire to make medical decisions concerning the embryo transfer and health of the fetus. The physician should always confirm that the parties are in agreement with respect to the number of embryos transferred, termination and selective reduction, and all other health precautions/procedures each party agrees to follow before any party commences medication in furtherance of an embryo transfer to a gestational surrogate.

Physicians should be aware that federal laws protect the gestational surrogate's right to medical autonomy, including her right to terminate a pregnancy. The US Supreme Court frowned upon the notion of restricting a pregnant woman's activities in *Planned Parenthood of Southeastern Pennsylvania v. Casey*, holding that a husband's consent could not be required in order for his wife to obtain an abortion, and noting in dicta that, "if the husband's interest in the fetus' safety is a sufficient predicate for state regulation, the State could reasonably conclude that pregnant wives should notify their husbands before drinking alcohol or smoking." *Planned Parenthood v. Casey* (1992). Requiring medical treatment or procedures raises even more complex issues. Although parties to surrogacy agreements typically agree that the gestational surrogate will submit to medical procedures (i.e., ultrasound, amniocentesis, Cesarean section, etc.), the US Supreme Court has held that a person has a constitutionally protected right to refuse unwanted medical treatment or procedures (Ross, 2013–2014) (<http://www.dcba.org/mpage/vol261213art1>).

Notwithstanding a surrogate's right to medical autonomy, the US Supreme Court has held that parents have a constitutionally protected fundamental liberty interest in procreating and making decisions about how to raise their children. *Skinner v. Oklahoma*, 316 U.S. 535 (1942) (striking down legislation allowing for sterilization, Court found that "marriage and procreation are fundamental to the very existence and survival of the race") (*Skinner v. Oklahoma*, 1942). See also, (*Santosky v. Kramer*, 1982) (natural parents have a fundamental liberty interest in "the care, custody and management of their child"). See also, (*Stanley v. Illinois*, 1972) (the right to conceive and raise one's children is deemed essential and the "basic civil rights of man"), citing (*Skinner v. Oklahoma*, 1942).

If this interest is found to extend to decision making with respect to a fetus, it could potentially override a gestational surrogate's right to medical autonomy. Although the right of intended parent(s) to protect a fetus gestated by a third party has not yet been addressed by legislation or case law, the US Supreme Court has considered whether a state could have an interest in protecting fetal life. *Roe v. Wade* is the landmark decision granting a pregnant woman, along with her physician, the right to make decisions with respect to her pregnancy, including the right to terminate prior to fetal viability. However, *Roe* also recognized that a state could have a compelling interest in the potential life of a fetus ("if the State is interested in protecting fetal life after viability, it may go so far as to proscribe abortion during that period, except when it is necessary to preserve the life or health of the mother.") *Roe v. Wade*, 410 U.S. 113, 163–164 (1973). (*Roe v. Wade*, 1973)

Although jurisdictions differ with respect to whether a state's interest in protecting fetal life can override a women's right to medical autonomy, the majority of states will not force a woman to undergo medical treatment against her will. "When state courts have ordered competent pregnant women to undergo invasive medical procedures against their will, the decisions have almost invariably involved situations where the refusal of treatment was clearly and undeniably placing a viable fetus at severe risk". Wald, Deborah, esq. And Black, Katherine, J.D. Candidate, (2011) *Surrogacy and a Pregnant Woman's Constitutional Right to Medical and Procreative Choice – A Brief Survey*, www.waldlaw.net/assets/files/ABA%20surrogacy%20article.10.2011.pdf (Wald & Black Katherine, 2011)

Each Party's intent with respect to termination, selective reduction, and other medical issues affecting a surrogacy arrangement should be discussed prior to proceeding with medical treatment. Best practices also include independent legal representation and psychological consultations. Only through the process of thorough and open discussion can parties to surrogacy arrangements determine whether they are an appropriate match. A gestational surrogate who is opposed to amniocentesis or a Cesarean section should not be matched with intended parent(s) who want these procedures. The parties are much better off discovering any disagreements early on in the process rather than having a dispute arise during fertility treatment or pregnancy.

Physician Conflicts of Interest in Egg Donation Arrangements

In most practices, the physician treats both the intended parent(s) and the egg donor. Although intended parent(s) and egg donors have a unified purpose: obtaining viable embryos that will result in a successful pregnancy and birth, the means to achieve this outcome has inherent conflicts. The primary goal of one patient, the intended parent(s), is to create as many embryos as possible, so he/she has multiple chances at achieving pregnancy. The primary goal of the second patient, the egg donor, is to preserve her own health while also completing her donation. Many intended parent(s) can only afford one cycle of egg donation, and have experienced tremendous psychological and financial hardship and loss even before the use of donor eggs is recommended. To further complicate matters, typically the physician has known and treated the intended parent(s) long before the egg donor becomes a patient, further enforcing the feeling of the egg donor being the "third party" in the assisted reproductive arrangement.

In addition, the laws, or lack thereof, perpetuate this conflict. Pursuant to federal law, clinic's have a legal requirement to report fertility success rates in order to "help infertility patients make informed decisions about assisted reproductive technology." (See <https://www.fertilityauthority.com/articles/fertility-clinic-success-rates>.)

There is no corollary requirement to report any statistics demonstrating the medical affects or outcomes for egg donors (i.e., complications, amount of drugs used or number of eggs retrieved, etc.). In fact, the only federal regulations that do exist deal solely with truth in advertising (reporting statistics) and safety testing (i.e., to prevent donors from transmitting communicable diseases to recipients per FDA requirements). Although a few states have protective legislation (see, i.e., California, Arizona, and New York), and the American Society for Reproductive Medicine (ASRM), a self regulating association, has recommendations for informed consent, there are no federal laws that regulate informed consent, or what information must be provided to women who wish to donate their eggs (Cahn & Collins, 2014a).

Moreover, because reporting is required, and the higher the success rate the better the advertising for the clinic, the reporting mandate creates a financial incentive for clinics to achieve positive fertility outcomes and birth rates (Bass et al., 2014); (*Fertility Clinic Success Rate and Certification Act (1992)*). In short, all of these issues raise the question of whether the medical needs of the egg donor may be inadvertently overlooked in order to maximize the chances of a successful pregnancy.

Physicians should always focus on the best interests of both intended parent(s) and egg donors and balance the potential for conflicting desires. It may be helpful for reproductive physicians to look to physicians who work with living organ donors, as organ donation may be the most analogous situation to gamete donation (although without the ability to receive compensation). Federal laws governing organ donation require an independent donor advocate to make sure organ donors understand the risk they will be taking—with no medical benefit (*The Organ Procurement and Transplantation Network*, 2017).

Donor Gametes: Disclosure?

One of the most challenging issues for intended parents pursuing gamete donation is the decision of whether or not to disclose to the child that he/she was conceived with the help of a third party donor. Patients worry about how their child will react to this information, and whether the child will feel confused about her identity or whether knowing will jeopardize the child's ability to bond with her parents. "Simply put, parents want to know whether to tell their children of their genetic origins and if they do tell their children, how to talk to them about this complex subject" (Wilkins & Carole, n.d.) (Disclosure Issues Fact Sheet). Although there are no laws in the United States providing donor offspring the legal right to know the identity of her gamete provider, there has been a shift in practice in recent years moving away from strictly anonymous donor arrangements to more open exchanges of information between the intended parent(s) and the donor (though anonymous email exchanges or the donor Sibling Registry, see <https://www.donorsiblingregistry.com/>).

Mental health professionals agree that secrets in families are damaging and that children are better off knowing from a young age that they are not genetically related to one (or sometimes both) of their parents. Although donor conception has traditionally looked to adoption practices, including the dated belief that an adopted child did not need to know her origins, the adoption field has changed dramatically, and it is now well accepted that adopted children should have the right to know they are adopted and the identity of their birth parents (Cahn, 2014).

Several useful sources exist to obtain information regarding donor gametes and disclosure to donor conceived children (see <https://www.dcnetwork.org/>, www.resolve.org, www.path2parenthood.org, www.pved.org).

Legal Considerations

Lawyers practicing in this area spend much of their time drafting contracts between intended parent(s) and the third party provider, as well as drafting and reviewing consent forms between each patient and the fertility clinic. The ultimate purpose of these

documents is the creation of a child. They are not simple exchanges of property rights, and, therefore, the ART attorney must carefully explore and reflect their client's wishes, with detailed consideration of ethical issues inherent in these types of arrangements. Acknowledging the complexity of these arrangements and advising patients to consult with competent legal representation may be the most important advice a physician can provide to patients (and their third party patient) embarking on this family building journey ([Crocker & Debele, 2015](#)).

Sperm Donation

By the 1970s most states in the United States had passed laws addressing parentage rights with respect to children conceived from donor sperm. Although state laws differ with respect to how parentage is established, most state legislatures have found that sperm donation statutes will not apply to the birth of a child conceived by means of sexual intercourse. See ([Uniform Parentage Act, 2002](#)) ("This article does not apply to the birth of a child conceived by means of sexual intercourse"). The method by which the sperm is donated is often a determinative factor in establishing whether the sperm provider is a donor or parent. Many states require the sperm be donated through a licensed physician, while other states recognize a home artificial insemination (e.g., at the time of this article, in Texas, Kansas and Wisconsin the sperm must be provided through a licensed physician, while in Illinois and Florida a home insemination is permitted). Some state laws require the intended father's written consent, while others allow for implied consent. Some states require the parties to be married and some specifically terminate the rights of the donor. Failure to comply with the state statute can result in unintended consequences. For example, a Kansas sperm donor who gratuitously donated his sperm to a lesbian couple via a home insemination kit was later sued by the Kansas Department of Children and Families for child support—even though the parents of the child agreed that he was NOT a father and should not have any financial responsibility for the child. It took more than 4 years for the Kansas courts to relieve the sperm donor of child support obligations ([State of Kansas Ex. Rel. vs. W.M., 2016](#)). In short, complying with relevant statutes/case law is extremely important to protect the intent of the parties and parentage rights to the child.

Egg Donation

Although building families through egg donation is a much more recent medical advancement, by 2011 egg donation had already increased to 10% of all ART cycles ([Practice Committee of the American Society for Reproductive Medicine, 2014](#)). Laws pertaining to egg donation have been slow to follow, with only a handful of states specifically addressing egg donation ([Swain, 2014](#)).

Unlike sperm donation, where most states have passed laws and the main concern is correctly adhering to the law, in egg donation arrangements most states are silent on the issue of parentage, and although parentage laws typically consider the woman who gives birth to a child as proof that she is the child's mother, an intended mother has no legal guarantee that she will be considered the legal mother of the child. Because egg donation carries greater medical risk and is much more expensive and time consuming for the donor, many fertility clinics have put in place additional steps the participants must follow to attempt to make sure each party understands the medical, psychological and legal risks. For example, many clinics require independent legal counsel, a mental health consultation and detailed informed consent disclosures (although true informed consent may be somewhat lacking for many egg donors—see above). Many arrangements are governed by direct agreements between the egg donor and the intended parent(s). Entering into a direct agreement allows each party the opportunity to set forth his/her expectations of how the donor arrangement will work, both before any medications commence and during the egg retrieval process. Perhaps most important, it provides a legal framework for the parties to consider the potential for future contact if a medical reason arises, or should the child be curious about the donor's identity.

Unlike many European countries, compensating an egg donor is not prohibited in the United States. However such compensation must be premised on the gamete provider's time and inconvenience and should not be tied to the number or quality of gametes donated, or the ultimate outcome of the cycle. Paying for bodily parts is strictly prohibited (see [NOTA, 1984](#)). While a compensated sperm donor typically earns one lump sum payment for his "time and inconvenience" at the time of his deposit, an egg donor typically earns her compensation in phases as her "time and inconvenience" is spread out over several weeks. This allows for the possibility of an egg donor changing her mind or becoming disqualified part way through a cycle—resulting in uncertainty as to the amount of compensation she should receive. The direct agreement can set forth the agreed upon financial terms between the parties to avoid disagreement about payment terms should the arrangement terminate before the egg retrieval.

In sum, although there has been very little litigation in this area, intended parents and egg donors should meet with independent legal counsel to make sure they are protecting their rights and the rights of the donor conceived child.

Gestational Surrogacy

The ability to give birth to a child for someone who is unable to carry has been one of the most important medical advancements in third party reproduction. The earliest form of surrogacy, referred to as "traditional surrogacy" occurs when the surrogate is genetically related to the child, and is typically accomplished through intrauterine insemination. Today, as a result of in vitro fertilization, the more common and legally acceptable form of surrogacy is "gestational surrogacy", when the transferred embryo is not genetically related to the surrogate. Because the traditional surrogate is genetically related to the child she is carrying, issues surrounding traditional surrogacy are socially and psychologically complex, and most laws do not recognize the parental status of intended parent(s) working with a traditional surrogate (thus requiring an adoption proceeding). Gestational surrogacy has become an

acceptable practice to achieve parentage in the U.S., and several states have laws protecting the sanctity of these arrangements. However, a few states have laws strictly prohibiting gestational surrogacy, and a few impose criminal sanctions to all participants (including professionals). Failure to comply with the specific law can result in unintended and expensive consequences for both the intended parent(s) and the gestational surrogate and her spouse, as well as potential liability for the treating professionals.

Different states allow for different types of surrogacy arrangements. Some require that the intended parent(s) be genetically related to the child, while other states allow gestational surrogacy arrangements with a donated embryo (where neither intended parent is related to the child). While some states, including Illinois, make it easy to place both names of the intended parents on the original birth certificate at the time of birth with no court involvement (so long as strict statutory requirements are complied with), other states make the process more difficult, and require an adoption proceeding or adjudication of parentage. Making matters more complex, in some arrangements the parties reside in different states and it may be challenging to determine which state law should apply. Before commencing any medical treatment it is imperative the each participant consult with independent counsel to make sure the contemplated arrangement has legal protection and that the parties are complying with all legal requirements imposed by the relevant jurisdiction (see <http://www.creativefamilyconnections.com/us-surrogacy-law-map>, <http://familybuilding.resolve.org/fertility-scorecard/>).

References

- Bass C and Gregorio J (2014) Conflicts of interest for physicians treating egg donors. *American Medical Association Journal of Ethics* 16(10): 822–826. <http://journalofethics.ama-assn.org/2014/10/pfor2-1410.html>
- Cahn, N. (2014). *Do Tell! The Rights of donor Conceived Offspring*. https://www.hofstralawreview.org/wp-content/uploads/2014/08/CC1.Cahn_final2_.pdf.
- Cahn, N., & Collins, J. (2014a). *AMA Journal of Ethics, Virtual Mentor*, 16(1), 49–56. <http://journalofethics.ama-assn.org/2014/01/hlaw2-1401.html>.
- Crockin, S. L., & Debele, G. A. (2015). Ethical Issues in Assisted. Reproduction: A Primer for Family. Law Attorneys www.aaml.org/sites/default/files/MAT202_4.pdf.
- Fertility Clinic Success Rate and Certification Act (1992). 42 USC 263a-1.
- National Organ Transplant Act of 1984, 42 U.S. Code Section 274e.
- Planned Parenthood v. Casey, (1992). 505 U.S. 833, 898.
- Practice Committee of the American Society for Reproductive Medicine (2014) Repetitive oocyte donation: A committee opinion, *Repetitive oocyte donation—ASRM*.
- Roe v. Wade, (1973). 410 U.S. 113, 163–164.
- Ross, H. E. (2013–2014). Gestational surrogacy in Illinois: Contracting the Unknown. *The Journal of the Dupage County Bar*, 26, 16–22. <http://www.dcba.org/mpage/vol261213art1>.
- Santosky v. Kramer (1982). 455 U.S. 745, 753–54.
- Skinner v. Oklahoma (1942). 316 U.S. 535.
- Stanley v. Illinois (1972). 405 U.S. 645, 652.
- State of Kansas Ex .Rel. vs. WM (2016) (Dist. Court Kansas 11/22/2016 Case No. 2007-DM-568). <http://cjonline.com/news/2016-11-28/shawnee-county-judge-topeka-sperm-donor-william-marotta-not-legally-child-s-father>.
- Swain MA (2014) Oocyte donation: Legal aspects, Springer Science & Business Media: New York, 2014, http://www.springer.com/cda/content/document/cda_downloadaddocument/9781461471684-c1.pdf?SGWID=0-0-45-1446034-p175011520.
- The Organ Procurement and Transplantation Network (2017) Guidance for the informed consent of living donors. [http://optn.transplant.hrsa.gov/ContentDocuments/Uniform Parentage Act \(2002\) 2002 Uniform Parentage Act \(adopted by 11 states\)](http://optn.transplant.hrsa.gov/ContentDocuments/Uniform%20Parentage%20Act%20(2002)%20Uniform%20Parentage%20Act%20(adopted%20by%2011%20states).pdf).
- Wald D and Black Katherine JD Candidate (2011) Surrogacy and a Pregnant Woman's Constitutional Right to Medical and Procreative Choice – A Brief Survey, www.waldlaw.net/assets/files/ABA%20surrogacy%20article.10.2011.pdf.
- Lieber Wilkins, Carole MA, MFT, Talking to Your Children, <http://www.resolve.org/family-building-options/donor-options/talking-to-your-children.html>.

Further Reading

- Cahn, N., & Collins, J. (2014b). Fully informed consent for prospective egg donors. *AMA Journal of Ethics, Virtual Mentor*, 16(1), 49–56. <http://journalofethics.ama-assn.org/2014/01/hlaw2-1401.html>.

EVALUATION

Overview: Evaluation and Male Reproductive Medicine

Samuel Ohlander, University of Illinois-Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Evolutionary theory correlates reproductive potential with self-worth. The male purpose is to pass their genes to the next generation. However simplified or antiquated this theory is, it is because of the belief that subfertility equates to inadequacy that physicians must approach the male fertility evaluation with sensitivity. It is a disease with physical, psychosocial, and economic implications, and most often must be evaluated in the context of a couple.

The normal couple carries pregnancy rates by intercourse of 20%–25% per month, 75% by 6 months, and 90% by 1 year (Spira, 1986). Approximately 15% of couples experience infertility, defined as the inability to conceive after 1 year of unprotected intercourse. In 40%–50% of these cases there is a male contribution, with approximately 20% solely male factor (Thonneau et al., 1991).

Often times, the male fertility evaluation is the first medical encounter that a man has had since being evaluated by his pediatrician. It is important to understand that a lack of diagnosis does not mean an absence of pathology. The chapters that follow will focus on the evaluation of the male. It is critical to consider the overall health of the patient, as subfertility has shown to be associated with other medical comorbidities, furthermore other comorbid conditions may offer explanation to reproductive dysfunction. The male fertility potential relies upon hormonal hemostasis, successful spermatogenesis, and intact erectile and ejaculatory function. Each component must be evaluated to establish an understanding of the patient's reproductive health.

Ultimately, the goal of evaluating the infertile male, and that which will be discussed in the following chapters, can be broken down into: 1. How to identify factors that may be modified to improve fertility potential. This requires an understanding of the male anatomy and physiology, particularly the hypothalamic-pituitary-testicular axis. 2. What diagnostic tools exist, and when they should be utilized in an evaluation. There is continuous evolution of diagnostic tools, many of which we as fertility experts are still trying to understand how best to interpret and utilize. 3. How to identify comorbidities and understand the role that existing pathology, and subsequent intervention, may play on male reproductive health. 4. How to approach care with financial consciousness. While health care economics is constantly evolving, and as such it is not a focus of this text, it is a significant factor in management of the infertile couple and must be a topic of consideration and discussion with our patients.

References

- Spira, A. (1986). Epidemiology of human reproduction. *Human Reproduction*, 1, 111–115.
- Thonneau, P., Marchand, S., Tallec, A., Ferial, M. L., Ducot, B., Lansac, J., Lopes, P., Tabaste, J. M., & Spira, A. (1991). Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988–1989). *Human Reproduction*, 6, 811–816.

Clinical Evaluation of the Subfertile Male

Julie A Szymaniak and Martin Kathrins, Brigham and Women's Hospital, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Epidemiology

Infertility is defined clinically as a failure to achieve pregnancy after one year of having intercourse without birth control. When the female partner is older than 35 years, the infertile period is defined as 6 months or more. An estimated 48.5 million couples are affected by infertility worldwide, corresponding to a rate of 8–15% of childless couples.(WHO) The burden of infertility has remained relatively unchanged over the past two decades.(Mascarenhas et al., 2012) In the United States, about 15% of couples are unable to conceive after 1 year of unprotected sex.(Jarow, 2011) It has been often thought of as woman's problem, but in fact 20% of infertility cases are caused by male reproductive issues and an additional 30–40% of cases are due to both male and female issues.(Jarow, 2011) Thus, the evaluation and treatment of the male partner is a critical component of infertility.

History / Risk factors

The successful production and delivery of sperm requires a seamlessly functioning endocrine system via the hypothalamic-pituitary-gonadal axis as well as unobstructed passage through the male genitourinary tract. There are a plethora of conditions that can result in male infertility, and a thorough history taking is essential in the evaluation of a subfertile male. This includes inquiring into a patient's medical and surgical history, review of medications, exposure to occupational and environmental toxins, infections (both genitourinary and/or febrile), previously diagnosed pediatric conditions, and a detailed sexual history.

When taking a thorough history, special attention should be paid to risk factors that predispose the patient to erectile dysfunction, which is defined as the inability to maintain a sufficient penile erection for satisfactory sexual intercourse. These include increasing age and medical conditions such as diabetes mellitus, obesity, cardiovascular disease, hypertension, dyslipidemia, and benign prostatic hyperplasia. The patient's medications should also be reviewed, as certain antihypertensive agents and psychotherapeutic drugs have been associated with erectile dysfunction. Exposure to spermatotoxic agents should be assessed. For example, drugs that increase the ratio of estrogen to testosterone have been shown to impact sperm production. These include anti-androgens, corticosteroids, antiretroviral protease inhibitors, and exogenous estrogen. Exposure to chemotherapy and radiation, as well as prior genitourinary tract infections, should also be queried. Childhood genitourinary diseases, such as testicular torsion and cryptorchidism (or undescended testes) may also impact reproductive outcomes later in life. Lastly, assessing a couple's sexual activity to ensure optimal timing of intercourse, avoidance of spermatotoxic lubricants, and ejaculatory function is important.

Physical Exam

A general physical exam may be helpful in elucidating the cause of a male's infertility. The lack of secondary sexual characteristics, such as pubic hair, or the presence of gynecomastia (breast enlargement) can suggest inadequate androgenization. The scrotum should be examined for the presence of masses or swelling. The testes should be measured for size, as a testicle that is < 4.6 cm in length or 20 mL in volume is associated with spermatogenic dysfunction.(Brannigan and Laborde, 2016) The vas deferens must be evaluated on both sides, as absence of the vas deferens on one side suggests a possible embryologic malformation, indicating possible renal agenesis. Absence of the vas deferens on both sides, or congenital bilateral absence of the vas deferens (CBAVD), is associated with cystic fibrosis disease or carrier status and warrants a genetic workup for the male and female partners. The penis should be inspected and the location of the urethral meatus should be noted. The groin should be assessed for the presence or absence of an inguinal hernia. A digital rectal examination may also be performed to evaluate the prostate and seminal vesicles.

Laboratory Evaluation

Endocrine assessment includes evaluating levels of total testosterone, estradiol, and LH and FSH. Spermatogenesis, or the development of sperm cells within the male reproductive organs (testes), relies on central and testicular hormonal feedback mechanisms. Gonadotropin-releasing hormone (GnRH) is released by the hypothalamus and causes the anterior pituitary to secrete gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These act locally within the testes, with LH acting on Leydig cells to produce testosterone and FSH stimulating Sertoli cells to produce sperm cell precursors. Germ cells interact intimately with Sertoli cells and modulate the release of FSH. Thus, FSH can be used as an indirect measure of germ cell volume. Combined with testicular size, FSH can help differentiate between a male with azoospermia (complete absence of sperm in ejaculate) due to obstruction versus spermatogenic impairment. Men with obstructive azoospermia have low FSH levels with larger testes while men with spermatogenic dysfunction have high FSH levels with smaller testes.

When analyzing semen samples, specifically sperm concentration, there is no absolute cut off to discern the infertile from the fertile male, aside from complete azoospermia. However, typically sperm counts in the 5th percentile or lower likely represent infertility, whereas men with sperm counts in the 50th percentile or higher will likely be able to conceive. Such a semen analysis should be performed twice to account for significant variability among bulk semen parameters within a given male patient. Semen volume can also be of clinical importance. Semen hypovolemia is defined as a volume of <1.0 mL. In such a setting, post-orgasmic urinalysis should be obtained, which can help differentiate between ejaculatory ductal obstruction and retrograde ejaculation.

Medical Management

Hormonal disorders account for a small number of cases of male infertility (<3%), and can be divided into disorders of hormonal deficiency and hormonal excess.

Hormonal Deficiency

Gonadotropin deficiency causes hypogonadotropic hypogonadism, a condition in which low levels of LH and FSH lead to testosterone deficiency. The condition may be congenital, such as Kallmann's syndrome, which is associated with dysfunction of the olfactory system, or acquired, such as pituitary tumors or trauma. This condition can be treated with replacement of gonadotropins, by using agents such as human chorionic gonadotropin (hCG), human menopausal gonadotropin (hMG), or recombinant follicle-stimulating hormone (r-FSH). hCG is an LH agonist that stimulates intratesticular testosterone production by Leydig cells. hMG and r-FSH stimulate spermatogenesis by supporting Sertoli cells. Anti-estrogen agents, such as clomiphene citrate, are also used. These work by binding to estrogen receptors in the hypothalamus and anterior pituitary gland, thereby blocking estrogen's negative feedback inhibition of gonadotropin release. The goal of treatment with the above-mentioned agents is to increase testosterone levels to normal and to optimize spermatogenesis.

Alternatively, hypergonadotropic hypogonadism is caused by primary testicular dysfunction. In this condition, gonadotropins are inappropriately elevated due to the lack of negative feedback from testosterone, estradiol, and inhibin from the testes. Again, spermatogenesis is impaired due to low levels of testosterone. The condition can be congenital, such as Klinefelter Syndrome diagnosed when a supranumerary X chromosome is noted on a karyotype, or acquired, such as following chemotherapy, radiation therapy, or genital mumps infection. Medical treatment of hypergonadotropic hypogonadism is not well standardized, but may include aromatase inhibitors, gonadotropins, and anti-estrogen agents. Aromatase inhibitors work by inhibiting the enzyme aromatase, which converts testosterone into estradiol. By inhibiting the conversion of testosterone, higher levels of it should be available to stimulate spermatogenesis. Gonadotropin agents such as hCG, hMG, and r-FSH can be used in combination with aromatase inhibitors and anti-estrogen agents rather than as single agents. Similarly, anti-estrogen agents are not used alone in treatment of hypergonadotropic hypogonadism. Both classes of these drugs aim to augment the already elevated levels of gonadotropins.

Hormonal Excess

Hormonal excess is typically seen in patients receiving exogenous androgens, such as anabolic steroids. The use of these agents causes suppression of intratesticular testosterone production and thereby decreases or stops spermatogenesis. Treatment involves stopping exogenous steroids, and some men may require additional hormonal therapy. After cessation of exogenous androgens, spermatogenesis may resume within 3 months, although it may take up to 3 years.

High levels of prolactin can lead to disruption of the hypothalamic-pituitary-gonadal axis, as prolactin excess inhibits the hypothalamic secretion of GnRH. Causes of prolactin excess include prolactinomas (tumors of the pituitary), hypothyroidism, liver disease, stress, and certain medications. Treatment involves addressing the underlying cause as well as the use of dopamine agonists, such as bromocriptine. These agents work by blocking prolactin secretion by the pituitary.

Erectile Dysfunction

Impaired sexual function can impact fertility by hindering a couple's ability to successfully engage in intercourse. About one-fifth of men presenting for treatment of infertility have erectile dysfunction. (Shindel et al., 2008) As mentioned above, several risk factors and comorbidities are associated with erectile dysfunction. It can be caused by organic pathologies, such as decreased blood flow or nerve impairment, or by psychological issues. Treatment includes addressing underlying medical issues, patient counseling and medical therapy with oral or local agents. If a patient is not satisfied with his erectile function with oral or local agents, then referral to a male sexual health specialist is indicated.

Ejaculatory Dysfunction

In order for a couple to successfully conceive through sexual intercourse, antegrade ejaculation (forward flow) of semen must occur. Antegrade ejaculation requires the sympathetically-induced emission of sperm and bladder neck closure with somatic nerve-controlled pelvic floor muscle contractures. When diagnosing and treating ejaculatory dysfunction, it is helpful to divide the condition into retrograde ejaculation and failure of seminal emission.

Retrograde ejaculation is the backwards flow of semen into the bladder during ejaculation. It should be suspected in patients with low semen volume or absence of ejaculation during orgasm. Conditions associated with retrograde ejaculation include congenital abnormalities of the bladder neck, neurologic injury, retroperitoneal lymph node dissection, and diabetes mellitus. (Wein et al., 2012) Evaluation of retrograde ejaculation includes analyzing post-orgasmic urinalysis. In patients with sperm noted in that urinalysis, a diagnosis of retrograde ejaculation can be made. Treatment includes alpha-adrenergic agonists, such as pseudoephedrine, and tricyclic antidepressant agents, such as imipramine, which act to close the bladder neck during climax by stimulating sympathetic nerves. Failure of emission is suspected in patients who do not have sperm on post-orgasmic urinalysis. Typically these patients fail with medical therapy, and often require sperm extraction procedures. Such patients should be evaluated with a transrectal ultrasound to evaluate the seminal vesicle and ejaculatory duct anatomy.

Infection and Inflammation

Infection and inflammation of the male genitourinary tract can have a detrimental effect on sperm function and viability. Patients may present with infection, inflammation, or both. Infectious organisms include *Escherichia coli*, mycoplasma, ureaplasma, and sexually transmitted bacteria, such as chlamydia trachomatis and Neisseria gonorrhea. Clean-catch sperm cultures should be collected and treated with antibiotics or antivirals if found to be positive, however such cultures are often contaminated. Results should be interpreted with caution. Additionally, inflammation may be present without an infection. Leukocytospermia, or white blood cell counts of > 1 million/mL, may have an adverse effect on sperm function, although this is controversial. The suggested mechanism is through reactive oxygen species, which are released by white blood cells and cause cellular damage. Proposed treatments include anti-inflammatory medications, antioxidants, or anti-histamines to reduce oxidative-stress induced sperm damage.

Surgical Management

Surgical intervention can be used to treat certain conditions resulting in male infertility, and can be divided into procedures that optimize spermatogenesis, improve sperm delivery, and retrieve sperm. (Brannigan and Hotaling, 2016).

Surgical procedures that optimize spermatogenesis involve varicocele correction. Varicoceles are abnormal dilations in the pampiniform plexus of veins within the spermatic cord. Although they are common and occur in about 15% of males, the majority of varicoceles do not cause infertility. Only palpable varicoceles have been shown to be associated with spermatogenic decline and are surgically treated in the setting of abnormal semen parameters.

Procedures that optimize sperm delivery do so by relieving ejaculatory duct obstruction (EDO) or by re-establishing ductal patency after a prior vasectomy. Semen analysis can help determine the diagnosis of EDO. With EDO, semen volume is low given that most of the ejaculate volume is seminal vesicle fluid, which will be unable to pass into the urethra. Furthermore, without seminal vesicle secretions, the ejaculate is often acidic ($\text{pH} < 7$) and lacks fructose.

Sperm retrieval procedures range from needle aspiration of sperm through the skin to open surgical exploration of the testicle to find viable sperm. If a patient is azoospermic due to obstruction, then successful retrieval rates of up to 100% can be expected.

Conclusion

Male factor infertility commonly contributes to a couple's inability to conceive. Therefore, the male partner should always undergo a fertility evaluation with referral to a male reproductive specialist in the setting of any abnormalities.

References

- Brannigan, R. E., & Hotaling, J. M. (2016). Infertility: Surgical Management [Online]. Linthicum, Maryland: American Urological Association. Available: http://www.auanet.org/university/core_topic.cfm?coreid=110. (Accessed 27 June 2017).
- Brannigan, R. E., & Laborde, E. L. (2016). Infertility: Medical Treatment [Online]. Linthicum, Maryland: American Urological Association. Available: http://www.auanet.org/university/core_topic.cfm?coreid=109. (Accessed 27 June 2017).
- Jarow, J. (2011). *Optimal Evaluation of the Infertile Male Best Practices Statements*. Linthicum, Maryland: American Urological Association.
- Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., & Stevens, G. A. (2012). National, regional, and global trends in infertility prevalence since 1990: a systematic analysis of 277 health surveys. *PLoS Med*, 9, e1001356.

- Shindel, A. W., Nelson, C. J., Naughton, C. K., Ohebshalom, M., & Mulhall, J. P. (2008). Sexual function and quality of life in the male partner of infertile couples: prevalence and correlates of dysfunction. *J Urol*, 179, 1056–1059.
- Wein, A. J., Kavoussi, L. R. & Campbell, M. F. 2012. *Campbell-Walsh urology / editor-in-chief, Alan J. Wein; [editors, Louis R. Kavoussi ... et al.]*, Philadelphia, PA, Elsevier Saunders.

Further Reading

WHO. *Global prevalence of infertility, infecundity and childlessness* [Online] World Health Organization Geneva Available: <http://www.who.int/reproductivehealth/topics/infertility/burden/en/> [Accessed June 27, 2017].

Male Reproductive Endocrinopathy: Evaluation

Tolulope Bakare and Neha Malhotra, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

In 20%–40% of infertility cases, male factor is either solely responsible, or a contributory factor, respectively (Thonneau et al., 1991). While one, office-based analysis, showed that only 1.5% of male infertility cases are finally diagnosed with endocrinologic abnormalities, a working understanding of the possible endocrinopathies is essential to evaluation of the infertile male (Sigman et al., 2009). Endocrine disorders leading to infertility are often correctable and may need further treatment outside the realm of fertility.

History and Physical Exam

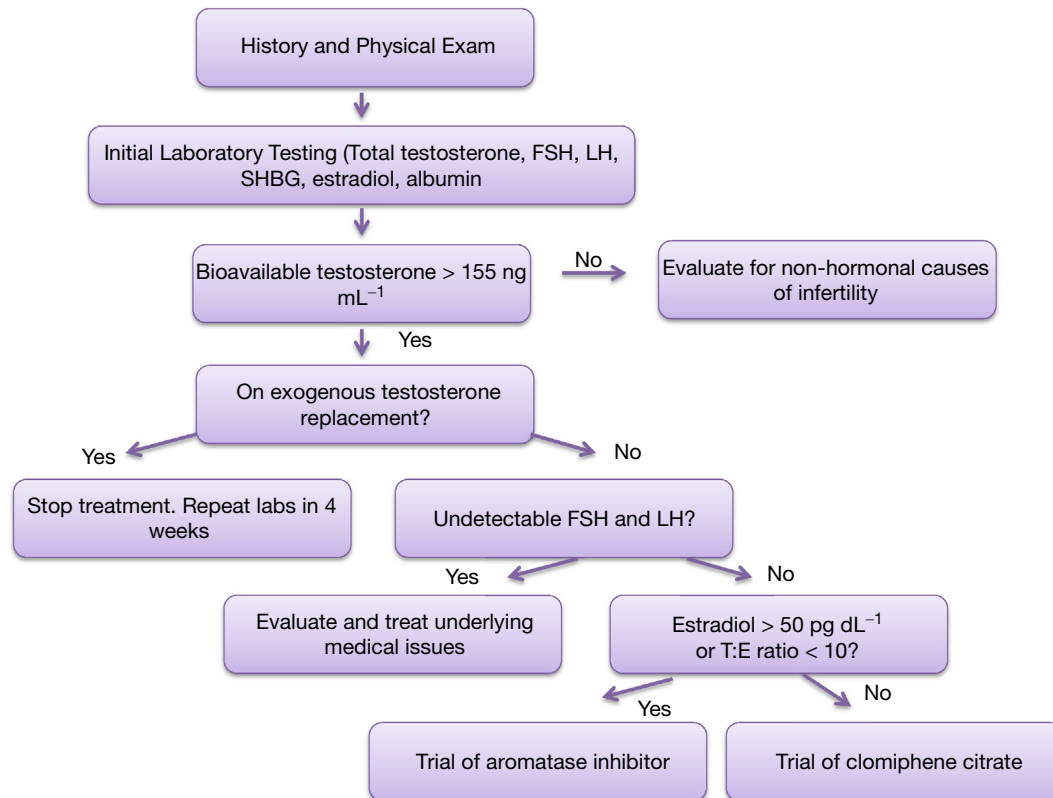
The history and physical exam is integral to the evaluation of the infertile male. In addition to a standard history, a reproductive history should include duration of infertility, prior fertility, developmental history (including childhood viral illness), sexual history (including sexual transmitted infections), genital or pelvic trauma, radiation history, coital frequency, use of lubricant, gonadotoxin exposure as well as familial fertility history (Jarow et al., 2002). Prior fertility rules out a number of congenital endocrinologic issues. A thorough medication history may reveal medications that may alter or disrupt normal endocrine function. Endocrine mimicking and disrupting chemicals can alter the balance of testosterone to estrogen and interfere with the hypothalamic-pituitary-gonadal axis. These include prescription medications such as antiandrogens (bicalutamide, flutamide, finasteride, and dutasteride), spiro-nolactone, protease inhibitors (saquinavir, indinavir, nelfinavir, ritonavir), nucleoside reverse transcriptase inhibitors (stavudine, zidovudine, lamivudine), exogenous testosterone, estrogen or steroids as well as histamine₂-blockers (cimetidine) (Bowman et al., 2012). Opioids suppress luteinizing hormone (LH) release and also disrupt the hypothalamic-pituitary-gonadal axis (Subiran et al., 2011). As opioids become increasingly commonly prescribed and abused, it is critical to ask about their use during the medical history. Other medications such as antifungals (ketoconazole), calcium-channel blockers, first general antipsychotics and chemotherapy may also affect the hypothalamus-pituitary-gonadal axis. Nonprescription drugs, such as marijuana and alcohol can also cause a decrease in androgens and worsening of bulk semen parameters. Surgical history should be noted for cryptorchidism, hypospadias repair, vasectomy, as well as pituitary resection. Physical exam should note the degree of virilization and secondary sex characteristics such as body habitus; hair pattern and gynecomastia. Shape, consistency, and size (in volume or testicular long axis) of testis should be recorded as well as cryptorchidism, varicocele, and whether or not the vasa are palpable bilaterally as well as the epididymal consistency. The testes volume is measured with an orchidometer or calipers. Testes size < 15 cm³ by Prader orchidometer are associated with abnormally low sperm concentration, and may be associated with a low testosterone production (Sigman et al., 2009). The penis should also be examined for meatal position. While rare, micropenis is often associated with endocrinologic abnormalities and should also be noted. Digital rectal exam should be performed to rule out masses. Besides discerning the etiology of infertility, a thorough medical evaluation is essential as 1.1%–1.3% of men presenting to an infertility clinic will be diagnosed with previously unknown, serious medical illness (Kolettis and Sabanegh, 2001; Honig et al., 1994). These men must further be counseled that their infertility in itself may be associated with future metabolic illness such as diabetes (hazard ratio [HR] 1.3, 95% confidence interval [CI] 1.10–1.54) and ischemic heart disease (HR 1.48, 95% CI 1.19–1.84) (Eisenberg et al., 2016).

Initial Laboratory Testing

After a complete history and physical exam, initial laboratory testing should be undertaken. Often, men will be referred with a semen analysis, but if not, one should be obtained. Men are typically instructed to wait 1–2 days after ejaculation to obtain an analysis; however, in a recent single-center, retrospective review of over 15,000 men it was found that longer abstinence time was not associated with improved sperm concentration, total sperm count or total motile count for men with oligospermia (Keihani et al., 2017; Levitas et al., 2005). While some authors recommend endocrinologic evaluation only if there is low sperm concentration, impaired sexual function or signs and symptomatic of an endocrinopathy, we recommend a screening androgen profile for all patients as a certain percentage of men with normal sperm density will have difficulty conceiving and an identifiable cause on endocrine evaluation (Jarow et al., 2002). Male androgen profile includes follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, total testosterone, sex-hormone binding globulin (SHBG), and albumin (the latter two to calculate the bioavailable testosterone). Androgen profile should be obtained in the morning, as testosterone is released in pulsatile fashion and peaks in the morning and a single random measurement of testosterone serum level may not accurately reflect the mean serum concentration. This initial evaluation can give a glimpse into possible underlying etiologies of infertility (see Table 1). Some authors advocate for routinely checking prolactin levels at the initial evaluation. If thyroid disorder is known or suspected, thyroid-stimulating hormone (TSH) should also be ordered. Similarly, if diabetes mellitus is known or suspected, consideration should be given to checking a fasting glucose or hemoglobin A1c level.

Table 1 Endocrinologic etiologies of male factor infertility

<i>Etiology</i>	<i>Testosterone</i>	<i>FSH</i>	<i>LH</i>
Normal spermatogenesis	Normal	Normal	Normal
Abnormal spermatogenesis	Normal	↑/Normal	Normal
Hypogonadotropic hypogonadism	↓	↓	↓
Hypergonadotropic hypogonadism	↓/Normal	↑	↑

**Fig. 1** Initial evaluation of the infertile male: hormonal dysfunction. Adapted from Kathrins, M. & Niederberger, C. (2016). Diagnosis and treatment of infertility-related male hormonal dysfunction *Nature Reviews Urology* **13**(6), 309–323.

If there are abnormalities of these initial screening tests, further investigation must be undertaken (see Fig. 1). If the bioavailable testosterone (as calculated from the total testosterone, SHBG and albumin is $> 155 \text{ ng mL}^{-1}$, nonendocrinopathy causes of infertility should be considered. The ratio of testosterone to estradiol (T:E) can be used to consider different treatment options such as aromatase inhibitors (T:E < 10) or nonsteroidal selective estrogen receptor modulators (T:E > 10). Specific endocrinopathies are discussed later on in this section.

Hypothalamic-Pituitary-Gonadal Axis

In order to understand the etiologies and implications of these laboratory abnormalities, the hypothalamic-pituitary-gonadal axis must be understood (see Fig. 2). Gonadotropin releasing hormone (GnRH) is synthesized in the preoptic nucleus in the anterior hypothalamus and then released in the hypophyseal portal bloodstream where it is carried to the pituitary gland. By activation of the GnRH receptor a signal cascade is initiated, which stimulated the synthesis and secretion of FSH and LH from the anterior pituitary. FSH and LH are also known as gonatropins and share the same alpha subunit; the beta subunit makes them distinct from one and other and interacts with their respective membrane bound receptors. Gonadotropin release is dependent on pulsatile activation of the GnRH receptor. Continuous secretion of GnRH essentially turns off FSH and LH secretion. FSH and LH then stimulate testicular function. FSH stimulates spermatogenesis in the Sertoli cells, as well as synthesis of Inhibin B. Whereas LH stimulates the Leydig Cells to increase expression of 17 β -hydroxysteroid dehydrogenase, which regulates the conversion of androstenedione to testosterone. Testosterone exists in both the free form, and bound to SHBG as well as albumin. Free and albumin bound testosterone

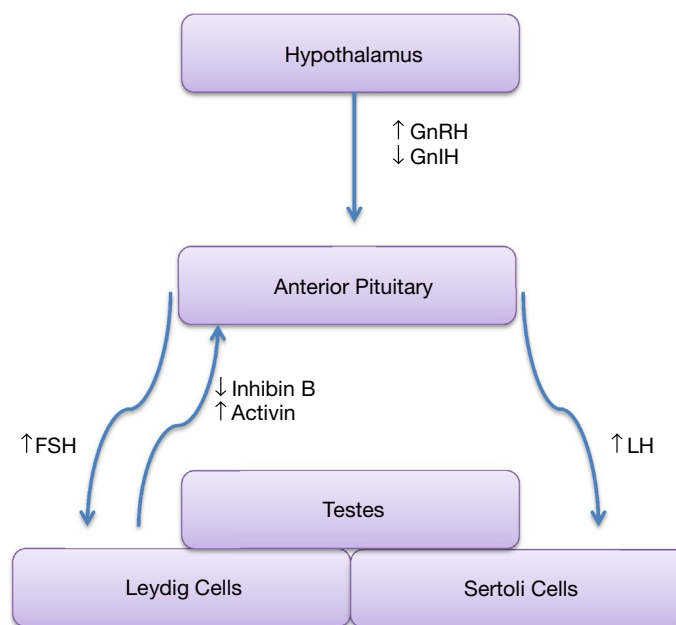


Fig. 2 Hypothalamic-pituitary-gonadal axis. GnRH, gonadotropin release hormone; GnIH, gonadotropin inhibiting hormone FSH, follicular stimulating hormone; LH, luteinizing hormone. Adapted from Kathrins, M. & Niederberger, C. (2016). Diagnosis and treatment of infertility-related male hormonal dysfunction. *Nature Reviews Urology* **13**(6), 309–323.

is considered to be active. Leydig cells are a site of steroidogenesis and also synthesis dihydrotestosterone as well as estradiol. Testosterone is also converted into dihydrotestosterone in the prostate, as well as to a lesser degree in the epididymis, through the action of 5α -reductase. Testosterone is peripherally converted to estradiol through the action of aromatase, principally in the adipose tissues. Excess gonadotropin or hormone causes feedback, which shuts off the system. For example, exogenous testosterone use results in an overabundance of testosterone, which is then converted into estradiol by aromatase. Estradiol inhibits LH secretion by the anterior pituitary and GnRH synthesis by the hypothalamus. Similarly, Inhibin B inhibits secretion of FSH by the anterior pituitary. Aberrations of this hypothalamic-pituitary-gonadal axis are discussed in detail further in this section.

Endocrinopathies of Male Infertility

Hypogonadism

Primary

Hypergonadotropic hypogonadism is characterized by low (hypogonadic) or normal testosterone in the setting of elevated FSH and LH (hypergonadotropic). This is also known as primary testicular failure or primary hypogonadism. This may be associated with gonadotoxic exposure such as mumps orchitis, radiation, chemotherapy, trauma or torsion; though this will usually be revealed early in the work-up of infertility.

Klinefelter syndrome one of the most common causes of primary testicular failure. Men with Klinefelter typically have a 47, XXY karyotype with mosaicism present in approximately 10% of these patients. Klinefelter should be suspected in especially tall men with gynecomastia, small, firm testis but many patients do not have this phenotype. Diagnosis is confirmed by aneuploidy on karyotype. Mosaicism is associated with less severe phenotypes and these men may be fertile. Successful pregnancy has been achieved using extracted sperm and then intracytoplasmic sperm injection (ICSI) even in nonmosaic Klinefelter patients (Greco et al., 2013).

Secondary

Hypogonadotropic hypogonadism is characterized by low testosterone (hypogonadic) as well as low FSH and LH (hypogonadotropic). This is known as secondary testicular failure or secondary hypogonadism and warrants further evaluation. If prolactin was not previously checked, serum prolactin levels should be ordered in the case of low testosterone with low gonadotropins. Prolactin can be a very volatile molecule and a single elevated result should prompt a repeat. If prolactin levels are truly high and symptoms are present, MRI should be ordered to evaluate for a pituitary tumor. Symptoms of a clinically significant pituitary adenoma usually include visual field changes (from compression of the optic chiasm), headache and erectile dysfunction. Typically, treatment involves pituitary resection; however, radiation and medical therapy with cabergoline and bromocriptine are also options (Brugh and Lipshultz, 2004). Pituitary tumors are typically not metabolically active and hypogonadotropic effect is caused by mass effect

on the anterior pituitary, change in blood supply and negative feedback on the hypothalamus. While pituitary adenomas may cause secondary hypogonadotropic hypogonadism, surgery for these adenomas can cause the same. Pituitary resection and trauma are acquired forms of secondary testicular failure. Certain medications, such as selective serotonin reuptake inhibitors, commonly prescribed for depression, may cause hyperprolactinemia as well. Again, highlighting the need for a thorough history in cases of male infertility.

Kallman syndrome is a genetic form of hypogonadotropic hypogonadism associated with a mutation of the KAL gene on Xp (Layman et al., 1992). This gene is associated with normal migration of hypothalamic neurons to the midline, and as such, patients tend to have anosmia and may also have cleft lip or cleft palate. While genetic testing is available, there are mutations associated with Kallman syndrome that are not readily identified and as such, a negative genetic test cannot rule out Kallman syndrome. Idiopathic hypogonadotropic hypogonadism is presumed to have an unrecognized genetic mutation.

Congenital Adrenal Hyperplasia

Congenital adrenal hyperplasia (CAH) is caused by enzyme mutations in the steroid synthesis pathway such that there is decreased production of glucocorticoid. Severity depends on which particular enzyme is deficient or mutated. As there is decreased glucocorticoid present and thus decreased feedback on the pituitary, there is an increase in adrenocorticotrophic hormone (ACTH) and overstimulation of the adrenal gland. This overstimulation causes hypersecretion of adrenal androgens and leads to negative feedback on the pituitary and decreased production of LH (Bonaccorsi et al., 1987). As a result, LH is typically found to be low in CAH patients; FSH can be high due to adrenal rest tissue present in the testis (King et al., 2016). Adrenal rests may be suggestive of inadequate replacement of glucocorticoids. In females, in infancy, CAH can be life threatening but in males it may not present until much later as either precocious puberty, or even later as infertility. If CAH is suspected, diagnosis is confirmed by plasma testing for hydroxyprogesterone and urinary excretion of 17-ketosteroid.

Exogenous Steroid Use

Exogenous steroid use may also be associated with secondary testicular failure. Exogenous testosterone (and the converted by-product estradiol) feedback and shut off production of GnRH and LH. Without pulsatile GnRH there can be no FSH synthesis and so spermatogenesis ceases. Similarly, as there is no LH, intratesticular testosterone synthesis stops. These patients may have testicular atrophy on exam. Discontinuation of exogenous steroids should allow for wear off in approximately 4 weeks, but cases have been reported of unmasking subfertility and having persistent hypogonadotropic hypogonadism (Jarow and Lipshultz, 1990). Recovery of spermatogenesis has been demonstrated; with 67% of men recovering at 6 months, 90% within 12 months, 96% within 16 months, and 100% within 24 months (Liu et al., 2006).

References

- Bonaccorsi, A. C., Adler, I., & Figueiredo, J. G. (1987). Male infertility due to congenital adrenal hyperplasia: Testicular biopsy findings, hormonal evaluation, and therapeutic results in three patients. *Fertility and Sterility*, 47, 664–670.
- Bowman, J. D., Kim, H., & Bustamante, J. J. (2012). Drug-induced gynecomastia. *Pharmacotherapy*, 32, 1123–1140.
- Brugh, V. M., 3rd, & Lipshultz, L. I. (2004). Male factor infertility evaluation and management. *The Medical Clinics of North America*, 88, 367–385.
- Eisenberg, M. L., Li, S., Cullen, M. R., & Baker, L. C. (2016). Increased risk of incident chronic medical conditions in infertile men: Analysis of United States claims data. *Fertility and Sterility*, 105, 629–636.
- Greco, E., Scarselli, F., Minasi, M. G., Casciani, V., Zavaglia, D., Dente, D., Tesarik, J., & Franco, G. (2013). Birth of 16 healthy children after ICSI in cases of nonmosaic Klinefelter syndrome. *Human Reproduction*, 28, 1155–1160.
- Honig, S. C., Lipshultz, L. I., & Jarow, J. (1994). Significant medical pathology uncovered by a comprehensive male infertility evaluation. *Fertility and Sterility*, 62, 1028–1034.
- Jarow, J. P., & Lipshultz, L. I. (1990). Anabolic steroid-induced hypogonadotropic hypogonadism. *The American Journal of Sports Medicine*, 18, 429–431.
- Jarow, J. P., Sharlip, I. D., Belker, A. M., Lipshultz, L. I., Sigman, M., Thomas, A. J., Schlegel, P. N., Howards, S. S., Nehra, A., Damewood, M. D., Overstreet, J. W., Sadovsky, R., & Male Infertility Best Practice Policy Committee of the American Urological Association Inc. (2002). Best practice policies for male infertility. *The Journal of Urology*, 167, 2138–2144.
- Keihani, S., Craig, J. R., Zhang, C., Presson, A. P., Myers, J. B., Brant, W. O., Aston, K. I., Emery, B. R., Jenkins, T. G., Carrell, D. T., & Hotaling, J. M. (2017). Impacts of abstinence time on semen parameters in a large population based cohort of subfertile men. *Urology*, 108, 90–95.
- King, T. F., Lee, M. C., Williamson, E. E., & Conway, G. S. (2016). Experience in optimizing fertility outcomes in men with congenital adrenal hyperplasia due to 21 hydroxylase deficiency. *Clinical Endocrinology*, 84(836), 830.
- Kolettis, P. N., & Sabanegh, E. S. (2001). Significant medical pathology discovered during a male infertility evaluation. *The Journal of Urology*, 166, 178–180.
- Layman, L. C., Wilson, J. T., Huey, L. O., Lanclos, K. D., Plouffe, L., Jr., & McDonough, P. G. (1992). Gonadotropin-releasing hormone, follicle-stimulating hormone beta, luteinizing hormone beta gene structure in idiopathic hypogonadotropic hypogonadism. *Fertility and Sterility*, 57, 42–49.
- Levitas, E., Lunenfeld, E., Weiss, N., Friger, M., Har-Vardi, I., Koifman, A., & Potashnik, G. (2005). Relationship between the duration of sexual abstinence and semen quality: Analysis of 9,489 semen samples. *Fertility and Sterility*, 83(1686), 1680.
- Liu, P. Y., et al. (2006). Rate, extent, and modifiers of spermatogenic recovery after hormonal male contraception: An integrated analysis. *Lancet*, 367, 1412–1420.
- Sigman, M. M., Lipshultz, L. I., & Howards, S. S. (2009). Office evaluation of the subfertile male. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn). New York: Cambridge University Press.
- Subiran, N., Casis, L., & Irazusta, J. (2011). Regulation of male fertility by the opioid system. *Molecular Medicine*, 17, 846–853.
- Thonneau, P., Marchand, S., Tallec, A., Ferial, M. L., Ducot, B., Lansac, J., Lopes, P., Tabaste, J. M., & Spira, A. (1991). Incidence and main causes of infertility in a resident population (1,850,000) of three French regions (1988–1989). *Human Reproduction*, 6, 811–816.

Ejaculatory Dysfunction: Evaluation and Pathophysiology

Saturnino Luján, Clínica IVI Valencia, Valencia, Spain; and Hospital Universitari i Politècnic La Fe, Valencia, Spain
Ramón Rogel and Enrique Broseta, Hospital Universitari i Politècnic La Fe, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Seminal vesicle The seminal vesicles are a pair of tube-like glands. They are found behind the bladder of males. They make about 70% of the content of semen (also called seminal fluid).

Sympathomimetics Sympathomimetic drugs mimic the effects of sympathetic activation and cause smooth muscle contraction and vasoconstriction.

Abbreviations

DM Diabetes mellitus

EEJ Electroejaculation

IUI Intrauterine insemination

L Lumbar

MS Multiple sclerosis

PVS Penile vibratory stimulation

S Sacral

SVs Seminal vesicles

T Thoracic

The Anatomy and Physiology of Ejaculation

Various anatomical structures, all of which are controlled by the central and peripheral nervous systems, participate in the formation of semen. The testicles only produce 1%–2% of the total volume of semen while the seminal vesicles (SVs) produce up to two-thirds of the volume of the ejaculate. In addition, the SVs produce two essential components of the ejaculate, namely fructose and seminogelins, which facilitate the coagulation of the semen (Ohl et al., 2008). The prostate produces a third of the volume of the ejaculate as well as prostate specific antigen (PSA). PSA is a proteolytic enzyme that acts on the seminogelins to produce seminal fluid. To produce the total volume of the ejaculate, the bulbourethral or Cowper glands are also involved. These glands produce a clear mucosal secretion which is liberated during sexual stimulation. This secretion neutralizes the traces of acid from the urine which could otherwise damage the sperm (Chughtai et al., 2005). Once the seminal liquid is formed, it is deposited in the posterior urethra through the ejaculatory ducts. The internal sphincter or bladder neck, which is located proximal to the ejaculatory ducts, contracts during the emission of semen to avoid retrograde ejaculation toward the bladder. The external sphincter, located distal to the ducts, must open during ejaculation to allow the semen to exit the body. This entire process is coordinated by the central nervous system. The brain receives erotic stimuli and the signals originating in the upper central nervous system descend to the sympathetic thoracolumbar nerve centers (T10–L2), which provoke the contraction not only of the SVs and vas deferens, but also of the internal sphincter/bladder neck. Along with this latter action to stop any retrograde ejaculation, a series of involuntary rhythmic contractions of the periurethral muscles occurs, provoking a pulsating, projectile ejaculation, causing the propulsion of liquid semen from the penis. This mechanism goes into effect upon stimulation of the dorsal nerves of the penis, which reach the spinal medulla at the S2–4 level. The efferent vessels originated at this level travel along the pudendal nerve to the periurethral muscles, causing them to contract. The integrity of this arc reflex can be assessed through an exploration of the bulbocavernosus reflex. Sperm is not stored in the SVs, where the biochemical conditions could damage them and lower their motility. Rather, spermatozoa are stored in the epididymal tail. Only in cases of prolonged abstinence, partial obstruction of the ejaculatory ducts, or spinal cord injuries are sperm stored in the SVs (Ohl et al., 1999). In response to sexual stimulation, they rapidly move through the vas deferens during the emission of semen. The sperm which remain in the vas deferens are not released and they return to the epididymal tail.

Pathologies Associated With Ejaculatory Dysfunction

Iatrogenic

Retroperitoneal surgery

This type of surgery, which includes retroperitoneal lymphadenectomy, comprises one of the potential treatments for testicular tumors, both in stage I and stage II as well as in cases of residual retroperitoneal masses after chemotherapy (Basiri et al., 2013).

This type of tumor occurs primarily in young men and, as a result, it is a frequent reason for specialist consults for secondary ejaculation dysfunction due to this type of surgery. Other surgical procedures of this sort must also be taken into account, including aortic surgery. During resection, the nerves from both the sympathetic chain ganglia and the hypogastric plexus are eliminated, which in turn eliminates the efferent stimuli for the emission of semen and the closure of the internal sphincter/bladder neck. Recent technical modifications to this type of lymphadenectomy try to preserve these nerves ([Donohue et al., 1990](#)), with the most important zones being the preaortic hypogastric plexus below the lower mesenteric artery and the postganglionic sympathetic nerves at the T10-L2 level. One study comparing this innovative method to the classic surgical technique found that 99% of patients who underwent the former maintained anterograde ejaculation compared to 89% who underwent the classic surgery that did not preserve the nerves ([Beck et al., 2010](#)). Nevertheless, [Basiri et al. \(2013\)](#) analyzed the outcomes of modified lymphadenectomy with nerve preservation and found that only 61% of patients had anterograde ejaculation after treatment. With this type of surgery, the sacral afferents that provide sensitivity to the penis and glans remain intact, as do the sacral efferents, which produce the periurethral contractions necessary for the expulsion of semen. In this case, patients maintain the ability to achieve orgasm, although there may be no emission of semen due to the absence of contractions in the vas deferens, SVs, and ejaculatory ducts, which keeps the seminal liquid from reaching the prostatic urethra. This is called a “dry” orgasm.

Pelvic surgery

Ejaculatory alterations are a common problem after colorectal surgery. In one retrospective study, 47% of 207 patients who had undergone surgical treatment for a rectal tumor later presented with anejaculation ([Nishizawa et al., 2011](#)). This is because both the sympathetic and the parasympathetic nerves are located close to the mesorectal fascia. The total mesorectal cleavage technique preserves this entire autonomous plexus, leading to fewer cases of ejaculatory alteration. Alternatively, the use of laparoscopic techniques likewise decreases the likelihood of ejaculatory dysfunction ([Nagpal and Bennett, 2013](#)).

Prostate surgery

The most common causes of retrograde ejaculation due to anatomical changes are simple prostatectomy and transurethral prostatic procedures such as transurethral resection, photovaporization, and neck incisions. All these procedures are indicated for the treatment of secondary obstructive symptoms due to the enlargement of the prostate and they all produce “involuntary” damage to the fibers of the external sphincter and the bladder neck, which become incompetent, thus contributing to retrograde ejaculation. In the past, Y–V (Young–Dees) bladder neck plasty was widely used for cases of voiding dysfunctions and incontinence in pediatric patients. This technique is no longer used, but patients who underwent this intervention in the past may need fertility treatments.

Pharmacology

The alpha-blocker drugs used to treat lower urinary tract infections caused by an enlarged prostate may produce retrograde ejaculation as an adverse side effect. Another group of drugs that produce ejaculatory alterations are high blood pressure medications, antipsychotic drugs, and antidepressants.

Neurological Diseases

The main problem for patients with multiple sclerosis (MS) is erectile dysfunction. Nevertheless, ejaculatory problems are also frequent and include premature ejaculation (60%), retrograde ejaculation, anejaculation (33%), and delayed ejaculation ([Previnaire et al., 2014](#)). Because the sympathetic center for ejaculation is located in the thoracolumbar segments of the spinal cord, the effects that MS has on this part of the body are the cause of these dysfunctions ([Zivadinov et al., 1999](#)).

Diabetes Mellitus

The autonomic neuropathy (both sympathetic and parasympathetic) as well as the micro cardiovascular disease common in patients with long-term diabetes mellitus (DM) ([Phillips et al., 2014](#)) causes various ejaculatory alterations, such as anejaculation and retrograde ejaculation. In these patients, the former is caused by a lack of peristalsis in the vas deferens and SVs, which hinders the deposition of semen in the posterior urethra. However, retrograde ejaculation is the most frequent ejaculatory dysfunction in this cohort ([Gaubay et al., 2013](#)). The closure of the internal sphincter/bladder neck is controlled by the sympathetic nerve fibers. The neuropathy that DM produces in these fibers reduces the pressure of the closure at the moment that semen is released, thus leading to retrograde ejaculation. One study carried out on a cohort of young men with DM (mean age: 36 years) found that 6% suffered anejaculation or retrograde ejaculation ([Dinulovic and Radonjic, 1990](#)). In another study, out of 500 men who consulted a physician for infertility, only 5 (1.1%) had type I diabetes ([Sexton and Jarow, 1997](#)). To date, the real incidence of retrograde ejaculation or anejaculation among men with DM remains unclear ([Gaubay et al., 2013](#)).

Spinal Cord Injuries

The type of ejaculatory dysfunction in patients with spinal cord injuries depends on both the level and the degree of injury. For example, in cases of *high spinal cord injuries* (complete cervical or thoracic injury), the communication between the nerve centers of the central nervous system and the spinal cord center for ejaculation, known as the sympathetic thoracolumbar center, is blocked,

with the arc reflex remaining intact at the sacral level. In these cases, the patient may experience reflex erections. If ejaculation occurs, it will also be a reflex reaction. In the case of *low spinal cord injuries* below the sympathetic centers, when ejaculation occurs, it tends to be weak and drooling.

Idiopathic

In some patients it is impossible to determine the exact physiological cause of ejaculatory dysfunction, fundamentally cases of anejaculation. Although an underlying neurological or physical problem may exist without having been diagnosed, most of these cases have a psychological origin (Ohl et al., 2008). In these cases, a patient's medical history may be revealing. Many reach orgasm and have ejaculations when masturbating, but not during sexual intercourse (Perelman and Rowland, 2006). These patients are often unable to reach orgasm during sexual relations because of frequent masturbation or the use of idiosyncratic masturbation techniques. Modifying the stimulation techniques used and reducing the frequency with they masturbate often reverses the problem (Perelman and Rowland, 2006). If, despite sex therapy, problems with anejaculation persist, electroejaculation (EEJ) or vibrostimulation techniques may be used (Perelman and Rowland, 2006). Alternatively, sperm may be collected surgically to be used later in assisted reproduction techniques.

Evaluation of Ejaculatory Dysfunctions

For these patients, obtaining an accurate medical history is essential, especially because patients often confuse the symptoms of erectile dysfunction with ejaculatory dysfunction. An accurate medical or surgical history can help identify risk factors, such as retroperitoneal surgery or a history of DM. Likewise, any medications taken by the patient should be analyzed to assess whether they could be altering ejaculation. During the physical examination, testicle size should be assessed since it can indicate problems with spermatogenesis (e.g., a longitudinal axis smaller than 4.5 cm) (Schoor et al., 2002), or, if there are one or two vas deferens, it could be a possible case of obstructive azoospermia. In addition, the position of the urethral meatus should be checked to ensure that any infertility is not due to the anomalous outflow of semen due to hypospadias. As for complementary testing, follicle stimulating hormone and testosterone levels should be examined to help determine whether there is a problem with spermatogenesis. Glycemic levels should also be tested to identify any unidentified underlying pathologies, such as DM. In cases where the semen is not expelled or where the sperm volume in the spermogram is found to be low (< 1.5 mL) despite maintaining the ability to achieve orgasm, the post-orgasm urine should be analyzed to rule out retrograde ejaculation, which is taken to be the presence of 10–15 sperm per high-growth field after centrifugation of the urine (Sabanegh and Agarwal, 2012). If the post-orgasm urine does not contain sperm, a transrectal sonogram should be performed to rule out any obstruction of the ejaculatory ducts.

Treatment of Ejaculatory Dysfunctions

Drugs

If the patient is taking alpha-blockers or antidepressants, these should be discontinued and tests should be carried out to check whether the ejaculatory dysfunction is solved. Administration of sympathomimetics may be useful in certain circumstances as they transform retrograde ejaculation into anterograde ejaculation through contraction of the internal sphincter/bladder neck. In the absence of any spinal cord injury or anatomical changes in the urethra, ejaculation can be induced by various drugs (Table 1; Jungwirth et al., 2012). In addition, and as an alternative, the patient should be told to try to ejaculate with a full bladder so that the pressure is increased at the level of the internal sphincter/bladder neck, thus favoring anterograde ejaculation (Crich and Jequier, 1978). In cases where anterograde ejaculation is achieved, drugs can be taken in cycles during the week prior to ovulation and discontinued during the rest of the partner's cycle or taken several hours before intercourse. Collecting post-orgasm urine to use in subsequent assisted reproduction techniques should be recommended when the patient cannot stop the medication responsible for retrograde ejaculation or when the drug prescribed to produce anterograde ejaculation either has no effect or produces serious side effects, including dryness of the mucous membranes and hypertension.

Table 1 Drugs used to provoke ejaculation in cases of retrograde ejaculation

Drug	Dose
Imipramine	25 mg before intercourse or 25–75 mg/8 h
Midodrine	5 mg/8 h
Pseudoephedrine	60 mg every 6 h
Ephedrine	25–50 mg before intercourse or 10–15 mg four times day
Brompheniramine	8 mg/12 h

Sperm should be obtained in coordination with the partner's ovulation period. The urine should be alkalinized to obtain a pH of 7.2–7.8. In order to do this, the patient should take 1–2 g of sodium bicarbonate every 6 h, starting 48 h before intercourse, and then extract a sample of post-orgasm urine. The bladder may also be catheterized to instill 10–50 mL of a buffered medium suitable for sperm, such as F-10 or Ham's medium. The catheter is then removed and, after ejaculation, the patient is catheterized again to collect the intravesical contents, thus avoiding all contact between the sperm and the urine (Mahadevan et al., 1981). It is important to use non-spermicidal lubricants and urethral catheters not made of silicone in order to prevent sperm adhesion (Fode et al., 2012). If the product obtained is not of sufficient quality for intrauterine insemination (IUI), fresh semen should be used, or, alternatively, it should be frozen for use in in vitro techniques, such as intracytoplasmic sperm injection or ICSI. If none of the methods used is successful, sperm should be obtained directly from the testes (testicular sperm extraction or percutaneous sperm aspiration) or the epididymis (microsurgical sperm aspiration) for later use in assisted reproduction techniques.

Penile Vibratory Stimulation

This technique is intended to cause ejaculation by provoking sufficient stimulation through vibrations at the level of the frenulum in the glans of the penis. Patients who may benefit from this technique are those who have suffered a spinal cord injury, but who maintain the sacral afferents and efferent, the thoracolumbar sympathetic efferent fibers, and the communication between the sacrum and the thoracolumbar segment intact. In this sense, the ideal candidates are patients with a spinal cord injury above T10. The technique should be performed at least 6 months after the injury. Penile vibratory stimulation (PVS) produces reflex ejaculation in 94% of patients with a T1–T6 lesion and in 67% of patients with a T6–T12 lesion (Bird et al., 2001). Patients with lower spinal cord injuries who thus present with an altered arc reflex only have a 15% success rate with PVS (Sonksen, 2003). In patients in whom standard PVS has failed, the use of two vibrostimulators at the same time, one on the frenulum and one on the dorsal portion of the glans, can recover ejaculation in 22% of cases (Brackett et al., 2007). The vibration parameters are key to achieving an ejaculation. Sonksen et al. (1994) has determined that vibration amplitude of 2.5 mm at a frequency of 100 Hz is optimal for inducing ejaculation. The vibrator is placed on the frenulum until ejaculation occurs (Fig. 1). Previous administration of phosphodiesterase inhibitor drugs may be beneficial in achieving ejaculation following PVS (Giuliano et al., 2008). Midodrine (7.5 mg) may also be used 120 min before PVS. Vibration cycles are generally run for 2–3 min followed by 1 min of rest. Several cycles are repeated until ejaculation is achieved. The technique is considered to have failed when sperm have not been obtained in either of two separate PVS procedures performed at least 1 week apart. The ejaculate is collected in a sterile flask that can be used in different ways depending on the sperm parameters of the semen obtained. If there are more than 4 million mobile spermatozoa, either intravaginal insemination or IUI are good options because of their low cost and low invasiveness (Van Voorhis et al., 2001). Couples may also perform PVS at home. The procedure should coincide with the partner's ovulation as determined by means of the temperature method or with the aid of kits that measure LH in urine. The ejaculate thus obtained is injected into the vagina. This technique has a success rate of 25%–65% (Fode et al., 2012). The other option, IUI, must be



Fig. 1 Penile vibrator located on the frenulum.

performed in a doctor's office and is successful in one out of three couples in which the male has a spinal cord injury (Fode et al., 2012). Side effects of PVS include irritation of the glans and autonomic dysreflexia. Dysreflexia occurs mainly in patients with a spinal cord injury above T6. Any stimulus that causes a sympathetic discharge, including bladder distention, can cause this reaction. Symptoms include sweating, facial redness, bradycardia, and high blood pressure. If a blood pressure spike occurs during the procedure, it should be stopped immediately and the patient should be sat upright. If there is a previous history of autonomic dysreflexia, 20 mg of sublingual nifedipine should be administered 15 min before PVS (Steinberger et al., 1990). This procedure is contraindicated in patients with severe heart disease or untreated high blood pressure.

Electroejaculation

The only device approved for this technique is the Seager electroejaculator (Dalzell Medical Systems, The Plains, VA, USA) (Figs. 2 and 3). Transrectal electrical stimulation at the level of the prostate and the SVs leads to the patient ejaculating. As in PVS, autonomic dysreflexia may be triggered with this technique; therefore, the same procedures must be followed for prevention and treatment. The bladder must be emptied prior to the procedure and 40 mL of a special buffered substance, such as F10, should be administered. The patient is placed in the lateral decubitus position. A rectoscopy should then be performed to identify rectal lesions that would contraindicate the procedure. Other contraindications include the patient having a pacemaker or taking anticoagulants. At the end of the procedure, a final examination should be made to detect possible lesions caused by EEJ. During the procedure itself, the patient's heart rate and blood pressure should be monitored constantly. The probe of the electrostimulator is inserted into the rectum with the electrodes directed toward the prostate and SVs (Fig. 2). Stimulation is achieved with a wavy pattern. First, a 5 V current is applied for 5 s and then stopped abruptly, followed by a 20 s pause. The cycle is repeated (5 s of current, 20 s of rest), but increasing the current 1–5 V each time until reaching 20–30 V (Brackett et al., 2002; Fig. 3). The Seager electroejaculator determines the intrarectal temperature and will automatically stop the procedure if the temperature exceeds 38°C. The ejaculation will occur in either a projectile or a drooling fashion, making it necessary to press on the urethra (a technique called "milking"). At the end of the procedure, the patient should be probed to catch any retrograde ejaculation that may have reached the bladder. To this end, a non-spermicidal probe and non-spermicidal lubricant are used. EEJ is the method of choice for patients with low spinal cord injuries, flaccidity in the lower limbs, and/or absence of the bulbocavernosus reflex. Because these patients have altered sensitivity, it is not necessary to perform the procedure under general anesthesia. Patients with high spinal cord injuries in whom PVS has failed are also candidates for EEJ. If there is no rectal sensitivity and if autonomic dysreflexia can be reversed with nifedipine, the procedure can be performed without general anesthesia. In patients with cervical lesions or with a history of dysreflexia, EEJ should be performed under general anesthesia. With regard to results, the largest published series on the use of this procedure (Brackett et al., 2010) found that in 210 patients with spinal cord injuries who underwent EEJ a total of 953 times (a mean of 4.5 times per patient), sperm suitable for insemination or other, more complex techniques was obtained in 90% of the cases. However, EEJ is not only used for patients with spinal cord injuries. It has also been used, for example, for cryopreservation of semen in a pediatric population that is unable to masturbate or in patients who do not masturbate for cultural or religious reasons (Berookhim and Mulhall, 2014). It can also be used in patients who present with anejaculation after a retroperitoneal lymphadenectomy, where it has been shown to achieve good results (Hsiao et al., 2012).

Therapeutic Protocol for Obtaining Sperm

PVS should be the technique of choice. In cases of failure, EEJ can be performed. EEJ, together with IUI, is a cost-effective procedure if performed without general anesthesia (Ohl et al., 2001). However, in cases where anesthesia is required (e.g., in patients without spinal cord injuries, with incomplete or cervical lesions, or with a tendency to dysreflexia), IUI is not effective and in vitro



Fig. 2 Probes of the electrostimulator.



Fig. 3 Seager Model 14 electroejaculator.

fertilization (IVF) should be used. In this latter group of patients, it is thus more advisable to perform surgical extraction of sperm under local anesthesia and then to perform IVF through ICSI.

Conclusion

- In patients with ejaculatory disorders it is essential to know the patient's surgical history, which medications he is taking, and whether there are any underlying neurological or metabolic diseases.
- In patients with low seminal volume (< 1.5 mL), the presence of sperm in the urine after orgasm should be checked to rule out or confirm retrograde ejaculation.
- If there is low seminal volume and no retrograde ejaculation, a transrectal ultrasound should be performed to assess any obstruction of the ejaculatory ducts.
- Sympathomimetic drugs act at the level of the internal sphincter/bladder neck, thus favoring antegrade ejaculation.
- Vibratory stimulation of the penis is especially indicated in patients with high spinal cord injuries while EEJ is indicated in patients with low spinal cord injuries.

References

- Basiri, A., Ghaed, M. A., Simforoosh, N., Tabibi, A., Danesh, A., Nouralizadeh, A., et al. (2013). Is modified retroperitoneal lymph node dissection alive for clinical stage I non-seminomatous germ cell testicular tumor? *Urology Journal*, 10(2), 873–877.
- Beck, S. D., Bey, A. L., Bihle, R., & Foster, R. S. (2010). Ejaculatory status and fertility rates after primary retroperitoneal lymph node dissection. *Journal of Urology*, 184(5), 2078–2080.
- Berokkhim, B. M., & Mulhall, J. P. (2014). Outcomes of operative sperm retrieval strategies for fertility preservation among males scheduled to undergo cancer treatment. *Fertility and Sterility*, 101(3), 805–811.
- Bird, V. G., Brackett, N. L., Lynne, C. M., Aballa, T. C., & Ferrell, S. M. (2001). Reflexes and somatic responses as predictors of ejaculation by penile vibratory stimulation in men with spinal cord injury. *Spinal Cord*, 39(10), 514–519.
- Brackett, N. L., Ead, D. N., Aballa, T. C., Ferrell, S. M., & Lynne, C. M. (2002). Semen retrieval in men with spinal cord injury is improved by interrupting current delivery during electroejaculation. *Journal of Urology*, 167(1), 201–203.
- Brackett, N. L., Kafetsoulis, A., Ibrahim, E., Aballa, T. C., & Lynne, C. M. (2007). Application of 2 vibrators salvages ejaculatory failures to 1 vibrator during penile vibratory stimulation in men with spinal cord injuries. *Journal of Urology*, 177(2), 660–663.
- Brackett, N. L., Ibrahim, E., Iremashvili, V., Aballa, T. C., & Lynne, C. M. (2010). Treatment for ejaculatory dysfunction in men with spinal cord injury: an 18-year single center experience. *Journal of Urology*, 183(6), 2304–2308.
- Chughtai, B., Sawas, A., O'Malley, R. L., Naik, R. R., Ali Khan, S., & Pentyala, S. (2005). A neglected gland: a review of Cowper's gland. *International Journal of Andrology*, 28(2), 74–77.
- Crich, J. P., & Jequier, A. M. (1978). Infertility in men with retrograde ejaculation: the action of urine on sperm motility, and a simple method for achieving antegrade ejaculation. *Fertility and Sterility*, 30(5), 572–576.
- Dinulovic, D., & Radonjic, G. (1990). Diabetes mellitus/male infertility. *Archives of Andrology*, 25(3), 277–293.
- Donohue, J. P., Foster, R. S., Rowland, R. G., Bihle, R., Jones, J., & Geier, G. (1990). Nerve-sparing retroperitoneal lymphadenectomy with preservation of ejaculation. *Journal of Urology*, 144(2 Pt 1), 287–291. discussion 91–2.
- Fode, M., Krogh-Jespersen, S., Brackett, N. L., Ohl, D. A., Lynne, C. M., & Sonksen, J. (2012). Male sexual dysfunction and infertility associated with neurological disorders. *Asian Journal of Andrology*, 14(1), 61–68.
- Gaunay, G., Nagler, H. M., & Stember, D. S. (2013). Reproductive sequelae of diabetes in male patients. *Endocrinology and Metabolism Clinics of North America*, 42(4), 899–914.
- Giuliano, F., Rubio-Aurioles, E., Kennelly, M., Montorsi, F., Kim, E. D., Finkbeiner, A. E., et al. (2008). Vardenafil improves ejaculation success rates and self-confidence in men with erectile dysfunction due to spinal cord injury. *Spine*, 33(7), 709–715.

- Hsiao, W., Deveci, S., & Mulhall, J. P. (2012). Outcomes of the management of post-chemotherapy retroperitoneal lymph node dissection-associated anejaculation. *BJU International*, 110(8), 1196–1200.
- Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., et al. (2012). European Association of Urology guidelines on male infertility: the 2012 update. *European Urology*, 62(2), 324–332.
- Mahadevan, M., Leeton, J. F., & Trounson, A. O. (1981). Noninvasive method of semen collection for successful artificial insemination in a case of retrograde ejaculation. *Fertility and Sterility*, 36(2), 243–247.
- Nagpal, K., & Bennett, N. (2013). Colorectal surgery and its impact on male sexual function. *Current Urology Reports*, 14(4), 279–284.
- Nishizawa, Y., Ito, M., Saito, N., Suzuki, T., Sugito, M., & Tanaka, T. (2011). Male sexual dysfunction after rectal cancer surgery. *International Journal of Colorectal Disease*, 26(12), 1541–1548.
- Ohl, D. A., Menge, A. C., & Jarow, J. P. (1999). Seminal vesicle aspiration in spinal cord injured men: insight into poor sperm quality. *Journal of Urology*, 162(6), 2048–2051.
- Ohl, D. A., Wolf, L. J., Menge, A. C., Christman, G. M., Hurd, W. W., Ansbacher, R., et al. (2001). Electroejaculation and assisted reproductive technologies in the treatment of anejaculatory infertility. *Fertility and Sterility*, 76(6), 1249–1255.
- Ohl, D. A., Quallich, S. A., Sonksen, J., Brackett, N. L., & Lynne, C. M. (2008). Anejaculation and retrograde ejaculation. *Urologic Clinics of North America*, 35(2), 211–220. viii.
- Perelman, M. A., & Rowland, D. L. (2006). Retarded ejaculation. *World Journal of Urology*, 24(6), 645–652.
- Phillips, E., Carpenter, C., & Oates, R. D. (2014). Ejaculatory dysfunction. *Urologic Clinics of North America*, 41(1), 115–128.
- Previnaire, J. G., Lecourt, G., Soler, J. M., & Denys, P. (2014). Sexual disorders in men with multiple sclerosis: evaluation and management. *Annals of Physical and Rehabilitation Medicine*, 57(5), 329–336.
- Sabanegh, E., & Agarwal, A. (2012). Male infertility. In A. J. Wein (Ed.), *Campbell-Walsh urology* (10th edn., pp. 616–646). Philadelphia, PA: Elsevier.
- Schoor, R. A., Elhanbly, S., Niederberger, C. S., & Ross, L. S. (2002). The role of testicular biopsy in the modern management of male infertility. *Journal of Urology*, 167(1), 197–200.
- Sexton, W. J., & Jarow, J. P. (1997). Effect of diabetes mellitus upon male reproductive function. *Urology*, 49(4), 508–513.
- Sonksen, J. (2003). Assisted ejaculation and semen characteristics in spinal cord injured males. *Scandinavian Journal of Urology and Nephrology. Supplementum*, 213, 1–31.
- Sonksen, J., Biering-Sorensen, F., & Kristensen, J. K. (1994). Ejaculation induced by penile vibratory stimulation in men with spinal cord injuries. The importance of the vibratory amplitude. *Paraplegia*, 32(10), 651–660.
- Steinberger, R. E., Ohl, D. A., Bennett, C. J., McCabe, M., & Wang, S. C. (1990). Nifedipine pretreatment for autonomic dysreflexia during electroejaculation. *Urology*, 36(3), 228–231.
- Van Voorhis, B. J., Barnett, M., Sparks, A. E., Syrop, C. H., Rosenthal, G., & Dawson, J. (2001). Effect of the total motile sperm count on the efficacy and cost-effectiveness of intrauterine insemination and in vitro fertilization. *Fertility and Sterility*, 75(4), 661–668.
- Zivadinov, R., Zorzon, M., Bosco, A., Bragadin, L. M., Moretti, R., Bonfigli, L., et al. (1999). Sexual dysfunction in multiple sclerosis: II. Correlation analysis. *Multiple Sclerosis*, 5(6), 428–431.

Erectile Dysfunction: Evaluation and Pathophysiology

Tolulope Bakare and Ramy Abou Ghayda, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The National institute of health Consensus Development Conference on Impotence in 1992 defined male erectile dysfunction as the inability to achieve or maintain an erection sufficient for satisfactory sexual performance and vaginal intercourse ([NIH Consensus Conference, 1993](#)). The Massachusetts Male Aging Study (MMAS), an observational survey of non-institutionalized subjects, which became a landmark study in the field, found that in men aged 40–70 years, 52% of men reported that they were somehow affected by this problem, with different intensity ([Feldman et al., 1994](#)). A successful erection is only achieved and maintained by the interplay of complex interrelated psychological, vascular, hormonal and neurological processes ([Montague et al., 1996](#)). Normal sexual function in males entails of four stages: sexual desire or libido, erection, ejaculation and orgasm, and detumescence or penile flaccidity.

Penile Anatomy and Normal Physiology of Penile Erection

The penis is composed of two corpora cavernosa and the corpus spongiosum that surrounds the urethra; together they form a three cylindrical system. The tunica albuginea, a tough and thick sheath, surrounds and supports the erectile tissues and plays a major role in erection. It is composed of a bi-layer of collagen and elastic fibers. The inner circular layer contains the intercavernosal columns that prevent the corporal bodies from overexpansion. The outer longitudinal layers provide support and course the penis from its glans to its base at the proximal crura.

The internal iliac artery branches into the internal pudendal artery that constitutes the main arterial vascular supply. The internal pudendal artery in turn branches into the dorsal, bulbourethral, and cavernous arteries. The cavernous arteries supply the spongy trabecular tissues trapping the blood during erection.

The penile venous drainage is through subtunical venules and emissary veins exiting the tunica albuginea and draining the corporal bodies. These emissary veins unite to form circumflex, periurethral, and superficial dorsal veins ([Awad et al., 2010](#)).

The innervation of the penis is both autonomic and somatic. Autonomic innervation is through the spinal cord and peripheral ganglia and is composed of two elements. The sympathetic nerves arises from T11-L2, they travel in the pelvic nerves, mainly pudendal nerves. The parasympathetic nerves arise from S2-S4; they travel in the hypogastric nerves and end up in the pelvic plexus. Erection is thus stimulated by pelvic plexus and cavernous nerve and inhibited by the sympathetic trunk. The autonomic nerves merge to form the cavernous nerves. The motor and sensory nerves are primarily responsible for sensation and the contraction of the bulbocavernosus and ischiocavernosus muscles ([Dean and Lue, 2005](#)).

Erection starts by external stimulation. This stimulation will lead to secretion of nitric oxide (NO) from the nonadrenergicnon-cholinergic (NANC) endothelial nerve endings. The NO release will lead to the penile smooth muscle relaxation, increasing blood flow and reducing cavernosal arterioles resistance ([Musicki and Burnett, 2006](#)). Erection is achieved by the engorgement of the corpora cavernosa sinusoids with blood. The increase in shear stress contributes further to the erection through the secretion of NO by the penile tissue endothelium ([Burnett, 1995](#).) Detumescence occurs secondary to phosphodiesterases enzyme in degrading cGMP and cAMP.

Causes of ED

The causes of ED can be grouped into two main categories: psychogenic (due to stress, performance anxiety or other psychological disorders) or organic (hormonal, neurovascular, drug induced or anatomical etiologies) ([Table 1](#)). Many patients might present with a combination of both etiologies. Men suffering from psychogenic ED are usually younger in age and report a specific incidence

Table 1 Organic causes of ED

<i>Cause</i>	<i>Example</i>
Vasculogenic	Atherosclerosis, sinusoidal fibrosis
Neurogenic	Spinal cord injury, multiple sclerosis, pelvic surgeries
Endocrinological	Diabetes, hypogonadism, hyperprolactinaemia
Drug-induced	Antidepressant, antipsychotic, recreational drugs
Local factors	Penile fracture, Peyronie's disease
Systemic diseases	Renal failure, cardiovascular diseases

([Shamloul and Ghanem, 2013](#)).

Table 2 Common prescription drugs associated with ED

<i>Drug class</i>	<i>Example</i>
Cardiac drugs	Digoxin, Amiodarone
Diuretics	Spironolactone, Thiazide
Antihypertensives	Beta Blockers, Verapamil, Clonidine
Antidepressants	Selective serotonin reuptake inhibitors, Tricyclic antidepressants
Hormones	Antiandrogen, Ketoconazole, LH releasing hormones agonists
Histamine receptor 2 antagonists	Ranitidine, Cimetidine
Recreational drugs	Alcohol, marijuana, cocaine

(Muneer et al., 2014).

after which they developed ED. They typically have normal nocturnal and morning erections and normal erections during masturbation. Organic ED is any disturbance in the process or coordination of the neurovascular and biochemical/hormonal component responsible for the achievement of penile tumescence.

History and Physical Examination

Before any effective management for ED a thorough history must be obtained. The physician should start with obtaining a detailed past medical history, psychosocial history, surgical history, and a list of prescribed medications (Table 2). A thorough medical history should be asked including but not limited to diabetes, cardiovascular disease, and lower urinary tract symptoms. The focus should be then turned to trying to differentiate between organic versus psychogenic cause by determining the onset, duration, and course of dysfunction. Standardized questionnaire may be used to assess the intensity of the problems. The two most used and informative questionnaire are International Index of Erectile Function and the Sexual Health Inventory for Men (Rosen et al., 1999.) Because erectile dysfunction can be a strong predictor of cardiovascular disease, all men presenting with ED should be screened for any cardiac symptoms. Physical exam includes exam checking pulses and sensations, signs for any trauma or scars, secondary sex characteristics. A more focused genital exam should be done for penile size, fibrosis and elasticity, testicular size and consistency. Rectal exam is reserved for patients over 50.

Treatment

Patients with mild ED have been shown to benefit from life style modification of risk factors, like smoking cessation, prevention of obesity and regular exercise (Gupta et al., 2011.) Treatment options include phosphodiesterase 5 inhibitors that may be oral, intra-cavernosal or intraurethral. Other options include vacuum assisted devices, and penile surgery (Table 2).

Oral phosphodiesterase 5 inhibitors (PDE5I) are the first line treatment for erectile dysfunction. Phosphodiesterase type 5 inhibitors prevent the breakdown of cyclic guanosine monophosphate (cGMP) to GMP in the cavernosal smooth muscle. This way, the activity of cGMP is prolonged decreasing the intracellular calcium stores facilitating smooth muscle relaxation via nitric oxide in both cavernosal arteries and muscle and subsequent rigid erections (Shamloul and Ghanem, 2013.) They are considered safe although they are contraindicated in patients prescribed nitrates and patients in whom vasodilation is not advisable, because of the risk of severe hypotension.

The side effects are most of the time well tolerated by patients, with headaches and flushing being the most common (Eardley et al., 2010.)

Testosterone can be used to treat erectile dysfunction, but its use has been limited to hypogonadic patients suffering from ED. In patients with low testosterone concentrations dual therapy of PDE5I and testosterone has been studied with promising results (Jain et al., 2000.)

Yohimbine, considered to be a sexual enhancer, is thought to contain active ingredients that work as a presynaptic alpha 2 adrenoceptors antagonist in the brain and spinal cord. Side effects include tachycardia, blood pressure changes, hallucinations and dizziness. Even though the active metabolite of *Butea superba* is a butenine, its mechanism is still unclear till this day (Muneer et al., 2014.)

Intracavernosal and Intraurethral Therapy

They are considered second line therapy. Their use leads to a predictable and fast response. Patients self-inject them into the penile corpora cavernosa after a period of supervised training. Treatment consists most commonly of prostaglandins E1 analogue (alprostadil), other agents such as papaverine, phentolamine, and vasoactive intestinal polypeptide have been used. Their mechanism of action is to increase smooth cell relaxation by increase intracellular cyclic AMP. Therapy side effects include priapism and penile

fibrosis. Alprostadil is used in intraurethral therapy as a pellet. Reported side effects include penile and urethral pain and priapism (Shamloul and Ghanem, 2013.)

Vacuum Devices

Vacuum devices can be used alone or in combination of therapy. Their mechanism of action depends on the increasing blood flow to the corpora cavernosa with continuous negative suction. Side effects can be serious including skin necrosis, penile pain and numbness (Cookson and Nadig, 1993) These devices are not widely used because of patients discomfort and the unnatural approach to achieve erection.

Penile Surgery

Penile surgery, consisting of prosthesis implantation, is the third line of therapy for ED. It is reserved for patient with refractory ED failing all other available therapy. Its complications are rare but devastating, including infection and device malfunction (Carson et al., 2000.)

References

- Awad, A., Alsaid, B., Bessede, T., Droupy, S., & Benoît, G. (2010). Evolution in the concept of erection anatomy. *Surgical and Radiologic Anatomy*, 33(4), 301–312. <https://doi.org/10.1007/s00276-010-0707-4>.
- Burnett, A. L. (1995). Role of nitric oxide in the physiology of erection. *Biology of Reproduction*, 52(3), 485–489.
- Carson, C. C., Mulcahy, J. J., & Govier, F. E. (2000). Efficacy, safety and patient satisfaction outcomes of the AMS 700CX inflatable penile prosthesis: results of a long-term multicenter study. AMS 700CX Study Group. *The Journal of Urology*, 164, 376–378.
- Cookson, M. S., & Nadig, P. W. (1993). Long-term results with vacuum constriction device. *The Journal of Urology*, 164, 290–294.
- Dean, R. C., & Lue, T. F. (2005). Physiology of Penile Erection and Pathophysiology of Erectile Dysfunction. *Urologic Clinics of North America*, 32(4), 379–395. <https://doi.org/10.1016/j.ucl.2005.08.007>.
- Eardley, I., Donatucci, C., Corbin, J., et al. (2010). Pharmacotherapy for erectile dysfunction. *The Journal of Sexual Medicine*, 7, 524–540.
- Feldman, H. A., Goldstein, I., Hatzichristou, D. G., Krane, R. J., & McKinlay, J. B. (1994). Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. *The Journal of Urology*, 151, 54–61.
- Gupta, B. P., Murad, M. H., Clifton, M. M., Prokop, L., Nehra, A., & Kopecky, S. L. (2011). The effect of lifestyle modification and cardiovascular risk factor reduction on erectile dysfunction: a systematic review and meta-analysis. *Archives of Internal Medicine*, 171, 1797–1803.
- Jain, P., Rademaker, A. W., & McVary, K. T. (2000). Testosterone supplementation for erectile dysfunction: results of a meta-analysis. *The Journal of Urology*, 164, 371–375.
- Montague, D. K., Barada, J. H., Belker, A. M., et al. (1996). *The American Urological Association Erectile Dysfunction Clinical Guidelines Panel Report on The Treatment of Organic Erectile Dysfunction*. Baltimore, MD: American Urological Association.
- Muneer, A., Kalsi, J., Nazareth, I., & Arya, M. (2014). Erectile dysfunction. *BMJ*, 348.
- Musicki, B., & Burnett, A. (2006). eNOS function and dysfunction in the penis. *Experimental Biology and Medicine*, 231(2), 154–165.
- NIH Consensus Conference. (1993). Impotence. NIH Consensus Development Panel on Impotence. *JAMA*, 270, 83–90.
- Rosen, R. C., Cappelleri, J. C., Smith, M. D., Lipsky, J., & Peña, B. M. (1999). Development and evaluation of an abridged, 5-item version of the International Index of Erectile Function (IIEF-5) as a diagnostic tool for erectile dysfunction. *International Journal of Impotence Research*, 11, 319–326.
- Shamloul, R., & Ghanem, H. (2013). Erectile dysfunction. *The Lancet*, 381(9861), 153–165. [https://doi.org/10.1016/s0140-6736\(12\)60520-0](https://doi.org/10.1016/s0140-6736(12)60520-0).

Orgasmic Dysfunction

Tolulope Bakare and Ramy Abou Ghayda, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The American Psychiatric Association Diagnostic and Statistical Manual of Mental Disorders, 5th edition, DSM-V-TR, defined orgasmic disorders as a persistent or recurrent delay in, markedly reduced intensity, or absence of orgasm during a sexual experience. These symptoms must have been present for at least 6 months and occurs on at least 75% or more of sexual activity interactions and causing marked distress or interpersonal difficulty (Tobia, 2013). This definition gave the treating physicians the flexibility in judging and assessing women's orgasmic capacity based on the patient's socio-demographics and previous sexual experiences (Ishak et al., 2010). Of note this definition does not apply if the symptoms are caused directly and solely by an organic medical condition, a substance abuse or drug induced. This condition should not be accounted better by another Axis 1 disorders (other than sexual dysfunction disorders) like mood or anxiety disorders. The American Psychiatric Association subdivides further the disorder into lifelong type versus acquired type; generalized type versus situational type; and due to psychological factors versus due to combined factors (Tobia, 2013).

Evaluation

History and Physical Exam

A comprehensive review of sexual history is key element in establishing the diagnosis. The physician should take note of all the factors that could affect or cause orgasmic dysfunction, starting with patients' demographics (age, socio-cultural background), comorbidities (anatomical or physiologic), psychological history, history of physical or sexual abuse, prescribed medication, substance abuse to reach finally interpersonal relationship with the partners. The physician should next characterize the orgasmic dysfunction and build a detailed description of it. It is essential to differentiate orgasmic disorders from other sexual dysfunction like arousal or desire disorders (Lewis et al., 2004). Anatomical abnormalities that might cause orgasmic dysfunction (like lichen sclerosis) should be ruled out by a careful focused physical exam.

Laboratory Evaluation

Laboratory evaluation is usually not necessary in diagnosis or management of orgasmic disorders.

Male Orgasmic Disorder

Normal Ejaculation in Men

This complex reflex is dependent on the sympathetic thoracolumbar nerve fibers originating from T10 to L2 and somatic nerve fibers originating from S2 to S4. Mechanical stimulation of the glans penis sends somatic input to this reflex through the dorsal nerve of the penis. Higher cerebral centers have a vital input and play a role in coordination and achievement of ejaculation. These centers include the midbrains, thalamus and hypothalamus, and the cortex. (Giuliano and Clement, 2005). Before ejaculation happens, the contractions of the smooth muscles transport the sperm stored in the epididymis to the vas deferens. Sympathetic input leads then to the coordinated contraction of the vas deferens moving the sperm to the ejaculatory ducts reaching the seminal vesicles. The somatic nerve fibers cause rhythmic contractions of the pelvic and periurethral muscles carrying the sperm and its seminal plasma fluid into the urethra projecting it to the outside. Retrograde ejaculation is prohibited by the efferent sympathetic fibers input, T10–L2, leading to the closure of the bladder neck and opening of the external urethral sphincter (Thomas, 1983).

Male orgasmic disorders are part of the male sexual dysfunction, which include erectile dysfunction, premature ejaculation, delayed orgasm, and anorgasmia. Premature ejaculation (PE) is defined in the DSM V as the persistent or recurrent pattern of ejaculation occurring during partnered sexual activity within approximately 1 min following vaginal penetration and before the individual wishes it. This symptom must have been present for at least 6 months and must be experienced on almost all or all occasions of sexual activity. It causes clinically significant distress in the individual (Table 1). Delayed ejaculation (DE) is defined by a marked delay in ejaculation or a marked infrequency or absence of ejaculation on almost all or all occasions of partnered sexual activity without the individual desiring delay, persisting for at least 6 months and causing significant distress to the individual (American Psychiatric Association, 2013). Anorgasmia is defined as the perceived absence of orgasm, independent of the presence of ejaculation.

Table 1 Classification of PE

<i>Premature ejaculation subtype</i>	<i>Possible underlying causes</i>
Lifelong	Biological or functional disturbances
Acquired	Medical and/or psychological disturbances
Variable	Normal variant
Subjective	Cultural beliefs or social misconceptions

From: McMahon, C., Abdo, C., Incrocci, L. (2004). Disorders of orgasm and ejaculation in men. *The Journal of Sexual Medicine* 1, 58–64.

Etiologies of Orgasmic Disorder May be Organic or Psychogenic

Organic Etiologies of Male Orgasmic Disorders

Spinal cord injuries are the main organic cause leading to male orgasmic disorders.

These injuries are responsible for the disruption of either the sympathetic and/or parasympathetic input. Iatrogenic injury of these nerves during pelvic or retroperitoneal surgery also may contribute to the interruption of neurogenic impulses responsible for orgasm (Maizels, 1998). Diseases affecting the peripheral nervous system, like multiple sclerosis and diabetes mellitus also play a causative role in orgasmic dysfunction. Medications that may lead to orgasmic dysfunction in the male, including antipsychotic, antidepressant, and antihypertensive (McMahon et al., 2004).

Psychogenic Etiologies of Male Orgasmic Disorders

Most of the literature consists of expert opinion or case reports. Perelman's theory blames some masturbation habits leading to the patient's preference for masturbation over other sexual experience and intercourse (Rowland et al., 2004). Other theories include discrepancy between the male sexual fantasies and their reality, or the excessive concerns with partner sexual pleasure (Rowland et al., 2004). Some psychological etiologies of delayed ejaculation may include sexual performance anxiety, a history of sexual abuse or sexual trauma during childhood, repressive sexual education or religious constraints (Jenkins and Mulhall, 2015). Hypothetical etiologies include substance abuse, obesity and psychological disorders.

Management of Male Orgasmic Disorder

Studies have shown that while treating PE, combining pharmacotherapy and psychotherapy is superior as an intervention to drugs alone. The gold standard in PE pharmacological therapy is selective serotonin reuptake inhibitors, which are used off label, such as dapoxetine. Other safe drugs exist with less efficacy including local anesthetics, tramadol, phosphodiesterase inhibitors, and alpha-1 adrenoceptors antagonists (Abdel-Hamid et al., 2016). Multiple drug treatment have been used for DE including alpha 1 adrenergic receptors agonists, psychotropic medications, oxytocin and Bethanechol (Abdel-Hamid et al., 2016).

Female Orgasmic Dysfunction

Normal Ejaculation in Women

Women's orgasm is achieved by erotic stimulation of either genital, non-genital sites, or both. In the female brain, multiple areas are responsible for integration and regulation of orgasm, including the paraventricular nucleus of the hypothalamus, the periaqueductal gray region of the midbrain, the hippocampus, and the cerebellum (Meston et al., 2004). In orgasmic dysfunction central and peripheral pathophysiology have been involved. The sympathetic nervous system activation increased vaginal impulse amplitude and blood flow in response to erotic stimulation in normal patients and patients with altered sexual desires, but not for patients with anorgasmia (Meston and Gorzalka, 1996). The pudendal nerve transmits clitoral stimulation to the spinal cord and the brain, on the other hand vaginal stimulation are transmitted via pudendal, hypogastric, and pelvic nerves. The vagus nerve has been showed to provide an alternative orgasmic pathway, bypassing spinal cord control (Meston et al., 2004).

Etiologies of Female Orgasmic Disorder

Many of the general medical condition that affects the patients' quality of life or mental wellbeing are associated with sexual dysfunction. Orgasmic disorders have been reported in multiple sclerosis, chronic kidney diseases, fibromyalgia, and atherosclerosis. Anatomic pelvic pathologies and pelvic surgeries have lead to orgasmic disorders; this has been demonstrated in patients with stress urinary incontinence and patients who underwent hysterectomy.

Table 2 Possible treatment for female orgasmic dysfunction

<i>Treatment</i>	<i>Possible benefit</i>
Hormone replacement therapy Tibolone	Promising results in post-menopausal women
Partial androgen replacement using testosterone	Used in anorgasmic post-menopausal women
Phosphodiesterase inhibitors	Mixed results
Oxytocin and midodrine	Promising results
Bremelanotide	Effective but with adverse side effects
Eros-Clitoral device	Only FDA approved treatment

From: Ishak, W. W., Bokarius, A., Jeffrey, J. K., Davis, M. C., & Bakhta, Y. Disorders of orgasm in women: A literature review of etiology and current treatments. *The Journal of Sexual Medicine*, 7(10), 3254–3268.

Drug induced orgasmic disorders is very often seen, and it is caused by the side effects of these medications. The most culprits ones are the psychotropic drugs. Stimmel et al. reported anorgasmia in almost one third of patients prescribed selective serotonin reuptake inhibitors (SSRIs) (Stimmel and Gutierrez, 2006). It has been found that these side effects on orgasmic dysfunction are directly related to the dosage of the medication. Although moderate alcohol consumption have a sexually stimulant effect, heavy consumption was correlated to orgasmic dysfunction. Hormonal abnormalities or changes during menopause have been associated with orgasmic disorders. Orgasmic dysfunction can have a genetic factor. In a study done by Dunn et al. it was found that difficulty-achieving orgasm was hereditary in about 34%–45% depending on the nature of the sexual experience (Dunn et al., 2005).

The psychosocial aspect plays a major role in orgasmic disorders. Body and genital image contribute to orgasmic dysfunction (Ackard et al., 2000). Anorgasmia has been linked to negative experience and feelings of desperation among women who suffers from this condition. A history of previous sexual abuse may also cause generalized feeling of sexual dissatisfaction and higher incidence of orgasmic dysfunction. Socio-demographics factors may also play an important role in contributing to orgasmic disorders, with education, social status and age being among the most important factors (Meston et al., 2004).

Orgasmic dysfunction is the second most common reported sexual dysfunction in women. Because women and couples are reluctant and embarrassed to discuss sexual issues, orgasmic disorders are under-reported and rarely addressed despite its hypothetical high prevalence. It is thus the responsibility of the treating physician to always assess patient's sexual function. Assessment instruments and questionnaire can help the physicians in their evaluation.

Management of Female Orgasmic Dysfunction

Only few clinical trials have studies efficient and reproducible medical therapy for orgasmic disorders in women, and the FDA has not approved any to this date. The physicians have both psychological and pharmacological intervention to intervene. Some of these interventions are extrapolated from treatment of other female sexual disorders (Table 2.) In postmenopausal women, hormone replacement therapy has shown promising results in restoring orgasm (Basson, 1999). In patients with drug induced orgasmic disorders, stopping these medication, switching to a different family of drugs with less sexual disorder side effect or using strategies like drug holidays and adjunct therapy are all possible approaches (Stimmel and Gutierrez, 2006). Buspirone and Yohimbine have been used as adjunct therapy or as SSRI therapy replacement (Basson, 1999). Although they cause less sexual dysfunction side effects, their benefit on orgasmic dysfunction is controversial. Bremelanotide, a melanocortin 1 and 4 receptor agonist, has been demonstrated to increase orgasm score measured by standardized questionnaires, and was concluded to be effective therapy (Safarinejad, 2008). The drug was not approved because it caused of elevated blood pressure. Dietary supplements and natural remedies have been shown to be beneficial in some studies, even though they lack universal approval regarding efficiency and safety.

The FDA has only approved the Eros-Clitoral device for the treatment for the female orgasmic disorder. This device increases the clitoral blood flow by gentle vacuum.

A short-term therapy with cognitive behavioral therapy can lead to a more positive thoughts regarding sexuality and subsequently increase orgasm (Heiman, 2000). Examples of such therapies include directed masturbation and sensate focus exercises. It has been well established that masturbation plays a beneficial role in women suffering from orgasmic dysfunction, and these women had a higher chance of achieving orgasm than non-masturbators (Struck and Ventegodt, 2008). Kegel exercise have been studies with mixed results, with the most benefit found in female suffering with additional urinary incontinence and women not suffering from the psychological etiology of orgasmic disorders (Ishak et al., 2010).

References

- Abdel-Hamid, I. A., Elsaied, M. A., & Mostafa, T. The drug treatment of delayed ejaculation. *Translational Andrology and Urology*, 2016, 5(4), 576–591. <https://doi.org/10.21037/tau.2016.05.05>
- Ackard, D. M., Kearney-Cooke, A., & Peterson, C. B. (2000). Effect of body image and self-image on women's sexual behaviors. *The International Journal of Eating Disorders*, 28, 422–429. [https://doi.org/10.1002/1098-108X\(200012\)28:4<422::AID-EAT10>3.0.CO;2-1](https://doi.org/10.1002/1098-108X(200012)28:4<422::AID-EAT10>3.0.CO;2-1).

- American Psychiatric Association. (2013). *Diagnostic and Statistical Manual of Mental Disorders* (5th edn.). Arlington, VA: American Psychiatric Association.
- Basson, R. (1999). Androgen replacement for women. *Canadian Family Physician*, 45, 2100–2107.
- Dunn, K. M., Cherkas, L. F., & Spector, T. D. (2005). Genetic influences on variation in female orgasmic function: A twin study. *Biology Letters*, 1, 260–263. <https://doi.org/10.1098/rsbl.2005.0308>.
- Giuliano, F., & Clement, P. (2005). Neuroanatomy and physiology of ejaculation. *Annual Review of Sex Research*, 16, 190–216.
- Heiman, J. R. (2000). Orgasmic disorders in women. In S. R. Leiblum, & R. C. Rosen (Eds.), *Principles and practice of sex therapy* (3rd edn., pp. 118–153). New York: The Guilford Press.
- Ishak, W. W., Bokarius, A., Jeffrey, J. K., Davis, M. C., & Bakhta, Y. (2010). Disorders of Orgasm in Women: A Literature Review of Etiology and Current Treatments. *The Journal of Sexual Medicine*, 7(10), 3254–3268. <https://doi.org/10.1111/j.1743-6109.2010.01928.x>.
- Jenkins, L. C., & Mulhall, J. P. (2015). Delayed orgasm and anorgasmia. *Fertility and Sterility*, 104, 1082–1088. <https://doi.org/10.1016/j.fertnstert.2015.09.029>.
- Lewis, R. A., Fugl-Meyer, K. S., Bosch, R., et al. (2004). Epidemiology/risk factors of sexual dysfunction. *The Journal of Sexual Medicine*, 1(1), 35–39. <https://doi.org/10.1111/j.1743-6109.2004.10106.x>.
- Maizels, M. (1998). Normal and anomalous development of the urinary tract. In P. C. Walsh, & M. F. Campbell (Eds.), vol. 2. *Campbell's Urology* (pp. 1545–1600). Philadelphia, PA: W. B. Saunders Co.
- McMahon, C., Abdo, C., & Incrocci, L. (2004). Disorders of orgasm and ejaculation in men. *The Journal of Sexual Medicine*, 1, 58–64. <https://doi.org/10.1111/j.1743-6109.2004.10109.x>.
- Meston, C. M., & Gorzalka, B. B. (1996). Differential effects of sympathetic activation on sexual arousal in sexually dysfunctional and functional women. *Journal of Abnormal Psychology*, 105, 582–591. <https://doi.org/10.1037/0021-843X.105.4.582>.
- Meston, C. M., Levin, R. J., Sipski, M. L., Hull, E. M., & Heiman, J. R. (2004). Women's orgasm. *Annual Review of Sex Research*, 15, 173–257.
- Rowland, D., Keeney, C., & Slob, A. (2004). Sexual response in men with inhibited or retarded ejaculation. *International Journal of Impotence Research*, 16, 270–274. <https://doi.org/10.1038/sj.ijir.3901156>.
- Safarinejad, M. R. (2008). Evaluation of the safety and efficacy of bremelanotide, a melanocortin receptor agonist, in female subjects with arousal disorder: A double-blind placebo-controlled, fixed dose, randomized study. *The Journal of Sexual Medicine*, 5, 887–897. <https://doi.org/10.1111/j.1743-6109.2007.00698.x>.
- Stimmel, G. L., & Gutierrez, M. A. (2006). Sexual dysfunction and psychotropic medications. *CNS Spectrums*, 11, 24–30. <https://doi.org/10.1017/S1092852900026730>.
- Struck, P., & Ventegodt, S. (2008). Clinical holistic medicine: Teaching orgasm for females with chronic anorgasmia using the Betty Dodson method. *Scientific World Journal*, 8, 883–895. <https://doi.org/10.1100/tsw.2008.116>.
- Thomas, A. J., Jr. (1983). Ejaculatory dysfunction. *Fertility and Sterility*, 39, 445–454.
- Tobia, W. W. (2013). DSM-5 Changes in Diagnostic Criteria of Sexual Dysfunctions. *Reproductive System & Sexual Disorders*, 02(02), 122. <https://doi.org/10.4172/2161-038x.1000122>.

Comorbidities Associated With Male Infertility

Brent M Hanson and James M Hotelling, University of Utah, Salt Lake City, UT, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Hypogonadism A condition in which the production of sex hormones and germ cells is inadequate.

Hypothalamus The part of the brain situated below the thalamus which functions to regulate body temperature, metabolic processes, and other autonomic activities. The hypothalamus produces gonadotropin-releasing hormone (GnRH) which plays an important role in fertility.

In vitro fertilization A procedure in which eggs from a woman's ovaries are retrieved, fertilized with sperm in a laboratory, and then transferred back to the uterus as an embryo.

Metabolic syndrome A group of metabolic risk factors linked to insulin resistance and associated with increased risk of cardiovascular disease. Components of the diagnosis include increased waist circumference, elevated triglycerides, low HDL cholesterol, hypertension, and impaired fasting glucose.

Pituitary gland A small endocrine gland attached to the base of the brain, consisting of an anterior and a posterior lobe. The pituitary gland releases luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which assist in reproductive function.

Primary testicular failure A congenital, developmental or genetic defect in sperm production by the seminiferous tubules, which may be complete or partial.

Sperm morphology A parameter assessed during semen analysis that measures the percentage of sperm with a normal appearance.

Sperm motility A parameter assessed as part of a semen analysis that measures the ability of sperm to swim in a forward direction.

Vas deferens The duct through which semen is carried from the epididymis of each testicle to an ejaculatory duct.

Background

Among infertile couples, approximately 20% of cases can be attributed solely to an abnormality in the male partner. Male factor infertility is also identified as a contributing factor in up to 50% of infertile couples ([American Society for Reproductive Medicine, 2015](#)). There are multiple causes for male infertility, including blockage or absence of the vas deferens, problems related to the hypothalamus or pituitary gland, primary testicular failure, and dysfunction of the seminiferous tubules. Male infertility may also result from serious underlying medical conditions. Causes of male infertility may be either congenital or acquired. Unfortunately, an identifiable cause for male infertility is not always present, with approximately 40%–50% of male infertility cases being classified as idiopathic or unexplained ([Fritz and Speroff, 2011](#)).

The primary test to achieve a diagnosis of male factor infertility is a semen analysis. This test involves collection of a semen sample followed by laboratory evaluation of sample volume, concentration, motility, and morphology. Clinical values have been established to categorize men as either fertile or subfertile based on semen analysis findings. Men with abnormal semen analysis parameters generally undergo further medical evaluation to characterize the exact nature of the problem.

While the majority of infertile men present to fertility specialists solely for evaluation and treatment of their reproductive issues, there is increasing evidence that a diagnosis of infertility has a broader health impact that must also be addressed. Comorbidities associated with male infertility may not only influence a man's ability to father a child, but also his life expectancy and overall well-being ([Ventimiglia et al., 2016](#)). One of the challenges with evaluating associations between infertility and comorbid health conditions is that the diagnosis of many health conditions may be chronologically separate from the diagnosis of infertility. However, the trend toward delaying fatherhood in many Western countries may result in more men who present for an infertility evaluation with concomitant medical conditions with a known detrimental effect on fertility ([Ventimiglia et al., 2016](#)). When evaluating associations between infertility and comorbidities, confounding factors such as obesity and smoking are known to negatively impact both semen quality and overall health. This can make it difficult to identify causal relationships and draw definitive conclusions from data. Nevertheless, research is ongoing to evaluate specific health risks that infertile men may face later in life ([Ventimiglia et al., 2015](#)). The idea that semen analysis parameters may be used as a biomarker of overall health or a predictor of mortality is gaining acceptance as understanding of this topic increases. See [Table 1](#) for a list of key studies evaluating the relationship between male infertility and somatic health.

The general health status of men with infertility appears to be poorer than their fertile counterparts. Male infertility has been associated with higher rates of urogenital infections, autoimmune diseases, metabolic disorders such as diabetes, chronic kidney disease, and liver disease. Men with infertility are also at higher risk of developing ischemic heart disease, alcohol abuse,

Table 1 Key studies evaluating male infertility and somatic health

<i>Publication title</i>	<i>Author</i>	<i>Year</i>	<i>Comorbidity evaluated</i>	<i>Journal</i>
Male fertility and skin diseases	Abdel-Nasser and Zouboulis	2016	Dermatologic conditions	<i>Reviews in Endocrine & Metabolic Disorders</i>
Klinefelter syndrome: cardiovascular abnormalities and metabolic disorders	Calogero et al.	2017	Klinefelter syndrome, cardiovascular disease	<i>Journal of Endocrinological Investigation</i>
Obesity, male infertility, and the sperm epigenome	Craig et al.	2017	Obesity	<i>Fertility and Sterility</i>
Increased risk of incident chronic medical conditions in infertile men: analysis of United States claims data	Eisenberg et al.	2016	Hypertension, diabetes, hyperlipidemia, renal disease, pulmonary disease, liver disease, depression, cardiovascular disease, alcohol/drug abuse, anxiety, bipolar	<i>Fertility and Sterility</i>
Relationship between semen production and medical comorbidity	Eisenberg et al.	2014	Endocrine, circulatory, genitourinary, skin disorders	<i>Fertility and Sterility</i>
Relationship between paternal somatic health and assisted reproductive technology outcomes	Eisenberg et al.	2016	Neurologic, respiratory, musculoskeletal, endocrine, mental disorders	<i>Fertility and Sterility</i>
Semen quality, infertility, and mortality in the United States	Eisenberg et al.	2014	Mortality risk	<i>Human Reproduction</i>
The relationship between male BMI and waist circumference on semen quality: data from the LIFE study	Eisenberg et al.	2014	BMI, obesity	<i>Human Reproduction</i>
Risk of diabetes according to male factor infertility: a register-based cohort study	Glazer et al.	2017	Diabetes	<i>Human Reproduction</i>
Male infertility and risk of cancer	Rogers et al.	2017	Testicular cancer, prostate cancer, melanoma, non-Hodgkin lymphoma	<i>Seminars in Reproductive Medicine</i>
Male reproductive health and prostate cancer risk	Walsh et al.	2011	Prostate cancer	<i>Current Opinion in Urology</i>
Infertility as a proxy of general male health: results of a cross-sectional survey	Ventimiglia et al.	2015	Charlson comorbidity index (CCI)—multiple factors	<i>Fertility and Sterility</i>
Comorbidities and male infertility: a worrisome picture	Ventimiglia et al.	2016	Age, multiple comorbidities	<i>Current Opinion in Urology</i>

and drug abuse (Eisenberg et al., 2014). Associations between a diagnosis of male infertility and other negative health outcomes are strongest in men who undergo long term follow-up, emphasizing the fact that a comorbid diagnosis may come several years after an initial diagnosis of infertility (Eisenberg et al., 2014). Whether there is a cause and effect relationship between infertility and specific disease processes remains unclear, but recent data seem to indicate that men with infertility would benefit from discussions and interventions related to overall health. To date, exact mechanisms to explain connections between somatic health issues and infertility are lacking. However, approximately 15% of the male genome is involved in reproduction, so it is plausible that defects resulting in many health conditions may be linked to problems with fertility (Craig et al., 2017).

Health Risks Associated With Klinefelter Syndrome

As noted previously, causes of male factor infertility may be either congenital or acquired. Table 2 demonstrates somatic health risks in men with certain acquired forms of male infertility. One of the most commonly encountered congenital forms of male infertility is a genetic abnormality known as Klinefelter syndrome. Klinefelter syndrome is one of the most common causes of primary testicular failure, affecting approximately 1 in 1000 males. This disease process results from an additional X chromosome in men, mostly commonly presenting as a 47,XXY karyotype. Men with this condition generally have severely reduced sperm counts and underdeveloped testes. This abnormality is associated with significant health comorbidity and a life expectancy which is shortened by somewhere between 2 and 5 years. Men with Klinefelter syndrome are at increased risk to develop cerebrovascular disease which can ultimately lead to a stroke. Cardiovascular congenital anomalies such as aortic valve disease are also more prevalent in men with this condition. Incidence of thrombotic clots and leg ulcers appears to be elevated in men with Klinefelter syndrome, and they appear to have an increased risk of developing endocrine or metabolic diseases such as obesity, type 2 diabetes, and metabolic syndrome. Cardiovascular health may be at least partially improved with testosterone replacement therapy, although the effect of testosterone therapy leading to long term improvement in health outcomes remains a topic

Table 2 Somatic health risks associated with congenital causes of male infertility

<i>Congenital causes of infertility</i>	<i>Somatic health risks</i>
Klinefelter syndrome (primary testicular failure)	Cerebrovascular disease and stroke Shortened lifespan Valvular heart disease (aortic) Obesity Type 2 diabetes Metabolic syndrome Thrombotic clots Ulcers of the extremities Psychosocial and behavior problems
Congenital bilateral absence of the vas deferens	Variable manifestations of cystic fibrosis Pulmonary disease Pancreatic insufficiency and diabetes Malnutrition Sinusitis and bronchitis
Kallman syndrome	Cryptorchidism Testicular tumors
Cryptorchidism	Testicular cancer
Dermatologic syndromes ^a	DeSanctis Cacchione—retarded growth and sexual development, mental retardation, deafness, ataxia, quadraparesis Poikiloderma Congenitale—cataracts, bone defects, hypogonadism, hair growth defects, osteosarcoma H syndrome—heart anomalies, hyperpigmentation, hypertrichosis, short stature, hepatosplenomegaly, insulin dependent diabetes Ichthyosis—diabetes, cholesterol metabolism problems, limb defects

^aOther rare skin disorders which have a negative impact on fertility have also been documented.

of debate (Calogero et al., 2017). Men with Klinefelter syndrome may also suffer from psychosocial morbidity and behavior problems, with specific challenges such as lack of insight, poor judgment and impaired ability to learn from adverse experience. Long term testosterone replacement therapy does appear to be beneficial in this specific population to reduce psychologic and behavior issues (Simm and Zacharin, 2006).

Absence of the Vas Deferens

Congenital or acquired abnormalities of the vas deferens can cause obstruction in normal sperm transport. Approximately 1%–2% of infertile men are found to have congenital bilateral absence of the vas deferens. This finding is nearly universally related to mutations in the gene responsible for cystic fibrosis (CFTR gene). Interestingly, many men who are diagnosed with infertility related to abnormalities within the CFTR gene do not display the classic respiratory and pancreatic manifestations that are typical with cystic fibrosis. More than 95% of patients with cystic fibrosis are infertile, and in patients with clinically significant disease, progressive obstructive lung disease with bronchiectasis, frequent hospitalizations for pulmonary infections, pancreatic insufficiency, malnutrition, recurrent sinusitis, and bronchitis are common. In patients with outright cystic fibrosis, diabetes related to pancreatic failure typically results (Fritz and Speroff, 2011). Men with absence of the vas deferens who desire pregnancy require assisted reproductive technology which involves extraction of sperm or testicular tissue to obtain sperm cells which are then used in fertilization.

The Relationship Between Infertility and Malignancy

With nearly 40%–50% of cases of male infertility falling into the unexplained or idiopathic category, it is not uncommon for otherwise healthy individuals to be diagnosed with infertility. It is important to recognize that despite the overall healthy nature of these men, the diagnosis of infertility may place them at increased risk for comorbidities in the future (Eisenberg et al., 2016a). Table 3 outlines specific health risks that men with acquired infertility face.

Early studies evaluating connections between infertility and health outcomes focused primarily on cancer, with rates of specific types of cancer found to be increased in infertile men compared to the general population. The association between infertility and testicular cancer has been well-documented in multiple studies conducted in Europe and United States. Testicular cancer incidence is somewhere between 3 and 20 times greater in men with abnormal semen analysis parameters when compared with men from the general population who were matched for age and race (Eisenberg et al., 2016a). Males with abnormalities of testosterone

Table 3 Somatic health risks associated with acquired causes of male infertility

<i>Acquired causes of infertility</i>	<i>Somatic health risks and reproductive impact</i>
Generalized male infertility	Increased rates of malignancy: testicular cancer, colorectal cancer, melanoma, prostate cancer Increased rates of cardiovascular disease: stroke, myocardial infarction, peripheral vascular disease, cardiac mortality Increased rates of drug and alcohol abuse Increased rates of depression, anxiety, certain psychologic disorders Increased risk of obesity, diabetes, and metabolic syndrome
Obesity	Diabetes or insulin resistance Hyperlipidemia Hypercholesterolemia Hypertension Peripheral vascular disease Abnormal testicular thermoregulation Decreased testosterone synthesis Erectile dysfunction Decreased quality of sperm Increased sperm DNA fragmentation
Obstructive sleep apnea	Decreased luteinizing hormone (LH) production Higher rates of infertility Decreased serum testosterone
Environmental factors	Smoking: lung cancer, coronary heart disease, heart attack, stroke, peripheral vascular disease, cerebrovascular disease Alcohol abuse: liver cirrhosis, hypertension, coronary heart disease, stroke, gastrointestinal cancer Drug abuse: various negative health impacts depending on specific drug
Cardiovascular disease	Hypertension Peripheral vascular disease Non-ischemic heart disease Increased cardiovascular mortality
Dermatologic problems	Negative health impact associated with specific cause of dermatologic disease: relationship seen between infertility and skin disorders related to sarcoidosis, zinc deficiency, HIV, leprosy, erythema nodosum leprosum, and other specific disorders

production such as Kallman syndrome are known to have higher rates of cryptorchidism, or failure of testicular descent during fetal development. The failure of the testes to descend can either be unilateral or bilateral and is associated with impaired sperm production and increased rates of testicular tumors and cancer. However, even in men with normal testicular descent, a diagnosis of infertility still conveys an elevated risk of testicular cancer ([Fritz and Speroff, 2011](#)). While data is less convincing for other types of malignancy, rates of colorectal cancer, melanoma, and prostate cancer may also be elevated in infertile men.

Metabolic Syndrome and Infertility

The link between infertility and the metabolic syndrome has been heavily researched, with components of the metabolic syndrome consistently found to result in impaired fertility. Men who have been diagnosed with insulin resistance and diabetes have been shown to have higher rates of abnormal semen parameters, erectile dysfunction, and difficulty with ejaculation. Men who present to a physician due to issues with infertility also appear to have higher rates of baseline diabetes than the general population. Hyperlipidemia is more common in men with infertility, and elevated body mass index correlates with impaired semen production. Evaluation of men with hypertension, peripheral vascular disease, and nonischemic heart disease also demonstrates increased rates of abnormal semen parameters. Diabetes, hyperlipidemia, obesity, and hypertensive disorders are strongly associated with increased cardiovascular mortality ([Glazer et al., 2017](#)). The severity of the comorbid disease process appears to be inversely correlated with sperm quality. Hormonal, developmental, lifestyle, and genetic factors have all been proposed as explanations for the associations described earlier. However, it is important to remember that the majority of men presenting for an initial fertility workup are relatively young and may not carry diagnoses associated with the metabolic syndrome. While a current diagnosis of diabetes, hypertension, hyperlipidemia, or cardiovascular disease will have an immediate negative impact on a man's sperm quality, the majority of men with infertility will not meet diagnostic criteria for the metabolic syndrome at the time of their infertility presentation. Exact elevations in risk have not been clarified for each of these disease processes, but the risk that an infertile man will develop conditions such as diabetes, hyperlipidemia, obesity, or hypertension later in life appears to be elevated. The correlation between male infertility and a subsequent diagnosis of diabetes has been one of the areas which has undergone substantial research, with a reported 30% increased risk of developing diabetes later in life among infertile men compared to the baseline population ([Craig et al., 2017](#)).

The Impact of Obesity on Fertility

The topic of obesity as it relates to infertility warrants detailed discussion due to the widespread nature of obesity. Rates of obesity among reproductive aged men have tripled in the United States since the 1970s. Currently, greater than 33% of the population over 20 years of age are considered obese. The negative impact of obesity on male fertility is clear, with worsening rates of male fertility seen as body mass index increases. Obese men are at greater risk to develop hypogonadism, impaired sperm development, and erectile dysfunction. Disease states which are associated with obesity, such as sleep apnea and diabetes, may also lead to abnormalities within the endocrine system that can affect testosterone levels within the testicles. In the United States, approximately 3.4% of the population is affected by both obesity and diabetes. High levels of intratesticular testosterone are necessary for normal sperm production, so even small decreases in serum testosterone that are seen in obese men can result in drastic drops in levels of intratesticular testosterone. The mechanism for lower levels of testosterone in obese men is related to increased conversion of testosterone to estradiol and estrone, which are forms of estrogen. Adipocytes, which are the fat cells within the body, appear to play a key role in this conversion of testosterone to various types of estrogen. With higher fat stores, more testosterone is converted to estrogen, altering the overall testosterone levels of obese men (Craig et al., 2017).

Sleep apnea, a common problem seen in obese men, leads to fragmentation of the normal sleep cycle. Lack of proper sleep can decrease nocturnal production of luteinizing hormone (LH). LH is required for the production of normal circulating testosterone levels, and in men with sleep apnea, drops in circulating testosterone may lead to impaired fertility. Increased lower abdominal fat, thigh fat, and scrotal fat may also act as insulators of the testicles and impair normal testicular thermoregulation. With impaired thermoregulation of the testicles, testosterone synthesis as well as spermatogenesis are negatively affected. The ability to maintain an erection is necessary to achieve spontaneous conception, and obesity is unfortunately strongly correlated with erectile dysfunction. Of men who report erectile dysfunction, 79% are obese (Craig et al., 2017). The cause of erectile dysfunction in obese men is multifactorial, but decreased testosterone levels as well as increased inflammatory cytokines such as tumor necrosis factor alpha and interleukin 6 are believed to play a role in disrupting normal vascular function required to maintain a penile erection. Obesity appears to have a direct effect on the quality of sperm produced as well. Obese men are at higher risk to have lower sperm counts, lower numbers of progressive motile sperm, and elevated rates of sperm DNA fragmentation. High rates of sperm DNA fragmentation are associated with a worsened functional status of the sperm (Craig et al., 2017).

A Possible Association Between Dermatologic Conditions and Infertility

A correlation has been proposed between certain skin diseases and higher rates of semen abnormalities. While this correlation has not been studied extensively in the scientific literature, it appears that certain acquired skin diseases may have a negative impact on sperm quality, predominantly as the result of orchitis or epididymal obstruction. Acquired infections with dermatologic manifestations that have been correlated with diminished fertility include leprosy, HIV, erythema nodosum leprosum, sarcoidosis, Langerhans cell histiocytosis, and zinc deficiency. Medications such as methotrexate, spironolactone, and finasteride which are used to treat various dermatologic conditions and autoimmune diseases may also contribute to impaired fertility in men with skin disorders. Numerous congenital dermatologic syndromes (De Santis Cacchione syndrome, poikiloderma congenital, H syndrome, ichthyosis, and others) have also been correlated with diminished male fertility (Abdel-Naser and Zouboulis, 2016). While data is relatively sparse, this connection is worth mentioning since more information may become available in the future.

The Impact of Infertility on Offspring

It is becoming increasingly apparent that there are health risks associated with male factor infertility which may extend to the offspring of infertile men. Table 4 outlines specific effects of various comorbidities on the health of offspring. Awareness of this fact is important in the counseling of men who present for an infertility consultation. The study of epigenetics has led to interest in whether disease states or environmental exposures within an individual may have detrimental effects on future generations. In the case of obesity, the children of obese fathers may be predisposed to abnormalities related to reproductive function. Animal models have demonstrated altered metabolic function as well as increased rates of sperm abnormalities in the offspring of obese fathers (Craig et al., 2017). Human data is less prevalent, but early findings suggest that obesity within a father may lead to detrimental changes in sperm function, embryogenesis, and possibly overall health in future generations.

There also appears to be a complex relationship between male infertility and congenital malformations in offspring. Research from a large population database demonstrated an increased risk of death due to congenital malformations in first degree relatives of men who underwent semen analysis as part of an infertility work-up. While some of this increased risk may be attributed to the female partner, infertility within couples seems to result in increased risks to future offspring. Importantly, a correlation between semen quality and childhood cancer mortality in offspring has not been established (Hanson et al., 2017). When determining how to prevent health consequences for future generations, management of disease processes within the infertile patient appears to hold promise. For example, studies evaluating children born before and after maternal gastrointestinal bypass surgery for obesity demonstrated significant differences in receptor signaling for insulin, leptin, and other glucoregulatory pathways within the two groups of children. Improvements in endocrine function were noted in the group born to mothers who had previously undergone

Table 4 Familial health risks associated with male infertility

Medical comorbidity/intervention	Effect on offspring or reproductive outcomes
Paternal obesity	• Alterations in DNA methylation at the insulin-like growth factor 2 gene in newborns • Worsened epigenetic profiles in metabolic genes of offspring • Metabolic derangements in offspring
Poor semen quality	• No apparent increase in cancer risk to offspring
Male infertility	• Possible increased risk of death in offspring due to congenital malformations
Maternal gastric bypass surgery for obesity	• Improvements in receptor signaling for insulin, leptin, and glucoregulatory pathways seen up to 18 years after conception • Improvements in endocrine function of offspring
Paternal mental health disorders	• Children born at earlier gestational age
Nervous system diseases	• Lower average pregnancy rates with assisted reproductive technology (23% vs. 30%) • Lower live birth rates (15% vs. 23%)
Paternal endocrine disorders	• Lower birth weights of offspring (2970 g vs. 3210 g)
Paternal respiratory diseases	• Lower fertilization rates with assisted reproductive technology (61% vs. 64%)
Paternal musculoskeletal diseases	• Lower fertilization rates with assisted reproductive technology (61% vs. 64%)

gastric bypass surgery (Craig et al., 2017). More research is necessary in this area, but if specific disease processes result in impaired health for offspring, correction of the disease process prior to conception may eliminate a substantial health burden for future generations.

Assisted Reproductive Technology Outcomes

Many men with a diagnosis of infertility ultimately receive treatment with assisted reproductive technology in order to achieve pregnancies with their partners. Techniques such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been utilized to overcome various types of male infertility. There is an increasing body of data suggesting that women with comorbid conditions such as obesity, diabetes, and thyroid disease may have lower rates of success with assisted reproductive technology. Comparatively less is known about the impact of comorbid conditions on assisted reproductive outcomes in male patients. The male contribution to assisted reproductive technology has historically been minimized, but increased attention is being placed on the role of sperm in assisted reproduction in order to optimize fertility outcomes. Evaluation of fertilization and pregnancy rates following the use of assisted reproductive technology in infertile men with specific comorbidities has offered insight into the impact that these conditions have on the ability to achieve pregnancy with medical assistance.

Based on emerging data, possible relationships do exist between overall male health and success rates with IVF. In men with neurologic diseases, lower pregnancy rates and lower live birth rates have been demonstrated following IVF cycles. Lower fertilization rates were also seen in men with respiratory diseases and musculoskeletal diseases. Men with disorders of the endocrine system appeared to father smaller children compared to men undergoing IVF without an endocrine abnormality, and men with psychiatric disease or mental health disorders appeared to have children born at an earlier gestational age. Assisted reproduction cycles of obese men appear to have higher rates of miscarriage and lower clinical pregnancy and live birth rates. Specifically in obese men, the use of ICSI to achieve pregnancy may mitigate some of these poor outcomes (Eisenberg et al., 2016b). Refer to Table 4 for a summary of the impact of comorbidities on assisted reproductive outcomes. To date, the exact cause for these poor obstetrical outcomes remains unknown and data remains somewhat controversial. Future research will hopefully provide meaningful information regarding whether treatment or adequate management of underlying comorbid conditions can reduce negative reproductive outcomes in men who undergo interventions with assisted reproductive technology.

References

- Abdel-Naser, M. B., & Zouboulis, C. C. (2016). Male fertility and skin diseases. *Reviews in Endocrine and Metabolic Disorders*, 17(3), 353–365.
- American Society for Reproductive Medicine. (2015). Diagnostic evaluation of the infertile male: a committee opinion. *Fertility and Sterility*, 104(5), 1116–1126.
- Calogero, A. E., Giagulli, V. A., Mongioi, L. M., et al. (2017). Klinefelter syndrome: cardiovascular abnormalities and metabolic disorders. *Journal of Endocrinological Investigation*, 3, 1–8.
- Craig, J. R., Jenkins, T. G., Carrell, D. T., et al. (2017). Obesity, male infertility, and the sperm epigenome. *Fertility and Sterility*, 107(4), 848–859.
- Eisenberg, M. L., Li, S., Behr, B., et al. (2014). Relationship between semen production and medical comorbidity. *Fertility and Sterility*, 103(1), 66–71.
- Eisenberg, M. L., Li, S., Cullen, M. R., & Baker, L. C. (2016a). Increased risk of incident chronic medical conditions in infertile men: analysis of United States claims data. *Fertility and Sterility*, 105(3), 629–636.
- Eisenberg, M. L., Li, S., Wise, L. A., et al. (2016b). Relationship between paternal somatic health and assisted reproductive technology outcomes. *Fertility and Sterility*, 106(3), 559–565.
- Fritz, M. A., & Speroff, L. (2011). *Clinical gynecologic endocrinology and infertility* (8th edn). Philadelphia, PA: Lippincott Williams & Wilkins.

- Glazer, C. H., Bonde, J. P., Giwercman, A., et al. (2017). Risk of diabetes according to male factor infertility: a register-based cohort study. *Human Reproduction*, 9, 1–8.
- Hanson, H. A., Mayer, E. N., Anderson, R. E., et al. (2017). Risk of childhood mortality in family members of men with poor semen quality. *Human Reproduction*, 32(1), 239–247.
- Simm, P. J., & Zacharin, M. R. (2006). The psychosocial impact of Klinefelter syndrome – a 10 year review. *Journal of Pediatric Endocrinology and Metabolism*, 19(4), 499–505.
- Ventimiglia, E., Capogrosso, P., Boeri, L., et al. (2015). Infertility as a proxy of general male health: results of a cross-sectional survey. *Fertility and Sterility*, 104(1), 48–55.
- Ventimiglia, E., Montorsi, F., & Salonia, A. (2016). Comorbidities and male infertility: a worrisome picture. *Current Opinion in Urology*, 26(2), 146–151.

Comorbidities, Modifiable Risk Factors, and Erectile Dysfunction

Dorota J Hawksworth and Arthur L Burnett, Johns Hopkins Medical Institutions, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Atherosclerosis Arteriosclerotic vascular disease, commonly known as a “hardening” of arteries. Due to chronic inflammation, arterial wall thickens and ultimately results in a plaque formation. Chronically enlarging plaque leads to lumen obstruction and eventual decreased supply of blood to downstream tissues, further resulting in chronic ischemia. Same plaque may rupture and result in a sudden obstruction of the blood vessel, resulting in tissue infarction (death). Most commonly, this process is encountered during a heart attack (myocardial infarction) or a stroke.

Corpus cavernosum Cavernous body of the penis which forms a paired structure (corpora cavernosa) and runs along the length of the penis from the pubic bone to the glans penis. The corpora are comprised of a spongy erectile tissue divided by the connective tissue, forming hollow “caverns” throughout. During erection, muscles in the trabecular septa relax, the spongy tissue fills with blood and the corpora enlarge in length and girth, further “trapping” the blood and resulting in penile erection.

Externa Outermost layer of a blood vessel, also known as adventitia. Composed of collagen fibers.

Hemoglobin A1c Also known as glycated hemoglobin is a form of hemoglobin measured to identify the 3-month average plasma glucose concentration. This blood test is used to evaluate glycemic control over the previous 3 months. In diabetes mellitus, higher levels of glycated hemoglobin correspond to a poorer control of blood glucose levels.

Intima Innermost layer lining a blood vessel. It is composed of a layer of endothelial cells supported by a connective tissue (internal elastic lamina) and comes in direct contact with the blood flow.

Media Middle layer of a blood vessel. It lies between the intima and the externa and is composed of smooth muscle fibers and elastic supportive tissue.

Nitric oxide synthase (NOS) Enzyme catalyzing production of nitric oxide from L-arginine. In mammals, it exists as three isoforms: neuronal NOS (nNOS) expressed in neurons, endothelial NOS (eNOS) expressed in endothelial cells, and inducible NOS (iNOS) present in all cell types. All three NOS isoforms have been identified in the corpus cavernosum, with nNOS responsible for initiation and eNOS sustaining erection.

Introduction

Erectile dysfunction (ED), defined as the consistent inability to achieve or maintain penile erection sufficient for satisfactory sexual intercourse, is a common condition in aging men and significantly affects their quality of life (Anon, 1993). This condition has been reported to afflict 52% of male adults between 40 and 70 years of age in the United States and about 322 million men worldwide (Aytac et al., 1999; Johannes et al., 2000). As its incidence increases with each decade of life, and the average duration of life has been progressively increasing, a corresponding increase in the number of men reporting ED should also be expected.

ED was previously believed to be mainly of psychogenic origin. Currently, comorbidities such as cardiovascular disease, diabetes mellitus (DM), hypertension, dyslipidemia, hypogonadism, lower urinary tract symptoms (LUTS), metabolic syndrome, and depression have been described as primary risk factors for the development of ED. Additionally, modifiable lifestyle factors, including physical inactivity, smoking, alcohol consumption, poor diabetes control, and obesity, have been associated with ED. A fine balance between comorbid conditions and lifestyle modification must be carefully maintained in order to prevent or improve ED (Fig. 1).

Comorbidities

Aging

Aging is a well-established risk factor for development and severity of ED. This relationship has been previously demonstrated in several epidemiological studies, to include the Baltimore Longitudinal Study of Aging and the Massachusetts Male Aging Study (Shock et al., 1984; Feldman et al., 1994). According to the Rancho Bernardo Study, ED increases from 16% in men younger than 50 years to >71% in men who are 70 years or older (Barrett-Connor, 2005). Aging may affect all the components of the erectile process, including nerves, arteries, veins, cavernosal tissue, and the hormonal milieu.

Benign Prostatic Hyperplasia/Lower Urinary Tract Symptoms

LUTS associated with benign prostatic hyperplasia (BPH) and ED are highly prevalent in older men. Evidence suggests that both may have a common pathophysiology. Several epidemiological studies report that LUTS/BPH and their negative impact on quality

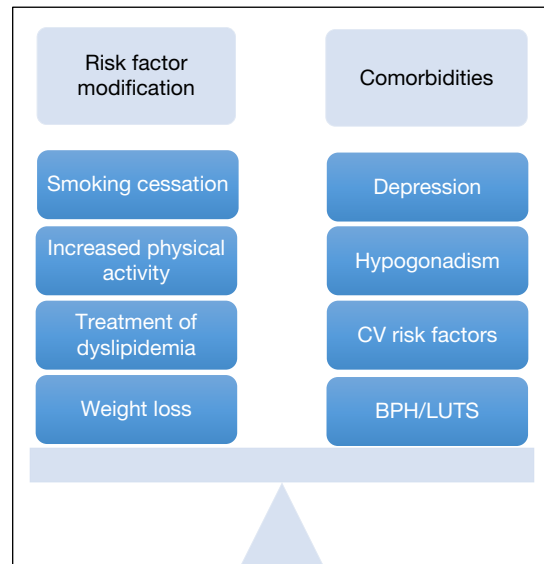


Fig. 1 Erectile function as balance between comorbidities and risk factor modification.

of life are independent risk factors for the development or exacerbation of ED. Moreover, quality of life indices reported by patients are significantly worse in those with simultaneous LUTS/BPH and ED. Experimental receptor mapping studies and animal models of bladder outlet obstruction support the hypothesis that LUTS/BPH partially results from increased sympathetic tone in the pelvis. This further results in down-regulation of corporal smooth muscles, thus impairing erections (Kobayashi et al., 2011; Lin et al., 2012).

The development of BPH has been linked to the systemic dysfunction represented by the metabolic syndrome. Prostate growth has been correlated with glucose intolerance (elevated plasma insulin levels and presence of noninsulin-dependent diabetes), central obesity/elevated body mass index (BMI), hypertension and dyslipidemia. Since all the processes representing the metabolic syndrome are associated with endothelial damage and dysfunction, they further provide a valid causal link between LUTS and ED (Barbosa et al., 2013).

Atherosclerosis and Cardiovascular Disease

The process of atherosclerosis begins in childhood, remains asymptomatic through early adulthood and becomes clinically evident in middle age. Endothelial dysfunction represents the initial, early phase of atherosclerosis. During this phase, the bioavailability of vasodilators, mainly nitric oxide (NO), decreases, shifting the balance in favor of endothelial vasoconstrictors. During the intermediate phase, thickening of the intima and the media and early plaque formation begin. Once the progressive plaque build-up reaches about 40% of the arterial diameter, narrowing of the arterial lumen begins. At this point, the late phase of atherosclerosis begins, and the obstructive vascular changes result in symptomatic vascular disease (see Fig. 2). Since penile erection is largely a vascular process, vasculogenic ED results from early changes to the endothelium-mediated smooth muscle relaxation and/or from the late occlusion of the cavernosal arteries (Montorsi et al., 2009).

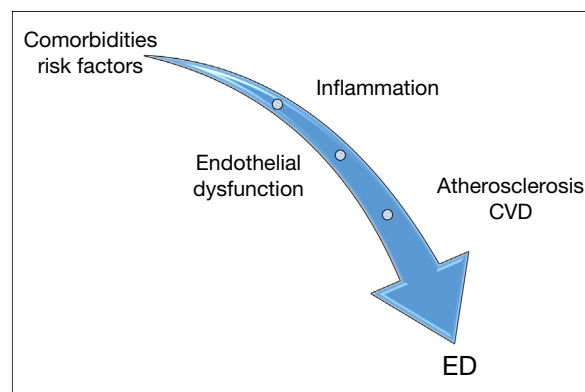


Fig. 2 Summary of processes resulting in ED.

ED is frequent in patients with coronary vascular disease (CVD) with prevalence ranging between 47% and 75%. The two conditions share the same risk factors leading to atherosclerosis, to include hypertension, elevated serum lipid levels, fatty diet, obesity, physical inactivity and smoking. The ED rate is 22% in patients with acute coronary syndrome (ACS) and one vessel disease, increases to 55% in patients with ACS and multi-vessel disease, and it further rises to 65% in those with chronic coronary syndrome (Vlachopoulos et al., 2013). Some patients, however, report ED before diagnosis of CVD. Prospective studies demonstrate that about 19% of men with ED suffer from subclinical CVD and in those patients ED precedes CVD by an average of 2–3 years. Currently, this phenomenon is best explained by the “artery size” hypothesis, in which the smaller penile arteries suffer obstruction earlier than the larger coronary arteries (Montorsi et al., 2005, 2006). Alternative explanations may include differences in metabolic mechanisms as well as increased permeability of endothelial cell–cell junctions in cavernosal tissue, resulting in further insufficiency (Musicki et al., 2015).

Hypertension

Hypertension is considered one of the most hazardous cardiovascular risk factors, and it is a frequent comorbidity of men with ED (Burchardt et al., 2000). ED is found in about 68% of hypertensive men between 34 and 75 years of age. Its severity among hypertensive subjects is associated with age, duration of hypertension, and presence of peripheral vascular disease. Long-standing hypertension has been shown to increase oxidative stress and endothelial cell injury. The resulting endothelial dysfunction and impaired vasodilation of the cavernosal vasculature are the hallmarks of the initial atherosclerotic process.

Medical therapy for hypertension is quite often blamed for erectile problems, especially when there is a temporal coincidence of starting symptoms and initiation of antihypertensive drugs. Recent systematic analysis of trials evaluating erectile function concluded that only thiazide diuretics and beta-blockers may influence erectile function. ACE inhibitors, angiotensin receptor blockers, and calcium channel antagonists were reported to have no relevant or even a positive effect on erectile function (Grimm et al., 1997). Favorable effects on NO synthase and oxidative stress have been shown with Nebivolol (Bystolic®, Allergan, Parsippany, NJ) pointing out a mechanism for improvement of erectile function. Experimental studies have demonstrated an enhancement of endothelial function in aorta and corpus cavernosum with a significant reduction in penile oxidative stress and collagen content, protected cavernosal tissue against structural changes, and increased expression of endothelial NO synthase (Baumhake et al., 2008).

Dyslipidemia

Both lipid disorders and ED are common problems in older men (Seftel et al., 2004). The association between hyperlipidemia and ED is originally attributed to atherosclerosis and resulting insufficiency in penile arterial inflow. More recently, the importance of cavernosal relaxation in the erectile process has been shown, and impairment of endothelium-dependent relaxation in men with hypercholesterolemia has been firmly established.

Dyslipidemia may contribute to ED by affecting endothelial and smooth muscle cells of the penis. Oxidized low-density lipoprotein is a causative factor for the impaired relaxation response of the corpus cavernosum. Elevated serum cholesterol and reduced high-density lipoprotein (HDL) cholesterol levels are associated with an increased risk of ED.

Epidemiological studies have found that the decrease in HDL and elevation of total cholesterol are correlated with ED. Studies have also shown that arterial stenosis and occlusion caused by hyperlipidemia may be attributed to the advanced-stage mechanism of ED induced by hyperlipidemia. Dyslipidemia may damage a man's erectile function at an early stage by affecting the endothelial cells and smooth muscles of the penis and the peripheral nerves responsible for penile erection (Gandaglia et al., 2014).

Diabetes Mellitus

Approximately 35%–75% of men with type 2 DM experience erectile problems. ED occurs 5–10 years earlier in men with diabetes than in age-matched controls and increases progressively with age (Fonseca and Jawa, 2005).

ED in diabetic men has a multifactorial etiology. Hyperglycemia and increasing age result in glycation of elastic fibers and subsequent impairment of cavernosal relaxation. Advanced end-products of glycation lead to increased concentration of reactive oxidizing substances and reduced NO production thus further causing impairment in vasodilation. Diabetic neuropathy further affects normal neural transmission from the spinal cord. Duration and severity of ED correlate with glycemic control, and hemoglobin A1c has been identified as an independent predictor for ED (Romeo et al., 2000).

Hypogonadism

Testosterone plays a major role in the control of sexual function. It contributes to endothelial and smooth muscle homeostasis via reduction of pro-inflammatory markers in the vascular tissue of penile and cardiovascular systems. Low testosterone levels have pro-inflammatory and pro-apoptotic effects on endothelial cells. This chronic inflammation further impairs endothelial function, leading to a pro-thrombotic state and atherosclerotic vascular remodeling. Thus, low androgen levels are associated with reduced nocturnal, morning and sex-induced erections, reduced decreased ejaculate volume and delayed ejaculation. Low testosterone levels are also associated with an increased risk of metabolic syndrome, DM and CVD, independent of age and obesity (Allan and McLachlan, 2010; Wang et al., 2011).

Depression

Depression is a common comorbidity associated with ED and represents an affective disorder with a lifetime prevalence of 16% (Kessler et al., 2003). Its hallmark symptoms include feelings of sadness, loss of interest in pleasurable activities (to include sex), loss of appetite, sleep disturbances, fatigue, and inability to concentrate. Depressed men report primarily low libido, orgasmic dysfunction and ED. They have lower sexual desire, poorer self-image and less sexual satisfaction.

A clear link between depression and ED was established by the landmark Male Massachusetts Aging Study (MMAS), in which men who had untreated depression were almost twice as likely to report ED than their counterparts with normal mood (Araujo et al., 1998). Depression and anxiety about sexual performance can cause ED, which in turn makes performance anxiety even worse. Thus, the two conditions reinforce each other and can result in a vicious cycle. The underlying mechanism correlating them on a physiological or biochemical level has not been well established. Goldstein (2000) described a biological “triad model” to further explain this relationship and link both ED and depression to CVD. Depression is a well-established risk factor for development of CVD (Musselman et al., 1998). Mental stress results in increased release of catecholamine hormones, and those in turn impair cavernosal muscle relaxation and promote ED.

Since hypogonadism and depression share common symptoms, such as loss of libido, fatigue, dissatisfaction, and poor appetite, studies suggest that low testosterone may be a link between depression and ED.

Medical antidepressant therapy can further exacerbate existing ED. Prospective trials demonstrate that Paroxetine (Paxil®, Apotex Corp, Weston, FL) causes ED at a rate of 40%. Animal studies demonstrate that Paroxetine is an inhibitor of NO synthase and thus decreases penile production of NO and depresses erectile response of cavernosal nerves (Finkel et al., 1996; Angulo et al., 2001). Since Paroxetine has the highest rate of sexual side effects, patients discontinue it much more often than Sertraline or Citalopram (Mullins et al., 2005). When treating patients with these concomitant diseases, care should be taken in choice and proper dose adjustment of the antidepressant. Furthermore, early use of phosphodiesterase type 5 inhibitor (PDE-5i) therapy is very effective in these men and can even improve mild depression symptoms.

Modifiable Risk Factors

Diabetes Mellitus and the Metabolic Syndrome

Reaven postulated that insulin resistance and its compensatory hyperinsulinemia predisposed patients to hypertension, hyperlipidemia, and glucose intolerance and thus were the underlying causes of CVD. This unique pathophysiological condition, called the “metabolic” or “insulin resistance” syndrome, is now well defined and recognized by the World Health Organization (WHO) and the Third Report of the National Cholesterol Education Program’s Adult Treatment Panel (ATP III) (Reaven, 1988).

Currently, no direct pharmacologic therapy for treatment of the metabolic syndrome is available. Lifestyle changes, including dietary adjustments and exercise leading to weight loss, are the cornerstones of therapy. Lifestyle change has been shown to not only prevent DM, but also to improve endothelial function and decrease markers of inflammation.

Drugs shown to improve endothelial function in patients with DM include insulin and insulin sensitizers, HMG—CoA reductase inhibitors, folic acid, ACE inhibitors, Niaspan, L-arginine, and PDE-5 inhibitors.

Animal models of hypercholesterolemia demonstrate that derangements in both endothelium- and neurogenic-dependent cavernosal relaxation are reversible once total plasma cholesterol levels are normalized through dietary changes. Treating dyslipidemia medically can also have a beneficial effect on ED. PDE-5i’s are now considered to be first line treatment for ED, and there is evidence that statins improve responses to these drugs.

Obesity and Sedentary Lifestyle

A BMI of 28 kg/m² or higher and sedentary lifestyle are strong predictors of ED, and obesity is a significant independent predictor of increasingly severe ED. Although obesity was not included in Reaven’s primary list of disorders caused by insulin resistance, he acknowledged that it, too, was correlated with insulin resistance or hyperinsulinemia, and that the obvious “treatment” for what he termed “syndrome X” was weight maintenance (or weight loss) and physical activity.

Physical activity is significantly inversely associated with ED. In the Health Professionals Followup Study, men who ran for nearly 90 min/week or did rigorous outdoor activity for 180 min/week had a 30% reduced risk of developing ED. The study further demonstrated that irrespective of exercise intensity, being overweight or obese increased the risk of ED (Bacon et al., 2006).

Esposito et al. demonstrated that a diet rich in fruits, vegetables, olive oil, whole grain, legumes, and walnuts was associated with improvement in erectile function in patients with the metabolic syndrome (Esposito et al., 2004, 2006).

In summary, regular long-term physical training and interval training as well as the consumption of Mediterranean diet yield improvement in endothelial function and further reduction in markers of inflammation, thus improving erectile function.

Smoking

Cigarette smoking is a known risk factor for ED and increases the likelihood of moderate or complete ED twofold. Clinical and basic science studies provide strong evidence that smoking affects penile erection by impairing endothelium-dependent smooth muscle

relaxation and increasing overall oxidative stress damage. Smoking exposure also results in impairment of penile arterial flow and may result in an acute vasospasm of the penile arteries. The association of ED with risk factors such as CVD and hypertension appears to be amplified by cigarette smoking. Studies suggest a dose–response association between smoking and ED with a statistically significant effect observed with ≥ 20 pack-years of exposure. Non-smokers, exposed to long-term second-hand smoke, are at moderate risk of developing ED. In middle-aged men, long-term vascular effects of smoking may persist even after smoking cessation. Thus, smoking cessation in young adulthood may be needed to reduce the risk of ED (Derby et al., 2000; Feldman et al., 2000; Verze et al., 2015).

Alcohol Use

Chronic excessive alcohol intake may have an irreversible adverse effect on erectile function, as it has been demonstrated to damage the neurogenic reflex arc involved in the erectile process. Change in drinking habits or even many years of complete sobriety are not associated with erectile function improvement. This lack of conclusive data may be also related to under-reporting, as men with high alcohol intake are very unlikely to participate in any risk reduction studies (Kostis et al., 2005).

Conclusion

ED is prevalent among men and its presence is often an indicator of systemic disease. The literature indicates that each patient developing ED should be considered a cardiac patient until proven otherwise. Endothelial dysfunction is the key feature in the early phase of ED, and evidence is accumulating in favor of ED as an independent predictor of future cardiovascular events. Cardiologic assessment and aggressive treatment of underlying risk factors to ameliorate the disease process should be offered to every patient complaining of ED. Addressing modifiable risk factors frequently improves a patient's overall health and increases lifespan. The literature suggests that smoking cessation, treatment of dyslipidemia, weight loss, making healthier food choices, and increased physical activity will improve erectile function in many patients. A man's doctor can help him make such changes by referring him to a smoking cessation program, a nutritionist, a fitness expert, or any other appropriate specialist, if necessary.

References

- Anon. (1993). NIH Consensus Conference. Impotence. NIH consensus development panel on impotence. *JAMA*, 270, 83–90.
- Allan, C. A., & McLachlan, R. I. (2010). Androgens and obesity. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 17, 224–232.
- Angulo, J., Peiro, C., Sanchez-Ferrer, C. F., Gabancho, S., Cuevas, P., Gupta, S., & Saenz de Tejada, I. (2001). Differential effects of serotonin reuptake inhibitors on erectile responses, NO-production, and neuronal NO synthase expression in rat corpus cavernosum tissue. *British Journal of Pharmacology*, 134, 1190–1194.
- Araujo, A. B., Durante, R., Feldman, H. A., Goldstein, I., & McKinlay, J. B. (1998). The relationship between depressive symptoms and male erectile dysfunction: cross-sectional results from the Massachusetts Male Aging Study. *Psychosomatic Medicine*, 60, 458–465.
- Aytac, I. A., McKinlay, J. B., & Krane, R. J. (1999). The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences. *BJU International*, 84, 50–56.
- Bacon, C. G., Mittleman, M. A., Kawachi, I., Giovannucci, E., Glasser, D. B., & Rimm, E. B. (2006). A prospective study of risk factors for erectile dysfunction. *The Journal of Urology*, 176, 217–221.
- Barbosa, J. A., Muracca, E., Nakano, E., Assalin, A. R., Cordeiro, P., Paranhos, M., Cury, J., Srougi, M., & Antunes, A. A. (2013). Interactions between lower urinary tract symptoms and cardiovascular risk factors determine distinct patterns of erectile dysfunction: a latent class analysis. *The Journal of Urology*, 190, 2177–2182.
- Barrett-Connor, E. (2005). Heart disease risk factors predict erectile dysfunction 25 years later (the Rancho Bernardo Study). *The American Journal of Cardiology*, 96, 3M–7M.
- Baumhake, M., Schlimmer, N., Buyukarsar, K., Arikian, O., & Bohm, M. (2008). Nebivolol, but not metoprolol, improves endothelial function of the corpus cavernosum in apolipoprotein e-knockout mice. *The Journal of Pharmacology and Experimental Therapeutics*, 325, 818–823.
- Burchardt, M., Burchardt, T., Baer, L., Kiss, A. J., Pawar, R. V., Shabsigh, A., de la Taille, A., Hayek, O. R., & Shabsigh, R. (2000). Hypertension is associated with severe erectile dysfunction. *The Journal of Urology*, 164, 1188–1191.
- Derby, C. A., Mohr, B. A., Goldstein, I., Feldman, H. A., Johannes, C. B., & McKinlay, J. B. (2000). Modifiable risk factors and erectile dysfunction: can lifestyle changes modify risk? *Urology*, 56, 302–306.
- Esposito, K., Giugliano, F., & Di Palo, C. (2004). Effect of lifestyle changes on erectile dysfunction in obese men: a randomized controlled trial. *JAMA*, 291, 2978–2984.
- Esposito, K., Ciotola, M., Giugliano, F., De Sio, M., Giugliano, G., D'Armiento, M., & Giugliano, D. (2006). Mediterranean diet improves erectile function in subjects with the metabolic syndrome. *International Journal of Impotence Research*, 18, 405–410.
- Feldman, H. A., Goldstein, I., Hatzichristou, D. G., Krane, R. J., & McKinlay, J. B. (1994). Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. *The Journal of Urology*, 151, 54–61.
- Feldman, H. A., Johannes, C. B., Derby, C. A., Kleinman, K. P., Mohr, B. A., Araujo, A. B., & McKinlay, J. B. (2000). Erectile dysfunction and coronary risk factors: prospective results from the Massachusetts Male Aging Study. *Preventive Medicine*, 30, 328–338.
- Finkel, M. S., Laghrissi-Thode, F., Pollock, B. G., & Rong, J. (1996). Paroxetine is a novel nitric oxide synthase inhibitor. *Psychopharmacology Bulletin*, 32, 653–658.
- Fonseca, V., & Jawa, A. (2005). Endothelial and erectile dysfunction, diabetes mellitus, and the metabolic syndrome: common pathways and treatments? *The American Journal of Cardiology*, 96, 13M–18M.
- Gandaglia, G., Briganti, A., Jackson, G., Kloner, R. A., Montorsi, F., Montorsi, P., & Vlachopoulos, C. (2014). A systematic review of the association between erectile dysfunction and cardiovascular disease. *European Urology*, 65, 968–978.
- Goldstein, I. (2000). The mutually reinforcing triad of depressive symptoms, cardiovascular disease, and erectile dysfunction. *The American Journal of Cardiology*, 86, 41F–45F.
- Grimm, R. H., Jr., Grandits, G. A., Prineas, R. J., McDonald, R. H., Lewis, C. E., Flack, J. M., Yunis, C., Svendsen, K., Liebson, P. R., & Elmer, P. J. (1997). Long-term effects on sexual function of five antihypertensive drugs and nutritional hygienic treatment in hypertensive men and women. Treatment of Mild Hypertension Study (TOMHS). *Hypertension*, 29, 8–14.

- Johannes, C. B., Araujo, A. B., Feldman, H. A., Derby, C. A., Kleinman, K. P., & McKinlay, J. B. (2000). Incidence of erectile dysfunction in men 40 to 69 years old; longitudinal results from the Massachusetts Male Aging Study. *The Journal of Urology*, 163, 460–463.
- Kessler, R. C., Berglund, P., Demler, O., Jin, R., Koretz, D., Merikangas, K. R., Rush, A. J., Walters, E. E., & Wang, P. S. (2003). The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA*, 289, 3095–3105.
- Kobayashi, K., Kato, R., Hisasue, S., Yamashita, S., Tanaka, T., Masumori, N., & Tsukamoto, T. (2011). Animal model for the study of the relationship between lower urinary tract symptoms/bladder outlet obstruction and erectile dysfunction. *International Journal of Urology*, 18, 710–715.
- Kostis, J. B., Jackson, G., Rosen, R., Barrett-Connor, E., Billups, K., Burnett, A. L., Carson, C., 3rd, Cheitlin, M., Debusk, R., Fonseca, V., Ganz, P., Goldstein, I., Guay, A., Hatzichristou, D., Hollander, J. E., Hutter, A., Katz, S., Kloner, R. A., Mittleman, M., Montorsi, F., Montorsi, P., Nehra, A., Sadovsky, R., & Shabsigh, R. (2005). Sexual dysfunction and cardiac risk (the Second Princeton Consensus Conference). *The American Journal of Cardiology*, 96, 313–321.
- Lin, W. Y., Chang, P. J., Lin, Y. P., Wu, S. B., Chen, C. S., Levin, R. M., & Wei, Y. H. (2012). Increased penile expression of transforming growth factor and elevated systemic oxidative stress in rabbits with chronic partial bladder outlet obstruction. *International Journal of Andrology*, 35, 79–85.
- Montorsi, P., Ravagnani, P. M., Galli, S., Rotatori, F., Briganti, A., Salonia, A., Rigatti, P., & Montorsi, F. (2005). The artery size hypothesis: a macrovascular link between erectile dysfunction and coronary artery disease. *The American Journal of Cardiology*, 96, 19M–23M.
- Montorsi, P., Ravagnani, P. M., Galli, S., Rotatori, F., Veglia, F., Briganti, A., Salonia, A., Deho, F., Rigatti, P., Montorsi, F., & Fiorentini, C. (2006). Association between erectile dysfunction and coronary artery disease. Role of coronary clinical presentation and extent of coronary vessels involvement: the COBRA trial. *European Heart Journal*, 27, 2632–2639.
- Montorsi, P., Ravagnani, P. M., Galli, S., Ali, S. G., Briganti, A., Salonia, A., & Montorsi, F. (2009). The triad of endothelial dysfunction, cardiovascular disease, and erectile dysfunction: clinical implications. *European Urology Supplements*, 8, 58–66.
- Mullins, C. D., Shaya, F. T., Meng, F., Wang, J., & Harrison, D. (2005). Persistence, switching, and discontinuation rates among patients receiving sertraline, paroxetine, and citalopram. *Pharmacotherapy*, 25, 660–667.
- Musicki, B., Bella, A. J., Bivalacqua, T. J., Davies, K. P., Disanto, M. E., Gonzalez-Cadavid, N. F., Hannan, J. L., Kim, N. N., Podlasek, C. A., Wingard, C. J., & Burnett, A. L. (2015). Basic science evidence for the link between erectile dysfunction and cardiometabolic dysfunction. *The Journal of Sexual Medicine*, 12, 2233–2255.
- Musselman, D. L., Evans, D. L., & Nemeroff, C. B. (1998). The relationship of depression to cardiovascular disease: epidemiology, biology, and treatment. *Archives of General Psychiatry*, 55, 580–592.
- Reaven, G. M. (1988). Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes*, 37, 1595–1607.
- Romeo, J. H., Seftel, A. D., Madhun, Z. T., & Aron, D. C. (2000). Sexual function in men with diabetes type 2: association with glycemic control. *The Journal of Urology*, 163, 788–791.
- Seftel, A. D., Sun, P., & Swindle, R. (2004). The prevalence of hypertension, hyperlipidemia, diabetes mellitus and depression in men with erectile dysfunction. *The Journal of Urology*, 171, 2341–2345.
- Shock, N. W., et al. (1984). *Normal human aging: The Baltimore longitudinal study of aging*. Bethesda, MD: National Institute on Aging (DHHS/NIH).
- Verze, P., Margreiter, M., Esposito, K., Montorsi, P., & Mulhall, J. (2015). The link between cigarette smoking and erectile dysfunction: a systematic review. *European Urology Focus*, 1, 39–46.
- Vlachopoulos, C., Jackson, G., Stefanadis, C., & Montorsi, P. (2013). Erectile dysfunction in the cardiovascular patient. *European Heart Journal*, 34, 2034–2046.
- Wang, C., Jackson, G., Jones, T. H., Matsumoto, A. M., Nehra, A., Perelman, M. A., Swerdloff, R. S., Traish, A., Zitzmann, M., & Cunningham, G. (2011). Low testosterone associated with obesity and the metabolic syndrome contributes to sexual dysfunction and cardiovascular disease risk in men with type 2 diabetes. *Diabetes Care*, 34, 1669–1675.

Effects of Exogenous Androgenic Agents on Male Reproduction

Stanton Honig and Campbell Bryson, Yale School of Medicine, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Androgen steroid hormones responsible for male sexual sex characteristics and other bodily functioning.

Apoptosis controlled and regulated cell death, a naturally occurring event.

Azoospermia complete lack of sperm in two separate semen samples.

Dysmorphic atypically formed.

Gonadotropin hormones secreted by the pituitary which stimulate the gonads, notable luteinizing hormone and follicle-stimulating hormone.

Hypogonadism reduction or absence of hormone or physiological activity related to the gonads.

Hypogonadotropic hypogonadism hypogonadism related to impaired secretion or function of gonadotropins.

Spermatogenesis the production and development of mature spermatozoa.

Oligospermia sperm concentration which is lower than normal in semen sample.

Normal Androgen Effects on Human Male Fertility

Testosterone plays a key role in the fertility of the adult male. In order for sperm to develop, intratesticular testosterone (ITT) (levels of testosterone specifically in the testes) has to concentrate to levels much higher than found in circulation. Lack of ITT can cause oligospermia or even lead to a complete loss of sperm in the semen (azoospermia) (Coviello et al., 2004). Circulating testosterone levels are commonly checked for patients to evaluate for hypogonadism, but this number can be artificially inflated from exogenous sources, and may not reflect adequate ITT levels necessary for spermatogenesis (Mulhall and Hsiao, 2014).

The hypothalamic-pituitary-gonadal (HPG) axis is a system where hormone production in the testes of males (e.g. testosterone) is controlled by a tightly regulated system initiated in the brain (see Fig. 1). This is covered in more detail in other chapters, but in brief: Gonadotropin releasing hormone (GnRH) is secreted episodically from the hypothalamus and travels via the hypophyseal portal system to the anterior pituitary. There, gonadotropic cells are stimulated to episodically release luteinizing hormone (LH)

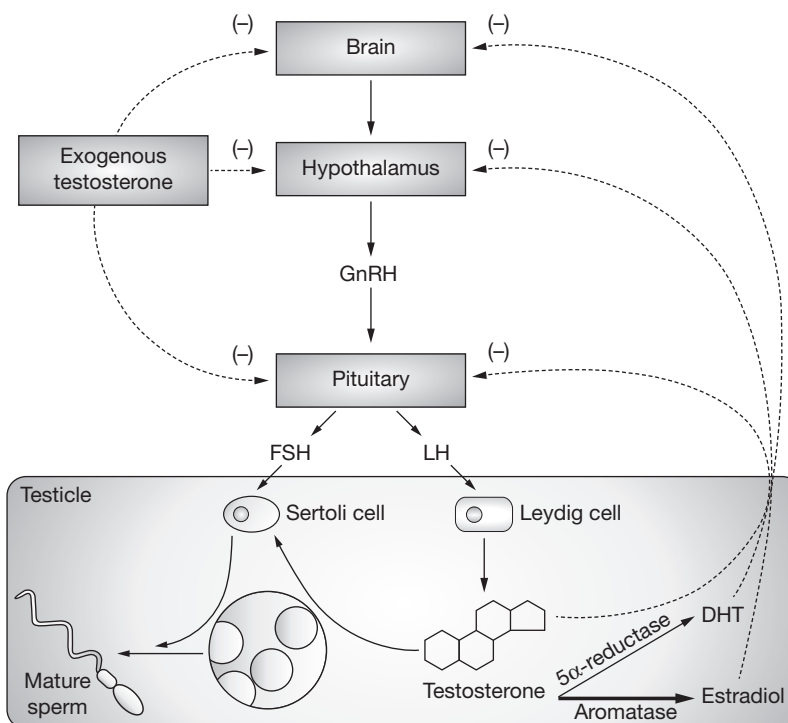


Fig. 1 Hypothalamic pituitary gonadal axis. Taken from Roth et al., 2008.

and follicle stimulating hormone (FSH). These hormones are released into the blood stream and have specific effects on testes cells. FSH acts on sertoli cells in the testicles to produce androgen binding protein (ABP), and stimulates production of sperm. The importance of sertoli cells on spermatogenesis is multifactorial: they have a central role in maintaining the blood-testis barrier, and supply nutrients to the growing immature spermatocytes. LH acts on the interstitial leydig cells of the testis to stimulate testosterone secretion. This testosterone is partially sequestered by ABP in the sertoli cells, with resulting important effects on spermatogenesis. The remaining testosterone is released into the blood stream. Systemic circulating testosterone has effects on multiple physiological and psychological factors, notably on muscle mass, bone health, sexual functioning and overall energy levels. Testosterone can be converted to estrogen via aromatase in peripheral tissues. It can also be converted to 5-alpha dihydrotestosterone (DHT) a potent androgen via 5-alpha reductase, which can also act on the androgen receptor.

Testosterone which becomes bound to ABP in the testicle acts on intracellular androgen receptors found in sertoli cells with resulting DNA activation and transcription of several proteins which promote spermatogenesis, and prevent spermatocyte apoptosis or cell death. Testosterone is actively sequestered in the seminiferous tubules and can have a concentration 25–100 times higher than in the blood circulation. The exact concentration of ITT required for spermatogenesis has not been defined, however, studies have shown that intratesticular levels similar to serum levels are not sufficient.

Acting as negative feedback, testosterone circulates back to both the hypothalamus and the anterior pituitary and directly inhibit further release of GnRH, FSH and LH. Estrogen also acts to inhibit the hypothalamus, and possibly the anterior pituitary (Roth et al., 2008). The stimulation of sertoli cells by FSH also causes secretion of inhibin B which acts on the anterior pituitary, inhibiting release of FSH. Thus, a balanced homeostasis occurs in the adult male for production of testosterone (Fronczak et al., 2012).

Exogenous Androgens and Their Effects on Male Fertility

Men who have low circulating testosterone levels (hypogonadism) are commonly treated with exogenous androgens to improve the systemic effects of testosterone, such as muscle mass, bone health, sexual functioning and energy levels. The prevalence of hypogonadism in the age group 20–45 has been estimated at 3–8% (Behre et al., 2016). However, the number of men being diagnosed with hypogonadism is increasing, with a four fold increase in men diagnosed and subsequently treated with exogenous testosterone in the age group 18–45 between 2003 and 2013 (Dohle et al., 2003). Returning the circulating testosterone levels to normal exogenously can have benefits on mood, muscle mass, and energy, however, it has a direct inhibitory effect on LH and FSH production via the normal feedback loop. The reduction in LH prevents the production of testosterone from leydig cells, and the reduction in FSH prevents the formation of ABP. The end result being that intratesticular levels of testosterone falls drastically. This reduction in ITT prevents spermatogenesis from occurring. Thus, patients treated with exogenous hormone therapy may have the benefits of systemic hormones, but have the unwanted or unexpected side effect of infertility. Unfortunately, many practitioners including urologists are unaware of this serious effect. Similarly, many young men use exogenous androgens to use as an exercise supplement, reportedly 1–6% of high school seniors (Depenbusch et al., 2002, Shaw et al., 2010).

Interest in the use of exogenous testosterone to inhibit spermatogenesis prompted its evaluation as a contraceptive agent in two landmark trials by the WHO in 1990 and 1996. Intramuscular testosterone was given to men weekly, with a 98% efficacy of reducing sperm to at least oligospermic levels temporarily, and 77% had a continued azoospermia while on the testosterone supplements (WHO, 1990, 1996). A more recent study involving 320 men were treated with norethisterone enanthate (form of progesterone) and testosterone IM injections every 8 weeks had a 95.9% efficacy rate at reducing sperm levels to < 1 million/mL within 24 weeks, and upon cessation of treatment there was a 94.8% reversibility of suppression within 1 year (Behre et al., 2016).

In patients who desire future fertility, studies show that in most patients, withdrawing the hormone therapy can increase circulating LH/FSH and subsequently return spermatogenesis. This takes a variable length of time, between 3 months and 2 years. However, these patients will lose the benefit of normalized systemic androgen levels, unless another method to stimulate hormone production is used.

Exogenous Products Affecting Androgen Production and Fertility

Synthetic testosterone (gel, cream, transdermal patch, buccal tablets, intramuscular injections, subdermal implants): commonly prescribed to male patients who are symptomatic and hypogonadal and not interested in future fertility. Synthetic testosterone acts in the same way as endogenous testosterone, and has feedback inhibition on the HPG axis, can be converted to DHT and estrogen as above. Levels are consistently monitored by physicians. Care must be made with any topical application to apply it to areas which would not normally be contacted by people in whom increased androgens could be dangerous, namely children and women. Otherwise premature virilization may occur.

Anabolic androgenic steroids (AAS): the benefit of exogenous steroid hormones on muscle mass and energy levels has been known since the early 1900s, and it was quickly adopted into use as a performance enhancing drug in athletes. Use in amateur male athletes is increasing, with an estimated 3.0% to 4.2% lifetime prevalence (Ramasamy et al., 2015). Unfortunately, AAS is associated with multiple medical issues including: testicular atrophy, low endogenous testosterone production, sexual dysfunction, gynecomastia (enlarged painful breast development), liver dysfunction, alopecia (hair loss), high blood pressure, dyslipidemia. Similar to exogenous synthetic testosterone therapy, AAS inhibits release of GnRH and LH/FSH and can cause oligo- or azoospermia. Users frequently get AAS over the internet, and so are not made aware of side effects or risks of their use. Men

identified as using AAS must be carefully counseled as to these risks, and specialized care via an andrologist or endocrinologist should be sought.

Selective Estrogen Receptor Modulators (SERMs): exogenous medications which act as competitive antagonists at the estrogen receptor. By blocking the negative inhibitory effect of estrogen (formed by aromatization of testosterone) on the hypothalamus, and perhaps the anterior pituitary, SERMs increase the circulating levels of LH and FSH with subsequent increases in testosterone levels. Data on improvement in semen quality is mixed. Tamoxifen and clomiphene citrate are the most commonly used medications in this class, and are FDA approved for breast cancer therapy and in female fertility, respectively. It is used off label in men. Multiple small prospective studies have showed a daily or every other day dose schedule of clomiphene citrate in hypogonadal men can be effective, and cost effective (Rao et al., 2017), in increasing circulating testosterone to desired levels (Shabsigh et al., 2005, Shabsigh et al., 2005). A 3 year follow up showed that patients had no adverse effects and had sustained improved levels (Katz et al., 2012).

Aromatase inhibitors: aromatase is a CYP450 enzyme found in liver, testes, brain and adipose tissue and catalyzes the synthesis of estrogen from testosterone. Inhibitors of aromatase, including anastrozole and letrozole have classically been used in breast cancer therapy to limit estrogen formation. Their use in men is not FDA approved, but has been used to prevent estrogenic feedback on the HPG axis and limit the side effects of increased estrogen, notably gynecomastia (Shabsigh et al., 2005). Side effects of their use include concern for worsening bone health. Some studies have shown an increased risk of clotting disorders including pulmonary embolism and stroke (Lycette et al., 2006).

Human chorionic gonadotropin (hCG): An endogenous protein made in men and women, it is comprised of an alpha and beta subunit. The alpha unit is identical to the alpha unit of both LH and FSH, and the beta unit is identical to the LH beta unit excepting 24 additional amino acids. hCG can stimulate the LH receptor and have downstream upregulation of testosterone production, increasing both circulating testosterone and IIT. This makes it a useful therapy in treating patients who have hypogonadotropic hypogonadism, as they in theory should have normally functioning testicular tissue, though may have atrophied from prolonged gonadotropin deficiency (Shabsigh et al., 2005). It is not FDA approved for hypogonadism of aging or hypergonadotropic hypogonadism. A retrospective review of 26 hypogonadal men treated with exogenous testosterone and low dose hCG intramuscular doses every other day showed sustained increased circulating testosterone levels and they all maintained their semen parameters (Shabsigh 2005). A small prospective study on men with hypogonadism treated with hCG and human menopausal gonadotropin (hMG) were able to induce spermatogenesis, maintain testosterone levels, and maintain sperm production over a 2 year period of followup (Depenbusch 2002). Hsieh et al. showed that a combination of testosterone and intermittent hCG can also maintain spermatogenesis (Hsieh 2013).

Further Reading

- Anon. (1990). Contraceptive efficacy of testosterone-induced azoospermia in normal men. World Health Organization task force on methods for the regulation of male fertility. *Lancet (London, England)*, 336(8721), 955–959.
- Behre, H. M., Zitzmann, M., Anderson, R. A., Handelsman, D. J., Lestari, S. W., McLachlan, R. I., et al. (2016). Efficacy and safety of an injectable combination hormonal contraceptive for men. *The Journal of Clinical Endocrinology and Metabolism*, 101(12), 4779–4788.
- Buckley, W. E., Yesalis, C. E., Friedl, K. E., Anderson, W. A., Streit, A. L., & Wright, J. E. (1988). Estimated prevalence of anabolic steroid use among male high school seniors. *Journal of the American Medical Association*, 260(23), 3441–3445.
- Coviello, A. D., Bremner, W. J., Matsumoto, A. M., Herbst, K. L., Amory, J. K., Anawalt, B. D., et al. (2004). Intratesticular testosterone concentrations comparable with serum levels are not sufficient to maintain normal sperm production in men receiving a hormonal contraceptive regimen. *Journal of Andrology*, 25(6), 931–938.
- Dabaja, A. A., & Schlegel, P. N. (2014). Medical treatment of male infertility. *The Translational Andrology and Urology*, 3(1), 9–16.
- Depenbusch, M., von Eckardstein, S., Simoni, M., & Nieschlag, E. (2002). Maintenance of spermatogenesis in hypogonadotropic hypogonadal men with human chorionic gonadotropin alone. *European Journal of Endocrinology*, 147(5), 617–624.
- Dohle, G. R., Smit, M., & Weber, R. F. A. (2003). Androgens and male fertility. *World Journal of Urology*, 21(5), 341–345.
- Fronczak, C. M., Kim, E. D., & Barqawi, A. B. (2012). The insults of illicit drug use on male fertility. *Journal of Andrology*, 33(4), 515–528.
- Hsieh, T.-C., Pastuszak, A. W., Hwang, K., & Lipshultz, L. I. (2013). Concomitant intramuscular human chorionic gonadotropin preserves spermatogenesis in men undergoing testosterone replacement therapy. *The Journal of Urology*, 189(2), 647–650.
- Katz, D. J., Nabulsi, O., Tal, R., & Mulhall, J. P. (2012 Aug). Outcomes of clomiphene citrate treatment in young hypogonadal men. *BJU International*, 110(4), 573–578.
- Lycette, J. L., Luoh, S.-W., Beer, T. M., & Deloughery, T. G. (2006). Acute bilateral pulmonary emboli occurring while on adjuvant aromatase inhibitor therapy with anastrozole: Case report and review of the literature. *Breast Cancer Research and Treatment*, 99(3), 249–255.
- Moskovic, D. J., Katz, D. J., Akhavan, A., Park, K., & Mulhall, J. P. (2012). Clomiphene citrate is safe and effective for long-term management of hypogonadism. *BJU International*, 110(10), 1524–1528.
- Mulhall, J. P., & Hsiao, W. (2014). *Men's sexual health and fertility: A clinician's guide*. New York: Springer.
- Ramasamy, R., Armstrong, J. M., & Lipshultz, L. I. (2015). Preserving fertility in the hypogonadal patient: An update. *Asian Journal of Andrology*, 17(2), 197–200.
- Rao, P. K., Boulet, S. L., Mehta, A., Hotaling, J., Eisenberg, M. L., Honig, S. C., et al. (2017). Trends in testosterone replacement therapy use from 2003 to 2013 among reproductive-age men in the United States. *The Journal of Urology*, 197(4), 1121–1126.
- Roth, M. Y., Amory, J. K., & Page, S. T. (2008). Treatment of male infertility secondary to morbid obesity. *Nature Clinical Practice Endocrinology & Metabolism*, 4(7), 415–419.
- Shabsigh, A., Kang, Y., Shabsigh, R., Gonzalez, M., Liberson, G., Fisch, H., et al. (2005). Clomiphene citrate effects on testosterone/estrogen ratio in male hypogonadism. *The Journal of Sexual Medicine*, 2(5), 716–721.
- Shaw, N. D., Histed, S. N., Srouji, S. S., Yang, J., Lee, H., & Hall, J. E. (2010). Estrogen negative feedback on gonadotropin secretion: Evidence for a direct pituitary effect in women. *The Journal of Clinical Endocrinology and Metabolism*, 95(4), 1955–1961.
- Sjöqvist, F., Garle, M., & Rane, A. (2008). Use of doping agents, particularly anabolic steroids, in sports and society. *Lancet (London, England)*, 371(9627), 1872–1882.
- Taylor, F., & Levine, L. (2010). Clomiphene citrate and testosterone gel replacement therapy for male hypogonadism: Efficacy and treatment cost. *The Journal of Sexual Medicine*, 7(1 Pt 1), 269–276.
- WHO. (1996). World Health Organization Task Force on Methods for the Regulation of Male Fertility. Contraceptive efficacy of testosterone-induced azoospermia and oligozoospermia in normal men. *Fertility and Sterility*, 65(4), 821–829.

Medications With Male Reproductive Side Effects

Mary K Samplaski, University of Southern California, Los Angeles, CA, United States

Ajay K Nangia, University of Kansas Medical Center, Kansas City, KS, United States

© 2018 Elsevier Inc. All rights reserved.

Background

- Men of reproductive age commonly take medications.
- Testing on the reproductive effects of these medications is not standardized.
- Testing for all possible facets of reproductive impairment is challenging:
 - Alterations of the hypothalamic-pituitary-gonadal (HPG) axis
 - Effects on sperm production or function
 - Impaired sexual function

Anti-Inflammatories

- > 30 NSAID pills/month may result in lower sperm concentration and motility, and a higher percentage of dead sperm.
- Limited human data
- Animal data seems to suggest that occasional use is not harmful

Testosterone

- Exogenous testosterone will decrease sperm counts and should not be used in men trying to achieve a pregnancy.
- Anabolic steroid users often have supra-physiologic serum testosterone concentrations, which may further suppress sperm production.

Phosphodiesterase Inhibitors

- Studies on their relationship with sperm function have yielded varying results
- Sub-fertile men may have a more pronounced (and negative) response

Psychotropic Medications

- Known to affect sexual function: libido, erectile and ejaculatory function.
- Limited data on semen parameters and sperm function.
- SSRI and lithium use may result in a reduced sperm concentration, motility and morphology.
- SSRI use is correlated with an increase in sperm DNA fragmentation index.

Anti-Hypertensive Agents

- Most cause some degree of vasculogenic sexual dysfunction, which can indirectly impair fertility
- Germinal (testicular) angiotensin-converting enzyme (ACE) is found exclusively in post-meiotic male germinal cells, and its presence is correlated with sperm concentration and motility, sperm function and fertilization. However, men taking angiotensin-converting enzyme inhibitors (ACE-Is) do not seem to have altered semen parameters or fertilizing ability.

Antibiotics

- Limited data and the co-existing inflammation may also impact fertility
- There is data to suggest that aminoglycosides and ciprofloxacin may negatively impact semen parameters.

5 α -Reductase Inhibitors

- In healthy men, these medications will result in ~30% decrease total sperm counts.
- Some men have a heightened sensitivity and will have a more pronounced response.
- Some men may develop “Post Finasteride Syndrome.”

Alpha-Blockers

- Most will result in some degree of ejaculatory dysfunction, ranging from retrograde ejaculation to decreased ejaculatory volume or impaired force of seminal emission.
- May also alter libido and erectile function.

Chemotherapeutic Agents

- Multi-agent chemotherapeutic regimens will minimize single agent toxicity.
- High gonadotoxic risk: alkylating agents (cyclophosphamide, chlorambucil, mustine, ifosfamide), nitrogen mustard derivatives (busulfan, melphalan), chlormethine, procarbazine, dacarbazine, chlormethine, and the MOPP regimen (nitrogen-mustard, oncovin (vincristine), procarbazine, and prednisone).

- Biologic or targeted therapies may also have effects on male fertility, although limited data.

Transplant Medications

- Limited and conflicting human and animal data.

Background

Sixty-eight percent of men ages 18–44 years have used medications (prescription or over the counter), increasing to 81% in men 45–64 years of age. In addition, the quantity of medications used increases with age (Kaufman et al., 2002). Both these medications themselves and the underlying disease states may impair reproductive ability. Testing on the reproductive effects of these medications is not standardized and few human studies have been performed. In addition, testing for all possible facets of reproductive impairment is challenging, including through alterations of the hypothalamic-pituitary-gonadal (HPG) axis, effects on sperm production or function, or impaired sexual function.

Anti-Inflammatories

While anti-inflammatories are among the most commonly used medications, their effects in humans remain poorly studied. In theory, a relationship could be present as cyclooxygenase (COX) enzyme inhibitors play an important role in the regulation of reproductive functions in females. A 2003 study on nonsteroidal anti-inflammatory drugs (NSAID) from 1376 men found a non-significant 16% decrease in sperm concentration in men who reported consuming > 30 NSAID pills/month. Linear regression analysis found a correlation between NSAID doses and the percentage of dead cells, sperm motility and morphology (Martini et al., 2003).

Acetaminophen, or paracetamol, bears some structural similarities to estradiol and progesterone, and therefore has been investigated as a sex steroid modulator (Anderson et al., 1998; Cramer et al., 1998). There are no human studies on this medication. In the largest animal study, male rats were treated with paracetamol at 500 or 1000 mg/kg (maximum dosing in humans 15 mg/kg) for 30 days, and libido, ejaculatory ability and fertility were assessed (Ratnasooriya and Jayakody, 2000). After 30 days, there was a decline in libido, sexual vigor and sexual performance. Mating studies found a decrease in pregnancies, fertility index, implantation index and number of implants. These effects were thought to be due to reduced sperm counts, motility and a reduced sperm fertilizing potential. Histologically, treated rats had an apoptosis of spermatocytes and spermatids, and reduction in testicular weight. The effect of paracetamol on sperm fertilizing ability and miscarriages, was thought to be due to its lipid peroxidation effects, which may impact sperm capacitation ability (Wendel et al., 1979). All these effects were reversible (Ratnasooriya and Jayakody, 2000).

There have been several other smaller animal studies on acetaminophen/paracetamol. A study of 6 rats that were treated with a single dose of paracetamol found altered seminiferous tubules, Sertoli cells and spermatids after treatment (Yano and Dolder, 2002). Furthermore, treatment with medications to mitigate oxidative stress may negate paracetamol-induced reproductive damage (Aksu et al., 2016). Finally, male mice treated with oral acetaminophen had an increase in sperm abnormal morphology and a decrease in offspring pup weights (7% in controls vs. 16% in the high-dose group) (1997).

There have been similarly no studies of ibuprofen on semen parameters in humans. Mouse studies have shown that after treatment with low and high doses of ibuprofen, for 35, 70 and 105 days, all of the treated mice had an increase in spermatozoa with immature chromatin, and there was a decline in sperm concentration, motility and normal forms (Roodbari et al., 2015). Conversely, in mice exposed to hypoxic conditions, ibuprofen was actually protective against teratozoospermia and elevated sperm DNA fragmentation (Vargas et al., 2011).

Aspirin, or acetylsalicylic acid, has been investigated. In the only human study, healthy adult males who used moderate amounts of aspirin (> 500 mg/wk) had a moderate decrease in sperm motility (62% vs. 44%) (Stutz et al., 2004). A study looking at rats given aspirin at 12.5 mg/kg for 30 and 60 days found that treated animals had reduced sperm density, count and motility in the cauda epididymis and testis, and histologic evaluation found a reduced number of primary spermatogonial cells (Vyas et al., 2016). There are no animal studies on the effects of aspirin on male semen parameters or reproductive organs, but there are studies demonstrating its protective effect (Karaguzel et al., 2012), and as such, occasional use is likely not detrimental.

There is only a single study looking at the male reproductive toxicity effects of naprosyn. After treatment with naprosyn for 35 days, male rats had a reduction in sperm count (600×10^6 vs. 375×10^6) and motility (95% vs. 35%), and induced damage of seminiferous tubules without effecting plasma hormone levels (Uzun et al., 2015).

Exogenous Testosterone

Exogenous testosterone suppresses the HPG axis, which results in a profound suppression in spermatogenesis (Gonzalo et al., 2002), an effect which is more pronounced with intramuscular (vs. transdermal) formulations (Ko et al., 2012). The largest and

longest study looking at this found that after intramuscular testosterone replacement therapy for 30 months, 95% of men became azoospermic or severe oligospermic with a sperm concentration of < 1 million. It is reassuring that all but 2 men recovered spermatogenesis, with a median recovery time to baseline sperm production of 182 days. Conversely, it is concerning that 2 men did not recover spermatogenesis (Liu et al., 2006). These findings have been corroborated by other studies (Rahnema et al., 2014; Kim et al., 2013). Because of this, exogenous testosterone should not be used in men of reproductive age.

Although “exogenous testosterone” is often medically prescribed, the most commonly abused illicit anabolic steroids are types of exogenous testosterone. In this population, the serum testosterone concentrations are typically supra-physiologic, which may further suppress sperm production. Animal models have demonstrated that after anabolic steroid discontinuation, Leydig cells tend to proliferate but remain below regular counts, even after prolonged periods, suggesting a delay in recovery of testicular function after anabolic steroid use (Nagata et al., 1999). Likewise, even when hCG is used as an adjunct to anabolic steroids use, lower motility and poorer morphology is seen, even after anabolic steroids are discontinued (Karila et al., 2004).

Phosphodiesterase Inhibitors

Several PDE isoforms have been found in human sperm (Sinclair et al., 2000), and both sildenafil and vardenafil have been found in the ejaculate after oral administration (du Plessis et al., 2004). Studies on their relationship with sperm function have yielded varying results. In vitro studies have shown conflicting results regarding the effect of sildenafil on human sperm motility (du Plessis et al., 2004; Purvis et al., 2002; Aversa et al., 2000; Jannini et al., 2004), capacitation and the acrosome reaction (Lefievre et al., 2000; Burger et al., 2000). In vivo studies have shown that, asthenozoospermic and normozoospermic men treated with oral sildenafil had no difference in semen parameters or acrosome reaction rate (Yang et al., 2014). Similarly, a double-blinded, placebo controlled study of 200 men treated daily with daily sildenafil, vardenafil or placebo for 6 months, did not show any alterations in semen parameters or reproductive hormones (Jarvi et al., 2008).

Data for tadalafil are similarly heterogeneous. A human study of fertile men treated with tadalafil versus placebo showed no difference in semen or reproductive hormones (Hellstrom et al., 2003). However, in a small study of subfertile men, sildenafil resulted in an increase in sperm progressive motility (37.0% vs. 28.5%), but tadalafil resulted in a decrease in motility (21.5% vs. 28.5%) (Pomara et al., 2007). Finally, a recent study of fertility unaware men treated with tadalafil 5 mg daily for 12 weeks found that treated men actually had an increase in sperm counts and motility, making the daily use of tadalafil for benign prostatic hyper trophy reassuring (Corvasce et al., 2015). Due to small numbers of patients, these data are difficult to extrapolate from, but may indicate a differential response to tadalafil versus sildenafil, and that subfertile men may have a more pronounced response to these medications.

Psychotropic Medications

Psychotropic medications are known to affect sexual function especially libido, erectile and ejaculatory function. These agents may also affect the HPG axis at many levels, resulting in lower FSH and LH levels, hyperprolactinemia and hypogonadism, and other abnormalities (Hendrick et al., 2000). Limited data has shown a reduction in semen volume and sperm motility (Levin et al., 1981) after treatment with tricyclic antidepressants in vivo, and in vitro (Maier and Koinig, 1994). Phenothiazines, monoamine oxidase inhibitors and lithium can result in sexual performance issues (Serretti and Chiesa, 2011).

Selective serotonin reuptake inhibitors (SSRIs) are often used to treat premature ejaculation or performance-anxiety related ED. Their effects on male fertility are unclear. Long-term use may result in a 50–60% incidence of ED, delayed or absent ejaculation (Montejo et al., 2001). For studied medications, the order of this effect is: paroxetine $>$ fluoxetine $>$ sertraline (Montejo et al., 2001). SSRIs have also been shown to be spermicidal in vitro, likely from oxidative phosphorylation (Kumar et al., 2006). Three prospective cohort studies conducted in men exposed to SSRIs found altered semen parameters after as little as 3 months of exposure, including reduced sperm concentration, motility and morphology (2015). A rat study using fluvoxamine demonstrated resulted apoptosis in testicular tissue (Galal et al., 2016). Similarly, rats treated with dapoxetine had a dose-dependent decrease in sperm motility and normal forms, and an increase in fetal resorptions (ElMazoudy et al., 2015). SSRI use has also been associated with increased sperm DNA fragmentation (30.3% vs. 13.8% (Tanrikut et al., 2010)) and decreased sperm motility, independent of type of SSRI, but related to treatment duration (Safarinejad, 2008).

Rat models have identified a dose-dependent reduction in sperm concentration, motility, morphology and fecundity after treatment with lithium (Toghyani et al., 2013; Thakur et al., 2003), and histologically a loss of germ cell attachment (Zarnescu and Zamfirescu, 2006). Human studies on sperm and lithium are scant and show only an in vitro reduction in sperm viability (Levin et al., 1981).

Antihypertensive Medications

Most antihypertensive agents cause some degree of vasculogenic sexual dysfunction, which can indirectly impair fertility (Ko et al., 2002). In mice, β -blockers have been shown to result in reversible impaired sperm concentration, motility and morphology

(el-Sayed et al., 1998; Nusier et al., 2007). A recent study of rats treated with cisplatin +/- concurrent carvedilol found that carvedilol mitigated cisplatin-induced testicular and spermatogenic injury by reducing inflammatory burden, enhancement of steroidogenesis and spermatogenesis, and mitigation of testicular histopathological damage (Eid et al., 2016). Human studies are limited and conflicting. In vitro studies have found that propranolol may inhibit human sperm motility (White et al., 1995), however, in vivo studies have found no change in semen parameters (Mahajan et al., 1984).

Spironolactone reduces testosterone production, increases the conversion of testosterone to estrogen and impairs DHT receptor binding. In humans, it has been shown to cause gynecomastia and decrease sperm concentration and motility (Caminos-Torres et al., 1977).

While the calcium ion is involved in in vitro sperm motility, capacitation and the acrosome reaction (Morakinyo et al., 2011), the clinical effect of calcium channel blockers on semen parameters and sperm function is not as clear. In vivo murine studies of calcium channel blockers have found impaired in semen parameters, the acrosome reaction and zona penetration (Saha et al., 2000a), and decreased litter size (Morakinyo et al., 2011). Human in vitro incubation studies with diltiazem have shown that at higher doses there is a decline in sperm motility (Aaberg et al., 1989). In addition, a contraception study looking at vasal irrigation with diltiazem at the time of vasectomy resulted in a reduction in sperm motility, viability and cervical mucus penetration (Wood et al., 2003). However, in humans, no change in fertilization rate in in vitro fertilization (IVF) or non-IVF cycles was seen in men using calcium channel blockers (Katsoff and Check, 1997).

There have been no human studies on semen parameters in men treated with angiotensin-converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers, although animal studies suggest that they may play a role. Rat studies have found conflicting results on the impact of treatment with ACE-I on semen parameters (Okeahialam et al., 2006; Saha et al., 2000b). A single in vitro human sperm incubation study found a decrease in motility (Shibahara et al., 2001). From a physiologic perspective, angiotensin II has been found to localize to human sperm, and levels are correlated with sperm motility (Gianzo et al., 2016). In addition, the germinal (testicular) ACE is found exclusively in post-meiotic male germinal cells (Li et al., 2014). Its presence is correlated with sperm concentration and motility (Shibahara et al., 2001), as well as multiple other sperm functions (Zalata et al., 2012), and this enzyme is crucial for fertilization (Kondoh et al., 2005).

Antibiotics

Interpretation of the effect of antibiotics on male fertility can be challenging, as the co-existing inflammation may negatively impact male fertility. In addition, recent data are not available for most of these medications.

A 1998 in vitro study used sperm from normospermic men cultured with increasing concentrations of several antibiotics, with variable negative effects on vitality and motility (Hargreaves et al., 1998). There have been no human studies on the effects of penicillins and cephalosporins on human sperm, but in vitro animal studies suggest a decline in sperm production and motility (Timmermans, 1974; Antohi et al., 2011). Limited studies of effects of trimethoprim-sulfamethoxazole (TMP-SMX) generally show minimal effects on human sperm (Murdia et al., 1978; Merino and Carranza-Lira, 1995). In the only human study looking at this drug, erythromycin for the treatment of men with asthenospermia resulted in no change in semen parameters (Baker et al., 1984). Similarly, therapeutic doses of nitrofurantoin have not been shown to affect semen parameters (Albert et al., 1975).

Ciprofloxacin for the treatment of positive human male semen cultures has not been shown to adversely affect sperm (Merino and Carranza-Lira, 1995). However, in a 2015 review, total motile sperm count, testis weight and reproductive histology was decreased after treatment with ofloxacin (Khaki, 2015).

Doxycycline has been studied in infertile men with low-level leukocytospermia. In this population, treatment resulted in an increase in natural pregnancy rates (47% vs 20%) (Hamada et al., 2011).

Aminoglycosides, including neomycin and gentamicin, have been shown to negatively impact semen parameters. A remote article found that the administration of gentamicin prior to prostate surgery resulted in the cessation of meiosis at the primary spermatocyte level and in increase in abnormal forms (Timmermans, 1974). Likewise, the use of neomycin to treat urologic inflammatory conditions has demonstrated a negative impact on sperm count, concentration and motility (Schlegel et al., 1991). Rat studies have confirmed that gentamicin results in a decrease in sperm count, motility and viability (Khaki et al., 2008).

Sparse data exist for the antifungals, but the available data does show a decline in testicular function. Ketoconazole has been shown to result in an increase in serum LH and a decrease in serum testosterone (Irsy et al., 1991). It has also been shown to result in impaired sperm motility when administered orally to dogs and monkeys (Vickery et al., 1985). This is the only antifungal for which there is any data.

5 α -Reductase Inhibitors

The available literature shows that most (but not all) men will not experience negative reproductive effects after treatment with 5 α -reductase inhibitors (5ARIs). At full doses, both dutasteride (0.5 mg/day) and finasteride (5 mg/day) result in a reversible ~30% decrease total sperm counts (Amory et al., 2007). Similarly, finasteride (1 mg/day) has not been shown to alter semen parameters in fertile men (Overstreet et al., 1999). However, in both studies, a subgroup of approximately 5% of men demonstrated a heightened sensitivity, with decreases in total sperm count to <10% of baseline values (Overstreet et al., 1999; Amory et al.,

2007). Accordingly, in men presenting for fertility evaluation who had been treated with finasteride 1 mg daily, a mean 11.6-fold increase in sperm concentration was seen after finasteride discontinuation (Samplaski et al., 2013). Finally, a single case report has identified an increase in sperm DNA fragmentation rate with finasteride (Tu and Zini, 2011).

Men taking 5ARIs may also be at risk for “Post Finasteride Syndrome”, a constellation of sexual dysfunction, ED, diminished sexual desire and ejaculatory dysfunction (Gur et al., 2013; Traish et al., 2011; Irwig and Kolukula, 2011). In two 1-year studies, men taking finasteride 1 mg daily had more sexual adverse events than placebo (4.2 vs. 2.2%); decreased sexual desire (1.9 vs. 1.3%), ejaculatory dysfunction (1.2 vs. 0.7%) and ED (1.4 vs. 0.9%) (Kaufman et al., 1998; McClellan and Markham, 1999). Similar results have been reported for finasteride 5 mg daily (Wessells et al., 2003; Byrnes et al., 1995) (Nickel et al., 1996). Alarming, in some men the sexual dysfunction persists even after discontinuation of the 5ARI. After finasteride discontinuation, 20% of men reported persistent adverse sexual effects for > 6 years (Mondaini et al., 2007). Dutasteride has been shown to have a similar adverse sexual side effect profile (Clark et al., 2004; Gur et al., 2013; Debruyne et al., 2004; Roehrborn et al., 2002).

Alpha-Blockers

Alpha adrenoceptor blockers inhibit sympathetic smooth muscle innervation to the distal ureter, prostate and male reproductive tract, and may cause ejaculatory dysfunction, ranging from retrograde ejaculation to decreased ejaculatory volume or impaired force of seminal emission. Four subtypes of α_1 receptors have been described, and the variation in side effects likely relates to variable affinities for these receptors. These effects do seem to be reversible. In a randomized 3-way crossover study in normal men, >20% decrease in ejaculate volume was seen in 89.6% of men taking tamsulosin 0.8 mg, 20.8% of men taking alfuzosin and 12.5% of men taking placebo (Hellstrom and Sikka, 2006). A meta-analysis looking at sexual side effects with α -blockers, found that ejaculatory dysfunction was more common with α -blockers overall compared with placebo (OR: 5.88). Tamsulosin had an odds ratio of 8.58 ($P = 0.006$), silodosin had an odds ratio of 32.5 ($P < 0.0001$), and doxazosin and terazosin were similar to placebo (Gacci et al., 2014). For most other α -blockers, ejaculatory dysfunction is found in 0–1% to 1.5% of men (Chen et al., 2009; Kaplan, 2009).

Limited studies have looked at the effect of α -blockers on libido and erectile function. Decrease libido is found in 1–2% of men taking α -blockers, particularly with tamsulosin at the 0.8 mg dose (Roehrborn et al., 2008; Lepor et al., 2012). ED is found in 3.8% of men taking tamsulosin (Roehrborn et al., 2008). Studies on the rates of ED and altered libido in other α -blockers are lacking.

α -Blockers have also been shown to alter semen parameters in healthy men. Alfuzosin 10 mg daily resulted in an increase in sperm count of 46.2×10^6 , and tamsulosin resulted in a decrease by 54.6×10^6 . Both drugs resulted in a decline in sperm motility, 13.8% after tamsulosin and 0.4% after alfuzosin (Hellstrom and Sikka, 2009). Studies on semen parameters for other α -blockers are lacking.

Chemotherapeutic Agents

Chemotherapeutic agents may damage sperm via effects on spermatogonial stem cells, Sertoli or Leydig cells (Boekelheide, 2005). Stem cell damage may result in genetically abnormal sperm precursors, including sperm diploidy (Genesca et al., 1990; De Mas et al., 2001). While multi-agent chemotherapeutic regimens will minimize single agent toxicity, these regimens may also make it difficult to determine the toxicity of the individual agents (Meistrich, 2013).

Some of the most gonadotoxic regimens include alkylating agents (cyclophosphamide, chlorambucil, mustine, ifosfamide), nitrogen mustard derivatives (busulfan, melphalan), chlormethine, procarbazine, dacarbazine, chlormethine, and the MOPP regimen (nitrogen-mustard, oncovin (vincristine), procarbazine, and prednisone). Moderate risk chemotherapeutic agents include cisplatin, carboplatin, doxorubicin, BEP (bleomycin, etoposide, cisplatin) and ABVD (adriamycin, bleomycin, vinblastine, dacarbazine). Low risk chemotherapeutic agents include antimetabolites, topoisomerase inhibitors, vinca alkaloids (vincristine, vinblastine), methotrexate, dactinomycin, dacarbazine, mercaptopurine, and bleomycin (Pont and Albrecht, 1997; Howell and Shalet, 2001; Wallace et al., 2005).

The alkylating agent, cisplatin, is commonly used in the treatment of testicular cancer, and is considered highly gonadotoxic (Boekelheide, 2005; Fung and Vaughn, 2011). In 1191 testis cancer survivors, at 11 years follow-up, the median sperm concentration for men treated with surgery alone was $30 \times 10^6/\text{mL}$, versus $18 \times 10^6/\text{mL}$ in men treated with chemotherapy containing cisplatin $< 850 \text{ mg}/\text{m}^2$, and $1 \times 10^6/\text{mL}$ in men treated with in chemotherapy containing cisplatin $> 850 \text{ mg}/\text{m}^2$ ($p = 0.001$) (Brydoy et al., 2012). These treatments also correlated with post-treatment paternity (Brydoy et al., 2012). In some men, the only manifestation of gonadal insult seen is altered gonadotropin or testosterone levels, and up to 34% of testis cancer survivors receiving cisplatin-based chemotherapy will have lower testosterone levels than chemo-naïve patients (Brydoy et al., 2012; Huddart et al., 2005). This may secondarily affect male fertility by altering libido, erectile function and sexual satisfaction (Huddart et al., 2005; Fung and Vaughn, 2011).

Non-Hodgkin lymphoma was traditionally treated with the R-CHOP regimen (rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone), which may result in long-term azoospermia (da Cunha et al., 1984). The ABVD regimen was developed because of this. A recent multicenter, prospective study looked at the effect of lymphoma treatments on sperm up to 24 months after treatment (Bujan et al., 2014). After 24 months, >90% of men were expected to recover normal sperm count after ABVD, compared with 61% for CHOP (Bujan et al., 2014). Within the CHOP regimen, the agent with the most significant gonadotoxicity

is cyclophosphamide. In general, a cumulative dose of 19 g/m² will produce prolonged azoospermia (Rivkees and Crawford, 1988), but when used as part of a regimen, lower doses may result in oligospermia (Eghbali and Papaxanthos-Roche, 2010).

Biologic or targeted therapies may also have effects on male fertility. Animal studies on imatinib, a tyrosine kinase inhibitor (TKI), have shown conflicting results, with some demonstrating normal spermatogenesis, and others demonstrating decreased litter sizes and elevated gonadotropins (Nurmio et al., 2008; Schultheis et al., 2012). A study of 13 peri-pubertal boys taking imatinib long-term for chronic myeloid leukemia found no alternations in inhibin B or serum testosterone levels after treatment (Tauer et al., 2014). Limited data suggest that spermatogenesis is maintained with imatinib, although gynecomastia may be seen in up to 20% of men and seems to be associated with Leydig cell dysfunction (which is seen with most TKIs (Meistrich, 2013)) (Gambacorti-Passerini et al., 2003). Once treatment is completed, men treated with imatinib seem to produce normal pregnancies and offspring (Ault et al., 2006). One mouse study found that both imatinib and sorafenib adversely affected sperm count and motility, in a reversible fashion (Shetty and Bairy, 2015). Sunitinib treatment in rats was found to have no effect on copulation, fertility, conception indices, sperm concentration, motility or morphology (Coburn et al., 2012).

Transplant Medications

The effect of transplant agents on the male reproductive system is extremely limited and controversial. A survey on the fertility of 164 male renal transplant recipients who underwent a long-term treatment with cyclosporine A (1.2–3 mg/kg/d), azathioprine (50–75 mg/d) and prednisone (5–10 mg/d) found that long-term treatment with small-dose immunosuppression had no obvious effect on fertility (Xu et al., 2009). Semen parameters were not investigated. A different group looked at prednisone at varying doses (5, 12.5 or 25 mg daily for one month) to determine if treatment improved seminal parameters in infertile oligozoospermic patients with signs of accessory gland inflammation on genital ultrasound. At all doses there was an increase in sperm counts (Milardi et al., 2017). Furthermore, corticosteroids have been used in the treatment of anti-sperm antibodies for many years, and do not seem to be harmful to semen parameters (Ulcova-Gallova et al., 2000).

In rats, cyclosporine and sirolimus at post renal transplant doses have been found to significantly affect testicular development, the HPG axis, and result in profound testicular histological changes under light and electron microscopic examinations (Chen et al., 2013). Reduced total sperm counts, motility, and normal morphology were also seen (Chen et al., 2013). Tacrolimus only resulted in a mild reduction in sperm counts, without histological evidence of testicular injury (Chen et al., 2013). Conversely, a different rat study found that tacrolimus induced adverse histopathological changes in the seminiferous tubules, resulting in spermatogenic damage and reduction in the number of Sertoli cells (Caneguim et al., 2009).

There have been rare human studies performed. In a single case study, a male of reproductive age was initially placed on sirolimus and oligospermia resulted. When his immunosuppression was changed to tacrolimus, spermatogenesis recovered (Skrzypek and Krause, 2007). A larger study looked at the ejaculates of 37 kidney transplant recipients treated with either: FK506 (tacrolimus), mycophenolate mofetil and prednisone; or cyclosporine, azathioprine and prednisone (Cao et al., 2006). There were no changes in viability and velocity of average path between groups (Cao et al., 2006). Sperm morphology and curve line velocity in the FK506 group were higher and lower, respectively, than those in the cyclosporine group; These data suggest that FK506 may be less gonadotoxic than cyclosporine (Cao et al., 2006).

Conclusions

For an alarming number of medications there are limited human data, which may make counseling patients difficult. While many medications may affect male fertility by altering sexual performance, those that cause alterations in semen parameters and possible adverse outcomes in the offspring are more concerning. Couples should be counseled of these limitations and risks, and the appropriate precautions taken if possible based on this very limited data.

References

- (1997) Reproductive toxicology. Acetaminophen. *Environmental Health Perspectives*, 105(Suppl 1)(1997), 267–268.
- (2015) Semen abnormalities with SSRI antidepressants. *Prescribe International*, 24(2015), 16–17.
- Aaberg, R. A., Sauer, M. V., Sikka, S., & Rajfer, J. (1989). Effects of extracellular ionized calcium, diltiazem and cAMP on motility of human spermatozoa. *The Journal of Urology*, 141, 1221–1224.
- Aksu, E. H., Ozkaraca, M., Kandemir, F. M., Omur, A. D., Eldutar, E., Kucukler, S., & Comakli, S. (2016). Mitigation of paracetamol-induced reproductive damage by chrysin in male rats via reducing oxidative stress. *Andrologia*, 48, 1145–1154.
- Albert, P. S., Salerno, R. G., Kapoor, S. N., & Davis, J. E. (1975). The nitrofurans as sperm-immobilizing agents, their tissue toxicity, and their clinical application in vasectomy. *Fertility and Sterility*, 26, 485–491.
- Amory, J. K., Wang, C., Swerdloff, R. S., Anawalt, B. D., Matsumoto, A. M., Bremner, W. J., Walker, S. E., Haberer, L. J., & Clark, R. V. (2007). The effect of 5alpha-reductase inhibition with dutasteride and finasteride on semen parameters and serum hormones in healthy men. *The Journal of Clinical Endocrinology and Metabolism*, 92, 1659–1665.
- Anderson, B. J., Holford, N. H., Woollard, G. A., & Chan, P. L. (1998). Paracetamol plasma and cerebrospinal fluid pharmacokinetics in children. *British Journal of Clinical Pharmacology*, 46, 237–243.

- Antohi, E., Gales, C., & Nechifor, M. (2011). Pharmacological agents that affect sperm motility. *Revista Medico-Chirurgicală a Societății de Medici și Naturaliști din Iași*, 115, 1183–1188.
- Ault, P., Kantarjian, H., O'Brien, S., Faderl, S., Beran, M., Rios, M. B., Koller, C., Giles, F., Keating, M., Talpaz, M., & Cortes, J. (2006). Pregnancy among patients with chronic myeloid leukemia treated with imatinib. *Journal of Clinical Oncology*, 24, 1204–1208.
- Aversa, A., Mazzilli, F., Rossi, T., Delfino, M., Isidori, A. M., & Fabbri, A. (2000). Effects of sildenafil (Viagra) administration on seminal parameters and post-ejaculatory refractory time in normal males. *Human Reproduction*, 15, 131–134.
- Baker, H. W., Straffon, W. G., McGowan, M. P., Burger, H. G., De Kretser, D. M., & Hudson, B. (1984). A controlled trial of the use of erythromycin for men with asthenospermia. *International Journal of Andrology*, 7, 383–388.
- Boekelheide, K. (2005). Mechanisms of toxic damage to spermatogenesis. *Journal of the National Cancer Institute. Monographs*, 6–8.
- Brydoy, M., Fossa, S. D., Klepp, O., Bremnes, R. M., Wist, E. A., Bjoro, T., Wentzel-Larsen, T., Dahl, O., & for the Norwegian Urology Cancer Group (NUCG) III Study Group. (2012). Sperm counts and endocrinological markers of spermatogenesis in long-term survivors of testicular cancer. *British Journal of Cancer*, 107, 1833–1839.
- Bujan, L., Walschaerts, M., Brugnon, F., Daudin, M., Berthaut, I., Auger, J., Saias, J., Szerman, E., Moirand, N., Rives, N., & Hennebicq, S. (2014). Impact of lymphoma treatments on spermatogenesis and sperm deoxyribonucleic acid: A multicenter prospective study from the CECOS network. *Fertility and Sterility*, 102(667–674), e3.
- Burger, M., Sikka, S. C., Bivalacqua, T. J., Lamb, D. J., & Hellstrom, W. J. (2000). The effect of sildenafil on human sperm motion and function from normal and infertile men. *International Journal of Impotence Research*, 12, 229–234.
- Byrnes, C. A., Morton, A. S., Liss, C. L., Lippert, M. C., & Gillenwater, J. Y. (1995). Efficacy, tolerability, and effect on health-related quality of life of finasteride versus placebo in men with symptomatic benign prostatic hyperplasia: A community based study. CUSP Investigators. Community based study of Proscar. *Clinical Therapeutics*, 17, 956–969.
- Camino-Torres, R., Ma, L., & Snyder, P. J. (1977). Gynecomastia and semen abnormalities induced by spironolactone in normal men. *The Journal of Clinical Endocrinology and Metabolism*, 45, 255–260.
- Caneguiam, B. H., Cerri, P. S., Spolidorio, L. C., Miraglia, S. M., & Sasso-Cerri, E. (2009). Structural alterations in the seminiferous tubules of rats treated with immunosuppressor tacrolimus. *Reproductive Biology and Endocrinology*, 7, 19.
- Cao, Z. G., Liu, J. H., Zhu, Y. P., Zhou, S. W., Qi, L., Dong, X. C., Wu, B., & Lin, Z. B. (2006). Effects of different immunodepressants on the sperm parameters of kidney transplant recipients. *Zhonghua Nan Ke Xue*, 12, 405–407.
- Chen, Y., Li, H., Dong, Q., & Wang, K. J. (2009). Blockade of alpha 1A-adrenoceptor: A novel possible strategy for male contraception. *Medical Hypotheses*, 73, 140–141.
- Chen, Y., Zhang, Z., Lin, Y., Lin, H., Li, M., Nie, P., Chen, L., Qiu, J., Lu, Y., Chen, L., Xu, B., Lin, W., Zhang, J., Du, H., Liang, J., & Zhang, Z. (2013). Long-term impact of immunosuppressants at therapeutic doses on male reproductive system in unilateral nephrectomized rats: A comparative study. *BioMed Research International*, 2013, 690382.
- Clark, R. V., Hermann, D. J., Cunningham, G. R., Wilson, T. H., Morrill, B. B., & Hobbs, S. (2004). Marked suppression of dihydrotestosterone in men with benign prostatic hyperplasia by dutasteride, a dual 5alpha-reductase inhibitor. *The Journal of Clinical Endocrinology and Metabolism*, 89, 2179–2184.
- Coburn, A. M., Cappon, G. D., Bowman, C. J., Stedman, D. B., & Patyna, S. (2012). Reproductive toxicity assessment of sunitinib, a multitargeted receptor tyrosine kinase inhibitor, in male and female rats. *Birth Defects Research. Part B, Developmental and Reproductive Toxicology*, 95, 267–275.
- Corvasce, A., Albino, G., Leonetti, T., Buonomo, A. F., & Marucco, E. C. (2015). Once-a-day Tadalafil administration improves the spermogram parameters in fertile patients. *Archivio Italiano di Urologia, Andrologia*, 87, 210–213.
- Cramer, D. W., Harlow, B. L., Titus-Ernstoff, L., Bohlke, K., Welch, W. R., & Greenberg, E. R. (1998). Over-the-counter analgesics and risk of ovarian cancer. *Lancet*, 351, 104–107.
- da Cunha, M. F., Meistrich, M. L., Fuller, L. M., Cundiff, J. H., Hagemester, F. B., Velasquez, W. S., McLaughlin, P., Riggs, S. A., Cabanillas, F. F., & Salvador, P. G. (1984). Recovery of spermatogenesis after treatment for Hodgkin's disease: Limiting dose of MOPP chemotherapy. *Journal of Clinical Oncology*, 2, 571–577.
- De Mas, P., Daudin, M., Vincent, M. C., Bourrouillou, G., Calvas, P., Mieusset, R., & Bujan, L. (2001). Increased aneuploidy in spermatozoa from testicular tumour patients after chemotherapy with cisplatin, etoposide and bleomycin. *Human Reproduction*, 16, 1204–1208.
- Debruyne, F., Barkin, J., van Erps, P., Reis, M., Tammela, T. L., Roehrborn, C., Aria, A., & ARIA3001, ARIA3002 and ARIA3003 Study Investigators. (2004). Efficacy and safety of long-term treatment with the dual 5 alpha-reductase inhibitor dutasteride in men with symptomatic benign prostatic hyperplasia. *European Urology*, 46, 488–494. discussion 495.
- du Plessis, S. S., de Jongh, P. S., & Franken, D. R. (2004). Effect of acute in vivo sildenafil citrate and in vitro 8-bromo-cGMP treatments on semen parameters and sperm function. *Fertility and Sterility*, 81, 1026–1033.
- Eghball, H., & Papaxanthos-Roche, A. (2010). The impact of lymphoma and treatment on male fertility. *Expert Review of Hematology*, 3, 775–788.
- Eid, A. H., Abdelkader, N. F., Abd El-Raouf, O. M., Fawzy, H. M., & El-Denshary, E. S. (2016). Carvedilol alleviates testicular and spermatological damage induced by cisplatin in rats via modulation of oxidative stress and inflammation. *Archives of Pharmacological Research*, 39, 1693–1702.
- el-Sayed, M. G., el-Sayed, M. T., Elazab Abd el, S., Hafeiz, M. H., el-Komy, A. A., & Hassan, E. (1998). Effects of some beta-adrenergic blockers on male fertility parameters in rats. *Deutsche Tierärztliche Wochenschrift*, 105, 10–12.
- ElMazoudy, R., AbdelHameed, N., & ElMasry, A. (2015). Paternal dapoxetine administration induced deterioration in reproductive performance, fetal outcome, sexual behavior and biochemistry of male rats. *International Journal of Impotence Research*, 27, 206–214.
- Fung, C., & Vaughn, D. J. (2011). Complications associated with chemotherapy in testicular cancer management. *Nature Reviews. Urology*, 8, 213–222.
- Gacci, M., Ficarra, V., Sebastianelli, A., Corona, G., Serni, S., Shariat, S. F., Maggi, M., Zattoni, F., Carini, M., & Novara, G. (2014). Impact of medical treatments for male lower urinary tract symptoms due to benign prostatic hyperplasia on ejaculatory function: A systematic review and meta-analysis. *The Journal of Sexual Medicine*, 11, 1554–1566.
- Galal, A. A., Alam, R. T., & Abd El-Aziz, R. M. (2016). Adverse effects of long-term administration of fluvoxamine on haematology, blood biochemistry and fertility in male albino rats: A possible effect of cessation. *Andrologia*, 48, 914–922.
- Gambacorti-Passerini, C., Tornaghi, L., Cavnaghi, F., Rossi, P., Pecori-Giraldi, F., Mariani, L., Cambiaghi, N., Pogliani, E., Corneo, G., & Gnassi, L. (2003). Gynaecomastia in men with chronic myeloid leukaemia after imatinib. *Lancet*, 361, 1954–1956.
- Genesca, A., Miro, R., Caballin, M. R., Benet, J., Germa, J. R., & Egozcue, J. (1990). Sperm chromosome studies in individuals treated for testicular cancer. *Human Reproduction*, 5, 286–290.
- Gianzo, M., Munoa-Hoyos, I., Urizar-Arenaza, I., Larreategui, Z., Quintana, F., Garrido, N., Subiran, N., & Irazusta, J. (2016). Angiotensin II type 2 receptor is expressed in human sperm cells and is involved in sperm motility. *Fertility and Sterility*, 105, 608–616.
- Gonzalo, I. T., Swerdloff, R. S., Nelson, A. L., Clevenger, B., Garcia, R., Berman, N., & Wang, C. (2002). Levonorgestrel implants (Norplant II) for male contraception clinical trials: Combination with transdermal and injectable testosterone. *The Journal of Clinical Endocrinology and Metabolism*, 87, 3562–3572.
- Gur, S., Kadowitz, P. J., & Hellstrom, W. J. (2013). Effects of 5-alpha reductase inhibitors on erectile function, sexual desire and ejaculation. *Expert Opinion on Drug Safety*, 12, 81–90.
- Hamada, A., Agarwal, A., Sharma, R., French, D. B., Ragheb, A., & Sabanegh, E. S., Jr. (2011). Empirical treatment of low-level leukocytospermia with doxycycline in male infertility patients. *Urology*, 78, 1320–1325.
- Hargreaves, C. A., Rogers, S., Hills, F., Rahman, F., Howell, R. J., & Homa, S. T. (1998). Effects of co-trimoxazole, erythromycin, amoxicillin, tetracycline and chloroquine on sperm function in vitro. *Human Reproduction*, 13, 1878–1886.
- Hellstrom, W. J., Overstreet, J. W., Yu, A., Saikali, K., Shen, W., Beasley, C. M., Jr., & Watkins, V. S. (2003). Tadalafil has no detrimental effect on human spermatogenesis or reproductive hormones. *The Journal of Urology*, 170, 887–891.
- Hellstrom, W. J., & Sikka, S. C. (2006). Effects of acute treatment with tamsulosin versus alfuzosin on ejaculatory function in normal volunteers. *The Journal of Urology*, 176, 1529–1533.

- Hellstrom, W. J., & Sikka, S. C. (2009). Effects of alfuzosin and tamsulosin on sperm parameters in healthy men: Results of a short-term, randomized, double-blind, placebo-controlled, crossover study. *Journal of Andrology*, 30, 469–474.
- Hendrick, V., Gitlin, M., Altschuler, L., & Korenman, S. (2000). Antidepressant medications, mood and male fertility. *Psychoneuroendocrinology*, 25, 37–51.
- Howell, S. J., & Shalet, S. M. (2001). Testicular function following chemotherapy. *Human Reproduction Update*, 7, 363–369.
- Huddart, R. A., Norman, A., Moynihan, C., Horwich, A., Parker, C., Nicholls, E., & Dearnaley, D. P. (2005). Fertility, gonadal and sexual function in survivors of testicular cancer. *British Journal of Cancer*, 93, 200–207.
- Irsy, G., Kovacs, Z., & Rozsahegyi, J. (1991). Gonadotropin release in infertile men following ketoconazole administration. *International Journal of Clinical Pharmacology, Therapy, and Toxicology*, 29, 67–70.
- Irwig, M. S., & Kolukula, S. (2011). Persistent sexual side effects of finasteride for male pattern hair loss. *The Journal of Sexual Medicine*, 8, 1747–1753.
- Jannini, E. A., Lombardo, F., Salacone, P., Gandini, L., & Lenzi, A. (2004). Treatment of sexual dysfunctions secondary to male infertility with sildenafil citrate. *Fertility and Sterility*, 81, 705–707.
- Jarvi, K., Dula, E., Dreihobl, M., Pryor, J., Shapiro, J., & Seger, M. (2008). Daily vardenafil for 6 months has no detrimental effects on semen characteristics or reproductive hormones in men with normal baseline levels. *The Journal of Urology*, 179, 1060–1065.
- Kaplan, S. A. (2009). Side effects of alpha-blocker use: Retrograde ejaculation. *Revista de Urologia*, 11, S14–8.
- Karaguzel, E., Kutlu, O., Yulug, E., Mungan, S., Kazaz, I. O., Tok, D. S., & Ozgur, G. K. (2012). Comparison of the protective effect of dipyridamole and acetylsalicylic acid on long-term histologic damage in a rat model of testicular ischemia-reperfusion injury. *Journal of Pediatric Surgery*, 47, 1716–1723.
- Karila, T., Hovatta, O., & Seppala, T. (2004). Concomitant abuse of anabolic androgenic steroids and human chorionic gonadotrophin impairs spermatogenesis in power athletes. *International Journal of Sports Medicine*, 25, 257–263.
- Katsoff, D., & Check, J. H. (1997). A challenge to the concept that the use of calcium channel blockers causes reversible male infertility. *Human Reproduction*, 12, 1480–1482.
- Kaufman, D. W., Kelly, J. P., Rosenberg, L., Anderson, T. E., & Mitchell, A. A. (2002). Recent patterns of medication use in the ambulatory adult population of the United States: The Slone survey. *JAMA*, 287, 337–344.
- Kaufman, K. D., Olsen, E. A., Whiting, D., Savin, R., Devillez, R., Bergfeld, W., Price, V. H., Van Neste, D., Roberts, J. L., Hordinsky, M., Shapiro, J., Binkowitz, B., & Gormley, G. J. (1998). Finasteride in the treatment of men with androgenetic alopecia. Finasteride male pattern hair loss study group. *Journal of the American Academy of Dermatology*, 39, 578–589.
- Khaki, A. (2015). Assessment on the adverse effects of aminoglycosides and Flouroquinolone on sperm parameters and male reproductive tissue: A systematic review. *Iranian Journal of Reproductive Medicine*, 13, 125–134.
- Khaki, A., Novin, M. G., Khaki, A. A., Nouri, M., Sanati, E., & Nikmanesh, M. (2008). Comparative study of the effects of gentamicin, neomycin, streptomycin and ofloxacin antibiotics on sperm parameters and testis apoptosis in rats. *Pakistan Journal of Biological Sciences*, 11, 1683–1689.
- Kim, E. D., Crosnoe, L., Bar-Chama, N., Khera, M., & Lipshultz, L. I. (2013). The treatment of hypogonadism in men of reproductive age. *Fertility and Sterility*, 99, 718–724.
- Ko, D. T., Hebert, P. R., Coffey, C. S., Sedrakyan, A., Curtis, J. P., & Krumholz, H. M. (2002). Beta-blocker therapy and symptoms of depression, fatigue, and sexual dysfunction. *JAMA*, 288, 351–357.
- Ko, E. Y., Siddiqi, K., Brannigan, R. E., & Sabanegh, E. S., Jr. (2012). Empirical medical therapy for idiopathic male infertility: A survey of the American Urological Association. *The Journal of Urology*, 187, 973–978.
- Kondoh, G., Tojo, H., Nakatani, Y., Komazawa, N., Murata, C., Yamagata, K., Maeda, Y., Kinoshita, T., Okabe, M., Taguchi, R., & Takeda, J. (2005). Angiotensin-converting enzyme is a GPI-anchored protein releasing factor crucial for fertilization. *Nature Medicine*, 11, 160–166.
- Kumar, V. S., Sharma, V. L., Tiwari, P., Singh, D., Maikhuri, J. P., Gupta, G., & Singh, M. M. (2006). The spermicidal and antitrichomonas activities of SSRI antidepressants. *Bioorganic & Medicinal Chemistry Letters*, 16, 2509–2512.
- Lefevre, L., De Lamirande, E., & Gagnon, C. (2000). The cyclic GMP-specific phosphodiesterase inhibitor, sildenafil, stimulates human sperm motility and capacitation but not acrosome reaction. *Journal of Andrology*, 21, 929–937.
- Lepor, H., Kazzazi, A., & Djavan, B. (2012). Alpha-blockers for benign prostatic hyperplasia: The new era. *Current Opinion in Urology*, 22, 7–15.
- Levin, R. M., Amsterdam, J. D., Winokur, A., & Wein, A. J. (1981). Effects of psychotropic drugs on human sperm motility. *Fertility and Sterility*, 36, 503–506.
- Li, L. J., Zhang, F. B., Liu, S. Y., Tian, Y. H., Le, F., Wang, L. Y., Lou, H. Y., Xu, X. R., Huang, H. F., & Jin, F. (2014). Human sperm devoid of germinal angiotensin-converting enzyme is responsible for total fertilization failure and lower fertilization rates by conventional in vitro fertilization. *Biology of Reproduction*, 90, 125.
- Liu, P. Y., Swerdloff, R. S., Christenson, P. D., Handelsman, D. J., Wang, C., & Hormonal Male Contraception Summit Group. (2006). Rate, extent, and modifiers of spermatogenic recovery after hormonal male contraception: An integrated analysis. *Lancet*, 367, 1412–1420.
- Mahajan, P., Grech, E. D., Pearson, R. M., Ridgway, E. J., & Turner, P. (1984). Propranolol concentrations in blood serum, seminal plasma and saliva in man after a single oral dose. *British Journal of Clinical Pharmacology*, 18, 849–852.
- Maier, U., & Koinig, G. (1994). Andrological findings in young patients under long-term antidepressive therapy with clomipramine. *Psychopharmacology*, 116, 357–359.
- Martini, A. C., Molina, R. I., Tissera, A. D., Ruiz, R. D., & Fiol De Cuneo, M. (2003). Analysis of semen from patients chronically treated with low or moderate doses of aspirin-like drugs. *Fertility and Sterility*, 80, 221–222.
- McClellan, K. J., & Markham, A. (1999). Finasteride: A review of its use in male pattern hair loss. *Drugs*, 57, 111–126.
- Meistrich, M. L. (2013). Effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertility and Sterility*, 100, 1180–1186.
- Merino, G., & Carranza-Lira, S. (1995). Infection and male infertility: Effect of different antibiotic regimens on semen quality. *Archives of Andrology*, 35, 209–212.
- Milardi, D., Luca, G., Grande, G., Ghezzi, M., Caretta, N., Brusco, G., De Filpo, G., Marana, R., Pontecorvi, A., Calafiore, R., Foresta, C., & Garolla, A. (2017). Prednisone treatment in infertile patients with oligozoospermia and accessory gland inflammatory alterations. *Andrology*, 5, 268–273.
- Mondaini, N., Gontero, P., Giubilei, G., Lombardi, G., Cai, T., Gavazzi, A., & Bartoletti, R. (2007). Finasteride 5 mg and sexual side effects: How many of these are related to a nocebo phenomenon? *The Journal of Sexual Medicine*, 4, 1708–1712.
- Montejo, A. L., Llorca, G., Izquierdo, J. A., & Rico-Villademoros, F. (2001). Incidence of sexual dysfunction associated with antidepressant agents: A prospective multicenter study of 1022 outpatients. Spanish working Group for the Study of psychotropic-related sexual dysfunction. *The Journal of Clinical Psychiatry*, 62(Suppl 3), 10–21.
- Morakinyo, A. O., Iranloye, B. O., Daramola, A. O., & Adegoke, O. A. (2011). Antifertility effect of calcium channel blockers on male rats: Association with oxidative stress. *Advances in Medical Sciences*, 56, 95–105.
- Murdia, A., Mathur, V., Kothari, L. K., & Singh, K. P. (1978). Sulpha-trimethoprim combinations and male fertility. *Lancet*, 2, 375–376.
- Nagata, S., Kurosawa, M., Mima, K., Nambo, Y., Fujii, Y., Watanabe, G., & Taya, K. (1999). Effects of anabolic steroid (19-nortestosterone) on the secretion of testicular hormones in the stallion. *Journal of Reproduction and Fertility*, 115, 373–379.
- Nickel, J. C., Fradet, Y., Boake, R. C., Pommerville, P. J., Perreault, J. P., Afridi, S. K., & Elhilali, M. M. (1996). Efficacy and safety of finasteride therapy for benign prostatic hyperplasia: Results of a 2-year randomized controlled trial (the PROSPECT study). PROscar safety plus efficacy Canadian two year study. *CMAJ*, 155, 1251–1259.
- Nurmio, M., Kallio, J., Toppari, J., & Jahnukainen, K. (2008). Adult reproductive functions after early postnatal inhibition by imatinib of the two receptor tyrosine kinases, c-kit and PDGFR, in the rat testis. *Reproductive Toxicology*, 25, 442–446.
- Nusier, M. K., Bataineh, H. N., & Daradka, H. M. (2007). Adverse effects of propranolol on reproductive function in adult male mice. *Pakistan Journal of Biological Sciences*, 10, 2728–2731.
- Okeahialam, B. N., Amadi, K., & Ameh, A. S. (2006). Effect of lisinopril, an angiotensin converting enzyme (ACE) inhibitor on spermatogenesis in rats. *Archives of Andrology*, 52, 209–213.

- Overstreet, J. W., Fuh, V. L., Gould, J., Howards, S. S., Lieber, M. M., Hellstrom, W., Shapiro, S., Carroll, P., Corfman, R. S., Petrou, S., Lewis, R., Toth, P., Shown, T., Roy, J., Jarow, J. P., Bonilla, J., Jacobsen, C. A., Wang, D. Z., & Kaufman, K. D. (1999). Chronic treatment with finasteride daily does not affect spermatogenesis or semen production in young men. *The Journal of Urology*, 162, 1295–1300.
- Pomara, G., Morelli, G., Canale, D., Turchi, P., Caglieresi, C., Moschini, C., Liguori, G., Selli, C., Macchia, E., Martino, E., & Francesca, F. (2007). Alterations in sperm motility after acute oral administration of sildenafil or tadalafil in young, infertile men. *Fertility and Sterility*, 88, 860–865.
- Pont, J., & Albrecht, W. (1997). Fertility after chemotherapy for testicular germ cell cancer. *Fertility and Sterility*, 68, 1–5.
- Purvis, K., Muirhead, G. J., & Harness, J. A. (2002). The effects of sildenafil on human sperm function in healthy volunteers. *British Journal of Clinical Pharmacology*, 53(Suppl 1), 53S–60S.
- Rahnema, C. D., Lipshultz, L. I., Crosnoe, L. E., Kovac, J. R., & Kim, E. D. (2014). Anabolic steroid-induced hypogonadism: Diagnosis and treatment. *Fertility and Sterility*, 101, 1271–1279.
- Ratnasooriya, W. D., & Jayakody, J. R. (2000). Long-term administration of large doses of paracetamol impairs the reproductive competence of male rats. *Asian Journal of Andrology*, 2, 247–255.
- Rivkees, S. A., & Crawford, J. D. (1988). The relationship of gonadal activity and chemotherapy-induced gonadal damage. *JAMA*, 259, 2123–2125.
- Roehrborn, C. G., Boyle, P., Nickel, J. C., Hoefner, K., Andriole, G., Aria, A., & ARIA3001, ARIA3002 and ARIA3003 Study Investigators. (2002). Efficacy and safety of a dual inhibitor of 5-alpha-reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia. *Urology*, 60, 434–441.
- Roehrborn, C. G., Siami, P., Barkin, J., Damiao, R., Major-Walker, K., Morrill, B., Montorsi, F., & CombAT Study Group. (2008). The effects of dutasteride, tamsulosin and combination therapy on lower urinary tract symptoms in men with benign prostatic hyperplasia and prostatic enlargement: 2-year results from the CombAT study. *The Journal of Urology*, 179, 616–621. discussion 621.
- Roodbari, F., Abedi, N., & Talebi, A. R. (2015). Early and late effects of ibuprofen on mouse sperm parameters, chromatin condensation, and DNA integrity in mice. *Iranian Journal of Reproductive Medicine*, 13, 703–710.
- Safarinejad, M. R. (2008). Sperm DNA damage and semen quality impairment after treatment with selective serotonin reuptake inhibitors detected using semen analysis and sperm chromatin structure assay. *The Journal of Urology*, 180, 2124–2128.
- Saha, L., Bhargava, V. K., Garg, S. K., & Majumdar, S. (2000a). Effect of nimodipine on male reproductive functions in rats. *Indian Journal of Physiology and Pharmacology*, 44, 449–455.
- Saha, L., Garg, S. K., Bhargava, V. K., & Mazumdar, S. (2000b). Role of angiotensin-converting enzyme inhibitor, lisinopril, on spermatozoal functions in rats. *Methods and Findings in Experimental and Clinical Pharmacology*, 22, 159–162.
- Samplaski, M. K., Lo, K., Grober, E., & Jarvi, K. (2013). Finasteride use in the male infertility population: Effects on semen and hormone parameters. *Fertility and Sterility*, 100, 1542–1546.
- Schlegel, P. N., Chang, T. S., & Marshall, F. F. (1991). Antibiotics: Potential hazards to male fertility. *Fertility and Sterility*, 55, 235–242.
- Schultheis, B., Nijmeijer, B. A., Yin, H., Gosden, R. G., & Melo, J. V. (2012). Imatinib mesylate at therapeutic doses has no impact on folliculogenesis or spermatogenesis in a leukaemic mouse model. *Leukemia Research*, 36, 271–274.
- Serretti, A., & Chiesa, A. (2011). Sexual side effects of pharmacological treatment of psychiatric diseases. *Clinical Pharmacology and Therapeutics*, 89, 142–147.
- Shetty, S. D., & Bairy, L. K. (2015). Effect of sorafenib on sperm count and sperm motility in male Swiss albino mice. *Journal of Advanced Pharmaceutical Technology & Research*, 6, 165–169.
- Shibahara, H., Kamata, M., Hu, J., Nakagawa, H., Obara, H., Kondoh, N., Shima, H., & Sato, I. (2001). Activity of testis angiotensin converting enzyme (ACE) in ejaculated human spermatozoa. *International Journal of Andrology*, 24, 295–299.
- Sinclair, M. L., Wang, X. Y., Mattia, M., Conti, M., Buck, J., Wolgemuth, D. J., & Levin, L. R. (2000). Specific expression of soluble adenylyl cyclase in male germ cells. *Molecular Reproduction and Development*, 56, 6–11.
- Skrzypczek, J., & Krause, W. (2007). Azoospermia in a renal transplant recipient during sirolimus (rapamycin) treatment. *Andrologia*, 39, 198–199.
- Stutz, G., Zamudio, J., Santillan, M. E., Vincenti, L., De Cuneo, M. F., & Ruiz, R. D. (2004). The effect of alcohol, tobacco, and aspirin consumption on seminal quality among healthy young men. *Archives of Environmental Health*, 59, 548–552.
- Tanrikut, C., Feldman, A. S., Altemus, M., Paduch, D. A., & Schlegel, P. N. (2010). Adverse effect of paroxetine on sperm. *Fertility and Sterility*, 94, 1021–1026.
- Tauer, J. T., Ulmer, A., Glauche, I., Jung, R., & Suttrop, M. (2014). Long-term imatinib treatment does not cause testicular toxicity in male adolescents with chronic myeloid leukemia and in a juvenile rat model. *Klinische Pädiatrie*, 226, 169–174.
- Thakur, S. C., Thakur, S. C., Chaube, S. K., & Singh, S. P. (2003). Subchronic supplementation of lithium carbonate induces reproductive system toxicity in male rat. *Reproductive Toxicology*, 17, 683–690.
- Timmermans, L. (1974). Influence of antibiotics on spermatogenesis. *The Journal of Urology*, 112, 348–349.
- Toghyani, S., Dashti, G. R., Roudbari, N. H., Rouzbehani, S., & Monajemi, R. (2013). Lithium carbonate inducing disorders in three parameters of rat sperm. *Advanced Biomedical Research*, 2, 55.
- Traish, A. M., Hassani, J., Guay, A. T., Zitzmann, M., & Hansen, M. L. (2011). Adverse side effects of 5alpha-reductase inhibitors therapy: Persistent diminished libido and erectile dysfunction and depression in a subset of patients. *The Journal of Sexual Medicine*, 8, 872–884.
- Tu, H. Y., & Zini, A. (2011). Finasteride-induced secondary infertility associated with sperm DNA damage. *Fertility and Sterility*, 95(2125), e13–4.
- Ulcova-Gallova, Z., Bouse, V., Rokyta, Z., & Krizanovska, K. (2000). Effect of corticosteroids on sperm antibody concentration in different biological fluids and on pregnancy outcome in immunologic infertility. *Zentralblatt für Gynäkologie*, 122, 495–499.
- Uzun, B., Atli, O., Perk, B. O., Burukoglu, D., & Ilgin, S. (2015). Evaluation of the reproductive toxicity of naproxen sodium and meloxicam in male rats. *Human & Experimental Toxicology*, 34, 415–429.
- Vargas, A., Bustos-Obregon, E., & Hartley, R. (2011). Effects of hypoxia on epididymal sperm parameters and protective role of ibuprofen and melatonin. *Biological Research*, 44, 161–167.
- Vickery, B. H., Burns, J., Zaneveld, L. J., Goodpasture, J. C., & Bergstrom, K. (1985). Orally administered ketoconazole rapidly appears in seminal plasma and suppresses sperm motility. *Advances in Contraception*, 1, 341–353.
- Vyas, A., Ram, H., Purohit, A., & Jatwa, R. (2016). Adverse effects of subchronic dose of aspirin on reproductive profile of male rats. *Journal of Pharmaceutics (Cairo)*, 2016, 6585430.
- Wallace, W. H., Anderson, R. A., & Irvine, D. S. (2005). Fertility preservation for young patients with cancer: Who is at risk and what can be offered? *The Lancet Oncology*, 6, 209–218.
- Wendel, A., Feuerstein, S., & Konz, K. H. (1979). Acute paracetamol intoxication of starved mice leads to lipid peroxidation in vivo. *Biochemical Pharmacology*, 28, 2051–2055.
- Wessells, H., Roy, J., Bannow, J., Grayhack, J., Matsumoto, A. M., Tenover, L., Herlihy, R., Fitch, W., Labasky, R., Auerbach, S., Parra, R., Rajfer, J., Culbertson, J., Lee, M., Bach, M. A., Waldstreicher, J., & PLESS Study Group. (2003). Incidence and severity of sexual adverse experiences in finasteride and placebo-treated men with benign prostatic hyperplasia. *Urology*, 61, 579–584.
- White, D. R., Clarkson, J. S., Ratnasooriya, W. D., & Aitken, R. J. (1995). Complementary effects of propranolol and nonoxynol-9 upon human sperm motility. *Contraception*, 52, 241–247.
- Wood, B. L., Doncel, G. F., Reddy, P. R., & Sokal, D. C. (2003). Effect of diltiazem and methylene blue on human sperm motility, viability and cervical mucus penetration: Potential use as vas irrigants at the time of vasectomy. *Contraception*, 67, 241–245.

- Xu, L., Han, S., Liu, Y., Wang, H., Yang, Y., Qiu, F., Peng, W., Tang, L., Fu, J., Zhu, X. F., Ding, X., & Zhu, Y. (2009). The influence of immunosuppressants on the fertility of males who undergo renal transplantation and on the immune function of their offspring. *Transplant Immunology*, 22, 28–31.
- Yang, Y., Ma, Y., Yang, H., Jin, Y., Hu, K., Wang, H. X., Wang, Y. X., Huang, Y. R., & Chen, B. (2014). Effect of acute tadalafil on sperm motility and acrosome reaction: In vitro and in vivo studies. *Andrologia*, 46, 417–422.
- Yano, C. L., & Dolder, H. (2002). Rat testicular structure and ultrastructure after paracetamol treatment. *Contraception*, 66, 463–467.
- Zalata, A. A., Morsy, H. K., Badawy Ael, N., Elhanbly, S., & Mostafa, T. (2012). ACE gene insertion/deletion polymorphism seminal associations in infertile men. *The Journal of Urology*, 187, 1776–1780.
- Zarnescu, O., & Zamfirescu, G. (2006). Effects of lithium carbonate on rat seminiferous tubules: An ultrastructural study. *International Journal of Andrology*, 29, 576–582.

Imaging in Male Reproductive Medicine

Jared M Bieniek, Hartford Hospital, Hartford, CT, United States
Kirk C Lo, University of Toronto, Toronto, ON, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Gone are the mystical days of four bodily humors, now replaced with imaging techniques that allow us to peer into every microscopic crevice of the human body. Beginning in the 1890s, Wilhelm Röntgen and others began to systematically study the emission of “invisible rays” from cathode tubes, creating the first X-ray images. Ultrasound (US) waves were sent out and received by a transducer to create medical images beginning in the 1940s, revolutionizing many specialties. The Japanese duo Takahashi and Ouchi reported the initial urologic application of US in 1963 with transrectal imaging of the prostate (Takahashi and Ouchi, 1963). By capturing the Doppler effects of US waves, clinicians were further able to place a “stethoscope” on the testicles by 1976, evaluating for blood flow problems including testicular torsion (Watanabe et al., 1974).

Imaging modalities are particularly useful in medical conditions that may be difficult to diagnose or treat such as male infertility. Fecundity involves the complex interplay of various physiologic pathways of both male and female partners. The inability to conceive after 12 months of trying (or 6 months if female age ≥ 35) is not rare, affecting nearly 15% of the reproductive-aged population. Male factors are implicated in half of couples who are struggling to conceive which emphasizes the need for all men in affected couples to undergo their own specialized evaluation.

Initial male evaluation consists of a thorough medical history including sexual/reproductive history and physical examination. Examination by a male infertility specialist is important for evaluation of testicular size, consistency, and masses in addition to abnormalities of the sperm transport system, namely the epididymis and vas deferens. Evaluation for testicular varicose veins, also known as a varicocele, should also be performed. Laboratory testing should consist of at least one semen analysis according to the American Urologic Association (AUA) guidelines on male infertility (Jarow et al., 2010). Low or absent sperm concentrations (oligospermia and azospermia, respectively) may indicate an underlying pathology such as varicocele, obstruction of the reproductive tracts, or a testicular tumor.

The lack of robust laboratory diagnostic tools other than a semen analysis creates a void that medical imaging helps to fill. Shortly after the advent of Doppler imaging, urologists began correlating male infertility with imaging results, studying the relationships between US-proven varicoceles and semen parameters. Infertility specialists currently employ a variety of imaging modalities including standard X-ray/fluoroscopy, angiography, computed tomography (CT), US, and magnetic resonance imaging (MRI). These imaging studies aid the diagnosis of the subfertile male patient male by supplementing the clinical evaluation and identifying potentially correctable etiologies. The purpose of this article is to review the current application of medical imaging modalities in male reproductive evaluation and treatment.

Imaging Modalities

X-ray

Standard X-rays and fluoroscopy utilize ionizing radiation to capture static or real-time images, respectively, of internal anatomic structures. Angiography refers to a fluoroscopic technique for evaluation of blood vessels via injection of iodinated contrast agents. X-ray studies specific for male infertility evaluation include vasograms and seminal vesiculograms to rule out pelvic obstruction of the reproductive tract and venography for evaluation and treatment of varicoceles. Each of these studies are used selectively in male infertility evaluation.

Vasograms are most commonly employed during vasal reconstructive procedures to assess for downstream patency of the vas deferens and ejaculatory ducts. To perform a vasogram, a vasotomy is made in the operating room at the time of planned reconstruction and contrast injected antegrade with simultaneous fluoroscopy. Contrast should be visualized flowing through the vas deferens, into the ejaculatory duct, and entering the prostatic urethra. Sites of possible obstruction include the inguinal or pelvic vas deferens (often secondary to prior surgery) or ejaculatory duct. While some contrast may enter the seminal vesicles (SVs) with forceful injection, prominent filling of the SV suggests ejaculatory duct obstruction (EDO). Vasograms are not commonly used in current practice as obstruction can also be assessed with saline injection while assessing for back pressure or by passing the free end of a small monofilament suture until resistance is met. If distal pelvic obstruction is suspected preoperatively, imaging with transrectal ultrasound (TRUS) or MRI may obviate the need for vasography.

Seminal vesiculograms are similarly indicated for evaluation of distal obstruction, specifically at the level of the ejaculatory ducts. The study is performed by first placing a needle in the SV under TRUS guidance before injection of contrast and fluoroscopic evaluation. Useful in confirming the diagnosis of EDO, a seminal vesiculogram can also evaluate real-time treatment efficacy after transurethral resection of the ejaculatory ducts (TURED). Immediately following resection, contrast is injected again into the SV to ensure patency as evidenced by free flow across the resected duct.

Varicoceles represent a common etiology of male subfertility and can be found in approximately 15% of the general male population (Gorelick and Goldstein, 1993). The prevalence rises to 30% in men presenting with primary infertility and up to 80% with

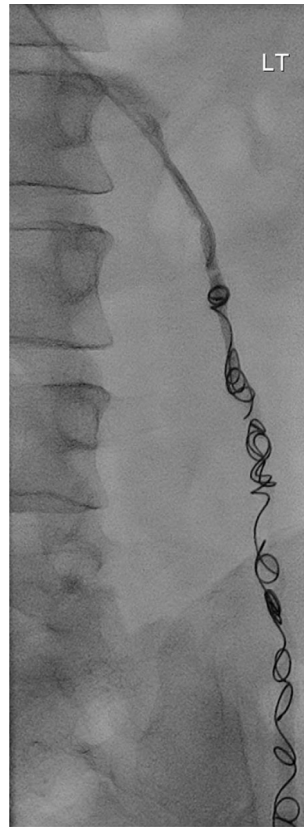


Fig. 1 Endovascular coils in the retroperitoneal left gonadal vein following varicocele embolization, note angiography catheter still present within upper gonadal vein.

secondary infertility. As the testicular veins enter the spermatic cord, they divide into a plexus of veins comprising the pampiniform plexus. These veins dilate excessively with Valsalva or upright positioning, causing retrograde blood flow back to the testis and palpably enlarged veins. Venography is an angiographic study utilizing fluoroscopy to image veins after the injection of contrast with the presence of large refluxing veins being diagnostic of a varicocele. While the most sensitive and specific test for varicocele diagnosis, venography is also the most invasive ([Jurewicz and Gilbert, 2016](#)). It is most useful in complex varicocele recurrences and is often performed in conjunction with angioembolization treatment where endovascular metal coils or sclerosing agents are deployed through percutaneous venous access to embolize, or block, a varicocele ([Fig. 1](#)). Risks to embolization include back pain, vascular injury, and coil migration. There is also a slightly higher risk of varicocele recurrence following embolization as compared with the current gold standard microsurgical subinguinal repair ([Halpern et al., 2016](#)).

Computed Tomography

CT similarly utilizes ionizing radiation but does so as the patient passes through a scanner, capturing X-rays from different angles and reformatting into axial, coronal, and sagittal images. MRI offers superior soft tissue resolution compared to CT, therefore it is not commonly used in male infertility evaluation. CT has a specific role in cross-sectional imaging for cases of nonreducing or solitary right varicoceles to rule retroperitoneal pathology which may be compressing the gonadal vein.

Ultrasound

The most common imaging modality used in the evaluation of male infertility is US owing to its low risk, minimal invasiveness, and overall cost effectiveness. US employs sound waves traveling through tissues at different speeds and reflected by interfaces back to a receiver to create a two-dimensional image. Increasing US frequency improves image resolution while sacrificing the depth of sound wave penetration (thus better for structures closer to the skin). US transducer arrays can be arranged in linear, curved, or other patterns for the desired application and probes built with one or more arrays in appropriate shapes for the anatomy of interest. Specific to male infertility, US can effectively evaluate testicular characteristics, ejaculatory duct patency, prostatic pathology, and penile vascular parameters in cases of erectile dysfunction.

Scrotal ultrasound

Scrotal US includes evaluation of the testes, epididymides, spermatic cord, vessels, and other scrotal structures. During the study, the scrotum is elevated on a towel or blanket and scanned with a high frequency (7–14 MHz) linear array transducer. Vascularity can be assessed with power and color Doppler flow modalities. Color Doppler imaging utilizes the Doppler effect to calculate fluid flow rate (usually blood) and displays flow in two colors, one signifying flow toward the probe and the other away. Power flow uses a single color to demonstrate flow within an organ with a monochromatic gradient to reflect the intensity of the flow rate.

Initial assessment of the testes should include measurements of the dimensions of each testis with total volume estimated using the Lambert formula ($L \times W \times H \times 0.71$). Size is not surprisingly correlated to the level of spermatogenesis since it is estimated that 85% of testis volume is involved in reproduction (Jurewicz and Gilbert, 2016). Normal testicular volume ranges from 15 to 25 cm³ with testicular atrophy diagnosed in many men with oligospermia or azoospermia. The echotexture of testicular parenchyma is normally smooth and homogeneous. Testicular tumors on the other hand appear as a discrete solid mass with heterogeneous features and are often palpable on physical examination. Some tumors may be incidentally discovered at the time of infertility evaluation given the known association with decreased semen parameters.

Microlithiasis refers to the presence of many echogenic foci within the testicular parenchyma creating a classic “starry sky” appearance (Fig. 2). While the etiology is unclear, microlithiasis is more commonly found in cases of male subfertility including testicular atrophy, cryptorchidism, and Klinefelter’s syndrome. Histologic review demonstrates calcification involving the seminiferous tubules which may underlie the relationship with reduced male fertility (Jurewicz and Gilbert, 2016). Concomitant testicular malignancies were noted in historical studies and thus microlithiasis was initially thought to be a risk factor for cancer development. Further research however has demonstrated that there is no evidence that microlithiasis represents a premalignant lesion and continued surveillance is no longer recommended (Jungwirth et al., 2012).

As with renal imaging, the increased availability of scrotal US has resulted in a greater frequency of incidental findings such as small testicular masses (STMs). Several series have shown that these subcentimeter hypoechoic lesions (Fig. 3) are typically benign

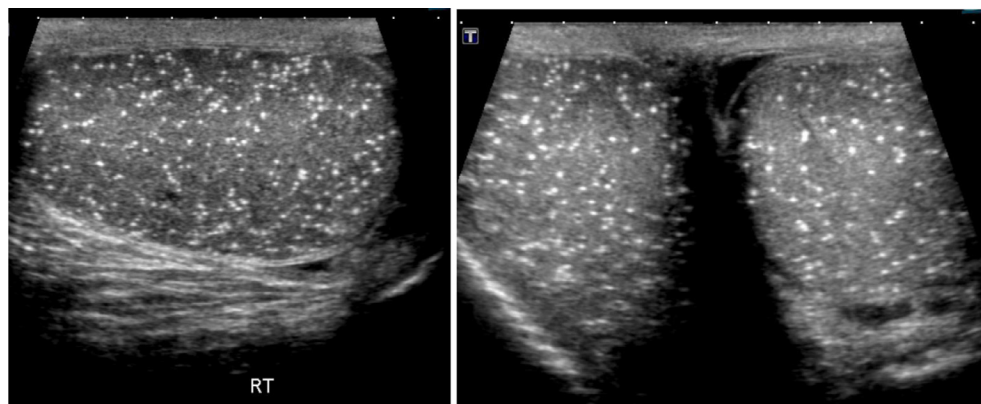


Fig. 2 Testicular microlithiasis present diffusely in bilateral testes on longitudinal (*left*) and transverse ultrasound (US) images (*right*).

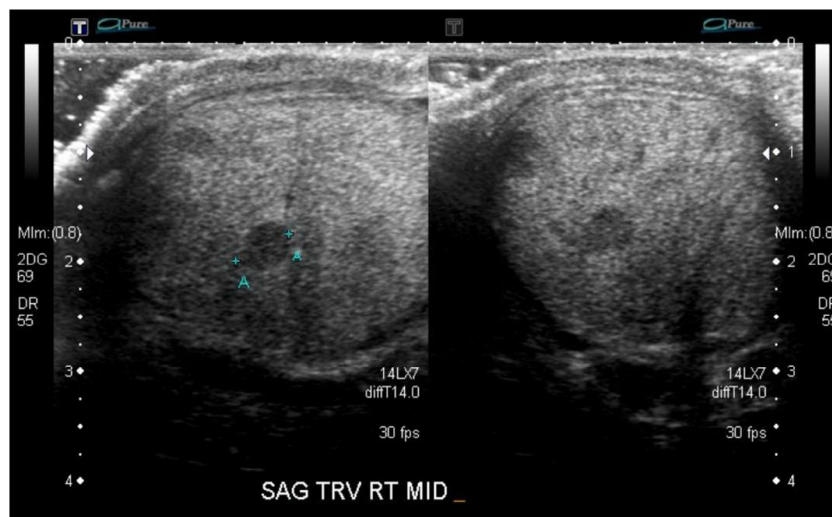


Fig. 3 Incidental intratesticular 5 mm hypoechoic lesion noted on male infertility evaluation with scrotal US.

and can be followed safely with surveillance strategies (Bieniek et al., 2015bb). If removed, many of these lesions prove to be Leydig cell tumors on final pathology, both in infertile and general populations. Maximal lesion size ≥ 5 mm with internal vascularity may suggest increased risk of malignancy. Contrast-enhanced US is a newer technique which uses injection of an air bubble solution with real-time imaging to create a contrast form of US imaging. This modality has a high predictive value for identifying malignancy among STMs, but hyperenhancement was not present in some malignant lesions in one study, suggesting that it is not ready for implementation in testicular imaging yet (Lock et al., 2011). Adrenal rests also present as intratesticular hypoechoic lesions in cases of untreated congenital adrenal hyperplasia (Jurewicz and Gilbert, 2016). US imaging additionally helps with tumor localization (via preoperative needle placement or intraoperative surgical guidance) when considering a partial orchiectomy in cases of STMs, solitary testis, or bilateral lesions. Partial orchiectomy allows for maximal testicular tissue preservation for testosterone production and spermatogenesis.

In assessing the testicles, the goal is not only to determine imaging characteristics but also to assess the underlying function such as spermatogenesis. Localization of focal sperm production may further assist with guidance for testicular sperm extraction in men with nonobstructive azoospermia (NOA). Focal areas of increased blood flow have been postulated to reflect underlying spermatogenesis with one study confirming increased sperm retrieval rates with US-directed biopsies (Nowroozi et al., 2015). Elastography is another novel US modality which evaluates internal tissue elasticity and offers additional diagnostic information. The elasticity of testicular tissue was studied in a sample of azoospermic men and a scoring system created with scores ≥ 3 out of 5 correlating with NOA diagnosis (Li et al., 2012).

The epididymides are bilateral tubular structures running longitudinally along the posterior aspect of the testicles and are involved in sperm transport and maturation. On US, they commonly contain anechoic rounded structures representing epididymal cysts. When these cysts contain sperm, they are referred to as spermatoceles. Solid masses within the epididymides are typically benign adenomatoid tumors though patients with von Hippel-Lindau syndrome classically present with epididymal papillary cystadenomas. Inflammation or infection involving the epididymis, also known as epididymitis, is often heralded by enlargement and increased epididymal vascularity. Though not often found at the time of infertility evaluation, a history of epididymitis may indicate scarring and obstruction contributing to a man's fertility struggles.

The spermatic cord is made up of testicular arteries, veins, vas deferens, and numerous lymphatics and is evaluated as part of a routine scrotal US study. As discussed previously, the internal spermatic veins form the pampiniform plexus within the upper scrotum and extend into the inguinal canal before traveling through the retroperitoneum and inserting into the left renal vein or inferior vena cava on the left and right, respectively. A varicocele is defined as palpably abnormal dilation of the pampiniform plexus (often described as a "bag of worms") present when a patient performs a Valsalva maneuver. Varicoceles are graded clinically as grade 1 which is palpable with Valsalva only, grade 2 which is easily palpable, or grade 3 which is visible before examination.

Present in many men presenting with infertility, varicoceles are typically found on the left side or bilaterally, rarely on the right side only. The difference in laterality is thought to be secondary to the longer length of the left gonadal vein and the angle of insertion into the renal vein creating greater flow resistance. Incompetent or absent venous valves allowing for retrograde flow is also theorized as a possible cause of varicoceles. Varicoceles may negatively impact sperm parameters, cause scrotal pain, or create an unwanted cosmetic appearance. Adolescent varicoceles specifically can retard testicular growth during puberty resulting in hypotrophy. The negative effects on spermatogenesis are thought to be secondary to increased testicular temperature (compromised countercurrent heat exchange), tissue hypoxia, increased reactive oxygen species, and reflux of testicular and adrenal metabolites (World Health Organization, 1992).

The US appearance of varicoceles is characterized by hypoechoic or anechoic tubular structures within the spermatic cord with reversal of flow on color Doppler imaging (Fig. 4). The maximal vein diameter with Valsalva, number of enlarged veins (single or multiple), and presence of retrograde flow with provocative maneuvers should be noted. The cutoff for enlarged vein diameter is somewhat debated but most groups agree that a maximal dimension > 3 mm with Valsalva defines a varicocele (Jungwirth et al., 2012). While clinical varicocele grade has been correlated with degree of spermatogenesis impairment, subclinical varicoceles found on US only are not clinically meaningful. Some data, however, has revealed that among palpable varicoceles a maximal vein size > 3.5 mm may be related to improved postvaricocelectomy outcomes as compared to maximal vein size < 3.5 mm (Bieniek et al., 2015aa). The ultimate role of US in varicocele evaluation remains to be defined but it should be considered for patients with difficult examinations, often secondary to body habitus, or for confirmation of a varicocele before treatment decision making according to the AUA guidelines (Jarow et al., 2010).

Penile Doppler ultrasound

Erectile dysfunction is defined as the persistent inability to achieve or maintain erections for completion of satisfactory sexual activity. ED can be secondary to a variety of etiologies with many cases resulting from vascular impairments such as arterial insufficiency or veno-occlusive dysfunction. Imaging evaluation of ED serves to differentiate vascular from nonvascular etiologies via a penile Doppler US study. The study requires induction of an erection, usually by intracavernosal injection of a vasodilatory medication such as alprostadil, papaverine, phentolamine, or some combination thereof. The cavernosal arteries are scanned at baseline and every 5–10 min until the induction of a fully rigid erection. The peak systolic velocity, end-diastolic velocity, and resistive index of each cavernosal artery should be evaluated. Normal peak systolic velocity should be > 35 cm s⁻¹ while end-diastolic velocity should be < 0 cm s⁻¹ indicating good blood flow into the penis to create an erection and adequate blood trapping to maintain it. Standard grayscale images may be obtained for evaluation of penile plaques, priapism, and other rare conditions such as possible penile fracture.

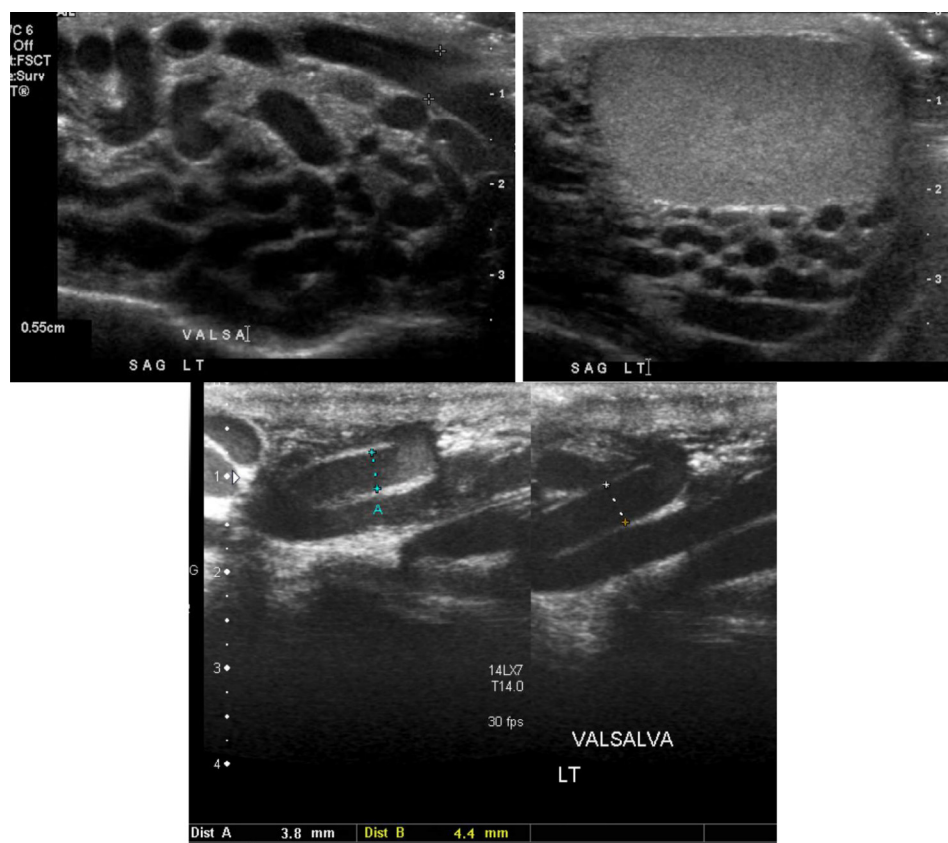


Fig. 4 Prominent left varicocele with numerous dilated veins superior (*top left*) and adjacent (*top right*) to the testis on US with measurement of increased vein diameter with Valsalva (*bottom*).

Transrectal ultrasound

A TRUS involves the placement of a transrectal probe for evaluation of male pelvic anatomy including the prostate, SVs, ampulla of the vas, ejaculatory ducts, and bladder neck. This study is especially useful for men with semen analysis findings suggestive of partial or complete EDO including low ejaculate volume, oligospermia or azospermia, acidic pH, and low or absent fructose levels (Jarow et al., 2010). Before performing a TRUS, the patient is asked to complete an enema to evacuate stool from the rectum to improve image quality. The patient is then positioned in lateral decubitus or lithotomy position and a transrectal probe inserted. Transrectal probes usually contain single or multiple linear or curved arrays in the 6–9 MHz frequency range.

It is important to evaluate for the presence of the SVs since they have a common Wolffian duct embryologic origin as the vas deferens and epididymis. Unilateral or bilateral absence of the vas deferens is typically associated with corollary absence of the SV and ampulla of the vas deferens. Rarely, men with a thin or atretic scrotal vas deferens may have absence of more distal structures due to abnormal embryologic development. Unfortunately there are no reconstructive options to repair congenital absence of the vas deferens but sperm retrieved from the epididymis or testicle can be successfully used with assisted reproductive techniques.

TRUS should evaluate for signs of EDO and possible etiologies including congenital (prostatic or ejaculatory duct cysts) and acquired (infections, stones, postprocedural stenosis) conditions. Routine sonographic evaluation of the prostate should also include review of the peripheral zone for any suspicious hypoechoic lesion suggestive of prostate cancer and any evidence of central zone nodular enlargement corresponding to benign prostatic hyperplasia. Prostatic cysts which may contribute to EDO include utricle and Müllerian duct cysts which are typically found in the midline and ejaculatory duct cysts which are located in a paramedian location. The ejaculatory ducts can be found alongside the verumontanum and should be <2.3 mm in maximal width on US (Smith et al., 2008). Dilation of the ejaculatory ducts, while not diagnostic, suggests an underlying obstruction (Fig. 5).

Obstruction of the ejaculatory ducts additionally results in dilation of the SVs with a transverse measurement >1.5 cm suggestive of obstruction (Smith et al., 2008). In unclear cases, functional tests for EDO such as ductal chromotubation or SV aspiration and vesiculography (discussed above) may be pursued. These tests increase specificity at the cost of increased invasiveness. TRUS-guided needle placement into the SV allows for fluid aspiration and microscopic evaluation for the presence of sperm, not normally found in the SVs. Aspirated sperm can be cryopreserved while planning further treatment choices. Duct chromotubation and seminal vesiculography similarly involve needle placement into the SV and injection of colored dye (diluted methylene blue) or contrast agent

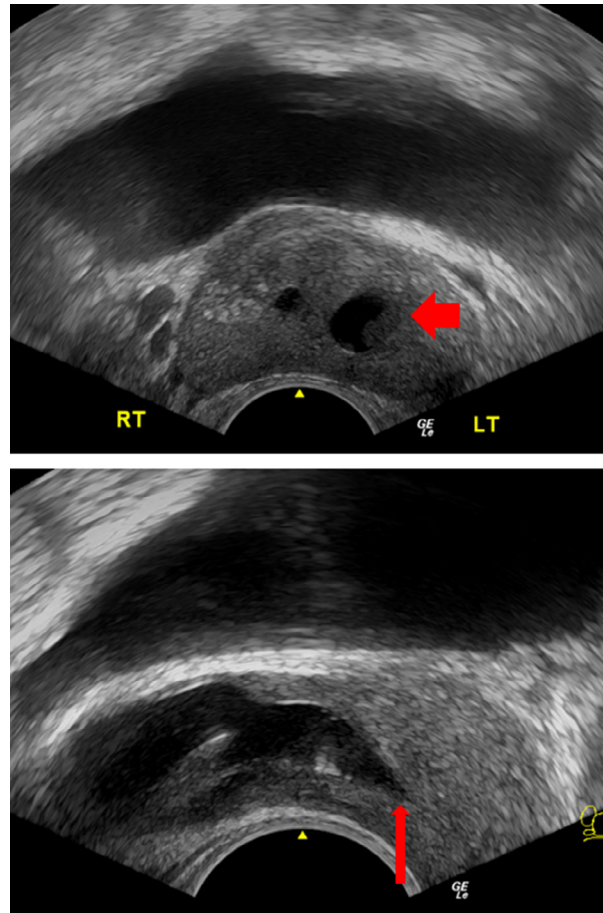


Fig. 5 Isolated left ejaculatory duct obstruction (EDO) confirmed on TRUS in transverse (*top*) and sagittal (*bottom*) images (wide arrow indicates obstructed duct in cross section, thin arrow points to “beaking” of obstructed distal duct).

injection, respectively. Each test can be performed for diagnosis and effective treatment evaluation following transurethral resection. Intraoperative TRUS is likewise suggested for transurethral resection of prostatic cysts to provide real-time guidance for improved safety and efficacy.

Retroperitoneal ultrasound

The retroperitoneal organs, in particular the kidneys, are located deeper within the body compared to the other genitourinary organs and are best evaluated with a lower US frequency setting of 2–5 MHz and a curved array probe. Positioning of the kidneys behind the lower ribs and under the diaphragm requires additional maneuvers for imaging including lateral positioning and breath holding. The AUA recommends evaluation for presence of congenital renal abnormalities in patients with Wolffian duct abnormalities such as congenital unilateral (CUAVD) or bilateral (CBAVD) absence of the vas deferens (Jarow et al., 2010).

During embryologic development, the ureteric bud emanates from the distal aspect of the Wolffian duct and improper regression of these structures can result in abnormalities of both the reproductive tract and ipsilateral kidney. Mutated cystic fibrosis genes can also be associated with congenital absence of the vas deferens but the kidneys are normally present in such cases. Renal agenesis is more commonly found with CUAVD than CBAVD though it occurs in a minority of such cases overall (Schlegel et al., 1996). Due to the possible association however, renal US is recommended for all patients with an absent vas deferens. In addition to renal evaluation, retroperitoneal US can assess for other retroperitoneal masses or lesions that may compress the gonadal vein and result in a varicocele. Imaging with CT or retroperitoneal US should be considered for any nonreducing or solitary right varicoceles.

Magnetic Resonance Imaging

MRI remains the most invasive and expensive imaging choice but offers superior soft tissue contrast, especially for male infertility evaluation involving cranial and pelvic anatomy. MRI works by inducing an oscillatory high strength magnetic field which reorients protons within tissues. As the field is turned off, the excited protons return to their resting state, emitting a radiofrequency signal measured by the receiving coil. These signals can be differentially processed to create standard T1, T2, and other sequence images.

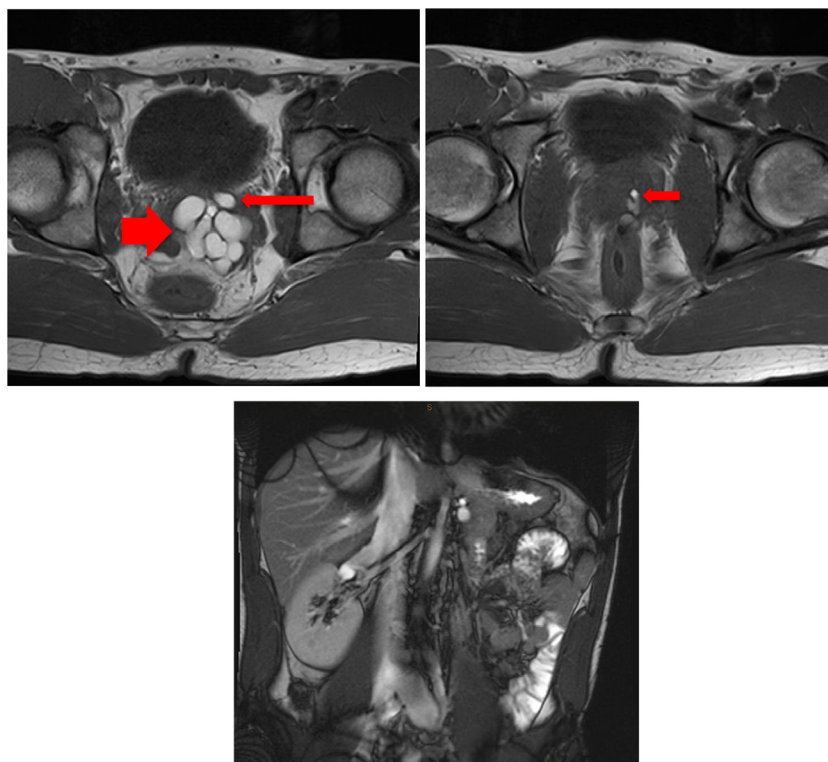


Fig. 6 Pelvic and abdominal MRI images in patient with Zinner syndrome demonstrating left SV cyst (*top left, wide arrow*) with ectopic ureteral remnant (*thin arrow*), dilated left ejaculatory duct (*top right, arrow*), and left renal agenesis (*bottom*).

Cranial MRI is useful in cases of hypogonadism with elevated prolactin to evaluate for pituitary adenomas. An adenoma typically causes significant prolactin elevation, at least multiple times the normal upper reference range value. Hyperprolactinemia inhibits pulsatile GnRH release resulting in decreased gonadotropin levels and downstream testicular signaling for testosterone production and spermatogenesis. Microadenomas may be amenable to medical management but macroadenomas should be referred for appropriate surgical evaluation.

When evaluating pelvic anatomy with MRI, the magnetic field strength is an important consideration. A higher strength magnet such as 3 Tesla should be utilized for improved image resolution. The use of an endorectal coil should be avoided if possible as it can compress and obscure pathologies, not to mention the intrinsic patient discomfort. An MRI study may be particularly useful to rule out an intra-abdominal testis in adult men presenting with an absent testicle and an unclear pediatric surgical history.

Pelvic MRI has been studied for the diagnosis of EDO but rates of detection were found to be similar to that for TRUS ([Engin et al., 2000](#)). Study authors suggested that MRI be reserved for complex or unclear cases such as men with Zinner syndrome. Zinner syndrome is a triad of Wolffian duct anomalies including renal agenesis, ipsilateral EDO, and SV cyst ([Fig. 6](#)) which can lead to subfertility, sexual dysfunction, or pelvic pain. Due to its rarity, Zinner syndrome has only been reported in small series with treatment typically consisting of either TURED or SV cyst excision. MRI can assist with surgical planning in similar challenging cases.

Conclusions

Medical imaging augments our diagnostic abilities for specific etiologies of male infertility incompletely evaluated with basic physical examination and laboratory testing. The most commonly used imaging modality is US, particularly scrotal and transrectal studies, for evaluation of varicoceles and reproductive tract obstruction. Specific attention should be made to rule out congenital anomalies which may affect a man's overall general health or be passed along to offspring. Certain treatment options such as SV aspiration, TURED, or varicocele embolization may also be performed with real-time imaging guidance to improve procedural safety and efficacy.

References

- Bieniek, J. M., Dhanjani, D., Samplaski, M., Grober, E. D., Lo, K. C., & Jarvi, K. A. (2015). In *Testicular vein size as measured by scrotal ultrasonography is independently predictive of varicocelectomy outcomes for men with clinical grade 1 and 2 varicoceles*American Society for Reproductive Medicine Annual Meeting, Baltimore, MD.
- Bieniek, J. M., Juvet, T., Margolis, M., Grober, E. D., Lo, K. C., & Jarvi, K. A. (2015). In *The prevalence and management of small testicular masses incidentally discovered on ultrasound evaluation of male infertility*American Society for Reproductive Medicine Annual Meeting, Baltimore, MD.

- Engin, G., Kadioglu, A., Orhan, I., Akdol, S., & Rozanes, I. (2000). Transrectal US and endorectal MR imaging in partial and complete obstruction of the seminal duct system. A comparative study. *Acta Radiologica*, 41, 288–295.
- Gorelick, J. I., & Goldstein, M. (1993). Loss of fertility in men with varicocele. *Fertility and Sterility*, 59, 613–616.
- Halpern, J., Mittal, S., Pereira, K., Bhatia, S., & Ramasamy, R. (2016). Percutaneous embolization of varicocele: technique, indications, relative contraindications, and complications. *Asian Journal of Andrology*, 18, 234–238.
- Jarow, J., Sigman, M., Kolettis, P. N., Lipshultz, L. R., McClure, D., Nangia, A. K., Naughton, C. K., Prins, G. S., Sandlow, J. I., & Schlegel, P. N. (2010). *The optimal evaluation of the infertile male: AUA best practice statement*. American Urological Association.
- Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., Krausz, C., & European Association of Urology Working Group on Male Infertility. (2012). European Association of Urology guidelines on Male Infertility: The 2012 update. *European Urology*, 62, 324–332.
- Jurewicz, M., & Gilbert, B. R. (2016). Imaging and angiography in male factor infertility. *Fertility and Sterility*, 105, 1432–1442.
- Li, M., Du, J., Wang, Z. Q., & Li, F. H. (2012). The value of sonoelastography scores and the strain ratio in differential diagnosis of azoospermia. *The Journal of Urology*, 188, 1861–1866.
- Lock, G., Schmidt, C., Helmich, F., Stolle, E., & Dieckmann, K. P. (2011). Early experience with contrast-enhanced ultrasound in the diagnosis of testicular masses: a feasibility study. *Urology*, 77, 1049–1053.
- Nowroozi, M. R., Ayati, M., Amini, E., Radkhah, K., Jamshidian, H., Delpazir, A., Ghasemi, F., & Rajabzadeh Kanafi, A. (2015). Assessment of testicular perfusion prior to sperm extraction predicts success rate and decreases the number of required biopsies in patients with non-obstructive azoospermia. *International Urology and Nephrology*, 47, 53–58.
- Schlegel, P. N., Shin, D., & Goldstein, M. (1996). Urogenital anomalies in men with congenital absence of the vas deferens. *The Journal of Urology*, 155, 1644–1648.
- Smith, J. F., Walsh, T. J., & Turek, P. J. (2008). Ejaculatory duct obstruction. *The Urologic Clinics of North America*, 35, 221–227.
- Takahashi, H., & Ouchi, T. (1963). The ultrasonic diagnosis in the field of urology. *Proceeding of Japan Society of Ultrasonic Medical*, 3, 7.
- Watanabe, H., Igari, D., Tanahashi, Y., Harada, K., & Saito, M. (1974). Development and application of new equipment for transrectal ultrasonography. *Journal of Clinical Ultrasound*, 2, 91–98.
- World Health Organization. (1992World). The influence of varicocele on parameters of fertility in a large group of men presenting to infertility clinics. *Fertility and Sterility*, 57, 1289–1293.

Further Reading

- Carmignani, L., Gadda, F., Mancini, M., Gazzano, G., Nerva, F., Rocco, F., & Colpi, G. M. (2004). Detection of testicular ultrasonographic lesions in severe male infertility. *The Journal of Urology*, 172, 1045–1047.
- Jarow, J. P. (1993). Transrectal ultrasonography of infertile men. *Fertility and Sterility*, 60, 1035–1039.
- Kolettis, P. N., & Sandlow, J. I. (2002). Clinical and genetic features of patients with congenital unilateral absence of the vas deferens. *Urology*, 60, 1073–1076.
- Modgil, V., Rai, S., Ralph, D. J., & Muneer, A. (2016). An update on the diagnosis and management of ejaculatory duct obstruction. *Nature Reviews. Urology*, 13, 13–20.
- Purohit, R. S., Wu, D. S., Shinohara, K., & Turek, P. J. (2004). A prospective comparison of 3 diagnostic methods to evaluate ejaculatory duct obstruction. *The Journal of Urology*, 171, 232–235. discussion 235–236.
- Sikka, S. C., Hellstrom, W. J., Brock, G., & Morales, A. M. (2013). Standardization of vascular assessment of erectile dysfunction: standard operating procedures for duplex ultrasound. *The Journal of Sexual Medicine*, 10, 120–129.
- Terris, M. K., & Klaassen, Z. (2013). Office-based ultrasound for the urologist. *The Urologic Clinics of North America*, 40, 637–647.
- Toren, P. J., Roberts, M., Lecker, I., Grober, E. D., Jarvi, K., & Lo, K. C. (2010). Small incidentally discovered testicular masses in infertile men—is active surveillance the new standard of care? *The Journal of Urology*, 183, 1373–1377.

Relevant Websites

- <http://uroweb.org/guideline/male-infertility/>—European Association of Urology guideline “Male Infertility”.
- <http://www.aium.org/resources/guidelines/urology.pdf>—American Institute of Ultrasound in Medicine “Practice Parameter for the Performance of an Ultrasound Examination in Practice of Urology”.
- [http://www.auanet.org/guidelines/male-infertility-optimal-evaluation-\(reviewed-and-validity-confirmed-2011\)](http://www.auanet.org/guidelines/male-infertility-optimal-evaluation-(reviewed-and-validity-confirmed-2011))—American Urologic Association Best Practice Statement for “Optimal Evaluation of the Infertile Male”.

ENVIRONMENT

Overview: Environment and Male Reproductive Medicine

Samuel Ohlander, University of Illinois-Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

The preceding chapters focused on the clinical evaluation of the male, highlighting diagnostic methodology to uncover pathophysiological abnormalities and their contributions to reproductive function. There is a broad range of historical information and examination criteria that a reproductive physician must consider in determining a male's reproductive fitness. We must not only consider the anatomic and physiologic make-up of the couple, but also the influences of their environment.

Human population exists across all cultures and climates, yet there are certainly aspects of regional lifestyle and vocational practices, such as diet, exercise, stress, shift work, and chemicals, that contribute to subfertility and adverse reproductive health. Similarly, the detrimental effects of obesity and malignancy on seminal quality are well established. This section will highlight the impact of industrial, agricultural, and technological evolution. Over the course of the last 40 years, seminal quality has dramatically decreased while the world has evolved. A recent meta-analysis reported a greater than 50% decline in total sperm count in Western men, unselected by fertility since 1973 (Levine et al., 2017). In a sense, we will be discussing the way that human kind has contributed to its own reproductive struggles.

The following chapter will focus on chemical exposures. While a basic understanding that certain chemicals can have an adverse effect on the testicles has existed for hundreds of years, our current knowledge remains limited. It is extremely difficult to control both the environment and the individual, and thus much of our scientific understanding of gonadotoxants is extrapolated from animal studies rather than well-constructed randomized, controlled human trials. Fortunately, male reproductive development and function is similar across species allowing for such inferences.

Increased public interest has stimulated greater investigation into chemical exposures. Researchers have identified directly gonadotoxic factors that impacted spermatogenesis, as well as parental insults that impacted fetal reproductive development in the womb. Colborn's proposed theory of endocrine-disrupting chemicals suggests that parental exposures to endocrine-disrupting chemicals impacts childhood development more than genetics (Colborn et al., 1993).

Yet not every cytotoxant has an adverse effect on reproductive development and function. Fortunately, the male anatomy contains a blood-testis barrier that is highly efficient at protecting testicular germ cells. The chemicals that we will discuss are those with properties allowing them to traverse the blood-testis barrier, or alternative means of reproductive insult, such as disruption of the endocrine axis.

Later chapters will focus on electromagnetic radiation and heat. With technologic evolution, public concern continues to rise over the potential adverse effects of electromagnetic waves from cell phones, computers, and other wireless devices. Due to the limited literature, controversy exists, but some observational studies have demonstrated dose dependent decreases in sperm quantity and quality. Along with the portability of technology has come a change of our workspace. People are no longer tied to electricity and desktops, but rather work from their laps. Given the highly temperature dependent environment of the testicle, much concern surrounds hyperthermia from portable devices.

While the type of the gonadotoxant or insult matters, often the reversibility of such exposures depends on timing, dose, potency, duration, and frequency. Pre-pubertal insults often impact the inherent development of the testes, leading to permanent damage, whereas adult exposures often may be reversed with removal of the insult. Most adult exposures contain a dose, potency, duration, or frequency dependent impact that dictates reversibility. Later chapters will discuss the relevance of exposures in greater depth.

Though not focus within this section, the contributions of lifestyle and personal habits on reproductive health are incorporated in the discussion throughout this volume. Most factors (diet, exercise, stress, smoking, alcohol) influence general health, which can then indirectly impact reproductive health.

Despite our incomplete understanding on much of the exact etiology, there is a general awareness of the potential detrimental health impacts from our ever evolving environment. We must continue to invest in well-designed studies to increase our knowledge, as subfertility and sexual dysfunction correlate to decreased quality of life.

References

- Colborn, T., Vom Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environmental Health Perspectives*, 101, 378–384.
- Levine, H., Jorgensen, N., Martino-Andrade, A., Mendiola, J., Weksler-Derri, D., Mindlis, I., Pinotti, R., & Swan, S. H. (2017). Temporal trends in sperm count: A systematic review and meta-regression analysis. *Human Reproduction Update*, 1–14.

Male Reproductive Toxicants: Electromagnetic Radiation and Heat

Beneranda S Ford-Glanton and David A Melendez, Saint Louis University, Saint Louis, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Human population in today's world lives surrounded by radiofrequency fields (RF) and electromagnetic radiation (EM) fields, transmitting almost all forms of electronic communication and data that humans produce every second. Mobile devices and laptop computers are EMR-emitting devices. The effect of mobile phone emitted radiation and heat on fertility is the subject of recent interest and investigations. Many studies have found a decrease in semen quality which has increased the focus on male reproductive health. Infertility affects approximately 15% of couples of reproductive age, and nearly half of these cases are linked to male fertility (Sharlip et al., 2002). Different harmful environmental influences have led to changes in semen analysis standards by reducing the lower limits of normal ranges, which were declared by the World Health Organization (2010). The possible negative impact of mobile phone radiation on sperm quality has been well established. While no certain conclusions can be drawn from current evidence, a growing number of studies indicate a decrease in male fertility associated with increased cellular phone usage (Agarwal et al., 2011) and laptop computers using Wi-Fi (Avendaño et al., 2012a). Here we review the current evidence regarding the effects of electromagnetic radiation and heat in male fertility.

Electromagnetic Radiation (EMR)

Modern society is ruled by the widespread use of mobile devices and computers and laptops with Wi-Fi function. It is nearly impossible to imagine our world today functioning without the use of these devices. Radiofrequency (RF) fields are part of the electromagnetic spectrum and are generally defined as covering the range of frequencies from 100 kHz to 300 GHz. Mobile phones use radio frequency waves, which are electromagnetic fields and, unlike ionizing radiation (e.g., X-rays and gamma rays) cannot break chemical bonds or cause ionization in the human body and therefore are less harmful. These are also known as forms of nonionizing radiation. However, there is evidence that suggests that sperm exposed to EMR can lose fertilizing abilities. Studies showed that specimens of human sperm exposed to EMR in a controlled setting had a decrease in rapid progressive sperm from 13% to 9%; a decrease in slow progressive sperm from 44% to 34% and an increase in immotile sperm from 36% to 51% (Erogul et al., 2006).

Mobile Phones as Toxicant to Fertility

Electromagnetic radiation emitted by mobile phones is one of the environmental toxicants that is capable of compromising male fertility by inducing a state of oxidative stress in the testes. This means that the ability of the testes to detoxify this type of radiation is impaired and the accumulation of these toxicants can cause cumulative damage. A correlation has been established between mobile phone radiation exposure, DNA-fragmentation level and decreased sperm motility (Gorpinchenko et al., 2014). The exact mechanism of the EMF interactions with our body is still controversial, although a few studies have suggested the involvement of degeneration of fats and the subsequent creation of highly reactive molecules in our body (Watanabe et al., 1997), as well as biochemically induced oxidative stress. Although the radio waves of cellular phones do not have enough energy to cause the ionization of atoms and molecules, the recent concerns over long-term exposure to the EMR emitted by mobile phones and other devices should be taken more seriously given the growing trend toward deterioration of the male germ line (spermatogenesis and sperm maturation) (Aitken and Koopman, 2004). The findings of Kesari et al. (2011) on antioxidants, malondialdehyde, histone kinase, micronuclei, and the sperm cell cycle are clear indications of an infertility pattern that is initiated due to an overproduction of cells that are already damaged. They also concluded that radiofrequency electromagnetic waves from commercially available cell phones may affect the motility and vitality of sperm.

Other studies suggest that people who use their cell phones >4 h a day may have lower sperm count and about 30% lower sperm motility than nonusers (Al-Damegh, 2012). Also, it was demonstrated that the percentage of normal sperm with no structural defects in high-level mobile users was half that of nonusers, and the rates of normal sperm with no defects were decreased with greater levels of cell phone use (Agarwal et al., 2008; Erogul et al., 2006). At the same time, sperm from long-time mobile users may have abnormal characteristics. Specifically, a study by Gutschi T, Mohamad Al-Ali B, Shamloul R, Pummer K, and Trummer H, compared the sperm of mobile users and nonmobile users. It was shown that 68% of the analyzed sperm from cell phone users had pathological morphology, compared to 58% of sperm from nonusers (Gutschi et al., 2011). Many men who talk on a cell phone using a Bluetooth device or other headset keep the phone in a pants pocket or clipped to a holster. This exposes their reproductive organs to cell phone radiation, and several studies have found lower sperm count and/or poorer sperm quality in men who use their phones this way than in those who do not (Kilgallon and Simmons, 2005).

Computers and Laptops: Toxicants to Fertility?

Laptops and computers use Wi-Fi to transmit data every second. These are EMR-emitting devices that have also been studied. Evidence suggests that prolonged use of these devices (> 4 h) can affect sperm motility and disrupt their DNA. [Avendaño et al. \(2012b\)](#) evaluated the effects of laptop computers, connected to local area networks through Wi-Fi, on human sperm. Their results demonstrated a significant decrease in sperm progressive motility and a significantly higher proportion of sperm with DNA fragmentation when samples were incubated for 4 h under the laptop. These differences were seen in comparison with portions of the same semen samples incubated under similar conditions, but outside the proximity of any computer or electronic device. The investigators speculated that the use of a laptop computer wirelessly connected to the internet and positioned near the male reproductive organs may decrease human sperm quality. Therefore, exposing the semen to radio frequency electromagnetic waves from laptops connected to the internet through Wi-Fi could have, basically, the same potential to damage spermatozoa as EMR emitted from a mobile phone.

The Effects of Heat

The testes are located outside the body to keep temperature below the core temperature, and it is well known that internal heating as in fever and external heating for short periods of time may result in a dramatic but temporary decrease of sperm count and other aspects of semen quality after a delay of some 6–8 weeks ([Mieusset and Bujan, 1995](#)). It is well documented that the sedentary work position is associated with an increased scrotal temperature. Men sitting at work for 8 h a day have on average 0.7°C increased scrotal temperature during the day in comparison with employees with < 8 h in the sedentary body position ([Hjollund et al., 2002](#)). Although an increase of scrotal temperature of this order of magnitude might be sufficient to impair the generation of sperm, reduced sperm count seems not related to sedentary work ([Rowley et al., 1974](#)). Likewise, it has been speculated that tight underwear could increase testicular temperature and a few studies have reported reduced sperm count related to the use of tight underwear and trousers, but with nonconclusive evidence.

There is no doubt that heat may affect male reproduction but whether heat exposures in current workplaces are of this magnitude is undetermined. The evidence for an adverse effect on male reproduction of several occupational and environmental exposures and toxicants, such as heat, ionizing radiation, inorganic lead, and welding operations is strongly supported in well-designed epidemiological studies. Occupational exposures have been divided into physical exposures (heat and radiation), chemical exposures (solvents and pesticides), psychological exposures (distress), and exposure to metals and welding.

An occupational exposure limit of 15 mSv per year has been adopted in several countries, and if this limit is not exceeded, testicular effects are unlikely. Millisievert is defined as the average accumulated background radiation dose to an individual for 1 year. Apparent testicular effects of high-frequency EMR (300 kHz–300,000 MHz including radar exposure and microwaves) that have been observed in earlier studies may result from testicular heating. So far, no consistent evidence indicates that nonionizing radiation interferes with male reproductive function unless the amount of energy is sufficient to disrupt testicular temperature regulation ([Jensen et al., 2006](#)).

EMR and Heat Effects: Irreversible?

EMR has a negative effect on testicular function through the creation of reactive molecules, or oxidative stress, and the concomitant disruption of the testicular ability to detoxify those molecules. Interestingly, few studies have evaluated the possible protective role of vitamins C and E in the EMR-induced oxidative stress in the testes, which may involve facilitating the restoration of normal testicular tissue and function ([Gorpinchenko et al., 2014](#)).

We know that testicular tissue is highly sensitive to ionizing radiation. Doses in the range of 0.11–15 Gy may temporarily reduce sperm counts, while 2 Gy may result in long-lasting or permanent absence normal sperm and sterility ([Rowley et al., 1974](#)). At higher doses (> 15 Gy), the Leydig cells, in charge of production and secretion of testosterone, may be affected. In addition, whole body irradiation can damage the regulation of hormones in the Brain, affecting the reproductive capability. However, more studies are necessary to evaluate if the additive effects of EMR-emitting devices and nonionizing radiation in sperm counts are reversible or irreversible. None of the above-mentioned studies have evaluated prospectively the influence of EMR irradiation on paternity. Such investigations require long-term design and many previously healthy volunteers. At the present time nobody knows the real intercommunication between the constant direct mobile phones, computers, laptops, and Wi-Fi-emitted irradiation effects on testes and developmental delays in the offspring.

Conclusion

Collectively, the research indicates that exposure to EMR radiation and heat may lead to decreases in sperm count, sperm motility, and vitality, as well as increases in indicators of sperm damage such as higher levels of reactive oxygen species, oxidative stress, DNA damage and changes in sperm morphology. However, there is no consensus on how these findings may affect human fertility in the years to come and if the effects caused by EMR and heat are reversible or irreversible. Well-designed investigations are needed to

evaluate the real consequences of long-term employment of EMR-emitting devices and long-time exposure to heat. Additionally, there are no published studies examining the effect of EMR radiation and heat on reproductive health in women. Such studies are much more difficult to carry out, since they often require invasive techniques. While it is certain that the evidence show a negative effect of radiofrequency fields (RF) and electromagnetic radiation (EM) fields on sperm studies, it is also evident avoidance of daily exposure to these invisible toxicants might be impossible in our time. Current recommendations should focus on limiting the amount of time we spend using our mobile phones, laptops and computers, especially during periods of times that couples are trying to conceive a child.

References

- Agarwal, A., Deepinder, F., Sharma, R. K., Ranga, G., & Li, J. (2008). Effect of cell phone usage on semen analysis in men attending infertility clinic: An observational study. *Fertility and Sterility*, 89(1), 124–128.
- Agarwal, A., Singh, A., Hamada, A., & Kesari, K. (2011). Cell Phones and male infertility: A review of recent innovations in technology and consequences. *International Brazilian Journal of Urology*, 37, 432–454.
- Aitken, R. J., & Koopman, P. (2004). Lewis SEM, seeds of concern. *Nature*, 432, 48–52.
- Al-Damegh, M. A. (2012). Rat testicular impairment induced by electromagnetic radiation from a conventional cellular telephone and the protective effects of the antioxidants vitamins C and E. *Clinics (São Paulo, Brazil)*, 67(7), 785–792.
- Avendaño, C., Mata, A., Sanchez Sarmiento, C. A., & Doncel, G. F. (2012a). Use of laptop computers connected to internet through Wi-Fi decreases human sperm motility and increases sperm DNA fragmentation. *Fertility and Sterility*, 97(1), 39–45.e2.
- Avendaño, C., Mata, A., Sanchez Sarmiento, C. A., & Doncel, G. F. (2012b). Use of laptop computers connected to internet through Wi-Fi decreases human sperm motility and increases sperm DNA fragmentation. *Fertility and Sterility*, 97(1), 39–45.e2.
- Eroglu, O., Oztas, E., Yildirim, I., Kir, T., Aydur, E., Komesli, G., et al. (2006). Effects of electromagnetic radiation from a cellular phone on human sperm motility: An in vitro study. *Archives of Medical Research*, 37(7), 840–843.
- Gorpinchenko, I., Nikitin, O., Banyra, O., & Shulyak, A. (2014). The influence of direct mobile phone radiation on sperm quality. *Central European Journal of Urology*, 67(1), 65–71.
- Gutsch, T., Mohamad Al-Ali, B., Shamloul, R., Pummer, K., & Trummer, H. (2011). Impact of cell phone use on men's semen parameters. *Andrologia*, 43(5), 312–316.
- Hjollund, N. H., Storgaard, L., Ernst, E., Bonde, J. P., & Olsen, J. (2002). The relation between daily activities and scrotal temperature. *Reproductive Toxicology*, 20(2), 209–214.
- Jensen, T. K., Bonde, J. P., & Joffe, M. (2006). The influence of occupational exposure on male reproductive function. *Occupational Medicine (London)*, 56(8), 544–553.
- Kesari, K. K., Kumar, S., & Behari, J. (2011). Effects of radiofrequency electromagnetic wave exposure from cellular phones on the reproductive pattern in male wistar rats. *Applied Biochemistry and Biotechnology*, 164(4), 546–559.
- Kilgallon, S. J., & Simmons, L. W. (2005). Image content influences men's semen quality. *Biology Letters*, 1(3), 253–255.
- Mieusset, R., & Bujan, L. (1995). Testicular heating and its possible contributions to male infertility: A review. *International Journal of Andrology*, 18, 169–184.
- Rowley, M. J., Leach, D. R., Warner, G. A., & Heller, C. G. (1974). Effect of graded doses of ionizing radiation on the human testis. *Radiation Research*, 1974(59), 665–678.
- Sharlip, I. D., Jarow, J. P., Belker, A. M., Lipshultz, L. I., Sigman, M., Thomas, A. J., et al. (2002). Best practice policies for male infertility. *Fertility and Sterility*, 77(5), 873–882.
- Watanabe, Y., Nakagawa, M., & Miyakoshi, Y. (1997) 1997. Enhancement of lipid peroxidation in the liver of mice exposed to magnetic fields. *Industrial Health*, 35(2), 285–290.
- World Health Organization. (2010). *WHO laboratory manual for the examination of and processing of human semen and sperm–cervical mucus interaction* (5th edn). Cambridge: Cambridge University Press.

Further Reading

- Environmental working group, Cell Phone Radiation Damages Sperm, Studies—Environmental working group.org
- Liu, K., Li, Y., Zhang, G., Liu, J., Cao, J., Ao, L., & Zhang, S. (2014). Association between mobile phone use and semen quality: A systematic review and meta-analysis. *Andrology*, 2(4), 491–501.

GENETICS

Overview Genetics and Male Reproductive Medicine

Robert D Oates, Boston University School of Medicine, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Congenital bilateral absence of the vas deferens The clinical condition resulting from an absence of the reproductive ductal structures that transport sperm from the testis to the ejaculate.

Intracytoplasmic sperm injection An advanced reproductive technique where an individual sperm is micro-injected into the cytoplasm of a harvested egg.

Klinefelter syndrome A constellation of infertility and mild hypogonadism resulting from an extra X chromosome being present, the karyotype is 47,XXY.

Primary ciliary dyskinesia A group of disorders manifested by, as one component of many, defects in sperm movement.

Y chromosomal microdeletion A small piece of the long arm of the Y chromosome is missing and the genes involved in sperm production that reside in that stretch are as well.

Introduction

Many cases of male reproductive deficiency were considered uncorrectable and the patient was deemed “sterile.” Evaluation was limited, not only by lack of any therapeutic options, but also by a dearth of any etiologic knowledge. However, the development of ever sophisticated and powerful advanced reproductive techniques (ART) provided treatment opportunities for some of these men, which coincided temporally with curious individuals coming into the nascent field of male reproductive medicine and surgery. The novel information and strategies to investigate and explore the human genome helped fuel studies trying to determine, as best possible, the genetic underpinnings of the conditions that were now being “treated,” or at least, compensated for. Was it appropriate, would the health of any conceived offspring be affected, does the genetic anomaly confer other health risks? All of these types of questions beg for answers and the field has moved steadily, if slowly, forward in trying to provide those answers.

Specific Chapters

Male reproductive deficiency can be due to defects in sperm production, defects in sperm quality (structure and/or function), defects in sperm transport through the reproductive ductal system or sperm genetic/epigenetic abnormalities limiting fertilization of embryo development ([Harnisch and Oates, 2012](#)). Each chapter in this section will touch on these, displaying the wide variety of diverse etiologies and presentations of male reproductive compromise.

Numerical chromosomal anomalies like Klinefelter syndrome (47,XXY) are actually quite common when looking at the causes of azoospermia and will be fully explored in Chapter Sperm Defects while Chapter Immunological Male Subfertility will detail the structural chromosomal defects that are also found in men with severe oligospermia or recurrent miscarriage ([Oates, 2012](#)). It is a common theme throughout these chapters that it is important to have determined the genetics behind the male’s reproductive deficiency prior to blindly just looking for sperm surgically or using ejaculate sperm as a male gamete source for advanced reproductive techniques. This can be no more clear than what will be described in Chapter Medical Conditions and Male Reproductive Medicine: An Overview—the *a priori* knowledge of an *AZF**a* or *AZF**b* microdeletion predicts that no sperm will be found on testicular exploration and that any invasive surgical options are not warranted or helpful for the couple. In addition, offspring health must always be viewed as important a concern as patient success. For example, when a man has an *AZF**c* microdeletion as the proximate cause of his sperm production deficiency, all sons will inherit this Y chromosome and will experience reproductive failure in their own future ([Rozen et al., 2012](#)).

Chapter Overview Treatment and Male Reproductive Medicine explores the connection of congenital bilateral absence of the vas deferens (CBAVD) and mutations in the “Cystic Fibrosis” genes ([Yu et al., 2012](#)). An odd association, it seems, but one well-grounded in molecular and cellular physiology. As will be discussed, before undertaking an ART cycle using harvested sperm, it is critical to understand the CFTR mutation status of both partners to prevent, if the situation was right for this, the birth of a child who will be burdened with the life-long morbidity (and mortality) of Cystic Fibrosis. Understanding the genetic basis of male

reproductive compromise is not about just answering the question of “why,” it is also about obtaining critical information to proactively and prospectively assist clinicians and couples attempting to navigate the trying and difficult world of infertility to have a healthy child. Chapters Paternal Age and Fertility Concerns and General Endocrine Therapy also describe anatomical abnormalities which can be responsible for failure of sperm transport to the ejaculate or even the success of coitus and intravaginal deposition of sperm (Van Batavia and Kolon, 2016).

A proper functioning endocrine system is critically important to optimizing sperm production and the other components necessary for male reproductive competency (libido, erection, and ejaculation). Both chapters Varicocele: Evaluation and Pathophysiology and Endocrine Stimulatory Therapy for Testis Sperm Extraction will explore the genetic determinants of disorders of the hypothalamic-pituitary axis and the consequent effect on male fertility (Dwyer et al., 2013).

Spermatozoal movement and ability to fertilize a waiting oocyte may be impaired to such a dramatic degree that the male (and couple) are rendered infertile. Some of these conditions can be overcome with technology while others are not. Chapters Medical Non-Endocrine-Targeted Therapies: Ejaculatory Dysfunction and Immunotherapy and Empiric Infertility Treatment will review these types of conditions, such as primary ciliary dyskinesia and globozoospermia in some detail (Davila Garza and Patrizio, 2013). Finally, the emerging field of epigenetics may herald an entirely new category of sperm deficiency that may not outwardly affect sperm number, sperm motility, sperm shape, sperm transport or sperm fertilizing ability. Rather, the earliest events of embryo formation and development are marred through heretofore unknown anomalies in the epigenome the sperm is delivering to the oocyte. Chapter Infectious and Inflammatory Male Infertility will enlighten the reader on this interesting field of inquiry (Jenkins et al., 2016).

References

- Davila Garza, S. A., & Patrizio, P. (2013). Reproductive outcomes in patients with male infertility because of Klinefelter's syndrome, Kartagener's syndrome, round-head sperm, dysplasia fibrous sheath, and 'stump' tail sperm: An updated literature review. *Current Opinion in Obstetrics & Gynecology*, 25(3), 229–246.
- Dwyer, A. A., Sykiotis, G. P., Hayes, F. J., Boepple, P. A., Lee, H., Loughlin, K. R., Dym, M., Sluss, P. M., Crowley, W. F., Jr, & Pitteloud, N. (2013). Trial of recombinant follicle-stimulating hormone pretreatment for GnRH-induced fertility in patients with congenital hypogonadotropic hypogonadism. *The Journal of Clinical Endocrinology and Metabolism*, 98(11), E1790–1795.
- Hamisch, B., & Oates, R. (2012). Genetic disorders related to male factor infertility and their adverse consequences. *Seminars in Reproductive Medicine*, 30(2), 105–115.
- Jenkins, T. G., Aston, K. I., Hotaling, J. M., Shamsi, M. B., Simon, L., & Carrell, D. T. (2016). Teratozoospermia and asthenozoospermia are associated with specific epigenetic signatures. *Andrology*, 4(5), 843–849.
- Oates, R. D. (2012). The natural history of endocrine function and spermatogenesis in Klinefelter syndrome: What the data show. *Fertility and Sterility*, 98(2), 266–273.
- Rozen, S. G., Marszałek, J. D., Irenze, K., Skaletsky, H., Brown, L. G., Oates, R. D., Silber, S. J., Ardlie, K., & Page, D. C. (2012). AZFc deletions and spermatogenic failure: A population-based survey of 20,000 Y chromosomes. *American Journal of Human Genetics*, 91(5), 890–896.
- Van Batavia, J. P., & Kolon, T. F. (2016). Fertility in disorders of sex development: A review. *Journal of Pediatric Urology*, 12(6), 418–425.
- Yu, J., Chen, Z., Ni, Y., & Li, Z. (2012). CFTR mutations in men with congenital bilateral absence of the vas deferens (CBAVD): A systemic review and meta-analysis. *Human Reproduction*, 27(1), 25–35.

Numerical Chromosome Abnormalities

Yetunde Ibrahim and James Hotaling, University of Utah, Salt Lake City, UT, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Human chromosomal abnormalities may be numerical or structural. Numerical chromosomal abnormalities or aneuploidies refer to an alteration in chromosomal number from the normal diploid and haploid complement of somatic cells and gametes respectively. These include trisomies, monosomies, triploidies and tetraploidies. On the other hand, structural chromosomal abnormalities include translocations, deletions and duplications. Aneuploidy can be either numerical or partial in nature involving either gain or loss of an entire chromosome. It is the leading cause of pregnancy loss and developmental disabilities in humans (Hassold and Hunt, 2001). It affects up to 5% of clinically recognizable pregnancies and it is estimated that up to 60% of conceptions are aneuploid but are spontaneously aborted even before a pregnancy is clinically recognized (Hassold et al., 1993).

Chromosomal aneuploidy typically occurs as the result of a meiotic nondisjunction event in gametes, precocious separation of chromatids or a post-zygotic mitotic nondisjunction event in the embryo (Hassold and Hunt, 2001; Hassold et al., 1993; Hassold and Hunt, 2009; Yanowitz, 2010; Fragouli et al., 2011; Gabriel et al., 2011; Kuliev et al., 2011) (Figs. 1 and 2). It has been established that advanced maternal age is the biggest risk factor for numerical chromosome abnormalities (Hassold and Hunt, 2009). Current knowledge suggests that most aneuploidy in early embryos is predominantly derived from female nondisjunction (~95%) or is mitotic in origin, with the exception of aneuploidy of the sex chromosomes which is paternal in origin in 50%–100% of cases (Tables 1 and 2; Hassold et al., 1993; Lue et al., 2001; Lanfranco et al., 2004; Herlihy et al., 2011; Abdel-Razic et al., 2012; Jacobs et al., 1965; Stochholm et al., 2006, 2012; Sarkar and Marimuthu, 1983; Tartaglia et al., 2010; Koeberl et al., 1995; Homer et al., 2010; Wilson et al., 1989; Suri et al., 1995; Hsu, 1996; Gunther et al., 2004; El Moussaif et al., 2011; Hsu, 1994; Peet et al., 1998; Patacchiola et al., 2012; Gropman et al., 2010; Tartaglia et al., 2011; Kassai et al., 1991; Ricci et al., 1968; Wood et al., 2011).

Nowadays, the advent of preimplantation genetic screening in addition to in vitro fertilization has enabled chromosomal complement of cultured embryos to be determined and allows for the selection against aneuploid embryos for embryo transfers. However, this technology is imperfect as there have been reports suggesting that on rare occasions, an embryo deemed aneuploid via testing but subsequently transferred resulted in a live birth without any chromosomal abnormality (Greco et al., 2015; Gleicher and Orvieto, 2017).

The following sections will summarize our current knowledge of numerical chromosomal abnormalities that impact male reproduction, some non-reproductive implications of these abnormalities, screening guidelines and potential future directions using our current technology.

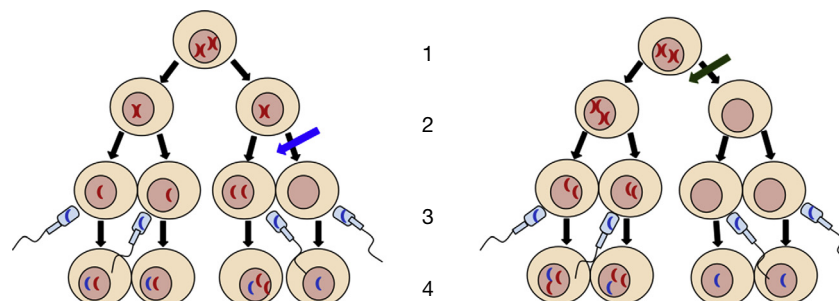


Fig. 1 Meiotic nondisjunction event.

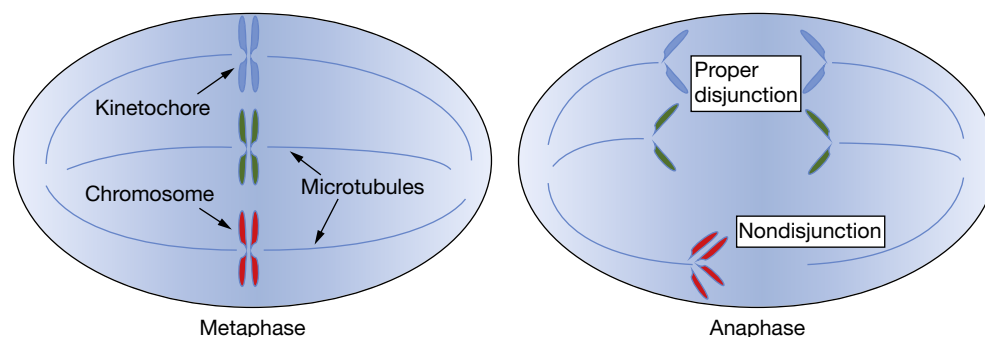


Fig. 2 Mitotic nondisjunction event.

Table 1 Numerical sex chromosome abnormalities

<i>Aneuploidy</i>	<i>Major clinical manifestations</i>	<i>Reproductive implication</i>
Klinefelter (47,XXY)	Tall stature, small testes, infertility	Infertile
47,XXY	Tall stature, mild delay in language and motor development	Most are fertile
Turner (45,X)	Short stature, facial dysmorphism, heart defects, streak gonads	Infertile
47,XXX	Most are physically normal. Some tendency to be tall	Most are fertile but premature ovarian failure can occur
45,X/46,XX Mosaic	Most common sex chromosome mosaicism. Milder clinical features of Turner	Some are fertile
45,X/46,XY Mosaic	Wide phenotypic spectrum with male phenotype being the most common presentation followed by ambiguous genitalia and lastly, female phenotype	Some are fertile, some have reduced fertility and some are infertile
49,XXXXY	Low nasal bridge with a wide or upturned tip, wide set eyes, epicanthal folds, skeletal anomalies, heart disease, severe hypogonadism, endocrine disorders	Infertile
49,XXXXX	Intellectual disability, craniofacial, cardiovascular and skeletal anomalies are variable	Infertile

Table 2 Suggested readings about numerical sex chromosome abnormalities

<i>Aneuploidy</i>	<i>Articles</i>
Klinefelter	Lue et al. (2001) , Lanfranco et al. (2004) , Herlihy et al. (2011)
47,XXY	Abdel-Razic et al. (2012) , Jacobs et al. (1965)
Turner	Stochholm et al. (2006) , Sarkar and Marimuthu (1983)
47,XXX	Tartaglia et al. (2010)
45,X/46,XX Mosaic	Koeberl et al. (1995) , Homer et al. (2010) , Wilson et al. (1989) , Suri et al. (1995) , Hsu (1996) , Gunther et al. (2004)
45,X/46,XY Mosaic	El Moussaif et al. (2011) , Hsu (1994) , 45,X/46,XY including Y chromosome rearrangements. Rarechromo.org
49,XXXXY	Peet et al. (1998) , Patacchiola et al. (2012) , Gropman et al. (2010) , Tartaglia et al. (2011)
49,XXXXX	Kassai et al. (1991) , Ricci et al. (1968) , Wood et al. (2011)

47,XXY—Klinefelter Syndrome (KS)

This is a syndrome that occurs in approximately 1–2 in 1000 live births ([Morris et al., 2008](#)) and is the most common cause of azoospermia in men ([Bojesen and Gravholt, 2007](#)). KS accounts for about two thirds of all chromosomal abnormalities observed in infertile men ([De Braekeleer and Dao, 1991](#)).

Etiology

In Utero, the XXY fetal testes has the normal complement of primordial germ cells. Due to a defect in Sertoli cells and germ cell communication during testes maturation, progressive loss of germ cells occurs during childhood such that by adulthood, no germ cells remain ([Lue et al., 2001](#)). In theory, the etiology of the nondisjunction in Klinefelter can be from maternal meiosis I or II or paternal meiosis II. You would expect that each of these situations would contribute to 33% of cases. However, nearly 50% of Klinefelter patients show a paternal origin ([Thomas and Hassold, 2003](#)) with some studies showing an increased frequency with advanced paternal age ([Lanfranco et al., 2004](#)).

Presentation

The spectrum of KS presentation is wide but all males with KS have small testes (<10cm³) and significantly elevated follicle stimulating hormone (FSH) and luteinizing hormone (LH). In early life, KS may be diagnosed in boys with behavioral disorders and long legs. It is important to distinguish KS from other forms of eunuchoidism that result in both long upper and lower extremities. The IQ of patients with KS typically falls within the normal range but tends to be lower than that of other siblings. Most patients present as adolescents with small firm testes and hypogonadism with varying degrees of androgen deficiency. Later in life, many males present at infertility centers with azoospermia ([Lanfranco et al., 2004](#)). Although interestingly, men with KS almost always have normal libido and no evidence of erectile dysfunction. Other non-reproductive sequela manifested in patients

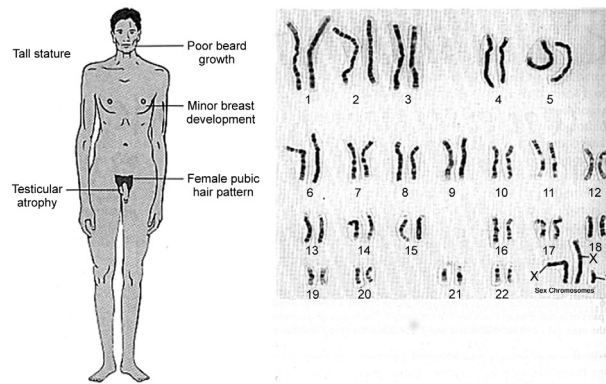


Fig. 3 Phenotype and Karyotype of a patient with Klinefelter syndrome.

with KS include expressive language difficulties, pulmonary diseases including bronchiectasis, chronic bronchitis and emphysema, systemic lupus erythematosus, varicose veins resulting in leg ulcers, hypothyroidism, metabolic dysfunction, osteoporosis, higher incidence of breast cancer, non-Hodgkin's lymphoma, extragonadal germ cell tumors and a decreased risk of prostate cancer (Anon, 1965; Volkl et al., 2006; Weiss et al., 2005; Swerdlow et al., 2005; Campbell et al., 1980; Scofield et al., 2008; Bojesen and Gravholt, 2007). Despite these wide array of clinical presentations, the exact mechanism behind many of these non-reproductive sequela remains unknown (Fig. 3).

Mechanism of Fertility Impairment

One reason for the wide phenotypic distribution of KS is that 10%–20% of men with KS are mosaics, typically 47,XXY/46,XY (Lanfranco et al., 2004). The greater the number of X chromosomes, the greater the phenotypic consequences, both gonadal and extragonadal (Paulsen et al., 1968). A small subset of non-Mosaic men with KS will have some sperm in their ejaculate. However, this does not translate to a normal hormonal profile as these men will still present with significantly elevated FSH levels in direct response to abnormal spermatogenesis. Likewise, LH levels are chronically elevated as the Leydig cells require maximal stimulation to produce testosterone. For these reasons, most men with KS have hypoandrogenism, and often hyperestrogenemia resulting in gynecomastia, which could increase in severity with age (Groth et al., 2013; Lanfranco et al., 2004; Oates, 2003; Wikstrom et al., 2006).

Implications of Non-reproductive Impairments

KS is a key risk factor for early-onset osteoporosis (Bojesen et al., 2011). The etiology is likely multifactorial but testosterone is thought to play a key role. It has been well defined that testosterone in men and estrogen in women play a vital role to bone health. Thus, in KS, testosterone deficiency can lead to decreased bone formation and resorption. Other possible mechanisms for osteoporosis in KS are abnormalities in CAG length, low levels of insulin-like factor 3 and X-chromosome inactivation (Ferlin et al., 2011; Wikstrom et al., 2006). Men with KS have also been shown to be susceptible to metabolic dysfunction including type 2 diabetes, decreased muscle, increased adiposity, decreased insulin sensitivity, decreased muscle strength and oxygen consumption capacity (Gravholt et al., 2011).

Diagnosis

The diagnosis of KS is usually made by determining the karyotype of peripheral leukocytes. Some mosaics will only be detected by karyotype of skin fibroblasts and occasionally of testicular biopsy specimen.

Management

Early detection of KS allows for early intervention for cognitive and behavioral disorders. Androgen replacement allows for development of masculine secondary sexual characteristics, improved self-esteem, strength and bone mineral density. With regards to fertility, the degree of testicular mosaicism is an important factor in determining spermatogenesis and fertility potential. The advent of intracytoplasmic sperm injection (ICSI) technology has led to an increased potential for fertility in patients with KS. Using testicular sperm extraction (TESE) technology, sperm can be successfully extracted from the testes and utilized in IVF/ICSI cycles. Results from this technology were however fraught with a high incidence of sex chromosome hyperploidy and autosomal aneuploidy in the sperm of Klinefelter men (Lim et al., 1999). A study by Yarali et al. demonstrated that pregnancy and implantation rates were comparable for a group of 33 patients with KS (39% and 23%) versus a group of 113 men with non-obstructive azoospermia and a normal karyotype undergoing TESE/ICSI (33% and 26%). Fertilization rates were also similar between the two groups. However, results from preimplantation genetic screening revealed that only 59% of the embryos from the KS patients had a normal karyotype (Yarali et al., 2009). Recently, a study by Vicdan et al. (2016) reported the ICSI outcomes using fresh and cryopreserved sperm obtained via TESE from azoospermic men with KS. In this group of 83 azoospermic men with KS, 24

live births were obtained. The authors found that the use of fresh and cryopreserved-thawed sperm in IVF/ICSI cycle outcomes were equally successful. In addition, no clinical or laboratory parameter predicted the presence of spermatozoa in patients with KS except the TESE procedure itself (Vidán et al., 2016). However, this study does not negate the concern over the increased risk of aneuploidy in KS men as it has been demonstrated that frequency of XY sperm, the main cause of aneuploidy in men with KS increases significantly with increasing paternal age (Arnedo et al., 2006). There is also evidence suggesting that spermatogenic potential decreases with advancing paternal age in men with KS (Aksela et al., 2006; Emre Bakircioglu et al., 2006; Foresta et al., 1999). This has led to the support of surgical sperm extraction through microTESE for sperm banking at a young age.

Trisomy 18p

Complete, isolated trisomy of the short arm of chromosome 18 is exceedingly rare. Only about 24 patients have been described in the literature (Orendi et al., 2013). This is caused by an unbalanced translocation involving the short arm of chromosome 18. Individuals with this rare syndrome have variable facial dysmorphism and mild to moderate cognitive impairment. A spermatogenic defect may be associated with Trisomy 18p. A recent case study reported on a 28-year-old male who presented with facial dysmorphism, mild intellectual disability and a symptom that had previously never been reported in the literature in this population; non-obstructive azoospermia. Cytogenetic analysis revealed an additional segment on the short arm of chromosome 14 (Jedraszak et al., 2015).

Unfortunately, the low numbers of reported cases of trisomy 18p makes it difficult to define a specific phenotype for this syndrome and it is possible that this finding of non-obstructive azoospermia in this one man is a chance event.

46,XX Testicular Disorder of Sex Development (DSD)

This is a very rare structural chromosomal aberration that affects 1/20,000 men with infertility (Ergun-Longmire et al., 2005). Although this syndrome will be covered in greater detail in another chapter, I have provided a brief review here due to similarities in phenotype with KS. The spectrum of individuals who are XX and have a disorder of sex determination have a range of phenotypes, from phenotypic males to all degrees of genital ambiguity. For the purpose of this article, we are focusing on patients with XX testicular DSD who are unambiguously male.

Etiology

The most common cause is an Xp:Yp translocation including SRY, the sex determining region, which initiates and maintains the male biologic program (Schiebel et al., 1997).

Presentation

Masculinized individuals with 46,XX DSD have been referred to as having *de la Chapelle syndrome*. There are some characteristics that are similar between men with 46,XX DSD and KS and these include small testes, azoospermia, gynecomastia and lack of secondary sexual characteristics. However, in 46,XX testicular DSD, phallus size is small to normal and patients have normal sexual function and intelligence (Bouayed Abdelmoula et al., 2003; Vorona et al., 2007).

Diagnosis

The diagnosis of 46,XX testicular DSD is often made during an investigation of infertility or delayed puberty. These patients have hypergonadotropic hypogonadism with elevated FSH and LH, decreased testosterone and dihydrotestosterone (DHT). FISH for the SRY gene can be performed and will be detected in 90% of phenotypic males with 46,XX testicular DSD.

Management

Phenotypic males with 46,XX testicular DSD who are SRY positive are not at increased risk for gonadoblastoma as they lack a complete Y chromosome. At puberty, most affected individuals require treatment with the male sex hormone testosterone to induce development of male secondary sex characteristics such as facial hair and deepening of the voice. Hormone treatment can also help prevent breast enlargement.

Reproductive Implication

46,XX DSD males are uniformly infertile because they lack the azoospermia factors (AZFa, AZFb, AZFc) required for normal spermatogenesis (Patrat et al., 2010). MicroTESE or other attempts at having biological children do not apply to these men. Referral to a genetic counselor is usually mandatory.

Genetic Screening

Genetic abnormalities can cause infertility by affecting sperm production or sperm transport. Structural chromosomal aberrations are up to 10 times more common in infertile men than fertile controls (3% in oligospermic men and 19% in men with non-obstructive azoospermia) (Bojesen and Gravholt, 2007; Chandley, 1998; Yoshida et al., 1997a,b). Men with nonobstructive azoospermia or severe oligozoospermia are at increased risk for having a genetic abnormality compared to fertile men. For these reasons, a karyotype is recommended by the American Society for Reproductive Medicine (ASRM), the American Urological Association (AUA) and the European Academy of Andrology (EAA) in all men with azoospermia or a total motile sperm count (<5 million/mL). Other guidelines, such as the international consortium of experts from 12 national scientific guidelines that produced a report in 2002, recommend a broader approach. They suggest offering a karyotype for men with a total motile sperm count below 10 million and for all couples who have not achieved a pregnancy after 1 year (Foresta et al., 2002). The tests consist of a gross examination of the autosomal and sex chromosomes and unfortunately, in the United States many insurance companies do not cover this expensive assay.

Looking Ahead: The Role of Preimplantation Genetic Screening

Preimplantation genetic screening (PGS) has revolutionized the field of reproductive medicine. Its goal is to detect de-novo aneuploidy and subchromosomal deletions and additions in embryos of couples undergoing IVF for subfertility reasons. Forty to sixty percent of preimplantation embryos are aneuploid and accounts for the most common embryo factor responsible for many implantation failures (Gianaroli et al., 1997). PGS allows for selection for euploid embryos and in theory, should reduce multiple pregnancy rates by allowing more efficient embryo transfer.

However, the hypothesis that PGS will improve implantations rates, pregnancy rates and live births while simultaneously reducing miscarriage rates has come under recent scrutiny. One of the essential assumptions surrounding the implementation of PGS over 20 years ago was that a single trophectoderm biopsy at the blastocyst stage is representative of the ploidy of the entire trophectoderm as well as the inner cell mass. This assumption fails to consider post-fertilization mitotic non-disjunction events, which can result in one embryo containing cells with different chromosomal complements i.e. a mosaic. While this issue is much less common nowadays because PGS is typically performed on blastocysts compared to cleavage stage embryos where the incidences of mosaicism are 3%–5% versus 50% respectively (Munne et al., 1994), the diagnostic accuracy is still not 100% (Dahdouh et al., 2015; Greco et al., 2015). The hypothesis is that even if PGS were to detect mosaicism, this does not necessarily mean that the fetus would be mosaic or abnormal as the biopsy may have removed those abnormal cells. Several reports have described healthy babies born after the transfer of embryos detected as aneuploid by PGS (Greco et al., 2015; Gleicher and Orvieto, 2017).

While this technology is imperfect, the incidence of mosaicism is still relatively low in blastocyst biopsies that it can be a significant tool in selecting against the transfer of embryos with numerical chromosomal abnormalities in subfertile couples undergoing IVF. We anticipate that with improvement in the current technology, we may be fortuitous and reduce the incidence of infants born with numerical chromosomal abnormalities within the subfertile population, which is already a population with a higher incidence of poor neonatal outcomes. We await further implementation of newer genetic tools and strengthening our current assays.

References

- Abdel-Razic, M. M., Abdel-Hamid, I. A., & Elsobky, E. S. (2012). Nonmosaic 47,XXX syndrome presenting with male infertility: case series. *Andrologia*, 44, 200–204.
- Aksglaede, L., Wikstrom, A. M., Rajpert-De Meyts, E., Dunkel, L., Skakkebaek, N. E., & Juul, A. (2006). Natural history of seminiferous tubule degeneration in Klinefelter syndrome. *Human Reproduction Update*, 12, 39–48.
- Anon. (1965). Case records of the Massachusetts General Hospital. Case 44-1965. *New England Journal of Medicine*, 273, 816–823.
- Arnedo, N., Templado, C., Sanchez-Blanco, Y., Rajmil, O., & Nogues, C. (2006). Sperm aneuploidy in fathers of Klinefelter's syndrome offspring assessed by multicolour fluorescent in situ hybridization using probes for chromosomes 6, 13, 18, 21, 22, X and Y. *Human Reproduction*, 21, 524–528.
- Bojesen, A., Birkebaek, N., Kristensen, K., Heickendorff, L., Mosekilde, L., Christiansen, J. S., & Gravholt, C. H. (2011). Bone mineral density in Klinefelter syndrome is reduced and primarily determined by muscle strength and resorptive markers, but not directly by testosterone. *Osteoporosis International*, 22, 1441–1450.
- Bojesen, A., & Gravholt, C. H. (2007). Klinefelter syndrome in clinical practice. *Nature Clinical Practice Urology*, 4, 192–204.
- Bouayed Abdelmoula, N., Portnoi, M. F., Keskes, L., Recan, D., Bahloul, A., Boudawara, T., Saad, A., & Rebai, T. (2003). Skewed X-chromosome inactivation pattern in SRY positive XX maleness: a case report and review of literature. *Annales de Génétique*, 46, 11–18.
- Campbell, W. A., Newton, M. S., & Price, W. H. (1980). Hypostatic leg ulceration and Klinefelter's syndrome. *Journal of Mental Deficiency Research*, 24, 115–117.
- Chandley, A. C. (1998). Chromosome anomalies and Y chromosome microdeletions as causal factors in male infertility. *Human Reproduction*, 13(Suppl. 1), 45–50.
- Dahdouh, E. M., Balayla, J., Audibert, F., Genetics, C., Wilson, R. D., Audibert, F., Brock, J. A., Campagnolo, C., Carroll, J., Chong, K., Gagnon, A., Johnson, J. A., Macdonald, W., Okun, N., Pastuck, M., & Vallee-Pouliot, K. (2015). Technical update: preimplantation genetic diagnosis and screening. *Journal of Obstetrics and Gynaecology Canada*, 37, 451–463.
- De Braekeleer, M., & Dao, T. N. (1991). Cytogenetic studies in male infertility: a review. *Human Reproduction*, 6, 245–250.
- El Moussaïf, N., Haddad, N. E., Iraqi, N., & Gaouzi, A. (2011). 45,X/46,XY mosaicism: report of five cases and clinical review. *Annales d'endocrinologie*, 72, 239–243.
- Emre Bakircioglu, M., Erden, H. F., Kaplancan, T., Ciray, N., Bener, F., & Bahceci, M. (2006). Aging may adversely affect testicular sperm recovery in patients with Klinefelter syndrome. *Urology*, 68, 1082–1086.
- Ergun-Longmire, B., Vinci, G., Alonso, L., Matthew, S., Tansil, S., Lin-Su, K., McElreavey, K., & New, M. I. (2005). Clinical, hormonal and cytogenetic evaluation of 46,XX males and review of the literature. *Journal of Pediatric Endocrinology & Metabolism*, 18, 739–748.

- Ferlin, A., Schipilliti, M., & Foresta, C. (2011). Bone density and risk of osteoporosis in Klinefelter syndrome. *Acta Paediatrica*, 100, 878–884.
- Foresta, C., Ferlin, A., Gianaroli, L., & Dallapiccola, B. (2002). Guidelines for the appropriate use of genetic tests in infertile couples. *European Journal of Human Genetics*, 10, 303–312.
- Foresta, C., Galeazzi, C., Bettella, A., Marin, P., Rossato, M., Garolla, A., & Ferlin, A. (1999). Analysis of meiosis in intratesticular germ cells from subjects affected by classic Klinefelter's syndrome. *The Journal of Clinical Endocrinology and Metabolism*, 84, 3807–3810.
- Fragouli, E., Alfarawati, S., Goodall, N. N., Sanchez-Garcia, J. F., Colls, P., & Wells, D. (2011). The cytogenetics of polar bodies: insights into female meiosis and the diagnosis of aneuploidy. *Molecular Human Reproduction*, 17, 286–295.
- Gabriel, A. S., Thornhill, A. R., Ottolini, C. S., Gordon, A., Brown, A. P., Taylor, J., Bennett, K., Handyside, A., & Griffin, D. K. (2011). Array comparative genomic hybridisation on first polar bodies suggests that non-disjunction is not the predominant mechanism leading to aneuploidy in humans. *Journal of Medical Genetics*, 48, 433–437.
- Gianaroli, L., Magli, M. C., Ferraretti, A. P., Fiorentino, A., Garrisi, J., & Munne, S. (1997). Preimplantation genetic diagnosis increases the implantation rate in human in vitro fertilization by avoiding the transfer of chromosomally abnormal embryos. *Fertility and Sterility*, 68, 1128–1131.
- Gleicher, N., & Orvieto, R. (2017). Is the hypothesis of preimplantation genetic screening (PGS) still supportable? A review. *Journal of Ovarian Research*, 10, 21.
- Gravholt, C. H., Jensen, A. S., Host, C., & Bojesen, A. (2011). Body composition, metabolic syndrome and type 2 diabetes in Klinefelter syndrome. *Acta Paediatrica*, 100, 871–877.
- Greco, E., Minasi, M. G., & Fiorentino, F. (2015). Healthy babies after intrauterine transfer of mosaic Aneuploid blastocysts. *The New England Journal of Medicine*, 373, 2089–2090.
- Gropman, A. L., Rogol, A., Fennoy, I., Sadeghin, T., Sinn, S., Jameson, R., Mitchell, F., Clabaugh, J., Lutz-Armstrong, M., & Samango-Sprouse, C. A. (2010). Clinical variability and novel neurodevelopmental findings in 49, XXXY syndrome. *American Journal of Medical Genetics. Part A*, 152A, 1523–1530.
- Groth, K. A., Skakkebaek, A., Host, C., Gravholt, C. H., & Bojesen, A. (2013). Clinical review: Klinefelter syndrome—a clinical update. *Journal of Clinical Endocrinology and Metabolism*, 98, 20–30.
- Gunther, D. F., Eugster, E., Zagar, A. J., Bryant, C. G., Davenport, M. L., & Quigley, C. A. (2004). Ascertainment bias in Turner syndrome: new insights from girls who were diagnosed incidentally in prenatal life. *Pediatrics*, 114, 640–644.
- Hassold, T., & Hunt, P. (2001). To err (meiotically) is human: the genesis of human aneuploidy. *Nature Reviews. Genetics*, 2, 280–291.
- Hassold, T., & Hunt, P. (2009). Maternal age and chromosomally abnormal pregnancies: what we know and what we wish we knew. *Current Opinion in Pediatrics*, 21, 703–708.
- Hassold, T., Hunt, P. A., & Sherman, S. (1993). Trisomy in humans: incidence, origin and etiology. *Current Opinion in Genetics & Development*, 3, 398–403.
- Herlihy, A. S., Halliday, J. L., Cock, M. L., & McLachlan, R. I. (2011). The prevalence and diagnosis rates of Klinefelter syndrome: an Australian comparison. *The Medical Journal of Australia*, 194, 24–28.
- Homer, L., Le Martelot, M. T., Morel, F., Amice, V., Kerlan, V., Collet, M., & De Braekeleer, M. (2010). 45,X/46,XX mosaicism below 30% of aneuploidy: clinical implications in adult women from a reproductive medicine unit. *European Journal of Endocrinology*, 162, 617–623.
- Hsu, L. Y. (1994). Phenotype/karyotype correlations of Y chromosome aneuploidy with emphasis on structural aberrations in postnatally diagnosed cases. *American Journal of Medical Genetics*, 53, 108–140.
- Hsu, L. Y. (1996). Prenatal diagnosis of 45,X/46,XX. *American Journal of Human Genetics*, 58, 634–636.
- Jacobs, P. A., Brunton, M., Melville, M. M., Brittain, R. P., & McClelland, W. F. (1965). Aggressive behavior, mental sub-normality and the XYY male. *Nature*, 208, 1351–1352.
- Jedraszak, G., Copin, H., Demailly, M., Quibel, C., Leclerc, T., Gallet, M., Benkhalfia, M., & Receveur, A. (2015). Azoospermia and trisomy 18p syndrome: a fortuitous association? A patient report and a review of the literature. *Molecular Cytogenetics*, 8, 34.
- Kassai, R., Hamada, I., Furuta, H., Cho, K., Abe, K., Deng, H. X., & Niihara, N. (1991). Penta X syndrome: a case report with review of the literature. *American Journal of Medical Genetics*, 40, 51–56.
- Koeberl, D. D., McGillivray, B., & Sybert, V. P. (1995). Prenatal diagnosis of 45,X/46,XX mosaicism and 45,X: implications for postnatal outcome. *American Journal of Human Genetics*, 57, 661–666.
- Kuliev, A., Zlatopolsky, Z., Kirillova, I., Spivakova, J., & Cieslak Janzen, J. (2011). Meiosis errors in over 20,000 oocytes studied in the practice of preimplantation aneuploidy testing. *Reproductive Biomedicine Online*, 22, 2–8.
- Lanfranco, F., Kamischke, A., Zitzmann, M., & Nieschlag, E. (2004). Klinefelter's syndrome. *Lancet*, 364, 273–283.
- Lim, A. S., Fong, Y., & Yu, S. L. (1999). Estimates of sperm sex chromosome disomy and diploidy rates in a 47,XXY/46,XY mosaic Klinefelter patient. *Human Genetics*, 104, 405–409.
- Lue, Y., Rao, P. N., Sinha Hikim, A. P., Im, M., Salameh, W. A., Yen, P. H., Wang, C., & Swerdloff, R. S. (2001). XXY male mice: an experimental model for Klinefelter syndrome. *Endocrinology*, 142, 1461–1470.
- Morris, J. K., Alberman, E., Scott, C., & Jacobs, P. (2008). Is the prevalence of Klinefelter syndrome increasing? *European Journal of Human Genetics*, 16, 163–170.
- Munne, S., Weier, H. U., Grifo, J., & Cohen, J. (1994). Chromosome mosaicism in human embryos. *Biology of Reproduction*, 51, 373–379.
- Oates, R. D. (2003). Clinical and diagnostic features of patients with suspected Klinefelter syndrome. *Journal of Andrology*, 24, 49–50.
- Orendi, K., Uhrig, S., Mach, M., Tschepper, P., & Speicher, M. R. (2013). Complete and pure trisomy 18p due to a complex chromosomal rearrangement in a male adult with mild intellectual disability. *American Journal of Medical Genetics Part A*, 161A, 1806–1812.
- Patacchiola, F., Sciarra, A., Di Fonso, A., D'Alfonso, A., & Carta, G. (2012). 49, XXXY syndrome: an Italian child. *Journal of Pediatric Endocrinology & Metabolism*, 25, 165–166.
- Patrat, C., Bienvenu, T., Janny, L., Faure, A. K., Fauque, P., Aknin-Seifer, I., Davy, C., Thiounn, N., Jouannet, P., & Levy, R. (2010). Clinical data and parenthood of 63 infertile and Y-microdeleted men. *Fertility and Sterility*, 93, 822–832.
- Paulsen, C. A., Gordon, D. L., Carpenter, R. W., Gandy, H. M., & Drucker, W. D. (1968). Klinefelter's syndrome and its variants: a hormonal and chromosomal study. *Recent Progress in Hormone Research*, 24, 321–363.
- Peet, J., Weaver, D. D., & Vance, G. H. (1998). 49, XXXY: a distinct phenotype. Three new cases and review. *Journal of Medical Genetics*, 35, 420–424.
- Ricci, N., Dallapiccola, B., Ventimiglia, B., Tiepolo, L., & Fraccaro, M. (1968). 48, XXXX-49, XXXX mosaic: asynchronies among the late-replicating X chromosomes. *Cytogenetics*, 7, 249–259.
- Sarkar, R., & Marimuthu, K. M. (1983). Association between the degree of mosaicism and the severity of syndrome in Turner mosaics and Klinefelter mosaics. *Clinical Genetics*, 24, 420–428.
- Schiebel, K., Winkelmann, M., Mertz, A., Xu, X., Page, D. C., Weil, D., Petit, C., & Rappold, G. A. (1997). Abnormal XY interchange between a novel isolated protein kinase gene, PRKY, and its homologue, PRKX, accounts for one third of all (Y+)XX males and (Y-)XY females. *Human Molecular Genetics*, 6, 1985–1989.
- Scofield, R. H., Bruner, G. R., Namjou, B., Kimberly, R. P., Ramsey-Goldman, R., Petri, M., Reveille, J. D., Alarcon, G. S., Vila, L. M., Reid, J., Harris, B., Li, S., Kelly, J. A., & Harley, J. B. (2008). Klinefelter's syndrome (47, XXY) in male systemic lupus erythematosus patients: support for the notion of a gene-dose effect from the X chromosome. *Arthritis and Rheumatism*, 58, 2511–2517.
- Stochholm, K., Bojesen, A., Jensen, A. S., Juul, S., & Gravholt, C. H. (2012). Criminality in men with Klinefelter's syndrome and XYY syndrome: a cohort study. *BMJ Open*, 2, e000650.
- Stochholm, K., Juul, S., Juel, K., Naeraa, R. W., & Gravholt, C. H. (2006). Prevalence, incidence, diagnostic delay, and mortality in Turner syndrome. *Journal of Clinical Endocrinology and Metabolism*, 91, 3897–3902.
- Suri, M., Kabra, M., Jain, U., Sanders, V., Saxena, R., Shukla, A., Singh, G. V., & Verma, I. C. (1995). A clinical and cytogenetic study of Turner syndrome. *Indian Pediatrics*, 32, 433–442.
- Swerdlow, A. J., Schoemaker, M. J., Higgins, C. D., Wright, A. F., Jacobs, P. A., & U.K. Clinical Cytogenetics Group. (2005). Cancer incidence and mortality in men with Klinefelter syndrome: a cohort study. *Journal of the National Cancer Institute*, 97, 1204–1210.

- Tartaglia, N., Ayari, N., Howell, S., D'epagnier, C., & Zeitler, P. (2011). 48,XXYY, 48,XXXY and 49,XXXXY syndromes: not just variants of Klinefelter syndrome. *Acta Paediatrica*, 100, 851–860.
- Tartaglia, N. R., Howell, S., Sutherland, A., Wilson, R., & Wilson, L. (2010). A review of trisomy X (47,XXX). *Orphanet Journal of Rare Diseases*, 5, 8.
- Thomas, N. S., & Hassold, T. J. (2003). Aberrant recombination and the origin of Klinefelter syndrome. *Human Reproduction Update*, 9, 309–317.
- Vicdan, K., Akarsu, C., Sozen, E., Buluc, B., Vicdan, A., Yilmaz, Y., & Biberoglu, K. (2016). Outcome of intracytoplasmic sperm injection using fresh and cryopreserved-thawed testicular spermatozoa in 83 azoospermic men with Klinefelter syndrome. *Journal of Obstetrics and Gynaecology Research*, 42, 1558–1566.
- Volkl, T. M., Langer, T., Aigner, T., Greess, H., Beck, J. D., Rauch, A. M., & Dorr, H. G. (2006). Klinefelter syndrome and mediastinal germ cell tumors. *American Journal of Medical Genetics. Part A*, 140, 471–481.
- Vorona, E., Zitzmann, M., Gromoll, J., Schuring, A. N., & Nieschlag, E. (2007). Clinical, endocrinological, and epigenetic features of the 46,XX male syndrome, compared with 47,XXY Klinefelter patients. *The Journal of Clinical Endocrinology and Metabolism*, 92, 3458–3465.
- Weiss, J. R., Moysich, K. B., & Swede, H. (2005). Epidemiology of male breast cancer. *Cancer Epidemiology, Biomarkers & Prevention*, 14, 20–26.
- Wikstrom, A. M., Dunkel, L., Wickman, S., Norjavaara, E., Ankarberg-Lindgren, C., & Raivio, T. (2006). Are adolescent boys with Klinefelter syndrome androgen deficient? A longitudinal study of Finnish 47,XXY boys. *Pediatric Research*, 59, 854–859.
- Wilson, M. G., Lin, M. S., Fujimoto, A., Herbert, W., & Kaplan, F. M. (1989). Chromosome mosaicism in 6,000 amniocenteses. *American Journal of Medical Genetics*, 32, 506–513.
- Wood, A., Kleis, L., Toriello, H., & Cemeroglu, A. P. (2011). Mosaic pentasomy X/tetrasomy X syndrome and premature ovarian failure. *Indian Pediatrics*, 48, 402–404.
- Yanowitz, J. (2010). Meiosis: making a break for it. *Current Opinion in Cell Biology*, 22, 744–751.
- Yarali, H., Polat, M., Bozdog, G., Gunel, M., Alpas, I., Esinler, I., Dogan, U., & Tiras, B. (2009). TESE-ICSI in patients with non-mosaic Klinefelter syndrome: a comparative study. *Reproductive Biomedicine Online*, 18, 756–760.
- Yoshida, A., Miura, K., Nagao, K., Hara, H., Ishii, N., & Shirai, M. (1997a). Sexual function and clinical features of patients with Klinefelter's syndrome with the chief complaint of male infertility. *International Journal of Andrology*, 20, 80–85.
- Yoshida, A., Miura, K., & Shirai, M. (1997b). Cytogenetic survey of 1,007 infertile males. *Urologia Internationalis*, 58, 166–176.

Y Chromosome Microdeletions

Ryan W Dobbs, Laurel Sofer, and Samuel Ohlander, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Determining the nature of the storage, replication, and transmission of our genetic material has long been a source of fascination and investigation for scientists. Following the identification of chromosomes as the carriers of genetic material, there has been a revolution in our understanding of genetic principles that underlie inheritance and reproduction. In humans, the genetic code is comprised of 23 pairs of chromosomes: 22 paired autosomes and the sex chromosomes, the latter of which consists of either a paired XX chromosome in women or one X and one Y chromosome in men.

In the early 20th century, researchers discovered that sex determination was due to the presence or absence of the Y chromosome (Hotaling, 2014). From an evolutionary standpoint, the Y chromosome is highly efficient, transmitting the entire male phenotypic pathway in a highly conserved amount of DNA. The Y chromosome is exclusively transmitted in reproduction through sperm and has a high baseline mutation rate due to the multiple cell divisions occurring during gametogenesis and the highly oxidative environment of the testis. Unlike autosomes and the paired sex chromosomes of women, the Y chromosome does not have a “backup” or paired copy of critical genetic material and is vulnerable to passing on deleterious alleles (alternative forms of a gene arising from mutations) because it is unable to recombine during meiosis and is directly passed from father to son. The consequence of this is that any loss or deletion of genetic material in the Y chromosome may have a significant reproductive impact.

Y Chromosome

The Y chromosome is one of the smallest chromosomes with the least number of genes of any chromosome. It is made up of several regions: the pseudoautosomal region (PAR) which lies on the short arm of the Y chromosome (Y_p), and the heterochromatic and euchromatic regions which lie on the long arm (Y_q). The PAR regions exchange genetic material with the PAR region on the X PAR region during meiosis. PAR1 and PAR2, located on the telomeres of Y_p and Y_q , respectively, represent 5% of the chromosome whereas the majority of the Y chromosome is made up of the nonrecombining Y (NRY). The region includes the SRY gene. Also known as the Male Specific Y (MSY) chromosome, this portion does not recombine with the X chromosome and composes 95% of the Y chromosome. Because of the fact that the Y chromosome has only one copy of its critical genetic material, any deletion or mutation of this material may have a severe impact on a man’s spermatogenesis potential. Microdeletions may occur when two palindromic sequences of the Y chromosome recombine inadvertently, deleting the intervening genetic material (Hotaling, 2014). A diagram of the Y chromosome is shown in Fig. 1.

Y Chromosome Microdeletions

Y chromosomal microdeletions (YCM) represent a family of genetic disorders that arise from spontaneous mutations in the Y chromosome as described above, and which are passed from father to son (Hotaling, 2014). Broadly speaking, these YCM occur as a result of deletions within portions of the Y chromosome which are responsible for carrying the genetic information necessary for spermatogenesis. These deletions impact production of a number of proteins necessary for normal sperm development and as such, men with YCM clinically present with a range of spermatogenic failure from oligozoospermia to complete azoospermia with severe male factor infertility (Hopps et al., 2003). As genetic abnormalities are associated with an estimated 15%–30% of cases of male factor infertility (Hotaling, 2014), YCM represent a critically important subset of men presenting with infertility, and understanding these microdeletions is vitally important for the proper counseling and care of these men.

While the gold standard for diagnosis of YCM is whole DNA sequencing of a patient’s Y chromosome, presently this technique is too expensive for routine research or clinical purposes. Instead, patients with unexplained infertility can be screened by harvesting genomic DNA from blood or lymphoblastoid cells and mixing these DNA samples with a series of known genetic markers for

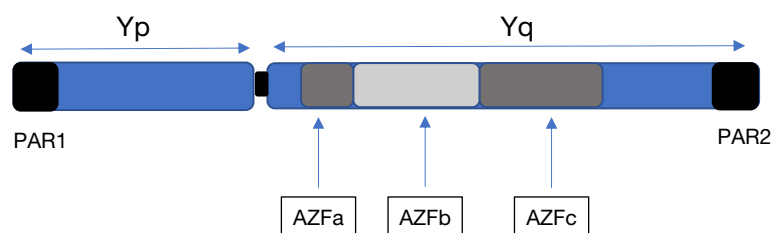


Fig. 1 AZF regions of the Y chromosome.

sequence tagged sites (STS) on the Y chromosome (Qureshi et al., 1996). These mixtures then undergo polymerase chain reaction amplification and gel electrophoresis. If a reaction fails to amplify following three amplifications, then the corresponding STS is considered to be absent and a YCM can be diagnosed. More recently, some investigators have described techniques with multiplex polymerase chain reactions to amplify several different DNA sequences to increase the speed and efficiency of YCM screening, detecting about 95% of microdeletions (Zhu et al., 2017).

Azoospermia Factor (AZF) Region Microdeletions

The most commonly implicated YCMs occur within a portion of the Y chromosome referred to as the azoospermia factor (AZF) region. Subregions within this AZF region located on the distal portion of the Y chromosome long arm are further divided into the AZFa (sometimes referred to as AZF1), AZFb, and AZFc (sometimes collectively grouped as AZF2) genes and serve critical functions in the production of sperm. An overview of AZFa, AZFb, and AZFc YCM are shown in Table 1. These deletions were first identified by Vogt et al. (1996), who identified a small subset of men with these specific YCM within a large cohort of men presenting for infertility evaluation. AZFa deletions remove two critical genes for spermatogenesis, USP9Y and DBY or DDX3Y. Loss of these genes result in absence of sperm and Sertoli cell only syndrome (Nutti and Krausz, 2008).

While the AZFb and AZFc regions were initially thought to be distinct, further investigation has demonstrated that these regions do have some overlap and may be deleted together (Hotaling, 2014). Microdeletions within different AZF regions have a profound impact on clinical outcomes and management. In a study of semen extraction by diagnostic testicular biopsy or testicular sperm extraction (TESE), Hopps et al. evaluated 78 men with AZF deletions, including three with AZFa deletion, 11 with AZFb, 42 with AZFc, 16 with AZFb + c and 6 with Yq (AZFa + b + c) deletions (Hopps et al., 2003). Within this group, all men with AZFa, AZFb, AZFb + c, and Yq deletions were found to be completely azoospermic. Men with isolated AZFc deletions had more favorable clinical outcomes, with sperm found in 75% (9/12) after TESE and 45% (9/20) on biopsy with an overall sperm extraction rate of 56.3% (Hopps et al., 2003). Further follow up of this cohort confirmed that all microdissection TESE performed for patients with AZFa, AZFb, AZFb + c, and Yq deletions failed to obtain sperm while sperm extraction was successful in 15/21 (71.4%) men with isolated AZFc YCM (Stahl et al., 2010). This rate of sperm extraction for men with isolated AZFc YCM was comparable to the 48.8% retrieval rate for 385 idiopathic azoospermic men without YCM who underwent TESE during the same time period (Stahl et al., 2010). In summation, AZFa and AZFb subregion YCM have a uniformly dismal prognosis for sperm retrieval and future fertility. Conversely, isolated AZFc YCM may present with either severe oligospermia or azoospermia, but the majority of these men will have sperm present at the time of TESE, which may be utilized for intracytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF). It is important to note that while spermatogenesis is impaired for men with isolated AZFc microdeletions that retrieved sperm are capable for fertilization and producing term pregnancies with favorable fertilization rates when compared to patients with non-obstructive azoospermia without gene deletions (Mulhall et al., 1997).

Other Y Chromosome Microdeletions: USP9Y and Gr/Gr Mutations

In addition to deletions, a number of genetic polymorphisms have been investigated to determine their role in male factor infertility. These include the USP9Y gene within the AZFa region of the Y chromosome, which has been associated with mild to severe oligospermia or azoospermia and subsequent male infertility in several studies (Sun et al., 1999; Ferlin et al., 2007). This USP9Y gene provides instruction for a protein called ubiquitin specific protease 9 (USP9), resulting in either cessation of production of the USP9 protein or in an abnormally short and nonfunctional version of the protein and resultant spermatogenic failure and Y chromosome infertility. While initial reports considered this mutation to be a cause of male factor infertility, more recent case reports have demonstrated spermatogenesis and normal sperm counts in men with USP9Y mutations (Luddi et al., 2009), and as such the previously described azoospermia and oligoasthenospermia cannot be completely due to the deletion of this gene and additional genetic or nongenetic factors may be contributing toward this clinical presentation. These reports are in line with studies reporting that the USP9Y serves to fine tune human spermatogenesis by improving the efficiency of sperm production (Krausz et al., 2006) rather than having an intrinsic function.

Similarly, the gr/gr mutation, a partial deletion within the AZFc region has also been identified as a potential risk factor for male factor infertility. Within one cohort of Italian men presenting with infertility, these partial AZFc deletions were associated with 5.3%

Table 1 AZF microdeletions

Mutation	Frequency	Genes affected	Prognosis
AZFa	Rare	DDX3Y and DBY or USP9Y	Sertoli only syndrome: no sperm
AZFb	Rare	RBMY1 PRY	No sperm
AZFc	10% of men with nonobstructive azoospermia, 1:4000 overall (Hotaling, 2014)	DAZ, gr/gr	Rarely sperm in ejaculate, 71.4% chance of finding sperm on mTESE. (Stahl et al., 2010)

of patients presenting with oligo or azoospermia while only 0.5% of fertile controls (Giachini et al., 2005). Conversely, some authors have failed to find statistically significant differences in gr/gr mutations between infertile patients and a fertile control group, and have postulated that gr/gr mutations represent a harmless polymorphism (Ravel et al., 2006). To address the discrepant findings in these studies, one meta-analysis found that gr/gr mutations (OR 1.81, 1.46–2.24 95% CI, $p < 0.01$) and a single MTHFR 677C->T polymorphism (OR 1.39, 1.15–2.69 95% CI, $p < 0.01$) were the only significant polymorphisms associated with male factor infertility (Tuttelmann et al., 2007). While comparisons between different infertility studies may be more difficult due to heterogeneous patient populations and varied clinical approaches, these authors concluded that “genetic studies performed so far emphasize the complexity of male infertility as a presumably polygenetic trait amended by environmental, lifestyle or occupational factors.” (Tuttelmann et al., 2007).

Implications for Fertility

Overall, 11% of men presenting to infertility clinics suffer from unexplained oligo- or azoospermia (Qureshi et al., 1996). Clinical management approaches for YCM and the decision to screen for YCM are highly clinician dependent. Because of the association of YCM with infertility, most abnormalities result de novo with new mutations on the Y chromosome within men with no family history of infertility. Men with a YCM who are able to father children with the assistance of ICSI or IVF should be counseled that this abnormality is passed on to male offspring. As daughters do not inherit a Y chromosome, they are not affected. The rates of YCM vary by population and by presenting symptoms. In one large prospective cohort of 3073 Italian men presenting to infertility clinic, a total of 99 men (3.2%) were identified with YCM (Ferlin et al., 2007). Patients who presented with nonobstructive azoospermia (52 of 625 men, 8.3%) were more likely to have a YCM than men presenting with severe oligospermia (as defined as a sperm concentration <5 million/mL, 47 of 1372 men, 3.4%). Within this cohort, only 2 men of the 99 identified with a YCM were found to have >2 million sperm/mL (Ferlin et al., 2007). This concentration thus may provide a reasonable cutoff for clinicians to consider further testing for YCM. In a similar study of American men presenting with sperm concentrations <5 million/mL, YCM were found in 149 of 1591 overall patients (9.4%), and 10.4% of azoospermic men and 10.1% of men with severe oligospermia (>0–1 million/mL) were found to have YCM within this cohort (Stahl et al., 2010). Given that there have been no reported cases of successful sperm retrieval in men with AZFa or AZFb region deletions, one must thoroughly discuss the reproductive prognostication. Donor sperm or adoption should be discussed as biological children are not possible by current reproductive techniques.

Summary

The discovery of YCMs and their contribution toward male factor infertility represent a breakthrough in the understanding of the genetic basis for spermatogenesis, but many questions still need to be answered. While it is clear that the AZFa and AZFb subregions contain critical information for normal fertility, their exact role remains unclear and further investigation is needed to determine the contributions of single gene deletions and polymorphisms toward spermatogenesis. Additionally, many researchers are working to improve the efficiency and accuracy of YCM diagnosis. Clinically, the ultimate goal is to provide a therapeutic intervention for men with AZFa and AZFb deletions to produce viable sperm; however, that remains an unrealized goal at this time. Hopefully understanding YCMs and their contribution toward the multifactorial etiology of male infertility will provide a window of knowledge that is critical toward driving the next generation of reproductive technology.

References

- Ferlin, A., Arredi, B., Speltra, E., et al. (2007). Molecular and clinical characterization of Y chromosome microdeletions in infertile men: a 10-year experience in Italy. *The Journal of Clinical Endocrinology and Metabolism*, 92(3), 762–770.
- Giachini, C., Guarducci, E., Longepied, G., et al. (2005). The gr/gr deletion(s): a new genetic test in male infertility? *Journal of Medical Genetics*, 42(6), 497–502.
- Hopps, C. V., Mielnik, A., Goldstein, M., Palermo, G. D., Rosenwaks, Z., & Schlegel, P. N. (2003). Detection of sperm in men with Y chromosome microdeletions of the AZFa, AZFb and AZFc regions. *Human Reproduction*, 18(8), 1660–1665.
- Hotaling, J. M. (2014). Genetics of male infertility. *The Urologic Clinics of North America*, 41(1), 1–17.
- Krausz C., Degl'Innocenti S., Nuti F., et al. (2006). Natural transmission of USP9Y gene mutations: a new perspective on the role of AZFa genes in male fertility. *Human Molecular Genetics* 15(18), 2673–81.
- Luddi, A., Margollicci, M., Gambera, L., et al. (2009). Spermatogenesis in a man with complete deletion of USP9Y. *The New England Journal of Medicine*, 360(9), 881–885.
- Mulhall, J. P., Reijo, R., Alagappan, R., et al. (1997). Azoospermic men with deletion of the DAZ gene cluster are capable of completing spermatogenesis: fertilization, normal embryonic development and pregnancy occur when retrieved testicular spermatozoa are used for intracytoplasmic sperm injection. *Human Reproduction*, 12(3), 503–508.
- Nuti, F., & Krausz, C. (2008). Gene polymorphisms/mutations relevant to abnormal spermatogenesis. *Reproductive BioMedicine*, 16(4), 504–513.
- Qureshi, S. J., Ross, A. R., Ma, K., et al. (1996). Polymerase chain reaction screening for Y chromosome microdeletions: a first step towards the diagnosis of genetically-determined spermatogenic failure in men. *Molecular Human Reproduction*, 2(10), 775–779.
- Ravel, C., Chantot-Bastaraud, S., El Houate, B., Mandelbaum, J., Siffroi, J. P., & McElreavey, K. (2006). GR/GR deletions within the azoospermia factor c region on the Y chromosome might not be associated with spermatogenic failure. *Fertility and Sterility*, 85(1), 229–231.
- Stahl, P. J., Masson, P., Mielnik, A., Marean, M. B., Schlegel, P. N., & Paduch, D. A. (2010). A decade of experience emphasizes that testing for Y microdeletions is essential in American men with azoospermia and severe oligozoospermia. *Fertility and Sterility*, 94(5), 1753–1756.
- Sun, C., Skaletsky, H., Birren, B., et al. (1999). An azoospermic man with a de novo point mutation in the Y-chromosomal gene USP9Y. *Nature Genetics*, 23(4), 429–432.

- Tuttelmann, F., Rajpert-De Meyts, E., Nieschlag, E., & Simoni, M. (2007). Gene polymorphisms and male infertility—a meta-analysis and literature review. *Reproductive BioMedicine*, 15(6), 643–658.
- Vogt, P. H., Edelmann, A., Kirsch, S., et al. (1996). Human Y chromosome azoospermia factors (AZF) mapped to different subregions in Yq11. *Human Molecular Genetics*, 5(7), 933–943.
- Zhu, X. B., Gong, Y. H., He, J., et al. (2017). Multicentre study of Y chromosome microdeletions in 1,808 Chinese infertile males using multiplex and real-time polymerase chain reaction. *Andrologia*, 49(5), e12662.

Translocations and Male Infertility

Bobby B Najari and Joseph P Alukal, New York University School of Medicine, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Genetic alterations can negatively impact male reproductive function in a number of ways. The hypothalamic-pituitary-gonadal axis can be negatively impacted by various chromosomal abnormalities (O'Flynn et al., 2010). Men with decreased sperm concentration are more likely to have chromosomal rearrangements (Clementini et al., 2005). In addition to impacting the quantity of sperm, chromosomal alterations can result in abnormal chromosomal segregation during meiosis, which can result in higher rates of sperm aneuploidy (Piomboni et al., 2014). Understanding chromosomal alterations and their impact on male fertility is important for all reproductive healthcare providers.

Chromosomal translocations are structural abnormalities of the chromosomes that are present in infertile men at a rate of 8.5 times the general population (Antonelli et al., 2000). There are two types of translocations, reciprocal and Robertsonian. Robertsonian translocations involve chromosomes in which the centromere is near the end of the chromosome. Reciprocal translocations are exchanges of genetic material between nonhomologous chromosomes. 46,XX male syndrome is often a result of a translocation of the SRY gene from the Y chromosome onto the paired (although not homologous) X chromosome. All of these chromosomal abnormalities have implications on both the reproductive potential of men who carry them, as well as the health of their future offspring.

Robertsonian Translocations

Robertsonian translocations occur between two acrocentric chromosomes, which are chromosomes in which the centromere is close to one end of the chromosome, resulting in a small (*p* for *petit*) length of genetic material beyond the centromere. There are six acrocentric chromosomes in the human genome: 13, 14, 15, 21, 22, and the Y chromosome. A Robertsonian event usually results in a genetic complement of 45 chromosomes due to the fusion of two long (*q*) chromosome arms and the loss of the corresponding two short arms.

In a retrospective review of 333 infertile men, 10 (3%) had Robertsonian translocations (Antonelli et al., 2000). Nine of these involved chromosome 13, and eight involved chromosome 14. Only 2 men of these 10 men had sperm concentrations greater than 20 million/mL, and 3 had concentrations below 5 million/mL, although no men had azoospermia. While all men had adequate percentages of sperm with normal morphology, only one man had sperm motility of at least 50%.

In another retrospective review of 2078 men undergoing IVF/ICSI, 11 (0.5%) had Robertsonian translocations (Clementini et al., 2005). The prevalence of these genetic alterations in the 1559 men with normal sperm concentrations was 0.3%, whereas 1.4% of the 514 men with sperm concentrations <20 million/mL had Robertsonian translocations. Unfortunately, motility was not commented on in this study, but its findings support the notion that this chromosomal abnormality is associated with diminished sperm production.

A more recent study compared 35 men with Robertsonian translocations to 57 men with normal karyotypes and partners with tubal factor female infertility (Pastuszek et al., 2015). While 71.9% of the men with a normal karyotype had sperm concentrations >15 million/mL, only 25.7% of the men with Robertsonian translocations had a normal sperm concentration. Furthermore, while men with a normal karyotype had an average of 40% progressive sperm motility, men with Robertsonian translocations only had 22% progressive motility.

While Robertsonian translocations can be associated with abnormal semen analysis parameters, patients are more concerned with birth rates. In a study of 367 couples with at least 2 miscarriages, Robertsonian translocations were found in 6 couples (1.6%), 4 of which were in the male partner (Kochhar and Ghosh, 2013). Four (67%) of these couples subsequently gave birth, with the remaining two couples having yet another miscarriage. Karyotypes of all six couples revealed two instances of transmission of the Robertsonian translocation: one resulting in a live birth, the other the product of a miscarriage. In a recent report of 2282 infertile men presenting to an andrology clinic in China, 6 (0.3%) had Robertsonian translocations (Guo et al., 2016). Interestingly, only one of these patients had a decreased sperm concentration, which may be relevant to the relatively high live birth rate of 83%.

Robertsonian translocations are associated with abnormal semen analysis profiles. While there are relatively few reports of this condition, it appears to be more common in men with low sperm concentration and low sperm morphology. It is less often associated with azoospermia. Fortunately, live birth rates in men presenting to reproductive medical clinics appear to be within normal ranges.

Reciprocal Translocations

Reciprocal translocations are a transfer of genetic material between homologous chromosomes. These are most commonly balanced exchanges, such that no genetic material is lost and individuals are phenotypically normal. While reciprocal translocations are the

most common structural chromosome abnormality in the general population (0.2% of newborns), infertile men are even more likely to have this abnormality (0.6%) (Mau-Holzmann, 2005). Patients with reciprocal translocation may have a normal phenotype, but this chromosomal abnormality can result in abnormal meiosis. The resulting sperm are more likely to have aneuploidy, which can manifest with recurrent pregnancy loss (Godo et al., 2013).

In a retrospective study of 333 infertile men from 2000, 6 (1.8%) had a reciprocal translocation. Only one of these men had a normal semen analysis. Four men had a sperm concentration less than 1 million/mL. All four men with severely low sperm concentrations also had 0% motility and 0% normal morphology.

In another retrospective review of 2078 men undergoing IVF/ICSI, 15 (0.72%) had reciprocal translocations. The frequency of reciprocal translocations increased as sperm concentration decreased. Whereas 7 (0.45%) of the 1559 men with normal sperm concentrations has a reciprocal translocation, 5 (1.69%) of the 295 men with mild oligospermia (5–20 million/mL), and 3 (2.1%) of the men with severe oligospermia had this chromosomal abnormality.

A more recent study compared 49 men with reciprocal translocations to 57 men with normal karyotypes and partners with tubal factor female infertility (Pastuszek et al., 2015). In contrast to the previous studies, the two groups did not differ in terms of sperm concentration or motility. This study may not have been powered to detect a significant difference in semen analysis parameters between these two groups.

In a study of 367 couples with at least two miscarriages, reciprocal translocations were found in 47 couples (12.8%), 16 of which involved the male partner. The subsequent live birth rate for these male carriers of reciprocal translocations was 61.5%. Of the 14 subsequent miscarriages, 4 (28.5%) were either trisomic or had unreciprocal translocations.

46,XX Male Disorder of Male Development

Patients with XX male syndrome have a 46,XX karyotype, and the disease has an estimated incidence of 1/20,000 male births (Abusheikha et al., 2001). However, the prevalence of this syndrome in men with azoospermia is 0.9% (Mau-Holzmann, 2005). Many patients are phenotypically normal men except for azoospermia, though 33% have gynecomastia, and 10% have hypospadias or ambiguous genitalia (Schweikert et al., 1982).

The most common mechanism for this syndrome is a translocation of the sex-determining region Y (SRY) gene, which encodes testis determining factor (TDF), from Yp to Xq (Page et al., 1987). These areas are known to be highly homologous (Van der Auwera et al., 1992). The gonads (testes) that develop due to the presence of SRY thus produce testosterone, which is why these patients can be phenotypically normal men. While the SRY gene is transferred to the X chromosome, the azoospermic factor region (AZF) and other genes required for normal spermatogenesis remain on the Y chromosome and are lost, explaining these patients' azoospermia.

Approximately 15% of men with 46,XX male syndrome are negative for the SRY gene (Anik et al., 2013). These individuals are more often undermasculinized and have ambiguous genitalia, and are diagnosed earlier than 46,XX SRY positive men. The male phenotype of 46,XX SRY negative individuals is likely due to mutations in autosomal or X-linked genes downstream from testis determining factor, such as SOX9 or DAX1 (Li et al., 2014). For example, multiple copies of the SOX9 gene can result in a 46,XX SRY negative male presentation (Cox et al., 2011).

The reproductive counseling of 46,XX male patients can be emotionally challenging. The potential for genetically-related offspring is nil. Due to the lack of a Y chromosome, and the relevant genes for spermatogenesis, attempts at testicular sperm extraction would be unsuccessful. This information is important to the patient from a prognostic standpoint, obviously, and reiterates the importance of a complete genetic evaluation including karyotype and Y-chromosome microdeletion in the patient presenting with non-obstructive azoospermia.

Conclusion

A variety of chromosomal abnormalities are relevant to the evaluation and treatment of male infertility. Translocations are one particular subset of chromosomal abnormalities that can be identified on karyotype. Both Robertsonian and reciprocal translocations can be associated with impaired semen analysis parameters. Reciprocal translocations are associated with a lower subsequent live birth rate, due to higher rates of sperm aneuploidy. 46,XX male patients can present as phenotypic males, but lack the potential for spermatogenesis.

References

- Abusheikha, N., Lass, A., & Brinsden, P. (2001). XX males without SRY gene and with infertility. *Human Reproduction*, 16(4), 717–718.
- Anik, A., Çatlı, G., Abacı, A., & Böber, E. (2013). 46,XX male disorder of sexual development: A case report. *Journal of Clinical Research in Pediatric Endocrinology*, 5(4), 258–260.
- Antonelli, A., Gandini, L., Petrinelli, P., et al. (2000). Chromosomal alterations and male infertility. *Journal of Endocrinological Investigation*, 23(10), 677–683.
- Clementini, E., Palka, C., Iezzi, I., Stuppia, L., Guanciali-Franchi, P., & Tiboni, G. M. (2005). Prevalence of chromosomal abnormalities in 2078 infertile couples referred for assisted reproductive techniques. *Human Reproduction*, 20(2), 437–442.
- Cox, J. J., Willatt, L., Homfray, T., & Woods, C. G. (2011). A SOX9 duplication and familial 46,XX developmental testicular disorder. *The New England Journal of Medicine*, 364(1), 91–93.

- Godo, A., Blanco, J., Vidal, F., & Anton, E. (2013). Accumulation of numerical and structural chromosome imbalances in spermatozoa from reciprocal translocation carriers. *Human Reproduction*, 28(3), 840–849.
- Guo, K. M., Wu, B., Wang, H. B., & Tian, R. H. (2016). Reproductive outcome of male carriers of chromosomal abnormalities: Multidisciplinary approach for genetic counseling and its implications. *Genetics and Molecular Research*, 15(4).
- Kochhar, P. K., & Ghosh, P. (2013). Reproductive outcome of couples with recurrent miscarriage and balanced chromosomal abnormalities. *The Journal of Obstetrics and Gynaecology Research*, 39(1), 113–120.
- Li, T. F., QY, W., Zhang, C., et al. (2014). 46,XX testicular disorder of sexual development with SRY-negative caused by some unidentified mechanisms: A case report and review of the literature. *BMC Urology*, 14, 104.
- Mau-Holzmänn, U. A. (2005). Somatic chromosomal abnormalities in infertile men and women. *Cytogenetic and Genome Research*, 111(3–4), 317–336.
- O'Flynn, O., Brien, K. L., Varghese, A. C., & Agarwal, A. (2010). The genetic causes of male factor infertility: A review. *Fertility and Sterility*, 93(1), 1–12.
- Page, D. C., Brown, L. G., & de la Chapelle, A. (1987). Exchange of terminal portions of X- and Y-chromosomal short arms in human XX males. *Nature*, 328(6129), 437–440.
- Pastuszek, E., Kiewisz, J., Kulwikowska, P. M., Lukaszuk, M., & Lukaszuk, K. (2015). Sperm parameters and DNA fragmentation of balanced chromosomal rearrangements carriers. *Folia Histochemica et Cytobiologica*, 53(4), 314–321.
- Piomboni, P., Stendardi, A., & Gambera, L. (2014). Chromosomal aberrations and aneuploidies of spermatozoa. *Advances in Experimental Medicine and Biology*, 791, 27–52.
- Schweikert, H. U., Weissbach, L., Leyendecker, G., Schwinger, E., Wartenberg, H., & Krück, F. (1982). Clinical, endocrinological, and cytological characterization of two 46, XX males. *The Journal of Clinical Endocrinology and Metabolism*, 54(4), 745–752.
- Van der Auwera, B., Van Roy, N., De Paepe, A., et al. (1992Van). Molecular cytogenetic analysis of XX males using Y-specific DNA sequences, including SRY. *Human Genetics*, 89(1), 23–28.

Identified Epigenetic Effects

Biren V Patel and James M Hotaling, University of Utah, Salt Lake City, Utah, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction to Epigenetics

Modern civilization is advancing at breakneck speed. For most of human history, the average life experience and accumulated wisdom changed little from generation to generation. But over the last half century – every decade, there have been fundamental changes to how we live and what we know. Biology has been at the forefront. One of the most important discoveries is DNA, the basic set of instructions for life. The discovery of its structure was heralded as a monumental leap in science. It is a basic language of only four letters – A (Adenine), G (Guanine), C (Cytosine) and T (Thymine) (Pray, 2008). The order of the four letters tells its message. It is heritable, passed down from generation to generation, only changed by mutation in the short run and evolution in the long run (Riggs et al., 1996).

Genetics and genomics, the study of DNA and its structure, are still in their infancy and marching forward. Newer technologies such as genetic engineering have exciting health possibilities. But scientists have also discovered a new field – epigenetics – which may be equally as important. The literal meaning of the prefix “*epi*” is “in addition to” in Greek (Casadesus and Low, 2006). In layman’s terms, epigenetics refers to all the heritable modifications of DNA without changing the sequence.

Review of Epigenetic Mechanisms

Epigenetic processes are those that alter the expression of DNA; they can also be inherited from parents. One can think of three sets signals which accomplish this goal: the epigenator, the initiator and the maintainer (Berger et al., 2009). The epigenator is the inciting event and it generally comes from the environment or diet but can also be a toxin or a secondary messenger molecule. The initiator then takes this event and transmits the signal to the specific location in the genome. The maintainer is the protein or set of proteins which sustain the appropriate DNA structure for future generations (Fig. 1).

Textbooks might slightly differ on the definitions of epigenetic mechanisms but they can be broken into three broad categories based on whether they effect DNA, histones or RNA. The most robustly studied epigenetic modification is DNA methylation. This is

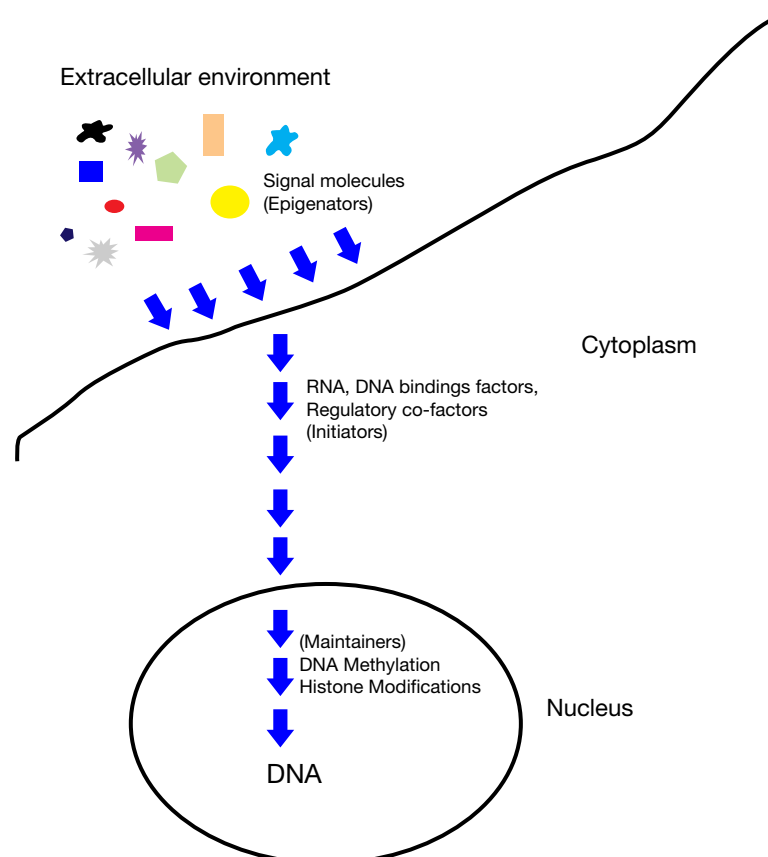


Fig. 1 The Epigenetic Pathway.

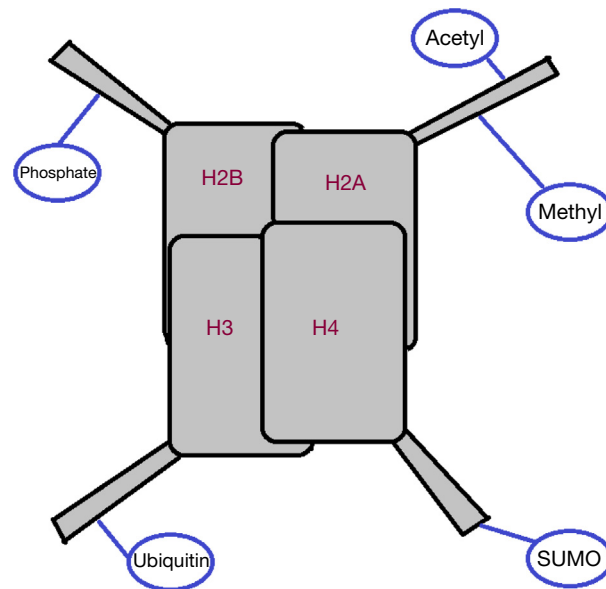


Fig. 2 Histone Epigenetics.

the process of adding a methyl group to a DNA base, usually Cytosine (to form 5-methylcytosine). In some rare instances, Adenine can also be methylated (Zemach et al., 2010). This modification is conducted by a set of proteins called DNA methyltransferases. Expression of DNA is generally silenced by methylation as the normal transcription machinery is unable to accurately carry out its function. The conformational change induced by the methylation alters the structure of DNA enough to prevent binding to otherwise available sites.

Histones, dimers or four proteins H2A, H2B, H3 and H4, form an octameric structure around which DNA wraps so that it may be packaged more densely in the nucleus. Histones are the scaffolding proteins of DNA and they can be epigenetically modified via methylation, acetylation, phosphorylation, ubiquitination or sumoylation. These modifications typically occur in the protruding tails which are more accessible to cell signals. Unlike DNA methylation which generally decreases the rate of transcription, histone modifications can either increase or decrease transcription.

Histone methylation and acetylation are carried out by histone methyltransferases and acetyltransferases, respectively. They typically occur on basic amino acids such as arginine, lysine and histidine (Greer and Shi, 2012; Lee and Workman, 2007). These modifications induce conformational change in DNA packing allowing or inhibiting access for transcription machinery. Histone phosphorylation occurs on serine, threonine or tyrosine amino acids and was originally discovered as part of the DNA repair pathway. Recently, it has been shown to have a crucial epigenetic component, interacting with other histone modifications to alter expression of DNA (Banarjee and Chakravarti, 2011). Ubiquitination and sumoylation are often grouped together but are unrelated. Ubiquitination involves the addition of ubiquitin, a small protein discovered several decades ago in the cell degradation pathway. SUMO stands for small ubiquitin-like modifier and is a catch-all category for small regulatory proteins which may act as epigenetic maintainers (Cao and Yan, 2012; Nathan et al., 2006) (Fig. 2). Compared to other epigenetic mechanisms, relatively little is known about ubiquitination and sumoylation.

RNA transcripts are also involved in epigenetic pathways. Messenger RNA (mRNA) can be alternatively spliced or modified (methylated) and descendants of the cells will have inherited these alterations. There are also non-coding RNA fragments (sRNAs) which have been shown to modulate gene expression (Howden et al., 2013). MicroRNAs (miRNAs) are another family of RNA molecules and they are even smaller than sRNA. Over 2000 have been discovered and each miRNA can potentially target up to 200 different mRNA transcripts for destruction (Knopik et al., 2012).

Clinical Applications of Epigenetics

Diet

An important epigenator is obesity which is approaching pandemic levels and affecting nearly two billion people worldwide (Philibert et al., 2012). It is well known that obesity leads to diabetes mellitus, cardiovascular disorders and endocrine dysfunction. These conditions directly contribute to male infertility. In a cross-sectional Australian study of 225 men, obesity was associated with decreased total sperm count (Kim et al., 2010). Body mass index (BMI) was seen to have a negative correlation with sperm concentration, motility and morphology in a prospective multicenter North American study (Salas-Huetos et al., 2017). The odds ratio of subfecundity (time to pregnancy) was increased to 1.18 (overweight) and 1.53 (obese) in a large Danish prospective birth cohort study of over 47,000 individuals (Craig et al., 2017).

Table 1 Summary of Systemic Reviews of Observational Studies (Salas-Huetos et al., 2017)

Summary of Findings	
Diet	Overall association on sperm parameters
Omega-3 fatty acids	Positive
Antioxidants	
Vitamin E	
Vitamin C	
β -carotene	
Selenium	
Zinc	
Cryptoxanthin	
Lycopene	
Vitamins	
Vitamin D	
Folate	
Saturated fatty acids	Negative
<i>trans</i> -Fatty acids	
Seafood	Positive
Fish	
Shellfish	
Poultry	
Cereals	
Vegetables	
Fruits	
Low-fat dairy	
Skimmed milk	
Processed meats	Negative
Soy foods	
Potatoes	
Full-fat dairy products	
Coffee	
Alcohol	
Sugar-sweetened beverages	
Sweets	

Obesity affects spermatogenesis, embryogenesis and offspring health via its epigenetic effects. Excess adipose tissue induces changes in DNA methylation and RNA content. Paternal obesity was found to modulate sperm microRNA and germ cell methylation status in the offspring of mice with diet-induced obesity (Fullston et al., 2013). This was a novel study where founder mice (F0 generation) were fed either a high-fat diet or a control diet for a period of 10 weeks. The F0 experimental mice were allowed to become obese without becoming diabetic and their descendants showed obesity and insulin resistance for two generations. A total of 414 different mRNAs and 11 microRNAs were altered in their descendants. Global methylation was also decreased 25% in their germ cells relative to the descendants from control diet mice.

In humans, pre-conception obesity has been shown to affect methylation patterns in future generations. Analysis of 79 newborn umbilical cord leukocytes showed hypomethylation at the Insulin-like Growth Factor 2 (IGF2) gene-associated regions in newborns with obese fathers (Greer and Shi, 2012). This particular gene was selected because in humans, it is normally paternally imprinted, that is only the copy from the father is expressed. Hypomethylation in these regions not only alters expression of a vital gene but it is associated with increased risks of Wilm's tumor, colorectal cancer and ovarian cancer. The mechanism of action is unknown. This study, like the previously described mouse study, demonstrates the impact of paternal obesity on offspring health.

Vitamins and nutrients also play a role in proper epigenetic programming. A recent systematic review of human observational studies showed that males adhering to a healthy diet could improve semen quality and fecundability (Riggs et al., 1996). A total of 1944 articles were identified and 35 were found to be relevant for the study. Only cross-sectional, case-control, prospective and retrospective studies were included. Diets rich in omega-3 fatty acids, anti-oxidants, vitamins and low in saturated and *trans*-fatty acids were found to be correlated with high quality semen parameters (see Tables 1 and 2). The quality of the sperm is directly related to healthy epigenetic programming.

Environment

Smoking causes abnormal DNA methylation patterns (Howden et al., 2013). A recent study comparing 51 smokers with 57 non-smokers showed that smoking was associated with significant biochemical changes in sperm DNA methylation. Many other studies have shown similar results. 37% of European reproductive age men are smokers, one of the highest rates of tobacco use in the world. A systematic review of 5865 men showed that the toxins from smoking are associated with reduced sperm count and motility

Table 2 Selected Observational Studies Investigating Nutrition and Sperm Quality

<i>Selected Large Association Studies on Nutrition and Sperm Quality</i>				
<i>Study</i>	<i>N</i>	<i>Design</i>	<i>Measured Outcomes</i>	<i>Conclusion</i>
Eslamian et al., 2012	169 normozoospermic controls; 72 asthenozoospermic men	Case-control; Food Frequency Questionnaire	Sperm parameters (volume, motility, morphology and concentration); hormone levels (FSH, LH, Testosterone and Prolactin)	Risk of asthenozoospermia was highest in upper third of processed meat intake; Lowest risk of asthenozoospermia in upper third fruit, vegetables, dark green vegetables, skim milk, poultry and sea food intake.
Minguez-Alarcon et al., 2012	215 healthy men	Cross –sectional; Food Frequency Questionnaire	Sperm parameters (volume, motility, morphology and concentration)	Positive association between increased intake of antioxidants cryptoxanthin, vitamin C, lycopene and β -carotene and total motile sperm count. The semen volume also increased with higher intakes of vitamin C.
Jensen et al., 2013	701 healthy men	Cross - sectional; Food Frequency Questionnaire	Sperm parameters (volume, motility, morphology and concentration)	Lower sperm concentration and total sperm count were associated with high intake of saturated fat in a dose-dependent manner.
Afeiche et al., 2014	155 men attending an infertility clinic	Cross – sectional; Food Frequency Questionnaire	Sperm parameters (volume, motility, morphology and concentration)	Low-fat dairy intake was associated with higher sperm concentration and progressive motility. Cheese intake was associated with lower sperm concentration among past or current smokers.

(Zheng et al., 2017). Toxins from smoking can directly damage sperm DNA, causing increased oxidative stress and increase pro-inflammatory cytokines. These changes are suspected to alter the epigenetic patterns in smokers. Smoking has been associated with altered expression of microRNA (Berger et al., 2009), altered histone modifications (Lim et al., 2005) and changes in phosphorylation (Xia et al., 2008).

Excessive alcohol use has numerous detrimental impacts on male reproduction. Superficially, it causes decreased penile tumescence and ejaculatory capability. Studies have assessed the effect of alcohol directly on spermatozoa in culture at levels comparable to what spermatozoa would be exposed to with social, moderate and heavy drinking (Casadesus and Low, 2006). Rising alcohol concentration was associated with significant decreases in sperm motility and morphology. Chronic alcohol intake has been shown to alter DNA methylation patterns (Wagner et al., 2007). In this study, over 485,000 CpG residues were assessed. These areas are regions of DNA with cytosine nucleotides adjacent to guanine nucleotides, by far the most common location for methylation. Six months of alcohol use was shown to cause widespread alterations in DNA methylation patterns. Alcohol has also been associated with abnormal histone acetylation (Ghezzi et al., 2013).

Ionizing radiation (IR) is another common environmental concern as it may cause widespread genomic instability (Lee and Workman, 2007). Humans are constantly being bombarded with radiation from nature (cosmic rays) and from man-made devices (medical imaging). Many cancers have been linked with radiation including thyroid, leukemia, lymphoma, breast and bladder. The main mechanisms of damage are from single and double stranded helical breaks, base alterations and cross links. These changes not only could be passed on to future generations but the damaged DNA itself can induce further epigenetic changes.

DNA methylation is altered, usually hypomethylated, in response to DNA damage. These changes in somatic cells have been seen to last decades. In germ lines, the aberrant changes are transferred to the next generation. Histones also play a large role in regulating access to damaged DNA. The phosphorylation of histone H2A is one of the most extensively studied modifications in regulating the reaction to double stranded DNA breaks. The phosphorylation of H2A allows unwinding of the histone-DNA complex, presumably to allow repair machinery to enter and in response to IR, the level of phosphorylation increases. There are also global changes in histone methylation and acetylation from IR. Small RNA molecules are also affected by IR-induced stress. Altered expression patterns of mRNA and miRNA have been seen in exposed males as well as their unexposed offspring (Eslamian et al., 2012).

Pesticides also play a significant role in epigenetic perturbation of the male epigenome. Their long-term somatic and male reproductive health implications had been largely unknown until recently. Pesticides are a heterogeneous category of molecules and can

Table 3 Summary of Studies Investigating Pesticides with Semen Quality

Summary of Studies Investigating Pesticides with Semen Quality			
	Author	n	Results
Organochlorines	Aneck-Hahn et al., 2007	311	DDE concentrations associated with decreased sperm concentration; DDE and DDT concentrations associated with decreased motility.
	Messaros et al., 2009	335	High total DDT-DDE exposure had higher odds ratio of low sperm concentration, low motility and poor morphology.
Organophosphates	Perry et al., 2011	152	Farmers with occupational pesticide exposure had higher risks for lower semen volume, lower concentration, higher abnormal morphology and decreased sperm motility.
	Perry et al., 2011	189	Men with abnormal sperm concentration and motility had higher levels of urinary DMP.
Pyrethroids	Ji et al., 2011.	240	Association between 3-PBA urine levels and decreased sperm concentration
	Xia et al., 2008	376	Higher urine 3-PBA concentration had higher odds of poorer sperm concentration

DDE: dichlorodiphenyldichloroethylene
DDT: dichlorodiphenyltrichloroethane
DMP: dimethylphosphate
3-PBA: 3-phenoxybenzoic acid

be grouped into categories: pyrethroids, organochlorines and organophosphates. One systematic review demonstrated that a number of pesticides negatively impacted sperm quality (Lee and Workman, 2007) (see Table 3). The most commonly reported finding with various pesticide classes was decreased sperm concentration.

The noxious effect of the pesticides on sperm quality also impact the epigenome (Filkowski et al., 2010). Organochlorines have been shown to alter global DNA methylation. In one study, low dose exposure to persistent organochlorines resulted in global hypomethylation in 86 otherwise healthy Koreans (Kim et al., 2010). Exposure to organochlorines and organophosphates has been shown to alter methylation patterns in rat brains and liver, respectively. Histone acetylation and miRNA expression changes in the presence of pesticides have also been documented (Collota et al., 2012).

Other environmental pollutants include bisphenol A (BPA) exposure, which has become a widespread problem. Many studies have demonstrated deleterious effects of BPA on male reproduction and epigenetic machinery (Zheng et al., 2017). Air pollutants (particulate matter, black carbon, benzene) and metals (arsenic, nickel, chromium, mercury) have also been examined as potential epigenators (Baccarelli and Bollati, 2009). Metals are also linked with oxidative stress which is known to cause direct DNA damage and trigger an inflammatory state. The cell responds to these triggers by altering access to DNA, blocking access to healthy regions and opening up damaged ones for repair. Reactive oxygen species directly damage key proteins in the epigenetic enzymatic steps (methyltransferases, acetyltransferases) rendering the proteins ineffective.

Arsenic toxicity has been shown to change miRNA expression (Marsit et al., 2006). Nickel is hypothesized to exert its mechanism by displacing magnesium, which normally plays a pivotal role in DNA stabilizing interactions. Nickel also enhances DNA and histone methylation while reducing histone acetylation. Likewise, chromium reduces histone phosphorylation, methylation and acetylation. In-utero mercury exposure is known to cause significant learning and developmental disorders (Baccarelli and Bollati, 2009).

Air pollution has also been associated with global epigenetic alterations. The three most common measures of air pollution include particulate matter, black carbon and benzene. Particulate matter is the sum of all small solid and liquid particles in the air. Black carbon is a byproduct of vehicle exhaust. Benzene is a pollutant from crude oil most commonly encountered at gas stations. These pollutants all cause widespread DNA hypomethylation.

Medications

One of the most exciting areas in biopharma is using small molecules to induce epigenetic changes. This has great potential to improve health outcomes for generations of descendants. Epigenetic mechanisms play a key role in many disease states. For example, hypermethylation of estrogen receptors can increase the risk of atherosclerosis. Neurological diseases, such as Rett syndrome, are the result of excessive DNA methylation which cause gene silencing and subsequent developmental delay. Many cancer pathways are influenced by DNA methylation and histone modification patterns which allow the tumorous cell to escape cell cycle check points (Heerboth et al., 2014).

Potential drug target mechanisms include inhibition of methylation and acetylation. There are many drugs in the pipeline. Some already exist. 5-azacytidine is a Cytosine analog which is incorporated into DNA. When DNA methyltransferases come to perform the methylation, an irreversible binding happens between the protein and 5-azacytidine. The drug has a unique structure which prevents dissociation of the enzyme. This complex is now targeted for destruction by the cell. Histone acetyltransferase inhibitors have potential to be used in many cancers and diseases. Curcumin, a naturally occurring HAT inhibitor, has been shown to inhibit cell growth and proliferation. Modified garcinol (extracted from fruit) and anacardiac acid (extracted from cashews) also act as HAT inhibitors and have been shown to induce apoptosis.

Thoughts on Future

The epigenome, representing the cross roads of environmental, lifestyle and inherited protein expression patterns, will likely drive much of the future research in reproductive medicine. This burgeoning field is just beginning to be translated into clinically actionable items. The future of the epigenome may hold the key to not only reproductive health but also the health of future generations.

References

- Afeiche, M., Bridges, N., Williams, P., et al. (2014). Dairy intake and semen quality among men attending a fertility clinic. *Fertility and Sterility*, 101, 1280–1287.
- Aneck-Hahn, N., Schulenburg, G. W., Bormman, M. S., Farias, P., & de Jager, C. (2007). Impaired semen quality associated with environmental DDT exposure in young men living in a malaria area in the Limpopo Province, South Africa. *Journal of Andrology*, 28, 423–434.
- Baccarelli, A., & Bollati, V. (2009). Epigenetics and environmental chemicals. *Current Opinion in Pediatrics*, 21, 243–251.
- Banarjee, T., & Chakravarti, D. (2011). A peek into the complex realm of histone phosphorylation. *Molecular and Cellular Biology*, 31, 4858–4873.
- Berger, S. L., Kouzarides, T., Shiekhattar, R., & Shilatifard, A. (2009). An operational definition of epigenetics. *Genes & Development*, 23, 781–783.
- Casadesus, J., & Low, D. (2006). Epigenetic gene regulation in the bacterial world. *Microbiology and Molecular Biology Review*, 70, 830–856.
- Cao, J., & Yan, Q. (2012). Histone ubiquitination and deubiquitination in transcription, DNA damage response, and cancer. *Frontiers in Oncology*, 2, 26. <https://doi.org/10.3389/fonc.2012.00026>.
- Collota, M., Bertazzi, P. A., & Bollati, V. (2012). Epigenetics and pesticides. *Toxicology*, 307, 35–41.
- Craig, J. R., Jenkins, T. G., Carrell, D. T., & Hotaling, J. M. (2017). Obesity, male infertility, and the sperm epigenome. *Fertility & Sterility*, 107, 848–859.
- Eslamian, G., Amirjannati, N., Rashidkhani, B., Sadeghi, M. R., & Hekmatdoost, A. (2012). Intake of food groups and idiopathic asthenozoospermia: A case control study. *Human Reproduction*, 27, 3328–3336.
- Filkowski, J. N., Illytsky, Y., Tamminga, J., et al. (2010). Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression. *Carcinogenesis*, 31, 1110–1115.
- Fullston, T., Ohlsson Teague, E. M., Palmer, N. O., et al. (2013). Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcription profile of testis and sperm microRNA content. *The Federation of American Societies for Experimental Biology*, 27, 4226–4243.
- Ghezzi, A., Krishnan, H. R., Lew, L., et al. (2013). Alcohol-induced histone acetylation reveals a gene network involved in alcohol tolerance. *PLoS Genetics*, 9, e1003986.
- Greer, E. L., & Shi, Y. (2012). Histone methylation: A dynamic mark in health, disease and inheritance. *Nature Reviews Genetics*, 13, 343–357.
- Heerboth, S., Lapinska, K., Snyder, N., et al. (2014). Use of epigenetic drugs in disease: An overview. *Genetics & Epigenetics*, 6, 9–19.
- Howden, B. P., Beaune, M., Harrison, P. F., et al. (2013). Analysis of the small RNA transcriptional response in multidrug-resistant staphylococcus aureus after antimicrobial exposure. *Antimicrobial Agents and Chemotherapy*, 57, 3864–3874.
- Jensen, T. K., Heitmann, B. L., Jensen, M. B., et al. (2013). High dietary intake of saturated fat is associated with reduced semen quality among 701 young Danish men from the general population. *American Journal of Clinical Nutrition*, 97, 411–418.
- Ji, G., Xia, Y., Gu, A., et al. (2011). Effects of non-occupational environmental exposure to pyrethroids on semen quality and sperm DNA integrity in Chinese men. *Reproductive Toxicology*, 31, 171–176.
- Knopik, V. S., Maccani, M. A., Francazio, S., & McGahey, J. E. (2012). The epigenetics of maternal cigarette smoking during pregnancy and effects on child development. *Development and Psychopathology*, 24, 1377–1390.
- Kim, K. Y., Kim, D. S., Lee, S. K., et al. (2010). Association of low-dose exposure to persistent organic pollutants with global DNA hypomethylation in healthy Koreans. *Environmental Health Perspectives*, 118, 370–374.
- Lee, K. K., & Workman, J. L. (2007). Histone acetyltransferase complexes: One size doesn't fit all. *Nature Reviews Molecular Cell Biology*, 8, 284–295.
- Lim, L. P., Lau, N. C., Garrett-Engle, P., et al. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, 433, 769–773.
- Marsit, C. J., Eddy, K., & Kelsey, K. T. (2006). MicroRNA responses to cellular stress. *Cancer Research*, 66, 9–19.
- Messaros, B. M., Rossano, M. G., Liu, G., et al. (2009). Negative effects of serum p,p'-DDE on sperm parameters and modification by genetic polymorphisms. *Environmental Research*, 109, 457–464.
- Minguez-Alarcon, L., Mendiola, J., Lopez-Espin, J., et al. (2012). Dietary intake of antioxidant nutrients is associated with semen quality in young university students. *Human Reproduction*, 27, 2807–2814.
- Nathan, D., Ingvarsdotter, K., Sterner, D. E., et al. (2006). Histone sumoylation is a negative regulator in saccharomyces cerevisiae and shows dynamic interplay with positive-acting histone modifications. *Genes & Development*, 20, 966–976.
- Philibert, R. A., Plume, J. M., Gibbons, F. X., Brody, G. H., & Beach, S. R. H. (2012). The impact of recent alcohol use on genome wide DNA methylation signatures. *Frontiers in Genetics*, 3, 54.
- Perry, M. J., Venners, S. A., Chen, X., et al. (2011). Organophosphorus pesticide exposures and sperm quality. *Reproductive Toxicology*, 23, 113–118.
- Pray, L. A. (2008). Discovery of DNA structure and function: Watson and Crick. *Nature Education*, 1, 100.
- Riggs, A.D., Russo, V.E., Martienssen R.A. (1996). Epigenetic regulation of gene mechanisms.vol. 32 Cold Spring Harbor Monograph Archive, Plainview, NY.
- Salas-Huetos, A., Bullo, M., & Salas-Salvado, J. (2017). Dietary patterns, foods and nutrients in male fertility parameters and fecundability: A systemic review of observational studies. *Human Reproduction Update*, 10, 1–19.
- Wagner, L., Lacz, B., Tamasko, M., et al. (2007). Cigarette smoke-induced alterations in endothelial nitric oxide synthase phosphorylation: Role of protein kinase C. *Endothelium*, 14, 245–255.
- Xia, Y., Han, Y., Wu, B., et al. (2008). The relation between urinary metabolite of pyrethroid insecticides and semen quality in humans. *Fertility and Sterility*, 89, 1743–1750.
- Zemach, A., McDaniel, I. E., Silva, P., & Zilberman, D. (2010). Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science*, 328, 916–919.
- Zheng, H., Zhou, X., Li, D., et al. (2017). Genome-wide alteration in DNA hydroxymethylation in the sperm from bisphenol A-exposed men. *PLoS One*, 12, e0178535.

Further Reading

- Bieniek, J. M., Kashanian, J. A., Deibert, C. M., et al. (2016). Influence of increasing body mass index on semen and reproductive hormonal parameters in a multi-institutional cohort of subfertile men. *Fertility & Sterility*, 106, 1070–1075.
- Donnelly, G. P., McClure, N., Kennedy, M. S., & Lewis, S. E. (1999). Direct effect of alcohol on the motility and morphology of human spermatozoa. *Andrologia*, 31, 43–47.
- Hossain, F., Ali, O., D'Souza, U. J., & Naing, D. K. S. (2010). Effects of pesticide use on semen quality among farmers in rural areas of Sabah, Malaysia. *Journal of Occupational Health*, 52, 353–360.
- Laggan, M., Tierling, S., Alkhaled, Y., et al. (2017). Aberrant DNA methylation patterns of human spermatozoa in current smoker males. *Reproductive Toxicology*, 71, 126–133.

- Martenies, S. E., & Perry, M. J. (2015). Environmental and occupational pesticide exposure and human sperm parameters: A systemic review. *Toxicology*, 307, 66–373.
- Merrifield, M., & Kovalchuck, O. (2013). Epigenetics in radiation biology: A new research frontier. *Frontiers in Genetics*, 4, 40.
- Ramlau-Hansen, C. H., Thulstrup, A. M., Nohr, E. A., et al. (2007). Subfecundity in overweight and obese couples. *Human Reproduction*, 22, 1634–1937.
- Sharma, R., Harlev, A., Agarwal, A., & Esteves, S. C. (2016). Cigarette smoking and semen quality: A new meta-analysis examining the effect of the 2010 World Health Organization Laboratory methods for the examination of human semen. *European Urology*, 70, 635–645.
- Soubry, A., Schildkraut, J. M., Murtha, A., et al. (2013). Paternal obesity is associated with IGF2 hypomethylation in newborns: Results from a newborn epigenetics study (NEST). *BioMed Central*, 11, 29.
- Stewart, T. M., Liu, D. Y., Garrett, C., et al. (2009). Recruitment bias in studies of semen and other factors affecting pregnancy rates in fertile men. *Human Reproduction*, 24, 2401–2408.
- Sundar, I. K., Nevid, M. Z., Friedman, A. E., & Rahman, I. (2014). Cigarette smoke induces distinct histone modifications in lung cells: Implications for the pathogenesis of COPD and lung cancer. *Journal of Proteome Research*, 13, 982–996.

Endocrine Genetic Defects

Ettore Caroppo, U.O. Fisiopatologia della Riproduzione Umana e P.M.A., Conversano, Italy

© 2018 Elsevier Inc. All rights reserved.

Glossary

AR Androgen receptor.

CAIS Complete androgen insensitivity syndrome.

CPHD Combined pituitary hormone deficiency.

DHT Dihydrotestosterone.

FSH Follicle-stimulating hormone.

FSHR Follicle-stimulating hormone receptor.

GnRH Gonadotropin-releasing hormone.

ICHH Isolated congenital hypogonadotropic hypogonadism.

IVF *In vitro* fertilization.

ICSI Intracytoplasmic sperm injection.

LH Luteinizing hormone.

MAIS Mild androgen insensitivity syndrome.

PAIS Partial androgen insensitivity syndrome.

T Testosterone.

Genetic Causes of Congenital Hypogonadotropic Hypogonadism

Idiopathic hypogonadotropic hypogonadism is a genetic disease that may be either the result of an isolated impaired secretion of gonadotropins due to lack of hypothalamic GnRH neurons or the consequence of the impaired production of pituitary hormones, but it may also occur as a feature of genetic syndromes resulting in multiorgan impairment. Isolated FSH and LH deficit due to genetic mutations have been also described, although in very rare cases.

Isolated Congenital Hypogonadotropic Hypogonadism

Isolated congenital hypogonadotropic hypogonadism (ICHH) is a rare disorder that is primarily caused by deficient production, secretion, or action of the gonadotropin-releasing hormone (GnRH) due to lack or deficient number of hypothalamic GnRH neurons, with complete absence of GnRH secretion being suggested by the presence of undetectable or narrowly gonadotropin secretion and/or by a pulsatile GnRH/LH secretion, and is clinically characterized by incomplete or absent puberty and infertility. ICHH can present solely as congenital GnRH deficiency (normosmic ICHH) or be associated with deficient sense of smell (anosmia) as in the condition termed Kallmann syndrome (KS), both conditions accounting for 40% and 60% of all ICHH, respectively ([Mitchell et al., 2011](#)).

The hypogonadotropic state is thought to be originated from impaired GnRH neuron migration from their origin in the olfactory placode to the hypothalamus, with anosmia (in KS) being the result of agenesis/dysgenesis of the olfactory bulb ([Balasubramanian et al., 2010](#)). Several mutated genes may affect the fate and migration of GnRH neurons, and therefore, both normosmic ICHH and KS may be monogenic, digenic, or oligogenic disorders.

Mutations in the KAL1 gene (now denoted as ANOS1) were the first characterized genetic defect reported to cause GnRH deficiency. KAL1 is located on Xp22.3 region, consists of 14 exons encompassing 220 kb of genomic DNA and encodes a modular extracellular matrix-associated 680 amino acid glycosylated protein termed anosmin-1. Anosmin-1 immunoreactivity in human embryos has been shown to be present in the olfactory placode as early as embryonic week 4.5 and is thought to be critical to attracting olfactory axons toward the forebrain. In its absence, this trajectory is lost, and GnRH neurons fail to reach the olfactory bulb initially and subsequently the hypothalamus. The incidence of ANOS1 mutation is approximately 10%–14% in familial and 8%–11% in sporadic KS patients ([Balasubramanian et al., 2010](#)).

GnRH neuron migrations may be affected by mutations in other genes/pathways ([Table 1](#)), namely, PROK2 (3p13) and PROKR2 (20p12.3), WDR11 (10q26.12), SEMA3A (7q21.11), NELF (9q34.3), FGFR1 (8p11.2-p11.1), and HS6ST1 (2q14.3). On the other hand, mutations in FGF8 (10q24.3) and CHD7 (8q12.2) may affect the neurogenic niche in the nasal area and craniofacial development. While mutations in ANOS1 and NELF are specific to KS, mutations in PROK2, PROKR2, WDR11, SEMA3A, FGFR1, HS6ST1, FGF8, and CHD7 may lead to both KS and normosmic ICHH ([Boehm et al., 2015](#)).

Postmigratory GnRH neurons are surrounded by a complex neuronal network of afferents, including kisspeptin-producing neurons that are essential for the different aspects of GnRH function, including the tonic feedback control of GnRH and/or

Table 1 List of genes associated with KS or normosmic ICHH

<i>Genes/pathways</i>	<i>Inheritance</i>	<i>Puberty</i>	<i>Reproductive phenotype^a</i>	<i>Nonreproductive phenotype^a</i>	<i>Response to GnRH therapy</i>	<i>Male reversibility</i>
Kal-1	X-linked	Absent/partial	Cryptorchidism, micropenis	Anosmia, unilateral renal agenesis	Variable	ND
FGF8	AD	Absent/partial	Cryptorchidism, micropenis	Cleft lip/palate, skeletal abnormalities, dental agenesis	Positive	ND
FGFR1	AD	Absent/partial	Cryptorchidism, micropenis	Cleft lip/palate, skeletal abnormalities, dental agenesis	Positive	Yes
PROK2	AR/AD	Absent/partial	Cryptorchidism, micropenis	Synkinesia	Positive	ND
PROKR2	AR/AD	Absent/partial	Cryptorchidism, micropenis	Synkinesia	Positive	Yes
CHD7	AD	Absent/partial	Cryptorchidism, micropenis	Hearing loss	Positive	
HS6T1	AD	Absent/partial	Micropenis	Cleft lip/palate	ND	Yes
WDR11	AD/AR	Absent/partial/complete	Cryptorchidism	Anosmia	ND	ND
SEMA3A	AR/AD	Absent	Cryptorchidism	Hyposmia	Variable	No
NELF	AR	Absent/partial	Cryptorchidism	Anosmia	ND	ND

AD, Autosomal dominant; AR, autosomal recessive; ND, not determined.

^aThe described phenotypes are observed in a variable proportion of patients.

From Kim, S.H. (2015). Congenital hypogonadotropic hypogonadism and kallmann syndrome: Past, present, and future. *Endocrinology and Metabolism* 30: 456–466; Mitchell, A.L., Dwyer, A., Pitteloud, N., Quinton, R. (2011). Genetic basis and variable phenotypic expression of Kallmann syndrome: Towards a unifying theory. *Trends in Endocrinology and Metabolism* 22, 249–258.

gonadotropin secretion and that have been demonstrated to undergo a dynamic process of prenatal and postnatal maturation that enables them to establish connections with GnRH neurons early in development (Boehm et al., 2015). Relevantly, inactivating mutations in genes encoding kisspeptin-1 (KISS1) and its receptor (KISS1R) halt pubertal development in humans. Mutations in TAC3 (encoding tachykinin-3) and TACR3 (encoding tachykinin receptor 3) in patients with ICHH have revealed that such members of the tachykinin family play a key role in the control of GnRH neurons. Also peripheral signals that convey information about metabolic status indirectly modulate GnRH neurosecretion, as demonstrated by the reproductive phenotype of patients with inactivating mutations in the genes encoding leptin (LEP) or its receptor (LEPR). Normosmic ICHH due to mutation in GnRH1 or GnRHR genes is very rare (Mitchell et al., 2011). The list of genes associated with normosmic ICHH is displayed in Table 2.

Combined Pituitary Hormone Deficiency

Combined pituitary hormone deficiency (CPHD) is characterized by the impaired growth hormone (GH) production with one or more other pituitary hormones deficit. For the purpose of this article, only those mutations leading to gonadotropin deficiency will be described.

Since the development of the pituitary gland is the result of the interaction between a number of signaling molecules and transcription factors, mutations in early transcription factors such as HESX1, Gli2, SOX3, and LHX3 are associated with complex pituitary phenotypes with extrapituitary manifestations including syndromic hypopituitarism, while mutations in later-acting transcription factors like PROP1 are associated with pituitary specific phenotypes (Table 3) (Giordano, 2016).

Table 2 List of genes associated with normosmic ICHH

<i>Genes/pathways</i>	<i>Inheritance</i>	<i>Puberty</i>	<i>Reproductive phenotype^a</i>	<i>Nonreproductive phenotype</i>	<i>Response to GnRH therapy</i>	<i>Male reversibility</i>
GnRH1	AR	Absent	Micropenis	None	Yes	ND
GnRHR	AR	Absent/incomplete/delayed	Cryptorchidism, micropenis	None	Variable	ND
KISS1	AR	Absent	Cryptorchidism, micropenis	None	ND	ND
KISS1R	AR/AD	Absent	Cryptorchidism, micropenis	None	ND	ND
TAC3	AR	Absent	Cryptorchidism, micropenis	None	ND	Yes
TACR3	AR	Absent	Cryptorchidism, micropenis	None	ND	Yes
LEP	AR	Absent/incomplete		Obesity	ND	ND
LEPR	AR	Absent/incomplete		Obesity	ND	ND

AD, Autosomal dominant; AR, autosomal recessive; ND, not determined.

^aThe described phenotypes are observed in a variable proportion of patients.

From Kim, S.H. (2015). Congenital hypogonadotropic hypogonadism and kallmann syndrome: Past, present, and future. *Endocrinology and Metabolism* 30: 456–466; Mitchell, A.L., Dwyer, A., Pitteloud, N., Quinton, R. (2011). Genetic basis and variable phenotypic expression of Kallmann syndrome: Towards a unifying theory. *Trends in Endocrinology and Metabolism* 22, 249–258.

Table 3 Genetic causes of combined pituitary hormone deficiency

<i>Gene</i>	<i>Inheritance</i>	<i>Pituitary hormone deficiency</i>	<i>Associated extrapituitary phenotypes</i>
PROP1	AR	FSH, LH, GH, PRL, TSH, ACTH	None
LHX3	AR	FSH, LH, GH, ACTH, TSH, PRL	Sensorineural hearing loss, limited neck rotation
LHX4	AR/AD	FSH, LH, GH, PRL, TSH, ACTH	Cerebellum anomalies
HESX1	AD/AR	GH to variable CPHD	Septooptic dysplasia
GLI2	AD	GH, PRL, TSH, FSH, LH, ACTH	Holoprosencephaly, craniofacial abnormalities, polydactyly
SOX3	X-linked	GH to variable CPHD	Intellectual disability, midline defects, infundibular hypoplasia

AD, Autosomal dominant; AR, autosomal recessive; CPHD, complete pituitary hormone deficiency.

From Giordano, M. (2016). Genetic causes of isolated and combined pituitary hormone deficiency. *Best Practice & Research Clinical Endocrinology & Metabolism* **30**, 679–691.

According to a large case series, the PROP1 mutations represent the most common known genetic cause of CPHD in sporadic (6.7%) and familial cases (48.5%). Two variants, c.301_302delAG and c150delA represent > 90% of the mutated alleles found in the Eastern European cohorts (Giordano, 2016). A recent study suggests that PROP1 is expressed in a progenitor cell that can develop into all anterior lobe cell types and that all hormone-secreting cell types of both the anterior and intermediate lobes are descended from PROP1-expressing progenitors (Davis et al., 2016).

HESX1 mutations at both the homozygous and the heterozygous states have been reported and may be associated with septooptic dysplasia, pituitary aplasia, or ectopic neurohypophysis. LHX3 mutations show very low mutation frequency both in sporadic (0.3%) and familial cases (11%). Homozygous LHX4 mutations are extremely rare and lethal due to severe panhypopituitarism and pulmonary failure, while patients with heterozygous mutations present with variable GH, TSH, ACTH, and gonadotropin deficiencies.

Genetic Syndromes Resulting in Multiorgan Impairment

Hypogonadotropic hypogonadism may be a feature of genetic syndromes resulting in multiple organ anomalies.

PNPLA6 mutations span a phenotypic variety characterized by variable combinations of clinical features including cerebellar ataxia, upper motor neuron involvement manifesting as spasticity and/or brisk reflexes, chorioretinal dystrophy associated with variable degrees of reduced visual function, and hypogonadotropic hypogonadism either in isolation or as part of anterior hypopituitarism. Less frequent features include peripheral neuropathy, impaired cognitive functioning, hair anomalies/scalp alopecia, and short stature. Some of these features can present in distinct clusters such as in Boucher–Neuhäuser syndrome, Gordon Holmes syndrome, Oliver–McFarlane syndrome, and Laurence–Moon syndrome (Table 4). PNPLA6-related disorders are inherited in an autosomal recessive manner; penetrance appears to be complete in individuals with biallelic PNPLA6 pathogenic variants (Synofzik et al., 2014).

NR0B1 is transcription factors expressed in the adrenal gland and reproductive axis during fetal development and in adult life. Mutations in genes encoding this factor are associated with primary adrenal insufficiency in early infancy or childhood, hypogonadotropic hypogonadism at puberty, and impaired spermatogenesis. Late onset form of this conditions presents with progressive or mild adrenal insufficiency and partial hypogonadotropic hypogonadism (HH).

Bardet–Biedl syndrome (BBS) is a rare genetic disorder with severe multiorgan impairment. Four to six primary features are needed for the diagnosis: retinal dystrophy, polydactyly, obesity, genital abnormalities, renal defects, and learning difficulties. Other (secondary) features may include brachydactyly/syndactyly, diabetes mellitus, developmental delay, speech deficit, dental defects, ataxia, olfactory deficit, and congenital heart disease. There are already 21 known BBS genes (BBS1–BBS20 and NPHP1): The mode of inheritance is autosomal recessive, but incomplete penetrance at least for some genes and/or types of mutations has been hypothesized.

CHARGE syndrome is characterized by a multitude of congenital aberrations due to CHD7 mutations. It is diagnosed in 1/10,000 live births. The clinical diagnosis of CHARGE syndrome is based on three out of four major diagnostic criteria (coloboma,

Table 4 PNPLA6-related disorders

<i>Syndrome's name</i>	<i>Hypogonadotropic hypogonadism</i>	<i>Cerebellar Ataxia</i>	<i>Chorioretinal dystrophy</i>	<i>Brisk reflexes</i>	<i>Trichomegaly</i>	<i>Peripheral neuropathy</i>	<i>Spastic paraplegia</i>	<i>Congenital/childhood hypopituitarism</i>
Boucher–Neuhäuser	X	X	X					
Gordon Holmes	X	X		X				
Oliver–McFarlane	X		X		X			X
Laurence–Moon	X	X	X			X	X	X

From Synofzik, M., Hufnagel, R.B., Züchner, S. (2014). PNPLA6-related disorders. In: Pagon, R.A., Adam, M.P., Ardinger, H.H., Wallace, S.E., Amemiya, A., Bean, L.J.H., Bird, T.D., Ledbetter, N., Mefford, H.C., Smith, R.J.H., Stephens, K., (eds.). *GeneReviews*® [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2017, [updated 2015 Jun 11].

choanal atresia, ear malformations, and cranial nerve dysfunction). Other features may include choanal atresia, cleft lip/palate, anosmia, genital anomalies, congenital heart defects, tracheoesophageal anomalies, intellectual disability, growth retardation, and hypogonadotropic hypogonadism.

Isolated FSH and LH Deficit

FSH is a dimeric pituitary glycoprotein hormone composed of an α -subunit, encoded by a single gene (CGA) that is common to FSH, LH, hCG, TSH, and a β -subunit. The α -subunit protein is noncovalently joined to the β -subunit to form a biologically active hormone, but it is the β -subunit that confers hormone specificity. The 4.26 kb human FSHB gene is located on chromosome 11p14.1.

Only three males with FSH β mutations have been described in humans to date. The first was homozygous for a Cys82Arg missense mutation, the second demonstrated homozygosity for the Val61X, and the third homozygosity for a Tyr76X nonsense mutation in the FSH beta gene. All had azoospermia with variable testis size (from prepubertal to nearly normal); virilization and pubertal development were normal in two, while one had no evidence of pubertal development. None had gynecomastia or cryptorchidism. All have low/undetectable FSH, unresponsive to GnRH administration, and high LH serum level with exaggerated response to GnRH, while testosterone was low, low normal, or normal. Testicular biopsies were available in two patients and showed a variable degree of seminiferous tubule dysfunction (Siegel et al., 2013).

The LHB subunit gene is located at chromosome 19q13.32. Inactivating mutations of the human luteinizing hormone beta-subunit gene have been described in five men, who had normal genitalia at birth but restrained pubertal development and hypogonadism. Spermatogenesis impairment, ranging from oligozoospermia to azoospermia, may be due to the lack of LH stimulation and low intratesticular testosterone. These patients respond to exogenous hCG administration.

Genetic Causes of Primary (Hypergonadotropic) Hypogonadism

Myotonic Dystrophy

Myotonic dystrophy type 1 (DM1) is a chronic, slowly progressive multisystem disorder caused by expansion of a CTG trinucleotide repeat in the noncoding region of the myotonic dystrophy protein kinase gene (MDPK) that affects skeletal and smooth muscle and the central nervous system, the eye, the heart, and the endocrine system. A Danish study (Ørngreen et al., 2012) on 97 patients with DM1 showed that 17% of the male DM1 patients had increased LH and low levels of plasma testosterone indicating absolute androgen insufficiency, while another 21% had increased LH but normal testosterone levels, indicating relative insufficiency. Numbers of CTG repeats correlated directly with plasma LH and tended to correlate with plasma testosterone levels.

Myotonic dystrophy type 2 (DM2) is caused by a (CCTG) $_n$ expansion in CNBP and is clinically characterized by myotonia (90% of affected individuals) and muscle dysfunction (82%) and less commonly by cardiac conduction defects, posterior subcapsular cataracts, type 2 diabetes mellitus, and testicular failure.

Gonadotropin Resistance

Hormone resistance is caused by inactivating mutations in hormone receptors or by inactivation of the hormone signaling pathways. FSH receptor (FSHR)-inactivating mutations are able to affect the FSH signaling only when they occur in homozygosity. Five men homozygous for the Finnish p.Ala189Val FSHR gene mutation have been identified to date. This 566C $\rightarrow$ T mutation, predicting an alanine to valine substitution, is located in exon 7 of the FSHR gene, in the region encoding the extracellular domain of the receptor molecule, and leads to a marked reduction in ligand binding and signal transduction by the mutated receptor, according to functional testing.

The affected men had normal puberty, small to normal testes volume (4–15 mL), normal testosterone levels, elevated FSH levels, and low inhibin B level, with normal-to-elevated LH levels. Sperm concentrations ranged from <0.1 million/mL to 42 million/mL, and two out of five men fathered two children each (Huhtaniemi and Alevizaki, 2006). This latter finding prompted the investigators to hypothesize that FSH contributes to the quality and quantity of spermatogenesis but is not mandatory for this process per se, as the normal androgen production may be sufficient to maintain spermatogenesis in these patients. This hypothesis, however, is contradicted by the finding of azoospermia in men with FSH β mutations; moreover, findings in female patients with the same FSHR mutation suggest that such mutation could permit some residual receptor activity.

The LH receptor gene mutations will be discussed in the “Genetic Causes of Disorders of Male Sexual Development” section.

Autoimmune Polyendocrine Syndrome Type I

Autoimmune polyendocrine syndrome type I (APS I) is a rare autosomal recessive disorder caused by mutations in a single gene on chromosome 21q22.3, named AIRE, characterized by multiple organ-specific autoimmunity and ectodermal manifestations. The disease usually begins in childhood with candidiasis, followed by hypoparathyroidism and thereafter by adrenal insufficiency (classical triad). Other associated conditions include thyroid autoimmune diseases, chronic active hepatitis, malabsorption,

pernicious anemia, alopecia, and primary hypogonadism, the latter being associated with the presence of autoantibodies against the side-chain cleavage enzyme.

Aromatase Gene Defects

The enzyme aromatase is a member of the cytochrome P450 superfamily and is encoded by the CYP19A1 gene located at chromosome 15q21.2. Both aromatase enzyme and the CYP19A1 gene are expressed in the testis, adipose tissue, skin, and brain and catalyze the conversion of androgens (androstenedione and testosterone) into estrogens (estrone and estradiol).

Aromatase deficiency is an extremely rare disorder in humans. Men with aromatase deficiency usually present after puberty with tall stature due to unfused epiphyses, delayed bone age, eunuchoid skeletal proportions, and bone demineralization; other features may include insulin resistance, obesity, and dyslipidemia. Maternal severe virilization during the early pregnancy may occur. FSH, LH, and testosterone level may be normal high or elevated; the testes volume may be small, normal, or higher than normal (macroorchidism); and spermatogenesis may be normal or impaired. Estrogen replacement may improve the bone, lipid, and glucose metabolism (Bulun, 2014).

Genetic Causes of Disorders of Male Sexual Development

Androgen Receptor Gene Mutations

The androgen receptor (AR) gene is located at Xq11–12 and comprises eight exons; part of exon 1 of the androgen receptor gene encodes for the large activation function domain, exons 2 and 3 form the highly conserved DNA-binding domain (DBD), and exons 4–7 and part of exon 8 encode the ligand-binding domain (LBD). The AR transcriptional activity is inversely proportional to the size of the CAG triplet repeat sequence of the N-terminal domains.

Androgen insensitivity syndromes are disorders resulting from complete (CAIS), partial (PAIS), or mild (MAIS) resistance to androgens in boys or men with normal testis determination. Hundreds of AR mutations have been discovered to date: mutations localized in exons that encode the DBD (2–3) and LBD (4–8) lead to CAIS or PAIS. About 80% of the mutations in DBD and the majority of those in the LBD are missense mutations (Jääskeläinen, 2012).

Patients with CAIS are phenotypically female, presents in infancy with inguinal hernia or labial swelling containing a testis, with blind-ending vagina. Müllerian duct regression, being androgen-independent, is generally normal, and the patients lack a uterus, fallopian tubes, and a cervix. Adult patients show tall stature, high testosterone and LH levels, and normal FSH. Testosterone is peripherally aromatized to estrogens, resulting in serum estradiol concentrations higher than the average levels seen in men but lower than those reported in women. After orchiectomy, serum FSH and LH levels increase further but are only partially suppressed by exogenous estrogen administration, suggesting that a complete resistance to the negative feedback orchestrated by the androgens at the hypothalamus and pituitary level may be present, together with a partial integrity of the negative feedback control effected by the estrogens. Diagnosis may be challenging in infancy: serum AMH level assay may be of help, as its levels are higher in infants with CAIS than in male infants (Hughes et al., 2012). An AR mutation is identified in > 95% of patients with CAIS.

The typical phenotype of PAIS patients is micropenis, severe hypospadias (perineoscrotal), and a bifid scrotum that might contain gonads.

Men with MAIS may be infertile but do not show genital anomalies. In rare cases, high-dose androgen treatment may restore sperm count. The length of AR CAG repeat has been associated with impaired spermatogenesis and seems to correlate with serum testosterone concentration (Jääskeläinen, 2012). MAIS can also manifest as bulbar and spinal muscular atrophy (Kennedy's disease): in addition to the classic neurological features (weakness and wasting of bulbar, facial, and limb muscles), patients have increased testosterone levels associated with gynecomastia and infertility. These patients display a very high AR CAG repeat number (> 38) (Hughes et al., 2012).

5 α -Reductase-2 Deficiency

The 5 α -reductase-2 gene is located on the short arm of chromosome 2 band 23. This gene's enzyme product is expressed in high levels in the epididymides, seminal vesicles, prostate, genital skin, and liver and catalyzes the conversion of testosterone (T) to dihydrotestosterone (DHT). More than 60 mutations of the 5 α -reductase-2 gene have been identified, including those affecting the three largest family ties (New Guinean, Dominican, and Turkish); the condition is inherited as autosomal recessive. Mutations in all fixed exons of the gene have been documented, ranging from point mutations to entire gene deletions: the resulting enzymatic dysfunction ranges from impaired binding of substrate and cofactor to the isozyme, blocked formation of a functional isozyme, or unstable isozyme, with consequent DHT deficiency due to decreased T to DHT conversion (Kang et al., 2014).

At birth, males may display genital ambiguity: The testes and male accessory ducts are present, but since external genitalia development is dependent on intracellular conversion of T to DHT, the urogenital sinus and genital tubercle fail to differentiate normally into the external genitalia, urethra, and prostate, so patients have clitoral-like phallus, bifid scrotum, blind-end vagina, and hypospadias; therefore, most are assigned a female gender. At puberty, the increase in testosterone production leads to virilization (increased muscle mass, penile enlargement, vocal cord enlargement, and scrotal pigmentation), prompting most to transition to the male gender. Adult subjects show sparsity of body hair and the lack of hairline recession but have normal height given

that skeletal maturation is testosterone-dependent. Hormone assay reveals normal-to-elevated T levels and elevated testosterone-to-DHT ratio. A variable degree of spermatogenesis impairment is observed in these patients, but paternity may be achieved through IVF/ICSI (Kang et al., 2014).

CYP17A1 Gene Defects

The CYP17A1 enzyme catalyzes two distinct enzymatic steps in the steroidogenic pathway, the 17 α -hydroxylase and 17,20-lyase. More than 90 mutations in CYP17A1 have been identified to date, including missense and nonsense mutations, insertions, deletions, and splice site variants (Kim et al., 2014). Mutations in the CYP17A1 gene cause a rare form of congenital adrenal hyperplasia (1% of cases) due to complete combined 17 α -hydroxylase/17,20-lyase deficiency or isolated 17,20-lyase deficiency. Mutations affecting the CYP17A1 steroid-binding domain have been found to result in combined 17 α -hydroxylase and 17,20-lyase deficiency, whereas mutations in the redox partner domain may result in isolated 17,20-lyase deficiency. Affected males (46 XY) show under-masculinization of the external genitalia and may present with arterial hypertension and hypokalemia due to accumulation of precursor steroids with mineralocorticoid activity.

LH/hCG Receptor Gene Mutations

The LHR gene is located on chromosome 2p21; inactivation of both alleles through homozygous or compound heterozygous loss-of-function mutations is required to provide a phenotypic effect. Homozygous inactivating LH/hCG receptor (LHCGR) gene mutations in male subjects (46 XY) have been rarely found and usually result in undermasculinization, with female phenotypes (blind-ending vagina and primary amenorrhea). The absence of breast development at the age of puberty represents the clearest phenotypic difference between this condition and complete androgen insensitivity syndrome due to AR inactivating mutation of the androgen receptor, where normal breast development occurs at puberty. Microscopic examination of the removed (inguinal or intraabdominal) gonads showed seminiferous tubules containing normal Sertoli cells, occasional immature germ cells, but no mature Leydig cells. Hormone assay reveals low testosterone level, high LH, and normal FSH. Milder forms of LHR inactivation where the receptor is only partially inactivated result in male phenotypes with micropenis and descended testes. Both phenotypically male and female patients are irresponsive to exogenous hCG administration. Heterozygous mutations leading to constitutive activation of the LHCGR cause gonadotropin-independent precocious puberty in males (Huhtaniemi and Alevizaki, 2006).

References

- Balasubramanian, R., Dwyer, A., Seminara, S. B., Pitteloud, N., Kaiser, U. B., & Crowley, W. F., Jr. (2010). Human GnRH deficiency: A unique disease model to unravel the ontogeny of GnRH neurons. *Neuroendocrinology*, 92, 81–99.
- Boehm, U., Bouloux, P. M., Dattani, M. T., de Roux, N., Dodé, C., Dunkel, L., Dwyer, A. A., Giacobini, P., Hardelin, J. P., Juul, A., Maghnie, M., Pitteloud, N., Prevot, V., Raivio, T., Tena-Sempere, M., Quinton, R., & Young, J. (2015). European consensus statement on congenital hypogonadotropic hypogonadism—Pathogenesis, diagnosis and treatment. *Nature Reviews Endocrinology*, 11, 547–564.
- Bulun, S. D. (2014). Aromatase and estrogen receptor α deficiency. *Fertility and Sterility*, 101, 323–329.
- Davis, S. W., Keisler, J. L., Pérez-Millán, M. I., Schade, V., & Camper, S. A. (2016). All hormone-producing cell types of the pituitary intermediate and anterior lobes derive from Prop1-expressing progenitors. *Endocrinology*, 157, 1385–1396.
- Giordano, M. (2016). Genetic causes of isolated and combined pituitary hormone deficiency. *Best Practice & Research Clinical Endocrinology & Metabolism*, 30, 679–691.
- Hughes, I. A., Davies, J. D., Bunch, T. I., Pastorski, V., Mastroyannopoulou, K., & MacDougall, J. (2012). Androgen insensitivity syndrome. *Lancet*, 380, 1419–1428.
- Huhtaniemi, I., & Alevizaki, M. (2006). Gonadotrophin resistance. *Best Practice & Research. Clinical Endocrinology & Metabolism*, 20, 561–576.
- Jääskeläinen, J. (2012). Molecular biology of androgen insensitivity. *Molecular and Cellular Endocrinology*, 352, 4–12.
- Kang, H. J., Imperato-McGinley, J., Zhu, Y. S., & Rosenwaks, Z. (2014). The effect of 5 α -reductase-2 deficiency on human fertility. *Fertility and Sterility*, 101, 310–316.
- Kim, Y. M., Kang, M., Choi, J. H., Lee, B. H., Kim, G. H., Ohn, J. H., Kim, S. Y., Park, M. S., & Yoo, H. W. (2014). A review of the literature on common CYP17A1 mutations in adults with 17-hydroxylase/17,20-lyase deficiency, a case series of such mutations among Koreans and functional characteristics of a novel mutation. *Metabolism*, 63, 42–49.
- Mitchell, A. L., Dwyer, A., Pitteloud, N., & Quinton, R. (2011). Genetic basis and variable phenotypic expression of Kallmann syndrome: Towards a unifying theory. *Trends in Endocrinology and Metabolism*, 22, 249–258.
- Ørngreen, M. C., Arlien-Søborg, P., Duno, M., Hertz, J. M., & Vissing, J. (2012). Endocrine function in 97 patients with myotonic dystrophy type 1. *Journal of Neurology*, 259, 912–920.
- Siegel, E. T., Kim, H. G., Nishimoto, H. K., & Layman, L. C. (2013). The Molecular basis of impaired follicle-stimulating hormone action: Evidence from human mutations and mouse models. *Reproductive Sciences*, 20, 211–233.
- Synofzik, M., Hufnagel, R. B., & Züchner, S. (2014). PNPLA6-related disorders. In R. A. Pagon, M. P. Adam, H. H. Ardinger, S. E. Wallace, A. Amemiya, B. L.J.H., T. D. Bird, N. Ledbetter, H. C. Mefford, S. R.J.H., & K. Stephens (Eds.), *GeneReviews*® [internet]. Seattle (WA): University of Washington, Seattle (1993–2017, updated 2015 Jun 11).

Further Reading

- Kim, S. H. (2015). Congenital hypogonadotropic hypogonadism and Kallmann syndrome: Past, present, and future. *Endocrinology and Metabolism*, 30, 456–466.

Disorders of Sex Determination and Development

Rodrigo L Pagani and Ramy Abou Ghayda, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Disorders of Gonadal Differentiation

Klinefelter Syndrome

Klinefelter syndrome is the most common major abnormality of sexual differentiation, and is characterized by males with at least one Y chromosome and at least two X chromosomes. Males with the classic form of Klinefelter syndrome have small, firm testes on physical examination, and histology shows seminiferous tubule degeneration, and hyalinization. Patients with Klinefelter syndrome presents with elevated serum levels of FSH and LH, and low to normal serum levels of testosterone, therefore secondary sexual development may be compromised. Patients have a predisposition for being taller than average and gynecomastia can be noteworthy during puberty. The vast majority of patients are azoospermic, and the presence of sperm suggests 46,XY/47,XXY mosaicism (Diamond and Yu, 2016).

Fertility has been demonstrated to be feasible with the use of microsurgical testicular sperm extraction followed by intracytoplasmic sperm injection with a success rate for sperm retrieval as high as 40%–66% (Ramasamy et al., 2009). Since Klinefelter syndrome patients have a depletion of germ cells at puberty, this has led some expert to advocate for sperm extraction during adolescence for fertility preservation, however this approach remains controversial (Gies et al., 2016). Furthermore, these patients may need androgen supplementation to enhance libido and in some patients, reduction mammoplasty may be required. Surveillance for testicular and breast carcinoma is also warranted.

46,XX Testicular DSD

This condition is characterized by normal testicular development, despite the lack of a normal Y chromosome in subjects who have two X chromosomes. Most patients present later in life with hypergonadotropic hypogonadism, gynecomastia, and azoospermia. Testicular histology shows Sertoli cell only pattern and hyalinization of seminiferous tubules (Vorona et al., 2007).

Two categories of patients with XX testicular DSD have been identified, those who are SRY positive (usually with translocation of SRY to the X chromosome) and rarely have genital abnormalities, and those who are SRY negative and more commonly have genital ambiguity (Ergun-Longmire et al., 2005). Since these patients lack most part of Y chromosome, including genes responsible for spermatogenesis, there has not been a role for assisted reproductive technique in these patients. Similar to Klinefelter syndrome, some patients may benefit from androgen replacement and reduction mammoplasty, since there is an increased risk for breast carcinoma and testis tumor.

Mixed Gonadal Dysgenesis

Mixed gonadal dysgenesis is characterized by a unilateral streak gonad with a contralateral, often dysgenetic, testis. A variant where two dysgenetic testes are present, rather than one dysgenetic testis and a streak gonad, is called partial gonadal dysgenesis. Most of patients have a 45,XO/46,XY karyotype and persistence of Müllerian structures associated with varying degrees of masculinization, including normal male genitalia. In the neonatal period, mixed gonadal dysgenesis is the second most common cause of ambiguous genitalia after congenital adrenal hyperplasia. Histologically, testicular hyalinization, germinal aplasia, and Leydig cell hyperplasia are the most common findings (Johansen et al., 2012). Typically, infertility due to azoospermia exists, however, one case of successful sperm extraction has been reported (Flannigan et al., 2014).

Patients with mixed gonadal dysgenesis have a higher risk of developing a gonadal tumor such as gonadoblastoma and dysgerminoma. They are also at increased risk for Wilms tumor, therefore the appropriate management of these individuals comprises of gender assignment, gonadectomy, and proper screening for Wilms tumor (Diamond and Yu, 2016).

Embryonic Testicular Regression and Bilateral Vanishing Testis

Embryonic testicular regression denotes loss of testicular tissue within the first trimester of pregnancy whereas bilateral vanishing testes syndrome implies individuals in whom male sexual differentiation occurred but loss of testicular tissue took place later in utero. These conditions are exemplified by patients with a 46,XY karyotype and anorchia. Embryonic testicular regression has a wide spectrum of phenotypes ranging from complete female to varying degrees of ambiguous genitalia, while in bilateral vanishing testis a normal male phenotype with micropallus, absent prostate, and empty scrotum is commonly seen. There are no fertility treatment options available, however these patients may benefit from androgen replacement to assure a normal pubertal growth spurt with normal secondary sex characteristics (Diamond and Yu, 2016).

Ovotesticular DSD

Formerly known as true hermaphroditism, ovotesticular DSD occurs in those who encloses both testicular and ovarian tissues, which may take the form of one ovary and one testis or, more commonly, one or two ovotestes. Approximately 60% of the patients with ovotesticular DSD show a 46,XX karyotype, whereas 33% are mosaics 46,XX/46,XY, and 7% have a 46,XY karyotype. Patients are classified according to the type and location of the gonads. Lateral cases (20%) have a testis on one side and an ovary on the other. Bilateral cases (30%) have testicular and ovarian tissue present bilaterally, usually as ovotestes. Unilateral cases (50%) have an ovotestis present on one side and an ovary or testis on the other. The most crucial aspect about dealing with ovotesticular DSD patients is gender assignment. Since most patients present with masculinized ambiguous genitalia, they are usually raised as a male. In these patients, all ovarian and müllerian tissue should be removed (Diamond and Yu, 2016). The ovarian portion of the ovotestis is frequently normal, whereas the testicular portion is typically dysgenetic, therefore only two studies have reported successful pregnancies with sperm harvested from azoospermic men (Younis et al., 2011; Sugawara et al., 2012).

46,XY DSD (Undermasculinized Male)

Leydig Cell Aplasia

This rare autosomal recessive disorder is characterized by normal 46,XY male karyotype with a variety of phenotypes depending on the genetic mutation, with severe forms presenting as normal-appearing female external genitalia, and milder forms with micropenis or hypospadias (Van Batavia and Kolon, 2016). Testes are usually palpable in the inguinal canals or labia. Serum LH levels are elevated and testosterone levels are usually low. This disorder represents a spectrum between absent Leydig cells and Leydig cells with abnormal LH receptor. Incomplete forms of the disorder may also be present in patients with normal male genitalia. Recently, it was reported the first successful sperm retrieval with successful in vitro fertilization that resulted in a live birth (Bakircioglu et al., 2014).

Steroidogenic Acute Regulatory Protein (StAR) Deficiency

Steroidogenesis is the process in which cholesterol is converted into testosterone. The first step is based on the conversion of cholesterol to pregnenolone, mediated by an enzyme known as cholesterol side-chain cleavage (P450_{scc}) enzyme, that when defective result in a condition called congenital lipoid adrenal hyperplasia (Diamond and Yu, 2016).

The steroidogenic acute regulatory protein (StAR) promotes cholesterol transport from the outer to the inner mitochondrial membrane and has been also associated as a cause of congenital lipoid adrenal hyperplasia (Saenger, 1997).

Individuals with this condition are subject to present with severe glucocorticoid deficiency early in life and progressive mineralocorticoid insufficiency resulting in hyponatremia, hyperkalemia, dehydration, acidosis, and collapse. In 46,XY individuals, mutations in StAR typically cause a marked deficiency in testosterone synthesis by fetal Leydig cells so that completely female genitalia is encountered. Testes may be localized in the abdomen, inguinal channel, or in the labia. A blind vaginal pouch is present and müllerian structures have degenerated (Bhangoo et al., 2005). Some patients have mutations that retain partial activity, hence normal pubertal development and adult sexual function may occur, and fertility has been reported (Metherell et al., 2009).

P450 Oxidoreductase (POR) Deficiency

P450 oxidoreductase (POR) plays a central role in electrons transfer from NADPH to P450 enzymes, and its mutation cause a complex steroidogenic spectrum including partial P450_{c17}, aromatase, and 21-hydroxylase deficiencies (Krone et al., 2012). This mutation was originally characterized in patients with Antley-Bixler syndrome, some of whom had ambiguous genitalia and abnormal steroidogenesis. Mildly affected males may present with normal or delayed pubertal development and hypogonadism, nevertheless fertility has not been reported in this group (Idkowiak et al., 2011).

3 β -Hydroxysteroid Dehydrogenase Deficiency

A 3 β -hydroxysteroid dehydrogenase deficiency affects steroidogenesis in both the adrenal glands and testes, usually presenting in newborn boys with small phallus, severe hypospadias, ambiguous genitalia and salt-wasting adrenal insufficiency. Spontaneous pubertal development has been reported but testicular biopsy usually shows spermatogenic arrest or germinal aplasia, although one case of paternity has been documented (Guercio et al., 2015).

21-Hydroxylase Deficiency

Congenital adrenal hyperplasia (CAH) is the most common cause of the 46,XX DSD (masculinized female) and ambiguous genitalia at birth and a deficiency of steroid 21-hydroxylase is responsible for 95% of cases of CAH. Of these patients with 21-hydroxylase deficiency, 75% of patients presents with virilization and aldosterone deficiency, while 25% of patients presents

only with virilization. Plasma levels of progesterone and 17-hydroxyprogesterone are markedly elevated as well as urinary levels of 17-ketosteroids and pregnanetriol (Diamond and Yu, 2016).

In both males and females with aldosterone deficiency, symptoms begin within the first few weeks after birth, with failure to regain birth weight, progressive weight loss, and dehydration. Males with CAH appear phenotypically normal, and those without salt wasting may show sexual and somatic precocity. Therefore, these children have enlargement of the penis accompanied by the appearance of pubic hair, acne, and deepening of the voice.

Men with classic CAH have reduced fertility compared with controls, as well as higher rates of abnormal semen analysis, which is likely due to the presence of testicular adrenal rest tumors and suppression of the hypothalamic–pituitary–gonadal axis by high systemic levels of androgens (Reisch et al., 2009). One study comparing men with CAH to controls found that fertility rates in the CAH men were approximately half of those seen in the controls (Falhammar et al., 2012). While testicular adrenal rest tumors are considered to be benign, they can cause infertility through compression of the seminiferous tubules leading to obstruction, decreased spermatogenesis, and peritubular fibrosis (Van Batavia and Kolon, 2016).

17 α -Hydroxylase Deficiency

17 α -Hydroxylase catalyzes the conversion of pregnenolone and progesterone to 17 α -hydroxypregnenolone and 17-hydroxyprogesterone respectively. In 46,XY with complete 17 α -hydroxylase deficiency, individuals have a normal external female phenotype with blind-ending vaginal pouch. In 46,XY with partial 17 α -hydroxylase deficiency, a variety of phenotype may be present, including hypospadias, and cryptorchidism. No cases of fertility have been reported (Diamond and Yu, 2016).

17,20-Lyase Deficiency

Differently from complete 17 α -hydroxylase deficiency, complete 17,20-lyase deficiency results in ambiguous genitalia at birth of a 46,XY individual. Diagnosis is suspected in undervirilized males with failure to develop secondary sexual characteristics and elevated gonadotropin levels (Diamond and Yu, 2016).

17 β -Hydroxysteroid Oxidoreductase Deficiency

17 β -Hydroxysteroid oxidoreductase is the last enzyme in the biosynthesis of testosterone and catalyzes the conversion of androstenedione to testosterone, DHEA to androstanedione, and estrone to estradiol. Since most individuals have a normal female phenotype at birth, female gender assignment is commonly made, however as these patients have well-differentiated testes located in the abdomen or inguinal canal, the phallus growth, and secondary male sexual characteristics develop during puberty (Diamond and Yu, 2016). For individuals reared as female, treatment includes gonadectomy and estrogen replacement. For male gender of rearing, treatment includes orchiopexy and external genitalia reconstruction. Infertility due to azoospermia is the rule in these boys.

Complete Androgen Insensitivity Syndrome

Patients with complete androgen insensitivity syndrome (CAIS) are characterized by female-appearing external genitalia with short, blind-ending vagina, absence of müllerian ducts, and bilateral abdominal or inguinal testes in a 46,XY individual. Many patients are diagnosed later in life due to primary amenorrhea or the finding of testes during hernia repair. Testicular development is severely abnormal and the potential for male fertility is extremely low.

As the risk of developing a testicular tumor increases with age, including seminoma or gonadoblastoma, some authors advocate the need for gonadectomy after puberty, mainly because at these ages, the testes start to produce estradiol, which results in the appropriate changes for secondary sexual characteristics (Ahmed et al., 2000). Nevertheless, each patient should receive an individualized approach discussing all the possible scenarios, and at this point in time, all patients support a female gender identity (Hughes et al., 2012).

Partial Androgen Insensitivity Syndrome

The classic phenotype for patients is partial androgen insensitivity syndrome (PAIS) is that of a male with perineoscrotal hypospadias, cryptorchidism, rudimentary wolffian duct structures, and gynecomastia. However, variable phenotypes ranging from external genital ambiguity to male infertility may be found, and management of these patients is individualized according to findings in the phenotype (Diamond and Yu, 2016).

Patients raised as female should undergo gonadectomy and surgical reconstruction of the external genitalia at the time of diagnosis, while patients reared as males require treatment of cryptorchidism, reduction of gynecomastia, and genital reconstruction. Fertility (both spontaneously and with assisted reproduction) is possible in individuals raised as male, whereas in patients raised as female are always infertile (Tordjman et al., 2014).

Mild Androgen Insensitivity Syndrome

Males with mild androgen insensitivity syndrome (MAIS) are usually phenotypically normal or may have a history of mild hypospadias. These patients are often diagnosed during workup for male factor infertility and they usually present with azoospermia or severe oligospermia, and normal to elevated testosterone levels (Hiort and Holterhus, 2003).

5 α -Reductase Deficiency

5 α -reductase is a microsomal enzyme that catalyzes the conversion of testosterone to dihydrotestosterone (DHT), which is the main androgen driving the masculinization of the urogenital sinus and external genitalia. However, DHT does not play a major role in spermatogenesis. Affected patients show ambiguous genitalia with hypospadias and cryptorchidism. Typically, the phallus is quite small, appearing as a normal or enlarged clitoris (Maimoun et al., 2011). At puberty, spontaneous virilization occurs and adult patients report male libido and erections. On endocrine evaluation these individuals have elevated mean plasma testosterone but low DHT levels. Semen analysis is characterized by extremely low volume, increased viscosity, and poor liquefaction, attributed to rudimentary prostate glands, and small seminal vesicles (Katz et al., 1996).

In 2008, one study compared testicular histopathology of boys affected with the disease to boys with isolated bilateral cryptorchidism, and demonstrated that the first group presented with normal germ cell count and Ad spermatogonia, while the latter group had reduced total germ cell counts with poor cell maturation into Ad spermatogonia (Hadziselimovic and Dessouky, 2008). Most of the affected individuals are infertile, although spontaneous proven fertility was reported, as well successful pregnancies following assisted reproductive techniques (Katz et al., 1996; Nordenskjöld and Ivarsson, 1998).

Persistent Müllerian Duct Syndrome

Defects in testicular antimüllerian hormone production as well as defects in antimüllerian hormone receptors lead to persistent müllerian duct syndrome (PMDS), characterized by patients with 46,XY karyotype and normal external genitalia but internal müllerian duct structures. Therefore, they present with unilateral or bilateral undescended testes, bilateral fallopian tubes, uterus, and an upper vagina draining into a prostatic utricle (Diamond and Yu, 2016).

As all patients are phenotypically males, treatment involves orchidopexy and resection of the rudimentary müllerian structures. Azoospermia is common due to cryptorchidism or to damage of testicular blood supply during surgical procedures (Josso et al., 2012).

References

- Ahmed, S. F., Cheng, A., Dovey, L., et al. (2000). Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome. *The Journal of Clinical Endocrinology & Metabolism*, 85, 658–665.
- Bakircioglu, M. E., Tulay, P., Findikli, N., Erzik, B., Gultomruk, M., & Bahceci, M. (2014). Successful testicular sperm recovery and IVF treatment in a man with Leydig cell hypoplasia. *Journal of Assisted Reproduction and Genetics*, 31, 817–821.
- Bhangoo, A., Gu, W. X., Pavlakis, S., et al. (2005). Phenotypic features associated with mutations in steroidogenic acute regulatory protein. *The Journal of Clinical Endocrinology & Metabolism*, 90, 6303–6309.
- Diamond, D. A., & Yu, R. N. (2016). Disorders of sexual development: Etiology, evaluation, and medical management. In A. J. Wein, L. R. Kavoussi, A. W. Partin, & C. A. Peters (Eds.), *Campbell-Walsh urology* (11th edn, pp. 3469–3497). Philadelphia, PA: Elsevier.
- Ergun-Longmire, B., Vinci, G., Alonso, L., et al. (2005). Clinical, hormonal, and cytogenetic evaluation of 46,XX males and review of the literature. *Journal of Pediatric Endocrinology and Metabolism*, 18, 739–748.
- Falhammar, H., Nystrom, H. F., Ekstrom, U., Granberg, S., Wedell, A., & Thoren, M. (2012). Fertility, sexuality, and testicular adrenal rest tumors in adult males with congenital adrenal hyperplasia. *European Journal of Endocrinology*, 166, 441–449.
- Flannigan, R. K., Chow, V., Ma, S., & Yuzpe, A. (2014). 45,X/46,XY mixed gonadal dysgenesis: A case of successful sperm extraction. *Canadian Urological Association Journal*, 8, 108–110.
- Gies, I., Oates, R., De Schepper, J., & Tounaye, H. (2016). Testicular biopsy and cryopreservation for fertility preservation of prepubertal boys with Klinefelter syndrome: A pro/con debate. *Fertility and Sterility*, 105, 249–255.
- Guercio, G., Costanzo, M., Grinspon, R. P., & Rey, R. A. (2015). Fertility issues in disorders of sex development. *Endocrinology & Metabolism Clinics of North America*, 44, 867–881.
- Hadziselimovic, F., & Dessouky, N. (2008). Differences in testicular development between 5-alpha-reductase 2 deficiency and isolated bilateral cryptorchidism. *The Journal of Urology*, 180, 1116–1120.
- Hiort, O., & Holterhus, P. M. (2003). Androgen insensitivity and male infertility. *International Journal of Andrology*, 26, 16–20.
- Hughes, I. A., Houk, C., Ahmed, S. F., & Lee, P. A. (2006). Consensus statement on management of intersex disorders. *Archives of Disease in Childhood*, 91, 554–563.
- Hughes, I. A., Werner, R., Bunch, T., et al. (2012). Androgen insensitivity syndrome. *Seminars in Reproductive Medicine*, 30, 432–442.
- Idkowiak, J., O'Riordan, S., Reisch, N., et al. (2011). Pubertal presentation in seven patients with congenital adrenal hyperplasia due to P450 oxidoreductase deficiency. *The Journal of Clinical Endocrinology & Metabolism*, 96, 453–462.
- Johansen, M. L., Hagen, C. P., DeMeyts, E. R., et al. (2012). 45,X/46,XY mosaicism: Phenotypic characteristics, growth, and reproductive function—A retrospective longitudinal study. *The Journal of Clinical Endocrinology & Metabolism*, 97, 1540–1549.
- Josso, N., Rey, R., & Picard, J. Y. (2012). Testicular anti-müllerian hormone: Clinical applications in DSD. *Seminars in Reproductive Medicine*, 30, 364–373.
- Katz, M. D., Kilgman, I., Cai, L. Q., et al. (1996). Paternity by intrauterine insemination with sperm from a man with 5[alpha]-reductase-2 deficiency. *New England Journal of Medicine*, 336, 994–998.
- Krone, N., Reisch, N., Idkowiak, J., et al. (2012). Genotype–phenotype analysis in congenital adrenal hyperplasia due to P450 oxidoreductase deficiency. *The Journal of Clinical Endocrinology & Metabolism*, 96, 453–462.

- Maimoun, L., Philibert, P., Cammas, B., et al. (2011). Phenotypical, biological and molecular heterogeneity of 5[alpha]-reductase deficiency: An extensive international experience of 55 patients. *The Journal of Clinical Endocrinology & Metabolism*, 96, 296–307.
- Metherell, L. A., Naville, D., Halaby, G., et al. (2009). Nonclassic lipoid congenital adrenal hyperplasia masquerading as familial glucocorticoid deficiency. *The Journal of Clinical Endocrinology & Metabolism*, 94, 3865–3871.
- Nordenskjold, A., & Ivarsson, S. A. (1998). Molecular characterization of 5 alpha-reductase type 2 deficiency and fertility in a Swedish family. *The Journal of Clinical Endocrinology & Metabolism*, 83, 3236–3238.
- Ramasamy, R., Ricci, J. A., Palermo, G. D., Gosden, L. V., Rosenwaks, Z., & Schlegel, P. N. (2009). Successful fertility treatment for Klinefelter's syndrome. *The Journal of Urology*, 182, 1108–1113.
- Reisch, N., Flade, L., Scherr, M., et al. (2009). High prevalence of reduced fecundity in men with congenital adrenal hyperplasia. *The Journal of Clinical Endocrinology & Metabolism*, 94, 1665–1670.
- Saenger, P. (1997). New developments in congenital lipid adrenal hyperplasia and steroidogenic regulatory protein. *Pediatric Endocrinology*, 44, 397–421.
- Sugawara, N., Kimura, Y., & Araki, Y. (2012). Successful second delivery outcome using refrozen thawed testicular sperm from an infertile male true hermaphrodite with a 46,XX/46,XY karyotype: Case report. *Human Cell*, 25, 96–99.
- Tordjman, K. M., Yaron, M., Berkovitz, A., Botchan, A., Sultan, C., & Lombroso, S. (2014). Fertility after high-dose testosterone and intracytoplasmic sperm injection in a patient with androgen insensitivity syndrome with a previously unreported androgen receptor mutation. *Andrologia*, 46, 703–706.
- Van Batavia, J. B., & Kolon, T. F. (2016). Fertility in disorders of sex development: A review. *Journal of Pediatric Urology*, 12, 418–425.
- Vorona, E., Zitzmann, M., Gromoll, J., Schuring, A. N., & Nieschlag, E. (2007). Clinical, endocrinological, and epigenetic features of the 46,XX male syndrome, compared with 47,XXY Klinefelter patients. *The Journal of Clinical Endocrinology & Metabolism*, 92, 3458–3465.
- Younis, J. S., Radin, O., Kerner, H., & Ben-Ami, M. (2011). Successful monozygotic twin pregnancy fathered by a male 46,XY true hermaphrodite. *Reproductive Biomedicine Online*, 22, 80–82.

Congenital Bilateral Absence of the vas Deferens

Gabriella Avellino and Robert D Oates, Boston University School of Medicine, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Congenital bilateral absence of the vas deferens The clinical condition resulting from an absence of the reproductive ductal structures that transport sperm from the testis to the ejaculate.

Cystic fibrosis transmembrane conductance regulator (CFTR) The protein product of the cystic fibrosis or *CFTR* gene.

Intracytoplasmic sperm injection An advanced reproductive technique where an individual sperm is micro-injected into the cytoplasm of a harvested egg.

Introduction

Congenital bilateral absence of the vas deferens (CBAVD), as a primary disorder, presents as low volume azoospermia and consequent infertility (Harnisch and Oates, 2012). The vasa deferentia constitute the major portion of the male reproductive ductal system and transport sperm from the testis to the ejaculate and are classically not able to be felt upon scrotal examination. CBAVD may be diagnosed as an isolated condition when the male has no recognizable pulmonary or pancreatic symptoms, but will always be present in boys and men with clinically diagnosed cystic fibrosis (CF). Asymptomatic CBAVD and classic CF are often two ends of a spectrum of genetic disease, as described more fully below. Finally, some cases of CBAVD coexist with abnormalities of the urologic tract, specifically unilateral renal agenesis and/or ectopy. CBAVD is found in 1%–2% of infertile men (Hotelling, 2014).

Phenotypic and Semen Analysis Findings in Men With CBAVD

Men with CBAVD have a normal hypothalamic-pituitary–gonadal axis. Libido and testicular function are fine as regards testosterone synthesis and sperm production (spermatogenesis). A brief review of male reproductive embryology is necessary in order to understand the anatomical findings and anomalies in CBAVD. During early embryogenesis and fetal development, each mesonephric duct (left and right) gives rise to two separate derivatives in and around week 7 of gestation. The most superior (anatomically) becomes the ureter and intrarenal collecting system while the most inferior (anatomically) gives rise to the distal two-thirds of the epididymis, the entire vas deferens, the seminal vesicle and the ejaculatory duct. The first portion of the epididymis (the caput—an entanglement of 6–8 efferent ducts) does not derive from the mesonephric duct and connects directly to the rete testis (the first collecting area for newly formed sperm within the testis). Therefore, the caput of the epididymis is always present in CBAVD, the more distal epididymides may be present to a quite variable degree, the vasa are absent by definition, and the seminal vesicles are typically deficient or non-functional.

The seminal vesicles produce the majority of the fluid found in the ejaculate and all of the alkalinity (Centola, 2014). The prostate is unaffected in CBAVD and secretes a small amount of acidic fluid. When the male is unaffected by any abnormalities, the volume and alkalinity of the seminal vesicle fluid overwhelms the small amount of prostatic fluid and so the ejaculate, as a final mixture, is of a “normal” volume and alkaline pH. In CBAVD, however, the seminal fluid consists of just a small amount of prostatic contribution which is acidic, averaging about 0.6 cm³ and pH of 6.5. There is no contribution from the seminal vesicles as they are absent or severely atretic and no sperm as the vasa deferentia are absent. In consequence, a low volume, acidic, azoospermic ejaculate should always raise suspicion of CBAVD, which is confirmed easily upon physical examination: adequately sized testes, at a minimum the caput epididymis is present, the vasa are not able to be palpated (Oates, 2012).

The Genetic Basis of Most Cases of CBAVD: Mutations in *CFTR*

CF is a common autonomic recessive disorder in Caucasians of Northern European descent, found in 1 in 2500–3500 neonates in the United States. Comparatively, it is diagnosed in about 1 in 17,000 African Americans and 1 in 31,000 Asian Americans (Farrell et al., 2017). Over 2000 mutations of the CF gene (also termed *CFTR* for its protein product (Cystic Fibrosis Transmembrane Conductance Regulator; located on the long arm of chromosome 7) have been discovered and annotated (Deignan and Grody, 2016). The most common mutation worldwide is referred to as deltaF508. The *CFTR* gene codes for a transmembrane chloride ion transport channel that helps to balance salt and water concentrations across the cell membrane and keep the secretions of certain epithelial cells in the lungs and pancreas, to name just a couple organs, thin and watery (Cholon and Gentzsch, 2017). When the total pool of cellular *CFTR* is poorly functional, the above mentioned secretions are thick and tenacious and eventually clog and obstruct the tubular system of the airways or the pancreas. During fetal development, *CFTR* also helps to maintain patency and

viability of the developing vas deferens, distal two-thirds of the epididymis and seminal vesicles. Again, when the total cellular functional pool of *CFTR* is reduced, these reproductive ductal structures may not continue their normal development and become atretic and, essentially, disappear.

The genotype–phenotype correlation, when there are defects in *CFTR* quantity or quality, is at the heart of understanding why some males have clinical CF with compromised pulmonary and pancreatic function as well as bilateral absence of the vasa deferentia while other males are perfectly healthy with normal pulmonary and pancreatic function but an absence of the vas deferens bilaterally and infertility. To have any form of recognizable disease, one must inherit a *CFTR* mutation from both parents, that is, a maternal mutation and a paternal mutation. It is the nature of each mutation and the combination of the two that determines the severity of the disease that the patient will exhibit. If a male inherits a *CFTR* mutation from his mother classified as “severe” and one from his father, also classified as “severe,” his total amount of adequately functioning cellular *CFTR* protein will be minimal, if not absent, and his secretions in the respiratory tree and pancreatic excurrent ducts will be viscous and glutinous leading to significant pulmonary and pancreatic disease. His vasa and seminal vesicles will also be absent. His clinical diagnosis will unfortunately be cystic fibrosis. However, if one of the two of those mutations is classified as “mild,” then his cellular *CFTR*, as an aggregate, may have enough quantity and quality to allow the lungs and pancreas to be relatively unaffected (his *CFTR* works “well enough” and his secretions in these organs/systems are thin and fluid “enough”) that he will not express disease until recognized to have CBAVD when presenting for infertility evaluation. The development and maintenance of the reproductive ductal structures is, therefore, the most sensitive indication of *CFTR* mutations in both maternally and paternally inherited alleles (Anguiano et al., 1992). Of course, an individual patient may present anywhere along the spectrum between those two extremes, for example, a male with newly diagnosed CBAVD who has a chronic history of sinusitis or bronchitis. It is the nature of the combination of mutations that determines the phenotype, and by extension the clinical diagnostic term, for the patient. Hence, when a male is diagnosed with CBAVD, it is necessary to obtain CF mutation analysis. Yu et al. provide a comprehensive review of the mutations found in CBAVD, the most common being the 5T allele (25%, considered “mild” in severity) while deltaF508 was the second most common at 17% of alleles (Yu et al., 2012). The combination of deltaF508 and 5T was found in 17% of CBAVD cases. However, the exact frequency and pattern shows skewing based upon inherited ethnicity (Xu et al., 2014). When CF mutation analysis is positive, family screening should also be carried out to inform the patient’s siblings (usually of reproductive age as well) of their risk of harboring one or two *CFTR* mutations. When positive, a new perspective on the etiology and optimal treatment of any heretofore unrecognized CF mutation spectrum coexisting illnesses such as sinusitis, pancreatitis, bronchitis may be realized. Even though the clinical diagnosis of CBAVD is relatively easy and straight forward to make with history and physical examination, the discovery of the underlying genetic basis is important for the above reasons and testing should not be felt unnecessary—it is often a helpful adjunct in cases of CBAVD.

Recently, a new genetic basis, involving *CFTR* function but not the *CFTR* gene, has been identified and will be important to define in selected cases. Patat et al. described four men with *CFTR*-negative CBAVD who were shown to have mutations in *ADGRG2*, which resides on the X chromosome and, therefore, would be maternally transmitted in X-linked fashion (Patat et al., 2016).

The Genetic Basis of a Few Cases of CBAVD: Maldevelopment of the Mesonephric Ducts

Reflecting back on the embryology of the reproductive ductal system, it is obvious that mutations in *CFTR* adversely affect the development of the vasa deferentia long after week 7 of gestation, that is, long after the separation of the two divisions of the mesonephric duct as the kidneys and ureters are normal in *CFTR* mutation directed CBAVD. However, a second broad genetic etiology exists in which there are putative genetic abnormalities which compromise mesonephric duct development at its earliest stages prior to week 7 of gestation. This insult derails normal morphogenesis of both the urinary and reproductive ductal derivatives of the mesonephric ducts. If the end result is bilateral renal agenesis and bilateral vasal agenesis, that individual will not be able to present later in life for an infertility evaluation, for obvious reasons. But if the affront is slightly less severe in totality, then one of the two renal/ureteral derivatives may form, allowing that individual to be healthy with one kidney and present later in adult life with infertility due to CBAVD (1 of the 4 “offspring” of the 2 mesonephric ducts matured and progressed but not the other 3). When *CFTR* mutation analysis is non-informative, it is necessary to perform renal ultrasonography to identify possible unilateral renal agenesis and counsel the patient and his partner accordingly (Schwarzer and Schwarz, 2012). From the data published by McCallum et al., it does not appear that it is likely that unilateral or bilateral renal agenesis will be transmitted to any conceived offspring, although it is possible (McCallum et al., 2001).

Fertility Options for Men With CBAVD

Prior to the advent of advanced reproductive technologies, CBAVD resulted in sterility. However, in the present day, harvested sperm from the epididymis or testis can be used in conjunction with intracytoplasmic sperm injection (ICSI) to achieve biological paternity (Elhanbly et al., 2015; van Wely et al., 2015). ICSI involves oocyte harvest after gonadotropin stimulation and in vitro placement of a single spermatozoan into the interior of the oocyte with a micropipette. Depending upon the philosophy of the practitioners involved, a limited number of embryos generated are transferred a variable number of days later. Since spermatogenesis is not generally an issue, sperm can be surgically retrieved from the most proximal portions of the epididymis (the caput;

		Male <i>CFTR</i> Mutation Status	
		$\Delta F508$	5T
Female <i>CFTR</i> Mutation Status	$\Delta F508$	$\Delta F508 / \Delta F508$ Cystic Fibrosis	$\Delta F508 / 5T$ CBAVD
	+	$\Delta F508 / +$ Carrier (unaffected)	$5T / +$ Carrier (unaffected)

Fig. 1 Punnet square of offspring genotype possibilities in men with *CFTR* mutation directed CBAVD and a female simple heterozygote partner. Embryos shown to be compound heterozygotes for delta F508/delta F508 would not be transferred as they will manifest full-blown CF while embryos that inherit only one mutation (simple heterozygotes; carriers) will be transferred and will become healthy individuals.

fferent ducts) or the testis tissue and will, when injected directly into an egg during ICSI, effect fertilization, embryo development and live birth. Whether the sperm are collected with an open scrotal operation as in microsurgical epididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESE) or testis sperm extraction (TESE) is generally a matter of patient, clinician, and embryologist choice, although there is data suggesting an improved overall outcome with epididymal sperm. What is not possible, is anything short of ICSI as the sperm harvested have limited capacity to carry out normal functions of the sperm in a natural setting. These include progressive motility, acrosome reaction and fertilization of an oocyte by penetrating the zona pellucida and the egg membrane. Once placed into the oocyte cytoplasm during ICSI, they work quite well.

It is necessary to screen the female partner for *CFTR* mutations as well. Approximately 1:25 people of Northern European descent is a carrier of a *CFTR* mutation. A carrier is asymptomatic, has only one *CFTR* allele affected while the other is normal, and is also known as a simple heterozygote. If the male carries two identifiable *CFTR* mutations, as is so often the case, and the female is a simple heterozygote, then 50% of the couple's embryos will have two *CFTR* mutations as shown in Fig. 1. Each embryo will always get one of the two *CFTR* mutation alleles the male possesses and will either get the mutated allele or the normal allele from the female partner. If the female partner is a carrier, then, each embryo can be genetically investigated and only those embryos that were NOT burdened with the maternal *CFTR* mutation transferred (Girardet et al., 2015). In this way, the couple's offspring will be obligate carriers but will not express *CFTR* mutation spectrum disease because they will not have abnormalities in both *CFTR* alleles.

Conclusion

Congenital bilateral absence of the vas deferens (CBAVD) is a clinical condition that typically presents at the time of an evaluation for infertility. An azoospermic semen specimen that is of low volume and an acidic pH is characteristic. Physical examination of the reproductive ductal structures and the testes reveal absence of the vas deferens and, always, at least the caput of the epididymis. Most cases of CBAVD are caused by mutations in both parental cystic fibrosis gene alleles (maternally and paternally inherited CF genes). Some cases are caused by defects in the development of the mesonephric ducts during embryogenesis that may result in associated renal agenesis. No known genetic etiology has yet been elucidated for this subset of CBAVD. Proper genetic screening is mandatory for patient, spouse and appropriate family members. Sperm can be successfully harvested in a number of different ways and used with great efficacy to generate pregnancies and healthy offspring. What was once impossible—pregnancy—is now routine.

References

- Anguiano, A., Oates, R. D., Amos, J. A., Dean, M., Gerrard, B., Stewart, C., Maher, T. A., White, M. B., & Milunsky, A. (1992). Congenital bilateral absence of the vas deferens. A primarily genital form of cystic fibrosis. *JAMA*, 267(13), 1794–1797.
- Centola, G. M. (2014). Semen assessment. *The Urologic Clinics of North America*, 41(1), 163–167.
- Cholon, D. M., & Gentzsch, M. (2017). Recent progress in translational cystic fibrosis research using precision medicine strategies. *Journal of Cystic Fibrosis* (Epub ahead of print).
- Deignan, J. L., & Grody, W. W. (2016). Molecular diagnosis of cystic fibrosis. *Current Protocols in Human Genetics*, 88, Unit 9 28.
- Elhanbly, S., El-Saied, M. A., Fawzy, M., El-Refaeey, A., & Mostafa, T. (2015). Relationship of paternal age with outcome of percutaneous epididymal sperm aspiration-intracytoplasmic sperm injection, in cases of congenital bilateral absence of the vas deferens. *Fertility and Sterility*, 104(3), 602–606.
- Farrell, P. M., White, T. B., Ren, C. L., Hempstead, S. E., Accurso, F., Derichs, N., Howenstine, M., McColley, S. A., Rock, M., Rosenfeld, M., Sermet-Gaudelus, I., Southern, K. W., Marshall, B. C., & Sosnay, P. R. (2017). Diagnosis of cystic fibrosis: consensus guidelines from the cystic fibrosis foundation. *The Journal of Pediatrics*, 181S, S4–S15 e1.
- Girardet, A., Ishmukhametova, A., Willems, M., Coubes, C., Hamamah, S., Anahory, T., Des Georges, M., & Claustres, M. (2015). Preimplantation genetic diagnosis for cystic fibrosis: The Montpellier center's 10-year experience. *Clinical Genetics*, 87(2), 124–132.
- Harnisch, B., & Oates, R. (2012). Genetic disorders related to male factor infertility and their adverse consequences. *Seminars in Reproductive Medicine*, 30(2), 105–115.
- Hotelling, J. M. (2014). Genetics of male infertility. *The Urologic Clinics of North America*, 41(1), 1–17.
- McCallum, T., Milunsky, J., Munarriz, R., Carson, R., Sadeghi-Nejad, H., & Oates, R. (2001). Unilateral renal agenesis associated with congenital bilateral absence of the vas deferens: Phenotypic findings and genetic considerations. *Human Reproduction*, 16(2), 282–288.
- Oates, R. (2012). Evaluation of the azoospermic male. *Asian Journal of Andrology*, 14(1), 82–87.

- Patat, O., Pagin, A., Siegfried, A., Mitchell, V., Chassaing, N., Faguer, S., Monteil, L., Gaston, V., Bujan, L., Courtade-Saidi, M., Marcelli, F., Lalau, G., Rigot, J. M., Mieusset, R., & Bieth, E. (2016). Truncating mutations in the adhesion G protein-coupled receptor G2 gene ADGRG2 cause an X-linked congenital bilateral absence of vas deferens. *American Journal of Human Genetics*, 99(2), 437–442.
- Schwarzer, J. U., & Schwarz, M. (2012). Significance of CFTR gene mutations in patients with congenital aplasia of vas deferens with special regard to renal aplasia. *Andrologia*, 44(5), 305–307.
- van Wely, M., Barbey, N., Meissner, A., Repping, S., & Silber, S. J. (2015). Live birth rates after MESA or TESE in men with obstructive azoospermia: Is there a difference? *Human Reproduction*, 30(4), 761–766.
- Xu, X., Zheng, J., Liao, Q., Zhu, H., Xie, H., Shi, H., & Duan, S. (2014). Meta-analyses of 4 CFTR variants associated with the risk of the congenital bilateral absence of the vas deferens. *Journal of Clinical Bioinformatics*, 4, 11.
- Yu, J., Chen, Z., Ni, Y., & Li, Z. (2012). CFTR mutations in men with congenital bilateral absence of the vas deferens (CBAVD): A systemic review and meta-analysis. *Human Reproduction*, 27(1), 25–35.

Non-CBAVD Extratesticular Ductal Disorders

Alexandra J Berger and Martin N Kathrins, Harvard University, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Extratesticular ductal disorders have been reported in up to 85% of patients with obstructive azoospermia (Hendry et al., 1983). Obstruction anywhere along the pathway of sperm and semen production and ejaculation can lead to infertility; this includes disruption of the epididymides, vasa deferentia, seminal vesicles or ejaculatory duct (Fig. 1, below). Such patients typically have normal or enlarged testicular volume and physical exam findings which may elucidate the origin of the obstruction (EU 2012). This includes enlarged or indurated epididymis with nodularity, absence of one or both vas deferens or a boggy or tender prostate concerning for prostatitis. The serum follicle stimulating hormone will often be within normal limits.

Epididymal disorders

Epididymal disorders are the most common causes of obstructive azoospermia, occurring in 65% of such cases (Schoysman, 1990; Matsumiya et al., 1994). These obstructions are typically acquired but can also be congenital. Acquired obstruction are most often infectious or inflammatory in etiology, but can also be due to scrotal trauma or prior scrotal surgery.

Epididymitis is a frequent cause of acquired epididymal obstruction. Acute or chronic epididymitis can lead to scarring which chronically occludes the epididymal ducts. The specific etiology of epididymitis is unclear in many patients and may be inflammatory or infectious. Chlamydia trachomatis is the most frequently acquired sexually transmitted infection and is thought to cause both acute and chronic post-infectious disease. Neisseria gonorrhoeae, E. coli, and other bacteria that cause urinary tract infections can also cause epididymitis. Granulomatous epididymitis due to tuberculosis is rare in the developed world but is a cause of infertility in countries with higher burden of disease. Non-infectious causes include generalized diseases such as Behcet's or sarcoidosis. Patients can develop chemical epididymitis from drugs such as amiodarone or due to retrograde flow of urine through the epididymis while exercising or during sexual intercourse, particularly in older men (Nickel, 2003).

Epididymal disjunction and epididymal or ductal atresia are congenital causes of epididymal obstruction and occur during fetal development. The testis and head (caput) of the epididymis both derive from the genital ridge. In contrast, the epididymal body (fundus) shares its origins with the vas deferens, developing from the mesonephric tubules and Wolffian duct. The epididymal caput and fundus join when the rete testis and mesonephric tubules come together starting at 12 weeks of fetal age. In epididymal disjunction, the efferent ducts fail to reach the testis, possibly because they do not properly coil and elongate (Kroovand and Perlmuter, 1981). This failure causes incomplete union of the epididymis and the testis, with the tail, caput or even the entire epididymis totally dissociated with the testis. Epididymal or ductal atresia is thought to occur due to a vascular accident associated with the involution of the mesonephric duct during this same time period (Dickinson, 1973). Both epididymal disjunction and atresia are closely associated with cryptorchidism and patency of the processus vaginalis and are often diagnosed at time of orchiopexy (Marshall and Shermeta, 1979; Kim et al., 2015).

Congenital epididymal obstruction also occurs in Young syndrome. Young – or sinusitis-infertility - syndrome is a rare disorder affecting the respiratory and reproductive systems. The disorder was first described in 1984 (Handelsman et al., 1984). Patients

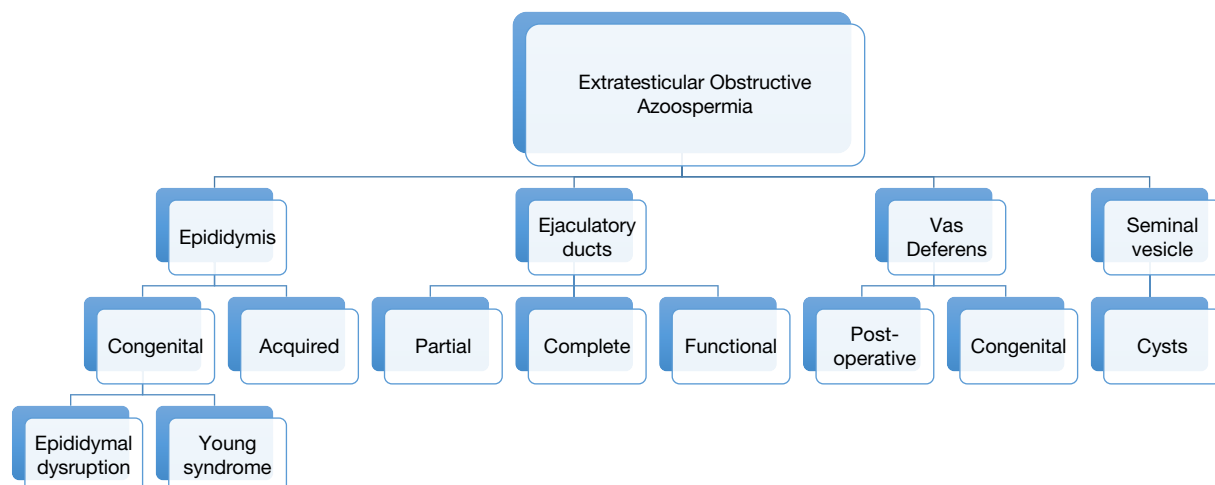


Fig. 1 Extratesticular obstructive azoospermia.

characteristically have chronic sinusitis, bronchiectasis and obstructive azoospermia caused by epididymal obstruction. Patients may also have structural sperm abnormalities in the axoneme which can cause motion abnormalities. It is believed that the disorder arises from abnormal ciliary function or mucous quality, however the exact mechanism remains unknown.

Patients with epididymal disruption or obstruction typically present with infertility and possibly scrotal pain. It is common for these patients to endorse a history of previous scrotal surgery, epididymo-orchitis, urinary tract infection or sexually transmitted infection. On physical exam, they may have an enlarged, indurated epididymis with palpable cysts. On semen analysis, the classic findings are azoospermia with a normal ejaculate volume. In patients with acquired obstruction, scrotal ultrasound may reveal an enlarged, hypoechoic epididymis consistent with epididymitis (Jhaveri et al., 2010). The diagnosis of epididymal obstruction is often difficult to make. This is especially true in patients with unilateral or partial obstructions. These patients may present not with azoospermia but oligo- or asthenozoospermia. The conclusive diagnosis of epididymal obstruction is typically made at the time of surgical exploration by performing a vasogram (Chan, 2013).

Patients with suspected epididymal obstruction are candidates for surgical reconstruction with vasoepididymostomy. In this procedure, an anastomosis is created between the vas deferens and the epididymis in order to bypass the area of epididymal obstruction. It is a technically challenging operation and more demanding than vasovasostomy (vas to vas anastomosis). During the procedure, fluid from the epididymis is extracted and examined for sperm content. The urologist continues with the vasoepididymostomy only if live sperm are visualized. Vasoepididymostomy is often not technically feasible in patients with congenital disruptions or ductal atresia and is most successful in patients with acquired obstructions. The group most likely to benefit are those with infectious obstructions. In one study, only 52% of patients with clinically suspected epididymal obstruction were identified to have an obstruction at the time of surgery; the remainder had other anatomic abnormalities and most of these were not amenable to repair. The post-operative patency rate ranges in the literature from 50 to 80% with about 60% of men after technically successful vasoepididymostomy having live sperm in their ejaculate (Berardinucci et al., 1998). The natural pregnancy rate is about 30% (Peng et al., 2012).

Vas deferens

Disruption of the vas deferens is almost always intentional, occurring after vasectomy. Another acquired cause of vas obstruction is iatrogenic injury following hernia repair, particularly when a mesh plug is employed. Infertility due to congenital disorders of the vas deferens are also possible.

Congenital unilateral absence of the vas deferens (CUAVD) is an infrequent cause of infertility. The prevalence of CUAVD in published studies is highly variable—from 0.08% to 1.25%. Most recently, a 2016 retrospective chart review of 23,000 patients undergoing vasectomy revealed only 82 men with likely UAVD, a prevalence of 0.36% (Miller et al., 2016). Even when present, the majority of patients are not subfertile. Among subfertile males with CUAVD, the majority have genitourinary anomalies of the contralateral testis or ducts (Schlegel et al., 1996). Just as in CBAVD, congenital unilateral absence of the vas deferens (CUAVD) is associated with CF mutations; the majority of these patients have concurrent occlusion of the contralateral vas and resultant infertility (Mickle et al., 1995). There is also a strong relationship between CUAVD and unilateral renal agenesis, especially in patients without CF mutations. Up to 1/3 of patients with CUAVD also have ipsi- or contralateral renal agenesis (Kolettis and Sandlow, 2002). Because of the strong association, all men with CUAVD should be screened with renal ultrasonography for concurrent renal agenesis. In the absence of such retroperitoneal pathology revealed by sonograms, genetic testing for cystic fibrosis carrier status should then be performed.

Ejaculatory Duct Obstruction

Ejaculatory duct obstruction (EDO) is an uncommon cause of OA, occurring in 1–5% of cases (Meacham et al., 1993; Matsumiya et al., 1994). The classic presentation of these patients is the triad of painful ejaculation, infertility and hematospermia. Patients may endorse a history of prior UTI, epididymitis, perineal trauma or testicular or perineal pain.

EDO can be congenital or acquired and is most commonly due to either cyst formation or postinflammatory change. Mullerian duct (prostatic utricle) or Wolffian duct (diverticular) cysts are typically congenital and occur medially in the prostate between the ejaculatory ducts, obstructing them. Prostatic cysts only rarely cause EDO. When not enlarged, the prostatic utricle is not visible on transrectal ultrasound (TRUS). When enlarged, however, utricle cysts can be visualized with TRUS and are often found incidentally during studies performed for other reasons. If symptomatic, these cysts can contribute to LUTS and cause pain, hematospermia, urinary tract infections, or infertility. Treatment is only recommended in symptomatic patients and consists of either cyst puncture or endoscopic marsupialization (Coppens et al., 2002).

The most common cause of postinflammatory EDO is urethral prostatitis. Postoperative adhesions after TURP are an iatrogenic cause. Other, rarer, causes of EDO include seminal vesicle calculi, calcifications near the verumontanum or congenital ejaculatory duct atresia. About 50% of patients with idiopathic EDO have CF mutations, therefore genetic screening is recommended in all patients with EDO of unclear cause (Jarvi et al., 1995).

Patients with EDO should undergo semen analysis, which typically reveals azoospermia with low semen volume, pH < 7.2 and absence of fructose in the specimen. It is important to screen patients with low ejaculate volume to ensure they are not taking medications that impair ejaculation. Patients presenting with either persistent or recurrent hematospermia should also be evaluated with TRUS. This is the gold standard in diagnosis of EDO. Enlarged seminal vesicles (width > 1.5 cm) or ejaculatory ducts (diameter

> 2.3 cm) on TRUS are suggestive of EDO, especially in combination with visualization of cyst or calcification in the area of the duct. TRUS is also useful to rule out malignancy, which is found in about 4% of patients with persistent hematospermia (Ahmad and Krishna, 2007).

While it was originally described in men with complete blockages, EDO is thought to represent a spectrum of disease. Patients with either unilateral or partial bilateral obstructions may present with oligoasthenozoospermia instead of azoospermia. The diagnosis and treatment of such a condition has not been rigorously studied yet. They may also have normal ejaculate volume. In contrast, patients with complete or functional complete bilateral obstructions present with low ejaculate and azoospermia.

EDO should be treated in men with bothersome ejaculatory or postejaculatory pain, infertility or recurrent/persistent hematospermia. The first step is cessation of all medications that reduce ejaculatory volume or lead to ejaculatory dysfunction. If this is not thought to be the cause, the standard of care for symptomatic patients is a transurethral resection of the ejaculatory ducts (TURED). This is an outpatient procedure during which a cystourethroscope with an electrocautery loop is used to resect the verumontanum in the midline. If the patient has a unilateral obstruction, the verumontanum can be resected only on that side. Another technique is to make multiple incisions in the verumontanum without resecting the structure. It is important to use electrocautery judiciously when achieving hemostasis during these procedures because of the risk of fulgurating and reobstructing the ejaculatory ducts. At the conclusion of the resection, milky fluid is often visualized refluxing from the open ducts; this is a sign that the procedure has been successful. Semen analysis values will improve as early as 2 weeks post-operatively and are followed until they normalize. In studies, 20%–30% of patients were able to achieve pregnancy after TURED with equal outcomes for patients with partial and complete obstruction. Common adverse events associated with TURED include epididymo-orchitis and watery ejaculate. Reobstruction also occurs in a significant minority of patients and may require re-TURED.

Seminal Vesicle

The seminal vesicles produce 80–90% of ejaculate volume, but vesicular obstruction or anomalies are a rare cause of infertility. Seminal vesicle cysts can be congenital and can cause blockage and therefore infertility. When they do occur, the cause is sometimes an ectopic ureter draining into the seminal vesicle from a dysplastic kidney. In fact, the seminal vesicle is the second most common place for ureteric ectopy in men after the prostatic urethra. This association exists because both the ureter and seminal vesicle are derived from a common embryologic mesonephric origin. Even when there is no ectopic ureter, congenital seminal vesicle cysts are associated with unilateral (usually ipsilateral) renal agenesis in the majority of cases (Coppens et al., 2002). These patients should therefore be screened with renal ultrasound (Jhaveri et al., 2010). Acquired seminal vesicle cysts are also possible; these are thought to be secondary to downstream obstruction due to prostatic enlargement, malignancy, or surgery.

The majority of cysts are small (<5 cm) and asymptomatic. Symptoms, if they arise, typically occur in patients between 20 and 40 years of age. Patients may experience dysuria, urinary frequency, perineal pain, epididymitis, post-ejaculatory or scrotal pain and infertility. In most cases, the cyst is palpable rectally. Imaging techniques - including TRUS and less often scan or vasovesiculography - may also be used to visualize the cyst.

Patients with symptomatically obstructing seminal vesicle cysts merit treatment. One option is aspiration of the cyst perineally, transabdominally or transectally. The cyst fluid can then be sent for evaluation for the presence of sperm to aid in diagnosis. Even if aspiration is immediately successful in decompressing the cyst, patients are at high risk of recurrence. Because of this, it is not considered first line management. Depending on the cyst location and size, it is sometimes possible to proceed with transurethral cyst deroofing. This technique has an excellent (75% in one study) success rate of alleviating patients' symptoms when technically possible. If inaccessible transurethrally or if the cyst recurs, open abdominal/pelvic surgery is an option and has an extremely high success rate, but is of course higher risk (Van den Ouden et al., 1998).

Conclusion

Obstruction anywhere along the path of semen formation and ejaculation can lead to infertility. The most common cause of obstructive azoospermia is epididymal obstruction due to infection. Obstructions of the vas deferens, seminal vesicles and ejaculatory ducts are less common but other causes of male infertility.

References

- Ahmad, I., & Krishna, N. S. (2007). Hematospermia. *The Journal of Urology*, 177(5), 1613–1618.
- Berardinucci, D., Zini, A., & Jarvi, K. (1998). Outcome of microsurgical reconstruction in men with suspected epididymal obstruction. *The Journal of Urology*, 159(3), 831–834.
- Chan, P. T. (2013). The evolution and refinement of vasoepididymostomy techniques. *Asian Journal of Andrology*, 15(1), 49.
- Coppens, L., et al. (2002). Adult müllerian duct or utricle cyst: Clinical significance and therapeutic management of 65 cases. *The Journal of Urology*, 167(4), 1740–1744.
- Dickinson, S. J. (1973). Structural abnormalities in the undescended testis. *Journal of Pediatric Surgery*, 8(4), 523–527.
- Handelsman, D. J., et al. (1984). Young's syndrome: Obstructive azoospermia and chronic sinopulmonary infections. *New England Journal of Medicine*, 310(1), 3–9.
- Hendry, W. F., Parslow, J. M., & Stedronska, J. (1983). Exploratory scrototomy in 168 azoospermic males. *British Journal of Urology*, 55(6), 785–791.
- Jarvi, K., et al. (1995). Cystic fibrosis transmembrane conductance regulator and obstructive azoospermia. *The Lancet*, 345(8964), 1578.

- Jhaveri, K. S., et al. (2010). The role of cross-sectional imaging in male infertility: A pictorial review. *Canadian Association of Radiologists Journal*, 61(3), 144–155.
- Kim, S.-O., et al. (2015). Epididymal anomalies in boys with undescended testis or hydrocele: Significance of testicular location. *BMC Urology*, 151, 108.
- Kolettis, P. N., & Sandlow, J. I. (2002). Clinical and genetic features of patients with congenital unilateral absence of the vas deferens. *Urology*, 60(6), 1073–1076.
- Kroovand, R. L., & Perlmutter, A. D. (1981). Congenital anomalies of the vas deferens and epididymis. In S. J. Hogan, & H. ESE (Eds.), *Pediatric Andrology* (pp. 173–180). Boston: Martinus Nijhoff Publishers.
- Marshall, F. F., & Shermeta, D. W. (1979). Epididymal abnormalities associated with undescended testis. *The Journal of Urology*, 121(3), 341–343.
- Matsumiya, K., Namiki, M., & Takahara, S. (1994). A clinical study of azoospermia. *International Journal of Andrology*, 17, 140–142.
- Meacham, R. B., Hellerstein, D. K., & Lipshultz, L. I. (1993). Evaluation and treatment of ejaculatory duct obstruction in the infertile male. *Fertility and Sterility*, 59, 393–397.
- Mickle, J., Milunsky, A., Amos, J. A., & Oates, R. D. (1995). Congenital unilateral absence of the vas deferens: A heterogeneous disorder with two distinct subpopulations based upon aetiology and mutational status of the cystic fibrosis gene. *Human Reproduction*, 10, 1728–1735.
- Miller, S., et al. (2016). Unilateral absence of vas deferens: Prevalence among 23,013 men seeking vasectomy. *International Brazilian Journal of Urology*, 42(5), 1010–1017.
- Nickel, J. (2003). Curtis. "chronic epididymitis: A practical approach to understanding and managing a difficult urologic enigma." *Reviews in Urology*, 5(4), 209.
- Peng, J., et al. (2012). Patency rates of microsurgical vasoepididymostomy for patients with idiopathic obstructive azoospermia: A prospective analysis of factors associated with patency—Single-center experience. *Urology*, 79(1), 119–122.
- Schlegel, P. N., Shin, D., & Goldstein, M. (1996). Urogenital anomalies in men with congenital absence of the vas deferens. *The Journal of Urology*, 155(5), 1644–1648.
- Schoysman, R. (1990). Vaso-epididymostomy—a survey of techniques and results with considerations of delay of appearance of spermatozoa after surgery. *Acta Europaea Fertilitatis*, 215(5), 239–245.
- Van den Ouden, D., et al. (1998). Diagnosis and management of seminal vesicle cysts associated with ipsilateral renal agenesis: A pooled analysis of 52 cases. *European Urology*, 33(5), 433–440.

Further Reading

- Heaney, J. A., Pfister, R. C., & Meares, E. M. (1987). Giant cyst of the seminal vesicle with renal agenesis. *American journal of Roentgenology*, 149, 139–140.

Primary Ciliary Dyskinesia

Gabriella Avellino and Robert D Oates, Boston University School of Medicine, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Axoneme The motor unit of the cilia and flagella.

Cilia The hairlike projections on the outside of many cells in the body.

Flagellum An elongated and modified cilium that helps propel a cell.

Intracytoplasmic sperm injection Placement of a single sperm into a harvested egg.

Introduction

Primary ciliary dyskinesia (PCD) is a genetically and phenotypically heterogeneous disease. Ciliary dysfunction and the resultant manifestations that arise characterize this autosomal recessive disorder. The moniker “Kartagener syndrome” is used to describe the clinical triad of situs inversus, chronic sinusitis and bronchiectasis, the underlying defect driving the pathophysiology to be in ciliary structure and function. In 1976, the appellation “immotile cilia syndrome” was used in common parlance to reflect the growing diversity of ciliary-based disease being recognized, one subtype still called “Kartagener syndrome.” Eventually, in addition to static, nonfunctional cilia, scientists observed cilia that were indeed moving in some affected patients, although in an uncoordinated and ineffective manner. Thus, the final and present change to “primary ciliary dyskinesia,” additionally further distinguishing these innate conditions from “secondary cilia deficiency” which occurs in response to chronic infection or inflammation (Leigh et al., 2009; Knowles et al., 2013).

The estimated prevalence of PCD is 1 in 10,000–20,000 births (Goutaki et al., 2017). Cilia have a myriad of crucial and necessary roles in embryonic and fetal development (e.g., proper left–right organ rotation and orientation), childhood health (e.g., proper mucociliary clearance in the respiratory tree and sinus tract), and procreation (e.g., proper sperm motility) (Leigh et al., 2009).

In a study based on an international PCD Cohort of 3013 patients, including 71% from European countries and 17% from America, 49% were male. The median current age of patients was 18 with a range of 1–92 years old. The largest age subgroup was 10–19-year-olds (38%), followed by 20–29-year-olds (18%) and 0–9-year-olds (17%). This group likely underrepresents adult patients and is limited by variability in care and diagnostic tools (Goutaki et al., 2017).

Basic Structure of Cilia and Flagella

Cilia and flagella are hair-like structures that have a configuration and purpose that have remained constant despite evolution over time and across species. Cilia are typically shorter than flagella at 5–10 μm , compared to 50–150 μm in length (Linck et al., 2016). Cilia can be classified into four different subtypes, distinguished by minute changes in axonemal substructure which determine ultimate function and role. The axoneme is the internal motor unit of a cilia/flagellum and is constructed of well-organized microtubules fashioned out of α - and β -monomers of tubulin that comprise the protofilaments (9 microtubule doublets, equally spaced, and centrifugally arranged around a central microtubule doublet pair—the “9 + 2” classic description) and their associated accessory elements (e.g., structural and mechanical braces as well as energy producing ATPases) (Leigh et al., 2009) (Fig. 1).

Motile Cilia (“9 + 2,” Dynein Arms)

Motile cilia facilitate the transport of fluids along the surfaces of upper and lower respiratory epithelium, central nervous ependymal lining, and fallopian tubes. The microtubules of motile cilia reside in a classic 9 + 2 pattern, that is, in cross-sectional image, nine pairs lie on the periphery in a circular fashion around a central pair of microtubules. A complex interaction of outer and inner dynein arm proteins convert the potential chemical energy stored within ATP to mechanical work creating shifting of microtubules relative to one another and, thus, motion. Additional key components are nexin links which connect the adjacent microtubule pairs one to another and radial spokes which secure the central microtubules to those in the periphery. There are approximately 200 cilia per respiratory epithelial cell that beat in a coordinated direction-specific fashion to allow for a stronger forward stroke than back stroke in order to move fluid and mucus and thus toxins and bacteria in a functionally appropriate direction (Knowles et al., 2013).

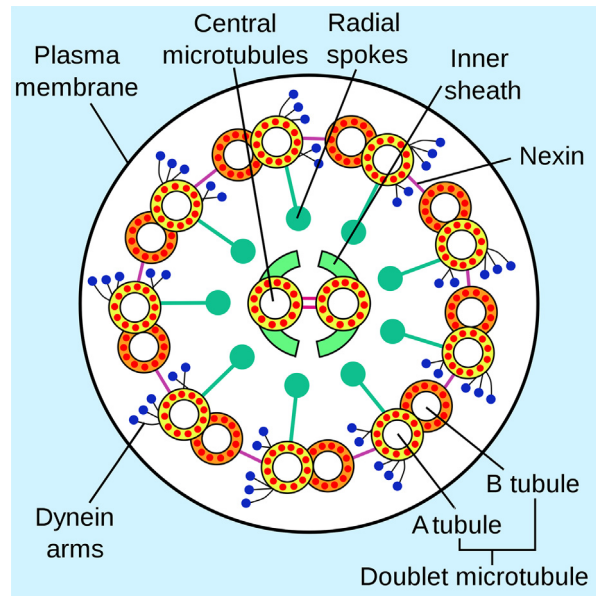


Fig. 1 A cross sectional diagram through a typical eukaryotic flagellum showing the 9 + 2 arrangement of the microtubules. Also shown are the dynein arms, the nexin links, and the radial spokes.

Nodal Cilia ("9 + 0," Dynein Arms)

Nodal cilia are critical at the embryonic stage to help direct proper right–left orientation and symmetry of the developing internal organs (heart and GI tract) as their movement creates a leftward directional flow of extracellular fluid. Nodal cilia lack the central doublet but do have the peripheral nine doublets and active dynein arms. Situs inversus totalis occurs when all internal organs mal-rotate while isolated dextrocardia refers to a situation in which the heart has rotated to the right while the abdominal viscera is oriented normally (Mirra et al., 2017).

Primary or Sensory Cilia ("9 + 0," No Dynein Arms)

Primary or sensory cilia are expressed on most cells, are nonmotile with a sensory function to receive and transduce local environmental signals. These cells contribute to signaling pathways and cell polarity. Sensory cilia have a structure of 9 + 0, similar to nodal cilia but are without dynein arms. They are found on most cells, and if defective can lead to various pathologies spanning multiple organs, for example, retinitis pigmentosa and adult polycystic kidney disease (Knowles et al., 2013).

Flagella ("9 + 2," Dynein Arms, Additional Periaxonemal Structural Elements)

The flagellum is a ubiquitous, conserved motile structure and is, essentially, the sperm tail in humans. Similar to motile cilia, flagella have a 9 + 2 axoneme, with nine outer microtubule doublets encircling a central doublet. However, the sperm tail contains a number of necessary, additional peri-axonemal elements that provide the structural foundation to generate forward propulsion including fibrous sheath proteins and outer dense fibers. Cilia sway back and forth in an "oar-like" fashion, while flagella beat with a "snake-like motion" (Linck et al., 2016).

Diagnosis of PCD

Due to its clinical heterogeneity, diagnosis can be challenging, expensive and often requires expertise. In 2017, a European task force composed of multidisciplinary clinicians and scientists published the *European Respiratory Society* Guidelines for the Diagnosis of Primary Ciliary Dyskinesia by defining the population for which PCD should be considered and for whom testing should be completed (Lucas et al., 2017). They specify a diagnostic algorithm which may include assessment of nasal nitric oxide (nNO), high-speed video light microscopy (HSVA), and transmission electron microscopy (TEM). The panel did not recommend genetic analysis as a diagnostic screening tool unless the above tests were normal or equivocal in the setting of a strong clinical suspicion. Currently recognized genetic defects diagnose only 50%–75% of cases. Genetic testing may be useful to augment a diagnostic pathway or to prepare for preimplantation genetic diagnosis (vide infra).

Transmission electron microscopy (TEM) evaluates ciliary ultrastructure. Multiple structural defects have been identified in respiratory cilia collected by nasal scrape or bronchial brush biopsy in patients with suspected PCD. Although this was once felt to be the

gold standard for diagnosis, TEM will not reveal an anomaly in approximately 30% of patients with PCD who have normal ciliary ultrastructure (Boon et al., 2014).

An alternative screening method involves evaluating nasal nitric oxide (nNO) levels. Most, but not all, patients with true PCD will have 10%–20% of normal levels. Results may be falsely negative in an acute upper respiratory infection and are not as reliable in children < 6 years of age. The mechanism for the difference in nNO levels between affected and unaffected patients has yet to be identified (Lucas et al., 2017).

A newer technique of high-speed video light microscopy (HVMA) allows for a nonstatic assessment of subtle alterations in ciliary wave form. Similar to TEM, the test is run *ex vivo* on nasal or bronchial respiratory epithelium. Different ultrastructural defects translate into specific patterns of ciliary dyskinesia that can be identified on HVMA (Lucas and Leigh, 2014).

Finally, a seven-point questionnaire (PICADAR) helps predict the likelihood of PCD in a patient referred for persistent wet cough. The variables include full-term gestational age, admittance to a neonatal unit, neonatal chest symptoms, persistent perennial rhinitis, chronic ear, and hearing symptoms, situs abnormalities, and presence of a cardiac defect. A score ≥ 10 predicts >90% chance of testing positive for PCD (Behan et al., 2016).

Additional future directions include immunofluorescence labeling of proteins in cilia to identify ultrastructural abnormalities and radioaerosol mucociliary clearance as an *in vivo* assessment.

Genetic Anomalies Underlying Ciliary and Axonemal Abnormalities in PCD

There are currently 40 known genes associated with PCD and multiple characterized mutations in these genes that code for structural and functional proteins in cilia—not just proteins in the axonemes itself but also proteins involved in the cytoplasmic preassembly of both outer and inner dynein complexes (Guo et al., 2017). In 1999 Pennarun et al. were the first to report a specific gene underlying PCD, through a candidate-gene approach identifying loss of function mutations in *DNAI1* leading to an absence of dynein arms within the axoneme (Pennarun et al., 1999). Deleterious alterations in *DNAI1* and anomalies in the molecular sequence of *DNAH5*, whose product contributes to the configuration of the outer dynein arm, are etiologic in 9% and 21% of PCD cases, respectively. As expected, mutations leading to abnormal outer dynein arm function will result in immotile or markedly slow cilia (Horani et al., 2016). At present, approximately 65% of patients with PCD have identifiable biallelic mutations. Guo et al. identified loss-of-function mutations in $\frac{3}{4}$ consanguineous families (*ARMC4*, *CCNO*, *DYX1C1*) and a missense mutation (*DNAI1*) in the last of the four families they reported on (Guo et al., 2017). Conversely, current genetic analysis will fail to identify a causal abnormality in up to 30% of patients.

The ultimate goal will be to associate the ultrastructural findings detailed on TEM, the specific ciliary dysfunction visualized on HVMA, the measured nNO values and the overall clinical and phenotypic picture of an individual patient with a defined PCD mutation profile. For instance, mutations in *DNAH11* reveal normal TEM findings, abnormal HSVA studies and low nNO. As reviewed by Mirra et al., mutations leading to abnormalities of the central doublet or radial spoke components, such as *RSPH1*, *RSPH3*, *RSPH4A*, *RSPH9*, and those that impact genes controlling the generation of multiple motile cilia, such as *MCIDAS* and *CCNO*, do not lead to laterality defects as nodal cilia do not have, nor require, a functional central doublet (Mirra et al., 2017). Males with *CCDC114* mutations, which impact the outer dynein arm docking factor have normal sperm motility. As the authors note, further genotype: molecular phenotype, genotype: functional phenotype, and genotype: anatomical phenotype correlations need to be pursued and codified.

Although primarily autosomal recessive, X-linked PCD has been described in association with mutations in *RPGR* and *PIH1D3*, the former being found in 20% of all cases of retinitis pigmentosa (Moore et al., 2006; Paff et al., 2017). Paff et al. report on four men with mutations in *PIH1D3*, either maternally inherited or *de novo*. As such, X-linked disease creates a sex-limited phenotypic expression pattern affecting predominantly, if not solely, males.

Prior to completing *in vitro* fertilization, many reproductive groups will perform carrier screening on one of the two members of a couple to determine if the offspring is at risk of an autosomal recessive disease. A few of the expanded tests available will check for *DNAH5*, *DNAI1*, and *DNAH11* (Horani et al., 2016). However, both members of these couples are asymptomatic and these tests have only a screening role. In the case of a symptomatic individual, variable numbers of mutations (up to 36 in one company's PCD panel) may be assayed for with the number increasing over time. "The yield of allele-specific approaches is low in PCD given the high genetic and allelic heterogeneity" (Lucas et al., 2017).

Clinical and Phenotypic Manifestations of PCD

Pulmonary and Respiratory Dysfunction

Defective cilia can lead to disease of the conducting airways, paranasal sinuses, and middle ear (Eustachian tube). As mucociliary clearance is part of a human's primary innate immunity, even though a nonspecific defense mechanism, the most prominent and distressing symptoms of PCD include recurrent to chronic upper and lower respiratory tract infections. Daily nasal congestion, year-round wet cough; chronic rhinorrhea with loss of ability to smell, and chronic otitis media is seen in over 75% of children with PCD and are the signs and symptoms that most often lead to evaluation and diagnosis (Mirra et al., 2017).

Situs Inversus Totalis

Situs inversus totalis describes the anatomical configuration of completely transposed thoracoabdominal viscera to a mirror position of normal, that is, the heart is rotated to the right side (dextrocardia) and the abdominal organs are flipped left-to-right and vice versa. This happens in approximately 50% of PCD cases, implying a random axis orientation when the normal embryonic, ciliary-directed signal is not present—a crucial function of cilia within the embryonic node. Heterotaxy represents a spectrum of laterality derangements of any of the internal thoracoabdominal organs and is seen in approximately 6% of individuals who do not have situs inversus totalis (Leigh et al., 2009). Certain regions of the brain, specifically the frontal and occipital petelia, may be subject to ciliary orientation while the lateralization of language may be controlled via a different mechanism (Kennedy et al., 1999).

Male Infertility

Most, but not all, men with PCD experience infertility as sperm motility is necessary for spermatozoa to swim up the female reproductive tract in order to meet and penetrate the oocyte. Often, ejaculate sperm count and morphology are within normal ranges but there is a complete absence of movement. Semen volume should be normal as the seminal vesicles, which are responsible for 70% of the ejaculate volume, are unaffected. The semen pH should be alkaline as all of the alkalinity arises from the secretions of the seminal vesicles which, again, are without defect or dysfunction. Testis size reflects sperm production capacity and so is normal. The epididymides and vasa are palpably present and unobstructed in PCD. The hypothalamic-pituitary-gonadal axis is optimal. It is the sperm axonemes themselves that are affected, thereby limiting sperm motility, propulsion, and forward progression, the proximate cause of the couple's infertility. Ji et al. provide a comprehensive description of the numerous genes (their name, axonemal location, the defect arising when they are dysfunctional, the infertility phenotype, and the chromosomal location) that have been identified so far that affect axonemal structure or function and result in, at least, male infertility (Ji et al., 2017).

Treatment Options for Reproductive Failure in Men With PCD

As reviewed by Ebner et al., data on fertility outcomes in couples with a male partner afflicted with PCD are limited and are generally small series or case reports (Ebner et al., 2015). However, what is clear is that intracytoplasmic sperm injection, an advanced reproductive technique in which an individual viable spermatozoon is placed directly into an individual oocyte via micromanipulation (ICSI), is necessary to effect fertilization, embryo development, and pregnancy. Although Davila Garza and Patrizio state, “the fertilization potential and live-birth rates are close to that obtained from nonspecific causes of sperm defects,” others would disagree (Davila Garza and Patrizio, 2013). It is unclear, therefore, whether sperm from the generic man with PCD will be able to generate these results consistently. Adjunctive techniques may be required to select viable sperm, since the most obvious sign—motility—is absent. These include selecting sperm to use by noting sperm tail coiling in a hypoosmotic solution which indicates viability, noting sperm tail flexibility upon flicking the sperm with the microinjection pipette which indicates viability, of stimulating the tiniest bit of motion with theophylline or pentoxifylline which indicates viability (Kordus et al., 2008). Some authors suggest the use of testicular sperm rather than ejaculate sperm as the source of the male gamete during ICSI, positing that it is more likely the embryologist will be able to select viable and healthy sperm from harvested testis tissue than from the mixture of viable and nonviable nonmotile sperm in the ejaculate. In this regard, an open operative approach to obtaining testis tissue or a percutaneous aspiration are both appropriate (Hayden et al., 2016). Finally, Ebner et al. report on using Ca^{2+} -ionophore to activate the oocyte, a normal function of the spermatozoan which, they speculate, may be deficient in some PCD cases (Ebner et al., 2015). They describe a twin live-birth to a couple in whom conventional ICSI did not result in fertilization but that Ca^{2+} -ionophore treatment did successfully. McLachlan et al. report on a variant of PCD in which not only were the sperm immotile but they were also structurally quite abnormal in the tail and midpiece (McLachlan et al., 2012). Testis tissue was used as the source of spermatozoa and they selected only relatively normal appearing gametes. No pre-ICSI genetic analysis was performed but the authors do caution that there would be concern of transmission to the offspring. In their case, the daughter born showed no evidence of an inherited ciliopathy.

As above, couples interested in conception should have genetic counseling. All children of individuals with PCD will at the very least, be obligate simple heterozygotes (carriers). If the partner of a PCD male is shown to be a carrier as well, preimplantation genetic screening may be applied to prevent delivery of an affected offspring. In the case of X-linked disease, couples may choose to only transfer male embryos (who will be free of disease as they inherit the paternal Y chromosome and not the paternal X chromosome) and not female embryos who will then be burdened with the potential, in their own future, to bear sons afflicted with PCD.

There is no cure for PCD. Treatment is not specific to PCD and is primarily symptomatic, based on strategies of care developed for patients with cystic fibrosis or idiopathic bronchiectasis. Treatment focus is to prevent exacerbations and slow the progression of pulmonary disease. Care includes clearance of airway by inhalers, nebulizers and chest physiotherapy and close monitoring for need of antibiotics. Exposure to inflammatory triggers, including second-hand smoke, should be limited. Patients should be up to date with immunizations, especially pneumococcal, and influenza. In addition to better symptom management, novel therapeutic strategies are being investigated. For example, identification of gene defects hopefully will allow for gene editing to reestablish gene function and consequently normal ciliary function and motility (Lai et al., 2016). Care is often multidisciplinary, requiring input from pediatricians, neonatologists, pulmonologists, chest physiotherapists, cardiologists, otolaryngologists, geneticists, radiologists, and urologists.

Conclusion

Primary ciliary dyskinesia is a group of disorders based upon structural and functional defects in the hair-like projections that are on many embryonic, fetal, and adult cells. As such, the respiratory and sinus systems are severely affected and typically lead to clinical diagnosis. There are a variety of tests being employed in an adjunctive fashion, with genetic testing playing only a confirmatory role in certain cases. Male fertility is often completely compromised as the motility of the sperm, necessary for the sperm to travel to and penetrate the egg, is absent or significantly deficient. Newer therapies, however, can help male patients achieve biological paternity. These include a variety of ways to identify viable and healthy sperm that can be placed, individually, inside a harvested egg to effect fertilization, embryo development and term birth. The genetic basis is varied as multiple genes are involved responsible for the numerous parts of the motor apparatus of the cilia and sperm tail. They are being identified and understood slowly but steadily with continued research. When contemplating treatment of male infertility in a PCD patient, it is important to recognize, if possible, the genetic mechanisms underlying the axonemal defect in that individual man so as to be able to prevent transmission to his offspring (or their offspring). The ultimate goal will be to link the clinical and phenotypic features to the genetic anomaly and provide more precise and directed treatment.

References

- Behan, L., Dimitrov, B. D., Kuehni, C. E., Hogg, C., Carroll, M., Evans, H. J., Goutaki, M., Harris, A., Packham, S., Walker, W. T., & Lucas, J. S. (2016). PICADAR: A diagnostic predictive tool for primary ciliary dyskinesia. *The European Respiratory Journal*, 47(4), 1103–1112.
- Boon, M., Smits, A., Cuppens, H., Jaspers, M., Proesmans, M., Dupont, L. J., Vermeulen, F. L., Van Daele, S., Malfroot, A., Godding, V., Jorissen, M., & De Boeck, K. (2014). Primary ciliary dyskinesia: Critical evaluation of clinical symptoms and diagnosis in patients with normal and abnormal ultrastructure. *Orphanet Journal of Rare Diseases*, 9, 11.
- Davila Garza, S. A., & Patrizio, P. (2013). Reproductive outcomes in patients with male infertility because of Klinefelter's syndrome, Kartagener's syndrome, round-head sperm, dysplasia fibrous sheath, and 'stump' tail sperm: An updated literature review. *Current Opinion in Obstetrics & Gynecology*, 25(3), 229–246.
- Ebner, T., Maurer, M., Oppelt, P., Mayer, R. B., Duba, H. C., Costamoling, W., & Shebl, O. (2015). Healthy twin live-birth after ionophore treatment in a case of theophylline-resistant Kartagener syndrome. *Journal of Assisted Reproduction and Genetics*, 32(6), 873–877.
- Goutaki, M. E., Maurer, F. S., Halbeisen, I., Amirav, A., Barbato, L., Behan, M., Boon, C., Casaulta, A., Clement, S., Crowley, E., Haarman, C., Hogg, B., Karadag, C., Koerner-Rettberg, M. W., Leigh, M. R., Loebinger, H., Mazurek, L., Morgan, K. G., Nielsen, H., Omran, N., Schwerk, S., Scigliano, C., Werner, P., Yiallourou, Z., Zivkovic, J. S., Lucas, C. E., Kuehni, P. C. D. I., Consortium, P. C. D. G., Swiss, D., & French Reference Centre for Rare Lung and C. Genetic Disorders of Mucociliary Clearance. (2017). The international primary ciliary dyskinesia cohort (iPCD cohort): Methods and first results. *The European Respiratory Journal*, 49(1).
- Guo, T., Tan, Z. P., Chen, H. M., Zheng, D. Y., Liu, L., Huang, X. G., Chen, P., Luo, H., & Yang, Y. F. (2017). An effective combination of whole-exome sequencing and runs of homozygosity for the diagnosis of primary ciliary dyskinesia in consanguineous families. *Scientific Reports*, 7(1), 7905.
- Hayden, R. P., Wright, D. L., Toth, T. L., & Tanrikut, C. (2016). Selective use of percutaneous testis biopsy to optimize IVF-ICSI outcomes: A case series. *Fertility Research and Practice*, 2, 7.
- Horani, A., Ferkol, T. W., Dutcher, S. K., & Brody, S. L. (2016). Genetics and biology of primary ciliary dyskinesia. *Paediatric Respiratory Reviews*, 18, 18–24.
- Ji, Z. Y., Sha, Y. W., Ding, L., & Li, P. (2017). Genetic factors contributing to human primary ciliary dyskinesia and male infertility. *Asian Journal of Andrology*, 19(5), 515–520.
- Kennedy, D. N., O'Craven, K. M., Ticho, B. S., Goldstein, A. M., Makris, N., & Henson, J. W. (1999). Structural and functional brain asymmetries in human situs inversus totalis. *Neurology*, 53(6), 1260–1265.
- Knowles, M. R., Daniels, L. A., Davis, S. D., Zariwala, M. A., & Leigh, M. W. (2013). Primary ciliary dyskinesia. Recent advances in diagnostics, genetics, and characterization of clinical disease. *American Journal of Respiratory and Critical Care Medicine*, 188(8), 913–922.
- Kordus, R. J., Price, R. L., Davis, J. M., & Whitman-Elia, G. F. (2008). Successful twin birth following blastocyst culture of embryos derived from the immotile ejaculated spermatozoa from a patient with primary ciliary dyskinesia: A case report. *Journal of Assisted Reproduction and Genetics*, 25(9–10), 437–443.
- Lai, M., Pifferi, M., Bush, A., Piras, M., Michelucci, A., Di Cicco, M., del Grosso, A., Quaranta, P., Cursi, C., Tantillo, E., Franceschi, S., Mazzanti, M. C., Simi, P., Saggese, G., Boner, A., & Pistello, M. (2016). Gene editing of DNAH11 restores normal cilia motility in primary ciliary dyskinesia. *Journal of Medical Genetics*, 53(4), 242–249.
- Leigh, M. W., Pittman, J. E., Carson, J. L., Ferkol, T. W., Dell, S. D., Davis, S. D., Knowles, M. R., & Zariwala, M. A. (2009). Clinical and genetic aspects of primary ciliary dyskinesia/Kartagener syndrome. *Genetics in Medicine*, 11(7), 473–487.
- Linck, R. W., Chemes, H., & Albertini, D. F. (2016). The axoneme: The propulsive engine of spermatozoa and cilia and associated ciliopathies leading to infertility. *Journal of Assisted Reproduction and Genetics*, 33(2), 141–156.
- Lucas, J. S., & Leigh, M. W. (2014). Diagnosis of primary ciliary dyskinesia: Searching for a gold standard. *The European Respiratory Journal*, 44(6), 1418–1422.
- Lucas, J. S., Barbato, A., Collins, S. A., Goutaki, M., Behan, L., Caudri, D., Dell, S., Eber, E., Escudier, E., Hirst, R. A., Hogg, C., Jorissen, M., Latzin, P., Legendre, M., Leigh, M. W., Midulla, F., Nielsen, K. G., Omran, H., Papon, J. F., Pohunek, P., Redfern, B., Rigau, D., Rindlisbacher, B., Santamaria, F., Shoemark, A., Snijders, D., Tonia, T., Titieni, A., Walker, W. T., Werner, C., Bush, A., & Kuehni, C. E. (2017). European Respiratory Society guidelines for the diagnosis of primary ciliary dyskinesia. *The European Respiratory Journal*, 49(1).
- McLachlan, R. I., Ishikawa, T., Osianlis, T., Robinson, P., Merriner, D. J., Healy, D., de Kretser, D., & O'Bryan, M. K. (2012). Normal live birth after testicular sperm extraction and intracytoplasmic sperm injection in variant primary ciliary dyskinesia with completely immotile sperm and structurally abnormal sperm tails. *Fertility and Sterility*, 97(2), 313–318.
- Mirra, V., Werner, C., & Santamaria, F. (2017). Primary ciliary dyskinesia: An update on clinical aspects, genetics, diagnosis, and future treatment strategies. *Frontiers in Pediatrics*, 5, 135.
- Moore, A., Escudier, E., Roger, G., Tamalet, A., Pelosse, B., Marlin, S., Clement, A., Geremek, M., Delaisi, B., Bridoux, A. M., Coste, A., Witt, M., Duriez, B., & Amselem, S. (2006). RPR is mutated in patients with a complex X linked phenotype combining primary ciliary dyskinesia and retinitis pigmentosa. *Journal of Medical Genetics*, 43(4), 326–333.
- Paff, T., Loges, N. T., Aprea, I., Wu, K., Bakey, Z., Haarman, E. G., Daniels, J. M., Sistermans, E. A., Bogunovic, N., Dougherty, G. W., Hoben, I. M., Grosse-Onnebrink, J., Matter, A., Olbrich, H., Werner, C., Pals, G., Schmidts, M., Omran, H., & Micha, D. (2017). Mutations in PIH1D3 cause X-linked primary ciliary dyskinesia with outer and inner dynein arm defects. *American Journal of Human Genetics*, 100(1), 160–168.
- Pennarun, G., Escudier, E., Chapelin, C., Bridoux, A. M., Cacheux, V., Roger, G., Clement, A., Goossens, M., Amselem, S., & Duriez, B. (1999). Loss-of-function mutations in a human gene related to Chlamydomonas Reinhardtii dynein IC78 result in primary ciliary dyskinesia. *American Journal of Human Genetics*, 65(6), 1508–1519.

Sperm Defects

Julio M Castañeda*, **Haruhiko Miyata***, and **Masahito Ikawa***, Osaka University, Osaka, Japan
Martin M Matzuk*, Baylor College of Medicine, Houston, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Reproduction and Its Global Implications

In the beginning of the 21st century, improvements in reproductive health was one of eight goals specified in the United Nations Millennium Development Goals (http://www.who.int/topics/millennium_development_goals/en/). A major aspect of reproductive health includes infertility that affects up to 15% of the population worldwide and causes emotional distress in couples and families. Access to contraceptive options was also outlined within the goal of reproductive health as unplanned pregnancies place undue burden on women and continued population growth places more strain on Earth's limiting resources. In this chapter, we will discuss sperm function and formation, and how basic research into spermatogenesis has provided insights in both infertility and potential male contraceptives.

Sperm Function

Spermatozoa (sperm) are remarkable cells considering their role and importance to sexually reproducing species. Sperm are delegated with the task of carrying the haploid genome of the male to the oocyte of the female, a journey along the mammalian female reproductive tract that is up to several thousand times the length of sperm. Remarkably, sperm undergo this journey in the absence of transcription and protein production. Of the millions of sperm, only a small number will reach the vicinity of the egg. These sperm must then penetrate the layer of ovarian granulosa cells and the zona pellucida surrounding the oocyte, and undergo the acrosome reaction to expose receptors that allow the sperm to bind the oocyte and promote fusion with the plasma membrane. Only after completion of their journey and fusion with the egg can development begin. Even in the case of healthy sperm, many sperm will not complete their task due to the strenuous journey. Any defect in sperm formation, production, or energy supply will exacerbate the difficulty of completing their journey. It is no surprise then that any problems in the production of healthy sperm lead to infertility. Conversely, disrupting sperm production and function has been an avenue to pursue male contraceptives, which we will discuss at the end of this section (Table 1).

Spermatogenesis 101

Spermatogonia and Mitosis

The production of sperm begins within the seminiferous tubules of the testis (Fig. 1A). Male germline stem cells (GSCs) are rare cells located at the periphery of the tubules. GSCs divide mitotically to replenish the stem cell pool and generate daughter cells that will

Table 1 Genes associated with sperm defects

Azoospermia
<i>CHD7</i> , <i>DAZ</i> , <i>SYCP3</i> , <i>SYCP1</i> , <i>MLH1</i> , <i>MYBL1</i> , <i>CREM</i>
<i>Chd7</i> , <i>Daz</i> , <i>Sycp3</i> , <i>Sycp1</i> , <i>Mlh1</i> , <i>Mybl1</i> , <i>Crem</i>
Oligozoospermia
<i>KLHL10</i>
<i>Klhl10</i>
Teratozoospermia
<i>AURKC</i> , <i>DPY19L2</i> , <i>PICK1</i> , <i>ZBPB</i> , <i>SPATA16</i>
<i>Dpy19l2</i> , <i>Pick1</i> , <i>Zbpb</i> , <i>Csnk2a2</i> , <i>Hrb</i> , <i>Gopc</i> , <i>Gba2</i> , <i>Spaca1</i>
Asthenozoospermia
<i>DNAH11</i> , <i>AKAP3</i> , <i>AKAP4</i> , <i>CATSPER1</i> , <i>CATSPER2</i>
<i>Akap4</i> , <i>Cabyr</i> , <i>Catsper1–4</i> , <i>Ppp3cc</i> , <i>Gapdhs</i> , <i>Ldhc</i>
Unexplained male fertility
<i>PLCZ</i>
<i>Adam2</i> , <i>Ace</i> , <i>Izumo1</i>

A list of genes mentioned in this article. Human genes are in uppercase and mouse genes are in lower case.

*All authors contributed equally to this work.

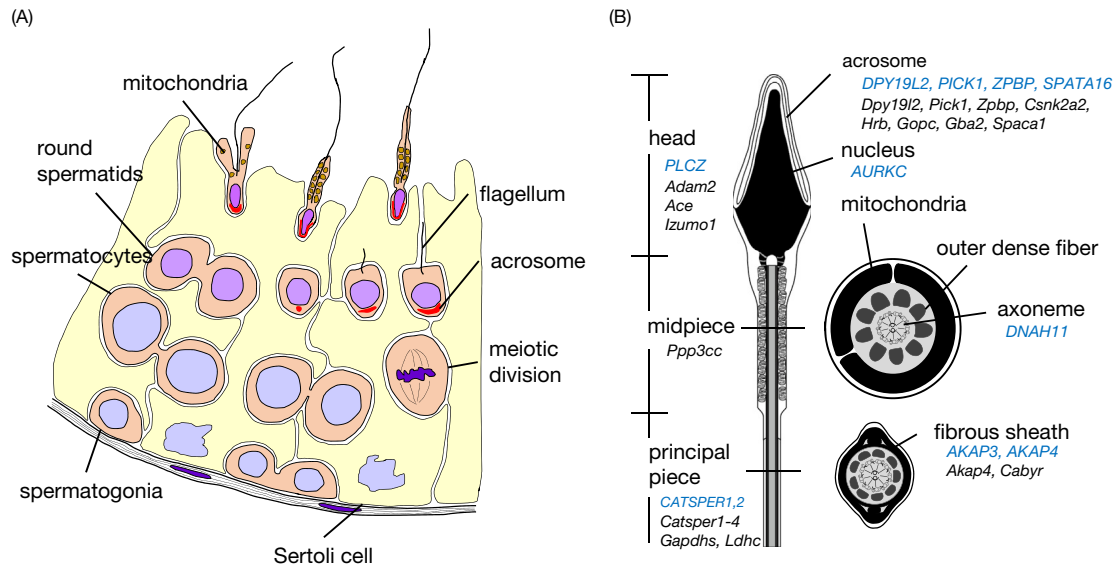


Fig. 1 Sperm formation and structure. (A) Sperm formation in the testis. A portion of the seminiferous tubule is shown. (B) Structure of a mature spermatozoon. Genes appearing in this chapter and important for the various structures are shown in uppercase (human) and lower case (mouse).

differentiate. The GSCs and their mitotically dividing daughter cells are referred to as spermatogonia (De Rooij, 2017). Spermatogonia will continue to undergo several rounds of mitosis with incomplete cytokinesis to generate a syncytium of cells connected by intracellular bridges. Intracellular bridges allow an exchange of materials and cellular signals that coordinate further development, and absence of intracellular bridges blocks sperm development prior to meiotic division (Greenbaum et al., 2006). In many systems, the dividing spermatogonia have been well characterized and named. For example, mouse GSCs and their initial daughter cell are referred to as A_{single} for a spermatogonia that is single and not interconnected with other cells. The A_{single} daughters divide to form a pair of joined cells called A_{paired} . With each subsequent division, the number of connected cells increases, while the developmental potential becomes restricted to the meiotic program. In the mouse, there can be 9–11 mitotic divisions of spermatogonia before proceeding to the next phase of spermatogenesis.

Spermatocytes and Meiosis

When spermatogonia finish dividing mitotically, the cells undergo a pre-meiotic S-phase and initiate meiosis. Meiotically dividing cells are referred to as spermatocytes (Russell et al., 1990). Spermatocytes begin to move away from the periphery of the seminiferous tubules and towards the lumen. In meiosis, homologous chromosomes pair and are held together by a protein complex called the synaptonemal complex. During the pairing of chromosomes, double strand break formation, break repair, and homologous recombination occur, generating genomic diversity by recombining parental chromosomes. In many species, recombination is required for meiosis to proceed properly. Recombination is important because the recombination spot, visualized as chiasmata, allows homologous chromosomes to properly align at the metaphase plate and divide, and is also responsible for generating genetic diversity that drives evolution. Once pairing and recombination have completed, spermatocytes undergo two rounds of division, with the first round separating homologues and the second round separating sister chromatids to generate haploid cells.

Spermiogenesis

The haploid cells generated by the meiotic divisions are called round spermatids due to their characteristic round shape. Round spermatids will differentiate into spermatozoa in a process called spermiogenesis (Russell et al., 1990). Multiple morphological changes occur during spermiogenesis which include flagellum formation, acrosome development, cytoplasmic loss, and reshaping of the nucleus. The flagellum of spermatozoa includes accessory structures in addition to the axoneme, the 9 + 2 microtubule core found in cilia and flagella in other eukaryotes. In mammalian systems, the flagellum can be divided mainly into the midpiece and principal piece (Fig. 1B). The midpiece is proximal to the nucleus and contains outer dense fibers surrounding the axoneme with tightly packed mitochondria. The principal piece is devoid of mitochondria but contains outer dense fibers surrounding the axonemal core, which in turn is encased by the fibrous sheath. Early in spermiogenesis, the acrosomal vesicle initially forms by the fusing of pro-acrosomal vesicles derived from the Golgi apparatus. As more vesicles fuse, the acrosomal vesicle grows and forms a cap-like shape (the acrosome) around the nucleus. Within the vesicle, there are proteases and receptors that allow for sperm-egg recognition. As spermatids progress through development, the nucleus and acrosome shift position to leave most of cytoplasm on one side. The cytoplasmic remnants (also called the residual body) will be lost at the end of spermiogenesis and be phagocytosed by

Sertoli cells. The loss of cytoplasm generates the streamline shape of spermatozoa. Midway through spermiogenesis, histone proteins are replaced by transition proteins, highly basic proteins, that are replaced themselves by protamines. Protamines are also highly basic proteins that allow for greater compaction of chromatin. Chromatin compaction along with the cytoskeleton generate the peculiar nuclear morphology of sperm. The tight compaction of the nucleus also prohibits transcription. In the final process of spermiogenesis, the spermatozoa are released into the lumen of the seminiferous tubules in a process called spermiation. Released spermatozoa are transported to the epididymis. During transit through the epididymis, spermatozoa acquire motility and fertilization ability. It is in the cauda epididymis where mature sperm are stored.

Defects in Sperm Number or Function

Azoospermia

Azoospermia is the condition of complete absence of sperm in the ejaculate. In infertile males, approximately 15% display azoospermia. Azoospermia can be subdivided into two broad categories, obstructive azoospermia (OA) and non-obstructive azoospermia (NOA). OA is the results of a blockage within the excurrent duct system. For example, mutations in *CFTR* result in congenital bilateral absence of the vas deferens in males homozygous for these mutations (Grangeia et al., 2007). NOA can result from primary, secondary, or incomplete testicular failure. Among males with azoospermia, 60% is due to OA and 40% due to NOA. Between OA and NOA, NOA presents with the most problems in conception rates using in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) because NOA is more often associated with spermatogenesis failure leading to production of few or no sperm.

Primary and secondary testicular failure result in the inability of testosterone production required for testis development; however, the source of the failure is what differentiates the two. Primary failure is the inability of the testis itself to produce testosterone while secondary failure results from defective hormonal signaling from the pituitary/hypothalamus axis which in turn instructs the testis to produce testosterone. Klinefelter Syndrome (XXY karyotype) is one condition that leads to primary testicular failure as the testis are underdeveloped with low levels of testosterone production. Very rare mutations in the chromatin protein CHD7 leads to CHARGE Syndrome, with one symptom being defective gonadotrophin releasing hormone production resulting in secondary testicular failure (Matzuk and Lamb, 2008).

Incomplete testicular failure presents with normal hormonal levels (i.e., FSH) with either small testis volume or normal testis volume. In these cases, maturation arrest and defective spermatogenesis can be present. Polysomy of the sex chromosomes (XXY, XYY, etc) and microdeletions on the Y chromosome are a common cause of NOA and were the first genetic abnormalities found in azoospermic patients (Miyamoto et al., 2015). Regions on the long arm of the Y chromosome that were commonly deleted in patients with azoospermia, are called azoospermia factor (AZF). Within this region, a testes specific RNA binding protein called Deleted in Azoospermia (*DAZ*) was identified as the first gene linked to patients with azoospermia. Since the discovery of *DAZ*, several genes in the AZF region and genes on the autosomes have been identified that lead to azoospermia when deleted. For example, deletion of *DBY*, a gene located in the AZF region, leads to seminiferous tubules devoid of developing gametes, which is referred to as a "Sertoli-cell only" phenotype. Mutations in genes essential to meiosis (such as *SYCP3*, *SYCP1*, *MLH1*, and *MYBL1*) lead to a meiotic arrest phenotype (Matzuk and Lamb, 2002, 2008). Genes essential in post-meiotic development show a round/elongated spermatid arrest. Deletions in *CREM*, considered the "master regulator" of round spermatid development, lead to an arrest in round spermatids (Matzuk and Lamb, 2002, 2008).

Oligozoospermia

Oligozoospermia is defined as decreased numbers of sperm in the ejaculate (<15 million sperm/mL), which is further subdivided depending on the number of sperm into mild (10–15 million sperm/mL), moderate (5–10 million sperm/mL), and severe (<5 million sperm/mL). Oligozoospermia can be considered as a less severe form of azoospermia, as the same mutations found in azoospermic patients can cause oligozoospermia in others. For example, the same deletions in the AZF region can also manifest as severe oligozoospermia in some patients. There are examples of gene deletions that have so far been specific to oligozoospermia. Deletions in *KLHL10* have been found in patients with oligozoospermia (Matzuk and Lamb, 2008).

Teratozoospermia

Teratozoospermia is a condition characterized by the presence of spermatozoa with abnormal morphology. In monomorphic teratozoospermia, macrozoospermia and globozoospermia are well-recognized categories (Coutton et al., 2015; De Braekeleer et al., 2015; Ray et al., 2017).

Macrozoospermia

Macrozoospermia is a very rare condition observed in <1% of infertile men and is characterized by oversized misshapen heads and multiple flagella. The spermatozoa of macrozoospermia patients often exhibit unusual number of ploidy, especially four sets of chromosomes instead of normal one set of chromosomes. In 2007, a genome-wide microsatellite analysis revealed mutations in *AURKC* in macrozoospermic men. *AURKC* is a component of the chromosomal passenger complex that regulates mitotic and

meiotic events including chromosome segregation. AURKC dysfunction leads to uneven chromosome segregation and improper cytokinesis during meiosis of spermatogenesis. Unlike human cases, *Aurkc* KO male mice are subfertile but exhibit defective nuclear chromatin condensation and acrosome detachment and malformation. This mild phenotype in mice may be due to compensation by *Aurkb* that is strongly expressed in the mouse testis.

Globozoospermia

Globozoospermia is also a very rare condition observed in less than 0.1% of infertile men and is characterized by round-headed spermatozoa lacking the acrosome and is accompanied by an abnormal nuclear shape and an unusual arrangement of the sperm mitochondria. Mutations in genes such as *DPY19L2*, *PICK1*, *ZBPB*, and *SPATA16* were reported in globozoospermic men and, among these genes, *DPY19L2* is mutated most frequently. The cellular function of *DPY19L2* was analyzed using *Dpy19l2* KO mice that also exhibit male sterility due to globozoospermia. *DPY19L2* is localized in the inner nuclear membrane of the spermatids and is essential for the stabilization of both the nuclear dense lamina and the junction between the nuclear envelope and the acrop-laxome, a structure that anchors the developing acrosome to the nuclear envelope. In addition to *Dpy19l2* KO mice, *Pick1* KO and *Zbpb* KO male mice are infertile due to globozoospermia, indicating that the process of acrosome formation is conserved well between humans and mice. There are more KO mouse lines that are reported to have globozoospermia such as *Csnk2a2*, *Hrb*, *Gopc*, *Gba2*, and *Spaca1* (Fig. 2A). So far, these genes have not been linked to human cases yet.

Asthenozoospermia

Asthenozoospermia is a condition characterized by reduced sperm motility (Coutton et al., 2015; Ray et al., 2017). Complete asthenozoospermia that shows 100% immotile spermatozoa is found at a frequency of 1 in 5000 men. Asthenozoospermia is often observed in men with primary ciliary dyskinesia (PCD), or immotile ciliary syndrome, because both cilia and sperm flagella utilize a common 9 + 2 axonemal structure for their motility. PCD is characterized by congenital impairment of mucociliary clearance and is estimated to be found at a frequency of 1 in 16,000 births. A multitude of genes that constitute the 9 + 2 axoneme were found to

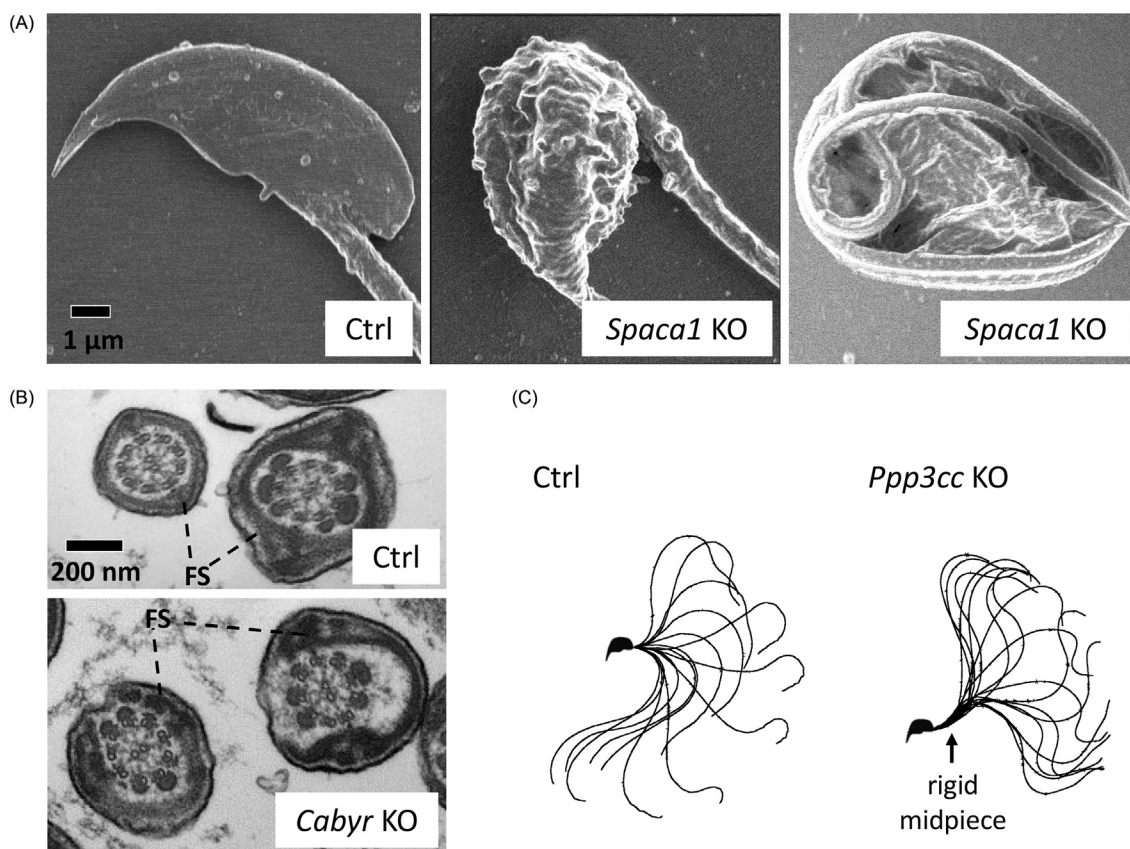


Fig. 2 Examples of sperm defects. (A) Globozoospermia. Sperm from control (Ctrl) and *Spaca1* KO mice were observed using scanning electron microscopy. *Spaca1* KO sperm show abnormal head morphology. (B) Dysplasia of the fibrous sheath. Sperm from Ctrl and *Cabyr* KO mice were observed using transmission electron microscopy. The fibrous sheath (FS) is disrupted in the *Cabyr* KO sperm. (C) Asthenozoospermia. Sperm motility of Ctrl and *Ppp3cc* KO mice were observed. Images of moving flagella were taken at 200 frames per second and were superimposed. *Ppp3cc* KO sperm exhibit impaired motility associated with a rigid midpiece.

be associated with PCD including asthenozoospermia. For example, mutations in *DNAH11* that composes a dynein arm, a motor apparatus in the axoneme, were found in PCD men.

Another cause of asthenozoospermia is dysplasia of the fibrous sheath (DFS). The fibrous sheath (FS) is an accessory structure that surrounds the axoneme in the principal piece of the flagellum. DFS is characterized by disrupted FS, leading to impaired sperm motility because not only does the FS regulate the degree of flagellar flexibility, but also acts as a scaffold for several signaling and/or metabolic pathways. Many components of FS have been identified, such as AKAP3, AKAP4, CABYR. While analyses of *Akap3* KO mice have not been reported yet, KO male mice of *Akap4* and *Cabyr* have been reported to be infertile due to disrupted FS organization (Fig. 2B), suggesting that mutations in these genes may cause DFS in humans. Indeed, deletions in *AKAP3* and *AKAP4* were described in some DFS patients.

In addition to structural defects found in the axoneme and fibrous sheath, functional defects in flagella may also cause asthenozoospermia (Ray et al., 2017). KO mouse lines that show normal flagellar structure but were infertile due to impaired sperm motility were reported in studies of mouse *Catsper1*, *Ppp3cc*, *Gapdhs*, *Ldhc* (Fig. 2C). CATSPER1 and PPP3CC are involved in calcium signaling, and GAPDHS and LDHC are metabolic enzymes. CATSPER1 is a component of a calcium channel localized in the principal piece of the flagellum and is necessary for hyperactivated motility. The pore-forming region of the calcium channel is thought to be composed of four proteins, CATSPER1–4. If any one of these proteins is deleted in mice, the whole channel is disrupted, leading to a decreased motility phenotype. In humans, mutations in *CATSPER1* and *CATSPER2* were identified in asthenozoospermic men.

Unexplained Male Infertility

When semen analyses are normal and female infertile factors are ruled out, the patients are categorized as having unexplained male infertility (UMI) (Esteves et al., 2014). UMI is observed at varying frequencies that range from 6% to 27%, suggesting evaluation criteria can account for the varying frequencies. As the name indicates, causes of UMI are unknown, but recent progress in basic research using KO mice reveals genetic factors that contribute to male infertility that cannot be identified by standard semen analysis. Because assisted reproductive techniques (ART) such as ICSI can often solve UMI, detailed analyses of the causes tend not to be performed. However, basic research including genome sequencing may lead to the development of new methods to evaluate UMI easily and inexpensively, which may help not only understand the causes but also promote cost-effective and low-invasive treatments.

Sperm migration in the female reproductive tract

There are KO mice that show normal sperm morphology and motility but whose spermatozoa cannot pass through the utero-tubal junction (UTJ) of the female reproductive tract (Okabe, 2015). So far, there are more than 10 genes reported, such as *Adam2* and *Ace*, disruption of which causes UTJ migration defects. The spermatozoa of these KO mice exhibit defective binding ability to the zona pellucida (ZP, an extracellular matrix that surrounds the oocytes) as well, suggesting that there is a common mechanism that regulates UTJ passage and ZP binding. When IVF was performed using cumulus-intact oocytes, the oocytes can be fertilized normally, indicating that the cause of infertility is the UTJ migration defect but not impaired ZP binding ability. Therefore, IVF may be an effective way for ART if mutations were found in these genes of infertile men.

Sperm-egg fusion

IZUMO1 is a single transmembrane protein localized to the sperm head (Inoue et al., 2011). The spermatozoa of *Izumo1* KO mice look normal and move vigorously but cannot fertilize oocytes due to impaired sperm-egg fusion. This infertility can be rescued by bypassing the fusion process with ICSI. By blocking human IZUMO1 function with an antibody against IZUMO1, the necessity of human IZUMO1 for sperm-egg fusion was demonstrated, but mutations in *IZUMO1* have not been reported in infertile men yet.

Oocyte activation

After fertilization, oocytes need to be activated by sperm-derived factors (Swann and Lai, 2016). There are two candidate activating protein factors, namely PLCZ and PAWP. Mutations in *PLCZ* were found in infertile men whose sperm fail to fertilize eggs even after ICSI because oocytes cannot be activated by the injected spermatozoa. In this case, it may be necessary to activate eggs artificially, for example, using *PLCZ* mRNA or strontium when performing ART. Intriguingly, *Plcz* or *Pawp* KO male mice were generated recently and were subfertile and fertile, respectively, suggesting that there are different oocyte-activating mechanisms that are not related to PLCZ or PAWP, at least in mice.

Summary and Prospects

From single spermatogonia to the streamlined, haploid spermatozoa, spermatogenesis is a complicated process that produces gametes capable of delivering the haploid male genome to the egg (Matzuk and Lamb, 2002, 2008). In some infertile men, problems in sperm production have been identified. Advances in genomic and exome sequencing has accelerated the discovery of genes associated with infertility observed in these men. The mouse has been a powerful model in assigning genes identified from patients as functioning in specific stages of spermatogenesis. The development of CRISPR/Cas9 genome editing technology has further

accelerated the production of gene ablation and point mutation studies in the mouse (Miyata et al., 2016). Research in identifying male infertility genes has increased the number of potential candidates for contraceptives. For example, a calcium signaling gene (PPP3CC), essential for male fertility, has been shown to be a potential contraceptive target (Miyata et al., 2015). FK506 and cyclosporin A are drugs that target the calcium signaling complex formed by PPP3CC. When administering these drugs, male mice become infertile after 2 weeks with no changes in mating behavior. Mice administered with these drugs produce sperm that have defects in motility, mimicking the asthenozoospermia seen in some infertile male patients. Remarkably, the defects in motility disappear in mice when the drug is withdrawn, leading to perfectly fertile mice. Unfortunately, calcium signaling is important in the immune system, and FK506 and cyclosporin A are powerful immunosuppressant drugs which precludes them from being utilized as a male contraceptive. Next generation drug screening offers the possibility of identifying more selective contraceptive drugs.

Despite these advances in understanding spermatogenesis and its role in explaining male infertility, many challenges remain. While basic research has explained some of the infertility seen in men, there is still a significant percentage of infertile men whose etiology is unknown. It is suspected that undiscovered genetic lesions/gene mutations will provide clues in explaining these cases. The problem of identifying genes essential for male fertility is confounded by the discovery that over 1000 genes are specifically expressed in the testis. We live in exciting times as CRISPR/Cas9 is increasing the pace of discovery in the complicated process that is sperm production and function (Miyata et al., 2016).

References

- Coutton, C., Escoffier, J., Martinez, G., Arnoult, C., & Ray, P. F. (2015). Teratozoospermia: Spotlight on the main genetic actors in the human. *Human Reproduction Update*, 21, 455–485.
- De Braekeleer, M., Nguyen, M. H., Morel, F., & Perrin, A. (2015). Genetic aspects of monomorphic teratozoospermia: A review. *Journal of Assisted Reproduction and Genetics*, 32, 615–623.
- De Rooij, D. G. (2017). The nature and dynamics of spermatogonial stem cells. *Development*, 144(17), 3022–3030.
- Esteves, S. C., Sharma, R. K., Gonsálvez, J., & Agarwal, A. (2014). A translational medicine appraisal of specialized andrology testing in unexplained male infertility. *International Urology and Nephrology*, 46, 1037–1052.
- Grangeia, A., Sá, R., Carvalho, F., Martin, J., Girodon, E., Silva, J., Ferráz, L., Barros, A., & Sousa, M. (2007). *Genetics in Medicine*, 9, 163–172.
- Greenbaum, M. P., Yan, W., Wu, M. H., Lin, Y. N., Agno, J. E., Sharma, M., Braun, R. E., Rajkovic, A., & Matzuk, M. M. (2006). TEX14 is essential for intercellular bridges and fertility in male mice. *PNAS*, 103, 4982–4987.
- Inoue, N., Ikawa, M., & Okabe, M. (2011). The mechanism of sperm-egg interaction and the involvement of IZUM1 in fusion. *Asian Journal of Andrology*, 46, 81–87.
- Matzuk, M. M., & Lamb, D. J. (2002). Genetic dissection of mammalian fertility pathways. *Nature Medicine*, 8, S41–S49.
- Matzuk, M. M., & Lamb, D. J. (2008). The biology of infertility: Research advances and clinical challenges. *Nature Medicine*, 14, 1197–1213.
- Miyamoto, T., Minase, G., Okabe, K., Ueda, H., & Sengoku, K. (2015). Male infertility and its genetic causes. *The Journal of Obstetrics and Gynaecology Research*, 41, 1501–1505.
- Miyata, H., Castaneda, J. M., Fujihara, Y., Yu, Z., Archambeault, D. R., Isotani, A., Kiyozumi, D., Kriseman, M. L., Mashiko, D., Matsumura, T., Matzuk, R. M., Mori, M., Noda, T., Oji, A., Okabe, M., Prunskaitė-Hyryläinen, R., Ramirez-Solis, R., Satouh, Y., Zhang, Q., Ikawa, M., & Matzuk, M. M. (2016). Genome engineering uncovers 54 evolutionarily conserved and testis-enriched genes that are not required for male fertility. *PNAS*, 113, 7704–7710.
- Miyata, H., Satouh, Y., Mashiko, D., Muto, M., Nozawa, K., Shiba, K., Fujihara, Y., Isotani, A., Inaka, K., & Ikawa, M. (2015). Sperm calcineurin inhibition prevents mouse fertility with implications for male contraceptive. *Science*, 350, 442–445.
- Okabe, M. (2015). Mechanisms of fertilization elucidated by gene-manipulated animals. *Asian Journal of Andrology*, 17, 646–652.
- Ray, P. F., Toure, A., Metzler-Guillemain, C., et al. (2017). Genetic abnormalities leading to qualitative defects of sperm morphology or function. *Clinical Genetics*, 91, 217–232.
- Russell, L. D., Ettlin, R. A., Hikim, A. P. S., & Clegg, E. D. (1990). *Histological and histopathological evaluation of the testis*. Clearwater, FL: Cache River Press.
- Swann, K., & Lai, F. A. (2016). Egg activation at fertilization by a soluble sperm protein. *Physiological Reviews*, 96, 127–149.

Further Reading

Yanagimachi, R. (1994). Mammalian fertilization. In Knobil, E. and Neill, J. D. (eds.) *The Physiology of Reproduction*. Vol. 1, pp. 189–317 New York: Raven Press.

MEDICAL CONDITIONS

Medical Conditions and Male Reproductive Medicine: An Overview

Isaac Lam and Robert E Brannigan, Northwestern University, Evanston, IL, USA

© 2018 Elsevier Inc. All rights reserved.

Glossary

- Azoospermic** Lack of viable sperm in semen.
- Bilateral** Involving both sides.
- Contralateral** Involving the opposite side.
- Ejaculatory duct** Formed by the seminal vesicle joining the vas deferens.
- Epididymis** Tube which connects testicle to vas deferens.
- History** Medical history of patient.
- Hernias** Organ abnormally rupturing through breach in tunica vaginalis.
- Hydroceles** Fluid accumulation in body cavity.
- Ipsilateral** Involving the same side.
- Karyotype** Appearance and number of chromosomes in eukaryotic cell nucleus.
- Libido** Sex drive.
- Physical exam** Physical investigation of patient body for disease.
- Spermatic cord** Structure that includes the vas deferens and tissue surround it.
- Spermatogenesis** Production of mature spermatozoa.
- Unilateral** Involving one side only.
- Urethra** Duct from the bladder that delivers urine out of the body.
- Vas deferens** Duct from the testicle to the ejaculatory duct that delivers sperm.

Introduction

Although the societal narrative of infertility typically revolves around women unable to bear children, studies have demonstrated that males contribute in approximately half of all infertility cases. The disease of infertility has a widespread impact, afflicting about one in every seven couples ([Agarwal et al., 2015](#)). This condition can be devastating to patients, causing excessive stress and low self-esteem, especially in cultures where self-worth or masculinity is closely tied to the ability to reproduce. In addition to the psychological consequences of infertility, the disease proves to be costly from an economic standpoint as well. The Urologic Diseases in America (UDA) Project estimates that in 2000 alone, \$17 million were used to treat male infertility. Accounting for the cost of assisted reproductive technologies for the female partner that often comes with treating male infertility, the total price tag of male reproductive medicine amounts to a substantial \$18 billion over the course of one year ([Meacham et al., 2007](#)).

In a normal couple, the chance of pregnancy with unprotected intercourse is about 25% per month and 90% per year. In contrast, nonazoospermic infertile couples have a drastically lower pregnancy rate at about 1–3% a month. Despite these low success rates, about a third of infertile couples can still eventually conceive without any treatment ([Dubey and DeCherney, 2012](#)). By definition, patients are typically considered infertile after a full year of unprotected sex without pregnancy. However, because most couples that have not conceived after 6 months will not be able to in the future, physicians often begin evaluating the patient at this point in time.

To help guide physicians and improve quality of care, the American Urological Association and the American Society for Reproductive Medicine have developed recommendations for the evaluation of male infertility, including history taking, the physical exam, semen analysis, and specialized testing. If etiology of the disease is known, the physician subsequently treats the underlying pathology. If etiology is unknown, the physician and patient typically discuss other available options such as intra-uterine insemination, in vitro fertilization, intracytoplasmic sperm injection, donor insemination, and adoption ([Gangel et al., 2002](#)).

Childhood Illnesses and Conditions

Certain childhood illnesses or developmental abnormalities can later present as infertility in male patients. This section of the article will provide an overview of some of these conditions, including torsion, cryptorchidism, and hypospadias.

Hypospadias and Epispadias

In some patients the opening of the urethra is abnormally positioned on either the ventral or dorsal side of the penis, conditions known as hypospadias and epispadias respectively. Both can lead to infertility if semen is deposited too distally in the vagina as a result of the anatomical abnormality. Although both conditions have seemingly similar names and presentations, the underlying pathophysiology is largely different. Hypospadias are typically caused by genetic polymorphic mutations and environmental factors such as maternal hypertension and low birth weight (Macedo et al., 2012). On the other hand, epispadias are the mildest form of the bladder–exstrophy–espadias complex (BEEC), which encompasses a variety of presentation from failed closure of the lower abdominal wall or bladder. Surgical treatments are available for both hypospadias and epispadias (Ludwig et al., 2009).

Testicular Torsion

Most commonly observed immediately after birth and during puberty, testicular torsion occurs when the spermatic cord twists, limiting blood supply to the testicle and thus causing ischemia. Torsion typically presents as an acute severe testicular pain often associated with nausea and vomiting. Interestingly, biopsies of the contralateral testicle in patients that have experienced torsion reveal abnormal findings as well, indicating that pre-existing testicular conditions may predispose patients to torsion. Treatment includes surgical detorsion to restore blood flow and orchidopexy (anchoring of the testicle to the dartos muscle) in order to minimize the risk of recurrent torsion (Shimizu et al., 2016).

Intersex or Disorders of Sexual Development

Genetic mutations in the sex chromosomes can lead to atypical development of internal or external reproductive organs. In the past, disorders of sexual development (DSD) were divided into four separate categories: male pseudohermaphroditism, female pseudohermaphroditism, true hermaphroditism, and gonadal dysgenesis. Currently, the nomenclature for characterizing DSDs includes the karyotype and the molecular basis of the defect. Treatment can range from hormonal to surgical, with the end goal of reversing the phenotypic abnormalities caused by the genetic defects (Kim and Kim, 2012).

Cryptorchidism

Although failure of the testes to descend is extremely common, the condition typically self resolves. Testes fail to descend in about 4% of newborns, but by age one, only slightly more than 1% of infants remain cryptorchid. For infants in whom the defect does not self-correct, orchiopexy is performed to permanently secure the testicle within the scrotal compartment. Cryptorchidism can present unilaterally or bilaterally, with the bilateral form presenting about twice as commonly as unilateral cryptorchidism. While unilaterally cryptorchid patients have fertility rates similar to the norm, bilaterally cryptorchid patients have substantially lower rates at around 50–60% (Fawzy et al., 2015).

Microductal Aplasia

Failure of the vas deferens to develop can occur unilaterally or bilaterally, each with a unique pathophysiological etiology. Congenital unilateral absence of the vas deferens (CUAVD) typically occurs due to abnormalities in mesonephric duct development. The mesonephric duct evolves into the epididymis, vas deferens, and ejaculatory duct, and thus CUAVD may present with defects in those anatomical parts as well. Because the mesonephric duct plays a role in renal development, CUAVD may suggest concurrent ipsilateral renal agenesis (Weiske et al., 2000).

In contrast, congenital bilateral absence of vas deferens (CBAVD) is typically associated with mutations in cystic fibrosis transmembrane conductance regulator (CFTR) or other genes that regulate mesonephric duct maturation. Cystic fibrosis is an autosomal recessive disease. While patients with just one abnormal copy of the CFTR gene typically do not have cystic fibrosis, they often have CUAVD or CBAVD. At this point in time, over 1000 CFTR genes have been identified. The most common gene mutation in patients with cystic fibrosis, CUAVD, and CBAVD is delta-F508 which involves a deletion of phenylalanine at the position of 508 in the protein sequence. Because gamete production remains normal in most patients with CBAVD, reproduction can still be achieved through ICSI using surgically extracted sperm (Weiske et al., 2000).

Systemic Diseases and Illnesses

Diabetes Mellitus

With 300 million individuals projected to have diabetes mellitus worldwide in 2025, the number of cases of diabetes-linked male infertility will continue to grow. Diabetes can lead to male infertility through a myriad of mechanisms. Leydig cells normally produce testosterone in response to luteinizing hormone (LH), but diabetes can result in dysfunction of Leydig cells leading to low testosterone levels, resultant high LH levels, and primary testicular failure. Secondary testicular failure, marked by low testosterone and LH levels, can also arise from diabetes via diabetes induced changes affecting the hypothalamic-pituitary-gonadal axis. In addition to causing endocrine changes, diabetes can alter sympathetic nerves innervating the reproductive system causing retrograde ejaculation or complete failure of emission. It is important to note that some studies have found that tight regulation of DM leads to diminished impact on male reproductive health (Sexton and Jarow, 1997).

Hypogonadism

Cases of low testosterone can be categorized into two types: hypergonadotropic hypogonadism and hypogonadotropic hypogonadism. As described in the previous section, hypergonadotropic hypogonadism occurs when Leydig cell dysfunction leads to low testosterone and high LH levels. In some cases this dysfunction can occur concomitantly with pituitary dysfunction, resulting in lower than expected LH levels. While treatment options for low testosterone include testosterone replacement therapy, this treatment should not be provided to a man actively trying to conceive as this treatment suppresses intratesticular testosterone levels, spermatogenesis, and commonly renders a patient infertile (Hohl, 2017).

Hypogonadotropic hypogonadism develops when the pituitary gland fails to secrete sufficient amounts of LH and follicle stimulating hormone (FSH). Kallman syndrome is a form of hypogonadotropic hypogonadism, a disease that commonly presents with loss of smell in addition to low serum LH and FSH levels. In most cases, patients are treated with human chorionic gonadotropin (hCG) and recombinant FSH (rFSH) to directly replace LH and FSH respectively. Treatment of milder forms of hypogonadotropic hypogonadism can sometimes include aromatase inhibitors and anti-estrogenic drugs, which have pituitary stimulating effects (Hohl, 2017).

Infection and Inflammation

Common infectious organisms that target the male reproductive system include *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella*, *Neisseria gonorrhoeae*, and *Chlamydia trachomatis*. These organisms can infect the urethra, prostate, epididymis, and testis, although infection of the testis occurs quite infrequently. Infection results in infertility through either functional or anatomical means. Functional impairment arises when an immunological response triggered by the infection induces spermatogenic dysfunction. Anatomical abnormalities leading to infertility can involve the narrowing of a duct in the reproductive system, e.g. the epididymis, vas deferens, or ejaculatory duct. Numerous studies have described the detrimental effects of inflammation on fertility in the setting of infection, but inflammation alone without clear evidence of infection can also have substantial negative effects on both sperm production and sperm function (Niederberger, 2012).

Cancer and History of Cancer Treatment

While it is probably more apparent that treatments targeting malignant growth can have deleterious effects on male reproductive health, cancer itself can impact fertility through various mechanisms. Spermatogenesis requires a precise balance maintained by the hypothalamus, pituitary gland, and testes that is often disrupted by cancer processes. Immune responses that help curb the progression of cancer can unfortunately have negative repercussions for fertility. Systemic conditions such as infection and malnutrition induced by cancer can upset homeostasis and ultimately damage the reproductive system. Even psychological issues such as anxiety and depression specifically linked to coping with cancer diagnoses have been demonstrated to increase the risk of sexual dysfunction (Brannigan, 2007).

As mentioned above, cancer treatments have the potential to impair the reproductive potential of oncologic patients. Since chemotherapy specifically suppresses the growth of rapidly proliferating cells, it is unsurprising that chemotherapy can damage the germinal epithelium as well. The degree to which reproductive ability is impaired by chemotherapy largely depends on the type of agent used, the drug dosage, and age of the patient. Alkylating agents such as cyclophosphamide have proven to be especially high risk for infertility. Impaired DNA function caused by these agents can result in genetic mutations in the germinal epithelium. While low doses of alkylating agents given to patients over a short period of time generally have reversible effects, higher doses and increased duration can lead to permanent damage (Brannigan, 2007).

Like chemotherapy, radiation therapy can damage the germinal epithelium. Because of the highly radiosensitive nature of the germ cells, fertility can be impacted by not only direct radiation but also scatter radiation from treatment of adjacent or nearby organs. Even just 2 Gy of radiation can damage spermatocytes and lead to irreversible azoospermia. Although many patients may regain spermatogenesis after treatment, some may experience a permanent reduction in sperm concentration or outright permanent and irreversible azoospermia (Brannigan, 2007).

Table 1 Medications that have potential detrimental effects on male fertility

<i>Medication/Drugs</i>	<i>Impact on fertility</i>
Diuretics, beta-blockers	Erectile dysfunction
Anti-psychotics	Lowered libido
Opioids	Hypogonadotropic hypogonadism
HAART	Impaired sperm motility and reduced ejaculate volume
Cannabis	Reduced testosterone levels
Tobacco	Damaged sperm DNA
Alcohol	Reduced androgen levels and impaired sperm parameters

References: [Brezina et al. \(2012\)](#), [Doulas and Douma \(2006\)](#), [Kulkarni et al. \(2014\)](#), [Niederberger \(2012\)](#), [Sigman et al. \(2009\)](#).

Medication and Drug Use

A summary of the impact of medications on male fertility can be found in [Table 1](#).

Antihypertensives

Erectile dysfunction from blood control medications is a common reason for patient nonadherence. Commonly used antihypertensive drugs such as diuretics and beta-blockers can result in erectile dysfunction, while ACE inhibitors and calcium antagonists less commonly have this side effect. Interestingly, some recent studies have shown that angiotensin II receptor blockers (ARBs) might have beneficial effects on reproduction as angiotensin II can contribute to the pathogenesis of erectile dysfunction ([Doulas and Douma, 2006](#)).

Antipsychotics

Antipsychotic drugs can reduce reproductive potential through several mechanisms. Dopamine plays a crucial role in maintaining libido, and most antipsychotic drugs function as dopamine antagonists, which can result in lower libido. Antipsychotic drugs can also elevate levels of prolactin which can lead to reduced sex drive in some patients. Additionally, studies have also shown that SSRIs specifically can cause anorgasmia and delayed or absent ejaculation in some patients ([Brezina et al., 2012](#)).

Opioids

Reduced release of LH and lowered levels of testosterone can result from opioid use, leading to hypogonadotropic hypogonadism. In addition, research has established that some endogenous opioid receptors reside in the testis, suggesting that opioids can impact spermatogenesis directly, although the mechanisms need further clarification. Discontinuation of opioid use typically leads to normalization of androgen levels ([Niederberger, 2012](#)).

Antiretrovirals

Numerous studies have found that highly active antiretroviral therapy (HAART) can result in impaired sperm motility, reduced ejaculate volume, abnormal sperm morphology, and other detrimental effects on mechanisms crucial for oocyte fertilization. Fortunately, despite these findings, most patients undergoing HAART often have preserved fertility ([Brezina et al., 2012](#)).

Occupational and Lifestyle Exposures

Recreational Drugs

Cannabis, tobacco, and alcohol use have all been shown to have negative repercussions for male fertility. Marijuana results in reduced testosterone levels in plasma proportional to the dose and duration of cannabis used. Cigarette smoking can impact the male reproductive system by inducing oxidative stress on semen with resultant damage to sperm DNA and reduction in sperm motility. Chronic alcohol use affects reproductive health by worsening sperm parameters and by reducing androgen levels ([Kulkarni et al., 2014](#)).

Heat

Scrotal temperature is regulated at 2–4 °C below body temperature primarily through its location outside of the body cavity and the countercurrent heat exchange produced by vasculature to and from the testes. The adverse effects of heat on the quality and output of sperm have been well documented, but the temperature at which thermal toxicity leads to infertility remains unclear. Studied sources of increased scrotal temperature range from clothes to body posture to laptop usage ([Thonneau et al., 1998](#)).

Pesticides and Insecticides

Research suggests that pesticides can impair male reproductive health. Dieldrin has been observed to cause defects in Leydig cell function, and both carbaryl and chlorpyrifos have been shown to damage DNA in the reproductive system. Even though counseling patients to consume local seems to be an intuitive solution to avoid the reproductive detriments of pesticides, one study actually found lower testosterone and LH levels as well as lower sperm concentration and aberrant sperm morphology in men who ate locally grown produce (Niederberger, 2012).

Pollution

A meta-analysis conducted in 2015 determined that air pollution has a deleterious impact on sperm motility, but not on other sperm parameters. Episodic exposure to high levels of air pollution has been shown to be associated with DNA fragmentation in sperm. Other studies have also linked air pollution to higher rates of miscarriage and lower rates of pregnancy (Deng et al., 2016).

Past Surgical History

A spectrum of surgical procedures including bladder neck, prostate, pelvic, and retroperitoneal surgery can result in ejaculatory dysfunction. Patients who experience retrograde ejaculation after surgery present with low-volume ejaculates and copious sperm in post-ejaculate urine. Treatment options include sympathomimetic drugs, electroejaculation, and recovery of sperm from postejaculatory urine or through surgical retrieval (Sigman et al., 2009).

Surgical repair of hydroceles and hernias in childhood can compromise fertility as well via obstruction of the vas deferens. Use of a surgical mesh during hernia repair can result in inflammation that can cause vasal obstruction. Other childhood procedures previously associated with male infertility include urethral valve ablation and bladder neck repair (Niederberger, 2012).

References

- Agarwal, A., Mulgund, A., Hamada, A., & Chyatte, M. R. (2015). A unique view on male infertility around the globe. *Reproductive Biology and Endocrinology*, 13, 37.
- Brannigan, R. E. (2007). Fertility preservation in adult male cancer patients. *Cancer Treatment and Research*, 138, 28–49.
- Brezina, P. R., Yunus, F. N., & Zhao, Y. (2012). Effects of pharmaceutical medications on male fertility. *Journal of Reproduction and Infertility*, 13, 3–11.
- Deng, Z., Chen, F., Zhang, M., Lan, L., Qiao, Z., Cui, Y., An, J., Wang, N., Fan, Z., Zhao, X., & Li, X. (2016). Association between air pollution and sperm quality: A systematic review and meta-analysis. *Environmental Pollution*, 208, 663–669.
- Doumas, M., & Douma, S. (2006). The effect of antihypertensive drugs on erectile function: A proposed management algorithm. *Journal of Clinical Hypertension (Greenwich, Conn.)*, 8, 359–364.
- Dubey, A. K., & DeCherney, A. H. (2012). *Infertility: Diagnosis, management and IVF*. Jaypee Brothers Medical Publishers: New Delhi.
- Fawzy, F., Hussein, A., Eid, M. M., El Kashash, A. M., & Salem, H. K. (2015). Cryptorchidism and fertility. *Clinical Medicine Insights. Reproductive Health*, 9, 39–43.
- Gangel, E. K. American Urological Association, Inc, American Society for Reproductive Medicine. (2002). AUA and ASRM produce recommendations for male infertility. American Urological Association, Inc. and American Society for Reproductive Medicine. *American Family Physician*, 65, 2589–2590.
- Hohl, A. (2017). *Testosterone*. New York, NY: Springer Science+Business Media.
- Kim, K. S., & Kim, J. (2012). Disorders of sex development. *Korean Journal of Urology*, 53, 1–8.
- Kulkarni, M., Hayden, C., & Kayes, O. (2014). Recreational drugs and male fertility. *Trends in Urology & Men's Health*, 5, 19–23.
- Ludwig, M., Ching, B., Reutter, H., & Boyadjiev, S. A. (2009). Bladder exstrophy-epispadias complex. *Birth Defects Research. Part A, Clinical and Molecular Teratology*, 85, 509–522.
- Macedo, A., Jr., Rondon, A., & Ortiz, V. (2012). Hypospadias. *Current Opinion in Urology*, 22, 447–452.
- Meacham, R. B., Joyce, G. F., Wise, M., Kparker, A., Niederberger, C., & Urologic Diseases in America Project. (2007). Male infertility. *The Journal of Urology*, 177, 2058–2066.
- Niederberger, C. S. (2012). Male infertility. In *Campbell-Walsh urology* (pp. 556–579). Philadelphia, PA: Elsevier Saunders.
- Sexton, W. J., & Jarow, J. P. (1997). Effect of diabetes mellitus upon male reproductive function. *Urology*, 49, 508–513.
- Sigman, M., Lipshultz, L. I., & Howards, S. S. (2009). Office evaluation of the infertile male. In *Infertility in the male* (pp. 153–176). Cambridge, UK: Cambridge University Press.
- Shimizu, S., Tsounapi, P., Dimitriadis, F., Higashi, Y., Shimizu, T., & Saito, M. (2016). Testicular torsion-detorsion and potential therapeutic treatments: A possible role for ischemic postconditioning. *International Journal of Urology*, 23, 454–463.
- Thonneau, P., Bujan, L., Multigner, L., & Mieusset, R. (1998). Occupational heat exposure and male fertility: A review. *Human Reproduction*, 13, 2122–2125.
- Weiske, W. H., Salzler, N., Schroeder-Printzen, I., & Weidner, W. (2000). Clinical findings in congenital absence of the vasa deferentia. *Andrologia*, 32, 13–18.

Further Reading

Male infertility. (2012). In C. S. Niederberger (Ed.), *Campbell-Walsh Urology* (pp. 556–579). Philadelphia, PA: Elsevier Saunders.

Immunologic Male Subfertility

Robert E Brannigan and Isaac Lam, Northwestern University, Feinberg School of Medicine, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

For about a century, sperm's potential to generate an immune response has been well recognized. In 1954, Rumke discovered the existence of anti-sperm antibodies and hypothesized that the antibodies may play a role in anomalous semen parameters and infertility (Meinertz, 1992). Since then, the knowledge base on the immune system's role in infertility has grown dramatically, but substantial gaps in understanding still remain. This article will first introduce the immune system for context, and then present an overview of the pathophysiology, diagnostics, and management of immune infertility.

The Immune System

Before delving into immunologic causes of male infertility, this section will give an overview of how the normal immune system functions in order to contextualize the rest of the article. The function of the normal immune system is to eliminate foreign material, or antigens. The body achieves this goal through two main mechanisms: innate immunity and adaptive immunity.

Innate Immunity

As the name implies, innate immunity refers to the part of the immune system that is programmed at birth. Cells involved in innate immunity include neutrophils, macrophages, and natural killer cells. Innate immunity reacts quickly and non-specifically to foreign antigens, taking only minutes to hours**** to mount a response. Innate immune cells use toll-like receptors (TLRs) to identify pathogen-associated molecular patterns (PAMPs) that commonly present on antigens such as the bacteria flagella or virus coats. Phagocytic cells such as macrophages and neutrophils then proceed to engulf and digest the identified antigens (Alberts, 2015).

Adaptive Immunity

Unlike innate immunity, adaptive immunity is highly specific to the invading pathogen. Antigen presenting cells (APCs) phagocytize the pathogen, then present antigens on type II major histocompatibility (MHC) proteins to T cells. When activated, the T cells either become cytotoxic T cells that destroy pathogens directly, or T helper cells that activate B-cells to produce antibodies. The released antibodies then attach to the pathogen and eliminate it through various mechanisms ranging from mast cell response to phagocytosis (Alberts, 2015).

Testicular Immunology

Because tolerance to self-antigens occurs during embryologic development and spermatogenesis does not begin until puberty, the immune system views sperm as foreign bodies. In order to prevent an autoimmune response, the testes are designed to be immunologically privileged, that is, immune reactions within the testes tend to be diminished relative to the same reactions that occur in other organ systems. Macrophages and lymphocytes are localized between seminiferous tubules but not within them, indicating that there is a blood-testis barrier that exists to create this distinction between the testicular immune system and the immune system of the rest of the body. This barrier is composed of two parts: the mechanical barrier and functional barrier (Walsh & Turek, 2009).

Mechanical Barrier

Sertoli cells found in the seminiferous tubules that primarily assist in spermatogenesis contribute to the mechanical barrier. Tight junctions, adherens junctions and gap junctions between Sertoli cells limit the migration of lymphocytes and large molecules such as immunoglobins. However, Sertoli cells alone are insufficient to maintain the blood-testis barrier as the density of junctions varies throughout the reproductive tract. For instance, the low density of junctions in the ductuli efferentes results in openings that allow entry of large molecules. Thus, a functional barrier is needed to complement the mechanical barrier (Cheng and Mruk, 2012).

Functional Barrier

The functional barrier is comprised of several mechanisms that down-regulate humoral and cell-mediated immunity. The vasculature of the seminiferous tubules assists with immunosuppression by ensuring lymphocytes do not accumulate in vulnerable portions of the germinal epithelium. Vulnerable regions of the germinal epithelium also house large numbers of T suppressor cells

that inhibit effector T cells and help produce immune tolerance at anatomically weak points of the seminiferous tubules. In addition, anomalies in human leukocyte antigen (HLA) proteins and sperm antigen-HLA associations may cause defective presentation of sperm antigens to lymphocytes, further contributing to the functional barrier (Cheng and Mruk, 2012).

Immunologic Infertility Mechanisms

Sperm Antigenicity

Most cases of germ line autoimmunity fall into one of two categories: testis-specific or anti-spermatogenic. In testis-specific autoimmunity, antigens induce a generalized immune response to the entire testicular organ leading to orchitis. The most common example of this phenomenon is mumps orchitis, a prevalent complication in post-pubertal men affected by mumps. The damage inflicted on testicular glands by the virus can cause inflammation of the parenchyma, interstitial lymphocyte infiltration, and seminiferous tubule separation. In the presence of these repercussions, mumps orchitis can lead to subfertility, including oligospermia, azoospermia, asthenospermia, and other defects. Although orchitis is not specific to the germinal epithelium, the general inflammation caused by orchitis often results in damage to epithelial and interstitial structures (Masarani et al., 2006).

In anti-spermatogenic autoimmunity, the immune response is specifically directed to the germinal epithelium. The resultant destruction of germ cell leads to a decline in spermatogenesis. A variety of sperm auto-antigens have been established using monoclonal antibody techniques. For instance, a sperm plasma membrane protein known as antigen 1 (FA-1) has been identified as an auto-antigen that regulates the interaction of sperm with zona pellucida, a glycoprotein layer surrounding oocytes. While antibodies against FA-1 do not impact sperm motility, their effects on sperm-zona pellucida interactions can still cause infertility (Coonrod et al., 1994).

Unfortunately, there are numerous limitations to the study of sperm antigenicity. A significant amount of current knowledge on sperm antigenicity stems from animal studies, which may not fully translate to human sperm antigenicity. In addition, sperm antigenicity is a highly dynamic phenomenon. At different stages of its maturity, sperm expresses a different set of antigens as it gain and loses antigens throughout development on the germinal epithelium. Lastly, cross-reactivity can be a confounding factor as well, as antibodies can bind to different molecules that share similar physical traits (Walsh and Turek, 2009).

Antibody-Mediated Sperm Autoimmunity

Although the immune system's ability to produce antibodies against sperm has been observed for a century, the exact clinical implications anti-sperm antibodies (ASA) have for male infertility remains uncertain. More than one out of ten males being evaluated for infertility have ASA, whether in the serum, seminal plasma, or bound to sperm (Turek and Lipshultz, 1994). Associations have been made between ASA and various conditions that result in the breach of the blood-testis barrier, but no causal link between ASA and infertility has been established. Some of these conditions include biopsy, vasectomy, testicular trauma, and other surgical procedures (Haas, 1987).

There are five different types of antibodies produced by the adaptive immune system including IgA, IgD, IgE, IgG, and IgM. Of the five only IgA, IgG, and IgM have demonstrated an ability to target sperm. IgMs have a large pentameric structure that restricts their ability to transudate to the seminal fluid, limiting the effects of IgM on infertility. IgA can be found in seminal fluid but typically does not present in the serum, and most IgGs in the seminal fluid are different from the IgGs in the serum. These two findings suggest that IgAs and IgGs that present in the testes are produced via local antigen inoculation and not systemic antigen inoculation (Haas et al., 1983; Uehling, 1971).

Antibodies may impair male fertility through various mechanisms. For instance, IgA bound to sperm has been shown to reduce sperm motility in the cervical mucus by binding to receptors in the mucus as well as causing agglutination of sperm. Female anti-sperm antibodies can impair the ability of sperm to penetrate the cervical mucus as well, quickly immobilizing sperm cells. Alternatively, antibodies can negatively impact fertility by binding to sperm and subsequently allowing phagocytosis or complement fixation of the sperm cells (McLachlan, 2002). Even if the sperm is not phagocytized or fixed by complement, the steric transformation of sperm caused by opsonization can interfere with sperm and egg interaction, as exemplified by the antibodies against FA-1 described earlier in this article. Some evidence even suggests that antisperm antibodies can lead to spontaneous abortions. In one study, zygotes derived from human sperm and animal oocytes expressed sperm-derived antigens shortly after fertilization, antigens that may leave the zygote vulnerable to opsonization (O'Rand, 1977).

Cell-Mediated Sperm Autoimmunity

In many cases of infertility, absence of antisperm antibodies suggests that cell-mediated autoimmune responses can be responsible for infertility as well. In spermatic cord torsion, necrosis and ischemia in the testis lead to release of significant amounts of sperm antigen. Despite this exposure to inoculum, the immune system does not produce significant levels of ASA in response, but a substantial amount of immune cell infiltration and inflammatory mediator release can be observed (Anderson et al., 1992). Studies have also demonstrated associations of elevated seminal white blood cell levels with abnormal semen parameters and impaired oocyte penetration (Anderson, 1990). However, not enough evidence exists to establish pyospermia as a cause of autoimmunity.

Cytokines produced by immune cells may play a role in infertility as well. Animal studies have demonstrated that sperm motility can be negatively impacted when the sperm is exposed to tumor necrosis factor and interferon gamma (Hill et al., 1987). However, evaluating the precise levels of cytokines in seminal fluid has proven to be difficult as prolific amounts of enzymes in seminal plasma quickly degrade the cytokines.

Immunologic Infertility Diagnostics

Antibody-Mediated Immunity

While there is no classic clinical vignette for antibody-mediated infertility, there are particular circumstances in which physicians should consider performing an ASA assay. These include sperm agglutination without infection, reduced sperm motility with a history of testis surgery or trauma, sperm shaking during sperm-cervical mucus contact testing, increased leukocyte levels, impaired mucus penetration on postcoital testing, and idiopathic infertility. When taking the history of the patient, the physician should ask about risk factors that would predispose the patient to antibody-mediated infertility, as well as attempt to rule out other causes before performing an ASA assay (Walsh and Turek, 2009).

The two most common tests used for ASA testing are the immunobead test (IBT) and the mixed agglutination reaction (MAR). The IBT functions by incubating fresh motile sperm with beads coated with antibodies against human immunoglobulins. Analysis of the test can determine the type of immunoglobulin bound to sperm, percentage of sperm with bound antibodies, and the location on sperm at which antibodies are present. Typically the threshold of significance is set to greater than 50% binding, although location of binding seems to play a role as well, for example, antibodies bound to the head of sperm have more significance than those bound to the tail. The MAR method uses a similar concept to the IBT. Fresh sperm is first incubated with beads or sheep erythrocytes labeled with human immunoglobulin. Then, anti-human immunoglobulins are added to the mixture. Results from IBT and MAR usually correspond fairly well. Other less commonly used methods include immobilization enzyme-linked immunosorbent assays (ELISA) and flow cytometry (McLachlan, 2002).

Cell-Mediated Immunity

Just as cell-mediated sperm autoimmunity is not as well understood as its antibody-mediated counterpart, diagnostics for cell-mediated immunity is relatively limited compared to testing for anti-sperm antibodies. The leukocyte migration inhibition factor (LMIF) assay, for example, can be used to measure sperm-leukocyte interaction***. Leukocytes are extracted from the serum of either partner and combined with sperm. The migration of leukocytes through a gel is then observed, with delayed migration indicating substantial sperm-leukocyte reaction. When applied to men with normal semen parameters, all assay results were negative. When applied to asthenospermic and oligoasthenospermic men, about half and one quarter had positive assay results respectively (Dimitrov et al., 1994). While the sensitivity of the assay may not be ideal, more accurate assays for evaluating cell-mediated immune infertility will likely develop as knowledge on sperm autoimmunity expands.

Immunologic Infertility Treatment

Corticosteroids

Immunosuppressive drugs reduce immune activity by dampening both the humoral and cellular components of the immune system. Thus far, however, few studies have been done on the impact of corticosteroids on immune fertility. One study conducted over 3 months found no significant difference between the control and experimental groups, whereas another study performed over 9 months indicated that treating patients with corticosteroids may lead to higher pregnancy rates (Hendry et al., 1990; Haas and Manganiello, 1987). This may suggest that long-term use of immunosuppressive drugs can contribute to improved fertility.

Intrauterine Insemination (IUI)

As described earlier in the article, sperm opsonized by antibodies often have a reduced ability to penetrate the cervical mucus. In intrauterine insemination, a small catheter is used to place the sperm in the uterus, bypassing the cervical mucus. Because opsonized sperm have a shorter lifespan, the timing of the procedure plays a key role in the success of IUI. Sperm washing prior to insemination also increases the chance of success by reducing the amount of antibodies bound to sperm. While there have been various studies suggesting the effectiveness of IUI for immune infertility treatment, no randomized controlled trials have been conducted on this issue thus far (Walsh and Turek, 2009).

In-Vitro Fertilization (IVF)

In IVF, both sperm and egg are harvested and the process of fertilization is performed in the laboratory. The new embryo is then placed into the uterus. Unfortunately, use of IVF as treatment for immune infertility seems to have a lower success rate compared to using IVF for other indications. Interestingly, studies observing the effects of immunoglobulin bound to sperm on IVF suggest that

the procedure has high success rates when sperm is only bound to either IgA or IgG, but these rates drop significantly when bound to both. In certain cases that IVF is not a viable option for overcoming immune infertility, intracytoplasmic sperm injection (ICSI) can be utilized. An individual sperm is extracted, immobilized, and injected into the egg cytoplasm. This procedure can specifically help those with immune infertility achieve fertility by allowing the sperm to bypass both the zona pellucida and oocyte membrane, both of which can have defective interactions with sperm due to ASA interference. However, studies have shown that sperm with ASA bound still result in lower quality embryos after ICSI compared to their ASA free counterparts (Walsh & Turek, 2009).

References

- Alberts, B. (2015). *Molecular biology of the cell*. New York, NY: Garland Science, Taylor and Francis Group.
- Anderson, D. J. (1990). Cell-mediated immunity and inflammatory processes in male infertility. *Archivum Immunologiae et Therapiae Experimentalis (Warsz)*, 38, 79–86.
- Anderson, M. J., Dunn, J. K., Lipshultz, L. I., & Coburn, M. (1992). Semen quality and endocrine parameters after acute testicular torsion. *The Journal of Urology*, 147, 1545–1550.
- Cheng, C. Y., & Mruk, D. D. (2012). The blood-testis barrier and its implications for male contraception. *Pharmacological Reviews*, 64, 16–64.
- Coonrod, S. A., Westhusin, M. E., & Naz, R. K. (1994). Monoclonal antibody to human fertilization antigen-1 (FA-1) inhibits bovine fertilization in vitro: Application in immunocontraception. *Biology of Reproduction*, 51, 14–23.
- Dimitrov, D. G., Urbanek, V., Zverina, J., Madar, J., Nouza, K., & Kinsky, R. (1994). Correlation of asthenozoospermia with increased antisperm cell-mediated immunity in men from infertile couples. *Journal of Reproductive Immunology*, 27, 3–12.
- Haas, G. G., Jr. (1987). Antibody-mediated causes of male infertility. *The Urologic Clinics of North America*, 14, 539–550.
- Haas, G. G., Jr., & Manganiello, P. (1987). A double-blind, placebo-controlled study of the use of methylprednisolone in infertile men with sperm-associated immunoglobulins. *Fertility and Sterility*, 47, 295–301.
- Haas, G. G., Jr., Schreiber, A. D., & Blasco, L. (1983). The incidence of sperm-associated immunoglobulin and C3, the third component of complement, in infertile men. *Fertility and Sterility*, 39, 542–547.
- Hendry, W. F., Hughes, L., Scammell, G., Pryor, J. P., & Hargreave, T. B. (1990). Comparison of prednisolone and placebo in subfertile men with antibodies to spermatozoa. *Lancet*, 335, 85–88.
- Hill, J. A., Haimovici, F., Politch, J. A., & Anderson, D. J. (1987). Effects of soluble products of activated lymphocytes and macrophages (lymphokines and monokines) on human sperm motion parameters. *Fertility and Sterility*, 47, 460–465.
- Masarani, M., Wazait, H., & Dinneen, M. (2006). Mumps orchitis. *Journal of the Royal Society of Medicine*, 99, 573–575.
- McLachlan, R. I. (2002). Basis, diagnosis and treatment of immunological infertility in men. *Journal of Reproductive Immunology*, 57, 35–45.
- Meinertz, H. (1992). Anti-sperm antibodies in the male: Detection and clinical impact. *American Journal of Reproductive Immunology*, 28, 110–116.
- O’Rand, M. G. (1977). The presence of sperm-specific surface isoantigens on the egg following fertilization. *The Journal of Experimental Zoology*, 202, 267–273.
- Walsh, T. J., & Turek, P. J. (2009). Office evaluation of the infertile male. In *Infertility in the male* (pp. 153–176). Cambridge, UK: Cambridge University Press.
- Turek, P. J., & Lipshultz, L. I. (1994). Immunologic infertility. *The Urologic Clinics of North America*, 21, 447–468.
- Uehling, D. T. (1971). Secretory IgA in seminal fluid. *Fertility and Sterility*, 22, 769–773.

Further Reading

- Walsh, T. J., & Turek, P. J. (2009). Office evaluation of the infertile male. In *Infertility in the male* (pp. 153–176). Cambridge, UK: Cambridge University Press.

Infectious and Inflammatory Male Infertility

Carlos Simón Rodríguez, Paula Charry Gónima, Juan Vicente García Cardoso, and Carmen González Enguita, Hospital Universitario Infanta Elena, Valdemoro, Spain; and Hospital Universitario Fundación Jiménez Díaz, Madrid, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

MAGI (male accessory gland infections) MAGI includes infections involving one or more of the following male genital organs or tracts: seminal vesicles, prostate, vas deferens, epididymis, testicles, urethra and Cowper glands.

Nomenclature

DFA Direct fluorescent antibody
EIA Enzyme immunoassay
FTA-ABS Fluorescent treponemal antibody-absorption test
GU Genitourinary
IFA Indirect fluorescent antibody
HCV Hepatitis C virus
HBV Hepatitis B virus
HIV Human immunodeficiency virus
HPV Human papilloma virus
MAGI Male accessory gland infections
PCR Polymerase chain reaction
PFGE Pulsed-field gel electrophoresis
REA Restriction enzyme analysis
RPR Rapid plasma reagin
STD Sexual transmitted disease
U Urealyticum ureoplasma urealyticum
VDRL Venereal Research Disease Laboratory
URS Unheated serum reagin

Introduction

Reproductive tract inflammation and infection are a common cause of male infertility, with a prevalence of 6%–10% on male infertile population. Infections can affect different sites of the male reproductive tract, such as testis, epididymis and the male accessory sex glands. Bacteria, viruses, fungi and parasites as well as inflammatory causes such as environment toxins, which are able to interfere in the human male's reproduction ([Table 1](#)). Most of the affected men are asymptomatic.

The following mechanism has been proposed to produce infertility ([Krause, 2008](#)).

Obstruction of the Reproductive Tract

The inflammation produced after a bacterial infection of the genital tract, can produce scars and consequently a stricture of the reproductive ducts. It is important to start a suitable antibiotic treatment to avoid these complications.

Stenosis of the epididymal duct may occur in the course of epididymitis, leading to a reduction of sperm count and finally to azoospermia. Urethritis can cause urethral scarring and stricture formation, leading to a decreased ejaculation volume and to a reduced fertility.

Impairment of Spermatogenesis

Epididymitis may frequently be associated with orchitis. Testicular tissue is very sensible to inflammatory changes, which can cause disturbances in a temporary or a definitive sperm production.

Table 1 Infections and inflammatory conditions related to male infertility*Infections diseases associated with infertility*

Bacteria	<i>Neisseria gonorrhoeae</i> <i>Chlamydia trachomatis</i> <i>Mycoplasma</i> <i>Ureoplasma</i> Non-STD bacteria like <i>E. coli</i> , <i>Enterococcus fecalis</i> , group B, <i>Streptococcus</i> and <i>Staphylococcus aureus</i>
Yeast	<i>Candida albicans</i>
Parasites	<i>Trichomonas vaginalis</i>
Virus	Hepatitis B virus Hepatitis C virus Human immunodeficiency virus Human papilloma virus Herpes virus
Inflammatory conditions associated with infertility	Prostatitis Epididymitis Urethritis Obesity and metabolic syndrome Chronic disease

Impairment of Sperm Function

MAGI (Male Accessory Gland Infections) can directly affect the spermatozoa and deteriorate sperm parameters.

Dysfunction of the Male Accessory Glands

The inflammation impairs the secretory function of the male accessory glands. The seminal concentrations of citric acid, L-carnitine, fructose, zinc concentration and alpha-glutamyltransferase are decreased in MAGI (Cooper et al., 1990).

Antisperm Antibodies

The presence of sperm antibodies reduces male infertility. They are usually formed after a disruption of the blood–testis barrier, in situations like testicular torsion, vasectomy or trauma. The role of antisperm antibodies in inflammation/infection processes is unclear (Marconi et al., 2009), but may be related to the chronicity of the inflammatory process (Bachir and Jarvi, 2014).

Pathogens Involved in Genital Tract Infection

Bacteria, viruses, fungi and parasites are able to interfere in male reproductive biology.

Bacteria***Neisseria gonorrhoeae***

This gram negative diplococci is a very common STD (sexual transmitted disease) in men. *N. gonorrhoeae* causes urethritis, prostatitis and less frequent epididymitis. The organism rarely has haematogenous spread. Gonorrhoeic urethritis is associated with urethral strictures that can decrease ejaculate volume. Men who developed epididymo orchitis, showed a strong impairment of testicular function 2 years later (Osegbe, 1991).

Chlamydia trachomatis

This organism is an intracellularly living bacterium. It is also one of the most common worldwide STD. The most common affections in males are urethritis and epididymitis, but they can be asymptomatic (Krause, 2008). It may impair male infertility

by causing scarring and obstruction along the male reproductive tract, and it can also cause sperm cell apoptosis (Wan et al., 2003).

Its role in infertility in asymptomatic individuals is not yet definitely determined (Ahmadi et al., 2016).

Mycoplasma and ureaplasma

Genital infections by *Mycoplasma hominis*, *Mycoplasma genitalium* and *Ureaplasma urealyticum* have been evaluated as causes of male infertility.

Ureaplasma urealyticum is the most common pathogen isolated from the genital tract of men with infertility. Studies have shown that men with infertility have higher frequencies of *U. urealyticum* detection in the semen than fertile men. Progressive motility and vitality are significantly lower in men infected by *U. urealyticum* positive than noninfected infertile men.

The presence of *Mycoplasma hominis* is also associated with a low total motility and with a total motile sperm count (Lee et al., 2013). *Mycoplasma genital* seems not to be associated with infertility (Huang et al., 2015).

Non STD bacteria

Non-STD bacteria like *E. coli*, *Enterococcus faecalis*, group B, *Streptococcus* and *Staphylococcus aureus* can cause male infertility. *E. coli* adheres to spermatozoa causing sperm agglutination (Diemer et al., 2000). The prevalence of bacteriospermia in men with infertility is approximately 15%. Bacteriospermia does not usually affect sperm cell parameters, but it is associated with a higher sperm DNA fragmentation (Domes et al., 2012).

Yeast

In vitro studies *Candida albicans* infection decreases sperm motility (Tuttle et al., 1977) and prevents fertilization after IVF (in vitro fertilization) and ICSI (intracytoplasmic sperm injection). The association of *Candida albicans* and male infertility is unclear, but it can possibly have a negative effect.

Parasites

Trichomonas vaginalis is the most important parasite involved in male infertility. It's a flagellated parasite often found as an occult resident of the genital tract of sexually active women and men. It binds to sperm head and flagella leading to a reduction of sperm motility and it increases agglutination (Benchimol et al., 2008).

Viruses

Besides the risk of horizontal or vertical transmission, viruses can negatively affect sperm parameters, fertilization and abortion rate (Garolla et al., 2013). It's known that chronic infection with HBV (hepatitis B virus) and HCV (hepatitis C virus) are associated with a deterioration of sperm parameters (count, motility and morphology) (Lorusso et al., 2010). An increase in oxidative stress has been observed, resulting in a major range of apoptosis and necrosis, versus noninfected patients (Moretti et al., 2008).

HIV (human immunodeficiency virus) impaired significantly sperm parameters. The sperm damage is correlated with CD4 count. Undetectable viral load improve the outcome of IUI/sperm washing in HIV(+) men (Nicolopoulos et al., 2004).

The influence on fertility of other viruses like human papilloma virus (HPV) and herpes virus is unknown. Hybridization technology have demonstrated in situ that HPV attaches to the equatorial region of the sperm head in semen (Schillaci et al., 2013), but the implication on male infertility is unclear (Yang et al., 2013; Luttmer et al., 2016).

Inflammatory Conditions Resulting in Male Infertility

Inflammation is the result of injury and tissue damage. When it has occurred, the presence of inflammatory cells (macrophages and lymphocytes) and proinflammatory cytokines affects sperm production (Azenabor et al., 2015). Many inflammatory noninfectious diseases can affect male infertility.

Prostatitis

Chronic prostatitis is the most common urologic condition in male patients younger than 50 years. Men with symptomatic noninfectious prostatitis has a reduced sperm motility and morphology (Leib et al., 1994; Pasqualotto et al., 2000).

Epididymitis

Acute epididymitis usually produces a temporary deterioration in sperm parameters in most of patients. Semen quality usually recovers after 6 months. Even though, azoospermia has been observed in some patients that can last more than 2 years from diagnosis (Bachir and Jarvi, 2014).

Orchitis

Most cases of orchitis are produced by infectious diseases (bacteria and virus), but it can also be produced by noninfectious causes, for example in autoimmune orchitis. Chronic inflammation of the testis may lead to impaired spermatogenesis and may reduce fertility.

Obesity and Metabolic Syndrome

Sperm parameters are impaired in obese men, specially sperm concentration. White adipose tissue is responsible for aromatase activity, that converts testosterone to estrogen. Obese men have decreased levels of testosterone and increased levels of estradiol. They also have increased oxidative stress (Katib, 2015).

Other Diseases Associated With Inflammatory Infertility

Spermatogenesis and testicular tissue are very sensible to inflammation and oxidative stress. Spermatozoa have limited levels of antioxidant defense and a limited ability of DNA-damage detection and repair mechanism (Bisht et al., 2017). Patients diagnosed of systemic diseases have increased levels of reactive oxygen species (ROS). Approximately 64% of people with cancer have a reduction in semen quality (Van Casteren et al., 2010).

Chronic diseases as inflammatory bowel disease are associated with infertility (Palomba et al., 2014).

Evaluation and Diagnosis of Genital Tract Infections

The clinical manifestation of the different infectious and inflammatories conditions determines the location in the genitourinary tract. In Table 2, there is the summary of the symptoms and diagnosis studies. Most STD can be diagnosed isolating pathogen in urethral exudate. Currently, semen culture is not recommended as a routine diagnostic test due to the high probability of urethra or skin contamination (Bachir and Jarvi, 2014). However, semen aerobic cultures can be considered in those men with high levels of sperm DNA fragmentation and antibiotic therapy can be provided to all of the patients with positive culture.

In chronic prostatitis, it is very difficult to find the microorganism with regular urine culture. In these cases, urine culture after prostatic massage according to Stamey technic can provide more specific microbiological information (Sibert et al., 1996).

When there is a high suspicion of genital infection and urinary cultures are negative, the tissue biopsy culture can render information. However, biopsies are contraindicated in acute epididymitis or orchiepididymitis (Schagdarsurengin et al., 2016).

Treatment

When genitourinary and reproductive tract infection is suspected, it is important to start a suitable antibiotic treatment immediately to avoid complications. Even though bacterial clearance by antibiotic therapy eradicates the pathogen and relieves pain symptoms, impaired semen parameters persist in 40%–80% of patients for up to 3 months and in 20% of cases beyond (Schagdarsurengin et al., 2016).

The most commonly used treatment for the *Neisseria gonorrhoeae* infection is the beta-lactamic for high sensibility. In cases of penicillin allergy, the fluoroquinolone is an option.

Tetracyclines are the first option for *Chlamydia trachomatis* infection. In the cases of *Treponema pallidum* penicillin is the election. For *Mycoplasma* and *Ureaplasma urealyticum* usually the Macrolides are the choice. For the Herpes virus the election is antivirals as Acyclovir oral or topic. In Table 2 the treatments are summarize.

In cases of obesity and metabolic syndrome, loss of weight and control of glycemic levels are recommended. It is important to control all the chronic conditions.

Table 2 Diagnosis and treatment of infections associated to male infertility

Agents	Clinics GU tract	Diagnosis	Treatment	Screening	Effect on male fertility
<i>Neisseria gonorrhoeae</i>	Urethritis, epididymitis, orchitis, infertility, disseminated gonococcal infection	Isolation pathogen in urethral exudate, detection of antigen or nucleic acid, DFA, EIA	• Ceftriaxon 250 mg IM q.d. • Azythromycin 1 g q.d. • Metronidazol 250 mg t.i.d./24 h • Fluoroquinolone q.d.	In specific populations with higher prevalence of <i>N. gonorrhoeae</i>	• Causes of strictures on the difference ducts (urethra, epididymo-orchitis)
<i>Chlamydia trachomatis</i>	Urethritis, epididymitis, orchitis, Reiter's syndrome, lymphogranuloma venerium, groin ulcers	Isolation pathogen (tissue culture or urethral exudate), direct detection of antigen (DFA, fluorescent tagged monoclonal antibodies), nucleic acid amplification (PCR), EIA	- Azythromycin 1 g q.d. - Doxycycline 100 mg b.i.d /7d. - Erythromycin - Fluoroquinolone	In specific populations with higher prevalence of <i>C. trachomatis</i>	• May impair sperm motility
<i>Treponema pallidum</i>	Primary ulcer with hard edges and local no pain adenopathy, condylomata lata	Dark-field microscopy, DFA PCR, EIA, FTA-ABS, VDRL, RPR, USR.	• Benzathine penicillin 2,4 mill IM q.d. • Doxycycline 100 mg b.i.d /14d.	In specific populations with higher prevalence of <i>T. pallidum</i>	• Possible attach to sperm cells. • Gummatous testicular lesions • Co-factor for transmission of HIV
<i>Mycoplasma genitalium</i>	Urethritis, urethral discharge	Isolation pathogen in urethral exudate, Culture, PCR, IFA	• Macrolides • Tetracyclines • Fluoroquinolone	Screening not suggested	
<i>Ureaplasma urealyticum</i> <i>Mycoplasma hominis</i> <i>Gardnerella vaginalis</i>	Urethritis, urethral discharge	Isolation pathogen in urethral exudate, PCR, IFA Dense aggregates of gram-negative bacilli on desquamated epithelial cells (Clue cells)	• Azythromycin 1 g q.d. • Doxycycline 100 mg b.i.d /7d. • Metronidazol 250 mg t.i.d./7 h	Screening not suggested	• Possible attach to sperm cells • May alter sperm parameters
HSV-2, HSV-1	Anogenital vesicular lesions and ulcer lesions	Zanck test, DFA, PCR, EIA, direct DNA detection	• Aciclovir 400 mg t.i.p/10d • Valaciclovir 500 mg b.i.p/10d	Screening not suggested	• May alter sperm parameters
HPV	Genital and anal warts; carcinoma of the penis	Pap smear, PCR, hybrid capture HPV DNA	• Famciclovir 250 mg t.i.p/10d • Imiquimod cream • Podophylin cream • Surgery	Screening not suggested	• May alter sperm parameters
<i>Trichomonas vaginalis</i>	Asymptomatic	Direct observation in the microscopy, demonstration of nucleic acid	• Metronidazol 2 g q.d.	Screening not suggested	• Binds to sperm head and flagella
<i>Candida albicans</i>	White superficial penis and foreskin	Culture, direct observation, PFGE, PCR	- Fluconazole 150 mg q.d. - itraconazole o clotrimazole cream	Screening not suggested	• In vitro: inhibitory effect in human sperm motility

GU, genitourinary; IFA, indirect fluorescent antibody; DFA, direct fluorescent antibody; EIA, enzyme immunoassay; FTA-ABS, fluorescent treponemal antibody-absorption test; PCR, polymerase chain reaction; REA, restriction enzyme analysis; PFGE, pulsed-field gel electrophoresis; VDRL, Venereal Research Disease Laboratory; RPR, rapid plasma reagin; URS, unheated serum reagin.

Conclusion

Infectious and inflammatory diseases of the genital tract are an important cause of infertility. A prompt diagnose and correct treatment can prevent further genitourinary complications and infertility.

References

- Ahmadi, M. H., Mirsalehian, A., & Bahador, A. (2016). Association of Chlamydia trachomatis with infertility and clinical manifestations: A systematic review and meta-analysis of case-control studies. *Infectious Diseases (London)*, 48, 517–523.
- Azenabor, A., Ekun, A. O., & Akinloye, O. (2015). Impact of inflammation on male reproductive tract. *The Journal of Reproduction and Infertility*, 16, 123–129.
- Bachir, B. G., & Jarvi, K. (2014). Infectious, inflammatory, and immunologic conditions resulting in male infertility. *The Urologic Clinics of North America*, 41, 67–81.
- Benchimol, M., De Andrade Rosa, I., Da Silva Fontes, R., & Burla Dias, A. J. (2008). Trichomonas adhere and phagocytose sperm cells: Adhesion seems to be a prominent stage during interaction. *Parasitology Research*, 102, 597–604.
- Bisht, S., Faiq, M., Tolahunase, M., & Dada, R. (2017). Oxidative stress and male infertility. *Nature Reviews. Urology*.
- Cooper, T. G., Weidner, W., & Nieschlag, E. (1990). The influence of inflammation of the human male genital tract on secretion of the seminal markers alpha-glucosidase, glycerophosphocholine, carnitine, fructose and citric acid. *International Journal of Andrology*, 13, 329–336.
- Diemer, T., Huwe, P., Michelmann, H. W., Mayer, F., Schiefer, H. G., & WEIDNER, W. (2000). Escherichia coli-induced alterations of human spermatozoa. An electron microscopy analysis. *International Journal of Andrology*, 23, 178–186.
- Domes, T., Lo, K. C., Grober, E. D., Mullen, J. B., Mazzulli, T., & Jarvi, K. (2012). The incidence and effect of bacteriospermia and elevated seminal leukocytes on semen parameters. *Fertility and Sterility*, 97, 1050–1055.
- Garolla, A., Pizzol, D., Bertoldo, A., Menegazzo, M., Barzon, L., & Foresta, C. (2013). Sperm viral infection and male infertility: Focus on HBV, HCV, HIV, HPV, HSV, HCMV, and AAV. *Journal of Reproductive Immunology*, 100, 20–29.
- Huang, C., Zhu, H. L., Xu, K. R., Wang, S. Y., Fan, L. Q., & Zhu, W. B. (2015). Mycoplasma and ureaplasma infection and male infertility: A systematic review and meta-analysis. *Andrology*, 3, 809–816.
- Katib, A. (2015). Mechanisms linking obesity to male infertility. *Central European Journal of Urology*, 68, 79–85.
- Krause, W. (2008). Male accessory gland infection. *Andrologia*, 40, 113–116.
- Lee, J. S., Kim, K. T., Lee, H. S., Yang, K. M., Seo, J. T., & Choe, J. H. (2013). Concordance of Ureaplasma urealyticum and mycoplasma hominis in infertile couples: Impact on semen parameters. *Urology*, 81, 1219–1224.
- Leib, Z., Bartoov, B., Eltes, F., & Servadio, C. (1994). Reduced semen quality caused by chronic abacterial prostatitis: An enigma or reality? *Fertility and Sterility*, 61, 1109–1116.
- Lorusso, F., Palmisano, M., Chironna, M., Vacca, M., Masciandaro, P., Bassi, E., Selvaggi Luigi, L., & Depalo, R. (2010). Impact of chronic viral diseases on semen parameters. *Andrologia*, 42, 121–126.
- Luttmer, R., Dijkstra, M. G., Snijders, P. J., Hompes, P. G., Pronk, D. T., Hubeek, I., Berkhof, J., Heideman, D. A., & Meijer, C. J. (2016). Presence of human papillomavirus in semen in relation to semen quality. *Human Reproduction*, 31, 280–286.
- Marconi, M., Pilatz, A., Wagenlehner, F., Diemer, T., & Weidner, W. (2009). Are antisperm antibodies really associated with proven chronic inflammatory and infectious diseases of the male reproductive tract? *European Urology*, 56, 708–715.
- Moretti, E., Federico, M. G., Giannarini, V., & Collodel, G. (2008). Sperm ultrastructure and meiotic segregation in a group of patients with chronic hepatitis B and C. *Andrologia*, 40, 286–291.
- Nicopoulos, J. D., Almeida, P. A., Ramsay, J. W., & Gilling-Smith, C. (2004). The effect of human immunodeficiency virus on sperm parameters and the outcome of intrauterine insemination following sperm washing. *Human Reproduction*, 19, 2289–2297.
- Osegbe, D. N. (1991). Testicular function after unilateral bacterial epididymo-orchitis. *European Urology*, 19, 204–208.
- Palomba, S., Sereni, G., Falbo, A., Beltrami, M., Lombardini, S., Boni, M. C., Fornaciari, G., Sassatelli, R., & La Sala, G. B. (2014). Inflammatory bowel diseases and human reproduction: A comprehensive evidence-based review. *World Journal of Gastroenterology*, 20, 7123–7136.
- Pasqualotto, F. F., Sharma, R. K., Potts, J. M., Nelson, D. R., Thomas, A. J., & Agarwal, A. (2000). Seminal oxidative stress in patients with chronic prostatitis. *Urology*, 55, 881–885.
- Schagdarsurengin, U., Western, P., Steger, K., & Meinhardt, A. (2016). Developmental origins of male subfertility: Role of infection, inflammation, and environmental factors. *Seminars in Immunopathology*, 38, 765–781.
- Schillaci, R., Capra, G., Bellavia, C., Ruvolo, G., Scazzone, C., VENEZIA, R., & Perino, A. (2013). Detection of oncogenic human papillomavirus genotypes on spermatozoa from male partners of infertile couples. *Fertility and Sterility*, 100, 1236–1240.
- Sibert, L., Grise, P., Boillot, B., Loulidi, S., & Guerin, J. G. (1996). Diagnostic value of Stamey's test in chronic prostatitis. *Progrès en Urologie*, 6, 107–111.
- Tuttle, J. P., Jr., Bannister, E. R., & Derrick, F. C. (1977). Interference of human spermatozoal motility and spermatozoal agglutination by Candida albicans. *The Journal of Urology*, 118, 797–799.
- Van Casteren, N. J., Boellaard, W. P., Romijn, J. C., & Dohle, G. R. (2010). Gonadal dysfunction in male cancer patients before cytotoxic treatment. *International Journal of Andrology*, 33, 73–79.
- Wan, C. C., Wang, H., Hao, B. J., Shang, X. J., & Huang, Y. F. (2003). Infection of chlamydia trachomatis and apoptosis of spermatogenic cells. *Zhonghua Nan Ke Xue*, 9, 350–354.
- Yang, Y., Jia, C. W., Ma, Y. M., Zhou, L. Y., & Wang, S. Y. (2013). Correlation between HPV sperm infection and male infertility. *Asian Journal of Andrology*, 15, 529–532.

Varicocele: Evaluation and Pathophysiology

Nikita Abhyankar and Samuel Ohlander, University of Illinois-Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

A varicocele is an abnormal dilation of the pampiniform plexus of veins in the scrotum, similar to the dilation of veins in the leg known as varicose veins (**Fig. 1**). Varicoceles are a common finding, occurring in 15% of all men. The prevalence of varicocele in primary infertility (defined as inability to conceive a first child after 1 year of unprotected intercourse) is 19%–41% and up to 80% of men with secondary infertility (defined as inability to conceive after unprotected intercourse for a year in an individual who has previously had a biological child) (**Witt and Lipshultz, 1993**). The relationship between infertility and varicocele is still incompletely understood, but current knowledge will be explored throughout this article.

Epidemiology

The prevalence of varicocele appears to increase with age during the childhood years. Researchers found <1% in boys aged 2–10 had varicoceles, 7.8% in boys aged 11–14 years and 14.1% in boys aged 15–19 years (**Akbay et al., 2000**). Other studies have similarly shown a rising prevalence with age in adult men with an increase of approximately 10% with every decade of life. This suggests a mechanism of venous incompetence worsening with age. There is also some indication of a higher incidence of varicose veins in the lower extremities of men with varicocele, perhaps suggesting a similar pathogenesis. The majority of varicoceles occur on the left side; however, around 50% of men have bilateral varicoceles (**Abdulmaaboud et al., 1998**). Isolated right sided varicoceles are less common (<5%) and should prompt further evaluation.

Somewhat counterintuitively, obesity has been found to negatively correlate with the presence of varicoceles. One theory for the reason is the increased difficulty of physical examination to identify the varicocele in obese individuals. Alternatively, common thought suggests that the increased adipose tissue in individuals with higher BMIs reduces potential compression of the renal vein since adipose has a lower density, thus, protecting against obstructed flow and venous congestion.

Several studies have shown that varicoceles do have a hereditary component. **Raman et al. (2005)** showed an eight times higher incidence in men with a first degree relative with a varicocele. While the correlation has been observed, the genetic etiology for this hereditary pattern has not yet been elucidated.

Other described risk factors for varicocele include congenital incompetence of valves in the spermatic veins or an acquired incompetence of the spermatic veins due to external compression of the gonadal veins in the retroperitoneum.

Etiology

Several theories exist for the cause of varicocele in men; however, there is not yet consensus on the exact cause. Absence of venous valves has been postulated as a contributing factor. The suggested mechanism for this is that absent valves leads to a higher back pressure in the gonadal vein, venous pooling, increased testicular temperature, accumulation of reactive oxidative species and, therefore, suboptimal spermatogenesis. It has been shown that in men overall, valves are absent in the left gonadal vein in 40% compared to 23% in the right gonadal vein (**Ahlberg et al., 1966**). Varicoceles are significantly more common on the left side, aligning with the valve theory. However, varicoceles can still occur in patients with competent valves.

Another theory for the etiology of varicoceles and the reason for left sided preponderance is anatomical. The left gonadal vein has a right-angle insertion into the left renal vein compared to the more oblique insertion of the right gonadal vein into the inferior vena

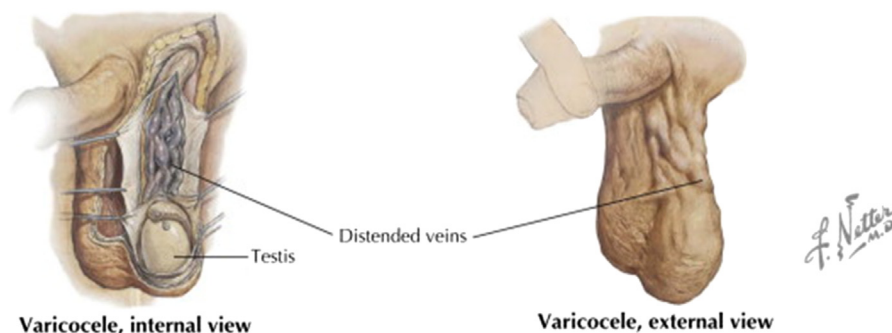


Fig. 1 Varicocele.

cava. In addition, the left renal vein has a higher pressure than the IVC, increasing back pressure and a higher venous tension in the left gonadal vein as compared to the right. This higher venous tension is thought to lead to venous pooling.

External compression of the gonadal veins can also contribute to varicocele formation. This compression may be due to a retroperitoneal mass. Patients with a right sided varicocele, or acute development of a varicocele should be evaluated for a retroperitoneal mass given the rarity of isolated right sided varicocele and their typical chronic development. Other causes of external compression of the gonadal vein include the “nutcracker syndrome.” Classically, anterior nutcracker syndrome is when the left renal vein is compressed between the aorta and the superior mesenteric artery (SMA) increasing the pressure in the left gonadal vein. This is thought to occur because the SMA branches from the aorta at an angle less than the usual 90 degree, resulting in compression of the left renal vein. A variant known as posterior nutcracker syndrome refers to when the left renal vein is retroaortic and is compressed between the aorta and vertebra. Nutcracker syndrome is believed to occur more commonly in patients with a lower BMI. Nutcracker syndrome, however, remains a rare cause of varicocele.

Varicocele—Evaluation

The American Society of Reproductive Medicine (ASRM) and American Urological Association (AUA) produced a joint report on best practice for varicocele in 2001. This report recommended evaluation of a male with a varicocele to include a history, physical examination and two semen analyses. This was further supported, though briefly, by the AUA’s best practice policy, optimal evaluation of the infertile male (Jarow et al., 2011). The lack of formal guidelines on management is attributed to the lack of well-designed studies and inconsistency of data on the impact of varicoceles on fertility and treatment outcomes.

Varicoceles are not usually symptomatic and are often discovered at the time of infertility evaluation. Symptoms that can occur include heaviness, dull aching pain, and warmth. These symptoms are usually worse with strenuous activity and improve on recumbency.

Physical examination remains the gold standard for diagnosis of varicocele. Examination is typically performed in a warm room in the supine position and then the standing position with and without Valsalva. Examination findings of palpable veins within the scrotal sac are classically described as a “bag of worms” (Fig. 1). Testicular atrophy, either unilateral or bilateral, can also occur due to varicocele. Varicoceles are graded according to the physical examination findings (Table 1).

The majority of varicoceles occur on the left side; however, around 50% of men have bilateral varicoceles. Isolated right sided varicoceles are less common (<5%) and should prompt evaluation as they may be found with situs inversus or retroperitoneal masses (Masson and Brannigan, 2014). Only palpable varicoceles have been associated with infertility, hence the AUA best practice statement recommended no ancillary diagnostic testing to identify subclinical varicocele (Jarow et al., 2011).

Two semen analyses should be obtained due to variability of semen parameters over time. It is recommended that the man abstains from ejaculation for 2–3 days prior to acquisition of the semen specimen. Overall men with varicoceles have been shown to have a stress pattern with reduced sperm concentration (oligo-), poor motility (astheno-), and poor morphology (terato-) compared to men without varicocele (Agarwal et al., 2016). This is known as oligoastheno-teratozoospermia. However, while classically described, not all men with varicocele have these abnormalities.

Pathophysiology

Varicocele has a higher prevalence in men presenting with infertility than those without. There is a higher rate of varicocele in men with secondary infertility as compared to primary infertility, and this is thought to be suggestive of a progressive disease course which worsens with time or age (Akbar et al., 2000).

The cause of these effects on testicular function is incompletely understood since some men experience subfertility from varicocele, while others do not. There are several factors that are thought to contribute to male infertility in men with varicocele; heat stress, hypoxia, oxidative stress, hormonal abnormalities, and exogenous intoxicants (Sheehan et al., 2014).

Physiologic temperature in the testicle is lower than that of the rest of the body. This is believed to be due to heat dissipation as a result of countercurrent blood flow in the pampiniform plexus around the testicular artery (Dahl and Herrick, 1959). In men with varicoceles, dilation of the pampiniform plexus veins alters the physiology, by allowing venous congestion and pooling. Human

Table 1 Varicocele grading

<i>Subclinical</i>	<i>Not present clinically, abnormality only seen on ultrasound</i>
Grade 1	Only present clinically when man performs Valsalva maneuver
Grade 2	Easily palpable, not visible
Grade 3	Visible through scrotal skin, easily palpable

Dubin, L., and Amelar, R.D. (1970). Varicocele size and results of varicocelectomy in selected subfertile men with varicocele. *Fertility and sterility* 21(8), 606–609.

studies have demonstrated a higher intra-testicular temperature and scrotal temperature in those with a varicocele compared to unaffected men (Goldstein and Eid, 1989). The mechanism of impairment in function from this elevated temperature is thought to be due to enzymatic activity in the testicle being suboptimal at a higher temperature. These affected enzymes appear to be principally involved in DNA synthesis. Fujisawa et al. (1988) demonstrated that levels of enzymes involved in DNA synthesis, were lower in infertile men with varicoceles when compared to controls. In addition to DNA synthesis, other enzymes have been shown to have altered activity in men with varicocele, also thought to be associated with the increased temperature. Other key enzymes involved in protein folding, function and sperm motility have also been shown to be affected by varicocele. One of these enzymes is the heat shock protein (HSP). HSPs are molecular chaperones that function to protect against misfolding of proteins due to heat stress and mediate homeostasis of protein degradation and synthesis. HSPs allow cells to survive in conditions where they otherwise might not (Feder and Hofmann, 1999). Several studies have shown differences in expression of various HSPs in men with varicoceles when compared to controls (Lima et al., 2006). Lima et al. showed HSP2A had a lower expression in adolescents with varicoceles and oligozoospermia when compared to adolescents with varicoceles but normozoospermia, as well as adolescents without varicoceles. Suggesting a role of HSP2A in the pathophysiology of infertility in men with varicocele.

Testicular capillary pressure has been shown in animal studies to be particularly low and steady. Therefore, an increase in venous hydrostatic pressure due to varicocele formation can lead to stagnation of the capillary flow and hypoxic-ischemic changes in the testicular tissue. Increased expression of hypoxia inducible factor HIF1- α have been shown in the spermatic cord veins men with varicoceles (Lee et al., 2006). HIF1- α is a protein considered to be a key regulator of the response to hypoxia, thus implying some degree of hypoxia in testicles affected by varicocele. It has been shown that infertile men with varicocele have a higher HIF1- α expression, suggesting hypoxia as a contributing factor for infertility. The reason for the differences between men with varicocele who are fertile versus not fertile is unclear.

An excess of reactive oxidative species (ROS) is also thought to be involved in the pathogenesis of varicocele. ROS occur as a product of oxygen metabolism and have been found to be an important for intracellular homeostasis and for various aspects of sperm function (Griveau and Le Lannou, 1997). These sperm functions include capacitation (the penultimate step in sperm maturation which occurs postejaculation), acrosome reaction (a reaction which allows the sperm to penetrate the outside layer of the egg) and hyperactivation (higher amplitude of sperm motility to aid egg penetration) (Griveau and Le Lannou, 1997). Several studies exist which have shown a higher level of ROS or oxidative stress in men with varicoceles (Barbieri et al., 1999). This excess of ROS has been associated with a higher degree of DNA fragmentation. DNA fragmentation has been quantified as the DNA fragmentation index (DFI), which is the proportion of DNA which is damaged. A higher DFI has been associated with reduced sperm motility, higher spontaneous abortion rates and possibly a lower pregnancy rate with assisted reproductive technologies (Zhao et al., 2014). Thus, this higher level of ROS in men with varicocele may be contributing to the resultant subfertility.

There is some evidence that varicocele may also affect Leydig cell function. Leydig cells in the testes produce testosterone, keeping the microenvironment with a very high concentration for sperm generation. It has been postulated that the difference in temperature leads to alterations in testosterone production by Leydig cells in men with varicoceles, affecting spermatogenesis. An early animal study in which unilateral varicocele was induced in rats showed lower testosterone concentration in bilateral testes as well as a reduction in two of the enzymes required for testosterone production (Rajfer et al., 1987). Recent publications evaluating men following varicocele repair have shown a significant increase in serum testosterone levels in men with preoperative hypogonadism (Dabaja and Goldstein, 2016). There is some controversy, however, on whether varicocelectomy should be performed for treatment of age related hypogonadism. It has been speculated that in older men with long term varicocele, the testicle may not be able to recover functionality to produce normal levels of testosterone again. However, there is recent data to dispute this and suggest that even older men with hypogonadism can have improvements in testosterone levels with varicocelectomy (Dabaja and Goldstein, 2016).

Ultimately, while many hypotheses exist, the exact cause of subfertility in men with varicocele and why some men are affected while others are not, remains unclear.

References

- Abdulmaaboud, M. R., et al. (1998). Treatment of varicocele: A comparative study of conventional open surgery, percutaneous retrograde sclerotherapy, and laparoscopy. *Urology*, 52(2), 294–300.
- Agarwal, A., et al. (2016). Effect of varicocele on semen characteristics according to the new 2010 World Health Organization criteria: A systematic review and meta-analysis. *Asian Journal of Andrology*, 18(2), 163–170. Available at <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4770480/>.
- Ahlberg, N. E., Bartley, O., & Chidekel, N. (1966). Right and left gonadal veins. *Acta Radiologica: Diagnosis*, 4(6), 593–601. Available at: <https://doi.org/10.1177/028418516600400601>.
- Akbay, E., et al. (2000). The prevalence of varicocele and varicocele-related testicular atrophy in Turkish children and adolescents. *BJU International*, 86(4), 490–493.
- Barbieri, E. R., et al. (1999). Varicocele-associated decrease in antioxidant defenses. *Journal of Andrology*, 20(6), 713–717.
- Dabaja, A. A., & Goldstein, M. (2016). When is a varicocele repair indicated: The dilemma of hypogonadism and erectile dysfunction? *Asian Journal of Andrology*, 18(2), 213–216.
- Dahl, E. V., & Herrick, J. F. (1959). A vascular mechanism for maintaining testicular temperature by counter-current exchange. *Surgery, Gynecology & Obstetrics*, 109(6), 697–705.
- Feder, M. E., & Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: Evolutionary and ecological physiology. *Annual Review of Physiology*, 61, 243–282.
- Fujisawa, M., et al. (1988). Deoxyribonucleic acid polymerase activity in the testes of infertile men with varicocele. *Fertility and Sterility*, 50(5), 795–800.
- Goldstein, M., & Eid, J. F. (1989). Elevation of intratesticular and scrotal skin surface temperature in men with varicocele. *The Journal of Urology*, 142(3), 743–745.
- Griveau, J. F., & Le Lannou, D. (1997). Reactive oxygen species and human spermatozoa: Physiology and pathology. *International Journal of Andrology*, 20(2), 61–69.

- Jarow, J., et al. (2011). The optimal evaluation of the infertile male: AUA best practice statement. *AUA Best Practice Statement*, 39.
- Lee, J.-D., Jeng, S.-Y., & Lee, T.-H. (2006). Increased expression of hypoxia-inducible factor-1 α in the internal spermatic vein of patients with varicocele. *The Journal of Urology*, 175(3 Pt 1), 1045–1048. discussion 1048.
- Lima, S. B., et al. (2006). Expression of the HSPA2 gene in ejaculated spermatozoa from adolescents with and without varicocele. *Fertility and Sterility*, 86(6), 1659–1663.
- Masson, P., & Brannigan, R. E. (2014). The varicocele. *The Urologic Clinics of North America*, 41(1), 129–144.
- Rajfer, J., et al. (1987). Inhibition of testicular testosterone biosynthesis following experimental varicocele in rats. *Biology of Reproduction*, 36(4), 933–937.
- Raman, J. D., Walmsley, K., & Goldstein, M. (2005). Inheritance of varicoceles. *Urology*, 65(6), 1186–1189.
- Sheehan, M. M., Ramasamy, R., & Lamb, D. J. (2014). Molecular mechanisms involved in varicocele-associated infertility. *Journal of Assisted Reproduction and Genetics*, 31(5), 521–526.
- Witt, M. A., & Lipshultz, L. I. (1993). Varicocele: A progressive or static lesion? *Urology*, 42(5), 541–543.
- Zhao, J., et al. (2014). Whether sperm deoxyribonucleic acid fragmentation has an effect on pregnancy and miscarriage after in vitro fertilization/intracytoplasmic sperm injection: A systematic review and meta-analysis. *Fertility and Sterility*, 102(4), 998–1005.e8.

Further Reading

- Dubin, L., & Amelar, R. D. (1970). Varicocele size and results of varicocelectomy in selected subfertile men with varicocele. *Fertility and Sterility*, 21(8), 606–609.
- Fisch, H., & Hyun, G. (2012). Varicocele repair for low testosterone. *Current Opinion in Urology*, 22(6), 495–498.
- Hendin, B. N., et al. (1999). Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *The Journal of Urology*, 161(6), 1831–1834.
- Lin, M.-H., et al. (2008). Sperm chromatin structure assay parameters are not related to fertilization rates, embryo quality, and pregnancy rates in in vitro fertilization and intracytoplasmic sperm injection, but might be related to spontaneous abortion rates. *Fertility and Sterility*, 90(2), 352–359.
- Mostafa, T., et al. (2001). Varicocelectomy reduces reactive oxygen species levels and increases antioxidant activity of seminal plasma from infertile men with varicocele. *International Journal of Andrology*, 24(5), 261–265.
- Sharma, R. K., & Agarwal, A. (1996). Role of reactive oxygen species in male infertility. *Urology*, 48(6), 835–850.

Paternal Age and Fertility Concerns

Ramón Rogel, Hospital Universitari i Politècnic La Fe, Valencia, Spain

Saturnino Luján, Hospital Universitari i Politècnic La Fe, Valencia, Spain; and Clínica IVI Valencia, Valencia, Spain

Enrique Broseta, Hospital Universitari i Politècnic La Fe, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

ACOG American College of Obstetricians and Gynecologists

ART Assisted reproduction techniques

ASD Autism spectrum disorders

bio-T bioavailable testosterone

DFI DNA fragmentation index

FGFR Fibroblast growth factor receptor

FSH Follicle stimulating hormone

HPG Hypothalamus-pituitary-gonadal axis

IVF In vitro fertilization

LH Luteinizing hormone

LTL Leukocyte telomere length

NHL Non-Hodgkin lymphoma

SHBG Sex hormone binding globulin

SOGC Society of Obstetricians and Gynecologist of Canada

Introduction

Men and women, because of their lifestyle, professional careers, late marriages and other factors, are delaying childbearing. In United States the birth rate in men older than 35 years has increased by 40% since 1980 (Alshahrani et al., 2014). Though maternal age has been widely investigated (Lansac, 1995), scarce information has been published about the role of paternal age in the conception progress and the potential consequences on the offspring. Advanced paternal age is believed to have a potential negative impact in fertilization rates, birth outcomes and childhood and adult health status (Table 1). Any man aged 40 years (y) or older at the time of conception is usually the age threshold considered for the definition of advanced paternal age. Investigate how paternal age may impair sperm and offspring development would help us to advice patients as well as to propose screening protocols to deliver healthy newborns (Conti and Eisenberg, 2016).

Semen Parameters

Modifications in semen parameters have been described as men grow older. Consensus exists about the fact of declination in volume, motility and normal morphology in men after 50. Kidd et al. analyzed 21 studies and stated declines ranging from 3% to 22% in semen volume, 3%–37% in motility and 4%–18% in normal morphology. Those data were reinforced by a meta-analysis published in 2015 underlining the decrease in semen volume, total sperm count, percent motility, percent progressive motility and percent normal sperm. The most controversial semen parameter is sperm concentration, which has been found to decrease with age in some studies and improves in some others (Plastira et al., 2007; Braham et al., 2011; Nijis et al., 2011).

Table 1 Pathologies and conditions with increased risk for the offspring of aged male

Achondroplasia	Centras Nervous System malignancies
Apert Syndrome	non-Hodgkin lymphoma
Crouzon Syndrome	Breast cancer
Pfeiffer Syndrome	Prostate Cancer
Klinefelter	Autism spectrum disorders
Syndrome	Schizophrenia
Leukemia	Bipolar disorder

The mechanisms to explain those changes in semen characteristics may be related to some anatomical structures. First, the seminal vesicle insufficiency could reduce semen volumen. Smooth muscular atrophy in the prostate as well as a decrease in water and protein content, may be responsible of the reduced semi volume and sperm motility (Kidd et al., 2001), which in addition might be altered by the inadequacy of the epididymal function with age.

Another situation that impairs semen parameters is the degeneration of the germinal epithelium status. Since Hendelsman et al. reported a decrease in testicular volume with age, another studies have proposed a difference in volumen of the 31% over 70 years when compared with men of 18–40 years (Handelsman and Staraj n.d.; Mahmoud et al. 2003). A reduction of Sertoli cell mass has been proposed to be responsible of the lower testicular volume in aged. In addition, thickening of basal membrane, narrowing and sclerosis of the tubular lumen, impaired blood supply and decreased in Leydig cells are other factors that contribute to explain the association of low testicular functions and age (Johnson et al. 1988).

Advanced paternal age may be a handicap to conceive because of sperm DNA damage, which relates with impaired fertility and abnormal embryo development, and with increased miscarriage rates. As much as an 88% is the estimated percentage of DNA damage is men between 60 and 80 years (Wyrobek et al., 2006). Several mechanisms have tried to explain how age may develop DNA damage proposing oxidate stress or inefficient apoptosis processes. Thus, DNA damage has been identified as predictor of low fertilization rates. Alshahrani et al. found a 24.4 $\pm$ 18.5% of sperm DNA damage in the group over 40 years of 472 non-azoospermic males seeking help for fertility issues, which was statistically significant when compared with the group under 40 years. (Alshahrani et al., 2014). Trying to clarify if the relationship between paternal age and DNA damage is independent of the relationship between paternal age and conventional semen parameters, Das et al. determined the DNA fragmentation index (DFI) in normozoospermic men under and over 40 years, and did so with another group of oligoasthenozoospermic men. In both groups the DFI of men over 40 years was significantly higher than in men under 40 years. Thus, in normozoospermia group, was around 15% over 40 years, and around 10% under 40 years (p 0.008). Another interesting finding of this author was the fact of the inverse correlation between percentage DFI with progressive sperm motility, supporting the idea that chromatin integrity and sperm motility are related (Das et al., 2013).

Natural Conception and Assisted Reproduction Techniques

Natural fecundity may be dependent on paternal age. 50% of couples succeed in conception within 3 months, reaching the 85% by the end of the first year (Bongaarts., 1975). Increasing male age is associated with significant decline in fertility reaching a fivefold longer time to pregnancy at age of 45 years (Hassan and Killick, 2003). Using time to conception to measure fecundity, Ford et al. determined an odds ratio for conception under 12 months of 0.51 for men over 40 years when compared to the group younger than 25 years (Ford et al., 2000). Another study concluded with 3287 couples that paternal age increase the time to pregnancy onset and conception difficulties when men were above 40 years (de La Rochebrochard and Thonneau, 2003). It has been proposed that advanced paternal age may modify time to conception, not only by biological issues, but because the reduced coital frequency also found in aging male. Defining spontaneous abortion as the death of fetus earlier than 20 weeks, and odds ratio os risk of having miscarriage increased to 1.80 in the group of men aged 40 to 64 years compared to 20 to 29 years and maintaining maternal age in the range from 20 to 29 years (de La Rochebrochard and Thonneau, 2003).

When the setting of assisted reproduction techniques (ART) is established, several publications, though no all of them, agree about the deleterious effect of paternal age. Robertshaw et al. found a 26% decrease in live birth rate with in vitro fertilization (IVF). When intrauterine insemination (IUI) is analyzed, when the male is over 35 years the pregnancy rate reduces by half (Robertshaw et al., 2014). A decline fertilization, blastocyst formation, implantation, and cryopreservation rates have been observed with advancing age. As well, in men with abnormal semen concentrations, pregnancy rates decrease 5% for each year of paternal age. This results were not that evident when adjusted to normozoospermic men. In contrast, Gallardo et al. found that increasing male age did not affect sperm characteristics or its ability to fertilize human eggs. Similarly, embryo development in vitro as well as implantation were not affected by age of the male when oocyte donor was restricted at 35 years or younger (Gallardo et al., 1996).

Hormones

Advanced age is associated with several hormonal changes that affect the hypothalamus-pituitary-gonadal axis (HPG), with the luteinizing hormone (LH) as a key participant in the regulation. LH alterations depend on the Leydig cell mass, sensitivity of the HPG to feedback inhibition, and decrease LH pulse amplitude. By the age of 75 years, bioavailable testosterone (bio-T) may be reduced by 50%. This bio-T has an important role binding to Sertoli cells maintaining essential intratesticular testosterone for spermatogenesis and preserving, thus, normal fertilization capability. In the same way, elevated follicle stimulating hormone (FSH) is usually found in male aging. Another hormone which is modified by age is sex hormone binding globulin (SHBG), that retains testosterone and make it inactive when bounded. SHBG increases with age explaining the disproportion between total serum testosterone and bio-T in the elder. Another conditions that may contribute to those hormonal alterations are metabolic syndrome, cirrhosis, HIV or use of 5 alpha reductase inhibitors, statins or betablockers (McBride et al., 2016; Ramasamy et al., 2015).

Genetic Implications

Unlike the ovarian function and its end with the menopause, spermatogenesis continues through life, which conditions differences in gametogenesis between the sexes. Each oocyte has 23 divisions with its 23 chromosome replications, and those do not go through any further DNA replication event. Before puberty, about 30 spermatogonial cell divisions occur in male. And after puberty, about 23 times per year, spermatogonial cells divide. This leads to spermatid chromosomes that have undergone as far as 610 replications by age 40 or 840 replications by age 50, with a remarkable increase in the number of de novo mutations with age (Kong et al., 2012). Lacking of a DNA repair system is common in elongated spermatids, immature and mature spermatozoa, worsening the situation.

Achondroplasia was the first genetic disorder related to paternal age, when observed by Weinberg as specially linked to last-born children of a sibship. Achondroplasia is the most common cause of dwarfism and affects, as an autosomal dominant disease, the fibroblast growth factor receptor 3 (FGFR3). FGFR2 is affected as well causing Apert syndrome, being one of the most studied disorders caused by paternal age, where the mutations 755C > G or 758C > G are found. Tiemann-Boege et al. marked interestingly that a 12-fold increased chance of fathering a child affected by achondroplasia was found in men of 50–54 years, while the relative risk of having sperm that carries the mutation was only doubled in them (Tiemann-Boege et al., 2002). Crouzon and Pfeiffer's syndromes are also related to FGFR2 and advanced paternal age. With that, the most common disorders affected by paternal age are autosomal dominant caused by mutations in FGFR2–3 genes, HRAS, PTPN11 and RET, conditioning disease like Apert, Crouzon, Pfeiffer and Muenke syndromes, achondroplasia, Costello and Noonan syndromes, multiple endocrine neoplasia type 2A and 2B (Conti and Eisenberg, 2016).

Aneuploidy is defined as a chromosomal numerical aberration and may occur as an error during meiosis of the gametes. Maternal errors are in origin of the vast number of human aneuploidy and, though every chromosome is candidate to nondisjunction, the 21 and sex chromosomes are described as the most commonly found. Trisomy 21 has been attributed to maternal aneuploidy in 93% of cases (Sartorelli et al., 2001). Regarding sex chromosomes, advanced paternal age has been related to an increase in XY sperm production. Lowe et al., when compared with the rate of XY sperm of 7.5/10000 in the 20 years group, determined an increase of 160% in men over 50 years. No evidence of age effect on frequencies of disomy X, Y or 21 were found (Lowe et al., 2001). An interesting point in relation to Klinefelter syndrome is the theory of the self-correcting aneuploidy, proposed to explain the fact of finding some foci of spermatogenesis in those patients. (Sciurano et al., 2009).

Malignancies

Parental age has been considered, as well, as a risk factor for malignancies. In fact, the increasing incidence of childhood cancer in the last decades has been attributed to improved detection methods and screening programmes, increased environmental risk factors and other epidemiological circumstances like paternal age. Yip et al. analyzed maternal and paternal age in a study in Sweden finding the most significant results in children under 5 years. Retinoblastoma was linked to maternal age. Leukemia was associated to both maternal and paternal age, but no association was found when maternal age was under 25 years, suggesting a main contribution of maternal factor. Central nervous system cancers risk was increased by paternal age, mainly to astrocytoma. Besides the association found, the proposed age ranges for the offspring may set a big contribution to generate and design screening campaigns focusing in the children group under 5 years (Yip et al., 2006).

Hematologic malignancies have been linked to paternal age. Lu et al. explained their finding of a 50% increased risk of non-Hodgkin lymphoma (NHL) in men over 40 years by several mechanisms. First of them, was the continuous replication of stem cells in male spermatogenesis comprising DNA damage. Aberrant epigenetic regulation may be altered by paternal age and might be another mechanism related to the increased incidence (Lu et al., 2010). In the literature, long leukocyte telomere length (LTL) in the offspring has been related, as well, as a predictor of increased NHL risk (Lan et al., 2009). Increased LTL may also have higher risk of breast cancer in daughters of old fathers (Arbeev et al., 2011). Telomeres are set at the end of the chromosomes and are repetitive sequences (TTAGGG) of non-coding DNA. Their function is to promote structural stability of the chromosomes and control cell division. They also have a role in cell durability, and in mice and human, long LTL showed evidence of increase longevity because its association with reduced risk of atherosclerosis. The aging and attrition of the telomere with successive cell division, avoids its natural protecting action for the chromosome, which becomes instable. Telomere shortening and malfunction produce cell apoptosis in the majority of cases. Not only leukocyte, muscle, skin and fat cells have been found to shorten their telomeres with increasing age. Telomerase is a protein with reverse transcriptase activity and adds TTAGGG sequences to the telomere when some repeats are lost by continuous cell division. This telomerase is highly found in germ cells and neoplastic cells. This controversy confronting longevity and cancer risks needs further studies to make this seemingly contradiction clear (Sharma et al., 2015).

Breast and prostate cancer are other malignancies proposed to have an association with maternal and paternal age. For female offspring, both maternal and paternal, have been related to breast cancer. But for male offspring, only paternal age have related to prostate cancer. Zhang et al. noted an overall increase of 70% in the risk of prostate cancer for sons sired by fathers over 38 years (Zhang et al., 1999).

Mental Health

An increasing number of papers have been recently published relating the paternal age to complex neuropsychiatric conditions like bipolar disorder, autism, schizophrenia, suicide attempt and, even, academic morbidity.

Autism spectrum disorders (ASD) are defined by impairment in social interaction, repetitive patterns of behavior and manifest difficulties to social communication or occupational development. ASD have been associated to paternal age and is usually recognized at early ages, usually in children under 3 years. Reichenberg et al. conducted their study in Israel and found that paternal age over 40 years, increased by 5.75 the autism risk in the offspring, when compared with fathers younger than 30 years. When considering age as a continuous variable, the odds of siring an autistic child doubled for each 10 years increase in paternal age (Reichenberg et al., 2006). In the same line, another important publication in Sweden, with more than 2,6 million individuals, reported an increased risk of having a diagnosis of ASD of 75% in the offspring of fathers over 45 years compared to father of 20 to 25 years. When accounting for all factors shared by siblings risk increased to 3.5 times when parental age was over 45 years (D'Onofrio et al. 2014).

The genetic implications for ASD include genetic, epigenetic and environmental factors but it is as strong as showed by the fact of finding a concordance rate of autism in monozygotic twins from 60% to 90% (Geschwind., 2009). But, not every author agrees with the theory of genetic mutation linked to paternal age to cause ASD. Those groups proposed other lines of causality like the potential difficulty for communicating with women or shyness and social impairment of those men, which may be related to any degree of ASD phenotypes found in these potential fathers (Puleo et al., 2008).

Finally in ASD, a transgenerational risk has been proposed as it has been reported a 1.79 increased risk of ASD for the offspring when maternal grandfather was over 50 years when he gave birth to his daughter. In the same line, risk goes to 1.67 for paternal grandfather. The high heritability of epigenetic factors has been pointed as an explanation (Frans et al., 2013).

Schizophrenia, being a disorder with an incidence of 10–22/100,000 persons per year, has been related as well to paternal age. Symptoms typically start at adolescence or early adulthood. Brown et al. in 2002 concluded that, when paternal age was considered as continuous variable, the analysis showed a strong trend to an association between paternal age and risk of adult schizophrenia spectrum disorders, proposing an adjusted rate ratio of 1.86 (adjusted to maternal age) for every 10 years increase in paternal age. If the focus was placed only in schizophrenia, the rate ratio was 1.89 (Brown et al., 2002). Another studied found an increased risk of psychosis (HR = 2.07) in advanced paternal age (D'Onofrio et al., 2014). The results of Sipos et al. with a cohort of 754.330 people born in Sweden found a hazard ratio for each 10 year increase in paternal age of 1.47 for schizophrenia and 1.12 for non-schizophrenic non-affective psychosis, confirming paternal age as a strong independent risk factor. A stronger association was found in subjects without a family history of schizophrenia, strongly supporting the theory of de novo mutations in the germ cells of older fathers (Sipos et al., 2004). Besides de novo mutations, parental constitutional factors like presence of schizophrenia spectrum disorders in either of the parents, have been proposed as an explanation. In the same line, environmental conditions like higher levels of psychosocial stress, physical illness in the father or becoming fatherless in the adolescence may have a role in the development of the disease (Brown et al., 2002).

Another psychiatric condition related to advanced paternal age is bipolar disorder, which is mainly diagnosed in the early adulthood. In the Sweden study by D'Onofrio et al. a HR of 7 was provided when adjusted for statistical covariates for men over 45 years (D'Onofrio et al., 2014). Another study analyzed 13,000 patients with bipolar disorder and found the offspring of men over 55 years were 1.37 times more likely to be diagnosed with bipolar disorder when compared with fathers under 25 years (Frans et al., 2013).

When considering cognitive capabilities, Aurox firstly associate the paternal age to impaired learning and conditioning in rats almost 35 years ago (Aurox., 1983). D'Onofrio et al. in their fixed-effects sibling models found an odds ratio of 1.59 for failing a grade and of 1.7 for low educational attainment for the offspring of fathers over 45 years when compared to those of 20–25 years (D'Onofrio et al., 2014). Another study showed paternal age was related to offspring intelligence, in this case nonverbal intelligence. The best scores were obtained by the group of fathers aged from 25 to 44 years, while fathers under 25 years or older than 44 years sired offspring with lower intelligence scores (Malaspina et al., 2005; Malaspina et al., 2015). Saha and colleagues published in 2009 two papers regarding how paternal age influenced neurocognitive outcomes and childhood behaviors. It was noticed that paternal age was associated with poorer scores in neurocognitive measures used (Bayles Scales, Stanford-Binet Intelligence Scale, Graham-Ernhart Block Sort Test, Weschler Intelligence Scale for Children and Wide Range Achievement Test). This results showed a linear progression when age of the fathers was grouped in 5 years intervals. Maternal age, conversely, was associated to higher scores in the same tests. The other publication by Saha et al. focused on childhood behaviors and concluded that advanced paternal age was associated with a significantly increased risk of adverse 'externalizing' behaviors (e.g. vandalism) at 7 years. For every 5 year increase in paternal age, the odds of higher 'externalizing' behaviors was increased by 12% even after adjusting for confounding factors. 'Internalizing' behavioral outcome was not associated with advanced paternal ages but was so with advanced maternal age (Saha et al., 2009a; Saha et al., 2009b).

Conclusions

Paternal age has been associated to many pathologies and conditions in the offspring, even in a transgenerational way. Nevertheless, there is no screening test aimed to detect those potentially deleterious factors in the aged father. Focused tests like sperm DNA damage may help to uncover a defect in spermatogenesis and be useful to select the most suitable method of ART. The clinical

practice guideline of the Society of Obstetricians and Gynecologists of Canada (SOGC) recommends in their last point of the list that “Advanced paternal age appears to be associated with an increased risk of spontaneous abortion and increased frequency of some autosomal dominant conditions, ASD and schizophrenia. Men over 40 years and their partners should be counseled about these potential risks when they are seeking pregnancy, although the risks remain small”. The American College of Obstetricians and Gynecologists (ACOG) also recommends counseling the partners with male age over 40 years. To date, despite the increased risk of disorders in the offspring, an old male’s partner pregnancy should be treated similar to any other pregnancy, according to the prenatal guidelines established by the American College of Medical Genetics (Kimberly et al., 2012; Toriello and Meck, 2008).

References

- Alshahrani, S., Agarwal, A., Assidi, M., et al. (2014). Infertile men older than 40 years are at higher risk of sperm DNA damage. *Reproductive Biology and Endocrinology*, 12(1), 103.
- Arbeev, K. G., Hunt, S. C., Kimura, M., Aviv, A., & Yashin, A. I. (2011). Leukocyte telomere length, breast cancer risk in the offspring: The relations with father's age at birth. *Mechanisms of Ageing and Development*, 132(4), 149–153.
- Auroux, M. (1983). Decrease of learning capacity in offspring with increasing paternal age in the rat. *Teratology*, 27(2), 141–148.
- Bongaarts, J. (1975). A method for the estimation of fecundability. *Demography*, 12(4), 645–660.
- Braham, S., Mehdi, M., Elghezal, H., & Saad, A. (2011). The effects of male aging on semen quality, sperm DNA fragmentation and chromosomal abnormalities in an infertile population. *Journal of Assisted Reproduction and Genetics*, 28(5), 425–432.
- Brown, A. S., Schaefer, C. A., Wyatt, R. J., et al. (2002). Paternal age and risk of schizophrenia in adult offspring. *The American Journal of Psychiatry*, 159(9), 1528–1533.
- Conti, S. L., & Eisenberg, M. L. (2016). Paternal aging and increased risk of congenital disease, psychiatric disorders, and cancer. *Asian Journal of Andrology*, 18(3), 420–424.
- D’Onofrio, B. M., Rickert, M. E., Frans, E., et al. (2014). Paternal age at childbearing and offspring psychiatric and academic morbidity. *JAMA Psychiatry*, 71(4), 432.
- Das, M., Al-Hathal, N., San-Gabriel, M., et al. (2013). High prevalence of isolated sperm DNA damage in infertile men with advanced paternal age. *Journal of Assisted Reproduction and Genetics*, 30(6), 843–848.
- de La Rochebrochard, E., & Thonneau, P. (2003). Paternal age over or equal to 40 years: An important risk factor for infertility. *American Journal of Obstetrics and Gynecology*, 189(4), 901–905.
- Ford, W. C., North, K., Taylor, H., Farrow, A., Hull, M. G., & Golding, J. (2000). Increasing paternal age is associated with delayed conception in a large population of fertile couples: Evidence for declining fecundity in older men. The ALSPAC study team (Avon longitudinal study of pregnancy and childhood). *Human Reproduction*, 15(8), 1703–1708.
- Frans, E. M., Sandin, S., Reichenberg, A., et al. (2013). Autism risk across generations: A population-based study of advancing grandpaternal and paternal age. *JAMA Psychiatry*, 70(5), 516–521.
- Gallardo, E., Simón, C., Levy, M., Guanes, P. P., Remohi, J., & Pellicer, A. (1996). Effect of age on sperm fertility potential: Oocyte donation as a model. *Fertility and Sterility*, 66(2), 260–264.
- Geschwind, D. H. (2009). Advances in autism. *Annual Review of Medicine*, 60(1), 367–380.
- Handelsman, D. J., & Staraj, S. (1985). Testicular size: The effects of aging, malnutrition, and illness. *Journal of Andrology*, 6(3), 144–151.
- Hassan, M. A. M., & Killick, S. R. (2003). Effect of male age on fertility: Evidence for the decline in male fertility with increasing age. *Fertility and Sterility*, 79(Suppl 3), 1520–1527.
- Johnson, L., Abdo, J. G., Petty, C. S., & Neaves, W. B. (1988). Effect of age on the composition of seminiferous tubular boundary tissue and on the volume of each component in humans. *Fertility and Sterility*, 49(6), 1045–1051.
- Kidd, S. A., Eskenazi, B., & Wyrobek, A. J. (2001). Effects of male age on semen quality and fertility: A review of the literature. *Fertility and Sterility*, 75(2), 237–248.
- Kimberly, L., Case, A., Cheung, A. P., et al. (2012). Advanced reproductive age and fertility: No. 269, November 2011. *International Journal of Gynaecology and Obstetrics*, 117(1), 95–102.
- Kong, A., Frigge, M. L., Masson, G., et al. (2012). Rate of de novo mutations and the importance of father's age to disease risk. *Nature*, 488(7412), 471–475.
- Lan, Q., Cawthon, R., Shen, M., et al. (2009). A prospective study of telomere length measured by monochrome multiplex quantitative PCR and risk of non-Hodgkin lymphoma. *Clinical Cancer Research*, 15(23), 7429–7433.
- Lansac, J. (1995). Delayed parenting. Is delayed childbearing a good thing? *Human Reproduction*, 10(5), 1033–1035.
- Lowe, X., Eskenazi, B., Nelson, D. O., Kidd, S., Alme, A., & Wyrobek, A. J. (2001). Frequency of XY sperm increases with age in fathers of boys with Klinefelter syndrome. *American Journal of Human Genetics*, 69(5), 1046–1054.
- Lu, Y., Ma, H., Sullivan-Halley, J., et al. (2010). Parents' ages at birth and risk of adult-onset hematologic malignancies among female teachers in California. *American Journal of Epidemiology*, 171(12), 1262–1269.
- Mahmoud, A. M., Goemaere, S., El-Garem, Y., Van Pottelbergh, I., Comhaire, F. H., & Kaufman, J. M. (2003). Testicular volume in relation to hormonal indices of gonadal function in community-dwelling elderly men. *The Journal of Clinical Endocrinology and Metabolism*, 88(1), 179–184.
- Malaspina, D., Reichenberg, A., Weiser, M., et al. (2005). Paternal age and intelligence: Implications for age-related genomic changes in male germ cells. *Psychiatric Genetics*, 15(2), 117–125.
- Malaspina, D., Gilman, C., & Kranz, T. M. (2015). Paternal age and mental health of offspring. *Fertility and Sterility*, 103(6), 1392–1396.
- McBride, J. A., Carson, C. C., & Coward, R. M. (2016). Testosterone deficiency in the aging male. *Therapeutic Advances in Urology*, 8(1), 47–60.
- Nijs, M., De Jonge, C., Cox, A., Janssen, M., Bosmans, E., & Ombelet, W. (2011). Correlation between male age, WHO sperm parameters, DNA fragmentation, chromatin packaging and outcome in assisted reproduction technology. *Andrologia*, 43(3), 174–179.
- Plastira, K., Msaouel, P., Angelopoulou, R., et al. (2007). The effects of age on DNA fragmentation, chromatin packaging and conventional semen parameters in spermatozoa of oligoasthenoteratozoospermic patients. *Journal of Assisted Reproduction and Genetics*, 24(10), 437–443.
- Puleo, C. M., Reichenberg, A., Smith, C. J., Kryzak, L. A., & Silverman, J. M. (2008). Do autism-related personality traits explain higher paternal age in autism? *Molecular Psychiatry*, 13(3), 243–244.
- Ramasamy, R., Chiba, K., Butler, P., & Lamb, D. J. (2015). Male biological clock: A critical analysis of advanced paternal age. *Fertility and Sterility*, 103(6), 1402–1406.
- Reichenberg, A., Gross, R., Weiser, M., et al. (2006). Advancing paternal age and autism. *Archives of General Psychiatry*, 63(9), 1026.
- Robertshaw, I., Khoury, J., Abdallah, M. E., Warikoo, P., & Hofmann, G. E. (2014). The effect of paternal age on outcome in assisted reproductive technology using the ovum donation model. *Reproductive Sciences*, 21(5), 590–593.
- Saha, S., Barnett, A. G., Foldi, C., et al. (2009a). Advanced paternal age is associated with impaired neurocognitive outcomes during infancy and childhood. *PLoS Med.*, 10(3), e40.
- Saha, S., Barnett, A. G., Buka, S. L., & McGrath, J. J. (2009b). Maternal age and paternal age are associated with distinct childhood behavioural outcomes in a general population birth cohort. *Schizophrenia Research*, 115(2–3), 130–135.
- Sartorelli, E. M., Mazzucatto, L. F., & de Pina-Neto, J. M. (2001). Effect of paternal age on human sperm chromosomes. *Fertility and Sterility*, 76(6), 1119–1123.
- Sciarano, R. B., Luna Hisano, C. V., Rahn, M. I., et al. (2009). Focal spermatogenesis originates in euploid germ cells in classical Klinefelter patients. *Human Reproduction*, 24(9), 2353–2360.

- Sharma, R., Agarwal, A., Rohra, V. K., Assidi, M., Abu-Elmagd, M., & Turki, R. F. (2015). Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. *Reproductive Biology and Endocrinology*, 13(1), 35.
- Sipos, A., Rasmussen, F., Harrison, G., et al. (2004). Paternal age and schizophrenia: A population based cohort study. *BMJ*, 329(7474), 1070.
- Tiemann-Boege, I., Navidi, W., Grewal, R., et al. (2002). The observed human sperm mutation frequency cannot explain the achondroplasia paternal age effect. *Proceedings of the National Academy of Sciences*, 99(23), 14952–14957.
- Toriello, H. V., & Meck, J. M. (2008). Professional practice and guidelines committee. Statement on guidance for genetic counseling in advanced paternal age. *Genetics in Medicine*, 10(6), 457–460.
- Wyrobek, A. J., Eskenazi, B., Young, S., et al. (2006). Advancing age has differential effects on DNA damage, chromatin integrity, gene mutations, and aneuploidies in sperm. *Proceedings of the National Academy of Sciences*, 103(25), 9601–9606.
- Yip, B. H., Pawitan, Y., & Czene, K. (2006). Parental age and risk of childhood cancers: A population-based cohort study from Sweden. *International Journal of Epidemiology*, 35(6), 1495–1503.
- Zhang, Y., Kreger, B. E., Dorgan, J. F., et al. (1999). Parental age at child's birth and son's risk of prostate cancer. The Framingham study. *American Journal of Epidemiology*, 150(11), 1208–1212.

TREATMENT: MEDICAL

Overview Treatment and Male Reproductive Medicine

Alayman Hussein, Minia University, Minia, Egypt

© 2018 Elsevier Inc. All rights reserved.

Semen analysis is the initial test used for evaluation of infertility in men. Based on the results of semen analysis, causes of male infertility are categorized into azoospermia, oligospermia, asthenospermia and teratospermia. Erectile dysfunction and ejaculatory disorders contribute to causes of male infertility in an indirect way and are categorized as coital causes of male infertility. In management of male infertility in men, we try to reach a specific cause underlying semen abnormalities or ejaculatory disorders and target it with the suitable line of treatment. Unfortunately, many abnormalities in semen remain without a clear definite cause and are called idiopathic.

We have three options for treating male infertility. The first option is to specifically treat the cause either surgically as we do in varicocele and genital duct reconstruction or medically as in hormonal treatment of hypogonadotropic hypogonadism. The second option is an empiric medical treatment for idiopathic semen abnormalities. The third option is assisted reproduction techniques (ART). ART is reserved for cases beyond, or had failed trials of, surgical repair or medical treatment. The choice of treatment depends on many parameters which include the expected success rate of the selected treatment, the cost of treatment and the age and fertility status of the partner.

Unless we have a definitely surgically correctable cause, medical treatment of male infertility is the first option for most of the cases. Medical treatments are either specific or empiric. Generally, the trial of medical treatment is required to cover at least 6 months, equivalent to two cycles of spermatogenesis, before judging its efficacy and before deciding to shift into another line of treatment. In the process of deciding the suitable line of treatment of any case, it is necessary to perfectly study the medical history of the patients and know in details all drugs that have been used. It is known that chemotherapy, exogenous androgens, anabolic steroids, alpha blockers and SSRIs may affect the fertility potentials, and it is required to put in consideration stopping or modulation of the use of these drugs and to take the required precautions on its use.

Azoospermia

Azoospermia is a complete absence of sperm in semen even after centrifugation of the specimen and examination of the pellet. It may be obstructive azoospermia due to genital duct obstruction with normal spermatogenesis, or functional azoospermia due to failure of the testis to produce sperm in a sufficient number to be demonstrated in semen and is called nonobstructive azoospermia. Treatment of obstructive azoospermia requires genital duct reconstruction or sperm retrieval for intra cytoplasmic sperm injection (ICSI). There is no role for medical treatment in obstructive azoospermia.

Nonobstructive azoospermia is a complete absence of sperm in semen due to a defect in sperm production which is either due to abnormalities in the level of the hormones which are required for normal spermatogenesis, namely FSH and testosterone or due to intrinsic testicular defect (Jarow and Zirkin, 2005). The choice of medical treatment in patients with azoospermia requires an initial evaluation of the hormones related to spermatogenesis.

Specific Medical Treatment

Low FSH, LH and testosterone in patients with features of hypogonadism is the key for the diagnosis of hypogonadotropic hypogonadism. Despite the requirement for testosterone for normal spermatogenesis, the administration of exogenous testosterone and other androgens have a negative feedback on hypothalamic pituitary gonadal axis and inhibit the secretion of FSH and LH from the pituitary gland and consequently decrease intratesticular testosterone. So, exogenous androgens should be avoided. Hormonal replacement with hCG and hMG in these patients help to restore the normal level of FSH and LH and stimulate Leydig cells in the testis to produce testosterone and stimulate spermatogenesis (Kim et al., 2013). Nonobstructive azoospermic men start to demonstrate sperm in their semen after long term treatment with injection of hCG and hMG (Liu et al., 2002). An alternative method for restoring the normal level of FSH and testosterone and successfully produce sperm in men with hypogonadotropic hypogonadism is the use of pulsatile injection of GnRH (Pitteloud et al., 2002).

In cases with low testosterone in azoospermic men with normally developed sexual features, we need more hormonal evaluation to reach the specific cause of the decreased testosterone level. Assessment of serum level of FSH, LH, prolactin, estradiol and thyroid hormones are required to reach the etiology and select the type of hormonal treatment.

Empiric Medical Treatment

High levels of FSH and LH with low or normal testosterone in azoospermic men indicate a primary testicular failure and there is no role of medical treatment. The only option for these cases is micro TESE and ICSI.

But, in case of normal or slightly high level of FSH and testosterone, we can try empiric medical treatment. Clomiphene citrate was tried in men with idiopathic nonobstructive azoospermia and sperm were demonstrated in the ejaculate of some men after its administration (Hussein et al., 2005). The idea of starting an empiric treatment in patients with nonobstructive azoospermia is based on the fact that Clomiphene, which successfully improves sperm production in some cases of oligospermia, might be useful to produce enough sperm for ICSI either by resulting in sperm identified in the ejaculate or potentially improving outcomes of TESE. These results were encouraging and opened the door for further studies that established the value of medical treatment of such cases with a considerable degree of success.

When clomiphene is applied to all nonobstructive azoospermic patients prior to TESE without any histopathological studies, excluding patients with baseline FSH more than one and half the upper limit of normal, the response to clomiphene is not identical in all patients. Patients differ in the dose and regimen required to achieve the target level of testosterone and FSH. Some patients do not reach the target level of serum testosterone and FSH even if we use the maximum dose of clomiphene. Some patients respond to clomiphene treatment by an obvious increase in FSH without increase in testosterone. Few patients respond to clomiphene by an unexpected decrease in testosterone which is also manifested by decrease in sexual desire.

Based on these findings, and as it is believed that optimizing the level of hormones rather than the type of the used drug is the most important factor for the success of the medical treatment, the empiric use of different drugs in nonobstructive azoospermia and evaluation of their hormonal response and their outcome is evaluated. A protocol showing the value of optimizing the level of FSH and testosterone in men with nonobstructive azoospermia to adequately stimulate spermatogenesis demonstrated around 10% chance of producing sperm in semen after around 6 months treatment (Fig. 1). In addition, it is noticed that an obvious increase in the likelihood of finding sperm in micro TESE in patients in whom sperm didn't show up in their ejaculate. The drugs that are used in this protocol are the same drugs that are popularly used in the treatment of oligospermia and the hypothesis of the success of treatment in this protocol is to change the type and dose of the drugs to increase the level of FSH and total testosterone to a target level adequate for stimulation of spermatogenesis (Hussein et al., 2013).

Oligospermia

Oligospermia is a decrease in the sperm concentration in semen below 15 million sperm per milliliter (WHO, 2010). Oligospermia is a defect in sperm production and may be due to surgically correctable causes like varicocele, exposure to thermal or chemical environmental factors suppressing spermatogenesis, hormonal factors, intrinsic testicular defect or idiopathic.

Specific treatment of oligospermia requires proper evaluation of the patient to identify its cause. Oligospermia may be multifactorial and the patients are informed that smoking, exposure to heat or chemicals and wearing tight clothes may be additional causes of oligospermia. We advise the patients to modify their life style and avoid such factors that inversely affect the process of normal spermatogenesis. We try to identify surgically correctable causes and treat it properly. We look for hormonal abnormalities that might suppress spermatogenesis and start the specific hormonal treatment to restore the normal levels of FSH and testosterone.

In idiopathic oligospermia, we use the same protocol we described above for idiopathic azoospermia (Fig. 1). Drugs that are commonly used for the treatment of oligospermia include.

Gonadotropin: Exogenous gonadotropin treatments include the use of hCG and hMG.

hCG, which is analogous to LH, stimulates the Leydig cell secretion of testosterone.

hMG has both LH and FSH activity. The reason for gonadotropin administration in idiopathic oligozoospermia is based on its observed efficacy in the treatment of hypogonadotropic hypogonadism. However, their effectiveness for treating normogonadotropic oligospermia is less clear (Siddiq and Sigman, 2002).

Antiestrogens, Clomiphene and Tamoxifene: The antiestrogens indirectly stimulate the secretion of FSH and LH by blocking estrogen receptors in the hypothalamus, which increases the release of GnRH. Clomiphene citrate is prescribed in many different protocols.

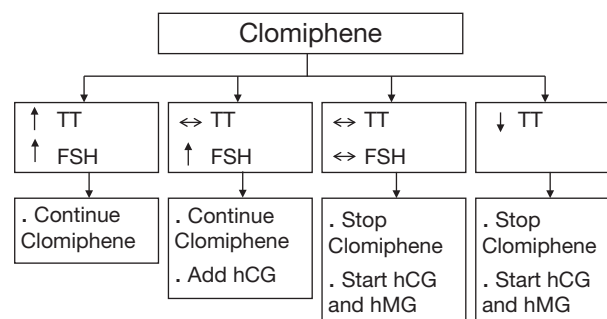


Fig. 1 A protocol of empiric use of drugs for optimizing spermatogenesis regulating hormones in nonobstructive azoospermic.

The commonly used dose of Clomiphene is a 25-mg daily oral dose. Higher doses may cause down-regulation of the hypothalamo-pituitary gonadal system (Heller et al., 1969). Men treated with clomiphene citrate consistently demonstrate an elevation in serum FSH, LH and testosterone levels. As a result, serum gonadotropins and testosterone must be monitored periodically to ensure that the testosterone level remains within normal limits, because higher levels may negatively influence spermatogenesis. Small number of patients may suffer deterioration in semen quality with antiestrogen therapy. Therefore, frequent semen analysis is essential during follow-up (Gilbaugh and Lipshultz, 1994). Side effects of Clomiphene are rare and include nausea, headache, dizziness, weight gain, alterations in libido, visual field changes, gynecomastia and allergic dermatitis. These side effects are usually mild and occur in less than 5% of patients (Siddiq and Sigman 2002). Tamoxifen citrate has less estrogenic activity than clomiphene citrate. Tamoxifen is taken orally in doses ranging from 10 to 30 mg per day. Side effects are similar to those seen with clomiphene but occur with lower frequency because of its weaker estrogenic properties.

Aromatase inhibitors: Aromatase enzyme converts the circulating testosterone into estrogen within fat cells. Markedly obese men may have an excessive endogenous conversion of testosterone into estrogen. Alteration in the ratios of estrogen and testosterone may decrease pituitary levels of LH and FSH and impair sperm production (Kulin and Reiter 1972). Normal fertile men have a testosterone/estradiol ratio above 15 and men with a ratio less than 10 usually have a defect in sperm production. Aromatase inhibitors block the conversion of testosterone to estrogen, thereby increasing testosterone level enhancing spermatogenesis. Raman and Schlegel used anastrozole and testolactone in 140 patients with oligospermia and low testosterone and a low Testosterone/Estradiol ratio and found a significant increase in sperm count and motility in addition to the increases level of testosterone (Raman and Schlegel, 2002).

Teratospermia

Teratospermia means increase in the percentage of sperm with abnormal form in semen. Normally, normal sperm with strict criteria in semen should be at least 4% (WHO, 2010). Teratospermia reflects a defect in sperm maturation in the testis. Testosterone is responsible for the late stages of spermatogenesis and its low level in the testis might be a cause of teratospermia. So, hormonal manipulation to restore a normal level of testosterone is our medical choice for treatment of teratospermia. Teratospermia may also be due to causes beyond medical treatment like varicocele and cases of prolonged exposure to spermatotoxins.

Asthenospermia

Asthenospermia is a decrease in the percentage of sperm with forward progressive motility below the reference limit of 32% (WHO, 2010). Many cases of asthenospermia have unexplained etiology and may be due to intrinsic sperm abnormalities with a limited treatment outcome. Smoking and exposure to environmental spermatoxins thermal, chemical, or radiation may affect sperm motility. Parallel to any medical or surgical treatment of male infertility, patients are advised to give up smoking and adjust their lifestyle affecting scrotal heating such as prolonged time spent seated and avoid any habitual, occupational or environmental exposure to excessive heat, chemicals, and radiation.

One of the common treatable causes of asthenospermia is varicocele. There is no role for medical treatment for varicoceles because it is an anatomical abnormality that requires a microsurgical repair. Most of the studies demonstrate a significant improvement in sperm motility and other semen parameters after microsurgical varicocelectomy (Chiba and Fujisawa, 2016).

Causes of asthenospermia requiring specific medical treatment include infection, antisperm antibodies, hyperviscid semen and its delayed liquefaction. One or more of the following lines of treatment are used for the treatment of patients with asthenospermia:

Antibiotics

Infection of the male genital system, when suspected, should be evaluated and properly treated without any delay. Acute Testicular infection and testicular abscess may cause destruction of the functioning seminiferous tubules and complete damage of the testis. Gonorrhea and severe genital duct infection may be complicated with its obstruction and may cause obstructive azoospermia if occur bilaterally.

Chronic prostatitis and genital duct infection may change the pH of semen, produce of seminal reactive oxygen species and create unsuitable media for sperm vitality, motility, and DNA integrity (Athayde et al., 2007). All men with elevated seminal white blood cell should be evaluated for a genital tract infection or inflammation. When prostatitis and genital duct infection is suspected, semen culture should be performed. Unexpectedly, most of leukocytospermic samples are microbiologically negative. The most commonly found gram positive and gram negative bacteria are *Streptococcus fecalis* and *Escherichia coli*, respectively (March and Isidori, 2002). Also, Chlamydia trachomatis and Ureaplasma urealyticum are often involved and are expected in cases of negative gram stain culture.

Once genital duct infection is diagnosed, antibiotic therapy is initiated according to the results of semen culture. The commonly used antibiotics that are used for the treatment of chronic prostatitis and genital duct infections are Doxycycline, tetracycline, fluoroquinolones (levofloxacin, ciprofloxacin, ofloxacin, norfloxacin), cotrimoxazole (sulfamethoxazole 800 mg, trimethoprim 160 mg) and macrolides such as erythromycin. These drugs are administered for 2–4 weeks. The objectives of such treatment are

to reduce or eradicate microorganisms, normalize inflammatory changes in prostatic secretions and semen and improve sperm parameters (Weidner et al., 1999).

Antioxidants

A small amount of reactive oxygen species (ROS) is necessary for the normal function of sperm. But, it is found that the rate of infertility is increased in men with seminal fluid containing a high level of ROS (Sharma and Agarwal, 1996). Although, the exact mechanism by which ROS affect sperm integrity and motility is not clearly defined, it is found that reducing seminal ROS levels is necessary for natural pregnancy and assisted reproductive technologies. The human body employs a number of mechanisms to minimize the damage caused by the ROS by many mechanisms. The presence of antioxidants in seminal plasma is the most important mechanism against the damaging effect of ROS (Agarwal and Prabakaran, 2005). Accordingly, clinicians used to prescribe antioxidants to improve sperm count and motility.

The use of antioxidants in the treatment of male infertility is completely empirical and there is no specific recommendation on its use to improve sperm count or motility. The commonly used antioxidants include vitamins A, C and E, zinc, folate, selenium, and carnitine. The beneficial effect of these drugs in treatment of male infertility is not certain (Ko et al., 2014).

Corticosteroids

Oral corticosteroids are commonly used to suppress antisperm antibody production, but its value in the treatment to improve pregnancy rates in male infertility is doubtful. The significance of ASA in infertile men is controversial and there is no standardized protocol for its treatment. The presence of antibodies against spermatozoa in the serum and/or in the seminal plasma or on the sperm surface may have a harmful effect in fertilization. Antisperm antibodies (ASA) can lead to immobilization and agglutination of spermatozoa and interfere with sperm-egg interaction (Veron et al., 2016). Common causes of ASA include previous genital tract infection, testicular biopsy, testicular trauma, testicular torsion, and vasectomy (Arap et al., 2007).

Mucolytics

On ejaculation, semen is coagulated due to its protein content secreted by the seminal vesicle. Normally, approximately 20–30 min after ejaculation, liquefaction of semen occurs by the proteolytic enzymes secreted by the prostate and sperm gain motility after being freed from the seminal clot. Hyperviscosity and delayed liquefaction of semen is seen due to prostatic infection and hypofunction of the prostate (Motrich et al., 2005).

Hyperviscosity and delayed liquefaction of semen impair normal sperm movement in the female reproductive tract and decrease the chance of fertilization and spontaneous pregnancy (Mandal and Bhattacharyya, 1987). Patients with hyperviscosity and delayed liquefaction of semen are treated with mucolytic drugs that are used in the treatment of cough such as *N*-acetylcysteine, carbocysteine and bromohexine. In addition, to the standard treatment of chronic prostatitis is indicated if leucocytes are detected in semen.

Coital Infertility

In this category of male infertility, the testes and genital duct are anatomically normal and produce normal quantity and quality of sperm. But, the process by which semen is ejaculated into the partner's vagina is not integrated. The problem may be due to erectile dysfunction in which the patient is unable to start the intercourse or may be due ejaculatory disorders in the form of anejaculation, retrograde ejaculation or extravaginal insemination due to premature ejaculation.

Erectile Dysfunction

Normal erection is required for normal intercourse with intravaginal ejaculation. The etiology of erectile dysfunction may be due to psychological, hormonal, neurological or vascular factors and may be due to a mix of two or more factors. Viagra (Sildenafil) was introduced, in 1998, as the first successfully specific oral medication for erectile dysfunction. Other oral medications for erectile dysfunction include Sildenafil, Tadalafil, Vardenafil, and Avanafil. All drugs are more or less similarly effective in treating men with erectile dysfunction and their side effects are generally accepted.

The WHO recommends using oral medication as a first line treatment of erectile dysfunction and it is necessary to define a clear diagnosis of erectile dysfunction before starting treatment. These drugs could be used with a degree of success in all causes of erectile dysfunction. On sexual stimulation, these drugs enhance the release of nitric oxide and cause relaxation of the cavernous smooth muscles and increase arterial blood flow and filling the cavernous bodies with blood.

The use of oral medications to treat erectile dysfunction is a doctor's decision according to the medical condition of the patient. These medications are risky when used in hypotensive patients, in patients with heart failure and in patients using drugs containing

nitrate. The choice of the type of oral medication is a patient preference base on clear information from the treating doctor about the advantages and disadvantages of each drug.

The effective dose of the drug is variable according to the degree of erectile dysfunction. Usually we start with a small dose in young patient and in patients with recent and mild form of erectile dysfunction. We can adjust the dose of the drug, or shift into another drug, according to the response to its initial dose.

Testosterone is a medication that might be needed to treat erectile dysfunction in cases with low serum testosterone. But, in patients seeking fertility, exogenous testosterone is contraindicated because it inhibits the hypothalamo-pituitary-gonadal access and suppresses the production of FHS, LH, and testicular testosterone, and all are required for normal spermatogenesis. In these cases we can use drugs that stimulate testicular production of testosterone and increase intratesticular testosterone. Examples of these drugs are hCG which is given to stimulate Leydig cells to produce testosterone and antiestrogens, such as Clomiphene and Tamoxifen, which increase the production of LH and consequently increase testicular production of testosterone.

Anorgasmia

Anorgasmia is a condition in which the patient has a normal erection and practice intercourse normally, but is unable to reach the stage of orgasm and ejaculation in his sex cycle. Anejaculation is usually a psychological disorder and requires a psychiatric consultation and psychotherapy.

Anejaculation

Emission is the deposition of seminal fluid into the posterior urethra. Emission is under sympathetic control and requires the integrity of the vas deferens, seminal vesicles, prostate, and bladder neck. Ejaculation is the expulsion of the seminal fluid through the urethra and depends on the function of the perineal striated musculature. For clinical purposes, the term ejaculation is often used to include both events (Revenig et al., 2014).

Ejaculatory failure or anejaculation is actually a failure of seminal emission. The patient reaches orgasm and feels the contraction of striated perineal muscles but with complete absence of ejaculate. Anejaculation may be psychogenic or neurogenic. It is usually seen in patients with spinal cord injury and in patients with a history of retroperitoneal lymph node dissection. It is also common in patients using alpha blocker drugs (Table 1).

In treatment of anejaculation, it is necessary to stop or change any medication that affects the sympathetic function. Medical therapy is used to enhance seminal emission and partially or completely restore the antegrade ejaculation. In case of no response to such medications, the other lines of treatment of anejaculation are vibratory stimulation or electroejaculation to get their sperm for the assisted reproductive techniques such as IUI or ICSI (Barazani et al., 2012).

Drugs used in the treatment of anejaculation include Pseudoephedrine hydrochloride (60 mg four times daily) and Imipramine hydrochloride (25 mg QID). Pseudoephedrine hydrochloride is a sympathomimetic agent that stimulates the contraction of ejaculatory muscles and improves antegrade ejaculation. It seems to be more effective when used over a few days, usually 10–14 days, rather than as a single dose before intercourse. Imipramine hydrochloride has an anticholinergic property and has been reported to be successful in treatment of anejaculation. It blocks the re-uptake of norepinephrine at the nerve terminals, potentiating the adrenergic activity. Human chorionic gonadotropin: Injections of human chorionic gonadotropin have been reported to increase the incidence of nocturnal emissions. It is used in treatment of anejaculatory patients who has low to normal gonadal hormones (Jiann, 2016).

Table 1 Etiology of anejaculation

Traumatic	Spinal cord injury
Iatrogenic	Posterior urethra injury
	Retroperitoneal lymph node dissection
	Aortic aneurysmectomy
	Colorectal surgery
	Sympathectomy
Pharmacological	Alpha Blockers used in treatment of BPH
	Antihypertensives
	Antipsychotics
	Antidepressants
	Alcohol
Metabolic and systemic	Diabetes mellitus
	Multiple sclerosis
Others	Bone marrow transplant
	Psychological
	Idiopathic

Table 2 Etiology of retrograde ejaculation

Anatomic	Neurologic	Pharmacologic
Bladder neck surgery	Diabetes mellitus	Antihypertensives
Prostatectomies	Retroperitoneal lymphadenectomy	Antipsychotics
Urethral strictures	Colorectal surgery	Antidepressants
Meatal stenosis	Abdominal aneurysmectomy	
Bladder neck trauma	Aortoiliac bypass surgery	
Posterior urethral trauma	Spinal cord injury	
Extrophy and epispadias		
Posterior urethral valves		
Ectopic ejaculatory duct		

Retrograde Ejaculation

Normally at emission, both the bladder neck and the external urethral sphincter are closed when semen is deposited in the posterior urethra. Then, the external urethral sphincter open as the pressure builds up in the posterior urethra, while the bladder neck remains closed and contraction of the bulbospongiosus and ischiocavernosus muscles occurs resulting in the antegrade ejaculation (Gosling et al., 1981). Any process interfering with adequate closure of the bladder neck will result in retrograde ejaculation. Table 2 demonstrate different causes of retrograde ejaculation.

The principle of treatment of retrograde ejaculation is to close the posterior urethra to allow only antegrade passage of semen. The posterior urethral sphincter is under control of the sympathetic system (alpha-adrenergic). Medical treatment of retrograde ejaculation is applicable only in patients with an anatomically intact bladder neck. Patients with anatomical abnormalities of the bladder neck secondary to surgical or endoscopic intervention are certainly not responsive to medical treatment. Treatment of retrograde ejaculation is initiated with the same drugs (Pseudoephedrine and Imipramine) outlined for treatment of anejaculation (Shoshany et al., 2017). In case of no response to such medications, the other choice of treatment is sperm retrieval from the bladder for assisted reproduction techniques.

Premature Ejaculation

Premature ejaculation is a persistent occurrence of ejaculation with minimal sexual stimulation before, on, or shortly after penetration and before the person and/or his partner wishes it. Premature ejaculation is considered an infertility problem if ejaculation occurs before vaginal penetration. Premature ejaculation is classified as primary (lifelong) characterized by onset from the first sexual experience or secondary (acquired) characterized by a gradual or sudden onset following normal ejaculation experiences before onset. Ejaculation is a sympathetic process and premature ejaculation is commonly seen in anxiety and stress. Erectile dysfunction may coexist in men with premature ejaculation and it is important to treat it first.

Various behavioral techniques have been beneficial in treating premature ejaculation in many cases and are advised for patients uncomfortable with pharmacological therapy. Behavioral techniques include the “stop-start” technique and the “squeeze” technique. The “stop-start” technique is a commonly advised program in which the partner stimulates the penis until the patient feels the urge to ejaculate. At this point, he and his partner stop and wait for the sensation to pass and then stimulation is resumed. In the “squeeze” technique, the partner applies manual pressure to the glans of the penis just before ejaculation until the patient loses his urge. Relaxation and distraction technique, at the time of feeling urge to ejaculate, is another behavioral technique may help men to delay ejaculation. Masturbation before anticipation of sexual intercourse is also a technique used by young men and helps to delay ejaculation at intercourse (Rowland and Cooper, 2011).

Pharmacological treatment of premature ejaculation is either topical or oral. On-demand topical anesthetic agents, applied shortly before intercourse, to the ventral surface of the glans have consistently shown efficacy in treating premature ejaculation (Atikeler et al., 2002). The antidepressants selective serotonin reuptake inhibitors are used to be used daily as oral medications for premature ejaculation. Dapoxetine is a short acting selective serotonin reuptake inhibitor and is specifically used on-demand for premature ejaculation (Russo et al., 2016).

References

- Agarwal, A., & Prabakaran, S. A. (2005). Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. *Indian Journal of Experimental Biology*, 43, 963–974.
- Arap, M. A., Vicentini, F. C., Cocuzza, M., Hallak, J., Athayde, K., Lucon, A. M., Arap, S., & Srougi, M. (2007). Late hormonal levels, semen parameters, and presence of antisperm antibodies in patients treated for testicular torsion. *Journal of Andrology*, 28(4), 528–532.
- Athayde, K. S., Cocuzza, M., Agarwal, A., Krajcir, N., Lucon, A. M., Srougi, M., & Hallak, J. (2007). Development of normal reference values for seminal reactive oxygen species and their correlation with leukocytes and semen parameters in a fertile population. *Journal of Andrology*, 28(4), 613–620.
- Atikeler, M. K., Gecit, I., & Senol, F. A. (2002). Optimum usage of prilocaine-lidocaine cream in premature ejaculation. *Andrologia*, 34(6), 356–359.

- Barazani, Y., Stahl, P. J., Nagler, H. M., & Stember, D. S. (2012). Management of ejaculatory disorders in infertile men. *Asian Journal of Andrology*, 14(4), 525–529.
- Chiba, K., & Fujisawa, M. (2016). Clinical outcomes of Varicocele repair in infertile men: A review. *World Journal of Men's Health*, 34(2), 101–109.
- Gilbaugh, J. H., & Lipshultz, L. I. (1994). Nonsurgical treatment of male infertility. An update. *Urology Clinic of North America*, 21(3), 531–548.
- Gosling, J. A., Dixon, Y. S., Critchley, H. O., & Thompson, S. A. (1981). A comparative study of the human external sphincter and periurethral levator ani muscles. *British Journal of Urology*, 53(1), 35–41.
- Heller, C. G., Rowley, M. J., & Heller, G. V. (1969). Clomiphene citrate: A correlation of its effect on sperm concentration and morphology, total gonadotropins, ICSH, estrogen and testosterone excretion, and testicular cytology in normal men. *The Journal of Clinical Endocrinology & Metabolism*, 29(5), 638–649.
- Hussein, A., Ozgok, Y., Ross, L., & Niederberger, C. (2005). Clomiphene administration for cases of nonobstructive azoospermia: A multicenter study. *Journal of Andrology*, 26(6), 787–792.
- Hussein, A., Ozgok, Y., Ross, L., Rao, P., & Niederberger, C. (2013). Optimization of spermatogenesis-regulating hormones in patients with non-obstructive azoospermia and its impact on sperm retrieval: A multicentre study. *British Journal of urology international*, 111(3, B), 110–114.
- Jarow, J. P., & Zirkin, B. (2005). The androgen microenvironment of the human testis and hormonal control of spermatogenesis. *Annals of the New York Academy of Sciences*, 1061, 208–220.
- Jiann, B. P. (2016). The office management of ejaculatory disorders. *Translational Andrology and Urology*, 5(4), 526–540.
- Kim, E. D., Crosnoe, L., Bar-Chama, N., Khera, M., & Lipshultz, L. I. (2013). The treatment of hypogonadism in men of reproductive age. *Fertility and sterility*, 99(3), 718–724.
- Ko, E. Y., Sabanegh, E. S. J., & Agarwal, A. (2014). Male infertility testing: Reactive oxygen species and antioxidant capacity. *Fertility and Sterility*, 102(6), 1518–1527.
- Kulin, H. E., & Reiter, E. O. (1972). Gonadotropin suppression by low dose estrogen in men: Evidence for differential effects upon FSH and LH. *The Journal of Clinical Endocrinology and Metabolism*, 35(6), 836–839.
- Liu, P. Y., Gebiski, V. J., Turner, L., Conway, A. J., Wishart, S. M., & Handelsman, D. J. (2002). Predicting pregnancy and spermatogenesis by survival analysis during gonadotrophin treatment of gonadotrophin-deficient infertile men. *Human Reproduction*, 17(3), 625–633.
- Mandal, A., & Bhattacharyya, A. K. (1987). Relationship between the coagulation-liquefaction property of human ejaculates and their volume, sperm count and motility. *Clinical Reproduction and Fertility*, 5(6), 367–371.
- March, M. R., & Isidori, A. (2002). New frontiers in the treatment of male sterility. *Contraception*, 65(4), 279–281.
- Motrich, R. D., Maccioni, M., Molina, R., Tissera, A., Olmedo, J., Riera, C. M., & Rivero, V. E. (2005). Reduced semen quality in chronic prostatitis patients that have cellular autoimmune response to prostate antigens. *Human Reproduction*, 20(9), 2567–2572.
- Pitteloud, N., Hayes, F. J., Dwyer, A., Boepple, P. A., Lee, H., & Crowley, W. F. J. (2002). Predictors of outcome of long-term GnRH therapy in men with idiopathic hypogonadotropic hypogonadism. *The Journal of Clinical Endocrinology and Metabolism*, 87(9), 4128–4136.
- Raman, J. D., & Schlegel, P. N. (2002). Aromatase inhibitors for male infertility. *Journal of Urology*, 167(1), 624–629.
- Revenig, L., Leung, A., & Hsiao, W. (2014). Ejaculatory physiology and pathophysiology: Assessment and treatment in male infertility. *Translational Andrology and Urology*, 3(1), 41–49.
- Rowland, D., & Cooper, S. (2011). Practical tips for sexual counseling and psychotherapy in premature ejaculation. *The Journal of Sexual Medicine*, 8(4), 342–352.
- Russo, A., Capogrosso, P., Ventimiglia, E., La Croce, G., Boeri, L., Montorsi, F., & Salonia, A. (2016). Efficacy and safety of dapoxetine in treatment of premature ejaculation: An evidence-based review. *International Journal of Clinical Practice*, 70(9), 723–733.
- Sharma, R. K., & Agarwal, A. (1996). Role of reactive oxygen species in male infertility. *Urology*, 48, 835–850.
- Shoshany, O., Abhyankar, N., Elyaguov, J., & Niederberger, C. (2017). Efficacy of treatment with pseudoephedrine in men with retrograde ejaculation. *Andrology*, 5(4), 744–748.
- Siddiq, F. M., & Sigman, M. (2002). A new look at the medical management of infertility. *Urology Clinic of North America*, 29(4), 949–963.
- Véron, G. L., Molina, R. I., Tissera, A. D., Estofan, G. M., Marin-Briggiler, C. I., & Vazquez-Levin, M. H. (2016). Incidence of sperm surface autoantibodies and relationship with routine semen parameters and sperm kinematics. *American Journal of Reproductive Immunology*, 76(1), 59–69.
- Weidner, W., Ludwig, M., Brahlér, E., & Schiefer, H. G. (1999). Outcome of antibiotic therapy with ciprofloxacin in chronic bacterial prostatitis. *Drugs*, 58(2), 103–106.
- WHO. (2010). *WHO Laboratory manual for the examination and processing of human semen* (5th ed). World Health Organization: Geneva.

General Endocrine Therapy

Rodrigo L Pagani and Ramy A Ghayda, University of Illinois at Chicago, Chicago, IL, United States
Jorge Hallak, University of São Paulo, São Paulo, Brazil

© 2018 Elsevier Inc. All rights reserved.

Steroidogenic Dysfunction

The terms hypergonadotropic hypogonadism, primary hypogonadism, and primary hypoandrogenism refer to impaired testosterone synthesis caused by Leydig cell dysfunction (Sokol, 2009). It is well established that spermatogenesis is highly dependent on intratesticular testosterone synthesis (Jarow and Zirkin, 2005), although hypoandrogenism is poorly associated with sperm parameters (Patel et al., 2015). The prevalence of hypoandrogenism in men with oligozoospermia and with azoospermia due to spermatogenic dysfunction have been observed to be approximately 43% and 45%, respectively (Sussman et al., 2008). For those hypogonadal men who desire to protect their future fertility, exogenous testosterone should be discouraged as it has contraceptive effects, therefore it is astonishing that 25% of urologists are still prescribing exogenous testosterone to treat hypoandrogenism with male factor fertility (Ko et al., 2012). Except for gonadotropins, all of these methods described below to increase intratesticular testosterone synthesis, are considered off-label. In cases where the patient has azoospermia associated with low testosterone concentrations and significantly elevated LH levels, currently the only feasible treatment is surgical sperm extraction.

Clomiphene Citrate

Clomiphene citrate, originally approved for the treatment of ovulatory dysfunction in women, is a selective estrogen receptor modulator (SERM) that competitively binds to estrogen receptors and blocks the negative feedback at the level of hypothalamus and the pituitary. As a result, it indirectly enhances LH and FSH excretion from the anterior pituitary, stimulating Leydig cells in the testis to produce testosterone (Ross et al., 1980). Clomiphene citrate is a racemic mixture of two isoforms—enclomiphene and zuclophene.

Clomiphene citrate is well tolerated by most patients. Although uncommon, adverse effects include headache, dizziness, nausea, vomiting, gynecomastia, weight gain and hypertension (Kim et al., 2013). There is also about 1.5% of patients who presents with blurred vision, photophobia, and diplopia that are normally reversible with cessation of the medication. However, a small percentage of men may develop a paradoxical decrease in testosterone or a negative impact on sperm parameters (Ross et al., 1980), therefore it is imperative to check the bioavailable testosterone of every patients after 2 or 3 weeks of medication, in order to evaluate if titration of the dosage is needed or if clomiphene must be discontinued and replaced by another medication.

Ross et al. (1980) found that more than 50% of the patients responded within the first 3 months of treatment with the rest of the cohort responding after 6–15 months. Similarly, Guay et al. (2003) observed an increase in free testosterone in all men with hypogonadism and erectile dysfunction after 4 months of treatment. In a recent study by Mazzola et al. (2014) nearly 40% of hypoandrogenic men on clomiphene citrate were non-responders (response defined as 4200 ng dL^{-1} improvement in total testosterone levels after 6 months of treatment) being testicular volume and baseline LH level predictive of a positive response. Although sperm concentration is not the best marker for fertility, there has been reported a $7.7 \times 10^6 \text{ mL}^{-1}$ increase in sperm concentration between clomiphene citrate administration versus control (Bridges et al., 2015).

Clomiphene citrate has been also used in patients with azoospermia due to spermatogenic dysfunction, as the increase of intratesticular testosterone may lead to sufficient sperm in the ejaculate or may improve the overall success rate of testicular sperm extraction. In two different studies done by the same group, it was demonstrated that 64.3% of the azoospermic patients with hypospermatogenesis or maturation arrest showed sperm in their ejaculate after clomiphene citrate therapy (Hussein et al., 2005), and that 57.7% of patients prescribed clomiphene citrate had a successful microsurgical sperm retrieval as opposed to 33.6% of the control group (Hussein et al., 2012).

Recently, enclomiphene citrate, one of the single isomers of clomiphene citrate that has a pure estrogen antagonism, has demonstrated positive preliminary results, increasing morning testosterone levels, LH, FSH, DHT and estrogen, while preserving spermatogenesis (Wiehle et al., 2014).

Aromatase Inhibitors

Aromatase is a cytochrome P450 enzyme present in many body organs, like testes, prostate brain, bones and adipose tissue. Aromatase inhibitors have been widely prescribed for infertile male with relative excess estrogen to testosterone levels, measured as testosterone/estradiol (T/E) ratio (Schlegel, 2012). Aromatase inhibitors act on the 2A6 and 2C19 subunits of aromatase enzyme complex to block the conversion of testosterone and androstenedione into estradiol and estrone respectively (Chehab et al., 2015). This will indirectly increase GnRH pulses, subsequently positively affecting the pituitary by increasing FSH, which will act on the Sertoli cells to enhance spermatogenesis, and LH, which will act on the Leydig cell to secrete testosterone (Chehab et al., 2015).

Anastrozole is a non-steroidal, 3rd generation aromatase inhibitor which bind reversibly to the aromatase enzyme complex. Raman and Schlegel (2002) compared testolactone and anastrozole and showed that the group treated with anastrozole had

statistically significant increases in the T/E ratio and improved semen parameters. Side effects of anastrozole therapy are rare, and they include decrease in libido in less than 5% of the patients, and clinically insignificant transient liver function test abnormalities in about 7.4% of the patients. Recently, anastrozole was reported to improve endocrine parameters in 95.3% of men with hypoandrogenism with low T/E ratio and a subset of patients displayed significantly improved sperm parameters (Shoshany et al., 2017).

Letrozole is also a non-steroidal, 3rd generation aromatase inhibitor. When comparing anastrozole to letrozole, it was demonstrated an increase in sperm concentration, motility, and morphologically normal sperm in the ejaculate of patients treated with either letrozole or anastrozole. Although, the increase in sperm concentration was numerically greater for patients treated with anastrozole, it was not statistically significant (Gregoriou et al., 2012). Side effects were similar to anastrozole including decreased libido, headache, and transient liver enzyme abnormalities (Gregoriou et al., 2012).

Currently, there are no prospectively randomized controlled trials, and the use of aromatase inhibitors remains off-label. Further studies are needed to better evaluate the effects of these drugs on spermatogenesis and to evaluate pregnancy and delivery rates post-treatment.

Gonadotropins

Spermatogenesis depends on the stimulation of the testis by the anterior pituitary gland gonadotropins secretion. Exogenous gonadotropins administration is the only FDA approved medical therapy for idiopathic male, but they are not widely prescribed due to their high cost and invasive method of administration. Gonadotropins therapy can be in the form of direct pituitary stimulation (GnRH) or exogenous replacement of LH and/or FSH.

Human chorionic gonadotropin (hCG) is an LH analog. hCG include a molecular weight $\sim 36,000$ Da (Da), with two subunits: α -subunit with gene location: 6q12-q21 and 92 aminoacids; and β -subunit with gene location: 19q13.32 and 145 aminoacids (Cole, 2010). It has similar biological activity in stimulating Leydig cells to secrete testosterone and it indirectly improves spermatogenesis by increasing intratesticular testosterone levels. It can be used in incremental dosage until target testosterone level is achieved. If after 2 months the testosterone level is still not adequate or if after 18 months spermatogenesis did not improve, many authors consider adding human menopausal gonadotropin (hMG), which contains both FSH and LH, and its dosage administration is incremental until sperm concentration is adequate (European Metrodin HP Study Group, 1998a). Another form of replacement of pituitary LH and FSH is the usage of its recombinant molecules (rLH and rFSH).

The gonadotropic replacement regimen is dependent on the onset of the hypogonadotropic hypogonadism. If it happened before puberty, FSH and LH analog are both needed because FSH has not primed the Sertoli cells. If on the other hand it happens post-pubertal, only LH analog is needed (Howles et al., 2007). They are not widely prescribed because of their high cost and invasive method of administration. Clinically they are reserved for infertile male who failed conventional therapy with clomiphene citrate and/or anastrozole, or for patient who developed severe side effects on these latter medications. A consensus regarding optimal starting dosage is still lacking.

A Cochrane review of six randomized clinical trials with patients on gonadotropins (Attia et al., 2013), confirmed the overall positive effect on the infertile male with live birth rate of 27% versus 0% and spontaneous pregnancy rate per couple of 16% versus 7% in placebos. A pregnancy rate of 13.4% was achieved versus 4.4% for those without treatment (odd ratio, 3.03; 95% confidence interval, 1.30–7.09).

In an old study, authors used transmission electron microscopy to evaluate the ultrastructural analysis of spermatozoa prior to the start of rFSH therapy and 6 weeks post-therapy and found that rFSH treatment resulted in a higher number of morphologically normal spermatozoa. The electron microscopic findings indicate that treatment with rFSH may be an effective way to improve sperm quality in cases with oligoasthenoteratozoospermia (Strehler et al., 1997). It was demonstrated that 89.3% of the men with complete isolated hypogonadotropic hypogonadism treated with hCG followed by rFSH were able ejaculate some sperm and that 64.3% of them were ejaculating over $1.5 \text{ M sperm mL}^{-1}$, with improvements in motility and morphology (European Metrodin HP Study Group, 1998b).

Gonadotropins have been used in patients with azoospermia due to spermatogenic dysfunction. Theoretically it might improve the results of testicular sperm extraction and it may help induce the appearance of the sperm in the ejaculate. This effect is achieved by “resetting” of the FSH and LH receptors in the Leydig and Sertoli through the high levels of exogenous gonadotropins, while there is a defective or inefficient quantities of bioactive FSH and LH (Wang et al., 1987). Multiple small studies have demonstrated clinically that treatment of NOA patients with recombinant FSH increased the chance of sperm retrieval on a second TESE, after a failed first attempt. Shiraishi et al. found a 22% success rate of retrieving sperm with 5000 IU treatment after a failed initial TESE (Shiraishi et al., 2011). Selman et al. performed a similar experiment with incremental dosage regime of rFSH and found similar positive rate of 22% (Selman et al., 2006). Ramasamy et al. demonstrated the same positive outcome in the Klinefelter group of patients (Ramasamy et al., 2009).

Pituitary Dysfunction

Hypogonadotropic hypogonadism occurs when the pituitary hormones LH and/or FSH are suppressed. One of the possible causes for hypogonadotropic hypogonadism is Kallmann syndrome, which consists in anosmia associated with decreased pituitary

function with autosomal dominant inheritance (although X-linked and autosomal recessive patterns were also observed) (Sokol, 2009). Treatment includes replacement of the pituitary hormones by exogenous gonadotropins that exhibit LH and/or FSH-like activities. In a recent publication in Japan, researchers looked at 55 adolescents with male hypogonadotropic hypogonadism. In regard to spermatogenesis, 76% of the patients treated with hCG-rFSH combined therapy showed positive results, though ranging in levels; impaired spermatogenesis was observed in some men with congenital hypogonadotropic hypogonadism while all men with acquired hypogonadotropic hypogonadism had favorable spermatogenesis (Sato et al., 2015).

Pituitary tumors and diseases that infiltrate its parenchyma, such as sarcoidosis, histiocytosis, hemochromatosis, and infections, may result in varying degrees of LH and FSH suppression. The most common kind of pituitary tumor that is related to male infertility secretes prolactin. In general, mild elevations of prolactin (below $50 \mu\text{g L}^{-1}$) do not warrant further evaluation (Niederberger, 2011). In cases where surgery is not indicated, dopamine agonists such as bromocriptine and cabergoline may be prescribed, with the last one showing fewer side effects (Klibanski, 2010).

Iatrogenic Dysfunction

The most common cause of testicular hypofunction is testosterone replacement therapy. The administration of exogenous testosterone leads to direct inhibition of the HPG axis as well as indirect inhibition through increased peripheral aromatization. Some substances also show steroid-like effects such as spironolactones, opioids, and phytoestrogens (Hotelling and Patel, 2014). Furthermore, as many as 20% of legally sold sports nutrition supplements contain synthetic anabolic-androgenic steroids (Ohlander et al., 2016). Anderson and Wu reported that 70% of men developed azoospermia or severe oligozoospermia after 18 months of exogenous testosterone therapy with only 85% experiencing recovery of spermatogenesis (Anderson and Wu, 1996). In men with azoospermia or severe oligozoospermia due to exogenous testosterone therapy, Wenker et al. observed that introduction of HCG in combination with clomiphene citrate, tamoxifen, anastrozole or recombinant FSH was responsible to demonstrate a return or improvement of spermatogenesis in 47 of the 49 subjects (Wenker et al., 2015).

Obesity also contributes to hormonal dysfunction, since estrogen excess from peripheral aromatization in adipose tissue can inhibit the pituitary function and spermatogenesis. It is also reported that abnormalities in the metabolism of IGF-1, glucocorticoid excess, and increased testicular temperature are factors that could cause pituitary and testicular dysfunction leading to hypospermatogenesis (Hotelling and Patel, 2014). Therefore, although there is no clinical evidence to date, weight loss theoretically could improve overall semen parameters.

References

- Anderson, R. A., & Wu, F. C. (1996). Comparison between testosterone enanthate-induced azoospermia and oligozoospermia in a male contraceptive study. II. Pharmacokinetics and pharmacodynamics of once weekly administration of testosterone enanthate. *The Journal of Clinical Endocrinology and Metabolism*, 81, 896–901.
- Attia, A., Al-Inany, H., & Proctor, M. (2013). Gonadotrophins for idiopathic male factor subfertility. *Cochrane Database of Systematic Reviews*, 8, Cd005071.
- Bridges, N., Trofimenko, V., Fields, S., et al. (2015). Male factor infertility and clomiphene citrate: A meta-analysis—The effect of clomiphene citrate on oligospermia. *Urology Practice*, 2, 109–205.
- Chehab, M., Madala, A., & Trussell, J. (2015). On-label and off-label drugs used in the treatment of male infertility. *Fertility and Sterility*, 103, 595–604.
- Cole, L. A. (2010). Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology*, 8, 102.
- European Metrodin HP Study Group. (1998a). Efficacy and safety of highly purified urinary follicle-stimulating hormone with human chorionic gonadotropin for treating men with isolated hypogonadotropic hypogonadism. *Fertility and Sterility*, 70, 256–262.
- European Metrodin HP Study Group. (1998b). Efficacy and safety of highly purified urinary follicle-stimulating hormone with human chorionic gonadotropin for treating men with isolated hypogonadotropic hypogonadism. *Fertility and Sterility*, 70, 256–262.
- Gregoriou, O., Bakas, P., Grigoriadis, C., et al. (2012). Changes in hormonal profile and seminal parameters with use of aromatase inhibitors in management of infertile men with low testosterone to estradiol ratios. *Fertility and Sterility*, 98, 48–51.
- Guay, A., Jacobson, J., Perez, J. B., Hodge, M. B., & Velasquez, E. (2003). Clomiphene increases free testosterone levels in men with both secondary hypogonadism and erectile dysfunction: Who does and does not benefit? *International Journal of Impotence Research*, 15, 156–165.
- Hotelling, J. M., & Patel, Z. (2014). Male endocrine dysfunction. *Urologic Clinics of North America*, 41, 39–53.
- Howles, C. M., Tanaka, T., & Matsuda, T. (2007). Management of male hypogonadotropic hypogonadism. *Endocrine Journal*, 54, 177–190.
- Hussein, A., Ozgok, Y., Ross, L., & Niederberger, C. (2005). Clomiphene administration for cases of nonobstructive azoospermia: A multicenter study. *Journal of Andrology*, 26, 787–791.
- Hussein, A., Ozgok, Y., Ross, L., Rao, P., & Niederberger, C. (2012). Optimization of spermatogenesis-regulating hormones in patients with non-obstructive azoospermia and its impact on sperm retrieval: A multicenter study. *British Journal of Urology International*, 111, 110–114.
- Jarow, J. P., & Zirkin, B. R. (2005). The androgen microenvironment of the human testis and hormonal control of spermatogenesis. *Annals of the New York Academy of Science*, 1061, 208–220.
- Kim, E. D., Crosnoe, L., Bar-Chama, N., Khera, M., & Lipshultz, L. I. (2013). The treatment of hypogonadism in men of reproductive age. *Fertility and Sterility*, 99, 718–724.
- Klibanski, A. (2010). Clinical practice. Prolactinomas. *New England Journal of Medicine*, 362, 1219–1226.
- Ko, E. Y., Siddiqi, K., Brannigan, R. E., & Sabanegh, E. S., Jr. (2012). Empirical medical therapy for idiopathic male infertility: A survey of the American Urological Association. *The Journal of Urology*, 187, 973–978.
- Mazzola, C. R., Katz, D. J., Lohmanieh, N., Nelson, C. J., & Mulhall, J. P. (2014). Predicting biochemical response to clomiphene citrate in men with hypogonadism. *Journal of Sex Medicine*, 11, 2302–2307.
- Niederberger, C. S. (2011). Clinical evaluation of the male. In C. S. Niederberger (Ed.), *An introduction to male reproductive medicine* (pp. 29–57). New York: Cambridge University Press.
- Ohlander, S. J., Lindgren, M. C., & Lipshultz, L. I. (2016). Testosterone and male infertility. *Urologic Clinics of North America*, 43, 195–202.

- Patel, D. P., Brant, W. O., Myers, J. B., et al. (2015). Sperm concentration is poorly associated with hypoandrogenism in infertile men. *Urology*, 85, 1062–1067.
- Raman, J., & Schlegel, P. (2002). Aromatase inhibitors for male infertility. *The Journal of Urology*, 167, 624–629.
- Ramasamy, R., Ricci, J. A., Palermo, G. D., Gosden, L. V., Rosenwaks, Z., & Schlegel, P. N. (2009). Successful fertility treatment for Klinefelter's syndrome. *The Journal of Urology*, 182, 1108–1113.
- Ross, L. S., Kandel, G. L., Prinz, L. M., & Auletta, F. (1980). Clomiphene treatment of the idiopathic hypofertile male: High-dose, alternate-day therapy. *Fertility and Sterility*, 33, 618–623.
- Sato, N., Hasegawa, T., Hasegawa, Y., et al. (2015). Treatment situation of male hypogonadotropic hypogonadism in pediatrics and proposal of testosterone and gonadotropins replacement therapy protocols. *Clinical Pediatric Endocrinology*, 24, 37–49.
- Schlegel, P. N. (2012). Aromatase inhibitors for male infertility. *Fertility and Sterility*, 98, 1359–1362.
- Selman, H., Santo, M. D., Sterzik, K., Cipollone, G., Aragona, C., & El-Danasour, I. (2006). Rescue of spermatogenesis arrest in azoospermic men after long-term gonadotropin treatment. *Fertility and Sterility*, 86, 466–468.
- Shiraishi, K., Ohmi, C., Shimabukuro, T., & Matsuyama, H. (2011). Human chorionic gonadotrophin treatment prior to microdissection testicular sperm extraction in non-obstructive azoospermia. *Human Reproduction*, 27, 331–339.
- Shoshany, O., Abhyankar, N., Mufarreh, N., Garvey, D., & Niederberger, C. (2017). Outcomes of anastrozole in oligozoospermic hypoandrogenic subfertile men. *Fertility and Sterility*, 107, 589–594.
- Sokol, R. Z. (2009). Endocrine evaluation. In L. I. Lipshultz, L. I. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). Cambridge, UK: Cambridge University Press.
- Strehler, E., Sterzik, K., De Santo, M., et al. (1997). The effect of follicle-stimulating hormone therapy on sperm quality: An ultrastructural mathematical evaluation. *Journal of Andrology*, 18, 439–447.
- Sussman, E. M., Chudnovsky, A., & Niederberger, C. S. (2008). Hormonal evaluation of the infertile male: Has it evolved? *Urologic Clinics of North America*, 35, 147–155.
- Wang, C., Dahl, K. D., Leung, A., Chan, S. Y., & Hsueh, A. J. (1987). Serum bioactive follicle-stimulating hormone in men with idiopathic azoospermia and oligospermia. *The Journal of Clinical Endocrinology & Metabolism*, 65, 629–633.
- Wenker, E. P., Dupree, J. M., Langille, G. M., et al. (2015). The use of HCG-based combination therapy for recovery of spermatogenesis after testosterone use. *Journal of Sexual Medicine*, 12, 1334–1337.
- Wiehle, R. D., Fontenot, G. K., Wike, J., et al. (2014). Enclomiphene citrate stimulates testosterone production while preventing oligospermia: A randomized phase II clinical trial comparing topical testosterone. *Fertility and Sterility*, 102, 720–727.

Endocrine Stimulatory Therapy for Testis Sperm Extraction

Moshe Shapiro and Samuel Ohlander, University of Illinois-Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Infertility, defined as the inability to conceive after 12 months of unprotected intercourse, affects roughly 15% of couples attempting to conceive (Cocuzza et al., 2013). Approximately 10%–15% of infertile males, and 1% of the general population, are azoospermic. Azoospermia is the absolute absence of sperm in the ejaculate. Prior to the development of intracytoplasmic sperm injection (ICSI) in 1989 and the utilization of testicular sperm extraction in 1995, men with azoospermia due to spermatogenic dysfunction (ASD), also commonly known as nonobstructive azoospermia, were unable to father their genetically own children. The only options for children were donor sperm or adoption. With the advent of ICSI, and the subsequent coupling with surgical sperm retrieval, single sperm harvested from the testicle were able to be manipulated and used for fertilization. Sperm develops in the seminiferous tubules, advances through the rete testis and then the epididymis, where it becomes capacitated and able to penetrate and fertilize an ovum (Bedford et al., 1973). ICSI directly injects the sperm into the ovum mechanically. This enables testicular sperm to be used for IVF to achieve pregnancies with similar success as compared to ejaculated sperm (Hussein et al., 2012). Further modification of the surgical retrieval utilizes a high power surgical microscope to intraoperatively identify dilated seminiferous tubules, or microsurgical testicular sperm extraction (microTESE), that have higher odds of obtaining sperm (Schlegel, 1999). It has been posited that endocrine manipulation of the hypothalamic-pituitary–testicular axis prior to surgical harvest can improve sperm retrieval rates and, in some patients, even induce sperm to appear in the ejaculate (Hussein et al., 2005). This article will discuss the means of endocrine stimulatory therapy prior to sperm retrieval.

Spermatogenesis

Spermatogenesis is the production of sperm. It occurs within the seminiferous tubules that comprise much of the testicular volume (Turek, 2016). The seminiferous tubules consist of germ cells, which are the progenitor cells for sperm, and support cells. These support cells include Sertoli cells, fibrocytes, and myoblasts. The complete cycle of spermatogenesis takes approximately 64 days (Clermont, 1972). Spermatogenesis occurs in a continuous and asynchronous manner, meaning, that at any one time, there are multiple different gametes at varying levels of maturity. For adequate spermatogenesis to occur, high levels of intratesticular testosterone are needed—typically at least greater than 10 times serum concentration and can approach greater than a hundredfold increase (Jarow and Zirkin, 2005). This high testosterone gradient compared to serum is maintained by local (testicular) production of testosterone by Leydig cells with the intratesticular testosterone kept locally high via the countercurrent gradient between the pampiniform venous plexus, with high testosterone levels diffusing to the testicular artery with lower testosterone levels (Jarow and Zirkin, 2005). When intratesticular testosterone levels decrease, spermatogenesis can be suppressed and may even cease altogether. Intratesticular testosterone levels are controlled by the multi-organ negative feedback regulatory system known as the hypothalamic-pituitary–testicular axis (HPTA) (Caroppo, 2009).

Hypothalamic-Pituitary–Testicular Axis

Male androgen levels and spermatogenesis are maintained by the HPTA (see Fig. 1). The HPTA is a finely tuned system controlled with multiple levels of stimulation and inhibition. Dedicated hypothalamic neurons secrete the peptide hormone gonadotropin releasing hormone (GnRH) in a pulsatile manner (Caroppo, 2009). The GnRH is transported through the portal system between the hypothalamus and the anterior pituitary. In the pituitary gland, extracellular receptors on gonadotrope secreting cells bind GnRH and, depending on the nature of the frequency of GnRH secretion, they secrete the gonadotropins follicle stimulation hormone (FSH) and luteinizing hormone (LH) (Caroppo, 2009). LH and FSH act in the testis to promote steroidogenesis and spermatogenesis, respectively. Testicular peptides and steroids then act upon the pituitary and hypothalamus with negative feedback. LH acts upon testicular Leydig cells to stimulate testosterone production, and FSH binds to Sertoli cells thereby promoting spermatogenesis. For normal spermatogenesis to occur, the HPTA must remain intact to maintain the appropriate intratesticular environment.

Azoospermia

Azoospermia is defined as the absence of any sperm in the semen, as opposed to aspermia, which is a lack of fluid where no anterograde specimen is produced at all. Azoospermia is diagnosed by centrifugation and microscopic evaluation of the pellet on two separate semen specimens to differentiate between cryptozoospermia, where small amounts of sperm are present (Corea et al., 2005). Azoospermia is typically categorized as either obstructive azoospermia, or, azoospermia due to spermatogenic dysfunction (ASD). Differentiation may often be delineated via thorough medical and surgical history (e.g., past vasectomy), though in less clear instances, laboratory evaluation may offer significant insight. In a patient with a FSH level of 7.6 mIU/mL

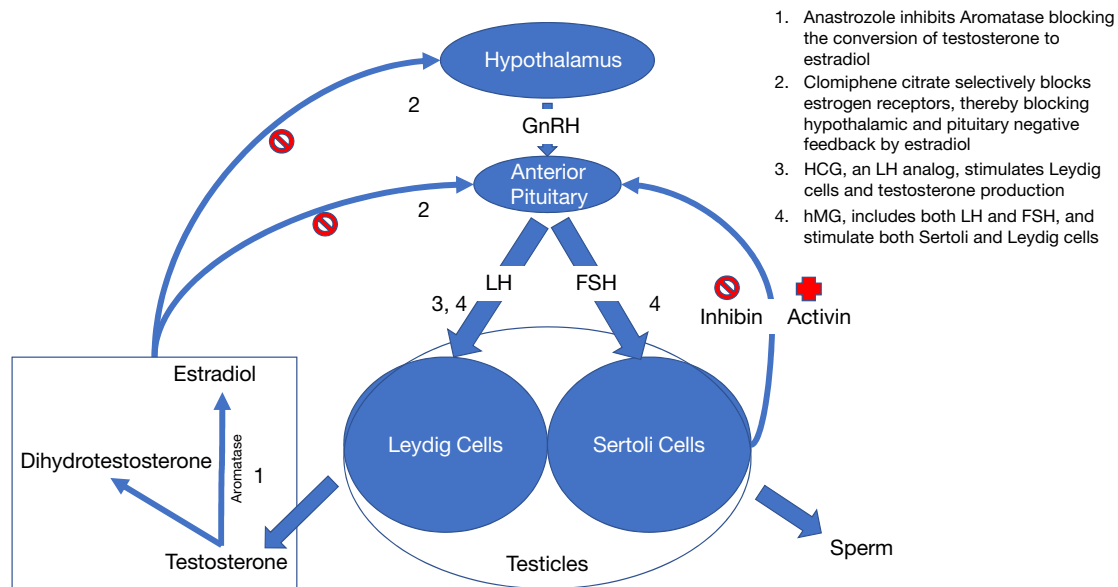


Fig. 1 Hypothalamic-pituitary-testicle axis.

- Anastrozole inhibits Aromatase blocking the conversion of testosterone to estradiol.
- Clomiphene citrate selectively blocks estrogen receptors, thereby blocking hypothalamic and pituitary negative feedback by estradiol.
- HCG, an LH analog, stimulates Leydig cells and testosterone production.
- hMG, includes both LH and FSH, and stimulate both Sertoli and Leydig cells.

or less, as well as testicular long axis of >4.6 cm, there is a 96% chance of azoospermia due to obstruction (Schoor et al., 2002). In patients with FSH levels >7.6 and testicular long axis length of <4.6 cm, there is an 89% chance of azoospermia due to spermatogenic dysfunction (Schoor et al., 2002). Given this data, diagnostic testicular biopsy is no longer routinely used and, if exam and serum assays indicate spermatogenic dysfunction, the physician may proceed confidently with discussions on surgical sperm retrieval and coupled assisted reproduction.

Microsurgical Testicular Sperm Extraction

For ASD, microTESE offers the greatest likelihood of successful sperm retrieval with retrieval rates of approximately 40%–60% (Esteves et al., 2011). The testicular parenchyma is divided into multiple compartments by septa into lobes, and within the lobes seminiferous tubules surround a central artery and the developing germ cells reside in each seminiferous tubule (Setchell and Brooks, 1988). The premise behind microTESE is that, even in an azoospermic man, there may be some pockets of spermatogenesis present within the testicle in some singular seminiferous tubules. Using high powered surgical magnification, these pockets of tubules are identified with the functional and sperm laden tubules appearing more gravid and fuller, as compared to the rest of the spermatogenic tubules.

MicroTESE became the gold standard for obtaining sperm in men with ASD when Schlegel showed sperm retrieval superiority with less testicular damage as compared to TESE without surgical microscope assistance (Schlegel, 1999). Despite this improved retrieval technique, 40%–60% of men with ASD will still be unable to father a biological child, thus there is impetus to improve the rate of retrieval and ability to predict success.

Medical Management for Azoospermia

Medical management of azoospermia is predicated on the theory that, increasing intratesticular testosterone will lead to clinically increased spermatogenesis (Jarow and Zirkin, 2005). In their seminal work, Hussein et al. showed that administration of clomiphene citrate, a selective estrogen receptor modulator, to men with spermatogenic dysfunction led to both the appearance of sperm in the ejaculate in some men, as well as improved sperm retrieval rates in patients undergoing microTESE (Hussein et al., 2012). Medical management acts by either direct stimulation of the testicles, or by inhibiting negative feedback leading to increased endogenous LH secretion with subsequent rise in serum and testicular testosterone (Boyle, 2009).

Indications for Endocrine Stimulatory Therapy

As most cases of male infertility are idiopathic in nature, management is typically empirical. In patients with a clear deficiency of the HPTA, such as in hypogonadotropic hypogonadism or exogenous testosterone use, medical management is clearly indicated. In

patients with an obstructive etiology, there is no deficiency in spermatogenesis, and, thus, stimulation is not indicated, and patients can be counseled to proceed directly to surgical extraction or reconstruction. In a patient with no fertility potential at all, such as with chromosome Y AZFa microdeletion, both endocrine stimulatory therapy and microTESE itself are contraindicated and futile (Oates and Lamb, 2009). However, since most patients presenting with ASD will have no identifiable cause, empiric therapy can and should be considered for those who are found to be hypogonadal. Unfortunately, there is no clear consensus on the appropriate testosterone cut point for the initiation of therapy. There is also variability in the use of total testosterone, free testosterone, and bioavailable testosterone for laboratory evaluation. Our recommendation is the use of bioavailable testosterone, calculated from any of the widely available online calculators that utilize serum testosterone, albumin, and sex hormone binding globulin (SHBG). In a study evaluating the use of bioavailable testosterone and sex hormone binding globulin, patients with both low overall testosterone and low bioavailable testosterone had worse sperm parameters (Ring et al., 2017). This difference was seen at the cutoff of a bioavailable testosterone of 156 ng/dL, despite patients with azoospermia being excluded from the study, this cutoff could be used to identify patients in whom endocrine stimulation prior to microTESE may be beneficial. Given that spermatogenesis takes approximately 2 months and endocrine stimulation can take up to 7 months for a response, great weight must be placed on factors other than the patient himself such as maternal age, timing of the female partner's cycle, as well as the availability of the IVF team, patient, and spouse.

Methods of Endocrine Stimulatory Therapy

Testicular endocrine stimulation is done either directly, to increase intratesticular testosterone and stimulate spermatogenesis, or indirectly, through suppression of negative feedback (Boyle, 2009). Optimally, in patients presenting with azoospermia due to spermatogenic dysfunction, an etiology of the azoospermia must be ruled out. In patients with a known etiology of their spermatogenic dysfunction, the goal of therapy is to restore the HPTA. When hypothalamic defects such as Kallman syndrome or idiopathic hypogonadotropic hypogonadism are present, stimulation of either the pituitary via gonadotropin releasing hormone (GnRH) or the testes themselves via FSH and human chorionic gonadotropin (HCG), a hormone endogenously produced by the placenta with an identical subunit and, thus, nearly biologically equivalent activity to LH, can be therapeutic targets (Dabaja and Schlegel, 2014). Another known cause of azoospermia due to spermatogenic dysfunction is exogenous testosterone administration. Exogenous testosterone administration leads to negative feedback at the level of the hypothalamus and pituitary decreasing LH and FSH secretion. This suppresses endogenous testicular production of testosterone and a decrease in intratesticular testosterone levels, impairing normal spermatogenesis. In a case series, these patients who had long-term exposure to exogenous testosterone were given HCG based regimens in addition to either a selective estrogen receptor modulator, an aromatase inhibitor or FSH. All the men tolerated the HCG regimen well, and 95.9% of men had return of spermatogenesis, with good sperm levels in the ejaculate (Wenker et al., 2015). In most men however, the etiology of their azoospermia is unknown, and treatment is empirical in nature, and seeks to stimulate the HPTA in the absence of a clear defect.

Currently, the only FDA approved drugs to treat male infertility are gonadotropins, including various formulations of LH and FSH (Chehab et al., 2015). GnRH is used in a pulsatile manner to induce endogenous pituitary secretion of the gonadotropins LH and FSH, though it is not available in the United States outside of specialty centers and clinical trials (Chehab et al., 2015). Men with Kallman syndrome or hypogonadotropic hypogonadism respond well to pulsatile GnRH therapy; however, there is little evidence to support its use in men with idiopathic spermatogenic dysfunction. Response to treatment can occur after 3 months of therapy. GnRH's utility is also limited by its scarce commercial availability, usually restricted to clinical trials and specialty centers, as well as a need for either a subcutaneous medication pump or frequent injections (Dabaja and Schlegel, 2014).

LH and its biological activity equivalent, human chorionic gonadotropin (HCG), are available in the US. Recombinant LH has a short half-life and has limited clinical utility compared to HCG, which has a much longer half-life and is more commonly used (Chehab et al., 2015). FSH is available in several formulations, as well as a purified urine product consisting of both LH and FSH, called human menopausal gonadotropin (hMG). In men with azoospermia due to spermatogenic dysfunction, HCG has been shown to reduce intratesticular fibrosis and increase Leydig cell size, compared to men with obstructive azoospermia (Oka et al., 2017). In men with hypopituitarism, gonadotropins are used. Initially starting therapy with HCG. If adequate androgenization occurs and the patient remains azoospermic, then FSH treatment can be introduced (Dabaja and Schlegel, 2014). Typically, a response can take up to 7 months to obtain sperm in the ejaculate and pregnancy occurs after an average of 28 months (Dabaja and Schlegel, 2014). In men with idiopathic spermatogenic dysfunction, there is lack of consensus regarding treatment with gonadotropins.

Selective estrogen receptor modulators (SERM), including clomiphene citrate (CC) and tamoxifen, act centrally at the hypothalamus and pituitary, where they disrupt the usual negative feedback of estrogen (Boyle, 2009). This leads to increased gonadotropins—mainly LH, with some FSH elevation as well (Hussein et al., 2012). SERMs are used to treat breast cancer and osteoporosis in women; however, their use in men to treat hypogonadism and infertility is off label. There is no clear consensus to the utility of SERMs in idiopathic infertility, with different meta-analysis having mixed results as to the efficacy of SERMs (Cocuzza et al., 2013; Willets et al., 2013). Regardless of findings regarding efficacy, CC and tamoxifen were well tolerated. Occasionally there can be a paradoxical effect from CC, with decreased testosterone levels after administration (Hussein et al., 2012). This may be due to clomiphene consisting of two isomers (*cis*) zuclomiphene and (*trans*) enclomiphene (Hill et al., 2009; Rodriguez et al., 2016). Zuclomiphene has been found to be an estrogen receptor agonist and enclomiphene is thought to competitively inhibit estrogen centrally (Rodriguez et al., 2016). It is also thought that the zuclomiphene component of CC may be responsible

for the estrogenic side effects of CC in men including the rare paradoxical response to CC. Enclomiphene citrate, under the trade name Androxal, is currently under development (Rodriguez et al., 2016). Additionally, there is a risk of pituitary enlargement and visual field disturbances (Chehab et al., 2015).

Estrogen in the male is a product of aromatized testosterone. Aromatase is present in adipose tissue as well as in the Leydig cells of the testes. Estrogen is the primary negative feedback molecule in the HPTA (Caroppo, 2009). High estrogen levels with low testosterone levels have also been shown to inhibit spermatogenesis. Aromatase inhibitors act by blocking peripheral conversion of testosterone into estrogen in adipose tissue and in the testes (Boyle, 2009; Schlegel, 2012). This leads to a cessation of negative HPTA feedback which induces increased secretion of gonadotropins. In a noncontrolled study, aromatase inhibitors such as anastrozole or letrozole improved patients' biochemical profile, semen parameters, and fertility (Schlegel, 2012). Patients who had the best response to aromatase inhibitors were men with a ASD or oligoasthenospermia, low testosterone and a low testosterone to estrogen ratio (Schlegel, 2012). Aromatase inhibitors are usually well tolerated; side effects include decreased libido, mildly elevated liver function tests, and nausea. Importantly decreased bone mineral density can occur due to lack of estrogen, hence long-term use of aromatase inhibitors in males is not recommended.

In a randomized controlled double blinded study comparing CC to anastrozole, CC was shown to raise serum testosterone levels significantly more than anastrozole; however, no difference in semen parameters or patient reported outcomes were seen (Helo et al., 2015). As CC selectively acts solely at the pituitary, bone density is maintained, and can be continued if the patient had been symptomatic from hypogonadism.

Procedural Outline

When testis endocrine stimulation prior to microTESE is indicated, a stepwise treatment can be implemented (see Fig. 2). However, as time may be of the essence due to advancing maternal age, frequently only one or two regimens are used prior to microTESE (Hussein et al., 2005). A common approach starts with an inexpensive, safe, oral medication, such as CC or anastrozole depending upon hormone profile, followed by symptomatic and laboratory evaluation of response after 2–4 weeks of therapy. If there is inadequate response, either due to side effects, lack of appropriate increase in serum testosterone, or disproportionate increase in serum estradiol, one can either increase the dose, switch medications, or add medications depending on the nature of the response. For example, a patient started on CC with good response of testosterone and normal estradiol can maintain the CC for at least 2–3 months before proceeding to microTESE (Hussein et al., 2005). If that patient were to have elevated estradiol, visual field disturbances, or an unchanged serum testosterone, they could switch to anastrozole, again measuring response. If the testosterone were unchanged, but FSH were elevated, then HCG could be added to spur testosterone production and so on, with the principle of safely raising testosterone and FSH. Fig. 2 provides an algorithm for implementation of therapy, though it ought to be noted that the algorithm is merely a guide, and patient specific management must be modified based upon tolerance, laboratory response, and reproductive timeline.

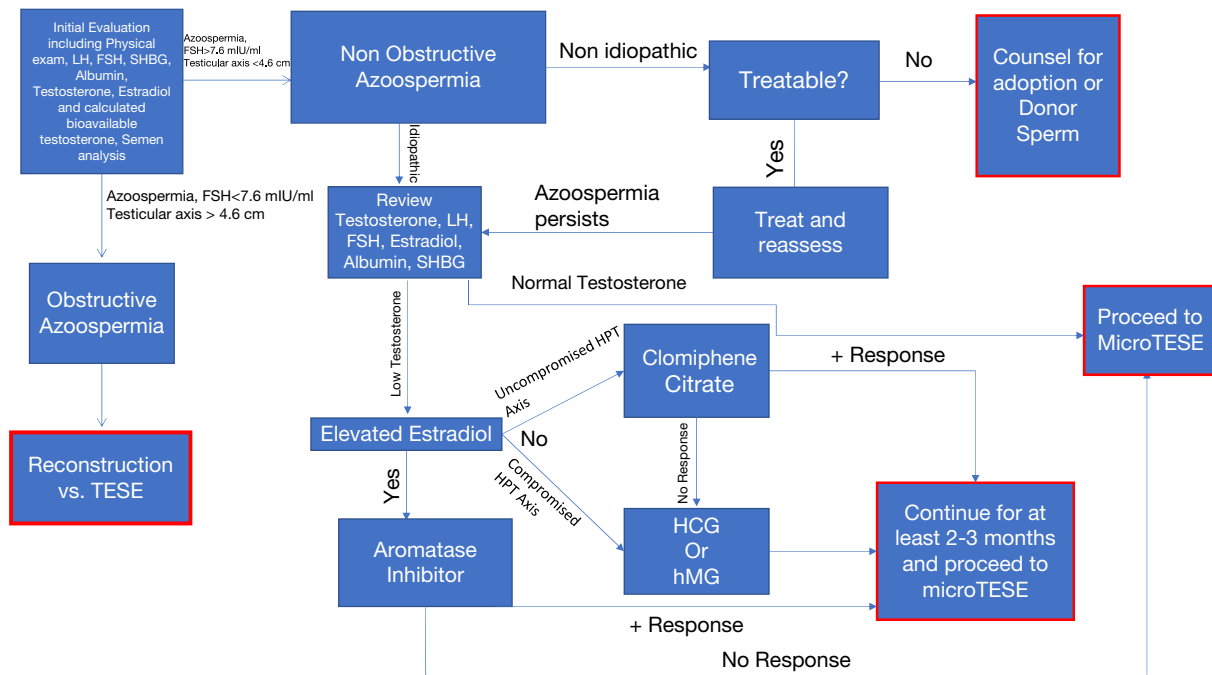


Fig. 2 Algorithm for endocrine stimulation prior to microTESE.

Outcomes

Several studies have debated the utility of endocrine stimulation of the testis to optimize sperm retrieval rates from microTESE (Boyle, 2009; Reifsnnyder et al., 2012; Hussein et al., 2012). An elegantly designed multi-center, prospective, randomized controlled trial demonstrated significant benefit, with improved rates of sperm retrieval after endocrine stimulation compared to non-stimulated controls with azoospermia due to spermatogenic dysfunction (Hussein et al., 2012). In this study, 612 patients were randomized to either a control, in which a microTESE was performed without any endocrine manipulation, or to an intervention cohort. Intervention included clomiphene citrate administration, with measured response guiding further interventions. Interventions included dose escalation of CC, addition of HCG, cessation of CC, and initiation of hMG in conjunction with HCG. All interventions were directed to a goal of a 50% increase of baseline FSH and serum testosterone levels of 600–800 ng/dL. In the intervention cohort, 10% of patients developed sperm in their ejaculate, with significantly improved sperm retrieval rates of 57% compared to 34% in the control patients (depending on regimen $P < 0.001$ – 0.05) (Hussein et al., 2012). Conversely, in the largest study supporting no benefit, the target testosterone was significantly lower, aiming for testosterone levels > 300 ng/dL (Reifsnnyder et al., 2012). Endocrine stimulation is well tolerated in general. Hence, the decision to proceed with endocrine stimulation prior to microTESE should include consideration of risks, benefits, costs, timing, and maternal age. However, in patients with diagnoses that are associated with irreversible infertility, such as certain chromosomal microdeletions, such therapy, as well as pursuing microTESE itself, would be inappropriate (Boyle, 2009).

Summary

Azoospermia due to spermatogenic dysfunction is a common cause of male factor infertility in patients presenting for an infertility evaluation. Studies have shown that with endocrine stimulatory therapy, there is an increased chance for successful sperm retrieval surgery. Endocrine stimulatory acts by raising intratesticular levels of testosterone produced by Leydig cells, improving the testicular histological environment and stimulating supportive Sertoli cells with FSH. Clomiphene citrate is frequently a first line, albeit off label, drug used to prompt testicular response, as well as HCG, anastrozole, and hMG. Sperm retrieval rates are improved in patients undergoing endocrine stimulatory therapy as compared to patients electing to proceed directly to surgical extraction, and some patients may even get sperm in the ejaculate. It is important to remember, however, that endocrine stimulatory therapy can take some time until titrated adequately, and, therefore, the treatment plan must take into consideration all factors including partners age and fecundity.

References

- Bedford, J. M., Calvin, H. I., & Cooper, G. W. (1973). The maturation of spermatozoa in the human epididymis. *Journal of Reproduction and Fertility*, 18, 199–213.
- Boyle, K. (2009). Nonsurgical treatment of male infertility. In L. I. Lipshultz, S. Howards, & C. Neiderberger (Eds.), *Infertility in the male* (pp. 438–453). Cambridge: Cambridge University Press.
- Caroppo, E. (2009). Male hypothalamic-pituitary-gonadal axis. In Lipshultz LI, S. Howards, Craig S. Niederberger. *Infertility in the Male* (4th edn, pp. 13–28). Cambridge: Cambridge University Press.
- Chehab, M., Medala, A., & Trussel, J. C. (2015). On-label and off-label drugs used in the treatment of male infertility. *Fertility and Sterility*, 103, 595–604.
- Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiology Review*, 198–236.
- Cocuzza, M., Alvarenga, C., & Pagani, R. (2013). The epidemiology and etiology of azoospermia. *Clinics (Sao Paulo)*, (Suppl 1), 15–26.
- Corea, M., Campagnone, J., & Sigman, M. (2005). The diagnosis of azoospermia depends on the force of centrifugation. *Fertility and Sterility*, 83, 920–922.
- Dabaja, A., & Schlegel, P. N. (2014). Medical treatment of male infertility. *Translational Andrology and Urology*, 3(1), 9–16.
- Esteves, S. C., Miyaoka, R., & Agarwal, A. (2011). Sperm retrieval techniques for assisted reproduction. *International Brazilian Journal of Urology*, 37, 570.
- Helo, S. E., Mechlin, J., et al. (2015). A randomized prospective double-blind comparison trial of clomiphene citrate and Anastrozole in raising testosterone in hypogonadal infertile men. *The Journal of Sexual Medicine*, 12(8), 1761–1769.
- Hill, S., Arutchelvam, V., & Quinton, R. (2009). Enclomiphene, an estrogen receptor antagonist for the treatment of testosterone deficiency in men. *Investigational Drugs*, 12(2), 109–119.
- Hussein, A., Ozgok, Y., Ross, L., & Niederberger, C. (2005). Clomiphene administration for cases of nonobstructive azoospermia: A multicenter study. *Journal of Andrology*, 26(6), 787–791.
- Hussein, A., Ozgok, Y., Ross, L., Rao, P., & Niederberger, C. (2012). Optimization of spermatogenesis-regulating hormones in patients with non-obstructive azoospermia and its impact on sperm retrieval: A multicentre study. *BJU International*, E110–E114.
- Jarow, J. P., & Zirkin, B. R. (2005). The androgen microenvironment of the human testis and hormonal control of spermatogenesis. *Annals of the New York Academy of Sciences*, 208–220.
- Oates, R. D., & Lamb, D. J. (2009). (2009) genetic aspects of infertility. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn, pp. 251–276). New York: Cambridge University Press.
- Oka, S., Shiraishi, K., & Matsuyama, H. (2017). Effects of human chorionic gonadotropin on testicular interstitial tissues in men with non-obstructive azoospermia. *Andrology*, 232–239.
- Reifsnnyder, J. E., Ramasamy, R., Husseini, J., & Schlegel, P. N. (2012). Role of optimizing testosterone before microdissection. *The Journal of Urology*, 532–537.
- Ring J, Welliver C, Parenteau M, et al (2017) The utility of sex hormone-binding globulin in hypogonadism and infertile males. *The Journal of Urology*, 1326–1331. <http://www.sciencedirect.com/science/article/pii/S0022534717300>.
- Rodriguez, K. M., Pastuszak, A. W., & Lipshultz, L. I. (2016). Enclomiphene citrate for the treatment of secondary male hypogonadism. *Expert Opinion on Pharmacotherapy*, 17(11), 1561–1567.
- Schlegel, P. N. (1999). Testicular sperm extraction: Microdissection improves sperm yield with minimal tissue excision. *Human Reproduction*, 14(1), 131–135.

- Schlegel, P. N. (2012). Aromatase inhibitors for male infertility. *Fertility and Sterility*, 98, 1359–1362.
- Schoor, R. A., Elhanbly, S., Niederberger, C. S., & Ross, L. S. (2002). The role of testicular biopsy in the modern management of male infertility. *The Journal of Urology*, 197–200.
- Setchell, B. P., & Brooks, D. E. (1988). Anatomy, vasculature, innervation and fluids of the male reproductive tract. In E. Knobil, & J. D. Neill (Eds.), *The physiology of reproduction* (pp. 753–836). New York: Raven Press.
- Turek, P. (2016). Male reproductive physiology. In A. Wein (Ed.), *Campbell-Walsh urology* (11th edn, pp. 516–537e.5). Philadelphia: Elsevier.
- Wenker, E. P., Dupree, J. M., Langille, G. M., et al. (2015). The use of HCG-based combination therapy for recovery of spermatogenesis after testosterone use. *The Journal of Sexual Medicine*, 12, 1334–1337.
- Willems, A. E., Corbo, J. M., & Brown, J. N. (2013). Clomiphene for the treatment of male infertility. *Reproductive Sciences*, 20, 739–744.

Further Reading

- Chua, M. E., Escusa, K. G., Luna, S., et al. (2013). Revisiting oestrogen antagonists (clomiphene or tamoxifen) as medical empiric therapy for idiopathic male infertility: A meta-analysis. *Andrology*, 1, 749–757.
- Niederberger, C. (2016). Male infertility. In A. Wein (Ed.), *Campbell-Walsh urology* (11th edn, pp. 556–579e.10). Philadelphia: Elsevier.

Medical Non-Endocrine-Targeted Therapies: Ejaculatory Dysfunction and Immunotherapy

Dane P Johnson, Washington University School of Medicine, St Louis, MO, United States

Jay I Sandlow, Medical College of Wisconsin, Milwaukee, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Ejaculation Disorders

Ejaculation Physiology, Anatomy and Dysfunction

Ejaculation is a complex process comprised of two phases: emission followed by expulsion. Following emission, during which time semen collects in the prostatic urethra, the bulbar urethra rapidly distends. Distension of the bulbar urethra triggers the expulsive phase of ejaculation, at which time the semen is rapidly propelled along the penile urethra by contraction of the bulbocavernosus muscle (Puppo & Puppo, 2016). Closure of the internal urethral sphincter is crucial to antegrade ejaculation, preventing retrograde flow of semen into the bladder.

The complex process of emission and expulsion is coordinated by sympathetic efferent fibers (T10-L3) (Jefferys et al., 2012). Spinal cord injuries, as well as peripheral neuropathies, including iatrogenic injury during retroperitoneal and pelvic surgery, can result in partial or complete bladder neck dysfunction (Goldwasser et al., 1983). Once bladder neck dysfunction occurs and retrograde ejaculation (RE) is noted, medical treatment can be offered to return antegrade flow during expulsion. Other patients may suffer from delayed or complete absence of ejaculation, while the last group of patients may have premature ejaculation (PE).

Medical Therapy of Retrograde Ejaculation

Medical treatment for RE is based on increasing bladder neck contractility, either through increasing sympathetic tone or decreasing parasympathetic activity. Tricyclic antidepressants, such as imipramine, along with alpha agonists, such as pseudoephedrine, have been found to increase the pressure profile in the posterior urethra (Goldwasser et al., 1983), successfully increasing antegrade ejaculation. Imipramine and pseudoephedrine are two medications commonly utilized for treatment of RE.

Pseudoephedrine is an α -adrenergic agonist that is readily absorbed from the gastrointestinal tract with a short onset of action and has a half-life of about 5–8 h (Kanfer et al., 1993). The maximum recommended dose is 240 mg each day. Sympathomimetic adverse events include various degrees of dizziness, sleep disturbances, weakness, restlessness, dry mouth, nausea, and sweating, and these can occur frequently in patients who respond to therapy (Kamischke & Nieschlag, 1999).

Imipramine is a tricyclic antidepressant whose response is mediated through both anticholinergic and slight alpha agonist effects. Dosing is typically 25–50 mg for 7 days prior to planned ejaculation or ovulation of partner. Side effects include dizziness, weakness, nausea or excessive sweating (Ochsenkühn et al., 1999).

The overall efficacy of these medications is variable, and studies evaluating their effect are limited to small non-randomized case studies. Overall, the efficacy for return of antegrade ejaculation is around 30% for pseudoephedrine and 35% for imipramine (see table for literature on success rates).

Paper	Drug	Sample size	AE achieved	% AE Achieved
Gilja et al. (1994)	Ephedrine sulfate	17	3	17.6
	Imipramine hydrochloride	14	2	14.3
Narayan et al. (1982)	Ephedrine sulfate	6	1	16.7
	Pseudoephedrine hydrochloride	7	1	14.3
	Imipramine hydrochloride	5	0	0
Okada et al. (1998)	Imipramine hydrochloride	6	3	50
Yuanhui. (2002)	Imipramine hydrochloride	17	5	29.4
Ochsenkühn et al. (1999)	Imipramine hydrochloride	11	11	100
Arafa & El Tabie, (2008)	Imipramine hydrochloride	33	10	38.5
	Pseudoephedrine hydrochloride	33	11	47.8
	Combined	33	16	61.5
Safarinejad, (2009)	Midodrine	128	18	29.5

Many prefer treatment of retrograde ejaculation with oral pharmaceuticals described above, as it allows the ability for spontaneous pregnancy. However, when antegrade ejaculation cannot be pharmacologically induced, bladder alkalization and sperm retrieval from postejaculatory urine is also an option.

Successful recovery of spermatozoa from postejaculatory urine requires careful regulation of both pH and osmolality of urine, and subsequent separation of motile sperm from bladder debris and other cells (Braude et al., 1987). Prior to ejaculation, the urine must be alkalized, which can be accomplished with either oral sodium bicarbonate or intravesicle instillation of Ringer's lactate

(LR) solution (Hotchkiss et al., 1954). To obtain retrograde ejaculated sperm without bladder catheterization, patients are instructed to drink solution containing 3 g/L sodium bicarbonate prior to ejaculation. Prior to ejaculation, patients must empty bladder and verify urinary pH has been neutralized. Following ejaculation, urine must be voided and collected within the hour (Jimenez et al., 1997). For urinary sperm retrieval using bladder irrigation, the bladder is first emptied and washed with LR solution via catheterization, and then a small volume (~30 mL) of the solution is instilled into the bladder. The catheter is removed, prior to ejaculation, and spermatozoa are obtained either by voiding or catheterization (Philippon et al., 2015).

Once the postejaculatory urine sample is obtained, it is centrifuged, and re-suspended in sperm washing buffer for use in IUI or ICSI only (sperm retrieved with this technique cannot be used to achieve spontaneous pregnancy). There are no studies examining the effectiveness of this approach to managing retrograde ejaculation (Mehta & Sigman, 2015), although pregnancy rates with the use of retrieved sperm for UI vary from 20% to 50% (Braude et al., 1987; Silva et al., 2000; Nikolettos et al., 1999; van der Linden et al., 1992; Shangold et al., 1990).

Medical Therapy of Delayed Ejaculation

Drug treatment of DE ejaculation has met with limited success. Many medications have been proposed as treatment strategies for delayed ejaculation, including cabergoline, amantadine, and even testosterone (Abdel-Hamid et al., 2016). These drugs can facilitate ejaculation by either a central dopaminergic (cabergoline), anti-serotonergic (bupropion), or oxytocinergic (oxytocin) mechanism of action or a peripheral adrenergic mechanism of action (midodrine) (Abdel-Hamid et al., 2016; Hsieh et al., 2012; Modell et al., 1997; Ishak et al., 2008). However, the evidence supporting pharmacological treatment of delayed ejaculation is relatively weak and limited to case reports, small case series and small trials (Abdel-Hamid et al., 2016). Currently, no drugs have been approved by regulatory agencies for this purpose and most drugs that have been identified for potential use have limited efficacy, impart significant side effects, or are as yet considered experimental in nature (McMahon, 2016).

Medical Therapy of Premature Ejaculation

Premature ejaculation (PE), is one of the most common male sexual disorders: According to the National Health and Social Life Survey, PE was reported to have a prevalence of 21% in men aged 18 to 59 in the United States (Laumann et al., 1999). In addition to sexual dissatisfaction, severe premature ejaculation that prevents successful coitus will likely impact a couple's ability to conceive naturally.

The exact etiology of PE is unknown and treatment is based on psychological, behavioral and pharmacological interventions. Behavioral and psychological therapy are often the first lines of treatment for patients presenting with PE. Psychotherapy includes both sexual therapy and relationship counseling, with the aim of addressing psychological and interpersonal issues that may be contributing to PE. Behavioral therapy involves physical techniques that have been developed to help men delay ejaculation. The "stop-start" technique involves the patient or his partner stimulating the penis until he feels the urge to ejaculate, then stopping until the sensation passes. Another behavioral therapy technique commonly employed involves the "squeeze" technique, where the patient squeezes the glans penis at the moment he feels the urge to ejaculate, until this sensation passes (Melnik et al., 2011). Randomized controlled trials evaluating these behavioral modifications demonstrated that these techniques can improve insertion-to ejaculation time and sexual satisfaction compared to controls. However, these studies have found that the addition of pharmacological therapies combined with behavioral therapies were better than either intervention alone (Cooper et al., 2015).

The serotonergic system is felt to act as a suppressor of ejaculation at the level of the hypothalamus (Porst, 2011) based on the anti-ejaculatory side effect of serotonin reuptake inhibitors (SSRIs) and other drugs that increase central activity of serotonin (Foreman et al., 1989). One of the most commonly associated side effect of SSRIs are delayed ejaculation and absent or delayed orgasm (Jannini et al., 2015), and these known side effects have been utilized in the off-label treatment of PE, in either on-demand doses or longer acting chronic courses.

Dapoxetine is a short acting SSRI developed specifically for the treatment of premature ejaculation (Dresser et al., 2006). In contrast to longer-acting SSRIs which can take 2 weeks or longer to reach steady state concentration (Hiemke & Härtter, 2000), dapoxetine has both a shorter time to onset and rapid elimination, with concentrations declining to <5% of peak value by 24 h (Modi et al., 2006). Further investigations have revealed dapoxetine to be a safe and effective treatment of PE (Porst et al., 2010), with mild side effects such as dizziness, nausea or diarrhea in 4–10% of patients (McMahon et al., 2011). Furthermore, men taking dapoxetine for PE have demonstrated no evidence of increased suicidal ideation or attempts, a side effect reported with other longer acting SSRI's (McMahon et al., 2011). With these attributes, dapoxetine has been approved and utilized for the treatment of PE in 50 countries worldwide (Pryor et al., 2006).

Longer-acting SSRIs such as paroxetine, fluoxetine, and sertraline, as well as the tricyclic antidepressant clonidine, may be utilized in the treatment of men with medication-refractory PE. Paroxetine exerts the strongest delay in ejaculation (Waldinger et al., 2004) when utilized in a daily dosing regimen. This regimen requires use 5–10 days prior to onset of effect, and an additional 2–3 weeks prior to full effect (McMahon et al., 2004). Similar to dapoxetine, these other SSRIs have been found to be safe and effective treatment for delaying ejaculation (Montague et al., 2004). Nausea, dry mouth, drowsiness and decreased libido are some common side effects of SSRI treatment, and long term use may be associated with weight gain and increased risk of developing Type 2 diabetes (Fava et al., 2000). Lastly, while the risk for suicidal ideation or suicide attempts has not been found to be increased

in non-depressed men on SSRIs for PE, there is a small increase in this risk in youths with depression on SSRIs (Khan et al., 2003). Therefore, caution is recommended in prescribing SSRIs to young adolescents with PE aged 18 or less.

Topical anesthetics can be applied to the penis prior to beginning intercourse, and can be applied with a condom or without. Residual topical anesthetics can also cause vaginal numbness, so men should be counseled to either wash clean any residual active topical agent or wear a condom during intercourse. Lidocaine/prilocaine cream (2.5 g) applied for 20 to 30 min prior to intercourse has been shown to increase latency time (Montague et al., 2004), with minimal side effects.

Immunotherapy Targeting Male Factor

Anti-sperm antibody (ASAB)-mediated male infertility is a rare cause of infertility, reported in 6–11% of infertility cases (McLachlan, 2002). However, when present, it is a potentially treatable cause. ASABs are felt to be formed in the epididymis, often in the presence of downstream obstruction (eg., vasectomy), infection/inflammation, or trauma. The IgG isotype is felt to be the most common, and important, of the isotypes seen. It appears that these Ab exert their effect in a variety of ways. Most think that they cause agglutination (sperm sticking together), thus inhibiting progressive forward motility, as well as clumping of the sperm in the cervical mucus. Others have reported reduced sperm viability due to cytotoxic ASABs, although this has been difficult to ascertain in vivo. Finally, impairment of the sperm-egg interaction has been demonstrated, with altered binding of the sperm to the zona pellucida. This has been demonstrated in conventional IVF. While there was also a theory of post-fertilization effects of ASABs, this has been disproven, as ICSI has been shown to be an effective treatment for ASABs, with no adverse effects on the fertilization rate or embryo development.

Diagnosis of ASABs is somewhat difficult, as they have been demonstrated in some fertile men. There are 2 methods for detection, direct and indirect. Direct methods identify ASABs on the sperm using anti-IgG antibodies. Indirect methods detect ASABs in body fluids (cervical fluid, blood, semen). The 2 most popular methods for direct testing are the immunobead test (IBT) and the mixed agglutination reaction (MAR). The strengths of the IBT are that the type of Ig, the percentage of sperm with beads attached and their location (head, mid-piece, tail) can be quantified. Reagent cost, time and non-specific binding are difficulties. Location of binding is important as well, as tail tip binding is of much less clinical significance than head or body binding. The MAR test methods uses latex beads or red blood cells labeled with human IgG A, M or G which are co-incubated with viable sperm following which anti-human Ig is added to cause agglutination with the sperm. Indirect testing is typically performed on the fluid in question using serologic tests to detect and quantify specific Ig isotypes.

The management of ASABs was previously difficult, as many treatments were empiric and anecdotal. However, with the advent of ICSI, most clinicians utilize this to overcome significant ASABs. There are situations; however, when couples are not open to IVF and wish to try medical therapy. There are 2 concepts used in the medical treatment of ASABs, either removal of the ASABs or suppression of their formation. The first method, removal, is rarely successful. ASABs bind to sperm prior to ejaculation; thus, attempts to wash them off are typically unsuccessful (they are bound by a nearly unbreakable covalent bond). Studies have explored prevention of ASAB formation through the use of immunosuppressive therapy. Most of these treatments utilize glucocorticoids, such as prednisone, in doses high enough to suppress the immune system, and are taken either cyclically or for several months. Unfortunately, the high rate of adverse events, particularly aseptic necrosis of the hip (reported in 1:150 cases), has limited the use of this treatment. One randomized study examined the use of immunosuppressive therapy with and without IUI and found the cumulative pregnancy rate with IUI to be 39% vs only 4% with timed intercourse. There were minimal side effects in this trial which used 40 mg of prednisone/day for 2 weeks, followed by 20 mg/day for 2 weeks (Robinson et al., 1995). While this report was promising, none of the couples had attempted IUI previously, thus bringing the impact of the steroid treatment into question. Thus, the utilization of ICSI has become the standard treatment for immunologic-mediated male infertility.

Conclusion

The non-endocrine medical treatment of male infertility has been hampered by the lack of randomized controlled trials for most of the conditions that it covers. Treatment of ejaculatory dysfunction is difficult and often results in the utilization of sperm retrieval with IVF. Similarly, the medical treatment of immunologic-mediated male fertility problems has not resulted in high success rates and ultimately results in the use of IVF/ICSI. While our understanding of many of the etiologies of male infertility is continually evolving, future medical therapy will likely be aimed at addressing the underlying causes.

References

- Abdel-Hamid, I. A., Elsaied, M. A., & Mostafa, T. (2016). The drug treatment of delayed ejaculation. *Translational Andrology and Urology*, 5(4), 576–591.
- Arafa, M., & El Table, O. (2008). Medical treatment of retrograde ejaculation in diabetic patients: A hope for spontaneous pregnancy. *The Journal of Sexual Medicine*, 5(1), 194–198. Epub 2007 Apr 13.
- Braude, P. R., Ross, L. D., Ockenden, K., et al. (1987). Retrograde ejaculation: A systematic approach to non-invasive recovery of spermatozoa from post-ejaculatory urine for artificial insemination. *British Journal of Obstetrics and Gynaecology*, 94(1), 76–83.
- Cooper, K., Martyn-St James, M., Cantrell, A., et al. (2015). Interventions to treat premature ejaculation: A systematic review short report. *Health Technology Assessment*, 19(21), 1–180. v-vi.

- Dresser, M. J., Desai, D., Modi, N. B., et al. (2006). Dapoxetine, a novel treatment for premature ejaculation, does not have pharmacokinetic interactions with phosphodiesterase-5 inhibitors. *International Journal of Impotence Research*, 18(1), 104–110.
- Fava, M., Judge, R., Koke, S. C., et al. (2000). Fluoxetine versus sertraline and paroxetine in major depressive disorder: Changes in weight with long-term treatment. *The Journal of Clinical Psychiatry*, 61(11), 863–867.
- Foreman, M. M., Hall, J. L., & Love, R. L. (1989). The role of the 5-HT₂ receptor in the regulation of sexual performance of male rats. *Life Sciences*, 45(14), 1263–1270.
- Gilja, I., Parazajder, J., Kovacic, M., et al. (1994). Retrograde ejaculation and loss of emission: Possibilities of conservative treatment. *European Urology*, 25(3), 226–228.
- Goldwasser, B., Madgar, I., Jonas, P., Lunenfeld, B., & Many, M. (1983). Imipramine for the treatment of sterility in patients following retroperitoneal lymph node dissection. *Andrologia*, 15 Spec No:588–591.
- Hiemke, C., & Härter, S. (2000). Pharmacokinetics of selective serotonin reuptake inhibitors. *Pharmacology & Therapeutics*, 85(1), 11–28.
- Hotchkiss, R. S., Pinto, A. B., & Kleegman, S. (1954). Artificial insemination with semen recovered from the bladder. *Fertility and Sterility*, 6(1), 37–42.
- Hsieh, T. C., Hollander, A. B., Walters, R. C., et al. (2012). Cabergoline for the treatment of male anorgasmia. *J Urol*, 187, e605 (Abstract) <https://doi.org/10.1016/j.juro.2012.02.2017>.
- Ishak, W. W., Berman, D. S., & Peters, A. (2008). Male anorgasmia treated with oxytocin. *The Journal of Sexual Medicine*, 5, 1022–1024, 00691.
- Jannini, E. A., Ciocca, G., Lenzi, A., et al. (2015). Premature ejaculation: Old story, new insights. *Fertility and Sterility*, 104(5), 1061–1073.
- Jefferys, A., Siassakos, D., & Wardle, P. (2012). The management of retrograde ejaculation: A systematic review and update. *Fertility and Sterility*, 97(2), 306–312. <https://doi.org/10.1016/j.fertnstert.2011.11.019>.
- Jimenez, C., Grizard, G., Boucher, D., et al. (1997 Sep). Birth after combination of cryopreservation of sperm recovered from urine and intracytoplasmic sperm injection in a case of complete retrograde ejaculation. *Fertility and Sterility*, 68(3), 542–544.
- Kamischke, A., & Nieschlag, E. (1999). Treatment of retrograde ejaculation and anejaculation. *Human Reproduction Update*, 5(5), 448–474.
- Kanfer, I., Dowse, R., & Vuma, V. (1993). Pharmacokinetics of oral decongestants. *Pharmacotherapy*, 13(6 Pt 2), 116S–128S.
- Khan, A., Khan, S., Brown, W. A., et al. (2003). Suicide rates in clinical trials of SSRIs, other antidepressants, and placebo: Analysis of FDA reports. *The American Journal of Psychiatry*, 160(4), 790–792.
- Laumann, E. O., Paik, A., & Rosen, R. C. (1999). Sexual dysfunction in the United States: Prevalence and predictors. *Journal of the American Medical Association*, 281(6), 537–544.
- McLachlan, R. I. (2002). Basis, diagnosis and treatment of immunological infertility in men. *Journal of Reproductive Immunology*, 57(1–2), 35–45.
- McMahon, C. G. (2016). Disorders of male orgasm and ejaculation. In A. J. Wein, L. R. Kavoussi, A. W. Partin, & C. A. Peters (Eds.), *Campbell-Walsh Urology* (11th ed). Philadelphia, PA: Elsevier. chap 29.
- McMahon, C. G., Abdo, C., Zin, Z. C., et al. (2004). Disorders of orgasm and ejaculation in men. *The Journal of Sexual Medicine*, 1(1), 58–65.
- McMahon, C. G., Althof, S. E., Porst, H., et al. (2011). Efficacy and safety of dapoxetine for the treatment of premature ejaculation: Integrated analysis of results from five phase 3 trials. *The Journal of Sexual Medicine*, 8(2), 524–539.
- Mehta, A., & Sigman, M. (2015). Management of the dry ejaculate: A systematic review of aspermia and retrograde ejaculation. *Fertility and Sterility*, 104(5), 1074–1081.
- Melnik, T., Althof, S., Riera, R., et al. (2011). Psychosocial interventions for premature ejaculation. *Cochrane Database of Systematic Reviews*, 8, CD008195.
- Modell, J. G., Katholi, C. R., Modell, J. D., et al. (1997). Comparative sexual side effects of bupropion, fluoxetine, paroxetine, and sertraline. *Clinical Pharmacology and Therapeutics*, 61, 476–487.
- Modi, N. B., Dresser, M. J., Gupta, S., et al. (2006). Single- and multiple-dose pharmacokinetics of dapoxetine hydrochloride, a novel agent for the treatment of premature ejaculation. *Journal of Clinical Pharmacology*, 46(3), 301–309.
- Montague, D. K., Jarow, J., Sharlip, I. D., et al. (2004). AUA guideline on the pharmacologic management of premature ejaculation. *The Journal of Urology*, 172(1), 290–294.
- Narayan, P., Lange, P. H., & Fraley, E. E. (1982). Ejaculation and fertility after extended retroperitoneal lymph node dissection for testicular cancer. *The Journal of Urology*, 127(4), 685–688.
- Nikolietos, N., Al-Hasani, S., Diedrich, K., et al. (1999). The outcome of intracytoplasmic sperm injection in patients with retrograde ejaculation. *Human Reproduction*, 14(9), 2293–2296.
- Ochsenkühn, R., Kamischke, A., & Nieschlag, E. (1999). Imipramine for successful treatment of retrograde ejaculation caused by retroperitoneal surgery. *International Journal of Andrology*, 22(3), 173–177.
- Okada, H., Fujioka, H., Kamidono, S., et al. (1998). Treatment of patients with retrograde ejaculation in the era of modern assisted reproduction technology. *J Urol*, 159(3), 848–850. Erratum in: *J Urol* 1998 May;159(5):1650.
- Philippon, M., Karsenty, G., Perrin, J., et al. (2015). Successful pregnancies and healthy live births using frozen-thawed sperm retrieved by a new modified Hotchkiss procedure in males with retrograde ejaculation: First case series. *Basic and Clinical Andrology*, 25, 5. <https://doi.org/10.1186/s12610-015-0021-4>.
- Porst, H. (2011). An overview of pharmacotherapy in premature ejaculation. *The Journal of Sexual Medicine*, 8(Suppl 4), 335–341.
- Porst, H., Vardi, Y., Wyllie, M., et al. (2010). Standards for clinical trials in male sexual dysfunctions. *The Journal of Sexual Medicine*, 7(1 Pt 2), 414–444.
- Pryor, J. L., Althof, S. E., Kell, S., et al. (2006). Efficacy and tolerability of dapoxetine in treatment of premature ejaculation: An integrated analysis of two double-blind, randomised controlled trials. *Lancet*, 368(9539), 929–937.
- Puppo, V., & Puppo, G. (2016). Comprehensive review of the anatomy and physiology of male ejaculation: Premature ejaculation is not a disease. *Clinical Anatomy*, 29(1), 111–119.
- Robinson, J. N., Forman, R. G., Barlow, D. H., et al. (1995). A comparison of intrauterine insemination in superovulated cycles to intercourse in couples where the male is receiving steroids for the treatment of autoimmune infertility. *Fertility and Sterility*, 63(6), 1260–1266.
- Safarinejad, M. R. (2009). Midodrine for the treatment of organic anejaculation but not spinal cord injury: A prospective randomized placebo-controlled double-blind clinical study. *International Journal of Impotence Research*, 21(4), 213–220. <https://doi.org/10.1038/ijir.2009.19>.
- Shangold, G. A., Cantor, B., & Schreiber, J. R. (1990). Treatment of infertility due to retrograde ejaculation: A simple, cost-effective method. *Fertility and Sterility*, 54(1), 175–177.
- Silva, P. D., Larson, K. M., Silva, D. E., et al. (2000). Successful treatment of retrograde ejaculation with sperm recovered from bladder washings. A report of two cases. *The Journal of Reproductive Medicine*, 45(11), 957–960.
- van der Linden, P. J., Nan, P. M., van Kooy, R. J., et al. (1992). Retrograde ejaculation: Successful treatment with artificial insemination. *Obstetrics and Gynecology*, 79(1), 126–128.
- Waldinger, M. D., Zwinderman, A. H., Olivier, B., et al. (2004). Relevance of methodological design for the interpretation of efficacy of drug treatment of premature ejaculation: A systematic review and meta-analysis. *International Journal of Impotence Research*, 16(4), 369–381.
- Yuanhui, X. (2002). Treatment of functional retrograde ejaculation with acupuncture and TCM herbal drugs. *Journal of Traditional Chinese Medicine*, 22, 286–287.

Empiric Infertility Treatment

G Luke Machen, Texas A&M Health Science Center College of Medicine, Bryan, TX, United States; and Baylor Scott & White, Temple, TX, United States

Stephanie E Harris, Baylor, Scott, & White Healthcare, Temple, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Asthenospermia Lower than normal sperm motility (<40% motile on analyses).

Azoospermia Semen abnormality in which no sperm is present in the ejaculate; to diagnose a patient as azoospermic, there must be no sperm in two separate semen analyses after centrifuged specimen is examined under a microscope.

Hypothalamus Portion of the brain that controls release of reproductive hormones. Releases gonadotropin-releasing hormone (GnRH) to stimulate the anterior pituitary gland to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH).

Idiopathic infertility Infertility in the absence of an identifiable cause.

Oligospermia Sperm concentration <15 million per mL.

Spermatogenesis Formation of sperm from germ cells located in the seminiferous tubule of the testicle.

Teratospermia Abnormal morphology of sperm (<4% sperm with abnormal form).

Introduction

Approximately 15% of couples are unable to conceive after 1 year of regular intercourse. It is estimated that the male at least contributes to the issue 50%–60% of the time, and is the sole cause in 20% of couples. Not infrequently, the male may have an abnormal semen analysis without an identifiable cause. In fact, in men with abnormal semen analyses parameters, the etiology is unknown around 25% of the time (Ko et al., 2012). In these instances, semen analyses will demonstrate decreased sperm counts, frequently associated with decreased motility and/or morphology. The ideal management of these individuals is not well defined (Kim and Schlegel, 2008). For the better part of the last century, varying medical treatments have been used off-label empirically despite the presence of mixed evidence supporting their use. A recent survey of 387 urologists found that approximately two thirds of providers utilized empiric medical treatment for idiopathic male infertility (Ko et al., 2012). The two main targets of modification include modification of the hypothalamic–pituitary–gonadal (HPG) axis and minimizing oxidative stress on spermatozoa. The objective of this chapter is to describe these treatments, the rationale for their use, and to summarize the evidence for and against them.

Hypothalamic–Pituitary–Gonadal Axis Modification

To adequately understand the rationale for the use of these empiric medications, it is necessary to briefly explain the control of reproductive hormones in the body, which are regulated via the HPG axis. Gonadotropin-releasing hormone (GnRH) is released in a pulsatile fashion from the hypothalamus and directly stimulates the anterior pituitary gland. In response, the pituitary gland releases luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which stimulates the production of testosterone and sperm in the testicle, respectively. Testosterone may be converted to estrogen, via a process catalyzed by aromatase. A negative feedback loop is in place, in which increased testosterone and thereby estrogen via aromatization, leads to a decrease in production of GnRH by the hypothalamus, and results in decreasing the release of LH and FSH (Kim and Schlegel, 2008). The empiric therapies discussed below involve manipulation of this cascade in some way.

Anti-estrogens

Perhaps the most prescribed and longest utilized forms of empiric therapy are the anti-estrogens, specifically clomiphene and tamoxifen. These medications are considered selective estrogen receptor modulators and function by blocking estrogen receptors, thereby inhibiting the negative feedback loop affecting production of GnRH by the hypothalamus. This serves to indirectly increase the secretion of LH and FSH by the anterior pituitary gland, with subsequent increases in testosterone and spermatogenesis. Unfortunately, the results of studies evaluating this effect in patients without hypogonadism have been mixed, with some demonstrating no benefit to treatment for idiopathic infertility and others having shown improved sperm counts and pregnancy rates (Kumar et al., 2006). Further, in a Cochrane database review of 10 studies analyzing around 740 patients on the effect of clomiphene and/or tamoxifen, there appeared to be a beneficial effect on testosterone, but there did not seem to be enough evidence to support their use for idiopathic infertility (Vandekerckhove et al., 2000). However, a more recent meta-analysis by Chua et al., which pooled

results from 11 randomized controlled trials employing either clomiphene or tamoxifen, demonstrated an odds ratio consistent with a 2.4 times greater chance of pregnancy, in addition to improvement in sperm concentrations and motility with anti-estrogens (Chua et al., 2013).

Use of anti-estrogens for idiopathic infertility remains off-label. Optimal dosing of these medications is not well established, however common doses administered are included in Table 1. Duration of therapy generally accounts for a spermatogenesis cycle of 74 days and studies suggest a large number of patients respond by 3 months, however some authors suggest treatment courses should not last longer than 12–15 months (Chebab et al., 2015). Clomiphene and tamoxifen are generally well tolerated by most patients. The most common side effects being gastrointestinal upset, dizziness, alopecia, gynecomastia, and weight gain. Additionally, it is important to note that some studies paradoxically demonstrate worsened sperm parameters with use, including decreased motility of sperm and worsened sperm counts. These improved with stoppage of the medication, although in a very small number the sperm counts remained decreased (Ramasamy et al., 2012).

Aromatase Inhibitors

Aromatase is an enzyme found in many locations in the body, including the testis and adipose tissue, which converts testosterone to estrogen. As mentioned previously, estrogen has an inhibitory effect on the hypothalamus in regards to production of GnRH via the negative feedback loop. Thus, by decreasing conversion of testosterone to estrogen, there should be an increase in androgen levels and spermatogenesis in the testis. This class of medications may be either steroidal or non-steroidal. The non-steroidal medications, including anastrozole and letrozole, tend to be more selective and therefore are more commonly used (Schiff et al., 2007). There is some evidence to suggest that the medication improves sperm parameters, however there is overall a dearth of high quality randomized controlled trials to support its use (Ramasamy et al., 2012). Intuitively, the medication would potentially be most effective in subfertile men with high estrogen levels.

As is the case with the anti-estrogens, aromatase inhibitor use is off-label for idiopathic infertility, and the ideal dosing regimen and duration is not well defined (Table 1). Frequently, the choice of an aromatase inhibitor versus an anti-estrogen is based off the ratio of testosterone to estrogen (T:E) in a patient. Specifically, aromatase inhibitors would be considered as therapeutic options in patients with higher estrogen levels and a T:E ratio of < 10 (Barazani et al., 2016). Side effects are infrequent, but can consist of transient elevations of liver enzymes and changes in libido. Long term, there may be a risk of osteopenia secondary to estrogen deficiency (Schiff et al., 2007).

Androgens

Androgens are known to play a central role in male sexual function and infertility, having demonstrated that testosterone encourages spermatogenesis. Furthermore, 20%–30% of male infertility patients are estimated to have low levels of testosterone (Ring et al., 2016). Perhaps intuitively, one could then infer that administration of exogenous testosterone could benefit semen parameters. However, due to the aforementioned negative feedback loop, exogenous testosterone actually serves to suppress the levels of intra-testicular testosterone, leading to azoospermia (Kim and Schlegel, 2008). Some investigators noticed that there seemed to be an increase in sperm counts after cessation of androgens, leading to theories that there may be some therapeutic utility of testosterone utilizing this “rebound effect” to treat male infertility. However, in meta-analysis and randomized controlled trials, this effect was not born out (Kumar et al., 2006). Thus, there is no role of androgen monotherapy in male infertility. Despite this, in a recent survey, around 25% of urologists reported prescribing testosterone for male infertility treatment, highlighting the need for increased education regarding empiric therapies for both patients and providers alike (Ko et al., 2012).

Table 1 Medications and commonly used dosages

Class	Medication	Dosage	Notes
Anti-estrogens	Clomiphene citrate	25 or 50 mg PO daily	Dose ranges used variable, reported from 12.5 to 400 mg. Intervals also vary and can include every other day dosing along with 25 day cycle followed by a 5 day break.
	Tamoxifen citrate	10–30 mg PO daily	Some controlled studies showed no effect with 10 and 20 mg doses
Aromatase inhibitors	Anastrozole	1 mg PO daily	Ideal frequency of administration unclear; ranges from weekly to daily
	Letrozole	2.5 mg PO daily	
Gonadotropins	hCG	1500 u SQ/IM 3 × per week	Can increase to 3500 u 3 × per week
	hMG	75 u IM 3 × per week	Titrate to 150 u if do not see response in sperm counts
	Recombinant human FSH (r-h FSH)	150 IU SQ two to three times per week	Can increase by 75 IU if no sperm present after 6 months

Gonadotropins

As described above, FSH and LH secreted by the anterior pituitary gland stimulate sertoli cells and leydig cells, respectively to produce sperm and testosterone. Thus it is postulated that abnormalities in gonadotropin secretion could contribute to male infertility. In fact, gonadotropins are the only FDA approved medication for male infertility, although specifically in situations of low FSH/LH and testosterone, known as hypogonadotropic hypogonadism (Chebab et al., 2015). Various analogues of exogenous FSH, LH, and pulsed GnRH exist. Common regimens are listed in Table 1. Typically, hCG (human chorionic gonadotropin, an LH analogue) monotherapy is the first line of treatment as this is more cost effective. hCG can lead to a dramatic increase of intra-testicular testosterone and therefore may be the only medication necessary to stimulate sperm production. If ineffective, analogues of FSH, either human menopausal gonadotropin (hMG) or recombinant human FSH (rh-FSH) can be used in addition to hCG (Chebab et al., 2015). hMG contains a mixture of LH and FSH purified from human urine and therefore has a theoretical concern about infection. The advantage of this medication is cost, as the recombinant product, which contains pure FSH, is significantly more expensive. In 2005, Foresta et al. analyzed the use of rh-FSH for 3 months on men with idiopathic oligoasthenozoospermia and in a sub group analysis found a benefit to sperm parameters and unassisted pregnancy rates (Foresta et al., 2005). Additionally, in 2013, a Cochrane review of six randomized controlled trials including around 450 patients seemed to suggest a benefit in live births and spontaneous pregnancy rates, although concluded that, while encouraging, more evidence was ultimately necessary (Attia et al., 2013).

Gonadotropin Releasing Hormone Therapy

Studies investigating the pregnancy rates in couples with men treated with pulsatile GnRH (5–20 mg per pulse every 2 h) for idiopathic oligoasthenoterazoospermia failed to demonstrate improvement over controls and given the high cost of this therapy it is seldom used clinically (Cocuzza and Agarwal, 2007).

Antioxidant Therapy

Reactive oxygen species (ROS), have long been a postulated target mechanism of male infertility and given the large amount of polyunsaturated fat in their cell membrane, spermatozoa are especially susceptible to damage via ROS as they have low levels of intracellular antioxidants and DNA repair enzymes. Therefore it is the antioxidant capacity of the semen that has been thought to be the protectant with antioxidant carotenoids, carnitine, folate, selenium, vitamin E, vitamin C, and zinc all being found in semen. There is evidence that infertile men have higher levels of ROS in their semen (Showell et al., 2014). These observations have led to interest in antioxidant treatment for male infertility. There are several studies that support the idea that antioxidants may improve the DNA integrity and function of sperm, although the exact mechanism of action and ideal regimen of supplementation is not well defined (Ramasamy et al., 2012). A Cochrane Review by Showell in 2014 concluded that with low quality evidence, antioxidants might have been effective in treating men with subfertility, as pregnancy rate and live birth rate were both improved but called for better evidence. What follows is a brief description of available supplements and the evidence supporting their use. In general, although further high quality studies are necessary, the side effects of these supplements are minimal, if any, and antioxidants are commonly used as an adjunct for empiric medical treatment of infertile males (Schiff et al., 2007).

Vitamin C

Vitamin C (or ascorbic acid) is a water-soluble antioxidant found in high concentrations in seminal plasma. It is postulated that it may limit oxidative DNA damage by neutralizing free radicals and protecting cell membrane degradation against ROS. Some studies have found seminal concentrations of vitamin C to correlate positively with sperm concentration and negatively with ROS. Further, vitamin C supplementation may decrease the level of DNA damage in sperm, although this has not definitively been shown to lead to improved semen parameters or pregnancy rates (Ahmadi et al., 2016; Agarwal and Sekhon, 2010).

Vitamin E

Vitamin E (α -tocopherol) is a lipophilic antioxidant usually found in cell membranes that is believed to inhibit ROS while enhancing the activity of other antioxidants. In fact, some randomized controlled trials did suggest a benefit in infertile males with high ROS levels, although others have found no improvement in semen parameters (Ahmadi et al., 2016; Agarwal and Sekhon, 2010).

Carnitine

Mostly obtained through diet, carnitine has been found to fend off ROS induced damage to cell membranes and DNA in sperm. In addition to its antioxidant properties, it is believed that carnitine plays a role in sperm maturation via enhancement of energy production, and therefore motility (Kumar et al., 2006). The highest concentrations of the compound exist in the epididymis.

Data exists to support the idea carnitine supplementation may improve semen motility, particularly in men with lower sperm motility at baseline. Some other studies have suggested that it may improve concentrations as well, although once again results have been somewhat mixed (Ahmadi et al., 2016).

Coenzyme Q10 (CoQ10)

CoQ10 functions in cellular respiration and energy generation. While its exact role in the treatment of male infertility is not entirely clear, it is postulated that it may lead to greater energy for mitochondria in sperm resulting in increased motility, and may scavenge free radicals and inhibit the formation of hydroperoxide, a ROS. Most studies on CoQ10 do show an improvement in semen parameters, although this did not necessarily lead to increased pregnancy or live birth rates (Ahmadi et al., 2016).

Zinc

The second most common metal in the body after iron, zinc has been shown to protect sperm from bacteria and chromosomal damage, as well as play a role in testicular development and maturation of sperm. For example, deficiency of zinc is associated with hypogonadism and incomplete sexual development in humans. There are several controlled and blinded studies that show an increase in sperm counts with zinc supplementation (Ahmadi et al., 2016).

Selenium

Selenium is necessary for development of the testicles and may protect against DNA damage in sperm, although the exact mechanism is unclear, although it is believed they play a role in maintain the structure of sperm. In fact, selenium deficiency may lead to worsening sperm morphology and motility. Some data suggests selenium, when combined with other supplements, may lead to improved motility and other parameters (Agarwal and Sekhon, 2010).

Glutathione/*N*-Acetyl Cysteine

Glutathione, as the most common non-protein thiol in the body, serves to scavenge ROS in sperm. Fertile men have been found to have more glutathione in their semen, and some data suggests worsening motility and morphology of sperm with low levels of glutathione. Its precursor, *N*-acetyl-cysteine (NAC), may be used as a supplement to augment the production of glutathione, and may have antioxidant properties itself. One study did find improved sperm concentration when NAC was given with selenium (Agarwal and Sekhon, 2010; Kumar et al., 2006).

Conclusion

As mentioned above, in a not insignificant cohort of men diagnosed with male factor infertility, no medically or surgically correctable etiology is identified. In these cases, it is reasonable to consider empiric medical treatment. The aims of this therapy are either to stimulate the HPA axis or prevent free radical damage to spermatozoa. These medications are generally well tolerated, and have been used for nearly half a century by many providers. It is important to note once again that Food and Drug Administration approval does not exist for the specific indication of male infertility for many of the medications. Further, despite a lack of large, high quality studies supporting their efficacy, empiric medical treatment is generally considered safe and reasonably priced means of improving ones odds of conception. As previously mentioned, the ideal patient to benefit from empiric treatment is not well defined. However, a recent study found that a nomogram based on body mass index, age, LH, as well as change in serum testosterone after initiating treatment could be predictive of a positive response to sperm counts using medical therapy (Barazani et al., 2016).

In conclusion, while further studies are necessary, empiric treatment is commonly used and may be beneficial to some men with idiopathic infertility. Patients should have informed discussions with their providers regarding the potential benefits and risks of the medications prior to use as well as discussions regarding proceeding with other forms of assisted reproductive techniques when no pregnancy is seen after reasonable trial period.

References

- Agarwal, A., & Sekhon, L. H. (2010). The role of antioxidant therapy in the treatment of male infertility. *Human Fertility (Cambridge)*, 13(4), 217–225.
- Ahmadi, S., Bashiri, R., Ghadiri-Anari, A., & Nadjarzadeh, A. (2016). Antioxidant supplements and semen parameters: an evidence based review. *International Journal of Reproductive Biomedicine*, 14(12), 729–736.
- Attia, A. M., Abou-Setta, A. M., & Al-Inany, H. G. (2013). Gonadotrophins for idiopathic male factor subfertility. *Cochrane Database of Systemic Reviews*, (8), CD005071. <https://doi.org/10.1002/14651858.CD005071.pub4>.
- Barazani, Y., Polackwich, A. S., & Sabanegh, E. S. (2016). A novel nomogram for prediction of spermatogenic improvement following empiric medical therapy for moderate-severe oligospermia. *Canadian Journal of Urology*, 23(1), 8135–8140.
- Chebab, M., Madala, A., & Trussell, J. C. (2015). On-label and off-label drugs used in the treatment of male infertility. *Fertility and Sterility*, 103, 595–604.

- Cocuzza, M., & Agarwal, A. (2007). Nonsurgical treatment of male infertility: specific and empiric therapy. *Biologics*, 1(3), 259–269.
- Chua, M. E., Escusa, K. G., Luna, S., Tapia, L. C., Dofitas, B., & Morales, M. (2013). Revisiting oestrogen antagonists (clomiphene or tamoxifen) as medical empiric therapy for idiopathic male infertility: a meta-analysis. *Andrology*, 1, 749–757.
- Foresta, C., Bettella, A., Garolla, A., Ambrosini, G., & Ferlin, A. (2005). Treatment of male idiopathic infertility with recombinant human follicle-stimulating hormone: a prospective, controlled, randomized clinical study. *Fertility and Sterility*, 84, 654–661.
- Kim, H. K., & Schlegel, P. N. (2008). Endocrine manipulation in male infertility. *Urologic Clinics of North America*, 303–318.
- Ko, E. Y., Siddiqi, K., Brannigan, R. E., & Sabanegh, E. S. (2012). Empirical medical therapy for idiopathic male infertility: a survey of the American Urological Association. *Journal of Urology*, 187, 973–978.
- Kumar, R., Gautam, G., & Gupta, N. P. (2006). Drug therapy for idiopathic male infertility: rationale versus evidence. *Journal of Urology*, 176, 1307–1312.
- Ramasamy, R., Stahl, P. J., & Schlegel, P. N. (2012). Medical therapy for spermatogenic failure. *Asian Journal of Andrology*, 14, 57–60.
- Ring, J. D., Lwin, A. A., & Kohler, T. S. (2016). Current medical management of endocrine-related male infertility. *Asian Journal of Andrology*, 18, 357–363.
- Schiff, J. D., Ramirez, M. L., & Bar-Chama, N. (2007). Medical and surgical management male infertility. *Endocrinology and Metabolism Clinics of North America*, 36, 313–331.
- Showell, M. G., Mackenzie-Proctor, R., Brown, J., et al. (2014). Antioxidants for male subfertility. *Cochrane Database of Systematic Reviews*, (12), CD007411.
- Vandekerckhove, P., Lilford, R., Vail, A., & Hughes, E. (2000). Clomiphene or Tamoxifen for idiopathic oligo/asthenospermia. *Cochrane Database of Systematic Reviews*, (2), CD000151.

Nutraceutical Therapy in Male Infertility

Pratik Kanabur and Ranjith Ramasamy, University of Miami, Miami, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Background

Overview

Global rates of male infertility range from 2.5% to 12%. Males are found to be solely responsible for 20%–30% of infertility cases and contribute to 50% of cases overall (Jungwirth et al., 2012). The management of male infertility includes assessing and identifying a patient's potential health problems. Decreased general health status has been associated with lower sperm concentration, lower total testosterone levels and higher follicle-stimulating hormone values. Specifically, nutrition and lifestyle factors, which are currently huge public health issues, play a critical role in the normal function of the reproductive system. Aside from evident factors such as smoking (Saleh et al., 2002) and heavy alcohol consumption (Condorelli et al., 2015), male obesity and high fat diets (Taha et al., 2016) have also been implicated in male infertility. One of the leading hypotheses for pathophysiological explanation for idiopathic male infertility is damage to the reproductive tract due to oxidative stress from reactive oxygen species (ROS). Studies say that anywhere from 30% to 80% of male factor infertility are due to damaging effects of oxidative stress (Ko et al., 2014).

Reactive Oxygen Species and Reactive Nitrogen Species

ROS represent a broad category of molecules that indicate the collection of radicals and nonradical oxygen derivatives. These molecules have an unpaired electron in their outer orbit, and thus are highly reactive and interact with a variety of lipids, proteins, and nucleic acids in the body (Agarwal et al., 2008). Free radicals include superoxide, hydroxyl radical, and hydroperoxyl. Nonradicals include hydrogen peroxide, singlet oxygen, iron oxygen complexes, and hypochlorite. A subclass of ROS is reactive nitrogen species (RNS) such as nitric oxide and peroxynitrate. These molecules, which are prominent in different areas of the male reproductive system, are responsible for contributing to nitrosative stress. RNS have multiple physiologic functions, which include regulation of multiple signaling pathways, assembly of the tight junctions in the blood testis barrier, production of hormones, and maintenance of vascular tone. Specifically RNS also are essential for conducting various sperm functions such as capacitation, acrosomal reaction, zona pellucida binding, as well as sperm motility, morphology, and viability (Doshi et al., 2012).

Mitochondria's Role in ROS

The mitochondria are responsible for balance of reactive oxygen species. Along with production of ATP, initiation of apoptosis, production of heat, and hereditary contribution, mitochondria produce ROS during the formation of superoxide in the electron transport chain. In sperm, 25–75 mitochondria are arranged in tubular structures in the mid piece and are responsible for sperm motility and overall functionality. Thus an intact mitochondrial genome and metabolome is essential for sperm functionality. While a controlled level of ROS is necessary, an imbalance between ROS and ROS scavenging by seminal antioxidants results in seminal oxidative stress which may lead to a decrease in motility, abnormal sperm morphology, and decreased ATP production leading to overall decreased sperm viability as well as increased sperm DNA damage (Amaral et al., 2013).

Sources of ROS

Human semen consists of different types of cells such as mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and epithelial cells. Of these, leukocytes (neutrophils and macrophages) and immature spermatozoa are the two main sources of ROS. ROS come from multiple extrinsic and intrinsic sources in the semen. Intrinsic sources include activated leukocytes from inflammation (i.e., diabetes, renal failure) and infection, immature spermatozoa with abnormal morphology, and varicoceles, which are abnormal dilations of the veins in the testicles. Extrinsic sources include smoking, alcohol, obesity, radiation exposure, and other environmental toxins such as pesticides, heavy metals, and plastics (Agarwal et al., 2016). These exposures are summarized in Table 1.

ROS and Male Infertility

In 30%–40% of cases, no male-infertility-associated factor is found. However, even in these cases of idiopathic normozoospermic male factor infertility, up to 25% of men have significantly higher levels of ROS when compared with fertile controls (Zini and Al-Hathal, 2011). In addition, the decreased levels of antioxidant capacity found in this population further synergize this state of oxidative stress. The delicate balance of oxidation and reduction is required for all major sperm functions such as capacitation, hyperactivation, and sperm-oocyte fusion; ROS may also disrupt sperm by fragmenting its DNA and interfering with normal chromatin packing. Normal chromatin compaction is required for sperm maturation during epididymal transit, capacitation, hyperactivation, acrosomal reaction, and sperm-oocyte fusion reaction, all of which are necessary for successful fertilization. Supraphysiologic ROS levels can affect sperm structural and functional integrity including motility morphology, count and viability. It can

Table 1 Sources of ROS

Category	Exposure	Results
Lifestyle	Smoking	Decrease in sperm motility and normal morphology
	Alcohol	Decrease in sperm concentration, motility, and normal morphology
	Obesity/high fat diet	Decrease in sperm concentration, motility, normal morphology, and vitality
Environmental	Air pollution	No significant changes in ejaculate volume, sperm concentration, motility, or normal morphology
	Pesticides	Decrease in sperm motility and normal morphology and an increase in pH of seminal fluid
	Plastics	Decrease in sperm concentration, motility, and normal morphology
Infection	Genitourinary	Decrease in sperm concentration
	Systemic	HIV: Leukocytospermia (increased white blood cells → increased ROS) and decreased sperm motility
		HBV: Decrease in ejaculate volume, sperm concentration, motility, vitality, and a decrease in pH of seminal fluid
Testicular	Varicocele	Decrease in sperm concentration
	Cryptorchidism	Decrease in sperm concentration, motility, normal morphology, and vitality
Chronic disease	Diabetes	Decrease in ejaculate volume
	Renal failure	Decrease in sperm concentration and motility
	Thyroid dysfunction	Hypothyroidism associated with statistically significant decrease in sperm concentration, motility, and normal morphology
Medications		Hyperthyroidism associated with statistically significant decrease in sperm motility
	Opioids	Decrease in sperm concentration, motility, and normal morphology
	SSRI	Decrease in sperm motility and normal morphology

induce apoptosis, resulting in low sperm counts characteristic of men with idiopathic infertility. Sperm DNA damage may also decrease fertilization rates, reduce implantation, impair embryonic development, and increase miscarriage/pregnancy loss and the potential for birth defects.

The spermatozoa and seminal plasma contain multiple protective antioxidants to protect spermatozoa from oxidative stress by scavenging the free radicals. These include both high molecular weight enzymatic antioxidants (superoxide dismutase, catalase and glutathione peroxidase) as well as nonenzymatic antioxidants (ascorbic acid, α -tocopherol, pyruvate, glutathione, L-carnitine, taurine, and hypotaurine)—which provide the bulk of antioxidant activity.

Nutraceutical Therapy

Overview of Nutraceuticals

Nutraceuticals are products derived from food that have health benefits, and may include amino acids, isolated nutrients, or dietary supplements. Many of these products have antioxidant properties and can help scavenge ROS. Since seminal oxidative stress may be due in part to deficiency in seminal antioxidants and there is a lack of serious side effects related to antioxidant therapy, nutraceutical therapy may be used in men with clinical subfertility, as well as in men with normal semen parameters who have been unable to conceive for 6–12 months.

Specific Nutraceuticals

Many different antioxidants have been investigated in the literature. It is important to distinguish between enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase and nonenzymatic antioxidants such as vitamins, proteins, glutathione, and ubiquinol. The different nutrients and their respective key role in infertility are summarized in [Table 2](#). Some of the important antioxidants studied in literature are discussed below. Overall, research has demonstrated that a decrease in the seminal levels of these antioxidants is associated with infertility. In addition, when given to men with infertility, these supplements or nutraceuticals can improve semen parameters.

CoEnzyme Q-10

CoEnzyme Q-10 (CoQ10) is an antioxidant molecule and a component of the mitochondrial respiratory chain, which participates, in aerobic cellular respiration. It plays an important role in energy metabolism, as well as functioning as a liposoluble chain-breaking antioxidant for cell membranes and lipoproteins ([Gvozdjaková et al., 2015](#)). At the cellular level, CoQ10 exists as a redox pair, that is, it is found in two forms—ubiquinone, the oxidized form, and ubiquinol, the reduced form—which are constantly switching back and forth as CoQ10 transfers a hydrogen in the electron transfer chain in the mitochondria. As a supplement there is no difference in taking ubiquinone or ubiquinol. Specifically, ubiquinol, which is the reduced state of CoQ10 is a strong lipophilic antioxidant that can regenerate other antioxidants. Ubiquinol inhibits organic peroxide formation in both the seminal fluid and seminal plasma, which reduces the oxidative stress to which sperm cells may be subjected ([Mancini and Balercia, 2011](#)). Thus

Table 2 Different nutraceutical therapies and role in infertility

<i>Ingredient</i>	<i>Key role in infertility</i>
Co-Enzyme Q-10	Energy, antioxidant, sperm parameter benefits
L-Carnatine	Energy, sperm parameter benefits
Lycopene	Antioxidant, sperm parameter benefits
Beta carotene	Antioxidant
Vitamin C	Antioxidant
Vitamin E	Antioxidant
Methylcobalmin	Sperm parameter benefits
Astaxanthine	Sperm parameter benefits
Alpha lipoic acid	Antioxidant
Zinc	Sperm parameter benefits
Selenium	Antioxidant

there is a strong positive correlation between CoQ10 concentration and sperm count and motility. Three clinical trials, summarized in a metaanalysis by Lafuente et al. have shown that supplementing infertile men with CoQ10 improved sperm concentration, motility, and concentration in the seminal plasma (Lafuente et al., 2013). However, this is no reported data on live births after treatment. Thus supplementation with CoQ10, while has not been proven to help increase pregnancy rates, has been shown to improve semen parameters.

L-Carnitine

L-Carnitine is a molecule that is necessary for beta-oxidation of fatty acids in the mitochondria and helps to continue the energy supply through the transfer of fatty acids from cytosol to mitochondria. It also protects DNA and cell membranes from the damage caused by ROS (Agarwal and Said, 2004). The highest concentration of L-carnitine in the human is in the epididymis, with the concentration being 2000 times greater than that in serum and infertile men have been found to have lower levels of seminal L-carnitine. Supplementation of L-Carnitine in infertile men demonstrated an improvement in sperm concentration, mobility, viability, and morphology (Sofimajidpour et al., 2016). In a metaanalysis by Zhou et al., supplementation with L-carnitine or L-acyl-carnitine versus placebo led to a significant improvement in pregnancy rate and sperm motility but not total sperm concentration or atypical sperm forms (Zhou et al., 2007). In addition, one study showed that in men with a grade 2 varicocele, which are varicoceles that are palpable on clinical examination, men who chose between 250 mg L-carnitine four times a day for 6 months or varicocelectomy showed improvement in sperm concentration, motility, and morphology similarly in both groups (Sofimajidpour et al., 2016).

Lycopene

Lycopene is a compound from the carotenoid family obtained through consumption of red-colored fruits and vegetables (Gajowik and Dobrzyńska, 2014). It is found in higher concentrations in semen and the concentration of lycopene was significantly decreased in infertile men (Ghyasvand et al., 2015). Lycopene is a lipophilic molecule that incorporates in the cell membrane to protect the sperm by preventing lipid peroxidation and also neutralizing ROS (Gajowik and Dobrzyńska, 2014). Studies have shown that supplementation with lycopene improved oxidative stress parameters such as DNA fragmentation index and sperm related parameters including sperm count, motility, and concentration. It did not, however, improve sperm morphology (Agarwal et al., 2014).

Vitamin E

Vitamin E is a major chain-breaking antioxidant in sperm membranes and this effect appears to be dose dependent. Naturally occurring vitamin E is actually a mixture of four tocopherols (alpha, beta, gamma, and delta) and four tocotrienols (again, alpha, beta, gamma, and delta). Most vitamin E supplements consist solely of a synthetic form of pure alpha tocopherol. Vitamin E scavenges the three major types of free reactive species, namely superoxide, hydrogen peroxide, and hydroxyl radicals (Kobori et al., 2014). In a randomized, double blind, placebo-controlled trial by Kessopoulou et al., in vitro functional tests of human spermatozoa improved after 3 months of alpha-tocopherol (600 mg/day) therapy (Kessopoulou et al., 1995). In another study by Suleiman et al., treatment with alpha-tocopherol reduced levels of malondialdehyde, a marker of lipid peroxidation, and 11 out of the 52 (26%) men treated were able to impregnate their spouses and 9 of those ended up in live births (Suleiman et al., 1996). While many studies have demonstrated a potential role for vitamin E in the management of male infertility, another randomized trial by Rolf et al. who used both 800 mg vitamin E and 1000 mg vitamin C failed to confirm these findings (Rolf et al., 1999).

Vitamin C

Vitamin C is another important chain-breaking antioxidant and is present at a higher concentration in seminal fluid than in plasma as well as being present in low but detectable amounts in sperm cells. Vitamin C neutralizes hydroxyl, superoxide, and hydrogen peroxide reactive species and prevents sperm agglutination, while preventing lipid peroxidation, recycling vitamin E, and protecting against DNA damage induced by hydrogen peroxide radicals (Angulo et al., 2011). It has been suggested that oral administration of

vitamin C with vitamin E significantly reduces hydroxyguanine levels in spermatozoa and also leads to an increased sperm count (Kobori et al., 2014; Rolf et al., 1999)

Zinc

Zinc is second only to iron as the most abundant element in human tissues. Although Zn is found in most types of foods such as red meat, white meat, fish, and milk, the World Health Organization estimates that one-third of the world's population is deficient in zinc. Zinc and citrate are excreted from the prostate gland as a low-molecular-weight complex; thus, it is estimated that the zinc levels in seminal plasma typically represent prostatic secretory function (Zhao et al., 2016). Since zinc is found in high concentrations of seminal fluid, it has numerous important functions, and it is essential for conception, implantation, and a favorable pregnancy outcome. On a more molecular level, zinc influences the fluidity of lipids and, thus, the stability of biological membranes. Zinc is a co-factor for superoxide dismutase, one of the important enzymatic antioxidants found in the semen. It also helps stabilize sperm chromatin. Lastly zinc has been found to play a regulatory role in the process of capacitation and the acrosome reaction (Gavella and Lipovac, 1998). Studies on the curative effects of zinc with respect to male infertility have shown that zinc supplementation can significantly increase the percentage of normal sperm morphology, sperm motility and semen volume. However, there were no significant effects of zinc supplementation on the sperm viability, sperm concentration, sperm count or percentage of abnormal sperm morphology (Zhao et al., 2016).

Selenium

Selenium (Se) is an essential element for normal testicular development, spermatogenesis, and spermatozoa motility and function. The predominant biochemical action of Se in both humans and animals is to serve as an antioxidant via the Se-dependent enzyme glutathione peroxidase and thus protect cellular membranes and organelles from peroxidative damage. In one study, infertile men with idiopathic asthenoteratospermia were treated with 200 µg of selenium with 400 U of vitamin E for at least 100 days. There was a 53% improvement in sperm motility, morphology, or both and an 11% spontaneous pregnancy rates in comparison with no treatment (Gavella and Lipovac, 1998). In another placebo controlled clinical trial carried out in Iran and Tunisia, selenium supplementation improved sperm counts, concentration, motility and morphology as well as sperm concentration in infertile men (Gavella and Lipovac, 1998).

N-Acetylcysteine

N-Acetyl cysteine is a naturally occurring compound, which comes from amino acid L-cysteine, and functions as a precursor of glutathione peroxidase. One study investigated the effect of selenium and N-acetyl-cysteine on infertile men with idiopathic oligo-asthenoteratospermia. These men were treated with the combinational therapy for 30 weeks. All semen parameters significantly improved with selenium and N-acetyl-cysteine treatment (Gavella and Lipovac, 1998).

Overall Effects of Nutraceuticals

Dosages(Ko and Sabanegh, 2014)

Although the doses that are prescribed vary between physicians and different sources, review of literature revealed a range of dosages that are recommended for various nutraceuticals (Table 3).

Cochrane review on overall effects of antioxidants (Showell et al., 2017)

The Cochrane review was a large literature review of multiple studies, aimed to quantify the effect of nutraceutical therapies on sperm motility, sperm count, and ability to achieve a pregnancy and/or a live birth. The results are summarized in Table 4.

Table 3 Doses of various nutraceuticals

<i>Supplement</i>	<i>Daily dose</i>
Vitamin E	300–1000 IU
Carnitines	1–2 g/day
Zinc	40 mg/day
CoEnzyme Q10	200–300 mg
N-Acetylcysteine	600 mg
Vitamin C	1000 mg
Vitamin D	1000–4000 IU
Selenium	60–400 µg
Glutathione	600 mg
Lycopene	10–30 mg

Table 4 Review on effects of various antioxidants

<i>Fertility improvement</i>	<i>Antioxidants used</i>	<i>Findings</i>
Live birth rate	Vitamin E; zinc	Increased
Pregnancy rate	L-Carnitine, L-acetytyl carnitine, co-enzyme Q10, magnesium, vitamin E plus vitamin C, zinc, combined antioxidants	Increased
Sperm DNA fragmentation	Vitamin E plus vitamin C	Decreased
<i>Sperm concentration</i>		
- < 3 months	Docosahexaenoic acid, magnesium, vitamin C plus E, <i>N</i> -acetylcysteine, pentoxifylline, L-carnitine	No difference
- 6 months	L-Carnitine, selenium, <i>N</i> -acetylcysteine, CoEnzyme Q10, pentoxifylline	Increased
- 9 months or more	L-Carnitine, CoEnzyme Q10	Increased
<i>Sperm motility</i>		
- 3 months or less	Vitamin C, vitamin E, L-carnitine, selenium, <i>N</i> -acerylcysteine, magnesium, zinc, docohexamandic acid, pentoxifylline	No difference
- 6 months	L-Carnitine, selenium, <i>N</i> -acetylcysteine, CoEnzyme Q10, vitamin E	Increased
- 9 months or more	L-Carnitine, L-acetylcarnitine, CoEnzyme Q10	Increased

Adverse effects(Arcaniolo et al., 2014)

Even though nutraceuticals are benign products, excess intake can lead to adverse effects. Review of the literature revealed various adverse effects from excess intake of nutraceuticals. Since men often take these medications for an extended period of time, it is important to ensure that the dosages are not large enough to cause adverse effects (Table 5).

Treatment**Ideal Couple**

Ideal couple should want to attempt natural conception or intrauterine insemination. For patients that want to proceed with intra cytoplasmic sperm injection or in vitro fertilization, surgical therapy may be preferred over administration of nutraceutical therapy.

Female Considerations

Full female workup should be considered. Female lab values should be within normal limits. This includes a normal AMH, a normal day 3 FSH, good follicle count. An anatomical workup with a hysterosalpingogram should be normal as well.

Male Considerations

Nutraceutical therapy is primarily intended for the 30%–40% of infertile men where no cause could be determined (idiopathic infertility). According to EAU Guidelines on male infertility, there is little evidence for empiric treatment using naturaceuticals. However, medical therapy should be reserved only in men with hypogonadotropic hypogonadism, which is low gonadotropins and sex steroid hormone levels in the absence of any problems in the hypothalamic-pituitary-gonadal axis.

Male sperm count should be between 5 and 15 million/cm³, which is an intermediate range of sperm. A patient is considered to have oligospermia with a concentration < 5 million/cm³. On the other hand a normal sperm count is > 15 million. Thus for men with sperm count that is decreased but not enough to be considered as oligospermia, nutraceutical therapy should be considered.

Sperm motility should be < 40% but > 20%. Sperm motility is more important for natural conception or intra uterine insemination but not as important for artificial techniques.

Male should also have no other correctible causes of abnormal semen parameters. These include varicoceles, cryptorchidism, infection, and inflammation. In general a male with idiopathic oligoasthenozoospermia is an ideal candidate for nutraceutical therapy. Overall these men who choose to take nutraceutical therapy will need to do so for over 6 months at the dosages required. The end goal of treatment with nutraceutical therapy is to help increase the motility of the sperm.

Conclusion

This article provides an overview of various causes of seminal oxidative stress, use of specific nutraceutical therapies to help in men with idiopathic infertility, and the selection of an ideal couple for nutraceutical therapy. The causes of oxidative stress include but are not limited to lifestyle, drugs, infections, chronic diseases, and iatrogenic causes. Various nutraceuticals have been studied in literature and include CoEnzyme Q10, carnitine, lycopene, vitamin E, vitamin C, zinc, and selenium. In a review of the literature

Table 5 Adverse effects on increased intake of various nutraceuticals

Supplement	Reported adverse effects
Vitamin E	GI distress, fatigue, muscle weakness, headache blurry vision, rash, bruising, bleeding complication (> 800 IU/d) Cardiovascular complications (> 400 IU/d)
Vitamin C (> 2000 mg/d)	Dyspepsia, headache, increased risk of nephrolithiasis
Vitamin A (> 50,000 IU/d)	Fatigue, irritability, mental status change, visual disturbances, vertigo Anorexia, GI distress, excessive sweating, myalgia/arthritis
Arginine	Hepatotoxicity, hypoplastic, anemia GI discomfort, hypotension, electrolyte abnormalities, renal insufficiency
Carnitine (> 4 g/d)	Increased bleeding risk, elevated glucose levels, worsening symptoms of sickle cell disease, asthma
CoEnzyme Q10	GI distress, seizures, malodorous body secretion
Glutathione	GI distress, loss of appetite, headache, skin rash
N-Acetyl-cysteine	Not adsorbed within GI tract GI distress, rash, fever, headache, drowsiness
Selenium	Hypotension, hepatic toxicity GI distress, nail changes, fatigue, irritability, hair loss, garlic breath/odor
Zinc	Metallic taste, muscle tenderness, tremors, facial flushing, hematologic changes, hepatic, and renal insufficiency
(> 200 mg/d)	GI distress, loss of appetite, dehydration, gastric ulceration, rash; headache
(> 450 mg/d)	Altered iron function, low copper levels, sideroblastic anemia, reduced immune function, reduced HDL levels

on nutraceutical therapies, it was found that taking these various supplements for over 6 months at certain doses increases chance to improve sperm count and motility. Overall, an infertile man with oligoasthenozoospermia is the best candidate for nutraceutical therapy.

References

- Agarwal, A., & Said, T. M. (2004). Carnitines and male infertility. *Reproductive Biomedicine Online*, 8(4), 376–384.
- Agarwal, A., Cocuzza, M., Abdelrazik, H., & Sharma, R. K. (2008). Oxidative stress measurement in patients with male or female factor infertility. *Transworld Research Network*, 37661(2), 195–218.
- Agarwal, A., Durairajanayagam, D., Ong, C., & Prashast, P. (2014). Lycopene and male infertility. *Asian Journal of Andrology*, 16(3), 420.
- Agarwal, A., Roychoudhury, S., Bjugstad, K. B., & Cho, C.-L. (2016). Oxidation-reduction potential of semen: What is its role in the treatment of male infertility? *Therapeutic Advances in Urology*, 8(5), 302–318.
- Amaral, A., Lourenco, B., Marques, M., & Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction*, 146(5), R163–R174.
- Angulo, C., Maldonado, R., Pulgar, E., Mancilla, H., Córdova, A., Villarreal, F., et al. (2011). Vitamin C and oxidative stress in the seminiferous epithelium. *Biological Research*, 44(2), 169–180.
- Arcaniolo, D., Favilla, V., Tiscione, D., Pisano, F., Bozzini, G., Creta, M., et al. (2014). Is there a place for nutritional supplements in the treatment of idiopathic male infertility? *Archivio Italiano di Urologia e Andrologia*, 86(3), 164.
- Condorelli, R. A., Calogero, A. E., Vicari, E., & La Vignera, S. (2015). Chronic consumption of alcohol and sperm parameters: Our experience and the main evidences. *Andrologia*, 47(4), 368–379.
- Doshi, S. B., Khullar, K., Sharma, R. K., & Agarwal, A. (2012). Role of reactive nitrogen species in male infertility. *Reproductive Biology and Endocrinology*, 10(1), 109.
- Gajowik, A., & Dobrzyńska, M. M. (2014). Lycopene – antioxidant with radioprotective and anticancer properties. A review. *Roczniki Państwowego Zakładu Higieny*, 65(4), 263–271.
- Gavella, M., & Lipovac, V. (1998 Nov). Vitro effect of zinc on oxidative changes in human semen. *Andrologia*, 30(6), 317–323.
- Ghyasvand, T., Goodarzi, M. T., Amiri, I., Karimi, J., & Ghorbani, M. (2015). Serum levels of lycopene, beta-carotene, and retinol and their correlation with sperm DNA damage in normospermic and infertile men. *International Journal of Reproductive Biomedicine (Yazd)*, 13(12), 787–792.
- Gvozdjaková, A., Kucharská, J., Dubravický, J., Mojto, V., & Singh, R. B. (2015). Coenzyme Q10, α -tocopherol, and oxidative stress could be important metabolic biomarkers of male infertility. *Disease Markers*, 2015, 1–6.
- Jungwirth, A., Giwercman, A., Tournaye, H., Diemer, T., Kopa, Z., Dohle, G., et al. (2012). European Association of Urology guidelines on male infertility: The 2012 update. *European Urology*, 62(2), 324–332.
- Kessopoulou, E., Powers, H. J., Sharma, K. K., Pearson, M. J., Russell, J. M., Cooke, I. D., et al. (1995). A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertility and Sterility*, 64(4), 825–831.
- Ko, E. Y., & Sabanegh, E. S. (2014). The role of nutraceuticals in male fertility. *The Urologic Clinics of North America*, 41(1), 181–193.
- Ko, E. Y., Sabanegh, E. S., & Agarwal, A. (2014). Male infertility testing: Reactive oxygen species and antioxidant capacity. *Fertility and Sterility*, 102(6), 1518–1527.
- Kobori, Y., Ota, S., Sato, R., Yagi, H., Soh, S., & Gaku Arai, H. O. (2014). Antioxidant cosupplementation therapy with vitamin C, vitamin E, and coenzyme Q10 in patients with oligoasthenozoospermia. *Archivio Italiano di Urologia e Andrologia*, 86.
- Lafuente, R., González-Comadrán, M., Solà, I., López, G., Brassesco, M., Carreras, R., et al. (2013). Coenzyme Q10 and male infertility: A meta-analysis. *Journal of Assisted Reproduction and Genetics*, 30(9), 1147–1156.
- Mancini, A., & Balercia, G. (2011). Coenzyme Q10 in male infertility: Physiopathology and therapy. *BioFactors*, 37(5), 374–380.
- Rolf, C., Cooper, T. G., Yeung, C. H., & Nieschlag, E. (1999). Antioxidant treatment of patients with asthenozoospermia or moderate oligoasthenozoospermia with high-dose vitamin C and vitamin E: A randomized, placebo-controlled, double-blind study. *Human Reproduction*, 14(4), 1028–1033.
- Saleh, R. A., Agarwal, A., Sharma, R. K., Nelson, D. R., & Thomas, A. J. (2002). Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: A prospective study. *Fertility and Sterility*, 78(3), 491–499.
- Showell, M. G., Mackenzie-Proctor, R., Brown, J., Yazdani, A., Stankiewicz, M. T., & Hart, R. J. (2014). Antioxidants for male subfertility. In M. G. Showell (Ed.), *Cochrane database of systematic reviews*. John Wiley & Sons, Ltd: Chichester, UK (cited 24 August 2017).

- Sofimajidpour, H., Ghaderi, E., & Ganji, O. (2016). Comparison of the effects of varicocelectomy and oral L-carnitine on sperm parameters in infertile men with varicocele. *Journal of Clinical and Diagnostic Research*, 10(4), PC07–PC10.
- Suleiman, S. A., Ali, M. E., Zaki, Z. M., el-Malik, E. M., & Nasr, M. A. (1996). Lipid peroxidation and human sperm motility: Protective role of vitamin E. *Journal of Andrology*, 17(5), 530–537.
- Taha, E. A., Sayed, S. K., Gaber, H. D., Abdel Hafez, H. K., Ghandour, N., Zahran, A., et al. (2016). Does being overweight affect seminal variables in fertile men? *Reproductive Biomedicine Online*, 33(6), 703–708.
- Zhao, J., Dong, X., Hu, X., Long, Z., Wang, L., Liu, Q., et al. (2016). Zinc levels in seminal plasma and their correlation with male infertility: A systematic review and meta-analysis. *Scientific Reports*, 6, 22386.
- Zhou, X., Liu, F., & Zhai, S. (2007). Effect of L-carnitine and/or L-acetyl-carnitine in nutrition treatment for male infertility: A systematic review. *Asia Pacific Journal of Clinical Nutrition*, 16(Suppl. 1), 383–390.
- Zini, A., & Al-Hathal, N. (2011). Antioxidant therapy in male infertility: Fact or fiction? *Asian Journal of Andrology*, 13(3), 374–381.

Alternative Reproductive Medicine

Rodrigo Lessi Pagani, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Acupuncture

In 1997, the National Institutes of Health (NIH) published a consensus statement stating that acupuncture is a promising modality for treating a wide range of conditions and that there was sufficient scientific evidence to subsidize the incorporation of acupuncture therapy into conventional medicine (Crimmel et al., 2001).

A fundamental concept in Chinese medicine is that two opposite but complementary forces (Yin and Yang) lie behind all natural phenomena and disease is considered the result of disharmony or imbalance of those forces. Acupuncture is part of traditional Chinese medicine, and its theory is based on the hypothesis that there are patterns of energy flow (Qi) through the body that are essential for health. The goal of acupuncture is to correct imbalances of the Qi and restore health through manual or electrical stimulations of needles inserted at points along the meridians that represents organs of the body. Another type of stimulation is moxibustion, which involves in the burning of the artemisia wool over specific points.

In the last decade of the past century, there were three controlled studies that showed promising results, but unfortunately, they were uncontrolled trials. In 1992, Gerhard et al. (Gerhard et al., 1992) reported mild but statistically significant improvement in sperm motility that was evident up to 10 weeks after the completion of acupuncture therapy. Progressive motility increased from 21% to 26% and total motility increased from 42% to 50%. Minghua (1993) reported a significant improvement not only in motility but also in sperm density. Sperm motility improved from 37% to 61% after acupuncture therapy. In 1997, Siterman et al. (1997) reported improvements in sperm viability and total motile sperm count (from 8.5 million to 19.3 million after 5 weeks of acupuncture therapy). Unfortunately, these studies did not encompass post-therapy evaluation up to or beyond the 90-day period needed for the maturation of spermatogonia to ejaculated spermatozoa.

In a prospective controlled study, Pei et al. (2005) reported a statistically significant improvement in the ultrastructural integrity of spermatozoa in 28 patients that receive acupuncture twice a week for 5 weeks. In another prospective controlled study, 19 patients with primary infertility with semen abnormalities were randomized into two groups that received acupuncture and moxibustion. The patients in the treatment group were stimulated at therapeutic points, while patients in the control group were stimulated at nontherapeutic points twice a week for 10 weeks. The authors reported a significant increase in the percentage of sperm with normal morphology (Gurfinkel et al., 2003). Dieterle et al. conducted a study where patients were randomized to receive either acupuncture or placebo acupuncture twice a week for 6 weeks. Placebo acupuncture was administered with non-penetrating needles in the same points used to manipulate the treated patients. The authors reported a significant effect of acupuncture on the percentage of total motile sperm (Dieterle et al., 2009).

A recent systematic review and metaanalysis that searched relevant studies in English, Korean and Chinese databases, including four randomized clinical trials (Dieterle et al., 2009; Liu et al., 2011; Wang et al., 2008; Shi, 2009) that compared acupuncture treatment against placebo, sham or no treatment, concluded that the acupuncture treatment might significantly improve the sperm motility and sperm concentration in infertile men with poor semen quality. However, there was no significant impact on the pregnancy rate (Jerng et al., 2014).

The underlying mechanism by which acupuncture improve the sperm parameters is yet to be determined. One possible role of acupuncture relies on the fact that it stimulates somatic afferent nerves and possibly modulates the autonomic nervous system and controls the hypothalamus-pituitary-gonadal axis (Franconi et al., 2011). Another possible factor is due to higher testicular blood flow in patients submitted to electroacupuncture, with possible increase in seminal antioxidant agents (Cakmak et al., 2008).

Herbal Medication

Herbal medications are a foundation of traditional Chinese medicine for male infertility. In the majority of cases, herbal formulas are prescribed as a decoction that may consist of a dozen or more individual ingredients. As most studies were published in the Chinese medical literature, the outstanding review and translation by Jiang and colleagues was used as a guide (Jiang et al., 2017).

Zhang et al. investigated the effect of Bushen Shengjing Decoction in 82 patients with azoospermia or severe oligospermia combined with intracytoplasmic sperm injection (ICSI) compared to 82 patients submitted to ICSI alone as controls. It was found that sperm density, sperm motility, sperm morphology, fertilization rate, and clinical pregnancy were higher in the treatment group, suggesting that Bushen Shengjing Decoction helped decrease semen ROS levels and improve sperm quality (Zhang et al., 2007).

In an effort to study the effect of herbal formula WuziYanzong Pill, Wang et al. divided 231 patients in three different groups: the first received only the herbal formula, the second was submitted to acupuncture alone, and the third received both treatments. There was an increase in sperm density, sperm vitality, and in the acrosome enzyme activity compared to pretreatment, with a more obvious improvement in the combined therapy group (Wang et al., 2008).

Several other studies with different types of herbal preparations have been reported. Sun et al. showed that herbal formula QingreYulin Decoction has antibiotic effect (Sun et al., 2006); Sun and Bao demonstrated that herbal formula Yikang Decoction has a role in the treatment of male immune infertility caused by antisperm antibodies (Sun and Bao, 2006); and Qi et al. suggested

that the use of herbal formula (Qi et al., 2001). An in vitro study, including 7 herbal decoctions and 37 individual herbs, concluded that they possess antioxidant and anti-estrogenic activities (Tempest et al., 2008).

The only randomized study conducted with an herbal preparation was reported Park et al. in 2016. The authors randomized 77 infertile men with varicocele in 4 different groups: placebo, Korean red ginseng, varicocelectomy with placebo, or varicocelectomy with Korean red ginseng. They concluded that the patients treated with Korean red ginseng with or without varicocelectomy showed significantly improved semen parameters in terms of sperm concentration, viability, motility, and morphology. Although the effects were more prominent in patients who also underwent varicocelectomy, Korean red ginseng monotherapy also showed significant improvement in semen parameters (Park et al., 2016).

Tai Chi and Qigong

Tai chi and qigong are Chinese mind-body practices that combine meditation with exercise to improve the flow of qi throughout the body. The main difference lies in the complexity of movements, with qigong being a simpler form utilizing repetitive body movements and breathing techniques. Tai chi originated as a martial art that has evolved to an exercise promoted for general health. The role of both techniques in male infertility is to integrate body, breath, and mind, to achieve mental clarity, body homeostasis, and heightened sexual energy and libido, but there are no specific study addressing their efficacy in improving parameters of male fertility (Yao and Mills, 2016).

Ayurvedic Medicine

In India, indigenous use of plant products has been an ancient practice and plays an essential part of healthcare system. Information about herbal or plant-based therapies and their efficacy has been documented in the Vedas and the knowledge has been transferred from one generation to another (Lohiya et al., 2016).

Withania somnifera (Indian ginseng) is reported to have antiserotogenic, antioncogenic, cardioprotective, anticoagulant properties and its roots is considered as an aphrodisiac and a tonic (Lohiya et al., 2016). Ambiye et al. showed a marked improvement in semen parameters and testosterone levels after 90 days of treatment with *W. somnifera* root extract in oligospermic men (Ambiye et al., 2013).

Another plant, *Tribulus terrestris* has been implicated in the treatment of male infertility. In a double-blind study, Sellandi et al. observed that administration of *T. terrestris* granules improved sperm count in 78% of the oligospermic patients. The possible mechanism of action is attributed to its ability to increase the production of LH, which subsequently increase testosterone levels (Sellandi et al., 2012).

Yoga

Yoga is a physical, mental, and spiritual discipline that originated in India. Yoga focuses on gentle stretching, exercises for breath control, and meditation. The role of yoga in male infertility focuses on reducing anxiety and optimizing erectile function (Sengupta et al., 2013).

Homeopathy

Homeopathy is a system of alternative medicine created in 1796 by Samuel Hahnemann. Homeopathy rationale sustains that a substance that causes the symptoms of a disease in healthy people would cure similar symptoms in sick people. The preparations are manufactured using a process called homeopathic dilution, in which a substance is repeatedly diluted in alcohol or distilled water.

In a prospective observational pilot study, 45 patients were treated with homeopathic drugs for an average of 10 months. Those patients with low alcohol consumption, nonsmokers, with less than five dental amalgam fillings, and no exposure to noxious substances at the workplace showed better results. Overall, patients with oligoasthenospermia and severe oligoasthenoteratospermia showed median improvement by 184% in their sperm count and by 114% in their sperm progressive motility (Gerhard and Wallis, 2002). The authors also reported a pregnancy rate of 15.6% and live birth rate of 11.1% in patients in the treatment group who were infertile for at least two years (Gerhard and Wallis, 2002).

References

- Ambiye, V. R., Langade, D., Dongre, S., Aptikar, P., Kulkarni, M., & Dongre, A. (2013). Clinical evaluation of the spermatogenic activity of the root extract of Ashwagandha (*Whitania somnifera*) in oligospermic males: A pilot study. *Evidence Based Complementary and Alternative Medicine*, 571420.
- Cakmak, Y. O., Akpinar, I. N., Ekincl, G., & Bekiroglu, N. (2008). Point and frequency-specific response of the testicular artery to abdominal electroacupuncture in humans. *Fertility and Sterility*, 90, 1732–1738.

- Grimmel, A. S., Conner, C. S., & Monga, M. (2001). Withered yang: A review of traditional Chinese medical treatment of male infertility and erectile dysfunction. *Journal of Andrology*, 22, 173–182.
- Dieterle, S., Li, C., Greb, R., Bartzsch, F., Hatzmann, W., et al. (2009). A prospective randomized placebo-controlled study of the effect of acupuncture in infertile patients with severe oligoasthenozoospermia. *Fertility and Sterility*, 92, 1340–1343.
- Franconi, G., Manni, L., Aloe, L., Mazzilli, F., Giambalvo Dal Ben, G., Lenzi, A., & Fabbri, A. (2011). Acupuncture in clinical and experimental reproductive medicine: A review. *Journal of Endocrinology Investigation*, 34(4), 307–311.
- Gerhard, I., & Wallis, E. (2002). Individualized homeopathic therapy for male infertility. *Homeopathy*, 91, 133–144.
- Gerhard, I., Jung, I., & Postneek, F. (1992). Effects of acupuncture on semen parameters/hormone profile in infertile men. *Molecular Andrology*, 4, 9–24.
- Gurfinkel, E., Cedenho, A. P., Yamamura, Y., & Srougi, M. (2003). Effects of acupuncture and moxa treatment in patients with semen abnormalities. *Asian Journal of Andrology*, 5, 345–348.
- Jerng, U. M., Jo, J. Y., Lee, S., Lee, J. M., & Kwon, O. (2014). The effectiveness and safety of acupuncture for poor semen quality in infertile males: A systematic review and meta-analysis. *Asian Journal of Andrology*, 16, 884–891.
- Jiang, D., Coscione, A., Li, L., & Zeng, B. Y. (2017). Effect of Chinese herbal medicine on male infertility. *International Review of Neurobiology*, 135, 297–311.
- Liu, L., Yue, Z. X., Fu, W., Zhang, L. J., & He, X. (2011). Effect of traditional Chinese drug combine with electric acupuncture on seminal plasma neutral alpha-1,4 glycosidase enzymes in patients with male infertility due to weak sperm. *Chinese Journal of Family Planning Gynecotokology*, 3, 32–34.
- Lohiya, N. K., Balasubramanian, K., & Ansari, A. S. (2016). Indian folklore medicine in managing men's health and wellness. *Andrology*, 48, 894–907.
- Minghua, P. (1993). Acupuncture treatment of male infertility with abnormal seminal fluid findings: A report of 39 cases. *International Journal of Clinical Acupuncture*, 4, 449–452.
- Park, H. J., Choe, S., & Park, N. C. (2016). Effects of Korean red ginseng on semen parameters in male infertility patients: A randomized, placebo-controlled, double-blind study. *Chinese Journal of Integrative Medicine*, 22, 490–495.
- Pei, J., Strehler, E., Noss, U., Abt, M., Piomboni, P., Baccetti, B., & Sterzik, K. (2005). Quantitative evaluation of spermatozoa ultrastructure after acupuncture treatment for idiopathic male infertility. *Fertility and Sterility*, 84, 141–147.
- Qi, G. C., Lu, J. K., & Kan, Q. L. (2001). Comparative clinical study on treatment of varicocele caused infertility by tongling granule and surgical operation. *ZhongguoZhong Xi Yi Jie He Za Zhi*, 21, 412–415.
- Sellandi, T. M., Thakar, A. B., & Baghel, M. S. (2012). Clinical study of *Tribulus terrestris* Linn. in oligozoospermia: A double-blind study. *Ayu*, 33, 356–364.
- Sengupta, P., Chaudhuri, P., & Bhattacharya, K. (2013). Male reproductive health and yoga. *International Journal of Yoga*, 6, 87–95.
- Shi, X. F. (2009). Effects of acupuncture and traditional Chinese medicinal for oligozoospermia and/or asthenozoospermia in male infertility. *China Modern Medicine*, 16, 115–116.
- Siterman, S., Eltes, F., Wolfson, V., Zabludovsky, N., & Bartoov, B. (1997). Effect of acupuncture on sperm parameters of males suffering from subfertility related to low sperm quality. *Archives of Andrology*, 29, 151–161.
- Smith, J. F., Eisenberg, M. L., Millstein, S. G., et al. (2010). The use of complementary and alternative fertility treatment in couples seeking fertility care: Data from a prospective cohort in the United States. *Fertility and Sterility*, 93, 2169–2174.
- Sun, Z., & Bao, Y. (2006). TCM treatment of male immune infertility – A report of 100 cases. *Journal of Traditional Chinese Medicine*, 26, 36–38.
- Sun, J., Zhou, A. F., & Ding, C. F. (2006). Clinical observation of Qingyeyulin decoction in treatment of male infertility caused by accessory gland infection. *ZhongguoZhong Xi Yi Jie He Za Zhi*, 26, 877–880.
- Tempest, H. G., Homa, S. T., Routledge, E. J., et al. (2008). Plants used in Chinese medicine for the treatment of male infertility possess antioxidant and anti-oestrogenic activity. *Systems Biology in Reproductive Medicine*, 54, 185–195.
- Wang, Z. Q., Huang, Y. Q., & Liang, B. (2008). Clinical observation on electroacupuncture and Chinese drug for treatment of oligospermia and asthenospermia of the male infertility patient. *Zhongguo Zhen Jiu*, 28, 805–807.
- Yao, D. F., & Mills, J. N. (2016). Male infertility: Lifestyle factors and holistic complementary, and alternative therapies. *Asian Journal of Andrology*, 18, 410–418.
- Zhang, H. Q., Zhao, H. X., & Zhang, A. J. (2007). Male infertility with severe oligospermia and azospermia treated by Bushen Shengjing decoction combined with intracytoplasmic sperm injection. *ZhongguoZhong Xi Yi Jie He Za Zhi*, 27, 972–975.

Medical Male Contraception

Morgan E Schubbe and Moshe Wald, University of Iowa, Iowa City, IA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Numerous methods for female contraception exist, including oral pills, implantable devices, transdermal patches, or intrauterine devices. However, many women are unable to use these methods due to intolerance or hormone-related side effects, leaving couples with limited options for safe and effective contraception. One area that has long been under investigation but is not yet available to the public is male medical contraception.

Male contraception can be broadly divided into three categories. First are methods that include physical barriers, such as condoms or vasectomy. Several other methods (both hormonal and nonhormonal) work by preventing the production of sperm. Lastly, there are agents that work by inhibiting sperm function or directly killing sperm.

Most contraceptive use among modern couples uses methods that place the burden solely on women. Currently, the only readily available methods of contraception for men include vasectomy and condoms. In 2015, male-directed methods accounted for 21% of contraceptive practice worldwide (United Nations, 2015). Since the latter part of the 20th century, the market has seen additions of new methods for use by women, including intrauterine devices. However, innovations in male methods have been limited to refinements in male condoms and improved vasectomy techniques. Many new methods of male contraception are currently in clinical trials, but not yet available in the United States.

This article will provide an overview of the methods of male contraception currently available commercially as well as those that are still experimental, as well as promising options that may be available in the future.

Currently Available Methods

Physical Barrier Methods

Condoms

Condoms remain the least expensive and most commonly used form of male contraception. They are relatively low cost, easy to use, have few to no side effects, and are the only method to also offer protection against sexually transmitted infections. According to the most recent National Survey of Family Growth (2006–10) male condoms had a failure rate of 13% within 1 year, down from 17% from the previous report in 2002 (Sundaram et al., 2017). This relatively high failure rate as well as perceived loss of pleasure are some of the main reasons men may not use condoms.

Vasectomy

Vasectomy is currently the most widely practiced surgical procedure for male contraception. Although it is safer, simpler, less expensive, and equally as effective as female sterilization, there is still a large discrepancy between female and male sterilization in many countries worldwide. The 2015 United Nations Population Division reported that male sterilization accounted for 10% or less of all sterilizations among couples (United Nations).

Vasectomy is a highly effective procedure with failure rates typically <1% (Sharlip et al., 2012). Failure is often due to lack of backup contraceptive use until azoospermia is confirmed or more rarely, late recanalization of the vas deferens. Most vasectomies are performed as outpatient procedures under local anesthesia with rapid recovery. The most common side effects include a 1%–2% risk of symptomatic hematoma and 3.4% incidence of infection. Additionally, 1 in 1000 men who undergo vasectomy will have long term pain requiring eventual medical or surgical intervention (Adams and Wald, 2009). Vasectomy is technically reversible, but is intended to be a permanent form of contraception.

Hormonal Methods

Methods of male hormonal contraception are the most widely studied, but remain unavailable for commercial use. Hormonal methods of contraception exert their effect by influencing a series of feedback loops on the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1).

The hypothalamus releases a hormone called gonadotropin-releasing hormone (GnRH) in a pulsatile manner. GnRH then travels through a local venous circulation to the anterior pituitary gland, where it stimulates the release of two important reproductive hormones called luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH are the primary pituitary hormones that regulate ovarian and testicular function.

In the testis, LH stimulates testosterone production within the Leydig cells. FSH binds to Sertoli cells and spermatogonial membranes and stimulates seminiferous tubule growth. When a male reaches puberty, secretion of LH and FSH from the anterior pituitary increases. The seminiferous tubules become the site of sperm production. This process is initiated when circulating testosterone and other local factors bind to the immature germ cells to induce their maturation.

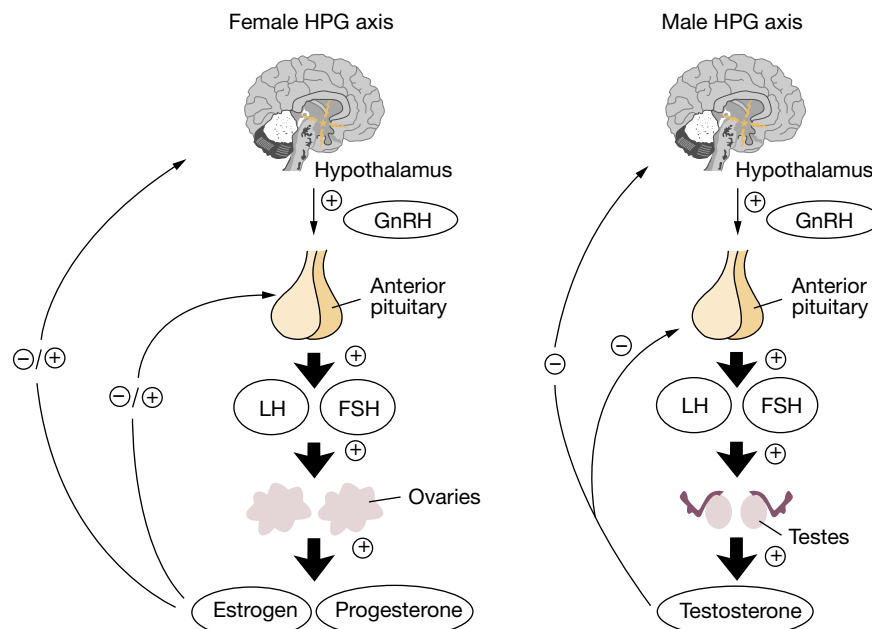


Fig. 1 Schematic representation of the hypothalamic-pituitary-gonadal (HPG) axes. From Kong L., et al. 2014. *International Journal of Molecular Sciences* **21**,253–269; MDPO Open Access.

The effects of exogenous testosterone on this axis work via a negative feedback loop to inhibit LH and FSH secretion from the pituitary gland. Decreased levels of LH and FSH in the testes lead to decreased production of endogenous testosterone, which is required for spermatogenesis. This effect can be augmented by the addition of progesterone analogues or GnRH antagonists, which will be discussed later in this article.

Overview of spermatogenesis

Spermatogenesis is a complex, hormonally regulated process. In humans, spermatogenesis begins in puberty and continues for life. The process is started when the hypothalamus secretes a substance called GnRH, which stimulates downstream release of FSH and LH that ultimately reach the Sertoli cells and germ cells in the testes.

The majority of testicular volume is made up of structures called seminiferous tubules. Sertoli cells can be found lining the seminiferous tubules. These cells are responsible for providing structural, functional, and metabolic support to the developing germ cells (sperm). The inside of the tubules is highly organized, with the developing germ cells following a sequential order of maturation as they progress toward the seminiferous tubule lumen where they are released. The spermatogonia is an early form of the germ cell, and as it grows and divides it progresses through a series of stages including primary spermatocyte, secondary spermatocyte, spermatids, and finally, mature spermatozoa.

The entire process of sperm production takes approximately 60–75 days. It takes an additional 10–14 days for the sperm to be transported through the reproductive tract from the seminiferous tubules, until they eventually reach the ejaculate. The concentration of sperm in normal ejaculate typically ranges from 15 to 150 million sperm per milliliter of ejaculate. The complete absence of sperm, called azoospermia, makes fertilization impossible and thus is the ultimate goal of male contraceptives. Another term, oligospermia, refers to sperm counts < 15 million per milliliter. The more severe the oligospermia (i.e., the lower the sperm count), the lower the chances of successful conception. Some investigational methods of male medical contraception have demonstrated varying levels of oligospermia. Few methods aside from surgical vasectomy actually achieve full azoospermia, so counts < 1 million sperm per milliliter are considered by some a reasonable and successful target for male contraceptives (Aaltonen et al., 2007).

Hormonal preparations are available in multiple formulations, including injectable, implantable, transdermal, and oral. Oral preparations of testosterone are prone to high first-pass metabolism, a phenomenon in which the concentration of the drug is greatly reduced by the time it reaches systemic circulation. This necessitates two to three time per day dosing and makes oral testosterone a less practical option. One of the most highly studied formulations is high-dose testosterone depot injections. Two large, multinational, multicenter studies sponsored by the World Health Organization published in the 1990s showed that once-weekly 200 mg intramuscular injections of testosterone enanthate (TE) produced azoospermia in 65% of men after 4 months with 0–0.8 pregnancies per 100 person-years and oligospermia (defined as < 3 million sperm/mL) in all but 2.2% with associated pregnancy rates of 8.1 per 100 person-years (World Health Organization, 1990, 1996). The combined failure rate of this regimen was 1.4 pregnancies per 100 person-years, comparable with the female contraceptive pill. The regimen was largely well tolerated with common reversible side effects including weight gain, acne, increased aggressiveness and libido, hypertension, depression, fatigue, decrease in

testicular volume, and decrease in HDL. The failure in 2% of patients to achieve sufficient oligospermia and the inconvenience of weekly injections were considered the major disadvantages.

The addition of progesterone derivatives to testosterone increases inhibition of spermatogenesis by further inhibiting the pituitary gland and has potential direct effect on germ cells. The synergistic effect of these two substances allows for lower total use of testosterone to achieve sufficient azoospermia in a shorter time period than testosterone alone. In one study, oral levonorgestrel, LNG (500 µm orally daily) was combined with TE (100 mg IM per week) for 6 months. The LNG-TE combination was superior to TE alone in terms of azoospermia (67% vs. 33%, respectively) and 94% of the men had sperm concentrations of < 1 million per milliliter compared with 61% of the TE-alone group (Bebb et al., 1996). Drawbacks to the LNG-TE regimen included greater weight gain and decreases in HDL cholesterol compared with the TE alone group.

Side effects of progestones include weight gain, lower HDL, lower sex-hormone binding globulin levels, and promotion of proinflammatory cytokines that have been linked to adverse cardiovascular events (Zitzmann et al., 2005).

Similar to progesterone derivatives, GnRH antagonists work synergistically with testosterone to inhibit spermatogenesis. These substances work by competitive inhibition at the GnRH receptor in the pituitary gland, inhibiting the production and release of FSH and LH necessary to produce testosterone. Though promising in theory, studies so far have been small and have not shown significant difference in efficacy over testosterone alone. Downsides of GnRH antagonists appear to be local skin reactions and high cost.

An interesting observation with hormonal methods is their varying efficacy based on ethnic background. In numerous studies, increased effectiveness of hormonal contraception has been seen in East-Asian populations with azoospermia rates approaching 90%–100%. Men in Europe, North America, and Australia have rates of azoospermia between 60% and 80% on the same regimen (World Health Organization, 1990, 1996). The reason for this difference has yet to be discovered, but may be due to differences in body fat distribution or genetic differences in hormone metabolism.

Nonhormonal Methods

The promise of nonhormonal methods of male contraception is becoming more appealing because they have no impact on testosterone and thereby may avoid side effects such as sexual dysfunction or weight gain. These nonhormonal alternatives may also be more easily dosed orally as they do not have the first pass metabolism of testosterone. These compounds work in a variety of ways at the cellular level including influencing gene expression, receptor inhibition, and physical disruption of essential cellular connections. Four of the most researched nonhormonal substances are reviewed here.

Adjudin is a recently discovered compound that acts by disrupting adhesion between spermatids and Sertoli cells, causing premature release of sperm and resulting in infertility. This is a locally acting agent with no apparent toxicity to Sertoli cells. Because it does not work on the HPG axis, FSH and LH levels are preserved and future fertility is maintained. Adjudin is currently being studied in animals and has shown promising results in rats. Potential effects on the liver and costs associated with modifying the compound are obstacles for testing in humans.

Another compound that inhibits spermatogenesis is WIN 18,446, a potent inhibitor of testicular retinoic acid, which is essential to spermatogenesis. In seminiferous tubules, both germ and Sertoli cells synthesize retinoic acid via enzymes called aldehyde dehydrogenases. The retinoic acid then binds to receptors (RARs) that regulate gene expression. Male mice with abnormal retinoic acid receptors (RAR) are sterile due to problems with spermatogenesis (Kastner et al., 1996). Benefits of WIN 18,446 include reversible inhibition that suppresses spermatogenesis while preserving normal testosterone levels with a relatively benign side effect profile.

Another option for more selective inhibition is BMS-189453, an oral agent that selectively inhibits nuclear RAR. Unlike WIN 18,446 which takes up to 16 weeks to produce azoospermia, BMS-189453 has onset of action within 1 month and shows effects lasting up to 4 months (Chung et al., 2011). Rat studies have demonstrated this compound to be safe and effective for inhibiting spermatogenesis at low doses with no discernable side effects.

One final testis-specific target is an inhibitor of the protein BRDT. This protein functions to reorganize hyperacetylated histones, interfering with DNA synthesis. DNA synthesis is crucial to the production and differentiation of spermatocytes, so inhibiting this process will prevent formation of sperm. Male mice with selective inhibition of BRDT have been found to have isolated infertility (Shang et al., 2007). These effects are reversible upon withdrawal of the medication and there have been no significant systemic or hormonal side effects reported.

New Technology on the Horizon

One of the most promising developments in male contraception is an injectable technique that has been under investigation since the 1970s. Termed RISUG (reversible inhibition of sperm under guidance), it involves injection of a synthetic substance directly into the vas deferens under direct visualization through a small scrotal incision. The result is formation of a porous polymer that disrupts sperm-cell membranes as they move through the vas, producing damaged, nonviable sperm.

This technique has been extensively studied in India with promising results. Men achieved azoospermia within 1–4 months, with no pregnancies reported over 6 months (Chaki et al., 2003). Successful reversibility has also been demonstrated in several animal studies.

RISUG is currently under preclinical investigation in the United States under the trademark name Vasalgel (Parsemus Foundation, Berkeley, CA). Vasalgel is an SMA acid polymer dissolved in DMSO that works by occluding the vas deferens. After injection, the SMA acid forms a hydrogel that adheres to the tissues and fills the vas lumen, acting as a mechanical barrier to the passage of

sperm. In a study of adult rabbits, the injection produced reliable, durable azoospermia for study duration of 12 months; the substance was then flushed with successful restoration of fertility (Waller et al., 2016). The contraceptive effects of Vasalgel were reversible either with bicarbonate injection or digital massage.

Although promising, this method is still in need of broader multicenter clinical trials in humans to ascertain true safety and efficacy. The main safety concern surrounding this method includes potential teratogenic effects on sperm.

Summary

Despite years of research into medical alternatives for male contraception, the only commercially available options remain condoms and vasectomy. An ideal contraceptive is low cost, easy to use, reversible, and with few to no side effects. Although many of the hormonal methods have shown promising efficacy, they can require frequent dosing and have a wide range of side effects that many men are unable to tolerate.

Some of the nonhormonal compounds currently under investigation appear to have high efficacy. They target more specific steps in spermatogenesis and have shown relatively few side effects in many animal studies. More studies are needed to fully evaluate the safety and efficacy of these specific inhibitors in humans.

Perhaps one of the most promising new technologies is RISUG. Though similar methods have been under investigation in India for many years, this technology is just starting to gain interest in the United States and is currently in preclinical investigations. A local injection has the benefit of limited to no systemic side effects and has proven methods of reversibility. It is performed as a one-time outpatient procedure that is likely to be well tolerated and potentially cost-effective.

At the present time, medical options for contraception remain those available to women. Many women are unable to shoulder this burden due to cost, accessibility, or inability to tolerate hormonal side effects. Additionally, studies have shown that many men do take an interest in contraceptive responsibility. It is important to keep supporting investigation of these male contraceptive alternatives.

References

- Aaltonen, P., Amory, J. K., Anderson, R. A., et al. (2007). 10th summit meeting consensus: Recommendations for regulatory approval for hormonal male contraception. *Journal of Andrology*, 28, 362–363.
- Adams, C. E., & Wald, M. (2009). Risks and complications of vasectomy. *The Urologic Clinics of North America*, 36, 331–336.
- Bebb, R. A., Anawalt, B. D., Christensen, R. B., et al. (1996). Combined administration of levonorgestrel and testosterone induces more rapid and effective suppression of spermatogenesis than testosterone alone: A promising male contraceptive approach. *The Journal of Clinical Endocrinology and Metabolism*, 81, 757–762.
- Chaki, S. P., Das, H. C., & Misro, M. M. (2003). A short-term evaluation of semen and accessory sex gland function in phase III trial subjects receiving intravasal contraceptive RISUG. *Contraception*, 67, 73–78.
- Chung, S. S., Wang, X., Roberts, S. S., et al. (2011). Oral administration of a retinoic acid receptor antagonist reversibly inhibits spermatogenesis in mice. *Endocrinology*, 152, 2492–2502.
- Kastner, P., Mark, M., Leid, M., et al. (1996). Abnormal spermatogenesis in RXR beta mutant mice. *Genes & Development*, 10, 80–92.
- Shang, E., Nickerson, H. D., Wen, D., Wang, X., & Wolgemuth, D. J. (2007). The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation. *Development*, 134, 3507–3515.
- Sharlip, I. D., Belker, A. M., Honig, S., et al. (2012). Vasectomy: AUA guideline. *The Journal of Urology*, 188, 2482–2491.
- Sundaram, A., Vaughan, B., Kost, K., et al. (2017). Contraceptive failure in the United States: Estimates from the 2006-2010 National Survey of family growth. *Perspectives on Sexual and Reproductive Health*, 49, 7–16.
- United Nations DESA Population Division. (2015). *World Contraceptive Use 2015*.
- Waller, D., Bolick, D., Lissner, E., Premanadan, C., & Gamerman, G. (2016). Azoospermia in rabbits following an intravas injection of Vasalgel. *Basic and Clinical Andrology*, 26, 6.
- World Health Organization task force on methods for the regulation of male fertility. (1990). Contraceptive efficacy of testosterone-induced azoospermia in normal men. *Lancet*, 336, 955–959.
- World Health Organization task force on methods for the regulation of male fertility. (1996). Contraceptive efficacy of testosterone-induced azoospermia and oligozoospermia in normal men. *Fertility and Sterility*, 65, 821–829.
- Zitzmann, M., Erren, M., Kamischke, A., Simoni, M., & Nieschlag, E. (2005). Endogenous progesterone and the exogenous progestin norethisterone enanthate are associated with a proinflammatory profile in healthy men. *The Journal of Clinical Endocrinology and Metabolism*, 90, 6603–6608.

Direct Application Therapy for Erectile Dysfunction

Jack Grinnan, University of Connecticut Health Sciences Center, Farmington, CT, United States

Jared M Bieniek, Hartford Hospital, Hartford, CT, United States

Stanton C Honig, Yale School of Medicine, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Penile erection is the end result of a culmination of physiologic events which allows for satisfactory sexual activity. With initial stimulation, sensory efferents trigger the release of nitric oxide (NO) from the nerve terminal with resultant arterial dilation and inflow of blood into the penis. This causes the smooth muscle of the penis to relax. As the spaces of the penis (corpus cavernosum) fill with arterial blood and an erection is achieved, veins exiting the corpora cavernosa are compressed, further preventing the egress of blood so that the erection is maintained. Detumescence occurs as sympathetic stimulation causes contraction of the smooth muscle and returns to baseline after sympathetic triggered ejaculation.

Multiple vascular, neurologic, and psychologic pathways are involved in the steps required for a satisfactory erection. Impeding factors cause many men to struggle with achieving and/or maintaining erections, also known as erectile dysfunction (ED), with more than half of men over age 40 reporting some degree of ED (Feldman et al., 1994). After a directed history and physical examination with specific attention to medications, initial treatment typically consists of sexual counseling (if indicated) and a trial of oral medications with a phosphodiesterase inhibitor. If these options do not result in adequate improvements in erections or cause unwanted side effects, second-line treatments involving medications or devices directed locally to the penis are employed. In individuals with recalcitrant ED failing other therapies, surgical treatment with penile prosthesis implantation is recommended.

This article focuses on direct application therapy for ED, or non-surgical treatment with a delivery mechanism that requires direct physical contact with the penis. As mentioned, direct application therapies are often used as second-line therapy choices although some modalities may be utilized in first-line treatment. Currently available options include vacuum-assisted erection devices, penile tension rings, intraurethral vasodilatory suppositories, intracavernosal vasoactive injections, topical medications, and low-intensity shockwave treatment. Each treatment will be reviewed with emphasis on indications, general clinical use, mechanisms of action, results, and complications of therapy.

Direct Application Therapies for ED

Vacuum Erection Device

The concept for vacuum-assisted erections was first described by John King, an American family physician, in 1874 (King, 1874). The patient with “impotency, with a diminution in the size of the male organ” was instructed to bath the genitals with a shampoo of rosemary, phosphorus, tincture of musk, and almond oil followed by application of a “glass exhauster.” In 1917, the Austrian physician Otto Lederer took the design one step further, creating a combination device which employed a constricting ring at the base of the penis to maintain the erection. After his own radical prostatectomy, Geddings Osbon Sr. invented the modern vacuum erection device (VED) that was ultimately approved by the Food & Drug Administration in 1982. Thereafter VEDs gained popularity and were the most commonly prescribed therapy for ED until the emergence of phosphodiesterase inhibitors (Oakley and Moore, 1998).

A VED functions by creating a negative pressure environment around the penis to draw venous blood into the cavernous spaces (Fig. 1). Before using the device, a well-lubricated penile constriction ring is loaded onto the base of the plastic or glass cylinder. The cylinder is subsequently placed over the penis and a manual or battery-operated pump is activated to create a vacuum until the penis is engorged. Once the desired erection is achieved, the constriction band is slipped off the cylinder at the base of the penile shaft to prevent venous outflow and maintain rigidity for sexual activity. VEDs are indicated as first- or second-line therapies for ED and can

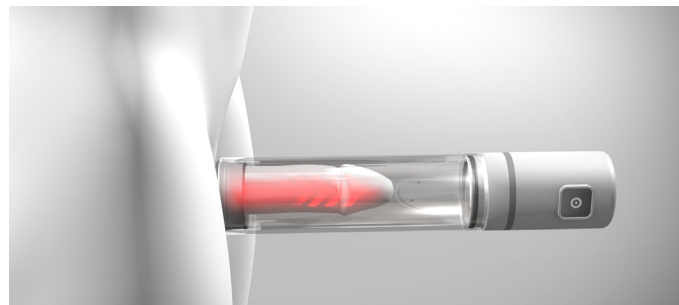


Fig. 1 Schematic of vacuum erection device. Image courtesy of OneMed Solutions.

be used as monotherapy or in combination with oral medications. In addition, a VED has been utilized in men with conditions contributing to corporal fibrosis or scarring (e.g., following radical prostatectomy or a prolonged episode of priapism) and may have a role in treating penile curvature as a result of Peyronie's disease.

VEDs have proven benefit for men with ED but efficacy is strongly dependent on proper usage of the device. Dedicated training sessions for patients learning to use a VED should be considered. With proper usage, up to 90% of men report satisfactory erections for penetrative intercourse (Montague, 2002). Continued usage at 2 years for those initially choosing a VED is up to 69%. Despite high initial success rates however, 86% of men will move on to other treatment choices for their ED when given additional options (Baniel et al., 2001).

Continued use of VEDs tends to be poor as many men struggle to integrate the cumbersome mechanical aspects of the device with their sex life. Some will discontinue the device secondary to quickly waning erections during intercourse, often the result of an inadequate seal of the constriction ring at the base of the penis. The seal of the ring may be improved by trimming pubic hair or using a lubricating jelly. Men with short or buried penises may be physically unable to achieve an adequate fit of the cylinder around the phallus secondary to anatomical challenges. It is also important to consider the natural anatomy of the penile corpora cavernosa which start in the glans penis, extend through the shaft, and insert under the pelvic rami. The constriction ring is placed at the base of the penis and holds blood in the penile section of corpora only, resulting in erectile instability or "hinging" as the proximal proximal corpora remain flaccid. Men also note an unnatural feel and appearance (cool and bluish) of the erection related to the retrograde venous inflow of blood in contrast to physiologic erections which contain arterial blood. Complications tend to be rare but may include petechiae and ecchymoses from VED usage. Concomitant usage of a VED in men on active anticoagulation is relatively contraindicated due to the theoretical increased risk of bruising and hematoma.

Penile Tension Rings

Constriction or tension rings were originally developed as a component of VEDs to aid in maintaining erections. The rings function in augmenting venous constriction to retain blood in the corpora for penile rigidity. Ideally, a penile tension system would be tight enough to constrict draining veins but still allow some arterial inflow into the corpora to achieve a maximally rigid erection. As such, constriction rings may also be utilized in men with innate venous dysfunction typically heralded by a complaint of strong rigid erections (either natural or with oral medications or penile injections) that quickly wane during intercourse.

Rings are available in a variety of sizes, shapes, and materials that together impart varying levels of penile constriction. Additionally, rings have varying designs to allow easier application and removal, a useful feature for men with limited dexterity. When purchasing a penile tension rings (and not a complete VED), it is important for men to purchase an appropriate application system that includes a loading cone and penile bushing/cylinder to allow for easy deployment.

Little research has focused on the success or satisfaction with penile tension rings alone or in conjunction with oral/injectable erectile medications. The major limiting factor for men using constriction rings is, again, cumbersome application of a mechanical device during sexual activity. Some men may experience mild discomfort with the ring in place or sensitivity to the material. If the ring is causing significant pain, it is likely not sized appropriately. Men may also complain that ejaculation is obstructed or limited with the ring in place. Blood gas analyses have demonstrated that penile ischemia begins approximately 30 min after applying the constriction ring, therefore it is not recommended to leave the device in place longer due to risk of necrosis and gangrene (Yuan et al., 2010). Metal rings should be avoided because they can require complex removal when associated with penile edema or swelling.

Urethral Suppositories

In an effort to avoid more invasive medication delivery to the penis, namely injections, intra-urethral suppositories were developed to deliver a vasoactive agent locally to the penis. As described later, alprostadil (a synthetic form of prostaglandin E1) was initially utilized as an injectable treatment for men with ED. Alprostadil acts by directly increasing cAMP levels, leading to arterial vasodilation and penile erection. The medication was placed in a dissolvable urethral suppository to work locally at the level of the penis without requiring a needle. MUSE® (Medicated Urethral System for Erections) was introduced as the first and only FDA approved alprostadil urethral suppository medication in 1996, 2 years before sildenafil would be approved.

The patient is required to initially urinate before using the urethral suppository to lubricate the urethra. The suppository comes in an applicator (Fig. 2) that is gently placed into the urethra and the deployment button pushed to release the suppository (Fig. 3). After removing the applicator, the penis should be rolled firmly for 10 s to assist in proper setting, dissolution, and distribution of the medication equally along the urethral epithelium. Pharmacologically, the medication acts through local diffusion of prostaglandin into the corporal tissues to initiate arteriolar vasodilation and penile erection. Erection typically occurs within 5–20 min after deployment. MUSE® is available in 125, 250, 500, and 1000 mcg dosages.

Urethral suppositories, like VEDs, are typically utilized as a second-line treatment choice for ED but can be used as a first-line treatment in select individuals (e.g., those with contraindications to phosphodiesterase inhibitors). After initial dose titration, 40%–65% of men with ED reported adequate erections for intercourse at home (Costabile et al., 1998; Padma-Nathan et al., 1997). Studies demonstrated success in men with ED secondary to various organic causes, though the series reporting up to 65% success excluded poorly controlled diabetics and spinal cord injury patients. Long-term urethral suppository compliance is poor with upwards of 30% of users discontinuing treatment within the first year (Nandipati et al., 2006).

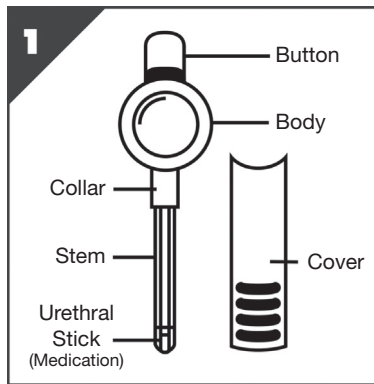


Fig. 2 Device for intraurethral placement of prostaglandin E1. Image courtesy of Mylan Pharmaceuticals Inc.

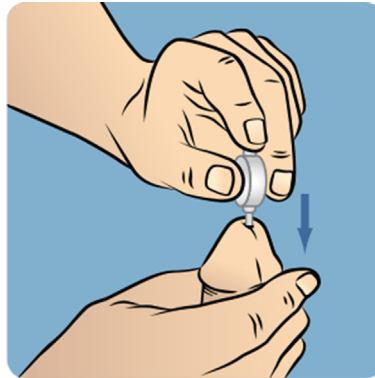


Fig. 3 Method of placement of intraurethral suppository. Image courtesy of Mylan Pharmaceuticals Inc.

Side effects from the use of urethral suppositories tend to be minimal. Men often experience mild discomfort with initial medication deployment with 5% reporting urethral trauma (Padma-Nathan et al., 1997). Nearly one third of men have penile pain or burning with intra-urethral alprostadil that is more pronounced at higher dosages. Rarely men may report dizziness thought to be secondary to the vasodilatory properties of the medication although no patients experienced clinical hypotension in the cited study. While less cumbersome than a VED, some men may find the suppository off-putting in its disruption to the natural flow of a romantic encounter. The same could be said of penile injections, however urethral suppositories do require extra effort to dissolve and distribute the medication. They are very expensive as well.

Intracavernosal Injections

Intracavernosal injection (ICI) therapy refers to direct injection of a vasodilatory medication or combination of medications into the corpus cavernosum to induce an involuntary erection. The technique was discovered in 1977 when papaverine was inadvertently injected into the cavernosum during a surgical shunting procedure and induced a rigid erection in a patient with known ED (Michael et al., 1997). After further pharmaceutical research, alprostadil for ICI was FDA-approved in 1995 and became the first prescription medication available for men with ED. ICI remains an attractive and highly successful option today for men desiring natural appearing erections with suboptimal efficacy or poor tolerance of oral phosphodiesterase inhibitors.

While original ICI studies reported the use of papaverine, the first FDA approved agent was alprostadil. Since then, there has been an introduction of other agents, namely phentolamine and atropine, to the injectable armamentarium. Alprostadil remains the most commonly used *single-agent* injectable under the trade names Edex® and Caverject®. After injection, alprostadil triggers direct corporal artery vasodilation by increasing cAMP levels. Papaverine is a nonspecific phosphodiesterase inhibitor which similarly increases intracellular levels of cAMP and cGMP. Phentolamine is an alpha-1 adrenergic antagonist that opposes sympathetic-mediated vasoconstriction of cavernosal arterial inflow, effectively keeping the arteries open.

Alprostadil injection alone may be insufficient for some men with vascular etiologies of ED and, thus, combinations of the vasoactive agents above have been developed. While combination therapy is not FDA approved, many physicians prescribe ICI mixtures to compounding pharmacies for treatment of ED. The combinations are referred to as Bimix (papaverine and phentolamine), Trimix (alprostadil, papaverine, and phentolamine), and Quadmix (alprostadil, papaverine, phentolamine, and atropine) based on the number of components of each. The combination and concentration is selected by the prescribing physician based on the

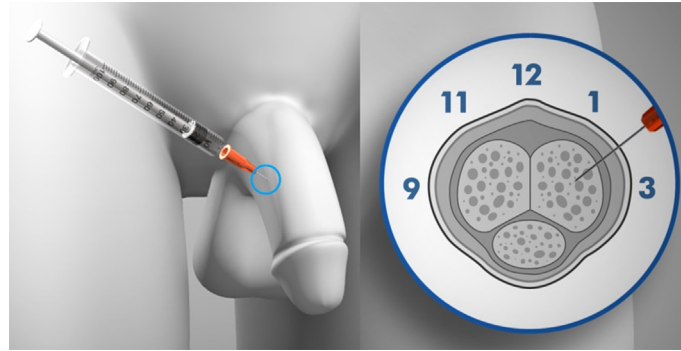


Fig. 4 Left: Schematic of intracavernosal injection of vasoactive agent. Right: Cross-sectional anatomy of injection placement. Image courtesy of OneMed Solutions.

severity of the patient's ED and medication side effect profile. Bimix, for example, does not include alprostadil which can cause burning penile discomfort in some men. While no standard dosing exists for these medications, they are typically implemented at low doses and titrated based on patient response.

Injectables are available in pre-filled auto-dosing syringes or standard medication vials. Patients should be instructed in proper medication preparation and cleaning of the skin with alcohol before injection. Only the amount prescribed should be injected, unless directed otherwise. The injection should be performed on the lateral aspect of the penile shaft, taking care to avoid visible veins and stay away from the glans and ventral (bottom side) penis where the urethra is and the dorsally (top side) where the blood vessels and nerves travel (Fig. 4). Temporary pressure should be held on the injection site with erection typically occurring within 5–15 min.

ICI is an ideal second-line treatment choice for men that have not achieved adequate results with other first-line treatment options (oral medications, VED, or urethral suppository) or is unable to tolerate associated side effects. Injections are very efficacious with approximately 85% of users achieving adequate erections after dose titration (Hanash, 1997). Despite high success rates, the discomfort with penile injection and other side effects lead many men to stop ICI therapy with data showing 40% stopping injections in the first year alone (Mulhall et al., 1999).

Side effects of penile pain, bleeding, and bruising are common but tend to be mild and well tolerated. Additional possible complications include penile burning, scar tissue, or penile curvature. Injection sites should be rotated in an effort to avoid scar formation. Priapism, or an erection lasting longer than 4 h, can occur if too much medication is injected and represents a *medical emergency*. When an erection remains rigid over an extended period of time, there is a lack of oxygenated blood flow and the corpora become increasingly ischemic with risk of worsening future ED. Initial testing and dose titration in the office is highly recommended to ensure a safe and effective transition to home ICI therapy.

Topical Therapies

Topical therapies for ED include various creams and gels that are applied to the penile skin or urethral meatus to induce an erection. Available products are composed of the familiar vasoactive agents discussed earlier. Vitaros[®] contains a prostaglandin E1 cream for perimeatal application and is commercially available throughout Europe for treatment of ED. There are no FDA approved topical treatments in the United States as of the writing of this article though some compounding pharmacies offer a gel formulation of Trimix. When the patient wishes to use the product (Vitaros[®], Trimix, or other), the product is gently applied with an applicator or finger to the penile skin (typically the glans) or meatus as directed. The glans is massaged with meatal application to promote absorption. Penile erection should occur within 5–30 min of application.

The theory behind topical creams/gels is that the vasoactive agents will be able to diffuse across the penile or urethral epithelium and enter the corpora cavernosum where they can induce erection. Active medications may be coupled to a fatty acid or ester meant to promote local diffusion through the urethral epithelium. Topical agents were developed in an effort to utilize local vasoactive medications while avoiding the discomfort and inconvenience of an injection or urethral suppository.

Several randomized controlled trials have demonstrated that topical therapies may serve a role in the treatment of ED (Hellstrom and Anaissie, 2016). Results are generally modest but men report a significant improvement in the ability to achieve intercourse with both subjective and objective increases in erectile function. A small percentage of men may experience local irritation with redness or pain at the site of application, and, rarely, the patient's partner may report similar symptoms secondary to transference during intercourse. Though exceedingly rare, symptomatic hypotension has been reported. This treatment is not utilized very often in general practice.

Low-Intensity Extracorporeal Shock Wave Therapy (LI-ESWT)

Extracorporeal shockwave therapy, known in urology for years for its ability to fracture stones, has shown some application in the form of low-intensity extracorporeal shockwave therapy (LI-ESWT) in the fields of orthopedics, trauma, and cardiac disease. It exerts

its effects via stimulation of vascular endothelial growth factor upregulation of the NO cascade, promoting angiogenesis. Treatment utilizes a focused electrohydraulic, electromagnetic, or linear piezoelectric shockwave lithotripter to apply 600–3000 low-intensity shocks along the shaft and crura of the penis. While treatment protocols have varied between series, patients have generally received individual treatments several times per week over a course of a few weeks.

Eight randomized controlled trials have evaluated the efficacy of LI-ESWT on improvements in erectile quality using validated questionnaires and scales of erectile hardness. A meta-analysis of available studies demonstrated a modest but significant improvement in erectile quality scores while the most recently published double-blinded, sham-controlled trial showed no benefit (Clavijo et al., 2017; Fojecki et al., 2017). As of yet, the efficacy of LI-ESWT has not been established and therefore is not widely offered unless under a research protocol. Due to possible angiogenic benefits, some LI-ESWT trials have specifically targeted phosphodiesterase inhibitor non-responders and post-prostatectomy ED with further research needed.

Side effects are rare with those reported including mild penile pain, irritation, and ecchymosis. The most efficacious treatment schedule still needs to be determined but will likely involve numerous treatments spaced out over several weeks. Many men may find the time required for therapy to be onerous and perhaps prohibitive for such a marginal benefit. The durability of any response to treatment is largely unknown. Future trials in LI-ESWT are still required to determine optimal shockwave strength, depth of penetration, and delivery schedule, and ultimately whether or not it has a place in the treatment algorithm for ED.

Summary

Many men move on from oral medications due to inadequate results or unwanted side effects such as headaches, flushing, or sinus congestion. In such cases, direct application therapies including VEDs, penile tension rings, urethral suppositories, ICI, or other investigational treatments are typically recommended. VEDs and penile tension rings represent mechanical options while urethral suppositories and ICI target vasodilatory medications to the corpora cavernosum. Each choice has proven efficacy with the highest success rates and most natural erections reported with ICI. Men should be encouraged to have an open dialogue with their physician about ED symptoms and how direct application therapies may improve their sexual quality of life while minimizing side effects. Treatment with topical agents (outside Europe) and LI-ESWT is still investigational but may offer an additional therapeutic strategy in the future.

References

- Baniel, J., Israilov, S., Segenreich, E., & Livne, P. M. (2001). Comparative evaluation of treatments for erectile dysfunction in patients with prostate cancer after radical retropubic prostatectomy. *BJU International*, 88, 58–62.
- Clavijo, R., Kohn, T., Kohn, J., & Ramasamy, R. (2017). Effects of low-intensity extracorporeal shockwave therapy on erectile dysfunction: a systematic review and meta-analysis. *Journal of Sexual Medicine*, 14(1), 27–35.
- Costabile, R. A., Spevak, M. A., Fishman, I., et al. (1998). Efficacy and safety of transurethral alprostadil in patients with erectile dysfunction following radical prostatectomy. *Journal of Urology*, 160(4), 1325–1328.
- Feldman, H. A., et al. (1994). Impotence and its medical and psychosocial results of the Massachusetts Male Aging Study. *Journal of Urology*, 151(1), 54–61.
- Fojecki, G., Tiessen, S., & Osther, P. (2017). Effect of low-energy linear shockwave therapy on erectile dysfunction – a double-blinded, sham-controlled, randomized clinical trial. *Journal of Sexual Medicine*, 14, 106–112.
- Hanash, K. A. (1997). Comparative results of goal oriented therapy for erectile dysfunction. *Journal of Urology*, 157, 2135–2138.
- Hellstrom, W., & Anaissie, J. (2016). Clinical use of alprostadil cream in patients with erectile dysfunction: a review. *Research and Reports in Urology*, 8, 123–131.
- King, J. (1874). *The American Family Physician: a guide to domestic health*. Cincinnati: Longley Brothers.
- Michael, V., Kramar, R., & Pospichal, J. (1997). Arterial epigastrico cavernous anastomosis for the treatment of sexual impotence. *World Journal of Surgery*, 1, 515–520.
- Montague, D. K. (2002). Nonpharmacologic treatment of erectile dysfunction. *Reviews in Urology*, 4, S9–S16.
- Mulhall, J. P., Jahoda, A. E., Cairney, M., et al. (1999). The causes of patient dropout from penile self-injection therapy for impotence. *Journal of Urology*, 162, 1291–1294.
- Nandipati, K. C., Raina, R., Agarwal, A., et al. (2006). Erectile dysfunction following radical retropubic prostatectomy: epidemiology, pathophysiology and pharmacological management. *Drugs & Aging*, 23(2), 101–117.
- Oakley, N., & Moore, K. T. (1998). Vacuum devices in erectile dysfunction: indications and efficacy. *British Journal of Urology*, 82, 673–681.
- Padma-Nathan, H., Hellstrom, W. J., Kaiser, F. E., et al. (1997). Treatment of men with erectile dysfunction with transurethral alprostadil. *New England Journal of Medicine*, 336, 1–7.
- Yuan, J., Hoang, A., Romero, C., et al. (2010). Vacuum therapy in erectile dysfunction – science and clinical evidence. *International Journal of Impotence Research*, 22, 211–219.

Further Reading

- Baltaci, S., et al. (1995). Treating erectile dysfunction with a vacuum tumescence device: a retrospective analysis of acceptance and satisfaction. *British Journal of Urology*, 76(6), 757–760.
- Bosshardt, R. J., et al. (1995). Objective measurement of the effectiveness, therapeutic success and dynamic mechanisms of the vacuum device. *British Journal of Urology*, 75(6), 786–791.
- Ganem, J. P., et al. (1998). Unusual complications of the vacuum erection device. *Urology*, 51(4), 627–631.
- Linnet, O. I., & Ogrinc, F. G. (1996). Efficacy and safety of intracavernosal alprostadil in men with erectile dysfunction. The alprostadil study group. *The New England Journal of Medicine*, 334(14), 873–877.
- McMahon, C. G., Samali, R., & Johnson, H. (1999). Treatment of intracorporeal injection nonresponse with sildenafil alone or in combination with triple agent intracorporeal injection therapy. *Journal of Urology*, 162(6), 1992–1997.

- Mulhall, J. P., et al. (1999). The causes of patient dropout from penile self-injection therapy for impotence. *Journal of Urology*, 162(4), 1291–1294.
- Nehra, A., et al. (2002). Rationale for combination therapy of intraurethral prostaglandin E(1) and sildenafil in the salvage of erectile dysfunction patients desiring noninvasive therapy. *International Journal of Impotence Research*, 14, S38–S42.
- Prabhu, V., et al. (2013). Long-term satisfaction and predictors of use of intracorporeal injections for post-prostatectomy erectile dysfunction. *Journal of Urology*, 189(1), 238–242.
- Shabsigh, R., et al. (2000). Intracavernous alprostadil alfadex is more efficacious, better tolerated, and preferred over intraurethral alprostadil plus optional actis: a comparative, randomized, crossover, multicenter study. *Urology*, 55(1), 109–113.
- Virag, R. (1982). Intracavernous injection of papaverine for erectile failure. *Journal of Urology*, 167, 1196.
- Virag, R., et al. (1991). Intracavernous self-injection of vasoactive drugs in the treatment of impotence: 8-year experience with 615 cases. *Journal of Urology*, 145(2), 287–292.
- Wein, A. J., Kavoussi, L. R., Campbell, M. F., & Walsh, P. C. (2015). *Campbell-Walsh urology*. Philadelphia: Elsevier.

Relevant Websites

- [http://www.auanet.org/guidelines/erectile-dysfunction-\(2005-reviewed-and-validity-confirmed-2011\)](http://www.auanet.org/guidelines/erectile-dysfunction-(2005-reviewed-and-validity-confirmed-2011))—American Urologic Association guideline on treatment of erectile dysfunction.
- <http://www.issm.info>—International Society for Sexual Medicine.
- <http://www.sexhealthmatters.org/>—Sex Health Matters.
- <http://www.smsna.org/V1/index.php>—Sexual Medicine Society of North America.

Medical Therapy of Peyronie's Disease

Alexandra J Berger and Martin N Kathrins, Harvard University, Cambridge, MA, USA

© 2018 Elsevier Inc. All rights reserved.

Introduction

Oral and intralesional medical therapies have been studied extensively in the treatment of Peyronie's Disease (PD). While there are no FDA approved oral therapies, pentoxifylline, colchicine, potassium aminobenzoate and co-enzyme Q10 all have some level of supportive evidence. Intralesional injections including collagenase clostridium histolytica (CCH), interferon α -2b, and to a lesser degree verapamil have also been investigated and found to have different levels of documented benefit. Mechanical traction has limited efficacy as an adjunct to these therapies.

PD begins with repeated microvascular trauma to the penile shaft caused by penile buckling in the erect or semi-erect state; this most often occurs during sexual activity. This trauma is typically minor and in many cases patients cannot recall any specific event. Following this repeated trauma, a cascade is initiated where there is significant extravascular protein deposition, fibrin trapping, macrophage recruitment and release of elastase. After a period of time, there is significant local scarring which leads to plaque formation and curvature of the penis.

The natural history of PD is variable; in many patients symptoms may resolve on their own with data suggesting about 90% of patients have improvement in pain one year after symptom onset. In about 50% of men, penile curvature either stabilizes or resolves at one year with the remainder of patients experiencing curvature worsening after the subacute period.

Oral Medications

Oral therapies used to treat PD are intended to reverse or reduce the underlying inflammatory pathophysiology of the condition. There is limited evidence to support the use of any of the currently existing oral medications which have been studied. The 2015 American Urological Association guidelines on Peyronie's disease listed pentoxifylline, colchicines, potassium aminobenzoate and co-enzyme Q10 as "possibly promising without sufficient evidence."

Pentoxifylline is a xanthine derivative nonselective phosphodiesterase inhibitor which is used to treat muscle pain in people with peripheral arterial disease. It has also been used off label to reduce fibrosis in a variety of conditions. Two recent studies—one randomized and one observational—demonstrated efficacy in the management of PD. However, data remains limited.

Colchicine is another anti-inflammatory medication that has been applied to the management of PD. Traditionally used to treat gout, the medication inhibits microtubular polymerization and therefore mitosis, and reduces neutrophil mobility and activity which leads to a net anti-inflammatory effect. While there is some data to support its use in the management of PD, a randomized placebo controlled study has not yet been performed.

Potassium aminobenzoate (Potaba) has been used since the late 1950s in the management of PD and has been deemed by the FDA as "possibly effective" in the treatment of the condition. The exact mechanism of the anti-fibrotic effects of this agent are as yet unknown, but they are believed to increase MAO-mediated serotonin degradation. While there have been two studies demonstrating its efficacy, its use is significantly limited by a poor side effect profile which includes nausea, anorexia, photosensitivity, anxiety, and difficulty concentrating.

Co-enzyme Q10 is a vitamin-like, anti-oxidant substance found in all aerobic eukaryotic cells and is a component of the electron transport chain participating in cellular respiration. The fully reduced form of Co-enzyme Q10 (ubiquinol) is also known to inhibit oxidative damage to polyunsaturated fatty acids and therefore exert an anti-inflammatory effect. Co-enzyme Q10 has been shown in one randomized control trial to reduce curvature and plaque size in patients with PD. However, there was no improvement in pain in these patients (Safarinejad, 2010).

Vitamin E, tamoxifen, procarbazine, omega 3 fatty acids and L-carnitine have all been investigated and found to be ineffective for the management of PD. Vitamin E (tocopherol) is an antioxidant which acts by scavenging oxygen free radicals; it was hypothesized that these antioxidant effects reduce fibrosis. While it is inexpensive, safe and has been used for this indication since the 1940s, recent literature does not support its use. Tamoxifen, a nonsteroidal antiestrogen, showed promise in one early 1990s study of PD. However subsequent placebo controlled reports on the medication have yet to demonstrate efficacy even when used in combination with other agents. Procarbazine is an alkylating chemotherapeutic agent used to manage CNS lymphoma, Hodgkin's lymphoma and high-grade gliomas. It was noted to have anti-fibrotic effects when used in Dupuytren's contracture and given the pathophysiologic similarities between this disorder and PD, it was subsequently studied for the latter condition. Unfortunately none of these studies demonstrated any efficacy and the extremely poor side effect profile of myelosuppression, hepatotoxicity, fatigue and GI distress limited additional investigation. Omega-3 fatty acids have known anti-inflammatory effects and are very well-tolerated but have been shown in multiple investigations, including a randomized placebo controlled trial, to have no benefit in patients with PD. L-Carnitine is an acetyl coenzyme A inhibitor which decreases free radical formation and therefore inflammation during times of stress. While it initially showed promise in reducing penile curvature, more robust trials did not demonstrate improvement with this medication.

Intralesional Therapy

Injection of intralesional therapies, including collagenase, interferon α -2b, and verapamil, is the mainstay of medical treatment for PD. Currently, only collagenase is FDA approved for PD, however all three medications have been shown to be both effective and relatively well tolerated.

Intralesional Collagenase *Clostridium Histolytica*

Intralesional collagenase *clostridium histolytica* (CCH) is currently marketed under the trade name Xiaflex. It was FDA approved for the treatment of PD in December 2013 for patients with palpable plaques and stable penile curvature greater than or equal to 30° at the start of therapy. CCH is an enzyme produced by the bacterium *clostridium histolytica* which breaks down collagen. It consists of a mixture of two collagenases, AUX-I and AUX-II. These act synergistically to cleave tropocollagen at different locations. CCH injections are considered extremely safe from a systemic standpoint because the enzymes do not enter the bloodstream. Instead, it is presumed that they stay at the point of injection where they are broken down by proteases. The medication is also approved for use in Dupuytren's contracture.

The earliest studies investigating CCH for PD began in the 1980s. The most robust data for CCH comes from two identical phase 3, randomized, double-blind, placebo-controlled studies—IMPRESS I & II—which demonstrated a 34% improvement in curvature when compared to placebo, who had only an 18.2% improvement. The average curvature reduction in existing literature is about 17°.

A single CCH treatment cycle consists of two injections which are given 48–72 h apart. A clinician must then perform modeling of the penis on a third office visit, and instruct the patient on how to do this himself at home. The modeling technique consists of two actions. First, there is a stretching activity performed when the penis is flaccid and a straightening exercise (gentle manual bending of the penis in the opposite direction of the curve) which is undergone when the penis is erect. The patient must perform daily modeling at home for approximately 6 weeks after they are instructed by their physician. Patients may receive a maximum of four cycles of CCH injections with approximately 6 weeks in between cycles. Patients are counseled not to have sexual intercourse in between the first and second injections of the treatment cycle and for 2 weeks after the second injection.

Patients most likely to benefit from CCH injections are those with stable disease, curvature between 30° and 90°, and with adequate erectile function either with or without the use of erectile aids. It is not as effective in men with calcified plaques. Furthermore, if the patient's predominant symptoms are pain or erectile dysfunction, CCH injections are unlikely to be of benefit.

The lack of systemic absorption of CCH injections makes this medication generally safe. The majority of patients experience penile ecchymosis but this is generally self-resolving. Other frequently reported mild or moderate adverse events include penile pain and penile swelling. Less than 1% of patients experience the more serious complications of penile hematoma requiring surgical intervention and corporal rupture. In studies, even these serious complications rarely required surgical intervention. In the IMPRESS trials, < 10% of treated patients either withdrew from treatment or were lost to follow-up.

Interferon α -2b

Interferon (INF) α -2b is an immunomodulator and cellular proliferation suppressor which is approved for management of hairy cell leukemia, follicular cell lymphoma, AIDS-related Kaposi sarcoma, malignant melanoma and chronic hepatitis B & C. Interferon α -2b reduces fibroblast proliferation and collagen synthesis, which have a natural application in PD considering its pathophysiology. It was first studied for use in PD in the mid-1990s and was shown in observational studies to stabilize or reduce plaque size in these patients. One subsequent randomized controlled trial demonstrated curvature improvement of around 13° (versus placebo improvement of 9°) as well as decreased plaque size, penile pain and penile hemodynamics. However, other randomized and observational studies have had mixed results with only some demonstrating benefit. Interferon has not been shown to improve erectile function. The current recommendation is that interferon α -2b injections are appropriate for patients with curvature > 30° without calcified plaques who have good erectile function (Nehra et al., 2015).

Interferon α -2b is injected intralesionally with frequencies ranging from once every 2 weeks to three times weekly. Treatment is typically for 12–24 weeks. Doses in the literature range from 1×10^6 to 5×10^6 units.

Unlike CCH injections, interferon α -2b has systemic effects. 40–100% of patients in the existing literature reported sinusitis and flu-like symptoms consisting of fever, chills and arthralgias. Typically, these effects are self-limiting and last < 48 h and can be managed with NSAIDs. Other adverse events, as with other intralesional therapies, include penile swelling and ecchymosis.

Verapamil

Verapamil is a calcium channel inhibitor and traditionally used to treat cardiac arrhythmias and angina. Calcium channel blockers are also known to alter the metabolism of fibroblasts, decrease collagen secretion and stimulate collagenase activity. Because of this, verapamil was proposed as a locally injectable agent for the management of PD. While the initial observational data on verapamil intralesional therapy was promising, the current evidence is weak. Multiple randomized controlled trials and observational studies have been performed with mixed results. While there is modest evidence to suggest verapamil improves penile pain and decreases curvature, many of the studies were performed on patients in the acute phase of PD when the clinical course is more uncertain and

variable. It is therefore difficult to extrapolate from this body of evidence to patients suffering from chronic PD. Current guidelines suggest verapamil can be considered in patients with more acute PD with the acknowledgement that the data to support its use is weak. Nicardipine is also under investigation, but is not currently recommended use in PD.

The dosing and frequency of verapamil administration varies widely in the literature. One typical regimen is a formulation of 10 mg of verapamil in 10 mL of normal saline injected into the Peyronie's plaque 6–12 times with 1–2 weeks in between each injection. It is suggested that the practitioner puncture the skin once, manipulating the needle in different directions to deliver the medication throughout the plaque. It is thought that the mechanical effect of manipulating the plaque with the injection needle is therapeutic in and of itself. Verapamil is considered very safe. Side effects consist primarily of penile hematoma and injection site discomfort. This is mostly due to the large volume of injection required. Systemic effects such as dizziness, nausea, loss of libido, weakness and sweating are rarely observed.

Other Intralesional Agents

There are limited studies on other intralesional agents for management of PD. However, none of these are widely used and they are largely investigational at this stage. Liposomal recombinant human superoxide dismutase (LrhSOD) has been found to be effective in very limited literature. Other medications under investigation include intralesional parathyroid hormone, steroids and iloprost. The existing data for these therapies is all observational and cannot be extrapolated to the population at large given small sample sizes and lack of control for natural history of the disease. There is also a body of literature on the use of intralesional agents in combination with oral agents, but this data is similarly varied and underpowered.

Mechanical Therapies

Penile traction therapy (PTT) has been investigated in multiple studies to treat PD either alone or in combination with oral or intralesional therapy. A penile traction device is composed of a plastic ring that fits around the glans of the penis and is attached to two movable rods that run down the sites of the penis. These rods apply tension longitudinally along the penile shaft, gently pulling and straightening over a period of months. Older data suggested modest improvement in penile length in patients who perform penile traction for 4.5–5 h per day. However, more recent studies have not found improved outcomes when traction is utilized in combination with CCH intralesional and other therapies. Nevertheless, these devices are commercially available for patient use and additional studies are ongoing.

Extracorporeal Shock Wave Therapy (ESWT)

Shockwave therapy has been studied in PD patients for many years. This treatment modality has been shown to be effective in management of orthopedic disorders such as epicondylopathies and given the pathophysiologic similarities between these conditions and PD it was hoped that ESWT would be similarly effective in patients with PD. While early data was promising, the bulk of the literature has not demonstrated efficacy of ESWT for PD. Many studies have failed to show improvement in curvature and plaque size. There is limited data to suggest ESWT may improve penile pain in patients with PD, but this may be an effect of the natural history of PD in that many patients initially have pain but this improves over time.

ESWT is associated with frequent adverse events which include localized petechial bleeding or bruising, urethral bleeding or transient hematuria and ecchymosis. A more severe risk is penile pain which has been reported in 2–4% of patients. Because of the poor side effect profile and lack of efficacy in reducing curvature and plaque size, ESWT is only recommended for well-selected patients to manage pain associated with PD.

Conclusion

Medical treatment modalities for PD include oral therapies, intralesional injections, penile traction therapy and ESWT. To date, intralesional CCH remains the mainstay of care for PD with only limited data to support the other therapeutic non-surgical options.

Further Reading

- Akkus, E., Carrier, S., Rehman, J., et al. (1994). Is colchicine effective in Peyronie's disease? A pilot study. *Urology*, 44, 2291.
- Akman, T., Sanli, O., Uluocak, N., et al. (2011). The most commonly altered type of Peyronie's disease deformity under oral colchicine treatment is lateral curvature that mostly shifts to the dorsal side. *Andrologia*, 43, 128.
- Aliperti, L. A., & Mehta, A. (2016). Peyronie's disease: Intralesional therapy and surgical intervention. *Current Urology Reports*, 17(9), 60.
- Alizadeh, M., Karimi, F., & Fallah, M. R. (2014). Evaluation of verapamil efficacy in Peyronie's disease comparing with pentoxifylline. *Global Journal of Health Science*, 6, 23.
- Biagiotti, G., & Cavallini, G. (2001 Jul). Acetyl-L-carnitine vs tamoxifen in the oral therapy of Peyronie's disease: A preliminary report. *BJU International*, 88(1), 63–67.

- Cakan, M., Demirel, F., Aldemir, M., et al. (2006). Does smoking change the efficacy of combination therapy with vitamin E and colchicines in patients with early-stage Peyronie's disease? *Archives of Andrology*, 52, 121.
- Carson, C. C., III, Sadeghi-Nejad, H., Tursi, J. P., Smith, T. M., Kaufman, G. J., Gilbert, K., et al. (2015). Analysis of the clinical safety of intralesional injection of collagenase clostridium histolyticum (CCH) for adults with Peyronie's disease (PD). *BJU International*, 116(5), 815–822. <https://doi.org/10.1111/bju.13120>.
- Chong, W., & Tan, R. B. W. (2016). Injectable therapy for Peyronie's disease. *Translational Andrology and Urology*, 5(3), 310.
- Dang, G., et al. (2004). Intralesional interferon- α -2B injections for the treatment of Peyronie's disease. *Southern Medical Journal*, 97(1), 42–47.
- Dhillon, S. (2015). Collagenase clostridium histolyticum: a review in Peyronie's disease. *Drugs*, 75(12), 1405–1412. <https://doi.org/10.1007/s40265-015-0441-7>.
- Essayan, D. M. (2001). Cyclic nucleotide phosphodiesterases. *The Journal of Allergy and Clinical Immunology*, 108(5), 671–680. <https://doi.org/10.1067/mai.2001.119555.11692087>.
- Gelbard, M., Goldstein, I., Hellstrom, W. J., McMahon, C. G., Smith, T., Tursi, J., et al. (2013). Clinical efficacy, safety and tolerability of collagenase clostridium histolyticum for the treatment of peyronie disease in 2 large double-blind, randomized, placebo controlled phase 3 studies. *The Journal of Urology*, 190(1), 199–207.
- Gelbard, M. K., et al. (1993). Collagenase versus placebo in the treatment of Peyronie's disease: A double-blind study. *The Journal of Urology*, 149(1), 56–58.
- Gelbard, M., et al. (2013). Clinical efficacy, safety and tolerability of collagenase clostridium histolyticum for the treatment of peyronie disease in 2 large double-blind, randomized, placebo controlled phase 3 studies. *The Journal of Urology*, 190(1), 199–207.
- Gontero, P., Di Marco, M., Giubilei, G., et al. (2009). Use of penile extender device in the treatment of penile curvature as a result of Peyronie's disease. Results of a phase II prospective study. *The Journal of Sexual Medicine*, 6, 2558.
- Kadioglu, A., Tefekli, A., Koksali, T., et al. (2000). Treatment of Peyronie's disease with oral colchicine: Long-term results and predictive parameters of successful outcome. *International Journal of Impotence Research*, 12, 169.
- Levine, L. A., Newell, M., & Taylor, F. L. (2008). Penile traction therapy for treatment of Peyronie's disease: A single-center pilot study. *The Journal of Sexual Medicine*, 5, 61468.
- Martinez-Salamanca, J. I., Egui, A., Moncada, I., et al. (2014). Acute phase Peyronie's disease management with traction device: A nonrandomized prospective controlled trial with ultrasound correlation. *The Journal of Sexual Medicine*, 11, 506.
- Minhas, S., & Mulhall, J. (Eds.). (2017). *Male Sexual Dysfunction: A Clinical Guide Custom Reprint*. John Wiley.
- Nehra, A., Alterowitz, R., Culkin, D. J., Faraday, M. M., Hakim, L. S., Heidelbaugh, J. J., et al. (2015). Peyronie's disease: AUA guideline. *The Journal of Urology*, 194(3), 745–753. <https://doi.org/10.1016/j.juro.2015.05.098>.
- Oosterlinck, W., & Renders, G. (1975). Treatment of Peyronie's disease with procarbazine. *British Journal of Urology*, 47, 219–220.
- Prieto Castro, R. M., Leva Vallejo, M. E., Regueiro Lopez, J. C., et al. (2003). Combined treatment with vitamin E and colchicine in the early stages of Peyronie's disease. *BJU International*, 91, 6522.
- Ralph, D. J., et al. (1992). The treatment of Peyronie's disease with tamoxifen. *BJU International*, 70(6), 648–651.
- Safarinejad, M. R., Hosseini, S. Y., & Kolahi, A. A. (2007a). Comparison of vitamin E and propionyl-L-carnitine, separately or in combination, in patients with early chronic Peyronie's disease: A double-blind, placebo controlled, randomized study. *The Journal of Urology*, 178, 1398–1403.
- Safarinejad, M. R. (2010). Safety and efficacy of coenzyme Q10 supplementation in early chronic Peyronie's disease: A double-blind, placebo-controlled randomized study. *International Journal of Impotence Research*, 22, 5298.
- Safarinejad, M. R. (2009). Efficacy and safety of omega-3 for treatment of early-stage Peyronie's disease: A prospective, randomized, double-blind placebo-controlled study. *The Journal of Sexual Medicine*, 6, 1743–1754.
- Safarinejad, M. R., Hosseini, S. Y., & Kolahi, A. A. (2007b). Comparison of vitamin E and propionyl-L-carnitine, separately or in combination, in patients with early chronic Peyronie's disease: A double-blind, placebo controlled, randomized study. *The Journal of Urology*, 178(4), 1398–1403.
- Smith, J. F., Shindel, A. W., Huang, Y. C., et al. (2011). Pentoxifylline treatment and penile calcifications in men with Peyronie's disease. *Asian Journal of Andrology*, 13, 2322.
- Strebel, R. T., et al. (2004). Extracorporeal shockwave therapy for Peyronie's disease does not correct penile deformity. *International Journal of Impotence Research*, 16(5), 448–451.
- Teloken, C., et al. "Tamoxifen versus placebo in the treatment of Peyronie's disease." *The Journal of Urology* 162.6 (1999): 2003–2005.
- Park, T. Y., et al. (2016). The efficacy of medical treatment of Peyronie's disease: potassium para-aminobenzoate monotherapy vs. combination therapy with tamoxifen, L-Carnitine, and phosphodiesterase type 5 inhibitor. *The World Journal of Men's Health*, 34(1), 40–46.
- Wegner, H. E., Andresen, R., Knipsel, H. H., et al. (1995). Treatment of Peyronie's disease with local interferon- α 2b. *European Urology*, 28, 236–240.
- Wegner, H. E., et al. (1997). Local interferon- α 2b is not an effective treatment in early-stage Peyronie's disease. *European Urology*, 32(2), 190–193.
- Wegner, H. E., et al. (1995). Treatment of Peyronie's disease with local interferon- α 2b. *European Urology*, 28(3), 236–240.
- Yafi, F. A., et al. (2015). The effect of duration of penile traction therapy in patients undergoing intralesional injection therapy for Peyronie's disease. *The Journal of Urology*, 194(3), 754–758.

TREATMENT: SURGICAL

Overview: Surgical Treatment and Male Reproductive Medicine

Samuel Ohlander, University of Illinois-Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Over the last 20 years, diagnostic advancements allowing for improved patient selection and refinement of surgical technique have revolutionized male reproductive care. We now know that non-obstructive azoospermia does not preclude a man from fathering biologic offspring. Our improved understanding of genetics has allowed us to counsel our patients as to the possibility of successful retrieval versus those in whom operative measure would be futile. Furthermore, utilization of the operative microscope in surgical sperm extraction allows for targeted retrieval with visualization of gravid tubules.

On the other hand, 5% of azoospermic men have an obstructive etiology ([Fertil Practice Committee, 2008](#)). Up to 6% of vasectomized men will seek reversal ([Sandlow and Nagler, 2009](#)). Reconstruction, along with sperm retrieval, remains at the cornerstone of male reproductive care. Our better understanding of vasal fluid aids intraoperative decision making. We continue to seek diagnostic criteria to provide preoperative predictors of success, though, unfortunately, an ideal formula still evades us. Vasoepididymostomy technique has undergone refinement from fistula creation to single-tubule end to side anastomosis to triangulation to two-suture intussusception with patency rates reaching 70%–90% and lower shut-down rates, though surgeon experience remains a critical factor ([Chan et al., 2005](#); [Kolettis and Thomas, 1997](#); [Schiff et al., 2005](#)).

A number of chapters in this section will focus on the surgical management of subfertility. Topics will range from reconstruction to oncofertility. At the crux of all discussion it must be highlighted that as our abilities evolve, so must our counseling. Intracytoplasmic sperm injection (ICSI) has allowed for fertilization with a single sperm. Many of the obstacles associated with severe male factor infertility may now be bypassed, though our understanding of associated genetic consequences is incomplete. Furthermore, more men are surviving cancer, subsequently living longer and desiring families. Future reproductive potential is required in survivorship discussions.

Other chapters will discuss the surgical management of erectile pathology. While reproductive potential is not dependent on erectile function, it does play a significant role in natural conception and sexual satisfaction. Penile prosthetics have been an established treatment option for refractory erectile dysfunction for over 40 years. Modifications have been made over time, but the basic technology and surgical principals have been preserved. Patient and partner satisfaction rates following implantation are incredibly high, owing not only to our surgical abilities, but perhaps more importantly to our patient selection and counseling. The same preoperative measures must be emphasized for management of penile curvature. Advancements in medical therapy for Peyronie's Disease has led to less utilization of operative management, yet surgery continues to have a significant role. Patient understanding of the surgical goal of functionality is paramount.

Finally, further chapters will focus on the very sensitive subjects of sexual development and gender reassignment. This text is not meant to impose ethical beliefs, but simply discuss surgical considerations. Though mentioned throughout this overview, patient selection and appropriate preoperative counseling may be of greatest importance here. For all aspects of reproductive care, physicians must be sensitive to the struggles of the patient. Issues of self-worth and personal identity correlate strongly with reproductive health. Our clinical evaluation and surgical management must not merely focus on what we have the ability to do, but rather what offers our patients the greatest potential for improved function and quality of life while maintaining minimal risk.

Ultimately, reproductive physicians must continue to invest time and resources into science, to better understand the pathologies we treat and the implications of our clinical and technologic advancements.

References

- Chan, P. T., Brandell, R. A. & Goldstein, M. (2005). Prospective analysis of outcomes after microsurgical intussusception vasoepididymostomy. *BJU International*, 96, 598–601.
- Fertil Practice Committee of American Society of Reproductive Medicine in collaboration with Society for Male Reproduction and Urology. (2008). The management of infertility due to obstructive azoospermia. *Fertility and Sterility*, 90, 121S–124S.
- Kolettis, P. N. & Thomas, A. J. Jr. (1997). Vasoepididymostomy for vasectomy reversal: A critical assessment in the era of intracytoplasmic sperm injection. *Journal of Urology*, 158, 467–70.
- Sandlow, J. I., & Nagler, H. M. (2009). Vasectomy and vasectomy reversal: Important issues. Preface. *Urologic Clinics of North America*, 36, xiii–xiv.
- Schiff, J., Chan, P., Li, P. S., Finkelberg, S., & Goldstein, M. (2005). Outcome and late failures compared in 4 techniques of microsurgical vasoepididymostomy in 153 consecutive men. *Journal of Urology*, 174, 651–655 (quiz 801).

Treatment: Surgical—Current Tools

Kevin A Ostrowski, University of Washington School of Medicine, Seattle, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Surgical treatment of male infertility involves three main objectives: improving fertility, identification and procurement of testicular sperm or restoration of fertility through vasectomy reversal. Data on these treatments is mostly retrospective in nature and all treatments will be discussed and evaluated.

Surgical Techniques to Improve Fertility

Varicocelectomy

Varicocele's are found in 15% of men and increases to 40% of men seen for a fertility evaluation (Clarke, 1966). The indications for treatment of a palpable varicocele is testicular pain, abnormal semen parameters with desire for fertility, and testicular atrophy on the same side as the varicocele. Newer articles have evaluated treating larger varicoceles to improve testosterone however this is provider preference (Practice Committee of the American Society for Reproductive Medicine, Society for Male Reproduction and Urology, 2014).

There are three main surgical treatment options for varicocelectomy: laparoscopic, subinguinal and retroperitoneal. The nonsurgical treatment option is coil embolization normally done by interventional radiology. Laparoscopy has the risk of entering the abdominal cavity and retroperitoneal varicocelectomy has a higher recurrence rate with most surgeons using an inguinal or subinguinal approach with use of optical magnification and Doppler ultrasound (US) of the artery (Practice Committee of the American Society for Reproductive Medicine, Society for Male Reproduction and Urology, 2014). The subinguinal approach is done through a 2 cm incision in the inguinal area. The cord is identified and the external and internal spermatic fascia are opened. The testicular artery is identified with use of a Doppler ultrasound and the lymphatics, artery and vas are preserved. All major veins are either clipped or tied. The incision is closed.

Infertility improvements are theorized to be from optimization of testicular temperature for spermatogenesis and reduction in reactive oxygen species. Significant data has shown improvement in DNA fragmentation after varicocelectomy (Smit et al., 2010; Alhathal et al., 2016). In addition, there are improvements in fecundity with natural pregnancy, intra-uterine insemination (IUI), and in-vitro fertilization (IVF) after varicocelectomy on the recent meta-analysis (Kirby et al., 2016).

Finally, varicocele surgery in non-obstructive azoospermic (NOA) patients is controversial but in published studies there can be sperm seen in the ejaculate in up 40% of men after varicocelectomy (Weedin et al., 2010). Even if patient's remain azoospermic after varicocele repair, there are improved sperm extraction rates (Kirby et al., 2016). However, not all patients will improve after surgery and it will take up to 3 months minimum to see effects of surgery making the partner's fertility very important in this decision making.

Vasectomy Reversal

6% of men who undergo vasectomy will have a vasectomy reversal (Sandlow and Nagler, 2009). At the time of vasectomy reversal fluid is evaluated from the testicular vas and the decision is made for vaso-vasostomy (VV) or vaso-epididymostomy (VE). There are many factors that go into the decision for VE including gross and microscopic fluid characteristics and length of time from vasectomy. If pasty fluid or creamy fluid with no sperm parts is encountered from the testicular vas then the decision is often made for a VE. This typically occurs with a longer interval from vasectomy (> 10 years) but it can happen at any time. By 15 years about 50% of vasectomy reversals have at least one side being a VE (Mui et al., 2014; Fuchs and Burt, 2002; Fuchs et al., 2016).

A VV has an overall >95% patency and >70% pregnancy rate (Ostrowski et al., 2015a; Belker et al., 1991). There is wide variability on published VE patency and pregnancy rates but newer studies show a patency rate of over 70% with a clinical pregnancy rate of around 50% (Chan, 2013; Schiff et al., 2005). Sperm is seen back in the ejaculate sooner with a VV than VE (over 75% of men have patency by 3 months). Often it will take longer to return sperm back in the ejaculate after VE and at 3 months 23% have sperm back vs. 62% at over one year (Yang et al., 2007).

If the couple has previously had children together the likelihood of pregnancy is much higher at over 80% (Ostrowski et al., 2015b). One of the most important factors associated with vasectomy reversal success is female partner age (Fuchs and Burt, 2002; Gerrard et al., 2007). There is no difference in patency or pregnancy rates in a modified one vs. two layer closure but some surgeons continue to do two layer closures to maintain the microsurgical skillsets required for VE (Nyame et al., 2016).

The procedure involves identification of the vasectomy defect and cutting into the vasal lumen. After this there is an evaluation of the vasal fluid from the testicular side and the decision for VV vs. VE is made. Patency of the abdominal vas is evaluated with flushing the vas. A VV anastomosis is performed under the operative microscope utilizing 9-0 and/or 10-0 suture. If a VE is required, the epididymis is evaluated to identify full tubules (ideally as distal on the epididymis as possible to increase tubule size). A specific epididymal tubule is identified and the vas is connected most commonly using a Berger end to side technique (Berger, 1998) or an intussusception technique (Chan, 2013).

Identification of Testicular Sperm

Fine Needle Aspiration (FNA) Testicular Mapping

NOA men are some of the most difficult patients to treat as they can have very small areas of spermatogenesis in the testicle, which combined with the use of ICSI, can cause a pregnancy. Identification of which men have sperm is difficult. In 1965 the first FNA of the testicles were performed on infertile men (Beliveau and Turek, 2011). FNA mapping of the testicle has good correlation with testicular biopsy (Beliveau and Turek, 2011).

FNA mapping includes up to 18 sites (Fig. 1) on each testicle after a spermatic cord block. Each site is evaluated for sperm production and patients are identified as sertoli-cell only, minimal spermatogenesis, or extensive spermatogenesis. Sertoli-cell only patients have very low probability of success with micro-TESE and various adoption options are discussed. Patients with very minimal spermatogenesis are offered micro-TESE knowing that the likelihood of success is almost 100% of finding sperm. Finally, patients with extensive spermatogenesis patients are offered TESA/TESE (Fig. 2).

FNA testicular mapping is performed under local anesthesia in the office typically. It involves bilateral spermatic cord blocks and scrotal skin blocks. A 23-gauge, 1" needle is inserted into a cameco holder and inserted into the template location. Suction is applied and 5–10 mm in-and-out movements are performed. The suction is released after 10–20 needle passes at each site and tissue fragments are expelled onto a slide, smeared, and immediately fixed in 95% ethyl alcohol. These slides are subsequently evaluated for spermatogenesis.

A clinical care pathway has been developed and when all FNA sites/map showed sperm success at TESA was 98% finding sperm, if > 2 sites showed sperm then directed TESE success was 90% finding sperm, finally if < 2 sites showed sperm then the micro-TESE success was 81% finding sperm (Arredondo et al., 2004). This allows for improved patient counseling and resource allocation by the patients before IVF.

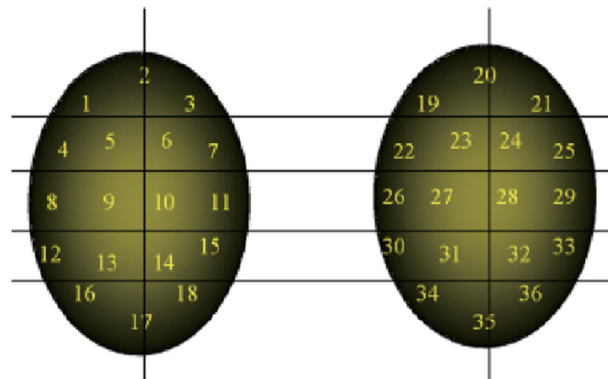


Fig. 1 A template FNA map of each testicle. Beliveau ME, Turek PJ. The value of testicular “mapping” in men with non-obstructive azoospermia. *Asian J Androl.* 2011;13(2):225–230.

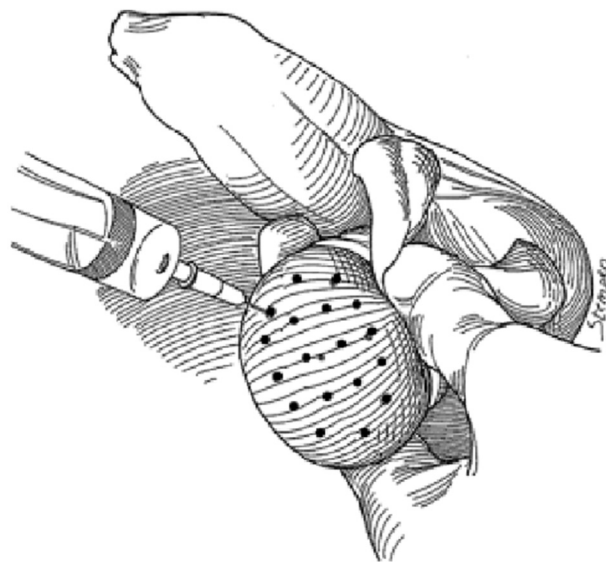


Fig. 2 A schematic drawing of FNA mapping with the testicle wrapped for fixation and the template drawn on the scrotal skin. Beliveau ME, Turek PJ. The value of testicular “mapping” in men with non-obstructive azoospermia. *Asian J Androl.* 2011;13(2):225–230.

Testicular Sperm Aspiration/Extraction (TESA/TESE)

Removal of testicular tubules allows for pregnancy with the use of ICSI. TESA or TESE can be successfully used to remove testicular tubules for use with ICSI in certain azoospermic patients. This is successful in patients who have obstructive azoospermia (OA) (Schoor et al., 2002) and sometimes in patients with NOA. Published reports of success with NOA ranges from 17% to 45% success of finding sperm (Deruyver et al., 2014). If FNA mapping shows sperm in all locations with significant sperm production then TESA or focused TESE can be successfully performed (Arredondo et al., 2004).

The procedure is performed after spermatic cord and local skin block. A TESA uses a large bore angio-catheter connected with extension tubing to a syringe. Suction is applied through a syringe to extract testicular tissue. This can be performed multiple times following the lobular planes of the testicle allowing for adequate tubule removal. The advantage of TESA is that it utilizes a minimally invasive approach allowing for improved patient recovery. There is no need to close the scrotal skin or tunica albuginea as this will close in it's own. A TESE uses an incision through the scrotal skin, dartos, tunica vaginalis and tunica albuginea. This allows for easy removal of testicular tissue but does require closure of all the above listed layers with absorbable suture.

Percutaneous/Microsurgical Epididymal Sperm Aspiration (PESA/MESA)

There is controversy regarding differences in IVF/ICSI success using epididymal and testicular sperm. A 2004 meta-analysis (Nicopoullos et al., 2004) and a 2008 Cochran review (Proctor et al., 2010) failed to identify any success differences between testicular or epididymal sperm.

Sperm from patients with OA from prior vasectomy, congenital bilateral absence of the vas deferens, or infection, can be obtained from epididymis for use with IVF/ICSI. PESA can be performed in the office under local and has an 80%–100% successful sperm retrieval rate depending on the study. MESA is performed ideally in the operating room and has a 95%–100% successful sperm retrieval rate with 15%–42% total motility depending on the study but is more invasive (Bernie et al., 2013).

After spermatic cord and local skin block, a PESA uses a small butterfly needle (usually 19 or 21 gauge) inserted through the skin transversely into the epididymis. With application of gentle suction epididymal fluid can be removed. This fluid is evaluated in real time to identify motile sperm for use with IVF/ICSI.

A MESA is performed in the operating room utilizing an operating microscope. An incision is made through the scrotal layers down to the epididymis. The epididymis is then examined under the operating microscope to identify full tubules. These tubules are opened and the fluid evaluated for sperm for IVF/ICSI (Fig. 3).

Micro-testicular sperm extraction (micro-TESE)

First described by Schlegel in 1999 (Schlegel, 1999), and has quickly become the gold standard for patients with Klinefelter's syndrome, NOA, or patients with very small quantities of sperm found on FNA mapping. Results vary from 43% to 70% depending on the report and circumstances (Deruyver et al., 2014).



Fig. 3 Depiction of MESA.

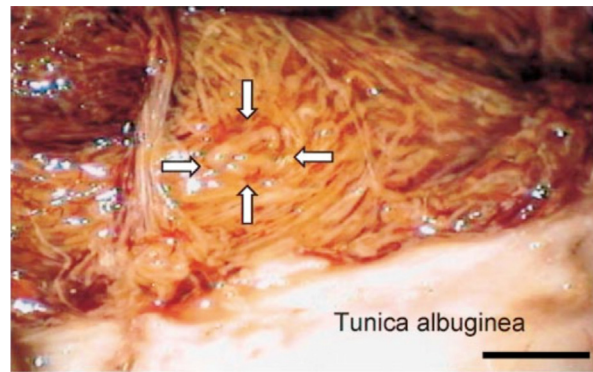


Fig. 4 Isolated area from original Micro-TESE article showing area of normal tubules surrounded by atrophic tubules.

This procedure is performed in the operating room with the use of an operative microscope. The testicle is delivered and the tunica albuginea is opened. The testicle is then sequentially evaluated with the use of an operative microscope for full seminiferous tubules (Fig. 4). These tubules are minced and evaluated for sperm in real time for use with IVF.

Conclusion

There are three main objectives for surgical treatment for male infertility: improving fertility, identification and procurement of testicular sperm or restoration of fertility through vasectomy reversal. There are many different modalities of surgical treatment for male infertility patients and the discussion, decision making and patient factors are often complex and stressful.

References

- Alhathal, N., Gabriel, M. S., & Zini, A. (2016). Beneficial effects of microsurgical varicocelectomy on sperm maturation, DNA fragmentation, and nuclear sulfhydryl groups: A prospective trial. *4*, 1204–1208.
- Arredondo, S., Shen, S. H., & Conaghan, J. T. P. (2004). A clinical care pathway for nonobstructive azoospermia based on testis biopsy and mapping data. *Fertility and Sterility*, *82*, S86.
- Beliveau, M. E., & Turek, P. J. (2011). The value of testicular “mapping” in men with non-obstructive azoospermia. *Asian Journal of Andrology*, *13*(2), 225–230.
- Belker, A. M., Thomas, A. J., Fuchs, E. F., Konnak, J. W., & Sharlip, I. D. (1991). Results of 1,469 microsurgical vasectomy reversals by the Vasovasostomy Study Group. *The Journal of Urology*, *145*(3), 505–511.
- Berger, R. E. (1998). Triangulation end-to-side vasoepididymostomy. *The Journal of Urology*, *159*, 1951–1953.
- Bernie, A. M., Ramasamy, R., Stember, D. S., & Stahl, P. J. (2013). Microsurgical epididymal sperm aspiration: Indications, techniques and outcomes. *Asian Journal of Andrology*, *15*(1), 40–43.
- Chan, P. T. (2013). The evolution and refinement of vasoepididymostomy techniques. *Asian Journal of Andrology*, *15*(1), 49–55.
- Clarke, B. G. (1966). Incidence of varicocele in normal men and among men of different ages. *Journal of the American Medical Association*, *198*(10), 1121–1122.
- Deruyver, Y., Vanderschueren, D., & Van der Aa, F. (2014). Outcome of microdissection TESE compared with conventional TESE in non-obstructive azoospermia: A systematic review. *Andrology*, *2*(1), 20–24.
- Fuchs, E. F., & Burt, R. A. (2002). Vasectomy reversal performed 15 years or more after vasectomy: Correlation of pregnancy outcome with partner age and with pregnancy results of in vitro fertilization with intracytoplasmic sperm injection. *Fertility and Sterility*, *77*(3), 516–519.
- Fuchs, M. E., Anderson, R. E., Ostrowski, K. A., Brant, W. O., & Fuchs, E. F. (2016). Pre-operative risk factors associated with need for vasoepididymostomy at the time of vasectomy reversal. *Andrology*, *4*(1), 160–162.
- Gerrard, E. R., Sandlow, J. I., Oster, R. A., Burns, J. R., Box, L. C., & Kolettis, P. N. (2007). Effect of female partner age on pregnancy rates after vasectomy reversal. *Fertility and Sterility*, *87*(6), 1340–1344.
- Kirby, E. W., Wiener, L. E., Rajanahally, S., & Crowell, K. C. R. (2016). Undergoing varicocele repair before assisted reproduction improves pregnancy rate and live birth rate in azoospermic and oligospermic men with a varicocele: A systematic review and meta-analysis. *Fertility and Sterility*, *106*(6), 1338–1343.
- Mui, P., Perkins, a., Burrows, P. J., Marks, S. F., & Turek, P. J. (2014). The need for epididymovasostomy at vasectomy reversal plateaus in older vasectomies: A study of 1229 cases. *Andrology*, *2*, 25–29.
- Nicopoulos, J. D., Gillings-Smith, C., Almeida, P. A., Norman-Taylor, J. G. I., et al. (2004). Use of surgical sperm retrieval in azoospermic men: A meta-analysis. *Fertility and Sterility*, *82*(3), 691–701.
- Nyame, Y. A., Babbar, P., Almassi, N., Polackwich, A. S., & Sabanegh, E. (2016). Comparative cost-effectiveness analysis of modified 1-layer versus formal 2-layer vasovasostomy technique. *The Journal of Urology*, *195*(2), 434–438.
- Ostrowski, K. A., Polackwich, A. S., Conlin, M. J., Hedges, J. C., & Fuchs, E. F. (2015a). Impact on pregnancy of gross and microscopic vasal fluid during vasectomy reversal. *The Journal of Urology*, *194*(1), 156–159.
- Ostrowski, K. A., Polackwich, A. S., Kent, J., Conlin, M. J., Hedges, J. C., & Fuchs, E. F. (2015b). Higher outcomes of vasectomy reversal in men with the same female partner as before vasectomy. *The Journal of Urology*, *193*(1), 245–247.
- Practice Committee of the American Society for Reproductive Medicine, Society for Male Reproduction and Urology. (2014). Report on varicocele and infertility: A committee opinion. *Fertility and Sterility*, *102*(6), 1556–1560.
- Proctor, M., Johnson, N., Am, V. P., & Phillipson, G. (2010). Techniques for surgical retrieval of sperm prior to intra-cytoplasmic sperm injection (ICSI) for azoospermia (Review). *Cochrane Database of Systematic Reviews*, *2*, CD002807.

- Sandlow, J. I., & Nagler, H. M. (2009). Preface. *The Urologic Clinics of North America*, 36(3), xiii–xiv.
- Schiff, J., Chan, P., Li, P. S., Finkelberg, S., & Goldstein, M. (2005). Outcome and late failures compared in 4 techniques of microsurgical vasoepididymostomy in 153 consecutive men. *The Journal of Urology*, 174(2), 651–655. quiz 801.
- Schlegel, P. N. (1999). Testicular sperm extraction: microdissection improves sperm yield with minimal tissue excision. *Human Reproduction*, 14(1), 131–135.
- Schoor, R. A., Elhanbly, S., Niederberger, C. S., & Ross, L. S. (2002). The role of testicular biopsy in the modern management of male infertility. *The Journal of Urology*, 167(1), 197–200.
- Smit, M., Romijn, J. C., Wildhagen, M. F., Veldhoven, J. L. M., Weber, R. F. A., & Dohle, G. R. (2010). Decreased sperm DNA fragmentation after surgical varicocelectomy is associated with increased pregnancy rate. *The Journal of Urology*, 183(1), 270–274.
- Weedin, J. W., Khera, M., & Lipshultz, L. I. (2010). Varicocele repair in patients with nonobstructive azoospermia: a meta-analysis. *The Journal of Urology*, 183(6), 2309–2315.
- Yang, G., Walsh, T. J., Shefi, S., & Turek, P. J. (2007). The kinetics of the return of motile sperm to the ejaculate after vasectomy reversal. *The Journal of Urology*, 177, 2272–2276.

Surgical Treatment of Varicocele

David T Greenwald and Samuel Ohlander, University of Illinois-Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Clinical varicocele Varicocele that is palpable on physical examination with or without Valsalva.

Inguinal Area of the groin or lower abdominal wall, with the thigh inferiorly, the pubic tubercle medially, and the anterior superior iliac spine superolaterally.

Laparoscopic Surgical technique where operations are performed through small incisions using a long, thin fiber-optic instrument.

Valsalva maneuver Exhalation against a closed airway. Often referred to as simply “Valsalva” and commonly described as “bearing down.”

Introduction

Varicocele is dilated and/or incompetent veins that drain blood from the testicle, and has been associated with impairment of spermatogenesis and Leydig cell function. It is thought that venous pooling results in significant elevations in testicular temperature leading to progressive and duration-dependent testicular impairment. Varicoceles are found in ~15% of general population, 35% of men with primary infertility, 75%–81% of men with secondary infertility ([World Health Organization, 1992](#)).

Indications for Intervention

Therapeutic management of a varicocele is indicated for a symptomatic, clinically palpable varicocele. Classification of grade is based upon whether or not Valsalva maneuver is necessary for palpation, though all palpable varicoceles are considered clinically relevant ([Table 1](#)). Subclinical varicoceles, or those that are not palpable on exam but merely identified via imaging, have not been found to be clinically relevant and thus repair is not indicated ([Jarow et al., 1996](#)). Primary symptoms may be infertility, either primary or secondary, or varicocele induced pain. For those with infertility, most often there is a degree of spermatogenic dysfunction as identified by decreased counts on semen analysis. The classic stress pattern from a varicocele is oligoasthenoteratospermia, which includes decreased counts (oligo), poor motility (astheno), and poor morphology (terato) ([Saypol, 1981](#)). However, not all seminal abnormalities are present in each case. Management is also indicated in certain patients with varicocele induced pain refractory to more conservative measures. Counseling plays an important role in the management of varicocele-induced pain, as surgical repair does not guarantee resolution of the pain ([Pasqualotto et al., 2006](#)). Additionally, though very controversial at this point in time, the application of management to age related androgen deficiency has been described. The dose dependent impairment of Leydig cell dysfunction has been demonstrated when comparing testosterone levels in men with varicoceles to age matched controls.

In the pediatric population, varicocele repair is indicated when there is a palpable varicocele with testicular size discrepancy of greater than 15% between the affected testicle and the contralateral testicle ([Lipshultz and Corriere, 1977](#)). The affected testicle may undergo catch-up growth after repair of the varicocele ([Kass and Belman, 1987](#)). As the noxious effect of varicocele on spermatogenesis is dose related, varicolectomy in the adolescent is also considered to prevent future fertility issues. As it is uncertain which pediatric patients with varicoceles will have a fertility problem, repair in this scenario is still controversial.

Management options include observation, surgical intervention (scrotal, retroperitoneal, laparoscopic, inguinal, subinguinal, microsurgical), and non-surgical radiographic options (percutaneous venous occlusion, antegrade sclerotherapy).

Table 1 Varicocele grading ([Dubin and Amelar, 1970](#))

Subclinical	Not present clinically, abnormality only seen on ultrasound
Grade 1	Only present clinically when man performs Valsalva maneuver
Grade 2	Easily palpable, not visible
Grade 3	Visible through scrotal skin, easily palpable

Special Considerations

Some men who present for vasectomy reversal are found to have significant varicoceles. Varicocelectomy in this scenario presents a challenge. With ligation of the spermatic veins in varicocelectomy, the vasal veins function as the only remaining venous return, but after vasectomy the vasal veins are likely compromised. One must assure to preserve some avenue for venous return, as well as the testicular artery (the primary remaining blood supply). This is accomplished with the preservation of the cremasteric veins using the microsurgical technique. The recurrence rate, however, was noted to be higher with preservation of the cremasteric veins and periaarterial veins, when compared to non-vasectomized men where these veins were ligated. Instead of performing simultaneous vasectomy reversal and varicocelectomy, a safer approach is to proceed with vasovasotomy or vasoepididymostomy first, assess the semen quality post-op, and perform varicocelectomy at least 6 months later if necessary. This allows for arterial and venous neovascularization across the anastomotic line.

For infertile couples considering assisted reproductive techniques, there is some evidence varicocelectomy improves outcomes. A study on varicocelectomy before intrauterine insemination (IUI) showed that although there was a slight decrease in sperm motility after varicocelectomy, there was a significant increase in pregnancy and live birth rates. This retrospective review of couples with male factor infertility that underwent IUI included men with varicocele and abnormal semen parameters, and compared outcomes of varicocelectomy versus no varicocelectomy (Daitch et al., 2001). Similarly, infertile couples where the male had a varicocele who used IVF/ICSI have shown improved pregnancy and live birth rates after varicocelectomy when compared to the same cohort without varicocelectomy (Kirby et al., 2016).

As it takes 3 months for sperm to mature, there is a time lag after varicocelectomy to observe for improvement in semen quality. For older couples with infertility, this delay must be factored into decision-making on whether or not to pursue treatment, as assisted reproductive techniques may yield more immediate results without surgical repair of the varicocele. This is of particular importance to couples in which the female has advanced maternal age and/or limited ovarian reserve.

Surgical Intervention

Scrotal

Scrotal approaches for varicocelectomy have been described historically though rarely play a role in present day practice. Scrotal varicocelectomies involve a transscrotal incision with dissection down to the spermatic cord, isolation of the vasculature, and ligation and/or excision of the varicocele. These approaches ultimately were not successful as the testicular artery is entwined with the pampiniform plexus at the level of the scrotum, and there are a large number of veins necessary for complete treatment, often leading to incomplete ligations and high recurrence rates (see Table 2). Given the intimate venous/arterial relationship, testicular atrophy often resulted from damage to the testicular artery.

Open Retroperitoneal

The retroperitoneal approach for varicocelectomy involves ligation of the internal spermatic veins proximally, near the left renal vein or IVC. The incision is at the level of the internal ring, dissection through the external and internal oblique muscles, to achieve exposure of the testicular artery and veins in the retroperitoneum near the level of the ureter. At this point the artery is distinct and there are limited venous tributaries converging together to form the gonadal vein.

This approach is more commonly used in pediatrics, but largely has been replaced by more minimally invasive therapeutic options. It may be advantageous in patients with previous inguinal surgery, where there is a higher risk of testicular artery or ilioinguinal nerve injury. This approach has a few notable technical challenges when compared to alternative approaches. The approach involves working in a deep hole. The testicular artery can be as small as 1 mm in diameter and, thus, hard to identify. Use of Doppler is paramount, as it is in all surgical approaches to varicoceles. Additionally, the veins have to be identified and ligated within the

Table 2 Complication rates after varicocelectomy

<i>Surgical technique</i>	<i>Recurrence (%)</i>	<i>Hydrocele (%)</i>
Retroperitoneal ^a	15–45	3.0–6.4
Open inguinal ^b	5–15	3–30
Microscopic inguinal/subinguinal ^c	0.6–1.0	1.3–1.6
Laparoscopic ^d	3–18	12–22
Radiographic ^e	4–11	0

^aReitelman et al. (1987), Watanabe et al. (2005).

^bSzabo and Kessler (1984).

^cGoldstein et al. (1992), Glassberg et al. (2008).

^dGlassberg et al. (2008), Al-Said et al. (2008).

^eMitchell et al. (1985), Matthews et al. (1992).

retroperitoneum as inability to fully mobilize the vessels leaves the surgeon unable to deliver them through the wound. It is also challenging to correctly identify and preserve lymphatics, resulting in a post-op hydrocele rate of 7%–33% (see [Table 2](#); [Szabo and Kessler, 1984](#)).

The rate of varicocele recurrence after the retroperitoneal approach is ~15% (see [Table 2](#); [Watanabe et al., 2005](#)). It has been reported higher in pediatrics, with a recurrence rate of 15%–45% ([Reitelman et al., 1987](#)). One hypothesis is that the fine periaarterial plexus of veins around the testicular artery, which have been shown to communicate with the larger spermatic veins, is spared, resulting in retrograde flow and pooling of venous blood after ligation of the other larger tributaries. [Kass and Marcol \(1992\)](#) described intentional ligation of the testicular artery and the periaarterial veins to reduce recurrence rate. In children, intentional arterial ligation at time of retroperitoneal varicolectomy can lead to reversal of varicocele associated testicular atrophy, with collateral arterial supply supporting the testicle. Another potential source of recurrence is the presence of parallel inguinal or retroperitoneal collateral. These veins exit the testis and bypass the ligated retroperitoneal vein(s), re-joining the internal spermatic vein proximal to the ligation. A third hypothesized source of recurrence is dilated cremasteric veins, as these are not identified via the retroperitoneal approach and have been implicated as a cause of recurrent and persistent varicocele after surgical repair.

Laparoscopic

The laparoscopic varicolectomy, similar to open retroperitoneal approach, involves ligation of the internal spermatic veins proximally, near the left renal vein or IVC. Exposure of the internal ring is obtained via transperitoneal laparoscopic access. Visualization of the internal spermatic veins, the vas deferens, the testicular artery, and nearby lymphatics allows for careful ligation of the veins while preserving the neighboring structures. Sparing the lymphatics has led to reduction in post-op hydrocele formation in pediatric patients (see [Table 2](#); [Glassberg et al., 2008](#)).

The reported rate of recurrence for all patients is 2.9%–18% (see [Table 2](#); [Glassberg et al., 2008](#)). An advantage to the minimally invasive approach is that the same laparoscopic access can be used for bilateral varicocele repair. Laparoscopic single site has also been described for children and adults, with shorter post-op pain and recovery time with similar post-op semen quality and complication rate. This technique carries additional risks versus open technique, including damage to abdominal viscera. Furthermore, minimally invasive techniques carry increased cost when compared to open approaches, and modifications utilizing the operative robot have increased costs over that of standard laparoscopic management.

Inguinal

The open inguinal and subinguinal approaches allow access to the spermatic cord structures with the benefit of improved surgical visualization. The cord structures are mobilized and delivered through the incision to better identify the veins, artery, vas deferens, and lymphatics. In addition, the external spermatic and gubernacular veins can be identified and ligated. These can otherwise bypass the cord and be a potential source of recurrence. The traditional inguinal approach involves an incision over the inguinal canal, dissection through the external oblique aponeurosis, delivery of the spermatic cord by freeing cremasteric attachments, identification and isolation of the cord structures to ligate the spermatic veins and preserve the vas deferens/testicular artery/lymphatics. Incidence of post-op hydrocele is 3%–15% (see [Table 2](#); [Szabo and Kessler, 1984](#)), often attributed to inadvertent ligation of lymphatics. The rate of testicular artery injury is unknown, but as it is the largest artery to the testicle, injury can lead to testicular atrophy, even with preservation of the vasal and cremasteric arteries.

Surgical modification with utilization of the operative microscope for varicolectomy has led to marked improvement in rates of post-op hydrocele formation due to better identification and preservation of lymphatics, with a reported rate of post-op hydrocele less than 1% (see [Table 2](#); [Cayan et al., 2000](#)). In addition, the testicular artery can be better identified and preserved. Again, utilization of the microdoppler is paramount in classifying the vasculature correctly. The inguinal approach is preferred in pediatrics, solitary testes, tight/low external ring, short cord, or high lying testis. In pediatric patients, the testicular artery is especially small and thus harder to identify. Approaching the artery inguinally increases the surgeon's ability to correctly identify it as there have been limited branches compared to a more distal approach. Additionally, there are fewer venous branches as tributaries have coalesced to an often more manageable number of veins that require management.

The procedure starts with locating and marking the location of the external ring. The incision is made at the level of the external ring and carried down through Campers and Scarpas fascia using blunt dissection and electrocautery. In the inguinal approach the external oblique aponeurosis is cleaned off and opened to the external ring, parallel to the fibers. The ilioinguinal nerve is identified and gently swept away from the cord. The spermatic cord is then gently freed up and delivered through the incision by releasing the cremasteric attachments. Once delivered, the cord may be encircled with a penrose drain to elevate it from the canal, and also can be used to exclude the ilioinguinal nerve from the operative field.

Once the cord is delivered, the operating microscope is brought over the surgical field. Under magnification, the external and internal spermatic fascias are opened, and the cord is inspected using the microdoppler to identify the arteries. Identified arteries are dissected free from surrounding structures, and encircled with a vessel loop for identification. When an artery is difficult to identify, it is often found adherent to the underside of a large vein. Also the testicular artery can go into spasm from surgical manipulation, rendering the microdoppler ineffective. Application of papaverine irrigation, a vasodilator, can restore normal arterial pulsations and aid in identification. Veins are stripped free of lymphatics, and ligated with clips or 4-0 silk. Bipolar cautery may be utilized for smaller veins, especially those adherent to the testicular artery. The vas and the accompanying pair of vasal veins are

preserved. If the vasal vein is dilated beyond 3 mm, it is dissected free and ligated, as only one vasal vein is sufficient for testicular venous return. Cremasteric arteries are found in the majority of cases, usually between a pair of accompanying veins. The veins are to be ligated, and the artery preserved. At the conclusion of the dissection, the cremasteric muscle fibers, testicular arteries, cremasteric arteries, the vas and vasal vessels, lymphatics, and nerves are preserved.

Some surgeons elect to deliver the testicle in order to directly visualize and ligate scrotal or gubernacular collateral veins, as these have been identified as the cause of up to 10% of varicocele recurrence. With gentle upward traction on the cord, the testis can be delivered through the incision to directly visualize all venous drainage from the testicle. Once the testicle is delivered, external spermatic veins and gubernacular veins can be identified and ligated. If an incidental hydrocele is identified when the testicle is delivered, as is the case in 15% of varicoceles, it can be repaired concurrently. Of note there is an immediate and transient elevation of venous pressure after varicolectomy which increases the chance of post-op hematoma.

The external oblique is closed with running 3-0 absorbable suture. Then for either approach, closure of Scarpas fascia and Campers fascia with interrupted 3-0 absorbable suture. Skin closure with 4-0 or 5-0 running subcuticular monofilament absorbable suture, reinforced with steri strips or surgical skin glue. Patient is fitted with scrotal support and fluff dressing.

Subinguinal

In the subinguinal approach, the technique is simply a modification of the inguinal technique described above. The incision is made inferior to the external ring and access to the spermatic cord is obtained without opening the inguinal canal. The cord is identified, pulled cephalad, and delivered through the incision. Identification and ligation of the vessels proceeds as done in the inguinal approach. Given that this approach precludes the need for opening the external oblique aponeurosis, it often leads to less post-op pain and a more rapid recovery. However, at the subinguinal level there are more venous tributaries, and often the artery has divided into smaller branches. In addition the arterial pulsations can be dampened due to compression by the edge of the external ring, so care must be taken to assure the cord is investigated under relaxed exposure. Due to these complexities, the operating microscope is generally used for this approach. The subinguinal approach is preferred in patients with prior inguinal surgery, obesity, lax/high external ring, or circumstances of a long cord with a low-lying testis. After previous inguinal surgery, the spermatic cord is often scarred and adherent to the external oblique aponeurosis, thus placing the cord at risk of injury when opening the fascia in the inguinal approach.

Radiographic

Radiographic options also have been described for diagnosis and treatment of varicoceles. Prior to widespread use of microsurgical technique, intraoperative venography previously was used to better identify venous collaterals, which, if left untreated, could be the source of varicocele recurrence.

Radiographic balloon or coil occlusion of internal spermatic veins can be used for treatment of varicocele. It involves access to the femoral or internal jugular vein, via a small cut down incision w/local anesthesia, and cannulation of the internal spermatic veins to deploy the occlusion device. Sclerotherapy can be employed for occlusion of smaller collaterals. Successful treatment was accomplished in 75%–90% of patients undergoing radiographic occlusion with a 4%–11% rate of recurrence (see [Table 2](#); [Matthews et al., 1992](#)). There is no risk of testicular artery injury with this approach. Disadvantages of the procedure include a relatively high rate of initial failure (10%–25%), increased length of procedure (1–3 h for radiographic occlusion versus approximately 45 min for surgical repair), need for radiation exposure (which infertile patients often seek to avoid), and rare serious complications (migration of coil/balloon into renal vein causing loss of kidney, or pulmonary embolism of coil/balloon, femoral vein thrombosis) ([Matthews et al., 1992](#)).

Anterograde scrotal sclerotherapy for treatment of varicocele was originally described by Tauber in 1988 in Germany. It involves access via cannulation of a scrotal vein, with similar rates of success and failure as radiographic balloon dilation ([Minucci et al., 2004](#)).

Complications

Hydrocele is the most common complication after varicocele, ranging from 3% to 33% depending on the technique (see [Table 2](#)). [Szabo and Kessler \(1984\)](#) analyzed the protein composite of the hydrocele fluid and demonstrated that lymphatic obstruction was the likely source of post-varicolectomy hydrocele formation. The addition of the operating microscope enables a surgeon to improve the identification and preservation of lymphatics, which greatly reduces the risk of post-op hydrocele ([Glassberg et al., 2008](#)). There is no current data to suggest post-varicolectomy hydroceles negatively impact fertility, and, thus, they are managed the same as simple hydroceles.

The testicular artery supplies roughly two-thirds of the testicular blood supply, with the remaining third from collateral blood flow from the cremasteric and vasal arteries. Testicular artery injury can lead to testicular atrophy and loss. The true rate of testicular artery injury is unknown. The usage of operating magnification with a microscopic Doppler vastly improves the identification of the testicular artery and decreases the rate of injury. Children likely have a better potential for compensatory increased blood flow to the vasal and cremasteric vessels when compared to adults, and are less likely to have testicular atrophy after testicular artery ligation.

Recurrence of varicocele after varicolectomy ranges from 0.6% to 45% (Al-Kandari et al., 2007), but more specifically, can be stratified by operative approach (see Table 2). Recurrence after retroperitoneal reported 15%–45%, conventional inguinal 5%–15%, microscopic inguinal/subinguinal 1%, laparoscopic 3%–15%, radiographic 15%–25%. Recurrence in essence is from residual venous collaterals—periarterial, parallel inguinal or retroperitoneal, transscrotal have all been reported. The microsurgical approach with delivery of the testis, to address scrotal collaterals, lowers the incidence below 1%.

Results

Varicolectomy for male factor infertility results in significantly improved semen analysis in 60%–80% of men, and reported pregnancy rates of 20%–60% after varicolectomy (Marmar et al., 2007). In one review of recent studies on varicolectomy in male factor infertility, Richardson et al. found that the majority of studies showed an improvement in sperm density, motility, and morphology after varicolectomy. Despite the improvement in semen parameters, the reported average pregnancy rate was 39.35%, which is similar to previously reported fecundity outcomes (Richardson et al., 2008). A randomized trial of infertile men with varicoceles demonstrated a marked improvement in pregnancy rate 1-year post-op: a pregnancy rate of 44% in surgery group versus 10% in an untreated control group (Abdel-Meguid et al., 2011). For azoospermic men with palpable varicocele, undergoing a microsurgical varicolectomy lead to a return of sperm in 60% of subjects (Matthews et al., 1998). When comparing laparoscopic to microsurgical technique, one trial did not show any difference in fertility outcome (Bryniarski et al., 2017). However, a meta-analysis comparing microsurgical, laparoscopic, and open approaches showed significantly better pregnancy rate and semen parameters with the microsurgical approach (Yuan et al., 2017). For patients with varicocele pain, the majority of patients report improvement after varicolectomy. Yaman et al. (2000) found that 88% of patients had complete resolution of their pain. Also, varicolectomy in infertile men with low testosterone have resulted in improvement in serum testosterone levels.

Conclusion

The treatment of varicocele has improved overtime with technological advancement and the addition of new approaches, most recently the microsurgical technique. During this evolution there has been a measured improvement in outcomes and decrease in complications.

References

- Abdel-Meguid, T., Al-Sayyad, A., Tayib, A., et al. (2011). Does varicocele repair improve male infertility? An evidence-based perspective from a randomized, controlled trial. *European Urology*, 59(3), 455–461.
- Al-Kandari, A. M., Shabaan, H., et al. (2007). Comparison of outcomes of different varicolectomy techniques: open inguinal, laparoscopic, and subinguinal microscopic varicolectomy: a randomized clinical trial. *Urology*, 69(3), 417–420.
- Al-Said, S., Al-Naimi, A., et al. (2008). Varicolectomy for male infertility: a comparative study of open, laparoscopic and microsurgical approaches. *Journal of Urology*, 180(1), 266–270.
- Bryniarski, P., Taborowski, P., Rajwa, P., et al. (2017). The comparison of laparoscopic and microsurgical varicolectomy in infertile men with varicocele on paternity rate 12 months after surgery: a prospective randomized controlled trial. *Andrology*, 5(3), 445–450.
- Cayan, S., Kadioglu, T. C., Tefekli, A., et al. (2000). Comparison of results and complications of high ligation surgery and microsurgical high inguinal varicolectomy in the treatment of varicocele. *Urology*, 55(5), 750–754.
- Daltch, J. A., Bedaiwy, M. A., Pasqualotto, E. B., et al. (2001). Varicolectomy improves intrauterine insemination success rates in men with varicocele. *Journal of Urology*, 165(5), 1510–1513.
- Dubin, L., & Amelar, R. D. (1970). Varicocele size and results of varicolectomy in selected subfertile men with varicocele. *Fertility and Sterility*, 21, 606–609.
- Glassberg, K. I., Poon, S. A., et al. (2008). Laparoscopic lymphatic sparing varicolectomy in adolescents. *Journal of Urology*, 180(1), 326–330.
- Goldstein, M., Gilbert, B. R., et al. (1992). Microsurgical inguinal varicolectomy with delivery of the testis: an artery and lymphatic sparing technique. *Journal of Urology*, 148(6), 1808–1811.
- Jarow, J. P., Ogle, S. R., & Eskew, L. A. (1996). Seminal improvement following repair of ultrasound detected subclinical varicoceles. *Journal of Urology*, 155(4), 1287–1290.
- Kass, E. J., & Belman, A. B. (1987). Reversal of testicular growth failure by varicocele ligation. *Journal of Urology*, 137(3), 475–476.
- Kass, E. J., & Marcol, B. (1992). Results of varicocele surgery in adolescents: a comparison of techniques. *Journal of Urology*, 148(2 Pt. 2), 694–696.
- Kirby, E. W., Wiener, L. E., Rajanahally, S., et al. (2016). Undergoing varicocele repair before assisted reproduction improves pregnancy rate and live birth rate in azoospermic and oligospermic men with a varicocele: a systematic review and meta-analysis. *Fertility and Sterility*, 106(6), 1338–1343.
- Lipshultz, L. I., & Corriere, J. N., Jr. (1977). Progressive testicular atrophy in the varicocele patient. *Journal of Urology*, 117(2), 175–176.
- Marmar, J. L., Agarwal, A., et al. (2007). Reassessing the value of varicolectomy as a treatment for male subfertility with a new meta-analysis. *Fertility and Sterility*, 88(3), 639–648.
- Matthews, G. J., Matthews, E. D., et al. (1998). Induction of spermatogenesis and achievement of pregnancy after microsurgical varicolectomy in men with azoospermia and severe oligoasthenospermia. *Fertility and Sterility*, 70(1), 71–75.
- Matthews, R. D., Roberts, J., et al. (1992). Migration of intravascular balloon after percutaneous embolotherapy of varicocele. *Urology*, 39(4), 373–375.
- Minucci, S., Mazzoni, G., et al. (2004). Evolution in varicocele sclerosing treatment: the ante/retrograde (A/R) approach. *Archivio Italiano Urologia e Andrologia*, 76(1), 29–33.
- Mitchell, S. E., Shuman, L. S., et al. (1985). Biliary catheter drainage complicated by hemobilia: treatment by balloon embolotherapy. *Radiology*, 157(3), 645–652.
- Pasqualotto, F. F., Sobreiro, B. P., et al. (2006). Induction of spermatogenesis in azoospermic men after varicolectomy repair: an update. *Fertility and Sterility*, 85(3), 635–639.
- Reitman, C., Burbige, K. A., et al. (1987). Diagnosis and surgical correction of the pediatric varicocele. *Journal of Urology*, 138(4 Pt. 2), 1038–1040.

- Richardson, I., Grotas, A. B., & Nagler, H. M. (2008). Outcomes of varicocelectomy treatment: an updated critical analysis. *Urologic Clinics of North America*, 35(2), 191–209. viii.
- Saypol, D. C. (1981). Varicocele. *Journal of Andrology*, 2, 61–71.
- Szabo, R., & Kessler, R. (1984). Hydrocele following internal spermatic vein ligation: a retrospective study and review of the literature. *Journal of Urology*, 132(5), 924–925.
- Watanabe, M., Nagai, A., et al. (2005). Minimal invasiveness and effectivity of subinguinal microscopic varicocelectomy: a comparative study with retroperitoneal high and laparoscopic approaches. *International Journal of Urology*, 12(10), 892–898.
- World Health Organization. (1992). The influence of varicocele on parameters of fertility in a large group of men presenting to infertility clinics. *Fertility and Sterility*, 57, 1289–1293.
- Yaman, O., Ozdiler, E., Anafarta, K., et al. (2000). Effect of microsurgical subinguinal varicocele ligation to treat pain. *Urology*, 55, 107–108.
- Yuan, R., Zhuo, H., Cao, D., et al. (2017). Efficacy and safety of varicocelectomies: a meta-analysis. *Systems Biology in Reproductive Medicine*, 63(2), 120–129.

Further Reading

- Lee, J. S., Park, H. J., et al. (2007). What is the indication of varicocelectomy in men with nonobstructive azoospermia? *Urology*, 69(2), 352–355.
- Penn, I., Mackie, G., et al. (1972). Testicular complications following renal transplantation. *Annals of Surgery*, 176(6), 697–699.
- Sayfan, J., Adam, Y. G., et al. (1980). A new entity in varicocele subfertility: the cremasteric reflux. *Fertility and Sterility*, 33(1), 88–90.
- Steckel, J., Dicker, A. P., et al. (1993). Relationship between varicocele size and response to varicocelectomy. *Journal of Urology*, 149(4), 769–771.

Vasovasostomy

Christopher Wu, University of Toronto, Toronto, ON, Canada

Keith Jarvi, University of Toronto, Toronto, ON, Canada; Murray Koffler Urologic Wellness Centre, Toronto, ON, Canada; and Lunenfeld-Tanenbaum Research Institute, Toronto, ON, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Male Reproductive Tract Anatomy

The male reproductive tract is comprised of multiple parts, including the paired testes, the epididymis, vas deferens, seminal vesicles, ejaculatory ducts, and prostate. All these components serve to produce, transport, and deliver viable sperm to the female reproductive tract, which allows for human reproduction.

The testes are paired ovoid organs located in the scrotum, with a normal sized testis ranging from 15 to 25 mL in volume. Sperm are produced within numerous coiled tubes known as seminiferous tubules in the testis. Within the seminiferous tubules lie germ cells (spermatogonia, spermatocytes, and spermatids) and Sertoli cells which support the sperm maturation process. Aside from these tubules, there are testosterone producing cells in the testis called Leydig cells. The sperm after being produced and undergoing a maturation process are released from the seminiferous tubules to subsequently exit the testes for transportation through the epididymis.

The epididymis is a comma shaped structure composed of thin tubules that cups the posterior aspect of the testis. The sperm as they travel through the epididymis undergo numerous changes including the development of functional motility and the capacity to fertilize oocytes.

After maturing in the epididymis, the sperm enter the vas deferens, a tubular structure ranging 30–35 cm in length that carries sperm from the epididymis to the ejaculatory duct deep within the male pelvis. The external diameter of this structure ranges from 1.5–3 mm, with an internal (lumen) diameter ranging from 0.2–0.7 mm in diameter. It is generally easily palpated within the scrotal skin.

Fluid from the vas deferens, epididymis and testis is then joined by secretions from the seminal vesicles (SV) and prostate to form the ejaculate. From 65% to 80% of the volume of ejaculate fluid is made by the SV, while approximately 25% arises from the prostate. These secretions aid in support the sperm as they enter the female reproductive tract ([McDougal et al., 2011](#)).

Means of Male Birth Control

There are numerous methods of contraception, or preventing pregnancy, involving the male reproductive tract.

One of the simplest methods for contraception involves interrupting sexual activity and withdrawing the penis prior to ejaculation. This is known as coitus interruptus or the withdrawal technique. Studies have shown a failure rate of up to 24% per year with this method ([Black et al., 2015a](#)).

Another method of contraception is through a barrier method such as a condom. A condom is a flexible sheath that is slipped over the penis prior to intercourse. The sheath then prevents any ejaculated fluid from entering the female partner's body. Barrier contraception also has the added benefit of preventing the transmission of sexually transmitted diseases. The failure rate of condoms are 18% per year with typical use ([Black et al., 2015b](#)).

Vasectomy is the only present form of permanent male contraception. This procedure involves physically disrupting the vas deferens, blocking passage of any sperm from the testes into the ejaculatory duct. This procedure is usually done under local anesthetic and has an early failure rate of 1%–2% ([Sharlip et al., 2012](#)). After no sperm has been demonstrated on a semen analysis postvasectomy, late failure rates are 1 in 2000. This is generally a very well tolerated and safe procedure with no impact on sperm production, sexual functioning or testicular function.

History

The history of the vasectomy procedure is marred by controversy, with applications ranging from therapeutic, eugenic, to punitive ([Sheynkin, 2009](#); [Kim and Goldstein, 2009](#)). The first reported vasectomy for contraception was reportedly performed in 1899 by Dr. Harry Sharp, the chief physician at the Indiana Reformatory. This procedure was performed on an inmate as an alternative to stop excessive masturbation. A semen sample was performed after this and the inmate was found to be sterile ([Sheynkin, 2009](#)). Early on, aside from contraception, there were medically questionable indications for vasectomies, including preventing inflammation of the epididymis in the setting of prostate enlargement, or for purposes of mental rejuvenation. Furthermore, surgical sterilization was once used on the criminal, the insane or perverse, to prevent them from producing offspring. Mass sterilization was also performed by the Nazis during World War II for the purpose of racial limitation or extermination. Due to these political, religious, and medical implications, considerable interest in the vasectomy reversal (VR) procedure was raised in the 1940s, though this was still fraught with technical challenges and low success rates.

One of the first reported cases of a re-anastomosis of severed vasa deferens was by J. Lynn Thomas, a consulting surgeon from Cardiff in 1904 ([Thomas, 1904](#)). He described a patient that had both vas severed by his wife in a semicomatose state after a fit of

jealousy. An oblique union of the testicular vas to the urethral vas was described. The procedure resulted in a pregnancy, thus was considered a success.

Subsequently in 1919, Quinby published a report on a successful vasovasostomy in a man who had purposely undergone bilateral vas resections 8 years prior (O'Connor, 1948). This repair was repaired over a strand of silkworm gut as a stent and left in place for 10 days. Vincent O'Connor, the urologist who assisted Quinby, subsequently published on a series of 14 vasectomized patients utilizing the same technique, resulting in a patency rate of 64%.

Numerous techniques soon arose on macroscopic vas anastomoses, with varying suture materials and certain practices using intraluminal stents consisting of suture or steel wires. Up to a 50%–70% patency rate was reported in literature.

Sherman Silber, an American urologist was the first to report on the use of a microsurgical vasovasostomy (VV) in the USA in 1975 (Kim and Goldstein, 2009). He performed a two-layer anastomosis using 9–0 and 10–0 nylon sutures. Earl Owen, an Australian plastic surgeon also independently performed the first VV in Australia at around the same time. The development of the microsurgical technique has laid the foundation for modern microsurgical approaches.

Re-Establishing Fertility Following a Vasectomy

With the vasectomy established as a safe, effective, and voluntary method of birth control, approximately 500,000 vasectomies are performed per year in the United States (Eisenberg et al., 2009). Out of this population, up to 6% of this population will consider some technique to re-establish their fertility (Potts et al., 1999). Other causes of a “vasectomy” such as hernia repairs, trauma and infections are far less common causes of vasal obstruction.

Consideration to re-establish fertility following a vasectomy has been found to be most frequently requested due to divorce and remarriage. Other changes in social circumstances including death of a spouse, death of a child, or a change of mind about family size are also reported reasons for vasectomy reversal (Potts et al., 1999).

Re-establishing fertility following a vasectomy/vasal obstruction could be accomplished by a

- (1) vasectomy reversal or
- (2) sperm retrieval from the testis or epididymis coupled with injection of the sperm into an oocyte (intra-cytoplasmic sperm insertion or ICSI),

How to Choose Between a Vasectomy Reversal or Sperm Aspiration with ICSI to Re-Establish Fertility: Risks and Benefits

The patients choose based on their assessment of risks of the procedures, costs, success rates and personal preferences. Overall, pregnancy rates following a vasectomy reversal are between 40% and 90% (see below) while pregnancy rates following one cycle of sperm aspiration with ICSI are 16%–40% (Pavlovich and Schlegel, 1997). Based strictly on the medical cost per live birth, Schlegel et al. found that a vasectomy reversal had an average cost of \$25,475 versus \$72,521 for sperm retrieval-ICSI, factoring the costs of potential complications as well as delivery costs (Pavlovich and Schlegel, 1997). However, patients often choose between the two procedures not based on the cost per child alone, but on personal preferences (such as **timing**: pregnancies are usually earlier with ICSI than with vasectomy reversal or **risk assessment**: competing risks of more surgery with a vasectomy reversal compared to a sperm aspiration, but higher risks with a pregnancy established through ICSI than a spontaneous pregnancy).

A balanced presentation of the birth rates, risks of the procedures and costs helps couples decide to choose either vasectomy reversal or sperm aspiration with ICSI.

Prediction of Outcomes of VR Prior to Surgery

The main measures for technical and functional success in VV are patency rates and pregnancy rates respectively. In the era of microsurgical reconstruction, outcome studies for VV have shown patency rates ranging from 75% to 100% and pregnancy rates from 45% to 73% (Herrel et al., 2015; Belker et al., 1991). A recent study on microsurgical vasovasostomy for vasectomy reversal, looked at a total of 31 studies conducted from 1980 until 2014 and examined outcomes (Herrel et al., 2015). An overall patency rate of 89.4% was noted and a pregnancy rate of 73% was calculated from grouping all of these studies together, though these numbers should be interpreted with caution due to the varied definitions of patency and pregnancy.

Multiple studies have further identified possible factors that affect the success of VV. These can be further divided into factors prior to the surgery (preoperative factors) and factors identified at the time of surgery (intraoperative factors).

Preoperative factors that have been studied include the time elapsed since vasectomy (obstructive interval), prior VV, prior fertility, prior surgery in the groins, length of the vasal stump, and age of the partner.

One of the largest and most comprehensive studies on vasectomy reversal was the Vasovasostomy Study Group (VVSG), a multi-center, multi-surgeon study that aimed to look at outcomes of the procedure (Belker et al., 1991). In this study, one of the factors that independently influenced success rates of the vasectomy reversal from this study was obstructive interval. This study showed that if the VV is performed within 3 years from vasectomy, the patency rate was as high as 97%, with a pregnancy rate of 76%. At more than 15 years out from the vasectomy, the patency rate dropped to 71% and the pregnancy rate decreased to 30%.

The presence of prior vasectomy reversal also negatively impacts both patency and pregnancy rates, with a higher chance of converting to a more complex reconstruction, such as connecting the tubules in the epididymis to the vas directly in a procedure called

an vasoepididymostomy (VE) (Hollingsworth et al., 2007). The VVSG also found that in patients with repeat reversal procedures, patency rates dropped from 86% to 75% and pregnancy rates fell from 52% to 43% (Belker et al., 1991). Another study by Hernandez and Sabanegh found that there was a unilateral VE rate of 73% in a repeat vasectomy reversal procedure versus 4% in the initial procedure. They also found that patency and pregnancy rates were impacted with a repeat procedure, at 79% and 31% respectively (Hernandez and Sabanegh, 1999).

The length of the postvasectomy testicular vasal remnant has also been found to impact reversal success rates. One study showed that a testicular vasal remnant length of less than 2.7 cm predicted for an intraoperative finding of no whole sperm in the vasal fluid. In this study, patients with whole sperm found intraoperatively were found to have patency and pregnancy rates of 90% and 54% respectively, versus 60% and 30% respectively in those with no whole sperm found (Witt et al., 1994).

One of the most significant factors affecting pregnancy rates after vasectomy reversal is the age of the female partner. One study on vasectomy reversals found that though postoperative patency remained similar, pregnancy rates dropped from 56% to 14% if the female partner was 40 years of age or older (Gerrard et al., 2007). This should be taken into account when counseling patients about vasectomy reversal success rates, especially since it takes on average 12 months for conception to occur after a successful reversal.

The presence of a sperm granuloma, a lump created by extravasated sperm after the vasectomy, has been found to be an independent predictor of vasectomy reversal success, with a reported patency rate of 95% in men with at least a unilateral sperm granuloma versus 75% in those without. It is thought that sperm extravasation from the testicular end reflects venting of high pressure away from the epididymis. This has been shown to have a favorable impact on VV patency rates (Boorjian et al., 2004). Operative scars from prior scrotal and groin surgeries should also be assessed, as these may suggest previous, inadvertent injury to the vas.

Numerous intraoperative factors that predict success rate have been studied. These include the length of the vas left on the testicular end, the presence of an inflammatory response to the sperm after vasectomy (sperm granuloma), and surgeon experience.

One of the key intra-operative findings to provide prognosis of success of a VR is the finding of the “quality” of fluid and the microscopic findings of the vasal fluid.

As per the landmark VVSG study, the color and quality of the fluid expressed from the testicular end of the vas deferens can often predict microscopic findings of the vasal fluid (Belker et al., 1991). The results of both gross appearance and microscopic examination will inform the surgeon of how to proceed with the procedure. In the study, a grade ranging from 1 to 5 was given based on microscopic findings of the vasal fluid, with grade 1 showing mainly normal, motile (moving) sperm, grade 2 showing mainly normal, nonmotile sperm, grade 3 showing mainly sperm heads, grade 4 showing only sperm heads, and grade 5 showing no sperm at all. The patency rate and pregnancy rates based on fluid grading were also outlined in the VVSG study, with good success rates with VV for grades 1–4 (Table 1).

Vasectomy Reversal to Treat Postvasectomy Pain Syndrome

In patients with prior vasectomy, 1%–2% of patients will have chronic (lasting greater than 3 months) postprocedural scrotal pain or discomfort that interferes with quality of life and requires medical attention (Tan and Levine, 2016). The onset of this condition can range from 7 to 24 months after the procedure (Leslie et al., 2007). Signs and symptoms of this postvasectomy pain syndrome (PVPS) can include point tenderness of the vas and/or epididymis, induration or enlargement of the vas, and pain with straining and dyspareunia (Tan and Levine, 2016). This etiology for this condition still remains unclear, but one possible explanation is thought to be vasal congestion and backpressure (Nangia et al., 2000). It then seems intuitive that a vasovasostomy, which eliminates the pressure from vasal obstruction, would help with PVPS. Though current data is mostly from small series at single institutions, several studies have shown improvement in close to 100% of patients undergoing vasovasostomy for PVPS and complete resolution of pain in 50%–70% of patients (Nangia et al., 2000; Polackwich et al., 2015; Horovitz et al., 2012).

Procedure

The vasectomy reversal is one of the most technically demanding procedures in urology, requiring precise alignment of the inner diameter of the vas deferens. Given the delicate nature of the structures involved and the degree of precision required, optical magnification is necessary. Though some surgeons perform macrosurgical anastomoses with magnifying optical loupes, it is generally accepted that microsurgical reconstruction produces superior outcomes, thus microsurgical training with an operating microscope is recommended.

Table 1 Reported patency and pregnancy rates based on vasal fluid quality (Belker et al., 1991)

<i>Vasal fluid quality</i>	<i>Patency rate</i>	<i>Pregnancy rate</i>
Grade 1 (most normal, motile)	94.3%	63.2%
Grade 2 (most normal, nonmotile)	90.5%	53.9%
Grade 3 (most heads without tails)	95.7%	50%
Grade 4 (only heads without tails)	75.3%	44.3%
Grade 5 (no sperm)	60.2%	30.8%

With the vasovasostomy procedure itself, the basic surgical principles that apply to all connections of tubular structures remain the same. First off, precise realignment of the inner diameter of the vas is paramount. The alignment of the inner, or mucosal, layer of the vas deferens minimizes the risk of leakage of sperm and subsequent inflammatory reaction. The diameters of the testicular end and abdominal ends of the vas deferens may be markedly discrepant, especially with prolonged obstructive intervals, making this challenging. Furthermore, unlike blood vessels, there are no clotting factors or platelets present in vasal fluid, making a leak-proof connection all the more important, and entirely dependent on the quality of the suturing.

Other principles include careful, atraumatic handling of the ends of the vasa, with little room for error as improperly placed sutures may damage the ends, and improper tension on the sutures can easily tear the mucosa. It is also important to preserve the blood supply of the ends of the vas, supplied by a network of small blood vessels that run along the surface. Ideally the ends of the vas that are connected together should exhibit some bleeding, reflecting good blood supply which is necessary for proper healing. The connection between the vas also needs to be performed in a tension free manner. Studies have shown that with connections under tension, sperm may return in the ejaculate transiently, but ultimately the connection will scar down again and fail.

Numerous techniques exist for vasectomy reversals and ultimately the technique chosen depends on surgeon experience as well as intra-operative factors. The basic tenets of vasectomy reversal remain the same, with the aim to reestablish patency from the testicle to the ejaculatory duct.

In all cases of prior vasectomy, there will be a buildup of scar tissue at the site where the vas deferens was occluded. This scar is usually readily palpable through the scrotal skin. The approach to the opening in the scrotum varies (from mini-incision approaches to larger openings), but this approach does not seem to change the success rates of the surgery. Bilateral vertical scrotal incisions are then made at the level of the scar. A ring clamp can be used to immobilize the scar tissue and overlying skin to aid in dissection. Both the testicular and abdominal ends of the vas must subsequently be mobilized away from the scar. It is important to isolate a portion of the vas away from the scar that is healthy, this is the best chance to ensure a good quality connection. Once this is performed, fluid should be milked out from the testicular end and examined microscopically for the presence of sperm.

Once sperm is confirmed in the vasal fluid, the abdominal end of the vas may be evaluated by irrigating the cut end with sterile saline using a blunt, 25-gauge IV catheter tip. The free flow of fluid into the abdominal vas confirms patency. Most surgeons do not routinely perform a vasogram while performing a VR.

The two most common microsurgical techniques used to connect the ends of the vas deferens are either the modified one-layer anastomosis or two-layer anastomosis. Most surgeons will use nylon suture material for the connection as it is less likely to cause inflammation of the surrounding tissues. It is also important to use bipolar cautery to seal off bleeding vessels intraoperatively. Bipolar cautery is a method of electrical energy delivery to the tissues where the current runs only from one side of a pair of forceps to the other side. This allows for concentrated energy delivery to the target tissues and minimizes scattering of energy to undesired areas. This is especially important in vasectomy reversals as this helps to preserve the integrity of the tissues at the ends of the vas.

The two-layer anastomosis was the initial microsurgical technique described by both Silber and Owen ([Kim and Goldstein, 2009](#); [Silber, 1977](#)). In this technique, the inner diameter of the vasa are connected together first in a single layer comprising of approximately six circumferential sutures. The needle in each of these sutures enters the lumen of the vas and exits out partway along the cut edge, bringing together only the inner diameter. Subsequently, a second layer of circumferential sutures are placed just in the outer muscular layer of the vas deferens, bringing the outer diameter together.

A modified one-layer technique was subsequently first described by Ira Sharlip, whereby a full thickness suture is placed through the vasa at the 12, 3, 6, and 9 o'clock positions ([Sharlip, 1981](#)). The intervening spaces were then closed with a suture placed through the muscular layer only. This technique was found to shorten the OR time for the case.

As per the VVSG study, they compared the modified 1 versus 2 layer anastomoses and no differences in patency were discovered ([Belker et al., 1991](#)) ([Fig. 1](#)).

If no sperm is found on microscopic examination, even after flushing out the testicular end of the vas with a syringe tip (Grade 5), then vasoepididymostomy should be considered, though good results can still be obtained if the quality of the fluid is watery ([Belker et al., 1991](#)) ([Table 2](#)).

The epididymis should be carefully inspected under the microscope to look for dilated epididymal tubules, which are enlarged ducts within the epididymis that suggest an obstruction closer to the testicle. If dilated epididymal tubules are visualized, the abdominal vas deferens should be connected to an epididymal tubule, a procedure known as the vasoepididymostomy.

The vasoepididymostomy is one of the most technically demanding procedures in microsurgery. Absolute precision is necessary in order to create a connection between the diameter of a dilated epididymal tubule measuring 0.15–0.2 mm to the abdominal end of the vas deferens. In this procedure, a small opening is made in the tunica of the epididymis, revealing numerous epididymal tubules. After this, the end of the vas deferens is anastomosed to the side of the epididymal tubule using, in general, one of two techniques. With the intussusception technique, an opening in the side of an epididymal tubule is made and then the open tubule is pulled inside the end of the vas deferens and sutured in place with 10–0 nylon sutures ([Chan et al., 2005](#)). The open technique, the mucosa of the opening in the side of the epididymal tubule is anastomosed to the mucosa of the vas deferens to complete the end to side anastomosis ([Shekarriz and Pomer, 1991](#)). This technique should only be performed by surgeons with the requisite microsurgery training and high procedural volumes, as good results are dependent on experience and training.

Given that there is a known failure rate for vasectomy reversals regardless of technique used, an option of sperm banking of any intraoperative sperm that are discovered should be discussed with the patient. This further opens up options for in-vitro fertilization in the case that sperm does not return in the ejaculate.

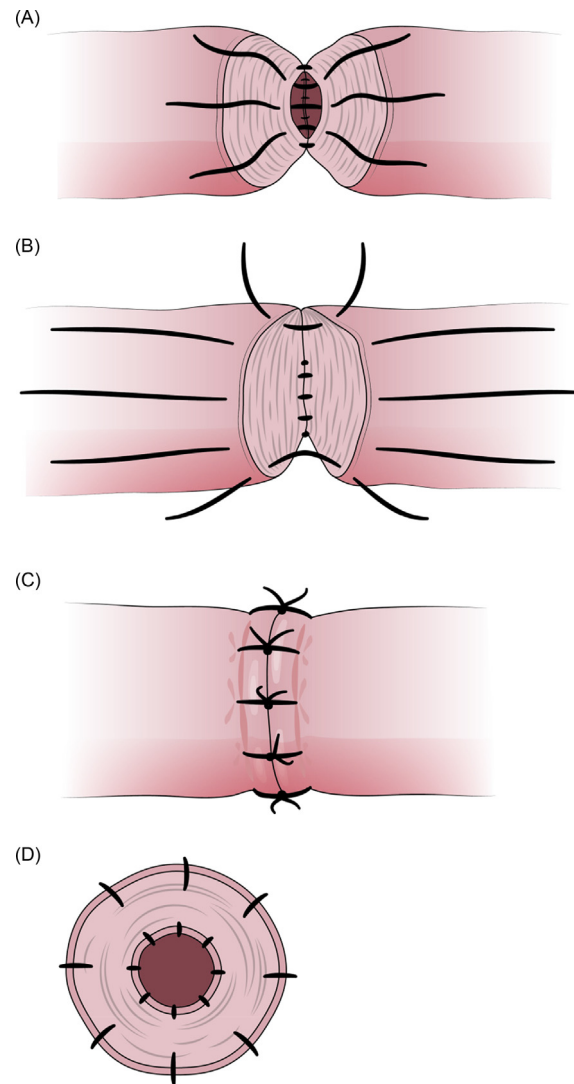


Fig. 1 Illustration of a microscopic 2-layer vasovasostomy. (A) The placement of 6, 10–0 nylon sutures circumferentially through the inner mucosal layer. (B) The placement of multiple 9–0 nylon sutures through the muscularis layer of the vas, forming the second layer. (C) The completed anastomosis. (D) A cross-sectional view of the completed anastomotic sutures.

Table 2 Reported patency and pregnancy rates based on gross appearance of vasal fluid (Belker et al., 1991)

<i>Vasal fluid gross appearance</i>	<i>Patency rate</i>	<i>Pregnancy rate</i>
Watery	91%	49%
Thin, opalescent	93%	59%
Creamy, thick	70%	45%
No fluid	88%	54%

Complications

The complication rates in vasectomy reversal are uncommon, with the most common complication being bleeding from the surgical site, causing a buildup of clotted blood within the scrotum, also known as a scrotal hematoma (Practice Committee of American Society For Reproductive Medicine, 2008). This complication is of particular concern as this may cause pressure on the fragile connection and also release substances in the tissue that may compromise healing and promote scarring. The management of post-operative hematomas after vasectomy reversal varies, with some centers managing this with observation alone, and other centers evacuating and draining these should they arise. The risk of scrotal hematomas is minimized through careful dissection of the tissues and meticulous sealing of any visualized bleeding vessels with electrical current.

With the use of antibiotics prior to the start of the procedure, wound infections are fairly rare. However, should they arise, they should be treated aggressively to prevent compromise of the fragile reversal.

Compromise of the testicular blood supply is also a rare but serious complication of vasectomy reversal. The blood vessels supplying the testicle are usually avoided in the procedure, but should any doubt arise, the presence of the testicular artery can be verified by using a Doppler ultrasound probe in complex cases.

Sperm granulomas can arise postoperatively, indicating that some vasal fluid has leaked out of the anastomosis and caused an inflammatory response. This unfortunately predicts for eventual obstruction of the reversal. The risks of this can be minimized by emphasizing meticulous technique, with accurate realignment of healthy ends of the vas with good blood supply.

Postoperative Care

At the completion of the procedure, fluff gauze and scrotal support are used overlying the dressings. All cases are performed with a single dose of perioperative antibiotics. Patients are counseled to apply an ice pack intermittently for the first few days for comfort and to decrease swelling in the area. They are discharged home with acetaminophen with codeine for analgesia. They are instructed to avoid sexual and strenuous activity for 3–4 weeks. We then see them at 6 weeks for a postoperative visit and to organize a semen analysis to check for patency. If no sperm has returned in the ejaculate by 6 months, then a repeat VV or VE should be considered.

References

- Belker, A. M., Thomas, A. J., Jr., Fuchs, E. F., KONNAK, J. W., & Sharlip, I. D. (1991). Results of 1,469 microsurgical vasectomy reversals by the Vasovasostomy Study Group. *The Journal of Urology*, 145, 505–511.
- Black, A., Guilbert, E., Costescu, D., Dunn, S., Fisher, W., Kives, S., Mirosh, M., Norman, W. V., Pymar, H., Reid, R., Roy, G., Varto, H., Waddington, A., Wagner, M. S., & Whelan, A. M. (2015a). Chapter 4 natural family planning. *Journal of Obstetrics and Gynaecology Canada*, 37, S5–11.
- Black, A., Guilbert, E., Costescu, D., Dunn, S., Fisher, W., Kives, S., Mirosh, M., Norman, W. V., Pymar, H., Reid, R., Roy, G., Varto, H., Waddington, A., Wagner, M. S., & Whelan, A. M. (2015b). Chapter 5 barrier methods. *Journal of Obstetrics and Gynaecology Canada*, 37, S12–24.
- Boorjian, S., Lipkin, M., & Goldstein, M. (2004). The impact of obstructive interval and sperm granuloma on outcome of vasectomy reversal. *The Journal of Urology*, 171, 304–306.
- Chan, P. T., Brandell, R. A., & Goldstein, M. (2005). Prospective analysis of outcomes after microsurgical intussusception vasoepididymostomy. *BJU International*, 96, 598–601.
- Eisenberg, M. L., Henderson, J. T., Amory, J. K., Smith, J. F., & Walsh, T. J. (2009). Racial differences in vasectomy utilization in the United States: Data from the national survey of family growth. *Urology*, 74, 1020–1024.
- Gerrard, E. R., Jr., Sandlow, J. I., Oster, R. A., Burns, J. R., Box, L. C., & Kolettis, P. N. (2007). Effect of female partner age on pregnancy rates after vasectomy reversal. *Fertility and Sterility*, 87, 1340–1344.
- Hernandez, J., & Sabanegh, E. S. (1999). Repeat vasectomy reversal after initial failure: Overall results and predictors for success. *The Journal of Urology*, 161, 1153–1156.
- Herrel, L. A., Goodman, M., Goldstein, M., & Hsiao, W. (2015). Outcomes of microsurgical vasovasostomy for vasectomy reversal: a meta-analysis and systematic review. *Urology*, 85, 819–825.
- Hollingsworth, M. R., Sandlow, J. I., Schrepferman, C. G., Brannigan, R. E., & Kolettis, P. N. (2007). Repeat vasectomy reversal yields high success rates. *Fertility and Sterility*, 88, 217–219.
- Horovitz, D., Tjong, V., Domes, T., Lo, K., Grober, E. D., & Jarvi, K. (2012). Vasectomy reversal provides long-term pain relief for men with the post-vasectomy pain syndrome. *The Journal of Urology*, 187, 613–617.
- Kim, H. H., & Goldstein, M. (2009). History of vasectomy reversal. *The Urologic Clinics of North America*, 36, 359–373.
- Leslie, T. A., Illing, R. O., Cranston, D. W., & Guillebaud, J. (2007). The incidence of chronic scrotal pain after vasectomy: A prospective audit. *BJU International*, 100, 1330–1333.
- McDougal, W. S., Wein, A. J., Kavoussi, L. R., Novick, A. C., Partin, A. W., Peters, C. A., & Ramchandani, P. (2011). *Campbell-Walsh Urology Review*. Canada: Elsevier.
- Nangia, A. K., Myles, J. L., & Thomas, A. J. (2000). Vasectomy reversal for the post-vasectomy pain syndrome: A clinical and histological evaluation. *The Journal of Urology*, 164, 1939–1942.
- O'Connor, V. J. (1948). Anastomosis of vas deferens after purposeful division for sterility. *Journal of the American Medical Association*, 136, 162.
- Pavlovich, C. P., & Schlegel, P. N. (1997). Fertility options after vasectomy: A cost-effectiveness analysis. *Fertility and Sterility*, 67, 133–141.
- Polackwich, A. S., Tadros, N. N., Ostrowski, K. A., Kent, J., Conlin, M. J., Hedges, J. C., & Fuchs, E. F. (2015). Vasectomy Reversal for Postvasectomy Pain Syndrome: A Study and Literature Review. *Urology*, 86, 269–272.
- Potts, J. M., Pasqualotto, F. F., Nelson, D., Thomas, A. J., Jr., & Agarwal, A. (1999). Patient characteristics associated with vasectomy reversal. *The Journal of Urology*, 161, 1835–1839.
- Practice Committee of American Society For Reproductive Medicine. (2008). Vasectomy reversal. *Fertility and Sterility*, 90, S78–82.
- Sharlip, I. D. (1981). Vasovasostomy: Comparison of two microsurgical techniques. *Urology*, 17, 347–352.
- Sharlip, I. D., Belker, A. M., Honig, S., Labrecque, M., Marmar, J. L., Ross, L. S., Sandlow, J. I., & Sokal, D. C. (2012). Vasectomy: AUA guideline. *The Journal of Urology*, 188, 2482–2491.
- Shekarriz, M., & Pomer, S. (1991). Microsurgical vasoepididymostomy: A comparison between the end-to-side anastomosis and the invagination technique. *Urological Research*, 19, 285–287.
- Sheynkin, Y. R. (2009). History of vasectomy. *The Urologic Clinics of North America*, 36, 285–294.
- Silber, S. J. (1977). Microscopic vasectomy reversal. *Fertility and Sterility*, 28, 1191–1202.
- Tan, W. P., & Levine, L. A. (2016). An overview of the management of post-vasectomy pain syndrome. *Asian Journal of Andrology*, 18, 332–337.
- Thomas, J. L. (1904). A Method for Anastomosing a Severed Vas Deferens. *British Medical Journal*, 1, 13–14.
- Witt, M. A., Heron, S., & Lipshultz, L. I. (1994). The post-vasectomy length of the testicular vasal remnant: A predictor of surgical outcome in microscopic vasectomy reversal. *The Journal of Urology*, 151, 892–894.

Vasoepididymostomy

Ryan Flannigan and Marc Goldstein, Center for Male Reproduction & Microsurgery, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Vasoepididymostomy is considered to be the most challenging microsurgical procedure in male reproduction. It is the reconstructive means to anastomose the vas deferens to an epididymal tubule. This technique is used in obstructive azoospermia, where obstruction occurs at the level of the epididymis. The earliest forms of vasoepididymostomy described in 1903, involved opening several epididymal tubules and the vas deferens, and hoping the lumina would fistulize. In 1918, Lespinasse attempted the first deliberate anastomosis of the epididymal and vas deferens (Chan et al., 2003). Due to the microscopic size of the vas deferens lumen (300–500 μm) and the epididymal tubule (150–250 μm), success was limited until the introduction of optical magnification for the procedure by Silber (1978). Since, several variations in technique have been trialed. We will discuss the most contemporary and successful techniques in this article.

Diagnosing Epididymal Obstructive Azoospermia

Etiology

Epididymal obstruction may be congenital or acquired. Congenital forms of epididymal obstruction include hypoplasia associated with cystic fibrosis transmembrane regulatory protein (CFTR) and unilateral or bilateral absence of the vas deferens (CBAVD). Acquired causes of epididymal obstruction include: infectious etiologies, that is, epididymitis, scrotal, or pelvic trauma, vasal obstruction with epididymal blowout, or direct epididymal injury (Wosnitzer et al., 2014). The latter two etiologies are often iatrogenic. The most common being hydrocelectomy, spermatocelectomy, inguina hernia repair, vasectomy, percutaneous epididymal sperm aspiration (PESA), microsurgical epididymal sperm aspiration (MESA) (Fuchs and Burt, 2002).

History and Physical Exam

A thorough history and physical examination are crucial for identification and localization of epididymal or vasal obstruction. A focused reproductive history of both the male and their partner is essential. The clinician should determine if this is primary or secondary infertility, and if either partner has contributed to a pregnancy or been surprised in the absence of a pregnancy with unprotected intercourse. Features on history which may suggest epididymal obstruction include: sexually transmitted infection or epididymitis, scrotal surgery, that is, vasectomy and number of years since vasectomy, orchidopexy, spermatocelectomy, hydrocelectomy, previous attempts at epididymal or vasal reconstruction, inguinal surgery, that is, inguinal hernia repair (especially with mesh), varicocelectomy, excision of lipoma. Furthermore, one should enquire about scrotal or pelvic trauma, or additional pelvic surgeries which may compromise the intraabdominal portion of the vas deferens such as radical prostatectomy, or renal transplant.

On physical examination, the clinician should perform a full reproductive exam including degree of virilization, midline defects, abdominal, and genital assessment. Special attention should be drawn to identifying surgical scars to the abdomen, inguinal region, and scrotum. With a warm, relaxed scrotum, testicles should be palpated for size, firmness, and asymmetries. Epididymal fullness or induration is often present with obstructive azoospermia. Cord structures should be assessed for presence of the vas deferens, vasal defects, and sperm granuloma if previous vasectomy, varicocele, spermatocele, lipoma, and inguinal hernia.

Investigations

Among men with two azoospermic semen analyses, obstructive etiology may be identified by history and physical exam. Low volume semen analyses or ejaculates with a pH of <7.2 should lead one to suspect congenital absence of the vas deferens, or in the presence of palpable vasa, partial absence or ejaculatory duct obstruction (EDO) and a transrectal ultrasound should be performed for diagnosis. EDO is discussed at further depth elsewhere in this text.

Investigations beyond the semen analysis serve to determine if the man has active spermatogenesis. Hormone profiles may identify men with low testosterone, which could be due to hypogonadotrophic hypogonadism (low T, FSH, and LH), or hypergonadotrophic hypogonadism (low T, high FSH >7.6 mIU/mL, and LH). Serum FSH and testicular size are important predictors for differentiating OA from NOA; in one study, a FSH of <7.6 mIU/mL and testicular long axis of >4.6 cm predicts OA in 96% of cases (Schoor et al., 2002). Genetic evaluation for the patient with azoospermia and primary infertility includes a karyotype, γ -microdeletion, and CFTR gene assessment if one or both vas deferens are nonpalpable, or ejaculatory duct obstruction is suspected. In patients with primary infertility, spermatogenesis must be confirmed prior to reconstruction of the excurrent ductal system. A serum anti-sperm antibody assay may be ordered including IgG, IgA, and IgM to assess for active spermatogenesis. The area under the curve (AUC) for ROC curves were 0.92, 0.85, and 0.67 respectively. This translates to a sensitivity and specificity of 85% and 97% respectively for IgG, while the specificity, positive predictive value and positive likelihood ratio for IgA were 99%, 99%, and 70% respectively (Lee et al., 2009). If there are no antisperm antibodies, or if the FSH is abnormally high >7.6 mIU/mL, then confirmation of

obstruction is warranted prior to reconstruction. This may be performed in advance of a planned surgical procedure, or, it may be performed at the beginning of a case with intraoperative microscopic inspection of the epididymis. If markedly dilated tubules are seen, obstruction is confirmed. If the epididymal tubules are flat, a testis biopsy is necessary, with intraoperative microscopic examination of the testicular tissue for presence of sperm. In this case, the patient must be counseled and consented for the different potential procedures depending on the findings. For instance, microscopic testicular extraction of sperm is required if there is abnormal sperm production on the diagnostic biopsy, or reconstruction is required if obstruction is confirmed. Men with secondary infertility due to vasectomy, and proven fecundity, do not need additional confirmation of spermatogenesis if physical exam reveals normal testis volume and consistency and a hormone profile reveals normal serum FSH. A positive serum antisperm antibody assay also suffices to confirm obstruction.

Localizing the Obstruction

Once OA is diagnosed, identifying the level of obstruction requires assessment of history, physical exam, and semen analyses as discussed earlier. EDO is suspected with low semen volume, pH <7.2 and absence of fructose in the semen. TRUS should be performed to assess for seminal vesicle dilation and midline cysts. In the absence of EDO, the level of obstruction is limited to the vas deferens or epididymis. Features on history may identify seminal vesicle side vasal obstruction associated with inguinal surgeries such as hernia repairs, or pelvic surgery such as renal transplant or prostatectomy. Definitive determination of the level of obstruction is imperative to ensure optimal result. This must be determined intraoperatively. First, the surgeon should visualize the epididymis for signs of dilated epididymal tubules and a transition of dilated to nondilated tubules. The transition signifies epididymal obstruction is probable. Next, the surgeon performs a vasotomy and samples fluid from the testicular side of the vas. Presence of abundant sperm or sperm parts confirms patency of the epididymis. Next, a vasogram toward the seminal vesicle side and testicular vasal fluid sampling. The vasogram initially consists of injecting normal saline or lactated ringer's solution through a 24-gauge angiocatheter. Lack of resistance and absence of reflux suggest patency. If resistance or reflux is met, the surgeon may place a 2-0 monofilament suture tail in the vasal lumen on the seminal vesicle side to determine where the suspected obstruction is present. This is diagnostic if it stops in the middle of the hernia scar. A formal vasogram with radio-opaque contrast and intraoperative X-ray may be performed if there is uncertainty regarding the location of the distal obstruction. The surgeon must also sample fluid from the testicular lumen to determine if sperm are present at the level of the vasotomy, indicating lack of epididymal obstruction. Visual appearance of the fluid provides insight to the presence of patency or obstruction; however, microscopic analysis is most definitive. Clear copious fluid suggests an unobstructed epididymis, while thick, toothpaste-like fluid, devoid of sperm, is suggestive of obstruction. Creamy yellow fluid may possess sperm parts and suggest epididymal patency. The fluid is then placed on a glass microscope slide and examined under 400× magnification using bright-field microscopy intraoperatively. The presence of sperm or sperm parts indicates epididymal patency. If no sperm or only rare sperm heads are present, the surgeon must sequentially sample fluid from the epididymis in a stepwise fashion, using a 10-0 needle until sperm or abundant sperm parts are identified. In our experience, we sample every 3-5 mm to identify the most distal site of vasal or epididymal patency, since more distal reconstructions portend to better postoperative patency rates.

Managing Epididymal Obstruction

Once epididymal obstruction has been identified, two management options exist: sperm retrieval for assisted reproductive techniques (ART) or reconstruction in the form of vasoepididymostomy (VE). The former may occur percutaneously via percutaneous epididymal sperm aspiration (PESA) or microsurgical epididymal sperm aspiration (MESA), [Fig. 1](#). Here, couples must be counseled and be willing to undergo ART to obtain pregnancy. PESA may be performed under local anesthetic, using a 21-26-gauge needle inserted percutaneously into the epididymis while withdrawing on the syringe and thus, aspirating sperm. A small volume of fluid (~0.1 mL) is retrieved, and yields thousands to millions of sperm among 61%-96% of men. MESA requires a general anesthetic. Here, the testis is delivered, and under 20-25× magnification with use of an operating microscope, the caput of the epididymis is reflected from the testis, exposing the 7-11 efferent ducts; an efferent duct is selected and punctured. A micropipette is then used to collect the epididymal fluid through capillary action. Sperm is successfully retrieved in 96%-100% of cases yielding 15-95 million sperm. Reconstructive options for epididymal obstruction is a viable option for a skilled microsurgeon and consists of a VE.

Cost-Effectiveness

Men with epididymal obstruction have the choice of sperm retrieval and subsequent in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) or VE. The latter appears to be more cost-effective with an estimated cost per live delivery, in 1997 dollars, of \$31,099 based upon an 85% VE patency rate and 36% live birth rate. Meanwhile, sperm retrieval via MESA and subsequent IVF/ICSI had a cost per live delivery rate of \$51,024. This particular study considered both direct and indirect costs to the associated procedures ([Kolettis and Thomas, 1997](#)). Several other studies have supported vasal reconstruction for men with previous vasectomies as being more cost effective compared to sperm retrieval and IVF/ICSI; however, these series include vasovasostomies which characteristically have higher patency rates compared to VE's.

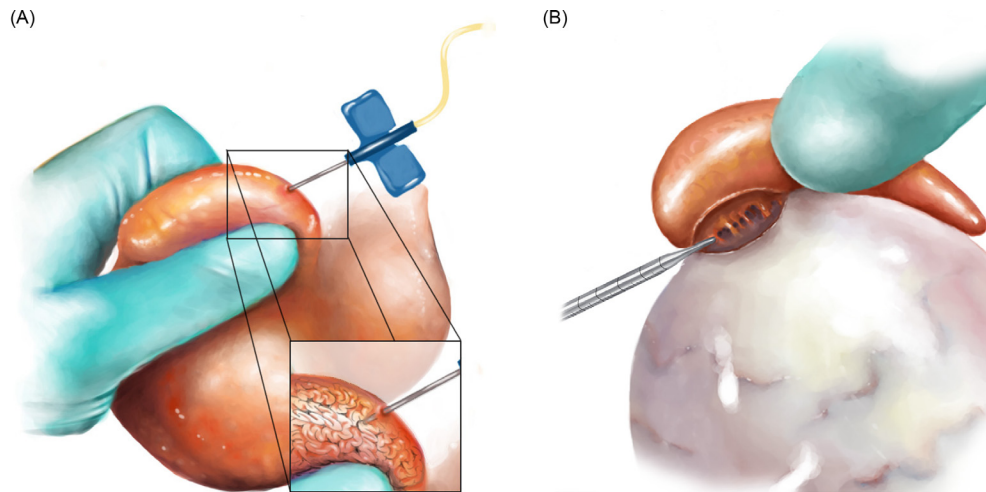


Fig. 1 (A) Percutaneous epididymal sperm aspiration (PESA). Under local anesthetic (skin and cord block), a 21–26 gauge needle is inserted to the epididymis, and epididymal fluid is aspirated for the presence of sperm. (B) Microsurgical epididymal sperm aspiration (MESA). Under general anesthetic, the testis is delivered and the tunica vaginalis is opened exposing the epididymis. The caput of the epididymis is reflected gently by incising the tunica albuginea at the junction of the testis and caput. The efferent ducts are visualized, and one is punctured. A micropipette is used to aspirate the sperm containing fluid by capillary action.

Vaso-Epididymostomy Surgical Principles

Surgical Incision and Mobilization

Vertical hemiscrotal incisions should be made in the mid to upper scrotum, this affords the ability to extend the incision cranially if additional dissection of the abdominal vas deferens is necessary. The testis is delivered with the tunica vaginalis intact. The vas deferens is then identified and separated from the rest of the spermatic cord with a Penrose drain at the site of previous vasectomy or at the junction of the straight and convoluted vas if the patient has not previously undergone vasectomy. The operating microscope is then used to carefully dissect the peri-vasal vessels off of the vasal surface at the site of planned vasotomy.

Identifying the Site of Obstruction

A hemivasotomy is performed and the vasal fluid is assessed visually, and smeared onto a glass slide and examined under $400\times$ bright-field microscope for the presence of spermatozoa. **Table 1** provides an overview of vasal fluid findings and procedure necessary. If thick white toothpaste-like fluid with no sperm is found in the case of a previous vasectomy, then a VE is indicated. Here, the epididymal tubules must be carefully evaluated starting at caudal end of the epididymis. A tubule is then punctured and the fluid is examined under the bright-field microscope. This is repeated moving 2–3 mm proximally until sperm is identified. If the line of demarcation is clearly seen with dilated tubules above and collapses below, one can go straight to set up for a vasoepididymostomy.

Preparing for the Anastomosis

Once a dilated tubule above the obstruction is identified, the site of anastomosis is prepared by excising a 2.5 mm ellipse of tunica overlying the favorable, dilated tubules. The abdominal vas deferens must have adequate length for a tension free anastomosis. The posterior vasal sheath is then sutured to the cranial portion of the tunical window to prevent tension during the anastomosis.

Table 1 Intraoperative testicular end vasal fluid is sampled at the time of vasal reconstruction for OA

<i>Vasal fluid</i>	<i>Microscopic findings</i>	<i>Surgical procedure indicated</i>
Copious clear fluid	No sperm	Vasovasostomy
Cloudy fluid	Intact sperm	Vasovasostomy
Creamy yellow fluid	Sperm heads	Vasovasostomy
Thick white toothpaste-like fluid	No sperm	Vasoepididymostomy
Dry vas with no granuloma	No sperm	Vasoepididymostomy
Dry vas with granuloma at vasectomy site	Barbotage fluid reveals sperm	Vasovasostomy

The decision to proceed with vasovasostomy compared to vasoepididymostomy is made by examining the appearance of the vasal fluid and the microscopic findings.

Unique Considerations

Obstruction of Both Epididymis and Abdominal Vas Deferens

In this scenario, the vasal fluid from the hemi-vasotomy demonstrates an absence of spermatozoa and injection of normal saline is met with resistance to the abdominal vas deferens. The tail of a 2–0 monofilament suture may be used to cannulate the abdominal vas and locate the site of obstruction; if localization is unclear, a formal vasogram should be performed to determine the site of obstruction. If the obstruction is at the level of the inguinal canal, we recommend sperm retrieval if the couple is willing to undergo IVF/ICSI, closing the hemi-vasotomy, and abandoning the VE. If the hemi-vasotomy is small and vessels preserved, then inguinal vasovasostomy may be considered during the initial procedure with plans for a delayed scrotal VE in 6 months. This period of time will allow the inguinal vasovasostomy site to heal and reconstitute vascularization to the intervening vas deferens. Performing both a scrotal VE and an inguinal VV would result in devascularization of the intervening segment of vas deferens. In a second scenario, with the site of distal obstruction localizing to the ejaculatory ducts, we recommend management of the EDO first, and if successful, subsequent VE.

Crossed Vasoepididymostomy

Men that have a clear asymmetry in testis quality, that is, atrophic or absent unilateral testis, accompanied with a contralateral vasal obstruction, that is, inguinal hernia repair, renal transplant, then a crossed vasal reconstruction may be considered. Here, the better quality testis is confirmed to be obstructed, while the poor quality testis has a patent abdominal end of the vas deferens as confirmed intraoperatively. The optimal procedure to facilitate sperm returning to the ejaculate is to anastomose the testicular end of the vas deferens or epididymis (based upon microscopic analysis of vasal fluid results) of the best quality testis to the patent vas deferens from the side of the atrophic testis. Here, the testicular vas deferens is tunneled through a copious opening in the scrotal septum for a tension free anastomosis; if vasal length for is a concern, the better testis can be transposed to the contralateral side.

Varicocele and Vasoepididymostomy

In the event that a patient presents with both epididymal obstruction and a varicocele, it is recommended to treat the epididymal obstruction first. By correcting the obstruction, the patient has the opportunity to develop sperm in the ejaculate and obtain paternity without requiring a varicocele repair (VR). However, if pregnancy is still not achieved due to presumed implications from the varicocele, we recommend waiting 6 months to perform the VR. We advocate against performing simultaneous VE and VR due to the venous congestion at the site of anastomosis. This congestion is unlikely to aid in healing the delicate anastomosis.

Vasoepididymostomy Surgical Techniques

Several techniques for VE are discussed in this section. End-to-end and end-to-side techniques were among the first described. More contemporary techniques include intussuscepting the epididymal tubule into the vasal lumen using two or three sutures. In a randomized controlled trial using an animal model, Chan and colleagues determined the highest patency rate among the longitudinal intussuscepted vasoepididymostomy (LIVE) two suture technique. Regardless of technique, the microsurgeon must practice strong principles of surgery for optimal results. These include, tension free and water-tight anastomosis, atraumatic tissue handling, precision of needle placement, square knots, a bloodless operating field, and preservation of anatomy while optimizing necessary exposure.

End-to-End Technique

An end-to-end (EE) anastomosis to a specific tubule is the original technique for VE as described by Silber (1978). The epididymis is cut in cross-section starting caudal and moving toward the caput systematically. Bipolar cautery is used to control bleeding vessels, and the epididymal fluid is assessed under a microscope for the presence of sperm. If no sperm is identified, a more proximal section is made. If sperm is found, the epididymis is milked until only one tubule continues to ooze clear fluid, this fluid is sampled once more to ensure sperm is present prior to the anastomosis. The epididymal tubule is then anastomosed to the vasal lumen with four to six, 9–0 or 10–0 sutures placed inside-out on the epididymal tubule to the corresponding location on the vas deferens, Fig. 2. A second, muscular layer anastomosis is performed to achieve a watertight anastomosis.

End-to-Side Technique

Most contemporary techniques beyond the conventional end-to-side (ES) use this approach. However, in the conventional ES technique, no intussusception is performed. Here, an epididymal tubule with sperm is identified as mentioned in section “Identifying the Site of Obstruction.” The selected epididymal tubule is then punctured with a microknife or microscissors and the fluid is tested again to confirm sperm. Methylene blue may be used to differentiate the out and inner surface of the epididymal tubule. Three to six double-armed 10–0 sutures are then placed inside-out in the epididymal tubule, then to the corresponding location in an inside-out fashion, Fig. 3. A second anastomotic layer is performed with 9–0 sutures from the muscularis of the vas deferens to the cut tunical edge on the epididymis.

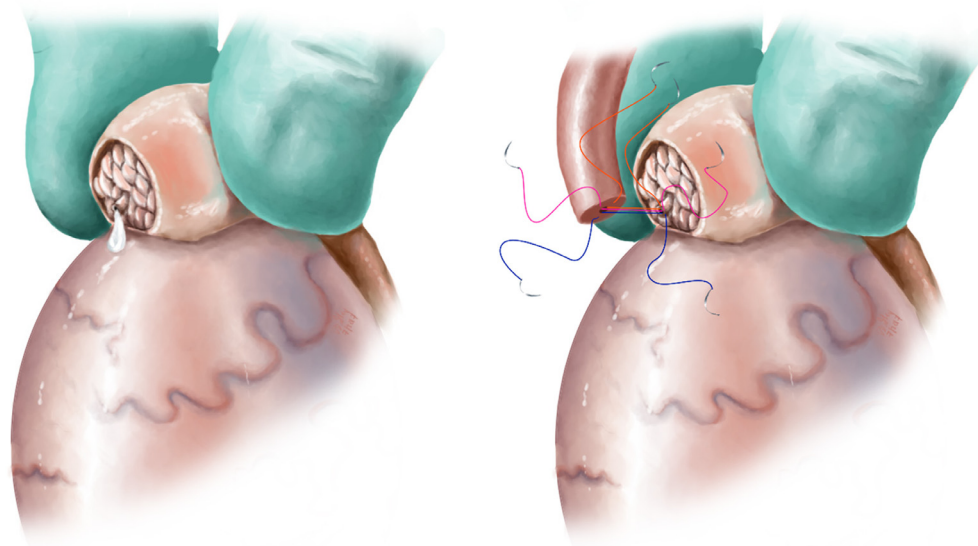


Fig. 2 End-to-end vasoepididymostomy. Here, the epididymal tubule that contains to leak fluid is likely the patent tubule containing sperm. This fluid may be tested under a microscope for presence of sperm. Four to six 9–0 or 10–0 sutures have been used for this anastomosis. A second layer of 9–0 sutures are then placed peripherally.

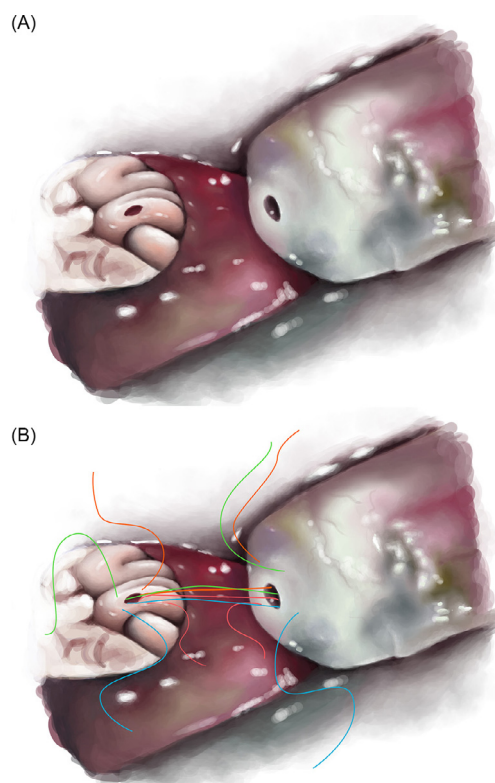


Fig. 3 End-to-side vasoepididymostomy. Here, the epididymal tubule that is dilated and contains sperm is opened with a 15° microknife or microscissors (A). Three to six 9–0 or 10–0 sutures are then used for the lumen anastomosis (B). A second layer of 9–0 sutures are then placed peripherally.

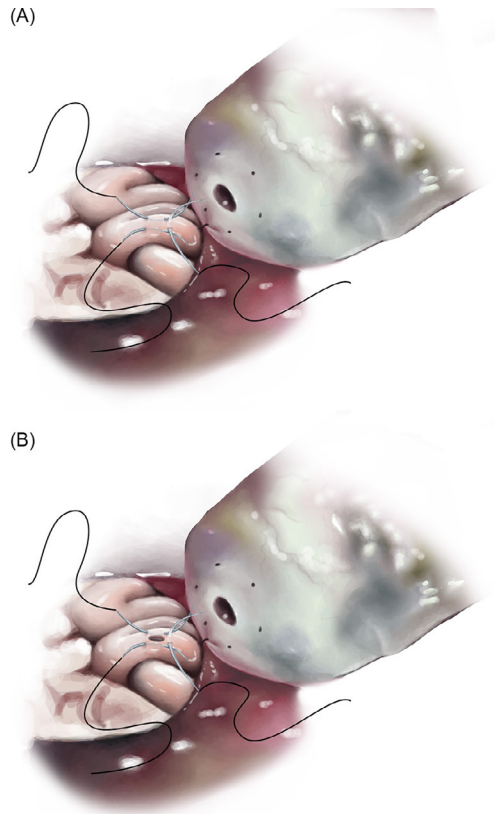


Fig. 4 Intussuscepted three-suture triangulation. Here, the dilated epididymal tubule containing sperm is isolated. Three double-armed 10-0 sutures are placed and partially pulled through the tubule in a triangulating orientation. A 15° microknife is then used to incise the lumen between the sutures. The needles are then pulled through and placed to the corresponding locations of the vas deferens inside to out. Upon tying these sutures, the epididymal tubule is intussuscepted into the vasal lumen. A second layer of 9-0 sutures are then placed from the outer edge of the vas deferens to the tunica of the epididymis circumferentially.

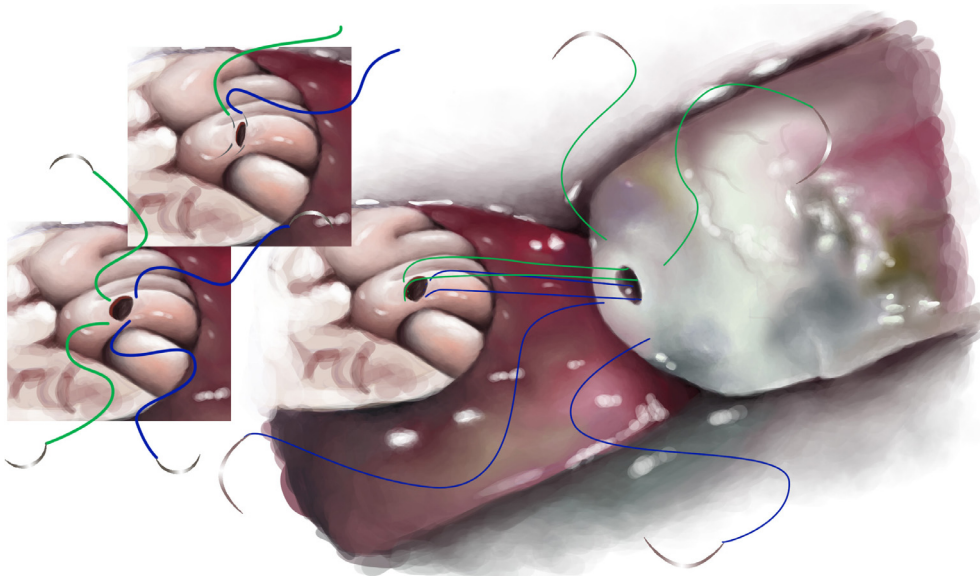


Fig. 5 Intussuscepted two-suture transverse technique. Here, the dilated epididymal tubule containing sperm is isolated. Two double-armed 10-0 sutures are placed and partially pulled through the tubule in a transverse orientation. A 15° microknife is then used to incise the lumen between the sutures. The needles are then pulled through and placed to the corresponding locations of the vas deferens inside to out. A 9-0 suture is then placed opposite to the original approximating 9-0 suture and tied. This opposes the vasal lumen and the epididymal opening, allowing for avoidance of tension to the epididymal anastomosis. Upon tying these sutures, the epididymal tubule is intussuscepted into the vasal lumen. A second layer of 9-0 sutures are then placed from the outer edge of the vas deferens to the tunica of the epididymis circumferentially.

Intussuscepted Three-Suture Triangulation Technique

The intussuscepted three-suture technique was the first technique to utilize intussusception and was first described by [Berger \(1998\)](#). This technique uses the same approach to isolate the epididymal tubule of choice as the above ES technique. This anastomosis however, differs from the conventional ES technique. Here, three double-armed 10-0 suture needles are placed partially through the epididymal tubule in a triangular fashion, prior to incising the tubule. Since the needles are larger diameter than the suture connected to the needle, leaving the needle in the tubule allows the tubule to maintain fullness for more precise placement of the 2nd and 3rd sutures, [Fig. 4](#). Following placement of all three sutures, the tubule is incised with a 15° microknife, and the sutures are then placed inside-out to the vasal lumen, intussuscepting the opening of the tubule into the vasal lumen. The sutures are gently placed on traction until the tail of the suture begins to move, and are then tied. A second layer of 9-0 sutures are placed from the muscularis of the vas deferens to the tunical edge for a watertight closure.

Intussuscepted Two-Suture Transverse Technique

Marmar first described the two-suture transverse intussusception technique ([Marmar, 2000](#)). This technique involves the same preparation as the three-suture intussusception technique; however, two doubled-armed 10-0 sutures are placed perpendicular to the length of the epididymal tubule, and kept only partially drawn through, [Fig. 5](#). A 15° microknife is then used to make a transverse incision between the two needles. The ends of the 10-0 sutures are then placed inside out to the corresponding locations in the vas

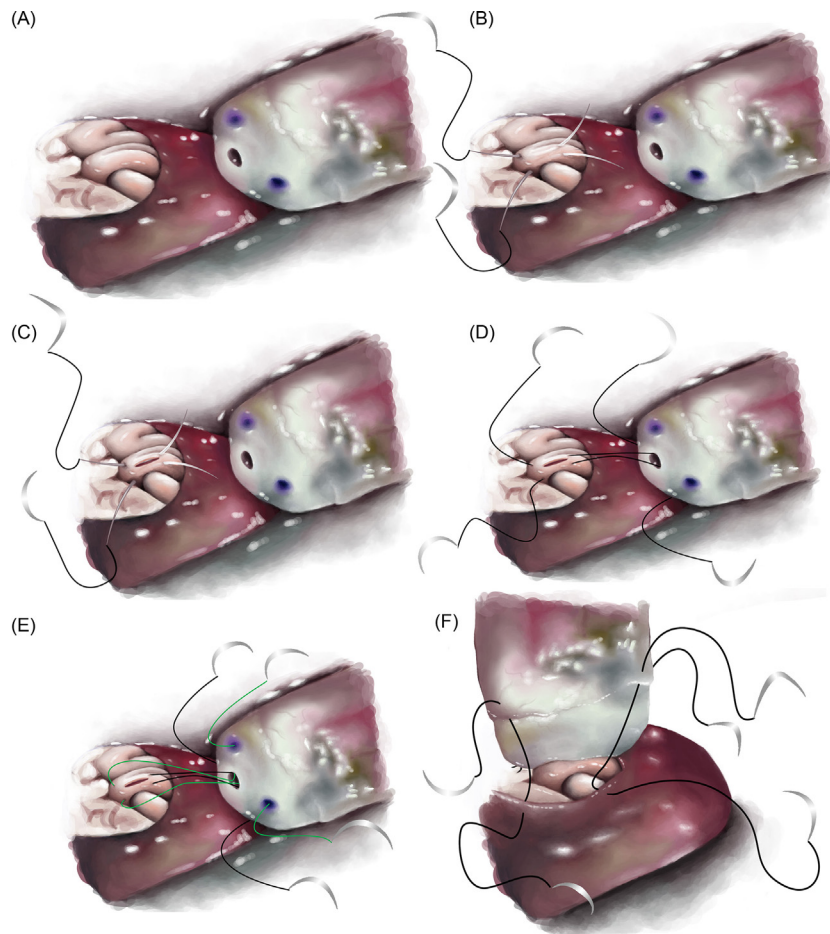


Fig. 6 Longitudinal intussuscepted vasoepididymostomy (LIVE). Here, the dilated epididymal tubule containing sperm is isolated. A 9-0 suture is placed on the posterior side of the vas deferens and tied to the corresponding side of the epididymal tunica to bring the vas in close approximation and avoid any tension. Four microdots are placed at the junction of the mucosa to muscularis of the vas deferens to plan the vasal stitches. Two double-armed 10-0 sutures are placed and partially pulled through the tubule in a longitudinal orientation. A 15° microknife is then used to incise the lumen between the sutures. The needles are then pulled through and placed to the corresponding locations of the vas deferens inside out. A 9-0 suture is then placed opposite to the original approximating 9-0 suture and tied. This opposes the vasal lumen and the epididymal opening, allowing for avoidance of tension to the epididymal anastomosis. Upon tying these sutures, the epididymal tubule is intussuscepted into the vasal lumen. A second layer of 9-0 sutures are then placed from the outer edge of the vas deferens to the tunica of the epididymis circumferentially.

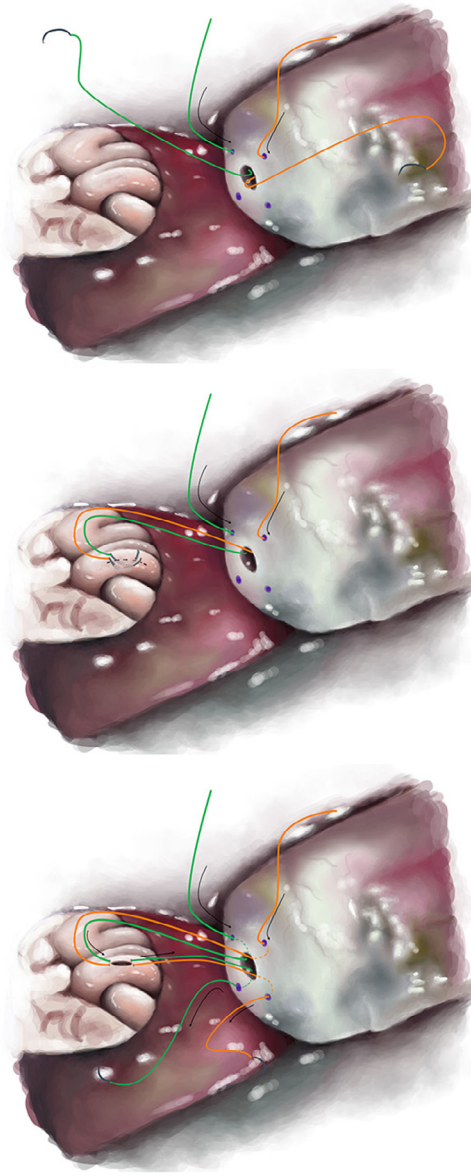


Fig. 7 Single arm suture longitudinal intussuscepted vasoepididymostomy (LIVE). Here, the dilated epididymal tubule containing sperm is isolated. A 9-0 suture is placed on the posterior side of the vas deferens and tied to the corresponding side of the epididymal tunica to bring the vas in close approximation and avoid any tension. Four microdots are placed at the junction of the mucosa to muscularis of the vas deferens to plan the vasal stitches. Two single arm sutures are placed outside-in from the microdots out through the lumen. The needles are then placed in a direction back toward the vas deferens in the epididymal tubule in a longitudinal fashion and partially pulled through the tubule. A 15° microknife is then used to incise the lumen between the sutures. The needles are then pulled through and placed to the corresponding locations of the vas deferens inside to out. A 9-0 suture is then placed opposite to the original approximating 9-0 suture and tied. This opposes the vasal lumen and the epididymal opening, allowing for avoidance of tension to the epididymal anastomosis. Upon tying these sutures, the epididymal tubule is intussuscepted into the vasal lumen. A second layer of 9-0 sutures are then placed from the outer edge of the vas deferens to the tunica of the epididymis circumferentially.

deferens. A second layer of interrupted 9-0 sutures are placed from the muscularis of the vas deferens to the tunical edge as with the three suture technique.

Intussuscepted Two-Suture Longitudinal Technique

Preferred technique, Chan, Li, and Goldstein described the double-armed longitudinal intussuscepted vasoepididymostomy (LIVE) technique and is the authors preferred technique ([Chan et al., 2003](#)). It involves the same preparation as the above two and three suture techniques. At our institution, we emphasize the importance of a consistent setup. Using a microtip surgical marker, we place four microdots at the transition of mucosa to muscularis of the vas deferens to separate planning from execution of needle

placement. In the LIVE technique, use of double-armed and single-armed sutures has been described. In the standard double-arm technique, two double-armed 10-0 sutures are placed longitudinally in the epididymal tubule, but not pulled through (Fig. 6). The needle is 70 μm in diameter, but the suture is only 17 μm , so if the needles are pulled through before both are placed, and the tubules incised, sperm will leak from the suture holes and the tubule will collapse, obviating a major benefit of the intussusception technique. A longitudinal incision between the sutures is then made with a 15-blade microknife and the suture ends are carefully pulled through the epididymal tubule and placed in the respective positions in the vas deferens inside-to-out through the microdots. However, in the single-arm technique, the 10-0 suture must first be placed outside-to-in, through the microdot, and out of the lumen, Fig. 6. The needles are then placed longitudinally in the epididymal tubule, and partially withdrawn. The tubule is opened as in the double-arm technique, then the single arm sutures are placed to the corresponding locations on the vas deferens inside-to-out. The single arm technique may be used where double-armed sutures are not available, Fig. 7. Prior to tying the 10-0 sutures, one end is pulled until the other end begins to move; at this point, it may be assumed that the slack has been removed and an appropriately intussuscepted anastomosis has been created. The second layer of 9-0's are then placed in an interrupted fashion as above to prevent leakage and subsequent sperm granuloma which may cause a late failure. We prefer the LIVE technique to the two-suture transverse technique since there is less risk of inadvertently transecting the epididymal tubule. Furthermore, Chan et al. performed a randomized trial in an animal model that demonstrated best patency rate among the LIVE technique (Chan et al., 2003).

Outcomes and Complications

For the LIVE technique, patency results have varied (48%–84%), but the highest reported rate was 84% at Weill Cornell, with a paternity rate of 40%, 31% of which required in vitro fertilization or intracytoplasmic sperm injection using fresh ejaculated sperm (Chan et al., 2005). Semen analyses may be ordered at 1 month postoperatively then every 3 months thereafter. Up to 60% of men will have sperm return to the ejaculate within 1-month post operatively. Mean sperm counts following LIVE are 12.8 (0.01–80) million with 21% motility (0%–30%) (Chan et al., 2005). Patients may develop a late failure where motile sperm was initially present, and they later become azoospermic. This has been estimated to occur at a rate of 8%–30% (Schiff et al., 2005). Thus, it is important to consider cryopreservation of ejaculated motile sperm postoperatively at first presentation in the event of an unfortunate late failure. Other potential complications include: scrotal hematoma, failure of anastomosis, wound infection, epididymorchitis, orchalgia or more rarely testicular atrophy or fibrosis of the epididymis. These complications are not common but should always be discussed with the patient prior to surgery at the time of informed consent. Diligent surgical technique can mitigate many of these complications, and never hesitate to place a Penrose for 24–48 h to prevent scrotal hematoma. Other important considerations are to maintain patient body temperature throughout the case and ensure the patient is well padded and positioned to avoid a position injury. These may occur with the arms outstretched for a long period of time, as such anesthesia should massage the arms and shoulders or safely reposition the arms between sides for a short break during the case.

Summary

Vasoepididymostomy is both cost effective compared to immediate in vitro fertilization and an effective surgical procedure to relieve epididymal obstruction in men wishing to achieve paternity. The LIVE technique has been well adopted internationally, with success using both single and double-armed sutures. A high regard for surgical principles are necessary for optimal results when performing vasoepididymostomies.

Acknowledgments

Medical Illustrator: Vanessa Dudley.

Source of Funding: Frederick J. and Theresa Dow Wallace Fund of the New York Community Trust; American Urology Association New York Section E. Darracott Vaughan MD, Research Scholar Award.

References

- Berger, R. E. (1998). Triangulation end-to-side vasoepididymostomy. *Journal of Urology*, 159(6), 1951–1953.
- Chan, P. T., Li, P. S., & Goldstein, M. (2003). Microsurgical vasoepididymostomy: A prospective randomized study of 3 intussusception techniques in rats. *Journal of Urology*, 169(5), 1924–1929.
- Chan, P. T., Brandell, R. A., & Goldstein, M. (2005). Prospective analysis of outcomes after microsurgical intussusception vasoepididymostomy. *BJU International*, 96(4), 598–601.
- Fuchs, E. F., & Burt, R. A. (2002). Vasectomy reversal performed 15 years or more after vasectomy: Correlation of pregnancy outcome with partner age and with pregnancy results of in vitro fertilization with intracytoplasmic sperm injection. *Fertility and Sterility*, 77(3), 516–519.
- Kolettis PN, Thomas AJ, Jr. Vasoepididymostomy for vasectomy reversal: A critical assessment in the era of intracytoplasmic sperm injection. *Journal of Urology* 1997;158(2):467–470.
- Lee, R., Goldstein, M., Ullery, B. W., et al. (2009). Value of serum antisperm antibodies in diagnosing obstructive azoospermia. *Journal of Urology*, 181(1), 264–269.

- Marmar, J. L. (2000). Modified vasoepididymostomy with simultaneous double needle placement, tubulotomy and tubular invagination. *Journal of Urology*, 163(2), 483–486.
- Schiff, J., Chan, P., Li, P. S., Finkelberg, S., & Goldstein, M. (2005). Outcome and late failures compared in 4 techniques of microsurgical vasoepididymostomy in 153 consecutive men. *Journal of Urology*, 174(2), 651–655. quiz 801.
- Schoor, R. A., Elhanbly, S., Niederberger, C. S., & Ross, L. S. (2002). The role of testicular biopsy in the modern management of male infertility. *Journal of Urology*, 167(1), 197–200.
- Silber, S. J. (1978). Microscopic vasoepididymostomy: Specific microanastomosis to the epididymal tubule. *Fertility and Sterility*, 30(5), 565–571.
- Wosnitzer, M., Goldstein, M., & Hardy, M. P. (2014). Review of azoospermia. *Spermatogenesis*, 4, e28218.

Non-Microsurgical Testis Sperm Extraction

Paul J Turek, The Turek Clinic, San Francisco, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Azoospermia is the absence of spermatozoa in the semen on two centrifuged semen samples. It affects 1% of the general population and up to 15% of infertile men (Jarow et al., 1989). Azoospermia takes two forms: obstructive conditions due to blockages within the reproductive tract, and nonobstructive disorders generally due to testicular failure. With the advent of in vitro fertilization (IVF) in 1978 and intracytoplasmic sperm injection (ICSI) in 1992, the opportunity for men with both types of azoospermia to conceive has increased dramatically (Palermo et al., 1992).

A Cochrane meta-analysis suggests that there is currently insufficient evidence to recommend a specific sperm retrieval technique over another for either obstructive or nonobstructive azoospermia (Van Peperstraten et al., 2006). In other words, based on our current knowledge, there is no “best” way to get sperm from the testicle. This has opened the door to the development of a variety of techniques, including minimally invasive procedures, to optimize both the safety and success of sperm retrieval. The biggest “safety” issue with sperm retrieval procedures is surgically-induced low testosterone or hypogonadism. During testicular sperm retrieval procedures, hormone producing Leydig cells are removed along with sperm producing cells. In addition, collateral damage to nearby testicular tissue from bleeding and devascularization during procedures can also induce hypogonadism. This is especially true in cases of nonobstructive azoospermia, in which men may be borderline hypogonadal at baseline, and large, invasive sperm retrieval procedures can result in the need for lifelong testosterone replacement. In addition, as multiple IVF-ICSI cycles may be needed for pregnancy or family building success, clinicians should develop *efficient* sperm retrieval techniques that *maximize* yield and *minimize* procedure extent and morbidity (Turek et al., 1999). With these concepts in mind, we review various testicular sperm retrieval procedures currently employed in azoospermic men.

Testis Sperm Retrieval With Azoospermia

The first use of testicular sperm with ICSI represented a great leap forward for the field, as it demonstrated that sperm do not require epididymal maturation to fertilize oocytes. Testicular sperm extraction is now used routinely in obstructive azoospermia and is essential for nonobstructive azoospermia (NOA).

Testis Sperm Retrieval With Obstructive Azoospermia

Testicular sperm in obstructed patients can be retrieved by needle aspiration (testicular sperm aspiration or TESA), by percutaneous biopsy or by open surgical biopsy (testicular sperm extraction or TESE). TESA is performed on an outpatient basis under local anesthesia with or without IV sedation (Fig. 1). It may be timed to occur with oocyte harvest, or it can be done in advance with cryopreservation. The technique involves stabilization of the testicle in the scrotal sac between the thumb, index, and middle fingers after local spermatic cord block is given. Next, a butterfly or angiocatheter needle is directed percutaneously into the testicular parenchyma through the skin (Marmar and Benoff, 2005). If an angiocatheter needle is used, the hollow needle is withdrawn, leaving the soft plastic catheter sheath within the testicle. Negative pressure is applied either directly with a syringe connected to the angiocatheter or by using arterial tubing attached to the catheter on one end and to the syringe on the other. The catheter or needle is passed back and forth within the substance of the testicle while maintaining the vacuum seal. When sufficient tissue is collected, the suction is released and the needle or catheter removed from the testicle. Using air or wash medium, aspirated tissue is flushed into a Petri dish or collection tube containing media. After fine dissection with scissors or scalpels, the tissue-fluid is examined with microscopy to confirm sperm presence. Repeat sperm aspiration can be performed on the same or opposite testicle as needed (Donoso et al., 2007). Pressure applied to the aspiration site is sufficient for hemostasis. The recovery period is 24 h. Complications include bleeding (1%) and infection (1%).

An alternative to TESA for testicular sperm retrieval involves percutaneous or open surgical biopsy, termed testicular sperm extraction (TESE). The timing of TESE is similar to TESA and is either coincident with oocyte harvest or performed in advance with sperm cryopreservation. The percutaneous TESE technique involves stabilization of the testicle as described with TESA. However, instead of using a butterfly needle, a biopsy gun loaded with a 14-gauge needle is used to obtain core biopsies of the testicle (Tuuri et al., 1998). Aided by a skin puncture by scalpel, the biopsy needle is advanced through the skin and into the testicular tunica albuginea either at the lower or upper pole of the testicle and is directed parallel to the long axis of the testicle. After firing the biopsy gun, the excised tissue core is placed in culture medium for processing. The main risk with testis tissue retrieval by biopsy gun is hematoma, which occurs in 1%–5% of cases as assessed by ultrasound (Tuuri et al., 1998).

Open surgical testicle biopsy (TESE) is a third option for obstructive azoospermia (Fig. 2). This is generally performed through a “window technique” in which the testicle and scrotum are stabilized and the scrotal skin incised with 1 cm incision (Coburn and Wheeler, 1991). The tunica vaginalis is opened and the tunica albuginea of the testicle incised parallel to the visible surface vessels. Testicular tissue is excised and processed for IVF-ICSI similar to TESA samples. The tunica albuginea and scrotum are closed with

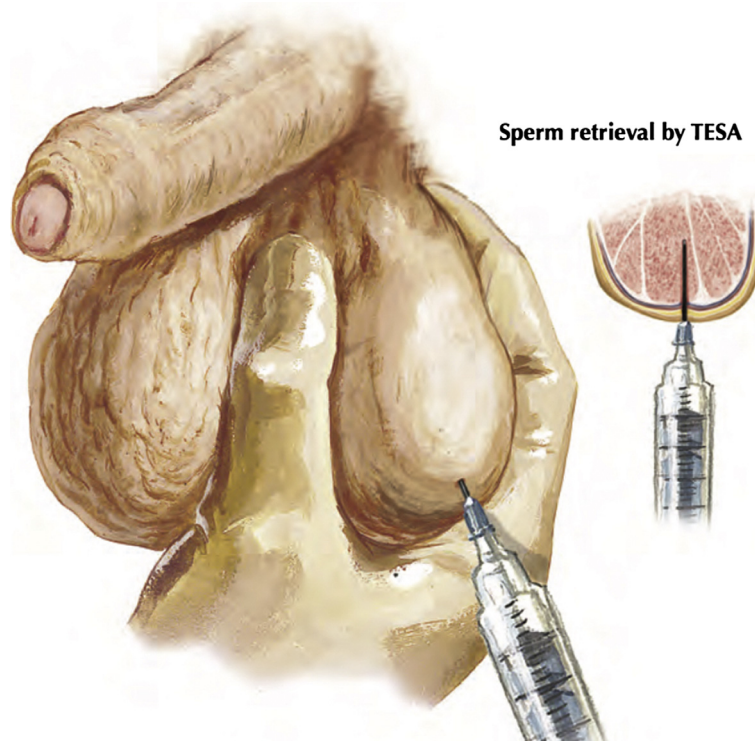


Fig. 1 Testis sperm aspiration (TESA) is performed by needle biopsy. Adapted with permission from Smith J and Turek PJ. Reproductive medicine. Netter's Images. 2nd edn, Vol 1. Elsevier Press, Philadelphia. 2011.



Fig. 2 Testis sperm extraction (TESE) is performed by incisional biopsy. Adapted with permission from Smith J and Turek PJ. Reproductive medicine. Netter's Images. 2nd edn, Vol 1. Elsevier Press, Philadelphia. 2011.

absorbable sutures. The recovery period is 24–48 h and procedural risks are low but include infection (<1%), bruising and bleeding (<5%).

In studies that have examined intratesticular bleeding after testis sperm retrieval, a risk factor for hypogonadism, the risk appears to be higher with open (29%) compared to percutaneous (7%) biopsy techniques ([Harrington et al., 1996](#)). A more worrisome adverse effect from testicular sperm extraction is reduction in testosterone production after large or repeated procedures.

Testis Sperm Retrieval With Nonobstructive Azoospermia

Nonobstructive azoospermia (NOA) underlies most cases of azoospermia. The physical examination typically reveals testicular atrophy and the hormonal analysis demonstrates elevated follicle stimulating hormone (FSH) levels. Unlike the findings from

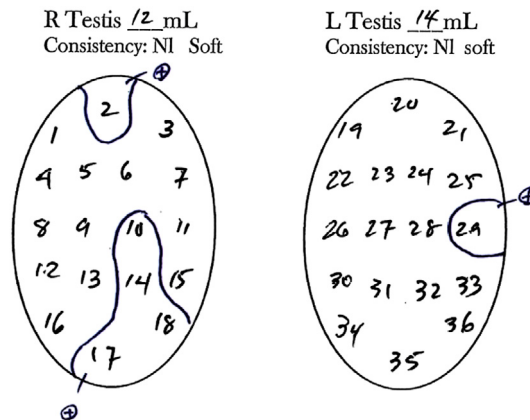


Fig. 3 Demonstration of the focal or “patchy” nature of sperm production in the NOA testis. This figure was derived from fine needle aspiration “mapping” of the testis. Numbers indicate mapped sites within the testis. Circles and lines show where sperm were found at a density of 1 sperm/hpf on cytological smear. Numbered areas without circles showed Sertoli cell-only on cytological review.

obstructive azoospermia, only about half of NOA cases will have mature testicular sperm for ICSI (Kahraman et al., 1996; Tournaye et al., 1997). Furthermore, clinical parameters such as testicular size, hormone levels, and testis biopsy patterns do not accurately predict whether a testicular procedure will yield sperm (Tournaye et al., 1996; Mulhall et al., 1997).

It is also well known that sperm production in NOA testes may be “focal” or “patchy” in nature (Turek et al., 2000) (Fig. 3). This makes testicular sperm aspiration (TESA) less effective than TESE procedures for successful sperm retrieval. Despite the opportunity to sample more testis tissue with TESE vs. TESA procedures, they can still fail to find sperm if performed in blind or random fashion. To address the problem of focal sperm production in NOA, several strategies have been developed to find sperm while minimizing testicular damage.

Multibiopsy testicular sperm extraction (TESE)

The multibiopsy TESE technique was developed in 1997 to maximize successful sperm retrieval by increasing sample size to increase the likelihood of finding sperm. Compared to a single tunical incision in simple TESE procedures, multibiopsy TESE involves several tunical incisions (up to 15) with tissue extraction until sufficient sperm is obtained (Fig. 4). This procedure can be performed in advance of ICSI and all sperm frozen for later use or can be timed to oocyte harvest. The original description of the technique in $n = 21$ patients found sperm in 70% of patients (Mulhall et al., 1997). A more recent study ($n = 741$ patients) examined the optimal number of biopsies needed to find sperm using the multibiopsy approach on a single testicle and found that sperm retrieval rates ranged from 44% for a single biopsy to 58% for four biopsies. Adding a fifth biopsy on the contralateral testis increased overall yield by only 1% (Dadkhah et al., 2013). Other contemporary studies among a total of almost 300 NOA men revealed sperm retrieval rates of 47%–48% using a multibiopsy approach (Schwarzer et al., 2013; Sacca et al., 2016).

Soon after the description of the multibiopsy TESE, in 1998 Schlegel developed microdissection TESE that allowed for improved localization of testis with the aid of intraoperative microscopy (Ostad et al., 1998). This technique will be discussed elsewhere. However, comparison purposes, there is thought to be an increase in sperm retrieval rates of about 11%–18% using microdissection rather than conventional single TESE (Schwarzer et al., 2013; Schlegel, 1999). Two systematic reviews have been published recently that compare sperm retrieval outcomes with conventional TESE and microdissection TESE (Deruyver et al., 2014; Bernie et al., 2015). In the European study, 62 studies were analyzed and sperm retrieval was observed to be higher in the microTESE (43%–63% sperm retrieval rate) than in the conventional TESE group (17%–45% sperm retrieval rate) (Deruyver et al., 2014). However, in patients with uniform maturation arrest biopsy histology, there was no benefit to mTESE vs. TESE procedures. Interestingly, this histology-specific finding mirrors prior reports that found that mTESE lacks superiority to TESE in cases of maturation arrest histology as seminiferous tubules tend to be of uniform size, reducing the ability to optically discriminate sperm-containing tubules (Amer et al., 2000; Takada et al., 2008). In the U.S. paper, a review of 15 studies, mTESE was deemed 1.5-fold more likely to find sperm than conventional TESE (Bernie et al., 2015). It should be noted however, that no randomized, prospective studies have been performed that directly compare the two sperm retrieval techniques.

As described earlier, a significant safety issue in men with non-obstructive azoospermia is hypogonadism after sperm retrieval procedures. It has been reported that if fewer TESE biopsies are taken, there is a less pronounced testosterone decrease (Silber, 2000). However, even with its more precise tissue dissection, microdissection TESE can have measurable effects on androgen balance: among experienced surgeons, serum testosterone levels recover to baseline in only 50%–85% of patients after 1 year (Everaert et al., 2006; Takada et al., 2008; Ishikawa et al., 2009; Dabaja and Schlegel, 2013). Because of the effects of TESE on androgen balance, and the lack of randomized data comparing various sperm retrieval techniques, some have advocated using a “stepwise” multibiopsy approach to finding sperm in which a single TESE sample is taken, followed by a micro-TESE using the same testicular

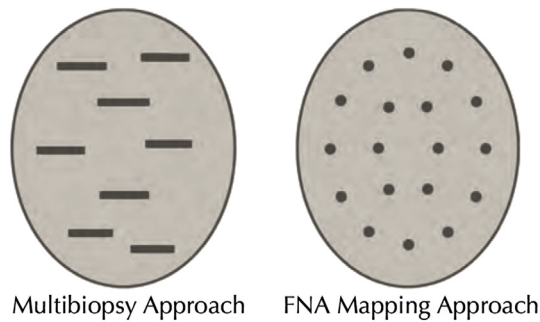


Fig. 4 Schematic drawing of the incisions made in the testicular tunical albuginea in the multibiopsy TESE (left) and FNA mapping (right) techniques. Permission from Smith J and Turek PJ. Reproductive medicine. Netter's Images. 2nd edn, Vol 1. Elsevier Press, Philadelphia. 2011.

incision, followed by a multibiopsy approach on the opposite testis if needed (Franco et al., 2016). This highlights the need for innovative strategies that can (a) improve sperm localization, and (b) reduce the extent of TESE procedures in NOA men.

Fine needle aspiration mapping and “map-guided” TESE

First published in 1997, fine needle aspiration (FNA) map-guided TESE is a two-step nonmicrosurgical procedure that first localizes and then retrieves sperm in the testes (Turek et al., 1997). First, sperm is identified through nonsurgical FNA testicular “mapping,” and then sperm retrieval follows, and is “guided” by the map findings (Figs. 5 and 6). This results in a high chance of successful sperm retrieval by focusing procedures on precise areas within testicles that are known to have sperm, thereby minimizing procedural invasiveness (Beliveau and Turek, 2011). Liken this concept to using GPS to find the shortest way to travel somewhere before beginning the trip. Performed under local anesthesia, FNA mapping involves taking a templated set of aspirated tissue samples, ranging in number from 4/testicle in a simple map to 18/testicle in a compound map (Fig. 4). Aspirated seminiferous tubules undergo routine Papanicolaou staining and are examined by a cytopathologist for sperm (Ljung, 1992). As it is incisionless, recovery is less than 24 h and an average of two pain pills are taken post-procedure. Reported rates of finding sperm in NOA patients with FNA mapping range from 47% to 68% depending on the population studied (Turek et al., 2000; Shefi et al., 2009; Beliveau and Turek, 2011).

If sperm are found on FNA mapping, the couple is counseled to proceed with sperm retrieval and IVF-ICSI. The method of testis sperm extraction is governed by the findings from the mapping procedure (Fig. 6). In a series ($n = 105$) of mapped NOA cases, 44% of patients needed TESA, 33% required TESE, and 23% used microdissection-TESE to retrieve sufficient sperm for all eggs at ICSI, demonstrating the ability of diagnostic mapping to reduce the need for microdissection-TESE sperm retrievals in NOA men

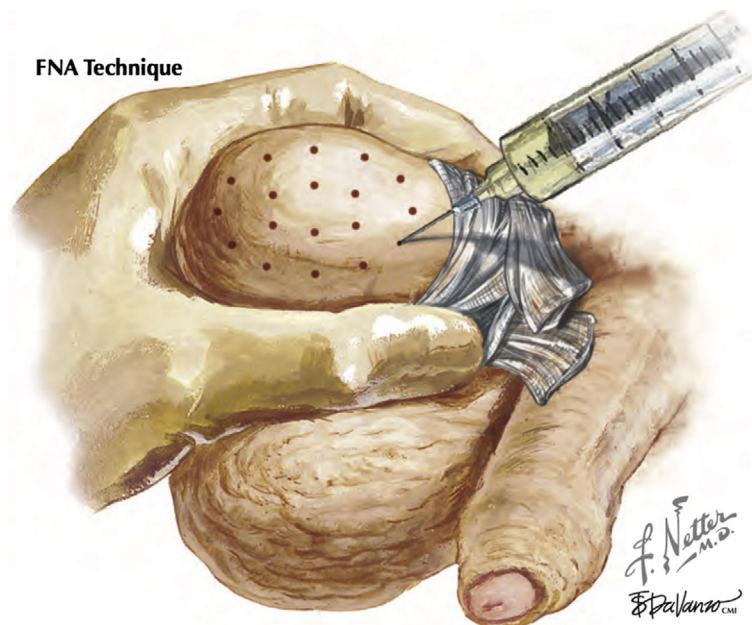


Fig. 5 Illustration of the technique of fine needle aspiration (FNA) mapping of the testicles. Permission from Smith J and Turek PJ. Reproductive medicine. Netter's Images. 2nd edn, Vol 1. Elsevier Press, Philadelphia. 2011.

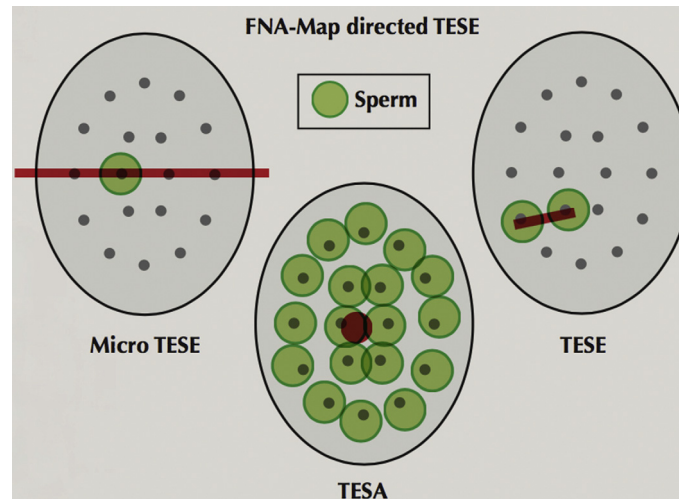


Fig. 6 Approach to FNA map guided sperm retrieval. Green circles are geographical areas of the testicle with mature sperm. Red lines and circles are planned incisions or aspiration sites. Note how sperm retrieval can be targeted to sperm positive map sites to minimize extent of sperm retrieval procedures. Permission from Smith J and Turek PJ. Reproductive medicine. Netter's Images. 2nd edn, Vol 1. Elsevier Press, Philadelphia. 2011.

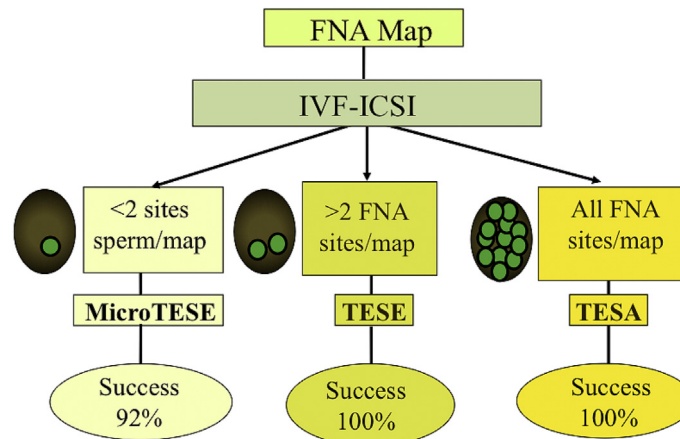


Fig. 7 A schematic showing the clinical pathway for sperm retrieval after initial diagnostic FNA Mapping in $n = 96$ patients. Overall success (sufficient sperm retrieved for all eggs at IVF-ICSI) occurs in 95% of men.

(Beliveau and Turek, 2011). In addition, all microdissection cases in this series were unilaterally performed. Overall, sufficient sperm (enough for all eggs at IVF) is obtained at retrieval after FNA mapping in 95% of cases (Fig. 7). Recently, FNA mapping has also been shown to be particularly effective in finding testicular sperm in cases of cryptozoospermia and failed microdissection TESE procedures (Turek et al., 2016a,b). By significantly curtailing the use of microdissection yet maintaining high sperm yields in NOA cases, sperm mapping could theoretically limit post-surgical hypogonadism. Indeed, the safety profile of the FNA approach has been confirmed in both human and animal studies (Dahlbom et al., 1997; Turek et al., 2000; Gouletsou et al., 2010). Recently described variations on the sperm mapping concept include single-site, percutaneous, multipass tissue sampling (Jensen et al., 2016) and single-seminiferous tubule biopsy through open micropuncture (Shah, 2001).

Conclusions

From an evidence-basis, several microsurgical and nonmicrosurgical sperm retrieval techniques exist and no single procedure is considered the "best" from a risk-reward profile for either obstructive or non-obstructive azoospermia. A summary comparison of these techniques is outlined in Fig. 8. Although it is not difficult to retrieve sperm in obstructed cases, it can be very difficult to find sperm in NOA, as sperm production may be "patchy" or "focal." Unfortunately, no prospective, randomized controlled trials guide our sperm retrieval strategies for NOA patients. However, since ICSI is not always successful, it behooves reproductive urologists to develop and apply retrieval techniques that are reliable, of low morbidity, and that harvest sufficient sperm to enable multiple ICSI attempts without repetition.

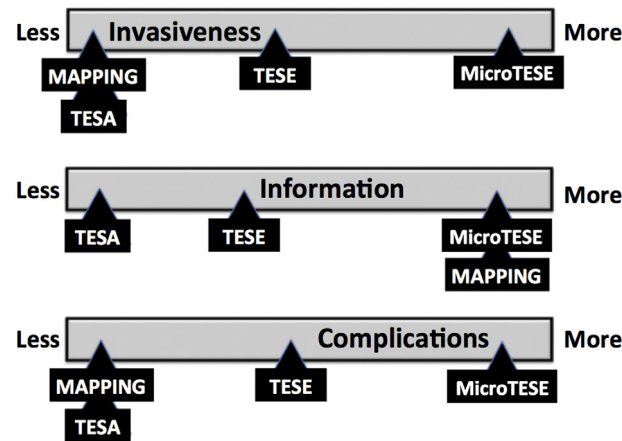


Fig. 8 A schematic comparison of various sperm retrieval techniques based on information obtained, invasiveness and complications. *Note:* Mapping = FNA mapping; MicroTESE = microdissection TESE.

Key Points

- All men with obstructive azoospermia and a majority of men with nonobstructive azoospermia will have sperm for biological pregnancies.
- There is currently insufficient high-level evidence to recommend any specific sperm retrieval technique over another for either obstructive or nonobstructive azoospermia.
- The onus is on clinicians to develop sperm retrieval techniques that maximize yield and minimize procedure numbers and morbidity.

References

- Amer, M., Ateyah, A., Hany, R., & Zohdy, W. (2000). Prospective comparative study between microsurgical and conventional sperm extraction in non-obstructive azoospermia: Follow-up by serial ultrasound examinations. *Human Reproduction*, 15, 653–656.
- Beliveau, M. E., & Turek, P. J. (2011). The value of testicular 'mapping' in men with non-obstructive azoospermia. *Asian Journal of Andrology*, 13, 225–230.
- Bernie, A. M., Mata, D. A., Ramasamy, R., & Schlegel, P. N. (2015). Comparison of microdissection testicular sperm extraction, conventional testicular sperm extraction, and testicular sperm aspiration for nonobstructive azoospermia: A systematic review and meta-analysis. *Fertility and Sterility*, 104, 1099–1103.
- Coburn, M., & Wheeler, T. M. (1991). In L. I. Lipshultz, & S. S. Howards (Eds.), *Infertility in the male* (2nd edn, pp. 223–253). Philadelphia: Mosby Year Book.
- Dabaja, A. A., & Schlegel, P. N. (2013). Microdissection testicular sperm extraction: An update. *Asian Journal of Andrology*, 15, 35–39.
- Dadkhah, F., Hosseini, S. J., Sadighi Gilani, M. A., Farrahi, F., Amini, E., & Kareminejad, B. (2013). Optimal number of biopsies and impact of testicular histology on the outcome of testicular sperm extraction. *Urology Journal*, 10, 795–801.
- Dahlbom, M., Makinen, A., & Suominen, J. (1997). Testicular fine needle aspiration cytology as a diagnostic tool in dog infertility. *The Journal of Small Animal Practice*, 38, 506–512.
- Deruyver, Y., Vanderschueren, D., & Van der Aa, F. (2014). Outcome of microdissection TESE compared with conventional TESE in non-obstructive azoospermia: A systemic review. *Andrology*, 2, 20–24.
- Donoso, P., Tournaye, H., & Devroey, P. (2007). Which is the best sperm retrieval technique for non-obstructive azoospermia? A systematic review. *Human Reproduction Update*, 13, 539–549.
- Everaert, K., De Croo, I., Kerckhaert, W., Dekuyper, P., Dhont, M., Van der Elst, J., De Sutter, P., Comhaire, F., Mahmoud, A., & Lumen, N. (2006). Long term effects of microsurgical testicular sperm extraction on androgen status in patients with nonobstructive azoospermia. *BMC Urology*, 6, 9–12.
- Franco, G., Scarselli, F., Casciani, V., De Nunzio, C., Dente, D., Leonardo, C., Greco, P. F., Greco, A., Minasi, M. G., & Greco, E. A. (2016). Novel stepwise micro-TESE approach in nonobstructive azoospermia. *BMC Urology*, 16, 20.
- Gouletsou, P. G., Galatos, A. D., Leontides, L. S., & Sideri, A. I. (2010). Impact of fine or large needle aspiration on the dog's testis: *In vitro* ultrasonographic, bacteriological, gross anatomy and histological assessment. *Theriogenology*, 74, 1604–1614.
- Harrington, T., Schauer, D., & Gilbert, B. (1996). Percutaneous testis biopsy: An alternative to open testicular biopsy in the evaluation of the subfertile man. *The Journal of Urology*, 156, 1647–1651.
- Ishikawa, T., Yamaguchi, K., Chiba, K., Takenaka, A., & Fujisawa, M. (2009). Serum hormones in patients with nonobstructive azoospermia after microdissection testicular sperm extraction. *The Journal of Urology*, 182, 1495–1499.
- Jarow, J. P., Espeland, M. A., & Lipshultz, L. I. (1989). Evaluation of the azoospermic patient. *The Journal of Urology*, 142, 62–65.
- Jensen, C. F., Ohl, D. A., Hiner, M. R., Fode, M., Shah, T., Smith, G. D., & Sonksen, J. (2016). Multiple needle-pass percutaneous testicular sperm aspiration as first-line treatment in azoospermic men. *Andrology*, 4, 257–262.
- Kahraman, S., Özgür, S., Alataş, C., Aksoy, S., Taşdemir, M., Nuhoğlu, A., Taşdemir, I., Balaban, B., Biberöğlu, K., Schoysman, R., Nijs, M., & Vanderzwalmen, P. (1996). Fertility with testicular sperm extraction and intracytoplasmic sperm injection in non-obstructive azoospermic men. *Human Reproduction*, 11, 756–760.
- Ljung, B.-M. (1992). In L. G. Koss, S. Woyke, & W. Olszewski (Eds.), *Techniques of aspiration and smear preparation* (pp. 12–34). New York: Igaku-Shoin.
- Marmar, J. L., & Benoff, S. (2005). The safety of ultrasonically guided testis aspiration biopsies and efficacy of use to predict varicocelectomy outcome. *Human Reproduction*, 20, 2279–2288.
- Mulhall, J. P., Burgess, C. M., Cunningham, D., Carson, R., Harris, D., & Oates, R. D. (1997). Presence of mature sperm in testicular parenchyma of men with nonobstructive azoospermia: Prevalence and predictive factors. *Urology*, 49, 91–96.

- Ostad, M., Liotta, D., Ye, Z., & Schlegel, P. N. (1998). Testicular sperm extraction for nonobstructive azoospermia: Results of a multibiopsy approach with optimized tissue dispersion. *Urology*, 52, 692–696.
- Palermo, G., Joris, K., Devroey, P., & Van Steirteghem, A. C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, 340, 17–18.
- Sacca, A., Pastore, A. L., Roscigno, M., Naspro, R., Pelluchi, F., Fuschi, A., Maruccia, S., Territo, A., Pisano, F., Zanga, L., Capitano, E., Carbone, A., Fusi, F., Chinaglia, D., & Pozzo, L. F. (2016). Conventional testicular sperm extraction (TESE) and non-obstructive azoospermia: Is there still a chance in the era of microdissection TESE? Results from a single non-academic community hospital. *Andrology*, 4, 425–429.
- Schlegel, P. N. (1999). Testicular sperm extraction: Microdissection improves sperm yield with minimal tissue excision. *Human Reproduction*, 14, 131–135.
- Schwarzer, J. U., Steinfatt, H., Schleyer, M., Kohn, F. M., Fielder, K., von Hertwig, I., Krusmann, G., & Wurfel, W. (2013). No relationship between biopsy sites near the main testicular vessels or rete testis and successful sperm retrieval using conventional or microdissection biopsies in 220 non-obstructive azoospermic men. *Asian Journal of Andrology*, 15, 795–798.
- Shah, R. S. (2001). Operative sperm retrieval for ART. In M. L. Goenka, & D. Goenka (Eds.), *Recent advances in infertility management* (pp. 179–183). Guwahati: Goenka.
- Shefi, S., Kaplan, K., & Turek, P. J. (2009). Analysis of spermatogenesis in non-obstructive azoospermic and virtually azoospermic men with known testicular pathology. *RBMonline*, 18, 460–464.
- Silber, S. J. (2000). Microsurgical TESE and the distribution of spermatogenesis in non-obstructive azoospermia. *Human Reproduction*, 15, 2278–2284.
- Takada, S., Tsujimura, A., Ueda, T., Matsuoka, Y., Takao, T., Miyagawa, Y., Koga, M., Takeyama, M., Okamoto, Y., Matsumiya, K., Fujioka, H., Nonomura, N., & Okuyama, A. (2008). Androgen decline in patients with nonobstructive azoospermia after microdissection testicular sperm extraction. *Urology*, 72, 114–118.
- Tournaye, H., Liu, J., Nagy, P. Z., Camus, M., Goossens, A., Silber, S., Van Steirteghem, A. C., & Devroey, P. (1996). Correlation between testicular histology and outcome after intracytoplasmic sperm injection using testicular spermatozoa. *Human Reproduction*, 11, 127–132.
- Tournaye, H., Verheyen, G., Nagy, P., Ubaldi, F., Goossens, A., Silber, S., Van Steirteghem, A. C., & Devroey, P. (1997). Are there any predictive factors for successful testicular sperm recovery in azoospermic patients? *Human Reproduction*, 12, 80–86.
- Turek, P. J., Cha, I., & Ljung, B.-M. (1997). Systematic fine-needle aspiration of the testis: Correlation to biopsy and results of organ “mapping” for mature sperm in azoospermic men. *Urology*, 49, 743–748.
- Turek, P. J., Nudell, D. M., & Conaghan, J. (1999). Methods of epididymal sperm retrieval: A urologic perspective. *Assisted Reproduction Reviews*, 9, 60–64.
- Turek, P. J., Cha, I., Ljung, B.-M., & Conaghan, J. (2000). Diagnostic findings from testis fine needle aspiration mapping in obstructed and non-obstructed azoospermic men. *The Journal of Urology*, 163, 1709–1716.
- Turek, P. J., Christensen, E., Tam, R., Prasad, K. C., & Cha, I. (2016a). Finding sperm in men after failed microdissection TESE procedures. *Fertility and Sterility*, 105(2), e41.
- Turek, P. J., Christensen, E., Tam, R., Prasad, K. C., & Cha, I. (2016b). The probability of obtaining testicular sperm in cases of cryptozoospermia. *Fertility and Sterility*, 105, e42.
- Tuuri, T., Moilanen, J., Kaukoranta, S., Mäkinen, S., Kotola, S., & Hovatta, O. (1998). Testicular biopsy gun needle biopsy in collecting spermatozoa for intracytoplasmic injection, cryopreservation and histology. *Human Reproduction*, 14, 1274–1278.
- Van Peperstraten, A., Proctor, M. L., Johnson, N. P., & Philipson, G. (2006). Techniques for surgical retrieval of sperm prior to ICSI for azoospermia (review). *Cochrane Database of Systematic Reviews*, 3, CD002807.

Microsurgical Testis Sperm Extraction

Tolulope Bakare, University of Illinois, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Assisted reproductive techniques have become an effective means of managing male-factor infertility. The advancement of in vitro fertilization (IVF) using intracytoplasmic sperm injection (ICSI) has led to the development of more sophisticated sperm retrieval techniques which has increased the number of men with male factor infertility who successfully father children. Conventional testicular sperm extraction has been used in management of these patients. This procedure involves the creation of a small window in the testis and random biopsy is obtained and evaluated for sperm. If sperm is found, it is then used in in vitro fertilization. In order to minimize tissue removed during conventional testicular sperm extraction (TESE), and to maximize the amount of sperm collected from the testicle, microsurgical testicular sperm extraction (micro-TESE) was developed. This technique (described below) originally described in 1999 has been proven to enhance the number of sperm extracted in specifically in men with spermatogenic dysfunction when compared to random biopsies (Schlegel, 1999). The technique is also used in extracting sperm when conventional sperm extraction methods have resulted in no sperm. Although, the incision for a micro-TESE is larger than conventional TESE, less intra-testicular reaction including scarring, and calcifications, has been noted on ultrasound in patients who undergo micro-TESE compared to conventional TESE (Ramasamy et al., 2005).

Technique

A midline scrotal incision is made through the raphe with electrocautery, entering into the hemiscrotum. The testicle is delivered within its tunica vaginalis and the tunica vaginalis is entered in a controlled fashion. The testicle is inspected for any gross abnormalities. The testicle may then be packed with moist gauze and the steps may be repeated on the contralateral side. The surgical microscope is then brought and docked into position, to perform the rest of the procedure under surgical magnification. A transverse incision is made through the tunica albuginea, away from any subcutaneous vessels to maintain the integrity of the testicular blood supply. The surgeon's nondominant hand holds the everted testicle while the dominant hand carefully and systematically dissects the extruded seminiferous tubules under high-power magnification (Fig. 1). Selected areas were identified which were notably more prominent and full with regard to tubular size than other areas within the testicle (Fig. 2). These tubules are removed bluntly with forceps and placed in the sperm cryopreservation medium, and sent to the embryologist, who examines the tissue and extracts sperm. Hemostasis is achieved with the judicious use of bipolar electrocautery. The tunica albuginea is closed with a running suture. The procedure is also performed on the contralateral testicle. The tunica vaginalis is closed on either side with a running suture. Each testicle is then returned into the hemiscrotum in the correct orientation, and the wound is closed. A compressive dressing may be applied to the base of the groin.

Post Surgical Care

Post operative instructions include application of ice packs to the surgical site for up to 48 h post operative, no heavy lifting that is, greater than 20 lb for 2–3 weeks post operatively, and no sexual activity for minimum of 48–72 h. Patients may resume sexual activity after that per their discretion. Pain medication is prescribed on a as needed basis. Oral antibiotics may be given for 3–5 days, but a single dose given during surgery may be sufficient (Akalin, 2002).



Fig. 1 Schematic representation of the microdissection TESE procedure.



Fig. 2 Dissected testicular tissue during microsurgical testis sperm extraction with prominent tubules.

Outcomes of Microsurgical Testicular Sperm Extraction

The ability to identify seminiferous tubules containing developing germ cells, which appear larger and more opaque under the microscope, has resulted in increased successful sperm retrievals using this technique. The chances of finding spermatozoa in patients with spermatogenic dysfunction has been shown to increase from 45% to 63% (Schlegel, 2009). Comparative assessments performed also revealed significantly higher sperm retrieval rates with micro-TESE when compared to conventional TESE (47% vs. 30%) (Amer et al., 1999). However, the success of sperm extraction depends on the etiology of male-factor infertility, the skill and experience of the surgeon, and the processing of the specimen after retrieval. When compared to patients with obstructive azoospermia, results of surgical sperm retrieval are lower in patients with nonobstructive azoospermia or spermatogenic dysfunction. This is due to the abundance of sperm in obstructive azoospermia patients. These patients can be treated with a wide variety of techniques and typically do not warrant microsurgical extraction. In contrast, Nonobstructive azoospermia also known as spermatogenic dysfunction is characterized by impairment of the endocrine and/or exocrine functions of the testis that is, impaired spermatogenesis. In a few instances, spermatogenic failure may result from hypogonadism, which may result from hypothalamic, pituitary or testicular disorders. More commonly however, nonobstructive azoospermia results from testicular failure with borderline to low serum testosterone. Medical therapies that increase serum testosterone may also increase intratesticular testosterone. However, it is uncertain if low testosterone in these patients predicts retrieval rates with microsurgical sperm extraction. In patients with Klinefelter's syndrome, men with a low baseline testosterone who were treated and responded to therapy had a 77% chance of sperm retrieval compared to 55% of men who did not respond to medical therapy (Ramasamy et al., 2009). Y chromosome microdeletions may also affect outcomes of sperm extraction. Men with complete deletions of the AZFa or AZFb regions typically have no chance of sperm retrieval during surgery and it is not recommended to perform micro-TESE procedures (Schlegel, 2009). However, men with AZFc region deletions alone may have a 67% chance of sperm retrieval after micro-TESE (Dabaja and Schlegel, 2013). Other factors that influence outcomes include history of chemotherapy, and cryptorchidism. Patients undergoing chemotherapy are counseled on sperm banking before chemotherapy. In men with cryptorchidism, sperm retrieval is successful if orchiopexy was performed an average of 10 years earlier when compared to men with failed retrieval (Raman and Schlegel, 2003).

Sperm obtained from micro-TESE must be used in intracytoplasmic injection (ICSI)/in vitro fertilization. This is because testicular sperm has not matured and cannot maneuver to the egg as in typical reproduction. The testicular sperm once extracted may be used fresh or frozen. Some embryology labs express concern over cryopreservation of testis sperm due to concern for increased in vitro fertilization failure rates. The freeze-thaw process may result in rupture of the acrosomal and plasma membrane of the sperm, as well as free radical injury that may result in DNA damage (Agca and Critser, 2002). However, the advantages which include the use of sperm obtained in multiple cycles of in vitro fertilization, and allowing the couple the option of waiting for ovarian stimulation and oocyte retrieval while sperm retrieval is undertaken (Fukunaga et al., 2001). Interestingly, recent analysis found no difference in pregnancy rates with fresh surgically retrieved sperm and cryopreserved surgically retrieved sperm. There was also no difference in fertilization rates in these different situations (Ohlander et al., 2014).

Conclusion

Advancement in in vitro fertilization through intracytoplasmic injection (ICSI) has led to continued desire to increase knowledge and improve surgical techniques for sperm retrieval in infertile men. These advances have significantly improved the quality of life of these patients. As not all sperm retrieval techniques are beneficial for certain causes of infertility, it is imperative to be adept in the various techniques and what patient the technique benefits. The primary etiology of the infertility, type of procedure performed and

the ability to adequately retrieve sperm from seminiferous tubules all contribute to the success rate of retrieval. Microsurgical testicular sperm extraction has been proven to be superior when compared to conventional testis sperm extraction technique especially in men with spermatogenic dysfunction.

References

- Agca, Y., & Critser, J. K. (2002). Cryopreservation of spermatozoa in assisted reproduction. *Seminars in Reproductive Medicine*, 20, 15–23.
- Akalin, H. E. (2002). Surgicalprophylaxis:theevolutionof guidelines in an era of cost containment. *The Journal of Hospital Infection*, 50(Suppl A), S3–7.
- Amer, M., Haggag, S. E., Moustafa, T., Abd El-Naser, T., & Zohdy, W. (1999). Testicular sperm extraction: Impact of testicular histology on outcome, number of biopsies to be performed and optimal time for repetition. *Human Reproduction*, 14, 3030–3034.
- Dabaja, A. A., & Schlegel, P. N. (2013). Microdissection testicular sperm extraction: An update. *Asian Journal of Andrology*, 15(1), 35–39.
- Fukunaga, N., Haigo, K., Kyono, K., & Araki, Y. (2001). Efficiency of using frozen-thawed testicular sperm for multiple intracytoplasmic sperm injections. *Journal of Assisted Reproduction and Genetics*, 18, 634–637.
- Ohlander, S., Hotaling, J., Kirshenbaum, E., Niederberger, C., & Eisenberg, M. L. (2014). Impact if fresh versus cryopreserved testicular sperm upon intracytoplasmic sperm injection pregnancy outcomes in med with azoospermia due to spermatogenic dysfunction: A meta-analysis. *Fertility and Sterility*, 101(2), 344–349.
- Raman, J. D., & Schlegel, P. N. (2003). Testicular sperm extraction with intracytoplasmic sperm injection is successful for the treatment of nonobstructive azoospermia associated with cryptorchidism. *The Journal of Urology*, 170, 1287–1290.
- Ramasamy, R., Yagan, N., & Schlegel, P. N. (2005). Structural and functional changes to the testis after conventional versus microdissection testicular sperm extraction. *Urology*, 65(6), 1190–1194.
- Ramasamy, R., Ricci, J. A., Palermo, G. D., Gosden, L. V., Rosenwaks, Z., et al. (2009). Successful fertility treatment for Klinefelter's syndrome. *The Journal of Urology*, 182, 1108–1113.
- Schlegel, P. N. (1999). Testicular sperm extraction: Microdissection improves sperm yield with minimal tissue excision. *Human Reproduction*, 14, 131–135.
- Schlegel, P. N. (2009). Nonobstructive azoospermia: A revolutionary surgical approach and results. *Seminars in Reproductive Medicine*, 27, 165–170.

Further Reading

- Sigman, M. M., Lipschultz, L. I., & Howard, S. S. (2009). Office evaluation of the subfertile male. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.

Sperm Retrieval From the Scrotal Extratesticular Ductal System

Sarah C Vij and Edmund Sabanegh, Jr., Glickman Urologic and Kidney Institute, Cleveland, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Anejaculation The inability of a male to ejaculate.

Cryopreservation The process by which biological tissues are preserved by cooling to very low temperatures.

Electroejaculation A procedure to obtain semen from male mammals or humans involving placement of an electric probe in the rectum and delivering a voltage to initiate an ejaculation.

Obstructive azoospermia A physical obstruction of the male genital tract that prevents sperm from reaching the ejaculate.

Penile vibratory therapy A procedure to obtain semen from male mammals or humans involving placement of a mechanical vibrating device at the base of the glans penis to initiate an ejaculation with an intact ejaculatory reflex.

Spermatogenesis The process involving development of spermatozoa from spermatogonial stem cells through mitosis and meiosis.

Patient Population and Indications

Sperm can be obtained from men with an obstructive etiology of male infertility with various sperm retrieval techniques when surgical reconstruction is not an option. Obstructive azoospermia is defined as a failure of the sperm to reach the ejaculate due to obstruction in the ductal system. These men typically have normal spermatogenesis—the abnormality is blockage to transport. Vasectomy is the most common etiology of obstructive azoospermia and most of these men are candidates for vasectomy reversal to reconstitute continuity from the testis to the ejaculate. However, there are other causes of obstructive azoospermia that are not amenable to surgical reconstruction. The etiology of obstruction in these men can be congenital or acquired. Such patients are those with congenital bilateral absence of the vas deferens, bilateral partial aplasia of the vas deferens, failed surgical reconstruction, or those with vasal or epididymal obstruction (typically iatrogenic due to vasectomy) that cannot be reconstructed surgically. Additionally, men with anejaculation due to neurologic or psychogenic disorders may be offered these techniques if normal ejaculation is not restored after other treatment modalities have been tried or if the patient prefers. The sperm retrieval techniques discussed in this article enable these men to conceive with in vitro fertilization, a type of assisted reproductive technique. Epididymal sperm are more motile than testicular sperm which confers a theoretical advantage to the techniques discussed in this article as compared to methods to obtain sperm from the testis although fertilization rates using a type of in vitro fertilization called intracytoplasmic sperm injection (ICSI) do not appear to be better using epididymal rather than testicular sperm.

Microsurgical Epididymal Sperm Aspiration

Microsurgical epididymal sperm aspiration (MESA) is a traditional microsurgical approach for obtaining sperm in patients with obstructive azoospermia. Microsurgical skill set and general anesthesia are required. As a result, the overall cost of MESA is higher than percutaneous epididymal sperm aspiration (PESA). A small incision is made in the upper scrotum and the epididymis is identified and examined under the operating room microscope. A dilated epididymal tubule is identified and incised with a micro-knife. The emanating fluid is examined under a microscope. If no sperm are found, the site is closed with microsuture and another site more proximal (closer to the testicle) is found. When motile sperm are found, a micropipette is placed next to the tubule and sperm are drawn in to the pipette which has been preloaded with sperm cryopreservation media. Gentle massage of the testicle and epididymis can be applied to increase yield. Several micropipettes are typically used. Pregnancy rates approach 60% when ICSI is used. Sperm can be used fresh or cryopreserved for future cycles. This technique involves an incision in the skin; therefore, there is a small risk of wound infection and hematoma.

Percutaneous Epididymal Sperm Aspiration

PESA is a procedure that can be performed under local anesthesia without a microsurgical skill set. The procedure involves placing a butterfly needle in to the head of the epididymis after administration of local anesthetic. Negative pressure is applied until a milky white fluid is visualized. The tubing is then clamped and the system is flushed with sperm media in to a collection tube. The procedure can be repeated until sufficient yield is obtained. The percutaneous approach obviates the need to perform a skin incision therefore avoiding the associated morbidity such as infection and pain; however, a small risk of hematoma is still present.

Percutaneous puncture can cause epididymal obstruction due to scarring which may preclude future reconstruction if this is pursued. PESA sperm yield is lower than MESA although usually sperm numbers are adequate for ICSI.

Vasal Aspiration

Vasal aspiration can be performed using percutaneous or open technique. Percutaneous vasal aspiration is performed with local anesthetic. The vas deferens is held between the surgeon's thumb and forefinger in the scrotum. A 21-gauge sharp needle is used to pierce the vas deferens. A 23-gauge blunt needle is then passed through the sharp needle, aiming toward the epididymis. The blunt needle has both a side hole and an end hole. It is connected to a syringe with sperm preservation fluid which is injected gently in to the vas deferens followed by gentle suction. This fluid is then used for sperm cryopreservation or immediate assisted reproductive techniques.

Open vasal aspiration requires a small incision over the spermatic cord in the superior scrotum. The vas deferens is exposed and isolated. The vas deferens is then partially transected with a fine blade. A microaspiration catheter is inserted in the testicular end of the vas deferens. Sperm preservation media is injected gently and aspirated. If sufficient fluid does not return, epididymal massage can be performed. The vas deferens is then closed with microsuture—this can be done with the microscope or loupe magnification depending on surgeon experience. Vasal aspiration may create an obstruction from the procedure itself which would preclude future surgical reconstruction if the patient is considering this option in the future.

Summary

Men with obstructive azoospermia or anejaculation are candidates for several sperm retrieval techniques including PESA, MESA, and vasal aspiration. Each technique has specific risks and benefits, and success rates vary among them.

Further Reading

- Leung, H., Mira, J., & Hsiao, W. (2014). Updates on sperm retrieval techniques. *Translation Andrology and Urology*, 3, 94–101.
- Levine, L., & Fakouri, B. J. (1998). Experience with vasal sperm aspiration. *The Journal of Urology*, 159, 1551–1553.
- Qiu, Y., Wang, S., Yang, D., & Wang, L. (2003). Percutaneous vasal sperm aspiration and intrauterine insemination for infertile males with anejaculation. *Fertility and Sterility*, 79, 618–620.
- Wein, A. J. (2016). *Campbell-Walsh urology* (11th edn.). Philadelphia, PA: Elsevier.

Penile Vibratory Stimulation

Charles M Lynne, Emad Ibrahim, and Nancy L Brackett, University of Miami Miller School of Medicine, Miami, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction: What Is Penile Vibratory Stimulation (PVS) and What Are Its Uses?

PVS, for the purposes of this chapter, is the application of a vibrator(s) to the penis in order to elicit a reflex ejaculation in men who are anejaculatory. Its main application is in the field of fertility management in men who have suffered a spinal cord injury (SCI) or suffer from a congenital disease or a disease or process in the spinal cord which results in neurogenic anejaculation.

History

The history of the use of PVS might be described by answering the question: "What good is sperm retrieval if we don't know what to do with the sperm after we get it?" The history of the use of sperm retrieval techniques in men with SCI-related ejaculatory dysfunction is interwoven with the use of artificial insemination, primarily intravaginal insemination, in veterinary/animal husbandry practice; the development of current assisted reproductive technologies (ARTs); the current age of information and communication; and the emergence of an informed SCI population. John Hunter is credited with the first artificial insemination in humans in the late 1700s. It was, in fact, an intravaginal insemination of sperm in a couple where the husband had hypospadias (Gutmacher, 1943). However, artificial insemination of this type plays a greater role in animal husbandry and the conservation of endangered species than in the management of couples who have difficulties in conceiving. (An exception exists in human infertility practice, where intravaginal insemination using ejaculates obtained using PVS in men with SCI is used successfully by many practitioners.)

In more current times, 1978 is considered a pivotal year. The world was told by Steptoe and Edwards of the first human live birth after IVF. Prior to this time, there were many descriptions of intravaginal artificial insemination and at least one report of intra-uterine insemination (IUI). In this 1941 report, 6 of 148 women became pregnant via IUI. Publications in the 1940s and 1950s described improvements in sperm preparation for insemination and sperm freezing. This led to the notion that infertile men could become biologic fathers. The later success of IVF in the 1980s spurred renewed interest in the use of IUI as well, based in part on what was learned in the earlier studies of sperm preparation (Gutmacher, 1943; Ombelet and Van Robays, 2015).

In 1981, Brindley described his experience with penile vibratory stimulation (PVS) and electroejaculation (EEJ) in men with spinal cord injuries (Brindley, 1981a,b). Although he was not the first, he was considered a champion of sperm retrieval in men with SCI. In 1987, a review coauthored by Stephen Seager, DVM, reviewed five previous reports of the use of electroejaculation. Seager described his own results in spinal cord injured patients (Halstead et al., 1987). Dr. Seager also developed a much needed tool: a reliable, safe, and eventually FDA-approved device for performing EEJ in humans. He had a significant prior experience in the use of EEJ in animals. The reliability and reproducibility of the EEJ device, allowed for the first time, collection of semen for the accumulation of data to study the condition of infertility in men with SCI.

Prior to the 1990s, the use of vibratory stimulation to the penis had been described, but had been used only sporadically by researchers and clinicians to obtain an ejaculate. In 1994, Sonksen showed that there was an optimal vibrator head frequency and amplitude in the application of PVS, which resulted in improved sperm quality versus non-optimized vibrators (Sonksen et al., 1994). These findings led to the development of the Ferticare device, which was specifically engineered to induce ejaculation in men with neurogenic anejaculation. In patients who were responsive to PVS, this method proved to be easier, quicker, and produced a higher quality ejaculate than EEJ (Sonksen et al., 1994).

More recent developments have further enhanced treatment of infertility in men with SCI. By the late 1980s, the internet was considered mainstream at most universities in the US, and mainstream in most US households by the late 1990s. The flow of information about any subject became as easy as entering queries into ever-improving applications and search engines. Second, the Americans with Disabilities Act, ADA, was signed into law in 1990, and persons with SCI began to seek the care they were entitled to. More recently, another FDA-approved device, the ViberecX3 (Reflexonic, Frederick, MD, USA), has been introduced for use in sperm retrieval in men with SCI. The ViberecX3 uses two vibrating pads (Fig. 1).

Why PVS?

There are two well-established and well-studied methods for sperm retrieval in men who have neurogenic anejaculation, EEJ and PVS. EEJ is successful in producing an ejaculate in over 95% of the spinal cord injured population. In common usage however, it is reserved for those persons who do not respond to PVS. EEJ is discussed in detail in another chapter. Compared to EEJ, PVS is easier to use, can produce an ejaculate in the majority of men with SCI lesions at T10 or above, and produces antegrade ejaculates with a higher percentage of motile sperm. In carefully selected and trained couples, it can be performed at home for use in intravaginal insemination (Ibrahim et al., 2016b).

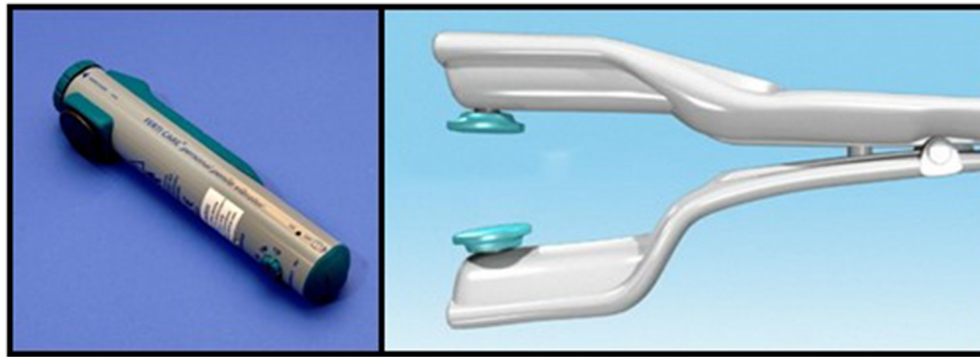


Fig. 1 Optimized vibrators for PVS: left: Ferticare; right: Vibrect X-3.

SCI: The Neurogenic Anejaculation Paradigm

SCI is the paradigm for illustrating the neural pathways necessary for normal ejaculation and the sequelae of neurogenic dysfunction in those areas. Normal ejaculation depends on intact neural pathways for the coordinated components of ejaculation, emission, and expulsion. Emission for the most part relies on sympathetic nervous output, T10–L2. Expulsion for the most part relies on parasympathetic output, S2–S4. The dorsal nerve of the penis, a branch of the pudendal nerve, S2–S4, is a necessary component providing somatic sensory input into the ejaculatory reflex arc. Motor branches of the pudendal nerve to the pelvic floor and external urinary sphincter are involved in the expulsive phase as well. A spinal generator of ejaculation located approximately at L3 orchestrates the somatic, sympathetic, and parasympathetic innervated anatomical structures participating in ejaculation. When the spinal cord is intact, supra-spinal input, both facilitative and inhibitory, moderate this complex reflex activity (Fode et al., 2015).

Some things become apparent in looking at this scenario. Persons with spinal cord injuries rostral to T10 should have their entire reflex components intact and respond to an adequate penile vibratory stimulus. These persons are the ideal candidates for PVS. Persons with injuries to the cord below T10 should be poor or no responders to an adequate stimulus. Persons with injuries to the lower sacral segments, or to the pudendal nerve may not respond to any stimulus if their lesion precludes transmission of the stimulus into the spinal cord.

The stimulus produced via sexual activity, usually is not enough on its own to initiate a reflex ejaculation in the SCI patient; hence only about 10% of men with injuries at or rostral T10 report the ability to ejaculate by masturbation or sexual intercourse. The stimulus produced by an optimized vibrator, however, will induce a reflex ejaculation in up to 85% of such patients, and 15%–17% of men whose injury is below T10 (Brackett et al., 2010). The intensity of stimulus necessary to induce ejaculation in men with SCI is considered painful to most neurologically intact men.

A Word of Caution

The overwhelming majority of patients who will respond to PVS will do so within the first 12 months post injury, however, some will not respond for up to 18 months post-injury. In practice, we do not delay attempting a trial of PVS if the patient so desires early after injury, but he is counseled that if there is no response, we will make another attempt in 3–6 months.

The Procedure

Prior to the actual performance of the PVS procedure, patient should have a history and physical exam. The presence or absence of a bulbocavernosus reflex and hip flexor reflex should be determined (see the section on results), the patient should be pre-treated for autonomic dysreflexia (see below), and the bladder prepared for sperm retrieval if needed as described below. The patient should be advised that certain somatic responses are often seen as the PVS stimulation progresses. These usually occur below the level of injury and may include sweating, flushing, other symptoms of autonomic dysreflexia, and greatly increased levels of spasticity. If the intercostal muscles are involved, the patient may experience a feeling of tightness in the chest or a feeling of difficulty breathing. There may be strong abdominal contractions which sometimes will become rhythmic just before ejaculation. An erection may occur immediately prior to ejaculation but is not necessary for ejaculation to occur. After the procedure is over, the patient may complain of a decrease in both muscle tone and spasticity, which occasionally may be disturbing to them. This period of flaccidity may last from minutes to hours and its occurrence is not predictable. For such patients, it may be advisable for someone to accompany the patient and drive him home. Alternatively, the patient should be prepared to wait until his normal muscle tone reappears.

Autonomic Dysreflexia

Autonomic dysreflexia (AD) is a condition wherein the thoracolumbar sympathetic nervous system reacts to a stimulus in an unmitigated and unregulated manner. This may result in symptoms ranging from mild discomfort to extremely severe and occasionally damaging hypertensive crises. This situation occurs in persons with spinal cord injuries at or above the level of T6, interrupting the supraspinal input which normally moderates this reflex response. The stimulus may be as simple as a full bladder, or may be triggered by external stimuli such as application of an intense vibrator to the penis as in PVS. It has been our practice to pre-medicate all patients, whose level of injury is T6 or higher, with the administration of nifedipine prior to starting the procedure. Other methods of management such as the use of nitro paste or Prazosin have been employed successfully.

Some patients may experience uncomfortable symptoms at what would otherwise be considered modest increases in blood pressure, whereas other patients may have startlingly high elevations in blood pressure with no symptoms. Patients' self-reporting of having experienced autonomic dysreflexia may often be inaccurate. For these reasons we have observed a few simple rules during the conduct of a PVS trial. First, all patients are monitored continuously with pulse and blood pressure readings taken each minute after the trial has started. Second, the trial will be discontinued if the patient experiences any uncomfortable symptoms, regardless of the blood pressure reading, and will be discontinued if the blood pressure reaches a preset limit, regardless of the symptoms. Finally, all patients that have a spinal cord lesion at T6 or rostral will be pretreated with nifedipine (20 mg initially). A wider ranging discussion of autonomic dysreflexia can be found in an excellent article by [Krassioukov et al. \(2009\)](#).

Bladder Preparation

Although the majority of PVS procedures will result in little or no retrograde component, if an ejaculate is obtained that is considered poor or inadequate for its intended purpose, the practitioner may wish to determine if any additional sperm can be retrieved from the bladder. If the practitioner knows that this may be the only visit he will have with the patient and the intended use for this visit's ejaculate will be for the purpose of an ART procedure, it is advisable then to prepare the bladder and obtain a post ejaculatory sample for inspection.

Details of the PVS Procedure

Generally two medical personnel will be needed to perform a PVS procedure. One person will steady the penis and apply the vibrator while a second person holds the sterile specimen cup close to the urethral meatus to collect the ejaculate. The collector usually is positioned on the opposite side of the person who applies the vibrator. Blood pressure should be monitored each minute of the procedure. This is best accomplished with a device that allows automatic recordings at time intervals that can be set by the operator. We set the alarms at systolic BP of 140 mmHg and diastolic of 100 mmHg. The meatus and glans should be cleaned with soap and water or an alcohol swab that allows it to dry in order to minimize the chance of contaminating the specimen and injuring the sperm.

Prior to the procedure, the patient is usually transferred to an exam table. Almost any position on the table is acceptable as long as the operators have comfortable access to the genitalia. (In some patients, it may be possible for the PVS procedure to be performed with the patient seated or reclined in his wheelchair, although this is a bit more cumbersome.) The vibrator is applied to the glans in an area that allows the collector access to the meatus. Some patients may experience stronger sensations in a particular area of the glans. In general, however, we have not noticed any difference in response if the vibrator is placed on the dorsum of the glans (the most convenient place for collection), versus the frenulum (more problematic when collection is involved) ([Fig. 2](#)).



Fig. 2 PVS performed with one vibrator.

The vibrator is applied for 2–3 min then stopped and the skin of the glans inspected for any early signs of damage or abrasion. Caution should be exercised in the amount of pressure used when applying the vibrator to the penis in men who have an underlying penile prosthesis. Additionally, in men who wear external urinary collection devices one should be aware the skin of the glans may be chronically inflamed to some degree and thus more sensitive to the abrasive effects of the vibrator. If necessary, the procedure can continue for up to 10–12 min so long as there is no damage to the skin of the glans. In practice, the majority of the men will ejaculate in less than 2 min.

Results and Clinical Applications

With the exception of select couples who may be able to learn and use intravaginal insemination at home, most couples require some form of assisted reproductive technique, such as intrauterine insemination or in-vitro insemination, to achieve pregnancy. In these cases, all other things being equal, the choice of technique is usually driven by the total number of motile sperm available in the ejaculate. Sperm concentration and sperm motility is typically higher in antegrade ejaculates produced by PVS compared to EEJ (Table 1).

Wherever possible, antegrade versus retrograde ejaculates are preferred for use in the ART. Experience with a large number of men with SCI has allowed us to develop an algorithm (Fig. 3) for semen retrieval in these men with an emphasis on identifying as many men as possible who could be candidates for PVS (Brackett et al., 2010).

Some men may not ejaculate on their first trial with an optimized vibrator, that is a single Ferticare. The reader's attention is directed to the portion of the algorithm contained within the black rectangular outline. This section of the algorithm deals with the most critical information, that is, how to identify the men who fail to ejaculate with their first trial of PVS that might benefit from a second trial of PVS with the use of two vibrators. The algorithm is based on the presence or absence of the bulbocavernosus reflex (BCR), the hip flexor reflex (HR), level of injury (LOI), and the presence of certain somatic responses seen during the first trial (Bird et al., 2001).

The Critical Information in More Detail

The first level of determination: Level of injury (LOI)

Cervical: If the patient's LOI is at a cervical segment, the presence or absence of the BCR or HR is no more predictive of a response than the LOI itself.

T1–T6: If the patient's LOI is between T1 and T6, the presence of at least one reflex is favorable. The absence of both reflexes is predictive of no response to PVS.

T7–T12: If the patient's LOI is between T7 and T12, those with no BCR are poor candidates and should go to EEJ.

The next level of determination: Somatic responses

While many somatic responses are commonly seen in men undergoing PVS, the following are seen more often in men who respond to PVS versus non-responders: piloerection; withdrawal responses; new or increased extremity spasms; thigh abduction. When the LOI and somatic response criteria are used to identify men who may be responders to PVS, more than 20% of men who initially fail a trial with one Ferticare vibrator will be found to respond to either a second trial of one vibrator or a trial using the application of two vibrators simultaneously (Fig. 4).

If one is assiduous in making all the efforts in identifying all candidates for PVS, the success rate of ejaculation using PVS is 86% if the LOI is T10 or higher, and 15% if the LOI is T11 or lower (Brackett et al., 2010).

Sperm Quality

In any given trial of PVS in which an antegrade ejaculate is obtained, no sperm will be seen approximately 10% of the time; 5 million or less total motile sperm (TMS) will be seen approximately 25% of the time; greater than 20 million will be seen approximately 45% of the time and 5–20 million TMS will be seen approximately 20% of the time (Kafetsoulis et al., 2006). The relevance of the numbers of motile sperm seen in an ejaculate is that, in general, most practitioners will be comfortable attempting IUI with

Table 1 Comparison by method of antegrade semen quality in men with SCI

	Control (non-SCI)	Semen quality in antegrade ejaculates		
		Masturbation (SCI)	PVS (SCI)	EEJ (SCI)
No. of subjects	61	43	243	158
Mean volume $\pm$ SEM (cc)	3.0 $\pm$ 0.2	2.2 $\pm$ 0.2	1.9 $\pm$ 0.1	1.2 $\pm$ 0.1
Mean count/cm ³ $\times 10^6 \pm$ SEM	82.0 $\pm$ 5.4	83.3 $\pm$ 0.3	77.4 $\pm$ 5.0	49.9 $\pm$ 5.2
Mean % motility $\pm$ SEM	58.0 $\pm$ 1.5	36.9 $\pm$ 3.3	25.9 $\pm$ 1.2	15.0 $\pm$ 1.2

ALGORITHM FOR SPERM RETRIEVAL IN MEN WITH SPINAL CORD INJURY

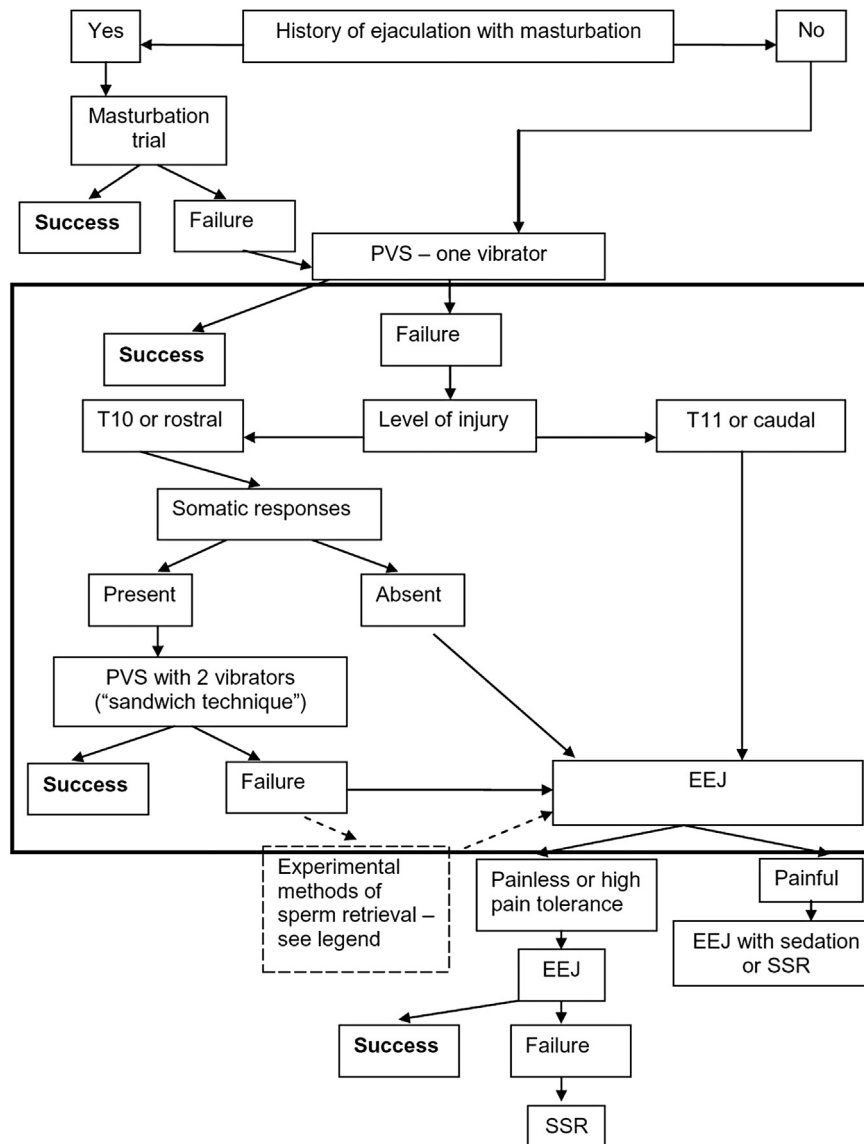


Fig. 3 Algorithm for sperm retrieval: Attention is paid to the information within the outlined box.



Fig. 4 PVS performed with two vibrators.

the number of motile sperm inseminated starting at 5 million; lower numbers of motile sperm being used for IVF/ICSI. For most of the couples who have intravaginal insemination recommended, 15–20 million motile sperm will be a starting point.

Pregnancy Rates

Pregnancy rates following assisted reproduction procedures using ejaculated sperm from men with SCI are similar to those seen in non-injured men with male factor infertility. For IUI, one study by our group found per cycle fecundity rate of 7.9%, similar to the rates published for non-injured men with male factor infertility. Likewise, another study compared IVF/ICSI fertility rates per couple in men with SCI to those with non-injured male partners and found them essentially the same, 58.1% versus 57.9%. While there are no strict guidelines relating to the procedure, published studies of at home intra-vaginal insemination in couples with a male partner with SCI report pregnancy rates of 25%–70% (Ibrahim et al., 2016a; Kathiresan et al., 2011a,b).

References

- Bird, V. G., Brackett, N. L., Lynne, C. M., Aballa, T. C., & Ferrell, S. M. (2001). Reflexes and somatic responses as predictors of ejaculation by penile vibratory stimulation in men with spinal cord injury. *Spinal Cord*, 39, 514–519.
- Brackett, N. L., Lynne, C. M., Ibrahim, E., Ohl, D. A., & Sonksen, J. (2010). Treatment of infertility in men with spinal cord injury. *Nature Reviews. Urology*, 7, 162–172.
- Brindley, G. S. (1981a). Electroejaculation: Its technique, neurological implications and uses. *Journal of Neurology, Neurosurgery, and Psychiatry*, 44, 9–18.
- Brindley, G. S. (1981b). Reflex ejaculation under vibratory stimulation in paraplegic men. *Paraplegia*, 19, 299–302.
- Fode, M., Ohl, D. A., & Sonksen, J. (2015). A step-wise approach to sperm retrieval in men with neurogenic anejaculation. *Nature Reviews. Urology*, 12, 607–616.
- Guttmacher, A. F. (1943). The role of artificial insemination in the treatment of human sterility. *Bulletin of the New York Academy of Medicine*, 19, 573–591.
- Halstead, L. S., Vervoort, S., & Seager, S. W. (1987). Rectal probe electrostimulation in the treatment of anejaculatory spinal cord injured men. *Paraplegia*, 25, 120–129.
- Ibrahim, E., Brackett, N. L., & Lynne, C. M. (2016a). Advances in the management of infertility in men with spinal cord injury. *Asian Journal of Andrology*, 18, 382–390.
- Ibrahim, E., Lynne, C. M., & Brackett, N. L. (2016b). Male fertility following spinal cord injury: An update. *Andrology*, 4, 13–26.
- Kafetsoulis, A., Brackett, N. L., Ibrahim, E., Attia, G. R., & Lynne, C. M. (2006). Current trends in the treatment of infertility in men with spinal cord injury. *Fertility and Sterility*, 86, 781–789.
- Kathiresan, A. S., Ibrahim, E., Aballa, T. C., Attia, G. R., Lynne, C. M., & Brackett, N. L. (2011a). Pregnancy outcomes by intravaginal and intrauterine insemination in 82 couples with male factor infertility due to spinal cord injuries. *Fertility and Sterility*, 96, 328–331.
- Kathiresan, A. S., Ibrahim, E., Aballa, T. C., Attia, G. R., Ory, S. J., Hoffman, D. I., Maxson, W. S., Barrionuevo, M. J., Lynne, C. M., & Brackett, N. L. (2011b). Comparison of in vitro fertilization/intracytoplasmic sperm injection outcomes in male factor infertility patients with and without spinal cord injuries. *Fertility and Sterility*, 96, 562–566.
- Krassioukov, A., Warburton, D. E., Teasell, R., & Eng, J. J. (2009). A systematic review of the management of autonomic dysreflexia after spinal cord injury. *Archives of Physical Medicine and Rehabilitation*, 90, 682–695.
- Ombelet, W., & Van Robays, J. (2015). Artificial insemination history: Hurdles and milestones. *Facts, Views & Vision in ObGyn*, 7, 137–143.
- Sonksen, J., Biering-Sorensen, F., & Kristensen, J. K. (1994). Ejaculation induced by penile vibratory stimulation in men with spinal cord injuries. The importance of the vibratory amplitude. *Paraplegia*, 32, 651–660.

Sperm Retrieval From the Bladder

Nancy L Brackett, Emad Ibrahim, and Charles M Lynne, University of Miami Miller School of Medicine, Miami, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

In normal ejaculation, sperm exit the urethral meatus during orgasm (antegrade ejaculation), and very few, if any sperm enter the urinary bladder (retrograde ejaculation). Certain medical conditions, however, lead to retrograde ejaculation, and this condition may interfere with the man's ability to initiate a pregnancy by sexual intercourse. If pregnancy is a goal, it may be necessary to retrieve sperm from the bladder for use in assisted conception procedures. This article will describe methods of retrieving sperm from the urinary bladder.

Retrograde Ejaculation

Normal ejaculation is mediated by a combination of peripheral, cerebral, and spinal sensory and motor neuron pathways. Sympathetic nerves arising from the T10–L2 spinal level mediate contraction of the emission phase of ejaculation in which the prostate and seminal vesicles deposit seminal fluid into the posterior urethra, with simultaneous closure of the bladder neck to prevent retrograde flow of semen (Thomas, 1983). Somatic nerves arising from the S2–S4 spinal level mediate the expulsion phase of ejaculation in which pulsatile contraction of the bulbocavernosus and pelvic floor muscles, together with relaxation of the external urinary sphincter to promote antegrade seminal emission (Thomas, 1983). During the expulsive phase of ejaculation, the internal urethral sphincter (the bladder neck) closes to prevent semen from entering the bladder. If the bladder neck fails to close adequately, semen can reflux into the bladder. This condition is known as retrograde ejaculation.

A man with the condition of retrograde ejaculation may have low-volume or no antegrade ejaculate. Consequently, little or no sperm may be available for conception by sexual intercourse. Medical conditions that can contribute to retrograde ejaculation include:

- Surgery, such as bladder neck surgery, retroperitoneal lymph node dissection or prostate surgery.
- Side effect of certain medications used to treat high blood pressure, prostate enlargement, or depression.
- Nerve damage caused by a medical condition, such as diabetes, multiple sclerosis, Parkinson's disease, or spinal cord injury.

Initial Diagnosis

Typically, an initial diagnosis of retrograde ejaculation is made. This diagnosis is followed by decisions regarding if, when, and how to treat the retrograde ejaculation. The initial diagnosis of retrograde ejaculation includes a postejaculatory urinalysis (PEU). The PEU is performed by asking the patient to masturbate until he feels sexual climax. Any antegrade ejaculate (even a small volume) that accompanies the climax should be collected in a specimen cup. The specimen cup should be nontoxic to sperm. The patient is instructed to then urinate into one or more additional specimen cups. Prior to performing masturbation for the PEU procedure, it is helpful for the patient to urinate without completely emptying. This step will help reduce the volume of postejaculatory urine that must be processed for sperm isolation.

The most accurate PEU results are usually obtained when masturbation and urine collection is performed in the clinic. The patient performs these activities privately in a designated semen collection room, or other appropriately-outfitted procedure room. The clinic setting allows personnel the most rapid access to the patient's urine specimen for processing and analysis. If it is not possible for the patient to perform masturbation and urine collection in the clinic, then the patient may perform these activities at home and bring the specimen cups to the clinic for analysis. Specimen collection times should be noted, because prolonged exposure of sperm to urine can be detrimental to sperm quality. For the initial diagnosis of retrograde ejaculation however, the quality of sperm may be less critical than simply noting the quantity of sperm in the urine. Future collections of sperm from the bladder can be optimized for assisted conception procedures.

Analyzing Sperm Collected From the Bladder

If the patient has properly collected his specimen for PEU, there will be one or more specimen cups of the patient's urine voided after he achieved orgasm. In addition, if the patient produced an antegrade ejaculate upon climax, there will be a specimen cup containing his antegrade semen specimen. The antegrade semen specimen should be analyzed according to the laboratory's routine protocol for semen analysis. The retrograde ejaculate should be analyzed by first measuring the total volume of urine voided. Depending on this total volume, all of the urine, or a portion of the urine, should be centrifuged and the pellet examined for sperm. Calculations are then made (see below) to determine the total sperm count in the retrograde ejaculate. The reason total sperm count

is calculated, rather than sperm concentration (sperm count/cm³ semen), is that the volume of semen released into the bladder is unknown during retrograde ejaculation. Therefore, in terms of sperm count, only the total number of sperm obtained from the bladder can be determined.

Calculating Total Sperm Count From the Bladder

To calculate the total number of sperm obtained from the bladder, the pellet of centrifuged urine is examined. The sperm count/cm³ of urine pellet is determined. This value is then multiplied by the volume of the pellet. This value is then multiplied by the portion of total voided urine it represents.

For example:

If the total volume of voided urine = 100 cm³.

If the volume of urine centrifuged = 20 cm³.

If the volume of the urine pellet = 0.4 cm³.

If the sperm count/cm³ urine pellet = 8 million sperm.

The calculation is: 8 million sperm × 0.4 cm³ pellet = 3.2 million sperm in 20 cm³ of urine = 16 million total sperm in 100 cm³ of urine (i.e., total sperm count in the retrograde ejaculate).

Treating Retrograde Ejaculation

After a diagnosis of retrograde ejaculation has been made, the physician will consult with the patient about the patient's goals. If pregnancy by sexual intercourse is a goal, then a concomitant goal will be to improve the volume of antegrade ejaculate produced by the patient. Medical therapies are typically recommended for this purpose. For example, oral sympathomimetics have been successful in restoring antegrade ejaculation in 50%–100% of cases, and these agents are often considered first-line therapy for the treatment of retrograde ejaculation ([Mehta and Sigman, 2015](#)).

Preparing the Bladder for Retrograde Ejaculation

When retrograde ejaculation is not responsive to medical therapy, and the patient wishes to achieve pregnancy, urinary sperm retrieval is the most commonly described technique to obtain sperm for use in assisted conception procedures. When a procedure is planned in which sperm retrieved from the bladder will be used to attempt pregnancy, then it is important to maximize the quality of sperm that is retrieved from the bladder. The urine in the bladder can be detrimental to the quality of sperm that comes into contact with it during retrograde ejaculation ([Voge et al., 2016](#)). Specifically, the acidic pH of urine, among other variables, can be detrimental to sperm motility ([Makler et al., 1981](#)). Consequently, alkalization of the bladder is often attempted prior to procedures in which sperm retrieval from the bladder are planned. Systemic approaches to alkalization include oral administration of agents such as acetazolamide, baking soda, and potassium citrate ([Mehta and Sigman, 2015](#)). Additionally, in patients who can tolerate urinary catheterization, placement of sperm-washing medium may be instilled into the empty bladder before ejaculation. This step should be performed immediately (within 10 min) prior to the ejaculation procedure.

Ejaculation Procedure

To maximize the number of sperm available for assisted conception, the man should abstain from ejaculation for 2–5 days prior to the ejaculation intended for assisted conception. If the man is aspermic, he should be specifically instructed to abstain from bringing himself to orgasm for 2–5 days prior to the ejaculation intended for assisted conception. This interval of abstinence has been shown to optimize sperm counts between ejaculations ([World Health Organization, 2010](#)). Immediately (within 5 min) prior to performing masturbation to produce the retrograde ejaculate, the patient should be instructed to urinate into the toilet until his bladder is nearly empty. Next, in a private collection room, the patient should masturbate until he feels sexual climax. If any antegrade ejaculate is produced, he should collect it in a specimen cup that is nontoxic to sperm. Next, the patient should urinate into an additional specimen cup. If that cup becomes full, he should urinate into a second cup, etc. Rarely are more than two cups needed if the patient has urinated prior to masturbation.

Isolating the Sperm From the Urine Specimen

When sperm from a retrograde ejaculate are intended for use in an assisted conception procedure, it is best for the patient to perform masturbation in the clinic versus at home. By doing so, the postejaculatory urine can be processed as rapidly as possible to isolate the sperm from urine, and thereby reduce the exposure time and detrimental effect of urine on sperm. Prior to processing the urine, the total volume of urine voided into the specimen cups should be measured. Next, the total volume of urine should be centrifuged for the purpose of obtaining all of the available sperm from the bladder for use in the assisted conception procedure. A brief and gentle centrifugation, such as 300 × g for 5 min, is typically sufficient to isolate the sperm into a pellet. The total sperm count can be

calculated as described above. In addition, sperm motility should be determined according to the laboratory's protocol for assessing sperm motility. Ultimately, the total motile sperm count can be calculated ($\text{total sperm count} \times [\% \text{ of motile sperm}/100]$). The total motile sperm count is often used for deciding which assisted conception procedure will be used to achieve pregnancy. Options may include intrauterine insemination (or possibly even intravaginal insemination), in addition to in vitro fertilization, with or without intracytoplasmic sperm injection. Further processing of the retrograde semen specimen may be necessary, depending on the laboratory's protocols. For example, a swim up procedure or gradient processing may be used to further isolate the most motile sperm for use in assisted conception.

Pregnancy Rates

No studies have compared the effectiveness of various regimens of alkalization on sperm motility or on pregnancy outcomes. Pregnancy rates with the use of sperm retrieved from the bladder have been reported. For example, pregnancy rates with intrauterine insemination vary from 20% to 50% (Mehta and Sigman, 2015). In vitro fertilization and intracytoplasmic sperm injection are options for couples that fail repeated cycles of intrauterine insemination, and for couples where a concomitant female factor may also be present. There is a need for better-quality prospective studies specifically designed to compare treatment options for aspermia. In cases where sperm are retrieved from postejaculate urine, there is an additional need for studies comparing reproductive outcomes following intrauterine insemination versus in vitro fertilization with or without intracytoplasmic sperm injection.

Surgical Options

A review by Mehta and Sigman notes that surgical options for the treatment of retrograde ejaculation are limited to reconstruction of the bladder neck and have been described by only two small series. Although both series noted highly successful outcomes, surgical treatment is largely historical and should be reserved as the last course of therapy for patients with retrograde ejaculation and must be weighed against urinary sperm retrieval and assisted reproductive technologies (Mehta and Sigman, 2015).

Men With Spinal Cord Injury

Men with spinal cord injury represent a unique group of patients with retrograde ejaculation. Their neurological damage results in an inability to ejaculate by sexual intercourse or masturbation in 90% of patients (Brackett et al., 2010). Consequently, those who wish to become biological fathers must have their sperm retrieved by medical procedures. Semen collection by medically assisted ejaculation should be attempted first, prior to sperm retrieved by surgery from reproductive tissues. Higher numbers of motile sperm can usually be obtained from the ejaculate versus from reproductive tissues such as the testis or epididymis. When higher numbers of motile sperm are available, then more options for assisted conception are possible.

For example, 5 million or more motile sperm can be obtained from most assisted ejaculation procedures of men with spinal cord injury (Brackett et al., 2010). This number of motile sperm is sufficient for most practitioners to consider intrauterine insemination, or possibly even intravaginal insemination. In contrast, when <5 million motile sperm are available (the number usually obtained with surgical sperm retrieval) the couple usually must undergo in vitro fertilization with intracytoplasmic sperm injection to achieve pregnancy. The procedure of in vitro fertilization (with or without intracytoplasmic sperm injection) is more costly than the procedures of intravaginal insemination or intrauterine insemination, that is, \$10,000–\$15,000 per attempt of in vitro fertilization versus \$0–\$1500 per attempt of intravaginal insemination or intrauterine insemination. In addition, the procedure of in vitro fertilization can add risk to the female partner relative to intravaginal insemination or intrauterine insemination (Winston and Hardy, 2002). For these reasons, it is recommended that the ejaculate (antegrade and/or retrograde) be examined before performing surgical sperm retrieval in patients with spinal cord injury.

Assisted Ejaculation in Men With Spinal Cord Injury

To obtain the ejaculate from an anejaculatory man with spinal cord injury, the methods of penile vibratory stimulation or electroejaculation are often used (Ibrahim et al., 2016a). These procedures are described in detail in other articles, and will be only briefly summarized here. The method of penile vibratory stimulation should be attempted prior to the method of electroejaculation because, relative to electroejaculation, penile vibratory stimulation is less invasive, is preferred more by patients, and results in a higher number of motile sperm in the antegrade ejaculate (Ibrahim et al., 2016b).

Penile vibratory stimulation is performed by applying, to the glans penis, a vibrating device which delivers mechanical stimulation to induce ejaculation. The device should be optimized to deliver an amplitude of 2.5 mm. This amplitude results in a higher response rate than lower amplitudes (Sonksen et al., 1994; Brackett et al., 2010). If the patient fails to respond to the application of one device, then two devices may be applied. In the two-device method, the penis is sandwiched between one device placed on the dorsum of the glans penis and one device placed on the frenulum of the glans penis. This "sandwich" method can salvage failures to one device (Brackett et al., 2007).

Patients who fail to respond to the application of one or two vibrators should be referred for the alternative assisted ejaculation procedure of electroejaculation. The method of electroejaculation is performed by inserting a probe into the patient's rectum. Current is delivered through electrodes positioned on the probe. Increasing amounts of current result in ejaculation. Detailed information about the application of penile vibratory stimulation and electroejaculation are provided in other articles.

Retrograde Ejaculation in Men With Spinal Cord Injury

The methods of penile vibratory stimulation or electroejaculation can result in retrograde ejaculation in men with spinal cord injury. Retrograde ejaculation is more likely to occur with electroejaculation than with penile vibratory stimulation (Brackett et al., 1997). Consequently, in electroejaculation procedures performed to obtain sperm for diagnosis or assisted conception, it is recommended that the bladder be prepared to optimize sperm quality obtained from the bladder. Preparation of the bladder may be similarly performed prior to penile vibratory stimulation procedures, however, retrograde ejaculation may not occur in many cases of penile vibratory stimulation. The practitioner's judgment is the best guide for deciding on preparation of the bladder prior to penile vibratory stimulation. In our work with 3540 penile vibratory stimulation procedures in 671 men with spinal cord injury, we have found that presence or absence of retrograde ejaculation is a consistent finding, trial to trial, within each patient. Therefore, if retrograde ejaculation is present in the first two trials of penile vibratory stimulation in a particular patient, we will prepare the bladder on future trials of penile vibratory stimulation in that patient. In contrast, if retrograde ejaculation is absent in the first two trials of penile vibratory stimulation in a particular patient, we will usually not prepare the bladder on future attempts of penile vibratory stimulation in that patient.

Preparing the Bladder for Retrograde Ejaculation in Men With Spinal Cord Injury

Most men with spinal cord injury are unable to void spontaneously. Therefore, urinary catheterization is necessary to drain the patient's bladder to prepare it for retrograde ejaculation. Urinary catheterization can be performed by the patient or by medical personnel assisting with the ejaculation procedure. Sterile technique should be used for urinary catheterization. A nonspermicidal lubricant should be used. After the urine has been drained, the catheter should be left in place, and 25–50 cm³ of sperm washing medium instilled through the catheter into the bladder. The catheter is then withdrawn from the bladder. The bladder should be prepared immediately (not more than 10 min) before the ejaculation procedure, to minimize accumulation of urine. Some practitioners may opt to also treat the patient with systemic alkalization. Our experience has shown that systemic alkalization may lead to gastric upset in some men with spinal cord injury, and provide little or no alkalization secondary to the instillation of sperm washing medium into the bladder (unpublished data). Consequently, we do not routinely treat men with spinal cord injury with systemic alkalization.

For patients whose bladders are managed with suprapubic catheters, the following may be done to prepare the bladder for retrograde ejaculation. First, the bladder should be lavaged with aliquots of normal saline until no sediment is seen in the fluid. Lavage should be repeated once or twice with the sperm washing medium. Finally, 25–50 cm³ of sperm washing medium should be left in the bladder. The suprapubic tube is clamped during the ejaculation procedure. A collection cup should be held at the meatus as the suprapubic tube does not preclude antegrade ejaculation.

Collecting the Retrograde Ejaculate in Men With Spinal Cord Injury

After the assisted ejaculation procedure is concluded, the bladder contents should be drained as soon as possible to process the retrograde specimen and isolate the sperm from the urine. Urinary catheterization using sterile technique should be performed to drain the bladder contents, with a nonspermicidal lubricant to help insert the catheter into the urethral meatus. With the catheter left in place, another 25–50 cm³ of sperm washing medium can be used to lavage the bladder to extract any residues of ejaculate that may have fallen to the floor of the bladder and were thus not captured easily during the initial draining. We perform a second lavage if the first lavage contents are cloudy. For our purposes, we label the cup containing the initial bladder drain contents as "R1" (for retrograde fraction 1) and the lavaged fractions as "R2" and "R3." Often, the semen quality of a lavaged fraction (R2 and R3) is better than the initial fraction (R1).

Isolating Sperm From the Bladder Contents in Men With Spinal Cord Injury

After an ejaculation procedure is complete, the urine should be obtained and processed as soon as possible to minimize exposure of sperm to urine. The various fractions collected (R1, R2, R3, etc.) should not be combined before processing because one of the fractions may have superior sperm quality for use in assisted conception, that is, using sperm from the superior fraction may be optimal for the chosen assisted conception procedure (versus using sperm from all of the fractions).

To isolate the sperm from the urine, pipet the urine into centrifuge tubes labeled R1, R2, R3, etc., as appropriate. Centrifuge at 300 × g for 5 min to pellet the sperm. Examine the pellets from R1, R2, R3 separately, and decide which pellets contain the sperm

that will be used in the assisted conception procedure. Depending on the laboratory's protocols, additional processing, such as a swim up procedure or a density gradient procedure, may be necessary to isolate the most motile sperm for use in assisted conception.

Conclusion

Retrograde ejaculation can interfere with a man's ability to achieve pregnancy by sexual intercourse. An initial diagnosis of retrograde ejaculation includes a postejaculatory urinalysis. Once the diagnosis of retrograde ejaculation is made, decisions are discussed with the patient regarding treatments. For patients who wish to attempt pregnancy by sexual intercourse, treatments that attempt to reverse retrograde ejaculation are recommended. When these treatments are not effective, then sperm retrieval from the urinary bladder may be performed to obtain semen for assisted conception. Men with spinal cord injury represent a specialized group of patients with retrograde ejaculation. Most men with spinal cord injury cannot ejaculate by sexual activity or void voluntarily. Semen retrieval procedures may be performed to obtain semen for assisted conception. These procedures may result in retrograde ejaculation. Bladder preparation prior to semen retrieval can optimize the quality of sperm obtained from the bladder. More studies are needed that compare pregnancy outcomes using sperm retrieved from retrograde versus antegrade semen specimens.

References

- Brackett, N. L., Ibrahim, E., Iremashvili, V., Aballa, T. C., & Lynne, C. M. (2010). Treatment for ejaculatory dysfunction in men with spinal cord injury: An 18-year single center experience. *The Journal of Urology*, 183, 2304–2308.
- Brackett, N. L., Kafetsoulis, A., Ibrahim, E., Aballa, T. C., & Lynne, C. M. (2007). Application of 2 vibrators salvages ejaculatory failures to 1 vibrator during penile vibratory stimulation in men with spinal cord injuries. *The Journal of Urology*, 177, 660–663.
- Brackett, N. L., Padron, O. F., & Lynne, C. M. (1997). Semen quality of spinal cord injured men is better when obtained by vibratory stimulation versus electroejaculation. *The Journal of Urology*, 157, 151–157.
- Ibrahim, E., Brackett, N. L., & Lynne, C. M. (2016a). Advances in the management of infertility in men with spinal cord injury. *Asian Journal of Andrology*, 18, 382–390.
- Ibrahim, E., Lynne, C. M., & Brackett, N. L. (2016b). Male fertility following spinal cord injury: An update. *Andrology*, 4, 13–26.
- Makler, A., David, R., Blumenfeld, Z., & Better, O. S. (1981). Factors affecting sperm motility. VII. Sperm viability as affected by change of pH and osmolarity of semen and urine specimens. *Fertility and Sterility*, 36, 507–511.
- Mehta, A., & Sigman, M. (2015). Management of the dry ejaculate: A systematic review of aspermia and retrograde ejaculation. *Fertility and Sterility*, 104, 1074–1081.
- Sonksen, J., Biering-Sorensen, F., & Kristensen, J. K. (1994). Ejaculation induced by penile vibratory stimulation in men with spinal cord injuries. The importance of the vibratory amplitude. *Paraplegia*, 32, 651–660.
- Thomas, A. J., Jr. (1983). Ejaculatory dysfunction. *Fertility and Sterility*, 39, 445–454.
- Voge, J., Varner, D. D., Blanchard, T. L., Meschini, M., Turner, C., Teague, S. R., Brinsko, S. P., & Love, C. C. (2016). The effects of urine concentration, and cushion centrifugation to remove urine, on the quality of cool-stored stallion sperm. *Theriogenology*, 86, 1294–1298.
- Winston, R. M., & Hardy, K. (2002). Are we ignoring potential dangers of in vitro fertilization and related treatments? *Nature Cell Biology*, 4(Suppl), s14–s18.
- WORLD Health Organization. (2010). *WHO Laboratory manual for the examination and processing of human semen*. Geneva: WHO Press.

Male Oncofertility and Sperm Cryopreservation

Ohad Shoshany and Chen Shenhar, Rabin Medical Center, Petah Tiqwa, Israel

© 2018 Elsevier Inc. All rights reserved.

Glossary

ART Assisted reproductive technologies.

TESA Testicular sperm aspiration. A method of retrieving sperm using needle aspiration and negative pressure.

TESE Testicular sperm extraction. Sperm retrieval from an open biopsy of testicular tissue.

ICSI Intracytoplasmic sperm injection. Introduction of processed sperm into an egg, enabling fertilization with sperm of unsatisfactory quantity and quality.

Oligospermia Low sperm count. According to the most recent WHO definitions (2010), a sperm concentration of 15 million per milliliter or less in the human ejaculate. Oligospermia is correlated with a lower pregnancy rate.

Introduction

It is widely recognized that cancer survivors are at a significant risk of infertility due to their disease and treatment related toxicities. Chemotherapy and radiotherapy incur deleterious effects on spermatogenesis and may result in temporary or permanent gonadal toxicity. There were an estimated 14.1 million cancer cases around the world in 2012 (of these, 7.4 million cases were in men), and this number is expected to increase to 24 million by 2035. In the US, approximately 9% of cancers in males were diagnosed in patients under the age of 45, and up to 1.1% are under the age of 20 (Siegel et al., 2014). Marked advances in the field of oncology have led to increased survival in young men with cancer. For example, between 2001 and 2012, the five-year survival rate for male cancer patients under the age of 45 in the US was over 75%. Improved survival rates, together with the explosive breakthroughs in the field of assisted reproduction, drove patients and caregivers to extend their focus toward fertility preservation. Since the introduction of sperm cryopreservation in the 1960s and the developments in assisted reproduction in the 1970–1980s, fertility preservation for cancer patients has been practiced sporadically. Increasingly, guidelines of various associations unequivocally called for a discussion of fertility preservation with all cancer patients of reproductive age (and with parents or guardians of children and adolescents) if infertility is a potential risk. The growing interest in this field has culminated in the coining of a new term in 2006: “oncofertility.” This term describes the integration of different specialties into a new subspecialty focused on the reproductive future for cancer survivors. This article will focus on the various aspects of oncofertility in the male cancer patient, its significance, optional treatment modalities, and future perspectives.

The Effects of Cancer and Cancer Therapies on Male Fertility

The Effects of Cancer on Male Fertility

Men with certain types of cancer may be at increased risk of lower semen parameters, and consequently, subfertility (Moss et al., 2016). Testis cancer, lymphoma, and leukemia patients are more likely to present with lower motile sperm counts compared to patients with other types of cancer, and are at higher risk of azoospermia. There are multiple possible causes for poor sperm quality in the oncological patient, including endocrine disturbances (disruption of the hypothalamic-pituitary-gonadal axis, production of hormones by the cancerous tissue), cytotoxic autoimmune response and other systemic effects of cancer, disease related catabolic state or dietary deficiencies, local tumor effects, and inborn impairment in the spermatogonial germ cells (Williams et al., 2009).

Table 1 summarizes the possible negative effect of cancer and its treatments on various physiologic mechanisms related to fertility.

The Effects of Radiation Therapy on Male Fertility

Radiation is employed in the treatment of various types of cancers, such as brain, bladder, rectal, testicular, or hematologic cancers. Spermatogenesis is extremely sensitive to radiation induced damage. Direct radiation to the testis at a dose as low as 0.1 Gray (Gy) may damage spermatogonia and disrupt spermatogenesis (Agarwal et al., 2014). More commonly, damage to the testis occurs through scatter from radiation directed at other structures or whole body radiation, rather than direct radiation to the testis (Meistrich, 2013). Unfortunately, even though protective lead shields are used, some scatter radiation is unavoidable (Moss et al., 2016). Several factors influence the degree of gonadotoxicity, including radiation source, treatment field, total dose, and fractioning. Radiation induces gonadotoxic damage in a dose-dependent manner, and immature cells are more susceptible. A radiation dose <0.8 Gy may result in transient oligospermia, while higher doses more commonly cause transient azoospermia (0.8–2 Gy) or prolonged, at times irreversible, azoospermia (>2 Gy and >4 Gy) (Shalet, 1993). Much higher doses (20 Gy)

Table 1 Physiological fertility functions and possible mechanisms for temporary or permanent negative effect of cancer and its treatments

Fertility function	Tumor effect	Chemotherapy	Radiation	Surgery
Central GnRH, LH, FSH production	hCG produced by tumor	- There is no strong direct evidence to implicate chemotherapy as a cause of permanent dysfunction of the anterior pituitary - Androgen deprivation therapy suppresses the androgen hormonal axis	Cranial/brain radiation	Brain surgery
Spermatogenesis, sperm maturation, DNA compaction	- Testicular cancer associated with testicular development defects - Antisperm antibodies	- Spermatogonial stem cell damage - Direct damage to sperm DNA	Testicular radiation or scatter from abdominal/pelvic radiation	Unilateral or bilateral orchiectomy
Androgen production		- Higher doses than those needed to impair spermatogenesis may cause Leydig cell dysfunction and hypogonadism	- Higher doses than those needed to impair spermatogenesis may cause Leydig cell dysfunction and hypogonadism	Bilateral orchiectomy
Sexual response and erectile function	- Depression and loss of libido - Catabolic state	- Autonomic nerve damage that may result from chemotherapy with vincristine or platinum-based drugs can result in erectile dysfunction - Chemotherapy induced hypogonadism, especially due to alkylating agents, may result in sexual dysfunction - Systemic effects of chemotherapy (Fatigue, gastrointestinal, cutaneous, etc.) may also impair sexual function	- Pelvic radiation causing penile microvascular damage and erectile dysfunction - Brain radiation and loss of libido	- Spinal surgery - Pelvic surgery (e.g., radical prostatectomy, low anterior resection)
Ejaculation	- Direct local obstruction of vasa deferentia by tumor - Spinal or pelvic nerve involvement by tumor	- Autonomic nerve damage (see above) may also cause ejaculation disorder - Chemotherapy induced hypogonadism may result in low ejaculate volume	- May cause varied ejaculatory disorders: low ejaculate volume, anejaculation, discomfort during ejaculation, and hematospermia	- Pelvic surgery causing obstruction of vasa deferentia or pelvic nerve damage - Retroperitoneal or spinal surgery causing nerve damage

FSH, Follicular stimulating hormone; GnRH, Gonadotropin releasing hormone; hCG, Human chorionic gonadotropin; LH, Luteinizing hormone.

are required to impair Leydig cell activity, including testosterone production. The time needed for the recovery of sperm counts also depend on radiation dose. The use of fractionated radiation, often employed to reduce the toxic effect on healthy cells, is actually more harmful to spermatogenesis. Repetitive radiation doses damage reserve stem cells activated after the initial insult, resulting in significant spermatogenic injury and greater delay in spermatogenic recovery. Radiation may also interfere with fertility by impairing erectile function and ejaculation through microvascular damage to either spongiform tissue circulation or nerve supply. Finally, the extent of damage to the spermatogonial stem cells DNA from ionizing radiation is unclear, but may further jeopardize future fertility (Osterberg et al., 2014).

The Effects of Chemotherapy on Male Fertility

Similarly to radiation, chemotherapy targets proliferating cancer cells but is also detrimental to the rapidly dividing cells in the germinal epithelium. The cytotoxic agents penetrate the blood-testes barrier, damage the spermatogonial stem cells, disrupt spermatogonial differentiation, and may also directly damage sperm DNA. Leydig cells are much more resistant to chemotherapeutic insult, but at high doses Leydig cells injury and hypogonadism may ensue. The extent of the damage is largely dependent on the types of chemotherapeutic agents and their dose. It is generally accepted that alkylating agents, nitrogen mustard derivatives, and cisplatin are the most harmful drugs. On the other hand, antimetabolites such as methotrexate and mercaptopurine may cause

temporary suppression of spermatogenesis but are unlikely to have a lasting effect. Although higher doses of chemotherapy are more likely to cause persistent azoospermia, we are yet unable to predict gonadotoxicity severity for a specific individual, and post-therapy recovery of spermatogenesis remains unpredictable. Combination chemotherapies or the combination of chemotherapy and radiation are often employed in the management of various oncological diseases. The risk for prolonged or permanent azoospermia may vary significantly between treatment protocols. For example, different combinations given to Hodgkin's disease patients carry different risk: MOPP (mechlorethamine, oncovin, procarbazine, and prednisone) may result in azoospermia in 90% of patients up to 4 years after treatment and a high risk for permanent sterility, while ABVD (adriamycin, bleomycin, vinblastine, and dacarbazine) is associated with temporary azoospermia, generally resolving at 18 months. Sperm counts often decline in the first 6 months after the start of chemotherapy. Generally, age at time of chemotherapy is not a prognostic factor, and chemotherapy is equally damaging to spermatogenesis in children, adolescents, and adults. Sperm count recovery relies on the regeneration of stem-cell spermatogonia and may take up to 5 years.

The Effects of Surgery on Male Fertility

Surgery by itself can impair fertility, mainly through ejaculatory obstruction, ejaculatory dysfunction, or erectile dysfunction. The gonad itself may be removed by radical orchiectomy in testicular cancer. Furthermore, some testis cancer patients undergo retroperitoneal lymph node dissection, risking an injury to the sympathetic nerves and consequent retrograde ejaculation or anejaculation. While meticulous nerve sparing and modified template primary RPLND performed by a high-volume surgeon may reduce this risk to a minimum of 1%–5%, postchemotherapy RPLND is notoriously more difficult, with a risk of ejaculatory dysfunction up to 75%. Pelvic surgeries such as radical prostatectomy and radical cystectomy purposely disrupt the genital ducts (by cutting the vasa deferentia), and cause ejaculatory obstruction. Moreover, any radical pelvic surgery may cause erectile dysfunction via injury to the pelvic or cavernous nerves.

Table 2 summarizes the risks for infertility for different types of cancer treatments.

Fertility Preservation—What are the Needs

As the number of cancer survivors grow, it has become increasingly important to address quality of life issues in these patients. Cancer survivors experience numerous short and long term physiological and psychological sequelae of their cancer treatment. Among the possible consequences of cancer and its treatment in men are disruption of normal psychosexual development, erectile or ejaculatory dysfunction, and disruption of normal spermatogenesis. Future fertility and health of future biological children are considerable concerns for adolescents and young adults (AYA) cancer survivors, both before and after treatment. Despite their concerns, young cancer survivors often desire to sire biological children and wish to discuss reproduction and fertility preservation with their physician. Moreover, older men should not be assumed to have completed reproduction as more and more men become fathers at increasing age due to life choices or initiating a second family after a divorce or the death of a spouse.

Importantly, fertility preservation was shown to have an added psychological benefit. Two thirds of the patients that cryopreserved sperm reported drawing significant encouragement from the act. Many patients who did not bank sperm (and their caregivers) report regret and guilt. Nonetheless, implementation of patient education and fertility preservation counseling as a mainstay of oncological treatment was slow to occur. Information provided by health care professionals regarding risk of infertility and options for fertility preservation is often inadequate. Physicians may be disinclined to bring up the subject because of lack of knowledge, time constraints, or their own discomfort. Young patients (and caregivers) may have difficulties comprehending the potential implications, or may be too overwhelmed by their new diagnosis to address the subject. In a 2012 survey conducted by the LIVESTRONG foundation, only about a quarter of AYAs took steps to preserve their fertility before their cancer treatment began. Similarly, 30%–40% of patients report physicians did not discuss fertility issues with them prior to treatment, and only 50% were given the opportunity to bank sperm.

Over the past decade, there has been tremendous progress and substantial advocacy for fertility preservation. Aware of the barriers and difficulties, guidelines of a growing number of professional societies and organizations worldwide endorse early discussion of infertility risk with cancer patients and recommended sperm cryopreservation as a mean of fertility preservation in men. However, there is still room for improvement. A survey conducted among oncologists in 2009 revealed that < 25% of them reported to adhere to the ASCO guidelines for fertility preservation, and 50% have never discussed the risk of infertility or discussed it with less than a quarter of their patients (Quinn et al., 2009). Accordingly, a growing number of groups and organizations advocating for fertility preservation have emerged, providing education and support to cancer patients (see list of relevant web pages).

Strategies for Fertility Preservation

Currently, cryopreservation of sperm is the established method for fertility preservation in men. When needed, banked sperm can be thawed and used for assisted reproductive techniques (ART). The simplest way to acquire sperm is through masturbation and ejaculation. However, some patients are unable or unwilling to provide ejaculated semen specimen, and will require other form of

Table 2 Male infertility risk associated with cancer treatments

<i>Risk and duration of azoospermia</i>	<i>Radiation</i>	<i>Chemotherapy</i>	<i>Surgery</i>
High risk of prolonged or indefinite azoospermia	- Total body irradiation - Testicular radiation dose ≥ 6 Gy in boys, ≥ 1.2 Gy in adult males - Cranial/brain radiation ≥ 40 Gy	- Alkylating agents—as a single agent (Cyclophosphamide), or combination protocols (e.g., Procarbazine, Mustine, and/or Chlorambucil as in COPP, MOPP, or MVPP), or in combination with irradiation (e.g., Cyclophosphamide, Procarbazine, Chlorambucil) - Specific alkylating agents in prepubertal treatment (BCNU, CCNU) cause Azoospermia in adulthood - High dose alkylating agents for bone marrow transplant (e.g., containing Melphalan or cyclophosphamide $\pm$ busulfan) - Likely cause azoospermia (but always given with other gonadotoxic agents): Busulfan, Ifosfamide, BCNU, Nitrogen mustard, Actinomycin D	- Removal of both testicles - Removal of pituitary gland - Nonnerve-sparing RPLND
Intermediate risk, or azoospermia for 5 years	- Testicular radiation dose ~ 1 Gy (from scattering of abdominal/pelvic radiation)	- BEP (2–4 cycles) - Cisplatin - Carboplatin - Alkylating agents in lower doses combinations, e.g., cyclophosphamide in CHOP - Hormonal treatments (e.g., for prostate cancer)	- Removal of one testicle - Procedures within the pelvis (prostate, bladder, lower large intestine, rectum)
Low risk, or temporary reduction in sperm counts	- Testicular radiation dose 0.2–0.7 Gy	- Alkylating agents (dacarbazine, cyclophosphamide, and/or mustine) in lower-dose combinations, e.g., ABVD, OEPA, VACOP-B. - Nonalkylating combinations (e.g., VEEP) - Drugs that have a temporary effect alone but have an additive effect when combined with more gonadotoxic agents: Doxorubicin, Thiotepa, Cytosine arabinoside, Vinblastine, Vincristine. - Drugs that have a temporary effect, with a possible additive effect when combined with gonadotoxic agents: Amsacrine, Bleomycin, Dacarbazine, Daunorubicin, Epirubicin, Etoposide, Fudarabine, Fluorouracil, 6-Mercaptopurine, Methotrexate, Mitoxantrone, Thioguanine	- Nerve-sparing RPLND
No risk	- Testicular radiation dose < 0.2 Gy (from scattering) - Radioactive iodine	- Prednisone - Interferon-α	
Unknown risk		- Irinotecan - Oxaliplatin - Monoclonal antibodies (e.g., Bevacizumab, Cetuximab, Trastuzumab) - Tyrosine kinase inhibitors (e.g., Erlotinib, Imatinib) - Taxanes	

ABVD, Adriamycin (doxorubicin), bleomycin, vinblastine, and dacarbazine; BEP, Bleomycin, etoposide, and cisplatin; BCNU, Carmustine; CHOP, Cyclophosphamide, hydroxydaunomycin, oncovin, and prednisone; COPP, Cyclophosphamide, oncovin, procarbazine, and prednisone; CCNU, Lomustine; MOPP, Mechlorethamine, oncovin (vincristine), procarbazine, and prednisone; MVPP, Mustine, vinblastine, procarbazine and prednisone; OEPA, Oncovin, etoposide prednisone, and adriamycin (doxorubicin); RPLND, Retroperitoneal lymph node dissection; VACOP-B, Vinblastine, doxorubicin, prednisone, vincristine, cyclophosphamide, and bleomycin; VEEP, Vincristine, etoposide, epirubicin, and prednisone.

Data from: Moss, J. L., et al. (2016). Male adolescent fertility preservation. *Fertility and Sterility* **105** (2), 267–273; Lee, S. J., et al. (2006). American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *Journal of Clinical Oncology* **24** (18), 2917–2931; Howell, S. J., Shalet, S. M. (2005). Spermatogenesis after cancer treatment: Damage and recovery. *Journal of the National Cancer Institute. Monographs* (34): 12–17.

collection. Adolescents present a special challenge to banking sperm. There have been reports of sperm cryopreservation in adolescents as young as 11. However, the age of spermatogenesis is variable, and may be as late as age 15 years in normal males. Adolescents also present other challenges such as immaturity or inhibitions, and some may not have attempted self-stimulation previously. Several small cohorts report successful banking of sperm in 67%–83% of adolescents (Adank et al., 2014).

Collection of Ejaculated Sperm

The default method of sperm acquisition is through masturbation. Simply put, self-stimulation is inexpensive and safe, and usually provides sperm with the best quality. Sperm should be retrieved before gonadotoxic treatment is initiated. Optimally, the patient should abstain for 2–3 days before providing a semen sample. Occasionally, more than one sample is required to reach an adequate number of cryopreserved vials. The number of banked samples is variable, and depends on the quality of the semen sample, the degree of urgency in which treatment is advised, the andrology laboratory's protocol and experience, and the patient's desire. However, with current advances in ART, specifically intracytoplasmic sperm injection (ICSI), even one low quality ejaculate can yield sufficient sperm for conception of a biological child.

Some men are unwilling to produce a semen sample through masturbation. Several religions strictly disallow masturbation, not even in the context of fertility preservation. Possible solutions include the collection of postcoital semen or the use of a specially designed nonspermicidal perforated condom (allowed due to a tiny perforation at the end of the condom, which theoretically allows for some of the sperm to reach the partner while maintaining most of the sample in the condom), with the caveat being lower specimen quality. Other men will opt for medical solutions (designed for ejaculatory dysfunction) discussed in the next section.

Collection of Sperm From Patients With Ejaculatory Dysfunction

Patients who are unable to provide antegrade ejaculate through self-stimulation will require medical assistance to produce a semen sample. The different aetiologies of anejaculation and retrograde ejaculation, and a detailed description of treatment options such as penile vibratory stimulation (PVS) and electroejaculation (EEJ), are provided elsewhere. The suitable technique is decided according to the unique situation of the oncological patient. PVS is relatively simple and inexpensive, and can provide a good and quick solution for sperm acquisition with minimal delay of oncological treatment. Medical treatment with sympathomimetic agents for retrograde ejaculation is also simple but may require at least 36–48 h of treatment before providing a sample. Another treatment option for patients with retrograde ejaculation is the retrieval of sperm from a post-ejaculatory urine sample. EEJ necessitates more sophisticated and expensive equipment. It usually requires general anesthesia, and can be performed concomitantly with other procedures such as implanting a venous access device. Several small cohorts reported sperm retrieval rates of 48–68% in cancer patients, both adults and adolescents, undergoing EEJ prior to cancer treatment ([Adank et al., 2014](#); [Berookhim and Mulhall, 2014](#)). Eventually, if these methods fail to provide sperm, surgical extraction remains a viable option.

Collection of Sperm in Azoospermic Patients

Reported prevalence of azoospermia at presentation is variable, reported to range 1% to 15% in several large cohorts, contingent on cancer type. Azoospermic men will require some form of surgical retrieval of sperm. Uniquely, in azoospermic testis cancer patients a procedure to extract sperm can be performed concomitant with orchiectomy either *in vivo* or *ex vivo*. This procedure is referred to as “onco-TESE” (Oncological Testicular Sperm Extraction). Several authors described extracting sperm from the resected testis and, through ICSI, achieving pregnancies and live births of healthy children. A recent study found that the overall likelihood of presence of sperm in the tumor bearing testis is ~60%, suggesting that onco-TESE is a viable sperm extracting method ([Shoshany et al., 2016](#)). The importance of obtaining sperm through onco-TESE is underlined by the observation that up to 9% of men who have an apparently normal contralateral testis become azoospermic after orchiectomy. Onco-TESE was also performed successfully in azoospermic patients with other malignancies. TESE may also be an option in azoospermic cancer survivors who do not have banked sperm, as focal spermatogenesis is still possible if some spermatogonial stem cells survived the gonadotoxic treatment. A review of results from five centers found an overall sperm recovery rate of 44% in patients with azoospermia undergoing TESE after chemotherapy ([Picton et al., 2015](#)).

Cryopreservation of Sperm

Cryopreservation of sperm in cancer patients presents few unique challenges. Not infrequently, sperm produced by cancer patients at the time of diagnosis is of poor quality. In the process of cryopreservation, sperm parameters are further negatively affected. Motility may decrease by 25%–75% after thawing. Dilution with cryoprotectant reduces sperm concentration. Further detrimental effects have been suggested such as an increase of sperm DNA fragmentation and decondensation, as well as alterations of other sperm parameters that may be relevant for the fertilization process. Historically, with the use of slow freeze, approximately 50% of sperm survive the freezing process. Moreover, sperm from patients with certain types of cancer such as testicular cancer or leukemia show a markedly decreased survival after freezing ([Nangia et al., 2013](#)). Newer methods of cryopreservation, such as vitrification, are explored to improve post-thaw survival in samples of low sperm concentration. The reduced post-thaw quality may result in a need for a more demanding ART, as some patients who were intra-uterine insemination (IUI) candidates before cryopreservation, may require in-vitro fertilization (IVF) or ICSI when using their thawed banked sperm. At times, it may be difficult to find motile sperm in thawed specimens, possibly compromising ICSI outcome. Cryopreserved sperm can theoretically last indefinitely and reportedly used successfully after 28 years to conceive healthy offspring. The American society for reproductive medicine states

that caregivers should receive patient instructions on the handling of cryopreserved sperm in the case of patient death or unavailability ([Ethics Committee of American Society for Reproductive Medicine, 2013](#)). [Fig. 1](#) provides an algorithm for sperm banking and utilization.

Fecundity in Male Cancer Survivors

Overall Fecundity

Although many cancer survivors maintain their fertility, several studies show decreased fecundity in these men. The Childhood Cancer Survivor Study (CCSS) is a large cohort of five-year cancer survivors with various malignancies. One study reviewing male survivors reported that non-surgically sterile men were less likely to sire a pregnancy compared with their siblings, with a hazard ratio (HR) of 0.56 (95% CI – 0.49 to 0.63) ([Green et al., 2010](#)). Poor prognostic factors included radiation dose > 7.5 Gy to the testes, treatment with cyclophosphamide or procarbazine, and higher cumulative alkylating agents dose. However, patients with none of these risk factors did not have a significantly reduced likelihood of siring a pregnancy. A later review of the CCSS cohort ([Chow et al., 2016](#)), focused on a specific group of male survivors not treated with radiation, found an overall decrease in pregnancy rates (HR 0.63 [0.58–0.68]), which was also shown in men not exposed to alkylating or DNA interstrand cross-linking drugs (HR 0.72 [0.66–0.80]). A Norwegian cohort of similar magnitude studied childbearing in cancer survivors. Compared with age matched controls, postcancer births were 30% lower ([Cvancarova et al., 2009](#)). A large Finnish cohort of over 25,000 cancer survivors reported a 0.57 relative probability of having a first child ([Madanat et al., 2008](#)). Evidently, fecundity is lower in male cancer survivors, and some may require ART to conceive. As the return of spermatogenesis after chemotherapy or radiotherapy is unpredictable, it is uncertain who will need to use his cryopreserved sperm. Banked sperm may also be used in the post treatment time frame, in which there are significant risks for conceiving naturally (see “reproduction safety”). Although most studies suggest that only a minority (usually < 10%–15%) of men will use their stored specimens, for these men this may be the only way to sire a biological child.

ART Success Rate in Cancer Survivors

The advent of ICSI revolutionized the treatment of male infertility in general, and cancer survivors in particular, by allowing fertilization with virtually no lower limit on sperm quantity. Furthermore, ICSI was shown to achieve similar results with the use of fresh or cryopreserved sperm. Our knowledge of ART success in male cancer survivors is scarce and is based on small retrospective cohorts, mostly describing success with cryopreserved sperm. A recent systematic review reported on the use and effectiveness of sperm banking programs for cancer patients ([Ferrari et al., 2016](#)). A total of 30 studies were included. For IVF, the aggregated data analysis included 654 IVF cycles and showed a clinical pregnancy rate and a live birth or ongoing pregnancy rate per cycle of 30% and 25%, respectively. The cumulative rate of men achieving parenthood was 49%. These results are in line with IVF results in the general population. Retrospective cohorts comparing IVF results of cancer patients to other couples with male infertility also found similar success rates ([Garcia et al., 2015](#)).

Reproductive Safety in Men During and After Cancer Treatment

Concerns for reproductive safety are based on the potential mutagenic effects of cancer treatment. Both chemotherapy and radiation negatively impact DNA structure and stability. This DNA damage may theoretically have genetic implications for the offspring such as congenital malformations or cancer. Damage to sperm is short lived, as new sperm are constantly being produced. However, damage to the spermatogonial stem cells may incur a long lasting or permanent risk of implications on offspring. The harmful potential of cancer treatment is demonstrated through numerous studies reporting higher sperm aneuploidy rates, higher sperm DNA fragmentation and chromatin defects, as well as animal studies showing increased genetic translocations, mutations, and congenital malformations in offspring after exposure to chemotherapeutic agents. In addition, concerns regarding permanent changes to the epigenome have been raised recently. Researchers found that adolescents’ exposure to chemotherapy can permanently reprogram the spermatogenic stem cell epigenome with the potential to promote epigenetic inheritance to the next generation ([Shnorhavorian et al., 2017](#)). Recent ASCO recommendation state that men should be advised of a potentially higher risk of genetic damage in sperm collected after initiation of therapy ([Loren et al., 2013](#)).

Risk in the Immediate Posttreatment Timeframe

Two reproductive risks are to be considered in the immediate chemotherapy aftermath. First, a theoretical risk of the presence of chemotherapeutic agents in the ejaculate, being transmitted to the partner. Although it was estimated that the female’s expected blood exposure would be three orders of magnitude lower than the male’s total exposure, a potential risk cannot yet be ruled out ([Choy and Brannigan, 2013](#)). Second, studies have reported increased rates of sperm aneuploidy and DNA fragmentation to persist for up to 24 months after treatment ([Choy and Brannigan, 2013](#)). Currently, there is no consensus on the period considered safe for reproduction after cancer treatment. The only available guidelines are provided by the European Society for Medical Oncology. This group advocates deferral of childbearing for at least 12 months after cancer therapy, as a low-grade recommendation

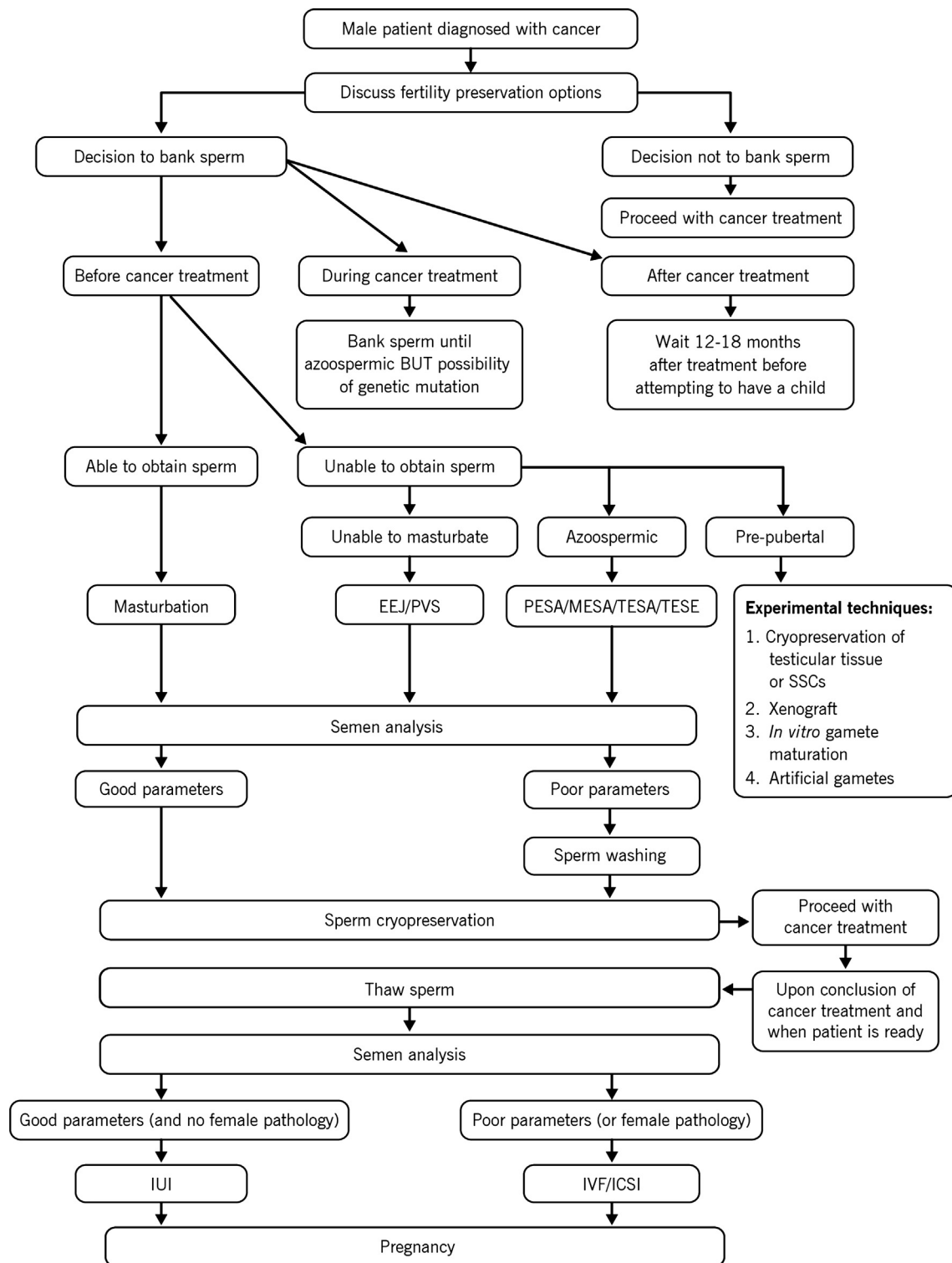


Fig. 1 Fertility preservation counseling and management in male cancer patients. Algorithm showing the process of sperm banking from initial diagnosis of cancer to possible methods of sperm collection, followed by sperm cryopreservation and thawing, and depending on the sperm parameters obtained, its use in suitable assisted reproduction techniques. *EEJ*, Electro-ejaculation; *PVS*, Penile vibrostimulation; *PESA*, Percutaneous epididymal sperm aspiration; *MESA*, Microsurgical epididymal sperm aspiration; *TESA*, Testicular sperm aspiration; *TESE*, Testicular sperm extraction; *IVF*, In vitro fertilization; *ICSI*, Intracytoplasmic sperm injection; *IUI*, Intrauterine insemination. With permission from: Agarwal, A., Ong, C., Durairajanayagam, D. (2014). Contemporary and future insights into fertility preservation in male cancer patients. *Translational Andrology and Urology* 3 (1), 27–40.

based on low level of evidence. Other researches recommend an extended period of 18–24 months, especially in patients who became azoospermic, indicating the spermatogonial stem cells may have been affected (Agarwal et al., 2014; Nangia et al., 2013).

Risk in Natural Conception

So far, most studies on children born to men with a history of cancer were reassuring (Ethics Committee of American Society for Reproductive Medicine, 2013; Lee et al., 2006). Unlike children born to female cancer survivors, offspring of male cancer survivors did not show a significant increased obstetric risk, perinatal morbidity, or decreased birth weight (Nagarajan and Robison, 2005). Several studies reported that the risk for congenital abnormalities was comparable to that of children of noncancer population. However, a recent large study from Danish and Swedish registries of over 1.77 million children reported conflicting results (Stahl et al., 2011). The authors found an increased overall risk of major congenital abnormalities in offspring of male cancer survivors (RR = 1.17; 95% CI 1.05–1.31). It is possible that studies to date were not sufficiently powered, and the conflicting results calls for further research.

Risk in ART

There are no good quality studies reporting on reproductive safety in male cancer patients utilizing ART. Two small cohorts reporting on ART success in male cancer patients reported the birth of 37 and 20 children, and all children were reportedly healthy with no congenital malformations or childhood malignancies (Chan et al., 2001; Ping et al., 2014). The aforementioned Danish and Swedish registries study (Stahl et al., 2011) found that the risk of congenital abnormalities was not modified by the mode of conception (natural vs. ART). Furthermore, a similar prevalence of major congenital abnormalities was observed in children born using fresh post-treatment sperm and those conceived using cryopreserved pre-treatment sperm. Up until now, the literature suggests that children born following IVF/ICSI have only modestly increased risk of most malformations, cancers, and birth defects and no evidence of impaired psychosocial development (Ethics Committee of American Society for Reproductive Medicine, 2013). It is still debatable if those risks are conferred by the ART technique, or are the manifestation of the genetic makeup of parents with subfertility or over diagnosis due to a more stringent follow up. However, other risks should be considered such as increased risk for multiple births and increased risk of perinatal problems such as low birth weight, prematurity, and perinatal mortality (Ethics Committee of American Society for Reproductive Medicine, 2013). Some cancer survivors will resolve to use their pretreatment cryopreserved sperm. In general, there is no evidence of an increased risk of adverse outcomes if cryopreserved rather than fresh sperm are used for ART.

Risk of Cancer in the Offspring

In the absence of a heritable cancer syndrome there does not appear to be an increased prevalence of malignancies in children born to men treated for cancer (Lee et al., 2006; Madanat-Harjuoja et al., 2010). The American Society of Clinical Oncology guidelines recommend that men should be advised of a possible, not yet quantifiable, higher risk of genetic damage in sperm of men with cancer or that underwent cancer treatment (Lee et al., 2006). A possible higher risk of cancer in the offspring conferred by ART is a contested subject, but should also be considered. Men who have a heritable genetic form of cancer with a known mutation may wish to use PGD or prenatal diagnosis to reduce the risk of the birth of a child with a similar disorder.

Future Perspectives

Worldwide, great effort has been made by the scientific community to try and develop new methods to preserve or reconstitute fertility, particularly in prepubertal patients who cannot cryopreserve sperm. Currently, there are no established clinical methods to achieve this goal. It is estimated that each year in the US alone there are >4000 prepubertal males facing cancer treatment and unable to cryopreserve sperm. A growing number of institutions are now offering these patients to cryopreserve testicular tissue or cell suspensions, with the hope that one of the currently experimental techniques will mature and be clinically available in the near future. These technologies include manipulation of spermatogonial stem cells in order to transplant them back into the testis or to grow mature sperm ex vivo, manipulation of testicular tissue via grafting (autologous or xenograft) or organ culture, induction of pluripotent stem cells to mature into germ cells, and gene therapy (Gassei and Orwig, 2016). These experimental technologies are fraught with technical and ethical obstacles, as well as safety concerns such as re-introducing malignant cells to the patient, transmission of zoonotic viruses, and risk for the offspring's genetic health (Gassei and Orwig, 2016).

References

- Adank, M. C., et al. (2014). Electroejaculation as a method of fertility preservation in boys diagnosed with cancer: A single-center experience and review of the literature. *Fertility and Sterility*, 102(1), 199–205. e1.
- Agarwal, A., Ong, C., & Durairajanayagam, D. (2014). Contemporary and future insights into fertility preservation in male cancer patients. *Translational Andrology and Urology*, 3(1), 27–40.

- Berookhim, B. M., & Mulhall, J. P. (2014). Outcomes of operative sperm retrieval strategies for fertility preservation among males scheduled to undergo cancer treatment. *Fertility and Sterility*, 101(3), 805–811.
- Chan, P. T., et al. (2001). Testicular sperm extraction combined with intracytoplasmic sperm injection in the treatment of men with persistent azoospermia postchemotherapy. *Cancer*, 92(6), 1632–1637.
- Chow, E. J., et al. (2016). Pregnancy after chemotherapy in male and female survivors of childhood cancer treated between 1970 and 1999: A report from the childhood cancer survivor study cohort. *Lancet Oncology*, 17(5), 567–576.
- Choy, J. T., & Brannigan, R. E. (2013). The determination of reproductive safety in men during and after cancer treatment. *Fertility and Sterility*, 100(5), 1187–1191.
- Cvancarova, M., et al. (2009). Reproduction rates after cancer treatment: Experience from the Norwegian radium hospital. *Journal of Clinical Oncology*, 27(3), 334–343.
- Ethics Committee of American Society for Reproductive Medicine. (2013). Ethics Committee of American Society for Reproductive Medicine. Fertility preservation and reproduction in patients facing gonadotoxic therapies: A committee opinion. *Fertility and Sterility*, 100(5), 1224–1231.
- Ferrari, S., et al. (2016). Sperm cryopreservation and reproductive outcome in male cancer patients: A systematic review. *Reproductive Biomedicine Online*, 33(1), 29–38.
- Garcia, A., et al. (2015). Assisted reproductive outcomes of male cancer survivors. *Journal of Cancer Survivorship*, 9(2), 208–214.
- Gassei, K., & Orwig, K. E. (2016). Experimental methods to preserve male fertility and treat male factor infertility. *Fertility and Sterility*, 105(2), 256–266.
- Green, D. M., et al. (2010). Fertility of male survivors of childhood cancer: A report from the childhood cancer survivor study. *Journal of Clinical Oncology*, 28(2), 332–339.
- Lee, S. J., et al. (2006). American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. *Journal of Clinical Oncology*, 24(18), 2917–2931.
- Loren, A. W., et al. (2013). Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *Journal of Clinical Oncology*, 31(19), 2500–2510.
- Madanat, L. M., et al. (2008). Probability of parenthood after early onset cancer: A population-based study. *International Journal of Cancer*, 123(12), 2891–2898.
- Madanat-Harjuoja, L. M., et al. (2010). Risk of cancer among children of cancer patients—A nationwide study in Finland. *International Journal of Cancer*, 126(5), 1196–1205.
- Meistrich, M. L. (2013). Effects of chemotherapy and radiotherapy on spermatogenesis in humans. *Fertility and Sterility*, 100(5), 1180–1186.
- Moss, J. L., et al. (2016). Male adolescent fertility preservation. *Fertility and Sterility*, 105(2), 267–273.
- Nagarajan, R., & Robison, L. L. (2005). Pregnancy outcomes in survivors of childhood cancer. *Journal of the National Cancer Institute. Monographs*, (34), 72–76.
- Nangia, A. K., Krieg, S. A., & Kim, S. S. (2013). Clinical guidelines for sperm cryopreservation in cancer patients. *Fertility and Sterility*, 100(5), 1203–1209.
- Osterberg, E. C., et al. (2014). Current practices in fertility preservation in male cancer patients. *Urology Annals*, 6(1), 13–17.
- Picton, H. M., et al. (2015). A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. *Human Reproduction*, 30(11), 2463–2475.
- Ping, P., et al. (2014). Fertility outcome of patients with testicular tumor: Before and after treatment. *Asian Journal of Andrology*, 16(1), 107–111.
- Quinn, G. P., et al. (2009). Physician referral for fertility preservation in oncology patients: A national study of practice behaviors. *Journal of Clinical Oncology*, 27(35), 5952–5957.
- Shalet, S. M. (1993). Effect of irradiation treatment on gonadal function in men treated for germ cell cancer. *European Urology*, 23(1), 148–151. discussion 152.
- Shnorhavorian, M., et al. (2017). Differential DNA methylation regions in adult human sperm following adolescent chemotherapy: Potential for epigenetic inheritance. *PLoS One*, 12(2), e0170085.
- Shoshany, O., et al. (2016). Predictors of spermatogenesis in radical orchiectomy specimen and potential implications for patients with testicular cancer. *Fertility and Sterility*, 106(1), 70–74.
- Siegel, R., et al. (2014). Cancer statistics, 2014. *CA: A Cancer Journal for Clinicians*, 64(1), 9–29.
- Stahl, O., et al. (2011). Risk of birth abnormalities in the offspring of men with a history of cancer: A cohort study using Danish and Swedish national registries. *Journal of the National Cancer Institute*, 103(5), 398–406.
- Williams, D. H. T., et al. (2009). Pretreatment semen parameters in men with cancer. *Journal of Urology*, 181(2), 736–740.

Relevant Websites

<http://www.livestrong.org/we-can-help>.
www.SaveMyFertility.org.

Vasectomy

Shu Pan and Stanton C Honig, Yale School of Medicine, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

ASA Antisperm antibodies
AUA American Urological Association
CV Conventional vasectomy
FI Fascial interposition
MIV Minimally invasive vasectomy
NSV No-scalpel vasectomy
PPA Primary progressive aphasia
PVSA Post vasectomy semen analysis

Introduction

Surgical sterilization has always been a reliable option for contraception in both men and women. Vasectomy is an outpatient procedure performed by most practicing urologists and select general practitioners. An estimated 350,000 procedures were performed in the United States in the year 2002 according to data extrapolated from National Survey of Family Growth ([Eisenberg and Lipshultz, 2010](#)), and a physician survey placed the incidence at over 526,000 per year ([Barone et al., 2006](#)). Vasectomy ranks as one of the most cost effective means of contraception, with the cost of approximately \$400–\$700 for patients in the United States ([Trussell et al., 2009](#)). Yet, despite comparable efficacy and its minimally invasive nature, vasectomy trails behind its female counterpart, tubal ligation. Trends in Contraceptive Use 2015, a data set published by the United Nations, revealed that male sterilization accounts for a mere 2.4% when compared to a prevalence of 19.2% for female sterilization ([United Nations, Department of Economic and Social Affairs, Population Division, 2015](#)). In fact, with the exception of northern Europe and Canada, vasectomy represents a small fraction of surgical sterilization performed.

History

Cooper performed the first documented vasectomy on a dog in 1823, but the first vasectomy performed on a human is less certain, although some credited R. Harrison, who completed the procedure in hopes of achieving prostate atrophy ([Leavesley, 1980](#)). In the ensuing period of late 19th and early 20th century, forced male sterilization played a part in the eugenical sterilization policy with passage of statutes in 12 states in the United States. Over 1400 sterilizations of institutionalized persons were performed between 1907 and 1917, as a means of “preventing the spread of defected germ plasm.” These laws were ultimately overturned in 1918 ([Reilly, 1983](#)). It was not until the time of World War II that vasectomy was regarded as a form of birth control.

Perioperative Considerations

Factors Influencing the Choice of Vasectomy

Numerous factors influence the selection of surgical contraception. Interestingly, a study performed on married couples seeking sterilization revealed that while the decision making process is shared between the couple, the final model entailed predominantly wife-only variables. This emphasizes the significance of spousal influence on men seeking and ultimately undergoing vasectomies ([Miller et al., 1991](#)). In the United States, men who are of non-Hispanic ethnicity and who have visited family planning clinics were more likely to select vasectomy. On the other hand, men with a lower education level were more likely to report their partners as undergoing tubal ligations ([Anderson et al., 2010](#)). The latter phenomenon is likely a reflection of the model of health care in the United States; the author of the study postulated that higher rates of tubal ligation in women of lower socioeconomic status may be related to the Medicaid coverage for sterilization in the post-partum period.

Preoperative anxiety certainly exists as one of the barriers to vasectomy. A study has shown that a group of men seeking vasectomy displayed a mean anxiety level of 3.5 out of a 10 point scale, where the most common reasons for anxiety were anticipated pain (27%) and fear of the unknown (23%) ([Sandlow et al., 2001](#)). The most prominent reason compelling men to select vasectomy over their partner obtaining tubal sterilization is because it is safer and simpler, reported by 62% of the men surveyed. An

additional 14% stated that it was their turn to shoulder the responsibility for contraception, and 22% cited that their partners disliked other contraceptive measures (Barone et al., 2004).

Preoperative Counseling and Evaluation

Preoperative counseling and evaluation is an important component of planning prior to surgical sterilization. The minimum age of requirement for vasectomy is usually 18 years matching that of age of consent, but this may vary depending on the country, state, or jurisdiction involved. While the majority of men seeking vasectomy reports a straight forward decision making process, up to 45% of men felt that the decision was a difficult one (Balde et al., 2006). As such, the clinician is obligated to schedule a consultation session to adequately address the patient's concerns and to discuss the necessary and minimum concepts associated with surgical contraception. Ideally a face to face encounter, the consultation may also utilize telephone or even electronic methods of communication that involves a two-way flow of information. The physical examination, with emphasis on the genitourinary system, may also be deferred to the day of the procedure. It may also be prudent to have the patient's partner present during the preoperative appointment but this is not a requirement. According to the American Urological Association, the patient should be made aware of the following concepts included in Table 1 (Sharlip et al., 2012). The above list constitutes the bare minimum points of discussion prior to vasectomy, but there are certainly additional non-essential, but nevertheless, important factors that may require dialog. These are covered in detail in the AUA guidelines on Vasectomy.

Surgical Procedure

Surgical Approach and Mechanism of Sterility

As mentioned prior, vasectomy is usually an outpatient procedure, most often performed in the office setting. Two surgical steps are important: vasal isolation and vasal occlusion. Prior to vas isolation, local anesthesia consistent of lidocaine and bupivacaine is administered intradermally followed by deeper infiltration to the perivasal sheath, which contains abundant neurovascular tissue (Fig. 1). Adjunctive topical anesthetics such as creams or sprays may be employed, but the evidence on their efficacy remains conflicting. Oral or intravenous anxiolytics may be used for patients who report a high level of pre-procedural anxiety. Rarely, general anesthesia or conscious sedation are reserved for patients who have unfavorable anatomy or simply cannot tolerate the discomforts of the procedure.

Conventional vasectomy (CV) was the most practiced technique prior to the introduction of minimally invasive instruments and their associated surgical refinements. CV is performed by making one midline scrotal incision or bilateral scrotal incisions using a scalpel. Incisions usually measure between 1.5 and 3.0 cm and the vas deferens is exteriorized using a surgical clamp.

Minimally invasive vasectomy (MIV) was developed in recent years and facilitated by the advent of specialized instrumentation. MIV has the following defining characteristics: (1) incisions/openings in the scrotal skin that measure ≤ 10 mm, either as a singular midline scrotal opening or bilateral openings and (2) minimal dissection of the vas and perivasal tissue. Single versus bilateral incisions usually depend on physician preference and influenced by patient anatomy. The use of a single opening or incision may reduce procedure time and the incidence of adverse events, but a large observational study examining 1800 patients did not perform statistical testing (Castillo Jimeno et al., 1992). In general, the single incision vasectomy localizes the surgical opening to the midline just below the penoscrotal junction (or halfway between the junction and the top of the testes) whereas the bilateral approach places the incision at the level of the penoscrotal junction. Experts recommend these surgical sites to aid in the isolation of the straight portion of the vas deferens. Moreover, for the single opening vasectomy, the surgeon has to ensure that the same vas is not isolated and occluded in two locations, while leaving the contralateral vas unoccluded.

As a form of MIV, the no-scalpel vasectomy (NSV) was developed in 1974 in China by Dr. Li Shunqiang and has since gained worldwide acceptance. The seminal publication by Li et al. describes the procedure in detail (Li et al., 1991), but a brief description of the procedure will be included here. NSV makes use of the vas ring clamp to isolate the vas, perivasal tissue, and overlying skin (Fig. 2). A small defect is then created in the skin with the vas dissector, measuring < 10 mm, and the anterior vasal sheath is spread

Table 1 AUA guidelines—minimum and necessary concepts to be discussed during a preoperative evaluation for vasectomy

Necessary concepts

1. Vasectomy as a method of contraception is considered permanent
2. It does not produce immediate sterility and so family planning is necessary until vasal occlusion is confirmed
3. Vasectomy is not 100% reliable. The failure rate associated with vasectomy is approximately 1 in 2000
4. Repeat vasectomy is necessary in $< 1\%$ of vasectomies provided that a technique known to have low failure rate has been used
5. The patient should refrain from sexual intercourse or ejaculation for 3–7 days
6. There are options for fertility after vasectomy but they tend to be expensive and have lower rates of success
7. As with all surgeries, there are risks of bleeding and infection
8. There is risk of chronic pain after vasectomy
9. There are alternatives, both permanent and non-permanent

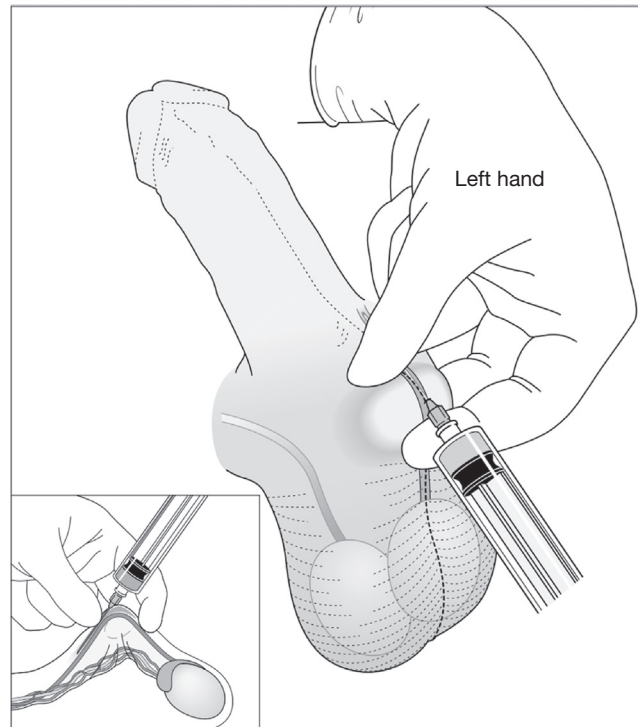


Fig. 1 Administration of local anesthetic.

open (**Fig. 3**). The vas deferens is then isolated and elevated from the skin opening using a supination maneuver and re-purchased using the ring clamp. The residual posterior sheath tissue, containing much of the neurovascular contents, is then dissected off. Vasal occlusion in the form of division, with or without segmental excision, is performed last.

The mechanism of sterility in vasectomy is vasal occlusion, the blockage of the flow of sperm through the lumen of the vas deferens. This can be achieved through several different techniques, most involving the division and/or excision of a segment of the vas. The only method of vas occlusion without division is the extended electrocautery, or Marie Stopes International technique, but it is rarely used in the United States. This method in particular employs full thickness electrocoagulation of the anterior vasal wall with a partial thickness coagulation of the posterior wall, for a length of approximately 2.5–3 cm ([Black et al., 1989](#)). Failure rate after this technique is comparable to other methods of vas occlusion, as demonstrated retrospective study with over 41,000 patients, and a reoperation rate of 0.46%–0.7% ([Black and Francome, 2002](#)).

There are several techniques of vasal occlusion employed after division of the vas. Commonly, the vas ends may be occluded with ligature or clips (**Fig. 4**). Thermal energy may also be applied to the luminal mucosa to achieve occlusion. Fascial interposition (FI), which is the suturing of the internal spermatic fascia to intervene between the divided vas ends, serves to prevent possible recanalization, and may be used in tandem with the above occlusive techniques. A notable variation is the open ended vasectomy, where the testicular end of the severed vas is left unoccluded. The intended benefit of this technique is to reduce the risk of post vasectomy pain by decreasing the back pressure in the epididymis. The open ended technique may also facilitate the formulation of sperm granuloma at the transected end of the vas ([Shapiro and Silber, 1979](#)). When the open ended technique is used, FI is

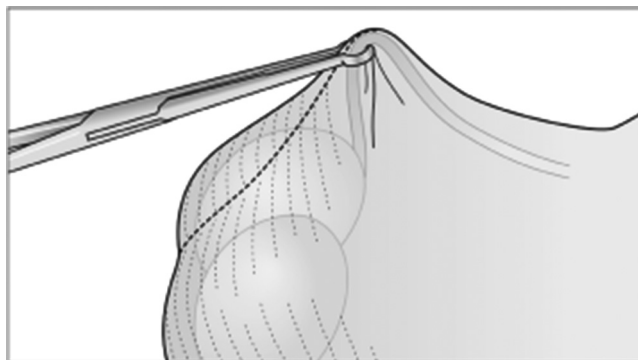


Fig. 2 Isolation of the vas during a no-scalpel vasectomy.

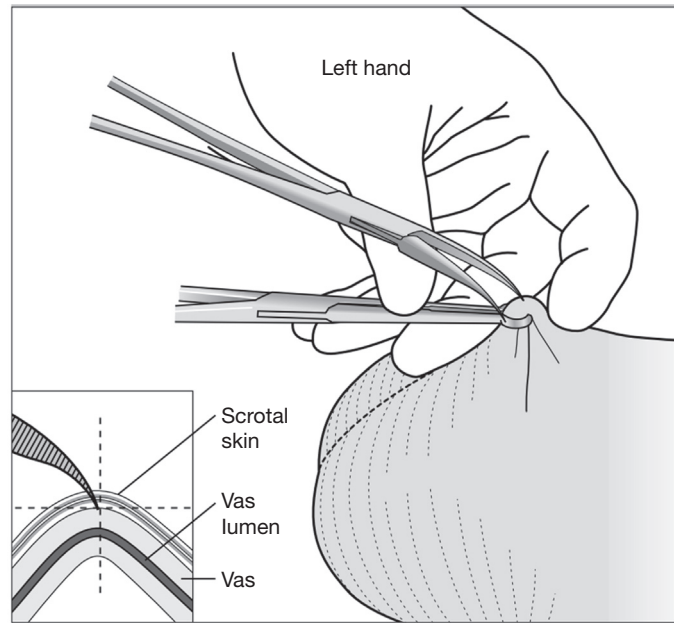


Fig. 3 Dissection during the no-scalpel vasectomy.

indicated to reduce the likelihood of recanalization. Additionally, some practitioners also excise a short segment of the divided vas, but the AUA expert panel found no convincing evidence to support the superiority of division/excision over division alone ([Sharlip et al., 2012](#)).

The AUA guidelines found that mucosal cautery with or without FI has the lowest failure rate.

Post-operative Considerations

Post Vasectomy Semen Analysis

A post vasectomy semen analysis (PVSA) is obtained to document the clearance of motile sperm before the man can rely on vasectomy alone for contraception. There is substantial variability in the time period required for the clearance of sperm, a process that is seemingly affected by anatomy, age, and possibly the number of ejaculations post vasectomy. Non-motile sperm may persist in the ejaculate for months after vasectomy ([Arango Toro et al., 1993](#)), and this may be due to the presence of residual sperm in the seminal vesicles, ampulla of the vasa and/or micro recanalization ([Freund and Couture, 1982](#)). Persistent motile sperm in the PVSA is concerning, and it prompts careful follow up. It may be caused by the erroneous double occlusion of the same vas deferens or early re-canalization. In most cases, however, semen samples provided 3 months after vasectomy have either no sperm or a small number of non-motile sperm. From a laboratory standpoint, PVSA should be conducted without centrifugation of the

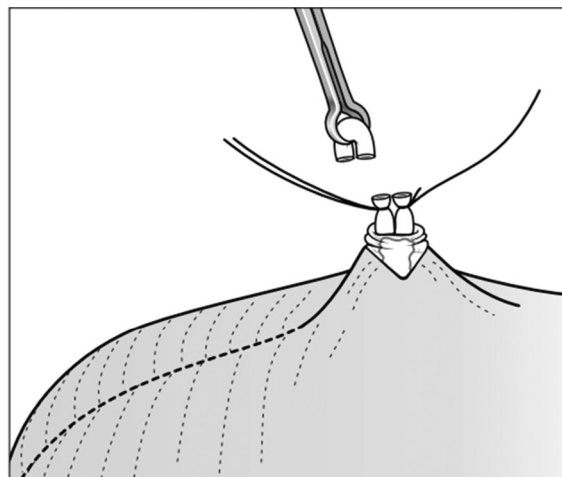


Fig. 4 Division of the vas and ligation of the two ends.

sample. Despite previous society guidelines that supported the use of routine centrifugation of azospermic semen samples, a recent study has shown that uncentrifuged semen analysis retains a sensitivity of 99.3% and negative predictive value of 99.8% for identifying samples with $\leq 100,000$ sperm/mL (World Health Organization, 2010; Hancock et al., 2002; Steward et al., 2008). The use of centrifuged specimen may detect the presence of sperm without clinical significance and prompt unnecessary testing or repeat vasectomies (Sharlip et al., 2012). According to the AUA, a PVSA should be performed 8–16 weeks post procedure and the patient may stop using other family planning measures when one uncentrifuged fresh specimen reveals azoospermia or rare non-motile sperm ($\leq 100,000$ sperm/mL) (Sharlip et al., 2012; Chawla et al., 2004).

Failure of vasal occlusion likely occurred should motile sperm persist 3–4 months after vasectomy. Repeat PVSA's are recommended until 6 month post vasectomy because up to half of the men with detected motile sperm ultimately achieve delayed vasectomy success (Labrecque et al., 2005). The physiologic process of both recanalization versus delayed success have not been completely elucidated, but may be related to post vasectomy inflammatory process and fibrosis.

Complications/Adverse Sequelae

The rate of complications following vasectomy is quite low. As with all surgical procedures, vasectomy carries with it risks of infection, bleeding, and damage to adjacent tissues. Bleeding, along with the formation of a scrotal hematoma, is a well-known risk of risk of vasectomy and has been estimated to 1%–2% in most series (de los Rios Osorio and Castro Alvarez, 2003; Kumar et al., 1999; Rees, 1973). Surgical site infection is rare and current recommendations do not support the use of perioperative antibiotics unless the patient falls into the high risk category, such as diabetes or an immunocompromised state. Chronic scrotal pain can manifest after vasectomy, presenting as a detriment to the overall quality of life. A retrospective cohort study compared men who had undergone vasectomy to non-vasectomized controls found that 6% of cases versus 2% of controls reported scrotal pain of sufficient bother that medical advice was sought. Notably, none of the individuals who elected for vasectomy later regretted their decision (Morris et al., 2002). In a well-designed prospective study, 593 respondents were surveyed before and after vasectomy. New onset scrotal pain was detected in 14.7% of the men approximately 7-month post vasectomy with a mean visual analog score of 3.4/10. Most importantly, four men (0.9%) reported pain that was quite severe and adversely affecting their quality of life (Leslie et al., 2007). Given the relative difficulty of studying post vasectomy orchalgia and the limited number of well-designed prospective studies, the AUA expert panel has reviewed available data to estimate the incidence of chronic scrotal pain severe enough to affect quality of life at 1%–2% (Sharlip et al., 2012).

Long-Term Risks

Multiple retrospective studies were performed to evaluate the possible linkage between vasectomy and coronary heart disease, hypertension, strokes, but the summary body of evidence does not point to any association (Beaglehole et al., 1985; Chi et al., 1990; Rosenberg et al., 1986; Tang et al., 1988; Massey et al., 1984). A limited number of studies actually suggest that men who had undergone vasectomy reported better states of health compared to their counterparts.

Primary progressive aphasia (PPA) is a language based dementia, fitting under the broader category of frontotemporal degeneration. One case control study was prompted by the report of a 43-year-old male who reported progressive word finding difficulty, correlating his onset of symptoms in the post vasectomy period. The authors compared 47 men with PPA to 57 men with no cognitive impairment and found that the age adjusted rate of vasectomy was significantly higher (40% vs. 16%, $P = 0.02$). Their hypothesis is that antisperm antibodies (ASA) that are produced after vasectomy may share antigenic epitopes with the brain, triggering an immunologic response akin to a paraneoplastic process (Weintraub et al., 2006). Interestingly the study did not measure the levels of ASA between case and controls, which would have certainly bolstered their hypothesis. A later study conducted in patients with Alzheimer's disease did not detect an association between the presence of ASA and cognitive function (Han et al., 2010). One must keep in mind that even if an association exists, the causality cannot be proven in a retrospective fashion.

From an oncologic perspective, meta-analysis of available data failed to demonstrate an association between vasectomy and testicular cancer (Sharlip et al., 2012). The AUA also performed an exhaustive review of available literature published on the correlation between vasectomy and prostate cancer. Over 30 studies spanning several decades were evaluated and the overwhelming negative findings lead the panel to conclude that vasectomy does not predispose men to develop clinically significant prostate cancer. Since the publication of the panel recommendations, additional research has been conducted on this subject. Nayan et al. published a large scale matched cohort study, which included over 320,000 men who had undergone a vasectomy matched 1:1 with men who did not. Some 3400 cases of prostate cancer were identified, and after adjustment for measures of health seeking behavior, no association was found (HR 1.02, 95% CI 0.95–1.09) (Nayan et al., 2016). Research originating from the United States also affirmed the above findings; among the Cancer Prevention Study II cohort of over 360,000 men, no association was found between vasectomy and prostate cancer mortality (Jacobs et al., 2016).

Future Directions

There remains a number of topics related to vasectomy that warrants further research. Certainly the basic science of the immunologic cascade after surgical sterilization remains to be elucidated. While numerous technical advances have been made in the recent

decades, surgeons should still strive for continued refinement, in the hopes of reducing patient discomfort and post-operative complications. Perhaps even more important, providers should focus on the areas of patient education, counseling, and general dissemination of vasectomy as a viable, safe, and reliable method of contraception. Rectifying the relative under-utilization of male sterilization will not only benefit men and their partners, but it may also deliver cost savings for health care system.

References

- Anderson, J. E., et al. (2010). Contraceptive sterilization use among married men in the United States: results from the male sample of the National Survey of Family Growth. *Contraception*, 82(3), 230–235.
- Arango Toro, O., et al. (1993). Post-vasectomy semen in 313 males. Statistical analysis, medical aspects, legal implications. *Archivos Españoles de Urología*, 46(1), 29–34.
- Balde, A., Legare, F., & Labrecque, M. (2006). Assessment of needs of men for decision support on male sterilization. *Patient Education and Counseling*, 63(3), 301–307.
- Barone, M. A., et al. (2004). Characteristics of men receiving vasectomies in the United States, 1998–1999. *Perspectives on Sexual and Reproductive Health*, 36(1), 27–33.
- Barone, M. A., et al. (2006). Vasectomy in the United States, 2002. *Journal of Urology*, 176(1), 232–236. discussion 236.
- Beaglehole, R., et al. (1985). Risk factors for coronary heart disease: a case-control study. *The New Zealand Medical Journal*, 98(774), 131–134.
- Black, T., & Francome, C. (2002). The evolution of the Marie Stopes electrocautery no-scalpel vasectomy procedure. *Journal of Family Planning and Reproductive Health Care*, 28(3), 137–138.
- Black, T. R., et al. (1989). The percutaneous electrocoagulation vasectomy technique—a comparative trial with the standard incision technique at Marie Stopes House, London. *Contraception*, 39(4), 359–368.
- Castillo Jimeno, J. M., et al. (1992). Unique incision vasectomy: review of 1,800 cases. *Archivos Españoles de Urología*, 45(1), 63–64.
- Chawla, A., Bowles, B., & Zini, A. (2004). Vasectomy follow-up: clinical significance of rare nonmotile sperm in postoperative semen analysis. *Urology*, 64(6), 1212–1215.
- Chi, I. C., et al. (1990). Vasectomy and non-fatal acute myocardial infarction: a hospital-based case-control study in Seoul, Korea. *International Journal of Epidemiology*, 19(1), 32–41.
- de los Rios Osorio, J., & Castro Alvarez, E. A. (2003). Analysis of 5000 vasectomies at a family planning clinic in Medellín-Colombia. *Archivos Españoles de Urología*, 56(1), 53–60.
- Eisenberg, M. L., & Lipshultz, L. I. (2010). Estimating the number of vasectomies performed annually in the United States: data from the National Survey of Family Growth. *Journal of Urology*, 184(5), 2068–2072.
- Freund, M. J., & Couture, M. (1982). The presence of spermatozoa in the semen of vasectomized men. *Journal of Andrology*, 3, 313–319.
- Han, C., et al. (2010). Lack of association between antisperm antibodies and language dysfunction in Alzheimer's disease. *Archives of Gerontology and Geriatrics*, 50(3), 338–340.
- Hancock, P., McLaughlin, E., & British Andrology Society. (2002). British Andrology Society guidelines for the assessment of post vasectomy semen samples (2002). *Journal of Clinical Pathology*, 55(11), 812–816.
- Jacobs, E. J., et al. (2016). Vasectomy and prostate cancer incidence and mortality in a large US cohort. *Journal of Clinical Oncology*, 34(32), 3880–3885.
- Kumar, V., et al. (1999). An evaluation of the no-scalpel vasectomy technique. *BJU International*, 83(3), 283–284.
- Labrecque, M., St-Hilaire, K., & Turcot, L. (2005). Delayed vasectomy success in men with a first postvasectomy semen analysis showing motile sperm. *Fertility and Sterility*, 83(5), 1435–1441.
- Leavesley, J. H. (1980). Brief history of vasectomy. *Family Planning Information Service*, 1(5), 2–3.
- Leslie, T. A., et al. (2007). The incidence of chronic scrotal pain after vasectomy: a prospective audit. *BJU International*, 100(6), 1330–1333.
- Li, S. Q., et al. (1991). The no-scalpel vasectomy. *Journal of Urology*, 145(2), 341–344.
- Massey, F. J., Jr., et al. (1984). Vasectomy and health. Results from a large cohort study. *JAMA*, 252(8), 1023–1029.
- Miller, W. B., Shain, R. N., & Pasta, D. J. (1991). Tubal sterilization or vasectomy: how do married couples make the choice? *Fertility and Sterility*, 56(2), 278–284.
- Morris, C., Mishra, K., & Kirkman, R. J. (2002). A study to assess the prevalence of chronic testicular pain in post-vasectomy men compared to non-vasectomised men. *Journal of Family Planning and Reproductive Health Care*, 28(3), 142–144.
- Nayan, M., et al. (2016). Vasectomy and risk of prostate cancer: population based matched cohort study. *BMJ*, 355, i5546.
- Rees, R. W. (1973). Vasectomy: problems of follow up. *Proceedings of the Royal Society of Medicine*, 66(1 Pt 1), 52–54.
- Reilly, P. (1983). The surgical solution: the writings of activist physicians in the early days of eugenical sterilization. *Perspectives in Biology and Medicine*, 26(4), 637–656.
- Rosenberg, L., et al. (1986). The risk of myocardial infarction 10 or more years after vasectomy in men under 55 years of age. *American Journal of Epidemiology*, 123(6), 1049–1056.
- Sandlow, J. I., et al. (2001). Psychological correlates of vasectomy. *Fertility and Sterility*, 75(3), 544–548.
- Shapiro, E. I., & Silber, S. J. (1979). Open-ended vasectomy, sperm granuloma, and postvasectomy orchialgia. *Fertility and Sterility*, 32(5), 546–550.
- Sharlip, I. D., Belker, A. M., Honig, S., Labrecque, M., Marmar, J. L., Ross, L. S., Sandlow, J. I., & Sokal, D. C. (2012). Vasectomy, 2015 [cited 2017 May 30]. Available from: [https://www.auanet.org/guidelines/vasectomy-\(2012-amended-2015\)#x3371](https://www.auanet.org/guidelines/vasectomy-(2012-amended-2015)#x3371).
- Steward, B., Hays, M., & Sokal, D. (2008). Diagnostic accuracy of an initial azoospermic reading compared with results of post-centrifugation semen analysis after vasectomy. *Journal of Urology*, 180(5), 2119–2123.
- Tang, G. H., et al. (1988). Vasectomy and health: cardiovascular and other diseases following vasectomy in Sichuan province, People's Republic of China. *International Journal of Epidemiology*, 17(3), 608–617.
- Trussell, J., et al. (2009). Cost effectiveness of contraceptives in the United States. *Contraception*, 79(1), 5–14.
- United Nations, Department of Economic and Social Affairs, Population Division. (2015). *Trends in Contraceptive Use Worldwide 2015 (ST/ESA/SER.A/349)*.
- Weintraub, S., et al. (2006). Vasectomy in men with primary progressive aphasia. *Cognitive and Behavioral Neurology*, 19(4), 190–193.
- World Health Organization, WHO laboratory manual for the examination and processing of human semen. 5th edn. 2010. Geneva: World Health Organization. xiv, 271 p.

Relevant Websites

[http://www.auanet.org/guidelines/vasectomy-\(2012-amended-2015\)](http://www.auanet.org/guidelines/vasectomy-(2012-amended-2015)).
<https://www.engenderhealth.org/pubs/family-planning/vasectomy.php>.

Penile Prosthesis for Erectile Dysfunction

Culley C Carson, University of North Carolina, Chapel Hill, NC, United States

© 2018 Elsevier Inc. All rights reserved.

While erectile dysfunction (ED) has plagued men since ancient times, effective treatment has only been available since the late 20th century. The era of implantable devices for ED began with the development of silicone-based prosthetic materials in the late 1960s (Carson, 2005). Modern penile prosthetic devices were first developed in the early 1970s when Small et al. (Small et al., 1975) along with Scott et al. (Scott et al., 1973) reported the implantation of penile prosthetic devices into the corpora cavernosa to fill the corpora cavernosa and provide a physiologically functional erection with good cosmetic results. These devices have undergone revisions and redesigns between the early prosthetic devices and the currently implanted inflatable penile prostheses. Current inflatable prosthetic devices have improved mechanical reliability and reduced infection risk. These current devices can be divided into semirigid and multiple-component inflatable penile prostheses of which there are two- and three-piece models available. (See Table 1.)

Semirigid rod available today are the successors of the devices designed in the 1970s. These devices, while easier to implant, have few advantages over the newer inflatable devices because infection and mechanical malfunction rates are similar. The semirigid devices usually consist of a central metal core and a silicone elastomer rod a series of disks held in position by a central cable. The latter design facilitates positioning of the implant between uses. The overwhelming preference in the United States is for the three piece inflatable implant.

The three-piece inflatable penile prostheses vary in construction from three-layer silicon/Dacron/Lycra/silicone to a single layer of silicon or Bioflex. Options include girth expansion and/or length elongation in the AMS 700 LGX device. Device structural modifications have decreased mechanical malfunction rates from greater than 30% to less than 5%. The most significant recent advance is the coating of three-piece implants to reduce infection risk.

The three-piece inflatable penile prostheses continue to be the most satisfactory prostheses once they are implanted. These prosthetic devices produce the most natural appearing erection in girth, length, and with satisfactory rigidity and excellent flaccidity for optimal concealment. They also have advantages for many patients with complex penile implantations because the flaccid position removes pressure from the corpora cavernosa and decreases the possibility of erosion in these complex patients.

To improve ease of surgical implantation and remove a portion of the prosthesis placed within the abdominal region, two-piece prostheses were designed. Because these two-piece inflatable prostheses remove the separate reservoir, additional fluid is available either by a larger scrotal pump or a combination of proximal cylinder and pump reservoir. Although these devices provide adequate erection in many patients, the limited reservoir capacity decreases flaccidity and may, in some patients, diminish rigidity. These prostheses are especially difficult to deflate in patients with small penises and frequently provide inadequate rigidity of patients with longer penises. While less optimal than three-piece devices, these two-piece implants may be acceptable for patients in whom reservoir placement is difficult or contraindicated. Such patients as renal transplant recipients and patients who have undergone significant radical pelvic exenteration procedures may benefit from two-piece devices.

Diagnosis of ED

The diagnosis of erectile dysfunction can be obtained by history (Montorsi et al., 2010). The clinician should determine whether the erectile dysfunction is situational or constant, whether the degree of dysfunction is partial or total, and also include the details of the relationship with the partner. Any coexisting conditions such as diabetes, vascular disease, smoking, medications (especially steroids, hormones, antihypertensives, or antidepressants), and use of alcohol or drugs should be identified. Physical examination should include the genitalia including the testes, hair distribution, femoral and distal lower-extremity pulses, and the penis for Peyronie's plaques or other abnormalities.

Indications

While there are several penile prosthesis designs available for implantation, not all patients with ED are candidates for penile prosthesis implantation. Penile prostheses are generally a procedure of last resort for those men failing more conservative measures such

Table 1 Available penile prostheses

<i>Semirigid Rods</i>	<i>Inflatable</i>
AMS 600 (AMS)	<i>Three piece</i> 700 CX (AMS) 700 LGX(AMS)
Malleable (Coloplast)	Alpha 1 (Coloplast)
Dura II (AMS)	<i>Two piece</i> Ambicor (AMS)

as oral PDE5 inhibitors, vacuum erection and cavernosal injection therapy (Hellstrom et al., 2010). For those men in whom penile prostheses are suggested, careful counseling with the patient and his partner before penile implant procedures will limit many of the problems with postoperative dissatisfaction. Once the discussion and demonstration of penile implant varieties has been carried out, patients can be counseled about the most appropriate penile prosthesis. Patients may choose a specific prosthetic type based on their needs and preferences (Hellstrom et al., 2010). Younger patients with normal manual dexterity and patients who wear stylish, form-fitting, athletic clothing or who shower in public at a health club often choose a three-piece inflatable penile prosthesis because appearance in the flaccid position is better than other designs. Similarly, patients with Peyronie's disease, secondary implantation, or significant peripheral neuropathy such as occurs in severe diabetes are best served with an inflatable penile prosthesis because interior tissue pressures are diminished between uses and the possibility of extrusion is diminished.

For patients in whom the convenience of inflation and deflation are not important, the risks and mechanical malfunctions may outweigh the disadvantage of a malleable penile prosthesis. Such patients as paraplegics who require external urinary collection device, men with inadequate manual dexterity, or those with significant obesity may be better served with a malleable penile prosthesis.

Surgical Approach

Surgical implantation of penile prostheses can be carried out using a variety of surgical approaches and incisions. Semirigid and malleable prostheses can be implanted through a distal penile approach. Multiple-piece prostheses, however, can be implanted by the infrapubic or penoscrotal approach. While individual surgeons have a variety of rationales for each of these approaches, there does not appear to be any clear advantage in patient satisfaction or outcome of the two approaches. Patient anatomy may dictate appropriate choice. Patients with previous abdominal surgical procedures where reservoir placement is difficult may be better served with an infrapubic approach while patients with massive obesity may be better approached through a penoscrotal incision. Two-piece devices, because there is no separate reservoir, are best implanted through a penoscrotal incision.

A distal penile approach is usually the best approach for insertion of a semirigid or mechanical penile prosthesis. This incision heals well, allows complete corporeal dilation, and facilitates rod placement.

The infrapubic approach allows better visualization of the three piece prosthesis reservoir placement than the penoscrotal approach. However, because of the proximity of the dorsal neurovascular bundle in the infrapubic approach, injury is possible, resulting in decreased distal penile sensation in some patients. The infrapubic approach is usually carried out with a horizontal incision approximately one finger breadth below the symphysis pubis, allowing implantation with an easily concealed incision once the pubic hair regrows. In patients with significant obesity or a previous midline incision, however, a midline incision can be used.

Three-piece inflatable penile prostheses, as well as, semirigid and two-piece prostheses can be implanted by a transverse or vertical penoscrotal incision. This approach has distinct advantages in obese patients and is the most common approach for routine three piece penile prosthesis implantation. In men with a penoscrotal skin web, the horizontal skin incision can be closed in a midline fashion to enhance functional penile length.

Perioperative antibiotic treatment is critical in diminishing incidence of perioperative infection and prosthetic removal. The Inhibizone (American Medical Systems, Minnetonka, MN) coating consisting of rifampin and minocycline or the lubricious coating of the Titan implant (Coloplast Inc., Minneapolis, MN) which allows antibiotics to adhere to the bioflex have reduced infection risk by 3 fold. An initial perioperative dosage of an antibiotic agent effective against the most common infectious pathogens should be administered 1–2 h prior to surgery and continued for 24 h postoperatively. Surgical guidelines suggest a choice of an aminoglycoside with a first-generation cephalosporin, ampicillin/sulbactam a cephalosporin alone, vancomycin, or a fluoroquinolone is appropriate for prophylaxis of the most common infections from *Staphylococcus epidermidis* (Hellstrom et al., 2010). The use of antibiotic-coated penile implants, while reducing the incidence of postoperative infection, does not preclude the need for systemic, perioperative antibiotics. The penile prosthesis remains deflated for 4 weeks while healing occurs. Prior to activation, the patient is advised to retract the pump into his scrotum on a daily basis and tight underwear and athletic supports are avoided to maintain pump position. A return clinic visit for activation of the device is carried out once discomfort has resolved. Patients are advised to inflate and deflate the device on a daily basis for 10–12 weeks to allow tissue expansion around the prosthesis. Most patients can then begin use of their device 4–6 weeks after implantation.

Complications

Despite careful counseling, many patients enter penile prosthesis procedures with expectations that cannot be met by penile prosthesis surgery. Complaints about decreased penile length compared with preimplant state, decreased penile sensation, "coolness" of the penis and glans penis, and chronic pain, as well as partner dissatisfaction, are among the complaints patients may voice despite adequate surgical implantation and satisfactory mechanical functioning. Fortunately, these complaints are unusual and more than 90% of patients report satisfaction with their prostheses (Carson, 2003). Many patients who are dissatisfied with their penile prostheses will benefit from sexual counseling or continued counseling assistance from the implanting surgeon to be sure that they are able to operate the device satisfactorily and understand its use.

Most patient's dissatisfaction results from difficulty with operation of the device and unrealistic expectations. Preoperative discussions with patients should include the concept that penile prostheses do not create normal erections but only support the penis for sexual activity. Penile prosthesis surgery brings about the ability to resume sexual functioning and vaginal penetration, but decreased penile sensation, length, and engorgement may result. Patients should also be advised that a penile prosthesis will not improve libido or ejaculation. Patients frequently report delayed or difficult ejaculation initially following penile prosthesis surgery. This delay is primarily a result of inadequate preparation, stimulation, and psychological adjustment to the prosthesis. Most patients require 3–6 months of prosthesis use with careful attention to presexual stimulation before ejaculation routinely returns to preoperative levels. Because the prosthesis neither improves nor detracts from preoperative ejaculatory ability, patients must be counseled regarding their preoperative ejaculatory ability before prosthesis placement.

The most worrisome postoperative complication is infection, which occurs in fewer than 10% of all patients (Carson et al., 2000). Perioperative prosthetic infections can, however, occur at any time in the postoperative period in patients with penile or other prosthetic devices. Patients continue to be at risk for hematogenously seeded infections from gastrointestinal, dental, or urologic manipulations as well as remote infections. Patients must be counseled to request antibiotic coverage if remote infections occur. Most periprosthetic infections are caused by Gram-positive organisms such as *S. epidermidis*, but fungi or Gram-negative organisms such as *Escherichia coli* and *Pseudomonas* are also common culprits. Severe gangrenous infections with a combination of Gram-negative and anaerobic organisms have also been identified and frequently result in significant disability and tissue loss. Patients at increased risk for perioperative infections include diabetics, patients undergoing penile straightening procedures or circumcision with prosthetic implantation, patients with urinary tract bacterial colonization, and immunocompromised patients, such as posttransplant patients. Spinal cord injury patients have also been reported to have a specially increased risk of infections, with rates reported as high as 15%.

Appropriate treatment of periprosthetic infections requires early and immediate identification with institution of parenteral antibiotic therapy and early prosthesis removal (Carson, 2003). Conservative treatment would dictate a healing period of 3–6 months followed by repeat prosthesis implantation. Satisfactory results with prosthesis removal, a 5–7 day course of antibiotic irrigation, followed by additional replacement has been reported for selected patients. Better long-term results with no additional morbidity can be achieved with the prosthesis salvage technique reported by Mulcahy (Carson, 2003). This technique—which requires removal of the infected implant and vigorous irrigation using solutions of antibiotics, provadone iodine, hydrogen peroxide, and a repeat of these solutions—is successful in more than 75% of infected prostheses (Carson, 2003).

The most common complication of penile prosthesis function is mechanical malfunction. Mechanical malfunction has declined from rates as high as 61% to levels below 5% since the 1970s (Scott et al., 1973; Carson, 2003). Aneurismal dilation of inflatable cylinders, both American Medical Systems and Mentor, tubing kinking, reservoir leakage, and pump malfunction have been limited by device modifications. Fluid leak, however, continues to be a problem for many inflatable penile prostheses. These mechanical malfunctions require replacement of the leaking portion of the inflatable portion of the prosthesis. If a nonfunctioning prosthesis has been in place more than 4 years, however, it is usual practice to replace the entire device to reduce further mechanical malfunction (Hellstrom et al., 2010).

Semirigid rod penile prostheses are associated with fewer mechanical problems; the most common complication associated with these prostheses is cylinder erosion through skin or urethra. Prosthesis fracture or breakage has been reported and patients may return 6–8 years postimplantation with complaints of decreased rigidity of their semirigid rod, indicating fracture of central prosthetic cylinder wires. These wire fractures cannot usually be appreciated radiographically unless the prosthesis is put on stretch once it has been explanted. Replacement of these devices is indicated when patients note decreased rigidity. Prosthesis extrusion or erosion is most common in diabetics and spinal cord injury patients, especially those requiring urinary management with catheter placement or of condom catheter collection devices.

Extrusion can also occur beneath the penile skin distally from vigorous dilation or remotely from trauma or repeated use. These extrusions are characterized by distal penile pain with use. Correction can be carried out with the use of a patch graft, but a better approach is rerouting with no grafting material. Rerouting is associated with less infection and pain and a reduced recurrence rate.

Results

Long term function and use have been confirmed in studies of patients with implants as long as 10 years (Hellstrom et al., 2010; Carson et al., 2000). While partner satisfaction literature is sparse, patients have a greater than 90% satisfaction and those with functioning devices for more than 5 years used them 27 times monthly (Carson et al., 2000). Other studies have confirmed that patient satisfaction is greater than any other ED treatment modality (Carson, 2005).

References

- Carson, C. C. (2003). Diagnosis, treatment and prevention of penile prosthesis infection. *International Journal of Impotence Research*, 15(Suppl 5), S139–46.
- Carson, C. C. (2005 Nov). Penile prosthesis implantation: Surgical implants in the era of oral medication. *The Urologic Clinics of North America*, 32(4), 503–509.
- Carson, C. C., Mulcahy, J. J., & Govier, F. E. (2000). Efficacy, safety and patient satisfaction outcomes of the AMS 700CX inflatable penile prosthesis: Results of a long-term multicenter study. AMS 700CX study group. *The Journal of Urology*, 164, 376–380.

- Hellstrom, W. J., Montague, D. K., Moncada, I., Carson, C., Minhas, S., Faria, G., & Krishnamurti, S. (2010). Implants, mechanical devices, and vascular surgery for erectile dysfunction. *The Journal of Sexual Medicine*, 7(1 Pt 2), 501–523.
- Montorsi, F., Adaikan, G., Becher, E., Giuliano, F., Khoury, S., Lue, T. F., Sharlip, I., Althof, S. E., Andersson, K. E., Brock, G., Broderick, G., Burnett, A., Buvat, J., Dean, J., Donatucci, C., Eardley, I., Fugl-Meyer, K. S., Goldstein, I., Hackett, G., Hatzichristou, D., Hellstrom, W., Incrocci, L., Jackson, G., Kadioglu, A., Levine, L., Lewis, R. W., Maggi, M., McCabe, M., McMahon, C. G., Montague, D., Montorsi, P., Mulhall, J., Pfaus, J., Porst, H., Ralph, D., Rosen, R., Rowland, D., Sadeghi-Nejad, H., Shabsigh, R., Stief, C., Vardi, Y., Wallen, K., & Wasserman, M. (2010). Summary of the recommendations on sexual dysfunctions in men. *The Journal of Sexual Medicine*, 7(11), 3572–3588.
- Scott, F. B., Bradley, W. E., & Timm, G. W. (1973). Management of erectile impotence. Use of implantable inflatable prosthesis. *Urology*, 2, 80–82.
- Small, M. P., Carrion, H. M., & Gordon, J. A. (1975). Small-carrier penile prosthesis. New implant for management of impotence. *Urology*, 5, 479–486.

Further Reading

- Mulcahy, J. J. (2000). Long-term experience with salvage of infected penile implants. *The Journal of Urology*, 163, 481–482.

Vascular Surgery for Erectile Dysfunction

Po-Cheng Huang, National Taiwan University Hospital Hsin-Chu Branch, Hsin-Chu, Taiwan
Geng-Long Hsu, Hsu's Andrology and National Taiwan University, Taipei, Taiwan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Angioplasty An endovascular treatment specific to artery while balloon tamponade and arterial stent are always used in one setting.

Deep dorsal vein arterialization A hybrid surgery for erection restoration by increasing penile arterial supply using donor artery and meanwhile the circumflex veins were ligated to limit the venous drainage.

Electrocautery A device can deliver electrical energy to coagulate vascular lumen and prevent the blood vessels from bleeding.

Erectile dysfunction A newer term to replace the older one impotence which is defined as inability either to attain or maintain a rigid erection for satisfactory coitus.

Penile arterial insufficiency A situation that penile supply artery is dysfunctional. The supplying artery fails to adequately feed the sinusoidal tissue, in turn, unable to attain a full erection.

Penile arterial reconstruction A kind of reconstruction surgery to restore the penile arterial supply to meet the sinusoidal demands. The surgery purpose is to restore blood supply, therefore no destruction is allowed.

Penile veno-occlusive Dysfunction Penile sinusoidal tissues sustain blood perfusion from 2 to 3 to 60–80 mL/min to flaccid and erection status respectively. It is, therefore, understandable that abundant blood drains back to systemic circulation. Inability either to attain or maintain a rigid erection occurs if a penile veno-occlusive dysfunction occurs.

Penile vascular surgery Surgery method involves penile vascular system including penile arterial or venous surgery.

Penile vascular intervention A general term of penile endovascular treatment managed by the physician including methods used by the surgeon, interventional radiologist.

Penile venous embolization A measurement in an attempt to block the penile venous drainage by hindering the vessel from vascular endothelium by implanting coil, band ligation or scleronizing agent injection.

Penile venous ligation A ligation of penile erection related veins.

Penile venous sclerotherapy Any method in an attempt to reduce the penile venous drainage speed by using sclerosing agents.

Penile venous stripping A term to describe a thorough removal of erection related veins while the exits of emissary veins are firmly ligated.

Suction apparatus A device to suck bleeding blood away in order to clearly see the operative field during surgery.

Penile Arterial Surgery for Patient with Erectile Dysfunction

Innovative Penile Vascular Anatomy and Erection Physiology

The human beings have been in its current penile anatomy for some three thousand centuries. In traditional medical literature, it is consistently depicted that a single circular tunica layer and one deep dorsal vein flanked by paired dorsal arteries between Buck's fascia and the tunica albuginea of the corpora cavernosa. We may question how the veno-occlusive mechanism elucidates if no outer tunic layer exists? Thus it is likely that we may still not thoroughly understand its anatomy and erection mechanism and so does the reconstructive surgery derived from this anatomical knowledge.

Recent studies substantiate a model of the tunica albuginea of the corpora cavernosa as a bi-layered structure with a 360° complete inner circular layer and a 300° incomplete outer longitudinal coat spanning from the bulbospongiosus and ischiocavernosus proximally and extending continuously into the distal ligament within the glans penis. The entire outer layer of the penis and the above two muscles could be collectively categorized as skeletal components. The inner layer contains and supports the intracavernous sinusoids, the erection-related veins, and artery which could collectively be allocated as the smooth muscle components. Human beings are peculiar within the erect animal family in that we possess an os analog associated with some proportionally large and extraordinarily extensible corpora cavernosa and however, man does not possess an os penis which is present in all quadriceps; the actual bony portion that provides penile rigidity. The erectile capability of the human penis largely depends on sinusoids in the glans penis, the corpus spongiosum, and the corpora cavernosa which are also exclusively responsible for erection rigidity.

Thus, human penis evolves from a rigid body into a hydraulic system. Albeit human may be proud of this penile hydraulic design at no expense of awkward locomotion for an erect animal. It seems that the human corpora cavernosa are vulnerable to encounter erectile dysfunction (ED) which is defined as inability either to attain or maintain a rigid erection for satisfactory coitus.

The importance of a functional penile artery is the prerequisite for rigid erection. A rigid erection of the corpora cavernosa is initiated by either local stimulation or central drive if there are healthy corpora cavernosa in which a unique dual circulatory route is characteristic. In addition to the regular vascular system for nutrition via the capillaries, there is a system for erectile function in

which sinusoids shunt directly from arterial to sinusoidal space and then venous channels bypassing the capillaries. In human corpora cavernosa, therefore, it accounts for 2–3 mL/min to 60–80 mL/min in accordance with flaccid and erection status respectively. The main source of blood supply to the penile sinusoids originates from the internal pudendal artery which is the end branch of the internal iliac artery, although accessory contributions may arise from the external iliac, obturator, vesical, and femoral arteries. The internal pudendal artery gives off branches of bulbourethral, cavernosal and dorsal artery along exiting from the pudendal canal. The sinusoids of the corpora cavernosa are primarily supplied by the cavernosal artery and secondary by the dorsal artery (Fig. 1).

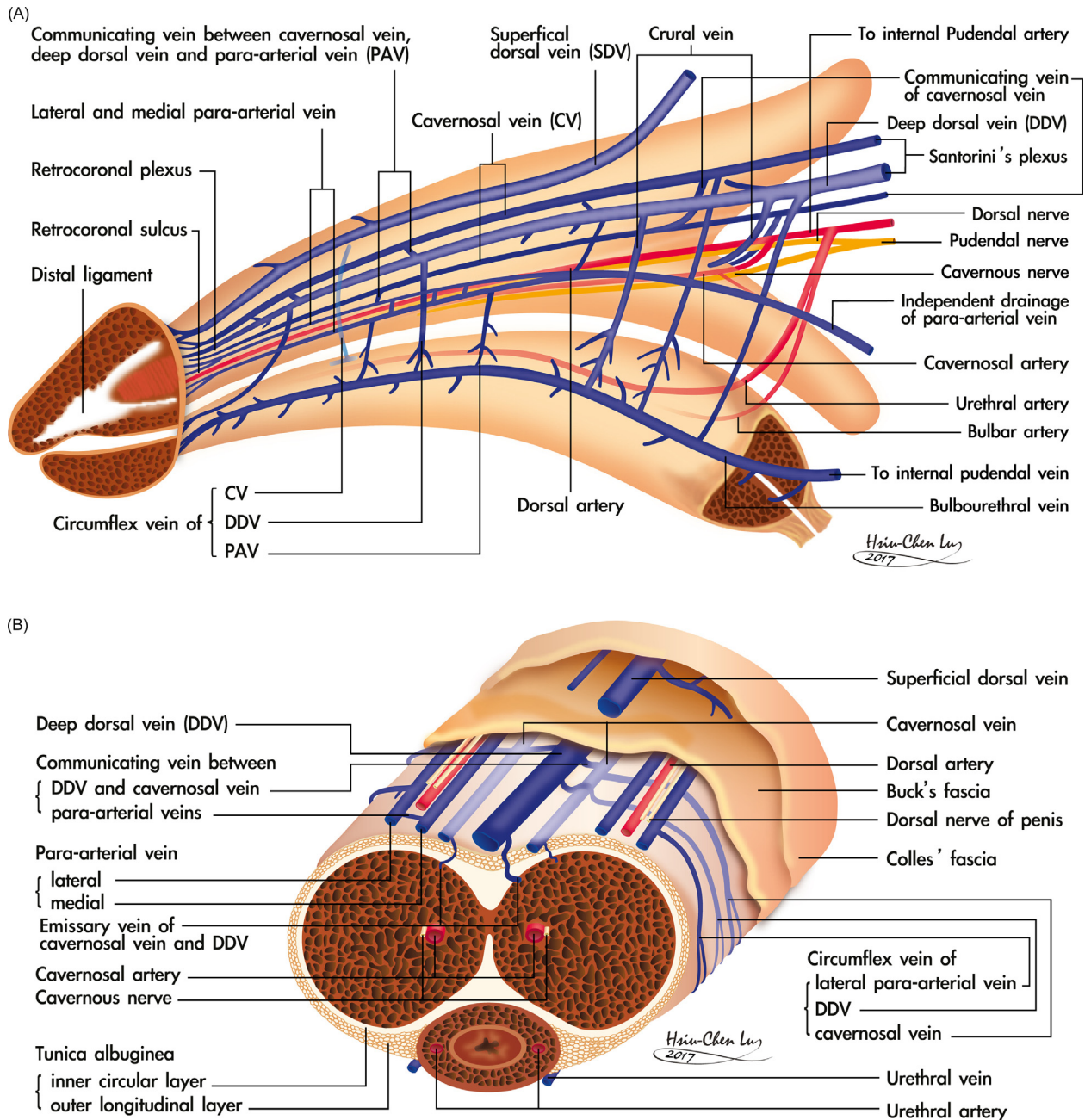


Fig. 1 Schematic illustration showing vascular system in the human penis. (A) Upper Lateral view: The deep dorsal vein consistently in the median position, receives the blood of the emissary veins from the corpora cavernosa and of the circumflex vein from the corpus spongiosum. It is sandwiched by cavernosal veins, although these lie in a deeper position. Bilaterally each dorsal artery is sandwiched by its corresponding medial and lateral para-arterial veins respectively. Note that the lateral para-arterial vein merges with the medial one proximally. The deeper color of the veins indicates the deepest group of the vasculature. The internal pudendal artery gives branch to bulbo-urethral artery and then the cavernosal artery which is the major supplier of the CC as well as dorsal artery supplying the glans penis. (B) Cross-section of the mid-portion: Note that the number of veins is seven rather than that of the traditionally described one, although it becomes four at the level of penile hilum because a merge takes place in each pair of the nomenclature veins. The erection-related veins are arrayed in an imaginary arc on the dorsal aspect of the tunica albuginea. The penile artery does not always accompany fellow veins.

Two types of branches arise from the cavernosal artery. Firstly, the outer capillaries which are responsible for penile nutrition supply the smooth component, skeletal component and nerve fibers. This system signifies that the corpora cavernosa are also susceptible to long hours in the erectile state. Secondly, and for fulfilling erection function, the inner helicine arteries open directly into cavernous spaces without entering capillaries, which are then emptied into the post cavernous venules. These inner arteries are shaped like corkscrews and allow the penis to elongate and enlarge without compromising flow.

The sinusoidal blood drains directly to sub-tunica venous plexus, subsequently passing through the tunica albuginea to the emissary veins, and then to the deep dorsal veins (DDV), cavernosal veins (CVs) and para-arterial veins (PAVs) separately. DDV, CVs and PAVs are collectively called erection related veins (ERVs) because they drain the sinusoidal blood independently. Thus DDV, CVs and PAVs own their specific emissary veins. This new understanding of penile venous anatomy is highly significant. Not only do we consider the traditional deep dorsal vein significant, but also the cavernosal vein, which distributes through the entire penile length and the para-arterial veins. The deep dorsal vein that lies consistently in the median position receives blood from the circumflex veins of the corpus spongiosum and from the emissary veins of the corpora cavernosa. Emissary veins run between the inner and outer layers of the tunica for a short distance, often piercing the outer layer bundles in an oblique manner. Therefore, and significantly, these emissary veins can be easily occluded by the shearing action elicited by the inner circular and outer longitudinal layers of the tunica albuginea.

The DDV is sandwiched by cavernosal veins that are coalesced to one at the penile base. Bilaterally each dorsal artery is sandwiched by the medial and lateral para-arterial vein respectively. Veins from the glans penis form a retrocoronal plexus that drains predominantly into the deep dorsal vein, the urethral veins, and the corpus spongiosum. The deep dorsal vein courses proximally in the midline between the two corpora cavernosa and empties into the periprostatic plexus. The superficial dorsal vein drains the skin and the subcutaneous tissue superficial to Buck's fascia and may have direct shunts to the corpora cavernosa. This, in turn, drains into the superficial external pudendal vein and is frequently seen in young male with ED. If and only if the erectile function of corpora cavernosa is healthy, the penis not only expands in its girth but also increases in its length, resulting in sufficient rigidity with sexual arousal.

Multiple layers of smooth muscle surround the helicine branches. This muscle is contracted while flaccid, allowing only small amounts of blood into the lacunar spaces. After the appropriate stimulus, muscle relaxation occurs and the arteries dilate and straighten, increasing blood flow and dilating the lacunar spaces, in turn, applying pressure against the outer longitudinal layer of the tunica albuginea to reduce the blood drainage, which eventually results in a rigid erection. Application of real world physics would assume that the corpora cavernosa is an ideal vessel to apply Pascal's Law which states that pressure applied to any part of an enclosed fluid at rest is transmitted undiminished to all walls of the containing vessel.

Pathophysiology of Erectile Dysfunction

There is a current consensus of erectile dysfunction pathophysiology which includes psychological disturbance, hormonal imbalance, vascular insufficiency, neurological deficit, sinusoidal fibrosis, pharmacological adverse's effect and metabolism disease. In other words, healthy male with normal sexual function must be under psychological health, an intact neurological system, a normal hormonal profile, an absence of adverse drug influence, a lack of systemic diseases, a functional artery and healthy intracavernous tissues. However, the end-organ penis just requires arterial sufficiency, normal sinusoidal tissue, and functional veins. Although there is no agreement on what would be the major contributor, there is a bias towards endothelial function due to the dramatic effects demonstrated by phosphodiesterase-5-inhibitors (PDE5 ↓) on erectile dysfunction.

There are, however, recent hemodynamic studies on fresh and defrosted human cadavers whereby rigid erections were unexceptionally reproducible despite the lack of endothelial activity. Constant low-pressure perfusion was used to mimic arterial inflow, and the staged removal of ERVs produced increasingly more rigid erections. Could it be that ERVs may, in fact, be the predominant factor that underlies erectile rigidity? Certainly, and in the light of our increased understanding of the penile vasculature, it would at least warrant re-evaluation of the role and even a viable option of venous surgery for erectile dysfunction in addition to rudimentary penile artery reconstruction. In summary, the corpora cavernosa, skeletal muscle components unite smooth muscle components to meet the requirements for an erection and only allow vascular and nervous tissue to communicate with the systemic circulation. Their anatomical relation is seemingly like a cluster of the grape when the emissary vein is regarded as the grape trunk and each sinusoid as a fruit. The rigid erection of the corpora cavernosa overall depends on cooperation among healthy sinusoids, a normal tunica, functional arteries, and competent veins. Thereafter vascular dysfunction is an important cause of male erectile dysfunction. It can be classified as veno-occlusive dysfunction, arterial insufficiency, or mixed. Accordingly, the best resort may be penile venous surgery, arterial reconstruction, and venous/arterial reconstruction respectively.

Penile Artery Surgery

In general, arterial vasculogenic causes of ED increase with age and is most prevalent in those with risk factors for atherosclerosis such as diabetes, hypertension, cigarette smoking, hyperlipidemia and obesity. Traumatic events, which occur more often in younger men, are due to pelvic trauma, straddle injury or even perineal insult such as long time bicycling. Albeit it is not above

controversy, documentation of arterial insufficiency can be noted via color Doppler ultrasonography, if peak systolic velocity is <25 cm/s or simple prostaglandin E1 test if the response fails to reach rigid erection.

Angiography, the benchmark for all arterial investigations, is reserved for those males who may resort their ED to penile arterial reconstructive surgery, concurrent embolization or angioplasty for treatment of arterio-venous fistula or stenosis respectively.

In 1973, Michal published firstly an article of penile arterial reconstruction in an attempt to enhance arterial flow to corpora cavernosa. He debuts revascularization surgery by the anastomosis of the inferior epigastric artery (IEA) to the corpora cavernosa. Subsequently, the deep dorsal vein arterializations, either without extensive ligation of the circumflex veins or with venous ligation, were introduced by Virag and Haudi in 1981 and 1986 respectively. In an effort to achieve more favorable outcomes varied modifications were performed, such as Furlow–Fisher procedure in which IEA was made in an end-to-side anastomosis to the DDV while several ligations were undertaken at the lateral circumflex veins, proximal and distal location (Fig. 2). It aims at increasing penile arterial inflow and limiting the venous drainage. Postoperative outcomes of arterial revascularization surgery are varied due to multifaceted causes, patient selection, surgical instruments and surgical technique. In general, its merit is limited, without long term benefits (Table 1). Furthermore, complication rate occurs in about 25% of patients who undergo penile revascularization surgery. Immediate postoperative arterial hemorrhage may result from disruption of the microvascular anastomosis with hematoma formation. The potential risks of these techniques, such as spongy necrosis and hyperesthesia of the glans, cannot be ignored. In addition, a complication rate of wound infection, inguinal hernias, loss of penile length, and decreased penile sensitivity cannot be avoided. Particularly, the major complications are priapism and glans hyperemia which deter continuing existence. Not surprisingly, it has been regarded as experimental among most urological surgeons. Although it is not beyond controversy, penile arterial revascularization surgery has been studied by several teams and supports its utility in young males suffered from arterial trauma, and in the older male with localized arterial occlusive disease (Fig. 2A). Endovascular angioplasty with stenting is also recommended as a minimally invasive option for this disease entity.

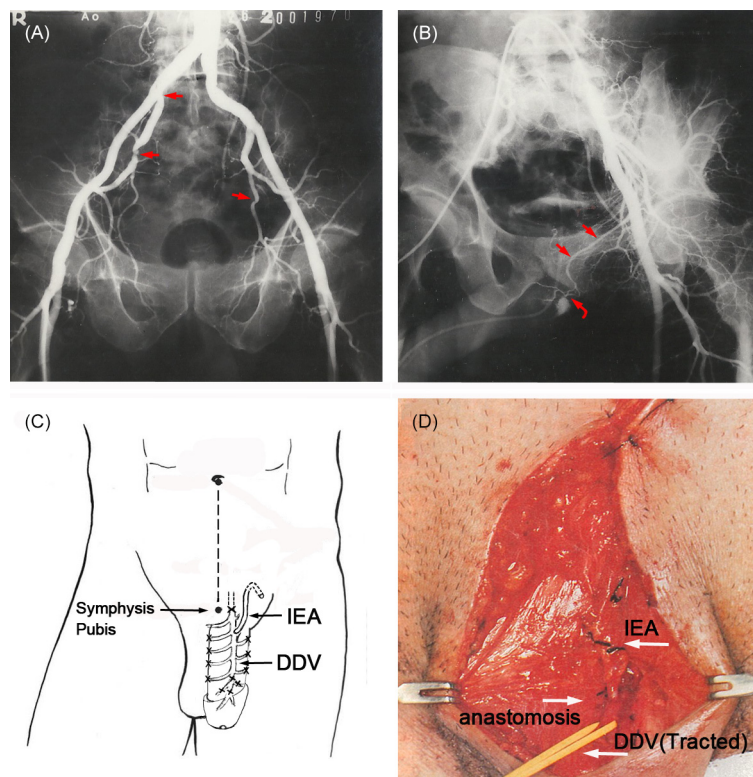


Fig. 2 Selective internal iliac arteriography. (A) An aortogram disclosed marked stenosis (arrows) of internal iliac artery bilaterally. It should not be prognostic for arterial revascularization. (B) A similar phase arteriogram showed a blockage of penile dorsal and cavernosal artery, whereas the spongiosal artery appeared diffusely profuse to the corpus spongiosum after a straddle injury in a 32-year-old man. Is it similar to an arterio-venous malformation? The Foley catheter (arrow) was clamped. The pudendal artery was well demonstrated (arrows), therefore, was this male a good candidate for arterial reconstruction? (C) Illustration of Furlow–Fisher procedure. The inferior epigastric artery is fashioned to the deep dorsal vein which is ligated distally, proximally and lateral locations of the circumflex veins. (D) Photo of an already DDV (yellow traction) arterializations with IEA. The microvascular anastomosis was performed.

Table 1 Outcomes from penile revascularization surgery in recent two decades

<i>Author/publication</i>	<i>Patient No/age(y)</i>	<i>Procedure</i>	<i>Follow-up (months)</i>	<i>Intercourse success rate</i>
Jarow/1996	11	DDVAA ^a	50	92
Lukkarinen/1997	24	Hauri/F–F ^b	N/A ^c	77
Manning/1998	62	Virag/Hauri	41	54
Sarramon/1997		DDVAA	17	
Kawanishi/2004	114	Hauri/F–F ^b	36–60	63
Vardi et al./2004	51	N/A	70.8	85.9
Kayigil et al./2008	52/28.5	F–F ^b	22.1	48
Munarritz et al./2009	71/30.5		34.5	
Kayigil et al./2012	125/43.2	F–F ^b	73.2	63.6– > 92.8

^aDDVAA denotes deep dorsal vein arterilization and arterial reconstruction.

^bF–F is an abbreviation of Furlow–Fisher procedure.

^cN/A stands for non-applicable.

Penile Venous Surgery for Patient with Erectile Dysfunction

Conventional Penile Venous Ligation Surgery

In 1873, the Italian Francesco Parona injected varicosity dorsal penile vein of an impotent young patient with hypertonic saline in order to cause sclerosis and reduce the excessive venous outflow by this. Subsequently, surgical dorsal vein ligation or resection was practiced by several American doctors, e.g. Henry Raymond and James Duncan each in 1895, Joe Wooten in 1902 and mainly Frank Lydston in 1908, who reported on 100 resections. Beginning in the 1930's Oswald Swinney Lowsley combined simple dorsal vein plication with a surgically more advanced perineal crural technique in which he plicated the bulbocavernosus and ischiocavernosus muscles with several mattress sutures. Penile venous surgery (PVS), however, remained unpopular until 1980's when the majority of patients with organic erectile dysfunction were subject to veno-occlusive dysfunction (VOD), the procedure took place and prevailed accordingly. The approach was expanded from initial procedures involving single vessel ligation of the deep dorsal vein to more elaborate techniques in which excision of the deep dorsal vein, cavernous vein, as well as the crural vein were described. It appears that the offending veins of VOD are merely a single DDV with tributaries, additionally crural veins and cavernous veins which drain the penile hilum.

Importantly, though anatomically the penis is in the realms of urology, the delicate microsurgery is beyond the traditional urology training, which may result in an inadequate skill level leading to just a single ligation at the expense of electrocautery influence. Not surprisingly penile venous ligation has been almost abandoned because the consensus on this type of treatment for erectile dysfunction (ED) is short term success of 1 to 2 years, without a sustainable long-term outcome. In addition to the lack of sustained functional improvement, irreversible deformity and permanent numbness of the penis after surgery were frequently noted and considered to be unacceptable complications. Failure to achieve a long-term merit for ED and the seemingly, unavoidable, adverse side effects have discouraged many surgeons from applying venous surgery for patients with ED. However, irreversible numbness results from nerve damage, and penile deformity is a result of either fibro-skeleton alteration or loss of extensibility and sliding capability of layered tissues from electrocoagulation-induced fibrosis. Should then neither nervous nor fibrous tissues not be innocent if PVS is exclusively targeting on venous structures? Should the venous removal be sufficient if all conventional penile venous anatomy has exclusively depicted a single DDV and tribute veins nearby the penile hilum?

Consensus of Penile Vascular Surgery

Penile arterial revascularization is considered to be unproven and controversial. In 1996, it was the first consensus reached when the AUA (American Urological Association) in its guidelines considered penile venous and arterial surgery in men with arteriosclerotic disease as investigational and should be performed only in a research setting with long-term follow-up available. The proceedings of the First Paris International Consultation on Erectile Dysfunction in 1999, the second in 2003 and the third in 2009 were in tandem with the AUA guidelines. Similarly, in 2005, the AUA Update on the Management of ED, and the ISSM's Standard Practice in Sexual Medicine textbook maintained the consensus. However, it may deserve reappraisal after a review of arterial reconstructions reported after 2004 (Table 1), including Kawanishi and Vardi in 2004, Kayigil in 2008, Munarritz in 2009 and Kayigil in 2012. Interestingly, researchers unexceptionally report positive long term outcomes when both penile arterial and venous reconstruction are incorporated, suggesting arterial reconstruction alone is not sufficient to long term restoration of ED.

For investigating the vascular contribution exclusively, hemodynamic studies have been performed on both fresh and defrosted human male cadavers since 2001 to 2013. In each case, a rigid erection was unexceptionally attainable under very low infusion rate following venous removal. This clearly has significant ramifications in relation to vascular reconstruction and its role in treating ED patients. Albeit there are no comparative prospective randomized studies assessing outcome of penile revascularization surgery for arteriogenic ED. Based on the evidence in the literature, this surgery may be offered to men below the age of 55 who are

non-smokers, non-diabetic, show no evidence of venous leakage, and demonstrate an isolated stenosis of the internal pudendal artery in particularly to young patients who have sustained focal arterial injury due to trauma. Regarding penile venous surgery for patients with VOD, it is rejected by most medical community due to disappointing outcomes and unavoidable complication such as permanent penile numbness and penile deformity. Although, use of electrocautery is routine in surgery, it is a major avoidable hazardous in penile venous surgery while penile sinusoids are too delicate to sustain a back fire. The second reason for unsuccessful-conventional PVS is incomplete removal of offending veins for VOD if the PVS refers to the conventional penile venous anatomy. Both residual veins and electro-cautery effects are readily demonstrated in those patients whom underwent PVS somewhere internationally since 2001.

Chronological Development of a Refined Penile Venous Stripping

The advancements in our understanding of penile anatomy, in our surgical instruments and our surgical techniques have resulted in our performing on an ambulatory basis PVS on over 3000 men since 1986 (Table 2). After microsurgery drill on rat and human cadaveric dissection of the entire penile structure in 1985, neither a Bovie nor a sucker has been used with our penile venous stripping techniques. In 1986, we introduced penile venous ligation of the proximal DDV in an initial study of 8 males. This simple procedure was replaced by venectomy very soon thereafter and then by venous stripping which was based on the single DDV conventional penile venous anatomy until 1999. The rate of successful intercourse improved from 50% to 91% in the past decade.

During 1999, 35 patients underwent repeat cavernosography because of the gradual decrease in the erectile capability that occurred in a period of 6 months to 7 years postoperatively. Imaging demonstrated some excessive veins - cavernosal and para-arterial veins, which were further confirmed in cadaveric dissections. By then, the revolutionary edition of erection-related veins in the human penis was regarded as a blueprint for venous stripping surgery. The latest method of penile venous reconstructive surgery is based on a circumferential incision plus a median pubic longitudinal approach via a specific key instrument with an acupuncture assisted local anesthesia on an ambulatory basis. Since the penile venous surgery is exclusively directed at the veins, damage to non-venous soft tissues has been avoided. Tissues like nerve, artery, and lymphatic vessels are spared. Since June 1988, we have performed the penile venous stripping procedure as an outpatient surgery under local anesthesia. Neither a Bovie nor a suction apparatus has been required in the entire procedure, resulting in minimal or no injury to any tissue other than the veins. The incision is small and delicate.

The latest refined penile venous stripping

In medical history, it is rare to encounter a surgical procedure like venous surgery for restoring erectile function that has sustained such an extended period of disrepute. For over a century, the merit for conducting penile venous surgery to treat ED has never been firmly established. It was believed to be indicated only for <1% of patients with ED secondary to veno-occlusive dysfunction (VOD). Recent studies, however, show that VOD may be more prevalent in patients with ED, and even in those with ED ascribed to penile arterial insufficiency. Thus, the prevalence of VOD may be greater than expected suggesting that a larger proportion of

Table 2 Chronological refinement in penile venous stripping surgery methods since 1986

Methods	No.	Age (Year)	Time period	Op. time (hours)	IR* (%)	SR* (%)	Follow-up period (years)	Anatomy blueprint
Ligation	8	22–58	Jun. 1986 Aug. 1987 Sep. 1986	0.5–2.0	<50.0	0.0	5.0–17.0	Multiple ligation of single DDV*
Stripping	23	19–68	May 1987 Jun. 1987	2.0–5.0	80.0	52.5	5.0–32.0	Venous stripping as much as possible
Stripping	245	19–83	April 1991 May 1992	2.2–3.1	67.8	NA	6.0–32.0	Venous stripping under local anesthesia since 1988
Stripping	1207	22–82	Aug. 1997 Sep. 1997	2–3	69.7	57.6	8.0–32.0	Single DDV* with its branches
Stripping	615	23–83	July 2000 Aug. 2000	2–5	85.0	64.6	8.0–32.0	IIEF* available since 1998
Stripping	378	19–81	Nov. 2003 Jan. 2004	2.1–5.0	90.4	76.6	5.1–8.2	Suspected penile venous anatomy
Stripping	235	20–91	Jan. 2009 Feb. 2009	2.1–6.2	90.8	77.8		<i>Newfound penile venous anatomy</i>
Stripping	103		Jan. 2011 Feb. 2011	4.2–8.0	88.7	68.7		DDV*, CV* & PAV*
Ultimate	283	20–75	Aug. 2016	2.1–7.5	95.7	85.3		Without well-trained assistant
Total	3097	19–91	1986–2015	0.5–8.0	<50–95.7	0–85.3	5–32.0	Unpublished data, ultimate method of USPTO* patent

*The DDV, CV, PAV, IIEF, USPTO, IR & SR are an abbreviation for the deep dorsal vein, cavernosal veins, para-arterial veins, international index of erectile function, The United States Patent and Trademark Office, improvement rate and satisfaction rate respectively.

patients with ED may be suitable for venous surgery. In contrast to the feared potential recurrence of conventional penile venous surgery, <25% of our 3000 patients on whom the operation was performed have complained of a recurrence. The overall outcomes are commensurate with method refinement over several decades.

The most advanced method of penile venous stripping—as depicted on the United States Patent and Trademark Office (USPTO) website—for treating impotence resulting from veno-occlusive dysfunction. A circumferential incision was first made while a circumcision was completed if required (Fig. 3). A more extensive degloving of those tissue layers superficial to the Colles' fascia was then undertaken. A milking manipulation is helpful to enhance the visibility of the DDV which is stripped proximally with a pull through technique via an opening made on the buck's fascia from opening to opening on the Buck's fascia until the penile base using a 6-0 nylon suture. Likewise the paired cavernosal veins (CVs) were stripped. For accessing the deep seated venous vessels a median longitudinal pubic incision skin was used to continue the venous stripping. The paired proximal stumps of the DDV and the CVs served as a guide, and the DDV was then firstly thoroughly stripped and then ligated with a help of 85° hemostat as far as the infra-pubic angle. Similarly, the deep-seated CVs are managed. The paired para-arterial veins (PAVs) which sandwich the dorsal arteries bilaterally were meticulously ligated rather than stripped. Smaller veins between the tunica albuginea and the Buck's fascia were identified by squeezing the sinusoids of the corpora cavernosa. The wound was closed with 5-0 chromic catgut and 6-0 nylon sutures. A compression dressing was placed such that it encircled the penile shaft. Follow-up cavernosograms is routinely undertaken immediately postoperatively or intraoperatively if required (Fig. 4). Postoperative cavernosograms categorically confirmed that the CCs are an ideal chamber for intracorporeal fluid retention in all patients, particularly, the penile crura was unexceptionally stronger radiopaque than that of femoral cortex (Fig. 4D, E & F vs. A & C).

Despite those drawbacks of conventional penile venous surgery, our penile venous stripping has been developed with the inspiration of patients' positive response in tandem with the advances of penile tunical and venous anatomy associated with the erection

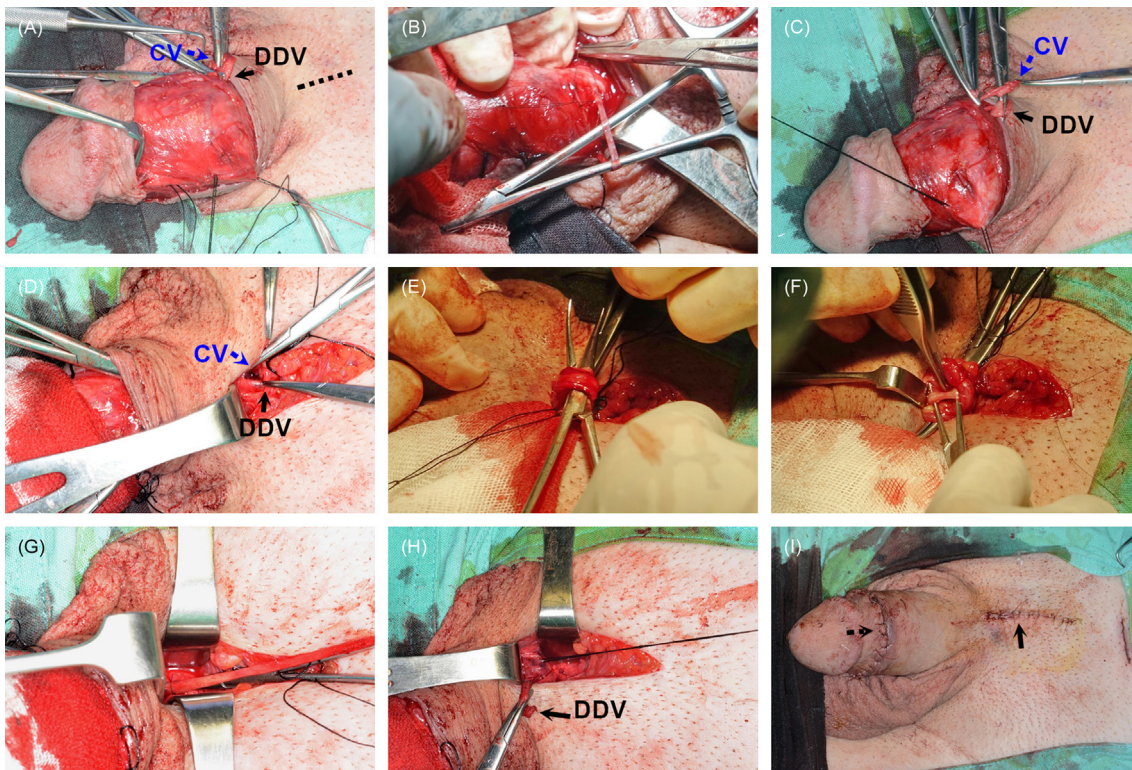


Fig. 3 Photos of the ongoing penile venous stripping process. (A) A longitudinal pubic incision was marked (dotted line) and preputial approach was performed to access the offensive veins, DDV (arrow) and CV (dotted arrow), which were managed and clamped with hemostat respectively. Note that tag suture was made at the junction between the corpus spongiosum and corpora cavernosa for fixation of the circumflex veins. The visibility of the deep dorsal vein (DDV) was enhanced by employing a milking manipulation, mimicking a squeeze applied to a balloon, of the corpora cavernosa. (B) The circumflex vein was managed while each emissary vein was ligated with 6-0 nylon closest to the outer tunical layer. Eventually, the venous channel was free from blood. (C) The venous plexus of DDV (arrow) and CV (dotted arrow) was stripped by 4–5 pull-through maneuvers until the pubic level encountered. (D) The venous plexus was passed underneath via a public longitudinal approach to relay the penile venous stripping. (E) The venous stump was relayed smoothly. Note the DDV (arrow in D) and CV (dotted arrow in D) were managed in an entire group in this case. (F) The first branch of the DDV was managed. (G) 7–9 big branches of DDV were managed one by one until the infra-pubic angle was met. (H) The DDV (arrow) was managed completely, then the CV was likewise managed while 5–8 branches were firmly ligated with 6-0 nylon. (I) Finally, the preputial (dotted arrow) and longitudinal pubic (arrow) wounds were fashioned with 5-0 chromic sutures respectively. Note that neither an electrocautery nor a suction apparatus was required in the entire procedures.

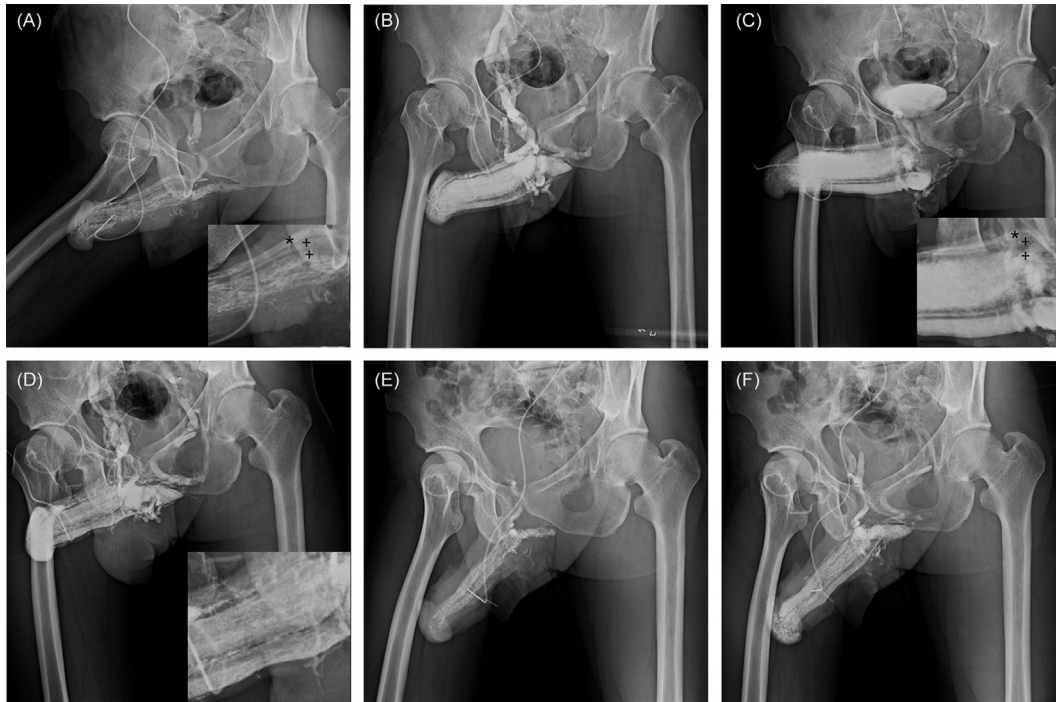


Fig. 4 Dural pharmaco-cavernosography of veno-occlusive dysfunction and cavernosographic evidence of erection restoration. (A) The first cavernosogram was obtained while a 10-mL diluted iohexol solution was intracavernously injected, resulting in the immediate opacification of the DDV (*asterisk*), CV (*cross*) and then preprostatic plexus. Note the volcano-like eruption of emissary veins. (B) A cavernosogram was obtained after further injection of the 10-mL solution, while the drainage veins were remarkable at the penile hilum. (C) A pharmacocavernosograms was obtained 20–30 min after 20 μ g of prostaglandin E1 (test) was intracavernously injected. The bulking of drainage veins were relatively prominent despite a rigid erection attained. Thus a veno-occlusive dysfunction was documented. (D) An intraoperative cavernosogram disclosed most offensive veins was stripped but an accessory cavernosal veins and several circumflex veins were left. (E) An immediate postoperative cavernosogram showed the penile venous stripping was complete. (F) Further intracavernous injection confirms an ideal milieu to apply intracavernosal fluid retention. Note D, E and F were postoperative ones in which the penile crura was unexceptionally stronger radiopaque than that of femoral cortex.

mechanism since 1986. We applied chronologically refined stripping techniques in a large patient population under acupuncture-assisted local anesthesia on an ambulatory basis. We consistently encounter patients seeking this treatment because of poor functional outcomes and unexpected adverse effects of prior penile vascular interventions internationally. Although currently we recommend this treatment only to patients who are nonresponders to phosphodiesterase-5 (PDE-5) inhibitors, we always fail to decline this sort of patient. Penile venous surgery has likely been innocently condemned; the abandonment would be more appropriate if its justification shifted from the surgery itself to the method of surgical manipulation. We heretofore suggest its ban should be lifted to help males that suffer from ED. Surely its efficacy and safety warrant well-structured, randomized, and controlled research by surgeons who know the anatomy well and are well-equipped with microvascular surgery skill without the necessity of electrocautery.

Varied Endovascular Intervention for Patient with Erectile Dysfunction

Penile endovascular interventions, both angioplasty and venous embolization, aim to treat VOD or arterial insufficiency via limiting the outflow of blood or increasing arterial inflow in the penis, respectively. It may be technically easy for physicians to implant, owing to simplicity and reproducibility, and it is a minor procedure for patient because of low morbidity. Consequently, pudendal stents and penile venous embolization with or without sclerotherapy have been repeatedly introduced for treating VOD and arterial insufficiency correspondingly. It is effective and safe in some vascular diseases. On the elusive ED issue, what great news to patients if endovascular treatment of vasculogenic erectile dysfunction works. Among options for venous treatment, penile venous embolization is an old paradigm newly revisited by the intervention radiologist. It is commonly believed to be minimally invasive and safe.

Refractory ED has prompted five international males to seeking our penile venous stripping since late 2012 despite coil venous embolization with sclerotherapy was performed elsewhere. Stunningly, uncontrolled coils not only lodged in pulmonary artery, but also made a right cardiac ventricle perforation (Fig. 5A) which is confirmed by a contrast computerized tomography. An acute chest pain occurred in a 25-year-old male resulting from the uncontrolled coils traveling to right pulmonary artery just in seven days after

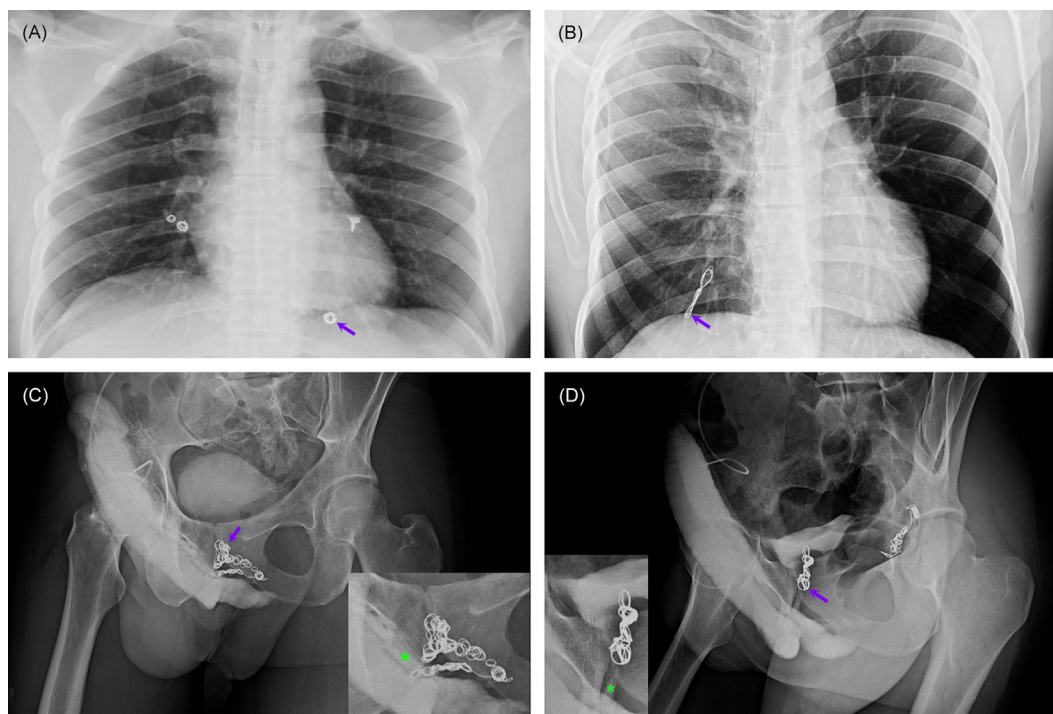


Fig. 5 Imaging of patients received prior coil embolization and sclerotherapy elsewhere internationally. (A) Coils migrated to lodge in left and right pulmonary artery respectively, and one coil perforated the right cardiac ventricle (*purple arrow*) which is further confirmed by a contrast computerized tomography. (B) The inserted coils (*purple arrow*) migrated to right lower pulmonary artery, which caused an acute chest pain. (C) An insufficient rigidity of erectile penis suffered a 25-year-old patient albeit dozens of coils (*purple arrow*) were inserted in an attempt to limit the penile venous drainage. Note the venous buckle (*green asterisk*) was remarked. (D) The preprostatic veins (*green asterisk*) were blocked and engorged despite many coils were inserted (*purple arrow*).

coils inserted (**Fig. 5B**). On limiting the VOD drainage, an insufficiently erectile rigidity resulting from blockage of the deep dorsal vein and cavernosal veins distal to the preprostatic plexus (**Fig. 5C**) and a consistently unacceptable erection quality resulting from failing venous blockage was noted (**Fig. 5D**). Therefore, the efficacy of endovascular therapy is not beyond controversy, and its safety may not be sustainable. This implies that a penile venous embolization coil is at risk of migrating throughout the body once it is inserted in the main common drainage channel of corpora cavernosa.

Accordingly, the efficacy of venous embolization warrants research because it just treats vascular channels outside the corpora cavernosa. Pulmonary migration of coils inserted for treatment of erectile dysfunction caused by venous leakage was reported in 1993. This can lead to symptoms such as acute chest pain and chronic cough. An uncontrolled coil is capable of traveling more than just the pulmonary arteries, and may also perforate the ventricular wall. The tension of embolization coils varies by manufacturer, as does the coils' penetration ability. So potentially severe morbidity of coils, though rare, should be borne in mind, and patients should be informed of this possibility in choosing coil embolization for treating ED resulting from veno-occlusive dysfunction.

The past 3 decades have seen an explosion of new technologies, devices, gadgets and application of state-of-the-art tools to medicine and surgery professionals. Penile vascular reconstructive surgery and endovascular intervention are significantly beneficial from this trend. It ought to be promising in the near future if and only if the sound method is used via valid instruments and appropriate technology.

Summary

New insights into penile tunical and venous anatomy, and erection physiology have been made in recent decades. They have underpinned penile morphological reconstructive, penile endovascular intervention as well as penile vascular surgery. Although vascular reconstructive surgery is still regarded as experimental, anatomical-based techniques of vascular reconstruction should be reappraised. Arterial reconstruction has been shown to be beneficial to young healthy males who sustain trauma. Older healthy ED males are more likely to benefit if the vascular surgery includes both arterial revascularization and veno-arterIALIZATION. The reproducible refined penile venous stripping procedure which has been developed and refined over several decades is showing promise as a viable solution for veno-occlusive dysfunction across all ages.

Acknowledgements

We wish to thank Directors Hsiu-Chen Lu and Chih-Cheng Lu for illustrations, Mrs. Hsin-Chen Wu and Ying-Hui Chen for their preparations of photos for this manuscript.

Further Reading

- Banya, Y., et al. (1989). Two circulatory routes within the human corpus cavernosum penis: A scanning electron microscopic study of corrosion casts. *The Journal of Urology*, 142(3), 879–883.
- Chen, S. C., et al. (2005). The progression of the penile vein: Could it be recurrent? *Journal of Andrology*, 26(1), 56–63.
- Gratzke, C., et al. (2005). Anatomy, physiology, and pathophysiology of erectile dysfunction. *The Journal of Sexual Medicine*, 7(1 Pt 2), 445–475.
- Halliday, D. (1997). Pascal's principle, fluids. In D. Halliday, R. Resnick, & J. Walker (Eds.), *Fundamentals of physics* (pp. 355–356). New York: John Wiley.
- Hsieh, C. H., et al. (2005). Penile veins play a pivotal role in erection: The hemodynamic evidence. *International Journal of Andrology*, 28(2), 88–92.
- Hsu, G. L. (2011). Physiological approach to penile venous stripping surgical procedure for patients with erectile dysfunction. Google patents; US 8,240,313 B2, <http://www.google.com/patents/US20110271966>.
- Hsu, G. L., et al. (1992). The three-dimensional structure of the human tunica albuginea: Anatomical and ultrastructural level. *International Journal of Impotence Research*, 4, 117–129.
- Hsu, G. L., et al. (2003). Penile venous anatomy: An additional description and its clinical implication. *Journal of Andrology*, 24(6), 921–927.
- Hsu, G. L., et al. (2012). Penile veins are the principal component in erectile rigidity: A study of penile venous stripping on defrosted human cadavers. *Journal of Andrology*, 33(6), 1176–1185.
- Hublin, J. J., et al. (2017). New fossils from Jebel Irhoud, Morocco and the pan-African origin of Homo Sapiens. *Nature*, 546(7657), 289–292.
- Kayöglü, O., Okulu, E., Aldemir, M., & Onen, E. (2012). Penile revascularization in vasculogenic erectile dysfunction (ED): Long-term follow-up. *BJU International*, 109(1), 109–115.
- Kawanishi, Y., Kimura, K., Nakanishi, R., Kojima, K., & Numata, A. (2004). Penile revascularization surgery for arteriogenic erectile dysfunction: The long-term efficacy rate calculated by survival analysis. *BJU International*, 94(3), 361–368.
- Montague, D. K., et al. (1996). Clinical guidelines panel on erectile dysfunction: Summary report on the treatment of organic erectile dysfunction. *The Journal of Urology*, 156, 2007–2011.
- Montorsi, F., Sarteschi, M., Maga, T., Guazzoni, G., Fabris, G. F., & Rigatti, P. (1998). Functional anatomy of cavernous helicine arterioles in potent subjects. *The Journal of Urology*, 159(3), 808–810.
- Munarriz, R., Uberoi, J., Fantini, G., Martinez, D., & Lee, C. (2009). Microvascular arterial bypass surgery: Long-term outcomes using validated instruments. *The Journal of Urology*, 182(8), 643–648.
- Rowe, C., Ganick, S., Munarriz, R., et al. (2010). Traumatic vasculogenic erectile dysfunction: Role of penile microarterial bypass surgery. *Current Urology Reports*, 11(6), 427–431.
- Sáenz de Tejada, I., et al. (2004). Physiology of erectile function. *The Journal of Sexual Medicine*, 1(3), 254–265.
- Vardi, Y., et al. (2004). Evaluation of penile revascularization for erectile dysfunction: A 10-year follow-up. *International Journal of Impotence Research*, 16(2), 181–186.

Surgical Correction for Peyronie's Disease and Anatomic Abnormalities of the Penis

M Ryan Farrell, Peter Tsambarlis, and Laurence A Levine, Rush University Medical Center, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Background

Initial descriptions of a disorder of penile curvature were first noted in the 13th century by Borgognoni and Saliceto followed in 1561 by Fallopius and Vesalius. Later, in 1743, Francois de la Peyronie further described and treated a condition involving rosary beads of scar tissue along the dorsal aspect of the penis with associated dorsal curvature during erections that now carries his name—Peyronie's disease (PD) (De la Peyronie, 1743).

PD is regarded as an acquired wound healing disorder of the tunica albuginea of the corpora cavernosa that results in the formation of a fibrotic, inelastic scar (Smith, 1966). The scar tissue causes penile deformity that commonly includes curvature, shortening, indentation, hinge effect, painful erections and erectile dysfunction (ED). The perceived narrowing and loss of length can have a substantial psychological impact leading to depressive symptoms and negatively impacting relationships (Rosen et al., 2008; Smith et al., 2008).

Epidemiology

The incidence of PD has been reported in 3%–9% of men (Schwarzer et al., 2001; Mulhall et al., 2004). However, given the psychosocial implications of this condition and the resulting reluctance of patients to report penile deformity and seek treatment, the actual incidence of PD may, in fact, be higher.

Men in their 5th decade of life have the highest prevalence of PD. Further, the incidence of PD increases with age as demonstrated by a large European survey by Schwarzer et al. (2001). Over 4000 men responded and 1.5% of men age 30–39 years reported an area of penile induration, which increased to 6.5% in men over 70 years old. It is speculated that the increased incidence of PD with age is the result of contributions from comorbid conditions that also have increased prevalence with age and include diabetes, hypertension, and hyperlipidemia.

Etiology and Pathophysiology

The etiology of PD has not been completely elucidated. PD is thought to be initiated by penile trauma or microtrauma that causes subtunical bleeding. In genetically susceptible individuals, this results in a localized inflammatory response that includes tumor growth factor beta. The subsequent cascade of cytokines and free radicals, cell cycle dysregulation, genotypic changes, and biological transformation of the tunica albuginea leads to fibronectin and collagen deposition in the extracellular matrix and ultimately scar formation. The resulting inelastic segment of the tunica albuginea leads to the characteristic penile deformity of PD (El-Sakka et al., 1997; Mulhall et al., 2002; Nachtsheim and Rearden, 1996; Schiavino et al., 1997; Cantini et al., 2008; Ryu et al., 2009). While 13% of patients may experience some spontaneous regression of PD, the lack of normal scar remodeling results in 47% of men experiencing no change in deformity over time, and 40% experiencing disease progression (Gelbard et al., 1990). One important characteristic of this wound healing disorder is that the scar does not resolve due to absent remodeling. This has been shown to be associated with inactive metalloproteinases with elevated tissue inhibitors of matrix metalloproteinase (TIMP) (Del Carlo et al., 2008).

Most men with PD receive conservative therapy as initial treatment. Various modalities exist including oral medication, topical agents, intralesional injections, electromotive drug administration, extracorporeal shock wave therapy, and penile traction (Gholami et al., 2003; Larsen and Levine, 2012). However, when the disease is in the stable phase, surgery remains the gold standard modality, as it offers the most reliable and definitive treatment for PD.

Other Abnormalities of the Penis

Chordee represents a pathology, which is unique from PD. For the sake of this discussion “chordee” will be the term used to describe congenital curvature of the penis, chordee without hypospadias, and monosymptomatic curvature. As such, this discussion is also referring to curvature of the penis with the urethral meatus in an orthotopic location. Critical to this discussion is the ability to differentiate chordee from PD. At a simplistic level, chordee is congenital and, by definition, PD is acquired. The etiology of chordee is believed to be a dysplasia of the penile corporal tunica albuginea which reduces elasticity around the dysplastic area. When the affected male obtains an erection the dysplastic portion of the tunica albuginea does not expand to the same degree as the surrounding normal tissue. This results in a curve towards the dysplastic area (Nyirády et al., 2008). This is distinct from the fibrotic scarring process described above.

The classic presentation is during puberty when the affected male becomes more aware of the aesthetic appearance of his erection. A subset of these men have a curve which is severe enough to impair or prevent successful penetrative intercourse. These are the men who warrant evaluation for potential surgical intervention. Nesbit was the first to describe surgical correction of chordee, which involved excision of ellipses of tunic on the convex side of the curvature followed by transverse suturing (Nesbit, 1965). The Heineke-Mikulicz technique was a modification where longitudinal incisions are made in the corpora on the convex side followed by transverse suturing (Yachia, 1990). The authors, however, advocate for a tunica albuginea plication (TAP), which will be described in greater detail in the following sections. Other congenital pediatric abnormalities of the penis such as extrophy, penile duplication, and micropenis are beyond the scope of this article.

Preoperative Evaluation

A thorough preoperative evaluation is necessary for surgical planning and includes a patient history, physical exam, and diagnostic imaging. A detailed history should include questions regarding time of onset, preceding trauma, pain, curvature, shortening, hinge effect, indentation, and erectile function. Between 8% and 40% of men with PD, 20% in our experience, recall a traumatic event that preceded the onset of curvature (Tal et al., 2012; Jarow and Lowe, 1997; Chung et al., 2012). Patients should be asked to subjectively describe their erection quality on a scale of 0–10, with 7 being defined as an erection that is adequate for penetration with manual assistance. Erect penile curvature should be assessed from the patient perspective to evaluate individual preoperative perceptions. It has been shown that only 20% of patients accurately describe their curvature, with 56% overestimating and 26% underestimating curvature by a mean of 20 degrees from the objective measurement (Bacal et al., 2009). Further, patient described penile shortening should be investigated. Shortening is reported in 70% of patients and can range from 1 to 10 cm in length lost, making this particularly distressing for patients (Rosen et al., 2008; Smith et al., 2008).

An understanding of erectile function prior to and following the development of PD is critical for operative planning. ED may be related to the pathophysiology of PD itself, the psychological effect of PD, or the result of pre-existing comorbidities including diabetes, peripheral vascular disease, and smoking (Rosen et al., 2008; Levine and Coogan, 1996).

The physical exam is directed at the penis. The plaque should be palpated and its location noted. Stretched flaccid penile length is measured over the dorsal aspect of the penis from the pubis to the corona (Wessells et al., 1996). An evaluation of stretched penile length can be useful to track progression of disease or for comparison after treatment.

Diagnostic imaging with dynamic duplex ultrasound with vasoactive injection of papaverine, prostaglandin E1, or trimix is essential in order to determine appropriate management. At this point, several characteristics can be objectively assessed including erect deformity, curvature, plaque calcification, indentation, hinge effect, and erectile response (Ralph et al., 2010).

Indications for operative intervention for men with PD are based on expert opinion and include: stable disease—at least 1 year from onset and at least 6 months with stable deformity, inability to engage in penetrative intercourse due to deformity and/or inadequate rigidity, failure of non-surgical management, extensive plaque calcification, and patient desire for the most rapid and definitive corrective modality following disease stabilization. Penile pain is a relative contraindication to surgery, with the exception of pain resulting from curvature-associated torque on the erect penis (Ralph et al., 2010).

There is a well established algorithm for the operative management of PD (Levine and Larsen, 2013; Levine and Lenting, 1997). For men with adequate rigidity for penetrative intercourse with or without pharmacotherapy, tunica albuginea plication (TAP) versus plaque incision or excision and grafting (PEG) can be considered. TAP is most appropriate for patients with curvature < 60–70 degrees, no hourglass or hinge effect, and anticipated loss of erect length < 20%. In contrast, PEG can be utilized for individuals with curvature > 60–70 degrees, extensive plaque calcification, and/or presence of hinge effect resulting in an unstable erection. Importantly, candidates for PEG must have strong preoperative erections, as there is an increased risk of postoperative ED that should be discussed with the patient prior to proceeding with the operation.

Men with ED who do not respond to pharmacotherapy should be considered for placement of a penile prosthesis in an effort to improve rigidity and curvature. Should there be residual curvature after placement of the prosthesis, manual modeling can be attempted (Wilson and Delk, 1994). If a curve of > 30 degrees remains, an incision of the tunica albuginea can be performed over the point of maximum curvature. If the incision results in a defect > 2 cm, a biograft should be placed, which prevents herniation of the prosthesis and prevents cicatrix contracture (Levine and Dimitriou, 2000).

Preoperative counseling is necessary to establish patient expectations for a successful surgery. The possibility of persistent curvature, decreased rigidity, loss of penile length and diminished sensation should be discussed. A successful straightening procedure involves establishing a functionally straight penis, which is defined as a residual deformity of 20 degrees or less (Ralph et al., 2010). Decreased rigidity occurs in up to 50% of men and may require treatment with PDE5 inhibitors, injection therapy, vacuum devices, or subsequent placement of a penile prosthesis. The occurrence of decreased postoperative rigidity depends on preoperative erection quality. Overall, > 5% of patients experience diminished postoperative rigidity, while among patients with suboptimal preoperative rigidity, the incidence increases to > 30% (Taylor and Levine, 2007). Because penile shortening is already a particularly troublesome occurrence for patients with PD, a discussion involving the possibility of further loss of length from corrective surgery is important. Loss of length is more likely with TAP than with PEG. Approximately 20% of men report decreased penile sensation following surgery. Hyperesthesia or diminished sensation in the acute postoperative period generally resolves in 6–12 months (Taylor and Levine, 2008; Ralph et al., 2010).

Surgical Management of Peyronie's Disease

Shortening Procedures

Technique

The principle of TAP is to shorten the longer or convex side of the tunica albuginea in order to achieve the length of the shorter or concave side. After general anesthesia is administered, the lower abdomen, genitalia and perineum is shaved, prepped, and draped creating a sterile field. IV antibiotics are administered in accordance with American Urological Association guidelines and generally involve use of a 1st generation cephalosporin or alternatively clindamycin (Wolf et al., 2012). The stretched flaccid penile length is measured from the pubis to the corona and an artificial erection is induced with injection of saline or a vasoactive agent into the corpora cavernosa. The erect penis is then evaluated noting degree of curvature, location of point of maximum curvature, as well as narrowing, and hinge effect if present. A circumferential, circumcising incision is made 1.5–2.0 cm proximal to the corona and the penis is degloved allowing for exposure of Buck's fascia along the entire length of the shaft. Buck's fascia is then elevated from the tunica albuginea in the area of anticipated plication on the convex side.

At this point, a number of plication techniques are available to the surgeon. The Nesbit procedure involves excision of a transverse elliptical wedge of tunica albuginea on the contralateral side of the point of maximum curvature. The defect is closed transversely with permanent suture (Andrews et al., 1999). Alternatively, the Yachia procedure can be utilized, which incorporates the Heineke–Mikulicz technique (Yachia, 1990, 1993). A full thickness, vertical, 0.5–1.5 cm incision is made through the tunica albuginea contralateral to the point of maximum curvature and closed transversely. The 16 dot procedure involves plication with Lembert-type permanent suture without incising the tunica albuginea (Brant et al., 2007). Finally, the Duckett–Baskin technique as modified by Levine may be utilized (Baskin and Duckett, 1994; Levine, 2006). This involves making two parallel transverse incisions opposite the point of maximum curvature 1.0–1.5 cm in length and 0.5–1.0 cm apart, depending on the desired degree of shortening. The incisions are made through the outer longitudinal fibers of the tunica albuginea leaving the inner circular fibers intact and the tissue between incisions is thinned. The goal is to reduce the redundant tunic between the incisions that occurs after plication, while reducing the likelihood of postoperative ED. Plication is performed transversely using a single permanent suture in an inverted mattress fashion and Lembert stitches with absorbable suture can be used to further reinforce the plication. The authors advocate for use of a limited number of permanent sutures, as we have found that there are fewer palpable nodules with no increased risk of recurrent curvature.

Once plication has been completed and hemostasis has been achieved, an artificial erection can be induced to ensure the deformity is adequately corrected. Buck's fascia is then closed. The dartos fascia and superficial tissues are closed along the subcoronal incision. We utilize ropivacaine to perform a penile block. Xeroform gauze is placed over the incision and a Coban (3M, Maplewood, MN) wrap is placed from distal to proximal over the penile shaft for light compression. Fluff dressing is placed over the penis and scrotum and covered with light compression underwear. The patient is often discharged on the same day as surgery. Dressings are removed on postoperative day 3.

Outcomes

Successful straightening following TAP can be expected in 29%–93% of patients, with patient satisfaction in 65%–96%. Success appears to be similar across the various plication techniques, however, shortening is not corrected. Loss of length has been reported in 18%–100% of patients following TAP and is more likely in men with ventral curvature that is > 60 degrees (Greenfield et al., 2006). Additionally, hinge effect is not addressed. Instead, performing TAP in patients with hinge effect may result in an unstable penis.

Complications with TAP include ED in 3%–38%, recurrent curvature of > 30 degrees in 8%–20%, penile narrowing or induration in 0%–17%, suture granuloma or bothersome knots in 0%–34%, diminished penile or glans sensation in 0%–48%, hematoma 0%–9%, phimosis in 0%–5%, and urethral injury in 0%–1% (Levine and Larsen, 2013; Tornehl and Carson, 2004).

Lengthening Procedures

Technique

PEG is indicated for men with strong preoperative erections and greater disease complexity including greater degree of curvature (> 60–70 degrees), presence of hinge effect, significant narrowing, or extensive plaque calcification. The procedure is initiated after administration of general anesthesia, along with shaving, preparing, and sterile draping of the lower abdomen, genitalia, and perineum. IV antibiotics are administered as above with the TAP procedure according to American Urological Association guidelines (Wolf et al., 2012). The stretched flaccid penile length is measured and the erect penis is evaluated following intracavernosal injection of a vasodilating agent (PGE1 or combination alprostadil, papaverine, and phentolamine). A circumcising incision is made and the penis is degloved.

In cases of dorsal curvature, Buck's fascia is elevated off the dorsolateral aspect of the penile shaft to expose the point of maximum curvature. The neurovascular bundle is then elevated off the dorsal aspect of the penile shaft. With the penis fully erect, the point of maximum curvature is again identified and the area of tunica albuginea is excised leaving a square or rectangular defect. The length of the lateral longitudinal defect should be equal on both sides to correct curvature. The corners of the excised tunic are darted radially to prevent cicatrix contracture and stay sutures are placed in each of the corners as well as at the midpoint transversely both proximally and distally. After appropriate sizing, the selected graft material is sutured to the tunica albuginea first via the stay

sutures and then in a running fashion. An artificial erection is again established and significant residual curvature (<30 degrees) may be further corrected via TAP.

Given the concern for erectile dysfunction resulting from excision of large plaques, plaque incision and grafting (PIG) can be utilized as an alternative to PEG. Once Buck's fascia and the dorsal neurovascular bundle are elevated, a relaxing H shaped incision is made to expand the tunica albuginea and a graft is placed (Gelbard, 1995; Lue and El Sakka, 1998). This may be best used when there is no significant hourglass indentation.

Following PEG or PIG, Buck's fascia is closed with running suture on each of the ventrolateral aspects of the penis and the skin is closed. Our approach to penile block and dressing is as described above for the TAP operation.

Graft material

Multiple graft materials have been utilized including vein (Montorsi et al., 2000), synthetic material (Licht and Lewis, 1997), tunica vaginalis (Das, 1980), dura mater (Sampaio et al., 2002), porcine small intestine submucosa (SIS) (Knoll, 2001), and cadaveric human or bovine pericardium (Usta et al., 2003; Egydio et al., 2002; Chun et al., 2001). The optimal graft has properties similar to that of native tunica albuginea including comparable strength, elasticity, thickness, and pliability. Further, it should be inexpensive, easy to manipulate, carry a low risk of transmitting infection, and should elicit minimal tissue reaction (Gur et al., 2011; Kadioglu et al., 2007). Allograft use requires graft harvesting from a second surgical site with associated comorbidities and complications and is used less commonly as a result. Synthetic materials including polytetrafluoroethylene (PTFE, Teflon) and polyethylene terephthalate (PTFE, Dacron) have fallen out of favor due to the risk of transmitting infection, postoperative inflammation, and resulting fibrosis (Devine et al., 1997; Brannigan et al., 1998).

Currently, the most common grafts in use are the processed cadaveric human pericardium (Tutoplast, Coloplast US, Minneapolis, MN) and the porcine SIS (Surgisis ES, Cook Urological, Spencer, IN) (Hellstrom, 1994; Hellstrom and Reddy, 2000; Knoll, 2001; Levine and Estrada, 2003). We prefer human cadaveric pericardium because it holds suture well and is strong, thin, and there are no reports of rejection or infection in over 2 million cases where this graft has been used. The tissue is processed into an acellular matrix, which elicits native tissue growth with minimal inflammatory response (Tornehl and Carson, 2004). Chun et al. reported that 92% of patients were able to achieve an erection rigid enough for successful coitus with or without assistance (Chun et al., 2001). SIS grafts are also processed into an acellular matrix with similar properties to cadaveric pericardium. However, graft contraction can occur leading to recurrent curvature in 37%–57% of cases (Santucci and Barber, 2005; John et al., 2006; Breyer et al., 2007; Kovac and Brock, 2007; Taylor and Levine, 2008). When sizing the graft to the measured defect in the tunica albuginea, cadaveric pericardium should be no more than 10% larger, while the SIS graft should be at least 25% larger.

There are multiple tissue engineered graft materials under investigation including autologous tissue engineered, adipose tissue-derived stem cell seeded SIS, and human acellular matrix grafts (Ma et al., 2012; da Silva et al., 2011; Schultheiss et al., 2004). Tissue engineered grafts may represent the future of lengthening procedures for PD, however, further investigation is required before these grafts can be utilized in clinical practice.

Outcomes

ED is a common complication of lengthening procedures. Often, men with excellent preoperative rigidity are able to maintain spontaneous erectile function postoperatively. However, men with moderate preoperative rigidity may develop ED postoperatively and require pharmacotherapy—men with poor preoperative erections will continue to have poor erections postoperatively. Postoperative ED has been reported at 0%–30% overall (Taylor and Levine, 2008; Cormio et al., 2009). The risk of postoperative ED can be minimized by limiting trauma to the cavernosal tissue, thereby maintaining the veno-occlusive mechanism between the corpora cavernosa, the tunica albuginea, and the graft.

Successful straightening following PIG and PEG has been shown in 63%–100% with patient satisfaction in 41%–96% (Taylor and Levine, 2008). Recurrence depends on the type of graft material that is used and ranges from 0% to 16% (Cormio et al., 2009; Usta et al., 2003). Shortening has been reported in 0%–40% and can be minimized with the use of penile traction devices postoperatively. Finally, diminished penile sensation has been described in 0%–16% and likely results from injury to the dorsal nerve bundle (Usta et al., 2003; Licht and Lewis, 1997; Montorsi et al., 2000).

Postoperative management

Diligent postoperative rehabilitation can optimize surgical outcomes by reducing the risk of further penile shortening, recurrent curvature, and development of ED. Massage and stretch therapy can be started 2 weeks postoperatively. The patient grasps the penis by the glans, places it on gentle stretch, and massages the penile shaft twice daily for 5 minutes. External penile traction devices are recommended as well to limit further penile shortening. Additionally, nightly low dose PDE5 inhibitors beginning 1 week postoperatively induce vasodilation in an effort to reduce contraction and support incorporation of the graft.

Erectile Dysfunction in PD

Concurrent ED in the setting of PD is a clear indication for placement of an inflatable penile prosthesis (Levine and Lenting, 1997; Kendirci and Hellstrom, 2004; Ralph and Minhas, 2004; Mulhall et al., 2005; Levine and Dimitriou, 2000). Multiple straightening maneuvers can be undertaken at the time of prosthesis placement (manual modeling, tunical plication, tunical incision with or without grafting). All patients are prepped via a 10 min betadine scrub followed by a chloraprep (Carefusion, San Diego, CA) finish.

Antibiotics are selected based on the AUA best practice guidelines. If there are no contraindications, vancomycin (15 mg/kg) and gentamicin (5 mg/kg) are used (Wolf et al., 2012). We prefer a penoscrotal incision in the setting of a PD but an infrapubic approach can be used as well. Blunt dissection is carried down to the corpora and the overlying attachments are dissected away. 2-0 PDS (Ethicon, Somerville, New Jersey) stay sutures are then placed into the bilateral corpora. A self retaining retractor is then placed and maintained until all components of the prosthesis are implanted. A 3 cm incision is then made in each corpus cavernosum between the stay sutures. The space is dilated with Brook's dilators (Coloplast, Humlebæk, Denmark) and measured both proximally and distally to determine the appropriately sized cylinders. After being prepared on the back table, the prosthesis is placed into the dilated corpora with the assistance of a Furlow inserter (AMS, Minnetonka, Minnesota). After closing the corporotomies, straightening maneuvers can be undertaken. These will be reviewed in the following section.

After completion of the straightening procedures, the scrotal pump and reservoir are placed. The pump is placed into a midline subdartos pouch in an anterior and dependent position. For placement of the reservoir, the external inguinal ring is identified. The Space of Retzius is entered sharply with the aid of a Jorgenson scissors. The curved scissor tips point away from the vessels, bladder, and bowel (Levine and Larsen, 2014). The space is digitally dilated, and the reservoir is placed. The tubing is connected via the connection system provided by the manufacturer. Finally, the scrotum is closed in layers and then with Dermabond (Ethicon, Somerville, New Jersey). A "mummy wrap" is placed around the penis and the Foley catheter is removed (Henry, 2009).

Manual modeling

Manual modeling is performed after placement of the cylinders and closure of the corporotomies. With the penile prosthesis inflated, shodded clamps are placed on the cylinder tubing. One finger is placed over each corporotomy, and the penis is grasped firmly. Strong gradual pressure is then applied in the direction opposite the curve. It is recommended this be held for 60–90 s, but in practice this can prove quite difficult; 30 s seems to suffice. The curvature is continually reassessed with cylinders inflated and repeated until the residual curve is <30 degrees. If a curve <30 degrees is unattainable with modeling alone, additional maneuvers can be undertaken (Levine et al., 2010).

Tunical incision

A tunical incision should be performed if residual curve after modeling exceeds 30 degrees or there is noticeable indentation. The shaft is degloved via a circumcising incision. The point of maximal curve is identified and Buck's fascia is carefully elevated in this area exposing the plaque (Levine and Dimitriou, 2000). Incision with cautery at <30 W (for the Coloplast Titan) is performed with periodic cylinder inflation and modeling to expand the tunical defect until adequate straightening and/or girth enhancement is accomplished.

Grafting

All defects over 2 cm should be grafted to minimize risk of cylinder herniation, hemotoma formation, or recurrent curvature (Levine and Dimitriou, 2000; Carson and Levine, 2014). Graft selection was reviewed above. Notably dermal grafts should not be used in this setting due to an increased risk of infection (Chun et al., 2001). The graft is generously sized to the defect and secured in place with 4-0 PDS suture (Ethicon, Somerville, New Jersey). Recently, equine collagen fleece patch grafts (TachoSil, Baxter) have been reported, which are adhesive and therefore no suturing is needed.

Tunical plication prior to prosthesis placement

It should be noted that plication prior to (but during the same operation) inflatable prosthesis placement has been reported. This is ideal for dorsal curves and is performed via a penoscrotal incision. The plication sutures can be placed as described above. Once adequate straightening has been achieved, an inflatable penile prosthesis can be placed via the same penoscrotal incision (Chung et al., 2014).

Outcomes

Management of PD and ED via placement of a penile prosthesis and subsequent straightening maneuvers yields a high satisfaction rate. In a study out of the authors' institution, 84% of patients were satisfied with their treatment after undergoing: IPP placement alone (4%), IPP with modeling (79%), tunical incision (4%), or tunical incision and grafting (12%). The goal of surgery, regardless of the approach, is functional straightness which is defined as an erection which is both rigid and straight enough to participate in penetrative sexual intercourse. Patient counseling is paramount in communicating this goal to the patient (Levine et al., 2010).

The most common complaint associated with penile prosthesis placement (and the majority of surgeries performed on the penis) is loss of length. This concern, however, can be exacerbated in PD patients as many men have already lost length due to the underlying disease process (Montague, 2007). This has been successfully countered with the use of traction therapy. When used for 3–4 hours per day for 3–4 months prior to prosthesis placement, patients were able to at least maintain their preoperative penile length. Some men gained up to 2 cm of length using this regimen (Levine and Rybak, 2011).

References

- Andrews, H. O., al-Akraa, M., Pryor, J. P., & Ralph, D. J. (1999). The Nesbit operation for congenital curvature of the penis. *International Journal of Impotence Research*, 11, 119–122.
- Bacal, V., Rumohr, J., Sturm, R., Lipshultz, L. I., Schumacher, M., et al. (2009). Correlation of degree of penile curvature between patient estimates and objective measures among men with Peyronie's disease. *The Journal of Sexual Medicine*, 6, 862–865.
- Baskin, L. S., & Duckett, J. W. (1994). Dorsal tunica albuginea plication for hypospadias curvature. *The Journal of Urology*, 151, 1668–1671.
- Brannigan, R. E., Kim, E. D., Oyasu, R., et al. (1998). Comparison of tunica albuginea substitutes for the treatment of Peyronie's disease. *The Journal of Urology*, 159, 1064–1068.
- Brant, W. O., Bella, A. J., & Lue, T. F. (2007). 16-Dot procedure for penile curvature. *The Journal of Sexual Medicine*, 2, 277–280.
- Breyer, B. N., Brant, W. O., Garcia, M. M., et al. (2007). Complications of porcine small intestine submucosa graft for Peyronie's disease. *The Journal of Urology*, 177(2), 589–591.
- Cantini, L. P., Ferrini, M. G., Vernet, D., Magee, T. R., Qian, A., et al. (2008). Profibrotic role of myostatin in Peyronie's disease. *The Journal of Sexual Medicine*, 5, 1607–1622.
- Carson, C. C., & Levine, L. A. (2014). Outcomes of surgical treatment of Peyronie's disease. *BJU International*, 113(5), 704–713.
- Chun, J. L., McGregor, A., Krishnan, R., & Carson, C. C. (2001). A comparison of dermal and cadaveric pericardial grafts in the modified Horton-Devine procedure for Peyronie's disease. *The Journal of Urology*, 166, 185–188.
- Chung, P. H., Francis Scott, J., & Morey, A. F. (2014). High patient satisfaction of inflatable penile prosthesis insertion with synchronous penile plication for erectile dysfunction and Peyronie's disease. *The Journal of Sexual Medicine*, 11(6), 1593–1598.
- Chung, E., et al. (2012). Penile Doppler sonographic and clinical characteristics in Peyronie's disease and/or erectile dysfunction: an analysis of 1500 men with male sexual dysfunction. *BJU International*, 110, 1201–1205.
- Cornio, L., Zucchi, A., Lorusso, F., Selvaggio, O., Fioretti, F., et al. (2009). Surgical treatment of Peyronie's disease by plaque incision and grafting with buccal mucosa. *European Urology*, 55, 1469–1475.
- da Silva, F. G., Filho, A. M., Damião, R., & da Silva, E. A. (2011). Human acellular matrix graft of tunica albuginea for penile reconstruction. *The Journal of Sexual Medicine*, 8, 3196–3203.
- Das, S. (1980). Peyronie's disease. Excision and autografting with tunica vaginalis. *The Journal of Urology*, 124, 818–819.
- De la Peyronie, F. (1743). Sur quelques obstacles qui opposent a l'ejaculation naturelle de la semence. *Mémoires de l'Académie royale de chirurgie*, 1, 423–434.
- Del Carlo, M., Cole, A. A., & Levine, L. A. (2008). Differential calcium independent regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by interleukin-1beta and transforming growth factor-beta in Peyronie's plaque fibroblasts. *The Journal of Urology*, 179(6), 2447–2455.
- Devine, C. J., Jr., Somers, K. D., Jordan, G. H., et al. (1997). Proposal: trauma as the cause of Peyronie's lesion. *The Journal of Urology*, 157, 285–290.
- Egydio, P. H., Lucon, A. M., & Arap, S. (2002). Treatment of Peyronie's disease by incomplete circumferential incision of the tunica albuginea and plaque with bovine pericardium graft. *Urology*, 59, 570–574.
- El-Sakka, A. I., Hassoba, H. M., Pillarisetty, R. J., Dahiya, R., & Lue, T. F. (1997). Peyronie's disease is associated with an increase in transforming growth factor-beta protein expression. *The Journal of Urology*, 158, 1391–1394.
- Gelbard, M. K. (1995). Relaxing incisions in the correction of penile deformity due to Peyronie's disease. *The Journal of Urology*, 154, 1457–1460.
- Gelbard, M. K., Dorey, F., & James, K. (1990). The natural history of Peyronie's disease. *The Journal of Urology*, 144, 1376–1379.
- Gholami, S. S., Gonzalez-Cadavid, N. F., Lin, C. S., Rajfer, J., & Lue, T. F. (2003). Peyronie's disease: a review. *The Journal of Urology*, 169, 1234–1241.
- Greenfield, J. M., Lucas, S., & Levine, L. A. (2006). Factors affecting the loss of length associated with tunica albuginea plication for correction of curvature. *The Journal of Urology*, 175, 238–241.
- Gur, S., Limin, M., & Hellstrom, W. J. (2011). Current status and new developments in Peyronie's disease: medical, minimally invasive and surgical treatment options. *Expert Opinion on Pharmacotherapy*, 12(6), 931–944.
- Hellstrom, W. J. (1994). The use of prosthetic materials in the surgical management of Peyronie's disease. *International Journal of Impotence Research*, 6(Suppl. 2), D32.
- Hellstrom, W. J., & Reddy, S. (2000). Application of pericardial graft in the surgical management of Peyronie's disease. *The Journal of Urology*, 163(5), 1445–1447.
- Henry, G. D. (2009). Surgical techniques: The Henry Mummy Wrap™ and the Henry Finger Sweep™ surgical techniques. *The Journal of Sexual Medicine*, 6(3), 619–622.
- Jarow, J. P., & Lowe, F. C. (1997). Penile trauma: an etiologic factor in Peyronie's disease and erectile dysfunction. *The Journal of Urology*, 158, 1388–1390.
- John, T., Bandi, G., & Santucci, R. (2006). Porcine small intestinal submucosa is not an ideal graft material for Peyronie's disease surgery. *The Journal of Urology*, 176, 1025–1029.
- Kadioglu, A., Sanli, O., Akman, T., et al. (2007). Graft materials in Peyronie's disease surgery: a comprehensive review. *The Journal of Sexual Medicine*, 4, 581–595.
- Kendirci, M., & Hellstrom, W. J. G. (2004). Critical analysis of surgery for Peyronie's disease. *Current Opinion in Urology*, 14(6), 381–388.
- Knoll, L. D. (2001). Use of porcine small intestinal submucosa graft in the surgical management of Peyronie's disease. *Urology*, 57, 753–757.
- Kovac, J. R., & Brock, G. B. (2007). Surgical outcomes and patient satisfaction after dermal, pericardial, and small intestinal submucosal grafting for Peyronie's disease. *The Journal of Sexual Medicine*, 4, 1500–1508.
- Larsen, S. M., & Levine, L. A. (2012). Review of non-surgical treatment options for Peyronie's disease. *International Journal of Impotence Research*, 24(1), 1–10.
- Levine, L. A. (2006). Penile straightening with tunica albuginea plication procedure: TAP procedure. In L. A. Levine (Ed.), *Peyronie's disease: a guide to clinical management* (pp. 151–160). Totowa, NJ: Humana.
- Levine, L. A., Benson, J., & Hoover, C. (2010). Inflatable penile prosthesis placement in men with Peyronie's disease and drug-resistant erectile dysfunction: a single-center study. *The Journal of Sexual Medicine*, 7(11), 3775–3783.
- Levine, L. A., & Coogan, C. L. (1996). Penile vascular assessment using color duplex sonography in men with Peyronie's disease. *The Journal of Urology*, 155(4), 1270–1273.
- Levine, L. A., & Dimitriou, R. J. (2000). A surgical algorithm for penile prosthesis placement in men with erectile failure and Peyronie's disease. *International Journal of Impotence Research*, 12(3), 147–151.
- Levine, L. A., & Estrada, C. R. (2003). Human cadaveric pericardial graft for the surgical correction of Peyronie's disease. *The Journal of Urology*, 170, 2359–2362.
- Levine, L. A., & Larsen, S. M. (2013). Surgery for Peyronie's disease. *Asian Journal of Andrology*, 15(1), 27–34.
- Levine, L. A., & Larsen, S. M. (2014). Peyronie's disease reconstruction: simple and complex. In *Advanced male urethral and genital reconstructive surgery* (pp. 585–614). New York: Springer.
- Levine, L. A., & Lenting, E. L. (1997). A surgical algorithm for the treatment of Peyronie's disease. *The Journal of Urology*, 158(6), 2149–2152.
- Levine, L. A., & Rybak, J. (2011). Traction therapy for men with shortened penis prior to penile prosthesis implantation: a pilot study. *The Journal of Sexual Medicine*, 8(7), 2112–2117.
- Licht, M. R., & Lewis, R. W. (1997). Modified Nesbit procedure for the treatment of Peyronie's disease: a comparative outcome analysis. *The Journal of Urology*, 158, 460–463.
- Lue, T. F., & El Sakka, A. I. (1998). Venous patch graft for Peyronie's disease. Part I. Technique. *The Journal of Urology*, 160, 2047–2049.
- Ma, L., Yang, Y., Sikka, S. C., Kadowitz, P. J., Ignarro, L. J., et al. (2012). Adipose tissue-derived stem cell-seeded small intestinal submucosa for tunica albuginea grafting and reconstruction. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 2090–2095.
- Montague, D. K. (2007). Penile prosthesis implantation: size matters. *European Urology*, 51(4), 887–888.
- Montorsi, F., Salonia, A., Maga, T., et al. (2000). Evidence based assessment of long-term results of plaque incision and vein grafting for Peyronie's disease. *The Journal of Urology*, 163, 1704–1708.

- Mulhall, J. P., Ahmed, P., & Anderson, M. (2004). Penile prosthetic surgery for Peyronie's disease: defining the need for intra-operative adjuvant maneuvers. *The Journal of Sexual Medicine*, 1, 318.
- Mulhall, J. P., Anderson, M. S., Lubrano, T., & Shankey, T. V. (2002). Peyronie's disease cell culture models: phenotypic, genotypic and functional analyses. *International Journal of Impotence Research*, 14, 397–405.
- Mulhall, J., Anderson, M., & Parker, M. (2005). ORIGINAL RESEARCH—SURGERY: A surgical algorithm for men with combined Peyronie's disease and erectile dysfunction: functional and satisfaction outcomes. *The Journal of Sexual Medicine*, 2(1), 132–138.
- Nachtsheim, D. A., & Rearden, A. (1996). Peyronie's disease is associated with an HLA class II antigen, HLA-DQ5, implying an autoimmune etiology. *The Journal of Urology*, 156, 1330–1334.
- Nesbit, R. M. (1965). Congenital curvature of the phallus: report of three cases with description of corrective operation. *The Journal of Urology*, 93, 230.
- Nyirady, P., Kelemen, Z., Bánfi, G., Rusz, A., Majoros, A., & Romics, I. (2008). Management of congenital penile curvature. *The Journal of Urology*, 179(4), 1495–1498.
- Ralph, D., Gonzalez-Cadavid, N., Miron, V., Perovic, S., et al. (2010). The management of Peyronie's disease: evidence based 2010 guidelines. *The Journal of Sexual Medicine*, 2359–2374.
- Ralph, D. J., & Minhas, S. (2004). The management of Peyronie's disease. *BJU International*, 93(2), 208–215.
- Rosen, R., Catania, J., Lue, T., Althof, S., Henne, J., et al. (2008). Impact of Peyronie's disease on sexual and psychosocial functioning: qualitative findings in patients and controls. *The Journal of Sexual Medicine*, 5, 1977–1984.
- Ryu, J. K., Piao, S., Shin, H. Y., Choi, M. J., Zhang, L. W., et al. (2009). IN-1130, a novel transforming growth factor-beta type I receptor kinase (activin receptor-like kinase 5) inhibitor, promotes regression of fibrotic plaque and corrects penile curvature in a rat model of Peyronie's disease. *The Journal of Sexual Medicine*, 6, 1284–1296.
- Sampaio, J. S., Fonseca, J., Passarinho, A., Christino, J., & Mendes, J. (2002). Peyronie's disease. Surgical correction of 40 patients with relaxing incision and duramater graft. *European Urology*, 41, 551–555.
- Santucci, R. A., & Barber, T. D. (2005). Reabsorbable extracellular matrix grafts in urologic reconstruction. *International Brazilian Journal of Urology*, 31(3), 192–203.
- Schiavino, D., Sasso, F., Nucera, E., Alcini, E., Gulino, G., et al. (1997). Immunologic findings in Peyronie's disease: a controlled study. *Urology*, 50, 764–768.
- Schultheiss, D., Lorenz, R. R., Meister, R., Westphal, M., Gabouev, A. I., et al. (2004). Functional tissue engineering of autologous tunica albuginea: a possible graft for Peyronie's disease surgery. *European Urology*, 45, 781–786.
- Schwarzer, U., Sommer, F., Klotz, T., et al. (2001). The prevalence of Peyroni's disease: results of a large survey. *BJU International*, 88(7), 727–730.
- Smith, B. H. (1966). Peyronie's disease. *American Journal of Clinical Pathology*, 45, 670–678.
- Smith, J. F., Walsh, T. J., Conti, S. L., Turek, P., & Lue, T. (2008). Risk factors for emotional and relationship problems in Peyronie's disease. *The Journal of Sexual Medicine*, 5, 2179–2184.
- Tal, R., et al. (2012). Peyronie's disease in teenagers. *The Journal of Sexual Medicine*, 9, 302–308.
- Taylor, F. L., & Levine, L. A. (2007). Peyronie's disease. *The Urologic Clinics of North America*, 34(4), 517–534.
- Taylor, F. L., & Levine, L. A. (2008). Surgical correction of Peyronie's disease via tunica albuginea plication or partial plaque excision with pericardial graft: long-term follow up. *The Journal of Sexual Medicine*, 5, 2221–2228. discussion 2229–2230.
- Tornehl, C. K., & Carson, C. C. (2004). Surgical alternatives for treating Peyronie's disease. *BJU International*, 94(6), 774–783.
- Usta, M. F., Bivalacqua, T. J., Sanabria, J., Koskal, I. T., Moparty, K., & Hellstrom, W. J. (2003). Patient and partner satisfaction and long-term results after surgical treatment for Peyronie's disease. *Urology*, 62, 105–109.
- Wessells, H., Lue, T., & McAninch, J. (1996). Penile length in the flaccid and erect states: guidelines for penile augmentation. *The Journal of Urology*, 156, 995–997.
- Wilson, S. K., & Delk, J. R., 2nd (1994). A new treatment for Peyronie's disease: modeling the penis over an inflatable penile prosthesis. *The Journal of Urology*, 152, 1121–1123.
- Wolf, J. S., Bennett, C. J., Dmochowski, R. R., Hollenbeck, B. K., Pearle, M. S., & Schaefer, A. J. (2012). Urologic surgery antimicrobial prophylaxis. In *American Urological Association*.
- Yachia, D. (1990). Modified corporoplasty for the treatment of penile curvature. *The Journal of Urology*, 143, 80.
- Yachia, D. (1993). Corporeal plication for surgical correction of Peyronie's disease. *The Journal of Urology*, 149, 869.

Reconstructive Therapy for Disorders of Sex Development

Neha R Malhotra, University of Illinois at Chicago, Chicago, IL, United States

Earl Y Cheng, Ann & Robert H. Lurie Children's Hospital of Chicago, The Feinberg School of Medicine at Northwestern University, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Overview and Initial Diagnosis

Understanding Differences of Sex Development

Disorders of sex development (DSD), previously known as intersex conditions, are broadly defined as “congenital conditions in which development of chromosomal, gonadal, or anatomical sex is atypical (Lee et al., 2006).” This encompasses a heterogeneous group of conditions such as congenital adrenal hyperplasia (CAH), mixed gonadal dysgenesis, and androgen insensitivity syndromes but even broader definitions may include proximal forms hypospadias. Classification of DSD has evolved over the years; some terms regarding the gonadal morphology have persisted, such as mixed gonadal dysgenesis, while others have changed (see Fig. 1). Current classification includes: sex chromosome DSD (also known as disorders of gonadal differentiation), ovotesticular DSD (formerly known as true hermaphrodite), 46, XY DSD (undermasculinized males) and 46, XX DSD (masculinized females) (Hughes et al., 2006). These terms, and the term DSD itself, are under much scrutiny. Recent studies have shown that patients and families affected by these conditions have negative views of commonly used DSD terminology (Johnson et al., 2017b). In this section, DSD will be considered as *differences* of sex development, rather than disorders. While terminology continues to evolve, it is essential to discuss with patients and families their preferred terminology, and to use this not only in discussions with the family, but in the medical record, if possible (Table 1).

Initial Diagnosis

The initial work-up of DSD is often triggered in the nursery or neonatal ICU when a neonate is noted to have ambiguous genitalia. Genitalia may be overtly ambiguous or findings may be more subtle, such as clitoromegaly, posterior labial fusion, inguinal mass in a female, nonpalpable testis or perineal hypospadias. Later presentations may include primary amenorrhea in a female. In the

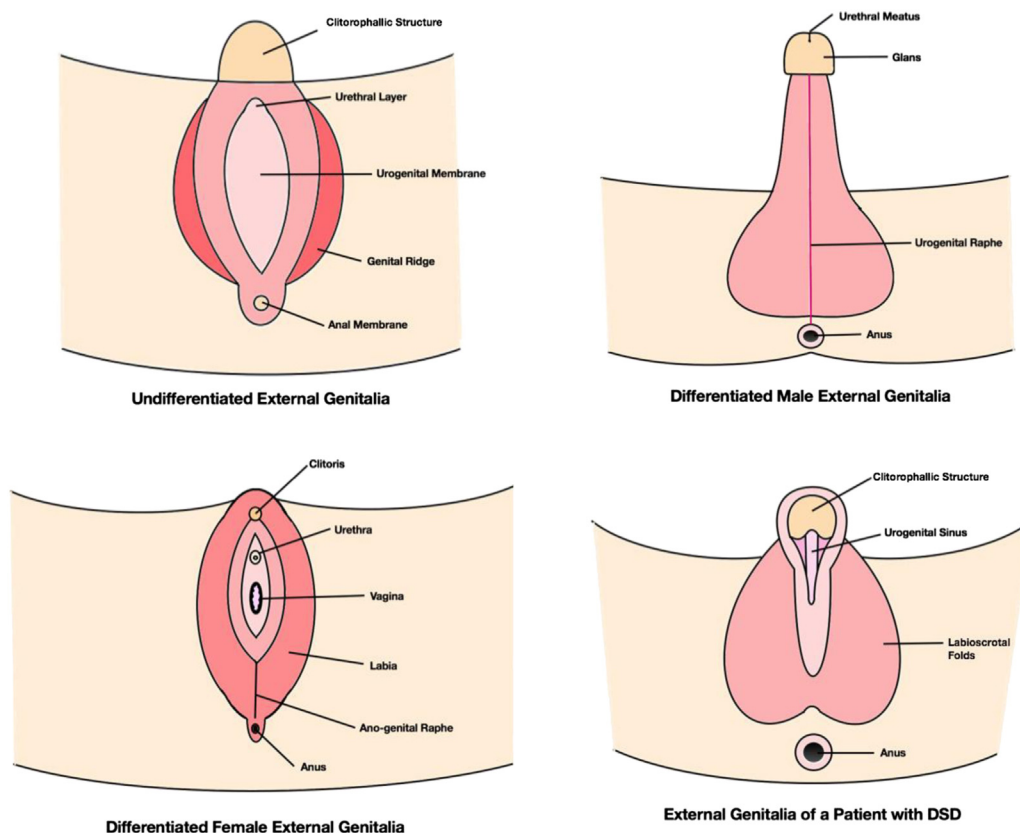


Fig. 1 Comparison of undifferentiated, differentiated and DSD external genitalia.

Table 1 Current nomenclature of disorders of sex development (DSD)

Current	Previous
Disorders of sex development (DSD)	Intersex
46,XY DSD	Male pseudohermaphrodite
	Undervirilized XY male
	Undermasculinized XY male
46,XX DSD	Female pseudohermaphrodite
	Overvirilized XX female
	Masculinized XX female
Ovotesticular DSD	True hermaphrodite
Sex chromosome DSD	Sex chromosome mosaicism, Gonadal dysgenesis

Adapted from Hughes, I. A., Houk, C., Ahmed, S. F., Lee, P. A. and Lawson Wilkins Pediatric Endocrine Society/European Society For Pediatric Endocrinology Consensus, G. 2006. Consensus statement on management of intersex disorders. *Journal of Pediatric Urology*, 2, 148–62.

neonatal period, gender assignment, and use of gender specific pronouns should be avoided until a decision is made as to the sex of rearing. Currently, it is recommended that the multidisciplinary team, in close collaboration with the family, should strive for eventual gender assignment (Lee et al., 2006; Lee et al., 2016). Though it should be recognized and discussed that some families may opt to not assign a binary gender. At all times during the work-up and management of DSD, open communication is essential. Participation by the family should be actively encouraged and all concerns should be respected and addressed.

Evaluation of a neonate

Once a patient with potential DSD is identified, a multidisciplinary team should be assembled to assist with the diagnosis and management. The team should include the primary care service, endocrinology, urology, surgery or gynecology, genetics, and social services or psychology. If available, or when the situation demands, ethics should also be involved. The role of nursing staff as part of the team should not be undervalued as they spend more time with the family and may have insight other team members do not. As always, evaluation should begin with a comprehensive history. This includes a detailed prenatal and family history, as such DSD are heritable.

Physical examination

When possible, physical exam should be performed with as many members of the team as possible to prevent repeat physical and psychological trauma to the patient and family. Photography may be helpful in preventing repeated exams and to evaluate changes in response to therapy. In newer electronic medical records, photographs can be included in the patient chart.

Assessment of the external genitalia should document the size and structure of the genital tubercle (also known as the clitorophallic structure), the location of the urethral opening, the morphology of the labioscrotal folds, ano-genital distance, as well as presence and location of gonads (see Fig. 1). The genital tubercle is the undifferentiated structure that develops into either the penis in a male or the clitoris in a female (Martinez-Mora, 1994). Evaluation should include the size (in stretched length and width), the length, width, and shape of the glans, as well as depth of the urethral groove (Malone et al., 2012). Any curvature should also be recorded. The shape and position of the urethral meatus, or the opening of the urethra, should be noted. In hypospadias, the meatus may located on glans, shaft or perineum (Lee et al., 2016). This is discussed further in detail in the masculinizing surgery section. The labioscrotal folds, which may be referred to as the genital folds, should be assessed for symmetry, fusion, pigmentation, and rugation. They should also be palpated for presence of gonads. Any other physical abnormalities unrelated to the external genitalia should also be evaluated, such as abnormal facies. This baseline examine is essential to initial diagnosis as well as future surgical planning.

Laboratory evaluation

Laboratory evaluation should be done in close consultation with endocrinology. A karyotype should be sent when DSD is suspected; though this will typically take a few days to result. Together, physical examination and karyotype may suggest a diagnostic classification, but other testing may be necessary. Electrolytes should be obtained immediately as salt-wasting CAH can be an emergency. CAH is a group of autosomal recessive conditions in which there is an enzymatic mutation in the steroid synthesis pathway (see Box 1). Testosterone, dihydrotestosterone and 17-hydroxyprogesterone should also be measured. Elevated 17-hydroxyprogesterone in a neonate is pathognomonic for CAH (Table 2).

Gonadotropin levels (follicle stimulating hormone [FSH] and luteinizing hormone [LH]) should also be measured, in addition to anti-Müllerian hormone (AMH, also known as Müllerian inhibiting substance [MIS]). While AMH is secreted in small amounts from ovarian granulosa cells and present in girls, circulating concentrations are much higher in boys and are suggestive of male gender. AMH is secreted by the Sertoli cells of the testis and confirms presence of testicular tissue, even in the absence of palpable gonads. FSH and LH should be normal or slightly elevated in steroid synthesis disorders, such as CAH, or androgen insensitivity but will be markedly elevated in sex chromosome DSD with dysgenetic gonads, such as Turner or Klinefelter

Box 1 Congenital adrenal hyperplasia (CAH)

Inheritance	Autosomal recessive
Etiology	Gene mutation in enzymes of the steroid synthesis pathway (most common mutation: 21-hydroxylase)
Symptoms	Vary based on which enzyme is mutated and thus which steroid is deficient or in excess Lack of mineralocorticoids: salt-wasting, dehydration, vomiting Lack of androgens/estrogens: ambiguous genitalia, undermasculinization in males, infertility in females Excess androgens: ambiguous genitalia, overmasculinization in females, precocious puberty, infertility
Diagnosis	Serum electrolytes, Serum 17 α -hydroxyprogesterone (newborn screen), Adrenocorticotrophic hormone (ACTH) stimulation test

Table 2 DSD diagnoses by classification

<i>Sex Chromosome DSD</i>	<i>46,XX DSD</i>	<i>46,XY DSD</i>
45,X (Turner Syndrome)	Disorders of Ovarian Development Ovotesticular DSD Testicular DSD <i>SRY</i> ⁺ <i>SOX9</i> Duplication Gonadal Dysgenesis	Disorders of Testicular Development Complete gonadal dysgenesis (Swyer syndrome) Partial gonadal dysgenesis Gonadal regression Ovotesticular DSD
47,XXY (Klinefelter Syndrome)	Disorders of Androgen Excess Fetal (Congenital Adrenal Hyperplasia) 21-hydroxylase deficiency 11- β -hydroxylase deficiency Fetoplacental Aromatase deficiency P450 oxidoreductase deficiency (PORC) Maternal Luteoma Exogenous androgen supplementation	Disorder of Androgen Synthesis or Effect Androgen biosynthesis defect 17-hydroxysteroid dehydrogenase deficiency 5- α -reductase deficiency Steroidogenic acute regulatory (StAR) protein mutation Defect in Androgen Action Complete androgen insensitivity syndrome (CAIS) Partial androgen insensitivity syndrome (PAIS) Luteinizing hormone receptor defects Leydig cell hypoplasia Leydig cell aplasia Disorders of anti-Müllerian hormone and receptor
45,X/46,XY (Mixed Gonadal Dysgenesis, Ovotesticular DSD)	Other Cloacal exstrophy Vaginal atresia Müllerian duct aplasia-renal agenesis-cervicothoracic somite dysplasia (MURCS)	
46,XX/46,XY (Chimeric, Ovotesticular DSD)		

Adapted from Lee, P. A., Houk, C. P., Ahmed, S. F., Hughes, I. A., International Consensus Conference on Intersex Organized by THE Lawson Wilkins Pediatric Endocrine, S. and The European Society for Pediatric, E. 2006. Consensus statement on management of intersex disorders. *International Consensus Conference on Intersex. Pediatrics*, **118**, e488–500.

Syndromes (Lee et al., 2016). If the diagnosis is still not clear from initial testing, further analysis may be required with human chorionic gonadotropin (hCG) or adrenocorticotrophic hormone (ACTH) stimulation testing. This should be done in close collaboration with an endocrinologist. Though not first line testing, complete genetic sequencing can be useful to identify specific genes associated with DSD and to reveal Y-chromosome material not assessed by conventional karyotyping. It is important to assess the presence of Y-chromosome material, as this increases the risk of gonadoblastoma in female patients with Turner Syndrome or mixed gonadal dysgenesis (MGD). This may be done with polymerase chain reaction (PCR) testing. In some cases, despite thorough physical exam and laboratory evaluation, the specific diagnosis may remain unclear.

Imaging

Ultrasound is the mainstay of radiologic imaging in the evaluation of DSD. This both avoids the radiation of computerized tomography (CT) and the anesthesia required in neonates to undergo magnetic resonance imaging (MRI). Ultrasound allows for visualization of gonads and assessment of Müllerian structures, such as the uterus and Fallopian tubes. It further allows for evaluation of the kidneys and adrenal glands, as these may be affected in conditions such as Turner Syndrome. If ultrasound is non-diagnostic, MRI may be necessary to further delineate the anatomy. Historically, genitograms, or fluoroscopic studies of the uro-genital system were performed; however these are no longer routine as endoscopic evaluation is far more helpful in defining the anatomy (Vanderbrink et al., 2010). Endoscopy allows for an understanding of internal structures in relation to one another and may assist in surgical planning. In select cases, laparoscopy may also be required to identify and evaluate internal structures. This also has the advantage of allowing for gonadal biopsy, which may be necessary for final diagnosis.

Evaluation of an adolescent

Though most cases of DSD are recognized in the neonatal period, some may not be detected until the time of expected puberty as they are not associated with ambiguous genitalia. The most common presentation is primary amenorrhea in a female. Patients with complete androgen insensitivity syndrome (CAIS) often present in this fashion (see **Box 2**). As with neonates, a detailed history and physical is essential to evaluation of the adolescent with suspected DSD. Adolescence is a time of change and the psychosocial needs of the patient should be considered and respected. History should include developmental history, including milestones and learning disabilities as well as any menstrual history in females, including cyclic abdominal pain. Physical examination should include evaluation of external genitalia as well as that of secondary sexual characteristics such as breast development and hair growth patterns. Some diagnosis, such as Turner syndrome and Klinefelter may be suspected on the basis of physical examination alone (see **Box 3**). Though a comprehensive discussion of all DSD is beyond the scope of this chapter, a list of the most common DSD can be found in **Table 3**.

Surgical Considerations

Controversy Regarding the Timing of Surgical Management

Gender assignment must be done with great care as it may have irreversible psychosocial consequences for the patient. As previously discussed, gender assignment should be done in conjunction with a multi-disciplinary team assessment and open discussion with

Box 2 Androgen insensitivity syndromes

	Complete androgen insensitivity syndrome (CAIS) Affects only 46,XY males; 46,XX females are minimally affected Most common cause of 46,XY DSD (undermasculinized male)	Partial androgen insensitivity syndrome (PAIS)
Inheritance	X-linked recessive	X-linked recessive
Etiology	Complete inability of cells to respond to androgens due to mutation in androgen receptor	Partial inability of cells to respond to androgens due to mutation in androgen receptor
External Genitalia	Feminine	Partially masculinized
Diagnosis	Karyotype, testosterone and dihydrotestosterone (DHT) with physical exam	Karyotype, testosterone, DHT, human chorionic gonadotropin (hCG) stimulation test, androgen receptor gene mutation testing

Box 3 Sex chromosome DSD

	Turner syndrome (45,X)	Klinefelter syndrome (47,XXY)	Mixed gonadal dysgenesis (MGD) (45,X/46,XY)
Presentation	Short stature Webbed neck Broad chest Differential blood pressure (aorticcoarctation) Heart murmur (valvular anomalies) Primary amenorrhea	Tall stature Learning disabilities Small firm testis Primary infertility	Variable Ambiguous genitalia Primary infertility
Diagnosis	Karyotype	Karyotype	Karyotype

Table 3 Causes of infertility in DSD

Abnormal gonadal development; premature gonadal failure
Gonadectomy (iatrogenic)
Abnormal hormonal milieu with resultant impairment of gamete production
Assumed infertility, due to discordance between assigned gender and gonadal type
Anatomical barriers to natural conception

Adapted from Finlayson, C., Fritsch, M. K., Johnson, E. K., Rosoklija, I., Gosiengfiao, Y., Yerkes, E., Madonna, M. B., Woodruff, T. K. and Cheng, E. 2017. Presence of Germ Cells in Disorders of sex development: Implications for fertility potential and preservation. *The Journal of Urology*, 197, 937–943.

the family regarding the pros and cons of gender assignment (male, female and unassigned). Regardless of gender assignment, parents need to be aware of the risk of gender dysphoria later in life and the small possibility of gender conversion (Chowdhury et al., 2015; Reiner and Kropp, 2004). Once gender has been assigned, additional discussion of possible surgical management and reconstructive treatment can be undertaken. The aims of surgery are to restore functional genital anatomy for future penetrative intercourse and reproduction, if possible, reduce the risk of gonadal cancer, foster development of individual identity, avoid stigmatization due to atypical anatomy (Mouriquand et al., 2016).

Historically, early surgical treatment was pursued to “normalize” children with ambiguous genitalia. However, today there is increased advocacy against early surgery so as to allow children to participate in the decision making process. The guiding surgical principle is to avoid early irreversible surgery, especially when the assigned gender may be controversial. On the other hand, patients with a diagnosis such as CAH have been shown to have very high rates of female gender identity and low rates of gender dysphoria in adulthood (Binet et al., 2016). As such, many would agree with proceeding with early surgery for many forms of CAH. While delaying irreversible surgical procedures does preserve the child’s autonomy, this decision is not without difficulty for the patient and family. Though lessening in the era of transgender and DSD advocacy, there is still significant societal pressure to fall into the binary norm of either male or female. A child raised to be different in this way may have trouble fitting in at school and in sports. The family will also be subject to psychosocial stressors associated with having a child who is different. At present, there is limited data on the impact of raising a child as neither male nor female (Rangecroft, 2003). As there is little evidence regarding the long-term outcomes of children who do not undergo early surgery, it is imperative to openly discuss this controversy with families.

Preoperative Preparation

Once the decision has been made to proceed with surgery, regardless of timing, the patient must be appropriately prepared for surgery. Patients with endocrine aberrations should be seen by the endocrinology service for metabolic stabilization. They may require peri-operative stress dose steroids. For complex cases, anesthesia should be involved in early preoperative planning. If the child is old enough to understand the implications of surgery, technical aspects should be explained at the appropriate level and assent obtained. Support staff, such as child life, should also be utilized throughout the peri-operative process. The psychosocial stressors of the diagnosis and of the surgery must be kept in mind and psychological services should play a central role for both the patient and the family.

Antibiotics should be given based on the procedure. Typically in the cases of laparoscopy or orchiopexy, single dose antibiotics to cover skin flora are given. In cases of feminizing genitoplasty with vaginoplasty, broad spectrum antibiotics are given for several days peri-operatively and the patient may need a mechanical bowel preparation with either an enema or an osmotic agent such as GoLyte[®]. Antibiotic use for hypospadias repair is being studied and is an issue of ongoing debate (Faasse et al., 2017). There is less standardization of antibiotic prophylaxis for genitoplasty and considerations should be given to prior history of urinary tract infection, asymptomatic positive cultures and local antibiotic resistance patterns.

Masculinization Surgery (Hypospadias Repair)

In cases of DSD, masculinization surgery primarily entails hypospadias repair and orchiopexy. Orchiopexy has been well described elsewhere. Hypospadias is a condition in which the urethral meatus is located proximal to the normal location at the tip of the glans. It is often associated with some degree of ventral curvature, known as chordee. The urethral meatus can be located anywhere from the glans, to the perineum. In general, the most distal the meatus, the lesser degree of chordee, and the more proximal the meatus, the greater the degree of chordee. In DSD, proximal hypospadias is more common. The meatus is located somewhere along the proximal shaft, peno-scrotal junction or perineum and is associated with functional problems, such as inability to void standing up and inability to have penetrative sexual intercourse. Proximal hypospadias is technically more challenging to repair than distal hypospadias. Goals of hypospadias repair are a straight penis, good sexual function, the ability to stand to void, a satisfied *adult* patient and parent. Ideally, there will be a slit-like meatus on the glans, a cone shaped glans with a well defined coronal sulcus, no penile curvature, as well as an appropriate anatomic relationship between the penis and the scrotum.

Surgical considerations

Proximal hypospadias is technically more challenging than distal hypospadias repair as tissues are underdeveloped and more dysplastic, especially in cases of DSD. Various techniques have evolved over the years. Though somewhat debated, the timing of hypospadias repair is far less controversial than feminizing genitoplasty. Most surgeons recommend repair between 6 and 18 months of age, in line with recommendations (American Academy of Pediatrics, 1996).

As with other complex surgical procedures, high volume surgeons and centers have been associated with better outcomes (Chowdhury et al., 2007). Patients should be referred to appropriate tertiary care centers for management of hypospadias in the context of DSD. There is no standard surgical procedure for proximal hypospadias repair. Many authors use preoperative testosterone to increase the size of the glans and vascularity of the penile tissue. Some studies have shown improved outcomes with use of testosterone; while others have shown increased rates of complications as testosterone has been associated prolonged wound healing (Kaya et al., 2008; Wright et al., 2013). Other work has suggested that boys with proximal hypospadias may have relative androgen resistance, limiting the utility of testosterone supplementation (Snodgrass et al., 2014). The use of testosterone supplementation in proximal hypospadias repair remains controversial and is left to the discretion of the surgeon and family.

Historically, two-stage repairs were recommended for proximal hypospadias. Then, in the 1980s, the single stage repair was shown to be feasible with acceptable results. However, over the past 10–20 years many surgeons reintroduced two-stage repairs to due to suboptimal short-and long-term results with the one stage repair (Gong and Cheng, 2017). Complications include suboptimal cosmesis, early wound breakdown and dehiscence, urethral stricture, diverticulum or fistula formation. Late complications such as recurrent chordee, urethral stricture and p erectile dysfunction are also well documented. As such, the two-stage repair is in vogue once again.

Surgical technique

While there is no standard technique for proximal hypospadias, most experts would agree that surgeons can consider a single stage repair in cases where chordee is <30 degrees and there is a healthy urethral plate (Gong and Cheng, 2017). However, in most cases of DSD with proximal hypospadias, the severity of the curvature will require a two-stage repair.

The procedure begins with a circumcising incision around the dorsal and lateral surfaces of the penile shaft and then a U-shaped ventral incision to the proximal aspect of the meatus (Gong and Cheng, 2017). Chordee is released by degloving the penis just above Buck's fascia. Residual curvature is then assessed. If residual chordee is <30 degrees dorsal plication usually suffices, but if residual chordee is >30 degrees the urethral plate will need to be divided. Transverse incisions of the corporal body (also known as fairy cuts) can be attempted, but if curvature persists corporal body grafting may be required. Small intestine submucosa is commonly used as graft material (Kropp et al., 2002). Once chordee has been addressed, a decision must be made as to how to create the neourethra. Skin flap or grafts, or buccal mucosa may be used. These grafts or flaps are used to cover the urethral defect on the ventral surface of the shaft and form the new urethral plate. This new urethral plate is left open and allowed to heal. The patient continues to void from the proximally located meatus. At the second stage, this newly vascularized urethral plate will be tubularized to create a single continuous urethra. The second stage typically occurs six or more months later (Gong and Cheng, 2017). Once the urethra has been tubularized, typically in two layers, a vascularized flap is placed over it, either from the dartos of the penis or the tunica vaginalis of the testis. This skin is then closed over this layer.

Feminization Surgery (Feminizing Genitoplasty)

Feminization surgery, commonly known as feminizing genitoplasty, consists of clitoroplasty, vaginoplasty and labioplasty. These procedures may be done individual, or together in one combined procedure.

Timing

Discussions as to timing of feminizing genitoplasty are rife with controversy. Though many in the medical community support early genitoplasty, there are those who universally oppose all gender surgery on legal and ethical grounds (Diamond and Garland, 2014). Opponents of early surgery focus on the principle of autonomy and the legal rights of the patient to make his or her decision regarding his or her body. On the other hand, in a case-control study of patients and parents involved in infant feminizing genitoplasty for CAH, nearly 90% of patients and 100% of parents support early surgery (Binet et al., 2016). Another retrospective study has shown that, despite some difficulties with sexual function, patients mostly preferred early surgery (Fagerholm et al., 2011).

Individuals that support early clitoroplasty express concern that girls who have not undergone surgery and have clitoromegaly may feel embarrassed or be made fun of in the changing room and this could lead to social withdrawal (Creighton et al., 2012). Often, the decision for early clitoroplasty is driven by the parents and their desire for a “normal” appearance of the external genitalia. This highlights the need for a multidisciplinary team that can offer not only the scientific rationale, but also the social support required to make the best decision for the child. The timing of vaginoplasty is also controversial. Proponents of early vaginoplasty offer the tangible benefits of using urogenital sinus mucosa for infant genitoplasty (Creighton et al., 2012). If the urogenital sinus is closed without concomitant creation of a vagina, this mucosa may not be available for use later. Similarly, if infant clitoroplasty is performed and vaginoplasty is not done at the same time, the clitoral foreskin is lost. The clitoral foreskin is a key component of an inversion vaginoplasty. However, the vagina is nonfunctional in childhood and delaying vaginoplasty allows the child to be involved not only in the decision making process but also the pre and post-operative care. This is especially important as vaginal dilation is necessary postoperatively and cannot be done without the willing participation of the child. Further follow-up is needed to elucidate long-term cosmetic and functional results as well as sexual satisfaction of feminizing genitoplasty.

Technique

Clitoroplasty

The goal of clitoroplasty is to reduce the size of the genital tubercle to give a feminine appearance without compromising future sexual function. Since some forms of reduction clitoroplasty carry the risk of decreased genital sensation, consensus guidelines recommend against clitoroplasty in the cases of mild virilization, reserving the procedure for more severe virilization, typically done in conjunction with vaginoplasty (Lee et al., 2006). As with other genitoplasty procedures, there is much controversy as to the optimal technique for reduction and anchoring of the genital tubercle.

Typically, the clitoris is degloved and the clitoral foreskin divided vertically to be reconstructed into the labia minora. This can be done as a Y-V plasty. Care is taken not to injure the dorsal neurovascular bundles as these provide sensation to the clitoris. The clitoris is reduced in size by excising the distal erectile tissue through ventral incisions on the corpora, without excising the corporal body and tunica albuginea itself. The glans tissue is then recessed under the pubic bone. A recent modification has been proposed in

which the corpora are divided from one and other and each corpus is then rotated inferior and laterally to be hidden beneath the labial folds (Pippi Salle et al., 2007). This allows for preservation of the corpora in the case of desired reversal of surgery in the future.

Vaginoplasty

Vaginoplasty techniques vary greatly as the anatomy and relationships of the vagina and urethra vary. The goal of vaginoplasty is to create a vagina suitable for sexual intercourse as well as to allow for unobstructed menstruation. In cases of DSD, there may not be two distinct openings for the urethra and vagina on the perineum, instead there is a common channel with a single opening on the perineum. The common channel is the fused urethra and vagina, though the bladder and uterus remain separate. Anatomy in which the vagina and urethra are separate until the very distal aspect near the introitus is known as a low confluence urogenital sinus. By contrast, in a high confluence sinus, the urethra and vagina form one common channel until a proximal location near the bladder and cervix. Low confluences sinuses are technically easier to repair. In high confluence sinuses, urinary continence becomes a concern, as the length of the vagina from the common channel is closely related to the bladder neck continence mechanism.

The rationale for early surgery is that tissues are more amenable to mobilization, there is decreased surgical morbidity and the child tolerates the surgery with less psychosocial trauma, as they often do not remember the surgery later in life. However, high rates of vaginal stenosis are associated with early surgery, as vaginal dilation is not appropriate in the infant period. Those that advocate for later surgery cite lower rates of stenosis as vaginal dilation can be performed, but tissues are harder to mobilize later in life and surgery may be associated with higher morbidity. Surgeons should discuss with the family realistic expectations for early versus delayed surgery (Alizai et al., 1999).

Due to the technical challenge of these surgical repairs, several techniques have emerged. While there is no consensus as to which is the optimal repair, total and partial urogenital sinus mobilization have become common in past few decades and have largely replaced flap vaginoplasties (Braga et al., 2006). Elucidation of specific techniques is beyond the scope of this chapter but a brief comparison is provided below. In total urogenital mobilization surgery, the urethral-vaginal confluence is mobilized as a single unit all the way down to provide two separate perineal openings. In comparison, partial urogenital mobilization preserves the pubo-urethral ligament and has been posited to improve long term continence (Rink et al., 2006). In cases of a high confluence, where the urethral-vaginal confluence will not reach the perineum the vagina can be mobilized off the urogenital sinus and down to the perineum (Creighton et al., 2012; Salle et al., 2012). In very severe cases, the vagina can be approached posteriorly, by opening the anterior wall of the rectum; this is known as the anterior sagittal transrectal approach (ASTRA) and is done in prone positioning.

Labioplasty

Labioplasty may be done at the time of clitoroplasty or at the time of vaginoplasty. The rugated, fused scrotal skin is used to construct the labia majora (Creighton et al., 2012). The labia minora are constructed from the clitoral foreskin as described above.

Gonadal Surgery

Another increasingly controversial issue is the fate of the gonads. Historically, patients at risk for germ cell tumors underwent prophylactic gonadectomy but as a more nuanced understanding of malignancy risk and fertility potential has emerged, this paradigm is being challenged. Gonadal surgery is now being delayed or altogether deferred (Spoon et al., 2017). Prior to a discussion of gonadal surgery there must be an understanding of the current state of fertility potential and malignancy risk for patients with DSD.

Fertility potential

Only 14% self-identified DSD patients have children, as compared to 40% of their counterparts in the same age group (Slowikowska-Hilczek et al., 2017; Fleming, 2016). Rates of fertility, and fertility potential, vary by DSD. Infertility, or decreased fertility potential, in patients with DSD may be multifactorial (see Table 3). Patients with DSD have not only abnormal gonadal development, but also an abnormal hormonal milieu (Finlayson et al., 2016a). This leads to impaired gamete production and high rates of early gonadal failure. Due to increased risk of malignancy in certain DSD, many patients may be candidates to undergo gonadectomy. This itself is an iatrogenic cause of infertility, as potential gametes are removed. There may also be assumed infertility due to discordance between assigned gender and gonadal type (Finlayson et al., 2016a). Educating pubertal and postpubertal patients as to their unique external and internal genitalia is critical to an understanding of sexuality and fertility. Potential for fertility must be considered in light of the particular diagnosis, the severity of the hormonal imbalances as well as prior surgical or hormonal treatment. Fertility potential for some DSD conditions are discussed below.

Rates of fertility in CAH vary by gender. Women with severe, salt-wasting forms of CAH have been reported to have rates of fertility ranging from 0% to 10% whereas women with simple masculinizing forms have been reported at 33%–50% (Van Batavia and Kolon, 2016). Other studies have shown that glucocorticoid treatment improves rates of fertility in CAH females (Bidet et al., 2010). Conversely, approximately 50% of males with CAH are fertile.

Rates of fertility may vary by assigned gender and fertility potential may change as a result of prior treatments and therapies. Females with partial androgen insensitivity (PAIS) typically undergo gonadectomy, and thus loss of fertility potential. Whereas if they are raised male, they receive hormonal treatment and maintain their fertility potential. Women with CAIS have limited

natural fertility potential as they lack Müllerian structures. Patients with 5-alpha reductase deficiency reared as male may have potential fertility through the use of TESE and ICSI but those raised as female have no fertility potential as they lack internal female genitalia (Van Batavia and Kolon, 2016).

Fertility potential in sex chromosome DSD is complex and often dependent on the particular histology of the gonads at a given point in time. Patients with Turner and Klinefelter syndrome undergo progressive gonadal failure, but do have functional tissue and have been shown to have spontaneous conception, albeit at low rates (Slowikowska-Hilczer et al., 2017; Kathrins and Kolon, 2016). Similarly, a patient with ovotesticular DSD may have functional oocytes and thus the potential for reproduction using assisted reproductive technology (ART) (Slowikowska-Hilczer et al., 2017). Biopsies may help to guide discussions of fertility potential in certain patients. Realistic expectations regarding future fertility should be provided to parents prior to gender assignment as this may affect their decision in gender rearing.

Information regarding potential fertility should be discussed with families in light of current reproductive technology. However, as technology advances, options for fertility preservation may evolve and this too should be discussed. Previously it was felt that patients with dysgenetic gonads did not have fertility potential. More recent analysis of gonads and biopsy specimens has shown presence of viable germ cells in ovotestes, dysgenetic testis and even 15% of streak gonads (Finlayson et al., 2016a). These viable germ cells decline with age and as such, fertility potential may be lost if early preservation is not undertaken. At this time, cryopreservation of sperm, oocytes and even embryos is well-established for postpubertal patients, but work is underway to mature prepubertal gonocytes in vitro for future use (Johnson et al., 2017a). Working with clinicians in OncoFertility—a field combining oncology and reproductive medicine to improve the reproductive future of cancer survivors—who are well-versed in fertility preservation is critical for advancing the field of fertility preservation in DSD (Finlayson et al., 2016b). As technologies are being developed, open communication with families about current and future possibilities for fertility is crucial.

Risk of malignancy

Malignancy potential is also an important consideration in discussing gonadal surgery. As with fertility potential, the risk of malignancy varies by diagnosis. DSD patients can be at risk for gonadoblastoma, germ cell tumors such as seminoma, nonseminoma, as well as carcinoma in situ, now called germ cell neoplasia in situ (GCNIS), though the risk may be lower than once presumed. The risk of germ cell tumors is linked to the presence of Y chromosomal material, particularly the testis specific protein (TSPY) (Wolffenbuttel et al., 2016; Cools et al., 2011). Other markers such as octamer binding protein (Oct 3/4), a transcription factor, and cKIT ligand, have also been linked to germ cell malignancies (Wolffenbuttel et al., 2016; Kathrins and Kolon, 2016). As such karyotyping, fluorescence in situ hybridization (FISH) and immunohistochemical analysis may be required to accurately gauge and discuss malignancy potential. Judicious use of open and percutaneous biopsies may improve the assessment of the risk of malignancy by providing tissue for diagnosis and staining. Malignancy potential for some DSD are discussed below.

Risk of malignancy in DSD varies by diagnosis. Gonadoblastoma is estimated to occur in 7%–10% of women with Turner Syndrome and known Y chromosomal material (Gravholt et al., 2000). Traditionally, men with Klinefelter were thought to be at increased risk of seminoma, but newer evidence suggests that this may not be true, with two studies reporting no Klinefelter patients with testicular cancer (Accardo et al., 2015; Swerdlow et al., 2005). In CAIS, risk of malignancy has been reported to be 0.8%; whereas females with PAIS have a 15% risk (Pleskacova et al., 2010). Increased awareness of these rates of malignancy has changed the paradigm around gonadal surgery for these DSD. Previously in cases of CAIS, gonadectomy was done early in life, but it has since been realized that these functional gonads carry a low risk of malignancy. This, coupled with the benefit of providing an adequate hormonal milieu for spontaneous puberty, has led some to delay gonadectomy until after puberty, if not defer it altogether. Conversely, as females with PAIS are at a greater risk of malignancy early gonadectomy is still recommended. Research into the risk of malignancy allows for more informed and shared decision making by the patient and family.

All DSD are not linked to risk of germ cell tumors. Some patients, such as females with congenital adrenal hyperplasia, are not at increased risk of gonadal malignancy, as they have two normal ovaries, and no gonadal surgery is recommended or required. Males with CAH are at risk of testicular or paratesticular adrenal rests if hormone supplementation is inadequate but these “tumors” are nonmalignant and regress with appropriate treatment (Kathrins and Kolon, 2016). Discussion of the risk of malignancy of a particular DSD should be based on current epidemiologic data and individual histology, if available, to allow for decision making regarding early or deferred gonadal surgery.

Surgical considerations

While perhaps operatively less complex than hypospadias repair or feminizing genitoplasty, gonadal surgery has become increasingly intellectually challenging as new technology evolves regarding potential fertility preservation and new data emerges on the specific risk of malignancy potential. Where an ovotestis may have once been removed, a patient must now consider her 2.6% risk of malignancy against the very real possibility that she has functional oocytes and could have a child through ART (Slowikowska-Hilczer et al., 2017; Pleskacova et al., 2010). Decisions regarding gonadal surgery, particularly gonadectomy, pertain to both patients identified as male and those identified as female. The aim of gonadal surgery was once to prevent malignancy, but now gonadal function and fertility are of increasing importance. Furthermore, the importance of hormone production from gonads, even if discordant with the gender of rearing, should not be overlooked, as these promote growth, bone health and puberty.

Conclusion

DSD is a heterogeneous group of conditions in which chromosomal, gonadal or anatomical sex development is atypical. Management of DSD should be undertaken in a multidisciplinary, patient centered team with the goal of shared decision-making. Surgical considerations must be made in light of fertility and malignancy potential as well as the patient and families goals and desires. Further research continues as to optimizing surgical techniques and maximizing future fertility in these patients.

References

- Accardo, G., Vallone, G., Esposito, D., Barbato, F., Renzullo, A., conzo, G., Docimo, G., Esposito, K., & Pasquali, D. (2015). Testicular parenchymal abnormalities in Klinefelter syndrome: A question of cancer? Examination of 40 consecutive patients. *Asian Journal of Andrology*, 17, 154–158.
- Alizai, N. K., Thomas, D. F., Lilford, R. J., Batchelor, A. G., & Johnson, N. (1999). Feminizing genitoplasty for congenital adrenal hyperplasia: What happens at puberty? *The Journal of Urology*, 161, 1588–1591.
- American Academy of Pediatrics. (1996). Timing of elective surgery on the genitalia of male children with particular reference to the risks, benefits, and psychological effects of surgery and anesthesia. *Pediatrics*, 97, 590–594.
- Bidet, M., Bellanne-Chantelot, C., Galand-Portier, M. B., Golmard, J. L., Tardy, V., Morel, Y., Clauin, S., Coussieu, C., Boudou, P., Mowzowicz, I., Bachelot, A., Touraine, P., & Kuttent, F. (2010). Fertility in women with nonclassical congenital adrenal hyperplasia due to 21-hydroxylase deficiency. *The Journal of Clinical Endocrinology and Metabolism*, 95, 1182–1190.
- Binet, A., Lardy, H., Geslin, D., Francois-Fiquet, C., & Poli-Merol, M. L. (2016). Should we question early feminizing genitoplasty for patients with congenital adrenal hyperplasia and XX karyotype? *Journal of Pediatric Surgery*, 51, 465–468.
- Braga, L. H., Lorenzo, A. J., Tatsuo, E. S., Silva, I. N., & Pippi Salle, J. L. (2006). Prospective evaluation of feminizing genitoplasty using partial urogenital sinus mobilization for congenital adrenal hyperplasia. *The Journal of Urology*, 176, 2199–2204.
- Chowdhury, M. M., Dagash, H., & Pierro, A. (2007). A systematic review of the impact of volume of surgery and specialization on patient outcome. *The British Journal of Surgery*, 94, 145–161.
- Chowdhury, T. K., Laila, K., Hutson, J. M., & Banu, T. (2015). Male gender identity in children with 46,XX DSD with congenital adrenal hyperplasia after delayed presentation in mid-childhood. *Journal of Pediatric Surgery*, 50, 2060–2062.
- Cools, M., Pleskacova, J., Stoop, H., Hoebeke, P., Van Laecke, E., Drop, S. L., Lebl, J., Oosterhuis, J. W., Looijenga, L. H., Wolffenbuttel, K. P., & Mosaicism Collaborative, G. (2011). Gonadal pathology and tumor risk in relation to clinical characteristics in patients with 45,X/46,XY mosaicism. *The Journal of Clinical Endocrinology and Metabolism*, 96, E1171–80.
- Creighton, S., Chernausek, S. D., Romao, R., Ransley, P., & Salle, J. P. (2012). Timing and nature of reconstructive surgery for disorders of sex development - introduction. *Journal of Pediatric Urology*, 8, 602–610.
- Diamond, M., & Garland, J. (2014). Evidence regarding cosmetic and medically unnecessary surgery on infants. *Journal of Pediatric Urology*, 10, 2–6.
- Faasse, M. A., Cheng, E. Y., & Farhat, W. A. (2017). Re: Advantages of Reduced Prophylaxis after Tubularized Incised Plate Repair of Hypospadias: S. Zeiai, A. Nordenskjöld and M. Fossum J Urol 2016;196:1244-1249. *The Journal of Urology*, 197, 264–265.
- Fagerholm, R., Santtila, P., Miettinen, P. J., Mattila, A., Rintala, R., & Taskinen, S. (2011). Sexual function and attitudes toward surgery after feminizing genitoplasty. *The Journal of Urology*, 185, 1900–1904.
- Finlayson, C., Fritsch, M. K., Johnson, E. K., Rosoklija, I., Gosiengfiao, Y., Yerkes, E., Madonna, M. B., Woodruff, T. K., & Cheng, E. (2016a). Presence of germ cells in disorders of sex development: Implications for fertility potential and preservation. *The Journal of Urology*, 197, 937–943.
- Finlayson, C., Johnson, E. K., Chen, D., Dabrowski, E., Gosiengfiao, Y., Campo-Engelstein, L., Rosoklija, I., Jacobson, J., Shnorhavorian, M., Pavone, M. E., Moravek, M. B., Bonifacio, H. J., Simons, L., Hudson, J., Fechner, P. Y., Gomez-Lobo, V., Kadakia, R., Shurba, A., Rowell, E., & Woodruff, T. K. (2016b). Proceedings of the working group session on fertility preservation for individuals with gender and sex diversity. *Transgend Health*, 1, 99–107.
- Fleming, J. (2016). Gallup analysis: Millennials, Marriage and Family. *Gallup News*. Retrieved from Gallup News website: <http://news.gallup.com/poll/191462/gallup-analysis-millennials-marriage-family.aspx>.
- Gong, E. M., & Cheng, E. Y. (2017). Current challenges with proximal hypospadias: We have a long way to go. *Journal of Pediatric Urology*, 13(5), 457–467.
- Gravholt, C. H., Fedder, J., Naeraa, R. W., & Muller, J. (2000). Occurrence of gonadoblastoma in females with Turner syndrome and Y chromosome material: A population study. *The Journal of Clinical Endocrinology and Metabolism*, 85, 3199–3202.
- Hughes, I. A., Houk, C., Ahmed, S. F., Lee, P. A., & Lawson Wilkins Pediatric Endocrine Society/European Society For Paediatric Endocrinology Consensus, G. (2006). Consensus statement on management of intersex disorders. *Journal of Pediatric Urology*, 2, 148–162.
- Johnson, E. K., Finlayson, C., Rowell, E. E., Gosiengfiao, Y., Pavone, M. E., Lockart, B., Orwig, K. E., Brannigan, R. E., & Woodruff, T. K. (2017a). Fertility preservation for pediatric patients: Current state and future possibilities. *The Journal of Urology*, 198, 186–194.
- Johnson, E. K., Rosoklija, I., Finlayson, C., Chen, D., Yerkes, E. B., Madonna, M. B., Holl, J. L., Baratz, A. B., Davis, G., & Cheng, E. Y. (2017b). Attitudes towards “disorders of sex development” nomenclature among affected individuals. *Journal of Pediatric Urology*, 13(6), 608.e1–608.e8.
- Kathrins, M., & Kolon, T. F. (2016). Malignancy in disorders of sex development. *Translational Andrology and Urology*, 5, 794–798.
- Kaya, C., Bektic, J., Radmayr, C., Schwentner, C., Bartsch, G., & Oswald, J. (2008). The efficacy of dihydrotestosterone transdermal gel before primary hypospadias surgery: A prospective, controlled, randomized study. *The Journal of Urology*, 179, 684–688.
- Kropp, B. P., Cheng, E. Y., Pope, J. C. T., Brock, J. W., Koyle, M. A., III, Furness, P. D., Weigel, N. D., III, Keck, R. W., & Kropp, K. A. (2002). Use of small intestinal submucosa for corporal body grafting in cases of severe penile curvature. *The Journal of Urology*, 168, 1742–1745. discussion 1745.
- Lee, P. A., Houk, C. P., Ahmed, S. F., Hughes, I. A., & International Consensus Conference on Intersex organized by the Lawson Wilkins Pediatric Endocrine Society and the European Society for Paediatric Endocrinology. (2006). Consensus statement on management of intersex disorders. International Consensus Conference on Intersex. *Pediatrics*, 118, e488–500.
- Lee, P. A., Nordenstrom, A., Houk, C. P., Ahmed, S. F., Auchus, R., Baratz, A., Baratz Dalke, K., Liao, L. M., Lin-Su, K., Looijenga, L. H., Mazur, T., III, Meyer-Bahlburg, H. F., Mouriquand, P., Quigley, C. A., Sandberg, D. E., Vilain, E., Witchel, S., & Global, D. S. D. U. C. (2016). Global disorders of sex development update since 2006: Perceptions, approach and care. *Hormone Research in Paediatrics*, 85, 158–180.
- Malone, P. S., Hall-Craggs, M. A., Mouriquand, P. D., & Caldamone, A. A. (2012). The anatomical assessment of disorders of sex development (DSD). *Journal of Pediatric Urology*, 8, 585–591.
- Martinez-Mora, J. (1994). *Textbook of intersexual states: Disorders of sex differentiation*. Barcelona: Doyma.
- Mouriquand, P. D., Gorduza, D. B., Gay, C. L., Meyer-Bahlburg, H. F., Baker, L., Baskin, L. S., Bouvattier, C., Braga, L. H., Caldamone, A. C., Duranteau, L., El Ghoneimi, A., Hensle, T. W., Hoebeke, P., Kaefer, M., Kalfa, N., Kolon, T. F., Manzoni, G., Mure, P. Y., Nordenskjöld, A., Pippi Salle, J. L., Poppas, D. P., Ransley, P. G., Rink, R. C., Rodrigo, R., Sann, L., Schober, J., Sibai, H., Wisniewski, A., Wolffenbuttel, K. P., & Lee, P. (2016). Surgery in disorders of sex development (DSD) with a gender issue: If (why), when, and how? *Journal of Pediatric Urology*, 12, 139–149.

- Pippi Salle, J. L., Braga, L. P., Macedo, N., Rosito, N., & Bagli, D. (2007). Corporeal sparing dismembered clitoroplasty: An alternative technique for feminizing genitoplasty. *The Journal of Urology*, 178, 1796–1800. discussion 1801.
- Pleskacova, J., Hersmus, R., Oosterhuis, J. W., Setyawati, B. A., Faradz, S. M., Cools, M., Wolffenbuttel, K. P., Lebl, J., Drop, S. L., & Looijenga, L. H. (2010). Tumor risk in disorders of sex development. *Sexual Development*, 4, 259–269.
- Rangecroft, L. (2003). Surgical management of ambiguous genitalia. *Archives of Disease in Childhood*, 88, 799–801.
- Reiner, W. G., & Kropp, B. P. (2004). A 7-year experience of genetic males with severe phallic inadequacy assigned female. *The Journal of Urology*, 172, 2395–2398. discussion 2398.
- Rink, R. C., Metcalfe, P. D., Kaefer, M. A., Casale, A. J., Meldrum, K. K., & Cain, M. P. (2006). Partial urogenital mobilization: a limited proximal dissection. *Journal of Pediatric Urology*, 2, 351–356.
- Salle, J. L., Lorenzo, A. J., Jesus, L. E., Leslie, B., AlSaid, A., Macedo, F. N., et al. (2012). Surgical treatment of high urogenital sinuses using the anterior sagittal transrectal approach: A useful strategy to optimize exposure and outcomes. *Journal of Urology*, 187(3), 1024–1031. <https://doi.org/10.1016/j.juro.2011.10.162>.
- Slowikowska-Hilczar, J., Hirschberg, A. L., Claahsen-Van Der Grinten, H., Reisch, N., Bouvattier, C., Thyen, U., Cohen Kettenis, P., Roehle, R., Kohler, B., Nordenstrom, A., & DSD, L. G. (2017). Fertility outcome and information on fertility issues in individuals with different forms of disorders of sex development: Findings from the dsd-LIFE study. *Fertility and Sterility*, 108, 822–831.
- Snodgrass, W. T., Villanueva, C., Granberg, C., & Bush, N. C. (2014). Objective use of testosterone reveals androgen insensitivity in patients with proximal hypospadias. *Journal of Pediatric Urology*, 10, 118–122.
- Spoor, J. A., Oosterhuis, J. W., Hersmus, R., Biermann, K., Wolffenbuttel, K. P., Cools, M., Kazmi, Z., Ahmed, S. F., & Looijenga, L. H. J. (2017). Histological assessment of gonads in DSD: Relevance for clinical management. *Sexual Development*.
- Swerdlow, A. J., Schoemaker, M. J., Higgins, C. D., Wright, A. F., Jacobs, P. A., & Group, U. K. C. C. (2005). Cancer incidence and mortality in men with Klinefelter syndrome: A cohort study. *Journal of the National Cancer Institute*, 97, 1204–1210.
- Van Batavia, J. P., & Kolon, T. F. (2016). Fertility in disorders of sex development: A review. *Journal of Pediatric Urology*, 12, 418–425.
- Vanderbrink, B. A., Rink, R. C., Cain, M. P., Kaefer, M., Meldrum, K. K., Misseri, R., & Karmazyn, B. (2010). Does preoperative genitography in congenital adrenal hyperplasia cases affect surgical approach to feminizing genitoplasty? *The Journal of Urology*, 184, 1793–1798.
- Wolffenbuttel, K. P., Hersmus, R., Stoop, H., Biermann, K., Hoebeke, P., Cools, M., & Looijenga, L. H. (2016). Gonadal dysgenesis in disorders of sex development: Diagnosis and surgical management. *Journal of Pediatric Urology*, 12, 411–416.
- Wright, I., Cole, E., Farrokhvar, F., Pemberton, J., Lorenzo, A. J., & Braga, L. H. (2013). Effect of preoperative hormonal stimulation on postoperative complication rates after proximal hypospadias repair: A systematic review. *The Journal of Urology*, 190, 652–659.

Further Reading

- Villanueva, C., Granberg, C., & Bush, N. C. (2014). Objective use of testosterone reveals androgen insensitivity in patients with proximal hypospadias. *Journal of Pediatric Urology*, 10, 118–122.

Surgical Reassignment From Female to Male

Ervin Kocjancic, University of Illinois Hospital and Health Science System, Chicago, IL, United States

Valerio Iacovelli, University of Rome Tor Vergata, Rome, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

Gender dysphoria (GD; *Diagnostic and Statistical Manual of Mental Disorders*, Fifth Edition) is characterized by a marked discrepancy between one's birth-assigned sex and one's gender identity and expression and is associated with immense bodily and emotional distress (Gooren, 2011). Some people with this condition, in order to manage the discrepancy or imbalance they experience, desire to possess the secondary sexual characteristics of the opposite sex (Brown, 1990). Not all transgender persons have gender dysphoria. For those who do, medical and surgical therapy can play a pivotal role in relieving their psychological discomfort (Monstrey et al., 2001; Imbimbo et al., 2009; Vujovic et al., 2009).

Gender reassignment surgery is indicated for the treatment of gender dysphoria. Patients who suffer gender dysphoria feel that their birth gender is somehow “wrong” and seek to adopt the opposite gender role. To facilitate this change, many patients seek surgery so that their bodies resemble their chosen gender. Gender reassignment surgery refers to all surgical procedures that a patient wishes to receive to resemble the appearance of the opposite gender. Sex reassignment surgery is part of gender reassignment surgery and refers only to the reconstruction of the genital area.

Currently, the guidelines on gender reassignment are published by the World Professional Association for Transgender Health (WPATH), and the standards of care are updated regularly and available for download from the WPATH website (World Professional Association, 2015a, b).

Epidemiology

Although early estimates on the prevalence of gender dysphoria were focused on identification of individuals for gender confirmation surgery, it was later realized that some individuals neither desired, nor were candidates for, such surgery (Kockott and Fahrner, 1987).

Early estimates of the prevalence of transsexualism were 1 in 37,000 biological males and 1 in 107,000 biological females (Roberto, 1983). Interestingly, approximately three times as many biological males as compared with biological females sought genital surgery. This historical discrepancy may exist for multiple reasons, including less accessibility, and more complicated—and expensive—surgical options available for biological females. However, there has been an increase in the number of biological females seeking genital surgery. More recent data estimate that 1 in 11,900 adult biological males and 1 in 30,400 adult biological females undergo genital procedures (PJ et al., 1996).

The true prevalence of gender dysphoria is likely much greater than these early estimates. With increased advocacy, acceptance, and access to care, individuals are now able to seek medical attention in an atmosphere free from harassment or shame.

Etiology

The cause of gender dysphoria remains unknown (Ettner, 2007). Research has investigated genetics, hormonal influences, family dynamics, and psychoanalytical observations as possible contributing causes (Selvaggi and Bellringer, 2011).

Existing theories on the pathogenesis of the disorder were based mainly on psychological perspectives, without supportive evidence based data (Selvaggi and Bellringer, 2011). These theories mainly focused on the role of nurture (environmental and external psychological factors) as the cause of gender identity disorder. By contrast, studies from the past two decades advocate a neurological basis for gender identity with sexual morphological differentiation of the brain (Selvaggi and Bellringer, 2011; Zhou et al., 1995; Kruijver et al., 2000; Swaab et al., 2002). These researchers have found that the volume of the central subdivision of the bed nucleus of the stria terminalis—a brain area that is essential for sexual behavior—is larger in men than in women, with similar findings in transsexual individuals (FtM transsexuals have the same size in this specific brain area as their male counterparts, and vice versa) (Selvaggi and Bellringer, 2011; Zhou et al., 1995; Kruijver et al., 2000; Swaab et al., 2002). In a 2008, another sex differentiated area in the brain, the hypothalamic uncinate nucleus, was demonstrated to have a relationship with gender identity (Garcia-Falgueras and Swaab, 2008).

Most probably, the etiology of transsexualism is both biological (nature) and dependent on external psychological influence (nurture) (Selvaggi and Bellringer, 2011). Although the etiology of transsexualism remains unknown, most specialists concede that psychotherapy aimed at “adjusting the mind to the body” is not an effective treatment on its own: no demonstrable, successful conversions of transsexual persons via psychotherapy alone have been observed (Meyer et al., 2001). Most professionals concur that gender reassignment surgery, or “adjusting the body to the mind”, is the best way to assist individuals with severe gender dysphoria who desire this procedure (Cohen-Kettenis and Kuiper, 1984; Kuiper and Cohen-Kettenis, 1988).

Disease Management

Medical Therapy

Masculinizing hormone therapy is initiated to induce virilization. The effects of ongoing testosterone therapy are multiple and, most relevant to genital reconstruction, include clitoral hypertrophy. The primary hormonal treatment is a testosterone preparation.

Before initiating hormone therapy, a psychosocial assessment by a qualified mental health or medical practitioner should be performed. The goal of endocrine therapy is to change secondary sex characteristics in order to reduce gender dysphoria and/or facilitate a physical presentation consistent with the individual's sense of self. Although specific hormonal regimens may vary between centers, the surgeon should be familiar with the possible side effects of hormonal therapy and how they relate to the surgical care of the transgender patient. These possible side effects include issues related to liver function, risk of venous thromboembolism, electrolyte imbalance, drug–drug interactions.

Masculinization through hormonal therapy for transmen follows general principles of hormone replacement for treatment of male hypogonadism. Both intramuscular and transdermal testosterone preparations are available and may be used to achieve testosterone values in the normal male range (typically 400–1000 ng/mL) (Hembree et al., 2009).

Complete blood count (CBC) and liver function level should be monitored because testosterone therapy can result in erythrocytosis and elevation in liver enzymes. Other risks of testosterone therapy include hypertension, excessive weight gain, salt retention, lipid changes, cystic acne, and adverse psychological changes (Hembree et al., 2009).

Preoperatively, transmen should discontinue their testosterone 2 weeks before gender confirmation surgery. Testosterone can be converted to estradiol in adipose tissue by the aromatase enzyme and this could augment the risk of postoperative venous thromboembolism.

Patients should not restart their testosterone therapy until they are ambulatory after surgery (Schechter, 2017).

Surgical Therapy

The World Professional Association for Transgender Health recommends that female-to-male transgender genital reconstruction proceed in accordance with the following criteria: “[if] persistent gender dysphoria is documented, [if] the patient has capacity to make informed consent [and] has reached legal age (if younger follow the standard of care for children and adolescents), [if] significant medical or mental health concerns are present they must be well controlled, [if] the patient has undergone 12 continuous months of hormone therapy (unless there is a medical contraindication or the patient is unwilling), and [if] the patient has lived continuously for at least 12 months in the gender role that is congruent with their gender identity” (Selvaggi et al., 2012).

Surgical procedures for the natal female subject being affirmed as a male subject (hereto referred to as female-to-male FtM transgender surgery) can be divided in genital reconstruction (bottom surgery), often paired with a combination of other hormonal and surgical interventions (e.g., urethroplasty, hysterectomy, oophorectomy) and male chest contouring (nongenital surgery or “top surgery”) and facial feminization (Frey et al., 2017).

Nongenital Surgery (Schechter, 2017; Frey et al., 2017)

Chest surgery

The chest wall is the main area in which additional surgery may be required, as the possession of female breasts all but precludes satisfactory passing in male role, other than by using tight binding. This procedure, commonly performed before genital surgery, involves bilateral subcutaneous mastectomies, liposuction of the chest, and repositioning and resizing of the nipple–areola complex, when necessary. Several different techniques are used, and the choice of technique depends on the volume of breast parenchyma, degree of breast ptosis, position and size of the nipple–areola complex, and degree of skin elasticity.

Mastectomy needs to be carried out by a surgeon with wide experience in FtM mastectomy. The exact technique will depend on the volume of breast tissue, and the skin excess and elasticity (Monstrey et al., 2008). Hormone therapy is discontinued 2 weeks before surgery. Small, nonptotic breasts can often be treated with periareolar incisions (limited incision). In these cases, the nipple may be reduced by a wedge resection of the lower pole, but the areola is not repositioned. A small amount of tissue is left beneath the nipple–areola to preserve viability. Circumareolar (purse-string) or vertical incisions with free nipple–areola grafts can be used for larger breasts with mild ptosis requiring smaller amounts of skin removal. Finally, traditional transverse inframammary crease incisions (double incision) with free nipple areola grafts may better serve individuals with larger breast volumes and increased breast ptosis requiring large amounts of skin removal. Finally, the nipple itself and the diameter of the areola are often reduced. When done properly, the results may be very satisfactory, but poor technique can lead to unacceptable cosmetic results. Minor revisions to ameliorate the final cosmetic outcome are often required (Monstrey et al., 2008). Additional complications include, but are not limited to, hematoma, seroma, infection, delayed healing, loss of nipple grafts, asymmetry, and underresection and overresection of tissue leading to chest wall contour abnormalities.

Gynecology

Patients will also require hysterectomy and ovariectomy, because of the potential risk of endometrial carcinoma with protracted testosterone use, and are usually accomplished laparoscopically. However, access for the vaginal route may be limited in nulliparous patients on testosterone therapy. Most transmen undergoing genital surgery will have undergone a hysterectomy

and oophorectomy at least 3 months before either metoidioplasty or phalloplasty. The short blind-ended vagina can be left in place or removed.

Genital Surgery (Schechter, 2017; Frey et al., 2017)

The aims of FtM genital surgery are to permit micturition in a standing position, provide bulk inside underwear and swimwear for cosmetic appearance, as well as enable sexual function, with erection, and penetrative intercourse. Ideally, these aims should be achieved with preservation of genital sensation sufficient for orgasm, minimal scarring, and a good cosmetic outcome. Not all patients require all of the aims to be fulfilled; interestingly, the ability to urinate in a standing position is the most common request.

The goal of genital surgery in transmen may range from clitoral release (metoidioplasty), with or without urethral lengthening, to creation of a penis, capable of sexual penetration. Transmen undergoing genital surgery discontinue their hormone therapy 2 weeks before surgery and complete a bowel preparation the day before surgery. Before surgery, sequential compression devices are placed and intravenous antibiotics are administered. Chemoprophylaxis for venous thromboembolism is administered subcutaneously.

Metoidioplasty

The procedure entails lengthening the hormonally hypertrophied clitoris by release of the suspensory ligament and resection of the ventral chordee. In order to allow for urination while standing, the natal female urethra is lengthened with the use of labia minora, vaginal musculomucosal flaps, and buccal mucosal grafts. The native urethral plate may be divided to elongate the neophallus; however, this division necessitates additional urethroplasty to recreate a functional neourethra (Stojanovic and Djordjevic, 2015; Djordjevic and Bizic, 2013). Alternatively, the urethral plate may be left intact by using a W incision anterior to and above the urethra (Hage, 1996). Local flaps for urethral reconstruction include dorsal clitoral flaps, which incorporate a superior buttonhole to allow passage of the flap over the neophallus, and inferior labia minora flaps (Stojanovic and Djordjevic, 2015; Hage, 1996). Reconstruction of the bulbar urethra can be bolstered or extended by an anterior vaginal wall flap (Stojanovic and Djordjevic, 2015; Djordjevic and Bizic, 2013). On completion of the urethroplasty, the clitoral bulbs around the neourethra are closed (Stojanovic and Djordjevic, 2015).

Additional masculinization of the lower abdominal and pubic areas may be performed with mons resection. This procedure, performed through a Pfannenstiel incision, is designed to resect and reposition excess fatty tissue often found overlying the natal female pubis. Anchoring of Scarpa fascia to the rectus sheath aids with stabilization of the pubic tissue. The mons resection is typically performed during placement of the testicular prostheses, so as not to communicate with the suprapubic tube, placed at the time of the initial metoidioplasty surgery. The mons resection helps to create the illusion of a longer phallus following metoidioplasty.

Although metoidioplasty may permit urination while standing, it does not allow placement of an erectile device, and it is unlikely that individuals undergoing metoidioplasty will be able to engage in penetrative intercourse (Perovic and Djordjevic, 2003). In addition, scrotal reconstruction following metoidioplasty positions the scrotum in a lateral, rather than an anterior, position. As such, patients may describe their testicles as being positioned “between their legs” rather than “in front of their legs.” Patients are hospitalized for 2–3 days to allow for pain control, intravenous antibiotics, and incisional care. Urination through the penis typically begins about 3 weeks after the procedure, following assessment of the urethral conduit. The suprapubic catheter is removed once the patient demonstrates effective transurethral voiding. Complications following metoidioplasty include, but are not limited to, hematoma, delayed healing, infection, and urethral fistula and/or stricture.

The metoidioplasty procedure is less complex compared with phalloplasty, avoids the disadvantage of additional scarring at remote donor sites, and may be converted to a phalloplasty at a later date, if desired. For some transmen, these considerations are sufficient to warrant the choice of metoidioplasty over phalloplasty.

As previously mentioned, some individuals who have undergone metoidioplasty request subsequent conversion to a phalloplasty. In such circumstances, the glans penis, representing the former glans clitoris, is de-epithelialized and separated from the reconstructed urethra. In addition, one of the dorsal clitoral nerves is harvested for later coaptation to the medial antebrachial cutaneous nerve of the forearm. The glans-urethral construct is transposed subcutaneously and anchored in position to the pubic symphysis. The now redundant skin of the penile shaft, representing the former skin of the labia minora, is excised. If possible, the scrotum is repositioned with anterior advancement of the previous scrotoplasty. The remainder of the procedure follows the principles of the phalloplasty outlined in the next section.

Phalloplasty

Phalloplasty represents the most complete genitoperineal transformation for transmen. Phalloplasty techniques may be divided into pedicled flaps and free flaps. Pedicle flaps transfer tissue, typically from the thigh, groin, or lower abdomen to reconstruct the penis, whereas free flaps involve the microsurgical transfer of tissue from a remote location.

The suprapubic phalloplasty is a pedicled flap technique and gives reasonable results (Bettocchi et al., 2005). A flap is raised on the anterior abdominal wall and rolled into a tube, which becomes the penis. A combination of vaginal epithelium and skin can then be used to create a neourethra, although this tube is seldom of sufficient length to open at the tip of the new phallus and typically opens ventrally. Nevertheless, it does allow micturition in the standing position. The clitoris is left in position at the

base of the new phallus to provide sexual sensation. If erectile function is required, and tissue is sensate, an inflatable prosthesis can subsequently be used.

Abdominal phalloplasties have largely been replaced in modern clinical practice by free-flap techniques. The radial forearm free flap remains the gold standard for phallic reconstruction. This procedure transfers a full thickness flap, including the radial artery, venae comitantes, cephalic vein, and lateral and medial antebrachial cutaneous nerves, from the forearm to reconstruct the penis and urethra. The perineal reconstruction is performed with construction of the fixed, or membranous, urethra, from the vaginal and vestibular lining, as well as scrotal reconstruction with labia majora flaps. Urethral complications, including both strictures and fistulae, are not uncommon, and additional, secondary procedures performed at a later date are required for placement of testicular implants and the erectile prosthesis. Usually, phalloplasty is performed with two surgical teams. One team performs the vaginectomy, colpocleisis, creation of the membranous urethra, scrotoplasty, and placement of a suprapubic tube, while the second team harvests and shapes the forearm flap, then closes the forearm donor site. The forearm flap is divided into two parts; a “tube within-a-tube” technique is used to create the neophallus. The urethra is created from tubularization of the ulnar border of the flap, and the skin of the penile shaft is created from the skin of the radial border of the flap. After detachment from the donor site, the neophallus is put into position on the pubic symphysis; the urethral anastomosis is performed first and then the blood vessels are anastomosed. Nerve wraps are placed over the neurotomy. Placement of penrose drains in each groin and layered closure of the skin complete the procedure.

Closure of the forearm donor site is performed by reapproximation of the muscle bellies over a closed suction drain. A skin graft may be applied to the forearm donor site, or, alternatively, a bilayer wound matrix may be placed. If a wound matrix is used, a skin graft is performed at a later date. Most often, negative-pressure wound therapy (NPWT) in conjunction with a topical antimicrobial dressing is used to dress the forearm donor site. A light compression wrap is then applied. Postoperatively, patients are maintained on a variable duration of bed rest with both transurethral and suprapubic catheters. The phallus is maintained in a vertical position with a soft gauze dressing. Urination through the penile urethra may begin as early as 3 weeks following phalloplasty.

Perhaps the next most used technique for phalloplasty is the use of tissue from the thigh, known as the anterolateral thigh (ALT) flap. Similar to the forearm technique, tissue, including the lateral femoral cutaneous nerve, may be transferred from the thigh to construct the penis. A musculocutaneous latissimus dorsi (MLD) flap may also be used for phalloplasty. Two downsides of this flap are the lack of a sensory nerve in the donor tissue and potential bulk of the constructed phallus.

Testicular implants and penile prostheses can be placed at a secondary procedure. Although the testicular implants may be placed as early as 3 months following phalloplasty, the penile prosthesis is typically placed 9 to 12 months after the phalloplasty. This amount of time allows the development of protective sensation, which is important for retention of the erectile device. Anchoring of the prosthesis can be a challenge and often requires fixation to the pubic bone. Anchoring of the prosthesis may be performed with suture fixation to the periosteum of the pubis, or, alternatively, bone anchoring. In addition, the prostheses can be wrapped with an acellular dermal matrix to help form a “pseudo corpora cavernosa.”

Complications

Unfortunately, phalloplasty in FtM surgery carries a high complication rate (overall >40%) (Monstrey et al., 2009). Complications following phalloplasty can be divided into early and late categories. Early complications tend to be flap-related and include delayed wound healing and flap failure. Additional early complications include venous thromboembolism, bleeding, and infection.

Most late complications are urologic in nature. Other late complications can be related to the erectile prosthesis; these include erosion, either through the shaft skin or urethra, dislodgement, and mechanical failure. Furthermore, disruption of the neurotomy can occur during placement of the prosthesis, resulting in loss of sensation to the phallus.

Urethral problems occur commonly after phalloplasty (Hoebeker et al., 2005), and additional procedures may be required. Complications include urinary tract infection, urethrocutaneous fistula, urethral stricture, urethral diverticula, and hair within the reconstructed urethra. Symptoms may include a weak urinary stream, leakage of urine from the penile shaft or scrotum, dysuria, urinary frequency, incomplete emptying, and inability to urinate (Hoebeker et al., 2005).

Conclusion

The vast majority of existing information on outcomes in female-to-male genital reconstruction is considered low-quality evidence (Sutcliffe et al., 2009). Existing studies on patient satisfaction are limited by a general lack of validated, standardized methods, a paucity of controlled studies, little prospective data collection, and poor response rates in long-term follow-up studies.

Gender reassignment surgery is one of the most challenging surgical disciplines. In FtM surgery, the gold standard technique has not yet been identified and, depending on a patient's request, a different surgical approach should be used. Furthermore, very few centers have the experience of, and subsequently can offer, different techniques for FtM gender reassignment. Moreover, complications are frequent and limitations to the ideal reconstruction are present with every technique used. The complex psychological background of the patients and their expectations further challenge gender reassignment surgeons. Thus, a very skilled, dedicated and cooperative team is necessary to achieve good results.

References

- Bettocchi, C., Ralph, D. J., & Pryor, J. P. (2005). Pedicled pubic phalloplasty in females with gender dysphoria. *BJU International*, 95, 120–124.
- Brown, G. R. (1990). A review of clinical approaches to gender dysphoria. *The Journal of Clinical Psychiatry*, 51(2), 57–64.
- Cohen-Kettenis, P., & Kuiper, B. (1984). Transseksualiteit en psychotherapie [Dutch]. *Tijdschrift voor Psychotherapie*, 3, 153–166.
- Djordjevic, M. L., & Bizic, M. R. (2013). Comparison of two different methods for urethral lengthening in female to male (metoidioplasty) surgery. *The Journal of Sexual Medicine*, 10, 1431–1438.
- Etner, R. (2007). *The etiology of transsexualism*. New York: The Haworth Press.
- Frey, J. D., Poudrier, G., Chiodo, M. V., & Hazen, A. (2017). An update on genital reconstruction options for the female-to-male transgender patient: A review of the literature. *Plastic and Reconstructive Surgery*, 139(3), 728–737.
- Garcia-Falgueras, A., & Swaab, D. F. (2008). A sex difference in the hypothalamic uncinate nucleus: Relationship to gender identity. *Brain*, 131(Pt 12), 3132–3146.
- Gooren, L. J. (2011). Care of transsexual persons. *The New England Journal of Medicine*, 364, 1251–1257.
- Hage, J. J. (1996). Metoidioplasty: An alternative phalloplasty technique in transsexuals. *Plastic and Reconstructive Surgery*, 97, 161–167.
- Hembree, W. C., Cohen-Kettenis, P., Delemarre-van de Waal, H. A., et al. (2009). Endocrine treatment of transsexual persons: An Endocrine Society clinical practice guideline. *The Journal of Clinical Endocrinology and Metabolism*, 94(9), 3132–3154.
- Hoebeker, P., Selvaggi, G., Ceulemans, P., et al. (2005). Impact of sex reassignment surgery on lower urinary tract function. *European Urology*, 47(3), 398–402.
- Imbimbo, C., Verze, P., Palmieri, A., et al. (2009). A report from a single institute's 14-year experience in treatment of male-to-female transsexuals. *The Journal of Sexual Medicine*, 6(10), 2736–2745.
- Kockott, G., & Fahrner, E. M. (1987). Transsexuals who have not undergone surgery: a follow-up study. *Archives of Sexual Behavior*, 16(6), 511–522.
- Kruijver, F. P., et al. (2000). Male-to-female transsexuals have female neuron numbers in a limbic nucleus. *The Journal of Clinical Endocrinology and Metabolism*, 85, 2034–2041.
- Kuiper, B., & Cohen-Kettenis, P. (1988). Sex reassignment surgery: A study of 141 Dutch transsexuals. *Archives of Sexual Behavior*, 17, 439–457.
- Meyer, W., 3rd., et al. (2001). The Harry Benjamin International Gender Dysphoria Association's standards of care for gender identity disorders, sixth version. World Professional Association for Transgender Health [online]. <http://www.wpath.org/documents2/socv6.pdf>.
- Monstrey, S., Hoebeker, P., Dhont, M., et al. (2001). Surgical therapy in transsexual patients: A multidisciplinary approach. *Acta Chirurgica Belgica*, 101(5), 200–209.
- Monstrey, S., et al. (2008). Chest wall contouring surgery in female-to-male (FTM) transsexuals: A new algorithm. *Plastic and Reconstructive Surgery*, 121, 849–859.
- Monstrey, S., et al. (2009). Penile reconstruction: Is the radial forearm flap really the standard technique? *Plastic and Reconstructive Surgery*, 124, 510–518.
- Perovic, S. V., & Djordjevic, M. L. (2003). Metoidioplasty: A variant of phalloplasty in female transsexuals. *BJU International*, 92, 981–985.
- PJ, V. K., Gooren, L. J., & Megens, J. A. (1996). An epidemiological and demographic study of transsexuals in the Netherlands. *Archives of Sexual Behavior*, 25(6), 589–600.
- Roberto, L. G. (1983). Issues in diagnosis and treatment of transsexualism. *Archives of Sexual Behavior*, 12(5), 445–473.
- Schechter, L. S. (2017). *Surgical Management of the Transgender Patient* (1st Edition). Elsevier, Inc., ISBN 978-0-323-48089-5
- Selvaggi, G., & Bellringer, J. (2011 May). Gender reassignment surgery: An overview. *Nature Reviews. Urology*, 8(5), 274–282.
- Selvaggi, G., Dhejne, C., Landen, M., & Elander, A. (2012). The 2011 WPATH standards of care and penile reconstruction in female-to-male transsexual individuals. *Advances in Urology*, 2012, 581712.
- Stojanovic, B., & Djordjevic, M. L. (2015). Anatomy of the clitoris and its impact on neophalloplasty (metoidioplasty) in female transgenders. *Clinical Anatomy*, 28, 368–375.
- Sutcliffe, P. A., Dixon, S., Akehurst, R. L., et al. (2009). Evaluation of surgical procedures for sex reassignment: A systematic review. *Journal of Plastic, Reconstructive & Aesthetic Surgery*, 62, 294–306. discussion 306.
- Swaab, D. F., Chun, W. C., Kruijver, F. P., Hofman, M. A., & Ishuina, T. A. (2002). Sexual differentiation of the human hypothalamus. *Advances in Experimental Medicine and Biology*, 511, 75–105.
- Vujovic, S., Popovic, S., Sbutega-Milosevic, G., et al. (2009). Transsexualism in Serbia: A twenty-year follow-up study. *The Journal of Sexual Medicine*, 6(4), 1018–1023.
- World Professional Association for Transgender Health press release May 26, 2010. Available at: [http://www.wpath.org/uploaded_files/140/files/depsychopathologisation 5–26-10 on letterhead.pdf](http://www.wpath.org/uploaded_files/140/files/depsychopathologisation%205-26-10%20on%20letterhead.pdf). Accessed December 1, 2015a.
- World Professional Association for Transgender Health Standards of Care. Available at: [http://www.wpath.org/uploaded_files/140/files/Standards of Care, V7 Full Book.pdf](http://www.wpath.org/uploaded_files/140/files/Standards%20of%20Care,%20V7%20Full%20Book.pdf). 2012. Accessed December 1, 2015b.
- Zhou, J. N., Hofman, M. A., Gooren, L. J., & Swaab, D. F. (1995). A sex difference in the human brain and its relation to transsexuality. *Nature*, 378, 68–70.

Further Reading

- Gooren, L. J. (2014). Management of female-to-male transgender persons: Medical and surgical management, life expectancy. *Current Opinion in Endocrinology, Diabetes, and Obesity*, 21, 233–238.

MENTAL HEALTH, ETHICS AND THE LAW

Psychological Aspects of Male Reproductive Medicine

Amanda Kohlmeier and Susan Klock, Northwestern University Feinberg School of Medicine, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Infertility, the inability of a couple to achieve pregnancy after 12 months of unprotected intercourse with regular, ovulatory cycles, affects approximately 1 in 10 couples of reproductive age. Of those, approximately 30%–40% of the cases are attributed to male factor infertility. Even though male factor infertility contributes to a significant percentage of infertility cases, the research on the psychological aspects of male infertility is disproportionately low. The purpose of this article is to provide an overview of the psychological aspects of male reproductive medicine and male infertility and its treatment.

The infertility journey begins after a couple has been trying unsuccessfully to conceive for many months and then see their physician to begin a fertility work up. The female partner typically has a physical exam, blood tests to check her hormone levels and other diagnostic tests to determine her fertility status. The male partner will also undergo blood tests and a semen analysis to determine if there is a male factor involved. The semen analysis can yield several results in relation to volume, total sperm count, sperm concentration, motility and morphology. **Table 1** provides information with reference ranges for normal semen analysis results (Cooper et al., 2010).

When abnormal results are found on the semen analysis they can occur in one or several aspects of the semen parameters. Some common male factor diagnoses are:

- (1) Azoospermia: complete absence of sperm from the ejaculate, present in about 1% of all men and 10%–15% of infertile men.
- (2) Aspermia: complete absence of seminal fluid emission upon ejaculation.
- (3) Oligospermia: low sperm count, defined by the World Health Organization (WHO) as concentrations less than 15 million sperm/mL, which is consistent with the 5th percentile for fertile men. Oligospermia is further classified as:
 - (a) Mild: concentrations 10–15million sperm/mL;
 - (b) Moderate: concentrations of 5–10 million sperm/mL and;
 - (c) Severe: less than 5 million sperm/mL.

Differentiation of azoospermia from severe oligospermia is accomplished by examination of the pellet of a centrifuged semen sample on at least two occasions.

- (4) Teratozoospermia, also known as teratospermia, is a semen alteration in which there is a large number of spermatozoa with abnormal morphology.

There can be many causes of male factor infertility but most causes are unknown. Common causes of male infertility include genetics, infection, trauma, varicocele, meningitis, diabetes, fever, alcohol or drug abuse, exposure to chemotherapy, and/or radiation, pharmaceuticals that interact with testosterone. Some causes may be reversible, others are not but can be overcome with treatment, such as in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI).

Psychological Research on Male Infertility

Despite the cause, the psychological impact of male infertility is significant. Emotional distress, such as depression, anxiety or sexual dysfunction are commonly reported after a diagnosis of male factor infertility. In addition, sexual performance anxiety can develop and further affect a man's psychological state. Even though male factor infertility contributes to a significant percentage of infertile

Table 1 Normative semen parameters (Cooper et al., 2010)

Volume	1.5 mL (1.4–1.7)
Total sperm count (count × volume)	39 million per ejaculate (33–46)
Sperm concentration (number per milliliter)	15 million/mL (12–16)
Vitality (live spermatozoa)	58% live (55–63)
Progressive motility (forward movement)	32% (31–3)
Total motility	40% (38–42)
Morphology (normal shape)	4% (3%–4%)

One-sided lower reference limits, the 5th centiles (95th percent confidence intervals).

cases, the research on the psychological aspects of infertility are disproportionately focused on women. [Petok \(2006\)](#) provides a thorough overview of the psychological reaction to male infertility including theoretical perspectives and cultural perspectives.

In terms of the research on the psychological impact of male infertility, [Luk and Loke \(2015\)](#) conducted a systematic review of the literature on the impact of infertility on psychological well-being. In their review they identified three studies that specifically focused on men's adjustment to infertility. One study by [Ahmadi et al. \(2011\)](#) assessed symptoms of depression in a sample of 114 infertile men in Iran. Ahmadi et al. noted that in traditional cultures, fatherhood is regarded as the equivalent of manhood. Therefore, there can be a stigma against infertile men as being "futile and useless" and their masculinity is "questionable." In order to assess the psychological impact of infertility among men, the authors assessed demographic information and depression in a series of men presenting to an infertility center. Using the Beck Depression Inventory, they found that 43% of the sample had depressive symptoms consistent with severe depression. Having less education (lower than a 12th grade education) was associated with greater depressive symptomatology. These rates are comparatively high compared to the 15% reported by [Drosdzol and Skrzypulec \(2009\)](#) and 5% reported by [Volgsten et al. \(2008\)](#) in European samples. [Gao et al. \(2013\)](#) found that in a sample of 1468 Chinese infertile men, 38% endorsed symptoms of anxiety and 15% reported symptoms of depression. Moreover, 19% of infertile men reported experiencing premature ejaculation and 18% suffered from erectile dysfunction. [Mikkelsen et al. \(2013\)](#) reported 28% of their sample of 210 Danish men with infertility reported that reduced sperm quality affected their perceptions of their masculinity and adversely affected their sense of well-being. In contrast to these findings, [Holter et al. \(2007\)](#) found no difference in psychological adjustment between men with male factor infertility compared to men with other causes of infertility prior to beginning IVF or ICSI treatment.

[Williams and Zappert \(2006\)](#) noted that grief reactions are common after a diagnosis of infertility. As [Mahlstedt \(1985\)](#), in her classic article describing the psychological aspects of infertility, described the psychological adjustment can be conceptualized through a loss and grief framework. The losses identified with male factor infertility include: (1) loss of relationship(s) with spouse, fertile friends, family members; (2) loss of health; (3) loss of sexual satisfaction; (4) loss of status in social community and; (5) loss of self-esteem. These losses are frequently expressed during counseling with men after the diagnosis of a male factor infertility.

Clinical Description—Grief and Loss

Infertile men frequently discuss feeling fearful that their infertility will undermine their relationship with their wife or significant other because it will prevent their partner from fulfilling their important life goal of having a child. Additionally, this loss is sometimes expressed as giving the partner permission to leave the relationship in order to pursue a relationship with a fertile man. Other losses related to relationships are the changes in relationships with fertile family or friends. Men often feel reluctant to talk to their male friends about their infertility diagnosis due to the association of infertility with virility and masculinity. Men may also remove themselves from family gatherings and interactions where the topic of having children will come up because they do not want to have to disclose or explain their infertility situation to others. Particularly in our pro-natal culture, "when are you going to have a baby?" is a common question for couples soon after they get married or begin living together. This common, seemingly benign, question can prompt anxiety, withdraw and frustration to a man with infertility.

Another loss associated with male factor infertility is loss of health. Men are frequently required to undergo several blood tests, semen analyses and possible other procedures, such as testicular biopsies, in the course of the diagnosis and treatment of infertility. The frequent trips to the clinic, the procedures themselves and the medical interventions to create a pregnancy can all contribute to the medicalization of reproduction and the perception of being "sick" in otherwise healthy men.

A loss that has received significant attention has been the loss of sexual satisfaction and sexual self-esteem. Several researchers have documented the occurrence of erectile dysfunction, diminished sexual interest and activity following a diagnosis of male factor infertility ([Luk and Loke, 2015](#)). Some men describe feeling like "what is the point?" of having sexual intercourse after their diagnosis because the reproductive aim of intercourse has been negated. For men with oligospermia, timed intercourse can increase performance demand and anticipatory anxiety which detracts from actual performance, thus creating a cycle of pressure to perform at a specific time, anticipatory anxiety and subsequent sexual difficulties. Additionally, assisted reproductive treatments often require the man to produce a semen sample at home or in the clinic to use for intrauterine insemination or IVF. The timed, clinical, nature of producing these samples as well as the pressure to produce a "good" sample are all contributors to the concerns about sexual functioning.

The other losses, such as the loss of self-esteem and the perceived loss of status in one's social community have received less attention in the literature on the psychological aspects of male infertility but are evident in clinical interactions with male infertility patients. One study, by [Nachtigall and his colleagues](#) reported that fertility is central to a man's sense of gender identity and that infertility may pose a threat to masculinity ([Nachtigall et al., 1992](#)). These authors found that almost two thirds of infertile men that they interviewed reported being preoccupied with their loss of physical potency and felt stigmatized related to their loss of masculinity that infertility caused. Additionally, as men consider various pathways to parenthood (IVF/ICSI, donor sperm use, adoption) or child-free living, they frequently consider these options in the context of their social community. "What would people think? is a common starting question related to status within their social community as men consider sharing their infertility situation with others or begin talking with others about child-free living, adoption or donor sperm use. The perceived loss of status men experience as they consider not having children is significant. Men frequently discuss feeling like they will not be as

respected or considered independent until they have children of their own. Nongenetic options for parenting (adoption, donor sperm use) frequently lead to concerns of not being the “real” father of a child and perceived loss of status as a father to a non-genetically related child.

Psychological Factors Influencing Male Fertility

In recent years, psychological stress has been postulated to affect male infertility by affecting testosterone levels and subsequent spermatogenesis. Nargund (2015) provides a review of this topic and possible mechanisms of action for both acute and chronic stress. Stress could affect fertility via the hypothalamic–pituitary–gonadal axis. Clinical studies investigating the interaction of stress and infertility have yielded conflicting results. Some studies have shown reduced sperm concentration and changes in other semen parameters during times of stress (exams, war, work stress). Other studies do not find this effect. Additionally, behaviors used to cope with stress (smoking, drinking alcohol, using drugs) can also affect semen parameters. Li et al. (2011) conducted a review of socio-psycho-behavioral factors and semen quality. In their review of 57 studies, they investigated several factors that were hypothesized to affect semen quality. These included: age, body mass index (BMI) psychological stress, smoking, alcohol and caffeine consumption, passive smoking, and sedentary lifestyle. Based on the results of their review, they concluded that age is negatively associated with semen volume. They also concluded that psychological stress could reduce sperm density and progressive motility and increase the number of abnormal sperm. Specifically, they found that job stress, life events or social support related stress could adversely affect semen quality. Smoking was found to be a risk factor for all semen parameters. Alcohol consumption was associated with lower semen volume. The effect of BMI and caffeine consumption was considered uncertain on the five most common sperm parameters. It should be noted that the studies reviewed by Li et al. included samples of fertile and infertile men, and varied in sample size and semen parameters assessed. The largest number of studies addressed the impact of smoking on semen and indicated a clear impact. Smoking is clearly a public health problem and is a behaviorally modifiable variable which can affect male fertility.

Male Psychological Response to Treatment (IVF)

After a diagnosis of male infertility, the couple may consider treatment options to address the infertility problem in order to achieve a pregnancy. One of the most common treatments for male factor infertility is IVF with or without ICSI. The psychological aspects of IVF have been studied extensively (see Verhaak et al., 2007 for a review) but these studies have predominantly focused on women. An exception to this is a recent review by Martins et al. (2016) who examined studies on men’s adjustment to infertility treatment to determine if male psychological adjustment to unsuccessful infertility treatment varies over time and if there are any psychological protective or risk factors that are related to psychological maladaptation. Martins et al. identified 12 studies that met their criteria of quantitative, longitudinal design and measurement of psychological adjustment. The study time frames varied from 4 weeks to 5 years post treatment.

In three studies looking at men’s adjustment after unsuccessful treatment, Martins et al. found that men reported an increase in depressive symptoms within a few weeks after the baseline assessment. Two studies measured anxiety in men after a diagnosis of azoospermia. In general, they found lowered levels of anxiety from baseline to 4 weeks after diagnosis. In terms of coping strategies, it was found that the use of active coping strategies decreased and the use of religiousness and seeking meaning coping increased in men 4 months after the infertility work-up. In terms of social support, men reported less social support and more negative reactions and comments from family and friends 1 year after beginning infertility treatment.

Psychological Support and Counseling During Male Infertility Treatment

After a diagnosis of male infertility, the couple is confronted with a decision about whether to pursue medically assisted reproduction (MAR) (IVF, ICSI, donor sperm use). Urologists or fertility specialists frequently refer their patients to a mental health professional with expertise in infertility counseling. The initial counseling can be focused on helping process the emotional reaction to the infertility diagnosis and discussing the decision whether to pursue MAR. Wischmann (2013) sites clinical research with recommendations for increasing the utilization of counseling by infertile men. These recommendations include:

- (1) Provide pre-treatment educational brochures about men, for men
- (2) Explain the potential benefits of counseling to both partners
- (3) Introduce psychological counseling and support before treatment begins
- (4) Make direct contact and connection with male patients
- (5) Present counseling and psychological support as an integrated part of infertility treatment

These suggestions can ease the burden for male patients and facilitate the delivery of psychological care. Given the concern about depression, anxiety or sexual dysfunction, the use of a psychological screening tool, such as the FertiQol (Boivin et al., 2011) or the SCREENIVF (Verhaak et al., 2010) can be used to identify men who may benefit from a psychology counseling referral.

If the couple decides to pursue IVF, with or without ICSI, it is helpful to have one or more counseling sessions to discuss the treatment process, including the steps in the treatment (injectable fertility medications, ultrasound monitoring and blood tests, oocyte retrieval, embryo creation and transfer, waiting for pregnancy test results). This helps the male patient increase his treatment knowledge and subsequently provide more focused support for his partner during treatment. During pre-treatment counseling the male partner can also express his feelings of sadness, loss, and guilt and those feelings can be processed and discussed with his partner. Then counseling can progress to a discussion of coping during IVF and ways the man can use instrumental coping skills to be engaged in the treatment process and support his partner during the treatment. Developing a treatment calendar, helping administer the medication injections, checking in via texts or phone calls and setting aside time for the couple to discuss their thoughts and feelings about treatment can all be adaptive coping strategies to use during IVF. The mental health professional can model and role play ways in which the partner can be supportive and help manage the treatment stress.

In the case of azoospermia, where the only option to create a pregnancy is donor sperm use, the role of psychological counseling and support is also important. In this context, the patient and his partner are typically seen for one or more sessions of counseling to prepare them for this unique family building options. [Klock and Maier \(1991\)](#) have previously described the topics to be covered during these types of consultations. The consultations begin with the mental health professional helping the man process his feelings about the diagnosis and its cause. His wife or partner can also express her sadness, frustration and support for her partner as they reconsider the joint goal of parenting. If they decide against child-free living, then the mental health professional can review the various types of family building options, including donor sperm use, adoption or foster parenting. The advantages and disadvantages of each family building option can be discussed.

If the decision is made to pursue donor sperm use, then specific psychological and social issues need to be considered before beginning with treatment. First, the man must examine his thoughts and beliefs about fatherhood. This usually begins with a recollection of his own relationship with his father, its strengths and weaknesses, and the patient's perception of the importance of genetic versus relationship bonds with his father. The next topic is typically the man's perception of the importance of the genetic connection or the relationship connection in the development of a paternal relationship with a child (nature vs. nurture). Men who feel that the preponderance of the father-child relationship is predetermined by the genetic relationship may have a more difficult time accepting donor sperm use and often opt not to undergo this treatment. If the genetic connection is extremely important to the patient, then the use of a related (brother, cousin) sperm donor may be discussed. The medical and psychological screening of known gamete donors is provided by the American Society for Reproductive Medicine (ASRM) practice committee ([ASRM, 2013](#)). If a known donor is not possible or preferred, then an anonymous donor can be selected from a sperm bank. The selection of a donor is another topic for pre-treatment counseling. Typically, the couple selects a donor that has physical resemblance to the patient in terms of ethnic background, height, hair color, eye color, and other characteristics. In addition, sometimes the patient selects a donor that physically resembles him by using a photo matching process. Alternatively, some patients do not want to see photos or other additional materials provided by the donor because they feel it interferes with their ability to bond with the potential child.

An important topic in the donor sperm counseling is the discussion of privacy and disclosure. This refers to the discussion with the prospective parents about their plans to tell the child, family and friends about the child's donor sperm origin. The discussion of these two topics is interrelated because the plan to tell the child is often closely related to plans to tell others. Couples may tell family and friends about the infertility diagnosis and therefore feel they "have to" tell about the use of donor sperm once the wife or partner becomes pregnant. The decision to tell others directly relates to whether they tell the child. A follow-up study of donor sperm parents found that most parents regretted telling other people because they felt that they had to tell the child for fear of the child finding out from someone other than the parents ([Klock et al., 1994](#)). An additional concern about telling others is that parents worry that their child may be angry or hurt if he/she found out that others knew of their conception story before they did. While most couples intend to tell their child about their donor conception origin, counseling by a mental health professional can provide a neutral place to discuss the decision and to practice how to tell the child. Parents often feel that they "don't have the words" to use to describe this unique form of family building to a pre-schooler or young child. Related to the disclosure discussion is the decision of whether or not to use an identity release donor. An identity release donor is a donor who agrees to provide identity related information to the donor conceived child when the child reaches the age of 18. This is an increasingly common occurrence in the field of donor sperm use and has led to the development of many half-sibling family groups.

For all of these psychological support and counseling services, provision of service by a mental health professional with expertise in infertility is a must. The detailed, specific fund of knowledge about infertility and its psychological concomitants is needed in order to provide optimal psychological services. Guidelines for the qualifications of mental health professionals are provided in [Mental Health Professional Group of the American Society for Reproductive Medicine \(2006\)](#). In addition, support information for patients about donor sperm use can be found at [RESOLVE.org](#) (a patient advocacy website), [ASRM.org](#) and [BICA.org](#) (British Infertility Counseling Association).

Conclusion

In conclusion, male infertility affects a significant proportion of couples wishing to become pregnant. While historically the focus on the psychological aspects of infertility has been focused predominantly on women, in the past several years more emphasis has been placed on understanding the man's experience and to increase the provision of support to infertile men as they pursue their

family building goals. While men may process and cope with the diagnosis of infertility differently than women, their experiences deserve equal attention, sensitivity and focus in the clinical and research realms.

References

- Ahmadi, H., Montaser-Kouhsari, L., Nowroozi, M., & Bazargan-Hejazi, S. (2011). Male infertility and depression: a neglected problem in the Middle East. *Journal of Sexual Medicine*, 8, 824–830.
- Boivin, J., Takefman, J., & Braverman, A. (2011). The Fertility Quality of Life (FertiQoL) tool: development and general psychometric properties. *Fertility and Sterility*, 96, 409–415.
- Cooper, T. G., Noonan, E., von Eckardstein, S., Auger, J., Baker, H. W., Behre, H. M., et al. (2010). World Health Organization reference values for human semen parameters. *Human Reproduction Update*, 16, 231–245.
- Drosdzol, A., & Skrzypulec, V. (2009). Depression and anxiety among Polish infertile couples. *Journal of Psychosomatic Obstetrics and Gynecology*, 30, 11–20.
- Gao, J., Zhang, X., Su, P., Liu, J., Shi, K., Hao, Z., Zhou, J., & Liang, C. (2013). Relationship between sexual dysfunction and psychological burden in men with infertility: a large observational study in China. *Journal of Sexual Medicine*, 10(8), 1935–1942.
- Holter, H., Anderheim, L., Bergh, C., & Moller, A. (2007). The psychological influence of gender infertility diagnosis among men about to start IVF and ICSI treatment using their own sperm. *Human Reproduction*, 22, 2559–2565.
- Klock, S. C., & Maier, D. (1991). Guidelines for the provision of psychological evaluations for infertility patients at the University of Connecticut Health Center. *Fertility and Sterility*, 56, 680–685.
- Klock, S., Jacob, M., & Maier, D. (1994). A prospective study of donor insemination recipients: secrecy, privacy, and disclosure. *Fertility and Sterility*, 62, 477–484.
- Li, Y., Lin, H., Li, Y., & Cao, J. (2011). Association between socio-psycho-behavioral factors and male semen quality: a systematic review and meta-analyses. *Fertility and Sterility*, 95, 116–123.
- Luk, B., & Loke, A. Y. (2015). The impact of infertility on the psychological well-being, marital relationships, sexual relationships, and quality of life of couples: a systematic review. *Journal of Sex & Marital Therapy*, 41(6), 610–625.
- Mahlstedt, P. (1985). The psychological component of infertility. *Fertility and Sterility*, 43, 335–346.
- Martins, M., Basto-Pereira, M., Pedro, J., Peterson, B., Almeida, V., Schmidt, L., & Costa, M. (2016). Male psychological adaptation to unsuccessful medically assisted reproduction treatments: a systematic review. *Human Reproduction Update*, 22, 466–478.
- Mental Health Professional Group of the American Society for Reproductive Medicine. (2006). Qualifications guidelines for mental health professionals in reproductive medicine. In S. N. Covington, & L. H. Burns (Eds.), *Infertility counseling: a comprehensive handbook for clinicians* (2nd edn, pp. 559–560). Cambridge: Cambridge University Press.
- Mikkelsen, A., Madsen, S., & Humaidan, P. (2013). Psychological aspects of male fertility treatment. *Journal of Advanced Nursing*, 69, 1977–1986.
- Nachtigall, R., Becker, G., & Wozny, M. (1992). The effects of gender-specific diagnosis on men's and women's response to infertility. *Fertility and Sterility*, 57, 113–121.
- Nargund, V. (2015). Effects of psychological stress on male infertility. *Nature Reviews Urology*, 12, 373–382.
- Petok W. (2006). The psychology of gender-specific infertility diagnosis. In Covington, S.N. & Burns, L.H. (eds.) *Infertility counseling: a comprehensive handbook for clinicians*, 2nd edn. pp. 37–60, Cambridge University Press, Cambridge.
- Practice Committee of the American Society for Reproductive Medicine (ASRM) and the Practice Committee of the Society for Assisted Reproductive Technology. (2013). Recommendations for gamete and embryo donation: a committee opinion. *Fertility and Sterility*, 99, 47–62.
- Verhaak, C. M., Smeenk, J., Nahuis, M., Kremer, J., & Braat, D. (2007). Long term psychological adjustment to IVF/ICSI treatment in women. *Human Reproduction*, 22, 305–308.
- Verhaak, C., Lintsen, A., Evers, A., & Braat, D. (2010). Who is at risk of emotional problems and how do you know? Screening women going for IVF treatment. *Human Reproduction*, 25, 1234–1240.
- Volgsten, H., Skoog Svanberg, A., Ekselius, L., Lundkvist, O., & Sundstrom Promaa, I. (2008). Prevalence of psychiatric disorders in infertile women and men undergoing in vitro fertilization treatment. *Human Reproduction*, 23, 2056–2063.
- Williams KE, Zappert LN. 2006. Psychopathology and psychopharmacology in the infertile patient. In Covington, S.N. & Burns, L.H. (eds.) *Infertility counseling: a comprehensive handbook for clinicians*, 2nd edn. pp. 97–115, Cambridge University Press, Cambridge.
- Wischmann, T. (2013). "Your count is zero" – counselling the infertile man. *Human Fertility*, 16, 35–39.

Legal and Ethical Aspects of Male Reproduction

Lawrence S Ross, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

In the past fifty years there have been significant advances in the science and technology of male reproductive medicine and surgery. These advances, coupled with the rapidly expanding development of advanced reproductive technologies, and changes in societal attitudes toward same sex relationships and issues of gender identification, have created legal and ethical challenges for those who wish to parent children. This chapter explores these topics and attempts to educate the reader on the present status of these problems with emphasis on the male.

Sperm Donation, Storage, and Use

Artificial insemination for reproduction in humans was first performed in 1790. It remained the sole method of reproductive technology until the early 1950s when in-vitro fertilization (IVF) was successful in animals. Bavister B.D., (2002) Early history of in vitro fertilization. *Reproduction* Aug;124 (2):181–96. (Bavister, 2002) The first human birth resulting from IVF using fresh ejaculated sperm was reported in 1978. Steptoe P.C., Edwards R.G., (1978) Birth after the reimplantation of a human embryo. *Lancet*, 1978; 12;2:366 (Steptoe and Edwards, 1978).

Key to the development of IVF as an option for male reproductive disorders, including severe oligospermia and azoospermia, was the ability to successfully freeze and thaw sperm. Although this concept was envisioned in the late 18th century, the first human pregnancy using inseminated frozen/thawed sperm occurred in 1953 and the first live birth was reported in 1963. Banking of cryopreserved sperm from anonymous donors for use by couples in which no sperm can be obtained from the male partner, began in the 1970s. Since that time the use of donor sperm, where autologous sperm is not available, has expanded to gay single women and men, and all LGBT people wishing to have children. Women desiring pregnancy and child bearing using sperm cryopreserved from a deceased partner add to the complexity of male reproductive treatment options.

The U.S. Food and Drug Administration regulates reproductive tissue (sperm and eggs) intended for donation. People intending to use donor sperm should be advised to use sperm only from banks that have registered with and follow FDA guidelines. This information can be accessed on line. CFR - Code of Federal Regulations Title 21 - FDA (Cfr N.d.).

Legal Aspects of Sperm Donation

There are multiple complex issues surrounding sperm donation and very little law addressing these issues. What laws exist are on a state by state basis. In most states with sperm donor laws, the donor has no rights, obligations, or interest with the child born resulting from assisted reproductive technology (ART), which includes artificial insemination or IVF; and the child born as result of ART has no rights, obligations, or interest with the sperm donor. In the case of artificial insemination these legal protections may only exist if the insemination is done by a licensed medical professional. Self-insemination with a donor's sperm may not protect the donor, woman, couple, or child from issues of paternity, child support, or visitation rights. In some states, if a child is born resulting from use of donor sperm, the husband may not be recognized by the state as the father. Similar issues can occur with egg or embryo donation and surrogacy births. 'Ross & Zuckerman, LLP' <http://www.infertilityattorney.com/articles.html> (Infertilityattorney, N.d.). The Uniform Parentage Act of 2002 is an attempt to deal with these issues. 'Uniform Parentage Act' www.uniformlaws.org/shared/docs/parentage/2016AM_AmendedParentage_Draft.pdf (Uniformlaws, N.d.).

A more complex and controversial issue is posthumous reproduction using sperm cryopreserved after death. Land S., Ross L.S., (2002) Posthumous reproduction: current and future status. *Urologic Clinics of North America*; 29 (4), 863–871 (Land and Ross, 2002) Post-mortem reproduction occurs in nature. The female praying mantis kills her mate during copulation. In humans the concept of producing offspring using sperm after the death of a man is a subject of both legal and ethical debate. In addition, there are numerous technical issues regarding the extraction of sperm: how long is the sperm viable after death; should the sperm be extracted from the vas deferens, epididymis, or testis? Various reports have shown that sperm are still functional for intervals of 24–30 h after death. In theory, ejaculated sperm could be obtained from a patient deemed brain dead but kept on life support for the harvest of other organs, but such retrieval has rarely been reported.

The prevalence of and actual use of post-mortem sperm for procreation are anecdotal. The true number of offspring conceived with post-mortem sperm is unknown. The legal issues surrounding posthumous reproduction are similar to those affecting other "organ" donations, but can be infinitely more complex. Patel Z.P., Ross H.E., Ross L.S., (2012) Request for posthumous fatherhood with perimortem surgical sperm retrieval. *Assisted Reproduction Techniques: Challenges and Management Options*; 353 (Patel et al., 2012).

Most guidance is determined state by state. The Illinois Uniform Anatomical Gift Act states: "Any individual who is of sound mind and has reached the age of 18 may give all or any part of his or her body as an anatomical gift. Such a gift, of course, would take effect only upon the individual's death. Making such a gift in advance eliminates the need to obtain consent from anyone else.

If an individual has not provided for an anatomical gift before death, another person, as listed in the priority below, may make an anatomical gift on behalf of the decedent (regardless of age), after or immediately before death:

1. The decedent's spouse.
2. The decedent's adult sons or daughters.
3. Either of the decedent's parents.
4. Any of the decedent's adult brothers or sisters.
5. The guardian of the decedent at the time of his/her death.
6. Any person authorized or under obligation to dispose of the body."

Of interest, sperm is rarely referenced in such state guides, or is specifically excluded. Illinois Anatomical Gift Act (755 ILCS 50/) <http://www.ilga.gov/legislation/ilcs/ilcs5.asp?ActID=2114&ChapterID=60>(lga, N.d.)

The number of potential legal issues associated with donor sperm are considerable. They include suits that have been filed for mistaken labeling of specimens involving race or ethnicity of the donor, undisclosed familial genetic issues, failure of laboratory systems to protect cryopreservation leading to lost sperm samples, and transmission of HIV or other infections. Among the most interesting was a case in which the wife of a prominent and wealthy man secretly banked a specimen of her husband's sperm. She informed the laboratory personnel that her husband was too busy to come to the lab and she would bring in the specimen with a signed and notarized contract from her spouse. Her plan was to have the husband murdered, become pregnant with his sperm, and lay claim to his considerable estate. The plan was foiled when the lab called the husband because the second year's storage fee was not paid. The wife offered an undercover police officer \$5000 to carry out the murder, was arrested, convicted at trial, and sentenced to 30 years in prison. *The Heir, His wife, and the Hit Man*, by Nancy Millman, *Chicago Magazine* 1999;48:57–9, 116–9. See, <http://www.chicagomag.com/Chicago-Magazine/1982-2000-1982/February-1999-Table-of-Contents/> (Chicago, 1999). Most semen laboratories today require donors to appear in person with valid ID, and produce the specimen on site.

Ethical Aspects of Sperm Donation

The use of donated sperm raises a number of ethical issues. With regard to use for reproduction these include, but are not limited to: 1) rights of the donor; 2) rights of clients (purchasers of sperm); 3) criteria for selection of donors; 4) amount of sperm a single man can donate; 5) costs associated with use of donor sperm.

The rights of donors include the right of anonymity and protection from claims of parenthood or financial obligations from offspring or parents. Clients purchasing sperm from commercial sperm banks have the right to expect that donors have had screening for potential transmission of communicable genetic abnormalities, sexually transmitted diseases including HIV, gonorrhea, syphilis, HPV, CMV, and most recently, Zika virus. Testing for or family history of potentially adverse genetic disorders are a frequent concern for recipients of donor sperm. Most sperm banks have arbitrary selection criteria for age range, height, and education. Virtually all commercial banks include race, ethnic origin, and religion of donors. One bank, the Repository for Germinal Choice which existed in California from 1980 to 1999 was known as the "Nobel prize sperm bank", although only one Nobel prize winner, William Shockley, donated sperm. 'The "Genius Babies" and How They Grew'. See, www.slate.com/articles/life/seed/2001/02/the_genius_babies_and_how_they_grew.html (Slate, N.d.).

Most selection criteria are chosen to attempt to provide recipients with some assurance that they and their potential offspring will be protected from transmissible diseases, and be able to choose desired physical, religious, ethnic, educational, and racial characteristics. Some ethicists raise concerns regarding the phenomenon of promoting "designer" offspring as well as the specter of eugenics. Concerns of the number of specimens a donor can provide and the number of recipients for sperm raise issues of consanguinity. There is little to no regulation for the number of specimens a man may donate. The number of children a single donor has or may have fathered is subject to speculation and there are no reliable statistics available.

'Delivery Man': 9 Sperm-Donation Questions You're Too Embarrassed to Ask'. See, <http://healthland.time.com/2013/11/22/delivery-man-the-9-sperm-donation-questions-youre-too-embarrassed-to-ask>(Healthland, N.d.).

The costs associated with donor reproduction also raise ethical questions. Are sperm and egg donors providing their gametes for reasons of altruism or profit? Could such motives affect or compromise the honesty of donors in the selection process? Donor insemination costs may range from \$300 to \$4000 depending on source of the sperm with lower cost using the male partner's sperm compared to anonymous donor sperm. Typical costs for egg donation can range from \$12,000 to \$20,000 and having a child by surrogacy can be considerably more.

'American Pregnancy Association, Donor Insemination'. See, <http://americanpregnancy.org/infertility/donor-insemination/> (Americanpregnancy, N.d.). 'ConceiveAbilities, Surrogate Mother Pay'. See, <https://www.conceiveabilities.com/surrogates/surrogate-mother-pay>(Conceiveabilities, N.d.).

Other special areas of ethical concern for sperm donation have developed in recent years. These require physicians caring for certain groups of patients to think about future reproductive needs when working with these patients. Medical oncologists must understand and think about the therapies they prescribe and their potential effects on reproductive tissues. Adults who need chemotherapy, radiation, or surgery should be educated and advised to consider preservation of their gametes when possible.

Cryopreservation of sperm can be accomplished quickly and rarely delays oncologic therapy. Egg freezing has advanced in recent years but the process is invasive and requires planning. It must be offered early in the oncology diagnostic workup.

Transgender people contemplating hormonal therapy and/or surgical reassignment should be counseled about their potential desire to parent children as should gay men with conditions that may affect their reproductive potential, including varicoceles. Since varicoceles are found in up to 50% of men with abnormal semen parameters they should be told that there is rarely a significant health concern requiring surgery but it is a common cause of decline in fertility. Recently, the author saw a 20 year old gay male with a large varicocele who wanted to know if he needed surgery. When told the only strong indication would be decline in sperm concentration and motility which can lead to infertility, he said "you know I'm gay". He was asked if he ever thought about the possibility of having a child as a single parent or with a partner and wanting such a child with his genetic material. He was pleased to be alerted to thinking about the issue.

The explosion of marketing for testosterone supplementation for low testosterone ("low T") in aging men has raised ethical concerns in the medical establishment and in government. Data from insurance carriers has shown a steady 10 year rise in prescriptions for supplemental testosterone preparations. Experience with testosterone abuse in athletes brought attention to the deleterious effects of testosterone supplements on male fertility and other research has been suggestive of additional risks including cardiovascular problems and concern in patients with prostate cancer. Because testosterone supplementation has been available to some men without adequate medical consultation or education as to risks (trainers, workout facilities, "Low T" clinics without physicians trained in the proper indications and risks of such therapy) increasing numbers of men of reproductive age have been compromised by such treatment. The magnitude of the problem has recently been examined by the Centers for Disease Control and Prevention (CDC) and a consortium of reproductive urologists. Rao PK, Boulet SL, Mehta A, Hotaling J, Eisenberg ML, Honig SC, et al. (2017) Trends in testosterone replacement therapy use from 2003 to 2013 among reproductive-age men in the United States. *The Journal of Urology* 197 (4), 1121–1126 (Rao et al., 2017).

Recent research has demonstrated the successful development of sperm and oocytes from mouse embryonic and induced pluripotent stem cells. Some progress has been made in the differentiation of human germ cells from human embryonic stem cells and pluripotent stem cells. Given the direction of such research it is theoretically possible to envision the creation of human embryos in the laboratory. Such embryos could be used for research or ultimately for reproductive purposes. The ethical concerns of such developments are being considered internationally. Ishii, T, Reijo Pera, R.A., Greely, T.E. (2013) Ethical and legal issues arising in research on inducing human germ cells from pluripotent stem cell. *Cell Stem Cell*. Aug.; 2 (1): 145–8. See, <http://www.sciencedirect.com/science/article/pii/S1934590913003135> (Sciencedirect, N.d.; Ishii et al., 2013).

References

- Bavister, B. D. (2002). Early history of in vitro fertilization. *Reproduction*, 1242, 1896.
- Cfr-N.d. Code of Federal Regulations Title 21- Fda.
- Americanpregnancy, N.d. <http://americanpregnancy.org/infertility/donor-insemination/>.
- Conceiveabilities, N.d. <https://www.conceiveabilities.com/surrogates/surrogate-mother-pay>.
- Healthland, N.d. <http://healthland.time.com/2013/11/22/delivery-man-the-9-sperm-donation-questions-youre-too-embarrassed-to-ask/>.
- Ilga,N.d. <http://www.ilga.gov/legislation/ilcs/ilcs5.asp?actid=2114&chapterid=60>.
- Infertilityattorney N.d. <http://www.infertilityattorney.com/articles.html>.
- Sciencedirect, N.d. <http://www.sciencedirect.com/science/article/pii/S1934590913003135>.
- Ishii, T., Reijo Pera, R. A., & Greely, T. E. (2013). Ethical and legal issues arising in research on inducing human germ cells from pluripotent stem cells. *Cell Stem Cell*, 2(1), 145–148.
- Land S, Ross LS. (2002) Posthumous reproduction: Current and future status. *Urologic Clinics of North America*: 29(4), 863–871
- Patel, Z. P., Ross, H. E., & Ross, L. S. (2012). Request for posthumous fatherhood with perimortem surgical sperm retrieval. *Assisted Reproduction Techniques: Challenges and Management Options.*, 353–359.
- Rao, P. K., Boulet, S. L., Mehta, A., Hotaling, J., Eisenberg, M. L., Honig, S. C., et al. (2017). Trends in testosterone replacement therapy use from 2003 to 2013 among reproductive-age men in the United States. *The Journal of Urology.*, 197(4), 1121–1126.
- Stepptoe, P. C., & Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. *Lancet*, 12(2), 366.
- Chicago, 1999. The heir, his wife, and the hit man, *Chicago Magazine*; 48:57–9, 116–9.
- Slate, N.d., www.Slate.Com/Articles/Life/Seed/2001/02/The_Genius_Babies_And_How_They_Grew.Html.
- Uniformlaws, N.d. www.uniformlaws.org/shared/docs/parentage/2016AM_AmendedParentage_Draft.pdf.

MALE REPRODUCTIVE CANCER AND DISEASE

Benign Prostatic Hyperplasia (BPH)

Ervin Kocjancic, University of Illinois Hospital and Health Science System, Chicago, IL, United States

Valerio Iacovelli, University of Rome Tor Vergata, Rome, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

Benign prostatic hyperplasia (BPH), is one of the causes of the lower urinary tract symptoms (LUTS) and a common diagnosis among the aging male population with increasing prevalence. The understanding of the LUTS as a functional unit, and the multifactorial etiology of associated symptoms, means that LUTS now constitute the main focus, rather than the former emphasis on BPH ([European Association of Urology, 2016](#); [Gratzke et al., 2015](#); [McVary et al., 2011](#)). LUTS present with dysfunction of both storage and emptying phase as well as the post micturition signs. These symptoms are nonspecific in their presentation and multifactorial in origin. LUTS are a progressive and age related but not sex- or organ-specific. They cause bother and impair quality of life ([Agarwal et al., 2014](#)).

Traditionally, LUTS have been related to bladder outlet obstruction (BOO) as a result of benign prostatic obstruction (BPO), which is often caused by benign prostatic enlargement (BPE) resulting from the histologic condition benign prostatic hyperplasia (BPH) ([Abrams et al., 2002](#)). Several recent studies have shown, however, that LUTS are not necessarily related to prostatic size. Major attention is currently oriented to the bladder wall; bladder dysfunctions, such as detrusor underactivity, detrusor overactivity and other functional abnormalities ([van Koeveeringe et al., 2014](#)).

As the world population ages, the incidence and prevalence of BPH and LUTS have rapidly increased ([Lepor, 2005](#)). Historical studies have examined nonmodifiable risk factors—including age, genetics and geography—that have important roles in the etiology of BPH and BOO ([Parsons, 2010](#)). However, several modifiable risk factors present new opportunities for treatment and prevention ([Chughtai et al., 2016](#)).

Epidemiology

Approximately 50% of men > 50 years of age will have pathological evidence of BPH, with this number increasing to > 80% as men reach their eighth decade of life and older ([Berry et al., 1984](#)).

LUTS are strongly associated with aging and progress dynamically: for some individuals LUTS persist and progress over long time periods, and for others they remit ([Société Internationale d'Urologie \(SIU\), Lower Urinary Tract Symptoms \(LUTS\), 2013](#)).

Several studies have examined the geographical variations in BPH and LUTS prevalence. Men from Southeast Asia have significantly smaller prostate volumes than men from western countries. Although differences in geographical prevalence for prostate disease (including BPH) have been documented, the reasons for such differences are poorly understood. However, these differences could be due to genetic factors and/or environmental differences ([Jin et al., 1999](#)).

Etiology

Many modifiable and nonmodifiable factors can increase the risk of development and progression of BPH and LUTS.

Age

BPH and LUTS increase with age, which has been confirmed by numerous studies ([Berry et al., 1984](#)). Prostate volume increases at a median rate of 0.6 mL per year. Although symptom severity cannot be correlated directly with prostate volume, having a large prostate volume is a risk factor for the development of LUTS. Thus, the incidence of LUTS also increases with age. That is, larger prostates are associated with increased risks of urinary retention, increased future need for surgery and clinical progression of BPH ([Société Internationale d'Urologie \(SIU\), Lower Urinary Tract Symptoms \(LUTS\), 2013](#)).

Metabolic Syndrome

Many studies have investigated associations between metabolic syndrome and BPH-related LUTS (BPH-LUTS) and results of several studies support possible roles for the individual components of the syndrome in the development of BPH-LUTS ([Vignozzi](#)

et al., 2016). Metabolic syndrome includes hypertension, dyslipidaemia, glucose intolerance, central obesity and insulin resistance with compensatory hyperinsulinaemia (De Nunzio et al., 2012).

- *Obesity and BPH–LUTS.* Obesity, and in particular visceral obesity, is often comorbid with BPH (Parsons, 2010; Vignozzi et al., 2016). More importantly, the risk of LUTS progression also increased as a function of obesity (BMI ≥ 35 kg/m²), visceral adiposity (waist circumference > 42 in., 106.7 cm). The influence of obesity on prostate overgrowth was observed also in early adulthood. Overall, these findings support the concept that the treatment of obesity might be important in the prevention and treatment of LUTS (Vignozzi et al., 2016).
- *Glucose intolerance and BPH–LUTS.* A possible association between BPH and glucose intolerance or overt T2DM has also been noted (Hammarsten et al., 1998). In particular, in patients with BPH, a history of T2DM increased the risk of moderate–severe LUTS twofold (Joseph et al., 2003). Interestingly, individuals with insulin resistance who develop compensatory hyperinsulinaemia might directly and/or indirectly affect several molecular signaling pathways that promote prostatic growth and inflammation (Nandeesha et al., 2006).
- *Dyslipidaemia and BPH–LUTS.* Circulating total cholesterol and HDL cholesterol are positively and negatively associated with prostate enlargement, respectively (Nandeesha et al., 2006). Furthermore, it has been shown that men with hypertriglyceridaemia had decreased likelihood of storage LUTS improvement during the follow-up period, and greater abdominal fat mass was associated with storage LUTS progression. In the same study, lower HDL cholesterol levels, as well as lower testosterone and higher oestradiol levels, were associated with worsening of voiding LUTS (Martin et al., 2014).
- *Hypertension and BPH–LUTS.* The role of hypertension is debated. Increased blood pressure seems to be associated with an increased odds ratio for surgical treatment of BPH (Gann et al., 1995).

Although insulin resistance and visceral obesity are considered to be the main pathophysiological aberrations of metabolic syndrome, a number of other factors, such as a proinflammatory state and sex steroid imbalance, could also be involved in its pathogenesis and clinical manifestation. In particular, chronic low-grade inflammation has been recognized as a central pathogenic mechanism underlying the development of individual metabolic syndrome components. In addition, in the past decade, clinical and preclinical evidence has confirmed that chronic inflammation also has a determining role in inducing BPH–LUTS (Fibbi et al., 2010; Vignozzi et al., 2014). That is, an inflammatory insult is the most plausible candidate bridging the mechanistic gap between metabolic syndrome and BPH–LUTS (Fibbi et al., 2010; Vignozzi et al., 2014).

Genetics

To date few genetic studies have focused on BPH. A recent study reported that variants in 2q31 and 5p15 are associated with aggressive (defined by multiple criteria) BPH (Qi et al., 2013). Furthermore, twin studies have suggested that heritability is an important determinant of disease severity in BPH, including the development of LUTS.

Pathogenesis

The pathophysiology of BOO in men with BPH has been attributed to both static (prostatic tissue) and dynamic (smooth muscle) factors. BPH is a diagnosis based on histological findings of proliferating stromal and epithelial cells within the prostatic transition zone. The exact etiology of BPH is unclear; however, much epidemiological evidence has led to the hypothesis that a sex steroid imbalance and an inflammatory process are the key drivers of not only the development but also the progression of BPH (Fibbi et al., 2010; Vignozzi et al., 2014).

Testicular androgens are required in the prostate for the development of BPH. The enzyme steroid 5 α -reductase 2 which is bound to the nuclear membrane, converts testosterone into dihydrotestosterone (DHT), the principal androgen in the prostate accounting for 90% of total prostatic androgen. Testosterone and its derivatives interact with the androgen receptor (AR), which upon binding translocates to the nucleus and binds to the androgen response element (ARE), promoting the expression of genes encoding various growth factors (including keratinocyte growth factor (KGF), epidermal growth factor (EGF) and insulin-like growth factors (IGFs). In stromal cells, most of the testosterone is converted to dihydrotestosterone (DHT), which then acts in an autocrine manner to promote stromal proliferation. DHT can also diffuse into the adjacent epithelial cell to act in a paracrine manner. DHT produced peripherally in the liver and the skin can also diffuse from the circulation into the prostate to act in an endocrine manner. BPH probably develops as a result of an imbalance between mechanisms that regulate cell death and cell proliferation. Growth factors such as KGF, EGF, and IGFs, which are AR target genes, are probably involved, as is transforming growth factor- β (TGF β), which is negatively regulated by androgens (Chughtai et al., 2016; Roehrborn, 2008).

The metabolic syndrome influences LUTS–BPE development and progression, having direct inflammatory effects within the prostate (Vignozzi et al., 2014). By acting as antigen-presenting cells or as targets of Toll-like receptor agonists, prostatic stromal cells can actively contribute to the prostate-specific inflammatory process, resulting in the production of proinflammatory cytokines and chemokines, which perpetuate chronic inflammation (Vignozzi et al., 2014). Activation of Toll-like receptors and production of proinflammatory chemokines, such as IL-6 and IL-8, further promote prostate cell hyperplasia through direct proliferative action of IL-8 or through the release of other growth factors, such as FGF-2 (Vignozzi et al., 2014). Activated prostatic cells

also stimulate CD4+ lymphocyte proliferation and prompt their differentiation towards a TH1/TH17 phenotype, which shifts an acute inflammatory response towards a chronic autoimmune inflammatory disease (Vignozzi et al., 2016).

Bladder Structure and Function

The bladder is sensitive to prostatic conditions. BOO caused by BPH has been shown to affect bladder structure and function. The response of the bladder to obstruction is primarily an adaptive one. Initially, BOO can lead to the development of smooth muscle hypertrophy in the bladder^{62,63}, which is associated with intracellular and extracellular changes in smooth muscle cells (Levin et al., 2000). Such changes may lead to detrusor overactivity and decreased compliance, resulting in patients experiencing symptoms of frequency and urgency as well deterioration in the strength of the urinary stream and incomplete emptying. Trabeculation (thickening) of the bladder wall is the predominant endoscopic finding in these patients.

Diagnostic Evaluation

As BPH is a common condition affecting a considerable proportion of men, screening and prevention of the disease are not pertinent.

Several key diagnostic tools and investigations are used to diagnose BPH in men who present with LUTS. The European Association of Urology and the American Urological Association have published guidelines for the investigation of LUTS related to BPH (European Association of Urology, 2016; Gratzke et al., 2015; McVary et al., 2011).

Medical History

The importance of assessing the patient's history is well recognized. The aim of obtaining a medical history is to identify potential causes of LUTS and relevant comorbidities, such as medical (e.g., diabetes mellitus or insipidus, renal disease, heart failure, sleep apnoea) and neurological diseases. It is useful to review current medication, and assess lifestyle habits, as well as emotional and psychological factors. Potential erectile and other forms of sexual dysfunction should be investigated (validated symptom questionnaires are helpful).

Symptom Score Questionnaires

Symptom scores are helpful in quantifying LUTS and in identifying which type of symptoms are predominant, yet they are not disease-, or age-specific. Several questionnaires are available (International Prostate Symptom Score IPSS, International Consultation on Incontinence Questionnaire ICIQ-MLUTS, Danish Prostate Symptom Score DAN-PSS, AUA Symptom Score), all of which are sensitive to symptom changes and treatment monitoring.

Frequency Volume Charts and Bladder Diaries

The recording of volume and time of each void by the patient is referred to as a frequency volume chart (FVC). Inclusion of additional information such as fluid intake, use of pads, activities during recording, or symptom scores is termed a bladder diary (Abrams et al., 2002). Parameters that can be derived from the FVC and bladder diary include: daytime and night-time voiding frequency, total voided volume, the fraction of urine production during the night (nocturnal polyuria index), and volume of individual voids.

Physical Examination and Digital-Rectal Examination (DRE)

A physical examination should be performed, particularly focusing on the urinary tract. The suprapubic region should be examined for signs of bladder distension. The penis should be examined for evidence of phimosis, meatal stenosis or abnormal penile lesions.

A neurological examination should also be performed on the lower limbs to rule out a possible underlying neurological condition that might result in urinary symptoms. A DRE should be performed on all patients with LUTS. A DRE facilitates the estimation of prostate volume and can also assess the shape and consistency of the prostate and identify the presence of firm or hard areas or nodules, which can raise suspicions of prostate cancer.

Urinalysis

Urinalysis (dipstick or sediment) is an inexpensive test that must be incorporated in the primary evaluation of any patient presenting with LUTS to determine conditions such as urinary tract infection and diabetes mellitus on the basis of abnormal findings (haematuria, proteinuria, pyuria, glucosuria, ketonuria, positive nitrite test). There is limited evidence, yet general expert consensus that the benefits outweigh the costs.

Prostate-Specific Antigen (PSA)

The level of PSA has been shown to reflect prostate volume. The higher the PSA level, the greater the likelihood of an enlarged prostate—with a 69% chance of having a prostate volume of > 40 mL with a PSA level of 4.1–7.0 ng per mL (Bohnen et al., 2007). However, PSA testing should not be routine; the benefits and risks of testing should be discussed with the patient. In particular, the test is used in prostate cancer diagnostics, therefore, the possibility of a need for a prostate biopsy and associated risks should be discussed. The European Association of Urology recommends that PSA testing should only be performed if it can assist in decision making in patients at risk of BPH progression.

Renal Function Measurement

Renal function may be assessed by measurement of serum creatinine or calculation or determination of the estimated glomerular filtration rate (eGFR). Hydronephrosis, renal insufficiency, and urinary retention appear with greater prevalence in patients with symptoms or signs of BPO. In addition, patients with renal insufficiency have a higher risk of developing postoperative complications compared to those with normal renal function.

Uroflowmetry and Postvoid Residual Urine (PVR)

Urinary flow rate assessment is a basic noninvasive urodynamic test that is widely used to evaluate LUTS. Key parameters are $Q_{\max}$, voided volume, and flow pattern. Uroflowmetry parameters should ideally be evaluated when the voided volume is > 150 mL. It is recommended that at least two flow rates should be obtained. The diagnostic accuracy of uroflowmetry for detecting BOO varies considerably and is substantially influenced by diagnostic threshold values. A $Q_{\max}$ threshold of 10 mL/s had specificity of 70%, PPV of 70%, and sensitivity of 47% for BOO; thus, uroflowmetry alone is unsuitable for detection and quantification of BOO. Low $Q_{\max}$ can arise as a consequence of BOO, detrusor underactivity, or an underfilled bladder. Thus, uroflowmetry is limited as a diagnostic test because of the inability to discriminate underlying mechanisms in men with low $Q_{\max}$. Specificity can be improved by repeated flow-rate testing in individual patients. Uroflowmetry can be used to monitor treatment outcomes and correlate symptoms with objective findings.

Postvoid residual urine (PVR) can be measured by transabdominal ultrasonography, a bladder scan, or catheterization. The AUA states that determination of the PVR volume is optional in the initial diagnostic assessment and during subsequent monitoring as a safety parameter, whereas the EAU guidelines state that measurement of PVR volume in men with LUTS should be a routine part of the assessment (European Association of Urology, 2016; Gratzke et al., 2015; McVary et al., 2011).

Imaging

The routine use of upper tract imaging is not recommended for patients with BPH (European Association of Urology, 2016; Gratzke et al., 2015; McVary et al., 2011); however, it might be helpful in patients who have a urinary tract infection, urolithiasis, renal insufficiency and/or haematuria. Patients with LUTS and haematuria should be investigated. The prostate can be imaged by several modalities including TRUS, CT and MRI (European Association of Urology, 2016). TRUS or transabdominal ultrasonography are the most easily performed, although TRUS is more accurate for assessing prostate volume. TRUS is also useful in determining the degree of intravesical prostatic protrusion, which is the distance between the bladder neck and the tip of the prostate median lobe. The degree of intravesical prostatic protrusion has been shown to correlate with the severity of BOO on urodynamics. Routine use of urethrocystoscopy is not required for patients with uncomplicated LUTS (which include those without a history of urinary tract infections, haematuria, bladder stones, predominantly irritative symptoms or a secondary pathology such as bladder cancer) according to the AUA (McVary et al., 2011). Evaluation of a prostatic middle lobe in urethrocystoscopic findings is necessary to determine the indication for certain interventional treatments (European Association of Urology, 2016).

Urodynamics and Video-Urodynamics

In male LUTS, the most widespread invasive urodynamic techniques employed are filling cystometry and pressure flow studies (PFS). The major goal of urodynamics is to explore the functional mechanisms of LUTS and to identify risk factors for adverse outcomes (for informed/shared decision-making). Although urodynamic pressure–flow investigations are a second line studies, they might be necessary to measure the relative contribution of the bladder and the prostate to lower urinary tract dysfunction. Pressure–flow studies are the only method to determine if LUTS are due to BOO or due to an underactive detrusor. The EAU recommends that men < 50 years of age or those > 80 years of age who are being considered for surgery should undergo pressure–flow studies. If a patient had a previous unsuccessful surgical treatment for BPH, pressure–flow studies should be performed to assess detrusor function (European Association of Urology, 2016; Gratzke et al., 2015).

Videourodynamics (synchronous x-ray imaging and filling of the bladder with contrast medium for cystometry and PFS) may be used when there is uncertainty regarding the mechanisms of voiding LUTS. The test provides additional anatomical information.

Disease Management

Treatment options for men with LUTS start at watchful waiting and progress through medical to surgical interventions.

Conservative Treatment: Watchful Waiting, Behavioral and Dietary Modifications

Many men with LUTS are not troubled enough by their symptoms to need drug treatment or surgical intervention. All men with LUTS should be formally assessed prior to any allocation of treatment in order to establish symptom severity and to differentiate between men with uncomplicated (the majority) and complicated LUTS. Watchful waiting includes advice about lifestyle changes that can help to ameliorate or circumvent symptoms. These changes include advice about volume, type and timing of liquids consumed, avoidance of caffeine (a diuretic), abstinence of alcohol consumption in the evening and regulation of bowel movements with avoidance of constipation ([European Association of Urology, 2016](#)).

Pharmacological Treatment

The aim of medical therapy is to improve symptoms, lower the risk of progression and improve quality of life. Guidelines and algorithms are available to help selection ([European Association of Urology, 2016](#)).

- *α 1-Adrenoceptor antagonists (α 1-blockers)* ([European Association of Urology, 2016](#)). The medical therapies target are adrenergic receptor subtypes: α 1A-adrenoceptor which is the predominate receptor in prostate stromal smooth muscle and bladder neck, α 1B-adrenoceptor and the α 1D-adrenoceptor. Blockade adrenoceptors results in smooth muscle relaxation and subsequent improvement in the passage of urine. α 1-blockers are often considered the first line drug treatment of male LUTS because of their rapid onset of action, good efficacy, and low rate and severity of adverse events ([Lepor, 2007](#)). However, α 1-blockers do not prevent occurrence of urinary retention or need for surgery. Ophthalmologists should be informed about α 1-blocker use prior to cataract surgery. Elderly patients treated with nonselective α 1-blockers should be informed about the risk of orthostatic hypotension. Sexually active patients treated with selective α 1-blockers should be counseled about the risk of retrograde ejaculation.
- *5 α -reductase inhibitors* ([European Association of Urology, 2016](#)). Two 5 α -reductase inhibitors (5-ARIs) are available for clinical use: dutasteride and finasteride. Finasteride inhibits only 5 α -reductase type 2, whereas dutasteride inhibits 5 α -reductase types 1 and 2 with similar potency (dual 5-ARI). 5-ARIs act by inducing apoptosis of prostate epithelial cells leading to prostate size reduction of about 18%–28% and a decrease in circulating PSA levels of about 50% after 6–12 months of treatment. Treatment with 5-ARIs should be considered in men with moderate-to-severe LUTS and an enlarged prostate (> 40 mL) and/or elevated PSA concentration (> 1.4–1.6 ng/mL). 5 α -reductase inhibitors can prevent disease progression with regard to acute urinary retention and the need for surgery. Due to the slow onset of action, they are suitable only for long-term treatment (years). Their effect on the serum PSA concentration needs to be considered in relation to PCA screening.
- *Muscarinic receptor antagonists (antimuscarinics) and Beta-3 agonist* ([European Association of Urology, 2016](#)). Muscarinic receptors are involved in the modulation of detrusor contractility; inhibition of these receptors reduces smooth muscle tone and relieves filling phase symptoms. In the human detrusor M2 and M3 receptors are predominant (M2:M3 ratio 3:1, but the M3 subtype is functionally more important). Available agents include nonselective antagonists, such as oxybutynin, and selective antagonists, such as solifenacin and tolterodine ([Abrams et al., 2006](#)). Not all antimuscarinics have been tested in elderly men, and long-term studies on the efficacy of muscarinic receptor antagonists in men of any age with LUTS are not yet available. In addition, only patients with low PVR volumes at baseline were included in the studies. These drugs should therefore be prescribed with caution, and regular reevaluation of IPSS and PVR urine is advised. Men should be advised to discontinue medication if worsening voiding LUTS or urinary stream is noted after initiation of therapy. For patients who have a poor response to anti-muscarinic drugs or who have previously experienced adverse effects with these drugs, agonism of the β 3-adrenoceptor might be an alternative treatment option ([Thiagamoorthy et al., 2016](#)). Beta-3 adrenoceptors are the predominant beta receptors expressed in the smooth muscle cells of the detrusor and their stimulation is thought to induce detrusor relaxation. Indeed, mirabegron has recently been made available for men with moderate-to-severe LUTS who mainly have bladder storage symptoms in the United States and Europe.
- *Phosphodiesterase 5 inhibitors (PDE5Is)* ([European Association of Urology, 2016](#)). Phosphodiesterase type 5 (PDE5) negatively regulates smooth muscle contraction by hydrolysing cGMP (which is crucial to muscle relaxation via its effects on intracellular calcium levels). Accordingly, inhibition of PDE5 leads to the relaxation of smooth muscle in the bladder neck, urethra and prostate ([Gacci et al., 2012](#)). To date, only tadalafil 5 mg once daily has been officially licensed for the treatment of male LUTS with or without ED. The meta-regression suggested that younger men with low body mass index and more severe LUTS benefit the most from treatment with PDE5Is. There is limited information on reduction of prostate size and no data on disease progression.
- *Plant extracts—phytotherapy* ([European Association of Urology, 2016](#)). Herbal drug preparations are made of roots, seeds, pollen, bark, or fruits. There are single plant preparations (mono-preparations) and preparations combining two or more plants in one pill (combination preparations). The most widely used plants are *Cucurbita pepo* (pumpkin seeds), *Hypoxis rooperi* (South African star grass), *Pygeum africanum* (bark of the African plum tree), *Secale cereale* (rye pollen), *Serenoa repens* (syn. Sabal serrulata; saw palmetto) and *Urtica dioica* (roots of the stinging nettle). Nowadays, there are not any specific

recommendations on phytotherapy for the treatment of male LUTS due to product heterogeneity, a limited regulatory framework, and methodological limitations of the published trials and meta-analyses.

- *Combination therapies* (European Association of Urology, 2016). Compared with α 1-blockers or 5-ARI monotherapy, combination therapy results in a greater improvement in LUTS and increase in $Q_{\max}$ and is superior in prevention of disease progression. However, combination therapy is also associated with a higher rate of adverse events. Combination therapy should therefore be prescribed primarily in men who have moderate-to-severe LUTS and are at risk of disease progression (higher prostate volume, higher PSA concentration, advanced age, higher PVR, lower $Q_{\max}$, etc.). Combination therapy should only be used when long-term treatment (more than 12 months) is intended and patients should be informed about this. Discontinuation of the α 1-blocker after 6 months might be considered in men with moderate LUTS (Roehrborn et al., 2010). Combination treatment of an α 1-blocker with a muscarinic receptor antagonist is helpful in patients with moderate-to-severe LUTS if relief of storage symptoms has been insufficient with monotherapy with either drug (Filson et al., 2013). However, measuring PVR is recommended during combination treatment.

Surgical Treatment

Many surgical options are available for men with BPH. Surgical treatment is usually required when patients have experienced recurrent or refractory urinary retention, overflow incontinence, recurrent UTIs, bladder stones or diverticula, treatment-resistant macroscopic haematuria due to BPH/BPE, or dilatation of the upper urinary tract due to BPO, with or without renal insufficiency (absolute operation indications, need for surgery).

Additionally, surgery is usually needed when patients have not obtained adequate relief from LUTS or PVR using conservative or medical treatments (relative operation indications). The choice of surgical technique depends on prostate size, comorbidities of the patient, ability to have anesthesia, patients' preferences, willingness to accept surgery-associated specific side-effects, availability of the surgical armamentarium, and experience of the surgeon with these surgical techniques.

- *Transurethral resection of the prostate (TURP) and transurethral incision of the prostate (TUIP)* (European Association of Urology, 2016). TURP removes tissue from the transition zone of the gland. TUIP involves incising the bladder outlet without tissue removal. This technique may replace TURP in selected cases. TURP and TUIP are effective treatments for moderate-to-severe LUTS secondary to BPO. The choice should be based primarily on prostate volume (<30 mL and 30–80 mL suitable for TUIP and TURP, respectively). No studies on the optimal cut-off value exist but the complication rates increase with prostate size. Bipolar TURP (B-TURP) addresses a major limitation of monopolar TURP (M-TURP) by allowing performance in normal saline. The choice of B-TURP should be based on equipment availability, surgeon's experience, and patient's preference.
- *Open prostatectomy* (European Association of Urology, 2016). It is the oldest surgical treatment for moderate-to-severe LUTS secondary to BPO. Obstructive adenomas are enucleated using the index finger, approaching from within the bladder (Freyer procedure) or through the anterior prostatic capsule (Millin procedure). It is used for substantially enlarged glands (>80–100 mL). Open prostatectomy is the most invasive surgical method but it is an effective and durable procedure for the treatment of LUTS/BPO. In the absence of an endourological armamentarium including a holmium laser or a bipolar system, OP is the surgical treatment of choice.
- *Laser treatments of the prostate* (European Association of Urology, 2016). Different types of lasers are available: holmium:yttrium-aluminium garnet (Ho:YAG) laser, the 532 nm (Greenlight) laser, Diode laser, Thulium:yttrium-aluminium-garnet laser. Laser operations are surgical procedures that require experience and relevant endoscopic skills. The experience of the surgeon is the most important factor affecting the results and the overall occurrence of complications. In general, follow-up data showed efficacy and safety outcomes similar to TURP but long term results are pending.
- *Prostatic stents* (European Association of Urology, 2016). A prostatic stent requires a functioning detrusor. Permanent stents are biocompatible, allowing for epithelialisation. Temporary stents do not epithelialise and may be either biostable or biodegradable. Temporary stents can provide short-term relief from BPO in patients temporarily unfit for surgery, or after minimally invasive treatment. Due to common side effects and a high migration rate, prostatic stents have a limited role in the treatment of moderate-to-severe LUTS. Temporary stents can provide short-term relief from LUTS secondary to BPO in patients temporarily unfit for surgery or after minimally invasive treatment.
- *Prostatic urethral lift* (European Association of Urology, 2016). The prostatic urethral lift (PUL) represents a novel minimally invasive approach under local or general anesthesia. Encroaching lateral lobes are compressed by small permanent suture-based implants delivered under cystoscopic guidance (Urolift[®]) resulting in an opening of the prostatic urethra that leaves a continuous anterior channel through the prostatic fossa ranging from the bladder neck to the verumontanum. Long-term studies are needed to evaluate the duration of the effect in comparison to other techniques.

References

- Abrams, P., Cardozo, L., Fall, M., et al. (2002). The standardisation of terminology of lower urinary tract function: report from the Standardisation Sub-committee of the International Continence Society. *Neurourology and Urodynamics*, 21, 167–178.

- Abrams, P., et al. (2006). Muscarinic receptors: Their distribution and function in body systems, and the implications for treating overactive bladder. *British Journal of Pharmacology*, 148, 565–578.
- Agarwal, A., Eryuzlu, L. N., Cartwright, R., Thorlund, K., Tammela, T. L., Guyatt, G. H., Auvinen, A., & Tikkinen, K. A. (2014). What is the most bothersome lower urinary tract symptom? Individual- and population-level perspectives for both men and women. *European Urology*, 65(6), 1211–1217.
- Berry, S. J., Coffey, D. S., Walsh, P. C., & Ewing, L. L. (1984). The development of human benign prostatic hyperplasia with age. *The Journal of Urology*, 132(3), 474–479.
- Bohnen, A. M., Groeneveld, F. P., & Bosch, J. L. (2007). Serum prostate-specific antigen as a predictor of prostate volume in the community: The Krimpen study. *European Urology*, 51, 1645–1652.
- Chughtai, B., Forde, J. C., Thomas, D. D., Laor, L., Hossack, T., Woo, H. H., Te, A. E., & Kaplan, S. A. (2016). Benign prostatic hyperplasia. *Nature Reviews Disease Primers*, 2, 16031.
- De Nunzio, C., Aronson, W., Freedland, S., Giovannucci, E., & Parsons, J. (2012). The correlation between metabolic syndrome and prostatic diseases. *European Urology*, 61, 560–570.
- European Association of Urology (2016) EAU guidelines on management of non-neurogenic male lower urinary tract symptoms (luts), incl. benign prostatic obstruction (BPO). EAU <http://uroweb.org/wp-content/uploads/EAU-Guidelines-Management-of-non-neurogenic-male-LUTS-2016.pdf>.
- Fibbi, B., Penna, G., Morelli, A., Adorini, L., & Maggi, M. (2010). Chronic inflammation in the pathogenesis of benign prostatic hyperplasia. *International Journal of Andrology*, 33, 475–488.
- Filson, C. P., Hollingsworth, J. M., Clemens, J. Q., & Wei, J. T. (2013). The efficacy and safety of combined therapy with α -blockers and anticholinergics for men with benign prostatic hyperplasia: A meta-analysis. *The Journal of Urology*, 190, 2153–2160.
- Gacci, M., et al. (2012). A systematic review and meta-analysis on the use of phosphodiesterase 5 inhibitors alone or in combination with α -blockers for lower urinary tract symptoms due to benign prostatic hyperplasia. *European Urology*, 61, 994–1003.
- Gann, P. H., et al. (1995). A prospective study of plasma hormone levels, nonhormonal factors, and development of benign prostatic hyperplasia. *Prostate*, 26, 40–49.
- Gratzke, C., Bachmann, A., Descasez, A., Drake, M. J., Madersbacher, S., Mamoulakis, C., Oelke, M., Tikkinen, K. A., & Gravas, S. (2015). EAU guidelines on the assessment of non-neurogenic male lower urinary tract symptoms including benign prostatic obstruction. *European Urology*, 67(6), 1099–1109.
- Hammarsten, J., Hogstedt, B., Holthuis, N., & Mellstrom, D. (1998). Components of the metabolic syndrome—Risk factors for the development of benign prostatic hyperplasia. *Prostate Cancer and Prostatic Diseases*, 1, 157–162.
- Jin, B., Turner, L., Zhou, Z., Zhou, E., & Handelsman, D. (1999). Ethnicity and migration as determinants of human prostate size. *The Journal of Clinical Endocrinology and Metabolism*, 84, 3613–3619.
- Joseph, M. A., et al. (2003). Risk factors for lower urinary tract symptoms in a population-based sample of African-American men. *American Journal of Epidemiology*, 157, 906–914.
- Lepor, H. (2005). Pathophysiology of benign prostatic hyperplasia in the aging male population. *Revista de Urología*, 7, S3–S12.
- Lepor, H. (2007). Alpha blockers for the treatment of benign prostatic hyperplasia. *Revista de Urología*, 9, 181–190.
- Levin, R. M., et al. (2000). Obstructive response of human bladder to BPH versus rabbit bladder response to partial outlet obstruction: a direct comparison. *Neurourology and Urodynamics*, 19, 609–629.
- Martin, S., Lange, K., Haren, M. T., Taylor, A. W., & Wittert, G. (2014). Risk factors for progression or improvement of lower urinary tract symptoms in a prospective cohort of men. *The Journal of Urology*, 191, 130–137.
- McVary, K. T., Roehrborn, C. G., Avins, A. L., Barry, M. J., Bruskewitz, R. C., Donnell, R. F., Foster, H. E., Jr., Gonzalez, C. M., Kaplan, S. A., Penson, D. F., Ulchaker, J. C., & Wei, J. T. (2011 May). Update on AUA guideline on the management of benign prostatic hyperplasia. *The Journal of Urology*, 185(5), 1793–1803.
- Nandeesh, H., Koner, B. C., Dorairajan, L. N., & Sen, S. K. (2006). Hyperinsulinemia and dyslipidemia in nondiabetic benign prostatic hyperplasia. *Clinica Chimica Acta*, 370, 89–93.
- Parsons, J. K. (2010). Benign prostatic hyperplasia and male lower urinary tract symptoms: Epidemiology and risk factors. *Current Bladder Dysfunction Reports*, 5, 212–218.
- Qi, J., et al. (2013). Genetic variants in 2q31 and 5p15 are associated with aggressive benign prostatic hyperplasia in a Chinese population. *Prostate*, 73, 1182–1190.
- Roehrborn, C. G. (2008). Pathology of benign prostatic hyperplasia. *International Journal of Impotence Research*, 20, S11–S18.
- Roehrborn, C. G., et al. (2010). The effects of combination therapy with dutasteride and tamsulosin on clinical outcomes in men with symptomatic benign prostatic hyperplasia: 4-year results from the CombAT study. *European Urology*, 57, 123–131.
- Société Internationale d'Urologie (SIU), Lower Urinary Tract Symptoms (LUTS). (2013). In C. Chapple, & P. Abrams (Eds.), *An international consultation on male LUTS*.
- Thiagamoorthy, G., Kotes, S., Zacchè, M., & Cardozo, L. (2016). The efficacy and tolerability of mirabegron, a β 3 adrenoceptor agonist, in patients with symptoms of overactive bladder. *Therapeutic Advances in Urology*, 8, 38–46.
- van Koeveeringe, G. A., Rademakers, K. L., Birder, L. A., Korstanje, C., Daneshgari, F., Ruggieri, M. R., Igawa, Y., Fry, C., & Wagg, A. (2014). Detrusor underactivity: Pathophysiological considerations, models and proposals for future research. ICI-RS 2013. *Neurourology and Urodynamics*, 33(5), 591–596.
- Vignozzi, L., Gacci, M., & Maggi, M. (2016). Lower urinary tract symptoms, benign prostatic hyperplasia and metabolic syndrome. *Nature Reviews. Urology*, 13(2), 108–119.
- Vignozzi, L., et al. (2014). Benign prostatic hyperplasia: A new metabolic disease? *Journal of Endocrinological Investigation*, 37, 313–322.

Further Reading

- Loeb, S., Kettermann, A., Carter, H. B., Ferrucci, L., Metter, E. J., & Walsh, P. C. (2009). Prostate volume changes over time: Results from the baltimore longitudinal study of aging. *The Journal of Urology*, 182(4), 1458–1462.

Prostate Cancer: Overview, Detection, Treatment

Daniel M Moreira and Michael R Abern, University of Illinois at Chicago, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

5-Alpha reductase Enzyme responsible for the conversion of testosterone to its biologic active form (dihydrotestosterone).

Dihydrotestosterone Biologic active form of testosterone

Prostate-specific antigen (PSA) Is a member of the kallikrein-related peptidase family and is secreted by the epithelial cells of the prostate gland.

Testosterone The primary male sex hormone produced by the testis and in the adrenal cortex

Overview

Prostate cancer is the leading cancer (29% of all cancers) and the third most common cause of cancer-related death in men in the United States. It is estimated 1 in 7 men will develop prostate cancer in their lifetimes and 1 in 38 will die of prostate cancer. Although prostate cancer can be a lethal disease, most men diagnosed with prostate cancer do not die from it. Indeed, the overall 5-, 10-, and 15-year survival rates are 100%, 98%, and 95%, respectively. Several risk factors associated with the development of prostate cancer have been described:

- Age: Older age is associated with prostate cancer. Only 1 in 10,000 under age 40 will be diagnosed with prostate cancer, compared to 1 in 39 for ages 40–59, 1 in 14 for ages 60–69.
- Race and ethnicity: African-American and Caribbean men of African ancestry have a higher risk of being diagnosed with prostate cancer. African-American men are also more likely to die of prostate cancer than Caucasian men.
- Family history and genetics: Prostate cancer risk increases according to the number of family members with prostate cancer, degree of relatedness, and the age at which they were diagnosed. For example, the risk of prostate cancer is doubled when the father is affected compared to a 5-fold increased risk when more than 2 first-degree relatives are diagnosed with cancer. Moreover, several prostate cancer susceptibility genes have been proposed including BRCA1, BRCA2, and HNPCC genes.
- Other factors: diet, obesity, lifestyle, and chemical exposures have been suggested as potential risk factors but the evidence is less clear. Some studies suggested a link between vasectomy and higher risk of prostate cancer, but this has been debunked by recent evidence showing no association.

Data on prostate cancer prevention is sparse. Five-alpha reductase inhibitors have been shown to reduce the overall risk of prostate cancer, but there were concerns about increase in the incidence of more aggressive tumors and mortality data is unknown. Diet and lifestyle have been proposed as potential areas where preventive measures could be developed, but so far there is no definitive evidence of a protective effect of lifestyle and dietary changes (Lee et al., 2017).

By far, the most common histological type of prostate cancer is adenocarcinoma. Prostate adenocarcinomas are typically composed of small glands that are back to back, with little or no intervening stroma. Cytologic changes include enlarged round, hyperchromatic nuclei that have a single prominent nucleolus. Less differentiated carcinomas have fused glands, solid nests, or sheets of tumor cells, and many tumors have two or more of these patterns. Prostate cancer is graded according to the Gleason grading system based on the pattern of growth. There are 5 grades (from 1 to 5) based upon the architectural patterns. The two most common patterns are reported and added to calculate the final score (ranging from 2 to 10).

Detection

Most patients with early prostate cancer are asymptomatic. In some cases, however, prostate cancer can cause local symptoms including obstructive and irritative urinary symptoms such as weak stream, increased urinary frequency, and dysuria, as well as hematuria. The presence of symptoms often suggests locally advanced disease as these can be the results of direct local invasion of the cancer into the urethra and/or bladder. Metastatic prostate cancer to the bone can cause pain and predispose patients to fractures. Although early prostate cancer typically has no signs on physical exam, abnormal digital rectal exam (DRE) such as the presence of induration or nodule in the prostate are the most common physical exam findings suggestive of prostate cancer.

Given the lack of reliable symptoms and signs to detect early prostate cancer, most cases are detected based on abnormal prostate-specific antigen (PSA). PSA is a member of the human kallikrein proteins, primarily secreted in the seminal fluid by the epithelial cells of the prostate acini. It is produced for the ejaculate and is responsible for liquifying the semen and potentially dissolving the cervical mucus allowing the free movement of sperm. PSA is present in small quantities in the serum in both bound and unbound forms that can be measured. Serum PSA levels vary with age, race, and prostate volume. In addition, prostate

inflammation, infection, and cancer can cause PSA to rise. On average, 30% of patients with elevated PSA have prostate cancer diagnosed on biopsy.

Prostate cancer screening with PSA and DRE has been the topic of much debate. Although the disease is theoretically ideal for screening given its long presymptomatic phase, accurate diagnostic tests, and effective treatment options, two large randomized trials have questioned the efficacy of screening in reducing cancer mortality. Most medical societies recommend a risk-stratified approach to prostate cancer screening where age, family history, and life expectancy should be taken into consideration.

Prostate biopsy is the most frequently employed technique to detect prostate cancer. The two most common approaches are transrectal and transperineal. Both approaches are typically guided by transrectal ultrasound which allows a uniform spatial separation and sampling of the regions of the prostate. Although there are several variations, most biopsy schemes sample the apex, mid-gland, and base of both right and left prostate lobes. Axial imaging of the prostate, especially magnetic resonance (MRI), has been gaining a bigger role in the management of prostate cancer. For example, MRI can identify suspicious lesions in the prostate that can be targeted by biopsies or focal treatments as well as aid in local staging by identifying signs of extraprostatic extension, seminal vesicle invasion, and enlarged lymph nodes. X-ray, CT scan, and nuclear medicine bone scan are used for the diagnosis of metastatic disease.

The natural history of prostate cancer is very heterogeneous. While some tumors have a slow and indolent disease course, others have a quick and aggressive progression leading to metastasis and death. Thus, it is important to risk stratify patients to determine patients who have a high-risk disease and may benefit from more aggressive management including radiotherapy and surgery and those who have low-risk disease and may potentially benefit from a watchful waiting and surveillance. Multiple risk stratification schemes have been described. Most of these schemes rely on clinical information such as the PSA, Gleason grade, and clinical staging including physical examination and imaging. Biomarkers based on gene expression of the tumor sample obtained on biopsy is an emerging area of research for risk stratification. The TNM staging system for prostate cancer, which correlates with the risk of disease progression, is presented in **Tables 1** and **2**. Of note, the stage is divided into clinical (based on clinical parameters such as digital rectal exam, PSA, and imaging) and pathological (based on pathology of the prostate gland after radical prostatectomy).

Table 1 Prostate cancer TNM staging

Stage	Clinical	Pathological
Primary tumor (T)		
Tx	Primary tumor cannot be assessed	No evidence of primary tumor
T0	No evidence of primary tumor	
T1	Clinically inapparent tumor neither palpable nor visible by imaging	
T1a	Tumor incidental histologic finding in 5% or less of tissue resected	
T1b	Tumor incidental histologic finding in more than 5% of tissue resected	
T1c	Tumor identified by needle biopsy (for example, because of elevated PSA)	Organ confined Unilateral, one-half of one side or less Unilateral, involving more than one-half of side but not both sides Bilateral disease Extraprostatic extension or seminal vesicle invasion Extraprostatic extension or microscopic invasion of bladder neck Seminal vesicle invasion Invasion of rectum, levator muscles, and/or pelvic wall
T2	Tumor confined within prostate	
T2a	Tumor involves one-half of one lobe or less	
T2b	Tumor involves more than one-half of one lobe but not both lobes	
T2c	Tumor involves both lobes	
T3	Tumor extends through the prostate capsule	
T3a	Extracapsular extension (unilateral or bilateral)	
T3b	Tumor invades seminal vesicle(s)	
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall	
Regional lymph nodes (N)		
NX	Regional lymph nodes were not assessed	Regional nodes not sampled
N0	No regional lymph node metastasis	No positive regional nodes
N1	Metastasis in regional lymph node(s)	Metastases in regional node(s)
Distant metastasis (M)		
M0	No distant metastasis	
M1	Distant metastasis	
M1a	Nonregional lymph node(s)	
M1b	Bone(s)	
M1c	Other site(s) with or without bone disease	

Source: Permission from <http://www.uicc.org/resources/tnm> & <http://www.wileyanduiicc.com/>.

Table 2 Prostate cancer TNM staging

Groups	T	N	M	PSA	Gleason
I	T1a-c	N0	M0	PSA < 10	Gleason ≤ 6
	T2a	N0	M0	PSA < 10	Gleason ≤ 6
	T1-2a	N0	M0	PSA unknown	Gleason unknown
IIA	T1a-c	N0	M0	< 20	Gleason 7
	T1a-c	N0	M0	10 ≥ PSA < 20	Gleason ≤ 6
	T2a	N0	M0	10 ≥ PSA < 20	Gleason ≤ 6
	T2a	N0	M0	PSA < 20	Gleason 7
	T2b	N0	M0	PSA < 20	Gleason ≤ 7
	T2b	N0	M0	PSA unknown	Gleason unknown
IIB	T2c	N0	M0	Any PSA	Any Gleason
	T1-2	N0	M0	PSA ≥ 20	Any Gleason
	T1-2	N0	M0	Any PSA	Gleason ≥ 8
III	T3a-b	N0	M0	Any PSA	Any Gleason
IV	T4	N0	M0	Any PSA	Any Gleason
	Any T	N1	M0	Any PSA	Any Gleason
	Any T	Any N	M1	Any PSA	Any Gleason

Treatment

Treatment decisions for prostate cancer are based on tumor aggressiveness (e.g., grade), stage, patient's life expectancy, and the ability of each therapy to ensure disease-free survival, maximizing quality-of-life while minimizing side effects. Localized prostate cancer is typically treated with active surveillance, radical prostatectomy, radiotherapy, or focal therapies. Metastatic prostate cancer is usually treated with systemic therapies. Given some of these therapies may interfere with fertility by either reducing or abolishing spermatogenesis and/or blocking sperm transport, some patients may be candidates for sperm banking before the initiation of treatment.

Watchful Waiting

Patients with prostate cancer are often older and many have comorbidities. Moreover, several prostate tumors have a very long and indolent course. Many untreated patients with prostate cancer die of causes other than prostate cancer. This raises the question of whether some patients with prostate cancer should be treated, especially considering that many treatments pose risks and side effects. The watchful waiting strategy involves deferring treatment until the patient becomes symptomatic due to local symptoms or metastatic disease. It aims at maintaining quality of life while avoiding side effects. It is an appropriate treatment choice for older patients with limited life expectancy and indolent tumors.

Active Surveillance (AS)

AS involves actively monitoring the course of prostate cancer with the expectation to deliver curative treatment if and when the disease progresses. It typically involves the use of serial prostate biopsies to monitor prostate cancer grade and volume in order to identify disease progression. Different from watchful waiting, AS is an appropriate treatment for younger patients with seemingly indolent disease with the goal to defer treatment and its potential side effects. Patients should be followed closely and treatment (typically surgery, radiation, or focal therapies) should start promptly when the cancer progresses to avoid missing the chance for cure.

Radical Prostatectomy (RP)

RP involves the surgical removal of the entire prostate and seminal vesicles. When indicated, a pelvic lymphadenectomy is done at the same time. There are several surgical approaches including open retropubic, open transperineal, laparoscopic, and robot-assisted. RP is considered an appropriate therapy for patients with clinically localized prostate cancer that can be completely excised surgically, who have life expectancy greater than 5 years and have no serious comorbidities that would contraindicate an elective operation. The most common risks and side effects associated with this procedure include bleeding, infection, damage to nearby structures, urinary incontinence, and erectile dysfunction. The prognosis of patients treated with RP depends on the pathological stage and grade. For example, patients with organ-confined disease have a 70%–85% disease-free survival at 10 years compared to 20%–30% for those with extraprostatic extension and positive surgical margins.

Radiotherapy

Radiotherapy involves the delivery of high-energy radiation to treat disease. It can be given both externally (external beam radiation) and/or internally (brachytherapy). Prior to treatment, the prostate is mapped using imaging tests (CT or MRI scan) and a treatment plan (simulation) is established. The most commonly used techniques of external beam radiation include

three-dimensional conformal radiotherapy (radiation beams are then shaped and aimed at the prostate from several directions), intensity-modulated radiotherapy (the intensity of the beams is adjusted to limit the doses reaching nearby normal tissues), and proton beam radiotherapy. Brachytherapy can be delivered permanently (low-dose) by placing radioactive pellets in the prostate, or temporally (high-dose) by placing where radioactive needles in the prostate for only a few moments and then removing them. Androgen deprivation therapy for a few months is typically given along with radiotherapy. The most common side effects of radiotherapy for prostate cancer include radiation proctitis, radiation cystitis, urethral stricture, and erectile dysfunction. The prognosis of patients treated with radiotherapy is comparable to those treated with RP when matched by pathological stage and grade.

Focal Therapies

The rationale for focal therapies of the prostate is to treat the tumor while sparing the normal prostate. Although there are several means of delivering energy to kill the tumor, the most used technique is cryotherapy. Freezing of the prostate is accomplished by using a multiprobe cryosurgical device. Multiple probes are placed percutaneously under transrectal ultrasound guidance, and the temperature is lowered to -25 to -50°C . Potential side effects include recurrence/persistence of the cancer in the untreated prostate, hematuria, urinary incontinence, erectile dysfunction, and injury to surrounding structures. Although short-term studies showed promising results, long-term oncologic results are unknown.

Systemic Therapies

Several systemic agents are available to treat prostate cancer. These treatments are commonly used for locally advanced and metastatic disease.

Androgen Deprivation Therapy (ADT)

Androgens are required for normal growth and function of the prostate. They also stimulate prostate cancer cells growth. Lowering androgen levels or stopping them from getting into prostate cancer cells often makes prostate cancer to shrink or grow more slowly for a time. ADT is currently the initial treatment for metastatic prostate cancer. Although most tumors respond to ADT in the beginning, over the course of months to years most tumors become refractory to ADT and are called castration-resistant prostate cancer (CRPC). Several types of ADT can be used to treat prostate cancer:

- Bilateral orchiectomy (surgical castration): removes 95% of the testosterone which is produced by the testicles. It does not suppress the androgens produced by adrenal or by peripheral conversion of other steroids.
- Luteinizing hormone-releasing hormone (LHRH) agonists and antagonists: by blocking the release or action of LHRH these drugs inhibit luteinizing hormone and consequently the production of testosterone by the testicles. Currently, these are the most prescribed ADT for prostate cancer.
- CYP17 inhibitors: block CYP17A1 enzymes that are expressed in the testicle, adrenal, and prostate tumors causing a decrease in circulating and tissue levels of androgens such as dehydroepiandrosterone (DHEA), testosterone, and dihydrotestosterone (DHT).
- Antiandrogens: are a class of drugs that blocks the androgen receptor (AR). By competing for binding to the androgen receptor, antiandrogens reduce the ability of androgens to promote prostate cancer cell growth. They are frequently used in combination with orchiectomy or a LHRH agonist or antagonist (called combined androgen blockade or total androgen blockade).
- Estrogens: Although estrogens are also able to inhibit testosterone production by the testicles, they are rarely used today because of their side effects.

Chemotherapy

The use of cytotoxic chemotherapy is usually used in cases of metastatic disease, especially CRPC. The most commonly prescribed drugs are: docetaxel, cabazitaxel, mitoxantrone, and estramustine.

Immunotherapy

Several immunomodulatory agents are being investigated. Currently only Sipuleucel-T is approved for the treatment of metastatic prostate cancer. It works through activating antigen-presenting cells to stimulate T-cell immune response targeted against prostatic acid phosphatase, an antigen that is highly expressed in most prostate cancer cells.

Reference

Lee, D. J., Mallin, K., Graves, A. J., Chang, S. S., Penson, D. F., Resnick, M. J., et al. (2017). Recent changes in prostate cancer screening practices and prostate cancer epidemiology. *Journal of Urology*. <https://doi.org/10.1016/j.juro.2017.05.074>.

Further Reading

Anon, & AJCC. (2016). *AJCC cancer staging manual*. New York, NY: Springer Science + Business Media.

Carter, H. B., Albertsen, P. C., Barry, M. J., Etzioni, R., Freedland, S. J., Greene, K. L., et al. (2013). Early detection of prostate cancer: AUA Guideline. *Journal of Urology*, 190(2), 419–426.

- Epstein, J. I., Egevad, L., Amin, M. B., Delahunt, B., Srigley, J. R., Humphrey, P. A., et al. (2016). The 2014 International Society of Urological Pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma: Definition of grading patterns and proposal for a new grading system. *The American Journal of Surgical Pathology*, 40(2), 244–252.
- Siegel, R. L., Miller, K. D., & Jemal, A. (2017). Cancer statistics, 2017. *CA: A Cancer Journal for Clinicians*, 67(1), 7–30.
- Wein, A. J., Kavoussi, L. R., & Campbell, M. F. (2012). *Campbell-Walsh urology*. In L. R. Kavoussi, A. J. Wein, et al. (Eds.) (10th edn.). Philadelphia, PA: Elsevier Saunders.

Relevant Websites

<http://www.auanet.org/guidelines>.
<https://www.cancer.org/cancer/prostate-cancer.html>.
<https://www.cdc.gov/cancer/prostate/>.
<https://medlineplus.gov/prostatecancer.html>.
https://www.nccn.org/professionals/physician_gls/f_guidelines.asp.
<https://seer.cancer.gov/statfacts/html/prost.html>.

Testicular Cancer

Hari T Vigneswaran and Michael Abern, University of Illinois Hospital and Health Sciences System, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Background

Although a rare tumor in the general population, testicular cancer is the most common solid organ cancer in males aged 15–44. Testicular cancer can often be treated successfully, with five-year survival of 99% for tumors localized to the testicle and 96% for cancer spread to regional retroperitoneal lymph nodes. Testicular cancer treatment has served as a model for the utilization of the multi-modal and multi-disciplinary care of cancer. Since the discovery of platinum-based chemotherapy in the 1980s, cure rates for advanced testicular cancer have increased from 5% to as high as 90%, when cure is defined as cancer remission for 5 years or greater. However, with advancements in treatment and therefore improved survival, due to their young age patients are at risk for long-term treatment induced toxicity and fertility concerns.

Epidemiology and Etiology

Epidemiology

Approximately 8000 men are diagnosed with testicular cancer per year in the United States. Since 1975 the incidence of testicular cancer has increased world-wide from 2.9 per 100,000 to now 5.1 per 100,000. The highest rates in the world span the United States, Western Europe, Scandinavia, and New Zealand. Non-Asian whites are affected five times as much as blacks and four times as much as Asians and approximately 75% more than Hispanic whites. Interestingly, Hispanic whites have noted the highest increase in incidence and have been predicted to surpass the rates among non-Hispanic whites by 2026. The differences in rates observed by race and ethnicity may be in part heritable, however, the rise in incidence across all groups suggest a potential role for environmental factors as well.

Etiology

The etiology of testicular cancer is thought to be multifactorial including several known risk factors. Men with cryptorchidism are at four to six times more likely to develop cancer in the ipsilateral testicle. The risk is highest if the testis is retained intra-abdominally, less so if within the inguinal canal and further decreased risk when the patient undergoes surgical repair with orchidopexy before puberty. Interestingly, these patients also have increased risk in the contralateral descended testis. Other risk factors include a family history of testicular cancer, testicular microlithiasis, Klinefelter Syndrome, history of mumps orchitis, personal history of testicular cancer in the contralateral testis, HIV infection, and early onset of puberty, and testicular dysgenesis syndrome.

Clinical Evaluation

Presentation

The most common presentation of testicular cancer is painless swelling or a mass of the testis. However, testicular cancer can present with pain in up to 45% of patients if there is intratesticular infarction, hemorrhage, torsion of the neoplasm or simultaneous acute infection of the epididymitis. Other presentations include nodularity felt to the testicle or the sensation of fullness or ache in the scrotum. Incidental trauma to the scrotum can reveal a subtler mass which may lead to the eventual diagnosis. Signs of β -hCG-producing tumors include gynecomastia or breast tenderness in approximately 10% of patients. In certain patients, 5% of cases are detected during an evaluation for infertility. In 25% of instances, the patient can present with symptoms of disseminated cancer including back pain suggestive of retroperitoneal disease, shortness of breath, hemoptysis or chest pain suggestive of pulmonary or mediastinal disease.

Physical Exam and Scrotal Ultrasound

A thorough history and physical exam must be obtained to help differentiate a testis tumor from other causes of testicular or scrotal pathology including such as hydrocele, spermatocele, varicocele, hematocele, or epididymo-orchitis. For the evaluation of scrotal or testicular pathology the scrotal ultrasound is an extension of the physical examination. The sensitivity of ultrasound for detecting testicular cancer is approximately 96%. Ultrasound is widely available, inexpensive and noninvasive. Once visualized sonographically an intratesticular mass is testicular cancer until proven otherwise. Testicular masses can appear hypoechoic or heterogeneous, or with increased vascularity. Epididymitis is the most commonly mistaken diagnosis made when patients present with testicular cancer and delays can last several months. Delays in diagnosis can lead to stage progression and therefore the need for more aggressive treatment so it is important to have a high degree of suspicion in any man age 15–44 years with a testis mass, retroperitoneal mass, or supraclavicular mass.

Serum Tumor Markers

When testicular cancer is suspected beta-human chorionic gonadotropin (β -hCG), alpha-fetoprotein (AFP) and lactate dehydrogenase (LDH) should be measured in the serum. It is important to note that the absence of abnormal serum tumor markers does not preclude the diagnosis of testicular cancer. Typically, β -hCG levels are elevated in non-seminomatous germ cell tumors (NSGCT) with significant levels >5000 IU/L. However, approximately 20% of seminomatous GCT contain syncytiotrophoblastic cells that secrete β -hCG. Marijuana use and hypergonadotropic hypogonadism can also result in elevated β -hCG levels. The marker AFP is commonly elevated (50–70%) in embryonal and yolk sac tumors, but can also be elevated with other malignancies including hepatocellular carcinoma and pancreatic cancer. Although LDH is less specific than β -hCG and AFP, the marker is expressed on chromosome 12 and levels correlate directly to bulk of disease in GCT. While less commonly used, placental alkaline phosphatase (PLAP) is a marker for seminoma and is present in most advanced seminomas. In general tumor marker levels will vary depending on the histologic cell type and stage of disease as seen in [Table 1](#). When elevated initially serum tumor markers also have utility after orchiectomy and chemotherapy for staging and to surveil for relapse. The circulating half life of β -hCG is 24–36 h, AFP at 5–7 days, and LDH at 24 h. After a complete response to orchiectomy or chemotherapy, serum tumor markers are expected to normalize after five half lives and therefore should be measured at the appropriate time interval.

Testis Cancer Histology and Biology

Pathophysiology

Germ cells are the only cells in the body that have half the amount of chromosomes, undergo both mitosis and meiosis and in males produce the gamete, sperm. Testicular GCT make up 95% of all testicular tumors followed by sex cord tumors comprising 5%,

Most GCTs are thought to arise from precursor lesions called intratubular germ cell neoplasia (previously incorrectly classified as carcinoma in situ). These lesions may be due to arrested germ cell development which are thought to lay dormant until stimulated by increased testosterone after puberty. Although environmental factors play a role, certain genes have been associated with GCT. A commonly mutated gene called the KIT ligand gene is amplified and found in excess on Chromosome 12 with 70–80% of GCTs having an extra chromosome 12.

Histology

See [Table 1](#) for the different histological types of testicular cancer. GCT are comprised of three histologic types: seminomas (SGCT), nonseminomas (NSGCT) and spermatocytic tumors. Seminomatous and nonseminomatous tumors comprise 99% of all GCT. The seminomas are the most common type of GCT comprising approximately 65%. SGCT generally occur in older patients between 30–40s as compared to NSGCT. SGCT first arise from intratubular germ cell neoplasia and have the ability to transform into NSGCT.

NSGCT are comprised of embryonal, yolk sac, choriocarcinoma, and teratoma and mixed histologies. Mixed elements of seminomas and nonseminomas are treated as nonseminomas. Embryonal Carcinoma is the most undifferentiated cell type of NSGCT and has the potential to differentiate to teratoma and other types of NSGCTs. Although choriocarcinoma is rare, it is very aggressive, spreads hematogenously and can present with metastasis to the lung, liver, and brain. Importantly, metastatic tumor of this type are prone to hemorrhage both spontaneously and after chemotherapy. Choriocarcinoma is associated with elevated β -hCG and due to the tumor marker cross reactivity with thyroid and luteinizing receptors, sometimes patients can present with hyperthyroidism and hyperandrogenism. Yolk sac tumors (YST) primarily occur in infants when in pure form, however mixed NSGCT commonly have elements of YST. The presence of AFP is associated with YST. Teratoma contain at least two of the three embryologic germ cell layers: endoderm, mesoderm, and ectoderm. In testicular GCT these tumors can exist in matured or

Table 1 Testicular Cancer Histology

<i>Tumor type</i>	<i>Histologic sub-type</i>	<i>Frequency</i>	<i>Serum tumor markers</i>	<i>Most common age of onset</i>
Germ cell	Spermatocytic seminoma	Less than 1%	-	65
	Seminoma	65%	β -hCG	30-40
	Yolk sac	Less than 1%	AFP	Less than 10
	Choriocarcinoma	Less than 1%	β -hCG	20-30
	Embryonal	40-50%	AFP, β -hCG	25-35
	Teratoma	40-50%		25-35
Sex cord or stromal	Leydig cell	3%	Testosterone, estradiol	5-10, 30-60
	Sertoli cell	1%	Testosterone, estradiol	Any age
	Granulosa Cell	1-3%	-	Pre-pubertal
Other	Gonadoblastoma	0.5%	-	Less than 30
	Primitive neuroectodermal tumors (PNET)	Less than 1%	-	Previous diagnosis of testicular cancer

immature form, however there is no clinical difference between the two. Although teratoma appears benign on histopathology, in advanced NSGCT, metastatic teratoma is frequently found. Importantly, these metastatic tumors found in retroperitoneal lymph nodes are resistant to chemotherapy.

Sex cord or stromal cells are the supporting cells of the germ cells which help to make and transport sperm. Gonadal stromal tumors are of the leydig, sertoli, or granulosa cell type and <10% of these tumors are malignant, however presentation may be indistinguishable from GCT.

Clinical Management of Testicular Cancer

Orchiectomy

Once testicular cancer is suspected prompt diagnosis and treatment is necessary. A radical inguinal orchiectomy is typically performed within 1–2 weeks to fully remove the testicle and spermatic cord as it meets the abdominal cavity. Trans-scrotal orchiectomy and biopsy are contraindicated because this both leaves part of the spermatic cord potentially harboring cancer and alters the lymphatic drainage pattern for the testicle and cancer. Rarely, testis sparing removal of the tumor is attempted for a mass <2 cm in maximum dimension occurring in a solitary testicle, in both testicles, or when there is a high suspicion for benign histology. At the time of radical orchiectomy, a testicular prosthesis can be placed, however in children the prosthesis is placed after the completion of puberty so the proper size can be determined.

Staging

Testicular cancer staging is described using the American Joint Committee on Cancer TNMS classification (tumor, node, metastasis, and serum marker) and relies on imaging of the chest, abdomen, and pelvis, and other organs as dictated by symptoms, as well as the nadir value of the serum tumor markers post-orchiectomy. Computed tomography (CT) and magnetic resonance imaging (MRI) are commonly used imaging modalities. Positron emitted tomography (PET) scans have limited utility in testicular cancer and are generally reserved for residual masses after chemotherapy for SGCT.

Stage 1 testicular cancer is cancer confined to the testis with or without lymphatic or vascular invasion and without regional lymph node involvement. GCT follow a predictable progression of disease with via lymph drainage to the regional retroperitoneal lymph nodes. Stage 2 cancer is classified as any primary tumor with this regional lymph node invasion including the abdominal nodes that are near the aorta and the inferior vena cava at the level below the kidneys. Lastly, stage 3 describes testicular cancer that has spread beyond the primary tumor and regional lymph nodes to distant sites such as the lungs, mediastinum, bones, non-regional lymph nodes, or brain. When tumor markers persist after orchiectomy the disease is up-staged to S disease (stage 1S, 2S, 3S).

Primary Management

After orchiectomy, initial CT imaging, and serum tumor marker measurements, the stage and histology of the cancer is determined. See Fig. 1 for treatment management paradigms after orchiectomy. For Stage 1 seminoma there are primarily three options. The first option is imaging and serum marker surveillance, which requires a reliable patient and strict follow up. <20% of patients with stage 1 SGCT will relapse on surveillance. Another option is adjuvant abdominal external beam radiation therapy (XRT) to the retroperitoneal lymph nodes. Toxicity from radiation includes secondary non-GCT malignancies such as leukemia, increased risk of cardiac disease, and radiation induced kidney disease. However, after XRT the risk of relapse for stage 1 SGCT is 3%. Alternatively, one to two cycles of the chemotherapeutic, carboplatin, is available for stage 1 disease and can reduce the relapse rate to 2%. Carboplatin causes immunosuppression, nausea, fatigue, and in some instances infertility, however it is without the risk of XRT induced cardiomyopathy.

The typical chemotherapy regimen is, etoposide and cisplatin with or without bleomycin (EP or BEP). For stage 2 SGCTs with lymph nodes <5 cm in size chemotherapy with BEP or abdominal XRT to the RPLN is the indicated treatment. When either regional lymph node disease increase past 5 cm or stage 3 disease occurs a course of three BEP cycles or four EP cycles is indicated. Bleomycin can lead to pneumonitis and pulmonary fibrosis, etoposide can lead to myelosuppression, mucositis, and dose related risk of leukemia, and cisplatin can cause neuropathy, and renal and ototoxicity. SGCT are generally highly radio- and chemo-sensitive. Residual masses >3 cm, require surgical resection with RPLND (see below).

In terms of stage 1 nonseminomas, there are also three options. Active surveillance with serum testing and imaging will result in a relapse rate of approximately 30% of stage 1 NSGCT. The most predictive features of NSGCT relapse is lymphovascular invasion of the primary tumor. Adjuvant chemotherapy or primary retroperitoneal lymph node dissection (RPLND) are preferred for clinical stage 1 NSGCT thought to be at higher risk of relapse or in situations where active surveillance is not feasible. The relapse rate after two cycles of BEP is <3%, with most relapses occurring in the retroperitoneum and usually containing teratoma.

RPLND is a diagnostic and therapeutic resection of the regional lymph nodes. Ejaculatory dysfunction (ED) can occur after surgery due to the close proximity of the post ganglionic sympathetic nerves and hypogastric plexus within the field of dissection.

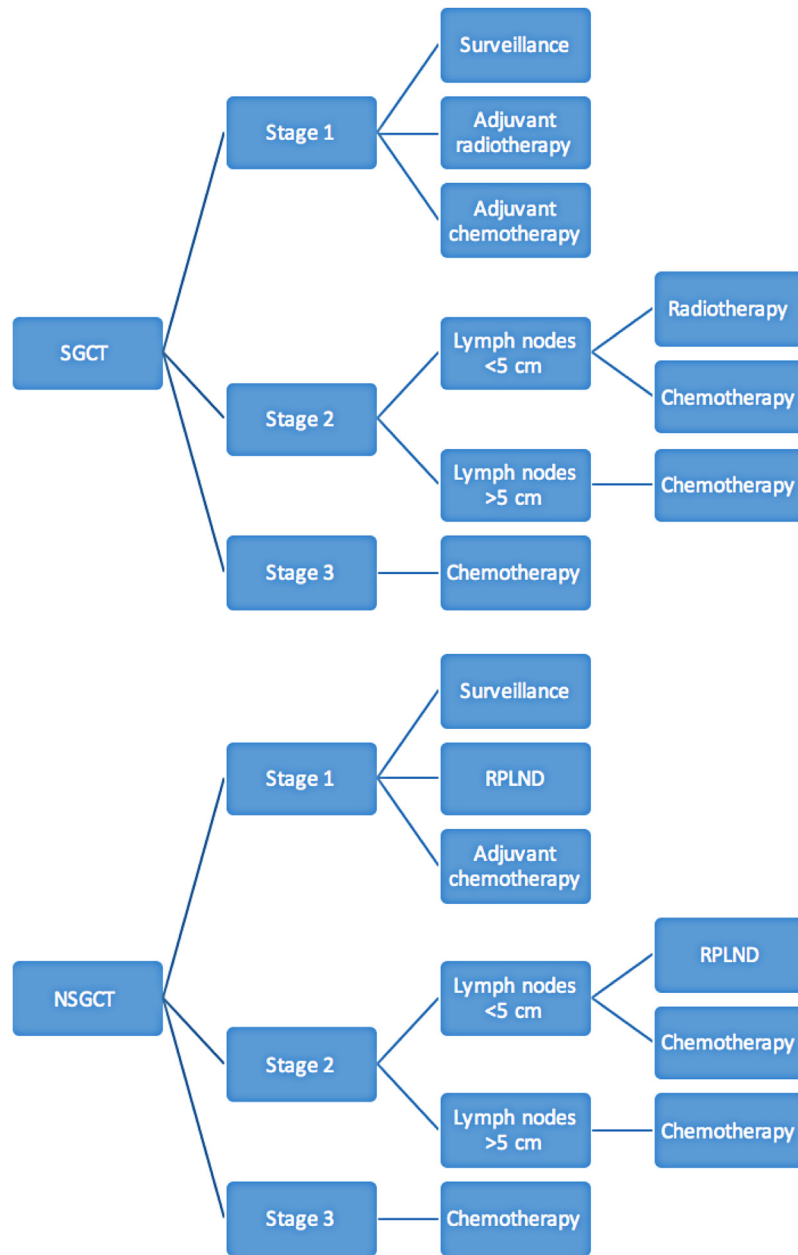


Fig. 1 Treatment paradigm for TNMS staging of testicular cancer.

Nerve sparing surgery can be achieved and when selected appropriately, maintains ejaculatory function in up to 99% of individuals. Apart from ED, risks of RPLND include postoperative tachycardia from sympathetic discharge, chylous ascites, vascular injury, bowel obstruction, and rarely death. The relapse rate after primary RPLND is reduced to approximately 10%. If the lymph nodes are benign or one lymph node contains a tumor focus < 2 cm in size, surveillance is preferred. More tumor involvement of the lymph nodes necessitate chemotherapy. After RPLND, most relapses occur outside of the retroperitoneum and are treated with chemotherapy.

For stage 2 NSGCTs with lymph nodes < 5 cm, either nerve sparing RPLND, a full course with 3 cycles of BEP or 4 cycles of EP chemotherapy are available options. If lymph nodes are > 5 cm (stage 2C) or serum tumor markers persist after orchiectomy (stage 1S), a full course chemotherapy is the preferred treatment. Residual retroperitoneal masses > 1 cm in size are resected with salvage RPLND. For stage 3 NSGCT, three cycles of BEP or four cycles of EP are first administered. Salvage surgical excision, including RPLND is used for residual masses > 1 cm. In stage 2C and stage 3 NSGCTs teratoma is found in approximately 35% of residual lesions, necrosis or fibrosis is found in approximately 45% of residual lesions, and malignancy are found in approximately 15% of residual lesions.

Fertility in Testis Cancer Patients

Men who are interested or undecided about fathering children are recommended to undergo cryopreservation of sperm. Cryopreservation should be done prior to radiation, RPLND, or chemotherapy. Although the etiology is not clearly identified, at the time of diagnosis of testicular cancer, approximately 50% of men are oligospermic and 10% are azoospermic, while after orchiectomy about 50% recover normospermia. Since chemotherapy is an excellent treatment for GCT, the effect on germ cells causes almost every patient to become azoospermic. If the patient had normal sperm parameters prior to treatment within 2 years of treatment completion, 50% will recover to normal levels and by years 80% of men will recover. Radiation is slightly more toxic to spermatogenesis and can take a few years longer to recover. Damage to the sympathetic nerves of ejaculation after non-nerve sparing RPLND may result in ED in 80% of patients.

Further Reading

- Friedlander, T. W., et al. (2014). Testicular Cancer. In *Abeloff's clinical oncology* (pp. 1506–1533). New York: Churchill Livingstone.
- Ghazarian, A. A., et al. (2017). Future of testicular germ cell tumor incidence in the United States: Forecast through 2026. *Cancer*, 123(12), 2320–2328.
- Gilligan, T. (2015). Quality of life among testis cancer survivors. *Urologic Oncology*, 33(9), 413–419.
- Gilligan, T., et al. (2017). Testicular cancer, version 2.2017. *J national comprehensive cancer network*.
- Hanna, N. H., & Einhorn, L. H. (2014). Testicular cancer—discoveries and updates. *New England Journal of Medicine*, 371(21), 2005–2016.
- Kamran, S. C., et al. (2017). Contemporary treatment patterns and outcomes for clinical stage IS testicular cancer. *European Urology*.
- Risk, M. C., & Porter, C. R. (2009). Management of non-germinal testicular tumors. *World Journal of Urology*, 27(4), 507–512.
- Sheinfeld, J. and G.J. Bosl (2016). Abdominal Medicine, Surgery of Testicular Tumors. Abdominal Key
- Wein, A.J. et al. (2011). Campbell-Walsh Urology. (Book 4) Saunders. Philadelphia 4, 4320.

Content and Volume Overview

Carlos Simón, Department of Obstetrics & Gynecology, Valencia University, Valencia, Spain

Craig Niederberger, Department of Urology, University of Illinois at Chicago, College of Medicine, Chicago, Illinois, USA

© 2018 Elsevier Inc. All rights reserved.

Assisted Reproductive Technologies in Reproductive Medicine are presented in volume 5 throughout 82 different chapters. This volume first describes the technological process that must be developed to perform low and high complexity ART, from the drugs used for controlled ovarian stimulation, monitoring, oocyte retrieval and luteal support. Then the different technologies are presented. Hand in hand with the clinical evaluation of the male is the laboratory, as sperm is easily accessible. In the male portion of volume 5, a thorough treatment of the andrology laboratory is presented with every aspect currently measurable. Unfortunately, the great challenge of male reproductive evaluation is that while we can observe the exterior of the male gamete and some of its biological milieu, we are unable to look within the sperm to truly understand its reproductive potential. Promising technologies on the horizon include chromosomal structure, epigenetics, and Raman microspectroscopy, and these are reviewed. The volume continues with a comprehensive view of the IVF laboratory from setting, quality control and accreditation to the detailed analysis of the embryological processes from oocyte insemination, fertilization to embryo development with morphokinetics, and mechanical assessment. The genetic and epigenetic interrogation of gametes, embryos and maternal endometrium is explained in the next section. Cryobiology has revolutionized how reproductive medicine is performed nowadays, deserving a detailed analysis of cryopreservation of sperm, oocytes, embryos and ovarian tissue for both oncological and social reasons. The different current clinical strategies used in IVF, proven and unproven, that affect ovulation, endometrial receptivity and embryo transfer are also presented followed by ART in special medical conditions and possible complications, ending with the clinical follow-up of ART pregnancies and babies from prenatal screening to pediatric follow-up. Since ART treatment impose an economic burden to the infertile population, we introduce in this volume the cost-effectiveness studies of high versus low complexity reproductive treatments as well as the genetics studies associated with IVF. The support team as well as the modern ethical dilemmas and worldwide legislation in ART is then presented. The final section portrays the potential contribution of advance cell therapy, organ bioengineering, gene editing and epigenetic research in the future development of ART.

TECHNICAL PROCESSES

Mild IVF Stimulation

Daniel Bodri, London Women's Clinic, Marylebone, London

Nick Macklon, London Womens' Clinic, Marylebone, London; and University of Copenhagen, Copenhagen, Denmark

© 2018 Elsevier Inc. All rights reserved.

Introduction

It was clear to the pioneers of IVF that inefficiencies in the laboratory procedures of fertilization and embryo culture could in part be overcome by ensuring that large number of oocytes were available from each treatment cycle: indeed this was necessary in order to provide a realistic chance of generating a viable embryo for transfer into the uterus. However, generating large numbers of oocytes from a single cycle met over-riding the physiological processes that govern normal cyclic follicle recruitment and subsequent mono-follicular dominance.

This was considered to require high doses of exogenous FSH, which became duly introduced in IVF practice, with the aim of maximizing the number of oocytes obtained from a single stimulation cycle. This approach achieved some success, with higher pregnancy rates being reported in comparison to unstimulated cycles. However, it soon became apparent that such an approach came with some disadvantages. The first to be noted was the high risk of premature ovulation, arising from the high levels of estrogen generated by multiple growing follicles, thus reaching a level capable of triggering an LH surge before any of the follicles had reached maturity. In order to prevent this, it became necessary to block the LH surge. This required the down regulation of the pituitary gland for some 2 weeks prior to starting exogenous FSH, using daily injections of a GnRH agonist, which in turn resulted in menopause like symptoms as many women thus treated suffered the effects of hypoestrogenism before levels rose under FSH stimulation.

This so called "long protocol" quickly became established as the standard approach to stimulating the ovaries in order to obtain sufficient oocytes to provide a reasonable chance of obtaining an embryo for transfer. But it was long, expensive, burdensome, and exposed the patient to the potentially lethal complication of ovarian hyperstimulation syndrome, which commonly occurred.

It soon became apparent that this approach merited re-evaluation. In 1996 the founding pioneer of IVF, Robert Edwards, co-published an influential editorial making the case for this ([Edwards et al., 1996](#)).

It was argued that IVF was not simply about obtaining a positive pregnancy test at any cost, but rather this aim should be achievable without exposing the woman to excessive therapeutic burden or risk.

The call was not ignored and a number of groups started devising new approaches that sought to find the optimal balance between safety and effectiveness, based on the premise that softer stimulation would generate fewer but relatively high quality oocytes. These approaches have come to be known as mild IVF, or in cases where little or no ovarian stimulation is applied, natural cycle IVF.

The last 20 years have seen an extensive literature emerge describing the principles and practice of mild IVF, and we are now benefiting from a clearer understanding of the advantages and disadvantages of these approaches, and which patients might most benefit. In this article we will review the development of mild approaches, the debate as to their efficacy and how they might be used in clinical practice.

Mild Ovarian Stimulation in IVF

The Aim of Mild Stimulation

Essentially, mild approaches to IVF seek to find the optimal balance between producing sufficient high quality oocytes to provide a good chance of having an embryo transfer and possibly more to freeze for later use, while not producing so many as to expose the patient to unnecessary side effects, risks, and costs. The optimal number of oocytes to be obtained with this aim in mind remains subject to debate. The analysis of large databases has indicated that pregnancy rates are highest after the long protocol when 12–15 oocytes are obtained ([Van der Gaast et al., 2006](#); [Sunkara et al., 2011](#)). In contrast, a meta-analysis of studies reporting outcomes after a mild stimulation revealed the optimal number to be six to eight oocytes ([Verberg et al., 2009a](#)). It is likely the cohort of patient achieving this number after mild stimulation would have generated 15 or so oocytes after the long protocol, and that both therefore represent the optimal response to a given stimulation regimen.

The scientific principles behind the mild stimulation concept have been previously outlined ([Macklon et al., 2006](#)). As shown in [Fig. 1](#), after the inter-cycle rise in endogenous FSH has initiated cyclic recruitment of the available FSH sensitive follicles, exogenous FSH is given in doses sufficient to maintain the level above the threshold required to stimulate follicle development for a period sufficient to maintain the "FSH Window" open in order to allow six to eight follicles to reach maturity. This is contrast to the

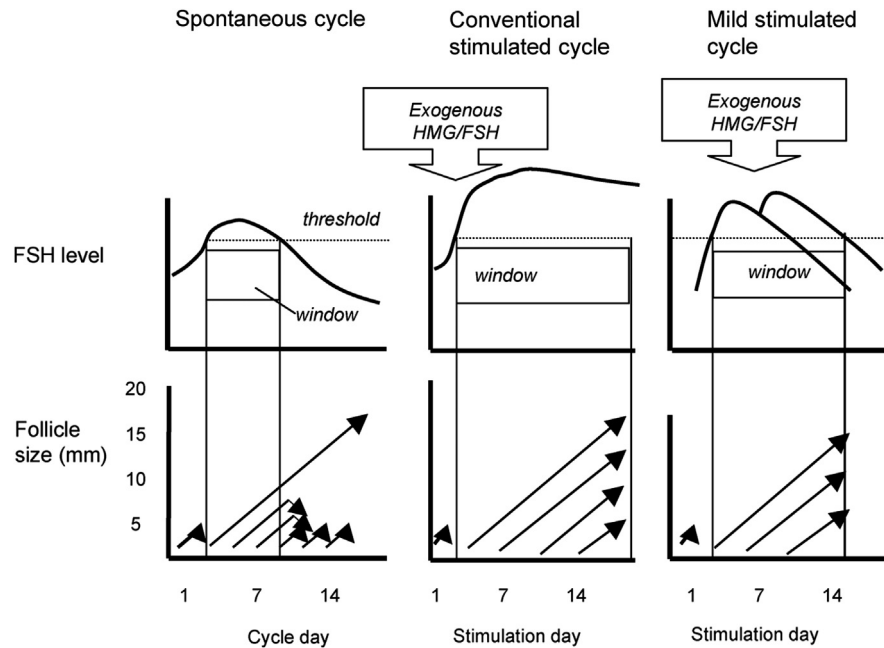


Fig. 1 The principles of mild stimulation. In contrast the conventional stimulated cycles, mild stimulation harnesses the physiological FSH rise and supplements with exogenous FSH to maintain the level of FSH just above the necessary threshold and for the period of time necessary to allow a small number of oocytes to reach maturity. Adapted from Macklon, N. S., Stouffer, R. L., Giudice, L. C. and Fauser, B. C. (2006). The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocrine Reviews* **27**, 170–207.

traditional approach that raised FSH levels well above the required threshold level, and maintained the FSH window open long enough for all available oocytes (most of which were destined to enter atresia) to reach maturity.

The key development that made this approach possible was the clinical availability of GnRH antagonists. In contrast to GnRH agonists, these could be administered for just a short period from the mid follicular phase and still be effective in preventing a premature LH surge and loss of the oocytes prior to surgical retrieval. This meant that the spontaneous inter-cycle FSH rise was not suppressed, and could instead be harnessed to initiate follicle growth. The need for exogenous FSH to raise levels above the threshold was therefore less, and since fewer oocytes were required, the FSH window needed to be held open for a shorter period of time, requiring fewer days of injection.

One of the leading groups to develop this approach was that of Bart Fauser in Rotterdam and Utrecht. Their studies demonstrated first that it was the duration of the FSH window rather than the level of FSH above the threshold that was the key determinant of the number of oocytes to reach maturity (Schipper et al., 1998). They then showed that exogenous FSH could be delayed until day 5 of the cycle and yet still enable multi-follicle development. However, one of their key findings was that while fewer numbers of oocytes were obtained after mild stimulation, the quality of the resulting embryos was higher. This was clearly illustrated by the observation that women obtaining fewer than four oocytes after stimulation with the long protocol rarely achieved a pregnancy, whereas half of the pregnancies occurring in the mild stimulation group occurred in women who had generated less than four oocytes (Hohmann et al., 2003).

The benefits of mild stimulation on the quality of the oocytes and embryos was confirmed by a much cited study which showed the rate of chromosomal mosaicism to be less prevalent in embryos derived from mild stimulation versus the long protocol (Baart et al., 2007; Fig. 2). These studies set the scene for a large prospective randomized trial comparing a mild approach which combined lighter stimulation with single embryo transfer (SET), against the standard approach of harder, longer stimulation followed by double embryo transfer (DET). The hypothesis of this study was that the former approach could deliver the same chance of providing a healthy singleton birth within a year of starting treatment as standard approaches but with fewer complications, fewer multiple births, lower costs, and less stress to the patients. This study showed that cumulative singleton live births were not significantly different after performing four mild SET versus three conventional IVF attempts (Fig. 3). Moreover, the mild approach was shown to be associated with reduced stress and drop out from treatment. (Fig. 4). A conceptually similar 4-year clinical trial involving 564 patients who were randomized to receive mild ovarian stimulation with clomiphene and gonadotropins coupled with single embryo transfer versus conventional ovarian stimulation with the long agonist protocol coupled with (mainly) double embryo transfer was subsequently reported (Zhang et al., 2010). Although cumulative live birth rates were in favor of the conventional approach (63% vs. 49%), the very mild approach tested was also associated with the absence of OHSS, reduced rate of multiples, and reduced gonadotropin use.

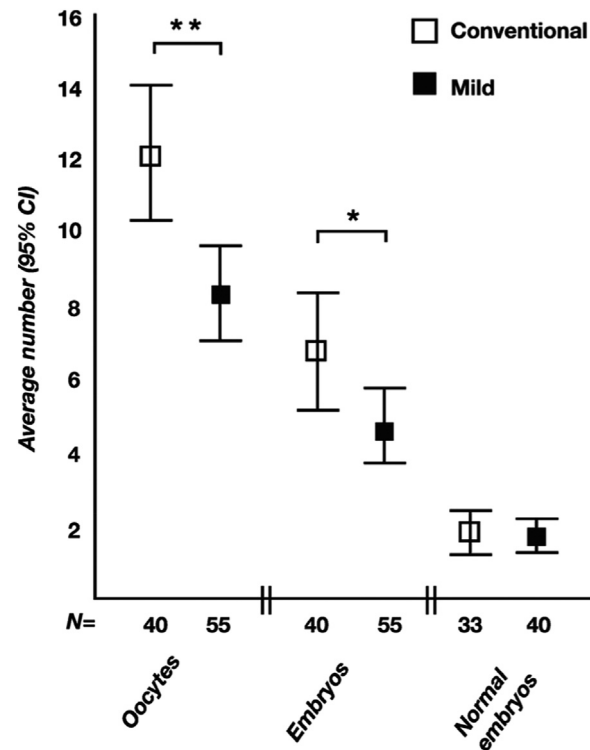


Fig. 2 Oocyte and embryo yield and embryos analyzed by fluorescent in situ hybridization (FISH) as chromosomally normal on the basis of FISH results from one cell following conventional and mild stimulation. Reproduced from Baart, E. B., Martini, E., Eijkemans, M. J., Van Opstal, D., Beckers, N. G., Verhoeff, A., Macklon, N. S. and Fauser, B. C. (2007). Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: A randomized controlled trial. *Human Reproduction* **22**, 980–988.

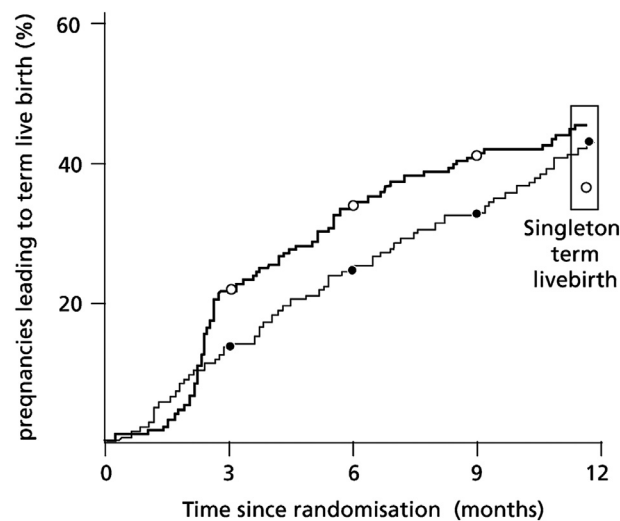


Fig. 3 The cumulative term live-birth rate within 12 months after starting IVF treatment. Mild: mild ovarian stimulation with GnRH antagonist and single embryo transfer. Standard: standard ovarian stimulation with GnRH agonist long protocol along with the transfer of two embryo. Reproduced from Heijnen, E. M., Eijkemans, M. J., De Klerk, C., Polinder, S., Beckers, N. G., Klinkert, E. R., Broekmans, F. J., Passchier, J., Te Velde, E. R., Macklon, N. S. et al. (2007). A mild treatment strategy for in-vitro fertilisation: A randomised non-inferiority trial randomized trial. *Lancet* **369**, 743–749.

Alternatives to Low Dose FSH

Clomiphene citrate is an anti-estrogen that blocks feedback at the pituitary gland causing FSH level to rise. Its advantages over FSH include its oral route of administration, low price, and widespread availability. An ovarian stimulation protocol combining CC with gonadotrophins could lead to a reduction in the amount of gonadotrophins required due to the combined synergistic

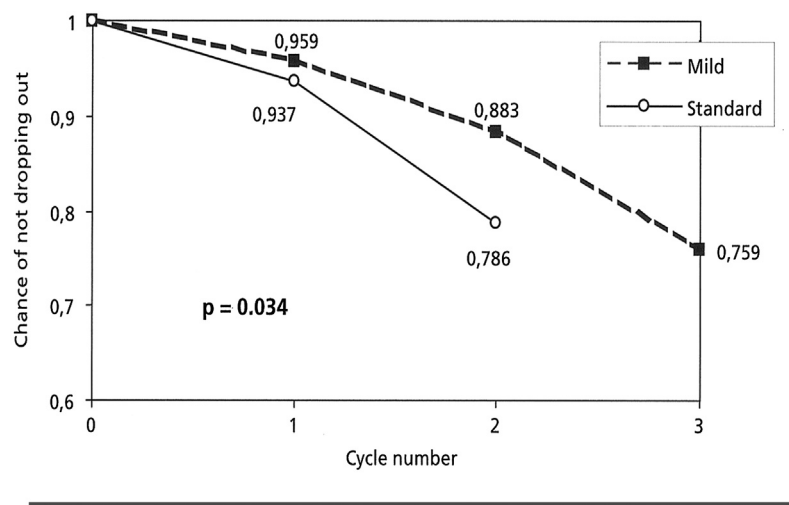


Fig. 4 The chance of not dropping out of further treatment after a failed first, second, and third cycle of IVF treatment. Those women who failed to conceive after undergoing mild stimulation IVF were more likely to continue with another cycle than those who failed after conventional ovarian stimulation. Reproduced from Verberg, M. F. G., Macklon, N. S., Nargund, G., Frydman, R., Devroey, P., Broekmans, F. J., Fauser, B. C. J. M. (2009). Mild ovarian stimulation for IVF. *Human Reproduction Update* **15**, 13–29.

effects. Additionally, because gonadotrophins may counter the anti-estrogenic effects of the CC on the endometrium which may account for the relatively low embryo implantation rates observed, this combination might lead to improved pregnancy rates compared with CC alone.

Aromatase inhibitors selectively inhibit the conversion of androgens to estrogens in granulosa cells of developing ovarian follicles, resulting in a subsequent increase in intra-ovarian androgens and absence of a rise in estrogens. Due to a reduced estrogen feedback and resulting increased endogenous gonadotrophin secretion, the need for exogenous gonadotrophins is reduced when aromatase inhibitors are administered in the early follicular phase. Aromatase inhibitors may therefore serve a similar purpose as CC in mild stimulation protocols (Verberg et al., 2009a).

In recent years mild or “mini” IVF has become successfully established in certain countries such as Japan where the “Kobe protocol” has attracted much attention. This protocol uses oral contraceptive pill pre-treatment, extended daily clomiphene citrate dosing at 50 mg until triggering of final oocyte maturation with a GnRH agonist, and a daily dose of 75–150 units of FSH from cycle day 4 to 7. In recent randomized controlled trial comparing this protocol to the long protocol lower cumulative live birth rates were observed (49% vs. 63%) but OHSS was eliminated (Zhang et al., 2016).

Mild Stimulation in 2018

The case for mild approaches is attractive and it has gained a number of proponents. However, while the last 10 years has seen a move away from high dose long protocol regimens, adoption of a true mild treatment regimens into routine clinical practice has been slow. In part this reflects the deeply held view that fewer oocytes meant fewer pregnancies, and a caution in adopting a new paradigm for IVF treatment. While some later studies have questioned the concept of an optimal number of oocytes above which outcomes deteriorate, the notion that within the cohort of oocytes available for stimulation, only a few can generate viable embryos, and these can be obtained through milder stimulation has recently been reinforced (Arce et al., 2014). Some 10 years after the publication of the studies showing the potential of the mild approach, these debates continued. However, the underlying message has influenced the field and led to an overall reduction in the doses of FSH being used, and the duration of ovarian stimulation. The new “standard” approach to IVF is remarkably similar to the “mild approach” first advocated by Edwards, Fauser, and others. In 2018, mild stimulation has established its place as an attractive option for those wishing IVF treatment with a good chance of conceiving a singleton pregnancy within a realistic time frame while avoiding the burden, intensity, and costs of conventional approaches (Fauser, 2017).

Given the potential advantages of milder stimulation, it is not surprising that the idea of taking this to its logical conclusion and that “natural” cycle IVF, the approach which finally led to the birth of the first IVF baby, enjoyed renewed interest.

Natural Cycle IVF

This minimalistic approach relies on endogenous FSH to stimulate monofollicular development, and triggering of final oocyte maturation by either a spontaneous LH surge or the administration of hCG. Early approaches relying on the former required intensive cycle monitoring which placed a considerable burden on both patients and the clinic. The first large reported series also

highlighted the low efficiency of this approach. Of 162 treatment cycles, only 89 (55%) reached embryo transfer and only 9 (5.6% per started cycle) resulted in live births (Zayed et al., 1997).

A response to these disappointing outcomes, as well as the logistic challenges posed by cycle monitoring in this way was to propose that final oocyte maturation be triggered by giving an hCG injection to mimic a timed LH surge. A subsequent extensive review which analyzed the efficiency of ncIVF treatment in 20 studies published between 1989 and 2001 involving 1800 cycles showed that successful oocyte recovery rate varied greatly between studies (30%–96%) (Pelinck et al., 2002).

A retrospective review comparing outcomes after spontaneous LH surge or with the use of hCG injection suggested a modest margin of benefit after the former. In the LH group (534 cycles), eggs were collected in 81% of cycles whereas in the hCG group (241 cycles) this was 76% (Lenton, 2007). Moreover, a randomized clinical trial in intrauterine insemination treatment showed that ongoing pregnancy rates were significantly higher (23% vs. 11%) in spontaneously versus hCG-triggered cycles (Kyrou et al., 2012).

These possible differences need to be weighed against the inconvenience of twice daily LH monitoring and most clinics prefer hCG triggering because it permits the timing of oocyte retrievals and is convenient both for the patients and the clinic's staff. However, the risk of premature ovulation remains a concern and this has led to the development of "modified" natural cycles. They still rely on endogenous FSH to stimulate the growth of a single follicle, but like mild stimulation regimens, employ GnRH antagonists to prevent a premature LH surge, thus diminishing the risk of premature ovulation and permitting the triggering by exogenous hCG at a convenient time, thus greatly simplifying oocyte retrieval scheduling. In a pilot study in which GnRH antagonists were administered daily from when the leading follicle reached 12–14 mm and concomitantly daily doses of 150 IU hMG were given to maintain follicular stimulation (Rongieres-Bertrand et al., 1999). Of 44 cycles in <37-year-old patients, 91% resulted in oocyte retrievals, 50% in embryo transfers, and there were 5 (11.4%) ongoing pregnancies. These results were considered encouraging enough for this new protocol to become the treatment of choice for many centers offering "natural" cycle IVF.

In a study comparing outcomes after modified ncIVF versus conventional IVF in 134 patients under 35 years of age, 56% of the ncIVF cycles led to embryo transfer 15% to clinical pregnancies, compared with 45% after conventional stimulation (Phillips et al., 2007). In older patients (between 35 and 38 years), the corresponding rates were much lower at 3.7% versus 34% respectively. The authors concluded that although with modified ncIVF, the cancellation rate is very high it might be offset by the fact that the mild treatment option is less hard on patients, could be repeated in each month, and costs less. The largest published series on modified ncIVF by Pelinck et al. (2007) reported the outcome of 1048 cycles on a cumulative basis in patients who were offered up to 9 treatment cycles. Per cycle ongoing pregnancy rates were 7.9% but reached 44.4% cumulatively.

Although to date no significant studies comparing the non-drug natural cycle with the GnRH antagonist-based "modified" natural cycle IVF protocol, the available data indicates that the overall cycle outcome is similar to protocols that only use exogenous hCG to trigger final oocyte maturation. This may be explained by the reported limited ability of GnRH antagonists to entirely prevent premature LH surges in the context of a non-stimulated cycles with relatively low steroid levels. Indeed it has been suggested that an increased GnRH antagonist dose might be necessary to completely prevent the occurrence of LH surges and given the relatively limited advantage offered, the cost effectiveness of this approach is debated (Reyftman et al., 2007). In a small study in eight volunteers treated with transdermal E2 with or without daily GnRH antagonist co-treatment (Messinis et al., 2010), GnRH antagonists were unable to block the occurrence of LH surge induced by supra-physiologic E2 levels. It was suggested that GnRH antagonists behave differently during ovarian stimulation and non-stimulated cycles with an additional role of other endocrine factors, such as the putative gonadotropin-surge attenuating factor (Messinis et al., 2010).

A systematic review of the efficacy of natural cycle IVF (Pelinck et al., 2002) summarized the findings of 20 studies comprising a total of 1800 initiated ncIVF cycles. This review included studies where HCG was the only drug used for induction of oocyte maturation and where it was possible to calculate ongoing pregnancy rates per started cycle from published data. This review concluded that only 45.5% of initiated cycles reached embryo transfer resulting in a 7.2% ongoing pregnancy rate per initiated cycle. However, there was a great variation among studies and embryo transfer and ongoing pregnancy rates per started cycle varied between 22%–80% and 0%–16%, respectively. The authors argued that further refinements such as the use of GnRH antagonists for controlling LH surges or NSAID to prevent premature ovulation might further increase the efficiency of ncIVF treatments, but the added value of these interventions remains to be demonstrated.

More recently, Roesner et al. (2014) reviewed the 5-year experience of their center in Germany involving a natural cycle IVF approach in which only hCG was administered to trigger final oocyte maturation, and compared it with the reported outcomes of 28 ncIVF studies published between 1995 and 2012. Clinical pregnancy rates per embryo transfer varied widely between 10% and 56%; figures consistent with those derived by Pelinck et al. (10%–50%). During the 10-year period that separates the publication of these two literature reviews, there was no apparent improvement in the overall efficiency of natural cycle IVF treatments.

The overall low per cycle efficiency of natural cycle IVF approach can be ameliorated by repeating successive treatment attempts. A number of studies have reported cumulative success rates following (modified) natural cycle approach. The earlier UK study reported the outcome 181 cycles performed in 52 infertile women (Nargund et al., 2001). Although per cycle live birth rate was only 8.8%, by performing four treatment attempts a cumulative live births rate of 32% was reportedly achieved. A large study from Groningen reported the cumulative outcome of 1048 modified natural cycle IVF (mncIVF) cycles performed in 256 patients (Pelinck et al., 2007). The participants, all under 37 years of age, were offered a maximum of 9 cycles, although on average 4.1 attempts were made. Ongoing pregnancy rates were 7.9% per cycle were reported but cumulative rates reached 44.4%. These data provided further support to the notion that modified ncIVF may represent a valuable treatment alternative to conventional ovarian stimulation when outcomes are considered over a course of treatment rather than per cycle.

Table 1 Challenges of natural cycle IVF

Challenges	Possible solutions	Advantages/drawbacks
Uncontrolled LH surge	Flexible scheduling GnRH antagonists Low-dose clomiphene	Very challenging Additional cost, inefficient? Endometrial effect?
Premature ovulation	NSAIDs	Cheap, efficient
Empty follicle	Follicular flushing	Time consuming
Immature oocyte	In vitro maturation	Low efficiency
Mandatory single embryo transfer	Optimized ultrasound-guided embryo transfer technique	Requires training
Low per cycle efficiency	Repeated cycles Patient selection	Risk of drop-out Limits patients access

Adapted from Bodri, D. (2017). Natural cycle IVF with the spontaneous LH surge. In: Chian, R. C. et al. (eds.) *Development of in vitro maturation for human oocytes*. Springer International Publishing AG.

A recently published 3-year cohort study also reported cumulative success from a program based uniquely on ncIVF and minimal ovarian stimulation (Bodri et al., 2014). Although this cohort was a mixture of different mild treatment approaches, natural cycle IVF still represented 57% of all treatment attempts. Crude cumulative live birth rates were favorable in young patients (65% and 60% in <35 and <38-year-old patients, respectively), still acceptable at intermediate age (39% for patients between 38 and 40 years of age) and declined gradually in >40-year-old infertile patients. Moreover, a plateau was also detected after approximately four to six treatment cycles.

A recent registry-based study from the United States yielded further insights into the efficiency of ncIVF treatment in different age groups. The review presented data on all 795 unstimulated IVF cycles that were registered in the SART (Society for Assisted Reproductive Technologies) database during 2006 and 2007 (Gordon et al., 2013). However, the average number of unstimulated cycles per reporting center was <10 per year and hence only represented a tiny fraction (1.5%) of all treatment cycles performed at each center. Limited experience and a selected (poor prognosis) population may have adversely affected reported outcomes. Indeed many clinics will offer natural cycle IVF primarily to poor prognosis, older patients with diminished ovarian reserve and poor egg quality for whom stimulated IVF offers little added value.

When offered to good prognosis patients outcomes can be better. The authors of the SART analysis reported embryo transfer and live birth rates per started cycle in <35 years patients of 54% and 15%, respectively, declining to 42% and 9% in 38–40-year-old patients and further in patients >40-year old. However, compared to stimulated IVF implantation rates per embryo were higher overall, suggesting some benefits from the avoiding the supraphysiological estradiol levels associated with ovarian stimulation. Taken together, these data suggest that ncIVF should be considered in younger patients (<38 years) rather than limiting it to older patients in whom all other treatment options have failed.

The above findings derived from the US registry were corroborated by a recent retrospective analysis of 469 natural cycle IVF cycles performed in 164 poor responding patients (Polyzos et al., 2012). In comparison to stimulated cycles, the authors found embryo transfer and live birth rates to be significantly reduced, concluding that natural cycle IVF does not represent any substantial benefit to this group.

A number of challenges relating to natural cycle continue to be encountered and these are summarized in Table 1.

Conclusions

The origins of mild and natural cycle IVF go back to the very early days of clinical IVF. However, it has required an increase in the efficiency of assisted conception technologies, together with an appreciation of the risks, costs, and burden of conventional stimulation protocols to reawaken interest in this lighter approach to treatment. While they continue to represent a relatively small proportion of IVF cycles worldwide, the advantages are widely recognized and have led to a reconsideration of standard clinical practices, with an increasing trend to using lower doses of FSH and stimulation regimens designed to limit the risk of complications of IVF. A number of patients remain attracted to these milder approaches for the reasons outlined in this article; a less invasive, less costly more natural treatment that reduces the disruptive side effects of ovarian stimulation with exogenous gonadotropins while still offering for many a reasonable chance of receiving a high quality embryo, and an acceptable chance of conceiving a health pregnancy over a number of cycles (Nargund et al., 2017).

References

- Arce, J. C., Andersen, A. N., Fernández-Sánchez, M., Visnova, H., Bosch, E., García-Velasco, J. A., Barri, P., de Sutter, P., Klein, B. M., & Fauser, B. C. (2014). Ovarian response to recombinant human follicle-stimulating hormone: A randomized, antimüllerian hormone-stratified, dose-response trial in women undergoing in vitro fertilization/intracytoplasmic sperm injection. *Fertility and Sterility*, 102(6), 1633–1640.

- Baart, E. B., Martini, E., Eijkemans, M. J., Van Opstal, D., Beckers, N. G., Verhoeff, A., Macklon, N. S., & Fauser, B. C. (2007). Milder ovarian stimulation for in-vitro fertilization reduces aneuploidy in the human preimplantation embryo: A randomized controlled trial. *Human Reproduction*, 22, 980–988.
- Bodri, D., Kawachiya, S., De Brucker, M., Tournaye, H., Kondo, M., Kato, R., et al. (2014). Cumulative success rates following mild IVF in unselected infertile patients: A 3-year, single-centre cohort study. *Reproductive Biomedicine Online*, 28(5), 572–581.
- Edwards, R. G., Lobo, R., & Bouchard, P. (1996). Time to revolutionize ovarian stimulation. *Human Reproduction*, 11, 917–919.
- Fauser, B. C. J. M. (2017). Patient-tailored ovarian stimulation for in vitro fertilization. *Fertility and Sterility*, 108, 585–591.
- Gordon, J. D., Dimattina, M., Reh, A., Botes, A., Celia, G., & Payson, M. (2013). Utilization and success rates of unstimulated in vitro fertilization in the United States: An analysis of the Society for Assisted Reproductive Technology database. *Fertility and Sterility*, 100(2), 392–395.
- Hohmann, F. P., Macklon, N. S., & Fauser, B. C. (2003). A randomized comparison of two ovarian stimulation protocols with gonadotropin-releasing hormone (GnRH) antagonist cotreatment for in vitro fertilization commencing recombinant follicle-stimulating hormone on cycle day 2 or 5 with the standard long GnRH agonist protocol. *The Journal of Clinical Endocrinology and Metabolism*, 88, 166–173.
- Kyrou, D., Kolibianakis, E. M., Fatemi, H. M., Grimbizis, G. F., Theodoridis, T. D., Camus, M., Tournaye, H., Tarlatzis, B. C., & Devroey, P. (2012). Spontaneous triggering of ovulation versus HCG administration in patients undergoing IUI: A prospective randomized study. *Reproductive BioMedicine Online*, 25(3), 278–283. <https://doi.org/10.1016/j.rbmo.2012.05.005>.
- Lenton, E. A. (2007). Natural cycle IVF with and without terminal HCG: Learning from failed cycles. *Reproductive Biomedicine Online*, 15(2), 149–155.
- Macklon, N. S., Stouffer, R. L., Giudice, L. C., & Fauser, B. C. (2006). The science behind 25 years of ovarian stimulation for in vitro fertilization. *Endocrine Reviews*, 27, 170–207.
- Messinis, I. E., Vanakara, P., Zavos, A., Verikouki, C., Georgoulas, P., & Dafopoulos, K. (2010). Failure of the GnRH antagonist ganirelix to block the positive feedback effect of exogenous estrogen in normal women. *Fertility and Sterility*, 94(4), 1554–1556.
- Nargund, G., Waterstone, J., Bland, J., Philips, Z., Parsons, J., & Campbell, S. (2001). Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. *Human Reproduction*, 16(2), 259–262.
- Nargund, G., Datta, A. K., & Fauser, B. C. J. M. (2017). Mild stimulation for in-vitro fertilization. *Fertility and Sterility*, 108, 558–567.
- Pelink, M. J., Hoek, A., Simons, A. H., & Heineman, M. J. (2002). Efficacy of natural cycle IVF: A review of the literature. *Human Reproduction Update*, 8(2), 129–139.
- Pelink, M. J., Vogel, N. E., Arts, E. G., Simons, A. H., Heineman, M. J., & Hoek, A. (2007). Cumulative pregnancy rates after a maximum of nine cycles of modified natural cycle IVF and analysis of patient drop-out: A cohort study. *Human Reproduction*, 22(9), 2463–2470.
- Phillips, S. J., Kadoch, I. J., Lapensee, L., Couturier, B., Hemmings, R., & Bissonnette, F. (2007). Controlled natural cycle IVF: Experience in a world of stimulation. *Reproductive Biomedicine Online*, 14(3), 356–359.
- Polyzos, N. P., Blockeel, C., Verpoest, W., De Vos, M., Stoop, D., Vloeberghs, V., et al. (2012). Live birth rates following natural cycle IVF in women with poor ovarian response according to the Bologna criteria. *Human Reproduction*, 27(12), 3481–3486. 22940767.
- Reyftmann, L., Dechaud, H., Loup, V., Anahory, T., Brunet-Joyeux, C., Lacroix, N., et al. (2007). Natural cycle in vitro fertilization cycle in poor responders. *Gynécologie, Obstétrique & Fertilité*, 35(4), 352–358.
- Roesner, S., Pflaumer, U., Germeyer, A., Montag, M., Strowitzki, T., & Toth, B. (2014). Natural cycle IVF: Evaluation of 463 cycles and summary of the current literature. *Archives of Gynecology and Obstetrics*, 289(6), 1347–1354.
- Rongieres-Bertrand, C., Olivennes, F., Righini, C., Fanchin, R., Taieb, J., Hamamah, S., et al. (1999). Revival of the natural cycles in in-vitro fertilization with the use of a new gonadotrophin-releasing hormone antagonist (Cetrorelix): A pilot study with minimal stimulation. *Human Reproduction*, 14(3), 683–688.
- Schipper, I., Hop, W. C., & Fauser, B. C. (1998). The follicle-stimulating hormone (FSH) threshold/window concept examined by different interventions with exogenous FSH during the follicular phase of the normal menstrual cycle: Duration, rather than magnitude, of FSH increase affects follicle development. *The Journal of Clinical Endocrinology and Metabolism*, 83(4), 1292–1298.
- Sunkara, S. K., Rittenberg, V., Raine-Ferring, N., Bhattacharya, S., Zamora, J., & Coomarasamy, A. (2011). Association between the number of eggs and live birth in IVF treatment: An analysis of 400135 treatment cycles. *Human Reproduction*, 26, 1768–1774.
- Van der Gaast, M. H., Eijkemans, M. J., van der Net, J. B., de Boer, E. J., Burger, C. W., van Leeuwen, F. E., Fauser, B. C., & Macklon, N. S. (2006). Optimum number of oocytes for a successful first IVF treatment cycle. *Reproductive Biomedicine Online*, 13, 476–480.
- Verberg, M. F., Eijkemans, M. J., Macklon, N. S., Heijnen, E. M., Baart, E. B., Hohmann, F. P., Fauser, B. C., & Broekmans, F. J. (2009a). The clinical significance of the retrieval of a low number of oocytes following mild ovarian stimulation for IVF: A meta-analysis. *Human Reproduction Update*, 15, 5–12.
- Zayed, F., Lenton, E. A., & Cooke, I. D. (1997). Natural cycle in-vitro fertilization in couples with unexplained infertility: Impact of various factors on outcome. *Human Reproduction*, 12(11), 2402–2407.
- Zhang, J., Chang, L., Sone, Y., & Silber, S. (2010). Minimal ovarian stimulation (mini-IVF) for IVF utilizing vitrification and cryopreserved embryo transfer. *Reproductive Biomedicine Online*, 21(4), 485–495.
- Zhang, J. J., Merhi, Z., Yang, M., Bodri, D., Chavez-Badiola, A., Repping, S., & van Wely, M. (2016). Minimal stimulation IVF vs conventional IVF: A randomized controlled trial. *American Journal of Obstetrics and Gynecology*, 214(96).

Further Reading

- Bodri, D., Chian, R. C., et al. (2017). Natural cycle IVF with the spontaneous LH Surge. In *Development of in vitro maturation for human oocytes*. Cham, Switzerland: Springer International Publishing AG.
- De Klerk, C., Heijnen, E. M., Macklon, N. S., Duivenvoorden, H. J., Fauser, B. C., Passchier, J., & Hunfeld, J. A. (2006). The psychological impact of mild ovarian stimulation combined with single embryo transfer compared with conventional IVF. *Human Reproduction*, (3), 721–727.
- Heijnen, E. M., Eijkemans, M. J., De Klerk, C., Polinder, S., Beckers, N. G., Klinkert, E. R., Broekmans, F. J., Passchier, J., Te Velde, E. R., Macklon, N. S., et al. (2007). A mild treatment strategy for in-vitro fertilisation: A randomised non-inferiority trial randomized trial. *Lancet*, 369, 743–749.
- Verberg, M. F., Eijkemans, M. J., Heijnen, E. M., Broekmans, F. J., de Klerk, C., Fauser, B. C., & Macklon, N. S. (2008). Why do couples drop-out from IVF treatment? A prospective cohort study. *Human Reproduction*, 23(9), 2050–2055.
- Verberg, M. F. G., Macklon, N. S., Nargund, G., Frydman, R., Devroey, P., Broekmans, F. J., & Fauser, B. C. J. M. (2009b). Mild ovarian stimulation for IVF. *Human Reproduction Update*, (15), 13–29.

Drugs Used for Controlled Ovarian Stimulation

Carlo Alviggi, Alessandro Conforti, Pasquale De Rosa, Raffaele Riccardi, Alessandra Abbamondi, and Giuseppe De Placido,
Department of Neuroscience, Reproductive Science and Odontostomatology, University of Naples Federico II, Naples, Italy

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

AFC Antral follicle count
AMH Anti müllerian hormone
ART Assisted reproductive technology
BMI Body mass index
CHO Chinese hamster ovary
COS Controlled ovarian stimulation
r-hFSH Recombinant-human follicle stimulating hormone
FSH Follicle stimulating hormone
hMG Human menopausal gonadotropin
GnRH Gonadotropin releasing hormone
hCG Human chorionic gonadotropin
IVF In vitro fertilization
LH Luteinizing hormone
IU International unit

Introduction

Controlled ovarian stimulation (COS) is crucial in assisted reproductive technologies (ART). The aim of COS is to promote the simultaneous maturation of multiple follicles in order to collect the adequate number of oocytes that can be subsequently used for in vitro fertilization. This goal is obtained by the use of gonadotropin medications, which induce multiple follicular growth. During COS, medications that suppress pituitary function, such as gonadotropin releasing hormone (GnRH) analogues, are prescribed in order to prevent an unwarranted ovulation before the scheduled oocytes retrieval. The ovulation of the recruited follicles is triggered only at the end of the ovarian stimulation with specific drugs with luteinizing hormone (LH) activity. In **Fig. 1** is illustrated the history of gonadotropin formulations.

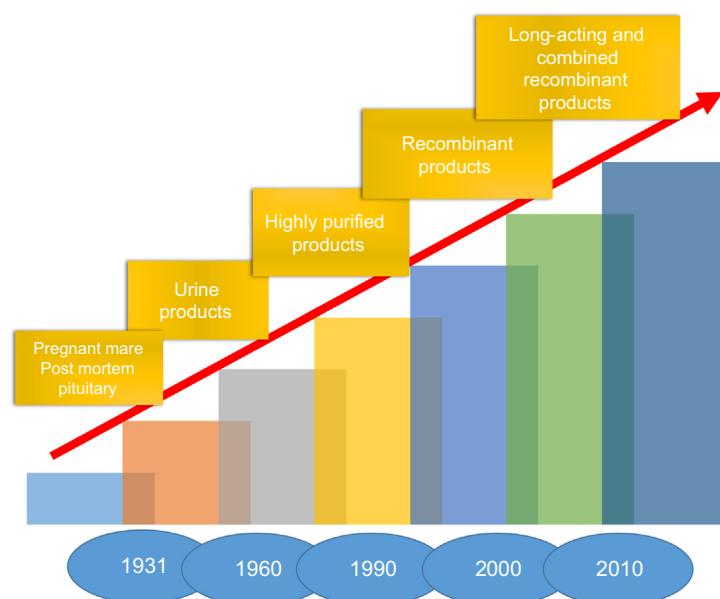


Fig. 1 The history of gonadotropin formulations.

In summary, COS requests the use of three classes of drugs:

- Follicle-stimulating drugs (recombinant or menopausal gonadotropins);
- Modulating-ovulation drugs (GnRH agonist and GnRH antagonist);
- Ovulation-inductor drugs (hCG and GnRH agonist);

Table 1 reported the pharmacological properties of the most common products available in the market.

Medications for Controlled Ovarian Stimulation

Follicle-Stimulating Drugs

Follicle-stimulating gonadotropins can be divided in:

- Recombinant gonadotropins, synthesized through genetic engineering techniques (recombinant DNA technique): follitropin- α , follitropin- β , corifollitropin- α , lutropin- α .
- Menopausal gonadotropins, extracted from menopausal women's urines, containing (follicle stimulating hormone) FSH and LH in different proportions (menotropin) or containing only FSH (urofollitropin).

Follicle stimulating gonadotropins induce follicle growth by binding to specific membrane receptors, localized in both theca and granulosa cells.

Menotropin

Menotropin is extracted from the urine of postmenopausal women (Lunenfeld, 2004). Early preparations contained varying levels of FSH, LH, and human chorionic gonadotropin (hCG). Currently, new purification techniques resulted in FSH and LH activities standardized at 75 IU for each type of gonadotropin (ASRM, 2008). Human menopausal gonadotropin (hMG) have both FSH and LH activity, but the latter is primarily derived from the hCG component in postmenopausal urine, which is concentrated during purification (Lunenfeld, 2004). Occasionally, hCG is added to induce the desired level of LH-like biological activity (Lunenfeld, 2004). Currently, hMG are commercially available at FSH:LH ratio of 1:1.

FSH is fundamental for follicular recruitment in the first stages of folliculogenesis, while LH is important for ovarian steroidogenesis and is involved in physiological events that promote the growth of an appropriate preovulatory follicle in the last stages of folliculogenesis.

In view of that, menotropin, having a double activity (FSH and LH), induces the growth and the development of ovarian follicles as well as the gonadic steroids in women with primitive ovarian deficiency. After an intramuscular or subcutaneous administration, the menotropin is excreted mainly by the kidney.

Furthermore, according with LH action in steroidogenesis strengthening, estradiol levels associated with menotropin treatment are higher than those associated with the one based on recombinant FSH (r-hFSH) in IVF cycles. This must be considered during stimulation. It is available in the pharmaceutical form of solution or powder with dosages of: 75 IU, 600 IU and 1200 IU.

Table 1 Pharmacological properties of the most common products available in the market

Gonadotropin preparation	FSH activity	LH activity	Route of administration	Route of elimination	Half-life
Menopausal gonadotropin	75 or 600 or 1200 IU	75 or 150 IU	i.m. or s.c.	Via kidneys	30 h (s.c.) 27 h (i.m.)
Urofollitropin	75 or 150 or 225 or 300 IU	Negligible	i.m.	Via liver and kidneys	35–40 h
Follitropin α	75 or 150 or 450 or 900 or 1050 IU	0	s.c.	Via liver and kidneys	24 h
Follitropin β	100 or 150 or 200 or 900 IU	0	i.m. or s.c.	Via liver and kidneys	26.9–43.9 h (dose dependent)
Corifollitropin α	100–150 μ g	0	s.c.	Via kidneys	65–70 h
Lutropin α	0	75 IU	s.c.	< 5% of dose excreted renally as unchanged drug	10–12 h
Urinary hCG	0	1000 or 2000 or 5.000 or 10.000 IU	i.m.-s.c.	Via liver and kidneys	29–36 h
Recombinant hCG	0	250 μ g	s.c.	$\cong$ 10% of dose excreted renally as unchanged drug	29–36 h

s.c.: subcutaneous; i.m.: intramuscular; h: hour; IU: international unit; μ g: microgram

Due to individual variability of ovarian response to the exogenous gonadotropins treatment, it is not possible to define a uniform dosing schedule. The recommended initial dose is usually 150–225 IU per day for at least the first 5 days, but it could be modified during the stimulation on the base of estradiol levels or follicles growth. Generally, the maximum dose should not exceed 450 IU per day and for no more than 20 days.

Urofollitropin

The urofollitropin belongs to the highly-purified gonadotropins family. It is also extracted by postmenopausal women's urines, but unlike hMG it is mainly characterized by the FSH action. This highly purified formulation contains less than 0.1 IU of LH and 5% of urinary proteins and could be administrated subcutaneously.

Urofollitropins are indicated for ovulation induction in women affected by chronic anovulation, although they could be also used for controlled ovarian stimulation treatments in women who underwent in vitro fertilization.

Posology depends on patient's needs and should be evaluated on a case-by-case basis. For example, in women affected by chronic anovulation, it is recommended a dosage between 75 and 150 IU per day. Among the women candidates for in vitro fertilization techniques, the initial dose ranges from 150 to 225 IU per day. The maximum recommended dose should not exceed 450 IU per day.

The preparations currently available can be used in subcutaneous or intramuscular form, in solution or powder form at dosages of 75 IU, 150 IU, 225 IU, 300 IU.

Follitropin alpha

The Follitropin α is a recombinant molecule of natural FSH (follicle-stimulating hormone) synthesized by Chinese hamster ovary (CHO) cell line into which has been inoculated a gene able to synthesize human FSH. In detail, FSH α and β subunits are incorporated into DNA of host CHO cells using specific plasmid vectors containing FSH gene and bacterial segments (Lunenfeld, 2004). After subcutaneous injection, the absolute bioavailability is about 70%. The initial and terminal half-lives after the administration of 150 IU recombinant FSH are 2 h and 24 h respectively (Leao and Esteves, 2014). Delivery systems include pen-shaped administration devices or lyophilized powder formulation (ASRM, 2008).

In patients undergoing COS for ART, the commonly used regimen requires the administration of 100–225 IU per day and could be progressively calibrated according to patient's response.

Follitropin beta

The Follitropin β is synthesized, like the Follitropin α , through the DNA recombinant technique from a Chinese hamster's line of ovarian cells, edited with human FSH genetic subunits.

Purification process and glycosylation are different in Follitropin α and Follitropin β formulation (Olijve et al., 1996). Nonetheless, these molecular differences do not have significant impact on clinical practice (ASRM, 2008).

In the same way as Follitropin α , Follitropin β can be used to induce follicular growth in case of chronic anovulation and could be adopted in controlled ovarian stimulation before in vitro fertilization.

Follitropin β is administrated subcutaneously and its peak level is reached within 12 h from administration. The absolute bioavailability is about 77% and an elimination half-life of 26.9–43.9 h (Table 1).

Follitropin β is available in liquid (pens/cartridges devices) or in lyophilized powder formulation (ASRM, 2008).

In controlled ovarian stimulation protocols, an initial dose of 100–225 IU per day is recommended. Afterward, any dosage modification is required on the basis of individual ovarian response.

Corifollitropin alpha

Corifollitropin α is a molecular hybrid obtained by the fusion of human Chorionic Gonadotropin β subunit carboxyl-terminal peptide with human FSH β chain (Balen et al., 2004). The addition of carboxyl-terminal peptide, which has 4 connected oligosaccharidic chains, provides a slower absorption and a longer plasmatic half-life while maintaining the same pharmacodynamic profile (Boime and Ben-Menahem, 1999).

The molecule acts exclusively on FSH receptors, missing of a LH-like activity. The plasmatic peak is reached 44 h after the administration and the resulting absolute bioavailability equals 58%.

The Corifollitropin α has an elimination half-life of 65 h and has a primarily renal clearance (Verbost et al., 2011).

The pharmacokinetic profile after a single injection is characterized by a higher stimulant FSH activity in the first 2 days of stimulation, followed by a gradual reduction of activity.

To date, the Corifollitropin α is available at the dosages of either 100 or 150 micrograms. The latter is suggested for women weighing more than 60 Kg or older than 36 years (Devroey et al., 2009, 2004).

Because of a higher half-life, one single subcutaneous injection of the recommended dose can replace the first 7 daily injections of r-hFSH. The stimulation usually starts on the 2nd–3rd day of menstrual cycle with a single subcutaneous injection. Afterwards, the stimulation can be further continued through the addition of exogenous FSH either in recombinant or in menopausal form at variable doses, according to ovarian response.

The main issue concerning the use of Corifollitropin α is the impossibility to adapt dosage during the ovarian stimulation in the first 7 days of stimulation (Patil, 2014). Moreover, in patients with a high risk of developing an excessive response to the ovarian

stimulation, its use should be avoided (Patil, 2014). On the other hand, in patients with normal response to the treatment, the Corifollitropin α can be a valid and a better patient-friendly alternative to the conventional treatments.

Lutropin α

Lutropin α is a recombinant human luteinizing hormone (r-hLH) glycoprotein, composed by two subunits α e β linked by non-covalent bonds.

According to the “two cells/two gonadotropins” hypothesis, LH and FSH are both essential for folliculogenesis. LH binds, a receptor shared with the hCG in both theca and granulosa cells.

The supplementation of LH could be of benefit in specific subgroups of women undergoing ART (Alviggi et al., 2011). For instance, recombinant LH in association with FSH is recommended for the induction of follicular growth in adult women with hypogonadotropin hypogonadism (Dhillon and Keating, 2008).

The role of LH is not limited to the support of androgenic production, but it also seems important in the biological functions carried out by granulosa. Data show how LH can improve the quota of cAMP in late proliferative phase in this compartment. Hence, the hypothesis of an expression of LH receptors in the granulosa (Jeppesen et al., 2012).

A recent meta-analysis pointed out how, in women in advanced reproductive age, the supplementation with recombinant LH can result in a significant improvement of implantation and pregnancy rate (Hill et al., 2012).

Moreover, LH administration increases the number of recovered oocytes and IVF outcome, compared with the sole increase of FSH dosage in women with a stagnation in follicular growth during COS (Ferraretti et al., 2004; De Placido et al., 2005). These women, identified as *hypo-responders*, represent approximately the 10%–12% of normogonadotropic women who underwent IVF (Alviggi et al., 2013).

After subcutaneous administration, the absolute bioavailability of Lutropin α is about 60% and has a terminal half-life of about 10–12 h, compared to 35–40 h of menopausal gonadotropin formation (Leao and Esteves, 2014). Clearance is about 2 L/h and less than 5% is eliminated with urines.

Recombinant LH has been used for clinical use since 1993, in vials designed to deliver 75 IU. A new combination of recombinant FSH plus recombinant LH was introduced in 2007 at 2:1 ratio. This new formulation shows similar bioequivalence of follitropin α and lutropin α administrated alone (Picard et al., 2008).

Ovulation Modulating Drugs

The administration of gonadotropins during COS is associated to an increased risk of spontaneous early LH surge that could result in an unwarranted ovulation. The use of GnRH analogues suppresses hypothalamic-pituitary-gonadal axis and prevents the early LH surge, providing a longer duration and higher power compared to the endogenous GnRH. There are two types of gonadotropin releasing hormones analogues adopted during in vitro fertilization: GnRH agonist and GnRH antagonist.

GnRH agonist

The use of GnRH agonist has been considered a milestone in preventing LH surge until the beginning of 2000. The GnRH agonist initially binds to its pituitary receptors, determining a transient increase of gonadotropins secretion, called *flare-up* effect. Subsequently, the continuous stimulation of pituitary receptors provokes a *down regulation* that results in the suppression of gonadotropins production.

GnRH antagonists

GnRH antagonists were launched at the beginning of the century. Compared to GnRH agonists, they cause an immediate block of gonadotropins release through a competitive antagonism for the receptor site. Requiring shorter time for pituitary suppression, they could be administrated in the late follicular phase (stimulation day 7–8) or when the size of leading follicles is around 13–14 mm. GnRH antagonists are administrated in a single (3 mg depot-formulation) or in multiple dose regimen (0.25 microgram daily).

Ovulation-Inductor Drugs

Human chorionic gonadotropin (hCG)

hCG is an hormone produced by the placenta during the pregnancy. It is characterized by a similar structure to LH and it is used to induce follicles maturation and to trigger ovulation, which occurs approximately 36 h after administration.

Currently, urinary preparations are the most used at dosage of 10,000 UI or 5,000 IU and are administrated intramuscularly when at least one follicle exceeds the dimensions of 17–18 mm.

Recombinant forms of hCG are also available and, likewise other recombinant gonadotropin, derive from genetically form Chinese hamster's line of ovarian cells though recombinant technologies. Comparing with urinary form, recombinant hCG is characterized by higher purity that makes this medication suitable for subcutaneous administration (Leao and Esteves, 2014). Pharmacogenetic profiles do not differ from urinary formulations with linearity over a dosage range of 500 IU and 20,000 IU and half-life of 30 h (Trinchard-Lugan et al., 2002).

In case of in vitro fertilization, the time of hCG administration is crucial; as a matter of fact the oocyte pick up should be performed about 36 h after the administration.

According to the last meta-analysis, there is no difference between recombinant or urinary forms in terms of ongoing pregnancy rates or risk of ovarian hyperstimulation (Youssef et al., 2016).

GnRH analogues

This new approach was proposed to reduce the risk of ovarian hyper stimulation syndrome (OHSS) onset during ovarian stimulation. OHSS is a potential life-threatening complication that can occur following the administration of hCG at the time of ovulation induction.

In the protocols of ovarian stimulation with GnRH antagonists, the analog is able to unseat the antagonist molecule on the pituitary, inducing a FSH and LH increase and finally the follicular maturation. When the diameter of a follicle is 17–18 mm, ovulation can be induced using a dosage of 0.2 mg subcutaneously.

Despite a decrease of OHSS risk, a reduction of pregnancy rate has been observed as a consequence of the lack of hCG activity during the luteal phase. Therefore, various strategies have been proposed to increase pregnancy rates in the protocols that use GnRH analogues as triggering:

- Administration of low doses of hCG (1500 UI) starting from the day of oocyte *pick-up*.
- Support of luteal phase with high doses of estradiol and progesterone in variable doses and formulations.
- Freezing of oocytes and embryos collected that could be used in a different cycle.

Stimulation Protocols

For many years, the most common used GnRH analogues for pituitary suppression in COS were GnRH agonist medications. More recently, new regimens based on GnRH antagonist have been introduced. Compared with GnRH agonist, these drugs induce an immediate pituitary suppression and are associated with shorter duration of the treatment, lower amount of gonadotropin required and lower risk of ovarian hyperstimulation syndrome (OHSS). Nonetheless, pregnancy and live-birth rate are comparable between GnRH agonist and GnRH antagonist regimen (Al-Inany et al., 2016).

Mild Protocol

The “mild” stimulation strategy has been proposed as an alternative to the standard stimulation protocol, in which a relevant amount of exogenous gonadotropins is spent. This strategy follows the theory that the stimulation with the minimum concentrations of drugs could promote the recruitment of good quality follicles at the expense of a reduced amount. Mild protocol offers several advantages such as a patient-friendly approach and a reduced risk of ovarian hyperstimulation consequences. Nonetheless, this strategy is usually criticized considering that the retrieval of less oocytes may impair IVF success. It is usually carried out by a low dose of exogenous FSH (100–150 IU/day) from day 4 of a spontaneous menstrual cycle (Fauser et al., 2010; Casano et al., 2012).

Long Protocol

Long protocol is widely used in IVF, however, short protocols with antagonist medication are becoming more and more frequent. It is characterized by subcutaneous or intramuscular administration of GnRH agonist via depot or daily formulation, starting from the 21st–24th day of previous menstrual cycle (late luteal phase). Ovarian stimulation starts when pituitary suppression is confirmed by low estrogenic plasmatic levels and no ultrasound evidence of any anomalous ovarian cysts, which usually occurs after 14–15 day of treatment. GnRH agonist administration ends the day of ovulation triggering.

Ultralong Protocol

This protocol is roughly similar to the long one, except that GnRH agonist is administrated for two or more consecutive cycles preceding ovarian stimulation. It is recommended in patients with history of endometriosis and adenomyosis. As a matter of fact, a recent meta-analysis demonstrated that this strategy could offer a significant improvement in clinical pregnancy rates in such patients (Sallam et al., 2006).

Short and Ultra-Short Protocols

In short and ultra-short protocols the administration of agonists (0.1 mg/day) starts from day one or two of the menstrual cycle (in conjunction with the commencement of ovarian stimulation) to the day of ovulation induction (short protocol), or just for 3 days (ultra-short protocol) (Maheshwari et al., 2011). The aim is to encourage the initial phase of stimulation through massive release of endogenous FSH and LH (“flare-up” effect). Bearing in mind such characteristics, these protocols has been widely adopted in *poor responders* patients (LoutRADIS et al., 2008). However, in general IVF population, there is a moderate quality evidence that short/ultra-short protocols are associated with lower clinical pregnancy rates than long one (Siristatidis et al., 2015).

Antagonists Protocols

In this protocol pituitary suppression starts after the administration of gonadotropins in the final phase of ovarian stimulation. In the fixed protocol the antagonist is administered at a scheduled day of stimulation (5th–7th), while in the flexible one the antagonist starts on the basis of follicular growth (i.e., 13–14 mm) or estradiol levels. In *poor responders*, some authors report an increased compliance to treatment reduced gonadotropins doses consumption and duration (Loutradis et al., 2008). In *hyper-responders*, others have shown that this protocol reduces the risk of OHSS and cycle interruption (Youssef et al., 2016).

Cancer Patients Protocols

As a consequence of the better survival rates, the number of women who survived after cancer has progressively increased and the quality of life is gaining importance. In that sense, fertility preservation should be encouraged in women with interest in future pregnancies. Among fertility preservation strategies, both embryo and oocyte cryopreservation after COS are the most widespread. Nonetheless, it is very important to minimize estrogen levels that usually raise during conventional COS in patients suffering from estrogen-dependent cancer (i.e., breast cancer). With this purpose, the concomitant administration of aromatase inhibitors is able to limit estrogens levels without compromising the ovarian response to exogenous gonadotropins (Azim et al., 2008).

Individualized Controlled Ovarian Stimulation

Variability in the infertile women population excludes the possibility of a single approach towards controlled ovarian stimulation. As explained above, modern technology has led to the development of new drugs, treatment options and quantitative methods that allow an individualized approach to IVF.

The personalization of treatments requires a comprehensive evaluation of several important aspects. First of all, age remains the best predictive factor of gametes euploidy rate. As a matter of fact, it was estimated that the percentage of abnormal embryos/oocytes dramatically increases in women over 35 years old. Strategies to improve the number of vital and euploid embryos in those women represent the most intriguing challenge nowadays considering that more and more women seeking ART are in advanced age. On the other hand, ovarian reserve markers, namely, FSH, anti-Müllerian hormone and antral follicle count (AFC), are also considered the most accurate predictors of ovarian reserve and could be successfully used to guide controlled ovarian stimulation.

The AFC (*Antral follicle count*) consists in the count of antral follicles performed with a transvaginal ultrasound probe by the 5th day of the cycle. It provides a direct quantitative measure of ovarian response to COS and it is related with the number of oocytes retrieved. Although a standardized method has been proposed (Broekmans et al., 2010), the inter- and intra-operators variability still represents a relevant drawback of this procedure.

Anti-Müllerian hormone (AMH) is the main hormonal biomarker used to assess ovarian reserve. It is produced by preantral and small antral follicles and it reflects the number of primordial follicles that characterizes the gonadotropin-independent phase of folliculogenesis.

Given the importance of AFC and AMH in the evaluation of ovarian response, several *cut-off* values based on these two markers were proposed to predict the risk of poor or hyper response to COS (Alvigi et al., 2016a; La Marca and Sunkara, 2014; Choi et al., 2011). Finally, there is an emerging evidence in literature which suggests that the ovarian sensitivity to exogenous gonadotropins could be also influenced by specific genotype profiles. Indeed, some studies report that polymorphisms of FSH receptor and the β -chain of LH may affect the success of COS (Alvigi et al., 2013, 2015, 2016b). If these data will be confirmed, an individual pharmacogenomic approach may allow to calibrate COS on the basis of individual genotype characteristics.

Acknowledgment

The authors would like to acknowledge Gabriele Basile for the final linguistic revision of the manuscript.

References

- Al-Inany, H. G., Youssef, M. A., Ayeleke, R. O., et al. (2016). Gonadotropin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database of Systematic Reviews*, 4, Cd001750.
- Alvigi, C., Andersen, C. Y., Buehler, K., et al. (2016a). A new more detailed stratification of low responders to ovarian stimulation: From a poor ovarian response to a low prognosis concept. *Fertility and Sterility*, 105, 1452–1453.
- Alvigi, C., Clarizia, R., Mollo, A., Ranieri, A., & De Placido, G. (2011). Who needs LH in ovarian stimulation? *Reproductive Biomedicine Online*, 22, S33–S41.
- Alvigi, C., Conforti, A., Caprio, F., et al. (2016b). In Estimated Good Prognosis Patients Could Unexpected “hyporesponse” to Controlled Ovarian Stimulation be Related to Genetic Polymorphisms of FSH Receptor? *Reproductive Sciences*, 23, 1103–1108.
- Alvigi, C., Conforti, A., & Esteves, S. C. (2015). Impact of mutations and polymorphisms of gonadotropins and their receptors on the outcome of controlled ovarian stimulation. In S. Ghumman (Ed.), *Principles and practice of controlled ovarian stimulation in ART*. India: Springer.
- Alvigi, C., Pettersson, K., Longobardi, S., et al. (2013). A common polymorphic allele of the LH beta-subunit gene is associated with higher exogenous FSH consumption during controlled ovarian stimulation for assisted reproductive technology. *Reproductive Biology and Endocrinology*, 11.
- Asrm. (2008). Gonadotropin preparations: Past, present, and future perspectives. *Fertility and Sterility*, 90, S13–20.

- Azim, A. A., Costantini-Ferrando, M., & Oktay, K. (2008). Safety of fertility preservation by ovarian stimulation with letrozole and gonadotropins in patients with breast cancer: A prospective controlled study. *Journal of Clinical Oncology*, 26, 2630–2635.
- Balen, A. H., Mulders, A. G., Fauser, B. C., et al. (2004). Pharmacodynamics of a single low dose of long-acting recombinant follicle-stimulating hormone (FSH-carboxy terminal peptide, corifollitropin alfa) in women with World Health Organization group II anovulatory infertility. *The Journal of Clinical Endocrinology and Metabolism*, 89, 6297–6304.
- Boime, I., & Ben-Menahem, D. (1999). Glycoprotein hormone structure-function and analog design. *Recent Progress in Hormone Research*, 54, 271–288. discussion 288-9.
- Broekmans, F. J., De Ziegler, D., Howles, C. M., et al. (2010). The antral follicle count: Practical recommendations for better standardization. *Fertility and Sterility*, 94, 1044–1051.
- Casano, S., Guidetti, D., Patriarca, A., et al. (2012). MILD ovarian stimulation with GnRH-antagonist vs. long protocol with low dose FSH for non-PCO high responders undergoing IVF: A prospective, randomized study including thawing cycles. *Journal of Assisted Reproduction and Genetics*, 29, 1343–1351.
- Choi, M. H., Yoo, J. H., Kim, H. O., et al. (2011). Serum anti-Müllerian hormone levels as a predictor of the ovarian response and IVF outcomes. *Clinical and Experimental Reproductive Medicine*, 38, 153–158.
- De Placido, G., Alviggi, C., Perino, A., et al. (2005). Recombinant human LH supplementation versus recombinant human FSH (rFSH) step-up protocol during controlled ovarian stimulation in normogonadotrophic women with initial inadequate ovarian response to rFSH. A multicentre, prospective, randomized controlled trial. *Human Reproduction*, 20, 390–396.
- Devroey, P., Boostanfar, R., Koper, N. P., et al. (2009). A double-blind, non-inferiority RCT comparing corifollitropin alfa and recombinant FSH during the first seven days of ovarian stimulation using a GnRH antagonist protocol. *Human Reproduction*, 24, 3063–3072.
- Devroey, P., Fauser, B. C., Platteau, P., et al. (2004). Induction of multiple follicular development by a single dose of long-acting recombinant follicle-stimulating hormone (FSH-CTP, corifollitropin alfa) for controlled ovarian stimulation before in vitro fertilization. *The Journal of Clinical Endocrinology and Metabolism*, 89, 2062–2070.
- Dhillon, S., & Keating, G. M. (2008). Lutropin alfa. *Drugs*, 68, 1529–1540.
- Fauser, B. C., Nargund, G., Andersen, A. N., et al. (2010). Mild ovarian stimulation for IVF: 10 years later. *Human Reproduction*, 25, 2678–2684.
- Ferraretti, A. P., Gianaroli, L., Magli, M. C., et al. (2004). Exogenous luteinizing hormone in controlled ovarian hyperstimulation for assisted reproduction techniques. *Fertility and Sterility*, 82(6), 1521.
- Hill, M. J., Levens, E. D., Levy, G., et al. (2012). The use of recombinant luteinizing hormone in patients undergoing assisted reproductive techniques with advanced reproductive age: A systematic review and meta-analysis. *Fertil Steril*, 97, 1108–14.e1.
- Jeppesen, J. V., Kristensen, S. G., Nielsen, M. E., et al. (2012). LH-receptor gene expression in human granulosa and cumulus cells from antral and preovulatory follicles. *The Journal of Clinical Endocrinology and Metabolism*, 97, E1524–31.
- La Marca, A., & Sunkara, S. K. (2014). Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: From theory to practice. *Human Reproduction Update*, 20, 124–140.
- Leao, R. B., & Esteves, S. C. (2014). Gonadotropin therapy in assisted reproduction: An evolutionary perspective from biologics to biotech. *Clinics*, 69, 279–293.
- Loutradis, D., Vomvolaki, E., & Drakakis, P. (2008). Poor responder protocols for in-vitro fertilization: Options and results. *Current Opinion in Obstetrics & Gynecology*, 20, 374–378.
- Lunenfeld, B. (2004). Historical perspectives in gonadotrophin therapy. *Human Reproduction Update*, 10, 453–467.
- Maheshwari, A., Gibreel, A., Siristatidis, C. S., & Bhattacharya, S. (2011). Gonadotrophin-releasing hormone agonist protocols for pituitary suppression in assisted reproduction. *Cochrane Database of Systematic Reviews*. Cd006919.
- Olijve, W., De Boer, W., Mulders, J. W., & Van Wezenbeek, P. M. (1996). Molecular biology and biochemistry of human recombinant follicle stimulating hormone (Puregon). *Molecular Human Reproduction*, 2, 371–382.
- Patil, M. (2014). Gonadotrophins: The future. *Journal of Human Reproductive Sciences*, 7, 236–248.
- Picard, M., Rossier, C., Papasouliotis, O., & Lugan, I. (2008). Bioequivalence of recombinant human FSH and recombinant human LH in a fixed 2:1 combination: Two phase I, randomised, crossover studies. *Current Medical Research and Opinion*, 24, 1199–1208.
- Sallam, H. N., Garcia-Velasco, J. A., Dias, S., & Arici, A. (2006). Long-term pituitary down-regulation before in vitro fertilization (IVF) for women with endometriosis. *Cochrane Database of Systematic Reviews*. Cd004635.
- Siristatidis, C. S., Gibreel, A., Basios, G., Maheshwari, A., & Bhattacharya, S. (2015). Gonadotrophin-releasing hormone agonist protocols for pituitary suppression in assisted reproduction. *Cochrane Database of Systematic Reviews*. Cd006919.
- Trinchard-Lugan, I., Khan, A., Porchet, H. C., & Munafò, A. (2002). Pharmacokinetics and pharmacodynamics of recombinant human chorionic gonadotrophin in healthy male and female volunteers. *Reproductive Biomedicine Online*, 4, 106–115.
- Verbost, P., Sloot, W. N., Rose, U. M., et al. (2011). Pharmacologic profiling of corifollitropin alfa, the first developed sustained follicle stimulant. *European Journal of Pharmacology*, 651, 227–233.
- Youssef, M. A., Abou-Setta, A. M., & Lam, W. S. (2016). Recombinant versus urinary human chorionic gonadotrophin for final oocyte maturation triggering in IVF and ICSI cycles. *Cochrane Database of Systematic Reviews*, 4, CD003719.

Role of Agonists and Antagonists in COS

Efstratios M Kolibianakis and Julia K Bosdou, Aristotle University of Thessaloniki, Thessaloniki, Greece

© 2018 Elsevier Inc. All rights reserved.

Introduction

Although the first baby born after in vitro fertilization (IVF) in 1978 was conceived in a non-stimulated cycle (Stephoe and Edwards, 1978), it was soon realized that pregnancy rates were disappointingly low following such an approach (Edwards et al., 1980). Thus, it seemed reasonable that an increase in the probability of pregnancy might result from an increase in the number of embryos transferred. For that purpose, it was necessary to induce multifollicular development that would allow the retrieval of multiple oocytes and the creation of multiple embryos. This could be achieved by stimulating the ovaries with the use of gonadotropins (Garcia et al., 1983; Trounson et al., 1981).

Gonadotropin use, however, was frequently associated with the occurrence of premature luteinizing hormone (LH) surge. This was accompanied by ovulation before oocyte retrieval leading to cycle cancellation in approximately one out of five women (Janssens et al., 2000; Loumaye, 1990).

To prevent a premature rise in LH, gonadotropin-releasing hormone (GnRH) analogues were introduced in ovarian stimulation (Fleming et al., 1982), following the revolutionary work of Schally (Schally et al., 1971); Guillemin (Ling et al., 1973).

GnRH Agonists

The mechanism of action of GnRH agonist involves its binding to the GnRH receptor in the pituitary. This will induce the release of follicle stimulating hormone (FSH) and LH (flare-up effect), increasing, at the same time, the number of GnRH receptors present (up-regulation). However, prolonged activation of GnRH receptors by GnRH agonist leads to desensitization and consequently to suppressed gonadotrophin secretion.

Through the 1980s, 1990s and until the early 2000s, a period known as the GnRH agonist era, downregulation in ovarian stimulation for IVF was exclusively accomplished with the use of GnRH agonists (Marcus and Ledger, 2001). The use of GnRH agonists for pituitary downregulation and inhibition of premature LH surge resulted in significant improvements in IVF cycles, including a decreased risk of cycle cancellation, retrieval of more oocytes and an increased availability of good quality embryos for transfer. Consequently, a higher probability of pregnancy compared to cycles with no suppression of premature LH surge was achieved (Hughes et al., 1992).

The most common protocols using GnRH agonist for suppressing premature LH surge are the “long protocol”, the “short protocol” and the “ultrashort protocol”. The long protocol involves administration of GnRH agonist for approximately 2 weeks before gonadotrophin administration is initiated. According to this protocol, GnRH agonist is administered either in the early follicular (day two of the cycle), or in the mid-luteal phase (usually day 21) of the preceding cycle. Follicular initiation of GnRH agonist in a long protocol, however, has been associated with a higher risk of functional ovarian cysts.

The short and ultrashort GnRH agonist protocols take advantage of the initial flare-up effect, occurring after administration of GnRH agonist on day two of the cycle. Duration of GnRH agonist administration last for 3 days in the ultrashort protocol and until triggering of final oocyte maturation by human chorionic gonadotrophin (hCG) administration in the short protocol (Ng and Trew, 2012).

Based on a recently published Cochrane review (Siristatidis et al., 2015), no significant difference was found between the long and the short protocol regarding live birth (OR 1.60, 95% CI: 0.85–3.03; 4 RCTs, $n = 295$ women) and ongoing pregnancy rate (OR 1.21, 95% CI: 0.82–1.78; 8 RCTs, $n = 681$ women). Similarly, no difference was observed between the long and the ultrashort protocol (OR 1.78, 95% CI 0.72–4.36; one RCT, $n = 150$ women) or between long luteal and long follicular phase GnRH agonist protocols (OR 1.89, 95% CI 0.87–4.10; one RCT, $n = 223$ women) in terms of live birth/ongoing pregnancy rate (Siristatidis et al., 2015).

GnRH Antagonists

Inhibition of premature LH surge by GnRH antagonist is achieved by a completely different mechanism compared to GnRH agonists. GnRH antagonists bind competitively to the GnRH receptors in the hypophysis. This competitive blockade of the receptors prevents endogenous GnRH stimulatory effects on the pituitary (Reissmann et al., 1995). Consequently, the secretion of gonadotrophins is decreased within hours after GnRH antagonist administration, while the occurrence of a flare up effect is avoided. Discontinuation of GnRH antagonists leads to immediate, rapid recovery of the pituitary-gonadal axis (Kenigsberg et al., 1984).

GnRH antagonist use, however, was initially associated with severe allergic reactions (Hahn et al., 1985) that inhibited their use in ovarian stimulation. Despite this, GnRH antagonistic molecules kept on developing and in the early 2000s, the third generation of GnRH antagonists that lacked histamine release problems was introduced in clinical practice (Duijkers et al., 1998; Mannaerts

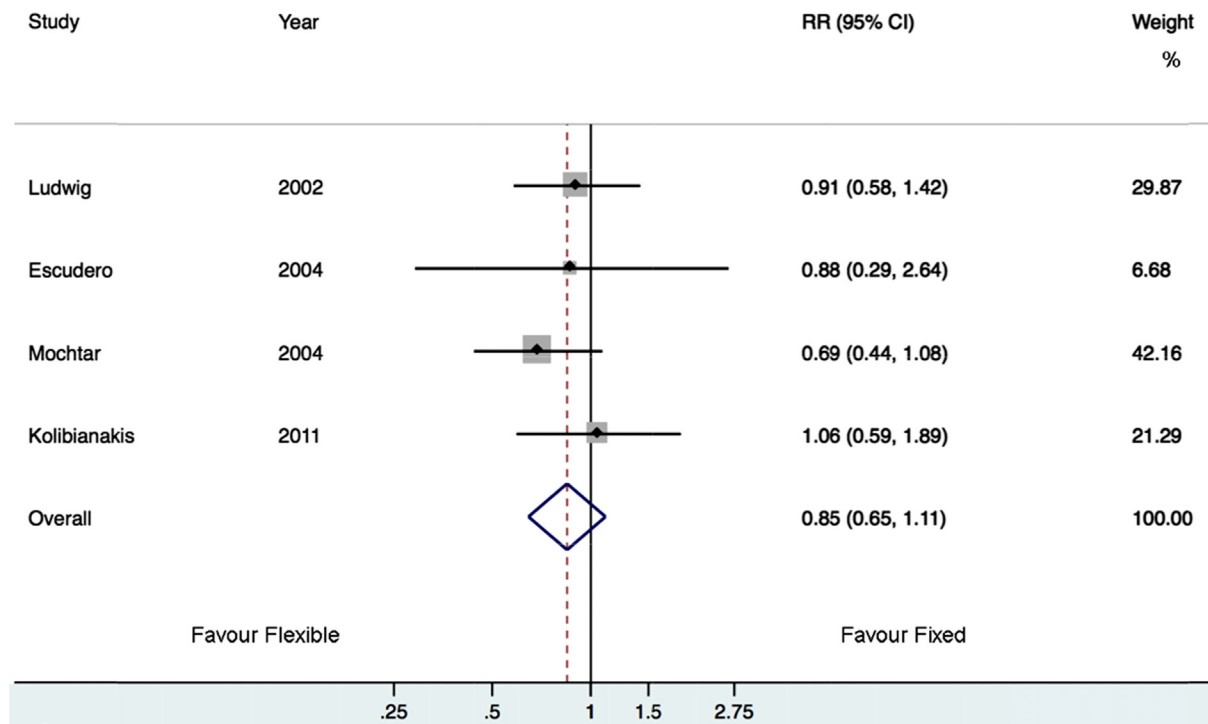


Fig. 1 Meta-analysis of RCTs comparing fixed versus flexible GnRH antagonist protocols.

and Gordon, 2000). Two types of third generation GnRH antagonists have been developed: Ganirelix (Organon, Oss, Netherlands) (Rabinovici et al., 1992) and Cetrorelix (ASTA-Medica, Frankfurt/M, Germany) (Klingmuller et al., 1993).

GnRH antagonists can be administered according to a multiple-dose scheme (Diedrich et al., 1994), where the antagonist is administered daily from stimulation day 5 or 6 onwards. Based on dose-finding studies, the optimal dose for the daily-dose GnRH antagonist scheme is 0.25 mg for both Cetrorelix and Ganirelix (Albano et al., 1997; Tgd-fs, 1998). GnRH antagonist can be initiated in either a fixed (day 6 of stimulation) or in a flexible (using ultrasound and/or hormonal criteria) protocol. A meta-analysis of four RCTs (Escudero et al., 2004; Kolibianakis et al., 2011; Ludwig et al., 2002; Mochtar and Dutch Ganirelix Study, 2004) comparing fixed versus flexible GnRH antagonist administration did not show a significant difference in clinical pregnancy rate between the two protocols (OR: 0.85, 95% CI: 0.65 to 1.11) (Kolibianakis et al., 2012b) (Fig. 1).

GnRH Agonists Versus GnRH Antagonists

The advantages and disadvantages of GnRH agonist/antagonists in ovarian stimulation for IVF have been described extensively in the literature (Table 1). Based on data from the German Registry, GnRH antagonist usage increased with time from 14.1% in 2000 to 67.0% in 2015, replacing gradually GnRH agonists as the analogue of choice for suppressing premature LH rise (D.I.R., 2000–2015) (Fig. 2).

Although the advantages of GnRH antagonists as compared to GnRH agonists were evident since their introduction in clinical practice in the early 2000 (Table 1), this was met with a lot of skepticism mainly due to doubts regarding the efficacy for pregnancy achievement. However, with time, it was made clear that the probability of pregnancy was not dependant on the type of analogue used (Al-Inany et al., 2011; Kolibianakis et al., 2006).

The most recent meta-analysis by the Cochrane group, comparing GnRH agonists with GnRH antagonists including 45 RCTs (Al-Inany et al., 2011), suggested, in addition, that a significantly decreased incidence of ovarian hyperstimulation syndrome (OHSS) was present with GnRH antagonists as compared to GnRH agonists (OR: 0.43; 95% CI: 0.33 to 0.57) (Al-Inany et al., 2011).

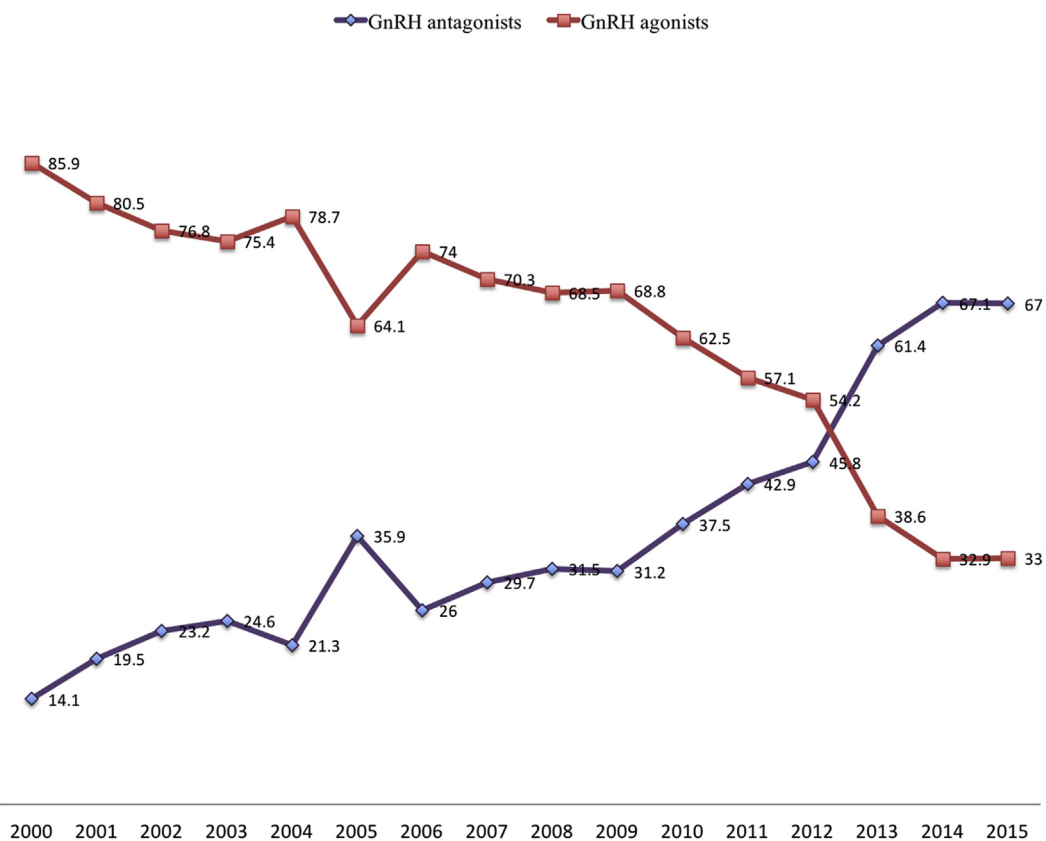
In line with this meta-analysis, the largest comparative randomized trial between the two analogues published in 2016 (Toftager et al., 2016), including 1050 women < 40 years of age undergoing a first cycle of IVF/ICSI showed that live birth rate per randomized patient was similar between the two analogue protocols compared (GnRH antagonist: 22.8% versus GnRH agonist: 23.8%; difference: – 1.0; 95% CI: – 6.3 to + 4.3; $P = 0.70$), whereas the incidence of severe OHSS was significantly lower in the GnRH antagonist versus the GnRH agonist protocol (5.1% vs 8.9%; difference: – 3.8; 95% CI: – 7.1 to – 0.4; $P = 0.02$, respectively) (Toftager et al., 2016).

Even though the risk of developing severe OHSS is significantly decreased in GnRH-antagonist protocols when hCG is used for triggering final oocyte maturation (Al-Inany et al., 2006; Al-Inany et al., 2011; Toftager et al., 2016), severe OHSS can still occur, especially in high responder patients.

Table 1 Advantages and disadvantages of GnRH agonist and antagonist for COS (Ludwig et al., 2002; Al-Inany and Aboulghar, 2001, Kolibianakis et al., 2006)

GnRH analogue	Advantages	Disadvantages
GnRH agonists	1. Follicle cohort synchronization 2. Retrieval of more oocytes	1. Initial increase in the secretion of gonadotrophins (flare-up effect) 2. Lengthy downregulation period 3. Estrogen deficiency symptoms during the downregulation period 4. Ovarian cyst formation 5. OHSS occurrence following hCG administration.
GnRH antagonists	1. Immediate action within a few hours after GnRH antagonist administration 2. No flare-up effect 3. Rapid reversibility of antagonist action 4. No ovarian cyst formation 5. No hypo-estrogenic side effects 6. Shortened duration of ovarian stimulation 7. Elimination of OHSS occurrence (potential for avoiding hCG for triggering final oocyte maturation)	1. Less flexibility regarding cycle programming as compared with the long, but not with the short, GnRH agonist protocol 2. OHSS occurrence following hCG administration although significantly decreased compared to GnRH agonists.

LH, luteinizing hormone; OHSS, ovarian hyperstimulation syndrome; hCG, human chorionic gonadotropin.

**Fig. 2** Proportion of cycles performed with either GnRH agonists or antagonists. Based on data published by the D.I.R. (2000–2015).

The replacement of hCG by GnRH agonist for triggering final oocyte maturation, followed by freezing all embryos and deferring transfer in subsequent cycles, appears to be currently the safest approach regarding the occurrence of severe OHSS resulting in its elimination (Griesinger et al., 2007; Kolibianakis et al., 2012a). It has to be noted that the option of replacing hCG with GnRH agonist is not feasible in GnRH agonist cycles and represents the main advantage of GnRH antagonists over GnRH agonists considering patients' safety (Kolibianakis et al., 2012a).

In conclusion, GnRH antagonists have rendered IVF treatment more patient friendly, requiring less days of treatment compared to GnRH agonists with similar efficacy to GnRH agonists regarding the probability of live birth. In addition, elimination of OHSS occurrence by GnRH agonist triggering and freezing of all embryos, feasible only in GnRH antagonist cycles, has led to the exclusive use of this protocol in high responder patients such as women with polycystic ovarian syndrome (PCOS). Taking into account all the advantages of GnRH antagonists, there is probably no much doubt that GnRH antagonists are the obvious analogue of choice for a safe, effective and patient friendly stimulation, gradually leading the GnRH agonist era to an end.

References

- Albano, C., et al. (1997). Comparison of different doses of gonadotropin-releasing hormone antagonist Cetrorelix during controlled ovarian hyperstimulation. *Fertility and Sterility*, 67, 917–922.
- Al-Inany, H., & Aboulghar, M. (2001). Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Database Systematic Reviews*, CD001750.
- Al-Inany, H. G., et al. (2006). Gonadotrophin-releasing hormone antagonists for assisted conception. *Cochrane Database of Systematic Reviews*, CD001750.
- Al-Inany, H. G., et al. (2011). Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database of Systematic Reviews*, CD001750.
- DIR, Deutsches IVF Register (2000–2015) Jahrbuch, <http://www.deutsches-ivf-register.de>.
- Diedrich, K., et al. (1994). Suppression of the endogenous luteinizing hormone surge by the gonadotrophin-releasing hormone antagonist Cetrorelix during ovarian stimulation. *Human Reproduction*, 9, 788–791.
- Duijkers, I. J., et al. (1998). Single and multiple dose pharmacokinetics and pharmacodynamics of the gonadotrophin-releasing hormone antagonist Cetrorelix in healthy female volunteers. *Human Reproduction*, 13, 2392–2398.
- Edwards, R. G., et al. (1980). Establishing full-term human pregnancies using cleaving embryos grown in vitro. *British Journal of Obstetrics and Gynaecology*, 87, 737–756.
- Escudero, E., et al. (2004). Comparison of two different starting multiple dose gonadotropin-releasing hormone antagonist protocols in a selected group of in vitro fertilization-embryo transfer patients. *Fertility and Sterility*, 81, 562–566.
- Fleming, R., et al. (1982). A new systematic treatment for infertile women with abnormal hormone profiles. *British Journal of Obstetrics and Gynaecology*, 89, 80–83.
- Garcia, J. E., et al. (1983). Human menopausal gonadotropin/human chorionic gonadotropin follicular maturation for oocyte aspiration: Phase II, 1981. *Fertility and Sterility*, 39, 174–179.
- Griesinger, G., et al. (2007). Triggering of final oocyte maturation with gonadotropin-releasing hormone agonist or human chorionic gonadotropin. Live birth after frozen-thawed embryo replacement cycles. *Fertility and Sterility*, 88, 616–621.
- Hahn, D. W., et al. (1985). Reproductive/endocrine and anaphylactoid properties of an LHRH-antagonist, ORF 18260 [ac-DNAL1(2), 4FDPhe2,D-Trp3,D-Arg6]-GnRH. *Life Sciences*, 37, 505–514.
- Hughes, E. G., et al. (1992). The routine use of gonadotropin-releasing hormone agonists prior to in vitro fertilization and gamete intrafallopian transfer: A meta-analysis of randomized controlled trials. *Fertility and Sterility*, 58, 888–896.
- Janssens, R. M., et al. (2000). Dose-finding study of triptorelin acetate for prevention of a premature LH surge in IVF: A prospective, randomized, double-blind, placebo-controlled study. *Human Reproduction*, 15, 2333–2340.
- Kenigsberg, D., et al. (1984). Medical hypophysectomy: I. Dose-response using a gonadotropin-releasing hormone antagonist. *Fertility and Sterility*, 42, 112–115.
- Klingmuller, D., et al. (1993). Hormonal responses to the new potent GnRH antagonist Cetrorelix. *Acta Endocrinologica*, 128, 15–18.
- Kolibanakis, E. M., et al. (2006). Among patients treated for IVF with gonadotrophins and GnRH analogues, is the probability of live birth dependent on the type of analogue used? A systematic review and meta-analysis. *Human Reproduction Update*, 12, 651–671.
- Kolibanakis, E. M., et al. (2011). Fixed versus flexible gonadotropin-releasing hormone antagonist administration in in vitro fertilization: A randomized controlled trial. *Fertility and Sterility*, 95, 558–562.
- Kolibanakis, E. M., et al. (2012a). GnRH agonist for triggering final oocyte maturation: Time for a critical evaluation of data. *Human Reproduction Update*, 18, 228–229. author reply 229–230.
- Kolibanakis, E., et al. (2012b). GnRH antagonists in ovarian stimulation for IVF. In 5th edn, D. K. Gardner, A. Weissman, C. M. Howles, & Z. Shoham (Eds.), 2. *Textbook of Assisted Reproductive Techniques: Clinical Perspectives*. Boca Raton, FL: CRC Press.
- Ling, N., et al. (1973). Direct sequence determination of ovine luteinizing hormone releasing factor by mass spectrometry. *Biochemistry*, 12, 5305–5310.
- Loumaye, E. (1990). The control of endogenous secretion of LH by gonadotrophin-releasing hormone agonists during ovarian hyperstimulation for in-vitro fertilization and embryo transfer. *Human Reproduction*, 5, 357–376.
- Ludwig, M., et al. (2002). Tailoring the GnRH antagonist cetrorelix acetate to individual patients' needs in ovarian stimulation for IVF: Results of a prospective, randomized study. *Human Reproduction*, 17, 2842–2845.
- Mannaerts, B., & Gordon, K. (2000). Embryo implantation and GnRH antagonists. GnRH antagonists do not activate the GnRH receptor. *Hum Reprod*, 15, 1882–1883.
- Marcus, S. F., & Ledger, W. L. (2001). Efficacy and safety of long-acting GnRH agonists in in vitro fertilization and embryo transfer. *Human Fertility (Cambridge, England)*, 4, 85–93.
- Mochtar, M. H., & Dutch Ganirelix Study G. (2004). The effect of an individualized GnRH antagonist protocol on folliculogenesis in IVF/ICSI. *Human Reproduction*, 19, 1713–1718.
- Ng, C., & Trew, G. (2012). Endocrinological insights into different in vitro fertilization treatment aspects. *Expert Review of Endocrinology and Metabolism*, 7, 419–432.
- Rabinovici, J., et al. (1992). Endocrine effects and pharmacokinetic characteristics of a potent new gonadotropin-releasing hormone antagonist (Ganirelix) with minimal histamine-releasing properties: Studies in postmenopausal women. *The Journal of Clinical Endocrinology and Metabolism*, 75, 1220–1225.
- Reissmann, T., et al. (1995). Development and applications of luteinizing hormone-releasing hormone antagonists in the treatment of infertility: An overview. *Human Reproduction*, 10, 1974–1981.
- Schally, A. V., et al. (1971). Isolation and properties of the FSH and LH-releasing hormone. *Biochemical and Biophysical Research Communications*, 43, 393–399.
- Siristatidis, C. S., et al. (2015). Gonadotrophin-releasing hormone agonist protocols for pituitary suppression in assisted reproduction. *Cochrane Database of Systematic Reviews*, CD006919.
- Stephoe, P. C., & Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. *Lancet*, 2, 366.
- The Ganirelix dose-finding study group (Tgd-fs G). (1998). A double-blind, randomized, dose-finding study to assess the efficacy of the gonadotrophin-releasing hormone antagonist ganirelix (org 37462) to prevent premature luteinizing hormone surges in women undergoing ovarian stimulation with recombinant follicle stimulating hormone (Puregon). *Human Reproduction*, 13, 3023–3031.
- Toftager, M., et al. (2016). Risk of severe ovarian hyperstimulation syndrome in GnRH antagonist versus GnRH agonist protocol: RCT including 1050 first IVF/ICSI cycles. *Human Reproduction*, 31, 1253–1264.
- Trounson, A. O., et al. (1981). Pregnancies in humans by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. *Science*, 212, 681–682.

Monitoring COS and HRT Cycles

Basil C Tarlatzis, Aristotle University of Thessaloniki, Thessaloniki, Greece
Konstantinos Dafopoulos, University of Thessaly, Larissa, Greece

© 2018 Elsevier Inc. All rights reserved.

Monitoring COS

The basic principle in ovarian stimulation for IVF (usually termed as superovulation or controlled ovarian stimulation-COS) is the retrieval of an adequate number of oocytes, in order to produce enough embryos and select the best of them to transfer in the uterine cavity, thus maximizing the probability of pregnancy. The stimulation of the ovaries is carried out through the exogenous administration of gonadotropins subcutaneously, although endogenous production of gonadotropins by the per os administration of clomiphene citrate or aromatase inhibitors is also possible. In modern assisted reproduction, the exogenous gonadotropins are mainly used for COS and it is clear that their efficacy is independent of their origin, since the live birth rates are comparable between urinary and recombinant gonadotropins ([van Wely et al., 2011](#)).

During the course of COS, it is mandatory to block the endogenous LH surge and the subsequent luteinization of the developing follicles. This LH surge is premature, namely it occurs when the sizes of the leading follicles are small, and is induced by the increasing levels of estradiol that is secreted from the stimulated follicles. For the blockade of the premature LH surge, the GnRH analogues are administered (agonists or antagonists) according to the respective protocols. Although both protocols are similar in terms of efficacy, the GnRH antagonist is safer than the agonist protocol, since it is related to lower ovarian hyper stimulation syndrome (OHSS) rate ([Al-Inany et al., 2016](#)).

The duration of the COS in the IVF/ICSI cycle is about 2 weeks and the monitoring is of paramount importance to obtain an adequate number of oocytes, avoiding poor or hyper ovarian responses. The quantification of the adequate number of retrieved oocytes is based on data from retrospective analyses of high numbers of IVF/ICSI cycles showing that the live birth rate increases with increasing number of retrieved oocytes up to 15 ([Sunkara et al., 2011](#)), while today it is generally accepted that poor and hyper responses are the retrieval of less than 4 and more than 15 or 20 oocytes, respectively ([Ferraretti et al., 2011](#), [Broer et al., 2013](#)). Furthermore, monitoring the COS will define the optimal time to give human chorionic gonadotrophin (HCG) for final oocyte maturation and finally avoid cycle cancellation which imposes significant psychological and financial burdens on patients.

In the long GnRH agonist protocol, the GnRH agonist daily injections usually start on day 21 of the previous cycle for pituitary down regulation and continues until the day of HCG administration. Once the pituitary down regulation is confirmed by low serum estradiol, progesterone and LH levels, usually after 12–14 days of GnRH agonist administration, the stimulation with daily gonadotropin injections starts. In the GnRH antagonist protocol, daily gonadotropin stimulation starts on the second day of the cycle and the daily injections of GnRH antagonist are added from day 5 onwards, until the day of HCG administration. However, today there are several variations of these protocols used in COS practice ([Tarlatzis et al., 2006](#)).

The first and most important step to approach the goal of adequate number of retrieved oocytes is the determination of the starting dose of gonadotropins. This is based on the blood levels of AMH, measured irrespectively of the day of the menstrual cycle, or the estimated antral follicle count (AFC) of both ovaries by transvaginal ultrasound in the early follicular phase before the treatment cycle. The AMH and AFC are the best biomarkers of ovarian reserves and predict more accurately the ovarian response to COS ([La Marca and Sunkara, 2014](#)). This AMH- or AFC-tailored individualization of COS is generally accepted today and the concept is that the higher the AMH levels or the AFC are, the lower the starting dose of gonadotropins should be given. Although some multifactorial models, taking into account the AMH, FSH, AFC and female age, have been proposed to select the starting dose of gonadotropins, these have not been fully validated as yet and in general, a starting dose of 200–225 IU, 300 IU, or 150 IU are used for expected normo-, poor or hyper-responders, respectively. The cutoff levels of AMH vary between studies and are also dependent on the assay used for its measurement. For example, the AMH levels measured with the manual DSL assay have been suggested to be 1 or 2.2, 5 or 15.6, and 15 or 28.6 ng/mL for expected poor, normal and hyper-responders, respectively. With the estimation of AMH levels by the modern automated assays, starting doses of Follitropin delta have been suggested to be 12 mg/d for AMH < 15 pmol/L and 0.10–0.19 mg/kg/d for patients with AMH ≥ 15 pmol/L ([Nyboe Andersen et al., 2017](#)). A multifactorial model, considering AMH levels, day 3 FSH levels, and female age, to tailor the starting dose of follitropin alpha in the first IVF cycle, has been shown to increase the proportion of patients with an optimal response, defined as the retrieval of 8–14 oocytes ([Allegre et al., 2017](#)).

Usually after 4 days of ovarian stimulation, the estradiol levels and/or the number of selected follicles (> 10 mm) are measured to quantify the ovarian response. If the response is adequate, the same dose of gonadotropins is maintained, while in cases of low or high response the dose is increased or decreased, respectively. The range of adequate ovarian response is not well defined and the cutoff level of estradiol suggesting a hyper-response is about 600 pg/mL. However, the cutoff levels studied as yet, are of limited prognostic value for the OHSS. Even the strategy of coasting, which means the withdrawal of gonadotropins administration while the GnRH analogue is given for less than 4 days, is an ineffective way to prevent OHSS, while a significantly lower yield of oocytes is obtained. On the other hand, the increase of FSH dose above 300 IU daily does not increase the number of retrieved oocytes in poor responders.



Fig. 1 Ultrasonographic measurement of developing follicles in COS.

In order to ensure safety and schedule the timing of HCG administration, ultrasound monitoring of the number and size of the developing follicles continues usually on a basis of alternate days, although even daily monitoring may be necessary. The diameter of each follicle is measured in two perpendicular planes and the average diameter is then calculated (**Fig. 1**).

The follicles grow at a rate of 2–3 mm per day and the average number of ultrasound scan monitoring is about two to three until the criteria for HCG administration are accomplished. Monitoring blood levels of estradiol together with ultrasound scanning is a usual practice, although there is no evidence to support that it improves pregnancy rate and decreases the OHSS rate compared to ultrasound monitoring alone ([Kwan et al., 2014](#)). Studies have shown that in the GnRH antagonist protocol, the number of follicles on the day of HCG administration have a higher predictive value for OHSS than the estradiol levels. If more than 18–19 follicles > 11 mm are measured in both ovaries on the day of HCG, the sensitivity and specificity for severe OHSS prediction are about 80% ([Griesinger et al., 2016](#)).

Endometrial thickness assessment at the time of transvaginal ultrasound scan monitoring has become part of standard monitoring during COS. The endometrial thickness is determined as the maximum distance between the two outer edges of the endometrial image on a longitudinal section of the uterus. Many studies have shown that an endometrial thickness below 8 mm on the HCG day is associated with lower pregnancy rates compared with thicker endometrium. Besides thickness, the sonographic pattern may be of clinical significance. During the COS, the increase in thickness of a three-layer pattern (**Fig. 2**) is due to estradiol levels increase, reflecting normal follicular dynamics, and the presence of this pattern on the day of HCG seems to be related to higher pregnancy rates in various studies.

Serum progesterone levels measurement may provide important information during monitoring of COS cycles. The premature rise of progesterone during the late follicular phase of the COS cycle may affect the endometrium and the window of implantation, resulting in asynchrony between the endometrium and the developing embryo. It is generally accepted that a progesterone level above 1.5 ng/mL on the day of HCG administration for the final oocyte maturation has a negative impact on pregnancy rates. For this reason, in cases with progesterone elevation it is advisable to cryopreserve embryos and transfer them in a future frozen-thawed embryos cycle ([Venetis et al., 2013](#)).

Human chorionic gonadotropin is used to trigger final oocyte maturation in IVF/ICSI cycles. HCG occupies the LH receptors and, when given exogenously, it actually mimics the function of the endogenous LH surge. Today it is clear that the effectiveness and safety of the recombinant HCG is comparable to the urinary HCG. Usually, HCG is given when three or more follicles reach the size of 17 mm. Prolongation of the follicular phase, i.e., triggering 2 days later after this criterion is met, in the GnRH antagonist protocol seems to be associated with lower ongoing pregnancy rates. The dose of urinary HCG is usually 5000–10,000 IU and the dose of recombinant HCG is 250 µg. The pregnancy rate is similar whether 5000 or 10,000 IU are given, while in high risk patients for OHSS doses as low as 3000 IU have been used without, however, enhanced safety. Doses below 2000 IU are ineffective to trigger final oocyte maturation.

Women with the polycystic ovary syndrome (PCOS) is a special group of IVF/ICSI patients, since the OHSS rate is about five times higher in these cases compared to patients with normal ovaries. Regarding the prediction of OHSS, such as in normal women, high AMH levels are related to increased risk for OHSS. PCOS women are hyper responders during COS and the monitoring



Fig. 2 Ultrasonographic appearance of the three-layer endometrium.

implemented is similar to that used in all women undergoing IVF/ICSI. Live-birth rates after COS for IVF/ICSI in women with PCOS seem to be comparable with those of women with other diagnoses, such as endometriosis, unexplained infertility or male factor infertility.

Many PCOS women have oligomenorrhea or amenorrhea and, therefore, the scheduling of COS with an induced menstruation is needed. This may be performed by the oral administration of combined contraceptives or progestins. However, the pregnancy rate after cycle scheduling with combined contraceptives may be lower compared to progestin induced scheduling. Obesity, depending on the population, may be prevalent in 40%–80% of PCOS patients, deteriorating the pregnancy rate and increasing the miscarriage rate. The GnRH antagonist is the protocol of choice, due to about 50% decrease in the risk of OHSS compared to the GnRH agonist protocol and comparable pregnancy rates. Moreover, it allows the induction of final oocytes maturation with GnRH agonist instead of HCG, thus eliminating practically the occurrence of OHSS (Tarlitzis et al., 2017).

Monitoring HRT Cycles

Frozen-thawed embryo transfer (FET) is an important part of IVF/ICSI treatment and involves the freezing of surplus embryos originating from a fresh cycle that may be transferred in the properly prepared endometrium of subsequent cycles, thus increasing the cumulative pregnancy rate after an oocyte retrieval. The modern practice of elective single embryo transfer with high availability of frozen embryos for subsequent FET cycles, results in low multiple birth rate and reduced overall costs of the IVF/ICSI treatment. With the advent of vitrification, the survival of frozen embryos and the pregnancy rates have been improved compared to the earlier slow freezing technique. This resulted in higher implementation of this technique for segmentation of IVF or “freeze-all” protocols that are used to prevent the occurrence of late onset OHSS due to pregnancy. Furthermore, the vitrification of embryos may be of paramount importance in cases of serum progesterone rise on the day of HCG administration that deteriorates the pregnancy rate of the fresh cycle.

The aim in FET cycles is the synchronization between the developmental stage of the embryo and the prepared endometrium, in order to maximize pregnancy rates. This may be performed with monitoring the natural cycle or with hormone replacement therapy (HRT) in an artificial cycle.

The natural cycle FET is feasible only in women with regular ovulatory menstrual cycles. In this case, the endometrium is prepared by the secretion of endogenous estradiol from the leading follicle in the follicular phase and the detection of the spontaneous LH surge with blood or urine measurements, which signifies the onset of the luteal phase. Specifically, when the mean diameter of the leading follicle is at least 14 mm, morning urinary LH is monitored, a method convenient but not accurate enough to determine the exact timing of ovulation. Daily blood measurements of LH provide more accurate information about the increase of serum LH levels and the detection of the onset of the endogenous LH surge. Usually, FET is scheduled 5 or 7 days after detecting the LH surge for Day-3 or Day-5 embryos, respectively, given a minimal endometrial thickness of about 7 mm. The advantage of natural cycle FET is that no medications are used but there are also significant disadvantages, such as accurate determination of serum LH levels needs the daily or even twice a day measurements, while the commercial kits for urine LH detection seem to have a significant risk of false-negative results.

A modification of the natural cycle is triggering with 5000 IU of HCG, instead of the endogenous LH surge, usually when the mean diameter of the leading follicle is ≥ 17 mm and minimal endometrial thickness of 7 mm, while FET is performed similarly

to the natural cycle, i.e., the day of HCG injection corresponds to the day of the LH surge onset (increase in LH levels). In natural cycle FET, with spontaneous LH surge, the luteal phase may or may not be supported. Luteal phase support may be beneficial in terms of pregnancy rates, and usually starts 3 or 5 days before embryo transfer by administering progesterone vaginally or intramuscularly (IM) until the 5th week of pregnancy. In natural cycle FET with HCG induction, luteal phase support with progesterone does not seem to be beneficial probably due to the luteotropic effect and the long half life of HCG.

The HRT or artificial cycle protocols include the administration of exogenous estrogen and progesterone, with or without co-treatment with GnRH agonists. It is obvious that the HRT cycle may be applied in ovulatory and anovulatory women and also in women with ovarian quiescence, such as donor oocyte recipients with premature ovarian failure. The preparation of the endometrium for FET may be identical whether the aim is a cycle with the patient's vitrified oocytes or a cycle with fresh or vitrified donor oocytes or embryo donation.

A common practice for endometrial preparation is the oral administration of estradiol since it is simple and well-tolerated. On cycle day 2 or 3, after a transvaginal ultrasound scan ensures that there are no ovarian cysts, oral estradiol valerate administration starts. The dose of estradiol pills increases gradually, 2 mg/day on days 1–4, 4 mg/day on days 5–8, and 6 mg/day from day 9 onward, although various dosing schemes may be applicable. After 10–12 days of estrogen administration, the endometrial thickness is determined with transvaginal ultrasound. Estradiol is given at the same dose until the endometrial thickness on ultrasound is about 8 mm, which is usually accomplished after 10–14 days of treatment, but if the endometrium fails to respond, the duration of treatment may be extended. It should be noted that after oral administration, estradiol is metabolized by the intestinal mucosa and liver (first-pass effect), while with the transdermal route more stable steady-state levels of estradiol are produced. In cases, when with oral administration of estradiol an appropriate endometrial thickness is not attained, then it may be beneficial to shift to the transdermal route in a next cycle. It is rare that both oral and transdermal routes fail to achieve adequate endometrial thickness, but in these cases the vaginal route of estradiol administration may be helpful (Paulson, 2011).

Once the target of an endometrial thickness of about 8 mm is met, progesterone is added, while estradiol is continued, for the number of days proportional to the stage of development of the embryo that will be transferred. The day that the ultrasound scan detects the appropriate endometrium, it is also important to check that no dominant follicle nor signs of ovulation exist, since in such cases the endogenous progesterone may impact on the endometrium inducing secretory changes and distorting its synchronization with the developmental stage of the transferred embryo. Regarding the optimal synchronization for maximizing embryo implantation, it seems to be day 3 or 4 of progesterone administration for day-3 embryos, and day 5 or 6 of progesterone treatment for day-5 embryos. Progesterone is administered either vaginally, subcutaneously or intramuscularly. With the IM route the highest serum levels are achieved, while with vaginal administration the endometrial tissue levels are highest. However, both routes are similarly effective in terms of success rate in FET cycles. Usually, natural micronized progesterone is given vaginally at a dose of 400 mg every 12 h (suppositories) or 90 mg daily (gel) and is continued until either a serum pregnancy test result is negative, or in case of success, until 12 weeks of pregnancy. Alternatively, natural progesterone-in oil may be given IM at the dose of 50 mg daily or natural progesterone in aqueous solution SC at the dose of 25 mg/day.

Gonadotropin suppression with GnRH agonists has also been used in FET cycles on the basis that, although estradiol treatment in the early follicular phase inhibits ovulation, the complete pituitary suppression may not be achieved and some follicular activity can still occur, with possible adverse effects on implantation. Usually, pituitary down-regulation starts with daily administration of a GnRH-agonist from the mid-luteal phase (day 21) of the menstrual cycle. On the first day of subsequent menstruation, the endometrial preparation with administration of estradiol is initiated as described above. However, it seems that pregnancy rates are similar whether the FET cycles are supplemented or not with GnRH agonists.

Many studies as yet have investigated which is the best method of endometrium preparation in FET and it seems that all appear to be equally effective regarding pregnancy rates (Groenewoud et al., 2013). Therefore, patients' preferences play an important role in the selection of the most appropriate strategy.

References

- Al-Inany, H. G., Youssef, M. A., Ayeleke, R. O., et al. (2016). Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database of Systematic Reviews*, 4, CD001750.
- Allegra, A., Marino, A., Volpes, A., et al. (2017). A randomized controlled trial investigating the use of a predictive nomogram for the selection of the FSH starting dose in IVF/ICSI cycles. *Reproductive Biomedicine Online*, 34, 429–438.
- Broer, S. L., Dölleman, M., van Disseldorp, J., et al. (2013). Prediction of an excessive response in vitro fertilization from patient characteristics and ovarian reserve tests and comparison in subgroups: An individual patient data meta-analysis. *Fertility and Sterility*, 100, 420–429.
- Ferraretti, A. P., La Marca, A., Fauser, B. C., et al. (2011). ESHRE consensus on the definition of 'poor response' to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Human Reproduction*, 26, 1616–1624.
- Griesinger, G., Verweij, P. J., Gates, D., et al. (2016). Prediction of ovarian hyperstimulation syndrome in patients treated with corifollitropin alfa or rFSH in a GnRH antagonist protocol. *PLoS One*, 11, e0149615.
- Groenewoud, E. R., Cantineau, A. E., Kollen, B. J., Macklon, N. S., & Cohlen, B. J. (2013). What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis. *Human Reproduction Update*, 19, 458–470.
- Kwan, I., Bhattacharya, S., Kang, A., & Woolner, A. (2014). Monitoring of stimulated cycles in assisted reproduction (IVF and ICSI). *Cochrane Database of Systematic Reviews*, 8, CD005289.
- La Marca, A., & Sunkara, S. K. (2014). Individualization of controlled ovarian stimulation in IVF using ovarian reserve markers: From theory to practice. *Human Reproduction Update*, 20, 124–140.

- Nyboe Andersen, A., Nelson, S. M., Fauser, B. C., et al. (2017). Individualized versus conventional ovarian stimulation for in vitro fertilization: A multicenter, randomized, controlled, assessor-blinded, phase 3 non inferiority trial. *Fertility and Sterility*, 107, 387–396.
- Paulson, R. J. (2011). Hormonal induction of endometrial receptivity. *Fertility and Sterility*, 96, 530–535.
- Sunkara, S. K., Rittenberg, V., Raine-Fenning, N., et al. (2011). Association between the number of eggs and live birth in IVF treatment: An analysis of 400135 treatment cycles. *Human Reproduction*, 26, 1768–1774.
- van Wely, M., Kwan, I., Burt, A. L., et al. (2011). Recombinant versus urinary gonadotrophin for ovarian stimulation in assisted reproductive technology cycles. *Cochrane Database of Systematic Reviews*, 2, CD005354.
- Tarlatzis, B. C., Fauser, B. C., Kolibianakis, E. M., Diedrich, K., Rombauts, L., & Devroey, P. (2006). GnRH antagonists in ovarian stimulation for IVF. *Human Reproduction Update*, 12, 333–340.
- Tarlatzis, B. C., Bosdou, J., & Kolibianakis, E. M. (2017). Elimination of OHSS by GnRH agonist and freezing embryos. In B. Rizk, & J. Gerris (Eds.), *Complications and outcomes of assisted reproduction* (1st edn., pp. 149–163). Cambridge: Cambridge University Press.
- Venetis, C. A., Kolibianakis, E. M., Bosdou, J. K., & Tarlatzis, B. C. (2013). Progesterone elevation and probability of pregnancy after IVF: A systematic review and meta-analysis of over 60 000 cycles. *Human Reproduction Update*, 19, 433–457.

Oocyte Retrieval

Michael M Alper, Boston IVF, Waltham, MA, United States

© 2018 Elsevier Inc. All rights reserved.

History of Oocyte Retrieval

In the early research years of IVF, there were no techniques available to easily extract eggs from the ovaries. With the advent of laparoscopy in the 1970s, the ovaries became more easily accessible and, in fact, Steptoe and Edwards used laparoscopy to obtain eggs for the first successful IVF case in 1978. Laparoscopy involves the placement of a pencil-size instrument just below the umbilicus to visualize the pelvic organs. Despite the breakthrough with laparoscopy, the technique required intubated anesthesia and also carried significant risks such as bladder and bowel injury, amongst other complications. Laparoscopy essentially limited IVF to the hospital setting, which resulted in the process being somewhat complex.

The advent of diagnostic ultrasound in the mid-1980s led to major advances in oocyte retrieval. Initially, only abdominal ultrasound probes were available and the only way to reach the ovaries was to place a needle (protected by a bladder catheter) into the bladder via the urethra and then piercing the bladder to reach the follicles. Trans-vesicle follicular puncture under abdominal ultrasound-guidance was less involved than laparoscopy but carried risks of hematuria and urinary tract infection. The development of the vaginal ultrasound probe led to the modern techniques used today.

The Modern Technique of Oocyte Aspiration

The natural cycle typically produces a single follicle and the efficiency of IVF is limited with only one egg/follicle. Most IVF programs use daily gonadotropin (FSH) injections to induce multiple ovarian follicles to develop. After 10 or so days of stimulation, the ovaries have multiple follicles typically of various sizes. Most programs target at least 10–15 follicles to form. Also, most follicles that measure 16–18 mm on ultrasound examination have mature eggs within them. When an adequate number of such follicles are present, the hormone hCG (human chorionic gonadotropin) is typically administered to induce final egg maturation and the oocyte retrieval is scheduled 35 h later. The hCG injection is often referred to as the “trigger shot.” (Note that hCG is biologically similar to luteinizing hormone, the hormone that induces egg maturation and ultimately ovulation in a natural cycle).

Before vaginal surgery it is important to cleanse the vagina to reduce the likelihood of introducing bacteria into the pelvic cavity. Although bacteriostatic solutions are used to prepare the vagina in most gynaecologic surgeries, solutions that are potentially toxic to oocytes must be avoided in IVF surgeries. For this reason, most IVF practices simply use saline to irrigate the vagina before egg retrieval in an attempt to remove as much debris and bacteria as possible. Studies have demonstrated that even though this approach does not use an antiseptic *per se*, the technique is effective.

The vaginal ultrasound probe is draped in a sterile cover and introduced into the vagina. Since the ovaries are positioned just behind the vagina, they are typically easily seen with the vaginal ultrasound transducer. A needle guide on the vaginal probe is used to position and guide the needle to traverse the vaginal wall (see Fig. 1).

Manufacturers produce oocyte aspiration needles of various sizes; most use 16–17 gauge needles. The aspiration needle itself is attached to fine tubing that ends in the cap of a test tube. The test tube cap has another tube that is also connected to suction so that the fluid in the needles is suctioned and collected in the test-tube. Most often, the surgeon controls a foot peddle that turns the suction on and off as the follicles are entered and the needle removed.



Fig. 1 Egg retrieval procedure demonstrating vaginal ultrasound probe with needle guide and egg retrieval needle with tubing (leading to test tube for egg collection).

Upon reaching the ovary, each follicle is aspirated in sequence. The needle tip is aimed at the center part of the follicle and the follicle can be seen to collapse around the needle tip as suction is applied. The typical straw-color fluid from each follicle is collected in the test-tube to be analyzed by the laboratory technician who searches through the fluid to find the microscopic egg. The technician looks through all the fluid collected and provides a running count of the number of oocytes retrieved.

Special Considerations

There are several issues that should be considered to optimize egg collection. The suction pressure must be correct. If the suction pressure is too low, then the flow of fluid will be reduced and leakage around the needle may occur and the follicle potentially lost. If the pressure is too high, then the rapid flow of fluid could damage the oocyte complex. It appears that a pressure of 140 mm seems best. Also, the eggs must be kept at a constant temperature as they wait to be discovered in the follicular fluid; the test tubes should sit in a warmer while waiting to be analyzed.

Also, it is important to minimize the number of times that the vaginal wall is pierced (to reduce infection and bleeding) so re-positioning the needle once past the vaginal wall is often used. Often just one puncture of the vaginal mucosa can result in aspiration of many follicles by simply angling the needle beyond the vaginal puncture site.

Some clinicians prefer to flush the follicles with media after initial aspiration in an attempt to improve the chances of retrieving the egg. Some needles are designed with an extra channel to allow for flushing. Studies have not demonstrated the value of flushing the follicles. Even patients with few follicles do not seem to have more eggs when the follicles are flushed (Mok-lin et al.). The disadvantage of flushing is that it adds considerable time to the procedure.

Various methods have been employed to make the egg retrieval comfortable for patients. Most programs use either conscious sedation or general anesthesia. Others have used local para-cervical blocks, acupuncture, and vaginal lidocaine. Centers using general anesthesia typically use propofol due to its short-acting characteristics and excellent amnesia. A Cochrane review evaluated 21 trials of pain control in women undergoing oocyte retrieval. The studies compared conscious sedation, general anesthesia, acupuncture, paracervical block, and patient controlled anesthesia. The conclusion was that all the various approaches worked to some extent. The use of more than one technique was associated with better pain relief. Of note is that the studies were not amenable to a meta-analysis due to design.

Risks and Complications

The risks of ultrasound-guided egg retrieval are finite and fortunately not that common. Bleeding is one of the most common complications of oocyte retrieval. The reason is that smaller blood vessels do not appear on ultrasound so it is not uncommon for a blood vessel between the vaginal wall and the ovary to be punctured inadvertently. Most of the time, if blood is seen emanating from the vagina it can be stopped with pressure. However, blood accumulating in the pelvic cavity is not typically noticed.

A study by Bodri and colleagues of 4052 retrievals revealed complications related to oocyte retrieval occurred in 17 patients (0.42%). The complications included intraabdominal bleeding ($n = 14$), severe pain ($n = 2$), and ovarian torsion ($n = 1$). Fourteen of the patients were hospitalized (0.35%) and six donors (0.15%) required surgical intervention. Pelvic infections, and injury to pelvic structures, nor anesthesia complications were not observed in this series.

A study by Ludwig and co-workers reviewed complication of 1058 egg retrievals. There were no complications from sedation or general anesthesia. Vaginal bleeding was observed in 2.8% of procedures, without any cases of intraabdominal bleeding. An injury of pelvic structures (a ureteral lesion) occurred in one case. No cases of pelvic infection occurred, but there was one case of unexplained fever was observed. Regarding pain, 3% of patients experienced severe to very severe pain after the egg retrieval and 2% of patients were still suffering from severe pain 2 days after the procedure. The pain level increased with the number of oocytes retrieved. About 0.7% of patients required hospitalization related to the egg retrieval.

Pelvic infection is another possible complication of egg retrieval. Despite a paucity of studies to demonstrate the value of prophylactic antibiotics, most IVF programs appear to use them in many or all cases. A study by Egbase and colleagues showed that prophylactic antibiotics administered routinely to women at the time of oocyte retrieval were associated with a reduction in positive microbiology cultures of embryo catheter tips 48 h later in 78% of patients. The implantation and clinical pregnancy rates were significantly lower in the women with positive microbial catheter-tip cultures. In a study of oocyte donors, 526 oocyte donors who received prophylactic antibiotics for oocyte retrieval were compared with a group of 625 patient controls that did not. Infection after retrieval was 0.4% in patients who did not receive prophylaxis compared to 0% in the group who received antibiotics.

Patients with endometriomas (especially if aspirated at the time of egg retrieval) may be at particularly high risk for infection. The reason is that the endometriotic fluid may act as a "culture" media for bacteria should they be introduced by the needle at the time of aspiration.

Other rare complications may occur. Urologic injury involving the ureter can be difficult to diagnose especially when masked by ovarian discomfort from stimulation. Fortunately, ureter and bladder injuries remain uncommon.

Pain management postoperatively is typically taken care of with acetaminophen or similar products. Patients having with difficulty increasing pain should be carefully evaluated for bleeding or ovarian accidents such as ovarian torsion. Patients with low pain tolerance or those with chronic pelvic pain should be evaluated carefully before surgery.

Summary

Vaginal oocyte retrieval is an important part of IVF. Current techniques use vaginal ultrasound guidance to pass a needle into the follicle. The needle is connected to a test-tube, which collects the eggs. Vaginal oocyte retrieval requires anesthesia and several methods have been promoted including general anesthesia and conscious sedation. The main complication from the procedure is bleeding. Prophylactic antibiotics may reduce the occurrence of infection.

Further Reading

- Bodri, D., Guillén, J. J., Polo, A., Trullenque, M., Esteve, C., Coll, O., et al. (2008). Complications related to ovarian stimulation and oocyte retrieval in 4052 oocyte donor cycles. *Reproductive Biomedicine Online*, 17(2), 237–243.
- Egbase, P. E., Udo, E. E., & Grudzinskas, J. G. (1999). Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet*, 354(9179), 651–652.
- Kwan, I., Bhattacharya, S., Knox, F., & Mcneil, A. (2005). Pain relief for women undergoing oocyte retrieval for assisted reproduction. *Cochrane Database of Systematic Reviews*, 3.
- Ludwig, A. K., Glawatz, M., Griesinger, G., Diedrich, K., & Ludwig, M. (2006). Perioperative and post-operative complications of transvaginal ultrasound-guided oocyte retrieval: Prospective study of >1000 oocyte retrievals. *Human Reproduction*, 21(12), 3235–3240.
- Mok-lin, E., Brauer, A. A., Schattman, G., Zaninovic, N., Rosenwaks, Z., & Spandorfer, S. (2013). Follicular flushing and in vitro fertilization outcomes in the poorest responders: A randomized controlled trial. *Human Reproduction*, 28(11), 2990–2995.
- Van Os, H. C., & Roozenburg, B. J. (1992). Vaginal disinfection with povidon iodine and the outcome of in-vitro fertilization. *Human Reproduction*, 7(3), 349–350.

Luteal Phase Support in IVF/ICSI- and Frozen Embryo Transfer-Cycles

Barbara Lawrenz and Human M Fatemi, IVI Middle East, Abu Dhabi, UAE

© 2018 Elsevier Inc. All rights reserved.

Introduction

The time between ovulation and onset of menses or, in case of conception, the establishment of a pregnancy is called “luteal phase”. After ovulation, the formation of the corpus luteum will occur under the influence of LH (luteinising hormone). The corpus luteum can be characterized as a timely limited endocrine organ, producing estradiol and up to 40 mg progesterone/day. Under the influence of progesterone the endometrium undergoes secretory transformation and also local vasodilatation and relaxation of the uterine muscle is promoted.

After conception the developing embryo will secrete human chorionic gonadotrophin (hCG). hCG produced by the embryo will maintain the secretory activity of the corpus luteum due to the structural similarity between hCG and LH and therefore by activating the same receptor. The corpus luteum will continue to produce progesterone until the shift of the progesterone production to the placenta has taken place at around 9 weeks of pregnancy.

Preconditions for a pregnancy are a functional embryo at blastocyst developmental stage, a receptive endometrium and synchrony between the embryo and the endometrium. Failure to achieve receptivity and synchrony results in infertility and this might be a limiting factor for success in IVF (In vitro-fertilization) – treatment.

Endometrial receptivity depends from the time of progesterone exposure after sufficient exposure to estrogen. The time frame in which the endometrium is receptive and able to support trophoblast–endometrial interactions is called “window of implantation” (WOI). The WOI is very limited in time and in a natural and idealized 28-day-cycle, it is thought to occur during a time around day 22–day 24. Previously it was assumed, that the WOI is more or less constant in time in all women. However lately it was shown, that the WOI can differ and displacement of the WOI might cause infertility, especially in the patients with repeated implantation failure.

Steroid Production of the Ovary in Natural and Stimulated Cycles

Estradiol and progesterone are essential for human reproduction in terms of providing the adequate endometrium for implantation. By administering estradiol and progesterone for endometrial preparation, pregnancies can be achieved with oocyte donation even in women without ovaries.

In a natural cycle, estradiol synthesis increases progressively from the dominant follicle and initiates LH (luteinising hormone) surge. Even before the LH-surge, a small increase in progesterone levels is seen, which reflects the increasing LH pulse amplitude and frequency leading up to the surge. A LH surge of 24–36 h is sufficient to initiate the resumption of oocyte meiosis, luteinization of granulosa cells, ovulation, and the initial phase of CL development. Progesterone and 17 α -hydroxyprogesterone (17 α -OHP) plasma concentrations increase rapidly after the LH surge or administration of human chorionic gonadotropin (hCG), indicating the beginning of granulosa and theca cell luteinization. The corpus luteum is producing up to 40 mg of progesterone per day and additional a significant amount of androgens and estradiol. This is unique to the corpus luteum of many primates, including humans (Devoto et al., 2009).

Progesterone biosynthesis occurs in two enzymatic steps: the conversion of cholesterol to pregnenolone (P5), a process which is catalyzed by the enzyme cytochrome P450_{scc} and further on its subsequent conversion to progesterone, through catalyzation by 3 β -hydroxy-steroid-dehydrogenase (3 β HSD). Under the influence of CYP 17, progesterone is further metabolized to androgens in the thecal cells. During early follicular phase, the enzymatic activity necessary to convert 17-OH-progesterone to androstenedione is absent or very low therefore concentrations of progesterone and estradiol rise with the increasing follicular diameter (Yding Andersen et al., 2011).

In a natural cycle with the development of a single dominant follicle, FSH-levels decline towards ovulation (Fleming and Jenkins, 2010). In IVF, multifollicular development is achieved by administration of daily high gonadotropin concentrations and in a normal/high responder, ovarian stimulation will result in a large number of growing follicles. Each follicle will contribute to the progesterone in the systemic circulation, therefore patients with more oocytes have high estradiol concentrations and significantly higher progesterone.

Without conception luteolysis will occur due to a lack of hCG support in the natural cycle and the corpus luteum will undergo a process of regression which leads to a decrease in progesterone production. In case of conception, the trophoblast will produce hCG, preventing the corpus luteum from regression and maintaining the progesterone production until placental progesterone production is adequate.

Influence of Progesterone on the Endometrium in Natural and Stimulated Cycles

Noyes et al. studied already in 1950 the different histological appearances of the endometrium, depending on the influence of estrogen or progesterone. Under the influence of progesterone, the epithelial glands and vasculature continue to grow and become

spiral. As the endometrial thickness remains relatively unchanged, this process results in a denser endometrium. By describing the histology for each specific day after ovulation, Noyes established in 1975 the classic endometrial dating paradigm that still serves as the gold standard for clinical evaluation of luteal function. An endometrial biopsy is considered to be “out of phase” in case it shows a difference of > 2 days between the histologic dating and the actual day after ovulation.

Comparison of endometrial steroid receptors and proliferation index between natural cycles and GnRH-agonist/hMG-stimulated cycles for IVF have revealed distinct alterations in endometrial maturation. On the day of oocyte pick-up procedure (OPU) in stimulated cycles, a more advanced secretory endometrial maturation combined with reduced estrogen receptors (ER) and PR and a low proliferation index in glands and stroma has been found (Bourgain et al., 2002).

It is assumed that the supraphysiological levels of estradiol and progesterone during stimulation lead to a reduced number of estradiol receptors (ER) and progesterone receptors (PR) and a low proliferation index in glands and stroma. Those endometrial alterations might affect the implantation. Patients with a progesterone level above 1.5 ng/mL on the day of hCG-administration have a different endometrial gene expression profile when compared to the gene expression pattern of patients below this threshold. These changes might explain the impairment of endometrial receptivity in the presence of elevated progesterone, reflected in the lower pregnancy rates reported in the literature (Bosch et al., 2003).

Endocrine Profile in IVF-Cycles After Oocyte Retrieval, Depending on the Type of Final Oocyte Maturation

Retrieval of mature oocytes after ovarian stimulation requires ovulation induction to mimic the LH-surge. Whereas in GnRH (gonadotropin-releasing-hormone)-agonist protocols final oocyte maturation has to be performed by the administration of hCG, in GnRH-antagonist-protocols the use of GnRH-agonist is also possible. Due to the specific way of action of hCG and GnRH-agonist, the endocrine profiles after OPU are different. In patients, triggered with either hCG alone or GnRH-agonist plus 1500 IU hCG, progesterone-levels on day 5 after oocyte retrieval procedure were highest and in patients after GnRH-agonist only and without any luteal phase support, were lowest. This difference is caused by the fact, that after hCG-application progesterone production of the theca cells is sustained to approximately at least 5 days due to the LH-activity and the half-life-time of hCG of > 24 h. Low levels of endogenous LH after GnRH-agonist trigger lead to low progesterone levels and can therefore cause severe luteal phase insufficiency (Fatemi et al., 2013).

Luteal Phase Support in Assisted Reproductive Techniques (ART)

A defective luteal phase occurs in almost all patients who undergo ovarian stimulation for ART and different theories have been discussed. Meanwhile the reason for the luteal phase insufficiency after ovarian stimulation is thought to be the multifollicular follicle growth during the follicular phase. The resulting supraphysiological luteal levels of progesterone and estradiol inhibit the LH secretion by the pituitary via negative feedback action (Fauser and Devroey., 2003).

To counterbalance luteal phase insufficiency after ovarian stimulation, luteal phase support (LPS) has to be applied. The possibilities are either direct substitution of progesterone via different routes of administration or stimulation of the remaining corpora lutea to sustain progesterone production.

Ways of Progesterone Administration

Oral Progesterone

Natural progesterone has been shown to be ineffective in inducing a sufficient secretory transformation after oral intake as it is rapidly metabolized. Synthetic progesterone derivatives are associated with side-effects, especially regarding the impact on the lipids and on the psyche, which will limit their use. To improve absorption and bioavailability progesterone can be micronized. However, even higher doses of micronized progesterone have failed to induce sufficient secretory transformation.

Progesterone i.m

Progesterone is rapidly absorbed after i.m. injection, therefore high progesterone plasma concentrations are reached after approximately 2 h and peak concentrations will be achieved after around 8 h. It seems that the i.m. site of the injection serves as a depot with progesterone accumulation in the fat tissue, resulting in sustained serum concentrations of progesterone (Nillius and Johansson, 1971). However the disadvantage of i.m. application of progesterone is the painful injection and the fact that daily injections are required to sustain sufficient progesterone concentrations. Additional swelling, redness and even sterile abscess formation are not uncommon side-effects and therefore lead to an increase of the physical treatment-burdens.

Vaginal Progesterone

After vaginal application, progesterone plasma concentrations reach maximal levels after approximately 3–8 h and then fall continuously over the next 8 h. Vaginal progesterone is cleared out of the circulation faster compared to i.m. progesterone, therefore higher

dosages of vaginal progesterone are needed to achieve luteal phase plasma levels. In most cases 300–600 mg of progesterone is administered daily, divided into two or three dosages, depending on the formula, like tablets, creams or suppositories. Vaginal absorption is influenced by the kind of formula preparation and is enhanced after previous oestrogenisation.

The serum levels measured after vaginal application are lower compared to i.m. application and sometimes even lower than measured in a natural cycle. Independently from the measured progesterone level, adequate secretory endometrial transformation is achieved after vaginal progesterone use.

Obviously, vaginally administered progesterone exerts a direct local effect on the endometrium before entering the systemic circulation. This is called “first uterine pass”-effect. Until now, the mechanism of the “first uterine pass”-effect is not fully understood and different ways of action are discussed: absorption of progesterone into the rich venous or lymphatic vaginal system and/or possibly countercurrent transfer between utero-vaginal lymph vessels or veins and arteries and direct drug diffusion through the tissues or even due to intraluminal transfer from the uterus to the vagina, similar to sperm transport. As the vaginal route is effective in providing sufficient luteal phase support and additional almost without any side-effects, it is nowadays the preferred route of progesterone administration. In case the increased discharge is not tolerated by the patient, rectal administration is also effective. Side-effects of rectal administration could be constipation and flatulence.

Human Chorionic Gondotropin for Luteal Phase Support

Due to the structural similarities of hCG and LH, hCG is able to activate the same receptor like LH. After hCG-application, no luteolysis will occur as the corpora lutea will be stimulated and progesterone production will be maintained. For a long time, the hCG-administration was the standard luteal phase support. The disadvantage is the possible development of ovarian hyperstimulation syndrome, especially in good responder patients (Ludwig and Diedrich, 2001).

Timing of Luteal Phase Support With Progesterone

Supraphysiological hormonal levels will suppress through the negative feedback of the hypothalamic-pituitary axis endogenous LH, which will lead finally to declining and low progesterone levels after OPU. To avoid a progesterone deficiency and therefore a negative impact on the endometrium, LPS has to be initiated before the endogenous progesterone levels are decreasing or low. On the other hand, preovulatory exposure of the endometrium to progesterone may have a negative impact on the endometrial receptivity.

Start of LPS before oocyte retrieval lead to a decreased likelihood of pregnancy, when compared to start on the day of oocyte retrieval. Initiation of progesterone on the evening of oocyte retrieval versus start on days 1–3 after oocyte retrieval did not lead to difference in clinical pregnancy rate. However, starting progesterone administration on day 6 will decrease the likelihood of pregnancy. Therefore, initiation of LPS between the evening of OPU and day 3 after OPU seems to be ideal (Connell et al., 2015).

Duration of Luteal Phase Support

In early pregnancy, lack of endogenous LH will be balanced by rapidly increasing amounts of hCG from the developing embryo. Progesterone production will shift from the corpora lutea towards the placenta between 7 and 9 weeks. Studies, which evaluated the duration of progesterone administration after a positive pregnancy test did not find any influence on the miscarriage rate and the delivery rate when progesterone application was continued or discontinued for 3 weeks after positive pregnancy test (Nyboe et al., 2002).

Luteal Support After GnRH-Administration for Final Oocyte Maturation

The use of GnRH-agonist for final oocyte maturation is meanwhile common in high responder patients as OHSS can almost completely be avoided. However, the first large randomized controlled trials after the introduction of GnRH-agonist for final oocyte maturation in GnRH-antagonist protocols reported a very poor reproductive outcome. It was assumed, that induced severe luteolysis, which develops due after the application of GnRH-agonist, cannot be counterbalanced using standard luteal phase support with “only” progesterone administration (Humaidan et al., 2005). To prevent luteolysis, administration of hCG or high doses of steroids is considered to be essential.

Luteolysis will occur when LH support is withdrawn for ≥ 3 days from the primate corpus luteum, hence it can be rescued if LH activity is reinitiated within 3 days corpus luteum function, so corpus luteum viability can be preserved without LH support for at least 72 h (Hutchison and Zeleznik, 1985).

Different treatment options have been described to maintain progesterone production from the corpora lutea and rescue the luteal phase. As GnRHa-induced luteolysis can be reverted by administration of hCG, one treatment concept is the application of low-doses of hCG during luteal phase. The rescue of the corpora lutea with hCG is dose dependent, and some studies suggested a hCG dose of ≥ 1500 IU, however also the application of low-dose HCG is effective in normalizing the reproductive outcome. Despite low dosages of hCG, OHSS can still occur in some patients.

OHSS can be almost completely avoided by the use of GnRH-agonist (200 mg of GnRH-agonist nasal spray twice daily; a total of 400 mg/d) for LPS after GnRH-agonist trigger without exogenous progesterone and midluteal progesterone levels of around 190 nmol/L (approximately 59.7 ng/mL) can be achieved (Bar-Hava et al., 2016).

Concept of Individualized Luteal Phase Support After Final Oocyte Maturation With GnRH-Agonist Trigger

Over the last years, ovarian stimulation for IVF-treatment is progressively individualized towards the patients' characteristics by the use of the ovarian reserve parameters anti-Muellerian-hormone (AMH) and ultrasound-based antral follicle count (AFC). However, until now LPS is performed uniformly in almost all patients.

Previously it was demonstrated, that GnRH-agonist trigger for final oocyte maturation would lead to severe luteolysis (Fatemi et al., 2013). However, luteolysis is not following the same pattern in all patients and some patients don't develop luteolysis at all. To avoid unnecessary administration of hCG with the consecutive risk of OHSS-development, a new concept of luteal coasting needs to be developed. Therefore, knowledge on predictive factors regarding the extend of the anticipated luteolysis with a possible subsequent development of luteal phase insufficiency without hCG-administration is needed. Additional randomized prospective trials have to evaluate different approaches of luteal phase support using low-dosage hCG or hCG-free approaches.

Luteal Phase Support in Frozen Embryo Transfer Cycles

With the improvement of the cryoconservation-techniques in the IVF-laboratory worldwide more and more frozen embryo-transfer (FET) or warmth oocyte embryo transfer-cycles are performed. This techniques could lower the treatment burden of the patient as in the event of surplus oocytes or embryos, this technique can improve the chance of a pregnancy without performing a new ovarian stimulation. Also in cases with the need of additional preimplantation genetic screening or diagnosis this technique is helpful in respect to the time needed for the genetic analysis.

In order to achieve a pregnancy, synchronization of embryo development and the endometrium is crucial. A simple way of endometrium preparation is the use of the natural cycle with endogenous production of estradiol of the growing follicle. In order to plan the correct timing for embryo thawing and transfer the LH-surge has to be detected. By the use of a natural cycle for FET, significantly higher pregnancy rates in the natural cycle are achieved as compared to modified natural cycles with application of hCG. For the natural cycle, patients have to be monitored closely by blood tests to detect ovulation and plan the thawing and transfer of the embryo(s) accordingly. To define ovulation, LH surge was deemed to have begun when the concentration rose by 180% above the latest serum value available in that patient and continued to rise thereafter. Day 1 after the LH rise, a drop of E2 and concomitant rise of $P > 1.5$ nmol/L confirmed the ovulation (Fatemi et al., 2010). Unfortunately, measurement of LH in the urine is too unreliable for accurate planning of the FET.

Contrary to a natural cycle, hCG is administered to induce ovulation and luteinization in a modified natural cycle. However, hCG-administration to end the follicular phase might have a negative effect on endometrium receptivity, as modified natural cycles result in significant lower pregnancy rates compared to natural cycles.

A frequently used approach for FET is a hormonal-replacement cycle with the administration of exogenous estradiol and progesterone. The advantages of this approach are seen in a reduced risk for cycle-variation and smoothening of the work-flow in the IVF-laboratory, as planning of the embryo-transfer is possible. Hormonal replacement cycle can be performed with or without co-treatment with GnRH agonists.

Until now, the best approach for FET is still under discussion and there is an urgent need for randomized controlled trials to compare the correctly conducted natural cycle with correct determination of LH-surge and the hormonal replacement cycle.

References

- Bar-Hava, I., Mizrachi, Y., Karfunkel-Doron, D., Omer, Y., Sheena, L., et al. (2016). Intranasal gonadotropin-releasing hormone agonist (GnRHa) for luteal-phase support following GnRHa triggering, a novel approach to avoid ovarian hyperstimulation syndrome in high responders. *Fertility and Sterility*, 106, 330–333.
- Bosch, E., Valencia, I., Escudero, E., Crespo, J., Simón, C., Remohí, J., & Pellicer, A. (2003). Premature luteinization during gonadotropin-releasing hormone antagonist cycles and its relationship with in vitro fertilization outcome. *Fertility and Sterility*, 80, 1444–1449.
- Bourgain, C., Ubaldi, F., Tavaniotou, A., Smits, J. M., Steirteghem, V., et al. (2002). Endometrial hormone receptors and proliferation index in the periovulatory phase of stimulated embryo transfer cycles in comparison with natural cycles and relation to clinical pregnancy outcome. *Fertility and Sterility*, 78, 237–244.
- Connell, M. T., Szatkowski, J. M., Terry, N., DeCherney, A. H., Propst, A. M., et al. (2015). Timing luteal support in assisted reproductive technology: A systematic review. *Fertility and Sterility*, 103, 939–946.
- Devoto, L., Fuentes, A., Kohen, P., Cespedes, P., Palomino, A., et al. (2009). The human corpus luteum: Life cycle and function in natural cycles. *Fertility and Sterility*, 92, 1067–1079.
- Fatemi, H. M., Kyrou, D., Bourgain, C., Van den Abbeel, E., Griesinger, G., et al. (2010). Cryopreserved-thawed human embryo transfer: Spontaneous natural cycle is superior to human chorionic gonadotropin-induced natural cycle. *Fertility and Sterility*, 94, 2054–2058.
- Fatemi, H. M., Polyzos, N. P., van Vaerenbergh, I., Bourgain, C., Blockeel, C., et al. (2013). Early luteal phase endocrine profile is affected by the mode of triggering final oocyte maturation and the luteal phase support used in recombinant follicle-stimulating hormone–gonadotropin-releasing hormone antagonist in vitro fertilization cycles. *Fertility and Sterility*, 100, 742–747.

- Fauser, B. C., & Devroey, P. (2003). Reproductive biology and IVF: Ovarian stimulation and luteal phase consequences. *Trends in Endocrinology and Metabolism*, 14, 236–242.
- Fleming, R., & Jenkins, J. (2010). The source and implications of progesterone rise during the follicular phase of assisted reproduction cycles. *RBMonline*, 21, 446–449.
- Humaidan, P., Bredkjaer, H. E., Bungum, L., Bungum, M., Grøndahl, M. L., et al. (2005). GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: a prospective randomized study. *Human Reproduction*, 20, 1213–1220.
- Hutchison, J. S., & Zeleznik, A. J. (1985). The corpus luteum of the primate menstrual cycle is capable of recovering from a transient withdrawal of pituitary gonadotropin support. *Endocrinology*, 117, 1043–1049.
- Ludwig, M., & Diedrich, K. (2001). Evaluation of an optimal luteal phase support protocol in IVF. *Acta Obstetrica et Gynecologica Scandinavica*, 80, 452–466.
- Nillius, S. J., & Johansson, E. D. (1971). Plasma levels of progesterone after vaginal, rectal, or intramuscular administration of progesterone. *American Journal of Obstetrics and Gynecology*, 110, 470–477.
- Nyboe, A. A., Popovic-Todorovic, B., Schmidt, K. T., Loft, A., Lindhard, A., et al. (2002). Progesterone supplementation during early gestations after IVF or ICSI has no effect on the delivery rates: A randomized controlled trial. *Human Reproduction*, 17, 357–363.
- Yding Andersen, C., Bungum, L., Nyboe Andersen, A., & Humaidan, P. (2011). Preovulatory progesterone concentration associates significantly to follicle number and LH concentration but not to pregnancy rate. *Reproductive Biomedicine Online*, 23, 187–195.

Further Reading

- Beckers, N. G., Macklon, N. S., Eijkemans, M. J., Ludwig, M., Felberbaum, R. E., et al. (2003). Nonsupplemented luteal phase characteristics after the administration of recombinant human chorionic gonadotropin, recombinant luteinizing hormone, or gonadotropin-releasing hormone (GnRH) agonist to induce final oocyte maturation in in vitro fertilization patients after ovarian stimulation with recombinant follicle-stimulating hormone and GnRH antagonist cotreatment. *The Journal of Clinical Endocrinology and Metabolism*, 88, 4186–4192.
- Devroey, P., Polyzos, N. P., & Blockeel, C. (2011). An OHSS-free clinic by segmentation of IVF treatment. *Human Reproduction*, 26, 2593–2597.
- Fatemi, H. M., Popovic-Todorovic, B., Papanikolaou, E., Donoso, P., & Devroey, P. (2007). An update of luteal phase support in stimulated IVF cycles. *Human Reproduction Update*, 13, 581–590.
- Fatemi, H. M. (2009). The luteal phase after 3 decades of IVF: What do we know? *Reproductive Biomedicine Online*, 19, 4331.
- Montagut, M., Santos-Ribeiro, S., De Vos, M., Polyzos, N. P., Drakopoulos, P., et al. (2016). Frozen-thawed embryo transfers in natural cycles with spontaneous or induced ovulation: The search for the best protocol continues. *Human Reproduction*, 31, 2803–2810.
- Noyes, R. W., Hertig, A. T., & Rock, J. (1975). Dating the endometrial biopsy. *American Journal of Obstetrics and Gynecology*, 122, 262–263.
- Tavaniotou, A., Smits, J., Bourgain, C., & Devroey, P. (2000). Comparison between different routes of progesterone administration as luteal phase support in infertility treatments. *Human Reproduction Update*, 6, 139–148.
- Simon, C., Martón, J. C., & Pellicer, A. (2000). Paracrine regulators of implantation. *Bailliere. Best Practice and Research: Clinical Obstetrics and Gynaecology*, 14, 815–826.

TECHNOLOGIES

Ultrasonography in IVF

Roger A Pierson, University of Saskatchewan, Saskatoon, SK, Canada

© 2018 Elsevier Inc. All rights reserved.

Ultrasonographic imaging is an integral part of clinical care in the assisted reproductive technologies. It is difficult to imagine how IVF was done before we had the ability to visualize the female reproductive organs easily. In the not-to-distant past, IVF was done using laparoscopic retrieval of oocytes, monitoring ovarian stimulation cycles only by hormonal assay of systemic estradiol levels, and embryos were transferred back into a uterus when we had no real idea about the physiologic status of the endometrium. Only clinical touch was used to guide the placement of the embryo transfer catheter. Ultrasonographic imaging in the hands of the individuals performing the assisted reproductive technology (ART) procedures has delivered us from those uncertainties. The quality and quantity of the information we receive from the images is an essential part of every IVF procedure. Visualization has been an intrinsic aspect of the incredible increases in ART success rates. It is important to remember that the integrated understanding of anatomy, physiology, endocrinology, and pathology we have gained with imaging in the patients undergoing IVF are as important as the incredible increase in knowledge and practice in the embryology laboratories. The confluence of, and synergy among, technologies used in ART care have greatly increased the probabilities of successful pregnancies (Pierson, 2017).

The essential uses of ultrasonography in IVF are in ovarian assessment, monitoring the course of ovarian stimulation protocols, visually-guided retrieval of oocytes, endometrial assessment, and visually-guided embryo transfer. The use of imaging in each of these areas also provides a springboard for new research areas that may be incorporated into clinical care. Awareness of new frontiers is essential to progress in ART and in understanding the changes that will surely come. We rely so heavily on imaging in general gynecology, basic infertility workups and early obstetrical care that it is a challenge to narrow the focus to IVF alone. However, ultrasonography has forever changed our understanding of female reproduction. This article is a synopsis of imaging in IVF integrated into a framework within which the highest quality of care may be provided for patients who require ART to complete their families.

Ovarian Assessment

Monitoring the Course of Ovarian Stimulation

Ovarian stimulation protocols vary tremendously and have evolved from fairly simplistic administration of exogenous hormones derived from urinary sources to sophisticated blends of GnRH analogs, recombinant FSH and LH and other compounds (Cohen, 2003). The protocols are undergoing constant refinement and are soon likely to be tailored to the physiologies of individual patients. The common denominator in all ovarian stimulation protocols is that ultrasonography is used to monitor their effects on the ovaries of each patient. High-resolution transvaginal ultrasonography allows a rapid, noninvasive and highly visual approach to following the fates of individual follicles and cohorts of follicles. When combined with our knowledge of natural ovarian physiology with concomitant assessment of circulating estradiol concentrations and oocyte development, imaging allows determination of the optimal timing for induction of the final stages of folliculogenesis and oogenesis and oocyte retrieval in each patient undergoing ovarian stimulation.

Ovarian stimulation protocols are designed to overwhelm the endogenous physiologic mechanism of selection for a single dominant follicle, obviate atresia in the cohort of other follicles recruited into the follicular wave and foster and sustain the development of many follicles to an imminently preovulatory state so that competent oocytes may be retrieved for IVF. Ultrasonography is essential in determining the numbers and fates of follicles stimulated and to tailor the ovarian response to the optimal number and quality of oocytes desired. In many cases, information such as the difference in ultrasonographically determined diameter between the largest follicle and first and other subordinate follicles may be used to assess the progress and quality of the stimulation. The follicular response of each woman, the number of oocytes desired and clinical assessment of the risk of ovarian hyperstimulation dictate modifications to the stimulation protocol. It is important to note that the expected linear relationship between circulating estradiol concentrations and follicular diameter may not exist during ovarian stimulation and that all follicles do not contribute equally to the estradiol concentrations in circulation.

Human chorionic gonadotrophin (hCG) is usually administered to trigger the final phases of follicular maturation when the largest follicle first attains a predetermined diameter (e.g., 18 mm to 20 mm). The timing of administration of hCG or recombinant LH administration is critical in initiating the final stages of oocyte maturation and establishing the time for oocyte retrieval which will yield the highest quality oocytes in the proper stage of development with the highest probability of fertilization. The relationships between follicle size and oocyte maturity remain poorly elucidated; however, oocyte competence and maturity play important roles in the ability of the resulting embryos to develop to the blastocyst stage. The timing of hCG administration varies among programs based upon individual clinician's feel for the stimulation cycle and laboratory logistics. Most commonly, 5000 IU or

10,000 IU hCG is administered, although recombinant LH also is utilized. Oocyte retrieval is then scheduled for 30–34 h thereafter. Many programs now use only ultrasonographic monitoring to determine the course of ovarian stimulation. Estradiol monitoring during the stimulation protocol seldom changes the timing of hCG administration and has not appeared to affect pregnancy rates or the risks of ovarian hyperstimulation syndrome (Lass, 2003).

The morphological characteristics, individual physiologies and most appropriate diameters of follicles that produce optimal oocytes prepared for fertilization remains the subject of intensive research. Mature oocytes yield the highest fertilization rates in standard IVF cycles; however, the wide-spread use of intracytoplasmic sperm injection (ICSI) and progress in understanding the potential of in vitro maturation and/or fertilization of immature oocytes necessitates reassessment of what constitutes an optimal oocyte. The role of ultrasonography in IVM-IVF protocols revolves around the optimal timing of oocyte retrieval for optimal fertilization and cleavage.

Ovulation in natural cycles has been reported from follicles as small as 14 mm and oocytes collected in IVF cycles from small follicles may fertilize well; oocytes have been aspirated from follicles <10 mm in diameter and matured in vitro to increase the number of transferable embryos. Through research in animal models, we know that there appears to be a correlation between computer-assisted ultrasound image attributes of follicles and the ability of the oocyte to fertilize; however, similar studies in humans have apparently not been completed. The natural synergy among these observations is the start of an emerging area of research.

Evaluation of growth rates for individual follicles may be a useful measure for prediction of the number of follicles that may develop during ovarian stimulation protocols. This information is equally important when assessing the risks of ovarian hyperstimulation. In the past, follicular growth rates during induced cycles were observed to be faster than those of natural cycles. However, a mathematical equation developed to equate follicular growth rate to follicular age was used to conclude that the growth rates of individual follicles in spontaneous cycles were similar to those recruited by exogenous gonadotropins. Reduced growth rates of follicles were observed in cycles where a pregnancy was established and it was concluded that growth rate was a more useful characteristic for prediction of ovulation than follicular diameter. Follow up work does not appear to have been done. It will be logistically challenging to combine daily detailed ultrasonographic measurements of individually mapped follicles with per follicle outcomes from the embryo laboratory and final pregnancy outcomes. New imaging based studies tracking the development trajectories of individual follicles are required.

Color flow and power flow Doppler imaging have been used to assess the state of relative maturity of dominant follicles. Intrafollicular influences on oocyte development such as the developmental competence of the oocyte, oocyte metabolism and mitochondrial function may be mediated, at least in part, by peri-follicular vascularity. It has been hypothesized that blood flow to the follicle is developmentally important for the generation of a normal follicle and oocyte (Borini et al., 2001). Preliminary studies using various indices of follicle vascularity support the notion that women who received embryos originating from oocytes nurtured in well-vascularized follicles had a higher pregnancy rate than women who received embryos derived from oocytes from more poorly vascularized follicles.

Assessment of Ovarian Follicular Reserve

Changes in demographic trends in the age at first pregnancy in our society have continued to yield more and more women seeking pregnancy when they are older and consequently less fertile. It is well documented that fertility declines progressively as age advances (The American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and The Practice Committee of the American Society for Reproductive Medicine, 2014). In IVF, much attention is on assessment of the ovarian reserve. Ultrasonography is a significant part of the tests now being used to investigate follicular dynamics in aging women as are detailed endocrine based tests (Practice Committee of the American Society for Reproductive Medicine, 2015). A decrease in the ovarian reserve, or number of follicles capable of being stimulated, is a primary reason for declining fertility. Similarly, the ovarian response to exogenous gonadotrophin stimulation also decreases, but the range of individual variation is extremely wide and it is well known that age is only a rough guesstimate of the ovarian reserve and hence the ovarian stimulation response.

There are several tests of “ovarian reserve” (Practice Committee of the American Society for Reproductive Medicine, 2015). Ultrasonography may be used to estimate the number of antral follicles at specific times of the menstrual cycle and provides useful information. Ultrasound assessments take place using antral follicle counts or measurement of ovarian volume. Early follicular phase antral follicle counts, typically done on day 3–day 7 postmenstruation, may be used to predict the number of follicles likely to develop during ovarian stimulation. Women having fewer than 5 follicles under 10 mm in diameter before ovarian stimulation begins have a relatively poor prognosis for success. Studies to determine the extent to which antral follicle counts correlate with endocrinologic measures of ovarian reserve (e.g., cycle day 3 follicle-stimulating hormone (FSH) and estradiol concentrations) remain to be widely confirmed. Ovarian volume assessments are based on the presumption that there is a significant correlation between the population of primordial follicles remaining in the ovary and the volume of the ovary, measured using either 2- or 3-dimensional ultrasonography. A clear relationship has been demonstrated between decreased ovarian volume and antral follicle counts and advancing age combined with increased FSH. There remains a good deal of work yet to do in order to standardize the imaging based assessments; however, ultrasonography remains an important aspect of ovarian reserve estimation and relating this knowledge to successful ovarian stimulation cycles.

Ovarian Hyperstimulation Syndrome

Ovarian hyperstimulation syndrome (OHSS) is an exaggerated ovarian response to ovarian stimulation that can develop into a life-threatening complication. In women with the disorder, ultrasonography demonstrates grossly enlarged ovaries containing

numerous large follicular cysts with thin, highly hyperechoic borders, and dramatically increased local blood flow (Battaglia et al., 2004). The ovaries may enlarge to diameters in excess of 10 cm and echotexture interpreted as intrafollicular hemorrhage in some of the large cysts frequently may be observed. In severe cases, the ovaries may become too large to visualize in their entirety with transvaginal ultrasonography and transabdominal imaging must be used. Serial TVUS during ovarian stimulation cycles and careful tailoring of the dose of exogenous gonadotrophins has helped to limit the risk of OHSS; however, prevention of OHSS is multifactorial and requires ovarian stimulation protocols tailored to patient specifics and close monitoring of the ovarian response. Clinicians take an active role in the prevention of OHSS by abandoning the treatment cycle, coasting strategies during stimulation, the use of low doses of ovulation triggering agents and/or cryopreserving all embryos and single embryo replacement in subsequent cycles. When OHSS does occur, torsion of an enlarged ovary is a complication that must be kept in mind. When torsion is suspected, color flow Doppler imaging can help to establish an early and accurate diagnosis.

Computer Assisted Ultrasonographic Imaging of Follicular Development

Computer-assisted image analysis is expected to have the potential to aid in the identification of healthy versus atretic follicles in natural and ovarian stimulation cycles. Physiologically dominant ovarian follicles are identifiable by ultrasonography at approximately day 7 post menstruation in unstimulated cycles and ovulatory and nonovulatory follicles were identifiable in ovulation induction cycles (Baerwald et al., 2012). The image attributes of ultrasonographic images of normal preovulatory follicles include thick, low-amplitude walls and a gradual transformation zone at the fluid-follicle interface. The walls of preovulatory follicles are characterized by increased heterogeneity, increased wall breadth and a more gradual transformation at the fluid-follicle wall interface. Atresia is characterized by thin walls, high numerical pixel value (bright) signals and highly variable signals from the follicular fluid. Evaluation of the acoustic characteristics indicative of viability and atresia combined with the elucidation of the stage of oocyte development for optimal fertilization is an active area of research that has significant implications for development of safer and more effective ovarian stimulation protocols.

Ultrasound-Guided Oocyte Retrieval

The most compelling use of imaging in IVF has been the tremendous advances facilitated by transvaginal retrieval of oocytes. Oocyte retrieval was a technology limiting step in the early days of IVF. Retrievals were done laparoscopically or using ultrasound guidance from transurethral, transvesicular or transabdominal imaging. Transvaginal imaging and concerted efforts to develop effective, accurate tracking of the needles used for follicle aspiration was probably the single most important step in making IVF as safe and effective as it is. Clinical complications appear to be rare.

Retrieval of oocytes in IVF cycles is now routinely performed under TVUS guidance. An aspiration needle is introduced through a guide attached to a transvaginal probe and is inserted into first one ovary, then the other, via the vaginal fornices. Almost all aspiration needles now in use have a small band of highly reflective surface near the tip of the needle to facilitate visualization as the needle enters the ovary and once it is in the follicles (Fig. 1A). The highly reflective echoic tip of the needles makes identifying their path quite easy and the path of the needle may be accurately visualized within biopsy guidelines imposed on the ultrasound screen as it is guided into each ovarian follicle. The needle tip can be observed directly as it is maneuvered within the ovaries and into each follicle (Fig. 1B). The follicular fluid containing the oocyte/cumulus complex is then aspirated by application of gentle suction. The walls of the follicle collapse as the fluid is aspirated and the needle moved within the follicle to ensure that all of the follicular fluid is withdrawn (Fig. 1C).

There are two main types of aspiration needles used for oocyte retrieval, single and double lumen needles. Single lumen needles typically have a smaller diameter and tend to cause less discomfort. In many, if not most, IVF centers follicle aspirations are done using single lumen needles and no follicle flushing. The double lumen needles were developed for a technique involving constant infusion of oocyte collection media into the follicle at the same time as the follicular fluid was being removed. The double lumen flushing technique was thought to increase the turbulence within the follicle, assist in dislodging the oocyte-cumulus complex from the follicle wall and increase the chances of oocyte collection. Single lumen needle flushing techniques may also be used. In this technique, all of the follicular fluid is first aspirated from the follicle and the follicle is then refilled with collection medium and re-aspirated. A back and forth motion on the plunger of the infusion syringe may be used to increase the turbulence of flow. Follicular flushing increases the procedural time required and no significant differences have been observed in live birth rate, clinical pregnancy rates, and the number of oocytes retrieved between flushing and nonflushing techniques for oocyte retrieval.

New types of follicle aspiration needle sets are under development and smaller diameter needles are under evaluation. Needle diameter does not affect oocyte yield, however, the time required for oocyte retrieval procedures was prolonged when smaller diameter needles were used. Similarly, equivalent oocyte retrieval numbers have been obtained using needles designed for aspiration of small follicles in an in vitro maturation (IVM) program.

Unsuccessful oocyte retrieval following apparently normal ovarian stimulation reportedly occurs in 1%–7% of cycles—the so-called “empty follicle syndrome.” The etiology appears to be multifactorial and may involve both technical and biological mechanisms. However, there is significant controversy over whether or not the syndrome actually exists and the nature of its prevalence and putative causative factors.

The complication rates of transvaginal ultrasound-guided oocyte retrieval are reportedly extremely low and almost all procedures are performed under conscious sedation on an outpatient basis.

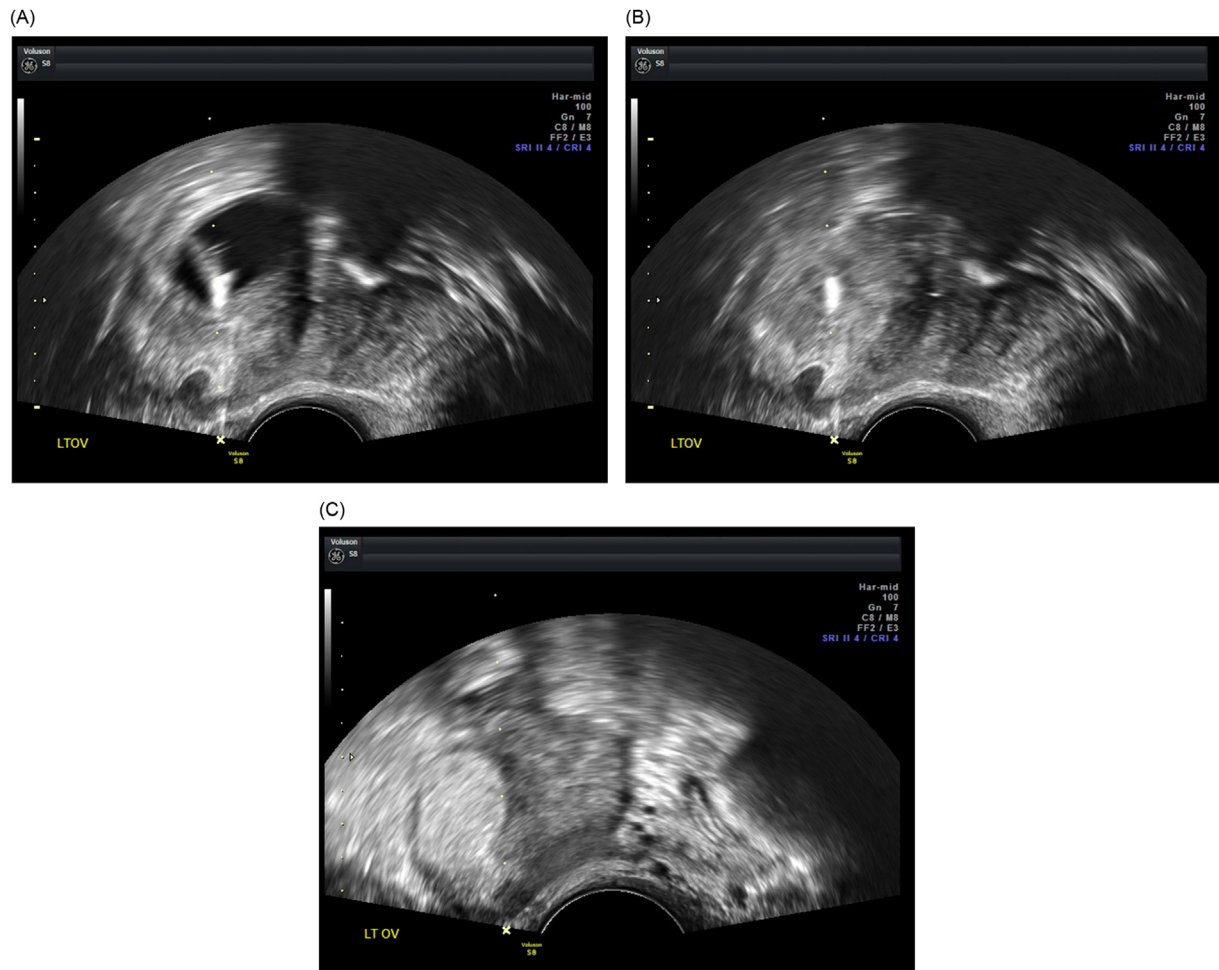


Fig. 1 Ultrasound images taken during oocyte retrieval (A,B,C). (A) The highly echogenic band around the distal end of the needle and the tip of the follicle aspiration needle are clearly visualized in the superior most follicle. The needle is maneuvered within the ovary to aspirate all follicles. (B) Following complete evacuation of the follicular fluid, the echoic tip of the needle is visualized within the apposed walls of the collapsed follicle. (C) The hyperechoic area of the former follicle is visualized following follicle aspiration and removal of needle. Images courtesy of Alex Hartman. Reprinted with permission from Pierson, R. A. (2017). *Ultrasonography in IVF*. In: Gardner, D., Simón, C. (eds.) *Handbook of in vitro fertilization* (4th edn.), pp. 81–103. London, UK: CRC Press. ISBN 9781498729390.

Assessment of the Endometrium at Embryo Transfer

Endometrial Thickness and Pattern for Assessing Endometrial Receptivity

Ultrasonography has been used, with varying degrees of success, to correlate the probability of pregnancy in ovarian stimulation–ovulation induction cycles and IVF cycles (Bonilla-Musoles et al., 2013). Most imaging studies attempt to predict the probability of implantation based on simple biophysical measurements of the endometrium. A thicker endometrium was observed on the day of oocyte retrieval in women who conceived during that cycle. In other studies, no correlation was observed among endometrial pattern, thickness, estradiol levels, number of oocytes retrieved, or progesterone level on the day of embryo transfer. Contradictory reports, and a lack of correlation between ultrasonographic endpoints and histologic staging of the endometrium in women undergoing IVF can be interpreted to mean that ultrasonography using simple biophysical measures is not sensitive enough to be useful in predicting endometrial receptivity and the probability of implantation with the exception of a strong negative correlation when the endometrium is thin. It is also possible that inconsistencies in the day on which measurements were done among the many studies, differences in measurement techniques and the use of a wide variety of ovarian stimulation protocols which affect endometrial development differently have played a role in our seeming inability to interpret the data. Consensus is that implantation is more likely to occur as long as the endometrial thickness is >6 mm.

Collections of fluid are sometimes found within the uterine lumen on the day of embryo transfer. Approximately 5% of cycles may be compromised by the presence of lumen fluid accumulation at some time during the IVF cycle procedures and in 2% of the cases the fluid accumulations persist until the day of embryo transfer. The pregnancy rate among women with fluid accumulations is markedly lower than for those who did not exhibit intraluminal fluid. Fluid accumulations have been found in almost three times

as many women with tubal factor infertility compared with other causes. Although luminal fluid collection does not appear to be a common problem in IVF cycles, it does appear to have a negative impact on implantation and pregnancy rates.

Spectral Doppler and Color Flow Doppler Ultrasonography

The history of Doppler ultrasonography of the uterine arteries in the literature is confusing because of many reports failed to differentiate between spectral Doppler and color flow Doppler imaging. Early studies tend to be based upon spectral Doppler examinations, which are a means of evaluating the resistance to blood flow using calculations of the pulsatility index (PI), resistance index (RI), $V_{\max}$ or the systolic to diastolic ratio (S/D ratio). Color flow Doppler and power flow Doppler imaging are means of turning motion, either toward or away from the transducer in the case of color flow Doppler, or motion in any direction in the case of power flow Doppler, into a visually detectable color overlay on the two-dimensional ultrasound image.

Initially, attempts to determine if evaluation of blood flow in the uterine arteries could be useful were based on RI to look for differences in uterine receptivity. No differences were found among women who conceived and those who did not. However, elevated PI in the uterine artery has been associated with lower pregnancy rates. Assessments of uterine artery RI have remained inconclusive, except that absent or low diastolic flow was associated with failure to conceive. Uterine artery vascular impedance measured by RI has not been found to be useful for predicting the probability of pregnancy. Some ultrasonographically detectable criteria were observed to be associated with negative pregnancy outcomes; however, no prognostic value has been observed in any measurement of vascular perfusion.

Power flow Doppler ultrasonography has been used to investigate endometrial receptivity and flow calculations showed that evidence of motion indicative of vascular flow of $<5 \text{ mm}^2$ were associated with lower pregnancy rates. Endometrial and subendometrial perfusion are reportedly impaired in women with unexplained infertility. In one report, spiral arterial flow and uterine artery flows were not different between pregnant and nonpregnant women; however, if spiral arterial flow could not be detected, no conceptions were observed.

Imaging-Based Uterine Scoring System

Imaging-based scoring systems to predict uterine sensitivity based on uterine biophysical profile system have been proposed. The scoring systems were designed by assigning “points” for various criteria and then adding the cumulative columns to compute a score. Comparisons of uterine scores in conception with nonconception cycles demonstrated no differences in any criteria measured, including endometrial thickness, endometrial pattern, PI, RI, color Doppler, and other vascular indices. However, in more recent work, a new endometrial scoring system based up two-dimensional imaging parameters and creation of a 3-dimensional mathematical surface for visual analysis has shown promising results in the ability to predict the probability of implantation from images taken on the day of oocyte retrieval.

Three-Dimensional Imaging of the Endometrium

Three-dimensional (3D) ultrasonography first became available in the late 1990s and 3D is now a part of almost all high-end imaging systems. There are several methods used to provide 3D information and there are no studies comparing the same endpoints with different imaging systems (Pierson, 1999). Descriptive studies of endometrial development during the menstrual cycle have been done and provide an excellent reference for the normal changes in 3D volumes. The prospects for predicting the probability of implantation in IVF programs have now extended into 3D exploration of endometrial receptivity and optimal embryo replacement location.

The role of three-dimensional volumes as predictors of endometrial receptivity and implantation is contradictory (Alcazar, 2006). Endometrial volumes compared among women who conceived and those who did not showed that pregnancy and implantation rates were lower in women with volumes of $<2 \text{ mL}$. No pregnancies were established when endometrial volumes were $<1 \text{ mL}$. However, a contemporary study found no relationship between 3D volume of the endometrium and conception. Nor was a correlation found among estradiol levels, endometrial thickness, or endometrial volume leading to the conclusion that there was no predictive value for conception in assessing endometrial volume. Endometrial thickness and endometrial volumes were not correlated with probability of pregnancy; however, 3D power flow Doppler indices used to measure endometrial perfusion may have some predictive value although definitive studies do not appear to have been completed. Spiral artery blood flow measurements in 3D had positive predictive value when performed on the first day of ovarian stimulation, while women who became pregnant had lower RI and a higher 3D flow index than those that did not. Taken together, these observations provide rationale for further investigation of 3-dimensional ultrasonography in all of its iterations, however, it is clear a predictive index is beyond the limits of our current technology.

Motion Analysis

Motion analysis, or direct measurement of subendometrial contractions, is a method of evaluating the endometrium based on the observation that the uterus and endometrium are in constant motion (van Gestel et al., 2003). Objective assessment of these contractions has now been applied to assisted reproduction cycles. Endometrial contractions may have a predictive effect on the

probability of pregnancy in IVF cycles. Women with a higher frequency of uterine contractions were found to have lower pregnancy rates. However, contradictory evidence also has been reported. Exogenous progesterone has demonstrably reduced uterine contractility on the day of embryo transfer and it has been hypothesized that progesterone supplementation before embryo transfer may improve endometrial receptivity by lowering the possibility that embryos might be expelled from the uterus by contractions. Uterine contractility at the time of blastocyst transfer was lower and reached a nadir 7 days after hCG administration in IVF cycles. Lower amplitude and frequency of contractions is hypothesized to facilitate blastocyst implantation. The effects of progesterone on uterine contractions have been demonstrated by the observation that higher progesterone concentrations correlated with lower amplitude and frequency uterine contractions. It was suggested that progesterone could be administered to reduce endometrial contractions and have a positive impact on pregnancy rates although this hypothesis does not appear to have been critically tested. The results of pharmaceutical intervention remain under investigation at this time.

Ultrasound-Guided Embryo Transfer

Ultrasonographic imaging is frequently used to guide the placement of the embryo transfer catheter in an effort to facilitate optimal embryo placement and enhance the probability of a successful pregnancy (Mansour and Aboulghar, 2002). Transabdominal ultrasound guidance is a more common means of directing the embryo transfer catheter, however, transvaginal scanning may also be used. It is also important to note that ultrasound guidance of embryo replacement does not prevent the establishment of an ectopic gestation. The recent advent of easy to use and relatively inexpensive three-dimensional ultrasonography has facilitated a new wave of inquiry into the utility of 3D imaging to guide the embryo transfer catheter (Gergely et al., 2005). Early impressions are that 3D imaging may be beneficial in identifying the site of optimal embryo placement with respect to anatomic variations in individual women.

Clinical and laboratory preparations for embryo transfer are the same, regardless of whether the transfer is to be ultrasound guided or not. Patients are placed in the lithotomy position and the cervix exposed using a bivalve speculum. Mucus and secretions are removed using culture media and the tip of the transfer catheter is introduced into the os cervix. The addition of transabdominal ultrasound imaging simply involves placement of the transducer, typically using a 3–4 MHz large aperture probe, on the lower abdomen and pelvis in the sagittal plane and imaging the full sagittal plane of the uterus and cervix through a full bladder window (Rijnders et al., 2000; Tang et al., 2001; Matarras et al., 2002) (Fig. 2). Most standard embryo transfer catheters are easily visualized as a pair of highly echogenic lines within the cervix; however, transfer catheter systems are undergoing constant refinement and new systems have been developed to increase the ease of imaging.

Once the catheter has been identified, the tip may be carefully guided through the uterine lumen using real-time imaging. Once the clinician attains the optimal place with the uterus, the embryos are gently expelled into the lumen. Opinion on exactly what optimal placement means is varied. The fluid droplet containing the embryos is visualized as a very small hypoechoic blip deposited at the tip of the transfer catheter. Specular reflection artifacts may sometimes be helpful in its identification (Fig. 2B).

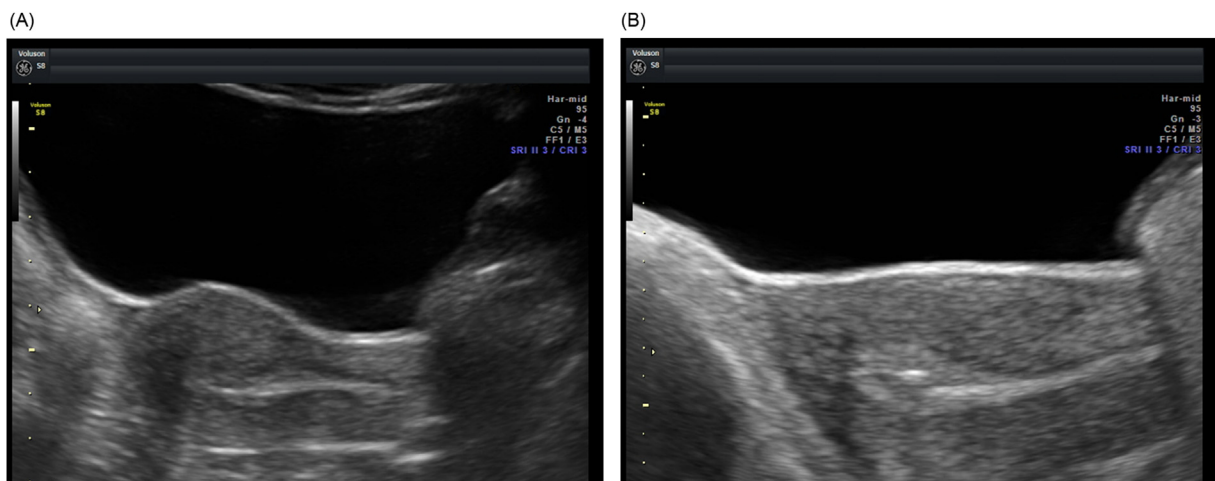


Fig. 2 Mid-sagittal view of the uterus imaged with transabdominal ultrasonography during embryo transfer (A,B). (A) The bladder is fluid-filled, hypoechoic and fills the upper aspect of the image. The uterus is seen in mid-sagittal plane and the transfer catheter is visualized as the highly echogenic line in the middle of the uterus. (B) Enlargement showing the microdroplet of media containing the embryo visualized approximately 6 mm from the endometrial/myometrial interface at the fundal aspect of the uterus (hyperechoic focal point). Specular echoes enhance the ability to visualize the microdroplet. The tip of the embryo transfer catheter is visualized to the right of the microdroplet as it is slowly removed from the lumen. The images document optimal replacement of the embryos. Image courtesy of Dr. Alex Hartman. Reprinted with permission from Pierson, R. A. (2017). Ultrasonography in IVF. In: Gardner, D., Simón, C. (eds.) *Handbook of in vitro fertilization* (4th edn.), pp. 81–103. London, UK: CRC Press. ISBN 9781498729390.

Transvaginal ultrasound guidance is done in a similar fashion, except that a probe designed for intracavitary use is introduced through the speculum and placed into contact with the anterior vaginal fornix. The transfer catheter is visualized and the tip guided to the optimal uterine location for embryo deposition. No differences in pregnancy rates were observed when transabdominal versus transvaginal ET catheter guidance was evaluated. The choice of imaging approach is left to individual clinicians assessment of uterine position, parity and their own level of comfort with the procedure.

There is a measure of controversy regarding the usefulness of ultrasonographic guidance during embryo transfer versus nonvisually guided clinical touch. Some clinicians prefer to rely on ultrasound guidance for mock transfers in cycles before IVF and embryo transfer and clinical touch in the actual procedure using physical measurements taken during the mock cycle. Others prefer to use ultrasound guidance for all procedures, and still others make a decision regarding its use based on whether or not the transfer is likely to be classified as easy or difficult. Three- and four-dimensional ultrasonography have been posited as an optimal means of identifying a “maximal implantation point (MIP)” (Gergely et al., 2005). It was reported that identification of the MIP was easily and reliably done using ultrasonographically identifiable anatomic markers that could be individualized for each patient and the ET catheter guided to it.

Concluding Remarks

Ultrasonography has provided us with direct visual access to all of the events involved in natural human reproduction and has allowed us to elucidate our natural reproductive processes. When we contemplate the incredible progress made in our ability to understand assisted reproduction, it seems strange to envision ART care without ultrasound imaging. We take for granted the ability to see the effects of ovarian stimulation and optimize the protocol for individual patients, easy accessibility of the ovaries for oocyte retrieval, direct visualization of the endometrium at the time of embryo transfer and embryo replacement under direct visual guidance.

One of the most joyous occasions we have in our work is when we are able to confirm pregnancy in our patients. There is little that approaches the drama in the imaging suite when we can point out an embryo to our patients and listen to the heartbeat of their new life. The combined contributions to ART made by the dramatic advances made in the embryo laboratory, enhanced clinical knowledge, and advanced imaging techniques enrich the not only our discipline and scientific knowledge, but also the lives of many couples who have struggled with infertility.

References

- Alcazar, J. L. (2006). Three-dimensional ultrasound assessment of endometrial receptivity: A review. *Reproductive Biology and Endocrinology*, 4, 56.
- Baerwald, A. R., Adams, G. P., & Pierson, R. A. (2012). Ovarian antral folliculogenesis during the human menstrual cycle: A review. *Human Reproduction Update*, 18(1), 73–91.
- Battaglia, C., Mancini, F., Persico, N., Zaccaria, V., & de Aloysio, D. (2004). Ultrasound evaluation of PCO, PCOS and OHSS. *Reproductive Biomedicine Online*, 9(6), 614–619.
- Bonilla-Musoles, F., Raga, F., Osborne, N. G., Castillo, J. C., & Bonilla, F., Jr. (2013). Endometrial receptivity: Evaluation with ultrasound. *Ultrasound Quarterly*, 29(1), 3–20.
- Borini, A., Maccolini, A., Tallarini, A., Bonu, M. A., Sciajno, R., & Flamigni, C. (2001). Perifollicular vascularity and its relationship with oocyte maturity and IVF outcome. *Annals of the New York Academy of Sciences*, 943, 64–67.
- Cohen, J. A. (2003). Short review of ovarian stimulation in assisted reproductive techniques. *Reproductive Biomedicine Online*, 6(3), 361–366.
- Gergely, R. Z., DeUgarte, C. M., Danzer, H., Surrey, M., Hill, D., & DeCherney, A. H. (2005). Three dimensional/four dimensional ultrasound-guided embryo transfer using the maximal implantation potential point. *Fertility and Sterility*, 84(2), 500–503.
- Lass, A. (2003). Monitoring of in vitro fertilization-embryo transfer cycles by ultrasound versus by ultrasound and hormonal levels: A prospective, multicenter, randomized study. *Fertility and Sterility*, 80(1), 80–85.
- Mansour, R. T., & Aboulghar, M. A. (2002). Optimizing the embryo transfer technique. *Human Reproduction*, 17(5), 1149–1153.
- Matorras, R., Urquijo, E., Mendoza, R., Corcostegui, B., Exposito, A., & Rodriguez-Escudero, F. J. (2002). Ultrasound-guided embryo transfer improves pregnancy rates and increases the frequency of easy transfers. *Human Reproduction*, 17(7), 1762–1766.
- Pierson, R. A. (1999). Three-dimensional ultrasonography of the embryo and fetus. In R. Jaffe, & T. H. Bui (Eds.), *Fetal and neonatal ultrasonography* (pp. 317–326). New York: Parthenon Publishing Group.
- Pierson, R. A. (2017). Ultrasonography in IVF. In D. Gardner, & C. Simón (Eds.), *Handbook of in vitro fertilization* (4th edn., pp. 81–103). London, UK: CRC Press, ISBN 9781498729390.
- Practice Committee of the American Society for Reproductive Medicine. (2015) Practice Committee of the American Society for Reproductive Medicine. Testing and interpreting measures of ovarian reserve: A committee opinion. *Fertility and Sterility*, 103(3), e9–e17.
- Rijnders, R. J., Mol, B. W., & Broekmans, F. J. (2000). Use of ultrasound guidance during embryo transfer. *Human Reproduction*, 15(11), 2449.
- Tang, O. S., Ng, E. H., So, W. W., & Ho, P. C. (2001). Ultrasound-guided embryo transfer: A prospective randomized controlled trial. *Human Reproduction*, 16(11), 2310–2315.
- The American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and The Practice Committee of the American Society for Reproductive Medicine. (2014). Female age-related fertility decline, Committee Opinion No. 589. *Fertility and Sterility*, 101(3), 633–634.
- van Gestel, I., Uij, M. M., Hoogland, H. J., & Evers, J. L. (2003). Endometrial wave-like activity in the non-pregnant uterus. *Human Reproduction Update*, 9(2), 131–138.

Further Reading

- Triwityakorn, A., Suwajanakorn, S., Pruksananonda, K., Sereepapong, W., & Ahnonkitpanit, V. (2003). Correlation between human follicular diameter and oocyte outcomes in an ICSI program. *Journal of Assisted Reproduction and Genetics*, 20(4), 143–147.

Egg and Embryo Donation

Irene Woo and Richard J Paulson, Keck School of Medicine of the University of Southern California, Los Angeles, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Egg and embryo donation are now standard components of care offered by infertility specialists. Initially intended as therapy for women with premature ovarian failure, or those with heritable genetic defects, the use of egg donation has been extended to women with a variety of defects in egg production or function. The number of donor egg cycles has significantly increased in the past decade; the North American Society for Assisted Reproductive Technology (SART, 2015) registry reported 20,435 cycles in 2015. Currently, women with age-related decline in fertility represent the largest group of women undergoing treatment with egg donation. Embryo donation is also a practical option for couples with female and male factor infertility who may need both egg and sperm donation. Frequently with in vitro fertilization (IVF) practices, more embryos are created than can be used. Couples who have completed their reproductive attempts may desire to donate the cryopreserved embryos to other individuals attempting to build a family (ASRM, 2013c). In 2015, SART reported 1428 cycles using donated embryos.

History

The first reports of human egg donation appeared in 1983. Trownson et al. (1983) reported a pregnancy after the donation of an oocyte by an infertile woman undergoing IVF. The donor had undergone controlled ovarian stimulation (COS) and the recipient had ovulated at the same time as the donor underwent egg retrieval. One of the recovered oocytes was donated to the recipient, and was inseminated with the recipient's husband's sperm. The resulting embryo was then transferred to the recipient, who became pregnant, but then miscarried. In a subsequent attempt, a single oocyte was again donated by an IVF patient, this time to a woman with premature ovarian failure (Lutjen et al., 1984), whose uterus was prepared with a combination of oral estradiol and vaginal progesterone. The recipient became pregnant and carried the pregnancy to term, thus becoming the first successful instance of egg donation.

During the same time period, a group working at Harbor-UCLA in the United States developed a technique called "ovum transfer." In this process, the egg donor was inseminated with the recipient partner's sperm at the time of the luteinizing hormone (LH) peak. Donors and recipients were matched on the basis of synchronous ovulation. Five days later, the donor's uterine cavity was lavaged in an effort to recover the fertilized blastocyst, which was then transferred to the uterus of the infertile patient. The technique resulted in a pregnancy and subsequent delivery of a child genetically similar to the donor (Bustillo et al., 1984). Unfortunately, this technique was inefficient and efforts to increase the embryo yield by administering COS to the donors led to retained pregnancies in the donors (Sauer et al., 1989a).

Egg donation quickly became the obvious treatment of choice for women with premature ovarian failure. The landmark event of pregnancy in a woman without ovarian function substantiated the hypothesis that exogenous estrogen and progesterone could reliably produce a receptive endometrium (Devroey et al., 1989a,b). In women with residual ovarian function, donor and recipient cycles could thus be synchronized with gonadotropin-releasing hormone (GnRH) agonists and the recipient endometrium stimulated with exogenous steroids (Sauer et al., 1989b, 1991). These essential principles have remained the key components of the current practice of egg donation.

The first published study on embryo donation was a report of an embryo created with donated sperm and donated eggs made specifically for the recipient (Van Steirteghem et al., 1987). When the first true embryo donation occurred is not known, however in the mid-1980s, it was in common usage (Devroey et al., 1989a,b). During this time, the term "embryo adoption" was also coined. While some may use the term embryo adoption and embryo donation synonymously, the transfer of an embryo is legally considered a property transfer and not an adoption (Wurmbbrand, 1986). The American Society for Reproductive Medicine (ASRM) ethics committee discourages the use of the term "adoption" as inaccurate (ASRM, 2016). Nevertheless, there are several "embryo adoption agencies" in the USA, which serve to match prospective donors and recipients and allow for the transfer of embryos which would otherwise be discarded. The medical and technical aspects of embryo donation and similar to those of oocyte donation, and may be simpler without the need for cycle coordination if one is using cryopreserved embryos.

Indications

Women whose eggs are not capable of producing a pregnancy may use egg donation as a means to become pregnant. In addition to premature ovarian failure, indications for egg donation include cases of poor egg quality presumed from multiple failed cycles of conventional IVF, women of advanced reproductive age with associated diminished ovarian reserve, and cancer survivors with premature ovarian failure secondary to chemotherapy. Patients with gonadal dysgenesis are appropriate candidates for egg donation. Those with Turner's Syndrome may conceive, but are at higher risk of obstetrical complications (Hagman et al., 2013). Other patients may choose egg donation to avoid passing heritable genetic diseases to their children, although

preimplantation genetic diagnosis (PGD) is increasingly being applied in these situations. In certain social situations such as same-sex male couples, use of donor oocytes and gestational surrogates is also indicated. For couples with both female and male factor infertility that would require both an oocyte and sperm donor, embryo donation is a practical option. Embryo adoption is also more cost effective than coordinating a fresh oocyte donor with in vitro fertilization (Finger et al., 2010).

Prognostic Indicators

Donor Age

The chronological age of the egg donor is one of the principal factors in the overall success and live birth outcome of oocyte recipient cycles (Sauer et al., 1990; Navot et al., 1991). The mean donor age in the United States is approximately 26–28 years (Kawwass et al., 2013). In a large population study, the highest live birth rate in recipients was achieved in cycles with donors ≤ 34 years of age, while recipients with donors aged 35–39 were significantly less likely to achieve a live birth (Wang et al., 2012). Egg donors are usually healthy and without a history of fertility treatment themselves. Whereas prior fertility in a donor is not necessary, previous successful cycles may improve the prognosis in subsequent ones (Harris et al., 2002). Similarly, for embryo donation, the age of the female at the time of the original oocyte retrieval is one of the main influence on success.

Recipient Age

Recipient age does not appear to decrease the probability of pregnancy success with egg donation or embryo (Paulson, 2014). Physiological observations have shown that uterine blood flow and the endometrial response to exogenous steroids do not appear to diminish with age (Sauer et al., 1993a). In a large clinical study, similar rates of pregnancy and live birth rates were achieved among fresh oocyte recipients aged <35 , 35–39, 40–44, and ≥ 45 years (Wang et al., 2012). An analysis of the national registry data suggested a modest decline in live birth rates after 45 with still poorer outcomes after age 50 (Toner et al., 2002; Yeh et al., 2014). However other studies have shown similar pregnancy success with oocyte donation after the age of 50 (Sauer et al., 1993b; Paulson et al., 1997).

Recipient Factors

Egg donation is successful irrespective of most causes of infertility in the recipient (Garcia-Velasco et al., 2003; Paulson et al., 1997; Harris et al., 2002). The main exceptions are associated with uterine pathology. For instance, Asherman's Syndrome or previous radiation therapy may negatively impact uterine receptivity (Teh et al., 2014). Analogously, uterine fibroids, specifically submucosal fibroids or fibroids that distort the cavity, have been shown to decrease embryo implantation rates and therefore should be resected (Shokeir et al., 2010). Hydrosalpinges should be also be removed as these have been shown to decrease implantation rates and increase the risk of infection as well as ectopic pregnancy in recipients of egg donation (Cohen et al., 1999). Neither endometriosis nor adenomyosis in recipients appear to negatively impact embryo implantation (Pellicer et al., 2001; Martinez-Conejero et al., 2011). Obesity has been associated with decreased odds of pregnancy in women undergoing IVF with autologous oocytes, however its effect on oocyte donation models have been variable (Soares et al., 2008). A recent systematic review noted no negative impact of recipient BMI on embryo implantation, miscarriage, or live births (Jungheim et al., 2013). Heavy tobacco smoking has been shown to reduce uterine receptivity with a significantly lower pregnancy rates in oocyte donation models and therefore patients should be advised to quit smoking (Soares et al., 2007).

Repetitive Cycles

Among recipients with a normal uterine cavity, one failed cycle does not necessarily negatively impact the outcome in subsequent cycles—pregnancy rates are comparable on a per cycle basis (Paulson et al., 1997). Cycle fecundability also appears to be unaltered by the number of cycles that the donor has undergone. The donor response to gonadotropin therapy, the number of oocytes produced, and pregnancy rates have been reported to be unimpaired after successive stimulation cycles (Jain et al., 2005).

Oocyte Donor Screening

Guidelines for donor screening are periodically updated and published by the American Society for Reproductive Medicine (ASRM, 2013c). The guideline attempts to incorporate information from the US Center for Disease Control and Prevention (CDC), the US Food and Drug Administration (FDA), and the American Association of Tissue Banks (AATB). However individual state practice requirements may differ and these must be followed. Oocyte donor screening generally includes a personal and sexual history and physical examination, with the intent of excluding those women at high risk for HIV, other sexually transmitted disease, transmissible spongiform encephalopathy, or recent confirmed or suspected West Nile Virus infection (ASRM, 2005, 2013c). For a complete list of screening questions and exclusions, see "Uniform Donor Application" at www.sart.org. The FDA also requires the following laboratory tests be performed within 30 days of oocyte collection: serum screening for syphilis, hepatitis B and C,

HIV-1 and HIV-2. *Neisseria Gonorrhoeae*, Chlamydia, and blood type and Rh factor are also recommended. Psychological screening should include a family, sexual, substance use, and psychiatric history along with assessment of stability, motivation to donate, life stressors and coping skills, and interpersonal relationships. The counselor should confirm that the donor has been informed of the medical treatment, discuss the psychological risks of egg donation, and evaluate for signs of coercion.

Genetic screening should elicit any personal or family history of any Mendelian disorders, major malformations due to multifactorial causes, or known karyotypic abnormalities. A screening test for cystic fibrosis is recommended in all donors, and heterozygotes may be included as a donor if the recipient is aware and the recipient's partner tests negative (Zenke and Chetkowski, 2002). Specific ethnic groups known to be at high risk for carrying certain recessive genes should undergo additional screening tests. It is not appropriate to screen donors for adult onset conditions (such as cancer predisposition, Huntington disease, etc.) without full consent of donor including formal genetic counseling (ASRM, 2013c).

Recipient Screening

As with donors, the ASRM periodically updates its guidelines for the screening of recipients (ASRM, 2013c). Routine screening includes a medical and reproductive history, physical exam, and standard preconception testing and counseling. Blood type and Rh factor, rubella and varicella titers (with vaccination if not immune) along with infectious disease screening is often obtained in addition to standard preconception laboratory evaluation. Recipients should undergo counseling regarding the potential psychological implications of becoming parents as a result of egg donation (ASRM, 2004).

Prior to embryo transfer the uterine cavity should be carefully evaluated and any pathology corrected. Evaluation of the endometrial cavity can be achieved by saline-injection sonography (Lindheim and Sauer, 1998), hysteroscopy, or hysterosalpingography (HSG). The advantage of HSG lies in its ability to visualize the fallopian tubes and thus detect the presence of hydrosalpinges. Furthermore, potential recipients over the age of 45 should undergo thorough medical evaluation (including cardiovascular testing) and a high-risk obstetric consultation due to concerns for obstetrical complications in the very advanced maternal age women. Turner's syndrome patients should undergo echocardiography or thoracic MRI because the risk of death from aortic rupture or dissection during pregnancy may be 2%. Any significant cardiac abnormality in these patients is an absolute contraindication for attempting pregnancy (ASRM, 2012).

Embryo Donor Screening

Eligibility of the donated embryo is based on the eligibility of the gamete donors. Anonymous donors must meet all the FDA screening and testing requirement for the respective sperm donor and oocyte donor. However, often the embryos donated are created for sexually intimate partners desiring to become pregnant and only at the conclusion of their reproductive attempts are the additional embryos donated. In these situations, donors may be rescreened and tested as per FDA recommendation-but recipients must be informed that the testing was performed after the embryos were created and cryopreserved. Besides signing an informed consent relinquishing all rights of the embryo, donors should not receive compensation for the donated embryos (ASRM, 2013c).

Stimulation

Oocyte donors undergo controlled ovarian stimulation in a similar manner to that of conventional IVF. As with conventional IVF, the use of antagonists in donor cycles reduces the amount of gonadotropins required and duration of treatment (Sauer et al., 1997). Studies comparing agonists and antagonists in oocyte donation cycles have shown comparable pregnancy and live birth rates achieved with either protocol (Bodri et al., 2006). In oocyte donation cycles, an antagonist protocol also has the advantage of the option to use GnRH agonist as an alternative to an HCG trigger for oocyte maturation in patients with a high risk of OHSS (Erb et al., 2010).

In our clinic, donors are often prescribed oral contraceptives prior to stimulation in order to coordinate with the recipient cycle. Oral contraceptives are begun on day 3 of menses, and donors are evaluated on the fourth pill free day to confirm down-regulation by ultrasound evaluation of the ovaries and endometrium. Stimulation is achieved using human menopausal gonadotropins alone or in conjunction with recombinant FSH. Patients return on the fifth day of stimulation for an ultrasound and measurement of serum estradiol. Depending on provider preference, the GnRH antagonist may be given either as a fixed protocol (starting on the sixth day of stimulation) or as a flexible protocol (starting when the leading follicle reaches approximately 14 mm in size). Once two follicles achieve 18 mm in size, ovulation is triggered with a GnRH agonist and oocyte retrieval is performed approximately 34–35 h later.

Donor cycles require vigilance on the part of the nursing staff to ensure adherence to the protocol. Donors should also be encouraged to abstain from unprotected intercourse from the onset of stimulation until the following menses, and prescribed some form of contraceptives to initiate with menses to prevent undesired pregnancy.

Donor Safety

It is rare for oocyte donors to have any serious complications with the stimulation or retrieval. < 1% of donors experience events requiring hospitalization or emergent intervention during or following aspiration as a result of any cause, including severe ovarian hyperstimulation syndrome (OHSS), reactions to anesthesia, pelvic infection, and intraperitoneal hemorrhage (ASRM, 2014). The use of GnRH agonist triggers with an antagonist protocol as previously described has virtually eliminated the development of OHSS. Furthermore, since donors do not conceive during the cycle, they have the advantage of avoiding pregnancy-related exacerbations of OHSS (Morris and Paulson, 1994). Patients that are at high risk for OHSS may be treated prophylactically with cabergoline (Tang et al., 2016). In our practice, donors at risk for OHSS are also evaluated in our office 2 days after follicle aspiration. If necessary, additional intravenous fluids (1000–2000 mL of normal saline) may be administered. Rarely, paracentesis may be required in cases of severe OHSS (Aboulghar et al., 1990).

Little is known about the effect of egg donation on future health and fertility.

Large studies have shown no increase in ovarian cancer risk between subfertile women who received treatment for infertility and subfertile women who were not treated (Venn et al., 1999) or when compared to women in the general population (Siristatidis et al., 2013). Data on breast cancer risk following fertility treatment has been more controversial with some studies showing a minimal increased risk (Reigstad et al., 2015) while others showing no increased risk when compared to other subfertile women (Lerner-Geva et al., 2012). However, no studies have evaluated these risks in oocyte donors, who in general are healthy and do not suffer from subfertility. Given the absence of clear evidence, ASRM recommends limiting donors to six cycles (ASRM, 2014).

Recipient Cycle

The objective of the recipient cycle is to prepare the endometrium for implantation and to synchronize endometrial progress with embryo development. Studies to date have repeatedly shown that estradiol (E2) and progesterone (P) are the only hormones needed to generate a receptive endometrial environment (Navot et al., 1986; Paulson, 2011). Our practice utilizes the regimen depicted in Table 1. If there are concerns regarding the endometrium's ability to produce an adequate response to the exogenous steroids, a practice cycle may be performed. In a practice cycle, the preparation of the recipient endometrium with E2 and P is identical to the actual recipient cycle except on the 7th day of progesterone administration, an endometrial biopsy is performed. The biopsy may document a lack of adequate E2 priming or out-of-phase endometrium. When practice cycles provide information on adjusting timing of embryo transfer, improved pregnancy rates have been noted (Potter et al., 1998).

Recipients with ovarian function should undergo pituitary down-regulation with a GnRH agonist to avoid untimely uterine bleeding associated with fluctuating steroid levels, whereas those with ovarian failure can initiate a cycle at any time.

There are a variety of estradiol regimens, using different routes of administration as well as duration of administration. Despite the extensive hepatic first-pass effect, oral E2 is the most commonly utilized preparation. Oral E2 is converted to estrone (E1) and estrone sulfate (E1S), both weak estrogens with lower binding affinity for the estrogen receptors when compared to E2. Parenteral routes: transdermal, IM, or vaginal can bypass this hepatic first-pass effect. Although transdermal absorption of E2 may be highly variable, it produces a stable steady-state level of serum E2 (Paulson, 2011). Vaginal E2 administration results in high endometrial tissue levels due to local uptake but the serum levels are also very high (Tourgeman et al., 1999; Tourgeman et al., 2001) and it is therefore usually reserved for patients when the other routes are not effective.

Estrogen for endometrial priming can also be provided in a variable or fixed regimen with comparable efficacy. In our clinic, we increase the E2 to mimic the natural pattern of E2 levels in the circulation (see Table 1). Fixed regimens using 4 or 8 mg oral E2 per day without GnRH agonist downregulation have been used to successfully achieve an endometrial thickness > 6 mm within 5–7 days and with good pregnancy rates (Shapiro et al., 1993). Prolonged duration of E2 therapy does not appear to have a negative impact as E2 administration of 4–5 weeks have resulted in pregnancies (Tourgeman et al., 2001). A Cochrane review and a 2013 systematic review reported there was insufficient data to support one particular protocol for endometrial preparation over another (Glujovsky et al., 2010; Groenewoud et al., 2013).

Following estrogen priming, progesterone prepares the endometrium for implantation. E2 stimulates both endometrial proliferation and the induction of progesterone receptors. Therefore, an endometrial biopsy taken during a practice cycle that shows little or no progestational effect has, in our experience, been primarily reflective of inadequate estrogen priming rather than

Table 1 Standard estrogen and progesterone replacement regimen (Authors' institution)

	<i>Micronized estradiol (oral administration)</i>	<i>Micronized progesterone (vaginal administration)</i>
Days 1–7	2 mg BID	
Days 8–14	2 mg TID	
Days 15–28 (through 13th week of gestation)	2 mg BID	200 mg TID

inadequate progesterone delivery to the endometrium. This effect may be observed in women who are hypogonadal with long stretches of amenorrhea with lack of E2 stimulation, or after prolonged hormonal replacement with combination estrogen and progesterone. It is probable that prolonged continuous progesterone stimulation of the endometrium produces a profound downregulation of estrogen receptors. These women may all require extended E2 priming to attain an appropriate response prior to progesterone supplementation.

Progesterone is started in the recipient either on the evening of the day of donor oocyte retrieval or 1 day after in order to synchronize endometrial progress with embryo development (Glujovsky et al., 2010). It is generally believed that there is a “threshold level” of serum progesterone that must be achieved for an orderly luteinization of an adequately primed endometrium. However a precise cut off value has not yet been established, though it is commonly accepted to be > 5 ng/mL (Paulson, 2011).

Progesterone, for the purpose of endometrial preparation, is primarily administered by the intramuscular or vaginal routes. Oral progesterone is ineffective because it is rapidly metabolized in the liver. In addition, progesterone is susceptible to metabolism by 5α -reductase in the skin, making transdermal delivery unrealistic due to the large size of the patch that would be required to provide adequate serum levels of the hormone.

Daily intramuscular injections of progesterone produce high luteal phase serum levels of progesterone and reassurance that the progesterone has been appropriately administered. Many programs in the United States still utilize this mode of delivery with a dose of 50–100 mg daily. Even lower doses of 25–50 mg per day results in a secretory endometrium and good pregnancy rates (Tavaniotou et al., 2000).

The advantage of the vaginal route of administration is that the patient does not have to give herself daily injections. Vaginal progesterone has generally been formulated in three ways: micronized progesterone powder encapsulated, a silastic ring which gradually releases progesterone, and a cream formulation. The most frequently used form is the vaginal micronized progesterone inserts which rapidly disintegrate and the released progesterone is absorbed by the vaginal epithelium. Compared to intramuscular administration of progesterone, vaginal progesterone achieves a lower peak serum progesterone levels, but higher endometrial tissue levels, and reaches a steady state in a shorter amount of time (Paulson et al., 2014). Most patients prefer the vaginal route over the pain and inconvenience of daily IM injections (Yanushpolsky et al., 2010).

Oocyte and Embryo Cryopreservation

The ASRM guideline recommends using the age of the egg donor to determine the appropriate number of embryos to transfer (ASRM, 2013a). For donors < 35 years of age the physician and patient should strongly consider elective single embryo transfers (eSET) in an effort to reduce the risk for multiples. The abundant number of oocytes typically retrieved in an oocyte donor cycle and the subsequent surplus embryos may all be cryopreserved as in conventional IVF. Patients may later choose to donate their remaining embryos to an embryo donation program.

The improved cryopreservation technology has also been applied towards commercial “egg banks.” These entities theoretically provide recipients of egg donation with more choices in selecting a donor, extra flexibility in timing, and potentially lower cost. As of 2012, 600 clinical pregnancies were achieved with frozen donor oocytes from seven commercial egg banks identified in the US, with still an estimate of 3130 oocytes from 294 donors stored for future use (Quaas et al., 2013).

Pregnancy and Neonatal Outcomes

Pregnancy and live birth rates after oocyte donation have risen steadily. In the 2015 SART report, fresh donor oocyte cycles achieved a live birth rate of 50.4%, compared to frozen donor oocyte cycles 38.4%. Thawed embryos using donor eggs had a live birth rate of 39.5%, and donated embryos 38.1%.

Oocyte donation cycles were associated with significantly higher rates of multiple gestations (13.9%) compared to women < 35 years using her own eggs (10.6%), and compared to baseline population risk (3%) (CDC, 2012; Kawwass et al., 2013; Kissin et al., 2015). Pregnancy loss after visualization of fetal heart motion has been reported to occur in 5.7% of singleton pregnancies after oocyte donation (Legro et al., 1995). In multiple gestation, the risk of fetal loss appears to positively correlate with the number of gestational sacs, however miscarriage rates (loss of all gestational sacs) do not appear to be increased and most losses occur prior to the 9th week gestation (Rodriguez-Gonzalez et al., 2002). The probability of spontaneous absorption of 1 or more embryos is approximately 19%–28% (Legro et al., 1995; Rodriguez-Gonzalez et al., 2002). Elective multi-fetal reduction from triplets to twins and from twins to singletons have been shown to improve perinatal outcomes in terms of gestational age and birth weight (Haas et al., 2015).

Besides the increased risk of multiples, oocyte donation in itself is an independent risk factor for pregnancy complications, mainly preeclampsia (Table 2) (Malchau et al., 2013). Many studies have shown oocyte donation increases risk for preeclampsia even when controlling for ICSI (Tranquilli et al., 2013), twins (Sekhon et al., 2014), maternal age (Le Ray et al., 2012), and history of chronic hypertension (Levron et al., 2014). One theory is that there is inadequate immunoprotection of the fetoplacental unit (van der Hoorn et al., 2014).

Perinatal mortality and neonatal malformation rates are similar to the general population. A recent study noted good perinatal outcomes in donor oocyte cycles—with pregnancies resulting in term births and low incidences of low birth rate (Kawwass et al.,

Table 2 Prevalence of antenatal and delivery complications during oocyte donation

	<i>Pregnancy induced hypertension</i>	<i>Gestational diabetes mellitus</i>	<i>First trimester vaginal bleeding</i>	<i>Preterm delivery</i>	<i>Cesarean section</i>	<i>Preeclampsia</i>
Singletons						
Abdalla et al. (1998) (<i>n</i> = 105)	21%	—	—	13%	—	—
Soderstrom-Anttila et al. (1998a,b) (<i>n</i> = 39)	29%	—	—	13%	51%	—
Sheffer-Mimouni et al. (2002) (<i>n</i> = 134)	27.6%	23.9%	43.3%	14.9%	72%	—
Stoop et al. (2012) (<i>n</i> = 148)	17%	7.5%	21.8%	11.6%	50%	10.2%
Malchau et al. (2013) (<i>n</i> = 215)	15.8%	—	—	—	59.5%	9.8%
Levron et al. (2014) (<i>n</i> = 139)	25%	16%	—	9% (<i><</i> 34 weeks)	85%	9.3%
All pregnancies						
Pados et al. (1994) (<i>n</i> = 52)	32.7%	—	34.6%	1.9%	63.5%	—
Soderstrom-Anttila et al. (1998a,b) (<i>n</i> = 51)	31%	12%	53%	30%	57%	—
Le Ray et al. (2012) (<i>n</i> = 104)	—	—	—	29.7%	61.4%	19.2%
Stoop et al. (2012) (<i>n</i> = 205)	19.1%	7.4%	20.6%	19.1%	58.9%	11.8%
Tranquilli et al. (2013) (<i>n</i> = 26)	11.5%	3.8%	7.5%	50%	—	19.2%
van Dorp et al. (2014) (<i>n</i> = 110)	46.9%	—	—	—	53.5%	25.7%

Table 3 Prevalence of neonatal complications in singleton pregnancies after oocyte donation

<i>Study</i>	<i>Perinatal mortality</i>	<i>Neonatal malformations</i>	<i>Low birth weight</i>	<i>Small for gestational age</i>
Pados et al. (1994) (<i>n</i> = 52)	1.7%	0%	—	—
Remohi et al. (1997) (<i>n</i> = 188)	2.7%	1%	—	—
Abdalla et al. (1998) (<i>n</i> = 105)	—	—	18%	15%
Soderstrom-Anttila et al. (1998a,b) (<i>n</i> = 41)	3.3%	5.1%	10%	5%
Sheffer-Mimouni et al. (2002) (<i>n</i> = 134)	0%	2.2%	14.9%	7.6%
Stoop et al. (2012) (<i>n</i> = 148)	2%	—	8.8%	—
Malchau et al. (2013) (<i>n</i> = 244)	—	10.3% (<i>n</i> = 215)	10.7%	6.6%
van Dorp et al. (2014) (<i>n</i> = 61)	3.3%	3.3%	8.3%	—

2013). Small studies have observed infants conceived after oocyte donation were at higher risk for low birth weight and preterm births (Table 3) (Pados et al., 1994; Abdalla et al., 1998; Soderstrom-Anttila et al., 1998b; Sheffer-Mimouni et al., 2002) however after controlling for preeclampsia, neonatal outcomes were similar between oocyte donation cycles and traditional IVF (Malchau et al., 2013; van Dorp et al., 2014). In a 5 year follow-up study of children conceived with oocyte donation, all were healthy with similar growth and developmental rates to children in the general population (Soderstrom-Anttila et al., 1998a).

Advanced Reproductive Age

Even in women over the age of 50, pregnancies can be achieved using donor oocytes and with the appropriate endometrial preparation (Sauer et al., 1993b,c). However with the ability to facilitate pregnancies in the advanced maternal age, medical, and ethical concerns should also be addressed. Precycle screening of women of advanced reproductive age should comprise a thorough medical and physical evaluation including a cardiovascular stress testing (Table 4). All recipients, particularly menopausal women, should also undergo a practice hormone replacement cycle with an endometrial biopsy to confirm adequate estrogen priming and subsequent luteinization of the endometrium.

Large studies on obstetrical and perinatal outcomes in the very advanced maternal age are limited. While some studies noted higher incidences of gestational diabetes, preeclampsia, and cesarean section rates in this older age group (Table 5) (Kort et al., 2012; Jackson et al., 2015) other studies have also noted similar obstetrical risk between donor oocyte recipients in advanced maternal age and younger recipients (Krieg et al., 2008; Kort et al., 2012). In one study of 77 postmenopausal women 50 years or older using donated eggs, pregnancy rates, multiple gestation rates, and spontaneous abortion rates were similar to those of younger recipients (Paulson et al., 2002). In another recent study, subanalysis comparing women ≥ 45 years who conceived via donor oocyte or autologous IVF cycles, both groups had similar rates of postpartum hemorrhage, cesarean section, gestational age, and birth weights (Jackson et al., 2015). Therefore it appears that oocyte donation in itself does not further increase obstetrical or perinatal risks in advanced maternal age women.

Table 4 Screening tests for prospective recipients of oocyte donation over the age of 45 years

Medical
Complete blood count
Blood chemistry panel
Thyroid-stimulating hormone
Fasting cholesterol panel
Glucose tolerance test
Coagulation parameters
Urinalysis
Papanicolaou test
Mammogram
Chest roentgenogram
Treadmill and baseline electrocardiogram
Colonoscopy (if over 50)
Reproductive
Transvaginal ultrasound
Endometrial biopsy and practice embryo transfer (day 21 of practice cycle)
Hysterosalpingogram or saline injection sonography
Infectious disease screen
HIV
HTLV I/II
VDRL
HBsAg
Hep C Ab
Preconceptual and psychosocial counseling

Table 5 Obstetrical outcomes after oocyte donation to women of advanced reproductive age

Study (n = number of women)	Age of subjects	Preeclampsia	Gestational diabetes	Cesarean section rate
Paulson et al. (2002) (n = 77)	≥ 50	35%	20%	78%
Porreco et al. (2005) (n = 50)	> 45	42%	8%	64%
Borini et al. (1995) (n = 34)	≥ 50	22%	11%	—
Sauer et al. (1996) (n = 162)	≥ 45	5.4% ^a	8%	64.8%
Antinori et al. (2003) (n = 1150)	≥ 45	1.1% ^b	0.8%	75%
Jackson et al. (2015) (n = 120)	≥ 45	—	—	70.8%
Kort et al. (2012) (n = 101)	≥ 50	23% ^c	8.9%	81%
Le Ray et al. (2012) (n = 104)	≥ 43	19.2%	7.7%	61.4%

^aGestational hypertension 10.8%.^bGestational hypertension 11.8%.^cInclusive of all hypertensive disorders.

Support for egg and embryo donation in postmenopausal women have expounded on the greater gender equality and reproductive freedom. Counter-arguments have focused on the psychologic impact on the children and concerns regarding the physical and parenting capabilities of these older parents (Shufaro and Schenker, 2014). In a study comparing parenting stress and physical functioning in women who conceived after the age of 50 compared to women in their 30s, advanced maternal age was not found to reduced parenting capacity (Steiner and Paulson, 2007).

The ASRM ethics committee has indicated it is reasonable to offer oocyte and embryo donation services for appropriately screened patients between the ages of 50 and 54 years without any persistent psychological, medical or obstetrical concerns (ASRM, 2013b).

Summary

Oocyte donation, designed to overcome blocks to fertility caused by oocyte problems is a logical extension of the technology of IVF. Additionally, embryo donation is a practical option for couples with female and male factor infertility who may need both egg and sperm donation. Stimulation of the oocyte donor is similar to standard IVF, yet may be less complicated, since poor responder protocols are rarely needed, and concerns about hyperstimulation are essentially eliminated with an antagonist protocol with gonadotropin agonist trigger. Preparation of the recipient endometrium and synchronization between oocyte donor and recipient

are also achieved with relative ease. Recipients of cryopreserved embryo donation are even simpler, requiring only endometrial preparation without need for coordination with the donors. Donors as well as recipients should be medically screened and offered psychosocial counseling to address issues of third-party parenting. Since oocyte and embryo donation bypasses most age-related block to conception in women of advanced reproductive age, it is possible to establish pregnancies in menopausal women over 50 years of age. Perinatal outcomes after oocyte donation appear similar to those after traditional assisted reproductive technologies, with a possible increase in the incidence of hypertensive disorders of pregnancy.

References

- Abdalla, H. I., Billett, A., Kan, A. K. S., Baig, S., et al. (1998). Obstetric outcome in 232 ovum donation pregnancies. *British Journal of Obstetrics and Gynaecology*, 105(3), 332–337.
- Aboulghar, M. A., Mansour, R. T., Serour, G. I., & Amin, Y. (1990). Ultrasonically guided vaginal aspiration of ascites in the treatment of severe ovarian hyperstimulation syndrome. *Fertility and Sterility*, 53(5), 933.
- Antinori, S., Gholami, G. H., Versaci, C., Cerusico, F., et al. (2003). Obstetric and prenatal outcome in menopausal women: A 12-year clinical study. *Reproductive Biomedicine Online*, 6, 257–261.
- ASRM. (2004). Psychological assessment of gamete donors and recipients. *Fertility and Sterility*, 82(1), S18–9.
- ASRM. (2005). American Society for Reproductive Medicine/Society for Assisted Reproductive Technology position statement on West Nile virus. *Fertility and Sterility*, 83(2), 527–528.
- ASRM. (2012). Increased maternal cardiovascular mortality associated with pregnancy in women with turner syndrome. *Fertility and Sterility*, 97(2), 282–284.
- ASRM. (2013a). Criteria for number of embryos to transfer: A committee opinion. *Fertility and Sterility*, 99(1), 44–46.
- ASRM. (2013b). Oocyte or embryo donation to women of advanced age: A committee opinion. *Fertility and Sterility*, 100(2), 337–340.
- ASRM. (2013c). Recommendations for gamete and embryo donation: A committee opinion. *Fertility and Sterility*, 99(1), 47–62.
- ASRM. (2014). Repetitive oocyte donation: A committee opinion. *Fertility and Sterility*, 102(4), 964–966.
- ASRM. (2016). Defining embryo donation: An ethics committee opinion. *Fertility and Sterility*, 106(1), 56–58.
- Bodri, D., Vernaeve, V., Guillen, J. J., Vidal, R., Figueras, F., & Coll, O. (2006). Comparison between a GnRH antagonist and a GnRH agonist flare-up protocol in oocyte donors: A randomized clinical trial. *Human Reproduction*, 21(9), 2246–2251.
- Borini, A., Bafaro, G., Violini, F., Bianchi, L., et al. (1995). Pregnancies in post-menopausal women over 50 years old in an oocyte donation program. *Fertility and Sterility*, 63, 258–261.
- Bustillo, M., Buster, J. E., Cohen, S. W., Thomeycroft, I. H., et al. (1984). Nonsurgical ovum transfer as a treatment on infertile women. Preliminary experience. *JAMA*, 251, 1171–1173.
- CDC (2012) Assisted Reproductive Technology National Summary Report, Available from: <http://www.cdc.gov/art>.
- Cohen, M. A., Lindheim, S. R., & Sauer, M. V. (1999). Hydrosalpinges adversely affect implantation in donor oocyte cycles. *Human Reproduction*, 14(4), 1087–1089.
- Devroey, P., Camus, M., van den Abbeel, E., van Waesberghe, L., Wisanto, A., & van Steirteghem, A. C. (1989a). Establishment of 22 pregnancies after oocyte and embryo donation. *British Journal of Obstetrics and Gynaecology*, 96(8), 900–906.
- Devroey, P., Smits, J., Camus, M., Wisanto, A., et al. (1989b). Synchronization of donor's and recipient's cycles with GnRH analogues in an oocyte donation programme. *Human Reproduction*, 4(3), 270–274.
- Erb, T. M., Vitek, W., & Wakim, A. N. (2010). Gonadotropin-releasing hormone agonist or human chorionic gonadotropin for final oocyte maturation in an oocyte donor program. *Fertility and Sterility*, 93(2), 374–378.
- Finger, R., Sommerfelt, C., Freeman, M., Wilson, C. K., Wade, A., & Daly, D. (2010). A cost-effectiveness comparison of embryo donation with oocyte donation. *Fertility and Sterility*, 93(2), 379–381.
- Garcia-Velasco, J. A., Isaza, V., Caligara, C., Pellicer, A., et al. (2003). Factors that determine discordant outcome from shared oocytes. *Fertility and Sterility*, 80, 54–60.
- Gluajovsky, D., Pesce, R., Fiszbajn, G., Sueldo, C., Hart, R. J., & Ciapponi, A. (2010). Endometrial preparation for women undergoing embryo transfer with frozen embryos or embryos derived from donor oocytes. *Cochrane Database of Systematic Reviews*, 1, Cd006359.
- Groenewoud, E. R., Cantineau, A. E., Kollen, B. J., Macklon, N. S., & Cohen, B. J. (2013). What is the optimal means of preparing the endometrium in frozen-thawed embryo transfer cycles? A systematic review and meta-analysis. *Human Reproduction Update*, 19(5), 458–470.
- Haas, J., Mohr Sasson, A., Barzilay, E., Mazaki Tovi, S., Orvieto, R., Weisz, B., Lipitz, S., & Yinon, Y. (2015). Perinatal outcome after fetal reduction from twin to singleton: To reduce or not to reduce? *Fertility and Sterility*, 103(2), 428–432.
- Hagman, A., Loft, A., Wennerholm, U. B., Pinborg, A., Bergh, C., Aittomäki, K., Nygren, K. G., Bente Romundstad, L., Hazekamp, J., & Soderstrom-Anttila, V. (2013). Obstetric and neonatal outcome after oocyte donation in 106 women with turner syndrome: A Nordic cohort study. *Human Reproduction*, 28(6), 1598–1609.
- Harris, S. E., Faddy, M., Levett, S., Sharma, V., et al. (2002). Analysis of donor heterogeneity as a factor affecting the clinical outcome of oocyte donation. *Human Fertility*, 5, 193–198.
- Jackson, S., Hong, C., Wang, E. T., Alexander, C., Gregory, K. D., & Pisarska, M. D. (2015). Pregnancy outcomes in very advanced maternal age pregnancies: The impact of assisted reproductive technology. *Fertility and Sterility*, 103(1), 76–80.
- Jain, A., Robins, J. C., Williams, D. B., & Thomas, M. A. (2005). The effect of multiple cycles in oocyte donors. *American Journal of Obstetrics and Gynecology*, 192(5), 1382–1384.
- Jungheim, E. S., Schon, S. B., Schulte, M. B., DeUgarte, D. A., Fowler, S. A., & Tuuli, M. G. (2013). IVF outcomes in obese donor oocyte recipients: A systematic review and meta-analysis. *Human Reproduction*, 28(10), 2720–2727.
- Kawwass, J. F., Monsour, M., Crawford, S., Kissin, D. M., Session, D. R., Kulkarni, A. D., & Jamieson, D. J. (2013). Trends and outcomes for donor oocyte cycles in the United States, 2000–2010. *JAMA*, 310(22), 2426–2434.
- Kissin, D. M., Kulkarni, A. D., Mneimneh, A., Warner, L., Boulet, S. L., Crawford, S., & Jamieson, D. J. (2015). Embryo transfer practices and multiple births resulting from assisted reproductive technology: An opportunity for prevention. *Fertility and Sterility*, 103, 954–961.
- Kort, D. H., Gosselin, J., Choi, J. M., Thornton, M. H., Cleary-Goldman, J., & Sauer, M. V. (2012). Pregnancy after age 50: Defining risks for mother and child. *American Journal of Perinatology*, 29(4), 245–250.
- Krieg, S. A., Henne, M. B., & Westphal, L. M. (2008). Obstetric outcomes in donor oocyte pregnancies compared with advanced maternal age in vitro fertilization pregnancies. *Fertility and Sterility*, 90(1), 65–70.
- Le Ray, C., Scherier, S., Anselm, O., Marszałek, A., Tsatsaris, V., Cabrol, D., & Goffinet, F. (2012). Association between oocyte donation and maternal and perinatal outcomes in women aged 43 years or older. *Human Reproduction*, 27(3), 896–901.
- Legro, R. S., Wong, I. L., Paulson, R. J., Lobo, R. A., et al. (1995). Multiple implantation after oocyte donation: A frequent but inefficient event. *Fertility and Sterility*, 63(4), 849–853.

- Lerner-Geva, L., Rabinovici, J., Olmer, L., Blumstein, T., Mashlach, S., & Lunenfeld, B. (2012). Are infertility treatments a potential risk factor for cancer development? Perspective of 30 years of follow-up. *Gynecological Endocrinology*, 28(10), 809–814.
- Levron, Y., Dviri, M., Segol, I., Yerushalmi, G. M., Hourvitz, A., Orvieto, R., Mazaki-Tovi, S., & Yinon, Y. (2014). The 'immunologic theory' of preeclampsia revisited: A lesson from donor oocyte gestations. *American Journal of Obstetrics and Gynecology*, 211(4), 383.e1–383.e5.
- Lindheim, S. R., & Sauer, M. V. (1998). Upper genital-tract screening with hysterosonography in patients receiving donated oocytes. *International Journal of Gynecology & Obstetrics*, 60, 47–50.
- Lutjen, P., Trounson, A., Leeton, J., Findlay, J., et al. (1984). The establishment and maintenance of pregnancy using in vitro fertilization and embryo donation in a patient with primary ovarian failure. *Nature*, 307, 174–175.
- Malchau, S. S., Loft, A., Larsen, E. C., Aaris Henningsen, A. K., Rasmussen, S., Andersen, A. N., & Pinborg, A. (2013). Perinatal outcomes in 375 children born after oocyte donation: A Danish national cohort study. *Fertility and Sterility*, 99(6), 1637–1643.
- Martinez-Conejero, J. A., Morgan, M., Montesinos, M., Fortuno, S., Meseguer, M., Simon, C., Horcajadas, J. A., & Pellicer, A. (2011). Adenomyosis does not affect implantation, but is associated with miscarriage in patients undergoing oocyte donation. *Fertility and Sterility*, 96(4), 943–950.
- Morris, R. S., & Paulson, R. J. (1994). Ovarian hyperstimulation syndrome: Classification and management. *Contemp. Obstetrics and Gynecology*, 39, 43.
- Navot, D., Laufer, N., Kopolovic, J., Rabinowitz, R., et al. (1986). Artificially induced endometrial cycles and establishment of pregnancies in the absence of ovaries. *The New England Journal of Medicine*, 314(13), 806–811.
- Navot, D., Bergh, P. A., Williams, M. A., Garrisi, J., et al. (1991). Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. *Lancet*, 337(8754), 1375–1377.
- Pados, G., Camus, M., Van Steirteghem, A., Bonduelle, M., et al. (1994). The evolution and outcome of pregnancies from oocyte donation. *Human Reproduction*, 9(3), 538–542.
- Paulson, R. J. (2011). Hormonal induction of endometrial receptivity. *Fertility and Sterility*, 96(3), 530–535.
- Paulson, R. J. (2014). Does the age of the recipient influence the probability of pregnancy among recipients of oocyte donation? *Fertility and Sterility*, 101(5), 1248–1249.
- Paulson, R. J., Hatch, I. E., Lobo, R. A., & Sauer, M. V. (1997). Cumulative conception and live birth rates after oocyte donation: Implications regarding endometrial receptivity. *Human Reproduction*, 12(4), 835–839.
- Paulson, R. J., Boostanfar, R., Saadat, P., Mor, E., Tourgeman, D. E., Slater, C. C., Francis, M. M., & Jain, J. K. (2002). Pregnancy in the sixth decade of life: Obstetric outcomes in women of advanced reproductive age. *JAMA*, 288(18), 2320–2323.
- Paulson, R. J., Collins, M. G., & Yankov, V. I. (2014). Progesterone pharmacokinetics and pharmacodynamics with 3 dosages and 2 regimens of an effervescent micronized progesterone vaginal insert. *Journal of Clinical Endocrinology and Metabolism*, 99(11), 4241–4249.
- Pellicer, A., Navarro, J., Bosch, E., Garrido, N., et al. (2001). Endometrial quality in infertile women with endometriosis. *Annals of the New York Academy of Sciences*, 943, 122–130.
- Porreco, R. P., Harden, L., Gambotto, M., & Shapiro, H. (2005). Expectation of pregnancy outcome among mature women. *American Journal of Obstetrics and Gynecology*, 192, 38–41.
- Potter, D. A., Witz, C. A., Burns, W. N., Brzyski, R. G., & Schenken, R. S. (1998). Endometrial biopsy during hormone replacement cycle in donor oocyte recipients before in vitro fertilization-embryo transfer. *Fertility and Sterility*, 70(2), 219–221.
- Quaas, A. M., Melamed, A., Chung, K., Bendikson, K. A., & Paulson, R. J. (2013). Egg banking in the United States: Current status of commercially available cryopreserved oocytes. *Fertility and Sterility*, 99(3), 827–831.
- Reigstad, M. M., Larsen, I. K., Myklebust, T. A., Røbsahm, T. E., Oldereid, N. B., Omund, A. K., Vangen, S., Brinton, L. A., & Storeng, R. (2015). Risk of breast cancer following fertility treatment—a registry based cohort study of parous women in Norway. *International Journal of Cancer*, 136(5), 1140–1148.
- Remohi, J., Gartner, B., Gallardo, E., Yalil, S., et al. (1997). Pregnancy and birth rates after oocyte donation. *Fertility and Sterility*, 67, 717–723.
- Rodriguez-Gonzalez, M., Serra, V., Garcia-Velasco, J. A., Pellicer, A., et al. (2002). The "vanishing embryo" phenomenon in an oocyte donation programme. *Human Reproduction*, 17(3), 789–802.
- SART. (2015). www.SART.org.
- Sauer, M. V., Anderson, R. E., & Paulson, R. J. (1989a). A trial of superovulation in ovum donors undergoing uterine lavage. A trial of superovulation in ovum donors undergoing uterine lavage. *Fertility and Sterility*, 51(1), 131–134.
- Sauer, M. V., Paulson, R. J., Macaso, T. M., Francis-Hernandez, M., et al. (1989b). Establishment of a nonanonymous donor oocyte program: Preliminary experience at the University of Southern California. *Fertility and Sterility*, 52(3), 433–436.
- Sauer, M. V., Paulson, R. J., & Lobo, R. A. (1990). A preliminary report on oocyte donation extending reproductive potential to women over 40. *The New England Journal of Medicine*, 322(17), 1157–1160.
- Sauer, M. V., Stein, A. L., Paulson, R. J., & Moyer, D. L. (1991). Endometrial responses to various hormone replacement regimens in ovarian failure patients preparing for embryo donation. *International Journal of Gynecology & Obstetrics*, 35(1), 61–68.
- Sauer, M. V., Miles, R. A., Dahmouh, L., Paulson, R. J., et al. (1993a). Evaluating the effect of age on endometrial responsiveness to hormone replacement therapy: A histologic, ultrasonographic, and tissue receptor analysis. *Journal of Assisted Reproduction and Genetics*, 10(1), 47–52.
- Sauer, M. V., Paulson, R. J., & Lobo, R. A. (1993b). Pregnancy after age 50: Application of oocyte donation to women after natural menopause. *Lancet*, 341(8841), 321–323.
- Sauer, M. V., Paulson, R. J., & Lobo, R. A. (1993c). Reversing the natural decline in human fertility: An extended clinical trial of oocyte donation to women of advanced reproductive age. *Journal of the American Medical Association*, 268(10), 1275–1279.
- Sauer, M. V., Paulson, R. J., & Lobo, R. A. (1996). Oocyte donation to women of advanced reproductive age: Pregnancy results and obstetrical outcomes in patients 45 years and older. *Human Reproduction*, 11, 2540–2543.
- Sauer, M. V., Paulson, R. J., & Lobo, R. A. (1997). Comparing the clinical utility of GnRH antagonist to GnRH agonist in an oocyte donation program. *Gynecologic and Obstetric Investigation*, 43(4), 215–218.
- Sekhon, L. H., Gerber, R. S., Rebarber, A., Saltzman, D. H., Klauser, C. K., Gupta, S., & Fox, N. S. (2014). Effect of oocyte donation on pregnancy outcomes in in vitro fertilization twin gestations. *Fertility and Sterility*, 101(5), 1326–1330.
- Shapiro, H., Cowell, C., & Casper, R. F. (1993). The use of vaginal ultrasound for monitoring endometrial preparation in a donor oocyte program. *Fertility and Sterility*, 59(5), 1055–1058.
- Sheffer-Mimouni, G., Mashlach, S., Dor, J., Levran, D., et al. (2002). Factors influencing the obstetric and perinatal outcome after oocyte donation. *Human Reproduction*, 17(10), 2636–2640.
- Shokeir, T., El-Shafei, M., Yousef, H., Allam, A. F., & Sadek, E. (2010). Submucous myomas and their implications in the pregnancy rates of patients with otherwise unexplained primary infertility undergoing hysteroscopic myomectomy: A randomized matched control study. *Fertility and Sterility*, 94(2), 724–729.
- Shufaro, Y., & Schenker, J. G. (2014). The risks and outcome of pregnancy in an advanced maternal age in oocyte donation cycles. *Journal of Maternal-Fetal & Neonatal Medicine*, 27(16), 1703–1709.
- Siristatidis, C., Sergeantanis, T. N., Kanavidis, P., Trivella, M., Sotiropoulos, M., Mavromatis, I., Psaltopoulou, T., Skalkidou, A., & Petridou, E. T. (2013). Controlled ovarian hyperstimulation for IVF: Impact on ovarian, endometrial and cervical cancer—a systematic review and meta-analysis. *Human Reproduction Update*, 19(2), 105–123.
- Soares, S. R., Simon, C., Remohi, J., & Pellicer, A. (2007). Cigarette smoking affects uterine receptiveness. *Human Reproduction*, 22(2), 543–547.
- Soares, S. R., Velasco, J. A., Fernandez, M., Bosch, E., Remohi, J., Pellicer, A., & Simon, C. (2008). Clinical factors affecting endometrial receptiveness in oocyte donation cycles. *Fertility and Sterility*, 89(3), 491–501.

- Soderstrom-Anttila, V., Sajaniemi, N., Tiitinen, A., & Hovatta, O. (1998a). Health and development of children born after oocyte donation compared with that of those born after in-vitro fertilization, and parents' attitudes regarding secrecy. *Human Reproduction*, 13(7), 2009–2015.
- Soderstrom-Anttila, V., Tiitinen, A., Foudila, T., & Hovatta, O. (1998b). Obstetric and perinatal outcome after oocyte donation: Comparison with in-vitro fertilization pregnancies. *Human Reproduction*, 13(2), 483–490.
- Steiner, A. Z., & Paulson, R. J. (2007). Motherhood after age 50: An evaluation of parenting stress and physical functioning. *Fertility and Sterility*, 87(6), 1327–1332.
- Stoop, D., Baumgarten, M., Haentjens, P., Polyzos, N. P., De Vos, M., Verheyen, G., Camus, M., & Devroey, P. (2012). Obstetric outcome in donor oocyte pregnancies: A matched-pair analysis. *Reproductive Biology and Endocrinology*, 10, 42.
- Tang, H., Mourad, S., Zhai, S. D., & Hart, R. J. (2016). Dopamine agonists for preventing ovarian hyperstimulation syndrome. *Cochrane Database of Systematic Reviews*, 11, Cd008605.
- Tavaniotou, A., Smits, J., Bourgain, C., & Devroey, P. (2000). Comparison between different routes of progesterone administration as luteal phase support in infertility treatments. *Human Reproduction Update*, 6(2), 139–148.
- Teh, W. T., Stern, C., Chander, S., & Hickey, M. (2014). The impact of uterine radiation on subsequent fertility and pregnancy outcomes. *BioMed Research International*, 2014, 482968.
- Toner, J. P., Grainger, D. A., & Frazier, L. M. (2002). Clinical outcomes among recipients of donated eggs: An analysis of the U.S. national experience, 1996–1998. *Fertility and Sterility*, 78(5), 1038–1045.
- Tourgeman, D. E., Gentzchein, E., Stanczyk, F. Z., & Paulson, R. J. (1999). Serum and tissue hormone levels of vaginally and orally administered estradiol. *American Journal of Obstetrics and Gynecology*, 180(6 (Pt 1)), 1480–1483.
- Tourgeman, D. E., Slater, C. C., Stanczyk, F. Z., & Paulson, R. J. (2001). Endocrine and clinical effects of micronized estradiol administered vaginally or orally. *Fertility and Sterility*, 71(1), 200–202.
- Tranquilli, A. L., Biondini, V., Talebi Chahvar, S., Corradetti, A., Tranquilli, D., & Giannubilo, S. (2013). Perinatal outcomes in oocyte donor pregnancies. *Journal of Maternal-Fetal & Neonatal Medicine*, 26(13), 1263–1267.
- Trounson, A., Leeton, J., Besanko, M., Wood, C., et al. (1983). Pregnancy established in an infertile patient after transfer of a donated embryo fertilised in vitro. *British Medical Journal*, 286, 835–838.
- van der Hooft, M. L., van Egmond, A., Swings, G. M., van Beelen, E., van der Keur, C., Tirado-Gonzalez, I., Blois, S. M., Karumanchi, S. A., Bianchi, D. W., Claas, F. H., & Scherjon, S. A. (2014). Differential immunoregulation in successful oocyte donation pregnancies compared with naturally conceived pregnancies. *Journal of Reproductive Immunology*, 101–102, 96–103.
- van Dorp, W., Rietveld, A. M., Laven, J. S., van den Heuvel-Eibrink, M. M., Hukkelhoven, C. W., & Schipper, I. (2014). Pregnancy outcome of non-anonymous oocyte donation: A case-control study. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 182, 107–112.
- Van Steirteghem, A. C., Van den Abbeel, E., Braeckmans, P., Camus, M., Khan, I., Smits, J., Staessen, C., Van Waesberghe, L., Wisanto, A., & Devroey, P. (1987). Pregnancy with a frozen-thawed embryo in a woman with primary ovarian failure. *The New England Journal of Medicine*, 317(2), 113.
- Venn, A., Watson, L., Bruinsma, F., Giles, G., & Healy, D. (1999). Risk of cancer after use of fertility drugs with in-vitro fertilisation. *Lancet*, 354(9190), 1586–1590.
- Wang, Y. A., Farquhar, C., & Sullivan, E. A. (2012). Donor age is a major determinant of success of oocyte donation/recipient programme. *Human Reproduction*, 27(1), 118–125.
- Wurmbrand, M. J. (1986). Frozen embryos: Moral, social, and legal implications. *Southern California Law Review*, 59(5), 1079–1100.
- Yanushpolsky, E., Hurwitz, S., Greenberg, L., Racowsky, C., & Hornstein, M. (2010). Crinone vaginal gel is equally effective and better tolerated than intramuscular progesterone for luteal phase support in in vitro fertilization-embryo transfer cycles: A prospective randomized study. *Fertility and Sterility*, 94(7), 2596–2599.
- Yeh, J. S., Steward, R. G., Dude, A. M., Shah, A. A., Goldfarb, J. M., & Muasher, S. J. (2014). Pregnancy outcomes decline in recipients over age 44: An analysis of 27,959 fresh donor oocyte in vitro fertilization cycles from the Society for Assisted Reproductive Technology. *Fertility and Sterility*, 101(5), 1331–1336.
- Zenke, U., & Chetkowski, R. J. (2002). Inclusion of heterozygotes for cystic fibrosis in the egg donor pool. *Fertility and Sterility*, 78(3), 557–561.

SEMEN ANALYSIS

Understanding Bulk Seminal Parameters

Grace M Centola, Reproductive Laboratory and Tissue Bank Consultant, Port St. Lucie, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Although the semen analysis is not a test of actual fertility, it provides the clinician with ample information to determine if there is a male factor affecting the inability of the couple to conceive. The routine semen analysis is an accurate measurement of sperm production and maturation, and the ejaculatory process (Centola, 1996; Lewis, 2007; Sikka and Hellstrom, 2016). Sperm fertility, that is., ability to fertilize an egg and subsequent processes, cannot be established by a routine semen analysis, and therefore more advanced tests often must be used to determine actual fertility (Lewis, 2007; Sikka and Hellstrom, 2016). The physician interprets the qualitative and quantitative semen analysis results and determines if further testing is warranted.

There is large biological variation in semen quality which is generally uncontrollable, and explains the individual variations seen between and within males (Keel, 2006; Castilla et al., 2006; WHO, 2010). A minimum of two semen analyses should be done to account for variation within individuals and between specimens.

A third analysis might be necessary if the previous analyses show different results.

It is important that the specimen be collected properly, methods standardized and results properly interpreted (Sigman et al., 2009; Poch and Sigman, 2010; Bjorndahl et al., 2010; Centola, 2011). Each lab report should list the normal or reference ranges, which will assist the physician with interpretation of the results. The normal range is obtained from published standards (such as WHO, 1999 or WHO, 2010), or an intra-laboratory range established by analysis of a local population of fertile men with pregnant partners (Bjorndahl, 2010; Niederberger, 2011).

Threshold values for sperm concentration, motility and morphology can classify the male as fertile or subfertile, or of indeterminate fertility (Guzick et al., 2001). The only way to determine that sperm are able to fertilize an egg is through in vitro fertilization or a current pregnancy.

Interpretation and Understanding of Bulk Seminal Parameters

The abstinence period, the method of collection and the time from collection to analysis will influence the results of the semen analysis. Therefore, specific instructions are provided to minimize inaccurate results. It is important that instructions are followed, and the laboratory is informed of any nonconformances since these may affect the results and invalidate the analysis.

The entire ejaculate should be collected into an aseptic or sterile specimen container provided by the laboratory. It is preferable to collect in a private room provided at the laboratory facility. However, if this is not possible, a specimen can be collected off-site provided that the specimen can be maintained at body temperature and delivered to the laboratory within 30–45 min of ejaculation. Coitus interruptus is not a reliable means to collect a specimen for analysis since the first part of the ejaculate is easily lost, and there may be cells and bacteria from the vaginal fluid contaminating the semen specimen (WHO, 2010). Over the counter latex condoms should not be used since they contain toxic chemicals that interfere with sperm motility (Jones et al., 1986; WHO, 2010).

If any portion of the ejaculate is lost or not collected, it should be reported to the laboratory performing the test (WHO, 2010). The first part of the ejaculate is the sperm-rich fluid, and therefore loss of this part will influence the results. If the sperm are kept in the raw ejaculate for an extended time, motility may decrease, and thus the time of collection and time of analysis are important to interpretation of the results.

A 2–3 day period from the last ejaculation is a standard abstinence time. Others have suggested an abstinence time of 2–7 days (WHO, 2010). It is not better to have a longer abstinence thinking that a longer time is better. A prolonged abstinence may cause increased numbers of dead, dying and nonmotile sperm (Centola, 2011).

Liquefaction and Viscosity

Upon collection, the semen is coagulated as a result of proteins from the seminal vesicles (Rothman and Reese, 2009; Turner, 2009; Bjorndahl et al., 2010). Between 30 and 45 min after specimen collection, and usually within 15–20 min, the semen liquefies due to enzymes present in the semen from the prostate gland (Rothman and Reese, 2009; Turner, 2009; Bjorndahl et al., 2010). With liquefaction, the ejaculate becomes thinner, more liquid and watery. If the ejaculate does not properly liquefy, abnormalities, including inflammation or infection of the accessory sex glands should be considered (Turner, 2009; Bjorndahl et al., 2010). Loss of part of the ejaculate, and hence loss of the fluid contributed by the seminal vesicles or prostate gland can also result in abnormal liquefaction.

Decreased motility may result from incomplete liquefaction (Rothman and Reese, 2009). Lack of, or partial liquefaction can also impair contact of the ejaculate and the sperm with the female's cervix following coitus.

Viscosity is the consistency or fluidity of the ejaculate. It is important to note that determining viscosity is very different from determining liquefaction. If the semen demonstrates discreet droplets when expelled from a disposable pipette, the viscosity is considered normal. However, if the semen does not form discreet droplets, falling thick and stringy from a pipette, there is increased viscosity. High viscosity can interfere with accurate semen analysis. Motility may also be reduced by increased viscosity. If the semen is viscous, the sperm will not make effective contact with the cervix. Although not the same as formation of a coagulum and liquefaction, viscosity can be related to disturbed accessory gland function, including inflammation and infection (Bjorndahl et al., 2010). Increased viscosity can also be related to dehydration or lack of fluid intake. Additional semen analyses and further evaluation may be warranted to determine if the increased viscosity is an isolated finding or persistent.

Color

A normal ejaculate appears as gray to white and opaque. A clear ejaculate may indicate a reduced sperm concentration, or azoospermia (Centola, 2011). Abnormal color may indicate the presence of red blood cells (pink or red-brown in color) (WHO, 2010). The presence of red blood cells may simply be due to skin abrasion during collection or an infection of one of the accessory sex glands (Bjorndahl et al., 2010). Infection, jaundice or vitamins and certain drugs may result in a yellow-colored semen (WHO, 2010). A yellowish color can also be related to prolonged abstinence (Bjorndahl et al., 2010). An unpleasant odor might also be related to infection or extended storage (age) of the specimen before lab analysis. Similarly, additional semen analyses and further evaluation may be warranted to determine if this is an isolated finding.

Volume and pH

The fluid making up the ejaculate is contributed by the accessory sex glands, primarily the prostate gland, which contributes 20%, the seminal vesicles, which contributes 70%, and a small portion from the bulbourethral glands and epididymis (Bjorndahl et al., 2010; WHO, 2010). Approximately 10% of the ejaculate volume is contributed by the testicles. The seminal vesicles provide alkaline fluid, while the prostate gland provides acidic fluid (Oates, 2012). The normal reference range for semen volume is 1.5–6.0 mL (WHO, 2010).

Clinical relevance becomes apparent only when the semen volume is equal to or less than 1.0 mL (Niederberger, 2011). Low semen volume can indicate dysfunction of the accessory sex glands (i.e., seminal vesicles, prostate), ejaculatory duct blockage, congenital absence of the vas deferens, or incomplete collection of the specimen (Poch and Sigman, 2010; WHO, 2010; Centola, 2011; Niederberger, 2011). Stress during collection or ejaculatory failure may also affect semen volume (Bjorndahl et al., 2010). Retrograde ejaculation, which occurs when the semen passes into the bladder rather than outward through the urethra, could result in low ejaculate volume or no antegrade ejaculation and thus no semen (Poch and Sigman, 2010; Niederberger, 2011).

High volume is often seen in inflammation or infection of the accessory glands, particularly the prostate (Bjorndahl et al., 2010; WHO, 2010). High volume results in dilution of the sperm, resulting in a lower concentration (million per milliliter). High volume can also result in impaired contact with the cervix and loss of the ejaculate from the vagina. Low semen volume may result in the spermatozoa being impaired by the acidic vaginal environment and poor contact with the cervix. Reduced contact with the cervix and loss of semen from the vagina can also occur with high semen volume (Bjorndahl, 2010).

The semen pH reflects the balance between the secretions from the seminal vesicles and prostate gland (WHO, 2010). In cases of azoospermia, pH may point towards accessory gland dysfunction (Rothmann and Reese, 2009). Low volume and low pH with no sperm usually indicates obstruction or absence of the vas deferens (Sikka and Hellstrom, 2016; Oates, 2012). Semen pH increases over time and with exposure to ambient air (Rothman and Reese, 2009; WHO, 2010). Thus, pH might have little useful information especially if the volume, concentration and motility are close to or within normal limits. Currently, most semen analyses do not include measurement of pH.

Concentration and Motility

Concentration and motility are probably the most important of the bulk seminal parameters. The term oligozoospermia refers to low sperm count and asthenozoospermia refers to reduced motility. Cryptozoospermia refers to sperm counts that are so low as to be nearly azoospermic (Niederberger, 2011).

The sperm concentration, or sperm density, refers to the numbers of sperm per unit of volume (milliliter) of semen. The total sperm count, which is the concentration times the semen volume, is thought to be more relevant than sperm concentration (Rothmann and Reese, 2009). The total count (number of spermatozoa per ejaculate) provides a measure of testicular function and the patency of the ejaculatory tubules (Rothmann and Reese, 2009; WHO, 2010). The total motile count (often referred to as TOMO) is also an important and relevant measurement, taking into consideration the sperm concentration, motility and semen volume. The lower reference limit for sperm concentration is 15 million sperm per milliliter (range 12–16) (WHO, 2010). The reference value for total sperm count is 39 million (WHO, 2010). The thresholds for sperm concentration were previously reported as 13.5 million/mL and 48 million/mL (Guzick et al., 2001), which means that in men with a sperm concentration greater than the threshold, any medical or surgical treatment is unlikely to significantly increase the odds of conception (Niederberger, 2011). Pregnancy rates

will be decreased if sperm counts are below the reference values of 15–20 million per milliliter, but only below 5 million per milliliter will fertility be significantly reduced (Bjorndahl, 2010; Poch and Sigman, 2010; Niederberger, 2011).

Besides incomplete sample collection, sperm count can be affected by abstinence time, and external factors such as general illness and fever, medications, recreational drug use and environmental or occupational exposures (Bjorndahl et al., 2010). Low sperm count can be caused by hormonal problems. Elevated FSH hormone with low sperm count can indicate testicular failure. Chromosome microdeletions, specifically Y-chromosome deletions, should also be considered as causative of severely low sperm count or azoospermia (Oates and Lamb, 2009; Niederberger, 2011).

Sperm motility is more difficult to assess than sperm numbers, since motility can be highly subjective. Furthermore, motility is often inaccurately reported by labs which infrequently perform semen analyses, and by technicians unfamiliar with the appearance of motile sperm under a microscope (Niederberger, 2011). The sperm motility is usually reported as total percent motility. A classification of progressive motility, that is movement that is rapid and mostly in a linear pattern can also be helpful to interpretation. Rapid progressive motility is an important functional property of sperm related to fertility and fertilization success (Bjorndahl et al., 2010). The WHO classifies motility as “progressive motility” where sperm are actively moving either linearly or in a large circle regardless of speed, “nonprogressive motility” which includes all other patterns of movement where the sperm move but do not progress, and no motility or movement at all (WHO, 2010; Niederberger, 2011). The classification of the total motility reported by the laboratory should be defined on the lab report. Usually, the total motility includes both progressive and nonprogressive motility, and is calculated as the number of motile sperm divided by the total number counted, expressed as percent. Along with progressive and nonprogressive motility, the quality of sperm movement is often classified according to a number scale (0–4) or letter scale (type a to d). The lower reference limit for total motility is 40% and for progressive motility is 32% (WHO, 2010). The fertility thresholds have been reported between 32% and 63% (Guzick et al., 2001).

Sperm motility is affected by many factors, including but not limited to the length of holding time from collection to analysis, and medical factors, such as presence of a varicocele, exposure of the testes to increased temperature and environmental exposures. A varicocele is an enlargement or dilatation of the veins of the testicles (Nagler and Grotas, 2009). Low motility may also be caused by prolonged abstinence, genital infection and inflammation of the accessory sex glands, antisperm antibodies and sperm structural defects (Sigman et al., 2009; Bjorndahl et al., 2010; Niederberger, 2011). Complete absence of motility can be caused by ultrastructural defects in the sperm midpiece or tail, a condition referred to as immotile cilia syndrome or Kartagener’s syndrome (Sigman et al., 2009; Bjorndahl et al., 2010).

Vitality or viability assessment can be a check on accuracy of low motility measurement, and allows the lab to distinguish complete immobility due to defects in the sperm midpiece or tail and other factors (Mortimer, 1994; Bjorndahl, 2010). In cases of low motility, especially in samples with less than 40% motility (WHO, 2010) (some laboratories use less than 25% as a threshold to perform a viability stain), a stain can be used to determine if the nonmotile sperm are metabolically alive. Sperm that have damaged membranes, and are thus dead, will take up the vital stain. The total number of live sperm by dye exclusion is obtained by multiplying the percentage of live sperm by the total sperm count.

In such cases, the reduced viability might be due to factors such as toxic exposure, exposure to temperature extremes, antisperm antibodies or structural defects (Sigman et al., 2009; Bjorndahl, 2010). A high percentage of live but immotile sperm may indicate structural defects, but a high percentage of immotile and nonviable sperm may indicate testicular (epididymal) dysfunction (WHO, 2010).

Morphology

Assessment of sperm morphology remains an important part of the routine semen analysis. The term, teratozoospermia refers to increased abnormal sperm forms. While some report sperm morphology as predictive of outcome in assisted reproduction (Kruger et al., 1988), others question its relevance (Centola, 1996, 2011; Centola and Jeyendran, 2009; Rothman and Reese, 2009; Bjorndahl, 2010), specifically because of the wide variations in morphology assessment between laboratories. Two types of morphology classification have been used, a strict morphology also referred to as “Kruger” morphology, and a less strict method referred to as WHO morphology (Kruger et al., 1988; WHO, 1999; Niederberger, 2011). The most recent version of the WHO manual endorsed a stricter method of classification (WHO, 2010; Niederberger, 2011).

A normal sperm is defined as one with a smooth, oval head and a clearly visible acrosomal cap. The sperm tail is 45–50 μm long with no breaks or sharp bends (Bjorndahl et al., 2010; WHO, 2010). Sperm defects are classified as head, midpiece and tail defects. Head defects include small or large head, elongated or tapered head, acrosomal defects, duplicate heads and amorphous heads, which are those with defects not falling into the previously indicated categories. Midpiece defects include bent necks, thick or thin necks, or if there is off-center insertion of the tail into the midpiece. Tail defects include bent tails (bent at any area along the tail length), short tails, coiling of the tail or coiled tips of the tail. In rare cases, all sperm can show giant heads, pin heads or uniformly round heads with no acrosomal cap (Niederberger, 2011). In the case of absent acrosomes, assisted reproduction, particularly ICSI is the only treatment.

Using the strict method of classification, any abnormality even if considered borderline normal versus abnormal, is classified as abnormal. The previous less strict method would classify such a sperm as normal. The current reference value for normal morphology is 4% (WHO, 2010). The thresholds for normal morphology have been reported as 9% and 12% normal forms (Guzick et al., 2001).

The majority of cases of increased abnormal sperm have no medical explanation. However, varicocele, extremes in temperature and, infrequently, genetic defects can cause sperm morphological defects (Oates and Lamb, 2009; Nagler and Grotas, 2009; Niederberger, 2011). Toxic and environmental exposure can also cause morphological defects (Katz, 1991; Rothmann and Reese, 2009). For example, increased tapered sperm heads may be a result of varicocele, duplicate heads, round heads or pin-headed sperm might be related to testicular trauma, toxic exposure or genetic defects (Katz, 1991; Nagler and Grotas, 2009; Oates and Lamb, 2009; Poch and Sigman, 2010; Niederberger, 2011). The percentage of abnormal sperm and the type of abnormalities can assist the physician in determining a treatment regimen for the infertile couple (Centola and Jeyendran, 2009). Even in cases of severe teratozoospermia, fertilization is possible (Poch and Sigman, 2010), although pregnancy rates in these cases are low even with assisted reproductive procedures (Sigman et al., 2009). It is important to remember that consistent high percentage of abnormal forms can be clinically significant, whereas isolated increased abnormal forms without other abnormal parameters are probably clinically insignificant (Niederberger, 2011).

Conclusion

A thorough history and physical exam, and multiple semen analyses will assist the physician in determining if further testing, such as hormone or genetic testing is needed. The physician will determine if medical or surgical intervention might increase the fertility potential of the male. Together with information from evaluation of the female partner, male evaluation will direct the clinician to an individualized treatment plan for the couple. Options might also include assisted reproductive procedures such as artificial insemination, and IVF/ICSI.

References

- Bjorndahl, L., Mortimer, D., Barratt, C. L. R., Castilla, J. A., Menkveld, R., Kvist, U., Alvarez, J. G., & Haugen, T. B. (2010). Basic semen analysis. In *A practical guide to basic laboratory andrology*. New York: Cambridge University Press.
- Bjorndahl, L. (2010). Semen analysis: Essentials for the clinician. Clinical evaluation and treatment of male factor infertility. In D. T. Carrell, & C. M. Peterson (Eds.), *Reproductive endocrinology and infertility integrating modern clinical and laboratory practice*. New York: Springer.
- Castilla, J. A., Alvarez, C., Aguilar, J., Gonzalez-Varea, D., Gonzalvo, M. C., & Martinez, L. (2006). Influence of analytical and biological variation on the clinical interpretation of seminal parameters. *Human Reproduction*, 21(4), 847–851.
- Centola, G. M. (1996). Basic semen analysis. In G. M. Centola, & K. A. Ginsburg (Eds.), *Evaluation and treatment of the infertile male*. New York: Cambridge University Press.
- Centola, G. M., & Jeyendran, R. S. (2009). Strict criteria for sperm morphology. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Centola, G. M. (2011). Semen: Analysis and processing. In C. Niederberger (Ed.), *An introduction to male reproductive medicine*. New York: Cambridge University Press.
- Guzick, D. S., Overstreet, J. W., Factor-Litvak, P., Brazil, C. K., Nakajima, S. T., Coutifaris, C., Carson, S. A., Cisneros, P., Steinkampf, M. P., Hill, J. A., Xu, D., Vogel, D. L., & National Cooperative Reproductive Medicine Network. (2001). Sperm morphology, motility and concentration in fertile and infertile men. *The New England Journal of Medicine*, 345(19), 1388–1393.
- Jones, D. M., Kovacs, G. T., Harrison, L., Jennings, M. G., & Baker, H. W. (1986). Immobilization of sperm by condoms and their components. *Clinical Reproduction and Fertility*, 4(6), 367–372.
- Katz, D. F. (1991). Human sperm as biomarkers of toxic risk and reproductive health. *Journal of NIH Research*, 3, 63–67.
- Keel, B. A. (2006). Within and between subject variation in semen parameters in infertile men and normal semen donors. *Fertility and Sterility*, 85, 128–134.
- Kruger, T. F., Acosta, A. A., Simmons, K. F., Swanson, R. J., Matta, J. F., & Oehninger, S. (1988). Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertility and Sterility*, 49(1), 112–117.
- Lewis, S. E. (2007). Is sperm evaluation useful in predicting human fertility? *Reproduction*, 134(1), 31–40.
- Mortimer, D. (1994). *Practical Laboratory Andrology*. New York: Oxford University Press.
- Nagler, H. M., & Grotas, A. B. (2009). Varicocele. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Niederberger, C. (2011). Clinical evaluation of the infertile male. In C. Niederberger (Ed.), *An introduction to male reproductive medicine*. New York: Cambridge University Press.
- Oates, R. A. (2012). Evaluation of the azoospermic male. *Asian Journal of Andrology*, 14(1), 82–87.
- Oates, R. D., & Lamb, D. J. (2009). Genetic aspects of infertility. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Poch, M. A., & Sigman, M. (2010). Clinical evaluation and treatment of male factor infertility. In D. T. Carrell, & C. M. Peterson (Eds.), *Reproductive endocrinology and infertility integrating modern clinical and laboratory practice*. New York: Springer.
- Rothmann, S. A., & Reese, A. A. (2009). Semen analysis. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Sigman, M., Lipshultz, L. I., & Howards, S. S. (2009). Office evaluation of the subfertile male. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Sikka, S. C., & Hellstrom, W. J. G. (2016). Current updates on laboratory techniques for the diagnosis of male reproductive failure. *Asian Journal of Andrology*, 18(3), 392–401.
- Turner, T. (2009). The epididymis and accessory sex organs. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- WHO. (1999). *World Health Organization laboratory manual for the examination of human semen and sperm cervical mucus interaction* (3rd edn.). Cambridge: Cambridge University Press.
- WHO. (2010). *World Health Organization WHO laboratory manual for the examination and processing of human semen* (5th edn.). Cambridge: Cambridge University Press.

Laboratory Evaluation of Bulk Seminal Parameters

Grace M Centola, Reproductive Laboratory and Tissue Bank Consultant, Port St. Lucie, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The semen analysis is the initial test for evaluation of the male partner. It is an important test which provides the clinician with useful information on the fertility potential of the male partner, but is not a test of fertility (Centola, 1996, 2011). The routine semen analysis is an accurate measurement of sperm production and maturation, and the ejaculatory process (Centola, 1996; Lewis, 2007; Sikka and Hellstrom, 2016). Sperm fertility, that is, ability to fertilize an egg and subsequent processes cannot be established by a routine semen analysis, and therefore more advanced tests must be used to determine fertility potential (Lewis, 2007; Sikka and Hellstrom, 2016). A semen analysis should be performed by an andrology laboratory, which specializes in semen analysis and advanced sperm testing. The basic techniques of semen analysis established over 50 years ago are still used for routine practice (Pacey, 2012). Most recently home sperm analysis kits, which provide information on the presence of sperm in the ejaculate, are available at most independent pharmacies.

A minimum of two separate specimens, provided at appropriate intervals should be analyzed to account for inherent specimen variation (WHO, 1999, 2010; Poch and Sigman, 2010; Sigman et al., 2009). There is large biological variation in semen quality which is largely uncontrollable, and explains the individual variations seen between and within males (Keel, 2006; Castilla et al., 2006; WHO, 2010). Method of collection, abstinence period, and circumstances of delivery to the laboratory are important for the laboratory results to be accurate and reliable (Centola, 1996, 2011; Bjorndahl, 2010; Bjorndahl et al., 2010; Sigman et al., 2009). Standardized methods for semen analysis are part of good laboratory practice and include quality control, proficiency testing, and technician competency evaluations (Castilla et al., 2006; Bjorndahl, 2010; Drobnis, 2016).

Laboratory Evaluation

The results of semen analyses are influenced by many factors. The clinical laboratory must provide specific instructions regarding abstinence period, method of collection, and holding temperature to minimize inaccurate analyses. It is extremely important that the specimen be collected properly, methods standardized, and results properly interpreted (Sigman et al., 2009; Bjorndahl et al., 2010; Centola, 2011; Agarwal et al., 2016a). The entire ejaculate should be collected into an aseptic or sterile specimen container provided by the laboratory. If any portion of the ejaculate is lost or not collected, it should be reported to the laboratory technician (WHO, 2010). The first part of the ejaculate is the sperm-rich part of the specimen; therefore, loss of this part will influence the results. Generally, 2–3 days abstinence from the last ejaculation is a standard abstinence time. Others have suggested an abstinence time of 2–7 days (WHO, 2010). Each laboratory must provide information regarding abstinence period in addition to method of collection. In most cases, a longer abstinence period may result in increased numbers of dead, dying, and nonmotile sperm, thus not an accurate picture of semen quality (Centola, 2011).

Specimens are collected using masturbation in a private room at the laboratory facility (Sigman et al., 2009; WHO, 2010). If a specimen can be delivered to the laboratory within a specified timeframe, a specimen can be produced off-site. Special nontoxic condoms might also be used by those who are not able to collect by masturbation (Sigman et al., 2009; Poch and Sigman, 2010). These condoms are available commercially or at some andrology laboratories. Ordinary latex condoms should not be used since they contain toxic chemicals that interfere with sperm motility (Jones et al., 1986; WHO, 2010). Coitus interruptus is not a reliable means to collect a specimen for analysis since the first part of the ejaculate is easily lost, and there may be cells and bacteria from the vaginal fluid contaminating the semen specimen (WHO, 2010).

If collected off site, the specimen must be delivered within 30–45 min of ejaculation. Specimens should be maintained at body temperature (preferably) or room temperature during transport to the laboratory. The specimen should not be exposed to extreme temperatures which will affect sperm motility (WHO, 2010). Upon receipt, the technician will place the properly labeled specimen container in a 37°C incubator or at room temperature until analysis.

Upon collection, the semen is a coagulated mass, but with time (usually within a maximum of 30–45 min post collection), the semen liquefies, becomes thinner, more liquid and watery (WHO, 2010). Liquefaction is facilitated by gentle mixing of the specimen container. The laboratory should note if the specimen is not completely liquefied by 1 h after collection. The semen analysis should begin soon after liquefaction, preferably 30 min after collection but not longer than 60 min to prevent decrease in sperm motility with prolonged exposure to the seminal fluid (WHO, 2010).

If the semen specimen remains as a coagulated mass by 1 h after collection, the laboratory can add buffered medium, perform repeated mixing or passage through a large bore syringe. Treatment with an enzyme such as bromelain dissolves the coagulum but may affect results (WHO, 2010). Such methods, and if effective or not, should be recorded on the laboratory report. The presence of gelatinous granules in the liquefied semen does not appear to have any clinical significance, although mucus strands may interfere with the analysis (WHO, 2010).

Initial Assessment

Semen consistency

Following liquefaction, the semen sample is aspirated into a wide-bore disposable pipette to determine the consistency or viscosity of the ejaculate. It is important to note that determining viscosity is very different from determining liquefaction. The semen is drawn into the disposable pipette and allowed to drop out of the pipette back into the container. If the sample demonstrates discreet drops, the viscosity is considered normal. However, if the semen does not form discreet droplets, falling as thick and stringy, there is increased viscosity. The viscosity can be rated on a scale (0 = normal to 4 = extremely viscous), or by using qualitative terms, such as normal, moderate, extreme. A viscous specimen interferes with accurate analysis of motility and concentration, but most importantly can inhibit contact with, and transport through, the female reproductive tract.

Semen color

The normal ejaculate appears as gray to white and opaque. A clear ejaculate may indicate a reduced sperm concentration or azoospermia (Centola, 2011). The presence of red blood cells will cause the semen to appear red-brown or pink. Bacterial infection may cause the semen to appear green or yellow, and certain medications or vitamins may also turn the semen a yellow color (WHO, 2010; Centola, 2011). The next steps in the semen analysis are the measurement of the semen volume or amount, the sperm count, motility, and normal forms or morphology.

Semen volume

The fluid of the ejaculate is contributed by the accessory sex glands, primarily the prostate gland and the seminal vesicles, with a small amount from the bulbourethral glands and epididymus (Bjorndahl et al., 2010; WHO, 2010). Approximately 10% of the ejaculate volume is contributed by the testicles, 70% by the seminal vesicles, and 20% from the prostate gland. The seminal vesicles provide alkaline fluid, while the prostate gland provides acidic fluid (Oates, 2012). The semen volume is measured by pipetting the semen into a graduated serological pipette (Rothmann and Reese, 2009; Bjorndahl, 2010) or use of other types of laboratory measuring devices such as graduated cylinders or graduated centrifugation tubes (Bjorndahl, 2010). Due to inherent inaccuracies with such methods, obtaining a weight of both the empty specimen container and the container with semen is considered by some to be a preferred method to determine volume (Bjorndahl, 2010; WHO, 2010). The density of the semen is consistent at 1 g/mL; therefore, the weight of the empty container is subtracted from the (heavier) weight of the container with semen, resulting in the semen volume (WHO, 2010). The lower reference limit for semen volume is 1.5 mL (1.4–1.7) (WHO, 2010); others report a range of 1.5–6.0 mL (Sikka and Hellstrom, 2009).

Semen pH

Measurement of semen pH is no longer routinely performed, particularly if sperm are present (Rothmann and Reese, 2009; Centola, 2011; Oates, 2012). Semen pH varies with time after ejaculation and exposure to ambient air. Therefore, if performed, the pH should be measured as soon as possible after liquefaction. The lower reference value for semen pH is 7.2 (WHO, 2010).

Microscopic Assessment

Following the above measurements, the semen is thoroughly mixed by swirling of the specimen cup and/or drawing into and expelling out of a disposable pipette. A drop of semen is placed on a clean glass slide and covered with a thin glass coverslip. The slide is examined for the presence of sperm agglutination or clumping, bacteria, and the presence of other types of cells such as round cells and white blood cells (Rothmann and Reese, 2009; Bjorndahl, 2010; WHO, 2010; Agarwal et al., 2016a,b,c). The type of round cells can be classified by examining a stained slide (WHO, 2010).

Sperm motility and sperm concentration

Sperm concentration refers to the number of sperm per unit volume (milliliter) of semen and the total sperm count is the total number of sperm in the entire ejaculate (sperm concentration times semen volume) (Sigman et al., 2009; WHO, 2010). Sperm concentration is determined using specialized counting chambers such as the improved Neubauer hemocytometer, a Makler chamber, or disposable slide chambers (Mortimer, 1994; Centola, 2011; Bjorndahl, 2010; WHO, 2010; Agarwal et al., 2016a). The hemocytometer was used for many years to determine sperm count and still is used in many hospital laboratories. The hemocytometer, however, required that an aliquot of the semen be fixed so that the total number of sperm can be counted. The need for performing these counts in duplicate requires additional labor and time to clean and load the chamber, introducing variations, and errors (Rothmann and Reese, 2009).

Specialized chambers for determining sperm count have been developed and are routinely used in the andrology laboratory. The counting chambers do not require dilution and have a depth appropriate for sperm allowing determination of motile and nonmotile sperm on the same chamber (Rothmann and Reese, 2009; Sikka and Hellstrom, 2016). The disposable slides have two built-in chambers for replicate counts (Seaman et al., 1996; Brazil et al., 2004; Agarwal et al., 2016a,b,c). The Makler chamber is a reusable chamber for concurrent measurement of sperm count and motility (Makler, 1980; Seaman et al., 1996). The Makler chamber requires precise specimen loading and handling and is cleaned following each use. The numbers of motile and nonmotile sperm are recorded within the boundaries of the slide grid, and the count and motility calculated.

A minimum of 200 spermatozoa should be counted, although for low count specimens, counting of 100 sperm may be used. Highly concentrated specimens need dilution to accurately determine the sperm count and motility. However, with experienced technicians, the count and motility can be estimated, and this might be sufficient for the clinician to formulate a treatment plan. A report of “greater than 50 or 100 million sperm” might be acceptable to the clinician.

Determination of accurate sperm count in specimens with severely low concentration can be inaccurate and potentially misleading. In such specimens, a standard reporting of “< 1 million sperm/mL” can be used, with notation of percent and quality of motility. When few, rare or no spermatozoa are seen on a wet mount slide, the specimen should be centrifuged to determine if any sperm are present (WHO, 2010). In these cases, a report might indicate “few sperm seen,” or “1 per high power field,” for example, which may be enough information for the clinician to formulate a plan.

There are advantages and disadvantages to the sperm counting chambers. However, it is important to validate any chamber the laboratory chooses to use and to perform daily quality control for each chamber type. Significant variability between chambers has been reported, emphasizing the need for laboratory standardization and quality control (Seaman et al., 1996; Rothmann and Reese, 2009).

The lower reference limit for sperm concentration is 15 million sperm per milliliter (range 12–16) (WHO, 2010).

Sperm motility should be evaluated immediately after semen liquefaction, usually within 30–45 min of ejaculation (WHO, 2010). The semen specimen is mixed well, and a drop is immediately removed and placed on a clean glass slide. If the disposable or Makler chamber is used, the chamber is loaded and both the sperm count and motility can be concurrently determined (see above). The numbers of motile sperm and the numbers of nonmotile sperm are counted out of at least 200 sperm (Bjorndahl, 2010; WHO, 2010; Agarwal et al., 2016a,b,c). The percentage motility is determined by dividing the number of motile sperm by the total number (motile + nonmotile) counted, times 100%.

To account for sampling errors and heterogeneity of the sample, the sperm motility and the sperm count should be determined in separate duplicate samples on two different slides (Bjorndahl, 2010; Sikka and Hellstrom, 2016). If the replicates are >10% different, a third slide should be examined. The most difficult part is to determine the moving sperm especially in a high count specimen. Laboratory technicians are rigorously trained to recognize the motile sperm and to count each sperm only once which can be difficult when motile sperm are rapidly moving all over the microscopic field.

Motility is assessed in four categories—nonmotile, motile but nonprogressive, slowly progressive, and rapidly progressive (Mortimer, 1994; Bjorndahl, 2010; WHO, 2010; Sikka and Hellstrom, 2016). Progressive motility is defined as progressive movement usually in a linear direction (straight progression) or in a large circle, regardless of speed of progression (WHO, 2010). Motile but nonprogressive movement is seen with slow, sluggish, or weak forward movement (Mortimer, 1994). Rapid progression is defined as those sperm moving in a rapid linear movement. Different number (0–4) or letter (a–d) scales may be used by laboratories to determine the quality of the movement in each category, although this is difficult to define accurately without bias (Mortimer, 1994; WHO, 2010). After counting all motile sperm in each category, the percentages of each can be determined. The total percent motility with a motility scale and the sperm count are then reported.

The lower reference limit for total motility is 40% and for progressive motility is 32% (WHO, 2010).

Sperm vitality or viability

Sperm vitality or viability is an assessment of the live versus dead sperm based on a dye exclusion method. In a specimen that shows a good motility, the assumption is made that the motile sperm are viable (Bjorndahl, 2010), and performing a vitality stain might not be necessary.

The live/dead staining is important to be performed for specimens that present with <40% progressively motile sperm (WHO, 2010). In such a specimen, the question arises if the nonmotile sperm are alive, but not motile, and could potentially be used for assisted reproduction. Vitality assessment is also a check on accuracy of motility measurement, and allows the lab to distinguish complete immobility due to defects in the sperm midpiece or tail (Mortimer, 1994; Bjorndahl, 2010). Vitality is assessed using a dye exclusion method, where the live sperm with intact membranes do not take up the dye and the dead sperm with damaged membranes take up the stain (Mortimer, 1994; Bjorndahl, 2010; WHO, 2010). Live cells can be assessed using several vital dyes, which include eosin Y, trypan blue, or a fluorochrome Hoeschst 33258 (Mortimer, 1994; WHO, 2010). The most commonly used technique is the eosin staining method, which includes a counterstain, nigrosin, which allows an easier way to distinguish the background and the sperm heads (WHO, 2010; Agarwal et al., 2016c).

After the specimen is mixed, a small amount is removed, and mixed with the stain in a small test tube. After mixing, a drop is smeared on a clean glass slide and allowed to air dry. The cells stained pink are dead, and the unstained clear cells are live cells. Replicate slides are prepared and assessed, and the mean is reported as percent live sperm.

A second, less common routine vitality test is the hypoosmotic swelling (HOS) test, which assesses the ability of the sperm cells to swell in a special hypoosmotic buffer (Mortimer, 1994). The HOS test is especially helpful when choosing sperm for ICSI because no toxic stain or fixative is used (WHO, 2010). When sperm are mixed with the hypoosmotic buffer, those with intact membranes show swelling in the tail and midpiece sections (WHO, 2010).

The lower reference limit for vitality using either method is 58% (range 55–63) (WHO, 2010).

Sperm morphology

Assessment of sperm morphology remains an important part of the routine semen analysis. While some report sperm morphology as predictive of outcome in assisted reproduction, others question its relevance (Centola, 1996, 2011; Centola and Jeyendran, 2009;

Rothmann and Reese, 2009; Bjorndahl, 2010). The percentage of abnormal sperm and the abnormalities can assist the physician in determining a treatment regimen for the infertile couple (Centola and Jeyendran, 2009).

Sperm morphology is one of the most confusing and difficult parts of the semen analysis (Rothmann and Reese, 2009; Bjorndahl, 2010; Sikka and Hellstrom, 2016). There are many factors which contribute to this confusion, including but not limited to variation between laboratories, variation between technicians within a lab, multiple classification methods, multiple slide preparation and staining methods, not to mention the variability in the sperm cells themselves.

A number of stains have been used to prepare a morphology slide, including the Papanicolaou stain, Wright-Giemsa, and Diff-Quick stains, some of which are sold in kit form (Mortimer, 1994; Centola, 2011; Centola and Jeyendran, 2009; Bjorndahl, 2010; Rothmann and Reese, 2009; WHO, 2010; Agarwal et al., 2016b). An accurate morphology cannot be obtained using an unstained wet mount slide (Centola and Jeyendran, 2009). By preparing a stained slide of a semen smear, the structure of the sperm cells can be observed under the microscope. A simple normal or abnormal classification with optional tally of the types of abnormalities is the most recommended method of determining sperm morphology (WHO, 2010).

Several classification methodologies are used to determine morphology. These include the WHO methods (WHO, 1999, 2010) and Strict methods (Menkveld et al., 1990; Kruger et al., 1988; Kruger and Franken, 2004; Agarwal et al., 2016b).

A minimum of 100 sperm are observed at high magnification on a stained slide. Only sperm with a tail are counted. The technician then rates each sperm cell as normal or abnormal. Most types of abnormal sperm are easily recognized, the difficulty arises in deciding that a normal appearing sperm cell is actually normal. Each sperm cell must show a uniform, smooth, and oval head; a regular and slender midpiece; and a thin tail which is estimated at 10 times the head length (WHO, 2010). Defective sperm include large, small, tapered or irregularly shaped head; bent or thin midpiece; and short, broken, coiled, or multiple tails.

The strict classification of sperm morphology uses stricter criteria to determine a normal sperm. A microscope eyepiece reticle is used to measure the head, midpiece, and tail. Each part of the sperm must be uniform and precise in size, with no defects. A normal sperm is one which conforms to four strict criteria (Kruger et al., 1988; Kruger and Franken, 2004; Centola and Jeyendran, 2009; Agarwal et al., 2016b). Any sperm cells not conforming to the four criteria, including borderline or questionable, are classified as abnormal when using strict criteria. Earlier versions of the WHO manual classified borderline as normal, while later versions classified such as abnormal (WHO, 1999, 2010).

It takes significant training and experience to reliably assess sperm morphology in an accurate and reproducible way. The normal reference value for sperm morphology is 4% (range 3.0–4.0) (WHO, 2010). Strict criteria reference ranges are as follows: <4% normal, severely impaired function; 4%–14% normal, questionably impaired; > 14% normal, potentially normal fertility (Kruger et al., 1988; Kruger and Franken, 2004; Centola and Jeyendran, 2009; Agarwal et al., 2016b).

Conclusion

Routine semen analysis provides significant information to the clinician managing the infertile couple. Information on sperm production and maturation, including morphology, allow the physician to potentially diagnose the cause of infertility or at least request additional testing of both partners. Andrology testing is classified as high complexity testing and requires trained staff with appropriate education (Drobnis, 2016). In order for a laboratory to provide reliable and precise information, each lab must establish and validate the methodology used and participate in proficiency testing to insure all staff are proficient in these methods. Furthermore, laboratory state and federal licensing, and, internal quality assurance, and improvement processes are essential (Centola, 2016).

References

- Agarwal, A., Gupta, S., & Sharma, R. (2016a). Basic semen analysis. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility*. Switzerland: Springer International Publishing.
- Agarwal, A., Gupta, S., & Sharma, R. (2016b). Sperm morphology stain (Diff-Quik). In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility*. Switzerland: Springer International Publishing.
- Agarwal, A., Gupta, S., & Sharma, R. (2016c). Eosin-Nigrosin staining procedure. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility*. Switzerland: Springer International Publishing.
- Bjorndahl, L. (2010). Semen analysis: Essentials for the clinician. Clinical evaluation and treatment of male factor infertility. In D. T. Carrell, & C. M. Peterson (Eds.), *Reproductive endocrinology and infertility integrating modern clinical and laboratory practice*. New York: Springer.
- Bjorndahl, L., Mortimer, D., Barratt, C. L. R., Castilla, J. A., Menkveld, R., Kvist, U., Alvarez, J. G., & Haugen, T. B. (2010). Basic semen analysis. In *A practical guide to basic laboratory andrology*. New York: Cambridge University Press.
- Brazil, C., Swan, S. H., Tollner, C. R., Treece, C., Drobnis, E. Z., Wang, C., Redmon, J. B., & Overstreet, J. W. (2004). Study for Future Families Research Group. Quality control of laboratory methods for semen evaluation in a multicenter research study. *Journal of Andrology*, 25(4), 645–656.
- Castilla, J. A., Alvarez, C., Aguilar, J., Gonzalez-Varea, D., Gonzalvo, M. C., & Martinez, L. (2006). Influence of analytical and biological variation on the clinical interpretation of seminal parameters. *Human Reproduction*, 21(4), 847–851.
- Centola, G. M. (1996). Basic semen analysis. In G. M. Centola, & K. A. Ginsburg (Eds.), *Evaluation and treatment of the infertile male*. New York: Cambridge University Press.
- Centola, G. M. (2011). Semen: Analysis and processing. In C. Niederberger (Ed.), *An introduction to male reproductive medicine*. New York: Cambridge University Press.
- Centola, G. M. (2016). Licensing and accreditation of the andrology laboratory. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility*. Switzerland: Springer International Publishing.

- Centola, G. M., & Jeyendran, R. S. (2009). Strict criteria for sperm morphology. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Drobnis, E. (2016). Competency assessment in andrology laboratory. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility*. Switzerland: Springer International Publishing.
- Jones, D. M., Kovacs, G. T., Harrison, L., Jennings, M. G., & Baker, H. W. (1986). Immobilization of sperm by condoms and their components. *Clinical Reproduction and Fertility*, 4(6), 367–372.
- Keel, B. A. (2006). Within and between subject variation in semen parameters in infertile men and normal semen donors. *Fertility and Sterility*, 85, 128–134.
- Kruger, T. F., & Franken, D. R. (2004). *Atlas of human sperm morphology evaluation*. London: Taylor and Francis.
- Kruger, T. F., Acosta, A. A., Simmons, K. F., Swanson, R. J., Matta, J. F., & Oehninger, S. (1988). Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertility and Sterility*, 49(1), 112–117.
- Lewis, S. E. (2007). Is sperm evaluation useful in predicting human fertility? *Reproduction*, 134(1), 31–40.
- Makler, A. (1980). The improved ten-micrometer chamber for rapid sperm count and motility evaluation. *Fertility and Sterility*, 33, 337–338.
- Menkveld, R., Stander, F. S. H., Kotze, T. J., Kruger, T. F., & van Zyl, J. A. (1990). The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Human Reproduction*, 5, 586–592.
- Mortimer, D. (1994). *Practical laboratory andrology*. New York: Oxford University Press.
- Oates, R. A. (2012). Evaluation of the azoospermic male. *Asian Journal of Andrology*, 14(1), 82–87.
- Pacey, A. A. (2012). Assessment of male factor. *Best Practice and Research: Clinical Obstetrics and Gynaecology*, 26, 739–746.
- Poch, M. A., & Sigman, M. (2010). Clinical evaluation and treatment of male factor infertility. In D. T. Carrell, & C. M. Peterson (Eds.), *Reproductive endocrinology and infertility integrating modern clinical and laboratory practice*. New York: Springer.
- Rothmann, S. A., & Reese, A. A. (2009). Semen analysis. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Seaman, E. K., Goluboff, E., BarChama, N., & Fisch, H. (1996). Accuracy of semen counting chambers as determined by use of latex beads. *Fertility and Sterility*, 66(4), 662–665.
- Sigman, M., Lipshultz, L. I., & Howards, S. S. (2009). Office evaluation of the subfertile male. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Sikka, S. C., & Hellstrom, W. J. G. (2009). Tests for antisperm antibodies. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn.). New York: Cambridge University Press.
- Sikka, S. C., & Hellstrom, W. J. G. (2016). Current updates on laboratory techniques for the diagnosis of male reproductive failure. *Asian Journal of Andrology*, 18(3), 392–401.
- World Health Organization. (1999). *WHO laboratory manual for the examination of human semen and sperm cervical mucus interaction* (4th edn.). Cambridge: Cambridge University Press.
- World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th edn.). Cambridge: Cambridge University Press.

Further Reading

- Katz, D. F. (1991). Human sperm as biomarkers of toxic risk and reproductive health. *Journal of NIH Research*, 3, 63–67.

CASA—Computer-Aided Sperm Analysis

Sharon T Mortimer, Oozoa Biomedical, West Vancouver, BC, Canada
Christopher J De Jonge, University of Minnesota, Minneapolis, MN, USA

© 2018 Elsevier Inc. All rights reserved.

Background

The most distinctive attributes of the mature sperm cell are its streamlined shape and its ability to move independently. This motility is critical for a spermatozoon to not only reach the site of fertilization in the woman's oviduct, but also to negotiate the layers of cumulus cells surrounding the oocyte, and then to penetrate the zona pellucida in order to fertilize the oocyte.

Perhaps surprisingly, not all human spermatozoa are motile, and because it is important for fertility, sperm motility is therefore an integral part of any clinical semen analysis. Even the most basic semen analysis provides an estimate of the proportion of sperm that are motile (i.e. show at least some movement of the tail), while more sophisticated analyses also report the proportion with progressive motility (i.e. able to move from one location to another). There is a relationship between the proportion of progressively motile spermatozoa in a semen sample and the likelihood of that man being fertile. There is also evidence that the concentration of seminal spermatozoa with certain movement patterns (kinematic parameters) is related to the same endpoint.

Determining the relative speed of movement of spermatozoa can be achieved in real-time by highly-trained laboratory scientists, but determining the kinematics of each sperm trajectory requires some sort of image capture and analysis. Historically, this was achieved with the use of photo- and cine-micrographic recordings of sperm movement. The film was then replayed slowly and the position of each sperm cell in successive frames traced onto paper. Although motility, and therefore the spermatozoon's trajectory, is a product of the movement of the sperm tail, it was the position of the sperm head that was traced, as it is the most prominent feature of the spermatozoon and the most likely to stay in focus. Once the trajectory of each spermatozoon in the image had been reconstructed, the tracings were analyzed to determine the speed of movement of each cell, and to derive other kinematic values (e.g. [Katz and Dott, 1975](#); [David et al., 1981](#)). As videomicrography became more accessible, recordings of sperm movement were made on videotape, and the tracks reconstructed manually on plastic sheets attached to the video monitor.

The kinematic values that are most commonly used today were developed by the research group in Kremlin-Bicetre in Paris ([David et al., 1981](#)). As the field expanded, a range of definitions and terminologies were developed for these kinematic values, leading to a significant amount of confusion. In a series of consensus meetings (reported in [Mortimer, 1990](#)), standardized terminology and definitions were developed and these were adopted by the World Health Organization for presentation in their laboratory manuals since 1992 ([Fig. 1](#)). These kinematic values are:

- Curvilinear velocity (VCL; $\mu\text{m/s}$), the length of the actual path followed by the spermatozoon as a function of time, reported to 1 decimal place;
- Average-path velocity (VAP; $\mu\text{m/s}$), the length of the smoothed path followed by the spermatozoon as a function of time, reported to 1 decimal place;
- Straight-line velocity (VSL; $\mu\text{m/s}$), the "net space gain" (distance between the first and last track points) as a function of time, reported to 1 decimal place;
- Linearity ($\text{LIN} = \text{VSL}/\text{VCL} \times 100$; %), a comparison of the lengths (and therefore relative shapes) of the straight-line and curvilinear paths, reported as an integer percentage;
- Straightness ($\text{STR} = \text{VSL}/\text{VAP} \times 100$; %), a comparison of the lengths (and therefore relative shapes) of the straight-line and average paths, reported as an integer percentage;
- Wobble ($\text{WOB} = \text{VAP}/\text{VCL} \times 100$; %), a comparison of the lengths (and therefore shapes) of the average and curvilinear paths, reported as an integer percentage;
- Amplitude of lateral head displacement (ALH; μm), the full width of the curvilinear path (rather than a true amplitude), reported as either a mean or maximum value, to 1 decimal place; and
- Beat cross frequency (BCF; Hz), a de facto expression of the flagellar beat frequency, calculated as the number of times the curvilinear path crosses the average path as a function of time, reported to 1 decimal place.

Originally, the kinematic values were derived empirically for each manually-reconstructed sperm trajectory, meaning that any experiment requiring sperm movement analysis involved many hours of labor-intensive track reconstruction and analysis.



Fig. 1 Kinematic values. Sperm head trajectory showing the derivation of the paths used in velocity calculations (i.e. for VCL, VAP, and VSL) as well as for the ratios (LIN, STR, and WOB) and beat-cross frequency (BCF). Track width is indicated, demonstrating how ALH is determined. Explanations for each of these terms is given in the text.

However, the development of the personal computer in the 1980s and the arrival of digitizer cards meant that these processes could be automated, with data delivered in a fraction of the time. These advances led to the development of commercial CASA instruments. Although there is a range of CASA instruments available now, they all share the general configuration of an optics system (either a stand-alone microscope or microscope components built into the instrument itself), a camera, a more-or-less stand-alone computer (incorporating a digitizer if an analog camera is used), and a display screen. For integrated systems, such as the Hamilton Thorne IVOS, the computer system also has I/O (i.e., input/output) functions to control features such as specimen temperature, illumination intensity, etc.

Technology

The first commercial CASA instruments were developed by CellSoft (Cryo Resources, New York, NY, USA) and by Hamilton-Thorn Research (Beverly, MA, USA) in the mid-1980s. The general concepts were similar for both instruments, with a 0.5–1 s video grab of sperm movement down the microscope (representing 15–30 consecutive images, assuming an image sampling frequency of 30 Hz), followed by identification of each sperm cell in the field of view, reconstruction of each sperm track (based on the movement of the sperm head), and derivation of the kinematic values for each track. Image sampling frequency refers to the number of images per second that are analyzed, expressed as Hz. The oldest CASA systems used 25 or 30 Hz, depending on the video system used (PAL or NTSC), rather than 50 or 60 Hz, the minimum image sampling frequency to describe the trajectory effectively (Mortimer et al., 1988).

The resolution of the images at that time was not good, and so there were problems with other cells, or debris, being identified as sperm cells. In addition, the computing power was quite low, so in this first generation of CASA instruments it still took several minutes (or even longer) for the reconstruction and kinematic analysis steps for each field.

Over the next 30 years, improvements in computing power and image acquisition have been reflected in the development of vastly improved CASA instruments. Now, it is possible to acquire 60 images per second and with almost instantaneous assessment of the kinematic values for each cell in the field. Significantly faster camera “shutter speeds” mean it is also now possible to image the sperm tail, so it might not be too much longer before it is possible to make direct assessments of flagellar movement, rather than relying on assessment of sperm head movement as a proxy.

In the meantime, we continue to rely upon sperm head assessment as the general approach for routine CASA assessments. To ensure that the sperm head can be imaged successfully, negative-high (NH) phase contrast optics are used when acquiring the images for assessment. NH optics show the sperm head as a white dot against a black background, so it is much more obvious. Once the machine has identified a white dot, it determines whether it is a sperm head based on dimensions pre-set by the user (the “gates”). In the most modern instruments, the machine also performs a perimeter assessment to identify a tail, which gives further confirmation that this is, in fact, a sperm head. Once each sperm cell in the field of view has been identified, the program then sets a zone of probability around each sperm head, which is a user-defined estimate of the maximum distance it would be likely (or possible) for the sperm head to travel from one image to the next (Fig. 2). When the next image in the sequence is assessed, the program identifies the most likely relationship between the sperm heads in the previous image and the sperm heads in the current image, and then connects the images to create a path for each spermatozoon. This is repeated for all of the images acquired in the sequence, and then the kinematic values are calculated for each trajectory.

There are some problems that can arise with this approach. For example, if cells enter or leave the field during the sequence, how can these trajectories be assessed? For cells that leave the field, it is possible to set a minimum number of images that are assessed before a trajectory can be included in the analysis, so any that meet this criterion could still be included. For cells that enter the field after the first image has been acquired, their trajectories are not included in the assessment, as there was no zone of probability established in the initial sequence analysis.

Similarly, in the earlier versions of CASA, if two or more cells were in the same zone of probability, it was not possible to be certain whether the correct trajectory would be followed, or whether different part-trajectories had been joined up in error. The more modern systems have solutions for this problem, including vector analysis of trajectories, relying on the assumption that each spermatozoon is more likely to continue in its established direction of travel. Of course, this is not necessarily going to be true if the sperm cells collide with each other, as the collision itself could force a change in direction. This potential problem is one of the reasons that there is a restriction on the sperm concentration in samples to be assessed by CASA, typically in the range of 60–80 million/mL (depending on the CASA instrument).

In addition to sperm concentration, there are several other technical factors that can affect the accuracy and applicability of CASA results, including chamber depth and image sampling frequency. Ideally, all the spermatozoa in the original field of view will remain in focus for as long as they stay in the field. If a sperm head were to go out of focus, the apparent change in the size of the head, or the brightness or contrast, could cause it to fall outside the expected limits, and the CASA instrument would truncate the track at that point. Choosing the magnification to use is a balance between ensuring the image is large enough to allow discrimination between sperm heads and other cells or debris, while maintaining a sufficient depth of focus. For human sperm samples, a 10× objective is typically used, due to the relatively small size of the sperm head, but this restricts the chamber depth to around 20 µm to ensure the spermatozoa remain in focus. Sperm in semen have a relatively low-amplitude flagellar wave, so 20 µm depth does not restrict their movement patterns. However, spermatozoa that have been removed from seminal plasma and washed into culture medium have a much broader range of flagellar movement, with amplitudes of up to 10 µm (i.e., 20 µm from peak to trough). In this case, a preparation depth of 30 µm would be preferable, to reduce the risk of contact

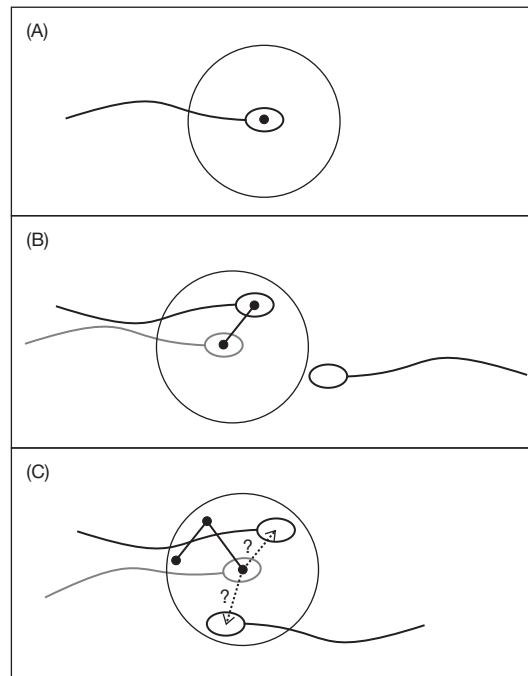


Fig. 2 Zone of probability. Once the CASA instrument has identified a sperm cell, it assigns a centroid at the center of the sperm head (shown as a black dot), and defines a “zone of probability” for where the sperm cell is expected to be in the next image (A). The CASA instrument then checks the next image to determine whether a sperm head is in the zone of probability. When it locates one, it assigns a centroid and a trajectory is started (B). If there is more than one sperm head in the zone of probability (C), the CASA instrument must either determine which is the more likely to be the sperm cell that is being tracked (for example via vector analysis), or truncate the trajectory at that point, with the associated risk of losing that trajectory from the analysis.

between the sperm tail and the chamber walls, but this requires the user to confirm whether too many sperm tracks were being truncated.

Given all of the technological requirements for effective CASA, it is not surprising that quality control is a critical aspect in ensuring that the instrument is working properly and that the results obtained are reproducible. In addition, some certification and licensing agencies require that quality control procedures be performed every day before any other assessments are done. For example, variations in the illumination intensity and in the gate sizes used to define the size of the sperm head can affect the ability of the CASA instrument to identify sperm correctly, which is a major factor in ongoing QC/QA. There are a number of solutions for this type of assessment, including the use of beads or fixed sperm suspensions of known concentration, or the analysis of standard recordings of sperm with known concentration and motility values, or the manual inspection of individual CASA-derived trajectories of spermatozoa from fresh semen samples to ensure that the instrument is correctly identifying all of the sperm cells in the field of view. Each approach has advantages and disadvantages, so the approach chosen for each lab should address the aspects of CASA assessment that are the most relevant to the outcomes being reported.

Because CASA supports the assessment of thousands of individual sperm trajectories, a large amount of data are generated for each sample. To save the user from needing to interpret all of this information, most CASA instruments report sample-averaged values in a single screen. While this is of value for sample-related sperm parameter values, such as concentration, % motile, and concentration of progressively motile spermatozoa, it is not useful for the kinematic parameters, as statistically they are not normally distributed. Indeed, each kinematic parameter usually has a wide range of values within a sample, so simply presenting the mean does not provide any useable information. Even worse, if users take the average of the mean kinematic values collected over several microscope fields, the standard deviation (SD) will be very low, thereby accidentally hiding any indication of variability in the measures. As a result, international consensuses have consistently called for kinematic values to be reported as either median and range or as the proportion of sperm tracks that meet pre-determined threshold values, such as the proportion of motile spermatozoa with $VCL \geq 100 \mu\text{m/s}$ (e.g. [ESHRE Andrology Special Interest Group, 1998](#)). Presenting the kinematic data in this way allows meaningful comparisons between test and control samples, and provides a better indication of the effect of an intervention on sperm function.

Research Applications

CASA is an extremely powerful tool for studying changes in sperm motility in response to experimental interventions, such as exposure to pharmaceuticals or to environmental toxicants, and in reproductive epidemiology studies ([Perreault, 2002](#)). In these applications, researchers are able to determine the relative effect of the exposures on the proportion of progressively motile sperm in a sample, as well as on aspects of sperm function, such as hyperactivated motility.

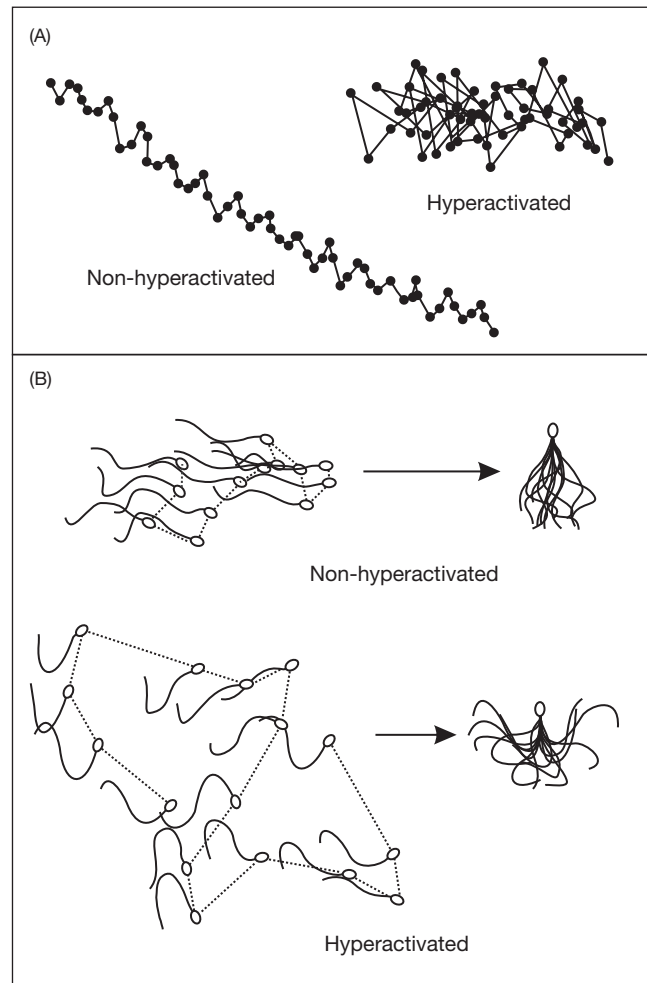


Fig. 3 Flagellar and head traces (HA vs non-HA). (A) Demonstrates the difference between the sperm head trajectories of a non-hyperactivated spermatozoon and a hyperactivated spermatozoon, drawn using the same magnification. Although CASA follows the sperm head, it is the sperm tail wave that determines the head trajectory. (B) Shows the difference between the tail movement patterns of hyperactivated and non-hyperactivated spermatozoa, as traced (left side of panel), and in a composite (right side of panel), drawn using the same magnification.

Hyperactivated (HA) motility, first described by [Yanagimachi \(1970\)](#), is characterized by a whiplash-type of flagellar movement, and is a concomitant of sperm capacitation, a range of maturational changes that confer to the spermatozoon the capacity to fertilize an oocyte. HA is induced by one or more signaling factors present in the ampulla of the periovulatory female reproductive tract, triggering Ca^{2+} influx in the sperm flagellum via CatSper1 Ca^{2+} protein channels ([Suarez, 2008](#)), and there is evidence that HA motility is a critical factor in sperm-egg interaction (reviewed by [Mortimer, 1997](#)). There is a significant difference in the range of flagellar movement of HA and non-HA spermatozoa, and this translates to differences in the sperm head trajectories ([Fig. 3](#)). As a result, HA motility cannot be reliably identified by eye, so CASA is required. The complexity of the trajectories also means that there isn't any single kinematic parameter that can define HA motility reliably, and so Boolean arguments are used. The definition for HA motility in the rodent uses a combination of the kinematic parameters VSL, ALH and LIN ([Suarez 2008](#)), while the definitions for human spermatozoa use a combination of VCL, ALH and LIN, or VCL and D (fractal dimension) ([Mortimer et al., 1998, 2015](#)).

CASA is also of value in the objective assessment of sperm morphology, particularly in species with relatively large sperm heads. Abnormal morphology is an indication of perturbations in the development of spermatozoa in the testes, and so it can serve as a functional marker of exposure to reproductive toxicants, and a predictor of reduced fertility. This information is invaluable in research studies of the effects of new pharmaceuticals, and in environmental exposure studies.

Clinical Applications

Although the concept of CASA arose from the need to study sperm movement, there has long been a hope that CASA could provide a reliable, objective method of semen analysis for clinical diagnostic use. While this is already a standard approach for a range of

animal species, this has not been the case for human semen assessments. This difference is primarily due to the relatively homogeneous nature of semen from domesticated and other animal species, which contains mostly motile spermatozoa with few other cell types, and the very heterogeneous nature of human semen, which contains many immotile spermatozoa as well as a range of other cell types and debris. As a result, it is relatively simple for the CASA instrument to accurately identify and track sperm heads in animal semen, whereas in human semen there has been a high risk of mis-identification of sperm heads, with the associated risk of under- or over-estimating sperm concentration and % motile. However, if human semen analysis by CASA was used to estimate the concentration of progressively motile spermatozoa in the ejaculate, the results would be much more accurate.

This functional approach to semen analysis is based on the observation that semen samples that contained at least 25×10^6 progressively motile sperm/ml (defined as trajectories that met the criteria: VAP $> 25 \mu\text{m/s}$ AND STR $> 80\%$ AND ALH $> 2.5 \mu\text{m}$) were more likely to have normal results in sperm-cervical mucus penetration tests, while samples that did not meet these criteria had abnormal results (Mortimer et al., 1986). The ability of spermatozoa to penetrate cervical mucus is a critical factor in fertility in vivo in many species, including human. The mucus in the cervix represents a physical barrier to the entry of spermatozoa into the female reproductive tract, so spermatozoa that are unable to penetrate cervical mucus are therefore unable to participate in fertilization.

Future Applications of CASA

As the image acquisition capabilities and computing power increase, perhaps in concert with artificial intelligence that will support machine learning, there will be more applications for CASA in research and clinical settings. It is likely that these applications will predominantly be using fixed and stained spermatozoa, providing a fast, objective method for the assessment of whole-cell morphology/morphometry. Multiparametric evaluation of sperm morphology provides an index-based estimate of the average number of abnormalities per abnormal sperm, giving an indication of the quality of testicular function in sperm production. This type of evaluation requires the assessment of not only the sperm head, but also the midpiece and tail. Until now, imaging the midpiece and tail has been a challenge, but CASA instruments are now gaining this capability and so it will be possible in the near future to perform this assessment automatically and objectively once the results have been validated against reference methods.

Other assays that could be performed by CASA in the future include sperm vitality assessment and sperm chromatin dispersion, which provides an indication of sperm DNA integrity.

Another future application of CASA is the use of a smart phone to perform sperm motility and concentration assessments (Kanakasabapathy et al., 2017). Again, the results need to be verified against those obtained using reference methods, but it is possible that this use of technology will allow sperm assessment in the absence of a laboratory.

References

- David, G., Serres, C., & Jouannet, P. (1981). Kinematics of human spermatozoa. *Gamete Research*, 4, 83–95.
- ESHRE Andrology Special Interest Group. (1998). Guidelines on the application of CASA technology in the analysis of spermatozoa. *Human Reproduction*, 13, 142–145.
- Kanakasabapathy, M. K., Sadasivam, M., Singh, A., et al. (2017). An automated smartphone-based diagnostic assay for point-of-care semen analysis. *Science Translational Medicine*, 9(382). <https://doi.org/10.1126/scitranslmed.aai7863>.
- Katz, D. F., & Dott, H. M. (1975). Methods of measuring swimming speed of spermatozoa. *Journal of Reproduction and Fertility*, 45, 263–272.
- Mortimer, D., Pandya, I. J., & Sawers, R. S. (1986). Relationship between human sperm motility characteristics and sperm penetration into cervical mucus *in vitro*. *Journal of Reproduction and Fertility*, 78, 93–102.
- Mortimer, D., Serres, C., Mortimer, S. T., & Jouannet, P. (1988). Influence of image sampling frequency on the perceived movement characteristics of progressively motile human spermatozoa. *Gamete Research*, 20, 313–327.
- Mortimer, D. (1990). Objective analysis of sperm motility and kinematics. In B. A. Keel, & B. W. Webster (Eds.), *Handbook of the laboratory diagnosis and treatment of infertility* (pp. 97–133). Boca Raton, FL: CRC Press.
- Mortimer, S. T. (1997). A critical review of the physiological importance and analysis of sperm movement in mammals. *Human Reproduction Update*, 3, 403–439.
- Mortimer, S. T., Swan, M. A., & Mortimer, D. (1998). Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Human Reproduction*, 13, 2139–2146.
- Mortimer, S. T., van der Horst, G., & Mortimer, D. (2015). The future of computer-aided sperm analysis. *Asian Journal of Andrology*, 17, 545–553.
- Perreault, S. D. (2002). Smart use of computer-aided sperm analysis (CASA) to characterize sperm motion. In B. Robaire, B. Hinton, & M.-C. Orgebin-Crist (Eds.), *The epididymis: From molecules to clinical practice. A comprehensive survey of the efferent ducts, the epididymis and vas deferens* (pp. 459–471). New York: Springer.
- Suarez, S. S. (2008). Control of hyperactivation in sperm. *Human Reproduction Update*, 14, 647–657.
- Yanagimachi, R. (1970). The movement of golden hamster spermatozoa before and after capacitation. *Journal of Reproduction and Fertility*, 23, 193–196.

Home Tests for Semen☆

Yoshitomo Kobori, Dokkyo Medical University, Saitama, Japan

© 2018 Elsevier Inc. All rights reserved.

Introduction

Worldwide reports suggest that infertility affects 15%–20% of couples of reproductive age and about 50% of these are accountable to the male partner. While men with infertility represent a significant percentage of the infertile population, public awareness of this fact is limited at best. Literature and the other media have often neglected the male component of reproduction other than its sexual nature. Wider access to male infertility assessment may help to resolve this deficiency.

Semen analysis is the key element in the diagnosing the reproductive potential of a man with fertility in question. In current practice men must use a clinic or other hospital facility to have their semen analyzed. Once at the clinic, they are asked to produce a semen sample, which is then analyzed by staff at the clinic. Many subjects are not comfortable with this procedure, which they often find embarrassing and expensive.

A diagnostic test that men could perform in the privacy of their own home could lead to substantial improvement in this area. Many devices were invented to solve these problems. In this article, we show the commercialized devices to test semen sample at home **Table 1**.

Home Semen Microscope Evaluation Kits

Over-the-counter microscopes that are directly marketed to the consumer are also available to perform home semen analysis. Some companies produce and sell kits that include a conventional microscope, slides, and other accessories to analyze semen sample. A Medline literature review did not reveal studies that have evaluated the performance of these kits compared with semen analysis performed by a laboratory.

Chromatographic Immunoassay ~ SpermCheck Fertility®

The SpermCheck Fertility test is a rapid qualitative test designed to detect the presence of sperm in human semen. This test was approved by the US FDA. The test employs solid-phase chromatographic immunoassay technology and consists of a immune-chromatographic strip in a single cassette. The test strip is calibrated to give a positive result if the sperm concentration is 15×10^6 sperm/mL or greater. The device employs a pair of monoclonal antibodies which bind distinct, nonoverlapping epitopes on the sperm acrosomal antigen SP-10. Similar kinds of immune-chromatographic strips are available (**Fig. 1**).

Table 1 Summary of home semen test

Type of testing	Method	Evaluation items	Limitation	Cost	The available number of times
Microscope evaluation kit	Conventional microscope	Sperm concentration Sperm motility	High cost, Conflicting/ lacking data Must count sperm by themselves	USD \$100–500	Unlimited
SpermCheck®	Chromatographic immunoassay	Sperm concentration 5×10^6 sperm/mL and above 20×10^6 sperm/mL and above	Fail to evaluate sperm motility	USD \$39.99	1 test
Trak®	Centrifuge	Three categories of sperm concentration low (≤ 15 M/mL), moderate (15–55 M/mL), and optimal (> 55 M/mL)	Fail to evaluate sperm motility	USD \$199.99	4 tests
YO®	Smartphone based device	Motile sperm concentration as normal (above 6.0 M/mL) or low (below 6.0 M/mL)	Supportive data lacking	USD \$49.95	2 tests
Men's loupe®	Ball lens microscope with smartphone	Sperm concentration Sperm motility	Must count sperm by themselves	USD \$15	4 tests

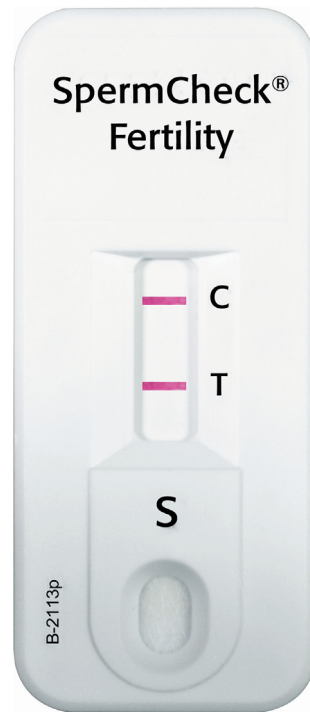


Fig. 1 SpermCheck fertility kit.

Centrifugal Method ~ Trak® Male Fertility Testing System

The Trak® Male Fertility Testing System (Sandstone Diagnostics, Livermore, CA) is a centrifugal microfluidic device for measuring sperm concentration in the home. The device, which recently received 510(k) clearance from the US FDA, provides linear sperm concentration measurement with three category (two cutoff) semiquantitative results: low (≤ 15 M/mL), moderate (15–55 M/mL), and optimal (> 55 M/mL). This categorical approach combines the WHO threshold with an evidenced-based reference value for faster time to pregnancy (**Fig. 2**).

The Trak device comprises a battery-powered reusable instrument (Engine), single-use test cartridges (Props), and various consumables. The Props use centrifugal force to process a defined volume of semen and visually display sperm concentration.

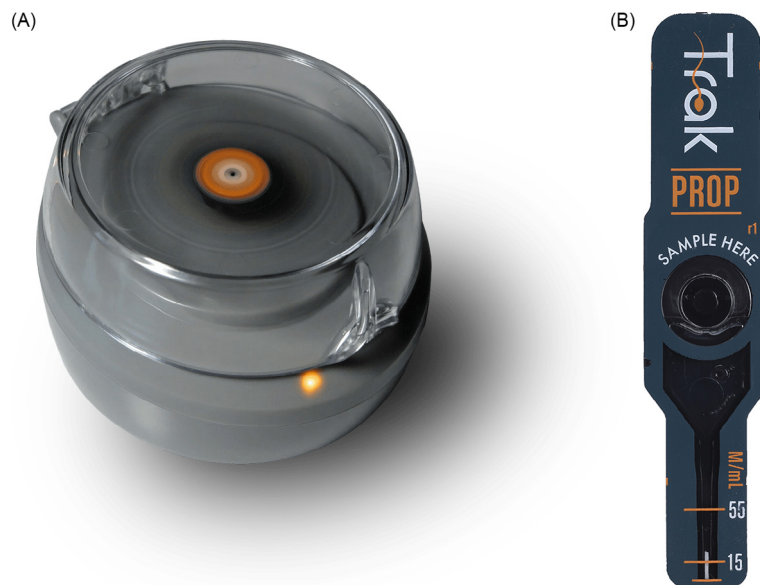


Fig. 2 (A) Trak® engine. (B) Trak® prop.

To operate the system, a user collects a semen sample in an enzyme-coated collection cup that promotes liquefaction (viscosity reduction). The user transfers approximately 0.25 mL of semen to the Prop inlet chamber, attaches the Prop to the Engine, and closes the lid to initiate the spin sequence (~6.5 min at 7000 rpm). As the Prop spins, a precise volume of semen is metered by centrifugal action from the sample inlet into the metering chamber, and sperm are compacted into a narrow channel at the end of the Prop. When the spin sequence is complete, the sperm cells form a visible, measurable white column that is proportional to the concentration of sperm in the sample and visually interpreted by the user. The system also includes an external control comprising a bead suspension formulated to simulate Trak results at a calibrated sperm concentration. Users may run the control on a Prop to verify proper operation of the system components, as well as to practice the assay protocol before testing a semen sample.

The Trak System includes multiple tests and encourages users to track changes in sperm concentration over time. A companion mobile app (Trak: Sperm Health & Fertility) provides users with personalized recommendations on health and lifestyle changes that may improve sperm concentration and chances of conception.

Smartphone Based Home Semen Test

YO® Home Sperm Test

The YO® Home Sperm Test was developed by the same team at Medical Electronic Systems that developed the SQA line of automatic semen analyzers used in over 4000 hospital labs, universities, and IVF centers globally. YO was cleared by the FDA in late 2016 and received a CE mark in early 2017. This system is comprised of a clip which attaches to the top of the smartphone thereby turning it into a microscope, a fixed cover slip plastic test slide that is inserted into the clip along with liquefaction powder (chymotrypsin) used to accelerate the liquefaction of the sample. YO measures motile cell concentration (moving cells) with 97% accuracy and reports results as normal (above 6.0 M/mL) or low (below 6.0 M/mL). The user can view and store a live video of his sperm and can receive an automatic test result within 2 min after liquefaction (Fig. 3).

Ball Lens Microscope ~ Men's Loupe®

A Dutch scientist, Antonie van Leeuwenhoek, first discovered the spermatozoon in 1677 using a ball lens microscope that he had invented. This simple device consisting of ball lens microscope (Leeuwenhoek's microscope) paired with a state-of-the-art smart phone equipped with a camera of the kind found in nearly all commercial smart phones. Sperm concentration and motility calculated with ball lens showed a very strong correlation with the CASA results (Fig. 4).

This microscope has been commercialized as "Men's loupe" (Tenga Health Care, Tokyo, Japan). The advantage of this device is very cheap due to a simple structure.

Smartphones have great potential to analyze semen because they are portable, contain excellent digital cameras and can be easily attached to a microscope. A ball lens microscope is inexpensive and easy to use for acquiring digital microscopic movies. Given its small size and weight, the device can support testing for male fertility at home or in the field, making it much more convenient and



Fig. 3 YO® Home sperm test.



Fig. 4 Men's loupe®.

economical than current practice. In addition, microscopy using smartphones offers additional capabilities such as telediagnosis, store-and-forward imaging, live-streaming of video, and the development of software enabling complex image analyses. This ball lens microscope provides an easy solution for global users to rapidly screen male infertility.

Conclusion

The advent of such new technologies and the democratization of fertility testing may upend the traditional approach to couples. Now, with such ease of access to home screening for abnormal bulk semen parameters, the male partner may pursue formal evaluation by a reproductive specialist before the female partner. With the use of a high-quality home semen test, even if the results are only semiquantitative, traditional attitudes toward male-factor infertility are already changed. Infertile couples will begin to initiate their own evaluations. Due to its accessibility, home semen testing may offer the potential to expand the public understanding of male-factor infertility.

Further Reading

- Björndahl, L., Kirkman-Brown, J., Hart, G., et al. (2006). Development of a novel home sperm test. *Human Reproduction*, 21, 145–149.
- Brezina, P. R., Habert, E., & Wallach, E. (2011). At home testing: Optimizing management for the infertility physician. *Fertility and Sterility*, 95, 1867–1878.
- Coppola, M. A., Klotz, K. L., Kim, K. A., et al. (2010). SpermCheck fertility, an immunodiagnostic home test that detects normozoospermia and severe oligozoospermia. *Human Reproduction*, 25, 853–861.
- Kanakasabapathy, M. K., Sadasivam, M., Anupriya Singh, A., et al. (2017). An automated smartphone-based diagnostic assay for point-of-care semen analysis. *Science Translational Medicine*, 9, eaai7863.
- Kobori, Y., & Kathrins, M. (2017). What is coming next for home semen testing? *Fertility and Sterility*, 107, 340.
- Kobori, Y., Pfanner, P., Prins, G. S., et al. (2016). Novel device for male infertility screening with single-ball lens microscope and smartphone. *Fertility and Sterility*, 106, 574–578.
- Medical Electronic Systems. Check your swimmers with the YO home sperm test. <http://www.yospermtest.com>. (Accessed May 2017).
- Schaff, U. Y., Fredriksen, L. L., Epperson, J. G., et al. (2016). Novel centrifugal technology for measuring sperm concentration in the home. *Fertility and Sterility*, 107, 358–364.

☆ *Change History:* October 2017. Yoshitomo Kobori updated the text, figures and table.

Laboratory Evaluation of Azoospermia

Grace M Centola, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Azoospermia is defined as the complete absence of sperm cells (spermatozoa) in the ejaculate. Approximately 1% of all men suffer from azoospermia, with up to 15% of men found to have no sperm (Jarow et al., 1989; Oates, 2012; Gudeloglu and Parekattil, 2013).

The causes of azoospermia are varied but include genetic (congenital), obstructive or nonobstructive, and unknown or idiopathic reasons. Genetic causes of azoospermia include hormonal abnormalities, Y chromosome microdeletions, absence of any sperm-producing cells (referred to as Sertoli cell only syndrome), and dysfunction of these sperm-producing cells (Gudeloglu and Parekattil, 2013). Obstructive azoospermia occurs in 15%–20% of azoospermic men and is caused by obstruction or blockage of the tubules or ducts that convey the sperm from the testicles to the outside. This includes ejaculatory dysfunction which prevents the semen from passing through the ducts and tubules during the ejaculatory process. Nonobstructive azoospermia occurs when the absence of sperm results from defects in sperm production. Congenital bilateral absence of the vas deferens (CBAVD) occurs when the vas deferens (the tube through which the sperm are transported) is missing from birth. Retrograde ejaculation, in which the semen is transported backward into the bladder rather than outward through the urethra, is also a form of nonobstructive azoospermia. In the case of retrograde ejaculation, sperm production is usually normal and the problem is often neurological, prohibiting antegrade or outward passage of sperm through the vas deferens. In most cases of retrograde ejaculation, sperm can be retrieved from the urine and used for assisted reproduction. Additional acquired causes of obstructive azoospermia include testicular trauma, infections including mumps orchitis, reproductive tract tumors, medications and recreational drug use, surgery and systemic diseases such as liver cirrhosis and kidney failure which prevent ejaculation through normally occurring ducts and tubules (Gudeloglu and Parekattil, 2013).

A thorough history and physical exam, as well as hormone evaluation, and semen analyses are important steps in the evaluation and diagnosis of azoospermia and could assist in formulation of an appropriate treatment plan, if possible, for these individuals.

Laboratory Evaluation of the Azoospermic Male

Azoospermia is diagnosed following microscopic examination of the semen. Generally two to three separate semen specimens will be examined by trained laboratory technologists. Each semen specimen should be collected 3–4 weeks apart and delivered to the laboratory within 30–45 min after ejaculation. Alternately, the specimens can be collected by masturbation in a private room provided at the laboratory facility.

The laboratory technologist will then prepare a wet-mount slide of the well-mixed semen specimen by placing a drop of the semen on a glass slide and covering with a thin glass coverslip. The specimen smear on the slide is carefully examined using a light microscope for the presence of spermatozoa. If no sperm are found on the slide, a second slide will often be prepared using a new drop of well-mixed semen. Additionally, a semen smear can be preserved and stained to confirm the absence of sperm and permanently document the laboratory findings.

If no sperm are found after scanning all areas of the slides, the semen can be centrifuged into a small pellet, and the pellet is examined for the presence of sperm (Jaffe et al, 1998; Corea et al, 2005; WHO, 1999). The pellet, containing all cellular components as well as noncellular debris, is then smeared onto a clean glass slide and carefully examined under the microscope for the presence of sperm. If even a low number of sperm is found, semen specimens may be frozen and stored for later use in assisted reproduction (Jaffe et al, 1998; Corea et al, 2005; WHO, 2010; Oates, 2012). When few sperm are found, the condition is referred to as severe oligozoospermia or cryptozoospermia (WHO, 2010; Gudeloglu and Parekattil, 2013).

Centrifugation of a semen specimen is recommended in cases of post vasectomy semen analysis (WHO, 1999). The WHO (2010) laboratory manual and the American Urological Association (AUA) (Sharlip et al, 2017) suggest that centrifugation is not necessary to confirm the presence of rare nonmotile sperm, suggesting that careful slide examination of the raw semen may be sufficient (Korthorst et al., 2010). Furthermore, Steward and colleagues (2008) concluded that examination of an uncentrifuged, raw semen specimen is sufficiently reliable for identifying >100,000 sperm. Since centrifugation might interfere with sperm motility, the AUA suggests that clinically relevant numbers of sperm can be identified without centrifugation (Sharlip et al, 2017).

Nonetheless, if no sperm are observed after careful examination of the raw semen specimen (duplicate slides as mentioned above), it is the standard and acceptable laboratory procedure to examine a centrifuged semen specimen for the presence or absence of sperm in order to correctly and reliably report a diagnosis of azoospermia.

Two other important parameters of the semen analysis—semen volume and pH—can provide useful information as to the cause of azoospermia (Oates, 2012). Approximately 10% of the ejaculate volume is contributed by the testicles, 70% by the seminal vesicles, and 20% from the prostate gland. The seminal vesicles provide alkaline fluid, while the prostate gland provides acidic fluid. Hence, the semen is alkaline since the seminal vesicles provide the majority of the fluid in the normal ejaculate (Oates, 2012). A low volume, low pH (acidic), azoospermic semen specimen is primarily made up of fluid from the prostate gland, suggesting absence of the seminal vesicles, bilateral absence or blockage of the vas deferens, or bilateral blockage of the ejaculatory ducts (Oates, 2012).

Alternately, in an azoospermic male, if the semen volume is normal, and the semen is alkaline, the seminal vesicles are presumably functional and duct obstruction is not a consideration. A blockage may be at a level closer to the testicles, or sperm production might be compromised in the testicle. Careful examination of centrifuged semen with a diagnosis of azoospermia can, in the case of normal volume and alkaline semen, confirm testicular failure. If even a few sperm are found in an ejaculate, testicular production of sperm is confirmed, albeit reduced, and blockage of the ducts, or absence of the vas deferens would not be possible (Oates, 2012).

Precise laboratory examination of both raw and centrifuged semen, measurement of semen volume and pH, along with evaluation of complete medical history, physical exam and hormone analyses, can confirm a diagnosis of azoospermia or complete absence of sperm in the ejaculate. Following such a diagnosis, a treatment algorithm can be individualized for each patient for purposes of reproduction.

References

- Corea, M., Campagnone, J., & Sigman, M. (2005). The diagnosis of azospermia depends on the force of centrifugation. *Fertility and Sterility*, 83(4), 920–922.
- Gudeloglu, A., & Parekattil, S. J. (2013). Update in the evaluation of the azoospermic male. *Clinics*, 68(S1), 27–34.
- Jaffe, T. M., Kim, E. D., Hoekstra, T. H., & Lipshultz, L. I. (1998). Sperm pellet analysis: A technique to detect the presence of sperm in men considered to have azoospermia by routine semen analysis. *Journal of Urology*, 159, 1548–1550.
- Jarow, J. P., Espeland, M. A., & Lipshultz, L. I. (1989). Evaluation of the azoospermic patient. *Journal of Urology*, 142(1), 62–65.
- Korthorst, R. A., Consten, D., & Van Rooijen, H. J. (2010). Clearance after vasectomy with a single semen sample containing < than 100 000 immotile sperm/mL: Analysis of 1073 patients. *BJU International*, 10511.
- Oates, R. A. (2012). Evaluation of the azoospermic male. *Asian Journal of Andrology*, 14(1), 82–87.
- Sharlip, I. D., Belker, A. M., Honig, S., Labrecque, M., Marmar, J. L., Ross, L. S., Sandlow, J. I., & Sokal, D. C. (2017). *American Urological Association Guideline—Vasectomy*. <http://www.auanet.org/education/guidelines/vasectomy.cfm>.
- Steward, B., Hays, M., & Sokal, D. (2008). Diagnostic accuracy of an initial azoospermic reading compared with results of post-centrifugation semen analysis after vasectomy. *Journal of Urology*, 180, 2119.
- World Health Organization. (1999). *WHO laboratory manual for the examination and processing of human semen* (4th edn.). Geneva: World Health Organization.
- World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th edn.). Geneva: World Health Organization.

Seminal Hypo- and Hypervolemia

Rajasingam S Jeyendran and Milica Ivanovic, Androlab Inc., Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Semen volume is the quantity of ejaculate produced and represents the sum of secretions from the accessory sex glands (seminal fluid) as well as sperm and other cellular components. Seminal fluid is comprised mainly of secretions by the seminal vesicles and prostate gland, with a small amount from the bulbourethral glands and epididymis. The seminal vesicles contribute approximately 75% of the ejaculate volume; the prostate provides another 20% and the remaining secretions, spermatozoa, and non-sperm cells make up approximately 5% of the ejaculate. During ejaculation, the bulbourethral gland secretions are emitted first, representing a small drop of fluid. Subsequently, the prostatic secretions as well as the majority of the spermatozoa are ejaculated, followed by the seminal vesicle secretions. By obtaining a “split ejaculate” (collecting the first and subsequent fractions of an ejaculate in two different containers), these secretions can be roughly separated.

An adequate volume of ejaculate during coitus is necessary to transport the spermatozoa to or near the cervix. An abnormal volume may help indicate why a couple has failed to conceive and thus recording the ejaculate volume is an important component of a semen analysis report. Similarly, the total volume of semen is used to calculate the total number of sperm in the ejaculate.

Even if the sperm concentration and motility are well within the acceptable limits, given the nature and consistency of the ejaculate especially if it is viscous, it can be difficult to measure the semen volume accurately. A precise measurement by weighing the sample and then calculating the volume from the weight is advocated by [WHO laboratory manual \(2010\)](#). However for Practical reasons we recommend the semen volume to be measured to the nearest 0.1 mL with a graduated pipette, centrifuge tube, or syringe.

For clinical interpretation, one needs to define what constitutes an abnormal ejaculate volume. [WHO laboratory manual \(2010\)](#) provides a lower reference value of 1.5 mL based on 1941 fertile men. In the author's own laboratory analyzing ejaculates from 3690 male spouses of infertile couples 701 (19%) had less than 1.5 mL. A deeper review of the results revealed a wider distribution within this group; 15 (0.4%) men produced no ejaculate or less than 0.1 mL and 93 (2.5%) men produced less than 0.5 mL.

It is generally thought that the seminal plasma is supportive to spermatozoa rather than being essential for their function, but the biochemical analysis of the seminal plasma can be useful for diagnostic purposes. Individual components of seminal plasma are more than likely not essential for sperm function so that they are not useful as fertility indicators but only to assess the reason if the semen volume is affected. In the author's own experience and clinical interpretation of semen analysis results ([Jeyendran, 2000](#)) it is recommended that the ejaculate volume be classified as:

- Aspermia and Anejaculate: no ejaculate following orgasm.
- Hypovolemia if the ejaculate is 0.5 mL or less.
- Hypervolemia if the ejaculate is more than 6.0 mL.

Outline

Semen volume may identify fertility impairing pathology of the male.

Aspermia and Anejaculate

This condition is defined as the absence of an antegrade ejaculate following orgasm or a very small semen volume (less than 0.1 mL) following ejaculation ([Kamischke and Nieschlag, 1999](#); [Mieusset et al, 2017](#)). In this situation, voided urine sample following masturbation must be evaluated for the presence of fresh sperm. If spermatozoa are present, then retrograde semen flow back into the bladder has occurred and the condition is referred to as retrograde flow of semen (commonly and erroneously referred to as: “retrograde ejaculation”). Normally, the urinary sphincter is closed during ejaculation to prevent retrograde semen flow into the bladder. If the sphincter fails to contract for whatever reason, the ejaculate may flow back into the bladder rather than proceed through the urethra. The following underlying causes can produce this condition:

- transurethral or open surgical resection of the bladder neck or prostate
- bilateral sympathectomy
- bilateral retroperitoneal lymphadenectomy
- extensive pelvic surgery (particularly proctectomy and colectomy)
- diabetic visceral neuropathy
- antihypertensive drugs that block sympathetic tone
- spinal cord injury

Hypovolemia

The hypovolemia is clinically relevant since it may affect fertility of the male. It is often due to factors that can be broadly classified into artifactual, behavioral, biological, psychogenic, and pathologic causes ([Roberts and Jarvi, 2009](#)).

Note: Hypovolemia has a minimal or no effect on spermatozoon fertilizing potential.

Artifactual Causes

The sexual stimulatory signals are modulated from higher brain centers, and therefore semen quality and quantity depend in part on the degree of psychological arousal.

Studies have demonstrated that collection of semen during actual coitus (using a seminal pouch) yields higher ejaculate quality and quantity than that obtained through masturbation in the laboratory environment.

An insufficient period of sexual abstinence between ejaculations will influence the ejaculate volume.

[WHO \(2010\)](#) reference value for semen volume is based on 2–7 days of sexual abstinence, but it is generally recommended the ejaculate be collected after 2–3 days of sexual abstinence.

Incomplete collection (partially missing the container) or spillage following sample collection will affect the semen volume determination.

Behavioral Causes

Recreational drugs such as heroin or methadone acting through higher brain centers may indirectly influence semen volume ([Nudell et al, 2002](#)).

Semen volume tends to decrease with increasing cigarette consumption ([Pasqualotto et al, 2006](#)).

Biological Causes

Patient age appears to affect the semen volume. As men age, the semen volume decreases ([Eskenazi et al, 2003](#)).

A 50-year-old man may have a 20% less semen volume compared to a 30-year-old man.

Psychogenic Causes

Anorgasmia due to anxiety, stress, disapproval, and embarrassment about masturbation may lead to partial ejaculation. Appropriate counseling or repeat collection on a different day may be advisable ([Roberts and Jarvi, 2009](#)).

Under certain emotional stress such as the recent diagnosis of cancer, one may produce poor ejaculate quality prior to semen analysis and cryopreservation.

Pathologic Causes

Hypovolemia without spermatozoa in the semen, and with a pH less than 7.4 could be due to ejaculatory duct obstruction or congenital absence of the seminal vesicles ([Roberts and Jarvi, 2009](#)).

Semen chemistries should be investigated. Such symptoms are associated with either partial or complete absence of fructose (produced by the seminal vesicles). However, normal zinc and acid phosphatase content with acidic pH (produced by the prostate gland) should be present.

Hypovolemia with spermatozoa present in semen and with a pH less than 7.4 could be due to obstruction of the seminal vesicular opening by a mucus-like plug, producing a high sperm concentration ([Perez-Pelaez et al, 1988](#)).

The obstructing plug may dissolve spontaneously, causing resumption of normal ejaculate volumes; but more often than not the obstacle actually enlarges, causing azoospermia over time.

Stricture of the seminal vesicular duct, probably due to inflammation, may also result in this condition.

Hypovolemia without (or low numbers of) spermatozoa in semen and with pH more than 7.8 could be due to hypoandrogenism leading to impaired spermatogenesis, while some fructose may still be present ([Jeyendran, 2000](#)).

Hypovolemia with spermatozoa present in the semen and with a pH greater than 7.8 could be due to a partial or incomplete retrograde flow of semen.

Post ejaculate; voided urine must be examined ([Kamischke and Nieschlag, 1999](#); [Mieusset et al, 2017](#)).

This condition may also be due to accessory sex gland impairment caused by inflammation or cancer (especially if the pH is more than 9.0; [Jeyendran, 2000](#)).

Other factors that appear to affect ejaculate volume include dietary zinc deficiency ([Hunt et al, 1992](#)), HIV -1 infection ([Bujan et al, 2007](#)), Klinefelter syndrome ([Aks glaede et al, 2009](#)), and certain drugs such as Silodosin and Tamsulosin ([Nudell et al, 2002](#)).

Hypervolemia

There is no clear cut point above which the seminal volume becomes pathologic, but the upper limit for normal has been reported to be 6 mL (Eliasson, 1976; Cooke et al, 1995; Jeyendran, 2000; Zhang et al, 2015). The determination of hypervolemia is generally not relevant clinically but should be noted. It is often due to either a long period of sexual abstinence or accessory sex gland fluid overproduction. (In the author's own laboratory experience, 4% of the men produced more than 6 mL; the largest volume measured from a single ejaculate was 15.8 mL.)

Note: Hypervolemia has a minimal or no effect on spermatozoon fertilizing potential.

With the increasing interest in sperm function testing and nearly routine use of intracytoplasmic sperm injection with assisted reproductive technologies, clinicians treating male infertility are sometimes only interested in obtaining sperm from the male rather than identifying or correcting any underlying etiology and pathology. This notion may suggest that the determination of ejaculate volume may no longer be important. It should be emphasized however that the measurement of semen volume is an essential part of routine semen analysis since it may help identify and correct fertility impairing pathology which may allow couples to conceive without resorting to advanced assisted reproductive technologies.

References

- Aksglaede, L., Jørgensen, N., Skakkebaek, N. E., & Juul, A. (2009). Low semen volume in 47 adolescents and adults with 47, XXY Klinefelter or 46, XX male syndrome. *International Journal of Andrology*, 32(4), 376–384.
- Bujan, L., Sergerie, M., Moinard, N., Martinet, S., Porte, L., Massip, P., Pasquier, C., & Daudin, M. (2007). Decreased semen volume and spermatozoa motility in HIV-1-infected patients under antiretroviral treatment. *Journal of Andrology*, 28(3), 444–452.
- Cooke, S., Tyler, J. P., & Driscoll, G. L. (1995). Hyperspermia: The forgotten condition? *Human Reproduction*, 10(2), 367–368.
- Eliasson, R. (1976). Semen analysis and laboratory workup. In A. T. K. Cockett, & R. L. Urry (Eds.), *Male Infertility: Workshop, treatment and research* (pp. 169–188). New York: Grune and Stratton.
- Eskenazi, B., Wyrobek, A. J., Slotter, E., Kidd, S. A., Moore, L., Young, S., & Moore, D. (2003). The association of age and semen quality in healthy men. *Human Reproduction*, 18(2), 447–454.
- Hunt, C. D., Johnson, P. E., Herbel, J., & Mullen, L. K. (1992). Effects of dietary zinc depletion on seminal volume and zinc loss, serum testosterone concentrations, and sperm morphology in young men. *The American Journal of Clinical Nutrition*, 56(1), 148–157.
- Jeyendran, R. S. (2000). *Interpretation of semen analysis results: A practical guide* (pp. 26–31). Cambridge, United Kingdom: Cambridge University Press.
- Kamischke, A., & Nieschlag, E. (1999). Mini symposium Non-surgical sperm recovery: Part II. Treatment of retrograde ejaculation and anejaculation. *Human Reproduction Update*, 5(5), 48–474.
- Mieusset, R., Walschaerts, M., Isus, F., Almont, T., Daudin, M., & Hamdi, S. M. (2017). Diagnosis of partial retrograde ejaculation in non-azoospermic infertile men with low semen volume. *Plos One*, 12(1), 1–14.
- Nudell, D. M., Monoski, M. M., & Lipshultz, L. I. (2002). Common medications and drugs: How they affect male fertility. *The Urologic Clinics of North America*, 29(4), 965–973.
- Pasqualotto, F. F., Sobreiro, B. P., Hallak, J., Pasqualotto, E. B., & Lucon, A. M. (2006). Cigarette smoking is related to a decrease in semen volume in a population of fertile men. *BJU International*, 97(2), 324–326.
- Perez-Pelaez, M., Jeyendran, R. S., & Malvar, T. (1988). A case report: Obstruction at the colliculus seminalis. *Andrologia*, 20(5), 447–449.
- Roberts, M., & Jarvi, K. (2009). Steps in investigation and management of low semen volume in the infertile man. *Canadian Urological Association Journal*, 3(6), 479–485.
- WHO laboratory manual for the examination and processing of human semen (5th edn.). (2010). Geneva, Switzerland: World Health Organization, WHO Press. pp. 15 and 224.
- Zhang, Y., Zhong, J., & Zhu, W. (2015). Evaluation on sperm parameters of ejaculates with hyperspermia. *Journal of Reproduction and Contraception*, 26(3), 131–134.

Laboratory Evaluation of Leukocytospermia

Grace M Centola, Reproductive Laboratory and Tissue Bank Consultant, Port St Lucie, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Normal semen specimens from fertile men will contain varying amounts of debris and other cell types as well as live and dead sperm. Leukocytes, predominately polymorphonuclear leukocytes (granulocytes or neutrophils) are present in all semen specimens (Tomlinson et al., 1992; Moskovtsev et al., 2007; WHO, 2010). When an unstained smear on a slide is observed under a microscope, it is not possible to distinguish whether the round cells are leukocytes or immature sperm cells, both of which appear as round cells and of similar size. When more than one round cell is seen under the microscope in multiple high power magnified areas, special staining is usually done to distinguish the WBCs from other types of cells (Mortimer, 1994; WHO, 2010). Generally, the presence of one round cell seen in the high magnification fields is a concentration of approximately one million WBCs per ml, which is commonly accepted as a normal level for WBCs in semen (WHO, 2010; Sikka and Hellstrom, 2016). Leukocytospermia is defined as excessive numbers of WBCs or leukocytes in the semen (WHO, 2010), in excess of one million/ml.

The clinical significance of semen white blood cells (WBCs) remains controversial (Moskovtsev et al., 2007; Palermo et al., 2016). Increased seminal WBCs may suggest a urogenital infection or inflammation (Pentyala et al., 2007; Duan et al., 2014; Fraczek et al., 2016; Palermo et al., 2016). An elevated leukocyte count, even as high as 30%, however, has been observed in the absence of urogenital inflammation or bacterial infection (Palermo et al., 2016). Negative effects of increased WBCs on sperm concentration and motility, sperm function and male fertility have been reported (Fedder, 1996; Arata de Bellabarba et al., 2000; Moskovtsev et al., 2007). A decrease in sperm concentration, motility and normal morphology, and an increase in oxidative stress has been seen with increased semen WBCs (Moskovtsev et al., 2007; Fraczek et al., 2016). Furthermore, increased semen WBCs was correlated with increased presence of abnormal sperm, particularly, with head damage, and midpiece and tail defects (Aziz et al., 2004). However, increased WBCs in the semen on the day of oocyte retrieval did not influence outcomes in in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Yilmaz et al., 2005; Ricci et al., 2015), while other studies have shown negative effects (Tomlinson et al., 1992; Lackner et al., 2008; Seshadri et al., 2012). Interestingly, moderate leukocyte concentration (< one million/ml) is considered normal and has been associated with increased fertilization and pregnancy rates (Barraud-Lange et al., 2011; Palermo et al., 2016).

Laboratory Evaluation of Leukocytospermia

Several methods have been used to distinguish between leukocytes, immature sperm cells and other round cells in semen (Mortimer, 1994; WHO, 2010; Ricci et al., 2015). Methods used to identify the types of round cells in the semen include special staining, cytochemical analysis, and labeling with special antibodies (Moskovtsev et al., 2007; WHO, 2010). Seminal leukocytes can be differentiated from immature sperm cells by differences in coloration when stained, and on the size and shape of the cell nucleus (Johanisson et al., 2000; WHO, 2010). Neutrophils, or granulocytes, the most common type of WBCs in semen, are characterized by the presence of granules in the cell cytoplasm, which will be visible after using special stains.

Staining

A stained slide is routinely used to determine normal and abnormal spermatozoa (see Laboratory Evaluation of Morphology). However, a morphology slide can also be used to estimate the presence of multi-nucleated immature sperm cells, polymorphonuclear cells (showing distinctive lobulated nucleus), and lymphocytes (large round or kidney shaped nucleus) all of which show a distinctive coloration and nuclear structure on a stained slide (Mortimer, 1994; Johanisson et al., 2000; WHO, 2010).

Common stains used to differentiate round cells (leukocytes and immature sperm cells) in semen are Papanicolaou, Shorr and Giemsa stains (Menkveld and Kruger, 1998; Johanisson et al., 2000; Moskovtsev et al., 2007; WHO, 2010). Using these stains, leukocytes appear blue in color, while immature sperm appear pink (WHO, 2010). Some granulocytes demonstrate fewer cytoplasmic granules, and a round or kidney shaped nucleus. The polymorphonuclear leukocytes (PMNs) have a characteristic large lobulated nucleus, with inter-nuclear bridges connecting the lobes, while immature sperm actually have multiple nuclei in one cell. On a stained slide, these differences can be readily seen and the leukocyte concentration estimated.

Peroxidase Staining

Since granulocytes are the majority of WBCs in semen, a routine method to distinguish these cells from other round cells is a stain that shows peroxidase activity in the cytoplasmic granules (Johanisson et al., 2000; WHO, 2010). The characteristic granules can be stained using o-toluidine which stains the peroxidase in the granules. Peroxidase positive cells stain brown, while negative cells are unstained. It is important to note that other types of WBCs (i.e., lymphocytes) do not have peroxidase-positive granules,

and thus, will not stain brown using this type of methodology (Menkveld and Kruger, 1998; Johanisson et al., 2000). Since the granulocytes are the majority of leukocytes in the semen, an estimate of the total WBC concentration can be made using the peroxidase staining. The concentration of the peroxidase-positive leukocytes is made based on the cells with brown-stained granules and the sperm concentration. It is important to note that the peroxidase stain method does not enumerate the non-granular WBCs and the immature sperm cells both of which contribute to the round cell concentration in the semen. Laboratory test kits are commercially available for determination of the granulocyte concentration (LeucoScreen, Vitrolife, Inc., Englewood, CO, USA; FertiPro N.V., Belgium).

Advanced Assays

Additional advanced testing can be used to distinguish between leukocytes, immature sperm cells or other round cells, although these tests are not routinely performed in the clinical andrology laboratory. The significance of these tests is questionable, and may not provide additional information to the clinician useful for management of the infertile male. One advanced test determines binding of unique surface proteins on the round cell membrane. One such protein is called CD45, which is present on the surface of leukocytes (Duan et al., 2014; Palermo et al., 2016). The surface protein on the round cell membrane can be tagged with a stain or label which would then be detected by specialized equipment. Similar assays include monoclonal antibody binding to leukocytes and alkaline phosphatase staining (Mortimer, 1994).

Flow cytometry has also been used for precise counting of seminal leukocytes, and has been considered more sensitive and specific than staining methods, particularly the peroxidase method (Ricci et al., 2015). Flow cytometry uses laser technology to excite probes bound to the surface of the cell. The probes give off light which is then detected by an electronic detection instrument (Perticarari et al., 2007; Ricci et al., 2015). Flow cytometry provides a simple, rapid, and highly accurate method for evaluation of several semen parameters, including leukocyte concentration (Perticarari et al., 2007). However, flow cytometry is not a standard instrument in clinical andrology laboratories, and hence, the importance of this technique for determination of semen leukocytes has not been realized.

Conclusion

Even though the clinical significance of seminal round cells has been questioned, the presence of an abnormally elevated concentration of round cells should be further evaluated. Approximately 15% of male infertility cases present with infectious etiologies (Jung et al., 2016), and, may or may not be accompanied by elevated leukocytes. It has been shown that an elevation of the semen WBC concentration may be deleterious to sperm count, motility and morphology, and increase oxidative stress on the sperm (Pentyala et al., 2007; Jung et al., 2016). Accurate determination of the WBC concentration in semen allows the clinician to determine a course of treatment. Antibiotic and antioxidant administration has become a widely accepted treatment to reduce semen WBCs, and the effects on sperm concentration, motility and oxidative stress (Lackner et al., 2006; Pentyala et al., 2007; Fraczek et al., 2016; Jung et al., 2016).

References

- Arata de Bellabarba, G., Tortolero, I., Villarroel, V., Molina, C. Z., Bellabarba, C., & Velazquez, E. (2000). Nonsperm cells in human semen and their relationship with semen parameters. *Archives of Andrology*, 45, 131–136. PEROXIDASE.
- Aziz, N., Agarwal, A., Lewis-Jones, I., Sharma, R. K., & Thomas, A. J., Jr. (2004). Novel associations between specific sperm morphological defects and leukocytospermia. *Fertility and Sterility*, 82, 621–627.
- Barraud-Lange, V., Pont, J. C., Ziyat, A., Pocate, K., Sifer, C., Cedrin-Dumerin, I., Fechtali, B., Ducot, B., & Wolf, J. P. (2011). Seminal leukocytes are good Samaritans for spermatozoa. *Fertility and Sterility*, 96(6), 1315–1319.
- Duan, Y. G., Zhang, Q., Liu, Y., Mou, L., Li, G., Gui, Y., & Cai, Z. (2014). Dendritic cells in semen of infertile men: Association with sperm quality and inflammatory status of the epididymis. *Fertility and Sterility*, 101(1), 70–77.
- Fedder, J. (1996). Nonsperm cells in human semen: With special reference to seminal leukocytes and their possible influence on fertility. *Archives of Andrology*, 36, 41–65.
- Fraczek, M., Hryhorowicz, M., Gill, K., Zarzycka, M., Gaczarzewicz, D., Jedrzejczak, P., Bilinsha, B., Piasecka, M., & Kurpisz, M. (2016). The effect of bacteriospermia and leukocytes on conventional and nonconventional semen parameters in healthy young normozoospermic males. *Journal of Reproductive Immunology*, 118, 18–27.
- Johanisson, E., Campana, A., Luthi, R., & de Agostini, A. (2000). Evaluation of 'round cells' in semen analysis: A comparative study. *Human Reproduction Update*, 6(4), 404–412.
- Jung, J. H., Kim, M. H., Kim, J., Baik, S. K., Koh, S.-B., Park, H. J., & Seo, J. T. (2016). Treatment of leukocytospermia in male infertility: A systematic review. *World J. Men's Health*, 34(3), 165–172.
- Lackner, J. E., Herwig, R., Schmidbauer, J., Schatzl, G., Kratzik, C., & Marberger, M. (2006). Correlation of leukocytospermia with clinical infection and the positive effect of antiinflammatory treatment on semen quality. *Fertility and Sterility*, 86(3), 601–605.
- Lackner, J. E., Mark, I., Sator, K., Huber, J., & Sator, M. (2008). Effect of leukocytospermia on fertilization and pregnancy rates of artificial reproductive technologies. *Fertility and Sterility*, 90, 869–871.
- Menkveld, R., & Kruger, T. F. (1998). Sperm morphology and male urogenital infections. *Andrologia*, 30(1), 49–53.
- Mortimer, D. (1994). *Practical laboratory andrology*. New York: Oxford University Press.
- Moskovtsev, S. I., Willis, J., White, J., & Mullen, J. B. M. (2007). Leukocytospermia: Relationship to sperm deoxyribonucleic acid integrity in patients evaluated for male factor infertility. *Fertility and Sterility*, 88(3), 737–740.
- Palermo, G. D., Neri, Q. V., Cozzubbo, T., Cheung, S., Pereira, N., & Rosenwaks, Z. (2016). Shedding light on the nature of seminal round cells. *PLoS*, 11(3), e0151640.
- Pentyala, S., Lee, J., Annam, S., Alvarez, J., Veerajay, A., & Yadiapalli, N. (2007). Current perspectives on pyospermia: A review. *Asian Journal of Andrology*, 9, 593–600.

- Perticarari, S., Ricci, G., Granzotto, M., Boscolo, R., Possobon, C., Guarnieri, S., Sartore, A., & Presani, G. (2007). A new multiparameter flow cytometric method for human semen analysis. *Human Reproduction*, 22(2), 485–494.
- Ricci, G., Granzotto, M., Luppi, S., Giolo, E., Martinelli, M., Zito, G., & Borelli, M. (2015). Effect of seminal leukocytes on in vitro fertilization and intracytoplasmic sperm injection outcomes. *Fertility and Sterility*, 104(1), 87–93.
- Seshadri, S., Flanagan, B., Vince, G., & Lewis-Jones, D. I. (2012). Leukocyte subpopulations in the seminal plasma and their effects on fertilisation rates in an IVF cycle. *Andrologia*, 44, 396–400.
- Sikka, S. C., & Hellstrom, J. G. (2016). Current updates on laboratory techniques for the diagnosis of male reproductive failure. *Asian Journal of Andrology*, 18(3), 392–401.
- Tomlinson, M. J., Barratt, C. L., Bolton, A. E., Lenton, E. A., Roberts, H. B., & Cooke, I. D. (1992). Round cells and sperm fertilizing capacity: The presence of immature germ cells but not seminal leukocytes are associated with reduced success of in vitro fertilization. *Fertility and Sterility*, 58, 1257–1259.
- WHO. (2010). *World Health Organization laboratory manual for the examination and processing of human semen* (5th Edition). World Health Organization.
- Yilmaz, S., Koyuturk, M., Kilic, G., Alpak, O., & Aytoz, A. (2005). Effects of leukocytospermia on semen parameters and outcomes of intracytoplasmic sperm injection. *International Journal of Andrology*, 28, 337–342.

Laboratory Evaluation of Antisperm Antibodies

Grace M Centola, Reproductive Laboratory and Tissue Bank Consultant, Port St Lucie, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The presence of antisperm antibodies in semen, blood (serum), cervical mucus, vaginal secretions and in follicular fluid which surrounds the egg, can be a significant cause of infertility. The clinical relevance of such antisperm antibodies, however, remains controversial, since these antibodies can be present in both fertile and infertile men (Chiu and Chamley, 2004; Zini et al., 2011; Sikka and Hellstrom, 2009). Numerous publications report antisperm antibodies in infertile men ranging from 9 to 36% (Naz, 2004), 9–13% (Chiu and Chamley, 2004), 5–15% (Ombelet et al., 2009; Zini et al., 2011) and 10–30% (Sikka and Hellstrom, 2009). As many as 60% of vasectomized men have serum antisperm antibodies (Parslow et al., 1983; Sikka and Hellstrom, 2009). Yet sperm antibodies have been found in as many as 3% of fertile men and 4% of fertile women (Chiu and Chamley, 2004; Ombelet et al., 2009).

Antisperm antibodies can cause a decrease in sperm concentration and motility, as well as a longer seminal fluid liquefaction time (Cui et al., 2015). Sperm antibodies have been shown to cause decreased sperm motility, clumping or agglutination of sperm, impairment of sperm-cervical mucus penetration, impaired sperm-egg interaction and post-fertilization events (Lombardo et al., 2001, 2004; Bjorndahl et al., 2010; Zini et al., 2011; Restrepo and Cardona-Maya, 2013; Vasquez-Levin et al., 2014; Cui et al., 2015). Furthermore, the presence antisperm antibodies has resulted in decreased fertilization rates (45–34%) and decreased pregnancy rates (11% versus 44%) when the male partner has sperm antibodies as compared to those who do not have sperm antibodies (Chiu and Chamley, 2004; Vasquez-Levin et al., 2014).

When performing a semen analysis, the laboratory will scan a slide to determine the quality of motility and progression of the sperm. Antisperm antibodies may be present when spermatozoa show a decrease in percent motility with an increase in non-progressive motile sperm (shaking in place, “tic-toc” movement). Furthermore, sperm antibodies may cause sperm to stick to each other (head to head, tail to tail, or in a mixed way) (WHO, 2010). Antisperm antibodies can also cause repeated abnormal sperm-cervical mucus interaction testing (PCT). Testicular trauma or injury, persistent leukocytospermia and repeated genital infections may also result in sperm antibodies (Sikka and Hellstrom, 2009).

Antisperm antibodies are only relevant if there is significant interference with fertilization-related processes (Naz, 2004; Sikka and Hellstrom, 2009, 2016; WHO, 2010). However, the nature and identity of antigens on the sperm surface that interfere with the fertilization process are not clear (Bjorndahl et al., 2010).

In the presence of significant agglutination and/or unexplained reduced sperm motility, testing should be considered to determine if these abnormalities are caused by the presence of sperm antibodies. Some laboratories include antibody screening as part of routine semen analysis regardless of the appearance of agglutination or immobility of the sperm. Several tests are available to assess the presence of immunological infertility. Testing includes direct tests, that is, test for the presence of antibody directly on the sperm surface, and indirect tests, that is., test for the presence of antibody in body fluids such as serum, cervical mucus, follicular fluid.

Direct Tests for Antisperm Antibodies

Current direct sperm antibody tests include the mixed antiglobulin reaction (MAR) test and the immunobead binding test (IBT). These tests are performed on a fresh semen specimen and requires at least 200 motile sperm for the test to be valid (Bjorndahl et al., 2010).

The MAR test is an inexpensive, quick and sensitive screening test (Bjorndahl et al., 2010; WHO, 2010). In this test, a “bridging” second antibody is used to attach antibody-coated latex beads to sperm that have surface antibodies (Sikka and Hellstrom, 2009; Bjorndahl et al., 2010; WHO, 2010). An earlier MAR test used sensitized red blood cells before the antibody-coated beads were developed (Mortimer, 1994; Sikka and Hellstrom, 2009).

Fresh, unwashed sperm are mixed on a glass slide with a drop of the antibody-coated latex beads and a drop of antiserum. After approximately 2–3 min, the slide is examined under the microscope. Sperm that have attached latex beads are those that are antibody positive. Although the MAR test is simple and cost-effective, it does not show the location of the antibody on the sperm surface or the class of antibody. This location and class of antibody is important in determining the clinical significance of the antisperm antibodies. In the MAR test, when 10% or more of sperm are clumped with latex beads, a more specific test, such as the Immunobead Binding Test (IBT) should be done (Sikka and Hellstrom 2009, 2016). Others suggest a value of 50% clumping in a MAR test for a test to be considered positive. Several kits for MAR testing are available commercially for laboratory use.

The Immunobead Binding Test (IBT) is a more specific antibody test which identifies the class or type of antisperm antibody and the binding site on the sperm surface (head, midpiece, tail) by using different size or color polyacrilamide beads corresponding to the immunological class of antibody (referred to as immunoglobulin type A (IgA) and immunoglobulin type G (IgG)). In this test, a drop of washed sperm is mixed with a drop of the individual type of bead. After a 2–3 min incubation, the slide is observed under a microscope (Sikka and Hellstrom, 2009; Bjorndahl et al., 2010; WHO, 2010). The beads will adhere to those sperm that have

antibodies on their surface. When greater than 20% of sperm have at least two beads attached, the test is considered a positive test. The test, however, is clinically significant only when greater than or equal to 50% of the motile sperm have attached beads (Sikka and Hellstrom, 2009, 2016; Bjorndahl et al., 2010; WHO, 2010). IBT kits are commercially available for clinical laboratory use.

Indirect Tests for Antisperm Antibodies

Indirect antibody tests are used to detect antisperm antibodies in sperm-free fluids such as serum, seminal plasma, cervical mucus and follicular fluid (WHO, 2010; Vazquez-Levin et al., 2014). An antibody negative donor sperm sample is incubated with the test fluid. This sperm specimen is then tested using a direct test (IBT) to see if sperm antibodies in the fluid bind to the sperm.

A known antibody negative sperm sample is washed and a fraction of motile sperm is recovered for use in the test. The motile sperm are incubated with the heat-inactivated test fluid for 1h. Next the sperm are washed to remove the test fluid, and are then mixed with immunobeads as in a direct IBT. As in the direct IBT, the sperm with attached beads are positive for sperm antibodies and those with no attached beads are negative (Sikka and Hellstrom, 2009, 2016; Bjorndahl et al., 2010; WHO, 2010). Both the class of antibody and the location on the sperm (head, midpiece, tail) are recorded and used to determine the test result. In the indirect test, at least 50% of the motile sperm must have attached beads in order for the test to have clinical significance (Bjorndahl et al., 2010; WHO, 2010). Known negative and positive samples, such as serum from a known positive and a known negative male, must also be tested as control samples.

Conclusion

The presence of antisperm antibodies is not a clear-cut indicator of infertility since fertile men and women can present with sperm antibodies. The presence of antisperm antibodies can be evaluated using a direct test on sperm or an indirect test using other body fluids. At least 50% of the sperm must show positive for antisperm antibodies in order for the test to be clinically significant. A report of a clinically significant positive antisperm antibody test should be followed by additional tests such as the sperm-cervical mucus interaction test, or compared to results of a post-coital test. However, antisperm antibodies may still cause infertility by affecting sperm-egg interaction, with no impairment of sperm penetration of the cervical mucus. Treatment for antisperm antibodies include drug treatment (immunosuppressive drugs), intrauterine insemination and IVF and ICSI. ICSI has been shown to be the primary choice for treatment of immunological infertility, especially in severe cases and with head-bound antibodies (Lombardo et al., 2001, 2004; Naz, 2004; Zini et al., 2011).

References

- Bjorndahl, L., Mortimer, D., Barratt, C. L. R., Castilla, J. A., Menkveld, R., Kvist, U., Alvarez, J. G., & Haugen, T. B. (2010). *Extended semen analysis. A practical guide to basic laboratory andrology*. New York: Cambridge University Press.
- Chiu, W. W.-C., & Chamley, L. W. (2004). Clinical associations and mechanisms of action of antisperm antibodies. *Fertility and Sterility*, 83(3), 529–535.
- Cui, D., Han, G., Shang, Y., Liu, C., Xia, L., Li, L., & Yi, S. (2015). Antisperm antibodies in infertile men and their effect on semen parameters: A systematic review and meta-analysis. *Clinica Chimica Acta*, 444, 29–36.
- Lombardo, F., Gandini, L., Lenzi, A., & Dondero, F. (2004). Antisperm immunity in assisted reproduction. *Journal of Reproductive Immunology*, 62, 101–109.
- Lombardo, F., Gandini, L., Dondero, F., & Lenzi, A. (2001). Antisperm immunity in natural and assisted reproduction. *Human Reproduction Update*, 7(5), 450–456.
- Mortimer, D. (1994). *Practical laboratory andrology*. New York: Oxford University Press.
- Naz, R. K. (2004). Modalities for treatment of antisperm antibody mediated infertility: Novel perspectives. *American Journal of Reproductive Immunology*, 51(5), 390–397.
- Parslow, J. M., Royle, M. G., Kingscott, M. M. B., Wallace, D. M. A., & Hendry, W. F. (1983). The effects of sperm antibodies on fertility after vasectomy reversal. *American Journal of Reproductive Immunology*, 3, 28–33.
- Restrepo, B., & Cardona-Maya, W. (2013). Antisperm antibodies and fertility association. *Actas Urológicas Españolas*, 37(9), 571–578.
- Sikka, S. C., & Hellstrom, W. J. G. (2009). Tests for antisperm antibodies. In L. I. Lipshultz, S. S. Howards, & C. S. Niederberger (Eds.), *Infertility in the male* (4th edn). New York: Cambridge University Press.
- Sikka, S. C., & Hellstrom, W. J. G. (2016). Current updates on laboratory techniques for the diagnosis of male reproductive failure. *Asian Journal of Andrology*, 18(3), 392–401.
- Vasquez-Levin, M. H., Marin-Briggiler, C. I., & Veaute, C. (2014). Antisperm antibodies: Invaluable tools toward the identification of sperm proteins involved in fertilization. *American Journal of Reproductive Immunology*, 72(2), 206–218.
- World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th edn.). Geneva, Switzerland: World Health Organization.
- Zini, A., Fahmy, N., Belzile, E., Ciampi, A., Al-Hathal, N., & Kotb, A. (2011). Antisperm antibodies are not associated with pregnancy rates after IVF and ICSI: Systematic review and meta-analysis. *Human Reproduction*, 26(6), 1288–1295.

Further Reading

- Ombelet, W., Vandeput, H., Janssen, M., Cox, A., Vossen, C., Pollet, H., Steeno, O., & Bosmans, E. (1997). Treatment of male infertility due to sperm surface antibodies: IUI or IVF? *Human Reproduction*, 12, 1165–1170.

Laboratory Evaluation of Reactive Oxygen Species

Ashok Agarwal, American Center for Reproductive Medicine, Cleveland, OH, United States

Chak-Lam Cho, Kwong Wah Hospital, Kowloon, Hong Kong

Rakesh Sharma, American Center for Reproductive Medicine, Cleveland, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chemiluminescence Is the emission of light as a result of a chemical reaction.

Colorimetry Is a technique used to test the concentration of a colored compound in a solution by measuring absorbance of a specific wavelength of light.

Free radical Is an atom, molecule or ion that has an unpaired valence electron. The unpaired electrons make free radicals highly reactive chemically towards other substances.

Peroxidation Is a type of reaction in which oxygen atoms are formed leading to oxidation to the greatest extent especially in formation of a peroxide.

Posttranslational modifications Regulates protein functions by chemical modifications after translation. The reactions include phosphorylation, ubiquitination, methylation, acetylation, glycosylation and nitrosylation, carbonylation.

Proteomics Refers to the application of biotechnology in analyzing the structure, function, and interactions of proteins produced by the genes in a particular biological system.

Redox Is short for reduction–oxidation reaction. It refers to a chemical reaction in which oxidation states of atoms are changed. The reaction involves the transfer of electrons between chemical species resulting in reduction process and a complementary oxidation process.

Introduction

Free radicals are normally formed during the intermediate steps of aerobic metabolism and a physiological level of free radicals is essential for successful fertilization by regulating sperm capacitation (Sharma and Agarwal, 1996). Free radicals are highly unstable due to the presence of unpaired electron(s). They attempt to gain electron(s) from other molecules in their vicinity and often result in a chain reaction. Reactive oxygen species (ROS) are a group of radical and non-radical derivatives of oxygen. The superoxide anion radical ($\cdot\text{O}_2^-$) is the most common ROS that is produced by spermatozoa. It is formed when an extra electron is added to molecular oxygen (O_2). The superoxide anion can be converted to other non-radical, such as, hydrogen peroxide (H_2O_2), which is relatively stable but still a powerful oxidant. Some free radicals containing nitrogen atoms such as nitrogen oxide (NO), peroxynitrite (NO_3^-), nitrous oxide (N_2O) and peroxynitrous acid (HNO_3) are called reactive nitrogen species. Reactive nitrogen species are often considered to be a subclass of ROS. ROS are very reactive in the presence of amino acids, lipids, and nucleic acids (Sharma and Agarwal, 1996).

The possible implication of ROS in male infertility have been reported since 1943 (MacLeod 1943). Since then, the detrimental effect of high ROS on sperm concentration, motility, morphology (Aziz et al., 2004), and DNA damage (Desai et al., 2009) have been described. Also, there are studies showing elevated ROS levels in 30%–80% of infertile men (Agarwal et al., 2006). The delicate balance in the cellular environment is maintained by the presence of scavenging system via enzymatic and nonenzymatic antioxidant pathways. Oxidative stress (OS) is the result of imbalance between production of ROS and the protective scavenging capacity of the antioxidants. It is more likely a result of a supraphysiological level of ROS rather than a low level of neutralizing antioxidants. Defective spermatozoa and leukocytes are the major sources of ROS in human semen (Kessopoulou et al., 1992).

Therefore, accurate assessment of seminal OS levels is a vital tool in the evaluation of infertile male. Laboratory evaluation of ROS is broadly divided into direct and indirect assays. Direct methods include cytochrome *c* reduction test, electron spin resonance, nitroblue tetrazolium technique, and chemiluminescence. Indirect methods measure the oxidized products by ROS. They include the Endtz test, measurement of lipid peroxidation products, measurement of sperm DNA fragmentation, and detection of post-translational modifications of proteins. In this chapter, the more widely used direct and indirect assays for measurement of ROS will be discussed in detail. Some tests, e.g. the Endtz test and sperm DNA fragmentation tests, will be discussed in other chapters. We will also present measurement of total antioxidant capacity (TAC) as another measure of OS status. Emerging techniques including measurement of oxidation–reduction potential (ORP) and proteomic analysis will be introduced.

Fig. 1 summarizes the different sources and deleterious effects of ROS. It also illustrates the different targets for ROS assays.

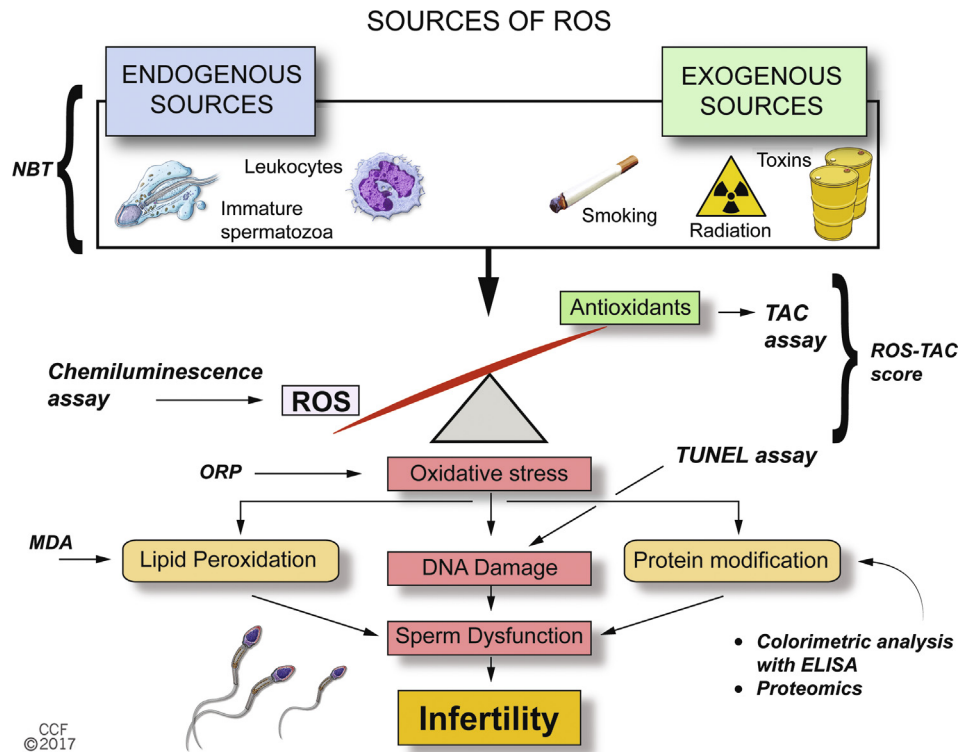


Fig. 1 Sources and adverse effects of ROS, and target of different OS assessment methods.

Direct Measurement of ROS

This ROS assay is the most widely utilized method for OS assessment. The amount of oxidation within the sperm cell membrane reflects the net oxidative imbalance between ROS production and the antioxidant concentration in semen (Agarwal et al., 2007). Direct measurements of ROS generally offer accurate results. However, their clinical application is often limited by the high cost (Agarwal et al., 2015). The more commonly used methods of nitroblue tetrazolium test (NBT) and chemiluminescence assay are discussed in more detail in the following sessions.

Nitroblue Tetrazolium Test

Nitroblue tetrazolium test or NBT test measures ROS generated by both spermatozoa and leukocytes. NBT becomes reduced by free oxygen radicals forming a blue-black compound, formazan. The reaction can be monitored spectrophotometrically or the stained cells can be scored histochemically. The NBT reaction reflects the ROS generating activity in the cytoplasm of cells. Moreover, results of NBT staining strongly correlate with ROS levels assessed by chemiluminescence (Esfandiari et al., 2003). Source of ROS, i.e. spermatozoa or leukocytes, can be distinguished by scoring the relative density of formazan granules. Different sources of ROS imply and point towards different pathological conditions. The test is readily available and inexpensive. The high sensitivity of the test potentially serves a good preliminary assessment tool for infertile men with suspected high ROS as the underlying etiology.

Chemiluminescence Assay

The chemiluminescence assay is a luminescence-based technique. Emitted light resulting from a chemical reaction between chemical reagents and oxidizing compounds is depicted and quantified by a luminometer (Fig. 2A). It is expressed as relative light units per second per 10^6 sperms ($\text{RLU s}^{-1} 10^{-6} \text{ sperms}$). A total of 11 tubes, including 3 blank, 3 negative control, 2 patient sample and 3 positive control, are labeled and analyzed (Fig. 2B). Different probes have been used in chemiluminescence assays. Each probe specifically measures a different type of free radicals in the sample, i.e. extracellular and/or intracellular. Luminol and lucigenin are among the most widely used probes. Luminol can measure the global level of ROS under physiological conditions. Both extra- and intracellular ROS including hydrogen peroxide and oxygen radicals are measured. On the other hand, lucigenin measures only extracellular ROS and it is specific for superoxide anions. Although the test measures both extra- and intracellular ROS with high sensitivity and specificity, it cannot measure multiple markers simultaneously and levels of ROS decline with time after ejaculation.

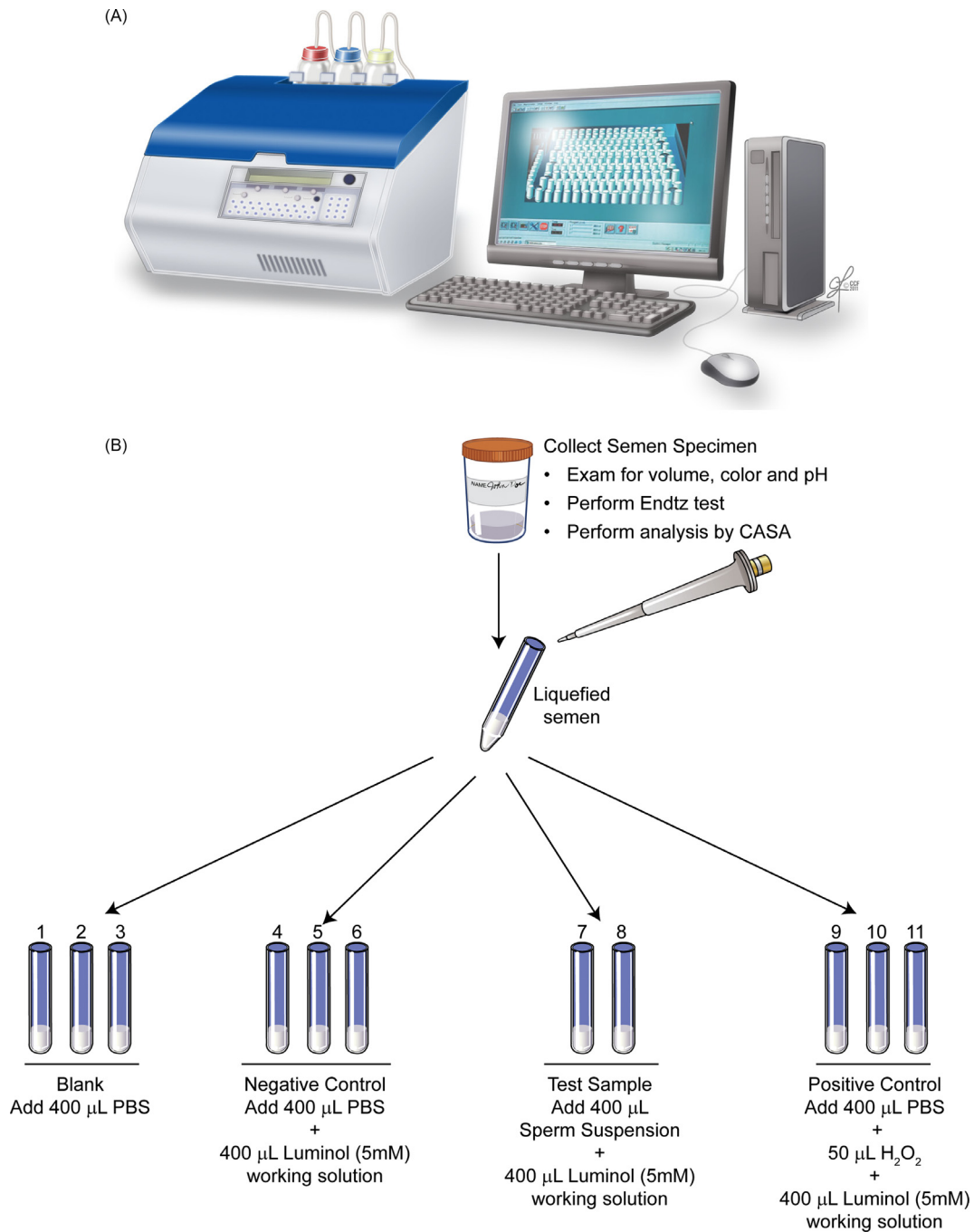


Fig. 2 Setup of direct ROS assay by chemiluminescence technique. (A) Luminometers for chemiluminescence and (B) preparation of tubes of ROS measurement.

Several studies have demonstrated that ROS levels measured by chemiluminescence may be able to distinguish good quality from poor quality samples. A sensitivity of 76.4%, specificity of 53.3%, positive predictive value of 82.1% and negative predictive value of 44.5% in differentiating infertile men from controls could be achieved with a cutoff value of $102.2 \text{ RLU s}^{-1} 10^{-6} \text{ sperm}$ (Agarwal et al., 2015).

Chemiluminescent assays can be used in patients with unexplained or idiopathic infertility. The results correlate with sample quality and also fertilizing potential of sperm in-vitro and in-vivo (Aitken et al., 1991). Patients who demonstrate high ROS levels in seminal ejaculate may benefit from antibiotic (in case of infection) or antioxidant treatments aiming at reducing ROS.

Indirect Assay of ROS

Unlike the direct methods, indirect assays measure the stable downstream end products of the peroxidative process or DNA damage. Indirect assays have the advantage of providing information of post-hoc ROS-related damage in addition to the value as a surrogate marker of OS level.

Measurement of Lipid Peroxidation

Polyunsaturated fatty acids are particularly susceptible to ROS. Breakdown of polyunsaturated fatty acids into lipid peroxides happens under OS. Aldehydic products including 4-hydroxynonenal (4-HNE), 8-isoprostane (IsoP) and malonaldehyde (MDA), are present as byproducts during the process of lipid peroxidation. They are relatively stable and act as a measure of the damage elicited to lipids by free radicals. Assessment of MDA by the thiobarbituric acid-reactive substance (TBARS) assay by colorimetry is the most commonly adopted method. The adduct formation between MDA and thiobarbituric acid is measured by color change depicted by microplate reader and converted using a standard curve. The test is relatively simple but non-specific.

Measurement of Post-Translational Modifications of Proteins

ROS can alter structure and functional integrity of specific proteins by reacting with amino acids. Several post-translational modification of proteins, including carbonylation and nitrosylation, commonly lead to dysfunctional proteins. Protein oxidation can be measured by colorimetric analysis with ELISA. The test is non-specific since most ROS will react with several amino acids to yield multiple products.

Measurement of Total Antioxidant Capacity (TAC)

It is feasible to measure levels of individual enzymatic or non-enzymatic antioxidants in a semen sample. However, TAC assesses the cumulative reducing ability of all antioxidants present in the semen and is the more commonly used technique. The reaction of seminal antioxidants against an oxidative reagent is measured by using a colorimeter or spectrophotometer, depending on the substrate (Agarwal et al., 2014; Roychoudhury et al., 2016). The colorimetric assay with the use of 2,20-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) is most widely used among multiple TAC assays. The capacity of the seminal antioxidants in the sample to inhibit ABTS oxidation is compared to that of standard Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and the result is expressed as micromoles of Trolox equivalent and the test is also known as Trolox equivalent antioxidant activity (TEAC) assay.

The lack of predictive value limits the application of TAC alone to routine infertility assessment. Its limitation is addressed by a recent study which established a cutoff for TAC assay. A diagnostic cutoff value of 1947 μM Trolox in seminal plasma distinguishes the prevalence of OS in infertile patients compared with healthy men with a sensitivity of 59.5% and specificity of 63.0% (Roychoudhury et al., 2016). The method of colorimetric TAC assay has been standardized and a kit has been used successfully with seminal plasma (Mahfouz et al., 2009).

ROS-TAC Score

The score is a novel measure of OS and minimizes the variability of ROS and TAC assays alone. Standardized values are generated from ROS and TAC results from healthy controls and a scale is created using principal component analysis and controls as reference points. The measure is superior to ROS or TAC alone in differentiating fertile from infertile men. Infertile patients have significantly lower ROS-TAC score. It is also reported that a lower score in infertile men is correlated with higher risk of prolonged inability to conceive (Sharma et al., 1999).

Proteomic Analysis

Proteomics is a large scale study of proteins by using bioinformatics tools. The use of proteomics in studying human biological system is gaining popularity in recent years and opens a new research area. Proteomic analysis in spermatozoa and seminal plasma measures ROS-induced protein alterations and demonstrates the change in sperm function as a result of post-translational modification of proteins. Global change in proteomic profiling of human spermatozoa and seminal plasma under OS conditions has been studied. It is shown that infertile patients have differential expression of proteins in relation to variations in ROS level (Ayaz et al., 2015). The identification of differentially expressed proteins in infertile men represent potential markers for ROS-induced male infertility. The understanding of function of differentially expressed proteins may reveal the underlying etiological relationship between OS and male infertility.

Redox Potential/ORP

Measurement of ORP represents an indirect assay of OS status by measuring the redox balance in a given biological system. The result reflects the overall oxidant and antioxidant activity in a sample. It differs from other assays that, rather than measuring a few markers, all known and unknown oxidants and antioxidants are measured in real time with a single test. Changes in redox potential in various body fluid has been correlated with severity of illness and mortality (Rael, 2009).

The introduction of the MiOXSYS System (Aytu BioScience, Inc.) presents a novel and comprehensive way to measure seminal OS (Fig. 3). ORP provides a snapshot of redox potential by applying a low voltage current to the sample. The results are expressed as ORP 10^{-6} sperm mL^{-1} after normalizing the sperm concentration. By using the MiOXSYS System, only a small volume of 30 μL of liquefied neat semen is required and the whole process takes less than 4 min. Sensitivity and specificity of 70.4% and 88.1% has been reported with ORP cutoff at 1.57 mV 10^{-6} sperm (Agarwal et al., 2017). Furthermore, the ORP measurement is not affected by the age of the specimen for up to 2 h. Also, fresh or frozen semen samples or seminal plasma can be used with consistent results. The positive association of ORP with conventional semen parameters has been clearly demonstrated (Zorn et al., 2003).

The clinical uses of ORP are many fold as a ready-to-use ORP assay kit potentially provides a fast screening tool in office. The result may be valuable in guiding further investigation and management plan. The measurement of ORP in cryopreserved semen samples may predict success of assisted reproductive technology. The test is also helpful in selecting patients to treatment of OS, as well as monitoring treatment response. The measurement of ORP addresses the drawback of other OS assays by providing a multi-faceted full picture of OS status. It may prove itself an easily applicable and comprehensive measure of OS in the assessment of infertile male. Table 1 compares the various more commonly used techniques in measuring seminal OS.

Types of Samples for ROS Measurement

ROS measurement can be applied to various types of semen samples. On the other hand, it is important to know the type of semen sample used in order to interpret the test result correctly.

Neat (or Unprocessed) Seminal Ejaculate

Seminal ejaculate comprises of both spermatozoa and secretions from various male sex glands. It also contains round cells and epithelial cells, and in pathological conditions, leukocytes. The setting is ideal in indicating the de novo levels of OS in the whole ejaculate.

'Wash and Resuspend' Sample

The seminal plasma which confers protection to spermatozoa is removed from a liquefied semen specimen by centrifugation. Sperm pellet is then washed and resuspended in sperm wash medium. Only cellular components including spermatozoa, round cells, white blood cells and debris are analyzed.

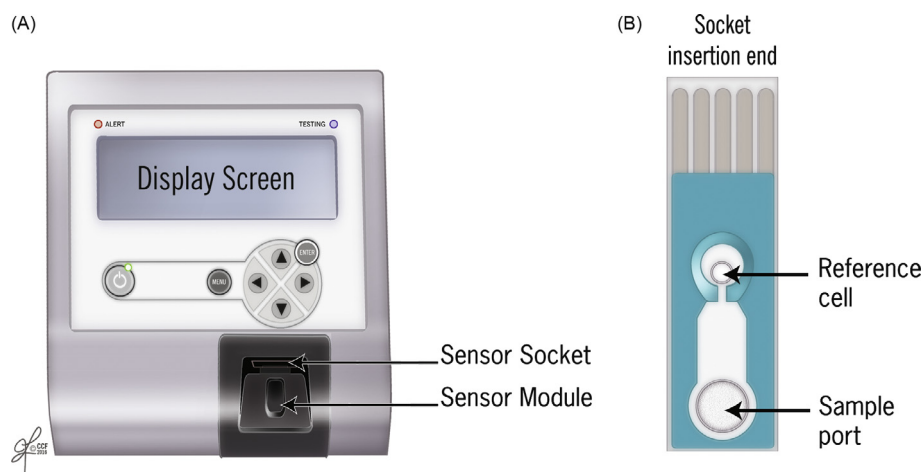


Fig. 3 MiOXSYS analyzer and sensor for measurement of oxidation–reduction potential: (A) analyzer and (B) sensor.

Table 1 Advantages and disadvantages of common techniques in oxidative stress measurement

Assay	Advantages	Disadvantages
Nitroblue tetrazolium test (NBT)	- Measures ROS generated by both spermatozoa and leukocytes - Source(s) of ROS can be differentiated, i.e. spermatozoa and/or leukocytes - High sensitivity - Inexpensive	- Scoring of relative density of formazan granules inside spermatozoa and leukocytes histochemically requires expertise - Possible inter-observer variability - Not a quantitative measure of ROS
Reactive oxygen species (ROS) by chemiluminescence	- Global level of ROS under physiological conditions can be assessed by luminol probe - Different systems of probe can be applied to the system - High sensitivity and specificity - Reference value has been reported	- Test results are affected by variables including semen age, semen volume, repeated centrifugation and background luminescence - Time-consuming - Requires expensive equipment
MDA (malonaldehyde)	- Measures byproduct of lipid peroxidation which is relatively stable compared to ROS - The measurement of MDA-TBARS (thiobarbituric acid reactive substances) adduct by colorimetry is relatively simple	The result is non-specific and provides post hoc measure only
TAC (total antioxidant capacity)	- Measures cumulative reducing ability of all antioxidants in seminal plasma - Rapid colorimetric method is available	- Measures non-enzymatic antioxidants only - Requires expensive microplate reader - The predictive value has yet to be established
ROS - TAC score	Better predictor compared to ROS and TAC alone by minimizing variability	- Requires statistical modeling - Provides a prediction of oxidative stress in a sample, but not a direct measure of ROS nor TAC
ORP (oxidation–reduction potential)	- Measures all known and unknown oxidants and antioxidants - Provides real-time assessment of redox balance - Quick and relatively simple test with ready-to-use sensor and analyzer - Applicable to various types of samples including seminal ejaculate or seminal plasma, both fresh and frozen - Requires small volume of semen for analysis - Test result not affected by semen age for up to 2 h	- Affected by viscosity of the sample - Does not provide information on individual oxidant and antioxidant which lead to the imbalanced redox state

Sample after Sperm Preparation Techniques

Swim up method select spermatozoa with high motility for ROS detection. The seminal plasma is removed from sample by centrifugation before swim up. Supernatant obtained after swim up is centrifuged again and sperm pellet resuspended for ROS measurement.

The use of double density gradient separates immature and mature spermatozoa in a sample by centrifugation. Immature sperm with poor motility and abnormal morphology, and mature sperm with good motility and normal morphology can be individually studied for ROS levels.

Conclusion

Oxidative stress is a result of imbalance between ROS levels and the scavenging antioxidant capacity. Elevated ROS levels are present in 30%–80% of infertile men. Its implication in the etiology of male infertility and sperm dysfunction resulting in lipid peroxidation of the cell membrane, sperm DNA damage and apoptosis in spermatozoa has been reported. Elevated ROS represents a common mediator between various disease conditions and decreased reproductive potential. Therefore, development of a simple and reliable technique of ROS measurement is important in the evaluation of infertile men. Direct ROS assays, including nitroblue tetrazolium test and chemiluminescence test, are the most widely used methodology for ROS measurement. The NBT assay is able to differentiate the source of ROS, i.e. spermatozoa and/or leukocytes. Chemiluminescence tests are robust and measure different intra- and extracellular ROS by using different probes. These tests are highly sensitive and specific. However, the result is affected by multiple factors including semen age. Chemiluminescence is also limited by the use of expensive equipment and technical expertise. On the other hand, indirect ROS assays measure the stable downstream products as a result of ROS-induced damage. Measurement of lipid peroxidation and post-translational protein modification are some of the techniques. The assessment of total antioxidant capacity provides a multifaceted assessment of OS and may confer more comprehensive information in combination with ROS measurement in the form of ROS–TAC score. Novel approaches including measurement of proteomics and oxidation–reduction potential opens new possibilities for evaluation of OS. They also offer an opportunity in understanding the nature and deleterious effect of ROS on male reproductive function.

Acknowledgement

The work on this manuscript was supported by the American Center for Reproductive Medicine, Cleveland Clinic, USA.

References

- Agarwal, A., Said, T. M., Bedaiwy, M. A., Banerjee, J., & Alvarez, J. G. (2006). Oxidative stress in an assisted reproductive techniques setting. *Fertility and Sterility*, 86, 503–512.
- Agarwal, A., Prabhakaran, S. A., & Sikka, S. C. (2007). Clinical relevance of oxidative stress in patients with male factor infertility: Evidence based analysis. *AUA Update Series*, 26, 1–12.
- Agarwal, A., Virk, G., Ong, C., & du Plessis, S. S. (2014). Effect of oxidative stress on male reproduction. *The World Journal of Men's Health*, 32(1), 17.
- Agarwal, A., Ahmad, G., & Sharma, R. (2015). Reference values of reactive oxygen species in seminal ejaculates using chemiluminescence assay. *Journal of Assisted Reproduction and Genetics*, 32, 1721–1729.
- Agarwal, A., Roychoudhury, S., Sharma, R., et al. (2017). Diagnostic application of oxidation-reduction potential assay for measurement of oxidative stress: Clinical utility in male factor infertility. *Reproductive Biomedicine Online*, 34, 48–57.
- Aitken, R. J., Irvine, D. S., & Wu, F. C. (1991). Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *American Journal of Obstetrics and Gynecology*, 164, 542–551.
- Ayaz, A., Agarwal, A., Sharma, R., et al. (2015). Impact of precise modulation of reactive oxygen species levels on spermatozoa proteins in infertile men. *Clinical Proteomics*, 12, 4.
- Aziz, N., Saleh, R. A., Sharma, R. K., et al. (2004). Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertility and Sterility*, 81, 349–354.
- Desai, N., Sharma, R., Makker, K., Sabanegh, E., & Agarwal, A. (2009). Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men. *Fertility and Sterility*, 92, 1626–1631.
- Esfandiari, N., Sharma, R. K., Saleh, R. A., Thomas, A. J., Jr., & Agarwal, A. (2003). Utility of the nitroblue tetrazolium reduction test for assessment of reactive oxygen species production by seminal leukocytes and spermatozoa. *Journal of Andrology*, 24, 862–870.
- Kessopoulou, E., Tomlinson, M. J., Barrat, C. L. R., et al. (1992). Origin of reactive oxygen species in human semen spermatozoa or leukocytes. *Journal of Reproduction and Fertility*, 94, 463–470.
- MacLeod, J. (1943). The role of oxygen in the metabolism and motility of human spermatozoa. *American Journal of Physiology*, 138, 512–518.
- Mahfouz, R., Sharma, R., Sharma, D., Sabanegh, E., & Agarwal, A. (2009). Diagnostic value of the total antioxidant capacity (TAC) in human seminal plasma. *Fertility and Sterility*, 91, 805–811.
- Rael, L. T. (2009). Injury severity and serum amyloid A correlate with plasma oxidation-reduction potential in multi-trauma patients: A retrospective analysis. *Scandinavian Journal of Trauma, Resuscitation and Emergency Medicine*, 17, 57.
- Roychoudhury, S., Sharma, R., Sikka, S., & Agarwal, A. (2016). Diagnostic application of total antioxidant capacity in seminal plasma to assess oxidative stress in male factor infertility. *Journal of Assisted Reproduction and Genetics*, 33, 627–635.
- Sharma, R. K., & Agarwal, A. (1996). Role of reactive oxygen species in male infertility. *Urology*, 48, 835–850.
- Sharma, R., Pasqualotto, F., Nelson, D., Thomas, A., Jr., & Agarwal, A. (1999). The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Human Reproduction*, 14, 2801–2807.
- Zorn, B., Vidmar, G., & Meden-Vrtovec, H. (2003). Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. *International Journal of Andrology*, 26, 279–285.

Further Reading

- Agarwal, A., Gupta, S., & Sharma, R. (2016). Reactive oxygen species (ROS) measurement. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility* (1st edn., pp. 155–163). Switzerland: Springer.
- Agarwal, A., Gupta, S., & Sharma, R. (2016). Oxidative-reduction potential measurement in ejaculated semen samples. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility* (1st edn., pp. 165–170). Switzerland: Springer.
- Agarwal, A., Gupta, S., & Sharma, R. (2016). Antioxidant measurement in seminal plasma by TAC assay. In A. Agarwal, S. Gupta, & R. Sharma (Eds.), *Andrological evaluation of male infertility* (1st edn., pp. 171–179). Switzerland: Springer.
- Agarwal, A., & Wang, S. M. (2017). Clinical relevance of oxidation-reduction potential in the evaluation of male infertility. *Urology*, (Feb 15) <https://doi.org/10.1016/j.urolgy.2017.02.016> (Epub ahead of print).

Sperm Morphology

Susan A Rothmann and Anna-Marie Bort, Fertility Solutions, Cleveland, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Role of Sperm Morphology

Semen analysis is a “gateway” laboratory test for male fertility evaluation, providing an initial screening test for assessing male reproductive health. Its components of spermatozoa (sperm) count, motility and morphology indirectly measure testicular health, spermatogenesis, spermiation and some sperm functional properties and can direct diagnostic or therapeutic paths for fertility treatment. Sperm morphology involves classification of sperm into a variety of types based on shape and structures that can be seen in stained semen smears using high magnification (1000×) brightfield microscopy.

Historically, sperm morphology is a predictive measure of fertility potential and treatment outcome. It is associated with fertility and pregnancy rates for natural and assisted fertilization (Aziz et al., 1996; Burr et al., 1996; De Vos et al., 2003; Francavilla et al., 1990; Elizur et al., 2004; Guzik et al., 2001; Hauser et al., 2001; Kruger et al., 1986, 1988; Lindheim et al., 1996; Menkveld et al., 1996; MacLeod, 1962; MacLeod and Gold, 1951; Toner et al., 1995), including fertilization and implantation outcomes in assisted fertilization (Aziz et al., 1996; Bourne et al., 1995; De Vos et al., 2003; Kruger et al., 1988; Osawa et al., 1999; Porcu et al., 2003; Salumets et al., 2002). It is also associated with in vitro measures of sperm binding to the oocyte zona pellucida and hyaluronic acid (Bastiaan et al., 2003; Liu and Baker, 1992, 2003; Liu et al., 2003; Menkveld and Kruger, 1996; Menkveld et al., 2003; Prinosilova et al., 2009; von Bernhardt et al., 1990). These relationships made sperm morphology a tool for choosing therapies such as varicocelectomy (Schatt et al., 1998), intra-cytoplasmic sperm injection (ICSI) or in vitro fertilization (IVF) (Porcu et al., 2003; Liu et al., 2003).

Sperm morphology also is a useful indicator of exposure to environmental and occupational toxicants such as perchlorethylene, lead, mercury, perfluorochemicals and many chlorinated or brominated hydrocarbons (Buck Louis et al., 2014; Katz, 1991; Moorman et al., 2000; Royster et al., 2000; Schrader et al., 1987, 1992). Morphology remains unchanged during overnight shipping making it a useful parameter in clinical studies requiring remote sample collection (Royster et al., 2000).

Basic Human Sperm Anatomy

The mature spermatozoan has three main regions (Fig. 1). The head of a human sperm is essentially all nucleus, tightly compacted into a very small elliptical shape approximately 4–5.5 µm long and 3 µm wide. For comparison, a red blood cell is 7–8 µm in diameter, nearly twice as large as the sperm head. The sperm head is covered partially by the acrosome, a cap-shaped organelle that contains enzymes and receptors for oocyte binding and fertilization (Fig. 2). The midpiece is 1–1.5 times the length of the head and contains dense outer longitudinal fibers that encircle a central axoneme, surrounded by a spiral array of mitochondria that provides ATP for flagellar movement (Figs. 2 and 3). The principal piece or tail is a 45–50 µm long flagellum used for propulsion through the female reproductive tract and penetration of the oocyte. Cytoplasmic remnants that represent errors in spermiation can often be seen attached to any part of the sperm but are most commonly seen on the midpiece. Immature sperm precursors can be observed in semen as spermatids or spermatocytes when differentiation is disrupted.

Sperm Classification Schemes

Many sperm classification schemes have been described during the last 60–70 years, but two main types are used now in clinical practice, Tygerberg Strict or Traditional Non-Strict. Validation of classification schemes typically compared the percentage of normal sperm to pregnancy or fertility treatment outcome, showing that normal sperm were ubiquitous in men of proven fertility and

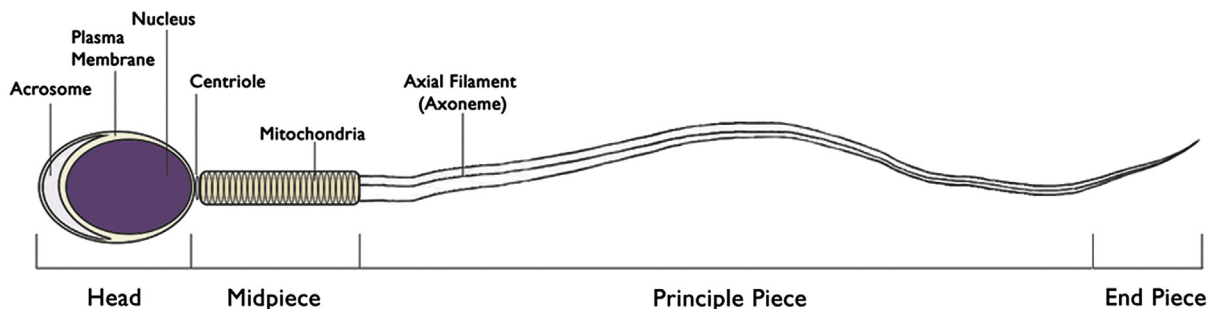


Fig. 1 Basic anatomy of a human spermatozoan.

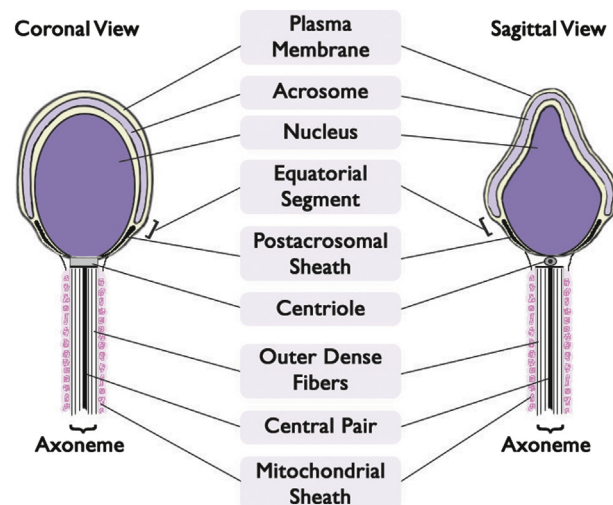


Fig. 2 Cross section of sperm in coronal and sagittal views.

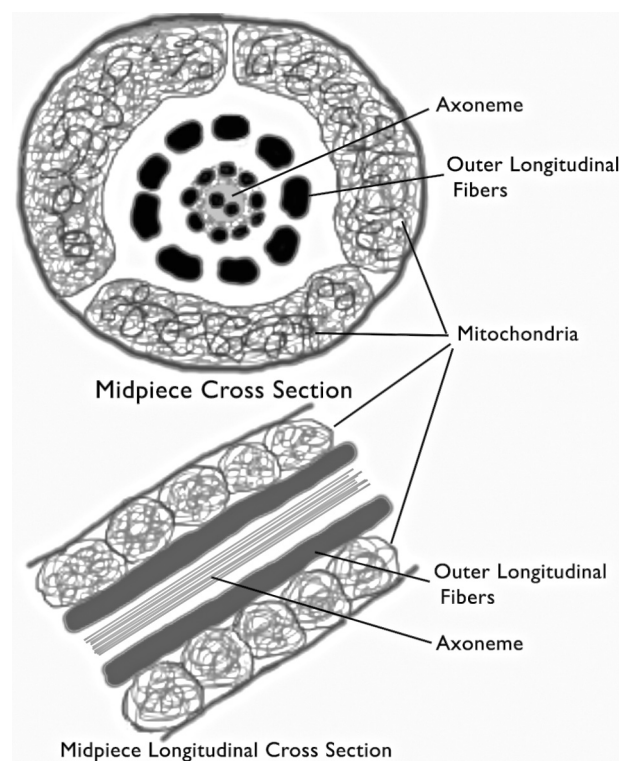


Fig. 3 Diagram of the human sperm midpiece.

relatively infrequent in infertile men (Kruger et al., 1988; MacLeod and Gold, 1951; Menkveld et al., 1990, 1991; Rehan et al., 1974). Table 1 summarizes the main differences between these types of classifications.

The first of the Traditional schemes was published by MacLeod (1962) with subsequent similar variations (Adelman and Cahill, 1989; David et al., 1975; Eliasson, 1971; Freund, 1966; World Health Organization (WHO), 1980, 1987, 1992). Normal was defined as an idealized sperm cell with an oval head, straight and regular midpiece with a tail approximately 10 times the length of the head (Fig. 4). Borderline normal sperm were classified as normal. These schemes have lower reference limits of 30%–50% normal and medians of 60%–80%.

In the mid-1980s, Roelof Menkveld in Tygerberg South Africa took a different approach to classification (Menkveld et al., 1990, 1991). Assuming that the morphologically-uniform post-coital sperm present in upper endocervical mucus represented a functional subset of ejaculated sperm, he used this homogeneous population of mostly oval-headed sperm as a reference normal prototype, defined very critically. He also defined two specific variants as borderline normal. Unlike previous schemes, the Tygerberg Strict

Table 1 Sperm classification schemes

	<i>Traditional criteria</i>	<i>Strict criteria</i>
Names	MacLoed, Eliason, David WHO 1st–3rd	Tygerberg, Menkveld, Kruger WHO 4th and 5th
Borderline normal classification	Normal	Abnormal
Lower reference limit	30%–50%	4%
Median	60%–80%	14%

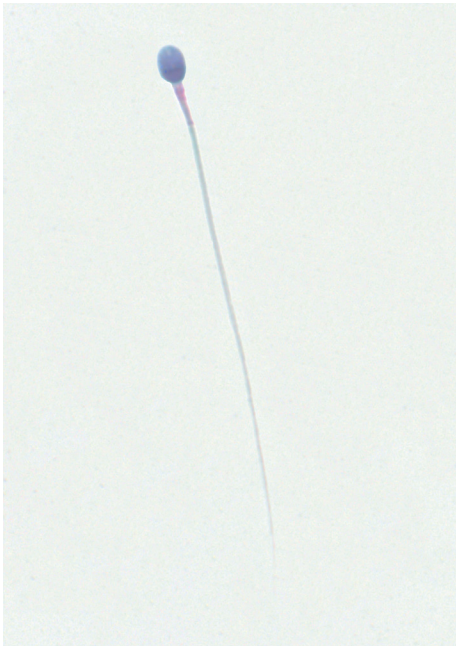


Fig. 4 Photomicrograph of prototypic normal human sperm stained with modified Papanicolaou Stain.

Criteria classified borderline as abnormal. The scheme consequently has a lower reference limit of 4% normal and a median of 14% (Cooper et al., 2010). The Tygerberg Strict Criteria became the recommended classification scheme in the Fourth and Fifth Editions of the WHO semen analysis manuals (World Health Organization, 1999, 2010).

Problems in Test Performance Have Led to Loss of Predictive Value

In spite of its known value, sperm classification is difficult to learn and perform in a consistent manner. Proficiency testing results demonstrate a wide variety of expertise where the variation among participating labs often encompasses most of the clinical range from 0% to 100% normal forms (Keel et al., 2000; Kinzer et al., 1998; Morbeck et al., 2008). Why is sperm morphology so difficult to perform in a standard way? Unlike many other species, human sperm are extremely pleiomorphic in several respects: many subtle variations of normal exist and many irregular and abnormal forms are present (Freund, 1966; Katz et al., 1986). Identification of different morphologic forms is difficult when they display continuous variation rather than precise and unique typologies (Bedford et al., 1973; Freund, 1966; Katz et al., 1986; Souchier et al., 1978), as exemplified in Fig. 5. A discrete form (e.g., oval or round) along the morphologic spectrum may be readily identifiable, but intermediate variations can be difficult to recognize and categorize (Fig. 6).



Fig. 5 Geometric progression of forms from round to elliptical to pyriform illustrates the difficulty of identifying discrete differences at individual points along the spectrum.



Fig. 6 Diagrammatic representation of the dilemma of categorizing a shape that is not clearly abnormal or normal.

Although Menkveld did not intend to define only visually “perfect” sperm as normal, as the system was adopted over time, many analysts became much more conservative in their classification, assigning normal only to “perfect” sperm. As a consequence, the degree of “strictness” varies from lab to lab, as shown in proficiency test results (Keel et al., 2002). When there is subjective application of classification schemes, individual observers or laboratories often develop variations, making comparison of results from different labs impossible (Baker et al., 1994; Chong et al., 1983; Davis and Gravance, 1994; Davis et al., 1995; Freund, 1966; Keel, 2004).

The authors’ own NIH funded research highlights the difficulty obtaining classification consensus (Bort et al., 2014; Rothmann and Bort, 2015). In a survey of 99 international experts, good agreement was present when the sperm had an obvious abnormality (Fig. 7). However, when the sperm was not grossly abnormal, agreement was terrible, with equal numbers of analysts classifying a cell in the three possible categories of normal, borderline or abnormal. Most experts reported classification using Strict Criteria but clearly there was no standard application in use.

As if classification variation was not a big enough problem, the names of various physicians and organizations (e.g. Kruger, WHO 4th and 5th Edition Manuals) who endorsed Menkveld’s Tygerberg Strict Criteria began to be used as the scheme name, creating an impression that multiple Strict schemes exist. However, they are all the same scheme using the same references to Menkveld’s original work. The name confusion is evident in proficiency testing programs that erroneously stratify answers by these many names, and often these different categories have different results and ranges of answers, which makes no sense.

Compounding the problem, Menkveld’s discrete definitions of two types of sperm with specific characteristics that should be classified as borderline normal sperm were lost on most laboratory analysts, who now typically classify anything not “perfect”

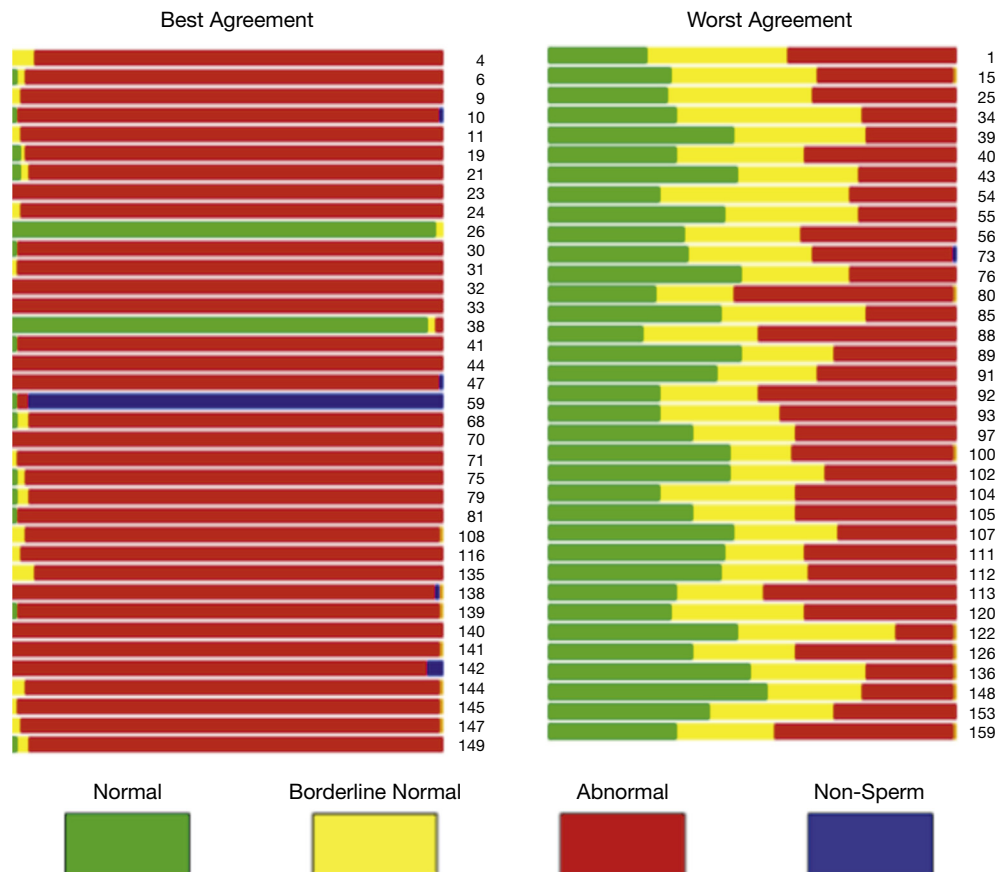


Fig. 7 Expert agreement on sperm classification. Each bar includes classification of a sperm photograph by 99 international analysts. The size of each color within the bar represents the cumulative number of analysts classifying in a specific way as shown by the color. Numbers to the right of each bar indicate photograph number.

(by their definition) and not clearly abnormal into the borderline normal category. Since these forms are combined into the abnormal category in Strict classification, the result is downward classification drift to the point where in many labs, few normal sperm are identified even in the semen of known fertile men (Menkveld, 2010).

The world-wide drop in ability to discern normal from abnormal forms led to the loss of predictive value for sperm morphology in many labs. At Mayo Clinic, a strong relationship between percent normal sperm and intra-uterine insemination outcome was completely lost as their lab analysts became more and more conservative in their classification (Morbeck et al., 2011). The problem is universal enough that some authors suggest that sperm morphology has no relevance in semen analysis (Gatimel et al., 2017). To the contrary, the problem is a profound loss of good laboratory practice throughout andrology laboratories.

The Solution—A Rational Method of Classification

Dr. Matthew Freund wrote 50 years ago “human sperm morphology has suffered from being subjective, qualitative, non-repeatable, and difficult to teach to students and technicians” (Freund, 1966). Numerous attempts to standardize sperm classification have met with limited successes and more often outright failures. All of these stem from the same problem, the lack of a consistent method or process for systematically evaluating descriptive characteristics. So how is this accomplished in other fields? Taxonomists and pathologists typically use algorithms to standardize classifications of organisms and cell types (Winston, 1999). Most sperm analysts use non-systematic practices of classification, based on different hierarchies of analysis and often different sperm morphology descriptors. Often analysts decide a sperm is normal or abnormal based on cursory examination. To develop a standard method, the authors’ research led to a novel heuristic sperm classification algorithm that could be used with existing definitions for the Tygerberg Strict Criteria Scheme or any of the Traditional Schemes.

The algorithm is a 12 query dichotomous key, that is, it uses twelve sequential binary questions that ask whether a specific characteristic is present (Fig. 8). If yes, classification is determined. If no, the analysis proceeds to the next query. If none of the abnormal or borderline normal traits are present, the sperm is classified as normal (Rothmann et al., 2013; Bort et al., 2014; Rothmann and Bort, 2015). The queries impose a standard method of classification that reduces subjective speculation about whether a sperm is normal or abnormal.

Morphology results using the Algorithm closely match the 2010 WHO reference distributions with a median of 14%. It produces reproducible results between technologists, permits simultaneous Strict and Traditional classification and reduces the tendency for overly conservative analysis that has diminished sperm morphology’s predictive value. Results obtained using the Algorithm showed predictive value associated with fecundity in the NIH Life Study (Buck Louis et al., 2014). Using prospectively measured time to pregnancy, Strict and Traditional morphology were associated with significantly shorter time to pregnancy fecundability odds ratios (FOR) > 1. Significantly longer time to pregnancy with FORs < 1 were observed for amorphous and round sperm heads and neck/midpiece abnormalities. Workshops at the American Society of Andrology demonstrated that the method is easy to learn and improves consensus (Bort et al., 2014).

Specific Sperm Abnormalities

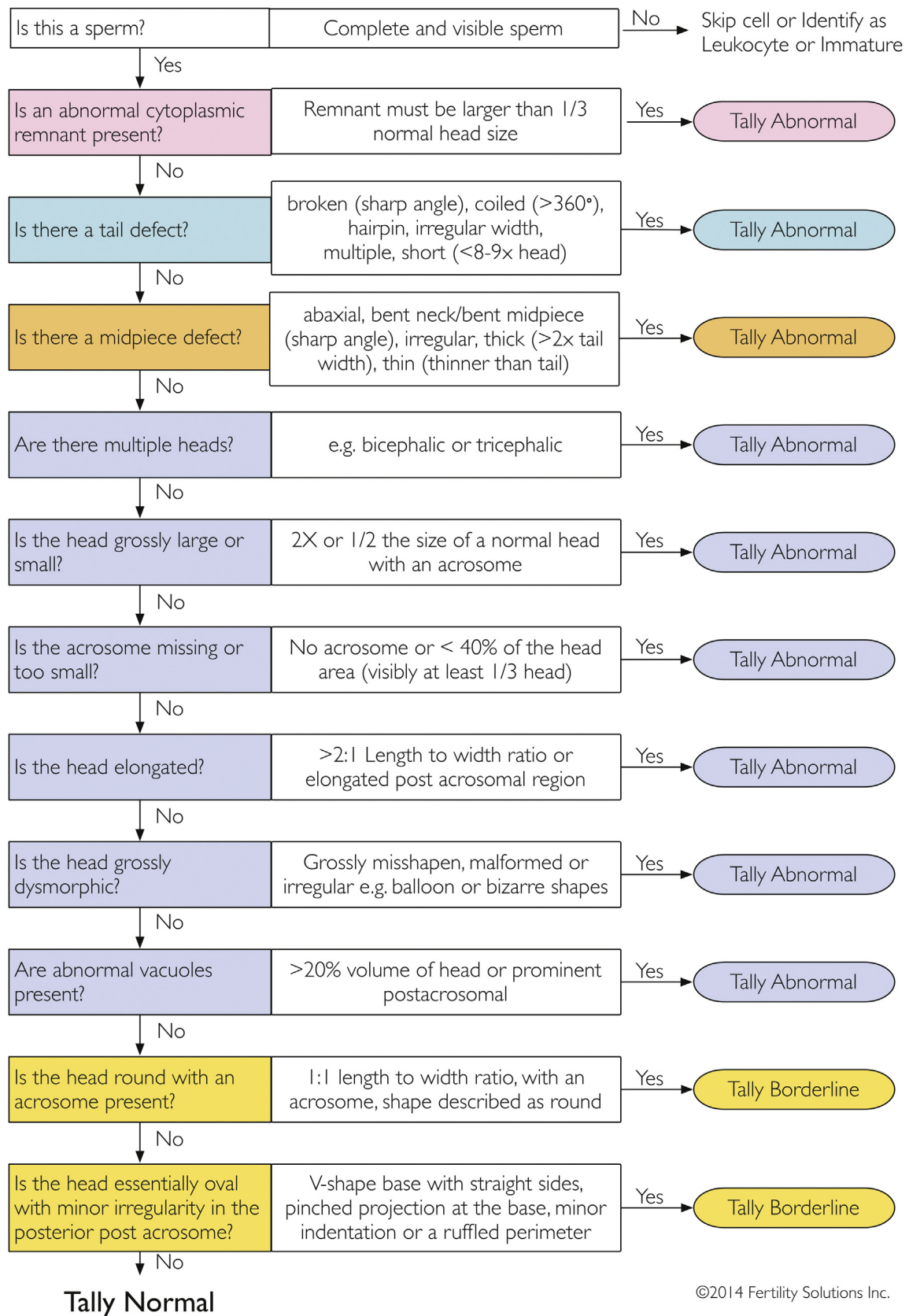
Although the most recent edition of the WHO Manual did not advocate differential analysis of the sperm, many authors disagree (Amann, 2010; Auger et al., 2016; Eliasson, 2003; Menkveld, 2010). Some sperm morphology variants are absolutely linked to pathology and increasingly are linked to specific human genes (Carrell, 2007; Krausz and Sassone-Corsi, 2005; Golas et al., 2008). As more are identified, the value of performing a sperm morphology differential should be increased because it will serve as a screen for investigating high probability genes. Specific sperm anatomic defects are summarized below. Many sperm have more than one abnormality. Various types of multi-entry differentials can be used to classify all defects or the predominant one in each of the three regions of each sperm (head, midpiece and tail) plus any abnormal cytoplasmic remnant.

Cytoplasmic Remnant

Cytoplasmic remnants, also termed cytoplasmic droplets and excess residual cytoplasm, should normally be removed from the round spermatid during passage through the epididymis. Cytoplasmic remnants smaller than one-third the head area of a normal sized sperm are considered normal, but remnants larger than one-third that size are abnormal. Excess cytoplasm reduces sperm function due to release of reactive oxygen species (Cooper, 2011). Remnants are usually located in the neck/midpiece region, but occasionally appear along the tail or around the head.

Tail

Many abnormalities of the sperm flagellum are found, including bent or broken, hairpin, short, coiled and thick. When seen in large numbers, they may be associated with athenozoospermia (poor motility). Several genes have been implicated in defective tail morphology. Multi-flagellate sperm have been linked to a variety of chromosomal aberrations (Sun et al., 2006).



©2014 Fertility Solutions Inc.

Fig. 8 Sperm confirm algorithm for morphology classification.

Midpiece

Thick midpieces are 1.5–2 times wider than normal and typically resemble a tapered golf tee shape. Ultrastructurally, they show mitochondrial misalignment and are associated with low sperm aster formation rates, probably due to dysfunction of the centrosome contained within the midpiece (Ugajin et al., 2010). Thin midpieces occur when the mitochondrial sheath loosens and separates from the underlying axoneme, exposing the thinner filamentous core (Ben Khelifa et al., 2014; Chemes et al., 1998). These disorders have been linked to a variety of gene mutations. Midpieces that are bent or have abaxial orientation to the tail have disordered or missing annulus (Lhuillier et al., 2009).

Multiple Heads

Bicephalic and tricephalic sperm arise from non-disjunctive events and failure of cytokinesis. Depending on the stage affected, the entire sperm including midpiece and tail also may be replicated.

Elongated Heads

Elongated heads where the length is two times the width or greater result from sperm maturation arrest during spermiation. Elongation can be manifested as an overall elongated shape like a cigar, pyriform where the post-acrosome is elongated to a point, or long dumbbell shape. Elongated sperm are more common in men who have experienced extensive febrile illness or hyperthermia associated with prolonged hot bath exposures (Eliasson, 1971; MacLeod and Gold, 1951; Mieusset et al., 1987).

Head Size

Head size abnormalities are associated with lower fecundity. Sperm with macrocephalic heads have a high frequency of polyploidy that arises from early non-disjunctive events (Sun et al., 2006). The rare syndrome of 100% macrocephaly is associated with mutations in the aurora kinase (AURKC) gene (El Kerch et al., 2011). A reversible form of macrocephaly is associated with sulphasalazine (Cosentino et al., 1984). Sperm with small sperm heads usually have small acrosomes leading to lower fertilization potential (Menkveld et al., 2003). Sperm size defects are found in higher numbers in men with testicular germ cell tumors (Auger et al., 2016).

Acrosome Absence or Size

When the acrosome is absent, the sperm usually has a spherical shape, appearing round on the stained smear. When most or all of the sperm are round without acrosomes, a rare clinical condition called globozoospermia is present. These sperm cannot penetrate the ovum but can be fertile if injected into the ovum using ICSI. Several gene mutations have been identified in globozoospermia, especially deletions of DPY19L2 on chromosome 12 that plays a role in acrosome formation and sperm elongation (Coutton et al., 2015; Elinati et al., 2012; Kosciński et al., 2011; Zhu et al., 2013).

In critical settings, measurement may be required. Small acrosomes that are less than one third of the head area are associated with premature sperm cell death (Menkveld et al., 2003) and early embryo development failure (Kihaila et al., 2003). Large acrosomes appear to have the same functional ability as normal size acrosomes (Menkveld et al., 2003).

Dysmorphic Heads

The presence of bizarre shaped heads with severe deformities and irregularities is common in human ejaculates but rarely observed in cervical mucus.

Vacuoles

Vacuoles are voids and craters in the surface of the sperm. The presence of a few small vacuoles is not abnormal, but multiple vacuoles that occupy > 20% of the head area and prominent vacuoles in the post acrosome are associated with reduced fertilizing capacity. Unfortunately, vacuoles can be induced by air drying, leading to significant over-classification of the abnormality in semen smears that are not fixed immediately while the semen is still wet. If vacuoles are found in all sperm and every ejaculate, procedures for smearing and fixation should be modified.

Borderline Normal Heads

Menkveld defined specific head shapes for this category with slight deviation from oval that include round heads with an acrosome or heads with minor irregularity in the post-acrosome ("V" shape without elongation, pinched projection at base of head, or irregular perimeter (Menkveld et al., 1990). All are observed in sperm that exhibit binding to hyaluronan in vitro (Prinosilova et al., 2009), suggesting that they have normal function.

Laboratory Practice

No matter what the classification scheme or method to employ it, good laboratory practice is the underpinning of quality sperm morphology. Unfortunately, it is often lacking in semen analysis (Chong et al., 1983). Automation has been largely unsuccessful, in part because sperm are so small compared to the other cells typically analyzed in the clinical laboratory, and because the market for such instrumentation is small. Manual microscopy remains the most reliable method.

Before the cells can be examined, the semen must be smeared correctly and fixed while wet to reduce artifacts of air drying like vacuolization and to improve semen adherence to the slide. Alcohol-based spray fixatives should be applied immediately after the smear is made. The gold standard for staining sperm morphology smears is modified Papanicolaou stain, which does not stain the background protein and mucoid material in the seminal plasma, giving excellent contrast to the sperm features and making sperm fine structures easier to discriminate. Many common stains commonly used for semen analysis are appropriate for blood but not semen, such as Wright Giemsa or commercial quick stains. These stain the background seminal plasma, dramatically lowering contrast and often obscuring features and structures, including tails.

Usefulness of morphology also depends on good training and a rigorous quality control (QC) program to establish and monitor consistency and agreement among technologists, as required by regulatory and accreditation agencies (Franken et al., 2000). One smear analyzed repeatedly over long periods of time shows classification stability and gives trending data that can be lost if too many QC smears are rotated (Rothmann et al., 2013; Rothmann and Bort, 2015). If the lab reference range differs from the WHO reference interval and median, the QC smear results should be established by a lab with techniques that produce with WHO 5th distribution of values. Virtual smears and high quality photographs of individual sperm cells allow exact comparison among multiple analysts and can help prevent drift and shift in classification.

Reference Limits

Reference intervals give an expected range of values of a given parameter within a defined population group. The median, upper and lower limits can be calculated from the group's results. For sperm morphology, the upper limit is not known to have negative consequences and is disregarded. The lower limit defines the value that is considered abnormal and is usually the lower 5th or 10th centile, depending on the rigor of testing and confidence in the population.

In 2010, reference intervals for sperm morphology using the Tygerberg Strict Criteria were published, based on retrospective review of publications that were considered comparable (Cooper et al., 2010). Sperm morphology of "Fertile Fathers," men whose partners became pregnant within 12 months of unprotected intercourse, had a lower (5th centile) reference limit of 4% normal forms and a median of 15% normal. The study also defined limits for a "General Population" group of men whose fertility was not known (Table 2).

A laboratory's range of values should encompass the spectrum described by the reference population. For Strict classification, 95% of results should be higher than 4%–5% normal with a median of 14%–15% normal. Too many analysts and physicians misinterpret lower reference limit as the median. Although men who are undergoing fertility evaluation with their partner will have a downward shift in the distribution of percent normal sperm, it is highly unlikely that all of them will be below the lower reference limit, especially if fertilization and pregnancies are occurring. Labs whose normal morphology never exceed 5%–10% normal should recalibrate their classification.

Summary

Sperm morphology is an important component of male reproductive health evaluation with predictive value when carefully performed. The consequences of incorrect sperm classification can be expensive, invasive treatment or inappropriate focus on finding pathology in the female partner. The need for improvement in morphology performance is essential. With intra-cytoplasmic sperm injection the most common treatment of male infertility, abnormal genes that cause sperm morphology defects could be transmitted to offspring. Rational classification methods based on standardized algorithms, high quality microscopic techniques and good quality control could restore and improve the predictive value of sperm morphology classification and its utility as a screening tool for male reproductive health.

Table 2 Normal sperm morphology reference limits for strict criteria

Reference group	# Men in reference group	Lower reference limit	Median	Total normal in ejaculate (million per mL)
Fertile fathers with time to pregnancy 12 months or less	1851	4%	15%	37
Men unscreened for fertility (general population)	137	5%	14%	29

Data from Cooper TG, Noonan E, Eckardstein S, Auger J, Gordon Baker HW Behre H, Haugen TR, Kruger T, Wang C, Mbizvo MT, Vogelson KM. (2010) World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 16, 231–245 and World Health Organization. (2010) *WHO Laboratory Manual for the Examination and Processing of Human Semen, 5th Edition*. Geneva, Switzerland: WHO Press

References

- Adelman, M. M., & Cahill, E. M. (1989). *Atlas of Sperm Morphology*. Chicago: American Society of Clinical Pathologists.
- Amann, R. P. (2010). Tests to measure the quality of spermatozoa at spermiation. *Asian Journal of Andrology*, 12, 71–78.
- Auger, J., Jouannet, P., & Eustache, F. (2016). Another look at human sperm morphology. *Human Reproduction*, 31, 10–23.
- Aziz, N., Buchan, I., Taylor, C., Kingsland, C. R., & Lewis-Jones, I. (1996). The sperm deformity index: A reliable predictor of the outcome of oocyte fertilization in vitro. *Fertility and Sterility*, 66, 1000–1008.
- Baker, D. J., Paterson, M. A., Klaassen, J. M., & Wyrick-Glatzel, J. (1994). Semen evaluations in the clinical laboratory: How well are they being performed? *Laboratoriums Medizin*, 25, 509–514.
- Bastiaan, H. S., Windt, M. L., Menkveld, R., Kruger, T. F., Oehninger, S., & Franken, D. R. (2003). Relationship between zona pellucida-induced acrosome reaction, sperm morphology, sperm-zona pellucida binding, and in vitro fertilization. *Fertility and Sterility*, 79, 49–55.
- Bedford, J., Calvin, H., & Cooper, G. W. (1973). The maturation of spermatozoa in the human epididymis. *Journal of Reproduction and Fertility*, 18(Suppl), 199–213.
- Ben Khelifa, M., Coutton, C., Zouari, R., Karaouzene, T., Rendu, J., Bidart, M., Yassine, S., Pierre, V., Delaroche, J., Hennebicq, S., Grunwald, D., Escalier, D., Pernet-Gallay, K., Jouk, P. S., Thierry-Mieg, N., Touré, A., Arnoult, C., & Ray, P. F. (2014). Mutations in DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple morphological abnormalities of the sperm flagella. *American Journal of Human Genetics*, 94, 95–104.
- Bort, A. M., Rothmann, S. A., Quigley, J. R., & Pillow, R. L. (2014). Sperm morphology using a novel dichotomous key algorithm improves analysis stability, reproducibility and teachability. *Andrology*, 2(Suppl), 103.
- Bourne, H., Richings, N., Liu, D. Y., Clarke, G. N., Harari, O., & Baker, H. W. (1995). Sperm preparation for intracytoplasmic injection: Methods and relationship to fertilization results. *Reproduction, Fertility, and Development*, 7, 177–183.
- Buck Louis, G. M., Sundaram, R., Schisterman, E. F., Sweeney, A. M., Lynch, C. D., Kim, S., Maisog, J., Gore-Langton, R., Eisenberg, M. I., & Chen, Z. (2014). Semen quality and time to pregnancy: The longitudinal investigation of fertility and the environment study. *Fertility and Sterility*, 101, 453–462.
- Burr, R. W., Sieberg, R., Flaherty, S. P., Wang, X. J., & Matthews, C. D. (1996). The influence of sperm morphology and the number of motile sperm inseminated on the outcome of intrauterine insemination combined with mild ovarian stimulation. *Fertility and Sterility*, 65, 127–132.
- Carrell, D. T. (2007). The genetics of male infertility in the era of genomics: Tools for progress. In D. T. Carrell (Ed.), *The genetics of male infertility* (pp. 3–27). Totowa: Humana Press.
- Chemes, H. E., Olmedo, S. B., Carrere, C., Osés, R., Carizza, C., Leisner, M., & Blaquier, J. (1998). Ultrastructural pathology of the sperm flagellum: Association between flagellar pathology and fertility prognosis in severely asthenozoospermic men. *Human Reproduction*, 13, 2521–2526.
- Chong, A. P., Walters, C. A., & Weinrieb, S. A. (1983). The neglected laboratory test: The semen analysis. *Journal of Andrology*, 4, 280–282.
- Cooper, T. G. (2011). The epididymis, cytoplasmic droplets and male fertility. *Asian Journal of Andrology*, 13, 130–138.
- Cooper, T. G., Noonan, E., Eckardstein, S., Auger, J., Gordon Baker, H. W., Behre, H., Haugen, T. R., Kruger, T., Wang, C., Mbizvo, M. T., & Vogelstein, K. M. (2010). World Health Organization reference values for human semen characteristics. *Human Reproduction Update*, 16, 231–245.
- Cosentino, M. J., Cher, W. Y., Takihara, H., & Cockett, A. T. (1984). The effects of sulfasalazine on human male fertility potential and seminal prostaglandins. *The Journal of Urology*, 132, 682–686.
- Coutton, C., Escoffier, J., Martinez, G., Arnoult, C., & Ray, P. R. (2015). Teratozoospermia: Spotlight on the main genetic actors in the human. *Human Reproduction Update*, 21, 455–485.
- David, G., Bisson, J. P., Czyglik, F., Jouannet, P., & Gernigon, C. (1975). Anomalies morphologiques du spermatozoïde humain. Propositions pour un système de classification. *Journal de Gynécologie, Obstétrique et Biologie de la Reproduction*, 4, 17–36.
- Davis, R. O., & Gravance, C. G. (1994). Consistency of sperm morphology classification methods. *Journal of Andrology*, 15, 83–91.
- Davis, R. O., Gravance, C. G., & Overstreet, J. W. (1995). A standardized test for visual analysis of human sperm morphology. *Fertility and Sterility*, 63, 1058–1063.
- De Vos, A., Van De Velde, H., Joris, H., Verheyen, G., Devroey, P., & Van Steirteghem, A. (2003). Influence of individual sperm morphology on fertilisation, embryo morphology, and pregnancy outcome of intracytoplasmic sperm injection. *Fertility and Sterility*, 79, 42–48.
- El Kerch, F., Lamzouri, A., Laarabi, F. Z., Zahi, M., Ben Amar, B., & Sefiani, A. (2011). Confirmation of the high prevalence in Morocco of the homozygous mutation c.144delC in the aurora kinase C gene (AURKC) in the teratozoospermia with large-headed spermatozoa. *Journal de Gynécologie, Obstétrique et Biologie de la Reproduction*, 40, 329–333.
- Eliasson, R. (1971). Standards for the investigation of human semen. *Andrologia*, 3, 549–564.
- Eliasson, R. (2003). Basic semen analysis. In P. Matson (Ed.), *Current topics in andrology* (pp. 35–89). Perth: Ladybrook Publishing.
- Elinati, E., Kuentz, P., Redin, C., Jaber, S., Vanden Meerschaut, F., Makarian, J., Kosciński, I., Nasr-Esfahani, M. H., Demirel, A., Gurgan, T., Louanjli, N., Iqbal, N., Bisharah, M., Pigeon, F. C., Gourabi, H., De Briel, D., Brignon, F., Gitlin, S. A., Grillo, J. M., Ghaedi, K., Deemeh, M. R., Tanhaei, S., Modarres, P., Heindryckx, B., Benkhalifa, M., Nikiforaki, D., Oehninger, S. C., De Sutter, P., Muller, J., & Viville, S. (2012). Globozoospermia is mainly due to DPY19L2 deletion via non-allelic homologous recombination involving two recombination hotspots. *Human Molecular Genetics*, 21, 3695–3702.
- Elizur, S. E., Levron, J., Seidman, D. S., Kees, S., Levran, D., & Dor, J. (2004). Conventional in vitro fertilization versus intracytoplasmic sperm injection for sibling oocytes in couples with mild oligoteratoasthenozoospermia and couples with normal sperm. *Fertility and Sterility*, 2, 241–243.
- Francavilla, F., Romano, R., Santucci, R., & Poccia, G. (1990). Effect of sperm morphology and motile sperm count on outcome of intrauterine insemination in oligozoospermia and/or asthenozoospermia. *Fertility and Sterility*, 53, 892–897.
- Franken, D. R., Barendsen, R., & Kruger, T. F. (2000). A continuous quality control program for strict sperm morphology. *Fertility and Sterility*, 74, 721–724.
- Freund, M. (1966). Standards for the rating of human sperm morphology: A cooperative study. *International Journal of Fertility*, 11, 97–118.
- Gatimel, N., Moreau, J., Parinaud, J., & Leandri, R. D. (2017). Sperm morphology: Assessment, pathophysiology, clinical relevance, and state of the art in 2017. *Andrology*, 5, 845–862.
- Golas, A., Dzieza, A., Kuzniarz, K., & Styra, J. (2008). Gene mapping of sperm quality parameters in recombinant inbred strains of mice. *The International Journal of Developmental Biology*, 52, 287–293.
- Guzick, D. S., Overstreet, J. W., Factor-Litvak, P., Brazil, C. K., Nakajima, S. T., Coutifaris, C., Carson, S. A., Cisneros, P., Steinkampf, M. P., Hill, J. A., Xu, D., Vogel, D. L., & National Cooperative Reproductive Medicine. Network. (2001). Sperm morphology, motility and concentration in fertile and infertile men. *The New England Journal of Medicine*, 345, 1388–1398.
- Hauser, R., Yogev, L., Botchan, A., Lessing, J. B., Paz, G., & Yavetz, H. (2001). Intrauterine insemination in male factor subfertility: Significance of sperm motility and morphology assessed by strict criteria. *Andrologia*, 33, 13–17.
- Katz, D. F. (1991). Human sperm as biomarkers of toxic risk and reproductive health. *Journal of NIH Research*, 3, 63–67.
- Katz, D. F., Overstreet, J. W., Samuels, S. J., Niswander, P. W., Bloom, T. D., & Lewis, E. L. (1986). Morphometric analysis of spermatozoa in the assessment of human male fertility. *Journal of Andrology*, 7, 203–210.
- Keel, B. A. (2004). How reliable are the results from the semen analysis? *Fertility and Sterility*, 82, 41–44.
- Keel, B. A., Quinn, P., Schmidt, C. F., Serafy, N. T., Jr., Serafy, N. T., Sr., & Schalue, T. K. (2000). Results of the American Association of Bioanalysts national proficiency testing programme in andrology. *Human Reproduction*, 15, 680–686.
- Keel, B. A., Stemberge, T., Pineda, G., & Serafy, N. T., Sr. (2002). Lack of standardization in performance of the semen analysis among laboratories in the United States. *Fertility and Sterility*, 7, 603–608.

- Kihaille, P., Hirotsuru, K., Kumasako, Y., Misumi, J., & Utsunomiya, T. (2003). Fertilization rates of small-headed sperm in conventional IVF and ICSI. *Archives of Andrology*, 49, 327–329.
- Kinzer, D. R., Caruso, C., Quigley, J., & Rothmann, S. A. (1998). Sperm morphology analysis: Problems as demonstrated by proficiency testing. *Journal of Andrology*, 19(suppl), 54.
- Koscinski, I., Elinati, E., Fossard, C., Redin, C., Muller, J., Velez de la Calle, J., Schmitt, F., Ben Khelifa, M., Ray, P. F., Kilani, Z., Barratt, C. L., & Virile, S. (2011). DPY19L2 deletion as a major cause of globozoospermia. *American Journal of Human Genetics*, 88, 344–350.
- Krausz, C., & Sassone-Corsi, P. (2005). Genetic control of spermiogenesis: Insights from the CREM gene and implications for human infertility. *Reproductive Biomedicine Online*, 10, 64–71.
- Kruger, T. F., Menkveld, R., Stander, F. S. H., Lombard, C. J., van der Merwe, J. P., van Zyl, J. A., & Smith, K. (1986). Sperm morphologic features as a prognostic factor in vitro fertilization. *Fertility and Sterility*, 46, 1118–1123.
- Kruger, T. F., Acosta, A. A., Simmons, K. F., Swanson, R. J., Matta, J. F., & Oehninger, S. (1988). Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertility and Sterility*, 49, 112–117.
- Lhuillier, P., Rode, B., Escalier, D., Lorès, P., Dirami, T., Bienvenu, T., Gacon, G., Dulioust, E., & Touré, A. (2009). Absence of annulus in human asthenozoospermia: Case report. *Human Reproduction*, 24, 1296–1303.
- Lindheim, S., Barad, D., Zinger, M., Witt, B., Amin, H., & Cohen, B. (1996). Abnormal sperm morphology is highly predictive of pregnancy outcome during controlled ovarian hyperstimulation and intrauterine insemination. *Journal of Assisted Reproduction and Genetics*, 13, 569–572.
- Liu, D. Y., & Baker, H. W. (1992). Morphology of spermatozoa bound to the zona pellucida of human oocytes that failed to fertilize in vitro. *Journal of Reproduction and Fertility*, 94, 71–84.
- Liu, D. Y., & Baker, H. W. (2003). Disordered zona pellucida induced acrosome reaction and failure of in vitro fertilization in patients with unexplained infertility. *Fertility and Sterility*, 79, 74–80.
- Liu, D. Y., Garrett, G., & Baker, H. W. (2003). Low proportion of sperm can bind to the zona pellucida of human oocytes. *Human Reproduction*, 18, 2382–2389.
- MacLeod, J. (1962). A possible factor in the etiology of human male infertility; preliminary report. *Fertility and Sterility*, 13, 29–33.
- MacLeod, J., & Gold, R. Z. (1951). The male factor in fertility and infertility. IV. Sperm morphology in fertile and infertile marriage. *Fertility and Sterility*, 2, 394–414.
- Menkveld, R. (2010). Clinical significance of the low normal sperm morphology value as proposed in the fifth edition of the WHO Laboratory manual for the examination and processing of human semen. *Asian Journal of Andrology*, 12, 47–58.
- Menkveld, R., & Kruger, T. F. (1996). Basic semen analysis. In A. A. Acosta, & T. F. Kruger (Eds.), *Human spermatozoa in assisted reproduction* (pp. 53–71). Camforth, England: Parthenon Publishing.
- Menkveld, R., Stander, F. S. H., Kotze, T. J., & van Zyl, J. A. (1990). The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Human Reproduction*, 5, 586–592.
- Menkveld, R., Oettle, E. E., Kruger, T. F., Swanson, R. J., Acosta, A. A., & Oehninger, S. (1991). *Atlas of human sperm morphology*. Baltimore: Williams and Wilkins.
- Menkveld, R., Rhemrev, J. P. T., Franken, D. R., Vermeiden, J. P. W., & Kruger, T. F. (1996). Acrosomal morphology as a novel criterion for male fertility diagnosis: Relation with acrosin activity, morphology (strict criteria), and fertilization in vitro. *Fertility and Sterility*, 65, 637–644.
- Menkveld, R., El-Garem, Y., Schill, W., & Henkel, R. (2003). Relationship between human sperm morphology and acrosomal function. *Journal of Assisted Reproduction and Genetics*, 20, 432–438.
- Mieusset, r., Bujan, L., Mansat, A., Pontonnier, F., & Grandjean, H. (1987). Effects of artificial cryptorchidism on sperm morphology. *Fertility and Sterility*, 47, 150–155.
- Moorman, W. J., Cheever, K. L., Skaggs, S. R., Clark, J. C., Turner, T. W., Marlow, K. L., & Schrader, S. M. (2000). Male adolescent exposure to endocrine-disrupting pesticides: Vinclozolin exposure in peripubertal rabbits. *Andrologia*, 32, 285–293.
- Morbeck, D., Klug, M., Fredrickson, J., Rollene, N., Hughes, P., & Krenik, A. (2008). Virtual sperm morphology images as a tool to build consensus among technologists and to identify specific morphologic criteria that affect grading variability. *Journal of Andrology*, 39 (Abstract Suppl).
- Morbeck, D. E., Leonard, P. H., Al, W., Shimek, K. M., EVA, B., & Coddington, C. C. (2011). Sperm morphology: Classification drift over time and clinical implications. *Fertility and Sterility*, 96, 1350–1354.
- Osawa, Y., Sueoka, K., Iwata, S., Shinohara, M., Konayashi, N., Kuji, N., & Yoshimura, Y. (1999). Assessment of the dominant abnormal form is useful for predicting the outcome of intracytoplasmic sperm injection in the case of severe teratozoospermia. *Journal of Assisted Reproduction and Genetics*, 16, 436–442.
- Porcu, G., Mercier, G., Boyer, P., Achard, V., Banet, J., Vasserot, M., Melone, C., Salas-Magnan, J., D'Ercole, C., Chau, C., & Guichaoua, M. R. (2003). Pregnancies after ICSI using sperm with abnormal head-tail junction from two brothers: Case report. *Human Reproduction*, 18, 562–567.
- Prinosilova, P., Kruger, T., Sati, L., Okavukcu, S., Vigue, L., Kovanci, E., & Huszar, G. (2009). *Reproductive Biomedicine Online*, 28, 177–183.
- Rehan, N. E., Sobrero, A. J., & Fertig, J. W. (1974). The semen of fertile men: Statistical analysis of 1,300 men. *Fertility and Sterility*, 26, 408–413.
- Rothmann, S. A., & Bort, A. (2015). *SpermConfirm morphology classification method*. Cleveland: Fertility Solutions.
- Rothmann, S. A., Bort, A., Quigley, J. R., & Pillow, R. (2013). Sperm morphology classification: A rational method for schemes adopted by the World Health Organization. *Methods in Molecular Biology*, 927, 27–37.
- Royster, M. O., Lobdell, D. T., Mendola, P., Perreault, S. D., Selevan, S. G., Rothmann, S. A., & Robbins, W. A. (2000). Evaluation of a container for collection and shipment of semen with potential uses in population-based, clinical, and occupational settings. *Journal of Andrology*, 21, 478–484.
- Salumets, A., Suikkari, A. M., Mols, T., Soderstrom-Anttila, V., & Tuuri, T. (2002). Influence of oocytes and spermatozoa on early embryonic development. *Fertility and Sterility*, 78, 1082–1087.
- Schatt, E. C., Hirschberg, S. J., Fallick, M. L., Lipschultz, L. I., & Kim, E. D. (1998). Varicocelectomy improves sperm strict morphology and motility. *The Journal of Urology*, 16, 1338–1340.
- Schrader, S. M., Ratcliffe, J. M., Turner, T. W., & Hornung, (1987). The use of new field methods of semen analysis in the study of occupational hazards to reproduction: The example of ethylene dibromide. *Journal Occupational Medicine*, 29, 963–966.
- Schrader, S. M., Chapin, R. E., Clegg, E. D., Davis, R. O., Fourcroy, J. L., Katz, D. L., Rothmann, S. A., Toth, G., Turner, T. W., & Zinaman, M. (1992). Laboratory methods for assessing human semen in epidemiologic studies: A consensus report. *Reproductive Toxicology*, 6, 275–279.
- Souchier, C., Czyba, J., & Grantham, R. (1978). Difficulties in morphologic classification of human spermatozoa. *The Journal of Reproductive Medicine*, 21, 244–248.
- Sun, F., Ko, D., & Martin, R. H. (2006). Is there a relationship between sperm chromosome abnormalities and sperm morphology? *Reproductive Biology and Endocrinology*, 4, 1–5.
- Toner, J. P., Mossad, H., Grow, D. R., Morshedi, M., Swanson, R. J., & Oehninger, S. (1995). Value of sperm morphology assessed by strict criteria for prediction of the outcome of artificial (intrauterine) insemination. *Andrologia*, 27, 143–148.
- Ugajin, T., Terada, Y., Hasegawa, H., Nabeshima, H., Suzuki, K., & Yaegashi, N. (2010). The shape of the sperm midpiece in intracytoplasmic morphologically selected sperm injection relates to sperm centrosomal function. *Journal of Assisted Reproduction and Genetics*, 27, 75–81.
- von Bernhardt, R., de Ioannes, A. E., Blanco, L. P., Herrera, E., Bustos-Obregon, E., & Vigil, P. (1990). Round-headed spermatozoa: A model to study the role of the acrosome in early events of gamete interaction. *Andrologia*, 22, 12–20.
- Winston, J. E. (1999). *Describing Species: Practical Taxonomic Procedure for Biologists*. New York: Columbia University Press.
- World Health Organization. (1980World). *Who laboratory manual for the examination of human semen and sperm-cervical mucus interactions*. Singapore: Press Concern.
- World Health Organization. (1987World). *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions* (2nd edn.). Cambridge, UK: Cambridge University Press.
- World Health Organization. (1992World). *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions* (3rd edn.). Cambridge, UK: Cambridge University Press.

- World Health Organization. (1999World). *WHO Laboratory Manual For The Examination Of Human Semen And Sperm-Cervical Mucus Interactions* (4th edn.). Cambridge, UK: Cambridge University Press.
- World Health Organization. (2010World). *WHO Laboratory manual for the examination and processing of human semen* (5th edn.). Geneva, Switzerland: WHO Press.
- Zhu, F., Gong, F., Lin, G., & Lu, G. (2013). DPY19L2 gene mutations are a major cause of globozoospermia: Identification of three novel point mutations. *Molecular Human Reproduction*, 19, 395–404.

Further Reading

- Auger, J. (2010). Assessing human sperm morphology: Top models, underdogs or biometrics? *Asian Journal of Andrology*, 12, 36–46.

Laboratory Evaluation of Sperm in Cervical Mucus

Grace M Centola, Reproductive Laboratory and Tissue Bank Consultant, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Determination of the capacity of the sperm to pass through the woman's cervical mucus at the time of intercourse is an important adjunct for clinicians to use in diagnosis and management of the infertile couple. The choice of which additional test(s) to perform, and when to perform them, are dependent on the initial evaluation of the male and female partners.

With natural conception, the sperm are deposited at the cervix, must swim through the cervical mucus into the uterus, and pass into the oviducts or fallopian tubes to arrive at the site of fertilization. Since the cervical mucus is the first barrier for the sperm entering the female reproductive tract, assessment of the interaction between the sperm and the cervical mucus can be an important step in fertility diagnostics.

An in vivo test of sperm–cervical mucus interaction is referred to as the postcoital test (PCT), Sims test, Sims–Huehner test, or Huehner test. In vitro laboratory assessment of sperm–cervical mucus interaction is referred to simply as a simple slide test or Kremer Test, and the capillary tube test or Kurzrock–Miller test (Mortimer, 1994; WHO, 2010).

Cervical mucus is conducive to sperm penetration only for a short time at the middle part of the female's ovulation cycle, from approximately day 9 of a normal 28-day cycle to the day of ovulation (WHO, 2010). At most times during a woman's cycle, the cervical mucus is thick and does not allow easy penetration by motile sperm. However, at midcycle, under the influence of hormones such as estrogen, the cervical mucus changes, becoming thinner and more abundant allowing easy passage of the motile sperm (WHO, 2010). It is at the midcycle time when both the quality of the cervical mucus and the activity of the sperm can be assessed. "Spinnbarkeit" is a term used to describe the elasticity or stretchability of the mucus, and "ferning" is the visual appearance of midcycle cervical mucus that is dried on a glass slide. Both Spinnbarkeit and ferning show characteristic patterns in midcycle cervical mucus as compared to other times during a woman's cycle. Abnormal amount and quality of midcycle cervical mucus is a cause for concern. Abnormal numbers and/or motility of sperm in normal midcycle cervical mucus are also a cause for concern. Both may be indications to attempt to improve the mucus with medications, or bypass the hostile cervical mucus to deposit motile sperm directly into the uterus (intrauterine insemination; IUI) or other forms of assisted reproduction which are very common treatment options.

Postcoital Test

A PCT is performed by the gynecologist or reproductive endocrinologist to determine if the midcycle cervical mucus is conducive to sperm penetration. Instructions are provided for ovulation timing with the assumption that at, or right before midcycle, the cervical mucus is receptive to sperm penetration and survival. The couple is instructed to have intercourse, and the female partner to appear at the physician's office 3–4 h after intercourse. Alternately, the couple is instructed to have intercourse the night before the scheduled test, and the cervical mucus is checked at the physician's office the next morning (WHO, 2010).

A smear of the cervical mucus is taken by the physician and examined under a microscope. There should be an adequate amount or volume of the cervical mucus, and the consistency should be watery minimally viscous midcycle mucus (Moghissi, 1976; WHO, 2010). The cervical mucus should show adequate stretching, Spinnbarkeit, and is scored as 0 (low stretchability) to 3 (9 cm or more stretching). Ferning is scored after a sample of mucus is air dried on a glass slide. Once mucus has dried on the slide, crystallization presents as a fern-like appearance, with branching of the "ferns." Ferning is scored on a scale of 0 (no fern formation) to 3 (significant fern stems) (Moghissi, 1976; WHO, 2010). The cervical mucus is rated as good mucus which favors sperm penetration, or unfavorable (Moghissi, 1976; Bush et al., 1997; WHO, 2010).

The mucus smear is also examined for the presence of sperm. It is desirable to see 10 or more sperm per high power field examined. Additionally, the motility of the sperm in the mucus is rated as progressive, motile but nonprogressive, or immotile. The PCT is negative if no sperm are found in the mucus. Rapidly motile sperm seen in the cervical mucus some 9–14 h after intercourse indicates that the cervical mucus is good quality and not hostile to sperm. It is then assumed that sperm would sufficiently progress through the cervix and into the uterus and fallopian tubes (Moghissi, 1976; Bush et al., 1997; WHO, 2010). Considering the results, the physician will interpret a negative PCT to result from incorrect midcycle timing, possible lack of intravaginal ejaculation, ejaculatory dysfunction, and abnormal semen analysis (if an analysis had not been previously performed). A negative PCT should be repeated to confirm these findings.

Laboratory Tests for Sperm–Cervical Mucus Interaction

Laboratory or in vitro tests of sperm–cervical mucus interaction are performed following a negative PCT, and to determine if the "problem" is with the sperm or the cervical mucus. Such tests compare the female partner's cervical mucus with donor cervical mucus, and the male partner's sperm with donor sperm in cross-over testing. Commercial laboratory test kits are also available

for use in sperm–cervical mucus interaction testing (Sharara et al., 1994; Niederberger et al., 2006). The concentration of sperm that penetrates into the cervical mucus, rather than how far the sperm has traveled in the mucus, has been suggested as a laboratory-based sperm function test (Aitken, 1993; Ola et al., 2003).

In vitro slide tests include a simplified slide test called the Kurzrock–Miller test and a capillary tube test or Kremer test (Kremer, 1965; Mortimer, 1994; WHO, 2010). The male partner's sperm is tested with both the female partner's and donated midcycle cervical mucus. Good quality donor sperm is also tested with samples of midcycle mucus. Bovine cervical mucus or synthetic gels have been substituted for human cervical mucus in these slide tests (WHO, 2010; Ivic et al., 2002). In vitro slide tests include a simplified slide test called the Kurzrock–Miller test and a capillary tube test or Kremer test (Kremer, 1965; WHO, 2010) (Mortimer, 1994).

The Kurzrock–Miller test is a simple slide test in which a drop of semen is placed against a drop of midcycle cervical mucus under a coverslip (Mortimer, 1994; WHO, 2010). Within a few minutes, the seminal fluid pushes into the cervical mucus, and a lead sperm can be seen swimming into the mucus. After 30 min, the slide is examined under a microscope to determine if, and how far, the motile sperm penetrated into the cervical mucus. A normal result is seen when multiple progressively motile sperm are found in the mucus. An abnormal result is seen when little or no sperm progress into the cervical mucus, or, if the sperm do penetrate the mucus interface, the sperm are immotile or “shaking” in place with no forward progression (Mortimer, 1994; WHO, 2010).

The capillary tube test or Kremer test measures the ability of sperm to penetrate into a column of donor and patient cervical mucus (Kremer, 1965; Mortimer, 1994; WHO, 2010). A capillary tube containing the test and donor mucus is placed into a test tube containing a small amount of the semen (male partner in one tube, donor sperm in another tube). Following incubation for 1–2 h, the motility, migration, and penetration of the sperm into the mucus are noted. The results are classified based on the numbers of sperm that penetrated into the mucus, the distance of passage into the mucus, and the time the sperm remained motile.

Presently, the simple assessment of the interaction between the sperm and cervical mucus using in vivo and in vitro methods is infrequently used, but nonetheless, these tests can provide useful information in evaluation of the infertile couple. Most recently, the role of the sperm–cervical mucus penetration tests, and the PCT have been questioned and remain controversial in the era of evidence-based medicine (Pavone et al., 2011) and the frequently used assisted reproductive procedures such as intrauterine insemination.

References

- Aitken, R. J. (1993). Sperm function tests and fertility. *Fertility and Sterility*, 60(2), 319–323.
- Bush, M. R., Walmer, D. K., Couchman, G. M., & Haney, A. F. (1997). Evaluation of the postcoital test in cycles involving exogenous gonadotropins. *Obstetrics and Gynecology*, 89(5), 780–784.
- Ivic, A., Onyeaka, H., Girling, A., Brewis, I. A., Ola, B., Hammadieh, N., Papaioannou, S., & Barratt, C. L. (2002). Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Human Reproduction*, 17, 143–149.
- Kremer, J. (1965). A simple sperm penetration test. *International Journal of Fertility*, 10, 209–215.
- Moghissi, K. S. (1976). Postcoital test: Physiologic basis, technique, and interpretation. *Fertility and Sterility*, 27(2), 117–129.
- Mortimer, D. (1994). *Practical laboratory andrology* (pp. 186–188). New York: Oxford University Press.
- Niederberger, C. S., Lamb, D. J., Glinz, M., Lipshultz, L. I., & Scully, N. F. (2006). Tests of sperm function for evaluation of the male: Penetrak and Tru-Trax. *Journal of Andrology*, 29(1), 69–75.
- Ola, B., Afnan, M., Papaioannou, S., Sharif, K., Bjorndahl, L., & Coomarasamy, A. (2003). Accuracy of sperm-cervical mucus penetration tests in evaluating sperm motility in semen: A systematic quantitative review. *Human Reproduction*, 18(5), 1037–1046.
- Pavone, M. E., Hirshfeld-Cytron, J. E., & Kazer, R. R. (2011). The progressive simplification of the infertility evaluation. *Obstetrical & Gynecological Survey*, 66(1), 31–41.
- Sharara, F. I., Beatse, S. N., Bailey, S. A., Neal, G. S., Coddington, C. C., 3rd, & Scott, R. T., Jr. (1994). Characterization of Tru-Trax-Trax in vitro penetration testing of cervical mucus. *Human Reproduction*, 9(11), 2027–2031.
- World Health Organization. (2010). *WHO laboratory manual for the examination and processing of human semen* (5th edn.). Geneva, Switzerland: World Health Organization.

Laboratory Evaluation of Sperm–Ovum Interaction

Mónica H Vazquez-Levin and Gustavo L Verón, Institute of Biology and Experimental Medicine (IBYME), National Scientific and Technical Research Council (CONICET), Buenos Aires, Argentina

© 2018 Elsevier Inc. All rights reserved.

"In chaos, there is fertility"

Anaïs Nin

Introduction

Mammalian fertilization is a seemingly chaotic but exquisitely organized biological process that requires a delicate progression of events toward sperm and oocyte encounter at the fertilization site, located at the upper segment of the fallopian tubes (oviductal ampulla in nonhuman mammals). Therein, male and female gametes assemble to accomplish fertilization and to initiate embryogenesis.

In recent years, great steps have helped researchers to elucidate the innermost phenomena in this chaos, but there is still a long way to go. Sperm that have completed morphogenesis in the testis and maturation while they transit through the epididymis, are ejaculated and deposited in the female reproductive tract. In their journey to fertilization, they must undergo a complex series of changes collectively known as sperm capacitation to develop full fertilizing competence. At the fertilization site, sperm meet the cumulus oocyte complex (COC). The cumulus is a cellular structure composed of granulosa cells that protect the oocyte and help trapping high quality sperm required for fertilization. Afterward, sperm interact with the *zona pellucida* (ZP), an acellular matrix of glycoproteins that surrounds the oocyte and gives species-specificity to the interaction with the male gamete. Sperm that have undergone the acrosome reaction (AR) penetrate the ZP and reach the oocyte plasma membrane (oolemma), to which they bind and fuse, followed by nuclear decondensation (Yanagimachi, 2012).

The interest for the mechanisms that rule fertilization referred to ancient times, when Hippocrates (460–370 years BCE) argued that both men and women contributed to form the embryo. Later on, Aristotle (384–322 BCE) proposed that women provided fertile ground for the male seed to grow. By the 17th century, studies already recognized that men produce spermatozoa, as A. van Leeuwenhoek first described in 1667 the human sperm. Over 150 years later, in 1827, K. von Bare discovered eggs in canine and human ovaries and, in 1875, O. Hertwig described sea urchin zygote formation after sperm–oocyte fusion. The 20th century witnessed numerous advances based on many new experimental tools available to better study these events, among them optical and electron microscopy, antibodies and other biochemical tools, in vitro models and, more recently, cloning, stem cell technologies, molecular biology and gene manipulation, including transgenesis and gene knock out models. All of them played important roles toward understanding the molecular basis of fertilization and revolutionized modern biology. These tools have been available and used due to the contribution of numerous passionate researchers worldwide, among whom R. Yanagimachi stands out for his tireless, innovative, inspiring, and outstanding work about mammalian fertilization. Dr. Yanagimachi published over 300 outstanding reports using rodent models in several topics: sperm capacitation, the mechanism and site of acrosome reaction, in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), cloning, among many breakthroughs in reproductive biology. Our humble and respectful tribute is dedicated to this outstanding pioneer scientist.

In 1978, human IVF finally broke through as a clinical practice with R. Edwards and P. Steptoe first living childbirth after an IVF, setting the bases for clinical infertility management, just as ICSI did in 1992 with G. Palermo. Currently, IVF and ICSI are worldwide standard procedures in clinical practice and tools used to unravel molecular mechanisms and identify molecules involved in sperm–oocyte interactions (Yanagimachi, 2012).

Based on the advances accomplished in the field of mammalian fertilization, a series of laboratory tests were developed in the last decades, aimed at evaluating human sperm fertilizing potential by mimicking some of the events that sperm have to overcome in their journey. A brief summary of the most relevant sperm–ovum interaction assays is summarized below.

Laboratory Evaluation of Sperm–Ovum Interaction

Sperm Interaction With the Cumulus Oophorus: The Hyaluronan Binding Assay

The first coating that sperm go through to reach the oocyte is the cumulus oophorus, a multilayered cell vestment composed of granulosa cells immersed in an extracellular matrix of hyaluronic acid (HA) (or hyaluronan), which grants the viscoelastic properties of this matrix. Sperm express HA receptors that bind to the cumulus. HA seems to play an important role during fertilization by selecting mature sperm with intact acrosomes and normal sperm morphology, minimal DNA fragmentation, and low frequency of chromosomal aneuploidies. These HA properties provided the basis for the hyaluronan binding assay (HBA) (Huszar et al., 2003).

Briefly, in the HBA a sperm suspension is placed in contact with a drop of suitable medium coated with HA, resulting in high quality sperm cells attached to the coated surface depicting vigorous tail movement, while unfit spermatozoa remain free. After sorting each population by transferring the unattached sperm into a new medium drop, a hyaluronan binding score (HBS) can be calculated as $HBS = (\text{bound sperm} / \text{total sperm}) \times 100$ (Nasr-Esfahani and Marziyeh, 2013) (Fig. 1). In a prospective study, the HBA test

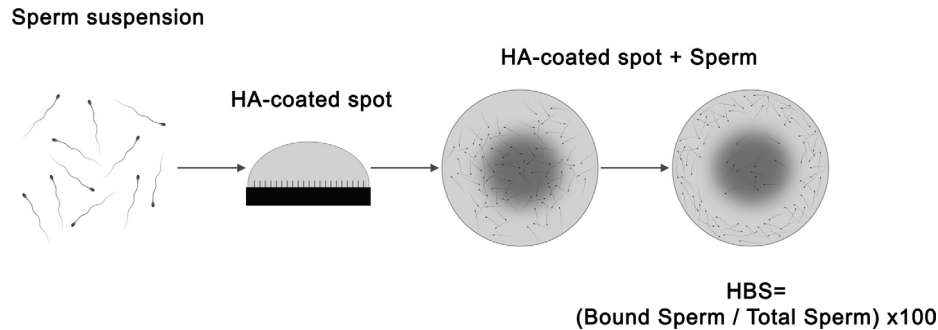


Fig. 1 Hyaluronan binding assay. A sperm suspension is placed over a HA-coated spot. Spermatozoa with vigorous tail movement are located in the inner area of the droplet, while unfit spermatozoa remain free. The hyaluronan binding score (HBS) is calculated as $HBS = (\text{bound sperm} / \text{total sperm}) \times 100$. HBA is also a suitable assay to select high quality spermatozoa prior to ICSI, the denoted physiological ICSI (PICSI).

was helpful in distinguishing semen samples suitable for IVF or ICSI (Pregl Breznik et al., 2013). In a later study done on patients undergoing ICSI treatment, HBA results correlated with fertilization rates and biochemical pregnancy values but not with parameters from routine semen analysis and clinical pregnancy rates (Esterhuizen et al., 2015). HBA has been also found suitable to select high quality sperm prior to ICSI, the denoted physiological ICSI (PICSI); this way, a high quality sample is assured. In this regard, a meta-analysis reported in 2016 on seven studies and over 1400 cycles revealed that HBA performance prior to ICSI improved embryo quality and implantation rates, while no enrichment in fertilization and pregnancy rates was found. Moreover, an analysis of prospective studies only showed an improvement in embryo quality. Authors emphasized the need of more studies to determine the benefit of the procedure in patients treated with ICSI (Beck-Fruchter et al., 2016).

There are currently two commercial alternatives for the HBA, one from Biocoat, USA and another from MidAtlantic Diagnostics Inc., USA.

Laboratory Procedure

1. Sperm suspension preparation
 - a. Calculate sperm concentration using a counting chamber.
 - b. Add suitable sperm culture medium to adjust sperm concentration to $0.2 - 1 \times 10^6$ sperm cells/mL.
2. Hyaluronic acid coating
 - c. Place a 10–50 μL droplet of hyaluronic acid (HA) with sterile water (1:40) in a tissue culture dish and let it dry.
 - d. Wash the coated spots with 15–75 μL of suitable medium.
3. Binding assay
 - e. Replace the washing medium droplet with 50 μL of a sperm suspension containing $1 - 5 \times 10^4$ sperm cells. Incubate 5–10 min.
 - f. *Diagnostic purposes:* calculate HA-bound sperm cells, characterized by a vigorous tail movement without progressive motility (unbound motile spermatozoa move without restriction). Nonmotile spermatozoa are not taking into account for the HBA report.
Therapeutic purposes: Select and detach HA-bound sperm cells for ICSI procedure using the ICSI-injecting pipette, and inject into oocytes.
4. Scoring
 - g. Count the number of motile sperm bound and unbound to the HA matrix using a phase contrast microscope. HBA score (%) is calculated as the number of bound motile sperm divided by the total number of motile sperm (multiplied by 100). The sum of bound and unbound spermatozoa counted is expected in the range of 150–200 to achieve good assay precision.

Note: The WHO Manual (Fifth Edition) does not describe this test.

(Adapted from Nasr-Esfahani, M. H., Marziyeh, T. (2013). Sperm selection for ICSI using the hyaluronic acid binding assay. Methods in Molecular Biology 927, 263–268.)

Sperm Interaction With the Zona Pellucida: The Hemizona Assay and the Sperm Zona Binding Ratio Assay

The ZP is an extracellular glycoprotein matrix formed by the oocyte during growth. Four ZP proteins (ZP1–ZP4) have been characterized in humans. Sperm binding and penetration of the ZP is critical for successful fertilization. In 1976, a procedure was described to assess human sperm–oocyte interaction (Overstreet and Hembree, 1976). Even though this assay was originally designed to evaluate sperm penetration of human ZP, it also provided the basis for a sperm–ZP binding assay. 12 years later, two assays were reported. Firstly, Burkman et al. (1988) developed the hemizona assay (HZA). The procedure involves ZP microdissection into equal halves and incubation of each half with the same concentration of Control (fertile men) or Test sperm (a patient sample in a clinical set-up; control sperm treated with a specific agent in a basic research set-up) (Fig. 2A). The HZA results are expressed as the hemizona index (HZI), calculated as follows: $HZI = \text{number of bound sperm from the Test sample} / \text{number of bound sperm from the Control sample}$ ($HZI = 1$ in cases when Test sperm have the same binding ability as

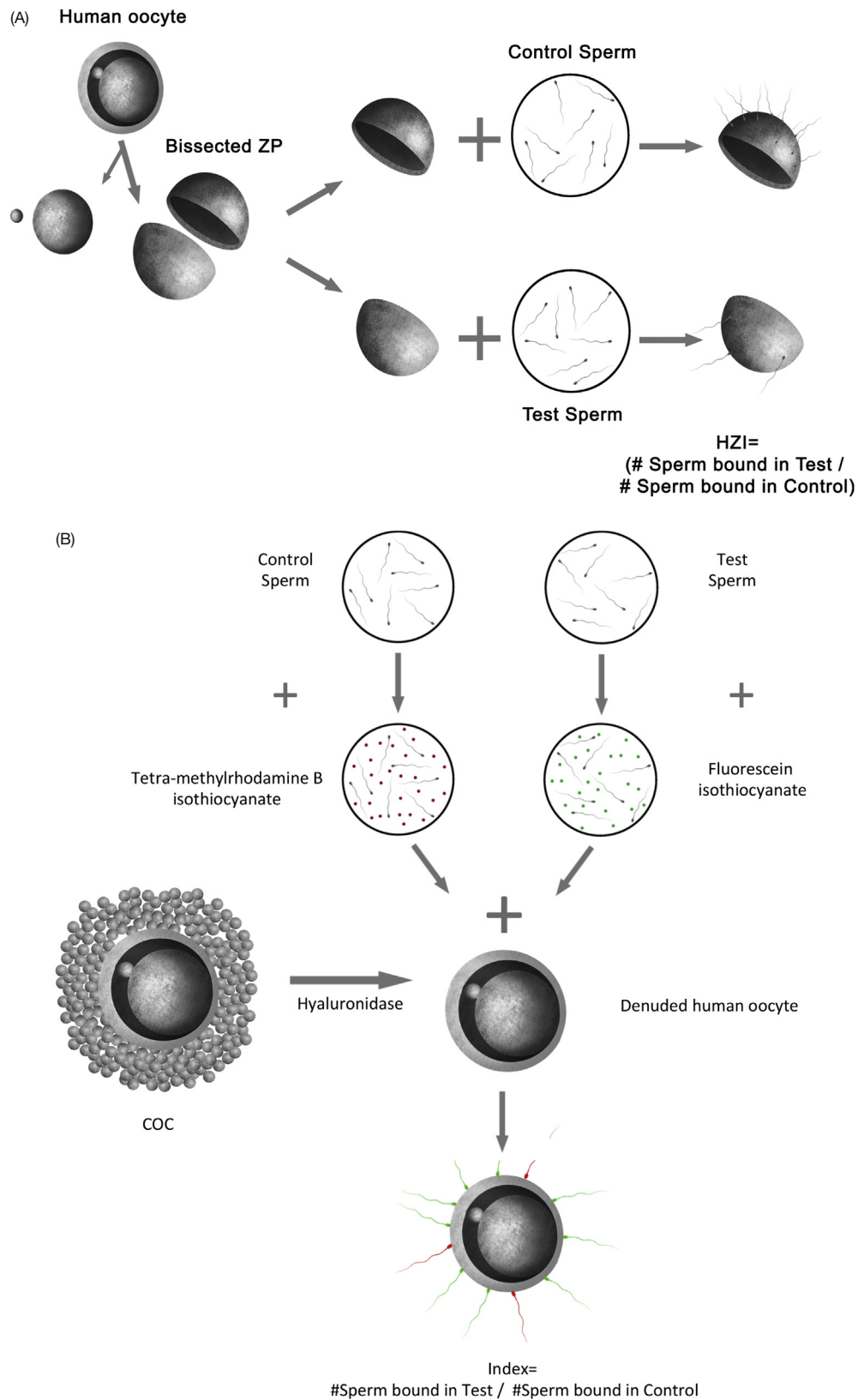


Fig. 2 Sperm–ZP interaction assays. (A) Hemizona assay. Discarded unfertilized or nonliving inactivated human oocytes are cut by half, and each HZ is co-incubated with Control or Test selected-motile sperm placed in culture medium droplets. After a 4-h incubation period, loosely bound sperm are removed by pipetting and the number of sperm bound to each hemizona is recorded. (B) Sperm zona binding ratio assay. Control and Test selected-motile sperm are incubated in culture medium droplets containing fluorescent dyes. Discarded unfertilized or nonliving inactivated human oocytes are co-incubated with a mix of both fluorescent selected-motile suspensions. After a 4-h incubation period, loosely bound sperm are removed by pipetting and the number of each fluorescent type sperm bound to the ZP is recorded.

Controls). Secondly, Liu et al. (1988) reported the development of a competitive sperm–ZP binding assay called sperm zona binding ratio (SZPB ratio) assay. The assay involves Control sperm labeling with a fluorescent dye (e.g., tetra-methylrhodamine B isothiocyanate) and Test sperm labeling with another fluorescent dye (e.g., fluorescein isothiocyanate), and incubation of both sperm samples with an inactivated human denuded oocyte (Fig. 2B). The number of sperm from the Test and Control cell suspensions bound to the same ZP are counted and reported as a ratio. Few or no sperm bound to the ZP is usually indicative of a sperm defect(s). The SZPB ratio assay uses a threshold similar to that of the HZA.

Both tests have shown to highly predict IVF outcome, as revealed from individual studies and from meta-analyses. Prospective clinical studies reported a cut-off HZI value of 30 as predictive of IVF outcome. In intrauterine insemination procedures, an HZI <30 was associated with a lower pregnancy rate compared with patients with HZI >30, showing a 90% sensitivity and a 78% specificity in cases of male factor infertility, although it was not found useful in couples with unexplained infertility (reviewed in Oehninger et al., 2014).

While the procedure by Liu does not require a micromanipulator to dissect the ZP, it does need a fluorescence microscope. Moreover, since sperm–ZP interaction is species-specific, only human ZP can be used for the abovementioned assays. Despite the difference in their methodologies, both assays include an internal positive control; this is highly relevant, considering that oocytes used in the assay may have abnormalities in their ZP-binding properties. Oocytes used are obtained from failed IVF procedures, as well as from ovaries surgically removed or from autopsies, and inactivated during storage in salt-based solutions.

Studies using the sperm–ZP interaction assays revealed the ability of the oocyte matrix to select sperm with normal morphology. Based on these observations, researchers have proposed the use of a simple and inexpensive method of sperm binding to the ZP of immature sibling oocytes as a procedure to select high-quality sperm for ICSI procedures (Liu, 2011). Controlled clinical trials in larger numbers of subjects are needed to confirm these findings.

Laboratory Procedure: The Hemizona Assay

1. Sperm preparation
 - a. Wash the sperm suspension two times with suitable medium.
 - b. Allow the washed sperm to swim-up for an hour.
2. Hemizonae preparation
 - c. Grasp a discarded or nonliving oocyte with a holding pipette and cut by half with a microscalpel blade.
 - d. Dislodge the ooplasm by pipetting.
3. Hemizona assay
 - e. Adjust the sperm concentration to 10^4 sperm/mL.
 - f. Place two 50 μ L droplets of the sperm suspension under oil (Control and Test samples).
 - g. Add one hemizona of a pair to each droplet and incubate for 4 h.
 - h. Rinse each hemizona by vigorously pipetting them in washing medium.
4. Scoring
 - i. Count the number of motile sperm bound to each hemizona matrix using a phase contrast microscope. The HZI score is calculated as the ratio between the number of bound motile Test sperm divided by the number of motile Control sperm (multiplied by 100 when expressed as percentage).

Note: While the WHO manual (4th Edition) described the sperm–ZP binding assays, the 5th Edition only briefly refers to these tests.

(Adapted from Burkman, L. J., Coddington, C. C., Franken, D. R., Kruger, T., Rosenwaks, Z., Hogen, G. D. (1988). The hemizona assay (HZA): Development of a diagnostic test for the binding of human spermatozoa to human hemizona pellucida to predict fertilization potential. Fertility and Sterility 49, 688–695.)

Sperm Acrosome Reaction: The AR Tests (ZIAR and ARIC)

The AR is an exocytotic event that must take place before the spermatozoon penetrates the oocyte vestments to accomplish fertilization. The AR involves fusion of the sperm plasma membrane and the outer acrosomal membrane, exposure of the inner acrosomal membrane and acquisition of fusogenic properties of the plasma membrane at the equatorial segment. Despite the long time evidence favoring the occurrence of AR once sperm attached to the ZP, findings using genetically modified rodents indicate that the physiological AR may occur prior to sperm–ZP contact.

The incidence of spontaneous AR in ejaculated human sperm is low (~5%–10%), and it increases after capacitation and AR induction. There are several physiological agents (e.g., follicular fluid, progesterone, ZP glycoproteins) that induce AR. Since Ca^{++} influx is one of the AR initiating events, Ca^{++} ionophores (e.g., ionomycin and A23187) can trigger this event, but results may not relate to those obtained from the ZP-induced AR. The sperm acrosomal status can be assessed by microscopy or flow cytometry with fluorescently labeled lectins that bind to acrosomal contents, such as *Pisum sativum* agglutinin or, alternatively, antibodies against acrosomal proteins (WHO, 1999; WHO, 2010) (Fig. 3).

The ZP-induced AR (ZIAR) assay can be done by incubating sperm with acid-solubilized ZP, assessing the results using fluorescent dyes and calculating the sperm response as the difference between the ZIAR (stimulated) and the spontaneous (unstimulated) AR results. Unfortunately, this test is limited by the restricted availability of human ZP. The AR after ionophore challenge (ARIC) is calculated using the same criteria as the ZIAR. A clinical cut-off value of 15% has been reported for both assays. Patients may have

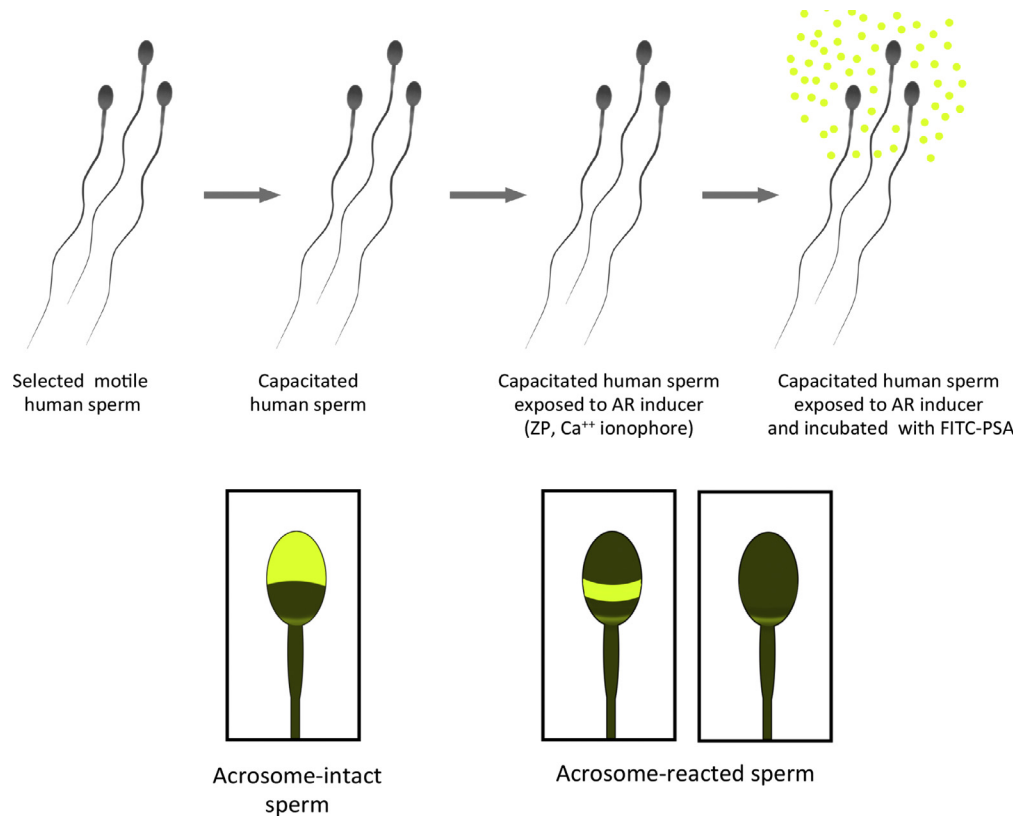


Fig. 3 Induced acrosome reaction assay. A selected-motile sperm suspension is exposed to capacitating conditions and Ca⁺⁺ ionophore A23187. Ionophore-exposed spermatozoa are incubated with [FITC]-PSA fluorescent dye for 1 h, and acrosome status is recorded: acrosome-intact sperm, acrosome-reacted sperm or other patterns.

two types of AR defects: premature AR (> 20% of spermatozoa exhibiting spontaneous AR) or insufficient AR (poor response to AR stimuli). Several studies have shown that ZIAR and ARIC can help predicting fertilization at conventional IVF, but the predictive values vary among studies (reviewed in Sigman et al., 2009; Oehninger et al., 2014).

The AR allows sperm penetration of the ZP and fusion to the oolemma. Thus, determining the acrosome reacting ability is important in the diagnostic and therapeutic approach to couples undergoing conventional assisted reproductive technology treatment. In cases of a poor acrosome reaction, ICSI therapy may be advised. Unfortunately, since most centers indicate ICSI as primary ART procedure, the AR assay is rarely used.

Laboratory Procedure: Induced Acrosome Reaction Assay

1. Sperm preparation
 - a. Calculate sperm concentration after semen liquefaction and washing.
 - b. Incubate 1×10^6 motile sperm for 3 h in sperm capacitating medium.
2. Sperm induction of acrosome reaction and staining
 - c. Add 10 μ M of A23187 calcium ionophore (alternative: *zona pellucida*) and incubate for 15 min to induce acrosome reaction.
 - d. Stop the reaction by adding 100 μ L of 3% (v/v) glutaraldehyde or 70% (v/v) ethanol.
 - e. After washing, stain fixed sperm placed onto microscope slides using fluorescent lectins, such as *Pisum sativum* agglutinin, coupled to fluorophores, for example, Fluorescein IsoThioCyanate, as [FITC]-PSA for an hour at 4°C.
 - f. Wash each slide with water and mount in ethanol-soluble medium.
3. Scoring
 - g. Assess the percentage of acrosome-reacted sperm (Test and Control): View the slide with fluorescence optics at 400 $\times$ magnification with oil immersion at 450–490 nm excitation.
 - h. Categories:
 - Acrosome-intact: sperm with brightly fluorescent head (half or more).
 - Acrosome-reacted: sperm with poor fluorescent signal at the equatorial segment or none at all.
 - Others (Acrosome-reacting): sperm with “patchy” fluorescent signal in the acrosomal cap (not shown in Fig. 3).

Note: The WHO Manual (Fourth and Fifth Edition) present a detailed protocol for the assessment of the sperm acrosomal status and the ionophore induced AR. (Adapted from WHO Manual, Fifth Edition).

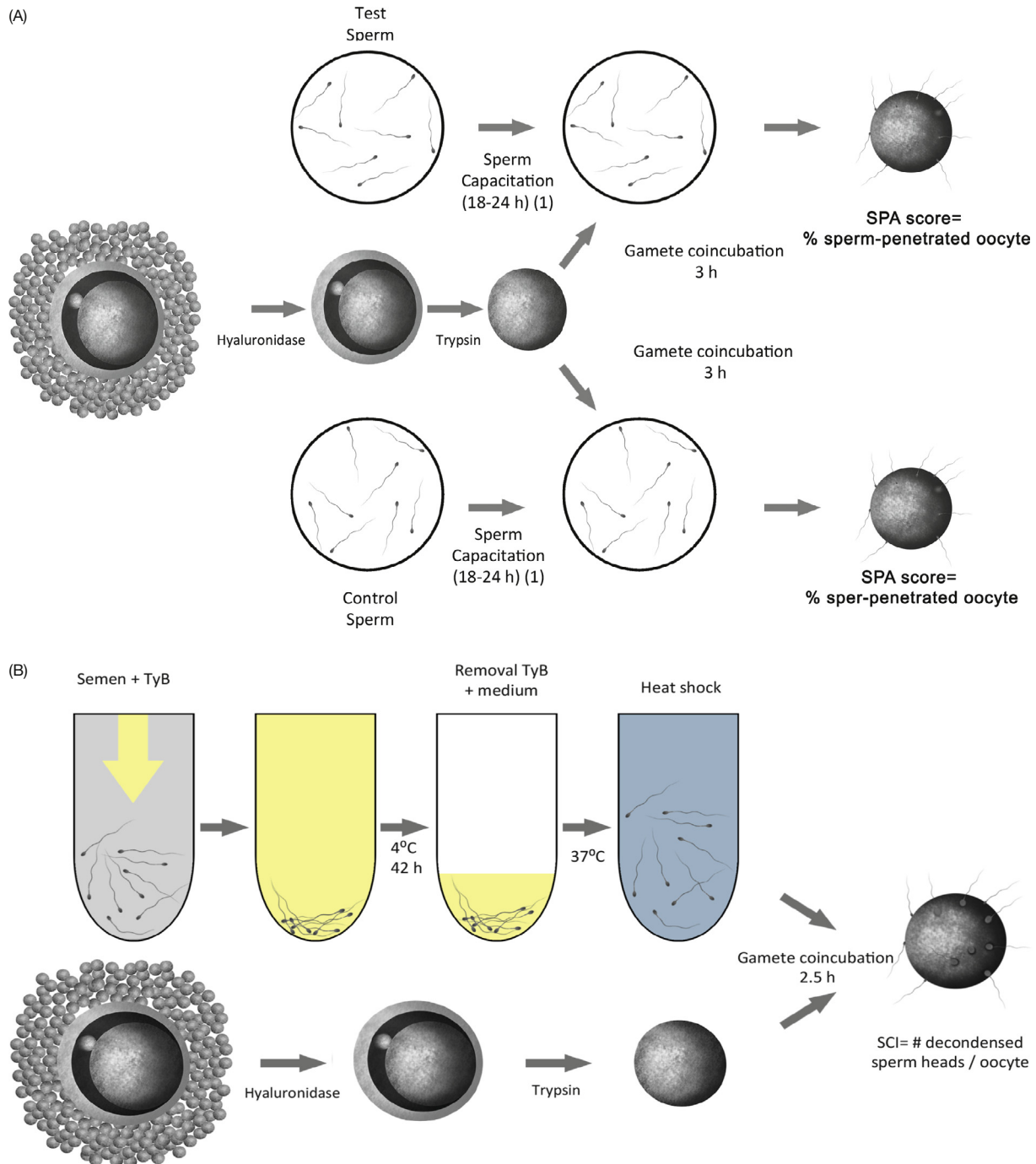


Fig. 4 Sperm penetration assay. (A) Standard and ionophore-induced procedures for sperm treatment. A selected-motile human sperm suspension is capacitated for 18–24 h. (1) Alternatively, capacitated spermatozoa are exposed to Ca^{++} ionophore A23187 for 1 h. Meanwhile, hamster COC are treated with hyaluronidase (to remove cumulus cells) and trypsin (to remove the ZP). Capacitated or Ca^{++} ionophore A23187-exposed sperm are incubated with ZP-free oocytes for 3 h, after which loosely bound sperm are washed out, and oocytes are mounted to score the % of oocytes with at least one decondensed sperm. (B) Modified procedure using Test-Yolk buffer (TYB) conservation. A semen sample is mixed with TYB and stored at 4°C for 42 h. Then, the TYB is removed and sperm are resuspended in a suitable medium (e.g., BWW) at 37°C (heat shock). Hamster COC are treated with hyaluronidase and trypsin. Sperm recovered after the heat shock are incubated with ZP-free oocytes for 2.5 h, after which loosely bound sperm are washed out, and oocytes are mounted to score the number of decondensed sperm heads in each oocyte. A parallel assay is done with the control semen sample (fresh or previously cryopreserved).

Sperm Interaction With the Oolemma: The Zona-Free Hamster Egg Sperm Penetration Assay

Sperm that have completed the AR, penetrate the ZP, and reach the perivitelline space. There, they come in close contact, bind and fuse, to the oolemma. As mentioned previously, the ZP provides species specificity, thus preventing cross-species fertilization. Investigations done back in 1976 by Dr. R. Yanagimachi and coworkers demonstrated the promiscuous behavior of hamster oocytes. In their study, they showed that ZP removal was sufficient to allow fusion and nuclear decondensation in the cytoplasm of sperm from several species, including humans. Based on these observations, the report described the development of the sperm penetration (SPA) assay, also known as the zona-free hamster egg–human sperm penetration assay (ZP-free SPA), using denuded hamster ova.

This test is one of the most sensitive measures of sperm function available, since sperm that had undergone the AR and penetrated the ZP, reach the perivitelline space and bind and fuse to the oolemma. Despite the fact that SPA does not measure sperm interaction with the ZP, it evaluates sperm ability to undergo the AR and provides information on the fusogenic nature of capacitated sperm plasma membrane at the equatorial segment and the ability to accomplish nuclear decondensation in the oocyte's cytoplasm. Depending on the protocol, it also measures the spermatozoa's ability to capacitate under the conditions assayed.

Conventional SPA relies on the occurrence of spontaneous AR in populations of sperm incubated in vitro for prolonged time periods, depicting false negative results, with failure in the SPA but success in the IVF procedure. Consequently, a modification of the standard procedure was introduced, using calcium ionophore-stimulated sperm to increase the amount of acrosome-reacted sperm and reduce the false negatives. In both classical and modified protocols, the percentage of ova that show at least one decondensed sperm head is determined, being positive when at least 10% and 50% penetrated oocytes are scored, respectively (Fig. 4). Also, a modified SPA procedure was developed, named “the optimized SPA.” The protocol introduces a modification on sperm handling to produce a vast subpopulation of acrosome-reacted sperm cells, which is accomplished by a combination of sperm storage at 4°C in a solution enriched in egg-yolk components that intercalate in the sperm plasma membrane followed by a heat shock procedure that induces a massive sperm AR. This protocol modification resulted in oocyte polyspermy in control sperm suspensions, reducing false negatives and consequently increasing assay sensitivity. Using this protocol, a lower limit of positive assay was established at five decondensed sperm heads per oocyte, defined as sperm capacitation index or SCI (reviewed by Lamb, 2010).

A report published in 2013 compared the results of the SPA and HZA. On one hand, the SPA showed wide parameters to predict IVF outcome, most likely because of differences in methodology and cut-off values used in each study. On the other hand, HZA methodology correlated well with IVF outcome and demonstrated better positive/negative predictive power. The report suggested the use of HZA in a sequential fashion with semen analysis and potentially other bioassays in an IVF setting (reviewed by Oehninger et al., 2014).

Nowadays, the SPA is rarely practiced in the clinical andrology laboratories, but it is still used for research focused on unrevealing the molecular basis of sperm–oolemma binding and fusion. Interestingly, despite its use to predict failure of sperm decondensation in ICSI using a variation of the procedure (reviewed by Lamb, 2010), the SPA test has not expanded in the ICSI era.

Laboratory Procedure: Sperm Penetration Assay

1. Sperm preparation
 - 1.1. Standard procedure
 - a. Subject the semen sample to motile sperm selection by density-gradient centrifugation or swim-up procedures.
 - b. Adjust the concentration of the selected sperm suspension to 1×10^6 sperm/mL.
 - c. Incubate the sample under capacitating conditions for 18–24 h in 0.5 mL of suitable medium.
 - 1.2. Optional incorporation of calcium ionophore A23187
 - d. Adjust the concentration to 5×10^6 sperm/mL.
 - e. Incubate separate sample aliquots with 1.25 and 2.5 μ M of calcium ionophore A23187 for 3 h.
 - f. Wash and adjust the concentration to 3.5×10^6 sperm/mL.
 - 1.3. Optional Test-Yolk Buffer (TYB) conservation and processing
 - g. Within the hour of collection, dilute the semen samples in tubes with an equal volume of TYB at room temperature.
 - h. Immerse the tubes in room temperature water and place them in a refrigerator.
 - i. Remove the TYB supernatant after 42 h and dilute in suitable medium at 37°C.
 - j. Retrieve motile sperm from the top third of the supernatant and adjust the concentration to 3.5×10^6 sperm/mL.
 - k. Place a 50 μ L droplet of the suspension into a Petri dish.
2. Oocyte preparation
 - a. Inject immature hamsters with 30 IU of PMSG intraperitoneally.
 - b. Inject the same hamsters with 40 IU of hCG after 48–72 h.
 - c. Sacrifice the hamsters 18 h after hCG injection.
 - d. Recover the COC by extracting and dissecting the oviduct.
 - e. Place the retrieved COC into droplets containing hyaluronidase (1 g/L) for 10 min to separate the oocyte from cumulus cells.
 - f. Rinse the oocytes and place them in droplets of suitable medium.
 - g. Treat the oocytes with trypsin (1 g/L) for 1 min to remove the ZP.
 - h. Wash the oocytes three times with suitable medium.

3. Sperm penetration assay:

- a. Place up to five oocytes per droplet and co-incubate them for 3 h.
- b. Thoroughly wash the oocytes to remove loosely bound spermatozoa.
- c. Place small droplets with washed oocytes on microscope slides and cover them with coverslips held by small wax-petroleum jelly pillars.
- d. Calculate the number of decondensed sperm heads per oocyte, and the percentage of penetrated oocytes by phase-contrast microscopy examination.

Note: The WHO Manual (Fourth and Fifth Edition) present a detailed protocol for the ZP-free hamster egg sperm penetration assay.

(Adapted from WHO Laboratory Manual for the Examination and Processing of Human Semen, Fifth Edition; and Handbook of the Laboratory Diagnosis and Treatment of Infertility, Keel & Webster, CRC Press).

Concluding Remarks

Sperm–oocyte interaction has been extensively studied in the last 50 years, and several approaches have been used to contribute to understanding the molecular basis of fertilization. The tests described above were developed in the last decades in parallel with in vitro fertilization and early embryonic transfer procedures. Being aware of the limited predictive value of semen analysis on sperm fertility potential, researchers developed these set of in vitro sperm–ovum interaction assays. These tests were also used to identify entities involved in the molecular mechanisms of fertilization. With the advent of ICSI, the evaluation and treatment of the infertile male dramatically changed. The procedure requires a single live sperm to be injected in the oocyte cytoplasm, bypassing all events tested to predict fertility potential. Although it was initially indicated for severe male factor infertility, ICSI was soon applied to all pathologies. ICSI offspring health is now being evaluated, finding abnormalities that may be related to the lack of an adequate sperm selection procedure.

Authors still believe that the abovementioned tests may be beneficial toward the selection of the appropriate ART procedure, to avoid the use of ICSI as a sole procedure to treat infertility. In addition, they may be used as tools to select sperm for ICSI, specifically, the HBA and the sperm–ZP binding assays. While the HBA is based on a reagent, the ZP–sperm assays require human ZP, which limits the assay usage. The research in this field should turn toward a large production of recombinant bioactive ZP–glycoproteins expressed in heterologous systems and couple them to a solid phase platform, replacing the scarce natural source. These “ZP-like” structures will then be used to assess sperm-binding potential and eventually to recover bound sperm for microinjection purposes. Finally, these assays should be used in a basic set-up to identify molecular biomarkers of sperm–ovum interaction that will become a powerful complement in the future Andrology-patient workout. There is still a lot of work ahead.

References

- Beck-Fruchter, R., Shalev, E., & Weiss, A. (2016). Clinical benefit using sperm hyaluronic acid binding technique in ICSI cycles: A systematic review and meta-analysis. *Reproductive Biomedicine Online*, 32, 286–298.
- Burkman, L. J., Coddington, C. C., Franken, D. R., Kruger, T., Rosenwaks, Z., & Hogen, G. D. (1988). The hemizona assay (HZA): Development of a diagnostic test for the binding of human spermatozoa to human hemizona pellucida to predict fertilization potential. *Fertility and Sterility*, 49, 688–695.
- Esterhuizen, A. D., Franken, D. R., Bosman, E., Rodrigues, F. A., Van Rensburg, J. H., Van Schouwenburg, J. A., & Lombard, C. (2015). Relationship between human spermatozoa–hyaluronan-binding assay, conventional semen parameters and fertilisation rates in intracytoplasmic spermatozoa injection. *Andrologia*, 47, 759–764.
- Huszar, G., Ozenci, C. C., Cayli, S., Zavadzki, Z., Hansch, E., & Vigue, L. (2003). Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status. *Fertility and Sterility*, 79(Suppl 3), 1616–1624.
- Lamb, D. J. (2010). Semen analysis in 21st century medicine: The need for sperm function testing. *Asian Journal of Andrology*, 12, 64–70.
- Liu, D. Y. (2011). Could using the zona pellucida bound sperm for intracytoplasmic sperm injection (ICSI) enhance the outcome of ICSI? *Asian Journal of Andrology*, 13, 197–198.
- Liu, D. Y., Lopata, A., Johnston, W. I., & Baker, H. W. (1988). A human sperm–zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertility and Sterility*, 50, 782–788.
- Nasr-Esfahani, M. H., & Marziyeh, T. (2013). Sperm selection for ICSI using the hyaluronic acid binding assay. *Methods in Molecular Biology*, 927, 263–268.
- Oehninger, S., Franken, D. R., & Ombelet, W. (2014). Sperm functional tests. *Fertility and Sterility*, 102, 1528–1533.
- Overstreet, J. W., & Hembree, W. C. (1976). Penetration of zona pellucida of non-living human oocytes by human spermatozoa in vitro. *Fertility and Sterility*, 27, 815–831.
- Pregl Breznik, B., Kovačić, B., & Vlasićević, V. (2013). Are sperm DNA fragmentation, hyperactivation, and hyaluronan-binding ability predictive for fertilization and embryo development in vitro fertilization and intracytoplasmic sperm injection? *Fertility and Sterility*, 99, 1233–1241.
- Sigman, M., Baazeem, A., & Zini, A. (2009). Semen analysis and sperm function assays: What do they mean? *Seminars in Reproductive Medicine*, 27, 115–123.
- WHO, 1999. *WHO Manual for the examination of human semen and sperm–cervical mucus interaction*, 4th edn.
- WHO Manual. 2010. *Examination and processing of human semen*, 5th edn.
- Yanagimachi, R. (2012). Fertilization studies and assisted fertilization in mammals: Their development and future. *The Journal of Reproduction and Development*, 58, 25–32.
- Yanagimachi, R., Yanagimachi, H., & Rogers, B. J. (1976). The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. *Biology of Reproduction*, 15, 471–476.

Laboratory Evaluation of Sperm Chromatin Structure

Luke Simon and Douglas T Carrell, University of Utah School of Medicine, Salt Lake City, UT, United States

© 2018 Elsevier Inc. All rights reserved.

What Is a Normal Sperm Chromatin?

In somatic cells, the DNA is bound to histones in a loose loop like manner to facilitate transcriptional activities. However, in sperm, transcription is inactive and the DNA is organized in a specific tight-coiled manner with the help of protamines (Oliva, 2006). The exchange in nuclear proteins allows a very tightly packaged chromatin, which facilitates the sperm head into streamline structure and also protect against oxidative damage. The chromatin is packed in a very precise fashion that allows the embryonic genome to easily access the genetic information soon after fertilization (Nanassy and Carrell, 2008). The sperm of fertile men is characterized to have an intact DNA, organized in a compact fashion and is capable of decondensation soon after fertilization.

How Does the Sperm Chromatin Get Damaged?

Number of factors has been reported to induce sperm DNA/chromatin damage. The main factor for chromatin damage is oxidative stress, which is an increase in the production of free radicals or reactive oxygen species (ROS) as a byproduct of normal cellular mechanism (Aitken and De Iuliis, 2007). It is observed that patients with leukocytospermia are showed to have high levels of oxidative stress. Leukocytospermia is a condition where the level of leukocytes increase in the semen as a result of genital tract infection or inflammation. When ROS comes in contact with sperm DNA, it can result in a base modification or single strand break. Cigarette smoking is showed to induce oxidative stress by overproduction of ROS (Aitken and De Iuliis, 2007). Patients with leukemia, testicular cancer, diabetic and Hodgkin's disease are showed to have increased level of sperm DNA damage. Treatments for diseases using cytotoxic drugs and radiation therapy could also affect the sperm DNA integrity.

Environmental and occupational exposures to chemicals, radiation and pesticides are known to increase sperm DNA damage. Studies have showed that intensive physical exercise could result in scrotal heating, inducing sperm chromatin damage (Aitken and De Iuliis, 2007). Defects in the spermatogenetic process such as abnormal chromatin packing may also result in sperm DNA damage. Approximately 85% of the histones are replaced to protamines, but high level of histone may suggest abnormal chromatin packaging and nuclear maturity defects (Oliva, 2006). In addition, abnormal chromatin packaging may render the sperm vulnerable to oxidative stress mediated DNA damage.

Finally, laboratory treatment and protocols are also known to affect chromatin integrity. For example, sperm preparation technique such as density gradient centrifugation (commonly used during assisted reproduction procedure) is showed to generate ROS. Another well-known example is the sperm cryopreservation protocols (where sperm is stored in liquid nitrogen for future use) is also showed to induce DNA damage (Thomson et al., 2009).

Evaluation of Sperm Chromatin Integrity

The stability of sperm chromatin/DNA integrity is important for a successful conception in vivo as well as in vitro. Assisted reproductive treatment (ART) outcomes such as, fertilization rates, embryo quality and development rates, implantation rates, clinical pregnancy outcomes, miscarriage and live birth rates are showed to be associated with sperm DNA damage. Advances in molecular tools have resulted in the development of assays that could measure the level of DNA damage in each sperm or the percentage of DNA damaged sperm in an ejaculate. The ability of such assays to accurately estimate the level of sperm DNA damage depends on its principle and biological properties of these assays. The available assays that evaluate sperm DNA integrity can be broadly classified into two types, direct and indirect methods. The direct method includes, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, in situ nick translation assay and the comet assay, and the level of DNA damage could be measured in individual sperm. The indirect method of assays included sperm chromatin structure assay (SCSA) and sperm chromatin dispersion (SCD) assay, these methods measure the susceptibility of DNA to be damaged. Some staining methods are commonly used to evaluate the nuclear proteins (histones and protamines) packaging in sperm, such as aniline blue assay, toluidine blue assay, and chromomycin A₃ assay. These staining methods measure the compaction of the sperm chromatin structure and nuclear maturity, and serve as indirect methods to measure chromatin integrity. These direct, indirect and staining methods have been frequently used in andrology research to evaluate a correlation between sperm DNA integrity with male infertility. Recent evidence suggests that the direct method of sperm chromatin evaluation assay is more closely associated with male infertility and, therefore, researchers have suggested that these assays should be introduced into the routine andrology laboratory diagnosis (Simon et al., 2011).

Comet Assay

The comet assay, also known as single cell gel electrophoresis is one of the simplest methods to measure sperm DNA integrity by quantifying the single and double strand breaks. The principle of the assay is that the sperm nuclear DNA is separated in the electric field based on their charge and size which can be viewed using a fluorescent stain that can bind to DNA. During the comet assay, the intact DNA remains in the comet head, whereas short fragments of DNA strands migrate into the tail (Singh et al., 1988). The resulting images under the microscope resembles a comet, consisting of a head and tail (see Fig. 1). The amount of DNA damage in the sperm is directly proportional to the extent of DNA in the comet tail. In addition, the comet assay also measures the diameter of the comet head, olive tail moment and the comet tail length, etc., as these parameters are also associated with sperm DNA damage.

The comet assay is being used to assess DNA damage in a number of cell types including sperm and in a wide range of studies. The methodology of the assay involves the following steps. First, the sperm cells are fixed in agarose gel and treated with detergents to remove the cell membrane. Then the nuclear proteins are removed, which help in the decondensed of sperm DNA. The naked DNA is now subjected to electrophoretic, where in the electric field the DNA fragments containing negative charge migrates towards the positive electrode through the agarose. Following electrophoresis, the DNA is stained using fluorescent dyes and visualized using fluorescence microscopy. The comet assay can be performed in a neutral or alkaline environment: in the neutral condition (pH: 7) only DNA with double strand breaks are measured, while in the alkaline condition (pH: 13), the double stranded DNA is unwound into single strands and hence both single- and double-strand breaks are measured (Singh et al., 1988).

Advantages of the Comet Assay

The comet assay is a cheap and one of the most sensitive techniques available to measure sperm DNA damage. The level of sperm DNA damage measured by the comet assay is closely associated to other direct method such as TUNEL assay (Shamsi et al., 2008). The comet assay can be used to measure DNA damage in a variety of cell types, including sperm. The assay requires only a few of cells to perform the procedure. The data is measured using a software and hence its more precise and less prone to experimental bias. DNA damage measured by the comet assay is closely associated to male infertility and to various end points of ART outcomes (Simon et al., 2017).

Limitations of the Comet Assay

The comet assay lacks a standardized protocol to measure DNA damage in sperm, which makes it difficult to fully understand and relate the results from different research groups (Tarozzi et al., 2007). The assay is known to induce damage at the alkaline labile sites and hence it is difficult to discriminate between endogenous DNA damage with that of induced DNA breaks. When measuring DNA damage in somatic cells, presence of residual RNA could increase the background intensity and affect the results. The assay is also criticized for underestimation of DNA damage due to entangling of DNA strands. Incomplete chromatin decondensation in sperm, could reduce the level of DNA strand breaks to be revealed (Shamsi et al., 2008). Overlapping comet tails reduces the accuracy of the assay. Smaller tail fragments are lost in the agarose gel or too small to be visualized under the microscope. The assay is extremely laborious and is known to have high level of inter-laboratory variation.

Sperm Chromatin Structure Assay (SCSA)

The SCSA is the largest commercial available test to detect sperm DNA damage and was first reported in 1980 (Evenson et al., 1980). The assay protocol includes acid denaturation of sperm chromatin followed by acridine orange staining method and the intensity of the fluorescence is measured by a flow cytometer to determine sperm with DNA damage from that of normal sperm. The principle of SCSA is that the acridine orange stain binds to double stranded DNA to emit green fluorescence and the same stain binds to single

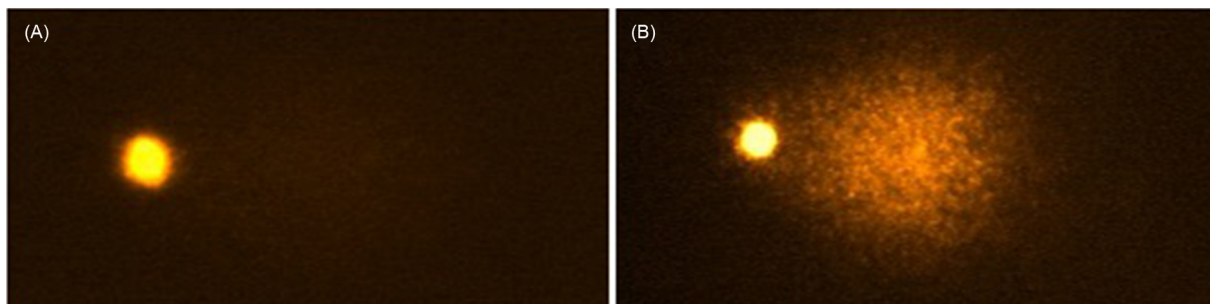


Fig. 1 Sperm DNA integrity measured by the alkaline comet assay: (A) sperm with high DNA integrity, (B) sperm with low DNA integrity exhibiting a typical “comet tail.”

stranded DNA to emit red fluorescence. Therefore, during acid denaturation of sperm chromatin, the separation of double stranded DNA into single strand DNA and such separation initiates at the site of the single and double strand breaks. The extent of DNA damage in the cell is determined by measuring the metachromatic shift from green fluorescence to red fluorescence after the acid treatment (Evenson et al., 1980). Therefore, normal cells will emit green fluorescence, while the DNA damaged cells will emit red fluorescence. The most important output parameter of SCSA is the DNA fragmentation index (% DFI), which represents the population of cells with DNA damage.

SCSA is an indirect method to measure DNA damage as it measures the susceptibility of sperm DNA to denaturation. Due to use of flow cytometry, it is possible to measure large number of sperm per sample making the technique highly reproducible. SCSA also measures highly DNA stainable (HDS) cells, which are described as the sperm population with increased accessibility of double-stranded DNA to the dye, mainly due to abnormalities in the replacement of histones with protamines (Evenson and Jost, 2000).

Advantages of SCSA

Sperm DNA damage measured by SCSA is known to be more constant over a longer period of time when compared with the traditional semen parameters. The consistency of the test makes it useful in most epidemiological studies (Shamsi et al., 2008). Freezing of semen samples does not affect the test, which allows samples to be batched for convenience or could be used in multi-center trials. The assay determines the percentage of sperm with DNA damage. Several clinical studies have shown its usefulness in associating the level of DNA damage measured by SCSA with male infertility. It is simple and rapid assay to analyze thousands of sperm. Researchers have found a threshold value of 30% DFI and 15% HSD above which men are likely to be infertile (Evenson and Jost, 2000). Several clinical studies have shown its usefulness in associating SCSA parameters with ART outcomes.

Limitations of SCSA

The SCSA does not give information about the extent of DNA damage in individual sperm. The assay requires expensive equipments for analysis. The assay protocol especially the acid denaturation step is time sensitive. DNA damage measured after density gradient centrifugation using SCSA is not predictive for ART outcomes (Shamsi et al., 2008). Sperm chromatin is highly coiled in nature, therefore absence of DNA decondensation step underestimates the level of DNA damage in sperm, as the acid denaturation occurs only at the periphery of the sperm nucleus.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

TUNEL assay is a direct method of DNA damage measurement. It is widely known to quantify DNA damage in somatic cells and can be efficiently used in sperm. The assay incorporates deoxyuridine triphosphate (dUTP) at single and double strand DNA breaks in a reaction catalyzed by the template-independent enzyme, terminal labels deoxynucleotidyl transferase (Gorczyca et al., 1993). The incorporated dUTP are labeled with fluorescent markers and hence the breaks can be quantified by flow cytometry or fluorescent microscopy. The TUNEL assay resembles the in situ nick translation assay in a number of technical aspects, but the template-independent enzyme used here helps to identify both single and double strand DNA breaks. The sperm DNA damage, measured by TUNEL assay, has a good stability with time and it is possible to measure baseline damage in both fertile and subfertile men (Sergeie et al., 2005). Sperm DNA fragmentation measured by the TUNEL assay is a valuable indicator of male fertility status and has been demonstrated to predict ART outcomes.

Advantages of TUNEL Assay

The assay has a simple protocol to simultaneously detect both single and double strand DNA breaks. Similar to SCSA freezing sperm samples does not affect the results of TUNEL assay. The popularity of TUNEL assay is justified by good quality control parameters, and low intra and inter observer variability. TUNEL assay can be performed using both fluorescent and light microscopy, depending on the type of labeling method used to visualize DNA breaks (Shamsi et al., 2008). The fluorescence labeling technique eliminates the problems associated with dye fading which occurs during conventional microscopic method, thereby giving the technicians more time to analyze a larger subset of samples. The coupling of the TUNEL assay with flow cytometry makes it possible to evaluate thousands of sperm in a short period of time, thus enhancing the accuracy and reproducibility of the technique.

Limitations of TUNEL Assay

TUNEL assay is not programmed to quantify the magnitude of DNA damage within a single cell, it only estimates the number of cells with DNA damage in a population of cells, termed as TUNEL positive cells (Shamsi et al., 2008). The light microscopy version of TUNEL assay is simple but the background staining decreases the efficiency of this method. The fluorescence labeling protocol requires highly sophisticated and expensive instruments. Due to a lack of useful threshold values and standardized protocol, the assay unreliable for routine clinical use (Simon et al., 2014). Extensive training is required for laboratory technicians to obtain a consistent and reproducible data. Due to the unique nature of the sperm chromatin organization and absence of a decondensation

step during the assay protocol, the TUNEL assay has been criticized as possibly only labeling the periphery of the sperm nucleus, and hence possibly underestimating the level of sperm DNA damage.

In Situ Nick Translation

The in situ nick translation (ISNT) assay is a modified form of TUNEL which quantifies the incorporation of biotinylated deoxyuridine triphosphate (dUTP) at single-stranded DNA breaks in a reaction that is catalyzed by the template-dependent enzyme, DNA polymerase I ([Irvine et al., 2000](#)). Unlike the TUNEL assay which utilizes template independent end labeling, nick translation can only be used to detect single strand DNA breaks. INST is a direct method of DNA damage measurement.

Advantages of ISNT

The assay identifies sperm that contain low and variable levels of endogenous DNA damage and is positively associated with protamine deficiency. Due to the efficiency of the DNA polymerase enzyme which is used in the assay, the single strand nicks are incorporated with labeled dUTP and hence a low levels of fluorescence in the sperm can be detected ([Shamsi et al., 2008](#)).

Limitations of ISNT

The clinical value of the nick translation assay is severely limited because no correlation has been proven with fertility outcomes during in vitro and in vivo studies. Other assays such as TUNEL and comet assays show better sensitivity when compared with ISNT assay, as ISNT assay measures only single strand DNA breaks. Furthermore, single strand breaks can be more easily repaired by the embryo when compared to double strand DNA breaks and hence the value of assay as a diagnostic tool is limited ([Shamsi et al., 2008](#)).

Sperm Chromatin Dispersion (SCD) Assay

The SCD assay, also known as Halo test, has been described as a simple and inexpensive method to analyze sperm DNA damage. It is based on the principle that sperm with fragmented/damaged DNA fail to produce the characteristic halo ([Fernandez et al., 2003](#)). The methodology of the test includes the following steps. First the sperm is fixed in an agarose matrix on a glass slide and allowed to solidify. The agarose matrix holds the sperm on a slide in a suspension-like environment. The slides are then treated with detergent to remove the cell membrane and nuclear proteins, followed by acid denaturation of the double stranded DNA. The removal of nuclear proteins results in nucleoid with a central core and a peripheral halo of dispersed DNA loops, which can be viewed using a DNA stain. The sperm nuclei with increased DNA damage produce very small or no halos, whereas normal sperm is able to release their DNA loops forming large halos ([Fernandez et al., 2003](#)).

Advantages of SCD Assay

This assay is a simple, fast, highly reproducible and inexpensive technique, where DNA damage as reflected by the size of the halos can be accurately determined. This assay does not rely on color or fluorescence intensity hence it is simple to analyze with light microscopy. The assay does not require the use of any complex and expensive instruments, as it can be carried out with equipment available in the andrology laboratory (such as a light microscope). The test endpoints are non-dispersed or dispersed halos, which can be easily distinguished by a laboratory technician. Therefore, the SCD assay has a potential to be routine used in the clinical laboratory for sperm chromatin evaluation ([Shamsi et al., 2008](#)).

Limitations of SCD Assay

The assay has been reported to have low-density nucleoids which are fainter with less contrasting images. Frequently, the peripheral limit of the halo, where the chromatin is even less dense, may not be accurately discriminated from the background. All the halos are not in the same plane hence, the use of software to analyze can result in misreading due to unfocused halos ([Shamsi et al., 2008](#)). More importantly, sperm tails are not preserved during the assay protocol; therefore, discriminating sperm from other contaminant cells is problematic. Another major disadvantage this assay is the lack of correlation between halo size with male fertility status and ART outcome, making this assay less suitable for diagnostic purposes.

Aniline Blue Assay

The aniline blue staining method is commonly used to study the chromatin maturity or chromatin compaction of sperm, as it is able to detect sperm chromatin defects related to their nucleoprotein content ([Auger et al., 1990](#)). The stain specifically binds to

histones and leaving the protamines unstained. Specifically, the stain binds to the amino acids region rich in lysine. Histones are basic proteins and are rich in lysine residues, whereas protamines are arginine and cysteine rich proteins. During spermatogenesis, the histones in the sperm nucleus are replaced to protamines. Approximately, 15% of the histones are kept in the nucleus at specific regions of the sperm chromatin. Therefore, increase in aniline blue staining correspond to increase in the level of histone retention, such sperm are considered as immature sperm with defective chromatin organization, whereas mature protamine-rich nuclei remain unstained.

The methodology of aniline blue stain is simple. Briefly, sperm sample are smeared on glass slides and air-dried. Sperm is then fixed with 4% formaldehyde solution and then stained with 5% aqueous aniline blue solution mixed with 4% acetic acid (pH: 3.5) for 5 min. The excessive stain is then removed by introducing the slide to a stream of water. The slides are air-dried and viewed under a light microscope.

Advantages and Limitations of Aniline Blue Assay

This staining method is very simple, inexpensive, rapid and the stained sperm can be viewed under a light microscope. The stain is able to differentiate between histones and protamines in sperm nucleus and hence it is able to identify immature sperm. The aniline blue staining method is an indirect method to study sperm chromatin status as the stain binds to chromatin bound histones but not to sperm chromatin.

Toluidine Blue Assay

Toluidine blue is a basic nuclear stain and is commonly used for metachromatic and orthochromatic staining of chromatin. This stain binds to the phosphate residues of sperm chromatin structure at places where the chromatin is free and not bound to histones or protamines. Therefore, the stain measures the availability of the free sperm chromatin DNA phosphate residues, which depends on both the protamination and DNA integrity of sperm. Dark purple stained nucleus correspond to abnormal sperm whereas, light blue stained nucleus correspond to normal sperm (Tsarev et al., 2009).

The methodology of toluidine blue staining is simple and consists of the following steps. Sperm samples are smeared on glass slides and then air-dried. The slides are then fixed with freshly prepared 96% ethanol–acetone (1:1 ratio) for 30 min and hydrolyzed with 0.1 N HCl for 5 min, and thoroughly rinsed with water. The slides are then stained with 0.05% toluidine blue stain prepared with 50% McIlvain's solution (citrate phosphate buffer, pH: 3.5) for 10 min. Slides were then rinsed briefly in water, dehydrated in tertiary butanol, cleared with xylene and mounted with DPX (water-insoluble embedding medium for histological preparations).

Advantages and Limitations of Toluidine Blue Assay

This staining method is very simple, inexpensive, rapid and the stained sperm can be viewed under a light microscope. The stain is able to differentiate sperm with abnormal chromatin packing from sperm with normal chromatin structure. The stain is very stable and hence it is a reliable method for assessing the sperm chromatin. The toluidine blue staining method is an indirect method to study sperm DNA integrity as the stain binds to regions of chromatin not bound to histones or protamines.

Chromomycin A₃ Assay

The chromomycin A₃ stain is used to identify sperm nucleus with poor chromatin organization. The stain binds to guanine–cytosine specific residues, which are present at the minor groove of the sperm chromatin. This stain and protamines compete for the same binding sites in the sperm chromatin. Therefore, high chromomycin stained sperm is a strong indicator of sperm with abnormal protamine packing (Iranpour et al., 2000).

The methodology of chromomycin staining method includes the following steps. Sperm samples are smeared on a glass slides and then air-dried. The sperm is then fixed in methanol-glacial acetic acid (3:1 ratio) for 20 min and then air-dried. The slides are then stained with 100 µL of chromomycin staining solution (0.25 mg/mL in McIlcaine buffer, pH: 7.0 containing 10 mM MgCl₂). The slides are then rinsed with PBS and mounted with buffered glycerol (1:1 ratio of PBS-glycerol). Under a fluorescent microscope, the sperm stained bright yellow are abnormal sperm (chromomycin positive), while the sperm stained dull yellow are chromomycin negative, or sperm with normal chromatin.

Advantages and Limitations of Chromomycin A₃ Assay

This staining method is simple, inexpensive and rapid but it requires complex instrumentation, such as a fluorescence microscopy and qualified technician to perform the experiment. Based on the stain intensity sperm can be differentiated into sperm with abnormal and normal chromatin packing, but the assay is criticized to have a low degree of separation. The stain is not stable for a long period of time and hence the slides should be read immediately or it can be refrigerated overnight. This is an indirect method to study sperm chromatin integrity as the assay reveals the amount of sperm chromatin not bound to protamines.

Conclusions

Simple and effective assays are available to assess sperm chromatin. Assays can distinguish single and double strand DNA strand breaks, as well as the protamination process. Standardization of assays and the establishment of consensus assays has not yet been accomplished, nevertheless, the assays have generally been shown to have clinical relevance and usefulness.

A comparison of principle and methodological aspects of sperm DNA damage assays

DNA damage assays	Methodology	Principle
Comet assay	Direct	Sperm DNA is subjected to electric field, intact DNA remains in the comet head, whereas DNA fragments migrate to the tail
Sperm chromatin structure assay	Indirect	The acridine orange stain binds to double stranded DNA to emit green fluorescence and to single stranded DNA to emit red fluorescence
TUNEL assay	Direct	Incorporates deoxyuridine triphosphate at the site of single and double strand DNA breaks in a reaction catalyzed by the template-independent enzyme
In situ nick translation	Direct	Incorporates biotinylated deoxyuridine triphosphate at the site of single-stranded DNA breaks in a reaction that is catalyzed by the template-dependent DNA polymerase enzyme
Sperm chromatin dispersion assay	Indirect	Sperm nuclei with increased DNA damage produce very small or no halos, whereas normal sperm is able to release their DNA loops forming large halos
Aniline blue assay	Indirect	The aniline blue stain specifically binds to histones, leaving the protamines unstained
Toluidine blue assay	Indirect	The toluidine blue stain binds to the phosphate residues of sperm chromatin at places where the chromatin is free and not bound to histones or protamines
Chromomycin A ₃ assay	Indirect	The chromomycin A ₃ stain and protamines compete for the same binding sites (guanine–cytosine specific residues), which are present at the minor groove of the sperm chromatin

References

- Aitken, R. J., & De Iuliis, G. N. (2007). 'Origins and consequences of DNA damage in male germ cells. *Reproductive Biomedicine Online*, 14, 727–733.
- Auger, J., Mesbah, M., Huber, C., & Dadoune, J. P. (1990). Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. *International Journal of Andrology*, 13, 452–462.
- Evenson, D., & Jost, L. (2000). Sperm chromatin structure assay is useful for fertility assessment. *Methods in Cell Science*, 22, 169–189.
- Evenson, D. P., Darzynkiewicz, Z., & Melamed, M. R. (1980). Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, 210, 1131–1133.
- Fernandez, J. L., Muriel, L., Rivero, M. T., Goyanes, V., Vazquez, R., & Alvarez, J. G. (2003). The sperm chromatin dispersion test: a simple method for the determination of sperm DNA fragmentation. *Journal of Andrology*, 24, 59–66.
- Gorczyca, W., Traganos, F., Jesionowska, H., & Darzynkiewicz, Z. (1993). Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. *Experimental Cell Research*, 207, 202–205.
- Iranpour, F. G., Nasr-Esfahani, M. H., Valojerdi, M. R., & al-Taraihi, T. M. (2000). Chromomycin A3 staining as a useful tool for evaluation of male fertility. *Journal of Assisted Reproduction and Genetics*, 17, 60–66.
- Irvine, D. S., Twigg, J. P., Gordon, E. L., Fulton, N., Milne, P. A., & Aitken, R. J. (2000). DNA integrity in human spermatozoa: relationships with semen quality. *Journal of Andrology*, 21, 33–44.
- Nanassy, L., & Carrell, D. T. (2008). Paternal effects on early embryogenesis. *Journal of Experimental & Clinical Assisted Reproduction*, 5, 2.
- Oliva, R. (2006). Protamines and male infertility. *Human Reproduction Update*, 12, 417–435.
- Sergerie, M., Laforest, G., Boulanger, K., Bissonnette, F., & Bleau, G. (2005). Longitudinal study of sperm DNA fragmentation as measured by terminal uridine nick end-labelling assay. *Human Reproduction*, 20, 1921–1927.
- Shamsi, M. B., Kumar, R., & Dada, R. (2008). Evaluation of nuclear DNA damage in human spermatozoa in men opting for assisted reproduction. *Indian Journal of Medical Research*, 127, 115–123.
- Simon, L., Lutton, D., McManus, J., & Lewis, S. E. (2011). Sperm DNA damage measured by the alkaline Comet assay as an independent predictor of male infertility and in vitro fertilization success. *Fertility and Sterility*, 95, 652–657.
- Simon, L., Liu, L., Murphy, K., Ge, S., Hotaling, J., Aston, K. I., Emery, B., & Carrell, D. T. (2014). Comparative analysis of three sperm DNA damage assays and sperm nuclear protein content in couples undergoing assisted reproduction treatment. *Human Reproduction*, 29, 904–917.
- Simon, L., Zini, A., Dyachenko, A., Ciampi, A., & Carrell, D. T. (2017). A systematic review and meta-analysis to determine the effect of sperm DNA damage on in vitro fertilization and intracytoplasmic sperm injection outcome. *Asian Journal of Andrology*, 19, 80–90.
- Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research*, 175, 184–191.
- Tarozzi, N., Bizzaro, D., Flamigni, C., & Borini, A. (2007). Clinical relevance of sperm DNA damage in assisted reproduction. *Reproductive Biomedicine Online*, 14, 746–757.
- Thomson, L. K., Fleming, S. D., Aitken, R. J., De Iuliis, G. N., Zieschang, J. A., & Clark, A. M. (2009). Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Human Reproduction*, 24, 2061–2070.
- Tsarev, I., Bungum, M., Givercman, A., Erenpreisa, J., Ebessen, T., Ernst, E., & Erenpreiss, J. (2009). Evaluation of male fertility potential by toluidine blue test for sperm chromatin structure assessment. *Human Reproduction*, 24, 1569–1574.

Sperm Chromatin Structure Analysis and Clinical Correlations

Denis Vaughan and Denny Sakkas, Boston IVF, Waltham, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Male infertility is thought to be the root cause or, at least, a contributing factor in 30%–40% of infertile couples. In 1992, the report of a successful pregnancy using intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992) revolutionized the ability to treat male factor infertility and enabled the field to overcome a large proportion of male factor regardless of the specific etiology. These impressive advances have however led to concerns regarding the quality of paternal genome being introduced into the egg when treating these patients. Even when considering the central importance of the paternal genome, sperm is still seen as a minor factor when assessing the infertile couple. Current practice most often comprises a brief medical history and a single semen analysis (SART Committee Report, 2015).

Mammalian sperm cells are, by far, one of the most specialized cells in the human body. Firstly, spermatogenesis entails multiple key cellular functions, including mitosis, meiosis, a complex remodeling of the nuclear chromatin and then a final cellular metamorphosis leading to the stripping of most of the cytoplasm and creation of a tail. Secondly, sperm are designed to accomplish a very difficult mission, involving several consecutive phases, each one independent and highly critical, traversing both the male and female reproductive tracts, burrowing through the egg vestments and completing pronuclear formation. The different components of a spermatozoon each play a crucial role during the conception process (Gagnon, 1999). For example, the head contains DNA/chromatin, which needs to be correctly condensed and decondensed at specific moments; the mid-piece, which contains the energy-generating mitochondria and; lastly, the flagellum transforms energy into movement. In addition, sperm possess all the mechanisms necessary for oocyte recognition and fusion, as well as intracellular structures and factors influencing early embryo development and division (Krawetz, 2005).

DNA/Chromatin Integrity

DNA integrity has been the most studied molecular feature in sperm. Although it is clear that sperm with abnormal DNA/chromatin integrity exist in ejaculates, the clinical utility of this information is controversial.

Sperm DNA fragmentation originates from a multitude of processes, comprising both endo- or exogenous factors and including defective spermatogenesis, abortive apoptosis, protamine defects, ROS attack, different medical pathologies (cancer, varicocele, infections), age, lifestyle influences or working conditions. Sperm has few repairing mechanisms, especially when compared to the demonstrated ability of the oocytes to repair limited damage (Sakkas et al., 2002; Seli and Sakkas, 2005; Sakkas and Alvarez, 2010; Esbert et al., 2011; Meseguer et al., 2011). Several tests are available in order to measure sperm DNA damage, and their application has been discussed extensively (Ribas-Maynou et al., 2013; Schlegel and Paduch, 2005; ASRM Guideline, 2013). This measurable damage may include single or double strand DNA damage or minor chromatin anomalies. The majority of current tests are capable of detecting only gross anomalies and are unable to detect more subtle anomalies. In addition, the lack of standardization among laboratories negatively contributes to the implementation of these techniques on a global scale. These confounders likely influence the results provided by laboratory reports, regarding the effect of DNA damage on sperm function and have resulted in the absence of an accurate and reproducible analysis to predict reproductive success according to DNA damage.

Despite these inconsistencies and concerns, there is a general consensus regarding the link between sperm DNA fragmentation and male infertility. The general fear exists of inadvertently microinjecting a DNA-damaged sperm during ICSI, thereby resulting in worse embryo quality and lower reproductive results, either in pregnancy achievement or on increased miscarriage rates (Bungum, 2012; Collins et al., 2008; Evenson et al., 1999; Fernandez et al., 2000). There have also been studies postulating concerns regarding the child's future health, following implementation of these assisted reproduction techniques (Fernandez-Gonzalez et al., 2008; Ahmadi and Ng, 1999a; Zini and Libman, 2006; Zini et al., 2008). Exactly to which extent DNA fragmentation decreases reproductive results is undetermined and there are conflicting data in the literature as to the utility of the tests (Niederberger, 2011; Payne et al., 2005). However, it seems sufficiently justified that selection of DNA-intact sperm to be utilized in ART will potentially improve results.

Sperm Chromatin Anomalies and Animal Models

Arguably, the most critical component in relation to successful in vitro fertilization or intracytoplasmic sperm injection is the sperm nucleus. In animal studies the relationship between an abnormal sperm nucleus, in particular the DNA or chromatin packaging, has been shown to have negative effects on reproductive outcome and progeny (Hales et al., 1992; Robaire and Hales, 2003; Anway et al., 2005). Some of the most intriguing studies have shown that, when sperm are exposed to gamma radiation prior to insemination, the proportion of nuclear DNA-strand breaks increased when sperm were subjected to between 5 and 100 GY. Interestingly, when the irradiated sperm are used to fertilize, live birth rate decreased dramatically, from over 50% in controls to 0%–20% when sperm were exposed to high doses of radiation (Ahmadi and Ng, 1997, 1999b). In other studies, disruption of

the paternal nuclear DNA has even led to transgenerational effects on offspring, in particular when endocrine disruptors are investigated (Skinner, 2016).

Sperm Chromatin Testing in the Human

With the known impact of sperm nuclear DNA in animal studies, it was subsequently postulated that testing the chromatin of human sperm would assist in the diagnosis and treatment of male factor infertility. Over 30 years ago, Evenson et al. (1980) demonstrated a relationship between mammalian sperm chromatin heterogeneity and fertility in human. The same group went on to perform the Georgetown Study (Evenson et al., 1999), which correlated sperm DNA integrity with pregnancy outcome. They described an analysis which they termed the Sperm Chromatin Structure Assay (SCSA). They provided SCSA values of human semen samples from 165 presumably fertile couples wishing to achieve pregnancy over 12 menstrual cycles. SCSA data from the male partners of 73 couples (group 1) achieving pregnancy during months 1–3 were compatible with “high fertility.” These SCSA values were significantly different from both couples (group 3) achieving pregnancy in months 4–12 or those who failed to achieve pregnancy ($P < .001$). Of the numerous sperm DNA integrity tests now available, the SCSA has become the most utilized test of sperm chromatin. The tests that are most commonly used to assess the sperm nucleus are described in the following paragraphs.

SCSA (Sperm Chromatin Structure Assay)

In this technique, the presence of DNA fragmentation is indirectly evaluated by examining the susceptibility of the sperm DNA to acid denaturation in situ, followed by staining with acridine orange (Evenson, 1990). By using flow cytometry, around 5000–10,000 sperm can be assessed in a few seconds. The percentage of red sperm (DNA fragmentation index or DFI) represent those sperm with denatured DNA, which in a normal semen sample should be under 27%–30% (Evenson et al., 1980; Virro et al., 2004). Specific SCSA-software can then determine the ratio between normal and abnormal sperm.

TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling)

This technique allows the evaluation of single or double stranded DNA damage. It measures the presence of 3′OH free groups, which are products of broken DNA strands. It is based on the fact that the enzyme “deoxynucleotidyl transferase” (TdT) allows for labeling of the DNA nicks through deoxyuridine triphosphate (dUTP). Similar to the SCSA, a fluorescent signal bound to the enzyme is then used to quantitate thousands of sperm using flow cytometry.

Comet Assay

This technique utilizes electrophoresis, and the principle that the fragmented DNA has a greater migrating velocity toward the anode of the electrophoretic field compared to that of intact DNA. This assay can be done at a neutral or alkaline pH, but may lead to an overestimation of broken DNA in the sperm (Shamsi et al., 2010). The reference ranges reported in the literature vary widely among authors (Tamburrino et al., 2012), as there is no standardized protocol.

SCD (Sperm Chromatin Dispersion or Halo Test)

The SCD or Halo technique identifies a differential in decondensation of the chromatin between those sperm with fragmented DNA and intact DNA. This is achieved by using an acid treatment followed by deproteinization, so that those sperm with fragmented DNA would not release DNA loops and do not generate a chromatin dispersion halo. Those sperm without DNA fragmentation demonstrate a big halo that corresponds to the DNA loops. It has been reported that, using this technique, the chances of achieving a pregnancy is very low if at least 30% fragmentation is identified (Fernandez et al., 2005).

Clinical Validation

The validity of these various sperm chromatin tests and their reliable prediction of reproductive outcome has been controversial over the years. Initial studies related the tests to both natural fertility and the use of intrauterine insemination. Indeed, the Georgetown Study (Evenson et al., 1999) mentioned above, and subsequent studies on IUI provided good evidence as to the validity of sperm chromatin testing. Bungum et al. (2004), in particular, demonstrated that when IUI was performed the chance of pregnancy/delivery was significantly higher in the group with $DFI \leq 27\%$ than in patients with $DFI > 27\%$. The odds ratios (ORs) (95% confidence intervals) were 20 (2.3–117), 16 (1.9–137), and 14 (1.6–110) for biochemical pregnancy, clinical pregnancy, and delivery, respectively.

In the IVF setting, sperm DNA fragmentation has been associated with reduced fertilization rates (Liu and Baker, 1992), embryo quality (Seli et al., 2004), pregnancy rates (Evenson et al., 1999) and increased miscarriage rates (Carrell et al., 2003). The various methods used to test sperm DNA fragmentation such as the SCSA, the sperm chromatin dispersion (SCD) test, the TUNEL assay and

the Comet assay were recently compared in a systematic review and meta-analysis to assess their value of measuring sperm DNA fragmentation and predicting the chance of ongoing pregnancy with IVF or ICSI.

The recent study by Cissen et al. (2016) examined 658 unique studies, however only 30 met the inclusion criteria for the metaanalysis. They reported that, overall, sperm DNA fragmentation tests had a reasonable to good sensitivity. When they constructed a hierarchical summary receiver operating characteristic (HSROC) curve they only found that the TUNEL assay (area under the curve (AUC) of 0.71; 95% CI 0.66–0.74) and Comet assay (AUC of 0.73; 95% CI 0.19–0.97) had a fair predictive capability for the chance of pregnancy. In their analysis, the SCSA and the SCD test had poor predictive capacity. They concluded that, at present, the current sperm DNA fragmentation tests have limited capacity to predict the chance of pregnancy in the context of IVF and ICSI.

One area of increasing clinical interest, in relation to the sperm chromatin anomalies, is the data demonstrating that high levels of DNA fragmentation are associated with recurrent pregnancy loss (Carrell et al., 2003; Ruixue et al., 2013; Ribas-Maynou et al., 2012). It is possible that while DNA compromised sperm may be able to reach and fertilize the eggs, there may be subsequent detrimental effects on the development of the embryo and fetus.

The question has arisen regarding the difference in predictive capability of sperm fragmentation tests between IUI and IVF? One of the main reasons is that the sperm utilized for IVF or ICSI is prepared in a different manner. It could be argued that the preparation of sperm for IVF or ICSI removes many of the abnormal sperm which could contribute to higher sperm DNA fragmentation levels. For example, in our own studies (Tomlinson et al., 2001) we showed that density gradient centrifugation enriched samples by improving the sperm nuclear integrity (by 450%), when comparing the fresh semen sample to the postpreparation samples ($N = 140$: 9% in semen versus 2% postpreparation). In addition, selection of even grossly motile normal sperm by an embryologist performing ICSI may add another layer of sperm selection that would eliminate sperm with abnormal DNA integrity.

New Tests of DNA Integrity

A number of new methodologies are now being developed to provide a different perspective on sperm DNA integrity. One of these is based on genome-wide sperm deoxyribonucleic acid (DNA) methylation patterns. In a recent study by Aston et al. (2015) they evaluated whether male fertility status and/or embryo quality during in vitro fertilization (IVF) therapy can be predicted by the DNA methylation status of sperm. They examined normospermic IVF patients who were separated into two groups: patients who had generally good embryogenesis and a positive pregnancy ($n = 55$) and patients with generally poor embryogenesis ($n = 72$; 42 positive and 30 negative pregnancies) after IVF. Their analysis revealed that sperm DNA methylation patterns differed significantly and consistently between the two groups. They concluded that DNA methylation patterns may be predictive of embryo quality during IVF.

Conclusion

Although the correlation between sperm chromatin/DNA integrity and reproductive outcome is less clear in the human than in animals, the current data on human sperm DNA testing suggests the following:

- (i) the presence of DNA damage in a high proportion of spermatozoa from a raw semen sample, is associated with a decreased likelihood of fathering a child, regardless of semen analysis parameters (Bungum et al., 2004);
- (ii) While a specific threshold or percentage of DNA-damaged sperm that is incompatible with pregnancy has not been established, it may be used during the initial evaluation of the infertile couple to guide management regarding which couples should undergo fewer IUI cycles in favor of early IVF/ICSI (Bungum et al., 2004);
- (iii) current sperm-DNA integrity tests are better at predicting reduced success with natural and IUI attempts at pregnancy than attempts using IVF or ICSI (Bungum et al., 2004, 2008; Zini et al., 2008).

Although the clinical application of the numerous sperm DNA integrity tests is still controversial (Collins et al., 2008), it is undeniable that infertile men or men from infertile couples have a higher frequency of abnormal results from DNA integrity testing than fertile controls (Tomlinson et al., 2001). It is also becoming more apparent that removal of DNA damaged sperm from the IVF or ICSI prepared samples can improve outcomes for patients (Worrlow et al., 2013). There is also mounting evidence that sperm DNA integrity abnormalities in men are associated with higher miscarriage rates (Carrell et al., 2003; Ribas-Maynou et al., 2012; Ruixue et al., 2013).

The reason why ejaculated sperm possess chromatin and DNA anomalies still remains unclear. The possible mechanisms hypothesized include: apoptosis in the seminiferous tubule epithelium; defects in chromatin remodeling during the process of spermiogenesis; oxygen radical-induced DNA damage during sperm migration to and while in the epididymis; the activation of sperm caspases and endonucleases; damage induced by chemotherapy and radiotherapy; and the effect of environmental toxicants (Sakkas and Alvarez, 2010; Aitken and Curry, 2011).

IVF reproductive technologies have come a long way since the birth of the first baby born, utilizing IVF technology, in 1978. The technical progression from routine insemination to ICSI for treating many male infertility patients has changed the way we prepare sperm. It is now coming to light that the role of the paternal genome in influencing the success of IVF is more than once thought. We

now recognize that more importance should be placed on sperm preparation techniques and many research laboratories are now striving toward the development of novel techniques for sperm preparation and selection.

References

- Ahmadi, A., & Ng, S. C. (1997). Fertilization and development of mouse oocytes injected with membrane-damaged spermatozoa. *Human Reproduction*, 12, 2797–2801.
- Ahmadi, A., & Ng, S. C. (1999a). Developmental capacity of damaged spermatozoa. *Human Reproduction*, 14, 2279–2285.
- Ahmadi, A., & Ng, S. C. (1999b). Fertilizing ability of DNA-damaged spermatozoa. *Journal of Experimental Zoology*, 284, 696–704.
- Aitken, R. J., & Curry, B. J. (2011). Redox regulation of human sperm function: From the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxidants & Redox Signaling*, 14, 367–381.
- Anway, M. D., Cupp, A. S., Uzumcu, M., & Skinner, M. K. (2005). Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science*, 308, 1466–1469.
- ASRM Guideline. (2013). The clinical utility of sperm DNA integrity testing: A guideline. *Fertility and Sterility*, 99, 673–677.
- Aston, K. I., Uren, P. J., Jenkins, T. G., Horsager, A., Cairns, B. R., Smith, A. D., & Carrell, D. T. (2015). Aberrant sperm DNA methylation predicts male fertility status and embryo quality. *Fertility and Sterility*, 104, 1388–1397.
- Bungum, M. (2012). Sperm DNA integrity assessment: A new tool in diagnosis and treatment of fertility. *Obstetrics and Gynecology International*, 2012, 531042.
- Bungum, M., Humaidan, P., Spano, M., Jepson, K., Bungum, L., & Giwercman, A. (2004). The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Human Reproduction*, 19, 1401–1408.
- Bungum, M., Spano, M., Humaidan, P., Eleuteri, P., Rescia, M., & Giwercman, A. (2008). Sperm chromatin structure assay parameters measured after density gradient centrifugation are not predictive for the outcome of ART. *Human Reproduction*, 23, 4–10.
- Carrell, D. T., Liu, L., Peterson, C. M., Jones, K. P., Hatasaka, H. H., Erickson, L., & Campbell, B. (2003). Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Archives of Andrology*, 49, 49–55.
- Cissen, M., Wely, M. V., Scholten, I., Mansell, S., Bruin, J. P., Mol, B. W., Braat, D., Repping, S., & Hamer, G. (2016). Measuring sperm DNA fragmentation and clinical outcomes of medically assisted reproduction: A systematic review and meta-analysis. *PLoS One*, 11, e0165125.
- Collins, J. A., Barnhart, K. T., & Schlegel, P. N. (2008). Do sperm DNA integrity tests predict pregnancy with in vitro fertilization? *Fertility and Sterility*, 89, 823–831.
- Esbert, M., Pacheco, A., Vidal, F., Florensa, M., Riqueros, M., Ballesteros, A., Garrido, N., & Calderon, G. (2011). Impact of sperm DNA fragmentation on the outcome of IVF with own or donated oocytes. *Reproductive Biomedicine Online*, 23, 704–710.
- Evenson, D. P. (1990). Flow cytometric analysis of male germ cell quality. *Methods in Cell Biology*, 33, 401–410.
- Evenson, D. P., Darzynkiewicz, Z., & Melamed, M. R. (1980). Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, 210, 1131–1133.
- Evenson, D. P., Jost, L. K., Marshall, D., Zinaman, M. J., Clegg, E., Purvis, K., De Angelis, P., & Claussen, O. P. (1999). Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Human Reproduction*, 14, 1039–1049.
- Fernandez, J. L., Vazquez-Gundin, F., Delgado, A., Goyanes, V. J., Ramiro-Diaz, J., de la Torre, J., & Gosálvez, J. (2000). DNA breakage detection-FISH (DBD-FISH) in human spermatozoa: Technical variants evidence different structural features. *Mutation Research*, 453, 77–82.
- Fernandez, J. L., Muriel, L., Goyanes, V., Segrelles, E., Gosálvez, J., Enciso, M., Lafromboise, M., & De Jonge, C. (2005). Simple determination of human sperm DNA fragmentation with an improved sperm chromatin dispersion test. *Fertility and Sterility*, 84, 833–842.
- Fernandez-Gonzalez, R., Moreira, P. N., Perez-Crespo, M., Sanchez-Martin, M., Ramirez, M. A., Pericuesta, E., Bilbao, A., Bermejo-Alvarez, P., de Dios, H. J., de Fonseca, F. R., et al. (2008). Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biology of Reproduction*, 78, 761–772.
- Gagnon, C. (1999). *The male gamete: From basic science to clinical applications*. Vienna, IL: Cache River Press.
- Hales, B. F., Crosman, K., & Robaire, B. (1992). Increased postimplantation loss and malformations among the F2 progeny of male rats chronically treated with cyclophosphamide. *Teratology*, 45, 671–678.
- Krawetz, S. A. (2005). Paternal contribution: New insights and future challenges. *Nature Reviews. Genetics*, 6, 633–642.
- Liu, D. Y., & Baker, H. W. (1992). Sperm nuclear chromatin normality: Relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. *Fertility and Sterility*, 58, 1178–1184.
- Meseguer, M., Santiso, R., Garrido, N., Garcia-Herrero, S., Remohi, J., & Fernandez, J. L. (2011). Effect of sperm DNA fragmentation on pregnancy outcome depends on oocyte quality. *Fertility and Sterility*, 95, 124–128.
- Niederberger, C. S. (2011). Semen and the curse of cutoffs. *Journal of Urology*, 185, 381–382.
- Palermo, G., Joris, H., Devroey, P., & Van Steirteghem, A. C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, 340, 17–18.
- Payne, J. F., Raburn, D. J., Couchman, G. M., Price, T. M., Jamison, M. G., & Walmer, D. K. (2005). Redefining the relationship between sperm deoxyribonucleic acid fragmentation as measured by the sperm chromatin structure assay and outcomes of assisted reproductive techniques. *Fertility and Sterility*, 84, 356–364.
- Practice Committee of the American Society for Reproductive Medicine. (2015). Diagnostic evaluation of the infertile male: A committee opinion. *Fertility and Sterility*, 103, e18–e25.
- Ribas-Maynou, J., Garcia-Peiro, A., Fernandez-Encinas, A., Amengual, M. J., Prada, E., Cortes, P., Navarro, J., & Benet, J. (2012). Double stranded sperm DNA breaks, measured by Comet assay, are associated with unexplained recurrent miscarriage in couples without a female factor. *PLoS ONE*, 7, e44679.
- Ribas-Maynou, J., Garcia-Peiro, A., Fernandez-Encinas, A., Abad, C., Amengual, M. J., Prada, E., Navarro, J., & Benet, J. (2013). Comprehensive analysis of sperm DNA fragmentation by five different assays: TUNEL assay, SCSA, SCD test and alkaline and neutral Comet assay. *Andrology*, 1, 715–722.
- Robaire, B., & Hales, B. F. (2003). Mechanisms of action of cyclophosphamide as a male-mediated developmental toxicant. *Advances in Experimental Medicine and Biology*, 518, 169–180.
- Ruixue, W., Hongli, Z., Zhihong, Z., Rulin, D., Dongfeng, G., & Ruizhi, L. (2013). The impact of semen quality, occupational exposure to environmental factors and lifestyle on recurrent pregnancy loss. *Journal of Assisted Reproduction and Genetics*, 30, 1513–1518.
- Sakkas, D., & Alvarez, J. G. (2010). Sperm DNA fragmentation: Mechanisms of origin, impact on reproductive outcome, and analysis. *Fertility and Sterility*, 93, 1027–1036.
- Sakkas, D., Moffatt, O., Manicardi, G. C., Mariethoz, E., Tarozzi, N., & Bizzaro, D. (2002). Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biology of Reproduction*, 66, 1061–1067.
- Schlegel, P. N., & Paduch, D. A. (2005). Yet another test of sperm chromatin structure. *Fertility and Sterility*, 84, 854–859.
- Seli, E., & Sakkas, D. (2005). Spermatozoal nuclear determinants of reproductive outcome: Implications for ART. *Human Reproduction Update*, 11, 337–349.
- Seli, E., Gardner, D. K., Schoolcraft, W. B., Moffatt, O., & Sakkas, D. (2004). Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertility and Sterility*, 82, 378–383.
- Shamsi, M. B., Venkatesh, S., Tanwar, M., Singh, G., Mukherjee, S., Malhotra, N., Kumar, R., Gupta, N. P., Mittal, S., & Dada, R. (2010). Comet assay: A prognostic tool for DNA integrity assessment in infertile men opting for assisted reproduction. *Indian Journal of Medical Research*, 131, 675–681.
- Skinner, M. K. (2016). Endocrine disruptors in 2015: Epigenetic transgenerational inheritance. *Nature Reviews. Endocrinology*, 12, 68–70.
- Tamburrino, L., Marchiani, S., Montoya, M., Elia, M. F., Natali, I., Cambi, M., Forti, G., Baldi, E., & Muratori, M. (2012). Mechanisms and clinical correlates of sperm DNA damage. *Asian Journal of Andrology*, 14, 24–31.

- Tomlinson, M. J., Moffatt, O., Manicardi, G. C., Bizzaro, D., Afnan, M., & Sakkas, D. (2001). Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: Implications for assisted conception. *Human Reproduction*, *16*, 2160–2165.
- Virro, M. R., Larson-Cook, K. L., & Evenson, D. P. (2004). Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility*, *81*, 1289–1295.
- Worilow, K. C., Eid, S., Woodhouse, D., Perloe, M., Smith, S., Witmyer, J., Ivani, K., Khoury, C., Ball, G. D., Elliot, T., et al. (2013). Use of hyaluronan in the selection of sperm for intracytoplasmic sperm injection (ICSI): Significant improvement in clinical outcomes—Multicenter, double-blinded and randomized controlled trial. *Human Reproduction*, *28*, 306–314.
- Zini, A., & Libman, J. (2006). Sperm DNA damage: Clinical significance in the era of assisted reproduction. *CMAJ*, *175*, 495–500.
- Zini, A., Boman, J. M., Belzile, E., & Ciampi, A. (2008). Sperm DNA damage is associated with an increased risk of pregnancy loss after IVF and ICSI: Systematic review and meta-analysis. *Human Reproduction*, *23*, 2663–2668.

Semen Analysis: Assaying Sperm Epigenetics

Millissia Ben Maamar, Ingrid Sadler-Riggleman, and Michael K Skinner, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

5mC 5-Methylcytosine
ART Assisted reproductive techniques
CpG Cytosine/Guanine dinucleotide
CGI CpG islands
CHARM Comprehensive high-throughput arrays for relative methylation
ChIP Chromatin immunoprecipitation
DMRs Differentially methylated regions
DNA Deoxyribonucleic acid
DTT Dithiothreitol
Dnmts DNA methyltransferases
EDTA Ethylenediaminetetraacetic acid
HAT Histone acetyltransferases
HADCs Histone deacetylases
ISH In situ hybridization
MBD Methyl-CpG-Binding domain
MeDIP Methylated DNA immunoprecipitation
miRNAs microRNAs
MREs Methylation-sensitive restriction enzymes
ncRNAs Non-coding RNAs
PCR Polymerase chain reaction
qPCR Quantitative polymerase chain reaction
RNA Ribonucleic acid
RT-PCR Real-Time polymerase chain reaction
Sarkosyl Sodium lauroyl sarcosinate
SDS Sodium dodecyl sulfate

Introduction

The mammalian sperm contains a very specialized and singular epigenetic landscape that is essential for biology and fertility. DNA methylation, spermatozoal RNAs and nuclear protein composition are marks thought to be remnants of epigenetic transitions occurring during spermatogenesis even though some of them can influence fertilization and embryogenesis (Hammoud et al., 2009). The main difference between sperm and somatic cells is the replacement of 90%–95% of histone proteins with protamines, thus allowing a tighter DNA compaction with the formation of toroid structures necessary for sperm motility and protection of DNA from oxidation and fragmentation (Jenkins and Carrell, 2011; Oliva, 2006).

Epigenetics in normal cell function is well described and has been implicated in many diseases. Yet, its use in the field of fertility is only beginning to be explored even though it is still not well understood. The use of epigenetic signatures as potential markers for diseases related to fertility or as predictors in assisted reproductive techniques (ART) is currently being investigated. Because of the unique nature of the sperm epigenome and how the patterns found in mature sperm can possibly perturb spermatogenesis, some researchers believe that these marks could provide predictive insight on infertility and pregnancy outcomes.

Several techniques have been developed to study these different epigenetic marks (Table 1). Each of them can yield tremendous amounts of data about the patterns associated with different forms of fertility phenotypes. Furthermore, these techniques could potentially be useful as diagnostic tools in the foreseeable future and provide so much more data than the current tests used clinically to diagnose male fertility.

Table 1 Experimental approaches for profiling genome-wide epigenetic marks in sperm

Technique	Analysis	Advantages	Disadvantages
CHARM	Methylation	• Analysis of CpG sites genome-wide regardless of proximity to genes or CpG islands	• Moderate resolution • Limited regions near enzymes recognition sites
Affinity enrichment-based methods	Methylation	• Detection of DMRs within high or low CpG density regions • Can discriminate 5mC from 5hmC • No mutation introduced • More sensitive than MeDIP in regions with higher CpG density	• Low resolution • Biased toward hypermethylated regions
MeDIP	Methylation	• No mutation introduced • Specific to 5mC/5hmC depending on the antibody • More sensitive in regions with low CpG density	• Biased toward hypermethylated regions • No identification of single 5mC sites • No prediction of absolute methylation level
Bisulfite conversion-based methods	Methylation	• Evaluate methylation state of almost every CpG sites	• High cost • Bisulfite treatment can degrade DNA • No discrimination between 5mC and 5hmC
Single-cell methylome	Methylation	• Highly sensitive • Detect target CpG sites at high coverage with low number of sequence reads	• Bisulfite treatment can degrade DNA • No discrimination between 5mC and 5hmC • Poor coverage for imprinting loci
Pyrosequencing analysis ChIP	Methylation Histones	• Measurements of individual CpG dinucleotides • Very efficient immunoprecipitation	• Not a genome-wide approach • Not applicable to non-histone proteins • Risk of protein rearrangement during chromatin preparation and precipitation • May have selective digestion of particular chromatin domains during preparation
RNA extraction	ncRNAs	• Pure preparations of RNA	• Depending on the method used, small RNAs cannot be recovered

DNA Methylation

DNA methylation is one of the most studied epigenetic modifications. A methyl group will be added on the fifth carbon of cytosine (C) forming 5-methylcytosine (5mC) by catalysis of DNA methyltransferases (Dnmts) (Smith and Meissner, 2013). DNA methylation occurs mainly in CpG dinucleotides (CpGs) even though it can also be found, less frequently, in non-CpG context (Kim et al., 2014). DNA methylation has been associated with a large number of cellular processes such as X chromosome inactivation, embryonic development, genomic imprinting, transcriptional repression, alteration of chromatin structure, and transposon inactivation (Wilson et al., 2012). These methyl marks are heritable but more interestingly certain methylation patterns have transgenerational effects (Li et al., 2015b). DNA methylation is very different depending on the organism studied. In human embryonic stem cells for instance, DNA methylation occurs in up to 80% of CpGs with the remaining unmethylated CpG residues enriched in CpG islands (CGI) located at gene promoters (Chen et al., 2011). On the contrary, in invertebrates like *Drosophila*, methylation levels are extremely low (Lyko et al., 2000).

These important findings on DNA methylation would not have been possible without the development and advancement of experimental and computational profiling approaches.

Sperm DNA Extraction

Classic high quality DNA extractions developed for mammalian somatic cells are ineffective for sperm. In fact, unlike somatic cells, sperm DNA is highly compacted by the replacement of histones with sperm-specific proteins called protamines. These protamines and the disulfide bridges formed within and between protamines inhibit the extraction of sperm DNA by standard techniques (Griffin, 2013).

A wide variety of methods have been developed to extract DNA. In 1988, Bahnak et al. described a guanidinium protocol for the isolation of mammalian spermatozoa (Bahnak et al., 1988). This protocol was later modified by Hossain et al. replacing the lengthy ultracentrifugation steps with the use of isopropanol and proteinase K for digestion (Hossain et al., 1997). Commercial companies have also based their kits on this guanidine thiocyanate method for the isolation of RNA, DNA, and protein. But, these kits utilize toxic chemicals such as chloroform and phenol. This was counterbalanced by the development of column purification of the DNA following lysis.

In round spermatids, somatic histones are replaced by testis-specific transition proteins during spermiogenesis. To have a highly condensed chromatin, transition proteins are later replaced by protamines in the elongated spermatid which will then be phosphorylated (Brewer et al., 2002; Dadoune, 1995). Once the protamines bind to the DNA, most of the phosphate groups are removed and

cysteine residues become oxidized and form disulfide bridges that link the protamines together. During sperm DNA extraction, a supplemental dissociation of nucleoproteins from nucleic acids is required. Dithiothreitol (DTT) and 2-mercaptoethanol enable to cleave disulfide bonds and allow proteins to unfold completely. DTT is known to be more effective, less toxic, quicker and the odor is more tolerated than 2-mercaptoethanol. Sodium dodecyl sulfate (SDS) is routinely used in DNA extraction methods. Sodium lauroyl sarcosinate (Sarkosyl), like SDS is also used to denature proteins and disrupt biological membranes. However, Sarkosyl, on the opposite of SDS, has an excellent solubility in chaotropic solutions. Extraction buffers used contain EDTA which removes metal ions potentially present in the seminal fluid, or on the cell surface and will thus reduce contamination in the final DNA extract. The addition of isopropanol facilitates DNA precipitation. An incubation at room temperature with sodium citrate and alcohol will concentrate any remaining chaotropic salts into the solution while the DNA will stay precipitated. In fact, sodium ions in the presence of alcohol neutralize the negatively charged phosphate groups on the DNA backbone facilitating its precipitation.

The first studies on DNA methylation determined the methylation status of genes of interest and quantified the total amount of 5mC. Thanks to microarray hybridization technology, studies on DNA methylation were able to scale up to the genome-wide-level. Nowadays, next-generation sequencing platforms are able to construct genomic maps of DNA methylation at single-base resolution. Enzyme digestion, affinity enrichment and bisulfite conversion are currently used for that purpose. The most recent development in the epigenomic profiling is a single-cell methylome, and the use of third-generation sequencing in detecting DNA methylation in real time.

Restriction Enzyme-Based Methods

These techniques use methylation-sensitive restriction enzymes (MREs) such as *BstUI*, *HpaII*, *NotI*, and *SmaI* which cleave only unmethylated target sequences leaving then the methylated DNA intact. This MRE digestion coupled with sequencing technologies can predict genome-wide DNA methylation levels (Maunakea et al., 2010). During the sequencing step, the DNA fragments from this cleavage are size-selected and sequenced. The results reveal the locations of the unmethylated CpG sites within the recognition sites of the enzyme utilized (Li et al., 2015a).

In conclusion, MRE-Seq estimates the relative DNA methylation levels with a low coverage of the genome.

Comprehensive High-Throughput Arrays for Relative Methylation (CHARM)

This method uses the enzyme McrBC digesting methylated DNA to fractionate it. McrBC cleaves half of the methylated DNA and all the methylated CpG islands (CGI). This enables to relatively size-select unmethylated DNA and subsequently utilizes hybridization arrays. Thus, CHARM is able to detect differentially methylated regions (DMRs) at CGI shores (sequences up to 2 kb away from CGI) which are not detectable with methods such as methylated DNA immunoprecipitation (MeDIP).

Affinity Enrichment-Based Methods

As the MeDIP, these affinity enrichment-based methods use antibodies specific for 5mC or methyl-CpG-binding domain (MBD) proteins to enrich methylated DNA regions. This MBD protein-based approach relies on the capacity of MBD proteins to bind specifically to methylated DNA sequences followed by a microarray (MBD-chip) or sequencing (MBDCap-seq/MethylCap-seq) (Brinkman et al., 2010), methylated DNA capture by affinity purification technologies.

Methylated DNA Immunoprecipitation (MeDIPs)

Using an anti-methylcytosine antibody, MeDIP is able to immunoprecipitate DNA with methylated CpG sites. These DNA fractions enriched by MeDIP can be evaluated using tiling arrays (MeDIP-chip) or high-throughput sequencing (MeDIP-seq). This technique usually yields a resolution of 100–300 bp. MeDIP-seq generates the relative enrichment of methylated DNA across the genome instead of predicting the absolute DNA methylation level. Because of the low amount of DNA needed (as low as 1 ng), this method can be used to profile DNA methylation in minute DNA samples, rare cell types and microdissected tissues (Taiwo et al., 2012; Zhao et al., 2014). However, in MeDIP, CpG-rich fragments are more likely to be enriched than CpG-poor ones even if they are fully methylated, a computational correction to normalize CpG content across a wide range of CpG densities is always required (Down et al., 2008).

Bisulfite Conversion-Based Methods

This technique is based on the deamination of unmethylated C to uracil (U) residues on the genomic DNA, whereas methylated C residues remain unaffected. The U eventually converts to thymine (T) in a subsequent polymerase chain reaction (PCR). The methods based on this bisulfite conversion provide single-base resolution. When investigating specific DNA sequences or studying genome-wide methylation via a methylation array, whole-genome bisulfite sequencing and reduced-representation bisulfite sequencing are commonly used.

Single-Cell Methylome

Generally, most genome-wide DNA methylation profiling techniques need bulk cell populations as starting materials. It is also impossible to assess the methylation heterogeneity among individual cells. To overcome this issue, single-cell bisulfite-based techniques have been developed. To reduce DNA loss and provide information on approximately 1 million CpG sites within an individual mouse or human cell, steps of *MspI* digestion have been integrated to the bisulfite conversion. These techniques are ideal in studies with limited cell amounts and heterogeneous cell populations. They are especially practical for sperm cells, oocytes, primordial germ cells, and embryonic stem cells (Guo et al., 2015; Schwartzman and Tanay, 2015).

Pyrosequencing Analysis

Pyrosequencing provides an analysis of the methylation pattern at a specific site of 25–50 bp. It allows measurement of the methylation percentage of individual CpG dinucleotides, a certain flexibility in sequencing primer position to analyze any CpG sites. This technique is only used for specific sites and it is not possible to use it as a genome-wide approach as many regions of the genome cannot be analyzed with this protocol.

Third-Generation Sequencing

The epigenetics field is evolving rapidly and with it the technologies associated. Emerging third-generation sequencing such as single-molecule real-time sequencing or nanopore sequencing are perfect examples. The first technique enables to detect directly modifications by monitoring the activity of DNA polymerase during the incorporation of different nucleotides labeled with fluorescence into complementary DNA strands. In the second technique, single-strands of DNA are pulled by a phage DNA polymerase through a bacterial pore in single-nucleotide steps and the ion current through the pore is recorded (Laszlo et al., 2013).

All together, these third-generation sequencing techniques will allow researchers to make more discoveries on the different epigenetic modifications and could reveal novel functions of these epigenetic marks in gene expression. However, before their application is extended, the throughput and accuracy must be substantially improved before applying them to studies involving complex genomes (Yong et al., 2016).

Histone Modifications

Histones order DNA into structural units called nucleosomes. They are the major protein component of chromatin and are subject to several different post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation.

The positive charge of the lysine residue on histone tails usually leads to a tight association with negatively charged DNA. Histone acetyltransferases (HATs) are enzymes initiating the formation of euchromatin from heterochromatin. HATs catalyze the transfer of an acetyl group (CH_3CO) to a conserved lysine on the histone tail. Acetylation of histones typically leads to relaxed chromatin and is associated with activation of transcription. In contrast, histone deacetylases (HDACs) catalyze the removal of acetyl groups from histones, consequently leading to more tightly packaged chromatin. The recruitment of HATs and HDACs is tightly regulated by DNA binding proteins located near epigenetic target regions. The methylation of specific amino acids on histones can lead to the formation of heterochromatin with consequent repression of transcription.

The interaction between histones and DNA is most often studied using a technique known as chromatin immunoprecipitation (ChIP). This method requires the use of highly specific antibodies directed against DNA-binding proteins and can be followed by a number of nucleic acid analysis techniques, such as PCR, qPCR, sequencing, and microarray hybridization. ChIP determines whether certain histones are associated with specific genomic regions and also identifies regions of the genome associated with specific histone modifications—methylation and acetylation being the most studied ones.

Chromatin Immunoprecipitation (ChIP)

To map histone modifications, chromatin is normally isolated under either fixed or native conditions. Sperm is treated with a reducing agent in order to reduce and prevent intramolecular and intermolecular disulfide bonds from forming between cysteine residues. The sperm is subsequently resuspended in a lysis buffer containing some detergents, whose concentrations are crucial to an efficient chromatin shearing. This shearing can be proceeded by an enzymatic digestion (usually a micrococcal nuclease) or by a sonication method. The genomic DNA that is not protected by histones will be divided in fragments around 150–250 bp (150 bp being the size of one nucleosome). After that histones will be detected in the soluble chromatin fraction while protamines are spun down. This chromatin can be directly used in ChIP to map histone variants, modifications or retention in sperm. To do the immunoprecipitation, an incubation with an antibody against histones or histone modifications is used. Then the antibody–chromatin complexes will be captured using beads and the bound chromatin is eluted. The immunoprecipitated DNA is obtained by a phenol/chloroform extraction. The DNA associated with histones and histone modifications can be characterized by candidate specific quantitative PCR (qPCR) approaches or genome-wide high-throughput sequencing. However, even if qPCR is a valid

technique, because the isolated DNA is around 150 bp in size, designing one or both primers outside these small fragments can lead to mistaken conclusions (Hisano et al., 2013).

Non-Coding RNA

A very large number of transcripts do not appear to encode for proteins. These transcript species are known as noncoding RNAs (ncRNAs). For a long time, they have been considered as “junk” in the genome, but emerging evidence has highlighted that ncRNAs can have a wide variety of functions. Complex eukaryotes such as fungi, plants, and animals have >50%, a number rising to ~98.5% noncoding DNA in humans (Amaral et al., 2008). Non-coding RNAs can be classified according to length: the small ncRNAs (sncRNA) with a length <200bp; anything longer than that is referred to as long ncRNA (lncRNA). Examples of short ncRNAs include rRNA, microRNA (miRNA), siRNA, and Piwi-interacting RNA (piRNA). Both of these ncRNAs have been shown to be implicated in spermatogenesis (Bettegowda and Wilkinson, 2010; Chuma and Nakano, 2012; Lee et al., 2012; Yadav and Kotaja, 2014).

Small non-coding RNAs are generally identified by RT-PCR analysis, in situ hybridization, or small RNA sequencing studies. Besides, the recent development of microarray technologies has permitted the global analysis of spermatozoal microRNAs (miRNAs) allowing different research teams to determine different expression profiles between fertile and infertile men (Hamatani, 2011; Kotaja, 2014). Large non-coding RNAs are also predominant in sperm and can be analyzed in a sequencing analysis.

The sperm contains reduced cytoplasm, low amounts of full-length RNAs along with biologically degraded RNAs, and highly protamine-condensed DNA. Due to this complex nature, the isolation of RNA from sperm is unique compared to somatic cells.

To isolate total RNA, the sperm membrane and nucleoprotamine complex have to be completely dissolved. Variations of two methods have historically been used to prepare RNA from natural sources (e.g., tissue samples, whole organisms, cell cultures, bodily fluids): chemical extraction and immobilization on glass, often referred to as solid-phase extraction. Chemical extraction methods typically use highly concentrated chaotropic salts in conjunction with acidic phenol or phenol-chloroform solutions to inactivate RNases and purify RNA from other biomolecules. These methods provide very pure preparations of RNA. But, the RNA must typically be desalted and concentrated with an alcohol precipitation step. This alcohol precipitation does not quantitatively recover small nucleic acid molecules, making it ill-suited for the preparation of very small RNAs.

The second method, solid-phase extraction, is based on high salt or salt and alcohol to decrease the affinity of RNA for water and increase its affinity for the solid support used. However, this solid-phase purification of RNA does not effectively recover small RNAs.

Some companies have developed isolation procedures combining the advantages of organic extraction and solid-phase extraction while managing to recover small RNAs. The use of ethanol immobilizes the large RNAs through a glass-fiber filter and the small RNAs are collected in the filtrate. For non-coding RNAs (ncRNAs), an enrichment is usually necessary. Candidate-specific RT-PCR, microarrays or genome-wide high throughput sequencing analysis can be used to characterize the nature of these ncRNA.

Epigenetic Human Sperm Analysis

Clinical Potential Use

Epigenetics has been well studied in normal cell function and has also been implicated in many diseases (Momparker, 2003). Its use in fertility is however just starting to be explored. The multiple factors involved in epigenetics such as the variables involved, the poor description of normality as each cell type has unique signatures with some degree of variation and the type of epigenetic mark being assessed; make it challenging and hard to understand. With our current understanding of the epigenome, we cannot predict the potential biological impact of a change in DNA methylation (Jenkins et al., 2017). The emergence of extremely high resolution techniques enabling for instance a single cell resolution of complete transcriptomes, provides remarkable insight into epigenetics at the single cell resolution which can offer tremendous possibilities in both research and diagnostic development. Despite these limitations, many epigenetic signatures in sperm have been tightly associated with a number of sperm abnormalities and/or clinical outcomes such as protamine levels in sperm from fertile and infertile human males (Jenkins and Carrell, 2011). Using epigenetic signatures as potential markers for various fertility related diseases and/or as predictors for success in ART is becoming more widely explored (Aston et al., 2015; Jenkins et al., 2016; Jodar et al., 2015). The high resolution techniques enable to screen large portions of the epigenome and create a huge amount of data available which can help understand the relationship between the sperm epigenome and various fertility phenotypes (Hrdlickova et al., 2017). 684 RNA “elements” have been identified as being required to facilitate the best chances at successful IVF outcomes and the absence of even one of these elements has been shown to decrease the success rate in various fertility treatments (Jodar et al., 2015). Another study identified genetic markers which could be useful as a complement for classical semen analysis in order to identify sperm donors with less favorable in utero insemination reproductive outcome despite having normal semen parameters (Bonache et al., 2012). DNA methylation techniques have progressed greatly these last years and they are commonly used in the assessment of sperm DNA methylation patterns. For instance, Aston et al. identified in the mature sperm some methylation patterns which could predict the likelihood for a man to go through IVF or if a less invasive procedure might be effective (Aston et al., 2015).

It is well known that the current available diagnostic tools to establish a man's reproductive potential are lacking (WHO, 2010). Many researchers have highlighted the need to innovate and improve approaches with better predictive power for male infertility diagnosis purpose. Assessing the DNA methylation seems to be the easiest option as it remains remarkably stable throughout spermatogenesis (Hammoud et al., 2014) whereas RNA transcripts and histone retention/modifications change dramatically. Yet, discriminating one epigenetic mark rather than another is less likely to offer a better understanding in the etiologies of commonly seen sperm disorders. These techniques, even though not presently applicable clinically, will probably help develop predictive tools to assess an individual's ability to procreate.

References

- Amaral, P. P., Dinger, M. E., Mercer, T. R., & Mattick, J. S. (2008). The eukaryotic genome as an RNA machine. *Science*, 319, 1787–1789.
- Aston, K. I., Uren, P. J., Jenkins, T. G., Horsager, A., Cairns, B. R., Smith, A. D., & Carrell, D. T. (2015). Aberrant sperm DNA methylation predicts male fertility status and embryo quality. *Fertility and Sterility*, 104, 1388–1397.e5.
- Bahnak, B. R., Wu, Q. Y., Coulombel, L., Drouet, L., Kerbiriou-Nabias, D., & Meyer, D. (1988). A simple and efficient method for isolating high molecular weight DNA from mammalian sperm. *Nucleic Acids Research*, 16, 1208.
- Bettegowda, A., & Wilkinson, M. F. (2010). Transcription and post-transcriptional regulation of spermatogenesis. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 365, 1637–1651.
- Bonache, S., Mata, A., Ramos, M. D., Bassas, L., & Larriba, S. (2012). Sperm gene expression profile is related to pregnancy rate after insemination and is predictive of low fecundity in normozoospermic men. *Human Reproduction*, 27, 1556–1567.
- Brewer, L., Corzett, M., & Balhorn, R. (2002). Condensation of DNA by spermatid basic nuclear proteins. *The Journal of Biological Chemistry*, 277, 38895–38900.
- Brinkman, A. B., Simmer, F., Ma, K., Kaan, A., Zhu, J., & Stunnenberg, H. G. (2010). Whole-genome DNA methylation profiling using MethylCap-seq. *Methods*, 52, 232–236.
- Chen, P.-Y., Feng, S., Joo, J. W. J., Jacobsen, S. E., & Pellegrini, M. (2011). A comparative analysis of DNA methylation across human embryonic stem cell lines. *Genome Biology*, 12, R62.
- Chuma, S., & Nakano, T. (2012). piRNA and spermatogenesis in mice. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, 368, 20110338.
- Dadoun, J. P. (1995). The nuclear status of human sperm cells. *Micron*, 26, 323–345.
- Down, T. A., Rakyian, V. K., Turner, D. J., Flicek, P., Li, H., Kulesha, E., Gräf, S., Johnson, N., Herrero, J., Tomazou, E. M., et al. (2008). A Bayesian deconvolution strategy for immunoprecipitation-based DNA methylome analysis. *Nature Biotechnology*, 26, 779–785.
- Griffin, J. (2013). Methods of sperm DNA extraction for genetic and epigenetic studies. *Methods in Molecular Biology*, 927, 379–384.
- Guo, H., Zhu, P., Guo, F., Li, X., Wu, X., Fan, X., Wen, L., & Tang, F. (2015). Profiling DNA methylome landscapes of mammalian cells with single-cell reduced-representation bisulfite sequencing. *Nature Protocols*, 10, 645–659.
- Hamatani, T. (2011). Spermatozoal RNA profiling towards a clinical evaluation of sperm quality. *Reproductive Biomedicine Online*, 22, 103–105.
- Hammoud, S. S., Nix, D. A., Zhang, H., Purwar, J., Carrell, D. T., & Cairns, B. R. (2009). Distinctive chromatin in human sperm packages genes for embryo development. *Nature*, 460(7254), 473–478.
- Hammoud, S. S., Low, D. H. P., Yi, C., Carrell, D. T., Guccione, E., & Cairns, B. R. (2014). Chromatin and transcription transitions of mammalian adult Germline stem cells and spermatogenesis. *Cell Stem Cell*, 15, 239–253.
- Hisano, M., Erkek, S., Dessus-Babus, S., Ramos, L., Stadler, M. B., & Peters, A. H. F. M. (2013). Genome-wide chromatin analysis in mature mouse and human spermatozoa. *Nature Protocols*, 8, 2449–2470.
- Hossain, A. M., Rizk, B., Behzadian, A., & Thorneycroft, I. H. (1997). Modified guanidinium thiocyanate method for human sperm DNA isolation. *Molecular Human Reproduction*, 3, 953–956.
- Hrdlickova, R., Toloue, M., & Tian, B. (2017). RNA-Seq methods for transcriptome analysis. *Wiley Interdisciplinary Reviews RNA*, 8, e1364.
- Jenkins, T. G., & Carrell, D. T. (2011). The paternal epigenome and embryogenesis: Poising mechanisms for development. *Asian Journal of Andrology*, 13, 76–80.
- Jenkins, T. G., Aston, K. I., Meyer, T. D., Hotaling, J. M., Shamsi, M. B., Johnstone, E. B., Cox, K. J., Stanford, J. B., Porucznik, C. A., & Carrell, D. T. (2016). Decreased fecundity and sperm DNA methylation patterns. *Fertility and Sterility*, 105, 51-7.e1-3.
- Jenkins, T. G., Aston, K. I., James, E. R., & Carrell, D. T. (2017). Sperm epigenetics in the study of male fertility, offspring health, and potential clinical applications. *Systems Biology in Reproductive Medicine*, 63, 69–76.
- Jodar, M., Sendler, E., Moskovtsev, S. I., Librach, C. L., Goodrich, R., Swanson, S., Hauser, R., Diamond, M. P., & Krawetz, S. A. (2015). Absence of sperm RNA elements correlates with idiopathic male infertility. *Science Translational Medicine*, 7, 295re6.
- Kim, K. D., El Baidouri, M., & Jackson, S. A. (2014). Accessing epigenetic variation in the plant methylome. *Briefings in Functional Genomics*, 13, 318–327.
- Kotaja, N. (2014). MicroRNAs and spermatogenesis. *Fertility and Sterility*, 101, 1552–1562.
- Laszlo, A. H., Derrington, I. M., Brinkerhoff, H., Langford, K. W., Nova, I. C., Samson, J. M., Bartlett, J. J., Pavlenok, M., & Gundlach, J. H. (2013). Detection and mapping of 5-methylcytosine and 5-hydroxymethylcytosine with nanopore MspA. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 18904–18909.
- Lee, T.-L., Xiao, A., & Rennett, O. M. (2012). Identification of Novel Long Noncoding RNA Transcripts in Male Germ Cells, *Methods in Molecular Biology*; 825, pp. 105–114.
- Li, D., Zhang, B., Xing, X., & Wang, T. (2015a). Combining MeDIP-seq and MRE-seq to investigate genome-wide CpG methylation. *Methods*, 72, 29–40.
- Li, Q., Xu, W., Cui, Y., Ma, L., Richards, J., Li, W., Ma, Y., Fu, G., Bythwood, T., Wang, Y., et al. (2015b). A preliminary exploration on DNA methylation of transgene across generations in transgenic rats. *Scientific Reports*, 5, 8292.
- Lyko, F., Ramsahoye, B. H., & Jaenisch, R. (2000). DNA methylation in *Drosophila melanogaster*. *Nature*, 408, 538–540.
- Maunakea, A. K., Nagarajan, R. P., Bilienky, M., Ballinger, T. J., D'Souza, C., Fouse, S. D., Johnson, B. E., Hong, C., Nielsen, C., Zhao, Y., et al. (2010). Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature*, 466, 253–257.
- Momparler, R. L. (2003). Cancer epigenetics. *Oncogene*, 22, 6479–6483.
- Oliva, R. (2006). Protamines and male infertility. *Human Reproduction Update*, 12, 417–435.
- Schwartzman, O., & Tanay, A. (2015). Single-cell epigenomics: Techniques and emerging applications. *Nature Reviews. Genetics*, 16, 716–726.
- Smith, Z. D., & Meissner, A. (2013). DNA methylation: Roles in mammalian development. *Nature Reviews. Genetics*, 14, 204–220.
- Taiwo, O., Wilson, G. A., Morris, T., Seisenberger, S., Reik, W., Pearce, D., Beck, S., & Butcher, L. M. (2012). Methylome analysis using MeDIP-seq with low DNA concentrations. *Nature Protocols*, 7, 617–636.
- WHO (2010) World Health Organization WHO Ten chemicals of major public health concern. http://www.who.int/ipcs/assessment/public_health/chemicals_phc/en/ (access date 17 May 2015).

- Wilson, G. A., Dhami, P., Feber, A., Cortázar, D., Suzuki, Y., Schulz, R., Schär, P., & Beck, S. (2012). Resources for methylome analysis suitable for gene knockout studies of potential epigenome modifiers. *GigaScience*, 1, 3.
- Yadav, R. P., & Kotaja, N. (2014). Small RNAs in spermatogenesis. *Molecular and Cellular Endocrinology*, 382, 498–508.
- Yong, W.-S., Hsu, F.-M., & Chen, P.-Y. (2016). Profiling genome-wide DNA methylation. *Epigenetics and Chromatin*, 9, 26.
- Zhao, M.-T., Whyte, J. J., Hopkins, G. M., Kirk, M. D., & Prather, R. S. (2014). Methylated DNA immunoprecipitation and high-throughput sequencing (MeDIP-seq) using low amounts of genomic DNA. *Cellular Reprogramming*, 16, 175–184.

Raman Microspectroscopy

Grace M Centola, Reproductive Laboratory and Tissue Bank Consultant, Port St Lucie, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Standard assisted reproductive procedures allow an individual sperm to be injected into the egg. A sperm is chosen from a cell culture dish under a microscope by a technician based on the light microscopic appearance of the sperm. However, there has been no method to determine the internal structure of the cell nucleus and organelles, which may be abnormal or not functional even when the microscopic appearance of the sperm cell is normal. Sperm DNA damage is being rigorously studied, since this type of damage could be the cause of poor embryo quality, poor implantation, and poor development (Speyer et al., 2010; Mallidis et al., 2011).

Current microscopic methods are limited because they require that cells are fixed, stained with a toxic dye, and/or embedded in plastic and cut into thin pieces to examine under a microscope. Thus, damaged cells would not be able to be used clinically, and in the case of sperm, could not be used for assisted reproduction (Mallidis et al., 2011).

Raman microspectroscopy is a laser scattering technique that uses vibrational, rotational, and low-frequency laser scattering to detect the fingerprint of a cell's chemical composition (Mallidis et al., 2014). RMS detects the internal structure of molecules which comprise the "fingerprint" of a cell (Mallidis et al., 2011). For RMS, cells are air dried onto a glass slide and then examined using a special laser with filters and light scattering detectors. The spectral map at selected wavelengths is then imaged based on the area within the cell. Although the cells are dried onto the slide, current research focuses on refining the technique to allow the cells to remain functional after analysis (Mallidis et al., 2013; Liu et al., 2013; Ma et al., 2013).

Raman spectroscopy, combined with three-dimensional spatial resolution of confocal microscopy, can detect changes in specific molecules in the cell and thus assist in diagnosis and characterization of pathology (Mallidis et al., 2011, 2014). Normal and abnormal cells and organelles demonstrate a unique RMS spectrum. Thus RMS has been shown to detect oxidative damage to the nuclear DNA of individual human sperm (Sanchez et al., 2012; Sigman, 2012). Furthermore, Raman microspectroscopy has been used to distinguish sperm bound to the egg (i.e., functional) as compared to unbound sperm (Liu et al., 2013). Additionally, RMS has been shown to detect complete and incomplete sperm production in testicular tissue from men presenting with no sperm in the ejaculate (Ma et al., 2013; Liu et al., 2014).

Raman microspectroscopy is an accurate and reproducible procedure for the assessment of sperm DNA structure and the sites of nuclear DNA damage. It holds promise for resolving the issue of idiopathic male infertility and ultimately can assist in the selection of a "normal" sperm for use in assisted reproductive procedures.

References

- Liu, F., Zhu, Y., Liu, Y., Wang, X., Ping, P., Zhu, X., Hu, H., Li, Z., & He, L. (2013). Real-time Raman microspectroscopy scanning of the single live sperm bound to human zona pellucida. *Fertility and Sterility*, 99(3), 684–689.
- Liu, Y., Zhu, Y., Di, L., Osterberg, E. C., Liu, F., He, L., Hu, H., Huang, Y., Li, P. S., & Li, Z. (2014). Raman spectroscopy as an ex vivo noninvasive approach to distinguish complete and incomplete spermatogenesis within human seminiferous tubules. *Fertility and Sterility*, 102(1), 54–60.
- Ma, M., Yang, S., Zhang, Z., Li, P., Gong, Y., Liu, L., Zhu, Y., Tian, R., Liu, Y., Wang, X., Liu, F., He, L., Liu, Y., Yang, H., Li, Z., & He, Z. (2013). Sertoli cells from non-obstructive azoospermia patients show distinct morphology, Raman spectrum and biochemical phenotype. *Human Reproduction*, 28(7), 1863–1873.
- Mallidis, C., Wistuba, J., Bleisteiner, B., Damm, O. S., Grob, P., Wubbeeling, F., Fallnich, C., Burger, M., & Schlatt, S. (2011). In situ visualization of damaged DNA in human sperm by Raman microspectroscopy. *Human Reproduction*, 26(7), 1641–1649.
- Mallidis, C., Sanchez, V., & Wistuba, J. (2013). Raman microspectroscopy: A method with great promise but which also needs circumspection. *Human Reproduction*, 28(9), 2595–2596.
- Mallidis, C., Sanchez, V., Wistuba, J., Wubbeeling, F., Burger, M., Fallnich, C., & Schlatt, S. (2014). Raman microspectroscopy: Shining a new light on reproductive medicine. *Human Reproduction Update*, 20(3), 403–414.
- Sanchez, V., Redmann, K., Wistuba, J., Wubbeeling, F., Burger, M., Oldenhof, H., Wolters, W. F., Kilesch, S., Schlatt, S., & Mallidis, C. (2012). Oxidative DNA damage in human sperm can be detected by Raman microspectroscopy. *Fertility and Sterility*, 98(5), 1124–1129.
- Sigman, M. (2012). Refining the measurement of sperm DNA fragmentation. *Fertility and Sterility*, 98(5), 1123.
- Speyer, B. E., Pizzey, A. R., Ranieri, M., Joshi, R., Delhanty, J. D., & Serhal, P. (2010). Fall in implantation rates following ICSI with sperm with high DNA fragmentation. *Human Reproduction*, 25, 1609–1618.

IVF LABORATORY

Setting Up the IVF Laboratory

Jason E Swain, CCRM IVF Network, Lone Tree, CO, United States

Charles L Bormann, Massachusetts General Hospital, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Background

It is a daunting endeavor to design and equip a new IVF laboratory. Numerous factors must be considered, including space requirements and layout to accommodate laboratory staff, equipment and workflow. Proximity to other associated rooms, like collection rooms, laboratory supply storage, medical gas storage, operating/procedure rooms and transfer rooms, as well as gamete and cryostorage areas are also relevant factors.

Initial planning for estimated volumes, as well as the ability to expand in the future is desirable. Each facility will also have to consider unique requirements as space planning takes place, factoring for areas like hormone analysis, andrology, fertility preservation, third party reproduction, genetic counseling, etc. The range of patient services offered at the clinic impacts the ultimate size and layout of the space.

Although daunting, commonalities do exist between highly successful programs in terms of how the IVF laboratory is constructed and equipped. During construction, extra care should be taken to provide an environment with proper air quality, low in volatile organic compounds (VOCs). This is accomplished via selection of appropriate construction materials as well as a proper HVAC system to provide desired air quality. In addition, key “burn-in” steps are often taken prior to clinical use to ensure proper functioning of the space.

In this article we will discuss the common elements to establish and equip a modern IVF laboratory, including: workflow and space planning, HVAC and medical gases, construction management, construction materials and equipment, safety, security and risk management, and lastly, burn-in. Of paramount importance is constant oversight during the construction process to ensure key details are met. Once these requirements have been met, diligence and a thorough quality management program are required to ensure that optimal laboratory conditions remain intact.

Space and Workflow Planning

One of the first steps in undertaking the building of a new IVF laboratory includes compiling a list of core functions necessary for the space and then subsequently mapping the layout of the required areas, taking care to note requirement for proximity relative to each other and the ancillary functions of the clinic. The core functions within the IVF laboratory (media preparation, sperm processing, egg retrieval, cumulus cell removal, micromanipulation, embryo culture, and cryopreservation), should be mapped relative to each other to layout the space within the laboratory. Care should be taken to consider distance between key procedural areas and traffic flow to avoid areas of congestion and also improve efficiency. Within the laboratory, this includes avoiding areas where workflow/employee movement may cross and create a potentially hazardous encounter.

For ancillary functions related to the lab, such as patient reception/waiting, specimen collection rooms, operating room/retrieval rooms, embryo transfer rooms, lab storage, and medical gas storage; consideration should be given to proximity to the laboratory to improve the patient experience and consider efficiency of laboratory workflow.

Each functional area should be adequately supplied with essential services, such as plumbing, electrical/emergency power, appropriate gas lines, HVAC, lighting, emergency alarm system, and IT network architecture as needed.

Once key areas of need are identified and workflows mapped, consideration of space requirements can be adjusted based on patient volumes and number of staff. Within the laboratory, this includes number of hoods, incubators, microscopes, micromanipulators, lab supply storage, and computers; as well as sufficient office/desk space.

Additional considerations for space planning may include designating a space for training new laboratory staff or for research. This space may include a fully functional workstation, incubator, and micromanipulation setup which would allow staff to practice new skills without disrupting clinical workflow or inadvertently changing the settings of clinical lab equipment.

Construction

As part of the design and construction process, specific requirements should be provided to the architect and construction management teams.

Construction Materials

Low or no VOC materials must be used in IVF laboratory construction. This includes all flooring, paint, adhesives, insulation, counter tops, and furniture.

Insulation: Care to use proper insulation is needed, as chemicals like formaldehyde are often present. Presence of insulation on the direct periphery of the IVF lab should be avoided if possible. If unable due to presence of an outside wall, a formaldehyde-free product should be used. Another option includes use of mineral wool. Of note, insulation may be useful for use around rooms that may require sound dampening, such as collection rooms or offices near key patient areas.

Flooring: Welded instead of glued seams of sheet vinyl flooring are recommended for sanitary reasons as well as minimal VOC release. A low VOC adhesive is required when laying lab flooring, as well as flooring in surrounding areas.

Paints: Latex based non-VOC paints should be used on all painted surfaces in and around the IVF laboratory.

Cabinetry: Powder coated metal cabinetry or stainless steel is recommended to avoid concerns with laminate and particle board, which would likely contain adhesives with high levels of VOCs.

Doors: Solid metal doors are often recommended. Door closers are useful on certain doors, as well as door stops. Consideration should be given to function to determine which hardware is required. Some doors may require security (keyed locks, coded locks, or electronic locks) or automatic door openers. Some doors may benefit from windows to avoid collisions as staff enter and leave the lab.

Furniture: purchase all medical or lab grade chairs with vinyl coated seating surfaces easily cleaned from any contamination. Tables and countertop should be made of either Epoxy or Corian or some other hard material devoid of adhesives (avoid laminate).

HVAC

The heating and air conditioning system must be considered when designing a modern IVF laboratory. This critical aspect will control temperature, humidity, and air quality. It is helpful to decide early on whether a commercially available “plug-and-play” filtration system will be used or if the contractors will build a custom unit. Regardless of the choice, the system should be dedicated to the IVF laboratory, and achieve positive pressure in the lab. It is often recommended that Tyvek wrapping, a hard deck ceiling, gasketed lights and doors, and door sweeps are included to achieve full insulation of potential air seepage to obtain positive pressure. Air exchanges should be considered to maintain positive pressure, but also filtration to achieve the desired air quality. Filtration should include prefilters (MERV) and HEPA filtration to remove particulates. Importantly, filtration should also reduce VOC content and may include the use of UV light for photocatalytic oxidation or use of chemical filter beds with carbon and potassium permanganate mixtures. The placement of the filtration system is important and should be as close to the outlet air ducts in the lab as possible to avoid the risk of introducing contaminants in-line after the filters. Only metal ductwork that has been precleaned should be used. Ductwork should remain sealed during the construction process to avoid contamination. Ensure that a proper water supply is drawn to the HVAC unit to allow for humidity control. A special consideration for the IVF lab, which may not be present in other types of medical laboratories, is proper placement of air supply ducts that ensures they are not directly above/near ICSI stations or incubators, as this will impact temperature regulation of the stage warming units. Assessment of air quality after install and before clinical cases commence is recommended to ensure expected standards are met. This can be achieved through contracting with an air quality expert or via commercially available air quality measurement devices. Importantly, assessment of lab VOC levels should use ppb to detect relevant levels.

Medical Gases

Sufficient supplies of medical gases like N₂ and CO₂ must be provided for all planned equipment inside the IVF lab. Nitrogen generators or liquid supply tanks for nitrogen or CO₂ may also be utilized in lieu of smaller gas cylinders. During the design phase, verify that a medical gas room of sufficient size to hold the required gas supply is located outside, but near the IVF laboratory. Proximity near a freight elevator and away from patient care areas is recommended for ease of delivery and avoidance of noise. The medical gas room should contain safety features like gas cylinder storage racks or cages, as well as an active atmosphere venting system. An oxygen alarm is also required.

Gas manifolds will be required inside the medical gas room; either manual systems or automatic systems. If automatic tank switchers are utilized, it is recommended these are connected to emergency backup power. Ensure master shutoff valves and pressure alarms are included and placed in key locations.

Braized copper piping is often utilized to supply laboratory gases, though welded stainless steel may also be used. Plastic tubing can suffice if supply pressure is not too high.

Low-pressure regulators and barbed fittings at the gas outlets inside the IVF laboratory are often recommended, as this permits supply of varying pressure requirements to different incubator models. Ensure gas outlets are placed near the location of incubators or other equipment requiring gas supply and at a sufficient height to avoid issues with installation of the equipment (low enough to fit beneath air tables or high enough to clear incubators, etc.).

Design Control

Functional laboratory design plans (a drawing or specification) describing all the components, equipment, and space requirement and should be reviewed and approved by the lab director or other person in charge of laboratory facilities. While a professional team of architects, along with a general contracting firm experienced in medical facility build out will have previous experience navigating complex local, federal, and international regulatory standards; they may not have experience with requirements or preferences for IVF laboratories. Regular meetings should be held with the construction company and contractors, and a detailed record of meeting minutes and plan revisions should be kept.

An integral part of design control is ensuring that the final product conforms to requested specifications. Reviewing the plans with the technologists, medical doctors, and staff who will be working in the space can help ensure the new design is fit for the intended use. Small but important details, like the placement of light switches and outlets for all necessary equipment for performing daily operations, can be easily assessed by the staff that use these items every day, and can be expensive to move or change after construction is complete. A person with lab expertise should review each set of designs/drawings and then monitor progress onsite via regular visits throughout the duration of the build to ensure that requirements are met. It is not uncommon for changes to be made during construction or for mistakes to arise and only through vigilant oversight will these be identified and corrected.

The design plans should include a lab floor plan that maps workflows and includes all lab equipment; maintaining close proximity between micromanipulation workstations and other IVF work stations to all incubators. Equipment lists and planned locations of instruments (with dimensions), placement of lights, air vents, electrical and IT outlets should be considered for each room.

If windows are present, ensure black out blinds are available to help regulate light/heat within the laboratory. Incubators and ICSI workstations should be protected from direct sunlight to avoid potential issues with light and/or temperature regulation.

Lab office space is often recommended to be adjacent to or connected to the laboratory with a doorway to separate to allow easy access, but avoid bringing unnecessary items into the main laboratory that could impact growth conditions.

Design should also consider confirm size of doors if large equipment will need to be installed, pathways of delivery to the lab, proper swing of doors to avoid collisions and optimize space utilization.

Equipment

The available number and quality of critical equipment and instrumentation, such as incubators and micromanipulators, can play a role in the success of an IVF program. For incubators, accurate case-volume estimates for the laboratory are critical, and should include low oxygen incubators that are utilized appropriately to avoid overcrowding. This may include separate units that are dedicated to storing culture media, oil and equilibrating dishes and units dedicated for embryo culture. Backup units or redundancy of critical equipment should be also considered (ICSI microscopes, lasers, incubators, fridges, freezers, etc.) to avoid disruption to the lab in the event of equipment failure.

Regardless of equipment choices, a regular laboratory equipment maintenance quality system schedule and documentation is required. This includes hoods workstations, incubators, centrifuges, pipette calibration/validation, and other small instruments.

Technical staff will be performing the same motions hundreds of thousands of times, day in and day out. Other equipment considerations should be ergonomics, providing technical staff with adjustable control of equipment, with tilting eye pieces, equipment with arm support, and adjustable seating as appropriate.

Quality assurance programs are predicated on the ability to rapidly identify and correct any nonconformance to set tolerance ranges. All incubators (CO₂ and temperature) and cryostorage vessels (liquid nitrogen levels) should be monitored by an alarm system with ability to email and/or call staff in case of an emergency. Additionally, natural gas or diesel backup power generators should be included in the laboratory design and sufficient for more than 8 h of backup power supply of all critical lab equipment, as well as other patient care areas. Generators should be certified initially with a 2 h load bank test, and serviced semiannually maintenance/inspections and yearly load tests. Occasionally, if a generator is not feasible, UPS units are required on all crucial equipment, with supply long enough to permit corrective action in case of a power failure.

Burn-In and Space Preparation

After construction is complete, the laboratory should be wiped down with an approved disinfectant/cleaner, such as 3% hydrogen peroxide, and the walls, floors, cabinets, and other surfaces should be wiped several times over the course of the entire burn-in period. It is necessary to move equipment into the lab and clean and test it with appropriate bioassays prior to clinical use. Of critical importance is the burn-in period for the lab incubators. Incubators should be cleaned according to manufactures instruction and temperature raised to greater than 45°C for several days or up to 2 weeks to promote off-gassing. The lab temperature can be raised to help with off gassing of other laboratory items.

The burn-in period should document on the lab's QC sheets that proper environmental conditions, that is, correct temperature, gas levels, and functioning of all equipment has been met for several days. Additionally, air quality testing should

measure VOC and particle counts pre and post burn in and the differences documented in the QC records. The air quality testing should be performed by a professional company or by lab personnel if hand-held particle counter and VOC meters have been purchased.

Ultimately, the burn-in period will conclude by checking pH and other growth condition parameters and pretesting all culture supplies with appropriate bioassays to ensure proper incubator conditions are met prior to clinical use.

Conclusion

Design and equipping of the laboratory is of paramount importance in help establish a successful IVF program. This begins with careful planning of workflow and design considerations. Constant oversight is then required to ensure proper materials are used and design specifications adhered to throughout the construction process. Careful selection of equipment is required, with proper use and maintenance. Validation of operation via a burn-in period is required with appropriate testing prior to testing patient samples.

Further Reading

- Boone, W. R., Johnson, J. E., Locke, A. J., et al. (1999). Control of air quality in an assisted reproductive technology laboratory. *Fertility and Sterility*, 71(1), 150–154.
- Cohen, J., Gilligan, A., Esposito, W., et al. (1997). Ambient air and its potential effects on conception in vitro. *Human Reproduction*, 12(8), 1742–1749.
- Cohen, J., Gilligan, A., & Willadsen, S. (1998). Culture and quality control of embryos. *Hum Reprod*, 13(Suppl 3), 137–144. discussion 145–7.
- Hall, J., Gilligan, A., Schimmel, T., et al. (1998). The origin, effects and control of air pollution in laboratories used for human embryo culture. *Human Reproduction*, 13(Suppl 4), 146–155.
- Lagunov, A., Crowe, M., & Swain, J. E. (2017). Establishing and equipping a new IVF laboratory. In *Principles of IVF Laboratory Practice Optimizing Performance and Outcomes*. Cambridge: Cambridge University Press.
- Swain, J. E. (2014). Decisions for the IVF laboratory: comparative analysis of embryo culture incubators. *Reproductive Biomedicine Online*, 28(5), 535–547.
- Swain, J. E., & Lagunov, A. I. V. F. (2017). Incubator Handling. In *Standard Operational Procedures in Reproductive Medicine: Laboratory and Clinical Practice* (pp. 8–11). Boca Raton, FL: CRC Press.

Quality Management: Maintaining a Stable IVF Laboratory

Jay Patel and Catherine Racowsky, Brigham and Women's Hospital, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The ultimate goal of an IVF program is to help every infertility patient/couple achieve their dream of having a healthy child. While this might seem easy to accomplish, there are a considerable number of variables that play into the success of each IVF cycle (Fig. 1). On the one hand, there are the clinical variables including the genetic background of the man and woman, the environments to which they have been exposed which might affect their reproductive potential, their age, the manner in which the woman's ovaries are stimulated for oocyte (egg) collection, the efficiency of the oocyte retrieval, the expertise with which the embryo transfer is performed (Sigalos et al., 2017a, b) and uterine issues concerning establishment of pregnancy (Luke, 2017). On the other hand, there are numerous variables relating to the IVF laboratory, including the culture system used to maintain the cells in vitro, and the scope of technologies offered and the manner and standards to which they are performed (Gardner and Kelley, 2017).

The IVF laboratory should be considered as a highly complicated manufacturing plant in which the starting off materials, the oocytes (eggs) and sperm (i.e., the gametes), are brought together to result in the final product, the embryo. If preimplantation development proceeds normally, the embryo traverses developmental milestones from day 1 to day 6, from the 1-cell stage through to the hatching blastocyst stage, ultimately to implant around day 6.5 (Fig. 2).

As with any complex manufacturing plant, consistent assurance of product quality in the IVF lab is of paramount importance. Overlain on this principle of final product quality are three further challenges faced by an IVF laboratory. Firstly, most often gametes and embryos from several patients are under the care of an IVF laboratory simultaneously, therefore requiring a stringent policy for maintenance of identity of all samples from all patients at all times. There is absolutely no room for sample mix-up in an IVF laboratory. Secondly, in addition to this over-arching concern about patient safety, the IVF laboratory has the responsibility to maximize the inherent ability of all gametes and embryos at all times. The IVF laboratory is particularly challenged in this regard, given the

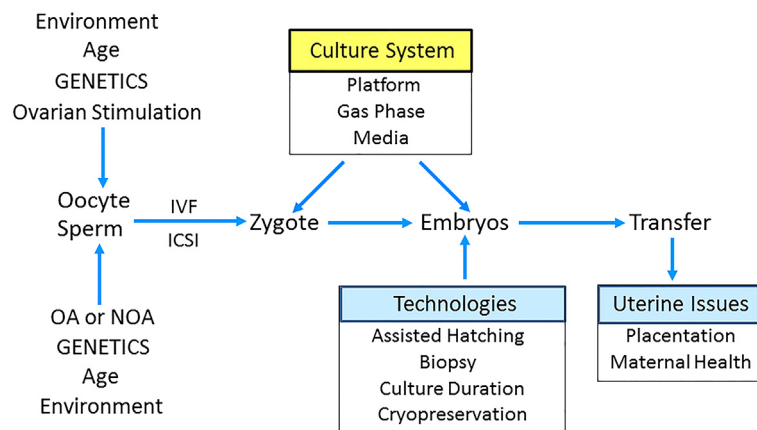


Fig. 1 Variables involved in the success of an IVF cycle. *IVF* = In vitro fertilization in which conventional insemination of oocytes is performed by adding sperm around the oocyte in the medium; *ICSI* = Intracytoplasmic sperm injection, in which a sperm is injected into an oocyte; *OA* = Obstructive azoospermia, a condition in which some portion of the male reproductive system is blocked, leading to absence of sperm in the ejaculate; *NOA* = nonobstructive azoospermia, in which there is no blockage, yet no sperm in the ejaculate.

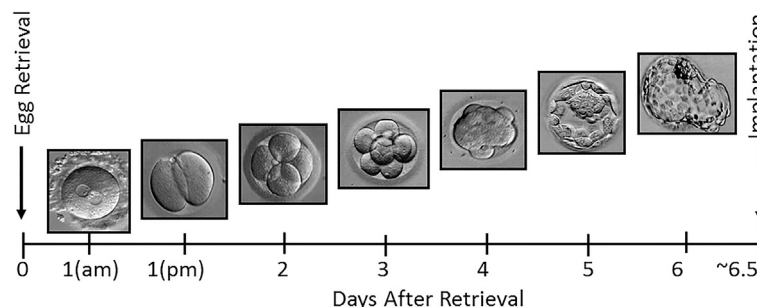


Fig. 2 Flow diagram showing the time-line of preimplantation development of the human embryo.

sensitivity of these cells to their environment (Cohen et al., 1997). Thirdly, the IVF laboratory must ensure that all procedures are performed exactly to the orders of the physician. As IVF laboratory technologies continue to evolve, more and more combinations and permutations of technologies are available, which inevitably lead to greater complexity in patient management and greater possibility of error.

With the above issues in mind, an IVF laboratory must have a comprehensive quality management program (QMP) in place. This program, a total quality management plan (TQMP), must ensure consistency in performance and allow for continuous improvement within a systematic and safe paradigm. In the United States, the Clinical Laboratory Improvement Amendments of 1988 (CLIA 88) mandates that all laboratories implement a QM program that monitors and evaluates overall quality of laboratory services at all times (Boone, 1992). In addition, our professional societies, the Society for Assisted Reproductive Technology/the American Society for Reproductive Medicine, and the European Society for Human Reproduction each has guidelines for the operation and management of an IVF laboratory (Practice Committee of American Society for Reproductive Medicine, Practice Committee of Society for Assisted Reproductive Technology, 2008; Magli et al., 2008).

In this article, we first consider the challenges to maintaining a stable IVF laboratory; we then review how to maintain a stable IVF laboratory; and finally we discuss paradigms for performing trials focused on quality improvement.

Challenges to Maintaining a Stable IVF Laboratory

Differences Between In Vivo and In Vitro Environments

Although we often refer to the environments in the lumen of the reproductive tract as being “fluid” (i.e., Fallopian tube “fluid” and uterine “fluid”), in fact they are anything but, consisting of a complexity of macromolecules including proteins and carbohydrates, as well as extracellular vesicles, ions and RNA molecules (Zhang et al., 2017). Indeed, the in vivo environment and the in vitro culture systems differ strikingly in fundamental ways (Fig. 3) and so it is probable that all currently used culture systems are nonphysiological, and that even the best is likely suboptimal for embryo development (Sunde et al., 2016). Therefore, despite continuing efforts to improve our culture technologies, and the plasticity of the human embryo to adapt to its environment, everything must be done to maintain a stable and reliable environment in the clinical IVF laboratory.

The Complexity of Procedures Performed in a Contemporary Clinical IVF Laboratory

The scope of lab technologies available today contrasts strikingly with that used for the first successful IVF birth of Louise Brown in 1978. For that success, only one oocyte in a natural cycle was retrieved without hormone priming, and fertilization was achieved by adding sperm to the culture medium surrounding the oocyte; transfer of the resultant embryo was done about 48 h later, late on day 2 (Steptoe and Edwards, 1978). For in those days, concerns about the probable sub-optimal culture environment for human embryo development led to the dogma that the embryo(s) should be taken out of the incubator and put into the uterus as quickly as possible. In contrast, clinical IVF nowadays typically involves retrieval of several oocytes, the formation of a number of embryos from either conventional insemination or ICSI, and use of one or more of a variety of other adjunct procedures, including extended culture of the embryos to day 5 or beyond. The complexity of procedures performed in the contemporary IVF laboratory is therefore considerable (Fig. 4), which makes it all the more necessary to have an all-encompassing and effective TQMP.

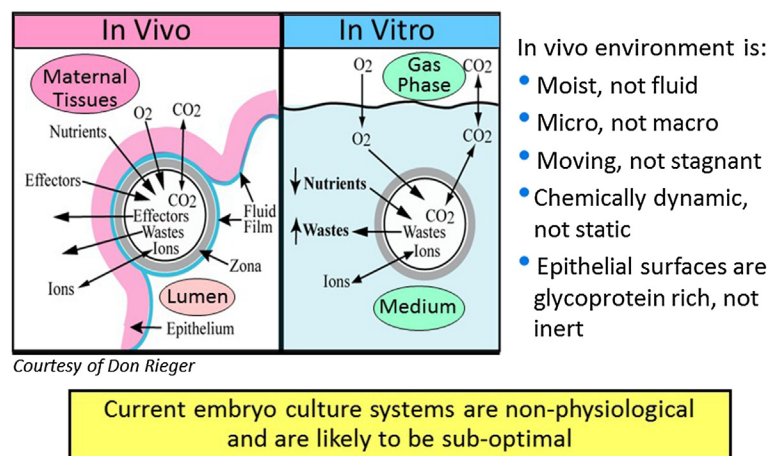


Fig. 3 Comparison of some of the major differences between the in vivo and in vitro environments. Reproduced with permission of LifeGlobal LLC.

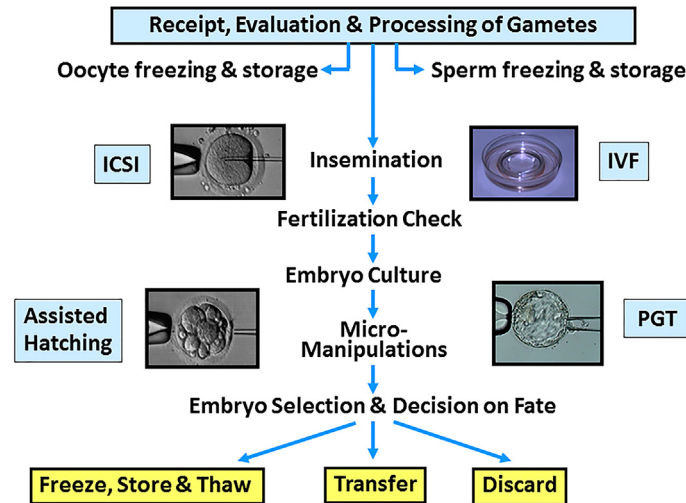


Fig. 4 The major procedures performed in the IVF laboratory. ICSI = Intracytoplasmic sperm injection, in which a sperm is injected into an oocyte; IVF = In vitro fertilization in which conventional insemination of oocytes is performed by adding sperm around the oocyte in the medium; PGT = preimplantation genetic testing, in which a few cells are removed (i.e., biopsied) from the embryo and are tested either for a known genetic mutation, or for the number of chromosomes present.

Sensitivity of the Cells Handled in the IVF Laboratory

Human gametes and embryos are exquisitely sensitive to any environmental perturbation including temperature fluctuations, light exposure, changes in the hydrogen ion concentration in the medium (measured by the acidity of the medium, known as the pH) and overall air quality (Boone et al., 1999; Hall et al., 1998). This is particularly the case for oocytes. For example, within minutes of only a 4°C drop in temperature, disassembly occurs of the meiotic spindle, the minute structure on which the chromosomes are held and aligned (Wang et al., 2001). Although the spindle re-polymerizes after recovery of the temperature, risks of error in chromosome realignment occur. Therefore, the importance of ensuring stable temperature control as a key component of maintaining a stable laboratory environment cannot be over-emphasized.

How to Maintain a Stable IVF Laboratory

As indicated in Fig. 5, there are numerous aspects to maintaining a stable IVF laboratory, which together fall into the three general principles and activities of quality control (QC), quality assurance (QA) and quality improvement (QI). All three principles are inter-related such that one independently is of no value without the remaining two. Each of these principles must be applied for protocol development and then for constant implementation and assessment of performance to ensure continuous stability of the laboratory.

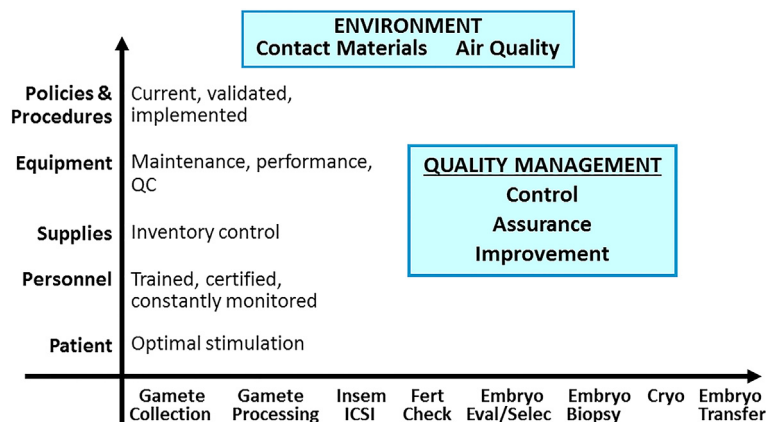


Fig. 5 The aspects and activities involved in running a stable IVF laboratory. QC = Quality Control; Insem = conventional insemination of oocytes, in which sperm are added to the medium surrounding the oocyte; Eval/Selec = Evaluation and Selection of embryos for transfer; Cryo = Cryopreservation, in which the embryos are frozen.

Quality Control (QC)

Quality control involves the collection of data that reflect the performance of the laboratory. To this end, data must be recorded that enable control for the effectiveness of methods, equipment, supplies, testing personnel and the environment to ensure the safe and efficacious function of the laboratory to the highest possible standards. Examples of key metrics for quality control in the IVF laboratory are shown in [Table 1](#). The data collections are undertaken at regular and defined intervals to prevent any ongoing problems that may lead to compromise in patient care ([Keck et al., 2009](#)). Of particular importance in this regard is the monitoring of the culture incubators, as any fluctuations in gas composition of the incubator atmosphere or the temperature of the machines can have disastrous results. Indeed, each incubator must be monitored daily, with successful operation being dependent on additional instrumentation including gas tanks, alarm systems, back-up power generators and the devices used to calibrate and measure the incubator settings. Activity logs are typically used in which acceptable ranges of performance are stipulated for each measured parameter ([Table 2](#)). All QC activities are run simultaneously with procedures to ensure that lab performance can be linked to clinical outcomes ([Boone et al., 2010](#)).

Quality Assurance (QA)

Quality assurance (QA) is the over-arching activity involving analysis of the data collected through QC and assessment of all other measures critical to appropriate running of a stable IVF laboratory. Through these comprehensive analyses, assurance is obtained that the protocol, equipment, supply/product, testing personnel and the environment are all performing within established targets and therefore performing to spec. The data analyses should be regular so as to catch any issue that may impact patient care in a timely fashion, whether this involves an equipment malfunction or subpar performance of an embryologist performing a particular procedure. In our laboratory, our standard key indicators of performance (KIPs) are the fertilization rate and the percentage of high quality of embryos on day 3 and day 5. Monitoring fertilization rate is particularly useful as it provides real-time insight into any variance in laboratory performance and one does not have to wait for weeks to see

Table 1 Key metrics for quality control in an IVF lab

<i>What</i>	<i>Action</i>
Protocols	Protocols written with regularly updates, and verification that personnel have read, understand and know them
Instrumentation performance	Recording performance of specific parameters (e.g., temperature at defined intervals)
Personnel performance	Adequate training and certification sign-off. Competency evaluations undertaken through external and internal proficiency testing
Patient data	Recording of key performance indicators (KIPs) with preidentified targets of performance. Standard KIPs in clinical IVF are: fertilization rates, embryo quality, and implantation rates
Environmental control	Recording of daily measures of air quality contaminants (e.g., particulates, CO ₂ , VOCs)
Patient identity (safety)	Documentation that two patient identifiers are on all samples. Documentation of use of checklists at key check points (e.g., oocyte retrieval, insemination, and transfer) to ensure patient safety
Supply inventory control	Documentation of dates that supplies are received, opened and expired, and documentation of suitability for use in clinical IVF with bioassay testing

Table 2 Incubator QC activity log

<i>Parameter</i>	<i>Frequency</i>	<i>Target</i>	<i>Acceptable Range/Supply</i>	<i>Corrective action</i>
CO ₂	Daily while in use	Based on recommended pH of Culture Media	±1% to achieve target pH	Adjust when out of range for two consecutive days
O ₂	Weekly	5%	±1%	Adjust when out of range for 2 consecutive days
Humidity	Daily while in use	95%	≥ 80%	Add sterile water
Temperature	Daily while in use	37°C	36.7°C–37.0°C	Adjust when out of range for two consecutive days
pH	Weekly	Based on media specifications	Based on media specifications	Adjust CO ₂ when out of range for two consecutive days
Alarm check	Monthly	Email and phone notification	100% coverage of alarmed instrumentation	Contact technical support
CO ₂ Tanks	Daily	Full back-up supply	> 1500 psi in primary tank	Order new tanks
Liquid N ₂	Daily	Full back-up supply	≥ 1 back-up tank available	Order new tanks
N ₂ tanks	Daily	Full back-up supply	≥ 1500 psi	Order new tanks
1-Cell mouse assay	Semi-annual	≥ 70% Blastocyst development	≥ 70% blastocyst development	Clean and sterilize incubator, repeat mouse assay

if pregnancy rates have been compromised. As with any KIP, acceptable targets should be set and a performance chart (p-chart) used to monitor performance. Fig. 6 shows the p-chart for weekly embryo quality rates (as assessed by the % of embryos having at least 7-cells on day 3) in our laboratory for 2106.

In addition to data analyses, other measures that are considered under the rubric of QA include maintenance of comprehensive and up-to-date laboratory protocols (with sign-off by testing personnel at least annually), evaluation of continuing medical education of testing personnel, integrity of result reporting, and auditing of treatments to ensure that the treatments performed reflect the orders made. Examples of key QA activities performed in an IVF laboratory are shown in Table 3. The overall purpose of QA is to ensure stable laboratory performance through constant surveillance that allows either confirmation of acceptable performance (obviously the desired result) or identification of any problems or errors that can then be dealt with through corrective action.

Quality Improvement

Quality improvement (QI) is the process undertaken to improve some specified aspect of laboratory performance. This may relate to the introduction of a new protocol, a new procedure to perform an established protocol or a new supply (Boone et al., 2010). Regardless, the IVF laboratory handles material that is irreplaceable, so great care must be taken to ensure that any such introduction will not compromise patient safety.

Establishment of written protocols for QC, QA, and QI are just the beginning of the process to ensure adequate quality management and maintenance of a stable IVF laboratory. Continued technical monitoring and improvement needs to be in place at all times. One way to do this is to perform mock cases or peer observations (Bormann and Racowsky, 2012). In mock cases, all aspects of the laboratory processes are performed by each staff member who is certified to perform those tasks. Protocols should be followed as if these were real cases. These mock cases will help identify problems that can be corrected before errors with clinical specimens occur. Peer observations play a similar role in which one embryologist observes another embryologist performing a task on a live case. Both members know the protocols and the task should be completed exactly as per written protocols. If there is a drift from the protocol, action can be taken to identify the drift as being detrimental to the process or not. If the drift is considered detrimental, steps need to be taken to ensure the written protocol is followed. If the deviation has a positive impact, steps can be taken to implement the new process among all team members. The more frequent the monitoring, the quicker the negative deviations are identified and eliminated and the more rapidly the positive ones are implemented. Due to the critical nature of all work in the IVF laboratory, failure at any juncture of the process has dire consequences. Therefore, the processes have to be airtight and unwavering at all times.

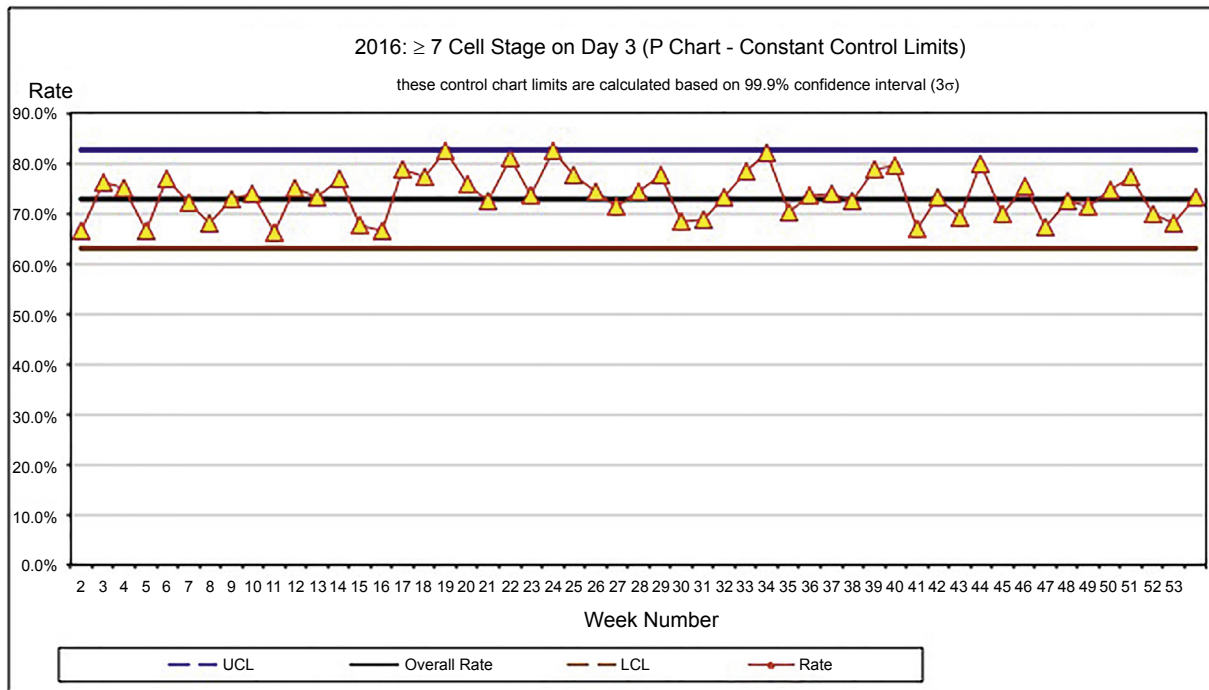


Fig. 6 An example of a performance chart for weekly embryo quality rates. This chart shows the percentage of embryos that reach at least to the 7-cell stage on day 3, which we have found gives a reliable guide of overall laboratory performance.

Table 3 Key quality assurance activities in the IVF lab

<i>What</i>	<i>Action</i>	<i>When</i>
Data review of instrumentation performance	Perform daily cross-checks and verifications that instruments are performing within acceptable targets for all measures	Daily
Data analyses	Analysis of collected data for Key Performance Indicators (KIPs) with preidentified targets of performance	Defined by the KIP: weekly for fertilization and embryo quality data; monthly for implantation rates
Personnel performance	Assessment of performance against identified metrics and against targets set in external and internal professional education activities	A minimum of twice annually
Environmental control	Monitoring of air quality with identified maximum targets of acceptability for individual measures (e.g., particulates, CO ₂ , VOCs etc.)	Weekly
Alarm monitoring	Monitoring of all culture incubators and frozen storage vessels	Daily, 24/7 with a rapid-response system in place; e.g., using an alarm phone tree
Patient identity (safety)	Identify checkpoints for identification and implementation of check-lists and double sign-offs. Use of mock cases to ensure patient safety. Confirm two patient identifiers on all samples. Perform mock cases with laboratory team to ensure safety	Before each patient sample is handled
Supply inventory control	Establish a supply management protocol for appropriate storage and stock rotation, and a method for quality control of supplies (e.g., using a mouse embryo bioassay to assess embryo development, or a sperm motility assay to assess motility after exposure to the new supply)	Before implementation in the IVF lab
Frozen tissue inventory control	Assurance of accurate log-keeping of tissue identity and location of all frozen oocytes, sperm, embryos, and ovarian tissue	Ongoing inventory inspection and verification of content and location at least annually

The Plan-Do-Check-Act Cycle

The central dogma of a total quality management plan demands continuous improvement of the systems and processes that will result in improvements in the product and/or services provided. In the case of the IVF laboratory, the TQMP helps improve the success of couples taking home a healthy child. There are many models that can guide setting up a strong TQMP. One that is used widely in many fields is the “Plan-Do-Check-Act” or PDCA Cycle or Shewart or Deming Cycle (Mortimer and Mortimer, 2005) (Fig. 7). The PDCA Cycle is based on the scientific method, and is a well-established simple model that can be implemented to monitor the laboratory’s performance and make quality improvements where needed (Bormann and Racowsky, 2012).

- *Plan*: In this step one looks at the current state of the processes and looks for areas for improvement. If a problem is identified, as much information as possible is gathered and an educated hypothesis is formed regarding possible causes. In this step, one needs to set objectives and processes to reach targeted outcomes; therefore it is wise to set measurable and attainable goals and, if possible, test the hypothesis on a small scale.
- *Do*: In this step, the plan is executed. Criteria are set for the studies, which are implemented on a trial basis to test the hypothesis. Data are collected for analysis during which all team members understand the changes implemented and that are being assessed.
- *Check*: In this step, the data collected from the previous step are analyzed. The effectiveness of the changes implemented is checked by looking at key performance indicators established in the Plan. Also, verification is done that the Plan was completed

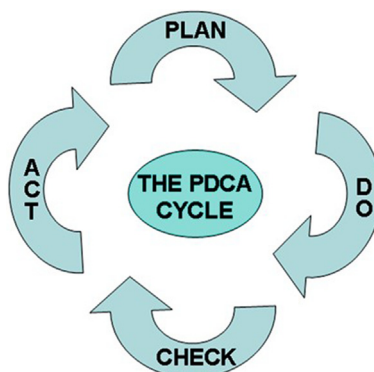


Fig. 7 The plan-do-check-act (PDCA) cycle. An approach used in a total quality management program to maintain and improve performance in a complex environment. Published with permission from Ginsburg, E.S. and Racowsky, C. (eds.) *In Vitro Fertilization: A Comprehensive Guide*. Springer: New York-Heidelberg-Dordrecht-London.

appropriately and there was no deviation from the Plan. Was the plan validated with the desired goals achieved? If so, the next step is undertaken. If not, the Plan and Do steps may need to be refined.

- *Act:* In this final step of the cycle, all of the differences from the actual and planned results are assessed. If the changes implemented showed an improvement, then these become the new standard and are implemented on a broader scale. If the results did not show an improvement, the current practice remains in place. These changes will be continuously monitored for possible improvements in a future PDCA cycle.

Patient and Tissue Identification

Patient safety in the IVF lab is of the upmost importance. This concept encompasses not only assurance that all patient samples at all times are maintained in appropriate conditions. Equally importantly, safety also incorporates the concept of patient and tissue identification. The IVF process is very stressful for the couple experiencing infertility. They are relinquishing their control in this intimate process to the highly trained staff of the IVF laboratory. They rely on the staff to make sure that the upmost care is taken to ensure that their gametes maintain their true identities. Unfortunately, albeit at exceedingly low frequencies, nightmarish scenarios have occurred such as transferring embryos to the wrong patient, insemination of oocytes with the wrong sperm (Pomeroy and Racowsky, 2012) and the mislabeling of embryos (Newman, 2000). In such circumstances, root cause analyses have indicated that there were major breakdowns in the sample identification process. The greatest fear in every IVF couple's mind is the possibility that their gametes or embryos are mixed up. This same fear is shared by every IVF laboratory and all checks and balances must be in place and implemented at all times to avoid such a disaster.

- *Rules and regulations governing patient and tissue identification:* Rules and regulations concerning the proper identification of patients and collection and handling of specimens should be part of any IVF manual. The College of American Pathologists (CAP) accredits IVF laboratories in the United States and this organization has a specific set of procedures in the General and Reproductive Laboratory Checklists that should be followed by all US IVF laboratories. One of the most important of these regulations is the requirement to have unique patient identification. This must be accomplished by having a minimum of two identifiers used to distinguish each patient and her specimens. This could be a combination of any number of the following: name, date of birth, medical record number, social security number, accession number, etc. In our lab, we use the patient name and a unique identifier comprised of the date of the oocyte retrieval (or oocyte or embryo thaw), followed by the order of the procedure that day. For example, if Jane Smith is the first patient to have her oocyte retrieval on August 22, 2017, her two identifiers would be [Smith, J and 082217-1]. In conjunction with patient identification, policies must be in place governing chain-of-custody of all specimens and safety pauses at critical steps such as oocyte retrieval, insemination, embryo biopsy, and embryo transfer. Two embryologists should sign off on all patient identifiers at these critical steps.
- *Protocols needed for airtight identification:* Airtight identification of patients and specimens is essential at all steps of the IVF process. Each lab needs to develop its own protocols to identify safety checkpoints based on the staffing and make-up of the lab that governs the day to day flow of laboratory operations. An excellent reference to identify the flow of the lab is the textbook by David Mortimer, "Quality and Risk Management in the IVF Laboratory" (Mortimer and Mortimer, 2005). This book contains flowcharts that can help identify points in the IVF process at which proper identification of patients and specimens is absolutely critical. Once the critical steps are identified (Table 4). The laboratory can write protocols and establish checklists to maintain the

Table 4 Steps in the IVF laboratory requiring patient and tissue identifications

Procedural step		Suggested identification steps			
Male ^a ID before sample collection	Patient ID with a government issued ID	Male signs verification of sample use	Male labels collection cup with ID		
Sperm sample hand-off to lab personnel	Patient ID at sample drop-off	Cup label verification	Chain of custody sign-off	Lab receiving	
Sperm preparation	Cup ID verification	Gradient tube ID	Wash tube ID	Final preparation ID	
Oocyte collection	Time out (i.e., surgical pause) for patient ID	Retrieval tubes ID	Retrieval dishes ID	Insemination dish ID	Incubator ID
IVF	Incubator ID	Sperm ID	Insemination dish/oocyte ID	Incubator ID	
ICSI	Incubator ID	Egg cleaning ID	ICSI dish ID	Sperm ID	Incubator ID
IVF Fertilization check	Incubator ID	Fertilization dish ID	Wash dish ID	Culture dish ID	Incubator ID
ICSI Fertilization check	Incubator ID	Culture dish ID	Incubator ID		
Embryo grading	Incubator ID	Culture dish ID	Incubator ID		
Assisted hatching/biopsy	Incubator ID	Culture dish ID	Incubator ID		
Embryo transfer	Patient ID	Culture dish/embryo ID	Transfer dish ID	Incubator ID	
Cryopreservation	Straw ID	Cane ID	Freeze dish ID	Incubator ID	Culture dish ID

^aID = Identification; two identifiers must be used at every identification step and two embryologists must sign off on both identifiers.

chain-of-custody and proper identification of each patient and sample. Checklists used at critical points of the IVF process are an extremely useful tool in reducing or eliminating procedural drift and the possibility of errors. (Gwande, 2009; Bormann, and Racowsky, 2012). Four critical steps in the general IVF process at which checklists are recommended are: sperm receiving/processing, egg retrieval, insemination and embryo transfer. An example of a template for the checklist for embryo transfer is shown in Fig. 8.

CHECKLIST FOR EMBRYO TRANSFER

(Embryologist participating in the transfer must check all boxes and initial below to confirm check completion)

Patient ID Sticker	Partner ID Sticker
--------------------	--------------------

HOOD SETUP

- ☐ Hood surface and microscope stage area clean
- ☐ Dissecting microscope turned on with oculars and focus adjusted ready for use
- ☐ HEPES pre-warmed in Inga placed in ER hood warming block
- ☐ Stripper pipette with new tips at hand
- ☐ Embryo transfer dish labeled with last name and Case ID

DATA RECORDING AND INCUBATOR CHECK

- ☐ Information relating to number of embryos to transfer recorded
- ☐ Patient name and Case ID on dishes confirmed

PREPARATION OF EMBRYOS FOR TRANSFER

- ☐ Cross-check link between label identifiers on home incubator and Name and Case ID on data record sheet
- ☐ Re-confirm Patient name and Case ID on transfer dish
- ☐ Confirmation of Embryo ID documented on data record sheet

PREPARATION OF EMBRYO PHOTOS

- ☐ Patient ID Sticker placed on each of the 2 photos
- ☐ Correct Patient ID Sticker name confirmed by 2nd embryologist

SURGICAL PAUSE

- ☐ Visual confirmation of patient ID from her hospital bracelet against the patient identifiers on the data record sheet
- ☐ Verbal confirmation with patient name and number of embryos to transfer as documented on data record sheet
- ☐ Documentation of presence of embryologist at surgical pause on data record sheet
- ☐ Documentation of confirmation of patient ID on data record sheet

PROCEDURE

- ☐ Visualization of patient name and Case ID by embryo transfer physician
- ☐ Hand-off or embryo transfer catheter loaded with embryo(s) to transfer physician
- ☐ Confirmation of absence of embryo in catheter immediately after transfer by rinsing the catheter
- ☐ Documentation that transfer completed on data record form

Initials: _____ // _____
 Transfer Embryologist 2nd Embryologist

Fig. 8 A template for a checklist used before and after an embryo is transferred into a patient. *ID* = Identification.

Trials Focused on Quality Improvement

The IVF laboratory is faced with two main challenges when testing a product or evaluating a protocol that may improve lab performance. First, human gametes and embryos are more sensitive than those of other species to environmental perturbations so use of animal tissue for product evaluation has limited utility. Second, accepting the premise that human gametes and embryos should be used for evaluation of any potential improvement, the evaluations have to be done with extreme care, recognizing that any compromise to patient care must be kept to an absolute minimum. In general, IVF laboratories will undertake a QI trial in two phases. In the first phase, samples are randomized within patients to the test condition or to the standard condition. If no adverse effects are observed in phase I and/or a benefit is suggested, the second phase is performed in which the patients themselves are then randomized.

Randomization of Samples Within Patients

In this design, the oocytes or sperm or embryos of each patient are randomized to one of two conditions and outcome variables are identified. For example, we might be interested in testing a new culture medium for growing embryos that has just come on the market. In this case, embryos on day 1 are typically randomized (by a coin toss or using a randomization numbers table) to the standard, currently used medium or the new medium and embryo development is assessed. The advantages of this design are (1) that any potential compromise to patient care is ameliorated because only a percentage of her cells are being exposed to the “test” medium; and (2) as the patient is serving as her own control, a matched paired analysis can be performed, thereby enabling a “cleaner” comparison between the two groups. The main disadvantage of this design is that only those embryos with a known outcome after transfer (i.e., either as implanted or not) can be included when analyzing developmental ability; in cases in which two embryos were transferred with one from the control and one from the test condition but only one implanted, it is unknown which medium can be attributed to the “implanter” so these cases have to be excluded.

Randomization of Patients

In this design, the patients themselves are randomized to the two conditions so that all the embryos from each patient are cultured either in our standard medium or in the new test medium. The advantage of this approach is that all transferred embryos are included in the analysis. However, disadvantages include (1) the time required to recruit a sufficient number of patients to demonstrate significance; and (2) possible harm to patients if any risks have not been identified in a phase I trial as described above.

Summary

Unlike most fields of medicine, the IVF laboratory plays a rather unique role in the management and treatment of patient care. For the physician team is dependent on a quality of IVF laboratory to provide the highest quality of care to their patients, while the laboratory staff are dependent on the physician team to bring in patients for their livelihood. It is therefore critical that the clinical and laboratory staff work in close collaboration with collegial and open communication to ensure that the ultimate goals of the IVF program are met at all times, for every single patient. To ensure that this synergistic clinical/laboratory rapport is successful, there must be trust on both sides, but particularly that the IVF laboratory is stable and providing the highest standards of patient care. To this end, the IVF laboratory must utilize a comprehensive and effective TQMP. All checks and balances must be in place to assure not only that effective QC and QA protocols are adhered to and updated, but also that a QI program is established that focuses on introducing improvements to patient care. All aspects of the QM program must be effectively managed through, for example, utilization of the PDCA cycle. With such systems in place, patient safety and identification will be assured at all times and the stability of the IVF laboratory will be maintained thereby ensuring that the developmental capabilities of all cells received are maximized.

References

- Boone, D. J. (1992). Literature review of research related to the Clinical Laboratory Improvement Amendments of 1988. *Archives of Pathology and Laboratory Medicine*, 116(7), 681–693.
- Boone, W. R., Johnson, J. E., Locke, A. J., Crane, M. M., & Price, T. M. (1999). Control of air quality in an assisted reproductive laboratory. *Fertility and Sterility*, 71(1), 150–154.
- Boone, W. R., Higdon, H. L., & Johnson, J. E. (2010). Quality management issues in the assisted reproduction laboratory. *Journal of Reproductive Stem Cell Biotechnology*, 1(1), 30–107.
- Bormann, C. L., & Racowsky, C. (2012). Ongoing quality assessment/improvement in clinical IVF. In E. S. Ginsburg, & C. Racowsky (Eds.), *In vitro fertilization: A comprehensive guide* (pp. 225–247). New York-Heidelberg-Dordrecht-London: Springer.
- Cohen, J., Gilligan, A., Esposito, W., Schimmel, T., & Dale, B. (1997). Ambient air and its potential effects on conception in vitro. *Human Reproduction*, 12(8), 1742–1749.

- Gardner, D., & Kelley, R. (2017). Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *Journal of Developmental Origins of Health and Disease*, 8(4), 418–435.
- Gwande, A. (2009). *The checklist manifesto: How to get things right*. New York: Metropolitan Books a division of Henry Holt and Company, LLC.
- Hall, J., Gilligan, A., Schimmel, T., Cecchi, M., & Cohen, J. (1998). The origin, effects and control of air pollution in laboratories used for human embryo culture. *Human Reproduction*, 13, 146–155.
- Keck, C. S., Sjoblom, C., Fischer, R., et al. (2009). Quality management in reproductive medicine. In D. K. Gardner, A. Weissman, C. M. Howles, & Z. Shohan (Eds.), *Textbook of assisted reproductive technologies* (3rd edn., pp. 435–445). United Kingdom: Informa Healthcare.
- Luke, B. (2017). Adverse effects of female obesity and interaction with race on reproductive potential. *Fertility and Sterility*, 107(4), 868–877.
- Magli, M. C., Van den Abbeel, E., Lundin, K., et al. (2008). Revised guidelines for good practice in IVF laboratories. *Human Reproduction*, 23(6), 1253–1262.
- Mortimer, D., & Mortimer, S. (2005). *Quality and risk management in the IVF Laboratory*. British Columbia: Cambridge University Press.
- Pomeroy, K. O., & Racowsky, C. (2012). Patient and tissue identification in the assisted reproductive technology laboratory. *Seminars in Reproductive Medicine*, 30(3), 173–181.
- Practice Committee of American Society for Reproductive Medicine, Practice Committee of Society for Assisted Reproductive Technology. (2008) Practice Committee of American Society for Reproductive Medicine. Revised guidelines for human embryology and andrology laboratories. *Fertility and Sterility*, 90, S45–59.
- Sigalos, G., Triantafyllidou, O. and Vlahos, N. (2017a). How do laboratory embryo transfer techniques affect IVF outcomes? A review of current literature. *Human Fertility* 20 (1), 3–13
- Sigalos, G., Triantafyllidou, O., & Vlahos, N. (2017b). How do laboratory embryo transfer techniques affect IVF outcomes? A review of current literature. *Human Fertility*, (1), 3–13.
- Steptoe, P. C., & Edwards, R. G. (1978). Birth after re-implantation of a human embryo. *Lancet*, 312(8085), 366.
- Sunde, A., Brison, D., Dumoulin, J., et al. (2016). Time to take human embryo culture seriously. *Human Reproduction*, 31(10), 2174–2182.
- Wang, W. N., Meng, L., Hackett, R. J., Odenbourg, R., & Keefe, D. L. (2001). Limited recovery of meiotic spindles in living human oocytes after cooling-rewarming observed using polarized light microscopy. *Human Reproduction*, 16(11), 2374–2378.
- Zhang, Y., Wang, Q., Wang, H., & Duan, E. (2017). Uterine fluid in pregnancy: A biological and clinical outlook. *Trends in Molecular Medicine*, 23(7), 604–614.

Further Reading

College of American Pathologists. (2017). *Reproductive laboratory checklist*. Northfield, IL: College of American Pathologists.

Relevant Websites

www.cap.org—College of American Pathologists.

Newman, A. (2000). Visiting rights denied in embryo mix-up case. New York Times <http://www.nytimes.com/2000/10/27/myregion/visiting-rights-denied-in-embryo-mix-up-case.html>.

The Preparation of Sperm

Denny Sakkas, Boston IVF, Waltham, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The preparation of sperm for infertility treatment was initially introduced for intrauterine insemination techniques. As far back as the 14th century there are accounts of Arab peoples using artificial insemination on horses. Even in the 3rd century AD, records show that Jewish thinkers were discussing the possibility of accidental or unintentional human insemination by artificial means. The first attempts at human artificial insemination by John Hunter are believed to have occurred in 1785, with a baby born the same year. Early reports of donor insemination were published in the *British Medical Journal* in 1945, and in 1955, four successful pregnancies using previously frozen sperm were reported. With the advent of in vitro fertilization (IVF) and the treatment of more complex male infertility cases the preparation of sperm has evolved.

Semen Analysis and Sperm Preparation

It is commonly accepted that the standard World Health Organization (WHO) criteria for sperm number, motility, and morphology are a good, although not always perfect, indication of a male's fertility status. The current criteria (WHO, 2010) have undergone various changes from the 1999 to 2010 WHO recommendations, whereby semen volume [2–1.5 mL], sperm concentration [20–15 million per mL], progressive motility [50%–32%], and normal forms [14%–4%] have all been decreased respectively. Although the current criteria suggest a certain volume, number, motility, and morphology it must be emphasized that this is an indication that the male may have difficulties to father a child rather than predicting fertility. For the preparation of sperm in cases of IVF, it is more a matter of maximizing the quality of sperm you prepare rather than the initial count in the semen. Furthermore, with the advent of intracytoplasmic sperm injection (ICSI) harvesting thousands or even millions of sperm is not always necessary.

This article will give a broad review of current laboratory technologies and procedures for the routine preparation of sperm from the semen.

Traditional Preparation of Semen Samples for IVF

Semen samples are routinely obtained from the male by masturbation into a sterile container. It is recommended for the male not to have ejaculated for 2–3 days prior to collection. This however is now coming under question. A recent study (Alipour et al., 2017) examined whether a short abstinence period of only 2 h yielded spermatozoa with better motility characteristics than samples collected after 4–7 days. They found that even though a significantly lower semen volume, sperm concentration, and total sperm counts in all motility subgroups is obtained, the significantly higher percentage of spermatozoa with better motility characteristics (velocity, progressiveness, and hyperactivation) in the second ejaculate, may provide and allow for a simpler and more effective selection of higher quality spermatozoa.

Preparation

For the purposes of an IVF cycle, a simple count and motility screen is normally sufficient, as the aim is to prepare the sperm for treatment rather than for diagnosis. Once collected, the semen is allowed to liquefy for up to 30–45 min. It can then be prepared using either a swim-up (SU) technique or by density gradient (DG) centrifugation. Sperm preparation for assisted conception by DG centrifugation routinely uses 0.5–1 mL volumes of a 40% suspension layered over 0.5–1 mL of a 90% suspension of a colloidal silica suspension in an isotonic salt solution mixed in human tubal fluid (HTF) media or a similar commercial media. Semen is overlaid on the 40% and 90% layers (Fig. 1) and then centrifuged for approximately 20 min (300g). Sperm in the 90% pellet are harvested, washed in HTF media and then stored in an incubator until use. Gradient systems use solutions with a higher density than semen to separate the debris, cells, microorganisms and nonmotile sperm from the motile ones. Cellular debris and nonmotile microorganisms will be trapped at the interphase between the two solutions. Among the dense solutions used are colloidal silica (Percoll, Puresperm), poly-sucrose (Ficoll, Ixaprep), and other dense solutions (Optiprep, Nycodenz). It is generally thought that the DG preparation will select more normal sperm than the swim-up method because it has a specific density of approximately 1.1 g mL^{-1} , however recovery may be poor in viscous semen and severe teratozoospermia, in particular when many small headed sperm are present as they will not be de-selected.

The swim-up method requires centrifugation of the ejaculate to form a pellet followed by layering of clean medium (0.5–1 mL) over the pellet (Fig. 1). The sperm are then allowed to swim freely up into the clean portion and are then harvested. This technique relies on the ability of the sperm to swim. This method is suitable for semen with high to moderate motility. Briefly, the semen is generally diluted with 1:2 ratio of culture medium and centrifuged at 250–300g for 5–10 min. The supernatant is removed leaving

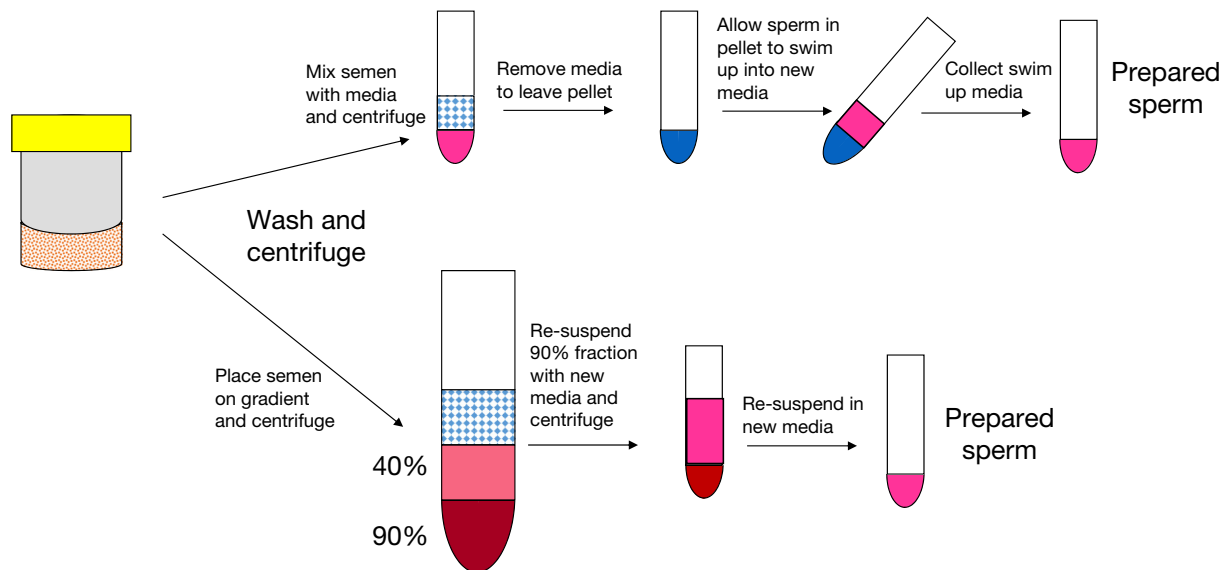


Fig. 1 Comparison of swim-up and density gradient for preparing sperm samples.

the pellet. The pellet can then either be layered under clean media or clean media can be carefully layered over the pellet. The tube is then left to stand for between 30 and 60 min at a 45 degree angle, which creates a larger surface area for sperm to swim-up. Once finished the clean media is taken up carefully without disturbing the lower layer and transferred into a new test tube. If a number of tubes are prepared per patient then to concentrate the sperm, the tubes can be pooled and centrifuged at 250–300g for 5–7 min. The supernatant is removed and the resultant pellet resuspended in 0.4 mL of media.

Finally, if the semen sample is very poor and no or only one to two sperm are seen during the analysis a simple wash can be prepared. This will mainly remove seminal plasma from the sample. One volume of semen is placed in a 15 mL test tube and diluted with twice the volume of culture medium. The tube is gently inverted to mix the components and then centrifuged at 250–300g for 5–10 min. The supernatant is removed and the pellet is resuspended in 2 mL of culture medium. Centrifugation can be repeated again if no sperm are seen. The pellet is then resuspended in about 0.4 mL of clean media. The same wash procedure is suitable for intracervical insemination.

The question of whether swim-up or density gradient provides a better sample has been scrutinized in many studies (Ng et al., 1992; Sakkas et al., 2000; Palini et al., 2017). In general, studies have shown that both are efficient in providing good sperm preparation. Recently Zhao et al. (2016) reported no significant difference between the DG and SU procedures, showing that both methods had lower DNA fragmentation, reactive oxygen species content, and sperm progressive motility. They also showed that sperm telomere length was improved with both techniques. In some other recent studies the argument has been made that swim-up is easier to perform and may improve pregnancy rates when used in comparison to DG.

Insemination and Intracytoplasmic Sperm Injection (ICSI)

Once the sample is prepared, the total motile sperm concentration is calculated using the volume and number of motile sperm. Insemination can then be performed by adding a predetermined amount to the droplet of culture medium containing the cumulus enclosed oocytes. Oocytes can be inseminated in groups or individually in droplets of embryo culture medium with between 50 and 500,000 motile sperm mL^{-1} overnight. Insemination is routinely performed between 1300 and 1500, allowing incubation overnight. There are some indications that embryo quality is improved when clinics have utilized a short insemination period (Kattera and Chen, 2003; Gianaroli et al., 1996) whereby the sperm are introduced for a period of 1–4 h only and then the oocytes are removed. With this approach, the oocytes are not exposed to radical oxygen species and other possible harmful factors generated by the large population of sperm overnight. If the sperm preparation is substandard in number and/or motility or if the patient has previously been identified to have fertilization issues then they will be a candidate for ICSI.

ICSI is now chosen as the standard method of insemination. Current rates of ICSI utilization in the USA are close to 70% of cycles. This has led to investigating whether it is over-used. A recent paper showed that routine insemination may actually yield more fertilized eggs on the whole when compared to ICSI (Boulet et al., 2015). Other concerns with ICSI still remain, in particular, with respect to its safety. Current data however indicates that children born after ICSI have not shown any differences when compared to children born from routine IVF or natural conception (Bonduelle et al., 1996; Leunens et al., 2008; Belva et al., 2011).

Relating Sperm Preparation With Sperm Analysis Methods

It is now becoming more apparent that sometimes a simple sperm preparation as a whole is not sufficient. The preparation method is therefore being tied in more and more with some type of sperm analysis which results in sperm selection. The most notable of these selection and preparation methods is the hyaluronan (HA) binding assay. The HA binding test ([Sbracia et al., 1997](#); [Huszar et al., 2006, 2007](#); [Jakab et al., 2005](#)) test provides information about the maturity of the sperm. Briefly, human sperm that bind to HA exhibit attributes similar to that of zona pellucida-bound sperm, including minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies ([Cayli et al., 2003](#); [Celik-Ozenci et al., 2003](#); [Jakab et al., 2005](#)). In preliminary results from a multicenter clinical trial, it was shown that when patients with less than 65% binding efficiency are screened and selected before ICSI, their success rates are improved by incorporating HA binding in their treatment ([Worriolow et al., 2012](#)).

Other sperm preparation technologies are also being developed. Magnetic activated cell sorting (MACS) of apoptotic sperm is based on the availability of colloidal superparamagnetic microbeads (50 nm diameter) conjugated with annexin V able to bind to phosphatidylserine residues (reviewed by [Said et al., 2008](#)). This technique has been utilized to separate dead and apoptotic spermatozoa ([Grunewald et al., 2001](#); [Lee et al., 2010](#); [Said et al., 2008](#)). Sperm cells are labeled with annexin-V magnetic beads and passed through a column, which is placed in the magnetic field of a MACS separator.

Reports have described the use of these techniques and the achievement of the first newborns. To date the data presented are limited, and there are few studies adequately addressing the benefits of MACS to improve clinical take home baby rate by selecting nonapoptotic sperm cells compared with standard sperm selection methods ([Dirican et al., 2008](#); [Polak de and Denaday, 2010](#); [Rawe et al., 2010](#)).

The other more promising technology is that of microfluidics to prepare sperm. This was initially introduced by Smith and colleagues ([Schuster et al., 2003](#)). Numerous groups have now started to introduce its use in IVF laboratories ([de Wagenaar et al., 2015, 2016](#); [Shirota et al., 2016](#)).

Of the numerous other methods investigated sperm membrane charge has also been used to enable better sperm preparation. Two methodologies are the Zeta potential ([Chan et al., 2006](#)) and an electrophoretic chamber ([Aitken et al., 2011](#)). Although the electrophoretic chamber appears promising ([Ainsworth et al., 2007](#)), neither has been tested to see if they convey significant clinical improvement. Unfortunately, of all the techniques developed to date there is no clear sperm preparation technique that has provided conclusive evidence of improving efficiency when compared to SU or DG.

Conclusion

IVF reproductive technologies have come a long way since the first birth in 1978. The technical progression from routine insemination to ICSI for treating many male infertility patients has changed the way we prepare sperm. It is now coming to light that the role of the paternal genome in influencing the success of IVF is more subtle than once thought ([Sakkas and Alvarez, 2010](#); [Jenkins and Carrell, 2012](#)). We are subsequently realizing that more effort should be made in sperm preparation techniques and many research laboratories are now putting more effort in developing novel methods for sperm preparation and selection.

References

- Ainsworth, C., Nixon, B., Jansen, R. P., & Aitken, R. J. (2007). First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa. *Human Reproduction*, 22, 197–200.
- Aitken, R. J., Hanson, A. R., & Kuczera, L. (2011). Electrophoretic sperm isolation: Optimization of electrophoresis conditions and impact on oxidative stress. *Human Reproduction*, 26, 1955–1964.
- Alipour, H., Van Der Horst, G., Christiansen, O. B., Dardmeh, F., Jorgensen, N., Nielsen, H. I., & Hnida, C. (2017). Improved sperm kinematics in semen samples collected after 2 h versus 4–7 days of ejaculation abstinence. *Human Reproduction*, 1–9.
- Belva, F., De Schrijver, F., Tournaye, H., Liebaers, I., Devroey, P., Haentjens, P., & Bonduelle, M. (2011). Neonatal outcome of 724 children born after ICSI using non-ejaculated sperm. *Human Reproduction*, 26, 1752–1758.
- Bonduelle, M., Wilkens, A., Buysse, A., Van, A. E., Wisanto, A., Devroey, P., Van Steirteghem, A. C., & Liebaers, I. (1996). Prospective follow-up study of 877 children born after intracytoplasmic sperm injection (ICSI), with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Human Reproduction*, 11(Suppl 4), 131–155.
- Boulet, S. L., Mehta, A., Kissin, D. M., Warner, L., Kawwass, J. F., & Jamieson, D. J. (2015). Trends in use of and reproductive outcomes associated with intracytoplasmic sperm injection. *JAMA*, 313, 255–263.
- Cayli, S., Jakab, A., Ovari, L., Delpiano, E., Celik-Ozenci, C., Sakkas, D., Ward, D., & Huszar, G. (2003). Biochemical markers of sperm function: Male fertility and sperm selection for ICSI. *Reproductive Biomedicine Online*, 7, 462–468.
- Celik-Ozenci, C., Catalanotti, J., Jakab, A., Aksu, C., Ward, D., Bray-Ward, P., Demir, R., & Huszar, G. (2003). Human sperm maintain their shape following decondensation and denaturation for fluorescent in situ hybridization: Shape analysis and objective morphometry. *Biology of Reproduction*, 69, 1347–1355.
- Chan, P. J., Jacobson, J. D., Corselli, J. U., & Patton, W. C. (2006). A simple zeta method for sperm selection based on membrane charge. *Fertility and Sterility*, 85, 481–486.
- de Wagenaar, B., Berendsen, J. T., Bomer, J. G., Olthuis, W., van den Berg, A., & Segerink, L. I. (2015). Microfluidic single sperm entrapment and analysis. *Lab on a Chip*, 15, 1294–1301.
- de Wagenaar, B., Dekker, S., de Boer, H. L., Bomer, J. G., Olthuis, W., van den Berg, A., & Segerink, L. I. (2016). Towards microfluidic sperm refinement: Impedance-based analysis and sorting of sperm cells. *Lab on a Chip*, 16, 1514–1522.

- Dirican, E. K., Ozgun, O. D., Akarsu, S., Akin, K. O., Ercan, O., Ugurlu, M., Camsari, C., Kanyilmaz, O., Kaya, A., & Unsal, A. (2008). Clinical outcome of magnetic activated cell sorting of non-apoptotic spermatozoa before density gradient centrifugation for assisted reproduction. *Journal of Assisted Reproduction and Genetics*, 25, 375–381.
- Gianaroli, L., Cristina, M. M., Ferraretti, A. P., Fiorentino, A., Tosti, E., Panzella, S., & Dale, B. (1996). Reducing the time of sperm–oocyte interaction in human in-vitro fertilization improves the implantation rate. *Human Reproduction*, 11, 166–171.
- Grunewald, S., Paasch, U., & Glander, H. J. (2001). Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. *Cell and Tissue Banking*, 2, 127–133.
- Huszar, G., Ozkavukcu, S., Jakab, A., Celik-Ozenci, C., Sati, G. L., & Cayli, S. (2006). Hyaluronic acid binding ability of human sperm reflects cellular maturity and fertilizing potential: Selection of sperm for intracytoplasmic sperm injection. *Current Opinion in Obstetrics & Gynecology*, 18, 260–267.
- Huszar, G., Jakab, A., Sakkas, D., Ozenci, C. C., Cayli, S., Delpiano, E., & Ozkavukcu, S. (2007). Fertility testing and ICSI sperm selection by hyaluronic acid binding: Clinical and genetic aspects. *Reproductive Biomedicine Online*, 14, 650–663.
- Jakab, A., Sakkas, D., Delpiano, E., Cayli, S., Kovanci, E., Ward, D., Ravelli, A., & Huszar, G. (2005). Intracytoplasmic sperm injection: A novel selection method for sperm with normal frequency of chromosomal aneuploidies. *Fertility and Sterility*, 84, 1665–1673.
- Jenkins, T. G., & Carrell, D. T. (2012). The sperm epigenome and potential implications for the developing embryo. *Reproduction*, 143, 727–734.
- Kattera, S., & Chen, C. (2003). Short coincubation of gametes in in vitro fertilization improves implantation and pregnancy rates: A prospective, randomized, controlled study. *Fertility and Sterility*, 80, 1017–1021.
- Lee, T. H., Liu, C. H., Shih, Y. T., Tsao, H. M., Huang, C. C., Chen, H. H., & Lee, M. S. (2010). Magnetic-activated cell sorting for sperm preparation reduces spermatozoa with apoptotic markers and improves the acrosome reaction in couples with unexplained infertility. *Human Reproduction*, 25, 839–846.
- Leunens, L., Celestin-Westreich, S., Bonduelle, M., Liebaers, I., & Ponjaert-Kristoffersen, I. (2008). Follow-up of cognitive and motor development of 10-year-old singleton children born after ICSI compared with spontaneously conceived children. *Human Reproduction*, 23, 105–111.
- Ng, F. L., Liu, D. Y., & Baker, H. W. (1992). Comparison of Percoll, mini-Percoll and swim-up methods for sperm preparation from abnormal semen samples. *Human Reproduction*, 7, 261–266.
- Palini, S., Stefani, S., Primiterra, M., Benedetti, S., Barone, S., Carli, L., Vaccari, E., Murat, U., & Feichtinger, W. (2017). Comparison of in vitro fertilization outcomes in ICSI cycles after human sperm preparation by density gradient centrifugation and direct micro swim-up without centrifugation. *JBRA Assisted Reproduction*, 21, 89–93.
- Polak de Fried, E., & Denaday, F. (2010). Single and twin ongoing pregnancies in two cases of previous ART failure after ICSI performed with sperm sorted using annexin V microbeads. *Fertility and Sterility*, 94, 351–358.
- Rawe, V. Y., Boudri, H. U., Alvarez, S. C., Carro, M., Papier, S., & Nodar, F. (2010). Healthy baby born after reduction of sperm DNA fragmentation using cell sorting before ICSI. *Reproductive Biomedicine Online*, 20, 320–323.
- Said, T. M., Agarwal, A., Zborowski, M., Grunewald, S., Glander, H. J., & Paasch, U. (2008). Utility of magnetic cell separation as a molecular sperm preparation technique. *Journal of Andrology*, 29, 134–142.
- Sakkas, D., Manicardi, G. C., Tomlinson, M., Mandrioli, M., Bizzaro, D., Bianchi, P. G., & Bianchi, U. (2000). The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. *Human Reproduction*, 15, 1112–1116.
- Sakkas, D., & Alvarez, J. G. (2010). Sperm DNA fragmentation: Mechanisms of origin, impact on reproductive outcome, and analysis. *Fertility and Sterility*, 93, 1027–1036.
- Sbracia, M., Grasso, J., Sayme, N., Stronk, J., & Huszar, G. (1997). Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa. *Human Reproduction*, 12, 1949–1954.
- Schuster, T. G., Cho, B., Keller, L. M., Takayama, S., & Smith, G. D. (2003). Isolation of motile spermatozoa from semen samples using microfluidics. *Reproductive Biomedicine Online*, 7, 75–81.
- Shirota, K., Yotsumoto, F., Itoh, H., Obama, H., Hidaka, N., Nakajima, K., & Miyamoto, S. (2016). Separation efficiency of a microfluidic sperm sorter to minimize sperm DNA damage. *Fertility and Sterility*, 105, 315–321.
- WHO. (2010). *World Health Organization Laboratory manual for examination of human semen* (5th edn). Cambridge: Cambridge University Press.
- Worilow, K. C., Eid, S., Woodhouse, D., Perloe, M., Smith, S., Witmyer, J., Ivani, K., Khoury, C., Ball, G. D., Elliot, T., et al. (2012). Use of hyaluronan in the selection of sperm for intracytoplasmic sperm injection (ICSI): Significant improvement in clinical outcomes—Multicenter, double-blinded and randomized controlled trial. *Human Reproduction*, 28(2), 306–314.
- Zhao, F., Yang, Q., Shi, S., Luo, X., & Sun, Y. (2016). Semen preparation methods and sperm telomere length: Density gradient centrifugation versus the swim up procedure. *Scientific Reports*, 6, 39051.

EMBRYOLOGY

Oocyte Treatment From Retrieval to Insemination

Laura Francesca Rienzi, Benedetta Iussig, and Filippo Maria Ubaldi, G.EN.E.R.A. Centres for Reproductive Medicine, Rome, Italy

© 2018 Elsevier Inc. All rights reserved.

A Brief Overview of Multifollicular Growth and Oocyte Retrieval

While the initial attempts of assisted reproductive technologies (ART) were conducted on spontaneous menstrual cycles, nowadays controlled ovarian hyperstimulation (COH) is widely used to stimulate multifollicular growth in order to increase oocytes availability for in vitro fertilization. This is accomplished with the administration of a cocktail of exogenous human gonadotropins from the early follicular phase to achieve supraphysiologic levels of circulating FSH, widening the so called “FSH window” and allowing to override the single dominant follicle selection (Pacchiarotti et al., 2016).

Follicular growth is then constantly monitored with ultrasound serial measurements of follicular size and determination of circulating estradiol concentration. When the follicles reach an appropriate stage of development, ovulation is induced with a surrogate LH surge, which stimulates the resumption of meiosis (consisting in the germinal vesicle-GV-breakdown and the consequent oocyte progression to the metaphase II-MII-stage), the retraction of corona cells processes and the mucidification and expansion of the cumulus cells layer (Russell and Robker, 2007).

While in spontaneous menstrual cycles the ovulated oocytes are then captured by fimbriae and released in the Fallopian tubes, during assisted reproductive treatments they are directly removed from the follicular microenvironment by means of surgical harvesting. The technical details of oocytes retrieval differ between centers, but basically it is performed transvaginally inserting a fine cave needle with ultrasound guidance to the ovaries and aspirating the follicular content by means of an external vacuum pump which modulates the aspiration pressure. Usually, this procedure is scheduled 34–36 h after ovulation triggering in order to avoid premature ovulation and subsequent cycle cancellation.

Don't Disturb the Gamete: Working Conditions

The follicular fluid containing the oocytes is collected into sterile tubes connected to the aspiration system (Fig. 1), then passed to the adjoining laboratory for further analysis. Any handling of the biological material outside the controlled environment of the incubators should be performed with extreme care.

The oocytes are susceptible to light exposure, so that this should be maintained to a minimum. Sunlight is the worst stressor to both oocytes and embryos, and even prolonged exposure of mammalian oocytes and embryos to ordinary cool or warm white fluorescent light is inadvisable. Incandescent light from the illuminators of ordinary optical microscopes would not produce any serious problem unless used excessively (Takenaka et al., 2007; Oh et al., 2007).

During the entire manipulation procedure, efforts should be made to minimize even transient temperature fluctuations that may have detrimental effects on oocyte developmental competence: in fact, only few minutes at room temperature cause meiotic spindle depolymerization, thus potentially increasing the risk of aneuploidy (Pickering et al., 1990). Temperature control can be easily

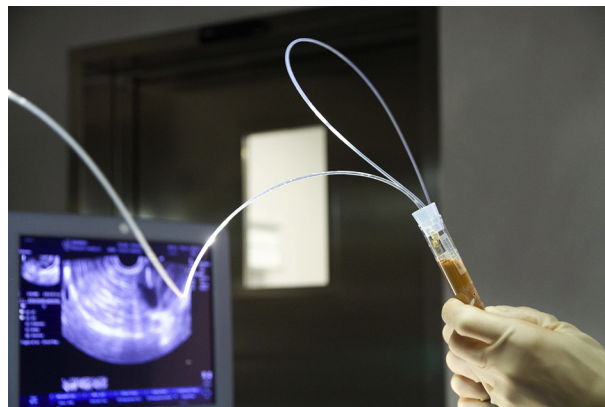


Fig. 1 Follicular fluid collected in a sterile tube during oocyte retrieval. All the tubes must be labeled with patient information and identification number in order to guarantee unambiguous identification.

accomplished using warmed surfaces and devices and limiting the time in which the biological material is kept at a temperature minor to 37°C. Since temperature is dispersed by plastic ware, it is recommended to calibrate the heated stages to a higher temperature, around 37.5–38°C.

Another key factor to keep controlled during manipulation outside incubators is pH. Intracellular pH (ipH) is necessary to maintain intracellular homeostasis. Since it is influenced by extracellular pH (epH), it is fundamental to maintain the optimum media pH relatively stable (Swain, 2010). This can be accomplished first limiting the time of manipulation under atmospheric conditions (low CO₂ concentration determines a rapid rise in the extracellular pH) and, secondly, guaranteeing fast oocyte relocation in an appropriate culture system (Swain 2010).

Finally, some authors found that dissolved oxygen levels during follicular aspiration and oocyte retrieval rise rapidly (Redding et al., 2006). Even if the precise O₂ level under natural conditions is still unknown and may be developmental stage-dependent, it is now ascertained that low oxygen level should be a principle for all mammalian embryo culture systems including humans. In fact, it has been proved to be beneficial with a significant increase in the number of newborns (Bontekoe et al., 2012). Since it takes more than 5 h for the oxygen to fall to oocyte and embryo safe levels, it is important to protect the culture from O₂ rising as well limiting once again air exposure.

Cumulus-Corona-Oocyte Complex Identification and Grading

Once in the laboratory, the follicular fluid is handed over to the embryologist, who pours it in prewarmed sterile plastic dishes in order to examine it carefully (Fig. 2). The follicular fluid contains many free granulosa cells, which derive from the follicle walls and are organized in amorphous clumps, and the cumulus-corona-oocyte complexes (CCOCs), the structures formed by the cumulus cells and the enclosed oocyte (Figs. 3 and 4). The entire CCOC identification procedure is performed under a laminar flow hood, at the stereomicroscope, which produces a three-dimensional visualization of the sample. The identified CCOCs are then cautiously transferred with a Pasteur pipette in an appropriate culture medium and cultured in incubator (humidified atmosphere, 37°C, 5%–6% CO₂, low oxygen tension) till conventional insemination, intracytoplasmic sperm injection (ICSI) or cryopreservation.

CCOC assessment generally represents the first line evaluation in the everyday work of an average IVF laboratory. Among cumulus cells (CCs), two different entities can be recognized: inner and outer cumulus cells, which differ for sensitivity and responsiveness to external stimuli. The innermost layer is also called “corona radiata”: it contacts the oocyte thanks to trans-zonal projections (TZPs) that form gap junctions with the oolemma, establishing a bidirectional communication fundamental for both follicle and egg growth (Albertini et al., 2001). At the time of oocyte retrieval, 34–38 h after ovulation triggering, oocytes are supposed to have reached full nuclear maturity (MII stage). The cumulus-corona mass of fully mature oocytes is expected to be highly expanded as a consequence of the secretion of hyaluronic acid, that interposes among the cells and separates them conferring to the complex a fluffy appearance (Testart et al., 1983). On the contrary, unexpanded compact CCs usually enclose immature oocytes, at the prophase (Germinal vesicle stage, GV) or metaphase stage of the first meiotic division (MI).

During the years there have been different attempts to define a grading system for objective and standard classification of cumulus-corona-oocyte complexes (Rienzi et al., 2011). The most applied criteria were those proposed by Lin et al. (2003), who produced a quite complete CCOCs evaluation scheme. In their proposal, CCOCs are divided in five different groups based upon detached granulosa cells (GCs) aspect, cumulus expansion, corona radiation and ooplasm features. In detail, the “mature” CCOCs are constituted by well-aggregated membrane granulosa cells, expanded cumulus, radiant corona, distinct zona pellucida



Fig. 2 Follicular fluid poured in a Petri dish during the CCOC identification procedure. All the dishes must be labeled with patient information and identification number in order to guarantee unambiguous identification. The embryologist works under flow laminar flood and manipulates the biological material with extreme care, paying particular attention to the maintenance of the correct culture conditions such as light exposure, temperature, pH and oxygen tension.

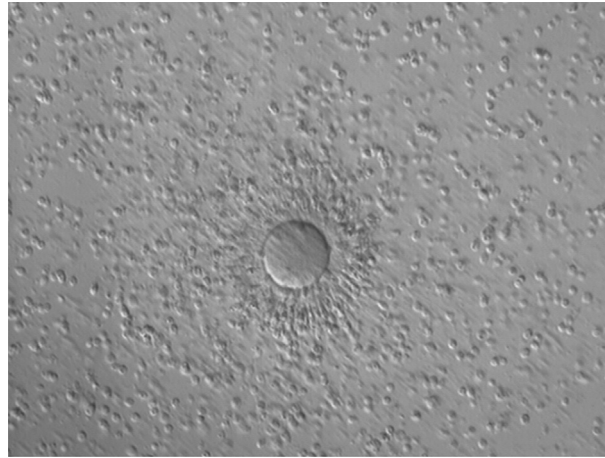


Fig. 3 Cumulus-corona-oocyte complex visualized at 100 $\times$ magnification. The cumulus mass is expanded and it is possible to identify the associated oocyte. Although not visualized in this particular focal plane, a first polar body is present in the perivitelline space. The ooplasm is clear.

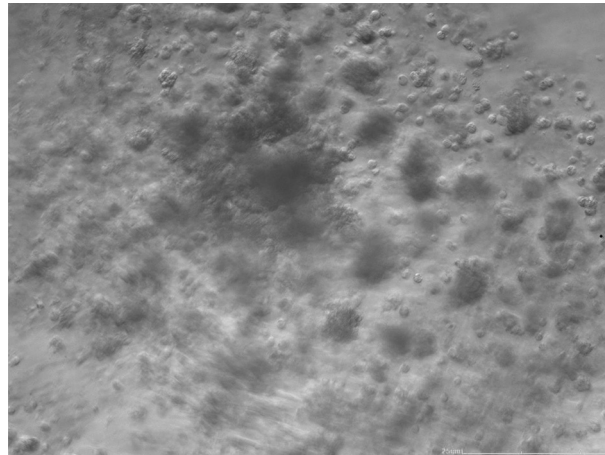


Fig. 4 Cumulus-corona-oocyte complex visualized at 100 $\times$ magnification. The cumulus mass is compact and dark GCs clumps are diffused. The associated oocyte is dark, obscured by the cells, and its precise maturational status is uncertain.

and clear ooplasm. On the contrary, “immature” CCOCs present compact and nonaggregated GCs, compact dense cumulus mass, adherent compact corona layer and presence of a germinal vesicle in the ooplasm (if visible). However, between these two CCOCs classification patterns, it is possible to identify one intermediate class, the so called “approximately mature,” characterized by expanded, well-aggregated membrana GCs, expanded cumulus mass but slightly compact corona. During oocyte pick-up, it is also possible to retrieve “postmature” CCOCs, with small and relatively nonaggregated membrana GCs, expanded cumulus with clumps, radiant corona radiata (yet often clumped, irregular or incomplete), visible zona pellucida and slightly granular or dark ooplasm. Finally, “atretic” CCOCs are composed by GCs with very small clumps of cells, rarely associated cumulus mass, clumped and very irregular corona radiata (if present), visible zona pellucida and dark and frequently misshapen ooplasm.

Notwithstanding, it has been suggested that in stimulated cycles cumulus expansion and CCOCs evolution doesn’t always run parallel to oocyte nuclear maturation, as observed in natural cycles, maybe because of a different sensitivity of the oocyte and the CCs to the stimulants, resulting in an asynchrony between the two processess ([Laufer et al., 1984](#); [Bar-Ami et al., 1989](#)).

More recently, the Alpha Scientists in Reproductive Medicine and the European Society of Human Reproduction (ESHRE) Special Group of Embryology concluded that, to date, there’s little evidence to support the role of CCOCs morphological analysis in the prediction of oocyte maturity and competence. As a consequence, the application of a simple binary score to define expanded CCs and radiating corona (point 1) ([Fig. 3](#)) or compact cumulus mass (point 0) ([Fig. 4](#)) appears more suitable for the purpose ([Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011](#)).

Conventional In Vitro Insemination

After CCOC identification, the oocytes can be inseminated through conventional insemination or intracytoplasmic sperm onjection (ICSI). In the first case, no more CCOC manipulation is required, since the CCOCs are incubated in the presence of a defined

concentration of motile spermatozoa. On the contrary, in the case of ICSI, it is necessary to remove the cumulus cells surrounding the oocytes (denudation) prior to perform injection.

Insemination timing should be decided evaluating the time elapsed from ovulation triggering and/or oocyte retrieval, assuming that a preincubation period may be beneficial for the accomplishment of complete oocyte maturation: normally, this correspond to 38–40 h after ovulation triggering or 3–4 h after oocyte retrieval (Ho et al., 2003).

Mature pre-ovulatory CCOCs are transferred with a Pasteur pipette to a convenient culture support, the choice of which is a matter of preference. Two main culture systems are commonly used: petri-dishes presenting microdrops of culture media under oil or, alternatively, four-well plates. In both cases, it is possible to culture the CCOCs singularly or co-culture them in small groups of 2–3 CCOCs. Whichever the culture system of choice is, it is advisable to use equilibrated media, preincubated in an incubator at 37°C, 5%–6% CO₂, low oxygen tension for a suitable period. Moreover, it is recommended to trace all the batches used for media as well as for any plastic support, in an effort to guarantee quality monitoring (ESHRE Guideline Group on Good Practice in IVF Labs, 2016).

The CCOCs are routinely inseminated exposing them to a prepared sample of progressively motile spermatozoa, the number of which must be optimized in order to increase the chance of normal fertilization. Normally, this corresponds to a concentration ranging between 0.1 and 0.5×10^6 progressively motile spermatozoa per ml (ESHRE Guideline Group on Good Practice in IVF Labs, 2016). It is recommended to suspend the final sperm preparation in a culture medium compatible with oocyte culture and fertilization, preferably containing glucose in order to allow sperm function (ESHRE Guideline Group on Good Practice in IVF Labs, 2016). Two different operators should check the correspondence of the gametes used, in order to guarantee their identity and to avoid hazardous mismatching.

The CCOCs-sperm suspension is then incubated overnight in incubator (humidified atmosphere, 37°C, 5%–6% CO₂, low oxygen tension) till the fertilization check, usually performed 16–18 h after insemination (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

References

- Albertini, D. F., Combelles, C. M., Benecchi, E., & Carabatsos, M. J. (2001). Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction*, 121, 647–653.
- Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. (2011). The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Human Reproduction*, 26, 1270–1283.
- Bar-Ami, S., Gitay-Goren, H., & Brandes, J. M. (1989). Different morphological and steroidogenic patterns in oocyte/cumulus-corona cell complexes aspirated at in vitro fertilization. *Biology of Reproduction*, 41, 761–770.
- Bontekoe, S., Mantikou, E., Van Wely, M., et al. (2012). Low oxygen concentrations for embryo culture in assisted reproductive technologies. *The Cochrane database of systematic reviews*, (1), CD008950. <https://doi.org/10.1002/14651858.CD008950.pub2>.
- ESHRE Guideline Group on good practice in IVF labs. (2016). Revised guidelines for good practice in IVF laboratories (2015). *Human Reproduction*, 31, 685–686.
- Ho, J. Y., Chen, M. J., Yi, Y. C., Guu, H. F., & Ho, E. S. (2003). The effect of preincubation period of oocytes on nuclear maturity, fertilization rate, embryo quality and pregnancy outcome in IVF and ICSI. *Journal of Assisted Reproduction and Genetics*, 20, 358–364.
- Laufer, N., Tarlatzis, B. C., DeCherney, A. H., et al. (1984). Asynchrony between human cumulus-corona cell complex and oocyte maturation after human menopausal gonadotropin treatment for in vitro fertilization. *Fertility and Sterility*, 42, 366–372.
- Lin, Y. C., Chang, S. Y., Lan, K. C., et al. (2003). Human oocyte maturity in vivo determines the outcome of blastocyst development in vitro. *Journal of Assisted Reproduction and Genetics*, 20, 506–512.
- Oh, S. J., Gong, S. P., Lee, S. T., Lee, E. J., & Lim, J. M. (2007). Light intensity and wavelength during embryo manipulation are important factors for maintaining viability of preimplantation embryos in vitro. *Fertility and Sterility*, 88, 1150–1157.
- Pacchiarotti, A., Selman, H., Valeri, C., et al. (2016). Ovarian stimulation protocol in IVF: An up-to-date review of the literature. *Current Pharmaceutical Biotechnology*, 17, 303–315.
- Pickering, S. J., Braude, P. R., Johnson, M. H., Cant, A., & Currie, J. (1990). Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertility and Sterility*, 54, 102–108.
- Redding, G. P., Bronlund, J. E., & Hart, A. L. (2006). The effects of IVF aspiration on the temperature, dissolved oxygen levels and pH of follicular fluid. *Journal of Assisted Reproduction and Genetics*, 23, 37–40.
- Rienzi, L., Vajta, G., & Ubaldi, F. (2011). Predictive value of oocyte morphology in human IVF: A systematic review of the literature. *Human Reproduction Update*, 17, 35–45.
- Russell, D. L., & Robker, R. L. (2007). Molecular mechanisms of ovulation: Co-ordination through the cumulus complex. *Human Reproduction Update*, 13, 289–312.
- Swain, J. E. (2010). Optimizing the culture environment in the IVF laboratory: Impact of pH and buffer capacity on gamete and embryo quality. *Reproductive Biomedicine Online*, 21, 6–16.
- Takenaka, M., Horiuchi, T., & Yanagimachi, R. (2007). Effects of light on development of mammalian zygotes. *Proceedings of the National Academy of Sciences of the United States of America*, 11, 14547–14548.
- Testart, J., Frydman, R., De Mouzon, J., Lassalle, B., & Belaisch, J. C. (1983). A study of factors affecting the success of human fertilization in vitro. I. Influence of ovarian stimulation upon the number and condition of oocytes collected. *Biology of Reproduction*, 28, 415–424.

Preparation and Evaluation of Oocytes for ICSI

Belén Aparicio-Ruiz, IVI-RMA, Valencia, Spain

Maria J de los Santos, IVI-RMA, Valencia, Spain; IVI Foundation, Valencia, Spain; and INCLIVA, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Oocyte Denudation

Opposite to conventional insemination, oocytes need to be denuded before performing sperm microinjection. The term *oocyte denudation* refers to the removal of the somatic cell layers that surround the oocytes. These cells are forming during the preovulatory growth, within the follicle. The oocyte will be surrounded by two different cell layers: *granulosa and thecal cells* that sustain oocyte nutrition and maturation providing essential metabolites, hormones, and growth factors (Fig. 1); all of it driven presumably by the oocyte which directs its own development as well as the follicle differentiation (Maggiulli et al., 2012; Albertini et al., 2001).

During oocyte pick up, oocytes will be aspirated from the preovulatory follicle environment and a new structure called cumulus oocyte complex COC will be obtained (Fig. 2). The COC is composed of the secondary oocyte, which should be arrested at metaphase II, surrounded by a multilayer of specialized granulosa cells called cumulus cells. They possess highly specialized trans-zonal cytoplasmic projections that pierce through the zona pellucida and form gap junctions at their tips with the oocyte (Albertini et al., 2001). The gap junctions will allow paracrine factors and low molecular weight factors to freely travel in both directions (Canipari, 2000; Feuerstein et al., 2006).

The timing between oocyte retrieval and oocyte denudation varies between different laboratories. Based on the on the ESHRE Guidelines for good practice in the IVF laboratory, the consensus was to wait for a consistent time interval of 4 ± 1 h between the time of trigger and the time of cumulus cell removal (de Los Santos et al., 2016).

What is commonly accepted is that a preincubation period in the incubator at 37°C and 5% CO₂ of at least 1 h is unavoidable. Rienzi et al. (1998) studied different preincubation timings concluding that a preincubation period between oocyte retrieval and ICSI of at least 3 h can improve the fertilization rate and embryo quality. This period might be necessary for some oocytes to reach full cytoplasmic maturity, leading to a higher activation rate upon microinjection. On the other hand, too long preincubation timings (in this case established as more than 9 h), resulted in embryos of lower quality (Rienzi et al., 1998). However, this can varies depending on different women's ranges of age, prospective randomized studies would be needed.

Oocytes are very vulnerable to fluctuations on temperature and pH (Swain, 2015; Swain et al., 2016; Will et al., 2011).

Before denudation, the presence of cumulus cells may provide to the oocytes a protective action against pH changes due to the presence of gap junctions. However once they are removed, the oocytes will be more sensitive. It has been shown that human oocytes have very limited capacity to combat alkalosis. On the other hand, the acidosis will require energy to be battled from the oocyte, consequently both alkalosis and acidosis should be avoided to decrease the stress at which the oocytes are exposed.

Concerning temperature, two aspects have to be taken into account, the actual temperature that will be achieved and the duration of the oocytes to be exposed to room temperature. Among the possible negative effect of temperature drops to room temperature such as impact of embryo metabolism, and embryo cleavage rate, others like the meiotic spindle stability and chromosome dispersion deserves special consideration (Pickering et al., 1990; Almeida and Bolton, 1995; Wang et al., 2001).

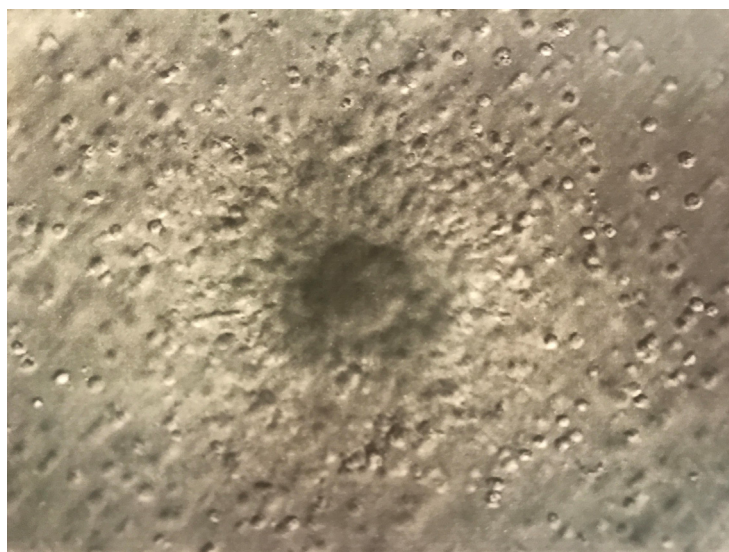


Fig. 1 Diagram showing the distribution of the oocyte and the cells surrounding it after oocyte retrieval.

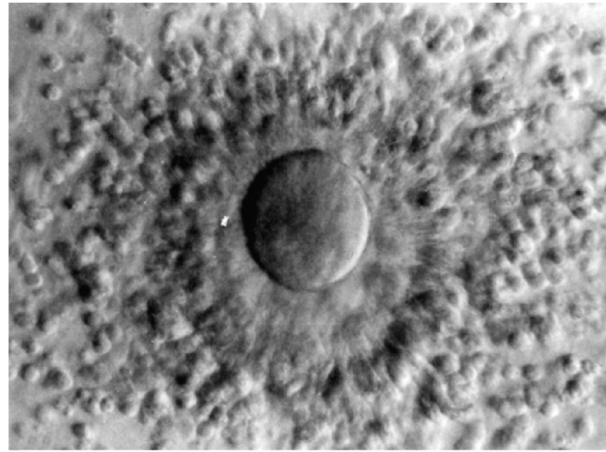


Fig. 2 Image of a cumulus oocyte complex after oocyte retrieval.

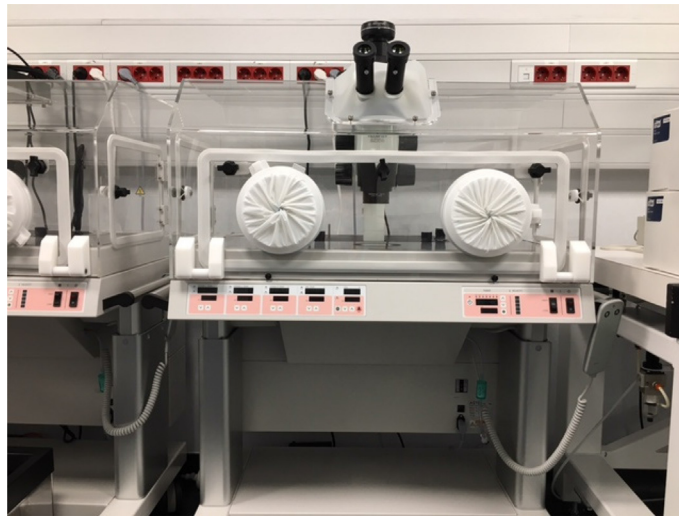


Fig. 3 ASTEC Smart Station is an integrated IVF Work Station which substantially enhances the working environment for IVF procedures.

For example, the exposure of oocytes to 34°C for 10–30 min decrease approx 20% the spindle recovery rate affecting fertilization ability as well. If the exposure is at room temperature, less than 50% of spindles will recover (Pickering et al., 1990).

Hence, the step of denudation has to be performed avoiding variations in both parameters. Handling conditions to allow a stable environment of 37°C and adequate CO₂ to control for pH are highly recommended. Laboratory settings using cabinets as shown in Fig. 3 are a very good option. Other approaches such as the use of buffered media and heated working surfaces may be used as well if the first option is not available.

The removal of the surrounding cumulus cells is achieved by a combination of both enzymatic and mechanical treatment.

Enzymatic Treatment

The egg released from the ovarian follicle is surrounded by the cumulus mass. Before ovulation takes place, in concomitance with oocyte maturation, LH induces some transformations in this cumulus cells. In response to this gonadotrophin the cumulus cells produce specific glucosaminoglycans, the secretion of which results in cumulus mucification and its expansion. The major component of the extracellular matrix secreted by the cumulus cells is hyaluronic acid.

Because of this, hyaluronidase is employed for enzymatic removal of these cells. This enzyme digests the hyaluronic acid interspaced between the cumulus cells (Mahadevan and Trounson, 1985). Until now, few studies have tested the possible effects of different concentrations of hyaluronidase in sibling-oocytes. The initial concentration used of this enzyme was established in 760 IU/mL, but it was found that this concentrations were too high and induced a high percentage of parthenogenetic activation of the mature oocytes. At the moment, the commonly used concentration of this enzyme in most IVF laboratories is 80 IU/mL. However, Van de Velde et al. developed a study that showed how a concentration as low as 10–8 IU/mL hyaluronidase can be used successfully to denude oocytes for microinjection (Van De Velde et al., 1997; Moura et al., 2017).

Mechanical Treatment

The enzymatic treatment is also helped by a mechanical action. The procedure needs to be carried out very gently to avoid any mechanical damage in the oocytes. Pricking of the oocyte has been shown to induce parthenogenetic oocyte activation (Iritani, 1991).

Once the oocytes have undergone the enzymatic contact with the hyaluronidase, changes in the diameter of the pipettes used are crucial for a final denudation.

To start with, the COC is too big and diameters of approximately 190 μm are required.

Once the enzyme has acted, a decrease in the diameter of the capilar to approximately 130–150 μm is needed, because the oocyte will still have some cells attached which can make it difficult to evaluate the final maturation. This smaller diameter needs to be used in clean drops of culture medium because oocytes should not spend more than 1 min in contact with the hyaluronidase. This critical timing and the knowledge of this critical step in the laboratory has been crucial to decide in our clinic that the maximum number of oocyte denudated in each dish has to be eight despite our experience as embryologists, and even less for embryologists with less experience.

Briefly, the dishes used in our laboratory to proceed to the decumulation of oocytes are shown in the Fig. 4.

In the Fig. 5 we illustrated two different ways of proceeding to oocyte denudation by two different embryologists in our laboratory basically based in a different organization or direction of the oocytes throughout the droplets.

The rationale behind the oocyte denudation before microinjection is to see the maturation stage of the oocytes in order to distinguish between those that suitable for being microinjected or not, or even to select some of them for in vitro maturation because in the presence of cumulus, visualization of the oocyte is very limited.

In addition to meiotic status, the morphology of the oocytes is also evaluated before ICSI being able to detect some abnormalities that may affect fertilization rates and embryo quality. This is important for embryologists to have more information especially in order to explain patients in cases where the results are not positive.

One of the key events that necessarily need to take place for successful fertilization is the resumption of meiosis in the oocyte. Although the majority of the oocytes (80%–90%) will be at metaphase II, since ovulation has been triggering, oocytes can be found at different maturation stages (Fig. 6).

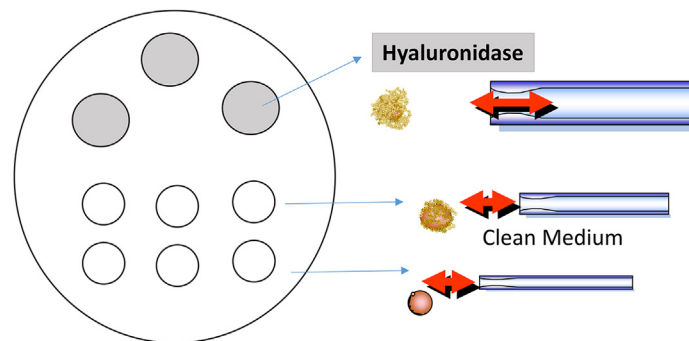


Fig. 4 Diagram showing the distribution of different drops in the dish and how oocyte denudation is performed.

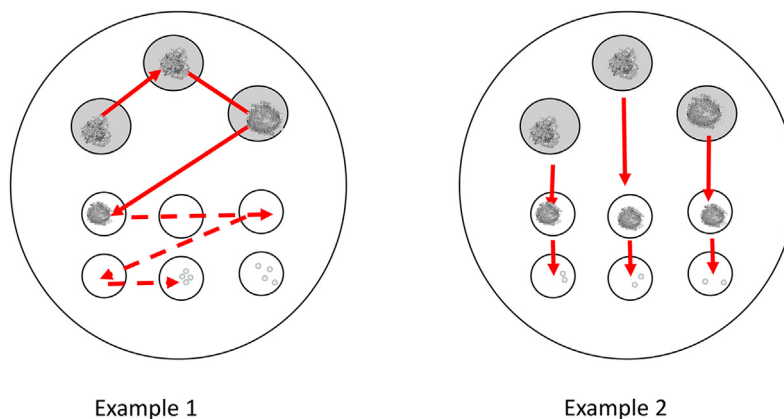


Fig. 5 Diagram showing different ways of performing oocyte denudation by different embryologists in our laboratory.



Fig. 6 Images of oocytes in different maturation stages.

Germinal vesicle (GV)

In this stage, oocytes are meiotically arrested. These are immature oocytes where a typical nuclear structure is perfectly visualized and are not used for microinjection.

Throughout the infancy of women, oocyte stay in this stage. When puberty arrives and women start with menstruation, at each cycle one (or rarely more) oocytes reinitiate the meiotic division.

Metaphase I

Meiosis has been reinitiated with what is known as the vesicle breakdown (GVB), however the appearance of the first polar body has not taken place yet.

With the reinitiating of meiosis the chromosomes condense and align on the newly formed meiotic spindle, and the pairs of homologous chromosomes segregate between the oocyte and the first polar body.

Metaphase II

All of the events have occurred and we have a mature fertilizable oocyte, completed at the metaphase of the second round of meiosis.

After checking for oocyte maturation status, ICSI will be performed on those oocytes that are presumably at metaphase II because the presence the first polar body (more detailed information about the ICSI procedure will be available on the upcoming chapters). However, we should always bear in mind that only by visualizing the meiotic spindle with the use of polarized light microscope, which evaluates the oocytes' spindles according to birefringence of living cells, can assured the maturation status of oocytes (Montag et al., 2006).

Eventually immature oocytes could be used for clinical purposes. In humans, in vitro maturation (IVM) occurs in 30%–50% of oocytes recovered from non-stimulated cycles (Cha et al., 1991) and in 60%–80% of germinal vesicle (GV) oocytes removed from stimulated ovaries (Goud et al., 2000; Escrich et al., 2011). An average of 75% of the denuded GV oocytes recovered from stimulated cycles matured spontaneously to MII during a period of 42.7 ± 2.4 h (Escrich et al., 2011) they can be fertilized and give live births although with low rate of efficiency.

References

- Albertini, D. F., Combelles, C. M., Benecchi, E., & Carabatsos, M. J. (2001). Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction (Cambridge England)*, 121(5), 647–653.
- Almeida, P. A., & Bolton, V. N. (1995). The effect of temperature fluctuations on the cytoskeletal organisation and chromosomal constitution of the human oocyte. *Zygote*, 3(4), 357–365.
- Canipari, R. (2000). Oocyte–granulosa cell interactions. *Human Reproduction Update*, 6(3), 279–289.
- Cha, K. Y., Koo, J. J., Ko, J. J., Choi, D. H., Han, S. Y., & Yoon, T. K. (1991). Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertility and Sterility*, 55(1), 109–113.
- Escrich, L., Grau, N., Mercader, A., Rubio, C., Pellicer, A., & Escribá, M. (2011). Spontaneous in vitro maturation and artificial activation of human germinal vesicle oocytes recovered from stimulated cycles. *Journal of Assisted Reproduction and Genetics*, 28(2), 111–117.
- Feuerstein, P., Cadoret, V., Dalbès-Tran, R., Guerif, F., & Royere, D. (2006). Le dialogue ovocyte–Cumulus. *Gynécologie Obstétrique & Fertilité*, 34(9), 793–800.
- Goud, A., Goud, P., Qian, C., Van Der Elst, J., Van Maele, G., & Dhont, M. (2000). Cryopreservation of human germinal vesicle stage and in vitro matured M II oocytes: Influence of cryopreservation media on the survival, fertilization, and early cleavage divisions. *Fertility and Sterility*, 74(3), 487–494.
- Iritani, A. (1991). Micromanipulation of gametes for in vitro assisted fertilization. *Molecular Reproduction and Development*, 28(2), 199–207.
- Maggiulli, R., Ubaldi, F., & Rienzi, L. (2012). *Oocyte denuding*. Practical Manual Of In Vitro Fertilization (pp. pp. 93–104). New York: Springer.
- de Los Santos, M. J., Apter, S., Coticchio, G., Debrock, S., Lundin, K., & Et, A. (2016). Revised guidelines for good practice in ivf laboratories (2015). *Human Reproduction*, 31(4), 685–686.
- Mahadevan, M. M., & Trounson, A. O. (1985). Removal of the cumulus Oophorus from the human oocyte for in vitro fertilization. *Fertility and Sterility*, 43(2), 263–267.
- Montag, M., Schimming, T., & Van Der Ven, H. (2006). Spindle imaging in human oocytes: The impact of the meiotic cell cycle. *Reproductive Biomedicine Online*, 12(4), 442–446.
- Moura, B. R., Gurgel, M. C., Machado, S. P., Marques, P. A., Rolim, J. R., Lima, M. C., & Salgueiro, L. L. (2017). Low concentration of hyaluronidase for oocyte denudation can improve fertilization rates and embryo quality. *Jbra Assisted Reproduction*, 21(1), 27–30.
- Pickering, S. J., Braude, P. R., Johnson, M. H., Cant, A., & Currie, J. (1990). Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertility and Sterility*, 54(1), 102–108.

- Rienzi, L., Ubaldi, F., Anniballo, R., Cerulo, G., & Greco, E. (1998). Preincubation of human oocytes may improve fertilization and embryo quality after intracytoplasmic sperm injection. *Human Reproduction (Oxford England)*, 13(4), 1014–1019.
- Swain, J. E. (2015). Optimal human embryo culture, *seminars*. In *Reproductive Medicine 2015* (pp. 103–117). New York, NY: Thieme Medical Publishers.
- Swain, J. E., Carrell, D., Cobo, A., Meseguer, M., Rubio, C., & Smith, G. D. (2016). Optimizing the culture environment and embryo manipulation to help maintain embryo developmental potential. *Fertility and Sterility*, 105(3), 571–587.
- Van De Velde, H., Nagy, Z. P., Joris, H., De Vos, A., & Van Steirteghem, A. C. (1997). Effects of different hyaluronidase concentrations and mechanical procedures for cumulus cell removal on the outcome of intracytoplasmic sperm injection. *Human Reproduction (Oxford England)*, 12(10), 2246–2250.
- Wang, W., Meng, L., Hackett, R. J., Odenbourg, R., & Keefe, D. L. (2001). Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Human Reproduction*, 16(11), 2374–2378.
- Will, M. A., Clark, N. A., & Swain, J. E. (2011). Biological Ph buffers in Ivf: Help or hindrance to success. *Journal of Assisted Reproduction and Genetics*, 28(8), 711–724.

ICSI Versus IMSI

Zsolt Peter Nagy, Reproductive Biology Associates, Atlanta, GA, United States

Pierre Vanderzwalmen, IVF Centers Prof. Zech–Bregenz, Bregenz, Austria; and Centre Hospitalier Inter Regional Cavell (CHIREC), Bruxelles, Belgium

© 2018 Elsevier Inc. All rights reserved.

Introduction to Assisted Fertilization

When the oocyte is fertilized by sperm “outside” of the body, it is called “in vitro” fertilization or assisted fertilization (Stephoe and Edwards, 1978). Robert Edwards together with Patrick Steptoe were the first to achieve a successful live birth in 1978 via “in vitro” fertilization using the “conventional insemination” procedure (Stephoe and Edwards, 1978). A little less than two decades later, in 1992 an alternative insemination procedure was developed, called intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992). These break through procedures followed decades—and even centuries of research/experiments on reproductive physiology, summarized well in the review by Clarke (Clarke, 2006). After the successful introduction of IVF in United Kingdom, very soon other countries also implemented the procedure and its use increased exponentially leading to an estimated 5 million babies born by 2015 thanks to assisted reproduction.

Conventional insemination was the only procedure performed in the “early days” of IVF (as no “viable” alternative approaches existed at the time); where oocyte(s) retrieved from follicle(s) in a natural or in a stimulated cycle were placed in a Petri dish and were mixed together with the processed sperm received from the male partner. Gametes were usually left in place for overnight to allow the sperm to enter the egg and initiate the fertilization. While this insemination procedure usually provided good fertilization and adequate outcomes for couples with no “male-factor” infertility, it frequently resulted in low or failed fertilization where sperm parameters were suboptimal (low concentration and/or low motility and/or low morphology).

Assisted Fertilization by Intracytoplasmic Sperm Injection (ICSI) and by Intracytoplasmic Morphologically Selected Sperm Injection (IMSI)

Due to the inconsistent or poor results with conventional IVF with mild and severe male-factor cases there was a need for a new type of insemination technique, using microinjection. There have been many different approaches explored to assist fertilization of oocytes, both in animal models and in human. Among the more studied options were partial zona dissection (PZD) and subzonal injection (SUZI), none providing significant improvement in oocyte fertilization. ICSI has also been investigated initially in animal models, however, it resulted in an extreme high degeneration rate typically when using rodent gametes (for review on the different microinjection techniques, please see the publication from van Rumste et al., 2000). Intriguingly, human gametes appeared to provide much better outcomes than it was observed in animal models, and when applied clinically, the first pregnancies were reported from the Brussels University (AZ-VUB) in 1992 (Palermo et al., 1992). Since its introduction, the use of ICSI has rapidly spread across the globe, and today it is used as a preferred way of oocyte insemination, frequently also in nonmale factor cases as it provides a more “secure” way for fertilization (Palermo et al., 2015).

Oocyte and Sperm Preparation for ICSI and IMSI

Timing of oocyte collection is typically performed 35–37 h after ovulation trigger. Cumulus and corona radiata cells have to be removed which is done by combination of enzymatic digestion (using hyaluronidase) and mechanical “stripping”, 2–3 h following egg retrieval.

Oocytes then observed by an inverted microscope at $\times 200$ magnification for nuclear maturity and for other morphological characteristics. Only those oocytes that are mature (i.e., extruded the first polar body) will be inseminated by ICSI or IMSI.

Sperm preparation is typically performed using a discontinuous gradient centrifugation (there are alternative options, including the more “historical” swim-up procedure) (Van der Zwalm et al., 1991). The aim of sperm preparation is to produce a clean suspension of motile sperm in culture medium that is compatible with both sperm and oocyte survival and fertilization. For ICSI, the prepared sperm is kept in suspension in a plastic tube until the microinjection procedure. For IMSI, a small aliquot of washed sperm is deposited into the IMSI dish (Fig. 1). The prepared sperm suspension will swim-out in a long “snake shape” drop before selection and oocyte injection. For IMSI, because more time needed for sperm selection, it is advised that sperm preparation is performed prior to oocyte retrieval.

Timing and Process of ICSI and IMSI Insemination

ICSI is usually performed 39–41 h after ovulation trigger (3 to 5 h after oocyte retrieval). Timing of microinjection relative to cumulus-corona cell removal does not seem to effect outcomes, however, most IVF programs will denude oocytes close to ICSI insemination. Microinjection of oocytes is carried out on the heated stage of the microscope using Hoffman Modulation Contrast System (as the best fit for plastic dishes) at $200\times$ or $400\times$ magnification. The microscope is typically equipped with coarse

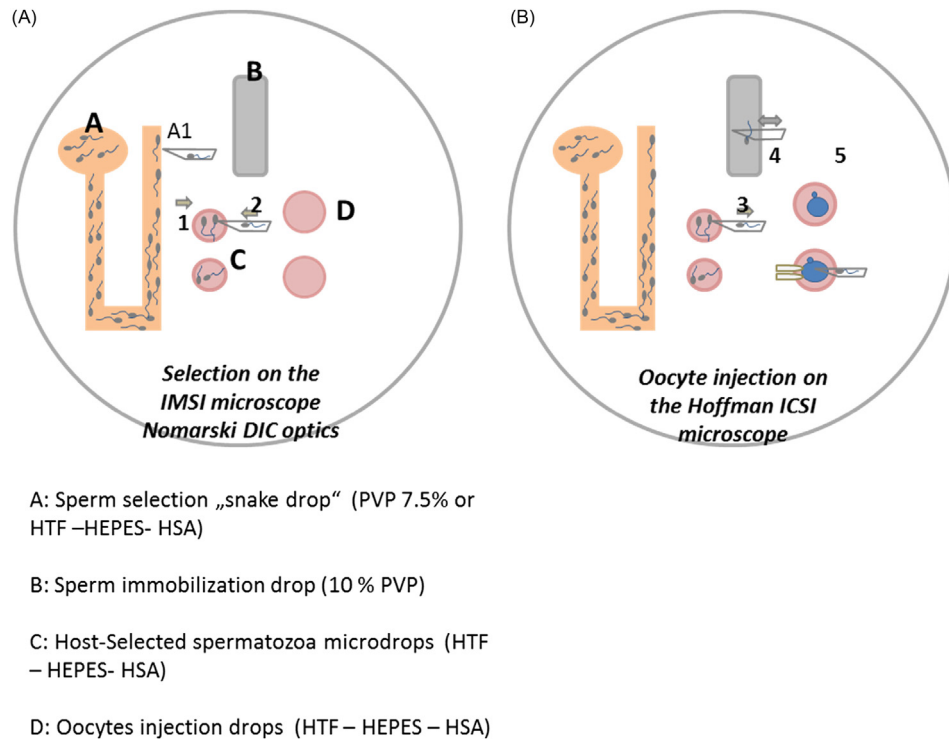


Fig. 1 Sperm selection with Nomarski DIC optics (2A) and oocyte Injection (2B) on the Hoffman ICSI microscope (2B).

positioning and hydraulic control (for fine movements) micro-manipulators, which are essential for microinjection (see Fig. 2). For ICSI, a single sperm is selected from the sperm-PVP drop of the ICSI dish (see Fig. 3) and aspirated in the injection pipette after breaking the sperm tail. Following, the oocyte is visualized and secured with the holding pipette, while the ICSI pipette is pushed through the zona pellucida, inside the cytoplasm, until membrane is broken, then the sperm is released inside the oocyte (see Fig. 4). For a more detailed description of ICSI set up and ICSI procedure, please, see publication from Joris et al. (1998).

Timing and process of IMSI insemination is somewhat different from ICSI insemination, as sperm selection takes longer. When several IMSI have to be performed, especially with severe oligo-teratozoospermia, the selection of spermatozoa and injection are performed on two different microscopes. On one microscope, that is equipped with Nomarski DIC optic, morphologically normal spermatozoa are first selected and isolated in micro-drops. Then oocyte injection is performed afterwards on the second microscope that is equipped with Hoffman modulation contrast (see Fig. 2). Alternatively, selection and injection of maximum two oocytes at a time are performed exclusively on the IMSI platform (using only one microscope). This approach is preferred when a previous MSOME (motile sperm organelle morphology examination) showed sufficient morphologically good quality semen so that the selection process would be less time consuming.

Sperm Selection for ICSI and IMSI

Sperm for ICSI is selected on the inverted microscope using $200\times$ or $400\times$ magnification, immediately right before performing microinjection. Most critically, a motile sperm should always be selected for ICSI (the only exception is when sperm has to be recovered from testicular tissue). It has been demonstrated that selecting/using nonmotile sperm for ICSI has a strong negative impact on outcomes, while any other basic sperm parameters have much less influence on results (Nagy et al., 1995). In the last decade, there have been several studies that aimed to improve results of ICSI using different methods of sperm selection. Those investigations include: annexin V magnetic activated cell separation (MACS), glass wool filtration; electrophoresis (surface charge based selection); hyaluronic acid binding (sperm “maturity” assessed by membrane characteristics). For details on the different sperm selection techniques, please, refer to the following reviews (Said and Land, 2011; Rappa et al., 2016).

Sperm selection for IMSI is based on high magnification evaluation of sperm morphology. Sperm morphology can be best assessed after fixing and staining the sperm cells (as it is done for semen evaluation), however, those sperm can no longer be used for microinjection. In order to counteract the problem of morphological evaluation on stained spermatozoa or with complex and high-tech systems; Bartoov (Bartoov et al., 2001) introduced in 2001 a new innovative noninvasive technique for a more precise morphological evaluation of motile spermatozoa in real time. With the use of Nomarski differential interference contrast optics (DIC), the so-called “motile-sperm organelle-morphology examination” (abbreviated as MSOME) changed the perception of how a viable spermatozoon suitable for injection should appear. The morphological characteristics of motile sperm nucleus



Fig. 2 Typical ICSI microscope (inverted), equipped with two coarse positioning manipulators (3D Motor Driven Coarse control Manipulator) and with two three-dimensional hydraulic remote-control micromanipulators. The holding and injection pipettes are fitted to a tool holder and are connected by Teflon tubing to a micrometer-type microinjector. The center of the microscope stage is heated to maintain physiological conditions during ICSI procedure.

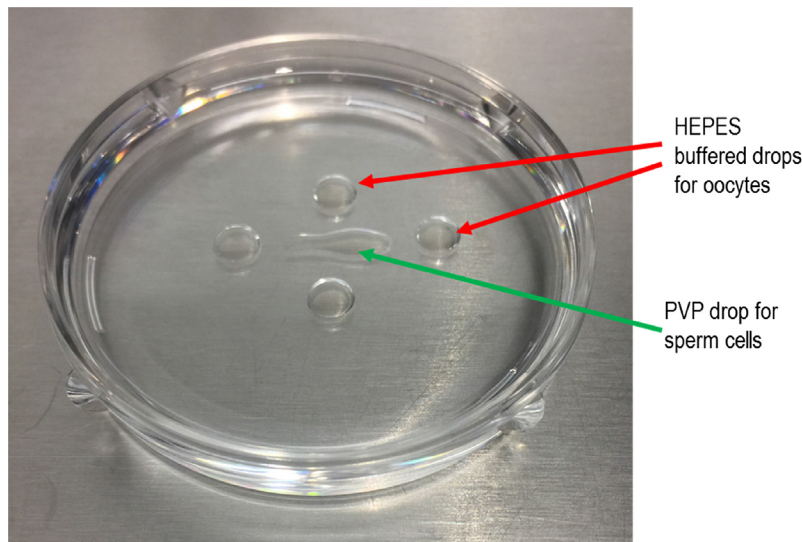


Fig. 3 Typical ICSI dish. Sperm is placed to the center drop (3–5 μL), which contains polyvinylpyrrolidone to slow down the sperm motility. The four side drops are HEPES buffered embryo culture drops (5–10 μL), where oocytes are placed. The drops are covered with paraffin oil to prevent evaporation from the drops.

according to the shape and chromatin content was defined. Particularly the fine morphology of the sperm head proved to be of major interest, namely fine nuclear abnormalities visible as craters, hollows or concavities which were defined as vacuole-like structures (VLS) are better detected and were identified as an abnormal, “thumbprint”-like nuclear concavity conferring an indirect representation of a pathological situation. Morphologically normal spermatozoa (class I) are selected using glass bottom dishes on an inverted light microscope equipped with Nomarski differential interference contrast optics (DIC), with dry or immersion $63\times$ or $100\times$ magnification objectives lens (corresponding to $630\times$ or $1000\times$ magnification in contrast to the $200\times$ or $400\times$ magnification with the “traditional” ICSI).

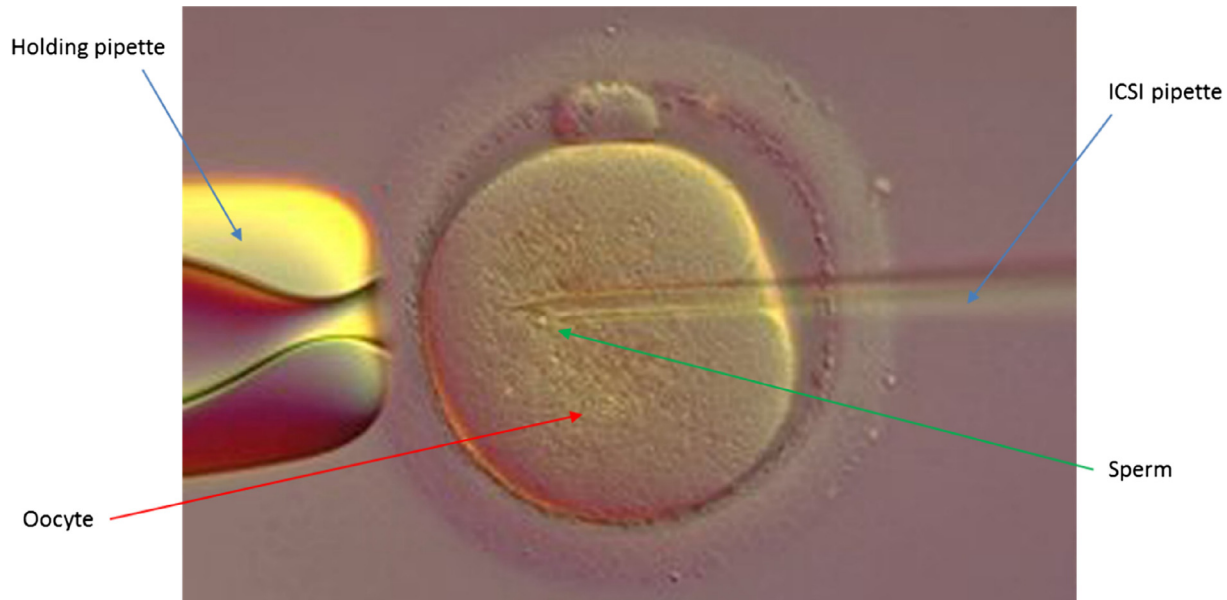


Fig. 4 ICSI procedure. The sperm is delivered deep inside the cytoplasm of the oocyte by the ICSI injection pipette, after passing through the zona pellucida and breaking the oolemma. Oocyte is kept in place by the holding pipette during the ICSI procedure.

Molecular Aspects of Vacuole Like Structure (VLS): Tangible Arguments to Select Sperm Free of Nuclear Defects

Since the implementation MSOME to assess sperm morphology, we are more anxious about the presence of large VLS and consider them as potential defects (Boitrelle et al., 2011). One crucial question to investigate is if VLS are the morphological manifestation of nuclear dysfunction. Undeniably, sperm carrying VLS were found to be related to nuclear dysfunction in terms of lower mitochondrial potential, loss of DNA integrity, higher aneuploidy rate and consistently related with chromatin condensation problem (Boitrelle et al., 2011; Perdrix et al., 2011). Anomalies of sperm chromatin packaging and abnormal chromatin remodeling during sperm maturation may impact DNA methylation levels. Indeed, the importance of re-packaging of the haploid genome before delivery in the oocyte is a crucial step for the establishment and maintenance of a viable pregnancy. By analyzing sperm DNA methylation and RNA transcripts in spermatozoa, a recent study showed the paternal contribution and the crucial role of sperm epigenetic in embryonic development (Kumar et al., 2013). Even though the indications for MSOME and the usefulness of IMSI are still under debate, the importance of selecting normal sperm free of VLS becomes obvious if epigenetic defects could be related with the presence of VLS. Epigenetic modifications in mature spermatozoa play an important role and may alter methylation profiles with the risk of fertilization failure, dysfunction of embryogenesis, preterm birth, low birth weight, congenital anomalies, autism, perinatal mortality (Schagdarsurengin et al., 2012). These altered methylation errors are subsequently transmitted to the embryo, conferring a potential risk of imprinting disorders to the offspring and for the next generation. It has also been observed that DNA methylation levels are significantly lower in normal spermatozoa compared with the group of spermatozoa carrying VLS. Out of these preliminary studies, we may assume that hypermethylation, source of epigenetic defects could be related with sperm morphology and presence of VLS.

Laboratory and Clinical Outcomes With ICSI Insemination

It has been demonstrated from the beginning, that ICSI insemination offers outstanding clinical and laboratory outcomes for patients who have male-factor infertility and/or had failed conventional insemination/IVF. Because of the excellent results with ICSI, intracytoplasmic sperm injection was fast adopted for routine use across the world. Furthermore, ICSI insemination today is used much more frequently than conventional/IVF insemination, and there are a number of IVF centers, where ICSI is the only technique used for insemination (to avoid occasional fertilization failure after conventional insemination, which can happen, even if sperm parameters are in the normal range). It has been demonstrated, that using ICSI, none of the basic sperm parameters have strong influence to the outcomes (Nagy et al., 1995), with the only exception of necrozoospermia (in which cases testicular biopsy is recommended to recover living sperm). The only other condition when ICSI results may be impaired is in the cases of total globozoospermia (or round-headed spermatozoa, which is a rare type of teratozoospermia characterized with multiple structural abnormalities, including the lack of acrosome). While ICSI is a more secure way than conventional insemination to obtain fertilization, — occasional fertilization failure can happen with ICSI too. In a study, analyzing 2732 ICSI cycles, total fertilization failure was observed in 76 cases (2.8%), however, most of those cases had an extreme low number of oocytes obtained, or immotile sperm or round-headed sperm (thus fertilization failure may not be because of the technique itself) (Liu et al., 1995).

Obstetrical and perinatal outcomes after ICSI have not raised concerns according to most studies published on the topic—despite that initially there were questions raised on intrinsic risks due to the technique. One of the argument was that ICSI is an “invasive” procedure which can damage the oocyte or some of its organelles (like the spindle) resulting either in degeneration of the oocyte, or possibly abnormal embryo development (or causing chromosomal alterations). A potentially confounding factor is that ICSI when used in male-factor infertility, the patient population itself may contribute to certain conditions observed in offspring. One example is when son(s) of fathers with strongly impaired sperm parameters are much more likely to have decreased sperm values as well.

When examining the incidence of fetal chromosomal anomalies, most studies observed a slightly (but significantly) higher rate of abnormalities (Gjerris et al., 2008). It is mostly agreed, that the slightly higher rate of prenatal chromosome anomalies after ICSI is likely related to the patient population requiring ICSI, and not due to the technique itself (Bonduelle et al., 2002).

Incidence of major malformation on children born after ICSI showed no difference compared to what was observed in the IVF or in the general population (Bonduelle et al., 1996). In a prospective study, a 2.6% rate of major congenital malformations was reported (23 out of 877 children born) (Bonduelle et al., 1996), however, some other studies observed a somewhat higher frequency of hypospadias and cryptorchidism (Massaro et al., 2015). More recent studies analyzing high number of patients, and also meta-analyses have not shown a meaningful increase in the incidence of birth defects of “ICSI children” (Yan et al., 2011; Lie et al., 2005), again suggesting, that the increased risk, if any, it is more likely being associated with the patient population and not the technique itself. Some studies looking at development and health of ICSI children at early ages, observe some differences in the health of children, but this is likely connected to the age (and other health/medical conditions) of the woman (and man) receiving the ICSI treatment (Belva et al., 2007; Fauser et al., 2014).

Regarding clinical outcomes following IMSI, there is not much information in the literature, as IMSI is a much more recently developed technique, and it is also not used very frequently. However, it is important to emphasize that animal data are absolutely unequivocal on this point and clearly indicate that DNA damage in the male germ line is potentially hazardous for the embryo and therefore for the resulting offspring. According to a recently published paper, sperm nucleus morphological normalcy, assessed at high magnification, could decrease the prevalence of de novo major fetal malformations in ICSI children (Cassuto et al., 2014).

ICSI or IMSI?—Potential Indications for IMSI

One of the most essential questions is not under which technical conditions the selection of spermatozoa should be recommended but rather if we have to consider to select the best morphologically normal spermatozoa and, if possible, excluding those carrying defects. But, for different reasons, there is undeniable evident skepticism about this method of spermatozoa selection. Originally, the platform to perform MSOME and IMSI was provided not only with an inverted microscope equipped with Nomarski differential interference contrast Optics and condenser, but, in addition, with a high definition digital video camera and a variable zoom lens that magnify the microscopic image up to 12,000× magnification. With such technical approach, a lot of professionals from the ART world claimed that IMSI is a nightmare because it is expensive, complicated to perform, time consuming, and finally for no specific indication. At the present, the easiest and most convenient way to perform MSOME in combination with ICSI is to use only an inverted light microscope equipped with Nomarski differential interference contrast optics, at 63× or 100× magnification objectives lens. After several years, the superiority of IMSI over ICSI is still a matter of debate and even to date randomized and well-powered studies to confirm a benefit of IMSI are limited and even depict conflicting results (Perdrix and Rives, 2013).

One of the main benefits of IMSI is the higher rate of blastocysts obtained per cycle when morphologically good quality spermatozoa are selected (Knez et al., 2013). Nowadays, with successful verification techniques, the full benefit of a better spermatozoa selection is highlighted, if we are able to produce more blastocyst per cycle giving the chance for the patient to achieve a pregnancy in successive vitrified-warmed embryo transfer cycle(s). It has also already shown that IMSI improves reproductive outcomes in cases of male factor infertility and/or previous failed ICSI attempts in terms of implantation and clinical pregnancy rates as compared with conventional ICSI (Nadalini et al., 2009). On the other hand, IMSI and conventional ICSI seemed to provide comparable laboratory and clinical results when an unselected infertile population was evaluated (De Vos et al., 2013). Semen quality might be more important with advancing maternal reproductive age,—a group of patients that have important relevance in ART. If we assume that a majority of nuclear defects reflected in vacuoles may be correlated with DNA integrity, we may have a more optimistic view in the sense that this damage brought into the zygote by the fertilizing spermatozoon may be effectively repaired by oocyte factors from young and/or good-quality oocytes (Gunes et al., 2015). Even though oocytes can repair sperm DNA damage; a threshold exists beyond which sperm DNA cannot be repaired. This mainly depends on the degree and the type of sperm DNA damage (single-stranded versus double-stranded damage). Also, oocytes whose DNA repair mechanisms are not functional (aging oocytes—in vitro culture conditions) or that have been damaged by endogenous (e.g., free radicals) or exogenous (e.g., radiation, environmental toxicants) factors might not be able to repair this damage. This is a strong argument in favor of an accurate sperm selection when we have to inseminate oocytes obtained from patients with advanced reproductive age.

Summary

Intracytoplasmic sperm injection (ICSI) was successfully introduced in the clinical practice in 1992, to overcome male-factor infertility and in failed conventional IVF cases. Today ICSI is used across the world in most IVF cases, for almost all indications of

infertility. ICSI has demonstrated highly consistent outcomes at the level of fertilization (frequently more reliable than conventional insemination), embryo development, and obtaining high rates of implantations and pregnancies. Importantly, it was also demonstrated, that ICSI outcomes are not influenced importantly by traditional sperm parameters, thus great results can be achieved even in the most extreme cases of male factor infertility, where virtual azoospermia or total astheno- or total teratozoospermia was diagnosed in the initial semen sample (from the ejaculate). Only one condition had a strongly negative influence on the result of ICSI, where immotile (presumably dead) spermatozoon was injected into the oocyte. Additionally, ICSI can be applied successfully also when fresh or frozen-thawed epididymal and testicular spermatozoa are used.

Recent studies indicated, that ICSI outcomes may be further improved if the sperm selected for microinjection is examined under very high magnification for morphological characteristics. This lead to the introduction of IMSI which provided the advantage for embryologists to realize that more attention has to be paid during sperm selection even in case of classical ICSI. The application of IMSI leads to more and potentially higher quality embryos/blastocysts and as a consequence it increases the chance to select the proper embryo for transfer with the highest implantation potential. The full benefit of using MSOME as an additional tool to ICSI procedure manifests itself when it is performed in combination with extended embryo culture of all fertilized oocytes.

Seeing that this simple, noninvasive technique still arises debates and skepticism exists about its efficiency, mainly due to a low number of controlled randomized studies published yet, one fundamental question is whether we should—with the knowledge that sperm vacuoles are related with abnormal chromatin packaging and possibly with DNA fragmentation—select spermatozoa with these defects for injection if we have only to change the optics? As far as we know, there is no reason for not selecting the morphologically best spermatozoa.

References

- Barthov, B., Berkovitz, A., & Eltes, F. (2001). Selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. *The New England Journal of Medicine*, 345(14), 1067–1068.
- Belva, F., Henriot, S., Liebaers, I., Van Steirteghem, A., Celestin-Westreich, S., & Bonduelle, M. (2007). Medical outcome of 8-year-old singleton ICSI children (born > or = 32 weeks' gestation) and a spontaneously conceived comparison group. *Human Reproduction*, 22(2), 506–515.
- Boitrelle, F., Ferfour, F., Petit, J. M., Segretain, D., Tourain, C., Bergere, M., et al. (2011). Large human sperm vacuoles observed in motile spermatozoa under high magnification: Nuclear thumbprints linked to failure of chromatin condensation. *Human Reproduction*, 26(7), 1650–1658.
- Bonduelle, M., Wilkens, A., Buysse, A., Van Assche, E., Wisanto, A., Devroey, P., et al. (1996). Prospective follow-up study of 877 children born after intracytoplasmic sperm injection (ICSI), with ejaculated epididymal and testicular spermatozoa and after replacement of cryopreserved embryos obtained after ICSI. *Human Reproduction*, 11(Suppl 4), 131–155. discussion 56–9.
- Bonduelle, M., Van Assche, E., Joris, H., Keymolen, K., Devroey, P., Van Steirteghem, A., et al. (2002). Prenatal testing in ICSI pregnancies: Incidence of chromosomal anomalies in 1586 karyotypes and relation to sperm parameters. *Human Reproduction*, 17(10), 2600–2614.
- Cassuto, N. G., Hazout, A., Bouret, D., Balet, R., Larue, L., Benifla, J. L., et al. (2014). Low birth defects by deselecting abnormal spermatozoa before ICSI. *Reproductive Biomedicine Online*, 28(1), 47–53.
- Clarke, G. N. (2006). A.R.T. and history, 1678–1978. *Human Reproduction*, 21(7), 1645–1650.
- De Vos, A., Van de Velde, H., Bocken, G., Eysenbosch, G., Franceus, N., Meersdom, G., et al. (2013). Does intracytoplasmic morphologically selected sperm injection improve embryo development? A randomized sibling-oocyte study. *Human Reproduction*, 28(3), 617–626.
- Fausser, B. C., Devroey, P., Diedrich, K., Balaban, B., Bonduelle, M., Delemarre-van de Waal, H. A., et al. (2014). Health outcomes of children born after IVF/ICSI: A review of current expert opinion and literature. *Reproductive Biomedicine Online*, 28(2), 162–182.
- Gjerris, A. C., Loft, A., Pinborg, A., Christiansen, M., & Tabor, A. (2008). Prenatal testing among women pregnant after assisted reproductive techniques in Denmark 1995–2000: A national cohort study. *Human Reproduction*, 23(7), 1545–1552.
- Gunes, S., Al-Sadaan, M., & Agarwal, A. (2015). Spermatogenesis, DNA damage and DNA repair mechanisms in male infertility. *Reproductive Biomedicine Online*, 31(3), 309–319.
- Joris, H., Nagy, Z., Van de Velde, H., De Vos, A., & Van Steirteghem, A. (1998). Intracytoplasmic sperm injection: Laboratory set-up and injection procedure. *Human Reproduction*, 13(Suppl 1), 76–86.
- Knez, K., Tomazevic, T., Vrtacnik-Bokal, E., & Virant-Klun, I. (2013). Developmental dynamics of IMSI-derived embryos: A time-lapse prospective study. *Reproductive Biomedicine Online*, 27(2), 161–171.
- Kumar, M., Kumar, K., Jain, S., Hassan, T., & Dada, R. (2013). Novel insights into the genetic and epigenetic paternal contribution to the human embryo. *Clinics (São Paulo, Brazil)*, 68(Suppl 1), 5–14.
- Lie, R. T., Lyngstadaas, A., Orstavik, K. H., Bakkevig, L. S., Jacobsen, G., & Tanbo, T. (2005). Birth defects in children conceived by ICSI compared with children conceived by other IVF-methods; a meta-analysis. *International Journal of Epidemiology*, 34(3), 696–701.
- Liu, J., Nagy, Z., Joris, H., Tournaye, H., Smits, J., Camus, M., et al. (1995). Analysis of 76 total fertilization failure cycles out of 2732 intracytoplasmic sperm injection cycles. *Human Reproduction*, 10(10), 2630–2636.
- Massaro, P. A., MacLellan, D. L., Anderson, P. A., & Romao, R. L. (2015). Does intracytoplasmic sperm injection pose an increased risk of genitourinary congenital malformations in offspring compared to in vitro fertilization? A systematic review and meta-analysis. *The Journal of Urology*, 193(5 Suppl), 1837–1842.
- Nadalini, M., Tarozzi, N., Distratis, V., Scaravelli, G., & Borini, A. (2009). Impact of intracytoplasmic morphologically selected sperm injection on assisted reproduction outcome: A review. *Reproductive Biomedicine Online*, 19(Suppl 3), 45–55.
- Nagy, Z. P., Liu, J., Joris, H., Verheyen, G., Tournaye, H., Camus, M., et al. (1995). The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Human Reproduction*, 10(5), 1123–1129.
- Palermo, G., Joris, H., Devroey, P., & Van Steirteghem, A. C. (1992). Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, 340(8810), 17–18.
- Palermo, G. D., Neri, Q. V., & Rosenwaks, Z. (2015). To ICSI or not to ICSI. *Seminars in Reproductive Medicine*, 33(2), 92–102.
- Perdrix, A., & Rives, N. (2013). Motile sperm organelle morphology examination (MSOME) and sperm head vacuoles: State of the art in 2013. *Human Reproduction Update*, 19(5), 527–541.
- Perdrix, A., Travers, A., Chelli, M. H., Escalier, D., Do Rego, J. L., Milazzo, J. P., et al. (2011). Assessment of acrosome and nuclear abnormalities in human spermatozoa with large vacuoles. *Human Reproduction*, 26(1), 47–58.
- Rappa, K. L., Rodriguez, H. F., Hakkarainen, G. C., Anchan, R. M., Mutter, G. L., & Asghar, W. (2016). Sperm processing for advanced reproductive technologies: Where are we today? *Biotechnology Advances*, 34(5), 578–587.

- van Rumste, M. M., Evers, J. L., Farquhar, C. M., & Blake, D. A. (2000). Intra-cytoplasmic sperm injection versus partial zona dissection, subzonal insemination and conventional techniques for oocyte insemination during in vitro fertilization. *Cochrane Database of Systematic Reviews*, 2, CD001301.
- Said, T. M., & Land, J. A. (2011). Effects of advanced selection methods on sperm quality and ART outcome: A systematic review. *Human Reproduction Update*, 17(6), 719–733.
- Schagdarsurengin, U., Paradowska, A., & Steger, K. (2012). Analyzing the sperm epigenome: Roles in early embryogenesis and assisted reproduction. *Nature Reviews. Urology*, 9(11), 609–619.
- Step toe, P. C., & Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. *Lancet*, 2(8085), 366.
- Van der Zwalmen, P., Bertin-Segal, G., Geerts, L., Debauche, C., & Schoysman, R. (1991). Sperm morphology and IVF pregnancy rate: Comparison between Percoll gradient centrifugation and swim-up procedures. *Human Reproduction*, 6(4), 581–588.
- Yan, J., Huang, G., Sun, Y., Zhao, X., Chen, S., Zou, S., et al. (2011). Birth defects after assisted reproductive technologies in China: Analysis of 15,405 offspring in seven centers (2004 to 2008). *Fertility and Sterility*, 95(1), 458–460.

In Vitro Maturation of Oocytes

Roger Hart, University of Western Australia, Perth, WA, Australia; and Fertility Specialists of Western Australia, Claremont, WA, Australia

Melanie Walls, Fertility Specialists of Western Australia, Claremont, WA, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

In the process of in vitro maturation of oocytes (IVM) oocytes are collected from a patient in an immature form, in a procedure similar to a standard in vitro fertilization (IVF) cycle oocyte retrieval procedure. In some instances, only a short duration of ovarian stimulation is performed and the immature oocytes collected are matured in the laboratory.

IVM was first performed in a human model, after animal experiments, by Bob Edwards in 1965. Oocytes were collected from unstimulated follicles after ovarian biopsy and spontaneously matured, and the first live birth was recorded in 1991, after oocyte collection of oocytes from unstimulated ovaries and culture in follicular fluid (Cha et al., 1991). The first birth subsequent to IVM after transvaginal oocyte retrieval occurred in 1994 (Trounson et al., 1994). However since these early successes with IVM were not been generally embraced by reproductive medicine physicians, perhaps due to the perceived poor success rates of the procedure, perceived difficulties in cycle management, and the challenging laboratory processes involved; however with improvements in the protocols excellent pregnancy rates began to be achieved (Junk and Yeap, 2012). However a consistent challenge has been the inability to achieve satisfactory endometrial preparation, hence many groups transferred multiple embryos, and other adopted an embryo freezing approach, to try to maximize pregnancy rates (Walls et al., 2015b).

In view of the increased safety and success rates of IVF in recent years the place of IVM as a fertility treatment has been questioned. The purported benefits of IVM are; that it avoids (or significantly reduces) the amount of drugs used for ovarian stimulation, oocytes can be collected earlier in a menstrual cycle for a woman who requires to rapidly proceed to a cycle of chemotherapy treatment, it completely avoids the risk of ovarian hyperstimulation syndrome (OHSS) (a serious cause of morbidity for a woman undergoing IVF), and for a woman with an estrogen sensitive tumor or a significant pro-thrombotic tendency it avoids the risk of high concentrations of oestradiol (Walls et al., 2015b).

Clinical Place of IVM

The ideal patient for an IVM protocol is a young woman with a high antral follicle count, such as found in a woman with polycystic ovary syndrome (PCOS); as such women are particularly at risk of OHSS, and IVM avoids this risk completely. Similarly, a young woman about to embark on cancer treatment which may compromise her subsequent fertility, particularly if she has an estrogen sensitive tumor, is ideally suited to undergo IVM treatment; as it avoids the high oestradiol concentrations found in standard IVF, it is a rapid treatment and enables a quick progression into oncology treatment, whilst enabling fertility preservation. Furthermore the laboratory process of IVM can be employed when a young woman undergoes oophorectomy for malignancy, ideally after a few days of follicular stimulating hormone (FSH); whereby immature oocytes can be retrieved in the laboratory enabling IVM and oocyte cryopreservation (Walls et al., 2015a).

Stimulation Protocols

Various approaches to the process of IVM stimulation protocols have been employed and documented and extensively debated in the literature (Dahan et al., 2016). Protocols differ with respect to the need for ovarian stimulation with FSH prior to oocyte retrieval; to ensure a cohort of follicles starts to develop until the lead follicle is 10–12 mm in diameter at the time of follicular aspiration, versus the “natural cycle” approach where follicular aspiration is performed without the use of any stimulation. Furthermore, there exists further debate as to the requirement to administer to the patient a “trigger” injection prior to follicular aspiration. Whilst the use of the trigger is vigorously debated (Dahan et al., 2016), the only randomized controlled trial of the use of vs not use of a trigger injection did not demonstrate any significant differences in embryological or clinical outcomes (Zheng et al., 2012). Our protocol is to commence ovarian stimulation with a few days of 150iu of FSH, aspirate of follicles at a maximum diameter of 12 mm, without the use of a trigger injection, using a double lumen needle, and the maturation of the oocytes in the laboratory. In a subsequent frozen embryo transfer cycle, a single blastocyst will be transferred, and has led to clinical pregnancy rates that are the same as those in a standard IVF cycle (Walls et al., 2015b).

Laboratory Procedures

Oocyte Collection and Maturation

Oocyte collection for IVM is a modified version of a standard egg collection procedure; however, clinicians need to be specially trained to puncture follicles of a much smaller size. Both double and single lumen needles can be used and the follicles may be solely aspirated and/or the follicles can be flushed to maximize oocyte yield (Walls et al., 2015a). Flushing solutions can comprise



Fig. 1 An immature germinal vesicle oocyte (GV).

of buffered cell culture media (such as Hepes), Hartmann's supplemented with heparin or commercially available, specialized flushing solutions. Aspiration pressure has been reported from 7 kpa (52.5 mmHg) to 200 mmHg. Immature germinal vesicle oocytes (GVs) can be identified by sight, by specially trained embryologists (Junk and Yeap, 2012) or the aspirates can be filtered through a mesh strainer. Due to their very small size and lack of cumulus oophorus, oocyte identification is more difficult in an IVM collection procedure compared to a standard IVF TVOA. Removal of the GV stage oocyte from the follicle leads to a decrease in gap junction communication between the coronal cells and the oocyte. In turn, this results in and a dramatic decrease in cyclic adenosine monophosphate (cAMP) activity and the potential for spontaneous progression to the metaphase II (MII) stage. Spontaneous maturation can be problematic as nuclear maturation does not necessarily reflect cytoplasmic maturation, resulting in asynchrony between these two crucial events. This can effect downstream progression, including successful fertilization and ongoing embryo development (Figs. 1 and 2).

As with standard embryo culture, a range of media have been employed for IVM maturation culture. There are currently two commercially available IVM media, Sage (Cooper Surgical, USA) and Medicult (Origio, Denmark) and blastocyst culture media has also been reported with high success rates (Junk and Yeap, 2012). Regardless of the base media composition, hormonal additives recombinant luteinizing hormone (LH) and human chorionic gonadotrophin (hCG) are currently an essential part of maturation culture. FSH and LH mediate oocyte maturation by acting on granulosa cell receptors to induce the intracellular rise in cAMP activity within the oocyte and the subsequent cAMP cascade promotes the resumption of meiosis and germinal vesicle breakdown. Reported LH concentrations range from 0.1 IU/mL to 0.75 IU/mL and FSH from 0.075 IU/mL or 0.1 IU/mL.

Culture media additives

Other media additives including epidermal growth factor (EGF) and insulin like growth factor (IGF-I), (Gomez et al., 1993), activin (Alak et al., 1998), and meiosis activating sterols (MAS) (Smits et al., 2007) have been reported with varying success and are rarely used in routine IVM culture.

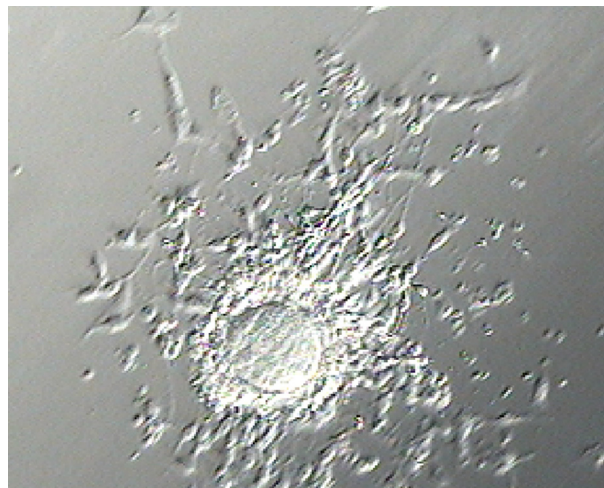


Fig. 2 In standard IVF—the oocyte is more readily identified than the oocyte in Fig. 1.



Fig. 3 Mature oocyte with expansion of the corona radiate.

Protein source in culture

Protein additives for IVM culture media include human follicular fluid (HFF) in concentrations ranging from 30% to 70% or more commonly, heat inactivated patient serum (maternal serum), ranging in concentrations from 10% to 20%. There have been reports of the use of fetal bovine serum (FBS) as concentrations of 10% or 20%; however, this is not advisable due to obvious ethical implications. The use of each of these additives carries with it the risk (however remote) of contributing contaminants to the embryo culture system and unknown concentrations of both positive and negative elements, which do not undergo rigorous quality assurance testing, unlike commercially available supplements and media.

Culture Timing

Following collection and transport to the laboratory, immature oocytes are cultured for timeframes between 24 and 40 h. Additionally, if a hCG trigger is used, culture times may vary to allow for insemination of oocytes according to the stages at which timing at which they are deemed mature. Following the designated period of maturation culture, oocytes should display expansion of the corona radiata (Fig. 3).

Fertilization and Embryo Culture

Fertilization in IVM has traditionally been performed using ICSI to overcome the potential for zona pellucida hardening. This is thought to be due to extended time in maturation culture and subsequently, lower fertilization rates occur; however, there is also evidence from a small sibling oocyte study suggesting fertilization rates to be comparable following a short 24 h maturation protocol (Walls et al., 2012).

Embryo culture following normal fertilization should not differ to that of standard IVF and continue according to the treating clinic's embryo culture protocol. Most IVM protocols employ cleavage stage culture and transfer/freezing; however, high rates of success have more recently been reported following day five to six, blastocyst culture (Junk and Yeap, 2012). There is some evidence to suggest that IVM derived embryos have a higher rate of early embryo arrest (Roesner et al., 2017) and therefore, blastocyst culture would allow for the selection of the most appropriate embryo for transfer and/or freezing for use in a frozen cycle.

Transfer and Cryopreservation

A freeze all approach is recommended in IVM protocols as implantation and live birth rates are significantly improved following frozen embryo transfers, as well as significantly lower rates of early miscarriage (Walls et al., 2015b). However, in fresh transfer cycles, endometrial priming with at least 6 days of estrogen initiated 2 days prior to TVOA and progesterone supplementation from the day of TVOA until the pregnancy test date, can also achieve high rates of implantation and ongoing pregnancy (Junk and Yeap, 2012).

Success Rates

Clinical pregnancy and live birth rates vary significantly between clinics and in the literature. Results are often difficult to compare due to variations in clinical protocols; however, using a freeze-all approach for IVM cycles implantation and live birth rates similar to standard IVF can be achieved, with similar long term outcomes for the children born (Shu-Chi et al., 2006).

Future Directions

The identification of oocyte secreted factors GDF9 and BMP-15 is an emerging area of IVM research (Mottershead et al., 2012) as well as cAMP modulators cilostamide and forskolin, as well as 3-isobutyl-1-methylxanthine (IBMX) which has shown to have no adverse implications for embryo chromosomal aneuploidy rates (Spits et al., 2015). Further large-scale trials into its effect on

human IVM success rates are required. Continued research into these factors may prove to be beneficial for IVM, should they prove to be successful culture media additives.

Conclusion

Several methodologies have been implemented in recent years to reduce the risk of OHSS; these include the administration of metformin, employing antagonist protocols and dopamine agonists (Cabergoline). Regardless, IVM is a safer alternative to traditional IVF, especially in a PCOS patient population due the complete elimination of the risk of OHSS. Additionally, IVM has the potential to treat a broader patient base including for fertility preservation and FSH resistant ovaries. While the research reporting on neonatal outcomes and developmental indicators have been positive (Shu-Chi et al., 2006), there is still a clear need for more research into the long-term outcomes for children born following IVM as well as large scale, multicentre, randomized control trials to more appropriately address the success rates of IVM compared to standard IVF treatment. Until that time, IVM should remain classified as an experimental treatment option.

References

- Alak, B. M., Coskun, S., Friedman, C. I., Kennard, E. A., Kim, M. H., & Seifer, D. B. (1998). Activin A stimulates meiotic maturation of human oocytes and modulates granulosa cell steroidogenesis in vitro. *Fertility and Sterility*, 70, 1126–1130.
- Cha, K. Y., Koo, J. J., Ko, J. J., Choi, D. H., Han, S. Y., & Yoon, T. K. (1991). Pregnancy after in vitro fertilization of human follicular oocytes collected from nonstimulated cycles, their culture in vitro and their transfer in a donor oocyte program. *Fertility and Sterility*, 55, 109.
- Dahan, M. H., Tan, S. L., Chung, J., & Son, W.-Y. (2016). Clinical definition paper on in vitro maturation of human oocytes. *Human Reproduction*, 31, 1383–1386.
- Gomez, E., Tarin, J., & Pellicer, A. (1993). Oocyte maturation in humans: The role of gonadotropins and growth factors. *Fertility and Sterility*, 60, 40–46.
- Junk, S. M., & Yeap, D. (2012). Improved implantation and ongoing pregnancy rates after single-embryo transfer with an optimized protocol for in vitro oocyte maturation in women with polycystic ovaries and polycystic ovary syndrome. *Fertility and Sterility*, 98, 888–892.
- Mottershead, D. G., Ritter, L. J., & Gilchrist, R. B. (2012). Signalling pathways mediating specific synergistic interactions between Gdf9 and Bmp15. *Molecular Human Reproduction*, 18, 121–128.
- Roesner, S., Dietrich, J. E., Weigert, J., Montag, M., Toth, B., & Strowitzki, T. (2017). Time-lapse imaging reveals differences in growth dynamics of embryos after in vitro maturation compared with conventional stimulation. *Fertility and Sterility*, 107, 606–612.E3.
- Shu-Chi, M., Jiann-Loung, H., Yu-Hung, L., Tseng-Chen, S., Ming-I, L., & Tsu-Fuh, Y. (2006). Growth and development of children conceived by in vitro maturation of human oocytes. *Early Human Development*, 82, 677–682.
- Smitz, J., Picton, H. M., Platteau, P., Rutherford, A., Cortvrindt, R., Clyde, J., Nogueira, D., Devroey, P., Lyby, K., & Gröndahl, C. (2007). Principal findings from a multicenter trial investigating the safety of follicular-fluid meiosis-activating sterol for in vitro maturation of human cumulus-enclosed oocytes. *Fertility and Sterility*, 87, 949–964.
- Spits, C., Guzman, L., Mertzaniou, A., Jacobs, K., Ortega-Hrepich, C., Gilchrist, R. B., Thompson, J. G., De Vos, M., Smitz, J., & Sermon, K. (2015). Chromosome constitution of human embryos generated after in vitro maturation including 3-isobutyl-1-methylxanthine in the oocyte collection medium. *Human Reproduction*, 30, 653–663.
- Trounson, A., Wood, C., & Kausche, A. (1994). In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertility and Sterility*, 62, 353.
- Walls, M., Junk, S., Ryan, J., & Hart, R. (2012). IVF versus ICSI for the fertilization of in vitro matured human oocytes. *Reproductive Biomedicine Online*, 25, 603–607.
- Walls, M. L., Douglas, K., Ryan, J. P., Tan, J., & Hart, R. (2015a). In vitro maturation and cryopreservation of oocytes at the time of oophorectomy. *Gynecologic Oncology Reports*, 13, 79–81.
- Walls, M. L., Hunter, T., Ryan, J. P., Keelan, J. A., Nathan, E., & Hart, R. J. (2015b). In vitro maturation as an alternative to standard in vitro fertilization for patients diagnosed with polycystic ovaries: A comparative analysis of fresh, frozen and cumulative cycle outcomes. *Human Reproduction*, 30, 88–96.
- Zheng, X., Wang, L., Zhen, X., Lian, Y., Liu, P., & Qiao, J. (2012). Effect Of hCG priming on embryonic development of immature oocytes collected from unstimulated women with polycystic ovarian syndrome. *Reproductive Biology and Endocrinology*, 10, 40.

Analysis of Fertilization

Basak Balaban, Vehbi Koc Foundation American Hospital Istanbul, Assisted Reproduction Unit, Istanbul, Turkey

© 2018 Elsevier Inc. All rights reserved.

Introduction

Fertilization in general can be described as the event where the reproductive human cells; the haploid spermatozoa fuses with the fully grown and mature oocyte at the metaphase II stage of development to form a new entity which is the embryo. Sperm–oocyte interaction in vivo begins with the transport of spermatozoa through the female reproductive tract, many acrosome-intact spermatozoa reaching the mature oocyte within an hour. Only selected group of spermatozoa are capable of successfully penetrating oocytes and they need to undergo certain modifications in the female genital tract such as; capacitation, hyperactivation, and acrosome reaction. The last step acrosome reaction begins at the time of spermatozoa binding to the zona pellucida of the oocyte which may be induced in vivo by cumulus cells and follicular fluid. Fusion then occurs between sperm plasma membrane in the region of the equatorial segment and the oolemma. The spermatozoa becomes immotile at this point as it's incorporated into the oocyte, and all of the spermatozoon, including the sperm tail is fused into the ooplasm. Fertilization is completed with the sequential steps as; the activation of the oocyte with membrane fusions, exocytosis of the cortical granules to form the block to polysperm and prevent further sperm entry, and the activation of the metaphase II leading to the formation of the second polar body and the appearance of pronuclei with the complement of cytoplasmic syngamy, and nuclear syngamy events whereby the genomes of both gametes are merged to form a new embryonic genome, forming the zygote (Kupker et al., 1998).

Even though the analysis of normal and abnormal fertilization is inaccessible in natural conception cycles, the capability of observation of particular events by in vitro fertilization from the time of fusion until the complement of embryo development provides valuable information that would relate with healthy ongoing pregnancy and offspring. As abnormal fertilization in in vitro applications can give rise to nonviable embryos, the non-invasive assessments of zygote and embryos can be clinically relevant in routine practice of assisted reproduction techniques.

Fertilization Process by Assisted Reproduction

Conventional IVF (In Vitro Fertilization)

As the natural barriers of spermatozoon to penetrate through the cervical mucus and follow the genital tract to reach the oocyte is bypassed by in vitro fertilization less concentration of motile spermatozoa is needed for fertilization. Despite this necessity the semen samples are still treated with special preparation techniques, to select the most progressive spermatozoa, and to eliminate the spermatozoa with functional disorders.

ICSI (Intra-Cytoplasmic Sperm Injection)

Single spermatozoa mainly selected by morphological appearance is micro-injected into the cytoplasm of the mature oocyte at metaphase II stage for the ICSI procedure thus the essential steps for spermatozoa to bypass for successful fertilization such as progressive movement, integrity of sperm functions for zona pellucida and oolemma penetration and fusion with oolemma remains less important. As the barriers of natural selection of healthy, viable, and chromosomally normal spermatozoa are bypassed by the direct injection of spermatozoa within the oocyte, the selection of the single spermatozoon plays a critical role for ICSI cycles (Sakkas, 2013).

Contribution of the Oocyte Characteristics on Fertilization and Further Embryo Development In Vitro

Assessment of human metaphase II mature oocytes in in vitro fertilization is mainly performed by the examination of the morphological characteristics. Despite the fact that the morphology of oocytes is difficult to evaluate in IVF cases as the cumulus corona cells surrounding the oocyte would eliminate precise examination, ICSI procedures allow the assessment of various morphological variations of metaphase II oocytes that could correlate with its fertilization capacity, embryo quality and development as well as the clinical and neonatal outcome. Optimal oocyte morphology is defined as an oocyte with spherical structure enclosed by a uniform zona pellucida, with a uniform translucent cytoplasm free of inclusions and a size-appropriate polar body (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). However Metaphase II-stage (MII) oocytes retrieved from patients after ovarian stimulation are known to show significant morphological variations that may affect the developmental competence and implantation potential of the derived embryo. Morphological abnormalities of the oocyte can be observed under two different subgroups; extracytoplasmic abnormalities and cytoplasmic abnormalities (Balaban and Urman, 2006; Balaban and Ebner, 2014).

Cytoplasmic abnormalities of MII oocytes include, different types and degrees of cytoplasmic granulations (slightly diffused or excessive whole/centrally located granulation) and appearance of refractile bodies, smooth endoplasmic reticulum clusters (sERCs), or vacuolization in the ooplasm. (Figs. 1A, B and 2A, B).

Despite the fact that the various degrees of diffused granulation in the cytoplasm does not seem to affect the fertilization capacity, embryo development and pregnancy outcome, condensed granulation that is centrally located within the cytoplasm with a clear border might have detrimental effect on the fertilization rates, embryo quality and more importantly chromosomal normality. Decreased pregnancy rates with such embryos, as well as increased abortion rates, are reported (Fig. 3A and B).

Fluid filled large vacuoles within the cytoplasm have a significant detrimental effect on the fertilization capacity of the oocyte, with a cut-off value suggested $> 14 \mu\text{m}$ in diameter. Large vacuoles are assumed to negatively affect a large proportion of the cytoskeleton (e.g., microtubules) which cannot function as it's supposed to. In addition to the detrimental affect on fertilization rates, large vacuoles can also negatively affect the embryo quality, blastocyst formation as well as the euploidy rates of the embryos obtained (Fig. 4A and B).

Presence of translucent vacuole like structures called as smooth endoplasmic reticulum clusters (sERC) are known to negatively affect the neonatal safety of the offspring and thus their utilization for fertilization in vitro is a continuous debate (Figs. 5A, B and 6A, B).

A variety of extracytoplasmic anomalies exist which in part negatively influence fertilization (consistency and thickness of the zona, polar body (Pb)1 decay and debris within the perivitelline space (PVS), blastulation (thickness of zona, Pb1 fragmentation), and pregnancy (thickness of zona, Pb1 morphology, debris in PVS). Characteristics of the zona pellucida and the PVS are most

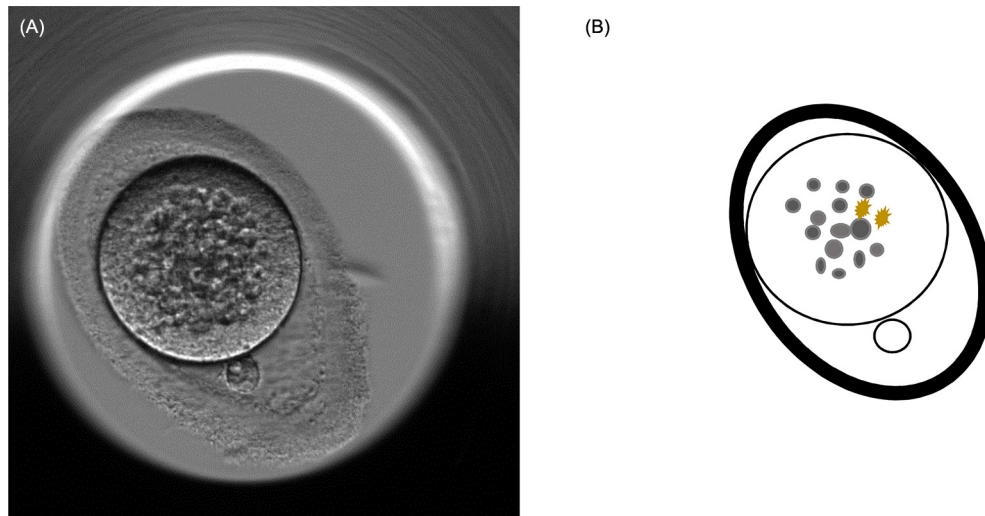


Fig. 1 (A and B) Centrally located granulation and refractile bodies.

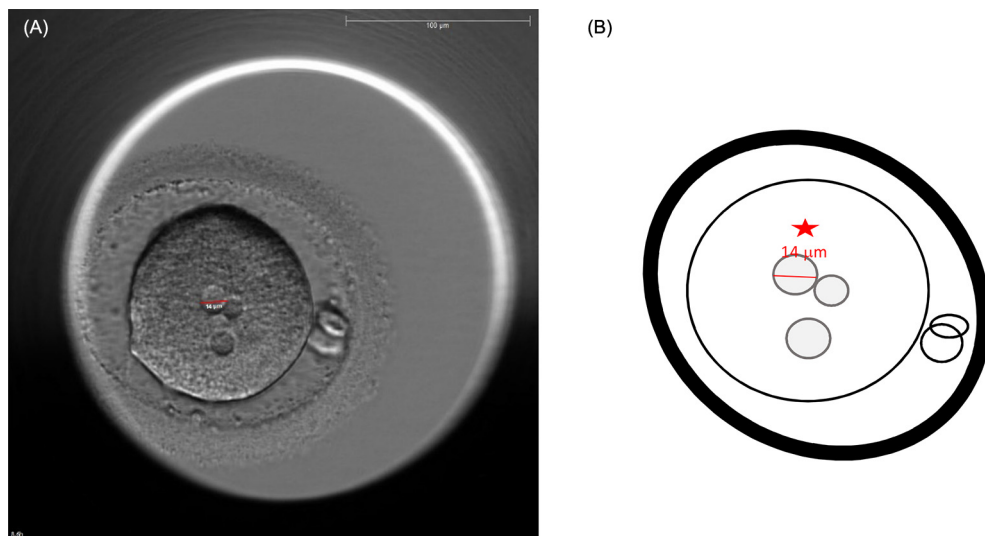


Fig. 2 (A and B) Multiple vacuoles of various sizes, *vacuole with $14 \mu\text{m}$ diameter, large perivitelline space.

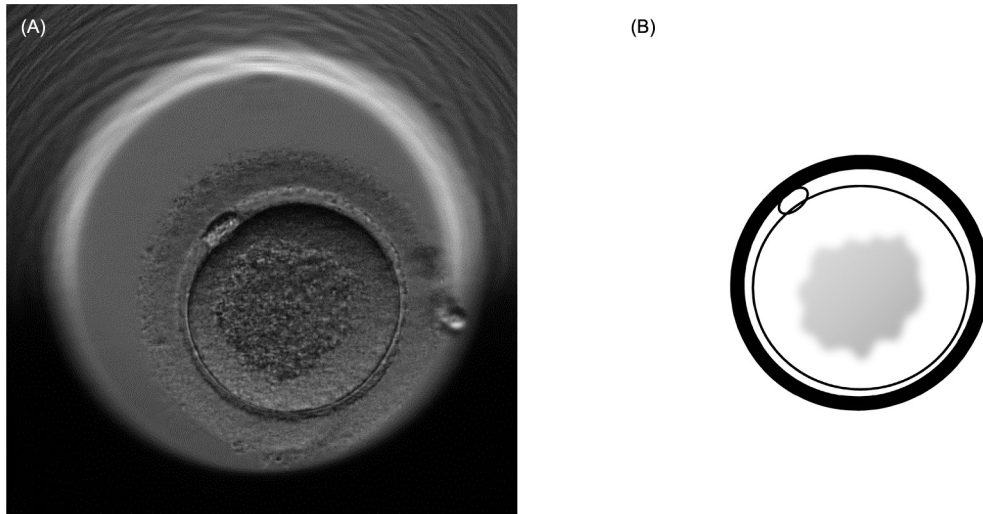


Fig. 3 (A and B) Centrally located condensed granulation.

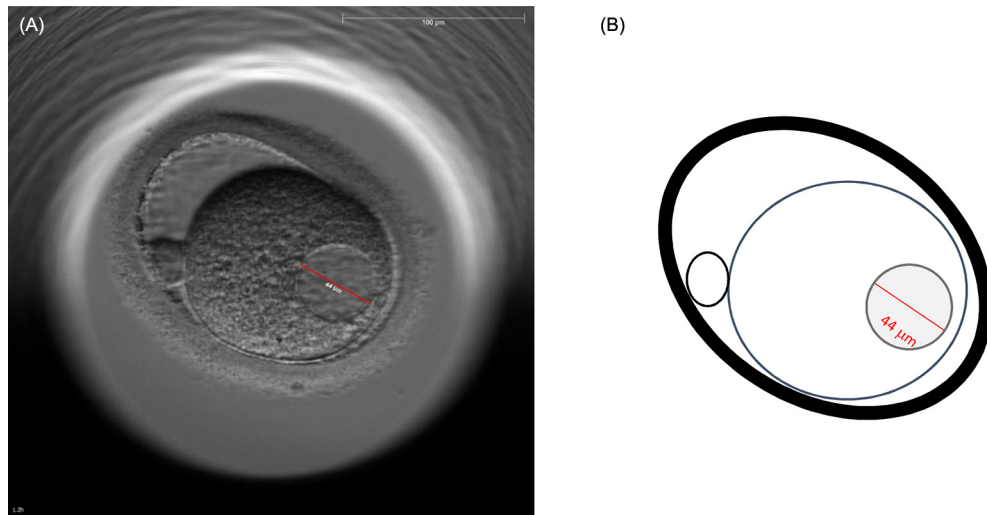


Fig. 4 (A and B) Large vacuole with 44 μm diameter, large perivitelline space.

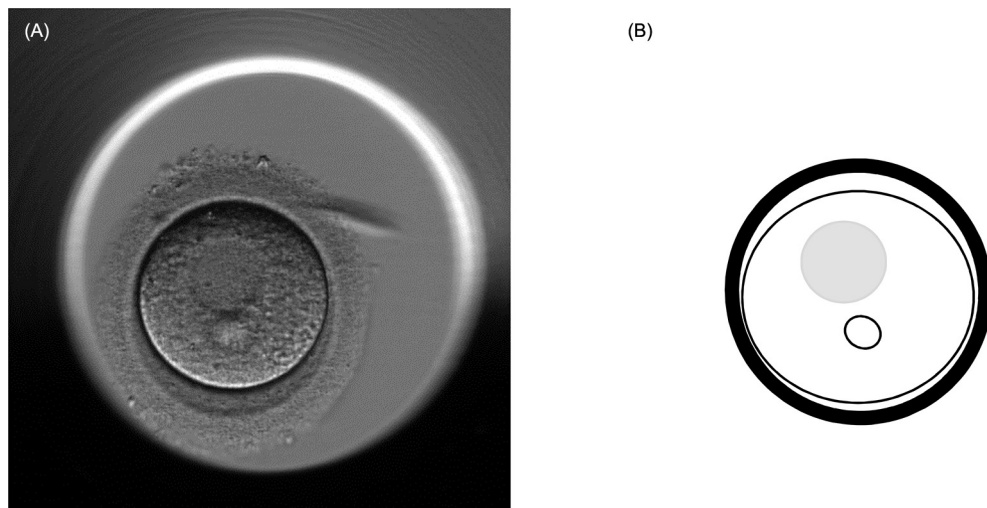


Fig. 5 (A and B) Translucent vacuole type structures—smooth endoplasmic reticulum clusters.

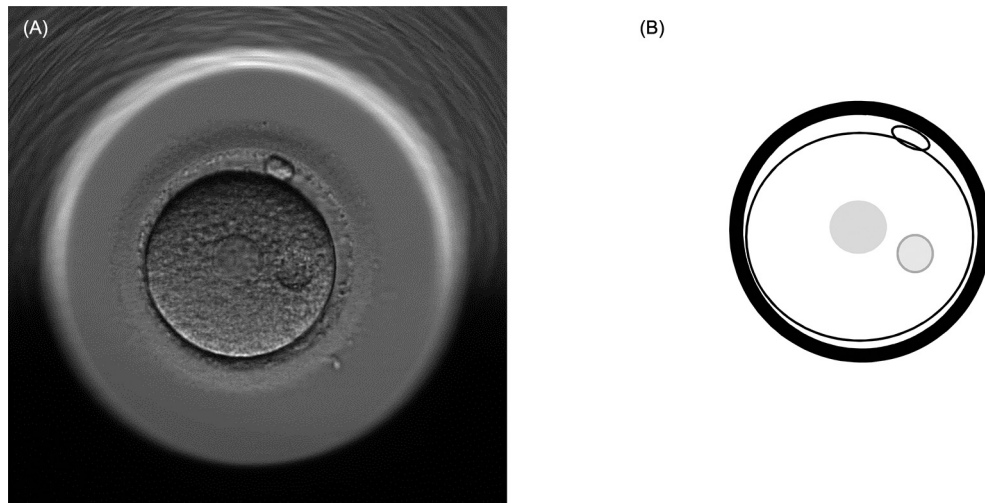


Fig. 6 (A and B) Smooth endoplasmic reticulum clusters.

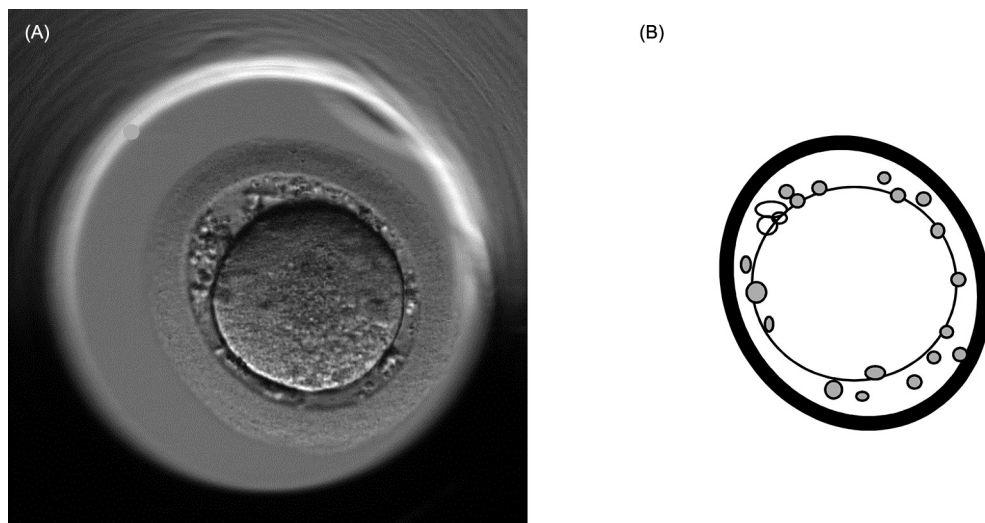


Fig. 7 (A and B) Debris in perivitelline space, fragmented first polar body.

probably associated with the health of the developing follicle, for example, its vascularization and oxygen content. Any disturbance during growth might severely alter oocyte morphology resulting in a pool of gametes with different prognoses (**Fig. 7A and B**).

Contribution of the Spermatozoon Characteristics on Fertilization and Further Embryo Development In Vitro

The effect of the spermatozoon, paternal genome on fertilization rates, embryo quality and especially the clinical outcome is less evident. All the natural barriers healthy spermatozoon with high fertilization capacity have to successfully achieve are bypassed and ignored during the ICSI procedure, for which a single spermatozoon is manually selected and inserted in the cytoplasm of the mature metaphase II oocyte. As the ICSI procedure is mainly applied for male factor infertility cases where poorer-quality spermatozoa is achieved, determination of defects that may adversely impact not only the fertilization, embryo viability and implantation potential but also the fetal outcome is of critical importance. Recent studies had shown that for the risk of neonatal and obstetric outcomes as well as congenital malformation rates for children born after the ICSI procedure for male factor infertility with ejaculated spermatozoon is not statistically different when compared with IVF treatments ([Belva et al., 2011](#)). For severe male factor infertility when epididymal or testicular spermatozoon is used overall neonatal health in terms of birth parameters, major anomalies and chromosomal aberrations of children born still seems reassuring in comparison to the outcome of children born after the use of ejaculated sperm ([Fedder et al., 2013](#)). More recent publications had also shown that ICSI with epididymal or testicular spermatozoon does not lead to more stillbirths or congenital malformations compared with ICSI using ejaculated

spermatozoon, and that the procedure is equally safe as even conventional IVF treatment (Guo et al., 2013). Despite this promising evidence, the seek for the selection of the ideal normal spermatozoa that would guarantee fertilization, good quality embryo and viable blastocyst development, and hence successful clinical outcome with the delivery of the healthy offspring, is still being investigated by novel technologies applicable in addition to classical morphological evaluation or as a solely method of selection with the aim of eliminating the utilization of spermatozoon with genetic integrity problems and DNA damage (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Fertilization Abnormalities by IVF

Abnormal fertilization by conventional in vitro fertilization is mainly associated with abnormal polyspermy, due to the dispermic oocyte penetration resulting in trippronuclear (3PN) oocytes. Reasons for the appearance of 3PN include; uncontrollable multiple sperm penetrations, fertilization by a diploid sperm or oocyte, inhibition of second polar body extrusion and formation of two female pronuclei (or possibly one diploid) together with a single sperm pronucleus (monospermic dignity). The identification and elimination of polyspermic oocytes are critical as it's known that significantly higher spontaneous abortions are expected from the transfer of embryos derived from triploid zygotes. In addition to the dispermia and digyny the origin of trippronuclear zygotes may also be connected with their chromosomal constitution. New micromanipulation techniques such as microsurgical removal of a single pronucleus may provide an opportunity to repair the abnormalities of trippronuclear zygotes, especially for patients with high rate of hyperploidy (Ivakhnenko et al., 2000). Recent study by Escriba et al. (2006) demonstrating heteroparental blastocyst production from microsurgically corrected trippronucleated human embryos from conventional IVF had also been a hope for future studies of correction micromanipulations for hyperploidy embryos.

Another frequently seen abnormal fertilization type in human oocytes after IVF procedure is the visualization of a single pronucleus at the time of assessment of fertilization, instead of distinct two pronuclei as a sign of both female and male gametes. Despite the fact that earlier studies had shown high diploidy rate for embryos derived from single pronucleus after standard IVF, with 50% of the blastomeres having Y chromosome and healthy deliveries reported after the transfer of embryos derived with mononucleated zygotes (assuming that the second pronucleus can be observed at a later reassessment), or the fusion of the two pronuclei might appear at static observations by a certain time period, recent molecular cytogenetic studies of human single pronucleate oocytes suggest a significantly lower rate of diploid embryos, hence it is not recommended to use such zygotes in assisted reproduction purposes (Andrea et al., 2000).

Fertilization Abnormalities by ICSI

As only one spermatozoa is injected within a mature oocyte in the ICSI procedure, the formation of the third pronucleus appears as a result of decondensation of the unextruded polar body. The failure of the second polar body extrusion might be connected to; damage to the metaphase plate region during ICSI, reorientation of the first meiotic spindle or damage to the oocyte cytoskeleton during sperm injection, and misorientation of the second meiotic spindle, high estradiol (E2) levels on the day of hCG administration, and origin of spermatozoa; utilization of testicular spermatozoon or spermatozoon from severe oligoasthenoteratozoospermic patients. Typically it is recommended to eliminate embryos derived from trippronuclear zygotes formed after ICSI as high rates of spontaneous abortions and mosaic embryos are reported.

A recent publication in which 10 relevant studies for the removal of the third pronucleus were reviewed, reported the transfer of corrected embryos attempted only for once and resulted in live birth. This study indicated that obstacles associated with the procedure such as the identification of the supernumerary pronucleus, the presence of two centrosomes in dispermic oocytes, and cytogenetically abnormal patterns after intracytoplasmic sperm injection are still to be clarified, possibly offering patients with exclusively abnormally or few normally fertilized oocytes to benefit from epronucleation to guarantee embryo transfer or increase the number of embryos that could be transferred (Rosenbusch, 2009).

The percentage of haploid embryos obtained from mononucleated zygotes after ICSI are reported to be higher and varying percentages from 10% to 30% of embryos had been shown to contain Y chromosome. It has been suggested that monopronucleated ICSI zygotes may usually be parthenogenetically activated and not fertilized. Mateo et al., in contrast reported that even though the majority of monopronuclear zygotes after ICSI result from fertilization, as all embryos obtained were chromosomally abnormal, a significantly low percentage of embryos developed to good quality blastocysts, suggesting the deselection of embryos derived from monopronuclear zygotes (Mateo et al., 2013). Some similar studies also revealed that after the analysis of chromosome status of ICSI embryos derived from monopronucleated zygotes, that the diploidy rate is low and thus such embryos should not be used in assisted reproduction treatments.

Examination of Pronuclear Morphology and It's Predictive Value for Clinical Outcome

One of the mostly cited and frequently used scoring schemes to assess the quality and viability of embryos to be transferred by looking to the pronuclear morphology of normally fertilized zygotes with two distinct pronucleus clarified in the oocyte cytoplasm

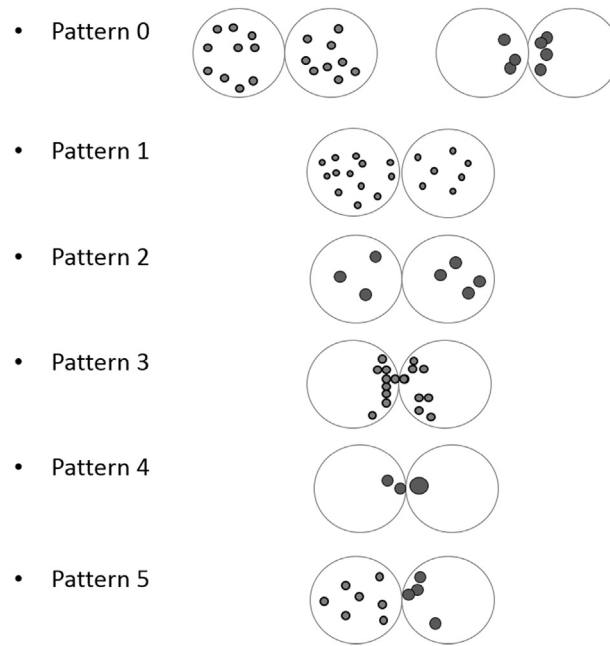


Fig. 8 Schematic presentation of pronuclear morphology corresponding to individual patterns. Pattern 0 corresponds to normal zygotes whereas all the others are asymmetrical scores (Tesarik and Greco, 1999).

was published by Tesarik and Greco (1999). The morphological parameters evaluated included number of nucleolar precursor bodies (NPB) and their distribution in each pronucleus defined as polarized or non-polarized. The distribution of NPB was considered polarized when all NPB present in a pronucleus were present in the pronuclear hemisphere whose pole was the point of contact with the other pronucleus it was considered non-polarized when at least one NPB was found in the opposite hemisphere. The relative size of both pronuclei (equal or unequal) and their position with regard to one another (in apposition versus at distance) were also noted. Five patterns based on these parameters were defined in the pronuclear grading scheme of Tesarik and Greco (Fig. 8).

The developmental fate analysis of zygotes with different patterns of pronuclear stage morphology had shown that these patterns can significantly predict the percentage of arrested embryos, embryos with multi-nucleated blastomeres (MNB), and good quality embryos. Pattern 0 which were zygotes giving rise to embryos transferred in 100% implantation cycles were defined as the ideal zygotes with; the number of NPB not showing big differences between both pronuclei, never differing by more than three, NPB being always polarized when they were fewer than seven and never polarized when they were more than seven in a pronucleus, the number of NPB in a pronucleus never fewer than three, and the distribution of NPB being either polarized or non-polarized in both pronuclei, but never polarized in one pronucleus and non-polarized in the other. All other patterns were defined as asymmetrically developing zygotes. Embryos originated from pattern 0 zygotes had significantly less percentage of arrested embryos, embryos with multi-nucleated blastomeres (MNB), significantly higher number of good quality embryos when compared with all other pattern groups. In a follow-up study they examined the clinical validation of their grading scheme and found in 380 fresh embryo transfer cycles that the clinical pregnancy and implantation rates were significantly higher for the group of patients who received embryos originated from pattern 0 zygotes, and that the transfer of only one pattern 0 embryo was sufficient for the optimal chance of pregnancy whereas transfer of two pattern 0 embryos mostly resulted in twin pregnancy (Tesarik et al., 2000). Balaban et al. (2001) also demonstrated that embryos showing an ideal pronuclear pattern 0 cleaved earlier and faster and resulted in better quality cleavage stage embryos and blastocysts. The incidence of blastocyst formation was very high; 72% in zygotes showing a 0 pattern, compared with 12.7% in zygotes with double abnormalities assessed by Tesarik and Greco grading scheme. In addition to better embryo quality and blastocyst formation, higher implantation and pregnancy rates were obtained when at least one blastocyst derived from a 0 pattern zygote was included in the set of embryos transferred. A more recent joint study by Balaban et al. (2004) also showed that both embryo cleavage characteristics (such as cleavage rate, embryo quality and blastocyst formation) and chromosome constitution of the embryo were related with pronuclear morphology. Embryos developing from zygotes with the normal PN pattern 0 cleaved faster and formed embryos with better morphology as compared with zygotes with abnormal pronuclear patterns. Aneuploidy rate of embryos derived from zygotes with the normal pronuclear pattern was significantly lower when compared with embryos derived from single or double pronuclear abnormality. Chromosomally normal embryos with the normal pattern 0 progressed to the blastocyst stage at a significantly higher rate than chromosomally normal embryos with single or double pronuclear abnormality. The same relationship applied to chromosomally abnormal embryos.

An alternative grading scheme that had also been widely cited and utilized is the one published by Scott and Smith (1998) which is based on empirical observations correlated with pregnancy. The basis of this grading scheme was a combination of pronuclear

size, nucleoli number and distribution, and cytoplasmic appearance that prospectively showed an increase on the incidence of implantation when utilized for day one embryo transfers and to select embryos for cryopreservation. As a continuum of this study, [Scott et al. \(2000\)](#) published a revised pronuclear grading scheme with the aim of better predictivity and easy and fast use for laboratory staff. The new grading scheme examined the cleavage stage and blastocyst stage embryo characteristics as well as their implantation potential in correlation with zygote morphology. The revised grading system was named as Z scoring scheme at which the zygotes were scored from Z1 to Z4, Z1, and Z2 representing the ideal zygotes that would give rise to implanting embryos. Similar to the previous scheme, the grading took into account the nuclear size and alignment, nucleoli number and distribution. The nucleoli needed to be aligned, or be beginning to align, at the pronuclear junction, between three and seven per nucleus with no more than one nucleolus difference between the nuclei and equal in size. Although the size of the nucleoli was not measured, zygotes with either very small pinpoint nucleoli or very large ones were designated as Z3. The study had shown that embryos derived from Z1 and Z2 scored zygotes have an higher rate of blastocyst formation. The pregnancy and implantation of embryos derived from Z1 and Z2 scored zygotes were also significantly higher ([Fig. 9](#)).

As in connection with the earlier hypothesis indicating that nucleolar patterns are closely linked to embryo viability, thereby reinforcing the relevance of polarity in human oocytes and embryos, it appears that pronuclear scoring can successfully be incorporated into the practice of embryo evaluation for prediction of implantation potential, and embryo normality of the individual embryo ([Balaban and Urman, 2003](#)). A recent systematic review on forty studies examining the effectiveness of zygote morphology evaluation in fresh IVF and ICSI cycles revealed that the majority of the evidence based clinical trials supported the utilization of the grading schemes by Tesarik and Greco, or Scott Z scoring system with minor modifications ([Nicoli et al., 2013](#)). This review also demonstrated that zygote morphology correlated significantly with embryo quality and cleavage, blastocyst stage, embryonic chromosome status, in a high proportion of the studies which assessed the specific outcome.

Timing of assessment of pronuclear scoring is of major importance as the pronuclear formation is a dynamic event that would eventually disappear by the beginning of the cleavage events of the embryos. The systematic review had shown that various timings differing from 14th to 18th hour post-insemination was being used in clinical trials for IVF and/or ICSI cases. Taking into the consideration the dynamic development of a human embryo, the heterogeneous results reported might be correlated to these timing differences of examination of pronuclear sizes, as well as polarization dynamics of the NPBs. In the time course leading to the initiation of pronuclear formation, zygotes arising from IVF are observed to be approximately an hour behind those arising from ICSI, and thus the widely used and recommended timing based on earlier studies for 16th–18th hour of assessment may not be appropriate time not only for the observation of visualization of normal fertilization with two distinct pronuclei and two polar bodies but also for the examination of pronuclear morphology. The Istanbul consensus workshop proceedings published by Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology suggested that the fertilization as well as pronuclear morphological characteristics of a zygote should be assessed at 17th with minus or plus an hour maximum flexibility ([Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011](#)). A simplified consensus grading scheme for pronuclear scoring was also suggested in the consensus paper. According to this grading system three categories were defined by which category 1 represented symmetrical zygotes that are equivalent to Z1 and Z2 as referred by Scott and Smith, category 2 represented non-symmetrical zygotes with all other arrangements of pronuclear appearances, and category 3 represented abnormal zygotes with 0 or 1 NPB within the pronuclei ([Figs. 10A, B and 11A, B](#)).

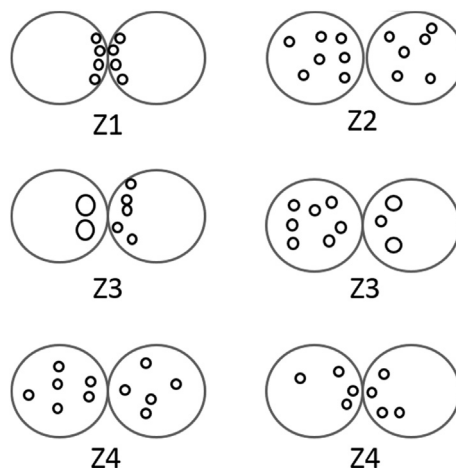


Fig. 9 Diagrammatic representation of Z-scoring by [Scott et al. \(2000\)](#). Z1 and Z2 represents normal zygotes whereas all the other arrangements are considered asymmetrical.

Consensus scoring system for pronuclei		
Category	Rating	Description
1	Symmetrical	Equivalent to Z1 and Z2
2	Non-symmetrical	Other arrangements, including peripherally sited pronuclei
3	Abnormal	Pronuclei with 0 or 1 NPB

Fig. 10 Alpha and ESHRE special interest group of embryology consensus grading scheme (2011) for pronuclear evaluation modified by Scott et al. (2000).

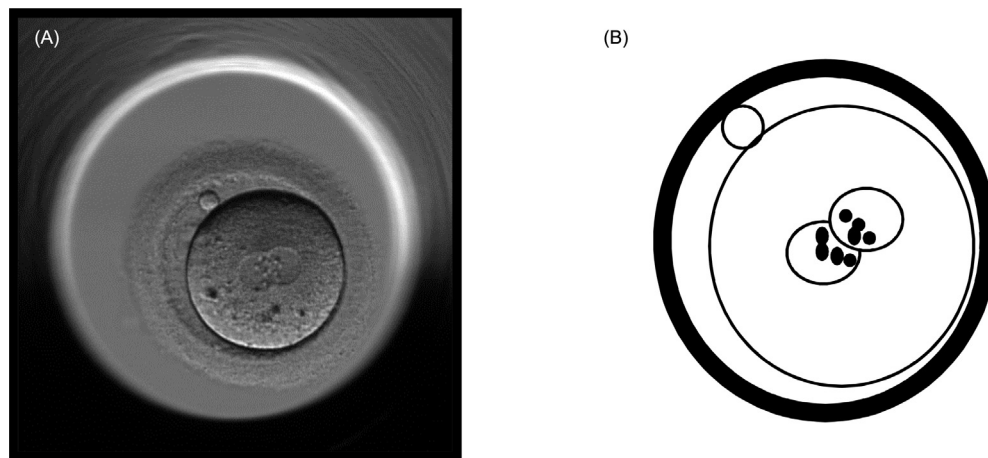


Fig. 11 (A and B) Category 1 pronuclear score—according to alpha and eshre special interest group of embryology consensus grading scheme (2011).

Dynamics of Fertilization, Pronuclear Development and Morphology

One of the major drawbacks of morphological evaluations and grading schemes of embryos, starting from the zygote level to the blastocyst stage, is that the assessment is performed at static developmental time points by microscopy. As the human embryo is a highly dynamic and complex entity the static evaluation of such a cell will always have limitations for viability and implantation competency assessments, by missing some of the important cleavage events that could be visualized by continuous monitorization. Time-lapse incubation systems have let the predictive value and objectivity of morphological grading schemes to be revisited, and open a debate for the necessity of revised scoring systems emphasizing the need for a new look on embryological parameters.

The study Montag et al. (2011) analyzed pronuclear morphology based on time-lapse imaging for the distribution of NPB at 14–15th, 16–18th, and 19–20th hours and found out that 14–15 h to 16–18 h similar pronucleus scorings were present in 75% zygotes, whereas 25% showed change in the patten which resulted in another pronuclear score. The change in the pronuclear score from 16–18 h to 19–21 h was 33.8%, whereas 66.2% remained in the similar pronuclear scores. The documented changes were mostly from an asymmetric distribution of NPB towards a symmetric or perfectly aligned distribution. The change from a symmetric to an asymmetric pattern was less common. These dynamic changes reported in the study can perhaps explain the contradictory situation in the literature where static observations for zygote morphology were reported. The prospective study by Azzarello et al. (2012) was performed on 159 embryos, all of which were transferred. The pronuclear morphology of the 46 embryos which resulted in live birth was compared with that of 113 embryos which resulted in no live birth. Pronuclear morphological assessment was performed on day of embryo transfer, using six different scoring systems at different times. The study showed that no embryo with pronuclear breakdown earlier than 2 h 45 min resulted in live birth, all six pronuclear assessment models showed no significant distribution of scores between the live birth and no live birth groups at the 16th and 18th hour post-fertilization and 40 min before pronuclear breakdown. The outcomes of assessments changed significantly over time and the time of pronuclear breakdown was found to be the optimal stage to evaluate pronuclear morphology. In agreement with published Works, this study also demonstrated that pronuclear morphology changes over time, showing that the single static observation is deficient in comparison to dynamic continuous observation. Even though the examination of the pronuclear morphology did not improve the embryo selection, the timing of pronuclear breakdown was suggested to be a more objective criteria for prediction of live birth. The study by Aguilar et al. (2014) compared the timings of early fertilization events by time-lapse imaging which were the second polar body extrusion,

first and second pronuclei appearance, pronuclear syngamy so called as abuttal, pronuclear movements within the cytoplasm, pronuclear morphology, pronuclear symmetry, pronuclear fading and the length of S-phase which is defined as the time from pronuclear appearance to pronuclear fading in implanted versus nonimplanted embryos in a 2 year cohort study. The timings at which second polar body extrusion (3.3–10.6 h), pronuclear fading (22–25.9 h) and length of S-phase (5.7–13.8) occurred were linked successfully to embryo implantation, and all the other parameters defined were unrelated with embryo implantation competency.

All these studies on the continuous observation of early fertilization events offer a new era to revisit the changes in fertilization phenomenon and the necessity of a more close examination of the dynamism of pronuclear morphological changes.

Conclusion

Successful fertilization process is a complex and highly dynamic biological phenomenon that includes several events which are closely correlated with the developmental capability and viability of the resulting embryo. The events in summary are the exclusion of the second polar body, the appearance and fading of the pronuclei, and the pronuclear syngamy which all happen before or after the conventional first embryo observation performed at 16–22 h. post insemination., most likely remaining unidentified when the assessment is performed at fixed time points (Balaban et al., 2017). Continuous observation of embryo development by time-lapse technology will not only provide valuable information for cleavage or blastocyst stage characteristics of the embryo but also may detect some critical abnormalities that start at the very early stage of the fertilization process. Despite the fact that the majority of clinical time-lapse studies identify information on the cleaved embryo, more future trials will guide us through the details of the human first cell cycle which may introduce alternative objective parameters for the selection of embryos with higher implantation probabilities that would lead us to successful clinical outcome.

References

- Aguilar, J., Motato, Y., Escriba, M. J., Ojeda, M., Munoz, E., & Meseguer, M. (2014). The human first cell cycle: Impact on implantation. *Reproductive Biomedicine Online*, 28, 475–484.
- Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. (2011). The Istanbul consensus workshop on embryo assessment: Proceedings of an expert meeting. *Human Reproduction*, 26, 1270–1283.
- Andrea, R., Sachs, B. A., & Ginsburg, E. S. (2000). Factors associated with the formation of triploid zygotes after intracytoplasmic sperm injection. *Fertility and Sterility*, 73, 1109–1114.
- Azzarello, A., Hoest, T., & Mikkelsen, A. L. (2012). The impact of pronuclei morphology and dynamics in live birth outcome after time-lapse culture. *Human Reproduction*, 27, 2649–2657.
- Balaban, B., & Ebner, T. (2014). Morphological selection of gametes and embryos: Oocyte. In M. Montag (Ed.), *A practical guide to selecting gametes & embryos* (pp. 81–96). Boca Raton London New York: CRC Press, Taylor&Francis Group.
- Balaban, B., & Urman, B. (2003). Embryo culture as a diagnostic tool. *Reproductive Biomedicine Online*, 7, 671–682.
- Balaban, B., & Urman, B. (2006). Effect of oocyte morphology on embryo development and implantation. *Reproductive Biomedicine Online*, 12, 608–615.
- Balaban, B., Urman, B., Isiklar, A., et al. (2001). The effect of pronuclear morphology on embryo quality parameters and blastocyst transfer outcome. *Human Reproduction*, 16, 2357–2361.
- Balaban, B., Yakin, K., Urman, B., Isiklar, A., & Tesarik, J. (2004). Pronuclear morphology predicts embryo development and chromosome constitution. *Reproductive Biomedicine Online*, 8, 695–700.
- Balaban, B., Gardner, D. K., & Simon, C. (2017). Handbook of in vitro fertilization (fourth edition). In *Analysis of fertilization*, ch. 12 (p. 170). 173. Abingdon, UK: CRC Press.
- Belva, F., De Schrijver, F., Tournaye, H., et al. (2011). Neonatal outcome of 724 children born after ICSI using non-ejaculated sperm. *Human Reproduction*, 26, 1752–1758.
- Escriba, M. J., Martin, J., Rubio, C., et al. (2006). Heteroparental blastocyst production from microsurgically corrected tripronucleated human embryos. *Fertility and Sterility*, 86, 1601–1607.
- Fedder, J., Loft, A., Parner, E. T., Rasmussen, S., & Pinborg, A. (2013). Neonatal outcome and congenital malformations in children born after ICSI with testicular or epididymal sperm: a controlled national cohort study. *Human Reproduction*, 28, 230–240.
- Guo, Y. H., Dong, R. N., YC, S., et al. (2013). Follow-up of children born after intracytoplasmic sperm injection with epididymal and testicular spermatozoa. *Chinese Medical Journal*, 126, 2129–2133.
- Ivakhnenko, V., Gieslak, J., & Verlinsky, Y. (2000). A microsurgical technique for anucleation of multipronuclear human zygotes. *Human Reproduction*, 15, 911–916.
- Kupker, W., Diedrich, K., & Edwards, R. G. (1998). Principles of mammalian fertilization. *Human Reproduction*, 13(suppl 1), 20–32.
- Mateo, S., Parriego, M., Boada, M., et al. (2013). In vitro development and chromosome constitution of embryos derived from monopronucleated zygotes after intracytoplasmic sperm injection. *Fertility and Sterility*, 99, 897–902.
- Montag, M., Liebenthron, J., & Koster, M. (2011). Which morphological scoring system is relevant in human embryo development? *Placenta*, 32(Suppl.3), 252–256.
- Nicoli, A., Palomba, S., Capodanno, F., et al. (2013). Pronuclear morphology evaluation for fresh in vitro fertilization(IVF) and intracytoplasmic sperm injection (ICSI) cycles: A systematic review. *Journal of Ovarian Research*, 6, 64.
- Rosenbusch, B. E. (2009). Selective microsurgical removal of a pronucleus from tripronuclear human oocytes to restore diploidy: Disgarded but valuable? *Fertility and Sterility*, 92, 897–903.
- Sakkas, D. (2013). Novel technologies for selecting the best sperm for in vitro fertilization and intracytoplasmic sperm injection. *Fertility and Sterility*, 99(4), 1023–1029.
- Scott, L. A., & Smith, S. (1998). The successful use of pronuclear embryo transfers the day after oocytes retrieval. *Human Reproduction*, 13, 1003–1013.
- Scott, L., Alvero, R., Leondires, M., & Miller, B. (2000). The morphology of human pronuclear embryos is positively related to blastocyst development and implantation. *Human Reproduction*, 15, 2394–2403.
- Tesarik, J., & Greco, E. (1999). The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Human Reproduction*, 14, 1318–1323.
- Tesarik, J., Junca, A. M., Hazout, A., et al. (2000). Embryos with high implantation potential after intracytoplasmic sperm injection can be recognized by a simple, non-invasive examination of pronuclear morphology. *Human Reproduction*, 15, 1396–1399.

Culture Systems for the Human Embryo

Thamara Viloria, José María De Los Santos, and Marcos Meseguer, IVI-Valencia, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

One of the successes of assisted reproduction techniques, it focuses on maintain optimal embryo viability, thus, an essential step during assisted reproduction treatments, is to keep the best in-vitro culture conditions of embryos and the optimization of this conditions, in order to select those with the highest developmental competence.

Two kinds of protocols, regarding culture media, are widely used in assisted reproduction laboratories. (A) Simple protocols, containing essential media for early embryo development; they are used for embryo culture from zygote to day 3 (Ertzeid et al., 1999; Shahine et al., 2011; Biggers and Summers, 2008); and (B) sequential protocols, to complete the development until blastocyst stage, providing more complex energy pathways required for these stage of development. Sequential culture can be made changing the medium to another with a different composition according embryo's requirements; or without change the medium composition, using a single step media with refreshment on day 3, or single step media without any refreshment for uninterrupted culture (Biggers and Summers, 2008; Balaban and Urman, 2005; Gardner, 2008; Hambiliki et al., 2011; Swain et al., 2016).

Continuous embryo culture in single-step media could have several important advantages, for example, offer the benefit of cost and simplified laboratory management, decreased embryonic stress, reduced pH and temperature fluctuations, compatibility with ever more popular time-lapse imaging culture systems (Costa-Borges et al., 2016; Sfountouris et al., 2017). However, culture conditions vary from lab to lab.

It is widely recognized that on assisted reproductive technology laboratories, in addition to the culture media, other components and conditions like gas phase, pH, culture vessel, incubation chamber, laboratory air quality, the training of embryologists and patient-own clinical characteristics, are very important, and its dynamic interaction with the culture system it is essential to provide a stable environment for embryos development (Swain et al., 2016; Swain, 2012, 2015). To this date, many different culture systems are development to improve the "in vitro" conditions, trying to mimic the physiologic environment that embryos have in the female reproductive tract. Thus, different platforms and systems have been developing to carry out an optimal culture of the human embryos. Table 1, summarize almost all culture systems that have been developed. Describe the most used, as well as the most recent and novel ones, is the objective of this article.

Microdrop Culture System

Conventional, flat sterile Petri dishes are the most common and traditional platform for embryo culture. Microdrops embryo culture, is one of the static system historically used by many assisted reproduction laboratories worldwide (Reed, 2012). The embryos are cultured in microdrops of variable volumes of medium, depending of the lab (10–50 µL) in Petri dishes, which are mostly covered with mineral oil to restrict evaporation. Then, dishes are placed into a standard incubator on controlled CO₂ and temperature conditions.

Physical culture environment, seems results important and may influence on embryo development. The way of embryo culture inside the drop (individually or by groups), could affect chemical environment because varying embryo spacing, being able to modify its development. Many efforts have been made to improve culture media and maintain the optimal microenvironment. However, most of these studies are made on animal models (Swain and Smith, 2011).

Table 1 Culture platforms

Statics	Ultra-microdrops
	Microdrops
	Submicroliter platforms
	Microwells
Dynamics	Microchannels
	Microfluids
	Shaking or rotation
	Vibration
Specialized	Tilting
	Agarose
	Hyaluronic acid
	Coculture
	Matrigel
	3-Dimensional matrix

On another hand, this kind of culture system, require more embryo manipulation, because occurs by manual pipetting and multiple observations are expected to give a better understanding of embryo development. The evaluation is made by the embryologist under microscopy outside incubator, almost every day during the embryo development. It is recognized that each observation involves handling and exposure of the embryos to suboptimal conditions outside the controlled environment.

Coculture System

Further optimizing culture conditions, for example by using improved culture media and paying exhaustive attention to the laboratory conditions, some groups have experimented with different coculture systems in the opinion that feeder cells would provide some factors missing from artificial culture media that would improve embryo quality (Eyheremendy et al., 2010).

The suggested beneficial effect of coculture system seems to fall on the fact of create a more physiologic environment, since includes the secretion of embryotrophic factors that may influence positively on embryo development. Multiple cell types have been used for this purpose, creating a surface that housing the embryos during the culture (Barmat et al., 1998; Mercader et al., 2006). Most recently, autologous endometrial cell monolayer coculture has yielded promising results for improved human embryo in vitro development (Rubio et al., 2000; Dominguez et al., 2010). However, there are controversial opinions about enhanced rates of blastocyst formation, and improve on implantation rates when embryos were cultured on the cells, or above them, compare to traditional culture systems. Although it has been used extensively in the past, it is a less widely used culture system, probably due to the delicate anterior process that epithelial and stromal endometrial human cells must have during their isolate and growth (Swain and Smith, 2011; Mercader et al., 2006; Simon et al., 1999).

Microfluids

At the beginning, microfluidic systems appeared to miniaturize devices designed for chemistry analysis and some molecular biology procedures. This technology is an emerging field and more recently, are being used as cell culture platforms. Even, some studies show possibilities to use them as an alternative for conventional ART techniques like sperm isolation, oocyte denudation, in vitro fertilization and embryo culture. In studies of assisted reproduction, microfluidics has some advantages like the very low consumption of culture media, in addition that the fluid in microchannels can be precisely predicted and controlled, among others (Han et al., 2010).

It is known that microchannels provide an environment that seems to be advantageous for embryos since mimic the active fluid situation that take place in vivo, compare to traditional static microdrop embryo culture methods. Microfluidics cell culture devices permits a gradual change of the culture medium, which might result in better embryo development and also reduces embryo manipulation. Although microdrop culture allows autocrine factors retention around embryo, it does not provide mechanical stimulation, and not mimicking the natural way of embryo culture. In vivo, the embryo goes through the Fallopian tube and is thus bathed in a flux of fluid of different composition, and the medium around the embryo change continuously, waste products are removed and probably the embryo senses the flux of medium and mechanical stimulation (Swain and Smith, 2011).

Microfluidics devices may be a new approach to solve this problem and might replace conventional human embryo culture in microdrops in the future. Probably microfluidics could reduce handling and disturbance of optimal culture conditions along each ART process, providing the suitable medium at the correct time and place and at the right flow rate in multiple channels at the same time, and all in one device with a minimal handling. Currently, there are very few microfluidic platforms that integrate all the IVF culture process in human embryos. However, to achieve this goal, some research groups are testing prototypes.

Time-Lapse Technology

Standard embryos assessments are based on subjective morphology evaluation, often with inter and intra observer variability, which joined to the increased handling and higher evaluation frequency, will expose the embryo to negative changes on important parameters that may affect its development such as temperature, humidity and gas composition (Meseguer et al., 2012).

Time-lapse is considered a novel noninvasive system and an important tool on ART, based on conventional incubators incorporating an image capture system which take pictures of the embryos in several focal plans at defined time intervals. The fusion of all the frames of each embryo generates a recording that lets visualizing the entire embryo development, in order to determine the exact moment in which each event occurs, with a better objectivity and precision since inter and intra observer variations is reduced and allows to detect any abnormal events that would normally occur between observations. In this way, embryo evaluation is done without taking them outside the incubator to minimize its manipulation while are maintaining on stable embryo culture conditions (Aparicio et al., 2013; Basile et al., 2015a). In another way, the use of kinetic markers can improve embryo selection and with an appropriate algorithm, can improve clinical results and increase implantation rates (Meseguer et al., 2011; Rubio et al., 2014; Basile et al., 2015a, 2015b).

At this time, more time-lapse incubators are available and being in use, because they represent a noninvasive tool for improving embryo selection without disturbing culture conditions (Meseguer et al., 2011). Morphokinetic evaluation may yield an important information for better embryo selection (Costa-Borges et al., 2016; Meseguer et al., 2011; Herrero and Meseguer, 2013).

Table 2 Time-lapse systems features


	Embryo-Scope	Geri	Primo Vision	Eeva	Miri	Embryo-Scope+
System	Vitrolife	Genea Biomedex	Vitrolife	Merck-Serono	ESCO Medical	Vitrolife
Optics	Bright field	Bright field	Bright field	Dark field	Bright field	Bright field
Focal planes	7	12	1	1	1	7
Embryo imaging	Pictures every 10'	Pictures every 10'	Pictures every 10'	Pictures every 5'	Pictures every 5'	Pictures every 10'
Capacity	6/system	6/system	1/camera	1/camera	6/system	15/system
Embryos per dish	12	16	16	12	14	16
Integrated incubator	Yes—Hybrid	Yes—Benchtop	No	No	Yes—Benchtop	Yes—Hybrid
Data analyses	Manual	Manual	Manual	Real Time	Manual	Manual
Selection algorithm	User defined	User defined	User defined	Yes	User defined	User defined

There are different alternatives of time-lapse systems available in the market. From those with single fully integrated equipment, for example, EmbryoScope®, Geri and Miri® TL; to those that introduce a microscope inside an available incubator, for example, Primo Vision® and The Eeva™ Test. **Table 2**, describes some features of the time lapse systems available.

Final and Future Considerations

At this time, our vision of some culture systems is like a big puzzle with few connecting pieces; thinking which of them could be the better one to maintain the best conditions for embryos development, and finally may improve our clinical results. However, these pieces will gradually joined and we can imagine the future. Therefore, it is perfectly conceivable visualize in this future devices with video time-lapse systems and probably with microfluidics technology without continuous embryo manipulation, that can replace current IVF platforms. Henceforth, embryologist tasks will also change because continuous gamete/embryo handling and assessment will no longer be necessary. New tasks can be clinically managed in IVF labs, such as supervise this possible automated procedures, to improve their technology/scientific background and to conduct active research with the embryological data generated by these new devices.

References

- Aparicio, B., Cruz, M., & Meseguer, M. (2013). Is morphokinetic analysis the answer? *Reproductive Biomedicine Online*, 27(6), 654–663.
- Balaban, B., & Urman, B. (2005). Comparison of two sequential media for culturing cleavage-stage embryos and blastocysts: Embryo characteristics and clinical outcome. *Reproductive Biomedicine Online*, 10(4), 485–491.
- Barmat, L. I., Liu, H. C., Spandorfer, S. D., Xu, K., Veeck, L., Damario, M. A., & Rosenwaks, Z. (1998). Human preembryo development on autologous endometrial coculture versus conventional medium. *Fertility and Sterility*, 70(6), 1109–1113.
- Basile, N., Caiazza, M., & Meseguer, M. (2015a). What does morphokinetics add to embryo selection and in-vitro fertilization outcomes? *Current Opinion in Obstetrics & Gynecology*, 27(3), 193–200.
- Basile, N., Vime, P., Florensa, M., Aparicio Ruiz, B., Garcia Velasco, J. A., Remohi, J., & Meseguer, M. (2015b). The use of morphokinetics as a predictor of implantation: A multicentric study to define and validate an algorithm for embryo selection. *Human Reproduction (Oxford, England)*, 30(2), 276–283.
- Biggers, J. D., & Summers, M. C. (2008). Choosing a culture medium: Making informed choices. *Fertility and Sterility*, 90(3), 473–483.
- Costa-Borges, N., Belles, M., Meseguer, M., Galliano, D., Ballesteros, A., & Calderon, G. (2016). Blastocyst development in single medium with or without renewal on day 3: A prospective cohort study on sibling donor oocytes in a time-lapse incubator. *Fertility and Sterility*, 105(3), 707–713.
- Dominguez, F., Gadea, B., Mercader, A., Esteban, F. J., Pellicer, A., & Simon, C. (2010). Embryologic outcome and secretome profile of implanted blastocysts obtained after coculture in human endometrial epithelial cells versus the sequential system. *Fertility and Sterility*, 93(3), 774–782.e1.
- Ertzeid, G., Dale, P. O., Tanbo, T., Storeng, R., Kjekshus, E., & Abyholm, T. (1999). Clinical outcome of day 2 versus day 3 embryo transfer using serum-free culture media: A prospective randomized study. *Journal of Assisted Reproduction and Genetics*, 16(10), 529–534.
- Eyheremendy, V., Raffo, F. G., Papayannis, M., Barnes, J., Granados, C., & Blaquier, J. (2010). Beneficial effect of autologous endometrial cell coculture in patients with repeated implantation failure. *Fertility and Sterility*, 93(3), 769–773.
- Gardner, D. K. (2008). Dissection of culture media for embryos: The most important and less important components and characteristics. *Reproduction, Fertility, and Development*, 20(1), 9–18.
- Hambiliki, F., Sandell, P., Yaldir, F., & Stavreus-Evers, A. (2011). A prospective randomized sibling-oocyte study of two media systems for culturing cleavage-stage embryos-impact on fertilization rate. *Journal of Assisted Reproduction and Genetics*, 28(4), 335–341.
- Han, C., Zhang, Q., Ma, R., Xie, L., Qiu, T., Wang, L., Mitchelson, K., Wang, J., Huang, G., Qiao, J., & Cheng, J. (2010). Integration of single oocyte trapping, in vitro fertilization and embryo culture in a microwell-structured microfluidic device. *Lab on a Chip*, 10(21), 2848–2854.
- Herrero, J., & Meseguer, M. (2013). Selection of high potential embryos using time-lapse imaging: The era of morphokinetics. *Fertility and Sterility*, 99(4), 1030–1034.

- Mercader, A., Valbuena, D., & Simon, C. (2006). Human embryo culture. *Methods in Enzymology*, 420, 3–18.
- Meseguer, M., Herrero, J., Tejera, A., Hilligsøe, K. M., Ramsing, N. B., & Remohí, J. (2011). The use of morphokinetics as a predictor of embryo implantation. *Human Reproduction*, 26(10), 2658–2671.
- Meseguer, M., Rubio, I., Cruz, M., Basile, N., Marcos, J., & Requena, A. (2012). Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: A retrospective cohort study. *Fertility and Sterility*, 98(6), 1481–1489.e10.
- Reed, M. L. (2012). Culture systems: Embryo density. *Methods in Molecular Biology*, 912, 273–312.
- Rubio, C., Simon, C., Mercader, A., Garcia-Velasco, J., Remohí, J., & Pellicer, A. (2000). Clinical experience employing co-culture of human embryos with autologous human endometrial epithelial cells. *Human Reproduction*, 15(Suppl 6), 31–38.
- Rubio, I., Galan, A., Larreategui, Z., Ayerdi, F., Bellver, J., Herrero, J., & Meseguer, M. (2014). Clinical validation of embryo culture and selection by morphokinetic analysis: A randomized, controlled trial of the EmbryoScope. *Fertility and Sterility*, 102(5), 1287–1294.e5.
- Sfontouris, I. A., Kolibianakis, E. M., Lainas, G. T., Venetis, C. A., Petsas, G. K., Tarlatzis, B. C., & Lainas, T. G. (2017). Blastocyst utilization rates after continuous culture in two commercial single-step media: A prospective randomized study with sibling oocytes. *Journal of Assisted Reproduction and Genetics*, 34(10), 1377–1383.
- Shahine, L. K., Milki, A. A., Westphal, L. M., Baker, V. L., Behr, B., & Lathi, R. B. (2011). Day 2 versus day 3 embryo transfer in poor responders: A prospective randomized trial. *Fertility and Sterility*, 95(1), 330–332.
- Simon, C., Mercader, A., Garcia-Velasco, J., Nikas, G., Moreno, C., Remohí, J., & Pellicer, A. (1999). Coculture of human embryos with autologous human endometrial epithelial cells in patients with implantation failure. *The Journal of Clinical Endocrinology and Metabolism*, 84(8), 2638–2646.
- Swain, J. E. (2015). Optimal human embryo culture. *Seminars in Reproductive Medicine*, 33(2), 103–117.
- Swain, J. E. (2012). Is there an optimal pH for culture media used in clinical IVF? *Human Reproduction Update*, 18(3), 333–339.
- Swain, J. E., Carrell, D., Cobo, A., Meseguer, M., Rubio, C., & Smith, G. D. (2016). Optimizing the culture environment and embryo manipulation to help maintain embryo developmental potential. *Fertility and Sterility*, 105(3), 571–587.
- Swain, J. E., & Smith, G. D. (2011). Advances in embryo culture platforms: Novel approaches to improve preimplantation embryo development through modifications of the microenvironment. *Human reproduction update*, 17(4), 541–557.

Human Embryo Development and Assessment of Viability

David K Gardner, University of Melbourne, Parkville, VIC, Australia; and Melbourne IVF, East Melbourne, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

The preimplantation period of human embryo development is marked by several key morphological, physiological and genetic changes, all of which occur within a 6-day period, after which the embryo implants into the endometrium of the uterus. From fertilization in the oviduct, to implantation in the uterus, the embryo develops from a totipotent single cell, to a multicellular blastocyst of over 100 cells, which contains two distinct cell populations. The outer layer of the blastocyst, which subsequently forms the placenta is referred to as the trophoblast and represents the first transporting epithelium of the conceptus. Within the fluid cavity, the blastocoel, resides an eccentrically placed group of cells known as the inner cell mass, and it is from this smaller group of cells that the fetus develops. During this period of life the embryo is free floating within the female tract and derives all of its nutrients from the surrounding fluids. This environment is characterized by nutrient and oxygen gradients as the embryo passes through the oviduct and uterus (Gardner et al., 1996; Ng et al., 2017), and becomes increasingly more complex in composition with the uterine environment providing an array of cytokines and growth factors (Hannan et al., 2011).

Development of the human embryo in vitro was significantly improved when culture media started to reflect the composition of the female reproductive tract, rather than being empirically derived (Gardner and Lane, 1997, 2017). Now it is possible to maintain the human embryo through to the blastocyst stage in culture, resulting in improved outcome for patients undergoing in vitro Fertilization (IVF) procedures. Analysis of embryo morphology, kinetics of development and the analysis of metabolic function have all been linked, to some degree, to pregnancy outcome. The future of human IVF will see the development and application of further biomarkers of developmental potential, potentially combined with the introduction of microfluidic devices, which are suited to single embryo/cell analyses (Thouas et al., 2013).

The Environments Created by the Female Reproductive Tract to Support the First Week of Life

The environments created within the lumen of the female reproductive tract are designed to support the stage-specific needs of the developing embryo, and are not simple serum-transudates (Leese, 1988). Hence, fluids within the oviduct and uterus are unique, complex and change with regards to the day of the menstrual cycle. Human oviduct fluid is comprised of high levels of albumin and mucins, and contains pyruvate (0.32 mM) and lactate (10.5 mM) and low levels of glucose (0.5 mM) (Gardner et al., 1996). Mammalian oviduct fluid is characterized by very high levels of specific amino acids including alanine, glutamate, glycine and taurine (Harris et al., 2005; Miller and Schultz, 1987), and has oxygen levels of between 5% and 8% (Gardner and Lane, 2017; Ng et al., 2017). In contrast, uterine fluid is somewhat more complex, characterized by the presence of glycosaminoglycans, especially hyaluronan and a rich reservoir of growth factors and cytokines. Uterine fluid has relatively high levels of glucose (3.15 mM), and low levels of pyruvate (0.1 mM) and lactate (5.87 mM) (Gardner et al., 1996). The level of oxygen in the uterus ranges from ~2.5% to 5% and is considered to drop close to 2% around the time of implantation (Gardner and Lane, 2017; Ng et al., 2017). As well as providing gradients of nutrients to the embryo, which also act as signals to the embryo (Gardner and Harvey, 2015), the embryo is exposed to numerous cytokines and growth factors, whose levels not only change with the day of the menstrual cycle, but also differ between fertile and infertile patients (Hannan et al., 2011), thereby indicating they have important roles in regulating embryonic development, differentiation and implantation. Hence, the environment to which the human preimplantation embryo is exposed to in vivo is anything but constant. As the biology of the developing embryo is considered in detail, the significance of the dynamic environment created within the maternal tract becomes evident.

Biology of Human Preimplantation Development

The human oocyte is the largest cell in the female, being ~110 µm in diameter, and is surrounded by a glycoprotein coat known as the zona pellucida. The zona serves not only to prevent polyspermy postfertilization, but also prevent premature attachment and possible invasion of the developing embryo into the wall of the oviduct. The zona is subsequently lost at the blastocyst stage through the action of both embryo and uterine-derived proteases. After Fertilization, the embryo must undergo the extrusion of the second polar body, decondensation of the sperm head, pronuclei formation, and finally the breakdown of the pronuclear membrane. The fertilized oocyte is totipotent, in that it can give rise to every cell type in the body. In the human preimplantation embryo, the first few divisions are referred to as restrictive mitoses, in which the cells (known as blastomeres) divide but the daughter cells are smaller. This is required to restore the cytoplasmic: nuclear ratio, which is abnormally high in the fertilized oocyte. The first of these divisions occurs around 18 h after fertilization, with subsequent divisions occurring approximately every 12 h, although there is variation between embryos (Meseguer et al., 2011). Up to the 8-cell stage, individual blastomeres are held in proximity by the zona, but are not joined per se. Indeed, a breach in the zona could lead to a loss of cells. In terms of their physiology, individual blastomeres lack sophisticated means of regulating their homeostasis, such as pH control, and are therefore more dependent

upon the maternal tract to provide the appropriate environment (Gardner and Lane, 2005; Lane and Gardner, 2005). Between the 8- to 16-cell stages, the embryo undergoes a significant change, with the formation of the first transporting epithelium, achieved through the creation of tight junctions between adjacent cells. This facilitates not only a means to control the internal environment of the embryo, but also creates an inside and out, with cells developing on the inside of the embryo being destined to become the fetus, while those cells on the outside become allocated to extra-embryonic membranes such as the placenta (Fleming and Johnson, 1988). Down the microscope, the appearance of individual blastomeres is lost at the 8- to 16-cell stage as the cells flatten or compact against each other, forming not only tight junctions at the apical surface of the cells, but also forming gap junction between cells. This process is aptly referred to as compaction, and the embryo becomes known as the morula. At this stage the embryo resembles a mulberry, and it is from this that the name morula was derived. Rather than continuing to develop as a solid ball of cells, the outer cells of the morula start to create a cavity through the action of Na/K ATPases on their basolateral membranes, thereby creating an ionic gradient, inducing the formation of a fluid-filled cavity, known as the blastocoel (Kidder and Watson, 2005). The embryo is then referred to as the blastocyst. Cells that have been developing on the inside of the embryo develop into a distinct group known as the inner cell mass (ICM), while the outer cells become the trophoblast (TE). While the pluripotent ICM cells are destined to form the fetus, the trophoblast cells have the incredible task of invading into the maternal endometrium lining the uterus, establishing a blood supply, and preventing the mother from rejecting the nonself embryo. Fig. 1 shows the spatial positioning of the human preimplantation embryo as it develops within the lumen of the female reproductive tract, while the detailed characteristics of human embryo development are illustrated in Fig. 2.

The complex dialogue that exists between the blastocyst and endometrium is far from understood, but what we can deduce is that cancers employ the same biological strategies as the trophoblast to invade a host tissue and establish its own vasculature (Gardner, 1998a,b, 2015). Indeed, analysis of the implantation process has much to inform us about cancers.

Dynamics of Embryo Physiology From the Fertilized Oocyte to the Blastocyst

From Fertilization to the 4 to 8-cell stage the human embryo is dependent upon stable mRNAs and proteins synthesized during oocyte maturation. Around this time the embryonic genome begins to be transcribed, and between the morula and blastocyst stages

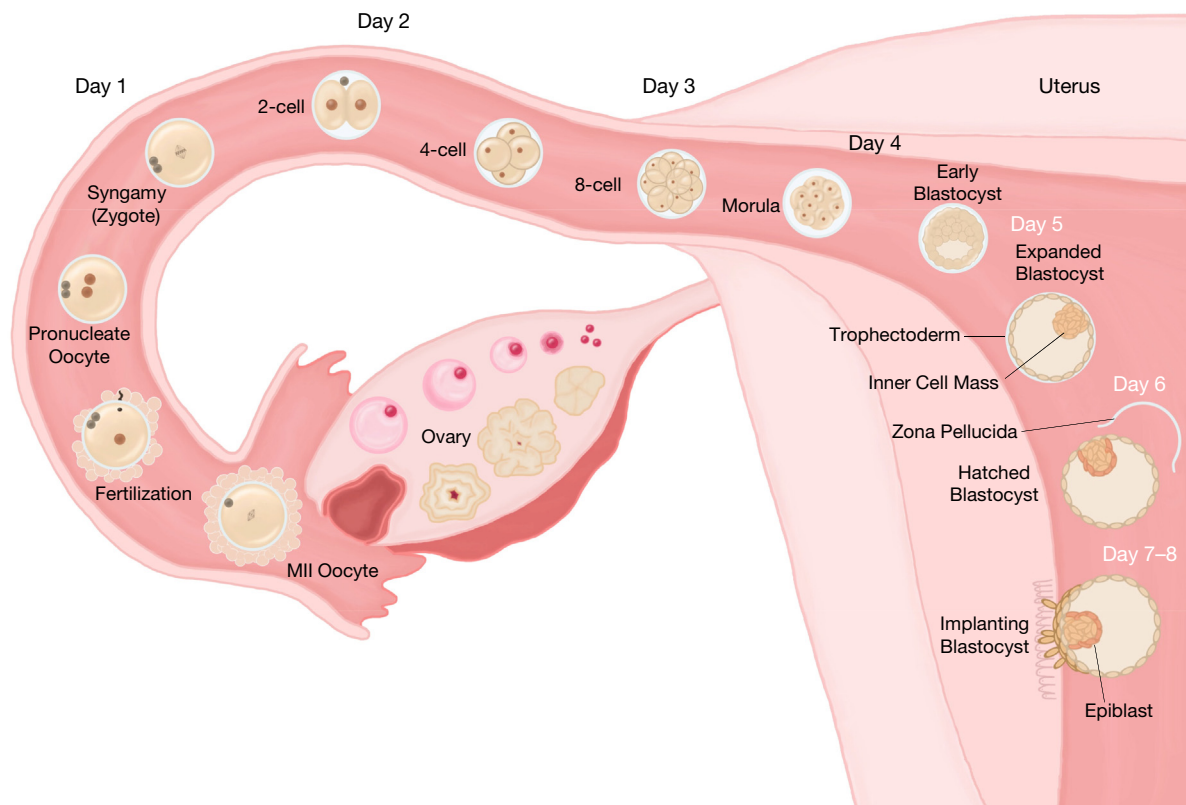


Fig. 1 Schematic of the human female reproductive tract and the relative position of the preimplantation embryos as it develops and differentiates. From fertilization in the ampulla of the oviduct, through to implantation in the endometrium 6–7 days later, the human embryo undergoes remarkable development and differentiation, all the while being exposed to an ever changing environment. The oviduct is characterized by relatively high levels of pyruvate and lactate and an oxygen concentration of around 8%. In contrast, uterine fluid is characterized by relatively high levels of glucose and lower concentration of oxygen. Further, the uterine environment is one containing numerous cytokines and growth factors.

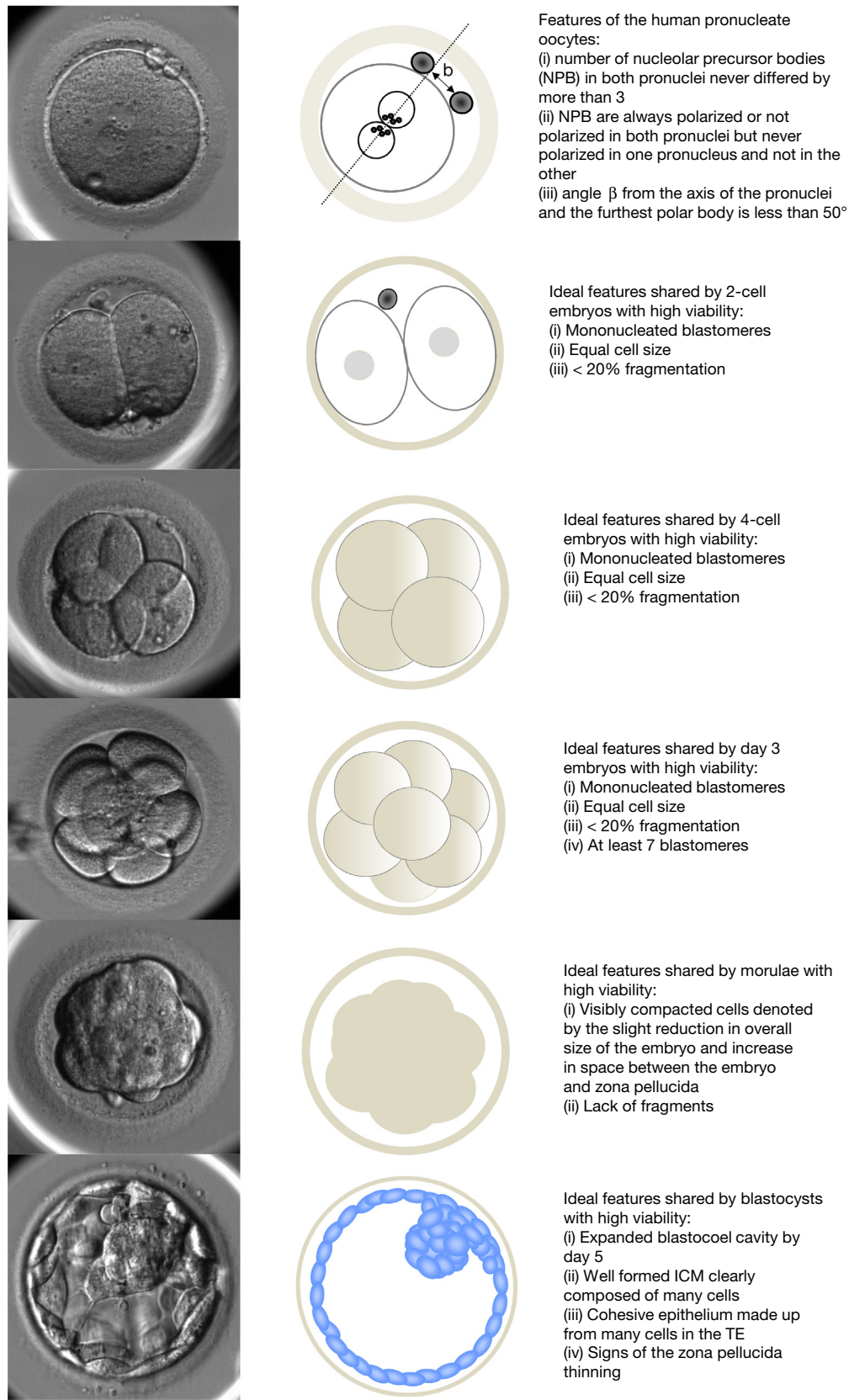


Fig. 2 Key morphological features of embryos with high viability. ICM: inner cell mass, TE: trophectoderm. Gardner, D.K., Balaban, B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and OMICS: Is looking good still important? *Molecular Human Reproduction* 22, 2016, 704–718 with permission.

there is a dramatic increase in embryonic gene expression, so that by the blastocyst stage the embryonic genome is active (Hamatani et al., 2006; Jukam et al., 2017). As the human embryo progresses through the preimplantation period, it undergoes changes in morphology, exhibits an exponential increase in cell number, and forms its blastocoel. Underlying all of this are remarkable changes in metabolism; the embryo changes the relative activity of specific pathways as a means of fulfilling the changing requirements as development proceeds. The metabolic transformation has typically been perceived as a means to an end with regards to developmental processes. However, it is now perceived that in some cases it is the metabolism of the embryo that regulates specific processes (Gardner, 2015; Gardner and Harvey, 2015), as will be elucidated.

The human oocyte is a truly remarkable cell given that it can reside in the ovary for up to 40 years and still give rise to a viable pregnancy. In order to achieve this, the oocyte exhibits a unique metabolism, one based primarily on low levels of oxidation of pyruvate, with little to no ability to utilize glucose. Oxygen consumption by the embryo remains low until after compaction (Kurosawa et al., 2016). The quiescent nature of the oocyte's existence means that its energy demands are extremely low. When a cell does not utilize energy, any ATP synthesized is not immediately used, and hence the cell is characterized by a high ATP:ADP ratio, and this is true of the oocyte (Leese et al., 1984). A direct consequence of this high ratio is the allosteric inhibition of a rate-limiting enzyme in glycolysis, phosphofructokinase (PFK), hence explaining why the oocyte cannot metabolize glucose. The fertilized oocyte shares the same metabolism as the unfertilized egg. Indeed, it is not until the 4- to 8-cell stage that the embryo can utilize glucose as an energy source. As the embryo commences its divisions, and cell number increases, there is a concomitant increase in the demand for energy, which is required not solely for cell proliferation, but also for the formation and maintenance of the blastocoel (the latter representing a constant energetic demand). Consequently, the absolute levels of ATP decline as it is rapidly utilized, resulting in a reduction in the ATP:ADP ratio, and a concomitant increase in AMP, both of which have a positive allosteric effect on PFK, and thereby facilitating a high flux of glucose through glycolysis. Oxygen consumption subsequently rises significantly upon formation of the blastocyst. However, rather than oxidizing all of the glucose consumed, the blastocyst exhibits an idiosyncratic trait referred to as aerobic glycolysis, where lactate is produced from glucose even when there is sufficient oxygen for its complete oxidation. This type of glucose metabolism was first described in cancers by Otto Warburg in 1923, and is hence referred to as the Warburg Effect. Warburg proposed that the high levels of lactate produced by tumors were a result of impaired mitochondrial function, however this does not appear to be the case. Certainly the mitochondria of the blastocyst have the capacity for oxidative metabolism. So what is the significance of this rather intriguing situation?

Although energetically unfavorable, aerobic glycolysis ensures that the biosynthetic arm of the pentose phosphate pathway (PPP) has maximum substrate availability at all times. As with all proliferating cells, there is a demand for biosynthetic precursor provision for membrane and nucleic acid synthesis. Glucose has a significant role in cellular biosynthesis, its carbon being required for the synthesis of triacylglycerols, phospholipids and as a precursor for complex sugars of mucopolysaccharides and glycoproteins. Hence, glucose is required by the morula and blastocysts stages not only for energy but to provide the intermediates for biosynthesis and the ribose moieties for RNA and DNA synthesis (Gardner, 1998a,b; Gardner and Wale, 2013). The complexities and dynamics of human embryo metabolism are highlighted in Fig. 3. Recently, the significance of aerobic glycolysis for the blastocyst implantation has been re-visited (Gardner, 2015). It is proposed that the human blastocyst has the capacity to create a microenvironment, characterized by high lactate and reduced pH, both of which facilitate the breakdown of the endometrial extracellular matrix, are involved in angiogenesis and actively help to modulate the local immune response to the blastocyst. It seems that embryonic metabolism is far more than energy production.

Human Embryo Culture

Given the dramatic differences in the physiology and metabolism of the embryo before and after compaction, optimizing the culture of the human preimplantation embryo has been challenging. However, through analysis of human female oviduct and uterine fluid, sequential media were developed, capable of supporting the human embryo throughout the preimplantation period (Gardner and Lane, 1997), leading to increased implantation rates (Gardner et al., 1998). Today, blastocyst transfer is considered the standard of care in human IVF. Of significance, oxygen is a powerful regulator of the preimplantation embryo, and hence whenever possible, human embryos should not be exposed to atmospheric oxygen (~20%), but rather cultured in the presence of more physiological levels, i.e., ~5%.

Analysis of Development and Viability: Morphology

What are the key morphological parameters during the cleavage stages that are associated with subsequent embryo viability and implantation potential post transfer? The percentage and localization of fragmentation, evenness of the blastomeres, multinucleation and rate of cleavage are features typically assessed to evaluate the quality of a cleavage stage embryo on day 2 and day 3 (Gardner and Balaban, 2016). The common characteristics of embryos with high viability include 4 or 5 blastomeres on day 2, and at least 7 blastomeres on day 3 after fertilization, with < 20% fragmentation and no signs of multinucleation. Rienzi et al. (2005) suggested a cumulative classification scheme based on the most essential morphological features that should be examined to evaluate a cleavage stage embryo. Morphological parameters selected were cleavage rate, blastomere symmetry, cytoplasmic appearance, extent of fragmentation and blastomere nucleus status. Such a grading scheme effectively predicted blastocyst formation and

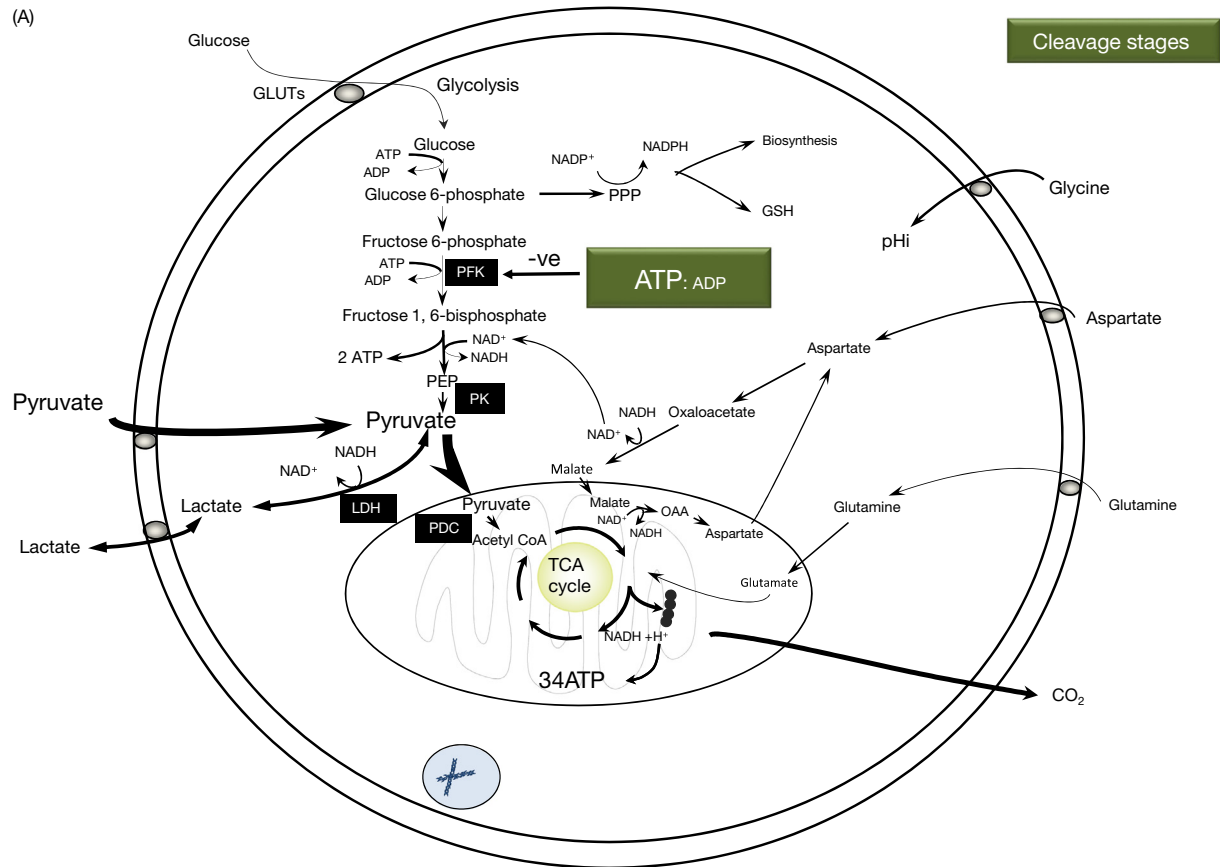


Fig. 3 (A) *Metabolism of the pronucleate oocyte and cleavage stage embryo.* Prior to compaction the embryo has a metabolism based around low levels of oxidation of pyruvate, lactate and specific amino acids. The ovulated oocyte is surrounded by, and is directly connected to, cumulus cells which actively produce pyruvate and lactate from glucose. This creates a high concentration of pyruvate and lactate and a low concentration of glucose around the fertilized oocyte. Upon dispersal of the cumulus cells, the human zygote and cleavage stage embryo still find themselves in a relatively high concentration of pyruvate (0.32 mM) and lactate (10.5 mM), and low levels of glucose (0.5 mM) within the ampulla. The early embryo is characterized by a high ATP:ADP level, which in turn allosterically inhibits PFK, thereby limiting the flux of glucose through the glycolytic pathway prior to compaction. Significantly, the relative abundance of nutrients affects the metabolism of the embryo. For example, the ratio of pyruvate:lactate in the surrounding environment directly affects the ratio of NADH:NAD⁺ in the embryo, which in turn controls the redox state of the cells and hence the flux of nutrients through specific energy generating pathways. Amino acids fill several niches in embryo physiology, such as the use of glycine as buffer of intracellular pH (pHi). Several amino acids are also utilized as energy sources by the early embryo such as glutamine and aspartate. Aspartate can be utilized through the malate-aspartate shuttle, the significance of which during embryo development we are only beginning to understand. The thickness of the lines represents the relative flux of metabolites through that pathway. GLUTs, glucose transporters; GSH, reduced glutathione; LDH, lactate dehydrogenase; OAA, oxaloacetate; PDC, pyruvate dehydrogenase complex; pHi, intracellular pH; PFK, phosphofructokinase; PK, pyruvate kinase; PPP, pentose phosphate pathway. (B) *Metabolism of the blastocyst.* After compaction the embryo exhibits greatly increased oxygen consumption and an increased capacity to use glucose as an energy source. The increase in oxygen consumption plausibly reflects the considerable energy required for the formation and maintenance of the blastocoel, while the increase in glucose utilization reflects an increased demand for biosynthetic precursors. Consequently, there is a reduction in the ATP:ADP ratio, and a concomitant increase in AMP, which will have a positive allosteric effect on PFK, thereby facilitating a higher flux of glucose through glycolysis. Rather than oxidize the glucose consumed, the blastocyst exhibits high levels of aerobic glycolysis. Although this may appear energetically unfavorable, it does ensure that the biosynthetic arm of the pentose phosphate pathway (PPP) has maximum substrate availability at all times. Activity of the PPP will ensure reducing equivalents are available for biosynthesis and ensure production of glutathione (reduced), a key intracellular antioxidant. In order for high levels of glycolysis to proceed the blastomeres need to regenerate cytosolic NAD⁺. This can be achieved through the generation of lactate from pyruvate. A second means of generating cytosolic NAD⁺ is through the activity of the malate-aspartate shuttle. Although it is evident that blastocysts do use aerobic glycolysis, it is proposed here that the significant increase in oxygen utilization at this stage of development could be largely attributed to the activity of the malate-aspartate shuttle and the resulting demand for oxygen to convert intramitochondrial NADH to ATP. Indeed many tumors that exhibit aerobic glycolysis also have high levels of malate-aspartate shuttle activity. Furthermore, inhibition of this shuttle has dire consequences for subsequent fetal development. The thickness of the lines represents the relative flux of metabolites through that pathway. ACL, acetyl-citrate lyase; GLUTs, glucose transporters; GSH, reduced glutathione; LDH, lactate dehydrogenase; OAA, oxaloacetate; PDC, pyruvate dehydrogenase complex; pHi, intracellular pH; PFK, phosphofructokinase; PK, pyruvate kinase; PPP, pentose phosphate pathway. Adapted from Gardner, D.K. and Wale P.L. Analysis of metabolism to select viable human embryos for transfer. *Fertility and Sterility* 99, 2013, 1062–1072 with permission.

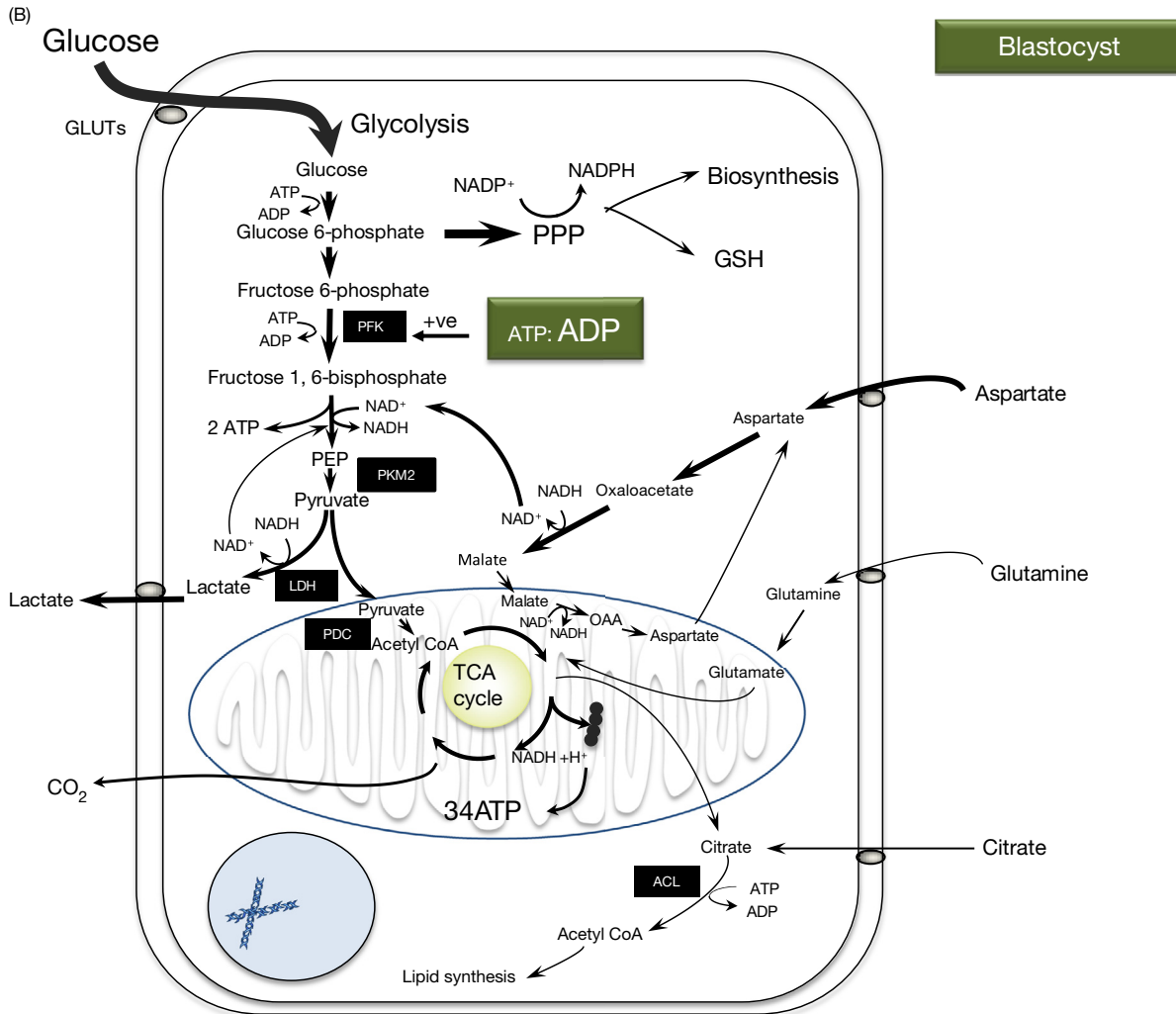


Fig. 3 (continued).

implantation. Day 1 pronuclear (PN) morphology and nucleolar precursor body (NPB) ratio, day 2 cell number, blastomere symmetry and nucleation and the ability to cleave from day 2 to day 3 appear to be the six most significant factors associated with subsequent fetal development.

Several studies have considered the relative weight of such cleavage stage morphological parameters and revealed that cell number, equal blastomere size and the number of mono-nucleated blastomeres on day 2 were the most predictive markers of blastocyst development and implantation potential. Rienzi et al., reported that cell number is the most critical factor determining the viability fate of the embryo, not only on day 2 but also on day 3, inferring that the optimal development can be defined as 4 or 5 cell on day 2 at 44–46 h post insemination, and more than 6 cells (7–9 cell) on day 3 at 66–68 h postinsemination (Rienzi et al., 2005). In agreement with such reports, the latest Cochrane review on cleavage stage versus blastocyst stage embryo transfer suggested that the most favorable group for blastocyst transfers were the good prognosis patient group that had higher number of 8 cell embryos on day 3 (Glujovsky et al., 2012). Evidence based data clearly demonstrates that several morphological features of the cleavage stages are associated with implantation potential and/or successful blastocyst formation.

Analysis of blastocyst morphology initially focused on the rate of expansion, and the determination of cell allocation to the two lineages of the inner cell mass (ICM) and trophoctoderm (TE). An advantage of assessing the blastocyst is that one is assessing the embryo proper (cleavage stage morphology reflects primarily oocyte competence). Determining the rate of expansion alone has physiological value, as the formation of the blastocoel is energetically demanding, and hence the embryo must possess a degree of metabolic competency to create the ionic gradient required to allow fluid to accumulate within the morula and coalesce to form a blastocoel. Analysis of the degree of expansion can be difficult after a blastocyst collapses. Interestingly, the thickness of the zona reflects each blastocyst's "expansion history," as an expanding blastocyst will induce changes to the shape of the surrounding zona, which does not revert back to its original form when the TE collapses.

Gardner and Schoolcraft (1999) therefore developed a comprehensive alphanumeric system, designed to incorporate the degree of blastocoel expansion, as well as the quality and cell number of the two cell lineages. Herein, blastocyst expansion rate is characterized by numbers from the early blastocyst to the hatched stage. Analysis of both the ICM and TE quality can then be performed for expanded blastocysts in which both cell types are clearly evident. ICM and TE quality were characterized by three grades, A to C. ICM grades: A, a tightly packed ICM with many cells; B, loosely grouped ICM with several cells, or C, very few cells and disorganized. TE grades: A, denoting TE with many cells forming a cohesive epithelium; B, few cells forming a loose epithelium; and C, very few large cells. (see Fig. 2 for the morphological characteristics of a 4AA blastocyst). Using this scoring system a strong correlation between the clinical outcome (implantation rates, pregnancy and twinning rates) and the morphological grade of blastocysts was established (Gardner et al., 2000). Subsequently, Ahlstrom and colleagues determined that although blastocyst expansion and ICM grade were significant predictors in both univariate and multivariate analyses, they were not predictors of live birth when analyzed by stepwise logistic regression, rather the TE was the most important determinant of successful transfer outcome (Ahlstrom et al., 2011). Such observations have been further supported by Ebner et al. (2016).

The physiology underlying the significance of the TE grade may reside in the significance of the TE during the initiation and progression of implantation, and that a healthy TE ensures acceptable implantation. A larger number of TE cells will be able to ensure greater signaling and interaction with the endometrium. Interestingly, although the TE produces human chorionic gonadotropin (hCG) as one of the earliest signals to the mother, it has recently been proposed that lactate production by the blastocyst, which will be derived primarily from the TE, has several key functions in regulating the implantation process (Gardner, 2015). These physiological roles endorse importance of a good TE grade in predicting implantation potential.

In spite of the importance of the TE grade, it remains important to include the ICM grade in order to determine true blastocyst quality, as it transpires that all three parameters of the blastocyst (degree of expansion, ICM and TE quality) are significantly associated with pregnancy and live birth rates (Van den Abbeel et al., 2013). Transfer of blastocysts with an “A” (top quality) grade ICM further reduces the incidence of pregnancy loss. Further, studies have also determined that ICM grade is positively associated with birth weight (Licciardi et al., 2015). Considering the quality of both the ICM and TE makes physiological sense as they do not exist in isolation, but rather coexist as a functional unit, whereby the TE which creates a unique environment for the ICM by the synthesis of blastocoel fluid, and the ICM itself that regulates the proliferation and activity of the TE (Ansell and Snow, 1975).

Analysis of Development and Viability: Screening for Aneuploidy

Through the development of blastocyst culture, it is now feasible to analyze biopsies taken from the TE of human embryos in order to determine the genetic constitution of the embryo (Schoolcraft et al., 2010). Technologies for the analysis of chromosome complement from a single or small group of cells has advanced significantly over recent years, and are now used worldwide to identify aneuploid embryos, thereby decreasing the time to pregnancy for patients of ages 38 and older (Gardner et al., 2015). However, even though this approach is able to identify aneuploid embryos to a high level of accuracy, not all euploid embryos are viable, and hence there remains considerable research into identifying biomarkers of viability (Sanchez et al., 2017).

Analysis of Development and Viability: Metabolism

Given the dynamics and complexity of embryonic metabolism, it is not surprising that should an embryo at any stage of development lose control of its energy production, i.e., if the flux of a specific nutrient through a metabolic pathway is altered to a significant degree, even for a brief period, then this is associated with significantly impaired development in culture and reduction of viability post transfer (Lane and Gardner, 2005). Proof of concept data was first reported in 1980 by Renard et al., who observed a positive relationship between glucose uptake by day 10 cow blastocysts and subsequent pregnancy after transfer (Renard et al., 1980). This relationship between blastocyst glucose uptake and the establishment of viable pregnancies was later confirmed in the mouse (Gardner and Leese, 1987). Early attempts to quantitate human embryo metabolism focused predominantly on the cleavage stages, given that it was not feasible to culture the human embryo to the blastocyst stage until the late 1990s. Hardy et al. (1989) reported that pyruvate uptake of embryos which arrested was lower than those which developed normally. With the development of more physiological media in the 1990s (Barnes et al., 1995; Gardner, 1994), it became possible to analyze the metabolism of the later stage embryo prior to transfer. Early studies revealed that blastocysts with the same alpha-numeric score, e.g., 3AA, from the same patient exhibited a wide range of glucose uptakes, indicating that morphological grade was not directly related to physiological status of the embryo (Gardner et al., 2001). Given the significance of glucose uptake by blastocysts in animal models, the uptake of glucose by individual human embryos on day 4 and 5, followed by single embryo transfer, allowed for the relationship between nutrient uptake and subsequent pregnancy to be established. Consistent with the animal data, glucose uptake by those embryos which were considered viable by the delivery of a healthy baby, exhibited a significantly higher glucose uptake on both day 4 and day 5 of development, thereby establishing in the human that embryo viability post compaction is positively associated with a high glucose uptake (Gardner et al., 2011) (Fig. 4). Furthermore, it was observed that female embryos consume significantly more glucose than males on day 4 of development. This phenomenon was previously reported in the mouse and attributed to the window in development in which both X-chromosomes are active in female embryos (Gardner et al., 2010; Gutierrez-Adan et al., 2006).

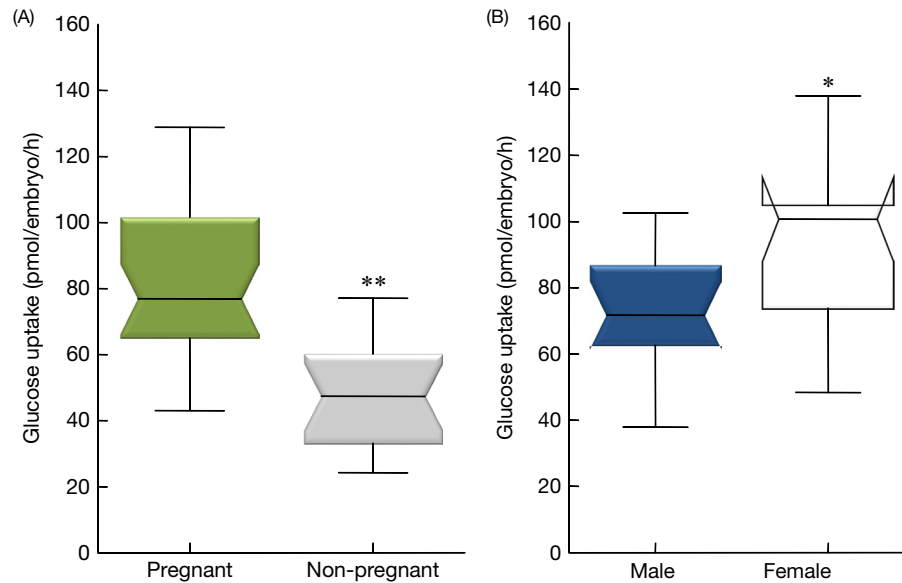


Fig. 4 Glucose uptake on day 4 of embryonic development and pregnancy outcome (positive fetal heart beat). Notches represent the interquartile range (50% of the data), whiskers represent the 5% and 95% quartiles. The line across the box is the median glucose consumption. Significantly different from pregnant; ** $P < 0.01$. (B) Glucose uptake by male and female embryos on day 4 of development. Significantly different from male embryos; * $P < 0.05$. From Gardner, D.K., Wale, P.L., Collins, R. and Lane M. (2011). Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Human Reproduction* **26**, 1981–1986 with permission.

As well as analysis of carbohydrates, Houghton et al. (2002) measured amino acid turnover by individual human embryos and observed different patterns of amino acid utilization between embryos that went on to form a blastocyst and those embryos that failed to develop. Those embryos that did develop, consumed more leucine from the culture medium, and the profiles of alanine, arginine, glutamine, methionine and asparagine flux were related to blastocyst formation (but not necessarily blastocyst quality). In a follow up study, Brison et al. observed changes in the levels of amino acids in the spent medium of human zygotes cultured for 24 h to the 2-cell stage. Asparagine, glycine and leucine were all significantly associated with clinical pregnancy and live birth (Brison et al., 2004). Subsequently it was determined that there also appear to be gender differences with regards to amino acid utilization (Picton et al., 2010; Sturme et al., 2010). Similar to the data on carbohydrate use, these data await validation in a larger patient population. Further, it is possible to use both data from time-lapse incubation, together with metabolic analysis of the embryo, to gain further insight into development and to facilitate selection models based on multiple parameters (Lee et al., 2015).

Conclusions

The preimplantation embryo resides within a changing environment in vivo, and undergoes dramatic morphological and physiological changes within the first 5 days of life, transforming from a single totipotent cell, to a blastocyst comprising of two distinct cell lineages. Underlying these changes are the transition from maternal to embryonic genome control, together with a shift from a metabolism based on low levels of oxidation of pyruvate, to one based on high levels of glucose uptake, which is subsequently metabolized by aerobic glycolysis (the Warburg effect). The commonality of glucose metabolism shared between blastocysts and cancers reflects their proliferation, and their ability to invade the endometrial tissue and establish a blood supply, while avoiding the maternal immune system (Gardner, 2015). Using physiological data, it has been possible to construct better culture media to support the development and viability of human embryos conceived through IVF, thereby increasing the overall success of this procedure. The ability to culture the human embryo to the blastocyst stage has not only led to higher success rates but facilitated the introduction of trophectoderm biopsy for preimplantation genetic screening. Analysis of embryonic kinetics and metabolism holds promise for identification of a viability marker to select embryos for transfer, and together with genetic screening, can help to reduce time to pregnancy for infertile couples.

References

- Ahlstrom, A., Westin, C., Reiser, E., Wikland, M., & Hardarson, T. (2011). Trophectoderm morphology: An important parameter for predicting live birth after single blastocyst transfer. *Human Reproduction*, **26**, 3289–3296.
- Ansell, J. D., & Snow, M. H. (1975). The development of trophoblast in vitro from blastocysts containing varying amounts of inner cell mass. *Journal of Embryology and Experimental Morphology*, **33**, 177–185.

- Barnes, F. L., Crombie, A., Gardner, D. K., Kausche, A., Lacham-Kaplan, O., Suikkari, A. M., Tiglias, J., Wood, C., & Trounson, A. O. (1995). Blastocyst development and birth after in-vitro maturation of human primary oocytes, intracytoplasmic sperm injection and assisted hatching. *Human Reproduction*, 10, 3243–3247.
- Brison, D. R., Houghton, F. D., Falconer, D., Roberts, S. A., Hawkhead, J., Humpherson, P. G., Lieberman, B. A., & Leese, H. J. (2004). Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Human Reproduction*, 19, 2319–2324.
- Ebner, T., Tritscher, K., Mayer, R. B., Oppelt, P., Duba, H. C., Maurer, M., Schappacher-Tilp, G., Petek, E., & Shebl, O. (2016). Quantitative and qualitative trophectoderm grading allows for prediction of live birth and gender. *Journal of Assisted Reproduction and Genetics*, 33, 49–57.
- Fleming, T. P., & Johnson, M. H. (1988). From egg to epithelium. *Annual Review of Cell Biology*, 4, 459–485.
- Gardner, D. K. (1994). Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biology International*, 18, 1163–1179.
- Gardner, D. K. (1998a). Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology*, 49, 83–102.
- Gardner, D. K. (1998b). Embryo development and culture techniques. In J. Clark (Ed.), *Animal breeding: Technology for the 21st century* (pp. 13–46). London: Harwood Academic.
- Gardner, D. K. (2015). Lactate production by the mammalian blastocyst: Manipulating the microenvironment for uterine implantation and invasion? *BioEssays*, 37, 364–371.
- Gardner, D. K., & Balaban, B. (2016). Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and OMICS: Is looking good still important? *Molecular Human Reproduction*, 22, 704–718.
- Gardner, D. K., & Harvey, A. J. (2015). Blastocyst metabolism. *Reproduction, Fertility, and Development*.
- Gardner, D. K., & Lane, M. (1997). Culture and selection of viable blastocysts: A feasible proposition for human IVF? *Human Reproduction Update*, 3, 367–382.
- Gardner, D. K., & Lane, M. (2005). Ex vivo early embryo development and effects on gene expression and imprinting. *Reproduction, Fertility, and Development*, 17, 361.
- Gardner, D. K., & Lane, M. (2017). Embryo culture systems. In D. K. Gardner, & C. Simon (Eds.), *Handbook of In Vitro Fertilization* (4th edn., pp. 205–244). Boca Raton: CRC Press.
- Gardner, D. K., & Leese, H. J. (1987). Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *The Journal of Experimental Zoology*, 242, 103–105.
- Gardner, D. K., & Schoolcraft, W. B. (1999). In vitro culture of human blastocyst. In R. Jansen, & D. Mortimer (Eds.), *Towards reproductive certainty: fertility and genetics beyond 1999* (pp. 378–388). Carnforth, UK: Parthenon Publishing.
- Gardner, D. K., & Wale, P. L. (2013). Analysis of metabolism to select viable human embryos for transfer. *Fertility and Sterility*, 99, 1062–1072.
- Gardner, D. K., Lane, M., Calderon, I., & Leeton, J. (1996). Environment of the preimplantation human embryo in vivo: Metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertility and Sterility*, 65, 349–353.
- Gardner, D. K., Schoolcraft, W. B., Wagley, L., Schlenker, T., Stevens, J., & Hesla, J. A. (1998). Prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Human Reproduction*, 13, 3434–3440.
- Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., & Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertility and Sterility*, 73, 1155–1158.
- Gardner, D. K., Lane, M., Stevens, J., & Schoolcraft, W. B. (2001). Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertility and Sterility*, 76, 1175–1180.
- Gardner, D. K., Larman, M. G., & Thouas, G. A. (2010). Sex-related physiology of the preimplantation embryo. *Molecular Human Reproduction*, 16, 539–547.
- Gardner, D. K., Wale, P. L., Collins, R., & Lane, M. (2011). Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Human Reproduction*, 26, 1981–1986.
- Gardner, D. K., Meseguer, M., Rubio, C., & Treff, N. R. (2015). Diagnosis of human preimplantation embryo viability. *Human Reproduction Update*, 21, 727–747.
- Glujovsky, D., Blake, D., Farquhar, C., & Bardach, A. (2012). Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database of Systematic Reviews*, 7, CD002118.
- Gutierrez-Adan, A., Perez-Crespo, M., Fernandez-Gonzalez, R., Ramirez, M., Moreira, P., Pintado, B., Loneragan, P., & Rizo, D. (2006). Developmental consequences of sexual dimorphism during pre-implantation embryonic development. *Reproduction in Domestic Animals*, 41(Suppl 2), 54–62.
- Hamatani, T., Ko, M., Yamada, M., Kuji, N., Mizusawa, Y., Shoji, M., Hada, T., Asada, H., Maruyama, T., & Yoshimura, Y. (2006). Global gene expression profiling of preimplantation embryos. *Human Cell*, 19, 98–117.
- Hannan, N. J., Paiva, P., Meehan, K. L., Rombauts, L. J., Gardner, D. K., & Salamonsen, L. A. (2011). Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology*, 152, 4948–4956.
- Hardy, K., Hooper, M. A., Handyside, A. H., Rutherford, A. J., Winston, R. M., & Leese, H. J. (1989). Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Human Reproduction*, 4, 188–191.
- Harris, S. E., Gopichandran, N., Picton, H. M., Leese, H. J., & Orsi, N. M. (2005). Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology*, 64, 992–1006.
- Houghton, F. D., Hawkhead, J. A., Humpherson, P. G., Hogg, J. E., Balen, A. H., Rutherford, A. J., & Leese, H. J. (2002). Non-invasive amino acid turnover predicts human embryo developmental capacity. *Human Reproduction*, 17, 999–1005.
- Jukam, D., Shariati, S. A. M., & Skotheim, J. M. (2017). Zygotic genome activation in vertebrates. *Developmental Cell*, 42, 316–332.
- Kidder, G. M., & Watson, A. J. (2005). Roles of Na,K-ATPase in early development and trophectoderm differentiation. *Seminars in Nephrology*, 25, 352–355.
- Kurosawa, H., Utsunomiya, H., Shiga, N., Takahashi, A., Ihara, M., Ishibashi, M., Nishimoto, M., Watanabe, Z., Abe, H., Kumagai, J., et al. (2016). Development of a new clinically applicable device for embryo evaluation which measures embryo oxygen consumption. *Human Reproduction*, 31, 2321–2330.
- Lane, M., & Gardner, D. K. (2005). Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reproduction, Fertility, and Development*, 17, 371–378.
- Lee, Y. S., Thouas, G. A., & Gardner, D. K. (2015). Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Human Reproduction*, 30, 543–552.
- Leese, H. J. (1988). The formation and function of oviduct fluid. *Journal of Reproduction and Fertility*, 82, 843–856.
- Leese, H. J., Biggers, J. D., Mroz, E. A., & Lechene, C. (1984). Nucleotides in a single mammalian ovum or preimplantation embryo. *Analytical Biochemistry*, 140, 443–448.
- Licciardi, F., McCaffrey, C., Oh, C., Schmidt-Saros, C., & McCulloh, D. H. (2015). Birth weight is associated with inner cell mass grade of blastocysts. *Fertility and Sterility*, 103, 382–387. e382.
- Meseguer, M., Herrero, J., Tejera, A., Hilligsoe, K. M., Ramsing, N. B., & Remohi, J. (2011). The use of morphokinetics as a predictor of embryo implantation. *Human Reproduction*, 26, 2658–2671.
- Miller, J. G., & Schultz, G. A. (1987). Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biology of Reproduction*, 36, 125–129.
- Ng, K. Y. B., Mingels, R., Morgan, H., Macklon, N., & Cheong, Y. (2017). In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: A systematic review. *Human Reproduction Update*, 1–20.
- Picton, H. M., Elder, K., Houghton, F. D., Hawkhead, J. A., Rutherford, A. J., Hogg, J. E., Leese, H. J., & Harris, S. E. (2010). Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Molecular Human Reproduction*, 16, 557–569.
- Renard, J. P., Philippon, A., & Menezes, Y. (1980). In-vitro uptake of glucose by bovine blastocysts. *Journal of Reproduction and Fertility*, 58, 161–164.
- Rienzi, L., Ubaldi, F., Iacobelli, M., Romano, S., Minasi, M. G., Ferrero, S., Sapienza, F., Baroni, E., & Greco, E. (2005). Significance of morphological attributes of the early embryo. *Reproductive Biomedicine Online*, 10, 669–681.
- Sanchez, T., Seidler, E. A., Gardner, D. K., Needleman, D., & Sakkas, D. (2017). Will noninvasive methods surpass invasive for assessing gametes and embryos? *Fertility and Sterility*, 108, 730–737.

- Schoolcraft, W. B., Fragouli, E., Stevens, J., Munne, S., Katz-Jaffe, M. G., & Wells, D. (2010). Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertility and Sterility*, 94, 1700–1706.
- Sturmey, R. G., Bermejo-Alvarez, P., Gutierrez-Adan, A., Rizos, D., Leese, H. J., & Loneragan, P. (2010). Amino acid metabolism of bovine blastocysts: A biomarker of sex and viability. *Molecular Reproduction and Development*, 77, 285–296.
- Thouas, G. A., Potter, D. L., & Gardner, D. K. (2013). Microfluidic devices for the analysis of gamete and embryo physiology. In D. K. Gardner, D. Sakkas, E. Seli, & D. Wells (Eds.), *Human gametes and Preimplantation embryos: Assessment and diagnosis* (pp. 281–299). New York: Springer.
- Van den Abbeel, E., Balaban, B., Ziebe, S., Lundin, K., Cuesta, M. J., Klein, B. M., Helmgard, L., & Arce, J. C. (2013). Association between blastocyst morphology and outcome of single-blastocyst transfer. *Reproductive Biomedicine Online*, 27, 353–361.

Further Reading

- Edwards, R. G. (2000). The role of embryonic polarities in preimplantation growth and implantation of mammalian embryos. *Human Reproduction*, 15(Suppl 6), 1–8.
- Gardner, D. K., & Kelley, R. L. (2017). Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *Journal of Developmental Origins of Health and Disease*, 1–18.
- Gardner, D. K., & Lane, M. (2014). Culture of viable mammalian embryos. In J. Cibelli, R. Lanza, K. Campbell, & M. D. West (Eds.), *Principles of cloning* (pp. 63–84). San Diego: Academic Press.
- Gardner, D. K., & Simon, C. (Eds.). (2017). *Handbook of in vitro Fertilization* (4th edn.). New York: CRC Press. ISBN-13: 978-1498729390.
- Gardner, D. K., Weissman, A., Howles, C., & Shoham, Z. (2017). *Textbook of assisted reproductive technologies: Laboratory and clinical perspectives* (5th edn.). London: Informa.
- Harvey, A. J., Rathjen, J., & Gardner, D. K. (2016). Metaboloepigenetic regulation of pluripotent stem cells. *Stem Cells International*, 2016, 1816525.
- Leese, H. J. (2012). Metabolism of the preimplantation embryo: 40 years on. *Reproduction*, 143, 417–427.
- Swain, J. E., Lai, D., Takayama, S., & Smith, G. D. (2013). Thinking big by thinking small: Application of microfluidic technology to improve ART. *Lab on a Chip*, 13, 1213–1224.
- Wale, P. L., & Gardner, D. K. (2016). The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Human Reproduction Update*, 22, 2–22.

Morphokinetic of Embryo Development

Ana Elena Palma Govea, IVI-RMA Panama, Panama City, Republic of Panama
Marcos Meseguer Escriba, IVI-RMA Valencia, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

One of the main failures in assisted reproduction is the selection of the best embryo, a single embryo that is able to implant and thus reduce the risk of adverse outcomes related to multiple gestations. This has led to the incorporation of new technologies in in vitro fertilization laboratories, which aim to find specific embryo viability markers to select the best embryo, with greater implantation potential, without having a negative impact on pregnancy rates.

Historically, the embryo selection has been based on the morphological characteristics that occur at different stages of development. The most commonly used technique for embryonic evaluation is the serial evaluation through a standard microscope at certain developmental times, because it is considered specific, sensitive, and safe technique (Cummins et al., 1986; Scott, 2003). But it has negative consequences for embryo development: culture stability, observation frequency, limited available information (Payne et al., 1997). This results in a high degree of observatory-dependent subjectivity.

All this has been associated with relatively low success rates (pregnancy rate ~30% per transfer) (Andersen et al., 2009).

The use of morphokinetic parameters, obtained through time-lapse systems, together with the morphological parameters used so far, seems to be the best formula for embryo selection.

Time-Lapse Technologies Available on the Market

There are several time-lapse options available on the market: Primo Vision (Vitrolife) and The Eeva Test (Merck) consist of a microscope that is placed into the standard incubator. Other technologies, such as EmbryoScope (Vitrolife), Geri (Genea Biomedex), and Miri TL (Esco Medical) consist of a whole system integrated in a single equipment.

It is based in specially designed instruments, which take images of embryos every 15–20 min automatically, without having to take the embryos outside the incubator to minimize manipulation.

This also provides additional info to the embryologist, being able to evaluate embryos from a dynamic point of view by studying exact timings of some important parameters of the embryo.

Table 1 provides a summary of the technical characteristics of the different time-lapse models available in the market.

Time-Lapse Imaging and Morphokinetic

Single point morphological assessment of embryos and gametes are still considered the gold standard around the world. Technologies that are used to define embryo implantation potential or endometrial receptivity are purely subjective and imprecise.

Nowadays, embryologists use a combination of the traditional and sequential embryo morphological assessment with the intention to increase the precision of the diagnostics.

The selection of embryos to be transferred is based on the morphological criterion established in the work of the IVF laboratory: cell number, % fragmentation, asymmetry (Fig. 1). However, the study of cellular activity during the early stages of embryonic development provides additional information that can later be used in the selection process (Basile et al., 2012).

Table 1 Summary of technical characteristics of the different models of time-lapse available in the market

Technical characteristics	Primo Vision (Vitrolife)	The Eeva test (Merck)	EmbryoScope (Vitrolife)	Geri (Genea Biomedex)	Miri TL (Esco Medical)
Optics	Bright field	Dark field	Bright field	Bright field	Bright field
Microscope/ incubator	Microscope that can be placed in standard incubators	Microscope that can be placed in standard incubators	Incubators with integrated time-lapse	Incubators with integrated time-lapse	Incubators with integrated time-lapse
Embryo imaging	Pictures every 10 s	Pictures every 5 s	Pictures every 10 s	Pictures every 5 s	Pictures every 5 s
Focal planes	1	1	7	10	4
Capacity—patients	1/camera	1/camera	6/system	6/system	6/system
Embryo culture	16 group culture	12 group culture	12 individually culture	16 individually culture	14 individually culture
Data analyses	Manual	Real time	Manual	Manual	Manual

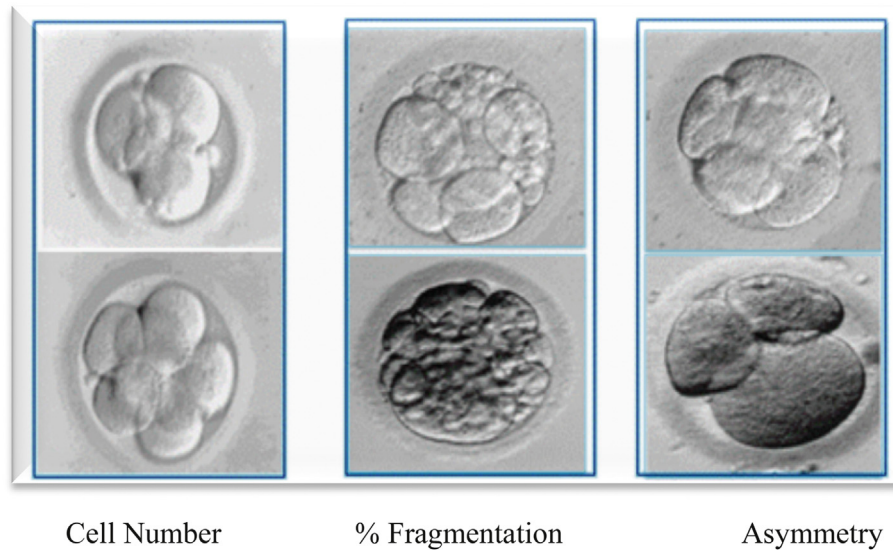


Fig. 1 Traditional morphological evaluation.

The first study based on time-lapse observations was published by [Payne et al. \(1997\)](#), where 50 fertilized eggs were observed. These eggs were chosen at random for time-lapse observation.

The study showed, it was possible to increase the time of observation, conserving the optimal conditions of culture.

Evaluations based on image analysis systems add objectivity to the process of embryo selection ([Basile et al., 2012](#)).

In contrast to the routine methods of embryonic evaluation traditionally used, time-lapse systems offer not only the precise determination of cell divisions but also the follow-up of morphological events such as appearance and resorption of fragments ([Van Blerkom et al., 2001](#); [Handarson et al., 2002](#)), formation of compaction, appearance of the blastocoe cavity and time of contraction of the blastocyst.

This helps us to avoid misinterpretations between a large fragment that could be confused with a blastomere, to categorize a 2PN fertilization when a third pronucleus may have been at some point and thus a series of events occurring throughout embryonic development.

The importance of the correlation between the number of cells and the stage of embryo development has been widely described. In recent years, the importance of knowing the time between fertilization and the first embryo division has become known, and can be used as an objective parameter that can be easily determined with some predictive value of embryonic viability ([Sakkas et al., 1998](#)).

In 1997, Shoukir was the first to demonstrate embryos had completed their division 25-h postinsemination (hpi) after conventional IVF had significantly better rates of gestation and implantation compared to those embryos that did not have been divided at this time.

In 1998, Sakkas published a study where he could verify that the results are the same as those published by Shoukir in embryos from ICSI cycles, where insemination times are more precise compared to traditional IVF.

In 2010, Wong et al. presented an analysis of 100 embryos that were cultured until day 5 or 6 of development, in which they correlated time-lapse imaging and molecular data throughout embryo development to the blastocyst stage. They found three morphokinetic parameters to predict blastocyst formation ([Cruz et al., 2012](#)) ([Fig. 2](#)):

- P1—Second cell cycle length: 14.3 ± 6.0 min (duration of the two-cell stage, CC2)
- P2—Synchrony of the second and third cell divisions: 11.1 ± 2.2 h (duration of the three-cell stage, S2) and
- P3—The duration of the second cell divisions: 1.0 ± 1.6 h (the cytokinesis giving rise to two daughter cells)

This study concluded that the development from the embryo to the blastocyst stage could be predicted with 94% sensitivity and 93% specificity after using these parameters. It was expected that embryos that did not meet one or more of these parameters would be stopped.

In 2011, Meseguer et al. published a study where optimal ranges were proposed for certain variables based in the analysis of 247 transferred embryos with known implantation (KID) that were culture in a time-lapse system, and it developed a hierarchical model that subdivided embryos into six categories from A to F. Categories from A to D were further subdivided into two subcategories (+) or (−).

The hierarchical classification begins with a morphological screening of the embryo cohort to eliminate those embryos that are clearly not viable (atresia, embryos that stop its development). These embryos are discarded and are not considered for transfer (category F).

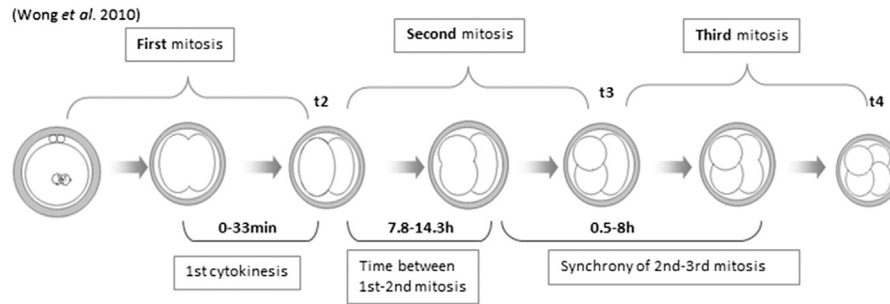


Fig. 2 Model algorithm proposed by Wong et al. (2010). Three parameters were found as predictors of blastocyst formation: P1—duration of the first cytokinesis, P2—interval between the end of the first mitosis and the initiation of the second mitosis, and P3—the synchrony between the second and third mitosis. Adapted from Aparicio, B., Cruz, M., Meseguer, M. 2013. Is morphokinetic analysis the answer? *Reproductive BioMedicine Online* 27, 654–663.

The next step is to rule out embryos that meet any of the following exclusion criteria (i) DC: direct cleavage from zygote to the three-blastomere embryo, defined as: $cc2 = t3 - t2 < 5$ h, (ii) UBS: uneven blastomere size at the two-cell stage during the interphase where the nuclei are observable or (iii) MN: multinucleation at the four-cell stage during the interphase where the nuclei are visible (category E) (Fig. 3).

The subsequent levels for the model follow a strict hierarchy based on the binary timing variables $t5$, $s2$, and $cc2$. The precise timings of numerous morphokinetic parameters were identified considering microinjection as period zero. First cleavage was considered finished when the newly formed blastomeres were completely separated by a clearly defined membrane. Absolute time of division into two cells was called $t2$, to three cells $t3$, to four cells $t4$, and to five cells $t5$. Cell cycle duration was also evaluated, the second cell cycle ($cc2$) being the duration of period as a two-blastomere embryo (i.e., $t3 - t2$) and the degree of synchrony of the second cell division ($s2$), defined as the duration of the transition period from a two-blastomere embryo to a four-blastomere embryo ($t4 - t3$), which corresponds with the duration as the period as a three-blastomere embryo (Basile et al., 2012).

On the condition that the value of $t5$ is within the optimum range (48.8–56.6 h), the embryo is categorized as A or B. If the value of $t5$ is outside the desirable range (or if $t5$ has not yet been observed at 64 h), the embryo is categorized as C or D. If the value of $s2$ is within the ideal range (≤ 0.76 h) the embryo is classified as A or C depending on $t5$; similarly, if the value of $s2$ falls outside the favorable range, the embryo is categorized as B or D depending on $t5$. Lastly, the embryo is categorized with the extra plus (+) if the value of $cc2$ is within the favorable range (≤ 11.9 h) ($A+/B+/C+/D+$) and categorized with a negative value ($A-/B-/C-/D-$) if the value for $cc2$ is outside the optimal range (Figs. 3 and 4).

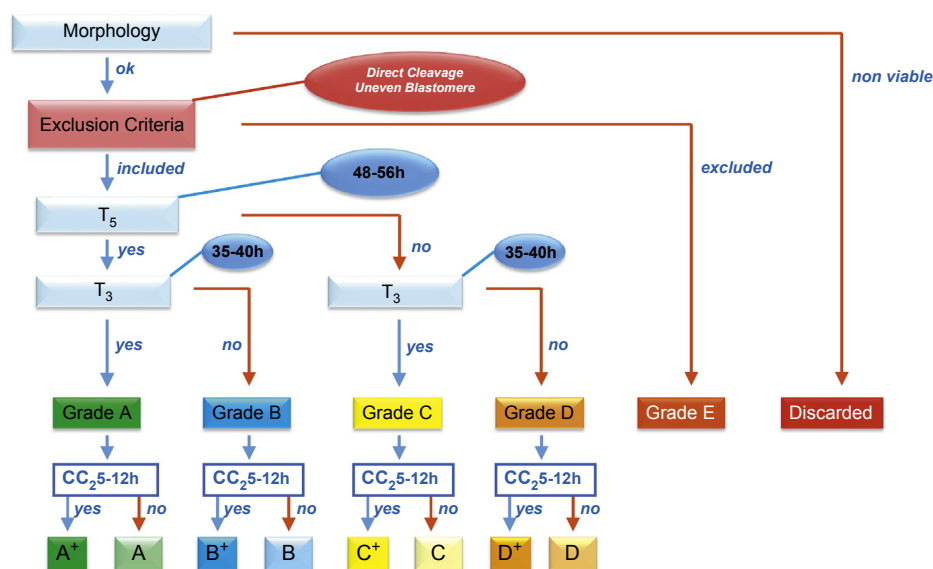


Fig. 3 Algorithm proposed by Meseguer et al. (2011). Developed a hierarchical model that subdivided embryos into six categories from (A) to (F). Four of these categories (A–D) were further subdivided into two subcategories (+) or (–).

(Meseguer *et al.* 2011)

Fig. 4 Model algorithm proposed by Meseguer *et al.* (2011). The model follows a strict hierarchy based on the binary timing variables t5, s2, and cc2. Adapted from Aparicio, B., Cruz, M., Meseguer, M. 2013. Is morphokinetic analysis the answer? *Reproductive BioMedicine Online* 27, 654–663.

In 2013, Conaghan *et al.* carried out a study where the utility of combining a time-lapse system with the traditional morphological evaluation for the selection of embryo was tested. Three distinct embryologists, from different centers, used two D3 evaluations (only morphology or morphology + time-lapse) to select which embryos in division would reach the blastocyst stage. As a result, when they used only morphology in D3, the three embryologists selected embryos with a specificity of 59.7%, 41.9%, and 79.5% and a positive predictive value (PPV) of 45.5%, 41.5%, and 50.5%. When time-lapse information was added to the D3 morphology, each embryologist improved his blastocyst selection to transfer with a specificity of 86.3% ($P < 0.0001$), 84.0% ($P < 0.0001$), and 86.6% ($P < 0.01$) and PPV of 56.3% ($P < 0.05$), 52.1% ($P < 0.05$), and 55.5% ($P = 0.34$).

In 2014, Rubio *et al.* published a study, where they validated the algorithm presented by Meseguer in 2011. In this study, 405 patients were chosen in the control group, in which the embryos were selected only by morphology and 438 patients in the study group, where the embryos were selected by the algorithm.

As a result, they had a significant increase in implantation rates in the study group (44.9%, 95% CI, 41.4–48.4) versus the control group (37.1%, 95% CI, 33.6–40.7).

In 2015, Basile *et al.* published a study presenting an improved version of the Meseguer algorithm. The study was divided into two phases. During the first phase, 754 KID embryos that had been selected only by morphological criteria were taken into account. They considered the morphokinetic data of these embryos and created an algorithm (variables: t3, cc2, and t5, criteria of exclusion: DC, UBS, and MN). In the next phase, the predictive capacity of this modern algorithm was tested, applying it to a fresh group of patients (885 cycles, embryos with known implantation = 1137).

As result, they obtained a significant decline in implantation rate when the embryo categories were reduced from A to E (A: 32%, B: 28%, C: 26.25%, D: 20.19%, and "E" 17% $P < 0.001$).

Is Morphokinetics Affected in Chromosomally Abnormal Embryos?

Basile *et al.* (2014) published a study combining time-lapse technology and PGS.

A total of 504 embryos were biopsied on day 3 of development and analyzed by arrayCGH, of these 143 had normal chromosomal content. Significant differences were observed between normal and abnormal embryos for t5, cc2, cc3, and t5–t2.

Embryos that were within the optimal range for t5 (47.2–58.2 h), cc3 (11.7–18.2 h), and t5–t2 (> 20.5 h) showed a significantly higher proportion of normal embryos than those outside these ranges.

The variables t5–t2 ($OR = 2853$; 95% CI, 1763–4616) and cc3 ($OR = 2095$; 95% CI, 1356–3238) were identified as the most relevant, related to normal chromosomal content. They created a hierarchical model that subdivided the embryos into four categories (A–D): if the value of t5–t2 falls within the optimal range, the embryo is categorized as A or B; if the value of t5–t2 falls outside the optimal range, the embryo is categorized as C or D. If the value of cc3 falls within the optimal range, the embryo is categorized as A or C depending on the value t5–t2; similarly, if the cc3 value falls outside the optimal range, the embryo is categorized as B or D, depending on t5–t2.

The algorithm increases the probability of selecting chromosomally normal embryos, although this by no means replaces the techniques of chromosomal analysis, such as array CGH (Fig. 5).

However, in that same year, Rienzi *et al.* (2014) published a study where a total of 455 blastocyst (day 5 or 6 development) were biopsied and analyzed performing real-time polymerase chain reaction.

In this study, they performed a hierarchical classification of euploid embryos based on the interval for completion of cleavage from 2 to 5 cells, and the duration of the third cell cycle (CC3) recently described by Basile *et al.* (2014).

Of 455 blastocysts analyzed, it was found euploidy in 186 (40.9%), single aneuploidy in 167 (36.7%), and complex aneuploidies in 102 (22.4%). Morphokinetic parameters of preimplantation embryo development were similar between euploid and aneuploid embryos.

No significant difference in the percentage of chromosomally normal embryos was found when embryos were divided into four categories according to the hierarchical classification proposed by Basile *et al.* (2014).

They concluded that PGS is currently the only diagnostic procedure for aneuploidy detection.

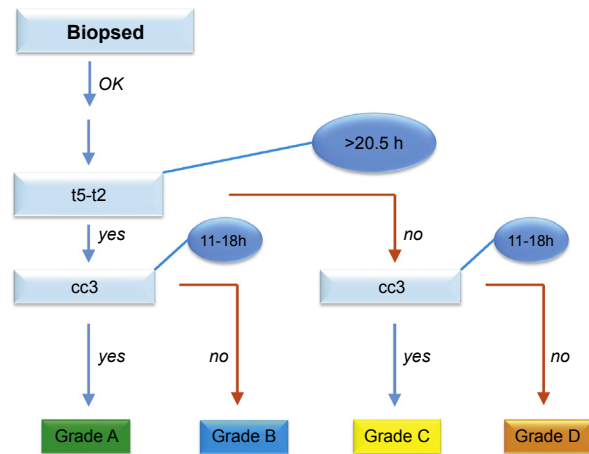


Fig. 5 An algorithm for embryo selection that classifies embryos from (A) to (D) with significant differences in the percentage of normal embryos. Based from Basile, N., Nogales, M.C., Bronet F., et al. 2014. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertility and Sterility* **101**, 699–704.

Conclusions

In the last years many studies have been published where it is shown that the selection of embryos through kinetic markers improves reproductive results. Time-lapse technology is no longer an experimental tool, since there are many studies that support it.

The study of embryo kinetics through time-lapse systems, continue to give us new markers for embryo selection and has become a powerful tool to know the cellular activity that we could not see before using standard microscopes.

Many studies prove that kinetic information in conjunction with traditional morphology reduces interobserver variability and significantly enhances the embryologist's ability to select viable embryos with high implantation potential.

There are still many studies underway to evaluate and confirm the power of embryo selection, using time-lapse systems to obtain even higher rates of single live births at home, reducing multiple pregnancies. That is our goal. Time-lapse technology represents a powerful tool in AR.

References

- Andersen, A. N., Goossens, V., & Bhattacharya, S. (2009). Assisted reproductive technology and intrauterine inseminations in Europe, 2005: Results generated from European registers by ESHRE. The European IVF Monitoring Program (EIM), for the European Society of Human Reproduction and Embryology (ESHRE). *Human Reproduction*, *24*, 1267–1287.
- Basile, N., Garcia-Velasco, J., & Meseguer, M. (2012). Evaluation of embryo quality: Time-lapse imaging to asses embryo morphokinesis. In D. Gardner, A. Weissman, C. Howless, & S. Seev (Eds.) (4th edn) *vol. 1. Textbook of assisted reproductive techniques: Laboratory perspectives* (pp. 254–265). Boca Raton, FL: Informa Healthcare.
- Basile, N., Nogales, M. C., Bronet, F., et al. (2014). Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetic analysis. *Fertility and Sterility*, *101*, 699–704.
- Basile, N., Vime, P., Florensa, M., et al. (2015). The use of morphokinetic as a predictor of implantation: A multicentric study to define and validate an algorithm for embryo selection. *Human Reproduction*, *30*(2), 276–283.
- Conaghan, J., Chen, A. A., Willman, S. P., et al. (2013). Improving embryo selection using a computer-automated time-lapse image analysis test plus day 3 morphology: Results from a prospective multicenter trial. *Fertility and Sterility*, *100*, 412–419.
- Cruz, M., Garrido, N., Herrero, J., et al. (2012). Timing of cell division in human cleavage-stage embryos is linked with blastocyst formation and quality. *Reproductive Biomedicine Online*, *25*, 371–381.
- Cummins, J. M., Breen, T. M., Harrison, K. L., et al. (1986). A formula for scoring human embryo growth rates in in vitro fertilization: Its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *Journal of in Vitro Fertilization and Embryo Transfer*, *3*, 284–295.
- Handarson, T., Löfman, C., Coull, G., et al. (2002). Internalization of cellular fragments in a human embryo: time-lapse recordings. *Reproductive Biomedicine Online*, *5*, 36–38.
- Meseguer, M., Herrero, J., Tejera, A., et al. (2011). The use of morphokinetic as a predictor of embryo implantation. *Human Reproduction*, *26*(10), 2658–2671.
- Payne, D., Flaherty, S. P., Barry, M. F., & Matthews, C. D. (1997). Preliminary observations on polar body extrusion and pronuclear formation in human oocytes using time-lapse video cinematography. *Human Reproduction*, *12*, 532–541.
- Rienzi, L., Capalbo, A., Stoppa, M., et al. (2014). No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor prognosis patients: A longitudinal cohort study. *Reproductive Biomedicine Online*, *30*, 57–66.
- Rubio, I., Galan, A., Larreategui, Z., et al. (2014). Clinical validation of embryo culture and selection by morphokinetic analysis: A randomized, controlled trial of the EmbryoScope. *Fertility and Sterility*, *102*(5), 1287–1294. e5.
- Sakkas, D., Shoukir, Y., Chardonnes, D., et al. (1998). Early cleavage of human embryos to the two-cell stage after intracytoplasmic sperm injection as an indicator of embryo viability. *Human Reproduction*, *13*, 182–187.
- Scott, L. (2003). The biological basis of non-invasive strategies for selection of human oocytes and embryos. *Human Reproduction Update*, *9*, 237–249.
- Van Blerkom, J., Davis, P., & Alexandre, S. (2001). A microscopic and biochemical study of fragmentation phenotypes in stage-appropriate human embryos. *Human Reproduction*, *16*, 719–729.
- Wong, C. C., Loeweke, K. E., Pan, S., et al. (2010). Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nature Biotechnology*, *28*, 1115–1121.

Embryo Biopsy: Polar Body, Cleavage Stage and Trophectoderm

Danilo Cimadomo, GENERA Centers for Reproductive Medicine, Roma, Italy; and GENETYX Molecular Biology Laboratory, Marostica, Italy

Maurizio Poli, REPROOMICS, Amsterdam, Netherlands

Valeria Romanelli, GENETYX Molecular Biology Laboratory, Marostica, Italy

Adriano Giancani, GENERA Centers for Reproductive Medicine, Roma, Italy; and GENETYX Molecular Biology Laboratory, Marostica, Italy

Laura Rienzi and Filippo Maria Ubaldi, GENERA Centers for Reproductive Medicine, Roma, Italy

Antonio Capalbo, GENERA Centers for Reproductive Medicine, Roma, Italy; and GENETYX Molecular Biology Laboratory, Marostica, Italy

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromosomal mosaicism Presence of two or more cell lines with different karyotypes within the same embryo. Either aneuploid mosaicism (two or more cell lines, all aneuploid) or euploid/aneuploid mosaicism (two or more cell lines, of which one is euploid) is possible.

Positive (or negative) clinical predictive value Rate of blastocysts predicted to be euploid (or aneuploid) after the comprehensive chromosome testing of a trophectoderm biopsy, that actually result in a full-term healthy life birth (or implantation failure).

Preimplantation Genetic Testing (PGT) Diagnostic methodology aimed at the detection of aneuploidies (PGT-A) and/or monogenic disorders (PGT-M) or chromosomal structural rearrangements (PGT-SR) in in vitro generated embryos.

Reproductive competence Potential of an embryo to result in a full-term live birth.

Preface

Preimplantation genetic testing (PGT) was introduced at the beginning of the 1990s as an alternative to prenatal diagnosis (PND) for the prevention of inheritable genetic disorders transmission in couples at increased reproductive risk. The first application of PGT was aimed at preventing the passing on of recessive X-linked disorders (e.g. adrenoleukodystrophy and X-linked mental retardation). To do this, sex chromosomes were detected and only female embryos were transferred ([Handyside et al., 1990](#)). Since then, genetic analysis techniques are undergoing continuous evolution and improvement. Indeed, we can report how it now encompasses the diagnosis of several recessive and dominant inheritable conditions. Given the recent increase of applications, as a basic concept, it is interesting to describe the difference between PGT for monogenic diseases (PGT-M) and chromosomal structural rearrangements (PGT-SR) and for aneuploidies (PGT-A).

PGT-M involves the detection of inheritable single gene mutations and/or unbalanced chromosomal aberrations. This type of testing is performed during an in-vitro fertilization (IVF) treatment cycle and it allows the identification of the embryos free of the genetic mutation that can be selected for transfer. To carry out this analysis, it is necessary that the cause of the genetic condition (mutation/chromosomal abnormality) had been identified in the affected parent. Nowadays, PGT-M can be considered a valid and established alternative to PND, as it can be employed for all genetic indications typically detected in PND. PGT-M is effective for single-gene disorders (genetic conditions that are the result of a single mutated gene), HLA typing, hereditary cancers-predisposing genes with variable penetrance (BRCA 1/2 status), late-onset genetic diseases (Huntington's Disease) and for some cases mitochondrial diseases. At present, over 4000 disorders have been linked to single-gene mutations; of these, the most common genetic conditions tested with PGT-M are Cystic Fibrosis, Tay-Sachs, Fragile X, Myotonic Dystrophy and Thalassemia. PGT-SR is an effective procedure for the detection of unbalanced derivatives from patients with chromosomal structural rearrangements. PGT-A is instead indicated for those couples at increased risk of producing embryos with chromosomal abnormalities, and can be employed also where no familial history of a genetic condition is present. Indications for PGT-A include:

- recurrent pregnancy loss (RPL), where patients have experienced 3 or more consecutive miscarriages after natural or assisted conception;
- repeated implantation failure (RIF), usually defined when patients have undergone 3 or more IVF treatments resulting in no embryo implantation;
- advanced maternal age (AMA), usually where the maternal age is above 35 years.

In PGT-A, embryo's karyotype is tested for the presence of aneuploidies (missing or additional part or entire chromosomes), the leading cause for implantation failure and increased miscarriage rate in women older than 35. The goal of PGT-A is to identify chromosomally-abnormal embryos and exclude them from transfer. By transferring only chromosomally-normal ones, the impact

of aneuploidies upon implantation failure and miscarriage can be excluded and the outcomes of IVF treatments can be significantly improved (Chen et al., 2015; Dahdouh et al., 2015).

Technical aspects and clinical implementation strategies of PGT techniques have evolved over the last decade. A critical issue for any PGT protocol is embryo biopsy. The biopsy procedure can be performed at different stages of embryo preimplantation development. Both embryo developmental stage at which the biopsy is performed and the characteristics of the cellular material retrieved have intrinsic features that impact diagnostic accuracy and post-biopsy embryo survival. Ideally, the gold-standard approach should be the least invasive and the most informative. Firstly, as for any new clinically-implemented technology, embryo viability and its reproductive potential must not be compromised. Secondly, the cellular material retrieved from the embryo must mirror the actual embryo genetic constitution with the highest possible accuracy, leading to a reliable genetic diagnosis and sufficient clinical positive predictive values.

In this chapter, the three main approaches for embryo biopsy will be examined: (1) polar body (PB) biopsy from the metaphase II oocyte and/or the zygote, (2) blastomere biopsy at the cleavage stage, and (3) trophectoderm (TE) biopsy at the blastocyst stage (see Fig. 1; Table 1 as a summary of the workflow of the different biopsy protocols, and Table 2 for pros and cons of each approach).

The ESHRE PGD consortium data from 2009 to 10 (Moutou et al., 2014) highlighted that blastomere biopsy alone accounted for almost 90% of the total number of biopsies, whilst TE biopsy was used in <1% of cases. Later, ESHRE data from 2012 to 13 showed a rapid shift from PB and cleavage stage biopsy procedures (approximately 2% and 75% respectively) towards blastocyst stage biopsy (increased to around 23% of all biopsies). This trend is foreseen to continue in the next years. In this chapter, the reasons behind this significant change in practice and the scientific evidence gained from preclinical and clinical studies over the last decade will be covered.

Blastomere Biopsy at the Cleavage Stage

Cleavage stage embryo biopsy is performed on Day 3 of in vitro development, when the embryo has between 6 and 10 cells. The procedure consists of opening the zona pellucida and collecting one blastomere that will be subjected to the genetic analysis (Table 1).

Zona opening can be performed by Tyrode acid, mechanical piercing, or laser-assisted methods. Clinical outcomes after the use of the three methods are similar, as it has been reported in several randomized controlled trials (RCTs) on sibling embryos (for a review see Cimadomo et al., 2016). However, the laser-assisted method is the most commonly used zona drilling technique, representing 75% of all biopsy procedures declared in the ESHRE PGD consortium data collection XII (Moutou et al., 2014). Probably, the reasons for the prevalence of laser-assisted method resides in its standardization and reproducibility, as it is less operator-dependent, less time-consuming and requires a shorter learning curve. Additionally, it has been shown that zona opening by laser does not appear to affect neither technical nor clinical outcomes (Taylor et al., 2010). Nevertheless, the procedure of zona pellucida opening can have an impact on subsequent preimplantation development affecting the hatching process and preventing embryo implantation. After zona pellucida opening, the removal of the selected blastomere is facilitated by the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free medium. The lack of these elements allows the loosening of cell-cell bonds, thus ensuring a less traumatic impact of the biopsy on the

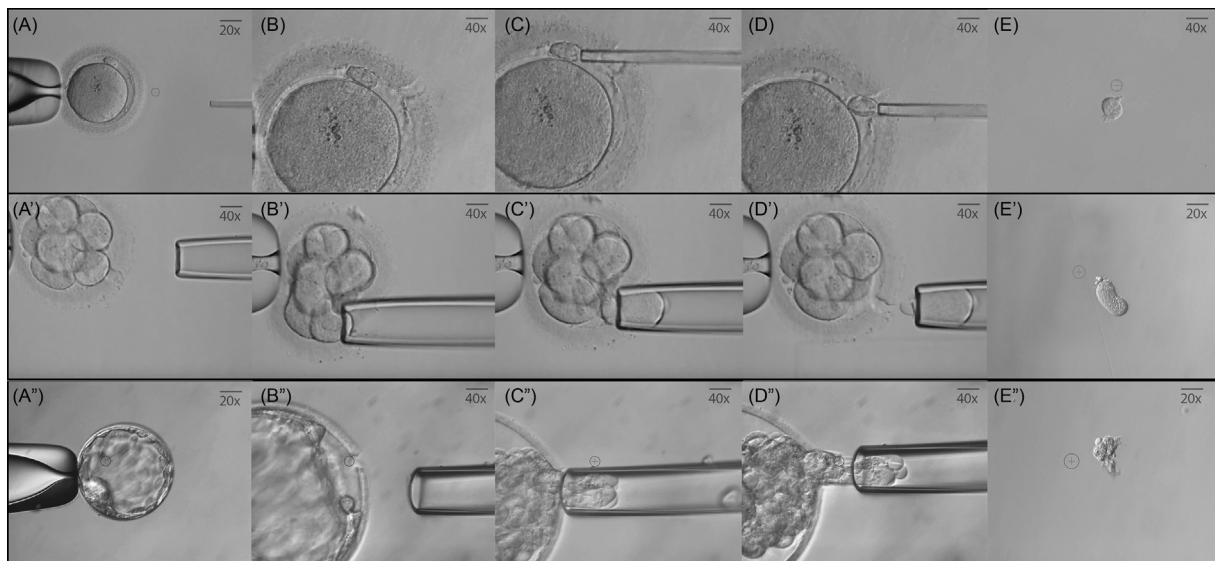


Fig. 1 Polar body biopsy at Day 0/1 of preimplantation development (A–E); cleavage stage biopsy at Day 3 of preimplantation development (A'–E'); trophectoderm biopsy at the blastocyst stage (A''–E''). Zona opening is always performed using laser. A holding pipette anchors the embryos, whilst the biopsic specimen is collected using a biopsy pipette. The magnifications of the pictures are 20 × and 40 ×.

Table 1 Workflow of the different biopsy approaches

	<i>Day 0 Egg collection Insemination</i>	<i>Day 1 fertilization check</i>	<i>Day 3 cleavage stage</i>	<i>Day 5 blastocyst</i>	<i>Day 6 blastocyst</i>	<i>Day 7 blastocyst</i>
Polar body biopsy	Biopsy of 1st polar body (prior to ICSI)	Biopsy 2nd polar body (post fertilization check)	Fresh transfer and/or cryopreservation	Fresh embryo transfer and/or cryopreservation		
Blastomere biopsy	–	–	Zona drilling and blastomere biopsy	Fresh embryo transfer and/or cryopreservation		
Trophectoderm biopsy approach 1	–	–	Zona drilling	TE biopsy and cryopreservation if no fresh transfer Slower embryos remain in culture	Fresh embryo transfer TE biopsy of additional embryos and cryopreservation	TE biopsy of additional embryos and cryopreservation
Trophectoderm biopsy approach 2	–	–	–	Sequential zona drilling, TE biopsy and cryopreservation if no fresh transfer Slower embryos remain in culture	Fresh embryo transfer Sequential zona drilling, TE biopsy of additional embryos and cryopreservation	Sequential zona drilling, TE biopsy of additional embryos and cryopreservation
Trophectoderm biopsy approach 3	–	–	–	Zona drilling + monitoring until the hatching of few TE cells, biopsy and cryopreservation if no fresh transfer Slower embryos remain in culture	Fresh embryo transfer Zona drilling + monitoring until the hatching of few TE cells, biopsy and cryopreservation if no fresh transfer	Zona drilling + monitoring until the hatching of few TE cells, biopsy and cryopreservation if no fresh transfer

embryo. However, negative effects of Ca^{2+} -depleted media have also been reported, with impacts on the compaction process, cytoskeleton remodeling and intercellular communication mechanisms (Sefton et al., 1996). Additionally, there is growing evidence that even at this early developmental stage, human embryos cells are already committed to a specific lineage and the removal of one random blastomere can interfere with cell differentiation processes and embryo's ability to correctly develop and implant. From an operational point of view, these variables are likely to reduce both clinical reproducibility and technical standardization of the approach. Moreover, one study demonstrated that zona pellucida drilling and blastomere removal compromise the hatching process and lead to the development of smaller and delayed blastocyst, with a thicker zona pellucida (Kirkegaard et al., 2012). Clear evidence about the detrimental effects of cleavage stage biopsy on embryo reproductive competence was provided by a randomized paired study performed by Scott et al. (Scott et al., 2013). In their investigation, they compared the implantation rate of sibling top quality embryos obtained from the same stimulation cycle, transferred in double ETs, where one of them was randomized for biopsy and the other used as paired control. The identity of the implanted embryo was confirmed by DNA fingerprinting performed on the biopsied cell and the fetal DNA. Thanks to this elegant design, they demonstrated a dramatic 39% relative decrease in implantation rate after blastomere biopsy, revealing that cleavage stage biopsy approach does compromise embryo reproductive competence.

Despite nowadays robust genetic technologies are available for the analysis of low DNA input samples, cleavage stage biopsy still suffers from technical and biological drawbacks associated with single cell analysis. Specifically, the fact that embryo's genome is represented in a single set derived from a single cell can give rise to technical problems, should some of the DNA be lost or degraded during blastomere collection. The lack of certain parts of the genome could lead to the generation of artifacts. This occurrence potentially causes false positive and false negative results, thus compromising the reliability of the diagnosis. These artifacts can give rise to erroneous analysis, as from a few loci to whole chromosomes could be under- or over-amplified. These types of misdiagnosis include: (i) Allele drop-out (ADO, random loss of one allele in favor of the other variant); (ii) Preferential amplification (PA,

Table 2 Comparison between the 3 different biopsy methods

<i>Polar body biopsy</i>	<i>Day 3 blastomere biopsy method</i>	<i>Day 5/6/7 TE biopsy method</i>
PROs • Analysis performed on waste products of female meiosis • Compatible with fresh ET after genetic diagnosis • Allowed in those countries where embryo biopsy is not.	• Vast experience worldwide • Embryos reach the cleavage stage in a synchronous fashion (day 3 of preimplantation development) • Standardized approach • Compatible with fresh ET	• Selection of TE cells to biopsy. The ICM is not involved • Robust diagnosis (5–10 cells are retrieved and analyzed) • High compatibility with TLM and single media culture systems • No impact on embryo reproductive competence • Highly cost-effective (biopsy of developmentally-competent embryos only) • Low amplification failure rate ($\approx 1\%$) • The IVF laboratory must be experienced in extended embryo culture and vitrification. • Limited experience worldwide
CONS • Analysis limited to the maternal genome (excludes paternal genome and potential mitotic errors) • Biopsy and analysis of both PBs required (time-consuming and not cost-effective procedure) • Technical issues associated to single cell-based analysis • High amplification failure rate ($\approx 10\%$)	• High impact on embryo reproductive competence • Technical issues associated with single cell-based analysis • Lowest diagnostic reliability • High amplification failure rate ($\approx 10\%$)	

ET, embryo transfer; TE, trophectoderm; ICM, inner cell mass; TLM, time lapse microscopy; PBs, polar bodies.

over-amplification of specific genomic regions or even of whole chromosomes); (iii) Chimerical DNA molecules formation; (iv) high rate of DNA amplification failure (Harton et al., 2011).

Furthermore, none of the contemporary molecular methods for single-cell analysis can distinguish between a cell in G1-, S- or G2/M-phase of the cell cycle. This limitation can inevitably give rise to false negative/positive results in the case a cell is at an intermediate point of the S-phase (DNA replication phase), when it is processed (Van Der Aa et al., 2013).

From a diagnostic point of view, one of the main limitations of cleavage stage biopsy is chromosomal mosaicism, which consists in the coexistence of two or more karyotypically different cell lines in the same embryo. This is caused by mitotic errors occurring in a cell at any point after the first cell division. This process is mainly induced by aberrant cell-replication mechanisms such as anaphase lagging, non-disjunction, endoreplication and structural events of DNA damage of chromatid/chromosome breakage leading to structural rearrangements (e.g. translocations) or segmental aneuploidies (Coonen et al., 2004; Daphnis et al., 2005). Mosaicism has been reported to reach its highest level at this stage of preimplantation development, but all the reported estimates of mosaicism are impacted by the low technical accuracy of the methods used to detect aneuploidy (e.g. FISH). In fact, the estimated rates that have been published to date range from 10 up to 90% (Capalbo et al., 2016b) and are likely overestimated, considering the much lower incidence of mosaicism detected in spontaneous conceptions (1.22%) and in IVF pregnancies (1.32%), where true fetal mosaicism accounts only for 0.3%–0.44% (Huang et al., 2009).

The limited predictive power for embryo implantation that derives from blastomere analysis was clearly highlighted in an important study where its positive clinical predictive value was assessed (Scott et al., 2012). This study showed that the rate of euploid embryos leading to a sustained implantation after a cleavage stage PGT-A diagnosis is lower than 30%.

In conclusion, despite the vast experience in its use worldwide, cleavage stage biopsy is going to be gradually abandoned, given the large evidence on the technical limitations of single cell analysis and the detrimental effect of the biopsy procedure on the embryo.

Polar Body Biopsy

Polar body (PB) biopsy is a diagnostic approach that was proposed as a valuable alternative to cleavage stage biopsy. The main reason behind this method was that embryonic aneuploidies are mainly derived from oocyte meiotic errors (Hassold and Hunt, 2001; Heffner, 2004). These chromosomal aberrations are accumulated during oocyte's meiotic arrest that lasts until ovulation and remain the main cause of the age-associated decrease of female fertility. Advantages of this approach include the fact that the analysis is performed on waste products of female meiosis without directly involving the embryo, and it is compatible with fresh ET after genetic diagnosis. Additionally, PB biopsy is the only PGT-A method that can be performed in those countries where embryo biopsy is not allowed at any stage of preimplantation development after fertilization.

PB biopsy consists in removing both the first and second PB, at different time points (the first before ICSI and the second at the time of fertilization check around 16 h after insemination) or by a simultaneous approach 16 h after ICSI or after the appearance of

the two pronuclei (PN) (Table 1). The detection of the PN prior to PB biopsy ensures that the zygote's meiotic process is concluded, thus minimizing the risk of enucleation.

The methods for zona drilling during PB biopsy are the same as previously described for blastomere biopsy.

Conflicting data have been reported when dealing with the impact of PB biopsy on subsequent embryo development and no comprehensive study is yet available on embryo implantation potential following PB biopsy. Some papers report no negative effect on oocyte/embryo quality parameters, including oocytes lysis and activation rates, embryo chromosome breakage incidence after calcium ionophore treatment and Tarkowski preparation or on neonatal outcomes. On the other hand, one study reported impaired preimplantation embryo development following PB biopsy, showing higher embryo fragmentation rate, lower embryo quality, higher cleavage arrest rate, and lower mean number of blastomeres on Day 3 compared to the control group (for a review see Cima-domo et al., 2016). However, data about embryo implantation potential was not reported in this study.

The ESHRE PGD Consortium data reports that, since its introduction, PB biopsy never exceeded 10%–15% of all the PGT-A/PGT-M procedures performed in Europe. Nowadays, its application is further decreasing probably due to the number of studies highlighting technical, economical, biological, and clinical limitations underlying this approach.

The main shortcoming of PB biopsy is that the genetic investigation is limited to the maternal genome, excluding both paternal genome contribution and potential post fertilization mitotic errors from the analysis. The reference study highlighting the biological and technical limitations of PB biopsy was published in 2013 (Capalbo et al., 2013a). It was designed as a sequential biopsy associated to aCGH analysis of PBs, blastomere and TE from the same embryo. The data generated led to a comprehensive view on chromosomal segregation patterns from female meiosis and throughout preimplantation development up to the blastocysts stage in advanced maternal age patients. Additionally, it provided the unique possibility to infer the accuracy of PB-based chromosome testing in predicting the chromosomal complement of the resulting embryos. Firstly, this study demonstrated that the procedure is time-consuming and not cost-effective, due to the need of investigating both polar bodies to have a prospect of the oocyte chromosomal contribution to the embryo. In fact, approximately half of oocyte-derived aneuploidies in embryos arise due to segregation errors in the second meiotic division (Ottolini et al., 2015). Secondly, PB diagnostic accuracy in detecting the karyotype of the resulting embryos was questioned. Specifically, on a chromosome basis, concordance between PBs and blastocyst chromosomal complement was as low as 60%. The authors reported high false positive and false negative rates in PB-based diagnoses due to the lack of information regarding paternally- and post-zygotic mitotically derived aneuploidies, as well as meiotic errors corrected at later stages of embryo development. These intrinsic biological and technical limitations of PB analysis compromise its diagnostic accuracy, potentially resulting in either the discarding of reproductively competent embryos, or in the transfer of chromosomally-abnormal ones. Other authors also described a consistent proportion ($\approx 20\%$) of chromosome segregation errors (Handyside et al., 2012) and 17% false positive rate in PB diagnoses, with chromosomal copy number changes in PBs not reflecting the karyotype of the ensuing zygote (Christopikou et al., 2013). Other concerns on PB-based PGT relate to the difficulty in detecting precocious separation of sister chromatids (PSSC) errors balancing in MII and causing reciprocal polar body aneuploidies (Forman et al., 2013).

In synthesis, all this evidence resulted in the gradual abandonment of PBs biopsy approach, in favor of the blastocyst stage one.

Trophectoderm Biopsy

There are three published TE biopsy protocols in the literature. The first was published by McArthur and colleagues in 2005 (McArthur et al., 2005), the second by Capalbo et al. in 2014 (Capalbo et al., 2014), and the third, which is an adaptation of the previous two, by Capalbo in 2016 (Capalbo et al., 2016a). The first method entails a laser-assisted zona pellucida drilling step at the cleavage stage, followed by extended culture to the blastocyst stage. On Day 5, expanded blastocysts (including those with herniating TE cells) can be biopsied, whilst cavitating morulas and early blastocysts are maintained in culture and biopsy is attempted 24 h later. The zona opening at the cleavage stage was thought to promote an artificial TE cells herniation on Day 5 or 6, thus facilitating the TE separation during the biopsy. Although this approach is still the most commonly used, it shows two important drawbacks: (i) The embryo is removed from the incubator twice; the first time, on Day 3 to open the zona pellucida, the second time, at the blastocyst stage when the biopsy is performed (Table 1) (hence, this approach entails an additional exposure of the embryo to suboptimal culture conditions, increasing the risk of impairing its preimplantation development, impacting zona pellucida thinning, blastocyst expansion and overall TE cells count); (ii) There is a risk that the ICM herniates from the opening produced on Day 3, making the biopsy a more challenging procedure.

The second protocol does not entail any manipulation on Day 3 of preimplantation development, so that the embryo is left undisturbed until the blastocyst stage. Blastocyst hatching is not artificially-induced and it is left to occur spontaneously. This strategy allows the selection of the portion of TE to biopsy, rather than having to target the cells extruded through the zona opening. In practice, Day 5 zona opening is performed when the ICM is clearly visible thus not to involve it in the biopsy process. Additionally, undisturbed culture conditions provide a good synchronization of the blastocysts to be biopsied on Day 5, 6 or 7 of preimplantation development, and it is well-suited with the use of a single media-based and/or a time-lapse microscopy-based culture system.

At last, for IVF centers that introduce this approach for the very first time, it can be initially adopted a Day 5/6 hatching strategy as described by Capalbo and colleagues in 2016. In this case, when the blastocyst is fully expanded is removed from the incubator and the zona pellucida is drilled using a laser, then the embryo is returned to the incubator. This intervention boosts the herniation of the TE cells close to the hole in the following hours. Subsequently, these cells are removed from the body of the blastocyst by

micromanipulation, combining gentle suction via the mouth-controlled pipette and dissection via laser beam. This alternative TE biopsy approach may be easier for unexperienced practitioners as it divides the procedure into two separate steps, whilst avoiding the drawbacks of Day 3 zona opening.

The paired randomized study by Scott and colleagues discussed above represents a landmark paper in this field (Scott et al., 2013). In this study, differently from embryos biopsied on Day 3, implantation rates of embryos biopsied on Day 5 were comparable to those of sibling non-biopsied blastocysts. This evidence about the safety of the procedure represented a milestone for the growing application of TE biopsy. The reasons for a higher stress-tolerance of the embryo at this stage of preimplantation development are mainly three: TE is the non-embryonic part of the blastocyst, the biopsy takes place after embryonic genome activation (Day 3) and a limited portion of the embryo biomass is removed (5–10 cells out of ≥ 200).

A diagnosis obtained from a randomly selected TE sample biopsy is extremely accurate. Blastocyst stage biopsy ensures a more robust diagnosis than previous approaches because multiple cells (5–10) are retrieved and analyzed from the embryo. Specifically, all validation studies published to date based on TE biopsy and comprehensive chromosomal screening (CCT), reported 98%–100% of correct prediction of meiotic errors (e.g., Capalbo et al., 2013a; Capalbo et al., 2013b; Fragouli et al., 2011).

Recently, the reliability of blastocyst stage aneuploidy testing using different CCT methods was assessed (Capalbo et al., 2015). This study was designed as a qPCR-based blinded reanalysis of several blastocysts previously diagnosed as aneuploid by aCGH. Whenever a discordant diagnosis was detected, a third biopsy and array-SNP analysis were conducted to define which platform was more prone to false positive/negative results. Importantly, on a chromosome analysis, a consistency of 99.8% between the three CCT methods was reported.

The presence of chromosomal mosaicism in preimplantation embryos, and its impact on the reliability of PGT-A diagnosis are at the center of current debate. In euploid/aneuploid mosaic embryos, aneuploid cells are randomly allocated across ICM and TE as showed by the almost full concordance between the chromosomal constitution of the two cell lineages in several studies (reviewed in Capalbo and Rienzi, 2017; Capalbo et al., 2016b). Moreover, a clear definition of the incidence of mosaicism in blastocyst staged embryos, and the risk of misdiagnosis associated, has been reported in at least four studies and reported to be around 4%–5% (Capalbo et al., 2016b).

A last interesting point deals with the implementation of blastocyst biopsy approach in the clinical practice. From an economic and logistic perspective, this approach currently represents the most cost-effective. Indeed, differently from PB and cleavage stage biopsies, blastocyst biopsy is performed only on developmentally-competent embryos (at least capable of blastocyst development), thus focusing operations and associated costs to the analysis of embryos with demonstrated developmental potential.

Laboratory Requisites for PGT

In the IVF laboratory, certain basic, nonetheless critical prerequisites are required to allow the safe implementation of a blastocyst stage biopsy approach. First, the IVF laboratory must be already experienced and set up for extended embryo culture. An appropriate number of incubators must be present to sustain the workload. It is recommendable not use the incubators at their maximum capacity as this would entail sub-optimal culture conditions (due to shorter recovery time for CO₂ and O₂ levels after each opening) and downstream effects on embryo development. Based on the most compelling scientific evidences recently published, the use of low oxygen tension should be considered mandatory for blastocyst culture (Wale and Gardner, 2016). Furthermore, blastocyst cryopreservation must be an already established, optimized and effective practice before blastocyst biopsy can be implemented in the laboratory. For these reasons, TE biopsy approach is probably perceived as more complicated, less reproducible and standardized than blastomere biopsy. For instance, embryos reach the blastocyst stage in an asynchronous fashion (Day 5–Day 7 of preimplantation development) and they are characterized by heterogeneous morphology. Nevertheless, several papers in the last decade showed no significant correlation between blastocyst aneuploidy rate and embryo developmental rate or static/dynamic morphological evaluation (Capalbo et al., 2014; Rienzi et al., 2015). These data indirectly suggest that every embryo that reaches the blastocyst stage should be biopsied, regardless their morphological grading.

Concluding Remarks and Future Perspectives

According to the data available in the literature, TE biopsy appears to be the most accurate and effective method for PGT purposes. Indeed, from ESHRE PGD consortium data, it is possible to appreciate how TE biopsy is gradually replacing both cleavage stage and PB biopsy approach, and its clinical implementation is expanding worldwide. Specifically, TE biopsy sharply increased from < 1% of the total number of biopsy procedures performed in Europe in 2009–10 to almost 23% in 2012–13. Indeed, over the last decade, PGT-A and PGT-M/SR application has sharply increased. Substantial improvements in embryo biopsy, and cytogenetic and molecular biology techniques were essential to for a more generalized implementation of these strategies. Further improvement will derive from the continuous monitoring of oocyte/embryo quality parameters and clinical outcomes to evaluate the accuracy and efficacy of different PGT methodologies.

Finally, non-invasive or less-invasive methods to perform preimplantation genetic testing are under investigation, namely the evaluation of liquid biopsies as blastocoel fluid and spent blastocyst media as alternative sources of embryonic DNA. Moreover,

novel information could arise in the next years to increase our predictive power upon implantation from static and dynamic blastocyst morphological evaluation in time-lapse, as well as proteomic/metabolomic screening of spent culture media.

References

- Capalbo, A., Bono, S., Spizzichino, L., Biricik, A., Baldi, M., Colamaria, S., Ubaldi, F. M., Rienzi, L., & Fiorentino, F. (2013a). Sequential comprehensive chromosome analysis on polar bodies, blastomeres and trophoblast: insights into female meiotic errors and chromosomal segregation in the preimplantation window of embryo development. *Human Reproduction*, 28, 509–518.
- Capalbo, A., & Rienzi, L. (2017). Mosaicism between trophectoderm and inner cell mass. *Fertility and Sterility*, 107, 1098–1106.
- Capalbo, A., Rienzi, L., Cimadomo, D., Maggiulli, R., Elliott, T., Wright, G., Nagy, Z. P., & Ubaldi, F. M. (2014). Correlation between standard blastocyst morphology, euploidy and implantation: An observational study in two centers involving 956 screened blastocysts. *Human Reproduction*, 29, 1173–1181.
- Capalbo, A., Romanelli, V., Cimadomo, D., Girardi, L., Stoppa, M., Dovere, L., Dell'edera, D., Ubaldi, F. M., & Rienzi, L. (2016a). Implementing PGD/PGD-A in IVF clinics: Considerations for the best laboratory approach and management. *J Assist Reprod Genet*, 33, 1279–1286.
- Capalbo, A., Treff, N. R., Cimadomo, D., Tao, X., Upham, K., Ubaldi, F. M., Rienzi, L., & Scott, R. T., Jr. (2015). Comparison of array comparative genomic hybridization and quantitative real-time PCR-based aneuploidy screening of blastocyst biopsies. *European Journal of Human Genetics*, 23, 901–906.
- Capalbo, A., Ubaldi, F. M., Rienzi, L., Scott, R., & Treff, N. (2016b). Detecting mosaicism in trophectoderm biopsies: Current challenges and future possibilities. *Hum Reprod*, 32, 492–498.
- Capalbo, A., Wright, G., Elliott, T., Ubaldi, F. M., Rienzi, L., & Nagy, Z. P. (2013b). FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. *Human Reproduction*, 28, 2298–2307.
- Chen, M., Wei, S., Hu, J., & Quan, S. (2015). Can comprehensive chromosome screening technology improve IVF/ICSI outcomes? A meta-analysis. *PLoS One*, 10, e0140779.
- Christopikou, D., Tsova, E., Economou, K., Shelley, P., Davies, S., Mastrominas, M., & Handyside, A. H. (2013). Polar body analysis by array comparative genomic hybridization accurately predicts aneuploidies of maternal meiotic origin in cleavage stage embryos of women of advanced maternal age. *Human Reproduction*, 28, 1426–1434.
- Cimadomo, D., Capalbo, A., Ubaldi, F. M., Scarica, C., Palagianio, A., Canipari, R., & Rienzi, L. (2016). The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. *BioMed Research International*, 2016, 7193075.
- Coonen, E., Derhaag, J. G., Dumoulin, J. C., Van Wissen, L. C., Bras, M., Janssen, M., Evers, J. L., & Geraedts, J. P. (2004). Anaphase lagging mainly explains chromosomal mosaicism in human preimplantation embryos. *Human Reproduction*, 19, 316–324.
- Dahdouh, E. M., Balayla, J., & Garcia-Velasco, J. A. (2015). Comprehensive chromosome screening improves embryo selection: A meta-analysis. *Fertility and Sterility*, 104, 1503–1512.
- Daphnis, D. D., Delhanty, J. D., Jerkovic, S., Geyer, J., Craft, I., & Harper, J. C. (2005). Detailed FISH analysis of day 5 human embryos reveals the mechanisms leading to mosaic aneuploidy. *Human Reproduction*, 20, 129–137.
- Forman, E. J., Treff, N. R., Stevens, J. M., Garnsey, H. M., Katz-Jaffe, M. G., Scott, R. T., Jr., & Schoolcraft, W. B. (2013). Embryos whose polar bodies contain isolated reciprocal chromosome aneuploidy are almost always euploid. *Human Reproduction*, 28, 502–508.
- Fragouli, E., Alfarawati, S., Daphnis, D. D., Goodall, N. N., Mania, A., Griffiths, T., Gordon, A., & Wells, D. (2011). Cytogenetic analysis of human blastocysts with the use of FISH, CGH and aCGH: Scientific data and technical evaluation. *Human Reproduction*, 26, 480–490.
- Handyside, A. H., Kontogianni, E. H., Hardy, K., & Winston, R. M. (1990). Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*, 344, 768–770.
- Handyside, A. H., Montag, M., Magli, M. C., Repping, S., Harper, J., Schmutzler, A., Vesela, K., Gianaroli, L., & Geraedts, J. (2012). Multiple meiotic errors caused by predivision of chromatids in women of advanced maternal age undergoing in vitro fertilisation. *European Journal of Human Genetics*, 20, 742–747.
- Harton, G. L., De Rycke, M., Fiorentino, F., Moutou, C., Sengupta, S., Traeger-Synodinos, J., Harper, J. C., & European Society of Human Reproduction and Embryology. (2011). ESHRE PGD consortium best practice guidelines for amplification-based PGD. *Human Reproduction*, 26, 33–40.
- Hassold, T., & Hunt, P. (2001). To err (meiotically) is human: the genesis of human aneuploidy. *Nature Reviews. Genetics*, 2, 280–291.
- Heffner, L. J. (2004). Advanced maternal age—How old is too old? *The New England Journal of Medicine*, 351, 1927–1929.
- Huang, A., Adusumalli, J., Patel, S., Liem, J., Williams, J., 3rd, & Pisarska, M. D. (2009). Prevalence of chromosomal mosaicism in pregnancies from couples with infertility. *Fertil Steril*, 91, 2355–2360.
- Kirkegaard, K., Hindkjaer, J. J., & Ingerslev, H. J. (2012). Human embryonic development after blastomere removal: a time-lapse analysis. *Human Reproduction*, 27, 97–105.
- Mcarthur, S. J., Leigh, D., Marshall, J. T., De Boer, K. A., & Jansen, R. P. (2005). Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertility and Sterility*, 84, 1628–1636.
- Moutou, C., Goossens, V., Coonen, E., De Rycke, M., Kokkali, G., Renwick, P., Sengupta, S. B., Vesela, K., & Traeger-Synodinos, J. (2014). ESHRE PGD Consortium data collection XII: Cycles from January to December 2009 with pregnancy follow-up to October 2010. *Human Reproduction*, 29, 880–903.
- Ottolini, C. S., Newnham, L., Capalbo, A., Natesan, S. A., Joshi, H. A., Cimadomo, D., Griffin, D. K., Sage, K., Summers, M. C., Thornhill, A. R., Housworth, E., Herbert, A. D., Rienzi, L., Ubaldi, F. M., Handyside, A. H., & Hoffmann, E. R. (2015). Genome-wide maps of recombination and chromosome segregation in human oocytes and embryos show selection for maternal recombination rates. *Nature Genetics*, 47(7), 727–735.
- Rienzi, L., Capalbo, A., Stoppa, M., Romano, S., Maggiulli, R., Albricci, L., Scarica, C., Farcomeni, A., Vajta, G., & Ubaldi, F. M. (2015). No evidence of association between blastocyst aneuploidy and morphokinetic assessment in a selected population of poor-prognosis patients: A longitudinal cohort study. *Reproductive Biomedicine Online*, 30, 57–66.
- Scott, R. T., Jr., Ferry, K., Su, J., Tao, X., Scott, K., & Treff, N. R. (2012). Comprehensive chromosome screening is highly predictive of the reproductive potential of human embryos: a prospective, blinded, nonselection study. *Fertility and Sterility*, 97, 870–875.
- Scott, R. T., Jr., Upham, K. M., Forman, E. J., Zhao, T., & Treff, N. R. (2013). Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertility and Sterility*, 100, 624–630.
- Sefton, M., Johnson, M. H., Clayton, L., & McConnell, J. M. (1996). Experimental manipulations of compaction and their effects on the phosphorylation of uvomorulin. *Molecular Reproduction and Development*, 44, 77–87.
- Taylor, T. H., Gilchrist, J. W., Hollowell, S. V., Hanshaw, K. K., Orris, J. J., Glassner, M. J., & Winger, J. D. (2010). The effects of different laser pulse lengths on the embryo biopsy procedure and embryo development to the blastocyst stage. *Journal of Assisted Reproduction and Genetics*, 27, 663–667.
- Van Der Aa, N., Cheng, J., Mateiu, L., Zamani Esteki, M., Kumar, P., Dimitriadou, E., Vanneste, E., Moreau, Y., Vermeesch, J. R., & Voet, T. (2013Van). Genome-wide copy number profiling of single cells in S-phase reveals DNA-replication domains. *Nucleic Acids Research*, e66, 41.
- Wale, P. L., & Gardner, D. K. (2016). The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Human Reproduction Update*, 22, 2–22.

Assisted Hatching: The Physiology of Blastocyst Hatching, the Use of Assisted Hatching in Assisted Reproduction, and a Survey of the Current Evidence Supporting its Use

Jonathan D Kort and Barry Behr, Stanford Fertility and Reproductive Health, Stanford University, Sunnyvale, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction to Physiologic Hatching

Normal preimplantation embryo development requires the developing blastocyst to hatch out of its glycoprotein “shell”—the zona pellucida—before the embryo can interact with and implant into the endometrium (Sun et al., 2012). The physiology of this process is a coupling of the development and expansion of the blastocoel cavity with the thinning, and eventual disruption of the zona pellucida (Gardner and Schoolcraft, 1999) as a result of lysins released by the embryo and endometrium (Montag et al., 2000). Cavitation and the consequent blastocoel result from gradients generated from ion transport by Na–K-ATPase activity in the trophoblast epithelium in conjunction with relative permeability of tight junctions between trophoblast cells (DiZio and Tasca, 1977; Gardner et al., 2000; Maitre et al., 2015). As a sodium gradient is generated by the Na–K-ATPase on the basolateral membrane, water follows and the blastocoel is created. As this process continues, hydrostatic pressure within the blastocoel increases resulting in blastocyst expansion and eventually hatching (Câmara et al., 2016) through the zona which has been concurrently thinned in response to lytic enzymes from the trophoblast and uterine cavity. Blastocyst expansion, a subjective assessment of the degree an embryo has hatched, is commonly incorporated into morphological assessments of embryo viability (Gardner et al., 2000) (Fig. 1).

The first component of the gardner/schoolcraft grading system (Gardner and Schoolcraft, 1999), the extent of blastocyst expansion and hatching, has shown to be predictive of embryo transfer outcomes in both fresh and frozen transfers (Goto et al., 2011; Gardner et al., 2000; Schoolcraft et al., 1999) with more expanded and/or hatching blastocysts at the time of assessment being more likely to lead to a successful pregnancy. As the elasticity and thickness of the zona pellucida varies between different patients' oocytes

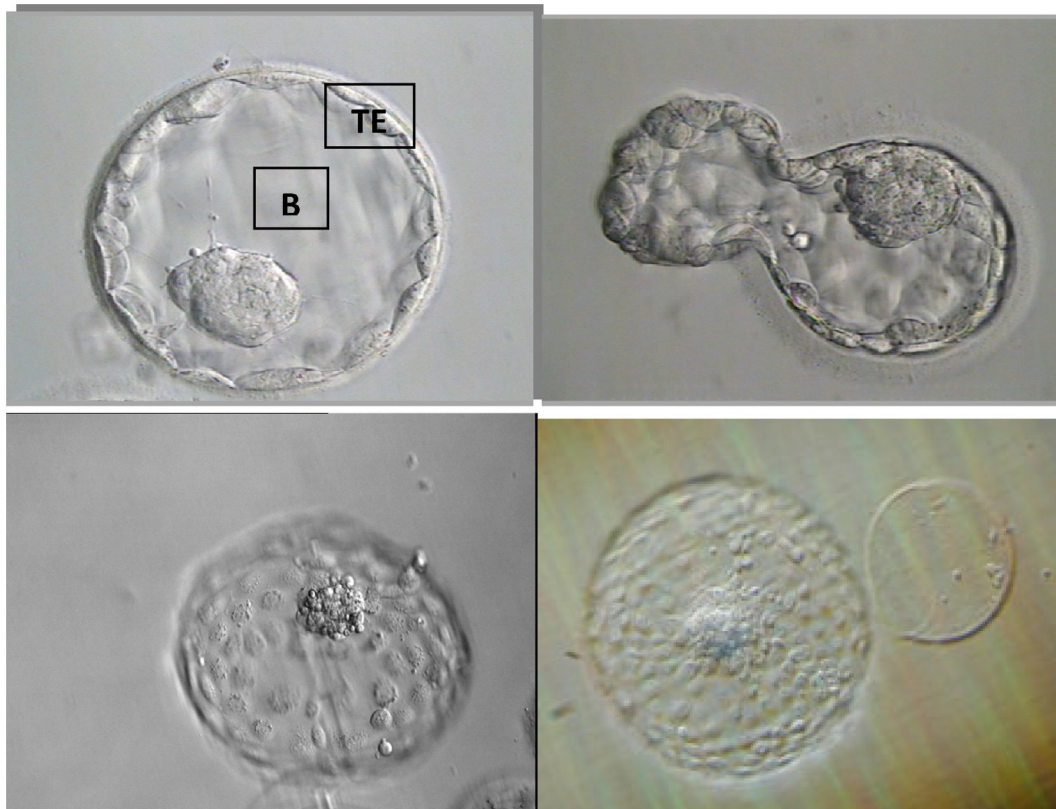


Fig. 1 Blastocysts at various stages of hatching. Top left: As the blastocoel expands, the zona pellucida is stretched thin. (B) Identifies the blastocoel. (TE) Identifies the trophoblast. Top right: Once the zona pellucida is breached the embryo begins to “hatch” out from the zona pellucida. The thickened zona in this hatching blastocyst suggests that the embryo underwent assisted hatching. Bottom left: The completely hatched blastocyst without a zona pellucida. Bottom right: The completely hatched blastocyst adjacent to its zona pellucida. From Barry Behr's (coauthor) Laboratory.

and embryos and changes throughout maturation, fertilization, and preimplantation development, some hypothesized that it was an abnormally thick or persistently rigid zona pellucida that explained the observed differences in clinical outcomes between less expanded and hatching blastocysts. Accordingly, a particularly restrictive zona pellucida could be the root cause of certain patients' infertility and poor outcomes with in vitro fertilization, not allowing the embryo to interface directly with the endometrium. This rationale formed the basis for using assisted hatching to overcome the theorized pathologic zona pellucida.

Assisted Hatching Techniques

Mechanical dissection: First described in clinical use in the 1980s by Cohen et al., assisted hatching is a micromanipulation procedure intended to help the developing embryo hatch from the zona pellucida so that it can interface directly with the endometrium (Cohen et al., 1990). While the zona is critical for early developmental events including fertilization and transport through the fallopian tubes (Bleil and Wassarman, 1980) and possibly even protection from microorganisms and the maternal immune system (Zhao and Dean, 2002), proponents of assisted hatching argue that many viable embryos are constrained by an abnormal zona impenetrable to the developing embryo. As these presumed viable embryos never have an opportunity to interface directly with the endometrium, the impenetrable zona pellucida causes these embryos' reproductive failure. As the zona is thought to have no beneficial role in implantation, its persistence was deemed only a potential hindrance to patients and facilitating the hatching process either by thinning or creating a complete opening in the zona has been presumed to be therapeutic. In the aforementioned initial trial, the zona was incised mechanically with a microneedle at two points and those patients who transferred embryos that were hatched had significantly higher implantation, clinical and ongoing pregnancy rates than patients who transferred embryos that had not undergone assisted hatching (Cohen et al., 1990).

Zona Drilling

Due to occasional trapping or damage to the developing embryo when using the microneedle method and inconsistencies in its application, the assisted hatching technique was then refined by some of the same authors who performed the zona breach by "drilling" the glycoprotein layer with acid tyrode's solution through an ICSI micropipette (Cohen et al., 1992), dissolving targeted areas in the glycoprotein layer. The practice of drilling with acid tyrode's had been previously used to remove or thin the zona pellucida to aid fertilization or facilitate embryo biopsy in animal models (Gordon and Talansky, 1986) as well as with early animal experimentation with assisted hatching (Malter and Cohen, 1989). During drilling, the embryo is stabilized with a holding pipette while a $\sim 10\text{ }\mu\text{m}$ micropipette is placed against the zona adjacent to an empty area of perivitelline space at the 3 o'clock position. Acid tyrode's is then slowly directed against the zona until an opening is seen. Some aim to create an opening in the zona as large as $30\text{ }\mu\text{m}$, while others describe a $10\text{--}13\text{ }\mu\text{m}$ inner breach and a $15\text{--}18\text{ }\mu\text{m}$ defect at the zona surface (Balaban et al., 2002; Jones et al., 2006). Others have advocated for thinning the zona or partial zona dissection rather than creating a complete opening (Khalifa et al., 1993). The heterogeneity in application of even a single method of assisted hatching makes it difficult to analyze the efficacy of the practice in general. Following zona drilling, embryos are rinsed repeatedly to wash away the acidic solution ($\text{pH} < 3$). Cohen's randomized controlled trial employing zona drilling did not benefit the general patient population, however a subanalysis confined to only patients with a relatively thickened zona pellucida ($\geq 15\text{ }\mu\text{m}$) demonstrated that patients who underwent zona drilling had significantly higher implantation rates than those who did not (Cohen et al., 1992). This supported the notion that assisted hatching benefits those patients who have poor prognoses due to an abnormally thick or restrictive zona pellucida. Advocates for therapeutic assisted hatching posited that the improved implantation rates observed for embryos subjected to the micromanipulation technique may be due to the overcoming the hardening associated with embryo culture in vitro (DeMeestere et al., 1997). Others argued that assisted hatching promoted earlier expansion and hatching of the blastocyst, potentially resynchronizing the developing embryos with the premature window of implantation encountered in fresh transfers after ovarian stimulation, while others explanations included the facilitating the exchange of growth factors and other messengers between the peri-implantation embryo and the endometrium (Nikas et al., 1999; Martins et al., 2011).

Laser Assisted Hatching

Due to observed improvements in pregnancy rates among poor prognosis patients, refinements in the hatching technique continued. The use of lasers to perform hatching is the most recent technique employed. Initially requiring direct contact of the pipette with the zona, more recent applications now offer noncontact laser diode methods which can be performed without any manipulation with holding or micropipettes, offering improved efficiency (Rink et al., 1996; Schöpper et al., 1999). Laser assisted hatching uses photoablation to create a breach in the zona. As the laser is highly absorbed by the media, the thermal effect is localized adding precision and consequent safety (Germond et al., 1996; Belil and Veiga, 2012). When applied to cleavage stage embryos prior to transfer, the embryos are placed in HEPES-buffered media on an inverted microscope capable of laser assisted hatching. An area of zona pellucida adjacent to an area between blastomeres is optimal for aiming the laser to minimize the risk of thermal injury to cells. Short pulse durations $< 5\text{ ms}$ and mild power $< 100\text{ mW}$ may also improve the safety profile of this practice (Douglas-Hamilton and Conia, 2001). In sibling embryo studies, subsequent embryo development after laser assisted hatching has shown to be comparable to those hatched with acid tyrode's (Jones et al., 2006) (Fig. 2).

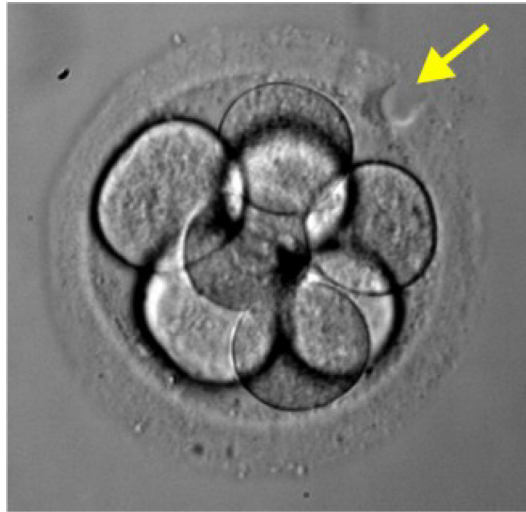


Fig. 2 Example of hatched cleavage stage embryo. Received from Hamilton Thorne.

Clinical Outcomes

Since its first incorporation into assisted reproduction, assisted hatching has been increasingly utilized, being incorporated in over half of day 3 fresh transfers and 22.8% of fresh blastocyst transfers. This increase utilization is most notable in the population of patients older than 38 years old or with a history of least two prior failed ART cycles (Kissin et al., 2014); however with the increasing incorporation of embryo biopsy in ART, patients undergoing PGS/PGD are likely the fastest growing patient population utilizing assisted hatching.

Despite its prevalent use by patients undergoing in vitro fertilization, the data supporting assisted hatching is inconsistent. It is unclear whether this is due to the heterogeneity among assisted hatching methods, applications, and indications studied, or whether its practice is not entirely justified. The most recent Cochrane review on assisted hatching analyzed 31 randomized controlled trials evaluating assisted hatching: the indications for assisted hatching in the trials were as diverse as the outcomes between trials. Among all studies included, the clinical pregnancy rate was significantly improved for the assisted hatching cohort (OR 1.13, 95% CI 1.01–1.27). Only nine randomized controlled trials reported live birth as an outcome, and analysis of these studies did not find any increased likelihood of live birth or clinical pregnancy attributed to assisted hatching (Carney et al., 2012). In another meta-analysis consisting of 28 randomized controlled trials, among all-comers there was a trend towards an increased pregnancy rate among patients whose embryos underwent assisted hatching (RR = 1.11, 95% CI = 1.00–1.24), although in the subgroup analysis, patients with fresh embryos transferred to women with previous repeated failure and frozen-thawed embryos transferred to unselected or non-poor prognosis women, there was a significantly increased pregnancy rate associated with assisted hatching. Among the 16 trials which reported live birth, there was not a significant benefit in the assisted hatching cohort however, the pooled analysis of 2 trials which facilitated a subgroup analysis of patients with previous repeated IVF failures did demonstrate a significantly improved live birth rate in the assisted hatching cohort (RR = 2.51, 95% CI = 1.06–5.96) (Martins et al., 2011). Based on this conflicting data, the practice committee of the American Society for Reproductive Medicine has concluded that there is insufficient evidence to conclude that assisted hatching improves live birth (Medicine and Technology, 2014), although some of the subgroup analyses from these studies still leaves its utility for patients with poor prognoses and previous repeated IVF failures open for debate.

Timing of Assisted Hatching

Due to the claimed benefit of alleviating viable embryos from an overly restrictive zona pellucida, assisted hatching has historically been incorporated into clinical practice at the cleavage stage before a cleavage stage embryo transfer on day 2 or day 3 of culture; however, there have also been studies advocating performing hatching at both the cleavage and blastocyst stages after thawing (Hiraoaka et al., 2007; Balaban et al., 2006; Wan et al., 2014). Additionally, with the advent of single step culture media and purported benefit of uninterrupted culture, performing assisted hatching at the fertilization check has even been described (Behr, unpublished data).

Use in Conjunction With Embryo Biopsy

As preimplantation genetic screening and diagnosis is being increasingly utilized by patients undergoing in vitro fertilization, techniques for embryo biopsy have become increasingly important. While cleavage stage embryo biopsy has largely been replaced by

trophectoderm biopsy, it is still occasionally used and penetrating the zona pellucida with one of the aforementioned assisted hatching methods at the time of biopsy is required. For patients planning trophectoderm biopsy, assisted hatching at the cleavage stage is frequently incorporated in order to maximize the number of embryos reaching the hatching or hatched blastocyst stage (Lee et al., 2015; Khalifa et al., 1993), facilitating an easier trophectoderm biopsy. While a safer embryo biopsy is the priority and chromosomal screening is likely a more sensitive screening tool than morphology based grading (Forman et al., 2013), by performing assisted hatching on these embryos it likely confounds the use of blastocyst expansion as an embryo selection method. This is demonstrated by the observation that blastocyst hatching status is not predictive of implantation after embryo biopsy (Rodriguez-Purata et al., 2016).

Adjuvant Treatments With Assisted Hatching

Since the first descriptions of incorporating assisted hatching into clinical use, investigators have been proponents of transfer recipients being treated with corticosteroids and antibiotics prior to embryo transfer (Cohen et al., 1990). Without an intact protective glycoprotein shell, advocates for these treatments were concerned that embryos with a breached zona may be particularly vulnerable to the maternal immune response or microbial contamination. Embryos that do not undergo assisted hatching must also hatch prior to implantation, and studies investigating the benefit of immunosuppression and prophylactic antibiotics have not consistently demonstrated a benefit (Lee et al., 1994; Polak de Fried et al., 1993). Despite inconsistent evidence, many providers have continued to provide antibiotics and corticosteroids to patients whose transferred embryos are exposed to the maternal environment prematurely due to assisted hatching, ICSI or embryo biopsy. In one randomized controlled trial, patients with a poor prognosis of success, previous implantation failures or transfers of cryopreserved embryos were randomized to receive assisted hatching with and without antibiotics and corticosteroids. The results were inconsistent, possibly due to the multiple sites involved and nuances of their hatching procedure and heterogeneous patient populations. In two sites, patients who underwent assisted hatching with a placebo for immunosuppression/antimicrobial treatment had a significantly lower pregnancy rate than those receiving hatching with corticosteroids and antibiotics possibly supporting their use; however, assisted hatching with corticosteroids and antibiotics did not result in a significantly improved implantation rate over control groups for any subgroup (Primi et al., 2004). More recently, a retrospective cohort study of 876 cycles involving the transfer of an embryo after assisted hatching, with and without corticosteroids and antibiotics. In this study, there was no difference in any clinical outcome between the two groups, causing the authors to conclude regarding their use: “old habits die hard” (Kaye et al., 2017). Further investigation is clearly needed to elucidate whether transfers outcomes of embryos with a breached zona pellucida are improved with immunosuppression and antimicrobial therapy.

Risk of Multiple Gestation Pregnancy/Monozygotic Twinning

From the first publications describing assisted hatching, authors have commented on the observed increase in multiple gestation pregnancies in cases employing this micromanipulation technique. Cohen et al. described a 17% triplet rate compared to a historical 2% triplet rate. While the number of embryos transferred is not described and this could theoretically result from an increased implantation rate, the authors note that monozygotic twinning could result if the inner cell mass divides when passing through the narrow opening in the zona pellucida (Cohen et al., 1990), as was observed in early animal experiments with assisted hatching (Malter and Cohen, 1989). Both a Cochrane meta-analysis of 31 randomized controlled trials and an analysis of 35,503 in vitro fertilization cycles from the Society for Assisted Reproduction Technology (SART) have demonstrated a significantly increased risk of multiple gestation pregnancy after assisted hatching, particularly for monozygotic twins which are associated with increased perinatal morbidity (Schieve et al., 2000; Carney et al., 2012). The Cochrane analysis demonstrated a 38% increase in the OR for multiple gestation pregnancy and monozygotic twins in the SART analysis had an OR 3.2 (95% CI 1.2–8.0) for the use of assisted hatching after controlling for potential confounders. The risks associated with multiple gestation pregnancies, particularly monozygotic multiple gestation are significant. While the absolute risk is low (0.33% monozygotic twin rate in cases with assisted hatching (Schieve et al., 2000)), the uncertain clinical benefit attributed to its use does question engaging any additional risk before undoing any favorable risk–benefit profile. Importantly, no congenital anomalies have been attributed to assisted hatching (Jwa et al., 2015).

Conclusion

Hatching is a critical step for embryo implantation. It is logical that assisting the hatching process for in vitro cultured embryos should improve outcomes—assuming the premise that viable embryos may be trapped in a pathologically thick or impassable zona pellucida. The variety of assisted hatching techniques, nuances of each method’s application, range of embryologist skill and experience, and the diverse indications for use, make it difficult to evaluate inconsistencies in attempts to reproduce favorable outcomes for patients undergoing assisted hatching. For patients with poor prognoses, included older patients with a history of repeated treatment failures, some studies suggest a significant improvement in pregnancy rates; however, larger studies with better

standardization of methods and patients are clearly needed to investigate whether assisted hatching improves the most important outcome, live birth rates.

References

- Balaban, B., Urman, B., Alatas, C., Mercan, R., Mumcu, A., & Isiklar, A. (2002). A comparison of four different techniques of assisted hatching. *Human Reproduction*, 17(5), 1239–1243.
- Balaban, B., Urman, B., Yakin, K., & Isiklar, A. (2006). Laser-assisted hatching increases pregnancy and implantation rates in cryopreserved embryos that were allowed to cleave in vitro after thawing: A prospective randomized study. *Human Reproduction*, 21(8), 2136–2140. <https://doi.org/10.1093/humrep/del097>.
- Bell, I., & Veiga, A. (2012). Assisted hatching in IVF. In Z. P. Nagy, A. C. Varghese, & A. Agarwal (Eds.), *Practical manual of in vitro fertilization: Advanced methods and novel devices* (pp. 445–451). New York, NY: Springer.
- Bleil, J. D., & Wassarman, P. M. (1980). Structure and function of the zona pellucida: Identification and characterization of the proteins of the mouse oocyte's zona pellucida. *Developmental Biology*, 76(1), 185–202.
- Câmara, D. R., Kastelic, J. P., & Thundathil, J. C. (2016). Role of the Na⁺/K⁺-ATPase ion pump in male reproduction and embryo development. *Reproduction, Fertility, and Development*. <https://doi.org/10.1071/RD16091>.
- Carney, S. K., Das, S., Blake, D., Farquhar, C., Seif, M. M., & Nelson, L. (2012). Assisted hatching on assisted conception (in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI)). *Cochrane Database of Systematic Reviews*, 12, CD001894. <https://doi.org/10.1002/14651858.CD001894.pub5>.
- Cohen, J., Elsner, C., Kort, H., Malter, H., Massey, J., Mayer, M. P., & Wiemer, K. (1990). Impairment of the hatching process following IVF in the human and improvement of implantation by assisting hatching using micromanipulation. *Human Reproduction*, 5(1), 7–13.
- Cohen, J., Alikani, M., Trowbridge, J., & Rosenwaks, Z. (1992). Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Human Reproduction*, 7(5), 685–691.
- DeMeestere, I., Barlow, P., & Leroy, F. (1997). Hardening of zona pellucida of mouse oocytes and embryos in vivo and in vitro. *International Journal of Fertility and Women's Medicine*, 42(3), 219–222.
- DiZio, S. M., & Tasca, R. J. (1977). Sodium-dependent amino acid transport in preimplantation mouse embryos. III. Na⁺–K⁺-ATPase-linked mechanism in blastocysts. *Developmental Biology*, 59(2), 198–205.
- Douglas-Hamilton, D. H., & Conia, J. (2001). Thermal effects in laser-assisted pre-embryo zona drilling. *Journal of Biomedical Optics*, 6(2), 205–213. <https://doi.org/10.1117/1.1353796>.
- Forman, E. J., Upham, K. M., Cheng, M., Zhao, T., Hong, K. H., Treff, N. R., & Scott, R. T. (2013). Comprehensive chromosome screening alters traditional morphology-based embryo selection: A prospective study of 100 consecutive cycles of planned fresh euploid blastocyst transfer. *Fertility and Sterility*, 100(3), 718–724. <https://doi.org/10.1016/j.fertnstert.2013.04.043>.
- Gardner, D. K., & Schoolcraft, W. B. (1999). Culture and transfer of human blastocysts. *Current Opinion in Obstetrics & Gynecology*, 11(3), 307–311.
- Gardner, D. K., Lane, M., Stevens, J., Schlenker, T., & Schoolcraft, W. B. (2000). Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertility and Sterility*, 73(6), 1155–1158.
- Germond, M., Nocera, D., Senn, A., Rink, K., Delacretaz, G., Pedrazzini, T., & Hornung, J. P. (1996). Improved fertilization and implantation rates after non-touch zona pellucida microdrilling of mouse oocytes with a 1.48 microm diode laser beam. *Human Reproduction*, 11(5), 1043–1048.
- Gordon, J. W., & Talansky, B. E. (1986). Assisted fertilization by zona drilling: A mouse model for correction of oligospermia. *The Journal of Experimental Zoology*, 239(3), 347–354. <https://doi.org/10.1002/jez.1402390306>.
- Goto, S., Kadowaki, T., Tanaka, S., Hashimoto, H., Kokeyuchi, S., & Shiotani, M. (2011). Prediction of pregnancy rate by blastocyst morphological score and age, based on 1,488 single frozen-thawed blastocyst transfer cycles. *Fertility and Sterility*, 95(3), 948–952. <https://doi.org/10.1016/j.fertnstert.2010.06.067>.
- Hiraoka, K., Fuchiaki, M., Horiuchi, T., Murakami, T., Kinutani, M., & Kinutani, K. (2007). Zona pellucida removal and vitrified blastocyst transfer outcome: A preliminary study. *Reproductive Biomedicine Online*, 15(1), 68–75.
- Jones, A. E., Wright, G., Kort, H. I., Straub, R. J., & Nagy, Z. P. (2006). Comparison of laser-assisted hatching and acidified Tyrode's hatching by evaluation of blastocyst development rates in sibling embryos: A prospective randomized trial. *Fertility and Sterility*, 85(2), 487–491. <https://doi.org/10.1016/j.fertnstert.2005.07.1314>.
- Jwa, J., Jwa, S. C., Kuwahara, A., Yoshida, A., & Saito, H. (2015). Risk of major congenital anomalies after assisted hatching: Analysis of three-year data from the national assisted reproduction registry in Japan. *Fertility and Sterility*, 104(1), 71–78. <https://doi.org/10.1016/j.fertnstert.2015.03.029>.
- Kaye, L., Bartels, C., Bartolucci, A., Engmann, L., Nulsen, J., & Benadiva, C. (2017). Old habits die hard: Retrospective analysis of outcomes with use of corticosteroids and antibiotics before embryo transfer. *Fertility and Sterility*, 107(6), 1336–1340. <https://doi.org/10.1016/j.fertnstert.2017.04.003>.
- Khalifa, E. A., Tucker, M. J., Hunt, P. P., & Hamidi, J. (1993). Improved hatching in mouse embryos brought about by combined partial zona dissection and co-culture. *Human Reproduction*, 8(4), 599–603.
- Kissin, D. M., Kawwass, J. F., Monsour, M., Boulet, S. L., Session, D. R., Jamieson, D. J., & National ART Surveillance System (NASS) Group. (2014). Assisted hatching: Trends and pregnancy outcomes, United States, 2000–2010. *Fertility and Sterility*, 102(3), 795–801. <https://doi.org/10.1016/j.fertnstert.2014.06.013>.
- Lee, K. A., Koo, J. J., Yoon, T. K., Do, B. R., Ko, J. J., & Cha, K. Y. (1994). Immunosuppression by corticosteroid has no effect on the pregnancy rate in routine in-vitro fertilization/embryo transfer patients. *Human Reproduction*, 9(10), 1832–1835.
- Lee, Y. S., Park, M. J., Park, S. H., Koo, J. S., Moon, H. S., & Joo, B. S. (2015). Effect of laser-assisted multi-point zona thinning on development and hatching of cleavage embryos in mice. *Clinical and Experimental Reproductive Medicine*, 42(2), 51–57. <https://doi.org/10.5653/ceerm.2015.42.2.51>.
- Maitre, J. L., Niwayama, R., Turlier, H., Nédélec, F., & Hiragi, T. (2015). Pulsatile cell-autonomous contractility drives compaction in the mouse embryo. *Nature Cell Biology*, 17(7), 849–855. <https://doi.org/10.1038/ncb3185>.
- Malter, H. E., & Cohen, J. (1989). Blastocyst formation and hatching in vitro following zona drilling of mouse and human embryos. *Gamete Research*, 24(1), 67–80. <https://doi.org/10.1002/mrd.1120240110>.
- Martins, W. P., Rocha, I. A., Ferriani, R. A., & Natri, C. O. (2011). Assisted hatching of human embryos: A systematic review and meta-analysis of randomized controlled trials. *Human Reproduction Update*, 17(4), 438–453. <https://doi.org/10.1093/humupd/dmr012>.
- Medicine, Practice Committee of the American Society for Reproductive, and Practice Committee of the Society for Assisted Reproductive Technology. (2014). Role of assisted hatching in in vitro fertilization: A guideline. *Fertility and Sterility*, 102(2), 348–351. <https://doi.org/10.1016/j.fertnstert.2014.05.034>.
- Montag, M., Koll, B., Holmes, P., & van der Ven, H. (2000). Significance of the number of embryonic cells and the state of the zona pellucida for hatching of mouse blastocysts in vitro versus in vivo. *Biology of Reproduction*, 62(6), 1738–1744.
- Nikas, G., Develioglu, O. H., Toner, J. P., & Jones, H. W. (1999). Endometrial pinopodes indicate a shift in the window of receptivity in IVF cycles. *Human Reproduction*, 14(3), 787–792.
- Polak de Fried, E., Blanco, L., Lancuba, S., & Asch, R. H. (1993). Improvement of clinical pregnancy rate and implantation rate of in-vitro fertilization-embryo transfer patients by using methylprednisone. *Human Reproduction*, 8(3), 393–395.
- Primi, M. P., Senn, A., Montag, M., Van der Ven, H., Mandelbaum, J., Veiga, A., Barri, P., & Germond, M. (2004). A European multicentre prospective randomized study to assess the use of assisted hatching with a diode laser and the benefit of an immunosuppressive/antibiotic treatment in different patient populations. *Human Reproduction*, 19(10), 2325–2333. <https://doi.org/10.1093/humrep/deh430>.

- Rink, K., Delacrétaz, G., Salathé, R. P., Senn, A., Nocera, D., Germond, M., De Grandi, P., & Fakan, S. (1996). Non-contact microdrilling of mouse zona pellucida with an objective-delivered 1.48-microns diode laser. *Lasers in Surgery and Medicine*, 18(1), 52–62. [https://doi.org/10.1002/\(SICI\)1096-9101\(1996\)18:1<52::AID-LSM7>3.0.CO;2-Q](https://doi.org/10.1002/(SICI)1096-9101(1996)18:1<52::AID-LSM7>3.0.CO;2-Q).
- Rodriguez-Purata, J., Gingold, J., Lee, J., Whitehouse, M., Slifkin, R., Briton-Jones, C., Copperman, A., & Sandler, B. (2016). Hatching status before embryo transfer is not correlated with implantation rate in chromosomally screened blastocysts. *Human Reproduction*, 31(11), 2458–2470. <https://doi.org/10.1093/humrep/dew205>.
- Schieve, L. A., Meikle, S. F., Peterson, H. B., Jeng, G., Burnett, N. M., & Wilcox, L. S. (2000). Does assisted hatching pose a risk for monozygotic twinning in pregnancies conceived through in vitro fertilization? *Fertility and Sterility*, 74(2), 288–294.
- Schoolcraft, W. B., Gardner, D. K., Lane, M., Schlenker, T., Hamilton, F., & Meldrum, D. R. (1999). Blastocyst culture and transfer: Analysis of results and parameters affecting outcome in two in vitro fertilization programs. *Fertility and Sterility*, 72(4), 604–609.
- Schöpfer, B., Ludwig, M., Edenfeld, J., Al-Hasani, S., & Diedrich, K. (1999). Possible applications of lasers in assisted reproductive technologies. *Human Reproduction*, 14(Suppl 1), 186–193.
- Sun, S. T., Choi, J. R., Son, J. B., Joo, J. K., Ko, G. R., & Lee, K. S. (2012). The effect of long zona dissection using ICSI pipettes for mechanical assisted hatching in vitrified-thawed blastocyst transfers. *Journal of Assisted Reproduction and Genetics*, 29(12), 1431–1434. <https://doi.org/10.1007/s10815-012-9872-1>.
- Wan, C. Y., Song, C., Diao, L. H., Li, G. G., Bao, Z. J., Hu, X. D., Zhang, H. Z., & Zeng, Y. (2014). Laser-assisted hatching improves clinical outcomes of vitrified-warmed blastocysts developed from low-grade cleavage-stage embryos: A prospective randomized study. *Reproductive Biomedicine Online*, 28(5), 582–589. <https://doi.org/10.1016/j.rbmo.2014.01.006>.
- Zhao, M., & Dean, J. (2002). The zona pellucida in folliculogenesis, fertilization and early development. *Reviews in Endocrine & Metabolic Disorders*, 3(1), 19–26.

GENETICS AND EPIGENETICS

Chromosomal Analysis of the Sperm

Lorena Rodrigo Vivó, IGENOMIX, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Asthenozoospermia Percentage of motile sperm below 40% (Cooper et al., 2010).

Autosomes The chromosomes identified in humans from number 1 to 22.

Azoospermia Absence of spermatozoa in ejaculate (Cooper et al., 2010).

Criptoospermia Sperm number below 1×10^6 per mL of ejaculate (Cooper et al., 2010).

Gonosomes The two sexual chromosomes identified in humans as X and Y.

Oligozoospermia Sperm number below 15×10^6 per mL of ejaculate (Cooper et al., 2010).

Teratozoospermia Number of sperm with normal shape below 4% (Cooper et al., 2010).

Introduction

Most of the factors associated with male infertility have a genetic component, such as chromosomal abnormalities and genetic defects that affect the reproductive system or spermatogenesis. Errors in synapsis, recombination, and DNA repair processes during male meiosis can generate abnormal segregation of homologous chromosomes at meiosis I or sister chromatids at meiosis II. Several checkpoint mechanisms that regulate the different stages of male meiosis can eliminate defective germ cells to ensure spermatozoa with a normal chromosome content. For this reason, spermatogenesis can be partially arrested in any of the maturation stages, resulting in oligozoospermia, or can be completely arrested, resulting in azoospermia. However, if these control mechanisms are deficient, any of the abnormal cell lines can escape the checkpoints and give rise to spermatozoa with numerical chromosome abnormalities such as aneuploidies or diploidies. Between 2% and 26% of infertile men with normal karyotype exhibit cytogenetic anomalies confined to the germ cell line (Vendrell et al., 1999; Egozcue et al., 2000), which makes sperm chromosome studies particularly relevant.

The first chromosome studies in sperm appeared in 1970 with the use of differential staining of specific chromosome regions. The total aneuploidy rate was estimated at 38%, with 1.4% for sex chromosomes and an average of 2% for autosomes. These rates were considered excessively high and were attributed to the low chromosomal specificity of the technique. In 1978, the development of a technique to fuse sperm with hamster oocytes without zona pellucida, which was standardized in 1982, provided information on the full chromosome content of sperm. However, this technique was complex and laborious, and was limited to the analysis only of spermatozoa capable to fertilize. The mid-1980s gave rise to the development of in situ hybridization techniques using specific DNA probes labeled with radioisotopes. Later, in the 1990s, the use of DNA probes labeled with nonradioactive isotopes allowed the visualization of sperm chromosomes by the fluorescence in situ hybridization (FISH) technique.

Chromosomal Analysis of Sperm by FISH

Using fluorescence DNA probes, FISH technique allows the analysis of numerical chromosome abnormalities of spermatozoa from the ejaculate, the epididymis, and the testis. Due to the nature of the spermatozoa, FISH protocol for sperm analysis requires the following steps.

Sperm Fixation

Before the hybridization, spermatozoa must be fixed maintaining their morphology and allowing permeability to the DNA probes. After centrifugation with sperm washing media, the supernatant containing the seminal plasma is discarded and the pellet with the spermatozoa is fixed with Carnoy solution (3 methanol: 1 glacial acetic acid). The fixed spermatozoa are spread on glass slides avoiding overlapping.

Nucleus Decondensation

Sperm heads have a tightly compacted nucleus due to the presence of disulfide bridges between protamines; this condensation of nuclear chromatin makes it inaccessible to DNA probes. To solve this problem, a pretreatment is performed by incubation with reducing agents and dehydration with ethanol. Reducing agents (e.g., DTT, dithiothreitol) break the disulfide bonds and produce nuclear chromatin decondensation allowing subsequent hybridization with DNA probes.

Denaturation and Hybridization

Double strand DNA denaturation of the sperm and FISH probes is carried out after incubation at high temperature (70°C–74°C). After denaturation, both DNAs are coincubated and hybridized to form a duplex of complementary strands. Hybridization protocols vary according to the type of FISH probe used, requiring different times and temperatures of hybridization (commonly between 4 and 16 h at 37°C–42°C).

FISH on sperm is commonly performed using centromeric, locus specific and subtelomeric fluorescent DNA probes. For segregation studies in structural rearrangements, such as translocations or inversions, specific combinations of these three types of probes are designed for each specific rearrangement. However, in carriers of numerical sex chromosome abnormalities and also in normal karyotype infertile males, the most widely analyzed are chromosomes 13, 18, 21, X and Y by using centromeric and locus specific probes.

Detection

The excess of DNA probes hybridized to unspecific complementary sequences is removed by astringent washes at high temperatures and low saline concentration (e.g., with SSC, saline sodium-citrate). Finally, a counterstain is applied to allow the visualization of the sperm nucleus (e.g., DAPI or DAPI/Antifade).

Signal Visualization and Evaluation

The hybridization signals are visualized using a fluorescent microscope equipped with specific filters for each fluorochrome. The evaluation is performed by counting the number of signals for each fluorochrome present in the nucleus of each spermatozoon. The spermatozoa are haploid cells containing one copy of each autosome and one sex chromosome, X-bearing, or Y-bearing. After the evaluation of the fluorescent signals using the criteria described by Blanco et al. (1996), the spermatozoa can be classified as (see Fig. 1):

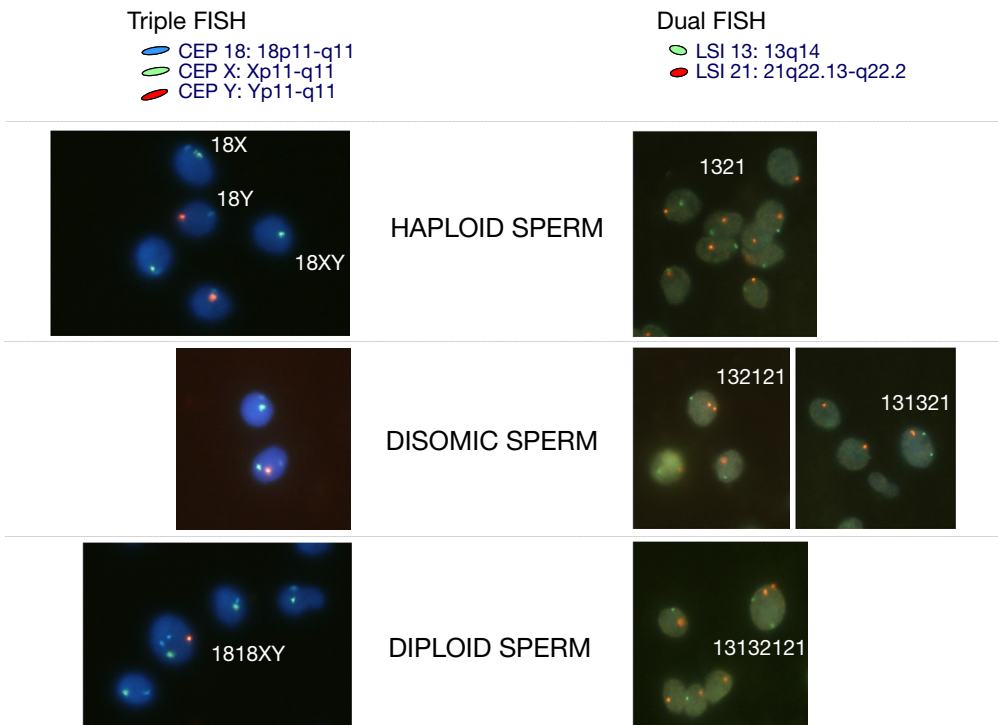


Fig. 1 Chromosomal analysis of the sperm by FISH: hybridization of the spermatozoa using a triple FISH with centromeric probes (CEP) for chromosomes 18, X and Y, and dual FISH with locus-specific probes (LSI) for chromosomes 13 and 21; evaluation of FISH signals using epifluorescence microscopy.

- Haploid-normal: when it shows one signal for each of the autosomes evaluated, and one signal for the sex chromosomes (X or Y).
- Disomic: with two signals for one of the chromosomes evaluated, and one signal for the remaining chromosomes evaluated.
- Diploid: with two signals for each of the chromosomes evaluated.

It is recommended to score only spermatozoa with clear hybridization signals, avoiding the analysis of cells with missing signals, as they could represent either nullisomies or hybridization failures.

After evaluation, a sperm sample is classified as abnormal when a significant increase of abnormal sperm (disomies and/or diploidies) is observed compared to the incidence observed in a control population of normozoospermic fertile males.

The total aneuploidy rate in normozoospermic males has been estimated in 6%, with 0.12% mean disomy for autosomes and 0.31% mean disomy for gonosomes (Egozcue et al., 1997). Due to this low aneuploidy rate, for clinical applications a minimal number of 1000 sperm per sample should be scored; nevertheless, this number may be limited in cases of low sperm count such as cryptozoospermic and azoospermic males.

Clinical Indications for Chromosomal Analysis in the Sperm

Currently, FISH in sperm is used in diagnosis protocols of male infertility, allowing the evaluation of the transmission risk of chromosomal defects to the offspring. Discriminating between infertile males with normal and abnormal karyotype, candidates for chromosomal analysis in the sperm would be the following.

Infertile Men With Abnormal Karyotype

Carriers of numerical abnormalities for sex chromosomes

Infertile males with Klinefelter (47,XXY) and 47,XYY syndromes are at risk of low sperm production with poor sperm quality and abnormal chromosome constitution. Blanco et al. (2001) described incidences of 1%–20% of spermatozoa with aneuploidies for the sex chromosomes and 1% diploid sperm in these males.

Carriers of structural chromosome abnormalities

Carriers of balanced chromosomal rearrangements such as Robertsonian or reciprocal translocations and inversions, even with a variable range of alterations during gametogenesis, resulting in normozoospermia, oligozoospermia, or even azoospermia. After spermatogenesis, the spermatozoa can be chromosomally unbalanced in a variable range. The incidence of unbalanced sperm for the chromosomes of the rearrangement is 10%–40% in Robertsonian translocations, 50%–65% in reciprocal translocations, and 1%–55% in inversion carriers (Antón et al., 2007).

Infertile Men With Normal Karyotype

Impaired meiosis in testicular analysis

Low recombination frequency in meiotic pachytene cells has been related to high aneuploidy frequency in sperm, mainly for the sex chromosomes (Ferguson et al., 2007). In fact, a significant correlation has been described between cells with sexual vesicles without recombination sites and sex chromosome disomy in sperm (Sun et al., 2008).

Impaired sperm parameters

Most of the studies performed in oligoasthenozoospermic males describe increased incidences of aneuploid and diploid sperm compared to normozoospermic men (Tempest et al., 2004). The sex chromosomes are the most affected, and the incidence of aneuploidy seems to be directly correlated with the severity of the oligozoospermia, being higher in patients with a sperm concentration lower than 5 million (Rubio et al., 2001; Martin et al., 2003). The same correlation has been observed in testicular sperm from azoospermic patients, mainly in those with nonobstructive azoospermia where up to 42% of the men have an abnormal FISH result (Rodrigo et al., 2011).

However, this correlation is not so clear regarding motility or morphology in sperm. Different studies on isolated asthenozoospermia have found no correlation with meiotic errors even when FISH analysis is performed on motile and nonmotile sperm of the same sample (Zeyneloglu et al., 2000). Nevertheless, a higher correlation with sperm meiotic defects has been observed in cases of isolated severe asthenozoospermia with specific deformities involving sperm flagella (Rives et al., 2005). Regarding isolated teratozoospermia, an increased incidence of aneuploidy and polyploidy seems to be associated with isolated teratozoospermia and specific morphological defects such as large-headed and multiple-tailed spermatozoa (Mateu et al., 2006).

Chemotherapy and radiotherapy treatments

Most chemotherapy or radiotherapy treatments have gonadal toxicity and affect spermatogenesis to a variable degree, depending on the type and duration of treatment. Reports indicate fivefold increases of diploid sperm and sperm with aneuploidies for autosomes and gonosomes after 6 months of treatment compared to their basal level and, in general, these rates decline to basal levels 18–24 months posttreatment (Tempest et al., 2008). Several studies have also described an association between Hodgkin's lymphoma

and impaired spermatogenesis, some of them finding a significant increase in aneuploid sperm before any treatment (Viviani et al., 1991). These data suggest that the emergence of cancer itself induces problems in meiosis.

Clinical history of unknown recurrent pregnancy losses

It has been described that approximately 66% of the abnormal karyotypes from miscarriages have a male origin. Meiotic abnormalities and sperm aneuploidy have been reported in recurrent pregnancy loss (RPL) patients (Ramasamy et al., 2015). Most of the reports describe increases in the incidence of sperm with sex chromosome disomy, and an increase in the diploidy rate was noted in a subset of patients with recurrent miscarriage after ovum donation (Rubio et al., 1999).

Clinical history of repetitive implantation failure

It is thought that oocyte fertilization by a chromosomally abnormal sperm may cause implantation failure. A study carried out in patients with three or more failed ICSI cycles reported that 31.6% of the patients had an increase in spermatozoa with sex chromosome disomy (Rubio et al., 2001). Further, later studies have related an abnormal FISH result in spermatozoa with a decrease in pregnancy and implantation rates in ICSI cycles (Nicolopoulos et al., 2008).

Previous pregnancy with chromosomopathy

Men with chromosomally abnormal offspring of paternal origin, such as Down syndrome (trisomy 21), Klinefelter syndrome (trisomy XXY), and Turner syndrome (monosomy X), have shown incidences of 1%–20% aneuploid sperm, with the altered chromosome affected (Blanco et al., 1998; Eskenazi et al., 2002; Tang et al., 2004).

Impact of Chromosomal Aneuploidies in Sperm

Impact on the Clinical Results

Most published studies related to sperm aneuploidy on infertile men have shown that men with increased frequencies of numerical sperm chromosomal abnormalities have lower pregnancy and implantation rates and higher miscarriage rate after IVF/ICSI than other infertile couples. Several groups have proposed preimplantation genetic testing for aneuploidy (PGT-A) as an alternative to improve the possibility of healthy pregnancies in couples with male infertility (Rubio et al., 2005).

The percentage of abnormal embryos ranged between 43% and 78% in patients with oligozoospermia and azoospermia in which an abnormal sperm FISH or an impaired meiosis were reported. Rodrigo et al. (2013) retrospectively analyzed the reproductive outcome of male factor infertility couples without history of recurrent miscarriage or implantation failure, who carried out sperm FISH analysis for chromosomes 13, 18, 21, X, and Y. IVF/ICSI cycles without embryo chromosomal analysis in couples with abnormal sperm FISH result showed significantly lower embryo transfer rates (64.0% vs. 84.8%), higher mean number of transferred embryos (2.3 ± 0.9 vs. 2.0 ± 0.6), and lower pregnancy (22.9% vs. 30.8%), and implantation rates (12.4% vs. 21.4%) than patients with normal sperm FISH result. However, PGT-A cycles with the embryo analysis of a battery of 9 chromosomes by FISH, showed higher pregnancy (39.7% vs. 28.3%) and implantation rates (33.8% vs. 21.4%) in patients with abnormal sperm FISH result than in patients with normal sperm FISH result. Interestingly, patients with normal sperm FISH analysis had similar clinical results regardless of IVF/ICSI or PGT-A; however, patients with abnormal sperm FISH analysis showed better pregnancy and implantation rates with PGT-A. Embryo aneuploidy screening of the 24 chromosomes in couples with male factor infertility offers even better clinical results, with 83.6% of cycles having at least one euploid embryo to transfer, resulting in a pregnancy rate per transfer of 62.9%, an implantation rate of 54.2% and a take-home baby rate of 50.9% (Rodrigo et al., 2014).

Impact on the Embryo Chromosomal Constitution

In translocation carriers, a correlation has been observed between the percentage of abnormal gametes and the percentage of abnormal embryos (Escudero et al., 2003). In normal karyotype patients, Sánchez-Castro et al. (2009) described a correlation between the percentage of aneuploid sperm and embryos, identifying a 65% of abnormal embryos in patients below 20 millions of sperm count and 3% of total sperm aneuploidy rate, whereas normozoospermic patients with 1.7% of total sperm aneuploidy rate gave rise to lower incidence of 41% of aneuploid embryos.

Additionally, Rodrigo et al. (2010) described a different effect on the embryo according to the type of sperm abnormality. Therefore, sex chromosome aneuploidies in sperm gave rise to aneuploid embryos compatible with life, whereas diploid sperm were more related to triploid embryos that mostly miscarry. In fact, at the offspring level several studies have reported increases in sperm chromosomal abnormalities associated to the chromosomopathies observed in their children. In a study conducted on two fathers of children with Down syndrome of paternal origin, the sperm disomy 21 rates were reported as 0.75% and 0.78% (Blanco et al., 1998). Similar studies in couples with miscarriages or children of carriers of sex chromosome abnormalities (Turner or Klinefelter syndrome) have reported high incidences of sperm aneuploidy for sex chromosomes, ranging from 0.20% to 24.7%.

Clinical Recommendations After Chromosomal Analysis of the Sperm

Taking into consideration the consequences that an increase of numerical chromosome abnormalities in sperm can have on the clinical results, infertility problems and genetic risk for the offspring, if the sperm sample shows an abnormal result, genetic counseling should be advice to the couple. Additionally, several clinical options can be offered, such as prenatal testing when slight increases in aneuploid or diploid sperm; PGT-A in the embryos when moderate-high levels of abnormal sperm; or sperm donation when severe meiosis impairment that results in extremely high increases in abnormal sperm.

References

- Antón, E., Vidal, F., & Blanco, J. (2007). Role of sperm FISH studies in the genetic reproductive advice of structural reorganization carriers. *Human Reproduction*, 22, 2088–2092.
- Blanco, J., Egozcue, J., & Vidal, F. (1996). Incidence of chromosome 21 disomy in human spermatozoa as determined by fluorescent in-situ hybridization. *Human Reproduction*, 11, 722–726.
- Blanco, J., Egozcue, J., & Vidal, F. (2001). Meiotic behaviour of the sex chromosomes in three patients with sex chromosome anomalies (47,XXY, mosaic 46,XY/47,XXY and 47,XXY) assessed by fluorescence in-situ hybridization. *Human Reproduction*, 16, 887–892.
- Blanco, J., Gabau, E., Gómez, D., et al. (1998). Chromosome 21 disomy in the spermatozoa of the fathers of children with trisomy 21, in a population with a high prevalence of down syndrome: Increased incidence in cases of paternal origin. *American Journal of Human Genetics*, 63, 1067–1072.
- Cooper, T. G., Noonan, E., Eckardstein, S. V., et al. (2010). World Health Organization reference values for human semen characteristics. *Human Reproduction Update*, 16, 231–245.
- Egozcue, J., Blanco, J., & Vidal, F. (1997). Chromosome studies in human sperm nuclei using fluorescence in-situ hybridization (FISH). *Human Reproduction Update*, 3, 441–452.
- Egozcue, S., Vendrell, J. M., García, F., et al. (2000). Increased incidence of meiotic anomalies in oligoasthenozoospermic males preselected for intracytoplasmic sperm injection. *Journal of Assisted Reproduction and Genetics*, 17, 307–309.
- Escudero, T., Abdelhadi, I., Sandalinas, M., & Munné, S. (2003). Predictive value of sperm fluorescence in situ hybridization analysis on the outcome of preimplantation genetic diagnosis for translocations. *Fertility and Sterility*, 3, 1528–1534.
- Eskenazi, B., Wyrobek, A. J., Kidd, S. A., et al. (2002). Sperm aneuploidy in fathers of children with paternally and maternally inherited Klinefelter syndrome. *Human Reproduction*, 17, 576–583.
- Ferguson, K. A., Wong, E. C., Chow, V., Nigro, M., & Ma, S. (2007). Abnormal meiotic recombination in infertile men and its association with sperm aneuploidy. *Human Molecular Genetics*, 16, 2870–2879.
- Martin, R. H., Rademaker, A. W., Greene, C., et al. (2003). A comparison of the frequency of sperm chromosome abnormalities in men with mild, moderate and severe oligozoospermia. *Biology of Reproduction*, 69, 535–539.
- Mateu, E., Rodrigo, L., Prados, N., et al. (2006). High incidence of chromosomal abnormalities in large-headed and multiple-tailed spermatozoa. *Journal of Andrology*, 27, 6–10.
- Nicopoulos, J. D., Gilling-Smith, C., Almeida, P. A., et al. (2008). The role of sperm aneuploidy as a predictor of the success of intracytoplasmic sperm injection? *Human Reproduction*, 23, 240–250.
- Ramasamy, R., Scovell, J. M., Kovac, J. R., et al. (2015). Fluorescence in situ hybridization detects increased sperm aneuploidy in men with recurrent pregnancy loss. *Fertility and Sterility*, 103, 906–909.
- Rives, N., Mousset-Simeon, N., Mazurier, S., et al. (2005). Primary flagellar abnormality is associated with an increased rate of spermatozoa aneuploidy. *Journal of Andrology*, 26, 61–69.
- Rodrigo, L., Mateu, E., Mercader, A., et al. (2014). New tools for embryo selection: comprehensive chromosome screening by array comparative genomic hybridization. *BioMed Research International*, 2014, 517125.
- Rodrigo, L., Peinado, V., Mateu, E., et al. (2010). Impact of different patterns of sperm chromosomal abnormalities on the chromosomal constitution of preimplantation embryos. *Fertility and Sterility*, 94, 1380–1386.
- Rodrigo, L., Rubio, C., Mateu, E., et al. (2013). FISH on sperm to identify infertile male patients with higher aneuploidy risk. *Human Reproduction*, 28, 458.
- Rodrigo, L., Rubio, C., Peinado, V., et al. (2011). Testicular sperm from patients with obstructive and nonobstructive azoospermia: Aneuploidy risk and reproductive prognosis using testicular sperm from fertile donors as control samples. *Fertility and Sterility*, 95, 1005–1012.
- Rubio, C., Gil-Salom, M., Simón, C., et al. (2001). Incidence of sperm chromosomal abnormalities in a risk population: Relationship with sperm quality and ICSI outcome. *Human Reproduction*, 16, 2084–2092.
- Rubio, C., Rodrigo, L., Pérez-Cano, I., et al. (2005). FISH screening of aneuploidies in preimplantation embryos to improve IVF outcome. *Reproductive Biomedicine Online*, 11, 497–506.
- Rubio, C., Simón, C., Blanco, J., et al. (1999). Implications of sperm chromosome abnormalities in recurrent miscarriage. *Journal of Assisted Reproduction and Genetics*, 16, 253–258.
- Sánchez-Castro, M., Jiménez-Macedo, A. R., Sandalinas, M., & Banco, J. (2009). Prognostic value of sperm fluorescence in situ hybridization analysis over PGD. *Human Reproduction*, 24, 1516–1521.
- Sun, F., Mikhaail-Philips, M., Oliver-Bonet, M., et al. (2008). Reduced meiotic recombination on the XY bivalent is correlated with an increased incidence of sex chromosome aneuploidy in men with nonobstructive azoospermia. *Molecular Human Reproduction*, 14, 399–404.
- Tang, S. S., Gao, H., Robinson, W. P., Ho Yuen, B., & Ma, S. (2004). An association between sex chromosomal aneuploidy in sperm and an abortus with 45,X of paternal origin: Possible transmission of chromosomal abnormalities through ICSI. *Human Reproduction*, 19, 147–151.
- Tempest, H. G., Homa, S. T., Dalakioridou, M., et al. (2004). The association between male infertility and sperm disomy: Evidence for variation in disomy levels among individuals and correlation between particular semen parameters and disomy of specific chromosome pairs. *Reproductive Biology and Endocrinology*, 2, 82.
- Tempest, H. G., Ko, E., Chan, P., et al. (2008). Sperm aneuploidy frequencies analysed before and after chemotherapy in testicular cancer and Hodgkin's lymphoma patients. *Human Reproduction*, 23, 251–258.
- Vendrell, J. M., García, F., Veiga, A., et al. (1999). Meiotic abnormalities and spermatogenic parameters in severe oligoasthenozoospermia. *Human Reproduction*, 14, 375–378.
- Viviani, S., Ragni, G., Santoro, A., et al. (1991). Testicular dysfunction in Hodgkin's disease before and after treatment. *European Journal of Cancer*, 27, 1389–1392.
- Zeyneloglu, H. B., Baltaci, V., Ege, S., Haberal, A., & Batioglu, S. (2000). Detection of chromosomal abnormalities by fluorescent in-situ hybridization in immotile viable spermatozoa determined by hypo-osmotic sperm swelling test. *Human Reproduction*, 15, 853–856.

Chromosomal Analysis of the Embryo: Preimplantation Genetic Screening

Carmen Rubio, IGENOMIX, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Aneuploidy Extra or missing individual chromosome/s.

Blastocyst Stage of preimplantation embryo development at day-5 or 6 after fertilization, with cell differentiation into the trophoctoderm and the inner cell mass.

Euploidy Normal content of chromosomes (23 pairs = 46 chromosomes).

Haploidy A single set of chromosomes ($23 \times 1 = 23$ chromosomes).

Monosomy Missing individual chromosome/s.

Mosaicism Coexisting cell lines with different chromosome constitution within an embryo.

Triploidy Extra set of chromosomes ($23 \times 3 = 69$ chromosomes).

Trisomy Extra individual chromosome/s.

Uniform aneuploidy All cells from an embryo display similar chromosome constitution.

Introduction

Aneuploidy is the most common genetic abnormality in human embryos. It is known that chromosomal abnormalities are implicated in first trimester spontaneous abortions. The incidence of chromosomal abnormalities decreases during the peri-implantation period in such a way that in livebirths, an incidence of 0.6% was observed, a 10-fold increase (6%) was present in stillborn and a 100-fold increase (60%) in spontaneous abortions. This pattern of loss of chromosomal abnormalities from implantation to birth may also operate during preimplantation embryogenesis with a progressive loss of abnormal embryos at specific stages in early development through developmental arrest and degeneration of abnormal embryos (Almeida and Bolton, 1996).

Cytogenetic evaluations in spontaneous abortions, made directly from fetal tissue have revealed an overall incidence of chromosomal abnormalities of 50%–70%. Analysis by chorionic villus sampling has showed higher percentage of chromosomal abnormalities (83%). Most of them are non-inherited numerical abnormalities (parents having a normal karyotype) that appear “de novo”. Only 4.7% of couples with two or more abortions include a carrier of a balanced structural abnormality.

In couples with normal karyotypes, the most common group of abnormalities in spontaneous abortions are autosomal trisomies (75.9%), followed by monosomy X (11.4%), triploidy (6.3%), and tetraploidy (3.8%). Chromosome 16 is the most common trisomy, followed by chromosome 22 and 21. More recently, high incidence of trisomy 15 has been also reported with lower incidence of other important autosomal trisomies, such as trisomy 13 and trisomy 18. It is important to point out that autosomal monosomies have been observed only rarely, despite autosomal monosomies should occur with the same frequencies as trisomies, therefore, they are considered to be responsible of embryo arrest or implantation failures.

The origin of the majority of autosomal trisomies is non-disjunction during maternal meiosis, and they are usually associated with a maternal age effect. Trisomy 16 is incompatible with postnatal survival and non-disjunction studies in spontaneous abortions showed maternal meiotic origin in all the cases, as well as for chromosome 22. Concerning trisomy 21, molecular studies have shown that 8%–9% of the cases were of paternal origin. Among the maternal errors, 75% were attributed to errors in meiosis I and 25% to errors in meiosis II. And about 5% of cases of trisomy 21 were probably due to mitotic nondisjunction (post-zigotic) in the early embryo. The origin of trisomy 18, was determined to be maternal in 91% of the cases, and 9% paternal. Trisomy 13 was the result of maternal errors in 88% of cases and paternal errors in 12% of cases. Monosomy for the X chromosome is the most common single genetic imbalance in first trimester spontaneous abortions. In these cases, the origin of the single X is usually maternal (80%), implying a paternal error exists during meiosis. Maternal sex chromosome mosaicism has been suggested to be responsible for a portion of monosomies 45,X. In 50% of 47,XXY trisomies and in 100% of 47,XYY trisomies, the origin was paternal non-disjunction. And finally, polyploidy occurs following the fertilization of a normal oocyte by more than one spermatozoa or due to meiotic errors during gametogenesis leading to an abnormal diploid oocyte or spermatozoa (Jacobs and Hassold, 1995).

In couples with known risk of aneuploidy, the frequent advice in the past was to offer prenatal diagnosis, either amniocentesis or chorionic villus sampling and more recently non-invasive prenatal testing (NIPT). In these cases, if the fetus is affected, the couple has to decide if they wish to continue with the pregnancy or undergo a termination. Preimplantation genetic diagnosis for aneuploidy screening (PGD-A) offers an alternative for these couples, where the genetic test is performed on the preimplantation embryo. In a PGD-A cycle, the couples should undergo an in vitro fertilization cycle (IVF) to generate embryos in vitro, the embryos are biopsied, and the genetic test is performed on the biopsied cells. PGD-A therefore allows the selection of chromosomally normal embryos to be transferred to the uterus, before the pregnancy is established.

Additionally, reproductive failure in some circumstances can be attributed to the presence of aneuploidy in sperm oocytes and embryos. Therefore, PGD-A can also increase pregnancy rates in infertile couples requiring IVF. This technique has also been called preimplantation genetic screening (PGS) and comprehensive chromosome screening (CCS), among other names.

Incidence of Aneuploidy and Mosaicism in the Preimplantation Embryo

Large datasets from comprehensive aneuploidy screening of preimplantation embryos demonstrate that over half of embryos produced by in vitro fertilization (IVF) are aneuploid (Fransasiak et al., 2014). Aneuploidy rates are higher in oocytes and embryos from women of advanced maternal age, probably stemming from meiotic recombination defects exacerbated by age (Nagaoka et al., 2012). More recently the presence of mosaicism in preimplantation embryos has been also described. Mosaicism refers to the presence of cells with different chromosomal genotypes within a single embryo. It can originate from the first embryo cleavage and can be identified in PGD-A of a blastocyst biopsy with analysis of several cells from the trophectoderm (day-5, day-6, or day-7 embryo biopsy). The first evidence of mosaicism in preimplantation embryos comes from re-analysis studies of aneuploid embryos, and its impact in good quality embryos should not be very high since the analysis of all cells from day-3 embryos or blastocysts showed similar results in all cells in about 97% of the embryos (Mir et al., 2013, 2016; Capalbo et al., 2013).

It is challenging to estimate the real incidence of mosaicism in preimplantation embryos because of technical limitations and because biopsies represent a small percentage of total number of cells of the embryo and will only partially represent the whole blastocyst, depending on the percentage and the distribution of euploid and aneuploid cells in the inner cell mass and trophectoderm. The bioinformatic analysis of sequencing data has difficulty clearly discriminating low-degree mosaicism from experimental noise related to the quality and quantity of the biological samples. There is consensus among most groups to report as mosaic embryos those with >30%–35% of estimated aneuploid cells.

More recently, some authors have proposed the possibility of transferring some types of mosaic embryos, with normal newborns resulting after the transfer into the uterus of mosaic blastocysts (Greco et al., 2015). However, the authors were cautious and state that additional clinical data must be obtained before this approach can be extended. Lately, Fragouli et al. (2017) reported that the transfer of mosaic embryos has significantly poorer clinical outcomes compared to a contemporary control group with the transfer of euploid embryos (ongoing pregnancy of 46.2% vs. 15.4%; $P = 0.003$). The authors concluded that embryo viability is compromised by the presence of aneuploid cells.

Technologies Applied to PGD-A: THE NGS ERA

Several methods allow study of aneuploidies in human embryos—from fluorescence in situ hybridization (FISH) that provides limited information for a few chromosomes, to single nucleotide polymorphism (SNP) arrays, quantitative polymerase chain reaction (qPCR), arrays comparative genomic hybridization (aCGH), and next-generation sequencing (NGS) to analyze all 23 chromosome pairs. The evolution of aneuploidy screening techniques has provided more information about genetic status of the embryo in addition to more reliable and faster results, which enable transfer of euploid embryos during the same cycle.

- **PGD-A version 1.0:** From the 1990s to 2010, FISH was performed on polar bodies or cleavage stage embryos. Numerous retrospective studies claim that the technique works, and thousands of cycles have been conducted worldwide (Munné et al., 1995). FISH assays use fluorescent nucleic acid probes complementary to DNA to visualize regions of interest. However, FISH cannot be performed on all chromosomes simultaneously; therefore, it is targeted to those chromosomes related to spontaneous miscarriages or compatible with live birth, such as chromosomes 15, 16, 17, 18, 21, 22, X, and Y. However, these chromosomes must be assessed over multiple rounds of hybridization, and informativity rates and accuracy of results depend of morphology and integrity of a single nucleus fixed onto a glass slide.
- **PGD-A version 2.0:** New technologies have facilitated the transition from FISH analysis of a limited number of chromosomes to analysis of all 23 chromosome pairs simultaneously in a single cell. Among these technologies, qPCR, SNP arrays, and aCGH have been most widely published to date and have been applied to polar bodies, day-3 and trophectoderm biopsies (Sermon et al., 2016). aCGH technology allows analysis of chromosome DNA copy number variations from an embryo compared to a reference sample. First, DNA from a single blastomere or 4–6 TE cells is amplified via whole genome amplification (WGA). Amplified DNA is then labeled with different fluorescent probes, combined, and hybridized onto a slide containing specific bacterial artificial chromosome (BAC) probes that span the length of chromosomes with ~1 Mb coverage. Chromosome loss or gain is revealed by the color of each spot after hybridization. Fluorescence intensity is detected using a laser scanner and data processing software, which can analyze whole chromosome aneuploidy and sub-chromosomal structural imbalances. SNP arrays also utilize an array setup, although they interrogate specific SNPs in the genome and compare this data to SNP patterns of maternal and paternal partners to arrive at a ploidy call. For qPCR, specific PCR primers amplify a limited section of each chromosome on all 23 chromosome pairs in replicates. By analyzing the relative amount of DNA from each PCR product, a ploidy status can be inferred and assigned to each chromosome.
- **PGD-A 3.0:** This could be considered the latest approach with the possibility to use NGS in a small number of cells from TE biopsies. Decreased cost of genome sequencing has positioned NGS as one of the most promising platforms to study not only

aneuploidies, but also mitochondrial DNA or gene disorders in simultaneous analyses (Fiorentino et al., 2014). For NGS, most extended protocols share the first steps with aCGH protocols, starting with WGA. A barcoding procedure follows this, in which different samples are labeled with unique sequences, so that they later can be mixed, sequenced, and matched to their original patient and embryo. This barcoding process allows 24–96 biopsies to be pooled in a sequencing run, optimizing cost per sequenced embryo. After sequencing, each sequence is aligned with a reference human genome, and copy number variations for whole chromosomes and small deletions/duplications are established using specific software (Vera-Rodríguez et al., 2016).

Clinical Results and Indications of PGD-A

PGD-A was introduced in clinical routine practice to improve pregnancy rates in sub-fertile couples, based on the assumption that high rates of chromosomal aneuploidy—frequently found in cleavage-stage embryos and blastocysts of these couples—were responsible for low pregnancy rates after IVF. Fig. 1, shows aneuploidy rates according to maternal age for blastocyst biopsies with the newest NGS technology. And Fig. 2 shows the percentage of cases with at least one euploid embryo after blastocyst biopsy according to maternal age (Igenomix data obtained from 12,000 cases and > 60,000 biopsied blastocyst).

The main goals for most indications are not only to increase implantation and pregnancy rates, but also to decrease miscarriages, risk of aneuploid offspring, and time to conceive. More recently, cost efficiency per healthy baby at home has also been considered, because with new PGD-A 3.0, blastocyst biopsy and NGS, PGD-A cost is no longer a limitation. There are many factors that contribute to patient-perceived determinants when choosing to accept or decline PGD-A: cost, religion, ethical values, social and family support, provider influences, and past reproductive experience. In light of these results, with the current trend toward single-embryo transfer (SET), it could be argued that failure to investigate chromosomal constitution of the preimplantation embryo to be transferred may raise ethical questions of its own.

Finally, with the gaining popularity of blastocyst biopsy, blastocyst vitrification after trophectoderm biopsy, and deferred transfer, PGD-A fits well into this clinical scheme. A recent study comparing fresh blastocyst transfer and frozen cycles shows improved implantation rate per transferred embryo, although the difference is not significant (75% vs. 67%). However, ongoing pregnancy rates (80% vs. 61%) and live birth rates (77% vs. 59%) are significantly higher with frozen samples compared to fresh transfer. Either transfer strategy can be a reasonable option, but there is a trend toward favoring deferred transfer (Coates et al., 2017). Fig. 3 shows clinical outcomes with PGD-A using blastocyst biopsy with deferred transfer (internal Igenomix data), compared to regular IVF/ICSI (www.sartcorsonline.com/2015).

The following are the most common current indications for PGD-A:

- Advanced maternal age (AMA)

AMA is the most common indication for PGD-A. Maternal age is a major factor in the prevalence of aneuploidy. A study performed in polar body biopsies reported that the rate of missegregation for most clinically relevant aneuploidies (chromosomes 13, 16, and 18) increases from 20% to 60% in women between the ages of 35 and 43 years (Kuliev et al., 2011). Most clinical IVF groups have traditionally considered AMA to be any patient older than 37 years, although recently there is a move to lower the cut-off to 35 years.

Incidence of aneuploidy according to maternal age

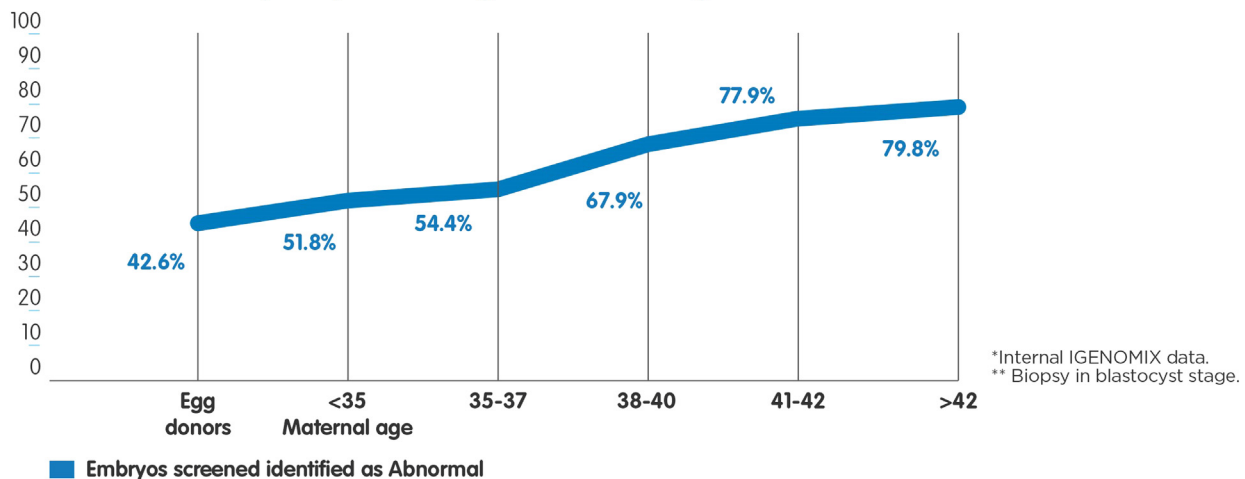


Fig. 1 Incidence of aneuploidy in trophectoderm biopsies analyzed in the laboratories of Igenomix.

Chance of transfer according to maternal age

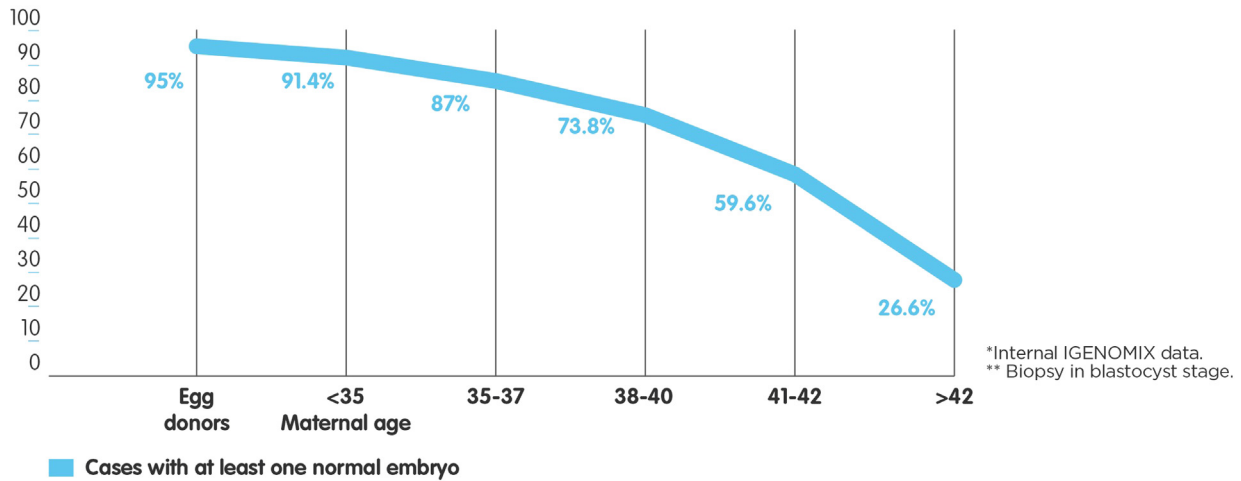


Fig. 2 Percentage of case with at least one euploid blastocyst for transfer. Data from trophoctoderm biopsies analyzed in Igenomix laboratories.

Ongoing pregnancy rate per embryo transfer

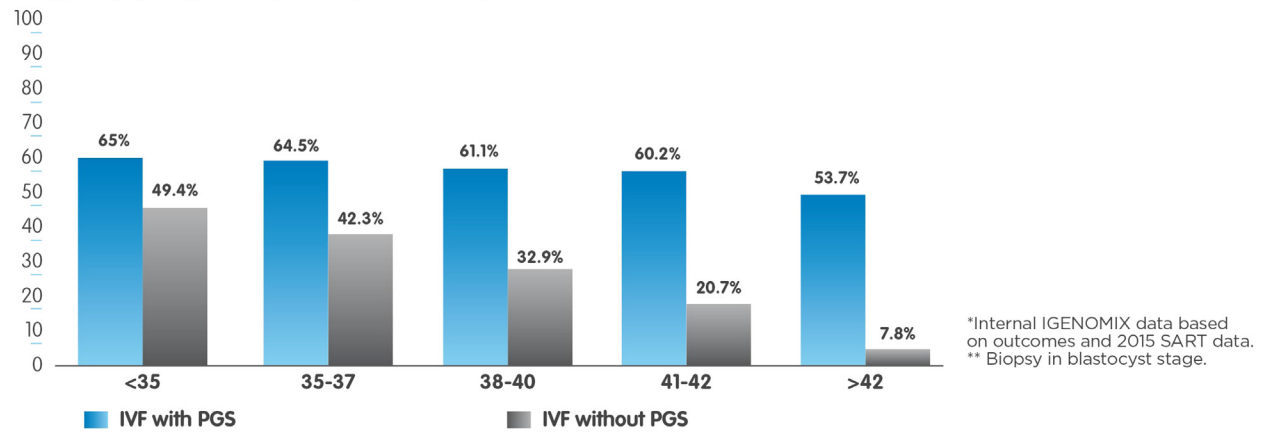


Fig. 3 Comparison of clinical pregnancy rates per embryo transference according to maternal age. The comparisons correspond to Igenomix data from trophoctoderm biopsies and the data from cycles without tophectoderm biopsy correspond to Sart data base (www.sartcorsonline.com).

Six randomized controlled trials (RCTs) were published for AMA patients using FISH technology to analyze a limited number of chromosomes. Three indicated that PGD-A offered no benefit (Mastenbroek et al., 2011). These studies were criticized by several authors who argued that the study methodologies had some important pitfalls, including patient inclusion criteria, embryo biopsy procedures, embryo culture conditions, and type of genetic analyses performed. On the other hand, three other studies described lower miscarriage rates and increased delivery rates (Schoolcraft et al., 2009; Rubio et al., 2013, 2017).

Without the ability to screen for aneuploidy, AMA patients with a high percentage of aneuploid embryos may be subjected to multiple unsuccessful embryo transfers for months, some of which may end in distressing miscarriages with the associated medical risks. With the introduction of NGS, PGD-A cost is becoming increasingly affordable and enables embryo chromosome analysis in IVF at a lower cost.

● Recurrent miscarriage (RM)

The definition of RM varies by country, but is generally considered the occurrence of 2–3 consecutive miscarriages with a gestational age up to 14 weeks. For PGD-A, other causes of miscarriage should be discarded before indicating this treatment, with a proper infertility work-up. PGD-A in these couples decreases miscarriage rates to those of general population and therefore increases delivery rates. Among couples suffering from RM, those with previous aneuploid miscarriages and couples with sperm chromosomal abnormalities showed even higher implantation rates and lower miscarriage rates (Rubio et al., 2009).

A retrospective case-control study reports PGD-A implantation rates of 52.63% compared to 19.15% in controls ($P = 0.001$) and almost double ongoing pregnancy rate (61.54% vs. 32.49%; $P = 0.0001$) (Keltz et al., 2013). In addition, a systematic review confirmed that PGD-A may lower miscarriage rates (Musters et al., 2011).

- Repetitive implantation failure (RIF)

RIF is defined as three or more failed IVF attempts or failed IVF treatments after cumulative transfer of > 10 good-quality embryos. RIF-defining criteria are not homogenous, and an exhaustive and comprehensive definition has not yet been reached. Therefore, RIF remains a challenge to clinicians because it can have multiple causes that are still poorly defined. Further, embryo and endometrial factors can play important roles in this condition.

There have been two RCTs published in RIF patients with conflicting results. One of them concluded that there were no significant differences in clinical pregnancy rates with PGD-A-FISH compared to controls (Blockeel et al., 2008). However, the second study that analyzed a few more chromosomes showed a clear trend toward better live birth rates with PGD-A-FISH (47.9% vs. 27.9%) (Rubio et al., 2013). For this indication, despite of age, other combined factors can predict a higher aneuploidy risk, such as low sperm concentration and low ovarian response (Garcia-Herrero et al., 2014).

- Severe male factor infertility (MF)

Couples with impaired sperm parameters have been proposed at increased risk of embryo aneuploidy. Oligozoospermia is associated with significant increases in sex chromosome disomy, chromosome 18 and 21 disomy, and percentage of diploid sperm, particularly in samples with markedly reduced sperm concentrations ($< 5 \times 10^6$ /mL spermatozoa). Such conditions might, in part, explain low implantation and high abortion rates observed in these patients (Rubio et al., 2001). Extreme teratozoospermia (Mateu et al., 2006), testicular sperm from non-obstructive azoospermia (Rodrigo et al., 2011) and from carriers of Y-microdeletions have been also related to increased sperm aneuploidy, mostly for sex chromosomes (Mateu et al., 2010).

Retrospective analysis of trophoctoderm biopsies in severe male infertility have shown significantly greater incidence of sex chromosome abnormalities compared to embryos derived from normal sperm samples. These results highlight severe MF infertility as a possible referral category for PGD-A (Coates et al., 2015).

- Previous trisomic pregnancy (PTP)

Some studies suggest that a PTP is associated with increased risk of another aneuploid conception. In 2009, De Souza and colleagues used register data from Australian population-based birth defects to establish whether the risk of trisomies 13, 18, and 21 (Patau, Edwards, and Down syndrome, respectively) and concluded that women who had had a previous trisomic pregnancy, particularly those <35 years old at the time of pregnancy, appear to have an increased risk of future trisomic pregnancies.

In relation to previous data, a more recent study stated that the incidence of chromosomal abnormalities in preimplantation embryos associated with a previous aneuploid miscarriage was significantly higher in individuals with a previous aneuploid conception (Al-Asmar et al., 2012).

- Good prognosis patients and SET

For patients with a good prognosis, trophoctoderm biopsy using aCGH has high potential to increase overall pregnancy rates in IVF programs and to decrease multiple pregnancies when single elective transfer (SET) is performed. The first RCT comparing blastocyst-stage SET with and without aCGH in good prognosis patients showed an aneuploidy rate of 44.9% among biopsied blastocysts, with a significantly higher clinical pregnancy rate in the PGD-A group (70.9% vs. 45.8%; $P = 0.017$), and no twin pregnancies. This study revealed the limitations of SET when conventional morphology is used alone, even in patients without an increased risk for aneuploidy, because the aCGH group implanted with greater efficiency and yielded a lower miscarriage rate than those without aCGH (Yang et al., 2012).

Key-Aspects in the Current Practice

- *Female age.* As described along the text, this is the main contributing factor to aneuploidy. However, once an euploid embryo is transferred, pregnancy rates remain high up to 43–44 years of age.
- *Male age.* There has been no clear association of paternal age with the incidence of aneuploidy or clinical outcome, however with the new technologies and the possibility to test for mosaicism and small deletions/duplications, this factor would need to be revisited.
- *Hormonal stimulation.* There is current agreement that despite the percentage of aneuploid embryos could increase with higher hormonal dosage and response, the absolute number of euploid embryos in a single cycle would not be impaired. In addition, with the possibility of blastocyst vitrification after biopsy, the potential negative impact of stimulation on the endometrium could be overcome.
- *Ovarian reserve.* This factor is not associated to aneuploidy, the percentage of aneuploid embryos is not related to ovarian reserve, but has a clear impact in the probability of obtaining an euploid embryo for transfer.
- *Sperm concentration.* As previously mentioned, when this value decrease below 2–5 millions of sperm/mL indicates an impairment of male meiosis with higher risk of sperm aneuploidy.
- *Day of biopsy.* The current approach is trophoctoderm biopsy on day-5/6 blastocyst, obtaining between 4 and 8 cells for a more accurate genetic analysis, including mosaicism detection.

- *Technology for chromosomal analysis.* The aim is to analyze all 23 pairs of chromosomes, with the proper technology to detect uniform aneuploidy and mosaicism and partial aneuploidies (small duplications/deletions).
- *Day of transfer.* Blastocyst vitrification after trophectoderm biopsy is becoming frequent, with a deferred transfer of a thawed blastocyst in a non-stimulated cycle. In PGD-A is scheduled with a fresh transfer, the most common approach is day-5 biopsy with the transfer on the morning of day-6. This latest approach has the limitation that only embryos developing to blastocyst stage on day-5 could be analyzed.

References

- Al-Asmar, N., Peinado, V., Vera, M., et al. (2012). Chromosomal abnormalities in embryos from couples with a previous aneuploid miscarriage. *Fertility and Sterility*, 98(1), 145–150.
- Almeida, P. A., & Bolton, V. N. (1996). The relationship between chromosomal abnormality in the human preimplantation embryo and development in vitro. *Reproduction, Fertility and Development*, 8, 235–241.
- Blockeel, C., Schutyser, V., De Vos, A., et al. (2008). Prospectively randomized controlled trial of PGS in IVF/ICSI patients with poor implantation. *Reproductive Biomedicine Online*, 17(6), 848–854.
- Capalbo, A., Wright, G., Elliott, T., et al. (2013). FISH reanalysis of inner cell mass and trophectoderm samples of previously array-CGH screened blastocysts shows high accuracy of diagnosis and no major diagnostic impact of mosaicism at the blastocyst stage. *Human Reproduction*, 28(8), 2298–2307.
- Coates, A., Hesla, J. S., Hurliman, A., et al. (2015). Use of suboptimal sperm increases the risk of aneuploidy of the sex chromosomes in preimplantation blastocyst embryos. *Fertility and Sterility*, 104(4), 866–872.
- Coates, A., Kung, A., Mounts, E., et al. (2017). Optimal euploid embryo transfer strategy, fresh versus frozen, after preimplantation genetic screening with next generation sequencing: A randomized controlled trial. *Fertility and Sterility*, 107(3), 723–730.
- De Souza, E., Halliday, J., Chan, A., Bower, C., & Morris, J. K. (2009). Recurrence risks for trisomies 13, 18, and 21. *American Journal of Medical Genetics*, 149(12), 2716–2722.
- Fiorentino, F., Birick, A., Bono, S., et al. (2014). Development and validation of a next-generation sequencing-based protocol for 24-chromosome aneuploidy screening of embryos. *Fertility and Sterility*, 101(5), 1375–1382.
- Fragouli, E., Alfarawati, S., Spath, K., et al. (2017). Analysis of implantation and ongoing pregnancy rates following the transfer of mosaic diploid-aneuploid blastocysts. *Human Genetics*, 136(7), 805–819.
- Franasiak, J., Forman, E., Hong, K., et al. (2014). The nature of aneuploidy with increasing age of the female partner: A review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertility and Sterility*, 101, 656–663.
- García-Herrero, S., Rodrigo, L., Mateu, E., et al. (2014). Embryo aneuploidy screening with CGH arrays in the absence of implantation. *Médecine de la Reproduction, Gynécologie Endocrinologie*, 16(2), 112–119.
- Greco, E., Minasi, M. G., & Fiorentino, F. (2015). Healthy babies after intrauterine transfer of mosaic aneuploid blastocysts. *The New England Journal of Medicine*, 373(21), 2089–2090.
- Jacobs, P. A., & Hassold, T. J. (1995). The origin of numerical chromosome abnormalities. *Advances in Genetics*, 33, 101–133.
- Keltz, M. D., Vega, M., Sirota, I., et al. (2013). Preimplantation genetic screening (PGD-A) with comparative genomic hybridization (CGH) following day 3 single cell blastomere biopsy markedly improves IVF outcomes while lowering multiple pregnancies and miscarriages. *Journal of Assisted Reproduction and Genetics*, 30(10), 1333–1339.
- Kuliev, A., Zlatopolsky, Z., Kirillova, I., et al. (2011). Meiosis errors in over 20,000 oocytes studied in the practice of preimplantation aneuploidy testing. *Reproductive Biomedicine Online*, 22(1), 2–8.
- Mastenbroek, S., Twisk, M., van der Veen, F., & Repping, S. (2011). Preimplantation genetic screening: A systematic review and meta-analysis of RCTs. *Human Reproduction Update*, 17, 454–466.
- Mateu, E., Rodrigo, L., Prados, N., et al. (2006). High incidence of chromosomal abnormalities in large-headed and multiple-tailed spermatozoa. *Journal of Andrology*, 27(1), 6–10.
- Mateu, E., Rodrigo, L., Martínez, M. C., et al. (2010). Aneuploidies in embryos and spermatozoa from patients with Y chromosome microdeletions. *Fertility and Sterility*, 94(7), 2874–2877.
- Mir, P., Rodrigo, L., Mercader, A., et al. (2013). False positive rate of an arrayCGH platform for single-cell preimplantation genetic screening and subsequent clinical application on day-3. *Journal of Assisted Reproduction and Genetics*, 30(1), 143–149.
- Mir, P., Mateu, E., Mercader, A., et al. (2016). Confirmation rates of array-CGH in day-3 embryo and blastocyst biopsies for preimplantation genetic screening. *Journal of Assisted Reproduction and Genetics*, 33, 59–66.
- Munné, S., Sultan, K. M., Weier, H. U., et al. (1995). Assessment of numeric abnormalities of X, Y, 18, and 16 chromosomes in preimplantation human embryos before transfer. *American Journal of Obstetrics and Gynecology*, 172(4 Pt 1), 1191–1199.
- Musters, A. M., Repping, S., Korevaar, J. C., et al. (2011). Pregnancy outcome after preimplantation genetic screening or natural conception in couples with unexplained recurrent miscarriage: A systematic review of the best available evidence. *Fertility and Sterility*, 95, 2153–2157.
- Nagaoka, S. T., Hassold, T., & Hunt, P. (2012). Human aneuploidy: Mechanisms and new insights into an age-old problem. *Nature Reviews Genetics*, 13(7), 493–504.
- Rodrigo, L., Rubio, C., Peinado, V., et al. (2011). Sperm from patients with obstructive and nonobstructive azoospermia: Aneuploidy risk and reproductive prognosis using testicular sperm from fertile donors as control samples. *Fertility and Sterility*, 95(3), 1005–1012.
- Rubio, C., Gil-Salom, M., Simón, C., et al. (2001). Incidence of sperm chromosomal abnormalities in a risk population: Relationship with sperm quality and ICSI outcome. *Human Reproduction*, 16(10), 2084–2092.
- Rubio, C., Buendía, P., Rodrigo, L., et al. (2009). Prognostic factors for preimplantation genetic screening in repeated pregnancy loss. *Reproductive Biomedicine Online*, 18, 687–693.
- Rubio, C., Bellver, J., Rodrigo, L., et al. (2013). Preimplantation genetic screening using fluorescence in situ hybridization in patients with repetitive implantation failure and advanced maternal age: Two randomized trials. *Fertility and Sterility*, 99(5), 1400–1407.
- Rubio, C., Bellver, J., Rodrigo, L., et al. (2017). In vitro fertilization with preimplantation genetic diagnosis for aneuploidies in advanced maternal age: a randomized, controlled study. *Fertility and Sterility*, 107, 1122–1129.
- Schoolcraft, W. B., Katz-Jaffe, M. G., et al. (2009). Preimplantation aneuploidy testing for infertile patients of advanced maternal age: A randomized prospective trial. *Fertility and Sterility*, 92(1), 157–162.
- Sermon, K., Capalbo, A., Cohen, J., et al. (2016). The why, the how and the when of PGS 2.0: Current practices and expert opinions of fertility specialists, molecular biologists, and embryologists. *Molecular Human Reproduction*, 22(8), 845–857.
- Vera-Rodríguez, M., Michel, C. E., Mercader, A., et al. (2016). Distribution patterns of segmental aneuploidies in human blastocysts identified by next-generation sequencing. *Fertility and Sterility*, 105(4), 1047–1055.
- Yang, Z., Liu, J., Collins, G. S., et al. (2012). Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: Results from a randomized pilot study. *Molecular Cytogenetics*, 5(1), 24.

Genetic Analysis of the Embryo: Preimplantation Genetic Diagnosis

Alan R Thornhill, Igenomix UK Limited, Guildford, United Kingdom; and University of Kent, Canterbury, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blastocyst An embryo that has developed for five to 7 days after fertilization and has two distinct cell types and a central cavity filled with fluid (blastocoel).

Chromosomal aneuploidy A condition in which a cell has an incorrect number of chromosomes.

Haplotyping A combination of alleles (for different genes) that are located closely together on the same chromosome and that tend to be inherited together.

Human Leucocyte Antigen (HLA) A gene complex encoding the major histocompatibility complex (MHC) proteins which are responsible for the regulation of the immune system in humans.

Karyomapping A genome-wide linkage based approach to assess disease status in embryos.

Monogenic Involving or controlled by a single gene.

Next generation sequencing (NGS) A catch-all term for high-throughput technologies enabling massively parallel or deep sequencing which has revolutionized genomic research.

Preimplantation genetic diagnosis (PGD) A technique using in vitro fertilization (IVF) to ensure that an embryo does not inherit a known genetic defect from either parent.

Preimplantation genetic screening (PGS) A technique used as part of an in vitro fertilization cycle which identifies chromosomal aneuploidy with the aim of selecting only chromosomally normal embryos to improve IVF outcomes.

Whole genome amplification (WGA) A catch-all term to describe any technology designed to amplify a limited genomic DNA sample so as to generate a new sample that is indistinguishable from the original but with a higher DNA concentration.

Background

For couples with known risk of transmitting a specific genetic condition to their offspring, preimplantation genetic diagnosis (PGD) is often viewed as an alternative to prenatal diagnosis because it avoids the difficult decision regarding termination of an affected pregnancy following a positive prenatal test. In addition, given the increasing age at which women are having children and the concomitant problems associate with delayed childbearing, PGD can also be considered as a means to increase the likelihood of, and reduce the time to, having an unaffected pregnancy, by selecting from multiple embryos. In brief, oocytes and/or preimplantation embryos obtained following in vitro fertilization (IVF) are analyzed and only those embryos free of the disorder under study are transferred to the uterus to achieve pregnancy.

The first successful application of PGD in humans involved sexing of embryos by polymerase chain reaction (PCR) to avoid males affected with an X-linked disorder. Gender was determined in single blastomeres by PCR using primers for amplifying Y-chromosome-specific DNA sequences and embryos identified as female were selectively transferred to the uterus ([Handyside et al., 1990](#)). Later, successful PGD was reported for cystic fibrosis, based on the amplification of a DNA fragment containing the causative mutation and its detection by fragment analysis ([Handyside et al., 1992](#)). Since then, PGD for single gene disorders has evolved to incorporate the detection of several mutations and multiple linked markers to improve both the accuracy and reliability of testing ([Fiorentino et al., 2006](#); [Harton et al., 2011a](#)). Despite the tiny amount of diagnostic material available and the ever-present dual threats of allelic dropout and contamination, technological advances coupled with best practice and rigorous quality assurance measures now deliver reliable results with an extremely low incidence of misdiagnosis ([Wilton et al., 2009](#); [De Rycke et al., 2017](#)).

Today, PGD indications have been expanded and PGD is performed in laboratories worldwide. The precise number of PGD cycles that have been performed to date can only be estimated. The European Society for Human Reproduction and Embryology (ESHRE) PGD consortium has collected data for PGD cycles since 1999, with over 12,000 PGD cycles performed with several thousand healthy children born. The most recent report described 3445 cycles for single-gene disorders, including human leucocyte antigen (HLA) typing ([De Rycke et al., 2017](#)). Although the ESHRE data represent only a partial record of the PGD cases conducted worldwide, they are indicative of the general trend in the field.

PGD requires a multidisciplinary approach with deep collaboration between the IVF clinic and the genetic laboratory, which can either be colocated within the same facility or, more frequently, remote. In this latter situation, an effective transportation and logistics operation must be in place; IVF treatment (controlled ovarian stimulation, oocyte retrieval and in vitro fertilization, embryo culture and embryo transfer) is carried out in the IVF clinic, and only the biopsied embryonic samples are transported to the reference laboratory conducting the PGD analysis.

Regarding clinical results of PGD, the last data collection carried out for the ESHRE consortium showed that 79% of PGD cycles resulted in an embryo transfer. The clinical pregnancy rate obtained for the cycles was 25% per oocyte retrieval and 31% per embryo transfer, giving an overall implantation rate of 23%. Finally, the delivery rate was 20% per oocyte retrieval and 26% per embryo transfer and the miscarriage rate was 11% (De Rycke et al., 2017). However, these clinical results should be considered with caution as the cycles were conducted in 2011–12, there may be significant differences in the clinical results between the different IVF centers within the consortium and some of the best centers may not submit data to the consortium.

PGD has become a routine, state-funded and regulated treatment in some countries providing a humane, effective and realistic option for at-risk families. Where PGD is regulated, it is generally limited to serious conditions with a high likelihood of transmission. For adult onset disorders, HLA matching, previously untested disorders or cases with borderline severity, it is sometimes necessary to apply to a specific body for approval. Elsewhere, it is not universally recognized or accepted for various religious, ethical and legal reasons (Soini, 2007).

This article will focus on PGD for single gene disorders, as the analysis of chromosomal rearrangements and generalized aneuploidy will be dealt with elsewhere in this book.

Indications for PGD

PGD is recommended when couples are at risk of transmitting a known genetic abnormality to their children and, therefore, the indications are similar to conventional prenatal diagnosis. PGD has mainly been used to diagnose and prevent well-defined autosomal recessive, autosomal dominant or X-linked single-gene disorders. The conditions for which PGD has been applied increase annually and, in theory, almost all single gene disorders that have been characterized can be diagnosed in the embryo. Other uses for PGD include gender selection, compatible HLA typing (aiming for a 'saviour sibling'), the identification of hereditary cancers with variable penetrance (e.g., BRCA 1, 2 status) and late onset genetic diseases; all of which have been considered controversial. While not exhaustive, the list of over 400 conditions in the UK approved for PGD by the fertility regulator (HFEA) is interesting, as all have had to pass a legal test for seriousness and likelihood of transmission (Human Fertilisation and Embryology Authority, 2017).

Single Gene Disorders

According to recent ESHRE PGD consortium data, the most common indications are for cystic fibrosis, spinal muscular atrophy and some hemoglobinopathies (De Rycke et al., 2015); all autosomal recessive disorders which involve the presence of two mutated copies from each healthy carrier parent.

For autosomal dominant conditions, one mutated copy of the gene is enough for a person to be affected. Myotonic dystrophy type 1, neurofibromatosis and Huntington's disease are the most frequently requested indications (De Rycke et al., 2015). In some cases, an affected person inherits the condition from an affected parent. In others, the condition may result from a new (de-novo) mutation in the gene and occurs in people with no family history of the disorder. When the patient has a de-novo mutation it is necessary to identify the molecular change causing the disease. Once the mutation has been characterized, this change must be analyzed directly in the embryo.

X-linked recessive disorders are mainly transmitted by healthy carrier mothers to their sons. In these cases, affected males will have no risk of affected offspring; all daughters will be obligate carriers, but no sons will be affected. PGD for X-linked disorders is mainly performed for Duchenne's muscular dystrophy, hemophilia and fragile-X syndrome (De Rycke et al., 2015). Although initially, sexing with FISH was widely applied for X-linked disorders and only female embryos selected for transfer, specific diagnosis of the molecular defect has important advantages and has replaced FISH. First, it facilitates the identification and subsequent transfer of those healthy male embryos that by FISH diagnosis would be discarded. Second, female carriers may be identified that can be excluded from transfer or not, according to patient wishes or center policy. This is significant for those X-linked dominant conditions (e.g., fragile-X syndrome) where carrier females can manifest disease symptoms.

Cancer Predisposition and Late-Onset Diseases

PGD is also offered to those couples where one partner carries a mutation predisposing to cancer or other late-onset disease. For cancer predisposition syndromes that are not fully penetrant and for which some form of therapeutic measures may be available, prenatal diagnosis and termination of pregnancy remain controversial and PGD appears as an attractive option, preventing the difficult decision of termination of an established pregnancy. In contrast, PGD for diseases that will not develop until adulthood, or for mutations that only confer a heightened risk, raises issues of how to balance the possible benefits of PGD for the future child against the risks of PGD and IVF for the patient. Despite these ethical concerns, the number of PGD cycles reported for these types of conditions is increasing, and the procedure has been performed for a number of diseases, including the common syndromes of genetic predisposition to colon and breast cancer (De Rycke et al., 2015, 2017; Rechitsky et al., 2002).

For some adult-onset conditions (e.g., Huntington's disease), patients with a family history of the disorder do not wish to know their genetic status in advance, but want to ensure that their children do not have the mutation. Unlike prenatal diagnosis, PGD for

Huntington's disease allows the status of the "potential" mutation carrier to be blinded, either by nondisclosure or the less ethically troublesome exclusion tests (Sermon et al., 2002). In the former, the mutation is analyzed but the results are not revealed to the patient. This approach has several practical and ethical problems including the requirement to withhold details of the IVF cycle to avoid any potential clues regarding the patient's carrier status. Moreover, a mock transfer might be necessary if no embryos are suitable for transfer so that the patients do not suspect they are carriers. In the exclusion test, those embryos inheriting the haplotype coming from the affected grandparent are ruled out for the transfer with the unfortunate consequence that, even if the patient is not a carrier, some unaffected embryos could be rejected.

HLA Matching

Another controversial but well-established indication is HLA matching in which PGD is used to help couples conceive a child (with or without a specific genetic risk themselves) who may donate compatible cord blood or hematopoietic stem cells for transplantation to save an affected sibling (Verlinsky et al., 2001), as HLA-identical donors are frequently unavailable. Hematopoietic stem cell transplantation (HSCT) from an HLA-identical donor is the best therapeutic option for genetic diseases affecting the hematopoietic and/or immune system in children (e.g., β -thalassemia, Fanconi anemia), and can also be an effective therapeutic option for acquired diseases (e.g., leukemia, acquired medullary aplasia).

PGD to select an embryo free of an inherited condition and HLA-matched to an existing affected child was first applied for Fanconi anemia in 2001 (Verlinsky et al., 2001) and has since been performed for a number of different hematopoietic diseases.

Where PGD/HLA testing is used to select embryos that are both HLA matched and free from specific disease, the chance of an embryo being both unaffected and a match is only 18.75% in the case of autosomal recessive conditions (e.g., beta-thalassemia), thus there are fewer embryos available for transfer and consequently poorer IVF success rates. Furthermore, many patients requesting preimplantation HLA typing are of advanced reproductive age, with corresponding reduced success, such that many patients require two or more attempts before delivering an HLA identical offspring. Counseling for such patients must include expectation management regarding realistic success rates, timelines and the possible requirement for bone marrow stem cells if cord blood is insufficient (Kahraman et al., 2014).

Diagnostic Methods

PCR and Fragment Analysis

A direct multiplex PCR approach using targeted primers designed specifically for the mutation of interest combined with primers for closely linked STR markers has been traditionally the gold standard to perform PGD for monogenic disorders. During pre-PGD work up, the analysis of polymorphic markers in DNA samples from patients and potentially informative relatives identifies which alleles are expected in the embryos, and the specific marker alleles which cosegregate with the mutation. This combined linkage and mutation detection approach improves accuracy, minimizing potential errors caused by undetected allele drop out (ADO) and/or contamination (Fiorentino et al., 2003). ADO refers to the amplification failure (or extreme preferential nonamplification) of one of the two alleles, making a heterozygous locus appear homozygous, and potentially leading to misdiagnosis.

Genotyping of the amplified products can be performed using many different strategies including amplification refractory mutation system, restriction enzyme digestion and real-time PCR, with minisequencing the most common method for point mutation detection (Fiorentino et al., 2003). In the minisequencing technique, a primer extension reaction is performed, allowing rapid and accurate detection of point mutations. The minisequencing primer is designed to anneal one base before the target site, and it can only be elongated with one specific dideoxynucleotide. The four different dideoxynucleotides are labeled with different fluorochromes, and the products analyzed on an automated DNA sequencing system.

The use of multiplex PCR for linkage markers alone (so-called preimplantation genetic haplotyping or PGH) has become widespread in PGD for monogenic disorders (Renwick et al., 2010) and HLA typing. The main advantage is that such protocols can be used for several couples, independent of the mutation they carry, thus saving time and resources in pre-PGD workups. A genome-wide linkage approach, karyomapping (Handyside et al., 2010), further expands on this concept using commercially available microarrays to provide an off-the-shelf accurate and reproducible semiautomated solution to PGD following a whole genome amplification step (see below).

Whole Genome Amplification (WGA)

The creation of robust, accurate multiplex protocols requires careful design, optimization and validation before clinical use, with a large investment of time and resources needed. The widespread adoption of whole genome amplification (WGA) has proved to be a practical and efficient alternative to direct testing to facilitate single cell testing for PGD and many commercially available products are now available (Huang et al., 2015). In general, WGA amplifies the entire 6 picograms of the single cell genome producing microgram quantities of DNA—enough for multiple downstream applications including standard PCR assays for haplotyping and the direct analysis of mutations in case of monogenic diseases. This can avoid the need to optimize multiplex PCR protocols on single

cells (Renwick et al., 2010; Handyside et al., 2010). Moreover, WGA allows combined PGD for single gene disorder or HLA typing with array comparative genomic hybridization (aCGH) or Next Generation Sequencing (NGS) for the detection of chromosomal imbalances using the same sample with the aim of improving clinical PGD results (Rechitsky et al., 2015). Finally, WGA facilitates repeat testing of samples for use in proficiency testing, validation or in the case of run failure.

Despite the large quantities of amplified DNA generated, WGA methods yield relatively high ADO rates overall from single cells (Huang et al., 2015). This problem can be circumvented with the application of enough linked markers to avoid misdiagnosis (e.g., with karyomapping) and/or reduced with the use of trophoctoderm biopsies instead of single cells because the former provides multiple cells in the biopsy sample, which is known to result in lower ADO rates.

Karyomapping

Historically, the growth of PGD has been limited by the need to develop individual protocols for different diseases and families. However, karyomapping is a universal, robust, off-the-shelf approach to PGD which uses a high-density single nucleotide polymorphism (SNP) array to accurately identify parental DNA haplotypes in embryonic samples. By genotyping the parents at several hundred thousand SNP sites throughout the genome, a set of highly informative SNP markers are identified for each of the four parental chromosomes (Handyside et al., 2010). The phase of the alleles for each informative SNP locus along each chromosome and linkage of the risk alleles with the parental chromosomes can then be established by reference to the genotype of a relative of known disease status (either affected or unaffected). The parental origin of each chromosome in the embryo is then ascertained by comparison with the genotype of the reference. A similar approach has been developed called haplarithmisis, but it is not yet commercially available (Zamani Esteki et al., 2015). Karyomapping's main advantage compared with the gold standard mutation plus linkage analysis method (described above) is that it is applicable to almost any familial single gene disorder, or any combination of loci, within the chromosome regions covered by informative SNP loci, eliminating the need to develop patient- or disease-specific tests (Handyside et al., 2010; Renwick et al., 2010). The main challenges of the karyomapping approach are that diagnosis is difficult when insufficient informative SNP markers are available (e.g., in some telomeric genes and in highly consanguineous families), or when pseudogenes are involved. Moreover, it cannot be used alone for de novo mutation cases or when other tested family members are not available to provide samples or are uninformative owing to recombination. In such cases, direct mutation testing from at least one embryo is necessary to establish phase i.e., identify on which chromosome the mutation is present (Konstantinidis et al., 2015).

However, this approach has several limitations (as described above), which need to be overcome as more carrier screening tests are performed that identify cocarriage of mutations in the same gene among couples with no family history or affected children—the historic source of referrals for PGD. At present, karyomapping is not commercially validated for aneuploidy screening and does not readily detect mitotic trisomies nor simple copy number variation. Nonetheless, use of karyomapping, for the most part, represents a significant advance over the current gold standard for PGD and will be a powerful tool to investigate parental origin and phase of origin of meiotic chromosome errors.

Next Generation Sequencing

Next generation sequencing (NGS) provides high-throughput and base pair resolution data, providing the analysis of multiple genetic loci and samples from different couples simultaneously. Moreover, NGS, as with karyomapping, allows the combined evaluation of aneuploidy and single-gene disorders from the same biopsy using a single platform.

A number of studies demonstrate the possibility of using NGS to test single cells for PGD (Treff et al., 2013; Sermon et al., 2016). One used DNA from a trophoctoderm biopsy with NGS that was consistent with two conventional methodologies of PGD (Treff et al., 2013). However, the major concern relating to NGS technology is that an insufficient sequencing depth may result in false positive or failure to identify a mutation (false negative) due to the presence of sequencing artifacts and ADO, respectively. Moreover, NGS has technical limitations in testing for dynamic mutations. Further studies are needed to evaluate this technology before its routine clinical use in PGD, although given its penetration in other diagnostic arenas NGS for PGD appears inevitable.

Combined PGD and Chromosomal Screening

Extensive evidence has revealed a high incidence of chromosomal abnormalities in human embryos obtained by assisted reproduction techniques leading to miscarriages and implantation failures (Sermon et al., 2016) and this is dealt with in more detail elsewhere in this book.

Couples presenting for PGD to avoid transmission of single gene disorders are not without these same problems despite the fact that they are often categorized as “fertile.” Indeed, many PGD patients may be of advanced age because they have already had affected children or pregnancies. For this reason, they can also benefit from the simultaneous analysis of the disease and the presence of aneuploidies, selecting for transfer those PGD “unaffected” and simultaneously euploid embryos. Indeed, reported PGD results have never been particularly high when reported together. The genetic basis of the disorder under test can affect the success

rates (as, for example, fewer embryos are available for transfer when testing a dominant condition versus a recessive one) and maternal age (as with routine IVF) is the single biggest predictor of a successful outcome. Since the main cause of IVF failure in advanced maternal age is chromosomal abnormality, it is rational to consider selection of only euploid embryos after PGD as a way to improve pregnancy rates per transfer and reduce miscarriage rates.

Several studies have shown significant improvement in rates of pregnancy and live births following testing for aneuploidy in patients undergoing IVF for infertility (reviewed in [Sermon et al., 2016](#)). The use of the WGA product provides a straightforward solution for performing any required test in the same biopsy material, allowing the simultaneous analysis of PGD and aneuploidy screening. Using this approach the first systematic study of PGD combined with the aneuploidy screening was published, demonstrating an increase in the pregnancy rate from 45.4% in the conventional PGD to 68.5% with combined aneuploidy screening and 3-fold miscarried reduction (5.5% vs. 15%) ([Rechitsky et al., 2015](#)).

Currently, it is possible to determine both monogenic diagnosis (plus HLA haplotyping) and aneuploidy detection by PGD using two different techniques on the same WGA product ([Rechitsky et al., 2015](#)). For this reason, a single assay using the same platform to detect simultaneously both chromosomal and monogenic disorders is desirable. Since both karyomapping and haplarithmism define unique sets of SNP markers for each of the four parental chromosomes, they represent such a method ([Handyside et al., 2010](#); [Zamani Esteki et al., 2015](#)), by allowing accurate identification of the region of interest containing the mutation and simultaneous high-resolution molecular cytogenetic analysis. Meiotic trisomies can be identified by the presence of both haplotypes from one parent in segments of the chromosome, resulting from the inheritance of two chromosomes with different patterns of recombination, in combination with a single haplotype from the other parent. Moreover, monosomies or deletions can be identified by the absence of one of the parental haplotypes ([Handyside et al., 2010](#); [Zamani Esteki et al., 2015](#); [Konstantinidis et al., 2015](#)). Both haplarithmism and karyomapping represent significant advances toward a universal PGD test (combining detection of specific single gene disorders and chromosomal aneuploidy), but both need further validation for aneuploidy detection against current gold standard methods.

Challenges, Limitations and Barriers to Access

Today, not all people who wish to have PGD can do so. This is mainly due to a lack of funding (PGD requires costly IVF and technologically advanced testing), limited access to treatment (not all IVF laboratories have biopsy skills or links with a suitable diagnostic laboratory) or in some cases legal restrictions, for example in Germany and Norway ([Soini, 2007](#)). It is essential that the disorder that is intended to be diagnosed has a comprehensive and accurate genetic characterization to at least identify the gene responsible for the condition. Some conditions, including autism, diabetes and heart disease, where the causes are either unidentified, polygenic or there are imprecise risk factors, are not suitable for PGD.

As PGD involves both an IVF procedure and a selection procedure based on genetic testing, it is important to estimate the number of unaffected embryos expected for transfer prior to starting the procedure. This number depends on the embryo quality and the theoretical risk according to the genetic disorder (e.g., recessive, dominant, sex-linked). The embryo's potential to implant depends mainly on the woman's age and the presence of additional viability factors. Chromosome aneuploidy is the major cause of IVF failure and miscarriage. To benefit patients, sufficient embryos should be analyzed to obtain unaffected embryos for transfer. Recent advances in vitrification procedures have made it feasible to batch oocytes or embryos to reach a minimum number of embryos for analysis. [Table 1](#) shows the rates of transferable embryos considering the inheritance patterns and the potential presence of aneuploidies (Igenomix, unpublished data).

One final significant point regarding PGD concerns patient and clinician expectation. PGD provides no guarantee of a completely healthy baby. Instead, PGD minimizes the risk for the disease for which the couple has a high risk of transmitting to their offspring. However, there remains a small risk of misdiagnosis due to the existence of mosaicism or technical limitations, and therefore couples should always be offered the possibility of prenatal diagnosis to confirm results. Moreover, couples may assume that a normal healthy child following successful PGD for a specific disorder is guaranteed, forgetting that a more cryptic disorder or

Table 1 Rates of transferable embryos considering the inheritance patterns and the potential presence of aneuploidies

<i>Indication</i>	<i>Unaffected embryos</i>	<i>PGS normal embryos^a</i>	<i>Transferable embryos</i>
Autosomal dominant	1/2	1/2	1/4
Autosomal recessive	3/4	1/2	3/8
X-linked recessive	3/4	1/2	3/8
X-linked dominant	1/2	1/2	1/4
HLA matching	1/4	1/2	1/8
HLA + AD	1/8	1/2	1/16
HLA + AR	3/16	1/2	3/32
HLA + X-linked	3/16	1/2	3/32

^a50% of blastocysts are expected to be PGS abnormal on average (Igenomix, unpublished data).

a de-novo mutation or abnormality which was not tested for may be present. Indeed, even with a genome-wide linkage based approach such as karyomapping, no other genes or mutations even within the same gene are routinely analyzed if there was no indication to do so. Furthermore, if chromosomes are not analyzed, an aneuploid but genetically unaffected embryo may be transferred. The entire procedure should always be explained in detail by a suitably qualified genetics professional before treatment to ensure that couples are informed about the potential risks and limitations (Harton et al., 2011b).

Future Perspectives for PGD

The rapid rise of lower cost, high-throughput and accurate next generation sequencing technology combined with the temporal separation of the biopsy and diagnostic procedures via improved embryo vitrification techniques has improved patient access to PGD, while providing more time for diagnosis and genetic counseling. The accuracy and reliability of PGD is improved when performing a multicellular trophectoderm biopsy from a blastocyst and this has become the gold standard approach. Improvements in noninvasive assessment of chromosomal aneuploidy in blastocoel fluid and spent culture medium could herald a new era in which no biopsy is necessary even for single gene disorders (Xu et al., 2016). Further technical improvements enabling the simultaneous analysis of single gene disorders and chromosome aneuploidy from the same sample may reduce costs to patients and improve implantation and rates of healthy live births whilst reducing miscarriage, time to pregnancy and patient dropout rates. The rapid uptake of pan-ethnic expanded preconception carrier screening for couples with no family history of specific genetic disease following professional guidelines, improved access and reduced cost, as well as government funding for PGD in many countries is certain to increase the number of PGD cases further. The increased demand for PGD should also drive standardization of best practice (Schoolcraft et al., 2017), and an evolution of existing guidelines (Harton et al., 2011a) to ensure that we learn from the challenges and mistakes of the past. Areas for focus include embryo selection for biopsy (a more inclusive approach), biopsy safety (safe laser use), sample preparation and identification (built in molecular assessment of contamination and embryo identification). Finally, recent advances in genome editing in embryos (Ma et al., 2017) bring ever closer the possibility of shifting the focus of PGD from “selection” to “correction” by repairing genetic defects in embryos, thereby “curing” previously incurable disorders including those with polygenic inheritance. Whatever the future holds for PGD, a robust, well-planned ethical and legal framework will continue to be important to ensure patient safety and public confidence.

Conclusions

PGD can be used to screen embryos for almost any kind of genetic disorder in which the genetic cause is characterized, thus the number of indications and total number of PGD cases increases year on year. With improving technologies, best practice guidelines and the adoption of external quality assessment programmes and laboratory accreditation (Harper et al., 2010), PGD analysis has reached a high level of accuracy, enabling multiple diagnoses from the same sample. Diagnosis of a monogenic disease can be combined with HLA typing and/or detection of chromosomal abnormalities to improve reproductive outcomes. The current strategy of blastocyst biopsy and vitrification, followed by later transfer of unaffected embryos in a thaw cycle, has superseded the cleavage stage biopsy and fresh transfer approach and will be further embedded as the gold standard for PGD following robust clinical trial data demonstrating the benefits of frozen-thaw versus fresh embryo transfer cycles. Despite all the advances made in PGD, one fundamental principle still remains true: successful PGD requires a platform of successful IVF.

References

- De Rycke, M., Belva, F., Goossens, V., et al. (2015). ESHRE PGD consortium data collection XIII: Cycles from January to December 2010 with pregnancy follow-up to October 2011. *Human Reproduction*, 30, 1763–1789.
- De Rycke, M., Goossens, V., Kokkali, G., et al. (2017). ESHRE PGD consortium data collection XIV-XV: Cycles from January 2011 to December 2012 with pregnancy follow-up to October 2013. *Human Reproduction*, 32, 1974–1994.
- Fiorentino, F., Biricik, A., Nuccitelli, A., et al. (2006). Strategies and clinical outcome of 250 cycles of preimplantation genetic diagnosis for single gene disorders. *Human Reproduction*, 21, 670–684.
- Fiorentino, F., Magli, M. C., Podini, D., et al. (2003). The minisequencing method: An alternative strategy for preimplantation genetic diagnosis of single gene disorders. *Molecular Human Reproduction*, 9, 399–410.
- Handyside, A. H., Harton, G. L., Mariani, B., et al. (2010). Karyomapping: A universal method for genome wide analysis of genetic disease based on mapping crossovers between parental haplotypes. *Journal of Medical Genetics*, 47, 651–658.
- Handyside, A. H., Kontogianni, E. H., Hardy, K., & Winston, R. M. (1990). Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. *Nature*, 344, 768–770.
- Handyside, A. H., Lesko, J. G., Tarin, J. J., Winston, R. M., & Hughes, M. R. (1992). Birth of a normal girl after in vitro fertilization and preimplantation diagnostic testing for cystic fibrosis. *New England Journal of Medicine*, 327, 905–909.
- Harper, J. C., Sengupta, S., Vesela, K., et al. (2010). Accreditation of the PGD laboratory. *Human Reproduction*, 25, 1051–1065.
- Harton, G. L., De Rycke, M., Fiorentino, F., et al. (2011a). ESHRE PGD consortium best practice guidelines for amplification-based PGD. *Human Reproduction*, 26, 33–40.
- Harton, G., Braude, P., Lashwood, A., et al. (2011b). ESHRE PGD consortium best practice guidelines for organization of a PGD centre for PGD/preimplantation genetic screening. *Human Reproduction*, 26, 14–24.

- Huang, L., Ma, F., Chapman, A., Lu, S., & Xie, X. S. (2015). Single-cell whole-genome amplification and sequencing: Methodology and applications. *Annual Review of Genomics and Human Genetics*, 16, 79–102.
- Human Fertilisation and Embryology Authority. (2017). PGD conditions. Table of PGD conditions currently approved and awaiting consideration by the HFEA. <https://www.hfea.gov.uk/pgd-conditions/>. (Accessed 28 November 2017).
- Kahraman, S., Beyazyurek, C., Yesilipek, M. A., et al. (2014). Successful haematopoietic stem cell transplantation in 44 children from healthy siblings conceived after preimplantation HLA matching. *Reproductive Biomedicine Online*, 29, 340–351.
- Konstantinidis, M., Prates, R., Goodall, N. N., et al. (2015). Live births following Karyomapping of human blastocysts: Experience from clinical application of the method. *Reproductive Biomedicine Online*, 31, 394–403.
- Ma, H., Marti-Gutierrez, N., Park, S. W., et al. (2017). Correction of a pathogenic gene mutation in human embryos. *Nature*, 548, 413–419.
- Rechitsky, S., Pakhalchuk, T., San Ramos, G., et al. (2015). First systematic experience of preimplantation genetic diagnosis for single-gene disorders, and/or preimplantation human leukocyte antigen typing, combined with 24-chromosome aneuploidy testing. *Fertility and Sterility*, 103, 503–512.
- Rechitsky, S., Verlinsky, O., Chistokhina, A., et al. (2002). Preimplantation genetic diagnosis for cancer predisposition. *Reproductive Biomedicine Online*, 5, 148–155.
- Renwick, P., Trussler, J., Lashwood, A., Braude, P., & Ogilvie, C. M. (2010). Preimplantation genetic haplotyping: 127 diagnostic cycles demonstrating a robust, efficient alternative to direct mutation testing on single cells. *Reproductive Biomedicine Online*, 20, 470–476.
- Schoolcraft, W., Meseguer, M., & Global Fertility Alliance. (2017). Paving the way for a gold standard of care for infertility treatment: Improving outcomes through standardization of laboratory procedures. *Reproductive Biomedicine Online*, 35, 391–399.
- Sermon, K., Capalbo, A., Cohen, J., et al. (2016). The why, the how and the when of PGS 2.0: Current practices and expert opinions of fertility specialists, molecular biologists, and embryologists. *Molecular Human Reproduction*, 22, 845–857.
- Sermon, K., De Rijcke, M., Lissens, W., et al. (2002). Preimplantation genetic diagnosis for Huntington's disease with exclusion testing. *European Journal of Human Genetics*, 10, 591–598.
- Soini, S. (2007). Preimplantation genetic diagnosis (PGD) in Europe: Diversity of legislation a challenge to the community and its citizens. *Medicine and Law*, 26, 309–323.
- Treff, N. R., Fedick, A., Tao, X., et al. (2013). Evaluation of targeted next-generation sequencing-based preimplantation genetic diagnosis of monogenic disease. *Fertility and Sterility*, 99, 1377–1384 e6.
- Verlinsky, Y., Rechitsky, S., Schoolcraft, W., Strom, C., & Kuliev, A. (2001). Preimplantation diagnosis for Fanconi anemia combined with HLA matching. *Journal of the American Medical Association*, 285, 3130–3133.
- Wilton, L., Thornhill, A., Traeger-Synodinos, J., Sermon, K. D., & Harper, J. C. (2009). The causes of misdiagnosis and adverse outcomes in PGD. *Human Reproduction*, 24, 1221–1228.
- Xu, J., Fang, R., Chen, L., et al. (2016). Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proceedings of the National Academy of Sciences of the United States of America*, 113, 11907–11912.
- Zamani Esteki, M., Dimitriadou, E., Mateiu, L., et al. (2015). Concurrent whole-genome haplotyping and copy-number profiling of single cells. *American Journal of Human Genetics*, 96, 894–912.

Mitochondria and Embryo Viability

Antonio Diez-Juan and Carlos Marín, IGENOMIX, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Mitochondria

Mitochondria play an important role in the generation of energy in eukaryotic cells, using the process of oxidative phosphorylation to derive energy (ATP) from carbohydrates and fatty acids. It is also important to remember that mitochondria it is not only energy factory as a central metabolic organelle, as a raw material factory, execute critical biochemical functions for the synthesis of anabolic precursor for fundamental cellular components, including amino acids, fatty acids, and nucleotides. Mitochondria contain their own genome, which encodes tRNAs, rRNAs, and some mitochondrial proteins. Ranging in size from 0.5 to 1.0 μm in diameter, these organelles have a double-membrane system consisting of inner and outer membranes separated by an intermembrane space. The outer mitochondrial membrane encloses the matrix (internal space) and contains a large number of proteins as transporters allowing the importation of mitochondrial proteins that encoded in the nucleus and synthesized in the cytoplasm, in addition multiple transporters that import and export numerous substrates and products for and from metabolic activity. The inner mitochondrial membrane, which is folded into structures (cristae) that increase the surface area, is less permeable, blocking the movement of ions and other small molecules allowing the generation of a membrane potential for energy production (Fig. 1).

Origin of Mitochondria in Eukaryotes

Eukaryotes are genetic chimeras. They have genes that they inherited vertically from their archaeobacterially associated host. Eukaryotes possess genes that they inherited vertically from the endosymbiont. Typical mitochondria harbor up to more than a 1000 proteins that are encoded in the nucleus. Throughout the course of eukaryotes origin, many genes were transferred from the genome of the mitochondrial endosymbiont to the genome (nuclear) of the host. This kind of endosymbiotic gene transfer is nothing unusual; endosymbiosis very often entails gene transfers from the endosymbiont to the host. It happened during the origin of plastids too, and it is still ongoing in our own genome: Mitochondrial DNA constantly escapes from the organelle and becomes integrated as copies into nuclear DNA. The vast majority of mitochondrial proteins are encoded by nuclear genes, and many of these are endosymbiotic acquisitions from the mitochondrial ancestor.

Similar to eukaryotes themselves, mitochondria have arisen only once in all of evolution. The top evidence for the single origin of mitochondria comes from a preserved set of genes preserved in the mitochondrial DNA across all known eukaryotic groups including plants. In the case of hydrogenosomes (which usually lack DNA) and mitosomes (which so far always lack DNA), the strongest evidence for their common ancestry with mitochondria is twofold.

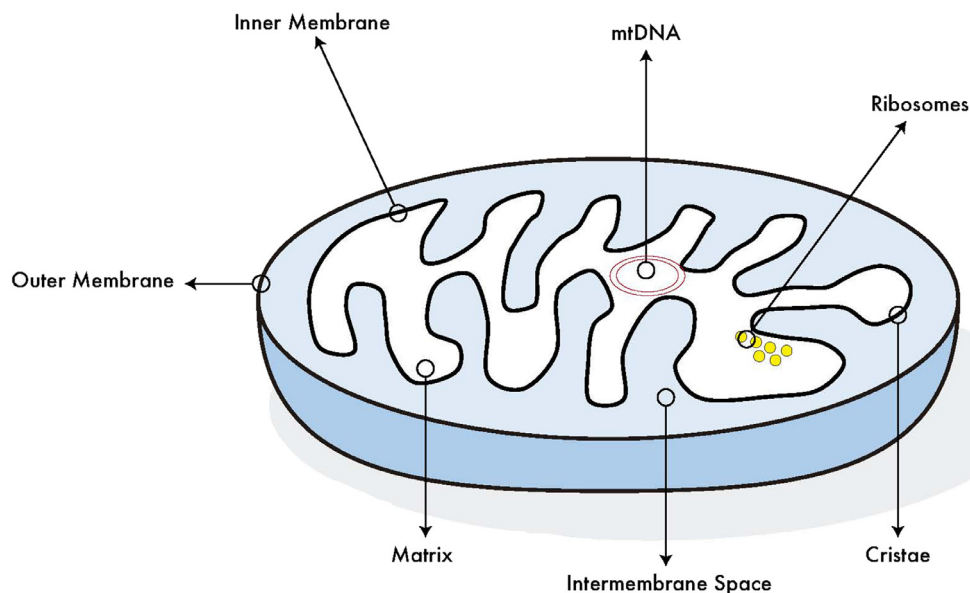


Fig. 1 Diagram of a mitochondrion. The different components of mitochondria are represented.

New Data Are Continuously Improving Our Views Regarding Mitochondrial Evolution

The autogenous hypothesis is less supported suggests that mitochondria were born by splitting off from a portion of DNA in the nucleus of the eukaryotic cell (Gray, 2012). The endosymbiotic hypothesis, proposed by Lynn Margulis, is the most official, it established the idea that mitochondria evolved from free-living bacteria via symbiosis within a eukaryotic host cell (Margulis, 1970).

The oldest eukaryotic microfossils date back 1.45 billion years. This date matches up to a time when the oceans were mostly anoxic because of the workings of marine H₂S-producing bacteria. Eukaryotes appeared and branched out in an environment where anoxia was common. The mitochondrial genome appears to have a monophyletic origin from α -Proteobacteria with Rickettsiales and several *Rickettsia*-like endosymbionts identified as the α -Proteobacterial order most closely related to mitochondria (Gray, 2012) (Fig. 2).

It is possible that the host that acquired the mitochondrion was a prokaryote. This is linked to the idea that the ancestral mitochondrion was a facultative anaerobe, perhaps similar in physiology to modern Rhodobacterales (Martin and Mentel, 2010). Although, existing data suggest that the mitochondrial genome originated from the eubacterial, not the archaeal, domain of life (specifically α -Proteobacterial). In fact, *Rickettsia prowazekii*, the etiologic agent of epidemic typhus, transmitted in the feces of lice, stands out as the most genetically similar to mitochondria.

Mitochondrial Is Not Only an ATP Factory

The top implicit mitochondrial role include ATP production by oxidative phosphorylation, β -oxidation of fatty acids, and metabolism of amino acids and lipids. In addition, mitochondria have different and complex functions, like participating in multiple cell signaling cascades. Proteins such as GTPases, kinases, and phosphatases are implicated in bi-directional communication connecting the mitochondria and the rest of the cell, contributing in the regulation of metabolism, cell-cycle control, embryonic development, antiviral responses, and cell death (McBride et al., 2006). Furthermore, there is an important role in the regulation of numerous proteins in response to calcium signaling. Mitochondria respond to calcium both as a calcium buffer and as the propagator of intracellular calcium waves during muscle contraction and synaptic vesicle release (McBride et al., 2006). Another example of the incorporation of mitochondria in signaling pathways is its fundamental role in the apoptotic cascade and cell death (Ott et al., 2007); indeed, mitochondrial fragmentation and cristae remodeling are essential steps for “cytochrome *c*” release and cell death (McBride et al., 2006).

Further, mitochondrial dysfunction is associated to genomic instability and abnormal RNA and DNA synthesis, since multiple enzymes responsible for metabolism of cytosolic ribonucleotides and deoxyribonucleotides are existing in mitochondria. For instance, serine, a major resource of one-carbon anabolic units, is necessary for the synthesis of glycine, thymidylate, methionine, epigenetic methylation reactions, and purine synthesis. Serine, is converted to glycine, and a methyl group is transferred from serine to tetrahydrofolate (THF), yielding glycine and 5,10-methylene-THF (Desler et al., 2010). In the mitochondria, 5,10-methylene-THF is transformed into formate in a series of enzymatic exchanges beginning with conversion into 5-methyl-THF and afterward into 10-formyl-THF. Cytosolic 10-formyl-THF is a fundamental one-carbon-unit donor for the de novo synthesis of purine

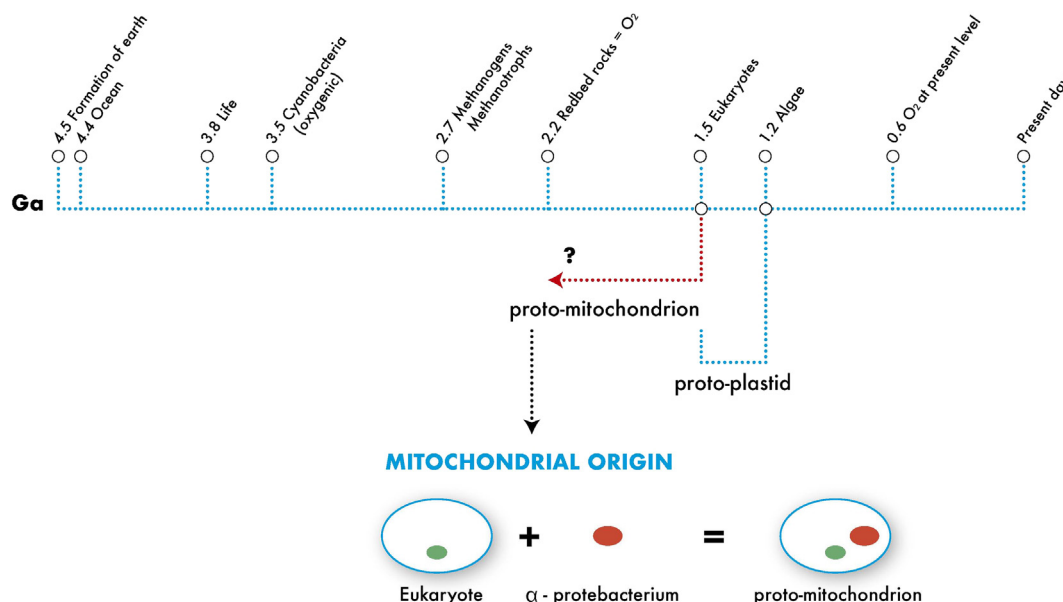


Fig. 2 Time line from the origin to present-day. The principal invasions giving rise to proto-mitochondrion are showed (Ga, billion years ago).

nucleotides (Desler et al., 2010). This route reveals the importance of mitochondria in DNA/RNA synthesis. In addition, mutations affecting the activity of the electron transport chain can lead to a damaging effect on cytosolic dNTP levels, originating instability in the nuclear genome (Desler et al., 2010).

In addition, reactive oxygen species (ROS) are reactive molecules that comprise various chemical species like superoxide anion, hydroxyl radical, and hydrogen peroxide. About 1%–2% of the molecular oxygen consumed in the course of physiological respiration is transformed into superoxide radicals (Ott et al., 2007). Mitochondrial ROS are produced in the electron transport chain through one-electron carriers interact straightly with mitochondrial proteins, lipids, and DNA. This results a plethora of oxidative modifications as lipid peroxidation, protein oxidation, and mitochondrial DNA (mtDNA) mutations. Without a doubt, as the majority of ROS are products of mitochondrial respiration, mitochondria are the major targets for their damaging.

Oxidative DNA injury affects replication and transcription of mtDNA. This inhibits mitochondrial function, which increases production of ROS. Therefore evolution have improved mitochondria defense system to detoxify ROS and repair ROS-induced damage. Numerous studies have suggested that mtDNA is more vulnerable to oxidative damage (Cui et al., 2012). Therefore, oxidative stress plays an important role in aging, and the modulation of this system directly affects the responsiveness of cells to go through.

Mitochondrial DNA

mtDNA is a circular DNA molecule of 16.5 Kb that is situated in the matrix. Its genetic code differs to some extent from the universal one, in that AUA codes for methionine and not isoleucine and UGA codes for tryptophan and not STOP. Also, mtDNA has no introns. In humans, 100–10,000 separate copies of mtDNA are usually present per cell and is dependent of mitochondrial activity. For example, power demanding tissues such as cardiac and skeletal muscle contain between 2000 and 10,000 thousands of copies of mtDNA per cell, with an average mtDNA copy number per mitochondrion between 1000 and 10,000 copies. Less energy-demanding tissues, like the lungs, have a typical amount between 200 and 3000 copies per cell and 50 and 300 per mitochondrion. In oocytes mitochondrial content has been estimated between 100 and 640 thousands, and it is assumed in general that each mitochondrion holds one or two genomes (reviewed by Van Blerkom, 2009) (Fig. 3A).

The mtDNA has a regulatory sequence, involved in replication and transcription, known as the D-loop. It also encodes 2 rRNAs (12S and 16S), 22 tRNAs, and 13 mRNAs, Sequence and organization of the human mitochondrial genome. There are also some proteins that are encoded by mtDNA and synthesized in the mitochondria. These proteins are fundamental subunits of the electron transport complexes of the electron transport chain (ETC): one subunit of complex III, seven subunits of complex I, three subunits of complex, and two subunits of complex V. If mtDNA genes are mutated, the functions of the ETC are affected; mutations in the nuclear genes encoding mitochondrial proteins can affect multiple mitochondrial functions (Fig. 3B). It is important to emphasize that mtDNA mutates faster than nuclear DNA due to its location near the ETC and the absence of protective histones. As a result, mtDNA is more exposed to the harmful effects of ROS.

Inheritance of Mitochondrial DNA and Bottleneck Selection

mtDNA is maternally inherited (Stewart and Larsson, 2014). Even though a zygote receives both maternal and paternal mtDNA at fertilization, the paternal mtDNA is specially targeted for elimination and removed from the cytoplasm of the zygote during very early embryogenesis (Sato and Sato, 2012).

The amount of mitochondria present in a metaphase II oocyte (Van Blerkom, 2009) correspond to only a small portion of the maternal mtDNA pool due to a genetic bottleneck that takes place in the course of oogenesis. In the primordial germ cells there is a great population of mtDNA that represents the maternal mtDNA pool. This is the starting point of the genetic bottleneck. At some stage in development of the germline, the maternal mitochondrial pool is subsampled to a quite small number resulting in only a small percentage of mtDNA being represent in the mature egg. In this way, the variety of mtDNA is restricted in each oocyte, and homoplasmy is promoted. Therefore, within a cohort of oocytes there are differences for the reason that the maternal mtDNA is arbitrarily segregated; in fact, in mtDNA diseases the level of mutant mtDNA differs in oocytes from the same patient (Marchington et al., 1998). Subsequent to the bottleneck, during oocyte maturation, there is an augment in mitochondrial content and mtDNA copy number (Fig. 4).

Mitochondrial Biogenesis and mtDNA Replication

mtDNA replication is mediated by a number of nuclear-encoded transcription and replication factors. These factors, together with mtDNA, form the mitochondrial nucleoid, which is in charge for the packaging, transcription, and replication of the mitochondrial genome. The nucleoid proteins include mitochondrial-specific polymerase gamma (which contains two subunits, POLGA and POLGB), mitochondrial transcription factor A (TFAM), mitochondrial RNA polymerase (mtRNA pol), mitochondrial transcription factor B (TFBM), helicase (Twinkle), key transcriptions factors (TFB1M, TFB2M), mitochondrial single-stranded DNA-binding protein (mtSSB), and mTERF. All of these are placed in the central region of the nucleoid; in contrast, ATAD3 is arranged

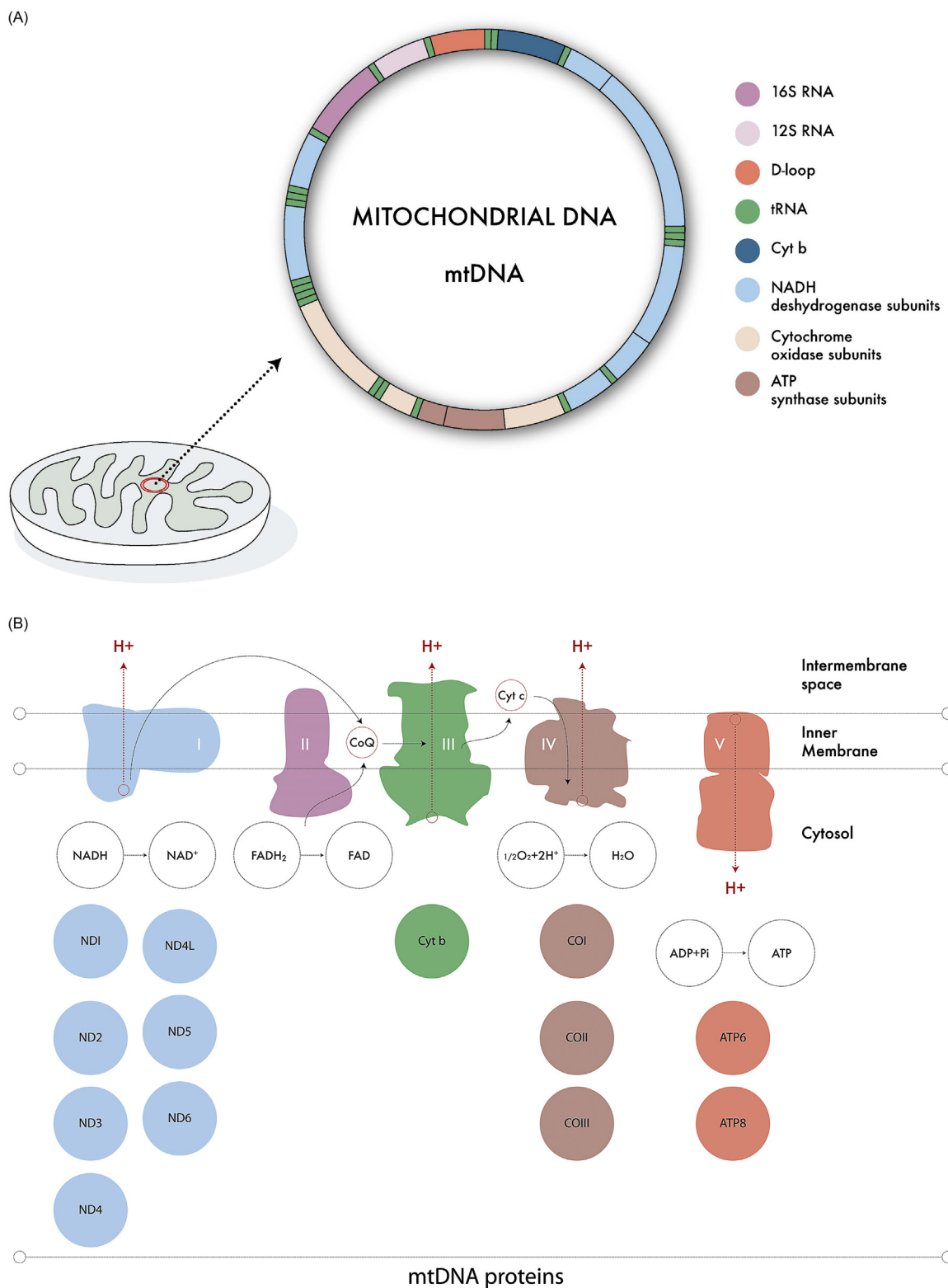


Fig. 3 (A) Scheme of mitochondrial DNA (mtDNA). mtDNA is composed by a D-loop (regulatory sequence) and 37 genes (22 tRNAs, 2 rRNAs, and 13 proteins). (B) The inner membrane contains the respiratory chain which is composed by five complexes. The 13 proteins encoded by mtDNA are distributed along the respiratory chain: seven in complex I, one in complex III, three in complex IV and two in complex V. Nuclear genome encodes Complex II entirely. An electrochemical gradient is generated across the inner membrane by H^+ pumps (Complexes I, III and IV). Finally, Complex V (ATP-synthase) pumps H^+ to the matrix and ATP is produced.

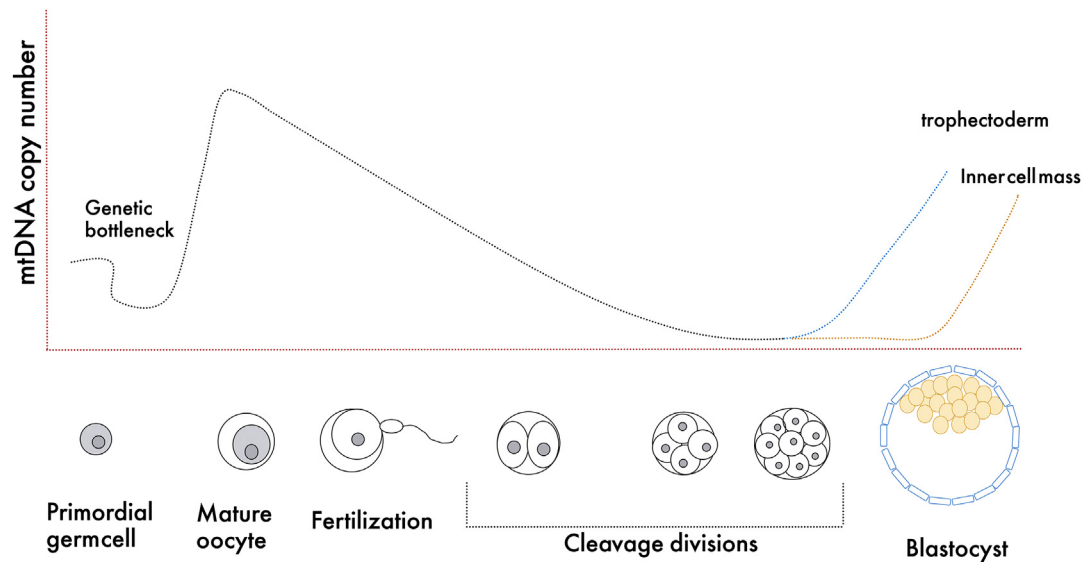


Fig. 4 Diagram of mtDNA copy number variation. During oogenesis, the copy number of mtDNA is reduced due to a genetic bottleneck. Subsequently, an amplification of the remaining fraction of mtDNA occurs. From the stage of mature oocyte, no further mtDNA replication befalls until blastocyst stage. Therefore, the number of mtDNA is being reduced during the cleavage division. mtDNA replication resumes at blastocyst stage, and it is thought that it starts before in the trophectoderm than in the inner cell mass.

Table 1 Principal proteins involved in mitochondrial DNA replication

Nucleoid protein	Function
POLGA	mtDNA polymerase, catalytic subunit
mTERF	Termination of transcription
mtSSB	Stabilizes mtDNA and stimulates twinkle activity
Twinkle	mtDNA unwinding
ATAD3	Backbone to the nucleoid
POLGB	mtDNA polymerase, accessory subunit. Forms an heterotrimer 2:1 with POLGA
mtRNA pol	Transcription of mtDNA, that generates RNA primer to mtDNA replication
TFAM	Transcription factor, starts replication
TFBM	Forms heterodimer with RNA polymerase, allowing specific transcription initiation

peripherally and acts as the backbone of the nucleoid (reviewed by St. John). **Table 1** summarizes the functions of mitochondrial nucleoid components.

TFAM starts replication; its binding to mtDNA induces structural modifications that result in exposure of the initiation region. Next, mtRNA pol synthesizes an RNA that becomes a primer used by POLGA to begin mtDNA replication. This process is supported by POLGB, which stabilizes POLGA and increases efficiency, and mtSSB and Twinkle, which mediate mtDNA unwinding. The mitochondrial genome replication differs from the organelle's replication and is controlled by signals modulated by cellular energetic demand, as results as previously explained copies of mtDNA are dependent of mitochondrial activity.

Studies in mouse models point out that, in standard situations, no further mtDNA replication takes place between the mtDNA amplification in the fertilized oocyte and the blastocyst stage: the total quantity of mtDNA remains constant from the one-cell oocyte through the early blastocyst stage (Piko and Taylor, 1987). Therefore, during cleavage divisions, the amount of mtDNA remains stable and mtDNA is apparently reduced with each cell division. As a result, the number of mtDNA molecules per cell is constantly diluted. At the blastocyst stage mtDNA replication is resumed (Thundathil et al., 2005); it is supposed to begin first in the trophectoderm (TE), then in the inner cell mass (ICM). In a pig model, mitochondrial-specific polymerase is increased in the outer edges of embryos at the morula stage, in the area of cells that are expected become TE. However, it is stable in the inner embryo, representing future ICM cells. Quantitative examination of ATP generation in mouse embryos also supports the idea that approximately 80% of ATP is generated in TE (Houghton, 2006).

Mitochondria a Shape Shifter Organelle

During oocyte growth and maturation, mitochondria are predominantly spherical to oval with a diameter $\leq 1 \mu\text{m}$ and are characterized by an increasingly dense matrix (Motta et al., 2000; Sathananthan and Trounson, 2000). Their appearance makes them seem inactive; in spite of this, some are generating ATP by oxidative phosphorylation (Van Blerkom, 2009). The organization remains unchanged until the eight-cell stage, when a reduction in matrix density happens gradually. During blastocyst differentiation, expansion, and hatching, mitochondria turn into progressively more elongated, show a less dense matrix, and have more abundant cristae, a clear sign of augmented metabolic activity (Motta et al., 2000; Sathananthan and Trounson, 2000).

Mitochondrial Function in the Early Embryo

Oxidative Stress and Early Embryo Development

As previously described, mitochondria generate ROS in the course of oxidative phosphorylation. These ROS produce oxidative stress if they are not controlled. Oxidative stress in an embryo is an inductor of apoptosis and embryo fragmentation during early development. Reasonably, then, the embryonic DNA should be protected from mitochondrial ROS, and this likely the evolutionary mechanism that explains why the early embryo presents little mitochondrial activity.

Mitochondria have a decisive role in apoptosis during early development; pro-apoptotic factors such as cytochrome *c* and apoptosis-inducing factor are out from the intermembrane space into the cytosol and activate caspase activation. In mouse zygotes go through in vitro culture, treatment with hydrogen peroxide (H_2O_2) induces cell death. Mitochondria are clearly involved in oxidative stress-induced cell death by liberation of mitochondrial pro-apoptotic factors. Oxidative stress is associated with the fragmentation rate of human embryos. H_2O_2 production is considerably higher in fragmented embryos compared to nonfragmented ones, and is connected with apoptosis and DNA fragmentation (Yang et al., 1998). However, the in vivo environment differs from the in vitro environment by the presence of defense mechanisms against ROS (Yang et al., 1998; Guerin et al., 2001). Antioxidants are present in the cumulus cells, (Bedaiwy et al., 2004) follicular fluid, ovarian tissue, fallopian tubes, and endometrium. They protect the oocyte and embryo by preventing ROS formation, capturing ROS, and supporting DNA repair (Agarwal et al., 2005). A study in which ROS levels were measured on the first day of in vitro culture after in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) showed that lower pregnancy rates are related to high ROS levels. Increased ROS levels are associated with low blastocyst, fertilization and cleavage rates, and high embryonic fragmentation only in ICSI cycles. This can be explained because in conventional IVF but not ICSI-only, cumulus cells are there and they may have antioxidant activity (Agarwal et al., 2005).

Mitochondrial Ca^{2+} Signaling and Fertilization

Fertilization of egg by sperm leads to metaphase II oocyte activation, resumption, and conclusion of meiosis, extrusion of the second polar body, and pronuclear formation. This activation produces zona pellucida and plasma membrane remodeling, cortical granule exocytosis and initiation of maternal mRNA translation. Intracellular Ca^{2+} signaling has a clear role during fertilization. Sperm-egg fusion conducts to an increase in cytosolic Ca^{2+} levels, going after a series of oscillations in Ca^{2+} levels (Armant, 2015). In the mouse, sperm-triggered Ca^{2+} waves created in the fertilized egg stimulate mitochondrial oxidative phosphorylation. In addition, mitochondrial oxidative phosphorylation is essential to maintain those Ca^{2+} oscillations. When a mouse egg is cultured in a medium devoid of the substrates required by mitochondria, it can be fertilized but the Ca^{2+} waves are repressed.

As fertilization involves ATP, it could be considered that ATP levels will decline during the process. Nevertheless, after fertilization in the mouse, no decline in ATP levels is observed. This denotes that energy supplies match the energy demand. Ca^{2+} signaling created by the entrance of the spermatozoa to the oocyte activates ATP production in mitochondria; thus, the energy required for this process is supplied (Armant, 2015), and small activity of oxidative phosphorylation are preserved when energy is not needed (Ramalho-Santos et al., 2009). In summary, the Ca^{2+} increase created by fertilization acts as a connection between cell energy necessities and energy production, that is, mitochondria. This activates the production of energy essential to undergo successive developmental stages, and when no energy is needed small levels of oxidative phosphorylation are preserved. In turn, mitochondria are fundamental to maintaining Ca^{2+} oscillations that are required to complete meiosis and begin embryo development.

Mitochondria Is a Stress Sensor

Mitochondria are capable to sense internal or environmental changes, such as diet and poisonous substances. Mitochondria indicate stress by membrane depolarization, changes in adenine nucleotide levels, ROS production, Ca^{2+} fluxes, permeability transition pore opening, and secretion of proteins/peptides. They also control bioenergetic, thermogenic, oxidative, and/or apoptotic responses so they can restore homeostasis (Fig. 5).

Severe exposure to stress intermediaries is linked with increase in mitochondrial biogenesis and changes of activity in the respiratory chain complexes, controlled production of ROS, thermogenesis, and apoptosis. In a chronic stress condition, the injure

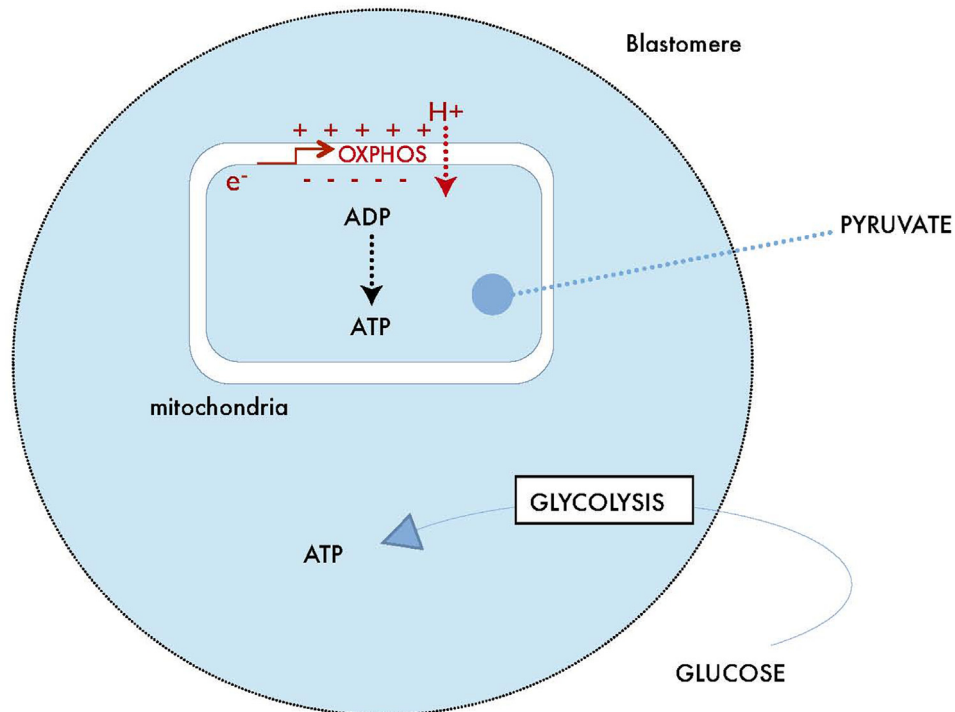


Fig. 5 Blastomere energy production. The energy required in the early embryo development can be produced by two possible mechanisms using different substrates: glycolysis (glucose) and oxidative phosphorylation (pyruvate). During the first stages of development the energy is produced in the mitochondria by oxidative phosphorylation (OXPHOS), at blastocyst stage there is a shift to ATP generation by glycolysis.

can surpass mitochondrial responses and produce abnormal mitochondrial biogenesis, respiratory chain dysfunction, decreased ATP production, increased ROS generation, lipid peroxidation, mitochondrial and nuclear DNA damage, and increased cell apoptosis (Manoli et al., 2007) (Fig. 6).

The “quiet embryo hypothesis” proposes that, by decreasing oxygen consumption (quiet metabolism) from the zygote to morula stage, the embryo confines the formation of ROS and there is less injury to the genome, transcriptome, and proteome. In contrast, the more “noisy” the embryo, the bigger the level of injury and activated demand for nutrients and energy (Fragouli et al., 2015; Leese, 2012). The embryo is likely reliant on energy accumulation throughout oocyte maturation, and only in a stress circumstances (reduced metabolic fuel) does the cellular machinery act in response to increase mtDNA copy number in an attempt to produce more energy. Thus, mitochondrial dysfunction is associated with mitochondrial hyperproliferation (Fragouli et al., 2015; Meldrum et al., 2016).

Mitochondrial biogenesis is mainly activated by increased energetic stress. It requires the control of several processes such as mtDNA synthesis, import and synthesis of nuclear-encoded proteins, and the assembly of proteins from nuclear and mitochondrial DNA. This regulation requires a set of nuclear transcription factors and coactivators, including PPAR coactivator 1a (PGC-1a) as the principal member. A drop in ATP activates energetic demands increasing the cellular adenosine monophosphate (AMP)/ATP ratio, which is sensed by AMP-activated protein kinase (AMPK). AMPK p activates PGC-1a, inducing mitochondria maturation increasing mtDNA copy number and cristae density (Meldrum et al., 2016).

An increase in mtDNA copy number in embryos would be indicative of metabolic stress. This stress could be connected to essential factors during oocyte maturation or could be in response to impaired respiratory capacities due to mtDNA mutations (Meldrum et al., 2016; Diez-Juan et al., 2015).

It has been demonstrated in human embryos that in response to impaired respiratory capacities due to mtDNA mutations, resulted in a reduced mtDNA copy number in germinal vesicles and MI metaphase oocytes gradual rise in mtDNA copy number from the germinal vesicle to the blastocyst stage (Monnot et al., 2013). Thus, initial oocyte mtDNA copy number could be independent of final values if there are factors affecting energetic or metabolic demands of the preimplantation embryo.

Mitochondria DNA Copy Number in Embryonic Implantation and Development

An important contributor to embryo viability is an adequate energy supply. This energy is provided by the accumulated mitochondria present in the oocyte, and only in cases of reduced metabolic fuel is there an increased mtDNA copy number (Meldrum et al., 2016; Diez-Juan et al., 2015).

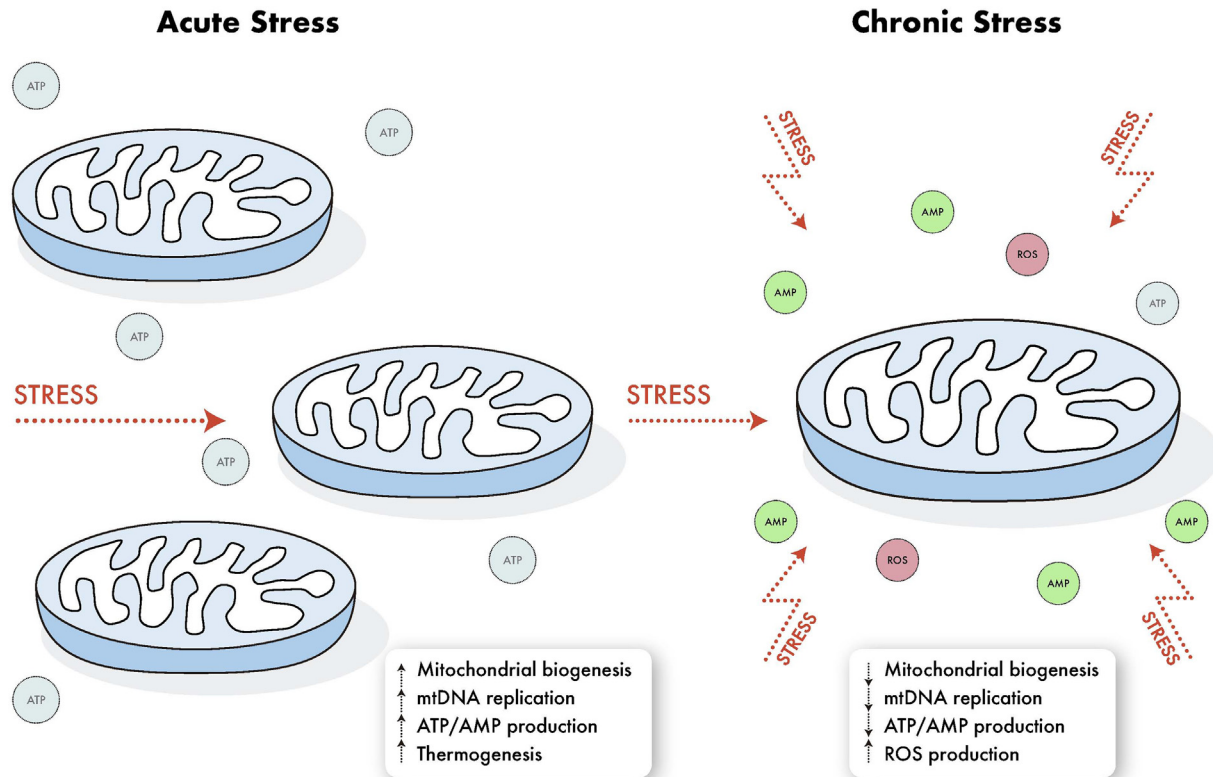


Fig. 6 The mitochondrial response to acute or chronic stress. Acute stress increases the energy cells demands. Thus, an increase of mitochondrial biogenesis and the enzymatic activity of selected subunits of the respiratory chain complexes is produced. Chronic stress to the mitochondrial homeostasis can exceed mitochondrial reserves provoking a decrease in mitochondrial biogenesis and ATP production, respiratory chain dysfunction and an increase in ROS generation and mitochondrial DNA damage.

Because mtDNA content remains stable during the first days of embryonic development, as shown in the mouse, the total amount of mtDNA must be split among cells during embryo division. On day six of development, each embryo cell should contain very few copies of mtDNA (Diez-Juan et al., 2015; Cummins, 1998) until mtDNA replication resumes after implantation. Disrupting the mouse gene for mitochondrial transcription factor A (low mitochondrial number in oocytes) does not affect fertilization or early embryonic development because the embryo continues with implantation and gastrulation. However, these embryos die by embryonic day (E) 10.5, indicating that the replication of mitochondria is not initiated until well after implantation (Van Blerkom, 2009; Meldrum et al., 2016; Larsson et al., 1998). The ability of embryos to implant suggests that oxidative phosphorylation is not required at this stage, or that the maternal contribution of functional mitochondria is sufficient.

New research has demonstrated that high mtDNA copy number in euploid embryos is indicative of lower embryo viability in terms of implantation potential (Diez-Juan et al., 2015), and aneuploid blastocysts contain significantly greater amounts of mtDNA. Further, increases in mtDNA content are associated with embryo loss (Fragouli et al., 2015). Manoli et al. (2007) assessed day 3 and day 5 embryos. The analysis of blastocysts revealed that blastocysts from older women had a higher mtDNA copy number, aneuploid blastocysts had a higher mtDNA copy number (independent of age), and euploid blastocysts that failed to implant had a significantly higher mtDNA copy number; and pregnancy did not occur when mtDNA copy number was above a relative threshold. Similar study at the same time, by Diez-Juan et al. (2015) assessed samples obtained from 205 cleavage stage embryos and 65 blastocysts. Similarly to the first study, a single blastomere from cleavage stage embryos and 5–10 blastomeres from blastocysts were tested for euploidy and mtDNA copy number, using aCGH and qPCR, respectively Diez-Juan et al. (2015) found that mtDNA copy number negatively correlated with implantation potential of euploid embryos, not only for blastocysts but also for cleavage stage embryos.

Final Remarks

Optimal maturation of the oocyte rest on its biological environment and governs embryo competence, since the embryonic genome is not activated until the cleavage stage and new mitochondria are not formed until blastulation. Environmental milieu like aging, oxidative stress, obesity, smoking, alcohol, and psychologic stress affects mitochondrial function and energy production affecting

ovarian reserve, chromosome segregation, and embryo competence. Mitochondrial metabolic stress can result in an abnormal compensatory increase in mitochondrial DNA, which can be assessed in biopsied blastomeres of trophectoderm as a predictive biomarker of implantation failure. Enhancing oocyte competence is a key intervention that promises to reduce the number of euploid embryos failing to produce viable deliveries, interventions that increases endogenous energy reserve and/or production should be a step forward to improve embryonic implantation and development in natural and clinical human reproduction.

References

- Agarwal, A., Gupta, S., & Sharma, R. K. (2005). Role of oxidative stress in female reproduction. *Reproductive Biology and Endocrinology*, 3, 28.
- Armant, D. R. (2015). Intracellular Ca^{2+} signaling and preimplantation development. *Advances in Experimental Medicine and Biology*, 843, 151–171.
- Bedaivvy, M. A., Falcone, T., Mohamed, M. S., Aleem, A. A., Sharma, R. K., Worley, S. E., et al. (2004). Differential growth of human embryos in vitro: Role of reactive oxygen species. *Fertility and Sterility*, 82(3), 593–600.
- Cui, H., Kong, Y., & Zhang, H. (2012). Oxidative stress, mitochondrial dysfunction, and aging. *J Signal Transduct.*, 2012, 646354.
- Cummins, J. (1998). Mitochondrial DNA in mammalian reproduction. *Reviews of Reproduction*, 3(3), 172–182.
- Desler, C., Lykke, A., & Rasmussen, L. J. (2010). The effect of mitochondrial dysfunction on cytosolic nucleotide metabolism. *Journal of Nucleic Acids*, 2010. <https://doi.org/10.4061/2010/701518>.
- Diez-Juan, A., Rubio, C., Marin, C., Martinez, S., Al-Asmar, N., Riboldi, M., et al. (2015). Mitochondrial DNA content as a viability score in human euploid embryos: Less is better. *Fertility and Sterility*, 104(3), 534–41.e1.
- Fragouli, E., Spath, K., Alfarawati, S., Kaper, F., Craig, A., Michel, C. E., et al. (2015). Altered levels of mitochondrial DNA are associated with female age, aneuploidy, and provide an independent measure of embryonic implantation potential. *PLoS Genetics*, 11(6), e1005241.
- Gray, M. W. (2012). Mitochondrial evolution. *Cold Spring Harbor Perspectives in Biology*, 4(9), a011403.
- Guerin, P., El Mouatassim, S., & Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update*, 7(2), 175–189.
- Houghton, F. D. (2006). Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst. *Differentiation; Research in Biological Diversity*, 74(1), 11–18.
- Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., et al. (1998). Mitochondrial transcription factor a is necessary for mtDNA maintenance and embryogenesis in mice. *Nature Genetics*, 18(3), 231–236.
- Leese, H. J. (2012). Metabolism of the preimplantation embryo: 40 years on. *Reproduction*, 143(4), 417–427.
- Manoli, I., Alesci, S., Blackman, M. R., YA, S., Rennert, O. M., & Chrousos, G. P. (2007). Mitochondria as key components of the stress response. *Trends in Endocrinology and Metabolism*, 18(5), 190–198.
- Marchington, D. R., Macaulay, V., Hartshorne, G. M., Barlow, D., & Poulton, J. (1998). Evidence from human oocytes for a genetic bottleneck in an mtDNA disease. *American Journal of Human Genetics*, 63(3), 769–775.
- Margulis, L. (1970). *Origin of eukaryotic cells*. New Haven, CT: Yale University Press.
- Martin, W., & Mentel, M. (2010). The origin of mitochondria. *Nature Education*, 3(9), 58.
- McBride, H. M., Neuspiel, M., & Wasiak, S. (2006). Mitochondria: More than just a powerhouse. *Current Biology*, 16(14), R551–60.
- Meldrum, D. R., Casper, R. F., Diez-Juan, A., Simon, C., Domar, A. D., & Frydman, R. (2016). Aging and the environment affect gamete and embryo potential: Can we intervene? *Fertility and Sterility*, 105(3), 548–559.
- Monnot, S., Samuels, D. C., Hesters, L., Frydman, N., Gigarel, N., Bulet, P., et al. (2013). Mutation dependence of the mitochondrial DNA copy number in the first stages of human embryogenesis. *Human Molecular Genetics*, 22(9), 1867–1872.
- Motta, P. M., Nottola, S. A., Makabe, S., & Heyn, R. (2000). Mitochondrial morphology in human fetal and adult female germ cells. *Human Reproduction (Oxford, England)*, 15(Suppl 2), 129–147.
- Ott, M., Gogvadze, V., Orrenius, S., & Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis*, 12(5), 913–922.
- Piko, L., & Taylor, K. D. (1987). Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Developmental Biology*, 123(2), 364–374.
- Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P., & Amaral, A. (2009). Mitochondrial functionality in reproduction: From gonads and gametes to embryos and embryonic stem cells. *Human Reproduction Update*, 15(5), 553–572.
- Sathananthan, A. H., & Trounson, A. O. (2000). Mitochondrial morphology during preimplantational human embryogenesis. *Human Reproduction (Oxford, England)*, 15(Suppl 2), 148–159.
- Sato, M., & Sato, K. (2012). Maternal inheritance of mitochondrial DNA: Degradation of paternal mitochondria by allogeneic organelle autophagy, autophagy. *Autophagy*, 8(3), 424–425.
- Stewart, J. B., & Larsson, N. G. (2014). Keeping mtDNA in shape between generations. *PLoS Genetics*, 10(10), e1004670.
- Thundathil, J., Filion, F., & Smith, L. C. (2005). Molecular control of mitochondrial function in preimplantation mouse embryos. *Molecular Reproduction and Development*, 71(4), 405–413.
- Van Blerkom, J. (2009). Mitochondria in early mammalian development. *Seminars in Cell & Developmental Biology*, 20(3), 354–364.
- Yang, H. W., Hwang, K. J., Kwon, H. C., Kim, H. S., Choi, K. W., & Oh, K. S. (1998). Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Human Reproduction (Oxford, England)*, 13(4), 998–1002.

Analysis of Human Endometrial Receptivity

Jose Miravet-Valenciano, IGENOMIX, Valencia, Spain

Inmaculada Moreno, IGENOMIX, Valencia, Spain; and Stanford University, Stanford, CA, United States

Carlos Simón, IGENOMIX, Valencia, Spain; University of Valencia, Valencia, Spain; and INCLIVA, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Biomarker A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.

Endometrial fluid Body fluid inside the uterine cavity that contains the molecules secreted from the endometrial glands to the uterine lumen. This fluid can be obtained by aspiration of the cavity by transcervically introduction of a thin and flexible catheter.

Endometrial receptivity Period within the menstrual cycle in which the endometrium acquires a functional status able to promote and support blastocyst implantation.

Endometrial receptivity analysis Is a personalized genetic test, developed and patented in 2009 by IGENOMIX, to diagnose the endometrial receptivity state in the window of implantation.

Genome-wide association studies Studies to identify DNA markers of variants across the genome related to a trait or disease. This kind of study has been lately proven useful for the prevention, diagnosis or treatment of several diseases.

NEXT-generation sequencing Modern technology that allow the sequencing of DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, revolutionizing the study of genomics and molecular biology.

“Omics” sciences General term for a broad discipline of science and engineering that analyze different molecules or processes at a large scale as well as the interactions of biological information objects in various “omes”: genome, transcriptome, proteome, lipidome, secretome, etc.

Personalized embryo transfer Novel therapeutic strategy used in patients with displaced window of implantation in which the embryo is transferred guided by the endometrial timing.

Pinopodes Smooth protrusions that arise from the apical surface of the luminal epithelium cells of the endometrium.

Recurrent implantation failure Clinical condition that prevents the final success of assisted reproduction. It is defined as three or more IVF cycles in which one or two morphologically high-grade embryos were transferred.

Window of implantation Period during the menstrual cycle in which the endometrium is ready to receive the embryo and start the pregnancy. This occurs around days 19–21 in each menstrual cycle of a fertile woman.

Abbreviations

ART Assisted reproductive technologies

BMI Body mass index

DNA Deoxyribonucleic acid

ERA Endometrial receptivity analysis

Eth Endometrial thickness

GWAS Genome-wide association studies

HRT Hormonal replacement therapy

IVF In vitro fertilization

LH Luteinizing hormone

miRNA micro-RNA

mRNA Messenger RNA

NGS Next-generation sequencing

pET Personalized embryo transfer

PG Prostaglandin

RIF Recurrent implantation failure

RNA Ribonucleic acid

SEM Scanning electron microscopy

uNK Uterine natural killer cells

WOI Window of implantation

Introduction

Endometrial receptivity is a physiological state in which the endometrium acquires an adhesive phenotype permitting embryo implantation, the initial step of pregnancy. Three main events are essential for the onset of a successful pregnancy: an euploid blastocyst ready to implant, a receptive endometrium, and correct embryo–maternal crosstalk (Cha et al., 2013). This allows the embryo to appose, attach, penetrate, and invade the endometrial barrier and gain access to essential nutrients required for proper growth and development. However, impaired synchronization between the embryo and endometrium will lead to implantation failure. The acquisition of endometrial receptivity occurs during a specific period of time known as the window of implantation (WOI) in the mid-secretory phase of the menstrual cycle (Hertig et al., 1956; Navot et al., 1991), but this WOI varies between patients. Thus, diagnosing endometrial receptivity plays a pivotal role in assisted reproductive technologies (ART) to avoid implantation failure and improve pregnancy outcomes.

Acquisition of Endometrial Receptivity

The endometrium is a hormonally regulated organ, and its receptive phenotype is achieved by activation of specific receptors for steroid ovarian hormones. During the follicular phase of the menstrual cycle, estrogen levels rise and stimulate endometrial cell proliferation. Then, after ovulation, the corpus luteum begins secreting progesterone promoting the physiological and molecular changes that prepare the endometrium to receive the developing embryo and maintain the pregnancy.

These changes affect different cell types in the endometrium, that is, the luminal and glandular epithelia and the stromal compartment. During the WOI, luminal epithelial cells undergo morphological remodeling leading to loss of polarity; apical microvilli known as pinopodes appear on the luminal surface; and adhesive molecules, such as integrins and mucins, and certain cytokines are upregulated. At the same time, the glandular epithelial cells increase in size and secrete the required factors to nurture the implanting embryo. Then, endometrial stromal cells begin differentiating in a process referred to as decidualization, which is characterized by acquisition of a rounded phenotype, increased storage of nutrients, accumulation of uterine natural killer cells, and vascular reorganization surrounding the site at which implantation is to occur (Evans et al., 2016). If the embryo does not implant, the decidualized endometrium is shed leading to menstruation and a new functional endometrial layer is regenerated in the next menstrual cycle (Fig. 1).

Technical Assessment of Endometrial Receptivity

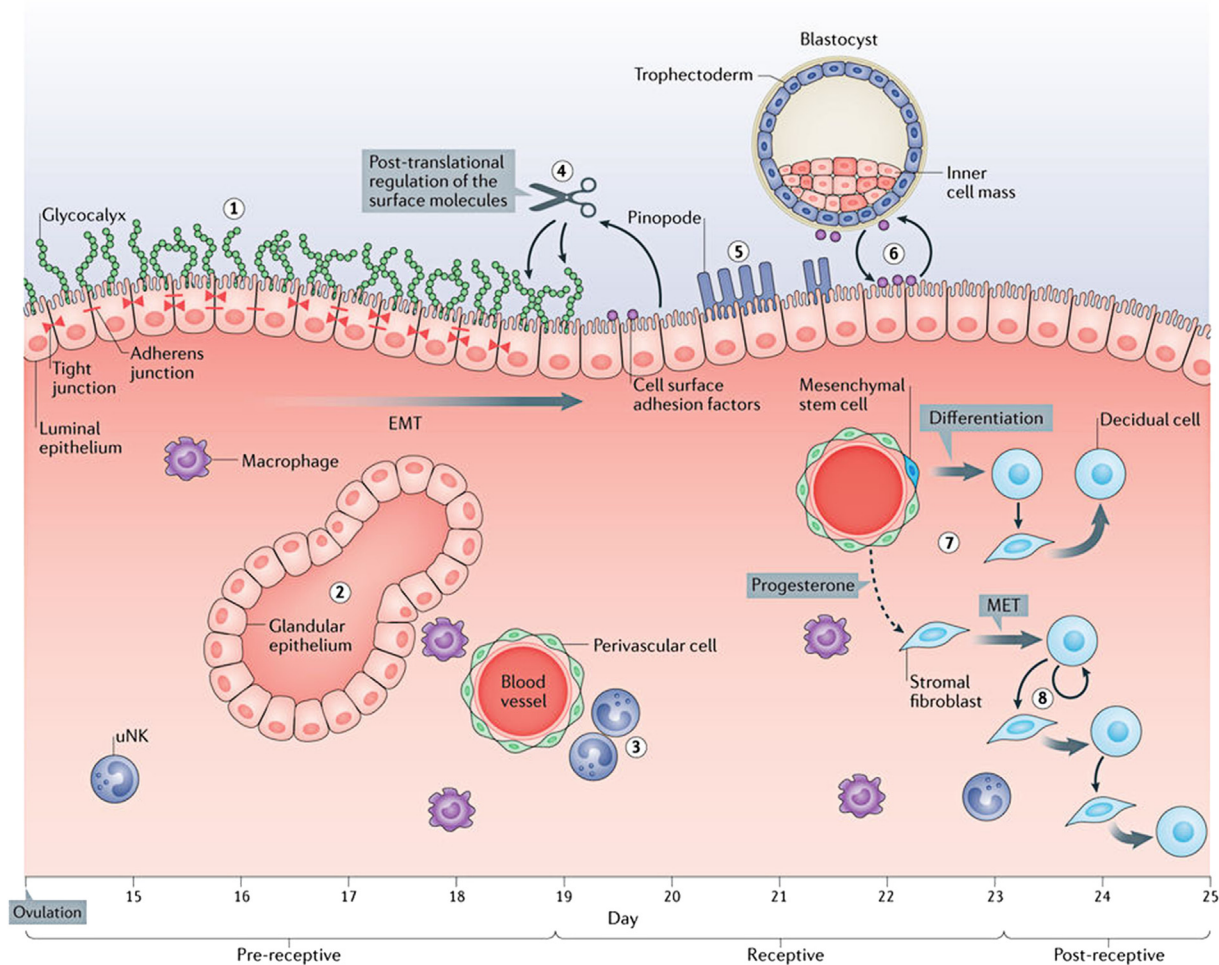
Because a receptive endometrium is mandatory for the initiation of pregnancy, several strategies have been proposed to identify the WOI (Aghajanova et al., 2008).

Morphological Evaluation of Endometrial Receptivity

Histology and immunohistochemistry

The morphological analysis of endometrial receptivity started with the histological dating of Noyes, based on features of the different endometrial compartments across the menstrual cycle (Noyes et al., 1950). These criteria have been used as a gold standard over six decades to assess the endometrial receptivity status. Nonetheless, subsequent retrospective, prospective, and randomized studies have questioned the accuracy and functional relevance of this method, leading to its discontinuation (see Miravet-Valenciano et al., 2015).

Immunohistochemical staining has been used since the 1990s to complement the analysis of endometrial dating by Noyes criteria. For this purpose, several markers of endometrial receptivity have been used to assess the abundance and localization of adhesion proteins, cell cycle progression of endometrial cells, or the regulation of immune cells in endometrial specimens. Because endometrial receptivity involves an adhesive phenotype, the abnormal expression of adhesion proteins has been studied as potential markers of uterine receptivity. In this regard, alpha-1, alpha-4, and beta-3 integrins are examined in women with unexplained infertility (Lessey et al., 1995) and constitute the basis of *E-tegrity* a clinical diagnosis test of endometrial receptivity (<http://www.etegritytest.com>). However, the association of beta-3 integrin with endometriosis (Lessey et al., 1994) is the main limitation of this test and may result in confounding results. In addition, the expression and subcellular localization of two cell-cycle proteins, cyclin E and cyclin-dependent kinase inhibitor p27, involved in endometrial cell mitosis have been used to determine the endometrial receptivity in donor ovum recipients (Kliman et al., 2006) and helped establish the rationale for the endometrial function test® (EFT®) (<http://klimanlabs.yale.edu/infertility/eft/>). Endometrial receptivity has also been analyzed by immunohistochemical detection of immune cells involved in maternal adaptation to the semiallogenic developing embryo, especially uterine natural killer (uNK) cells. Recently, it has been reported that high abundance of cytotoxic CD16(+) cells or the NKp46(+):CD56(+) ratio can be used as a marker of increased endometrial inflammation that correlates with implantation failure or pregnancy loss. However, the prognostic value of measuring total uNK cells or CD56(+) cells in endometrial specimens remains uncertain (Giuliani et al., 2014).



Nature Reviews | Endocrinology

Fig. 1 Acquisition of endometrial receptivity. (1) Before receptivity, the luminal epithelium is nonadhesive to the embryo. (2) The glandular epithelium becomes secretory. (3) Immune cells proliferate and migrate to the endometrium. (4) The luminal epithelial cells undergo modifications for the acquisition of the receptive phenotype: loss of polarization and increased surface adhesion molecules. (5) At initiation of receptivity, pinopodes appear on the surface of luminal cells. (6) The correct embryo–maternal communication is essential for implantation. (7) Decidualization of the endometrial stromal cells starts in perivascular regions. (8) Fibroblastic stromal cells acquire a rounded phenotype and secrete molecules that facilitates decidualization spread. Reprinted by permission from Macmillan Publishers Ltd.: *Nature Reviews Endocrinology* 12(11), 654–667, copyright 2016.

Scanning electron microscopy

Scanning electron microscopy (SEM) can distinguish the two types of cells, that is, the secretory and ciliated cells, present in the endometrial epithelium at the ultrastructural level. Ciliated cell morphology does not change significantly during the menstrual cycle. In contrast, the secretory cells undergo hormone-dependent morphological changes, including the protrusion of pinopodes on their apical surface during the WOI (Martel et al., 1991). For this reason, pinopodes have been proposed as morphological markers of endometrial receptivity. However, due to the technical complexity, price, and turnaround time of SEM, its use is limited to research. Moreover, the timing of pinopode formation, their function, and their value for diagnosing endometrial receptivity are controversial (Quinn and Casper, 2008).

Ultrasound

Ultrasound evaluation of the uterine cavity is a general practice in reproductive medicine. A triple-layered pattern and an endometrial thickness of 7–14 mm, measured by abdominal ultrasound, have been considered optimal for implantation, but a meta-analysis comparing ART outcomes in patients with endometrial thickness above or below 7 mm did not show differences between these two groups, indicating that endometrial thickness cannot itself be considered a receptive marker (Kasius et al., 2014).

Three-dimensional ultrasound has allowed the measurement of endometrial volume in correlation with endometrial receptivity reporting that an endometrial volume higher than 2 mL at the time of embryo transfer is a positive predictive variable of ART success.

Finally, the quantification of endometrial and subendometrial blood flow using color power Doppler sonography has been related to receptivity, showing that abundant endometrial flow correlates with better implantation and clinical pregnancy rates while undetectable blood flow is associated with implantation failure (Wang et al., 2010).

However, contradictory results have been found by other researchers concluding that there is no predictive value for these techniques.

Molecular Evaluation of Endometrial Receptivity

Biochemical markers

The endometrium proliferates in response to estrogen, and differentiation is dependent on progesterone secretion from ovarian follicles. Then, the preimplantation embryo enters the uterine cavity driven by L-selectin signaling. There, the antiadhesion molecule mucin prevents the blastocyst from attaching to the endometrium before reaching an implantation site, to which the embryo is attracted by increased levels of specific cytokines and chemokines. Finally, adhesion molecules help the blastocyst to implant.

Different molecules have been proposed as potential biomarkers of endometrial receptivity due to roles in the above processes as well as their differential abundance during the WOI compared to other phases of the cycle. These differential molecules can be the cause or the consequence of the morphological features of endometrial receptivity and involve: adhesion molecules, cytokines, chemokines, and growth factors (Table 1). However, single biomarkers do not often offer enough specificity to discriminate the correct endometrial receptivity period. Because of this, none of the aforementioned molecular markers have been successfully used as a sole marker for clinical management (Aghajanova et al., 2008).

High throughput techniques

Technological advances promise to improve the prediction of the WOI. Thus, molecular evaluation of endometrial receptivity has recently focused on complete profiles of molecules rather than specific biomarkers. This is possible thanks to the so-called “omics” sciences that have demonstrated a more accurate prediction of endometrial receptivity than those provided by morphological or visual analyses. In fact, a clinical transcriptomic array has displaced histological dating as the gold standard of endometrial receptivity in current clinical practice.

Proteomics

Proteomic approaches reflect cellular functions through the identification of differential levels of detectable proteins. Following the development of mass spectrometry, studies profiling protein expression in endometrial samples have improved our understanding

Table 1 Biochemical markers of endometrial receptivity

Type of molecule	Biomarker	Role/abundance in endometrial receptivity
Adhesion	L-selectin ligands	Expressed in luminal and glandular epithelium to receive L-selectin expressed by the trophectoderm cells of the blastocyst prior to implantation
	$\alpha 1\beta 1$ - and $\alpha 1\beta 4$ -integrins	Differentially overexpressed in receptive stage
	$\alpha V\beta 3$ -integrin	Specifically expressed during the window of implantation. It is associated with increased implantation and pregnancy outcomes
	Mucin	Antiadhesive protein expressed in the luminal epithelium to prevent attachment of embryo outside the contact sites
Cytokines	Cadherins	Involve in calcium-dependent cell-to-cell interaction
	IL-6	Overexpressed during the mid-secretory phase of the menstrual cycle in the luminal epithelium and recognized by IL-6 receptors expressed in the embryo
	LIF	Induces different transcriptional factors and signaling pathways leading to cell polarity changes, angiogenesis and stromal cell decidualization
Chemokines	IL-1	Expressed in epithelial and stromal cells during the WOI. Enhances the expression of $\alpha V\beta 3$ -integrin
	IL-8	Acts as a chemoattractant in luminal epithelium for endometrial receptivity
	MCP-1	Involved in immunomodulation and inflammation in the embryo–endometrial interphase
	RANTES	Mainly located in stromal cells during the WOI. RANTES receptors are expressed in the preimplantation embryo
Growth factors	HB-EGF	Induces expression of $\alpha V\beta 3$ -integrin in luminal epithelium. Influences glandular epithelium secretions
Others	Glycodelin A	Induced by progesterone. Modulates immune response to prevent rejection from the mother to the embryo to allow successful implantation
	Osteopontin	Ligand for $\alpha V\beta 3$ -integrin
	Calcitonin	Increases intracellular calcium to modulate cytoskeleton reorganization, epithelial cells remodeling and polarity
	HOXA 10	Transcription factor that regulates $\alpha V\beta 3$ -integrin expression

Reviewed in Achache, H. and Revel, A. (2006). Endometrial receptivity markers, the journey to successful embryo implantation. *Human Reproduction Update* 12(6), 731–746 and Miravet-Valenciano, J. A., Rincon-Bertolin, A., Vilella, F., and Simon, C. (2015). Understanding and improving endometrial receptivity. *Current Opinion in Obstetrics and Gynecology*, 27(3), 187–192.

of the biological processes underlying implantation. The first proteomic study comparing the proliferative and secretory endometrium identified five differentially expressed proteins, but the utility of those proteins in endometrial receptivity has not yet been evaluated (DeSouza et al., 2005). A later study comparing the proteomic profile of prereceptive (LH+2) versus receptive (LH+7) endometrial samples using differential in-gel electrophoresis and mass spectrometry identified 35 differentially expressed proteins (12 upregulated and 23 downregulated) in the receptive phase. Interestingly, two proteins associated with the cytoskeleton, Annexin A2 and Stathmin 1, showed consistent differential expression (Dominguez et al., 2009). Annexin A2 promotes fibrinolytic activity on the surface of vascular endothelial cells by assembling plasminogen and tissue plasminogen activator and by accelerating the generation of plasmin; Stathmin acts as a regulator of microtubule dynamics during cell-cycle progression. This is consistent with the required remodeling of the epithelial cells to achieve the receptive phenotype. The functional analysis of these proteins on a refractory endometrium (induced by the insertion of an intrauterine device) indicated that the labeling pattern for these proteins was similar to that present in the prereceptive endometrium, suggesting that Annexin A2 and Stathmin 1 are functionally involved in endometrial receptivity.

Lipidomics

The large-scale study of lipids secreted from the endometrial cells into the uterine cavity has also been used to find molecular markers of endometrial receptivity. Mass spectrometry has revealed that endocannabinoids, lysophosphatidic acid, and prostaglandins are the most important lipids in the uterus at the time of embryo implantation, meaning that the endometrium presents a specific pattern of lipids during the WOI. Specifically, the secretion of prostaglandins PGE2 and PGF2 α into the endometrial fluid is significantly higher during the mid-secretory phase of the menstrual cycle in healthy asymptomatic women (Vilella et al., 2013), suggesting that these two lipids could predict receptivity. Twenty-four hours before embryo transfer, the endometrial fluid of patients who became pregnant in their current IVF cycle contained higher levels of PGE2 and PGF2 α than endometrial fluid from patients who did not (Vilella et al., 2013).

Transcriptomics

The transcriptome, created by analyzing mRNA content, reflects the genes that are actively expressed at any given time in a specific cell population. It provides expression profiles that can accurately predict endometrial functionality as well as diagnostic or prognostic criteria. Genes involved in metabolism, glandular secretion, cell differentiation, cell communication, innate immune response, response to stress, response to wounding, cell adhesion, and regulation of proteolysis are more highly expressed during the WOI than during other phases of the menstrual cycle (reviewed in Gómez et al., 2015). These functional genomic studies have demonstrated that gene expression profiles differ depending on the phase of the cycle and hormonal treatment (Fig. 2).

The identification of sets of genes that reflect endometrial receptivity in natural cycles or in hormonal replacement cycles (HRT) lead to the development of a molecular diagnostic test, that is, Endometrial Receptivity Analysis (ERA) (Díaz-Gimeno et al., 2011). The ERA, first based on microarray data, is now performed using next generation sequencing (NGS) of 236 genes coupled to a computational predictor able to identify the optimal WOI of each patient (<https://www.igenomix.com/tests/endometrial-receptivity-test-era/>). The success of this test is based on its accuracy, which is superior to endometrial histology, and its reproducibility, as demonstrated in samples taken from the same patient up to 40 months apart (Díaz-Gimeno et al., 2013). This test is currently employed in clinical practice to identify the time frame in which each patient is receptive, thus allowing for personalized embryo transfer (pET) (Ruiz-Alonso et al., 2013).

Other studies have attempted to describe the transcriptomic profile of endometrial receptivity (Gómez et al., 2015). A recent meta-analysis found that 57 genes, including genes present in the ERA (i.e., SPP1, ANXA4, CLDN4, DPP4, GPX3, MAOA, and PAEP), were identified as potential receptivity biomarkers in multiple studies and are the most representative panel for predicting the WOI (Altmäe et al., 2017).

Cell-free RNA

Aspiration of the endometrial cavity was recently used for the analysis of potential markers of endometrial receptivity. The endometrial fluid (EF) is a complex biological fluid secreted by the endometrial glands. It provides nutrients for embryonic survival during the peri-implantation period and constitutes the microenvironment where the endometrial–blastocyst dialogue occurs before implantation. The molecular composition of EF has been investigated to identify markers of receptivity. A study analyzing the miRNA content of the EF throughout the menstrual cycle identified a set of 27 miRNAs differentially expressed during the WOI, with miR-30d being the most upregulated. This miRNA was shown to be internalized by trophoblast cells of the blastocyst, modulating blastocyst adhesion (Vilella et al., 2015). These results confirm the role of miRNAs in endometrial receptivity and their potential as biomarkers.

Clinical Assessment of Endometrial Receptivity

Recurrent Implantation Failure

A prospective clinical trial using ERA at the expected moment of receptivity found that 26% of patients with recurrent implantation failure (RIF) but only 12% of control patients (with one or no previous failed cycles) presented a displaced (advanced or delayed)

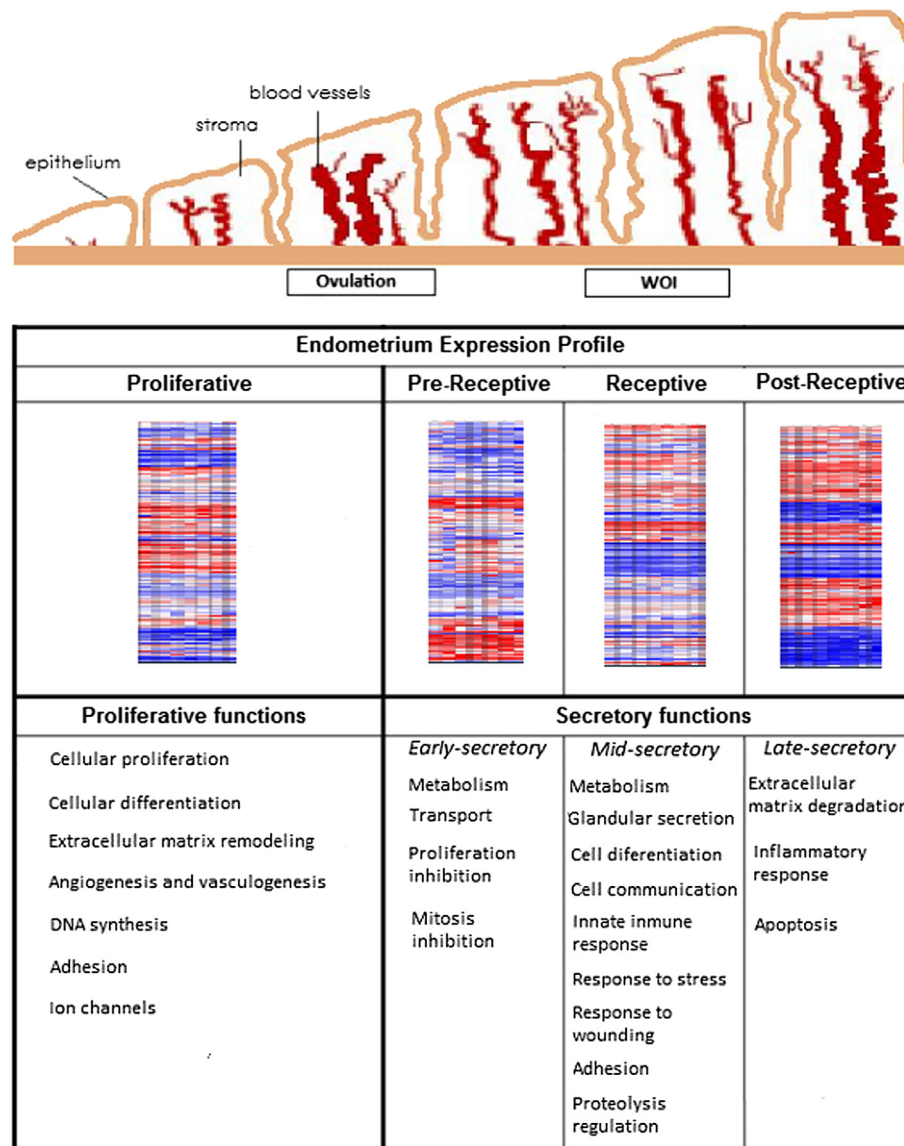


Fig. 2 Transcriptomic analysis of endometrial receptivity. Gene expression profiles of endometrial cycle can be used to analyze endometrial receptivity. Reprinted from Garrido-Gómez, T., Ruiz-Alonso, M., Blesa, D., Díaz-Gimeno, P., Vilella, F., Simon, C. (2013). Profiling the gene signature of endometrial receptivity: Clinical results. *Fertility and Sterility* **99** (4), 1078–1085, Copyright 2013, with permission from Elsevier.

WOI. Interestingly, when pET, guided by the ERA prediction of WOI, was performed on RIF patients, their clinical outcomes (implantation and pregnancy rates) increased to the levels observed in the control group, meaning that in those patients, the endometrial factor was responsible for the failed cycles. Thus, the diagnostic value of ERA was proven in clinical practice to improve pregnancy outcomes of women with RIF (Ruiz-Alonso et al., 2013).

Factors Influencing Endometrial Receptivity

Endometriosis

Endometriosis, a benign condition related to infertility, is characterized by the presence of endometrial cells outside the uterine cavity. Endometriosis does not affect embryo implantation in ovum recipients, meaning that the endometrial factor is not responsible for infertility in these patients. However, disruption of endometrial genes may be responsible for endometriosis (García-Velasco et al., 2015). To uncover the role of endometrial receptivity in endometriosis-associated infertility, ERA results of endometrial samples from infertile patients with different stages of endometriosis (stages I–IV) were compared to results of samples from patients without endometriosis. This pilot study showed that the transcriptomic profile of endometrial receptivity was similar in patients with or without endometriosis, regardless of the stage of the disease, indicating that endometriosis does not affect endometrial receptivity (García-Velasco et al., 2015).

Obesity

Obesity levels are increasing worldwide, and maternal obesity is linked to infertility. Obesity is associated with anovulatory problems, a lower implantation potential, increased miscarriage rates, and longer time-to-pregnancy in both spontaneous pregnancies and ART cycles. The poorer reproductive outcomes in obese patients may involve the egg, the embryo, the endometrium, or a combination. To clarify the role of endometrial receptivity in obesity-associated infertility, the ERA test was performed in infertile patients stratified by body mass index (BMI): normal-weight (BMI 18.5–24.9 kg m⁻²), overweight (BMI 25.0–29.9 kg m⁻²), and obesity class I (BMI 30.0–34.9 kg m⁻²), II (BMI 35.0–39.9 kg m⁻²), and III (BMI over 40.0 kg m⁻²). This study demonstrated that the transcriptomic profile of the endometrium during the WOI is altered in obese patients. The displacement of endometrial receptivity was more pronounced as BMI increased, in the proportion of non-receptive patients and in the levels of specific dysregulated genes (either up or downregulated). In conclusion, an altered endometrial receptivity may be a cause of infertility in obese patients (Comstock et al., 2017).

Ethnicity

Race or ethnicity may influence endometrial receptivity. The ERA test has been applied to patients of different ethnicities to elucidate whether differential genomic variants affecting ethnicity could influence the WOI. The transcriptomic profiles of endometrial biopsies of infertile women from the following ethnicities were compared: African ($n = 19$), Arabian ($n = 20$), Caucasian ($n = 1003$), East Asian ($n = 209$), Hispanian ($n = 45$), and South Asian ($n = 1244$). Most ethnic groups exhibited similar proportions of receptive and nonreceptive women; however, African women were distinct. In this group, there was a significant decrease in receptive patients with an apparent shift to a prereceptive phenotype. However in this study, the African women had significantly higher BMIs than the women from other groups, and this could bias the results obtained from the ERA test (see section **Obesity**). For this reason, further studies are required to clarify if ethnicity influences the acquisition and/or prediction of endometrial receptivity (Valbuena and Simon, unpublished data).

Endometrial thickness

A number of studies have investigated the association of endometrial thickness and receptivity, concluding that a thickness of 6 mm before progesterone administration in an HRT cycle indicates a receptive endometrium (see section **Ultrasound**). To corroborate these results using a reliable molecular method, ERA analysis was performed in retrospective endometrial samples classified by endometrial thickness at ultrasound into three groups: atrophic endometrium (Eth < 6 mm), normal endometrium (Eth 6–12 mm), and hypertrophic endometrium (Eth > 12 mm). A normal ratio of receptive and nonreceptive profiles were observed in samples from endometria with either normal or increased thicknesses. However, in samples from atrophic endometria, the percentage of nonreceptive profiles was significantly increased and accounted for more than 50% of the analyzed samples. Specifically, patients with atrophic endometrium presented a transcriptomic profile consistent with the prereceptive status, suggesting that the WOI in those patients is delayed due to insufficient growth of the endometrium (Valbuena and Simon, unpublished data).

Conclusions and Future Directions

Histological, biological, and physiological characteristics of the endometrium during the window of implantation are the result of transcriptomic and posttranscriptional changes. The ERA test is currently a clinically validated tool for diagnosing a displaced WOI and predicting the optimal time for pET, thus increasing the chances of successful implantation. However, the main limitation of the ERA test is that it requires an endometrial biopsy for analysis of the transcriptome. Future endometrial receptivity tests will ideally use minimally invasive techniques aimed at providing consistent results while avoiding pain and discomfort to the patients. The presence of RNA or single cells in the endometrial fluid will serve as the basis for developing noninvasive diagnostics and personalized ET without the need for a biopsy.

Technological advances in genetics may also lead to the development of less invasive diagnostic tools. Studies have already detected the association of single-nucleotide polymorphisms or genetic variants with several traits and diseases. Genome-wide association studies may help identify genetic variants in nonreceptive patients that are causative of a displacement of the WOI. This information could eventually be used to diagnose endometrial receptivity using blood samples. Furthermore, if such associations are identified, the relevant genes could be targets for novel lines of research the clinical management of infertile patients with endometrial factor.

References

- Aghajanova, L., Hamilton, A. E., & Giudice, L. C. (2008). Uterine receptivity to human embryonic implantation: Histology, biomarkers, and transcriptomics. *Seminars in Cell & Developmental Biology*, 19(2), 204–211.
- Altmäe, S., Koel, M., Võsa, U., Adler, P., Suhorutšenko, M., Laisk-Podar, T., & Aghajanova, L. (2017). Meta-signature of human endometrial receptivity: A meta-analysis and validation study of transcriptomic biomarkers. *Scientific Reports*, 7(1), 10077.
- Cha, J., Vilella, F., Dey, S. K., & Simón, C. (2013). Molecular interplay in successful implantation. In S. Sanders (Ed.), *Ten critical topics in reproductive medicine* (pp. 44–48). Washington, DC: Science/AAA.

- Comstock, I. A., Diaz-Gimeno, P., Cabanillas, S., Bellver, J., Sebastian-Leon, P., Shah, M., & Simon, C. (2017). Does an increased body mass index affect endometrial gene expression patterns in infertile patients? A functional genomics analysis. *Fertility and Sterility*, 107(3), 740–748.
- DeSouza, L., Diehl, G., Yang, E. C., Guo, J., Rodrigues, M. J., Romaschin, A. D., & Siu, K. W. (2005). Proteomic analysis of the proliferative and secretory phases of the human endometrium: Protein identification and differential protein expression. *Proteomics*, 5(1), 270–281.
- Diaz-Gimeno, P., Horcajadas, J. A., Martínez-Conejero, J. A., Esteban, F. J., Alama, P., Pellicer, A., & Simón, C. (2011). A genomic diagnostic tool for human endometrial receptivity based on the transcriptomic signature. *Fertility and Sterility*, 95(1), 50–60.
- Diaz-Gimeno, P., Ruiz-Alonso, M., Blesa, D., Bosch, N., Martínez-Conejero, J. A., Alama, P., & Simón, C. (2013). The accuracy and reproducibility of the endometrial receptivity array is superior to histology as a diagnostic method for endometrial receptivity. *Fertility and Sterility*, 99(2), 508–517.
- Dominguez, F., Garrido-Gomez, T., Lopez, J. A., Camafeita, E., Quinonero, A., Pellicer, A., & Simon, C. (2009). Proteomic analysis of the human receptive versus non-receptive endometrium using differential in-gel electrophoresis and MALDI-MS unveils stathmin 1 and annexin A2 as differentially regulated. *Human Reproduction*, 24(10), 2607–2617.
- Evans, J., Salamonsen, L. A., Winship, A., Menkhorst, E., Nie, G., Gargett, C. E., & Dimitriadis, E. (2016). Fertile ground: Human endometrial programming and lessons in health and disease. *Nature Reviews Endocrinology*, 12(11), 654–667.
- García-Velasco, J. A., Fassbender, A., Ruiz-Alonso, M., Blesa, D., Thomas, D. H., & Simon, C. (2015). Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. *Reproductive Biomedicine Online*, 31(5), 647–654.
- Giuliani, E., Parkin, K. L., Lessey, B. A., Young, S. L., & Fazleabas, A. T. (2014). Characterization of uterine NK cells in women with infertility or recurrent pregnancy loss and associated endometriosis. *American Journal of Reproductive Immunology*, 72(3), 262–269.
- Gómez, E., Ruiz-Alonso, M., Miravet, J., & Simón, C. (2015). Human endometrial transcriptomics: Implications for embryonic implantation. *Cold Spring Harbor Perspectives in Medicine*, 5(7), a022996.
- Hertig, A. T., Rock, J., & Adams, E. C. (1956). A description of 34 human ova within the first 17 days of development. *Developmental Dynamics*, 98(3), 435–493.
- Kasius, A., Smit, J. G., Torrance, H. L., Eijkemans, M. J., Mol, B. W., Opmeer, B. C., & Broekmans, F. J. (2014). Endometrial thickness and pregnancy rates after IVF: A systematic review and meta-analysis. *Human Reproduction Update*, 20(4), 530–541.
- Kliman, H. J., Honig, S., Walls, D., Luna, M., McSweet, J. C., & Copperman, A. B. (2006). Optimization of endometrial preparation results in a normal endometrial function test[®] (EFT[®]) and good reproductive outcome in donor ovum recipients. *Journal of Assisted Reproduction and Genetics*, 23(7–8), 299–303.
- Lessey, B. A., Castelbaum, A. J., Sawin, S. W., Buck, C. A., Schinnar, R., Bilker, W., & Strom, B. L. (1994). Aberrant integrin expression in the endometrium of women with endometriosis. *The Journal of Clinical Endocrinology & Metabolism*, 79(2), 643–649.
- Lessey, B. A., Castelbaum, A. J., Sawin, S. W., & Sun, J. (1995). Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertility and Sterility*, 63(3), 535–542.
- Martel, D., Monier, M. N., Roche, D., & Psychoyos, A. (1991). Hormonal dependence of pinopode formation at the uterine luminal surface. *Human Reproduction*, 6(4), 597–603.
- Miravet-Valenciano, J. A., Rincon-Bertolin, A., Vilella, F., & Simon, C. (2015). Understanding and improving endometrial receptivity. *Current Opinion in Obstetrics and Gynecology*, 27(3), 187–192.
- Navot, D., Scott, R. T., Droesch, K., Veeck, L. L., Liu, H. C., & Rosenwaks, Z. (1991). The window of embryo transfer and the efficiency of human conception in vitro. *Fertility and Sterility*, 55(1), 114–118.
- Noyes, R. W., Hertig, A. T., & Rock, J. (1950). Dating the endometrial biopsy. *Fertility and Sterility*, 1(1), 3–25.
- Quinn, C. E., & Casper, R. F. (2008). Pinopodes: A questionable role in endometrial receptivity. *Human Reproduction Update*, 15(2), 229–236.
- Ruiz-Alonso, M., Blesa, D., Diaz-Gimeno, P., Gómez, E., Fernández-Sánchez, M., Carranza, F., & Simón, C. (2013). The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. *Fertility and Sterility*, 100(3), 818–824.
- Vilella, F., Ramirez, L., Berlanga, O., Martinez, S., Alama, P., Meseguer, M., & Simón, C. (2013). PGE2 and PGF2 α concentrations in human endometrial fluid as biomarkers for embryonic implantation. *The Journal of Clinical Endocrinology & Metabolism*, 98(10), 4123–4132.
- Vilella, F., Moreno-Moya, J. M., Balaguer, N., Grasso, A., Herrero, M., Martínez, S., & Simón, C. (2015). Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. *Development*, 142(18), 3210–3221.
- Wang, L., Qiao, J., Li, R., Zhen, X., & Liu, Z. (2010). Role of endometrial blood flow assessment with color Doppler energy in predicting pregnancy outcome of IVF-ET cycles. *Reproductive Biology and Endocrinology*, 8(1), 122.

Further Reading

- Garrido-Gómez, T., Ruiz-Alonso, M., Blesa, D., Diaz-Gimeno, P., Vilella, F., & Simón, C. (2013). Profiling the gene signature of endometrial receptivity: Clinical results. *Fertility and Sterility*, 99(4), 1078–1085.

Relevant Websites

- E-tegrity—<http://www.etegrityttest.com/>
 EFT[®]—<http://klimanlabs.yale.edu/infertility/efl/>
 ERA—<https://www.igenomix.com/tests/endometrial-receptivity-test-era/>

Developmental Epigenetic Analysis of Sperm

Deepika Kubsad, Millissia Ben Maamar, and Michael K Skinner, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

ART Assisted reproductive technologies

DAZL Deleted in azoospermia like

DNA Deoxyribonucleic acid

DNMT DNA methyltransferases

GWAS Genome wide association studies

IVF In vitro fertilization

MTHFR Methyleneetrahydrofolate reductase

PGC Primordial germ cells

RNA Ribonucleic acid

TET Ten-Eleven translocation enzymes

Male Infertility

The definition of male infertility is the inability of a male to impregnate a fertile female. Infertility is a worldwide problem and according to Sharlip et al. affecting around 15% couples (Sharlip et al., 2002). Furthermore, nearly half of human infertility cases can be attributed to abnormal spermatogenesis (Kitamura et al., 2015). Genetic causes for male infertility are related to a limited number of cases in which altered seminal parameters (motility, morphology, and concentration) are directly associated with a genetic basis such as chromosome aberration or microdeletions at the Y chromosome (Jodar et al., 2017). The intricacies to define a genetic origin of this disease have led some researchers to suspect that both genetic and environmental factors contribute. The complex pathology, including the difficulty of isolating molecular abnormalities associated with this diagnosis makes it challenging to determine its underlying cause. However, recent research indicates the presence of epigenetic factors which may provide further insights into the pathogenesis of male infertility (Jenkins et al., 2017). The increased usage of human assisted reproductive technologies (ART) has created an enormous interest in identifying, diagnosing and potentially treating male infertility. In recent years the advancements in the field of ART have provided indirect evidence for the role of epigenetic mechanisms in male infertility (Kitamura et al., 2015).

Epigenetics

Epigenetics refers to changes in the phenotype caused by mechanisms other than changes in DNA sequences. Nearly all the cells in multicellular organisms share an identical genotype however, the diversity in cellular function is based on gene expression profiles that differ among individual cell types. Mechanisms such as DNA methylation, non-coding RNAs, chromatin remodeling and histone modification chemically and structurally alter the expression of genomic region (Tvrda et al., 2015). These various epigenetic signatures are specific to each individual cell type and make up the cell epigenome. The epigenome is thought to reflect the organism developmental history and past environmental influences. The dynamic and tissue specific nature of the epigenome allow for flexibility in gene expression which can lead to changes in phenotypes in the organism. Throughout the process of mitosis, majority of the epigenetic landscape is replicated from one cell to another. During reproduction, some but not all of the epigenome is reset in the embryo (Murphy et al., 2014). The remaining epigenetic signatures after the erasure event may allow the transfer of phenotypic traits on the subsequent generations (Tollefsbol, 2014). Thus, the epigenetic modifications of the germline (sperm and egg) can have long-term consequences as the altered epigenome can be transmitted to the embryo and this process is referred as transgenerational epigenetics (Guerrero-bosagna and Skinner, 2014).

Epigenetic transgenerational inheritance is a new and rising subfield which studies the impact of non-genetic inheritance on epigenomes, specifically, transcriptomes of all subsequently derived somatic cells in the embryo. The transcriptome consists of the small fraction of the genome corresponding to genes and protein coding regions of the genome that have an influential role in the development of the embryo (Guerrero-bosagna and Skinner, 2014). This field of study provides a mechanism through which exposures in previous generations influence the phenotypes of subsequent generations through epigenetic regulation. Germ cells are vehicles of inheritance that transmit genetic information from one generation to the next. Male and female mammalian germ cells inherently differ in their development, morphology and means of fertilization. Thus, it is not surprising that divergent epigenetic mechanisms accompany these cell types (Murphy et al., 2014).

DNA accessibility differs throughout an organism lifespan resulting in distinct sets of active genes varying at each developmental stage. Sperm cells are highly specialized. Their production occurs during spermatogenesis, a process involving extensive cellular, epigenetic and chromatin changes (Oliva and Castillo, 2011). Life cycle of the sperm begins at the embryonic stage as a specialized pool of somatic cells called primordial germ cells (PGC). PGC undergo an erasure of DNA methylation and histone replacement event, essential for proper sperm and offspring development (Murphy et al., 2014). The replacement of the majority of histones with protamines helps promote nuclear compaction, increases sperm motility and insulates the genome against harmful molecules in the female reproductive tract (Jenkins et al., 2017). After gonadal maturity, spermatogenesis ensues. Spermatogenesis takes place in the seminiferous tubules of the testes in three distinct phases: (i) an undifferentiated diploid male germ cell differentiates into two different types of spermatogonia. Spermatogonia type A replicates through mitosis and maintains the spermatogonia population in the testes. Spermatogonia type B can enter into meiosis and further differentiate into spermatozoa. (ii) Spermatogonia type B increase in size turning into primary spermatocytes. Further mitotic divisions convert the diploid spermatocytes into haploid spermatids. (iii) The round spermatids continue to undergo nuclear and morphological changes to become mature spermatozoa. Spermatozoa continues to mature as it transits along the epididymis in order to acquire both motility and fertilization potential to reach full maturation (Fig. 1) (Jodar et al., 2017). After the completion of sperm maturation, they are then able to swim and fertilize an oocyte. During the complex process of fertilization, sperm travels through the female reproductive tract, penetrates the cumulus oophorus, binds and enters the zona pellucida and then the oocyte. At this stage, there is a second epigenetic reprogramming event which involves DNA methylation erasure and histone reassembly following paternal DNA. The molecular reasons underlying the reassembly remain unknown. After this stage, the zygote initiates development and continues to totipotent conversion (Murphy et al., 2014).

DNA Methylation

One of the most studied epigenetic marks is DNA methylation. This methylation occurs on the cytosine residues in the cytosine phosphate guanine (CpG) dinucleotides. Methylation near promoter regions prevent the expression of that gene (Güneş and Kulaç, 2013). The process of initiation and the maintenance of these methylation marks is controlled by a family of proteins, the DNMT (DNA MethylTransferases) (Portela and Esteller, 2010).

Throughout the male germ cell development, there are two instances during which epigenetic reprogramming takes place: during primordial germ cell development and post fertilization in the zygote. The first epigenetic reprogramming event is initiated when the PGCs begin to proliferate and migrate to the genital ridges where the future gonads will reside. During the primordial germ cell

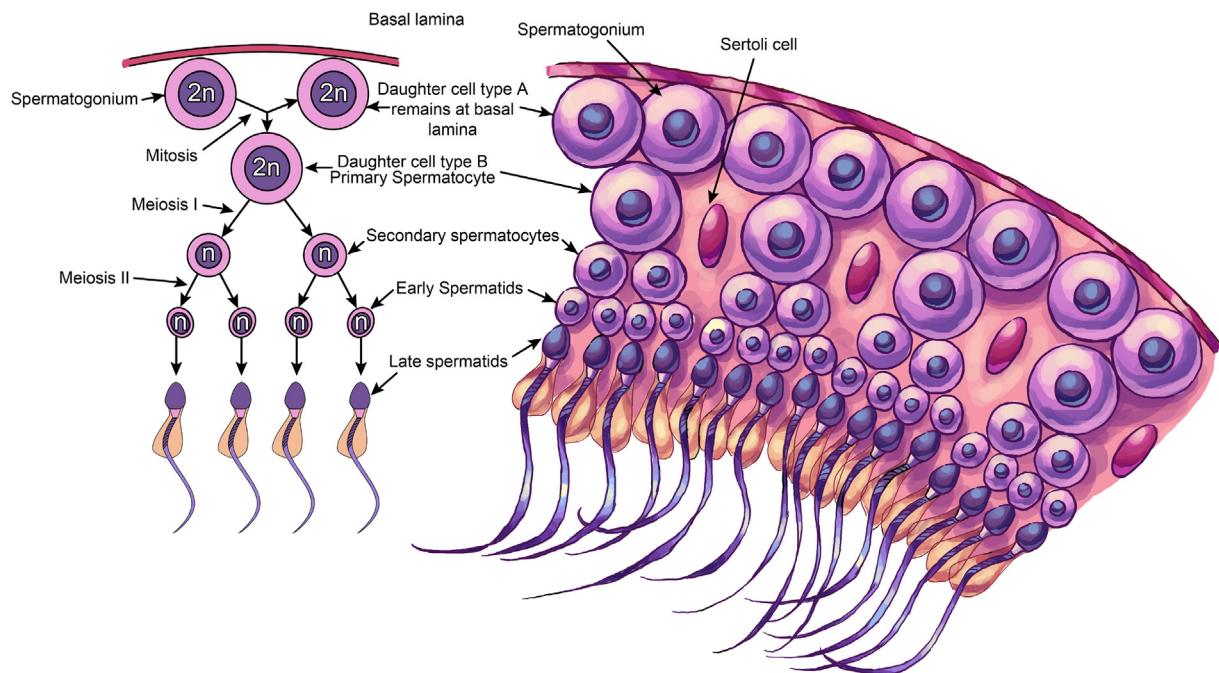


Fig. 1 The germ cells population lies on the basal lamina of the convoluted seminiferous tubules. Type A spermatogonia undergo mitosis: one of the daughter cells renew the stock of type A spermatogonia, the other becomes a type B spermatogonia. These divide and their daughter cells migrate towards the lumen. They differentiate themselves thereby into sperm cells up to the outer surface of the epithelium where the separation of the cytoplasm is not complete. Modified by Stephanie E. King from *The Reproductive System in Human Anatomy & Physiology*, 9th edn. Benjamin Cummings—An imprint of Addison Wesley Longman, Inc. 2012.

reprogramming event, the erasure of epigenetic marks differentiates somatic cell lineages and inserts new marks to promote sex specific imprinting patterns and germ cell function. The demethylation event occurs asynchronously across the genome suggesting the presence of regulatory mechanism to control the pace and location of DNA demethylation (Murphy et al., 2014). Although a large portion of the genome undergoes a rapid demethylation event, some portion of the genome retains methylation. The majority of the resistant loci consists of long terminal repeat transposable elements along with long and short interspersed elements. It is predicted that the methylation prevents the activation from these retroviral elements thus protecting the germline from their activation (Murphy et al., 2014).

The second reprogramming event occurs after fertilization when paternal DNA is demethylated and the methylation patterns are reset in the developing embryo (Fig. 2). After fertilization, DNA is demethylated and methylated in the developing embryo resulting in a reset of parental germlines. During this process, epigenetic marks specific to germ cells are reset for early embryonic pluripotency. This active removal of methylation is coordinated by Ten-eleven translocation enzymes (TET). The demethylation event of the zygote takes place prior to the first cell division and is thought to occur through active hydroxy-mediated demethylation. This reset is not complete thus some methylation marks are maintained in the developing embryo (Fig. 2). Recently, researchers have used genome wide association studies (GWAS) to identify specific loci that escape the reprogramming event. The maintenance of certain methylation patterns in the embryo are known as imprinted genes. These loci have become the foundation for identifying pathways that are biochemically regulated by epigenetics (Murphy et al., 2014). These GWAS have also indicated that epigenetic marks on the sperm not only facilitate its own development but also the development of the embryo (Murphy et al., 2014). In the developing embryo, regulatory processes such as genomic imprinting and X chromosome inactivation are continued to be influenced by gene processes including DNA methylation (Jenkins et al., 2017). Methylation events continue to occur throughout development in a tissue specific manner.

Chromatin Remodeling

The process of fertilization and movement of sperm along the female reproductive tract requires the coordination of many physiological events. Modifications of the sperm structure is essential as it progresses through potentially harmful environment in the female reproductive tract. Regulatory mechanisms following sperm maturation replaces 90%–95% of histones with protamines (Fig. 3) (Kim, 2014). The replacement of histones with small, arginine rich molecules known as protamine allows for compaction of the nucleus. The condensation of the sperm nucleus, with its decreased surface area, increases sperm motility and protects the sperm genome from oxidation and other chemicals in the female reproductive tract. The compact packaging of the genome prevents the binding of transaction factors prematurely. Immediately following fertilization, protamines are translocated with histones (Güneş and Kulaç, 2013).

Histone Modification

Histones—basic proteins rich in lysine and arginine—induce packaging of the DNA into structural units called nucleosomes. H2A, H2B, H3, and H4 histones are found in the nuclei of the nucleosomes (Fig. 3). These units are formed by combination of

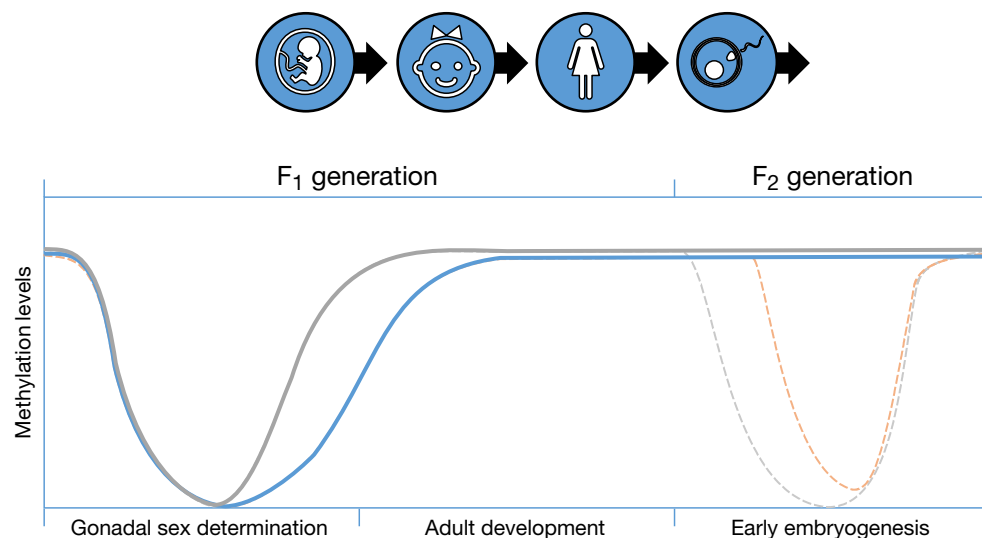


Fig. 2 DNA methylation levels throughout life. Modified from Jirtle, R.L. and Skinner, M.K. (2007). Environmental epigenomics and disease susceptibility. *Nature Reviews. Genetics* 8(4), 253–262.

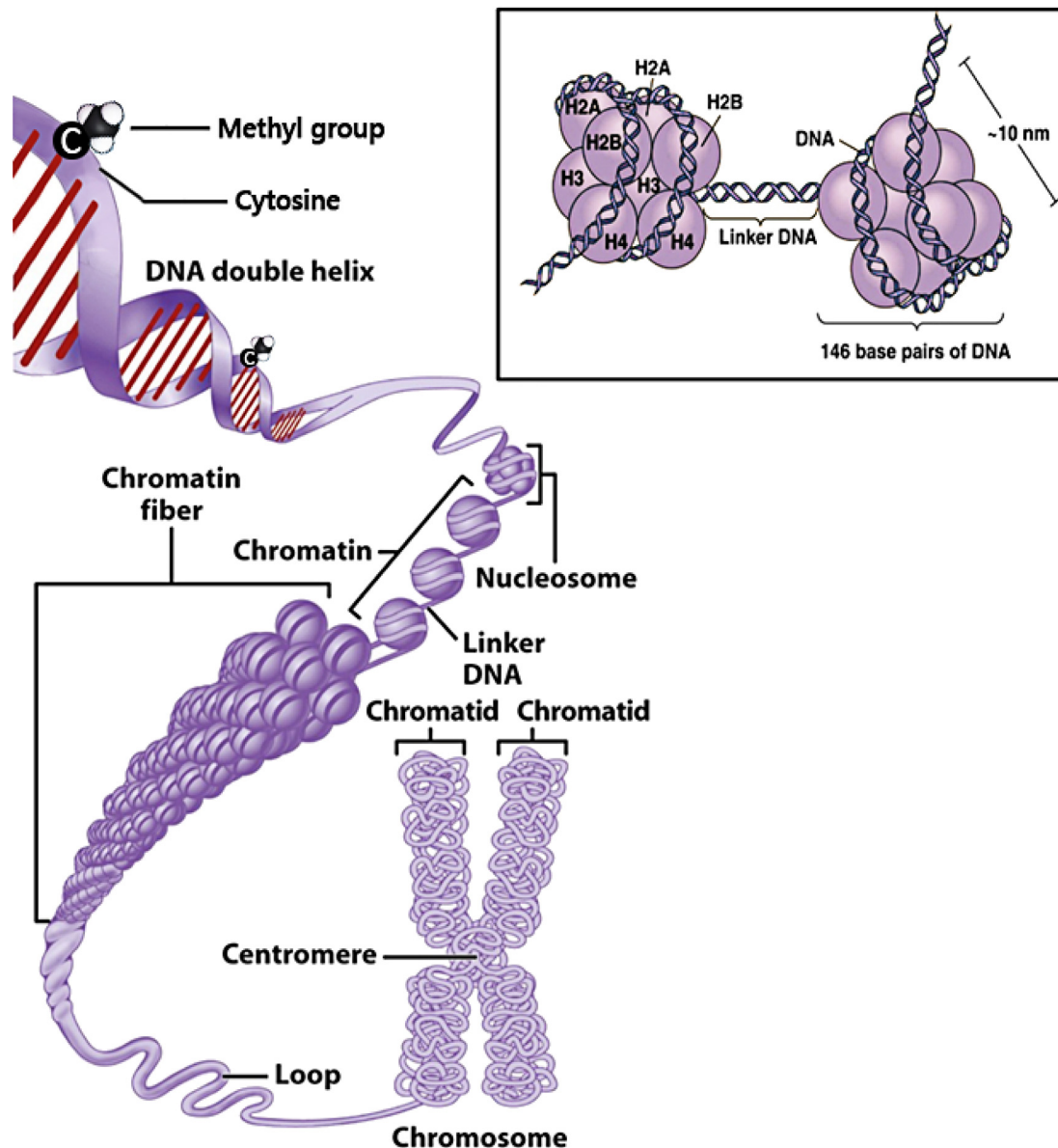


Fig. 3 Structure of DNA, chromatin, and histone. Chromatin is an instructive DNA scaffold that can respond to external cues to regulate the many uses of DNA. A principle component of chromatin that plays a key role in this regulation is the modification of histones. Histones are proteins found in eukaryotic cell nuclei. They package and order DNA into structural units called nucleosomes. Modified from Kim, Y.Z. (2014). Altered histone modifications in gliomas. *Brain Tumor Research and Treatment* 2, 7–21, and the cellular level of organization in *Principles of Anatomy and Physiology* 11th edn. by G. J. Tortora and B. H. Derrickson, John Wiley and Sons.

two H3–H4 dimers and two H2A–H2B dimers bound by H1 histone proteins (Campos and Reinberg, 2009). Histones can change the DNA-binding capacities and interaction of other regulatory factors with DNA. Histone modifications include acetylation, methylation, phosphorylation, and ubiquitination. During sperm formation, in the haploid phase of spermatogenesis where round spermatids mature into spermatozoa, histone variants are incorporated to restructure chromatin. In human, mouse and fly, testes-specific variants of H1, H2A, H2B, and H3 displace canonical histones prior to incorporation of transition proteins and protamines (Godde and Ura, 2009; Govin et al., 2004; Montellier et al., 2013). Some in vitro studies have demonstrated that some of these variants cause nucleosome destabilization (Güneş and Kulaç, 2013). Other histone variants mark specific chromatin domains. For example, in mouse spermatids, H2AL1/2 are incorporated into heterochromatic pericentric chromatin (Samson et al., 2014).

Acetylation of histone H3 and H4 usually leads to open chromatin configuration and active transcription by facilitating the binding of transcription factors. Deacetylation and methylation are generally correlated, they are associated with an inactivation of the transcription. A methylation of lysines localized on the histone tails is another regulatory mechanism. H3K9 and H3K27

histones are usually methylated and thus linked with inactivation. However, methylation of histones takes place both in active and inactive chromatin sites. When H3 and H4 histone protein tails undergo modifications, it sometimes regulates transitions between active and inactive chromatin states through a reverse mechanism called histone code.

Histone modification and changes in its composition play important roles in chromatin modifications. These modifications are required for normal meiotic process and later maturation of gametes. Following termination of meiotic divisions, the sperm cells undergo developmental changes. In fact, some histones on the X chromosome label and retain some methylated histones H3K9me2. A global remodeling in haploid spermatids occur at a lower rate. In mature sperm, H2B was found to be the most frequently expressed histone. H3K4me3 is usually found on genes regions involved in spermatogenesis while H3K4me2 in regions rich in developmental genes. Furthermore, the hypermethylation of histone H4 is responsible for the replacement of histones by protamines in haploid spermatids (Güneş and Kulaç, 2013).

Thus, the histone packaging process is an evolutionary and developmental procedure for spermatogenesis. DNA methylation and histone modification both play an important role in the regulation and control of gene expression.

Sperm Non-Coding RNA

The original observations suggest that DNA methylation alterations and histone modifications in the sperm were crucial; noncoding RNAs have, subsequently, also been shown to be involved (Skinner, 2014). Sperm is known to contain a diverse population of RNAs including non-coding RNAs which can be long or small (Skinner, 2015). Abundant small non-coding RNAs retained in spermatozoa include miRNAs and piRNAs. Some of them are essential to the early embryo development like miR-34c which is required for the first cellular division. Other non-coding RNAs include transposable elements, annotated lnc-RNAs, intronic retained elements, exonic elements, chromatin-associated RNAs, small-nuclear ILF3/NF30 associated RNAs, quiescent RNAs, mse-tRNAs, and YRNAs (Jodar et al., 2013). Some non-coding RNAs are known to act as epigenetic modifiers, such as histone modifications and DNA methylation, thus playing a role in transgenerational epigenetic inheritance.

This new area of study holds great promise for the development of this field. For example, comparing the differential ncRNAs profiles of infertile versus fertile individuals should help understand the regulatory pathways contributing to male infertility.

Clinical Issues

Aberrant epigenetic changes in male germ cells can lead to clinical issues. Abnormal methylation patterns and aberrant protamine insertions have been known to influence male fertility. CpG islands at gene promoter regions which are usually hypomethylated are especially susceptible to aberrant methylation for specific genes such as DAZL and MTHFR or imprinted loci. Epigenetic abnormalities in these regions are strongly associated with various forms of infertility and sperm defects in men (Carrell, 2012). Proper DNA methylation is especially important in imprinted genes as these genes are inherited directly from the parental germline and may have a transgenerational effect. Men with low sperm counts; oligospermic patients, (less than 10 million spermatozoa per 1 mL of semen) have been shown to contain a greater number of DNA methylation errors compared to men containing normal sperm counts. Furthermore, these imprinted loci are suspected to be inherited by the offspring during in vitro fertilization (IVF) treatments. Disorders due to defective imprinted gene expression can cause severe developmental defects including Angelman and Prader-Willi syndromes. Furthermore, children born from IVF treatments are found to have slightly greater incidents of imprinting disorders than children who were naturally conceived. This raises questions regarding the status of sperm methylome used in IVF treatments.

The use of assisted reproductive technologies have increased in recent years. Understanding the epigenetic processes underlying male factor infertility will aid proper diagnosis as well as, help develop sperm selection techniques used for IVF (Murphy et al., 2014).

Epigenetic dysfunction can also occur in spermatogenesis and may be a contributing factor in male infertility. The proper replacement of histones with protamines is a crucial step in male gametogenesis as reduced protamine levels are associated with oligospermic men. The differential DNA methylation patterns observed with male infertility is accompanied by altered protamine ratio and indicate the interaction between epigenetic phenotypes. Like the protamine, histones also carry programmatic information and thus can possibly contribute to male infertility. Histone retention has been found to occur sporadically throughout the genome, and loss of histone retention at imprinted loci is often associated with male infertility.

Epigenetic modifications are well studied in both DNA methylation and histone retention. Yet, the topic of chromatin remodeling and of non-coding RNAs have been understudied. More comprehensive understanding of epigenetic influences on male factor infertility can help further sperm selection techniques for artificial reproductive technologies.

References

- Campos, E. I., & Reinberg, D. (2009). Histones: Annotating chromatin. *Annual Review of Genetics*, 43, 559–599.
- Carrell, D. T. (2012). Epigenetics of the male gamete. *Fertility and Sterility*, 97, 267–274.

- Godde, J. S., & Ura, K. (2009). Dynamic alterations of linker histone variants during development. *The International Journal of Developmental Biology*, 53, 215–229.
- Govin, J., Caron, C., Lestrat, C., Rousseaux, S., & Khochbin, S. (2004). The role of histones in chromatin remodelling during mammalian spermiogenesis. *European Journal of Biochemistry*, 271, 3459–3469.
- Guerrero-bosagna, C., & Skinner, M. K. (2014). Environmentally induced epigenetic transgenerational inheritance of male infertility. *Current Opinion in Genetics & Development*, 26, 79–88.
- Güneş, S., & Kulaç, T. (2013). The role of epigenetics in spermatogenesis. *Turkish Journal of Neurology*, 39, 181–187.
- Jenkins, T. G., Aston, K. I., James, E. R., & Carrell, D. T. (2017). Sperm epigenetics in the study of male fertility, offspring health, and potential clinical applications. *Systems Biology in Reproductive Medicine*, 63, 69–76.
- Jodar, M., Selvaraju, S., Sandler, E., Diamond, M. P., Krawetz, S. A., & Reproductive Medicine Network. (2013). The presence, role and clinical use of spermatozoal RNAs. *Human Reproduction Update*, 19, 604–624.
- Jodar, M., Soler-Ventura, A., & Oliva, R. (2017). Semen proteomics and male infertility. *Journal of Proteomics*, 162, 125–134.
- Kim, Y. Z. (2014). Altered histone modifications in gliomas. *Brain Tumor Research and Treatment*, 2, 7–21.
- Kitamura, A., Miyauchi, N., Hamada, H., Hiura, H., Chiba, H., Okae, H., Sato, A., John, R. M., & Arima, T. (2015). Epigenetic alterations in sperm associated with male infertility. *Congenital Anomalies*, 55, 133–144.
- Montellier, E., Boussovar, F., Rousseaux, S., Zhang, K., Buchou, T., Fenaille, F., Shiota, H., Debernardi, A., Hery, P., Curtet, S., et al. (2013). Chromatin-to-nucleoprotamine transition is controlled by the histone H2B variant TH2B. *Genes & Development*, 27, 1680–1692.
- Murphy, K. E., Murphy, P. J., & Carrell, D. T. (2014). Epigenetic changes in the paternal Germline. In *Transgenerational epigenetics* (pp. 43–55). London: Elsevier Inc.
- Oliva, R., & Castillo, J. (2011). Proteomics and the genetics of sperm chromatin condensation. *Asian Journal of Andrology*, 13, 24–30.
- Portela, A., & Esteller, M. (2010). Epigenetic modifications and human disease. *Nature Biotechnology*, 28, 1057–1068.
- Samson, M., Jow, M. M., Wong, C. C. L., Fitzpatrick, C., Aslanian, A., Saucedo, I., Estrada, R., Ito, T., Park, S. K., Yates, J. R., et al. (2014). The specification and global reprogramming of histone epigenetic marks during gamete formation and early embryo development in *C. Elegans*. *PLoS Genetics*, 10, e1004588.
- Sharlip, I. D., Jarow, J. P., Belker, A. M., Lipshultz, L. I., Sigman, M., Thomas, A. J., Schlegel, P. N., Howards, S. S., Nehra, A., Damewood, M. D., et al. (2002). Best practice policies for male infertility. *Fertility and Sterility*, 77, 873–882.
- Skinner, M. K. (2014). Endocrine disruptor induction of epigenetic transgenerational inheritance of disease. *Molecular and Cellular Endocrinology*, 398, 4–12.
- Skinner, M. K. (2015). Endocrine disruptors in 2015: Epigenetic transgenerational inheritance. *Nature Reviews. Endocrinology*, 12, 68–70.
- Tollesbol, T. O. (2014). *Transgenerational epigenetics*. London: Academic Press/Elsevier.
- Tvrda, E., Gosalvez, J., & Agarwal, A. (2015). Epigenetics and its role in male infertility. In R. Watson (Ed.), *Handbook of Fertility: Nutrition, Diet, Lifestyle and Reproductive Health* (pp. 412–423). Ch. 36.

Epigenetics and Sperm Abnormalities

Timothy G Jenkins and Douglas T Carrell, University of Utah School of Medicine, Salt Lake City, Utah, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction to the Relationship Between Genetics, Epigenetics, and Gene Regulation

In individual multicellular organisms each cell is generally made up of identical DNA (aside from germ cells which only contain a haploid genome). Despite this, each unique cell type within that individual is able to arise using the same genetic blueprint as all other cell types and to facilitate normal functionality. In humans, there are more than 200 different, and highly unique, cell types in every individual that arise from the same genetic blueprint. Thus, a fundamental question in all of basic cell biology and genetics is simply, "How can so many diverse cell types arise from the same DNA code?" While the answer to this question is incredibly complex and in some cases yet to be fully elucidated, in short, the answer has to do entirely with gene regulation, not the genetic code itself. With the rate of advancement and focus on genetics following the sequencing of the human genome, it is important to note that genetics, while incredibly important, makes up only a portion of the story of human physiological function. Another key component to human physiological function is gene regulation, of which a key component is the epigenetic landscape.

The classic definition of an epigenetic mark is a molecule or modification, which can alter gene expression, is modifiable during the life of an individual, and is heritable. Under this definition, two major components of the epigenome exist, DNA methylation and histone modifications. Each of these marks can have profound impacts on gene regulation. DNA methylation typically occurs on the 5' carbon of cytosine nucleotides in the DNA, most frequently in the context of cytosine-phosphate-guanine (CpGs) dinucleotides. These CpGs are commonly enriched at the promoter region of genes and when the CpGs are methylated, they tend to inhibit transcription by blocking the binding of transcriptional machinery (Jaenisch and Jahner, 1984). There is a great deal of evidence that DNA methylation is influential in gene regulation in many other contexts beyond promoter methylation as well. Histone modifications also play a key role in gene regulation. In brief, histone tail modifications of different types help to drive gene inactivation or transcription. These modifications come in many flavors including ubiquitination, acetylation, methylation, etc. These types of modifications on both histones and CpGs play a key role in gene regulation (with both silencing and activation) and help to drive the differentiation of the various cell types in humans (Fig. 1).

Recently, a much more loose definition of epigenetics has been used to categorize other key contributors to gene regulation that are also highly modifiable in an individual over time but that are not directly heritable (at least in the same way DNA methylation and histone modifications may be). These marks include the many different species of RNA molecules. The main form of RNA that is assessed in this context are small, non-coding RNAs (ncRNA). There are many different forms of these RNAs including micro-RNAs, piRNAs, siRNAs, etc. There is even evidence that tRNA fragments may play a role in some epigenetic regulation. Though many of these marks do not fit into the conventional definition of epigenetic marks, for many reasons their roles and potential impact are so similar many researchers cluster these marks with the more commonly considered epigenetic signatures.

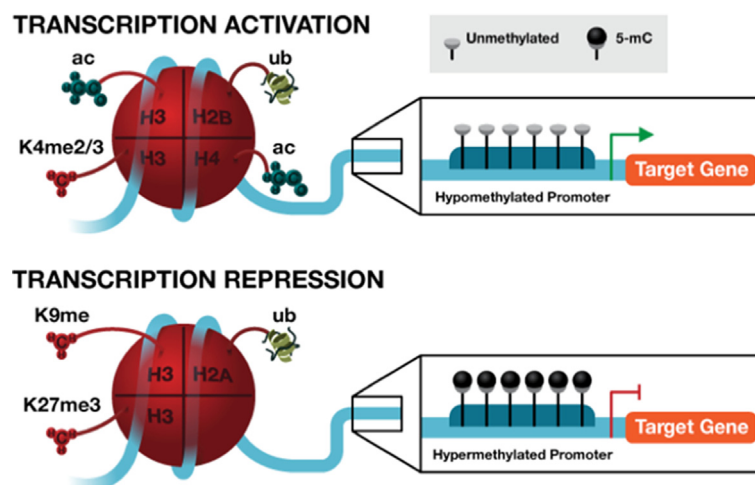


Fig. 1 Illustration depicts common epigenetic marks and associated gene activation or repression. The presence or absence of DNA methylation at gene promoters can regulate the activation or silencing of gene transcription. Similarly, histone modifications including ubiquitination (ub), monomethylation (me), dimethylation (me2), trimethylation (me3), and acetylation (ac) at various histone (H3, H2A, H4, H2B, etc.) can drive gene regulation. These histone modifications typically occur at histone tails on lysine residues (K4, K9, K27, etc.).

Sperm Epigenome

The mammalian sperm epigenome is among the most unique and specialized epigenetic landscapes known in biology. The sperm's unique epigenetic signatures are required to facilitate the normal function of this incredibly unique cell type. The human sperm epigenome specifically has been the focus of a great deal of study in recent years to better understand the nature of this unique epigenetic landscape and the impact of alterations to DNA methylation and histone modifications found therein. These studies have revealed a dynamic series of reprogramming events during spermatogenesis that make the sperm capable of its specialized function, namely the safe delivery of the paternal DNA blueprint through the female reproductive tract to fertilize the ovulated oocyte. To facilitate this functionality, the sperm nucleus becomes remarkably, highly condensed to 6–20 times more dense than somatic cell nucleosome-bound chromatin (Ward and Coffey, 1991). This compaction occurs as a result of the stepwise replacement of histone proteins with protamine proteins. The protamination process occurs throughout spermiogenesis, where histones (both canonical and testis specific variants) are replaced by transition proteins. These same transition proteins are then replaced by protamine proteins, which then form tight toroid structures to highly condense the sperm nucleus, facilitating motility and effective DNA protection from free radicals and other potential DNA damaging agents that the sperm are likely to encounter. Such tight packaging also generates a transcriptionally quiescent cell.

In addition to the massive chromatin reorganization that occurs throughout spermatogenesis, there are also quite unique DNA methylation signatures found in the mature sperm. It appears that these signatures are established early in spermatogenesis (perhaps as early as the adult germ line stem cell in humans) and are held relatively stable throughout sperm maturation. Many interesting DNA methylation profiles can be found in the mature sperm, but among the most interesting is the global hypermethylated state of the sperm DNA (most likely driven by methylation at repeat elements and other sites in the genome). This is believed to contribute to some degree to the quiescent state of mature sperm, though not all of the sperm genome is hypermethylated. In fact, there are often low methylation levels at gene promoters. Even more interesting is the enrichment of DNA demethylation intermediates, most notably 5-hydroxymethylcytosine (5-hmC), at the promoters of genes important in development. This enrichment is coupled with “bivalent” histone modifications at the same locations. These “bivalent” domains are defined by histone modifications that both promote and repress gene activation. It is believed that these domains, where both bivalent histone modifications and 5-hmC are enriched, are poised for gene activation. It is no surprise then to note that such domains are most commonly seen in embryonic stem cells. It is for this reason that many believe that the sperm epigenome is a direct and key contributor to establishing early embryonic development.

One of the most interesting facets of the sperm epigenome relates directly to the unique nature of the cell. In effect, sperm must acquire all of the epigenetic marks that are essential for its main function to deliver the DNA blueprint to the egg. However, the cell must also remain competent to drive and/or contribute to processes in the embryo. Because of the massive and dynamic reprogramming that occurs in the sperm and immediately following fertilization, the two roles of the sperm appear to be at odds with one another. Specifically, it is known that histone modifications are important regulators of gene activity but in sperm we also know that, through the process of protamination, the majority of the histones, and their associated modifications, are removed and replaced by protamine proteins. Further, immediately following fertilization there is a massive erasure of DNA methylation signatures. Taken in this context, there has historically been little interest in the contribution of the paternal epigenome to the embryo and beyond. Remarkably, recent evidence has shown that protamination and active zygotic demethylation are incomplete leaving some fraction of histones and DNA methylation marks in place. In fact, evidence suggests that not only do some histones escape protamination, but that the histones that are retained are enriched at gene promoters important in embryonic development (Hammoud et al., 2009). Thus, the previously held view that the sperm epigenome is of little significance has been largely dismissed.

As a result of the many roles of the epigenetic signatures in sperm, investigating these marks actually offers tremendous insight into both the history of the cell itself (spermatogenesis, etc.), as well as the potential for the generation of a successful pregnancy. Thus, the paternal epigenome offers unique opportunities for study (Fig. 2). In fact, sperm DNA methylation in particular offers the unique opportunity to observe the relative epigenetic environment in the adult germ line stem cell. This is due to the fact that the DNA methylation profile in sperm changes very little throughout the process of spermatogenesis. In fact, it appears that in mammals virtually no change occurs from the adult germ line stem cell all the way through to the mature sperm. Researchers are thus able to examine the nature of the epigenome and potentially be able to better diagnose the etiology of alterations seen in the mature sperm. Further, because the sperm epigenome appears to play a key role in the embryo, there is very real potential to identify signatures that

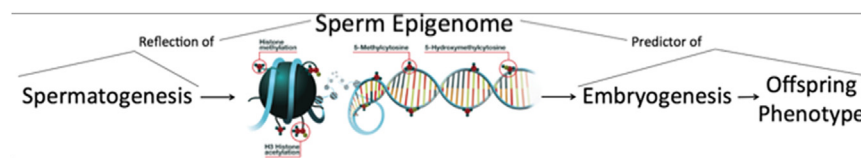


Fig. 2 Epigenetic signatures in the sperm are both reflective of the spermatogenesis process as well as potentially predictive of reproductive outcomes.

may be predictive of fertility in general, fertilization, embryo quality, and even offspring health. These potentials are what drive the great deal of recent interest in the sperm epigenome.

Sperm Epigenetics and Sperm Abnormalities

Many studies have been performed in an effort to understand the link between the sperm epigenome and various forms of male infertility phenotypes. Recent studies have observed alterations to global sperm DNA methylation profiles that appear to be associated with many forms of infertility including low sperm count and motility (Montjean et al., 2015). Interestingly, similar findings were seen when assessing far more high-resolution data of sperm abnormalities and DNA methylation signatures. Specifically, it was found using microarray technology that DNA methylation signatures were significantly altered in the sperm of patients who suffered from either low sperm viability or low motility (Jenkins et al., 2016a). Importantly, these epigenetic alterations were found at loci (particularly in the low viability cohort) that are important in meiosis and/or spermatogenesis (Jenkins et al., 2016a). A great deal of research has also suggested that DNA methylation at imprinted regions such as MEST, H19, and IGF2 are commonly altered in infertile men (Boissonnas et al., 2010; Poplinski et al., 2010). Such findings strongly supports the assertion the sperm methylome provides an excellent opportunity to observe changes that may have occurred early in spermatogenesis, which ultimately affect sperm physiological function.

Further studies have also shown alterations to histone modifications associated with various forms of infertility. In one study, it was shown that there were both alterations in histone modifications as well as in the regions in which histones were retained (through the process of protamination) in patients who had various forms of male infertility (Hammoud et al., 2011). Interestingly, in men who were infertile due to abnormal protamination it was found that methylation patterns were disrupted as well at the CREM promoter (Nanassy and Carrell, 2011). In a recent study of smokers it was shown that smoking affected histone retention, and that these individuals also displayed adverse fertility impacts (Hamad et al., 2014). Clearly, a great deal of data suggest that assessing the sperm epigenome may be informative in determining the etiology of the processes involved in the many forms sperm abnormalities.

Sperm Epigenetics and Fertility

While it is clear that altered sperm epigenetic patterns can affect normal sperm function, including everything from sperm viability, morphology, motility, and even general production (resulting in low sperm counts), the impact of the sperm epigenome on male fertility is less directly clear. However, we do have some recent evidence, suggesting that the impact of the sperm epigenome on fertilization and general male fertility is not only possible but quite probable. In a recent study of individuals attempting to conceive who had not been screened for any form of infertility, some interesting patterns were found that were associated with fecundity and sperm DNA methylation signatures. Specifically, the study compared individuals who were able to conceive within 2 months of attempting conception to individuals who were unable to achieve a pregnancy within 12 months of attempting to conceive. It was found that a small number of DNA methylation alterations occurred readily in the group that never conceived compared to the group that did conceive in the first 2 months of attempting conception (Jenkins et al., 2016b). Of particular interest was the location of these alterations. It was found that only two sites had altered methylation signatures, specifically HSPA1L and HSPA1B. These two tightly related genes are found in the same genomic neighborhood and also share similar functionality. The relationship of these functions to infertility specifically is not well understood, however the fact that across the entire genome these are the two most significantly altered genes that were identified is intriguing. Clearly, in addition to being associated with various forms of sperm abnormalities, the sperm epigenome is also associated with fertility and fecundity in general.

Sperm Epigenetics and Embryogenesis

As described above, the sperm epigenome appears to be not only reflect the process of spermatogenesis but is also poised to facilitate normal early embryogenesis. This hypothesis is based on data that suggests that both sperm DNA methylation signatures and histone modifications (or nuclear protein composition in general) appear to be poised to affect gene activation at genes essential for developmental processes in the embryo. Adding credence to this idea is the fact that in many important ways the epigenetic signatures in sperm reflect that of an early embryonic stem cell. With this in mind, it is not surprising then that alterations to the sperm epigenome may result in abnormal embryo development, which is particularly important in cases where this is followed closely such as in in vitro fertilization (IVF). In some animal models the association between the sperm epigenome and embryogenesis has been tested quite directly. In a number of early studies of sperm DNA methylation it was found that chemical alteration of the methylome (with the use of 5-azacytidine) resulted in decreased general fertility in mice, but also resulted in increases in embryo mortality (Seifertova et al., 1976). Further studies have shown, on the global scale that a similar treatment in both mouse and rat resulted in increased pre-implantation loss (Doerksen and Trasler, 1996; Kelly et al., 2003; Oakes et al., 2007). Clearly, the epigenetic profile in sperm are important for the early embryo.

In more direct tests of the impacts of alterations in sperm epigenetic profiles in humans, similar results were identified to those shown in other mammals. In a recent study, human sperm DNA methylation profiles were compared between men who sired embryos of varying degrees of quality through IVF (Aston et al., 2015). These individual samples were categorized based on both the quality of their embryos and also compared a fertile cohort. These results indicated that the DNA methylation signatures in sperm were quite predictive of an individual's fertility status and that there was also evidence to suggest that the sperm methylome could predict the likelihood of generating high quality embryos (good morphology and implantation potential) through IVF. While a great deal more work is required in this regard, it is intriguing that such patterns exist and that they may offer some degree of diagnostic utility. While not fully elucidated, from the available evidence it is clear that the sperm epigenome is not only impactful in respect to sperm function but that it is also important in normal embryonic development.

Sperm Epigenetics and Impact on the Offspring

The sperm epigenome is important in normal sperm function and even in early embryonic development, but there is also intriguing evidence to suggest that paternal epigenetic patterns have the capacity to affect offspring health. Specifically, epidemiological evidence has shown that offspring sired by older fathers have an increased incidence of neuropsychiatric disorders (including autism, schizophrenia, and bipolar disorder), some forms of cancer, and trinucleotide repeat disorders (Hare and Moran, 1979; Miller et al., 2011; Frans et al., 2013; Brunner et al., 1993; Oksuzyan et al., 2012; Yip et al., 2006). These data are quite striking and have brought to light the potential role that epigenetics may play in the process.

Two recent studies have examined this question directly in both human and mouse. In the mouse model it was shown that there are very distinct DNA methylation alterations that occur in the sperm methylome that occur with age and that there were associated alterations to offspring behavior that mimicked that of neuropsychiatric disease (Milekic et al., 2015). A year prior to this publication was a study in humans that showed strikingly consistent alterations to the sperm methylome at distinct regions throughout the genome. Of most interest in this specific study were the regions at which the sperm DNA methylation alterations occurred most frequently. It was found that the regions that were epigenetically impacted in the sperm genome were enriched at genes known to be associated with bipolar disorder and schizophrenia (Jenkins et al., 2014). Such findings were remarkable on their own, but taken into the context of chromosomal position these findings become even more intriguing. In the human study there appeared to be some enrichment of age-affected sites in sub-telomeric regions. Remarkably, it has been shown in mouse models that these same sub-telomeric regions are some of the few that escape the wave of methylation erasure and reprogramming that occurs in the zygote and primordial germ cells (Guibert et al., 2012). Taken together, there is real potential for the alterations identified in the sperm epigenome to actually impact the offspring, in this case, in a negative way.

Aging-associated epigenetic alterations to sperm DNA demonstrate one way in which the sperm epigenome can affect even the offspring. This represents only one of many sources of evidence which all point to the same conclusion that the sperm epigenome can impact the offspring and even future generations. In one recent study, it was shown that DNA methylation alterations at the POMC promoter in sperm induced by alcohol exposure in utero were transmitted over multiple generations through the paternal germ line (Govorko et al., 2012). Other studies have shown clear effects on the offspring as a result of other exposures in the father, which did not result in genetic mutations, but instead were the result of epigenetic alterations. One such study examined exposure to nicotine in male mice that sired offspring. The nicotine exposure in these males induced a protective phenotype (enhanced metabolic tolerance of the nicotine) in the offspring (Vallaster et al., 2017). Clearly, while many questions still remain, there is a great deal of evidence that establishes an important contribution to offspring phenotype and health from the father. It also appears quite likely that the mechanism driving this effect is at least in part the sperm epigenome.

Future Directions

Because of the far-reaching implications of epigenetic alterations in the sperm, from male fertility, embryo health, and even offspring phenotypic modifications, it is important that we focus a great deal of research effort to fully elucidating the mechanisms that underlie these processes. Because of the extremely unique nature of the marks (particularly in sperm) and tissue availability, there is a tremendous amount of potential for diagnostic tests, which screen epigenetic signatures. Some work has already been performed with good success. The nature of methylation data is that it provides a tremendous amount of information about the state of a given tissue type (normal or abnormal) and thus this data type lends itself very well to newly available machine learning approaches for predictive model generation. Such models have the potential to improve diagnostic testing in a field, male infertility, where such effective diagnostic testing is lacking.

While much work is still required, the available data clearly demonstrate that alterations to sperm epigenetic patterns are associated with various forms of abnormal sperm phenotypes, male infertility in general, abnormal embryogenesis, and even offspring health. Future work must rely on human research as well as animal models to fully elucidate the mechanisms that underlie the relationships between epigenetics and phenotypes. Further, because of the amount of data that can be generated in epigenetic analysis, predictive model generation will be a key component to future analyses.

References

- Aston, K. I., Uren, P. J., Jenkins, T. G., Horsager, A., Cairns, B. R., Smith, A. D., et al. (2015). Aberrant sperm DNA methylation predicts male fertility status and embryo quality. *Fertility and Sterility*, 104, 1388–1397. e1–5.
- Boissonnas, C. C., Abdalaoui, H. E., Haelewyn, V., Fauque, P., Dupont, J. M., Gut, I., et al. (2010). Specific epigenetic alterations of IGF2-H19 locus in spermatozoa from infertile men. *European Journal of Human Genetics*, 18, 73–80.
- Brunner, H. G., Bruggenwirth, H. T., Nillesen, W., Jansen, G., Hamel, B. C., Hoppe, R. L., et al. (1993). Influence of sex of the transmitting parent as well as of parental allele size on the CTG expansion in myotonic dystrophy (DM). *American Journal of Human Genetics*, 53, 1016–1023.
- Doerksen, T., & Trasler, J. M. (1996). Developmental exposure of male germ cells to 5-azacytidine results in abnormal preimplantation development in rats. *Biology of Reproduction*, 55, 1155–1162.
- Frans, E. M., Sandin, S., Reichenberg, A., Langstrom, N., Lichtenstein, P., McGrath, J. J., et al. (2013). Autism risk across generations: A population-based study of advancing grandpaternal and paternal age. *JAMA Psychiatry*, 70, 516–521.
- Govorko, D., Bekdash, R. A., Zhang, C., & Sarkar, D. K. (2012). Male Germline transmits fetal alcohol adverse effect on hypothalamic proopiomelanocortin gene across generations. *Biological Psychiatry*.
- Guibert, S., Forne, T., & Weber, M. (2012). Global profiling of DNA methylation erasure in mouse primordial germ cells. *Genome Research*, 22, 633–641.
- Hamad, M. F., Shelko, N., Kartarius, S., Montenarh, M., & Hammadeh, M. E. (2014). Impact of cigarette smoking on histone (H2B) to protamine ratio in human spermatozoa and its relation to sperm parameters. *Andrology*, 2, 666–677.
- Hammoud, S. S., Nix, D. A., Zhang, H., Purwar, J., Carrell, D. T., & Cairns, B. R. (2009). Distinctive chromatin in human sperm packages genes for embryo development. *Nature*, 460, 473–478.
- Hammoud, S. S., Nix, D. A., Hammoud, A. O., Gibson, M., Cairns, B. R., & Carrell, D. T. (2011). Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Human Reproduction*, 26, 2558–2569.
- Hare, E. H., & Moran, P. A. (1979). Raised parental age in psychiatric patients: Evidence for the constitutional hypothesis. *The British Journal of Psychiatry*, 134, 169–177.
- Jaenisch, R., & Jahner, D. (1984). Methylation, expression and chromosomal position of genes in mammals. *Biochimica et Biophysica Acta*, 782, 1–9.
- Jenkins, T. G., Aston, K. I., Pflueger, C., Cairns, B. R., & Carrell, D. T. (2014). Age-associated sperm DNA methylation alterations: Possible implications in offspring disease susceptibility. *PLoS Genetics*, 10, e1004458.
- Jenkins, T. G., Aston, K. I., Hotaling, J. M., Shamsi, M. B., Simon, L., & Carrell, D. T. (2016a). Teratozoospermia and asthenozoospermia are associated with specific epigenetic signatures. *Andrology*, 4, 843–849.
- Jenkins, T. G., Aston, K. I., Meyer, T. D., Hotaling, J. M., Shamsi, M. B., Johnstone, E. B., et al. (2016b). Decreased fecundity and sperm DNA methylation patterns. *Fertility and Sterility*, 105, 51–57. e1–3.
- Kelly, T. L., Li, E., & Trasler, J. M. (2003). 5-Aza-2'-deoxycytidine induces alterations in murine spermatogenesis and pregnancy outcome. *Journal of Andrology*, 24, 822–830.
- Milekic, M. H., Xin, Y., O'Donnell, A., Kumar, K. K., Bradley-Moore, M., Malaspina, D., et al. (2015). Age-related sperm DNA methylation changes are transmitted to offspring and associated with abnormal behavior and dysregulated gene expression. *Molecular Psychiatry*, 20, 995–1001.
- Miller, B., Messias, E., Miettinen, J., Alaraisanen, A., Jarvelin, M. R., Koponen, H., et al. (2011). Meta-analysis of paternal age and schizophrenia risk in male versus female offspring. *Schizophrenia Bulletin*, 37, 1039–1047.
- Montjean, D., Zini, A., Ravel, C., Belloc, S., Dalleac, A., Copin, H., et al. (2015). Sperm global DNA methylation level: Association with semen parameters and genome integrity. *Andrology*, 3, 235–240.
- Nanassy, L., & Carrell, D. T. (2011). Analysis of the methylation pattern of six gene promoters in sperm of men with abnormal protamination. *Asian Journal of Andrology*, 13, 342–346.
- Oakes, C. C., Kelly, T. L., Robaire, B., & Trasler, J. M. (2007). Adverse effects of 5-aza-2'-deoxycytidine on spermatogenesis include reduced sperm function and selective inhibition of de novo DNA methylation. *The Journal of Pharmacology and Experimental Therapeutics*, 322, 1171–1180.
- Oksuzyan, S., Crespi, C. M., Cockburn, M., Mezei, G., & Kheifets, L. (2012). Birth weight and other perinatal characteristics and childhood leukemia in California. *Cancer Epidemiology*, 36, e359–65.
- Poplinski, A., Tuttelmann, F., Kanber, D., Horsthemke, B., & Gromoll, J. (2010). Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. *International Journal of Andrology*, 33, 642–649.
- Seifertova, M., Vesely, J., & Enhanced, C. A. (1976). Mortality in offsprings of male mice treated with 5-azacytidine prior to mating. Morphological changes in testes. *Neoplasma*, 23, 53–60.
- Vallaster, M. P., Kukreja, S., Bing, X. Y., Ngolab, J., Zhao-Shea, R., Gardner, P. D., et al. (2017). Paternal nicotine exposure alters hepatic xenobiotic metabolism in offspring. *eLife*, 6.
- Ward, W. S., & Coffey, D. S. (1991). DNA packaging and organization in mammalian spermatozoa: Comparison with somatic cells. *Biology of Reproduction*, 44, 569–574.
- Yip, B. H., Pawitan, Y., & Czene, K. (2006). Parental age and risk of childhood cancers: A population-based cohort study from Sweden. *International Journal of Epidemiology*, 35, 1495–1503.

Cryopreservation of Sperm

Sherman Silber, Infertility Center of St. Louis, St Luke's Hospital, Chesterfield, MO, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The basic principles for cryopreservation of sperm are not different from cryopreservation of embryos, eggs, and ovarian tissue. However freezing with sperm is much easier because there is such a minute amount of water in this small, otherwise highly specialized gamete. In fact very little advance has been made in sperm freezing research, because the results seem to be so adequate with the simplest of methods (Keel and Webster, 1990; Mortimer, 1994; Morshedi, 1990; Oshima and Burger, 1993; Hallak et al., 2000; Bhattacharya et al., 2006; Hammadeh et al., 2001; Nallella et al., 2004; Freezing Medium - TYB with Glycerol and Gentamicin, 2016; Sadri-Ardekani and Atala, 2014; Sadri-Ardekani et al., 2009, 2011, 2013, 2014; Polge, 1957; Bunge and Sherman, 1953; Brinster, 2007; Brinster and Zimmermann, 1994; Bahadur et al., 2000; Brinster and Avarbock, 1994; Keros et al., 2007; Hovatta, 2001).

For embryo or egg freezing, much more precise methodology is required because 70% of these cells is water. When the intracellular water freezes, it crystalizes, and occupies a larger volume and is less dense. That is why ice cubes float. But it is also why cells are killed by freezing.

So all methods of cryopreservation rely on preventing intra cellular ice crystal formation. However I will describe, in addition to routine sperm cryopreservation, a simple new method for freezing individual sperm for difficult cases of TESE. Slow cooling results in preferential freezing of water on the outside of the cell, where the osmolality is lower. That raises the osmolality extracellularly. This in turn draws water out of the sperm making the inside even more hyperosmolar. Thus as the temperature cools gradually and ice forms preferentially on outside of the cell, that pulls water out of the cell, and then cryoprotectants enter the cell and lower the freezing point inside the sperm cell even further. Eventually the inside of the sperm actually vitrifies when the temperature is low enough and the osmolality is high enough in this extremely low volume cell.

This slow cooling rate for sperm here need not be so precise as it is with using a controlled rate freezing machine (like for slow freeze of embryos). In fact one can simply have a vial containing sperm mixed with cryoprotectants over an open liquid nitrogen dewar, vapor freeze it and then plunge it into the tank. Before holding over liquid nitrogen vapor, it can be first put in an ordinary refrigerator at 4°C. It is amazingly simple. It is the same principle as slow freezing of embryos but it does not have to be very precise, because the water content of sperm is so low. Despite the crudeness and lack of graduality of such sperm freezing and thawing protocols, the effectiveness of this vapor slow freeze approach for sperm cryopreservation is very reliable.

Methodology Details

For sperm freezing and simple testicular tissue freezing in sophisticated studies documented by Brinster, Sadri-Ardekani, Shinohara, and Van Pelt with sperm and spermatogonial stem cells (SSC'S), the best results were remarkably obtained with just this simple vapor freeze. A media containing test yolk buffer (Irvine Scientific (YP)), with glycerol and gentamycin is the usual favorite. It contains 20% egg yolk USDA certified, and 12% glycerol. The steps are as follows:

1. The sperm simply liquefies at room temperature.
2. Use a thawed 5 ml vial of medium.
3. Add liquefied sperm sample drop by drop very slowly over 30 seconds until there is a 50:50 ratio of semen or sperm to media, and mix slowly but very thoroughly. A failure to do this mixing slowly, and drop by drop, will result in complete failure.
4. For this manually assisted vapor freeze method we recommend the mixture should be placed in the refrigerator (2–5°C) for an hour before putting over the liquid nitrogen vapor.
5. Then after an hour plunge the vial into liquid nitrogen at –196°C for storage.

We have sent difficult samples to complex research programs all over the world with this simple approach of sperm freezing, and indeed testicular tissue freezing, and remarkably, this works well with no apparent need for any further improvement. So this simple sperm freeze protocol using glycerol and egg yolk, with vapor phase freeze followed by plunging, is quite adequate for sperm, and for testis tissue. We are benefited by the tiny water content of sperm and by the very small size of the sperm cell. Glycerol is a reasonable cryoprotectant for sperm, but the TEST yolk gives better results by stabilizing the membrane. These are not effective cryoprotectants for eggs or embryos.

Media for Sperm Freeze

Freezing medium TYB contains TEST-yolk buffer (TYB) to recover more motile sperm postthaw than glycerol-only media (Hallak et al., 2000; Nallella et al., 2004). TEST-yolk buffer is a semen extender and cryobuffer that can be paired with glycerol to achieve high sperm motility and viability. The egg yolk is from USDA certified virus-free flocks and washed from the sperm after cryopreservation. Freezing medium TYB is also the only CE-marked and FDA-approved sperm freezing media containing TYB and glycerol. As one of the most referenced sperm cryopreservation media worldwide, freezing medium TYB has become the routine. The media contains:

- 20% egg yolk from USDA certified SPF (virus free) laying flocks, heat inactivated at 56°C for 30 min
- 12% glycerol
- 10 µg/mL gentamicin sulfate

The most important use of sperm cryopreservation is for donor sperm banks, or for cases where the man wants to preserve his sperm before undergoing cancer treatment, or vasectomy. These would be all cases of men with normal spermatogenesis. However, when there is severe oligospermia in an infertile male, who would require IVF and ICSI with every few sperm, a more precise method is required, and here again it can be simple.

For freezing single sperm, or just a few or small number of sperm (e.g., derived from TESE with nonobstructive azoospermia), one can purchase a small platform with tiny microwells, and put just 1 µL of sperm freezing solution in each tiny well. Slip that into the ICSI dish and pick the individual sperm from the microdroplet of the ICSI dish, and drop them into the 1 µL droplet of cryoprotectants solution. Then plunge that small platform into liquid nitrogen directly, after holding in vapor.

Frozen Donor Sperm: Clinical Application

In the past, if the husband had no sperm in his ejaculate, the only solution was for the wife to have artificial insemination with donor sperm. Since we have developed the ICSI technique, there are now very few couples who would need to consider donor sperm, because the vast majority of previously hopeless cases of male infertility can now be treated successfully with ICSI. In most cases in which ICSI is not successful for dealing with male infertility, it is because of the wife's eggs, not the sperm. However, there are still a small number of cases in which no sperm at all can be found with TESE-ICSI, or in which the sperm are completely abnormal. Therefore, the need for donor sperm banks may have dwindled, but some couples will still have this need. The process of using donor sperm for couples for whom there is no alternative is as follows.

For counseling, in a sense, using donor sperm is really no different from adoption. It is simply a matter of adopting sperm. You're adopting the baby at a much earlier stage prior to conception. Using the sperm of a well-selected anonymous donor is the most realistic and sensible solution for the few couples who simply have no sperm at all, even in the testes. The use of donor sperm or donor eggs (or even donor sperm and donor eggs) has tremendous advantages over classic adoption for parental bonding and child development, not to mention less cost.

There has always been a heated debate about where a child's personality and abilities come from, its environment or its genes. After counseling many hundreds of couples who have used donor sperm and having seen the results over the last 30 years, as well as having studied rather extensively the early childhood development research coming from the Harvard and the Ypsilanti early childhood projects, I have some definite views that could be of benefit to couples who are facing this issue of whether to use donor sperm (or donor eggs). It is obviously preferable emotionally to do whatever is possible to allow patients to have their own genetic baby using the husband's sperm. However, if such a solution is not at hand, the following observations should be seriously considered.

The divorce rate among couples who choose to elect donor sperm is <1%, even though the general divorce rate in our population is over 50%. This is not because the decision to have a baby by donor insemination in some way holds the marriage together. Rather, couples who wind up choosing this route have a solid relationship and a good basis of communicating with each other. Couples who have any flaws or weaknesses in their ability to communicate on tough issues usually won't choose donor insemination. Couples who do use donor insemination are a select group that have an extraordinarily solid marriage. They can deal with a potentially divisive issue and come to a common understanding that makes them both happy. So if a couple decides to use donor sperm, it is a very good sign that the marriage is going to remain solid.

Couples will frequently ask how the baby will do when its genes come half from the mother and half from some stranger. Very often they will say, "Well, at least if we go this route, as opposed to adoption, the baby will be one half ours." This outlook is a strong reason *not* to have donor insemination. My observation has been that when the husband and the wife both accept the baby as being 100% theirs, and take the view that the genetic contribution is of no significance, then the father-infant bonding is completely normal.

Although there is a controversial view held in some circles that a child's personality, intelligence, and athletic ability are basically genetically transmitted and that there is only a partial contribution from parental rearing, evidence from most donor sperm and donor egg couples, and from early childhood education projects, strongly refutes this commonly held genetic bias. In truth, the child's personality, intelligence, and even athletic skill (though not size, hair color, eye color, or body build) are overwhelmingly related to how he or she was raised in the first several years of life.

Children in the first year and a half of life are, in many respects, like parrots. They learn by copying what they see around them. A personality tends to emerge more clearly as a sense of individual identity around the age of a year and a half, when language skills first become readily apparent. The way in which a nongenetic offspring mimics his or her rearing father, regardless of genetic origin, is so striking that these parents are sometimes shocked and do double takes when a friendly neighbor (who knows nothing about the child's genetic origin) comments admiringly on how the child is the spitting image of his father. In couples who elect donor insemination, most of the time the father's bonding with the infant is no different than if it were his own "genetic" child. So donor sperm banks are crucial for the happiness of those couples with azoospermia in which no sperm can be found even in the testis.

References

- Bahadur, G., Chatterjee, R., & Ralph, D. (2000). Testicular tissue cryopreservation in boys. Ethical and legal issues: Case report. *Human Reproduction*, 15, 1416–1420.
- Bhattacharya, J., et al. (2006). A comparative study on TEST-yolk buffer and human sperm preservation medium on post thaw characteristics of human sperm from pre-freeze specimens. *Fertility and Sterility*, 86, S200.
- Brinster, R. L. (2007). Male germline stem cells: From mice to men. *Science*, 316, 404–405.
- Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11303–11307.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Bunge, R. G., & Sherman, J. K. (1953). Fertilizing capacity of frozen human spermatozoa. *Nature*, 172, 767–768.
- Freezing Medium - TYB with Glycerol & Gentamicin. (2016). Retrieved from <http://www.irvinesci.com/products/90128-freezing-medium-tyb-with-glycerol-gentamicin?dpt=Assisted+Reproductive+Technology>.
- Hallak, J., et al. (2000). Cryopreservation of human spermatozoa: Comparison of TEST-yolk buffer and glycerol. *International Journal of Fertility*, 45(1), 38–42.
- Hamadeh, M., et al. (2001). Comparison between human sperm preservation medium and TEST-yolk buffer on protecting chromatin and morphology integrity of human spermatozoa in fertile and subfertile men after freeze-thawing procedure. *Journal of Andrology*, 22(6), 1012–1018.
- Hovatta, O. (2001). Cryopreservation of testicular tissue in young cancer patients. *Human Reproduction Update*, 7, 378–383.
- Keel, B. A., & Webster, B. W. (1990). *Handbook of the laboratory diagnosis and treatment of infertility*. Boca Raton, FL: CRC Press.
- Keros, V., Hultenby, K., Borgstrom, B., Fridstrom, M., Jahnukainen, K., & Hovatta, O. (2007). Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. *Human Reproduction*, 22, 1384–1395.
- Morshedi, M., et al. (1990). Cryopreserved/thawed semen for invitro fertilization: Results from fertile donors and infertile patients. *Fertility and Sterility*, 54, 1093–1099.
- Mortimer, D. (1994). *Practical laboratory andrology*. Oxford: Oxford University Press.
- Nallella, K. P., et al. (2004). Cryopreservation of human spermatozoa: Comparison of two cryopreservation methods and three cryoprotectants. *Fertility and Sterility*, 82, 913–918.
- Oshima, H., & Burger, H. G. (1993). *Current Topics in Andrology*. Japan Society of Andrology.
- Polge, C. (1957). Low-temperature storage of mammalian spermatozoa. *Proceedings of the Royal Society of London—Series B: Biological Sciences*, 147, 498–508.
- Sadri-Ardekani, H., & Atala, A. (2014). Testicular tissue cryopreservation and spermatogonial stem cell transplantation to restore fertility: From bench to bedside. *Stem Cell Research & Therapy*, 5, 68.
- Sadri-Ardekani, H., Mizrak, S. C., van Daalen, S. K., Korver, C. M., Roepers-Gajadien, H. L., Koruj, M., Hoving, S., de Reijk, T. M., de la Rossetta, J. J., van der Veen, F., de Rooij, D. G., Repping, S., & van Pelt, A. M. (2009). Propagation of human spermatogonial stem cells in vitro. *JAMA*, 302, 2127–2134.
- Sadri-Ardekani, H., Akhondi, M. A., van der Veen, F., Repping, S., & van Pelt, A. M. (2011). In vitro propagation of human prepubertal spermatogonial stem cells. *JAMA*, 305, 2416–2418.
- Sadri-Ardekani, H., Akhondi, M. M., Vossough, P., Maleki, H., Sedighnejad, S., Kamali, K., Ghorbani, B., van Wely, M., van der Veen, F., & Repping, S. (2013). Parental attitudes toward fertility preservation in boys with cancer: Context of different risk levels of infertility and success rates of fertility restoration. *Fertility and Sterility*, 99, 796–802.
- Sadri-Ardekani, H., Homburg, C. H., van Capel, T. M., van den Berg, H., van der Veen, F., van der Schoot, C. E., van Pelt, A. M., & Repping, S. (2014). Eliminating acute lymphoblastic leukemia cells from human testicular cell cultures: Apilotstudy. *Fertility and Sterility*, 2014(101), 1072–1078.

Fertility Preservation for Oncological Reasons

Atsuko Kusuhashi and Teresa K Woodruff, Northwestern University, Chicago, IL, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Based on a 2010–2012 survey, it is estimated that approximately 40% of men and women will be diagnosed with cancer at some point during their lifetime (Howlader et al., 2017). While the incidence of cancer has increased, recent improvements in cancer treatment have led to a decrease in cancer mortality rates. In 2014, the number of cancer survivors reached an estimated 14.5 million in the United States alone (Howlader et al., 2017), and over the last 50 years, the survival rates for pediatric cancers have risen from 10% to nearly 80% (Ward et al., 2014). These cancer survivors expect to have a future that may include the decision to start a family, an important part of life for both men and women. However, recent research has shown that certain cancer treatment regimens can damage ovarian follicles in females and spermatogonia in the testis of males, leading to compromised fertility. In addition to the typical factors that affect fertility, such as age, treatment-related factors include type and cumulative dose of chemotherapy, radiation dose, and type of surgery performed (Jeruss and Woodruff, 2009; De Vos et al., 2014). The gonadotoxic effect of various chemotherapeutic agents is diverse, may involve a variety of mechanisms, and is not well understood.

Young cancer patients deliberating a potentially gonadotoxic treatment regimen may wish to preserve their fertility prior to undergoing treatment. Fertility preservation for oncological reasons—termed oncofertility—is a complex process involving the coordination of a highly skilled, multidisciplinary team. To avoid a delay in cancer treatment, the options for fertility preservation need to be discussed and performed rather quickly following diagnosis. Collaboration between oncologists and reproductive endocrinologists is necessary to understand the patient's status and goals, and determine the best fertility preservation option. This collaboration is vital, as patients must be rapidly referred from the oncology clinic to reproductive endocrinologists to undergo fertility preservation procedures, and then transferred back to oncology to begin treatment. In addition, psychosocial care providers and other resources may be brought in to help patients and their families during the decision-making process.

Histological Background of Fertility Preservation

In the 1960s, when cancer was associated with high mortality rates, little attention was paid to a patient's future fertility—the focus was on survival (Noll et al., 2013). The 1970s introduced new treatments such as megavoltage radiation therapy and combination chemotherapy that dramatically reduced the mortality rate, but also led to an increase in severe long-term side effects including cardiovascular dysfunction, pulmonary dysfunction, muscle atrophy, endocrine complications, and infertility (Nielsen et al., 2017). Long-term endocrine effects included impairment of the hypothalamus, pituitary, thyroid and gonads; decreased bone mineral density; metabolic derangements leading to obesity and/or diabetes mellitus; and infertility (Chemaitilly and Cohen, 2017). While the endocrinological effects of cancer treatment can be minimized by hormone treatments, the loss of the reproductive function is irreversible. With the increased survival rates that characterized cancer treatment in the 1970s, it became important to consider patients' future quality of life—including fertility. The health care system started to pay closer attention to the long-term effects of treatment on cancer survivors and began to improve follow-up care to patients (The National Coalition for Cancer Survivorship, 1995). Multidisciplinary teams were established to provide cancer survivors with reproductive health management options in the early 2000s (Woodruff, 2015). In 2006, the American Society of Clinical Oncology (ASCO) published a clinical practice guideline on fertility preservation (updated in 2013), which recommend that all cancer patients diagnosed during their reproductive years should be informed about potential loss of fertility, and that oncologists should be prepared to discuss fertility preservation options and/or to refer all potential patients to appropriate reproductive specialists (Loren et al., 2013).

The Impact of Cancer Treatments on Fertility

The effect of cancer treatments on fertility—here defined simply as “the ability to reproduce”—depends on the age at the time of treatment, the radiation or chemotherapy protocol, the duration of exposure, and total cumulative dose administered (Ben-Aharon and Shalgi, 2012). Most chemotherapeutic agents work by affecting cell cycle division. Therefore, cancer drugs affect the proliferation of gametes (oocytes and sperm) in the maturation stage as well as the somatic cells that support these gametes (granulosa cells in the ovary and Sertoli cells in the testes) (Blumenfeld, 2012). Radiation therapy in fields near the pelvis can directly affect the testes and ovary (Albers et al., 2005; Petersen et al., 2002). Total body irradiation given in combination with myeloablative conditioning prior to bone marrow transplantation is associated with a high risk of gonadal failure in both males and females through effects on the hypothalamus and pituitary (Vakalopoulos et al., 2015; Cohen et al., 2008). Gonadal failure may affect all aspects of reproductive health, including pubertal development, hormone production, and sexual function in adults.

Cancer Treatment and Male Infertility

For males, gonadal dysfunction is due to direct toxic effects of chemotherapy and radiation therapy on spermatogonia, the primordial germ cells that mature into sperm during spermatogenesis. The production of mature sperm from spermatogonia occurs in the testes starting at puberty. Some chemotherapies, radiation therapy, and surgery can cause damage to the testes and spermatogonia, and reduce or stop sperm production (Biedka et al., 2016). The epithelium of the testis consists of Sertoli cells and spermatogenic cells, and is particularly vulnerable. Infertility may occur immediately or within a few months after cancer treatment and can last for months or years; in some cases, infertility is permanent (Trost and Brannigan, 2012). The extent and speed of recovery of spermatogenesis following a cytotoxic incursion are dependent on the agent used and the dose received (Kandeel, 2007). Germ cell failure is common in males treated with high cumulative doses of cyclophosphamide (7500 mg/m² or more) and more than 3 months of combination alkylating agent therapy (Blumenfeld, 2012; Vakalopoulos et al., 2015). Leydig cells, the interstitial cells adjacent to the seminiferous tubules that produce testosterone, are less susceptible to cytotoxic damage; as a result, post-treatment androgen insufficiency is rare (Jeruss and Woodruff, 2009). Although the prepubertal testis does not support spermatogenesis and cannot produce mature sperm, the primordial germ cells in the prepubertal testis are nevertheless sensitive to cytotoxic drugs; therefore, chemotherapy given to prepubertal males may impair their future fertility (Vassilakopoulou et al., 2016). The spermatogonia are extremely sensitive to radiation, regardless of age. Radiation therapy can lower sperm production when it is targeted towards the testicles, pelvis, pituitary gland, and brain, as can total body radiation (Trost and Brannigan, 2012; Ogilvy-Stuart and Shalet, 1993).

The surgical management of testicular cancer can profoundly affect fertility. Removal of both testicles stops sperm production forever, while orchiectomy has been shown to decrease median sperm concentration and total sperm count (Petersen et al., 1999). In addition, surgery of the prostate, bladder, large intestine, spine, or rectum can damage the nerves and cause ejaculatory dysfunction. These operations often cause retrograde ejaculation or the backflow of semen towards the bladder, resulting in little or no sperm emission during ejaculation. Even before treatment, some cancers, such as testicular cancer and Hodgkin's lymphoma, can themselves lower sperm counts (Vakalopoulos et al., 2015).

Cancer Treatment and Female Infertility

In females, ovarian function is based on the number of oocytes in the ovary. The germ cell population reaches a maximum of 6–7 million at approximately 20 weeks of gestation when primordial follicles containing oocytes begin to form. Primordial follicles constitute a finite pool of resting ovaries in the ovary. At the time of birth, the resting follicle pool decreases to approximately 1 million due to atresia, with a further reduction to 250,000 by the time of puberty. It does not increase thereafter, gradually decreasing from birth to menopause. The age at which ovarian function is ultimately lost is based on both the size of the primordial follicle pool and the rate of depletion of this pool (Gore et al., 2014). Certain cancer drugs directly act on the ovary and affect ovarian function, causing genetic damage to growing follicles and their enclosed oocytes or ovarian failure, early menopause, and other reproductive problems. Reduction in the number of follicles by cancer treatments can lead to a premature ovarian failure, defined as menopause before the age of 41 (Wallace et al., 2005; Green et al., 2009). Chemotherapy-related amenorrhea occurs in over 90% of patients who are either treated with high-dose chemotherapies, induction therapies before bone marrow transplantation, or total body irradiation (Fleischer et al., 2011). The incidence of chemotherapy-related amenorrhea depends on the type of chemotherapy regimen used. Alkylating agents (e.g., cyclophosphamide) damage DNA and induce apoptosis through covalent binding of alkyl groups to cellular macromolecules (Hudson, 2010). Alkylating agents are highly toxic to the ovary, and the primordial ovarian follicles are particularly sensitive to these agents. The health consequences of ovarian follicle damage by cancer therapies increases with age, most likely because older females have a smaller resting follicle pool—fewer primordial oocytes—than younger females. Oocyte quality also normally declines with age, making it more difficult for older females to conceive; age compounds the negative effects of cancer treatment on reproductive function and fertility. The toxicity of chemotherapies also extends to actively growing oocytes. It is therefore recommended that patients avoid conception for at least 6 months after completion of cancer treatment (Rodriguez-Wallberg and Oktay, 2014). Cancer surgery can also reduce fertility, either through removal of or damage to the reproductive organs. Treatments such as cranial radiation can impair hypothalamic–pituitary function and indirectly impair ovarian function, resulting in sex hormone deficiency (Gonfloni et al., 2009). Radiation to the pelvis may induce uterine fibrosis, which may complicate future pregnancies (Biedka et al., 2016; Critchley and Wallace, 2005).

Fertility Preservation

Technological advances in reproductive science and medicine have made fertility preservation a viable option for patients with cancer diagnosis (Fig. 1). The standard method of fertility preservation is to cryopreserve germ cells or embryos prior to undergoing cancer treatment. Cryopreservation involves cooling cells to very low temperatures to stop all biologic activity and preserve them for future use (ASRM). The first report of sperm cryopreservation for a patient with cancer was in the 1980s and the first case of a successful pregnancy with in vitro fertilization (IVF) using cryopreserved sperm from a man diagnosed with testicular malignancy was reported in 1985 (Rowland et al., 1985). Fertility preservation is much more complicated for females, and techniques to

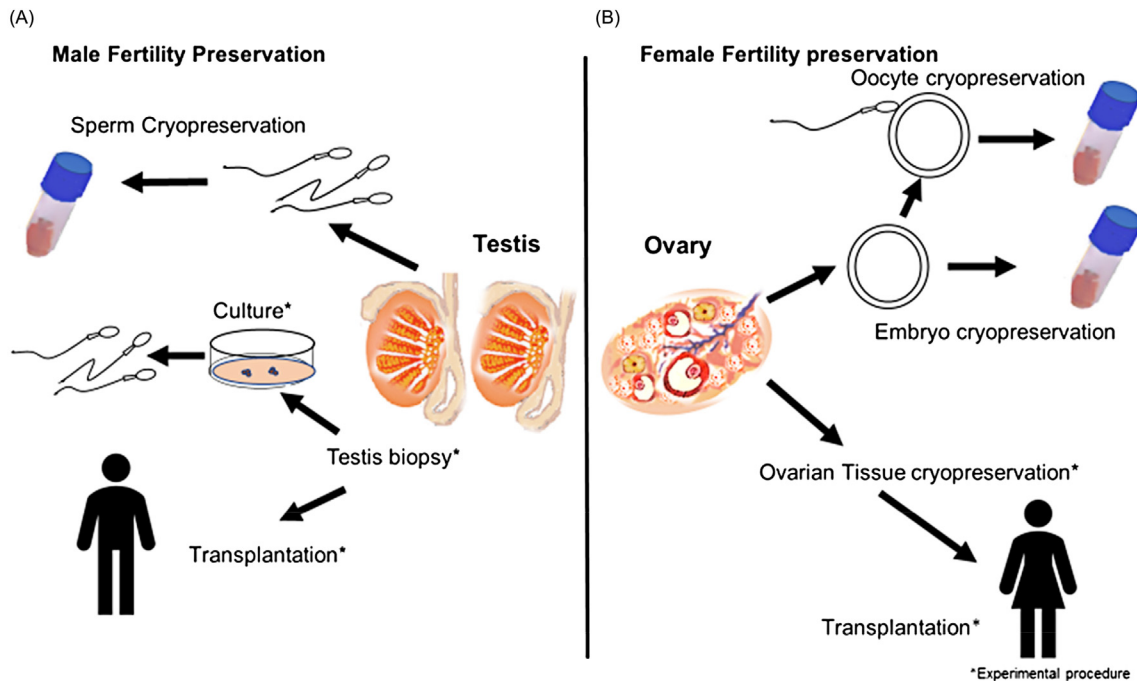


Fig. 1 Fertility preservation options for male and female. Fertility preservation options for post pubertal males involve collecting sperm. Sperm may be cryopreserved for later use. Investigational options for prepubertal boys include testicular tissue biopsy and cryopreservation. Oocyte or embryo cryopreservation are the most accepted approaches for female. Following cancer treatment, cryopreserved oocytes are thawed, fertilized by IVF, and transferred to the uterus as embryos.

cryopreserve oocytes, which are much larger than sperm, came about in 1986. Cryopreservation of ovarian tissue for transplantation after cancer treatment is a more recent development. Since then, fertility preservation clinical guidelines have been updated to include oocyte cryopreservation ([Ethics Committee of American Society for Reproductive Medicine, 2013](#); [Loren et al., 2013](#)), and the number of female patients undergoing fertility preservation procedures prior to cancer treatment has increased.

Fertility Preservation for Males

The testis is highly sensitive to radiation and chemotherapy at all ages; therefore, it is strongly recommended that sperm are cryopreserved prior to initiation of cancer treatment. The quality of the sperm sample and sperm DNA integrity may be compromised even after a single treatment session. Sperm cryopreservation (or “banking”) before starting cancer treatment is an established clinical option for male fertility preservation ([Ethics Committee of American Society for Reproductive Medicine, 2013](#)). Although cryopreservation is associated with a decrease in semen parameters when thawed, with advanced reproductive technologies (ART) such as IVF and intracytoplasmic sperm injection (ICSI), a low number of viable sperm is sufficient to achieve fertilization. The number of cancer survivors who have utilized ART with cryopreserved sperm are reported to be between 33% and 56%. Although the exact length of time that cryopreserved sperm remains viable is unknown, successful paternity has been demonstrated with sperm more than 21 years after cryopreservation ([Feldschuh et al., 2005](#)). When sperm collection for cryopreservation is not possible due to patient age, anatomy, or concerns about psychological impact, sperm can be removed using testicular sperm extraction (TESE) procedures ([Johnson et al., 2017](#)). For prepubertal patients who have not initiated spermatogenesis, cryopreservation of testicular tissue is another option, but remains experimental. Tissue can be obtained by multiple techniques, including testicular biopsy, and immature spermatogonia can be collected via microsurgical epididymal sperm aspiration (MESA), or TESE ([Brinster and Zimmermann, 1994](#); [Faes et al., 2013](#)). Researchers are now developing techniques to mature cryopreserved spermatogonia in vitro for use in IVF and transplant cryopreserved testicular tissue ([de Lambert et al., 2015](#)). In selected patients with testicular cancer, a partial orchiectomy has become an established option for fertility preservation.

Fertility Preservation for Females

Oocyte or embryo cryopreservation are the most accepted approaches for preservation of female fertility prior to cancer treatment. Both options require ovarian stimulation and ultrasound-guided oocyte retrieval for either freezing for later use or for IVF. Following cancer treatment, cryopreserved oocytes are thawed, fertilized by IVF, and transferred to the uterus as embryos. Oocyte

cryopreservation is particularly important for females who do not have a partner at the time of cryopreservation (Loren et al., 2013). In most countries, women who have a partner at the time of oocyte retrieval choose to create embryos for cryopreservation. In some countries, women can use sperm donors if they do not have a partner. Because ovarian stimulation with gonadotropins is needed to obtain more than one mature oocyte per cycle for cryopreservation of oocytes or IVF (Rodriguez-Wallberg and Oktay, 2014), this option is possible only if cancer treatment can be delayed and in women who do not have hormone-sensitive cancers, such as certain breast cancers (Blumenfeld, 2012). Ovarian tissue cryopreservation (OTC) is another fertility preservation option for female patients who need to start cancer treatment immediately or have hormone-sensitive cancers, or for pediatric cancer patients (Meirow et al., 1999; Fleischer et al., 2011) who cannot undergo ovarian stimulation. In 2004, the first live birth after transplantation of frozen-thawed ovarian cortex was reported by a Belgian group (Donnez et al., 2004). Strips of ovarian cortical tissue were cryopreserved prior to cancer treatment; the patient subsequently experienced ovarian failure and 6 years after treatment the frozen-thawed cortical strips were transplanted into the peritoneal window beneath the hilum of the inactive ovary. A follicle that had physiologically matured in the transplanted ovarian tissue was collected for IVF (Donnez et al., 2004). Although OTC is still considered an experimental technique, 60 live births after transplantation of cryopreserved ovarian tissue have been reported as of 2016 (Meirow et al., 1999). For patients with blood-borne malignancies or ovarian cancer, there is a potential risk of reintroducing cancer cells within the cryopreserved ovarian cortex; this risk must be taken into account before ovarian tissue transplantation.

New approaches in gynecologic oncologic surgery are being developed with the goal of preserving reproductive organ function without compromising survival. Patients with well-differentiated, low-grade, early-stage tumors or tumors with low malignancy are potential candidates for these procedures (Wallberg et al., 2009). Young women presenting with borderline ovarian tumors may be offered a single oophorectomy (Mangili et al., 2016). Another established surgical procedure for fertility preservation in young female patients is radical trachelectomy for early-stage invasive cervical cancer (Tomao et al., 2016).

Pediatric Fertility Preservation

Children with cancer suffer both short- and long-term side effects of cancer and its treatment, including future infertility (Meirow et al., 1999). Established methods of fertility preservation such as sperm cryopreservation in adult males and embryo and oocyte cryopreservation in adult females are not possible in prepubertal males and females because of their gonadal immaturity (Ben-Aharon and Shalgi, 2012) (Table 1). Puberty affects the growth and maturation of the gonads, and is associated with the initiation of spermatogenesis in male and follicular growth and ovulation in females. As in adults, gonadal function may be affected by cancer treatment in children, and may affect not only future fertility but also the growth and maturation of the gonads during juvenile development. In prepubertal patients, failure of pubertal development may be the first sign of gonadal failure (Thibaud et al., 1998). One challenge in fertility preservation for pediatric patients is that parents may feel uncertain about making fertility-related decisions on behalf of their child (Loren et al., 2013). The mean age of diagnosis of pediatric cancer patients is 6 years; in such situations, patients and their families are asked to consider future fertility. Several studies have confirmed that adult survivors of pediatric cancer wish they had been given more information and options about fertility at the time of diagnosis (Cvancarova et al., 2009). Therefore, discussing fertility preservation is critical for pediatric cancer patients' long-term quality of life.

Management

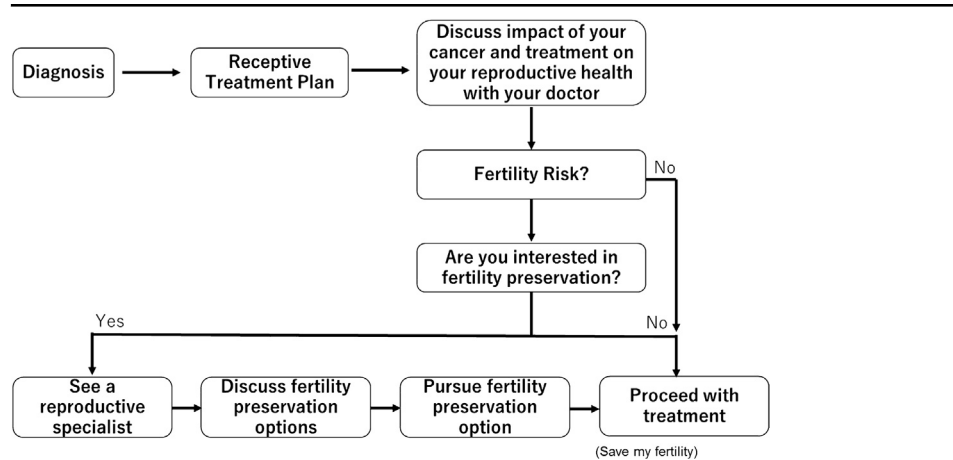
In most cases, decisions about fertility preservation need to be made before cancer treatment begins (Table 2). Since post-therapy recovery of gonadal function is unpredictable, it is important to inform patients facing potential treatment-related infertility of this possible side effect and all the options that are available to prevent it. When patients are counseled on fertility preservation for oncological reasons, they usually are still processing the life-changing cancer diagnosis and may be under pressure to initiate cancer treatment. Decision making about preserving fertility can add to the emotional burden at the time of diagnosis, but fertility preservation

Table 1 Fertility preservation options for adult and pre-pubertal girls and boys

Women	Girls	Men	Boys
• Oocyte cryopreservation • Embryo cryopreservation • Ovarian tissue cryopreservation^a • Conservative surgery • GnRH agonist^a	• Ovarian tissue cryopreservation^a • Conservative surgery • Ovarian transposition	• Sperm cryopreservation • Testicular sperm extraction (TESE) • Testicular tissue cryopreservation^a	• Conservative surgery • Testicular tissue cryopreservation^a

Established methods of fertility preservation such as sperm cryopreservation in adult males and embryo and oocyte cryopreservation in adult females are not possible in prepubertal males and females because of their gonadal immaturity.

^aExperimental procedure.

Table 2 The process of fertility preservation

Although patients have just received a life-changing cancer diagnosis, it is important to know the potential treatment-related infertility and all the options that are available to prevent it.

can also provide hope for a future. Resources and tools have been developed to help the multidisciplinary providers involved in fertility preservation in the oncological setting. Collaboration between oncologists and reproductive medicine providers is necessary for patients to receive timely access to fertility preservation options after a cancer diagnosis. In 2013, clinical guidelines changed the label of who is responsible for discussing fertility preservation by relabeling “oncologist” as “health care provider,” and extending this label to medical oncologists, radiation oncologists, gynecologic oncologists, urologists, hematologists, pediatric oncologists, and surgeons, as well as nurses, social workers and psychologists (Ethics Committee of American Society for Reproductive Medicine, 2013; Loren et al., 2013). Social workers and psychologists can be particularly helpful when a patient is distressed about potential infertility as a result of cancer or its treatment.

Other family building options after cancer include the use of gestational carriers, embryo donation, egg or sperm donation, and adoption (Loren et al., 2013). Focusing on the potential consequences of cancer treatment and infertility, and finding solutions to preserve or restore fertility and reproductive function, has become its own field of study. Advances in oncofertility research continue to improve and expand fertility preservation options for cancer survivors and focus on finding better ways to protect oocytes from the toxic effects of cancer treatments (Kim et al., 2016). Fertility preservation gives hope to cancer patients who will survive their disease by offering the option of preserving their fertility (Woodruff, 2015).

Acknowledgements

The authors thank Dr. Stacey C. Tobin for critical review of the manuscript. This work was supported by the Master of Science in Reproductive Medicine program, and the Center for Reproductive Health After Disease (P50 HD076188) from the National Center for Translational Research in Reproduction and Infertility (NCTRI).

References

- Albers, P., Albrecht, W., Algaba, F., Bokemeyer, C., Cohn-Cedermark, G., Horwich, A., Klepp, O., Laguna, M. P., & Pizzocaro, G. (2005). Guidelines on testicular cancer. *European Urology*, 48, 885–894.
- Ben-Aharon, I., & Shalgi, R. (2012). What lies behind chemotherapy-induced ovarian toxicity? *Reproduction*, 144(2), 153–163. <https://doi.org/10.1530/rep-12-0121>.
- Biedka, M., Kuzba-Kryszak, T., Nowikiewicz, T., & Zyromska, A. (2016). Fertility impairment in radiotherapy. *Contemporary Oncology*, 20, 199–204.
- Blumenfeld, Z. (2012). Chemotherapy and fertility. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 26, 379–390. <https://doi.org/10.1016/j.bpobgyn.2011.11.008>.
- Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, 91, 11298–11302.
- Chemaitilly, W., & Cohen, L. E. (2017). Diagnosis of endocrine disease: Endocrine late-effects of childhood cancer and its treatments. *European Journal of Endocrinology*, 176, R183–R203.
- Cohen, A., et al. (2008). Endocrinological late complications after hematopoietic SCT in children. *Bone Marrow Transplantation*, 41(Suppl. 2), S43–S48.
- Critchley, H. O., & Wallace, W. H. (2005). Impact of cancer treatment on uterine function. *Journal of the National Cancer Institute. Monographs*, 34, 64–68.
- Cvancarova, M., Samuelsen, S. O., Magelssen, H., & Fossa, S. D. (2009). Reproduction rates after cancer treatment: Experience from the Norwegian radium hospital. *Journal of Clinical Oncology*, 27, 334–343.
- De Vos, M., Smits, J., & Woodruff, T. K. (2014). Fertility preservation in women with cancer. *Lancet*, 384, 1302–1310.
- Donnez, J., Dolmans, M. M., Demylle, D., Jadoul, P., Pirard, C., Squifflet, J., Martinez-Madrid, B., & van Langendonck, A. (2004). Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet*, 364, 1405–1410.

- Ethics Committee of American Society for Reproductive Medicine. (2013). Fertility preservation and reproduction in patients facing gonadotoxic therapies: A committee opinion. *Fertility and Sterility*, 100, 1224–1231.
- Faes, K., Tournaye, H., Goethals, L., Lahoutte, T., Hoorens, A., & Goossens, E. (2013). Testicular cell transplantation into the human testes. *Fertility and Sterility*, 100, 981–988.
- Feldschuh, J., Brassel, J., Durso, N., & Levine, A. (2005). Successful sperm storage for 28 years. *Fertility and Sterility*, 84, 1017.
- Fleischer, R. T., Vollenhoven, B. J., & Weston, G. C. (2011). The effects of chemotherapy and radiotherapy on fertility in premenopausal women. *Obstetrical & Gynecological Survey*, 66, 248–254.
- Gonfloni, S., Di Tella, L., Caldarola, S., Cannata, S. M., Klinger, F. G., Di Bartolomeo, C., Mattei, M., Candi, E., De Felici, M., Melino, G., & Cesareni, G. (2009). Inhibition of the c-Abl-TAp63 pathway protects mouse oocytes from chemotherapy-induced death. *Nature Medicine*, 15, 1179–1185.
- Gore, A. C., Hall, J. E., & Hayes, F. J. (2014). Aging and Reproduction. In A. J. Zeleznik, & T. M. Plant (Eds.), *Knobil and Neill's physiology of reproduction*. St. Louis, MO: Elsevier.
- Green, D. M., Sklar, C. A., Boice, J. D., Jr., Mulvihill, J. J., Whitton, J. A., Stovall, M. and Yasui, Y. 2009. Ovarian failure and reproductive outcomes after childhood cancer treatment: Results from the Childhood Cancer Survivor Study. *Journal of Clinical Oncology* 27, 2374–2381.
- Howlader, N., Noone, A. M., Krapcho, M., Miller, D., Bishop, K., Kosary, C.L., Yu, M., Ruhl, J., Tatalovich, Z., Mariotto, A., Lewis, D. R., Chen, H. S., Feuer, E. J. and Cronin, K. A. (eds.) (2017). SEER Cancer Statistics Review, 1975-2014, Bethesda, MD: National Cancer Institute, https://seer.cancer.gov/csr/1975_2014/, based on November 2016 SEER data submission, posted to the SEER web site, April 2017.
- Hudson, M. M. (2010). Reproductive outcomes for survivors of childhood cancer. *Obstetrics and Gynecology*, 116, 1171–1183.
- Jeruss, J. S., & Woodruff, T. K. (2009). Preservation of fertility in patients with cancer. *The New England Journal of Medicine*, 360, 902–911.
- Johnson, E. K., Finlayson, C., Rowell, E. E., Gosiengfiao, Y., Pavone, M. E., Lockart, B., Orwig, K. E., Brannigan, R. E., & Woodruff, T. K. (2017). Fertility preservation for pediatric patients: Current state and future possibilities. *The Journal of Urology*, 198(1), 186–194.
- Kandeel, F. R. (2007). The effects of chemotherapy and radiotherapy on testicular function. In *Male reproductive dysfunction: Pathophysiology and treatment*. Boca Raton, FL: CRC Press.
- Kim, S. Y., Kim, S. K., Lee, J. R., & Woodruff, T. K. (2016). Toward precision medicine for preserving fertility in cancer patients: Existing and emerging fertility preservation options for women. *Journal of Gynecologic Oncology*, 27, e22.
- de Lambert, G., Poirot, C., Guerin, F., Brugieres, L., & Martelli, H. (2015). Preservation of fertility in children with cancer. *Bulletin du Cancer*, 102, 436–442.
- Loren, A. W., Mangu, P. B., Beck, L. N., Brennan, L., Magdalinski, A. J., Partridge, A. H., Quinn, G., Wallace, W. H., & Oktay, K. (2013). Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *Journal of Clinical Oncology*, 31, 2500–2510.
- Mangili, G., Somigliana, E., Giorgione, V., Martinelli, F., Filippi, F., Petrella, M. C., Candiani, M., & Peccatori, F. (2016). Fertility preservation in women with borderline ovarian tumours. *Cancer Treatment Reviews*, 49, 13–24.
- Meirow, D., Lewis, H., Nugent, D., & Epstein, M. (1999). Subclinical depletion of primordial follicular reserve in mice treated with cyclophosphamide: Clinical importance and proposed accurate investigative tool. *Human Reproduction*, 14, 1903–1907.
- Nielsen, K. M., Offersen, B. V., Nielsen, H. M., Vaage-Nilsen, M., & Yusuf, S. W. (2017). Short and long term radiation induced cardiovascular disease in patients with cancer. *Clinical Cardiology*, 40, 255–261.
- Noll, R. B., Patel, S. K., Embry, L., Hardy, K. K., Pelletier, W., Annett, R. D., Patenaude, A., Lown, E. A., Sands, S. A., & Barakat, L. P. (2013). Children's Oncology Group's 2013 blueprint for research: Behavioral science. *Pediatric Blood & Cancer*, 60, 1048–1054.
- Ogilvy-Stuart, A. L., & Shalet, S. M. (1993). Effect of radiation on the human reproductive system. *Environmental Health Perspectives*, 101(Suppl 2), 109–116.
- Petersen, P. M., Skakkebaek, N. E., Rorth, M., & Giwercman, A. (1999). Semen quality and reproductive hormones before and after orchiectomy in men with testicular cancer. *The Journal of Urology*, 161, 822–826.
- Petersen, P. M., Giwercman, A., Dugaard, G., Rorth, M., Petersen, J. H., Skakkebaek, N. E., Hansen, S. W., & von der Maase, H. (2002). Effect of graded testicular doses of radiotherapy in patients treated for carcinoma-in-situ in the testis. *Journal of Clinical Oncology*, 20, 1537–1543.
- Rodriguez-Wallberg, K. A., & Oktay, K. (2014). Fertility preservation during cancer treatment: Clinical guidelines. *Cancer Management and Research*, 6, 105–117.
- Rowland, G. F., Cohen, J., Steptoe, P. C., & Hewitt, J. (1985). Pregnancy following in vitro fertilization using cryopreserved semen from a man with testicular teratoma. *Urology*, 26, 33–36.
- The National Coalition for Cancer Survivorship. 1995. History of the National Coalition for Cancer Survivorship. 1995–2011 [Online]. Available: <http://www.canceradvocacy.org/about-us/our-history/> [Accessed April 18 2017].
- Thibaud, E., Rodriguez-Macias, K., Trivin, C., Esperou, H., Michon, J., & Brauner, R. (1998). Ovarian function after bone marrow transplantation during childhood. *Bone Marrow Transplantation*, 21, 287–290.
- Tomao, F., Peccatori, F., Del Pup, L., Franchi, D., Zanagnolo, V., Panici, P. B., & Colombo, N. (2016). Special issues in fertility preservation for gynecologic malignancies. *Critical Reviews in Oncology/Hematology*, 97, 206–219.
- Trost, L. W., & Brannigan, R. E. (2012). Oncofertility and the male cancer patient. *Current Treatment Options in Oncology*, 13(2), 146–160.
- Vakalopoulos, I., Dimou, P., Anagnostou, I., & Zeginiadou, T. (2015). Impact of cancer and cancer treatment on male fertility. *Hormones (Athens, Greece)*, 14, 579–589.
- Vassilakopoulou, M., Boostandoost, E., Papaxoinis, G., de La Motte Rouge, T., Khayat, D., & Psyrri, A. (2016). Anticancer treatment and fertility: Effect of therapeutic modalities on reproductive system and functions. *Critical Reviews in Oncology/Hematology*, 97, 328–334.
- Wallace, W. H., Anderson, R. A., & Irvine, D. S. (2005). Fertility preservation for young patients with cancer: Who is at risk and what can be offered? *The Lancet Oncology*, 6(4), 209–218. [https://doi.org/10.1016/s1470-2045\(05\)70092-9](https://doi.org/10.1016/s1470-2045(05)70092-9).
- Wallberg, K. A., Keros, V., & Hovatta, O. (2009). Clinical aspects of fertility preservation in female patients. *Pediatric Blood & Cancer*, 53, 254–260.
- Ward, E., DeSantis, C., Robbins, A., Kohler, B., & Jemal, A. (2014). Childhood and adolescent cancer statistics, 2014. *CA: A Cancer Journal for Clinicians*, 64, 83–103.
- Woodruff, T. K. (2015). Oncofertility: A grand collaboration between reproductive medicine and oncology. *Reproduction*, 150, S1–10.

Relevant Websites

Save My Fertility, 2015—<https://www.savemyfertility.org>
 The National Coalition for Cancer Survivorship, 1995—<https://www.canceradvocacy.org/>

Fertility Preservation in Women for Social Reasons

Karin Hammarberg, Monash University, Melbourne, Australia

© 2018 Elsevier Inc. All rights reserved.

The Social Context of Fertility Preservation in Women

Age of Childbearing Is Increasing in High-Income Countries

In most high-income countries, the age of childbearing is increasing. Many factors contribute to this including access to effective contraception and safe legalized abortion; life course changes including women's increased education and labor market participation; postponement of partnership formation; and structural factors including the increased cost of housing, economic uncertainty and the absence of supportive family policies such as paid maternity leave, flexible working arrangements, and access to high quality and affordable child care.

As women age their fertility declines. The monthly chance of pregnancy for couples where the woman is 35 years or younger is about 20% and 80%–90% achieve a pregnancy within 12 months. By age 40 the monthly chance has dropped to 5% and only 50% of couples conceive within 12 months. Age-related fertility decline is a cause of involuntary childlessness or having fewer children than planned. To overcome age-related infertility some people use assisted reproductive technology (ART) to conceive. However, as with spontaneous conception, the chance of conceiving with ART decreases with increasing female age and ART pregnancies are rare among women in their forties. To guard against age-related infertility women who plan to have children at an age when their fertility is likely to have declined can freeze their eggs (oocyte cryopreservation) when they are younger and use them in an assisted reproductive technology (ART) procedure when the time for childbearing is right for them.

Social Acceptability of Fertility Preservation for Nonmedical Reasons

Existing research and public discourse relating to childbearing focus almost exclusively on women and declining fertility rates are often portrayed as being the result of women delaying childbearing to pursue other life goals such as a career and travel. The term 'social egg freezing' is often used in the professional literature and in the media to describe women who freeze their eggs to avoid age-related infertility. This term implies that women make self-interested decisions about timing of childbearing. However, research indicates that the lack of a partner willing to commit to partnership and parenthood is the main reason for the decision to freeze eggs to postpone childbearing (Pritchard et al., 2017). Therefore, 'non-medical egg freezing' may be a more accurate and less judgemental term to describe fertility preservation to prevent age-related infertility.

Emerging research indicates that fertility preservation is becoming a socially acceptable option for women who anticipate age-related infertility and is likely to be used by an increasing number of women. Data from surveys show that most Swedish women aged 30–39 have a positive attitude towards egg freezing for non-medical reasons (Wennberg et al., 2016); about one in three women of reproductive age in Belgium would consider oocyte cryopreservation to extend their reproductive life (Stoop et al., 2011); and, among childless women aged 28–35 years who completed an online survey, the intention to freeze eggs was associated with feeling susceptible to infertility, considering egg freezing useful to achieve parenthood, and expecting to have children at a later age (ter Keurst et al., 2016). Since 2014 large companies such as Apple and Facebook have covered the cost of oocyte cryopreservation for their female employees. Some argue that rather than an altruistic gesture, this is most likely for the commercial benefit to the company of not having childbearing interrupt young talented women's careers.

Who Freezes Eggs for Nonmedical Reasons and Why?

Women who electively freeze their eggs are predominantly aged in their late thirties, well educated, socioeconomically advantaged, and single. Since there is considerable cost associated with egg freezing for non-medical reasons, being financially well-resourced is likely a pre-requisite for undergoing the procedure.

Stated reasons for freezing eggs include not having a partner with whom to have children; having a partner who is unwilling to be a father; wanting time to prepare financially, emotionally, and socially for single parenthood; investing in the future; and using egg freezing as an insurance against not finding a partner while still fertile (Pritchard et al., 2017).

Views on Benefits and Risks of Egg Freezing for Nonmedical Reasons

Opinions vary about the potential benefits and risks of fertility preservation for non-medical reasons. Some argue that it helps women by adding to their reproductive choices and allowing them to extend their reproductive lifespan. This in turn gives them more time to find a suitable partner and fulfill other life goals before having children and reduces their risk of age-related chromosomal abnormalities and miscarriage (Goold and Savulescu, 2009). Using their mathematical model, Devine et al. (2015) contend that for women who plan to delay childbearing until age 40 years, egg freezing at a younger age is more cost-effective than using ART at age 40. Stated potential risks of fertility preservation include that it might encourage women to delay childbearing beyond the natural reproductive lifespan and that this increases the risk of adverse pregnancy outcomes for both mother and baby (Radon et al., 2015). Others are concerned that women are 'misled to believe that the reproductive fountain

of youth is obtainable by freezing their eggs' (Schattman, 2016). Some make the case that, rather than encouraging egg freezing to avoid age-related infertility, family-friendly social policies that support people to have children during their most fertile years would be more helpful (Martin, 2017; Mertes, 2015).

Medical Aspects of Fertility Preservation in Women

What Egg Freezing Involves

Egg freezing has been used for several decades to preserve fertility in women who need cancer treatment which can diminish fertility. Freezing techniques have improved over time and with vitrification, the currently most commonly used technique, frozen eggs used in ART have a similar potential to result in a live birth as 'fresh' eggs (Argyle et al., 2016). With improved efficiency, egg freezing is advertised as an 'insurance' against age-related fertility decline and the number of healthy women who freeze their eggs to guard against future involuntary childlessness is increasing (Domingo et al., 2016).

The process of egg freezing involves a course of hormone injections to stimulate the ovaries to produce multiple eggs. Egg maturation is monitored with ultrasound and blood tests and when the eggs are mature they are retrieved in a transvaginal ultrasound-guided procedure under light anesthetic. The retrieved eggs are stored in liquid nitrogen until the woman returns to use them. At that time, they are thawed and inseminated with sperm from the woman's partner or a donor. If healthy embryos develop, one (sometimes two) is transferred to the uterus and any remaining embryos can be frozen for later use.

The Chance of a Live Birth After Egg Freezing

The two most important factors that determine the chance of having a baby from frozen eggs are the woman's age when the eggs are frozen and the number of eggs that are stored. The number and quality of eggs available for freezing is important because in every step there is a risk that some are lost. Of the eggs that are retrieved, some may not be suitable for freezing, some may not survive the freezing and thawing processes, and some may not fertilize or develop into normal embryos. Of the embryos that are transferred, only some will result in a pregnancy, and some pregnancies will miscarry.

The number and quality of the eggs that develop when the ovaries are stimulated decline with increasing age. A woman in her early thirties might have 15–20 eggs available for freezing after the hormone stimulation but for women in their late thirties and early forties the number is usually much lower. Also, as women age they are more likely to have eggs with chromosomal abnormalities. It is estimated freezing 10 eggs at age 35 years or younger gives women a 69% chance of having a live birth. This drops to 50% for women aged 37 and 30% for women aged 40 at the time of egg freezing (Goldman et al., 2017).

Using the Frozen Eggs

Egg freezing for non-medical reasons is a relatively new phenomenon and little is currently known about the reproductive outcomes of women who freeze their eggs. The limited existing evidence suggests that the number of women returning to use their eggs is still low (Cobo et al., 2016; Shenfield et al., 2017) and that most pregnancies that occur after egg freezing are a result of natural conception or an ART procedure using 'fresh' eggs (Hodes-Wertz et al., 2013; Hammarberg et al., 2017a). A study of 96 women who had stored their eggs found that only six had returned to use them, three of whom had had a baby as a result. Another 12 women had conceived naturally and six had conceived after an ART procedure using 'fresh' eggs. That almost half of the respondents had stored oocytes in the two years preceding the survey may, in part, explain why so few had used them. The main reasons for not using the stored eggs were not wanting to be a single parent, preferring to conceive naturally, and not wanting to use a sperm donor which indicates that women were still hoping to find a male partner willing to commit to parenthood (Hammarberg et al., 2017a).

Medical Risks of Egg Freezing

A small proportion of women have an excessive response to the fertility drugs that are used to stimulate the ovaries. In rare cases, this can cause ovarian hyperstimulation syndrome (OHSS), a potentially serious condition. Bleeding and infection are very rare complications of the egg retrieval procedure.

Egg freezing is still a relatively recent technology and the long-term health of babies born as a result is not known. However, it is reassuring that their health at birth appears to be similar to that of other children (Cobo et al., 2014).

Cost of Fertility Preservation

The cost of egg freezing varies between countries and between clinics. Around US\$10,000 is commonly quoted for hormone stimulation, egg retrieval and egg freezing. Older women often need more than one treatment cycle to have the 10–15 eggs needed to provide a reasonable chance of having a baby as a result. In addition, annual storage fees of US\$300–500 are common and when women return to use their eggs they are charged for the cost of thawing the eggs, fertilization of the eggs with partner or donor sperm, and embryo transfer.

Psychological Aspects of Fertility Preservation in Women

Information Needs of Women Who Freeze Eggs for Nonmedical Reasons

Fertility preservation is often promoted as an insurance against future childlessness. However, there is no guarantee that a live birth will result from frozen eggs. Most data relating to the chance of success using frozen eggs stem from egg donation programs. Because egg donors are on average younger than women who electively freeze eggs it is questionable whether data from egg donation programs can be extrapolated to women who freeze oocytes for non-medical reasons.

To make informed decisions about fertility preservation women need accurate and realistic information about the likelihood of a live birth; the cost of retrieval, storage, and later use of the eggs; and the known and potential risks of the procedure for the woman herself and any future children. While acknowledging that egg freezing should no longer be regarded as experimental, in 2013 the American Society for Reproductive Medicine (ASRM) cautioned that ‘data on the safety, efficacy, cost-effectiveness, and emotional risks of elective oocyte cryopreservation are insufficient to recommend elective oocyte cryopreservation’ and that ‘marketing this technology for the purpose of deferring childbearing may give women false hope and encourage women to delay childbearing’ (ASRM, 2013). But, even if comprehensive information is provided, women may still over-estimate the potential of frozen eggs to result in a birth. A qualitative study of women who had stored their eggs because they wanted to share parenthood with a future partner rather than be a single parent found that they dismissed information about limited success rates and potential risks and were overly optimistic about their own chance of success. And, although the women found the procedure very costly, they were willing to invest their money to increase their chances for shared parenthood (de Groot et al., 2016).

Psychological Needs of Women Who Freeze Eggs for Nonmedical Reasons

While data about the safety and efficacy frozen eggs is accumulating, evidence about the psychological needs of women who contemplate fertility preservation is limited. Nevertheless, based on evidence about optimal care in the context of ART, women who contemplate or undergo fertility preservation for non-medical reasons are likely to benefit from continuity of care, staff who understand the emotional impact of egg freezing, access to understandable and personally relevant treatment information, and specialized psychosocial care being offered before, during and after treatment.

In addition to the psychological difficulties inherent in undergoing ART treatment there are specific challenges that may confront women who freeze eggs for non-medical reasons that clinic staff need to be aware of and prepare women for. There are many reasons why eggs may remain unused including the woman conceiving naturally or with ART with ‘fresh’ eggs, not finding a partner and not wanting to be a single mother, or feeling too old to become a mother. If the eggs are not used women need to decide whether to donate them to another woman or discard them. Such a decision can be difficult, particularly for women who still want children but who are unable to through their circumstances. For women who decide to use the eggs and donor sperm to try to have a child, the complexities of donor conception and single parenthood need to be explored. Lastly, women who return to use their eggs may not be successful and regret spending time, effort and financial resources on freezing eggs. Also, their opportunities for experiencing motherhood may then be exhausted and they may grieve for the children they wanted but were unable to have.

Men’s Role in Reproductive Decision Making

The few studies that have gauged the reasons for freezing eggs to postpone childbearing report that most commonly it is not having a partner with whom to share parenting. Research shows that although men almost universally value parenthood, want and expect to become fathers, and aspire to have at least two children, they have inadequate knowledge about the limitations of female and male fertility and overestimate the chance of spontaneous and assisted conception (Hammarberg et al., 2017b). This may lead to unfulfilled parenthood aspirations for women and men. Childbearing and parenting are shared endeavors and better understanding of men’s contribution to childbearing decisions and the factors that influence this are crucial to informing policy and public education responses. Government policies and public education strategies aimed to support childbearing during the most fertile years are needed to reduce the personal and societal cost of infertility and ART use, including fertility preservation, and allow people to fulfill their parenthood goals.

References

- Argyle, C. E., Harper, J. C., & Davies, M. C. (2016). Oocyte cryopreservation: Where are we now? *Human Reproduction Update*, 22(4), 440–449.
- ASRM. (2013). Mature oocyte cryopreservation: A guideline. *Fertility and Sterility*, 99(1), 37–43.
- Cobo, A., García-Velasco, J. A., Coello, A., Domingo, J., Pellicer, A., & Remohí, J. (2016). Oocyte vitrification as an efficient option for elective fertility preservation. *Fertility and Sterility*, 105(3), 755–764.
- Cobo, A., Serra, V., Garrido, N., Olmo, I., Pellicer, A., & Remohí, J. (2014). Obstetric and perinatal outcome of babies born from vitrified oocytes. *Fertility and Sterility*, 102(4), 1006–1015.
- de Groot, M., Dancet, E., Repping, S., Goddijn, M., Stoop, D., van der Veen, F., & Gerrits, T. (2016). Perceptions of oocyte banking from women intending to circumvent age-related fertility decline. *Acta Obstetrica et Gynecologica Scandinavica*, 95(12), 1396–1401.

- Devine, K., Mumford, S., Goldman, K., Hodes-Wertz, B., & Druckenmiller, S. (2015). Baby budgeting: Oocyte cryopreservation in women delaying reproduction can reduce cost per live birth. *Fertility and Sterility*, 103(6), 1446–1453.
- Domingo, J., Cobo, A., & Pellicer, A. (2016). Oocyte cryopreservation. In N. Suzuki, & J. Donnez (Eds.), *Gonadal tissue cryopreservation in fertility preservation*. Japan: Springer.
- Goldman, R. H., Racowsky, C., Farland, L. V., Munne, S., Ribustello, L., & Fox, J. H. (2017). Predicting the likelihood of live birth for elective oocyte cryopreservation: A counseling tool for physicians and patients. *Human Reproduction*, 32(4), 853–859.
- Goold, I., & Savulescu, J. (2009). In favour of freezing eggs for non-medical reasons. *Bioethics*, 23(1), 47–58.
- Hammarberg, K., Collins, V., Holden, C., Young, K., & McLachlan, R. (2017b). Men's knowledge, attitudes and behaviours relating to fertility. *Human Reproduction Update*, 23(4), 458–480.
- Hammarberg, K., Kirkman, M., Pritchard, N., Hickey, M., Peate, M., McBain, J., Agresta, F., Bayly, C., & Fisher, J. (2017a). Reproductive experiences of women who cryopreserved oocytes for non-medical reasons. *Human Reproduction*, 32(3), 575–581.
- Hodes-Wertz, B., Druckenmiller, S., Smith, M., & Noyes, N. (2013). What do reproductive-age women who undergo oocyte cryopreservation think about the process as a means to preserve fertility? *Fertility and Sterility*, 100(5), 1343–1349.
- Martin, L. J. (2017). Pushing for the perfect time: Social and biological fertility. *Women's Studies International Forum*, 62, 91–98.
- Mertes, H. (2015). Does company-sponsored egg freezing promote or confine women's reproductive autonomy? *Journal of Assisted Reproduction and Genetics*, 32(8), 1205–1209.
- Pritchard, N., Kirkman, M., Hammarberg, K., McBain, J., Agresta, F., Bayly, C., Hickey, M., Peate, M., & Fisher, J. (2017). Characteristics and circumstances of women in Australia who cryopreserved their oocytes for non-medical indications. *Journal of Reproductive and Infant Psychology*, 35(2), 108–118.
- Radon, C. M., Borkar, A. A., & Homburg, R. R. (2015). Female fertility preservation: a fertile future? *The Obstetrician & Gynaecologist*, 17(2), 116–124.
- Schattman, G. L. (2016). A healthy dose of reality for the egg-freezing party. *Fertility and Sterility*, 105(2), 307.
- Shenfield, F., de Mouzon, J., Scaravelli, G., Kupka, M., Ferraretti, A. P., Prados, F. J., & Goossens, V. (2017). Oocyte and ovarian tissue cryopreservation in European countries: Statutory background, practice, storage and use. *Human Reproduction Open*, 1. <https://doi.org/10.1093/hropen/hox003>.
- Stoop, D., Nekkebroeck, J., & Devroey, P. (2011). A survey on the intentions and attitudes towards oocyte cryopreservation for non-medical reasons among women of reproductive age. *Human Reproduction*, 26(3), 655–661.
- ter Keurst, A., Boivin, J., & Gameiro, S. (2016). Women's intentions to use fertility preservation to prevent age-related fertility decline. *Reproductive Biomedicine Online*, 32(1), 121–131.
- Wennberg, A.-L., Rodriguez-Wallberg, K. A., Milsom, I., & Brännström, M. (2016). Attitudes towards new assisted reproductive technologies in Sweden: A survey in women 30–39 years of age. *Acta Obstetrica et Gynecologica Scandinavica*, 95(1), 38–44.

CLINICAL STRATEGIES

The Ovulation: Triggering Ovulation with hCG Versus GnRH-Agonist

Mustafa Bahceci and Fazilet Kubra Boynukalin, Bahceci Fulya IVF Center, Istanbul, Turkey

© 2018 Elsevier Inc. All rights reserved.

Molecular Similarity and Differences between hCG and LH

Pulsatile GnRH secretion in a critical range for frequency and amplitude is necessary for normal ovulatory function. GnRH has three positive action on gonadotropins; (i) synthesis and storage (ii) activation, and (iii) immediate secretion (Hoff et al., 1983). In normal menstrual cycle, exponential increase of estradiol secretion and small but distinct rise in progesterone levels in late follicular phase, LH levels increases 10-fold over 2–3 days while FSH levels increases fourfold. LH surge is mandatory for final maturation of the oocyte and rupture of the follicle. It is the critical component of normal menstrual cycle (Itskovitz et al., 1991).

LH and hCG have the same α -subunits, their main differences involve the sequence of the β -subunit. They share the same receptor, LH/CGR. LH/CGR, is expressed in different sites of the reproductive tract and extra-gonadal tissues and expression of this receptor is necessary for promoting oocyte maturation, fertilization, implantation, and early embryo development (Weissman et al., 1996). In consequence, hCG has been administered successfully for many decades for the endogenous LH surge to induce final oocyte maturation.

The biggest difference between LH and hCG is that hCG, has a circulating half-life of 37 h, while LH, has a circulating half-life of just 25–30 min (Cole, 2010). Following intramuscular (IM) administration of hCG has a slower plasma metabolic clearance consists of a rapid phase in the first 5–9 and a slower phase in the first 1–1.3 days after administration. Besides this difference, hCG demonstrates a stronger LH/CGR receptor binding affinity, probably due to differences in the carbohydrate moiety, and is much more potent (roughly six to eight times greater) than LH (Theofanakis et al., 2017).

Luteal Phase Physiology; Difference Between hCG Trigger and GnRH-a Trigger for Final Oocyte Maturation Trigger

In the normal menstrual cycle, the midcycle LH and FSH surge is a complex event which takes apart in late follicular phase by exponentially increasing estrogen concentrations in combination with a small, but distinct, rise in P. Exogenous FSH administration either for COH in IVF or for the treatment of anovulation, the timing of the endogenous LH surge is frequently interrupted. Therefore, exogenous hCG has been used for decades to replace endogenous LH during FSH stimulation protocols to induce ovulation, final oocyte maturation, and corpus luteum formation. In COS cycles triggered with hCG, endogenous LH secretion is negatively affected by supra-physiological steroid levels (estradiol and progesterone) and cause a disruption of the luteal phase. The physiological mid-cycle surge of LH and FSH last 48 h after its onset however the hCG-mediated LH activity extends several days into the luteal phase. The supraphysiologic hCG mediated LH activity stimulates multiple corpus luteum activity which causes high serum progesterone and estradiol. This hormonal milieu decreases the endogenous LH secretion by pituitary. Although large amount of granulosa cells being removed during oocyte pick-up procedure, hCG mediated corpus luteum activity promotes uterus for implantation. In the process of implantation, hCG levels start decreasing and endogenous progesterone productions are negatively influenced. Besides this, low endogenous LH levels leads to disruption of the luteal phase, causes requirement for exogenous progesterone supplementation is mandatory until the increase in endogenous hCG production from implanting embryo for luteal phase support (LPS) (Fauser and Devroey, 2003).

The synthesis of GnRH-a was succeeded in late 1970s and increased receptor affinity and decreased degradations was led with alterations or deletions of in amino acid at position 6 and 10. These changes resulted 100–200 times greater GnRH activity releasing LH and FSH from the pituitary (Casper, 2015). There are different GnRH-a commercially available leuprolide, buserolin, and triptoreline. There is no consensus on the type and dosage for ovulation trigger.

Gn-RHa trigger was first analyzed in 8 cases of timed intercourse or IUI cycles and ovulation was determined by follicle collapse on USG. The characteristics of luteal phase was seem to be similar to spontaneous ovulation (Lanzone et al., 1989). The use of GnRH-a trigger in IVF cycles was first reported in 1990 and midcycle LH surge was revealed. Mature oocytes were retrieved and pregnancy was resulted after luteal support with progesterone (Bentick et al., 1990). The first randomized study comparing the single bolus of 500 mg of leuprolide acetate to 5000 international units (IU) of hCG as the trigger for follicular and oocyte maturation described the difference between luteal phase. The serum LH and FSH levels were elevated for 34 h after GnRH-a administration, in contrast hCG administration resulted serum hCG levels were detectable till 6 days later without any rise in FSH levels. The results of this study reported that the release of administration of GnRH-a in an IVF cycle was a good alternative to complete the final stage of follicular maturation, resulting in the retrieval of fertilized oocytes, with normal embryo development

to hCG (Gonen et al., 1990). In 1992, a larger study in IVF cycles were randomized to receive 5000 IU of hCG, or 500 mg of leuprolide acetate, to trigger final follicular maturation. The luteal phase estradiol and progesterone levels were significantly lower and clinically short luteal phases, despite vaginal P administration was detected in GnRH-a triggered cycles (Segal and Casper, 1992).

In a randomized multicenter study, the efficacies of two different GnRH-a were compared with that of hCG for triggering final stages of oocyte maturation after COS for IVF using antagonist protocol. LH peak was resulted 4 h after both triptorelin and leuporelin administration. Within administration of GnRH-a 24 h LH and FSH levels were significantly higher in both GnRH agonist groups compared with hCG. Luteal phase progesterone and estradiol were significantly elevated in the hCG group. The mechanism under this circumstance is thought to be; GnRH-a trigger for final oocyte maturation causes a direct pituitary down-regulation and therefore, the remaining corpora lutea lack LH-stimulus and are not capable to sustain progesterone production for the luteal phase. The mean numbers of oocytes retrieved, the percentages of metaphase II oocytes and fertilization rates were similar in the triptorelin, leuporelin, and hCG group (Fauser et al., 2002). These findings support the effective induction of final oocyte maturation in both GnRH-a groups.

The spontaneous LH-surge in a natural cycle is characterized by an ascending phase of approximately 4 h, a peak plateau of 20 h, and a descending phase of 20 h whereas the GnRH-a-induced LH/FSH surge consists of two phases; a short ascending phase lasting > 4 h and a long descending phase lasting > 20 h and has a total duration between 24 and 36 h. The supraphysiologic luteal steroid levels produced by multiple corpora lutea, which directly inhibit LH secretion from the pituitary is the most important reason. In addition, GnRH-a triggering leads to a significant shorter in the luteal phase compared with hCG triggering. Most important reason for the abnormal luteal phase is early luteal LH levels remain significantly lower as compared to hCG triggering (Humaidan et al., 2005). GnRH-a trigger in oocyte donors was evaluated in a randomized trial and the mean duration of the nonsupplemented luteal phase after GnRH-a trigger in was as short as 4 days, compared with 13 days after hCG trigger, suggesting a markedly defective luteal function (Acevedo et al., 2006).

Molecular based studies demonstrated that the human ovary is a target for GnRH (Nathwani et al., 2000). Cultured granulosa cells, the GnRH agonist was revealed to mobilize Ca, activate mitogen protein kinases, increase c-fos mRNA expression, and decrease its steroidogenic capacity (Kang et al., 2001; Brus et al., 1997; Minaretzis et al., 1995; Hori et al., 1998). It was supposed that luteolysis would ultimately be due to the capacity of the GnRH agonist to initiate the intracellular cascade that drives the cell to programmed cell death or apoptosis.

GnRH-agonist for final oocyte maturation reported a very poor reproductive outcome due to the severe luteolysis and it was assumed that the extend of the induced luteolysis cannot be counterbalanced by using standard luteal phase support with only progesterone administration. However, lately several case reports demonstrate that luteolysis after GnRH-agonist trigger is not complete and varies, indicating distinct differences among patients. In a more recent study; it was reported that longer stimulation duration as well as a higher level of progesterone on the day of final oocyte maturation and more retrieved oocytes will result in higher levels of progesterone 48 h after oocyte retrieval and concluded that luteolysis after GnRH-agonist trigger is patient-specific and also luteal phase support requires individualization (Lawrenz et al., 2017).

Clinical Outcome; Safety and Efficacy of GnRH-a Triggered and hCG Triggered Cycles

During the last decade of the twentieth century, intensive pharmaceutical efforts reached the target of producing GnRH antagonists with proven clinical activity and few side effects. The worldwide common use of GnRH antagonist protocols has allowed the widespread use of GnRH-a for triggering final follicular maturation. It is accepted to be the major way to prevent ovarian hyperstimulation syndrome (OHSS).

OHSS is a potential morbidity and mortality undergoing fertility treatment, according to the most recent report by the European Society of Human Reproduction and Embryology (ESHRE) (Kupka et al., 2014). The predictive effect of GnRH-a trigger is due to the shorter half-life of the endogenously secreted LH compared to the long acting LH activity after an hCG trigger, which results in a defective corpus luteum formation (Engmann et al., 2008). Not only the shorter half-life, also follicular fluid composition VEGF, which may relate to reduction in the risk of OHSS, differed significantly between the GnRH-a and hCG trigger. Decrease in the release of VEGF is a major factor for the preventive effect of OHSS (Humaidan et al., 2011).

It has been reported that GnRH-a trigger has a higher oocyte maturation rate (Imoedemhe et al., 1991). EGF-like peptides have been shown to be potent mediators of oocyte maturation. Follicular fluid levels of amphiregulin (AR) and EGF-like peptide following GnRH-a trigger was reported to be closer to the natural cycle levels. The authors suggested that a bolus of hCG represented a supra-physiological signal for oocyte maturation compared with that of the natural cycle and GnRH-a trigger, and that this supra-physiological signal might have negative impacts on the oocytes and embryos (Humaidan et al., 2011).

The transcriptome of the somatic cells of the follicle was explored in patients who had final oocyte maturation with either hCG or GnRH-a and showed significant functional differences which may led to decreased OHSS and higher oocyte maturation rates (Borgbo et al., 2013).

Few studies have addressed failed trigger with the use of GnRH agonists. The highest reported failure rate for GnRH agonist trigger is 3.5% (Castillo et al., 2012). In a more recent study, risk factors for inadequate trigger response was analyzed. Two percent of patients triggered with GnRH agonist had an inadequate response and were retriggered with hCG. Low body mass index, low baseline LH, and higher total dosage of gonadotropins required for stimulation were associated with an increased risk of having an inadequate response to the GnRH agonist trigger (Chang et al., 2016).

The reproductive outcome of oocytes and embryos that are obtained from GnRH-a trigger in oocyte donation programs are good either fresh or frozen-thawed cycles (Acevedo et al., 2006; Bodri et al., 2009; Griesinger et al., 2007). The earlier studies suggested that high level hCG concentrations mediated an exaggerated intrafollicular shift in steroids from androgens, estradiol and progesterone and causes a detrimental effect on embryo development (Fanchin et al., 2001). Morphokinetic characteristics of embryos was analyzed in a retrospective cohort study and embryos from cycles GnRH-a triggered cleaved faster than embryos from hCG triggered cycles but the embryo quality was not significantly different (Muñoz et al., 2013).

Endometrial GnRH receptor expression was demonstrated by some studies and mid-luteal bolus of GnRH-a resulted improvement in implantation rates. It was hypothesized that GnRH receptor mediated IL-1b and VEGF production in endometrium improves implantation rates (Schachter et al., 2008). In contrast some studies reported a potential negative impact of hCG on endometrial receptivity. It was suggested precocious exposure of the endometrium to hCG, outside the timing of normal endometrial-embryonic communication, down-regulates the endometrial epithelial LHCG-R (Evans and Salamonsen, 2013). Also, after hCG trigger P levels peak significantly earlier than during natural cycle which may lead to impair endometrial receptivity.

The impaired luteal phase after GnRH-a trigger, is the major draw back in fresh cycles. The mean mid-luteal LH level of the natural cycle is nearly 4 times greater than GnRH-a trigger (6 IU/mL vs. 1.5 IU/mL). Lower LH levels are leading insufficient luteal phase and causes early luteolysis at the end implantation failure or early pregnancy loss occurs in fresh ET cycles using a standard LPS.

The first published Cochrane review comparing GnRH-a versus hCG trigger was published in 2010 and concluded that 'GnRH agonists should not be routinely used as a final oocyte maturation trigger in fresh autologous cycles because of lowered live birth rates and ongoing pregnancy rates' (Youssef et al., 2010). A more recent updated Cochrane review on GnRH-a trigger reached the same general conclusions of the previous review and stated that "GnRH agonist triggers significantly reduce the risk of ovarian hyperstimulation, but also lower the chance of pregnancy in fresh autologous IVF-ICSI treatment cycles compared with HCG. GnRH agonist use as an oocyte maturation trigger could be useful for women who choose to avoid fresh transfers (for whatever reason), women who donate oocytes to recipients or women who wish to freeze their eggs for later use in the context of fertility preservation" (Youssef et al., 2014). The last Cochrane analysis luteal phase support differs between studies included. The Cochrane review included a Forest plot in which a stratification for LPS with and without LH activity was performed. Importantly, in this analysis there was no significant difference in ongoing pregnancy rates between GnRH-a with modified LPS and hCG trigger, OR 0.89 (95% CI 0.65–1.21).

The optimal type of luteal phase support after GnRH-a trigger followed by fresh transfer is still subject to research and has not reached its final form. The physiological advantage of the GnRH-a trigger concept is clear but the defective luteal phase is a major concern. The early luteal phase support individualizations should be answered.

There are two different strategies for effective luteal phase support in GnRH-a triggered fresh cycles; (i) corpus luteum rescue (European Approach) and (ii) exogenous steroid support (American Approach).

Corpus Luteum Rescue

Today, minimum hCG dose needed to rescue the corpora lutea until the hCG produced by the implanting embryo covers the luteal LH activity deficiency without increasing the risk of OHSS does not known exactly.

In a randomized pilot study low dose hCG (1,500 IU) was administered at either 12 h or 35 h after GnRH-a trigger, followed by a standard LPS and compared with hCG trigger. Higher mid-luteal P levels and better pregnancy rates were reported in the 35 h group compared with the 12 h group. Importantly, no differences in pregnancy outcomes were seen between the GnRH-a trigger 35 h hCG group and hCG group. This results suggested that a refractory period may occur after the initial GnRH-a trigger, during which human granulosa cells do not respond to subsequent LH-like exogenous stimulation. This pilot study included 45 normal responder patients and suggested that the ideal timing of the 1500 IU hCG rescue bolus after GnRH-a trigger seemed to be at 35 h after trigger—immediately after oocyte retrieval (Humaidan et al., 2006).

In a retrospective analysis including high risk for OHSS reported that GnRH agonist trigger combined with 1500 IU hCG at the time of oocyte retrieval and subsequent estradiol and progesterone replacement in OHSS high-risk patients can facilitate fresh embryo transfer with high clinical pregnancy rates and a low risk of severe OHSS (Iodromiti et al., 2013).

A new protocol was initially explored prospectively in a small group of 12 OHSS high-risk patients, having 25 or more follicles > 11 mm. Patients were administered a bolus of 1,500 IU hCG following GnRH-a trigger, 2 h after the oocyte retrieval, resulting reassuring results as regards live birth rate (50%) and OHSS reduction (Humaidan, 2009). A large randomized controlled trial including a total of 302 patients was performed to corroborate these findings. Until now, this study is the largest comparing hCG triggering with 10,000 IU hCG with GnRH-a triggering supplemented with a bolus of 1500 IU hCG on the day of oocyte retrieval. This study reported a nonsignificant difference in live birth rates between GnRH-a triggering and hCG triggering of 24% and 31%, respectively, per patient started. No OHSS case was seen in the group of patients who had GnRH-a trigger final oocyte maturation, compared with 2% in the hCG group (Humaidan et al., 2010).

Two RCTs including a total of 390 patients compared GnRH-a trigger with hCG trigger, with the use of hCG supplementation. The primary outcome of the study was OHSS incidence in the group at risk of OHSS and secondary outcome was pregnancy rates. Thus, in patients with 14 follicles > 11 mm, who were considered to be at low OHSS risk, an additional bolus of 1500 IU hCG was administered on the day of oocyte retrieval + 5, in addition to the 35-h dose. Patients with 15–25 follicles > 11 mm received a single bolus of 1500 IU hCG at 35 h only. All patients were supplemented with standard luteal phase support. Patients with > 25 follicles were excluded from the study in an effort to set an upper cutoff level for the risk of OHSS development after fresh transfer. No OHSS was seen in the OHSS high-risk group (15–25 follicles) triggered with GnRH-a, versus 3% for those triggered with hCG. In contrast, two patients in the group considered to be at low OHSS risk (< 14 follicles) developed late-onset moderate OHSS (Humaidan et al.,

2013). Further fine-tuning was necessary to find the minimal dose of hCG administered to secure optimal pregnancy rates without increasing the OHSS incidence in the OHSS low-risk group.

rLH administration was used after GnRHa trigger combined with a standard LPS in a small proof-of-concept study. A total of 17 patients in the control group had a trigger with 6,500 IU hCG followed by a standard LPS. In the study group a total of 18 patients received a GnRHa trigger followed by a total of six doses of 300 IU rLH administered every second day during the luteal phase combined with a standard LPS. All patients underwent elective single blastocyst transfer. Similar implantation rates (25.0% vs. 26.7%, respectively) were seen in the two groups, and no OHSS case was reported in this study, consisting of only normal responder patients (Papanikolaou et al., 2011).

The exogenous steroid free luteal phase in GnRHa triggered IVF/ICSI cycles was explored in a proof of concept trial in which 93 normal responder patients were randomized to either GnRHa trigger (two study groups) or hCG trigger (control group). In both study groups, the luteal phase was supported with a daily subcutaneous bolus of 125 IU hCG until the pregnancy test was performed. No other LPS was used. In the hCG trigger control group a standard LPS, consisting of vaginally administered micronized P was given until the day of the pregnancy test. The authors reported no significant differences in neither pregnancy rates nor early pregnancy loss rates between groups. Four moderate late onset OHSS were seen in the control group (6%) and one in each of the study groups (3%) (Bar-Hava et al., 2016).

Exogenous Steroid Support

Luteal phase after GnRHa trigger with the use of luteal phase support similar to that used after hCG trigger reported poor results (Humaidan et al., 2005).

In a randomized controlled trial that all of the patients received 50 mg intramuscular (IM) progesterone (P) daily and three 0.1-mg estradiol (E2) transdermal patches every other day starting on the day after oocyte retrieval starting the evening after oocyte retrieval and continued until 10 weeks' gestation after GnRHa trigger with an excellent ongoing pregnancy rate of 53.3%. Serial serum E2 and P were measured on the day of embryo transfer, 1 week after oocyte retrieval, and weekly thereafter. The dose of transdermal E2 patches was increased, to a maximum of four 0.1-mg patches every other day, and/or addition of oral micronized E2 to maintain the serum E2 level > 200 pg/mL. The IM P dose was also increased, if necessary, to a maximum of 75 mg daily and/or addition of micronized vaginal P to maintain serum P level > 20 ng/mL (Engmann et al., 2008). Similar intensive luteal support protocol have also reported favorable results with fresh transfers (liodromiti et al., 2013).

In 2011, Devroey et al. reported a strategy to obtain an OHSS-Free Clinic, the segmentation concept. It consists of optimization of the ovarian stimulation, including GnRH agonist triggering in a GnRH antagonist cycle (segment A). Segment B then consists of optimum cryopreservation methods for oocyte or embryo vitrification. Segment C includes embryo replacement in a receptive, nonstimulated endometrium in a natural cycle or with artificial endometrial preparation (Devroey et al., 2011). The potential advantage of this method is that it provides a more physiologic environment in which ET can occur; this advantage could lead to better pregnancy rates and decreased maternal and perinatal morbidity (Barnhart, 2014).

In a retrospective cohort study, congenital anomalies, obstetrical complications, and neonatal complications in antagonist cycles where either GnRH-a or hCG was used for final oocyte maturation. There were no significant differences in the rate of congenital anomalies between GnRHa and hCG trigger (6.6% vs. 9.2%). There were also no differences in the maternal complications (27.6% vs. 20.8%) or neonatal complications (19.7% vs. 20.0%) between the GnRHa trigger and hCG trigger groups (Budinetz et al., 2014).

Conclusion

GnRH-a trigger has physiological advantages over HCG trigger and seems to be patient friendly. The major drawback of GnRH-a trigger is the most optimal luteal-phase support after GnRHa trigger is not clear and still under investigation. GnRHa trigger is likely to become the future gold standard trigger concept in IVF.

References

- Acevedo, B., Gomez-Palomares, J. L., Ricciarelli, E., & Hernandez, E. R. (2006). Triggering ovulation with gonadotropin-releasing hormone agonists does not compromise embryo implantation rates. *Fertility and Sterility*, 86, 1682–1687.
- Bar-Hava, I., Mizrach, Y., Karfunkel-Doron, D., Omer, Y., Sheena, L., Carmon, N., et al. (2016). Intranasal gonadotropin-releasing hormone agonist (GnRHa) for luteal-phase support following GnRHa triggering, a novel approach to avoid ovarian hyperstimulation syndrome in high responders. *Fertility and Sterility*, 106, 330–333.
- Barnhart, K. T. (2014). Are we ready to eliminate the transfer of fresh embryos in vitro fertilization? *Fertility and Sterility*, 102, 1–2.
- Bentick, B., Shaw, R. W., Iffland, C. L., Burford, G., & report, B. A. C. (1990). IVF pregnancy after induction of an ovulatory endogenous gonadotrophin surge using an LHRH agonist nasal spray. *Human Reproduction*, 5, 570–572.
- Bodri, D., Guillén, J. J., Galindo, A., Mataró, D., Pujol, A., & Coll, O. (2009). Triggering with human chorionic gonadotropin or a gonadotropin-releasing hormone agonist in gonadotropin-releasing hormone antagonist-treated oocyte donor cycles: Findings of a large retrospective cohort study. *Fertility and Sterility*, 91, 365–371.
- Borgbo, T., Povlsen, B. B., Andersen, C. Y., Borup, R., Humaidan, P., & Grøndahl, M. L. (2013). Comparison of gene expression profiles in granulosa and cumulus cells after ovulation induction with either human chorionic gonadotropin or a gonadotropin-releasing hormone agonist trigger. *Fertility and Sterility*, 100, 994–1001.
- Brus, L., Lambalk, C. B., de Koning, J., Helder, M. N., Janssens, R. M., & Schoemaker, J. (1997). Specific gonadotropin-releasing hormone analogue binding predominantly in human luteinized follicular aspirates and not in human pre-ovulatory follicles. *Human Reproduction*, 12, 769–773.
- Budinetz, T. H., Mann, J. S., Griffin, D. W., Benadiva, C. A., Nulsen, J. C., & Engmann, L. L. (2014). Maternal and neonatal outcomes after gonadotropin-releasing hormone agonist trigger for final oocyte maturation in patients undergoing in vitro fertilization. *Fertility and Sterility*, 102, 753–758.

- Casper, R. F. (2015). Basic understanding of gonadotropin-releasing hormone-agonist triggering. *Fertility and Sterility*, 103, 867–869.
- Castillo, J. C., Garcia-Velasco, J., & Humaidan, P. (2012). Empty follicle syndrome after GnRHa triggering versus hCG triggering in COS. *Journal of Assisted Reproduction and Genetics*, 29, 249–253.
- Chang, F. E., Beall, S. A., Cox, J. M., Richter, K. S., DeCherney, A. H., & Levy, M. J. (2016). Assessing the adequacy of gonadotropin-releasing hormone agonist leuprolide to trigger oocyte maturation and management of inadequate response. *Fertility and Sterility*, 106, 1093–1100.
- Cole, L. A. (2010). Biological functions of hCG and hCG-related molecules. *Reproductive Biology and Endocrinology*, 24, 102.
- Devroey, P., Polyzos, N. P., & Blockeel, C. (2011). An OHSS-free clinic by segmentation of IVF treatment. *Human Reproduction*, 26, 2593–2597.
- Engmann, L., DiLuigi, A., Schmidt, D., Nulsen, J., Maier, D., & Benadiva, C. (2008). The use of gonadotropin-releasing hormone (GnRH) agonist to induce oocyte maturation after cotreatment with GnRH antagonist in high-risk patients undergoing in vitro fertilization prevents the risk of ovarian hyperstimulation syndrome: A prospective randomized controlled study. *Fertility and Sterility*, 89, 84–91.
- Evans, J., & Salomonsen, L. A. (2013). Too much of a good thing? Experimental evidence suggests prolonged exposure to hCG is detrimental to endometrial receptivity. *Human Reproduction*, 28, 1610–1619.
- Fanchin, R., Peltier, E., Frydman, R., & de Ziegler, D. (2001). Human chorionic gonadotropin: Does it affect human endometrial morphology in vivo? *Seminars in Reproductive Medicine*, 19, 31–35.
- Fausser, B. C., & Devroey, P. (2003). Reproductive biology and IVF: Ovarian stimulation and luteal phase consequences. *Trends in Endocrinology and Metabolism*, 4, 236–242.
- Fausser, B. C., de Jong, D., Olivennes, F., Wramsby, H., Tay, C., Itskovitz-Eldor, J., et al. (2002). Endocrine profiles after triggering of final oocyte maturation with GnRH agonist after cotreatment with the GnRH antagonist ganirelix during ovarian hyperstimulation for in vitro fertilization. *Journal of Clinical Endocrinology and Metabolism*, 87, 709–715.
- Gonen, Y., Balakier, H., Powell, W., & Casper, R. F. (1990). Use of GnRH agonist to trigger follicular maturation for in vitro fertilization. *Journal of Clinical Endocrinology and Metabolism*, 71, 918–922.
- Griesinger, G., Kolibianakis, E. M., Papanikolaou, E. G., Diedrich, K., Van Steirteghem, A., Devroey, P., et al. (2007). Triggering of final oocyte maturation with gonadotropin-releasing hormone agonist or human chorionic gonadotropin live birth after frozen-thawed embryo replacement cycles. *Fertility and Sterility*, 88, 616–621.
- Hoff, J. D., Quigley, M. E., & Yen, S. S. C. (1983). Hormonal dynamics at midcycle: A reevaluation. *Journal of Clinical Endocrinology and Metabolism*, 57, 792–796.
- Hori, H., Uemura, T., & Minaguchi, H. (1998). Effects of GnRH on protein kinase C activity, Ca²⁺ mobilization and steroidogenesis of human granulosa cells. *Endocrine Journal*, 45, 175–182.
- Humaidan, P. (2009). Luteal phase rescue in high-risk OHSS patients by GnRHa triggering in combination with low-dose HCG: A pilot study. *Reproductive Biomedicine Online*, 18, 630–634.
- Humaidan, P., Ejdrup Bredkjaer, H., Bungum, L., Bungum, M., Grøndahl, M. L., Westergaard, L. G., et al. (2005). GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomised study. *Human Reproduction*, 20, 1213–1220.
- Humaidan, P., Bungum, L., Bungum, M., & Yding Andersen, C. (2006). Rescue of corpus luteum function with peri-ovulatory hCG supplementation in IVF/ICSI GnRH antagonist cycles in which ovulation was triggered with a GnRH agonist: A pilot study. *Reproductive Biomedicine Online*, 13, 173–178.
- Humaidan, P., Bredkjaer, H. E., Westergaard, L. G., & Andersen, C. Y. (2010). 1,500 IU human chorionic gonadotropin administered at oocyte retrieval rescues the luteal phase when gonadotropin-releasing hormone agonist is used for ovulation induction: A prospective, randomized, controlled study. *Fertility and Sterility*, 93, 847–854.
- Humaidan, P., Westergaard, L. G., Mikkelsen, A. L., Fukuda, M., & Andersen, C. Y. (2011). Levels of the epidermal growth factor-like peptide amphiregulin in follicular fluid reflect the mode of triggering ovulation: A comparison between gonadotropin-releasing hormone agonist and urinary human chorionic gonadotropin. *Fertility and Sterility*, 95, 2034–2038.
- Humaidan, P., Polyzos, N. P., Alsbjerg, B., Erb, K., Mikkelsen, A. L., Elbaek, H. O., et al. (2013). GnRHa trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Human Reproduction*, 28, 2511–2521.
- Imoedemhe, D. A., Sigue, A. B., Pacpaco, E. L., & Olazo, A. B. (1991). Stimulation of endogenous surge of luteinizing hormone with gonadotropin-releasing hormone analog after ovarian stimulation for in vitro fertilization. *Fertility and Sterility*, 55, 328–332.
- Itskovitz, J., Boides, R., Levron, J., Erlik, Y., Kahana, L., & Brandes, J. M. (1991). Induction of preovulatory luteinizing hormone surge and prevention of ovarian hyperstimulation syndrome by gonadotropin-releasing hormone agonist. *Fertility and Sterility*, 56, 213–220.
- Kang, S. K., Tai, C. J., Nathwai, P. S., Choi, K. C., & Leung, P. C. (2001). Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone in human granulosa-luteal cells. *Endocrinology*, 142, 671–679.
- Kupka, M. S., Ferraretti, A. P., de Mouzon, J., Erb, K., D'Hooghe, T., Castilla, J. A., et al. (2014). Assisted reproductive technology in Europe, 2010: Results generated from European registers by ESHREdagger. *Human Reproduction Oxford England*, 29, 2099–2113.
- Lanzzone, A., Fulghesu, A. M., Apa, R., Caruso, A., & Mancuso, S. (1989). LH surge induction by GnRH agonist at the time of ovulation. *Gynecological Endocrinology*, 3, 213–220.
- Lawrenz, B., Garrido, N., Samir, S., Ruiz, F., Melado, L., & Fatemi, H. M. (2017). Individual luteolysis pattern after GnRH-agonist trigger for final oocyte maturation. *PLoS ONE*, 12, e0176600. <https://doi.org/10.1371/journal.pone.0176600>.
- Iliodromiti, S., Blockeel, C., Tremellen, K. P., Fleming, R., Tournaye, H., Humaidan, P., et al. (2013). Consistent high clinical pregnancy rates and low ovarian hyperstimulation syndrome rates in high-risk patients after GnRH agonist triggering and modified luteal support: A retrospective multicentre study. *Human Reproduction (Oxford, England)*, 28, 2529–2536.
- Minaretzis, D., Jakubowski, M., Mortola, J. F., & Pavlou, S. N. (1995). Gonadotropin releasing hormone receptor gene expression in human ovary and granulosa-lutein cells. *Journal of Clinical Endocrinology and Metabolism*, 80, 430–434.
- Muñoz, M., Cruz, M., Humaidan, P., Garrido, N., Pérez-Cano, I., & Meseguer, M. (2013). The type of GnRH analogue used during controlled ovarian stimulation influences early embryodevelopmental kinetics: A time-lapse study. *Eur J Obstet Gynecol Reprod Biol.*, 168, 167–172.
- Nathwani, P. S., Kang, S. K., Cheng, K. W., Choi, K. C., & Leung, P. C. (2000). Regulation of gonadotropin-releasing hormone and its receptor gene expression by 17β-estradiol in cultured human granulosa-luteal cells. *Endocrinology*, 141, 1754–1763.
- Papanikolaou, E. G., Verpoest, W., Fatemi, H., Tarlatzis, B., Devroey, P., & Tournaye, H. (2011). A novel method of luteal supplementation with recombinant luteinizing hormone when a gonadotropin-releasing hormone agonist is used instead of human chorionic gonadotropin for ovulation triggering: A randomized prospective proof of concept study. *Fertility and Sterility*, 95, 1174–1177.
- Schachter, M., Friedler, S., Ron-El, R., Zimmerman, A. L., Strassburger, D., Bern, O., et al. (2008). Can pregnancy rate be improved in gonadotropin-releasing hormone (GnRH) antagonist cycles by administering GnRH agonist before oocyte retrieval? A prospective, randomized study. *Fertility and Sterility*, 90, 1087–1093.
- Segal, S., & Casper, R. F. (1992). Gonadotropin-releasing hormone agonist versus human chorionic gonadotropin for triggering follicular maturation in in vitro fertilization. *Fertility and Sterility*, 57, 1254–1258.
- Theofanakis, C., Drakakis, P., Besharat, A., & Loutradis, D. (2017). Human chorionic gonadotropin: The pregnancy hormone and more. *International Journal of Molecular Sciences*, 14, 18–25.
- Weissman, A., Lurie, S., Zalel, Y., Goldchmit, R., & Shoham, Z. (1996). Human chorionic gonadotropin: Pharmacokinetics of subcutaneous administration. *Gynecological Endocrinology*, 10, 273–276.
- Youssef, M. A., Van der Veen, F., Al-Inany, H. G., Griesinger, G., Mochtar, M. H., & van Wely, M. (2010). Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist assisted reproductive technology cycles. *Cochrane Database of Systematic Reviews*, 11, CD008046.
- Youssef, M. A., Van der Veen, F., Al-Inany, H. G., Griesinger, G., Mochtar, M. H., & van Wely, M. (2014). Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist assisted reproductive technology cycles. *Cochrane Database of Systematic Reviews*, 10, CD008046.

Ovulation: Dual Trigger

Sergio Cabanillas, IVI Valencia, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Assisted reproduction technology (ART) Is the technology used to achieve pregnancy in procedures such as fertility medication, in vitro fertilization and surrogacy. It is reproductive technology used primarily for infertility treatments, and is also known as fertility treatment. It mainly belongs to the field of reproductive endocrinology and infertility, and may also include intracytoplasmic sperm injection (ICSI) and cryopreservation. Some forms of ART are also used with regard to fertile couples for genetic reasons (preimplantation genetic diagnosis).

Follicle-stimulating hormone (FSH) Is a gonadotropin, a glycoprotein polypeptide hormone. FSH is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland, and regulates the development, growth, pubertal maturation, and reproductive processes of the body. FSH and luteinizing hormone (LH) work together in the reproductive system.

Luteinizing hormone (LH) Also known as lutropin and sometimes lutrophin is a hormone produced by gonadotropic cells in the anterior pituitary gland. In females, an acute rise of LH ("LH surge") triggers ovulation and development of the corpus luteum.

Introduction

One of the objectives of an in vitro fertilization cycle (IVF) is to retrieve an adequate number of mature oocytes to fertilize, thereby to optimize the chances of gestation. To trigger the release of mature oocytes, at the end of the controlled ovarian hyperstimulation (COH) protocol, human chorionic gonadotropin (hCG) is often administered as a surrogate LH surge. hCG exhibits structural and biological similarities to LH, and they bind and activate the same receptor, the LH/hCG receptor (Kessler et al., 1979). One major difference between them is that the half-life of natural LH is about 60 min, but that of hCG exceeds 24 h (Yen et al., 1968; Damewood et al., 1989).

The use of hCG has some adverse consequences. Indeed, hCG appears to negatively affect both endometrial receptivity (Simon et al., 1995; Forman et al., 1998; Simon et al., 1998) and embryo quality (Valbuena et al., 2001; Tavaniotou et al., 2002). Further, the sustained luteotrophic effect of hCG is associated with increased chances of ovarian hyperstimulation syndrome (OHSS), which is an iatrogenic complication of assisted reproductive technology (ART) (Practice Committee of the American Society for Reproductive Medicine, 2003). These negative outcomes have prompted explorations of alternative approaches to induce an LH surge.

One such alternative is the use of gonadotropin-releasing hormone (aGnRH) agonists. aGnRH administration triggers the release of endogenous luteinizing hormone (LH) (Gonen et al., 1990; Olivennes et al., 1996; Olivennes, 2001; Tay, 2002). Its use is applicable only in IVF cycles using COH treatment regimens in which the cycle has been down-regulated by a GnRH antagonist. Subsequent oocyte maturation triggering with GnRH agonists reduces the risk of OHSS due to quick and irreversible luteolysis (Kol, 2004). However, evidence suggests that using aGnRH as a final oocyte maturation trigger in fresh autologous cycles is associated with a significant reduction in the clinical pregnancy rate (Griesinger et al., 2006). This prompts questions about the value of substituting aGnRH for hCG.

To overcome this effect on pregnancy rates, a more recent development is the use of "dual trigger." The dual trigger approach combines a bolus of aGnRH simultaneous with hCG administration. This strategy captures the benefits of GnRH agonists and hCG in the induction of ovulation, but strives to improving pregnancy rates in patients without adding a risk of OHSS.

Luteinizing Hormone (LH) and Follicle-Stimulating Hormone (FSH) in Natural Cycles

The ovarian cycle requires a coordinated interaction along the hypothalamic-pituitary-ovarian axis. The pulsatile secretion of GnRH by the hypothalamus activates the synthesis of gonadotrophins (FSH and LH) by the anterior hypophysis. FSH and LH act directly on the ovary, promoting follicular maturation and the production and subsequent secretion of steroid hormones that act at the endometrial level (Sociedad Española de Fertilidad, 2017).

LH secretion induces the ovulatory cascade (ovulation occurs between 34 and 36 h after the start of the LH peak). It also re-starts the arrested meiotic development of the oocytes; promotes luteinization of granulosa cells in the dominant follicle, resulting in the production of progesterone; and provokes cumulus expansion and synthesis of prostaglandins essential for follicle rupture. (Fritz and Speroff, 2011) On the other hand, FSH induces nuclear maturation (resumption of meiosis) (Zelinski-Wooten et al., 1995), activity of LH receptors in granulosa cells (Zeleznik et al., 1974; Richards et al., 1976), and cumulus cell expansion

(Strickland and Beers, 1976; Eppig, 1979). FSH also has an important role in maintaining communication between cumulus cells and oocytes (Atef et al., 2005).

hCG and Ovarian Hyperstimulation Syndrome

OHSS is a medical condition affecting the ovaries of some women following an ART cycle. Most cases are mild, but rarely the condition is severe and can lead to serious illness or death (Shmorgun et al., 2011). The fundamental etiological factor in OHSS onset is hCG exposure, which increases vascular permeability by stimulating the production of vascular endothelial growth factor (VEGF) (Gómez et al., 2002). hCG exposure can result from endogenous (trophectoderm implantation) production or exogenous administration (pharmacological induction of ovulation).

We can distinguish two types of OHSS: early OHSS occurs 2–5 days after administration (exogenous) of hCG for the induction of ovulation; late OHSS is produced by embryonic implantation and secondary to the secretion (endogenous) of hCG by the trophoblast. Late OHSS is more severe and can last up to eight weeks. Importantly, this iatrogenic complication can be avoided by triggering ovulation with aGnRH instead of hCG in patients at high risk for OHSS.

Ovulation Induction With aGnRH

aGnRH is a viable alternative to hCG for triggering endogenous LH release (Gonen et al., 1990; Olivennes et al., 1996; Olivennes, 2001; Tay, 2002). However, it must be used only in those IVF cycles with COH treatment regimens including down-regulation by a GnRH antagonist. A single injection of aGnRH results in an acute release of LH and FSH, an effect called “flare up”. Serum LH and FSH levels rise after 4 and 12 h, respectively, and are elevated for 24–36 h. The amplitudes of the surges are similar to those observed in a natural cycle. However, in contrast to the natural cycle, the LH surge consists of two phases: a short ascending limb (>4 h) and a long descending limb (>20 h) (Youssef et al., 2014).

Triggering oocyte maturation with GnRH agonists reduces the risk of OHSS due to fast and irreversible luteolysis (Kol, 2004). This approach may result in better oocyte and embryo quality due to a more physiological LH and FSH surge (Humaidan et al., 2005). Importantly, though, evidence suggests that using aGnRH to provoke final oocyte maturation in fresh autologous cycles may lead to a significant reduction in the clinical pregnancy rate (Griesinger et al., 2006), a lower live birth rate, a lower ongoing pregnancy rate (pregnancy beyond 12 weeks), and a higher rate of early miscarriage (<12 weeks) (Griesinger et al., 2006; Youssef et al., 2014). These outcomes suggest that alternative approaches are needed to overcome the problems with hCG administration (OHSS) while maintaining the clinical outcomes of ART (pregnancy and birth rates).

Dual trigger

The dual trigger approach combines a bolus of aGnRH is delivered at the time of hCG administration. This method combines the advantages of both hCG and aGnRH induction regimens:

- (1) It decreases the risk of OHSS by decreasing the dose of hCG;
- (2) It is a more physiological cycle since there is the addition of an FSH peak induced by GnRH agonist, generating a larger number of mature oocytes (Griffin et al., 2014); hCG alone induces only an LH peak (Decleer et al., 2014);
- (3) It improves endometrial receptivity for releasing endometrial GnRH receptors (Schachter et al., 2008).
- (4) It extends the ovulation time after use of the induction caused by hCG, also improving oocyte maturation (Zilberberg et al., 2015); and
- (5) There is better luteal phase recruitment when there is a proven combined use of hCG with GnRH agonist (Shapiro et al., 2011).

This strategy has been studied in different patient groups with GnRH antagonist IVF protocols. In high responders, Shapiro et al. (2011) and Griffin et al. (2014) showed an improvement in pregnancy and implantation rate with respect to the single trigger with aGnRH; however, neither of those two studies resulted in complete elimination of the risk of OHSS. Among normo responders, Lin et al. (2013) and Kim et al. (2014) showed an improvement in pregnancy and implantation rates following dual trigger with respect to the single trigger with hCG; on the other hand, Decleer et al. (2014) reported no significant differences between dual trigger and single trigger in terms of pregnancy and implantation rates. Finally, in patients with previous failed cycles, Schachter et al. (2008) showed an increase in pregnancy and implantation rates following dual trigger. Griffin et al. (2014) found that, among patients with large numbers of immature oocytes, there was an increase in the % of oocytes and mature oocytes retrieved following dual trigger.

Conclusions

Dual trigger for final oocyte maturation is a good alternative to hCG alone after ovarian stimulation with recombinant FSH and LH and suppression of premature LH surge with GnRH antagonist. This approach may have particular benefit for poor

responder patients or patients with immature oocytes. However, the prognosis of these patients remains unfavorable, possibly due to a basic ovarian problem difficult to resolve. The use of dual trigger in high and normal responder patients, since the majority of studies show a benefit in terms of improving pregnancy and implantation rates, does not completely eliminate the risk of OHSS. Thus, special care must be taken with the indication of dual trigger among these patients. More prospective randomized studies will help better define the use of this approach. In particular, studies combining these IVF regimens with preimplantation genetic diagnosis of the embryos will help identify the impact of dual trigger on embryo quality.

References

- Atef, P. F., Christian, V., & Marc-Andre, S. (2005). The potential role of gap junction communication between cumulus cells and bovine oocytes during in vitro maturation. *Molecular Reproduction and Development*, 71(3), 358–367.
- Damewood, M. D., Shen, W., Zacur, H. A., Schlaff, W. D., Rock, J. A., & Wallach, E. E. (1989). Disappearance of exogenously administered human chorionic gonadotropin. *Fertility and Sterility*, 52(3), 398–400.
- Decler, W., Osmanagaoglu, K., Seynhave, B., Kolibianakis, S., Tarlatzis, B., & Devroey, P. (2014). Comparison of hCG triggering versus hCG in combination with a GnRH agonist: A prospective randomized controlled trial. *Facts Views Vis Obgyn.*, 6, 203–209.
- Eppig, J. J. (1979). FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature*, 281, 483–484.
- Forman, R., Fries, N., Tastart, J., Belaisch, J., Hazout, A., & Frydman, R. (1998). Evidence of an adverse effect of elevated serum estradiol concentrations on embryo implantation. *Fertility and Sterility*, 49, 118–122.
- Fritz, M. A., & Speroff, L. (2011). Regulation of the menstrual cycle. In *Clinical gynecologic endocrinology and infertility* (8th ed.). Philadelphia: Lippincott Williams & Wilkins.
- Gómez, R., Simón, C., & Remohí, J. (2002). Vascular endothelial growth factor receptor-2 activation induces vascular permeability in hyperstimulated rats, and this effect is prevented by receptor blockade. *Endocrinology*, 143(11), 4339–4348.
- Gonen, Y., Balakier, H., Powell, W., & Casper, R. F. (1990). Use of GnRH agonist to trigger follicular maturation for in vitro fertilization. *The Journal of Clinical Endocrinology and Metabolism*, 71, 918–923.
- Griesinger, G., Diedrich, K., Devroey, P., et al. (2006). GnRH agonist for triggering final oocyte maturation in the GnRH antagonist ovarian hyperstimulation protocol: A systematic review and meta-analysis. *Human Reproduction Update*, 12, 159–168.
- Griffin, D., Feinn, R., Engmann, L., Nulsen, J., Budinetz, T., & Benadiva, C. (2014). Dual trigger with gonadotropin-releasing hormone agonist and standard dose human chorionic gonadotropin to improve oocyte maturity rates. *Fertility and Sterility*, 102, 405–409.
- Humaidan, P., Bredkjær, H. E., Bungum, L., Bungum, M., Grøndahl, M. L., Westergaard, L., & Andersen, C. Y. (2005). GnRH agonist (buserelin) or hCG for ovulation induction in GnRH antagonist IVF/ICSI cycles: A prospective randomized study. *Human Reproduction*, 20(5), 1213–1220.
- Kessler, M. J., Reddy, M. S., Shah, R. H., & Bahl, O. P. (1979). Structures of N-glycosidic carbohydrate units of human chorionic gonadotropin. *The Journal of Biological Chemistry*, 254(16), 7901–7908.
- Kim, C. H., Ahn, J. W., You, R. M., Kim, S. H., Chae, H. D., & Kang, B. M. (2014). Combined administration of gonadotropin-releasing hormone agonist with human chorionic gonadotropin for final oocyte maturation in GnRH antagonist cycles for in vitro fertilization. *The Journal of Reproductive Medicine*, 59(1–2), 63–68.
- Kol, S. (2004). Luteolysis induced by a gonadotropin-releasing hormone agonists the key to prevention of ovarian hyperstimulation syndrome. *Fertility and Sterility*, 81(1), 1–5.
- Lin, M. H., FS, W., Lee, R. K., Li, S. H., Lin, S. Y., & Hwu, Y. M. (2013). Dual trigger with combination of gonadotropin-releasing hormone agonist and human chorionic gonadotropin significantly improves the live-birth rate for normal responders in GnRH-antagonist cycles. *Fertility and Sterility*, 100(5), 1296–1302.
- Olivennes, F. (2001). Induction of final oocyte maturation by a single dose of GnRH agonist after ganirelix treatment. *Gynecological Endocrinology*, 15(Suppl 2), 7.
- Olivennes, F., Fanchin, R., Bouchard, P., Taieb, J., & Frydman, R. (1996). Triggering of ovulation by a gonadotropin-releasing hormone (GnRH) agonist in patients pretreated with GnRH antagonist. *Fertility and Sterility*, 66, 151–153.
- Practice Committee of the American Society for Reproductive Medicine. (2003). Ovarian hyperstimulation syndrome. *Fertility and Sterility*, 80, 1309–1314.
- Richards, J. S., Ireland, J. J., Rao, M. C., Bernath, G. A., Midgley, A. R., & Reichert, L. E. (1976). Ovarian follicular development in the rat: Hormone receptor regulation by estradiol, follicle stimulating hormone and luteinizing hormone. *Endocrinology*, 99(6), 1562–1570.
- Schachter, M., Friedler, S., Ron-El, R., Zimmerman, A. L., Strassburger, D., Bern, O., & Razi, A. (2008). Can pregnancy rate be improved in gonadotropin-releasing hormone (GnRH) antagonist cycles by administering GnRH agonist before oocyte retrieval? A prospective, randomized study. *Fertility and Sterility*, 90, 1087–1093.
- Shapiro, B. S., Daneshmand, S. T., Garner, F. C., Aguirre, M., & Hudson, C. (2011). Comparison of "triggers" using leuprolide acetate alone or in combination with low-dose human chorionic gonadotropin. *Fertility and Sterility*, 95, 2715–2717.
- Shmorgun, D., Claman, P., Gysler, M., Hemmings, R., Cheung, A. P., Goodrow, G. J., Hughes, E. G., Min, J. K., Roberts, J., & Senikas, V. (2011). The diagnosis and management of ovarian hyperstimulation syndrome. *Journal of Obstetrics and Gynaecology Canada*, 31, 1156–1162.
- Simon, C., Cano, F., Valbuena, D., Remohí, J., & Pellicer, A. (1995). Clinical evidence for a detrimental effect on uterine receptivity of high serum estradiol concentrations in high and normal responders. *Human Reproduction*, 10, 2432–2437.
- Simon, C., Garcia Velasco, J. J., Valbuena, D., Peinado, J. A., Moreno, C., Rehmoji, J., et al. (1998). Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a folliclestimulating hormone step-down regimen. *Fertility and Sterility*, 70, 234–239.
- Sociedad Española de Fertilidad. (2017). *Manual práctico de estimulación ovárica. Capítulo 4: Maduración final ovocitaria: modalidades de tratamiento*. Concepto de doble descarga.
- Strickland, S., & Beers, W. H. (1976). Studies on the role of plasminogen activator in ovulation. In vitro response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. *The Journal of Biological Chemistry*, 251, 5694–5702.
- Tavaniotou, A., Albano, C., Smits, J., & Devroey, P. (2002). Impact of ovarian stimulation on corpus luteum function and embryonic implantation. *Journal of Reproductive Immunology*, 55, 123–130.
- Tay, C. C. (2002). Use of gonadotropin-releasing hormone agonist to trigger ovulation. *Human Fertility*, 5, G35–7.
- Valbuena, D., Martin, J., de Palbo, J. L., Remohí, J., Pellicer, A., & Simon, C. (2001). Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertility and Sterility*, 76, 962–968.
- Yen, S. S., Llerena, O., Little, B., & Pearson, O. H. (1968). Disappearance rates of endogenous luteinizing hormone and chorionic gonadotropin in man. *The Journal of Clinical Endocrinology and Metabolism*, 28(12), 1763–1767.
- Youssef, M. A., Van der Veen, F., Al-Inany, H. G., Mochtar, M. H., Griesinger, G., Nagi Mohesen, M., Aboulfotouh, I., & van Wely, M. (2014). Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database of Systematic Reviews*.
- Zeleznik, A. J., Midgley, A. R., & Reichert, L. E. (1974). Granulosa cell maturation in the rat: Increased binding of human chorionic gonadotropin following treatment with follicle-stimulating hormone in vivo. *Endocrinology*, 95(3), 818–825.

- Zelinski-Wooten, M. B., Hutchison, J. S., Hess, D. L., Wolf, D. P., & Stouffer, R. L. (1995). Follicle stimulating hormone alone supports follicle growth and oocyte development in gonadotrophin releasing hormone antagonist-treated monkeys. *Human Reproduction*, *10*(7), 1658–1666.
- Zilberberg, E., Haas, J., Dar, S., Kedem, A., Machtinger, R., & Orvieto, R. (2015). Co-administration of GnRH-agonist and hCG, for final oocyte maturation (double trigger), in patients with low proportion of mature oocytes. *Gynecological Endocrinology*, *31*, 145–147.

The Endometrium: Scratching

Claire Bourgain, Catholic University Leuven, Leuven, Belgium; and Imelda Hospital, Bonheiden, Belgium

© 2018 Elsevier Inc. All rights reserved.

Glossary

Asherman syndrome (Syn amenorrhea traumatica) condition named after Joseph G Asherman, first described in 1948, characterized by adhesion and fibrosis of the endometrium, following trauma. It results into absence of menstruation and infertility. It is frequently associated with curettage of the endometrium.

Angiogenesis Formation and growth of new blood vessels. This process is vital in the growth and maintenance of an organism, as well as in wound repair.

Biopsy Medical test involving sampling of cells or tissue for examination to determine the presence or extend of a disease.

Cochrane review The Cochrane Database of Systematic Reviews (CDSR) is the leading resource for systematic reviews in health care.

Cytokines A broad and loose category of small proteins (~5–20 kDa) that are important in cell signaling. Their release has an effect on the behavior of cells around them. They are different from hormones.

Decidua The part of the endometrium that in higher placental mammals undergoes special modifications in preparation for and during pregnancy and is cast off at parturition.

Endometrial stroma Part of the endometrial tissue that has a supportive and connective function. It contains mainly blood vessels, specialized supportive cells and immune cells.

Endometrium The endometrium is the inner lining of the uterus. It is a mucosal lining that changes in thickness and composition during the menstrual cycle. It is composed of two layers: the basal layer which is the deepest layer and will not change much during the cycle and the functional layer that will undergo changes in order to allow the implantation of an embryo. The functional layer is responsive to hormones. In the first part of the menstrual cycle, estrogen causes growth of the endometrium. In the second part of the cycle, progesterone induces differentiation to enable the embryo implantation.

Epithelium One of the basic types of animal and human cells, lining the surface of cavities and organs.

Hysteroscopy Procedure inspecting the uterine cavity by endoscopy through the uterine cervix. The procedure can be used for diagnostic and therapeutic intentions.

Implantation The process of attachment and invasion of an embryo in placental animals.

In-vitro fertilization (IVF) Assisted reproductive technique where one or more human eggs (oocytes) are combined with sperm outside the body to allow fertilization. The obtained embryo is transferred to the uterus to obtain a pregnancy.

Oocyte donation Procedure by which one women donates her eggs to another women in order to allow her to conceive.

Peritoneum The [serous membrane](#) that forms the lining of the [abdominal cavity](#).

Pipelle biopsy The Pipelle de Cornier is a flexible polypropylene device enabling a blind sample of the endometrium. It is largely used in the diagnosis of several endometrial diseases.

Recurrent implantation failure (RIF) The failure to achieve a pregnancy after 3 completed fresh embryo transfer cycles with good morphological embryo quality into a normal uterus.

Definition

Endometrial scratching is defined as ‘intentional damage to the endometrium, performed with the objective to improve reproductive outcome of women or couples desiring pregnancy’.

Introduction

Having a child is an important issue for many couples. Up to 10% of couples will not achieve pregnancy spontaneously. Many of them will seek help from fertility treatments in order to conceive. Success rate of infertility treatments is limited, as, at best, in-vitro fertilization (IVF) will lead to a live birth rate of only 30% per cycle ([Kupka et al., 2014](#)).

Each cycle, in the proliferative phase, the endometrium is build up under the influence of the ovarian hormone estrogen, until ovulation occurs. After ovulation, the ovarian corpus luteum will produce progesterone. This hormone initiates the secretory phase of the endometrium. In the presence of an embryo, implantation will occur during a short time span. This time span, also called implantation window, is the result of a precisely orchestrated modulation lasts for approximately 4 days. During this period, the endometrium is called ‘receptive’ to allow an embryo to implant.

Failure of IVF treatments is due to several factors, from which a lack of endometrial receptivity is thought to account for up to two-thirds of unsuccessful pregnancy outcomes. Further gain in pregnancy rates must, therefore be targeted from an improved endometrial environment.

One technique to enhance implantation is to perform an intentional injury to the endometrium prior to IVF treatment, also referred to as 'endometrial scratching',

According to a recent survey, up to 83% of clinicians already recommend some form of scratching procedure as part of routine clinical treatment for their patients (Lensen et al., 2016).

Endometrial Scratching: The Theory behind Biological Mechanism of Action

Different theories by which endometrial injury may interact with implantation have been proposed. Evidence is mainly derived from experiments in animal models and from laboratory in-vitro tissue culture of human endometrial tissue.

Experiments in Animal Models

The idea that local injury to the endometrium may be beneficial for pregnancy is an old concept. In guinea pigs, mice and rats, different types of injury have been studied. Already in 1907 (Loeb, 1907) reported that scratching the uterus of the guinea pig provoked a reaction of the endometrium that resulted in an improved receptivity. The same observations were made in mice and rats, if scratching was replaced by the injection of oil into the uterine horns. The presence of a thread in the uterine lumen, inserted for one day, provoked the same effect.

These early studies in rodents and guinea pigs all demonstrated a similar effect of the endometrium resulting from local injury. The stromal cells of the endometrium underwent a rapid decidual reaction, which was responsible for an increased implantation after embryo replacement.

More recently, effect of injury on another type of endometrial cells was observed. Experimental endometrial injury caused by injection of lipopolysaccharide into the peritoneum (Hyodo et al., 2011) resulted in a rapid increase in side population (SP) cells, which are equivalent to endometrial adult stem/progenitor cells. Stem cells are responsible the unique regenerative capacity of endometrial tissue. The effects of the injury were hormone-dependent, as the number of SP cells increased in the presence of estrogen and was blocked by progesterone.

In other experiments, it was demonstrated that injury has a long-term effect on the endometrium, persisting until the following menstrual cycle. Standardized local injury in mice uteri improved embryo implantation. The mice uteri were examined in the cycle that followed the injury. They showed a better morphology and also increased expression of specific implantation-related cytokines. From these observations in a mouse model, it was concluded that mechanical injury in a given cycle results into better endometrial receptivity and improves embryo implantation in following cycles (Zhang et al., 2015).

Experiments in Endometrial Tissue Cultures

In an attempt to understand the mechanism of endometrial injury, human endometrial tissue cultures can be prepared and observed in a laboratory. These so-called in-vitro cultures can be conditioned in a way that mimics an injury-like environment. Tumor necrosis factor (TNF-alpha) is a cytokine capable to initiate an inflammatory stimulus, similar to injury. When applied in in-vitro cultured human endometrial cells, recovered on a non-receptive cycle day (day12) and a receptive cycle day (day 21) of the menstrual cycle, TNF-alpha treated stromal cells were able to express cytokines that attract monocytes, a particular type of white blood cells. Subsequently, TNF-alpha induced further differentiation of these monocytes into dendritic cells, which are another type of immune cells. In turn, these dendritic cells stimulated the endometrial epithelial cells to produce several factors involved in embryo implantation: osteopontin and its receptors along with the up-regulation of genes involved in embryo adhesion (Fig. 1). At the same time, TNF-alpha caused a down-regulation of molecules that are present in the non-receptive endometrium. The hypothesis was made of a 'tissue memory' effect that may explain a favorable effect of the recruited monocytes in further treatment cycles.

In other experiments, injury increased the population of natural killer (NK) cells. NK cells are immune cells involved in the implantation process. They are crucial in stromal cell differentiation, immune modulation and angiogenesis during implantation. It has been shown that the number of NK-cells is decreased in IVF cycles. Thus, an injury stimulating an increase of these cells could be considered beneficial. However, the injury seemed to have no effect on angiogenesis, warning against an over-simplistic view on the potential clinical benefit.

Although tempting, results from experiments in animal models and cell cultures must be considered with caution before they can be extrapolated to the in-vivo human condition or applied in patient care.

It is not yet proven that experimental injury enhances the naturally initiated shift from a non-receptive to a receptive endometrial condition in humans. Also the 'tissue memory' hypothesis deserves further documentation.

Hypothesis of Action in Human

There are different hypothesis attempting to explain the clinical effects of endometrial scratching (Siristatidis et al., 2014). For each theory, pro's and contra's can be argued.

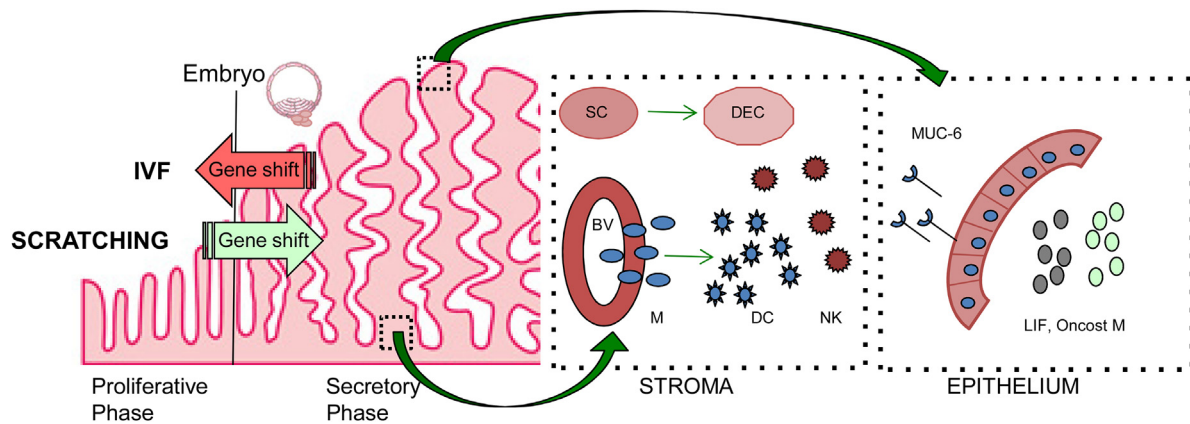


Fig. 1 Hypothesis of biological action of endometrial scratching in IVF patients: Scratching is thought to counter the gene shift induced by the IVF therapy. In the endometrial stroma (insert), scratching induces decidual (DEC) transformation of the stromal cells (SC). From the blood vessels (BV) there is an outflow of monocytes (M), transformed into dendritic cells (DC). Scratching also attracts natural killer cells (NK). In the endometrial epithelium (insert), the latter process will release cytokines involved in implantation (LIF, Oncost M) and decrease adhesion molecules (MUC-6) that interfere with embryo adhesion.

Injury Induces Local Decidual Changes in the Endometrium

A focal decidual reaction of the endometrial tissue in women has been described following the mechanical irritation caused by an embryo transfer catheter (Dallenbach-Hellweg et al., 1984). Various histological reaction patterns according to the timing of the catheter insertion were observed. If the catheter was inserted during the proliferative phase, no or minimal response was visible, whereas insertion in the secretory phase induced a focal decidual change of the endometrial stroma in the majority of the patients. Patients taking oral contraceptives showed no response to the catheter stimulus.

Pro: the human endometrium seems to be capable of focal, cycle phase-dependent and hormonal therapy-related response to mechanical stimulation. Contra: it is not certain that a local decidual change increases endometrial receptivity. Indeed, in a normal conceptive cycle, decida is not yet initiated during the limited time span by which the endometrium will accept an embryo that is about to implant. Decidual changes of the endometrium will only appear at a later stage of the luteal phase and will be a prerequisite for maintenance of an already established implantation. Therefore, artificially induced decidualization by injury may occur at a too premature time point.

Implantation According to the Wound-Healing Theory

It has been proposed that human embryo implantation is an inflammatory process bearing similarities to trauma, when white blood cells migrate from the blood vessels into the surrounding tissue (Gnainsky et al., 2015). Injury would thus induce secretion of wound-healing cytokines, which are similar to those beneficial for implantation.

Pro: theories claiming special attraction for the embryo to scar tissue of a cesarean section have been proposed.

Pro: wound-healing cytokines are associated with an inflow of certain immune cells, especially macrophages and dendritic cells. In women treated by IVF with a history of implantation failure, injury to the endometrium resulted into a statistically significant higher number of both types of immune cells. The immune cells enhance the production of cytokines in the endometrium. There is a positive correlation the levels of certain cytokines (namely, macrophage inflammatory protein 1B and TNF-alpha) and subsequent pregnancy rates in the following IVF cycle.

Contra: implantation of an embryo within scar tissue is a rare event and the majority of implantation sites following a cesarean section are located remotely from the scar. Moreover, the deep placental invasion into the myometrium (and beyond the scar tissue) is most likely to result from a failure rather than from an increased endometrial decidual response, thus also invalidating the previous hypothesis.

Theory of Injury-Induced Gene Expression Shift

It has been demonstrated that IVF therapies are responsible for an abnormal gene expression leading to a premature secretory change of the endometrium, which may cause the embryo to miss the implantation window.

This hypothesis assumes that injury restores a better embryo-endometrial synchrony and corrects the changes caused by the IVF therapy.

Pro: In a natural menstrual cycle, endometrial injury on cycle days 11–13 and 21–24 modulates several genes on day 20–21 of the next spontaneous cycle.

Contra: this backward development theory is likely to be over-simplistic, since endometrial advancement in IVF has only been described in the very first days of the secretory phase, but not at the time of presumed implantation. Furthermore, it also needs to be emphasized that no specific endometrial gene signature has yet been defined in IVF cycles with successful pregnancy outcome.

Taken together, there is substantial documentation on a short-term inflammatory response following intentional endometrial trauma, which recalls elements from the natural embryo implantation process. However, the evidence pointing to its exact implication in enhanced endometrial receptivity is fragmentary. A lasting effect of scratching in subsequent cycles needs yet to be clarified, all benefit remaining largely hypothetical.

Endometrial Scratching in Patients. What, Who, When and Why?

Evidence from Clinical Trials

Barash *et al.* (2003) was the first to postulate a beneficial effect of an endometrial biopsy taken before initiation of the IVF treatment cycle. He demonstrated a doubling in implantation, clinical pregnancy and live birth rates (2). More recently, over 1000 publications addressed the topic of endometrial scratching. Evidence derived from systematic reviews and meta-analysis (Nastri *et al.*, 2015) modestly favored a benefit of performing a local endometrial injury. The conclusions from these reviews indicate the greatest benefit in women with unexplained recurrent implantation failure (RIF). According to these conclusions, scratching should preferentially be performed in the cycle preceding the IVF treatment cycle. The technique of endometrial scratching is presented as an easy to perform and low-cost procedure, potentially increasing the IVF outcome.

There is severe criticism to these studies. Detractors point to methodological weaknesses of the presented data and a general lack of basic scientific evidence to prove a definite efficiency of endometrial scratching in improving the pregnancy outcome (Santamaria *et al.*, 2016).

In the general IVF population (i.e. patients without recurrent implantation failure) or in other procedures of assisted reproduction such as intra-uterine insemination or oocyte donation, scratching did not improve pregnancy outcome. In these patient groups, there is no indication to perform a scratching procedure. In view of these flaws, it is not possible to ascertain a beneficial effect of scratching and it is thus not recommended to implement the procedure in clinical practice unless more robust evidence becomes available. Besides defining which patient population may benefit from the scratching procedure, several other points such as tools, methods and timing for scratching also need to be standardized (Bourgain *et al.*, 2017).

Curiously, despite this lack of evidence, the procedure is already proposed in many fertility clinics for women with recurrent implantation failure. Several ongoing clinical trials are now trying to address the gaps in the utility of the scratching procedure (Lensen *et al.*, 2016; Lensen *et al.*, 2016). These are conducted in patients receiving fertility treatments or trying to conceive naturally. Further evidence of benefit must be awaited from those studies before the procedure can be implemented in routine practice.

Tools and Methods for Endometrial Scratching

There is no dedicated device to perform an endometrial scratching. Most commonly, an endometrial Pipelle biopsy device by Cornier is used for this purpose. A sample of the superficial layers of the endometrium is retrieved by suction and rotation. There is a need for standardization of the procedure, when applied to induce intentional injury. Until now, there is no consensus on the number of biopsies (single procedure or multiple procedures over different days). Neither is the technical method of intervention using the Pipelle biopsy standardized (360 degrees rotation with 4 up-and-down movements or scratching the whole endometrial cavity for a number of seconds). Alternative methods of injury include hysteroscopic irrigation with saline, endometrial damage provoked by a neonatal feeding tube, use of an endometrial biopsy catheter, a Novak curette or a grasping forceps with teeth. Although not included in the Cochrane definition of endometrial scratching, hysteroscopy can also be considered to cause some extent of endometrial injury.

The indication for endometrial injury is also poorly defined, as the procedure has been applied not only in patients with a normal cavity, but also in sub-fertile patients with the intention to treat intra-uterine anomalies such as polyps and fibroids. Even in the latter group, the benefit of systematic hysteroscopic surgical approach remains controversial.

Clearly, one cannot expect to draw consistent information from such highly divergent 'scratching' techniques in variable clinical indications.

It must also be mentioned that injury methods in clinical studies differ considerably from those applied in the experimental settings from which the different hypothesis of the biological actions of scratching were retrieved.

Another methodological gap in the current literature is the lack of a well-defined 'no injury' control population. Controls vary from no intervention to sham procedures, such as cervical biopsy or even hysteroscopy without biopsy. Especially the latter procedure may cause unintentional disturbance to the endometrial tissue.

There is an urgent need for guidelines to standardize the 'scratching' technique and to define the control population where no injury is performed.

When Is the Best Timing for Endometrial Scratching?

There appears to be no standardized clinical procedure reported for the best moment where endometrial scratching should be performed.

Among the different reports, there is a large variability concerning the timing of endometrial injury. Most studies advocate the intervention performed in the cycle preceding IVF, either in the proliferative or luteal phase or even both (Fig. 2). Less commonly,

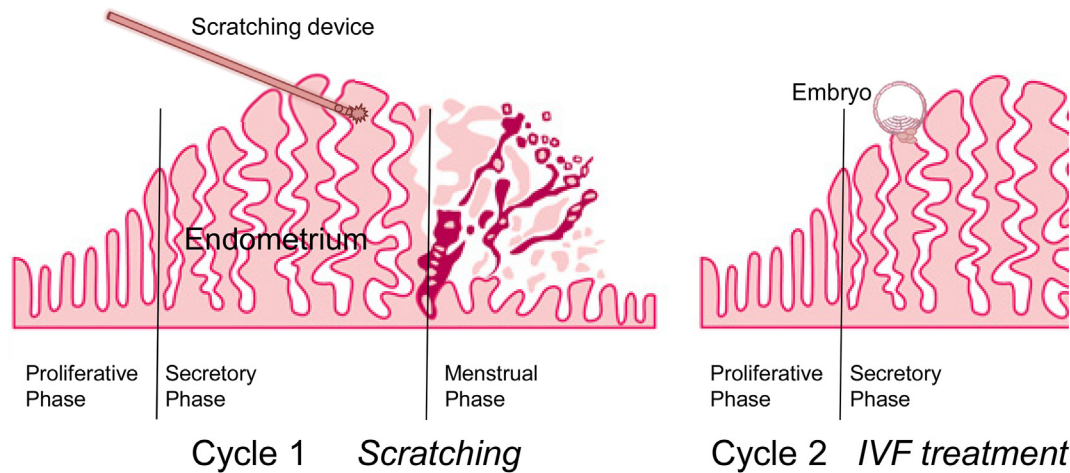


Fig. 2 The endometrial scratching is preferentially performed with a Pipelle biopsy device in the menstrual cycle that precedes the IVF treatment.

the injury is performed in the proliferative phase of the IVF cycle. A biopsy in the IVF cycle, closer to the implantation day is not recommended, being associated with a lower pregnancy outcome. Consensus guidelines upon the best timing to perform endometrial scratching still need to be developed.

Complications Due to Endometrial Scratching

Multiple pregnancy and miscarriage rates following endometrial scratching are very poorly documented and no conclusion can therefore be drawn from current literature.

Pain and bleeding are underreported and have only been assessed systematically in one study (Nastri et al., 2013), whereas elsewhere, only spontaneous pain complaint was reported. It is presumed that endometrial scratching is associated with a subjective increase in pain and a risk of bleeding. Theoretically, endometrial scratching in the luteal phase could be harmful to a spontaneous pregnancy, occurring at that moment. Therefore, couples are asked to refrain from intercourse in the cycle where scratching is performed. There is no documentation on the potential risk of Asherman syndrome. More systematic studies are warranted from a standardized 'scratching' procedure to address these adverse events.

Post-injury infection has not been reported. There is insufficient evidence to recommend the use of prophylactic antibiotics.

Overall, the procedure seems to be quite easy to perform in an out patient setting, to carry little risk and to be fairly well tolerated. Nevertheless it must be kept in mind not to propose a painful intervention at a supplementary cost to women without established added benefit.

Conclusions

Endometrial 'scratching' is an easy to perform and therefore tempting procedure to increase pregnancy outcome as part of a fertility treatment. To date, fragmentary and insufficient scientific evidence is available to prove a biological mechanism for a beneficial effect of local injury on endometrial receptivity. Curiously enough, despite this lack of proof, scratching is already proposed as a standard of care in numerous reproductive medicine units.

Clinical evidence must await further data from well-designed randomized trials to define the target patient population to whom the procedure could be of benefit. Standardized guidelines for a specific instrument, method and timing of scratching need to be established before implementation in routine practice.

References

- Barash, A., Dekel, N., Fieldust, S., Segal, I., Schechtman, E., & Granot, I. (2003). Local injury to the endometrium doubles the incidence of successful pregnancies in patients undergoing in vitro fertilization. *Fertility and Sterility*, 79, 1317–1322.
- Bourgain, C., Santos, R. S., & Blockeel, C. (2017). Endometrial scratching. In C. Simon, & L. Giudice (Eds.), *The endometrial factor: A reproductive precision medicine approach*. CRC Press.
- Dallenbach-Hellweg, G., Hohagen, F., & Hohlweg-Majert, P. (1984). Die histologische Reaktion des Endometriums auf Sondenreizung im Hinblick auf die Problematik des Embryonentransfers bei der In-vitro-Fertilisation. *Geburtshilfe und Frauenheilkunde*, 44(6), 381–387.
- Gnainsky, Y., Granot, I., Aldo, P., Barash, A., Or, Y., Mor, G., & Dekel, N. (2015). Biopsy-induced inflammatory conditions improve endometrial receptivity: the mechanism of action. *Reproduction*, 149, 75–85.

- Hyodo, S., Matsubara, K., Kameda, K., & Matsubara, Y. (2011). Endometrial injury increases side population cells in the uterine endometrium: A decisive role of estrogen. *The Tohoku Journal of Experimental Medicine*, 224, 47–55.
- Kupka, M. S., Ferraretti, A. P., de Mouzon, J., Erb, K., et al. (2014). Assisted reproductive technology in Europe, 2010: Results generated from European registers by ESHRE. *Human Reproduction*, 29(10), 2099–2113.
- Lensen, S., Martins, W., Nastri, C., & Farquhar, C. (2016). Pipelle for pregnancy (PIP): Study protocols for three randomized trials. *Trials*, 17, 216–229.
- Lensen, S., Sadler, L., & Farquhar, C. (2016). Endometrial scratching for subfertility: Everyone's doing it. *Human Reproduction*, 31(6), 1241–1244.
- Loeb, L. (1907). Über die experimentelle erzeugung von knoten von deciduagewebe in dem uterus des meerschweinchens nach stattgefundener copulation. *Zentralblatt für allgemeine Pathologie u. pathologische Anatomie*, 563–565.
- Nastri, C., Lensen, S., Gibreel, A., Raine-Fenning, N., Ferriani, R., Bhattacharya, S., & Martins, W. (2015). Endometrial injury in women undergoing assisted reproductive techniques. *Cochrane Database of Systematic Reviews*, 3, CD009517.
- Nastri, N., Ferriani, R., Raine-Fenning, N., & Martins, W. (2013). Endometrial scratching performed in the non-transfer cycle and outcome of assisted reproduction: A randomized controlled trial. *Ultrasound in Obstetrics & Gynecology*, 42(4), 375–382.
- Santamaria, X., Katzorke, N., & Simon, C. (2016). Endometrial 'scratching': what the data show. *Current Opinion in Obstetrics & Gynecology*, 2(4), 242–249.
- Siristatidis, C., Vrachnis, N., Vogiatzi, P., Chrelias, C., Quinteiro-Retamar, A., Bettocchi, S., & Glujovsky, D. (2014). Potential pathophysiological mechanisms of the beneficial role of endometrial injury in in vitro fertilization outcome. *Reproductive Sciences*, 21(8), 955.
- Zhang, X. H., Liu, Z. Z., Tang, M. X., Zhang, Y. H., Hu, L., & Liao, A. H. (2015). Morphological changes and expression of cytokine after local endometrial injury in a mouse model. *Reproductive Sciences*, 22(11), 1377–1386.

The Embryo Day 3 Versus Day 5 ET

Ashley W Tiegs and Jason Franasia, Reproductive Medicine Associates of New Jersey, Basking Ridge, NJ, United States; and Thomas Jefferson University, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

ART Assisted reproductive technology
FSH Follicle-stimulating hormone
IVF In vitro fertilization
LH Luteinizing hormone

Implantation: Physiology

In order to produce a viable pregnancy, the competence of both the embryo and the endometrium must be ensured. In murine models, the luminal epithelium remains non-receptive to implantation for the first 3 days following the union of spermatozoa with the oocyte, despite adequate preparation of the endometrium with ovarian-derived estrogen and progesterone. At the blastocyst stage, appositional contact with the luminal epithelium can be seen. It is at this time that an additional small surge of ovarian estradiol is required to induce the synthesis of cytokines involved with implantation, for example, leukemia inhibitory factor (LIF), a pro-inflammatory cytokine belonging to the interleukin-6 (IL-6) family. LIF is a glycoprotein which activates the Janus kinase—signal transducer and activator of transcription (JAK-STAT) pathway in the luminal epithelium. As a result, many transcription factors are produced, effecting a cascade of changes. Ultimately, due to LIF and other cytokines, the uterine luminal epithelium is transformed from a non-receptive to a receptive state ([Rosario and Stewart, 2016](#)).

The receptive endometrium often exhibits bulbous protrusions on the surface of the epithelial lumen and stromal cells that are primed to decidualize with application of the appropriate stimuli. Additionally, the receptive endometrium displays reorganization of the stromal extracellular matrix components that allows for decidualization and secretes stage-specific glycoproteins through the lumen. Once decidualization occurs, the basement membrane components appear within the matrix ([Yoshinaga, 1988](#)).

Once a receptive endometrium is established, it must then interact with the embryo. Adherence of the embryo to the endometrium is regulated by a family of cell adhesion molecules called integrins (ITG) ([Mangale and Reddy, 2007](#)). Specific integrins present on the luminal surface of the endometrium transduce cellular signals between uterine epithelial cells and trophoblastic cells. Specifically, α 5 β 1 integrin and integrin β 8 (ITGB8) are implicated in blastocyst attachment and can regulate vascular development via activation of vascular endothelial growth factor or transforming growth factor β 1 (TGFB1) ([Apparao et al., 2001](#); [Kumar et al., 2015](#)). Disruptions in the aforementioned complex sequence of events can lead to failure of implantation.

The impact of the immune system at the level of the endometrium has been identified to potentially play a role in determining the success or failure of implantation, even in the absence of infection. For example, imbalances in cytokines produced by T-lymphocyte helper cells-1 and 2 (T_H1 and T_H2 , respectively) can contribute to implantation failure, as pregnancy is considered a T_H2 -dominant state. Whether an imbalance occurs as a result of a failed pregnancy or is the cause of a failed gestation is not well-delineated at this time ([Fransia and Scott, 2017](#)). The role of natural killer (NK) cells on recurrent implantation failure has also been extensively studied, however, a meta-analysis of 12 studies did not find an association between pregnancy outcomes and peripheral NK cell activity or quantity or uterine NK density ([Tang et al., 2011](#)).

While the evidence for immunologic interference or assistance in reproductive success is conflicting, other endometrial factors have also been postulated to influence implantation. For example, an abnormal structure of the endometrial cavity, such as uterine hypoplasia, can result in impaired reproductive outcomes ([Berger and Goldstein, 1980](#)). The presence of infection, such as chronic endometritis, can also impact the receptivity of the endometrium. In such cases, an abnormal pattern of lymphocyte subsets are identified within the endometrium, leading to an abnormal endometrial microenvironment. This has been shown by some investigators to decrease reproductive potential ([Matteo et al., 2009](#); [Cicinelli et al., 2014](#)).

It should be noted that the physiology of embryonic and endometrial development can be altered. This is not due to a reproducible pathology in the processes described above. In these cases, it is likely that dyssynchrony exists between the endometrium and the embryo, precluding pregnancy in an otherwise normal endometrium and presumably normal embryo. For implantation to occur, the embryo and endometrium must not only be normal, but the window of embryonic implantation and window of endometrial receptivity must align. Synchrony implies that the endometrium is receptive at the time that the embryo is prepared to implant.

Assessment of the Embryo

With regard to implantation failure, investigators have traditionally evaluated the embryo and the endometrium in isolation. Since the time of the first embryo transfers, significant advances have been made in both laboratories and embryo culture systems. At present, most laboratories culture embryos to the cleavage stage, the blastocyst stage, or commonly, both. A 2016 Cochrane Database Systematic review of 27 randomized controlled trials (RCTs) of 4,031 couples compared the success of cleavage stage versus blastocyst transfers. Primary outcomes included live birth rates and cumulative clinical pregnancy rates. Secondary outcomes included multiple pregnancy, miscarriage, embryo cryopreservation and inability to produce an embryo for transfer. The authors found higher clinical pregnancy and live birth rates following fresh transfer in the blastocyst group as compared with cleavage stage (day 2 or 3) group (odds ratio (OR) 1.30, 95% confidence interval (CI) 1.14 to 1.47; 27 RCTs, 4031 couples; OR 1.48, 95% CI 1.20 to 1.82; 13 RCTs, 1630 couples, respectively). There was no difference seen between groups with regard to miscarriage (OR 1.15, 95% CI 0.88 to 1.50; 18 RCTs, 2917 couples) or multiple pregnancy (OR 1.05, 95% CI 0.83 to 1.33; 19 RCTs, 3019 couples). However, couples were two times more likely to have no embryos available for transfer in the blastocyst group (OR 2.50, 95% CI 1.76 to 3.55; 17 RCTs, 2577 couples). The quality of evidence was assessed using Grading of Recommendations, Assessment, Development and Evaluations (GRADE) methods and found to be low for most of the main comparisons in each study, with the major limitation being risk of bias. In most of the included studies, this risk was attributed to failure to describe acceptable methods of randomization. The authors of the review concluded an overall benefit of blastocyst transfer over cleavage stage transfer, although the impact on cumulative pregnancy and live birth rates could not be ensured given the inherent limitations of the 27 RCTs cited ([Glujovsky et al., 2016](#)).

A recent study from De Vos and colleagues also showed significantly higher live birth rates per started cycle after transfer of a blastocyst compared with a cleavage-stage embryo ([De Vos et al., 2016](#)). Additional findings included a reduced number of embryo transfer necessary until the first live birth when using blastocyst-stage embryos. Therefore, it is surmised that embryos with the capability to develop to the blastocyst stage in vitro demonstrate higher pregnancy potential. Additionally, if preimplantation genetic screening is to be used to select a euploid embryo for transfer, blastocysts tolerate this procedure without detriment, while cleavage stage embryos do not ([Scott et al., 1993](#)). In a randomized control trial using a paired design, the biopsy procedure itself was demonstrated to have no adverse impact on the pregnancy potential of the embryos.

An important addition to the consideration of extended embryo culture is the assessment of embryonic development for synchrony. This was not evaluated or discussed in the previously mentioned Cochrane review. The duration of culture required to reach the blastocyst stage is not uniform amongst embryos, even between those obtained within the same cycle and from the same patient. The time to develop to the blastocyst stage takes between 5 and 7 days. The only way to monitor embryos for blastulation is through assessment during extended culture. If blastulation occurs late, the endometrium may be no longer receptive to the embryo after a physiologically early progesterone stimulus, as we will see. Such dyssynchrony can be circumvented by the cryopreservation of embryos obtained from the controlled ovarian hyperstimulation cycle. A programed transfer cycle with a known progesterone start and transfer of a cryopreserved embryo (which was an expanded blastocyst at the time of vitrification) can ensure synchrony between the endometrium and the embryo chosen for transfer ([Franasiak et al., 2016](#)).

Assessment of the Endometrium

Our assessments of the endometrium continue to evolve. Endometrial biopsies were once commonly performed in efforts to diagnose a luteal phase defect, in which it was believed that low mid-luteal progesterone levels were responsible for conception and pregnancy failure. A later investigation by Scott *et al* revealed that endometrial dating by histologic criteria had poor intra-observer reliability, potentially explaining the conflicting reports published on the topic ([Scott et al., 1993](#); [Murray et al., 2004](#)). The Reproductive Medicine Network later confirmed these findings, reporting the greatest observer variability in histologic dating of the endometrium occurred when assessing the window of implantation in infertile women ([Myers et al., 2004](#)).

Furthermore, luteal serum progesterone levels have historically been measured to identify a luteal phase defect as a method for describing issues with endometrial physiology which can cause implantation issues. However, it has been demonstrated that serum progesterone levels vary widely throughout the luteal phase. Filicori *et al* demonstrated that luteal progesterone is characterized by a pulsatile pattern, as its production is dependent on luteinizing hormone (LH) ([Filicori et al., 1984](#)). Therefore, in a natural cycle, the serum progesterone level confers little predictive value.

Next, the advent of routine use of transvaginal ultrasound into clinical practice has allowed visualization of the endometrium throughout the menstrual cycle. Studies correlating ultrasound findings to endometrial histology have enhanced our understanding of the sonographic features of endometrial abnormalities. Many aberrations in endometrial development can easily be visualized on ultrasound, including the presence of a thin endometrium in the late follicular phase, a hyperechoic endometrium in the late follicular phase ([Fanchin et al., 2000](#)), or a hyper-contractile endometrium identified in the luteal phase. However, a 2014 systematic review and meta-analysis reported that endometrial thickness has limited ability to predict chances of conceiving in IVF cycles. A very thin endometrial lining (measuring <7 mm) appeared to be related to a lower chance of pregnancy, but such a finding occurs infrequently in isolation and therefore, adds limited prognostic value ([Kasius et al., 2014](#)).

Most recently, extensive work in transcriptomic profiling has demonstrated the possibility of classifying human endometrial receptivity at a molecular level. Ruiz-Alonso and colleagues published their success with the endometrial receptivity array (ERA),

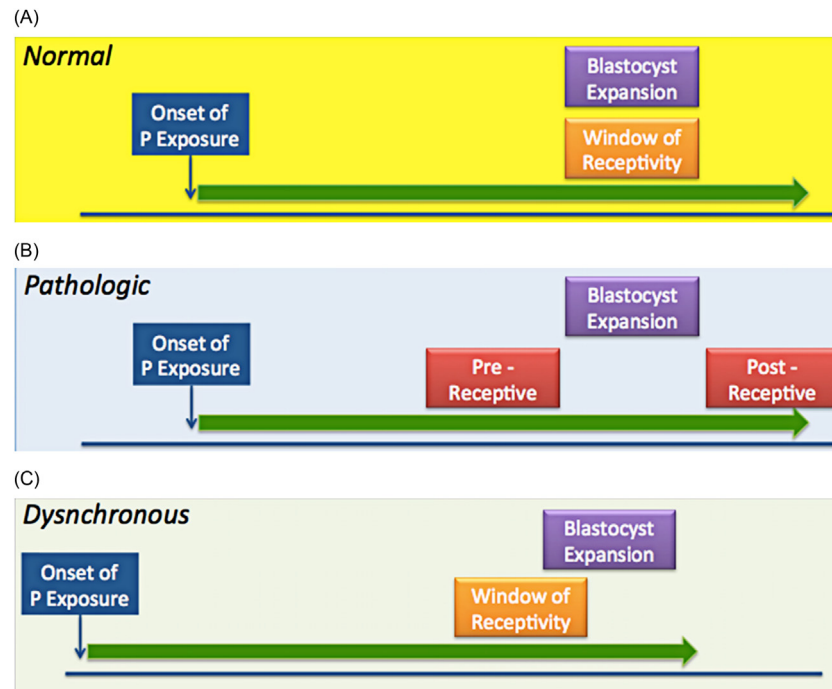


Fig. 1 The timing of the onset of the progesterone stimulus is critical to synchrony. In natural conception, secretory transformation begins and the window of endometrial receptivity opens several days later. This is synchronous with the timing of blastocyst development (A). In some patients, the window of implantation is pathologic; i.e., trending towards accelerated or retarded development (B). In ART cycles, endometrial-embryo dyssynchrony is usually not due to endometrial pathology, but rather, secondary to aberrant timing of the progesterone stimulus (C).

a molecular diagnostic tool containing 238 genes expressed differentially during the menstrual cycle with the capability to identify the endometrial receptivity of a sample. Unlike endometrial biopsy, this test has been shown to show intra-patient reproducibility and has shown success in defining a personalized window of implantation (Ruiz-Alonso et al., 2013).

Such assessments of the endometrium evaluate pathology. For example, the endometrial receptivity array can identify patients whose window of implantation is pathologic; that is, trending towards accelerated or retarded development (Fig. 1A). However, in ART cycles, endometrial-embryo dyssynchrony is usually not due to endometrial pathology, but rather, secondary to aberrant timing of the progesterone stimulus. The timing of the onset of the progesterone stimulus is critical to synchrony. Once progesterone reaches a threshold value after ovulation, secretory transformation begins and the window of endometrial receptivity opens several days later (Fig. 1B).

Synchrony and Dyssynchrony Between the Embryo and the Endometrium

In a natural cycle, the follicle facilitates coordination between the endometrium and the embryo. At approximately the same time that the spermatozoa meet the oocyte, a secretory transformation of the endometrium occurs secondary to a post-ovulation elevation in progesterone (Fig. 1C). This rise in progesterone serves as the stimulus that initiates the opening of the endometrium's window of receptivity. Development will proceed synchronously if the embryo and endometrium are both normal. In an assisted reproduction cycle, this coordination must be replicated.

The window of implantation can be divided into the possible window of implantation and the optimal window of implantation. Early studies posited that the window was 3–5 days in length. In a study by Navot et al, day-2 embryos were transferred between cycle days 16 through 24. Pregnancies were noted to result after transfers on days 16–20, with the majority resulting after transfer on cycle days 17–19 (Navot et al., 1991). Subsequent studies described a narrower window of implantation, with highest pregnancy rates occurring during a 2-day window (Prapas et al., 1998). In these subsequent studies, 2–6 days of progesterone was administered with subsequent transfer of day-2 embryos. Pregnancy rates were noted to decrease and abnormal placentation was found to increase at the later margin of the window (Prapas et al., 1998; Wilcox et al., 1999).

In a retrospective study, Bosch et al demonstrated that the endometrial window of implantation is influenced by the progesterone levels, as premature (late follicular) serum progesterone levels greater than or equal to 1.5ng/mL were associated with lower ongoing pregnancy rates (Bosch et al., 2010). Later meta-analysis data from Hamdine et al confirms reduced clinical outcomes with elevated late follicular progesterone rise (Hamdine et al., 2014). Santos-Ribiero et al subsequently described late follicular serum progesterone levels found to be either too high or too low were associated with reduced live birth rates after controlling for potential confounding factors (Santos-Ribiero et al., 2014).

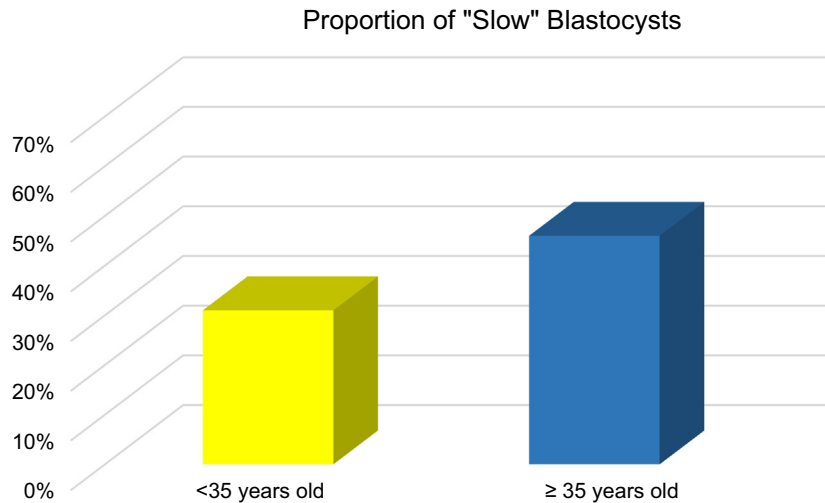


Fig. 2 No difference in outcomes seen between day 5 euploid embryos and day 6 euploid embryos after cryopreservation with subsequent thaw and transfer in a programmed cycle (Forman et al., 2013). In this study, blastulation occurred more commonly after day 5 in women age 35 and older.

Detrimental clinical outcomes associated with a premature rise in progesterone seen in ART cycles are related to an earlier shift in secretory transformation of the endometrium. The progesterone rise occurs earlier and more quickly in IVF cycles as compared with natural cycles. As a result, the window of implantation is shifted approximately 16–24 h earlier in ART cycles. If progesterone rises even earlier in the late follicular stage, the window of implantation correspondingly shifts forward. Unlike patients with pathologic endometrium (i.e., displaying consistent retardation or acceleration of endometrial development), shifts of the window occurring in stimulated cycles are not reproducible from cycle to cycle. Once this characterization of the stimulus for the window of implantation in ART cycles has been identified, attention can be turned to the role of the embryo in synchrony.

Embryonic development has been studied with regard to those that can mature to the blastocyst stage in vitro. Prior to routine cryopreservation of embryos with transfer in a delayed programmed cycle, embryos that blastulated later were associated with reduced fitness (Shapiro et al., 2008; Barrenetxea et al., 2005). In fresh embryo transfer cycles, Barrenetxea et al demonstrated significantly higher implantation rates with embryos that reached blastocyst stage by day 5 post-fertilization as compared with those blastulating at day 6 (Barrenetxea et al., 2005). However, Shapiro et al showed that this effect disappeared when outcomes of thawed cryopreserved day 5 and 6 embryos were compared after transfer within programmed transfer cycles (Shapiro et al., 2008). These findings point to dyssynchrony between the embryo and the endometrium as a possible confounder that may explain the decreased competence of embryos that blastulate later. This concept was also demonstrated by Forman and colleagues, noting no difference in outcomes between day 5 euploid embryos and day 6 euploid embryos after cryopreservation with subsequent thaw and transfer in a programmed cycle (Forman et al., 2013). In this study, blastulation occurred more commonly after day 5 in women age 35 and older (Fig. 2). Later blastulating embryos coupled with an earlier shift in the window of endometrial receptivity seen in ART cycles results in a high likelihood that dyssynchrony will occur. As a result, dyssynchrony can potentially explain the diminution of clinical outcomes seen in many older patients. Alternatively, embryos from younger women tend to blastulate early, falling within the window of endometrial receptivity and resulting in improved clinical outcomes.

Management of Dyssynchrony

Prevention of dyssynchrony involves active management of both follicular stimulation and the timing of embryo blastulation in extended embryo culture. Late follicular serum progesterone may be monitored for an elevation above a threshold at-risk value, likely unique to each laboratory. It may be possible to prevent premature progesterone rise by carefully adjusting the ovarian stimulation. In an analysis of 10,280 first IVF cycles, Werner et al showed that LH or low-dose hCG can prevent premature late follicular rises in serum progesterone when added to the stimulation regimen. Specifically, the ratio of LH to follicle-stimulating hormone (FSH) of 0.3–0.6 was found to be optimal to prevent progesterone rise in both high and low responders (Werner et al., 2014). Preventing premature rises in serum progesterone is important when a patient is scheduled for a fresh embryo transfer, however, this is no longer pertinent with a frozen/thawed embryo transfer. Many studies have demonstrated the safety and even benefit of cryopreservation of embryos with replacement in a separate programmed cycle. The benefits include improved synchrony between the embryo and endometrium, decreased risk of ovarian hyperstimulation syndrome and improved pregnancy and fetal outcomes (Cobo et al., 2012). The latter benefits are ascribed to be the result of normalization of the hormonal milieu (Moreno and Franasiak, 2017).

In summary, embryo implantation is a complex process. The physiology of uterine receptivity is different in natural versus IVF cycles and should be treated as such. We know that synchrony between the embryo and the window of endometrial receptivity is essential, and therefore, both components must be scrutinized and actively managed in order to achieve optimal pregnancy outcomes. An improvement in clinical outcomes has been seen with transfer of blastocysts as opposed to cleavage-stage embryos. Given the available evidence, improved outcomes seen after the transfer of blastocysts are likely secondary to improved synchrony between the endometrium and the embryo, which can only be attained with extended embryo culture. Furthermore, the endometrium can be programmed toward receptivity to the blastocyst if embryo cryopreservation with subsequent thaw and careful timing of transfer is employed.

References

- Apparao, K. B., Murray, M. J., Fritz, M. A., Meyer, W. R., Chambers, A. F., Truong, P. R., & Lessey, B. A. (2001). Osteopontin and its receptor $\alpha v \beta 3$ integrin are coexpressed in the human endometrium during the menstrual cycle but regulated differentially. *The Journal of Clinical Endocrinology and Metabolism*, 86, 4991–5000.
- Barrenetxea, G., Lopez de Larrea, A., Ganzabal, T., Jimenez, R., Carbonero, K., & Mandiola, M. (2005). Blastocyst culture after repeated failure of cleavage-stage embryo transfers: a comparison of day 5 and day 6 transfers. *Fertility and Sterility*, 83, 49–53.
- Berger, M. J., & Goldstein, D. P. (1980). Impaired reproductive performance in DES-exposed women. *Obstetrics and Gynecology*, 55, 25–27.
- Bosch, E., Labarta, E., Crespo, J., Simo n, C., Remohi, J., Jenkins, J., & Pellicer, A. (2010). Circulating progesterone levels and ongoing pregnancy rates in controlled ovarian stimulation cycles for in vitro fertilization: Analysis of over 4000 cycles. *Human Reproduction*, 25, 2092–2100.
- Cicinelli, E., Matteo, M., Tinelli, R., Pinto, V., Marinaccio, M., Indraccolo, U., Ziegler, D. D., & Resta, L. (2014). Chronic endometritis due to common bacteria is prevalent in women with recurrent miscarriage and confirmed by improved pregnancy outcome after antibiotic treatment. *Reproductive Sciences*, 21, 640–647.
- Cobo, A., de los Santos, M. J., Castello, D., Gamiz, P., Campos, P., & Remohi, J. (2012). Outcomes of vitrified early cleavage-stage and blastocyst-stage embryos in a cryopreservation program: evaluation of 3,150 warming cycles. *Fertility and Sterility*, 98, 1138–1146.
- Fanchin, R., Righini, C., Ayoubi, J. M., Olivennes, F., de Ziegler, D., & Frydman, R. (2000). New look at endometrial echogenicity: objective computer-assisted measurements predict endometrial receptivity in in vitro fertilization-embryo transfer. *Fertility and Sterility*, 74, 274–281.
- Filicori, M., Butler, J. P., & Crowley, W. F., Jr. (1984). Neuroendocrine regulation of the corpus luteum in the human. Evidence for pulsatile progesterone secretion. *The Journal of Clinical Investigation*, 6, 1638–1647.
- Forman, E. J., Franasiak, J. M., Hong, K. H., & Scott, R. T. (2013). Late expanding euploid embryos that are cryopreserved (CRYO) with subsequent synchronous transfer have high sustained implantation rates (SIR) similar to fresh normally blastulating euploid embryos. *Fertility and Sterility*, 100(Suppl), S99.
- Franasiak, J. M., Ruiz-Alonso, M., Scott, R. T., & Simon, C. (2016). Both slowly developing embryos and a variable pace of luteal endometrial progression may conspire to prevent normal birth in spite of a capable embryo. *Fertility and Sterility*, 105, 861–866.
- Franasiak, J. M., & Scott, R. T. (2017). Contribution of immunology to implantation failure of euploid embryos. *Fertility and Sterility*, 107, 1279–1283.
- Glujovsky, D., Farquhar, C., Quinteiro Retamar, A. M., Alvarez Sedo, C. R., & Blake, D. (2016). Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database of Systematic Reviews*, 30, CD002118.
- Hamdine, O., Macklon, N. S., Eijkemans, M. J., Laven, J. S., Cohlen, B. J., Verhoeff, A., van Dop, P. A., Bernardus, R. E., Lambalk, C. B., Oosterhuis, G. J., Holleboom, C. A., van den Dool-Maasland, G. C., Verburg, H. J., van der Heijden, P. F., Blankart, A., Fauser, B. C., Broekmans, F. J., & CETRO trial study group. (2014). Elevated early follicular progesterone levels and in vitro fertilization outcomes: A prospective intervention study and meta-analysis. *Fertility and Sterility*, 102, 448–454.
- Kasius, A., Smit, J. G., Torrance, H. L., Eijkemans, M. J., MOI, B. W., Opmeer, B. C., & Broekmans, F. J. (2014). Endometrial thickness and pregnancy rates after IVF: A systematic review and meta-analysis. *Human Reproduction Update*, 20, 530–541.
- Kumar, V., Maurya, V. K., Joshi, A., Meeran, S. M., & Jha, R. K. (2015). Integrin beta 8 (ITGB8) regulates embryo implantation potentially via controlling the activity of TGF- β 1 in mice. *Biology of Reproduction*, 92, 109.
- Mangale, S. S., & Reddy, K. V. (2007). Expression pattern of integrins and their ligands in mouse fetomaternal tissues during pregnancy. *Reproduction, Fertility, and Development*, 19, 452–460.
- Matteo, M., Cicinelli, E., Greco, P., Massenzio, F., Baldini, D., Fakagario, T., Rosenberg, P., Castellana, L., Specchia, G., & Liso, L. (2009). Abnormal pattern of lymphocyte subpopulations in the endometrium of infertile women with chronic endometritis. *American Journal of Reproductive Immunology*, 61, 322–329.
- Moreno, I., & Franasiak, J. M. (2017). Endometrial microbiota—new player in town. *Fertility and Sterility*, 108, 32–39.
- Murray, M. J., Meyer, W. R., Zaino, R. J., Lessey, B. A., Novotny, D. B., Ireland, K., Zeng, D., & Fritz, M. A. (2004). A critical analysis of the accuracy, reproducibility, and clinical utility of histologic endometrial dating in fertile women. *Fertility and Sterility*, 5, 1333–1343.
- Myers, E. R., Silva, S., Barnhart, K., Groben, P. A., Richardson, M. S., Robboy, S. J., Leppert, P., Coutifaris, C., & NICHD National Cooperative Reproductive Medicine Network. (2004). Interobserver and intraobserver variability in the histologic dating of the endometrium in fertile and infertile women. *Fertility and Sterility*, 82, 1278–1282.
- Navot, D., Scott, R. T., Drosch, K., Veeck, L. L., Liu, H. C., & Rosenwaks, Z. (1991). The window of embryo transfer and the efficiency of human conception in vitro. *Fertility and Sterility*, 55, 114–118.
- Prapas, Y., Prapas, N., Jones, E. E., Duleba, A. J., Olive, D. L., Chatziparasidou, A., & Vlassis, G. (1998). The window for embryo transfer in oocyte donation cycles depends on the duration of progesterone therapy. *Human Reproduction*, 13, 720–723.
- Rosario, G. X., & Stewart, C. L. (2016). The multifaceted actions of leukemia inhibitory factor in mediating uterine receptivity and embryo implantation. *American Journal of Reproductive Immunology*, 75, 246–255.
- Ruiz-Alonso, M., Blesa, D., Diaz-Gimeno, P., Gomez, E., Fernandez-Sanchez, M., Carranza, F., Carrera, J., Vilella, F., Pellicer, A., & Simon, C. (2013). The endometrial receptivity array for diagnosis and personalized embryo transfer as a treatment for patients with repeated implantation failure. *Fertility and Sterility*, 100, 818–824.
- Santos-Ribiero, S., Polyzos, N. P., Haentjens, P., Smits, J., Camus, M., Tournaye, H., & Blockeel, C. (2014). Live birth rates after IVF are reduced by both low and high progesterone levels on the day of human chorionic gonadotropin administration. *Human Reproduction*, 29, 1698–1705.
- De Vos, A., Van Landuyt, L., Santos-Ribiero, S., Camus, M., Van de Velde, H., Tournaye, H., & Verheyen, G. (2016). Cumulative live birth rates after fresh and vitrified cleavage-stage versus blastocyst-stage embryo transfer in the first treatment cycle. *Human Reproduction*, 31, 2442–2449.
- Scott, R. T., Snyder, R. R., Bagnall, J. W., Reed, K. D., Adair, C. F., & Hensley, S. D. (1993). Evaluation of the impact of intraobserver variability on endometrial dating and the diagnosis of luteal phase defects. *Fertility and Sterility*, 60, 652–657.
- Shapiro, B. S., Daneshmand, S. T., Garner, F. C., Aguirre, M., & Ross, R. (2008). Contrasting patterns in in vitro fertilization pregnancy rates among fresh autologous, fresh oocyte donor, and cryopreserved cycles with the use of day 5 or day 6 blastocysts may reflect differences in embryo-endometrium synchrony. *Fertility and Sterility*, 89, 20–26.
- Tang, A. W., Alfirevic, Z., & Quenby, S. (2011). Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. *Human Reproduction*, 26, 1971–1980.

- Werner, M. D., Forman, E. J., Hong, K. H., Franiak, J. M., Molinaro, T. A., & Scott, R. T. (2014). Defining the “sweet spot” for administered luteinizing hormone-to- follicle-stimulating hormone gonadotropin ratios during ovarian stimulation to protect against a clinically significant late follicular increase in progesterone: an analysis of 10,280 first in vitro fertilization cycles. *Fertility and Sterility*, 102, 1312–1317.
- Wilcox, A. J., Baird, D. D., & Weinberg, C. R. (1999). Time of implantation of the conceptus and loss of pregnancy. *The New England Journal of Medicine*, 34, 1796–1799.
- Yoshinaga, K. (1988). Uterine receptivity for blastocyst implantation. *Annals of the New York Academy of Sciences*, 54, 424–431.

Further Reading

- Cicinelli, E., Matteo, T. R., Lepera, A., Alfonso, R., Indraccolo, U., Marrocchella, S., Greco, P., & Resta, L. (2015). Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy. *Human Reproduction*, 30, 323–330.
- Franiak, J., Forman, E. J., Hong, K. H., Werner, M. D., Upham, K. M., & Scott, R. T., Jr. (2013). Investigating the impact of the timing of blastulation on implantation: Active management of embryo-endometrial synchrony increases implantation rates. *Fertility and Sterility*, 100(Suppl), S97.
- Scott, R. T., Upham, K. M., Forman, E. J., Zhao, T., & Treff, N. R. (2013). Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: A randomized and paired clinical trial. *Fertility and Sterility*, 100, 624–630.
- Shapiro, B. S., Daneshmand, S. T., Garner, F. C., Aguirre, M., & Hudson, C. (2013). Factors related to embryo-endometrium asynchrony in fresh IVF cycles increase in prevalence with maternal age. *Fertility and Sterility*, 100(Suppl), S287.

SPECIAL MEDICAL CONDITIONS

ART in Endometriosis

Graciela Kohls and Juan A Garcia-Velasco, IVI-RMA, Madrid, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

Endometriosis is one of the most common gynecological disorders and is a condition estrogen-dependant characterized by endometrial tissue located outside of the uterus, most commonly on the ovaries and peritoneum.

Reduced fertility and chronic pain are the two principal clinical features of endometriosis. Endometriosis is one of the main causes of infertility, although the actual relationship between the two is unclear.

The association between severe and advanced endometriosis and reduced fertility may be explained through the severe anatomical and functional abnormalities caused by advanced endometriosis in the female pelvis. The possibility that minimal or mild endometriosis may cause infertility is still a matter of debate.

Epidemiology

The prevalence of endometriosis in women of reproductive age is around 10%. In large studies the prevalence of endometriosis in fertile women appears to be lower (0.5–5%), whereas in infertile women it is higher (25%–40%). Moreover, the rate of infertility has been reported to be six to eight times higher in patients with endometriosis.

Diagnosis Delay

Endometriosis is a chronic and, in most cases, debilitating disease associated with a significant reduction of quality of life due to pain symptoms and/or infertility. In addition, several lines of evidence indicate that a significant number of women with endometriosis do develop comorbidities, such as depressive or anxiety disorders. Delaying the diagnosis of endometriosis clearly aggravates these problems. Early detection is of significant importance because many complications of this chronic disease, such as infertility, may be reversed or treated earlier. Several studies have demonstrated that the length of the time interval from onset of symptoms to diagnosis is surprisingly long (Table 1).

Several factors cause the delay of the diagnosis like widespread use of oral contraceptives that attenuate the symptoms, misdiagnosis, the normalization of menstrual pain, clinical examinations that are nondiscriminatory without transvaginal sonography (TVS), etc.

Risk Factors

Risk factors for endometriosis include obstruction of menstrual outflow (e.g., anatomic anomalies), exposure to drugs (diethylstilbestrol) in utero, prolonged exposure to endogenous estrogen (early menarche, late menopause, or obesity), short menstrual cycles,

Table 1 Diagnostic delay in years from symptoms to diagnosis across different countries

	<i>Diagnosis delay (years)</i>
USA	11.7
UK	7.9
Brazil	7
China	3.3
Italy	10.7
Germany and Austria	10.4
UK and Spain	6.7
Norway	6.7
Ireland and Belgium	4–5

low birth weight, and exposure to endocrine-disrupting chemicals found in various materials such as pesticides, metals, additives or contaminants in food, and personal care products. Twin and family studies suggest a genetic component. Consumption of red meat and trans fats is associated with an increased risk of laparoscopically confirmed endometriosis, and eating fruits, green vegetables, and $n - 3$ long-chain fatty acids is associated with a decreased risk. Prolonged lactation and multiple pregnancies are protective. Endometriosis is associated with increased risks of autoimmune diseases and ovarian endometrioid and clear-cell cancers, as well as other cancers, including non-Hodgkin's lymphoma and melanoma.

Relationship Between Endometriosis and Infertility

The monthly pregnancy rate in women in their mid-reproductive years without endometriosis is around 30%, whereas in those with endometriosis this figure is between only 2% and 10%. This reduced pregnancy rate may even apply to women with minimal endometriosis.

The epidemiological association between endometriosis and low fertility may indicate a causal relationship (Fig. 1), explained by several hypotheses:

- Disorders of ovulation and fertilization, reduced quality of the oocyte and embryo, and dysfunctional implantation.
- Immunological and inflammatory disorders of the peritoneal environment.
- Tubal occlusion
- Disorders of endometrial receptivity, for example due to insufficient hormonal stimulation and progesterone resistance

Diagnosis of Endometriosis

Symptoms

Many symptoms are associated with endometriosis, ranging from pelvic and intestinal complaints to referred pain. The clinician should always suspect endometriosis when any of the following signs are reported by women of reproductive age:

- Pelvic symptoms: pelvic pain typically associated with endometriosis is often intramenstrual (dysmenorrhea), progressive and of variable intensity. Painful sexual intercourse (dyspareunia), abdominal cramping, noncyclical pelvic pain, infertility, fatigue, and weariness are also symptoms of endometriosis.
- Intestinal complaints: periodic bloating, diarrhea/constipation, painful defecation (dyschezia), rectal bleeding.
- Urinary symptoms: pain (dysuria) and/or bleeding (hematuria) when urinating.
- Referred pain: legs, back (specially in adolescents), and shoulder.

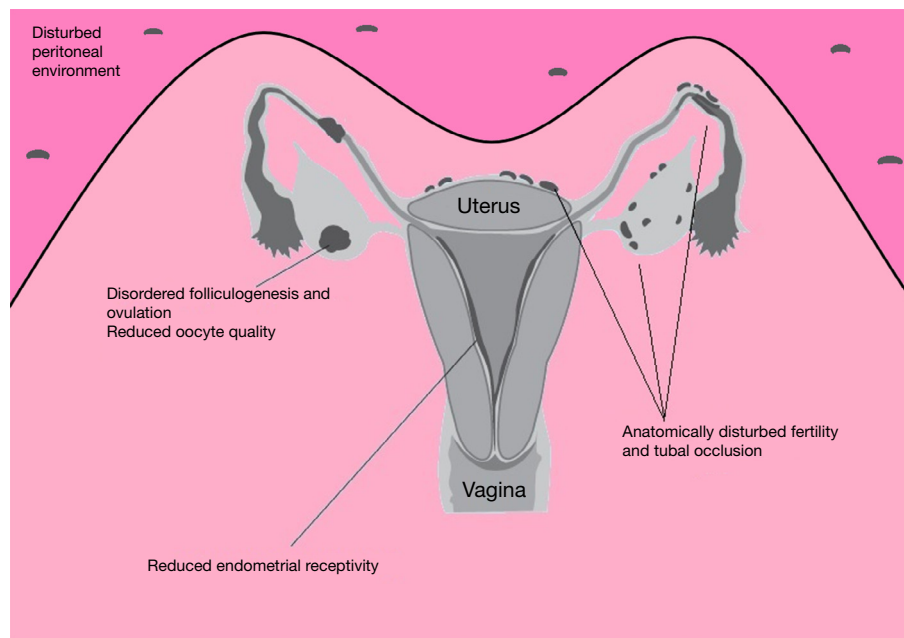


Fig. 1 Mechanisms that may link endometriosis with infertility.

Measuring pain is not an easy task because it is very subjective. Questionnaires and quality-of-life scales may be utilized. They may help to assess the impact of pain and treatment response.

Clinical Examination

A clinical examination may indicate endometriosis on the basis of pelvic adhesions that limit movement or cause pain during uterine or ovarian manipulation, ovarian cysts or palpable nodules.

As an important reminder, clinical examination may be inappropriate in some patients (adolescents, sexual abuse victims, certain religious beliefs) and could be very painful in some women. Other technologies could be of help in these situations. In some cases where vaginal exploration cannot be performed, rectal examination may be helpful.

The evidence in the medical literature is weak for diagnosing pelvic endometriosis with physical examination. Ultrasound and magnetic resonance imaging (MRI) technologies are more accurate at diagnosing ovarian and/or deep endometriosis.

Ultrasonography

Transvaginal ultrasound is not useful in the early stage of endometriosis but remains the best technique for diagnosing ovarian cystic endometriosis and it's useful for identifying and ruling out rectal endometriosis but should be performed by clinicians that are highly experienced.

Magnetic Resonance Imaging (MRI)

This is the most appropriate technique for the diagnosis of deep endometriosis, such as rectovaginal, bladder and ureteral forms. Laparoscopy may have blind spots and this is where MRI can have a key role. MRI can help to visualize those areas that may have escaped the laparoscopic field of view. Such areas include the retroperitoneal space or lesions obscured by dense adhesions. MRI may also be helpful to assess response to treatment and the recurrence of disease.

Laparoscopy

Visualization of the lesions outside the uterine cavity during the laparoscopy and histological verification of endometrial glands is the gold standard diagnostic test for pelvic endometriosis.

Controversy exists concerning whether laparoscopy should be performed in all patients when endometriosis is suspected. It could be argued that empirical treatment can be started without a definitive diagnosis, especially in young women or those who are not in need of diagnosis confirmation. Many women will not want to go through surgery if medical treatment relieves the pain. Conversely, arguments to perform a laparoscopy include the patient's wish to have a definitive diagnosis or advanced disease.

In cases of infertility and ovarian endometriosis, laparoscopy should be approached with caution for the procedure may change from a diagnostic tool into an operative event. Surgery, especially before ART treatment, should be envisioned only in specific circumstances.

Biomarkers

By definition, a biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention. Many studies have focused on identifying biomarkers on blood or urine samples.

May et al. in a comprehensive two article review cover many molecules that have been studied as potential biomarkers. It is not recommended at present to clinically use biomarkers for the diagnosis of endometriosis. New cytokines and miRNAs are being investigated.

By far, the most studied potential marker has been CA-125. A relationship exists between this marker and the presence of endometriosis. It may not be accurate in early stages of disease to distinguish between presence and absence of disease, but it may have higher accuracy at later stages. It also appears to be more commonly elevated with the presence of an endometrioma, but it is still not currently used to diagnose endometriosis. One potential future use may be for treatment monitoring.

Classification of Endometriosis

The most widely accepted endometriosis classification system is that of the American Society for Reproductive Medicine (ASRM). The scoring is used to determine the stages: I or minimal disease, II or mild, III or moderate and IV or severe disease. This system is based on the location, extent, depth of invasion and aspect of the lesions seen at the laparoscopy. The scoring assigned to each type of lesion is arbitrary and is currently not considered to be properly correlated to the symptoms and activity of the disorder but is useful to determining disease burden and management.

Differential Diagnosis of Endometriosis

Other different conditions of the reproductive tract can cause chronic pelvic pain such as adenomyosis, pelvic adhesions, pelvic inflammatory disease, congenital anomalies of the reproductive tract, and ovarian or tubal masses. It may also be related to other non-gynecologic conditions such as irritable bowel syndrome, interstitial cystitis, fibromyalgia, and other musculoskeletal disorders. A thorough evaluation should be carried out in order to exclude other causes of pain and specially in those women that have not responded with conventional therapy.

Treatment of Infertility Associated With Endometriosis

Medical Treatment

Temporary suppression of ovarian function using antigonadotropic agents is one treatment option for endometriosis-associated pain. However, none has demonstrated any efficacy in the restoration of fertility in infertile patients with endometriosis. The European Society of Human Reproduction and Embryology's *ESHRE guideline for the diagnosis and treatment of endometriosis* states that the suppression of ovarian function to improve fertility in patients with minimal or mild endometriosis is not effective and should not be offered for this indication alone. There is no evidence of its possible efficacy in more advanced stages of the disorder either.

Surgical Treatment

The overall objective of surgery is to excise or coagulate the peritoneal lesions, ovarian cysts, areas of deep endometriosis and adhesions. Laparoscopy is the safest and most effective technique. This procedure, although clearly indicated for the surgical treatment of pain caused by endometriosis, is more questionable for the treatment of infertility. Moderate and severe endometriosis may produce such distortion of the pelvis that restoration of the anatomy may be surgically impossible. Moreover, radical surgery that seeks to remove all endometriotic lesions may be detrimental to the ovarian functional reserve, which is already compromised by the endometriosis itself.

The ESHRE guideline concludes that ablation of endometriotic lesions to improve fertility in cases of minimal to mild endometriosis is more effective than diagnostic laparoscopy alone but there are insufficient clinical trials to determine whether surgical treatment of moderate to severe endometriosis improves the subsequent pregnancy rate. There is also no consensus on the indications for surgical treatment of endometriotic cysts in infertile patients. The presence of an endometriotic cyst in a patient undergoing assisted reproduction could reduce the ovarian reserve and the effectiveness of that treatment.

Combined Medical and Surgical Treatment

It has been proposed that preoperative administration of drugs effective at controlling endometriosis could increase the beneficial effect of surgical treatment on fertility. Medical treatment could facilitate excision, while reducing the surgical trauma, the duration of surgery, the formation of postoperative adhesions and the probability of subsequent recurrence. However, there is no proof that these treatments increase fertility after surgery to a greater extent than surgery alone; therefore their use is not recommended.

Postoperative medical treatment has the advantages of encouraging the involution of residual lesions following surgery and reducing the risk of spreading the disorder associated with intraoperative rupture of endometriomas. However, studies assessing the effect of postoperative treatment on fertility have found no additional benefits to those achieved by surgery alone. The ESHRE guideline therefore concludes that postoperative medical treatment offers no advantages in relation to surgical treatment.

Intrauterine Insemination (IUI)

The presence of endometriosis is thought to reduce the effectiveness of IUI, based on the results of studies in patients treated with artificial insemination using donor semen. This negative effect persists even after the treatment of endometriosis. Several randomized studies show that IUI associated with pharmacological stimulation increases fertility in patients with minimal to mild endometriosis in comparison with expectant management. IUI with ovarian stimulation is effective, but the role of unstimulated IUI is uncertain.

In Vitro Fertilization (IVF)

IVF is an appropriate treatment for patients who have advanced endometriosis with reduced ovarian reserve, or if tubal function is compromised or if there is male factor infertility, and/or other treatments have failed. IVF is the only viable method in cases of severe tubal adhesion-related disease or in the presence of large endometriomas. A recent study concluded that patients with endometriosis-associated infertility have pregnancy rates and birth rates similar to tubal factor controls during IVF treatments. The exception is women with endometriomas, who have lower success rates compared with peritoneal endometriosis and tubal infertility.

Women with endometrioma have a lower mean number of oocyte retrieved and require higher FSH dosage for ovarian stimulation, suggesting that their ovarian reserve is diminished prior to IVF. Women with endometriomas should be counseled regarding their increased risk of cycle cancellation.

When analyzing the impact of endometriosis on uterine environment there was no difference in pregnancy rates between women with endometriosis and tubal factor receiving donor oocytes, suggesting that endometriosis is not detrimental to embryo implantation (26, 27). Recently, we demonstrated that the endometrial receptivity gene signature during the window of implantation is similar in infertile women with and without endometriosis, also for different stages of endometriosis.

Another relevant aspect is whether patients with ovarian endometriosis who are scheduled for IVF would benefit from prior excision of the endometriomas. The chosen procedure should consider the specific condition of each patient, taking into account the likelihood of contributing to ovarian failure through intraoperative destruction of normal tissue, especially in previously operated patients. Whilst surgery did not seem to influence the live birth rate, surgical treatment of endometrioma prior to IVF could exert a further detrimental impact on ovarian reserve. There is therefore not one dogmatic recommendation as to whether women with endometrioma should or should not have surgical intervention prior to IVF.

In summary, there is no evidence that surgical treatment of endometriosis improves ovarian function or enhances the possibility of successful IVF. Based on current evidence, consideration should be given to individualize the care of these patients.

Conclusions

IVF is the most appropriate treatment for infertility in women with endometriosis, especially if there are coexisting causes for the infertility and/or other treatments have failed.

Unfortunately, IVF pregnancy rates are lower in women with endometriosis –oocytes have poorer quality- and diminished ovarian reserve –women with endometriosis produce lower number of oocytes-. Surgical removal of ovarian endometriotic cysts prior to IVF does not offer any additional benefit in terms of fertility outcomes and we should take into account that it could reduce even more the ovarian reserve. Surgery is indicated in cases of pelvic pain when women are young (under 35). Fertility preservation may be considered prior to surgery is ovarian function could be compromised.

Further Reading

- Arruda, M. S. (2003). Time elapsed from onset of symptoms to diagnosis of endometriosis in a cohort study of Brazilian women. *Human Reproduction*, 18(4), 756–759.
- Ballard, K., Lowton, K., & Wright, J. (2006). What's the delay? A qualitative study of women's experiences of reaching a diagnosis of endometriosis. *Fertility and Sterility*, 86(5), 1296–1301.
- de la Fuente, G., & Garcia-Velasco, J. A. (2010). Endometriosis and infertility. *Gynaecology Forum*, 15(2), 8–12.
- Diaz, I., Navarro, J., Blasco, L., Simón, C., Pellicer, A., et al. (2000). Impact of stage III–IV endometriosis on recipients of sibling oocytes: Matched case-control study. *Fertility and Sterility*, 74(1), 31–34.
- Dunselman, G. A. J., Vermeulen, N., Becker, C., Calhaz-Jorge, C., D'Hooghe, T., et al. (2014). ESHRE guideline: Management of women with endometriosis. *Human Reproduction*, 29(3), 400–412.
- Garcia-Velasco, J. A., Fassbender, A., Ruiz-Alonso, M., Blesa, D., D'hooghe, T., et al. (2015). Is endometrial receptivity transcriptomics affected in women with endometriosis? A pilot study. *Reproductive Biomedicine Online*, 31(5), 647–654.
- Garcia-Velasco, J. A., & Somigliana, E. (2009). Management of endometriomas in women requiring IVF: To touch or not to touch. *Human Reproduction*, 24(3), 496–501.
- Giudice, L. C. (2010). Endometriosis. *The New England Journal of Medicine*, 362(25), 2389–2398.
- Hadfield, R., Mardon, H., Barlow, D., & Kennedy, S. (1996). Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. *Human Reproduction*, 11(4), 878–880.
- Hamdan, M., Dunselman, G., Li, T. C., & Cheong, Y. (2015). The impact of endometrioma on IVF/ICSI outcomes: A systematic review and meta-analysis. *Human Reproduction Update*, 21(6), 809–825.
- Hudelist, G., Fritzer, N., Thomas, A., Niehues, C., Oppelt, P., et al. (2012). Diagnostic delay for endometriosis in Austria and Germany: Causes and possible consequences. *Human Reproduction*, 27(12), 3412–3416.
- Hughes, E., Brown, J., Collins, J. J., Farquhar, C., Fedorkow, D. M., et al. (2007). Ovulation suppression for endometriosis. *Cochrane Database of Systematic Reviews*, 3, CD000155.
- Jacobson, T. Z., Duffy, J. M., Barlow, D., Farquhar, C., Koninckx, P. R., et al. (2010). Laparoscopic surgery for subfertility associated with endometriosis. *Cochrane Database of Systematic Reviews*, 3, CD001398.
- Jansen, R. (1986). Minimal endometriosis and reduced fecundability: Prospective evidence from an artificial insemination by donor program. *Fertility and Sterility*, 46(1), 141–143.
- Kennedy, S. (2006). Should a diagnosis of endometriosis be sought in all symptomatic women? *Fertility and Sterility*, 86(5), 1312–1313.
- Kennedy, S., Bergqvist, A., Chapron, C., D'Hooghe, T., Dunselman, G., et al. (2005). ESHRE guideline for the diagnosis and treatment of endometriosis. *Human Reproduction*, 20(10), 2698–2704.
- May, K. E., Conduit-Hulbert, S. A., Villar, J., Kirtley, S., Kennedy, S. H., et al. (2010). Peripheral biomarkers of endometriosis: A systematic review. *Human Reproduction Update*, 16(6), 651–674.
- May, K. E., Villar, J., Kirtley, S., Kennedy, S. H., & Becker, C. M. (2011). Endometrial alterations in endometriosis: A systematic review of putative biomarkers. Vol. 17. *Human Reproduction Update*, 17(5), 637–653.
- Maiorana, A., Cicerone, C., & Niceta, M. A. L. (2007). Evaluation of serum CA 125 levels in patients with pelvic pain related to endometriosis. *The International Journal of Biological Markers*, 22(3), 200–202.
- Mol, B. W., Bayram, N., Lijmer, J. G., Wiegman, M. A., Bongers, M. Y., et al. (1998). The performance of CA-125 measurement in the detection of endometriosis: A meta-analysis. *Fertility and Sterility*, 70(6), 1101–1108.
- Nnoaham, K. E., Hummelshoj, L., Webster, P., d'Hooghe, T., de Cicco Nardone, F., et al. (2011). Impact of endometriosis on quality of life and work productivity: A multicenter study across ten countries. *Fertility and Sterility*, 96(2), 366–373.

- Opøien, H. K., Fedorcsak, P., Omland, A. K., Abyholm, T., Bjercke, S., et al. (2012). In vitro fertilization is a successful treatment in endometriosis-associated infertility. *Fertility and Sterility*, 97(4), 912–918.
- Ozkan, S., Murk, W., & Arici, A. (2008). Endometriosis and infertility. Epidemiology and evidence-based treatments. *Annals of the New York Academy of Sciences*, 1127, 92–100.
- Senapati, S., Sammel, M. D., Morse, C., & Barnhart, K. T. (2016). Impact of endometriosis on in vitro fertilization outcomes: an evaluation of the Society for Assisted Reproductive Technologies Database. *Fertility and Sterility*, 106(1), 164–171.
- Simón, C., Gutiérrez a, V.a., de los Santos, M. J., Tarin, J. J., et al. (1994). Outcome of patients with endometriosis in assisted reproduction: Results from in-vitro fertilization and oocyte donation. *Human Reproduction*, 9(4), 725–729.
- The Practice Committee of the American Society for Reproductive Medicine. (2004). Endometriosis and infertility. *Fertility and Sterility*, 81(5), 1441–1446.
- Toma, S. K., Stovall, D. W., & Hammond, M. G. (1992). The effect of laparoscopic ablation or danocrine on pregnancy rates in patients with stage I or II endometriosis undergoing donor insemination. *Obstetrics and Gynecology*, 80(2), 253–256.
- Tummon, I. S., Asher, L. J., Martin, J. S. B., & Tulandi, T. (1997 Jul). Randomized controlled trial of superovulation and insemination for infertility associated with minimal or mild endometriosis. *Fertility and Sterility*, 68(1), 8–12.
- Yap, C., Furness, S., & Farquhar, C. (2004). Pre and post operative medical therapy for endometriosis surgery. *Cochrane Database of Systematic Reviews*, 3, CD003678.

Infertility Treatment in PCOS

Ian Waldman and Richard S Legro, Penn State College of Medicine, Hershey, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Since first being described in 1935 by Stein and Leventhal (Stein, 1935), polycystic ovary syndrome (PCOS) is now known to be the most common endocrinopathy in reproductive aged women with multiple medical, metabolic, and reproductive implications. PCOS is a complex heterogeneous disorder and therefore appropriate diagnosis has been debated for many years. The most widely accepted diagnostic criteria are the 2003 Rotterdam criteria, which were subsequently endorsed by the European Society for Human Reproduction and Embryology and the American Society of Reproductive Medicine. The Rotterdam Criteria based the diagnosis of PCOS by the presence of oligo/anovulation, clinical or biochemical signs of hyperandrogenism, and ultrasound evidence of polycystic morphology. Diagnosis is based on the presence of two of the three while excluding other disorders, including Cushing's Syndrome, atypical congenital adrenal hyperplasia, androgen secreting tumors, thyroid disorders, and other disorders causing oligo/anovulation (Group REA-SPcw, 2004).

PCOS is the most common cause of irregular and decreased ovulation, and the most common cause of female infertility affecting 1 in 15 women (Azziz et al., 2004). There is no optimal treatment for infertility in the patient with PCOS and treatment should be individualized based on patient presentation. Optimizing outcome and decreasing risks should be the focus of treatment decision-making, but heterogeneity in study-design, diagnosis, and primary outcome (optimally live-birth rate) make comparisons difficult. This article will review the current treatment options for the infertile patient diagnosed with PCOS.

Life-Style Modification

Obesity is an epidemic worsening throughout the world, but is particularly problematic in the United States, with 38% of women currently afflicted (Ogden et al., 2015). Obesity has been determined to be an independent risk factor for infertility (Sim et al., 2014a; Sim et al., 2014b). As many as 80% of patient's with PCOS in the United States have a concomitant diagnosis of obesity, (Sam, 2007) thus making this an important and growing fertility management issue. The prevalence of obesity among reproductive-aged women is increased in most developed countries around the world.

Weight loss has been recommended as a first line treatment for obese women with infertility prior to attempting other methods of assisted reproductive technology (ART), secondary to the multiple negative effects obesity has on fecundity and fertility (Gesink Law et al., 2007). The implications of obesity are not solely related to infertility, but more significantly to worsening pregnancy outcomes. Obese women were found to have increased rates of stillbirth, gestational diabetes, hypertensive disorders, cesarean delivery, operative vaginal delivery, prolonged labor, shoulder dystocia, fetal neural tube defects, fetal cardiac defects, and neonatal death (Linné, 2004; Cedergren, 2004).

Obese women had a rate of menstrual disturbance three times more frequently than nonobese women and obese women were found to have a greater than $2.5 \times$ higher chance of ovulatory infertility (Norman et al., 2004; Rich-Edwards et al., 1994). Studies have set out to determine if a reduction in weight would lead to improvement in ovulation rates, pregnancy rates, and ultimately live-birth rates.

Studies have shown reasonable goals when recommending weight loss, and found as little as a 5% reduction in current weight, with resumption of ovulation (Crosignani et al., 2003). Likewise, as central adipose tissue is more metabolically active and disruptive to reproduction, a decrease in central adipose tissue improves ovulation rates (Palomba et al., 2008). Just as a decrease in 5% of body weight led to an improvement in ovulatory rates and also to improve pregnancy rates (Crosignani et al., 2003; Kiddy et al., 1992). The primary goal of recommending weight loss prior to attempting pregnancy is to improve pregnancy outcomes, as well as improve live-birth rates. Clark et al., in a prospective trial, found an improvement in miscarriage rate, spontaneous ovulation rate, pregnancy rate, and live birth rate. Women in this study lost an average of 10.2 kg/m^2 (Clark et al., 1998).

In a randomized prospective trial by Legro et al., evaluated preconception intervention with either oral contraceptive pills (OCP), lifestyle modification with a goal of 7% weight loss, or combination of the two. The intensive lifestyle intervention consisted of meal replacements to control caloric intake, antiobesity medication, brief psychological counseling and increase physical activity, indicated that meaningful weight loss can be obtained in a relatively short period (16 weeks) (Fig. 1). They were followed to determine increase in ovulation rates and subsequently live birth rates. Ovulation rates were found to be superior after weight loss with control group ovulatory rates of 46%, lifestyle change 60%, and combination 67%. Live birth rates were not found to be significantly different between the three groups, but after a post-hoc analysis combining the two weight loss groups they approached statistical significance with a *P* value of 0.05 (Legro et al., 2015). In a follow-up study by Legro et al., a secondary analysis of two randomized trials was performed to evaluate immediate treatment with ovulation induction compared with delayed treatment after lifestyle modification. Delaying treatment for lifestyle modification offered a significantly improved ovulation rate and live birth rate (Legro et al., 2016).

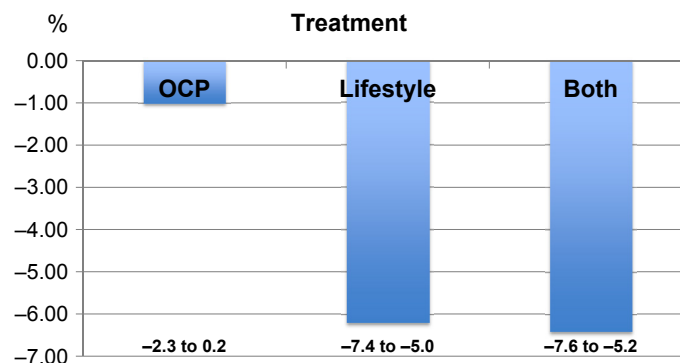


Fig. 1 Effects of a 16 week preconception intervention on weight from baseline. Numbers underneath the columns are 95% Confidence intervals. Significant weight loss was achieved with the intensive lifestyle modification with or without the use of oral contraceptives (OCP). From Legro, R. S., Dodson, W. C., Kris-Etherton, P. M., et al. (2015). Randomized controlled trial of preconception interventions in infertile women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism* **100**, 4048–58.

First-Line Treatment

Women with PCOS have a monthly spontaneous ovulation rate of 32%, (Azziz et al., 2001), and often require ovulation induction if lifestyle modification alone does not result in spontaneous pregnancy. There are currently two primary medications by which ovulation induction is executed, clomiphene and letrozole. Letrozole will likely in the near future become the first line treatment for ovulation induction in women with PCOS, but there remain in some countries regulatory barriers to its use. Thus we will begin with clomiphene as a first line therapy. Clomiphene is a selective estrogen receptor modulator (SERM) with partial agonist and antagonist effects. The primary method by which clomiphene works is by antagonistic effects on the hypothalamic estrogen receptors preventing negative feedback resulting in increased secretion of gonadotropin releasing hormone (GnRH) and subsequently altering gonadotropins favorably stimulating ovarian follicular development (Kerin et al., 1985).

Clomiphene has been used since the 1960's for ovulation induction and is considered the first line treatment for ovulation induction (Dickey et al., 1998). Studies have shown ovulation rates of up to 80%, (Messinis, 2005), pregnancy rates up to 60% (Imani et al., 2002), and live birth rates up to 22% (Group TEA-SPCW, 2008). In the Pregnancy in Polycystic Ovary Syndrome I (PPCOS 1) trial, a randomized controlled trial (RCT) found ovulation rates of 49%, pregnancy rates of 30%, and live-birth rates of 23% over six ovulation induction cycles (Legro et al., 2007). It is recommended to move on to a second or third line therapy after six failed cycles of ovulation induction with a single agent (Group TEA-SPCW, 2008).

The standard dose of clomiphene is to initiate with 50 mg/day for 5 days starting on cycle day 2–5 (there is no consensus on which cycle day to begin). This dose can be increased by 50 mg/day up to 150 mg/day and the lowest dose that initiates ovulation should be maintained for the recommended 6 cycles (Group TEA-SPCW, 2008). As many patients with PCOS are anovulatory, many practitioners initiate a menstrual withdrawal bleed with progestin. Cycle initiation with a progestin withdrawal bleed may decrease both pregnancy and live-birth rate, and therefore should not be performed prior to initiation of clomiphene (Diamond et al., 2012). Protocols, such as the stair step protocol advance the dose of clomiphene after a brief period of observation for follicular development without inducing a withdrawal bleed. Empirical evidence thus suggests that in the absence of abnormal bleeding or suspected endometrial pathology, that the dose of clomiphene (or letrozole) can be increased without routine induction of a withdrawal bleed. Of course periodic endometrial cycling is necessary over a longer period of time (3–6 months).

In a recent double blind RCT, Morin-Papunen et al., showed Metformin in combination with clomiphene for ovulation induction with obese and nonobese patients improves pregnancy (13.2% improvement, $P = 0.006$) and live-birth rates (13.1% improvement, $P = 0.014$) compared to placebo. Cox regression analysis showed the addition of metformin increased pregnancy rates 1.6 times (Morin-Papunen et al., 2012).

The most common adverse effects of clomiphene administration is hot flushes, headaches, and visual complaints, but is usually well tolerated (Group TEA-SPCW, 2008). The multiple pregnancy rate has been found to be up to 8% (Liu et al., 2014) and the potential risk of ovarian hyperstimulation syndrome (OHSS) is very low (Group TEA-SPCW, 2008; Brown and Farquhar, 2016).

Letrozole is an aromatase inhibitor that prevents conversion of androgens to estrogens. Its primary action is by suppressing estrogen production, thereby decreasing the negative feedback of estrogens in the hypothalamus leading to increase GnRH production and subsequent ovarian follicular development. The typical starting dose for letrozole is 2.5 mg/day on cycle days 3–7 and if ovulation does not occur, the dose should be increased to 5 mg/day with a maximum dose of 7.5 mg/day. If ovulation is confirmed and pregnancy does not occur, the dose should be repeated for up to 6 subsequent cycles.

Letrozole has been evaluated and initially postulated to have improved outcomes and side-effect profile in comparison to Clomiphene for ovulation induction. In a large RCT by Legro et al., there was found to be a statistically higher ovulation rate by 13.4%, and live birth rate by 8.4%, for Letrozole compared to Clomiphene (Fig. 2). No differences were found in congenital anomalies, pregnancy loss, or multiple pregnancy rates. Letrozole was associated with a higher incidence of fatigue and dizziness compared to Clomiphene (Legro et al., 2014). In a systematic review and meta-analysis, Letrozole was found to have increased

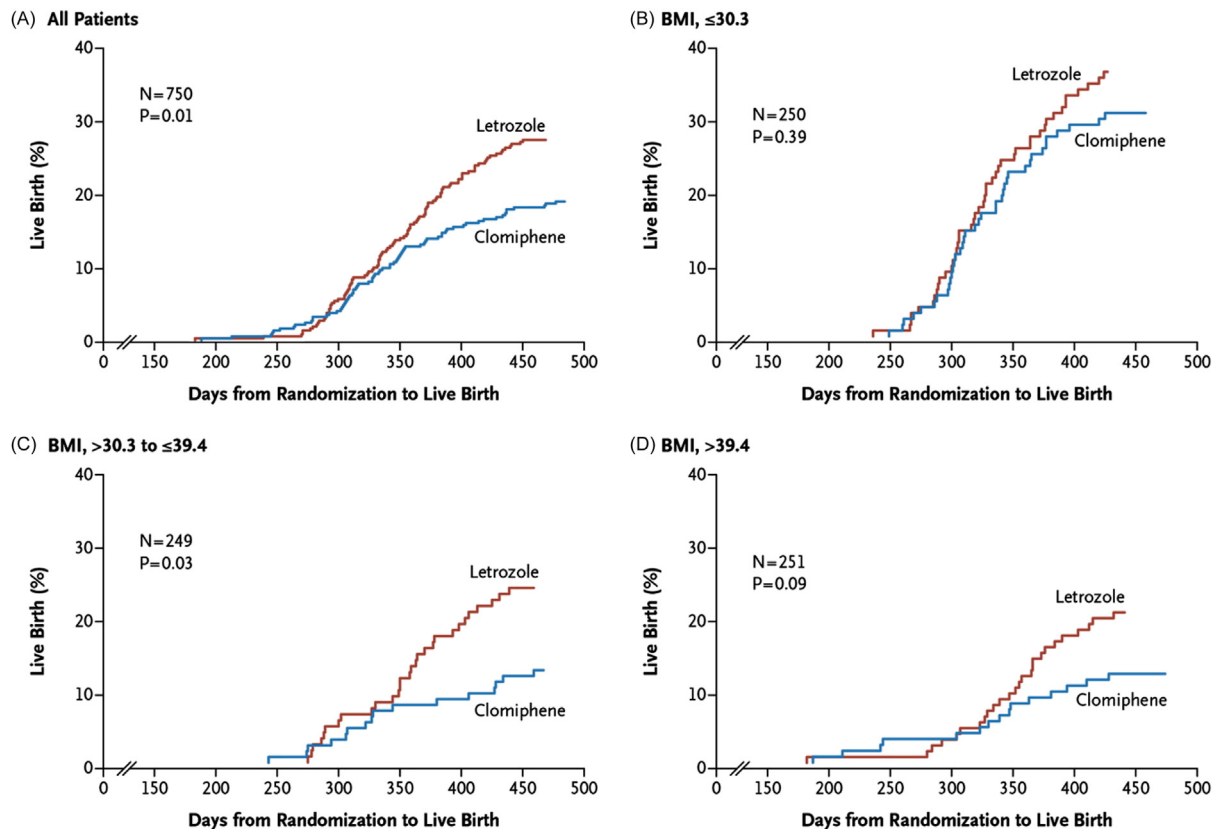


Fig. 2 Kaplan-Meier curves for Live Birth in the PPCOS II trial of letrozole vs. clomiphene. Live-birth rates are shown according to treatment group in Panel A and according to treatment group and maternal body-mass index (BMI, the weight in kilograms divided by the square of the height in meters), in thirds, in Panels B, C, and D. Letrozole improved the live birth rate by 44% over clomiphene with significant improvement or trends toward improvements at all weight classes. From Legro, R. S., Brzyski, R. G., Diamond, M. P., et al. (2014). Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. *The New England Journal of Medicine* 371, 119–129.

live-birth rates and decreased multiple pregnancy rate (Wang et al., 2017). Secondary to these findings, it has been suggested that Letrozole be used as a first line treatment in patients with PCOS for ovulation induction, but as of now, has not officially been adopted by ASRM or ESHRE.

Second-Line Treatment

Clomiphene and Letrozole resistance (i.e., failure to ovulate) is not uncommon among women with PCOS (Imani et al., 1998), affecting approximately 25% of patients challenged with clomiphene and 10% challenged with letrozole. Therefore low dose gonadotropin induction is considered the second-line in medical management for ovulation induction. In a prospective analysis, Eijkemans et al. found a singleton live birth rate of 56% with cumulative live-birth rates of 50% and 71% at 12 and 24 months, respectively (Eijkemans et al., 2003). There are multiple negative effects of gonadotropin induction, including increased risk of OHSS (Swanton et al., 2010), increased multiple pregnancy rates (Legro, 2016), higher cost (Homburg et al., 2012), and increased burden of medication usage, with no oral form available and need for regular ultrasound monitoring. The use of low-dose step-up follicle stimulating hormone (FSH) regimens have resulted in a markedly decreased rate of OHSS, as well as multiple pregnancy rate (Homburg and Howles, 1999).

We have thus far discussed lifestyle modification and medical treatments for the PCOS patient with infertility, but there does exist a surgical option for the clomiphene resistant patient, ovarian drilling. Surgical management for patients with PCOS was first described in the late 1930s by Stein and Leventhal in the form of ovarian wedge resection (CM, 1939), but has since been replaced with laparoscopic ovarian drilling, first described in the 1980s secondary to the concern for postoperative pelvic adhesive disease (Gjønnaess, 1984). Laparoscopic ovarian drilling is performed by creating multiple small perforations with either electrocautery or laser within the ovary. The mechanism of action is not completely understood, but it has been postulated the decrease in ovarian follicles secreting sex steroids decreases the negative feedback on FSH leading to upregulation within the hypothalamus and improved FSH secretion and resumption of ovulation. It is also plausible surgery increases blood flow to the ovaries and increases delivery of gonadotropins, but less likely that it improves insulin sensitivity (Abu Hashim et al., 2013).

In a Cochrane database systematic review of randomized controlled trials, Farquhar et al. evaluated outcomes in patients undergoing laparoscopic ovarian drilling compared with ovulation induction. They found a live-birth rate of 34% with no difference when compared with clomiphene and tamoxifen (a selective estrogen receptor modulator), gonadotropins, or aromatase inhibitors, but decreased compared with clomiphene and metformin. Gonadotropin ovulation induction showed a significantly increased multiple pregnancy rates compared with laparoscopic ovarian drilling. There were no cases of OHSS with ovarian drilling alone, and no difference in clinical pregnancy rates or miscarriage rates with ovarian drilling compared with medical management. There was no difference between unilateral or bilateral drilling with regard to ovulation rate, pregnancy rate, or live birth rate (Farquhar et al., 2012).

In long-term follow-up studies evaluating persistent ovulation rates after ovarian drilling found 7% in patients monitored for 3 years and 38% in those monitored for up to 9 years (Amer et al., 2002). The total pregnancy rate was found to be 82% at 2 years in another study (Felemban et al., 2000). These findings led Nahuis et al. to conclude that laparoscopic ovarian drilling is as effective as gonadotropins for ovulation induction, with the added benefit of decreasing need for other forms of artificial reproductive technology and increasing likelihood of chance for another child (Nahuis et al., 2011).

The major concern in ovarian wedge resection was postoperative adhesion formation and that concern continues for laparoscopic ovarian drilling. In a randomized trial performing second-look laparoscopy after ovarian drilling found ovarian adhesions in 60% of women with no difference in adhesion formation severity by number of drilled holes (Mercorio et al., 2008). The theoretical risk of infertility secondary to tubal occlusion from pelvic adhesive disease is not validated based on the fertility outcomes stated above, but does need adequately powered prospective trials to further elucidate this concern.

In Vitro Fertilization

If first and second line managements are unsuccessful in treating the infertile patient with PCOS, then another form of artificial reproductive technology exists which can be utilized: In vitro fertilization (IVF)/Intracytoplasmic sperm injection (ICSI). IVF/ICSI is the process whereby the ovaries are stimulated to form mature eggs that are ultimately retrieved and fertilized by sperm in the lab. The fertilized embryo is then implanted into the prepared uterus.

There is much debate regarding the ideal treatment method for controlled ovarian hyperstimulation with GnRH agonists having survived the test of time but now being replaced by antagonist protocols in high responders. The debate continues to exist with the significant risk of OHSS primarily in the PCOS patient population (Heijnen et al., 2006). The risk of OHSS is characterized by high rates of abdominal fluid (ascites), pleural and pericardial effusions, hemoconcentration, and electrolyte disorders (Delvigne et al., 1993). The ultimate goal is to improve, or at least maintain, the live-birth rate while decreasing the potential risk for severe OHSS.

The debate continues between agonist vs. antagonist protocols for ovarian hyperstimulation with conflicting reports. GnRH antagonist have begun to be preferred for controlled ovarian hyperstimulation secondary to newer studies and meta-analysis showing no difference in clinical pregnancy rate or abortion rate with a decrease in OHSS (Mancini et al., 2011). Lin et al., in a recent meta-analysis, also found no difference in clinical pregnancy rate and ongoing pregnancy rate in GnRH antagonist protocol while reducing the rate of severe OHSS (Lin et al., 2014). Pundir et al. found no difference in severe OHSS, but when pooled with moderate and severe OHSS there was a significant decrease (Pundir et al., 2012). Al-Inany et al. reported moderate evidence to suggest a decrease in OHSS with an antagonist protocol in a Cochrane review, (Al-Inany et al., 2016), while Singh et al. found similar pregnancy rates, as well as no difference in OHSS (Singh et al., 2014).

On top of the decision to perform an agonist or antagonist cycle, is the decision to undergo final oocyte maturation trigger with the traditionally used human chorionic gonadotropin (HCG) or a GnRH agonist (if using an antagonist protocol). The reason to perform one or the other is to continue to optimize the live-birth rate and attempt to decrease the rate of OHSS. Youssef et al. in a Cochrane review, found a significant decrease in the rate of OHSS when using a GnRH agonist trigger compared to an HCG trigger. The problem with this GnRh trigger are data suggesting a decrease in live-birth rate and ongoing pregnancy rate, while also increasing potential for early miscarriage rate. However these findings were for fresh autologous embryo transfers. When evaluating the data for donor-recipient cycles, there was no difference in live-birth rate or ongoing pregnancy rate (Youssef et al., 2014). This suggests that in a frozen embryo transfer, GnRH agonists could be considered for oocyte maturation without detriment to live-birth rate, while decreasing the risk of OHSS. An insufficient luteal phase was posited as the cause and subsequent treatment has including luteal phase support after GnRh agonist trigger. These observations also led to the hypothesis that elective frozen embryo transfer among high responders, such as women with PCOS may improve live birth rates.

This hypothesis was recently evaluated in a multicenter prospective randomized trial evaluating fresh versus frozen embryo transfers in patients with PCOS. They found a higher live-birth rate mediated primarily through a lower pregnancy loss rate, a markedly decreased risk of OHSS and a higher singleton birthweight among women who were randomized to the elective FET. However there was an increased risk of preeclampsia among these women and a trend toward increased neonatal deaths in the frozen-embryo group, but did not reach statistical significance (Chen et al., 2016). In a Cochrane review including the prior study, they found no difference in live-birth rate, a decrease in OHSS, and decrease in miscarriage rate for frozen embryo transfers compared to traditional IVF/ICSI cycles (Wong et al., 2017). In the Cochrane review, they reported significant risk of bias in the studies analyzed with unclear blinding, unit of analysis error, and absence of adequate termination rules. Secondary to these findings the debate regarding freeze all cycles versus traditional IVF/ICSI in women with PCOS remains, and further high quality studies are needed.

Conclusion

This chapter has reviewed the treatment and current controversies in the polycystic ovary syndrome patient with infertility. Much research is still necessary to optimize outcomes and decrease risks.

References

- Abu Hashim, H., Al-Inany, H., De Vos, M., & Tournaye, H. (2013). Three decades after Gjönnæss's laparoscopic ovarian drilling for treatment of PCOS; what do we know? An evidence-based approach. *Archives of Gynecology and Obstetrics*, 288, 409–422.
- Al-Inany, H. G., Youssef, M. A., Ayeleke, R. O., Brown, J., Lam, W. S., & Broekmans, F. J. (2016). Gonadotrophin-releasing hormone antagonists for assisted reproductive technology. *Cochrane Database of Systematic Reviews*, 4, CD001750.
- Amer, S. A., Gopalan, V., Li, T. C., Ledger, W. L., & Cooke, I. D. (2002). Long term follow-up of patients with polycystic ovarian syndrome after laparoscopic ovarian drilling: Clinical outcome. *Human Reproduction*, 17, 2035–2042.
- Azziz, R., Ehrmann, D., Legro, R. S., et al. (2001). Troglitazone improves ovulation and hirsutism in the polycystic ovary syndrome: A multicenter, double blind, placebo-controlled trial. *The Journal of Clinical Endocrinology and Metabolism*, 86, 1626–1632.
- Azziz, R., Woods, K. S., Reyna, R., Key, T. J., Knochenhauer, E. S., & Yildiz, B. O. (2004). The prevalence and features of the polycystic ovary syndrome in an unselected population. *The Journal of Clinical Endocrinology and Metabolism*, 89, 2745–2749.
- Brown, J., & Farquhar, C. (2016). Clomiphene and other antioestrogens for ovulation induction in polycystic ovarian syndrome. *Cochrane Database of Systematic Reviews*, 12, CD002249.
- Cedergren, M. I. (2004). Maternal morbid obesity and the risk of adverse pregnancy outcome. *Obstetrics and Gynecology*, 103, 219–224.
- Chen, Z. J., Shi, Y., Sun, Y., et al. (2016). Fresh versus frozen embryos for infertility in the polycystic ovary syndrome. *The New England Journal of Medicine*, 375, 523–533.
- Clark, A. M., Thornley, B., Tomlinson, L., Galletley, C., & Norman, R. J. (1998). Weight loss in obese infertile women results in improvement in reproductive outcome for all forms of fertility treatment. *Human Reproduction*, 13, 1502–1505.
- CM, S. I. F. (1939). Surgical treatment of bilateral polycystic ovaries. *American Journal of Obstetrics and Gynecology*, 38.
- Crosignani, P. G., Colombo, M., Vegetti, W., Somigliana, E., Gessati, A., & Ragni, G. (2003). Overweight and obese anovulatory patients with polycystic ovaries: Parallel improvements in anthropometric indices, ovarian physiology and fertility rate induced by diet. *Human Reproduction*, 18, 1928–1932.
- Delvigne, A., Dubois, M., Battheu, B., et al. (1993). The ovarian hyperstimulation syndrome in in-vitro fertilization: A Belgian multicentric study. II. Multiple discriminant analysis for risk prediction. *Human Reproduction*, 8, 1361–1366.
- Diamond, M. P., Kruger, M., Santoro, N., et al. (2012). Endometrial shedding effect on conception and live birth in women with polycystic ovary syndrome. *Obstetrics and Gynecology*, 119, 902–908.
- Dickey, R. P., Taylor, S. N., Rye, P. H., & Lu, P. Y. (1998). Future use of clomiphene in ovarian stimulation. A role for clomiphene in the 21st century? *Human Reproduction*, 13, 2361–2362.
- Eijkemans, M. J., Imani, B., Mulders, A. G., Habbema, J. D., & Fauser, B. C. (2003). High singleton live birth rate following classical ovulation induction in normogonadotrophic anovulatory infertility (WHO 2). *Human Reproduction*, 18, 2357–2362.
- Farquhar, C., Brown, J., & Marjoribanks, J. (2012). Laparoscopic drilling by diathermy or laser for ovulation induction in anovulatory polycystic ovary syndrome. *Cochrane Database of Systematic Reviews*, CD001122.
- Felemban, A., Tan, S. L., & Tulandi, T. (2000). Laparoscopic treatment of polycystic ovaries with insulated needle cautery: A reappraisal. *Fertility and Sterility*, 73, 266–269.
- Gesink Law, D. C., Maclellan, R. F., & Longnecker, M. P. (2007). Obesity and time to pregnancy. *Human Reproduction*, 22, 414–420.
- Gjönnæss, H. (1984). Polycystic ovarian syndrome treated by ovarian electrocautery through the laparoscope. *Fertility and Sterility*, 41, 20–25.
- Group REA-SPcw. (2004). Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Human Reproduction*, 19, 41–47.
- Group TEA-SPCW. (2008). Consensus on infertility treatment related to polycystic ovary syndrome. *Human Reproduction*, 23, 462–477.
- Heijnen, E. M., Eijkemans, M. J., Hughes, E. G., Laven, J. S., Macklon, N. S., & Fauser, B. C. (2006). A meta-analysis of outcomes of conventional IVF in women with polycystic ovary syndrome. *Human Reproduction Update*, 12, 13–21.
- Homburg, R., & Howles, C. M. (1999). Low-dose FSH therapy for anovulatory infertility associated with polycystic ovary syndrome: Rationale, results, reflections and refinements. *Human Reproduction Update*, 5, 493–499.
- Homburg, R., Hendriks, M. L., König, T. E., et al. (2012). Clomifene citrate or low-dose FSH for the first-line treatment of infertile women with anovulation associated with polycystic ovary syndrome: A prospective randomized multinational study. *Human Reproduction*, 27, 468–473.
- Imani, B., Eijkemans, M. J., te Velde, E. R., Habbema, J. D., & Fauser, B. C. (1998). Predictors of patients remaining anovulatory during clomiphene citrate induction of ovulation in normogonadotrophic oligoamenorrheic infertility. *The Journal of Clinical Endocrinology and Metabolism*, 83, 2361–2365.
- Imani, B., Eijkemans, M. J., te Velde, E. R., Habbema, J. D., & Fauser, B. C. (2002). A nomogram to predict the probability of live birth after clomiphene citrate induction of ovulation in normogonadotrophic oligoamenorrheic infertility. *Fertility and Sterility*, 77, 91–97.
- Kerin, J. F., Liu, J. H., Phillipou, G., & Yen, S. S. (1985). Evidence for a hypothalamic site of action of clomiphene citrate in women. *The Journal of Clinical Endocrinology and Metabolism*, 61, 265–268.
- Kiddy, D. S., Hamilton-Fairley, D., Bush, A., et al. (1992). Improvement in endocrine and ovarian function during dietary treatment of obese women with polycystic ovary syndrome. *Clinical Endocrinology*, 36, 105–111.
- Legro, R. S. (2016). Ovulation induction in polycystic ovary syndrome: Current options. *Best Practice & Research. Clinical Obstetrics & Gynaecology*, 37, 152–159.
- Legro, R. S., Barnhart, H. X., Schlaff, W. D., et al. (2007). Clomiphene, metformin, or both for infertility in the polycystic ovary syndrome. *The New England Journal of Medicine*, 356, 551–566.
- Legro, R. S., Brzyski, R. G., Diamond, M. P., et al. (2014). Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. *The New England Journal of Medicine*, 371, 119–129.
- Legro, R. S., Dodson, W. C., Kris-Etherton, P. M., et al. (2015). Randomized controlled trial of preconception interventions in infertile women with polycystic ovary syndrome. *The Journal of Clinical Endocrinology and Metabolism*, 100, 4048–4058.
- Legro, R. S., Dodson, W. C., Kunselman, A. R., et al. (2016). Benefit of delayed fertility therapy with preconception weight loss over immediate therapy in obese women with PCOS. *The Journal of Clinical Endocrinology and Metabolism*, 101, 2658–2666.
- Lin, H., Li, Y., Li, L., Wang, W., Yang, D., & Zhang, Q. (2014). Is a GnRH antagonist protocol better in PCOS patients? A meta-analysis of RCTs. *PLoS One*, 9, e91796.
- Linné, Y. (2004). Effects of obesity on women's reproduction and complications during pregnancy. *Obesity Reviews*, 5, 137–143.
- Liu, A., Zheng, C., Lang, J., & Chen, W. (2014). Letrozole versus clomiphene citrate for unexplained infertility: A systematic review and meta-analysis. *The Journal of Obstetrics and Gynaecology Research*, 40, 1205–1216.
- Mancini, F., Tur, R., Martinez, F., Coroleu, B., Rodriguez, I., & Barri, P. N. (2011). Gonadotrophin-releasing hormone-antagonists vs long agonist in in-vitro fertilization patients with polycystic ovary syndrome: A meta-analysis. *Gynecological Endocrinology*, 27, 150–155.

- Mercorio, F., Mercorio, A., Di Spiezio Sardo, A., Barba, G. V., Pellicano, M., & Nappi, C. (2008). Evaluation of ovarian adhesion formation after laparoscopic ovarian drilling by second-look minilaparoscopy. *Fertility and Sterility*, 89, 1229–1233.
- Messinis, I. E. (2005). Ovulation induction: A mini review. *Human Reproduction*, 20, 2688–2697.
- Morin-Papunen, L., Rantala, A. S., Unkila-Kallio, L., et al. (2012). Metformin improves pregnancy and live-birth rates in women with polycystic ovary syndrome (PCOS): A multicenter, double-blind, placebo-controlled randomized trial. *The Journal of Clinical Endocrinology and Metabolism*, 97, 1492–1500.
- Nahuis, M. J., Kose, N., Bayram, N., et al. (2011). Long-term outcomes in women with polycystic ovary syndrome initially randomized to receive laparoscopic electrocautery of the ovaries or ovulation induction with gonadotrophins. *Human Reproduction*, 26, 1899–1904.
- Norman, R. J., Noakes, M., Wu, R., Davies, M. J., Moran, L., & Wang, J. X. (2004). Improving reproductive performance in overweight/obese women with effective weight management. *Human Reproduction Update*, 10, 267–280.
- Ogden, C. L., Carroll, M. D., Fryar, C. D., & Flegal, K. M. (2015). Prevalence of obesity among adults and youth: United States, 2011–2014. *NCHS Data Brief*, 1–8.
- Palomba, S., Giallauria, F., Falbo, A., et al. (2008). Structured exercise training programme versus hypocaloric hyperproteic diet in obese polycystic ovary syndrome patients with anovulatory infertility: A 24-week pilot study. *Human Reproduction*, 23, 642–650.
- Pundir, J., Sunkara, S. K., El-Toukhy, T., & Khalaf, Y. (2012). Meta-analysis of GnRH antagonist protocols: Do they reduce the risk of OHSS in PCOS? *Reproductive Biomedicine Online*, 24, 6–22.
- Rich-Edwards, J. W., Goldman, M. B., Willett, W. C., et al. (1994). Adolescent body mass index and infertility caused by ovulatory disorder. *American Journal of Obstetrics and Gynecology*, 171, 171–177.
- Sam, S. (2007). Obesity and Polycystic ovary syndrome. *Obesity Management*, 3, 69–73.
- Sim, K. A., Partridge, S. R., & Sainsbury, A. (2014a). Does weight loss in overweight or obese women improve fertility treatment outcomes? A systematic review. *Obesity Reviews*, 15, 839–850.
- Sim, K. A., Dezarnaulds, G. M., Denyer, G. S., Skilton, M. R., & Caterson, I. D. (2014b). Weight loss improves reproductive outcomes in obese women undergoing fertility treatment: A randomized controlled trial. *Clinical Obesity*, 4, 61–68.
- Singh, N., Naha, M., Malhotra, N., Lata, K., Vanamail, P., & Tiwari, A. (2014). Comparison of gonadotropin-releasing hormone agonist with GnRH antagonist in polycystic ovary syndrome patients undergoing in vitro fertilization cycle: Retrospective analysis from a tertiary center and review of literature. *Journal of Human Reproductive Sciences*, 7, 52–57.
- Stein, I. F. L. M. (1935). Amenorrhea associated with bilateral polycystic ovaries. *American Journal of Obstetrics and Gynecology*, 29, 181.
- Swanton, A., Storey, L., McVeigh, E., & Child, T. (2010). IVF outcome in women with PCOS, PCO and normal ovarian morphology. *European Journal of Obstetrics, Gynecology, and Reproductive Biology*, 149, 68–71.
- Wang, R., Kim, B. V., van Wely, M., et al. (2017). Treatment strategies for women with WHO group II anovulation: Systematic review and network meta-analysis. *BMJ*, 356, j138.
- Wong, K. M., van Wely, M., Mol, F., Repping, S., & Mastenbroek, S. (2017). Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database of Systematic Reviews*, 3, CD011184.
- Youssef, M. A., Van der Veen, F., Al-Inany, H. G., et al. (2014). Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database of Systematic Reviews*, CD008046.

ART and Obesity

José Bellver, Instituto Valenciano de Infertilidad, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Why the Obese Population Undergoes ARTs more Frequently

Female obesity is related to a three-fold increased risk of subfertility (prolonged time to pregnancy) and infertility (no pregnancy achieved after at least one year of non-protected sexual intercourse). An inverse relationship between female body mass index (BMI) and fecundability has been demonstrated (Wise et al., 2010). Moreover, obesity in adolescence has been associated with a three to four-fold increase in the risk of lifetime nulligravidity and nulliparity (Polotsky et al., 2010). Classically, fertility problems in obese women were related to their high prevalence of ovulatory disorders (Rich-Edwards et al., 2002). However, more recent studies have highlighted that even obese women who present regular menses and ovulation have a reduced probability of conceiving. van der Steeg et al. (2008) showed that per each unit of increase in BMI above 29 kg/m² there was a 4% significant reduction in the cumulative pregnancy rate within a year, the same effect as an additional year of female age. Therefore, infertility in obese women does not seem to be caused only by the presence of disovulation, but also by alterations at the level of the oocyte, embryo and/or endometrium. In this way, obese women are more prone to initiate ARTs than normoweight women. Male overweight and obesity have also been related to increased infertility rates (Nguyen et al., 2007), with the combination of male and female excess weight representing poorer chances of spontaneous conception. Ramblau-Hansen et al. (2007) observed that the odds ratio for subfecundity (defined as time to pregnancy > 12 months) was higher as BMI increased in both men and women, and that this effect was maximum when both members of the couple were obese. In this way, male and female obesity influences natural conception negatively and independently, having an enhanced impact when both are present. Indeed, this is not an infrequent scenario, since partners often share similar lifestyle behaviors.

What Are the Results of ARTs in Obese Population

Many studies agree regarding a lower ovarian response in obese women after ovulation induction or controlled ovarian hyperstimulation (COH) for IVF. Different mechanisms have been implicated in this effect, such as hyperleptinemia, insulin-resistance or the increased volume of distribution of the drug through the body. Thus, the higher the BMI, the lower the ovarian response achieved, regardless of the drug used (clomiphene citrate or gonadotropins) (Bellver et al., 2006). The real question is whether the outcome of assisted conception is impaired by obesity once ovulation has been achieved in ovulation induction treatments or the oocytes have been recovered after COH in IVF.

Low Rank Assisted Conception (Scheduled Intercourse and Intrauterine Insemination)

The results of low rank assisted conception cycles in obese women are controversial. Some studies have reported that the chances of conception are similar to that in normoweight controls (Balén et al., 2006; Souter et al., 2011), but others have described reduced pregnancy rates (White et al. 1996; Koloszar et al., 2002; Al-Azemi et al., 2004). Many patients included in these studies presented polycystic ovary syndrome (PCOS), mild fertility problems -especially anovulation- and low-risk obesity (class I; 30–35 kg/m²), thus having a good prognosis. Therefore, the influence of obesity on reproduction could have been scarce. However, the central distribution of fat seems to be one of the most negative parameters on fertility. In fact, Zaadstra et al. (1993) showed a 30% significant reduction in the chances of patients conceiving after intrauterine insemination per each 0.1 unit of increase in the waist-to-hip ratio.

High Rank Assisted Conceptions (IVF with or without ICSI, and Ovum Donation)

Studies performed on IVF patients are more consistent, showing a significantly poorer outcome in obese women. Bellver et al. (2010) published the largest single center study to date about the influence of female BMI on IVF results, including 6500 IVF cycles with own oocytes. All the parameters of outcome (implantation, pregnancy and live birth rates) were significantly reduced from 30 kg/m² of BMI. Other later multicenter studies have confirmed these findings. Luke et al. (2011) analyzed 45,163 embryo transfers from 345 assisted reproduction clinics in the USA and found a significant reduction in clinical pregnancy and live birth rates as female BMI increased in a dose-dependent manner. Thus, the higher the female BMI, the higher the chances of not achieving a clinical intrauterine gestation or a live born. More recently, Provost et al. (2016a) and Kawwass et al. (2016), with 239,127 and 494,097 IVF cycles with own oocytes, respectively, again reported a significant reduction in clinical pregnancy and live birth rates, and a significant increase in spontaneous abortion rates with a rise in female BMI. To determine whether these results are due to a damage to the embryo or/and endometrium, oocyte donation undoubtedly constitutes the best human model, in which oocytes from healthy young donors controlled for weight are given to recipients with different BMIs. Using this model Cardozo et al. (2016) showed that oocytes from donors in the highest quartile of BMI in their institution (25.3–34.0 kg/m²) resulted in a significant reduction of clinical pregnancy rates in recipients adjusted for BMI, suggesting a deleterious clinical effect of BMI on the oocyte.

Bellver et al. (2013a) analyzed 9587 first cycles of ovum donation in which donors were normoweight. All the outcome parameters (implantation, pregnancy, clinical pregnancy, multiple pregnancy and live birth rates) decreased significantly when the BMI of the recipient was above 30 kg/m². Recently, Provost et al. (2016b) have reported similar results with >22,317 cycles of ovum donation, with outcomes being especially affected from 40 kg/m² of BMI upwards. These reports demonstrate a negative clinical effect of female BMI on endometrial receptivity.

Finally, male obesity also seems to impair IVF outcome. Some studies (Colaci et al., 2012; Petersen et al., 2013) and a recent meta-analysis (Campbell et al., 2015) have described reduced clinical pregnancy and live birth rates in couples in whom the male partner was obese, although the contribution of female BMI to this outcome has not always been adequately controlled.

Why the Results of Spontaneous and Assisted Conception Are Poorer in Obese Patients

Despite the fact that many endocrine and metabolic systemic alterations have been described in obese men and women, as well as local disturbances at the level of the cumulus-oocyte complex, embryo and spermatozoon, no obvious gamete or embryo damage has been described according to the conventional morphological criteria by which their quality is commonly assessed. In fact, despite the abovementioned clinical effects of male obesity on reproduction, no evident alteration of classical parameters of semen (MacDonald et al., 2010; McPherson & Lane, 2015) or sperm DNA fragmentation (Bandel et al., 2015) has been detected. Even in IVF/ICSI studies in which a clear negative effect of male BMI on live birth rates has been observed, no differences in embryo quality have been noted among different ranges of BMI (Colaci et al., 2012). This discrepancy has also been extensively addressed by dozens of studies that have attempted to ascertain the effect of female BMI on oocyte and embryo quality after IVF, but no consensus has been reached. In fact, in a study by Bellver et al. (2010) in which 6500 IVF cycles were included and 81,581 oocytes and their corresponding embryos were analyzed, no correlation was observed between female BMI and embryo quality on day 2 or 3 of embryo development (day of embryo transfer), despite the poorer results obtained in obese women. These findings indicate that the conventional method by which gametes and embryos are assessed in the andrology/IVF lab based on static morphological criteria is inadequate for determining the damage exerted by obesity. In light of this, new methods of assessment have been developed in recent years. Time-lapse analysis was used to evaluate whether the dynamic pattern of embryo division differed in embryos from normoweight or obese women, but no differences were detected (Bellver et al., 2013b). However, more recently, Bellver et al. (2015) observed a different metabolomic profile in the spent culture media of day 3 good quality embryos when the woman was obese, proving that the composition of metabolites that the embryo took from- or excreted into- the culture media was different, with a significant reduction of saturated fatty acids among the identified ones. This suggests that the function of embryos with similar morphology differs according to female BMI, which may account for the clinical results observed.

Table 1 Described consequences of female obesity for IVF results.

<i>Ovarian response</i>
Higher gonadotrophin requirement ("gonadotrophin resistance")
Longer period of ovarian stimulation
Higher cancellation rates
Higher incidence of follicular asynchrony
Lower bioavailability of injected hCG
Decreased periovulatory hCG intrafollicular concentrations
Lower peak of estradiol serum concentrations
<i>Oocytes, embryos and endometrium</i>
Reduced oocyte retrieval
Poorer oocyte quality
Lower mature oocytes
Lower fertilization rates
Poorer embryo quality
Impaired embryo metabolomics (culture media)
Lower incidence of embryo transfer
Lower mean number of transferred embryos
Reduced endometrial receptivity
<i>IVF outcome</i>
Lower implantation rates
Lower pregnancy rates
Higher preclinical miscarriage rates (biochemical pregnancies)
Higher clinical miscarriage rates
Increased risk of fetal defects
Increased complications in 2nd and 3rd trimesters of pregnancy (for mother and fetus)
Lower live birth rates

hCG, human chorionic gonadotrophin; *IVF*, in vitro fertilization.

Regarding the reduction in endometrial receptivity, Bellver et al. (2011) detected a different pattern of gene expression during the window of implantation (WOI; receptive phase of the endometrium) in obese women in comparison to normoweight controls, with a more dysregulated pattern when PCOS was associated. In fact, during the WOI 12 of the 25 genes most related to implantation at that time were expressed in the same way in both obese PCOS women and women with other refractory conditions, such as undergoing COH or carrying an inert intrauterine device. Recently, the same group (Comstock et al., 2017) has analyzed the transcriptomic profile of endometrial gene alterations during the WOI in infertile obese patients, detecting the dysregulation of several genes involved mainly in chemokine, cytokine, and immune system activity and in the structural extracellular matrix and protein-binding molecular functions.

To sum up, obesity impairs spontaneous and assisted conception in men and women, and has a synergistic effect when both members of the couple are obese. A deleterious effect on gametes, embryos and endometrium seems to exist, and is the focus of much current research. The best way to avoid the negative effects of excess weight on fertility is to achieve conception when BMI is below 30 kg/m², and, if possible, below 25 kg/m². This goal can also reduce the increased risk of congenital malformations, maternal and fetal pregnancy complications, and metabolic and non-metabolic diseases in the offspring, all of which are described in obese women.

Table 1 summarizes all the consequences of female obesity in assisted reproduction described in the current literature.

References

- Al-Azemi, M., Omu, F. E., & Omu, A. E. (2004). The effect of obesity on the outcome of infertility management in women with polycystic ovary syndrome. *Archives of Gynecology and Obstetrics*, 270, 205–210.
- Balen, A., Platteau, P., Andersen, A., et al. (2006). The influence of body weight on response to ovulation induction with gonadotrophins in 335 women with World Health Organization group II anovulatory infertility. *BJOG*, 113, 1195–1202.
- Bandel, I., Bungum, M., Richtoff, J., et al. (2015). No association between body mass index and sperm DNA integrity. *Human Reproduction*, 30, 1704–1713.
- Bellver, J., Busso, C., Pellicer, A., Remohí, J., & Simón, C. (2006). Obesity and assisted reproduction technology outcomes. *Reproductive Biomedicine Online*, 12, 562–568.
- Bellver, J., Ayllón, Y., Ferrando, M., et al. (2010). Female obesity impairs in vitro fertilization outcome without affecting embryo quality. *Fertility and Sterility*, 93, 447–454.
- Bellver, J., Martínez-Conejero, J. A., Labarta, E., et al. (2011). Endometrial gene expression in the window of implantation is altered in obese women especially in association with polycystic ovary syndrome. *Fertility and Sterility*, 95, 2335–2341.
- Bellver, J., Pellicer, A., García-Velasco, J. A., et al. (2013a). Obesity reduces uterine receptivity: clinical experience from 9,587 first cycles of ovum donation with normal weight donors. *Fertility and Sterility*, 100, 1050–1058.
- Bellver, J., Mifsud, A., Grau, N., Privitera, L., & Meseguer, M. (2013b). Similar morphokinetic patterns in embryos derived from obese and normoweight infertile women: a time-lapse study. *Human Reproduction*, 28, 794–800.
- Bellver, J., De Los Santos, M. J., Alamá, P., et al. (2015). Day-3 embryo metabolomics in the spent culture media is altered in obese women undergoing in vitro fertilization. *Fertility and Sterility*, 103, 1407–1415.e1.
- Campbell, J., Lane, M., Owens, J., & Bakos, H. (2015). Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. *Reproductive Biomedicine Online*, 31, 593–604.
- Cardozo, E., Karmon, A., Gold, J., Petrozza, J., & Styer, A. (2016). Reproductive outcomes in oocyte donation cycles are associated with donor BMI. *Human Reproduction*, 31, 385–392.
- Colaci, D., Afeiche, M., Gaskins, A., et al. (2012). Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization. *Fertility and Sterility*, 98, 1193–1199.e1.
- Comstock, I. A., Diaz-Gimeno, P., Cabanillas, S., et al. (2017). Does an increased body mass index affect endometrial gene expression patterns in infertile patients? A functional genomics analysis. *Fertility and Sterility*, 107, 740–748.e2.
- Kawwass, J., Kulkarni, A., Hipp, H., et al. (2016). Extremities of body mass index and their association with pregnancy outcomes in women undergoing in vitro fertilization in the United States. *Fertility and Sterility*, 106, 1742–1750.
- Koloszár, S., Daru, J., Kereszturi, A., et al. (2002). Effect of female body weight on efficiency of donor AI. *Archives of Andrology*, 48, 323–327.
- Luke, B., Brown, M. B., Stern, J. E., et al. SART Writing Group. (2011). Female obesity adversely affects assisted reproductive technology (ART) pregnancy and live birth rates. *Human Reproduction*, 26, 245–252.
- MacDonald, A. A., Herbison, G. P., Showell, M., & Farquhar, C. M. (2010). The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Human Reproduction Update*, 16, 293–311.
- McPherson, N. O., & Lane, M. (2015). Male obesity and subfertility, is it really about increased adiposity? *Asian Journal of Andrology*, 17, 450–458.
- Nguyen, R. H., Wilcox, A. J., Skjaerven, R., & Baird, D. D. (2007). Men's body mass index and infertility. *Human Reproduction*, 22, 2488–2493.
- Petersen, G. L., Schmidt, L., Pinborg, A., & Kamper-Jørgensen, M. (2013). The influence of female and male body mass index on live births after assisted reproductive technology treatment: a nationwide register-based cohort study. *Fertility and Sterility*, 99, 1654–1662.
- Polotsky, A. J., Hailpern, S. M., Skurnick, J. H., et al. (2010). Association of adolescent obesity and lifetime nulliparity- the Study of Women's Health Across the Nation (SWAN). *Fertility and Sterility*, 93, 2004–2011.
- Provost, M., Acharya, K., Acharya, C., et al. (2016a). Pregnancy outcomes decline with increasing body mass index: analysis of 239,127 fresh autologous in vitro fertilization cycles from the 2008–2010 Society for Assisted Reproductive Technology Registry. *Fertility and Sterility*, 105, 663–669.
- Provost, M., Acharya, K., Acharya, C., et al. (2016b). Pregnancy outcomes decline with increasing recipient body mass index: an analysis of 22,317 fresh donor/recipient cycles from the 2008–2010 Society for Assisted Reproductive Technology Clinic Outcome Reporting System Registry. *Fertility and Sterility*, 105, 364–368.
- Ramlau-Hansen, C. H., Thulstrup, A. M., Nohr, E. A., et al. (2007). Subfecundity in overweight and obese couples. *Human Reproduction*, 22, 1634–1637.
- Rich-Edwards, J. W., Spiegelman, D., Garland, M., et al. (2002). Physical activity, body mass index, and ovulatory disorder infertility. *Epidemiology*, 13, 184–190.
- Souter, I., Baltagi, L., Kuleta, D., Meeker, J., & Petrozza, J. (2011). Women, weight, and fertility: the effect of body mass index on the outcome of superovulation/intrauterine insemination cycles. *Fertility and Sterility*, 95, 1042–1047.
- van der Steeg, J. W., Steures, P., Eijkemans, M. J., et al. (2008). Obesity affects spontaneous pregnancy chances in subfertile, ovulatory women. *Human Reproduction*, 23, 324–328.
- White, D. M., Polson, D. W., Kiddy, D., et al. (1996). Induction of ovulation with low-dose gonadotropins in polycystic ovary syndrome: an analysis of 109 pregnancies in 225 women. *The Journal of Clinical Endocrinology and Metabolism*, 81, 3821–3824.
- Wise, L. A., Rothman, K. J., Mikkelsen, E. M., et al. (2010). An internet-based prospective study of body size and time-to-pregnancy. *Human Reproduction*, 25, 253–264.
- Zaadstra, B. M., Seidell, J. C., Van Noord, P. A., et al. (1993). Fat and female fecundity: prospective study of effect of body fat distribution on conception rates. *BMJ*, 306, 484–487.

Evaluation and ART of the Low Ovarian Responder Patient

Nikolaos P Polyzos, Dexeus University Hospital, Barcelona, Spain; Vrije Universiteit Brussel, Brussel, Belgium; and Aarhus University, Aarhus, Denmark

Pedro Barri Rague, Dexeus University Hospital, Barcelona, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

Low ovarian responders are considered women who fail to respond to ovarian stimulation and produce an adequate number of follicles and therefore oocytes. Low responders represent a very unique group of patients attending an infertility unit with certain difficulties associated with the diagnostic approach and the optimal treatment they should be offered. Available evidence suggests that they may represent a significant percentage of the population seeking infertility advice, reaching according to reports an incidence of 24% (Ubaldi et al., 2005).

Owing to this increased incidence, it is of paramount importance for the clinician not only to evaluate the etiology associated with low ovarian response, but also to provide a patient-tailored treatment in order to maximize the chances to achieve the delivery of a healthy child.

Etiology and Evaluation of Low Ovarian Response

Age and Low Ovarian Response

Age is probably the strongest element of treatment success in women seeking fertility advice. This is not only associated with decreased oocyte quality and increased aneuploidy rates, but also with the decreased pool of nongrowing follicles with advancing age. Previous reports have shown that the number of nongrowing follicles in the ovaries is dropping and this drop is accelerated after the age of 36 years (Wallace and Kelsey, 2010). Owing to this sequel of events, women of advanced age are at higher risk of experiencing low response to ovarian stimulation and subsequently retrieve fewer oocytes as compared with women of advanced age. Nevertheless, besides that age is apparently a very strong determinant of ovarian response, other factors may modify the association of age with ovarian response. Thus, it is not uncommon to identify excellent ovarian response in women of advanced age with specific phenotypes such as PCOS. Given that previous research has shown that women with PCOS have a delayed age at menopause (Li et al., 2016) and that these patients often present even at the age of 40 with a very good ovarian reserve (Tian et al., 2014), in such cases response to ovarian stimulation can be fairly optimal.

Iatrogenic Interventions and Low Ovarian Response

Ovarian surgery is a key approach of infertility management and has a beneficial effect in women with conditions such as endometriosis. Over the last decades it has been clearly shown that removal of endometriomas in combination with GnRH agonist depot supplementation can minimize the extent of endometriosis-associated inflammation and thus increased the chances of spontaneous pregnancy among infertile couples. Similarly, along with endometriosis, other clinical conditions such as the presence of dermoid cysts or other ovarian benign tumors require surgical management in order to alleviate pain-associated symptoms or due to the risk of malignancy in the future (e.g., borderline ovarian tumors). Still, in spite of the fact that over the years surgical techniques have evolved and sparing fertility ovarian surgery has been of paramount importance, recent evidence proves that such procedures are very often associated with low ovarian response (Raffi et al., 2012). Young women with a previous ovarian surgery often present with a severely diminished low ovarian reserve and thus a high risk to experience low response to stimulation. Consequently, and based on current evidence, the extent and the nature of surgery should always be carefully evaluated and reviewed based on patients' family status (Somigliana et al., 2015). In this context, the decision on whether surgery should be performed avoided or postponed until fertility preservation or family planning has been achieved, needs to be taken based on the risks the benefits of each procedure, but also based on the level of patient's ovarian reserve and age at the moment of the counseling (Somigliana et al., 2015).

Similarly, taking into account the substantial progress made in the treatment of certain malignancies, the percentage of "cancer survivors" has significantly increased over the years. Thus, it is very common to counsel women at their late 30s or early 40s desiring to have a child several years after the treatment of malignant disease. Available evidence demonstrated that chemotherapy and pelvic irradiation may have a severely dramatic reduction in ovarian reserve which unfortunately is irreversible (De Vos et al., 2014). This appears to be strictly associated with the type of chemotherapy used (Anderson and Cameron, 2007) and also with the level of ovarian reserve and the age of the patient at the time of treatment (Dezellus et al., 2017). Taking this into consideration, it is of paramount importance not only for fertility specialists, but also for gynecologists and oncologists to communicate this great risk for all women of reproductive age with a malignant disease requiring treatment and tailor the optimal management plan. Fertility preservation must be offered in these women, either through oocyte vitrification or/and by ovarian cortex cryopreservation, given that many of these patients will be free of disease in the future and may require achieving a pregnancy.

Genetics Causes of Low Ovarian Reserve

Low ovarian response in women of young reproductive age is a finding that should not be overlooked. Although a reduction in ovarian reserve and a low ovarian response should be anticipated with advancing age, occurrence of low response in your reproductive age should be further investigated. According to previous research, a woman whose mother had an early menopause has a sixfold increased risk of having early menopause (Morris et al., 2011; van Asselt et al., 2004), with estimates of this heritability for age at menopause ranging from 30% to 85% (van Asselt et al., 2004). Unfortunately, available evidence suggest that genetic causes can only explain <5% of the variance in the age of menopause (Perry et al., 2013) and thus it should be anticipated that genetic causes will be identified in women of young age who present with low ovarian response. Nevertheless, it is generally accepted that in young patients with low ovarian response further investigation is justified. This should undoubtedly include a karyotype analysis to exclude mainly the presence of any chromosomal abnormality such as Turner syndrome but also genotyping for the detection of FMR1 premutation (Visser et al., 2013; Rohr et al., 2008). Taking into account the higher incidence of impaired ovarian reserve and low ovarian response in these patients (Rohr et al., 2008) but also the consequences that may arise for the offspring from the presence of such genetic conditions, it is of paramount importance to screen patients at risk in order to provide adequate patient management.

Nevertheless, irrespective of the above-mentioned profound aetiological factors associated with low ovarian reserve, in great percentage of low responders the etiology remains unknown.

Assisted Reproduction Technologies of Low Ovarian Responders

Proper management of the low ovarian responder patient should always involve the development of a treatment plan. This not only includes the selection of the appropriate procedure but also the selection treatment protocol specifically tailored for each patient.

Intrauterine Insemination or Ovarian Stimulation for In Vitro Fertilization

The decision on whether low responders should be offered intrauterine insemination or IVF is apparently related to several factors such as the male partner sperm quality, the duration of infertility and of course the age of the woman. Although in young poor responders IUI could be the first treatment option in case of normospermia and a short duration of infertility, it is rather uncertain whether we shall proceed to such an approach in women of advanced age. A recent RCT has clearly shown that in women above 40 years old one cycle of IVF results in significantly higher pregnancy rates compared with three cycles of IUI and a shorter time to pregnancy (Goldman et al., 2014). Thus in these cases IVF should be the optimal procedure. Furthermore, we always need to take into consideration the level of ovarian reserve prior to proceeding (Freeman et al., 2012). Consequently, it may be considered more appropriate to proceed with IVF in women with very low AMH levels, even if they are younger, simply because evidence clearly demonstrate that these women have a higher risk of entering early menopause compared with women with normal AMH levels (Freeman et al., 2012).

Ovarian Stimulation Protocols for In Vitro Fertilization

Over the last 20 years several different stimulation protocols have been utilized for the treatment of poor ovarian responders. Still, despite the vast body of literature, it appears that treatment of poor ovarian responders always remains a burden for the clinician.

Downregulation protocol

The type of downregulation protocol has always been an issue of interest, with investigators supporting the advantage of one versus other. Despite that GnRH antagonists have been introduced into clinical practice much later, initially they were frequently introduced in poor ovarian responders. Today following the publication of several clinical trials it seems rather unlikely that the type of protocol plays a role for poor ovarian responders. Metaanalysis published in 2006 showed no difference either when comparing short flare-up agonist or long GnRH agonist protocol versus GnRH antagonist (Griesinger et al., 2006), whereas a more recent meta-analysis replicated the results by Griesinger (Xiao et al., 2013).

Type of gonadotropins

Similarly, the type of gonadotropin used does not appear to have any effect on the final reproductive outcome. Although several studies supported that potentially the use of LH activity may improve pregnancy rates in women of advanced reproductive age (Gizzo et al., 2015; Revelli et al., 2012), the most recent randomized controlled trial has shown that the addition of rLH to rFSH does not increase pregnancy rates in poor ovarian responders (Humaidan et al., 2017).

In the same context, recent cohort studies have clearly shown that the prognosis in poor ovarian responders fulfilling the Bologna criteria for poor response (Ferraretti et al., 2011) is always poor, with very low pregnancy rates irrespective of the type of gonadotropin used (Polyzos et al., 2014; Busnelli et al., 2015; La Marca et al., 2015).

Gonadotropin starting dose

Controversy regarding the appropriate starting dose is frequent among treating clinicians. Others believe that mild ovarian stimulation should be the aim for this difficult group of patients whereas others are proponents of strong stimulation with very high doses.

The current literature strongly supports that doses above 300 IU appear to be futile since no difference can be seen in pregnancy and live birth rates in women treated with very high doses ([Berkkanoglu and Ozgur, 2010](#); [Lefebvre et al., 2015](#)). Thus, the use of such doses is not recommended. Furthermore, a recent randomized study demonstrated that mild stimulation results in comparable results with high starting FSH doses ([Youssef et al., 2017](#)). Still, we certainly need to be cautious, especially in women who may have a good functional ovarian reserve with a good antral follicle count and had a previous low ovarian response. Given that we do have enough evidence now to demonstrate that cumulative live birth rates significantly increase with the number of oocytes ([Drakopoulos et al., 2016](#)) in such kind of patients with a good antral follicle count it may be more clinically relevant to aim to the maximum number of oocytes retrieved.

Natural Cycle IVF

Natural cycle IVF has been traditionally considered the valid treatment option for poor ovarian responders with very low functional reserve. In such women with very low antral follicle count, ovarian stimulation definitely has no actual role simply because there are no follicles to stimulate and recruit. Although natural cycle can be considered an option for the treatment of these patients, women should be aware that the anticipated outcome should be rather low. A large recent retrospective cohort in poor ovarian responders according to the Bologna criteria ([Ferraretti et al., 2011](#)) demonstrated disappointingly low live birth rates, significantly lowers compared with the outcome of natural cycle IVF in normal ovarian responders ([Polyzos et al., 2012](#)).

Accumulation of Oocytes

The strategy of accumulating oocytes in order to increase the outcome in poor ovarian responders has also been analyzed over the years. Although such an approach is a preferred strategy by many clinicians, we need to underscore that apparently the available evidence is rather weak. Based on the study by Cobo et al., it seems that potentially accumulation might be superior to only a fresh IVF cycle ([Cobo et al., 2012](#)), results should be interpreted with caution. The reason is not only the fact that results in that study appear comparable between accumulation and fresh embryo transfer in women less than 40 years old, but also because the patients sample in women more than 40 years old was limited.

Adjuvant Therapies

Owing to the disappointing results of most ovarian stimulation protocols in low ovarian responders, research has focused on adjuvant therapies, prior to or during ovarian stimulation in an attempt to increase the recruitable follicular cohort. The use of androgens and growth hormone has been the most wide used regimens. Although results from metaanalysis appeared promising, the body of available evidence is very small and the protocols used lack uniformity regarding the actual dose and duration of administration ([Gonzalez-Comadran et al., 2012](#); [Kolibianakis et al., 2009](#)).

Growth hormone

Early experimental studies have shown that growth hormone (GH) administration can stimulate the production of insulin-like growth factor (IGF) ([Doldi et al., 1996](#)). Thus, owing to the synergistic effects of IGF-1 with FSH on follicular development ([Adashi, 1992](#)), many hypothesizes that GH could stimulates follicular development and in this context increase oocyte quality. Results of randomized trials favored GH supplementation during ovarian stimulation with metaanalysis of randomized trials demonstrating that GH can increase pregnancy rates in low responders ([Kolibianakis et al., 2009](#)) and an updated metaanalysis 4 years later providing similar results with a three times higher odds for a pregnancy in favor of GH administration ([Duffy et al., 2010](#)). Nevertheless, the sample size of all trials was very limited and the quality not adequate in order to justify routine clinical use, especially taking into consideration the lack of safety data and its increased cost.

Androgen pretreatment

Androgen pretreatment in low responders undergoing IVF/ICSI has also been investigated as an option to increase the recruitable follicular.

Owing to early studies in primates demonstrating that testosterone can increase the number of primary follicles and the FSH receptor expression ([Vendola et al., 1998, 1999a,b](#); [Weil et al., 1998, 1999](#)) research has focused on the use of androgens in women with poor ovarian response. Although initial reports have shown that DHEA may be promising in these women, the most recent RCTs failed to show any improvement in pregnancy rates in poor responders as compared with placebo ([Yeung et al., 2014](#)).

On the contrary, data for testosterone appear to be rather more promising with early RCTs showing an increase in pregnancy rates compared with placebo or no treatment ([Gonzalez-Comadran et al., 2012](#)). Still, evidence is limited, sample sizes small and the optimal dose and duration of testosterone administration remains to be determined. Available evidence from animal

experiments and testosterone pharmacokinetics suggest administration of ~2 months of testosterone prior to initiation of ovarian stimulation at a dose around 5.5 mg per day and a multinational RCT is ongoing in order to provide clear evidence regarding the role of testosterone in low responder patients (Polyzos et al., 2016).

Conclusion

The diagnostic and therapeutic management of low ovarian responders remains one of the most challenging tasks for every clinician. Appropriate diagnostic approach and analysis of the etiological factors of diminished ovarian reserve and low response to stimulation is always essential as an initial approach for these women. Selection of treatment strategy is equally important and always should be associated with the level of ovarian reserve of the patient the age and the duration of the infertility. Although most of the available treatment protocols appear to be equally “ineffective” in low responders, functional ovarian reserve and the number of recruitable follicles should always guide the decision of the protocol selection. In women with low functional reserve current options are rather limited given that ovarian stimulation is unlikely to change the prognosis in these women. Future research on adjuvant supplementation, such as testosterone, prior to ovarian stimulation is needed in order to demonstrate whether such an approach can alter the prognosis of these women or whether oocyte donation should be the only realistic option for low ovarian responders with severely limited ovarian reserve.

References

- Adashi, E. Y. (1992). Intraovarian regulation: The IGF-I example. *Reproduction, Fertility, and Development*, 4(5), 497–504.
- Anderson, R. A., & Cameron, D. A. (2007). Assessment of the effect of chemotherapy on ovarian function in women with breast cancer. *Journal of Clinical Oncology*, 25(12), 1630–1631. Author reply 2.
- Berkkanoglu, M., & Ozgur, K. (2010). What is the optimum maximal gonadotropin dosage used in microdose flare-up cycles in poor responders? *Fertility and Sterility*, 94(2), 662–665.
- Busnelli, A., Papaleo, E., Del Prato, D., La Vecchia, I., Iachini, E., Paffoni, A., et al. (2015). A retrospective evaluation of prognosis and cost-effectiveness of IVF in poor responders according to the Bologna criteria. *Human Reproduction*, 30(2), 315–322.
- Cobo, A., Garrido, N., Crespo, J., Jose, R., & Pellicer, A. (2012). Accumulation of oocytes: A new strategy for managing low-responder patients. *Reproductive BioMedicine Online*, 24(4), 424–432.
- De Vos, M., Smits, J., & Woodruff, T. K. (2014). Fertility preservation in women with cancer. *Lancet*, 384(9950), 1302–1310.
- Dezellus, A., Barriere, P., Campone, M., Lemanski, C., Vanlemmens, L., Mignot, L., et al. (2017). Prospective evaluation of serum anti-Müllerian hormone dynamics in 250 women of reproductive age treated with chemotherapy for breast cancer. *European Journal of Cancer*, 79, 72–80.
- Doldi, N., Bassan, M., Bonzi, V., & Ferrari, A. (1996). Effects of growth hormone and growth hormone-releasing hormone on steroid synthesis in cultured human luteinizing granulosa cells. *Gynecological Endocrinology*, 10(2), 101–108.
- Drakopoulos, P., Blockeel, C., Stoop, D., Camus, M., de Vos, M., Tournaye, H., et al. (2016). Conventional ovarian stimulation and single embryo transfer for IVF/ICSI. How many oocytes do we need to maximize cumulative live birth rates after utilization of all fresh and frozen embryos? *Human Reproduction*, 31(2), 370–376.
- Duffy, J. M., Ahmad, G., Mohiyiddeen, L., Nardo, L. G., & Watson, A. (2010). Growth hormone for in vitro fertilization. *Cochrane Database of Systematic Reviews*, 1, CD000099.
- Ferraretti, A. P., La Marca, A., Fauser, B. C., Tarlatzis, B., Nargund, G., Gianaroli, L., et al. (2011). ESHRE consensus on the definition of ‘poor response’ to ovarian stimulation for in vitro fertilization: The Bologna criteria. *Human Reproduction*, 26(7), 1616–1624.
- Freeman, E. W., Sammel, M. D., Lin, H., & Gracia, C. R. (2012). Anti-müllerian hormone as a predictor of time to menopause in late reproductive age women. *Journal of Clinical Endocrinology and Metabolism*, 97(5), 1673–1680.
- Gizzo, S., Andrisani, A., Noventa, M., Manfe, S., Oliva, A., Gangemi, M., et al. (2015). Recombinant LH supplementation during IVF cycles with a GnRH-antagonist in estimated poor responders: A cross-matched pilot investigation of the optimal daily dose and timing. *Molecular Medicine Reports*, 12(3), 4219–4229.
- Goldman, M. B., Thornton, K. L., Ryley, D., Alper, M. M., Fung, J. L., Hornstein, M. D., et al. (2014). A randomized clinical trial to determine optimal infertility treatment in older couples: The Forty and Over Treatment Trial (FORT-T). *Fertility and Sterility*, 101(6), 1574–1581. e1–2.
- Gonzalez-Comadran, M., Duran, M., Sola, I., Fabregues, F., Carreras, R., & Checa, M. A. (2012). Effects of transdermal testosterone in poor responders undergoing IVF: Systematic review and meta-analysis. *Reproductive BioMedicine Online*, 25(5), 450–459.
- Griesinger, G., Diedrich, K., Tarlatzis, B. C., & Kolibianakis, E. M. (2006). GnRH-antagonists in ovarian stimulation for IVF in patients with poor response to gonadotrophins, polycystic ovary syndrome, and risk of ovarian hyperstimulation: A meta-analysis. *Reproductive BioMedicine Online*, 13(5), 628–638.
- Humaidan, P., Chin, W., Rogoff, D., D’Hooghe, T., Longobardi, S., Hubbard, J., et al. (2017). Efficacy and safety of follitropin alfa/lutropin alfa in ART: A randomized controlled trial in poor ovarian responders. *Human Reproduction*, 32(3), 544–555.
- Kolibianakis, E. M., Venetis, C. A., Diedrich, K., Tarlatzis, B. C., & Griesinger, G. (2009). Addition of growth hormone to gonadotrophins in ovarian stimulation of poor responders treated by in-vitro fertilization: A systematic review and meta-analysis. *Human Reproduction Update*, 15(6), 613–622.
- La Marca, A., Grisendi, V., Giulini, S., Sighinolfi, G., Tirelli, A., Argento, C., et al. (2015). Live birth rates in the different combinations of the Bologna criteria poor ovarian responders: A validation study. *Journal of Assisted Reproduction and Genetics*, 32(6), 931–937.
- Lefebvre, J., Antaki, R., Kadoch, I. J., Dean, N. L., Sylvestre, C., Bissonnette, F., et al. (2015). 450 IU versus 600 IU gonadotropin for controlled ovarian stimulation in poor responders: A randomized controlled trial. *Fertility and Sterility*, 104(6), 1419–1425.
- Li, J., Eriksson, M., Czene, K., Hall, P., & Rodriguez-Wallberg, K. A. (2016). Common diseases as determinants of menopausal age. *Human Reproduction*, 31(12), 2856–2864.
- Morris, D. H., Jones, M. E., Schoemaker, M. J., Ashworth, A., & Swerdlow, A. J. (2011). Familial concordance for age at natural menopause: Results from the breakthrough generations study. *Menopause*, 18(9), 956–961.
- Perry, J. R., Corre, T., Esko, T., Chasman, D. I., Fischer, K., Franceschini, N., et al. (2013). A genome-wide association study of early menopause and the combined impact of identified variants. *Human Molecular Genetics*, 22(7), 1465–1472.
- Polyzos, N. P., Blockeel, C., Verpoest, W., De Vos, M., Stoop, D., Vloeberghs, V., et al. (2012). Live birth rates following natural cycle IVF in women with poor ovarian response according to the Bologna criteria. *Human Reproduction*, 27(12), 3481–3486.
- Polyzos, N. P., Nwoye, M., Corona, R., Blockeel, C., Stoop, D., Haentjens, P., et al. (2014). Live birth rates in Bologna poor responders treated with ovarian stimulation for IVF/ICSI. *Reproductive BioMedicine Online*, 28(4), 469–474.
- Polyzos, N. P., Davis, S. R., Drakopoulos, P., Humaidan, P., De Geyter, C., Vega, A. G., et al. (2016). Testosterone for poor ovarian responders: Lessons from ovarian physiology. *Reproductive Sciences*. <https://doi.org/10.1177/1933719116660849>.

- Raffi, F., Metwally, M., & Amer, S. (2012). The impact of excision of ovarian endometrioma on ovarian reserve: A systematic review and meta-analysis. *Journal of Clinical Endocrinology and Metabolism*, 97(9), 3146–3154.
- Revelli, A., Chiado, A., Guidetti, D., Bongioanni, F., Rovei, V., & Gennarelli, G. (2012). Outcome of in vitro fertilization in patients with proven poor ovarian responsiveness after early vs. mid-follicular LH exposure: A prospective, randomized, controlled study. *Journal of Assisted Reproduction and Genetics*, 29(9), 869–875.
- Rohr, J., Allen, E. G., Charen, K., Giles, J., He, W., Dominguez, C., et al. (2008). Anti-Mullerian hormone indicates early ovarian decline in fragile X mental retardation (FMR1) premutation carriers: A preliminary study. *Human Reproduction*, 23(5), 1220–1225.
- Somigliana, E., Vignano, P., Filippi, F., Papaleo, E., Benaglia, L., Candiani, M., et al. (2015). Fertility preservation in women with endometriosis: For all, for some, for none? *Human Reproduction*, 30(6), 1280–1286.
- Tian, X., Ruan, X., Mueck, A. O., Wang, J., Liu, S., Yin, D., et al. (2014). Anti-Mullerian hormone levels in women with polycystic ovarian syndrome compared with normal women of reproductive age in China. *Gynecological Endocrinology*, 30(2), 126–129.
- Ubaldi, F. M., Rienzi, L., Ferrero, S., Baroni, E., Sapienza, F., Cobellis, L., et al. (2005). Management of poor responders in IVF. *Reproductive BioMedicine Online*, 10(2), 235–246.
- van Asselt, K. M., Kok, H. S., Pearson, P. L., Dubas, J. S., Peeters, P. H., Te Velde, E. R., et al. (2004). Heritability of menopausal age in mothers and daughters. *Fertility and Sterility*, 82(5), 1348–1351.
- Vendola, K. A., Zhou, J., Adesanya, O. O., Weil, S. J., & Bondy, C. A. (1998). Androgens stimulate early stages of follicular growth in the primate ovary. *Journal of Clinical Investigation*, 101(12), 2622–2629.
- Vendola, K., Zhou, J., Wang, J., & Bondy, C. A. (1999a). Androgens promote insulin-like growth factor-I and insulin-like growth factor-I receptor gene expression in the primate ovary. *Human Reproduction*, 14(9), 2328–2332.
- Vendola, K., Zhou, J., Wang, J., Famuyiwa, O. A., Bievre, M., & Bondy, C. A. (1999b). Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biology of Reproduction*, 61(2), 353–357.
- Visser, J. A., Hokken-Koelega, A. C., Zandwijken, G. R., Limacher, A., Ranke, M. B., & Fluck, C. E. (2013). Anti-Mullerian hormone levels in girls and adolescents with Turner syndrome are related to karyotype, pubertal development and growth hormone treatment. *Human Reproduction*, 28(7), 1899–1907.
- Wallace, W. H., & Kelsey, T. W. (2010). Human ovarian reserve from conception to the menopause. *PLoS One*, 5(1), e8772.
- Weil, S. J., Vendola, K., Zhou, J., Adesanya, O. O., Wang, J., Okafor, J., et al. (1998). Androgen receptor gene expression in the primate ovary: Cellular localization, regulation, and functional correlations. *Journal of Clinical Endocrinology and Metabolism*, 83(7), 2479–2485.
- Weil, S., Vendola, K., Zhou, J., & Bondy, C. A. (1999). Androgen and follicle-stimulating hormone interactions in primate ovarian follicle development. *Journal of Clinical Endocrinology and Metabolism*, 84(8), 2951–2956.
- Xiao, J., Chang, S., & Chen, S. (2013). The effectiveness of gonadotropin-releasing hormone antagonist in poor ovarian responders undergoing in vitro fertilization: A systematic review and meta-analysis. *Fertility and Sterility*, 100(6), 1594–1601. e1–9.
- Yeung, T. W., Chai, J., Li, R. H., Lee, V. C., Ho, P. C., & Ng, E. H. (2014). A randomized, controlled, pilot trial on the effect of dehydroepiandrosterone on ovarian response markers, ovarian response, and in vitro fertilization outcomes in poor responders. *Fertility and Sterility*, 102(1), 108–115. e1.
- Youssef, M. A., van Wely, M., Al-Inany, H., Madani, T., Jahangiri, N., Khodabakhshi, S., et al. (2017). A mild ovarian stimulation strategy in women with poor ovarian reserve undergoing IVF: A multicenter randomized non-inferiority trial. *Human Reproduction*, 32(1), 112–118.

Evaluation and Assisted Reproductive Technology of the High Responder Patient

Mazen R Fouany, Cole Memorial Hospital, Coudersport, PA, United States; and Lock Haven University, Coudersport, PA, United States

Fady I Sharara, Virginia Center for Reproductive Medicine, Reston, VA, United States; and George Washington University, Washington, DC, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

AFC Antral follicle count

AMH Anti Mullerian hormone

ART Assisted reproductive techniques

BMI Body mass index

FSH Follicle stimulating hormone

hCG Human chorionic gonadotropin

OHSS Ovarian hyperstimulation syndrome

PCOS Polycystic ovary syndrome

Introduction

Assisted reproductive techniques (ART) form the cornerstone for the management of infertility that includes male factor infertility, anovulatory causes, diminished ovarian reserve, tubal factor, endometriosis, and unexplained infertility (NICE, 2016). Controlled ovarian stimulation is the mainstay of IVF cycles to increase the pool of oocytes and subsequently embryos. Approximately, 1.5 million ART cycles per year have been reported and this number is expected to increase (ESHRE, 2016). Due to the significant variation in response to gonadotropins, reproductive endocrinologists have a significant interest in optimizing outcomes success rates and reducing the risk of ovarian hyperstimulation in patients.

Definition and Risk Factors

The definition of high responders helps designing the appropriate stimulation protocol, reducing the risk of OHSS, and plan appropriate interventions. While the Bologna criteria have been adopted for the poor responder patients, no such definition exists for high responders (Mascarenhas and Balen, 2017). That said, two criteria have been use: the first is serum estradiol > 6000 pg/mL on the day of trigger while the second is > 30 oocytes retrieved (Mascarenhas and Balen, 2017). When either one of these risk factors was present, there was a 33% chance of developing severe OHSS; however, if both risk factors were present, the risk of developing severe OHSS was 80%. Similarly, different cut-offs have also been used for serum estradiol levels on the day of trigger including 3000 and 4000 pg/mL. In 2002, Papageorgiou et al. included serum estradiol levels from 461 cycles in women aged < 35 years and defined high response as the 90th centile corresponding to 3444 pg/mL in their study. Reproductive Technology (SART) registry-based retrospective cohort study concluded that above a threshold of 15 oocytes, there is an increased risk of OHSS but a plateauing of live birth rate (Steward et al., 2015). In 2014, Arce and La Macra published a retrospective analysis revealing that potential high-responders were defined as having an initial anti-Müllerian hormone (AMH) value > 75th percentile (5.2 ng/mL). Arce et al. concluded that AFC and AMH are very sensitive markers of ovarian reserve identified to date, and are ideal in planning personalized stimulation protocols. These sensitive markers permit prediction of the ovarian response with reliable accuracy (Arce et al., 2014). Markers of ovarian reserve, in particular AMH and AFC, are critical in identifying those women who are likely to show a high response to controlled ovarian response. The diagnostic accuracy of basal AMH level for the prediction of OHSS has been shown to be good and comparable to the estradiol levels or number of ovarian follicles on the day of hCG administration (Lee et al., 2008), thus allowing for its use in routine clinical practice for preventative strategies. An AMH cutoff of 3.36 ng/mL had 90% sensitivity and 81% specificity for predicting OHSS (Lee et al., 2008). (Table 1)

Young women have a higher degree of ovarian responsiveness, which could explain the higher risk of hyper-response. Follicle-stimulating hormone (FSH) receptor polymorphisms also could influence ovarian response with activating mutations predisposing to hyper-response and inactivating mutations leading to infertility (Rizk, 2009).

According to the Royal College of Obstetrics and Gynecology, there are several predictors or risk factors for high response and they are the following (Royal College of Obstetrics and Gynecology, 2016) (Table 2):

Table 1 Importance of markers of high responder

<i>Risk factors for high responder</i>	<i>Importance</i>
AMH	+++
AFC	+++
FSH	++
Number of oocytes retrieved	++
Estradiol level	++
Age	+

+, Not very appropriate.

+++, very appropriate.

1. Previous history of OHSS
2. High antral follicle count (AFC). An antral follicle count (AFC) >20 correlates with a higher risk of OHSS (8 is for poor responders).
3. The presence of polycystic ovaries.
4. High anti-Mullerian hormone (AMH) levels. An AMH cutoff of 3.36 ng/mL had 90% sensitivity and 81% specificity for predicting OHSS.
5. Young age
6. Low body mass index (BMI)

Management of the High Responder

Because ovarian response is determined by the dose of gonadotropins, individualizing the dose of gonadotropins according to patient characteristics and risks factors is crucial to reduce the risk of hyper stimulation while still ensuring the retrieval of multiple oocytes. Many authors have published multiple dosing algorithms. [La Marca et al. \(2012, 2013\)](#) have published three normograms: the first including age and AFC, the second including age, AFC and FSH ([La Marca et al., 2013](#)) and third including age, AMH and FSH ([La Marca et al., 2012](#)). Another meta-analysis suggested that the optimal daily recombinant FSH stimulation dose is 150 IU/day in normal responders younger than 39 years undergoing IVF. Compared with higher doses, this dose is associated with a slightly lower oocyte yield, but similar pregnancy rates and embryo cryopreservation rates ([Sterrenburg et al., 2010](#)).

Other treatments have been studied in high responder patients to help reduce the risks. Such treatments include the following:

1. Metformin ([Mascarenhas and Balen, 2017](#))
2. Albumin ([Mascarenhas and Balen, 2017](#))
3. Hydroxyethyl starch ([Mascarenhas and Balen, 2017](#))
4. Cabergoline ([Mascarenhas and Balen, 2017](#))
5. Vascular endothelial growth factor blockers ([Mascarenhas and Balen, 2017](#))

Monitoring the ovarian response during ovarian stimulation allows providers to adjust the dosage of gonadotropins. Using GnRH antagonist-based protocols results in a 50% reduction in OHSS compared to long agonist protocols ([Toftager et al., 2016](#)). Also using GnRH antagonist cycles enables the use of a GnRH agonist, rather than hCG, as a trigger for final oocyte maturation. This will result in significant decrease in the risk of OHSS however, at the expense of a reduced pregnancy rate ([Youssef et al., 2014](#)), though recent data on using a low dose of HCG-add back (1500 IU every 3 days) resulted in comparable success rates ([Humaidan et al., 2013](#)). Two other techniques have emerged to help reduce the risks in the high responder patient. The first is segmentation of the IVF cycle, and the second is utilizing a modified luteal phase after agonist triggering to overcome the luteal phase deficiency.

The segmentation approach (also known as freeze-all) is best explained by routine use of antagonist cycles with GnRH agonist triggering followed by elective cryopreservation of oocytes or embryos and transferring them later in a frozen embryo transfer cycle.

Table 2 Risk factors for hyper response

<i>Risk factors</i>
Young age (RCOG, 2016)
Low BMI (RCOG, 2016)
High AMH (Arce et al., 2014)
High AFC (La Macra et al., 2013)
PCOS (Mascarenhas et al., 2017)
High serum estradiol (Steward et al., 2015 ; Papageorgiou et al., 2002)
Multiple follicles during the ovarian stimulation (Steward et al., 2015)

While reducing the risk of OHSS in the high responder patient, this approach results in longer time to pregnancy and increased cost (Devroey et al., 2011).

The utilization of luteal phase support after agonist triggering can help optimizing the luteal phase through intensive hormonal support with estrogen and progesterone or low dose hCG (Griffin et al., 2012).

Coasting is another technique used in high responder patients. This technique involves the temporary withdrawal of gonadotropins while continuing ovarian suppression for no more than 4 days while waiting a fall in serum estradiol levels. While there was a reduction in the mean number of oocytes retrieved, the clinical pregnancy and live birth rate were not affected (D'Angelo et al., 2011). Coasting however has been largely replaced by the above two treatment modalities.

In a large randomized control study including 1050 women allocated to either short GnRH antagonist or long GnRH agonist protocol, Toftager et al. concluded that the short GnRH antagonist protocol should be the protocol of choice for patients undergoing their first ART cycle in females younger than 40 years of age including both low and high responders. Patients at risk of OHSS particularly benefit from the short GnRH antagonist treatment with a 50% reduction compared to the long agonist protocol (Toftager et al., 2016).

Conclusions

High response to gonadotropins is a risk factor for ovarian stimulation for ART. In addition to the risk of OHSS, high response can adversely impact endometrial receptivity. The uses of GnRH agonists for triggering and segmental approach (freeze all) have been very effective in reducing the risk of OHSS. This has resulted in safer treatments of hyper responders without compromising the success rate of ART.

References

- Arce, J. C., Klein, B. M., & La Marca, A. (2014). The rate of high ovarian response in women identified at risk by a high serum AMH level is influenced by the type of gonadotropin. *Gynecological Endocrinology*, 30(6), 444–450.
- D'Angelo, A., Brown, J., & Amso, N. N. (2011). Coasting (with- holding gonadotrophins) for preventing ovarian hyperstimulation syndrome. *Cochrane Database of Systematic Reviews*, 2011(6), CD002811.
- Devroey, P., Polyzos, N. P., & Blockeel, C. (2011). An OHSS- Free Clinic by segmentation of IVF treatment. *Human Reproduction*, 26, 2593–2597.
- ESHRE. (2016). ART fact sheet. (2016, March 19). Retrieved from [https://www.eshre.eu/\\$/media/sitecore-files/Guide lines/ART-fact-sheet-2016.pdf?la1/4en](https://www.eshre.eu/$/media/sitecore-files/Guide%20lines/ART-fact-sheet-2016.pdf?la1/4en).
- Griffin, D., Benadiva, C., Kummer, N., Budinetz, T., Nulsen, J., & Engmann, L. (2012). Dual trigger of oocyte maturation with gonadotropin-releasing hormone agonist and low-dose human chorionic gonadotropin to optimize live birth rates in high responders. *Fertility and Sterility*, 97, 1316–1320.
- Humaidan, P., Polyzos, N. P., Alsbjerg, B., Erb, K., Mikkelsen, A. L., Elbaek, H. O., Papanikolaou, E. G., & Andersen, C. Y. (2013). GnRHa trigger and individualized luteal phase hCG support according to ovarian response to stimulation: Two prospective randomized controlled multi-centre studies in IVF patients. *Human Reproduction*, 28(9), 2511–2521.
- La Marca, A., Papaleo, E., Grisendi, V., Argento, C., Giulini, S., & Volpe, A. (2012). Development of a nomogram based on markers of ovarian reserve for the individualization of the follicle-stimulating hormone starting dose in in vitro fertilization cycles. *British Journal of Obstetrics and Gynecology*, 119, 1171–1179.
- La Marca, A., Grisendi, V., Giulini, S., Argento, C., Tirelli, A., Dondi, G., & Volpe, A. (2013). Individualization of the FSH starting dose in IVF/ICSI cycles using the antral follicle count. *Journal of Ovarian Research*, 6, 11.
- Lee, T. H., Liu, C. H., Huang, C. C., Wu, Y. L., Shih, Y. T., Ho, H. N., & Lee, M. S. (2008). Serum anti-Müllerian hormone and estradiol levels as predictors of ovarian hyperstimulation syndrome in assisted reproduction technology cycles. *Human Reproduction*, 23, 160–167.
- Mascarenhas, M., & Balen, A. H. (2017). The high responder: A review of pathophysiology and outcomes during IVF treatment. *Human Fertility*, 20, 1742–1755.
- NICE. (2016). Fertility problems: Assessment and treatment. Clinical Guideline [CG156]. National Institute for Health and Care Excellence. (2016, March 19). Retrieved from <https://www.nice.org.uk/guidance/cg156/chapter/1-recommendations>
- Papageorgiou, T., Guibert, J., Goffinet, F., Patrat, C., Fulla, Y., Janssens, Y., & Zorn, J. R. (2002). Percentile curves of serum estradiol levels during controlled ovarian stimulation in 905 cycles stimulated with recombinant FSH show that high estradiol is not detrimental to IVF outcome. *Human Reproduction*, 17, 2846–2850.
- Rizk, B. (2009). Symposium: Update on prediction and management of OHSS. Genetics of ovarian hyperstimulation syndrome. *Reproductive Biomedicine Online*, 19, 14–27. [https://doi.org/10.1016/S1472-6483\(10\)60041-7](https://doi.org/10.1016/S1472-6483(10)60041-7).
- Royal College of Obstetricians and Gynaecologists (2016). The Management of Ovarian Hyperstimulation Syndrome. RCOG Green-top Guideline No. 5. Retrieved from https://www.rcog.org.uk/globalassets/documents/guidelines/green-top-guidelines/gtg_5_ohss.pdf.
- Sterrenburg, M. D., Veltman-Verhulst, S. M., Eijkemans, M. J. C., Hughes, E. G., MacKlon, N. S., Broekmans, F. J., & Fauser, B. C. J. M. (2010). Clinical outcomes in relation to the daily dose of recombinant follicle-stimulating hormone for ovarian stimulation in in vitro fertilization in presumed normal responders younger than 39 years: A meta-analysis. *Human Reproduction Update*, 17(2), 184–196.
- Steward, R. G., Zhang, C. E., Shah, A. A., Yeh, J. S., Chen, C., Li, Y. J., & Muasher, S. J. (2015). High peak estradiol predicts higher miscarriage and lower live birth rates in high responders triggered with a GnRH agonist in IVF/ICSI cycles. *Journal of Reproductive Medicine*, 60, 463–470.
- Toftager, M., Bogstad, J., Bryndorf, T., Lossi, K., Roskaer, J., Holland, T., Praetorius, L., Zedeler, A., Nilas, L., and Pinborg, A. (2016). Risk of severe ovarian hyperstimulation syndrome in GnRH antagonist versus GnRH agonist protocol: RCT including 1050 first IVF/ICSI cycles. *Human Reproduction*, 31, 6, 1253–1264, 2016.
- Youssef, M. A., Van der Veen, F., Al-Inany, H. G., Mochtar, M. H., Griesinger, G., Nagi Mohesen, M., et al. (2014). Gonadotropin-releasing hormone agonist versus HCG for oocyte triggering in antagonist-assisted reproductive technology. *Cochrane Database of Systematic Reviews*, (10), CD008046.

COMPLICATIONS

Hemoperitoneum After Oocyte Retrieval and Severe Pelvic Infection

Raoul Orvieto, Sheba (Tel-Hashomer) Medical Center, Ramat Gan, Israel; and Tel-Aviv University, Tel-Aviv, Israel

© 2018 Elsevier Inc. All rights reserved.

Transvaginal ultrasound-guided aspiration of oocytes is a well-accepted and universally used method in assisted reproduction (Dicker et al., 1993). Its major advantages include easy access to ovarian follicles with excellent oocyte yield and good visualization of the major pelvic vessels. It is done as a day care procedure, either under intravenous analgesia and sedation or under general anesthesia, and is usually traumatic.

Nevertheless, there are some inherent risks, namely, puncture of blood vessels and hemoperitoneum, bleeding from the vaginal-vault puncture site, rupture of adnexal cystic masses, bowel perforation, trauma to pelvic organs, and pelvic infection. Maxwell et al. (2008) have reported on the incidence of both serious and minor complications in young women undergoing 886 oocyte retrievals for oocyte donation. While the rate of serious complications, which included ovarian hyperstimulation syndrome, ovarian torsion, infection, and ruptured ovarian cyst, was 0.7%, the rate of minor complications severe enough to prompt the donor to seek medical attention after retrieval was 8.5%.

The aim of the present article is to discuss comprehensively two of these complications: bleeding and pelvic infection.

Bleeding

During ultrasound-guided transvaginal oocyte aspiration, multiple punctures of the vaginal vault, or inappropriate handling and rotation of the ultrasound vaginal probe while inserting an aspiration needle through the vaginal vault can injure or tear the vaginal mucosa, ovaries, intra-abdominal organs, or blood vessels (Dicker et al., 1993; Tureck et al., 1993; Lenz et al., 1987; Wikland et al., 2011). Bleeding from the vaginal vault is a common consequence of ovum pick-up (OPU), with a reported incidence of 1.4%–18.4% (Tureck et al., 1993). The use of a thinner-tipped needle (0.9 mm in diameter) during OPU resulted in a significantly less vaginal bleeding (Lenz et al., 1987). In most cases, vaginal bleeding as a result of OPU stops spontaneously (Wikland et al., 2011). In cases where a local pressure with a sponge forceps or vaginal packing with a large gauze roll could not stop bleeding, suturing is required.

Intraperitoneal or Retroperitoneal Bleeding

Transvaginal oocyte aspiration can also cause bleeding if intraperitoneal or retroperitoneal pelvic blood vessels are injured or if there is damage to the fine vascular network surrounding the punctured ovarian follicle. The reported incidence of severe intra- or retroperitoneal bleeding varies from 0% to 1.3% (Dicker et al., 1993; Wikland et al., 2011; Aragona et al., 2011); Sauer (2001) reported on one case of intra-abdominal bleeding complicating aspiration of 1000 oocyte donors. Young, lean patients, those with polycystic ovary syndrome (PCOS) or a history of previous surgery were specifically demonstrated to be at much higher risk for this complication (Liberty et al., 2010; Zhen et al., 2010). Intraperitoneal bleeding tends to be severe with acute hemodynamic deterioration, whereas retroperitoneal bleeding usually has a later and more indolent presentation. Yih et al. (2001) studied serial complete blood counts before and after OPU in 93 in vitro fertilization (IVF) cycles and demonstrated a nonsignificant change in hematocrit levels, indicating that a clinically significant blood loss after OPU is actually uncommon.

Azem et al. (2000) described a patient who presented to the emergency room 10 h after OPU with severe lower abdominal pain, vomiting, and tenesmus. Examination revealed a distended abdomen with severe tenderness in the pouch of Douglas; on transvaginal sonography, a minimal, 3–4 cm collection of fluid was noted. Laparoscopy followed by laparotomy, which was performed on the basis of the clinical profile, revealed a retroperitoneal hematoma 7 cm in diameter. After evacuation and hemostasis, active bleeding from the mid-sacral vein occurred and was controlled by a metal clip. This case demonstrates the indolent course of retroperitoneal bleeding and physicians should be alerted to the possibility of retroperitoneal hematoma despite an absence of free fluid in the pouch of Douglas. Of notice, similar case with no significant intraperitoneal fluid collection was also described as a result from ureteral injury with the consequent uroretroperitoneum (Fiori et al., 2006).

Intraabdominal bleeding should be suspected immediately after OPU on the development of signs and symptoms of anemia—specifically, weakness, dizziness, dyspnea, or persistent tachycardia. Early management consists of intense hemodynamic monitoring, together with serial measurement of blood hemoglobin concentrations and ultrasonographic evaluation for the presence of intra-abdominal fluid. It should be emphasized, however, that intra-abdominal blood clots or retroperitoneal bleeding might be invisible even to an experienced ultrasound operator. A drop in hemoglobin concentration is an indication for prompt blood transfusion. If hemodynamic deterioration continues or acute abdominal pain develops, diagnostic laparoscopy or exploratory

laparotomy with subsequent hemostasis of the bleeding site(s) are required. The clinician must make sure to handle the fragile hyperstimulated ovaries very cautiously. Of notice, a longer time interval between OPU and surgical intervention was noted to put the patient at risk of ovariectomy (Nouri et al., 2014).

Dicker et al. (1993) described three cases of severe intra-abdominal bleeding from ovarian puncture sites during OPU, leading to acute abdominal complications. In two of the patients, symptoms developed 3 h after OPU (hemoglobin 9.0 g/100 mL and 8.1 g/100 mL, respectively), and laparoscopic drainage and hemostasis were sufficient. The third patient became symptomatic after 4 h (hemoglobin 7.3 g/100 mL) and required exploratory laparotomy and hemostasis in addition to the transfusion of four units of blood as a life-saving procedure. Later, Battaglia et al. (2001) reported severe intra-abdominal bleeding from the surface of both ovaries in a patient with coagulation factor XI deficiency. As expected, the patient became symptomatic 3 h after OPU and required laparotomy, partial resection of stuffed ovaries, and hemostasis. Physicians should be aware of the presence of concomitant coagulopathy and might therefore consider intense coagulation-factor replacement before or during abdominal exploration.

Massive delayed intra-abdominal hemorrhage was also reported following OPU (2 and 4 days later), in patients at risk for thromboembolic event, who concomitantly used therapeutic dose of low molecular weight heparin (LMWH) (Mashiach et al., 2013). These cases should direct physicians' attention and keep them alert, while conducting an IVF treatment to this subgroup of patients. Moreover, the authors recommended that the patient be kept in the ward for observation for at least 2–4 days following OPU.

Prevention and Treatment

Can we prevent severe intra-abdominal bleeding from ovarian puncture sites during OPU? In a cross-sectional retrospective study, Revel et al. (2011) questioned the utility of coagulation screening before OPU. Among the 1032 patients evaluated, they found that 534 coagulation tests were needed to prevent one case of bleeding associated with an abnormal coagulation test result. Moreover, while the use of color Doppler sonography during OPU was suggested to reduce the risk of blood vessel injury by the guiding needle (Shalev et al., 2004), its routine use could not predict all cases with moderate peritoneal bleeding.

Description of the intraoperative measures needed to control intra-abdominal hemorrhage is beyond the scope of this text, and the reader is referred elsewhere for a detailed review.

Pelvic Infection

Pelvic inflammatory disease (PID) is an infrequent complication of ultrasound-guided transvaginal aspiration of oocytes or ET, with a reported incidence of 0.2%–0.5% per cycle (Howe et al., 1988; Ashkenazi et al., 1994; Al-Kuran et al., 2008). Signs or symptoms of pelvic infection, such as pyrexia, continuous low abdominal pain, dysuria, or offensive vaginal discharge, are infrequent (Howe et al., 1988). However, this does not exclude occult, subclinical bacterial colonization, which may influence the success of the IVF–ET treatment, or slowly progress throughout pregnancy (Al-Kuran et al., 2008). Ashkenazi et al. (1994) evaluated the outcome of all IVF–ET procedures performed in their unit between 1986 and 1992. Of the 4771 patients who underwent transvaginal OPU, 28 (0.58%) had symptoms of PID within 1–7 days. The diagnosis was established by a rise in body temperature to 38°C for more than 48 h, signs of pelvic peritonitis on physical examination, leukocyte count of $> 12,000$ cells/ m^3 , and elevated erythrocyte sedimentation rate. All patients were admitted to hospital for treatment with intravenous antibiotics. Of notice, ovarian abscess following oocyte retrieval may manifest late during pregnancy with low grade fever or vague abdominal pain.

OPU can also lead to severe abdominal complications. Our group reported on nine patients (0.24%) with tubo-ovarian or pelvic abscess after transvaginal-guided OPU (Ashkenazi et al., 1994). Three patients required laparotomy and adnexectomy, whereas in six patients, culdocentesis was performed for adequate pelvic abscess drainage. Kelada and Ghani (2007) have described a case of bilateral ovarian abscesses following transvaginal oocyte retrieval, complicated by early signs of consumption coagulopathy. The latter is a serious and life-threatening complication of pelvic infection and sepsis, which should be diagnosed and corrected immediately.

Mechanisms Underlying Pelvic Infection

During transvaginal aspiration, accidental needle transport of cervicovaginal flora into ovarian tissue can cause unilateral or bilateral oophoritis, and accidental puncture of a contaminated or sterile hydrosalpinx can cause salpingitis. Some authors have attributed pelvic infection to infected endometriotic cysts or tuboovarian abscess after aspiration of endometriomas, or, rarely, to inadvertent puncture of the bowel. Pelvic infection in women with endometriosis was shown to be more serious and resistant to antibiotic treatment, and frequently required surgical intervention (Elizur et al., 2014). Pelvic infection can occur as a direct consequence of transcervical ET. This is evidenced by reported cases of PID following ET in an agonadal donor-egg recipient, or during cryopreserved ET (Friedler et al., 1996); it may also occur as a result of the reaction of a silent or persistent subclinical infection, as seen occasionally after hysterosalpingography. Another possible cause during ET is catheterization of the uterus, which may force bacteria-laden air or fluid into one or both tubes by a piston-like effect.

Effect of Acute Pelvic Infection on IVF–ET Outcome

The first study of the impact of pelvic infection on IVF–ET outcome was reported by [Ashkenazi et al. \(1994\)](#). We found that the number of oocytes recovered, fertilized, and cleaved in 28 patients undergoing IVF in whom PID developed was similar to that of a comparison group with mechanical infertility. However, there were no pregnancies in the PID group, as compared with the 23%–31% pregnancy rate per transfer in the whole group of patients treated by IVF, indicating that the appearance of PID at the critical time of implantation may cause a failure to conceive. This finding has several possible explanations, such as endotoxemia, the local inflammatory reaction or temperature elevation ([Ben-Rafael and Orvieto, 1992](#)).

Treatment

The potential for intraperitoneal bacterial contamination during transvaginal oocyte recovery is well known and has led to the routine use of prophylactic antibiotics and vaginal disinfection. [Meldrum \(1989\)](#) found no case of pelvic infection among 88 transvaginal retrievals with the use of intravenous cefazolin and vaginal preparation with povidone–iodine and saline irrigation; nor did [Tsai et al. \(2005\)](#) in patients with ovarian endometrioma, using only vaginal douching with aqueous povidone–iodine followed by normal saline irrigation. [Borlum and Maiggard \(1989\)](#) reported on two cases of serious pelvic infection in almost 400 transvaginal aspirations. They used only two vaginal douchings with sterile saline and noted that minimizing the number of repeated vaginal penetrations may have helped in lowering the risk of infection. However, the appropriate type of antibiotic administration, timing or duration of therapy, and the efficacy of therapy have not yet been established. Indeed, some authors claim that these measures may not only further reduce the incidence of PID after oocyte retrieval, but can even increase the risks of both an adverse reaction and of colonization with resistant organisms. Our experience with vaginal douchings with sterile saline in approximately 1100–1200 OPIs per year revealed a very low rate of PID after OPI. [Peters et al. \(1993\)](#) suggested that only women with a tubal abnormality and a history of pelvic infection should receive prophylactic antibiotics before oocyte aspiration, and also possibly after ET. Others have suggested that such patients may benefit from transabdominal or transvesical rather than transvaginal procedures ([Wren and Parson, 1989](#)).

It is also noteworthy that [Egbase et al. \(1999\)](#), in a study of the effect of prophylactic antibiotics in OPI on the endocervical microbial inoculation of the endometrium at ET, found that prophylactic antibiotics not only reduced the number of positive microbiology cultures of embryo catheter tips, but also significantly increased implantation and clinical pregnancy rates. On the other hand, in their prospective randomized study, [Peikrishvili et al. \(2004\)](#) could not demonstrate any beneficial effects of antibiotic prescription (amoxicillin & clavulanic acid 1 g/125 mg) for 6 days following oocyte retrieval on implantation, pregnancy, or miscarriage rates.

Pelvic inflammatory disease or tubo-ovarian abscess after OPI require accurate diagnosis and prompt treatment with broad-spectrum antibiotics. In the presence of a pelvic abscess that is larger than 8 cm or unresponsive to medication, transvaginal or percutaneous drainage is the treatment of choice, with or without ultrasound-guided intracavitary instillation of a combination of antibiotics. Patients who received antibiotics alone are more likely to require further surgical intervention when compared with patients who additionally received image-guided drainage ([To et al., 2014](#)). Sometimes surgical laparoscopy or laparotomy is needed to evacuate the abscess or remove the infected tubes or adnexae.

In summary, the appearance of PID at the critical time of implantation results in failure to conceive. This effect may be mediated by bacterial endotoxins, a local inflammatory reaction against bacteria with the involvement of cytokines that affect implantation and early embryonic development, or temperature elevation that directly affects the conceptus. Although the role of prophylactic antibiotics is still controversial, they can be considered in the presence of risk factors for PID; aspiration of hydrosalpinx or endometriomas during OPI might be a risk factor for infection and should be avoided. Furthermore, to prevent total failure, if PID develops before ET, cryopreservation and ET in subsequent cycles should be considered. However, if PID develops after ET, the bacterial infection and fever should be treated rigorously to prevent reproductive failure.

References

- Al-Kuran, O., Beitawi, S., & Al-Mehaisen, L. (2008). Pelvic abscess complicating an in vitro fertilization pregnancy and review of the literature. *Journal of Assisted Reproduction and Genetics*, 25, 341–343.
- Aragona, C., Mohamed, M. A., Espinola, M. S., et al. (2011). Clinical complications after transvaginal oocyte retrieval in 7,098 IVF cycles. *Fertility and Sterility*, 95, 293–294.
- Ashkenazi, J., Farhi, J., Dicker, D., et al. (1994). Acute pelvic inflammatory disease after oocyte retrieval: adverse effects on the results of implantation. *Fertility and Sterility*, 61, 526–528.
- Azem, F., Wolf, Y., Botchan, A., et al. (2000). Massive retroperitoneal bleeding: a complication of transvaginal ultrasonography-guided oocyte retrieval for in vitro fertilization-embryo transfer. *Fertility and Sterility*, 74, 405–406.
- Battaglia, C., Regnani, G., Giulini, S., et al. (2001). Severe intraabdominal bleeding after transvaginal oocyte retrieval for IVF-ET and coagulation factor XI deficiency: a case report. *Journal of Assisted Reproduction and Genetics*, 3, 178–181.
- Ben-Rafael, Z., & Orvieto, R. (1992). Cytokine involvement in reproduction. *Fertility and Sterility*, 58, 1093–1099.
- Borlum, K. G., & Maiggard, S. (1989). Transvaginal oocyte aspiration and pelvic infection. *Lancet*, 2, 53 (letter).
- Dicker, D., Ashkenazi, J., Feldberg, D., et al. (1993). Severe abdominal complications after transvaginal ultrasonographically-guided retrieval of oocytes for in vitro fertilization and embryo transfer. *Fertility and Sterility*, 59, 1313–1315.
- Egbase, P. E., Udo, E. E., Al-Sharhan, M., & Grudzinskas, J. G. (1999). Prophylactic antibiotics and endocervical microbial inoculation of the endometrium at embryo transfer. *Lancet*, 354, 651–652.

- Elizur, S. E., Lebovitz, O., Weintraub, A. Y., Eisenberg, V. H., Seidman, D. S., Goldenberg, M., & Soriano, D. (2014). Pelvic inflammatory disease in women with endometriosis is more severe than in those without. *The Australian & New Zealand Journal of Obstetrics & Gynaecology*, 54, 162–165.
- Fiori, O., Cornet, D., Darai, E., Antoine, J. M., & Bazot, M. (2006). Uro-retroperitoneum after ultrasound-guided transvaginal follicle puncture in an oocyte donor: a case report. *Human Reproduction*, 21, 2969–2971.
- Friedler, S., Ben-Shachar, I., Abramov, Y., et al. (1996). Ruptured tubo-ovarian abscess complicating transcervical cryopreserved embryo transfer. *Fertility and Sterility*, 65, 1065–1066.
- Howe, R. S., Wheeler, C., Mastroianni, L., Jr., et al. (1988). Pelvic infection after transvaginal ultrasound-guided ovum retrieval. *Fertility and Sterility*, 49, 726–728.
- Kelada, E., & Ghani, R. (2007). Bilateral ovarian abscesses following transvaginal oocyte retrieval for IVF: a case report and review of literature. *Journal of Assisted Reproduction and Genetics*, 24, 143–145.
- Lenz, S., Leeton, J., & Renou, P. (1987). Transvaginal recovery of oocytes for in vitro fertilization using vaginal ultra-sound. *Journal of In Vitro Fertilization and Embryo Transfer*, 4, 51–55.
- Liberty, G., Hyman, J. H., Eldar-Geva, T., et al. (2010). Ovarian hemorrhage after transvaginal ultrasonographically guided oocyte aspiration: a potentially catastrophic and not so rare complication among lean patients with polycystic ovary syndrome. *Fertility and Sterility*, 93, 874–879.
- Mashiach, R., Stockheim, D., Zolti, M., & Orvieto, R. (2013). Delayed intra-abdominal bleeding following trans-vaginal ultrasonography guided oocyte retrieval for in vitro fertilization in patients at risk for thrombo-embolic events under anticoagulant therapy [v1; ref status: awaiting peer review, <http://f1000r.es/1ufl>]. *F1000Research*, 2, 189.
- Maxwell, K. N., Cholist, I. N., & Rosenwaks, Z. (2008). The incidence of both serious and minor complications in young women undergoing oocyte donation. *Fertility and Sterility*, 90, 2165–2171.
- Meldrum, D. R. (1989). Antibiotics for vaginal oocyte aspiration. *Journal of In Vitro Fertilization and Embryo Transfer*, 6, 1–2.
- Nouri, K., Walch, K., Promberger, R., Kurz, C., Tempfer, C. B., & Ott, J. (2014). Severe haematoperitoneum caused by ovarian bleeding after transvaginal oocyte retrieval: a retrospective analysis and systematic literature review. *Reproductive Biomedicine Online*, 29, 699–707.
- Peikrishvili, R., Evrard, B., Pouly, J. L., & Janny, L. (2004). Prophylactic antibiotic therapy (amoxicillin+clavulanic acid) before embryo transfer for IVF is useless. Results of a randomized study. *Journal de Gynécologie, Obstétrique et Biologie de la Reproduction*, 33, 713–719.
- Peters, A. J., Hecht, B., Durinzi, K., et al. (1993). Salpingitis or oophoritis: What causes fever following oocyte aspiration and embryo transfer? *Obstetrics and Gynecology*, 81, 876–877.
- Revel, A., Schejter-Dinur, Y., Yahalomi, S. Z., et al. (2011). Is routine screening needed for coagulation abnormalities before oocyte retrieval? *Fertility and Sterility*, 95, 1182–1184.
- Sauer, M. V. (2001). Defining the incidence of serious complications experienced by oocyte donors: a review of 1000 cases. *American Journal of Obstetrics and Gynecology*, 184, 277–278.
- Shalev, J., Orvieto, R., & Meizner, I. (2004). Use of color Doppler sonography during follicular aspiration in patients undergoing in vitro fertilization may reduce the risk of blood vessel injury. *Fertility and Sterility*, 81, 1408–1410.
- To, J., Aldape, D., Frost, A., Goldberg, G. L., Levie, M., & Chudnoff, S. (2014). Image-guided drainage versus antibiotic-only treatment of pelvic abscesses: short-term and long-term outcomes. *Fertility and Sterility*, 102, 1155–1159.
- Tsai, Y. C., Lin, M. Y., Chen, S. H., et al. (2005). Vaginal disinfection with povidone iodine immediately before oocyte retrieval is effective in preventing pelvic abscess formation without compromising the outcome of IVF-ET. *Journal of Assisted Reproduction and Genetics*, 22, 173–175.
- Tureck, R. W., Garcia, C., Blasco, L., & Mastroianni, L. (1993). Perioperative complications arising after transvaginal oocyte retrieval. *Obstetrics and Gynecology*, 81, 590–593.
- Wikland, M., Blad, S., Bungum, L., Hillensjö, T., Karlström, P. O., & Nilsson, S. (2011). A randomized controlled study comparing pain experience between a newly designed needle with a thin tip and a standard needle for oocyte aspiration. *Human Reproduction*, 26, 1377–1383.
- Wren, M., & Parson, J. (1989). Ultrasound directed follicle aspiration in IVF. In C. Chen, S. L. Tan, & W. C. Cheng (Eds.), *Recent advances in the management of infertility* (pp. 105–181). New York: McGraw-Hill.
- Yih, M. C., Goldschlag, D., Davis, O. K., et al. (2001). Complete blood counts (CBC) after oocyte retrieval: what is normal? *Fertility and Sterility*, 76, S115–16.
- Zhen, X., Qiao, J., Ma, C., Fan, Y., & Liu, P. (2010). Intraperitoneal bleeding following transvaginal oocyte retrieval. *International Journal of Gynaecology and Obstetrics*, 108, 31–34.

Ectopic and Heterotopic Pregnancies

Lisa L Campbell and Andrew W Horne, University of Edinburgh, Edinburgh, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Ectopic pregnancy A pregnancy implanted at an abnormal location outside the normal uterine cavity, most commonly within the Fallopian tube in women.

Heterotopic pregnancy Concurrent intrauterine and ectopic pregnancy.

Human chorionic gonadotrophin The hormone produced by the trophoblast cells of the placenta; essential to maintain progesterone output from the corpus luteum in early pregnancy and detected in maternal blood or urine in a pregnancy test.

Laparoscopy Minimally invasive (keyhole) surgery where access to and visualization of the abdominal/pelvic cavity is achieved using an endoscope (telescope).

Laparotomy A large incision through the abdominal wall into the abdominal cavity.

Salpingectomy Surgical removal of a Fallopian tube.

Salpingostomy Incision in a Fallopian tube wall.

Nomenclature

ART Artificial reproductive technology

β -hCG Beta subunit of human chorionic gonadotrophin ("pregnancy hormone")

EP Ectopic pregnancy

FT Fallopian tube

HP Heterotopic pregnancy

IUP Intrauterine pregnancy

IVF In vitro fertilization

TVUSS Transvaginal ultrasound scan

Introduction

Ectopic pregnancy (EP) occurs when an embryo implants and develops outside of the endometrial-lined uterine cavity. The name derives from the Greek "ektopos" meaning "out-of-place" and was first described in AD 963 by Islamic surgeon Albucasis. In women, 1%–2% of all pregnancies are ectopic and in 95%–98% of cases, the pregnancy occurs in the Fallopian tube (FT; Fig. 1). Rarely, EPs develop at or in the utero-tubal junction (interstitial or cornual), ovary, cervix, myometrium, abdominal cavity or in a caesarean section scar. They are also occasionally bilateral. In respect to abdominal EPs, implantation to almost any mesentery, organ or ligamentous structure (including the broad ligament) has been described. Cornual pregnancies are a slight

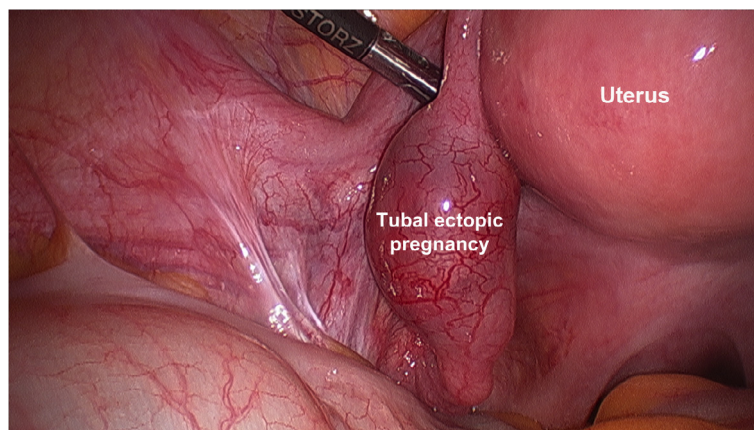


Fig. 1 Image of a tubal ectopic pregnancy taken at laparoscopy.

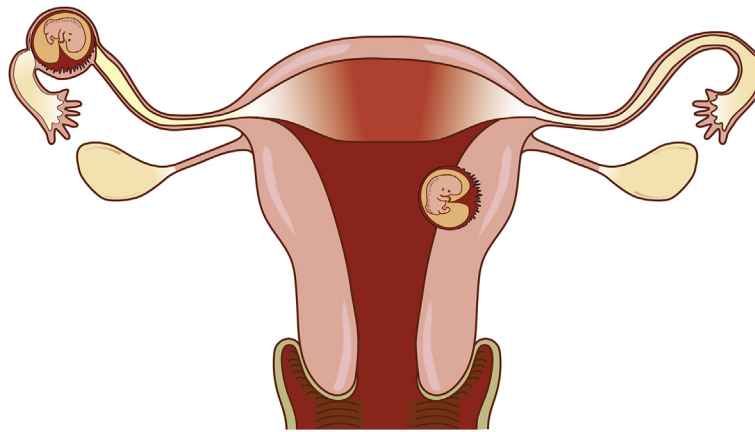


Fig. 2 Heterotopic pregnancy.

misnomer as they occur within an endometrial-lined cavity but this is within a developmentally abnormal uterus. EPs are almost exclusively incompatible with a live neonate, except in occasional cases of abdominal and caesarean section scar EPs. However, all EPs risk serious maternal morbidity, if not mortality, largely as a result of associated intraabdominal hemorrhage. In tubal EP, presence of hemorrhage is often referred to as a “ruptured” EP. This description is misleading as hemorrhage is more often a result of gradual trophoblast invasion of maternal vasculature and destruction of the tube wall as opposed to sudden tubal dehiscence. This article will focus on EPs in women but it is important to note that this phenomenon also occurs, although rarely, in other mammalian species (e.g., in rabbits, guinea pigs, and other primates). However, EPs in these species are almost exclusively abdominal, with only a small number of tubal pregnancies reported in other primates (Corpa, 2006). Heterotopic pregnancy (HP) refers to concurrent ectopic and intrauterine pregnancy (IUP) (Fig. 2). HP is essentially a rare variant of EP and presents similar diagnostic difficulties and management dilemmas as with other rare forms of EP (Talbot et al., 2011).

Incidence and Mortality

Globally, the incidence of EP has risen in the last few decades. However, it is likely this is largely due to improved detection and increased reporting. The Eighth Report of the Confidential Enquiries into Maternal Deaths in the United Kingdom covering the triennium 2006–2008, provides the most recent estimate of EP incidence in the UK at 11 per 1000 pregnancies (equivalent to 12,000 EPs per year). The incidence of HP is low at 1:25,000–30,000, except following ART where incidence is approximately 1%. The global incidence of EP is hard to estimate due to regional differences in registering of births and varying use of live birth (in lower income countries) or total number of pregnancies (Western world) as a denominator. Underreporting is probable in resource poor areas as the diagnosis can be missed or misclassified (in particular spontaneously resolving EPs will likely go unidentified) (Leke et al., 2004). In these lower resource countries the incidence is thought to be higher, at least partially attributed to increased prevalence of untreated sexually transmitted infections (Masha et al., 2017). In the UK, the most recent MBRRACE (Mothers and Babies: Reducing Risk through Audits and Confidential Enquires) report identified 12 women who died from early-pregnancy causes and in 9 of these cases cause of death was EP. In low resource countries, the majority of women present with ruptured EPs due to a lack of early pregnancy care, and as a result mortality is higher.

Etiology and Pathophysiology

The majority of tubal EPs are located in the ampulla (~70%) perhaps not surprisingly, as the fertilized embryo spends the majority of tubal transit time in this region. Other tubal EPs occur in the isthmus (~12%) or infundibulum/fimbria (~11%) (Bouyer et al., 2002). Currently, the ovary is the next most common site for EPs. The etiology of ovarian EP is largely unknown. Interstitial and cornual EPs deserve special mention as 33% present with rupture and they carry a 2%–2.5% mortality rate, much higher than other EPs. The terms interstitial and cornual EP are often used synonymously in the literature but they describe two separate entities. Interstitial EPs develop within the 1–2 cm uterine portion of the FT which lies within the thick muscular wall of the uterus. Cornual EPs are within an endometrial lined cavity but this is within a developmentally abnormal uterus (bicornuate, unicornuate, or septate), such that ongoing pregnancy is associated with uterine rupture. Intramural pregnancies carry the same risk of uterine rupture but are extremely rare, occurring at the site of previous myomectomy. Abdominal EPs result from retrograde efflux of an embryo or an unfertilized oocyte into the peritoneal cavity prior to implantation or follow expulsion of an implanted tubal EP through a breach in the FT wall. With the rise in caesarean deliveries and improved ultrasound resolution, the incidence of caesarean scar EPs has increased over the last few decades to 1:1800–2200. These pregnancies implant in the

endometrial- and myometrial-deficient scar tissue at the site of the previous uterine incision. Due to their scarcity, the natural history of caesarean scar EP is uncertain but there appears to be some overlap with placenta accreta. There are some reported cases of misdiagnosis or intentional pregnancy continuation with resulting emergency caesarean hysterectomy and delivery of a live, albeit premature, infant. However, uterine rupture is inevitable and most rupture before viability with serious associated maternal morbidity.

The development of tubal EPs are largely attributed to FT damage or dysfunction, although uterine and embryonic factors may also be involved (particularly in the case of IVF where physiological passage of the embryo through the FT is bypassed) (Shaw et al., 2010). Although epidemiological studies have identified strong correlations between certain risk factors and EP the causal mechanisms behind these correlations have not been fully elucidated. There exists a “chicken and egg” scenario as it is not possible, for obvious ethical and practical reasons, to discover if alterations in gene or protein expression in ex-vivo EP samples predisposed to, or resulted from, EP. Moreover, in up to 50% of cases, no risk factor is identified. Anything that can cause anatomical distortion or inflammation within the FT can pre-dispose to EP, for example, pelvic infection (particularly *Chlamydia trachomatis*), previous EP or previous tubal surgery. It is supposed that damage to the FT creates a proinflammatory environment, exposing extracellular matrix and cell adhesion proteins, which can encourage aberrant blastocyst attachment. In addition, functional alterations within the endothelial cilia, smooth muscle or tubal secretions that transport the embryo towards the uterus can lead to EP, such as those induced by cigarette smoking. Other risk factors associated with EP include: abdominal surgery (e.g., appendectomy, caesarean section), subfertility, IVF, endometriosis, advancing maternal age (particularly >35 years) or conception on contraception (intrauterine devices, the oral contraceptive pill, the morning after pill). In regards HP (EP in the presence of ongoing IUP), the single biggest risk factor is ART, particularly related to multiple embryo transfer following IVF or ovarian stimulation. Within this group, the same preexisting risk factors apply as for EP. As with EP, the extrauterine pregnancy in HP is most commonly tubal, although cervical, caesarean scar and even triplet pregnancy with tubal and cervical EP have been reported.

Clinical Presentation and Diagnosis

A diagnosis of EP/HP should be considered in any woman of reproductive age presenting with collapse (hypovolemic shock) and anemia, regardless of knowledge of pregnancy status. Indeed, in half of the UK cases of maternal mortality reported between 2009 and 2014, there was delay in initial diagnosis in the face of these signs. A “FAST” (Focused Assessment by Sonography for Trauma) scan and a catheter β -hCG test can quickly establish a diagnosis in the case of haemodynamic instability. In the Western world, the majority of EP/HPs present subacutely with one, some or none of the following symptoms:

- acute abdominal or pelvic pain that is often but not exclusively unilateral,
- vaginal spotting or bleeding,
- gastrointestinal disturbance (diarrhea/vomiting),
- dizziness,
- shoulder tip pain (resulting from diaphragmatic irritation).

Some of these symptoms may also indicate miscarriage, ongoing viable intrauterine pregnancy (IUP) or maybe unrelated to pregnancy (e.g., appendicitis). However, in all women of reproductive age with symptoms possibly related to EP/HP, an initial urinary β -hCG test is cheap, quick and vital. If positive, this can be followed with quantitative maternal serum β -hCG measurement and a transvaginal ultrasound scan (TVUSS).

TVUSS has a sensitivity of 87%–99% and a specificity of 94%–99% for diagnosis of EP (Elson et al., 2016). It is now so sensitive that a gestational sac at a diameter of <3 mm can be identified. A TVUSS in early pregnancy should include examination of the cervical canal, interstitial portions of the FT, both adenexa and the pouch of Douglas (to identify free fluid, indicating blood, in the peritoneal cavity) (Jurkovic and Mavrelos, 2007). Regardless of anatomical location, the majority of EPs appear as a heterogeneous solid swelling. It is less common to see a gestational sac +/- yolk sack and even rarer to see a fetus +/- cardiac activity. Occasionally a “pseudosac” is seen in the endometrial cavity and can be misdiagnosed as an IUP. This pregnancy-induced fluid accumulation can be differentiated from an IUP as it is centrally as opposed to eccentrically placed, does not contain a yolk sac and is not surrounded by a hyperechoic ring of decidua. A “textbook” TVUSS of a tubal EP identifies an empty uterus with an adenexal mass that moves separate to the ovary (“sliding sign”). This sign is absent in ovarian EP, where a clue to diagnosis is additional identification of a separate corpus luteum. Cervical EPs can be detected by identification of a “barrel shaped” cervix with a mass below the level of the internal os. Caesarean section scar EPs are often in a similar location, but will be anterior with thin or absent myometrium between the gestation and the bladder. In both instances it is important to identify surrounding color doppler flow to exclude a miscarrying gestation. Interstitial pregnancies can be differentiated from those within the uterine cavity as they are completely encased by myometrium. It should also be possible to identify the pregnancy at the lateral aspect of the “interstitial line” (a thin hyperechoic line representing the interstitial portion of the FT, extending from the uterine cavity through the myometrium). In cornual pregnancies, there may only be one “interstitial line” seen if the uterus is unicornuate. The gestational sac will be surrounded by myometrium but, in contrast to an interstitial pregnancy, there will be communication with the intrauterine cavity seen on TVUSS. Where a pregnancy is developing within a rudimentary horn, this will be seen separate and mobile from the uterus, perhaps attached by a vascular pedicle. In some cases, where accurate diagnosis by TVUSS is difficult (e.g., multifibroid uterus with distortion of the endometrial cavity) or detailed anatomical delineation for surgical planning is required, a magnetic resonance image should be obtained.

TVUSS is the investigation of choice to identify an EP and can detect an EP even at very low serum β -hCG levels. This is due to the aberrant nature of β -hCG secretion in an EP which is not necessarily related to the size of mass on TVUSS. As such, TVUSS should be undertaken regardless of β -hCG if EP is considered. That said, an IUP, if present, is only likely to be detected if β -hCG is > 1000 – 2000 IU/L. Where there is no obvious intra- or extrauterine pregnancy on TVUSS, the pregnancy is of unknown location (PUL) and serial serum β -hCG measures become important. PUL is a working diagnosis reached at first assessment in approximately 10% of women presenting with symptoms in early pregnancy. The differentials in this case include: ongoing IUP, failing IUP or EP. In a normal IUP, maternal serum β -hCG levels typically increase by $> 66\%$ in a 48 h period and IUP can then be confirmed by TVUSS. In EP or failing IUP, serum β -hCG levels can rise suboptimally, remain roughly static or fall. Serial β -hCG measures every 48 h, repeat TVUSS and repeat clinical assessments are required to reach a diagnosis. On some occasions, where it is difficult to differentiate between a failing IUP and an EP, inpatient monitoring may be necessary and sometimes an endometrial biopsy is obtained to identify if placental tissue (villi) are present within the uterine cavity, suggesting an IUP. Ultimately, diagnostic laparoscopy, with a view to treatment if an EP is positively identified, may be required. As yet there is no single biochemical test that can definitively identify an EP, nor discriminate between a failing IUP and an EP. Progesterone is sometimes included alongside β -hCG in assessment of women with possible EP but does not add any predictive power and is not recommended (Cartwright et al., 2009).

In HP, where, in the presence of an IUP seen on TVUSS, there is question about a concurrent EP, serial serum β -hCG measures are unhelpful as aberrant β -hCG production by an EP is masked by the IUP. In these cases, repeat TVUSS and in some cases MRI, is required. HP is a dangerous condition as detection of an IUP can distract from the presence of a concurrent EP. Symptoms that, in a woman of reproductive age, might signal an EP, such as pain and bleeding, are attributed to the IUP. A cystic adnexal mass on ultrasound may be discounted as a corpus luteum. As the majority of cases of HP follow ART, it is fortunate that a population of patients at risk of HP can be identified in which intensive monitoring is possible and already undertaken. However, in the general population, it is important to “think ectopic” if symptoms persist even if an IUP is identified.

Management of Tubal EP

Tubal EP can be managed surgically, medically or expectantly, depending on patient parameters and patient preference.

Surgical Treatment

Historically, EP had an extremely high mortality rate. Edinburgh born, Robert Lawson Tait, was the first surgeon to successfully perform salpingectomy for ruptured tubal EP. He was meticulous regarding sepsis (unusual at the time) and from 1883 he performed over 40 laparotomies with salpingectomy with only one maternal death. Over the next century, survival rates for EP improved dramatically with the advent of blood transfusion, urinary and serum β -hCG detection and ultrasound. Today, the majority of women with EP will still be managed surgically. In particular, surgery (following initial resuscitation) is the only safe treatment when a woman presents with haemodynamic compromise. Surgical management in a stable patient is also the only option if the current criteria for medical or expectant management (see below) are not met or the patient chooses surgical treatment. A laparoscopic approach is preferred, where surgical skills and equipment are available and the women’s clinical condition allows. Laparoscopy is associated with less postoperative pain, fewer complications, quicker recovery and shorter hospital stay than laparotomy. There has been much debate as to whether surgical removal of the tube containing the EP (salpingectomy) or evacuation of the products of conception through an incision in the tube (salpingostomy) has the best outcome in terms of future fertility. In a randomized controlled trial, salpingostomy, as opposed to salpingectomy, did not significantly improve future fertility prospects in women with a healthy contralateral tube (Mol et al., 2014). As salpingectomy is associated with fewer complications (e.g., bleeding from the tube) and does not require postoperative β -hCG monitoring to ensure resolution of residual trophoblast (present in 8% of cases), it is now the preferred approach in most instances. Salpingostomy or medical treatment is generally preferred in women with contralateral tubal disease unless they only plan to pursue IVF. In this instance, where presence of damaged tubes may reduce embryo implantation rate, increase early pregnancy loss and increase risk of EP, bilateral salpingectomy is sometimes performed after careful consultation. Tubal reanastomosis (following removal of the tubal segment containing the EP) has a high rate of EP recurrence and is not recommended. Clinical consensus of the NICE Ectopic Pregnancy and Miscarriage guideline development group is to recommend anti-D rhesus prophylaxis following surgery for EP or significant maternal hemorrhage.

Medical Treatment

Intramuscular methotrexate is currently the only medical treatment for EP. Methotrexate inhibits dihydrofolate reductase, preventing reduction of dihydrofolate to tetrahydrofolate; the latter is a requirement for purine and pyrimidine production during DNA synthesis. In 1956, Roy Hertz used methotrexate to treat choriocarcinoma. This was the first successful use of chemotherapy to cure a solid tumor. In the 1980s, high- and multiple-dose methotrexate regimes, with folinic acid supplementation, were used to treat tubal EPs. We now use a lower- and single-dose of 50 mg/m^2 without folinic acid supplementation, to treat small tubal EPs. Serum β -hCG is measured on days 1, 4, 7, 11, and weekly thereafter until β -hCG is < 15 IU/L (as tubal rupture has not been reported below this level). A 15% drop in β -hCG between days 4 and 7 has a positive predictive value for treatment success of 93%. If this

drop does not occur a further dose of methotrexate is administered. Resolution of the EP takes 2–7 weeks. This regime is based on a clinical trial by [Stovall and Ling \(1993\)](#) in 120 EP patients and is successful in 88% of cases. Where β -hCG does not decline or tubal rupture occurs (sometimes even despite a declining β -hCG) surgery is required. Although other protocols have been suggested, no well-powered comparative trials have been performed. Based on a few small trials, oral or direct intragestational injection have similar efficacy to intramuscular methotrexate but have, respectively, more side effects or are more invasive and are therefore not favored.

Methotrexate is inexpensive but, due to the follow-up required, only becomes cost effective in comparison with laparoscopic surgery when β -hCG is <1500 IU/L ([Mol et al., 2008](#)). It is cost neutral when β -hCG is <3000 IU/L and in most units this is the cut-off for methotrexate treatment of EP. Clinically, however, 80%–90% success rates are reported up to 5000 IU/L. Other ultrasound criteria must be met: tubal mass <3.5 cm diameter (larger masses can indicate intragestational hemorrhage); minimal free fluid on TVUSS; no fetal cardiac activity (as this increases treatment failure rate) and exclusion of a concurrent IUP. Clinically, the woman must be haemodynamically stable and report minimal pain. She must not be breastfeeding nor taking any medications that might interact with methotrexate and should be advised to avoid alcohol and sexual intercourse during treatment. A full blood count, liver and renal function are obtained before treatment to rule out any coincident infection; liver, renal, or hematological disorder; or immunodeficiency. Low dose methotrexate causes nonspecific abdominal pain in two thirds of women and rarely stomatitis, conjunctivitis or UV related dermatitis. Theoretically, myelotoxicity and acute respiratory distress syndrome are possible but unlikely using the “single-dose” protocol. Unfortunately randomized control trials comparing treatment success and effects on future fertility of single-dose intramuscular methotrexate with salpingectomy are lacking (most previous studies have compared with salpingostomy +/- methotrexate at the time of surgery). Information from retrospective cohort studies suggest salpingectomy may be associated with lower fertility rates than salpingostomy or methotrexate, but there is inevitable selection bias as salpingectomy is the treatment of choice in women with intraabdominal hemorrhage and larger EPs. Recent studies suggest combining methotrexate with the EGFR antagonist, gefitinib, may reduce the need for surgery and decrease the time to β -hCG resolution ([Skubisz et al., 2013](#)).

Expectant Management

As with IUPs, some EPs will resolve spontaneously. A recent prospective observational study has suggested that as many as 30% of EPs presenting to a UK early pregnancy unit will spontaneously resolve ([Mavrelos et al., 2013](#)). The authors conclude that women with a β -hCG below 1500 IU/L, EP <3 cm on USS, no haemoperitoneum, no or minimal abdominal pain and no fetal cardiac activity can be safely managed expectantly until serum β -hCG is negligible with a 71% success rate. Failure was most often due to a rising β -hCG, although 8% of women experienced worsening pain or intraperitoneal bleeding. These women did not require blood transfusion but this highlights the absolute necessity of easy access to healthcare, patient attendance for monitoring and patient understanding of the signs of tubal hemorrhage when considering expectant management. Where this is achievable, this option can be offered to eligible women. However, an economic assessment of expectant management in comparison with methotrexate or surgical treatment is yet to be performed. This is necessary given the requirement for frequent monitoring, and the increased failure rate in comparison with methotrexate. In addition, good quality evidence is currently lacking examining EP recurrence and IUP rates following expectant management.

Management of HP

The majority of HPs are managed surgically, ideally by laparoscopy, without instrumentation, and with minimal handling of the uterus. Salpingectomy is likely preferable over salpingostomy as the operation is easier, there are less complications and the situation does not permit monitoring for residual trophoblast. The rate of ongoing IUP in HP following treatment of the EP is about 60%. Systemic methotrexate is not offered in HP due to teratogenicity concerns and risk to viability of the IUP. Ultrasound guided potassium chloride/hypertonic solution injection into the gestational sac and/or aspiration is sometimes used in cornual or interstitial HPs where it is preferable to radical surgery.

Management of Nontubal EP

Rare forms of EP require personalized management strategies based on expert opinion as only case reports and small case cohorts exist in the literature. An ongoing Cochrane review may provide some evidence base for interventions in future ([Shen et al., 2014](#)). Surgical resection, preferably by laparoscopy is preferred in ovarian EP (wedge resection, partial, or complete oophorectomy), cornual and interstitial EP (cornuostomy, cornual resection, or hysterectomy) and abdominal pregnancies (ligation/embolisation of blood supply +/- removal of EP). Concurrent uterine embolisation in the case of cornual, interstitial or caesarean scar EP is often prudent and may allow for hysteroscopic or laparoscopic removal. Adjuvant methotrexate, either empirically or in the case of persistently high β -hCG postoperatively, can be a useful addition to surgery. Primary treatment with systemic methotrexate may reduce morbidity and improve fertility prospects where surgical treatment risks significant blood loss/hysterectomy such as in cornual, cervical, and caesarean scar EP. One study using single-dose intramuscular methotrexate for women with cornual pregnancy and with β -hCG <5000 IU/L, regardless of presence of fetal heart beat, was successful in 16/17 patients. Failure rate increases

with β -hCG > 10,000 IU/L but some success in multidose regimes, particularly in the case of caesarean scar EP, has been reported. As with surgical management of these rare variants of EP, the risk of sudden, significant hemorrhage despite medical treatment is present. Haemostatic techniques such as balloon tamponade and cervical cerclage may minimize this risk but occasionally emergency “rescue” surgery is required. Expectant management is only very occasionally undertaken where initial β -hCG is very low and declining.

Following Treatment

The psychological impact of EP on a woman or couple can be considerable. In the acute situation it can be easy to forget that these patients have lost a pregnancy and will experience the emotions associated with a miscarriage in addition to dealing with difficult decisions regarding treatment, implications for future fertility, and perhaps significant morbidity. Patient associations such as The Ectopic Pregnancy Trust can provide evidence-based information and support to those affected by EP. Women should be advised to expect bleeding or spotting that can last up to 6 weeks after treatment for EP. For most women, it is possible to advise that IUP rates following methotrexate, salpingectomy, or salpingostomy are 60%–80% within the following 2 years with a 5%–20% chance of EP recurrence. Women can try to conceive again as soon as they feel ready, except following methotrexate where women should be advised to avoid conception for 3 months due to concerns about teratogenicity. Following uterine surgery the advice should be tailored to the individual but is generally 3–6 months.

Conclusions and Future Research Directions

Worldwide, EP is still a leading cause of maternal morbidity and mortality in early pregnancy. Although it is possible, in about 50% of cases, to retrospectively identify specific risk factors, in most instances, screening for EP in early pregnancy is only instigated if the patient has previously suffered an EP. Research is required to understand the mechanisms behind the recognized epidemiological associations, but this is inhibited by the difficulty in differentiating cause from effect and the lack of an animal model of EP. Improvements in management of EP over the last few decades have attempted to minimize morbidity associated with treatments and maximize future reproductive potential. With the development of more potent therapeutics for EP (e.g., combination therapy with gefitinib), medical treatment may become the gold standard treatment for stable EP in future. However, improvements in management are hampered by the lack of a noninvasive diagnostic test for EP in early pregnancy and resultant diagnostic delays. A simple, cheap test with good sensitivity and specificity has the biggest potential to make a difference to outcomes for this condition. In the absence of that, improved periconception and early antenatal care is vital to reduce maternal morbidity and mortality, particularly in low resource countries.

References

- Corpa, J. M. (2006). Ectopic pregnancy in animals and humans. *Reproduction*, 131, 631–640.
- Talbot, K., Simpson, R., Price, N., & Jackson, S. R. (2011). Heterotopic pregnancy. *Journal of Obstetrics and Gynaecology*, 31, 7–12.
- Leke, R. J., Goyaux, N., Matsuda, T., et al. (2004). Ectopic pregnancy in Africa: A population-based study. *Obstetrics & Gynecology*, 103, 692–697.
- Masha, S., Wahome, E., Vanechoutte, M., et al. (2017). High prevalence of curable sexually transmitted infections among pregnant women in a rural county hospital in Kilifi, Kenya. *PLoS ONE*, 12, e0175166.
- Bouyer, J., Coste, J., Fernandez, H., Pouly, J. L., & Job-Spira, N. (2002). Sites of ectopic pregnancy: A 10 year population-based study of 1800 cases. *Human Reproduction*, 17, 3224–3230.
- Shaw, J. L., Dey, S. K., Critchley, H. O., & Horne, A. W. (2010). Current knowledge of the aetiology of human tubal ectopic pregnancy. *Human Reproduction Update*, 16, 432–444.
- Elson, C. J., Salim, R., Potdar, N., et al. (2016). Diagnosis and management of ectopic pregnancy. *BJOG*, 123, e15–e55.
- Jurkovic, D., & Mavrellos, D. (2007). Catch me if you scan: Ultrasound diagnosis of ectopic pregnancy. *Ultrasound in Obstetrics & Gynecology*, 30, 1–7.
- Cartwright, J., Duncan, W. C., Critchley, H. O., & Horne, A. W. (2009). Serum biomarkers of tubal ectopic pregnancy: Current candidates and future possibilities. *Reproduction*, 138, 9–22.
- Mol, F., van Mello, N. M., Strandell, A., et al. (2014). European surgery in ectopic pregnancy (ESEP) study group. Salpingostomy versus salpingectomy in women with tubal pregnancy (ESEP study): An open-label, multicentre, randomised controlled trial. *Lancet*, 383, 1483–1489.
- Stovall, T. G., & Ling, F. W. (1993). Single-dose methotrexate: An expanded clinical trial. *American Journal of Obstetrics & Gynecology*, 168, 1759–1762.
- Mol, F., Mol, B. W., Ankum, W. M., van der Veen, F., & Hajenius, P. J. (2008). Current evidence on surgery, systemic methotrexate and expectant management in the treatment of tubal ectopic pregnancy: A systematic review and meta-analysis. *Human Reproduction Update*, 14, 309–319.
- Skubisz, M. M., Horne, A. W., Johns, T. G., Nilsson, U. W., Duncan, W. C., Wallace, E. M., Critchley, H. O., & Tong, S. (2013). Combination gefitinib and methotrexate compared with methotrexate alone to treat ectopic pregnancy. *Obstetrics & Gynecology*, 122, 745–751.
- Mavrellos, D., Nicks, H., & Jamil, A. (2013). Efficacy and safety of a clinical protocol for expectant management of selected women diagnosed with a tubal ectopic pregnancy. *Ultrasound in Obstetrics & Gynecology*, 42, 102–107.
- Shen, L., Fu, J., Huang, W., Zhu, H., Wang, Q., Yang, S., & Wu, T. (2014). Interventions for non-tubal ectopic pregnancy (protocol). *Cochrane Database of Systematic Reviews*, 7.

Relevant Websites

www.ectopic.org.uk—The Ectopic Pregnancy Trust.

Miscarriage: Biochemical and Clinical

Manish Banker, Jayesh Patel, and Azadeh Patel, NOVA IVI Fertility, Ahmedabad, India

Jwal Banker, Shrimati Bhikhiben Kanjibhai Shah Medical Institute and Research Center, Vadodara, India

Arati Gupte-Shah, NOVA IVI Fertility, Ahmedabad, India

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

β-hCG (beta-hCG) It is a quantitative blood test to measure the level of human chorionic gonadotropin hormone (hCG) in the body. hCG is produced from the syncytiotrophoblast cells of pregnancy.

Introduction

“...I recall nothing which in times past has caused me more anxiety and doubt, or in regard to which I have found it more difficult to get any satisfactory rules from books, than the treatment of abortion.”

—T.G. Thomas, 1908.

Assisted reproductive technology (ART) is defined as all interventions that include the in vitro handling of both human oocytes and sperm or of embryos for the purpose of reproduction. This includes, but is not limited to, in vitro fertilization (IVF) and embryo transfer (ET), intracytoplasmic sperm injection (ICSI), embryo biopsy, preimplantation genetic testing (PGT), assisted hatching, gamete intrafallopian transfer (GIFT), zygote intrafallopian transfer, gamete and embryo cryopreservation, semen, oocyte and embryo donation, and gestational carrier cycles (Zegers-Hochschild et al., 2017).

For a woman or a couple hoping to have a child through ART, a positive pregnancy test seems like the first hurdle crossed in what will be a very long journey. Thus when after the first few days, the couple is told that the pregnancy has “failed,” there are numerous psychological repercussions that a patient goes through, along with the physical and clinical.

It is thus important to understand what miscarriage is, so as to know what can be done to prevent it.

Nomenclature

The word *abortion* is derived from the Latin word *aboriri*, which means to miscarry.

According to The International Glossary on Infertility and Fertility Care (2017), a spontaneous miscarriage/abortion is defined as spontaneous loss of an intra-uterine pregnancy prior to 22 completed weeks of gestational age (Zegers-Hochschild et al., 2017).

Different nomenclature defining events associated with early pregnancy and miscarriage described in Table 1.

Incidence

In natural cycles, up to 55% of conceptions are estimated to be lost due to implantation failure or pre-clinical (biochemical) miscarriage and approximately 15% lost in clinical miscarriage (Macklon, 2002). In a recent study by Boomsma et al. (2009) it was shown that in stimulated cycles, the contribution of implantation failure for the numbers of conception losses is higher (50%) than described for natural cycles (30%) while pre-clinical (biochemical losses) and clinical miscarriage is approximately 15% and 10%, respectively.

While reviewing other literature, biochemical pregnancy loss rate is 27% (54/200) after frozen embryo transfer (ET) and 22.1% (122/500) after fresh ET. Clinical abortion rate is 14.5% after frozen ET and 9% after fresh ET (Aflatoonian et al., 2010).

When reviewing the incidence based on type of oocyte and embryos, the incidence of clinical miscarriage in patients after transferring embryos using self-oocytes, donor-oocytes and vitrified-warmed embryos are 15.9%, 21.8%, and 25.2%, respectively (Banker et al., 2016).

If we look at the incidence of miscarriage following ART in different registries: according to Australian & New Zealand Assisted Reproduction Database (ANZARD) (2015) out of the 17,659 clinical pregnancies, 79.9% resulted in a delivery and 19.0% resulted in clinical miscarriage. The outcomes of 193 (1.1%) clinical pregnancies were not known because women could not be followed up or contacted by fertility centers.

According to FIVNAT registry (2015), out of 2149 pregnancies, 525 (24%) had spontaneous miscarriage.

Table 1 Nomenclature describing early pregnancy events

<i>Nomenclature</i>	<i>Definition</i>
Biochemical pregnancy (preclinical spontaneous abortion/miscarriage)	A pregnancy diagnosed only by the detection of HCG in serum or urine and that does not develop into a clinical pregnancy (Zegers-Hochschild et al., 2009)
Clinical miscarriage	Intrauterine pregnancy demise confirmed by ultrasound or histology (ASRM Practice Committee, 2013; Stephenson and Kutteh, 2007)
i. First-trimester miscarriage	Intrauterine pregnancy demise before 12 weeks of gestational age
Early miscarriage	Intrauterine pregnancy loss of < 10 weeks size on ultrasound (Kolte et al., 2014)
• Anembryonic miscarriage	Intrauterine pregnancy loss with a gestational sac but without a yolk sac or an embryo on ultrasound
• Yolk sac miscarriage	Intrauterine pregnancy loss with a gestational sac and yolk sac, without an embryo on ultrasound
• Embryonic miscarriage	Intrauterine pregnancy loss with an embryo without cardiac activity on ultrasound
Fetal miscarriage	Intrauterine pregnancy loss ≥ 10 weeks size with a fetus (≥ 33 mm) on ultrasound (Stephenson and Kutteh, 2007)
ii. Second-trimester miscarriage	Intrauterine pregnancy loss after 12 weeks of gestational age
Vanishing sac(s) or embryo(s)	Spontaneous disappearance of one or more gestational sacs or embryos in an ongoing pregnancy, documented by ultrasound (Zegers-Hochschild et al., 2017)

Miscarriage Types

Broadly, miscarriages are of two types: biochemical and clinical miscarriages.

Biochemical Miscarriage

Spontaneous pregnancy demise based on decreasing serum or urinary b-hCG levels, without an ultrasound evaluation.

In the general population, most cases of biochemical pregnancy remain undetected. Thus the incidence is different for these cases as compared to patients undergoing assisted reproductive treatment. In the latter group, more stringent monitoring and early testing allows us to detect transient β -hCG rises which would otherwise have been missed (Annan et al., 2013).

Numerous studies have advocated that ovarian stimulation alters the endometrial receptivity, hence increasing biochemical pregnancy rates (Kasius et al., 2014).

But a study by Zeadna et al. (2015) attempts to dispel this theory. Their study showed fertile controls who conceived spontaneously has a higher biochemical pregnancy rate (18%) as compared to women undergoing fresh or frozen embryo transfers (13.8%).

A study by Yang et al. (2015) reviewed over 10,000 cycles to show that biochemical pregnancy in the first IVF cycle is a negative predictor for future positive results.

Etiology (Annan et al., 2013)

The exact etiology of biochemical miscarriage after ART is unknown.

Numerous factors have been implicated:

1. Genetically abnormal embryos
2. Poor endometrial receptivity
3. Genetically abnormal oocyte
4. Sperm DNA damage
5. Stress

Pathogenesis

Soon after fertilization, or embryo transfer, the embryo begins to secrete β -hCG. The amount of β -hCG continues to rise as the trophoblastic reaction and embryo invasion continues. Hence a patient tests positive for pregnancy at this early stage. However, in case of a biochemical miscarriage, further growth of the embryo stops, thus preventing any clinical evidence of the pregnancy to be visible on ultrasound (Annan et al., 2013).

Clinical Miscarriage

A pregnancy which can be visualised on ultrasound is known as a clinical pregnancy. A spontaneous loss of this type of intra-uterine pregnancy prior to 22 completed weeks of gestational age is known as spontaneous miscarriage/abortion (Zegers-Hochschild et al., 2017).

Broadly, there are two types of clinical miscarriage (Fig. 1):

- i. First trimester miscarriage
- ii. Mid-trimester miscarriage

Types

- i. First trimester miscarriage (Kolte et al., 2014):

Spontaneous pregnancy demise before 12 weeks of gestational age is called first trimester miscarriage.

This can be divided into two parts: early miscarriage and fetal miscarriage

- Early miscarriage:

Early miscarriage is defined as intrauterine pregnancy loss of < 10 weeks size on ultrasound and it is divided in to three categories:

- (a) *Anembryonic miscarriage*:

It is an intrauterine pregnancy loss with a gestational sac but without a yolk sac or an embryo on ultrasound. In this case, the trophoblast has invaded the decidua but the embryonic disc has not developed.

- (b) *Yolk sac miscarriage*:

Intrauterine pregnancy loss with a gestational sac and yolk sac, without an embryo on ultrasound.

- (c) *Embryonic miscarriage*:

It is an intrauterine pregnancy loss with an embryo without cardiac activity on ultrasound.

- Fetal miscarriage:

It is defined as pregnancy loss ≥ 10 weeks size with a fetus (≥ 33 mm) on ultrasound.

- ii. Mid-trimester miscarriage:

Miscarriage/abortion resulting after 12 weeks of gestation is defined as mid-trimester miscarriage.

Etiopathogenesis of clinical miscarriage following ART

Causes/factors related to ART

1. *Ovarian stimulation*:

The use of exogenous gonadotrophins for controlled ovarian hyperstimulation (COH) as part of IVF has become a well-accepted practice; however, this can have an impact on oocyte and embryo quality and endometrial receptivity.

Early research suggested that the exposure of the developing oocyte to supraphysiological concentrations of gonadotrophins may disturb oocyte maturation and the completion of meiosis leading to chromosomal aneuploid oocytes and/or embryos (Hodges et al., 2002). Whereas animal studies seem to support an association between FSH exposure and embryonic aneuploidy, human studies have been more conflicting.

Possible mechanisms suggested either FSH-induced chromosome dysfunction in oocytes or recruitment of poorer-quality oocytes when the process of naturally selecting a dominant follicle is overridden with ovarian stimulation. If exogenous FSH

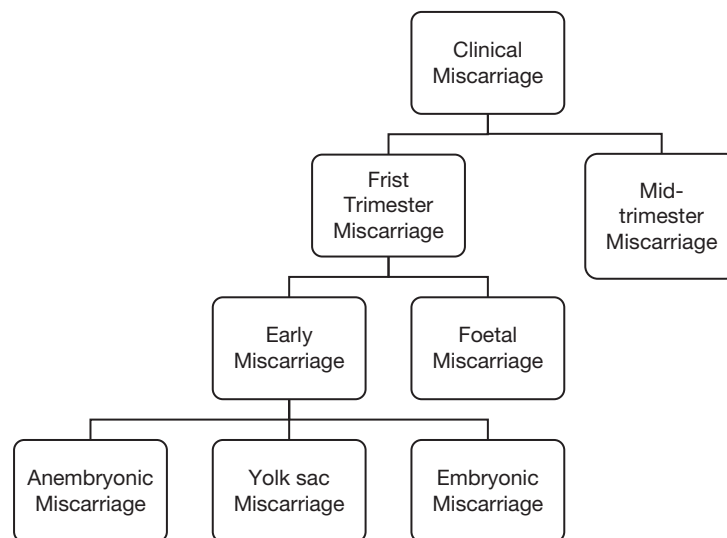


Fig. 1 Types of clinical miscarriage.

increases aneuploidy, it could lead to an increased miscarriage rate in patients undergoing IVF, because most miscarriages are the result of chromosomal abnormalities (Andersen et al., 2000; Lathi et al., 2007). But, Massie et al. (2011) concluded that the incidence of embryonic aneuploidy was not higher in pregnancies conceived with FSH stimulation compared with spontaneous conceptions in infertility patients. This suggests that exogenous gonadotrophins exposure does not increase the risk of aneuploidy.

Multiple ovarian follicle maturation by COH will produce supraphysiological serum estradiol (E2) levels that may induce morphologic (Kolb et al., 1997) and biochemical (Simón et al., 1996) endometrial alterations related to uterine receptivity without affecting embryo quality. Thus, with high E2 concentration, implantation and LBR (live birth rate) decreases without affecting miscarriage rate (Ji et al., 2013).

2. *Number of oocytes retrieved:*

There was a strong association between the number of oocytes retrieved and the clinical miscarriage rate. The miscarriage rate fell from 20% to 13% with increasing numbers of oocytes. Stepwise logistic regression ($P < 0.001$) identified three cut-points (4, 10, and 15 oocytes) at or beyond which the probability of clinical miscarriage fell. It failed to find any oocyte number above which the miscarriage rate rose again. The miscarriage rates were 20.0% (1–3 oocytes), 15.5% (4–9 oocytes), 13.8% (10–14 oocytes) and 13.1% (≥ 15 oocytes). After adjusting for female age, the effect of oocyte numbers on the clinical miscarriage rate was reduced, but not eliminated.

These findings conclude that poor responders have a higher clinical miscarriage indicating that poor ovarian response is associated with a parallel decline in both oocyte quantity and quality (Sunkara et al., 2014).

Though decline in the LBR (live birth rate) observed with higher number of eggs could be due to the deleterious effect of the raised serum E2 levels affecting at the level of embryo implantation (Valbuena et al., 2001; Mitwally et al., 2006; Joo et al., 2010) but not beyond that.

Ji et al. (2013) concluded that the fresh embryo LBR per started cycle increased with the number of retrieved oocytes up to Groups 2 and 3 (6–10 and 11–15 oocytes) and then (> 15 oocytes) decreased, because of the high number of cycles with all embryos being cryopreserved, in order to avoid moderate—severe OHSS in group 4 (> 15 oocytes). However, the cumulative LBR per started cycle continued to increase with oocyte number, as did the incidence of moderate—severe OHSS. There was no significant difference in the miscarriage rates among the patient groups.

3. *Frozen embryo transfer (FET):*

There is no difference of miscarriage rates between fresh and FET, though FET resulted in an increase in the clinical pregnancy rate (RR 1.31; 95% CI 1.10–1.56) and the ongoing pregnancy rate (RR 1.32; 95% CI 1.10–1.59) (Evans et al., 2014). But, on comparing different FET protocols, a higher miscarriage rate was observed in the HRT (hormone replacement therapy) group when compared to hCG or LH surge (21.2% vs. 12.9% vs. 11.1%, $P < 0.01$) (Cerrillo et al., 2017).

4. *Cryopreservation technique:*

Earlier, slow freezing technique was used to cryopreserve gametes/embryos. Recently vitrification technique has replaced this due to better results. According to Kaartinen et al. (2016), the clinical pregnancy and delivery rates were similar for both freezing methods. The clinical miscarriage rate in the slow-freezing group was higher (29% vs. 15.7%). Slow freezing and thawing has been detected to induce numerical chromosomal changes in human embryos, which could be explained by increased ice crystal formation. The embryo DNA integrity index was higher in vitrified than in slow-frozen blastocysts which could explain the lower miscarriage rate.

5. *Multiple pregnancy:*

More than one embryo is very often transferred in an IVF—ICSI cycle to improve the pregnancy rate. This leads to a higher incidence of twins, triplets and sometimes even high order multiple pregnancies.

According to Sullivan et al. (2013), the number of embryos transferred per ART cycle varied among countries. The average number of embryos transferred overall has decreased from 2.47 in 2002 to 2.35 in 2004 reflecting increase in single and double embryo transfers from 67.6% in 2002 to 73.2% in 2004. Positively, triple and quadruple embryo transfers decreased between 2002 and 2004 from 28.6% to 25.1% and 13.7% to 11.6%, respectively, and this has contributed to the decline in the trend of multiple deliveries following ART.

The lowest proportion of ≥ 4 fresh embryo transfers were reported from Australia/New Zealand at 0.4% then Europe at 3.3% and the highest from Asia at 42.5% with the Nordic countries reporting no embryo transfer cycles of ≥ 4 . The proportion of single embryo transfers (SETs) increased from with the highest reported by Sweden (67.4%), Belgium (48.9%) and then Finland (47.0%).

The proportion of twin deliveries marginally increased from 24.8% in 2003 to 25.1%, while the proportion of triplet deliveries continued to decrease, from 2.0% to 1.8%. However, these rates differed largely among countries; the percentage of twin births ranged from 5.6% in Sweden to 48.2% in Peru, and births of triplet and higher order multiples from, 1% in several countries (including Sweden, Belgium, Australia, New Zealand, Norway, Denmark, and France) to 10% in Lithuania and six Latin American countries (Argentina, Brazil, Ecuador, Guatemala, Mexico, and Venezuela).

Similar rates of multiple deliveries were seen for FET in 2003 and 2004, with twins at 17.0% and triplets or higher at 1.0%. The percentage of transfers with four or more embryos in fresh cycles increased slightly, to 11.6% from 10.8% in 2003.

According to Tummers et al. (2003), the overall incidence of spontaneous abortion—in twin pregnancies was 17.1% (12.1% vanishing twins and 5.0% complete miscarriages) and in singleton pregnancies was 21.7%. The incidence of miscarriage in the twin pregnancies, expressed per gestational sac, was 11.1%. Once fetal heart activity was present, the risk of abortion (per

gestational sac) was 7.3%, which is significantly lower than that in singleton pregnancies. Twin pregnancies after IVF have a better potential for survival than singleton pregnancies.

According to La Sala et al. (2016), overall clinical miscarriages (odds ratio [OR] 4.9, 95% confidence interval [CI] 3.4, 6.9) and live births (OR 0.2, 95% CI 0.1, 0.3) were, respectively, higher and lower when compared to patients who had one clinical twin pregnancy. The overall risk of perinatal complications was significantly higher in patients who had one twin delivery rather than patients who had two consecutive singleton deliveries. Thus, he concluded that compared with two consecutive singleton pregnancies, twin pregnancies are characterized by higher success rates but worse perinatal outcomes irrespectively of vanishing twin syndrome. Thus, reducing the number of embryos transferred should be encouraged for better perinatal outcome.

6. ART laboratory parameters:

● Air quality:

Filtration units have been shown to reduce airborne concentrations of toxic volatile organic compounds (VOC), chemical contaminants and particles in IVF laboratories. Control of air pollution in ART laboratory, operating and transfer rooms through the construction of cleanrooms equipped with carbon-impregnated filters [like Class 100 (ISO 5; $n = 248$) cleanroom] for air and incubators results in better good quality embryo formation, cleavage and pregnancy rates, and in lower spontaneous abortion rates (Esteves et al., 2004).

● Culture media:

The pregnancy rate and implantation rate in the group without HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] were statistically significantly higher than in the control group where media buffered with HEPES used. But no differences were observed for the number of embryos transferred, top-quality embryo rate transferred, or abortion rate between the two groups (Morgia et al., 2006).

● ICSI (intra-cytoplasmic sperm injection):

ICSI is suspected to increase the chromosomal aberrations as it bypasses natural selection mechanisms and may increase first trimester aneuploidy rates (Kim et al., 2010). Explanations for this include (i) physical or biochemical disturbances of the ooplasm or meiotic spindle, (ii) injection of biochemical contaminants, (iii) injection of sperm associated exogenous DNA, (iv) injection of sperm carrying a chromosomal anomaly, (v) transmission of genetic defects that may be related to the underlying male-factor infertility, (vi) male gametes with structural defects, (vii) anomalies of sperm activating factors, (viii) potential for incorporating sperm mitochondrial DNA, and (ix) female gamete anomalies.

Kim et al. (2010) concluded that out of total miscarriages, 52.62% were found to be cytogenetically abnormal among all patients, but no statistical difference was found between ICSI and conventional IVF group (54.3% v/s 55.3%, P 0.503).

General causes miscarriage

Many causes of abortion not specifically related to ART but can cause abortion in any pregnancy conceived naturally or through IUI or ART.

1. Chromosomal abnormalities (Agenor and Bhattacharya, 2015):

Almost 50% of early pregnancy losses take place due to chromosomal abnormalities, mostly aneuploidy. These maybe genetic, but more often than not, they arise de novo, due to aberrations in chromosomal structure during development.

- (a) *Trisomies*: Seen most often in cases of advanced maternal age (≥ 35 years), trisomy 21, 16, and 18 are known to be a cause of early pregnancy loss.
- (b) *Y-chromosome abnormalities*: Translocation in the Y-chromosome is commonly detected, leading to defective spermatogenesis.
- (c) *Factor V Leiden*: This mutation is genetically inherited, predisposing the affected individual to thrombosis.

Pre-implantation genetic screening (PGS) can be offered to rule out chromosomal aneuploidies in embryos before transfer.

2. *Antiphospholipid antibodies*: especially anticardiolipin antibodies and lupus anticoagulant have been linked with pregnancy loss, right from the implantation stage, through to late preterm labor. These antibodies are said to impair trophoblastic invasion of spiral arterioles, reducing the blood supply to the fetus, causing improper placental development, PIH and changes in fetal blood flow patterns. Aspirin and low molecular weight heparin are commonly used treatment option.
3. *Anatomical defects in the uterus*: These maybe both congenital and acquired. Defects like uterine septum, unicornuate uterus, distorted anatomy due to endometriosis, uterine fibroids, polyps and intrauterine synechiae all can cause pregnancy losses, especially 2nd trimester loss. Surgical correction, when appropriate helps to reduce the risk.
4. *Cervical incompetence*: Cervical incompetence, often undiagnosed until it is too late, is a common cause of recurrent and preventable mid-trimester pregnancy loss. Cervical cerclage is performed in second trimester to prevent such losses.
5. *Endocrine cause*: The main causes include poorly controlled diabetes, overt hyperthyroidism and hypothyroidism, hyperprolactinemia and luteal phase defect.
6. *Infections*: Infections can cause sporadic losses, never recurrent. The organisms responsible are Chlamydia, ureaplasma, mycoplasma, and toxoplasma.
7. *Environmental cause* (Gardella and Hill, 2000): Heavy metals such as mercury and lead, alcohol, organic solvents, and ionizing irradiation are confirmed teratogens which on exposure can lead to miscarriage. The suspected teratogens are caffeine (> 300 mg/day), and cigarette smoking, whereas the teratogenic potential of pesticides remains unknown.
8. *Maternal obesity* (Lashen et al., 2004): Obesity is associated with increased risk of first trimester and recurrent miscarriage. Weight loss should be encouraged for obese patients before ART (Sim et al., 2014).

9. Immunological factors (Agenor and Bhattacharya, 2015):

- (a) *HLA antigens*: HLA expression influences various stages of gestation. Its expression is associated with increased chances of an embryo implanting successfully as well as elevated embryo cleavage rates.
- (b) *Antisperm antibodies*: although these are mainly said to cause infertility, their role in miscarriages has also been studied, leading to lower pregnancy rates.
- (c) *Integrins*: these are adhesion molecules that facilitate cell to cell interactions, and are considered essential for the binding of a sperm to an oocyte. They are also essential for the process of implantation and later on for placental development. Lack of integrin expression can lead to miscarriages.
- (d) *LIF*: leukemia inhibitory factor is required in the endometrium to facilitate the process of implantation. Its absence or reduction has been linked to human reproductive failure.
- (e) *Cytokines*: Cytokines are involved in gamete development, trophoblast invasion, implantation, placental development, decidualization and immune tolerance to pregnancy.

Th1 cytokines are said to be cytotoxic, causing tissue injury, whereas Th2 cytokines promote antibody production and cell healing. Thus when the Th1:Th2 balance is disturbed in favor of the cytotoxic cytokines, pregnancy is affected.

- (f) *Natural killer cells*: increased NK cell activity, especially the uterine NK cells, as compared to the peripheral NK cells, has been implicated in impaired placental development leading to reproductive failure.
- (g) *Haemostatic pathways*: Proteins C, protein S, factor V Leiden, homocysteine, are some of the factors that have been considered responsible for pregnancy loss.

Management strategies are vast and detailed. In a nutshell, they involve detecting the underlying cause first, and then solving it to be able to take a pregnancy to term.

References

- Aflatoonian, A., MansooriMoghaddam, F., Mashayekhy, M., & Mohamadian, F. (2010). Comparison of early pregnancy and neonatal outcomes after frozen and fresh embryo transfer in ART cycles. *Journal of Assisted Reproduction and Genetics*, 27(12), 695–700.
- Agenor, A., & Bhattacharya, S. (2015). Infertility and miscarriage: Common pathways in manifestation and management. *Women's Health*, 11(4), 527–541.
- Andersen, A., Wohlfahrt, J., Christens, P., Olsen, J., & Melbye, M. (2000). Maternal age and fetal loss: Population based register linkage study. *BMJ*, 320(7251), 1708–1712.
- Annan, J., Gudi, A., Bhide, P., et al. (2013). Biochemical pregnancy during assisted conception: A little bit pregnant. *Journal of Clinical Medicine Research*, 5(4), 269–274.
- ASRM Practice Committee. (2013ASRM). Definitions of infertility and recurrent pregnancy loss: A committee opinion. *Fertility and Sterility*, 99, 63.
- Banker, M., Mehta, V., Sorathiya, D., Dave, M., & Shah, S. (2016). Pregnancy outcomes and maternal and perinatal complications of pregnancies following in vitro fertilization/ intracytoplasmic sperm injection using own oocytes, donor oocytes, and vitrified embryos: A prospective follow-up study. *Journal of Human Reproductive Sciences*, 9(4), 241.
- Boomsma, C., Kavelaars, A., Eijkemans, M., et al. (2009). Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF. *Human Reproduction*, 24(6), 1427–1435.
- Cerrillo, M., Herrero, L., Guillén, A., Mayoral, M., & García-Velasco, J. (2017). Impact of endometrial preparation protocols for frozen embryo transfer on live birth rates. *Rambam Maimonides Medical Journal*, 8(2), e0020.
- Esteves, S., Gomes, A., & Verza, S. (2004). Control of air pollution in assisted reproductive technology laboratory and adjacent areas improves embryo formation, cleavage and pregnancy rates and decreases abortion rate: Comparison between a class 100 (ISO 5) and a class 1.000 (ISO 6) cleanroom for micromanipulation and embryo culture. *Fertility and Sterility*, 82, S259–S260.
- Evans, J., Hannan, N., Edgell, T., et al. (2014). Fresh versus frozen embryo transfer: Backing clinical decisions with scientific and clinical evidence. *Human Reproduction Update*, 20(6), 808–821.
- Gardella, J., & Hill, J., III (2000). Environmental toxins associated with recurrent pregnancy loss. *Seminars in Reproductive Medicine*, 18(04), 407–424.
- Hodges, C., Ilagan, A., Jennings, D., et al. (2002). Experimental evidence that changes in oocyte growth influence meiotic chromosome segregation. *Human Reproduction*, 17(5), 1171–1180.
- Ji, J., Liu, Y., Tong, X., Luo, L., Ma, J., & Chen, Z. (2013). The optimum number of oocytes in IVF treatment: An analysis of 2455 cycles in China. *Human Reproduction*, 28(10), 2728–2734.
- Joo, B., Park, S., An, B., et al. (2010). Serum estradiol levels during controlled ovarian hyperstimulation influence the pregnancy outcome of in vitro fertilization in a concentration-dependent manner. *Fertility and Sterility*, 93(2), 442–446.
- Kaartinen, N., Kananen, K., Huhtala, H., Keränen, S., & Tinkanen, H. (2016). The freezing method of cleavage stage embryos has no impact on the weight of the newborns. *Journal of Assisted Reproduction and Genetics*, 33(3), 393–399.
- Kasius, A., Smit, J., Torrance, H., et al. (2014). Endometrial thickness and pregnancy rates after IVF: A systematic review and meta-analysis. *Human Reproduction Update*, 20(4), 530–541.
- Kim, J., Lee, W., Yoon, T., et al. (2010). Chromosomal abnormalities in spontaneous abortion after assisted reproductive treatment. *BMC Medical Genetics*, 11(1).
- Kolb, B., Najmabadi, S., & Paulson, R. (1997). Ultrastructural characteristics of the luteal phase endometrium in patients undergoing controlled ovarian hyperstimulation. *Fertility and Sterility*, 67(4), 625–630.
- Kolte, A., Bernardi, L., Christiansen, O., et al. (2014). Terminology for pregnancy loss prior to viability: A consensus statement from the ESHRE early pregnancy special interest group. *Human Reproduction*, 30(3), 495–498.
- La Sala, G., Morini, D., Gizzo, S., Nicoli, A., & Palomba, S. (2016). Two consecutive singleton pregnancies versus one twins pregnancy as preferred outcome of in vitro fertilization for mothers and infants: A retrospective case–control study. *Current Medical Research and Opinion*, 32(4), 687–692.
- Lashen, H., K. Fear, K. & Sturdee, D. (2004). Obesity is associated with increased risk of first trimester and recurrent miscarriage: Matched case-control study. *Human Reproduction*, 19(7), pp.1644–1646.
- Lathi, R., Mark, S., Westphal, L., & Milki, A. (2007). Cytogenetic testing of anembryonic pregnancies compared to embryonic missed abortions. *Journal of Assisted Reproduction and Genetics*, 24(11), 521–524.
- Macklon, N. (2002). Conception to ongoing pregnancy: The 'black box' of early pregnancy loss. *Human Reproduction Update*, 8(4), 333–343.
- Massie, J., Shahine, L., Milki, A., Westphal, L., & Lathi, R. (2011). Ovarian stimulation and the risk of aneuploid conceptions. *Fertility and Sterility*, 95(3), 970–972.
- Mitwally, M., Bhakoo, H., Crickard, K., et al. (2006). Estradiol production during controlled ovarian hyperstimulation correlates with treatment outcome in women undergoing in vitro fertilization–embryo transfer. *Fertility and Sterility*, 86(3), 588–596.

- Morgia, F., Torti, M., Montigiani, M., et al. (2006). Use of a medium buffered with *N*-hydroxyethylpiperazine-*N*-ethanesulfonate (HEPES) in intracytoplasmic sperm injection procedures is detrimental to the outcome of in vitro fertilization. *Fertility and Sterility*, 85(5), 1415–1419.
- Sim, K., Partridge, S., & Sainsbury, A. (2014). Does weight loss in overweight or obese women improve fertility treatment outcomes? A systematic review. *Obesity Reviews*, 15(10), 839–850.
- Simón, C., Mercader, A., Frances, A., et al. (1996). Hormonal regulation of serum and endometrial IL-1 α , IL-1 β and IL-1ra: IL-1 endometrial microenvironment of the human embryo at the apposition phase under physiological and supraphysiological steroid level conditions. *Journal of Reproductive Immunology*, 31(3), 165–184.
- Stephenson, M., & Kutteh, W. (2007). Evaluation and management of recurrent early pregnancy loss. *Clinical Obstetrics and Gynecology*, 50, 132–145.
- Sullivan, E., Zegers-Hochschild, F., Mansour, R., et al. (2013). International Committee for Monitoring Assisted Reproductive Technologies (ICMART) world report: Assisted reproductive technology 2004. *Human Reproduction*, 28(5), 1375–1390.
- Sunkara, S., Khalaf, Y., Maheshwari, A., Seed, P., & Coomarasamy, A. (2014). Association between response to ovarian stimulation and miscarriage following IVF: An analysis of 124 351 IVF pregnancies. *Human Reproduction*, 29(6), 1218–1224.
- Tummers, P., Sutter, P., & Dhont, M. (2003). Risk of spontaneous abortion in singleton and twin pregnancies after IVF/ICSI. *Human Reproduction*, 18(8), 1720–1723.
- Valbuena, D., Martin, J., de Pablo, J., et al. (2001). Increasing levels of estradiol are deleterious to embryonic implantation because they directly affect the embryo. *Fertility and Sterility*, 76(5), 962–968.
- Yang, R., Yang, S., Li, R., et al. (2015). Biochemical pregnancy and spontaneous abortion in first IVF cycles are negative predictors for subsequent cycles: An over 10,000 cases cohort study. *Archives of Gynecology and Obstetrics*, 292(2), 453–458.
- Zeadna, A., Son, W., Moon, J., & Dahan, M. (2015). A comparison of biochemical pregnancy rates between women who underwent IVF and fertile controls who conceived spontaneously. *Human Reproduction*, 30(4), 783–788.
- Zegers-Hochschild, F., Adamson, G., de Mouzon, J., et al. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009*. *Fertility and Sterility*, 92(5), 1520–1524.
- Zegers-Hochschild, F., Adamson, G., Dyer, S., et al. (2017). The international glossary on infertility and fertility care, 2017. *Human Reproduction*, 32(9), 1786–1801.

Further Reading

- Wilcox, A., Weinberg, C., O'Connor, J., et al. (1988). Incidence of early loss of pregnancy. *New England Journal of Medicine*, 319(4), 189–194.

CLINICAL FOLLOW-UP

Monitoring Art Outcomes: The Registries

Fernando Zegers-Hochschild, University Diego Portales, Santiago, Chile

G David Adamson, International Committee for Monitoring Assisted Reproductive Technology (ICMART), Palo Alto, CA, United States; Palo Alto Medical Foundation, Palo Alto, CA, United States; and Stanford University School of Medicine, Stanford, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Monitoring national and regional trends in utilization as well as safety and effectiveness of assisted reproductive technologies (ART) treatments constitutes an important task. Data generated systematically, incorporating heterogeneity in populations but large numbers of cases, have limitations. However, they can often serve as external quality control to be used by institutions performing ART in a given region, and also to compare themselves with different regions of the world. Additionally, monitoring trends in effectiveness and safety contributes to developing better health interventions and appropriate public policies. From a consumer/patient perspective, having access to a large number of cases helps empower infertile couples in their capacity to evaluate risks and benefits when requesting in vitro fertilization (IVF). It is often difficult for an infertile and sometimes vulnerable person to understand and to weigh the risks and benefits of offered treatments. Access to an objective and external database is often well received by infertile persons when deciding if and which treatment option should be undertaken, for example how many embryos to transfer, whether preimplantation genetic testing (PGT) should be undertaken, or even whether treatment should be undertaken at all.

Types of Art Registries

Registries can be *voluntary*, as in all Latin America, Asia and the Middle East or *obligatory* and therefore enforced by law or by other regulatory bodies. Such is the case in the United States, Australia, the United Kingdom, France, Belgium, and the Nordic countries. The major difference between these two systems is that when reporting is obligatory, national data include all or the vast majority of centers offering ART services; while in cases with voluntary registration, data reported are most of the time incomplete. There are exceptions such as Chile and Uruguay in Latin America; and also in larger populations such as Japan, where in spite of a non-obligatory reporting system, the majority of centers send their data to a centralized organization. Another characteristic seen exclusively in obligatory reporting systems is the possibility to publicly disclose the results from each participating institution, as it is in the United States.

Countries can also report their data through *national registries*, as in most European countries, United States, Australia and Japan; or via *regional registries* such as in Latin America and Africa. In these two continents, most countries report their national data, with information provided by a regional organization, such as REDLARA (www.redlara.com) for Latin America and ANARA (www.anara-africa.com) for Africa. The reason for this triangulation of data collection and data reporting has to do with difficulties encountered by many institutions to disclose their results to a potentially competing institution. Irrespective of how ART data are collected, it is very useful for countries to have access to their own data in order to generate evidence-based public policies and compare their practices and outcomes with other countries or regions.

From another perspective, registries can collect *cycle-based or individualized data*, also called case-by-case registry; this has the advantage that every individual treatment cycle can be followed from the start of stimulation or cycle monitoring until birth or miscarriage. This is the case in most European countries, Australia, US and Japan; and since 2010 in Latin America when the registry became the only regional registry with a cycle-based reporting system, which now includes 15 countries in the region. Today, South Africa is also reporting with individualized data collection; this system is now being expanded to other African countries through the African Network and Registry for Assisted Reproductive Technology (ANARA).

Another way of collecting ART data is through summary collection in which different treatment modalities are grouped into age groups, number of embryos transferred, fertilization technique, etc. In this case, very useful information is still collected but there are several limitations when interpreting data. For example, it is not possible to calculate cumulative events, such as the cumulative delivery rate of women age 37. In summary, registries you can add the number of deliveries of fresh transfers plus frozen embryo transfers (FET) of all women age 37–39 but it will not be possible to extrapolate the cumulative birth rate that a woman of a certain age has after transferring all the embryos that were generated and transferred during her fresh plus frozen transfers. Another big advantage of a cycle based registry is the possibility to associate birth outcome such as gestational age at delivery, weight of newborns, and malformation rate to any preceding event, for example type of stimulation protocol or whether embryo transfer (ET) was performed fresh during the stimulation cycle or delayed for a frozen–thaw cycle.

World Art Registry

The International Committee for Monitoring Assisted Reproductive Technology (ICMART) is the only registry reporting global data by country and region. This committee started as the International Working Group of Registers in Assisted Reproduction, generating the first world reports of procedures performed in 1989 (Lancaster, 1996); and later reorganized as ICMART. The data corresponding to ART treatment cycles performed every year are collected from five regional ART registries, compiled from national registry data in the case of Europe and North America, and from individual ART clinics gathered together in a regional registry in Latin America, Australia/New Zealand, and intermittently in the Middle East. Also, data are collected from national registries directly reporting to ICMART in several Asian countries such as Japan, India, Taiwan, Korea, and Vietnam; as well as in South Africa and Israel. Irrespective as to whether the national registry gathers information on a cycle based modality or in a summary modality, ICMART data collection and reporting system uses a summary modality.

The ICMART data collection uses forms describing the organization of each country's register, the practice of ART in terms of utilization, and the results of IVF, intracytoplasmic sperm injection (ICSI), and frozen embryo transfer (FET), and includes initiated cycles, follicular aspirations, ETs, clinical pregnancies, deliveries, and newborns. Whenever available, these variables are further classified according to the fertilization technique, woman's age, number of embryos transferred, and gestational age at delivery. Other forms describe pre implantation genetic testing (PGT), oocyte donation (OD), immediate complications for women, and congenital anomalies detected during the perinatal period. The ICMART forms for data collection can be found at www.icmartivf.org.

One of the major requirements for a global and systematic recording of reproductive health data is to have a common language. ICMART has been a leader in developing infertility glossaries, first to allow for standard registration of ART data, and most recently in partnership with numerous other international organizations, The International Glossary on Infertility and Fertility Care, 2017 has been developed and published (Zegers-Hochschild et al., 2017a,b). This serves as a basis for systematic recording of data and, among other advantages, a standard way of reporting outcomes.

Art From a Global Perspective

Utilization is Affected by Availability and Access

The global number of clinics reporting to ICMART has grown significantly. In the year 2000, 1400 clinics in 49 countries reported approximately 460,000 initiated cycles with an estimated 178,000 babies born (Adamson et al., 2006). In 2012, ART procedures were reported by 2551 clinics in 69 countries. The actual number of initiated cycles reported was 1,559,982, with an estimate above 1,700,000, and approximately 400,000 babies born (de Mouzon et al., 2012). This represents almost a 2.5 fold increase in these 12 years. Europe continues to perform the largest number of procedures, representing 47%, followed by Asia with 30% and North America with 12% (Fig. 1A). Furthermore, the number of babies born follows a similar pattern. Of an estimated 400,000 babies born in 2012, Europe contributes 45.2% followed by North America with 21.7% and Asia with 19.2% (Fig. 1B).

The higher proportion of births in North America compared with Asia results from, among other factors, a higher delivery rate (DR) per initiated cycle in the United States, as well as a higher proportion of multiple births. Although ART services are available in almost every country in the world, the majority of countries have access restricted to those who can afford out of pocket funding; an exception is countries where infertility treatment is provided free of cost or reimbursed. A description of how each country deals with fertility treatments can be found in an infertility survey published regularly by the International Federation of Fertility Societies (IFFS) (Surveillance, 2016). The number of ART procedures per million inhabitants (utilization) represents a measure of access. As seen in Fig. 2, countries with facilitated access to ART perform approximately 2500 cycles per million inhabitants compared with only hundreds in poorer countries with out of pocket funding. The major driver of utilization is affordability (Chambers et al., 2014).

Effectiveness

The effectiveness of ART treatments can be expressed in different ways. The clinical biomarker most frequently used is the clinical pregnancy rate (CPR); and although this is not a robust marker, it is widely used because infertility is defined as "the failure to achieve a clinical pregnancy"; therefore, pregnancy becomes the first successful step in an infertility treatment, irrespective of whether pregnancy is followed by miscarriage or the delivery of a healthy live birth. A more accurate marker is the delivery rate (DR) per initiated, aspirated or transferred cycle. There is overall agreement that the most accepted measure of success is the birth of a healthy singleton. However, when balance is placed between the birth of twins against the absence of pregnancy, twins is almost always considered a blessing—especially because most couples have limited chances in their lifetime, mainly due to cost, stress of treatment and age.

World trends in effectiveness over a 12-year interval

Between the year 2000 and 2012, the CPR and DR/aspiration have remained almost unaltered. At first this seems disappointing, considering major improvements in drugs for ovarian stimulation (OS), laboratory facilities and clinical knowledge. However, these small changes in CPR and DR are coupled with a significant drop in the average number of embryos transferred, from 2.6 embryos

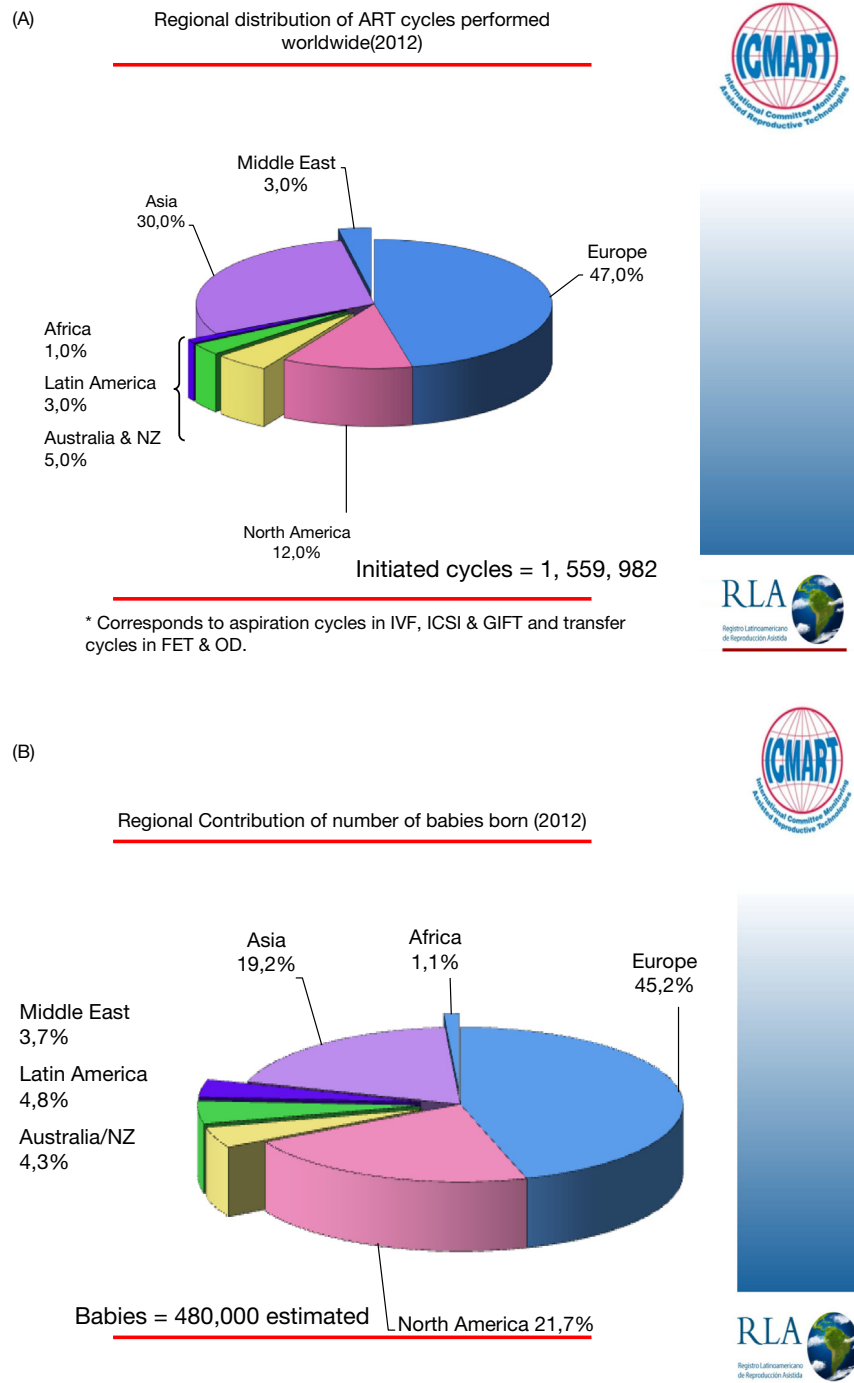


Fig. 1 (A) Regional contribution to ART initiated cycles performed worldwide in 2012. (B) Proportion of babies born from ART treatments by region in 2012.

in 2000 to 1.88 in 2012 (**Table 1**). This resulted in a significant reduction in the proportion of twins and, especially ≥ 3 babies, from 2.8% of births in 2000 to $< 1\%$ in 2012. Therefore, the major improvement in ART in this 12 years interval has been towards a better balance between effectiveness and safety.

Cumulative/Total delivery rate as the ultimate marker of effectiveness

Cumulative and total delivery rates or live birth rates ([Zegers-Hochschild et al., 2017a,b](#)) on a global basis are difficult to document since most countries report aggregate data to their own registries and then to ICMART, rather than cycle-based data. Despite this, estimated cumulative delivery rates can be extrapolated by adding all fresh plus FET cycles in women at certain age categories; this is

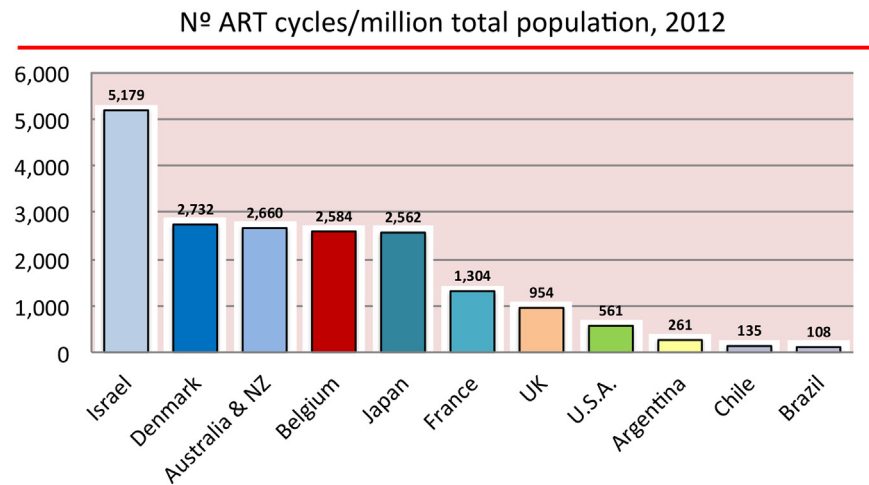


Fig. 2 Availability of ART treatments in a selected number of countries, expressed as number of cycles per million total population.

Table 1 Clinical pregnancy rate and delivery rate per aspiration in IVF/ICSI by year (2000, 2005, 2008, 2010, 2012)

Year	$\bar{X}$ ET	Procedure	CPR/Asp	DR/Asp
2000	2,6	CII (IVF)	26,7	18,6
		ICSI	27,7	20,4
2005	2,29	CII	29,8	20,3
		ICSI	28,9	19,2
2008	2,1	CII	26,1	19,1
		ICSI	28,7	18,9
2010	1,9	CII	25,4	19,1
		ICSI	26,8	20,0
2012	1,88	CII	24,4	17,7
		ICSI	24,9	17,9

$\bar{X}$ ET = mean of embryos transferred.

CPR/Asp = clinical pregnancy rate per aspiration.

DR/Asp = delivery rate per aspiration.

how it is done in the world reports published by ICMART (Dyer et al., 2016). It is important to note that cumulative delivery rates resulting from fresh plus frozen transfers have greater impact in younger women compared to women >40 and is also strongly affected by the number of oocytes collected in the fresh cycle (Toftagert et al., 2017). For example, data provided by the Latin American registry show that in women <35 delivery rates increase from 29.2% in fresh transfers to a 41.3% cumulative delivery rate when FET cycles are added. However, in women of 40–42 years, the increase is only from 14.5% to 17.6%, having much less clinical impact (Zegers-Hochschild et al., 2014). Similar results for cumulative live birth (CLBR) were recently reported in a large population extracted from the national registry of the United Kingdom. While CLBR in young women could reach up to 40%–67%, in women age 40 years or more the increment in CLBR was only from 5.9% to 9.8% (McLernon et al., 2016).

Reaching global consensus on how to calculate CLBR will be important in the future to enable the comparison of effectiveness within and across registries. Global markers of reproductive outcome must be understood as references to examine similarities and differences. It is only by carefully collecting, collating and analyzing country specific variables that one can really determine factors that influence the way ART is practiced and the outcomes obtained.

Safety

Issues of safety primarily concern multiple birth and ovarian hyperstimulation syndrome (OHSS). OHSS has only recently been monitored and reported globally; furthermore, lack of standardized definition of reportable occurrence has made interpretation of incidence difficult. However, increasing attention to prevention and documentation is occurring globally and data are being collected to follow its incidence more closely.

Lowering multiple births as a safety component has been progressively incorporated into ART management, but important differences persist among countries. The range of twin and triplet deliveries for 2012 was from 11% to 0.4%. Variations among

regions are shown in Fig. 3. Australia, Asia, and Europe have the lowest number of embryos transferred and, consequently, the lowest rate of multiple births. Globally, countries with the lowest proportion of twins and triplets are Japan with 3.9% twins and 0.1% triplets, followed by Sweden with 5.6% and 0.1% and Australia with 6.3% and 0.1%, respectively. The opposite extreme is also found in Europe where Serbia and Lithuania have 36.6% and 32.3% of twins respectively. The USA report 26.4% twins and 1.0% triplets while in Latin America, Argentina has the lowest rate of multiples, with 16.9% twins and 1.0% triplets, while Guatemala and Nicaragua report 31.6% and 41.9% twins, respectively, but reporting very few pregnancies.

The proportion of twins and triplets or more are a direct consequence of the number of embryos transferred. Fig. 4 shows the proportion of single, double and triple or more embryo transfers (SET, DET and TET, respectively) in countries mentioned above. There is a direct correlation between the number of embryos transferred and multiple pregnancy. Furthermore, there is a direct correlation between multiple gestation and perinatal mortality which, according to the Latin American registry (Zegers-Hochschild et al., 2014), increases from 23 to 35 per thousand from singletons to twins.

Multiple births not only result in poorer outcomes for mothers and babies but in greater long-term costs associated with caring for complicated pregnancies and preterm birth—a cost and responsibility that in high resourced countries is commonly taken care

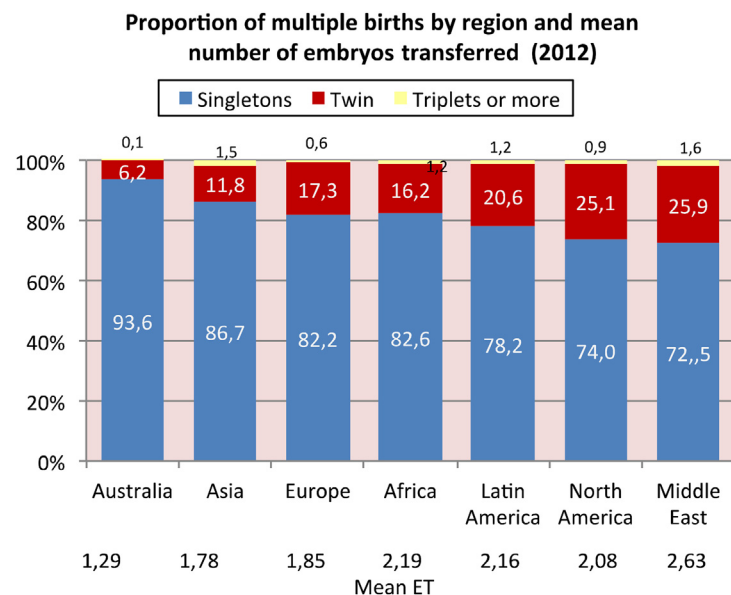


Fig. 3 Regional variation in the proportion of multiple births (twins and triplets or more) and its relationship with the mean number of embryos transferred. The proportion of triplets and a more can be affected by embryo reduction which is practiced in few regions in the world.

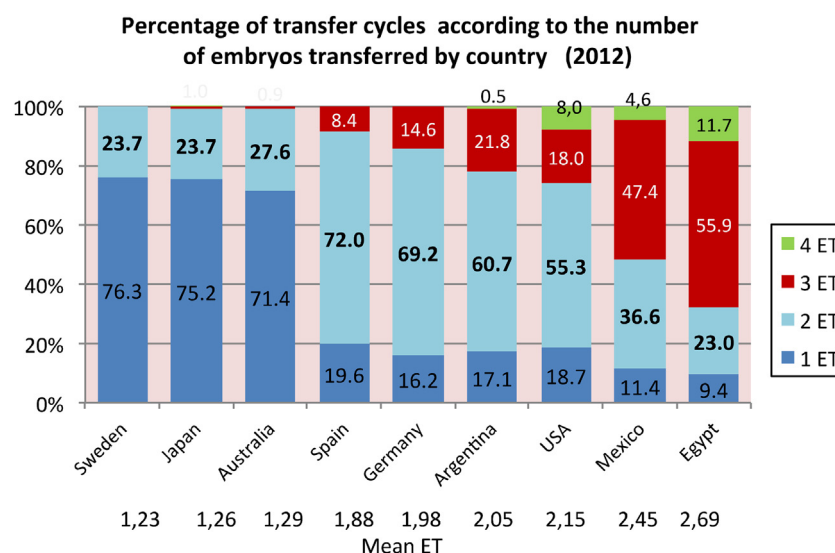


Fig. 4 Country variability in the proportion of single embryo transfer (SET), double embryo transfer (DET) and triple or more embryos transferred. There are big differences within regions, such as Sweden and Germany in Europe, or Argentina and Mexico in Latin America.

of by society through government health plans (Adamson et al., 2006); however, in less well-resourced countries multiple births usually create major financial burdens for affected patients and their households. This provides a strong argument for the development of public policies in which individuals and society as a whole share the costs and responsibilities that result from fertility treatments.

Improving The Data in Registries

Original data collection for registries was very rudimentary, consisting of written reports that were collected, collated and analyzed by hand with calculators. Subsequently, computers and computer programs have improved our ability to capture, collate and analyze data in a much more sophisticated fashion. Currently, progress is enabling electronic, cloud-based ART data collection and transfer of data electronically from one registry to another; this facilitates the development of combined databases.

The use of standardized definitions that are the same globally have continued to improve registry data. The most recent is The International Glossary on Infertility and Fertility Care, 2017 (Zegers-Hochschild et al., 2017a).

One of the most important aspects of obtaining good data is to have prospective reporting of cycles. This means that all ART cycles are entered at the time there is an intention to treat the patient: with fresh transfer ART cycles this is date on which ovarian stimulation is started; with frozen-thaw cycles this is the date on which medications are given to establish a “controlled” cycle or at which cycle monitoring is started in preparation for a frozen/thaw transfer in a natural cycle. With prospective reporting, all intended treatment cycles are counted, including those which may be canceled later for various reasons. This is critical if we are to know the “real world” experience of patients and the true “success” of ART. It also limits “cherry picking” of patients in which only those who respond well to ovarian stimulation medications are counted in ART results. This results in reported outcomes that appear better than that actually experienced by patients.

One of the advantages of electronic data collection is that software programs can be written into the data collection system to ensure accuracy, consistency and completeness of the data that are reported in different areas of the registry; this also reduces simple arithmetical errors.

Additionally, data verification programs can check for both expected and unexpected problems where the reported results do not make sense mathematically or biologically. These data can then be double checked at their source to ensure that accurate data are included in the registry.

Finally, electronic data collection can be used to run software programs to identify reporting entities that have outliers in terms of patient treatment or outcomes, for example, high multiple birth rates or very low miscarriage rates. This information can be used to assess programs needing attention with respect to quality of care and/or integrity of their data. Any outliers must be assessed by knowledgeable clinicians and scientists objectively, almost always in conjunction with the originator of the data, usually an ART clinic, but sometimes even a nation state.

Electronic data collection can be used to screen the data provided, and to identify those entities that might require on-site visits or more in-depth assistance to ensure quality data are submitted by that entity.

The Influence of New Technologies on Registries

Data collection is influenced by changes in technology. For example, with the increasing use of day 5/6 blastocyst embryo transfer, implantation rates improved because of the biologically-based higher implantation rates of blastocysts over cleavage stage embryos. Therefore, it is important for registries to report on whether embryo transfers are performed on day 3 or day 5 because this creates significant differences in pregnancy rates.

Intracytoplasmic sperm injection (ICSI) treats male factor infertility exceptionally well, but is not necessary when sperm function normally. Overall, live birth rates are probably slightly less with ICSI and birth abnormalities are slightly higher, so it is important to know the proportion of cycles that involve ICSI. This ranges from just over 50% in some countries to almost 100% in others.

Elective single embryo transfer (eSET) has been increasingly promoted over the past two decades in order to decrease multiple pregnancy rates; however, it is associated with lower pregnancy rates on the initial transfer of the embryo, although not lower cumulative pregnancy rates. Since different ART clinics and different countries and regions have incorporated eSET at different rates, there are very different proportions of patients treated with eSET or double embryo transfer (DET) or even higher number of embryos transferred. It is essential to know the proportion of eSET and average number of embryos transferred in order to interpret pregnancy rates.

Cryopreservation affects all aspects of ART. This impact has increased significantly with the advances in vitrification and markedly improved embryo and egg viability and increased pregnancy rates: eSET is performed more often; more embryos are cryopreserved; more cycles are frozen as opposed to fresh embryo transfer cycles; cycles are not canceled for ovarian hyperstimulation syndrome (OHSS) as often; more cycles are “freeze all” cycles because of high progesterone levels or less than optimal endometrium; more PGT is performed; more fertility preservation both of embryos and eggs is performed. All these changes in clinical protocols change the proportions of types of cycles and the pregnancy rates associated with them. Registry data can only be interpreted with an understanding of these technology and clinical protocol changes.

Freeze all cycles have created a special difficulty for interpreting registry data. Historically, the outcome of a cycle was relatively simple to report—stimulation drugs were given, eggs retrieved, embryos created and at least one replaced within a few days. Outcome was pregnancy or live birth per cycle start, egg retrieval or embryo transfer. However, with freeze all cycles there is no transfer, so the pregnancy rate is zero for a fresh transfer. Yet the first frozen/thaw transfer cycle will use, statistically, a better embryo than in a patient who already had the best embryo selected for the fresh transfer. If the transfer does not occur in the same year as the retrieval, this creates the need to report a cycle outcome in the year following the ovarian stimulation (cycle start/intended treatment) and the egg retrieval, markedly increasing the complexity of reporting. This problem is compounded when eggs or embryos are cryopreserved for fertility preservation and may not be used for several years (egg or embryo banking). Complex and standardized rules for linking the retrievals with the transfers in reporting such cycles need to be implemented by registries to deal with these issues so that the numbers reported in different registries are based on the same calculations. On a global basis, these complex issues are just beginning to be addressed.

PGT has also changed registries because essentially all cycles are cultured to blastocyst rather than just cleavage stage, generally all the embryos are cryopreserved for later use, and there is selection of embryos so that a higher quality embryo is chosen for transfer. However, PGT does not increase the reproductive capacity of the embryos created from a single transfer—rather, the cumulative pregnancy rate from all the embryos is reduced because of loss of reproductive potential from embryo biopsy, testing errors, harm from cryopreservation and mosaicism. These factors must be considered when interpreting the data in a registry.

Summary

Registry data collection is essential for our professional understanding of ART practice and to inform patients and policy makers. It is much more complex than many appreciate. For years registries were overlooked as an integral component of quality control for all stakeholders in ART. However, the importance of global ART reporting is now recognized. Fortunately, much improvement has occurred in the amount and quality of data collected and reported. However, many challenges remain. Ongoing commitment to improved data collection for ART registries is necessary so that we can continuously improve access, utilization, effectiveness and safety of ART.

References

- Adamson, G. D., de Mouzon, J., Lancaster, P., Nygren, K.-G., Sullivan, E., & Zegers-Hochschild, F. (2006). International Committee Monitoring Assisted Reproductive Technology. World collaborative report on in vitro fertilization, 2000. *Fertility and Sterility*, 85, 1586–1622.
- Chambers, G., Phuong Hoang, V., Sullivan, E., Chapman, M., Ishihara, O., Zegers-Hochschild, F., Nygren, K.-G., & Adamson, G. D. (2014). The impact of consumer affordability on access to assisted reproductive technologies and embryo transfer practices: An international analysis. *Fertility and Sterility*, 101(1), 191–198.
- Dyer, S., Chambers, G. M., de Mouzon, J., Nygren, K.-G., Zegers-Hochschild, F., Mansour, R., Ishihara, O., Banker, M., & Adamson, G. D. (2016). International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted Reproductive Technology 2008, 2009 and 2010. *Human Reproduction*, 31(7), 1588–1609.
- IFRS Surveillance. (2016). *Global Reproductive Health*, 1(1), 1–143. <https://doi.org/10.1097/GRH.0000000000000001>. Supplement Article, 7th Edition.
- Lancaster, P. A. (1996). Registers of in-vitro fertilization and assisted conception, 572 *Human Reproduction*, 11(Suppl 4), 89–104, 573.
- McLernon, D., Maheshwari, A., Lee, A., & Bhattacharya, S. (2016). Cumulative live birth rates after one or more complete cycles of IVF: A population-based study of linked cycle data from 178,898 women. *Human Reproduction*, 31(3), 572–581.
- de Mouzon, J., et al. (2012). International Committee Monitoring Assisted Reproductive Technology. In *World collaborative report on in vitro fertilization*. in preparation for Human Reproduction.
- Toftager, M., Bogstad, J., Løssl, K., Prætorius, L., Zedeler, A., Bryndorf, T., Nilas, L., & Pinborg, A. (2017). Cumulative live birth rates after one ART cycle including all subsequent frozen–thaw cycles in 1050 women: Secondary outcome of an RCT comparing GnRH-antagonist and GnRH-agonist protocols. *Human Reproduction*, 32(3), 556–567.
- Zegers-Hochschild, F., Schwarze, J. E., Crosby, J., Musri, C., Urbina, M. T., & on behalf of the Latin American Network of Assisted Reproduction (REDLARA). (2014). Assisted reproduction techniques in Latin America: The Latin American Registry. *Reproductive Biomedicine Online*, 35(2017), 287–295. <https://doi.org/10.1016/j.rbmo.2017.05.021>.
- Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., de Mouzon, J., Sokol, R., Rienzi, L., Sunde, A., Schmidt, L., Cooke, I. D., Simpson, J. L., & van der Poel, S. (2017a). The International Glossary on Infertility and Fertility Care. *Human Reproduction*, 1–16. <https://doi.org/10.1093/humrep/dex234>.
- Zegers-Hochschild, F., Adamson, G. D., Dyer, S., Racowsky, C., de Mouzon, J., Sokol, R., Rienzi, L., Sunde, A., Schmidt, L., Cooke, I. D., Simpson, J. L., & van der Poel, S. (2017b). The International Glossary on Infertility and Fertility Care, 2017. *Fertility and Sterility*, 108(3), 393–406.

Multiple Versus Singleton Births: Consequences and Preventions

Siwen Wang, Jinqun Xu, Yifeng Liu, and Dan Zhang, Zhejiang University, Zhejiang, China

© 2018 Elsevier Inc. All rights reserved.

Glossary

Feto-fetal transfusion syndrome (FFTS) FFTS is a complication of disproportionate blood supply. One baby will receive too much blood and the other will receive too little.

Gonadotropin Human gonadotropins are glycoprotein hormones secreted by anterior pituitary of brain (FSH and LH) or placenta (HCG) that work to regulate sexual development, sex hormone secretion, and reproductive function.

Gonadotropin-releasing hormone (GnRH) Peptides synthesized and secreted by hypothalamus to regulate the release of Gonadotropins.

Intrahepatic cholestasis of pregnancy (ICP) ICP is a medical condition in which cholestasis (bile cannot flow from the liver to the duodenum) occurs during pregnancy. It typically presents with intense itching, usually on the hands and feet.

Preeclampsia Preeclampsia is a disorder of pregnancy characterized by high blood pressure and signs of damage to another organ system, most often the liver and kidneys.

Premature rupture of the membranes (PROM) PROM is defined as rupture of membranes before the onset of labor.

Vascular anastomoses A connection between two or more blood vessels that are diverging or branching.

Zona pellucida A protective glycoprotein layer surrounding the oocytes in mammals.

Development of Multiple Pregnancies

The term “multiple births” refers to the delivery of two or more offspring from one pregnancy. Multiple births occur in almost all mammals and could be more common than singleton births in polytocous species. In contrast, multifetal pregnancies in low-ovulating mammalian species, such as cattle and primates, are often related with birth difficulties and health problems for both mother and newborns. This article will focus on human multiple pregnancies.

A multiple pregnancy may be the result of fertilization of more than one egg, or the division of a fertilized egg into identical fetuses, or a combination of both events. For example, twin fetuses can result from the fertilization of two separate ova, or the division of one fertilized ovum, referred to as dizygotic (fraternal) twins and monozygotic (identical) twins, respectively. Either or both processes may be involved in higher-order multiple pregnancies. There are various types of monozygous twins, depending on the timing of division. If the cleavage occurs within the first 72 h after fertilization, two distinct amnions and chorions develop. And this is termed as a dichorionic, diamnionic twin. In this case, either two placentas or a fused one develop. If zygotic splitting occurs during the blastocyst stage (between the fourth and eighth day of fertilization), twins share a placenta and chorion but have a separate amniotic sac, which is a monochorionic, diamnionic pregnancy. This type accounts for approximately 70% of all monozygous pregnancies. Least frequently, the zygotes split after the ninth day of fertilization when the amniotic sac is already differentiated, a monochorionic, monoamnionic twin pregnancy evolves. Incomplete division of zygotes after 13 days of embryogenesis results in conjoined twins. The development of monozygotic twins is shown in [Fig. 1](#).

The incidence of multiple pregnancies also varies with ethnicity, parity, heredity (predominantly maternal side) and nutritional factors ([Cunningham et al., 2014](#); [Luke and Brown, 2008](#)), whereas the frequency of monozygotic twin births is relatively constant worldwide.

The drastic surge in twinning rates over the past decades is largely attributable to the increasing use of infertility treatment, including drug-induced ovulation and in vitro fertilization (IVF), wherein more than one embryo is transferred into the uterus. It is estimated 30%–50% of multifetal pregnancies are conceived by assisted reproductive technology (ART) and about 35% of ART pregnancies are multiples ([Blondel and Kaminski, 2002](#); [Zhu et al., 2016](#)). In addition, monozygotic twinings are more commonly seen following ART procedures, as embryos are particularly susceptible to minor turbulences. Ovarian stimulation, zona pellucida manipulation, temperature fluctuations, blastocyst transfer, and prolonged embryos culture in vitro have been proposed as mechanisms contributing to the increased incidence of ART-associated monozygotic twins ([Aston et al., 2008](#)).

Deferred childbearing is another well-known risk factor for multiple pregnancies, independent of the application of ART ([Blondel and Kaminski, 2002](#); [Luke and Brown, 2008](#)). Women over 35 years of age are more likely to release more than one egg in a single menstrual cycle than younger counterparts. In general, there is an age-related fertility decline in both men and women, so the need of infertility treatment increases. Furthermore, older maternal age requires more aggressive therapies to achieve a pregnancy, which includes transferring more embryos.

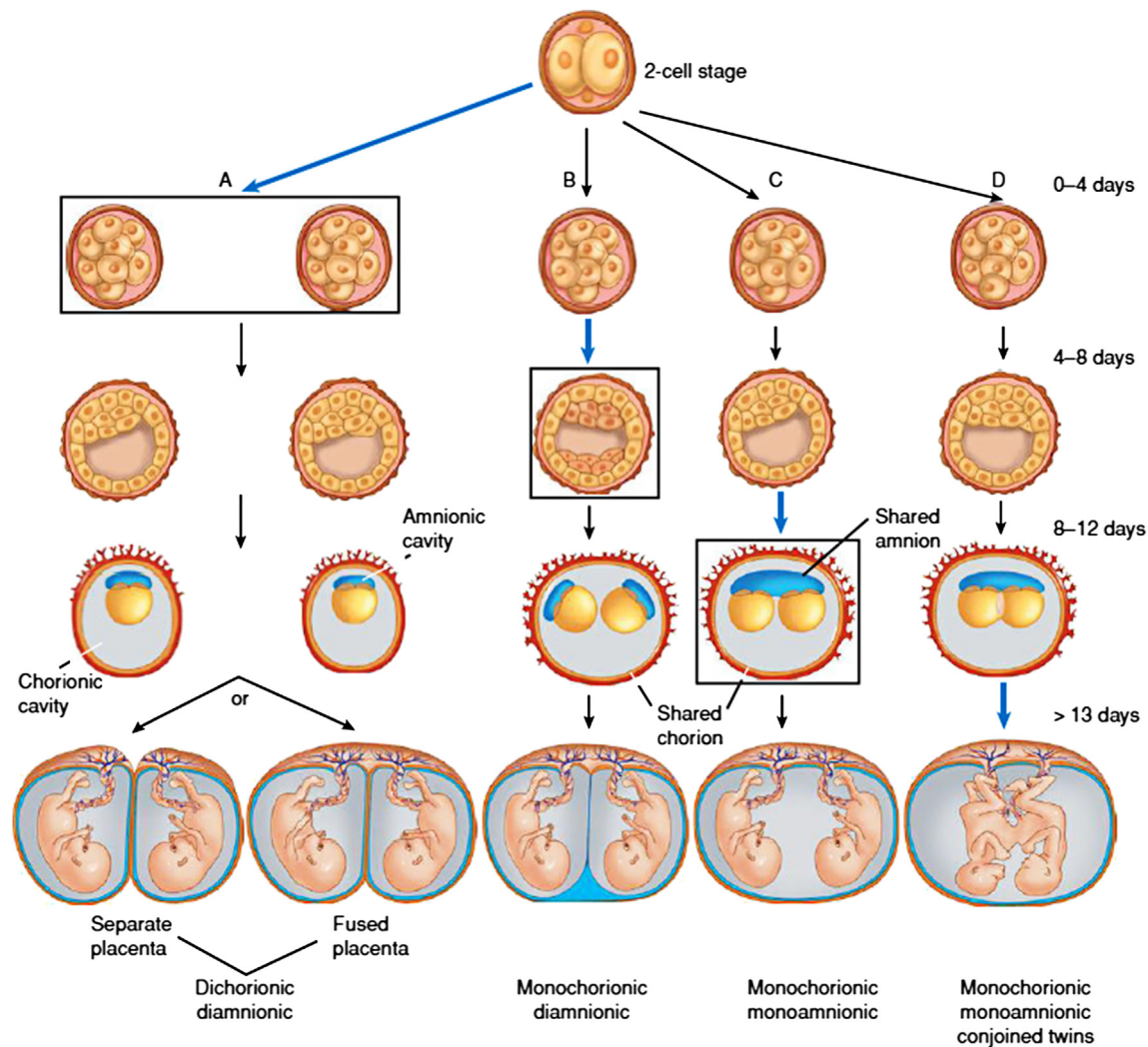


Fig. 1 Development of monozygotic twins. Cunningham, F. G., Leveno, K.J., Bloom, S.L., Spong, C.Y., Dashe, J.S., Hoffman, B.L., Sheffield, J.S. 2014. *Williams obstetrics*, 24th edn. McGraw-Hill Education, New York.

Maternal Adaptations to Multiple Gestations

Systemic physiologic changes take place to meet the needs of pregnancy. Although the nature of most adaptive changes is quite similar in multiple and singleton pregnancies, multiple pregnancies are featured with more hyper dynamic circulation as the maternal body is at even higher demands of support (blood, oxygen, and nutrition supply, and clearance of fetal waste) and protection.

Nausea and vomiting of pregnancy is more commonly seen in women carrying multiple fetuses because of comparatively higher serum hCG levels. Initiated by hormonal change, the blood volume expansion following sodium and water retention is also more significant in women with multiple fetuses.

In a typical singleton gestation, maternal diastolic blood pressure continues to drop until mid-pregnancy in response to the decrease in peripheral vessel resistance but will gradually recover to nonpregnant level by term, while systolic pressure is less affected (Norwitz et al., 2005). During multifetal pregnancies, however, a greater fall in diastolic pressure is observed as early as 8th week, along with a higher rise at term (Cunningham et al., 2014; Norwitz et al., 2005). Marked augment in cardiac output achieved by a greater increase in heart rate and stroke volume during multiple gestation serves to maintain the blood pressure and thus the perfusion to fetoplacental unit throughout pregnancy.

The greater enlargement of uterus during multiple pregnancies exerts stronger space occupying effects on adjacent organs, especially in advancing pregnancy. Thus the magnitude of respective changes is exaggerated. Enlarging uterus compresses large blood vessels (venae cava inferior and aorta); consequently result in edema, supine hypotension, and regional anesthesia. Gastrointestinal functions are compromised as the stomach and intestines are appreciably displaced by the expanding uterus.

Alterations in respiratory system are initially induced by the change in hormones and later by the anatomical changes in thoracic cavity secondary to abdominal distention (Cunningham et al., 2014; Norwitz et al., 2005). Both the hyperventilation caused by

elevated progesterone and the rise of diaphragm is more significant with multiple pregnancies as compared with singletons (Norwitz et al., 2005), leading to a further reduction in residual volume and an increment in tidal volume. But mother-to-bes carrying multiples claim to experience more breatheless moments (possibly associated with the direct effect of progesterone and the abnormally decreased abdominal muscle tone).

Maternal Complications of Multiple Pregnancies

As per discussed, multiple gestations are associated with more profound body changes, which predispose mothers to almost all potential pregnancy and obstetric complications. The risk of anemia, pregnancy-induced hypertension, preeclampsia-eclampsia, postpartum hemorrhage (PPH), premature rupture of membrane, intrahepatic cholestasis of pregnancy (ICP), cervical incompetence, diabetes mellitus, placental insufficiency, and cesarean delivery were substantially increased in women with high-order multiple gestations (Cunningham et al., 2014; Norwitz et al., 2005; Wen et al., 2004). A dose-response relationship (risk goes up with gestational plurality) is observed in certain complications, such as pregnancy-associated hypertension, diabetes mellitus, and placental abruption (Wen et al., 2004).

Hypertension

Being the second leading cause of maternal death, gestational hypertension, and preeclampsia can be very ominous if not diagnosed earlier. Epidemiological studies have demonstrated that despite lower blood pressure measured in mid-pregnancy, pregnancy-associated hypertension complicates 40% of twin pregnancies, 2–3 times higher than that in singleton pregnancies (McMullan et al., 1984). Apart from that, hypertensive disorders with multifetal pregnancies are characterized by earlier incidence and severer outcomes.

Postpartum Hemorrhage

Researchers found a twofold-increased risk of PPH and severe PPH (blood loss > 500 mL or 1000 mL within the first 24 h after termination of pregnancy, respectively) in women who had multiple births. The over-distended uterus and enlarged placental attachment area place mothers at greater risk of postpartum hemorrhage.

Preterm Premature Rupture of the Membranes (pPROM)

About 10% multiple pregnancies are complicated by pPROM (Norwitz et al., 2005), which is possibly caused by the over distention and subsequent hyperirritability of uterus.

Family Stress

The psychological well-being of parents cannot be neglected. Although couples raising multiples generally reported greater enjoyment, mothers are more likely to experience emotional distress and some may require psychotherapy (Blondel and Kaminski, 2002). Apart from that, one thing that also weighs on multiple-birth families is the financial stress thereafter.

Fetal and Neonatal Complications of Multiple Births

Multiple births represent a higher risk in fetal and neonatal complications, which may have a long-term effect on the growth and development of newborns. Notably, chorionicity is of greater importance in predicting the prognosis of multiples than zygosity (Cunningham et al., 2014). The incidence of perinatal complications is similar between dizygotic and dichorionic monozygotic twins. Nevertheless, monochorionic multiples are far more prone to structural anomalies, cord entanglement, and selective growth restriction. This excess in risk is, at least in part, due to the vascular anastomoses within placenta, which could result in a potential hemodynamic imbalance under pathological conditions. What's more, this shared circulation accounts for some unique complications in monochorionic twins, namely, fetofetal transfusion syndrome (FFTS), twin reversed arterial perfusion (TRAP), and acute hemorrhage of the survivor after the death of their co-twin (single intrauterine fetal demise, IURD).

Spontaneous Pregnancy Loss

The prevalence of miscarriage (unexpected pregnancy losses at ≤ 20 weeks or with a fetal weight < 500 g) in multiple pregnancies is seven times as that in singletons (Cunningham et al., 2014). This is not necessarily a negative trend, as spontaneous multifetal reduction conferred benefits of extending the duration of pregnancy to 34 weeks or longer (Skiadas et al., 2011). Monochorial placentation and male fetuses are of higher risk of spontaneous pregnancy loss (Norwitz et al., 2005).

Preterm Birth

Preterm birth (born before 37 gestational weeks) is the most common complication of multiple gestations. In an earlier study conducted in the US, over 50% of twins and nearly all higher-order multiples are born premature (Martin et al., 2012). By contrast,

only approximately 10% singletons are premature (Blondel and Kaminski, 2002). The risk of prematurity grows correspondingly with the number of fetuses in the pregnancy, and the length of gestation contrariwise. Premature labor could be spontaneous, induced by PPROM, or medically indicated. The increasing application of indicated premature births in recent years explains the corresponding increased premature birth rate in many developed countries. However, the effectiveness of this intervention to reduce neonatal mortality and morbidity is controversial.

Premature births remain the leading cause of perinatal adverse outcomes, contributing over 75% to perinatal mortality and morbidity (Norwitz et al., 2005). It is reported that preterm multiples are more likely to have cerebral dysfunction than their peers, while other studies have shown that the clinical outcome of preterm infants seems only to correlate with gestational ages, regardless of the etiologies of preterm delivery and plurality.

Intrauterine Growth Restriction

Intrauterine growth restriction (previously known as intrauterine growth retardation, IUGR) in multiple gestations is a common occurrence. Compared with singletons, multiples are at notably increased risk of IUGR for intrinsic (fetal characteristics) and extrinsic (maternal conditions, umbilical abnormality, and placental insufficiency) reasons. In general, the degree of growth restriction increases with fetal number. Delayed growth is closely associated with fetal death, inhibited neurodevelopment, and chronic diseases later in life.

Selective IUGR (sIUGR) refers to the growth discordance among multiples. Although the etiological causes of sIUGR in dichorionic twins vary, discordancy in monochorionic twins is mainly due to the unequal distribution of blood supply and hence nutrients and oxygen.

Low Birth Weight

Low birth weight (<2500 g at birth) is a risk factor for infant mortality and morbidity. Multiples are more likely to be low birth weight than singleton pregnancies due to higher incidence of preterm delivery and restricted intrauterine fetal growth.

Induced Twins Versus Natural Twins

More adverse outcomes are observed in induced twins compared with spontaneous twins, but only of slight difference. Multiple pregnancies following ART procedures represent a higher risk of spontaneous reduction, umbilical cord anomalies, stillbirth, prematurity, and low birth weight than naturally conceived ones (Skiadas et al., 2011; Lambalk, 2001). In another research, ART twins group was observed to have a significantly increased incidence of postpartum hemorrhage and small for date infant compared with natural twins group (Zhu et al., 2016) (Tables 1 and 2). Yet, it is not clear whether the impaired obstetric and neonatal outcomes of iatrogenic twins are associated with the artificial manipulation throughout the fertilization process, or the parental intrinsic characteristics responsible for their subfertility, or a combination of both.

Preventions of Multiple Births

The causes of multiple pregnancies are mostly patients' clinical characters (maternal age and sex hormone levels), ovarian response (number and size of follicles), and treatment methods (initial and total dose of gonadotropins, duration of ovarian stimulation, and number of embryos transferred). Therefore, physicians and patients could take measures to prevent the occurrence and the subsequent adverse effects of multiple births.

Maternal Age

Generally speaking, older maternal age is one of the most significant risk factors for multiple pregnancies. Besides, an increased risk of almost all pregnancy complications is observed in mothers aged over 35 carrying multiples. This fact can be seen through the changing trend of the ASRM Practice Committee's (the first society that issued the guidelines for multiple pregnancy) recommend as well (Murray and Norman, 2014): In Jan. 1998, the ASRM Practice Committee recommended that women younger than 35 years old should have only three embryos transferred. Those with an average prognosis (patients over 40 who had multiple previous embryo transfers without success) could have up to five embryos transferred. In Nov. 1999, the committee modified their recommendations and stated that patients with good prognosis (age under 35) should have no more than two embryos transferred, those with an average prognosis (35–40 years old) could have three transferred, and those with below-average prognosis (age older than 40 years or previous difficulties that could be identified) could have four transferred. In Jul. 2006, the committee changed the number of embryos transferred for patients with favorable prognosis under 35 into only one. Through the developing trend of the committee's recommendations, we could see that strict limitations on the number of embryos transferred are required, individual treatment plan should be modified according to patients' own conditions such as age, etc.

Number of Embryos Transferred

The most effective strategy for reducing multiple pregnancy rates in IVF cycles is to reduce the number of embryos transferred. Researches have shown that a mandatory single embryo transfer (SET) could result in a dramatic decrease of multiple pregnancy rates.

Table 1 Incidence of pregnancy and perinatal complications in ART and spontaneously pregnant groups with stratified analysis by birth plurality

	Total				Singletons				Twins			
	ART (<i>n</i> = 2641)	Controls (<i>n</i> = 5282)	<i>P</i>	Adjusted OR (95% CI)	ART (<i>n</i> = 1659)	Controls (<i>n</i> = 5193)	<i>P</i>	Adjusted OR (95% CI)	ART (<i>n</i> = 982)	Controls (<i>n</i> = 89)	<i>P</i>	Adjusted OR (95% CI)
Pregnancy complications												
GDM	309 (11.7)	342 (6.5)	< .001	1.99 (1.69–2.36)	214 (12.9)	334 (6.4)	< .001	2.23 (1.85–2.69)	95 (9.7)	8 (9.0)	1	—
Gestational hypertension	249 (9.4)	189 (3.6)	< .001	2.58 (2.11–3.15)	124 (7.5)	183 (3.5)	< .001	1.99(1.56–2.53)	125 (12.7)	6 (6.7)	.127	—
Preeclampsia	98 (3.7)	110 (2.1)	< .001	1.49 (1.12–1.98)	43 (2.6)	107 (2.1)	.21	1.71 (1.34–2.19)	55 (5.6)	3 (3.4)	.471	—
Mild preeclampsia	35 (1.3)	56 (1.1)	.315	—	15 (0.9)	55 (1.1)	.675	0.60 (0.33–1.09)	20 (2.0)	1 (1.1)	1	—
Severe preeclampsia	63 (2.5)	54 (1.0)	< .001	2.04 (1.40–2.96)	28 (1.7)	52 (1.0)	.035	1.52 (0.95–2.43)	35 (3.6)	2 (2.3)	.763	—
ICP	302 (11.4)	228 (4.3)	< .001	2.86 (2.39–3.42)	110 (6.6)	214 (4.1)	< .001	1.59(1.25–2.02)	192 (19.6)	14 (15.7)	.482	—
Perinatal complications												
Placenta previa	185 (7.0)	179 (3.4)	< .001	2.23 (1.79–2.78)	117 (7.1)	175 (3.4)	< .001	2.25 (1.75–2.89)	68 (6.9)	4 (4.5)	.508	—
Complete placenta previa	58 (2.2)	53 (1.0)	< .001	2.61 (1.78–3.83)	44 (2.7)	53 (1.0)	< .001	3.09 (2.05–4.66)	14 (1.4)	0	—	—
Partial placenta previa	12 (0.5)	8 (0.2)	.016	3.39 (1.36–8.46)	10 (0.6)	7 (0.1)	.002	5.15 (1.92–13.82)	2 (0.2)	1 (1.1)	.229	—
Marginal placenta previa	115 (4.4)	118 (2.2)	< .001	1.85 (1.42–2.41)	63 (3.8)	115 (2.2)	.001	1.60 (1.17–2.19)	52 (5.3)	3 (3.4)	.616	—
Placental abruption	40 (1.5)	16 (0.3)	< .001	5.06 (2.83–9.06)	21 (1.3)	15 (0.3)	< .001	4.43 (2.28–8.61)	19 (1.9)	1 (1.1)	1	—
pPROM	258 (9.8)	176 (3.3)	< .001	3.05 (2.48–3.74)	85 (5.1)	161 (3.1)	< .001	1.68 (1.27–2.22)	173 (17.6)	15 (16.6)	1	—
Abnormal placental cord insertion	105 (4.0)	167 (3.2)	.067	1.37(1.06–1.76)	69 (4.2)	163 (3.1)	.051	1.40 (1.05–1.87)	36 (3.7)	4 (4.5)	.568	—
Placental adherence	197 (7.5)	173 (3.3)	< .001	2.37(1.90–2.95)	115 (6.9)	168 (3.2)	< .001	2.21 (1.71–2.84)	82 (8.4)	5 (5.6)	.541	—
Postpartum haemorrhage	197 (7.5)	155 (2.9)	< .001	2.72 (2.18–3.41)	82 (4.9)	151 (2.9)	< .001	1.74 (1.30–2.32)	115 (11.7)	4 (4.5)	.025	2.819 (1.02–7.83)
Polyhydramnios	60 (2.3)	73 (1.4)	.005	1.79 (1.26–2.53)	36 (2.2)	71 (1.4)	.03	1.65 (1.09–2.50)	24 (2.4)	2 (2.3)	1	—
Oligohydramnios	127 (4.8)	344 (6.5)	.002	0.67 (0.54–0.83)	116 (7.0)	342 (6.6)	.572	—	11 (1.1)	2 (2.3)	.295	—

CI, confidence interval; OR, odds ratio; Data are *n* (%).GDM, gestational diabetes mellitus.

Zhu, L., Zhang, Y., Liu, Y., Zhang, R., Wu, Y., Huang, Y., Zhang, D. 2016. Maternal and live-birth outcomes of pregnancies following assisted reproductive technology: A retrospective cohort study. *Scientific Reports*, 6(1).

Table 2 Neonatal outcomes in ART and spontaneously pregnant groups with stratified analysis by birth plurality

	Total			Singletons			Twins		
	ART (n = 2641)	Controls (n = 5282)	P	ART (n = 1659)	Controls (n = 5193)	P	ART (n = 982)	Controls (n = 89)	P
Preterm labor	1024/2641 (38.8)	556/5282 (10.5)	< .001	291/1659 (17.5)	489/5193 (9.4)	< .001	733/982 (74.6)	67/89 (75.3)	1
Low birthweight	1321/3623 (36.5)	430/5371 (8.0)	< .001	170/1659 (10.2)	314/5193 (6.0)	< .001	1151/1964 (58.6)	116/178 (65.2)	.11
Macrosomia	121/3623 (3.3)	387/5371 (7.2)	< .001	120/1659 (7.2)	387/5193 (7.5)	.788	1/1964 (0.0)	0 (0.0)	–
Small for date infant	137/3623 (3.8)	63/5371 (1.2)	< .001	17/1659 (1.0)	43/5193 (0.8)	.451	120/1964 (6.1)	20/178 (11.2)	.016
1 min Apgar ≤ 7	178/3623 (4.9)	159/5371 (3.0)	< .001	64/1659 (3.9)	152/5193 (2.9)	.063	114/1964 (5.8)	7/178 (3.9)	.675
5 min Apgar ≤ 7	43/3623 (1.2)	13/5371 (0.2)	< .001	11/1659 (0.7)	13/5193 (0.3)	0.028	32/1964 (1.6)	0 (0.0)	–

Data are n (%).

Zhu, L., Zhang, Y., Liu, Y., Zhang, R., Wu, Y., Huang, Y., Zhang, D. 2016. Maternal and live-birth outcomes of pregnancies following assisted reproductive technology: A retrospective cohort study. *Scientific Reports*, 6(1).

The AFS Ethics Committee stated in 1994 that the number of embryos to be transferred should be limited to eliminate quadruplet pregnancy and keep the rate of triplets to 1%–2% of all pregnancies. This statement was made to account for the variability among patients and the variability of success in various programs (Murray and Norman, 2014).

However, SET could also cause a sharp decrease of pregnancy rates when embryo quality is poor. Thus, the selection of good quality embryos is critical in SET, and longer culture of embryos until day 5 is recommended whenever possible (Esinler et al., 2014). However, in some patients, day-5 embryo transfer (ET) is impossible due to some unfavorable factors, such as poor embryo quality. For these patients, day-3 ET is mandatory and day-3 double embryo transfer (DET) for the second cycle could be considered. DET for these patients could improve pregnancy rates. But one should not forget that the occurrence of multiple pregnancies is still a problem in DET cycles.

Besides, the eagerness to become pregnant among most patients, especially the aged ones, might lead them to downplay or ignore the risk of multiple pregnancies. Patients' willingness should of course be involved in making surgical plans. It's a dilemma for both doctors and patients to consider.

Mild Ovarian Stimulation

During the past 15 years, there has been a marked increase in the use of two treatments (used alone or in combination) for unexplained infertility: induction of ovulation induction, in which the ovaries are stimulated with exogenous gonadotropins to develop several dominant follicles, and intrauterine insemination (IUI), in which motile spermatozoa are suspended in culture medium and injected transcervically into the uterine cavity (Balasch, 2004). However, it shows a rising trend of gonadotropins usage, which would probably lead to the increment of multiple pregnancies. To prevent this, clomiphene citrate could be postulated as a treatment option, but its effects on women with unexplained infertility it not satisfying (Balasch, 2004).

Ovulation can be induced by a number of agents, including gonadotrophins, pulsatile gonadotrophin releasing hormone, aromatase inhibitors, and selective estrogen receptor modulators, commonly known as antioestrogens. Clomiphene citrate is the antiestrogen most commonly used for ovarian stimulation. Multiple pregnancy in clomiphene ovarian stimulation generally occurs because of multifollicular development. Preventing the need for ovarian stimulation will limit an individual multiple pregnancy risk. In controlled ovarian hyperstimulation and intrauterine insemination cycles, a protocol of 50 IU of recombinant FSH daily combined with the use of GnRH antagonists and a policy of strict cancellation based on echographic criteria are associated with a satisfactory pregnancy rate per initiated cycle and a low risk of high-order multiple pregnancies. Clinical workers could use ultrasound monitoring or serum monitoring to detect or prevent multiple ovulation.

A major criticism to the strategy of mild ovarian hyperstimulation is that it reduces the rate of conception, but this assumption should be supported by more prospective randomized studies.

Reduction of the Number of Fetuses

Selective termination of the fetuses has been used during multiple pregnancy treatment, but the acceptability of these options for the couple will depend on their social background and underlying beliefs. There is no available data from randomized trials to inform the risks and benefits of pregnancy reduction procedures for women with a multiple pregnancy so far (Dodd et al., 2015).

References

- Aston, K. I., Peterson, C. M., & Carrell, D. T. (2008). Monozygotic twinning associated with assisted reproductive technologies: A review. *Reproduction*, 136(4), 377–386.
- Balasch, J. (2004). Gonadotrophin ovarian stimulation and intrauterine insemination for unexplained infertility. *Reproductive Biomedicine Online*, 9(6), 664–672.
- Blondel, B. C. A., & Kaminski, M. (2002). Trends in the occurrence, determinants, and consequences of multiple births. *Seminars in Perinatology*, 26(4), 239–249.
- Cunningham, F. G., Leveno, K. J., Bloom, S. L., Spong, C. Y., Dashe, J. S., Hoffman, B. L., & Sheffield, J. S. (2014). *Williams obstetrics* (24th edn.). New York: McGraw-Hill Education.
- Dodd, J. M., Dowswell, T., & Crowther, C. A. (2015). Reduction of the number of fetuses for women with a multiple pregnancy. *Cochrane Database of Systematic Reviews*.
- Esinler, I., Bozdog, G., & Sokmensuer, L. K. (2014). Mandatory single embryo transfer policy dramatically decreases multiple pregnancy rates. *Journal of Obstetrics and Gynaecology Research*, 40(1), 75–79.
- Lambalk, C. (2001). Natural versus induced twinning and pregnancy outcome: A Dutch nationwide survey of primiparous dizygotic twin deliveries. *Fertility and Sterility*, 75(4), 731–736.
- Luke, B., & Brown, M. B. (2008). Maternal morbidity and infant death in twin vs triplet and quadruplet pregnancies. *American Journal of Obstetrics and Gynecology*, 198(4).
- Martin, J. A., Hamilton, B. E., Ventura, S. J., Osterman, M. J., Wilson, E. C., & Mathews, T. J. (2012). Births: Final data for 2010. *National Vital Statistics Reports*, 61(1), 1–72.
- McMullan, P. F., Norman, R. J., & Marivate, M. (1984). Pregnancy-induced hypertension in twin pregnancy. *British Journal of Obstetrics and Gynaecology*, 91(3), 240–243.
- Murray, S., & Norman, J. (2014). Multiple pregnancies following assisted reproductive technologies – A happy consequence or double trouble? *Seminars in Fetal and Neonatal Medicine*, 19(4), 222–227.
- Norwitz, E. R., Edusa, V., & Park, J. S. (2005). Maternal physiology and complications of multiple pregnancy. *Seminars in Perinatology*, 29(5), 338–348.
- Skias, C. C., Missmer, S. A., Benson, C. B., Acker, D., & Racowsky, C. (2011). Spontaneous reduction before 12 weeks gestation and selective reduction similarly extend time to delivery in in vitro fertilization of trichorionic-triamniotic triplets. *Fertility and Sterility*, 95(2), 596–599.
- Wen, S. W., Demissie, K., Yang, Q., & Walker, M. C. (2004). Maternal morbidity and obstetric complications in triplet pregnancies and quadruplet and higher-order multiple pregnancies. *American Journal of Obstetrics and Gynecology*, 191(1), 254–258.
- Zhu, L., Zhang, Y., Liu, Y., Zhang, R., Wu, Y., Huang, Y., & Zhang, D. (2016). Maternal and live-birth outcomes of pregnancies following assisted reproductive technology: A retrospective cohort study. *Scientific Reports*, 6, 35141.

Long-Term Pediatric Follow-Up of Babies Born After ART

Julie Barberet and Patricia Fauque, Laboratoire de Biologie de la Reproduction, Dijon, France

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

ART Assisted reproductive technologies
CNS Central nervous system
CP Cerebral palsy
DBP Diastolic blood pressure
DHEA DeHydroEpiAndrosterone
DXA Dual-energy X-ray absorptiometry
ICSI Intracytoplasmic sperm injection
IVF In vitro fertilization
LH Luteinizing hormone
NC Naturally conceived
SBP Systolic blood pressure

Introduction

There has been a rapid and constant increase in the number of reproductive medicine attempts. Indeed, since the birth of the first baby conceived by in vitro fertilization (IVF) almost 40 years ago (Louise Brown in 1978), more than 5 million children have been born by assisted reproductive technologies (ART) (Hyrapetian et al., 2014). They now comprise roughly 2%–3% of all births in Europe and United States. However, the safety of these techniques has not been fully demonstrated, especially in the long term. Most studies have investigated perinatal outcomes.

Metaanalyses have revealed an increased risk of birth defects in singleton and multiple ART deliveries compared with births following natural conception (Rimm et al., 2004; McDonald et al., 2005; Pandey et al., 2012; Wen et al., 2012; Hansen et al., 2013). The absolute risk of birth defects in ART children could reach around 6.5%–7% (Hansen et al., 2013).

However, there are currently few data about the long-term health and development of ART children. The difficulty is to provide a comprehensive summary of the data. Only a handful of studies were prospective and most presented methodological bias such as a small sample size, were uncontrolled or had an inappropriate control group, used subjective criteria or had incomplete reporting notably when the analysis covered a long period and others lacked adjustment for parameters which could affect the health and development of these infants (Fig. 1). Indeed, it is important to take into account parental characteristics (age, life-style,

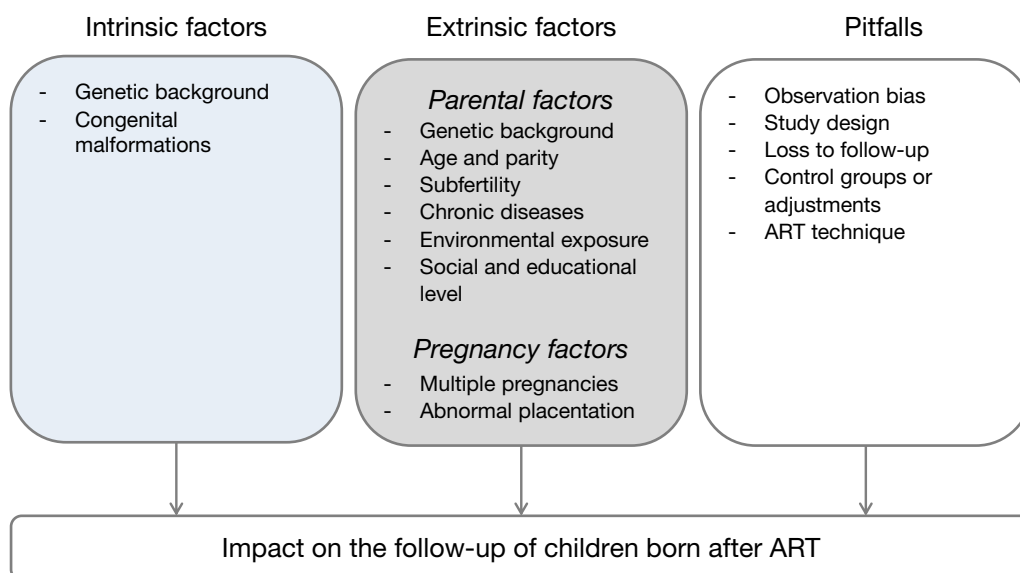


Fig. 1 Factors impacting the follow-up of children born after ART.

socio-economic status, chronic disease, smoking habits) and all of the factors related to the pregnancy (parity, obstetrical disease and, term of delivery).

In addition, very long follow-up periods needed to identify health problems such as metabolic disorders and cancers and thus led inevitably to surveillance bias.

Moreover, even though the practice of transferring single embryos is increasing, multiple pregnancy rates are higher in the context of ART. It is therefore extremely important to investigate the risk of adverse outcomes in singleton pregnancies only (Fig. 1).

The potential increased risks in ART infants could be a consequence of the ART procedure or inherent to the infertility problems per se. In humans, it is quite challenging to distinguish the responsibility of each of these factors. In addition, as several individualized methods are used during an ART procedure, it is difficult to isolate the impact of each technique.

Long-Term Health Outcomes

Growth and Pubertal Development

Several cohort studies have examined the growth and weight patterns of ART-conceived children, and have revealed conflicting results. Indeed, even though a few studies have reported that IVF children were significantly taller (Pruksananonda, 2001; Miles et al., 2007), the majority stated no significant difference in the growth of ART-conceived children versus naturally conceived (NC) children.

A study which followed children up to 3 years old found similar growth and development in ART and NC children and in multiple and singleton pregnancies (Yeung et al., 2016). These results have been confirmed in assessments at 8 years old (Belva et al., 2007) and 12 years old (Basatemur et al., 2010). However, the rare longitudinal studies reported a lower weight and height for ART-infants aged less than 6 months old and a growth catch-up at 3 (Ceelen et al., 2009) or 4 years old (Woldringh et al., 2011). These studies remain difficult to conduct as it is challenging to eliminate all confounding factors known to be associated with impaired childhood growth. Indeed, most studies included children that were born prematurely, small for gestational age (SGA) or born following multiple pregnancies, which are frequently associated with ART.

Concerning pubertal development, Ceelen et al. (2008a,b) found an advanced bone age and increased DHEAS and LH concentrations in pubertal IVF singleton girls whereas the pubertal timing was comparable with that in controls born to subfertile parents. Sakka et al. also found the increased DHEAS concentrations but only in SGA children (Sakka et al., 2010). However, the menarche, genital development in males and pubic hair development in males and females were similar in singleton ICSI- and NC-children (Belva et al., 2012).

General Health and Surgery

As recently reviewed, conflicting findings were also reported on the general health of ART-children (Kettner et al., 2015). Indeed, some studies did not find an increased risk of childhood illnesses (Pinborg et al., 2003; Place and Englert, 2003; Bonduelle et al., 2004; Belva et al., 2007; Knoester et al., 2008; Beydoun et al., 2010) or the consumption of hospital services (Belva et al., 2007; Knoester et al., 2008) up to the age of 5 years old for ART-infants as compared with NC-children. However, Hansen et al. reported relevant differences regarding the incidence of childhood illnesses, and hospital admissions up to the age of 3 years (Hansen et al., 2008). Later, another team confirmed that IVF-conceived children had more hospitalizations, and for a longer duration, than NC-children (Koivurova et al., 2007). Concerning childhood surgery, two studies found equal incidences in children conceived by ART and in NC-children (Pinborg et al., 2003; Ludwig et al., 2009). Moreover, a study based on questionnaires completed by young adults found that they were healthy and without any increased susceptibility to chronic diseases (Beydoun et al., 2010). However, an increased risk of urogenital surgery was observed among boys conceived by ART (Ludwig et al., 2009) but this was not confirmed by Knoester et al. (2008).

Vascular and Metabolic Disorders

Several studies have reported subclinical but significant cardiovascular changes in ART children compared to controls. Systemic blood pressure levels were reportedly higher among children conceived by IVF or ICSI than among NC-infants (Belva et al., 2007; Ceelen et al., 2008a,b) even after adjustments for birth weight, gestational age and the height of the children (Ceelen et al., 2008a,b). Similar results were observed in a small case-controlled series of IVF-conceived children (Sakka et al., 2010). In contrast, Belva et al. did not detect any difference even for resting blood pressure or after mild psychological stress in a cohort of 150 ICSI-conceived children as compared to 147 controls (Belva et al., 2007).

However, the studies on systemic and pulmonary vascular functions revealed significant differences between ART- and NC-offspring. Indeed, Sherrer et al. reported a 25% smaller flow-mediated dilation of the brachial artery, a faster pulse wave velocity, and a thicker carotid intima-media in ART-conceived children than in controls. Similarly, pulmonary artery pressure at high altitude was significantly higher (+30%) in ART offspring (Scherrer et al., 2012). Other studies also reported suboptimal cardiac diastolic function in the IVF-ICSI group, especially under identical stressful conditions (Valenzuela-Alcaraz et al., 2013; Zhou et al., 2014; von Arx et al., 2015; Liu et al., 2015). All of these data suggest that ART children may have underlying endothelial dysfunction (Fig. 2).

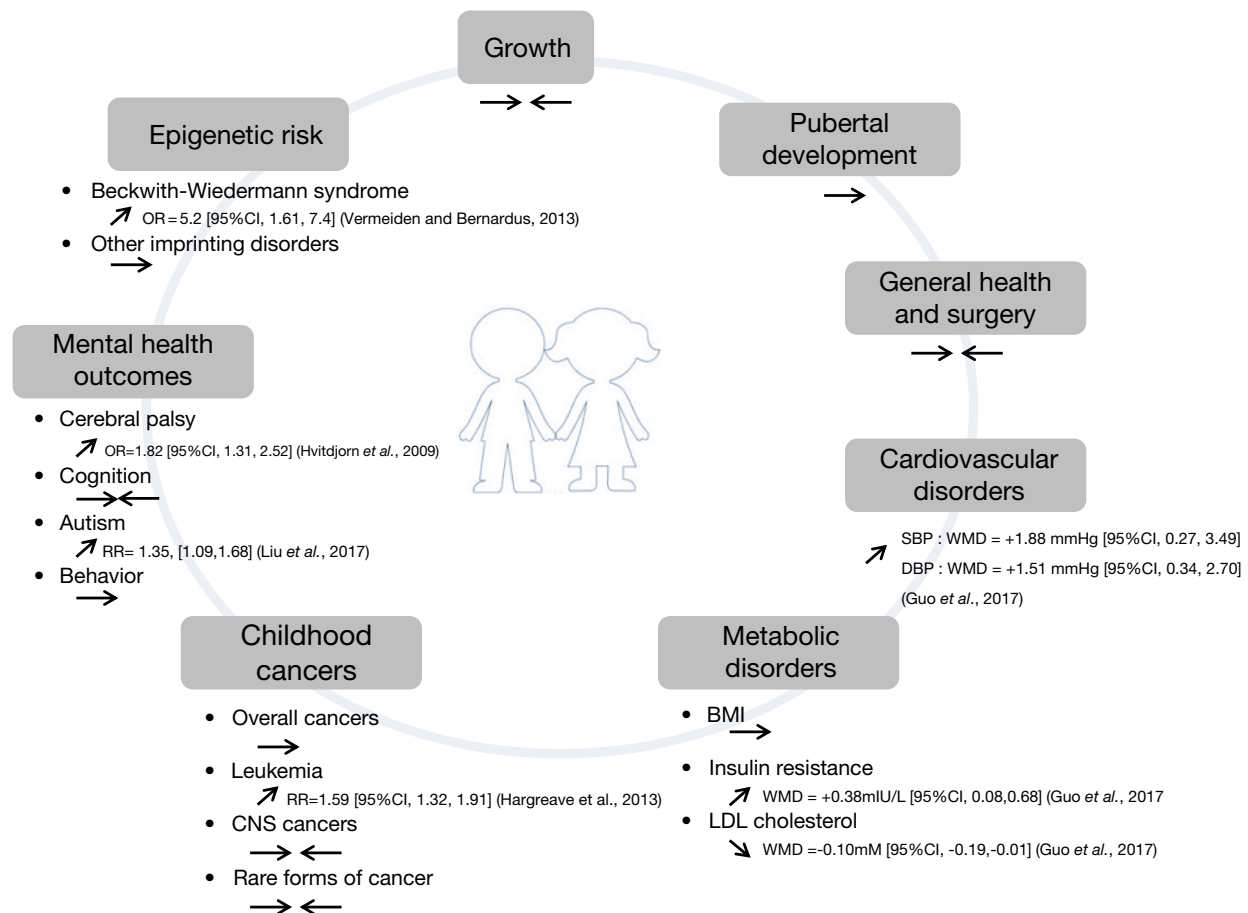


Fig. 2 Overview of health in ART infants as compared to controls (naturally conceived infants). → No effect. ↘ Decreased, with odds ratio (OR), relative risk (RR) or weighted mean difference (WMD). ↗ Increased, with odds ratio (OR), relative risk (RR) or weighted mean difference (WMD). → ← Discordant results. DBP, diastolic blood pressure; SBP, systolic blood pressure.

Concerning metabolic disorders, although IVF–ICSI offspring displayed comparable BMI (-0.04 kg/m^2 , $P = .20$), differences in body composition and signs of metabolic dysregulation were noted in ART-conceived children and adolescents (Guo et al., 2017). Ceelen et al. reported that IVF children had significantly more peripheral body fat assessed by skinfold thickness and dual-energy X-ray absorptiometry (DXA), even after adjustment for antenatal and parental factors, and despite minimal differences in BMI as compared to children born to subfertile parents (Ceelen et al., 2008a,b, 2009). Similar observations were made in pubertal ICSI singleton girls (Belva et al., 2012). Specifically, ICSI-girls aged 14 years old had increased peripheral, central, and total adiposity, while only increased peripheral adiposity was observed in ICSI singleton boys at an advanced pubertal stage. Conversely, Miles et al. found no difference in fat percentage by DXA between 4 and 10 year-old IVF children and NC controls (Miles et al., 2007).

Additionally, some reports have suggested that ART may also impair glucose metabolism, potentially as a result of increased adiposity. Two studies reported higher fasting glucose levels (Ceelen et al., 2008a,b; Pontesilli et al., 2015) and one, a lower peripheral insulin sensitivity in ART-conceived children (Chen et al., 2014) (Fig. 2). However, all of the other cohort studies did not report any difference in glucose and insulin levels according to the mode of conception (Sakka et al., 2010; Scherrer et al., 2012; Green et al., 2013; Chen et al., 2014).

Concerning lipids profiles, the results are reassuring as lower LDL cholesterol levels were reported in IVF children (weighted mean difference -0.10 mM ; 95%CI $[-0.19, -0.01]$) and triglycerides and HDL/total cholesterol levels were similar to those in controls (Guo et al., 2017).

Childhood Cancers

Most cohort studies that examined the cancer risk in ART children demonstrated that ART procedures were not associated with an increased risk of cancers overall (Reigstad et al., 2017). For instance, one data linkage study that included 25,782 ART children with a median follow-up of 6.9 years showed that ART children did not have a significantly increased incidence of cancer (Reigstad et al., 2016). A similar conclusion was reported in a Nordic cohort study over a longer follow-up (in 91,796 ART children over an average follow-up of 9.5 years—Sundh et al., 2014) as well as in an Israeli study (9042 ART children over a median follow-up of 9.3

years—Lerner-Geva et al., 2017). Only one of the larger cohort studies detected a significantly higher risk of overall cancer in children conceived by ART (OR 1.42; 95% CI [1.09, 1.87]) (Kallen et al., 2010). However, some studies have reported an increased risk of specific cancer types such as hematological, neurological, and rare cancers.

The two metaanalyses based on 25 and 23 cohort studies concluded that there was a higher risk of hematological cancers in children conceived after ART (Fig. 2) (Hargreave et al., 2013; Reigstad et al., 2017).

Data on neurological cancers are scarce. However, a large retrospective study reported an increased risk in ART children for central nervous system (CNS) tumors (adjusted HR 1.44; 95%CI [1.01, 2.05]) and malignant epithelial neoplasms (adjusted HR 2.03; 95%CI [1.06, 3.89]), after adjusting for country, maternal age, parity, year of birth, sex, gestational age, and birth defects (Sundh et al., 2014). Conversely, these findings were not confirmed by two other large cohort studies (Williams et al., 2013; Reigstad et al., 2016). However, as CNS cancer is most frequent in older children and adolescents, a longer follow-up is needed.

A few case reports have indicated a potential increased risk of retinoblastoma and hepatoblastoma in ART children (Marees et al., 2009; Williams et al., 2013; Lerner-Geva et al., 2017). However, it is difficult to draw any conclusions on these rare forms of cancer. In addition, some of these studies lacked statistical power and were limited by a short follow-up.

Mental Health Outcomes

Cerebral palsy (CP) is a rare but serious neuromuscular disorder that affects the development of movement and posture, with a prevalence of about 2 per 1000 live births. In several studies, a higher risk of CP was reported following ART (Pinborg et al., 2003; Lidegaard et al., 2005; Hvidtjørn et al., 2006, 2009; Klemetti et al., 2006; Goldsmith et al., 2017). The authors concluded that this could be explained by a higher frequency of preterm birth, multiple pregnancies or vanishing embryos in ART pregnancies (Hvidtjørn et al., 2005, 2009; Pinborg et al., 2005). As Zhu et al. did not find any effect of time to pregnancy on the risk of CP they concluded that the effect of ART treatments could be more important than that of parental subfertility. One metaanalysis on singletons reported an increased risk of CP after ART (OR 1.82; 95%CI [1.31, 2.52]) (Hvidtjørn et al., 2009).

The last review on cognitive outcomes in children conceived following ART reported discrepant results among five high-quality studies (Rumbold et al., 2017). Indeed, three studies found no difference (Pinborg et al., 2003; Wagenaar et al., 2009; Bay et al., 2013b) whereas two studies reported poorer cognitive ability in ART infants than in NC infants (Strömberg et al., 2002; Sandin et al., 2013).

Moreover, among studies that assessed the impact of the mode of in vitro fertilization and rated as high quality, one out of three found a 50% increase in the risk of mental retardation in children conceived by ICSI as compared with children conceived with IVF (Sandin et al., 2013).

Concerning the association between the use of ART and the risk of autism spectrum disorders (ASD), several studies, either case-control or cohort studies, have been inconsistent in their findings. Nevertheless, the recent review of Liu et al., which summarized 11 previous studies involving more than 8 million patients, concluded that ART is likely to be an independent risk factor for ASD in ART children ($RR = 1.35$; 95%CI [1.09, 1.68]) (Liu et al., 2017).

However, a recent review of the literature provided reassuring evidence on socio-emotional, cognitive, behavioral, and psychomotor development for preschool and middle childhood ART children (Bay et al., 2013a). Nevertheless, very few studies have assessed this development among teens, and their results were also inconsistent. On the other hand, Ilioi et al. reported that IVF-conceived adolescents did not show differences in emotional or behavioral problems as compared with NC controls (Ilioi and Golombok, 2015) (Fig. 2).

Epigenetic Risk

Even though contradictory findings exist, several reports pointed toward an increased risk of diseases caused by abnormal genomic imprinting. Indeed, the relative risk for the birth of a child with Beckwith–Wiedemann syndrome was 5.2 (95%CI [1.6, 7.4]) following ART compared with the risk in the normal population (Fig. 2). This excess of imprinting disorders following ART, as well as the fact that all ART-BWS cases typically occur through loss of DNA methylation in imprinting control regions raises the issue of a potential epigenetic risk induced by ART techniques. Concerning Silver–Russell syndrome, as it is extremely rare, it is difficult to draw any conclusions, but a positive association with IVF or ICSI treatment is likely. No significant associations were found between the incidence of Angelman and Prader–Willi syndromes and IVF or ICSI treatments. Fertility problems could be involved in these latter syndromes in ART children.

Conclusion

In summary, this review underlines discrepancies between results in the various health domains studied, making it difficult to draw reliable conclusions. The majority of studies did not have a robust methodology and included several potential confounders associated with the studied populations (Fig. 1). These may be due to differences in the ages investigated, the study period and design, the inclusion criteria of subjects and the comparison group, sample size, parental characteristics, and the ART technique employed. Thus, it is challenging to interpret and analyze the data as they are heterogeneous and prone to various biases.

Nonetheless, it seems that individuals conceived by ART may have an increased risk of cardiovascular and metabolic diseases, hematological cancers, cerebral palsy, and autism. Other health issues found in childhood, such as growth and pubertal development, need to be investigated further. It is also crucial to have studies with a longer follow-up and continuing into adulthood to assess the risk of late-onset diseases (such as oncogenic, cardiovascular, metabolic, and neurological diseases) and fertility. It is still unresolved whether the ART procedures or subfertility led to some of these changes. In addition, ART includes the use of several techniques such as ovarian stimulation, embryo culture (short or extended embryo culture up to the blastocyst stage, type of culture medium) and embryo transfer (fresh or frozen embryos). Notably, the culture medium (Zandstra et al., 2015; Bouillon et al., 2016) and the transfer of frozen embryos (Pinborg et al., 2014) could have an impact on birth weight or developmental age. It is thus important to distinguish their respective impact on the health of infants thus conceived.

Moreover, some of these changes could be related to multiple pregnancies and low birth weight, which are more frequent in ART pregnancies and associated with an increased risk of adverse obstetric and neonatal outcomes. In recent decades, it has been reported that these environmental changes during early development could have adverse effects on health in later life. Thus, further prospective, large and high-quality studies in the long term are necessary to ensure that ART methods, which are recognized as being effective, are also safe for the future health of adults and for future generations.

References

- von Arx, R., et al. (2015). Right ventricular dysfunction in children and adolescents conceived by assisted reproductive technologies. *Journal of Applied Physiology*, 118(10), 1200–1206.
- Basatemur, E., Shevlin, M., & Sutcliffe, A. (2010). Growth of children conceived by IVF and ICSI up to 12 years of age. *Reproductive Biomedicine Online*, 20(1), 144–149.
- Bay, B., Mortensen, E. L., & Kesmodel, U. S. (2013a). Assisted reproduction and child neurodevelopmental outcomes: A systematic review. *Fertility and Sterility*, 100(3), 844–853.
- Bay, B., et al. (2013b). Fertility treatment and risk of childhood and adolescent mental disorders: Register based cohort study. *BMJ*, 347, f3978.
- Belva, F., et al. (2007). Medical outcome of 8-year-old singleton ICSI children (born ≥ 32 weeks' gestation) and a spontaneously conceived comparison group. *Human Reproduction*, 22(2), 506–515.
- Belva, F., et al. (2012). Pubertal development in ICSI children. *Human Reproduction*, 27(4), 1156–1161.
- Beydoun, H. A., et al. (2010). A cross-sectional evaluation of the first cohort of young adults conceived by in vitro fertilization in the United States. *Fertility and Sterility*, 94(6), 2043–2049.
- Bonduelle, M., et al. (2004). Medical follow-up study of 5-year-old ICSI children. *Reproductive Biomedicine Online*, 9(1), 91–101.
- Bouillon, C., et al. (2016). Does embryo culture medium influence the health and development of children born after in vitro fertilization? *PLoS One*, 11(3), e0150857.
- Ceelen, M., et al. (2008a). Cardiometabolic differences in children born after in vitro fertilization: Follow-up study. *The Journal of Clinical Endocrinology & Metabolism*, 93(5), 1682–1688.
- Ceelen, M., et al. (2008b). Pubertal development in children and adolescents born after IVF and spontaneous conception. *Human Reproduction*, 23(12), 2791–2798.
- Ceelen, M., et al. (2009). Growth during infancy and early childhood in relation to blood pressure and body fat measures at age 8–18 years of IVF children and spontaneously conceived controls born to subfertile parents. *Human Reproduction*, 24(11), 2788–2795.
- Chen, M., et al. (2014). Altered glucose metabolism in mouse and humans conceived by IVF. *Diabetes*, 63(10), 3189–3198.
- Goldsmith, S., et al. (2017). Cerebral palsy after assisted reproductive technology: A cohort study. *Developmental Medicine and Child Neurology*. <https://doi.org/10.1111/dmcn.13577>.
- Green, M. P., et al. (2013). Phenotypic differences in children conceived from fresh and thawed embryos in vitro fertilization compared with naturally conceived children. *Fertility and Sterility*, 99(7), 1898–1904.
- Guo, X.-Y., et al. (2017). Cardiovascular and metabolic profiles of offspring conceived by assisted reproductive technologies: A systematic review and meta-analysis. *Fertility and Sterility*, 107(3), 622–631.e5.
- Hansen, M., et al. (2008). Admission to hospital of singleton children born following assisted reproductive technology (ART). *Human Reproduction*, 23(6), 1297–1305.
- Hansen, M., et al. (2013). Assisted reproductive technology and birth defects: A systematic review and meta-analysis. *Human Reproduction Update*, 19(4), 330–353.
- Hargreave, M., et al. (2013). Fertility treatment and childhood cancer risk: A systematic meta-analysis. *Fertility and Sterility*, 100(1), 150–161.
- Hvidtjorn, D., et al. (2005). "Vanishing embryo syndrome" in IVF/ICSI. *Human Reproduction*, 20(9), 2550–2551.
- Hvidtjorn, D., et al. (2006). Cerebral palsy among children born after in vitro fertilization: The role of preterm delivery—A population-based, cohort study. *Pediatrics*, 118(2), 475–482.
- Hvidtjorn, D., et al. (2009). Cerebral palsy, autism spectrum disorders, and developmental delay in children born after assisted conception: A systematic review and meta-analysis. *Archives of Pediatrics & Adolescent Medicine*, 163(1), 72–83.
- Hyrapietian, M., Loucaides, E. M., & Sutcliffe, A. G. (2014). Health and disease in children born after assistive reproductive therapies (ART). *Journal of Reproductive Immunology*, 106(Supplement C), 21–26.
- Ilioi, E. C., & Golombok, S. (2015). Psychological adjustment in adolescents conceived by assisted reproduction techniques: A systematic review. *Human Reproduction Update*, 21(1), 84–96.
- Kallen, B., et al. (2010). Cancer risk in children and young adults conceived by in vitro fertilization. *Pediatrics*, 126(2), 270–276.
- Kettner, L. O., et al. (2015). Assisted reproductive technology and somatic morbidity in childhood: A systematic review. *Fertility and Sterility*, 103(3), 707–719.
- Klemetti, R., et al. (2006). Health of children born as a result of in vitro fertilization. *Pediatrics*, 118(5), 1819–1827.
- Knoester, M., et al. (2008). Perinatal outcome, health, growth, and medical care utilization of 5- to 8-year-old intracytoplasmic sperm injection singletons. *Fertility and Sterility*, 89(5), 1133–1146.
- Koivurova, S., et al. (2007). Post-neonatal hospitalization and health care costs among IVF children: A 7-year follow-up study. *Human Reproduction*, 22(8), 2136–2141.
- Lerner-Geva, L., et al. (2017). Possible risk for cancer among children born following assisted reproductive technology in Israel: Lerner-geva et al. *Pediatric Blood & Cancer*, 64(4), e26292.
- Lidegaard, Ø., Pinborg, A., & Andersen, A. N. (2005). Imprinting diseases and IVF: Danish national IVF cohort study. *Human Reproduction*, 20(4), 950–954.
- Liu, H., et al. (2015). Association between assisted reproductive technology and cardiac alteration at age 5 years. *JAMA Pediatrics*, 169(6), 603–605.
- Liu, L., et al. (2017). Association between assisted reproductive technology and the risk of autism spectrum disorders in the offspring: A meta-analysis. *Scientific Reports*, 7, 46207.
- Ludwig, A. K., et al. (2009). Physical health at 5.5 years of age of term-born singletons after intracytoplasmic sperm injection: Results of a prospective, controlled, single-blinded study. *Fertility and Sterility*, 91(1), 115–124.
- Marees, T., et al. (2009). Incidence of retinoblastoma in Dutch children conceived by IVF: An expanded study. *Human Reproduction*, 24(12), 3220–3224.

- McDonald, S. D., et al. (2005). Perinatal outcomes of singleton pregnancies achieved by in vitro fertilization: A systematic review and meta-analysis. *Journal of Obstetrics and Gynaecology Canada*, 27(5), 449–459.
- Miles, H. L., et al. (2007). In vitro fertilization improves childhood growth and metabolism. *The Journal of Clinical Endocrinology & Metabolism*, 92(9), 3441–3445.
- Pandey, S., et al. (2012). Obstetric and perinatal outcomes in singleton pregnancies resulting from IVF/ICSI: A systematic review and meta-analysis. *Human Reproduction Update*, 18(5), 485–503.
- Pinborg, A., et al. (2003). Morbidity in a Danish national cohort of 472 IVF/ICSI twins, 1132 non-IVF/ICSI twins and 634 IVF/ICSI singletons: Health-related and social implications for the children and their families. *Human Reproduction*, 18(6), 1234–1243.
- Pinborg, A., et al. (2005). Consequences of vanishing twins in IVF/ICSI pregnancies. *Human Reproduction*, 20(10), 2821–2829.
- Pinborg, A., et al. (2014). Large baby syndrome in singletons born after frozen embryo transfer (FET): Is it due to maternal factors or the cryotechnique? *Human Reproduction*, 29(3), 618–627.
- Place, I., & Englert, Y. (2003). A prospective longitudinal study of the physical, psychomotor, and intellectual development of singleton children up to 5 years who were conceived by intracytoplasmic sperm injection compared with children conceived spontaneously and by in vitro fertilization. *Fertility and Sterility*, 80(6), 1388–1397.
- Pontesilli, M., et al. (2015). Subfertility and assisted reproduction techniques are associated with poorer cardiometabolic profiles in childhood. *Reproductive Biomedicine Online*, 30(3), 258–267.
- Pruksananonda, C. (2001) Growth and development of children conceived by intracytoplasmic sperm injection at King Chulalongkorn Memorial Hospital, Journal of the Medical Association of Thailand, 84 Suppl 1, pp. S76–85.
- Reigstad, M. M., et al. (2016). Risk of cancer in children conceived by assisted reproductive technology. *Pediatrics*, 137(3), e20152061.
- Reigstad, M. M., et al. (2017). Literature review on cancer risk in children born after fertility treatment suggests increased risk of haematological cancers. *Acta Paediatrica*, 106(5), 698–709.
- Rimm, A. A., et al. (2004). A meta-analysis of controlled studies comparing major malformation rates in IVF and ICSI infants with naturally conceived children. *Journal of Assisted Reproduction and Genetics*, 21(12), 437–443.
- Rumbold, A. R., et al. (2017). The impact of specific fertility treatments on cognitive development in childhood and adolescence: A systematic review. *Human Reproduction*, 32(7), 1489–1507.
- Sakka, S. D., et al. (2010). Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. *Fertility and Sterility*, 94(5), 1693–1699.
- Sandin, S., et al. (2013). Autism and mental retardation among offspring born after in vitro fertilization. *JAMA*, 310(1), 75–84.
- Scherrer, U., et al. (2012). Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation*, 125(15), 1890–1896.
- Strömberg, B., et al. (2002). Neurological sequelae in children born after in-vitro fertilisation: A population-based study. *The Lancet*, 359(9305), 461–465.
- Sundh, K. J., et al. (2014). Cancer in children and young adults born after assisted reproductive technology: A Nordic cohort study from the Committee of Nordic ART and Safety (CoNARTaS). *Human Reproduction*, 29(9), 2050–2057.
- Valenzuela-Alcaraz, B., et al. (2013). Assisted reproductive technologies are associated with cardiovascular remodeling in utero that persists Postnatally Clinical perspective. *Circulation*, 128(13), 1442–1450.
- Wagenaar, K., et al. (2009). Information processing, attention and visual-motor function of adolescents born after in vitro fertilization compared with spontaneous conception. *Human Reproduction*, 24(4), 913–921.
- Wen, J., et al. (2012). Birth defects in children conceived by in vitro fertilization and intracytoplasmic sperm injection: A meta-analysis. *Fertility and Sterility*, 97(6), 1331–1337.e4.
- Williams, C. L., et al. (2013). Cancer risk among children born after assisted conception. *New England Journal of Medicine*, 369(19), 1819–1827.
- Woldringh, G. H., et al. (2011). Weight of in vitro fertilization and intracytoplasmic sperm injection singletons in early childhood. *Fertility and Sterility*, 95(8), 2775–2777.
- Yeung, E. H., et al. (2016). Infertility treatment and children's longitudinal growth between birth and 3 years of age. *Human Reproduction*, 31(7), 1621–1628.
- Zandstra, H., et al. (2015). Does the type of culture medium used influence birth weight of children born after IVF? *Human Reproduction*, 30(3), 530–542.
- Zhou, J., et al. (2014). Association of Cardiac Development with assisted reproductive technology in childhood: A prospective single-blind pilot study. *Cellular Physiology and Biochemistry*, 34(3), 988–1000.

Ovarian Stimulation and the Long-Term Risk of Cancer

Enes Taylan and Kutluk Oktay, Innovation Institute for Fertility Preservation and In Vitro Fertilization, New York, NY, United States; and Yale University School of Medicine, New Haven, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Assisted reproductive technologies (ART) All technological tools, surgical procedures, and medical treatment modalities used to achieve pregnancy.

Cryopreservation A specific procedure in which various chemical solutions and anti-freezing molecules are used to preserve cells or tissues by cooling to very low temperatures below zero.

Gamete cryopreservation Cryopreservation of egg or sperm cells.

Gonadal tissue cryopreservation Cryopreservation of ovarian or testicular tissue.

In vitro fertilization (IVF) The process in which an oocyte and a sperm are fertilized outside of the body.

Intracytoplasmic sperm injection (ICSI) An advanced IVF technique in which a single sperm is directly injected into an oocyte cytoplasm.

Ovarian stimulation An ART technique introduced to induce development of multiple ovarian follicles using various fertility medications.

Preimplantation genetic diagnosis A genetic profiling technique used to screen embryos for specific genetic diseases prior to implantation.

Introduction

In 1972, for the first time, Patrick Steptoe and Robert Edwards developed a human embryo in a petri dish. In the next four decades, assisted reproductive technologies (ART) resulted in more than 6 million children born around the globe. For achieving a healthy pregnancy, ART consists of a numerous procedure including ovarian stimulation, in vitro fertilization (IVF), intra-cytoplasmic sperm injection (ICSI), gamete and embryo cryopreservation, gonadal tissue cryopreservation, and preimplantation genetic diagnosis. All these methods usually require stimulation of ovaries using special medications as an initial step towards a desired pregnancy. The goal of ovarian stimulation is to induce development of multiple follicles with mature oocytes in order to improve the chances obtaining a viable embryo. For this purpose, several fertility drugs have been introduced and utilized in the treatment of thousands of women with fertility issues including those with cancer history. Hence, it has been questioned whether inducing multiple ovulations by fertility drugs and alterations in endogenous sex steroid levels may have an association with an increased risk of developing cancer.

In this article, we review the current knowledge examining the relation between the use of fertility drugs for ovarian stimulation and the risk of cancer. In the first section, we summarize the available data on the risk of cancer occurrence after use of fertility drugs, and in the second section we evaluate the risk of recurrence in women with history of cancer.

Long-Term Risk of Cancer After Ovarian Stimulation With Fertility Drugs

The concept of ovarian stimulation using fertility drugs such as clomiphene citrate or gonadotropins is of the main premise behind infertility treatments. Fertility drugs can be used to restore cyclic ovulatory function in anovulatory infertile women or to stimulate development of multiple ovarian follicles for higher oocyte yield in IVF treatments. According to most recent statistical reports, approximately one million IVF cycles and even larger numbers of ovulation induction cycles are being performed every year in the United States. While the use of fertility drugs is becoming increasingly common, some clinical studies suggested a possible link between fertility drugs and various types of cancers such as breast, endometrium, ovary, cervix, colon, and thyroid (**Table 1**).

Breast Cancer

Breast cancer is the most commonly diagnosed cancer and is the leading cause of cancer-related death in women in the world. According to National Cancer Institute's SEER database from 2007 to 2011 on average more than 25,000 women are diagnosed with breast cancer before reaching the age of 45 years, each year in the United States. Although the central underlying etiological factors in the development of breast cancer are not fully understood, it is well acknowledged that there are multiple genetic and environmental factors that are predisposing. Advanced age, ethnicity, BRCA gene mutations, family history of breast cancer, and ionizing radiation are known risk factors. Among the others, exposure to estrogen has been suggested as a primary promoter of

Table 1 Currently available evidence for the suggested link between fertility drugs and various types of cancer

<i>Fertility medications</i>	<i>Drug effect</i>	<i>Investigated malignancies</i>	<i>Current evidence</i>
• Clomiphene citrate • Gonadotropins (rFSH, rLH, HMG)	• Multiple ovulation • Increased serum estradiol • Increased serum progesterone	• Breast cancer • Endometrial cancer • Ovarian cancer • Cervical cancer • Colon cancer • Malignant melanoma • Thyroid cancer • Non-Hodgkin lymphoma	No sufficient evidence for increased risk with fertility drugs

FSH, Follicle stimulating hormone; *rFSH*, recombinant FSH; *LH*, luteinizing hormone; *rLH*, recombinant LH; *HMG*, human menopausal gonadotropin.

breast cancer. During ovarian stimulation cycle, along with multiple developing follicles in the ovaries, endogenous production of estrogen is increased on average up to 10 times higher level than the levels in natural menstrual cycle. Therefore, stimulation of ovaries using fertility medications has been argued as an emerging risk factor for breast cancer development. However, ovarian stimulation is also associated with an increase in exposure to progesterone hormone via multiple ovulations. Parity, which is characterized as a state of high progesterone levels, is associated with a lower risk of breast cancer, hence some researchers proposed progestogens as effective treatment options in breast cancer. On the other hand, in some experimental studies, progesterone was reported to drive tumoral cell proliferation in breast cancer cell lines. Regardless of the controversial role of progesterone in breast cancer, it should be noted that fertility drug in single cycle use results in high levels of hormones for a short period of time and prolonged exposure must involve numerous cycles of fertility drugs.

Several studies have investigated the relationship between fertility drugs and breast cancer. The majority of those investigations, including large cohort studies with up to 30 years of follow-up, have either shown no significant increase in the risk of breast cancer or a decrease in risk following infertility treatments when compared with either women with infertility who did not undergo treatment with fertility medications or the general population. Based on the current evidence, fertility drugs appear not to be associated with a significant risk of breast cancer.

Gynecological Cancers (Endometrium, Cervix, and Ovary)

Endometrial cancer is the most common uterine cancer and its association with unopposed estrogen exposure is well established whereas progesterone is reported to be a protective factor. Hence, it is plausible to suggest that fertility drugs could either increase the incidence of endometrial cancer due to increased estrogen production or decrease the incidence of endometrial cancer secondary to the protective effect of progesterone released following ovulation during luteal phase. However, studies addressing the risk of endometrial cancer after use of fertility medications are also limited by methodological issues. First, infertility has been shown to be an independent risk factor for endometrial cancer regardless of fertility drug use. Second, most cohort studies reported on small numbers of women, short or incomplete follow-up, and inadequate methods to control for potential confounders such as anovulation, hormonal therapy, metabolic syndrome, and hysterectomy. Nevertheless, the majority of studies and meta-analyses showed that the overall use of fertility drugs, specifically clomiphene citrate, gonadotropins, and IVF treatment in general, is not associated with a significant increased risk for endometrial cancer.

Among all gynecological malignancies, ovarian cancer is the deadliest cancer and accounts for about 3% of all cancers in women, with approximately 20,000 cases diagnosed annually in the United States. Nulliparity is one of the main risk factors for the disease, hence women with infertility are considered to be at an increased risk for ovarian cancer. On the other hand, pregnancy and the use of ovarian suppressing medications such as oral contraceptive drugs have been observed to decrease the incidence of the disease. To be able to explain the biological mechanism behind the development of ovarian cancer, the theory of “incessant ovulation” has been proposed by Fathalla in 1971. This theory suggests that ovarian cancer primarily originates from the epithelium that covers ovarian surface and tumorigenesis is initiated by follicular rupture during ovulation which causes repeated damage and repair cycles in disrupted epithelial tissue. According to this hypothesis, years of prolonged and uninterrupted ovulation may increase the risk of

ovarian cancer by repetitive trauma to the epithelial cells. Following that logic, it was hypothesized that induction of multiple and repetitive ovulations with fertility drugs may increase the risk of ovarian cancer development. However, this long-standing theory has been recently challenged by the accumulating evidence that aggressive ovarian cancers originate in other pelvic organs and involve the ovary secondarily. The most recent studies revealed that the fallopian tube epithelium is the primary origin of high-grade serous ovarian cancers. Therefore, the incessant ovulation theory appears to have insufficient base to suggest a link between fertility drugs and ovarian cancer.

When the studies considering the relationship between fertility drugs and invasive ovarian cancer are analyzed, several methodological issues arise including lack of proper controls, failure to control confounding factors known to influence cancer risk, and lack of long-term follow up. Despite the earlier studies suggesting an association between fertility drugs and invasive ovarian cancer, recent investigations with better study design, as well as several systematic reviews and/or meta-analyses have shown no significant increase in the risk of invasive ovarian cancer following the use of fertility drugs when compared with infertile controls and/or with the general population.

Though there is insufficient evidence to establish a link between fertility drug use and invasive ovarian cancer, some studies have shown an association between the utility of fertility drugs and borderline ovarian tumors. Borderline ovarian tumors are of low malignant potential with a 95% 5-year-survival rate, and contrary to invasive ovarian cancers, they are more likely to be diagnosed in women of reproductive age. However, based on the available data, there is insufficient evidence to suggest that using fertility drugs increases the risk of borderline ovarian tumors.

Regarding cervical cancer, the second most common gynecological cancer, several studies found no increased risk with fertility drug use when compared to the general population as well as patients with infertility.

Other Types of Cancer

In addition to breast and gynecological cancers, researchers have also investigated whether use of fertility drugs may increase the risk of other types of cancer such as colon, thyroid, malignant melanoma, and non-Hodgkin lymphoma. Although in some experimental studies female sex steroids have been reported to cause altered tumoral progression in some of these cancers, majority of clinical studies have found no increase in the incidence of those cancers in patients undergoing IVF treatments compared to general population.

The Long-Term Risk of Recurrence in Cancer Patients After Ovarian Stimulation

Cancer is a global health problem and an important cause of morbidity and mortality in reproductive age women. Cancer treatments including chemotherapy and radiotherapy have been shown to damage ovarian follicles and cause premature ovarian failure. Additionally, increasing number of women delay childbearing due to various reasons that has made fertility preservation a major issue for young women with cancer. Hence, several treatment options for young women with cancer have been developed to preserve their future chance of having children. Embryo cryopreservation after IVF is currently considered as an established fertility preservation option, which offers the best chance of livebirth for women with a partner or single women who elect to use donor sperm. Cryopreservation of mature eggs is another fertility preservation option for women without a partner and those who not wishing to use donor sperm due to legal, ethical, or religious considerations. However, these established methods typically require controlled ovarian stimulation in order to obtain mature eggs. Since ovarian stimulation is known to elevate circulating estrogen and progesterone levels, it has been considered unsafe in women with estrogen-sensitive cancers.

Breast Cancer

The relation between breast cancer and estrogen is well-known and the major issue associated with the conventional ovarian stimulation protocols is elevated circulating estrogen levels due to the development of large number of follicles at once. Therefore, conventional stimulation treatments are considered unsafe in women with estrogen-sensitive breast cancer. As a response to these concerns, we have developed new and effective ovarian stimulation protocols for breast cancer patients using Tamoxifen and Letrozole, which are normally used in the endocrine treatment of estrogen sensitive cancer.

Tamoxifen is an estrogen receptor modulator and can be used either alone for ovulation induction or in combination with low dose gonadotropins for IVF. Letrozole is an aromatase inhibitor that reduces estrogen production by blocking the enzyme that converts androgens to estrogen. By reducing the circulating estrogen levels, aromatase inhibitors induce a surge in FSH production from the pituitary gland, which in turn stimulates ovarian follicle development. We found that letrozole in combination with gonadotropins can produce comparable or better IVF outcomes to conventional ovarian stimulation protocols while providing significantly lower estrogen levels and decreased gonadotropin requirements in premenopausal breast cancer patients. Furthermore, after short and mid-term follow up, we showed that letrozole-gonadotropin combination protocol was not associated with any reduction in disease free survival rates.

Gynecological Cancers

Gynecological cancers are commonly seen in the late stages of life; however, a significant number of affected women are of reproductive age. Young women are likely to present with earlier stage of gynecological cancer, which can be cured with fertility sparing

surgeries and treatments. For those women embryo and oocyte cryopreservation following ovarian stimulation are suitable options for fertility preservation. A currently experimental procedure, ovarian tissue cryopreservation and subsequent transplantation is also promising option for those who are not eligible for ovarian stimulation. Although current data on ovarian stimulation after gynecological cancer treatment is limited to very few case reports or case series, ART seems to not have any adverse effect on disease free survival. Furthermore, using letrozole protocol in ovarian stimulation may have protective effect in estrogen-sensitive gynecological cancers.

Other Types of Cancer

There is insufficient data regarding the use of fertility drugs and the recurrence of cancers other than breast and gynecological cancers.

Summary

Despite the plausibility of postulated biological mechanisms, several clinical studies examining the relationship between fertility drugs and cancer found conflicting results; some studies reporting a possible increased or decreased risk, while others showing no effect. It should be emphasized that examining the relationship between fertility drugs and cancer is a difficult task due to numerous confounding factors and methodological limitations. For example, while nulliparity is considered as a risk factor for development of breast, endometrial and ovarian cancer, successful fertility treatments should in theory reduce the risk of these cancers by enabling these women to become fertile. In addition, most studies lack of long-term follow-up to capture the age at which these diseases commonly occur. Another limitation is that the medications used in the treatment of infertility have changed over the past years, which makes the interpretation of long term data challenging. Finally, it is plausible that infertility and cancer may have shared pathways. We showed that women with BRCA mutations have accelerated ovarian aging. Hence the observations of cancer in an infertile population may simply be due to the factor that these two conditions are manifestations of the same underlying disorder, that is deficient DNA repair, rather than one causing the other.

In conclusion, controlled ovarian stimulation particularly in combination with aromatase inhibitor appears to be safe protocol for women who are concerned about the added estrogen exposure from fertility treatments. Current evidence suggests that infertile women may be at an increased risk for various types of cancer, however the use of fertility drugs for ovarian stimulation is not associated with a significant risk of occurrence or recurrence.

Further Reading

- Althuis, M. D., Scoccia, B., Lamb, E. J., et al. (2005). Melanoma, thyroid, cervical, and colon cancer risk after use of fertility drugs. *American Journal of Obstetrics and Gynecology*, 193, 668–674.
- Azim, A. A., Costantini-Ferrando, M., & Oktay, K. (2008). Safety of fertility preservation by ovarian stimulation with letrozole and gonadotropins in patients with breast cancer: A prospective controlled study. *Journal of Clinical Oncology*, 26, 2630–2635.
- van den Belt-Dusebout, A. W., Spaan, M., Lambalk, C. B., et al. (2016). Ovarian stimulation for in vitro fertilization and long-term risk of breast cancer. *JAMA*, 316, 300–312.
- Brinton, L. A., Scoccia, B., Moghissi, K. S., et al. (2014). Long-term relationship of ovulation stimulating drugs to breast cancer risk. *Cancer Epidemiology, Biomarkers & Prevention*, 23, 584–593.
- Calderon-Margalit, R., Friedlander, Y., Yanetz, R., et al. (2009). Cancer risk after exposure to treatments for ovulation induction. *American Journal of Epidemiology*, 169, 365–375.
- Ethics Committee of American Society for Reproductive Medicine. (2013). Fertility preservation and reproduction in patients facing gonadotoxic therapies: A committee opinion. *Fertility and Sterility*, 100, 1224–1231.
- Hannibal, C. G., Jensen, A., Sharif, H., & Kjaer, S. K. (2008). Risk of thyroid cancer after exposure to fertility drugs: Results from a large Danish cohort study. *Human Reproduction*, 23, 451–456.
- Kim, J., Turan, V., & Oktay, K. (2016). Long-term safety of letrozole and gonadotropin stimulation for fertility preservation in women with breast cancer. *The Journal of Clinical Endocrinology and Metabolism*, 101, 1364–1371.
- Loren, A. W., Mangu, P. B., Beck, L. N., et al. (2013). American Society of Clinical Oncology. Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. *Journal of Clinical Oncology*, 31, 2500–2510.
- Oktay, K., & Karlikaya, G. (2000). Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *The New England Journal of Medicine*, 342, 1919.
- Oktay, K., Buyuk, E., Davis, O., et al. (2003). Fertility preservation in breast cancer patients: IVF and embryo cryopreservation after ovarian stimulation with tamoxifen. *Human Reproduction*, 18, 90–95.
- Oktay, K., Buyuk, E., Veeck, L., et al. (2004). Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet*, 363, 837–840.
- Oktay, K., Hourvitz, A., Sahin, G., et al. (2006). Letrozole reduces estrogen and gonadotropin exposure in women with breast cancer undergoing ovarian stimulation before chemotherapy. *The Journal of Clinical Endocrinology and Metabolism*, 91, 3885–3890.
- Oktay, K., Turan, V., Titus, S., Stobezki, R., & Liu, L. (2015). BRCA mutations, DNA repair deficiency, and ovarian aging. *Biology of Reproduction*, 93(3), 67.
- Oktay, K., Bedoschi, G., Pacheco, F., Turan, V., & Emirdar, V. (2016). First pregnancies, live birth, and in vitro fertilization outcomes after transplantation of frozen-banked ovarian tissue with a human extracellular matrix scaffold using robot-assisted minimally invasive surgery. *American Journal of Obstetrics and Gynecology*, 214, 94.e1–94.e9.
- Oktay, K., Taylan, E., Sugishita, Y., & Goldberg, G. M. (2017). Robot-assisted laparoscopic transplantation of frozen-thawed ovarian tissue. *Journal of Minimally Invasive Gynecology*, 24, 897–898.
- Steptoe, P. C., Edwards, R. G., & Purdy, J. M. (1971). Human blastocysts grown in culture. *Nature*, 229, 132–133.
- Titus, S., Li, F., Stobezki, R., Akula, K., et al. (2013). Impairment of BRCA1-related DNA double-strand break repair leads to ovarian aging in mice and humans. *Science Translational Medicine*, 5, 172ra121.

- Turan, V., Bedoschi, G., Moy, F., & Oktay, K. (2013). Safety and feasibility of performing two consecutive ovarian stimulation cycles with the use of letrozole-gonadotropin protocol for fertility preservation in breast cancer patients. *Fertility and Sterility*, *100*, 1681–1685.e1.
- Zapardiel, I., Cruz, M., Diestro, M. D., Requena, A., & Garcia-Velasco, J. A. (2016). Assisted reproductive techniques after fertility-sparing treatments in gynaecological cancers. *Human Reproduction Update*, *22*, 281–285. Pii. dmv066.

Relevant Website

www.fertilitypreservation.org—Further information on fertility preservation options in cancer patients can be found online.

ECONOMICS

Cost-Effectiveness of High Versus Low Complexity ART Treatments

Nina Resetkova and Ashley Aluko, Beth Israel Deaconess Medical Center, Boston IVF, Waltham, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Cost effectiveness is an elusive topic within the pages of reproductive journals. Most clinicians and scientists have limited formal training in healthcare economics and often hesitate to read and interpret studies on cost effectiveness. In addition, many are skeptical of this area of research as conclusions can be based on variables chiefly selected to influence the desired outcome. However, there can be a lot of practical application drawn from well-constructed cost effectiveness studies. The examination of key issues within our field from the viewpoint of total cost to the system, to the patient, or to the clinic is a timely topic in this age of emerging new and often expensive technologies. This is especially relevant given the seemingly unending age of cuts to reimbursements in modern healthcare systems.

The purpose of this article is to compare the cost effectiveness of low and high complexity artificial reproductive technology (ART) treatment strategies. As new technologies and medications are brought to market, they should be critically compared to established therapies. In low resource settings in particular, healthcare costs are more trackable and have been frequently studied. However, with rising costs of care in developed nations, it is also important to consider the value proposition of ART innovations in these settings.

Although randomized controlled trials (RCT) are the gold standard for evaluating the efficacy of new treatments or interventions, they are labor and time intensive, and simply do not exist in certain subject areas. This article provides a broad overview of available studies of varying design and methodology, as these serve as a helpful initial study tool in evaluating the cost effectiveness of new treatments.

What is a Cost Effectiveness Analysis

Cost effectiveness analysis (CEA) is a form of economic analysis that compares the relative costs and outcomes of two or more courses of action. The most commonly used outcome measure is the quality adjusted life year (QALY). The QALY is a generic measure of health-related quality of life that takes into account both the quantity and quality of life generated by interventions.

Another important concept to understand when reading and interpreting CEA's is the ICER (incremental cost effectiveness ratio). An ICER is calculated to provide the net difference in costs divided by the net difference in benefits when the net costs and benefits are compared to a competing strategy. The concept of value is related to the ICER, since members of a society, social group, or workplace may place emphasis on certain metrics. For example, in some high resource countries efficacy is valued over cost effectiveness, and society is willing to pay more for a higher chance of cure, live birth, or other positive outcome.

CEA is often used in the field of health services, where it may seem inappropriate to monetize health effect. In contrast, cost benefit analysis (CBA) is favored in public services projects, such as the construction of a dam or road, where monetizing outcomes or effects is more straightforward. Notably, CBA does not take into account the quality of life of an individual. Other forms of economic analyses that may be applied in health care analysis include cost-utility analysis and direct analyses of associated costs.

Cost Effectiveness Analysis in Reproductive Medicine

In order to provide a methodical review of cost effectiveness studies in our field, we approached this article by grouping and analyzing studies according to diagnosis. You will find that certain topics have been more extensively studied than others, lending to more reliable input data (the probabilities entered into a cost effectiveness model). Generally speaking, the conclusions that can be generated from a CEA are as good as the quality of the data or assumptions that are made in constructing the model.

There are some research areas in which several papers have all come to similar conclusions, however there are other areas in which there has been extensive discussion with conflicting published results. Gaps in the topic areas discussed below reflect a lack of robust data, which logically extends that more research is needed to draw any further conclusions. We conclude with an overview of the cost-based studies performed in regions with emerging economies, the majority of which examine the necessity of intervention as compared to expectant management, or a low complexity intervention to one involving moderate to high complexity, such as an IVF laboratory. A review of strategies employed for cost containment in emerging economies can serve as a helpful reminder of which testing and treatments are truly essential.

Male Factor Infertility

Male factor infertility affects 15% of men and contributes to a couples' inability to conceive in approximately 50% of cases (Turek, n.d.). ART such as intrauterine insemination (IUI), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and artificial insemination with donor semen, has revolutionized treatment of male factor infertility. Though these technologies are widely utilized, there are few randomized controlled trials validating their cost effectiveness in the setting of male factor infertility.

Studies comparing the cost effectiveness of IUI and IVF in male factor subfertility and unexplained fertility yield conflicting results. Goverde et al. concluded that initiating fertility treatment with an IVF cycle is more cost effective than IUI followed by IVF (Goverde et al., 2000). On the contrary, Pashayan et al. found that IUI and IVF conferred a similar chance of a successful pregnancy, and deemed IUI the more cost effective approach (Pashayan et al., 2006). There are no clearly defined algorithms to determine which patients with male factor infertility are more likely to benefit from initial treatment with IUI or IVF, however it is logical that certain semen parameters portend a poor prognosis when IUI treatment is chosen as the first step. In a decision analysis model of couples with male subfertility, if the pre-wash total motile sperm count (TMSC) was over 3 million, the cost per live birth was lower for IUI with ovarian stimulation than for IVF. However, below a pre-wash TMSC of 3 million, IVF/ICSI treatment resulted in a lower cost per live birth (Moolenaar et al., 2015). This lends a helpful approach to treating couples with a mild male factor infertility when cost effectiveness is deemed a priority.

Fertility After Vasectomy

In patients who desire fertility after a vasectomy, vasectomy reversal is considered a less-costly alternative to IVF, especially when performed by a skilled microsurgeon. An early study on fertility options after vasectomy compared the cost per delivery after vasectomy reversal (\$25,475) and sperm retrieval plus ICSI (\$72,521). The authors calculated a delivery rate of 47% after a vasectomy and 33% after a single cycle of sperm retrieval and ICSI, and concluded that vasectomy reversal was more cost-effective (Pavlovich and Schlegel, 1997). This historical study, published in 1997, may have overestimated the cost and underestimated the effectiveness of ART, as innovations over the last 20 years have improved IVF outcomes and reduced procedural costs. Regardless, contemporary cost effectiveness data generated from decision analysis models still favors vasectomy reversal over ART (Robb and Sandlow, 2009; Lee et al., 2008; Meng et al., 2005). A 2005 study estimated the cost per delivery for vasectomy reversal versus sperm retrieval with ICSI to be \$38,983 versus \$39,506, respectively. In this study, in order for vasectomy reversal to be more cost effective than IVF with ICSI, a threshold patency rate of 78% had to be established. Parameters associated with higher patency rates include shorter obstructive interval and ability to perform bilateral vasovasostomy (Meng et al., 2005).

Although it appears as though vasectomy reversal is more cost effective than IVF, providers must carefully consider the female partners' reproductive capacity, interval of time that has passed since vasectomy, and the etiology of male factor infertility, as these factors play a role in the ability to conceive after a vasectomy reversal. Often, despite initial treatment with vasectomy reversal, couples with obstructive azoospermia due to vasectomy will go on to require IVF (Chiles and Schlegel, 2015). While vasectomy reversal can be considered a cost effective alternative to IVF, it is imperative to carefully select couples who would benefit most from this approach.

PCOS and Anovulation

Polycystic ovarian syndrome (PCOS) affects 5%–10% of reproductive aged women and is a major contributor to anovulatory infertility (Tarlitzis et al., 2008). Ovulation induction with clomiphene citrate (CC) is widely regarded as first line pharmacologic therapy due to low cost, ease of administration, favorable side effect profile, and infrequent need for ovarian response monitoring. Up to 80% of women with PCOS ovulate with CC, and of those who ovulate the conception rate is 22% per cycle (Tarlitzis et al., 2008). For patients who fail to conceive with CC, second-line options include exogenous gonadotrophins and laparoscopic ovarian surgery, with the latter performed less frequently due to potential complications of surgery (Tarlitzis et al., 2008). Administration of exogenous gonadotrophins for ovulation induction is more costly than CC and requires ovarian response monitoring to mitigate the risk of ovarian hyperstimulation syndrome (OHSS) and multiple gestation pregnancies (Eijkemans et al., 2005). In patients with anovulatory infertility who fail to conceive with CC, exogenous gonadotrophins, or laparoscopic ovarian surgery, IVF remains a viable, though significantly more costly, option (Tarlitzis et al., 2008).

Eijkemans et al. sought to develop an algorithm for an individualized strategy to treating anovulatory infertility in a cost effective manner. The authors determined that using a stepwise approach, with first CC, then gonadotrophins, and finally IVF was cost-effective for women less than 30 years old with normal androgen levels. In women over 30 years old with elevated androgen levels, proceeding with IVF (rather than gonadotropin treatment with IUI) when CC fails to produce conception was most cost effective (Eijkemans et al., 2005).

In a decision analysis model of women with PCOS who did not conceive after six cycles of CC, continuation of CC for six cycles followed by three cycles of IVF was the least costly scenario, with a two-year birth rate of 74%. Continuing CC for three cycles, followed by six to twelve cycles of gonadotropins then three cycles of IVF "in case of no live birth," was found to be more expensive, but also more efficacious (93%–98% live birth rate) (Moolenaar et al., 2014). One limitation of this study, and others within the area, is that it assumed a steady pregnancy rate over the course of IUI cycles, which does not mirror the attrition in success rates typically observed in clinical practice.

Aromatase inhibitors such as letrozole have been investigated for use in ovulation induction, either as an alternative to CC or for CC-resistant patients (Casper and Mitwally, 2012). An RCT comparing the cost effectiveness of letrozole and purified urinary FSH in CC-resistant PCOS demonstrated no difference in ovulation and clinical pregnancy rate, however FSH cycles were almost five times more expensive. Although in this study letrozole was found to be more cost effective than FSH in treating CC-resistant PCOS, further research on this topic is necessary (Hassan et al., 2017).

Tubal Factor

Options for women seeking fertility after tubal ligation include tubal anastomosis and IVF. Maternal age is the most important prognostic factor in pregnancy rates after tubal anastomosis (ASRM Committee Opinion, 2012), thus most studies on this topic stratify patients by age.

A recently published cost and efficacy comparison study found that tubal anastomosis was more cost effective than IVF for women under the age of 41. They reported that for women over age 40, tubal anastomosis remained the most cost effective option only when IVF costs exceeded \$30,000 per cycle. This particular study quoted the cost per pregnancy as \$16,315 (age < 35), \$23,914 (age 35–40), and \$218,742 (age > 40) after tubal anastomosis and \$32,814 (age < 35), \$45,839 (age 35–40), and \$114,445 (age > 40) after IVF (Messinger et al., 2015). This study focused on a United States-based population, however its re-analysis in different regions of the world would lead to valuable and likely differing conclusions based on varying inputs of cost of treatment, surgery, and IVF success rates.

Certain tubal ligation techniques, including those that do not require removing a large segment of fallopian tube, are more amenable to anastomosis than others. Hirschfield-Cytron et al. found that across all age groups, anastomosis after a favorable ligation technique (ex. clip, ring) was cost effective. In addition, tubal anastomosis, regardless of type of prior tubal ligation, was found to be more cost effective than IVF in all age groups, including women over age 40, as long as IVF costs exceed \$4500 (Hirschfeld-Cytron and Winter, 2013). These results contrast to the study by Messinger et al., which may reflect the differences in study design, as well as improved IVF success rates over the years between the studies were published.

While limited data suggests that tubal anastomosis is more cost effective than IVF, especially in younger women, there are no RCTs evaluating the relative cost effectiveness of each strategy. Treatment decisions should reflect an individualized approach because many parameters, specifically surgeon experience, maternal age, future pregnancy plans, and ovarian reserve, can impact the cost effectiveness of each technique.

Unexplained Infertility

Thirty percent of couples that fail to conceive are ultimately diagnosed with unexplained infertility (ASRM Practice Committee, 2006). These patients are often treated empirically with IUI, ovulation induction, or IVF, however expectant management remains a reasonable option with a shorter duration of infertility. A United Kingdom-based RCT of patients with unexplained infertility found that IUI and ovulation induction with CC was less cost effective than expectant management, with calculated costs per live birth of 72 pound sterling with expectant management, 1487 pound sterling with IUI, and 2611 pound sterling with CC (Wordsworth et al., 2011).

While expectant management may be more cost effective in certain situations, some patients, especially those with any concern for diminishing ovarian reserve, may be more suited to active management. Studies comparing the cost effectiveness of IUI and IVF in male factor subfertility and unexplained fertility yield conflicting results. While some studies suggest that starting treatment with IVF, as opposed to IUI followed by IVF, is more cost effective, others have found that IUI and IVF confer a similar chance of a successful pregnancy, favoring IUI as the more cost effective approach (Goverde et al., 2000; Pashayan et al., 2006).

Because poor fertilization may play a role in unexplained infertility, ICSI is sometimes performed in these cases. In one study that compared IVF, split IVF-ICSI, and ICSI in couples with unexplained infertility, IVF alone (\$13,842 per live birth, 38.8% likelihood of a live birth) was the most cost effective option if only one cycle was performed. The authors calculated an ICER of \$58,766 with split IVF-ICSI and all ICSI. They concluded that if two IVF cycles were necessary, split IVF-ICSI was the more cost effective approach (Vitek et al., 2013). The application of ICSI for IVF cycles with normal sperm parameters is controversial, thus decisions to institute treatment strategies should never be based solely on cost features, but instead requires attention and counseling on the risks and benefits of proposed strategies.

Interventions for treating patients with unexplained infertility range from very low cost (ex. expectant management) to high cost (ex. IVF with ICSI). With increasing interest in personalized medicine, further research is needed to guide clinicians in making economically-conscious decisions for these patients.

ART Versus Expectant Management, and Treatment in Low Resource Settings

The majority of couples with infertility reside in developing countries where advanced reproductive technology is either unavailable or prohibitively costly (Ombelet and Campo, 2007). Unfortunately, common causes of infertility in the developing world (i.e., tubal occlusion, severe oligospermia) are not amenable to conventional treatments such as CC and IUI, but instead require sophisticated, high complexity ART (Asemota and Klatsky, 2015; Dhont et al., 2011). Implementation of successful ART programs in low-resource settings necessitates a multifactorial approach to cost containment. This may involve simplifying the process of diagnosing

infertility, optimizing patient selection for treatment, maximizing non-IVF ART, utilizing alternatives to expensive ovarian stimulation protocols, and reducing laboratory and equipment costs. In addition, reducing costly complications such as ovarian hyperstimulation syndrome (OHSS) and multiple gestations should be emphasized (Ombelet and Campo, 2007; Teoh and Maheshwari, 2014).

A thorough medical history, in conjunction with simple and inexpensive investigative tools, often suffices in diagnosing infertility. Ombelet et al. proposed a one-step clinic model for basic infertility evaluation that utilizes infectious screening tests, hysterosalpingography and/or hysterosalpingo-contrast-sonography, and semen analysis. This initial workup will ideally identify most causes of infertility: ovulatory dysfunction, uterine malformations, tubal infertility, and male factor infertility (Ombelet and Campo, 2007).

Once initial diagnostic testing is completed, careful determination of which couples are most likely to benefit from expectant management can eliminate unnecessary interventions and costs. In a trial of couples with unexplained subfertility randomized to either 6 months of expectant management or immediate IUI with ovarian stimulation, there was no difference in pregnancy rate or time to ongoing pregnancy between groups. In fact, expectant management proved to be more cost effective, with an estimated saving of 2616 euros (Custers et al., 2012). When considering the cost effectiveness of IVF, one must not only take into account the procedural costs and anticipated success rates, but also a couple's chance of conception without treatment (Mol et al., 2000).

In patients who ultimately require IVF, natural cycle and low-dose ovarian stimulation protocols have been researched as potential alternatives to conventional IVF. Despite being low-cost and "patient-friendly," natural cycle IVF is not a widely utilized approach as its efficacy is limited by high cancellation rates due to premature LH surge and ovulation (Pelinck et al., 2002). Low-dose stimulation appears promising, but with lower success rates than conventional IVF (Asemota and Klatsky, 2015; Ombelet et al., 2008). In a study of couples undergoing IVF, those who underwent a low-dose stimulation protocol had similar clinical pregnancy rates, shorter gonadotrophin administration periods, and lower cost-per live birth (USD 13,200 versus 24,900) compared to those in the high-dose GnRH antagonist group. Low-dose stimulation was found to be more cost-effective, with the added benefit of a decreased incidence of OHSS (Chiles and Schlegel, 2015). Other options for low-dose stimulation protocols involve CC, letrozole, and gonadotrophins or recombinant FSH (Ombelet et al., 2008).

In addition to medications, laboratory and equipment fees are a major determinant of high IVF costs. Creative substitutions for expensive equipment can significantly reduce spending. For example, plastic incubators intended for newborns can be used in place of a laminar flow hoods for handling gametes and embryos (Asemota and Klatsky, 2015; Ombelet et al., 2008). Intravaginal and intrauterine fertilization and incubation methods have also been investigated (Ombelet et al., 2008). The INVO procedure, in which embryos are incubated within an intravaginal plastic device, there are comparable pregnancy rates to conventional IVF procedures (Lucena et al., 2012). The Walking Egg is a non-profit organization that pioneered a simplified IVF culture system which reduces the cost of a single IVF cycle to less than 200 euros (Ombelet, 2013).

There are several reports of successful ART programs in low-resource settings, including a public university hospital in Nigeria that offers IVF and ICSI. At this center, which has achieved a clinical pregnancy rate of 30% per cycle, patients are treated in batches to allow for bulk purchasing and reduced staff time. By implementing cost-saving measures, the cost per cycle for good responders is approximately \$3000—much less than the estimated cost of \$4000–5000 in other Nigerian clinics and \$12,000 in the United States. Sadly, in this particular center, even with these reduced costs, many couples were unable to afford a single attempt at IVF, and most who had an unsuccessful first cycle were unable to afford a second (Orhue et al., 2012). While affordable ART is possible in developing countries, further attention is necessary to make treatment of infertility more widely accessible to all.

Summary and Conclusions

As demonstrated in this article, there is extensive published data on cost effectiveness in reproductive medicine. However, it remains clear that studies evaluating fertility face specific challenges and limitations. From a 2014 paper by Goldhaber-Fiebert and Brandeau, the following were cited as criticisms of studies that affect fertility and childbearing, and were found to be consistently seen among the studies published over the preceding 40 years:

- Studies are heterogeneous in how they report outcomes
- Studies select outcomes that favor a cost effective conclusion
- Studies often report costs per immediate outcome
- Studies take different approaches to outcome inclusion
- QALY's related to pregnancy or fertility are often not addressed in interventions for which these are unintended/indirect outcomes

The paper recommended standardization in economic evaluations evaluating infertility and childbearing, with inclusion of all stakeholders, not just the individual carrying the pregnancy. The authors recommended the formation of consensus guidelines on how to count the impact of interventions that affect future fertility and childbearing (Goldhaber-Fiebert and Brandeau, 2015).

There are several base texts that provide excellent foundations for learning about how to construct and interpret cost effectiveness studies. Key resources include Drummonds Methods for the Economic Evaluation of Health Care Programmes (2015, Oxford University Press), as well as Muennig's Cost Effectiveness Analysis in Healthcare (2016, Wiley).

Increasing research interest, clinician awareness, and media attention to this area not only sparks further interest in cost effectiveness as a discipline of study within reproductive medicine, but also urges more providers to make care decisions with cost effectiveness in mind. In this vein, we would like to conclude with some key points to keep in mind when reading and interpreting CEA's. First, CEA studies are a moving target—as seen in some of the aforementioned studies, cost effectiveness of strategies can change over time. Second, one must remain cognizant of the fact that the current standard of care may not be cost effective. Third, it is worth reexamining processes proven to be cost effective in other countries, in order to see if previously held beliefs of what is cost effective still holds true.

Cost effectiveness studies can perhaps best be constructed from information derived from RCT's, but it is also true that cost based studies can help formulate the design of RCT's. RCT's are challenging to conduct in an area where patients can be very sensitive to cost, when there is an intrinsic belief that one treatment strategy is superior, and especially when the treatment in question is expensive to deliver. With increasing attention to the rising cost of care as addressed at the introduction of this article, it is worthwhile to consider calculating costs of treatment strategies in every RCT or comparable large scale study, even if that is not specifically related to the core focus or primary outcome of the study. The accumulation of more cost data can lead to downstream studies, help inform reporting about this important area, and derive data for future cost effectiveness studies.

References

- Asemota, O. A., & Klatsky, P. (2015). Access to infertility care in the developing world: The family promotion gap. *Seminars in Reproductive Medicine*.
- ASRM Committee Opinion. (2012). *Role of tubal surgery in the era of assisted reproductive technology*.
- ASRM Practice Committee. (2006). Effectiveness and treatment for unexplained infertility. The practice Committee of the American Society for reproductive medicine. *Fertility and Sterility*.
- Casper, R. F., & Mitwally, M. (2012). A historical perspective of aromatase inhibitors for ovulation induction. *Fertility and Sterility*.
- Chiles, K. A., & Schlegel, P. N. (2015). Vasectomy reversal must be the first step for a man who had a vasectomy and wants a children from a new marriage? Opinion: No. *International Brazilian Journal of Urology: Official Journal of the Brazilian Society of Urology*, 41, 1046–1048.
- Custers, I. M., et al. (2012). Long-term outcome in couples with unexplained subfertility and an intermediate prognosis initially randomized between expectant management and immediate treatment. *Human Reproduction*.
- Dhont, N., et al. (2011). Results of infertility investigations and follow up among 312 infertile women and their partners in Kigali, Rwanda. *Tropical Doctor*.
- Eijkemans, M. J., Polinder, S., Mulders, A. G., Laven, J. S., Habbema, J. D., & Fauser, B. C. (2005). Individualized cost-effective conventional ovulation induction treatment in normogonadotrophic anovulatory infertility (WHO group 2). *Human Reproduction*.
- Goldhaber-Fiebert, J. D., & Brandeau, M. L. (2015). Cost-effectiveness of interventions that affect fertility and childbearing intervention. *Medical Decision Making*.
- Goverde, A. J., McDonnell, J., Vermeiden, J. P., Schats, R., Rutten, F. F., & Schoemaker, J. (2000). Intrauterine insemination or in-vitro fertilization in idiopathic subfertility and male subfertility: A randomized trial and cost-effectiveness analysis. *Lancet*.
- Hassan, A., Shehata, N., & Wahba, A. (2017). Cost effectiveness of letrozole and purified urinary FSH in treating women with clomiphene citrate resistant PCOS: A randomized controlled trial. *Human Fertility*.
- Hirshfeld-Cytron, J., & Winter, J. (2013). Laparoscopic tubal reanastomosis versus in vitro fertilization: Cost-based decision analysis. *AJOG*.
- Lee, R., et al. (2008). Reassessing reconstruction in the management of obstructive azoospermia: Reconstruction or sperm acquisition? *Urologic Clinics North America*.
- Lucena, E., et al. (2012). INVO procedure: Minimally invasive IVF as an alternative option for infertile couples. *Scientific World Journal*.
- Meng, M. V., Greene, K. L., & Turek, P. J. (2005). Surgery or assisted reproduction? A decision analysis of treatment costs in male infertility. *Journal of Urology*.
- Messinger, L. B., et al. (2015). Cost and efficacy comparison of in vitro fertilization and tubal anastomosis for women after tubal ligation. *Fertility and Sterility*.
- Mol, B. W., et al. (2000). Cost-effectiveness of in vitro fertilization and embryo transfer. *Fertility Sterility*.
- Moolenaar, L. M., Nahuis, M. J., Hompes, P. G., van der Veen, F., & Mol, B. W. J. (2014). Cost-effectiveness of treatment strategies in women with PCOS who do not conceive after six cycles of clomiphene citrate. *Reproductive BioMedicine Online*.
- Moolenaar, L. M., Cissen, M., de Bruin, J. P., Hompes, P. G. A., Repping, S., van der Veen, F., & Mol, B. W. J. (2015). Cost-effectiveness of assisted conception for male subfertility. *Reproductive BioMedicine Online*.
- Ombelet, W. (2013). The walking egg project: Universal access to infertility care – From dream to reality. *Facts, Views & Vision in ObGyn*.
- Ombelet, W., & Campo, R. (2007). Affordable IVF for developing countries. *Reproductive Biomedicine Online*.
- Ombelet, W., et al. (2008). Infertility and the provision of infertility medical services in developing countries. *Human Reproduction Update*.
- Orhue, A. A., et al. (2012). In vitro fertilization at a public hospital in Nigeria. *International Journal of Gynecology & Obstetrics*.
- Pashayan, N., Lyratzopoulos, G., & Mathur, R. (2006). Cost-effectiveness of primary offer of IVF vs. primary offer of IUI followed by IVF (for IUI failures) in couples with unexplained or mild male factor subfertility. *BMC Health Services Research*.
- Pavlovich, C. P., & Schlegel, P. N. (1997). Fertility options after vasectomy: A cost-effectiveness analysis. *Fertility and Sterility*.
- Pelinck, M. J., et al. (2002). Efficacy of natural cycle IVF: A review of the literature. *Human Reproduction Update*.
- Robb, P., & Sandlow, J. I. (2009). Cost-effectiveness of vasectomy reversal. *Urologic Clinics North America*.
- Tarlatzis, B. C., et al. (2008). In *Consensus on infertility treatment related to polycystic ovary syndrome*Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. *Human reproduction*.
- Teoh, P. J., & Maheshwari, A. (2014). Low-cost IVF: Current insights. *International Journal of Women's Health*.
- P.J. Turek. Male infertility. Yen & Jaffe's reproductive endocrinology, 538–550.e2 (Chapter 24).
- Vitek, W. S., et al. (2013). Management of the first IVF cycle for unexplained infertility: A cost-effectiveness analysis of split IVF-ICSI injection. *Fertility and Sterility*.
- Wordsworth, S., et al. (2011). Clomifene citrate and IUI as first-line treatments for unexplained infertility: Are they cost-effective? *Human Reproduction*.

Further Reading

- Misso, M. L., Wong, J. L. A., Teede, H. J., Hart, R., Rombauts, L., Melder, A. M., Norman, R. J., & Costello, M. F. (2012). Aromatase inhibitors for PCOS: A systematic review and meta-analysis. *Human Reproduction*.

Cost Effectiveness of Preimplantation Genetic Testing for Aneuploidy (PGT-A)

David Jimenez, Igenomix SL, Valencia, Spain

© 2018 Elsevier Inc. All rights reserved.

Among the genetic tests that nowadays are available for the patients of assisted reproduction, PGT-A (Preimplantation Genetic Testing for Aneuploidies) is the one that might generate more doubts about its cost effectiveness. During the last years, there are doubt between using PGT-A and adding to the total cost of the treatment a significant part or repeating transfers until the patient becomes pregnant. Both approaches will have the same objective, find the euploid embryo. However, deeper analysis is required to understand all the variables in order to have an informed decision both, from the clinic and for the patient point of view.

PGT-A

There is consensus that PGT-A might provide clinical benefits in a certain group of patients going through assisted reproduction (i.e., advance maternal age). However, there are also some drawbacks:

1. Higher level of complexity from the operations at the IVF lab
2. Affordability and cost effectiveness
3. Possible reduction of the clinic income
4. Non transfer dilemma
5. Ethical or religious aspects

We intend to provide some clarity specially in the cost benefit of the PGT-A.

The inclusion of PGT-A in the assisted reproduction treatment increases the complexity of the clinical process specially in the IVF lab due to the need of performing a biopsy to the embryo. Currently, several groups are researching about the possibility to diagnose the embryo trough the spent culture media, however, no publication has confirmed this option yet. So, until this possibility is not confirmed with solid data, embryo biopsy is required to provide the chromosome diagnosis.

The biopsy is a critical step in the PGT-A process and must be performed by expert embryologist ([Munne et al., 2010](#); [Harton et al., 2011](#)). One of the main explanation for some studies that did not find a better clinical result in patients of PGT-A versus regular IVF treatment might be the deficiency of the biopsy. A deficient biopsy will increase the stress of the embryo and might cause the failure of the whole treatment ([Cimadomo et al., 2016](#); [Capalbo et al., 2016](#)) due to the reduction of survival rate of embryos after the biopsy ([Swain et al., 2016](#); [Rubio et al., 2009](#)). In expert hands, the survival rate should be almost 100%.

Including PGT-A in a clinical IVF treatment requires that clinics increase the investment and personnel to be able to manage the PGT-A process mainly at the laboratory of IVF but also to provide to patients a proper genetic counseling before they accept and sign the consent form.

Affordability is the main barrier among clinicians to recommend the test. In general, PGT-A can increase substantially the total cost of the treatment that it is consider already a very expensive procedure for most patients. PGT-A cost has decreased in the last years thanks to the launch of new technologies and the appearance of new reference labs that has created a more competitive environment in most of the countries around the world. Additionally, the new trend to make the transfers the following month with frozen embryos allows reference labs to optimize their processes increasing productivity ([Weinerman and Mainigi, 2014](#); [Shapiro et al., 2014](#); [Roque et al., 2015](#)) while decreasing their prices.

To understand the cost of the PGT-A, several variables must be taken into consideration: the biopsy cost, the type of cycle (frozen vs. fresh), the technology used, the margin added by the clinic, the cost of the service provider and the cost of transportation of the samples from the clinic to the reference lab. The main cost elements are described below:

1. Intracytoplasmic sperm injection (ICSI): Although most of the clinics today include ICSI as a routine practice for any IVF cycle, PGT-A can be done only with ICSI to reduce the risk of sample contamination that could cause a misdiagnosis in the result of the test ([Mir et al., 2013](#)). In case a clinic does not routinely perform ICSI, it must be considered an extra cost versus a regular cycle without PGT-A.
2. Cost of biopsy: It is one of the most relevant factors of cost together with the genetic cost from the reference lab. It might be different if the biopsy is performed in day three versus in blastocyst stage.
 - a. Day-3 biopsy: The general trend is to choose the blastocyst stage at day-5 or 6 to perform the biopsy. In day 3, the embryologist can perform the process for all the embryos at the same time and this contributes to a reduction of lab consumables and embryologist time reducing the overall cost at the IVF lab. However, the average number of embryos that will be send to the genetic lab for testing will be higher in comparison to the number in blastocyst ([Rubio et al., 2007](#)) and therefore the savings might be balanced by the additional embryos that requires genetic test. In general, the genetic cost will depend on the number of samples sent to the reference lab.

- b. Trophoctoderm biopsy: As described above, the number of embryos to test is lower than in day-3. However, the protocol in day-5 requires more time from the embryologist and more consumable at the IVF lab since not all embryos can typically be biopsied at the same time (Scott and Hong, 2013). This factor increases the complexity of the process and the overall resources.
3. Frozen versus fresh transfer: The type of transfer also has an impact on the overall cost (Weinerman and Mainigi, 2014; Shapiro et al., 2014; Roque et al., 2015). Some publications suggest that a differed transfer might increase pregnancy rates due to the improvement of the endometrium versus a stimulated one. A frozen transfer will generate additional cost:
 - a. Vitrification consumables used in the process of freezing the embryos.
 - b. Labor resources at the laboratory.
 - c. Transportation cost to deliver samples to the reference lab. In case of fresh transfer, the transportation must be faster and more expensive due to the limited time for the whole process. On the contrary, when the samples are related to a frozen transfer, they can be send using a regular courier service reducing the cost significantly and allows the grouping of samples from several patients in the same shipment.
 - d. PGT-A related to frozen cycles allows to optimize the workload at the IVF lab and at the genetic lab. Transfers on weekends can be eliminated, night shifts at the genetic lab are also minimized reducing the overall level of stress for the teams.
4. PGT-A technologies: During the last years several technologies have been used to diagnoses embryos:
 - a. FISH (fluorescence in situ hybridization): It allows to diagnose a limited number of chromosomes and it is very manual requiring an expert team to achieve a consistent diagnosis. Since the introduction of arrays and NGS (next generation sequencing), FISH has lost relevance and only a small number of genetic laboratories still use it due to its clinical limitations. In addition, some publications suggest that using technologies that allows screening of 24 chromosomes increases the pregnancy rate. FISH is a labor-intensive technology making it expensive to use.
 - b. Arrays and QPCR: Both technologies have a similar cost structure and achieve 24 chromosomes. They allow some level of automation and it does not require so much labor resources to run the tests.
 - c. NGS: Since 2015, NGS has become the gold standard from the diagnosis point of view and from the cost. It allows to run many samples (i.e., 96 samples per run) and it also allows to automate the process at the wet lab part but also at the diagnosis process.
5. Pricing models from the genetic reference labs: Thanks to the introduction of NGS and the increase of the competition, prices for PGT-A in general have decreased in recent years. New pricing models are introduced to facilitate patients to afford the service: price per embryo, flat fees per patient independent of the number of embryos, batching programs, etc.

As described above, there are several factors that impact the cost of the PGT-A for a patient. In the following section a cost benefit model is described to help patients to take inform decisions regarding the convenience of the PGT-A not only from the clinical point of view but also from the financial one.

Cost Benefit PGT-A Analysis

The objective is to understand from the cost point of view if it is worthy for a couple to invest in PGT-A. Main variables of the model that will affect the cost benefit are:

1. Female age: Advance maternal age patients tend to have a higher percentage of abnormal embryos than a young patient. Therefore, a tool as PGT-A that helps to identify the normal embryo will reduce unsuccessful transfers, saving money and disappointments for patients.
2. Previous miscarriages: According to some publications the risk of Asherman syndrome increases with previous miscarriages history.
3. Male factor: Some publication also suggests that males with male factor (below 2 million sperm/mL) will generate a higher percentage of abnormal embryos.
4. Embryos to be transferred is also very relevant for the model. Single embryo transfer will need more transfers to find a normal embryo.
5. Some other variables also need to be considered as probability of miscarriage, probability of D&C procedure, probability of Asherman syndrome.

After defining a table of average abnormal embryo per age based on RCTs (randomized control trials), and retrospective data from genetic labs database from thousands of embryos from different clinics, the model can start assessing the relevant information to understand the clinical implications of PGT-A and the cost benefit analysis.

In the cost benefit analysis, the number of transfers is very relevant for the model. Generally, IVF centers include one transfer in the initial cost and every additional one has an extra cost for patients that can range from 20% to 30% of the initial price, like the cost of a PGT-A.

Below in **Tables 1 and 2**, an example is described to illustrate the model with a patient of 38 years old. We have not included the cost related to days lost due to miscarriage neither the estimated cost related to its psychological burden.

1. Cycle cost: Includes all services related to the treatment including one transfer.
2. Medication cost: Stimulation drugs.

Table 1 Example with a 38 years old patient with high ovarian reserve, male with normal sperm and without previous miscarriage

<i>Clinical data</i>	
Female age	38
Male sperm	Normal
Type of biopsy	Trophectoderm
Previous miscarriage	No
Expected number of blastocyst	4
Embryos to be transferred	1

Table 2 Example of cost of the clinic

<i>Cost data (euros)</i>	
Cycle cost	6000
Medication cost	1200
Extra transfer cost	1950
PGT-A cost	1500
Biopsy cost	700
Cost of amnio or NIPT	750

3. Additional transfers: Number of additional transfers until pregnancy.
4. Biopsy cost charged by the clinic to the patient that typically is a fixed amount independently of the number of embryos. We do not consider the vitrification cost since it is the same in both strategies, with or without PGT-A.
5. PGT-A cost: Cost of the genetic analysis including transportation of samples.
6. Cost of amniocentesis or NIPT (noninvasive prenatal testing): In case a PGT-A is not performed, a prenatal test should be recommended due to advance maternal age risk.

The model generates the information covering two dimensions: (1) clinical information and (2) cost information (**Table 3**).

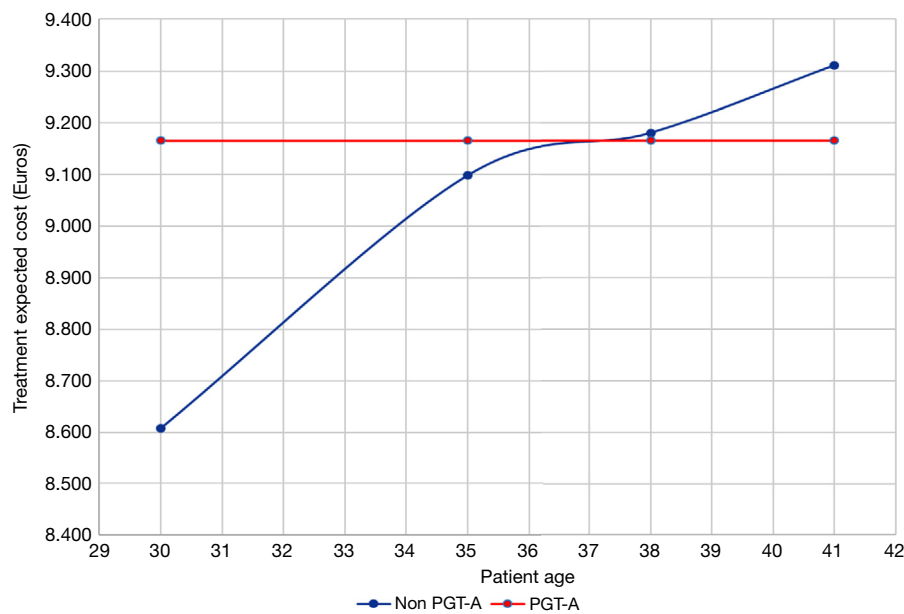
1. % Aneuploid embryos: One of the most important information to determine the model. PGT-A effectiveness increases directly related to the increase of the percentage of aneuploid embryos.
2. Expected number of euploid embryos: Assessing the ovarian reserves it is possible to make a good estimation of the number of blastocyst that a patient of 38 years old will have for biopsy. In this example, the patient has a high reserve so 4 blastocysts are expected and 1.52 normal embryos ($4 \times 38\%$), a bit less (1.49) for PGT-A.
3. Probability to select an euploid embryo at initial transfer: It is accepted that morphology cannot predict the chromosomal profile of embryos. Using statistics, we can estimate the probability of selecting 1 normal embryo out of 4 available knowing that 1.52 are normal. In this case, the patients have 38% to select the normal embryo at first try.
4. Estimated number of transfers to select a normal embryo: With the information above and using hypergeometric distribution formula, we can define the number of transfer requested to find the euploid embryo. This is the most important variable of the model. This couple will need in average two transfers to find the normal embryo versus only one for the PGT-A.
5. Multiple pregnancy probability: IVF community is trying to promote single embryo transfer to reduce multiple pregnancies due to (1) complications and additional cost in multiple pregnancies and (2) health advantage of single vs multiple pregnancy babies.

Table 3 Clinical information

<i>Clinical information</i>	<i>Non PGT-A</i>	<i>PGT-A</i>
% Aneuploid embryos	62%	62%
Expected number of euploid embryos	1.52	1.49
Probability to select an euploid embryo at initial transfer	38%	99%
Probability of pregnancy at first transfer	23%	59%
Miscarriage probability	36%	8%
Multiple pregnancy probability	1.4%	1.4%
D&C probability	18%	4%
Asherman syndrome risk probability	3%	0,3%
Estimated number of months before baby at home	19.5	16
Estimated number of transfers to select a normal embryo	2.0	1

Table 4 Cost benefit analysis

	Non PGT-A	PGT-A
Cycle cost	6000	6000
Medication	1200	1200
Additional transfers cost	1995	0
PGT-A cost	0	2200
D&C cost	292	65
NIPT cost	492	0
Total cost	9487	9465

**Fig. 1** Distribution of cost according to maternal age.

6. D&C probability: It is important to calculate the cost associated to the higher probability of miscarriage and therefore D&C required. This couple will have a significant reduction of the risk of miscarriage from 36% to 8% when using PGS-A and also a consequent reduction on the D&C from 18% to 4%. According to the American Pregnancy Association, half of the miscarriages will require a D&C procedure.
7. Asherman syndrome risk probability: Transferring aneuploid embryos might have some relevant health consequences that patients need to understand: miscarriage, D&C and also Asherman syndrome. In this example the patient will have a risk of 3% or 10 times higher than with PGT-A (Friedler et al., 1993).
8. Estimated number of months before baby at home: Additional transfer means additional time to find the euploid embryo and therefore additional time to have the baby at home. According to some studies, the difference of time to obtain a baby at home between PGT-A and non PGT-A treatments is 100 days.

The cost benefit analysis is shown in Table 4. Using the data in the table above, we can calculate what will be the expected cost for a treatment including and not including PGS-A. In this particular example, the model suggests that there is no difference between both strategies.

Repeating this exercise for several ages, the model shows that above 35 years old female, total average treatment cost will be lower when adding PGT-A to the IVF treatment (Fig. 1).

Conclusion

According to several publications PGT-A seem to bring several advantages (1) higher pregnancy rate per transfer, (2) reduction of the miscarriage risk and (3) shorter period to baby at home. PGT-A has also some disadvantages (1) cost and (2) potential over diagnosis of aneuploid embryos.

PGT-A is a complex procedure that involves a higher level of sophistication at the IVF lab and represents a higher stress for the embryo (Munne et al., 2010; Harton et al., 2011). However, with a proper level of expertise PGT-A does not represent a threat for the embryo.

Cost is the most important barrier for patients to adopt PGT-A. Although new technologies and higher competition among genetic labs are bringing cost down, there is still a general believe that attempting to have a baby in IVF with embryo genetic screening is more expensive than without PGT-A. However, after a cost benefit analysis we have seen that PGT-A is not more expensive since its cost is balanced by the saving of additional unsuccessful transfer.

References

- Capalbo, A., Ubaldi, F. M., Cimadomo, D., et al. (2016). Consistent and reproducible outcomes of blastocyst biopsy and aneuploidy screening across different biopsy practitioners: A multicentre study involving 2586 embryo biopsies. *Human Reproduction*, 31, 199–208.
- Cimadomo, D., Capalbo, A., Ubaldi, F. M., et al. (2016). The impact of biopsy on human embryo developmental potential during preimplantation genetic diagnosis. *BioMed Research International*, 2016, 7193075.
- Friedler, S., Margalioth, E. J., Kafka, I., & Yaffe, H. (1993). Incidence of postabortion intrauterine adhesions evaluated by hysteroscopy—A prospective study. *Human Reproduction*, 8, 442–444.
- Harton, G. L., Magli, M. C., Lundin, K., et al. (2011). ESHRE PGD consortium/embryology special interest group-best practice guidelines for polar body and embryo biopsy for preimplantation genetic diagnosis/screening (PGD/PGS). *Human Reproduction*, 26, 41–46.
- Mir, P., Rodrigo, L., Mercader, A., et al. (2013). False positive rate of an arrayCGH platform for single-cell preimplantation genetic screening and subsequent clinical application on day-3. *Journal of Assisted Reproduction and Genetics*, 30, 143–149.
- Munne, S., Wells, D., & Cohen, J. (2010). Technology requirements for preimplantation genetic diagnosis to improve assisted reproduction outcomes. *Fertility and Sterility*, 94, 408–430.
- Roque, M., Valle, M., Guimaraes, F., Sampaio, M., & Geber, S. (2015). Freeze-all policy: Fresh vs. frozen-thawed embryo transfer. *Fertility and Sterility*, 103, 1190–1193.
- Rubio, C., Rodrigo, L., Mercader, A., et al. (2007). Impact of chromosomal abnormalities on preimplantation embryo development. *Prenatal Diagnosis*, 27, 748–756.
- Rubio, C., Gimenez, C., Fernandez, E., et al. (2009). The importance of good practice in preimplantation genetic screening: Critical viewpoints. *Human Reproduction*, 24, 2045–2047.
- Scott, K. L., Hong, K. H., & Scott, R. T., Jr. (2013). Selecting the optimal time to perform biopsy for preimplantation genetic testing. *Fertility and Sterility*, 100, 608–614.
- Shapiro, B. S., Daneshmand, S. T., Garner, F. C., Aguirre, M., & Hudson, C. (2014). Clinical rationale for cryopreservation of entire embryo cohorts in lieu of fresh transfer. *Fertility and Sterility*, 102, 3–9.
- Swain, J. E., Carrell, D., Cobo, A., et al. (2016). Optimizing the culture environment and embryo manipulation to help maintain embryo developmental potential. *Fertility and Sterility*, 105, 571–587.
- Weinerman, R., & Mainigi, M. (2014). Why we should transfer frozen instead of fresh embryos: The translational rationale. *Fertility and Sterility*, 102, 10–18.

Cost-Effectiveness of Low-Cost IVF

Rachel Grimes Sprague and Gordon Wright Bates, University of Alabama Birmingham, Birmingham, AL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Clomid An oral medication used to treat infertility in women who do not ovulate.

Conventional IVF protocol Incorporates three commonly used protocols for ovarian stimulation that use higher doses of fertility medications, including agonist, antagonist, or flare and micro-flare protocols. This protocol typically requires closer patient observation given risk of OHSS, but produces a greater yield of oocytes.

Human menopausal gonadotrophins An injectable hormonally-active medication used for the treatment of infertility where they stimulate the ovaries to mature follicles, thus making women more fertile.

Intracytoplasmic sperm injection (ICSI) An IVF procedure in which a single sperm is injected directly into an egg. This procedure is typically performed in patients with defective sperm function.

Intrauterine insemination (IUI) A fertility treatment in which there is a deliberate introduction of sperm into a female's uterus or cervix for the purpose of achieving a pregnancy through in vivo fertilization by means other than sexual intercourse.

Low-cost IVF (LCIVF) A movement toward reproductive justice with primary goal of helping global infertility and access to treatment, especially in resource-poor settings.

Mild stimulation IVF Is a protocol for ovarian stimulation which is conducted in the women's natural menstrual cycle with smaller doses of stimulating fertility medications. It produces a lower yield of oocytes than the conventional stimulation protocol. Other names include mild-IVF or mini-IVF.

Natural cycle IVF No ovarian stimulatory drugs are administered and, therefore, the treatment cycle relies on the spontaneous development of one follicle production and aspiration.

Ovarian Hyperstimulation Syndrome (OHSS) A medical condition affecting women who take fertility medications which can lead to enlarged ovaries and ascites (or fluid accumulation). Most cases are mild, but rarely can be severe and/or lead to death.

'WE lab method This method utilizes simple, low cost interventions instead of expensive medical gases or high-tech incubation equipment and infrastructure typical of IVF labs in high resource settings.

The first successful pregnancy after in vitro fertilization (IVF) and the first birth from an in vitro-fertilized embryo were reported in 1976 and 1978 (Stephoe and Edwards, 1976, 1978). However despite being over 40 years since the development of IVF, the cost of treatment for patients remains significantly high. Furthermore, sophisticated technologies and expensive medications have been introduced increasing cost and limiting access despite the need. Globally, infertility is estimated to affect 48.5 million couples worldwide (Mascarenhas et al., 2012). Consequences of infertility are known to include stress, depression, low self-esteem, guilt, and marital problems including sexual problems, isolation, and abandonment. Cultural consequences include loss of legitimacy in society including inability to conduct public speaking and dismissal as a spouse (Hjelmstedt et al., 1999; Inhorn, 2003; Omurtag et al., 2009; Inhorn and Patrizio, 2015). Unfortunately, the portion of patients with infertility who seek medical care is only about half in either developed (56.1%) or developing countries (51.2%) with the percentage of patients actually receiving care substantially less at about 22.4% (Boivin et al., 2007), presumably due to limited or unavailable resources and the expensive cost (Eisenberg et al., 2010). The cost of IVF treatment is variable among different countries; however, is generally an expensive treatment. Per 2014 data, the average cost per one fresh IVF or intracytoplasmic sperm injection (ICSI) cycle was \$12.4K in the United States with Canada a close second at \$8.5K. Countries such as Australia, Brazil, Germany, Japan, Scandinavian countries, South Africa, and United Kingdom ranged from \$3–\$5K per single cycle (Collins et al., 1997; Maheshwari et al., 2009; Katz et al., 2011). With an average mean income in developed nations somewhere between \$40–\$60K, this is a significant financial cost. Comparatively, the worldwide average median annual household income is only \$10K and for those populations in third-world counties, infertility treatments may be impossible.

The majority of the costs of IVF are contributed to medication and laboratory costs, ultrasound procedures, IVF procedures (oocyte retrieval and embryo transfer), and expenditures for investigations. This does not include other extraneous costs such as charges from hospital stays, anesthesia, and administrative fees along with time lost from employment and travel costs (Collins, 2002; Wu et al., 2013).

Public funding for IVF treatment differs between countries with varying restrictions. Australia has publicly funded IVF treatment with no limitations on the number of previous cycles, maternal age, duration of subfertility, body mass index, or smoking status; however, patients are required to make expensive copayments for their treatments (Chambers et al., 2013; Harris et al., 2016). In the Netherlands, health insurance covers the performance of three IVF cycles in licensed IVF centers, irrespective of the cause of infertility. Other countries, such as the United Kingdom, provide limited public funding although with strict restrictions

(Maheshwari et al., 2009). On the contrary, the United States IVF is generally only available via private funding (Smith et al., 2011). However, in general, due to the high cost of IVF, few governments have been able or willing to subsidize IVF thereby limiting IVF primarily to the private medical sector. In patients with no infertility insurance coverage, the burden of the high fertility costs falls onto individual or couple (Collins, 2002; Spar, 2006).

Global attempts at lowering total costs of IVF emerged around 2001. Around this time, the World Health Organization (WHO) recommended that infertility be considered a global health problem (Ombelet et al., 2008). The organization aimed to increase awareness of infertility to national reproductive health education programs and services, to support the ethical acceptability of IVF, and to spread accessibility to infertility care in developing countries and other low-resource regions. In order to improve accessibility to all patients, there grew interest in exploring strategies improve the general affordability as this was deemed the largest barrier to access. In response to this announcement, the low-cost in vitro fertilization (LCIVF) initiative emerged.

LCIVF is a movement toward reproductive justice with primary goal of helping global infertility and access to treatment, especially in resource-poor settings (Ombelet et al., 2008; Hammarberg and Kirkman, 2013). The movement gained momentum in July of 2013 at the European Society of Human Reproduction and Embryology annual meeting. Willem Ombelet's nonprofit organization, The Walking Egg (WE), announced a newly invented LCIVF method based on a pilot clinical trial. The specialized LCIVF technique to that date had produced 12 healthy LCIVF babies with a total per-cycle cost as low as 200 Euros (ESHRE, 2013; Gallaher, 2013; Ombelet, 2013, 2014).

The outcomes of the WE study were based on case studies. The new LCIVF technique attempted to bypass need for a costly IVF laboratory by simplifying embryo culture methods and eliminating high-end equipment. Embryo cultures produced by the Walking Egg were simplified, low-cost and designed for easy transport (Ombelet et al., 2014; Van Blerkom et al., 2014). The method of IVF culturing is called 'WE lab method'. The method utilizes simple, low cost interventions instead of expensive medical gases or high-tech incubation equipment and infrastructure typical of IVF labs in high resource settings. The 'WE lab method' is a closed system with a connection between two test-tubes (see Fig. 1). The first tube contains inexpensive components of citric acid, sodium

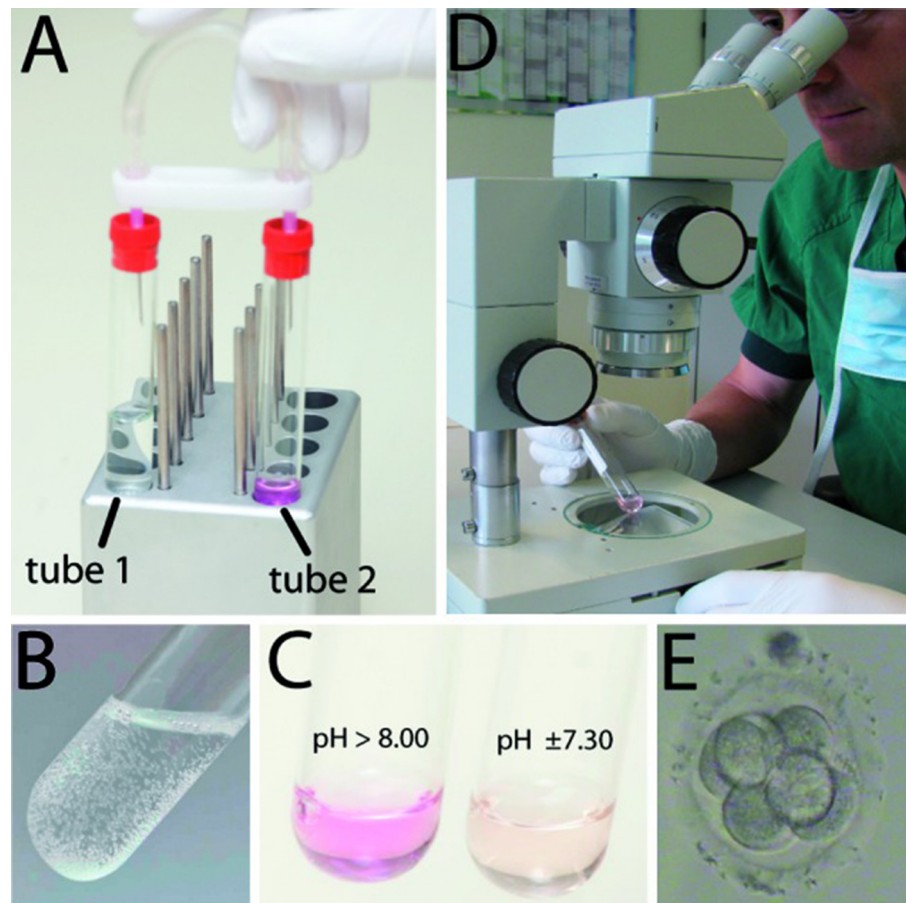


Fig. 1 The tWE-lab system: (A) connection between tube 1 with citric acid and sodium bicarbonate in water and tube 2 with IVF culture medium transfers enough CO₂ gas (B) produced by the chemical reaction between the acid and the base to equilibrate the pH of the medium to ± 7.30 . Fertilization and embryo scoring by looking through the glass tube (D,E). Borrowed from Ombelet, W., J. Van Blerkom, E. Klerkx, M. Janssen, N. Dhont, G. Mestdagh, G. Nargund and R. Campo (2014). The (t)WE lab Simplified IVF Procedure: First Births after freezing/thawing. *Facts Views Vis Obgyn* 6(1): 45–49.

bicarbonate, and water and the second tube contains the IVF culture medium and the embryo. The weak acid/base combination produces and transfers enough CO_2 gas to the culture medium tube to maintain a pH of around 7.3. This closed system utilizes this simple chemical reaction to prevent adverse effects of temperature or pH changes to the culture media. The 'WE lab method initial results showed that IVF methodology could be significantly simplified and result in successful pregnancies at low cost and with easily transportable culture media (Ombelet et al., 2014).

Although this novel approach piqued interest in LCIVF, there remained some hesitations and limitations. Most importantly, given the results were based mainly on three case studies from one laboratory, the 'WE lab method needed to be replicated in different laboratories and under field conditions to assess long-term safety issues, hidden infrastructure, verify success rates, and review personal costs prior to being fully implemented. The 'WE lab method could not replicate ICSI laboratory techniques in a low-cost format. It also did not address patients who require preimplantation genetic diagnosis and screening. Therefore, the 'WE lab method is not currently an alternative option for patients requiring these more specialized treatments and testing modalities.

Another recent low cost innovation is the use of intravaginal culture (IVC) with the INVOcell™ device (Fig. 2). This device functions as an incubator for IVF embryos prior to transfer. Oocytes and sperm are cocultured in a plastic cylinder that is inserted into the vagina for 5 days to form blastocyst. This natural form of incubation replaces expensive conventional incubators that require adjustments of gas composition, temperature, pH levels, and other embryo monitoring costs. Live birth rates per cycle were similar between the INVOcell device (55%) and conventional incubators (60%). Concerns have been raised whether abnormal fertilization may go undetected due to the reduced monitoring of the developing embryos. Although further studies are necessary, the low cost of the IVC using INVOcell™ has the potential to broaden access to assisted reproduction (Doody et al., 2016).

The LCIVF movement has since expanded from beyond simplifying procedures and laboratory equipment. Current strategies focus to decrease total cost including careful patient selection, simplifying investigative methods, reducing the cost of ovarian stimulation, and minimizing the complications of IVF in both developed and underdeveloped nations (Teoh and Maheshwari, 2014).

The cost-effectiveness of IVF has also been shown to be dependent on the couple-specific chances of treatment-independent conception. The LCIVF initiative, therefore, recommends careful patient selection to avoid unnecessary interventions for patients with likely poor IVF outcomes or in patients who would be able to have similar chances of conception with expectant management. The objective is to weigh the expected probability of successful conception with treatment against the chances of conception without treatment in order to determine the most economical patient population to whom IVF should be offered. To start, the prevention of unnecessary IVF, and therefore unnecessary costs, in populations in which expectant management would lead to similar conception results has been researched. In patients with unexplained infertility without tubal pathology or severe male-factor infertility, the natural conception rate can be >50% if expectant management is adopted (Custers et al., 2011; Kamphuis et al., 2014).



Fig. 2 The INVOcell™ utilizes the vagina as a natural form of incubation to support fertilization and embryo development prior to transfer. Borrowed from invobioscience.com.

Economic-modeling studies also reveal that in unexplained infertility in younger women, IVF treatment may not be cost effective within 3 years of trying to conceive. And in women >35 years of age with primary infertility, immediate IVF is more cost-effective than delay of IVF (Mol et al., 2000; Kamphuis et al., 2014). In Canada, where IVF is publicly funded without age limits, a study in 2015 supported the need for age eligibility criteria as the cost of fertility treatments significantly increased over the age 40 with low live birth rates. The study results noted that the mean treatment cost per birth (excluding medications) for a 40 year old was \$43,153 with live birth number of 105 compared to a 44 year old where total cost was \$597,800 with 0 live births (Ouhilal et al., 2015). Other studies address infertility type along with ideal treatment modality approach. Many patients may be candidates for less expensive options for fertility treatment including use of ovulation induction and/or intrauterine insemination (IUI). For instance in women with patent fallopian tubes, proceeding with IUI with/without clomid or human menopausal gonadotrophins (hMG) before IVF is more cost effective. However in women with blocked fallopian tubes, IVF-Embryo Transfer appears to be the most cost-effective modality and, therefore, no cost or time should be wasted proceeding with initial IUI attempts (Van Voorhis et al., 1997). Therefore, the overarching belief behind careful patient selection is to identify which patients are most ideal candidates for IVF—based on age, infertility type diagnosis, and timing of treatment—to provide the most cost-effective treatment approach.

Another method for limiting expenditures targets the simplification of baseline investigations and equipment use in the laboratory. The goal is to limit duplicated or unnecessary studies and to proceed with the most inexpensive option first. Studies have shown that practices involved in investigation of infertility are widely variable among developed counties and even among various practices in similar regions (Helmerhorst et al., 1995; Ojha et al., 2003). These differing investigation protocols can lead to significant differences in the cost of baseline investigations. The LCIVF initiative moves to have standardized protocols that focus on inexpensive, but adequate, baseline investigations for infertility. Firstly, male-factor fertility can compose up to 40% of infertility diagnoses in couples and initial evaluation with semen analysis is one of the most inexpensive costs that should be performed early in the investigation of couple infertility. An adequate history, pelvic examination, and pelvic ultrasound with antral follicle count (AFC) are also inexpensive assessments that are recommended. Ovarian reserve testing utilizes biomarkers and imaging techniques to determine the quantity of oocytes capable of fertilization. The goal of ovarian reserve testing is to assess reproductive potential and to predict an individual's response to ovulation induction agents. The most common biomarkers include basal follicle-stimulating hormone (FSH) plus estradiol levels or anti-Müllerian hormone (AMH). The cost per live birth was greatly reduced when ovarian reserve testing was used in comparison with initiating IVF treatment without any information of ovarian reserve. In addition, inducing ovarian stimulation without the predictive value of ovarian reserve can be dangerous given risk of ovarian hyperstimulation syndrome (OHSS) (Nardo et al., 2011). No single assessment of ovarian reserve has 100% sensitivity and specificity and, therefore, tests often are combined in an attempt to improve the prediction of poor outcomes. However, models of combined ovarian reserve tests do not significantly improve the ability to predict poor reproductive outcomes over single ovarian reserve tests (Practice Committee of the American Society for Reproductive, 2015). Furthermore, the use of multiple ovarian reserve tests may complicate the understanding of an individual's ovarian reserve and increase the expense of screening.

The value of using specific imaging techniques is more difficult to standardize as costs, availability, and treatment protocols differ in varying regions. For instance, in places like the United Kingdom where IVF is considered the most cost-effective treatment for all subfertility factors apart from anovulation, assessment of tubal patency and/or hysteroscopy may not be necessary as they will not affect treatment route (National Collaborating Centre for Women's and Children's Health (UK), 2013). And although laparoscopy is the goal standard for diagnosis of tubal patency, use of other imaging modalities prior including HSG and SIS are less expensive and less invasive options which should be considered anywhere that these are available. Options such as office endoscopes may be more beneficial in the near future as use prevents need for costly operating room expenditures.

The next step at expenditure reduction explores ovarian stimulation and medication costs. Movements at reducing costs of ovarian stimulation and medication costs have transitioned to natural cycle or mild stimulation protocol (or "mini-IVF") rather than conventional stimulation (Teoh and Maheshwari, 2014). The overall purposes are to reduce costs of medications, treatment invasiveness, and complications. In natural cycle IVF, no ovarian stimulatory drugs are administered and, therefore, the treatment cycle relies on the spontaneous development of one follicle production and aspiration. The cumulative probability of pregnancy is 46% with a live birth rate of 32% after four cycles of treatment (Nargund et al., 2001). Mild stimulation procedures have also been utilized which use low dose gonadotropins. Both the natural cycle and mild stimulation cycle IVF are significantly lower cost than conventional IVF by at least \$5K (Siristatidis et al., 2012; Crawford et al., 2016). Other clinical benefits include shorter duration of the protocol, simplicity of treatments/follow up, and reduced patient discomfort. Due to less follicular development, there are fewer required monitoring visits, including transvaginal ultrasound examinations and blood tests, than conventional IVF. Likewise, with use of lower dose or no gonadotropins, there is decreased risk of OHSS that can cause significant morbidity and potentially high costs for treatment. However, due to low ovarian response with subsequent high cancellation rate, the current evidence does not favor natural cycle or mild stimulation IVF (Pelinck et al., 2002; Ombelet et al., 2008). Natural cycle and mild stimulation IVF may prove costlier than conventional IVF in the long run due to the lower live birth rate per cycle and, therefore, overall need for more treatment cycles (Groen et al., 2013; Crawford et al., 2016). Another disadvantage of natural cycle and mild stimulation is the lower number of oocytes retrieved and, subsequently, the inability to store unused embryos. Thereby, this increases the number of necessary cycles along with the potential morbidity associated with oocyte retrievals. Natural cycle or mild stimulation protocols may be advantageous for select patient populations; however, full disclosure regarding potential benefits and risks, including lower per cycle birth rates, must be performed. Additional randomized control trials comparing natural cycle, mild stimulation, and conventional IVF may help clarify the ideal patient populations for each method.

Lastly, the LCIVF initiative directs attention to optimizing the safety of infertility treatments and minimizing the complications, especially OHSS and multiple pregnancies. As mentioned above, OHSS can be expensive to treat, however, more importantly can endanger the life of the patient. The moderate and severe forms of OHSS are typically treated in an inpatient setting and put patients at risk for complications such as hypovolemia, respiratory/circulatory shock, ovarian torsion, ovarian rupture, thrombophlebitis, and renal insufficiency. Aspiration of accumulated fluid from the abdominal and/or pleural cavities may also be necessary, thereby requiring input and assistance from a multidisciplinary team (Joint Society of Gynecologists of Canada-Canadian Fertility Andrology Society Clinical Practice Guidelines et al., 2011). The use of natural cycle and mild stimulation has been believed to help reduce incidence of OHSS due to the lower dose of fertility medications (Williams et al., 2002; Aboulghar and Mansour, 2003). Regarding multiple gestations, over the last 30 years there has been a 65% increase in the frequency of twins and a 500% increase in triplet and higher-order births, primarily due to the increased use of assisted reproductive technology. These multifetal births account for a high incidence of perinatal morbidity and mortality, including complications such as preeclampsia, preterm labor, preterm rupture of membranes, placental abruption, pyelonephritis, and postpartum hemorrhage (Cunningham et al., 2010; American College of Gynecologists Committee on Practice et al., 2004). In comparison to singletons, multi-fetal gestations are at least six times more likely to develop cerebral palsy (Pharoah and Cooke, 1996). In 2014, the costs associated with perinatal morbidity for IVF multiple gestations in the United States were estimated at \$6.3 billion (Allen et al., 2014). In the United Kingdom, it has been shown that 56% of the direct cost of IVF pregnancies is associated with multiple gestations after IVF (Ledger et al., 2006). Therefore, the practice of single-embryo transfer has been encouraged as it has shown to drastically reduce the incidence of multiple pregnancies (Ombelet et al., 2008; McLernon et al., 2010).

The various LCIVF initiatives hold great promise, especially to resource-poor regions and nations. However, the techniques and strategies are still in the formative stages. The advantages of current LCIVF advances include focusing on appropriate patient populations and treatment timing, shortening of stimulation time and medication cost, decreasing laboratory costs, and reducing chances of, and therefore morbidity associated with, OHSS and multiple pregnancies. It pushes for low-cost medication through different ovarian stimulation protocols, although with a disclaimer to patients regarding decreased life birth rate per cycle. The most significant breakthrough in cost-reduction is the invention of a simplified culture system (the 'WE lab method), which can reduce the laboratory cost up to 90%. LCIVF and the simplified culture system can be promising to patients, especially in resource-poor countries who may otherwise receive no treatment. Nonetheless, these LCIVF initiatives come with certain limitations. In developed countries, high-tech treatment programs/laboratories and easily available resources are already established and have been clinically validated and study-proven. Additionally, the implementation of age-limitations to infertility treatment comes at a psychological cost to reproductive liberties. The danger of low-cost IVF may also mean low quality of care or need for repeat cycles that in turn become more expensive.

Overall, the trend for the development of low-cost IVF is encouraging, especially to those who are financially disadvantaged or in underdeveloped nations. Further research is needed to decide the clinical and cost effectiveness of these initiatives on a larger scale. In an ideal world, cost should not be the main barrier to parenthood.

References

- Aboulghar, M. A., & Mansour, R. T. (2003). Ovarian hyperstimulation syndrome: Classifications and critical analysis of preventive measures. *Human Reproduction Update*, 9(3), 275–289.
- Allen, B. D., Adashi, E. Y., & Jones, H. W. (2014). On the cost and prevention of iatrogenic multiple pregnancies. *Reproductive Biomedicine Online*, 29(3), 281–285.
- American College of Obstetricians and Gynecologists Committee on Practice Bulletins-Obstetrics; Society for Maternal-Fetal Medicine; ACOG Joint Editorial Committee. (2004). ACOG practice bulletin #56: Multiple gestation: Complicated twin, triplet, and high-order multifetal pregnancy. *Obstetrics and Gynecology*, 104(4), 869–883.
- Boivin, J., Bunting, L., Collins, J. A., & Nygren, K. G. (2007). International estimates of infertility prevalence and treatment-seeking: Potential need and demand for infertility medical care. *Human Reproduction*, 22(6), 1506–1512.
- Chambers, G. M., Hoang, V. P., & Illingworth, P. J. (2013). Socioeconomic disparities in access to ART treatment and the differential impact of a policy that increased consumer costs. *Human Reproduction*, 28(11), 3111–3117.
- Collins, J. (2002). An international survey of the health economics of IVF and ICSI. *Human Reproduction Update*, 8(3), 265–277.
- Collins, J. A., Feeny, D., & Gunby, J. (1997). The cost of infertility diagnosis and treatment in Canada in 1995. *Human Reproduction*, 12(5), 951–958.
- Crawford, N. M., Sahay, K. M., & Mersereau, J. E. (2016). Mild stimulation versus conventional IVF: A cost-effectiveness evaluation. *Open Journal of Obstetrics and Gynecology*, 6, 180–188.
- Cunningham, G. L., Leveno, K. J., Bloom, S. L., Hauth, J. C., Rouse, D. J., & Spong, C. Y. (2010). *Williams Obstetrics*, 23rd edn. Chapter 39 Multifetal Gestation. New York: McGraw-Hill.
- Custers, I. M., Konig, T. E., Broekmans, F. J., Hompes, P. G., Kaaijk, E., Oosterhuis, J., Mochtar, M. H., Repping, S., van Wely, M., Steures, P., van der Veen, F., & Mol, B. W. (2011). Couples with unexplained subfertility and unfavorable prognosis: A randomized pilot trial comparing the effectiveness of in vitro fertilization with elective single embryo transfer versus intrauterine insemination with controlled ovarian stimulation. *Fertility and Sterility*, 96(5), 1107–1111. e1101.
- Doody, K. J., Broome, E. J., & Doody, K. M. (2016). Comparing blastocyst quality and live birth rates of intravaginal culture using INVOcell to traditional in vitro incubation in a randomized open-label prospective controlled trial. *Journal of Assisted Reproduction and Genetics*, 33(4), 495–500.
- Eisenberg, M. L., Smith, J. F., Millstein, S. G., Nachtigall, R. D., Adler, N. E., Pasch, L. A., Katz, P. P., & Infertility Outcomes Program Project, G. (2010). Predictors of not pursuing infertility treatment after an infertility diagnosis: Examination of a prospective U.S. cohort. *Fertility and Sterility*, 94(6), 2369–2371.
- ESHRE. (2013). "IVF for 200 euro per cycle: first real-life proof of principle that IVF is feasible and effective for developing countries.", from <http://www.eshre.eu/Londen2013/Media/Releases/Elke-Klerckx.aspx>.
- Gallaher, J. (2013). IVF as cheap as LE170, doctors claim., 2013, from <http://www.bbc.co.uk/health-23223752>.
- Groen, H., Tonch, N., Simons, A. H., van der Veen, F., Hoek, A., & Land, J. A. (2013). Modified natural cycle versus controlled ovarian hyperstimulation IVF: A cost-effectiveness evaluation of three simulated treatment scenarios. *Human Reproduction*, 28(12), 3236–3246.

- Hammarberg, K., & Kirkman, M. (2013). Infertility in resource-constrained settings: Moving towards amelioration. *Reproductive Biomedicine Online*, 26(2), 189–195.
- Harris, K., Burley, H., McLachlan, R., Bowman, M., Macaldowie, A., Taylor, K., Chapman, M., & Chambers, G. M. (2016). Socio-economic disparities in access to assisted reproductive technologies in Australia. *Reproductive Biomedicine Online*, 33(5), 575–584.
- Helmerhorst, F. M., Oei, S. G., Bloemenkamp, K. W., & Keirse, M. J. (1995). Consistency and variation in fertility investigations in Europe. *Human Reproduction*, 10(8), 2027–2030.
- Hjelmsstedt, A., Andersson, L., Skoog-Svanberg, A., Bergh, T., Boivin, J., & Collins, A. (1999). Gender differences in psychological reactions to infertility among couples seeking IVF- and ICSI-treatment. *Acta Obstetrica et Gynecologica Scandinavica*, 78(1), 42–48.
- Inhorn, M. C. (2003). Global infertility and the globalization of new reproductive technologies: Illustrations from Egypt. *Social Science & Medicine*, 56(9), 1837–1851.
- Inhorn, M. C., & Patrizio, P. (2015). Infertility around the globe: New thinking on gender, reproductive technologies and global movements in the 21st century. *Human Reproduction Update*, 21(4), 411–426.
- Joint Society of Obstetricians and Gynaecologists of Canada-Canadian Fertility Andrology Society Clinical Practice Guidelines, E. Reproductive, S. Infertility Committee of the Executive, O. Council of the Society of, C. Gynaecologists of, F. Board of the Canadian, S. Andrology, D. Shmorgun and P. Claman. (2011). The diagnosis and management of ovarian hyperstimulation syndrome. *Journal of Obstetrics and Gynaecology Canada*, 33(11), 1156–1162.
- Kamphuis, E. I., Bhattacharya, S., van der Veen, F., Mol, B. W., Templeton, A., & Evidence Based, I. V. F. G. (2014). Are we overusing IVF? *BMJ*, 348, g252.
- Katz, P., Showstack, J., Smith, J. F., Nachtigall, R. D., Millstein, S. G., Wing, H., Eisenberg, M. L., Pasch, L. A., Croughan, M. S., & Adler, N. (2011). Costs of infertility treatment: Results from an 18-month prospective cohort study. *Fertility and Sterility*, 95(3), 915–921.
- Ledger, W. L., Anumba, D., Marlow, N., Thomas, C. M., Wilson, E. C., & Cost of Multiple Births Study Group. (2006). The costs to the NHS of multiple births after IVF treatment in the UK. *BJOG*, 113(1), 21–25.
- Maheshwari, A., Scotland, G., Bell, J., McTavish, A., Hamilton, M., & Bhattacharya, S. (2009). The direct health services costs of providing assisted reproduction services in overweight or obese women: A retrospective cross-sectional analysis. *Human Reproduction*, 24(3), 633–639.
- Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., & Stevens, G. A. (2012). National, regional, and global trends in infertility prevalence since 1990: A systematic analysis of 277 health surveys. *PLoS Medicine*, 9(12), e1001356.
- McLernon, D. J., Harriid, K., Bergh, C., Davies, M. J., de Neubourg, D., Dumoulin, J. C., Gerris, J., Kremer, J. A., Martikainen, H., Mol, B. W., Norman, R. J., Thurin-Kjellberg, A., Tiitinen, A., van Montfoort, A. P., van Peperstraten, A. M., Van Royen, E., & Bhattacharya, S. (2010). Clinical effectiveness of elective single versus double embryo transfer: Meta-analysis of individual patient data from randomised trials. *BMJ*, 341, c6945.
- Mol, B. W., Bonsel, G. J., Collins, J. A., Wiegerinck, M. A., van der Veen, F., & Bossuyt, P. M. (2000). Cost-effectiveness of in vitro fertilization and embryo transfer. *Fertility and Sterility*, 73(4), 748–754.
- Nardo, L. G., Fleming, R., Howles, C. M., Bosch, E., Hamamah, S., Ubaldi, F. M., Hugues, J. N., Balen, A. H., & Nelson, S. M. (2011). Conventional ovarian stimulation no longer exists: Welcome to the age of individualized ovarian stimulation. *Reproductive Biomedicine Online*, 23(2), 141–148.
- Nargund, G., Waterstone, J., Bland, J., Philips, Z., Parsons, J., & Campbell, S. (2001). Cumulative conception and live birth rates in natural (unstimulated) IVF cycles. *Human Reproduction*, 16(2), 259–262.
- National Collaborating Centre for Women's and Children's Health (UK). (2013). Fertility Assessment and Treatment for People with Fertility Problems. In *NICE Clinical Guidelines*, No. 156. London: Royal College of Obstetricians & Gynaecologists.
- Ojha, K., Philips, Z., & Darne, F. J. (2003). Diagnosing infertility in a district general hospital: A case-note and cost analysis. *Human Fertility (Cambridge, England)*, 6(4), 169–173.
- Ombelet, W. (2013). The walking egg project: Universal access to infertility care - from dream to reality. *Facts Views Vis Obgyn*, 5(2), 161–175.
- Ombelet, W. (2014). Is global access to infertility care realistic? The walking egg project. *Reproductive Biomedicine Online*, 28(3), 267–272.
- Ombelet, W., Cooke, I., Dyer, S., Serour, G., & Devroey, P. (2008). Infertility and the provision of infertility medical services in developing countries. *Human Reproduction Update*, 14(6), 605–621.
- Ombelet, W., Van Blerkom, J., Klerkx, E., Janssen, M., Dhont, N., Mestdagh, G., Nargund, G., & Campo, R. (2014). The (t)WE lab simplified IVF procedure: First births after freezing/thawing. *Facts Views Vis Obgyn*, 6(1), 45–49.
- Omurtag, K. R., Styer, A. K., Session, D., & Toth, T. L. (2009). Economic implications of insurance coverage for in vitro fertilization in the United States. A review. *The Journal of Reproductive Medicine*, 54(11–12), 661–668.
- Ouhilal, S., Lachgar, H., Bissonnette, F., Buckett, B., Kadoch, I., St-Michel, P., & Mahutte, N. (2015). Public funding of IVF without age limits: a cautionary tale from Quebec. *Fertility and Sterility*, 104(3), e207.
- Pelink, M. J., Hoek, A., Simons, A. H., & Heineman, M. J. (2002). Efficacy of natural cycle IVF: A review of the literature. *Human Reproduction Update*, 8(2), 129–139.
- Pharoah, P. O., & Cooke, T. (1996). Cerebral palsy and multiple births. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, 75(3), F174–F177.
- Practice Committee of the American Society for Reproductive Medicine. (2015). Testing and interpreting measures of ovarian reserve: A committee opinion. *Fertility and Sterility*, 103(3), e9–e17.
- Siristaidis, C., Trivella, M., Chrelias, C., Sioulas, V. D., Vrachnis, N., & Kassanos, D. (2012). A short narrative review of the feasibility of adopting mild ovarian stimulation for IVF as the current standard of care. *Archives of Gynecology and Obstetrics*, 286(2), 505–510.
- Smith, J. F., Eisenberg, M. L., Millstein, S. G., Nachtigall, R. D., Sadetsky, N., Cedars, M. I., Katz, P. P., & Infertility Outcomes Program Project, G. (2011). Fertility treatments and outcomes among couples seeking fertility care: Data from a prospective fertility cohort in the United States. *Fertility and Sterility*, 95(1), 79–84.
- Spar, D. (2006). *The baby business: How money, science, and politics drive the commerce of conception*. Harvard Business School Press.
- Stephoe, P. C., & Edwards, R. G. (1976). Reimplantation of a human embryo with subsequent tubal pregnancy. *Lancet*, 1(7965), 880–882.
- Stephoe, P. C., & Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. *Lancet*, 2(8085), 366.
- Teoh, P. J., & Maheshwari, A. (2014). Low-cost in vitro fertilization: Current insights. *International Journal of Women's Health*, 6, 817–827.
- Van Blerkom, J., Ombelet, W., Klerkx, E., Janssen, M., Dhont, N., Nargund, G., & Campo, R. (2014). First births with a simplified culture system for clinical IVF and embryo transfer. *Reproductive Biomedicine Online*, 28(3), 310–320.
- Van Voorhis, B. J., Sparks, A. E., Allen, B. D., Stovall, D. W., Syrop, C. H., & Chapler, F. K. (1997). Cost-effectiveness of infertility treatments: A cohort study. *Fertility and Sterility*, 67(5), 830–836.
- Williams, S. C., Gibbons, W. E., Muasher, S. J., & Oehninger, S. (2002). Minimal ovarian hyperstimulation for in vitro fertilization using sequential clomiphene citrate and gonadotropin with or without the addition of a gonadotropin-releasing hormone antagonist. *Fertility and Sterility*, 78(5), 1068–1072.
- Wu, A. K., Elliott, P., Katz, P. P., & Smith, J. F. (2013). Time costs of fertility care: The hidden hardship of building a family. *Fertility and Sterility*, 99(7), 2025–2030.

THE SUPPORT TEAM

Patient Support in the ART Program

Sharon N Covington, Shady Grove Fertility, Rockville, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Overview

Infertility is a life crisis as old as time. Despite the mind-boggling technological advances in the diagnosis and treatment of impaired fertility, the one element that has remained constant is the profound emotional response a person or couple usually experiences when their desire to have a baby is thwarted. The psychic conflict produces an amalgamation of emotions related to loss and grief, as well as stress and distress, which is well documented throughout the literature (Covington and Burns, 2006).

The psychological understanding of infertility has shifted over the years with the growth of patient advocacy and evidence based approaches to care. In the early to mid-1900's, when no medical reason could be found for infertility, it was proposed that patient psychopathology was the etiological factor. This psychogenic model of infertility prevailed until the late 1970's when a shift occurred, partially through the advocacy work of Barbara Eck Menning, formulating that emotional distress is the consequence and not a cause of infertility (Covington and Burns, 2006). The psychological sequelae model identified numerous psychological factors, affecting all aspects of an individual's and couple's life, which result from infertility. The application of this model has led away from the individualistic perspective of the psychogenic model to a more holistic approach whereby interactions among individuals/couples and social/medical components must be factored into medical treatment. Further developments emerged with extensively reviewed research on the negative impact of infertility and its treatment on the well-being of patients that has resulted in the development of evidence-based interventions to support coping and resiliency (Boivin and Gameiro, 2015).

Assisted reproductive techniques (ARTs), while opening up expanded opportunities for the treatment of infertility, have generated their own psychological challenges for patients. For most couples, ARTs are the last, best option for having a child, often occurring after long months, and sometimes years, of treatment failure, and typically at tremendous emotional, physical, and financial cost. Patients entering ART programs usually do so with the burden of grief and disappointment from infertility, acting depressed, angry, tired, dependent, and anxious. Although emotionally depleted, couples are attracted to a technology that offers hope where, not so long ago, none existed. They find themselves drawn into a new emotional turbulence of contrasting feelings of hope and despair, which seem to be generated in part by the experience of the technology itself. The intensity and high-tech nature of ART create a stressful atmosphere, where the stakes are high and the chance of success may be relatively low.

There is an increasing awareness of the burden of infertility treatment, especially in the ART program. Issues related to psychological strain and distress, poor interactions between patient and clinic staff, and the physical drain of treatment itself are all considered to be reasons for discontinuation from medical assistance. When factored together, the single biggest reason for ending treatment before having a child, is not related to money or time, but rather the emotional cost of treatment and its affect has on one's life (Gameiro et al., 2012). Thus, identifying ways to reduce the emotional burden of treatment and provide better supportive care is fundamental in any ART program, with the goal being delivering good care and positive treatment outcome (Boivin et al., 2012). The recognition of treatment burdens has resulted in a growing consensus among all reproductive medical organizations that patients seeking assistance need a "patient-centered" approach, care that is focused on individual patient needs, while being responsive and respectful to patient preferences and values (Van Emple et al., 2011). Patient-centeredness is considered a core construct in quality medical care. Taking a patient-centered approach, this section will focus on providing support in the ART program by identifying levels of psychosocial care, types of services offered, and how best to provide access to resources.

Levels of Support Care

As noted, given the substantial research on the emotional consequences of infertility and ART treatment, it is clear that patients need psychological support as an integrated part of the medical treatment process. "Who is to provide the care?" is sometimes the question. As reproductive technology has advanced, so has the role of mental health professionals (MHPs), which include social workers, psychologists, psychiatrists, marriage and family therapists, licensed professional counselors, and psychiatric nurses, as important members of the reproductive medical team (Boivin and Gameiro, 2015). The specialization of "[in]fertility counseling" has evolved internationally, combining the areas of reproductive health psychology and reproductive medicine, providing skilled psychosocial care in all phases of treatment. The phrase "infertility counseling" itself has shifted over time to "fertility counseling" reflecting more positive terminology and broader reproductive health perspective regarding treatment of patients who may not be technically infertile (i.e., lesbian, gay, single, etc.) (Covington, 2015). Fertility counselors serve as a resource to patients and staff by providing specialized psychological services that support and enhance quality care. For example, the complex medical and

psychological issues in third-party reproduction have social and legal implications that must be assessed carefully, and warrant involvement of a qualified MHP experienced in fertility counseling. As part of patient preparation, the implications and psychosocial impact on the off-spring created by third-party reproduction must be considered, and assistance given to families dealing with these issues pre- and posttreatment.

Nevertheless, the responsibility for patient support in the ART program is the duty of all staff members, not just the domain of nurses or fertility counselors. Interactions with each staff member, from administrative to physician, influence a patient's perception of care and, in turn, his or her stress level. Good communication with sensitivity, warmth, patience, and responsiveness create an environment of support. Also, the general clinic routine and ambience reflect support and respect of patients when it is provided in an efficient, organized, clean, uncrowded, and esthetically pleasing atmosphere. All staff need to be sensitive to and knowledgeable about the psychological needs and stresses of ART patients (Gameiro et al., 2013). While the primary focus of physicians, nurses, laboratory scientists, and other healthcare staff is the medical diagnosis and treatment of infertility, it must also entail "treating the patient, not the disease", thus necessitating a patient-centered approach.

Routine Versus Specialized Care

Taking an evidence-based method, the European Society of Human Reproduction and Embryology (ESHRE) published extensive guidelines for routine psychosocial care for fertility staff (Gameiro et al., 2015). Underscoring the word "routine", the Guidelines state that psychosocial care must be the responsibility of all staff members with patient contact, and occur across the treatment spectrum from the beginning when the diagnosis process is started, throughout treatment phases, and at the end of treatment particularly when it is not successful. Routine care includes providing information, resources, self-help support tools and interventions, as well as overall positive clinic environment. Four domains of care are identified according to patient needs: behavioral (life style, nutrition, exercise, and compliance); relational (partner relationships, family, friends, and social network); emotional (anxiety, depression, quality of life and well-being); and cognitive (understanding of treatment, concerns, and knowledge). Specific recommendations are given on patient needs, identified as conditions necessary for a healthy experience, on these four domains all of which are supported by an extensive review of the literature.

While routine psychosocial care is the responsibility of all reproductive clinic staff in providing patient support, specialized psychosocial care must be provided by qualified MHPs trained in fertility counseling and psychotherapy. Despite the stressful consequences of infertility and ART, numerous studies have shown that the clear majority of patients are well adjusted, psychologically resilient and maintain adequate social function. For the roughly 20% of patients who are at risk of clinically significant emotional problems (Verhaak et al., 2010), psychosocial services should be provided trained fertility counselors ideally integrated in as part of the clinic staff (Covington and Burns, 2006; Covington, 2015). Specialized patient support services provided by trained fertility counselors may include psychosocial assessment and evaluation; implications, supportive and therapeutic counseling; and psychoeducational counseling.

Types of Support Services

ART patient support services are as routine as overall clinic administration, communication, and environment to specific specialized psychological services involving psychoeducation, assessment and psychosocial intervention. From the routine to the specialized, the methods of providing patient support services will be categorized for this discussion as clinic management; information and education; assessment; therapeutic counseling; and supportive counseling (see Fig. 1).

Clinic Management

The approach in which a clinic is managed, along with physical setting, is an essential aspect of patient support and a means of promoting better quality of care. Interactions between staff and patients as well as the overall environment of clinic operations impact patients on the most basic of levels—patients' stress levels, perception of care and emotions. In a systematic review of patient satisfaction studies, Dance et al., identified that clinic factors were significant in care and problematic in that the level of satisfaction was low (Dancet et al., 2010). Physical comfort was important as well as other factors such a caring relationship (good attitude, sensitivity, trustworthiness, etc.) and good technical skills (high quality information, counseling, best medical options, and comprehensive testing and treatments) provided by competent staff. Unfortunately, the consensus from the study reviews were that patients viewed clinic factors as greatly lacking.

Staff interpersonal skills that enhance good communication, empathy, and respect are a fundamental aspect of patient support, yet may be often overlooked or minimized. Simple interactions that personalize care, such as introducing oneself, identifying the reason for appointment, giving information about procedures, looking patients in the eye and asking about concerns provide patient support. Classes in effective communication are a means of training staff in skills that are as important as their clinical abilities, and should be offered to all staff with patient contact. Often staff feel inadequately trained to deal with difficult patient situations, from complaints, delivering bad news, to strong emotions. In addition, helping staff "find the right words" in various contexts of patient care can be very effective in helping patients deal with the burdens of treatment (Boivin et al., 2012).

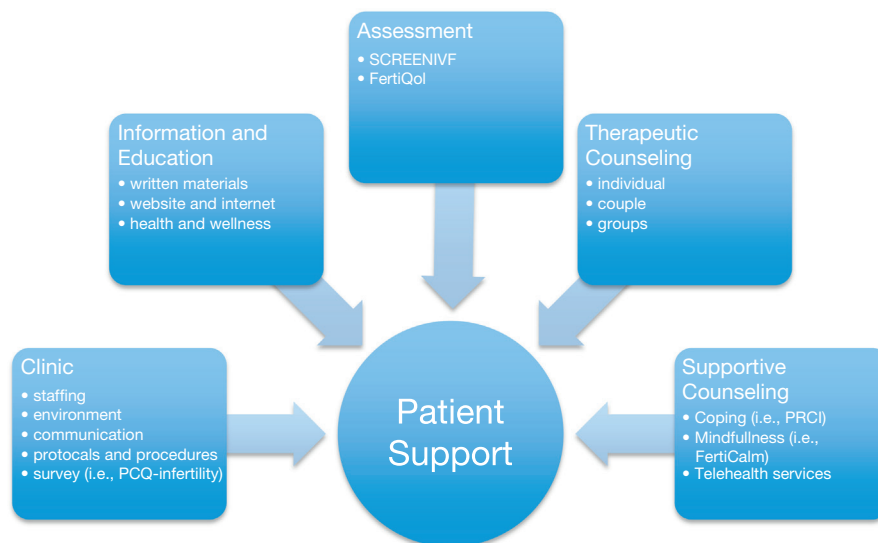


Fig. 1 Types of support services.

Communication training can also potentially help with staff turnover, as it can provide skills in dealing with difficult situations that may lead to compassion fatigue and burnout.

Clinic management should also consider the physical burden of treatment on patients and how protocols and procedures might be adjusted to lessen them. For some patients, the physical demands of treatment are more difficult to deal with than the emotional. Bodies being poked, prodded, and punctured with needles, instruments, medications, and intrusive procedures (often by people patients view as total strangers) are depleting, exhausting and painful. Considering medications and protocols that may be less invasive and physically disruptive, while allowing patients to participate in decision-making can be helpful. Boivin and colleagues reviewed a number of interventions which target treatment and clinic management demonstrated to be effective in patient centered care (Boivin et al., 2012). In addition, tools are now available to help clinics reliably survey their patient's experiences with focused fertility care. The Patient Centeredness Questionnaire-Infertility (PCQ-infertility) measures patient's particular experiences, rather than overall satisfaction, and thus can be tailored to improving clinic specific care (Van Emple et al., 2011). Reviewing periodically with such a tool can help clinics make adjustments to care and environment that are focused on patient needs and which encourage compliance with treatment.

Information and Education

Probably the most far-reaching opportunity for ART support is through patients' easy access to written information and education about the medical and psychological aspects of infertility. Patients rely heavily on the educational materials that document the process and procedure of ART, and search out information at the clinic, through the media (TV, magazines, books, etc.), and on the internet. Patients often identified informational materials as a primary source of support, after talking with spouse, family, or friends, and thus need assistance in accessing reliable and accurate information on their care and condition. The American Society of Reproductive Medicine (ASRM) has a section devoted to patient education, fact sheets, and educational videos that cover a wide range of reproductive health topics (www.reproductivefacts.org). Patient advocacy groups, such as RESOLVE (www.resolve.org), also have an abundance of information and resources on infertility and family building options. In addition, excellent material for patients who have or are using donor assistance in creating a family is available through the Victorian Assisted Reproductive Treatment Authority (www.varta.org.au) as well as other advocacy groups, such as the Donor Conception Network (www.dcnetwork.org).

Any information and treatment packets sent out to new patients should include material on the emotional aspects of infertility and on support resources available through the clinic, in the community, and via the Internet. For example, men are sometime forgotten in educational materials, particularly on coping, and a two-page booklet helping men prepare for a fertility workup, could be considered in these materials (Pook and Krause, 2005). A clinic's website is also an important source of support information and could connect to other internet resources, articles, advocacy groups, etc. for easy patient access.

Another area of education occurs in services that increase involvement with patients' overall health and well-being. A clinic may want to consider offering a "wellness program" that includes resources to enhance coping during treatment including acupuncture and yoga. In addition, nutritional programs that help patients address weight issues which can impact fertility treatment is often a needed area of support and intervention. At the very least, clinics need to identify referral sources within the nearby community and provide patients with written materials on where they can find sound professionals who provide services in these areas.

Assessment

The shift to patient centeredness has resulted in the development of a several psychosocial tools available to help identify patients' at risk for distress at the start of medical evaluation. Every patient brings a psychological history to infertility and some, as a result, may be more vulnerable to the experience. Being able to identify patients at risk before treatment begins (as well as during treatment) can assist in providing support and therapeutic interventions that will help in their care and the ability to comply with treatment. SCREENIVF is a validated, self-administered tool that provides specific information on those at risk, including a patient's vulnerability to depression, anxiety, fertility cognitions, and need for social support (Verhaak et al., 2010). The Fertility Quality of Life (FertiQoL) measures treatment quality (interactions with staff and quality of information) and treatment tolerability (effects on mood and disruptions to daily life), and proves to be an invaluable tool for clinicians. It is completed on line (with results sent to the clinician), currently available in 31 languages, and takes about 10–15 min to complete (www.fertiquol.org) (Boivin et al., 2011). Both these tools are at no cost. While there are a number of other validated, general self-screening inventories for depression and anxiety, the tools mentioned are specific to the infertility and its treatment.

Therapeutic Counseling

Another aspect of patient support services involves specialized intervention and treatment care for the consequences of infertility, or for underlying psychological disturbances that could affect medical treatment. Fertility counselors can provide treatment modalities of individual, couple, and group counseling to assist patients in understanding and handling the emotional sequelae of infertility; identifying and developing coping mechanisms to deal with treatment; managing the effects of infertility or psychosocial history on interpersonal functioning (anxiety, depression, etc.) and on marital, sexual, and social relationships; considering the implications of ART treatment; decision-making on treatment options and alternative family building; pregnancy and parenting following treatment; and ending treatment and building a life after infertility. Appropriate mental health referral sources for psychotherapy should be made available for patients with pre-existing emotional problems and those issues exacerbated by treatment. Unfortunately, research has found that while some patients experience significant symptoms of depression and anxiety, most do not receive referral for appropriate mental health services (Pasch et al., 2016).

Group counseling has been shown to be a highly effective, cost efficient intervention for producing positive change, particularly when education and skills training (e.g., relaxation techniques) are emphasized. Regular support group meetings, offered in the clinic and facilitated by a fertility counselor, help with the emotional isolation patients often feel with infertility. These groups can also provide a forum for learning coping strategies which ultimately allow patients to continue with treatment (Covington and Burns, 2006). As an example, groups can be an effective way to help patients change unhealthy behaviors (smoking, overweight, etc.) which effect treatment, though psychoeducation and peer support. In addition, groups can be used to prepare and educate patients about third-party reproduction, when required for treatment, and help “normalize” the experience in a way that individual or couple counseling cannot.

Supportive Counseling

Supportive counseling involves reproductive healthcare providers giving both advice (counsel) and comfort (console) to patients. Nurses often assume primary responsibility for patient support, yet all members of the treatment team have opportunities for advising and consoling. To begin, clear information needs to be provided to patients on what services are available and how they can access psychosocial support, whether through the clinic or outside resources. This information should be clearly displayed and included on all materials distributed to patients. Information on patient advocacy groups, such as RESOLVE, which offer peer support forums and web-based resources should also be included.

Patients need to be educated and offered easy tools to help in coping with treatment. These tools are self-administered and require minimal explanations from clinic staff to use. The two-week waiting period before a pregnancy test has been shown to be one of the most difficult times during the treatment cycle. The Positive Reappraisal Coping Intervention (PRCI) helps patients work on reappraising aspects of the infertility experience in a more positive way (possible benefits rather than focusing on the costs) which help to change feelings (Lancastle and Boivin, 2008). The PRCI is also helpful during other times of uncertainty, such as with patients experiencing recurrent miscarriage, and can be used throughout the day when anxiety or negative thoughts take over. The information can be placed on a card for easy use. In addition, a number of apps for android phones help patients with an array of mind-body techniques related to breathing, mediation, graduated relaxation, guided imagery, etc. *FertiCalm*, designed by two reproductive health psychologists, specifically targets the many aspects of infertility treatments with cognitive-behavioral techniques for coping.

Access to Resources

Support services can be delivered in the “here and now” of clinic interactions, operations, and on-site counseling as well as in the “virtual” reality of computer mediated technology. To begin, a clinic's website reflects the practice's patient centeredness and is an important source of medical and support information. Articles, blogs, seminars and webinars, and resource information help to

educate patients and convey a message about clinic philosophy in patient care. Many clinics now have Facebook pages for their patients to connect on a variety of infertility related topics (i.e., single women, PCOS, pregnancy loss, etc.). The clinic website serves an introduction to the practice and can serve as a springboard to connecting patients to other reliable internet sites for medical and psychosocial information.

Computer-based technology has become a powerful source of information and emotional support for patients. A scoping review of patient-focused internet based intervention studies in fertility care found a number of multimedia, interactive formats being applied for support, education, and promoting mental health (Aarts et al., 2012). Thus, the internet can provide an effective forum for intervention in helping patients manage the distress of infertility, yet receiving direction from the medical staff on reliable Internet sites for information is needed. Social media, such as Facebook and Twitter, have become resources for support, interaction, and information for infertile patients when managed by the clinic, as well as providing a marketing tool for the practice. Finally, providing Internet access to personal health records and medical data is increasingly being considered within reproductive clinics to improve patient empowerment and satisfaction with care (Van Empe et al., 2011).

When possible, clinics should have a trained MHP on-site to offer easy access to patients for specialized psychosocial care of individual, couple and group counseling. A clinic fertility counselor, also, has the ability to help in staff training on effective communication as well as managing staff stress levels which can lead to burnout and turnover. At the very least, clinics should have a list of trusted fertility counselors as referral sources for patients, and all counseling resources should be easily accessed within the clinic and on their website.

The increasing use of telehealth in patient care as well as the reality that patients may be traveling great distances for treatment, presents the opportunity to provide counseling and patient support via secure teleconferencing platforms. It is important to have staff who are competent and comfortable in using teleconferencing. While a number of servers exist that are HIPAA compliant, it is imperative for patients to understand that security and confidentiality cannot be completely ensure when using any internet-based technology. No doubt, the field of telehealth will continue to evolve and offer more opportunities for patients to receive support wherever they are located, while requiring clinics (and staff) to be current and proficient in these technologies.

Table 1 Support strategies across treatment

Before

- Educational classes presented by each member of the treatment team on IVF
- Pretreatment assessment tools (i.e., SCREENIVF and FertiQol)
- Pretreatment counseling session with an fertility counselor
- Psychosocial preparation and assessment of gamete donors, recipients, and surrogates with a fertility counselor
- Extensive written materials available and distributed on the medical, emotional, and financial aspects of ART
- Educational videos on the medical and emotional aspects of infertility and ART
- Support groups
- Educational and support materials available on clinic website, including podcasts, webinars, and social media outlets
- Stress management, relaxation, and guided imagery classes
- Resource lists of community support services including RESOLVE, Inc.

During

- Access to a fertility counselor and other team members
- Telephone support with a primary-care nurse
- If a patient has met with a fertility counselor before starting the cycle, a brief visit in the OR on retrieval and/or transfer day
- Stress management, relaxation, and guided imagery classes
- Complementary programs such as acupuncture, yoga, and nutrition counseling
- Coping materials for the two week wait or during times of uncertainty (i.e., PRCI)
- Computer-mediated technology, including clinic website with resource materials and interactive social media (e.g., Facebook and Twitter); online educational webinars, written materials identifying reliable Internet sites for information and support, and android phone Apps supporting coping techniques (i.e., FertiCalm)
- Support groups

After

- Psychosocial follow-up after a failed cycle or pregnancy loss
- Decision-making counseling regarding alternative therapies or ending treatment
- Counseling on alternative family building through adoption or third-party reproduction
- Counseling and support for the decision to remain child-free after infertility
- Counseling and preparation for multiple pregnancy, including selective reduction
- Counseling and follow-up for pregnancy after infertility, including support groups
- Counseling and follow-up for issues in parenting after infertility, including families created through donor gametes
- Support groups
- Patient feedback survey (i.e., PCQ-infertility)

Conclusion

Patient support is the responsibility of all who work in an ART program. Infertility is an emotionally exhausting, psychologically demanding experience for patients and, at times, their caregivers. Since ART is considered the most stressful of all infertility treatments, patients who undergo it need as much support psychologically as they do medically from their clinical team. It can begin before a patient steps through the door of a clinic requesting evaluation and may continue after the patient ends treatment, whether pregnant or not.

Table 1 identifies strategies which will assist practices in providing patient support services across the treatment spectrum. Integrating in these services as part of patient care and training all staff to be sensitive to the emotional needs of infertility patients will help to minimize the burdens of ART treatment, thus offering the best opportunity for helping patients achieve their goals in family building.

References

- Aarts, J. W., van den Haak, P., Nelen, W. L., et al. (2012). Patient-focused internet interventions in reproductive medicine: A scoping review. *Human Reproduction Update*, 18, 211–227.
- Boivin, J., Takefman, J., & Braverman, A. (2011). Fertility quality of life (FertiQoL) tool: Development and general psychometric properties. *Fertility and Sterility*, 96, 409–415.
- Boivin, J., Domar, A. D., Shapiro, D., et al. (2012). Tackling burden in ART: An integrated approach for medical staff. *Human Reproduction*, 27, 941–950.
- Boivin, J., & Gameiro, S. (2015). Evolution of psychology and counseling in infertility. *Fertility and Sterility*, 104, 251–259.
- Covington, S. N., & Burns, L. H. (Eds.). (2006). *Infertility counseling: A comprehensive handbook for clinicians* (2nd edn.). New York: Cambridge University Press.
- Covington, S. N. (Ed.). (2015). *Fertility counseling: Clinical guide and case studies*. Cambridge, UK: Cambridge University Press.
- Dancet, E. A., Nelen, W. L., Sermeus, W., et al. (2010). The patients' perspective on fertility care: A systematic review. *Human Reproduction Update*, 16, 467–487.
- Gameiro, S., Boivin, J., Peronace, L., & Verhaak, C. M. (2012). Why do patients discontinue fertility treatment? A systematic review of reasons and predictors of discontinuation in fertility treatment. *Human Reproduction Update*, 18, 652–669.
- Gameiro, S., Boivin, J., & Domar, A. (2013). Optimal *in vitro* fertilization in 2020 should reduce treatment burden and enhance care delivery for patients and staff. *Fertility and Sterility*, 100, 302–309.
- Gameiro, S., Boivin, J., Dancet, E., et al. (2015). ESHRE guideline: Routine psychosocial care in infertility and medical assisted reproduction—A guide for fertility staff. *Human Reproduction*, 30, 2476–2485.
- Lancastle, D., & Boivin, J. (2008). A feasibility study of a brief coping intervention (PRCI) for the waiting period before a pregnancy test during fertility treatment. *Human Reproduction*, 23, 2299–2307.
- Pasch, L. A., Holley, S. R., Bleil, M. E., et al. (2016). Addressing the needs of fertility treatment patients and their partners: Are they informed of and do they receive mental health services? *Fertility and Sterility*, 106, 209–215.
- Pook, M., & Krause, W. (2005). Stress reduction in male infertility patients: A randomized, controlled trial. *Fertility and Sterility*, 83, 68–73.
- Van Empe, I., Hermens, R., Akkermans, R., et al. (2011). Organization determinants of patient-centered fertility care: A multilevel analysis. *Fertility and Sterility*, 95, 513–519.
- Verhaak, C. M., Lintsen, A. M. E., Evers, A. W. M., & Braat, D. D. M. (2010). Who is at risk of emotional problems and how to know? Screening of women going for IVF treatment. *Human Reproduction*, 25, 1234.

The Role of the Mental Health Professional in the ART Clinic Setting

Alice D Domar, Domar Center for Mind/Body Health, Waltham, MA, United States; Boston IVF, Waltham, MA, United States; Beth Israel Deaconess Medical Center, Boston, MA, United States; and Harvard Medical School, Boston, MA, United States

Kristin L Rooney, Domar Center for Mind/Body Health, Waltham, MA, United States; and Boston IVF, Waltham, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Infertility is commonly misconstrued as a purely physiological issue, having little to do with the psychological state of the patient or her partner. While the primary doctors in charge of infertility are the obstetrician/gynecologist and the reproductive endocrinologist, the mental health professional (MHP) also plays a pivotal role in the world of infertility. MHPs are under-utilized by most infertility centers, with their role being restricted to just third party evaluations (third party referring to egg donation, sperm donation, gestational surrogacy, and/or embryo donation) in some centers. However, MHPs can and are a highly effective member of the infertility care team through patient interventions that decrease stress, which can lower the emotional burden for patients as well as potentially decreasing dropout rates and increasing pregnancy rates. In addition, being a nurse or other caregiver for couples struggling with infertility can lead to burnout and depression; providing mental health services to nurses may improve the infertility experience for patients and reduce nursing turnover due to burnout. Finally, the MHP can provide training in empathic skills communication to the entire medical team, including physicians, which could also lead to a lower burden of care in patients.

The role of the MHP in the infertility setting has changed dramatically over the past few decades. The “pioneer” practitioners focused on counseling by conducting psychoeducational sessions for patients facing treatment, identifying which patients expressed the most distress and offering therapy sessions in an effort to relieve at least some of that distress. They also educated the medical community that infertility patient care needed to focus on the whole patient, not just the physical aspects of the diagnostic workup and subsequent treatment.

Currently, most MHPs who specialize in reproductive medicine dedicate all or most of their time to third party patients. The American Society for Reproductive Medicine (ASRM) guidelines include recommendations or requirements that individuals and couples who are pursuing egg or sperm donation, surrogacy, or embryo donation receive counseling. Thus, many MHPs do not have the availability to see non-third party patients and/or the medical team does not think to refer them for counseling. This model of care does not benefit the vast majority of infertility patients or the health care team. The MHP needs to hold a more vital role in the care of our patients in order to improve the treatment experience, potentially increase fertility rates, as well as leading to decreases in dropout rates.

Stress in Infertility

The current research on mental health disorders in infertile women, as well as couples, is consistent and clear; women and men experiencing infertility report heightened levels of anxiety and/or depression. In fact, patients struggling with infertility have similar levels of anxiety and depression as patients with cancer, heart disease, or HIV positive status (Domar et al., 1993).

In a gold-standard study which utilized a structured psychiatric interview instead of a self-report questionnaire (in which many patients may “fake good” in order to appear able to handle treatment), women visiting a fertility center for the first time met with a psychiatrist. Of the 112 participants, 40% were diagnosed with a psychiatric disorder, the most common being anxiety and depression (Chen et al., 2004). In a larger study of IVF patients, utilizing a self-reported psychiatric questionnaire, 31% of females and 10% of males had a psychiatric diagnosis: depression, anxiety or both (Volgsten et al., 2008). The study also determined that most of the patients with the psychiatric disorders were undiagnosed and untreated, with only 21% receiving any treatment.

In regards to suicide risk, a study on 106 women presenting at an infertility clinic found that 9% of women reported having suicidal thoughts or attempts; women who had no or fewer children and had higher levels of depressive symptoms were most likely to report suicidal symptoms (Shani et al., 2016). Researchers suggest that infertility patients may also display symptoms of obsessive-compulsive disorder, psychoticism, and battle with substance abuse and eating disorders (De Berardis et al., 2014).

Stress and Treatment Termination

Until a series of studies was published in 2004, it was assumed that patients dropped out of treatment for only two reasons—either they didn’t have the money to undergo further treatment, or their physician recommended that they discontinue treatment due to a poor prognosis (called active censoring). In fact, finances are the most common reason for treatment termination in uninsured patients. However, recent research consistently demonstrates that the emotional burden of treatment is the most common reason given by insured patients who drop out of treatment.

Patient drop out is an issue worldwide. In the Netherlands, 32% of patients dropped out before completing three cycles, in Germany 39% dropped out after the first cycle, and in France more than a third dropped out after only one cycle (Troude et al., 2014). In Israel, couples are offered unlimited free IVF until they have two children, and their centers also experience patient

dropouts. A cohort-study showed that the main reasons for treatment termination in a cost-free environment like Israel were psychological burden and lost hope of success (Lande et al., 2015). In our most recent study, we surveyed 268 women who had insurance coverage for six IVF cycles but did not complete them, nor did they achieve a pregnancy. The most prevalent reason for discontinued treatment was that it was too stressful (41%) (Domar et al., 2016), which aligned with our prior research showing 39% dropped out due to stress (Domar et al., 2010).

There are many factors associated with treatment termination, including depressive symptoms prior to the first cycle, biochemical pregnancy or miscarriage from a previous cycle, older age, perception of poor prognosis, lost hope for success, and minimal family support. In order to decrease patient burden, Boivin et al. (2012) created a list of recommendations to support infertility patients undergoing IVF treatment. These include tailored educational materials, checklists to make sure all treatment worries are addressed, screening high risk patients for psychological issues, and implementing coping interventions for patients. It is also recommended to refer high risk patients for emotional support with a MHP who specializes in reproductive medicine, and to ensure that the partner is involved.

In general, the research suggests that the emotional and psychological needs of our patients are not being met. In a recent study of men and women undergoing IVF (Pasch et al., 2016), 57% of women and 32% of men reported depressive symptoms and 76% and 61% respectively reported symptoms of anxiety. Of the patients displaying psychiatric symptoms, only 21% of women and 11% of men reported that they had received mental health services; about 25% of participants stated that the fertility clinic had made information on mental health services available to them. The men and women who had reported significant depressive and/or anxiety symptoms, even those with a prolonged history of symptoms, were no more likely than other patients to have received information about mental health services. The authors concluded that emotional distress is common, most patients don't receive mental health services information or referrals, and most importantly, there is no targeted approach for those most in need.

Psychological Interventions for Infertility Patients

There have been numerous randomized controlled studies on the efficacy of psychological interventions for infertility patients. There are at least three meta-analyses which summarize the data. In the first (Boivin, 2003), the psychological interventions which were identified as being the most effective in reducing psychological distress were those which included specific coping strategies for reducing distress. In the second (Hammerli et al., 2009), the interventions which were identified as being the most effective were group sessions of at least six sessions. Finally, a recent meta-analysis (Frederiksen et al., 2015), indicated that psychological interventions which had a cognitive-behavioral therapy (CBT) focus were the most effective. Thus, if one combines the results of the three meta-analyses, a group intervention of at least six sessions, with focused coping strategy acquisition based on CBT should provide the best relief to women struggling with infertility.

The Mind/Body Program for Infertility was first launched in 1987 and there have been a number of RCT's to examine its effectiveness (Domar et al., 2000a,b, 2011). The 10-week group program includes relaxation exercises, cognitive strategies, lifestyle modification, as well as group support. Research indicates that participants experience significant reductions in psychological distress, stress-related physical symptoms such as insomnia and headaches, as well as increases in pregnancy rates. The clinical program is currently offered at numerous centers throughout the US, as well as internationally. Participants report improvements in all symptoms and approximately 55% conceive within 6 months.

Nursing Burnout Prevention

Another critical role of the MHP in the infertility clinic setting is in preventing nursing burnout. Burnout occurs when one feels overwhelmed by extreme prolonged stress. Many nurses begin to experience burnout as the stress of their job increases, and they then experience lower levels of motivation and dedication to their patients. In fact, prior findings suggest nurses leave their job or abandon the practice of nursing all together as a result (Constable and Russell, 1986).

Previous research suggests that the factors contributing to nursing burnout are due to low job enhancement, work pressure, and lack of supervisor support (Constable and Russell, 1986). In a survey of more than 700 nurses, it was determined that those who were motivated primarily by the desire to help others, rather than by enjoyment of the work, were more likely to burnout (Dill et al., 2016). Unfortunately, the characteristics that one looks for in a REI nurse—a deep sense of commitment and compassion, as well as being extremely detail-oriented—are also characteristics associated with the development of burnout.

In a 2016 survey of ART clinic nurses performed by ASRM, 80% reported that their workload had increased in the past year, more than 70% reported that their stress level had increased, 60%–70% reported that patient expectations/demands were the main contributors to nursing stress, and more than 70% had experienced burnout in their job setting (Tobias, 2016).

A survey from the European Society for Human Reproduction and Embryology (ESHRE) on staff burnout had similar findings (Boivin et al., 2017). The survey focused on three topics: work stressors, perceived sources of difficulties and understanding what, if any, workshops clinic staff be willing to attend. The findings of this survey concluded that time pressures, workload, and team management issues were among the top work stressors, while patients' unrealistic expectations, communication and counseling patients added to their stress. Survey respondents reported they felt they had to prioritize tasks from clinical duties and

administrative duties and did not have time to complete both to the fullest during the work day. Sadly, it was noted that there is not enough time to counsel patients and give them the attention and care they may need. As we know, infertile patients experience much distress during treatment, so referrals to a MHP are of even more importance since other staff may not be able to provide adequate attention to the patient's psychological needs.

Given the scope of infertility treatment, nurses are often presented with the burden of telling patients of a negative outcome. Having to share bad news on a regular basis can be emotionally draining. The role of the MHP can assist nurses by offering them coping techniques, or making themselves available to talk to if they have an especially difficult case.

Nurse-patient rapport is very important for patient satisfaction, and replacing nurses leads to problems in care. It is also very expensive to replace a nurse; it has been estimated that the cost to a practice to replace one nurse in the ART clinic setting is \$250,000 (Goldstein, 2016). MHP's can ensure that nurses have adequate preparation and support for their roles, provide opportunities where personal issues can be discussed, as well as guidance on when and how to refer a patient to a MHP (Payne and Goedeke, 2007). The 2016 ESHRE survey suggests that fertility clinic staff may be willing to attend workshops to reduce the burden of their job. The workshops that were of the most interest would focus on communicating and counseling with patients, reviewing difficult cases to improve success and dealing with patient expectations. Because infertility patients have a high prevalence of anxiety and depression, appropriate training for clinical staff working with patients is important. In addition, enhancing communication skills within the organization is beneficial to reducing staff burden. Infertility treatment is an emotional journey not only for the patient but for the clinic staff. Both the patients and staff have one common goal, to achieve a viable pregnancy, and negative outcomes can burden all parties involved in the patient's treatment.

Patient Centered Care/Empathic Communication

Finally, another benefit of having MHPs in the ART clinic setting is by reducing the burden of care through focusing more on patient expectations and communication. The importance of patient centered care is dramatically undervalued by physicians. In one study, 925 patients and 227 infertility physicians completed a questionnaire which asked about the relevant importance of travel time, pregnancy rate, physician attitude, continuity of physicians, and information on treatment (van Empel et al., 2011). The last three represent patient-centered care. Patients reported more value to patient-centeredness than physicians, and even rated patient centered care more important than pregnancy rates. While the physicians considered pregnancy rates to be of the utmost importance, patients considered patient-centered care to be of more importance. In fact, the lack of patient centered care was cited as the top non-medical reason for patients to change fertility clinics.

Patients respond well to effective physician communication; a study involving 13 infertility specialists reported on the differences in patient satisfaction before and after the physicians attended a weekend retreat which focused on empathic skills (García et al., 2013). Physicians were assessed by 2146 patients prior to and following their training. There were significant improvements in patient satisfaction on perceived information quality, communication skills and time spent for first visit. The intervention was so successful in terms of patient satisfaction with care that now every employee of the center receives this training.

MHPs are qualified to teach the other members of the health care team how to communicate empathetically. Research shows that three 60-min training modules can significantly impact a physician's ability to communicate with patients and can lead to increased patient satisfaction (Riess et al., 2012). Boivin et al. (2012) suggested that an integrated approach to fertility care may benefit both patients and clinic staff. By tending to the needs of fertility clinic staff, it may indirectly benefit patient wellbeing with regard to quality of life, compliance, and decrease drop outs.

The Boston IVF Model of Mental Health Integration

At Boston IVF, we feel that we have created a model that integrates MHPs in an efficacious and cost-effective manner. All third party sessions are provided by three social workers, who offer therapy sessions as well. The three Ph.D. psychologists on staff provide individual and couples counseling with a CBT approach. These visits are usually covered by insurance. The psychologists also offer the 10-week Mind/Body Program for Infertility which helps reduce distress among patients. Any patient in crisis is guaranteed to be able to meet with or speak with one of the psychologists within 60 min of the request, including on-site visits in the PACU. All patients with an abnormal prenatal ultrasound are offered a crisis intervention session within 60 min. Also, all patients who have experienced an unsuccessful treatment cycle are offered a free 30-min session with one of the psychologists. One of the psychologists is available for crisis counseling for any employee. All employees receive one free acupuncture session, and a 25% discount for subsequent sessions. "Stress lunches" are offered on a regular basis for any employee who wishes to learn stress management techniques.

This model of care meets the needs of patients and nurses alike. The nurses know that any patient in crisis will have their concerns addressed by a MHP immediately, lessening the burden on the nurses. Patients are aware that psychological resources are always available to them, often at no cost. This model is also cost-efficient for the infertility center. It is known that the burden of care is the most common reason given by insured patients for dropping out of treatment. Efficiently treating patients' emotional distress, combined with programs designed to decrease nursing burnout, both can combine to encourage patients to remain in treatment either until their physician recommends stopping for medical/prognosis reasons, or the patient conceives a healthy baby.

Conclusions

The mental well-being of infertility patients is often overlooked and can be equally important as the physical treatments. The three roles which the MHP can provide in the ART setting include providing the means to decrease patient distress, supporting nurses to prevent burnout, and teaching health care professionals how to communicate to patients in an empathic fashion.

Patients struggling with infertility may experience severe emotional distress, even including suicidal ideation. They need knowledgeable support throughout this process, as well as effective psychological interventions specifically aimed at infertility patients. The cost of treatment termination must be considered as well. Patients who drop out of treatment are unlikely to ever conceive, and the clinic loses a source of income. The MHP can provide such interventions designed to prevent these outcomes.

Nursing burnout can have a significant impact on patients and nurses. Nursing turnover is disruptive to patients and other staff members, is incredibly expensive for the clinic, and may have long-lasting repercussions for nurses' emotional and physical health. The MHP has the training to support nurses in an effort to prevent or decrease burnout.

Empathic communication is highly correlated to patient satisfaction. The MHP can train physicians, nurses, and support staff on the most effective forms of communication, designed to decrease patient distress.

While there may be a perception that the MHP is simply an added cost, with little return on investment, in fact the potential for the MHP to decrease costs cannot be overlooked. In addition, the return on investment in terms of patient and staff quality of life cannot be underestimated. Ideally, a MHP in the ART setting will lead to less patient distress, lower dropout rates, less nursing burnout, and increased patient satisfaction.

Acknowledgment

The authors would like to thank Adam Yecies for his assistance with the preparation of this manuscript.

References

- Boivin, J. (2003). A review of psychosocial interventions in infertility. *Social Science and Medicine*, 57, 2325–2341.
- Boivin, J., Domar, A., Shapiro, D., et al. (2012). Tackling burden in ART: An integrated approach for medical staff. *Human Reproduction*, 27, 941–950.
- Boivin, J., Bunting, L., Koert, E., Ieng, U. C., & Verhaak, C. (2017). Perceived challenges of working in a fertility clinic: A qualitative analysis of work stressors and difficulties working with patients. *Human Reproduction*, 32, 403–408.
- Chen, T. H., Chang, S. P., Tsai, C. F., & Juang, K. D. (2004). Prevalence of depressive and anxiety disorders in an assisted reproductive technique clinic. *Human Reproduction*, 19, 2313–2318.
- Constable, J. F., & Russell, D. W. (1986). The effect of social support and the work environment upon burnout among nurses. *Journal of Human Stress*, 12, 20–26.
- De Berardis, D., Mazza, M., Marini, S., et al. (2014). Psychopathology, emotional aspects and psychological counselling in infertility: A review. *La Clinica Terapeutica*, 165, 163–169.
- Dill, J., Erickson, R. J., & Diefendorff, J. M. (2016). Motivation in caring labor: Implications for the well-being and employment outcomes of nurses. *Social Science and Medicine*, 167, 99–106.
- Domar, A., Zuttermeister, P., & Friedman, R. (1993). The psychological impact of infertility: A comparison with patients with other medical conditions. *Journal of Psychosomatic Obstetrics and Gynecology*, 14, 45–52.
- Domar, A. D., Clapp, D., Slawsky, E., et al. (2000a). The impact of group psychological interventions on distress in infertile women. *Health Psychology*, 19, 568–575.
- Domar, A. D., Clapp, D., Slawsky, E. A., et al. (2000b). Impact of group psychological interventions on pregnancy rates in infertile women. *Fertility and Sterility*, 73, 805–811.
- Domar, A. D., Smith, K., Conboy, L., Iannone, M., & Alper, M. (2010). A prospective investigation into the reasons why insured United States patients drop out of in vitro fertilization treatment. *Fertility and Sterility*, 94, 1457–1459.
- Domar, A. D., Rooney, K. L., & Wiegand, B. (2011). Impact of a group mind/body intervention on pregnancy rates in IVF patients. *Fertility and Sterility*, 95, 2269–2273.
- Domar, A. D., Rooney, K., Rich, C., et al. (2016). Burden of care is the primary reason why insured women terminate IVF treatment. *Fertility and Sterility*, 106, e62–e63.
- Frederiksen, Y., Farver-Vestergaard, I., Skovgård, N. G., Ingerslev, H. J., & Zachariae, R. (2015). Efficacy of psychosocial interventions for psychological and pregnancy outcomes in infertile women and men: A systematic review and meta-analysis. *BMJ Open*, 28, e006592.
- García, D., Bautista, O., & Venereo, L. (2013). Training in empathic skills improves the patient–physician relationship during the first consultation in a fertility clinic. *Fertility and Sterility*, 99, 1413–1418.
- Goldstein, B. (2016). Presented at the annual SREI Fellows retreat, Park City, Utah.
- Hammerli, K., Znoj, H., & Barth, J. (2009). The efficacy of psychological interventions for infertile patients: A meta-analysis examining mental health and pregnancy rate. *Human Reproduction Update*, 15, 279–295.
- Lande, Y., Seidman, D., Maman, E., Baum, M., & Hourvitz, A. (2015). Why do couples discontinue unlimited free IVF treatments? *Gynecological Endocrinology*, 31, 233–236.
- Pasch, L. A., Holley, S. R., Bleil, M. E., et al. (2016). Addressing the needs of fertility treatment patients and their partners: Are they informed of and do they receive mental health services? *Fertility and Sterility*, 106, 209–215.
- Payne, D., & Goedeke, S. (2007). Holding together: Caring for clients undergoing assisted reproductive technologies. *Journal of Advanced Nursing*, 60, 645–653.
- Riess, H., Kelley, J. M., Bailey, R. W., Dunn, E. J., & Phillips, M. (2012). Empathy training for resident physicians: A randomized controlled trial of a neuroscience-informed curriculum. *Journal of General Internal Medicine*, 27, 1280–1286.
- Shani, C., Yelena, S., Reut, B. K., Adrian, S., & Sami, H. (2016). Suicidal risk among infertile women undergoing in-vitro fertilization: Incidence and risk factors. *Psychiatry Research*, 30, 53–59.
- Tobias, T. (2016). Presented at pre-congress course at ASRM, Salt Lake City, UT.
- Troude, P., Guibert, J., Bouyer, J., & de La Rochebrochard, E. (2014). Medical factors associated with early IVF discontinuation. *Reproductive Biomedicine Online*, 28, 321–329.
- van Empel, I. W., Dancet, E. A., Koolman, X. H., et al. (2011). Physicians underestimate the importance of patient-centredness to patients: A discrete choice experiment in fertility care. *Human Reproduction*, 26, 584–593.
- Volgsten, H., Skoog Svanberg, A., Ekselius, L., Lundkvist, O., & Sundström Poromaa, I. (2008). Prevalence of psychiatric disorders in infertile women and men undergoing in vitro fertilization treatment. *Human Reproduction*, 23, 2056–2063.

Acupuncture in the ART Clinic

Jeremy J Cottle and Alice D Domar, Harvard Medical School, Boston, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Acupuncture is a needle-based practice most often utilized to provide pain relief, local anesthesia, and treatment for illness, although it is claimed to have a plethora of other benefits. The first description of acupuncture is from China over 2000 years ago, where at the time it was believed that illness was caused when the life force known as “qi” couldn’t properly flow through the body, or when the two forms of qi “yin” and “yang” were imbalanced. It was thought that prodding certain points with fine needles would help restore the balance and flow of qi, causing the illness to subside. Similar theories of healing were prevalent in the Western world around the same time about the balance of “humors,” and Western doctors used to think people were sick due to an excess of blood and bile within the body. While blood-letting went out of style with the advent of modern medicine, acupuncture has made a strong comeback across the world since the 1950s as a tool used primarily for pain relief. It has also been used to treat depression, fibromyalgia, rheumatoid arthritis, high and low blood pressure, many varieties of musculoskeletal pain, and infertility (Paulus et al., 2002).

So far there have been few plausible theoretical physiological mechanisms for how this one treatment can be helpful for such a wide array of ailments. Whether or not acupuncture works is controversial. Even though over 3000 trials with attempted placebo-control groups have been conducted, there is still considerable debate over the efficacy of acupuncture.

The potentially analgesic, or pain-relieving, effects of acupuncture are believed to be triggered by the needles penetrating the skin, which then releases endorphins. Endorphins are hormones that, along with other neurotransmitters and endogenous opioid-like substances, can reduce pain and have been connected with the skin penetration of acupuncture (Wang et al., 2008). Other studies have found similar physiological effects of acupuncture, like one study published in *Nature Neuroscience* that found the local release of adenosine at the site of skin deformation from acupuncture needles (Goldman et al., 2010). This release of adenosine is linked with pain relief in acupuncture studies on mice, according to a 2014 review (Gorski, 2014). Acupuncture can also be anti-inflammatory (Kavoussi and Ross, 2007). A systematic review of 34 brain imaging studies suggests that the stress and pain reduction linked with acupuncture stimulation is due to modulated brain activity in the limbic system, somatosensory cortices, and other brain areas (Huang et al., 2012).

Despite these positive connections between acupuncture and health outcomes, there remains controversy over the quality of these claims. Systematic reviews have played an important role in the promotion of acupuncture for health benefits, but one review examined 88 Chinese acupuncture-related systematic reviews for quality, finding that the impact factor of over half the publishing journals was 0, and less than half reported assessing for publication bias (Ma et al., 2012). In fact, all trials originating in Asia were in favor of acupuncture. In comparison, only 53% of review trials in the United States showed favorable outcomes from acupuncture (Vickers et al., 1998). A 2006 meta-analysis published by the Journal of Internal Medicine found the results of acupuncture treatment could not be differentiated from the placebo treatment, suggesting that medical effects could really depend on a placebo response rather than a direct physiological impact (Ernst, 2006). A 2011 analysis of 57 systematic reviews showed similar findings, suggesting that there was little evidence supporting acupuncture as an effective form of pain relief, especially considering the lack of high-quality articles on the subject, the narrow scope of health benefits in those high-quality articles, and the potential for significant adverse effects, no matter how rare (Ernst et al., 2011). In 2012, however, a meta-analysis from the Memorial Sloan-Kettering Cancer Center came out in favor of acupuncture; the conclusions were that acupuncture had a significantly greater impact on chronic pain than placebo (Vickers et al., 2012). This was promising considering the addictive properties of pain-relieving medications which also have a large number of negative side effects.

While there is continued debate on the efficacy of acupuncture, when compared to other treatments for these ailments such as pain, acupuncture poses minimal risks, and has potential for great benefits.

Acupuncture and Female Infertility

Acupuncture has been used to increase fertility since its inception in China over 2000 years ago. In modern times, the cause of infertility is usually determined through a work-up and can be corrected/treated. However, some infertility patients do not receive a diagnosis and the causes of many failed IVF cycles are unknown, in which case many patients seek out alternative treatments in an effort to increase the chances of a pregnancy. The pioneering scientific study on the impact of acupuncture on infertility was done in 2002 (Paulus et al., 2002). This randomized controlled prospective study included 160 patients undergoing assisted reproductive treatments, randomly assigning patients to either an acupuncture or control group. The acupuncture patients received 25 min of acupuncture before and after embryo transfer, while the control group rested alone for equivalent amounts of time. There was a significantly higher clinical pregnancy rate in the acupuncture group (42.5%) compared to the control group (26.3%).

This ground-breaking study, frequently referred to as the Paulus study, utilizing the “Paulus protocol,” has radically changed how many infertility patients view acupuncture. After the study was published, infertility patients flocked to acupuncture clinics and at some infertility centers, patients demanded that acupuncture be delivered on site. Some clinics reported that more than

80% of their advanced reproductive technology (ART) patients were receiving acupuncture and some acupuncture clinics were overwhelmed by the demand (Hay and Domar, 2015). However, not all subsequent research has replicated the Paulus study. The impact of acupuncture on fertility/ART success remains a topic of frequent debate.

From a physiological standpoint, one theory as to why acupuncture may improve fertility outcomes is via neurotransmitters. Specifically, acupuncture can influence fertility by modulating gonadotropin levels, specifically in the case of electroacupuncture, which can then influence ovulation through the menstrual cycle (Maliqueo et al., 2015). Acupuncture may also inhibit “uterine central nerve activity” by stimulating blood flow to the uterus, therefore increasing chances of conception. A literature review on the impact of acupuncture on IVF outcome included in its conclusions that possible mechanisms of action were neuroendocrine, increased blood flow to the pelvic region, modulating cytokines, and/or the reduction of distress (Anderson et al., 2007). Finally, considering the role that the biological stress response plays in reducing chances of pregnancy, acupuncture can improve fertility outcomes by stimulating the production of endogenous opioids that inhibit that stress response through the central nervous system (Cho et al., 1997). Some researchers dismiss these theories as simply being a placebo effect.

To account for this easy dismissal, researchers often attempt to control for the placebo effect by randomly assigning participants to either acupuncture or control groups. The most common method for creating a placebo control group is via “sham” acupuncture, in which the patient and researchers, but not the acupuncturist, are blinded to whether the patient is receiving real or sham acupuncture (Moffet, 2009). Sham acupuncture either uses non-penetrating needles (needles which touch the skin, so the patient feels a pricking sensation but the needle doesn’t penetrate the skin) or needling at non-acupuncture points, where the practice will either not specifically address the condition being studied or not be placed on the meridians typically used for acupuncture.

This sham acupuncture has played an important role in casting doubt on traditional acupuncture practices involving meridians and needling locations, as a number of studies have not produced differences between acupuncture and control groups (Moffet, 2009). Due to these findings, researchers have suggested that the therapeutic effects of acupuncture are not specifically related to acupuncture; rather, they could be due to other variables such as the relaxing environment or a placebo effect (Moffet, 2009). The aforementioned 2014 review in *Nature Reviews Cancer* found that the act of penetrating skin at meridians for the purpose of redirecting the flow of *qi* may be unrelated to the health benefits of acupuncture, as is the frequency of needling (Gorski, 2014). This aligns with the view that acupuncture is successful due to a placebo effect rather than a directly physiological effect (Gorski, 2014).

Despite the rampant issues in differentiating between real and sham acupuncture, there *are* studies showing significant positive effects of real acupuncture above and beyond the effects of sham acupuncture. A 2013 study aimed to measure the unique effects of acupuncture on fertility among women struggling to conceive, including 84 infertile patients with a history of at least two unsuccessful IVF attempts (di Villahermosa et al., 2013). Using acupuncture, control, and sham groups, this study found significant differences in clinical pregnancy rates, such that the acupuncture group exhibited significantly higher rates than both the sham and control groups, which were not significantly different from one another. This supports the unique effect of acupuncture on women’s fertility above and beyond a mere placebo effect.

Although findings such as these are encouraging for the role of acupuncture in supporting fertility in patients undergoing in vitro fertilization, the literature remains divided on the efficacy of acupuncture to improve IVF outcomes. A 2008 meta-analysis examining 13 randomized controlled trials (RCT) on acupuncture and IVF outcome found that acupuncture did not lead to a significant increase in live birth rate or clinical pregnancy at multiple points during the IVF process (El-Toukhy et al., 2008). On the contrary, a meta-analysis conducted in 2012 on 24 trials found that acupuncture with IVF patients was associated with both a higher pregnancy rate and live birth rate, but only when the type of control was considered (Zheng et al., 2012). One early review of the literature concluded that there is some evidence to support the theory that acupuncture may be associated with higher pregnancy rates from IVF as well as improved quality of life (Anderson et al., 2007). Another meta-analysis of seven trials suggested that acupuncture administered at the time of embryo transfer can be linked with improved pregnancy rates and live birth rates; however, some of this was attributed to selection bias (Manheimer et al., 2008).

Other trials produced mixed findings as well, with each taking a slightly different approach. In a 2006 RCT on 228 subjects, results showed a pregnancy rate of 31% for the acupuncture group, whereas the sham acupuncture group exhibited a 23% pregnancy rate, although the difference was nonsignificant (Smith et al., 2006). In a more recent study, acupuncture was performed at different points during the IVF process (Shen et al., 2015). This study included three randomized groups, each of which received acupuncture: one group received acupuncture 25 min before and after embryo transfer (ET), the second group received acupuncture during the follicular phase of the cycle as well as 25 min before and after ET, and the final group only received acupuncture 30 min after ET. The results showed no significant improvements in the clinical pregnancy rate for two of the groups, where acupuncture was performed only at the time of ET or 30 min after ET. However, participants who received acupuncture during the follicular phase of the menstrual cycle as well as 25 min before and after ET exhibited significantly higher pregnancy rates.

A variation of traditional acupuncture is electroacupuncture (EA), where the needles are placed in the desired location and are then attached to an electrical stimulator. An early randomized controlled prospective study using EA on 44 IVF patients showed no significant differences in pregnancy between the two groups but did show that EA was associated with significant reductions in the pulsatility index of both uterine arteries (Ho et al., 2009). A subsequent non-randomized study on EA in 21 women with diminished ovarian reserve who received 12 weeks of treatment revealed significant decreases in follicle-stimulating hormone, an indication of improved ovarian reserve (Wang et al., 2016). These improvements continued 12 weeks after the cessation of treatment. There is ongoing research on the impact of EA on pregnancy rates in infertile women.

One study described how an invitation to participate in an RCT of acupuncture as an adjuvant to IVF was received by infertile women and how they processed their decision to participate. This study found that most women who agree to RCT infertility studies described themselves as active agents searching for a better outcome for their infertility or improved outcomes for women in the future. “Their decision to participate in an RCT was motivated by factors such as opportunity, novelty and a value of science and was made after weighing various risks and benefits”. This is an important aspect of these studies because these women in most of the studies are already less fertile than the typical woman who goes through an IVF cycle (De Lacey et al., 2017). In general, women who volunteer for acupuncture studies tend to be poorer prognosis patients; women with the best quality embryos do not seem to be as anxious to volunteer for research on acupuncture.

Perhaps one of the most important points that has received minimal attention is the quality of embryos which are transferred. Presumably, there is no intervention which has the capacity of transforming an abnormal embryo into a normal one. Thus, if the women who tend to volunteer to participate in acupuncture trials have a greater chance of having poorer quality embryos to transfer, when they participate in a RCT, there is less of an opportunity for acupuncture to have a significant impact, thereby producing a ceiling effect. The only way to accurately perform an RCT is to do so only with women with good quality embryos. In the original Paulus study (2002), this is exactly what researchers did; future research should attempt to replicate these conditions. Until there is more research which only includes women with good quality embryos, the question of the impact of acupuncture on IVF is unanswered.

Acupuncture and Male Infertility

The relationship between male infertility and acupuncture has also been evaluated in a number of studies. A recent Chinese study examined whether or not acupuncture can improve male sperm quality and therefore fertility outcomes for couples. This study found that acupuncture was more effective than Western Medicine alone and as effective as traditional Chinese medicine, and its effectiveness was enhanced when combined with traditional Chinese or western medicine in improving sperm count and quality (He et al., 2015). Contrary to these findings, a meta-analysis of four RCTs examining the effectiveness and safety of acupuncture for poor semen quality in infertile males recently found that most studies were unable to find a significant difference in sperm quality associated with acupuncture (Jerng et al., 2014). However, the authors attributed the lack of significant results to poor reporting, high levels of heterogeneity, and high risk of bias.

A 2016 study found acupuncture to be an effective treatment for patients with primary infertile varicocele (an enlargement of the veins within the scrotum) and semen abnormalities, producing similar results to Western varicocelectomy treatment (Kucuk et al., 2016).

Acupuncture, Stress, and Infertility

Stress is often cited as negatively impacting the ability to achieve a pregnancy. High levels of physical stress can increase the risk of miscarriage, and chronic life stress can make it more difficult to achieve pregnancy. Infertility patients may participate in acupuncture as a way to reduce their distress, and thus increase their chance of achieving a pregnancy.

Patients who experience infertility and its subsequent treatment have high rates of depression and anxiety. High levels of distress can lower the chances of a successful embryo transfer, and may increase the risk of miscarriage. One consistent result of acupuncture in the scientific literature is that it can be a useful tool for reducing distress, which in turn may improve fertility and IVF outcomes. A 2012 study on 43 patients undergoing IVF treatment found that acupuncture significantly lowered anxiety levels when compared to the control group after four weekly acupuncture sessions (Isoyama et al., 2012). A 2010 study conducted with 57 infertile patients undergoing IVF treatment found that an acupuncture regimen was associated with lower levels of distress both before and after embryo transfer when compared to a control group without acupuncture; this difference in stress levels may have improved pregnancy rates (Balk et al., 2010). Lower perceived stress has been shown to improve pregnancy rates at the time of embryo transfer. Women with lower stress and anxiety prior to oocyte retrieval and embryo transfer tend to have higher pregnancy and live birth rates (Turner et al., 2013).

Depression, anxiety, and life stress are all detrimental to sleep and a steady sleep schedule is also essential for fertility (Summa et al., 2012). Physical stress has the potential to disrupt circadian rhythms and cyclic melatonin, which are critical to the optimal functioning of ovaries and placenta (Reiter et al., 2014). Similarly, many animal studies have been done that show that an environmental perturbation of the circadian clock can disrupt pregnancies (Summa et al., 2012). Acupuncture has been shown to help sleep disorders such as insomnia and narcolepsy. It has also been shown that acupuncture is effective in improving sleep quality and prompting relaxation in people of all ages with residual or primary insomnia (Zuppa et al., 2015). Considering the role that sleep plays in pregnancy, acupuncture's effect on sleep could have an indirect effect on pregnancy rate.

A 2011 pilot RCT examined 32 women aged 20–45 years split into acupuncture or wait-list control groups, all with a diagnosis of infertility (Smith et al., 2011). Participants in the acupuncture group received six sessions of acupuncture over 8 weeks, and then completed the Fertility Problem Inventory (FPI) to measure psychosocial factors including infertility self-efficacy, anxiety, and infertility-related stress. After 8 weeks, women in the acupuncture group reported better psychosocial outcomes than the control group, as they exhibited significantly less social and relationship concern, as well as trends toward reduced infertility-related stress, improved fertility self-efficacy, and reduced anxiety.

Conclusions

Since the publication of the Paulus study in 2002, acupuncture has been seen by many patients as a way to enhance their chance of pregnancy from ART. Although the research on the impact of acupuncture on IVF outcome is still not definitive, there is enough data to warrant its inclusion in selected patients. Women with normal-appearing embryos can be encouraged to undergo acupuncture pre- and post-embryo transfer; there are virtually no side effects and the cost of these two sessions, approximately \$150, is minimal compared to the cost of an IVF cycle.

Boston IVF is one of the largest infertility centers in the United States. An integrative care center opened on site in 2006 and was expanded in 2017. The Domar Center for Mind/Body Health now shares a waiting room with ultrasound and phlebotomy, thus giving patients the impression that it truly is an integrative approach. Included in the integrative care center are four acupuncture rooms and the Center is located next to the operating and transfer rooms. The Center employs five acupuncturists, and it is open 365 days per year, i.e., when the BIVF operating/transfer rooms are open.

When a patient is scheduled for her embryo or blastocyst transfer, she is asked whether or not she would like to schedule pre- and post-transfer acupuncture. If she expresses an interest, she is transferred to the Domar Center and she can then schedule her appointment. She is instructed to come in for acupuncture a half hour before her scheduled transfer arrival time. She receives 25 min of acupuncture prior to and following the transfer, using the Paulus protocol. Approximately one third of all transfer patients end up receiving acupuncture.

It is unknown at the current time if acupuncture leads to an increase in pregnancy rates for our patients. However, patients consistently report feeling more relaxed as they prepare for and then recover from their transfer. Perhaps more importantly, for a small investment in time and money, these patients truly feel they are doing everything within their power to increase their odds of conceiving a healthy baby.

Acknowledgments

We would like to thank Kristin Rooney, BA, and Adam Yecies for their assistance with the preparation of this manuscript.

References

- Anderson, B. J., Haimovici, F., Ginsburg, E. S., Schust, D. J., & Wayne, P. M. (2007). In vitro fertilization and acupuncture: Clinical efficacy and mechanistic basis. *Alternative Therapies in Health and Medicine*, 13, 38–48.
- Balk, J., Catov, J., Horn, B., Gesci, K., & Wakim, A. (2010). The relationship between perceived stress, acupuncture, and pregnancy rates among IVF patients: A pilot study. *Complementary Therapies in Clinical Practice*, 16, 154–157.
- Cho, Z. H., Chung, S. C., Jones, J. B., et al. (1997). New findings of the correlation between acupoints and corresponding brain cortices using functional MRI. *Proceedings of the National Academy of Sciences of the United States of America*, 95, 2670–2673.
- De Lacey, S. L., Sanderman, E., & Smith, C. A. (2017). Acupuncture in reproductive medicine: The motivations of infertile women to participate in a randomised controlled trial. *Journal of Psychosomatic Obstetrics and Gynaecology*, 1–9.
- di Villahermosa, D., dos Santos, L., Nogueira, M., Vilarino, F., & Barbosa, C. (2013). Influence of acupuncture on the outcomes of in vitro fertilisation when embryo implantation has failed: A prospective randomised controlled clinical trial. *British Medical Journal*, 31, 157–161.
- El-Toukhy, T., Sunkara, S. K., Khairy, M., et al. (2008). A systematic review and meta-analysis of acupuncture in vitro fertilisation. *British Journal of Obstetrics and Gynecology*, 115, 1203–1213.
- Ernst, E. (2006). Acupuncture: A critical analysis. *Journal of Internal Medicine*, 259, 125–137.
- Ernst, E., Soo Lee, M., & Choi, T. Y. (2011). Acupuncture: Does it relieve pain and are there serious risks? A review of reviews. *Pain*, 152, 755–764.
- Goldman, N., Chen, M., Fujita, T., et al. (2010). Adenosine A1 receptors mediate local anti-nociceptive effects of acupuncture. *Nature Neuroscience*, 13, 883–888.
- Gorski, D. (2014). Integrative oncology: Really the best of both worlds? *Nature Reviews Cancer*, 14, 692–700.
- Hay, M., & Domar, A. (2015). Is acupuncture associated with improved IVF outcomes? *Biennial Review of Infertility*, 157–163.
- He, Y., Chen, C. T., Qian, L. H., et al. (2015). Acupuncture treatment of male infertility: A systematic review. *National Journal of Andrology*, 21, 637–645.
- Ho, M., Huang, L. C., Chang, Y. Y., et al. (2009). Electroacupuncture reduces uterine artery blood flow impedance in infertile women. *Taiwanese Journal of Obstetrics and Gynecology*, 48, 148–151.
- Huang, W., Pach, D., Napadow, V., et al. (2012). Characterizing acupuncture stimuli using brain imaging with fMRI—A systematic review and meta-analysis of the literature. *Public Library of Science*, 7, e32960.
- Isoyama, D., Cordts, E., Niewegen, A., et al. (2012). Effect of acupuncture on symptoms of anxiety in women undergoing in vitro fertilisation: A prospective randomised controlled study. *British Medical Journal*, 30, 85–88.
- Jerng, U., Jo, J., Lee, S., Lee, J., & Kwon, O. (2014). The effectiveness and safety of acupuncture for poor semen quality in infertile males: A systematic review and meta-analysis. *Asian Journal of Andrology*, 16, 884–891.
- Kavoussi, B., & Ross, E. (2007). The neuroimmune basis of anti-inflammatory acupuncture. *Integrative Cancer Therapies*, 6, 251–257.
- Kucuk, E., Binda, A., Boylu, U., Onol, F., & Gumus, E. (2016). Randomised clinical trial of comparing effects of acupuncture and varicocelelectomy on sperm parameters in infertile varicocele patients. *Andrologia*, 48, 1080–1085.
- Ma, B., Qi, G. Q., Lin, X. T., et al. (2012). Epidemiology, quality, and reporting characteristics of systematic reviews of acupuncture interventions published in Chinese journals. *Journal of Alternative and Complementary Medicine*, 18, 813–817.
- Maliqueo, M., Benrick, A., Alvi, A., et al. (2015). Circulating gonadotropins and ovarian adiponectin system are modulated by acupuncture independently of sex steroid or β -adrenergic action in a female hyperandrogenic rat model of polycystic ovary syndrome. *Molecular and Cellular Endocrinology*, 5, 159–169.
- Manheimer, E., Zhang, G., Udoff, L., et al. (2008). Effects of acupuncture on rates of pregnancy and live birth among women undergoing in vitro fertilisation: Systematic review and meta-analysis. *British Medical Journal*, 336, 545–549.

- Moffet, H. (2009). Sham acupuncture may be as efficacious as true acupuncture: A systematic review of clinical trials. *Journal of Alternative and Complementary Medicine*, 15, 213–216.
- Paulus, W., Zhang, M., Strehler, E., El-Danasouri, I., & Sterzik, K. (2002). Influence of acupuncture on the pregnancy rate in patients who undergo assisted reproduction therapy. *Fertility Sterility*, 77, 721–724.
- Reiter, R., Tamura, H., Tan, D., & Xu, X. (2014). Melatonin and the circadian system: Contributions to successful female reproduction. *Fertility and Sterility*, 102, 321–328.
- Shen, C., Wu, M., Shu, D., Zhao, X., & Gao, Y. (2015). The role of acupuncture in in vitro fertilization: A systematic review and meta-analysis. *Gynecologic and Obstetric Investigation*, 79, 1–12.
- Smith, C., Coyle, M., & Norman, R. (2006). Influence of acupuncture stimulation on pregnancy rates for women undergoing embryo transfer. *Fertility and Sterility*, 85, 1352–1358.
- Smith, C. A., Ussher, J. M., Perz, J., Carmady, B., & De Lacey, S. (2011). The effect of acupuncture on psychosocial outcomes for women experiencing fertility: A pilot randomized controlled trial. *Journal of Alternative and Complementary Medicine*, 17, 923–930.
- Summa, K., Viaterna, M., & Turek, F. (2012). Environmental perturbation of the circadian clock disrupts pregnancy in the mouse. *Public Library of Science*, 7, e37668.
- Turner, K., Reynolds-May, M., Zitek, E., et al. (2013). Stress and anxiety scores in first and repeat IVF cycles: A pilot study. *Public Library of Science*, 8, e63743.
- Vickers, A. J., Cronin, A. M., Maschino, A. C., Lewith, G., MacPherson, H., Victor, N., Foster, N. E., Sherman, K. J., Witt, C. M., & Linde, K. (2012). Acupuncture for chronic pain: Individual patient data meta-analysis. *Archives of Internal Medicine*, 172, 1444–1453.
- Vickers, A. J., Goyal, N., Harland, R., & Rees, R. (1998). Do certain countries produce only positive results? A systematic review of controlled trials. *Controlled Clinical Trials*, 19, 159–166.
- Wang, S. M., Kain, Z. N., & White, P. (2008). Acupuncture analgesia I: The scientific basis. *Anesthesia & Analgesia*, 106, 602–610.
- Wang, Y., Li, Y., Chen, R., Cui, X., & Liu, Z. (2016). Electroacupuncture for reproductive hormone levels in patients with diminished ovarian reserve: A prospective observational study. *Acupuncture in Medicine*, 34, 386–391.
- Zheng, C., Huang, G., Zhang, M., & Wang, W. (2012). Effects of acupuncture on pregnancy rates in women undergoing in vitro fertilization: A systematic review and meta-analysis. *Fertility and Sterility*, 97, 599–611.
- Zuppa, C., Prado, C. H., Wieck, A., et al. (2015). Acupuncture for sleep quality, BDNF levels and immunosenescence: A randomized controlled study. *Neuroscience Letters*, 587, 35–40.

ETHICS AND THE LEGISLATION

Global Changes in the Regulation of Reproductive Medicine

Tetsuya Ishii, Hokkaido University, Sapporo, Japan

© 2018 Elsevier Inc. All rights reserved.

Introduction

In 1884, an old type of infertility treatment was carried out: A Philadelphia physician inseminated a woman using sperm taken from his student (Yuko, 2016). This case of artificial insemination (AI) is believed to be the first instance of reproductive medicine involving a third-party. Nearly a century later, a UK woman, named Lesley Brown had suffered from infertility for 9 years due to complications of blocked fallopian tubes. She underwent a new reproductive technique, termed in vitro fertilization (IVF), successfully resulting in the birth of her daughter, Louise Joy Brown, in 1978 (Steptoe and Edwards, 1978). Developed countries are facing a rising average age of first birth, and advanced maternal age may affect fertility in several respects, including a compromised quality of eggs. IVF has also been used to treat such cases other than blocked fallopian tubes. Male infertility is generally associated with environmental factors, including stress, life habits, and drugs, as well as congenital factors. To assist fertility for men with few or poorly motile sperm, a reproductive technique involving the directly injection of one sperm into one egg was developed, termed intracytoplasmic sperm injection (ICSI). The first child born using ICSI was reported in 1992. Against such a backdrop, reproductive medicine, such as IVF and ICSI, has now spread across the globe.

IVF and ICSI involving a third-party have also been used to assist reproduction for medical and social reasons. Some infertile couples require a gamete donor or a surrogate mother if the partners cannot produce fertile eggs or sperm, or if a female partner has no functional uterus. Moreover, same-sex couples and men and women without a partner also make use of such third-party reproduction techniques for their family-building. However, the practice of reproductive medicine using donor gametes and/or a surrogate mother has raised ethical and social issues, particular in cross-border reproduction (Jackson et al., 2017). The sale of donor gametes (particularly eggs) has raised concerns over the exploitation and/or commodification of human reproductive cells. Some egg donors suffer health problems due to such as hormonal injection in egg retrieval. Furthermore, the use of IVF using donor gametes reduces the consistency of genetic relatedness in a legitimate birth. Disclosing to the resultant children the fact that they were conceived via donor gametes has been chief among the thorny issues raised, and some clashes have occurred between the surrogate mother and the genetic parents (ABC, 2017).

Preimplantation genetic diagnosis (PGD) involves the DNA testing of preimplantation embryos and was originally developed in order to avoid childbirths with genetic disease. Subsequently, PGD has been repurposed for social reasons in some countries, such as sex-selection to ensure that the child is a desired sex in a family or a socially preferred sex (Bayefsky, 2016).

Thus, complicated issues have occurred surrounding reproductive medicine. As to why this happened, in human society, reproduction implies family-building. Reproductive medicine can help meet the fertility and childbirth needs of homosexual couples as well as heterosexual couples. In addition, it can be used to help give birth to children of the desired number, gender, health or other characteristics. While, advances in biomedicine and the diffusion of reproductive medicine have progressed with astonishing speed. In response, some professional societies have established relevant guidelines, whereas many countries have struggled to enact relevant laws though social discussions.

The present article first describes the current state of regulation of reproductive medicine in 49 countries, then analyzes the changes from 2007 to 2016. Subsequently, it highlights important legal changes concerning specific techniques: IVF, gamete donation, surrogacy, PGD, and germline genetic modification. It also focuses on the regulation of reproductive medicine in G7 countries. Finally, future regulatory changes are discussed, with consideration of the recent advances in reproductive biomedicine.

Current State of Regulation of Reproductive Medicine

Because reproductive medicine can have a wide impact a society, its regulation should, ideally, be based on laws that are a national or regional norm with enforcement, such as the deprivation of a medical license, a fine, or imprisonment when violated. However, enacting law usually require a broad social consensus that takes at least several years. Moreover, the amendment to or the abolishment of enacted law can also take years. Social costs after the enactment, such as data registry, monitoring, inspection, and administration, are also necessary. In contrast, the establishment and amendment of guidelines on reproductive medicine is, in general less difficult, as such guidelines are typically established by professional societies or health authorities, not a national legislative assembly. Furthermore, such guidelines are intended to notify physicians of the medical standards and are largely not binding.

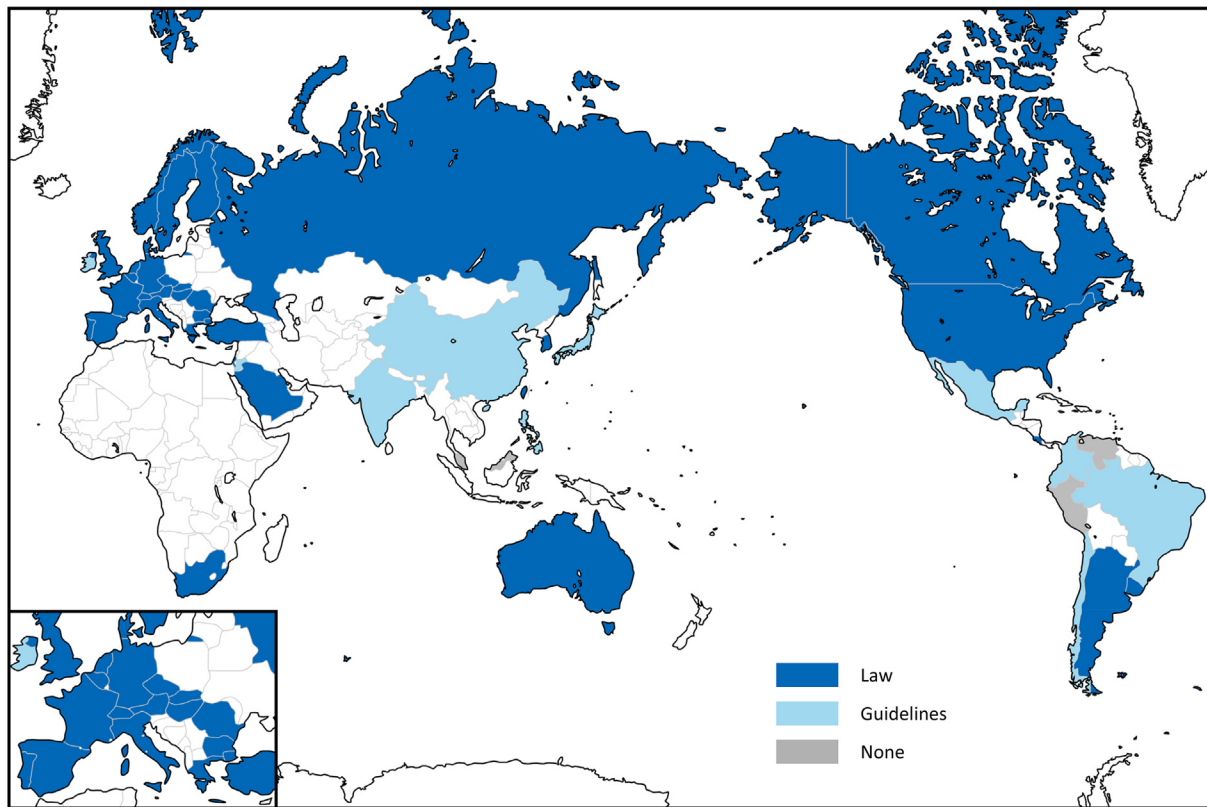


Fig. 1 The international regulatory landscape of reproductive medicine, as of 2016. This map was drawn according to the information in IFFS Surveillance reports 2007 and 2016. Only the regulatory state in Hong Kong was reassessed and judged “Law” in both 2007 and 2016.

The International Federation of Fertility Societies (IFFS) regularly surveys global activities regarding reproductive medicine. A total of 49 countries were selected among the countries that participated in the both IFFS Surveillance 2007 and 2016 (IFFS, 2007; IFFS, 2016). According to the IFFS Surveillance reports, such countries provided reliable data pertinent to the regulation of reproductive medicine. **Fig. 1** displays the regulatory state of reproductive medicine in these 49 countries, based on the 2016 report. The countries were categorized into three subcategories: 34 countries regulate reproductive medicine by law (69%), 12 regulate it by guidelines (24%: Brazil, Chile, China, India, Ireland, Japan, Mexico, Philippines, Singapore, Colombia, Ecuador, Jordan), and three have no relevant laws nor guidelines (6%: “None”: Malaysia, Peru, and Venezuela). Because approximately 200 countries currently exist worldwide, the data suggests that at least 17% of all countries have established laws regarding reproductive medicine. The regulatory map also shows that the 15 countries operating under “Guidelines” or no regulation (“None”) are largely located in South America and Asia (**Fig. 1**).

Changes in the Past Decade

Table 1 shows the regulatory changes in the 49 countries (**Fig. 1**), stratified by comparing the findings in IFFS Surveillance reports of 2007 and 2016. The regulatory state regarding the practice of reproductive medicine remained unchanged in the 37 countries (76%). However, the remaining 12 countries changed their relevant regulation (24%). In detail, 8 of 12 countries newly enacted laws regarding reproductive medicine. Interestingly, Finland, Portugal, Romania, and Uruguay newly enacted relevant laws, despite having no regulations at the Surveillance 2007. Of particular note, Costa Rica legally permitted IVF in 2016, despite having prohibited it for 16 years. Colombia, Ecuador, and Jordan newly established relevant guidelines.

In the past decade, the number of countries with laws pertinent to reproductive medicine has increased from 53% to 69% among the 49 countries (**Table 1**). However, Brazil, Chile, China, India, Ireland, Japan, Mexico, the Philippines, and Singapore have not changed their guidelines to laws. Malaysia, Peru, and Venezuela have continued to have no regulation of reproductive medicine.

Reproductive Medicine, Religion, and Regulation

Reproductive medicine is unique in medical disciplines. It may involve in two different individuals (typically a wife and a husband), but its direct subject is a prospective child. Additionally, reproductive medicine may also involve a third-party: a gamete donor or

Table 1 The changes in the regulation pertinent to reproductive medicine

According to the IFFS report 2007 and 2016		Countries (49)
37 countries unchanged regulation	Law (25)	Austria, Belgium, Bulgaria, Canada, Czech Republic, Denmark, France, Germany, Greece, Hungary, Israel, Italy, Hong Kong, Netherlands, Norway, Russia, Saudi Arabia, Slovak Republic, South Korea, Spain, Sweden, Switzerland, Taiwan, Turkey, United Kingdom
	Guidelines (9) "None" (3)	Brazil, Chile, China, India, Ireland, Japan, Mexico, Philippines, Singapore Malaysia, Peru, Venezuela
12 countries changed	Guidelines to law (4)	Argentina, Australia, South Africa, United States
	"None" to law (4)	Finland, Portugal, Romania, Uruguay
	Legal prohibition to legal permission (1) "None" to guidelines (3)	Costa Rica Colombia, Ecuador, Jordan

IFFS, International Federation of Fertility Societies; "None", implies having no law nor guidelines regarding reproductive medicine.

a surrogate mother. Generally, reproductive autonomy supports the practice of reproductive medicine. However, the complexity of parties concerned in third-party reproduction has made it difficult to discuss its appropriate use in society.

Religious beliefs play a large role in the manner of human reproduction or family-building in some countries. Thus, many religious groups have expressed their view toward reproductive medicine (Sallam and Sallam, 2016). Briefly, nearly all forms of Judaism, Hinduism, and Buddhism currently accept reproductive medicine. By contrast, Roman Catholicism does not accept any form of reproductive medicine, as they regard human embryos as humans and assert that a new human life must begin only through natural means and that artificial interventions at the inception of human life involve inherent dangers and are unacceptable. Most Orthodox Jews and Christians refuse third-party reproduction because they value blood lineage. Protestants, Anglicans, Coptic Christians, and Sunni Muslims accept most forms of reproductive medicine, so long as gamete or embryo donation are not involved. Likewise, Confucianism, which is associated with a preference for sons, still influences Chinese culture, and accepts all forms of reproductive medicine involving no third parties.

Thus, some religious groups largely accept reproductive medicine, while others condemn some or all types of reproductive medicine. Such religious beliefs have shaped the acceptable use of reproductive medicine in some countries, as reflected in relevant regulation.

Recent Regulatory Changes in Specific Techniques

IVF and ICSI

Based on reproductive autonomy, many countries legally permit IVF and ICSI, while some countries limit the maximum number of transferred embryos in order to prevent multiple pregnancies. For instance, relevant Spanish law stipulates that a maximum of three embryos can be transferred (Law on Assisted Human Reproduction Techniques 2006). In Turkey, only single embryo transfer (SET) is allowed for women <35 years of age in the first and second cycles of IVF, and a maximum of two embryos can be transferred to women ≥ 35 years of age or women <35 on the third or further cycles (Legislation concerning assisted reproductive treatment practices and centers 2010). Likewise, the Japan Society of Obstetrics and Gynecology (JSOG) adopts the SET policy (Takeshima et al., 2016).

Notably, Costa Rica became the only country explicitly prohibiting IVF in 2000 because its Constitutional Court ruled that IVF creates excess embryos and violates their right to life, strictly according to Roman Catholicism (Lemaitre and Sieder, 2017). In 2012, the Inter-American Court of Human Rights ruled that the IVF prohibition violates the right to privacy, liberty, personal integrity, and to form a family. On February 26, 2016, Costa Rica lifted its 16 years IVF ban after President Luis Guillermo Solís signed Executive Decree No. 39210-MP-S in 2015.

Reproduction With Gamete Donation

As mentioned in the Introduction, AI with donor sperm has been practiced for infertile people for over a century. With the advent of IVF, the reproductive use of donor eggs as well as sperm has been increasing for those suffering from infertility due to advanced reproductive age (particularly in women), cancer treatments, and unknown causes. Moreover, reproductive medicine involving gamete donation has recently been practiced for lesbian couples and single mothers, and it can also be used to aid gay couples and single fathers if surrogacy is available.

In the USA, gamete donation is liberally performed to satisfy such medical and social needs, and is generally allowed at the federal level, although the state of Louisiana prohibits the sale of human oocytes (La Rev. Stat 9:122). However, countries that permit reproduction using donor gametes are likely to face social issues due to inconsistencies between legitimate parenthood and genetic parenthood. For this reason, reproductive medicine involving gamete donation is typically regulated from three standpoints of intended parents, gamete donors, and resultant children. For intended parents, the parental authority in

reproduction using donor gametes is stipulated by such as civil law. Even in cases wherein gametes donated by a third-party are used, the woman who gives rise to the child is deemed a legitimate mother if statutory requirements are satisfied. Likewise, a legitimate husband is deemed a legitimate father if he acknowledges the child as his own. Gamete donors have no obligation to foster the resultant children, if they satisfy legal requirements, such as the abandonment of parental authority. In some countries, including the Czech Republic (Act on Specific Health Services 2011), Israel (Egg Donation Law 2010), and Spain (Law on Assisted Human Reproduction Techniques 2006), gamete donors must be anonymous. Moreover, the maximum number of gamete donations is frequently regulated to avoid consanguineous marriage between people born from an identical donor, and to protect them from health problems after egg retrieval, such as ovarian hyperstimulation syndrome. Some countries, such as Canada (Assisted Human Reproduction Act 2004), Czech Republic (the law shown above), India (National Guidelines for Accreditation, Supervision & Regulation of ART Clinics 2005), Taiwan (Artificial Reproduction Act 2007), and Israel (the law above), permit the reimbursement of necessary costs such as travel expenses and/or wage loss, even if gamete donation is understood to be an altruistic behavior. Other countries, such as China (The Human Assisted Reproductive Technology Specifications and the Human Assisted Reproductive Technology and Human Sperm Bank Ethical Principles 2003), prohibit any commercialization of donor gametes. For people born using donor gametes, some countries, such as Victoria state in Australia (Assisted Reproductive Treatment Amendment Act 2016), Sweden (Genetic Integrity Act 2006), Switzerland (Act on Medically Assisted Reproduction 1998), the Netherlands (Artificial Insemination: Donor Information Act 2002) and the UK (Human Fertilization and Embryology Act 1990), legally guarantee the right to access donor information, while such as the Czech Republic, Israel, and Spain do not grant any such rights as they prioritize donors' anonymity, as mentioned above.

Conversely, some countries have remained against reproductive medicine involving gamete donation. In Japan, the JSOG permits its members to perform AI using donor sperm, but forbids other third-party reproduction under their guidelines. Italy, where Roman Catholicism is the dominant religion, initially prohibited reproductive medicine gamete donation (Law on Rules in the Field of Medically Assisted Reproduction 2004). However, in 2014, the Constitutional Court lifted the ban on gamete donation by judgment 162/2014. Of particular note, Turkey, in which Sunni Muslims are dominant, prohibited all medical assistance in conception using donor gametes (Legislation Concerning Assisted Reproductive Treatment Practices and Centers 2010). The Turkish law also prohibits citizens from going abroad to seek donor gametes for reproduction. Probably, Turkey has the most strictest prohibitions against reproduction using donor gametes (Gürtin, 2016).

Surrogacy

Surrogacy is a form of third-party reproduction in which a woman consents to carry a pregnancy for intended parent(s) who cannot conceive for medical reasons or those who are a gay couple. There are two forms of surrogacy: traditional surrogacy and gestational surrogacy. Traditional surrogacy uses the surrogate mother's egg for conception. In contrast, gestational surrogacy is performed by transferring embryos made through IVF with eggs from the intended mother or a donor. Thus, the advent of IVF has assisted gestational surrogacy. However, pregnancy and gestation involve psychological burden and health risks for the surrogate mother. Moreover, the legal procedures for parenthood following surrogacy are complicated due to the typical legal assumption that a woman giving birth to a child is the legitimate mother of the child. Therefore, a surrogate mother is required to formally abandon parental authority, and the intended parent(s) then adopt the child born.

However, some clashes have occurred after childbirth in cases where a surrogate mother refuses to deliver the child to intended parents or where the intended parents refuse to accept the child (ABC, 2017). Surrogacy is likely to become more problematic, particularly in case involving payment to a surrogate mother. Commercial surrogacy has raised concerns, such as the exploitation and commodification of woman's bodies for client's family building. While, surrogacy can be altruistically performed in some countries, with the surrogate mother receiving no compensation other than reimbursement of medical and other expenses (Jackson et al., 2017). Altruistic surrogacy is sometimes considered among the relatives of an intended mother; as such, foreigners can experience difficulties in finding suitable surrogates.

Countries have responded differently to the legality of and payment for surrogacy in their policies. Some countries permit only altruistic surrogacy under relevant law, including Canada (the AHR Act 2004), Greece (such as Law on Enforcement of Medically Assisted Reproduction 2005), Israel (Surrogacy Law 1996), the Netherlands (guided by the Criminal Code), South Africa (Children's Act 2005), the UK (Surrogacy Arrangements Act 1985), and several states in Australia (except the Northern Territory). However, commercial surrogacy has been offered in several countries with lax regulations, including Georgia (Law on Health Protection 1997), Russia (Federal Law on the Fundamentals on Protection of Citizens' Health 2011), Ukraine (The Family code of Ukraine), and a few US states (Arkansas, California, Illinois, and Maryland). Other countries largely prohibit both types of surrogacy by law or under guidelines for medical, ethical, social, and religious reasons.

In Asia, important changes were recently made or attempted regarding the regulation of commercial surrogacy (Jackson et al., 2017). Thailand, which acknowledged itself as the "Health tourism hub of Asia," has gathered substantial attention from foreign (infertile) patients seeking surrogacy that is inaccessible in their home countries. However, international troubles recently occurred surrounding commercial surrogacy. As a result, Thailand legally banned commercial surrogacy for foreign tourists, by enacting the Protection of Children Born from Assisted Reproductive Technology Act 2015. In India, commercial surrogacy has been practiced since 2002. Although lax regulations had attracted foreigners seeking commercial surrogacy, clashes occurred between surrogate mothers and the intended parents. Thus, the Cabinet approved Surrogacy (Regulation) Bill 2016 to regulate commercial surrogacy.

Preimplantation Genetic Diagnosis (PGD)

The world's first PGD reported in 1990 from the UK intended to avoid the birth of children with X-linked genetic diseases by selecting female embryos through a genetic analysis of biopsied embryonic cells. Subsequently, PGD to select human embryos without a pathogenic mutation has spread worldwide. However, the role of PGD also increased at fertility clinics, raising ethical controversies. Although PGD has also been used to screen euploid embryos to increase the implantation rate and/or avoid miscarriage, the efficacy of the preimplantation genetic screening (PGS) is controversial and requires further clinical studies. Notably, the use of PGD for social sex-selection has raised concerns, such as the potential for the reinforcement of sex discrimination, particularly against women (WHO, 2017).

The USA has no federal regulations regarding the use of PGD at clinics (Bayefsky, 2016). Indeed, PGD are liberally practiced in the USA, including the embryo selection to avoid the birth of a child with genetic diseases, the screening of aneuploid embryos (PGS), social sex-selection, and "the selection of a disability". In Western Europe, many countries sanction PGD to avoid the birth of children with genetic disease but prohibit it in cases of, for example, social sex-selection under relevant law (Bayefsky, 2016).

Some countries prohibited the DNA testing of the human embryos for a long time. Germany prohibited PGD under the Embryo Protection Law 1990, but legally permitted it by enacting the Law regulating preimplantation genetic diagnosis 2011. At that time, Austria, Italy, and Switzerland, which are located close to each other, were the only remaining countries that prohibited PGD in Western Europe. Their predominant religion (Roman Catholicism) might have influenced their PGD-prohibitive policy. In 2015, Austria amended the Law on Reproductive Medicine 1992. Although the provision on the prohibition of PGD remained, the amendment granted the exceptional use of PGD in cases of serious conditions. In Italy, which had prohibited PGD with the enactment of Law 2004, the Constitutional Court ruled that couples who are carriers of genetic diseases have the right to undergo PGD in 2015. Additionally, Switzerland, which prohibited PGD under Reproductive Medicine Act 1998, recently held a referendum to lift the ban on PGD in 2016 after both the Federal Council and Parliament recommended it. Therefore, PGD for avoiding the birth of children with genetic disease is becoming widely accessible in Western Europe.

Recently, Asia also saw legal changes regarding the practice of PGD. In Thailand, PGD for social sex-selection had been offered to prospective parents including foreigners. However, Thailand legally banned social sex-selection by enacting the Protection of Children Born from Assisted Reproductive Technology Act 2015.

Germline Genetic Modification

Reproduction involving germline genetic modification has been regarded as taboo for clinical and ethical reasons, such as the serious risk to future generations, the transgression of the natural and divine laws, and the potential misuse for human enhancement for social purposes. Indeed, a legal survey performed in 2015 suggested that 74% of 39 countries prohibit human germline genetic modification by laws or guidelines (Ishii, 2017). In 2015, the UK became the first country that legalized a form of germline genetic modification under Canada. The permitted mitochondrial donation is germline mitochondrial DNA manipulation involving nuclear transfer to an enucleated donor egg or zygote made using a donor egg to reduce the load of pathogenic mitochondrial DNA, and prevent the onset of mitochondrial diseases in the offspring. Immediately after the legalization in the UK, a group led by a US physician first performed egg nuclear transfer to prenatally prevent the onset of mitochondrial disease in the USA and shipped the resulting embryo for transfer to their clinic in Mexico. Although it resulted in the birth of a boy, the legality of such a cross-border use of germline genetic modification is currently controversial (Palacios-Gonzalez and Medina-Arellano, 2017).

In 2015, a Chinese group reported basic research in which the correction of a pathogenic mutation in human embryos was attempted using a robust genetic engineering tool, known as genome editing. However, this attempt raised profound concern worldwide (Ishii, 2017). That same year, the USA established the Consolidated Appropriations Act 2016 Sec. 749, which prohibits the Food and Drug Administration from using the federal budget to review or approve applications for exemptions to the investigational use of biologics in which a human embryo is intentionally created or modified to include a heritable genetic modification. In 2016, Israel approved Amendment Law No. 3 Prohibition on Genetic Intervention (Human Cloning and Genetic Change in Reproductive Cells), 5776/2016 (valid till May 23, 2020). Despite the advent of genome editing techniques, such as CRISPR/Cas9, most countries will probably maintain their legal prohibition of human germline genetic modification.

Regulatory Situations in the G7 Countries

The regulation of reproductive medicine as of 2017 was compared in G7 countries, considering on their clinical activity and outcome according to the latest International Committee for Monitoring Assisted Reproductive Technologies (ICMART) world report 2010 (Dyer et al., 2016) (Table 2). These countries have made only three major changes in their regulation of reproductive medicine since 2010; Italy's legalization of gamete donation in 2014, the UK's legalization of mitochondrial donation in 2015 and the USA's legal ban of the FDA's use of the federal budget to review the clinical use of germline genetic modification in 2015.

Table 2 Regulatory state on reproductive medicine (RM) in G7 countries, as of October 2017

Country	Number of clinics ^a	Treatment cycles ^a	Babies/asp. cumul. (%) ^a	Reproduction using gamete donation (GD) is:	Surrogacy (S) is:	Preimplantation genetic diagnosis (PGD) is:	Germline genetic modification (GGM) is clinically:
Japan	591	242,833	19.9	Prohibited	Prohibited	Permitted (medical use only)	Prohibited
USA	474	176,214	59.2	Allowed	Permitted in some states even if commercial	Allowed (medical and social uses)	Prohibited (FDA's review banned)
Italy	202	56,419	20.7	Permitted	Prohibited	Permitted (medical use only)	Prohibited
Germany	124	75,701	32.6	Permitted (sperm donation)	Prohibited	Permitted (medical use only)	Prohibited
France	107	85,122	29	Permitted	Prohibited	Permitted (medical use only)	Prohibited
UK	72	57,482	39	Permitted	Permitted (altruistic only)	Permitted (medical use only)	Prohibited (except mitochondrial donation)
Canada	28	17,926	45.9	Permitted	Permitted (altruistic only)	Permitted (medical use only)	Prohibited

In Japan, RM is largely regulated by Japan Society of Obstetrics and Gynecology *guidelines* (GD, S, PGD) and Ministry of Health *guidelines* (GGM).

In the USA, RM is generally allowed at the federal level. The Fertility Clinic Success Rate and Certification Act 1992 requires clinics to annually provide data for all procedures to the Centers for Disease Control and Prevention. Some state laws guide the practice of GD and S. GGM is indirectly prohibited by the Consolidated Appropriations Act 2016.

Italy regulates RM by Law on Rules in the Field of Medically Assisted Reproduction 2004. However, in 2014, the Constitutional Court lifted the ban on GD.

Germany regulates RM primarily by the Embryo Protection Act 1990. PGD has been legal since Law 1990 was amended by the Law regulating preimplantation genetic diagnosis 2011.

France regulates RM by Law on Bioethics of 2011, Bioethics Law 2004, and Law 1994 on the Donation and Use of Elements and Products of the Human Body, Medically Assisted Procreation, and Prenatal Diagnosis.

The UK regulates RM by the Human Fertilization and Embryology Act 2008 and 1990 and the Surrogacy Arrangements Act 1985. Mitochondrial donation is permitted by the Human Fertilization and Embryology (Mitochondrial Donation) Regulations 2015.

Canada regulates RM primarily by the Assisted Human Reproduction Act 2004.

^aData in 2010 from Dyer, S., et al. (2016). *Human Reproduction* 31(7), 1588–1609.

These seven developed countries differ markedly in the number of fertility clinics and treatment cycles (Table 2). According to the ICMART world report 2010, Japan and the USA were the most active countries among the 60 countries examined, having hundreds of clinics and more than 100,000 treatment cycles per year. Of particular note, the total number of treatment cycles for 2010 in Japan was 242,833, which is approximately 14.8% of the total cycles 2010 across 60 countries. In Japan, the JSOG guidelines adopt a SET policy and prohibit reproductive medicine involving gamete donation, based on the request of the Ministry of Health. This is the direct opposite to the regulatory state in the USA, where gamete donation is largely allowed, and some states also permit commercial surrogacy. However, Japan showed a lower rate of childbirths per egg aspiration in 2010 (19.9%), than the USA (59.2%). The situation in Japan suggests the possibility that many patients repeatedly undergo IVF or ICSI using their own gametes. This rate is similar to that of Italy (20.7%), which is the third-most active country but had legally banned gamete donation until 2014, as discussed above. The rate of childbirths per egg aspiration ranges from 29% to 45.9% in the four western countries. Of note, Germany prohibits egg donation by the Embryo Protection Law. The gamete donation-prohibitive policies of Japan and Italy may partially account for the relatively low rate of childbirth following assisted reproduction.

With regard to regulations of surrogacy, the USA is unique among the seven developed countries, as some US states permit commercial surrogacy (Table 2). The other six countries wholly prohibit surrogacy or permit only altruistic surrogacy. Likewise, the USA allows the medical and social uses of PGD, whereas the other countries permit its use for only for medical purposes such as the avoidance of genetic disease in offspring (Table 2). In Japan, the JSOG guidelines permit PGD to avoid the birth of children with genetic conditions, but ban the practice of PGS for ethical reasons. Concerning the clinical use of human germline genetic modification, the UK is the first country that legalized mitochondrial donation. However, the other six countries largely ban it by law or guidelines (Table 2).

Future Regulatory Changes

As reproductive medicine has spread worldwide, its regulatory landscape has globally changed, reflecting ethical, religious, and social aspects. The number of countries with laws of reproductive medicine has gradually increased in the past decade, although some have not changed their relevant guidelines or continue to have no regulation of reproductive medicine.

In the early days of reproductive medicine, regulatory issues frequently occurred in reproductive medicine involving third parties. With advances in biomedicine, new issues will likely emerge in reproductive medicine involving no third parties, such as next-generation sequencing-PGS and germline genetic modification by genome editing. Such reproductive medicine can be justified by advocating parental autonomy as well as its safety in some countries. However, with the spread of cross-border reproduction, many countries will have to establish clear regulations on upcoming reproductive techniques, conducting deep discussions of its ethical, social, and religious implications. In so doing, the welfare of future children and parental autonomy will be weighted to shape appropriate regulation in each country and, hopefully, in a global society.

References

- ABC. (2017). "Baby Gammy", now Grammy, starts kindergarten amid tensions over charitable donations. <http://www.abc.net.au/news/2017-06-29/baby-gammy-starts-kindergarten-amid-tensions-over-donations/8585596>, accessed 10.10.17.
- Bayefsky, M. J. (2016). Comparative preimplantation genetic diagnosis policy in Europe and the USA and its implications for reproductive tourism. *Reproductive Biomedicine & Society Online*, 3, 41–47.
- Dyer, S., Chambers, G. M., de Mouzon, J., Nygren, K. G., Zegers-Hochschild, F., Mansour, R., Ishihara, O., Banker, M., & Adamson, G. D. (2016). International Committee for Monitoring Assisted Reproductive Technologies world report: Assisted reproductive technology 2008, 2009 and 2010. *Human Reproduction*, 31, 1588–1609.
- Gürtin, Z. B. (2016). Patriarchal pronatalism: Islam, secularism and the conjugal confines of Turkey's IVF boom. *Reproductive Biomedicine & Society Online*, 2, 39–46.
- IFFS. (2007). International Federation of Fertility Societies (IFFS) surveillance 2007. *Fertility and Sterility*, 87, S1–S67.
- IFFS. (2016). International Federation of Fertility Societies (IFFS) surveillance 2016. *Global Reproductive Health*, 1, 1–143.
- Ishii, T. (2017). Germ line genome editing in clinics: The approaches, objectives and global society. *Briefings in Functional Genomics*, 16, 46–56.
- Jackson, E., Millbank, J., Karpin, I., & Stuhmcke, A. (2017). Learning from cross-border reproduction. *Medical Law Review*, 25, 23–46.
- Lemaitre, J., & Sieder, R. (2017). The moderating influence of international courts on social movements: Evidence from the IVF case against Costa Rica. *Health and Human Rights*, 19, 149–160.
- Palacios-Gonzalez, C., & Medina-Arellano, M. J. (2017). Mitochondrial replacement techniques and Mexico's rule of law: On the legality of the first maternal spindle transfer case. *Journal of Law and the Biosciences*, 4, 50–69.
- Sallam, H. N., & Sallam, N. H. (2016). Religious aspects of assisted reproduction. *Facts, Views & Vision in ObGyn*, 8, 33–48.
- Steptoe, P. C., & Edwards, R. G. (1978). Birth after the reimplantation of a human embryo. *Lancet*, 2, 366.
- Takeshima, K., Jwa, S. C., Saito, H., Nakaza, A., Kuwahara, A., Ishihara, O., Irahara, M., Hirahara, F., Yoshimura, Y., & Sakumoto, T. (2016). Impact of single embryo transfer policy on perinatal outcomes in fresh and frozen cycles-analysis of the Japanese Assisted Reproduction Technology registry between 2007 and 2012. *Fertility and Sterility*, 105, 337–346.
- WHO. (2017). *World Health Organization (WHO): Gender and Genetics, Sex Selection and Discrimination*. <http://www.who.int/genomics/gender/en/index4.html>, accessed 06.10.17.
- Yuko, E. (2016). *The First Artificial Insemination Was an Ethical Nightmare in The Atlantic*. <https://www.theatlantic.com/health/archive/2016/01/first-artificial-insemination/423198/>, accessed 06.10.17.

Cross-Border Reproductive Care

Guido Pennings, Bioethics Institute Ghent, Ghent, Belgium

© 2018 Elsevier Inc. All rights reserved.

Introduction

Cross-border reproductive care (CBRC) (more commonly called “reproductive tourism”) refers to the phenomenon of people traveling from one country where treatment is unavailable for them to a country where it is available. There is a general lack of empirical data on the prevalence of CBRC. Most published data are based on gross estimates and the studies are often methodologically lacking. In the only European study to date, the size of the phenomenon was estimated around 24,000–30,000 cycles per year minimally (Shenfield et al., 2010). Detailed numbers on incoming patients only exist for Belgium (Pennings et al., 2009). Attempts have been made to get some insight into what is happening in the United States and Canada but these were not very successful for lack of cooperation by clinics.

The accumulation of small qualitative studies on patients shows that there are many different types of patients who make different trajectories for different reasons and under diverse conditions. The term CBRC covers a large set of heterogeneous activities. As a consequence, it is difficult to make broad statements on its characteristics. It is clear, however, that some preliminary conditions have to be present before the phenomenon can develop. The most important one is the availability of information. The internet allows people to look for alternatives and to find out where the solution can be obtained and under which conditions. Other helping factors are cheap flights, willingness of clinics to adapt protocols (e.g. part of the treatment can be done at home), and special arrangements for foreign patients (interpreters, insurance ...).

Reasons for Crossing Borders

The reasons why people seek infertility treatment abroad can be put in three categories: treatment cost, treatment quality and treatment availability. First, as for other types of medical tourism, the treatment cost is an important motivator. Most countries reimburse only a limited number of cycles (1–3) and as a consequence most patients have to pay out-of-pocket. A second possible motivator is treatment quality. This element may refer to numerous aspects of the treatment: success rate, length of the waiting lists, general attitude of the doctors, patient-friendliness of the treatment and privacy considerations. A third factor is the availability of treatment. Again several aspects can be discerned: treatment might not be available because of lack of expertise or equipment (preimplantation genetic diagnosis), certain groups of patients are excluded from treatment on the basis of certain characteristics (age, sexual orientation, marital status...) and certain interventions are forbidden by law because they are considered ethically unacceptable (such as oocyte donation and social sexing) (Pennings, 2004).

Often, several of these factors are intertwined. For instance, legislation that denies certain groups access to donor sperm (like lesbian couples in France) will also affect the waiting time for donor sperm in the country. In a study on the patients going to Belgium for infertility treatment, several instances of a direct correlation between regulation in the country of residence of the patient and the extent of the patient flow towards another country were found (Pennings et al., 2009). When the Netherlands abolished donor anonymity by law in 2004, the number of Dutch patients attending Belgian fertility clinics for donor sperm doubled because of the long waiting lists at home and patients who wanted anonymous sperm.

Law Evasion

Legal diversity is a necessary condition for CBRC. Within Europe, there are not two countries with similar rules on all aspects of medically assisted reproduction. The single most important driver of CBRC is law evasion (Shenfield et al., 2010). The attitudes towards CBRC are largely determined by people's attitude towards this reason. There are three possible manners in which states can respond to CBRC: coerced conformity, international harmonization and moral pluralism (Pennings, 2004).

Coerced conformity: Some people want the government to take steps to stop citizens from going abroad. However, such defensive laws would be impossible to reinforce as one cannot know with certainty that people go abroad for medically assisted reproduction and one cannot know when citizens return that they had treatment abroad (apart from foreign surrogacy of course because they have to bring in the child). Moreover, legislation on medically assisted reproduction is (mostly) limited by the territoriality principle, that is, the laws only apply to actions performed on the national territory unless explicitly stated otherwise.

International harmonization: Another possible reaction to legal diversity is legal harmonization. Especially within the European context, people believe that a European law on medically assisted reproduction would strongly reduce the number of movements. It is, however, highly unlikely that a European consensus could be reached. Legislation on assisted reproduction touches very personal and emotional domains such as family building, marriage, and child welfare. National legislation on such issues is molded by each country's cultural, political and historical background and without a European common cultural basis, a European law would lack a firm foundation.

Moral pluralism: The last regulatory position is that states enact moral pluralism regarding citizens who seek locally prohibited treatments in other jurisdictions. That means that, although the state prohibits certain services, it recognizes the right of citizens with a different opinion to pursue such treatments elsewhere. This position displays a pragmatic and tolerant attitude.

Gamete Shortage

Beside law evasion, gamete shortage is the most important driver of CBRC. The shortage generally has several causes among which the general attitude towards genetic relationships and the perception of gamete donation as an intrusion into the family. Moreover, most European countries spend very little money and effort on campaigns to promote gamete donation. The most likely explanation for this reticent position is the controversial nature of gamete donation (socially, psychologically and morally) reinforced by secrecy and embarrassment. It is not surprising that most countries struggle with a shortage. Self-sufficiency, an ideal put forward for organ and blood donation, would decimate the number of cross-border movements.

The situation is different for sperm and eggs. Many European countries obtain sperm from Danish sperm banks. It is well known that the Danish banks ship thousands of samples around the world every year. There is no precise empirical data but press briefings by these banks give a certain idea about the extent of the trade. In Belgium, for instance, about 60% of all sperm comes from Denmark. This fact is generally ignored also because of the invisibility of these movements, in contrast to patients moving. Countries that do not import sperm generally have waiting lists going from 1 to 2 years.

For eggs, the situation is generally worse and the waiting lists are longer. Given the effort required for women to donate, relatively few women volunteer for the task. In this case, most candidate recipients head for Spain. Half (52%, $n = 15,600$) of all egg donor cycles in Europe are performed in Spain (Kupka et al., 2016). Again, however, there is scarcity of reliable empirical data on cross-border movements. According to Hernandez and de la Fuente (2010), the number of non-resident cycles in Spain can be calculated around 5000–10,000 with a large incidence of egg donation.

According to many people, the success of the Spanish centers can be explained by the fact that they pay the donors. However, this can surely not be the only explanation. Spanish centers give 900€, not as payment but as compensation for loss of time, inconvenience, pain etc. Obviously 900€ is a lot of money and may work as an incentive for women to donate. However, a look at the “compensation” schemes of other countries shows that many other countries are paying approximately the same amount or more. In Belgium, Greece, Poland, Russia, United Kingdom, and Ukraine, sums comparable to the 900€ are provided to egg donors (Pennings et al., 2014).

The question of payment has been debated for decades. It is part of the larger evolution towards commercialization in medicine. Increasingly, market rules are infiltrating all aspects of health care. However, resistance against payment for body material is still strong in Europe. In the United States, no shortage of gametes exist but then payment is considered normal. Depending on the characteristics of the donor, the going rate is between 5000 and 10,000\$. In Europe, several statements by the European parliament have emphasized the gratuity rule. Also in the Tissues and Cells Directive of 2004, non-commercialization is put forward as a central principle. However, this Directive also states that ‘donors may receive compensation, which is strictly limited to making good the expenses and inconveniences related to the donation’. The main point of discussion is the part on inconveniences. If only proven expenses can be covered, one can label this reimbursement. In case of compensation for non-financial losses, one speaks of payment. The question then becomes when compensation turns into reward and remuneration (Nuffield Council on Bioethics, 2011). To avoid payment, only reimbursement of proven expenses would be acceptable. However, it is not clear why non-financial losses (time, inconveniences, pain, etc.) cannot be compensated. Still, when non-financial losses are compensated with money, the distinction with payment and wages becomes small, if not untenable. A possible solution might be to offer non-financial compensation such as free vacation, a voucher for a weekend trip or something similar.

Since many patients leave their country looking for gametes abroad, one part of the solution would be to recruit more donors at home. More of an effort should be made by all countries to become self-sufficient as far as donor gametes is concerned. Every donor recruited at home is a patient less who has to travel to another country to obtain gametes.

Main Risks and Challenges in CBRC

CBRC has a bad reputation and is usually presented as a problem. Many organizations warn their members about traveling to another country. An analysis of the statements of four major organizations (European Society of Human Reproduction and Embryology (ESHRE); Human Fertilization and Embryology Authority (HFEA); International Federation of Gynecology and Obstetrics (FIGO); and Infertility Consumer Support for Infertility (iCSI)) on the types of risks and challenges related to CBRC results in the following list of major risks: violation of or non-compliance with safety standards (multiple pregnancy, donor screening, ovarian hyperstimulation syndrome); violation of or non-compliance with quality standards (success rates); confidentiality of patient information (medical records); lack of or insufficient psychosocial and medical counseling (language and culture); financial implications (real cost, reimbursement...); differences in gamete donation arrangements and surrogacy (anonymity, legal rights and obligations); and legal recourse after treatment (malpractice, indemnification, insurance ...).

Several strategies have been developed to deal with these issues. The European Union has enacted laws and directives to guarantee safety of care and to protect patient rights. ESHRE published a good practice guide for cross-border reproductive care specifically directed at clinics and practitioners (Shenfield et al., 2011). Health insurers worldwide are offering portable insurance packages that provide financial and legal support for cross-border patients.

To avoid tendentious presentations, the assumed dangers of cross-border reproductive care should be corroborated by empirical evidence. At the moment, a lot of anecdotal evidence circulates on the internet as well as in academic literature but, given the possible conflict of interest of the sources of the stories, this evidence should be handled with care.

The literature shows that CBRC would hold a risk for three important values, that is, safety, autonomy, and justice.

Safety

Multiple pregnancy leads to a strong increase in obstetric complications, perinatal morbidity, maternal and child mortality rate, congenital malformations, pre-term birth, and long-term social, psychological and financial difficulties. The occurrence of this side effect of ART is a test case for the moral quality of the field. The most recent report of pregnancy rates in Europe after medically assisted reproduction indicates that there are still huge differences: in 2011, twin pregnancies after IVF or ICSI occurred in 4.9% of cases in Sweden, 22.1% of cases in Spain, and 41.5% of cases in Greece ([Kupka et al., 2016](#)). Whereas CBRC is often cited as leading to more multiple pregnancies, one might also argue that patients from countries with a high multiple pregnancy rate should, for their own and their child's safety, seek treatment in countries with a low multiple pregnancy rate. The conclusion is that whether or not CBRC jeopardizes patients' health depends on the starting conditions at home and on the general conditions in the destination country.

It seems that generally speaking most clinics in member states of the European Union follow good practice guidelines, partially imposed by the European Tissues and Cells Directive, and that risks for the patients are not significantly higher when they seek treatment elsewhere. For countries outside the European Union, things are less clear but still there are no studies showing that there are major reasons for concern as far as safety is concerned.

Autonomy

Autonomy refers to the right of the patient to make important decisions in his or her life in accordance with his or her life plans and values. Medical interventions generally qualify as important decisions and thus require permission from the patient. In the context of medical treatment, autonomy is generally operationalized in the informed consent. Two important aspects are involved here: the provision of information and counseling. A person can only make an informed decision when he received all the relevant information. More specifically, this implies information on success rates, complications, costs etc. This is a difficult job since these parameters frequently depend on the personal characteristics of the patient (age, body mass index, etc.) and may not be clearly known before the treatment. Nevertheless, there are reasons for concern. Most patients choose the clinic abroad through the internet. There are very few studies on the quality and reliability of information on websites of infertility clinics. However, as for all other types of commercial activities, websites are used to attract customers and there is no reason to assume that this would be different in assisted reproduction. Points that raise concerns are exaggerated success rates (mainly by taking a mean that completely ignores the increased age of foreign patients), downplaying of potential risks (especially when multiple pregnancies are concerned), and subtle commercial messages to convince patients. Several studies have indicated that a large percentage of patients use the internet as their prime source of information, although there are large international differences ([Shenfield et al., 2010](#)). However, despite these concerns, no empirical studies have demonstrated a real danger. Moreover, even if shortcomings would be demonstrated, this would still not count as an argument against cross-border care as such. Information could be provided by other, more reliable sources such as local physicians, patient organizations, etc. Another solution could be to have quality labels accorded through independent international organizations to clinics.

The second issue regards counseling especially for socially and psychologically more complex interventions such as gamete donation. It is important that patients receive counseling regarding the possible aspects, consequences, and psychological repercussions of such treatments. A possible problem here might be the language. Receiving counseling in a foreign language is certainly not ideal. Talking about personal, intimate, and difficult issues such as feelings, self-esteem and sexuality is hard in any language but even more so in a foreign tongue. One may appeal to interpreters but many psychologists are reluctant to do so. In addition, there may be a cultural gap: some things may not be discussable in certain cultures or between certain persons, others things may be seen as self-evident by the counselor but not by the patients. Two points should be made here: first, many larger clinics with significant numbers of foreign patients have hired doctors from several nationalities, precisely to accommodate these patients. They are welcomed, counseled and treated in their mother tongue. Second, if there are concerns about counseling for certain types of interventions, this should be an overall concern that applies to other treatments as well and also to patients of foreign origin resident in the country. So, although appropriate counseling is certainly relevant for the assessment of cross-border treatments, it should be evaluated in a broader perspective.

A final remark regarding autonomy: cross-border traveling allows patients who do not have access to a certain type of treatment at home to fulfill their child wish abroad. This brings an enormous increase in their autonomy. This advantage may easily outweigh the possible negative effects of treatment abroad.

Justice

Many countries reimburse only a limited number of IVF cycles (or none at all), and if these are unsuccessful or if patients want more children, additional cycles may become a serious financial burden. This burden might be considerably lighter if the patients seek

treatment abroad. The direct cost of an ART cycle is estimated around 10,000€ in the United States, 4500€ in the United Kingdom and 2500€ in Belgium (Connolly et al., 2010). There are two dimensions to justice: access and equality. Access means that people should not be discriminated, that is, should not be excluded on the basis of ethically irrelevant elements. One such element is wealth, at least in countries where infertility treatment is considered to be part of the basic health care package. Equality refers to the fact that all people should be treated equally.

The level of subsidization or public funding determines financial access and thus indirectly the overall access to infertility treatment. If infertility treatment is part of basic health care and a country does not provide a reasonable number of IVF cycles, only the rich will be able to access treatment. When people have to pay out-of-pocket at home, they may be able to pay a treatment abroad which they could never afford at home. If this is the case, justice as access to treatment may be improved for poor people by crossing the border.

The situation is completely different for patients who travel because they want to evade a restrictive legislation or because they want to avoid a long waiting list. There indeed those who have the financial means can travel while the others cannot. Those who use the argument of justice to reject the possibility of going abroad focus on the equality aspect. Equality can be established by making sure that either no one has access to treatment or that everyone has. So the restrictive way would be to try to prevent people from going abroad. That would install equality since no one (not even the rich) would have access. However, another way in which this inequality could be avoided would be to introduce portability of health insurance for all. That means that people who go abroad to obtain treatment that is not available at home would still be reimbursed by their health insurance. Most countries will try to avoid this scenario since they will see this as a form of complicity with wrongdoing. In fact, when the European Directive on patient mobility was discussed, countries explicitly demanded the guarantee that they would not be obliged to reimburse treatments that were not available in their territory. From the start, the member states wanted to keep a certain degree of control, both on health care expenditures and on the type of treatment they would be obliged to pay for abroad. It was specified that they would not have to reimburse ethically controversial medical services like euthanasia, DNA testing or IVF when these services are not allowed or not reimbursed at home (European Parliament and Council, 2009). Of course, another solution would be to adopt a more lenient legislation that does not exclude large groups in the population and does not force them to seek help in another country. In other words, rather than blaming the victim from finding a way out, one may reconsider the causes of the movements.

Conclusion

Cross-border reproductive care is a multifaceted phenomenon. The empirical evidence has demonstrated that it is difficult to compose a profile of the “reproductive tourist.” Moreover, the whole field is developing fast. International chains of clinics emerge, the introduction of European legislation specifically on patient mobility and portability of health insurance may lead to an increase in movements, and changes in national legislation could alter patient streams quite drastically. Since it is unlikely that the main causes of cross-border reproductive care will drop away any time soon, the first and minimal step now is to try to reduce harm to patients (and donors) as much as possible. This can be done by better regulation of clinics, improved control of safety standards, and objective information provided to patients. However, cross-border care is not necessarily a bad thing. Many risks and complications currently attributed to cross-border reproductive care are not really related to crossing borders. Still, since patients should have access to treatment at home, and since cross-border treatment frequently adds an extra burden to the already quite difficult project of infertility treatment, governments should at least look at the possibilities of eliminating the reasons why the citizens leave.

References

- Connolly, M. P., Hoorens, S., & Chambers, G. M. (2010). The costs and consequences of assisted reproductive technology: an economic perspective. *Human Reproduction Update*, 16, 603–613.
- European Parliament and Council (2009). Report on the proposal for a directive of the European Parliament and of the Council on the application of patients' rights in cross-border healthcare (COM(2008)0414 – C6-0257/2008–2008/0142(COD)). Committee on the Environment, Public Health and Food Safety.
- Hernandez, J., & de la Fuente, L. A. (2010). In *Cross border reproductive care in Spain* Presentation at the ESHRE meeting 'Cross border reproductive care.' May 14, 2010, Paris.
- Kupka, M. S., Ferraretti, A. P., de Mouzon, J., et al. (2016). The European IVF Monitoring Consortium (EIM) for The European Society of Human Reproduction and Embryology (ESHRE) (2016). Assisted reproductive technology in Europe, 2011: Results generated from European registers by ESHRE. *Human Reproduction*, 3, 233–248.
- Nuffield Council on Bioethics. (2011). *Human bodies: donation for medicine and research*. London: Nuffield Council on Bioethics.
- Pennings, G. (2004). Legal harmonization and reproductive tourism in Europe. *Human Reproduction*, 19, 2689–2694.
- Pennings, G., Autin, C., Decler, W., et al. (2009). Cross-border reproductive care in Belgium. *Human Reproduction*, 24, 3108–3118.
- Pennings, G., de Mouzon, J., Shenfield, F., et al. (2014). Socio-demographic and fertility-related characteristics and motivations of oocyte donors in eleven European countries. *Human Reproduction*, 29, 1076–1089.
- Shenfield, F., de Mouzon, J., Pennings, G., et al. (2010). Cross border reproductive care in six European countries. *Human Reproduction*, 25, 1361–1368.
- Shenfield, F., Pennings, G., De Mouzon, J., Ferraretti, A. P., & Goossens, V. (2011). ESHRE's good practice guide for cross-border reproductive care for centers and practitioners. *Human Reproduction*, 26, 1625–1627.

Modern Ethical Dilemmas in ART

Guido Pennings, Seppe Segers, and Heidi Mertes, Bioethics Institute Ghent, Gent, Belgium

© 2018 Elsevier Inc. All rights reserved.

Introduction

Medically assisted reproduction offers people an increasingly broad spectrum of techniques to have the child they long for. These techniques can be subdivided in two categories: those using the intended parents own genetic material/gametes and those using donor genetic material/gametes. For the large majority of the people, the applications in the latter category are “solutions of the last resort”: only if there is no way that a couple or person can have a genetically related child will a non-genetically related child be considered. However, almost all the techniques to bypass a defect of the reproductive system imply manipulations and modifications of which the consequences for the long term health of the future offspring are unknown. One recent example that caused quite some concern is mitochondrial transfer. Women at risk of transmitting a mitochondrial disorder to their offspring either transfer healthy mitochondria from a donor into their oocytes or transfer the nucleus of their egg into an enucleated donor oocyte. The health consequences of this manipulation are unknown. The important point is, however, that this issue could be avoided by using donor eggs. Still, despite the risks, one goes ahead with the development of the technique. The same applies to people with known increased risks of transmitting a genetic disease who go through multiple cycles of IVF and PGD while donor gametes would solve the issue. At the same time, the use of donor gametes is not self-evident or easy either. In the present climate in which much emphasis is put on the person of the donor, the importance of knowing one’s genetic origin in order to develop one’s identity and the strong value attached to the genetic relationship between the donor and the child, gamete donation is increasingly confronted with moral and psychological problems. Moreover, there is a shortage of donor gametes (in particular of eggs) in many countries, resulting in long waiting lists.

In this article we will look more closely at two applications that illustrate this general problem: oocyte cryopreservation for healthy women and stem cell derived gametes. Both examples show in different ways the importance of genetic parenthood.

Oocyte Cryopreservation for Healthy Women

One of the interventions in ART that has been the topic of ethical debate for several years now is oocyte cryopreservation in anticipation of age-related fertility decline, known as “social egg freezing”, “elective egg freezing”, “non-medical egg freezing” or “AGE banking” (whereby AGE is an acronym for Anticipated Gamete Exhaustion). Although the first healthy live birth from a frozen human egg cell dates back to 1986, egg freezing has long been so inefficient that it was hardly considered a valid treatment option. From 2004 onwards, however, there has been an explosion in research directed at oocyte cryopreservation (OC), leading to successful slow freezing and vitrification protocols for human egg cells with high survival rates after thawing and with reassuring preliminary data on the health of the resulting offspring (Cobo et al., 2014). There are several applications for this new technology: donor egg banking, but also autologous egg banking. The latter was introduced in the context of oncofertility, so that women fearing premature infertility due to disease, gonadotoxic medical treatments or irradiation therapies could bank their eggs before irreversibly losing them.

Soon there was also an interest in storing eggs for women not faced with a medical condition leading to premature infertility, but for those approaching the natural boundaries of their reproductive lifespan while being unable to start a parental project, for example due to lack of a partner. However, whereas the introduction of OC for oncofertility was met with much enthusiasm and optimism, the expansion of the option of OC to healthy women did not incite the same reactions (Mertes, 2013). Both the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) initially denounced the use of OC for healthy women, while endorsing it for medical conditions. In 2012, ESHRE changed its recommendations, saying that OC should also be available for non-medical reasons, whereas the ASRM remains cautious (ESHRE Task Force on Ethics and Law et al., 2012; Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Reproductive Technology, 2012).

Medical versus Social freezing: A Tenable Distinction?

A first important question is on which grounds a moral distinction can be made between “medical egg freezing” and “non-medical egg freezing”. If women are only allowed to preserve their fertility when they anticipate premature infertility, but not when they anticipate natural infertility, one needs to give a justification for this differential treatment. Several arguments are possible, but not convincing. A first one is that we should respect natural boundaries. But why? Is medicine not constantly interfering with the natural decline of our bodies? A second one is that only those who are unable to anticipate (and thus prevent) being infertile before embarking on parenthood are deserving of a medical intervention. But why? First, medicine is constantly curing preventable medical problems. Second, the “solution” of reproducing early in life is not an option for everyone: there are very good reasons for waiting to establish a family until certain side constraints are in place, such as having a partner, financial stability, et cetera.

Moreover, there is a large gray area between medical and non-medical reasons for egg banking. In which category do women who freeze their eggs due to a family history of early menopause (and a low ovarian reserve) belong? What about women who have undergone a cancer treatment in the past that has diminished, but not depleted their ovarian reserve and who want to store eggs after their treatment because they fear premature ovarian failure? In short, it is difficult to indicate what the crucial difference between the two applications (medical and non-medical) is and even more so why it would matter.

Reasons for Caution

Yet there are reasons to be cautious about the introduction of AGE banking, with the main fear being that this new technology is being “oversold”. [Cobo et al. \(2016\)](#) report cumulative live birth rates in women 35 or younger of respectively 15.4%, 40.8%, and 85.2% when freezing 5, 8 or 15 oocytes. The cumulative live birth rates achieved with 5, 8 and 11 oocytes for women 36 or older were 5.1%, 19.9% and 35.6% (in this group the additional benefit of banking more than 11 oocytes is very small). At present most women opting for AGE banking belong to the latter group, not the former, as reported average ages at the time of egg banking range from 36 to 38 years. Although these are good success rates compared to a subfertile population, this still means that most of these women have at best a 35% chance of achieving a healthy live birth (if their ovarian response is good enough to generate 11 oocytes). This contrasts with the image of egg freezing that commercial companies offering this technology create. Egg banking is said to “stop the biological clock” or offer “insurance” against infertility. The fear is that women will expect more of egg banking than the intervention can deliver and that many women will feel disappointed and betrayed when it turns out that the eggs they stored do not lead to a live birth. In this sense, an intervention that is being marketed as increasing women’s reproductive autonomy becomes an intervention that curtails reproductive autonomy and preys on desperate women.

Should this technology be marketed to young women then, with higher success rates? In fact, this may equally lead to an undermining of a woman’s intended reproductive life plan. When egg banking becomes a deliberate attempt to put parenthood “on hold” while other life goals are being pursued (possibly under pressure of employers), women may insufficiently be aware that egg banking in such a context jeopardizes a woman’s reproductive potential, rather than safeguarding it, as a woman’s chances of reproducing naturally at a younger age will always be better than her chances of reproducing with banked eggs at an older age. This fear became a lot less hypothetical when Facebook and Apple announced in 2014 that they would offer egg freezing to their female employees ([Mertes, 2015](#)).

What Does a Cautious Implementation Look Like?

The crucial question then becomes: how can this technology be implemented in a way that has maximal respect for reproductive autonomy and an acceptable utility rate? Regarding the utility rate the paradox is that the younger women freeze their eggs, the better quality the eggs will be, but the less chance that the woman will actually need them, as they have plenty of time to reproduce naturally. The older a woman is when she freezes her eggs, the worse their quality, but also the less chance she has of reproducing naturally (and the more chance to need her stored eggs). The ideal age for egg banking would therefore be between 30 and 35 ([Mertes and Pennings, 2011](#)). Also, women opting for egg banking should be counseled carefully about the drawbacks of AGE banking: about the success rates, tailored to their age/ovarian reserve; about the technicalities of the procedure, for example the fact that ICSI will be needed; about the maximal storage period, etc. Next to this practical counseling, it would also be beneficial to inspire candidates for AGE banking to critically reflect on the importance of (genetic) parenthood and on the prospect of the alternatives. This may imply that women approaching their forties or young women with many fertile years ahead of them will end up being discouraged from storing their eggs, while women who are most likely to benefit will be encouraged to bank. Given the possible biasing effect of commercial interests, it becomes increasingly important to ensure that counseling and information provision to women is as objective and complete as possible.

Stem Cell Derived Gametes

The in vitro creation of stem cell-derived (SCD) gametes promises new possibilities for human procreation, both via basic science applications and clinical use in assisted reproduction. The value of genetic relatedness is an important incentive in the development of this technique. Proof of principle for in vitro gametogenesis (IVG) is available in mice, and it is believed that the differentiation of functional human gametes from pluripotent stem cells will be possible in the “not too distant future” ([Cohen et al., 2017](#)). Creation of gametes from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) is the main focus. Other sources such as bone marrow stem cells and germline stem cells receive less (ethical) attention. We will first outline the possible reproductive applications of IVG and then the ethical challenges of both ESC-derived and iPSC-derived gametes.

Patient-Specific SCD-Gametes

The most important goal of IVG is to establish a genetic link between the parents-to-be and the future offspring. To establish a genetic link, patient-specific SCD-gametes are needed. This requires either the creation of a cloned embryo through somatic cell nuclear transfer (SCNT) from which ESCs are derived or the creation of induced pluripotent stem cells (iPSCs) from a somatic

cell of the patient, which will then be differentiated into gametes. It is also suggested that IVG would make equal genetic relatedness between the child and both partners possible for same-sex couples, but researchers remain skeptical about reprogramming stem cells to gametes of the opposite sex (Moreno et al., 2015). Moreover, if IVG is to lead to the creation of patient-specific SCD-gametes at all, the technique of SCNT or the technique to obtain iPSCs would have to be improved.

If it would become possible to create patient-specific SCD-gametes, it could also be combined with technologies to prevent certain diseases in the future offspring. IVG could enable the creation of a large gamete and embryo pool to select (through PGD) genetic traits. Alternatively, stem cells could be modified via genome editing, and then differentiated into gametes (Vassena et al., 2016). This can be seen as an additional benefit, although it also raises concerns about possible usage to select/edit non-disease related traits.

Non-Patient-Specific SCD-Gametes

Deriving gametes from ESCs of embryos created by fertilization is presumably more feasible and closer to clinical application than creating patient-specific SCD-gametes. This would require the use of donated spare IVF embryos, or embryos created from oocytes and sperm donated for this goal. However, these gametes would be non-patient-specific, and will thus not lead to shared genetic parenthood.

Yet, the value of parent-child genetic relatedness also plays a central role here. Non-patient-specific SCD-gametes could be used to create an SCD-gamete bank for “third party” assisted reproduction. One benefit of this scenario over “traditional” donor assisted reproduction is that the parents-to-be would not have to fear that the donor would claim parental rights based on his/her genetic link with the child, since the donor (the embryo) is destroyed in the process of gamete derivation (unless such rights would be accorded to the genetic grandparents, viz. the embryo donors). Also, the resulting child would have no “unknown” genetic progenitor to look for, which might be more comforting than knowing that one’s genetic parent is “out there”. It could, however, be countered that this benefit is undermined by the child’s inability to enrich his/her narrative by knowing that one of his/her genetic parents never existed as a person.

Non-patient-specific SCD-gametes could also be used for same-sex reproduction: a gamete derived from a fertilized embryo, created by combining a donor gamete with one of the partner’s gametes, could be combined with the other partner’s complementary gamete (Segers et al., 2017b). This would lead to a 50% genetic link between the latter partner and the child, while both the other partner and the donor would share 25% of the child’s DNA. Many would consider it an important advantage of this scenario that although a donor is still involved, it will not be possible to ascribe a greater parental status to him/her solely on the basis of his/her genetic contribution. The partner who contributed as much to the child’s genetic makeup as the donor, would outrank the donor given his/her role as a social parent and the intention to be a parent.

New Possibilities of Reproduction

Application of IVG for reproductive purposes could revolutionize human procreation. Apart from possible use in same-sex reproduction and treatments for infertile couples of reproductive age, IVG could allow anyone to produce gametes, regardless of age or relationship status (Smajdor and Cutas, 2015). If person-specific gametes could be created from stem cells, this could allow women to reproduce later in life, despite declining oocyte quality and even if they have already gone through menopause (see above). Prepubescent children would not be restricted by their age to have genetically related offspring either. IVG could also allow variations in the number of people involved in genetic parenting. Fusing someone’s natural gamete with a derived gamete from this same person could result in single genetic parenthood. Also multiple genetic parenting might become possible: gametes of multiple persons could be combined to create embryos, from which ESC-derived gametes could be established, which could again be recombined in as much cycles as needed to obtain a child who is genetically related to each of the participating persons. For a more extensive ethical discussion of these scenarios, see: Smajdor and Cutas (2015) and Segers et al. (2017a). In the final section, we will explore some of the general ethical concerns about IVG.

Main Ethical Concerns

For reproductive IVG, the main ethical issue is the safety risk to the offspring. At this stage, clinical application of IVG is unacceptable because of the potential high risk to the offspring’s physical wellbeing. This is especially the case for patient-specific SCD-gametes, as both the technique of SCNT and that of iPSCs derivation are hindered by incomplete reprogramming of the somatic cell nucleus to an embryonic state. The derivation of gametes from ESC-lines from embryos created by fertilization would be less complicated and therefore possibly safer. Still, this raises the question what standard of risk is acceptable to make genetic relatedness possible, or, in the case of non-patient-specific reproductive IVG, to avoid the attribution of parental status to the donor. It could be argued that, unless their life is not worth living, IVG offspring cannot be harmed by the parental choice to reproduce through IVG because without this decision they would not have existed at all. However, this conclusion runs counter to most people’s moral intuition. Instead the “reasonable welfare principle” has been suggested: this standard is adopted by the European Society of Human Reproduction and Embryology (ESHRE) and holds that the use of a reproductive technology is only acceptable if the resulting child will have a reasonably happy life (Pennings et al., 2007).

Even if IVG would be safe, it is worth contemplating that the endeavor of creating SCD-gametes for reproduction might reinforce the dogma of genetic relatedness. Even the use of non-patient-specific SCD-gametes could, at least implicitly, propagate the importance of genetic ties in a parent–child relationship. This is not morally wrong per se, and is also not unique to IVG, but it could send the incorrect message that a good parent–child relationship requires a genetic link. According to this reinforcement argument, IVG would create rather than meet demand, which is a fortiori important given the related questions of access, cost and resource allocation. Because IVG will probably not be affordable for everyone who could benefit from it, it could be argued that access to IVG should be state covered. It is questionable, however, whether the goals that are served by IVG are important enough to outweigh other claims on public resources. When this weighing is done, not only the goal of reproduction should be considered, but also the possible benefits of IVG in the research context.

A final argument is that IVG as such is unacceptable because it inherently involves embryo destruction. Embryo destruction is obvious if ESC-derived gametes are used (both patient-specific and non-patient-specific), but also the iPSC route might be troubling for those who oppose embryo destruction. While iPSC-derived gametes generally avoid the use of embryos, embryos will still have to be created in the research phase to ensure the safety of the technique. Many people find it less disrespectful to use spare IVF embryos (as they will be destroyed anyway), but those applications involving the destruction of embryos created from SCD-gametes that were differentiated from spare embryos will likely be opposed by those who object to the intentional creation/destruction of embryos. Only if the prohibition of embryo destruction is not regarded as an absolute rule, and if the importance of the goals of IVG (whether reproductive or scientific) outweigh the cons of creating and destroying embryos, can those who oppose embryo destruction accept IVG in general.

Conclusion

Having a genetically related child is a highly valued goal in many people's lives. Still, it is not easy to justify as the benefits of this wish are unclear. When the wish is accompanied by possible health risks for the offspring, society and the future parents will have to decide what risk is acceptable. In this balancing act, the existence of alternatives has to be taken into account. Donor gametes are such an alternative in most circumstances. However, this solution is more complicated from the psychosocial point of view given the increasing value attributed to genetic relationships.

References

- Cobo, A., Serra, V., Garrido, N., et al. (2014). Obstetric and perinatal outcome of babies born from vitrified oocytes. *Fertility and Sterility*, 102, 1006–1015.
- Cobo, A., García-Velasco, J. A., Coello, A., et al. (2016). Oocyte vitrification as an efficient option for elective fertility preservation. *Fertility and Sterility*, 105, 755–764.
- Cohen, I. G., Daley, G. Q., & Adashi, E. Y. (2017). Disruptive reproductive technologies. *Science Translational Medicine*, 9(372), eaag2959.
- ESHRE Task Force on Ethics and Law, Dondorp, W., de Wert, G., Pennings, G., et al. (2012). Oocyte cryopreservation for age-related fertility loss. *Human Reproduction*, 27, 1231–1237.
- Mertes, H. (2013). The portrayal of healthy women requesting oocyte cryopreservation. *Facts, Views & Vision in ObGyn*, 5, 141–146.
- Mertes, H. (2015). Does company-sponsored egg freezing promote or confine women's reproductive autonomy? *Journal of Assisted Reproduction and Genetics*, 32, 1205–1209.
- Mertes, H., & Pennings, G. (2011). Social egg freezing: For better, not for worse. *Reproductive Biomedicine Online*, 23, 824–829.
- Moreno, I., Míguez-Forjan, J. M., & Simón, C. (2015). Artificial gametes from stem cells. *Clinical and Experimental Reproductive Medicine*, 42, 33–44.
- Pennings, G., de Wert, G., Shenfield, F., et al. (2007). ESHRE task force on ethics and Law 13: The welfare of the child in medically assisted reproduction. *Human Reproduction*, 22, 2585–2588.
- Practice Committee of the American Society for Reproductive Medicine and Practice Committee of the Society for Reproductive Technology. (2012). Mature oocyte cryopreservation: A guideline. *Fertility and Sterility*, 99, 37–43.
- Segers, S., Mertes, H., de Wert, G., Dondorp, W., & Pennings, G. (2017a). Balancing ethical pros and cons of stem cell derived gametes. *Annals of Biomedical Engineering*. <https://doi.org/10.1007/s10439-017-1793-9>.
- Segers, S., Mertes, H., Pennings, G., de Wert, G., & Dondorp, W. (2017b). Using stem cell-derived gametes for same-sex reproduction: An alternative scenario. *Journal of Medical Ethics*. <https://doi.org/10.1136/medethics-2016-103863>.
- Smajdor, A., & Cutas, D. (2015). Background paper: Artificial gametes. Nuffield Council on Bioethics <http://nuffieldbioethics.org/wp-content/uploads/Background-paper-2016-Artificial-gametes.pdf>.
- Vassena, R., Heindryckx, B., Peco, R., et al. (2016). Genome engineering through CRISPR/Cas9 technology in the human germline and pluripotent stem cells. *Human Reproduction Update*, 22, 411–419.

FUTURE

Creation of Bioengineered Reproductive Organs: The End of Organ Transplantation?

Mats Hellström, University of Gothenburg, Gothenburg, Sweden

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

3D	3-Dimensional
ART	Assisted reproductive technology
EDC	Ethylcarbodiimide
eMSC	Mesenchymal stem cell-like stem cell source from the endometrium
ES	Embryonic stem
GFP	Green fluorescent protein
iPS	Induced pluripotent stem
MRKHS	Mayer-Rokitansky-Küster-Hauser syndrome
MSCs	Mesenchymal stem cells
NHC	Hydroxysuccinimide
SMC	Smooth muscle cells
STAT3	Signal transducer and activator of transcription 3

The Concept of Bioengineering

Bioengineering, or tissue engineering, is a research discipline in regenerative medicine that combines various types of cells and materials for the production of implants/grafts with the purpose to restore or improve tissue function. The largest fields within the bioengineering discipline are indisputably bone- and skin regeneration. However, cardiovascular-related tissue engineering solutions are also comprehensively investigated, e.g., for heart valves and blood vessels.

The discoveries of adult stem cells (both of hematopoietic- and of mesenchymal stem cell origin) in the 1960s generated a vast interest to harvest, grow and utilize these cells for various cell-based therapies and together with various biomaterials to improve construct function and adaptation after implantation. For example, mesenchymal stem cells (MSCs) have been used extensively due to their ability to participate in tissue repair and regeneration, and due to their unique immunomodulatory properties that reduces the host foreign body response to the biomaterial ([Hanson et al., 2014](#)). There is a large quantity of MSCs that resides in the bone marrow, but MSCs can be harvested from a wide variety of adult tissues which makes them attractive to use as a cell source for the generation of a patient-specific transplant. Other sources of mature cell types can be used, however the versatility of MSCs differentiation (e.g., they can mature into hard- or soft tissue cell types) and their ability to proliferate, usually surpass any other adult cell type contender. MSCs can be isolated directly from the patient, expanded and/or differentiated in vitro, and then be transplanted for the therapeutic purpose without causing a significant immune response. Other cell sources that are interesting to use for bioengineering is embryonic stem (ES) cells or induced pluripotent stem (iPS) cells. The use of ES cells is attractive since they have an ability to differentiate into any kind of adult tissue. However, there are some limitations since they are; (a) difficult to control and direct into the correct cell lineage, (b) known to cause cancer-like teratomas, (c) ethically debatable since they are of fetal origin, and (d) not patient-specific and may thus activate an immune response following transplantation. iPS cells are perhaps therefore a better cell source than fetal stem cells to use for bioengineering. iPS cells can be constructed by reprogramming adult cells taken e.g., from a skin biopsy. By inducing an overexpression of various transcription factors (e.g., the Yamanaka factors; Oct3/4, Sox2, cMyc, and Klf4), the cells are manipulated to reverse their differentiation state. These immature cells can then be used to generate many different tissue-specific cell types ([Takahashi and Yamanaka, 2006](#)). This discovery gave Dr. Yamanaka the Noble price in 2012. Thus, iPS cells can be used to create patient-specific bioengineered grafting material which would be immune tolerable after transplantation. However, the potential of iPS cells, and possible risks involved are still relatively unknown in the clinical setting.

Bioengineering for Reproductive Tract Disorders

Reproductive tract disorders and infertility often lead to substantial socio-psychological distress. These conditions may be acquired from birth (e.g., Mayer-Rokitansky-Küster-Hauser syndrome; MRKHS) or may be disease-induced (e.g., testicular infection, uterine fibroids or salpingitis) or consequences from treatments (e.g., chemotherapy- or radiotherapy induced sterility, hysterectomy or oophorectomy). While in some of these conditions, fertility can be established with standard assisted reproductive technology (ART), other conditions lead to permanent infertility. However, novel research that evaluates bioengineering solutions in animal studies may one day lead to new treatment protocols for these patient groups. These ideas include the use of grafted engineered tissues that facilitates sperm motility (vas deferens), sexual intercourse (corpus cavernosum; vaginal reconstruction), give growth support to maturing follicles (artificial ovaries), stabilizes the pelvic region using bioengineered meshes for prolapse treatment, or to a uterine wall defect during pregnancy (uterine tissue patches), or even the creation of a transplantable whole bioengineered uterus that may be able to replace a donor in a uterus transplantation setting ([Sadri-Ardekani and Atala, 2015](#); [Campo et al., 2017](#); [Hellström et al., 2017](#)).

Stem Cells for the Creation of Gametes

Different sources of stem cells have been used to create sperm cells for potential use in fertility treatments, both on animal- and human cells. For instance, spermatogonial stem cells can be harvested from the basement membrane of the seminiferous tubules in the testis, and are the origin of spermatogenesis. These cells can either be derived into mature and functional sperm cells in vivo (by re-transplantation) or by in vitro differentiation. These methods resulted in matured sperm cells successful of oocyte fertilization that led to offspring in zebra fish, mice and rats ([Hendriks et al., 2015](#)). Sperm cells were also derived from in vitro differentiated iPS cells, including human cells. However, due to ethical- and legal reasons, this work has not yet lead into any human births. These ideas however, may become future treatment options for sterile men where standard ART is unsuccessful. It is important to mention that alterations in the genome, or the epigenome may occur during the reprogramming ([Eguizabal et al., 2011](#)), thus rigorous testing should precede any clinical protocols.

Similarly, adult female cells (iPS cells) and stem cells (embryonic- and oogonial stem cells) were reprogramed and differentiated into oocytes ([Hendriks et al., 2015](#)). As for cancer treated young men, young women also risk sterility due to the negative side-effects from cancer treatment. These patients may benefit by cell reprogramming. However, this is a controversial topic since artificial oocytes also have been derived from male cells, and artificial sperm was created using a female cell source ([Hendriks et al., 2015](#)).

Bioengineering in the Male Reproductive Tract

Genitourinary tract abnormalities sometimes require surgical interventions, but sufficient autologous tissue for the reconstruction is often limited. For these reasons, a rabbit model was used to evaluate the construction of bioengineered corporal tissue derived from a biodegradable polymer scaffold which was seeded with corporal smooth muscle cells (SMC) and endothelial cells ([Chen et al., 2010](#)). Results from in vivo studies showed significantly more sperm after native mating, and only the treated animals were able to generate offspring. In another successful rabbit study, an obstructed vas deferens was repaired using tubular shaped encapsulated autologous myofibroblast-rich tissue ([Campbell et al., 2008](#)). These reports show the potentials of using bioengineering solutions for novel treatments of male reproductive tract disorders. However, more follow-up studies are required, including on larger animal models to evaluate the usefulness and safety of such constructs prior any clinical use.

Bioengineering in the Female Reproductive Tract

Ovarian Bioengineering

Fertility preservation methods for cancer treated women were successfully developed by Professor Donnez and his colleagues. Before the fertility-detrimental radio- and chemotherapy, fertility may be preserved in female patients by an ovarian cortex cryopreservation and a re-transplantation procedure. More specifically, the follicle-containing ovarian cortex can be isolated by minimal invasive laparoscopy prior to cancer treatment, and then cryopreserved in small rectangular tissue pieces which can be re-transplanted back to the patient after the successful cancer treatment. Thus, the patient re-acquires a part of her original follicle reserve that may otherwise have been lost due to the radio- and chemotherapy. Hormone production and oocyte maturation have shown to be reestablished and this method has now generated more than 100 healthy babies around the world ([Donnez and Dolmans, 2015](#); [Chiti et al., 2017](#)). However, this method cannot safely be performed on patients treated for blood-related cancers since they risk reintroducing the malignancy along with the cryopreserved ovarian tissue. Therefore, scientists now evaluate bioengineering solutions to support the growth of isolated early follicles. The advantage of transplanting isolated follicles rather than the ovarian cortex is that they do not contain any blood-related cancer cells and could therefore potentially be transplanted safely back to the treated patient.

Biomaterials made of various hydrogels (e.g., based on fibrin and/or alginate) work particularly well for ovarian bioengineering protocols in rodents. Multiple groups witnessed follicular maturation using such constructs and succeeded with the generation of healthy offspring using harvested functional oocytes from these grafts (Chiti et al., 2017). However, it is particularly challenging to construct an ovarian biomaterial suitable for human folliculogenesis since the volume of the primordial human follicle expands about 4.7×10^6 -fold before reaching the final mature stage of a follicular diameter of about 20 mm. Similar follicular expansion is seen in many other large mammals, which can be compared to the much lesser expansion seen in rodents (with an estimated 37-fold volume expansion) (Shea et al., 2014). During folliculogenesis, the biomaterial needs to be plastic enough to allow the expansion to occur, yet give structural support to the follicle to prevent collapse. Thus, improved biomaterials need to be developed. Lately, scaffold production through a method called decellularization received a lot of attention in the bioengineering field. This method is based on normal tissue which has been stripped from cells thorough a series of washing procedures. The remaining structure consists of tissue-specific extra cellular matrix low on immunogenic components that has the 3-dimensional (3D) shape of the original organ. Thus, this structure offers a biologically-based biomaterial that may avoid a negative immune response after transplantation and new cells should recognize it and be able to remodel it as required. This prospective biomaterial is only just starting to be evaluated for ovarian bioengineering applications (Laronda et al., 2015; Liu et al., 2017). An alternative to fibrin/alginate-, or to use decellularized tissue as an ovary scaffold is to use a 3D-printed construct. Recently, follicles isolated from green fluorescent protein (GFP) mice were applied to a 3D-printed gelatin scaffold crosslinked with an ethylcarbodiimide (EDC)-hydroxysuccinimide (NHC) solution. These follicles underwent successful folliculogenesis and matured oocytes could successfully be fertilized resulting in GFP-fluorescent mice pups (Laronda et al., 2017). When an improved ovarian-like biomaterial has been developed that can provide support to the vast follicular expansion required for larger mammals, a rapid progress to reach the clinic is expected.

Bioengineered Meshes in Pelvic Prolapse Surgery

Pelvic organ prolapse that results from childbirth affect up to 25% of all women and significantly reduces their quality of life. Current treatment protocols for severe pelvic organ prolapse include using meshes to surgically stabilize the pelvic region. However, about 15%–30% of these treatments fail and common problems include pain and mesh erosion into the bladder, vagina or bowel. Therefore, it has been proposed to use MSCs as a mean to improve the meshes biocompatibility (Emmerson and Gargett, 2016). Due to the MSCs' immunomodulatory and antiinflammatory properties, it may be advantageous to coat the meshes with these cells prior to implantation. Indeed, rodent studies using an MSC-like stem cell source from the endometrium (eMSC) proved beneficial (Ulrich et al., 2014). Ongoing studies on large domestic animals (ovine) will further examine if bioengineered meshes coated with autologous eMSCs can lead to clinical improvements in the near future (Emmerson and Gargett, 2016).

Partial Uterus Reconstruction

The existence of uterus-resident stem cells and the fact that the endometrium is a highly regenerative tissue may enable novel bioengineering applications to the uterus. Partial uterine repair may become clinically applicable and a desired treatment regimen since the prevalence of caesarean delivery is increasing resulting in significant uterine wall scarring. Similar injury also results from repeated uterine surgeries due to extensive myomectomy, adenomyomectomy or from resection of placental tumors. The creation of a bioengineered patient-specific uterus patch to cover/replace uterine scars may in these cases be used to increase the strength of the uterine wall. Uterine bioengineering could thus become a major advantage for the many women that otherwise risk life-threatening adverse pregnancy complications. Multiple groups reported progress in uterine tissue reconstruction using various semi-synthetic- and biological biomaterials (Campo et al., 2017; Hellström et al., 2017). For example, scaffold matrixes made up of collagen/matrigel, or collagen/silk were evaluated in vitro using static cell culturing methods with mouse-, rabbit- or human cells. These scaffolds allowed for some diffusion of the culture media into the deeper layers of the constructs which enabled the formation of a stratified morphological structure, similar to that of a normal uterine wall.

Decellularized tissues as extra cellular matrix scaffolds for uterine tissue have also been evaluated. Scaffolds created by decellularization provide some general advantages over synthetic scaffolds, for example they; (a) consist of the correct tissue specific extra cellular matrix composition, (b) have the physical 3D appearance of the original organ with the correct architecture for blood vessels and glandular ducts, (c) allow cells to be reintroduced and sustained in a perfusion bioreactor via the vascular conduits for organ regeneration, (d) can be remodeled by repopulated cells, and (e) provide guiding cues for tissue specific cell differentiation and migration (Fig. 1) (Crapo et al., 2011). For uterine tissue reconstruction, patches of decellularized rat- and human myometrium was first described (Young and Goloman, 2013). These protocols were developed using ethanol- and trypsin as cell removal agents, and then the scaffolds were re-seeded with human or rat myocytes. After about 50 days, coordinated contractions from the bioengineered tissues were noticed, suggesting some biological function. Santoso et al. showed that decellularized rat full-thickness uterus patches could be decellularized and then used as grafting material (without prior cellular reconstruction) (Santoso et al., 2014). Later, the same group members used the mouse model to show that the spontaneous tissue regeneration in the grafted area was Signal Transducer and Activator of Transcription 3 (STAT3) -dependent and estrogen- and progesterone independent (Hiraoaka et al., 2016). Recently, slices of human endometrium were decellularized and then reconstructed again using primary endometrium cells (Olaekan et al., 2017). This in vitro recreated endometrium was viable for up to 28 days and responded to hormonal stimuli. Collectively, these studies provide important clues on how to best create engineered constructs for partial uterine repair.

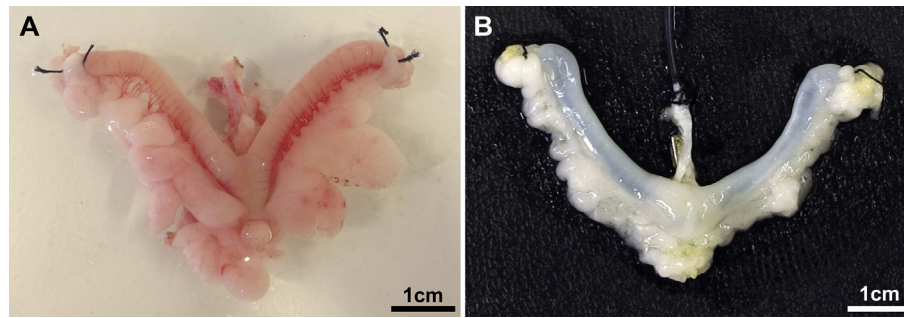


Fig. 1 The normal rat uterus (A) can be decellularized by perfusing detergents and other solutions through the vasculature (B). Including multiple washing procedures, this process takes about 5–10 days depending on the type of chemicals used.

Furthermore, they may also aid towards the development of a bioengineered whole-uterus that potentially could replace the need for a donor in a uterus transplantation setting.

Uterus Transplantation and Whole-Uterus Bioengineering

The pioneering work that led to the world's first baby born from a transplanted uterus started in 1999 by Professor Brännström's team on small animal models. As successful surgery techniques with proof-of-function were established, the team rapidly progressed to larger animal models that included pig, sheep and nonhuman primates. This translational research approach led to the successful clinical trial that began in 2013 that included nine female uterine factor infertile patients (eight with MRKHS, and one with previous hysterectomy) (Brännström, 2017; Brännström et al., 2015). Two of the patients had their grafts removed due to an infection and a thrombosis, respectively. However, today, 6 babies have been born and there are two ongoing pregnancies (week 34) from a total of 7 patients who received embryo transfers. This trial used live donors, and this may be one of several reasons for the successful outcome, since multiple centers have now begun uterus transplantation trials using diseased donors with less successful outcomes (reports from open media). The live donor approach has some major advantages, including; (a) the ability to choose a donor uterus with proven functionality, and (b) logistically advantageous to better prepare the donor- and recipient surgeries and minimize cold ischemic injuries. However, the risky donor surgery is challenging and is considerably long with an average of about 12 h (Brännström et al., 2014). Furthermore, the recipient needs pharmacological agents to suppress probable immune rejection events following the engraftment, and thus risk negative side effects such as accelerated arteriosclerosis, nephrotoxicity, hypertension, diabetes and reduced fertility. The possibility of creating a bioengineered patient-specific donor organ instead of using a live donor would eliminate these risk factors (Hellström et al., 2017).

For these reasons, protocols for whole uterus decellularization were developed, first on the rat (Hellström et al., 2014; Miyazaki and Maruyama, 2014), and then more recently also on the pig (Campo et al., 2017). In these reports, the uterus was efficiently decellularized by perfusing combinations of hyper- or hypotonic solutions and detergents through the organ's vasculature. Detailed analyses on the remaining uterus ECM scaffolds showed that the developed scaffolds were rich in collagen, elastin, fibronectin and other important ECM biomolecules (Fig. 2). Importantly, these perfusion procedures also maintained patent conduits for the vasculature (Hellström et al., 2014; Miyazaki and Maruyama, 2014), even down to the small capillaries (Campo et al., 2017). This enables anastomoses and future re-transplantation, including vascular anastomoses of the bioengineered uterus. Toxicity- and biocompatibility tests on these scaffolds were conducted by recellularization studies which showed that cells can attach and survive, but that the cell distribution was mainly limited to the peripheral parts of the scaffold, or in isolated pockets within the deeper layers (Hellström et al., 2017). When rectangular patches of the reconstructed rat uterus scaffolds were cut out and transplanted into a rat model

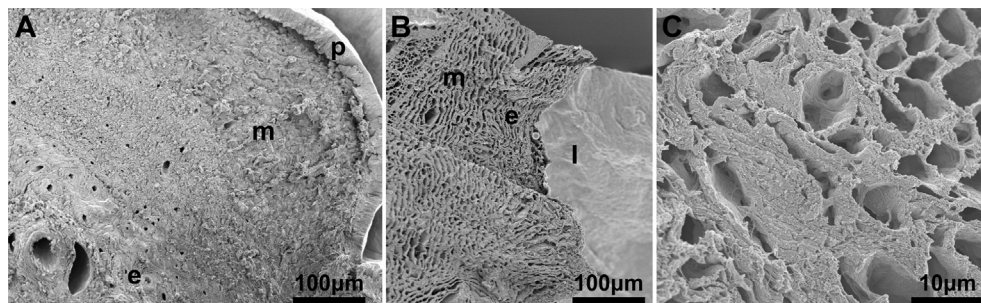


Fig. 2 The normal rat uterus is densely cell-populated as viewed here by scanning electron microscopy (A). Depending on the chemicals used for decellularization, the remaining porous structure (B) consists of tissue specific extra cellular matrix, rich in collagen, fibronectin, elastin and other important macromolecules that support cell growth (C). *p*, perimetrium; *m*, myometrium; *e*, endometrium; *l*, lumen.

with an impaired uterine wall, these patches showed to improve the healing and regeneration. Pregnancies were also reported from the operated animals, some fetuses even growing in the graft-area (Hellström et al., 2017; Miyazaki and Maruyama, 2014). This confirms that the constructs are strong enough to support full-term pregnancy. However, no placentation has yet been reported over the graft itself. This would have proved the functionality of the bioengineered constructs with respects to successful implantation and embryogenesis. Nevertheless, collectively these studies show great potential using uterine tissue engineering for partial uterus reconstruction, but additional animal studies are required to further optimize methods which can generate transplantable bioengineered whole-uterus constructs able to be grafted along with vascular anastomosis.

Conclusions

The production of an artificial ovarian structure that supports folliculogenesis as a mean to preserve fertility for young cancer treated women has shown great promise in studies on the mouse. Particularly when using biomaterials such as alginate, fibrin and 3D-printed gelatin. When improved scaffolds have been developed that can support the prominent follicular expansion characteristic to larger mammals, a rapid progress towards clinical use is expected.

Perhaps a more debatable route to preserve fertility includes using reprogrammed somatic cells, or isolated germ cells. These methods show potentials to one day offer fertility solutions to conceive genetically related children to sterile men and women. However, the overexpression of transcription factors and the re-differentiation procedures that takes place in an artificial in vitro environment has yet to be proved safe, also considering potential epigenetic changes.

Bioengineered uterine tissue patches have proved beneficial to repair and increase fertility in rats with a full thickness uterine wall injury. This rapid and promising progress in using bioengineered uterine patches in rodents show that the field is ready to move forward and evaluate these strategies in large animal models to see if these treatment regimens are translational to a clinical setting. However, large bioengineered patches need to be assessed further. This research will also contribute to the development of a whole bioengineered uterus that could possibly provide a major advantage for both the donor and the recipient in a uterus transplantation setting. So far, limitations in the reconstruction phase of the scaffolds need to be resolved. Improved recellularization is dependent on the scaffold composition, what cell types are used, how the cells are added to the construct, and under what conditions the recellularized scaffold is cultured in vitro. All these factors need further optimization to improve the outcomes of uterus bioengineering experiments.

Acknowledgments

The author thanks Professor Mats Brännström for correcting the manuscript and for the continuous research support. The author also acknowledges the Centre for Cellular Imaging at the University of Gothenburg and the National Microscopy Infrastructure, NMI for providing assistance in the scanning electron microscopy. This work was supported by Hjalmar Svensson-, Adlerbertska- and the Wilhelm och Martina Lundgrens Research Foundation, and the Swedish Science Research Council (Vetenskapsrådet; Grant No. 116008).

References

- Brännström, M. (2017). Uterus transplantation and beyond. *Journal of Materials Science. Materials in Medicine*, 28, 70.
- Brännström, M., Johannesson, L., Bokstrom, H., Kvarnstrom, N., Molne, J., Dahm-Kahler, P., Enskog, A., Milenkovic, M., Ekberg, J., Diaz-Garcia, C., Gabel, M., Hanafy, A., Hagberg, H., Olausson, M., & Nilsson, L. (2015). Livebirth after uterus transplantation. *Lancet*, 385, 607–616.
- Brännström, M., Johannesson, L., Dahm-Kahler, P., Enskog, A., Molne, J., Kvarnstrom, N., Diaz-Garcia, C., Hanafy, A., Lundmark, C., Marcickiewicz, J., Gabel, M., Groth, K., Akouri, R., Eklind, S., Holgersson, J., Tzakis, A., & Olausson, M. (2014). First clinical uterus transplantation trial: A six-month report. *Fertility and Sterility*, 101, 1228–1236.
- Campbell, G. R., Turnbull, G., Xiang, L., Haines, M., Armstrong, S., Rolfe, B. E., & Campbell, J. H. (2008). The peritoneal cavity as a bioreactor for tissue engineering visceral organs: Bladder, uterus and vas deferens. *Journal of Tissue Engineering and Regenerative Medicine*, 2, 50–60.
- Campo, H., Baptista, P. M., Lopez-Perez, N., Faus, A., Cervello, I., & Simon, C. (2017). De- and recellularization of the pig uterus: A bioengineering pilot study. *Biology of Reproduction*, 96, 34–45.
- Campo, H., Cervello, I., & Simon, C. (2017). Bioengineering the uterus: An overview of recent advances and future perspectives in reproductive medicine. *Annals of Biomedical Engineering*, 45(7), 1710–1717.
- Chen, K. L., Eberli, D., Yoo, J. J., & Atala, A. (2010). Bioengineered corporal tissue for structural and functional restoration of the penis. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 3346–3350.
- Chiti, M. C., Dolmans, M. M., Donnez, J., & Amorim, C. A. (2017). Fibrin in reproductive tissue engineering: A review on its application as a biomaterial for fertility preservation. *Annals of Biomedical Engineering*, 45(7), 1650–1663.
- Crapo, P. M., Gilbert, T. W., & Badylak, S. F. (2011). An overview of tissue and whole organ decellularization processes. *Biomaterials*, 32, 3233–3243.
- Donnez, J., & Dolmans, M. M. (2015). Ovarian cortex transplantation: 60 reported live births brings the success and worldwide expansion of the technique towards routine clinical practice. *Journal of Assisted Reproduction and Genetics*, 32, 1167–1170.
- Eguizabal, C., Montserrat, N., Vassena, R., Barragan, M., Garreta, E., Garcia-Quevedo, L., Vidal, F., Giorgetti, A., Veiga, A., & Izpisua Belmonte, J. C. (2011). Complete meiosis from human induced pluripotent stem cells. *Stem Cells*, 29, 1186–1195.
- Emmerson, S. J., & Gargett, C. E. (2016). Endometrial mesenchymal stem cells as a cell based therapy for pelvic organ prolapse. *World Journal of Stem Cells*, 8, 202–215.
- Hanson, S., D'souza, R. N., & Hematti, P. (2014). Biomaterial-mesenchymal stem cell constructs for immunomodulation in composite tissue engineering. *Tissue Engineering. Part A*, 20, 2162–2168.
- Hellström, M., Bandstein, S., & Brännström, M. (2017). Uterine tissue engineering and the future of uterus transplantation. *Annals of Biomedical Engineering*, 45(7), 1718–1730.

- Hellström, M., El-Akouri, R. R., Sahlbom, C., Olsson, B. M., Lenggqvist, J., Backdahl, H., Johansson, B. R., Olausson, M., Sumitran-Holgersson, S., & Brännström, M. (2014). Towards the development of a bioengineered uterus: Comparison of different protocols for rat uterus decellularization. *Acta Biomaterialia*, 10, 5034–5042.
- Hellström, M., Moreno-Moya, J. M., Bandstein, S., Bom, E., Akouri, R. R., Miyazaki, K., Maruyama, T., & Brännström, M. (2016). Bioengineered uterine tissue supports pregnancy in a rat model. *Fertility and Sterility*, 106, 487–496. e1.
- Hendriks, S., Dancet, E. A., Van Pelt, A. M., Hamer, G., & Repping, S. (2015). Artificial gametes: A systematic review of biological progress towards clinical application. *Human Reproduction Update*, 21, 285–296.
- Hiraoka, T., Hirota, Y., Saito-Fujita, T., Matsuo, M., Egashira, M., Matsumoto, L., Haraguchi, H., Dey, S. K., Furukawa, K. S., Fujii, T., & Osuga, Y. (2016). Stat3 accelerates uterine epithelial regeneration in a mouse model of decellularized uterine matrix transplantation. *JCI Insight*, 1(8), e87591.
- Laronda, M. M., Jakus, A. E., Whelan, K. A., Wertheim, J. A., Shah, R. N., & Woodruff, T. K. (2015). Initiation of puberty in mice following decellularized ovary transplant. *Biomaterials*, 50, 20–29.
- Laronda, M. M., Rutz, A. L., Xiao, S., Whelan, K. A., Duncan, F. E., Roth, E. W., Woodruff, T. K., & Shah, R. N. (2017). A bioprosthetic ovary created using 3D printed microporous scaffolds restores ovarian function in sterilized mice. *Nature Communications*, 8, 15261.
- Liu, W. Y., Lin, S. G., Zhuo, R. Y., Xie, Y. Y., Pan, W., Lin, X. F., & Shen, F. X. (2017). Xenogeneic decellularized scaffold: A novel platform for ovary regeneration. *Tissue Engineering. Part C, Methods*, 23, 61–71.
- Miyazaki, K., & Maruyama, T. (2014). Partial regeneration and reconstruction of the rat uterus through recellularization of a decellularized uterine matrix. *Biomaterials*, 35, 8791–8800.
- Olalekan, S. A., Burdette, J. E., Getsios, S., Woodruff, T. K., & Kim, J. J. (2017). Development of a novel human recellularized endometrium that responds to a 28 day hormone treatment. *Biology of Reproduction*, 96(5), 971–981.
- Sadri-Ardekani, H., & Atala, A. (2015). Regenerative medicine for the treatment of reproductive system disorders: Current and potential options. *Advanced Drug Delivery Reviews*, 82–83, 145–152.
- Santoso, E. G., Yoshida, K., Hirota, Y., Aizawa, M., Yoshino, O., Kishida, A., Osuga, Y., Saito, S., Ushida, T., & Furukawa, K. S. (2014). Application of detergents or high hydrostatic pressure as decellularization processes in uterine tissues and their subsequent effects on in vivo uterine regeneration in murine models. *PLoS One*, 9, e103201.
- Shea, L. D., Woodruff, T. K., & Shikanov, A. (2014). Bioengineering the ovarian follicle microenvironment. *Annual Review of Biomedical Engineering*, 16, 29–52.
- Takahashi, K., & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126, 663–676.
- Ulrich, D., Edwards, S. L., Su, K., Tan, K. S., White, J. F., Ramshaw, J. A., Lo, C., Rosamilia, A., Werkmeister, J. A., & GARGETT, C. E. (2014). Human endometrial mesenchymal stem cells modulate the tissue response and mechanical behavior of polyamide mesh implants for pelvic organ prolapse repair. *Tissue Engineering. Part A*, 20, 785–798.
- Young, R. C., & Goloman, G. (2013). Allo- and xeno-reassembly of human and rat myometrium from cells and scaffolds. *Tissue Engineering. Part A*.

Regenerative Medicine in Patients With Asherman's Syndrome and Endometrial Atrophy

Xavier Santamaria, Igenomix, Valencia, Spain; and IVI Barcelona, Barcelona, Spain

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

AFS American Fertility Society
AS Asherman's syndrome
ATMP Advanced therapy medicinal product
BMDSCs Bone marrow derived stem cells
CPCs Circulating progenitor cells
EA Endometrial atrophy
EMA European medicines agency
G-CSF Granulocyte-colony stimulating factor
HRT Hormonal replacement therapy
IGF-1 Insulin-like growth factor 1
MNCs Mono nuclear cells
MoA Mechanisms of action
MSC Mesenchymal stem cells
ODD Orphan drug designation
Thbs1 Thrombospondin
TNF- α Tumor necrosis factor α

Introduction

The endometrium is a tissue with a high capacity for renewal ("self renewal") regulated by hormones that undergoes almost complete changes of growth, differentiation, and shedding every 28 days during 400–500 cycles during a woman's reproductive life-time. This level of tissue regeneration is comparable to other high cellular turnover tissues such as the epidermis, gut epithelium, and/or bone marrow.

The biology of the menstrual cycle involves the hypothalamus, pituitary, ovary and finally the endometrium in a coordinated and complex sequence. During the menstrual phase, the endometrium undergoes changes and is sloughed in women due to a drop in progesterone and estrogen levels. The proliferative phase is defined as the period of time from the menstrual phase to ovulation. As estrogen levels begin to rise, the endometrial lining thickens leading to a proliferative pattern. Estrogen (spell the same way thru the article) triggers a proliferation of stroma and glands and elongation of the spiral arteries. The secretory phase corresponds from ovulation to through menstruation. After ovulation, progesterone levels rise in the early secretory phase which leads to the secretion of glycogen and mucus. In the mid-secretory phase, the endometrium becomes decidualized and receptive for a fertilized embryo to implant. Finally, in the late secretory phase, in the absence of pregnancy there is a drop in both estrogen and progesterone levels which causes the spiral arteries to vasoconstrict, leading to involution of the endometrium and menstruation.

Histologically, the endometrium is divided in two functional layers: the basal and functional layers. The functional layer responds to progesterone and estradiol and is completely shed during menstruation. On the other hand, the basal layer does not respond to hormones and also does not undergo desquamation and is responsible to regenerate the mucosa. The human endometrium is composed primarily of two main cell types, epithelial cells (luminal and glandular) and supporting mesenchymal cells (stromal cells). In addition endothelial cells and leukocytes are found within the endometrium. The endometrial–myometrial junction is irregular with no submucosal tissue to separate endometrial glandular tissue from the underlying smooth muscle of the myometrium. The ultimate function of the endometrium is allowing an embryo to implant. However, implantation is not an efficient process and natural conception rate in healthy young couples is only about 25% per cycle. Inadequate uterine receptivity seems to play an important role in these cases of implantation failure (Simon and Laufer, 2012).

An increasing number of studies which constitute the current knowledge about endometrium-derived stem cells (EDSCs) have been published in the last decade. These studies support the fact that stem cell play a significant role in the highly regenerative self-renewal capacity of the endometrium. These stem cells functions are regulated by the niche which is defined as the specific physiological microenvironment in which niche cells secrete molecules to regulate stem cell proliferation and cell fate decisions according to the tissue needs. The niche is crucial to maintain a correct homeostasis of the tissues and is mostly placed in the basal layer (Schwab and Gargett, 2007; Cervello et al., 2010) of the endometrium which is mostly vascularized by the spiral arteries. Moreover,

some endometrial stem cells reside in the functional layer of the endometrium next to the blood vessels in form of pericytes. In this sense, some groups have reported the presence of stem cells in menstrual blood.

A subgroup of cells with adult stem cell activity in the human endometrium have been described in the endometrium with capacity to differentiate into cell types of the three different germ layers such as chondrocytes, neurons (Wolff et al., 2011) and insulin-producing cells (Santamaria et al., 2011) that may contribute to the rapid endometrial regeneration following menstruation.

Bone marrow derived stem cells (BMDSCs) also contribute to the repair and regeneration of tissues and organs including murine and human endometrium (Du and Taylor, 2007; Cervelló et al., 2012; Ikoma et al., 2009). According to these findings, BMDSCs have the capacity to differentiate into fully functional stromal and epithelial endometrial cells. However, the BMDSC subpopulation(s) that promote(s) the repair of the endometrium remains unknown. Other studies have reported endometrial cells with a side population (SP) phenotype (that is, cells identified as having the capacity to extrude the DNA-binding dye Hoechst via the ATP-binding cassette transporter G2 (ABCG2) and that generally have a stem cell-like phenotype) that contribute to human endometrial regeneration in vitro and in vivo in animal models and human patients suggesting two different sources of stem cells: one endogenous source composed by resident endometrial stem cells and an other exogenous source composed by BMDSCs (Cervelló et al., 2012).

Certain pathological entities such as Asherman syndrome (AS) and endometrial atrophy (EA) share a common pathophysiology: the absence of functional endometrium that leads to infertility. Therefore, stem cell therapies targeting the endometrial niche with the ultimate aim to improve endometrial function may offer a promising possibility for treating AS and EA and participate in the regeneration of this pathological endometrium.

Asherman's Syndrome (AS) and Endometrial Atrophy (EA)

Asherman's syndrome (AS) is considered an acquired condition defined by the presence of intrauterine adhesions (IUAs). AS results when the bona fide endometrium is replaced with fibrotic tissues, causing the uterine walls to adhere to one another. This leads to symptoms such as menstrual abnormalities, pelvic pain, recurrent miscarriage, infertility, abnormal placentation and attendant psychological distress. The term AS should be applied when the signs and symptoms are present in women with established intrauterine adhesion although currently may be used only referring as intrauterine adhesions/scarring, synechiae or Fritsch syndrome (Zupi et al., 2015) indistinctly.

The prevalence of AS can vary significantly in different countries. This is because gynecological interventions may be a causative factors and in certain countries where gynecological care is not state of the art (for example, use of sharp, blunt or suction curettage for puerperal and postabortal evacuation) or where illegal interventions (for example, abortions) are performed at a higher frequency and/or to a lesser standard than in industrialized countries. In addition, other causative factors such as genital tuberculosis may also have a higher prevalence in some countries.

According to the European Medicines Agency (EMA), a rare disease has a prevalence lower than 5 out of 10,000 individuals. AS is included in the Orphanet database under the registry ORPHA137686, although the quality of the available epidemiological data are poor and the incidence and prevalence of are difficult to calculate. Recently the European Medicines Agency (EMA) has considered the prevalence of AS to be 4.4 cases/10,000 in the European Population (Committee for Orphan Medicinal Products, 2011). Danish studies and epidemiological public databases such as DESTATIS that provide information about the diagnosis in all the German hospitals support this prevalence.

Histologically, in AS the stroma is largely replaced with fibrous tissue and the glands are replaced by inactive cubocolumnar endometrial epithelium. The functional and basal layers are indistinguishable with the functional layer replaced by an epithelial monolayer unresponsive to hormonal stimulation and fibrotic synechiae forming across the cavity. The tissue is typically avascular, although thin-walled telangiectatic vessels can be observed.

Sometimes calcification can occur in the stroma and the glands may be cystically dilated. Different layers of the endometrium, myometrium or connective tissue may be involved in the resulting intrauterine adhesions. In the most severe cases, adhesions may be composed of collagen bundles, fibrous strips, or muscle with the same characteristics as normal myometrium. Biopsy specimens from patients with intrauterine adhesions compared with patients without intrauterine adhesions contain 50%–80% of fibrous tissue compared with 13%–20% in the uterine wall. This scarring limits uterine myometrial activity and reduces the perfusion of sex steroids resulting in atrophy.

Hysteroscopy is considered as the gold standard for diagnosis of AS and provides a real time view of the uterine cavity, allowing for a meticulous definition of the site, extent and character of any adhesions, and it is the optimum tool for assessing the endometrium. Currently, this technique can be performed in ambulatory setting with less discomfort than a HSG and also makes immediate treatment possible in some favorable cases (Conforti et al., 2013). Moreover, the magnification and the direct view of the adhesions allow for precise and safe therapy.

Fertility restoration after hysteroscopic treatment seems to be influenced by several factors such as menstrual pattern before and after the surgery, severity of adhesions and adhesions recurrence rate after treatment. An overall pregnancy rate from 40% to 63% was previously described and pregnancy rates ranged between 66% to 30%–35% in moderate to severe cases of AS in two prospective trials. Moreover, other retrospective studies have reported similar pregnancy rates for moderate and severe cases (Zikopoulos et al., 2004; Capella-Allouc et al., 1999; Pistofidis et al., 1996).

In addition to reduced fertility, other reproductive consequences of AS include recurrent miscarriage, intrauterine growth restriction, and placenta accreta.

Due to the possibility of adhesions recurrence, several different methods to prevent re-scarring after surgery for AS have been used, especially in cases of severe adhesions since a high rate of reformation of adhesions has been reported. Estrogen supplementation after surgery, or other additional therapies such as the insertion of intrauterine device (IUD), intrauterine balloon, hyaluronic acid among others has also been proposed. However, studies have not yet confirmed the method of treatment that is most likely to prevent recurrences and restore fertility.

On the other hand, endometrial atrophy (EA) is another rare condition in which the endometrium is too thin and unable to grow above 5–6 mm thickness (Senturk and Erel, 2008). Factors that can cause EA include functional (and usually reversible) causes such as prolonged use of oral contraceptives and tamoxifen, premature ovarian failure or Kallman Syndrome, but in many instances the cause of EA are unknown. The prevalence of this pathology is 0.5% of infertile women undergoing assisted reproductive treatments (ARTs). Patients suffering EA patients have a poor reproductive outcome despite many therapies have been attempted such as high doses of estradiol therapy, pentoxifyline and vitamin E, sildenafil, L-arginine or intrauterine perfusion of Granulocyte Colony Stimulation Factor (G-CSF). None of these treatments has proved to be fully effective.

Research related to endometrial stem cells is relevant to understand normal endometrial physiology. Novel stem cell based approaches may prove to be very useful for both EA and AS.

Regenerative Medicine

The endometrium has an intrinsic capacity of regeneration that normally occurs after menstruation or delivery. As previously explained, there is substantial evidence in literature that adult endometrial tissue contains epithelial progenitor cells and mesenchymal/stromal (MSC) cells. The niche of these stem cells could be the target of a specific therapy in order to regenerate the endometrial tissue in cases of dysfunctional/nonfunctional or atrophic endometrium. Therefore, stem cell therapy offers an attractive alternative to the treatment of these pathological conditions aiming to regenerate the endometrium. In this sense, several studies have evidenced the potential benefit of regenerative therapies in AS and EA. A case report of a severe AS treated with autologous stem cells isolated from the women's own bone marrow was firstly described (Nagori et al., 2011). The case consisted in woman with history of infertility and hypomenorrhea and that had been previously hysteroscopically treated for severe intrauterine adhesions with an IUD placed inside the uterus for 6 months. During that time, she also received therapy with combined estrogen and progesterone (ethinylestradiol and medroxyprogesterone). Finally, after failure of hormonal therapy in restoring endometrium, endometrial stem cells were implanted inside the uterus after curettage on the second day of menstrual cycle. A clinical pregnancy was obtained after a heterologous embryo transfer.

Our group performed a proof of concept study using CD133+ BMDSCs demonstrating that in the first 3 months, autologous cell therapy, using CD133+ BMDSCs in conjunction with hormonal replacement therapy, increased the volume and duration of menses as well as the thickness and angiogenesis processes of the endometrium while decreasing intrauterine adhesion scores. In this study functionality of the treated endometrium revealed pregnancy rates of 50% and five live-births out of four uncomplicated pregnancies.

Background on Stem Cell Therapy

Stem cells are undifferentiated cells with the ability to proliferate into undifferentiated cells both in vitro and in vivo (self-renewal) and to differentiate into mature specialized cells (43). Many adult tissues contain populations of stem cells that have the capacity for renewal after trauma, disease, or aging. The cells may be found within the tissue or in other tissues that serve as stem cell reservoirs. Embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells, and mesenchymal stem cells have been used to derive mature cell types for tissue regeneration and repair.

Adult bone marrow represents an important reservoir of stem cells including vascular progenitor cells. These stem cells from the bone marrow have the capacity to express different biomarkers including CD133/VEGFR2 which represent a subpopulation of cells with endothelial progenitor capacity and are known as EPCs. Furthermore, these cells can be mobilized to the peripheral circulation as circulating endothelial progenitors (CEPCs) and participate and improve neoangiogenesis afforded by preexisting endothelium as shown in different physiological and pathophysiological processes such as wound healing and limb ischemia, postmyocardial infarction, endothelialization of vascular grafts, atherosclerosis, retinal and lymphoid organ neovascularization, vascularization during neonatal growth and tumor growth. Additionally, CD133+ have the capacity to differentiate into nonhematopoietic cell lineages and display long-term repopulation potential in SCID mice (Rafii and Lyden, 2003).

Certain bone marrow derived stem cells have the capacity to engraft and fully differentiate into the endometrium as observed in the endometrium of women who had previously undergone a bone marrow transplantation of a HLA mismatch or male donor (Ikoma et al., 2009; Taylor, 2004).

Autologous bone marrow-derived CD133+ stem cell therapy has been used in some clinical trials for patients with chronic total occlusion and ischemia, myocardial infarction, hepatic fibrosis, and liver regeneration as well as bone regeneration showing an excellent safety profile. All these findings support the use of this subtype of stem cells to treat endometrial pathologies such as AS and EA and also highlight the potential use of stem cells to treat these incurable pathological conditions.

CD133 + Bone Marrow Derived Stem Cells

Human CD133 is a transmembrane glycoprotein of 865 amino acids with a total molecular weight of 120 kDa. This protein consists of an N-terminal extracellular domain, five transmembrane domains with two large extracellular loops, and a 59 amino acids cytoplasmic tail (51). CD133 is found in many cell lines and most adult tissues with the exception of mature peripheral blood leukocytes, although it is widely known that the expression of this biomarker is more restricted to undifferentiated cells that include endothelial progenitor cells, hematopoietic stem cells, fetal brain stem cells, kidney stem cells, prostatic epithelial stem cells and liver stem cells (Handgretinger et al., 2003).

CD133 expression is regulated by several extracellular or intracellular factors and usually correlates with changes of cell type and function. In this sense, conditions such as hypoxia, mitochondrial dysfunction or depletion of mitochondrial DNA induce a reversible up-regulation of CD133 expression. The number of CD133+ cells remain relatively constant within the bone marrow, blood and different tissues. However, when cells or tissues are damaged by chemical, physical or mutational causes, CD133+ progenitor or stem cells are activated to self-renew, proliferate and differentiate in order to repair this damage.

Cells derived from bone marrow coexpressing CD133+ and vascular endothelial growth factor receptor 2 (VEGFR2) represent a subpopulation of cells known as endothelial progenitor cells (EPCs) with endothelial progenitor capacity (Handgretinger et al., 2003) that can be mobilized to the circulation and can improve neoangiogenesis of preexisting endothelium.

CD133+ BMDSCs have been previously used in clinical trials for regenerative medicine in nonhematological applications (Rafi and Lyden, 2003) and have functionally contributed to neoangiogenesis during wound healing and limb ischemia, postmyocardial infarction, endothelialisation of vascular grafts, atherosclerosis, retinal and lymphoid organ neovascularization and tumor growth. For instance, cells isolated from the peripheral blood using CD133 antibodies have been able to enhance angiogenesis, astrogliosis, axon growth and functional recovery in a mouse spinal cord injury model. In another study, embedded CD133+ cells in atelocollagen gel within a silicone tube used to bridge a 15 mm defect in the sciatic nerve of athymic rats, regenerate within 8 weeks the nerves and the transplanted CD133+ cells differentiated into Schwann cells. Moreover, G-CSF-mobilized peripheral blood CD133+ cells enhanced histologically and functionally recovery from skeletal muscle injury in a rat model contributing to the environment for muscular regeneration.

Mechanisms of Action (MoA)

The EMEA has recently categorized CD133+ cells as an Advanced Therapy Medicinal Product (ATMP) with certain regenerative properties such as tissue remodeling, tissue regeneration and neoangiogenesis.

These effects are exerted through different MoA: Paracrine activity, endometrial stem cell differentiation/cell fusion, endometrial resident stem cell recruitment/activation and endothelial differentiation. Paracrine activity and endometrial resident stem cell recruitment/activation cause and immediate short-term effect and seem to work at larger scale compared to endometrial and endothelial differentiation that has a more stable long-term effect, but seem to be more residual MoA. Interestingly the paracrine effects can contribute to endothelial regeneration as well as endometrial resident stem cell recruitment/activation as shown in Fig. 1.

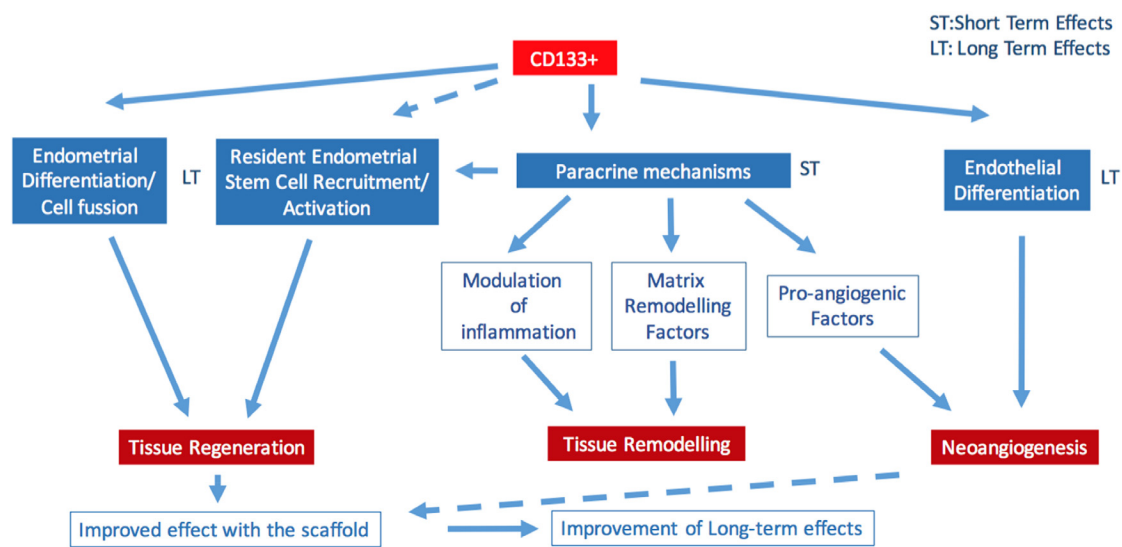


Fig. 1 Different MoA of CD133+ cells and interaction among them. The effect of CD133+ cells consists in tissue regeneration, tissue remodeling and neoangiogenesis. Some mechanisms act at short term whereas others have long term effects.

Preclinical Studies of CD133+ BMDSC in AS

Several AS model in rodents has been reported mostly based on physical damage of the endometrium and some preclinical studies have assessed the effect of stem cell therapy in these pathological conditions. In a recent study animals where AS was mechanically induced were divided into two groups (Cervello et al., 2015): In Group A, both uterine horns of the mice were damaged and human cells previously labeled with super paramagnetic iron oxide (SPIOs molecules) were injected intrauterine into only horn only whereas in Group B, only the left horn was damaged and human cells were administered by tail vein injection. Subsequently, uteri were excised after 90 (Group A) or 12 days (Group B) and subjected to Prussian blue, Ki67 and trichrome staining, as well as analyzed for paracrine gene expression. The cells used in this study where an aliquot of the cells extracted from the patients enrolled in the pilot study (Santamaria et al., 2016). Cell engraftment and localization were analyzed by Prussian blue staining, endometrial proliferation by Ki67 staining and quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) of marker genes.

Prussian blue staining was used to detect accumulation of iron deposits of the SPIO-labeled cells. Human labeled cells seemed to be mostly engrafted around small endometrial vessels in all damaged uterine horns regardless of the route of cell administration or timing. Quantification of iron-positive cells revealed that 0.59% and 0.65% of engrafted cells were present after intrauterine or tail vein injection, respectively. However, no cells were detected in spleen, liver and kidney. Therefore, we observed that, systemically or locally injected, CD133+ cells have the ability to home to and engraft selectively in the damaged endometrium.

Moreover, the epithelial glands of the CD133+ cells treated horns showed a statistically significant increase of Ki67+ cells in comparison with nontreated horns, regardless of the administration route. After intrauterine injection, the proliferation rate increased from $14\% \pm 10.37\%$ to $23.15\% \pm 10.89\%$ ($P < 0.01$) at 90 days after administration, whereas, after tail vein injection, Ki67+ cells increased from $6.92\% \pm 7.03\%$ to $20.55\% \pm 10.89\%$ ($P < 0.05$) 12 days after administration. The expression of several well-described paracrine molecules such as bone morphogenetic protein 6 (Bmp6), platelet-derived growth factor β (Pdgf β), thrombospondin 1 (Thbs1), Tumor necrosis factor α (TNF- α) and Insulin-like growth factor 1 (IGF-1) was compared by qRT-PCR. Statistical differences were seen in the up-regulation of Thbs1 (2.065 vs. 0.752 fold-change; $P < 0.05$), and the downregulation of IGF-1 (0.651 vs. 0.995 fold-change; $P < 0.05$) in the treated damaged horns in comparison with nontreated damaged horns in the intrauterine administration group (Group A).

These results suggest that human CD133 BMDSCs induced indirect proliferation of the neighboring endometrial cells in the damaged endometrium mainly at the epithelial compartment. However, the endometrial cell proliferation observed appears more related to the secretion of soluble factors acting by a paracrine fashion than a direct proliferation of the transplanted cells.

Other groups have also shown similar effects and stem cell engraftment and improvement in reproductive outcome in a rodent model of AS using total Bone Marrow Stem Cells (Alawadhi et al., 2014). Finally, an other group did not observe stem cell engraftment and highlighted the paracrine effect of these stem cell therapy whereas other groups have observed engraftment and full differentiation of these stem cells into stromal and epithelial cells (Ikoma et al., 2009; Taylor, 2004).

Clinical Studies of CD133+ BMDSC in AS and EA

Recently a prospective, experimental, noncontrolled, open labeled study in 18 patients aged 30–45 years with refractory AS or EA was reported (Santamaria et al., 2016). A total of 16 of these completed the study and the end point consisted in assessing the use of CD133+ BMDSC as a potential therapy to refractory cases of AS and EA. Prior to the enrolment, 8 out of 11 patients diagnosed with AS and all the patients diagnosed of EA had previously undergone a mean of 2 (range 1–9) hysteroscopy and 2 subsequent cycles of HRT after each hysteroscopy with no clinical neither sonographic improvements.

In this study an initial ecographical and hysteroscopic diagnosis was performed in proliferative phase. Uterine cavities were assessed following the American Fertility Society (AFS) Classification and adhesions were removed in AS with sharp and blunt scissors. All patients received Hormonal Replacement Therapy (HRT) immediately after the surgery for 2 months.

Then BMDSCs were mobilized into peripheral blood after administration of recombinant human granulocyte colony stimulating factor (G-CSF) at a dose of 10 $\mu\text{g/kg/day}$ for 5 days. G-CSF is a cytokine extensively used for this purpose in both autologous and allogeneic donors (59). One day after the last injection, isolation of the Peripheral Blood Mononuclear Cells by apheresis through peripheral venous access and then, CD133+ cells were isolated. A mean of 124.39 million cells (range 42–236) were obtained. These cells delivered into the spiral arterioles by catheterisation. As shown in Fig. 2, spiral arterioles lay next to the endometrial stem cell niche. Therefore, this delivery method represents an effective minimally invasive approach to homogeneously instill these cells next to the endometrial niche and seems to have an increased engraftment as Liu et al. has recently shown in rodent models.

Three months after the stem cell instillation, the endometrial cavity was re-scored with a second-look hysteroscopy and in some patients a third look hysteroscopy was performed 5–6 months after stem cell instillation. All the patients experienced an improvement in the scores and stages although their cavity was not completely normalized in all the cases. Specifically, all patients diagnosed with stage III AS improved to stage I, while one of the two patients affected with stage II showed a completely normalized endometrial cavity and the other improved to stage I. The remaining patients initially with stage I improved in qualifying score.

Clinically, all the treated patients showed a significant improvement in terms of duration and intensity of the menstrual period specially in the first 3 months after stem cell instillation (Fig. 3). However, this effect seemed to decrease progressively from a mean

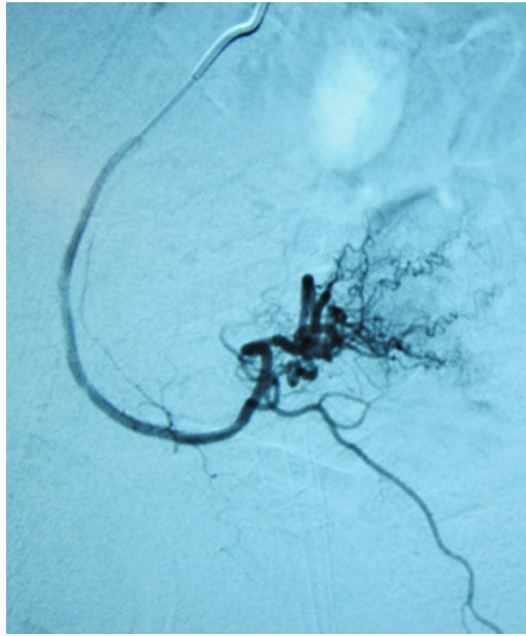


Fig. 2 Image of uterine artery catheterization. Spiral arteries that vascularize the basal layer of the endometrium are clearly visible when instillation is performed in the distal part of the uterine artery.

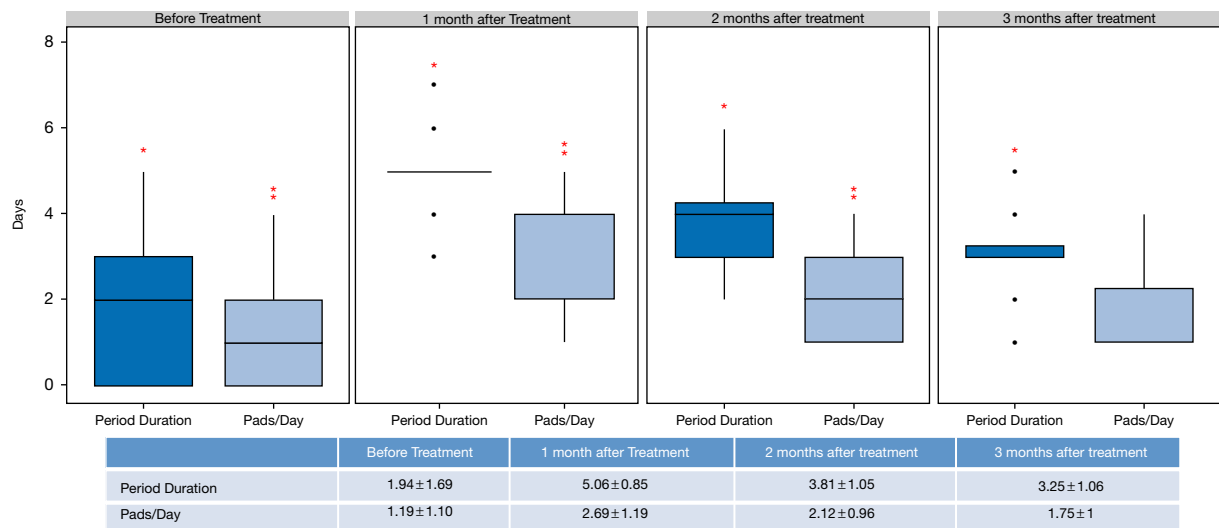


Fig. 3 Volume of the menstrual period before and after CD133-BMDSC treatment. All the patients required a larger number of pads/day right after the treatment, but the effect seemed to decrease with time.

of 5.06 days (ranging from 3 to 7) in the first month to 3.25 days (ranging from 1 to 3) in the third month after cell therapy. Menstrual volume assessed by number of pads/day used also decreased from a mean of 2.69 (ranging from 1 to 5) to 1.75 (ranging from 1 to 4).

Moreover, a statistically significant improvement in endometrial thickness was observed in AS patients from 4.3 mm (range from 2.7 to 5.0 mm) to 6.7 mm (range 3.1–12 mm; $P = 0.004$) whereas in the EA group improved from 4.2 mm (range 2.7 to 5.0 mm) to 5.7 mm (range 5–12 mm; $P = 0.03$) (Fig. 4).

CD133+ BMDSC correspond to circulating endothelial progenitors (CEPCs) with the capacity to improve neoangiogenesis. AS a matter of fact, a statistical significant increase in number of blood vessels in the endometrium was observed 3 months after stem cell instillation together with a histological improvement of the stromal and epithelial layers of the endometrium was clearly visible. However, this process seemed to decrease 6 months after the cellular therapy as observed with the duration and intensity of menses. Interestingly, no side effects related to treatment were observed during the study.

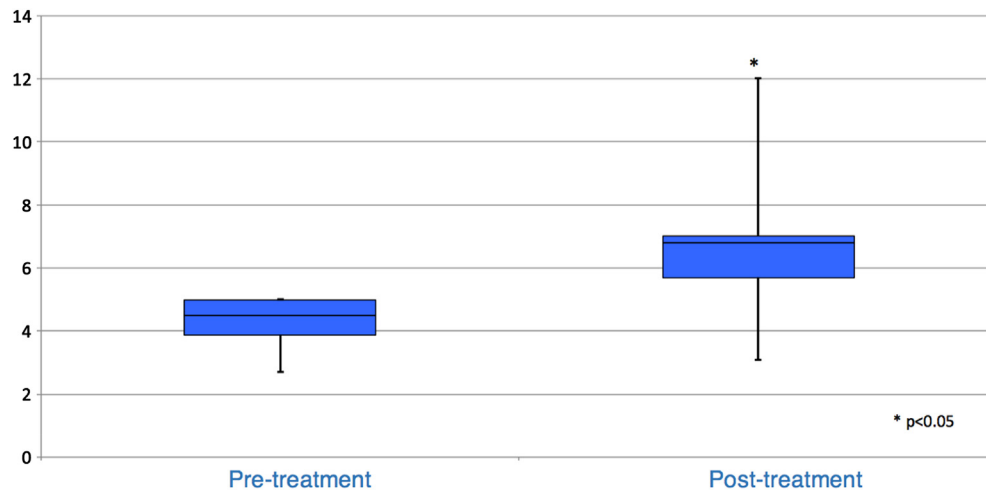


Fig. 4 Endometrial thickness measured in proliferative phase before and after stem cell therapy. In this study each patient acts as her own control. There is a statistical significant increase in the endometrial thickness after stem treatment compared to the measurements before CD133 + BMDSCs instillation.

This study was designed as a pilot study where each patient represented its own control after failed surgical and assisted reproductive treatments. The functionality of the treated endometrium was assessed through the reproductive outcome of patients who underwent ART after stem cell therapy. Briefly, three patients became pregnant spontaneously, 2, 4 and 19 months after cell therapy, resulting in two healthy babies born and a miscarriage at the 17th week due to a premature rupture of membranes, 2 weeks after a Corial Villous Sampling. Seven positive pregnancies were obtained after 14 embryo transfers, resulting in three biochemical pregnancies, one miscarriage at the 9th week due to a chromosomally abnormal embryo identified after the analysis of the products of conception, one ectopic pregnancy, and three healthy new born out of two patients since one was a twin pregnancy. In one patients, embryo transfer was canceled due to chromosomal abnormalities in all of the embryos and, in another case, transfer was not performed due to failure of cell therapy.

Other studies have also suggested (Nagori et al., 2011; Singh et al., 2014) positive results in treating AS with stem cell therapies with the aim to regenerate the endometrium. In the first reported case (Nagori et al., 2011), CD9, CD40 and CD90 autologous bone marrow stem cells were placed into the endometrial cavity to treat AS in a patient undergoing egg donation with success while another series of six cases described the direct placement of noncharacterized mononuclear stem cells into the sub-endometrial zone with a needle. In this latter study a statistically significant restoration of the endometrial thickness (ET) was reported compared to baseline measures ($P < 0.05$) with menstruation reported in 5/6 women (Singh et al., 2014). MNCs were isolated by a Ficoll density separation method and a volume of 3 mL of MNC were implanted in the subendometrial zone transmyometrial at 2–3 sites (fundus, anterior and posterior part) of the myometrium. However, no reproductive outcomes were assessed in this study.

Future Perspectives

On the whole, all these findings suggest regenerative medicine may play an important future role in the treatment of incurable infertility in endometrial pathologies such as AS and EA. It seems quite obvious that further studies are mandatory in order to better important issues such as dosage, MoA and refining the protocol. In this sense, the EMA has recently issued a positive opinion to label CD133 + cells as the first orphan drug designed (ODD) for the treatment of AS and phase I/II and phase III trials are planned in the near future.

Other important advances such as bioengineering, nanotechnology or development of biomaterials together with stem cell therapy may help in the future to optimize and improve the efficiency of these regenerative therapies.

References

- Alawadhi, F., Du, H., Cakmak, H., & Taylor, H. S. (2014). Bone marrow-derived stem cell (BMDSC) transplantation improves fertility in a murine model of Asherman's syndrome. *PLoS One*, 9(5), e96662. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24819371>.
- Capella-Alloc, S., Morsad, F., Rongieres-Bertrand, C., Taylor, S., & Fernandez, H. (1999). Hysteroscopic treatment of severe Asherman's syndrome and subsequent fertility. *Human Reproduction*, 14(5), 1230–1233. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/10325268>.
- Cervello, I., Gil-Sanchis, C., Mas, A., Delgado-Rosas, F., Martínez-Conejero, J. A., Galan, A., et al. (2010). Human endometrial side population cells exhibit genotypic, phenotypic and functional features of somatic stem cells. *PLoS One*, 5(6), e10964. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20585575>.

- Cervelló, I., Gil-Sanchis, C., Mas, A., Faus, A., Sanz, J., Moscardó, F., et al. (2012). Bone marrow-derived cells from male donors do not contribute to the endometrial side population of the recipient. *PLoS One*, 7(1).
- Cervello, I., Gil-Sanchis, C., Santamaria, X., Cabanillas, S., Diaz, A., Faus, A., et al. (2015). Human CD133(+) bone marrow-derived stem cells promote endometrial proliferation in a murine model of Asherman syndrome. *Fertility and Sterility*, 104(6), 1552–1553. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/26384164>.
- Committee for Orphan Medicinal Products. Public summary of opinion on orphan designation. Eur Med Agency. 2011;0(September):1–5. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Orphan_designation/2017/05/WC500227690.pdf
- Conforti, A., Alviggi, C., Mollo, A., De Placido, G., & Magos, A. (2013). The management of Asherman syndrome: A review of literature. *Reproductive Biology and Endocrinology*, 11, 118. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/24373209>.
- Du, H., & Taylor, H. S. (2007). Contribution of bone marrow-derived stem cells to endometrium and endometriosis. *Stem Cells*, 25(8), 2082–2086. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17464086>.
- Handgretinger, R., Gordon, P. R., Leimig, T., Chen, X., Bühring, H. J., Niethammer, D., et al. (2003). Biology and plasticity of CD133+ hematopoietic stem cells. *Annals of the New York Academy of Sciences*, 996, 141–151. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12799292>.
- Ikoma, T., Kyo, S., Maida, Y., Ozaki, S., Takakura, M., Nakao, S., et al. (2009). Bone marrow-derived cells from male donors can compose endometrial glands in female transplant recipients. *American Journal of Obstetrics and Gynecology*, 201(6), 608e1–8. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/19800602>.
- Nagori, C. B., Panchal, S. Y., & Patel, H. (2011). Endometrial regeneration using autologous adult stem cells followed by conception by in vitro fertilization in a patient of severe Asherman's syndrome. *Journal of Human Reproductive Sciences*, 4(1), 43–48. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/21772740>.
- Pistofidis, G. A., Dimitropoulos, K., & Mastrominas, M. (1996). Comparison of operative and fertility outcome between groups of women with intrauterine adhesions after Adhesiolysis. *The Journal of the American Association of Gynecologic Laparoscopists*, 3(4, supplement), S40. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/9074216>.
- Rafii, S., & Lyden, D. (2003). Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nature Medicine*, 9(6), 702–712. Available from <http://www.ncbi.nlm.nih.gov/pubmed/12778169>.
- Santamaria, X., Massasa, E. E., Feng, Y., Wolff, E., & Taylor, H. S. (2011). Derivation of insulin producing cells from human endometrial stromal stem cells and use in the treatment of murine diabetes. *Molecular Therapy*, 19(11), 2065–2071. Available from: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3222529&tool=pmcentrez&rendertype=abstract>.
- Santamaria, X., Cabanillas, S., Cervello, I., Arbona, C., Raga, F., Ferro, J., et al. (2016). Autologous cell therapy with CD133+ bone marrow-derived stem cells for refractory Asherman's syndrome and endometrial atrophy: A pilot cohort study. *Human Reproduction*, 31(5), 1087–1096. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/27005892>.
- Schwab, K. E., & Gargett, C. E. (2007). Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. *Human Reproduction*, 22(11), 2903–2911. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/17872908>.
- Senturk, L. M., & Erel, C. T. (2008). Thin endometrium in assisted reproductive technology. *Current Opinion in Obstetrics & Gynecology*, 20(3), 221–228. Available from: <http://www.embase.com/search/results?subaction=viewrecord&from=export&id=L351643508%5Cnhttps://doi.org/10.1097/GCO.0b013e328302143c%5Cnhttp://sfx.library.uu.nl/utrecht?sid=EMBASE&issn=1040872X&id=doi:10.1097%2FGCO.0b013e328302143c&atitle=Thin+endometriu>.
- Simon A, Laufer N. Assessment and treatment of repeated implantation failure (RIF). *Journal of Assisted Reproduction and Genetics*. 2012; 29(11): 1227–39. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/22976427>.
- Singh, N., Mohanty, S., Seth, T., Shankar, M., Bhaskaran, S., & Dharmendra, S. (2014). Autologous stem cell transplantation in refractory Asherman's syndrome: A novel cell based therapy. *J hum Reprod Sci*, 7(2), 93–98. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/25191021>.
- Taylor, H. S. (2004). Endometrial cells derived from donor stem cells in bone marrow transplant recipients. *JAMA*, 292(1), 81–85. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15238594>.
- Wolff, E. F., Gao, X. B., Yao, K. V., Andrews, Z. B., Du, H., Elsworth, J. D., et al. (2011). Endometrial stem cell transplantation restores dopamine production in a Parkinson's disease model. *Journal of Cellular and Molecular Medicine*, 15(4), 747–755. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/20406327>.
- Zikopoulos, K. A., Kolibianakis, E. M., Platteau, P., de Munck, L., Tournaye, H., Devroey, P., et al. (2004). Live delivery rates in subfertile women with Asherman's syndrome after hysteroscopic adhesiolysis using the resectoscope or the Versapoint system. *Reproductive Biomedicine Online*, 8(6), 720–725. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/15169591>.
- Zupi, E., Centini, G., & Lazzeri, L. (2015). Asherman syndrome: An unsolved clinical definition and management. *Fertility and sterility*, 104, 1380–1381.

Gene Editing Nuclear and Mitochondrial DNA

Cristina Eguizabal, Basque Center for Blood Transfusion and Human Tissues, Galdakao, Spain

Nuria Montserrat, Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain

Juan Carlos Izpisua Belmonte, Salk Institute for Biological Studies, San Diego, CA, United States

© 2018 Elsevier Inc. All rights reserved.

The Origin of Gene Editing Technology

DNA damage can occur in several situations: after exposure to ionizing radiation or chemotherapy, during DNA replication, or following experimental manipulation through the action of endonucleases. DNA double strand breaks (DSBs) are often injurious to the cell, causing genome instability and mutations. Luckily, the DNA repair machinery helps to repair this damage, thereby preventing the formation of DNA mutations that can cause disease. The ability of DNA damage to elicit DNA responses forms the basis of the gene-editing concept. There are two main repair pathways for DSBs: (1) nonhomologous end joining (NHEJ), and (2) homologous recombination (HR) (Fig. 1) (Ceccaldi et al. 2016).

The NHEJ pathways are active throughout the cell cycle and are the most common mechanism for DSB repair. However, these pathways are prone to errors and often result in genetic insertions or deletions, called indels. HR is initiated only in the S or G2 phases of the cell cycle, when a sister chromatid is available to provide a homologous donor template for the repair process. This process is referred to as homology-directed repair (HDR) in the context of an exogenous DNA repair template. This property of HR was used in the early 1980s to insert and to repair genes in mammalian cells by HDR. Later, these techniques were applied to mouse embryos to generate transgenic mice, and the era of genetic engineering was born. These important early studies were performed by Drs. Oliver Smithies, Mario Capecchi, and Martin Evans, who together pioneered HR-mediated gene editing in mouse embryos. They would later share the 2007 Nobel Prize in Physiology or Medicine “for their discoveries of principles for introducing specific gene modifications in mice by the use of embryonic stem cells”.

In the 1990s, gene targeting in mammalian cells using HR was further improved by Dr. Maria Jasin and colleagues when they used the yeast homing endonuclease I-SceI (Rouet et al., 1994). This enzyme generates site-specific breaks in chromatin engineered to contain the 18-bp rare restriction site for this enzyme. Importantly, this site is not found in the mouse or human genomes, so the I-SceI cleavage is highly specific. Cutting with this enzyme enhanced HR up to 500-fold at the target locus, foreshadowing the use of designer site-specific endonucleases to enhance gene editing. These next-generation endonucleases include the zinc finger nucleases (ZFNs) and the transcription activator-like effector nucleases (TALENs), which combined the DNA-binding specificity of zinc fingers or TALE transcription factors from plants, with the DNA cutting activity of the FokI endonuclease (Miller et al., 2011; Kim et al., 1996) (Fig. 1).

One of the major breakthroughs achieved in the field of gene editing occurred in the late 1980s, when a group of researchers discovered a strange topology at the 3' end of the alkaline phosphatase gene in *Escherichia coli*. This consisted of 32 nucleotide sequences, flanked by short invariable palindromic repeats—the first known description of a clustered regularly interspaced short palindromic repeats (CRISPR) array. In 2005, several laboratories demonstrated that the unique spacer regions found in CRISPR

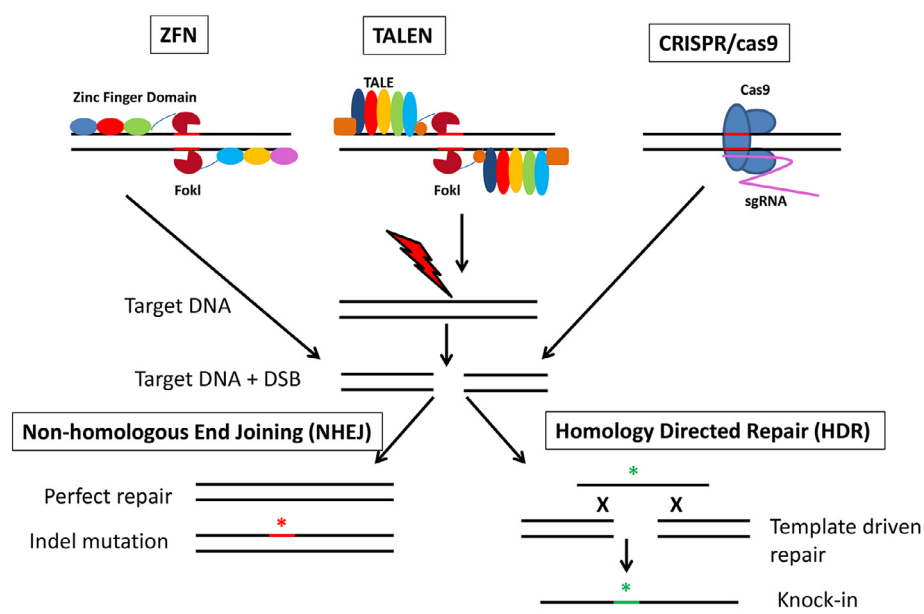


Fig. 1 Gene editing technologies to repair DNA DSBs (ZNFs, TALENs, and CRISPR/Cas9).

arrays actually mapped to phage genomes, hinting that CRISPR may serve as an adaptive immune response to phage infection through an RNA-guided process (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). The molecular mechanism of this immune response involves the transcription of CRISPR arrays into RNA, which is then cleaved and loaded into CRISPR-associated (Cas) proteins (Cas9). This RNA:protein complex then mediates RNA-guided dsDNA endonuclease activity. Furthermore, it was discovered cas9 can be reprogrammed to target novel sequences with an in vitro transcribed single guide RNA (sgRNA) (Fig. 1).

For many years, researchers had been searching for a tool for easily inducing mutations in a targeted fashion. While some progress had been made using engineered meganucleases, ZFNs, and TALENs, these systems had numerous limitations. They were either expensive, labor-intensive, or both, as the targeting mechanisms were based on protein-nucleic acid interactions, thereby requiring a custom-designed protein for each genetic locus of interest. The promise of RNA-guided nuclease activity afforded by CRISPR-based approaches led numerous groups to recognize immediately that this technology could potentially be used to induce targeted, DSBs in eukaryotes, which previously could only be accomplished with much difficulty. DSBs produced either by previously available technologies or CRISPR-based systems, are repaired by low-fidelity DNA repair pathways, leading to the production of indels—a class of mutations characterized by the random insertion or deletion of nucleotides at the site of the DSB.

The Differences Between Gene Editing Tools

As mentioned previously, there are four basic nuclease technologies: meganucleases, ZFNs, TALENs, and CRISPR/Cas9 nucleases. Differences are described in detail below (Porteus, 2015).

Engineered meganucleases are derived from a large family of natural homing endonucleases. A small number of these endonucleases have been designed to recognize natural target sites in the genome via a variety of strategies, including structure-based design and yeast surface display. Natural meganucleases have historically been the gold standard for specificity, but the challenge of engineering meganucleases for novel target sites has limited their translational development. Furthermore, the specificity of engineered meganucleases has not been fully evaluated.

Zinc-finger nucleases (ZFNs) are artificial proteins in which a zinc-finger DNA-binding domain is fused to the nonspecific nuclease domain from the enzyme *FokI*. To cut DNA efficiently, the nuclease domain must dimerize, a pair of ZFNs must be engineered for each target site, and these must be oriented correctly to allow dimerization. Zinc-finger-DNA binding domains can be engineered for novel target sites using a variety of strategies, including phage display, modular assembly, bacteria-based two-hybrid and one-hybrid systems, and combination approaches. Although ZFN design strategies are continuously being improved, engineering ZFNs that have high activity and high specificity to endogenous target sites remains a challenge. The best-quality ZFNs created using a combination of phage display and modular display have entered into clinical trials in which, for example, engineered T cells have been shown to be safe.

TAL effector nucleases (TALENs) are also artificial proteins. They share a similar structure to ZFNs in which an engineered DNA-binding domain is fused to the nuclease domain of the enzyme *FokI*. In TALENs, the DNA-binding domain is engineered by assembling a series of TAL repeats, with each repeat mediating the interaction with a single base through a two amino acid repeat variable di-residue (RVD) that can be described by a simple code. Thus, creating a highly active TALEN is much simpler than creating a highly active ZFN and simply involves using the code to assemble the correct TAL repeats needed to recognize a novel target sequence. In addition to the TAL repeats that use natural RVDs, TAL repeats that use engineered RVDs are now being used to create TALENs. These engineered RVDs may have increased specificity over natural RVDs, although this point remains to be further studied. As with ZFNs, a pair of TALENs must be engineered to recognize each target site of interest. Even TALENs that use TAL repeats containing natural RVDs may have better specificity than ZFNs.

CRISPR/Cas9 nucleases (CRISPR is an abbreviation of “Clustered Regularly Interspaced Short Palindromic Repeats”) are derived from a bacteria-based adaptive immune system. In contrast to the other three technologies, the CRISPR/Cas9 nuclease system does not derive specificity through protein–DNA interaction, but instead through RNA–DNA Watson–Crick base pairing. In the CRISPR/Cas9 system, a single-guide RNA (sgRNA) is designed such that the 20-bp recognition region of the sgRNA is identical to the desired target site. The target site must be nearby to a proto-spacer adjacent motif (PAM) sequence, which the Cas9 protein uses to identify target sites. The multifunctional Cas9 protein, in complex with the sgRNA, is able to unwind double-stranded DNA, to interrogate whether the guide strand is sufficiently identical to the target site, and to then create a rounded DSB if there is sufficient identity. Thus, CRISPR/Cas9 nucleases can be engineered very easily, and many of the designed nucleases (between one third and one half) are active at the desired target site.

The Potential Therapeutic Relevance of Genome Editing Technologies

Gene editing technologies are powerful tools for basic research, but the ultimate goal is to translate these systems into therapeutic applications. The potential use of gene editing in the clinic emerged from the idea that the best way to treat monogenic diseases would be to develop a method for correcting the disease-associated mutation, but that it would also be necessary to make more dramatic changes to the genome to cure diseases with multifactorial origins, which are more common. The use of genome editing

to cure monogenic disease is conceptually simple, but the true power of genome editing tools is that they provide a mechanism for making more sophisticated genomic changes, which can be used to cure more common diseases or to modify their course. In fact, there are currently several companies developing gene editing-based approaches to treat disease, including Editas Company, Intellia Therapeutics, CRISPR Therapeutics, OvaScience, and Sangamo Therapeutics.

Prospective Therapeutic Applications of NHEJ- and HR-Mediated Genome Editing

The use of ZFNs for therapeutic purposes has primarily been focused on common inherited monogenic disorders, such as severe combined immunodeficiency (SCID) and cystic fibrosis (CF), as well as acquired infections, such as human immunodeficiency virus (HIV) and hepatitis B virus (HBV). Studies have been conducted in a variety of human and animal cell lines to test systems for NHEJ-mediated gene disruption, and HDR-based gene correction (through the use of donor templates). ZFNs are among the most mature gene editing technologies, with the successful completion of early phase human clinical trials to treat HIV. A number of ZFN-based therapeutic applications have been investigated, including those to treat sickle cell anemia, hemophilia B, Duchenne muscular dystrophy (DMD), paroxysmal nocturnal hemoglobinuria (PNH), X-linked chronic granulomatous disease (XCGD), ataxia, retinitis pigmentosa (NARP), Leigh's syndrome, Down syndrome, Parkinson's disease, and HBV.

Similar to ZFNs, the therapeutic potential of TALENs has been demonstrated in cellular and animal models for a variety of genetic and acquired disorders. Examples of both gene disruptions and gene corrections by NHEJ and HDR, respectively, have been reported. While TALEN technologies are not as mature as those utilizing ZFNs, and no clinical trials have been initiated, a number of studies are currently underway. Including efforts to treat sickle cell anemia, DMD, alpha1-antitrypsin deficiency (A1ATD), polycythemia vera (PV), recessive dystrophic epidermolysis bullosa (RDEB), SCID, mitochondrial disease, malaria, HIV-1 resistance, HBV replication, HPV infections, and various cancers.

Even though use of the *CRISPR/Cas9* system as a gene editing technique was first reported in January 2013, significant progress has been made in the last 2 years to demonstrate therapeutic potential for several diseases, including sickle cell anemia, DMD, CF, A1ATD, PV, cataracts, Barth's syndrome, hereditary tyrosinemia type I (HTI), HIV-1 resistance/infection/immunization, HBV, Epstein-Bar virus (EBV), HPV/cervical cancer, osteosarcoma, and cardiovascular disease. This is likely because the simplicity and relative affordability of the system makes it accessible to a wide array of researchers. Looking at disease targeted for CRISPR/Cas9-, ZFN-, and TALEN-based approaches, there is a clear effort to evaluate the utility of these three gene editing technologies for treating inherited monogenic disorders, and for antiviral therapy. For the CRISPR/Cas9 system, the first clinical trials may still be years away, but recent in vivo studies, including some performed in non-human primate embryos, demonstrate rapid progression toward that goal.

Potential Applications of Gene Editing Technology in Reproductive Biology

There are several potential applications for the CRISPR/Cas9 technology in reproductive biology, including those that target pluripotent stem cells, gametes, or embryos (Vassena et al., 2016). The first theoretical option for preventing a genetic disorder in the progeny is to use gene editing to correct the mutation in pluripotent stem cells (induced pluripotent stem cells (iPSCs) or somatic cell nuclear transfer-human embryonic stem cells (SCNT-hESC)) that have been obtained from a patient with a specific disease. These type of cells are ideal for researching technologies for gene editing because can be grown easily. Following gene correction, these pluripotent stem cells must be differentiated in vitro toward oocytes or sperm for use in assisted reproductive technology (ART). The possibility of creating in vitro stem cell-derived gametes in mice was recently demonstrated by two revolutionary papers by Hayashi et al., and current research is being conducted to achieve this result in humans to overcome certain types of infertility (Vassena et al., 2015).

For female gametes, it has been demonstrated in mice that TALEN and CRISPR/Cas9 tools can be used to eliminate mtDNA molecules in oocytes or zygotes (Reddy et al., 2015; Wang et al., 2015). For males, it is currently very difficult to generate post-meiotic sperm from spermatogonial stem cells (SSCs) in vitro. Thus, it appears that SSCs are the better target for treating male infertility (specifically, the inability to generate mature sperm). Recent studies in mouse SSCs have nudged the field closer toward using gene editing to treat male infertility.

Finally, the efficiency of genomic editing is extremely low in embryos. This results from inefficient nuclease cutting and/or inaccurate DNA repair before the embryo undergoes cleavage. Although this is a major problem in generating mosaic embryos, several studies in different animal models have demonstrated the feasibility of gene editing in animals. In nonhuman primates, microinjection of CRISPR/Cas9 or TALENs into zygotes has led to the birth of modified offspring with genome-modification efficiencies that range from 0.5%–40.9% per injected zygotes. Very recently, CRISPR/Cas9 editing was performed in human zygotes to verify its specificity and fidelity. Also, a novel technique, called genome-editing via oviductal nucleic acids delivery (GONAD) has been effectively delivered to preimplantation embryos within the intact mouse oviduct using a simple electroporation method, resulting in the desired genetic modification in the embryo. Unfortunately, these edited embryos were mosaic (this has been seen in other model systems as well) and a substantial number of “off-target” mutations were identified (presumably from nonspecific activity of the CRISPR/Cas9 complex, intrinsic abnormalities of the embryos originating from the 3 pronuclei (3PN) zygotes, or a combination of both).

Recently, it has been published up to now a major milestone in the field of gene editing. Mitalipov's team addressed several steps to improve the precision and safety of the CRISPR/Cas9 technique. As we mentioned above, to date, the CRISPR/Cas9

technology used in embryos and pluripotent stem cells has frequently generated mosaics by repairing the mutation in some cells but also by introducing a high rate of unwanted off-target mutations or extra mutations in the targeted gene (nonhomologous repair). However, Mitalipov's team considerably improved on previous efforts by injecting the CRISPR/Cas9 components together with the patient sperm directly into normal MII oocytes. Until now, the Cas9 complex has been injected directly into the zygote. Remarkably, they now found high efficiency of homologous-repair, no evidence of off-target genetic changes, and only a single mosaic generated in an experiment involving 58 human embryos (Ma et al., 2017).

Obviously, more research in animal models is needed to improve the safety and efficiency of this method for germline correction through genome editing.

Gene Editing to Treat Germline Genetic Disorders: The Case of Mitochondrial Diseases

Mitochondrial diseases are among the most devastating inheritable diseases, with the most severe forms causing death shortly after birth. While there is currently no cure for mitochondrial diseases, advances in science have provided ways to prevent disease transmission. One mitochondrial-disease prevention method is preimplantation genetic diagnosis (PGD), a technique in which cells taken from in vitro fertilized (IVF) embryos prior to uterine implantation are screened to select healthy embryos. Specifically, PGD has been shown to significantly increase the probability of producing healthy offspring for heteroplasmic carriers of mitochondrial DNA (mtDNA) mutations. However, PGD is not a valuable test for all patients. While it provides information about the mtDNA, it offers no intervention when the mtDNA is found to be mutant, as is often the case with homoplasmic or heteroplasmic carriers close to the disease threshold. For this reason, the field of mitochondrial disease prevention is limited until technological interventions are ready for clinical use. In recent years, a novel method of mtDNA replacement therapy (MRT) has emerged as a promising approach for preventing mitochondrial disease transmission (Fogleman et al., 2016; Gómez-Tatay et al., 2017). There are currently four primary techniques for mtDNA replacement: (1) metaphase II spindle-chromosome complex (MII-SCC) transfer, (2) pronuclear (PN) transfer, (3) germinal vesicular (GV) transfer, and (4) polar body (PB) transfer (Fig. 2).

For *MII-SCC transfer*, the mature oocyte containing mutant mtDNA is progressed to metaphase II when the chromosomal material is arranged along the metaphase plate. The nucleus is then harvested and implanted into a healthy, enucleated donor oocyte (Fig. 2A). This technique allows for the newly constructed oocyte to be fertilized by a viable sperm after the transfer occurs or by activation of the reconstructed oocyte. However, because of the imprecise nature of the spindle complex, this technique carries the risk of extracting more cytoplasm and increasing the amount of mutated mtDNA that is transferred.

PN transfer is the process by which pronuclei from both the sperm and oocyte (before fusion) are removed from parental zygotes and placed in a donor zygote that was previously fertilized and enucleated (Fig. 2B). The advantage of this technique is that it allows for the extraction of two, well-defined pronuclei after the sperm has been introduced into the oocyte, potentially reducing the amount of cytoplasm that is transferred with the pronuclei and decreasing the carryover of mutated mtDNA. PN transfer, rather than MII-SCC transfer, may be the technique worth pursuing in future studies to achieve better outcomes for patients with high levels of mutant mtDNA. While it is difficult at this point to compare MII-SCC and PN transfer techniques in a meaningful way, both have advantages and show great promise.

GV transfer involves transferring a healthy, immature oocyte nucleus to a healthy donor egg. This is potentially another way to reduce the amount of inviable mtDNA, but more studies are required (Fig. 2C). GV transfer is performed during the arrested prophase of meiosis I, when two copies of each of the 23 pairs of chromosomes are present. Because of the large size of the nucleus at this stage, it is easier to successfully harvest. The reconstructed oocyte is then matured in vitro prior to fertilization, or fertilized with sperm. However, during GV harvest, small amounts of residual ooplasm may be transferred as well, thus it is possible that mutated mtDNA is present in the newly reconstructed oocyte.

The last method is *PB transfer*. The first polar body (PB1) is formed during egg maturation. In this process, the DNA duplicates so that the egg contains four chromosome sets. Of these, two will remain within the egg, while the other two will package, forming the PB1, which is extruded and will not be present in the resulting embryo. The second polar body (PB2) is formed during fertilization. One set of the remaining chromosomes is packaged, forming the PB2, while the other set will form the nuclear DNA of the embryo, together with the sperm DNA. Polar bodies contain very few mitochondria, which is an advantage for avoiding mitochondrial carryover. In PB transfer, the PB1 is transferred to an unfertilized enucleated donor egg (PB1T), or the PB2 is transferred to a half enucleated zygote (PB2T). Thus, the first strategy is strictly preventive, whereas the second is not (Fig. 2D).

Mitochondrial Gene Editing With TALENs

As we mentioned above, it is possible to use TALENs to specifically target mtDNA. These mito-TALENs can be used to cleave mutated mtDNA, and have already been used to selectively eliminate defective mtDNA in both unfertilized mouse eggs and in murine embryos. Moreover, these genetically modified mice gave birth to two successive generations of healthy mice. When mRNA encoding mito-TALENs was injected into an oocyte from a heteroplasmic mouse that carried two mtDNA haplotypes (NZB and BALB), mtDNA heteroplasmy shift was achieved. Furthermore, mito-TALENs successfully targeted and reduced the levels of mutated human mtDNA when injected into human patient cells that had been fused to a mouse oocyte (Reddy et al., 2015).

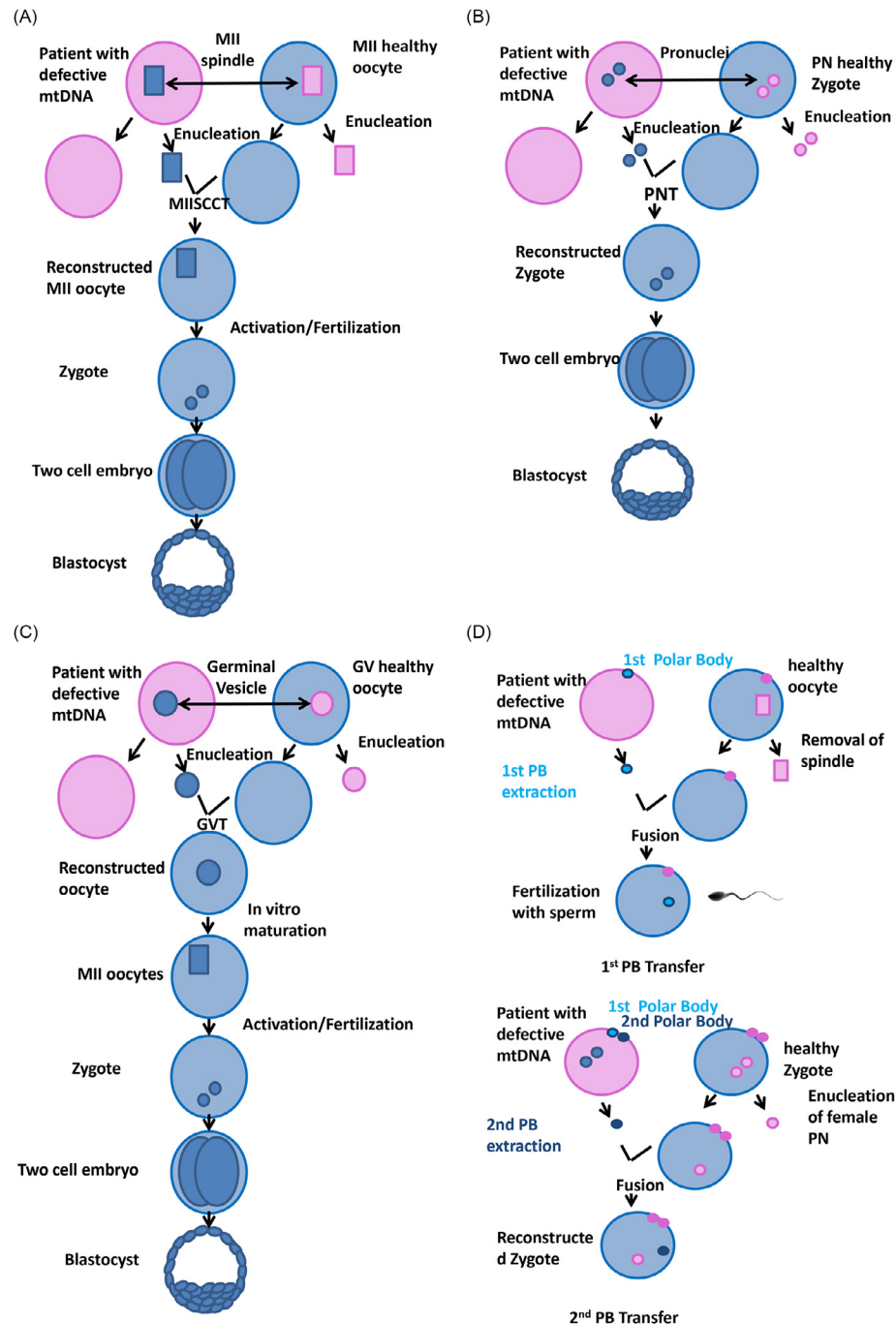


Fig. 2 mtDNA replacement techniques: (A) MII SSC transfer, (B) pronuclear transfer, (C) germinal vesicle transfer, and (D) first and second polar body transfer.

Mitochondrial Gene Editing With CRISPR/Cas9

We are only just beginning to enumerate the enormous possibilities offered by this new biotechnology tool, both in research and in a multitude of clinical applications, particularly in the field of germline genetic modification (mitochondrial diseases). This is reason enough to be curious about the curative potential of CRISPR/Cas9 technologies for carriers of both heteroplasmic and homoplasmic mtDNA mutations. While gene-editing may not affect all mutant mtDNA, it may modify enough to bring the individual below the disease threshold, conferring therapeutic benefits. Although there is considerable hesitation among the scientific community in using this technique in the germ line, studies using nonviable embryos have already been performed. These studies show that the technique works with low efficiency to generate on-target gene modification, but that off-target

mutations are also generated and the resulting embryos are mosaic, as mentioned before. CRISPR/Cas9 has already been successfully used to produce mitochondrial sequence-specific cleavage, as proof of concept that this technique can target specific mitochondrial genes. In the same study, researchers engineered a new version of the enzyme Cas9, mitoCas9, whose localization is restricted to the mitochondrial matrix. This is an important step, as it reduces the risk of off-target mutations in the embryo and prospective children.

CRISPR/Cas9 editing of embryonic mtDNA may represent a more socially acceptable alternative to “three-parent IVF.” Instead of combining the genetics of three individuals, this technique may enable a couple to conceive without requiring donor genetic material. Another advantage of trying to treat mitochondrial disease before implantation is the small number of cells; it may be easier to ensure a reduced mutation load if the embryo is in the 8- or 16-cell stage, rather than waiting to provide a therapeutic intervention after birth, when there are exponentially more cells.

Conclusions

The development of new technologies based on sequence-specific nucleases has resulted in: (1) greatly improved efficiency in targeting specific genomic loci, (2) greater ease in constructing donor vectors, (3) the possibility of genome editing with oligonucleotides, (4) direct genome editing by the electroporation of embryos, and (5) the possibility of multiplexed genome editing. This technological breakthrough makes the possibility of editing the human germline much more feasible than in the past, particularly for embryos that carry mitochondrial disorders. From our point of view, basic studies must be conducted in animal models to better understand mitochondrial biology before these technologies can successfully be used to edit mtDNA. For example, what are the mechanisms of nucleus/mitochondria communication and how is the transmission of mtDNA to the offspring regulated. In particular, what controls variable transmission of heteroplasmy, and how does this affect symptoms of mitochondrial diseases. Since animal studies have limitations in predicting outcomes in humans, subsequent research in humans will be necessary. Similarly, before CRISPR/Cas9 technology can be translated to the clinic, outstanding problems must be resolved, primarily mosaicism and off-target effects. Nevertheless, once technical issues are resolved, editing of the human germline to prevent the birth of a child with a mitochondrial defect should only be considered when already established methods, which do not entail manipulation of the genome (such as PGD), are not available.

Acknowledgments

Work in the laboratory of J.C.I.B. was supported by G. Harold and Leila Y. Mathers Charitable Foundation, The Leona M. and Harry B. Helmsley Charitable Trust, The Moxie Foundation, The Department of Defense (W81XWH-17-1-0552) and NIH (R01 AG36871).

References

- Bolotin, A., Quinquis, B., Sorokin, A., et al. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology*, 151, 2551–2561.
- Ceccaldi, R., Rondinelli, B., & D'Andrea, A. D. (2016). Repair pathway choices and consequences at the double-strand break. *Trends in Cell Biology*, 26(1), 52–64.
- Fogleman, S., Santana, C., Bishop, C., Miller, A., & Capco, D. G. (2016). CRISPR/Cas9 and mitochondrial gene replacement therapy: promising techniques and ethical considerations. *American Journal of Stem Cells*, 5(2), 39–52.
- Gómez-Tatay, L., Hernández-Andreu, J. M., & Aznar, J. (2017). Mitochondrial modification techniques and ethical issues. *Journal of Clinical Medicine*, 6(3), 25.
- Kim, Y. G., Cha, J., & Chandrasegaran, S. (1996). Hybrid restriction enzymes: Zinc finger fusions to Fok I cleavage domain. *Proceedings of the National Academy of Sciences of the United States of America*, 93(3), 1156–1160.
- Ma, H., Martí-Gutierrez, N., Park, S.-W., et al. (2017). Correction of a pathogenic gene mutation in human embryos. *Nature*. <https://doi.org/10.1038/nature23305>.
- Miller, J. C., Tan, S., Qiao, G., et al. (2011). A TALE nuclease architecture for efficient genome editing. *Nature Biotechnology*, 29(2), 143–148.
- Mojica, F. J., Díez-Villaseñor, C., García-Martínez, J., et al. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *60*(2), 174–182.
- Porteus, M. H. (2015). Towards a new era in medicine: therapeutic genome editing. *Genome Biology*, 16, 286.
- Pourcel, C., Salvignol, G., & Vergnaud, G. (2005). CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology*, 151(Pt 3), 653–663.
- Reddy, P., Ocampo, A., Suzuki, K., Luo, J., Bacman, S. R., Williams, S. L., Sugawara, A., Okamura, D., Tsunekawa, Y., Wu, J., Lam, D., Xiong, X., Montserrat, N., Esteban, C. R., Liu, G. H., Sancho-Martínez, I., Manau, D., Civico, S., Cardellach, F., Del Mar, O. C. M., Campistol, J., Zhao, H., Campistol, J. M., Moraes, C. T., & Izpisua Belmonte, J. C. (2015). Selective elimination of mitochondrial mutations in the germline by genome editing. *Cell*, 161(3), 459–469.
- Rouet, P., Smih, F., & Jasim, M. (1994). Introduction of double-strand breaks into the genome of mouse cells by expression of a rare-cutting endonuclease. *Molecular and Cellular Biology*, 14(12), 8096–8106.
- Vassena, R., Eguizabal, C., Heindryckx, B., Sermon, K., Simon, C., van Pelt, A. M., Veiga, A., & Zambelli, F. (2015). ESHRE special interest group Stem Cells. Stem cells in reproductive medicine: Ready for the patient? *Human Reproduction*, 30(9), 2014–2021.
- Vassena, R., Heindryckx, B., Peco, R., Pennings, G., Raya, A., Sermon, K., & Veiga, A. (2016). Genome engineering through CRISPR/Cas9 technology in the human germline and pluripotent stem cells. *Human Reproduction Update*, 22(4), 411–419.
- Wang, S., Yi, F., & Qu, J. (2015). Eliminate mitochondrial diseases by gene editing in germ-line cells and embryos. *Protein & Cell*, 6(7), 472–475.

Content and Volume Overview

Penny Swanson, NOAA Federal Fisheries, Northwest Fisheries Science Center, Seattle, WA, USA

Michael K Skinner, Center for Reproductive Biology, School of Biological Sciences, Washington State University, Pullman, WA, USA

© 2018 Elsevier Inc. All rights reserved.

Summary

Overview of Volume 6 of Comparative Reproduction involves the topics and sections of modes of reproduction, behavior, development and anatomy of reproductive organs, sex determination and differentiation, gamete structure, oogenesis and ovulation, maternal reserves, spermatogenesis, fertilization, endocrinology, seasonal reproduction, puberty and metamorphosis, pregnancy and parturition, phylogeny and reproduction, wildlife and lab animals, and reproductive technology.

Overview

Volume 6 is focused on Comparative Reproduction in all species and phylogeny when reproduction has been investigated. The focus is on nonmammalian species due to Volumes 1–5 being focused on mammalian reproduction. The first section of Volume 6 is Modes of Reproduction, which involves chapters on sexual reproduction, asexual/unisexual reproduction in plants and animals. Hermaphroditism, viviparity, and oviparity in vertebrates and invertebrates are presented. Reproductive strategies and tactics are reviewed.

The second section involves Reproductive Behavior in a variety of species, including birds, invertebrates, insects, fish, turtles, and mammals. The sexual behavior reviewed includes mate choice and sexual selection, courtship and mating, communication (e.g., song), migration and navigation, nesting, brooding, and parental behavior.

The Development and Anatomy of Reproduction section is reviewed with a focus on comparative analysis between species such as plants and insects. The mammary glands, placenta, and accessory sex glands are tissues compared between species. The Primordial Germ Cells, Sex Determination and Differentiation section involves a variety of species, including fish, *Drosophila*, vertebrates, amphibians, reptiles, birds, and insects. Primordial germ cell development, sex determination, environmentally induced sex determination, and sex reversal are reviewed in the different species. The Gamete Structure section reviews egg and sperm in vertebrates and invertebrates. The Oogenesis and Ovulation section reviews these developmental processes in fish, reptiles, birds, insects, and echinoderms. Oocyte maturation is compared, and impacts on reproduction are reviewed. The Maternal Reserves section reviews maternal RNA and vitellogenesis and yolk proteins in fish, *Drosophila*, birds, insects, crustaceans, and molluscs. The comparative reproduction of maternal reserves is reviewed.

The Spermatogenesis and Spermiogenesis section reviews the topic on fish, elasmobranchs, birds, insects, crustaceans, and molluscs. A detailed comparison is made for each to identify similarities and differences in male germ cell development. The Fertilization section compares the fertilization, sperm storage, and delayed fertilization in numerous species. The Endocrine Control of Reproduction section reviews the comparative cellular organization of pituitary gland in vertebrates. In addition, the endocrine control of reproduction in fish, birds, amphibians, reptiles, insects, crustaceans, and molluscs is reviewed.

A number of reproductive physiology topics are reviewed in the Seasonal Reproduction section for photoperiodism, in the Puberty and Metamorphosis Related to Reproduction section, and in the section on Pregnancy and Parturition in fish, birds, mammals, insects, reptiles, and amphibians. The Olfaction and Reproduction section reviews pheromones and olfactory imprinting and homing in fish, insects, and mammals.

The Overview of Reproduction by Phylogeny section reviews the topic on sponges, corals, jellyfish, flatworms, roundworms, *Caenorhabditis elegans*, rotifer, molluscs, insects, crustaceans, centipedes, sea stars, sea urchins, tunicates, fish, sharks, rays, sturgeons, bony fish, frogs, alligators, turtles, birds, monotremes, marsupials, mammals, and plants. This section provides an extensive comparative reproduction overview for each.

The Reproduction, Exotics/Zoo Animals/Wildlife section reviews reproduction in a variety of species. The reproduction in penguins, hyena, alligators, crocodiles, elephants, snakes, primates, and deer are presented. In addition, the General Reproduction in Lab Animals section presents common lab models and associated reproduction. This includes *C. elegans*, *Drosophila*, cockroaches, tobacco hornworm, sea urchin, zebrafish, and rodents.

The Genetics and Epigenetics in Life History and Reproduction section reviews several very interesting models. This includes honeybees, fish, oysters, and the genetic selection of reproductive traits in animals. The Energetics/Nutrition in Reproduction section reviews a various species such as fish and birds. Hibernation and lactation are other physiological processes reviewed.

The final section on Reproductive Technologies in nonhuman/nonprimate species examines a variety of technologies. Germ cell transplantation in fish is reviewed. Gamete preservation in a variety of species is reviewed. Reproductive cloning in a number of different species is also presented. Captive breeding in wildlife, mammals, birds, fish, amphibians, and reptiles is reviewed. Sex control and sterilization in species such as fish is presented. In addition, methods of sterilization and contraception in mammals and wildlife are reviewed.

The breadth, quality, and educational impact of Volume 6 of the Encyclopedia of Reproduction is solely due to the chapters' authors who are internationally recognized authorities on the topics and contributed significantly to the scientific advances in

the field. All are active researchers who have made major contributions in the area of Comparative Reproduction. The contributors to this volume are very much appreciated.

References

- Johri, B. M., & Srivastava, P. S. (2012). *Reproductive biology of plants*. Springer.
- Plant, T., & Zeleznik, A. (2014). *Knobil and Neill's physiology of reproduction*. Academic Press.
- Schatten, H., & Constantinescu, G. M. (2007). *Comparative reproductive biology*. Ames, Iowa: Wiley-Blackwell.
- Wooding, P., & Burton, G. (2008). *Comparative Placentation: Structures, functions and evolution*. Berlin Heidelberg: Springer.

MODES OF REPRODUCTION

Sexual, Unisexual and Asexual Reproduction in Animals – Causes and Consequences

Isa Schön, Royal Belgian Institute of Natural Sciences, Belgium; and University of Hasselt, Diepenbeek, Belgium

Koen Martens, Royal Belgian Institute of Natural Sciences, Brussels, Belgium; and University of Ghent, Ghent, Belgium

© 2018 Elsevier Inc. All rights reserved.

Glossary

Apomixis A form of parthenogenesis without meiosis, which is here replaced by cell division. It results in offspring being genetically identical to the mother and to each other, except for few mutations.

Automixis A form of parthenogenesis with a modified meiosis. The result can either be offspring being genetically similar to their mothers or different, depending on the mechanisms restoring the original number of chromosomes.

Chromosome Carrier of genetic information (DNA), visible as thread in the nucleus of a cell with a light microscope during certain cell stages.

Clonal Total genetic identity between parents and their offspring.

Fertilisation The fusion of egg and sperm cell.

Gametes In animals, these are egg and sperm cells that have only a single set of chromosomes and fuse during fertilisation.

Genome All genes or genetic material (DNA) present in an organism.

Genomic imprinting Genes are differently expressed (translated into proteins), depending on from which parent they originate.

Gynogenesis A combination of parthenogenesis with sexual reproduction, where sperm from males of a closely related species is only required to initiate cleavage of the egg but no actual fertilisation takes place.

Hemiclonal A reproductive mode where only the female part of the genome is passed on clonally (without meiosis) to the next generation.

Hermaphrodites Organisms that have both, male and female reproductive organs and can possibly reproduce with themselves.

Hybridogenesis A combination of parthenogenesis with sexual reproduction. The eggs that are produced by the females of one species are fertilized by the sperm of males from another species, resulting in hybrids. This process is also called hemiclonal.

Kleptogenesis An intermediate form between gyno- and hybridogenesis, with the occasional incorporation of genetic material coming from males of other species. The name comes from the fact that females “steal” the genetic material of these males.

Meiosis Special kind of cell division that leads to the reduction of chromosomes and is required prior to fertilisation and sexual reproduction.

Mutation Genetic change in the DNA, which can be beneficial or deleterious.

Parthenogenesis Reproductive mode where eggs develop into daughters without any male contribution.

Polyploidy Organisms with more than two sets of chromosomes. They often originate through hybridisation.

Sex in Animals is One Big Genetic Lottery!

The majority of all higher organisms reproduces sexually. In animals, sexual reproduction usually takes place with two parents (females and males) although there are also hermaphrodites which can sometimes reproduce through self-fertilisation. Myriads of other forms of sexual reproduction are known from plants, fungi and unicellular organisms – and such “weird sex” in all its aspects has been the topic of a special issue ([Aanen et al., 2016](#)) to which interested readers should refer.

In animals, sexual reproduction involves the fusion of two gametes consisting of a maternal egg cell and a paternal sperm cell. If these two cells fuse, the egg is fertilized, after which cleavage of the egg and embryonic development starts. Before fertilisation can take place, however, both gamete cells have to undergo special processes, including a special type of cell division called meiosis. While all normal cells in a eukaryotic body contain two (or multiple, see below) copies of each chromosome, egg and sperm cells mostly contain only a single set (called haploid nuclei), as otherwise the number of chromosomes would double with fertilisation in every generation. Humans, for example, have 23 pairs of chromosomes, fruit flies have only four. During meiosis, novel genetic

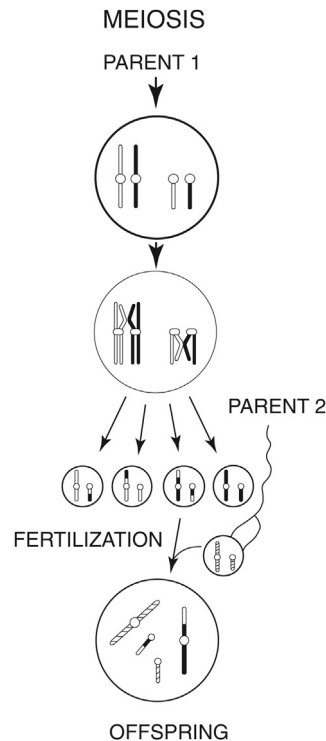


Fig. 1 The most important steps of meiosis. Different chromosomes are indicated by different size, different genes on each chromosome by different colours. In the second step, crossing over between the two chromatids of the same chromosome occur. The four gametes that are the product of meiosis from parent 1 are all genetically different. Redrawn from Martens, K. (Ed.), (1998). *Sex and Parthenogenesis, Evolutionary Ecology of Reproductive Modes in Non-Marine Ostracods*. Leiden: Backhuys Publishers.

combinations in the gametes are formed because chance decides how the (single) chromosomes are distributed over the four gametes that are the result of meiosis. Furthermore, different parts of homologue chromosomes can be exchanged during meiosis, for example, by crossing over, a process that is called recombination and provides additional means to generate novel genetic combinations (Fig. 1).

Subsequently to meiosis, also fertilisation itself generates novel genetic combinations. This is so, because the gametes of the two parents fuse, which can be genetically very different from each other. But also within each parent, not all gametes have the same genetic constitution so which sperm cell will fertilise which egg cell is to a large part determined by chance. As a result, sexual reproduction leads to offspring that differ genetically from each other and from their parents. Sexual reproduction thus causes a large amount of novel genetic variability each generation within populations and can be regarded as a kind of genetic lottery for the evolutionary success of subsequent generations.

In evolutionary terms, sexual reproduction is a costly process, not only because of the specialized mechanisms like meiosis that are involved, but also because of a variety of other reasons.

Firstly, there is the cost of mating as such. Mating is dangerous because when the two parents actively seek each other, they are exposed to predators, unfavourable environmental conditions etc. Sexual reproduction furthermore often includes complicated and costly courtship and mating behaviours, involving elaborate ornaments, mostly in males. Additionally, during mating, harmful parasites, bacteria, viruses etc. can be exchanged which can infect the other parent and transmit diseases.

Secondly, the two-fold cost of sex holds that fully asexual populations have an advantage as all (female) individuals directly produce offspring, while in sexual populations only half of the individuals (females) do so, males do not. Assuming equal numbers of females and males in a population, asexually reproducing populations will grow twice as fast (Maynard Smith, 1978). Additionally, asexuals should be better and faster colonizers because a single egg is sufficient to found a new population.

Despite its evolutionary cost, sex is still the most common reproductive mode in the animal kingdom and most animal species have sex during at least part of their life cycle. This represents an evolutionary paradox and more than 25 hypotheses have been put forward trying to solve this paradox, which remains to date “the queen of problems in evolutionary biology” (Bell, 1982; Schön and Martens, 2014). But several alternatives to sexual reproduction exist.

Unisexual Reproduction in Animals – Half Clones, Sperm Parasites and Sperm Thieves

In vertebrates, asexual reproduction with female-only populations is historically called unisexuality and does not involve fertilisation of the egg by sperm from males of the same species. About 100 unisexual vertebrate species are known (Avisé,

2008, 2015), and certain lizards, snakes and allies can reproduce fully asexually as described in the next paragraph. However, in contrast to ‘real’ asexual reproduction (see below), eggs in most unisexually reproducing animals cannot develop into viable offspring on their own. One could thus regard these types of unisexual reproduction as kinds of intermediate reproductive modes between sex and parthenogenesis. They probably evolved as a result of hybridisation between different species (see below). Hybridogenesis, one mode of unisexual reproduction, combines parthenogenesis with sexual reproduction. Females produce haploid eggs (with only one set of chromosomes), that are fertilized by males from another, usually closely related, species. Since only the female part of the genome is passed on to the next generation during meiotic egg division (the male chromosomes are discarded during the process), this process is also called hemiclinal (=half clonal and half sexual), and thus differs from fully sexual reproduction. This kind of reproduction occurs in various fish, salamanders and frogs (Avisé, 2008, 2015; see also chapters 15 & 16 in Schön *et al.* (2009)).

In another form of unisexuality, gynogenesis, the sperm of males from closely related species is still required to initiate egg cleavage but not for fertilisation. The male DNA is thus no longer incorporated into the egg, in contrast to what happens in hybridogenesis. Gynogenesis has also been called sperm parasitism, as it is detrimental to the males of the other species that waste their sperm. It is known from many vertebrates and is especially common in fish (see an overview in chapter 15 in Schön *et al.* (2009)) and certain salamanders (Avisé, 2015). In total, less than 0.1% of all vertebrates, excluding birds and mammals, reproduce unisexually. In bird and mammals, genomic imprinting appears to be the main reason why parthenogenesis is absent (see chapters 15 & 19 in Schön *et al.* (2009)).

Finally, there is also an intermediate form between hybrido- and gynogenesis, where occasionally, parts of the male genome “leak” into the offspring. The mechanisms and potential selective advantages of this phenomena, for which Avisé (2015) coined the very fitting name “kleptogenesis” or sperm thieves, are still not well understood.

Asexual Reproduction in Animals – Female Power!

There are at present different concepts of asexuality. One school holds that all reproduction by eggs should be considered sexual and only vegetative reproduction, where animals like flatworms that are cut into two, can survive and develop into two novel individuals, are truly asexual. A second approach defines asexuality as reproduction that might involve the production of gametes but without full meiosis and without fertilisation. We follow this second definition. Following this definition, asexuality in animals involves egg cells that can develop into (asexual) offspring without fusion with a sperm cell, a process also called parthenogenesis. Thus, truly asexual populations consist of females only.

There are many different kinds of asexual reproduction (Bell, 1982; Schön *et al.*, 2009). Some processes still include part of meiosis (“automixis”) while others have only mitotic cell divisions without reductions in chromosome numbers (“apomixis”- diploid cells remain diploid) and which generally result in genetically identical offspring or clones (Loxdale and Lushai (2003); chapter 9 in Schön *et al.* (2009)).

Because of the evolutionary cost of sex explained above, asexuality could be more advantageous than sex and many animals, especially invertebrates and certain unisexuals (see previous paragraphs), are able to reproduce asexually (Bell, 1982; Simon *et al.*, 2003; Schön *et al.*, 2009; Avisé, 2015), and do so for at least part of their life cycle. Asexual reproduction is thus not uncommon in animals (Simon *et al.*, 2003; Schön *et al.*, 2009), but it has a “twiggy” phylogenetic distribution (Bell, 1982). This has long been regarded as evidence for the fact that asexuals are by necessity short-lived and are, evolutionary speaking, dead ends of the tree of life. This view has meanwhile been challenged and closer inspection has revealed that there appears to be an even distribution of the age of asexuals (Neiman *et al.*, 2009) without a distinct gap between young and very old asexual taxa. Still, animal groups that have been asexual for millions of years (the so-called ancient asexuals) are very rare, and only four potential cases exist (see chapters 11–13 in Schön *et al.* (2009), Schwander *et al.* (2011)). These lineages are the ideal model organisms to study the evolutionary consequences of asexuality as we would expect that they have developed special mechanisms to overcome the disadvantages of the loss of meiosis and recombination, and its resulting loss of genetic diversity in populations.

The Origin and Maintenance of Unisexual and Asexual Reproduction

Genetic Factors

It is assumed that sexual reproduction was the ancestral condition in early animals. This means that all forms of asexuality and unisexuality described above have developed from sexual ancestors, and this can have been caused by both intrinsic and extrinsic factors or combinations thereof.

The evolution of parthenogenesis is a two-step process: firstly, meiosis needs to be suppressed, and secondly, eggs must be able to initiate cleavage and develop further without the trigger of fertilization. The loss of meiosis can be a relatively simple and spontaneous process, involving only few genetic changes. In animal genomes, no parthenogenetic genes *sensu stricto* have so far been identified as opposed to in plants, but in certain cladoceran crustaceans (Eads *et al.*, 2008), a small genomic alteration has indeed led to the loss of meiosis. With the increasing availability of genomic data from asexual animals, it is likely that more such examples will become known in the near future.

The second step, initiating cleavage and further development, is often seen as a gradual process. The following three steps can be recognised. (1) In hybridogenesis, sperm is still required for both egg cleavage and for further development. (2) In gynogenesis, foreign sperm is still required to initiate egg cleavage, but after that eggs can develop without fertilisation. (3) In parthenogenesis, eggs can start cleavage and develop further by themselves without any need for contact with sperm.

Unisexual and asexual reproduction is often a consequence of hybridisation, the mating of females and males from two different species. Such hybrids are often characterized by polyploidy (multiple sets of chromosomes) and can no longer reproduce sexually with normal meiosis (see above). Hybridisation is very common in plants and probably, also in animals, as the application of novel genomic techniques shows (for recent examples, see [Flot et al. \(2013\)](#) on bdelloid rotifers or [Blanc-Mathieu et al. \(2017\)](#) on root knot nematodes). Young (and hybridogenic) hybrids can have high genetic variability through the genetic material that is received from both parental species. This kind of hybrids thus potentially combines the benefits of both reproductive modes ([Bell, 1982](#)). In contrast, gynogenesis continuously omits the male part of the genome during egg division, while no males are present any more in fully parthenogenetic animals, both resulting in a loss of genetic variability in asexuals over time.

There are several other processes (apart from hybridisation) that can lead to asexuality but which also combine, at least to some extent, the advantages of sexual, unisexual and/or asexual reproduction. These include asexuals with very occasional input of male genetic material (kleptogenesis, see “unisexuals” above), and certain kinds of automictic parthenogenesis where parts of the meiotic processes and recombination still take place (see above “asexuality” and [Bell \(1982\)](#); chapter 1 in [Martens \(1998\)](#); chapter 4 in [Schön et al. \(2009\)](#)).

Environmental Factors

The reproductive mode can also be influenced by the environment. Decreasing temperatures and crowding conditions in populations are known factors triggering the switch from asexual to sexual reproduction in so-called cyclic parthenogenes. These have their name from the fact that populations go through alternative cycles of asexual and sexual reproduction. Sex is required to survive winter (aphids), or produce resting stages (cladocerans, rotifers; see chapters 14 & 15 in [Schön et al. \(2009\)](#)). Again, cyclic parthenogenesis is a way to combine the evolutionary advantages of both reproductive modes.

Another evolutionary avenue to mixed reproduction in certain species of non-marine ostracods is the existence of three genders, sexual and asexual females plus males ([Martens, 1998](#)). Often, these three genders co-occur ([Martens, 1998](#)). The physical closeness of asexual females and males promotes their mating and consequently, the generation of new asexual lineages through hybridisation, causing polyploid, asexual offspring with novel genetic input from their sexual relatives.

Finally, in many invertebrates, asexuality can also be caused by bacterial infections (see the recent review by [Ma and Schwander \(2017\)](#)), with *Wolbachia* being the best known and studied example ([Werren et al., 2008](#)). These bacteria are found in close contact with their hosts, for example, in the reproductive organs where they can arrest meiosis, kill males or change genetic males into phenotypic females.

The Paradox of Sex – Good and Bad Genes

More than 25 evolutionary hypotheses and combinations thereof have been put forward to explain why sex is so prevalent despite its evolutionary costs. The major hypotheses can be divided into two major categories – sex is required to generate “good genes” or to eliminate “bad genes” (see chapter 1 in [Martens \(1998\)](#), [Bell \(1982\)](#)).

Good Genes – Changing Environments and the Running Red Queen

All “good genes” hypotheses state that sex can bring beneficial gene combinations and genetic changes together very fast, within a single generation, and this through the processes of meiosis, recombination and fertilisation explained above. This is the main reason why sexuals are supposed to adapt much faster to changing environments than asexuals where beneficial genetic changes need to occur subsequently in the same clone, which can take a long time (Fisher-Muller accelerated evolution; [Fisher \(1930\)](#), [Muller \(1932\)](#)) (Fig. 2).

Especially in fluctuating or heterogeneous environments, sex should be favoured (see [Schön and Martens \(2014\)](#) for more details). Changing environments can include abiotic (temperature, salinity, pH etc.) as well as biotic factors. Biotic interactions are excellent examples where fast adaptations through sex are essential, for example for hosts trying to win the arms race against their (harmful) parasites ([Hamilton, 1980](#); [Bell, 1982](#)). [Van Valen \(1973\)](#) was the first to coin the term Red Queen for this kind of interactions, referring to the book “Through the looking glass”, where the Red Queen explains to Alice that in Wonderland, it “takes all the running one can do to stay in the same place”. Biologically speaking, this means that constant adaptation of hosts is required to counter new adaptations of their virulent parasites. Despite its wide applicability, the Red Queen hypothesis on its own for explaining the paradox of sex remains controversial (see chapter 7 in [Schön et al. \(2009\)](#)) (Fig. 3).

Bad Genes – Ratchets and Hatchets

Besides generating good genes, sex and recombination have also another very important biological function, namely to quickly eliminate deleterious genetic changes or mutations from the population. Two hypotheses predict that the loss of recombination in fully asexuals will eventually lead to their extinction over longer evolutionary time frames: Muller’s ratchet ([Muller, 1964](#)) and Kondrashov’s hatchet ([Kondrashov, 1993](#)). Muller compared the loss of mutation-free (or mutation-low) genotypes in asexual populations to a ratchet, which clicks every time when such an individual is lost (by chance). The ratchet only operates in one direction, meaning that the loss of mutation-free genotypes is irreversible in asexual lineages whereas they can be restored again in sexuals every generation through meiosis and recombination. Kondrashov’s theory predicts that selection will be less effective in asexuals to eliminate deleterious mutations (see [Schön and Martens \(2014\)](#)). Both hypotheses depend on a number of factors,

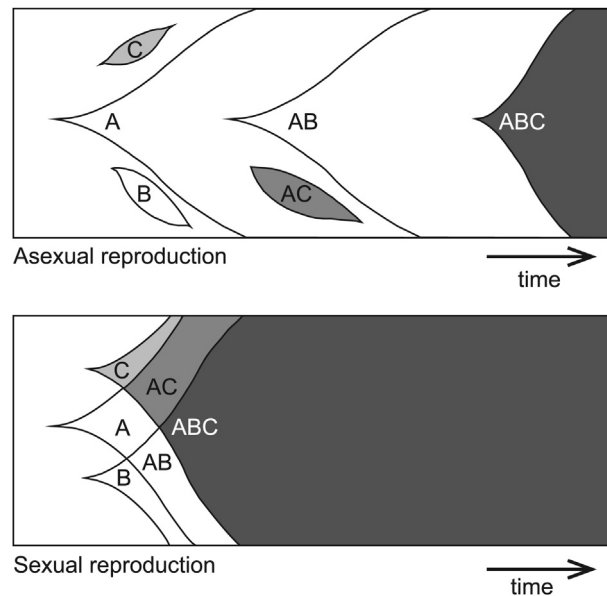


Fig. 2 Sketch describing Fisher-Muller accelerated evolution as one example of the “good genes” hypothesis. Novel beneficial mutations are indicated by different capital letters. While it takes a long time in asexuals (top part of figure) before the mutations A to C occur in the same individual, this process goes very fast in sexual reproduction (lower part of figure) because of meiosis. Redrawn from Martens, K. (Ed.), (1998). Sex and Parthenogenesis, Evolutionary Ecology of Reproductive Modes in Non-Marine Ostracods. Leiden: Backhuys Publishers.



Fig. 3 Alice and the Red Queen, taking all the running they can do to stay in the same place. Illustration from “Through the Looking Glass” by Lewis Carroll 1832–98, first published 1871.

for example population size and the rate of deleterious mutations, which is why their validity can only be ultimately tested when genome-wide data on deleterious mutation rates will have become available for many different sexual and asexual animals.

Pluralistic Approaches Combining the Red Queen and Bad Genes

Up to now, no single hypothesis has been able to explain the evolutionary advantages of sex by itself but the combination of several hypotheses might do so. Asexuals should not only suffer from “bad genes” but for example also from the Red Queen (West *et al.*, 1999). Such pluralistic approaches towards solving the paradox of sex have so far mainly been evaluated theoretically through modelling and their relevance to the living worlds still remains to be tested for most asexual animals.

Novel Evolutionary Mechanisms in Asexuals to get Good Genes and Prevent Bad Genes

A Little Sex Now and Then...

As described above, at least theoretically, animals would benefit the most if they can find a way to combine the evolutionary advantages of both reproductive modes sex and asexuality. We already described several types of reproductive modes where both reproductive modes are closely linked or follow each other during seasonal cycles. Rare sex is another way to allow asexuals to persist without suffering from the consequences of “good and bad genes”. Rare sex can for example occur through kleptogenesis as described above (“Unisexual”) and is known from hermaphroditic planarians and snails, frogs and reptiles (chapters 18, 19 and 20 in Schön *et al.* (2009)). However, the incidence of rare sex in a population must still have a meaningful effect, and thresholds for such minimal effects are debatable.

Weird Sex and Horizontal Gene Transfer

While most animals undergo sexual reproduction as described in the first paragraph above, it has been postulated that other forms of genetic exchange between individuals could also allow fully asexuals to persist without true sex and meiosis. In ancient asexual bdelloid rotifers, there is recent evidence for genetic exchange between individuals, while no males have ever been observed in these animals and recent genomic data reveal genome structures that are incompatible with the occurrence of classical meiosis (Flot *et al.*, 2013). Whether this genetic exchange can be explained by a form of weird sex only known from plant species (Signorovitch *et al.*, 2015) or through non-sexual, horizontal exchange between individual rotifers (Debortoli *et al.*, 2016) as it is long known from bacteria, remains to be further tested. Several studies report that up to 9% of the genome of asexual bdelloid rotifers seems to consist of alien genes originating from bacteria, fungi, plants and other animals (e.g., Gladyshev *et al.* (2008), Flot *et al.* (2013)). Horizontal gene transfer thus provides an important potential evolutionary mechanism for asexuals to acquire “good genes” without meiosis and recombination, although it remains to be tested with genomic data how frequently it has evolved in other asexuals. Asexual root knot nematodes, for example, also have parts of their genome derived from horizontal gene transfer.

One Genotype for all

Another way for asexuals to overcome the need to constantly adapt to changing environments is to develop a so-called General Purpose Genotype, which is advantageous for survival in many different circumstances (see chapter 6 in Schön *et al.* (2009)). Ecological studies have shown that such general purpose genotypes enable ancient asexual ostracods to survive in a wide range of temperatures and salinities (chapter 11 in Schön *et al.* (2009)). Such a genotype can only stay intact in fully asexual organisms, where there is no risk that meiosis and recombination can break it up.

Multiple Copies and Genetic Copy and Repair Machines

In hybrids, the presence of two different parental genomes increases overall genetic variability and their number of potential “good genes” (see above). Many asexuals (see above) are polyploid, with several copies of the same gene. Multiple copies can considerably slow down the accumulation of deleterious mutations as described in the “bad genes” hypotheses. In a similar way, gene duplication causing the presence of multiple genes with different functions can evolve, also increasing the overall genetic variability and the “good genes”. From genomic data, it seems that such so-called gene families are very abundant in asexual cladocerans and aphids, but the influence of the asexual reproductive mode on gene duplication or even multiplication needs to be verified by comparisons with close sexual relatives.

In certain asexual groups, alternative, non-meiotic genetic mechanisms have evolved to prevent the accumulation of deleterious mutations as proposed by the “bad genes” hypotheses. Gene conversion, for example, has been described for various asexual lineages, sometimes in very high frequencies. This mechanism converts two copies of the same homologue gene that usually exist on two different chromosomes into two identical copies. This way, the copy with the deleterious mutations can be overwritten. Genome-wide data on gene conversion will hopefully become available soon, to test how widespread this mechanism is in asexual animals. Also, DNA repair, actively repairing deleterious mutations, has been proposed as a possible biochemical mechanism in ancient asexuals (see chapter 11 in Schön *et al.* (2009)) and there is some evidence for this in ancient asexual bdelloid rotifers and ostracods, which needs to be further explored (Table 1).

Table 1 Summarizing the evolutionary advantages and costs of asexual reproduction and possible mechanisms countering the disadvantages. Disadvantages of asexual reproduction are highlighted in grey. For details, see the text above

<i>Advantages of asexuality</i>	<i>Costs of obligate asexuality</i>	<i>Mechanisms countering the evolutionary costs in asexuals</i>
Fast population growth	Good genes	Mixed reproduction, a little sex
No cost of mating	Fisher-Muller accelerated evolution	Horizontal gene transfer
	Slower adaptation to changing environments	Gene expansion
		General purpose genotype
No cost of meiosis (favourable gene combinations stay together)	Red Queen	Polyploidy
Fast & efficient colonization		Hybridisation
	Bad genes	Mixed reproduction, a little sex
	Muller's ratchet	Large population size
	Kondrashov's hatchet	Gene conversion
		Polyploidy
		Hybridisation
		DNA repair

Concluding Remarks

The paradox of sex remains one of the last unsolved puzzles in evolutionary biology. In many organisms, sexual as opposed to asexual reproduction is not a black and white picture, but mixed reproduction or a link between the two reproductive modes as in unisexuality is common. Evolutionary speaking, such mixed strategies combine the advantages of both modes of reproduction, and are thus expected. There seems furthermore to be large diversity of how sex and asexuality can be combined in different animal groups. As outlined in the last paragraph, we are also just starting to comprehend how different asexuals can counter the evolutionary costs of asexuality. With the increasing availability of genomic data, also from non-model organisms, we expect that our understanding of these evolutionary mechanisms will skyrocket in the coming years.

References

- Aanen, D., Beekman, M., Kokko, H. (Eds.), 2016. Weir sex: The underappreciated diversity of sexual reproduction. *Philosophical Transactions of the Royal Society*, vol. 371, issue 1706.
- Avise, J. C. (2008). *Clonality: The Genetics, Ecology, and Evolution of Sexual Abstinence in Vertebrate Animals*. Oxford: Oxford University Press.
- Avise, J. C. (2015). Evolutionary perspectives on clonal reproduction in vertebrate animals. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 8867–8873. <https://doi.org/10.1073/pnas.1501820112>.
- Bell, G. (1982). *The Masterpiece of Nature: The Evolution and Genetics of Sexuality*. Berkeley: University of California Press.
- Blanc-Mathieu, R., Perfus-Barbeoch, L., Aury, J. M., et al. (2017). Hybridization and polyploidy enable genomic plasticity without sex in the most devastating plant-parasitic nematodes. *PLoS Genetics*, 13, e1006777. <https://doi.org/10.1371/journal.pgen.1006777>.
- DeBortoli, N., Li, X., Eyres, I., et al. (2016). Genetic exchange among bdelloid rotifers is more likely due to horizontal gene transfer than to meiotic sex. *Current Biology*, 26, 723–732.
- Eads, B. D., Tsuchiya, D., Andrews, J., Lynch, M., & Zolan, M. E. (2008). The spread of a transposon insertion in *Rec8* is associated with obligate asexuality in *Daphnia*. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 858–863. <https://doi.org/10.1073/pnas.1119667109>.
- Fisher, R. A. (1930). *The Genetic Theory of Natural Selection*. Oxford: Clarendon Press.
- Flot, J. F., Hespeels, B., Li, X., et al. (2013). Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. *Nature*, 500, 453–457.
- Gladyshev, E. A., Meselson, M., & Arkhipova, I. R. (2008). Massive horizontal gene transfer in bdelloid rotifers. *Science*, 320, 1210–1213.
- Hamilton, W. D. (1980). Sex versus non-sex versus parasite. *Oikos*, 35, 282–290.
- Kondrashov, A. S. (1993). Classification of hypotheses on the advantage of amphimixis. *Journal of Heredity*, 84, 372–387.
- Loxdale, H.D., Lushai, G. (Eds.), 2003. Intracolonial genetic variation: Ecological and evolutionary aspects. *Biological Journal of the Linnean Society*, vol. 79. Oxford: Blackwell Publishing, pp. 1–208.
- Martens, K. (Ed.). (1998). *Sex and Parthenogenesis, Evolutionary Ecology of Reproductive Modes in Non-Marine Ostracods*. Leiden: Backhuys Publishers.
- Ma, W. J., & Schwander, T. (2017). Patterns and mechanisms in instances of endosymbiont-induced parthenogenesis. *Journal of Evolutionary Biology*, 30, 868–888. <https://doi.org/10.1111/jeb.13069>.
- Maynard Smith, J. (1978). *The Evolution of Sex*. Cambridge: Cambridge University Press.
- Muller, H. J. (1932). Some genetic aspects of sex. *American Naturalist*, 66, 118–138.
- Muller, H. J. (1964). The relation of recombination to mutational advance. *Mutational Research*, 1, 2–9.
- Neiman, M., Meirmans, S., & Meirmans, P. (2009). What can asexual lineages tell us about the maintenance of sex? The year in evolutionary biology 2009. *Annals of the New York Academy of Sciences*, 1168, 185–200. <https://doi.org/10.1111/j.1749-6632.2009.04572.x>.
- Schön, I., & Martens, K. (2014). Paradox of sex. In J. Losos (Ed.), *Oxford Bibliographies in Evolutionary Biology*. New York, NY: Oxford University Press. Available at: <http://www.oxfordbibliographies.com/view/document/obo-9780199941728/obo-9780199941728-0035.xml>.
- Schön, I., Martens, K., & van Dijk, P. (Eds.). (2009). *Lost Sex: The Evolutionary Biology of Parthenogenesis*. Dordrecht, The Netherlands: Springer Science + Business Media.
- Schwander, T., Henry, L., & Crespi, B. J. (2011). Molecular evidence for ancient asexuality in *Timema* stick insects. *Current Biology*, 21, 1129–1134. <https://doi.org/10.1016/j.cub.2011.05.026>.
- Signorovitch, A., Hur, J., Gladyshev, E., & Meselson, M. (2015). Allele sharing and evidence for sexuality in a mitochondrial clade of bdelloid rotifers. *Genetics*, 200, 581–590. <https://doi.org/10.1534/genetics.115.176719>.
- Simon, J.-C., Delmotte, F., Rispé, C., & Crease, T. (2003). Phylogenetic relationships between parthenogens and their sexual relatives: The possible routes to parthenogenesis in animals. *Biological Journal of the Linnean Society*, 79, 151–163. <https://doi.org/10.1046/j.1095-8312.2003.00175.x>.
- Van Valen, L. (1973). A new evolutionary law. *Evolutionary Theory*, 1, 1–30.
- Werren, J., Baldo, L., & Clark, M. E. (2008). *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, 6, 741–751. <https://doi.org/10.1038/nrmicro1969>.
- West, S. A., Lively, C., & Read, A. F. (1999). A pluralist approach to sex and recombination. *Journal of Evolutionary Biology*, 12, 1003–1012.

Sexual Reproduction vs Unisexual; Asexual Reproduction, Plants

Peter J van Dijk and Charles J Underwood, Keygene. N.V., Wageningen, The Netherlands

© 2018 Elsevier Inc. All rights reserved.

Glossary

Allopolyploid A polyploid where selective chromosome pairing and recombination occurs during meiosis for a given chromosome (AA/BB).

Apomeiosis The avoidance of reduction in chromosome number and genetic recombination that usually occurs in meiosis.

Autonomous endosperm Spontaneous development of the endosperm without fertilization of the central cell (opposite of pseudogamy or pseudogamous endosperm development).

Autopolyploid A polyploid where all copies of a given chromosome can pair and recombine randomly in meiosis (AAAA).

Double fertilization The process by which one sperm nucleus fuses with the egg cell nucleus and the second sperm nucleus fuses with the central cell nucleus.

Endosperm The endosperm is a component of the seed which surrounds and nourishes the embryo.

Hermaphrodite An organism that contains both male and female sexual organs.

Heterosis The phenomenon that the hybrid progeny of two parents is more vigorous than both parents.

Megagametophyte The seven-celled tissue that develops from the functional megaspore, containing the egg cell and the central cell; also called female gametophyte or embryo sac.

Megaspore Mother Cell (MMC) The cell that will undergo female meiosis resulting in four megaspores.

Megaspores The products of female meiosis of which generally three degenerate and one survives.

Parthenogenesis Fertilization-independent activation of embryogenesis in an egg cell.

Pseudogamy Endosperm development relies on fertilization of the central cell (opposite of autonomous endosperm development), while the embryo is asexual due to parthenogenesis.

Sexual Reproduction in Flowering Plants

Flowering Plant Life Cycle

The great majority of flowering plants reproduce through sexual seeds, which are the result of the fusion of female and male gametes. (Fig. 1). The life cycle of flowering plants is predominantly sporophytic with a short gametophytic generation, separated by meiosis. The female gametes (egg cells) of flowering plants are formed in the ovary. The male gametes (pollen, actually the megagametophyte, see below) are formed in the anthers of the stamens. Most plant species (~95%) are hermaphrodites and produce both female and male gametes, in the same individual in the same or in different flowers.

Gamete Formation

In diploid $2n$ plants female meiosis (megasporogenesis) in the Megaspore Mother Cell (MMC) gives rise to four haploid (n) megaspores, of which three degenerate (Fig. 1). From the remaining functional megaspore, the megagametophyte (also known as female gametophyte or embryo sac) develops by three mitotic divisions, at the mature stage consisting of 8 nuclei and 7 cells (one egg cell, two synergids, one central cell and three antipodals). The two polar nuclei of the central cell fuse, and form a $2n$ nucleus. Male meiosis (microsporogenesis) produces four haploid microspores, known together as tetrads. The nucleus of the microspore divides mitotically into a vegetative and a generative cell, the latter of which divides into two sperm nuclei. The microgametophyte (pollen) is released at a bi-nucleate or tri-nucleate stage.

Double Fertilization

Pollination is the process where pollen grains land on the stigma of a plant, after transport by biotic (insects, birds or bats) or abiotic (wind or water) vectors. The pollen grain germinates and forms a pollen tube that grows into the style eventually reaching the megagametophyte. A hallmark of sexual reproduction in flowering plants is the process of double fertilization which was independently discovered by Sergei Navashin and Léon Guignard in 1898 (Van Dijk and Ellis, 2016). Of the two sperm nuclei in the pollen tube, one fertilizes the egg cell ($n + n$) and the second the central cell nucleus ($2n + n$), forming the embryo and the endosperm, respectively (Fig. 1). The endosperm nourishes the embryo and is essential for the development of the seed, but does not contribute genetically to the next generation.

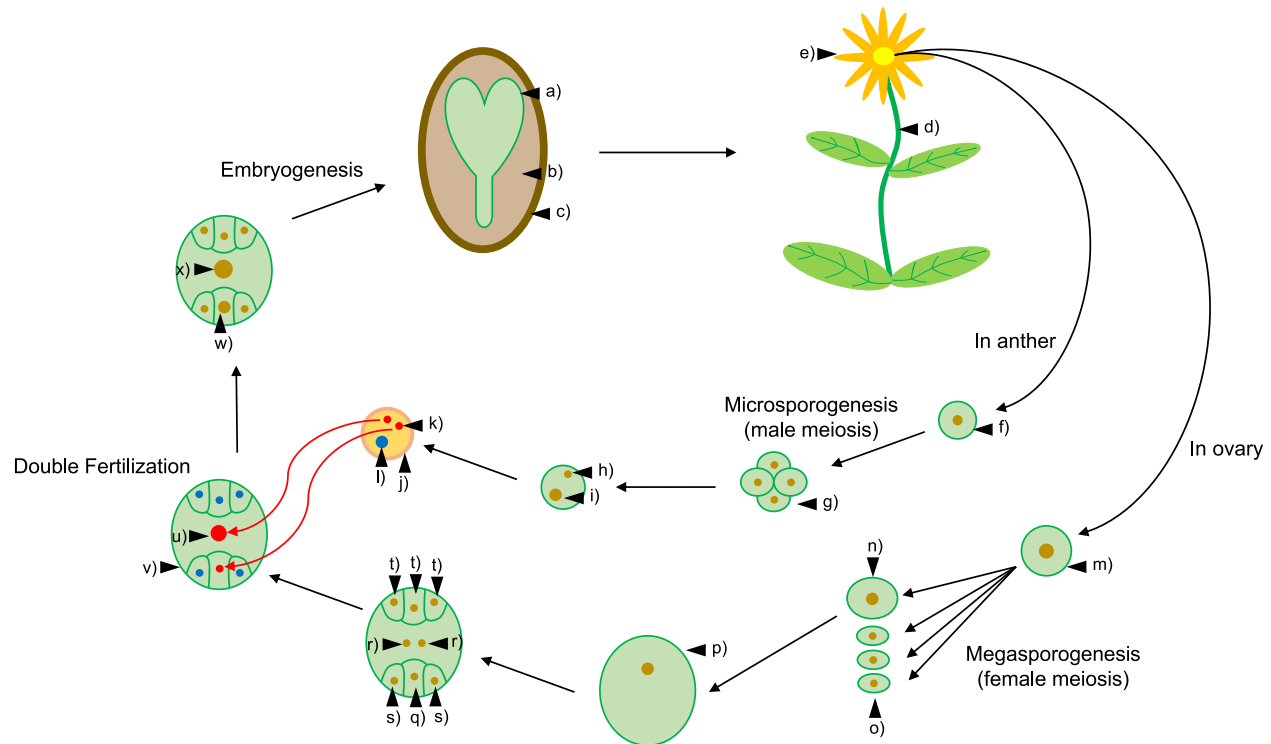


Fig. 1 Sexual reproduction in flowering plants. (a) mature embryo, (b) endosperm, (c) seed coat, (d) mature plant, (e) inflorescence, (f) microsporocyte (male meiocyte), (g) tetrad of microspores (male meiotic products), (h) generative cell, (i) vegetative cell, (j) mature pollen grain, (k) one of two sperm, (l) vegetative nucleus, (m) megaspore mother cell (MMC) or megasporocyte (female meiocyte), (n) functional megaspore, (o) one of three non-functional megaspores, (p) megagametophyte, (q) egg cell, (r) polar nuclei, (s) synergid cells, (t) antipodal cells, (u) central cell, (v) mature megagametophyte, (w) zygote, (x) endosperm nucleus, In the mature gametophytes, generative nuclei (nuclei that contribute genetic information to the seed) are depicted as red (●) and vegetative nuclei (nuclei that don't contribute genetic information to the seed) are depicted as blue (●).

Asexual Seed Production in Flowering Plants

Apomixis is a Modification of Sexual Reproduction

A relatively small number of plant species produce asexual seeds by modifying parts of sexual reproduction. These seeds are genetically identical to the mother plant. For the phenomenon in which sexual reproduction has been replaced by asexual reproduction, in 1908 Hans Winkler introduced the term "apomixis" (etymology: "apo" means "away" and "mixis" means "mixing" in Greek, hence "without mixing") (Nogler, 1984; Bicknell and Koltunow, 2004). Strictly speaking, in onions (*Allium*), bulbils (small bulblike organ of vegetative reproduction) in the inflorescence also fall under the apomixis definition. Nowadays apomixis is generally used in sensu stricto, that is, as seed-formation without gametes, thus synonymous with the term agamospermy, which is now obsolete.

Well-known apomicts are dandelions, blackberries and grasses such as Kentucky blue grass, Fig. 2 shows some common apomicts of the Compositae (Asteraceae) family. These are wild plants; apomixis does not occur in major agricultural crops, citrus being an exception.

The Discovery of Apomixis

In 1839, John Smith described three female plants of an Australian plant species *Cælebogyne ilicifolia* (now named *Alchornea ilicifolia*) that produced seeds in Kew Gardens in the absence of male plants (Van Dijk and Ellis, 2016). Other early claims of parthenogenesis could be explained by outcrossing (pollen contamination from adjacent plants) or by self-pollination (in hermaphrodites). For a long time, *Cælebogyne* was the only solid example of parthenogenesis in flowering plants – meiosis was not known in these days – until Eduard Strasburger showed that the embryos were of somatic origin and not from parthenogenetic development of the egg cell (thus no true parthenogenesis) (Van Dijk and Ellis, 2016). In 1898, Hans Oskar Juel demonstrated through cytological investigations on female plants of *Antennaria alpina* that seed development was the result of parthenogenetic development of the egg cell. Like in *Cælebogyne*, a female *Antennaria* specimen produced seeds in the absence of male plants.



Fig. 2 Three natural apomictic flowering plants. Inflorescences of three apomictic species from the Compositae family, which produce clonal seeds: (A) the common dandelion (*Taraxacum officinale*), (B) orange hawkweed (*Hieracium aurantiacum*) and (C) the annual fleabane (*Erigeron annuus*). © Peter van Dijk.

Apomixis can be demonstrated in hermaphroditic plant species by using male sterile plants (excluding self-fertilization), cytological investigations (observing parthenogenesis) and marker analysis of progeny (fixed heterozygosity).

Types of Apomixis

The formation of extra somatic embryos like in the case of *Cœlebogryne* described above is called sporophytic apomixis (**Fig. 3(D)**). Another name often used is nucellar embryony, referring to the origin of the embryo in the nucellus, the tissue that surrounds the female gametophyte. In nucellar embryony, alongside the sexual embryo a seed can contain several somatic embryos, and hence this form of apomixis is often called adventitious embryony. Examples of nucellar embryony include citrus and orchids.

In gametophytic apomixis, the other main class of apomixis, a gametophyte with an egg cell is formed. To produce clonal offspring that is genetically identical to the mother plant, the avoidance of meiosis, both meiotic recombination and chromosome number reduction (apomeiosis), and the development of the embryo without fertilization of the egg cell (parthenogenesis) are required. To date, gametophytic apomixis has been demonstrated in some 300 species, which is about 0.1% of the flowering plants.

Apomeiosis and parthenogenesis take place in different cells and at different times and thus are different elements of apomixis. At low frequencies, these apomixis elements also occur in sexual plants in the form of unreduced $2n$ egg cells (giving $2n + n$ offspring after fertilization by a haploid pollen grain) and haploid parthenogenetic offspring. However, these are rare non-recurrent phenomena, as opposed to the forms of highly penetrant, recurrent apomixis described here.

Gametophytic apomixis is further subdivided into diplospory and apospory, depending on the origin of the unreduced gametophyte. Diplospory most closely resembles sexual development (**Fig. 3(B)**). Here, the normal female meiosis with recombination and reduction is replaced by a mitotic division or by a restitution of meiosis I (the first meiotic division) without recombination. Two spores are formed, one of which progresses through three mitotic divisions to form an unreduced and non-recombined gametophyte with egg cell, which is genetically identical to the mother plant. The embryo then develops through parthenogenetic activation of the unreduced egg cell. Examples of diplospory are the Compositae genera *Taraxacum* (Dandelion) and *Erigeron*, *Boechera* (Brassicaceae) and *Tripsacum* and *Eragrostis*.

In the case of apospory (**Fig. 3(C)**), an Aposporous Initial (A.I.) cell (of sporophytic origin) is formed near the sexual megagametophyte, which subsequently undergoes three mitotic divisions resulting in an unreduced embryosac, whose egg cell develops parthenogenetically. There is competition between the aposporous and the sexual gametophyte. Usually, the sexual gametophyte is not fully developed, but in some cases the sexual egg cell also develops and, in addition to the apomictic, some sexual seeds may develop (called facultative apomixis, although partial apomixis is a better term, since the sexual egg cell will develop non-parthenogenetically and the aposporous egg cell will develop parthenogenetically). Examples of genera with apospory are: *Hieracium* (Compositae); *Hypericum* (Hypericaceae), in the grasses *Poa*, *Pennisetum*, *Paspalum* and *Cenchrus*.

A rare and curious form of gametophytic apomixis is described in a conifer *Cupressus dupreziana* (not angiosperm but a gymnosperm) (Pichot *et al.*, 2001). This is a case of so-called paternal apomixis, also known as surrogate mothers. The embryos are genetically identical to the father plant, which must have produced unreduced pollen that has developed parthenogenetically. Paternal apomixis has never been reported in angiosperms, while maternal apomixis has not been observed in gymnosperms. This may be related to maternal inheritance of cytoplasmic genes in seed plants and paternal inheritance in gymnosperms. Hence maternal apomicts in gymnosperms would lack mitochondria and chloroplasts as would be the case with paternal apomicts in angiosperms.

As described above, flowering plants have double fertilization and the sexual endosperm nurses the embryo. In many apomicts, endosperm development is still dependent on fertilization. This phenomenon is called pseudogamy: pollination by the father is necessary for proper endosperm and seed development, but the father has no genetic input in the offspring. In some apomicts, especially from the Compositae family (dandelion, hawkweed, *Erigeron*), the endosperm develops autonomously from the central cell, without fertilization.

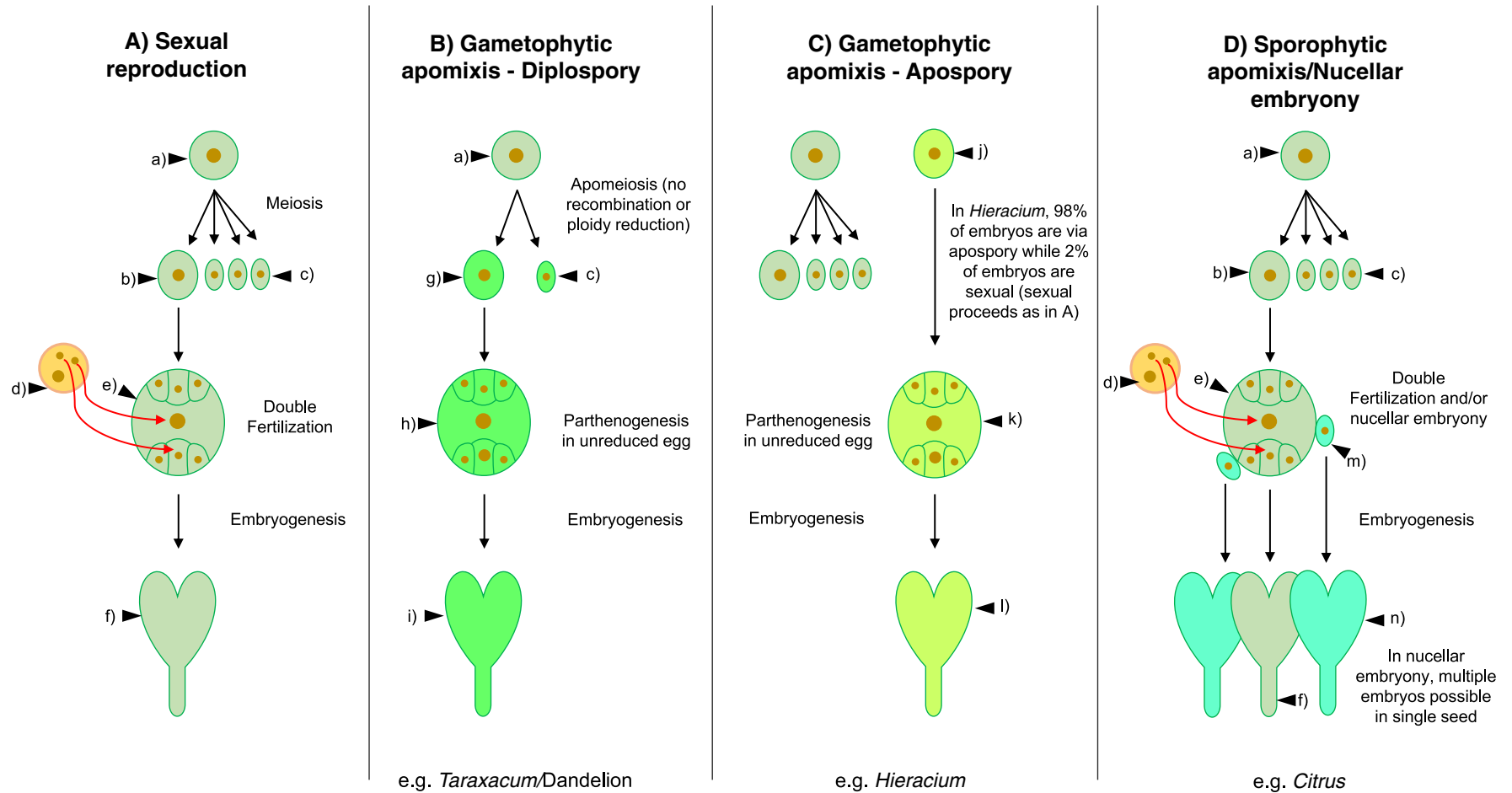


Fig. 3 Sexual and asexual reproduction in flowering plants. (a) megaspore mother cell (MMC) or megasporocyte (female meiocyte), (b) functional megaspore, (c) non-functional megaspore, (d) mature pollen grain, (e) mature megagametophyte, (f) mature sexual embryo, (g) non-recombined and unreduced functional megaspore, (h) unreduced mature megagametophyte, (i) mature unreduced embryo, (j) aposporous initial cell (sporophytic origin), (k) aposporous mature megagametophyte, (l) mature aposporous embryo, (m) nucellar cell (or other cell of sporophytic origin), (n) nucellar embryo (non-sexual embryo).

Epigenetic Reprogramming in Sexual and Apomictic Plant Species

Flowering plant germline cells are specified very late in development from somatic cells in reproductive organs, whereas mammalian germlines are specified during embryogenesis where they undergo widespread reprogramming of DNA methylation. Therefore epigenetic reprogramming in flowering plant germline cells can only take place immediately before or during reproductive development. Unlike in mice and humans, DNA methylation in the *Arabidopsis thaliana* male germline is largely maintained in CG and CHG contexts (where H=A, C or T), and only reprogrammed in CHH contexts (Kawashima and Berger, 2014). This validates work in *Arabidopsis*, *Linaria vulgaris*, and other species, that plants can inherit epialleles in a Mendelian manner. In the female *Arabidopsis* germline, inference from data collected from early embryo and endosperm stages suggests that DNA methylation is reprogrammed in the egg cell and central cell, and subsequently DNA methylation is restored during embryogenesis (Kawashima and Berger, 2014).

It is yet to be understood whether epigenetic reprogramming of the female germline occurs in apomictic species. Given that apomixis has arisen independently on many occasions it is possible that epigenetic reprogramming may occur to different extents and in different ways depending on the type of apomixis. A complete absence of epigenetic reprogramming in apomicts would lead to the accumulation of epimutations and epigenetic drift over generations which could contribute to reduced fitness and dying out of an individual clone. As gametophytic apomixis, for instance *Taraxacum*/Dandelion type diplospory, partially resembles sexual female development, it is possible that female epigenetic reprogramming may occur in a similar manner to sexual species. If female reprogramming would occur in apomicts as in sexual *Arabidopsis*, then it is unclear how apomicts overcome the requirement of pollen derived small RNAs which are thought to contribute to the re-establishment of DNA methylation in the early embryo and/or endosperm of sexual plants (Slotkin *et al.*, 2009). In contrast to gametophytic apomicts, sporophytic apomicts that do not pass through a gametophyte are probably least likely to have female reprogramming like in sexual *Arabidopsis*, but it is possible another form of epigenetic reprogramming has evolved in these species.

Correlations Between Apomixis and Other Phenomena

Apomixis and Polyploidy

In contrast to sporophytic apomixis, gametophytic apomixis is strongly associated with polyploidy. The only well documented diploid gametophytic apomict is the allodiploid apomict *Boechera holboellii* (Kantama *et al.*, 2007). The other known apomictic species are polyploid, both auto (AAAA) or allopolyploid (AA/BB). Polyploidization per se does not lead to apomixis. Many natural polyploid plant species are sexual and artificial polyploidization through colchicine treatment of diploid sexuals does not result in apomixis. Triploidy can only be maintained as the major ploidy type in a population when apomixis is present, because sexual triploids are highly infertile. Therefore natural triploid populations are always apomictic (e.g., dandelions). Theory predicts that apomicts accumulate deleterious mutations over long evolutionary time scales, because of chance events (Muller's Ratchet) or lack of effective purging of deleterious mutations (Van Dijk *et al.*, 2009). Polyploids have multiple allelic copies and are better buffered against a mutation load than diploids.

Apomicts always have living sexual diploid relatives. One reason why diploids are not apomictic may be that apomixis genetic factors are not passed through haploid pollen. In dandelions, markers linked to the diplospory allele are not transmitted through haploid pollen. A possible explanation is that recessive lethals linked to the apomixis genes are not masked in haploid pollen by sexual wild type homologs as in diploid pollen (Van Dijk *et al.*, 2009). In *Ranunculus*, *Hieracium* and *Erigeron*, for example, diploid apomicts can be made via culturing diploid microspores from tetraploid apomicts or reduced diploid egg cells of a polyploid facultative apomict (Nogler, 1984; Bicknell and Koltunow, 2004; Noyes and Wagner, 2014). In these cases a haploid life cycle phase is avoided.

Taxonomic and Phylogenetic Distribution of Apomixis

Apomixis has a broad taxonomic distribution. So far sporophytic apomixis has been found in 148 genera, apospory in 110 genera and diplospory in 68 genera (Hojsgaard *et al.*, 2014). Apomictic taxa typically form the twigs of the phylogenetic tree, suggesting that the current apomicts have arisen quite recently and that apomictic lines have a limited evolutionary life span. Assuming that apomixis in different genera originated independently, gametophytic apomixis evolved at least 178 times in the evolutionary history of the angiosperms. Most apomicts occur in the Compositae and the grass (Poaceae or Graminae) families. Sporophytic apomixis is quite common in the orchids. It is striking that in the large legume family (Fabaceae) no apomixis has been found.

Geographic Distribution

Apomicts and sexual relatives often differ in geographic distribution, a phenomenon described as "geographic parthenogenesis" by Vandel in 1928 (Hörandl, 2009). Apomicts often have a more peripheral and larger distribution than sexuals. This may be related to a better colonization ability when new territory became available in the past (e.g., at the end of the Pleistocene). In autonomous apomicts a single individual can build a new population, but for pseudogamous apomicts at least two founders are needed. Geographic parthenogenesis may also be an indirect effect of the strong correlation between apomixis and polyploidy, since sexual polyploids often have different geographic distributions than their sexual diploid relatives. Populations of apomicts are often

polyclonal with some clones being geographically widespread. For example the same dandelion apomictic clone was found in the Netherlands and in Denmark, spanning a distance of more than 600 km (Van Dijk *et al.*, 2009).

The Genetic Control of Apomixis

Most apomicts are hermaphrodites and produce both asexual seeds and sexual pollen. These pollen grains can fertilize related sexual plants and partial apomicts. Despite not knowing about the existence of apomixis, with his search for constant hybrids in hawkweeds (*Hieracium*), Gregor Mendel was the first to demonstrate that apomixis could be transferred through pollen grains (Van Dijk and Ellis, 2016).

The use of apomicts in crosses as pollen donors allows the study of the genetics of apomixis (Hand and Koltunow, 2014). In most species, apomixis appeared to be dominant to sexual reproduction, with apomicts having a simplex *Aaa* or *Aaaa* genotype thus with a single dose of genetically dominant and hemizygous apomixis factors.

The first genetic studies indicated a single locus for apomixis in which apomeiosis and parthenogenesis are jointly inherited. In later studies, especially in the Compositae family, separate unlinked loci for apomeiosis and for parthenogenesis were found. Later in some apomictic grasses uncoupling of apomeiosis and parthenogenesis was observed. It is therefore likely that the single apomixis locus found in other species contains different linked apomixis genes for different elements of apomixis.

Genetic marker studies from the late 1990s onwards showed that recombination around apomixis loci (during male meiosis) was strongly suppressed. In addition, markers in these areas were often hemizygous (only a single copy of a gene at a locus), indicating strong divergence between the sexual and the apomictic alleles. Chromosomal Fluorescent In Situ Hybridization (FISH) studies with BACs showed a hemizygous, heterochromatic Apomixis Specific Chromosomal Region (ASCR). Sequencing of apomixis locus BACs indicated that these ASCRs had a high density of transposable elements (Akiyama *et al.*, 2011).

The next step was the identification and cloning of apomixis genes. Suppression of recombination in ASCRs rendered the usual genetic map-based method unusable. Alternatively, a deletion mapping method of gamma radiation induced deletions was used in *Hieracium* (Catanach *et al.*, 2006). Deletion of the dominant apomixis alleles resulted in a reversal to sexuality, implying that sexual reproduction is default and suppressed by dominant apomixis genes. In *Pennisetum* random BACs from the ASCR were sequenced, identifying a gene homologous with BabyBoom, a known gene which causes somatic embryogenesis in *Arabidopsis* (Conner *et al.*, 2015). As in *Hieracium*, radiation-induced deletions were used in *Taraxacum*. A second EMS mutagenesis screen was used to identify via resequencing the diplospory gene within the diplospory locus.

In *Boechera*, apomixis specific gene expression was used in reproductive tissues for the identification of apomixis genes (Corral *et al.*, 2013). Association of genes with apomixis in broad wild germplasm has been used for the detection and confirmation of candidate genes in *Boechera*, *Hypericum*, *Taraxacum* and *Citrus*.

Engineering Apomixis in Sexual Plant Species

Alongside studies of apomictic species, forward and reverse genetics have been applied in sexual plants, like maize and *Arabidopsis*, to see whether apomictic-like behavior can be instigated. Interestingly, these studies have identified RNA interference (RNAi) pathway mutants as displaying apomictic characteristics. A large forward genetic screen has been carried out in maize to discover mutants that exhibit *Dominant non-reduction (dnr)* phenotypes, i.e., dominant mutations that produce unreduced egg cells. *dnr4*, one of six mutants, was mapped to a gene encoding an argonaute protein which bind to small RNAs and execute post-transcriptional and transcriptional gene silencing (PTGS and TGS) as part of the RNAi pathway (Singh *et al.*, 2011). A reverse genetic study in *Arabidopsis* has also identified mutations in *AGO9*, encoding an argonaute protein, and other genes that encode components of the RNAi pathway, that can lead to apospory like phenotypes in the female reproductive organ (Olmedo-Monfil *et al.*, 2010). The tantalizing connection between epigenetic reprogramming, RNAi and apomixis remains to be fully understood.

An alternative approach to introduce apomixis in sexual crop species is to understand the genetic and molecular basis of apomixis in natural apomicts and then use this knowledge to engineer apomixis in naturally sexual species. This approach has been successfully applied to transfer parthenogenesis from the grass *Pennisetum squamulatum* to a sexual pearl millet by way of transgenic introduction of a single BabyBoom-like gene (Conner *et al.*, 2015). This represents an important proof-of-principle that transgenic introduction of a single apomixis gene can dominantly introduce an element of apomixis in another species. Further understanding of apomixis in many different natural apomictic species will allow the development of a wide number of genetic options for apomixis engineering in crop species.

Apomixis and Plant Breeding

Introducing apomixis into sexual crop species will likely facilitate novel seed production and crop breeding approaches (Van Dijk *et al.*, 2016). For many crop species, including maize and tomato, first generation hybrids (F₁ hybrids) between two different parental lines are the most robust and highest yielding plants. The heterosis, or hybrid vigor, found in these hybrids is such that many phenotypes are markedly improved compared with either parent, i.e., they are transgressive. However, heterosis is lost in the

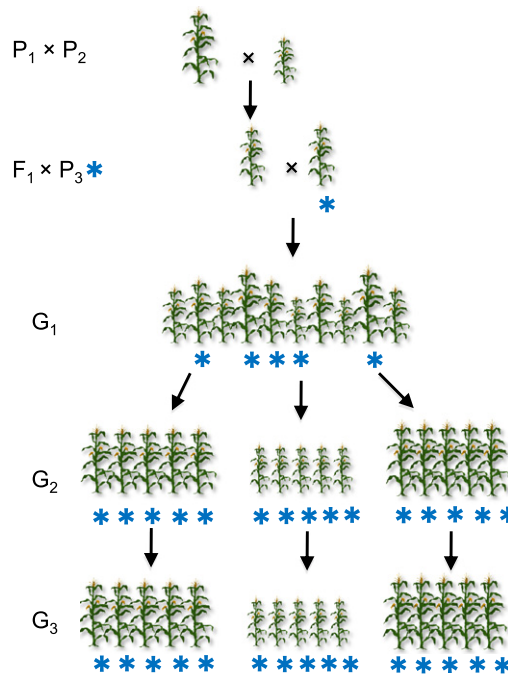


Fig. 4 Constant hybrid breeding. Two different inbred parental lines are crossed ($P_1 \times P_2$) to create an F_1 hybrid. The hybrid forms recombinant and reduced eggs and is pollinated by an additional parental line (P_3 *) which expresses dominant apomixis genes. The individual plants in the next generation (G_1) that contain the apomixis genes (marked with *) will reproduce asexually and can subsequently be maintained as clones in future generations allowing rapid testing of new lines.

next generations because of segregation. Practically speaking this means that seed producers must constantly generate new seed by way of sexual crosses of the two parental lines, which is an expensive process.

The introduction of engineered apomixis into sexual species like maize and tomato has the potential to remove the necessity for recurrent hybrid seed production by sexual crosses. Moreover, apomixis could facilitate the use of hybrids in crops like soybean where recurrent F_1 seed production is not currently feasible on a commercial scale due to a strong preference for self-fertilization. If a line expressing dominant apomixis genes is crossed with a non-apomictic line, then subsequent F_1 hybrids carrying the apomixis genes will reproduce as an apomict. In these F_1 hybrids the genetic information will be maintained in the same state in the next and subsequent generations. A recent study aimed to address whether apomixis in hybrids can allow true phenotypic stability. Using *Hieracium pilosella*, a natural apomictic species, apomictic hybrids between sexual and apomictic lines can be propagated a further 2 generations, and they stably maintained eighteen out of twenty traits, in eleven out of the twelve lines propagated (Sailer *et al.*, 2016). This stable phenotypic inheritance suggests that employing apomixis in agriculture is of great promise.

The introduction of apomixis in sexual plants also has the potential to significantly alter the process by which breeding is carried out. In conventional hybrid breeding where hybrid plants are used as the final crop, the genetic improvement is carried out at the level of the parental lines. In order for the hybrid of the parental lines to be consistent, the parental lines need to be inbred so that they become genetically homogenous. If the parental lines are genetically heterogeneous then the subsequent hybrids will be genetically different, and therefore will be phenotypically inconsistent which is undesirable. Constant hybrid breeding using apomixis genes, could take a different approach (Fig. 4). For instance, if an inbred line that contained apomixis genes was used to pollinate a hybrid of two different inbred lines (which would have produced a recombined and reduced egg cell) then you could produce a population of genetically heterogeneous plants that could be maintained as clones indefinitely. Phenotyping this large population and selecting the most promising plants could be quickly followed by small trials in the next generation, as all plants will be clones, and larger trials in the next generations. An additional potential advantage of introducing apomixis to sexual species is that it could guarantee seed production in the field, irrespective of weather conditions or the absence of pollinators.

The combination of apomixis breeding and simpler seed production would significantly reduce the time it takes from the original crossing of lines to being able to release a new elite variety.

References

- Akiyama, Y., Goel, S., Conner, J.A., *et al.*, 2011. Evolution of the apomixis transmitting chromosome in *Pennisetum*. BMC Evolutionary Biology 11, 289–305.
- Bicknell, R.A., Koltunow, A.M., 2004. Understanding apomixis: Recent advances and remaining conundrums. Plant Cell 16, S228–S245.
- Catanach, A.S., Erasmuson, S.K., Podivinsky, E., *et al.*, 2006. Deletion mapping of genetic regions associated with apomixis in *Hieracium*. Proceedings of the National Academy of Sciences of the United States of America 103, 18650–18655.

- Conner, J.A., Mookkan, M., Huo, H., Chae, K., Ozias-Akins, P., 2015. A parthenogenesis gene of apomict origin elicits embryo formation from unfertilized eggs in a sexual plant. *Proceedings of the National Academy of Sciences of the United States of America* 112, 11205–11210.
- Corral, J.M., Vogel, H., Aliyu, O.M., *et al.*, 2013. A conserved apomixis-specific polymorphism is correlated with exclusive exonuclease expression in premeiotic ovules of apomictic *Boechera* species. *Plant Physiology* 163, 1660–1672.
- Hand, M.L., Koltunow, A.M.G., 2014. The genetic control of apomixis: Asexual seed formation. *Genetics* 197, 441–450.
- Hojsgaard, D., Klatt, S., Baier, R., Carman, J.G., Horandl, E., 2014. Taxonomy and biogeography of apomixis in angiosperms and associated biodiversity characteristics. *Critical Reviews in Plant Sciences* 33, 414–427.
- Horandl, E., 2009. Geographical parthenogenesis: Opportunities for asexuality. In: Schön, I., Martens, K., Van Dijk, P.J. (Eds.), *Lost Sex: The Evolutionary Biology of Parthenogenesis*. New York, NY: Springer, pp. 161–186.
- Kantama, L., Sharbel, T.F., Schranz, E.M., *et al.*, 2007. Diploid apomicts of the *Boechera holboellii* complex display large scale chromosome substitutions and aberrant chromosomes. *Proceedings of the National Academy of Sciences of the United States of America* 104, 14026–14031.
- Kawashima, T., Berger, F., 2014. Epigenetic reprogramming in plant sexual reproduction. *Nature Reviews Genetics* 15, 613–624.
- Nogler, G.A., 1984. Gametophytic apomixis. In: Johri, B.M. (Ed.), *Embryology of Angiosperms*. Berlin: Springer, pp. 475–518.
- Noyes, R.D., Wagner, J.D., 2014. Dihaploidy yields diploid apomicts and parthenogens in *Erigeron* (Asteraceae). *American Journal of Botany* 101, 865–874.
- Olmedo-Monfil, V., Durán-Figueroa, N., Arteaga-Vázquez, M., *et al.*, 2010. Control of female gamete formation by a small RNA pathway in *Arabidopsis*. *Nature* 464, 628–632.
- Pichot, C., El Maâtaoui, M., Raddi, S., Raddi, P., 2001. Surrogate mother for endangered *Cupressus*. *Nature* 412, 39.
- Sailer, C., Schmid, B., Grossniklaus, U., 2016. Apomixis allows the transgenerational fixation of phenotypes in hybrid plants. *Current Biology* 26, 331–337.
- Singh, M., Goel, S., Meeley, R.B., *et al.*, 2011. Production of viable gametes without meiosis in maize deficient for an ARGONAUTE protein. *Plant Cell* 23, 443–458.
- Slotkin, R.K., Vaughn, M., Borges, F., *et al.*, 2009. Epigenetic reprogramming and small RNA silencing of transposable elements in pollen. *Cell* 136, 461–472.
- Van Dijk, P.J., De Jong, J.H., Vijverberg, K., Biere, A., 2009. An apomixis-gene's view on dandelions. In: Schön, I., Martens, K., Van Dijk, P.J. (Eds.), *Lost Sex: The Evolutionary Biology of Parthenogenesis*. New York, NY: Springer, pp. 475–493.
- Van Dijk, P.J., Ellis, T.H.N., 2016. The full breadth of Mendel's genetics. *Genetics* 204, 1327–1336.
- Van Dijk, P.J., Rigola, R., Schauer, S.E., 2016. Plant Breeding: Surprisingly less sex is better. *Current Biology* 26, R122–124. doi:10.1016/j.cub.2015.12.010.

Modes of Reproduction Verts: Hermaphroditism, Viviparity, Oviparity, Ovoviviparity: (General Definition With Examples)

Marvalee H Wake, University of California, Berkeley, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Clade A lineage of biological taxa that includes all the descendants of one common ancestor.

Direct development Laying well-yolked eggs terrestrially (including burrows, in trees, etc. Embryos develop through metamorphosis, juveniles of the adults hatch, and a free-living larval stage is absent.

Hermaphroditism The expression of both male and female reproductive function in a single individual.

Lecithotrophy Yolk is the nutrient material provided to embryos, etc., in retained eggs.

Oviparity Egg-laying.

Ovoviviparity Retention of fertilized eggs in or on the body of a parent, presumably with nourishment only from yolk, and birth at any stage of development from relatively late larva (usually into water) to fully metamorphosed juvenile.

Parthenogenesis A form of asexual reproduction in which embryos develop from an unfertilized ovum.

Placenta Organ by which fetuses obtain maternal nutrition in some species; features vascularized membranes (yolk sac, amnion, chorion) (sharks may have only the former; amniotes have all three); features close association and modification of local maternal oviductal circulation and that of the embryonic membranes.

Pseudoplacenta Organ for obtaining maternal nutrition post-yolk from the female parent; depending on the species, can be modified gills, extensions of the hindgut, etc.

Viviparity Live-birth; developing embryos retained in or on the body of a parent (usually the oviducts of the female parent), with or without post-yolk nutrients supplied by the parent, diverse means by which the fetuses obtain that nutrition (including placentas, pseudoplacentas, oral ingestion, etc.), and with birth of a fully metamorphosed juvenile (sometimes used broadly to indicate all modes of live-bearing).

Introduction

Modes of reproduction in vertebrate animals are many and diverse. Members of all of the major groups (Classes: Chondrichthyes (sharks, skates, and rays), Osteichthyes (bony fishes), Amphibia (frogs, toads, salamanders, newts, and caecilians), "Reptilia" (lizards and snakes, turtles, crocodilians, tuataras), "Aves" (birds) and Mammalia (monotremes (platypus and echidna, marsupials), and eutherian mammals)) have experimented with a number of ways of modifying eggs and sperm, and their unions, the places fertilized eggs develop, and many other features. (The reason "Reptilia" and "Aves" class names are in quotation marks is that, according to current taxonomy, some reptiles are more closely related to birds than they are to other reptiles (e.g., crocodilians are more closely related to birds than they are to lizards or turtles), so birds are included in the Class Reptilia.) The ancestral condition for vertebrates as a lineage is that reproduction occurs in water, fresh or marine; the females of the species develop and lay a relatively large number of eggs while with courting males; the males extrude sperm over the emerging eggs to fertilize them (external fertilization); then both parents abandon the egg mass so that the embryos develop and hatch into free-living, feeding larvae that subsequently metamorphose into juveniles that resemble the adults of their species. However, some to all members of each Class retain their fertilized eggs following internal fertilization (co-opting structures to transfer sperm, or developing new ones), carry the developing embryos in or on parts of their bodies for varying lengths of developmental time, and some provide nutrition to the growing embryos after they resorb their yolk, with the provision of special structures (e.g., placentas, pseudoplacentas, etc.) to provide uptake of those nutrients. The developing young, depending on the species, can be born as larvae or as fully metamorphosed juveniles. Some fishes, both marine and freshwater, and both chondrichthyans and teleost fishes, use modified fins of the males to fertilize eggs internally for the females, and teleosts, which lack the standard vertebrate oviducts, retain the fertilized eggs/embryos in the follicles or lumens of the females' ovaries until birth. Furthermore, the transition to increased terrestriality has permitted many amphibians, and of course most reptiles and all birds, to lay their eggs on land and even in trees to develop. With that increase in terrestriality, other amphibian and reptilian species, and marsupials and eutherian mammals, retain developing young in or on their bodies so that the aquatic larval stage is avoided. As they have "emerged from the water", many amphibian taxa have devised special ways of retaining their eggs – perhaps the majority of taxa keep their developing eggs/young in their oviducts, but some amphibians, especially frogs, keep their developing embryos in the stomachs of the maternal females, the vocal sacs of the fathers, pouches on the legs of the fathers, or in the skin of the back of the mothers, often in specialized pouches to contain them. Concomitantly, the developing fetuses of various species have developed ways of obtaining parental nutrition – oral ingestion, placentas, pseudoplacentas, etc. There are many different "natural experiments" among vertebrates, and some

have evolved repeatedly in different lineages in different Classes (see [Lombardi \(1998\)](#)). Brief characterizations of the major modes of reproduction in vertebrates follow and examples of the variations that have evolved among the Classes.

Oviparity

Oviparity, egg-laying, is considered to be the ancestral mode of reproduction for vertebrates. Ova (eggs) develop in the females' ovaries (in rare exceptions in males, such as a clade of toads, and pathologically in other vertebrates), mediated by such hormones as estrogen and progesterone, luteinizing hormone, etc., usually cyclically and often determined by seasonality (light, moisture, etc.). Egg sizes vary enormously, often within classes, families, and genera, but size usually characterizes each species. A major feature of egg development is vitellogenesis, the deposition of yolk in each ovum. The ancestral condition is characterized by the development of yolky eggs that are laid externally (in water or on land), often in large numbers, and fertilized externally during a diversity of forms of interactions with males. The yolk mass present in each ovum is typically sufficient to maintain the developing embryo until it hatches, when it becomes a free-living, usually aquatic, larva that can forage on its own until it metamorphoses. However, in direct-developers (see below) eggs sizes are usually large, so that the embryo is provisioned with enough yolk to carry it through metamorphosis to hatch as a juvenile. Conversely, many viviparous species (see below) have very small eggs with little yolk, correlated with their providing other forms of nutrition to the developing young (e.g., via a placenta, etc.). Numbers of eggs (clutch size) laid vary enormously; some fishes and frogs lay hundreds, even thousands, of eggs that are fertilized in water by one, sometimes more, males. While egg-laying is the ancestral condition retained by some members of all classes of vertebrates, (most fishes, most amphibians, most squamates, all turtles and crocodiles, all birds, and both species of monotremes among mammals), some are live-bearers (see below). Also, internal fertilization via a diversity of means of sperm transfer has evolved in most lineages – some fishes, using modified fins, a very few frogs, most salamanders, and all caecilians among amphibians, and all of the amniotes – those vertebrates (reptiles and birds, mammals) that surround their developing embryos with extra-embryonic membranes and then either lay the eggs/embryos or retain them in their oviducts for part or all of their development.

Viviparity

Viviparity (live-bearing) involves retaining fertilized eggs in or on a parent's body. This derived mode of reproduction is thought to have evolved in response to various stresses on free-living larvae, such as predation, limited food resources, drying, cold temperatures, etc. However, for many live-bearers the course of evolution is not clear, and certainly not the same for all. In fact, the common assumption is that live-bearers retain their internally fertilized eggs in the maternal female's oviducts; this is indeed the case in some teleost fishes, most sharks and rays, a few species of frogs and salamanders and several of caecilians, some lizards and snakes, and nearly all mammals. However, teleost fishes lack complete oviducts, and retain developing young in the follicles or the lumens of the ovaries and in one group (seahorses and relatives) the males retain the developing young in special abdominal pouches, and some frogs retain the eggs in pouches on the females' backs, in her stomach, or even in the vocal sacs or leg pouches of the male parents. In these frog examples, the eggs are fertilized externally, and the male either kicks the eggs into the back pouches of the female, or one of the parents turns and ingests the newly fertilized eggs – either the mom depositing the eggs in her stomach (the species is recently extinct, so the mechanism by which the developing young are retained without being digested is not known, save for evidence that the young secrete a prostaglandin that inhibits stomach acid production by the female) or the dad in his vocal sacs. Retention in squamate reptiles and in nearly all mammals is in the females' oviducts, at least for some time. Retention of eggs in oviducts and maintenance through to a juvenile state seems to be easy to do. Some estimates indicate that it has evolved more than 100 times in squamate reptiles; however, recent phylogenetic analyses question that number. Retention can occur either with the yolk being the only (or main) maternal nutrient provided, or with the maternal (rarely, but occasionally, the male) providing nutrients after the yolk is resorbed (see [Section Ovoviviparity](#)). Various mechanisms for provision of nutrients and their uptake by the developing young have evolved. Neither teleost fishes nor amphibians have placentas, at least in the sense of their being composed of extra-embryonic membranes. Some teleost fishes develop extensions of the hind part of the intestine or the gills that are highly vascularized and apparently used for nutrient uptake as well as gaseous exchange ([Wourms and Lombardi, 1992](#)). Among a very few frogs and salamanders, but several caecilians, the oviductal lining cells elaborate a nutrient material that the juveniles, hatched after their yolk is resorbed, ingest orally before birth, often using fetal teeth. It also has been suggested that the gills, tails, and skins of viviparous amphibians might be involved in nutrient uptake, but this has not been confirmed. The gestation period in amphibians varies according to species, but apparently is tied to seasonal/ecological variables, such as rain periods, temperature, and prey abundance. Both the obligately viviparous frog and salamander live at high altitudes, the frog on Mt. Nimba in Africa, the salamander in the Alps. Their reproductive periodicity is very different, however; the frog's gestation is 9 months long, and its endocrinology and embryonic development are specifically controlled in response to temperature and moisture. In contrast, the gestation period for the salamander can be anywhere from a few months to 4 or 5 years, apparently depending on the extremity of the cold of the winter seasons. Subspecies of a salamander have an important form of maternal nutrition in that oviductal embryos at a somewhat advanced stage of development are cannibalistic: they eat sibling eggs and often less-well developed embryos while in the oviduct, and the cannibals are born fully metamorphosed. This constitutes an unusual form of maternal nutrition (summarized by [Wake \(2015\)](#)). Some sharks and rays, and marsupials, at least early in development, employ the highly

vascularize yolk sac as a nutrient-accessing placenta. The placenta of squamate reptiles, many marsupials, and all eutherian mammals is composed of various arrangements of the amniotic and chorionic membranes, and associated assembly of the area of the oviduct in which the young develop (usually termed the uterus). In fact, although there are some differences in the relationship of the yolk, etc., the evolution of placental organization is very similar among squamates and eutherians. New information about the genetics of placental development and maintenance, first found among mammals, but now also occurring in squamates and seahorses, indicates that a common genetic basis exists in all of these diverse vertebrates for intra-oviductal maintenance, especially placentae. Typically, in the species in which the mother provides nutrients during gestation for a time after the yolk has been resorbed, it is correlated with the eggs having been provisioned with reduced amounts of yolk – the system has evolved such that the maternal nutrient contribution is a limited amount of yolk, followed by nutrition via oviductal secretion or via placentation for vascular provision (see [Blackburn \(2015\)](#), for a summary of the evolution of vertebrate viviparity).

Ovoviviparity

Ovoviviparity is a term that has been used to characterize a diversity of features in a diversity of taxa. It generally refers to modes of fertilized egg retention, often with live birth, such as egg retention but with eggs laid (some fishes, amphibians, reptiles, but usually considered a form of oviparity, in that eggs are laid), aplacental viviparity (as in certain elasmobranchs, teleosts, amphibians), pseudoviviparity (some teleost fishes), lecithotrophy (yolk nutrition of the retained developing young, without provision of post-yolk-resorption nutrients) as in some elasmobranchs, teleosts, amphibians, and reptiles. Some researchers use “ovoviviparity” to mean any of these conditions and the inherent discrepancies and even contradictions, largely because of differing assumptions and lack of coherent definition of the precise meaning of the term for the taxa studied ([Lodi, 2012](#)). Much of the difficulty is removed by explicit definition, as well as the recognition that a more precise term may be better applied to specific taxa and cases. For example, is a snake that lays eggs that hatch within 24 h of laying oviparous, despite the long-term (developmentally) egg retention? Is the snake whose eggshell breaks down 24 h before birth such that a juvenile is born clearly viviparous? In a strict sense, the answer is “yes”, but the terms do not give real clues as to the reproductive pattern. I find that a careful definition of “ovoviviparous” that includes two criteria: retention of ova with investments of yolk but no other provision of parental nutrition, and birth of free-living, autonomous offspring at virtually any stage of development, from early larva to fully metamorphosed young (often still with vestiges of yolk in the gut) is most appropriate. This definition of “ovoviviparity” then requires that taxa with the presence of aplacental or placental maternal nutrition and genetically/physiologically “fixed” birth following the completion of metamorphosis be considered “viviparous” because of the combination of these two characteristics. These definitions of ovoviviparity and viviparity work most usefully for live-bearing amphibians, in which the two features clearly distinguish the reproductive modes. Apparently all live-bearing frogs and salamanders, except one species each (see [Section Viviparity](#)), are ovoviviparous – yolk is the nutrient material. Reports of possible nutrient uptake in a few species still require demonstration of nutrient value of secretions of the paternal vocal sac in the former and the dorsal brood pouch in the latter. Two distantly related clades of frogs have evolved a similarly complex mode of ovoviviparity that involves a particular kind of courtship, followed by egg-laying and spermiation, then with the male parent kicking the fertilized eggs up onto the back of the maternal parent. Members of the genus *Pipa* in the aquatic New World family Pipidae have a complexly specific courtship that ends with the vent of the male appressed to that of the female so that sperm are deposited as each egg is delivered. The pair stays in copulation during the period of egg deposition and the male kicks them onto the back of the female that he is clasping. The female’s skin has proliferated and become highly vascularized owing to her reproductive hormones (estrogen and progesterone), so that the eggs lie in cups of skin tissue. The cups deepen as the embryos develop. The embryos in some species leave the parent’s back as late larvae and complete metamorphosis in streams or ponds, while in other species they are born as fully metamorphosed froglets. The other clade that has evolved back-brooding is even more complex. In the genus *Gastrotheca* in the terrestrial New World family Hemiphractidae, the female parent develops a pouch composed of the folded skin of her back into which the father frog, at the end of courtship, kicks each egg into the opening of the pouch. The pouch develops early in the life of the female in response to estrogen, and at breeding time proliferates and vascularizes as her estrogen and progesterone concomitantly accelerate egg development and ovulation. In some of the more than 100 species, the embryos reach a late-tadpole stage and emerge from the pouch into water to complete development (whereupon they assume a terrestrial life); in other species, the embryos are born as fully metamorphosed froglets, obviating any need for an aquatic larval period (summarized by [Wake \(2015\)](#)). The evolutionary convergence between the aquatic pipids and the terrestrial *Gastrotheca* is striking!

Direct Development

Direct development is a term sometimes used to indicate all forms of development in which a free-living, usually feeding, pre-metamorphic larval stage is absent, such that it thereby includes all forms of live-bearing. However, most researchers on vertebrate taxa use a more restricted definition of direct development, such that it is the condition in which eggs are laid on land, and development through metamorphosis occurs before hatching with fully developed juveniles emerging. This mode of development characterizes several lineages of frogs, salamanders, and caecilians. The elimination of a biphasic life cycle via this mode is considered to be a component of the evolution of terrestriality. The eggs are usually externally fertilized, and range in number between 4 or 5 and more than 100, depending on the species. The eggs are usually larger than those of biphasic relatives, owing to

the provision of a large yolk mass to nourish the embryos through metamorphosis. The term “direct development”, in this sense, is rarely (if ever) used for amniotes: egg-laying turtles, crocodilians, squamates, and birds that lay eggs on land that hatch as autonomous juveniles, even though post-hatching parental provisioning might be needed (e.g., in birds that are fed by parents for a time), or mammals.

Hermaphroditism

Among vertebrates, hermaphroditism, the expression of both male and female reproductive function in a single individual, is found only in teleost fishes and in frogs (the instances reported for other vertebrates are considered pathologies, as are those in some species of fishes and frogs). Hermaphroditism takes many forms – the distinction between functional and non-functional hermaphroditism is a major one. Hermaphroditism is usually identified by gonad morphology; there are three main groups or categories. One is synchronous hermaphrodites, in which mature testicular and ovarian tissues are present at the same time, and both produce sperm and ova, respectively. Typically, they are fishes that form spawning pairs, and one initially spawns eggs and the other fertilizes them, then the “fertilizer” deposits its eggs and the other fish fertilizes them, such that both fishes perform both “male” and “female” roles. In other hermaphrodites, the gonads have both ovum-producing and sperm-producing areas, but one or the other predominates during different phases. Sex change is genetically programmed. “Female first” (protogynous) species lay eggs initially, then shift to testis-dominance and sperm deposition. “Male first” (protandrous) species are the opposite, depositing sperm initially and shifting to egg development and deposition. Both types have a transitional period in which the gonads exhibit fairly well-developed ovarian and testicular tissues. Functional hermaphroditism is reported in several families of teleosts. It is most prevalent in tropical marine perciforms, in which it has evolved in 16 genera (including seahorses, gobies, damselfishes, parrotfishes, and wrasses), but it occurs in eels in two orders as well as in diverse members of other clades. Hermaphroditism is found almost exclusively in marine fishes; synbranchid eels are the only fresh water forms for which hermaphroditism has been documented. Highly speciose freshwater families do not include any confirmed instances of hermaphroditism, although they have evolved diverse morphologies and ecologies. Some frogs, especially bufonid toads, have long been known to have ovarian components associated with the testes of presumably male animals. The ova that are produced are usually resorbed. In a few species of frogs, old females sometimes reduce their ovaries and testicular tissue develops, occasionally producing mature sperm; typically, apparently female frogs have well developed ovaries and oviducts, and less well developed testicular tissue and sometimes seminal vesicles. Recently, evidence has shown that many species of frogs are affected by exposure to herbicides during their development such that they are demasculinized, with reduced testes and testosterone production, developing ovaries, modified larynges, and incapability of reproduction. The same phenomenon has been reported in several species of fishes as well, and attributed to consequences of runoff of agricultural water into natural streams and rivers. Furthermore, such induced hermaphroditism appears to be increasing world-wide, correlated with agricultural use of herbicides and other endocrine disruptors (see [Avisé \(2011\)](#)).

Parthenogenesis

Parthenogenesis is reproduction without fertilization, an ovum developing into a new individual without fertilization by a sperm. In vertebrates, parthenogenetic “species” are the result of modification of ovum development, usually changes in meiosis, leading to eggs produced with multiple sets of chromosomes. Some species are facultatively parthenogenetic, induced to produce asexually as ova that have undergone “meiotic error” are laid and develop into adults, but these females usually are sterile and cannot produce a new generation of offspring. They typically are evolutionary/ecological modifications of species that have “standard” reproductive modes, but have hybridized or undergone other speciation modes. In some cases, courtship by a male of a closely related species is required to induce ovum development, but his sperm do not fertilize the ovum. Often, this mode of reproduction produces all-female species, given that only ova develop. Consequently the broods of young are effectively clones of the mother. Several species of teleost fishes, at least three sharks, several lizards, a snake, and frogs and salamanders are known to be strictly parthenogenetic, while yet others are considered facultative or incomplete parthenogens. There are no known cases of natural parthenogenesis in mammals, but it has been artificially induced in rabbits, mice, pigs, and monkeys, often with consequent abnormal development. The low genetic variation and modification of size and other components of development typically cause reduced fitness of offspring in parthenogenetic species. The patterns of evolution of various forms of natural parthenogenesis in several vertebrates have been studied, and are diverse and complex (summarized by [Avisé \(2008\)](#)).

The complexity, variation, and frequent convergences of the evolution of vertebrate reproductive modes is exciting, and not yet fully understood.

References

- Avisé, J. C. (2008). *Clonality: The Genetics, Ecology, and Evolution of Sexual Abstinence in Vertebrates*. Oxford: Oxford University Press.
Avisé, J. C. (2011). *Hermaphroditism: A primer on the Biology, Ecology and Evolution of Dual Sexuality*. New York: Columbia University Press.
Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *Journal of Morphology*, 276, 961–990.

- Lodi, T. (2012). Oviparity or viviparity? That is the question. *Reproductive Biology*, 12, 259–264.
- Lombardi, J. (1998). *Comparative Vertebrate Reproduction*. New York: Springer.
- Wake, M. H. (2015). Fetal adaptations for viviparity in amphibians. *Journal of Morphology*, 276, 941–960.
- Wourms, J. P., & Lombardi, J. (1992). Reflections on the evolution of piscine viviparity. *American Zoologist*, 32, 276–293.

Further Reading

- Wake, M. H. (1992). Evolutionary scenarios, homology, and convergence of structural specializations for vertebrate viviparity. *American Zoologist*, 32, 355–363.

Modes of Reproduction in Fishes

John Godwin and Marshall Phillips, North Carolina State University, Raleigh, NC, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Arginine vasotocin (AVT) Nine amino acid neuropeptide that is the homologue of arginine vasopressin found in mammals. These hormones are released at the posterior pituitary gland, but also widely in the brain where they act as neuromodulators.

Aromatase Enzyme that is a member of the cytochrome P450 family of proteins and plays a key role in the biosynthesis of estrogens through metabolism of androgens such as testosterone. Teleost fishes exhibit two forms of aromatase produced from distinct genes and that are primarily active in the gonad and brain respectively.

Estradiol 17 β (E₂) The most important circulating estrogen in both teleost fishes and mammals, produced in the ovaries but also other tissues including brain through the action of the enzyme aromatase.

Gonadotropin Releasing Hormone (GnRH) Ten amino acid neuropeptide hormone produced in the preoptic area of the hypothalamus in fishes that sits 'atop' the hypothalamo-pituitary-gonadal axis; it is released at the anterior pituitary gland to regulate gonadotropin secretion and is a key regulator of reproduction.

Gonochorism A sexual pattern in which individuals mature as one sex and remain that sex.

Initial phase The first sexual phenotype seen in many protogynous species, often characterized by relatively drab colors and relatively low displays of aggression and courtship behavior.

11-Ketotestosterone (11KT) Potent androgenic steroid hormone in teleost fishes that is analogous to dihydrotestosterone in tetrapod vertebrates in terms of inducing the development of secondary sexual characters often associated with territoriality and courtship in large males.

Monoamine neurotransmitters These are important neural signaling molecules characterized by having an amino group connected to an aromatic ring. Important examples include serotonin, dopamine, and norepinephrine.

Oviparity A mode of reproduction in which females produce eggs that develop outside of the female's body.

Protandry A sexual pattern in which individuals mature as males and can then later change functional sex to become female.

Protogyny A sexual pattern in which individuals can mature as females and then later change functional sex to become secondary males. In *monandric* ('one male') protogyny, all secondary males first pass through a female stage. In *diandric* ('two males') protogyny, individuals can mature as either males or females and both can change from the initial phase (IP) to become the larger and typically colorful and aggressive terminal phase (TP) males.

Simultaneous Hermaphroditism A sexual pattern characterized by individuals possessing both mature ovarian and spermatogenic tissue within the same functional gonad.

Steroid receptors Steroid hormones act largely through intracellular steroid receptor proteins that bind hormone and then function as 'ligand activated transcription factors' to regulate gene expression in target cells. Teleosts have multiple forms of both the estrogen and androgen receptors.

Testosterone (T) Important androgenic steroid hormone in all classes of vertebrates; critically, this steroid often functions as a biosynthetic intermediate in estradiol or 11-ketotestosterone production.

Viviparity A mode of reproduction in which the fertilized eggs develop within the body of the female and offspring are born live.

Introduction

This article addresses modes of reproduction in fishes generally with an emphasis on one of the more dramatic examples of reproductive adaptation to social environment seen in fishes and, indeed, any animal – socially controlled functional sex change. Fishes show the broadest range of sexual patterns of any group of vertebrates. With respect to sex determination, this variation includes all-female unisexual species. Within sexual species, these patterns range from strict gonochorism where sex is determined by sex chromosomes to simultaneous hermaphroditism where individuals can alternate male and female function on a second-to-second basis. Between these extremes are species where:

- i) Sex is determined by social or physical factors prior to maturation, but then sex is fixed after maturation;
- ii) Species that mature as one sex, either male or female, and then change to become the other sex following maturation either as part of an ontogenetic sequence dependent on age or body size or in response to changes in their social environment; and
- iii) Species that can undergo serial adult sex changes in both directions in response to social environment.

In addition to variation in sex determination patterns, fishes also exhibit variation in terms of whether fertilization occurs externally or internally and whether the offspring develop inside or outside of the mother. Most fishes exhibit external fertilization in which females release either negatively buoyant eggs onto a substratum (e.g., rock, sand, or underwater vegetation) or

positively-buoyant eggs that instead undergo early development while floating in the water column. These floating eggs and typically later planktonic larvae can often be transported considerable distances from the location where they were fertilized by currents, particularly in large lakes and oceanic environments.

External fertilization involves the release of eggs by females and the development of these eggs externally is a pattern referred to as oviparity. Viviparity instead refers to the pattern where embryonic development occurs inside the female. For sexually-reproducing species, this is associated with internal fertilization. A full description of the variation across fishes is beyond the scope of this article, but the well-studied Poeciliid fishes illustrate viviparity and some of the variation that occurs within this pattern well. Poeciliid fishes, of which the guppy is a familiar example, are a speciose group of freshwater fishes originally found in Africa and tropical and warm temperate zones of North and South America, but which have now been introduced very widely around the world. Male poeciliids have an intromittent organ termed the gonopodium that used to transfer sperm to females during copulation. Development of embryos occurs internally and the young are born live as free-swimming juveniles. Interestingly, there is considerable variation in the degree to which embryos are provisioned with nutrients before fertilization (*lecithotrophy*) versus after fertilization through maternal transfer (*matrotrophy*) in these live-bearing species and even within closely related groups such as the genus *Poeciliopsis*. Matrotrophy involves development of a placental structure and can account for the bulk of nutrients contributing to embryonic growth in some poeciliid species. This variation in a complex reproductive trait across the Poeciliid fishes has made it a particularly interesting group for addressing the evolution of reproductive modes (see Pollux *et al.*, 2009).

Socially-Controlled Sex Change

The remainder of this article focuses on sex change in adult fishes. Many species that undergo sex change also exhibit alternate male phenotypes and these are fascinating reproductive adaptations, but a full treatment is beyond the scope of this article.

Sex change provides a useful natural experiment for understanding basic processes of sex determination and differentiation because key developmental events take place in manipulable adult animals. However, understanding sex change is also important from a practical perspective as many commercially important fish change sex. The adaptive significance, or 'why?', of sex change is generally better understood than the developmental and physiological mechanisms that underlie this process (i.e., 'how?'). When individuals can reproduce more effectively as one sex when small or young and as the other sex when larger or older, there is an obvious benefit from being able to change sex to maximize reproductive success for their body size. This is known as the 'Size Advantage Model' of sex change and it has been quite successful in explaining patterns of sex change in many species. Other models include the Low Density Model and high dispersal models, in which sex change offers an advantage to species in which the opportunity to encounter an individual of the opposite sex is rare.

While the Size Advantage model and other models offers a compelling explanation for the presence of sex change in many species of fishes where this pattern is observed, it is equally striking that many species exhibit similar patterns of reproductive success across body size (especially mating advantages for large males) and would be predicted to exhibit sex change according to the model, yet do not. This is true both within the fishes and especially for other vertebrate groups where sexual lability is both much rarer and restricted to early developmental periods if it occurs. What accounts for this variation?

In mammals, the sex of an individual is determined by sex chromosomes at fertilization and specifically the presence or absence of the gene SRY (for 'Sex determining Region of the Y chromosome'). The testis or ovaries then arise relatively early in development and their hormonal secretions (particularly testicular androgens) organize other tissues including the brain. By contrast, the gonads develop relatively late in fishes as compared with other tissues and often well after individuals have spent considerable time as independently living 'miniature adults'. Why does this matter in terms of environmental control of sex determination?

When the gonads develop very early, the brain is neither capable nor has the opportunity to gather and interpret environmental information that might be used to adaptively guide sex determination. By contrast, fishes often have ample opportunity to collect such information prior to gonadal development and, in the case of social influences, possess a well-developed brain to interpret it.

Socially controlled sex change was first described and is best characterized in tropical marine fishes associated with coral reefs. This is because sex change is relatively common in four families that are conspicuous and important parts of the fish community on reefs. These families are the wrasses (Labridae), parrotfishes (Scaridae), damselfishes (Pomacentridae including the anemonefishes, subfamily Amphiprioninae), and the gobies (Gobiidae). Socially controlled sex change is also found in some temperate species where studies have focused on wrasses and gobies.

Male-to-female adult sex change is termed protandry and is seen in several families of fishes. Socially controlled protandry is best described in the anemonefishes, an Indo-Pacific group of 26 species (25 in *Amphiprion* and one species of *Premnas*). The anemonefishes have been an object of fascination due to the symbiotic relationship they show with large tropical sea anemones and as stars in the movie *Finding Nemo* that featured Nemo, a 'boy' anemonefish, being raised by his single father Marvin. However, had the movie been biologically accurate, Nemo's father would have likely changed sex to become female following the disappearance of Nemo's mother and Nemo would have been like other anemonefishes, developing as an immature female who would likely have matured into a functional male to form a breeding pair in the social group (Fig. 1). This pattern of a breeding pair and up to several immature females forming social groups has now been described for several anemonefish species. The gonadal structure of males consists of peripheral active spermatogenic tissues surrounding immature ovarian tissue (oocytes in the previtellogenic stage of development, Fig. 2). This pattern is interesting because it illustrates the fundamentally 'female first' pattern

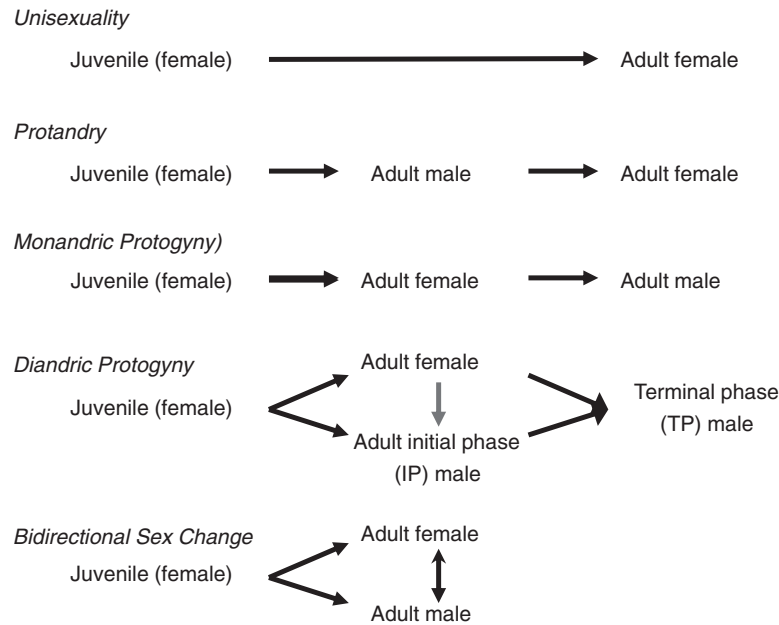


Fig. 1 Sexual patterns in fishes.

of development in sex changing fishes even when the adult sex change pattern is male-to-female (we can only speculate as to why the movie makers decided not to have Nemo's father change sex!).

The maturation of juveniles and sex change by breeding males appears to be prevented by aggressive dominance of large females in anemonefish. In the cinnamon anemonefish *Amphiprion melanopus*, dominant females rapidly and aggressively approach their male pair mates approximately 150 times a day. Disappearance or experimental removal of these females eliminates this inhibition and both the behavior and gonadal structure of the male begins to change rapidly. Male cinnamon anemonefish made experimentally dominant show significant increases in aggressive behavior within one day of the removal of their dominant female pair mates and extensive changes in gonadal structure with proliferation of oogonial like cells by ten days later. Replacement of spermatogenic tissue with ovarian tissue is complete by 20 days into the process.

The most common form of socially mediated sexual plasticity seen in reef fishes is female-to-male sex change or protogyny. Protogyny can take two forms. In monandric ('one male') protogyny, all individuals mature as females and only later potentially change to become males. In diandric protogyny, individuals develop initially as immature females, but may mature into the 'initial phase' (IP) as either males or females and either of these phenotypes can then undergo change to the typically brightly colored terminal phase males later (Figs. 1 and 3). Monandric protogyny occurs in some damselfishes (Pomacentridae) and is common in gobies and groupers with both monandric and diandric protogyny occurring in wrasses and parrotfishes. As predicted by the Size Advantage model discussed earlier, the adaptive significance of both monandric and diandric protogyny appears to be the high levels of reproductive success that accrue to large males who can monopolize access to females or spawning territories.

Perhaps the most dramatic examples of sexual plasticity are seen in gobies and some serranid fishes. A number of serranid species are simultaneous hermaphrodites, maintaining both mature ovarian and testicular tissue in the same gonad. The hamlets (genus *Hypoplectrus*) are native to the Caribbean Sea and alternate female and male behavior and spawning on a second-to-second time scale in a behavior termed 'egg trading'. Other species of small serranid sea basses maintain gonads with mature tissue of both sexes and may lose the ovarian tissue when they become socially dominant members of social groups able to monopolize mating.

A number of goby species are protogynous as discussed above for other families. However, gobies also show an additional degree of flexibility in species that exhibit bi-directional sex change. Species of *Gobiodon* and *Paragobiodon* are obligate residents of branching corals in the west Pacific that can change sex in either direction. This flexibility allows any two individuals to form a mating pair on a coral, likely a strong advantage for a very small-bodied species in which traveling large reef areas in search of mates could be very dangerous.

Steroid Hormone Patterns in Sex Changing Fishes

The effects of androgens in a sex changing species were examined as early as 1962 in the wrasse *Coris julis*, but measurements of circulating levels of sex steroid hormones had to wait for development of a technique termed 'radioimmunoassay' (RIA). Early studies using RIA showed that plasma levels of the primary estrogen in fishes (and mammals) 17-beta-estradiol (E_2) were elevated in female saddleback wrasses and declined dramatically at the onset of sex change at the same time that oocytes in the gonad degenerated (Fig. 4). Levels of E_2 remained very low across sex change and in terminal phase (TP) males. By contrast, a key

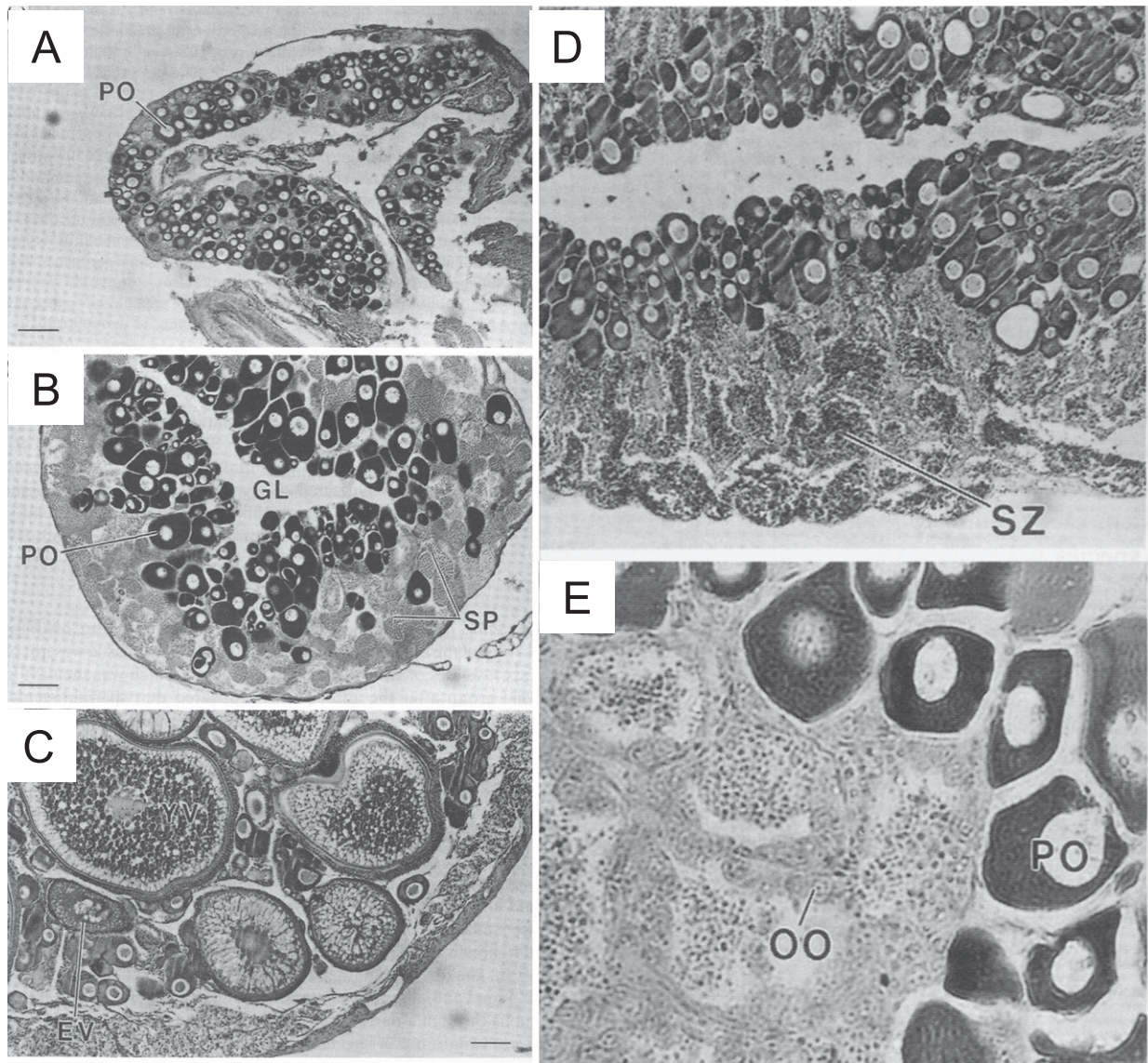


Fig. 2 Gonad structure in the anemonefish *Amphiprion melanopus* for juvenile non-breeders (A), breeding males (B), breeding females (C), and a sex changing fish ten days after removal of the dominant female (D) – low magnification, (E) – higher magnification. OO, presumptive oogonia appearing as ovary develops during sex change; PO, previtellogenic oocyte; SP, spermatogenic tissue (multiple stages); SZ, spermatozoa. Micrographs originally appeared in Godwin, J., 1994. Histological aspects of protandrous sex-change in the anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *Journal of Zoology* 232. 199–213.

androgen in fishes 11-ketotestosterone (11KT) was present at low levels in females and across sex change, only becoming elevated in TP males. Importantly, significant increases over female 11KT levels appeared to follow rather than precede or accompany the appearance of mature spermatogenic tissues. Similar sex steroid hormone patterns are seen in the stoplight parrotfish (*Sparisoma viride*) and several other sex changing species that have been examined, including goby and grouper species. In an interesting twist, the anemonefish *Amphiprion melanopus* also shows higher plasma E_2 levels in females and higher 11KT levels in males despite the direction of sex change being male-to-female and females being the larger and aggressively dominant sex (Fig. 5). A study in the protogynous bluebanded goby *Lythrypnus dalli* show a positive correlation between androgen levels and aggression in dominant females, but only when the male was removed from the social group, indicating that androgens may be responsible for inducing behavioral changes associated with female to male sex change.

The role of steroid hormones in the control of sex change is supported by many experiments manipulating either steroid hormones directly or their synthesis. Androgen administration is effective for inducing male sex determination in a wide range of fishes and this approach is widely used in aquaculture to produce faster-growing males. Beginning in 1955 with bluehead wrasses, androgen administration has also been used to successfully induce sex change in a number of sex-changing species. These include a number of wrasses, the stoplight parrotfish *S. viride*, the blackeye goby *Coryphopterus nicholsi*, and several species of groupers.



Fig. 3 Initial phase (top) and terminal phase males (bottom) of the bluehead wrasse, *Thalassoma bifasciatum*.

Blocking estrogen synthesis has similar effects to androgen administration in inducing female-to-male sex change. A key regulatory step in estrogen synthesis is at the enzyme aromatase, a member of the cytochrome P450 family of proteins. Fishes express two forms of this enzyme, known as gonadal and brain aromatase. Aromatase and estrogens generally appear to play key roles in sex determination in a range of species. Manipulations of estrogen signaling and aromatase activity are effective in manipulating the occurrence of sex change in several wrasses and grouper species as well as two goby species. For example, inhibiting aromatase action in the blackeye goby *C. nicholsi* induces sex change as effectively as 11KT implants. A recent experiment successfully induced sex change in the protogynous greasy grouper, *Epinephelus tauvina*, through administration of 17-methyl testosterone and an aromatase inhibitor and studies have indicated that gonadotropins modulate the androgen increase in that triggers sex change in the dusky grouper, *Epinephelus marginatus*.

The bi-directionally sex changing gobies in the genus *Gobiodon* are especially interesting in this respect. As noted above, these gobies can change sex in either direction to form heterosexual pairs on the corals where they reside. Implants of the aromatase inhibitor fadrozole induce females to become males and males to remain male even when paired with a larger male. Conversely, E_2 implants induce sex change in males paired with other males while E_2 -implanted females paired with other females did not change sex. Also consistent with a dominant role for estrogenic signaling in regulating sex change was a study where co-administering E_2 with androgens in threespot wrasses blocked the female-to-male sex change that would otherwise occur. In bluehead wrasses, E_2 implants prevented behavioral sex change in large females even when removal of more dominant fish created a socially permissive environment that normally promotes sex change. Taken together, these studies suggest estrogenic inhibition may be the critical controlling factor for gonadal sex change.

The environmental cues for socially controlled sex change must clearly first be perceived in the brain. Consistent with this expectation, the more rapid decreases in aromatase activity seen in the brain compared to the ovaries of sex changing *Lythrypnus dalli* gobies suggest a key early event in the sex change process is reduced estrogen signaling to neural structures.

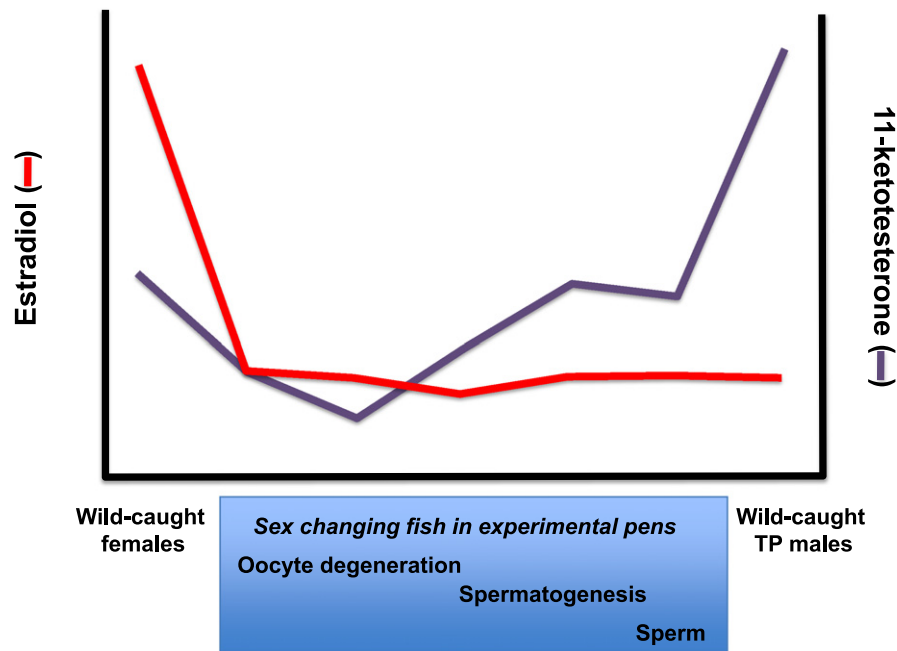


Fig. 4 Relative steroid hormone levels in plasma of saddleback wrasses shown for natural females and TP males as well as females undergoing socially-induced sex change in experimental pens. Redrawn from Nakamura, M., 2005. Sex change in coral reef fish. *Fish Physiology and Biochemistry* 31, 117.

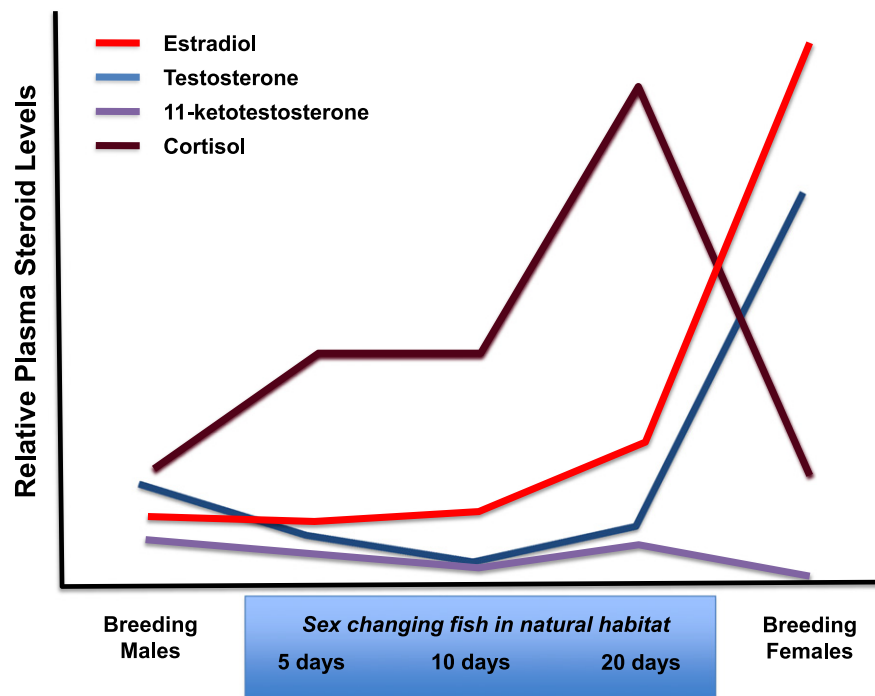


Fig. 5 Relative steroid hormone levels in plasma of the anemonefish *Amphiprion melanopus* in natural males and females and as males underwent protandrous sex change following experimental female removals from social groups. Relative levels of different hormones are depicted approximately to scale except for cortisol, where levels were substantially higher. Redrawn from Godwin, J.R., Thomas, P., 1993. Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *General and Comparative Endocrinology* 91(2), 144–157.

Other Steroid Hormones and Sex Change

Because the social regulation of sex change is often closely related to aggressive behavioral dominance, there has also been interest in the potential role of stress hormones. The key stress-related steroid in fishes is cortisol, as in humans, which is synthesized in the interrenal gland. Subordinate social status is quite often characterized by elevated cortisol levels in fishes and other species and elevated cortisol levels can also inhibit reproductive function. However, newly dominant and sex-changing anemonefish (*A. melanopus*) and bluebanded gobies actually show increases in cortisol levels during the sex change process. This led Solomon-Lane and colleagues to propose glucocorticoid signaling plays an important role in this process as with other examples of developmental transitions in vertebrates. Interestingly and consistent with this suggestion, prolonged administration of cortisol to female three-spot wrasses induced sex change, potentially by causing a decrease in the estrogen level that leads to sex change. However, stress effects can be exquisitely time sensitive and animals show physiological adaptation to chronic stressors in sometimes subtle ways, so more work remains to be done regarding the potential role of the endocrine stress axis in mediating sex change.

Neural Mechanisms of Sex Change

It has become clear that the often dramatic changes in behavior that accompany sex change are key parts of the change in sexual phenotype and may play an important role in stimulating the gonadal sex change process. This appreciation of the role of behavioral change as well as development of new techniques have shifted much of the focus in recent studies of sex change to neural and neuroendocrine mechanisms that may translate changes in social environment to changes in the gonads and other parts of the sexual phenotype.

The hypothalamus serves as a critical integrative center for both the reproductive axis and sexual behavior and is the site of gonadotropin-releasing hormone (GnRH) neurons which project to the pituitary gland. This brain region has therefore been a logical starting point for investigations of the neural mediation of sex change. Both correlative and experimental approaches support a role for the GnRH system in the sex change process. Working with bluehead wrasses in the early 1990s, Matthew Grober and colleagues described greater numbers of immunoreactive GnRH neurons in the hypothalamus of TP males than in that of females and that these numbers were increased in females by 11KT treatment. This pattern of greater numbers of GnRH neurons in males than females is also seen in the Ballan wrasse (*Labrus berggylta*) and in the protandrous anemonefish *Amphiprion bicinctus* (despite females being larger and aggressively dominant in anemonefishes). Expression of GnRH has not been closely monitored through the sex change process in any species, but findings from the cichlid *Astatotilapia burtoni* suggest this would likely be informative. This species shows socially regulated sexual development that involves rapid activation of preoptic area GnRH neurons when changes in social conditions are perceived. This rapid activation of GnRH neurons underlies the rapid activation of the HPG axis when subordinate males ascend to social dominance.

What mechanisms might induce a change in the activity of GnRH neurons? Changes in neural estrogen signaling due to reductions in brain aromatase activity at the onset of sex change and a potential role for stress hormones were discussed above. Another intriguing possibility is the monoamine neurotransmitters – serotonin, noradrenaline, and dopamine which vary in key forebrain areas across sex change in the saddleback wrasse with manipulative experiments further supporting this role. The neuropeptide hormone arginine vasotocin may also be important as studies in the grouper *Epinephelus adscensionis* revealed co-localization of AVT and GnRH in the preoptic area of the hypothalamus. Further studies have indicated that a related hormone, gonadotropin inhibitory hormone (GnIH) may also play an important role in the regulation of sex reversal, potentially by affecting GnRH signaling.

Before leaving the discussion of neural mediation of gonadal sex change, we discuss a study suggesting that variation in target tissue responsiveness to gonadotropins regulates sex change. The bidirectionally sex changing goby *Trimma okinawae* expresses both ovarian and testicular tissues, but with only one type being mature and active at a given time. Kobayashi and colleagues found that the active portion of the gonad expressed gonadotropin receptors at much higher levels than the inactive portion and that this higher expression was associated with greater responsiveness to gonadotropin. Exposure to visual cues that induce a change to the opposite sex also reversed the pattern of gonadotropin receptor expression within approximately twelve hours.

Neural Bases of Behavioral Sex Change

Successful sex change requires coordinated changes in a suite of phenotypic features. Behavioral sex change is often very rapid, occurring within one to several days in anemonefishes and blue banded gobies and within minutes of dominant male removal with cleaner and bluehead wrasses studied in nature. This short time scale suggests gonadal change does not drive behavioral change. Indeed, the reverse may be true. The role of the gonads, neural mediators of behavioral sex change, and potential connections between the behavioral and gonadal sex change are discussed in this section.

Surgically removing the gonads from female bluehead wrasses does not prevent behavioral sex change when those females are made socially dominant. While the gonads are not necessary for the behavioral components of sex change in this species, ovariectomized females do not develop TP male coloration on becoming dominant. This is presumably because they lack a source of 11KT, which is critical for male morphological secondary sexual characters. This lack of necessity for gonads in behavioral sex

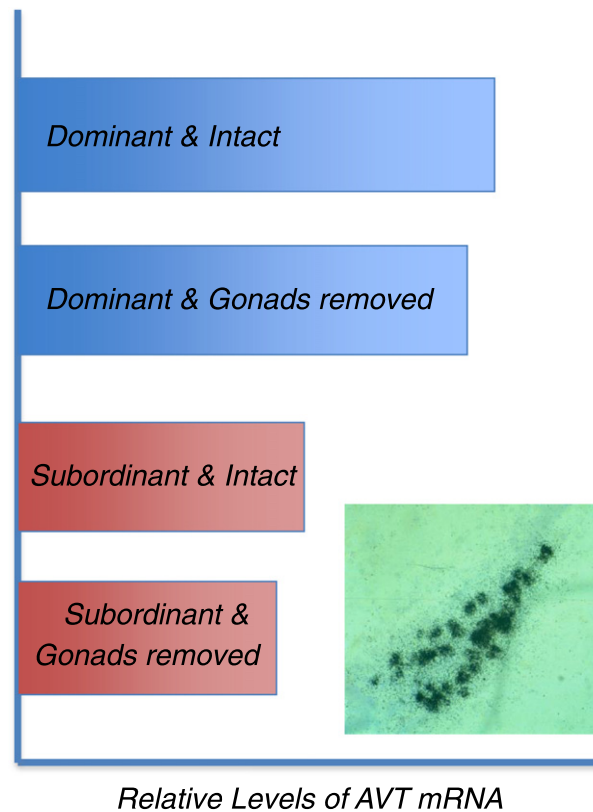


Fig. 6 Social status influences AVT mRNA levels in the preoptic area of the hypothalamus in bluehead wrasses while gonadal status does not. Dominant individuals were the largest in their social groups while subordinates were not. Intact animals underwent 'sham' operations to control for the surgical procedure. Inset shows AVT mRNA in the preoptic area visualized by the technique of in situ hybridization. Data from Semsar, K., Godwin, J., 2003. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. *Journal of Neuroscience* 23, 4386–4393.

change is generally consistent with often very rapid time course of this process and highlights the fact that important changes in steroid hormone signaling do not need to occur in the gonad itself.

What neural mechanisms do mediate behavioral changes during the sex change process? Two areas of focus have been monoamine neurotransmitters and neuropeptide hormones. Changes in monoamines were mentioned above and in saddleback wrasses (*T. duperrey*) changes are especially pronounced during the first week of sex change when social dominance for sex changing females is being established. During the first week in experimental groups, dominance interactions develop and accompanied by a number of alterations in monoaminergic signaling, although the patterns are complex across brain nuclei. In bluehead wrasses, serotonergic manipulations can decrease aggression in both laboratory and wild TP males, but no studies have closely examined the behavioral effects of manipulating monoamines during the sex change process in wrasses.

A second focus for studies of the neural mechanisms mediating behavioral sex change has been on neuropeptide hormones, especially arginine vasotocin (AVT). The first study to show differences in AVT neurons across sexual phenotypes in a sex changing fish was in the goby *T. okinawae*. In bluehead wrasse, TP males have significantly higher abundances of AVT mRNA in the preoptic area of the hypothalamus compared with females and these levels increase rapidly during experimentally induced sex change. Both the gonads and social interactions are changing as a female wrasse undergoes sex change; therefore, to tease these two factors apart, levels of AVT neurons in females changing sex with and without gonads and females that remain as subordinate females were compared. Social status affected AVT mRNA abundances, while gonadal status did not (Fig. 6). Behavioral effects of AVT also appear to depend on sexual phenotype. Neither courtship nor territorial behavior could be induced by AVT injections in females or female-mimic IP males. However, injection of AVT increases courtship in TP males and induces non-territorial TP males to establish territories. The only data available regarding neuron patterns in isotocin, a peptide closely related to oxytocin in mammals, for sex-changing species comes from blue banded gobies, where more isotocin neurons were found in females than males. Further exploration of isotocin actions in sex changing fishes would be valuable.

Summary

Our understanding of the physiological basis of sex change has increased rapidly in recent years. This is due to both technical advances in endocrine techniques and especially molecular biology as well as the identification and exploitation of powerful

model systems such as sex changing wrasses and bi-directionally sex changing gobies. Estrogen synthesis through the aromatase enzyme and the actions of the neuropeptides GnRH and AVT appear especially important in regulating sex change, but other physiological mediators are doubtless important contributors to the process. New developments such as RNA sequencing (RNAseq) are promising for future investigations of sex change. Advances in genomic methods have made it possible to employ these methods in non-model species and RNAseq has now been used to characterize changes in neural gene expression in the protandrous anemonefish *Amphiprion bicinctus* and the protogynous bluehead wrasse. The discovery of these mechanisms and development of synthetic models of the mechanistic basis of sex change from the perception of changed social environment to gonadal change should be the next stage in study of this.

Reference

Pollux, B.J.A., Pires, M.N., Banet, A.I., Reznick, D.N., 2009. The evolution of placentas in the fish family Poeciliidae – An empirical study of macroevolution. *Annual Review of Ecology, Evolution and Systematics* 40, 271–289.

Further Reading

- Black, M.P., Balthazart, J., Baillien, M., Grober, M.S., 2005. Socially induced and rapid increases in aggression are inversely related to brain aromatase activity in a sex-changing fish, *Lythrypnus dalli*. *Proceedings of the Royal Society B – Biological Sciences* 272, 2435–2440.
- Casas, L., Saborido-Rey, F., Taewoo, R., *et al.*, 2016. Sex change in clownfish: Molecular insights from transcriptome analysis. *Scientific Reports* 6, 35461. doi:10.1038/srep35461.
- de Mitcheson, Y.S., Liu, M., 2008. Functional hermaphroditism in teleosts. *Fish and Fisheries* 9 (1), 1–43.
- Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* 208 (3–4), 191–364.
- Fishelson, L., 1970. Protogynous sex reversal in fish *Anthias squamipinnis* (Teleostei, Anthiidae) regulated by presence or absence of a male fish. *Nature* 227, 90–91.
- Francis, R.C., 1992. Sexual lability in teleosts – Developmental factors. *Quarterly Review of Biology* 67 (1), 1–18.
- Ghiselin, M.T., 1969. The evolution of hermaphroditism among animals. *Quarterly Review of Biology* 44, 189–208.
- Godwin, J., 1994. Histological aspects of protandrous sex-change in the anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *Journal of Zoology* 232, 199–213.
- Godwin, J.R., Thomas, P., 1993. Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *General and Comparative Endocrinology* 91 (2), 144–157.
- Kline, R.J., Holt, G.J., Khan, I.A., 2016. Arginine vasotocin V1a2 receptor and GnRH-I co-localize in preoptic neurons of the sex changing grouper, *Epinephelus adscensionis*. *General and Comparative Endocrinology* 225 (2016), 33–44.
- Kroon, F.J., Liley, N.R., 2000. The role of steroid hormones in protogynous sex change in the blackeye goby, *Coryphopterus nicholsii* (Teleostei: Gobiidae). *General and Comparative Endocrinology* 118 (2), 273–283.
- Kroon, F.J., Munday, P.L., Westcott, D.A., Hobbs, J.P.A., Liley, N.R., 2005. Aromatase pathway mediates sex change in each direction. *Proceedings of the Royal Society B – Biological Sciences* 272 (1570), 1399–1405.
- Larson, E.T., Norris, D.O., Grau, E.G., Summers, C.H., 2003. Monoamines stimulate sex reversal in the saddleback wrasse. *General and Comparative Endocrinology* 130 (3), 289–298.
- Nakamura, M., Hourigan, T.F., Yamauchi, K., Nagahama, Y., Grau, E.G., 1989. Histological and ultrastructural evidence for the role of gonadal steroid hormones in sex change in the protogynous wrasse *Thalassoma duperrey*. *Environmental Biology of Fishes* 24, 117–136.
- Nakamura, M., 2005. Sex change in coral reef fish. *Fish Physiology and Biochemistry* 31, 117.
- Nozu, R., Nakamura, M., 2015. Cortisol administration induces sex change from ovary to testis in the protogynous Wrasse, *Halichoeres trimaculatus*. *Sexual Development* 9 (2), 118–124. doi:10.1159/000373902.
- Pollux, B.J.A., Pires, M.N., Banet, A.I., Reznick, D.N., 2009. The evolution of placentas in the fish family Poeciliidae – An empirical study of macroevolution. *Annual Review of Ecology, Evolution and Systematics* 40, 271–289.
- Robertson, D.R., 1972. Social control of sex reversal in a coral-reef fish. *Science* 177, 1007–1009.
- Semsar, K., Godwin, J., 2003. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. *Journal of Neuroscience* 23, 4386–4393.
- Solomon-Lane, T.K., Crespi, E.J., Grober, M.S., 2013. Stress and serial adult metamorphosis: Multiple roles for the stress axis in socially regulated sex change. *Frontiers in Neuroscience* 7, 210. doi:10.3389/fnins.2013.00210.
- Stoll, L.M., 1955. Hormonal control of the sexually dimorphic pigmentation of *Thalassoma bifasciatum*. *Zoologica* 40, 125–131.
- Warner, R.R., 1984. Mating behavior and hermaphroditism in coral-reef fishes. *American Scientist* 72, 128–136.

Mode of Reproduction: Invertebrate Animals

Thanumalaya Subramoniam, Sathyabama University, Chennai, India

© 2018 Elsevier Inc. All rights reserved.

Introduction

Invertebrates represent the animal assemblage that lack true vertebral column. They are the most numerous kinds of living animals, having marvelled the vertebrates by their sheer diversity of form and bodily organization. Whereas the basal metazoans such as the sponges, cnidarians and flatworms are simple in organization, other invertebrate phyla, like Arthropoda, Mollusca, and Echinodermata are much more complex, usually enveloped by armour-plated exoskeletons. Most invertebrates originated and diversified in the sea, before some of them migrated to freshwater and then, species like insects, invaded terrestrial environments. Their success in the colonization of every conceivable ecological niche both in water and on land is obviously attributed to their various reproductive modes, ranging from the primitive vegetative binary fission to evolutionarily advanced sexual reproduction.

Furthermore, this diversity in reproductive strategies could be correlated to the modes of development and life history patterns, together with their ecological adaptiveness. In many invertebrates, the development is indirect with the interception of a larva, which undergoes metamorphosis to reach the adult stage. Understandably, reproductive processes are inclusive of embryonic development and larval metamorphosis in these forms. In marine invertebrates, the larvae are planktonic, playing a major role in the dispersion of species, as the adults are normally sedentary, or sessile with limited mobility. Different types of larval forms in marine invertebrates are listed in Table 1. In terrestrial and freshwater environments, however, pelagic larval development is suppressed by virtue of the production of energy-rich yolky eggs, internal fertilization and sperm storage facility in the female. Reproduction in protists is excluded in this article, as it involves mainly mitotic division of the whole body, thus differing vastly from metazoan reproduction.

Asexual Reproduction

Although sex evolved early in animal evolution, agametic cloning from somatic tissue by asexual reproductive modes is prevalent in most of the soft-bodied invertebrates such as sponges, cnidarians, flatworms, annelids, and some echinoderms, as well as urochordates, a close relative of vertebrates. In sedentary corals, asexual reproduction is the clonal propagation in which an

Table 1 Larval forms of marine invertebrates

<i>Phylum</i>	<i>Class</i>	<i>Larval forms</i>
Porifera		Parenchymula, Amphiblastula
Cnidaria	Hydrozoa	Planula, Actinula
	Scyphozoa	Planula, Actinula
	Anthozoa	Planula
Ctenophora	Tentaculata	Cydidippid
	Nuda	Cydidippid
Platyhelminthes	Turbellaria	Muller
	Cestoda	Miracidium, Cercaria
	Trematoda	Miracidium, Cercaria
	Monogenea	Onchomiracidium
Nemertea		Pilidium
Annelida	Polychaeta	Trochophore, Nectochaeta
Mollusca	Gastropoda	Trochophore
	Bivalvia	Trochophore
Lophophorates	Phoronida	Actinula
	Bryozoa	Cyphonautes
Crustacea	Copepoda	Nauplius
	Cirripedia	Nauplius, Cypris
	Malacostraca	Zoea, Megalopa (Crab); Phyllosoma, Puerulus (Lobster); Nauplius, Meta nauplius, Mysis (Shrimp)
Echinodermata	Echinoidea	Pluteus
	Asteroidea	Dipleurula, Bipinnaria, Brachiolaria
	Ophiuroidea	Ophiopluteus
	Holothuroidea	Doliolaria
Hemichordata	Enteropneusta	Tornaria
Urochordata	Tunicata	Tadpole

Table 2 Taxonomic distribution of asexual reproduction in invertebrates

	Phylum	Class	Order	Species
1. Agametic cloning				
1a. Budding	Placozoa	Trichoplacoidea	Trichoplacida	<i>Trichoplax adhaerens</i>
	Cnidaria	Anthozoa	Scleractinia	<i>Acropora palmata</i>
	Ctenophora	Tentaculata	Lobata	<i>Bolinopsis infundibulum</i>
	Xenacoelomorpha	Acoela		<i>Convolutriloba macropyga</i> <i>Convolutriloba retrogemma</i>
	Bryozoa	Phylactolaemata		<i>Lophopus crystallinus</i>
1b. Fission	Placozoa	Trichoplacoidea	Trichoplacida	<i>Trichoplax adhaerens</i>
1b. i. Binary fission (Simple)	Echinodermata	Asteroidea	Forcipulatida	<i>Allostichaster polyplex</i>
	Cnidaria	Anthozoa	Actiniaria	<i>Haliplanella lineata</i>
1b. ii. Transverse fission	Xenacoelomorpha	Acoela		<i>Convolutriloba hastifera</i> <i>Convolutriloba longifissura</i>
	Platyhelminthes	Rhabditophora		<i>Girardia tigrina</i>
1b. iii. Longitudinal fission	Annelida	Oligochaeta	Tubificida	<i>Paranais litoralis</i>
	Acoelomorpha	Acoela		<i>Paratomella unichaeta</i>
	Platyhelminthes	Rhabditophora	Tubificida	<i>Planaria fissipara</i>
1c. Fragmentation	Ctenophora	Tentaculata	Lobata	<i>Mnemiopsis leidyi</i>
	Cnidaria	Anthozoa	Actiniaria	<i>Metridium senile</i>
	Nemertea	Anopla	Heteronemertea	<i>Lineus longissimus</i>
	Xenacoelomorpha	Acoela		<i>Convolutriloba retrogemma</i>
	Hemichordata	Enteropneusta	Enteropneusta	<i>Balanoglossus capensi</i>
2. Parthenogenesis	Platyhelminthes	Trematoda	Echinostomia	<i>Philophthalmus megalurus</i>
	Rotifera	Monogononta	Ploimida	<i>Brachionus calyciflorus</i>
	Arthropoda	Crustacea	Cladocera	<i>Daphnia pulex</i>
	Arthropoda	Insecta	Hemiptera	<i>Myzus persicae</i>
			Hemiptera (Aphididea)	<i>Rhopalosiphum padi</i>

organism gives rise to the production of genetically identical replicates (ramets) by budding. Conversely, unitary animals are those that have a single body per genet (a genetic individual arising from one zygote by mitosis), which reproduce only sexually. Animals that reproduce asexually are highly regenerative, compared to obligate sexual reproducers.

In invertebrates, adult stem cells play a pivotal role in regeneration and asexual reproduction. Unlike vertebrate systems, invertebrate stem cells are not housed within a regulatory microenvironment (niche). Stem cells involved in agametic cloning in invertebrate taxa are of different kinds: choanocytes in sponges; interstitial cells in cnidarians, neoblast cells in planarians, and haemoblast stem cells in ascidians (Urochordata). Some metazoans like nematodes, rotifers, gastrotrichs and insects lack somatic stem cells and hence the inability to undergo agametic cloning. Different kinds of asexual reproduction occurring in various invertebrate animals are listed in [Table 2](#).

Gemmule Formation in Sponges

All sponges (phylum Porifera) can regenerate their bodies and reproduce asexually using totipotent stem cells. In addition, many freshwater sponges use choanocytes to develop dormant overwintering gemmules under unfavourable conditions. On return of favourable conditions, such multicellular propagules reactivate and regenerate genetically identical replicas of the original sponge.

Fission, Budding and Fragmentation

In the simplest condition, asexual reproduction can take place by subdivision of an existing body into two or more multicellular parts, followed by regeneration of the missing parts. Placozoans, the basal metazoan animals without distinct tissues or organs, reproduce exclusively by fission, whereby two parts of the animal move away from each other until their connection is ruptured ([Fig. 1](#)). In cnidarians, asexual reproduction is coupled with the sessile existence of the adult. In the freshwater hydrozoan, *Hydra*, reproduction occurs almost exclusively by budding, suppressing gamete formation ([Fig. 2](#)). In the calyx region, the parent tissue cells continuously move towards the bud-forming area and get incorporated into the growing bud. Evidently, reorientation and repolarization of parent tissue play a major role in bud formation. In addition, epithelial stem cells that mediate the morphogenetic plasticity of the tissue in regeneration may also have a similar role in asexual budding.

In *Hydra*, a new polyp bud eventually becomes a separate individual clone. However, in the coral relatives of *Hydra*, the clones do not break off, but stay attached and become a branch as in plants. Such branching colonies are also found in the bryozoan, *Membranipora*. In some anemones, the adults autotomise (self-mutilate) a tentacle, which regenerates in to a small individual. In the scyphozoan cnidarian *Aurelia*, the polypoid scyphistoma undergoes horizontal fission (strobilation) into a stack of juvenile



Fig. 1 Budding in *Hydra vulgaris*. Seen on the right side is a well grown bud and on the left, is early stage bud. Photo courtesy of Carolina Biological Supply Company.

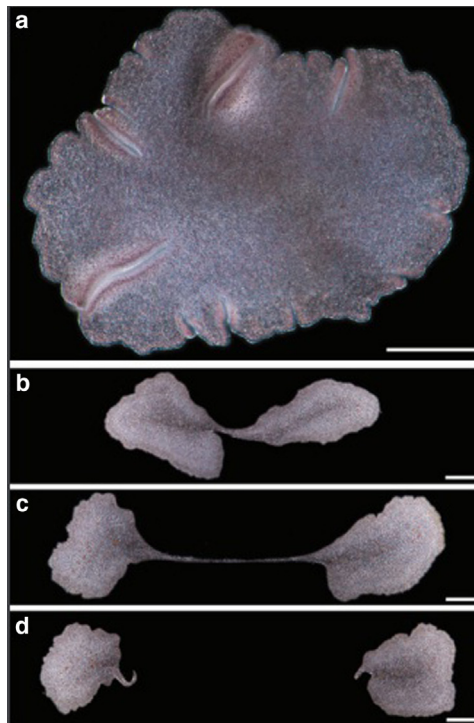


Fig. 2 Binary fission in the placozoan, *Trichoplax*. (a) Before fission, (b)–(d) *Trichoplax* progressing through asexual reproduction by fission. Reproduced from Srivastava, M., Begovic, E., Chapman, J., *et al.*, 2008. The *Trichoplax* genome and the nature of placozoans. *Nature* 454, 955–960.

medusas, called ephyra (**Fig. 3**). The ephyra transforms into male and female medusas, which produce planula larva by sexual reproduction. The planula then settles into a sedentary scyphistoma, thus continuing the life cycle. This is termed as alternation of generation or metagenesis. Whereas asexual reproduction produces genetically identical modules that may prolong the survival of the genotype, sexual reproduction enables genetic recombination and production of new coral genotypes to enhance fitness and survival of the species.

Scyphozoans also reproduce asexually by podocyst formation, under conditions of limited food supply. Podocysts are cysts produced beneath the pedal discs of polyps of scyphozoans. They excyst small polyps that develop into active scyphistomae, which are capable of producing further podocysts as well as medusas by strobilation.

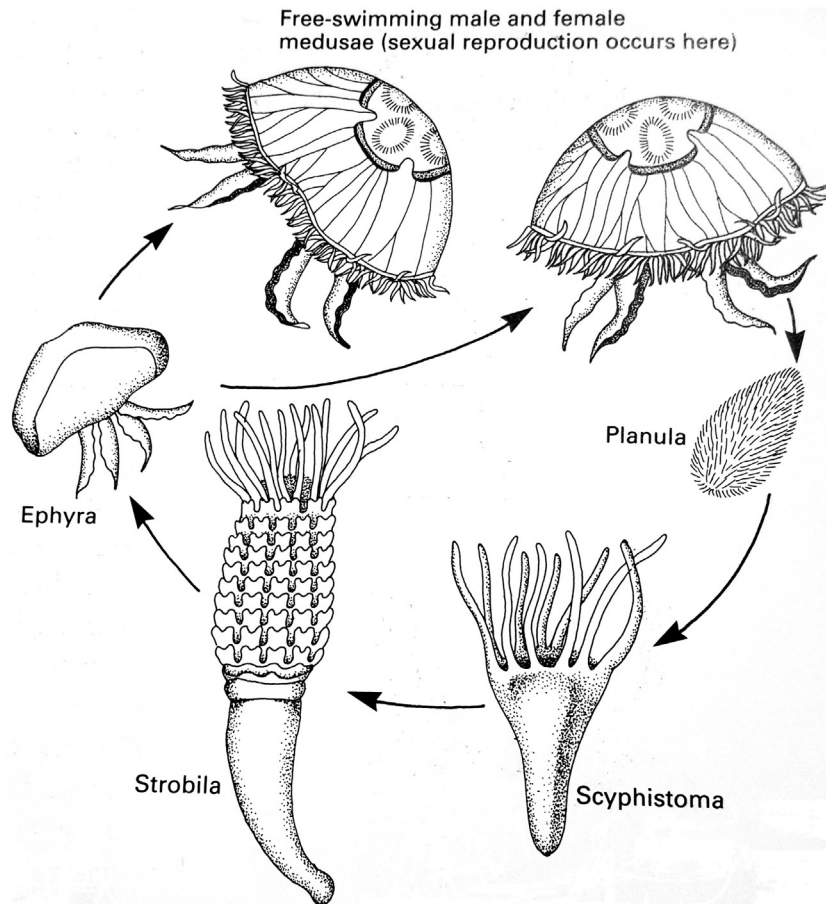


Fig. 3 Life cycle of *Aurelia* to demonstrate alternation of generation. Reproduced from Barnes, R.S.K., Calow, P., Olive, P.J.W, 1993. *The Invertebrates: A New Synthesis*, second ed. Oxford: Blackwell Science Ltd., p. 488.

In the azooxanthellate soft corals, fragmentation of the colony occurs by autotomy. New clonal colonies in the reef corals are also formed by colony fragmentation as a result of storm or wave impacts. In the anemone, longitudinal fission is the common mode of asexual reproduction, although transverse fission occurs in some forms.

Asexual reproduction in sea anemones also occurs by basal laceration, which involves regeneration from a small piece of tissue that typically includes all the three body layers. Different asexual modes of reproduction found in corals reflect the extraordinary ability of cnidarian cell lines to differentiate, dedifferentiate and redifferentiate, providing their tissues with remarkable developmental plasticity.

Budding is also common in flatworms (Platyhelminthes), which have excellent regenerative abilities. In the paratomic fission, new individuals differentiate in a chain-like fashion from a parent worm before separating from it, while in architomy, the body simultaneously fragments and only thereafter individuals differentiate from the pieces. The acoelous turbellarians release their progeny by budding from the posterior margin of the body. In the nemertean worm, belonging to genus *Lineus*, sexually immature adults undergo transverse fragmentation with subsequent regeneration. In the polychaete worm (Annelida), *Dodecaceria*, the adult worm fragments into several individual segments, each of which reconstitutes a new head and a tail by renewed segment proliferation.

Fissiparity and Budding in Echinoderms

The commonly occurring asexual mode of reproduction in sea stars and brittle stars is the division of the body across the disk, termed fissiparity. Each resulting part regenerates a complete individual which can split again. The population of the fissiparous sea star *Coscinasteria tenuispina* at Rio de Janeiro, Brazil, appears to be sustained only by fission, although these sea stars are capable of gamete production. Another asexual method is autotomy, by which a whole new animal is regenerated from a single arm or even part of an arm. Remarkably, fragmentation, fission or budding occur regularly in echinoderm larvae. Transverse fission has also been reported in the sea cucumber, *Holothuria edulis*.

Both exogenous and endogenous factors appear to be involved in regulating asexual and sexual reproduction in echinoderms. Whereas the asexual mode is related to small body size, sexual maturity is attained after an individual reaches a certain size.

Regeneration after fission is aided by existing pluripotent stem cells or de-differentiation of tissues into stem cells. Conversely, regeneration after autotomy, which takes place across predefined planes, involves the formation of an extensively proliferating blastema. However, both these regenerative processes are nerve-dependent and require neurotransmitters and neuropeptides, as growth factors.

Urochordates

The ascidian urochordates, sometimes called sea-squirts, are colonial marine invertebrates with remarkable budding capacity to form new entities from existing structures. Two types of stem cells (epithelial and blood-born) are employed in the budding of ascidians. Pallial budding (buds developed from thoracic body region) and stolonial budding (buds originating from distal tip of developing stolon) are derivatives of epithelial stem cells, whereas in vascular budding, the buds are produced by the totipotent haemoblast stem cells inside the vascular system.

Parthenogenesis

Parthenogenesis is the development of a new offspring from an unfertilized egg. Parthenogenetic lineages occur in many insect species, but are widespread among other invertebrate taxa (**Table 1**). It entails modification or absence of meiosis so that the eggs remain diploid and do not have to fuse with sperm to give rise to a diploid zygote. This kind of parthenogenesis is termed as apomictic, which produces genetically identical modules of the same genet (typical asexual reproduction). In contrast, automixis (meiotic parthenogenesis) restores diploidy by the fusion of the egg with the second polar body (e.g., free-living nematode, *Rhabditis*). Obviously, the resultant modules may not be the exact genetic replicates of the mother.

In insects, parthenogenesis may be thelytokous (female producing), arrhenotokous (male producing) or amphitokous (producing either sex). Haploid parthenogenesis is a special case in which the oocytes undergo regular meiotic division. If the eggs are fertilized, the offspring is a female, and if the eggs remain unfertilized, then parthenogenetic development results in a male offspring, which is haploid in its somatic tissues (e.g., the haplodiploid Hymenopteran insects). In social insects like the subterranean termite, *Reticuliter messperatus*, the queens produce new queens asexually by thelytokous parthenogenesis, but produce other colony members (workers and soldiers) by sexual reproduction. The parthenogenetic production of these new queens is achieved by the closing of the egg's micropyle (sperm gates) to prevent sperm entry. Yet another type, namely obligate parthenogenesis, occurs in bdelloid rotifers, in which sexual reproduction never takes place due to the lack of males in the population.

In the cladoceran rotifers and aphid insects, parthenogenesis occurs cyclically together with bouts of sexual reproduction. This is called cyclical parthenogenesis. In *Daphnia*, parthenogenetic reproduction takes place for one to several generations during favourable conditions, followed by sexual reproduction under unfavourable environments. The sexually produced long-lived dormant eggs hatch once favourable conditions return. By this alternation of generations, favourable environmental conditions can be exploited to increase the number of offspring by parthenogenetic reproduction, whereas the periodical appearance of one or more sexual generations will ensure genetic advantages such as increased heterosis, and re-assortment of genetic characters.

Sexual Reproduction

Sexual reproduction involves haploid gamete formation and their subsequent fusion (amphimixis), paving the way for genetic recombination in the newly formed genotypes to enhance fitness and survival. Over 99% of all invertebrates exhibit sexual reproduction at some stage in their lives. Sexuality has evolved early in basal metazoans like placozoans by the formation of gametic cells, even before the origin of a discrete gonad. At this level of sex cell origin, certain stem cell-like choanocytes (sponges) and interstitial cells (cnidarians) could give rise to or differentiate into both egg and sperm. In the flatworms, the gametes are loosely spread among the mesenchyme cells. Only in the coelomate invertebrates, true gonads are formed to give rise to sperm and eggs. Thus, in the majority of invertebrate animals, sexual reproduction is the main mode for the propagation of progeny.

Sexual Systems

Invertebrates have undergone dynamic species radiation, occupying diverse niches in both aquatic and terrestrial ecosystems. The correlated diversity in their morphological features and physiological adaptations has given origin to an array of sexual systems and reproductive modalities. Their highly variable patterns of reproduction have an underlying relationship with the manner in which sex determination and differentiation have taken place in different invertebrate taxa.

Although gonochorism (separate sex) is the principal mode of sexuality in invertebrates, alternative sexual systems also exist in the form of mixed-sex hermaphrodites (same sex). A hermaphrodite is an individual that produces functional male and female gametes in its lifetime. Approximately 65,000 species (about 6%) of known invertebrate animals are hermaphroditic with representation in nearly 50% of 85 invertebrate classes (**Table 3**). Unlike plants, hermaphroditism is considered to be a derived condition in invertebrate lineages. Using phylogenetic character mapping, it has been deduced that hermaphroditism is polyphyletic in invertebrate animals, having arisen from ancestral gonochorism on many separate occasions.

Hermaphroditism is rarely reported in insects, but crustaceans possess various categories of sexual systems (**Table 4**). Primitive groups of crustaceans such as notostracans and conchostracans show an extensive variation in their reproductive strategy that

Table 3 On the occurrence of hermaphroditism among invertebrate animals

Phylum	Lower taxon	Popular name	Hermaphroditism
Porifera		Sponges	Ubiquitous
Cnidaria	Hydrozoa	Hydras and hydroids	Rare
	Scyphozoa and Cubozoa	Jellyfish	Rare
	Anthozoa	Anemones and corals	Ubiquitous
Annelida	Polychaeta		Rare
	Pogonophora		Rare
	Gnathostomulida		Ubiquitous
	Oligochaeta	Earthworms, freshwater oligochaetes	Ubiquitous
	Hirudinea	Leeches	Ubiquitous
Arthropoda	Crustacea	Crabs, shrimps, crayfish, woodlice, copepods, barnacles	Rare
Tardigrada		Water bears	Rare
Sipunculida		Peanut worms	Rare
Ectoprocta		Bryozoans	Ubiquitous
Platyhelminthes	Cestoidea	Tapeworms	Ubiquitous
	Trematoda	Flukes	Ubiquitous
	Monogenea	Monogenean ectoparasites	Ubiquitous
	Turbellaria	Free-living flatworms	Ubiquitous
Nemertini		Ribbon worms	Rare
Chaetognatha		Arrow worms	Ubiquitous
Gastrotricha			Ubiquitous
Nematoda		Round worms	Present
Ctenophora		Sea walnuts, comb jellies	Ubiquitous
Phoronidea			Present
Brachiopoda		Lamp shells	Rare
Echinodermata		Starfish, brittle stars, sea urchins, sea cucumbers, sea lilies	Rare
Urochordata		Sea squirts, salps	Ubiquitous

Source: Modified from Michiels, N.K., 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. In: Birkhead, T.R., Møller, A.P. (Eds.), *Sperm Competition and Sexual Selection*. London: Academic Press, pp. 219–248 and Avise, J., 2011. *Hermaphroditism: A Primer on the Biology, Ecology, and Evolution of Dual Sexuality*. New York, NY: Columbia University Press.

may range from gonochorism to self-fertilizing hermaphroditism and parthenogenesis. The transition from gonochorism to hermaphroditism is also evident in the clam shrimp *Eulimnadia* (Conchostraca: Crustacea) by the occurrence of an extreme sexual type, androdioecy. Interestingly, the hermaphrodites of clam shrimp lack clasping appendages for mating and thus can only self-fertilize or mate with males. In malacostracan crustaceans, androgenic gland hormone controls sex differentiation and causation of different hermaphroditic conditions.

Among the various forms of mixed sex forms, simultaneous and sequential hermaphroditism is most common. Simultaneous hermaphrodite is an animal that produces ripe sperm and ova at the same time. Although self-fertilization (selfing) is possible in many simultaneous hermaphrodites, offering fertilization assurance, many invertebrates practise cross fertilization in order to gain genetic variability among the resultant offspring. The major genetic deterrent to selfing is however inbreeding depression. Therefore, many species of simultaneously hermaphroditic invertebrates have evolved genetic self-incompatible systems that inhibit self-fertilization. Sequential or successive hermaphroditism is another form of hermaphroditism, commonly found among invertebrates like corals, molluscs and crustaceans. Here, the individual produces both male and female gametes at different periods of its life. They are either male or female at a time with a brief transitional state, called 'intersexual' during the changeover phase.

Mating Systems

Mating brings together both male and female gametes, culminating in fertilization and production of a diploid zygote. In marine invertebrates, such as cnidarians, polychaetes, and echinoderms, broadcast spawning and external fertilization is the rule, favoured by the seawater medium that could support prolonged gamete survival, fertilization and early development. For example, in the reef-building corals that live in shallow waters of the tropical oceans, both gonochoristic and hermaphroditic species release the gametes into the open sea, where random mating (fusion of gametes) occurs. The fertilized eggs undergo development to release the planktonic planula larvae, which are dispersed to far-away locations for settlement. Both external fertilization and the possession of pelagic larvae in many marine invertebrates are considered to be a primitive pattern of reproduction, with a clear advantage of colonizing new habitats. Alternatively, internal fertilization, followed by brooding of the planula larvae within their polyps also takes place in gonochoristic and simultaneously hermaphroditic anthozoan corals. The brooded larvae released from the coral polyp search for a suitable site in the reef substratum for attachment and metamorphosis into a juvenile polyp.

Table 4 Different types of sexual systems present in crustaceans

Types of sexuality	Developmental sequence	Examples
Gonochorism	Juvenile $\bar{x}$ Primary male	Brachyuran crab – <i>Scylla serrata</i>
Androdioecy	Juvenile $\bar{x}$ Primary female	
	Juvenile $\bar{x}$ Primary male	Clam Shrimp – <i>Eulimnadia texana</i>
	Juvenile $\bar{x}$ Hermaphrodites	
Sequential hermaphroditism	Juvenile $\bar{x}$ Male phase $\bar{x}$ Female phase	Humpy shrimp – <i>Pandalus goniurus</i>
a. Simple protandry	Juvenile $\bar{x}$ Male phase $\bar{x}$ Female phase	Palaemonid shrimp – <i>Processa edulis</i>
b. Protandry (with primary females)	Juvenile $\bar{x}$ Primary female	
c. Protandry (with early maturing females)	Juvenile $\rightarrow$ Male phase $\rightarrow$ Female phase “early maturing” female phase	Pink shrimp – <i>Pandalus borealis</i>
d. Protogyny	Juvenile $\bar{x}$ Female phase $\bar{x}$ Male phase	Isopod – <i>Cyathura carinata</i>
Simultaneous hermaphroditism	Juvenile $\bar{x}$ Both functional male and female in the same individual	Mud shrimp – <i>Calocaris macandreae</i>
Protandric simultaneous hermaphroditism	Juvenile $\bar{x}$ Male phase $\bar{x}$ Simultaneous hermaphrodites	Monaco Shrimp – <i>Lysmata seticaudata</i>
Intersexuality		
a. Normal intersexuals (existing as a part of life cycle)	Juvenile $\rightarrow$ Male phase $\rightarrow$ Intersexuals (Transitory) $\downarrow$ Female phase (as in protandric hermaphroditism) Juvenile $\rightarrow$ Female phase $\rightarrow$ Intersexuals (Transitory) $\downarrow$ Male phase (as in the case of protogyny)	Crayfish – <i>Parastacus varicosus</i>
	Juvenile $\bar{x}$ Male phase $\bar{x}$ Intersexuals (due to incomplete feminization by parasites)	Isopod – <i>Cyathura carinata</i>
b. Epigenetic intersexuals (arising from epigenetic factors)	Juvenile $\bar{x}$ Female phase $\bar{x}$ Intersexuals (due to androgenic gland implants)	Amphipod – <i>Gammarus deubeni</i>
c. Sex intermediates	Juvenile $\bar{x}$ Sex intermediates (hormonal disruption by methyl farnesoate)	Blue crab – <i>Calinectes sapidus</i>
d. Gynandromorphism	Embryo $\bar{x}$ Bilateral gynandromorphism (due to the chromosomal mutation in the early embryonic stage)	Cladocera – <i>Daphnia pulex</i>
	Embryo $\bar{x}$ Bilateral gynandromorphism (due to chromosomal loss during early embryonic cleavage)	Brine shrimp – <i>Artemia franciscana</i>
		Lobster – <i>Homarus gammarus</i>

Source: Data based on Subramoniam, T., 2016. Sexual Biology and Reproduction in Crustaceans. London: Academic Press/Elsevier, p. 526.

Sperm Transfer Mechanisms

In terrestrial and freshwater invertebrates, release of unprotected sperm and egg into the medium is unsafe and hence fertilization must be internal, requiring direct pairing between male and female partners, often associated with complex copulatory behaviour. A feature associated with internal fertilization in invertebrate animals is the formation of sperm packets, called spermatophores, which prevent sperm loss during copulation. In the primitive insects and other cryptozoic arthropods, however, spermatophores seem to have evolved prior to the development of copulatory organs, as a means to transfer semen to effect indirect, contact-free transfer from male to female. In the more advanced insects, the spermatophores are replaced by liquid semen, transferred by well-formed penes. Spermatophores have also evolved independently in aquatic invertebrates such as crustaceans and mollusks in which they are deposited externally onto the female body or inserted into the seminal receptacles by way of copulatory organs.

Indirect sperm transfer has also been encountered in soil-dwelling polychaetes, semi terrestrial oligochaetes and leeches. In the hermaphroditic rhynchobdellid leeches, the tubular spermatophores are attached to the skin of their partners along with a hydrolytic secretion. The contraction of the spermatophore injects the sperm into the body cavity, where they reach the eggs by chemotaxis. Hypodermic insemination has also been reported in the onychophore, *Peripatus*, hermaphroditic sea slug, and some flatworms.

Mating Behaviour

Several mating behaviours have evolved in invertebrate animals in accordance with the modes of sperm transfer, the lifestyles and habitat conditions. Chemical communication by way of pheromones is also involved in the attraction of mates and the ensuing

courtship. Olfactory sex pheromones involved in the attraction of male moths over greater distances are well-known. Marine invertebrates release a water-soluble pheromone bouche to attract the opposite sex for mating and sperm transference. In the marine polychaetes, the swarming of epitokous (sexually mature) heteronereids is effected by the pheromone, inducing a nuptial dance and gamete release for external fertilization. Marine crustaceans such as the lobsters, crayfish and crabs, also use pheromones for communication and coordination of sexual activities.

Alternative Mating Strategy

Several studies have described variation in male mating behaviour among invertebrate animals. Any deviation from their common mating behaviour is considered as alternative mating strategy. In males, such deviant behaviours are associated with the assumption of reproductive phenotypes or morphotypes. Alternative mating strategy has apparently evolved in response to variation in the mating environment. The marine isopod *Paracerceis sculpta*, which breeds inside the spongocoel, has three genetically distinct male morphotypes, α male, β male, and γ male. The largest and dominant α male possesses elongated uropods and defends harems within the spongocoel. The smaller β males resemble females morphologically and invade the spongocoel by mimicking female behaviour. The γ male is tiny and adopts sneak mating tactics to gain access to females. The occurrence of dwarf males has been reported in many invertebrate species, in which they adopt several alternative mating strategies.

Types of Mating and Social Organization

Mating system refers to the procedures used in finding and securing a mate, the number of mates an individual acquires, the type of pair bonds, and the nature of parental care. The basic mating systems found in invertebrates are monogamy and polygamy. Although such mating behaviours have evolved in vertebrates to a high level, higher invertebrate groups like arthropods and molluscs practise them along with other behavioural traits such as parental care. Monogamy has been practised even among the hermaphroditic pair of a tube-dwelling polychaete worm, *Ophryotrocha diadema*, which lives together for several reproductive cycles. Mating events consist of one partner spawning eggs and the other fertilizing them. In this case of 'egg trading' both partners share brood care.

As in birds, social monogamy in certain coral-reef dwelling caridean shrimps has evolved into territorial cooperation and extended mate guarding by the male partner. In many crustaceans, mating occurs in the fresh moult stage when the female is receptive to males. Hence a new behavioural trait, namely pre-copulatory mate guarding by the males has evolved as a physiological necessity. Interestingly, in the sponge-dwelling snapping shrimp *Synalpheus*, social monogamy has given origin to advanced social systems, such as eusociality. Among other invertebrates, reproductive division of labour and cooperative care of young, resulting in eusocial condition has been well-defined only in social insects like honey bees and termite ants.

Unusual Reproductive Strategies

Polyembryony

Polyembryony, otherwise known as embryonic cloning, refers to the splitting of one sexually produced embryo into many offspring, which are genetically identical to each other but distinct from their parent(s), thus differing from asexual budding. Polyembryony occurs in a wide range of invertebrates, like cnidarians, bryozoans, insects and echinoderms (Table 5). In the cyclostome Bryozoa, cloning occurs by fission of blastula-stage embryo, with each blastomere becoming an individual offspring. In the classic example of the parasitic hymenopteran insects, the female wasp, *Copidosma*, oviposits one or two eggs inside the moth egg, which develops subsequently into a caterpillar larva. Inside the moth larva, the wasp's egg develops into a single compact mass of cells, called morula, which fragments into a large number of diminutive units that develop into two types of larvae. One type develops into a normal fertile adult, while the other type, called soldier larva, has a thin worm-like body, and becomes sterile.

Viviparity

In the great majority of invertebrate species, females lay eggs that develop and hatch in the external environment (oviparity). However, in some species females retain fertilized eggs inside the body and give birth to the young (viviparity). In the pulmonate terrestrial snails, viviparity exists in two forms: lecithotrophic viviparity (embryonic nutrition from egg yolk) and matrotrophic viviparity (nutrients supplied by maternal tissue). All species of scorpions are viviparous and the offspring are directly nourished by the mother during development. The young escape through the gonopore and crawl on to the mother's back for protection and parental care.

Insects are known to display various viviparous developments, but in a strange reproductive mode, termed hemocoelic viviparity, embryonic development occurs in the hemocoel of the mother, obtaining nutrients through their egg membrane by osmosis. In an interesting example, a parasitic female wasp injects her eggs into the ladybug. The eggs hatch and the larvae feed on the interior of the host insect. When the larva is ready to transform into a wasp, it comes out of the ladybug's abdomen and spins a cocoon, which is guarded by the ladybug from predators.

Table 5 Occurrence of polyembryony in invertebrates

Phylum	Class	Order	Species	
Cnidaria	Hydrozoa	Trachylina	<i>Pegantha</i> spp. <i>Cunina</i> spp.	
		Hydroida	<i>Cunioctantha</i> spp. <i>Polypodium hydriforme</i>	
Platyhelminthes	Monogenea	Gyrodactyloidea	<i>Gyrodactylus elegans</i>	
	Cestoidea	Eucestoda (subclass)	<i>Echinococcus</i> spp.	
Arthropoda	Trematoda	Digenea (subclass)	<i>Schistosoma</i> spp.	
	Cirripedia (Crustacea)	Rhizocephala	<i>Loxothylacus panopaei</i>	
	Insecta	Hymenoptera		
		– Encyrtidae		<i>Copidosoma</i> spp.
		– Platygastridae		<i>Platygaster</i> spp.
		– Braconidae		<i>Macrocentrus</i> spp.
– Dryinidae		<i>Macrocentrus</i> spp.		
Bryozoa	Stenolaemata	Strepsiptera	<i>Halictoxenos simplicis</i>	
		Cyclostomata	All species studied to date (e.g., <i>Crisia</i> spp.)	
Echinodermata	Asteroidea	Paxillosida	<i>Luidia</i> sp.	
	Ophiuroidea	Unidentified non-paxillosid		
		Ophiurida	<i>Ophiopluteus opulentus</i>	

Paedogenesis

In this reproductive mode, viviparity and neoteny (attainment of sexual maturity in larva) are combined. In the paedogenic insects, the ovaries of larvae become active and the eggs develop parthenogenetically. Paedogenesis often occurs in parasitoids, which live in the hemocoel of a host insect. Free-living insects (Homoptera) also exhibit paedogenesis. Here, the parthenogenetically developing larvae within the ovarioles of the mother contain embryos, so that a female aphid will contain her own grand-daughters.

Conclusion

The diversity in form and anatomical features of reproductive functions is truly reflected in the exhibition of numerous reproductive modes in invertebrate animals. The absence of phylogenetic relatedness in the occurrence of different reproductive strategies may imply their independent evolutionary origin among various invertebrate species. Nevertheless, the predominance of vegetative reproductive methods, ubiquitous or alternative to sexual propagation, furthermore indicates the co-evolution of both asexual and sexual reproductive processes in exploiting the environmental conditions. Another aspect of asexual reproduction in invertebrates is the occurrence and active participation of adult stem cells in agametic cloning of both colonial and unitary species such as the cnidarians. In *Hydra vulgaris*, induction of sexual reproduction leads to senescence and early death, whereas maintenance of asexual reproduction does not show any sign of aging. Constant self-renewal of stem cells during asexual reproduction and the loss of interstitial stem cells during gametogenesis are the proposed reasons for the above differences in their longevity. Stem cells of invertebrates demonstrate alkaline phosphatase activity (a marker of embryonic stem cells in vertebrates) and express *vasa*-like genes (as expressed in primordial germ cells), underpinning their importance in the evolution of asexual reproductive processes among various taxa. Significantly, invertebrate stem cells could become vital model systems to investigate molecular mechanisms underlying ageing and longevity in higher animals, including humans.

In invertebrates, sexual reproductive processes appear to be strongly influenced by various environmental factors. In particular, gametogenic cycles are initiated and synchronised with environmental changes by extrinsic or exogenous factors. In consequence, synchronised spawning within coral population enhanced fertilization success leading to increased planula production. For the marine invertebrates, seasonal changes in sea temperature, day length, wind or current patterns, lunar cycles or night irradiance are the major environmental factors to control reproduction and spawning. In terrestrial invertebrates like insects, a variety of extrinsic variables such as temperature, photoperiod, humidity, and food availability may influence egg production and fecundity.

The retention of primitive reproductive characteristics such as external fertilization and the development of planktonic larva in marine invertebrates demonstrate their evolutionary origin in the sea, which has changed little in physical and chemical properties since life began in it. Any change in the chemistry of seawater due to the predicted climate change could adversely affect reproduction in marine invertebrates. Conversely, terrestrialization has necessitated the development of certain modified reproductive traits such as sperm transfer via spermatophores, sperm storage in females, internal fertilization and the production of yolk-rich eggs. In spite of the widespread occurrence of sexual reproduction, invertebrates have effectively combined this mode with episodes of asexual reproduction in their complex life cycles.

Alternative Reproductive Phenotypes Within Species

Karen P Maruska, Julie M Butler, and Karen E Field, Louisiana State University, Baton Rouge, LA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction: Terms and Definitions

The terms reproductive “strategy” and “tactic” originated from concepts of evolutionary game theory, but their subsequent use in the literature is muddled, with flexible definitions depending on the context of individual research studies (see [Gross \(1996\)](#), [Maynard \(1982\)](#), [Oliveira et al. \(2008\)](#), [West-Eberhard \(2003\)](#)). In game theory, a “strategy” is typically defined as a program or set of decision rules that results in specific “tactics” (or phenotypes). Traditionally, strategies and their resulting tactics implies adaptive evolved function, but ultimate benefits or whether phenotypic traits arise from genotypic or environmental factors (or a combination of both) is typically not known. The concept of “alternative reproductive tactics”, which is widespread in the literature, refers to discontinuous phenotypic traits that allow an individual sex (males or females) to obtain fertilizations in alternative ways. While these traits can be behavioural, morphological, or physiological (or some combination of these), discontinuity is easiest to observe and measure for behavioural traits. Since the adaptive value and acting selection mechanisms for individual traits is often not initially known, it may be more useful to use the term “alternative phenotypes” or “alternative pathways” when referring to modes of reproduction. Therefore, a departure from game theory to use a fitness-neutral definition accounts for situations where adaptive value is not known, as may occur in early stages of the evolution of alternative phenotypes. Thus, alternative reproductive phenotypes (ARPs) involve individuals in the same life history stage using decision rules to allocate resources to one or another trait (or suite of traits) as a means of achieving the same goal. In reproduction, these alternatives are mutually exclusive (not expressed at the same time in an individual), often result from conditional expression of different solutions to reproductive competition, exist within a sex or population at one point in time, and have obtaining fertilizations as the common end goal. While ARPs can occur in any taxa, we focus our examples and discussion on the animal kingdom. The examples presented below are by no means exhaustive, but were chosen to highlight some of the diversity of ARPs displayed across animals.

Threshold Model for Expression of Alternative Phenotypes

Reproductive alternative phenotypes can be viewed as a threshold model, with plasticity between different traits triggered by a switch (see [Emlen \(2008\)](#), [Oliveira et al. \(2008\)](#), [West-Eberhard \(2003\)](#) for discussions) ([Fig. 1](#)). Alternative phenotypes can result solely from genetics (e.g. presence of a specific allele; allelic-switch), from environmental influences (conditional-switch; polyphenism), or from a combination of both genetic and environmental factors (combined-switch). For example, genetically similar individuals can adopt specific reproductive phenotypes based on differential exposure to factors such as population density or structure, abiotic conditions, social environment, and others. Further, many animals express a certain phenotype based on conditions encountered during development. This developmental plasticity can then dictate an individuals’ phenotypic propensity as a reproducing adult. The expression of alternative phenotypes is often governed by “threshold mechanisms”, in which individuals compare some internal sensitivity threshold to their surrounding environment and then “decide” which phenotypic traits to adopt. These condition-dependent threshold mechanisms involve a switch between phenotypic alternatives, and more often result in populations with dimorphic traits rather than ones that contain intermediate phenotypes. Some argue that alternative phenotypes can only evolve when there is selection against intermediate phenotypes (disruptive selection), because if phenotypes showed continuous variation, they would not be “alternative”. However, in nature, there are many examples of systems in which some individuals in a population have intermediate phenotypes that are more sensitive to a phenotypic switch (e.g., maintain traits closer to threshold); thus, condition-dependent threshold sensitivity itself can be a substrate for evolution.

The premise of the threshold mechanism is that individuals must assess their circumstances (via external sensory inputs or internal physiology), translate it into an internal signal for change (via changes in physiology such as hormone levels, neural function, or gene expression), and then compare this signal with a specified internal threshold level. This comparison of condition-dependent internal signals and threshold then mediates whether or not the individual will switch to an alternative phenotype to achieve fertilizations. These phenotypes can also be reversible or irreversible, which often differ in their developmental timing and biological level of trait expression. For example, reversible phenotypes typically occur during the adult stage with little delay (time between environmental assessment and decisions resulting in phenotypic switch), allowing animals to quickly switch back and forth between alternatives depending on social or abiotic conditions. As such, reversible traits are most commonly behavioural (e.g., dominant aggressive vs. subordinate submissive individuals), but can also be morphological (e.g., sexual ornamentation) or physiological (e.g., hormone levels in parental vs. non-parental individuals). For example, dominant and subordinate individuals coexist in populations, with diverse examples from beetles to primates. Within a population, dominant individuals typically have higher reproductive success, but changing social interactions and resource availability can cause individuals to switch back and forth between these phenotypes; sometimes many times within their lifespan ([Fig. 2](#)). In contrast, irreversible phenotypes have a longer delay, often occur earlier in development, and can generate dramatic morphological, physiological, and behavioural alternatives that have important consequences for reproduction later in life. Irreversible switches are most common in insects, where they occur prior to or during metamorphosis (e.g. switches from larval to adult stages, as in moths and butterflies), but they also exist in other taxa (e.g., thermal rearing conditions in zebrafish results in irreversible changes in adult thermal tolerance), and can

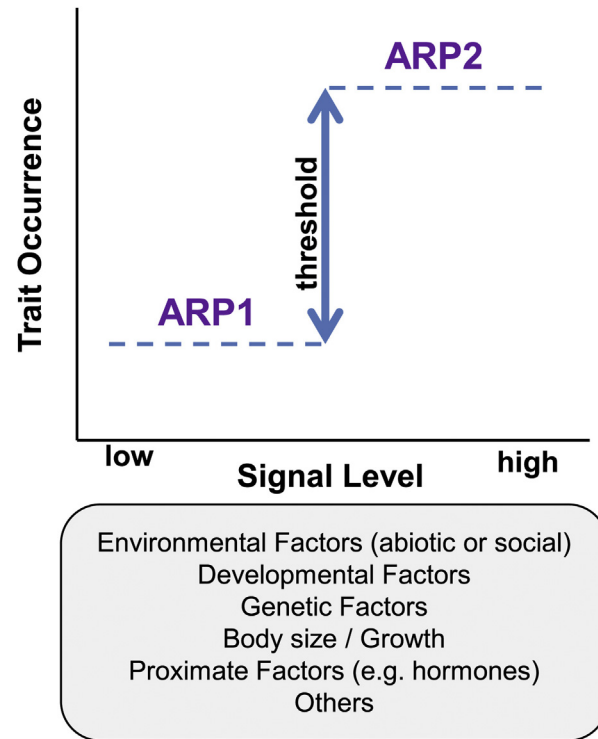


Fig. 1 Threshold mechanism for expression of alternative reproductive phenotypes (ARP). Individuals at signal conditions below a critical threshold express one phenotype (ARP1), with its accompanying suite of morphological, physiological, and behavioural traits. Individuals at conditions above this threshold express an alternative phenotype (ARP2). The signal conditions that mediate the switch between ARPs, and its timing, varies across species, but can include factors associated with an individual's environment (abiotic or social), development, genetics, body size, hormone levels, and others.

result from phenomena such as environment, stress, hormones, or competition during certain ontogenetic stages. Importantly, each step of the threshold assessment (detection, translation, and trait expression) leading to alternative phenotypes can be a substrate for selection and evolution.

Some General Categories of Alternative Phenotypes With Examples

In the sections below, we present some of the most common examples of ARPs, within broad major categories, to highlight their diversity across animal taxa. Many ARP categorization schemes are used in the literature (e.g. based on mechanism, function, whether an ARP is reversible or irreversible, etc.), and the ones chosen below represent only one approach (see [Oliveira et al. \(2008\)](#), [Shuster and Wade \(2003\)](#), [West-Eberhard \(2003\)](#) for discussion of other schemes). It is also important to recognize that some specific examples may be applicable to multiple categories (e.g. developmental, environment, and size-dependency), as many aspects of ARPs can be linked or overlap, particularly in the early stages of discovery. Further, additional category types may also exist and will likely be required as more examples in different species are revealed in the future.

Size- and Status-Dependent Alternative Reproductive Phenotypes

Some of the most common occurrences of ARPs across all animal taxa, and their accompanying threshold switches, are related to an individual's size or status. The most prevalent examples are found in males of a species ([Fig. 2](#)). We therefore focus this section as a brief taxonomic survey of male ARPs that are related to an individual's size and status to highlight their widespread occurrence in diverse animal groups ([Oliveira et al., 2008](#)), and then discuss examples of female ARPs in a subsequent section.

ARPs associated with body size occur across all orders of insects. For example, in the horned beetle *Onthophagus binodius*, large horned males make significant investment by helping females gather provisions prior to oviposition. In contrast, small hornless males do not help females and instead search for already provisioned females, often fertilizing about half of the offspring. These smaller males have larger testes and more sperm than horned males. In other species of dung beetle, both major and minor males help females. Larger males have higher success rates with females because they win male-male contests. Smaller males attempt to copulate with females by digging into a larger males' burrow when he leaves, where they are able to move about the burrow easily (avoiding larger males in the process) and mate with females.

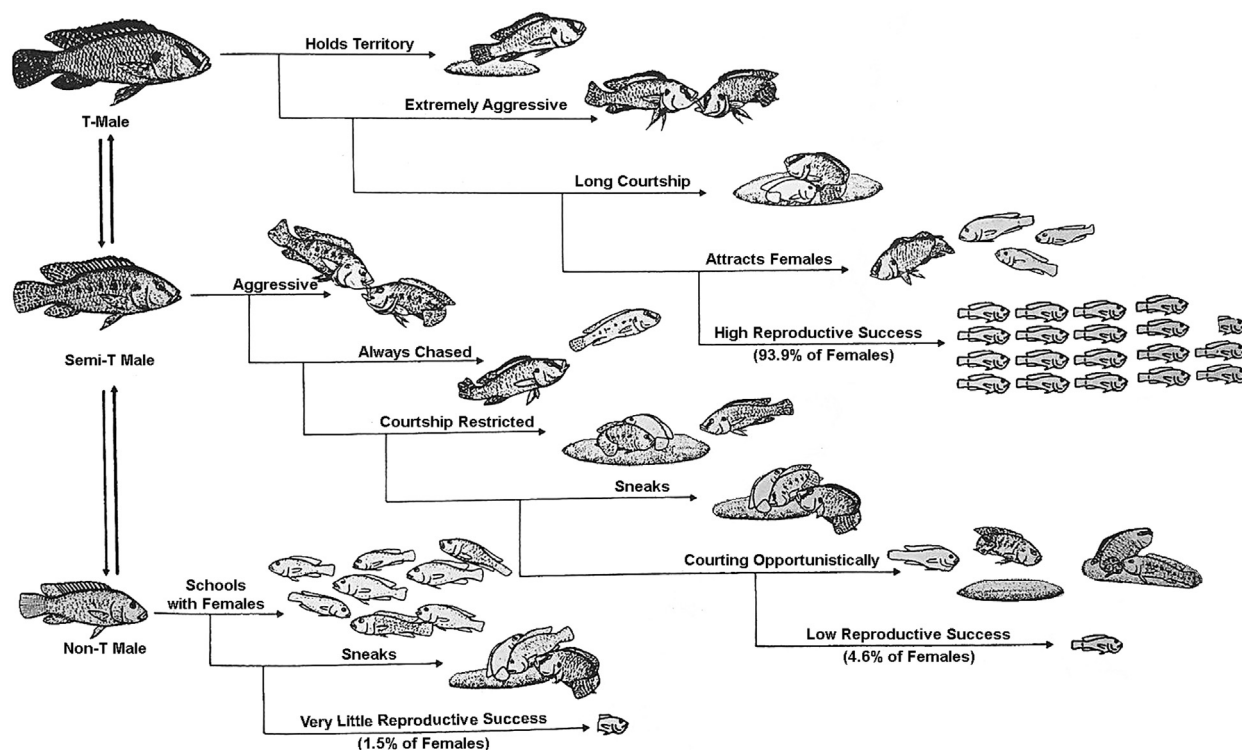


Fig. 2 Status-dependent male alternative reproductive phenotypes and relative reproductive success in the African cichlid fish *Pseudocrenilabrus philander*. Adoption of one of three alternative phenotypes (left) depends on relative male size and competitiveness. Territorial (T)-males aggressively defend spawning sites, perform extended courtship, and attract the majority of females, resulting in high reproductive success. Semi-T males perform aggressive opportunistic courtship and sneak spawning, with lower reproductive success. Non-T males resemble females in coloration and behaviour and use opportunistic sneak spawning that results in limited success. Individual males can reversibly switch between phenotypes. Reproductive success (shown by number of small fish at end of each arrow) is estimated from eggs spawned per spawning circumstance divided by the number of males that spawned (not genetic paternity studies). Original drawing is from Chan, T., 1987. *The role of male competition and female choice in the mating success of a lek-breeding Southern African cichlid fish Pseudocrenilabrus philander* (Pices: Cichlidae). PhD, Rhodes University, Grahamstown, South Africa. and figure is reproduced with permission from West-Eberhard, M.J., 2003. *West-Eberhard, M. J. 2003. Developmental Plasticity and Evolution*. New York: Oxford University Press.

Another classic invertebrate example of size-dependent ARPs occurs in horseshoe crabs, *Limulus polyphemus*. Small males arrive at mating beaches already clasped to females whom they spawn with, while older males arrive alone and patrol the area as satellites. Importantly, adoption of satellite behaviour is not a result of limited females, but depends on male age and size. The age or size at which individual males switch between pair-spawning and satellite behaviours may be governed by environmental conditions or female availability.

Among vertebrates, the most numerous and variable occurrences of ARPs appear in fishes. One reason for this is their indeterminate growth, which leads to large intra-sexual size dimorphism (ISD) within species. In species with large ISD, large males often employ a bourgeois tactic, monopolizing mate access and resources while smaller males are often parasitic. However, whether large ISD is a result of ARPs or vice versa is difficult to determine. Body size is the most widespread determinant of ARPs with larger males often at an advantage and smaller males sneaking to fertilize eggs. In some cases, as in cichlids and wrasses, piracy can occur when large males overtake several nests to fertilize eggs but leave other males to tend the nest. One of the most well-known size-related ARPs occurs in salmon (Fig. 3). Males can mature at 1–4 years of age and faster growing males spawn as sneaker males. Slower growing males (and all females) will migrate to sea for 1–3 years before returning to where they hatched. These slower growing anadromous males can weigh between 1–10 kg and develop a hooked lower jaw used in aggressive interactions with other males. They also court females and their success is often tied to body size. The sneaker males are significantly smaller and form their own hierarchies downstream of an anadromous male to attempt sneak spawning. Juvenile growth rate of males determines their reproductive phenotype and is influenced by genetics, parental life history, and habitat quality.

In amphibians such as frogs, most ARPs are conditional on either body size or social status, with relatively few known examples from only select species. Vocal communication is used by male frogs for mate attraction and is a mate choice determinant for females. Dominant males, or “callers”, use vocalizations to both attract females and defend territories while “satellite” males sit in silence near vocalizing males and intercept females or overtake sites occupied by caller males. Typically, males adopt either caller or satellite status for several nights, but switching within a single night has also been observed. The success rate of satellite males varies between species. For example, in *Bufo cognatus*, 95% of caller males are parasitized while only 2–14% of *Hyla picta* callers are

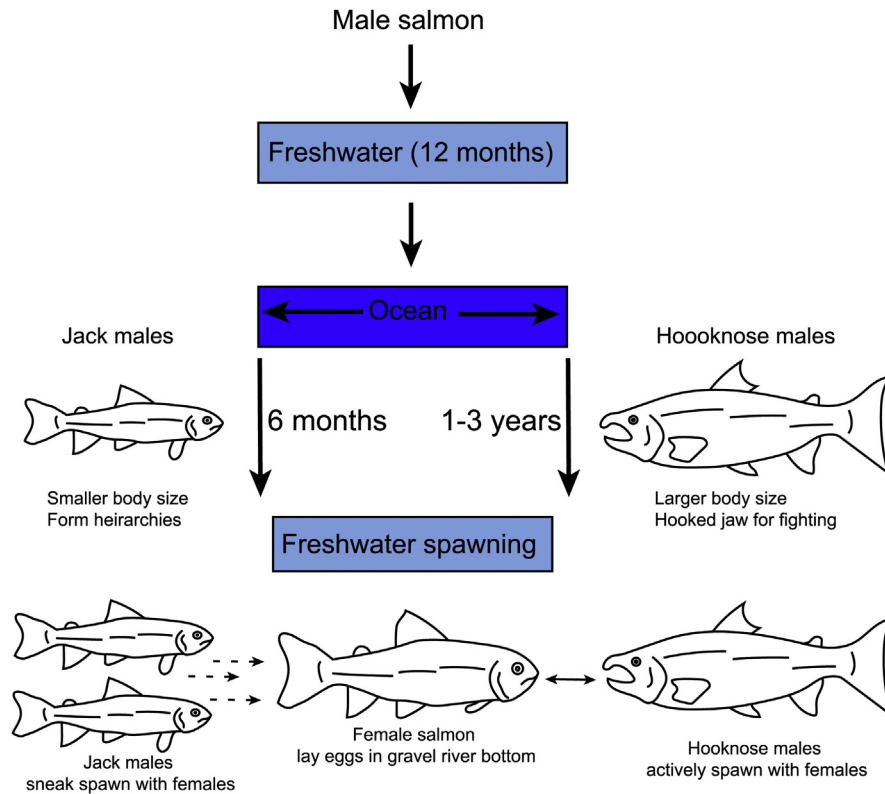


Fig. 3 Male Coho salmon exhibit size/status-dependent alternative reproductive phenotypes. After hatching in freshwater, all male (and female) salmon migrate to the ocean where they grow and mature. Smaller, fast-growing jack males remain at sea for approximately 6 months while larger, slow-growing males (and females) remain at sea for 1–3 years before turning to natal streams to spawn. The larger “hooknose” males develop a hooked jaw for fighting other males to facilitate spawning with females. The smaller jack males form hierarchies in freshwater where they attempt to sneak spawn with females. Growth rate of males, which has several influences, determines the males’ jack or hooknose status.

parasitized. In other species, such as *Rana clamitans*, smaller males wait for vacancies in oviposition sites rather than attempting to intercept females. In other species, smaller males may adopt satellite status as a tactic to pay off later in the breeding season. For example, satellite *Uperoleia rugosa* males often gain weight while in satellite status, increasing their chance to overtake territories of larger males later in the season. In other species, arrival time at breeding grounds may determine reproductive phenotype, rather than body size and/or condition. In *Physalaemus signifier*, for example, males that arrive early to territories vocalize to attract females and those that arrive later will not vocalize, regardless of body size and/or condition.

Social status is a common determinant of ARPs in lizards, which can be both conditional and influenced by genetics. For example, throat colour is often associated with dominance or aggression levels with lower status males employing female mimicry and/or sneaky behaviours. These approaches are often due to different endocrine pathways and/or genetics, and correlated with specific coloration (bright or cryptic colouring). In *Uta stansburiana*, sneaky males are smaller than more dominant males and exhibit traits such as yellow throat and distended abdomens that resemble those of gravid females. They will also flee from threats and are more cryptic in coloration compared to aggressive bright, dominant males. They do not hold territories, but reside near those of dominant males to sneak mating with females. These sneaky males, however, can switch to blue-throated dominant males with an increase in testosterone during the breeding season. However, only males with *by* genotype at the OBY locus can make this switch. Sneaker males with genotype *yy* are yellow-throated sneakers forever. However, only the absence of orange males (another dominant male morph) allows males to go from yellow to blue-throated.

The most well-studied ARP example in birds is that exhibited by the ruff (*Philomachus pugnax*), in which males are genetically determined to be either independents or satellites. Independent males are larger and can hold territories, while satellite males are smaller and cannot hold territories. Within the larger independent morph, males are either resident or marginal. Resident males hold a territory and marginal males do not, but try to take over those of resident males. Thus, while males are genetically fixed as satellite or independent, they can switch between resident and marginal within independent males. Satellite males make up 20% of the population, but account for only 10% of the copulations in this species.

Non-primate mammals also have a high prevalence of ARPs as a result of body size or social status. One example is seen in harem defence in which dominant males defend a harem of females. Subordinate males will adopt sneaking to attempt copulations with females when the dominant male is away or distracted. Examples of this harem defence polygyny occur in Northern and Southern elephant seals, plain zebras, and red deer. In elephant seals, smaller males that are of similar size to females will engage

in behavioural mimicry to sneak copulations. They will also wait until late in the breeding season to attempt mating when females are returning from the water and are not guarded. However the latter tactic is often unsuccessful because females have likely already mated. Territoriality based on status/size is also observed in several non-primate mammals. Dominant male bearded seals, for example, defend large aquatic territories in which they mate with females, while subordinate males roam these areas. In non-territorial mammal species, however, males will aggregate and pursue oestrous females. For example, dominant male raccoons will gather in feeding areas and tend to an individual female. Subordinate males, however, roam and encounter females away from dominant males. ARPs also exist in primates with complex social structures, and the reader is directed to the following review for examples (Setchell, 2008).

Environmental Influence on Expression of Alternative Reproductive Phenotypes

While many animals depend on abiotic cues to trigger the start of breeding season, the differences between breeding and nonbreeding (due to seasonality, environment, or cyclic reproductive cycles) animals are not considered alternative reproductive phenotypes because they occur at different points in time within the same individual. Abiotic environmental factors can, however, influence the proportion of animals within a population that are displaying each ARP at any given time. For example, female peacock wrasses mate with males that will care for their eggs. Because of this, females tend to mate with males that hold territories. A low abundance of nest availability (often due to environmental factors) causes females to mate outside the nest (low parental investment) instead of spending energy to find a suitable nest (high parental investment). This can change throughout the breeding season as nest availability changes. Similarly, different frequencies of high and low-parental investment are observed between populations inhabiting different environments.

Environmental factors can also cause more favourable conditions for certain phenotypes. For example, large and small-billed black-bellied seedcracker birds (*Pyrenestes ostrinus*) eat different types of seeds due to their different beak morphologies. Their food availability is affected by precipitation, causing them to nest at different times. Large-billed morphs nest early in the season while small-billed morphs nest late in the breeding season. Females mate randomly, independent of bill type, maintaining variation and preventing speciation. In the lizard *Urosaurus ornatus*, the environment determines whether satellite or nomadic tactics are used. Satellite males linger around other males' territories and under stressful environmental conditions, such as drought, will switch to nomadic males. However, this switch appears to be progesterone sensitivity-dependent and is therefore not solely environment-dependent.

Alternative reproductive phenotypes must occur in the same population at the same period in time. Because animals within a population are often exposed to the same environmental conditions, there are no true documented environmentally-induced ARPs in adults. However, environmental factors can have a big impact on the development of ARPs when juveniles and developing animals are exposed to them.

Developmental Influence on Expression of Alternative Reproductive Phenotypes

Conditions encountered during development can dictate an individual's reproductive phenotype as an adult. Most examples of true developmental ARPs exist in insects and are due to threshold switches that occur during development, initiating dramatic morphological, physiological, and behavioural alternatives with important consequences for adult reproduction. These can result from phenomena such as environment, stress, hormones, or competition during certain ontogenetic stages. The most common example of a developmental threshold mechanism involves hormone signals (e.g. juvenile hormone, ecdysone), where levels can be influenced by factors such as diet, temperature, and over-crowding. Hormones serve to link information from individuals' surroundings to their internal physiology during sensitive periods to initiate physiological or behavioural responses. In dung beetles (*Onthophagus* spp.), for example, juvenile hormone (JH) levels in larvae prior to metamorphosis help regulate small and large males along different developmental trajectories; large males produce horns used to guard tunnel entrances that allows them to monopolize females, while small males are hornless and adopt the alternative phenotype of sneaking into tunnels for mating opportunities (Emlen, 2008) (Fig. 4). In some species of butterflies, ecdysone (or ecdysteroid) levels during development also influence reproductive investment and life span in adults. Thus, many developmental switches operate by integrating environmental cues to phenotypic responses via hormonal (or neural) signals.

Alternative Reproductive Phenotypes in Females

Although less obvious and less frequently studied, female ARPs also exist in a variety of animals. While male ARPs are often related to access to females, females tend to use alternative phenotypes to boost their fertility or optimize the probability for success of a reproductive encounter. Female ARPs can be broadly divided into four categories (modified from Hensen and Warner (1997)): those relating to (1) parental care and investment, (2) mate choice, (3) resistance to harassment and coercion, and (4) mode of mating. Female ARPs are thought to be rarer than male ARPs, but more research is still needed, especially field studies, to determine whether they are truly less common or possibly just underreported.

Parental care and investment

The most well documented female ARP is brood parasitism. Commonly documented in fishes and birds, some females hold nests while others lay their eggs in others' nests. In the weakly electric fish *Eigenmannia virescens*, females create spawning nests in heavy

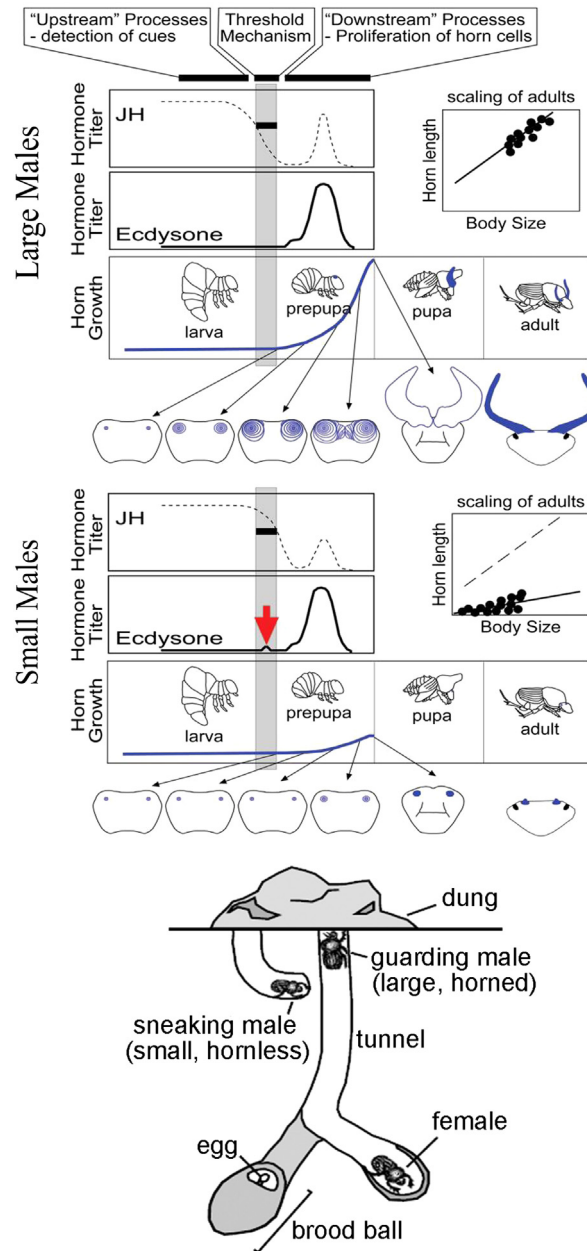


Fig. 4 Example of developmental and size-dependent alternative reproductive phenotypes in the beetle *Onthophagus taurus*. During the middle of the third larval instar of development, large and small males have different levels of juvenile hormone (JH). During a brief sensitive period before the cessation of feeding (vertical gray bar), large males have JH levels below a critical threshold (black horizontal line). Cells in the developing horns of these large individuals undergo a brief pulse of rapid proliferation during the prepupal period (blue curve), and these larvae mature into adult males with fully developed horns (inset at bottom). Small male larvae have JH concentrations above the threshold during the sensitive period, and these individuals also experience a brief pulse of ecdysone hormone (red arrow in B) resulting in only minimal proliferation of cells in the horns. Small males mature into adults with only rudimentary horns (inset at bottom). **Bottom illustration:** Female beetles dig tunnels beneath howler monkey dung, using it to provision to larvae inside. Large males with horns encounter females by 'guarding' the tunnel entrances while small males with rudimentary horns adopt a 'sneaking' approach to access females by digging side tunnels that intercept guarded tunnels below ground. Horns help guarding males block entrances from intruders, while they would hinder sneaking males by bumping against tunnel walls as they move underground. Adopted and reproduced with permission from Emlen, D.J., Hunt, J., Simmons, L.W., 2005. Evolution of sexual dimorphism and male dimorphism in the expression of beetle horns: phylogenetic evidence for modularity, evolutionary lability, and constraint. *Amer Nat.* 166 (Suppl 4) S42–68 and Emlen, D.J., 1997. Diet alters male horn allometry in the beetle *Onthophagus acuminatus* (Coleoptera: Scarabaeidae). *Proc R. Soc Lond B* 264, 567–574.

vegetation areas. Some females sneak through the plants and lay their eggs in another females' nest instead of their own, potentially driven by lack of males or inadequacy of their own spawning nest. In the mochokid catfish of Lake Tanganyika, eggs are deposited and incubated in the mouths of mouth brooding (retain developing young in their buccal cavity for the duration of development) cichlids along with the cichlid host's eggs (Fig. 5(A)). The catfish young hatch earlier and then feed on the host's developing young, exploiting both a food and refuge resource. Similarly, over 240 species of birds are known to parasitize others' nests. When females are unable to maintain their own nest, or their nest suffers from predation, they will lay their eggs in another's intact nest. The common cuckoo (*Cuculus canorus*), for example, is an obligate brood parasite. Females do not build their own nests, but instead, lay eggs that physically resemble those of the species they are parasitizing. Many bird species reject nonmimetic eggs, so cuckoo females must lay eggs in species-specific nests. This has resulted in cuckoos being divided into gentes based on the species' eggs they mimic. In the white-throated sparrow (*Zonotrichia albicollis*), both males and females have either a white or tan stripe over their eye and behaviour differences within each sex are associated with this phenotypic difference. White-striped females pursue conspecific brood parasitism and solicit copulations more often than tan-striped females.

Similar to brood parasitism, some fish species also display offspring dumping and kidnapping. Many mouth brooding cichlid fishes display parental care behaviours toward their young and, after release, will take the fully-developed offspring back into their mouth and carry them to safety. Instead of returning their young to their own nest, some fishes are observed depositing their offspring into another's nest for care. Conversely, some fishes travel to another's nest, and gather young into their mouths to care for as their own.

Mate choice and copying

Theoretical and experimental studies in fishes have demonstrated that some females mimic mate choice of others instead of making their own decisions. In guppies, this mate choice copying behaviour appears to be age-dependent. Older females are not influenced by younger females, but younger females will copy the choice of older females. Female sensory perception is often tied to reproductive state, and although not studied, could also be linked to female social status. Male katydid insects present females with resources during courtship. The females then fight with each other for the best males. In areas of high competition, females may develop larger or have increased sensitivity to male courtship songs. More research is needed to explore the plasticity in female sensory perception as it relates to ARPs.

Resistance to harassment and coercion

The most commonly observed female ARP in insects deals with the females' attempt to control male mating attempts. Some female damselflies mimic male appearance to avoid harassment, leading to lower mating but higher survival rate. Other female insects will hide from males to reduce harassment.

Some females are also more resistant to coercion than others. Male guppy fish will try to force copulation; however, females typically resist this coercion and require extensive courting. In the presence of predators, however, some females choose to forgo courtship and accept copulation without courting. Both phenotypes are present within a population, but the proportion of each depends on perceived predation risks.

Mode of mating

The most dramatic female ARPs are those between "breeding" and "nonbreeding" individuals. Some best-known examples occur in queen ants and wasps where a single queen, or sometimes multiple queens, is the sole reproducing female of the population

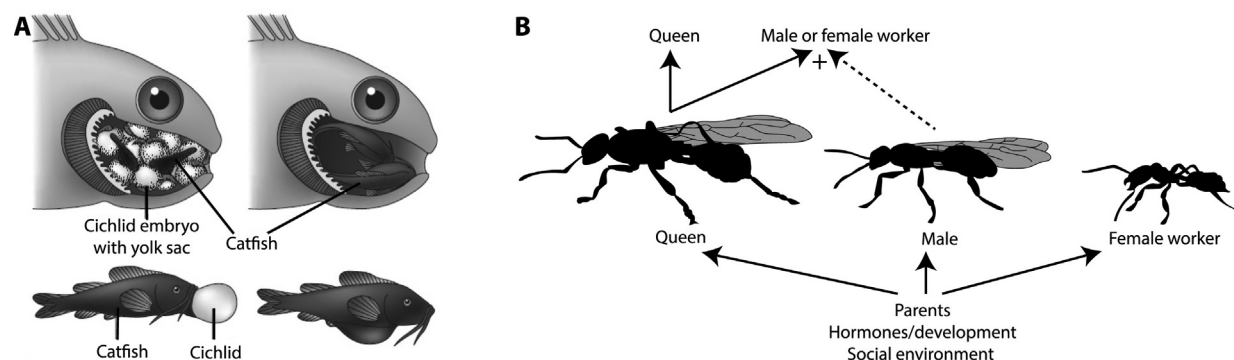


Fig. 5 Examples related to alternative reproductive phenotypes in females. (A) In Lake Tanganyika, the mochokid catfish will sometimes deposit their eggs with those of mouth brooding cichlids, causing the cichlid to pick them up with their own eggs and incubate them for development. The catfish young (black) develop faster and then eat the developing cichlids (gray). Reproduced with permission from Helfman, G., Collette, B.B., Facey, D.E., Bowen, B.W., 2009. *The Diversity of Fishes: Biology, Evolution, and Ecology*. Wiley-Blackwell. (B) Although variable between species, queen ants are typically the regulators of reproduction in their colonies. Due to a combination of their heredity, hormones, nutrition, development, and social environment, pupae develop into either a queen ant, male ant, or worker ant. Most queens are able to reproduce either on their own to produce more queens, or with males to produce more male or worker ants.

(Fig. 5(B)). This determination is primarily genotypic, but some research also suggests that diet at larval stages can manipulate future role. ARPs in some ant species can also be influenced by female size. For example, queen ants can choose to start a new colony or to found independent colonies using the existing colonies work force. Across ant taxa, smaller queens choose to utilize the existing work force and form dependent colonies, while larger queens found independent colonies.

Another commonly observed ARP in females is nest helpers. Some females assume the role of “breeders” while others are “helpers”. Position in this cooperative breeding can depend on several factors, including age, relatedness, and social status. In the cichlids *Neolamprologus brichardi/pulcher*, subordinate females often serve as nest helpers to breeding females. Being a helper may reduce the level of observed aggression and increase their opportunity for brood parasitism. Interestingly, the relative presence of helper females may be dependent on the abundance of available breeding substrate.

Females also display ARPs related to their mode of spawning. In the bluehead wrasse, females partake in both group- and pair-spawning. Some females mate consistently at group spawning sites, while others only spawn with a single male at pair-spawning sites. These alternative phenotypes appear to be size dependent, as nearly all females switch to pair-spawning when they reach a large size. Sneaking is another approach also observed in bluehead wrasse females. Damselfish *Chromis multilineata* females will also engage in sneaking behaviours depending on their condition. Males do not allow parasitized females into their nests. Instead, parasitized females sneak into a nest and quickly release their eggs before they are chased out. In primates, females often sneak copulate with subordinate or extra-group males, but will mate openly with dominant group males.

Yolk-provisioning strategies, egg retention, and variation in viviparity underlie most of the ARPs displayed by female reptiles. For example, female side-blotched lizards display one of two throat colours, yellow or orange, each with a different reproductive approach. Orange-throated females lay large clutches of small eggs, but yellow-throated females lay small clutches of large eggs.

Proximate Mechanisms of Alternative Reproductive Phenotypes

The initial threshold-based decisions that result in expression of ARPs require that individuals assess their surroundings. As such, knowledge of the proximate sensory, neural, and hormonal mechanisms that lead to ARPs is imperative to understand how and why ARPs develop within a species or population, and their consequences for reproductive success. Behavioural traits are more labile than morphological traits, and typically the first observable traits related to ARPs to appear; however, there are often physiological switches involved in the expression of these behaviours. For example, neural plasticity of brain circuits (e.g., synaptogenesis, excitatory/inhibitory neuron activation, neuromodulation, changes in gene expression or epigenetic factors), and changes in circulating hormones can alter reproductive behaviours in different contexts. This neural and hormonal plasticity can occur on different timescales, from rapid effects related to reversible conditional phenotypes (as in status-dependent phenotypes), to longer-term irreversible changes that occur during development or in a fixed sequence during adulthood (as in non-overlapping developmental trajectories of different reproductive male morphs in fishes like midshipman). Where ARPs are concerned, there is frequently also an uncoupling of neurobiological and gonadal traits in the expression of behavioural phenotypes. In many cases, the relative contribution of proximate mechanisms acting on multiple levels of biological organization, and whether they act independently or together to result in single or suites of alternative traits is unknown. There are numerous specific examples across animal taxa that illustrate the involvement of sensory, neural, hormonal, biochemical, epigenetic, and molecular level switches in ARP expression, and the reader is directed to the following sources: Bass and Forlano (2008), Oliveira *et al.* (2008). Understanding the proximate mechanisms that underlie ARP expression within a species can yield important insights relevant to their evolution. This includes identification of whether specific traits may have contributed to ARP trait expression or served as physiological constraints. Moving forward, it will be important to combine proximate mechanistic studies with ultimate selection and evolutionary approaches to gain a more complete understanding of the complexity of ARPs.

Evolution of Alternative Reproductive Phenotypes

The field of evolutionary biology seeks to understand mechanisms by which alternative phenotypes arise and persist in a population. While the question of how and why ARPs evolve is complex and a complete discussion is beyond the scope of this article (see Brockmann and Taborsky (2008a,b), Oliveira *et al.* (2008), Taborsky and Brockmann (2010), West-Eberhard (2003) for extensive discussions), we highlight below several of the main ways ARPs can evolve. First, the sensory systems that detect and perceive relevant changes in the environment (includes biotic and abiotic factors) can evolve. This can lead to changes in sensitivity to specific stimuli in one sensory channel, or shifts in the importance of stimuli among different sensory channels. For example, some butterfly species form pupae that match the colour of their substrates, and caterpillars can switch between alternative colours depending on sensory inputs such as photoperiod, colour of reflected light, or texture of the background substrate. Second, the physiological response mechanisms that link sensory detection to changes in physiology, such as expression changes in genes, proteins, and other signalling molecules, can evolve. These physiological changes are then revealed as alternative behaviours or morphologies if levels rise above the animal's internal switching threshold. Genetic changes in response thresholds can then alter the conditions under which alternative phenotypes are expressed. Lastly, components of the downstream signalling cascades that regulate the final condition-dependent expression of alternative traits can evolve. Importantly, each step in the process can involve a complex suite of genes that can act as substrates for evolution. However, it is also relevant to realize that phenotypic plasticity itself is a heritable trait

subject to selection and evolutionary change, and that alternative phenotypes can be viewed as a phase of evolution (West-Eberhard, 2003).

Why do alternative reproductive phenotypes evolve? ARPs are expected to exist in situations where different reproductive approaches have different benefits for acquiring fertilizations (see Fig. 2). When decision switch points for condition-dependent phenotypes are heritable and result in variable fitness, then the decision or threshold point can evolve. This becomes complicated because the decision point often relies on many variables that can vary in space and time, producing different benefits depending on environmental or social conditions. Brockmann and Taborsky (2008a,b) outline four general requirements for evolution of ARPs: (1) a mechanism by which distinct phenotypes can develop (e.g. threshold switch); (2) heritable variation in the mechanisms that control the ARPs; (3) selection that favours multiple rather than a single phenotype (e.g. disruptive selection); and (4) crossing fitness curves (points when individuals choose between alternatives with different associated trade-offs). Evolutionary processes can therefore act on underlying mechanisms, including decision rules, as well as on phenotypic flexibility (reversible or irreversible switches) or frequency within the population. Because of the higher reproductive investment in females, ARPs are expected to evolve more often in males. Moving forward, however, both sexes within a species should be taken into account to better understand the complex evolution of ARPs within individual species.

References

- Bass, A. H., & Forlano, P. M. (2008). Neuroendocrine mechanisms of alternative reproductive tactics: The chemical language of reproductive and social plasticity. In R. F. Oliveira, M. Taborsky, & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach* (pp. 109–131). Cambridge: Cambridge University Press.
- Brockmann, H. J., & Taborsky, M. (2008a). Alternative reproductive tactics and the evolution of alternative allocation phenotypes. In R. F. Oliveira, M. Taborsky, & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach* (pp. 25–51). Cambridge: Cambridge University Press.
- Brockmann, H. J., & Taborsky, M. T. (2008b). Alternative reproductive tactics and the evolution of alternative allocation phenotypes. In R. F. Oliveira, M. T. Taborsky, & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach* (pp. 25–51). Cambridge: Cambridge University Press.
- Emlen, D. J. (2008). The roles of genes and the environment in the expression and evolution of alternative tactics. In R. F. Oliveira, M. Taborsky, & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach* (pp. 85–108). Cambridge: Cambridge University Press.
- Gross, M. R. (1996). Alternative reproductive strategies and tactics: Diversity within sexes. *Trends Ecol. Evol.*, 11, 92–98.
- Hensen, S. A., & Warner, R. R. (1997). Male and female alternative reproductive behaviors in fishes: A new approach using intersexual dynamics. *Annu Rev Ecol Syst*, 28, 571–592.
- Maynard, S. (1982). *Evolution and the Theory of Games*. Cambridge: Cambridge University Press.
- Oliveira, R. F., Canario, A. V., & Ros, A. F. (2008). Hormones and alternative reproductive tactics in vertebrates. In R. F. Oliveira, M. Taborsky, & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach* (pp. 132–173). Cambridge: Cambridge University Press.
- Oliveira, R. F., Taborsky, M., & Brockmann, H. J. (2008). *Alternative Reproductive Tactics: An Integrative Approach*. Cambridge, MA: Cambridge University Press.
- Setchell, J. M. (2008). Alternative reproductive tactics in primates. In R. F. Oliveira, M. Taborsky, & H. J. Brockmann (Eds.), *Alternative Reproductive Tactics: An Integrative Approach* (pp. 373–398). Cambridge: Cambridge University Press.
- Shuster, S. M., & Wade, M. J. (2003). *Mating Systems and Strategies*. Princeton, N.J.: Princeton University Press.
- Taborsky, M., & Brockmann, H. J. (2010). Alternative reproductive tactics and life history phenotypes. In P. Kappeler (Ed.), *Animal Behavior: Evolution and Mechanisms* (pp. 537–586). Berlin/Heidelberg: Springer-Verlag.
- West-Eberhard, M. J. (2003). *Developmental Plasticity and Evolution*. New York: Oxford University Press.

Further Reading

- Dodson, J. J., Aubin-Horth, N., Theriault, V., & Paez, D. J. (2013). The evolutionary ecology of alternative migratory tactics in salmonid fishes. *Biol Rev*, 88, 602–625.
- Engqvist, L., & Taborsky, M. (2016). The evolution of genetic and conditional alternative reproductive tactics. *Proc R Soc B*, 283, 20152945.
- Faulkes, C. G., & Bennett, N. C. (2001). Family values: Group dynamics and social control of reproduction in African mole-rats. *Trends Ecol. Evol.*, 16, 184–190.
- Gangestad, S. W. (2007). Reproductive strategies and tactics. In L. Barrett, & R. Dunbar (Eds.), *Oxford Handbook of Evolutionary Psychology*. Oxford University Press. <https://doi.org/10.1093/oxfordhb/9780198568308.013.0023>.
- Gillette, M. T., & Folinsbee, K. E. (2012). Early menarche as an alternative reproductive tactic in human females: An evolutionary approach to reproductive health issues. [Review]. *Evol Psychol*, 10(5), 830–841.
- Heinze, J., & Keller, L. (2000). Alternative reproductive strategies: A queen perspective in ants. *Trends Ecol Evol*, 15, 508–512.
- Knapp, R. (2004). Endocrine mediation of vertebrate male alternative reproductive tactics: The next generation of studies. *Integrative Comparative Biol*, 43, 658–668.
- Moore, M. C. (1991). Application of organization-activation theory to alternative male reproductive strategies: A review. *Hormones Behavior*, 25, 154–179.
- O'Donnell, S. (1998). Reproductive caste determination in eusocial wasps (Hymenoptera: vespidae). *Annu. Rev. Entomol.*, 43, 323–346.
- Potts, G.W., Wootton, R.J., 1984. Fisheries Society of the British Isles & FSBI International Symposium, Plymouth, England, 1982. Fish Reproduction: Strategies and Tactics. Academic Press, London; Orlando.
- West-Eberhard, M. J. (1989). Phenotypic plasticity and the origins of diversity. *Annu Rev Ecol Syst*, 20, 249–278.
- Zink, A. G., & Lyon, B. E. (2016). Evolution of conspecific brood parasitism versus cooperative breeding as alternative reproductive tactics. *Am Naturalist*, 187, 35–47.

BEHAVIOR

Altricial and Precocial Development in Birds

Haruka Wada, Auburn University, Auburn, AL, United States

Sarah E DuRant, University of Arkansas, Fayetteville, AR, United States

FM Anne McNabb, Virginia Tech, Blacksburg, VA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Altricial Referring to a pattern of development in which birds are hatched at a relatively early stage of development and remain in the nest with parental care for some time.

Development All of the processes involved in the differentiation of specific tissues plus growth in body mass.

Ectotherm Animals that rely on the environment to regulate body temperature.

Endotherm Animals that internally generate heat to control body temperature.

Homeothermy Having a regulated, constant body temperature independent of changes in environmental temperature; endothermic animals use heat produced by metabolism and are homeotherms; ectotherms can also reach homeothermy through behavioral thermoregulation.

Precocial Referring to a pattern of development in which birds are hatched at a relatively advanced stage of development and are capable of leaving the nest.

Thermoregulation The regulation of constant body temperature by metabolic activities that produce or dissipate heat depending on the environmental temperatures to which the animal is exposed.

The terms altricial and precocial refer to patterns of development in which the young are hatched at a relatively early stage of development (altricial) or at a much more advanced stage of development (precocial). These terms also refer to a suite of developmental characteristics at hatch as well as to behavioral and parental care patterns. In general, altricial nestlings are still undergoing rapid tissue and organ maturation at hatching, thus altricial chicks are nest-dwelling (nidicolous) and dependent on parental care. In contrast, precocial chicks are nest-fleeing (nidifugous), i.e., they leave the nest soon after hatching or immediately after hatching, and are much more self-sufficient. Altricial development is the most common pattern of development among avian species with nearly 90% of birds hatching altricial nestlings. Although most birds can easily be categorized as either altricial or precocial, these terms represent the ends of a continuum and intermediate patterns can be subdivided into a more detailed classification. For instance, at the precocial end of the spectrum, hatchlings of very precocial species such as megapode birds are fully feathered as adults and hatchlings of some species can fly on the day they hatch out of their eggs. Examples of birds with altricial development include songbirds, pigeons, parrots, woodpeckers, and hummingbirds. Examples of precocial species include ducks, chickens, quail, grouse, and ostriches. Intermediate patterns include examples such as semiprecocial gulls and terns, which are able to leave the nest after a few days but stay in the general area, and semialtricial herons and hawks, which stay in the nest for several weeks but are capable of coordinated movements within the nest. It should be noted that mammals, like birds, also may be categorized as altricial or precocial with respect to their developmental patterns.

General Patterns of Altricial and Precocial Development

Altricial development in birds is characterized by hatching of the young at a relatively early stage of development. There is a general trend toward altricial species laying smaller eggs with lower energy content (for embryonic nourishment) and having shorter incubation periods. Thus, it appears that these species put less energy into egg production and incubation and more energy into post-hatching care of the nestlings. Altricial hatchlings typically are unfeathered or only sparsely feathered (i.e., they lack insulation), their eyes are not open, and they are incapable of coordinated body movements (Fig. 1(A)). A consequence of hatching relatively early in development is that the young are dependent on parental care and confined to the nest for some time after hatch.

The parents of altricial nestlings must provide food and protection until the nestlings are capable of flight (fledging) and of leaving the nest to assume independent life. The relative helplessness and vulnerability of altricial nestlings means they require considerable protection. These species often nest in locations that provide protection against predators, and parents may alternate

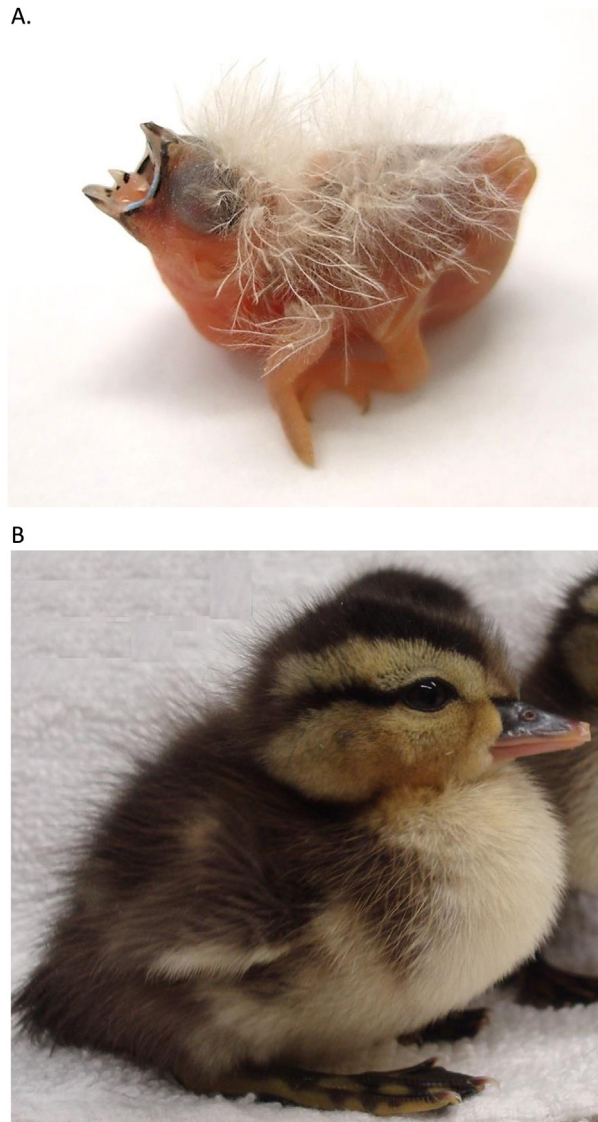


Fig. 1 General characteristics of altricial zebra finch nestling (A) and precocial wood duck duckling (B) at 1 or 2 days posthatching.

with each other in spending time on the nest and foraging for food for the young. An adult bird arriving at the nest is greeted by begging nestlings with wide-gaping mouths into which the parent regurgitates food it has captured and stored in its crop. Nestlings often have their crops greatly distended with food. Characteristically, the parents of altricial young spend most of their daylight hours supplying food to the nestlings for 1–3 weeks after the young hatch.

Precocial development in birds is characterized by hatching of the young at much more advanced stages of development than is the case for altricial birds. Precocial species generally lay larger eggs with a larger proportion of yolk and have longer incubation periods than altricial species. Thus, precocial hens have relatively higher energy use in egg production and incubation, but relatively less energy use in the care of chicks than do altricial birds. However, in some cases very mobile precocial young may require considerable attention on the part of parents, especially in cases in which predation is likely.

Precocial chicks typically look like miniature adults of the species; their body proportions and shape are generally like those of the adults (**Fig. 1(B)**). At hatching, precocial chicks typically are covered with down feathers, their eyes are open, their nervous systems are well developed, and they are capable of coordinated locomotion within minutes to hours after hatching. Precocial chicks typically begin to drink and eat within the first day after hatching. However, during hatching the yolk sac and its remaining nutrient stores are pulled into the body and are an important source of nutrition for the next several days.

Early learning of precocial chicks is facilitated by their “imprinting” to the mother. Imprinting is the process whereby a chick develops its sense of species identity by associating with its mother or another adult during a critical learning period in early post-hatching life. In precocial young that are capable of locomotion and independent feeding, this identification with the mother results

in behaviors that enhance the survival of young chicks that must learn about food sources and are vulnerable to predation or harsh environmental conditions. Thus, chicks imprinted to the mother will remain relatively close to her or other members of the same species, can follow and learn about food sources from adults, can respond to the mother's vocalizations, and will be brooded by her for protection from severe weather.

Growth and Development

Altricial nestlings typically have “distorted” body proportions and do not look like their parents. They have well-developed heads, large mouths with wide gapes, large crops, and distended abdomens due to a large visceral mass. Organ systems that must assume function during the perihatch period are the respiratory system, those of the gastrointestinal tract, and, to a lesser extent, the nervous system. The appendages of altricial nestlings are poorly developed and incapable of coordinated locomotion; their legs cannot support the body in an upright position. The wings must undergo considerable growth, maturation, and development of flight feathers before the young are capable of flight. Water content of the tissues is high until the tissues reach functional maturity and the proportion of body water (relative to dry solids) has been used as an index of the degree of maturation of both altricial and precocial young.

Precocial chicks have much more mature body development and the muscular, skeletal, and sensory/nervous systems are considerably more advanced at hatch than is the case for altricial nestlings. As in altricial young, the respiratory and gastrointestinal organs must have functional maturity from the perihatch period onward. In addition, the functional maturity of most other organ systems is also necessary for the relative independence of precocial chicks early in posthatching life.

Investigations of tissue differentiation and maturation in altricial vs precocial chicks have not shown the striking differences in key maturation events that might have been expected based on the organ system patterns described previously. For example, the beginning of cartilage to bone transition in many skeletal elements occurs at about the same developmental stage in altricial and precocial young. However, there may be differences in the extent of bone formation (ossification), with altricial nestlings having proportionately less ossification and precocial chicks having proportionately more at a given stage of posthatching development. Similar results have been found in tissues such as muscle.

The inverse relationship between tissue functional maturity and growth potential seems to be a key factor in the differences in growth of altricial and precocial species. Most avian species with altricial development have high growth rates posthatch and delayed maturation of function in many tissues. Precocial young, which have earlier maturation of many tissue functions, have slower body growth rates posthatch (Fig. 2).

Development of Temperature Regulation

The patterns of body temperature (thermoregulatory) development are strikingly different in altricial and precocial species of birds. Altricial young are hatched in an ectothermic or cold-blooded state. Although altricial nestlings are capable of detecting temperature change and some may shiver in the cold, their capacity for heat production is very limited. In addition, their insulation is often poor, so their capability for heat retention is also limited. Consequently, their body temperatures change with the temperature of the environment (except when brooded by the parents) and their metabolic rates are low. Altricial young remain ectothermic for some time (generally 1 or 2 weeks, depending on the species), then develop homeothermy (body temperature becomes constant) relatively rapidly. During their period of ectothermy, the nestlings increase in body size (which decreases their relative surface area) and they begin to grow an insulative feather covering (Fig. 2). When the ability of the nestlings to produce metabolic heat (endothermy) increases, both the relative reduction in surface area due to growth and the insulation provided by the feathers are important in

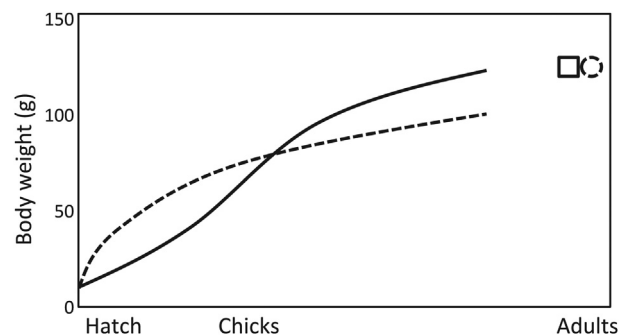


Fig. 2 Body weight patterns in altricial mourning doves (dotted line) and precocial Japanese quail (solid line) from hatching to 6 weeks of age. While Japanese quail reach sexual maturity in 6 weeks, mourning dove young do not reach sexual maturity until 13 to 14 weeks. Adult body weights of doves (●) and quail (□) are equivalent.

conserving metabolic heat for attaining homeothermy. Body temperatures begin to increase and the nestlings shiver vigorously in response to a decrease in the environmental temperature. These processes continue and typically result in the maintenance of constant body temperatures similar to those of adults by the time fledglings leave the nest.

Before altricial young can produce metabolic heat, they have some coping mechanisms for withstanding exposure to hot and cold temperatures. During exposure to hot temperatures, nestlings will gular flutter, which refers to rapid shallow “panting” that evaporates water and withdraws heat to cool the body, appears earlier than the mechanisms for dealing with cold in some altricial nestlings. Heat may potentially be of more danger to young confined to the nest than cold, since nestlings are resilient to short term decreases in body temperature.

In response to cold temperatures, altricial nestlings may huddle to reduce heat loss. Several altricial nestlings huddled in a group may develop “effective homeothermy” before each is capable of maintaining relatively constant body temperature as an isolated individual. Huddling as a group in the nest reduces heat loss, because less total surface area is exposed relative to the mass of the group than would be the case for isolated individuals. For nestlings that are in the process of developing their capacity for metabolic heat production, the reduced heat loss achieved by huddling is of considerable advantage and allows them to achieve high and stable body temperatures while in a group.

Prior to hatching, precocial chicks, which are much more mature metabolically than altricial chicks, can detect changes in incubation temperature. In response to cooling temperature, embryos can generate heat, however it is not enough to sustain egg temperature. Normally, parents brood eggs to maintain relatively constant temperatures in the nest and some precocial embryos near hatching can signal parents to warm them up. For instance, ring-billed gull embryos make distress calls when their body temperature drops below 36 °C. After hatching, thermoregulatory function matures and young begin to produce sufficient metabolic heat to regulate their body temperature well above the temperature in the environment. Precocial hatchlings further increase their metabolic rates by shivering in response to a decrease in environmental temperature. However, precocial hatchlings have limited capability to produce heat through metabolism, and the body temperatures maintained by young chicks are lower than those of adults. During the first few days to weeks posthatch, the chicks attain adult body temperatures and the range of environmental temperatures at which they can remain homeothermic is extended. Body growth (which reduces the relative surface area) and replacement of down by juvenile plumage with improved insulative qualities are both important factors in this refinement of their thermoregulatory ability. As in altricial young, huddling in groups or brooding by parents can be very important to precocial young during harsh weather conditions.

Development of Endocrine Control Systems

Hormones play important roles in the development and growth of individual tissues and of the body as a whole. Major hormones in controlling the development of individual tissues and determining the timing of developmental events are thyroxine and triiodothyronine (the hormones produced by the thyroid gland), corticosterone (a hormone produced by the adrenal glands), growth hormones, growth factors and sex steroids such as testosterone and estrogen. Of the thyroid hormones, triiodothyronine is considered to be responsible for most thyroid hormone actions. Thyroid hormones trigger differentiation (the development of individual tissue characteristics and functions) in a number of tissues and are necessary along with other hormones for general body growth. Thyroid hormones have developmental effects in all classes of vertebrate animals. In addition, in homeothermic birds and mammals, thyroid hormones are the key controllers of the high metabolic rates that are necessary for homeothermy.

The patterns of thyroid development are distinctly different in altricial and precocial birds, as might be expected by the differences in thermoregulatory development associated with the two modes of development, and given the importance of thyroid hormones in controlling metabolic heat production. In altricial embryos the thyroid gland shows little functional development during embryonic life and around hatching (the perihatch period). After hatching, the thyroid gradually increases its capability for hormone production and release, but the thyroid hormone concentrations in the blood remain low for several days. Then thyroid hormone concentrations in the blood increase and the young develop thermoregulatory responses to cooling and are able to maintain constant body temperatures (Fig. 3(a)).

In contrast to the relatively late thyroid development in altricial nestlings, the thyroid glands of precocial species attain a relatively high level of activity during the latter half of embryonic life. During the perihatch period, there are dramatic peaks of thyroid hormone concentrations in the blood and these high hormone concentrations are important in the initial thermoregulatory responses that appear at hatch. After the perihatch period, thyroid hormone concentrations in the blood decrease, then gradually increase to adult levels (Fig. 3(b)).

Corticosterone plays an important role in maintaining energy balance during stressful situations and facilitating developmental transitions. In precocial birds, corticosterone secretion from the adrenal glands increases during the latter part of the embryonic period and peaks around hatching (Fig. 4(b)). This peak in corticosterone along with thyroid hormones is thought to contribute to the initiation of the hatching process. Precocial birds can also secrete corticosterone in response to cold and hot temperatures and other stressors during the latter part of embryonic development. After hatching, corticosterone concentrations steadily drop over the course of several days and eventually reach concentrations similar to adults. In contrast, in altricial birds, circulating corticosterone levels are low early in the nestling period and the ability of the adrenal glands to secrete corticosterone gradually increases to adult-like levels by the time the nestlings fledge (Fig. 4(a)). In some species, fledglings have higher corticosterone levels than adults. It is unknown whether corticosterone also peaks around hatching in altricial birds. However, it is hypothesized that the gradual rise in corticosterone from hatching until fledging may be equivalent to a peak of corticosterone around hatching in precocial species,

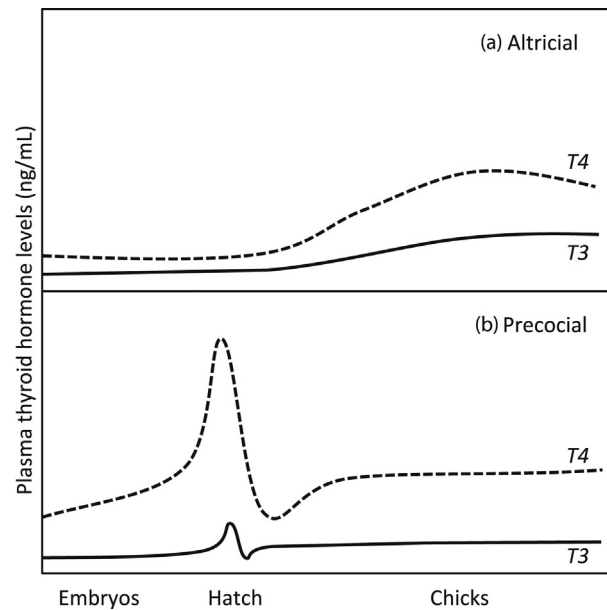


Fig. 3 Patterns of plasma concentrations of thyroid hormones (T4, thyroxine (dotted lines); T3, triiodothyronine (solid lines)) in altricial and precocial birds from mid-incubation to 2 or 3 weeks posthatch. The characteristic patterns shown here are for (a) altricial doves and (b) precocial quail. For references to the data on individual species, see McNabb, F.M.A., Olson, J.M., 1996. Development of thermoregulation and its hormonal control in precocial and altricial birds. *Poultry and Avian Biology Reviews* 7, 111–125 and McNabb, F.M.A., Scanes, C.G., Zeman, M., 1998. Endocrine control of development. In: Starck, J.M., Ricklefs, R.E. (Eds.), *Avian Growth and Development. Evolution Within the Altricial-Precocial Spectrum*. New York: Oxford University Press.

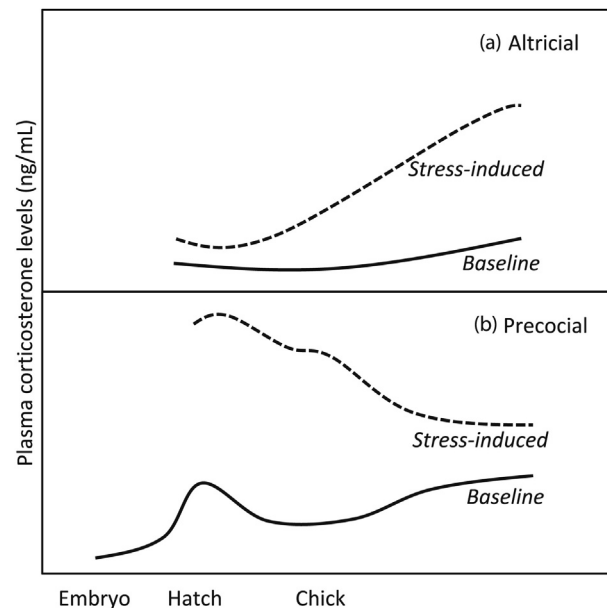


Fig. 4 Patterns of plasma concentrations of corticosterone in (a) altricial and (b) precocial birds. Solid lines represents baseline, non-stressed levels while dotted lines indicate levels after exposure to a stressor. For references to the data on individual species, see Wada, H., Cristol, D.A., McNabb, F.M.A., Hopkins, W.A., 2009a. Suppressed adrenocortical responses and thyroid hormone levels in birds near a mercury-contaminated river. *Environmental Science and Technology* 43 (15), 6031–6038; Wada, H., Salvante, K.G., Wagner, E., Williams, T.D., Breuner, C.W., 2009b. Ontogeny and individual variation in the adrenocortical response of zebra finch (*Taeniopygia guttata*) nestlings. *Physiological and Biochemical Zoology* 82 (4), 325–331; and Holmes, W.N., Redondo, J.L., Cronshaw, J. 1989. Changes in the adrenal steroidogenic responsiveness of the mallard duck (*Anas platyrhynchos*) during early post-natal development. *Comp. Biochemical Physiology* 92A, 403–408.

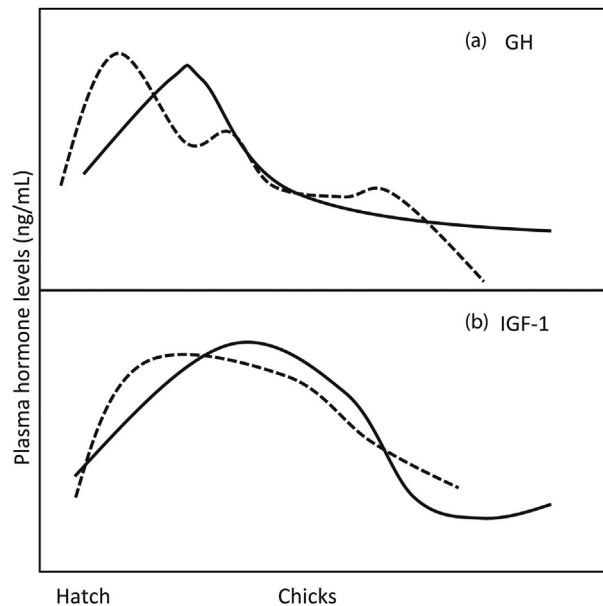


Fig. 5 Patterns of plasma concentrations of (a) growth hormone (GH) and (b) insulin-like growth factor-1 (IGF-1) in altricial and precocial birds. The characteristic patterns shown here are for altricial starlings (dotted lines) and precocial quail (solid lines) from hatch to 4 weeks of age. For references to data on other species see McNabb, F.M.A., Scanes, C.G., Zeman, M., 1998. Endocrine control of development. In: Starck, J.M., Ricklefs, R.E. (Eds.), *Avian Growth and Development. Evolution Within the Altricial-Precocial Spectrum*. New York: Oxford University Press.

aiding in the sudden increase in energetic demands related to leaving the nest. A rise in corticosterone during development is important for maturation of many organs, such as lungs, small intestine, liver, adrenals, and kidneys. For instance, corticosterone stimulates synthesis of surfactant in the lungs, which reduces surface tension and keeps the alveoli open.

The other hormones that are most important in growth and development are growth hormone, the insulin-like growth factors (IGF-1 and IGF-2), and insulin. Growth hormone exerts its effects on overall body growth (which is important in the development of temperature regulation, as indicated above) by stimulating the liver to produce IGFs that are released into the circulation and directly trigger growth in many tissues throughout the body. Circulating concentrations of growth hormone and IGFs have similar patterns, with some shifts in the time scale, in altricial and precocial birds (Fig. 5). Thus, circulating patterns of these growth-promoting hormones do not appear to be the key controlling factors in the different tissue/organ growth patterns observed in these two developmental modes. However, circulating patterns of growth factors may not provide all the relevant information because some tissues independently produce IGFs or other growth factors which have local effects within the tissue. Although insulin and corticosteroids are known to affect some aspects of development in chicken embryos, the effects of these hormones have not been studied systematically throughout development in either precocial or altricial birds. Likewise, differences in how thyroid hormones, growth hormone, or IGFs may be involved in triggering the different functional maturation rates of organs in altricial vs precocial embryos and chicks have not been investigated. Examples of known effects of these hormones on differentiation suggest such studies are likely to aid our understanding of these developmental modes.

Male songbirds or oscines sing songs to attract mates and defend their territory. Males learn species-specific songs during a critical window of development and the sex steroid, testosterone plays an important role in song learning. First, young males must hear the songs of the same species during the sensory period. During this phase, hearing song even for a brief period every day is enough for young males to memorize the tutor's song. Because birds learn rapidly and permanently, sensory learning of song in altricial songbirds is comparable to imprinting on individuals or objects in precocial species. Males then go through a phase where they produce generic and variable sound, similar to human babies babbling. As time passes, young start to produce more structured and less variable song until stereotyped songs are crystallized. As testes grow and secrete testosterone this triggers the crystallization of songs. It is unclear what role testosterone plays in the development of vocalizations in precocial birds.

Incubation Temperature and Avian Development

A key factor in the development of both altricial and precocial embryos is incubation temperature. Incubation temperature is closely linked with the duration of incubation, whereby eggs incubated at lower temperatures take longer to hatch than eggs incubated at higher temperatures. Embryos require a fairly narrow window of temperature for proper development to take place, although species differ in their tolerance to variable incubation temperatures. While it is well known that temperature affects hatching success of eggs for both altricial and precocial birds, more recent research in non-domesticated birds indicates that incubation temperature also affects a multitude of traits in the hatchlings, including size at hatching, growth rates, hormonal profiles, immune responses,

thermoregulation, and some evidence that incubation temperature affects overwinter survival. In general, lower incubation temperatures produce individuals with reduced physiological performance relative to individuals produced at warmer temperatures. Although incubation temperature appears to affect early growth and development of both altricial and precocial species, it is unclear if the two developmental modes differ in their responsiveness to temperature. For instance, altricial species hatch with much less mature tissues than precocial species. It may be that development and maturation remains sensitive to temperature during the post-hatch period in altricial species, but not in precocial species.

Further Reading

- De Groef, B., Grommen, S. V. H., & Darras, V. M. (2013). Hatching the cleidoic Egg: The role of thyroid hormones. *Front. Endocrinol.*, 4, 1–10.
- DuRant, S. E., Hopkins, W. A., Hepp, G. R., & Walters, J. R. (2013). Ecological, evolutionary, and conservation implications of incubation temperature-dependent phenotypes in birds. *Biol. Rev. Camb. Phil. Soc.*, 88(2), 499–509.
- McNabb, F. M. A. (2006). Avian thyroid development and adaptive plasticity. *Gen. Comp. Endocrinol.*, 147(2), 93–101.
- McNabb, F. M. A., Scanes, C. G., & Zeman, M. (1998). Endocrine control of development. In J. M. Starck, & R. E. Ricklefs (Eds.), *Avian Growth and Development. Evolution within the Altricial-Precocial Spectrum*. New York: Oxford University Press.
- Wada, H. (2008). Glucocorticoids: Mediators of vertebrate ontogenetic transitions. *Gen. Comp. Endocrinol.*, 156(3), 441–453.

Drosophila Sexual Behavior

Scott McRobert, Saint Joseph's University, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Alleles Alternative forms of a gene.

Conspecific Belonging to the same species.

Copulation Duration The time during which a pair of animals spends copulating.

Copulation Frequency The number of animals that mate during a mating experiment, expressed as a percentage.

Copulation Latency The time from the onset of courtship until the initiation of copulation.

Courtship Index The amount of time a male spends performing any of the courtship behaviors, expressed as a percentage.

Dipteran A large order of insects typically called flies. The term dipteran means “two wings.”

Ecdosion In insects, the act of emerging from a pupal case.

Female Choice A sexual system in which females are the ‘choosey’ sex, typically leading to the evolution of sexual displays in males. It is thought that females inspect the displays of potential sexual partners, choosing to mate only with the fittest males.

Fixed Action Patterns (FAP's) Highly stereotyped, innate behaviors that occurs in response to a very specific environmental cue, known as a Sign Stimulus.

Fore-tarsi The front pair of legs of an insect.

Genitalia The reproductive organs.

Genotype The entire genetic constitution of an organism.

Gravid The condition of carrying fertilized eggs.

Heterozygous The condition of having two different alleles for a specific gene.

Homozygous The condition of having identical alleles for a specific gene on both homologous chromosomes.

In copula The time during which a pair of flies are physically connected during mating.

Oviposit To lay eggs.

Phenotype The observable characteristics (morphology, behavior, etc.) of an individual. Often discussed as a result of specific genetic characteristics, or genotype.

Pheromone A chemical produced by one individual that alters the behavior of another organism.

Pomace flies Members of the family Drosophilidae, which typically feed on yeasts growing on decaying vegetation.

Sex Combs A set of distinct bristles found on the fore-tarsi of males, in some *Drosophila* species.

Sex-Linked Gene Any gene located on one of the sex chromosomes (typically the X or Y).

Sex Peptide One of the proteins carried in the seminal fluid of *Drosophila melanogaster* males.

Sign Stimulus A specific cue that elicits the production of a fixed action pattern.

Sinusoidal Variation according to a sine wave.

Tephritid Flies belonging to the family Tephritidae. Known as true fruit flies because many species in this group feed on live fruit and are, thus, agricultural pests.

Wildtype Phenotypically normal individuals, as found in nature. This term is often used in contrast to mutant individuals.

Introduction

Drosophila are small dipteran flies that are well known for their use in research and in teaching laboratories, typically in genetics courses. *Drosophilids* are often referred to as fruit flies, although this term can be misleading. True “fruit flies” (typically found in the family *Tephritidae*) are herbivores that feed on live fruit that is still on the vine (see Christensen and Foote (1960)). For this reason, true fruit flies are often agriculture pests. An example of a Tephritid would be *Ceratitis capitata*, also known as the Mediterranean fruit fly, or Med Fly. By contrast, most *Drosophilids* feed exclusively on fruit that has fallen and is rotting on the ground. Because of this, most species of *drosophila* (with a few exceptions) do not pose a threat to crops, which is one of the main reasons they have become such popular experimental organisms. *Drosophila* can be shipped around the world, from laboratory to laboratory, with no fear that escapees will damage local fruit crops. However, most scientists, students, and textbooks still refer to *Drosophila* as fruit flies. Better terms for these flies would be pomace flies, or vinegar flies, addressing their predilection for rotting fruit.

Drosophilids have become one of the most powerful model organisms in the scientific world, owing largely to a number of life history characteristics. They are small, allowing huge numbers to be kept in relatively small spaces (typically small jars and vials). They have a fast life cycle, typically moving from egg to sexually mature adult in just a couple of weeks. They breed readily in

captivity and produce large numbers of offspring from a single mating. They are easy to maintain on simple, relatively inexpensive, food. And, as noted above, most species in this group do not represent a threat to agricultural crops.

For all of the reasons noted above, *Drosophila* became the model organism for early genetics studies in the laboratory of Thomas Hunt Morgan. Around 1907, Morgan collected wild *Drosophila* from bananas placed outside his lab at Columbia University, then brought these animals in and raised them in milk bottles, pilfered from doorsteps in the area. The species Morgan caught is the now-famous *Drosophila melanogaster*, which would become one of the most powerful model organisms in scientific history.

Morgan's work involved the induction of mutations in *D. melanogaster*, and resulted in the discovery of the *white* mutation (abbreviated *w*), which alters the flies natural eye color (bright red) to a remarkably distinct, white. The discovery of this mutation marks a seminal moment in the history of the field of genetics. Morgan found that crosses using flies bearing the mutant *w* allele did not follow standard Mendelian ratios. Further study determined that the *w* gene was located on the X chromosome, thus leading to differences in the inheritance pattern between males and females. This was to be the discovery of the first sex-linked mutation (Morgan, 1910).

However, it was the work of one of Morgan's students, Alfred Sturtevant, that showed just how valuable *Drosophila* would become as a model organism for the study of animal behavior. Sturtevant noted that *Drosophila* males performed a series of display behaviors in response to *Drosophila* females (Sturtevant, 1915). These behaviors would later be described in more detail (see Spieth (1952), Bastock and Manning (1955)) and would be found to be critical to mating success in this species. *Drosophila* females who seem, initially, sexually unreceptive, will eventually become receptive to males who perform the entire sequence of sexual displays in an acceptable manner.

The Sexual System in *Drosophila*

Sexual behavior in *Drosophila* is based on a phenomenon known as female choice. This system, which is found in many animal species, centers around the fact that females are the 'choosy' sex, while males mate indiscriminately with any willing member of the opposite sex. The evolutionary basis of female choice appears to be related to the overall contribution by members of each sex to the next generation. Female contribution is generally greater than that of males, and may include the production of gametes (with eggs being more metabolically costly to produce than sperm), the cost of being pregnant, or gravid, and the potential expenditures associated with parental care (for species that have parental care). All these factors lead to a system in which females actively choose the males with whom they will mate, typically following some form of expression by the males that convinces the female that this male is evolutionarily fit. Often these male 'expressions' take the form of courtship behaviors that males perform in response to potential mates.

Male Behavior

In *Drosophila melanogaster*, male courtship involves a series of distinct behaviors. These behaviors often start with orientation, in the which the male assumes a position in which he is facing the female. If the female moves, the male chases her (known as following). He will attempt to contact her with one of his fore-tarsi (front legs) in a behavior known as tapping. Tapping may provide cues to the male by allowing him to sample chemical compounds on the female's body. The male also produces one of the most critical behaviors within the sexual display by extending and vibrating a single wing (usually the wing closest to the female). Wing vibration produces the courtship song (the behavior is known as singing), which involves a series of sound pulses that interrupt a humming sound (known as 'sine song'). The time interval between the pulses (known as the inter-pulse interval, or IPI) varies in a sinusoidal pattern, which appears to be species-specific and may aid the female in knowing that she is being courted by a conspecific male. The final behavior in the repertoire of *D. melanogaster* males is licking, in which the male attempts to contact the female with his mouth parts, or proboscis (possibly picking up chemicals from her body), and attempted copulation, in which the male curls his abdomen and attempts to contact the female's genitalia with his genitalia.

In terms of the function of courtship, the courtship song, more than any other behavior, has been shown to be crucial to increasing female receptivity to copulation. Females courted by wingless males (males whose wings have been surgically removed), will not copulate, or will take a much longer time to copulate. However, when the courtship song is played through a speaker, a female will copulate with a wingless male as quickly as she would with a normal, intact male. However, the song must be the correct song for her species. In tests with *D. melanogaster* and the closely-related *D. simulans*, females mated most quickly when they heard songs that incorporated the correct IPI for their species (Kyriacou and Hall, 1982).

Female Behavior

In many *Drosophila* species, females do not perform the complex series of display behaviors seen males. Instead, their role is to elicit courtship from males through the production of aphrodisiac pheromones. These chemicals stimulate *Drosophila* males, acting as the sign stimuli that trigger the fixed-action pattern of male courtship behavior. In *Drosophila melanogaster*, mature females produce a 27-carbon aphrodisiac pheromone whose chemical name is (Z,Z)-7, 11-heptacosadiene. This compound, when applied

to a small wad of cotton, can elicit courtship from males. However, visual cues are also important for normal sexual behavior. So, while blind mutant males can court, and wildtype males can court in the dark, these situations do not produce typical sexual behavior. Males in these circumstances have difficult time tracking the females when they run away.

For the most part, in a species like *Drosophila melanogaster*, females initially move away from a courting male. In fact, the majority of female sexual behaviors are related to their initial unwillingness to mate. Unreceptive females kick at courting males, and flick their wings at them. However, once pursued, the male's song appears to cause the female to slow down and stop performing rejection behaviors. Eventually a courted female stops altogether and, when receptive, opens vaginal plates to allow the male to mate with her. It appears that in terms of copulation, females are completely in control.

In many *Drosophila* species, copulation causes dramatic changes in the biology of the female. These changes are caused by chemicals provided by the male during copulation. In *D. melanogaster* the major effects are caused by a chemical known as sex peptide, which the male delivers to the female prior to delivering sperm. Within hours of copulation the effects of this chemical can be detected in the "mated female." She stops production of the aphrodisiac pheromone, causing her to be less attractive to other males. She also undergoes changes that make her less likely to be sexually receptive if a male does court her. These effects result in the female becoming an essentially non-sexual organism and last (again, in *D. melanogaster*) for about 5 days (see [Chapman et al. \(2003\)](#)).

The evolution of compounds like sex peptide, delivered by males, to females, during copulation, are likely a result of a phenomenon known as sperm competition. Since courtship is such a costly process for the males, in terms of time and energy, it is crucial that a male protect his investment in the next generation. If a recently-mated female was to copulate with another male, she might use his sperm preferentially over the sperm of the male who originally mated with her. Therefore, a system has evolved in males to provide chemicals that reduce the chances that the females with whom they mate will re-mate again quickly. The effects of compounds, like sex peptide, act for a period of days, during which the female lays eggs fertilized by the male that provided the sex peptide along with his sperm. She may lay hundreds of eggs before the effects of sex peptide wear off. She then becomes sexually attractive, and sexually receptive, again. The negative to this entire process is that sex peptide has been shown to shorten the life of females. Thus, we may someday see the evolution of systems within the female to counteract the effects of sex peptide.

Other Species

Drosophila melanogaster has become the most well-known *Drosophila* species, owing largely to chance as this was the species collected by Thomas Hunt Morgan. However, there is no reason to believe that *D. melanogaster* is the best representative of the entire *Drosophila* family. In fact, as would be expected in any taxonomic group, significant differences exist amongst *Drosophilids* in a number of systems, including reproductive behavior.

For example, the reproductive behavior of the species *Drosophila biarmipes*, found in Southeast Asia and India, differs dramatically from that of *D. melanogaster* (see [McRobert et al., 1997](#)). *D. biarmipes* males perform many of the same courtship behaviors as *D. melanogaster*, including orientation, following, tapping, wing extension and vibration, and attempted copulation. However, in addition to that repertoire of displays, *D. biarmipes* perform a distinctive wing display behavior in which they extend both wings, tilt them to the side, and circle in front of the female. And while the function of this behavior is not known, the conjecture is that they are literally showing their wings to the female. This may be important as the males have a single, distinct, black spot on each wing. And the wing spots are sexually dimorphic in that females do not have them. Since wing spots are not found in all *Drosophilids*, it seems possible that displaying the wing spots is necessary to show the female that she is being courted by a male of her species.

What's more, the sexual behavior of *D. biarmipes* has another dramatic difference when compared to *D. melanogaster*. This involves the post-mating behavior of females. We'll recall that *D. melanogaster* females undergo a series of changes following mating, including a reduction in sex appeal (mediated by sex peptide, which shuts off production of the aphrodisiac pheromone), and reduction in sexual receptivity, that last approximately five days. Following that period of time, *D. melanogaster* females regain their sexual attractiveness and their receptivity to courting males. In *D. biarmipes*, however, while females undergo a similar set of changes following mating, including loss of attractiveness and receptivity, the restoration of normal female behavior is quite different. While attractiveness returns, *D. biarmipes* females remain unreceptive to copulation for at least 14 days post-mating. This observation suggests that *D. biarmipes* females are capable of storing, and utilizing sperm for a longer period of time than that seen in *D. melanogaster*.

Interestingly, another Asian species, *D. suzukii*, which is closely related to *D. biarmipes*, shares many characteristics of the *D. biarmipes* reproductive behavior. *D. suzukii* males, who also have a single black spot on each wing, also perform the wing-display behavior. In addition, *D. suzukii* females also lose, then quickly regain, their sex appeal following mating. But, like the situation in *D. biarmipes*, *D. suzukii* females do not regain their sexual receptivity with 14 days of mating. The interest in the reproductive behavior of *D. suzukii* stems from the fact that this species is a true fruit fly, with females ovipositing in live fruits like blueberries, strawberries and cherries. *D. suzukii* has become a destructive invasive species causing huge losses in agricultural crops (see [Cini et al. \(2012\)](#)).

The Genetics of Reproductive Behavior in *Drosophila*

Since *Drosophila* became one of the most important model organisms for the study of genetics in the 20th Century, it seems reasonable that a fair amount has been discovered about the genes affecting sexual behavior in this species (see [Sokolowski \(2001\)](#)).

As techniques to create mutations and manipulate different aspects of the genome developed, some very creative studies came about in the field of behavioral genetics. These studies were more complex than standard genetic analysis, which often involves mutagenesis (creation of mutations through the feeding of chemicals that disrupt DNA replication), and then looking for visible mutations in the offspring (things like white eyes or missing wings). In behavioral genetics, the potential mutants must go through a screening process that involves observation of the behavior of the offspring to identify alterations in normal behavior (see [Vosshall \(2007\)](#)).

A number of genes have been identified that have an impact on normal sexual behavior in *Drosophila* (see [Greenspan and Ferveur \(2000\)](#)). Not all of these genes are, however, specifically involved in sexual behavior, but rather alter aspects of the fly which affect other systems and impinge on sexual behavior. For instance, the *white* mutation, mentioned earlier, affects vision. Males expressing the *white* mutation cannot see as well as normal flies and, while they identify females and initiate courtship through olfactory cues, they have a difficult time following when the female moves away. This affects their courtship success. So, while this gene has effects on courtship, it is not specifically a 'courtship gene.'

Other genes, again, not specifically sexual genes, can affect the male courtship song and, thus, affect sexual behavior. A mutation known as *raised* (*rsd*) affects thoracic muscles and causes the wings of a fly to be held straight up. Affected individuals cannot fly, and *rsd* males cannot extend and vibrate their wings to produce the courtship song. It has been shown that *rsd* males are less successful in their courtship of wildtype (normal) females, due to their inability to sing. However, *rsd* females mate at normal rates with *rsd* males, probably indicating a form of artificial selection within laboratory cultures.

Another mutation that affects the courtship song is known as *period* (*per*). The *per* gene is involved in all rhythmic activity within the fly, almost like an internal timer. Behaviors affected by *per* include wake/sleep cycles and the timing of emergence from the pupal case (eclosion). Another behavior that appears to be rhythmic is the courtship song. The intervals at which the male produces bursts of song oscillate sinusoidally according to a species-specific rhythm. Males expressing mutant *per* phenotypes perform abnormal song. Some *per* alleles result in a faster song rhythm, some *per* alleles result in a slower song rhythm, and some *per* alleles abolish the song rhythm completely.

A true courtship gene, however, is *fruitless* (abbreviated as *fru*). This gene appears to be the central, controlling gene, for male sexual behavior in *Drosophila*. The *fru* gene produces a protein (FruM) that is expressed in neurons within the brains of *Drosophila* males, but not in the brains of females. And it is signals from these neurons that result in the series of courtship behaviors that males perform in response to females. Abnormalities in the function of the *fru* gene lead to abnormalities in sexual behavior. For instance, males who are homozygous for the *fru* mutation court other males as strongly as they court females, often resulting in long lines of males courting each other. Furthermore, when *Drosophila* females were genetically engineered to express FruM in their brains, they performed male courtship behaviors (which are never seen in normal females).

Measuring Reproductive Behavior in *Drosophila*

Within the field of Animal Behavior, *Drosophila* are ideal organisms. They will produce complex reproductive behaviors, by both males and females, under the artificial conditions provided within the laboratory. Indeed, this would be rare in species that require specific environmental factors, such as natural food sources, or seasonal changes in temperature, light, or humidity. Creating captive conditions under which these species will perform courtship and mate is almost impossible. In addition, many species are simply so intimidated by captive conditions, or by the specter of a scientist watching them, that normal courtship and mating behaviors will never be performed. But this is not the case with *Drosophila*. If a single, sexually mature male is placed into a container (practically any container!), with a sexually mature female of the same species, he will almost certainly perform the entire complicated courtship display. It is then a simple matter of observation, either by naked eye, through a microscope, or by means of video camera, for the scientist to collect data on a wide variety of parameters. And thus, the reproductive behavior of *Drosophila* has been extensively studied.

Typical courtship studies often involve collection of very young, sexually immature, flies. This is easily done by "clearing" the stock bottles of all adults, then checking the bottles within a few hours. At that point, any adults in the bottle will have enclosed from their pupal cases since the clearing, and are thus known to be young, virgin flies. The young flies are then separated by sex (in many *Drosophila* species, males are morphologically distinct from females. For example, *D. melanogaster* males are slightly smaller than females, have dark pigmentation on the tip of their abdomens, and have a set of distinct bristles, called 'sex combs,' on their fore-tarsi), and males and females housed in separate vials until testing.

Assays on *Drosophila* courtship are performed when the flies are sexually mature (for *D. melanogaster* tests are often run at 2–5 days post-eclosion). At this point a male and female can be placed into an observation chamber (almost anything that contains the flies and enables the researcher to observe them will work – a glass vial, and small plastic well with a microscope cover slip across the top, etc.) and observed. To assess the ability of the male to court (which is sometimes important to examine the effects of mutations), or the female's ability to elicit courtship, the courtship index is determined by running a timer when the male is performing any of the courtship display behaviors within a designated observation period. For flies that mate, other measures can be determined, including copulation latency (the amount of time until copulation begins), copulation duration (the amount of time the pair remain *in copula*), and copulation frequency (the percentage of couples that mate within a given period of time). By running such assays, repeatedly, a tremendous amount of information can be gained on the courtship behavior of different *Drosophila* species, or on the effects of mutations, age, and environmental parameters such as temperature, on courtship.

References

- Bastock, M., & Manning, A. (1955). The courtship of *Drosophila melanogaster*. *Behaviour*, 8, 85–111.
- Chapman, T., Bangham, J., Vinti, G., et al. (2003). The sex peptide of *Drosophila melanogaster*: Female post-mating responses analyzed by using RNA interference. *Proc. Natl. Acad. Sci.*, 100, 9923–9928.
- Christensen, L. D., & Foote, R. H. (1960). Biology of fruit flies. *Ann. Rev. Ent.*, 5, 171–192.
- Cini, A., Ioriatti, C., & Anfora, G. (2012). A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. *Bull. Insectol.*, 65, 149–160.
- Greenspan, R. J., & Ferveur, J.-F. (2000). Courtship in *Drosophila*. *Ann. Rev. Gen.*, 34, 205–232.
- Kyriacou, C. P., & Hall, J. C. (1982). The function of courtship song rhythms in *Drosophila*. *Anim. Behav.*, 30, 794–801.
- McRobert, S. P., Adams, C. R., Wuttke, M., Frank, J., & Jackson, L. L. (1997). A comparison of female postcopulatory behavior in *Drosophila melanogaster* and *Drosophila biarmipes*. *J. Insect Behav.*, 10, 761–770.
- Morgan, T. H. (1910). Sex-limited inheritance in *Drosophila*. *Science*, 32, 120–122.
- Sokolowski, M. B. (2001). *Drosophila*: Genetics meets behavior. *Nat. Rev.*, 2, 879–890.
- Spieth, H. T. (1952). Mating behaviour within the genus *Drosophila* (Diptera). *Bull. An. Mus. Nat. Hist.*, 99, 401–474.
- Sturtevant, A. H. (1915). Experiments in sexual recognition and the problems of sexual selection in *Drosophila*. *J. Anim. Behav.*, 5, 351–366.
- Vosshall, L. B. (2007). Into the mind of a fly. *Nature*, 450, 193–197.

Behavior: Mate Choice and Sexual Selection

Leslie A Knapp, University of Utah, Salt Lake City, UT, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Mate choice can be simply defined as any pattern of behavior shown by members of one sex that leads to preferred mating with certain members of the opposite sex. Studies of mate choice and mating behavior have focused on a wide range of organisms. In some species, such as sage grouse and peacocks, courtship displays serve a critical role in attracting potential partners. These displays lead to mating opportunities and successful reproduction. In some cases, however, the display might not be comparable to other members of their sex and the potential partner may choose another, more attractive, partner. This type of mate choice is typically known as sexual selection. It has been used to explain mate choice in male and female animals and also men's and women's particular mating strategies.

The Evolution of Sexual Selection Theory and Its Relevance to Mate Choice

Sexual selection theory was put forward by Darwin (1871). Darwin's theory of evolution by natural selection concentrated only on adaptation in the context of particular environments. Elaborate traits and dangerous courtship rituals did not fit the model of natural selection. However, he recognized that some traits might actually threaten an individual's survival and, yet, those traits persisted. He called these traits secondary sexual characters. These traits function in reproduction but they are not necessary for reproduction. Brightly colored feathers are an example of secondary sexual characteristics but ovaries and testes are primary sexual characteristics.

Darwin argued that inherited traits which helped when competing for sexual mates will increase in frequency over time, even if they compromise survival. He called the process sexual selection and argued that it was not a subcategory of natural selection because it arises from differences in mating success specifically. Sexual selection can be anything from heightened senses when searching for a mate to decorative ornaments, weapons and signals.

According to Darwin, there were two types of sexual selection: mate choice and competition for mates. Elaborate traits were the consequence of competition and also choice for particular mates. He noted that these two strategies were not mutually exclusive. He recognized that male-male competition and female choice were interrelated because mate choice by one sex usually results in competition between the members of the other sex. The competition might be physical fights or aggressive displays directed toward competitors of the same sex or through indirect competition that resulted in gaining access to fertile females before other males.

More often than not, Darwin noted, males, rather than females, spent more time on mating competition and courtship behavior. He cited dozens of examples of species where males competed harder for mates than females did. He observed male-male competition among peacocks and other birds, for example. Male-male competition is known as intra-sexual selection. In these cases, males exhibit competitive behaviors and/or display physical features. Those with a competitive advantage typically achieve greater reproductive success by gaining access as many females as possible.

In many species males compete *before* copulation. In addition to elaborate displays, traits associated with male-male competition before copulation include body size and testes size. Body size is advantageous in fights, especially when fighting is important in gaining access to females. When males grow larger than females this is known as sexual dimorphism. In monogamous species, like ourselves, there is relatively little sexual dimorphism. If there is intense sexual selection, male canines may also be very large compared to females. This is another example of sexual dimorphism. Additional examples include horns, tusks, and spurs. Males may also compete with each other by choosing and guarding a particular territory or by building an attractive nest. As mentioned above, some males may also compete for mates by exhibiting traits that represent status. Classic examples in primates include male silverback gorillas (Harcourt and Stewart, 2007) and brightly colored male mandrills (Setchell and Dixon, 2001).

Other species may compete *after* copulation. Male-male competition also includes sperm competition and larger testicles are frequently observed in multi-male social groups that have equal access to fertile females. In species where females mate with multiple males, often in succession, males may need to produce large amounts of sperm to increase their reproductive success. Some examples of primates with large testes include chimpanzees (Boesch, 2009), woolly spider monkeys (Milton, 1985) and some species of howler monkeys (Dunn *et al.*, 2015). Millions of sperm can be produced repeatedly and, therefore, there is little investment in the production of male gametes. This has important consequences for male investment in physical and behavioral traits to attract potential mates (Table 1).

In species where multiple matings occur, a male cannot be certain that a female's offspring are fathered by him. So, generally, males also spend relatively little time caring for, and investing in, their offspring. Instead, investment in traits that are attractive to females will enhance a male's mating success. Based upon decades of research, Darwin's observation that males make greater effort to attract females and they often engage in fierce male-male competition holds true. Males also compete most intensely when female parental investment greatly exceeds that of males and/or when there are many more males than females in a social group.

One other way to capture a female's attention and increase the chance of her choosing him is with "nuptial gifts." Male bowerbirds construct a so-called "love nest" to attract females for mating. When males transfer resources to females this may affect how

Table 1 The Theory of Sexual Selection predicts that females and males have different reproductive strategies due to differences in reproductive resource availability

<i>Why male-male competition & female choice?</i>		
	<i>Female gametes</i>	<i>Male gametes</i>
Number	Few	Many
Size	Large	Small
"Cost"	"Costly"	"Cheap"
Subsequent Investment	High	Low

females produce eggs, choose partners, and even select sperm to fertilize eggs. Nuptial gifts are offered by males of numerous species, particularly insects, but there remains some debate regarding the primary function of food gifts on female reproduction and offspring survival. In some cases, a nuptial-gift-type display may be seen in some human populations.

Typically, it is believed that females are the ones who choose the partner and, as a rule, females do seem "choosy" when selecting a mate. One important reason is that females invest so much in each gamete compared to males. A female's egg is large, nutrient-filled and "expensive" to produce (Table 1). Studies of birds show that bird eggs can constitute 25% or more of a female's body weight. Mammalian ova are also limited in number and are produced infrequently. In species with internal fertilization the female carries the egg after fertilization. Since males are not anatomically tied to the egg they are typically thought to be "free" from parental care. Female mammals produce the placenta, while also providing nourishment for the embryo(s). Later, females are the ones that typically provide parental care and they produce milk after birth. In many bird species the female cares for the young after they hatch. In some species, like nonhuman primates, there is a prolonged juvenile period that requires further investment from the mother. In light of these factors, it should not be surprising that females carefully select their mates to lower the chance of losing their own reproductive investment.

A variety of factors may come into play when a female chooses a mate. It is well-documented that female birds prefer more intense or more varied courtships, favoring males with larger, more brightly colored ornamentation, more extreme rates of acoustical song or a combinations of these factors. More generally, factors can be broadly divided into direct benefits that increase female fecundity or lifespan and indirect benefits that increase the quality of offspring. One example of an indirect benefit is the production of sons that will be more attractive to females in the future, ideally these sons will inherit the attractive traits from their father. This is known as Fisher's effect (Fisher, 1930).

Females may prefer certain males for many different reasons. One preference put forward in the literature is "good genes," meaning the male has attributes that predict survivorship of offspring or possessing resources. This is known as the Zahavi handicap model (Zahavi and Zahavi, 1997). In this model, females choose males with handicaps because males with handicaps must have "good genes" in order to survive, even though they possess the fitness-reducing handicap. An example of a handicap is a peacock's tail because it may reduce fitness through the process of natural selection. It has been argued that the handicap model might evolve if the female's advantage of mating with males possessing "good genes" outweighs the cost of her offspring having to carry this handicap around in the future.

For the most part, elaborate female ornamentation has been overlooked because of the prevalence of elaborate displays in males. Females are generally not expected to invest in ornamentation unless the fitness benefits of the ornament exceed those from investing the resources directly into offspring. Circumstances under which mammalian females would benefit from honestly signaling their quality are limited. Research on nonhuman primates provide equivocal support that females signal their own quality through brightly colored sexual swelling during estrous (Higham and Petersdorf, 2017). When sex roles are reversed and males provide parental care, females exhibit elaborate and colorful displays to represent honest signals of quality.

Since Darwin's time, research on sexual selection has increased exponentially. Numerous models of mate choice evolution have been suggested. In addition to the Fisher model (females prefer a trait in males and male offspring inherit the trait to attract mates in subsequent generations), there is the Bateman model (1948) that emphasizes the difference between sexes in gamete size while making the point that male fitness would be highly variable and increased with each mating but female fitness was less variable and increased minimally. A comprehensive discussion of all mate choice models is beyond the scope of this article, however when considering human mate choice there are several models to highlight. These types of models focus on the information communicated by signals. For example, there is a condition-dependent indicator model arguing that females will prefer "good genes" which are a reliable indicator of genetic quality. There is also a genetic compatibility model that suggests females choose males who complement their own genome. The Trivers model (1972) states that the greater the level of parental investment required, the more stringent the criteria that required of potential mates. Another classic model relevant to sexual selection is the "Red Queen" hypothesis (Van Valen, 1973). This suggests a sort of co-evolutionary relationship between hosts and their parasites. The expectation of the "Red Queen" model is that sexual reproduction and recombination of genetic material from parents provides their offspring with the ability to evade infection with their novel combinations of genes.

“Marketing” and Mate Choice

Darwin, and researchers that followed him have, noted that intrasexual competition and intersexual mate choice are not mutually exclusive. If one individual draws the interest of a potential mate, a decision must be made by each party whether or not the individual is a potentially suitable mate. In most cases, the male will assess the female’s value and determine whether it is worth offering a resource incentive sufficient to prevent her from mating with another, alternative, male with whom he is, or could potentially be, in competition. Contrastingly, the female will assess this offer relative to offers potentially available elsewhere in order to determine if she should accept, or reject, the male’s offer. A male’s offer to a female is assumed to come at the expense of opportunities to mate with additional females. A female’s decision to accept the offer is assumed to prevent her from capitalizing on other mating opportunities.

For sexually reproducing species, it is important for individuals to accurately evaluate the phenotypic condition and genetic quality of potential mates. Individuals should also be adapted to display their own condition and quality, frequently through investment in sexual ornaments that serve as fitness indicators or costly signals. Ever since Darwin’s work on sexual selection, the bulk of research has focused on sexually dimorphic ornaments, usually male ornaments that evolve through female choice. Nevertheless, both sexes can show conspicuous, costly ornaments and elaborate courtship behaviors. Frequently, these types of ornaments or behaviors are non-dimorphic. They are said to serve as “species recognition markers” that arise through mutual mate choice to indicate genetic quality and phenotypic condition (Miller, 1998).

Human Mate Choice and Sexual Selection

In his book *The Descent of Man, and Selection in Relation to Sex*, Darwin argued that sexual selection played a major role in the evolution of humans and in the development of human populations. He noted that men’s and women’s mating strategies were highly variable and were the result of numerous interacting contextual cues. He explained how male-male competition for access to mates would work in humans. He specifically stated that sexual selection arises from differences in reproductive success caused by competition for access to mates. He noted that men were physically larger, taller, and stronger. He suggested that more body hair and a darker complexion might be the result of male-male competition. Darwin also pointed out that men’s and woman’s bodies were very different. Women are rounder and they generally have less body hair than men. He noted that women mature earlier and develop their secondary sexual traits, such as breasts, before men. He said that most men do not develop distinct sexual characteristics, such as beards, until they are fully mature.

Surprisingly, Darwin did not discuss how or why females made particular mate choices. At the time, it appears that he, and contemporary scholars, were less interested in the issue of female choice or perhaps they believed females made choices were based solely on the outcome of male-male competitions. Darwin did note, however, that female animals exercised mate choice and sexual selection through mate choice was comparable to artificial selection by humans. Interestingly, Darwin’s ideas put females in a very powerful evolutionary role, even though this was not widely appreciated by male Victorian biologists at the time.

For the most part, research over the past few decades has focused on mate choice as the primary mechanism for human sexual selection. Like other female mammals, women invest more in offspring than men do through gestation and nursing for up to several years. Before conception, women also expend energy in the process of gamete production. These investments are similar to what has been observed for other female mammals. When considering male investment, however, men invest heavily in offspring compared with most male mammals. This is especially true in Western culture with a history of sexual monogamy, which is relatively rare in other mammals. In humans, socially monogamous individuals pair up to mate and raise offspring at least temporarily.

How Do We Choose Our Partners?

Similar to other male mammals, men are usually larger, stronger, faster, and more physically aggressive than women. Although it might not be immediately apparent, the degree of sexual dimorphism in these traits rivals that of species with obviously intense male contests. Men may seem just modestly taller, larger and stronger when compared to women. However, when female body fat is discounted, sexual dimorphism is obvious. Men are about 50% larger than women and it has been suggested that this is the result of male-male competition. Interestingly, in humans we also see a basis for female competition since men invest heavily in offspring when compared to most other male mammals.

Visual cues for intrasexual competition, and intersexual attraction, played a major role in Darwin’s discussion of sexual selection. For the animal kingdom, he cited peacock’s tails and red male mandrill faces. More recent research provides us with examples of the relative importance of features, and colors, in humans. Symmetry, or the extent to which one-half of an image is the same as the other half, provides information about development and health. If individuals develop successfully, and symmetrically, in the face of environmental pressures, this is believed to indicate genetic quality. Some research reports that male facial symmetry and perceived masculinity correlate positively. Both are said to predict underlying genetic quality. Any deviation from perfect symmetry is believed to be a sub-optimal solution to issues that potentially include inbreeding, mutation, homozygosity, nutrition and/or pathogen load. Not all studies have found a relationship between symmetry and attractiveness. Nevertheless, preference for symmetry can be associated with benefits that include providing healthy genes to offspring and/or avoiding infection from potential partners.

While symmetry is a feature relevant for male and female faces, studies of specific features of male and female faces have also been examined with reference to mate choice. In some cases women prefer both slightly feminine-looking (Perrett *et al.*, 1998) and slightly masculine-looking (DeBruine *et al.*, 2006) male faces. At this point there is no consensus on this topic. Nevertheless, it has been suggested that facial masculinity represents markers of social and physical dominance. As a consequence, faces might advertise relevant information to mates and same-sex competitors by providing valuable information about mate quality and dominance. It has also been suggested that female preference may vary throughout the reproductive period. For example, females may prefer a masculine face when ovulating and then may prefer a more feminine face to help rear the offspring.

Feminine female faces are perceived as most attractive by males. These faces would be the product of high levels of estrogen and could communicate both fertility and good health. Research suggests that females may have evolved neotenic (baby-like) faces, high-pitched voices and fatty breasts and hips as sexual ornaments to attract male investment. Studies of male preferences also reveal that males prefer an “hourglass” shaped figure, known as a low waist to hip ratio (WHR). WHR reflects the measurement of the circumference of the waist and hips. WHR has been used as an indicator or measure of health, and also the risk of developing serious health conditions in the future. While WHR preferences may vary across human populations, WHR correlates with fertility in females and indicates pubertal onset, sex, fertility, hormonal irregularities, and/or differentiates male from female.

Another way to attract, or provide cues to, potential mates relates to the olfactory system. Humans are not known for their particularly keen sense of smell but numerous studies have reported that humans have distinct odor preferences, especially with reference to “attractiveness.” These studies followed on from research reporting that rodents prefer odors from genetically different members of the opposite sex. The preferred odors came from individuals that differed with respect to genes involved in immune response (Table 2). These genes are generally known as the major histocompatibility complex (MHC). Thus far, none of the relatively few MHC human odor preference studies have been replicated and there is no clear consensus among researchers.

There have been studies of odor preferences among women on and off the contraceptive pill. In these studies, women are asked to identify their body odor preference by smelling tee shirts worn by men over consecutive nights. Ten studies have also examined the degree of MHC dissimilarity between established couples (Table 2). Few of these studies found a relationship between MHC genetics and partner choice. Perhaps this is a consequence of the MHC being so variable in urban populations and, because of this, it could be difficult to find anything other than MHC dissimilarity except when studying small, closed populations. Thus far all of this research, while potentially interesting and important for contraception and infant health, has not yet clarified the relationship between odor preference and mate choice in humans. Importantly, there are potentially serious consequences of disrupting adaptive mate preferences by the use of hormonal contraceptives. These include fertility problems due to MHC allele-sharing among couples (Ober *et al.*, 1998) and high levels of MHC homozygosity in offspring successfully conceived.

In the early 1980s, Hamilton and Zuk (1982) hypothesized that females discriminate in favor of males bearing genotypes that provide them with resistance to parasites. An important feature of this model is that females select males according to indicator traits that reveal the mate’s infection status. Research on mate choice in birds and fish demonstrate that females can indirectly determine unhealthy males by selecting brightly colored mates, but this observation doesn’t always hold up. According to the “good genes” hypothesis, women should also be attracted to men who can provide the best genes for their offspring. If women choose mates with dissimilar MHC genotypes they are likely to produce offspring with a healthy immune response and thereby increase their own fitness. Although MHC similar partners may provide other resources, MHC dissimilar mates provide better genes. Instead of selecting for arbitrarily “good” genes, the search for heterozygosity. This argument is based on numerous studies of animals that demonstrate how MHC diversity is associated with resistance to parasites and pathogens.

Most studies have found that women are “attracted to” MHC diverse and MHC dissimilar potential mates. This is explained by referring to either the “good genes” or the “Red Queen” theories of sexual selection; although these are not mutually exclusive. When considering the “good genes” hypothesis, women may be attracted to MHC heterozygous men because these men are more likely to be healthy and to transmit better genes. In the context of MHC genes, the “Red Queen” hypothesis attributes the benefit of a polymorphic MHC to producing MHC heterozygous offspring to evade parasites.

Table 2 Studies of mate preference in humans provide inconclusive support for our reliance on odors, facial appearance or an MHC genetic basis for potential partners

Trait	Studies of Human Mate Choice		
	Number of studies	Number of individuals	Preference
Odor Preference	8	529	Disassortative N=5 None N=3
Facial Preference	7	497	Assortative N=1 MHC Heterozygosity N=2 None N=4
Partner Preference	10	6582	Assortative N=1 Disassortative N=2 None N=7

How Do Our Primate Relatives Choose Their Partners?

Sexual selection typically produces ornaments in response to mate choice and armaments in response to male–male competition. Unusually among mammals, many nonhuman primates exhibit color signals that may be related to one or both processes. Some primates are noteworthy for displaying brightly colored skin. At first glance, these colorful ornaments might appear analogous to those of birds and fish, where it might be aimed at attracting the opposite sex. A number of studies of primates, however, have shown that these traits indicate social status and are like many other mammalian sexually selected traits selected primarily through male–male competition. Primates that exhibit these color-based traits include mandrills, gelada baboons, drills, and vervet monkeys (see review in [Setchell, 2015](#)).

Whether these signals are also important in female mate choice is less clear. In rhesus macaques, for example, male coloration does not correlate with social status but is used instead for female mate choice. In mandrills, male coloration is involved in both male–male competition ([Setchell et al., 2008](#)) and female mate choice ([Setchell, 2005](#)). A study of drills found evidence that male coloration is involved in communicating social status and not in attracting females beyond the consequences of dominance rank ([Marty et al., 2009](#)). For either purpose, however, coloration serves an important purpose in the context of sexual selection.

Conclusion

Darwin, and his contemporaries, and indeed more recent biologists, have collated a substantial amount of evidence to support the concept of sexual selection. These data support the fundamental truth of Darwin's hypothesis, but many questions remain. Two areas that remain underexplored is female preference and female reproductive competition. Similarly, there is much to learn about the relationship between sexual-signal honesty, genotype and environment factors.

References

- Bateman, A. J. (1948). Intra-sexual selection in *Drosophila*. *Heredity*, 2, 349–368.
- Boesch, C. (2009). *The Real Chimpanzee: Sex Strategies in the Forest*. Cambridge University Press.
- Darwin, C. (1871). *The Descent of Man and Selection in Relation to Sex* (vol. 1998, p. 641). New York: Prometheus Books (originally published London 1871).
- DeBruine, L. M., Jones, B. C., Little, A. C., et al. (2006). Correlated preferences for facial masculinity and ideal or actual partner's masculinity. *Proc. Biol. Sci.*, 273(1592), 1355–1360.
- Dunn, J. C., Halenar, L. B., Davies, T. G., et al. (2015). Evolutionary trade-off between vocal tract and testes dimensions in Howler Monkeys. *Current Biology*, 25(21), 2839–2844.
- Fisher, R. A. (1930). *The Genetical Theory of Natural Selection*. Oxford: Clarendon Press.
- Hamilton, W. D., & Zuk, M. (1982). Heritable true fitness and bright birds: A role for parasites? *Science*, 218, 384–387.
- Harcourt, A., & Stewart, K. J. (2007). *Gorilla Society: Conflict, Compromise, and Cooperation Between the Sexes*. University of Chicago Press.
- Higham, J. P., & Petersdorf, M. (2017). Sexual Swellings. *The International Encyclopedia of Primatology*. John Wiley & Sons, Inc.
- Marty, J. S., Higham, J. P., Gadsby, E. L., & Ross, C. (2009). Dominance, Coloration, and Social and Sexual Behavior in Male Drills *Mandrillus leucophaeus*. *International Journal of Primatology*, 30, 807.
- Miller, G. F. (1998). How mate choice shaped human nature: A review of sexual selection and human evolution. In C. Crawford, & D. Krebs (Eds.), *Handbook of evolutionary psychology: Ideas, issues, and applications* (pp. 87–130). Lawrence Erlbaum.
- Milton, K. (1985). Mating patterns of woolly spider monkeys, *Brachyteles arachnoides*: Implications for female choice. *Behavioral Ecology and Sociobiology*, 17(1), 53–59.
- Ober, C., Hyslop, T., Elias, S., et al. (1998). Human leukocyte antigen matching and fetal loss: Results of a 10 year prospective study. *Human Reproduction*, 13, 33–38.
- Perrett, D. I., Lee, K. J., Penton-Voak, I. S., et al. (1998). Effects of sexual dimorphism on facial attractiveness. *Nature*, 394, 884–887.
- Setchell, J. M. (2005). Do female mandrills prefer brightly colored males? *International Journal of Primatology*, 26(4), 715–735.
- Setchell, J. M. (2015). Color in competition contexts in non-human animals. *Handbook of color psychology* (pp. 546–567). Cambridge: Cambridge University Press (Cambridge Handbooks in Psychology).
- Setchell, J. M., & Dixon, A. F. (2001). Changes in the secondary sexual adornments of male mandrills (*Mandrillus sphinx*) are associated with gain and loss of alpha status. *Horm. Behav.*, 39, 177–184.
- Setchell, J. M., Smith, T., Wickings, E. J., & Knapp, L. A. (2008). Social correlates of testosterone and ornamentation in male mandrills. *Hormones and Behavior*, 54, 365–372.
- Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed.), *Sexual selection and the descent of man* (pp. 136–179). Chicago: Aldine.
- Van Valen, L. (1973). A new evolutionary law. *Evolutionary Theory*, 1, 1–30.
- Zahavi, A.M., Zahavi, A.V., 1997. *The Handicap Principle: A Missing Piece of Darwin's Puzzle*. Oxford.

Courtship and Mating

Clodhna Quigley and Leonida Fusani, University of Veterinary Medicine, Vienna, Austria; and University of Vienna, Vienna, Austria

© 2018 Elsevier Inc. All rights reserved.

Courtship and Mating

What Is Courtship?

Courtship can be defined as the behavior used to obtain copulation with a partner, or to maintain reproductive interactions with an existing partner. From the shimmering tail of the peacock to the chorus of frog song during the mating season, many examples exist, including the extremely elaborate bowers created by male bowerbirds and the acrobatic dance routines performed by tropical birds such as manakins. It should be noted that courtship is particularly important for terrestrial animals for whom internal fecundation is necessary as gametes cannot survive outside of water. This places an emphasis on an agreement between partners in the mating context.

The ornaments and behavioral patterns that constitute courtship for a given species may differ considerably between the male and the female. It was such differences between the sexes that inspired Darwin (1882) to propose sexual selection as an explanation for the inheritance of such traits by means of the advantage they confer when it comes to competition for reproduction. As well as competition between males for access to females, he emphasized the role of female choice among prospective male mates based on their 'love-antics'. As such, courtship can be seen as the behavior on which mate choice is based. Indeed, the degree of difference in appearance between the sexes (sexual dimorphism) is a good indication of the degree of sexual selection within a species. The complexity of courtship behavior is also often related to strength of sexual selection.

Courtship displays are particular behavioral patterns that reappear within an individual animal in the reproductive context, with different individuals of the same species exhibiting similar behavior. In one of the earliest reports on courtship in the framework of animal behavior research, Julian Huxley (1914) described the typical sequential stages of the Great Crested Grebe's (*Podiceps cristatus*) joint choreographies that occur in the time between formation of a mating pair and copulation and successful fertilization. The male and female of a pair take part in several different recurring displays, all involving set patterns of mutual behavior, including head shaking, diving, and particular body postures. Huxley was intrigued that their bouts of courtship behavior did not lead directly to copulation and therefore could not be said to play a purely excitatory role. As the birds share parental care, he inferred that these 'ceremonies' were a self-exhausting expression of emotion that served to cement the pair bond. This underlines the dual role of courtship – as well as allowing an animal to choose the best mate, it can also strengthen the bond between a monogamous pair.

It is clear that such behavior is a central part of animal communication (see Patricelli and Hebets, 2016, for a recent overview). Courtship is usually a complex trait with multiple components, often spanning different sensory modalities and including motor behavior as well as acoustic, visual, olfactory, and/or tactile components. To date, there is no agreed-upon explanation for the existence of multiple courtship traits. Multisensory signals may function as redundant signals or backup systems, or as multiple independent messages, and may mutually amplify or otherwise interact with each other. Even within a sensory modality, different parameters of the signal can carry differing information. It has recently been proposed that the sensory and cognitive properties of the animals receiving the transmitted signals might exert an influence on the evolution of elaborate courtship displays, a theory known as sensory or perceptual bias (see Ryan and Cummings, 2013, for a review).

How Is It Studied?

Historically, the advent of Ethology in the post-war period saw a flourishing of studies on courtship. The founders of the discipline, and in particular Konrad Lorenz and Niko Tinbergen, realized that courtship contains highly ritualized patterns of behavior, often derived from other behavioral contexts such as aggression. The work of behavioral endocrinologists such as Frank A. Beach, Daniel Lehrman, and John Hutchison demonstrated that courtship is under the control of gonadal hormones and is typically activated in males by the rise in testosterone levels that occurs at the beginning of the breeding season. However, these studies also revealed that courtship is a dynamic process and changes in its structure may depend on the metabolization of circulating hormones within the brain.

Behavioral biology approaches its objects of investigation from four complementary points of view, aiming to explain the adaptive function of the behavior, its underlying mechanisms, its evolution, and its development within the animal's lifetime (Tinbergen, 1963; for a general introduction see Manning and Dawkins, 2014). In this article, we review courtship and mating in relation to each of these aspects, after discussing mating systems. It is worth noting that the majority of the work into courtship has been recently conducted within the framework of behavioral ecology, which focuses on the evolution of animal behavior, although proximate mechanisms of courtship had received considerable attention in the last quarter of the 20th century.

Methodologically, courtship has been studied using many different approaches. Empirical methods range from field research in the animals' natural habitat to laboratory work with captive animals. Both typically involve observational studies or other recordings of behavior, or collection of physiological data. The data describing aspects of courtship can then be subjected to a correlation analysis with mating success (number of mates acquired) and/or reproductive success (average number of offspring per mate). As it can be extremely difficult in fieldwork to track the outcome of copulation in terms of number of young and their survival, mating

success is often used as a proxy for reproductive success. This has been shown in some avian species to be a reasonable assumption. It is also possible to manipulate aspects of the environment (e.g., blocking access to a sapling in the arena of a courting manakin), or of the animal itself (e.g., accentuating ornaments like tail length, or using hormones to alter physiological state), and to use statistical analysis to quantify the effect of the manipulation. In the laboratory, preference tests are also possible where the dependent variable is the time spent by a test animal in proximity to one of multiple, simultaneously presented possible mates.

Thanks to technological advances in video and audio equipment, it is becoming increasingly popular to use artificial stimuli to examine the effect of an experimenter-controlled courtship display on animals' behavior or physiology. Artificial stimulus material is most commonly visual or auditory, and can take the form of either a previously recorded courtship display ('playback'), a manipulated version of a recorded display, or a completely synthesized display. These approaches provide much better experimental control of the behavior presented to test animals than do experiments using a stimulus animal, as well as access to traits beyond the range physiologically possible in stimulus animals, and a reduction in the number of experimental animals needed. In some cases artificial stimuli can be used in the field (e.g., loudspeakers can be used to relay courting calls), but they are usually more amenable to laboratory work (e.g., computers can be used to show computer-animated fish displays). A major advantage of using artificial stimuli is that the range of preference can be measured in the courted sex (usually females) and compared to the range of the trait naturally occurring in the courting sex. However, the fact that audio and video equipment is optimized for human sensory systems presents a serious limitation – although they may appear identical to the human experimenter, the artificial experimental stimulus and real-world stimulus might be perceived very differently by the target animal. Robotics has also been used to good effect in field studies, for example, to investigate the influence of male responsiveness to female behavior on mating success in a bowerbird species.

Mating Systems

Any population of animals can be characterized by its general behavioral strategy in obtaining mates. Mating systems can be subdivided by the number of mates acquired (monogamous vs. polygamous), and further distinguished by the manner in which mates are obtained, the division of labor involved in parental care of the young, and whether mating partners form a pair bond. The particular mating system that occurs can be well predicted when opportunities for reproduction are considered as a resource with costs and benefits (Emlen and Oring, 1977). While it may be beneficial to engage in polygamy in order to maximize the number of offspring, the costs of monopolizing mates must be taken into account – when opportunities to mate are limited, competition will accordingly be high to access and to defend access to mates. The two most important environmental factors that determine these costs and benefits are the spatial distribution of mates (or other relevant resources such as food or habitat), and the temporal availability of mates, taking into account the time required for parental investment in offspring.

Both males and females can be polygamous, but the number of species in which a male has multiple mates whereas the female has one or just a few is preponderant. This derives from a basic difference between gametes in the two sexes: with few exceptions, males produce large numbers of small, mobile sperms whereas females produce relatively few large, non-mobile eggs. The value of a single gamete is therefore considerably different for female and males, with the consequence that females are often much more choosy about their mates. In addition, in most terrestrial vertebrates, the time required to care for the young considerably limits the temporal availability of females as sexual partners, with the result that the operational sex ratio is typically skewed towards the male.

Monogamy is the prevalent mating system in birds, possibly due to the large parental investment required of males and females in production and incubation of the eggs, and feeding and care of precocial young. The cost of neglecting a set of offspring to court and mate with an additional partner is thought to outweigh the benefit. Although extra-pair copulations are known to occur in monogamous birds, the social mating system is monogamous when considered in terms of parental investment. Fidelity has been proposed to be advantageous in birds as repeated pairings show increased physiological synchronization, leading to faster and more efficient reproduction. In addition, reduced aggression during courtship is seen between familiar partners, and the correspondingly better physical condition of the mate may help maximize survival chances of offspring.

Polygamous mating systems include variants where individual males mate with multiple females (polygyny) or vice versa (polyandry). In some of these systems, access to mates is dealt with in intrasexual competition before courtship occurs. For example, in resource defense polygyny, males compete for control of territories and defend access to females by virtue of their territorial control of food or other resources. When it comes to elaborate courtship behavior, the most interesting mating system is male dominance polygyny, in which females and resources relevant to females cannot be monopolized, and males group together during the breeding season and are visited by females who will actively choose a mate. In the lek mating system, which is a type of male dominance polygamy, female fertility is not temporally synchronized and males will gather together and perform their courtship display for the entire breeding season. Females will visit the lek and may take several days to decide upon a mate, with the decision made on the basis of the male's courtship display. Typically, one or few males will be chosen most frequently, and many males will not mate at all. After copulation, the male contribution to the offspring is complete and the female leaves and raises the young alone. Prominent examples of this mating system are bowerbirds, who create elaborate decorated staging arenas as part of their display, and manakins, who perform acrobatic dance routines in forest arenas which they clear of leaves. The advantages to the female are clear: she can efficiently access a broad pool of candidate mates. Males in larger aggregates may profit from the cumulative signal of the group being more attractive to females in general, but although lower-ranked males will encounter females, their chance of success is extremely low.

Functional Aspects of Courtship

Why does courtship exist? Some taxa display courtship (see [Table 1](#)), but those who do not can still reproduce and survive perfectly well without it. Courtship is therefore not necessary, but it has several important advantages.

As mentioned above, courtship involves the agreement of the opposite partner in the mating context. The main purpose of courtship is to ensure that there are no errors in mating: males and females can use courtship behavior to recognize and restrict reproductive interactions to conspecifics of the opposite sex in order to avoid wasting resources invested in egg and sperm production. In insects, chemical signals often function as an aid to species and sex identification. For example, in most species of fruit flies, males decide whether to continue courting after tapping candidate mates with their forelegs to acquire a pheromone sample, in order to discriminate against flies of other species or conspecifics of the incorrect sex ([Spieth, 1974](#)). Within sexually monomorphic species, which have no outward features that allow differentiation between the sexes, courtship behavior can indicate the sex of an individual. For example, Columbidae are sexually monomorphic in appearance until they engage in courtship behavior.

As well as its importance for recognizing that a partner is of same species, the communicative exchange involved in courtship has implications for the evolution of new and distinct species (speciation) as a result of assortative mating via mate choice. If differing traits (e.g., red and blue pigmentation) and corresponding differing preferences for each trait arise within a species, assortative mating of red-preferring with red animals, and blue-preferring with blue animals can lead to reproductive isolation by color and then to speciation. For example, in cichlid fishes in which nuptial colors play an important role in courtship, evidence has been found for speciation driven in part by pigmentation-associated assortative mating on the basis of divergent evolution in female color vision ([Seehausen et al., 2008](#)). Indeed, the range of possible behaviors and/or the sensory bandwidth of an organism may play a crucial role in speciation. A greater dynamic range in possible courtship traits and sensory ability results in a higher potential for signals and preferences to diverge between populations, which can then lead to reproductive isolation of a behavior-preference combination. This is mechanistically related to sensory bias and has been proposed in anurans, where mating calls are important courtship signals, and the diversity of species is found to correlate with the complexity of the anatomy of the inner ear ([Ryan, 1986](#)).

Courtship displays are also a form of advertising. They indicate that the displaying individual is ready to mate, and are an attempt to attract prospective partners. Displays can communicate such information as reproductive status, age, and health. As such, it is thought that mate choice is based on the quality of the courting animal as communicated in the display, thereby allowing the choosing animal to access the highest quality available mate. In some animals, very elaborate displays are found which include exaggerated movements that do not correspond to the normal behavioral repertoire. A well-studied example is the golden-collared manakin (*Manacus vitellinus*). These small tropical birds are polygynous (see [Section Mating Systems](#)) and males congregate during the breeding season at a regularly used location in order to display to visiting females. Each male occupies and defends a small arena, which he keeps clear of leaves and rubbish, and which provides the staging area for his energetic courtship display involving choreographed high-speed leaps and twists, wingsnaps, and vocalizations (reviewed in [Fusani et al., 2014](#)). Sexual dimorphism has been found in the skeleton, musculature and brain of these birds, suggesting that males are specialized for planning and performing their acrobatic displays, while females may have more intricate visual processing for better evaluation of courtship.

Courtship can also play an important role in the facilitation of mating by coordinating the behavior of mates in space and time ([Bastock, 1967](#)). For example, courtship displays can aid in locating a possible partner. For small, solitary insects like grasshoppers, auditory signals are particularly useful in this regard, as they can operate over longer distances than visual or chemical information. Courtship behavior can also be used to indicate or even to facilitate arrival at the location of a suitable breeding site. This is

Table 1 Taxa with courtship

<i>Invertebrates</i>	In general, courtship is not common except for advanced arthropods, and is particularly uncommon when fertilization is external
Molluscs	Courtship has been well-characterized in terrestrial gastropods (slugs, snails) and some cephalopods (cuttlefish and squid, but not many octopods)
Insects	Courtship is uncommon but examples are found in some members of all subphyla. Most widely studied: drosophila, fireflies, moths, butterflies
Arachnids	Courtship is present in many species to allow the safe delivery of the spermatophore required for internal, indirect fertilization. Spiders and scorpions are well studied. Courtship may include nuptial gift of food. Sexual cannibalism is prevalent in some species.
Crustaceans	Courtship present mainly in malacostracans including crabs, lobsters, crayfish
<i>Vertebrates</i>	Extremely common in mammals and birds
Fish	Occurrence not only limited to species with internal fertilization. Diverse courtship displays, including olfactory, visual, acoustic, and electrical signaling. Many fish species (e.g., guppies, cichlids) are important animal models for sexual selection research in part because of their courtship and ornamentation
Amphibians	Elaborate courtship is known in salamanders with internal but not external fertilization; frogs and toads typically use simple courtship comprised of mating calls
Reptiles	Internal fertilization and courtship is common and well characterized in snakes, crocodilians, lizards, and turtles
Birds	Courtship is widespread and well studied, and very varied. In most species males perform the main part of the courtship display and it can be very elaborate
Mammals	Widespread in terrestrial and marine mammals

important in some fish species in which the eggs or future young require particular environmental conditions to survive. Elements of the display may furthermore help synchronize the physiological processes of both partners (e.g., fish in which both partners must simultaneously spawn; colonial birds in which synchronous laying reduces the effect of predation) or may be directly sexually stimulating and induce the courted partner to permit mating (e.g., insects such as *Drosophila* species, where females store sperm and the timing of fertilization is therefore not important to the male's reproductive success). Also, in most species females will indicate when they are ready to copulate.

Courtship Mechanisms

During courtship, stimuli from the potential mate, from the animal itself, and from the environment interact to have an effect on behavior. This includes seasonal effects driven by day length. The role of neuroendocrine mechanisms in reproductive behavior was elucidated by early animal experiments. The often dramatic changes in courtship behavior seen after castration made it clear that gonadal hormones play a central role in the organization of such behavior. The work of [Berthold \(1849\)](#) is often cited as the 'first' experiment in behavioral endocrinology. Berthold showed that male-typical behavior of young cockerels, including crowing, disappeared with castration but could be restored by re-implantation of the testes. A plethora of studies afterwards confirmed that treatment of immature or castrated animals with gonadal hormones can, in some cases, induce or restore courtship traits. Importantly, it has also been shown that some typically male behavior patterns can be induced in females treated with testosterone (including wingsnaps in female manakins). Although the brain circuits underlying sex-specific behavior are typically organized in a permanent manner during development, in some species the control system for such behaviors is present (to some or other degree of development) in both sexes, and the introduction of the appropriate amount of required hormones into the system is sufficient to activate the behavior via the central nervous system. The influence of gonadal hormones on the brain and its organization of courtship behavior is perhaps best understood in birds, as most species in this taxon possess courtship displays that use the acoustic or visual channels and are therefore particularly suitable to human observation (see [Fusani, 2008](#), for a review).

Historically, the ring dove (*Streptopelia risoria*) was one of the most important model species for behavioral and neuroendocrinology. Males display three different behaviors during courtship: the bowing display, in which they extend the head and bow low and parallel to the ground before rising again, simultaneously emitting a coo vocalization that differs slightly between individuals; the chasing display, in which they chase after the potential mate while vocalizing; and nest solicitation (also performed by females) in which they lower the head and raise the tail while cooing and flipping the wings. The first two behaviors are induced by testosterone, while the third is estrogen-related and is controlled in males by the aromatization in the brain of gonadal testosterone into oestradiol. Perhaps most importantly, the study of courtship and mating in ring doves revealed the behavior-mediated interaction between the various endocrine processes involved, as well as the importance of environmental stimuli including day length, availability of nesting material, presence of a potential mate ([Lehrman, 1964](#)), and the role of self-stimulation of the female by her own vocalizations.

However, we still know very little about the physiological mechanisms involved in how females decide with whom to mate. As already mentioned, courtship typically involves multiple ornaments which are shown during dynamic behavioral displays. It has previously been shown that combined visual and auditory displays induce a stronger physiological response in female ring doves than only auditory information ([Friedman, 1977](#)). The direction in which a courting male is facing also makes a difference to the observing female's physiological response, which emphasizes the importance of reciprocal social interaction between the two sexes during courtship. As already mentioned, one series of mating preference models states that females have a 'sensory bias' that evolved for reasons unrelated to sexual selection, and male ornaments 'exploit' such a bias. Regarding the neural circuitry, *Drosophila* is an excellent model organism as the brain is small. *Drosophila* behaviors and genetics are well explored, and new technologies such as optogenetics are already being used to investigate causal factors.

Evolution of Courtship

The proximate mechanisms of courtship are more fully understood when viewed in an evolutionary framework that takes into account the interplay between (typically male) courtship traits and (typically female) mate choice. Sexual selection research has mostly focused on the mechanisms through which female preferences evolved ([Andersson, 1994](#)).

Developmental Aspects

How do young animals acquire the behaviors used in courtship? On the one hand, certain behaviors seem highly stereotyped and 'innate', and need only to be activated appropriately, for example, via gonadal hormones, as seen in female birds who vocalize or move like courting males when exposed to testosterone. On the other hand, such behavioral patterns are only constituent parts of the overall courtship display, which we have noted is typically multi-component and is often clearly tailored to the audience during its execution. Bird song plays an important role in courtship of many passerines, and learning of song is a central part of development. Indeed, songbirds have been an extremely important model in neurobiological studies of learning.

In some species, more elaborate courtship displays involve targeted manipulations of the environment, for example, in bowerbirds or manakins. The development of such complex courtship behaviors has not yet been studied in great depth. Interestingly, males in some passerine bird species with sex-dimorphic plumage show delayed plumage maturation, retaining their juvenile coloring (similar to females) even after they are sexually mature. This is the case in golden-collared manakins, and although juvenile male manakins have been observed visiting the lek, it is not clear whether they learn any aspect of courtship by watching performing males.

A second important aspect is learning the appropriate audience to which a courtship display should be directed. For example, some species of *Drosophila* learn to discriminate between conspecifics and heterospecifics through experience.

Finally, it is of interest whether and how animals acquire the preferences used to choose among prospective partners. Two types of acquisition have been proposed: imprinting and learning. Imprinting is a particular form of learning resulting from early exposure to the parents or other conspecifics before or closely after birth during a rather short temporal window, and is thought to lead to rigid preference. For example, Japanese quail have been shown to acquire a preference for plumage coloration of prospective mates by imprinting (Bateson, 1978). In contrast, learning in later life by means of sexual or more general social experience, and/or continued exposure to some trait, is thought to correspond to flexible preferences. Experimental comparison of virgin females to more experienced females can provide evidence for which aspects of behavior or preference might be learned, while cross-fostering experiments have been used to investigate imprinting (see Verzijden *et al.*, 2012, for a review). A third means of influence of experience on the choosing sex's decision is also possible, namely mate-choice copying or non-independent mate choice, where the chosen mate depends primarily on which mate was chosen by previous individuals. This has been found to explain courtship outcomes in some species.

Conclusions

As Margaret Bastock concluded in her book on courtship 50 years ago, “we still have much to learn about courtship. Its significance and evolutionary origins are reasonably clear but details of its mechanisms are largely unknown. Probably the relationship between it and mating behavior differs from animal to animal and the processes involved must be studied separately in each species” (Bastock, 1967, p. 194). Although we have come further in elucidating some important mechanistic aspects in some species, much work remains to be done. New methods such as optogenetics that can be used to uncover the causal contributions of neural circuits to behavior are already being applied to investigate the mechanisms underlying courtship in mice, *Drosophila*, and songbirds. Besides technical advances, there is a need for theoretical developments in the interpretation of courtship signals. This is particularly important for elaborate displays, for which simple mechanisms based on sensory properties of the courted partners appear insufficient.

References

- Andersson, M. (1994). *Sexual Selection*. Princeton, NJ: Princeton University Press.
- Bastock, M. (1967). *Courtship: An Ethological Study*. New Brunswick: AldineTransaction.
- Bateson, P. (1978). Sexual imprinting and optimal outbreeding. *Nature*, 273(5664), 659–660.
- Berthold, A. A. (1849). Transplantation der Hoden. *Archiv für Anatomie, Physiologie und Wissenschaftliche Medizin*, 16, 42–46.
- Darwin, C. (1882). *The Descent of Man, and Selection in Relation to Sex* (second ed.). London: John Murray.
- Emlen, S. T., & Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science*, 197(4300), 215–223.
- Friedman, M. B. (1977). Interactions between visual and vocal courtship stimuli in the neuroendocrine response of female doves. *Journal of Comparative and Physiological Psychology*, 91(6), 1408–1416.
- Fusani, L. (2008). Testosterone control of male courtship in birds. *Hormones and Behavior*, 54(2), 227–233.
- Fusani, L., Barske, J., Day, L. D., Fuxjager, M. J., & Schlinger, B. A. (2014). Physiological control of elaborate male courtship: Female choice for neuromuscular systems. *Neuroscience and Biobehavioral Reviews*, 46(Pt. 4), 534–546.
- Huxley, J. S. (1914). The courtship-habits of the great crested grebe (*Podiceps cristatus*); with an addition to the theory of sexual selection. *Proceedings of the Zoological Society of London*, 84(3), 491–562.
- Lehrman, D. S. (1964). The reproductive behavior of ring doves. *Scientific American*, 211, 48–54.
- Manning, A., & Dawkins, M. S. (2014). *An Introduction to Animal Behavior*. Cambridge: Cambridge University Press.
- Patricelli, G. L., & Hebets, E. A. (2016). New dimensions in animal communication: The case for complexity. *Current Opinion in Behavioral Sciences*, 12, 80–89.
- Ryan, M. J. (1986). Neuroanatomy influences speciation rates among anurans. *Proceedings of the National Academy of Sciences of the United States of America*, 83(5), 1379–1382.
- Ryan, M. J., & Cummings, M. E. (2013). Perceptual biases and mate choice. *Annual Review of Ecology, Evolution, and Systematics*, 44(1), 437–459.
- Seehausen, O., Terai, Y., Magalhaes, I. S., *et al.* (2008). Speciation through sensory drive in cichlid fish. *Nature*, 455(7213), 620–626.
- Spieth, H. T. (1974). Courtship behavior in *Drosophila*. *Annual Review of Entomology*, 19, 385–405.
- Tinbergen, N. (1963). On aims and methods of ethology. *Zeitschrift für Tierpsychologie*, 20(4), 410–433.
- Verzijden, M. N., ten Cate, C., Servedio, M. R., *et al.* (2012). The impact of learning on sexual selection and speciation. *Trends in Ecology & Evolution*, 27(9), 511–519.

Songbirds and Singing

Eliot A Brenowitz, University of Washington, Seattle, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Age-limited learning Also known as sensitive-period learning. An ontogenetic pattern in which song learning is limited to the first year of life and new songs are not developed in adulthood.

Crystallization The third stage of song production in the sensorimotor phase of song learning, when birds produce a well-structured, stereotyped version of the conspecific song model to which they were exposed during the earlier memory acquisition phase.

Open-ended learning An ontogenetic pattern in which new song patterns continue to be developed beyond the first year of life, into adulthood.

Oscine A suborder of birds in the order Passeriformes, also referred to as “songbirds”.

Plastic song The second stage of song production in the sensorimotor phase of song learning. Plastic song is louder and better structured than subsong, but still variable in form.

Sensorimotor The second major phase of song learning, which involves ongoing comparison between a sensory model of song acquired during an earlier memorization phase and auditory feedback from a bird’s own production of song.

Subsong The initial stage of song production in the sensorimotor phase of song learning. Subsong is quiet, poorly structured, and very variable in form.

Template A sensory model of song acquired during the initial memory phase of song learning as a result of listening to songs produced by adult conspecific birds.

Songbird Systematics

There are about 10,000 species of living birds. Nearly 6000 species belong to the taxonomic order Passeriformes, which consists of the oscine suborder (the songbirds), and the suboscine suborder, which includes birds such as flycatchers, antbirds, and cotingas. When we speak of “songbirds,” we are referring to the oscine birds, such as finches, sparrows, and blackbirds, within this large order. The Passeriformes order is considered to be a single evolutionary lineage derived from a common ancestor (i.e., monophyletic), and the oscines and suboscines are each viewed as monophyletic lineages within the Passeriformes. There are approximately 4000 species of songbirds, essentially all of which use learned vocalizations for communication.

General Characteristics of Song Behavior

Song Structure

Songs have well-defined acoustic structures that are unique to each species. The structure of a song can be visualized using a sound spectrograph, which plots the sound frequencies against time (see [Fig. 1](#)). Song consists of different structural components. The simplest individual sounds are called song “notes.” A sequence of one or more notes that occurs together in a regular manner in song is called a song “syllable.” A series of one or more syllables that is repeated in song is referred to as a song “phrase.” A specific combination of phrases that occurs repeatedly is a song “type.”

There is great diversity among songbird taxa in the structural complexity of song behavior. In some species, such as the white-throated sparrow (*Zonotrichia albicollis*), a male produces only one simple type of song. At the other extreme, a male brown thrasher (*Toxostoma rufum*) has a repertoire of several thousand different types of songs. Most bird species have song repertoire sizes that fall between these extremes ([Beecher and Brenowitz, 2005](#); [Brenowitz and Beecher, 2005](#)).

Sex Differences in Song Production

Songbird species show extensive diversity in sex patterns of song behavior ([Ball et al., 2008](#)). At one extreme are species in which only males sing, such as the zebra finch (*Taeniopygia guttata*). At the other extreme are several tropical species in which females and males contribute equally to elaborate song duets. Between these extremes are species in which females sing, but less commonly and with less complexity than males ([Schlinger and Brenowitz, 2017](#)). In most species, song is a sexually dimorphic behavior; males sing more often and with greater complexity.

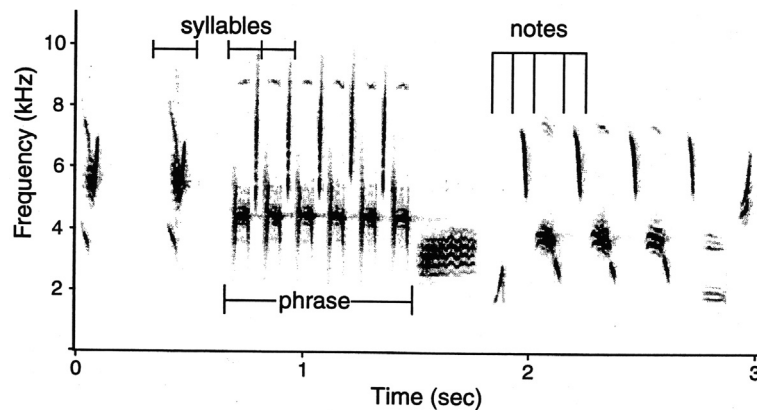


Fig. 1 Sound spectrogram of a single song produced by a male song sparrow (*Melospiza melodia*), showing a frequency vs. time representation. The simplest individual sounds in the song are *notes*. A sequence of one or more notes that occurs together in a regular manner in song is a *syllable*. A series of one or more syllables that is repeated in song is a *phrase*. The entire combination of phrases is a song *type* that is sung repeatedly. An individual male song sparrow has a *repertoire* of several different song types.

Behavioral Functions of Song

Song is important in the reproductive behavior of birds, and serves two main functions in this context (Catchpole and Slater, 2008). In many species, song is used to declare a territory from which other birds are aggressively excluded. Breeding occurs on the territory. Mating birds decreases their ability to deter intrusions by other birds. Both males and females may use song for territorial advertisement. Song may also be used by males to attract females to mate with them, as well as to stimulate the females' reproductive behavior and physiology.

Each of these functions of song may be influenced by the number of song types that a bird produces (i.e., the repertoire size). Large repertoires are more effective at deterring intrusions on a bird's territory than are small repertoires. Males with large repertoires may mate earlier in the breeding season, and/or have more mates than conspecific males with smaller repertoires. Large repertoires may also be more effective at stimulating female reproductive physiology and behavior. Female canaries (*Serinus canaria*), for example, build nests faster and lay more eggs in response to playback of a large song repertoire than they do to a small repertoire. These effects of large song repertoires on females can increase a male's reproductive success (Beecher and Brenowitz, 2005).

Song may serve other functions, in addition to territorial advertisement and mate attraction. There is often considerable individual variation in song structure, which can enable recognition of individual birds by vocal cues alone. Such individual recognition may be important for recognition of territorial neighbors, mates, kin, and members of a social group (Catchpole and Slater, 2008). Song may also play a role in maintaining dominance hierarchies in social species such as the brown-headed cowbird (*Molothrus ater*) (Kohn et al., 2013).

The rate at which birds sing may vary seasonally. In temperate zone bird species, song used in either territorial or mate-attraction contexts is produced at higher rates during the breeding season, and at lower rates or not at all outside the breeding season. In birds species residing in the tropics, song is often used for territorial defense throughout the year.

General Characteristics of Song Development

Stages of Song Learning

One of the most interesting features of song is that it is a learned behavior. Song development is characterized by distinct stages (Marler, 1997). During an initial "memory acquisition" phase, young birds acquire a sensory model of song by listening to adult conspecifics sing. Juvenile birds begin to translate this sensory memory to a motor pattern of song production at some later time, in what is referred to as the "sensorimotor" phase of song development. The sensorimotor phase consists of three stages. Initially birds produce "subsong," which is quiet, poorly structured, and very variable in form. With continued vocal practice, young birds improve their performance of song and progress to "plastic song," which is louder and better structured, but still variable in form. Eventually, song becomes "crystallized" in structure as the birds produce a stereotyped version of the sensory model to which they were exposed earlier. The specific age at which these different phases of song development occur varies considerably between species (Beecher and Brenowitz, 2005; Brenowitz and Beecher, 2005). Also, some species develop songs by improvisation, in addition to copying songs from adult conspecifics.

There is much species diversity in ontogenetic patterns of song development. At one extreme are species referred to as "age-limited" or "sensitive-period" learners, in which song learning is limited to the first year of life and new songs are not developed in adulthood. Examples of age-limited learners include the zebra finch and white-crowned sparrow (*Zonotrichia leucophrys*) (Immelmann, 1969; Marler, 1970). At the other extreme are "open-ended" learning species in which new song patterns can be developed

beyond the first year of life. Examples include the canary and European starling (*Sturnus vulgaris*) (Marler and Waser, 1977; Mountjoy and Lemon, 1995). The development of new songs by adults of open-ended species usually occurs in a restricted seasonal manner. Other species have ontogenetic patterns of song development that fall between the extremes of age-limited and open-ended learning.

Auditory Feedback is Required for Song Learning

In most species, juvenile birds must hear the song of conspecific adults if they are to develop normal song behavior. If a young bird is isolated from hearing the songs of conspecific adults, it will not develop normal song of its species. Young birds must also be able to hear themselves sing in order to develop a crystallized version of the conspecific song learned during the earlier memorization phase (Marler, 1997). If juvenile birds are deafened after the memorization phase, but before song crystallization, they will not develop normal song (Konishi, 2004). Translating the sensory memory of song to a motor program involves an ongoing comparison between a “template” of song stored during the memorization phase and auditory feedback from a bird’s own production of song: hence the description of this phase as being “sensorimotor.”

Auditory feedback may also be necessary for the maintenance of crystallized song in adults. Previously crystallized song structure can degrade within weeks to months after an adult bird is deafened. There is variation between species in the continued reliance on auditory feedback (Woolley, 2004).

Neural Control of Song Behavior

Pathways for Song Learning and Production

Song behavior in songbirds is controlled by a discrete network of interconnected nuclei in the forebrain. Two circuits that are involved in song learning and production are illustrated in Fig. 2. The motor circuit controls song production, and is also presumed to participate in the sensorimotor phase of song learning. This circuit consists of projections from the nidopallial nucleus HVC (proper name) to the robust nucleus of the arcopallium (RA) in the telencephalon. RA projects both to the dorsomedial part of the intercollicular nucleus (DM) in the midbrain (not shown in Fig. 2) and to the tracheosyringeal part of the hypoglossal motor nucleus in the brainstem (nXIIts). Motor neurons in nXIIts send their axons to the muscles of the sound-producing organ, the syrinx. The projection from RA onto the motor neurons in nXIIts is topographically organized according to the distribution of muscle groups within the syrinx. Neuronal activity in HVC and RA is synchronized with the production of sound by the syrinx (Fee et al., 2004). If nuclei in this motor pathway are inactivated, a bird is unable to produce song.

RA also projects to nucleus retroambiguus (RAm) and nucleus ambiguus (Am) in the medulla (Fig. 2). RAm contains respiratory related neurons that fire in phase with expiration. Am contains neurons that innervate the larynx. This pattern of descending projections from RA may be critical for coordination of syringeal, respiratory, and laryngeal muscle activity during song production

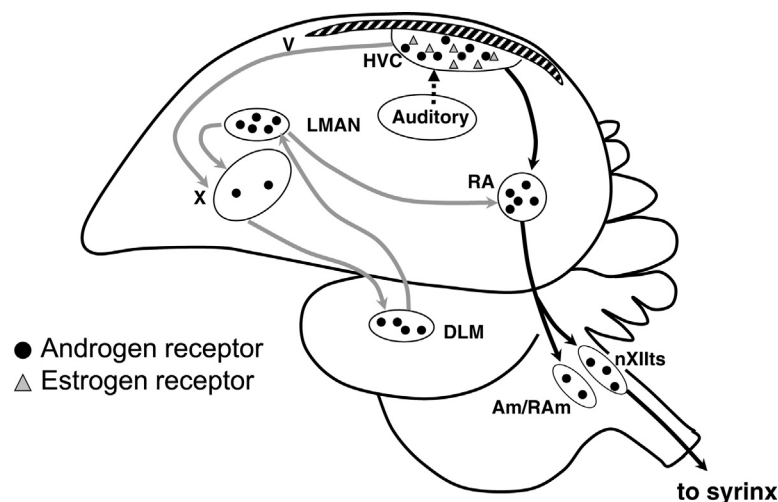


Fig. 2 A schematic sagittal drawing of the songbird brain showing projections of major nuclei in the song system and the distribution of steroid receptors. The descending motor pathway (black arrows) controls the production of song. The gray arrows indicate the anterior forebrain pathway that is essential for song learning. The dotted arrow indicates input to HVC from auditory regions in the nidopallium. Abbreviations: Am, nucleus ambiguus; DLM, medial portion of the dorsolateral nucleus of the medial thalamus; LMAN, lateral portion of the magnocellular nucleus of the anterior nidopallium; RA, robust nucleus of the arcopallium; RAm, nucleus retroambiguus; V, lateral ventricle; X, Area X of the medial striatum; nXIIts, tracheosyringeal part of the hypoglossal nucleus.

(Schmidt and Goller, 2016). Birds only produce song during expiration, and the larynx may filter the sound frequencies produced by the syrinx.

A second, anterior forebrain pathway is thought to be necessary for song learning and recognition (Fig. 2). This circuit consists of projections from HVC to Area X in the striatum, then to nucleus DLM in the thalamus, from DLM to the lateral portion of the magnocellular nucleus of the anterior nidopallium (LMAN), and finally to RA. LMAN neurons that project to RA also send collaterals to Area X, thus providing the potential for feedback within this pathway. The projections within this pathway are topographically organized. Inactivation of LMAN, DLM, or Area X in adults seems to have no effect on stable song, whereas the same lesions in juveniles prevent the development of normal song. This pathway continues to be important, however, in modulating song plasticity in adults induced by manipulations like deafening or denervation of the syringeal muscles. Lesions of LMAN prevent these changes (Brainard, 2008).

There are pronounced sex differences in the volume of HVC, RA, and Area X in species in which males sing more than females (Ball *et al.*, 2008). The extent of morphological sex differences in the song system of a given species is closely related to the degree to which the sexes differ in behavioral song complexity.

Auditory Responses in the Song Control Circuits

Auditory information is conveyed from field L in the nidopallium, which is the analog of the primary auditory cortex, to HVC and then to other nuclei in the song control circuits. Neurons in both the descending motor and anterior forebrain pathways show auditory responses to song stimuli, with the most pronounced selectivity to a bird's own song, and weaker responses to other conspecific songs, heterospecific songs, and artificial stimuli. This pattern of selectivity results from a bird's experience in hearing its own song. Neurons in HVC, Area X, and LMAN initially respond unselectively in young birds prior to the start of sensorimotor song learning. They become more selective as the bird proceeds through the sensorimotor stages to song crystallization. Within the anterior forebrain pathway there is a progressive increase in song selectivity as one proceeds from Area X to DLM to LMAN (Brenowitz and Woolley, 2004).

A Circuit for Song Perception and Memory

Subdivisions of the telencephalic auditory region field L also project to the caudomedial nidopallium (NCM) and the caudomesopallium (CM), which together are analogous to mammalian secondary auditory cortex. These two regions play a role in song perception and memory. There is strong induction of the immediate early gene *EGR1* in these regions in response to presentation of conspecific song, but only weak induction in response to heterospecific song or artificial sound stimuli. Neurons in NCM show stronger electrophysiological responses to playback of familiar conspecific songs. Both the ZENK induction and auditory responses in NCM decrease with repeated presentation of the same conspecific song. This decrease in responsiveness is reminiscent of habituation, and implies that neurons in NCM form a persistent memory of the stimulus song. This type of memory could be important in the context of recognizing the songs of territorial neighbors and mates (Brenowitz and Remage-Healey, 2016).

Hormone Effects

Steroid sex hormones affect song learning and production, and the juvenile development and adult plasticity of the song circuits. The pronounced effect of sex steroids on the song system is consistent with the close relationship between song behavior and reproduction.

Effects on Song Behavior

Proper timing and amount of exposure to estrogenic and androgenic hormones are necessary for normal song learning. Estrogenic and androgenic hormones may play opposing roles in song learning. Circulating estrogen concentrations are high during the memory acquisition phase of song learning in swamp sparrows (*Melospiza georgiana*), and may facilitate the plasticity of the song circuits that underlies this stage (Marler *et al.*, 1987). Testosterone, on the other hand, may terminate this plasticity. Young male birds that are castrated or treated with anti-androgenic agents can acquire sensory memories of song and progress to plastic song, but will not crystallize song unless they are treated with testosterone (Marler *et al.*, 1988). Chronic exposure of juvenile male zebra finches to testosterone severely impairs song learning (Korsia and Bottjer, 1991).

Adult song behavior is also influenced by sex steroids. Castration of adult males reduces the rate at which they sing, and treatment with exogenous steroids can reinstate song (Arnold, 1975). It appears that both androgenic and estrogenic hormones must be present for full song activation. In temperate zone birds, seasonal changes in circulating steroid levels are closely correlated with changes in the rate of song production. In some seasonally breeding species, such as the white-crowned sparrow, song continues to be produced at a lower rate after the breeding season, when circulating steroid concentrations are low (Brenowitz, 2008). In tropical songbirds, testosterone levels tend to be low throughout the year and song behavior appears to be largely independent of this hormone. These observations suggest that while circulating steroids may modulate the level of song behavior, these hormones are not required for song in all species.

Effects on the Song Control Circuits

The brain nuclei that control song are extremely sensitive to sex steroids and their metabolites (Schlinger and Brenowitz, 2017). Nuclear androgen receptors are present in the song nuclei HVC, RA, LMAN, Area X, DM, and nXlts, as well as in the muscles of the syrinx. Classical nuclear estrogen receptors are found in HVC and DM (Fig. 2).

Steroids influence the development of multiple attributes of the song system, whereas other traits appear to be under direct genetic regulation. Estrogens, and to a lesser extent androgens, stimulate masculine growth of the song system when they are injected into very young (1–3 week posthatching) female zebra finches. The primary source of estrogen synthesis that masculinizes the song system is the telencephalon rather than the gonads. Steroids induce growth of the overall volumes of HVC, RA, Area X and LMAN, stimulate increases in neuronal size and number, and alter biochemical properties and connectivity between neurons in these nuclei (Schlinger and Brenowitz, 2017).

Sex steroids also have pronounced effects on adult song control circuits. Seasonal changes in circulating testosterone levels induce plasticity in various morphological attributes of the song nuclei, including the size of the song nuclei and individual neurons, the spacing of neurons, the survival of mature neurons and the incorporation of newly generated neurons in HVC, the spontaneous electrical activity of RA neurons, and various synaptic attributes. These changes in the structure of the song nuclei are functionally related to seasonal changes in different aspects of song behavior, including song rate, stereotypy, and the development of new song patterns (Brenowitz, 1981).

Interaction with Neurotrophins

Steroids interact with brain-derived neurotrophic factor (BDNF) during development of the song system and in adult plasticity, including the addition of newborn neurons to the HVC and seasonal changes in structure and function of the song circuits (Brenowitz, 2013). Androgens, estrogens, and BDNF interact locally within HVC to influence cell proliferation and survival. The gene for BDNF has a hormone response element, and steroid/receptor complexes thereby regulate BDNF gene expression in the developing and adult song system. The trophic effect of testosterone on the addition of new neurons to HVC is mediated by BDNF activity. Steroid-BDNF interaction may also occur transsynaptically; testosterone increases the synthesis of BDNF in HVC, and BDNF protein is then released by HVC axons on to postsynaptic cells in nucleus RA where it has trophic effects on cell size and electrical activity.

Effects on Auditory Responses

Sex steroids influence activity in auditory regions in a sex-specific manner (Brenowitz and Ramage-Healey, 2016). Neurons within the auditory forebrain, including NCM, CM, and also the thalamic region IC (central nucleus of the inferior colliculus), exhibit elevated *EGR1* expression in response to song following treatment with estrogen in female but not male zebra finches. In NCM and CM this is due to decreased response to simple stimuli like tones, compared to complex stimuli like song, and this increases the selectivity for biologically relevant sounds. Similarly, estrogenic treatment increases the sensitivity and response strength of spike-timing neurons in field L to tones and conspecific songs in breeding female, but not male, white-crowned sparrows. These findings support the general idea that prolonged increases in circulating hormone levels that bring female birds into breeding condition also produce sex-specific changes in auditory responsiveness. Male song has its greatest behavioral salience for females when they are in breeding condition, and estrogens are a seasonal internal cue that provides an effective way of enhancing auditory function at that critical time of year.

References

- Arnold, A. P. (1975). The effects of castration and androgen replacement on song, courtship, and aggression in zebra finches (*Poephila guttata*). *Journal of Experimental Zoology*, 191, 309–326.
- Ball, G. F., Ritters, L. V., MacDougall-Shackleton, S. A., & Batlitzart, J. (2008). Sex differences in brain and behavior and the neuroendocrine control of the motivation to sing. In H. P. Zeigler, & P. Marler (Eds.), *Neuroscience of Birdsong* (pp. 320–331). Cambridge: Cambridge University Press.
- Beecher, M. D., & Brenowitz, E. A. (2005). Functional aspects of song learning in songbirds. *Trends in Ecology and Evolution*, 20, 143–149.
- Brainard, M. S. (2008). The anterior forebrain pathway and vocal plasticity. In H. P. Zeigler, & P. Marler (Eds.), *Neuroscience of Birdsong* (pp. 240–255). Cambridge: Cambridge University Press.
- Brenowitz, E. A. (1981). Territorial song as a flocking signal in red winged blackbirds (*Agelaius phoeniceus*). *Animal Behaviour*, 29, 641–642.
- Brenowitz, E. A. (2008). Plasticity of the song control system in adult birds. In H. P. Zeigler, & P. Marler (Eds.), *Neuroscience of Birdsong* (pp. 332–349). Cambridge: Cambridge University Press.
- Brenowitz, E. A. (2013). Testosterone and brain-derived neurotrophic factor interactions in the avian song control system. *Neuroscience*, 239, 115–123.
- Brenowitz, E. A., & Beecher, M. D. (2005). Song learning in birds: Diversity and plasticity, opportunities and challenges. *Trends in Neurosciences*, 28, 127–132.
- Brenowitz, E. A., & Ramage-Healey, L. (2016). It takes a seasoned bird to be a good listener: Communication between the sexes. *Current Opinion in Neurobiology*, 38, 12–17.
- Brenowitz, E. A., & Woolley, S. M. (2004). The avian song control system: A model for understanding changes in neural structure and function. In T. Parks, E. W. Rubel, A. Popper, & R. Fay (Eds.), *Plasticity in the Auditory System* (pp. 228–284). New York: Springer-Verlag.
- Catchpole, C. K., & Slater, P. J. B. (2008). *Bird song: Biological themes and variations* (second ed.). Cambridge, UK: Cambridge University Press.
- Fee, M. S., Kozhevnikov, A. A., & Hahnloser, R. H. (2004). Neural mechanisms of vocal sequence generation in the songbird. *Annals of the New York Academy of Sciences*, 1016, 153–170.

- Immelmann, K. (1969). Song development in the zebra finch and other estrildid finches. In R. A. Hinde (Ed.), *Bird Vocalizations* (pp. 61–77). Cambridge: Cambridge University Press.
- Kohn, G. M., King, A. P., Dohme, R., Meredith, G. R., & West, M. J. (2013). In the company of cowbirds, *Molothrus ater*: robust patterns of sociability predict reproductive performance. *Journal of Comparative Psychology*, 127, 40–48.
- Konishi, M. (2004). The role of auditory feedback in birdsong. *Annals of the New York Academy of Sciences*, 1016, 463–475.
- Korsia, S., & Bottjer, S. W. (1991). Chronic testosterone treatment impairs vocal learning in male zebra finches during a restricted period of development. *Journal of Neuroscience*, 11, 2362–2371.
- Marler, P. (1970). A comparative approach to vocal learning: Song development in white-crowned sparrows. *Journal of Comparative and Physiological Psychology*, 71(Suppl), 1–25.
- Marler, P. (1997). Three models of song learning: Evidence from behavior. *Journal of Neurobiology*, 33, 501–516.
- Marler, P., Peters, S., Ball, G. F., Duffy, A. M., Jr., & Wingfield, J. C. (1988). The role of sex steroids in the acquisition and production of birdsong. *Nature*, 336, 770–772.
- Marler, P., Peters, S., & Wingfield, J. (1987). Correlations between song acquisition, song production, and plasma levels of testosterone and estradiol in sparrows. *Journal of Neurobiology*, 18, 531–548.
- Marler, P., & Waser, M. S. (1977). Role of auditory feedback in canary song development. *Journal of Comparative and Physiological Psychology*, 91, 8–16.
- Mountjoy, D. J., & Lemon, R. E. (1995). Extended song learning in wild European starlings. *Animal Behaviour*, 49, 357–366.
- Schlinger, B. A., & Brenowitz, E. A. (2017). 2.12 – Neural and hormonal control of birdsong. In D. W. Pfaff, & M. Joëls (Eds.), *Hormones, Brain and Behavior* (third ed., pp. 255–290). Oxford: Academic Press.
- Schmidt, M. F., & Goller, F. (2016). Breathtaking songs: Coordinating the neural circuits for breathing and singing. *Physiology (Bethesda)*, 31, 442–451.
- Woolley, S. M. (2004). Auditory experience and adult song plasticity. *Annals of the New York Academy of Sciences*, 1016, 208–221.

Communication, Insects

Jan Buellesbach and Elizabeth Cash, University of California, Berkeley, CA, United States
Thomas Schmitt, University of Würzburg, Würzburg, Germany

© 2018 Elsevier Inc. All rights reserved.

Introduction

Communication is universal among all living organisms. From single cells to the most complex of plant and animal societies, information exchange is crucial for coordinating aggregation, food source location, avoidance of harm, and, maybe most importantly, reproduction. Broadly defined, communication is the exchange of information between individuals, usually a sender and a receiver (Bradbury and Vehrencamp, 2011). In general, the pathway between a sender and a receiver is defined as a channel (Greenfield, 2002). In animals, many different types of information are exchanged in various ways: The sender's current status (e.g., aggressive, dominant, or subordinate) can be conveyed, as well as the sender's affiliation to a certain sex, species, or group. Accurate information transfer in a reproductive context is pivotal for finding, attracting, securing, and eventually reproducing with suitable mates (Saetre *et al.*, 1997; Maan and Seehausen, 2011). Concerning insects, by far the most diverse group of animals (Chapman, 2009), communication has the difficult distinction of often being beyond our own human perception or even imagination (Greenfield, 2002). Despite this, insects display a variety of communication behaviors unparalleled in any other class of the animal kingdom (Hölldobler, 1984), matching their vast diversity (see Fig. 1 for a schematic example).

All insect communication can be subdivided into three basic categories: olfactory, acoustic, and visual (Greenfield, 2002). So far, electrical communication, as has been shown in fishes and some amphibians, could not be documented in either terrestrial or aquatic insects (Kramer, 1996). Furthermore, although other forms of energy can apparently be perceived by some insects, e.g., infrared radiation (Schmitz *et al.*, 1997; Schmitz and Bleckmann, 1998) and geomagnetism (Wiltschko and Wiltschko, 1995), no evidence has yet demonstrated that these modalities are used for communication (Greenfield, 2002). The relative importance of acoustic, visual, and olfactory signaling channels can vary widely in different insect taxa, with no particular species excelling in all three communication modalities (Lewis, 1984). However, olfactory communication appears to be the predominant signaling mode prevailing in most studied insect taxa so far (Greenfield, 2002). It is hypothesized that this dependence on and high preference for

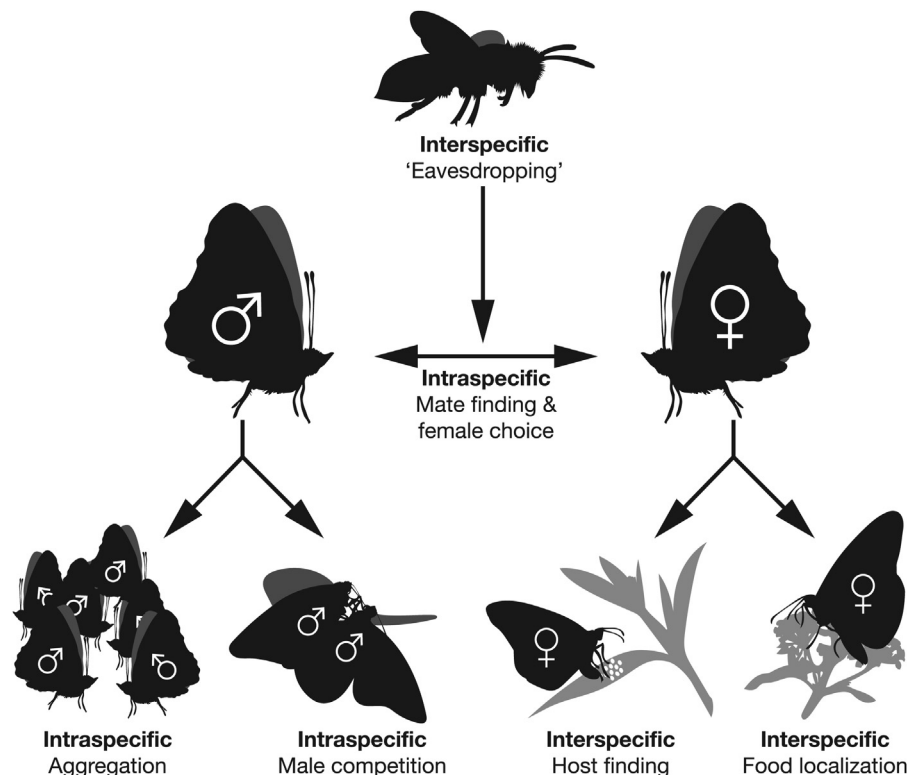


Fig. 1 Schematic overview of intra- and interspecific communication and information transfer in a hypothetical insect. Depicted is intraspecific communication between the sexes (i.e., mate finding and female choice) and within the sexes (most commonly occurring as aggregation or male competition) as well as interspecific communication as eavesdropping behavior (e.g., predators using sex pheromones as kairomones), host finding (e.g., parasitoids of eggs from other insect species), or food localization (e.g., phytophagous insects). All of these examples are further described in the main text with concrete examples in all three main insect communication modalities, acoustic, visual, and olfactory.

olfactory communication has evolved over the long period of adaptation to a terrestrial and partially airborne life style after the switch of ancestral insects from primarily marine environments (Missbach *et al.*, 2014).

Acoustic Communication

Among all terrestrial animals, only insects and vertebrates have evolved the capability to produce, detect, and behaviorally respond to 'sound', generally defined as wave motions originating from a source of vibration and transmitted through a medium, irrespective of that medium being air, water, or a solid substrate (Claridge, 2005). It has been proposed that sound production in insects can be subdivided into five categories (Ewing, 1989; Claridge, 2005):

Vibration ('Tremulation')

This category includes vibrations of insect body parts relatively unspecialized for sound production, e.g., the abdomen or wings. Wing vibration has evolved as a means for species-specific communication and mate recognition in many insect species, e.g., swarming mosquitos (Roth, 1948), *Drosophila* fruit flies (Bennet-Clark, 1971) and *Nasonia* jewel wasps (Diao, Ritchie, van de Zande, and Beukeboom, unpublished data). 'Tremulation' has been suggested as a more specific term for this type of sound production to differentiate it from the more general term 'vibration' (Claridge, 2005).

Percussion

Active percussion on either substrates or other body parts is distinct from the sound emissions resulting from tremulation that mostly originate as by-products. For instance, in Australian whistling moths (Bailey, 1978) and some cicadas (Boulard, 2005), percussion of one body part against another has apparently evolved into specific communication systems. In termites (Howse, 1964) and stoneflies (Stewart and Sandberg, 2005), sound signaling by percussion of the substrate with the tip of their abdomina is a well-known mechanism, whereas males of a certain bushcricket species (*Meconema*) actively stamp the substrate with one of their hind legs to produce patterned sound signals (Ragge, 1965).

Stridulation

The wide-spread and well-studied mechanism of stridulation constitutes the creation of friction by rubbing specialized body parts against each other in a regular pattern (Ewing, 1989; Claridge, 2005). Stridulation has been found in at least seven different insect orders, including Orthoptera (grasshoppers, locusts, and crickets), Hemiptera (true bugs), and Coleoptera (beetles), and in most of them, it apparently evolved several times independently, mostly in the context of sexual signaling and mate attraction (Claridge, 2005; Matthews and Matthews, 2010).

Click Mechanisms

The deformation of specialized membranous areas of the cuticle, i.e., a tymbal, is most commonly achieved by contraction and relaxation of special insect body musculature, resulting in a series of 'clicks' allowing for rapid repetitions of characteristic sound patterns (Wessel *et al.*, 2014). The best known example for this particular sound production are the loud mate attraction sounds of cicadas (Matthews and Matthews, 2010; Wessel *et al.*, 2014), but it has also been described in other insect groups such as certain Lepidopteran families (Sales and Pye, 1974).

Air Expulsion

An unusual and rare insect sound production mechanism, the function of these exhalatory sounds, most often expelled via the tracheal spiracles, is generally not well-understood. One of the best-known examples is the European hawkmoth or Death's Head Hawk, *Acherontia atropos*, which forcibly expels air through its mouthparts for a loud, distinctive sound (Busnel and Dumortier, 1959). Another example is the Madagascar hissing cockroach, *Gromphadorhina portentosa*, capable of producing loud hisses from a pair of modified spiracles. Adult males have been found to mostly hiss during courtship, copulation, and aggressive encounters, but also when disturbed (Nelson and Fraser, 1980).

The loudest insect sound detected so far has been measured in a certain African cicada species, *Brevisana brevis*, where it reaches up to 106 decibels and can maintain this level up to 50 cm from the source (Matthews and Matthews, 2010). In comparison, male Asian corn borer moths (*Ostrinia furnicalis*) use very low intensity courtship songs of only 46 decibels with a range of just 1 cm, produced by male-specific scales on their forewings and the mesothorax (Nakano *et al.*, 2008). Apparently, this very restrained acoustic communication behavior has evolved to form a private, close-vicinity communication channel between male and female moths as protection from interference of male competitors and potentially eavesdropping predators (Conner, 2014).

For sound detection in insects, in general, specialized stretch receptor organs need to be present called tympanal organs, fitted with an exterior membrane (tympanum) and an air cavity behind it for detecting the pressure of sound waves transmitted through air or water (Hoy and Robert, 1996). Since the tympanum needs to exceed a certain minimum diameter in order to be fully functional, i.e., uphold the extreme tension to respond to sound effectively, insect body size is generally considered a constraining factor (Fletcher, 1992; Rodríguez *et al.*, 2005). Indeed, smaller insects generally lack tympanal organs and thus the ability to actually hear sound over long distances traveling through air or water (Bennet-Clark, 1998; Roces and Tautz, 2001). However, if sound is

considered in its broader definition as wave motions originating from vibrations that can be transmitted through any kind of medium, most insects actually can detect short-distance vibrational signals through different kinds of solid substrates (Hoy and Yack, 2009). This has also been described as 'seismic communication', and it has been hypothesized that this apparently wide-spread but understudied form of insect communication has evolved from the capability to detect self-induced body movements through so-called 'proprioceptors'. This potentially explains the body-wide distributions of these receptor organs in most studied insect taxa so far, as opposed to the evolutionary and developmentally constrained cranial position of almost all known vertebrate ears (Hoy and Yack, 2009).

Visual Communication

Although insects tend to rely more heavily on their other sensory modalities, visual communication still plays a major role in certain insect taxa (Matthews and Matthews, 2010). Perhaps most prominently, in the orders Odonata and Lepidoptera, particularly sophisticated color vision has evolved. Whereas Lepidoptera also rely on olfactory communication, Odonata apparently have exploited visual communication as their primary signaling modality, possessing greatly reduced antennae whose removal does not seem to impair their capability of prey capture and navigation (Matthews and Matthews, 2010).

The compound eyes of insects are composed of single units called ommatidia which, in turn, are composed of a facet lens, a crystalline cone, surrounding visual pigment cells as well as a set of photoreceptors (Nilsson, 1989). The visual pigments of the photoreceptors determine the spectral sensitivity, whereas actual photodetection primarily takes place in the rhabdomeres, a cylindrical structure in the photoreceptors, where the absorbed light is transduced into a neural signal. Insect vision is generally based on three major photoreceptor classes, specialized on different wave lengths of the light spectrum (Smith and Goldsmith, 1990): near ultraviolet (300–400 nm), cyan/blue/violet (400–500 nm), and orange/yellow/green (500–600 nm). Insect compound eyes directly connect to the optical lobe, the visual center of the insect brain, processing the perceived light signals to detect motion, colors, or other patterns of interest (Stavenga, 2002).

Insect color vision has been particularly well-investigated in butterflies and the honey bee, with the three photoreceptor classes playing major roles for their exceptional capacity to detect and discriminate between very distinct color sets (Stavenga, 2002; Briscoe, 2008). Whereas both honey bees and butterflies rely heavily on color vision in food foraging, particularly concerning the distinction of a vast array of flower coloration patterns (Menzel and Backhaus, 1989; Stavenga, 2002; Kelber *et al.*, 2003), butterflies have also extensively exploited color preference and distinction in mate choice and intraspecific male-male interactions (Lederhouse and Scriber, 1996; Frentiu and Briscoe, 2008, see also Fig. 1). This is particularly evident in emerging patterns of apparent co-evolution between butterfly color perception capabilities and their vast and distinguished wing coloration patterns (Briscoe, 2008; Koshitaka *et al.*, 2008).

Another particularly well-investigated form of visual communication in insects occurs in the Coleopteran family Lampyridae (fireflies), where many nocturnally active species emit precisely timed photic signals consisting of distinctive and clearly visible bioluminescent flashes or glows to attract mates (Lloyd, 1969; Ohba, 2004; Lewis and Cratsley, 2008). Interestingly, in diurnal Lampyridae species, olfactory communication appears to be the more prominent signaling modality (Lloyd, 1972; De Cock and Matthysen, 2005).

Olfactory Communication

In contrast to visual and auditory signaling, whose emission and reception are largely governed by quantitative differences, chemical signals display a more discrete character (Symonds and Elgar, 2008). The ability to perceive specific chemical compounds is mostly dependent on the presence or absence of the respective receptor molecules, leading to a tight linkage of sender and receiver (Bargmann, 2006).

Chemical compounds released from an individual capable of modifying the behavior of another individual who perceives them are generally defined as semiochemicals (Regnier, 1971). They can be further subdivided according to their function either between individuals of the same species (i.e., intraspecific) or separate species (i.e., interspecific).

In intraspecific communication, chemical signals exchanged between conspecific individuals are generally defined as pheromones (Karlson and Lüscher, 1959), fundamental elements in sexual communication typically functioning to attract conspecific mating partners (e.g., Baker, 1989; Wyatt, 2003) and to signal fertility, reproductive status, and mate quality (e.g., Hardie and Minks, 1999; Johansson and Jones, 2007). Although females have long been assumed to be the main emitters of sex attractants (Antony and Jallon, 1982; Löfstedt, 1993; Wyatt, 2003), more recent studies have delivered empirical evidence that males are not mostly restricted to the role of receivers, but can be sex attractant emitters as well (Grillet *et al.*, 2006; Ruther *et al.*, 2007; Eltz *et al.*, 2008). Independent of which sex is emitter or receiver, both parts of the communication system are required to be tightly coordinated to ensure accurate and successful mutual attraction of the mating partners (Bargmann, 2006).

Semiochemicals used in interspecific communication are generally defined as allelochemicals (Nordlund and Lewis, 1976; Dicke and Sabelis, 1988), and can be further subdivided into three main types: kairomones, allomones, and synomones (Brown *et al.*, 1970; Regnier, 1971). Those benefitting the receiver, while usually putting the sender at a disadvantage, are commonly defined as kairomones; whereas the ones benefitting the sender through behavioral modification of the receiver, while having a neutral or disadvantageous effect on the latter, are known as allomones. Synomones, on the other hand, are semiochemicals that benefit both the sender and the receiver (Brown *et al.*, 1970; Regnier, 1971). Semiochemical parsimony refers to the phenomenon when

a particular semiochemical simultaneously fulfills several of the above-mentioned categories depending on the specific behavioral context induced in several different recipients. For instance, an intraspecific sex pheromone may also function as a kairomone luring a predatory species in addition to a conspecific mate (Gullan and Cranston, 2010; Matthews and Matthews, 2010).

The phenomenon and terminology of kairomones, in general, represents an interesting evolutionary conundrum: It has been argued that a semiochemical mediating an interaction that is ultimately detrimental to the emitter would be selected against (Blum, 1977; Dicke and Sabelis, 1988). However, if put into perspective, it appears to be important to take the benefit into account that the emitter might gain, and weigh it against the potential detrimental effect when it is interspecifically exploited as a kairomone (Nordlund and Lewis, 1976; Weldon, 1980). A well-investigated case study constitutes the aggregation pheromone of bark beetles, which are important and beneficial in intraspecific calling behavior to quickly exploit food sources and potential mating grounds, while simultaneously bearing the risk of interspecific interception by predators (Wood, 1982).

Allomones, on the other hand, benefit just the receiver, and thus provide a strictly one-sided selective advantage. Bolas spiders of the genus *Mastophora* provide an excellent example for this: Specialized to prey on male moths, female bolas spiders use a specific string of web with a ball of glue at the distal end, which they actively swing towards their prey in flight (Eberhard, 1977; Yeargan, 1988). Most strikingly, it has been shown that bolas spiders produce compounds identical to the female moth sex pheromones that they instill on their bolas, an allomone through chemical mimicry by definition (Stowe et al., 1987; Haynes and Yeargan, 1999).

A case-in-point study exemplifying the definition of synomones would be volatiles released by leguminous plants that are damaged by feeding and oviposition activity of the southern green stink bug (*Nezara viridula*). These volatiles, in turn, attract the egg parasitoid wasp *Trissolcus basalis* (Wollaston), a highly efficient natural enemy of *Nezara viridula* whose own larvae parasitize and eventually consume the eggs of the southern green stinkbug laid previously on the plants (Hoffmann et al., 1991; Ehler, 2002; Colazza et al., 2004). Thus, both the damaged plants as well as the parasitoid wasps benefit from the emitted volatile plant synomones (Colazza et al., 2004).

Interactions of Different Signaling Modalities

Though integrative empirical studies assessing and incorporating different communication modalities are relatively rare, several case studies provide intriguing examples on how various signaling modalities can interact for optimizing information transfer.

In certain species of the aforementioned fireflies, it has been shown that, although visual communication is used for long-range mate attraction through bioluminescent flashing signals, close-range mate and species assessment can be mediated through olfactory communication via contact sex pheromones in certain species (South et al., 2008).

Similarly, field crickets, a prime example of long-distance mate attraction through auditory signaling, have been shown to incorporate olfactory signaling in their sexual communication behavior as well. Thus, female crickets, attracted through male mate calls, have been shown to use chemical cues to preferentially mate with novel males (Ivy et al., 2005), to favor unrelated males (Simmons, 1991), and to discriminate dominant from subordinate males (Kortet and Hedrick, 2005).

Nest-founding females of the paper wasp, *Polistes dominulus*, are apparently capable of discriminating different shapes, numbers and sizes of black facial spots on their rival foundresses to assess their quality, but chemical contact pheromones have also been shown to play a crucial role in their nestmate recognition behavior (Dani et al., 2001; Matthews and Matthews, 2010).

In *Drosophila* fruit flies, all three main signaling modalities apparently play a role in their complex courtship and mating behavior, as visual, acoustic, and chemical signaling have all been shown to contribute to mate localization, assessment, and acceptance (Cobb and Ferveur, 1995; Greenspan and Ferveur, 2000; Marcillac and Ferveur, 2004).

A quite unique case of a different and unusual signaling behavior has been found in honey bees: First decoded over 70 years ago by Karl von Frisch, honey bees have evolved a complex 'dance language' to convey information about the location of distant food sources to their nestmates (von Frisch, 1946). This well-known behavior constitutes a characteristic figure eight shaped 'waggle dance' that returning scouting bees perform on the vertical wax combs inside the bee hive (von Frisch, 1946, 1967). Thus, information about the distance of a new food source is conveyed by the duration of the 'waggle phase' of the dance, whereas information of the direction of the food source is based on the orientation of the dancer's body position relative to gravity (von Frisch and Jander, 1957; Gould et al., 1970; Riley et al., 2005). This signaling behavior is apparently complemented with auditory cues providing additional and more complete information about the distance of potential food sources from the hive (Wenner, 1962).

Furthermore, particularly fascinating examples of combinations of communication modalities include cases where larger taxonomic boundaries are traversed between very different species. For instance, certain lepidopteran larvae and pupae show functionally similar acoustic communication patterns to their symbiotic ant hosts, although their respective calls are apparently produced by very different means (Devries et al., 1993; Travassos and Pierce, 2000; Barbero et al., 2012). Even larger taxonomic boundaries are bridged in warning coloration arms races between certain butterflies and their vertebrate predators, e.g., bats (Dunning et al., 1992) or birds (Endler and Mappes, 2004; Langham, 2004), not to mention the well-known mutualistic visual and olfactory communication between plants and their insect pollinators (e.g., Silberglied, 1979).

Outlook

Despite a handful of intriguing case studies, integrative investigations of different communication modalities are still mostly lacking, and bear huge potential for unraveling a more holistic view on the entire spectrum of signaling interactions. For instance,

in the Hymenoptera (ants, bees, and wasps), one of the most prominent and largest insect orders (Grimaldi and Engel, 2005; Sharkey, 2007), studies on auditory communication are woefully absent so far (Claridge, 2005). Also, in more general terms, insect auditory communication has only occasionally been studied in interspecific interactions, particularly predator-prey and parasite-host relationships. However, integrating auditory with other communication modalities would greatly aid our understanding of how different information transfer modes interact to provide the full sensory spectrum in signaling relationships (Claridge, 2005). With the vast technological advancements in recent decades, our ability to record, analyze, reproduce, and synthesize different types of conveyed information has been greatly augmented. Therefore, combining our knowledge from these very different fields could open up new and exciting venues in deciphering insect communication in all its fascinating aspects.

Acknowledgements

Special thanks to Joshua D. Gibson for helpful suggestions on the compilation of signaling modalities presented here.

References

- Antony, C., & Jallon, J. M. (1982). The chemical basis for sex recognition in *Drosophila melanogaster*. *J. Insect Physiol.*, 28, 873–880.
- Bailey, W. J. (1978). Resonant wing systems in the Australian whistling moth *Hecatesia* (Agaridae, Lepidoptera). *Nature*, 272, 444.
- Baker, T. C. (1989). Sex-pheromone communication in the Lepidoptera: New research progress. *Experientia*, 45, 248–262.
- Barbero, F., Patricelli, D., Witek, M., et al. (2012). Myrmica ants and their butterfly parasites with special focus on the acoustic communication. *Psyche*, 2012.
- Bargmann, C. I. (2006). Comparative chemosensation from receptors to ecology. *Nature*, 444, 295–301.
- Bennet-Clark, H. C. (1971). Acoustics of insect song. *Nature*, 234, 255.
- Bennet-Clark, H. C. (1998). Size and scale effects as constraints in insect sound communication. *Philos. Trans. R. Soc. Lond. Ser. B: Biol. Sci.*, 353, 407–419.
- Blum, M. S. (1977). Behavioral responses of hymenoptera to pheromones and allomones. In H. H. Shorey, & J. J. McKevey, Jr. (Eds.), *Chemical Control of Insect Behavior: Theory and Application* (pp. 149–167). New York: Wiley & Sons.
- Boulard, M. (2005). Acoustic signals, diversity and behaviour of cicadas (Cicadidae, Hemiptera). In S. Drosopoulos, & M. F. Claridge (Eds.), *Insect Sounds and Communication: Physiology, Behaviour, Ecology, and Evolution* (pp. 331–350). CRC Taylor & Francis Group.
- Bradbury, J. W., & Vehrencamp, S. L. (2011). *Principles of Animal Communication*. Sunderland, MA: Sinauer Associates.
- Briscoe, A. D. (2008). Reconstructing the ancestral butterfly eye: Focus on the opsins. *J. Exp. Biol.*, 211, 1805–1813.
- Brown, W. L., Eisner, T., & Whittake, R. H. (1970). Allomones and kairomones: Transspecific chemical messengers. *Bioscience*, 20, 21–22.
- Busnel, R. G., & Dumortier, B. (1959). Vérification par des méthodes d'analyse acoustique des hypothèses sur l'origine du cri du Sphinx *Acherontia atropos* (Linné). *Bull. Soc. Entomol. Fr.*, 64.
- Chapman, A. D. (2009). *Numbers of Living Species in Australia and the World*. Canberra: Australian Biological Resources Study.
- Claridge, M. F. (2005). Insect sounds and communication – An introduction. In S. Drosopoulos, & M. F. Claridge (Eds.), *Insect Sounds and Communication: Physiology, Behaviour, Ecology, and Evolution* (pp. 3–10). CRC Taylor & Francis Group.
- Cobb, M., & Ferveur, J.-F. (1995). Evolution and genetic control of mate recognition and stimulation in *Drosophila*. *Behav. Process.*, 35, 35–54.
- Colazza, S., Fucarino, A., Peri, E., et al. (2004). Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids. *J. Exp. Biol.*, 207, 47–53.
- Conner, W. E. (2014). Adaptive sounds and silences: Acoustic anti-predator strategies in insects. In B. Hedwig (Ed.), *Insect Hearing and Acoustic Communication* (pp. 65–79). London, UK: Springer.
- Dani, F. R., Jones, G. R., Destri, S., Spencer, S. H., & Turillazzi, S. (2001). Deciphering the recognition signature within the cuticular chemical profile of paper wasps. *Anim. Behav.*, 62, 165–171.
- De Cock, R., & Matthysen, E. (2005). Sexual communication by pheromones in a firefly, *Phosphaenus hemipterus* (Coleoptera: Lampyridae). *Anim. Behav.*, 70, 807–818.
- Devries, P. J., Cocroft, R. B., & Thomas, J. (1993). Comparison of acoustical signals in Maculinea butterfly caterpillars and their obligate host *Myrmica* ants. *Biol. J. Linn. Soc.*, 49, 229–238.
- Dicke, M., & Sabelis, M. W. (1988). Infochemical terminology: Based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol.*, 2, 131–139.
- Dunning, D. C., Acharya, L., Merriman, C. B., & Ferro, L. D. (1992). Interactions between bats and arctiid moths. *Can. J. Zool./Rev. Can. Zool.*, 70, 2218–2223.
- Eberhard, W. G. (1977). Aggressive chemical mimicry by a bolas spider. *Science*, 198, 1173–1175.
- Ehler, L. E. (2002). An evaluation of some natural enemies of *Nezara viridula* in northern California. *BioControl*, 47, 309–325.
- Eltz, T., Zimmermann, Y., Pfeiffer, C., et al. (2008). An olfactory shift is associated with male perfume differentiation and species divergence in orchid bees. *Curr. Biol.*, 18, 1844–1848.
- Endler, J. A., & Mappes, J. (2004). Predator mixes and the conspicuousness of aposematic signals. *Am. Nat.*, 163, 532–547.
- Ewing, A. W. (1989). *Arthropod Bioacoustics: Neurobiology and Behaviour*. Ithaca, New York: Comstock Publishing Associates.
- Fletcher, N. H. (1992). *Acoustic Systems in Biology*. New York: Oxford University Press.
- Frentiu, F. D., & Briscoe, A. D. (2008). A butterfly eye's view of birds. *Bioessays*, 30, 1151–1162.
- Gould, J. L., Henerey, M., & MacLeod, M. C. (1970). Communication of direction by the honey bee. Review of previous work leads to experiments limiting olfactory cues to test the dance language hypothesis. *Science*, 169, 544–554.
- Greenfield, M. D. (2002). *Signalers and Receivers: Mechanisms and Evolution of Arthropod Communication*. New York, USA: Oxford University Press.
- Greenspan, R. J., & Ferveur, J.-F. (2000). Courtship in *Drosophila*. *Annu. Rev. Genet.*, 34, 205–232.
- Grillet, M., Darteville, L., & Ferveur, J.-F. (2006). A *Drosophila* male pheromone affects female sexual receptivity. *Proc. R. Soc. Lond. Ser. B: Biol. Sci.*, 273, 315–323.
- Grimaldi, D. A., & Engel, M. S. (2005). *Evolution of Insects*. Cambridge: Cambridge University Press.
- Gullan, P. J., & Cranston, P. S. (2010). *The Insects: An Outline of Entomology*. Wiley-Blackwell.
- Hardie, J., & Minks, A. K. (1999). *Pheromones of Non-lepidopteran Insects Associated with Agricultural Plants*. Wallingford, UK: CABI Press.
- Haynes, K. F., & Yeaman, K. V. (1999). Exploitation of intraspecific communication systems: Illicit signalers and receivers. *Ann. Entomol. Soc. Am.*, 92, 960–970.
- Hoffmann, M. P., Davidson, N. A., Wilson, L. T., et al. (1991). Imported wasp helps control southern green stink bug. *Calif. Agric.*, 45, 20–22.
- Hölldobler, B. (1984). Evolution of insect communication. In T. Lewis (Ed.), *Insect Communication* (pp. 349–377). London: Academic Press.
- Howse, P. E. (1964). The significance of the sound produced by the termite *Zootermopsis angusticollis* (Hagen). *Anim. Behav.*, 12, 284–IN288.
- Hoy, R., & Yack, J. (2009). Hearing. In H. Resh, & R. T. Cardé (Eds.), *Encyclopedia of Insects* (pp. 440–446). Academic Press.
- Hoy, R. R., & Robert, D. (1996). Tympanal hearing in insects. *Annu. Rev. Entomol.*, 41, 433–450.
- Ivy, T. M., Weddle, C. B., & Sakaluk, S. K. (2005). Females use self-referent cues to avoid mating with previous mates. *Proc. R. Soc. Lond. Ser. B: Biol. Sci.*, 272, 2475–2478.
- Johansson, B. G., & Jones, T. M. (2007). The role of chemical communication in mate choice. *Biol. Rev.*, 82, 265–289.
- Karlson, P., & Lüscher, M. (1959). 'Pheromones': A new term for a class of biologically active substances. *Nature*, 183, 55–56.

- Kelber, A., Vorobyev, M., & Osorio, D. (2003). Animal colour vision – Behavioural tests and physiological concepts. *Biol. Rev.*, 78, 81–118.
- Kortet, R., & Hedrick, A. (2005). The scent of dominance: Female field crickets use odour to predict the outcome of male competition. *Behav. Ecol. Sociobiol.*, 59, 77.
- Koshitaka, H., Kinoshita, M., Vorobyev, M., & Arikawa, K. (2008). Tetrachromacy in a butterfly that has eight varieties of spectral receptors. *Proc. R. Soc. B: Biol. Sci.*, 275, 947–954.
- Kramer, B. (1996). *Electroreception and Communication in Fishes*. Jena, Lübeck, Ulm: Gustav Fischer Verlag, Stuttgart.
- Langham, G. M. (2004). Specialized avian predators repeatedly attack novel color morphs of heliconius butterflies. *Evolution*, 58, 2783–2787.
- Lederhouse, R. C., & Scriber, J. M. (1996). Intrasexual selection constrains the evolution of the dorsal color pattern of male black swallowtail butterflies, *Papilio polyxenes*. *Evolution*, 50, 717–722.
- Lewis, S. M., & Cratsley, C. K. (2008). Flash signal evolution, mate choice, and predation in fireflies. *Annu. Rev. Entomol.*, 53, 293–321.
- Lewis, T. (1984). *Insect Communication*. London: Academic Press.
- Lloyd, J. E. (1969). Flashes, behavior and additional species of nearctic Photinus fireflies (Coleoptera: lampyridae). *Coleopterists Bull.*, 23, 29–40.
- Lloyd, J. E. (1972). Chemical communication in fireflies. *Environ. Entomol.*, 1, 265–266.
- Löfstedt, C. (1993). Moth pheromone genetics and evolution. *Philos. Trans. R. Soc. Lond. Ser. B: Biol. Sci.*, 340, 167–177.
- Maan, M. E., & Seehausen, O. (2011). Ecology, sexual selection and speciation. *Ecol. Lett.*, 14, 591–602.
- Marcillac, F., & Ferveur, J.-F. (2004). A set of female pheromones affects reproduction before, during and after mating in *Drosophila*. *J. Exp. Biol.*, 207, 3927–3933.
- Matthews, R. W., & Matthews, J. R. (2010). *Insect Behavior*. Springer Science & Business Media.
- Menzel, R., & Backhaus, W. G. K. (1989). Color vision of honey bees: Phenomena and physiological mechanisms. In S. D.G., & H. R.C. (Eds.), *Facets of Vision* (pp. 281–297). Berlin Heidelberg New York: Springer.
- Missbach, C., Dweck, H. K., Vogel, H., et al. (2014). Evolution of insect olfactory receptors. *eLife*, 3, e02115.
- Nakano, R., Skals, N., Takanashi, T., et al. (2008). Moths produce extremely quite ultrasonic courtship songs by rubbing specialize scales. *Proc. Natl. Acad. Sci. USA*, 105, 11812–11817.
- Nelson, M. C., & Fraser, J. (1980). Sound production in the cockroach, *Gromphadorhina portentosa*: Evidence for communication by hissing. *Behav. Ecol. Sociobiol.*, 6, 305–314.
- Nilsson, D.-E. (1989). Optics and evolution of the compound eye. In D. G. Stavenga, & R. C. Hardie (Eds.), *Facets of Vision*. Berlin, Heidelberg, New York: Springer.
- Nordlund, D. A., & Lewis, W. J. (1976). Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *J. Chem. Ecol.*, 2, 211–220.
- Ohba, N. (2004). Flash communication systems of Japanese fireflies. *Integr. Comp. Biol.*, 44, 225–233.
- Ragge, D. R. (1965). *Grasshoppers, Crickets and Cockroaches of the British Isles*. London: Frederick Warne.
- Regnier, F. E. (1971). Semiochemicals – Structure and function. *Biol. Reprod.*, 4, 309–326.
- Riley, J. R., Greggers, U., Smith, A. D., Reynolds, D. R., & Menzel, R. (2005). The flight paths of honeybees recruited by the waggle dance. *Nature*, 435, 205.
- Roces, F., & Tautz, J. (2001). Ants are deaf. *J. Acoust. Soc. Am.*, 109, 3080–3082.
- Rodríguez, R. L., Schul, J., Coccoft, R. B., & Greenfield, M. D. (2005). The contribution of tympanic transmission to fine temporal signal evaluation in an ultrasonic moth. *J. Exp. Biol.*, 208, 4159–4165.
- Roth, L. M. (1948). A study of mosquito behavior. An experimental laboratory study of the sexual behavior of *Aedes aegypti* (Linnaeus). *Am. Midland Nat.*, 40, 265–352.
- Ruther, J., Stahl, L. M., Steiner, S., Garbe, L. A., & Tolasch, T. (2007). A male sex pheromone in a parasitic wasp and control of the behavioral response by the female's mating status. *J. Exp. Biol.*, 210, 2163–2169.
- Saetre, G. P., Mourm, T., Bures, S., et al. (1997). A sexually selected character displacement in flycatchers reinforces premating isolation. *Nature*, 387, 589–592.
- Sales, G., & Pye, D. (1974). *Ultrasonic Communication by Animals*. London: Chapman and Hall.
- Schmitz, H., & Bleckmann, H. (1998). The photomechanic infrared receptor for the detection of forest fires in the beetle *Melanophila acuminata* (Coleoptera: buprestidae). *J. Comp. Physiol. A*, 182, 647–657.
- Schmitz, H., Bleckmann, H., & Murtz, M. (1997). Infrared detection in a beetle. *Nature*, 386, 773–774.
- Sharkey, M. J. (2007). Phylogeny and classification of Hymenoptera. *Zootaxa*, 521–548.
- Silberglied, R. E. (1979). Communication in the ultraviolet. *Annu. Rev. Ecol. Syst.*, 10, 373–398.
- Simmons, L. M. (1991). Female choice and the relatedness of mates in the field cricket, *Gryllus bimaculatus*. *Anim. Behav.*, 41, 493–501.
- Smith, W. C., & Goldsmith, T. H. (1990). Phyletic aspects of the distribution of 3-hydroxyretinal in the class insecta. *J. Mol. Evol.*, 30, 72–84.
- South, A., Levan, K., Leombruni, L., Orians, C. M., & Lewis, S. M. (2008). Examining the role of cuticular hydrocarbons in firefly species recognition. *Ethology*, 114, 916–924.
- Stavenga, D. (2002). Colour in the eyes of insects. *J. Comp. Physiol. A*, 188, 337–348.
- Stewart, K. W., & Sandberg, J. B. (2005). Vibratory communication and mate searching behaviour in stoneflies. In S. Drosopoulos, & M. F. Claridge (Eds.), *Insect Sounds and Communication: Physiology, Behaviour, Ecology, and Evolution* (pp. 179–186). CRC Taylor & Francis Group.
- Stowe, M. K., Tumlinson, J. H., & Heath, R. R. (1987). Chemical mimicry: Bolas spiders emit components of moth prey species sex pheromones. *Science*, 236, 964–967.
- Symonds, M. R. E., & Elgar, M. A. (2008). The evolution of pheromone diversity. *Trends Ecol. Evol.*, 23, 220–228.
- Travassos, M. A., & Pierce, N. E. (2000). Acoustics, context and function of vibrational signalling in a lycaenid butterfly–ant mutualism. *Anim. Behav.*, 60, 13–26.
- von Frisch, K. (1946). Die Tänze der Bienen. *Österr. Zool. Zeit.*, 1, 1–48.
- Von Frisch, K. (1967). *The Dance Language and Orientation of Bees*. Harvard University Press.
- von Frisch, K., & Jander, R. (1957). Über den Schwänzeltanz der Bienen. *Z. vgl. Physiol.*, 40, 239–263.
- Weldon P., 1980. In Defense of Kairomone as a Class of Chemical Releasing Stimuli.
- Wenner, A. M. (1962). Sound production during the waggle dance of the honey bee. *Anim. Behav.*, 10, 79–95.
- Wessel, A., Mühlethaler, R., Hartung, V., Kuštor, V., & Gogala, M. (2014). The tymbal: Evolution of a complex vibration-producing organ in the tymbalia (Hemiptera excl. Sternorrhyncha). In R. B. Coccoft, M. Gogala, P. S. M. Hill, & A. Wessel (Eds.), *Studying Vibrational Communication* (pp. 395–444). Berlin, Heidelberg: Springer Berlin Heidelberg.
- Wiltshko, R., & Wiltshko, W. (1995). *Magnetic Orientation in Animals*. Berlin, Heidelberg: Springer-Verlag.
- Wood, D. L. (1982). The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol.*, 27, 411–446.
- Wyatt, T. D. (2003). *Pheromones and Animal Behaviour. Communication by Smell and Taste*. Cambridge: Cambridge University Press.
- Yeagan, K. V. (1988). Ecology of a bolas spider, *Mastophora hutchinsoni*: Phenology, hunting tactics, and evidence for aggressive chemical mimicry. *Oecologia*, 74, 524–530.

Migration and Navigation in Fish

Hiroshi Ueda, Hokkaido University, Sapporo, Japan; and Hokkaido Aquaculture Promotion Corporation, Sapporo, Japan

© 2018 Elsevier Inc. All rights reserved.

Migratory Fish

Among the roughly 30,000 species of fish, migratory species account for only 165 species. However, they show interesting and complex life histories, and acquire many important biological functions that make possible for successful migration and navigation. Moreover, most of migratory fishes are valuable fisheries resources. Migratory fish either alone or in schools make drifting migration from spawning area to nursery area for recruitment, feeding migration from nursery area to feeding area for growth, and spawning migration from feeding area to spawning area for reproduction (Fig. 1). In special case, drifting migration occurs again from spawning area to feeding area if adults could survive after reproduction. Some migratory fishes are semelparous species that all adults die after reproduction only one time for their lifetime (semelparity), and other migratory fishes are iteroparous species that adults produce offspring several times during their lifetime (iteroparity). These migrations form a migration loop to produce next generations continuously, and allow fishes not only to exploit different food availabilities and to adapt to environmental changes, but also to regulate population density and widen their distribution.

Classification of Fish Migration

Migratory fishes are classified into the diadromous species that migrate between freshwater and seawater, and the non-diadromous species that migrate either only in seawater or freshwater (Table 1). The diadromous species are divided into catadromous species (eel), anadromous species (salmon, sturgeon, American shad, and striped bass), and amphidromous species that is further divided into seawater species (Japanese sea bass) and freshwater species (ayu). These migrations are characterized by their hatching, growing, and spawning area in either freshwater or seawater. Amphidromous fishes that are special species of catadromous and anadromous species can grow both in freshwater and seawater (Fig. 2). The non-diadromous species are divided into oceanodromous species that are further divided into highly migratory species (tuna) and coastal migratory species (herring), and potamodromous species that are further divided into fluvial species (Mekong giant catfish) and lacustrine species (isaza) (Table 1). Some migratory species have amazing abilities to precisely migrate and navigate thousands of kilometers from their feeding area to their natal spawning area, and anadromous salmon is famous for its accurate homing to the natal spawning area in the rivers. Other migratory species have relatively wide spawning areas. Each typical fish migration is reviewed using representative species.

Catadromous Migration

Eel is a worm-water catadromous species, and divided into four species: American eel (*Anguilla rostrata*), European eel (*A. anguilla*), Japanese eel (*A. japonica*), and short-finned eel (*A. australis*). They begin their life in the ocean as larval form called “leptocephalus” that drift with the aid of the ocean current toward the estuaries of continental shelf where they metamorphose into transparent larval stage called “glass eels.” Many of glass eels make upstream migration into freshwater habitats where they grow into “yellow eels” for 5–20 years, and some become “sea-eels” that never enter freshwater spending their entire lifetime in the ocean (Tsukamoto *et al.*, 1998). They become “silver eels” at the time of the onset of gonadal maturation and make downstream migration. Adult eels make oceanic spawning migration to the spawning area, and all adults die after reproduction (semelparity).

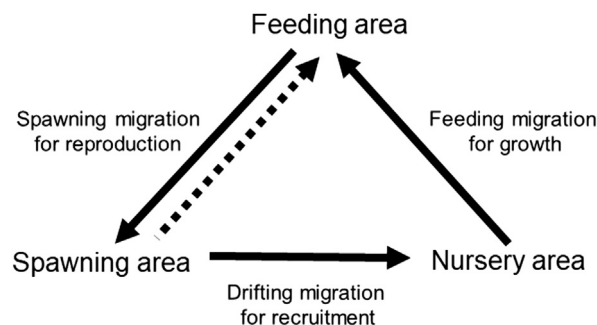


Fig. 1 Fish migration showing migration loop. Modified from Ueda, H., Tsukamoto, K., 2013. Physiology and Ecology of Fish Migration. CRC Press, Taylor & Francis Group. ISBN 978-1-4665-9513-2.

Table 1 Classification and representative species of migratory fish.

I. Diadromous species	
1. Catadromous species:	Eel
2. Anadromous species:	Salmon; Sturgeon; American shad; Striped bass
1) Sea-run form	
2) Reident form	
3. Amphidromous species	
1) Seawater species:	Japanese sea bass
2) Freshwater species:	Ayu (Sweet fish)
II. Non-diadromous species	
1. Oceanodromous species	
1) Highly migratory species:	Tuna
2) Coastal migratory species:	Herring
2. Potamodromous species	
1) Fluvial species:	Mekong giant catfish
2) Lacustrine species:	Isaza

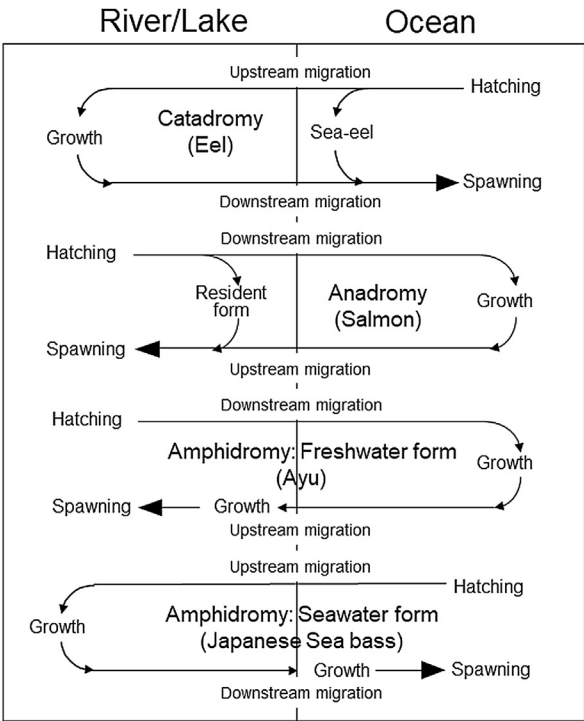


Fig. 2 Diadromous fish migration showing catadromous specie (eel), anadromous species (salmon), and amphidromous seawater species (Japanese sea bass) and freshwater species (ayu).

European eel

The spawning areas of European eel was found in the Sargasso Sea in 1920s, but many mysteries of juvenile and adult migration between the Sargasso Sea and freshwater habitats distributed along European countries are unsolved yet. Juveniles were reported to possess a magnetic map of the North Atlantic Ocean and use to target the Gulf Stream System (Naisbett-Jones *et al.*, 2017). The spawning migration of adults were observed by biotelemetry techniques revealing that they exhibited diel vertical migrations in deeper water during the day and shallower water at night at the swimming speeds from 3 to 47 km/day (Righton *et al.*, 2016).

Japanese eel

The spawning area of Japanese eel was discovered in the west of the Marian Islands where adults and newly hatched larvae were caught during new moon periods throughout the spawning season indicating that spawning occurs in shallower layers of 150–200 m (Tsukamoto *et al.*, 2011). However, there are many mysteries about how can adult eel migrate and navigate in ocean and find their spawning site using what kinds of sensory systems.

Anadromous Migration

Salmon is a cold-water anadromous species, and divided into Pacific salmon (genus *Oncorhynchus*) and Atlantic salmon (genus *Salmo*). There are seven Pacific salmon species and two Atlantic salmon species, and they show different complex life cycles.

Pacific salmon

In Pacific salmon, underyearling all juveniles of pink salmon (*O. gorbuscha*) and chum salmon (*O. keta*) make downstream migration in spring within a few months after emergence, and there is no resident form. Adult pink salmon and chum salmon make upstream migration in autumn within a few weeks of spermiation and ovulation after 2 years and 2–7 years oceanic feeding migration, respectively. In contrast, juveniles of sockeye salmon (*O. nerka*), Chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), and masu salmon (*O. masou*) stay in freshwater rivers or lakes for a few years, carry out smoltification (parr-smolt transformation) that is physiological changes obtaining seawater adaptability, and make downstream migration in spring. There are resident individuals that spend their whole life in rivers or lakes. Adults make upstream migration after several years of oceanic feeding migration in autumn at least several months prior to spermiation and ovulation (Fig. 3).

These six Pacific salmon are semelparous species. It is phylogenetically reported that pink salmon is the most advanced salmon species, while masu salmon is the most primitive species (Murata *et al.*, 1996). Pink salmon is also the most widely distributed species in the north Pacific Ocean and have the largest population size, while masu salmon appear to have the most restricted distribution to the west Pacific Ocean and the smallest population (Kaeriyama and Ueda, 1998). Although the homing accuracy of these salmon has not been compared in detail, it is believed that masu salmon return to their natal stream with the highest precision, and that pink salmon are more likely to stray into a non-natal stream. If most salmon might show a highly accurate homing to the natal stream, there would be little chance to enhance their distribution area as well as to increase their population size. And, they might encounter the dangerous possibility to reduce their genetic diversity. The relationship between salmon evolution and homing accuracy is one of the most interesting questions from a viewpoint of biological evolution.

Steelhead or steelhead trout (*O. mykiss*) is a sea-run form of rainbow trout, and an iteroparous species. Juveniles remain in freshwater for a few years before smoltification, and migrate to ocean. Adults make upstream migration to their natal spawning areas for breeding, and about 10% of adults survive and make drifting downstream migration again from spawning area to oceanic feeding areas in multiple years.

Atlantic salmon

In Atlantic salmon (*S. salar*) and sea trout (*S. trutta*) that is a sea-run form of brown trout, juveniles stay in freshwater for several years, and grow to smolt that make downstream migration to ocean where they do feeding migration for several years. There are some resident individuals that never enter to seawater spending their entire lifetime in freshwater. Adults make upstream migration to their natal spawning areas for breeding, and some can survive after spawning and make drifting downstream migration again from spawning area to oceanic feeding area in multiple years (Iteroparity).

The other anadromous species

The other anadromous species include sturgeon (four genera: *Acipenser*, *Huso*, *Scaphirhynchus*, and *Pseudoscaphirhynchus*), American shad (*Alosa sapidissima*), and striped bass (*Morone saxatilis*). They all spawn in freshwater, make downstream migration into seawater where they grow, and make upstream migration for reproduction.

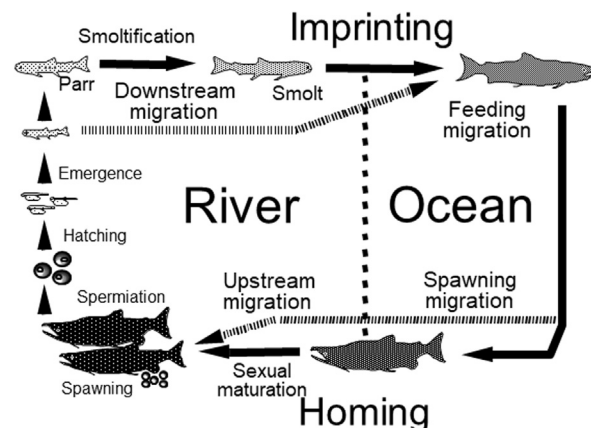


Fig. 3 Life history of two different types of Pacific salmonid species. Dotted line: chum and pink salmon; Solid line: sockeye, Chinook, coho and masu salmon. Ueda, H., Tsukamoto, K., 2013. Physiology and Ecology of Fish Migration. CRC Press, Taylor & Francis Group. ISBN 978-1-4665-9513-2

Amphidromous Migration

Seawater species

Japanese sea bass (*Lateolabrax japonicus*) is a seawater species of amphidromous fish, native to the west Pacific Ocean, and spawn in coastal sea in winter. Juveniles inhabit in seawater, brackish water, and freshwater where they grow from spring to autumn, and make upstream migration in rivers to avoid high water temperature. Adults make downstream migration to coastal sea for reproduction.

Freshwater species

Ayu or sweetfish (*Plecoglossus altivelis*) is a freshwater species of amphidromous fish, and spawn in rivers in autumn. Juveniles make drifting downstream migration to coastal sea where they feed planktons in winter. Young fish make upstream migration in the lower and middle reaches where they feed attached algae, spawn in autumn, and die after reproduction (semelparity).

Oceanodromous Migration

Highly migratory species

Tuna is divided into true tuna species (genus *Thunnus*) and other tuna species (four genera: *Allothunnus*, *Auxis*, *Eurhynchus*, and *Katsuwonus*). The genus *Thunnus* is further divided into two subgenera: the bluefin group and the yellowfin group. There are five species of relatively large size bluefin group: albacore tuna (*T. alalunga*), southern bluefin tuna (*T. maccoyii*), bigeye tuna (*T. obesus*), Pacific bluefin tuna (*T. orientalis*), Atlantic bluefin tuna (*T. thynnus*), and three species of relatively small size yellowfin group: blackfin tuna (*T. atlanticus*), longtail tuna (*T. tonggol*), yellowfin tuna (*T. albacares*).

Tunas are endothermic species that can maintain their body temperature of certain parts of body (skeletal muscles, eyes, and brain) above the temperature of ambient seawater, which supports faster swimming speeds (up to 75 km/h) and survival in cooler ocean environments. They distribute throughout the oceans of the world, generally in tropical and temperate waters at latitudes ranging between about 45° north and south of the equator. They carry out migrations of significant distances across oceans for feeding and reproduction. They are iteroparous species, and their relatively large spawning areas in the ocean are different among species.

Coastal migratory species

Herring is divided into three species: Arucanian herring (*Clupea bentincki*), Atlantic herring (*C. harengus*), and Pacific herring (*C. pallasii*). They make migration in school from open ocean to coastal sea in intertidal and sub-tidal environments, and spawn in variety seawater environments at different seasons among species. Eggs are laid on the seabed (rock, stones, gravel, sand) or submerged vegetation (eelgrass or seaweed), stick in layers of clumps to gravel, seaweed, or stones.

Potamodromous Migration

Fluvial species

Mekong giant catfish (*Pangasianodon gigas*) is a critically endangered species and native to the Mekong basin. They are recognized as the world's largest freshwater species weighting up to 350 kg. Although little is known about their migrations in the Mekong River, they congregate during the beginning of the rainy season and migrate upstream to spawn in the Mekong River.

Lacustrine species

Isaza (*Gymnogobius isaza*) is an endemic species in Lake Biwa, Japan. They are freshwater pelagic species, inhabit offshore areas in the lake, and migrate to the shore to spawn under stones in spring. Males care for the eggs in their nest.

Physiological Mechanisms of Migration and Navigation in Fish

Migratory fishes possess many important essential biological functions that make possible for a long distance migration and navigation between their nursery habitats, feeding habitats, and spawning habitats in their complex life histories. Especially, salmon are recognized for their amazing abilities to memorize their natal river information during downstream migration, carry out thousands kilometer feeding migration in the ocean for many years, and make homing migration precisely to their natal rivers for reproduction. Physiological mechanisms of imprinting and homing migration of Pacific salmon were reviewed using three different research approaches: Homing behavior of adult chum salmon from the Bering Sea to Hokkaido as well as lacustrine sockeye salmon and masu salmon in Lake Toya (serves as a model ocean) were examined using physiological biotelemetry techniques; Hormone profiles in the brain-pituitary-thyroid (BPT) and brain-pituitary-gonad (BPG) axes of chum and sockeye salmon were analyzed using endocrinological techniques; Olfactory memory formation and retrieval of natal stream odors in Pacific salmon were investigated using neurobiological techniques (Ueda, 2016). Finally, impacts of global climate changes on these physiological mechanisms of migratory fish are reviewed.

Physiological Biotelemetry Researches on Adult Salmon Homing Migration

The recent rapid development of biotelemetry technologies, such as ultrasonic and radio telemetry, data logging, and pop-up satellite telemetry, makes it possible to continually observe the underwater behavior of migratory fish in open water (Hussey *et al.*, 2015). Japanese adult chum salmon homing behavior from the central Bering Sea to the east coast of Hokkaido, Japan was examined using propeller data loggers, which recorded swimming speed, depth, and temperature (Tanaka *et al.*, 2005). The first recorded swimming profiles of homing chum salmon in the oceanic phase revealed that chum salmon had travelled with an average swimming speed, depth, and temperature of 62 ± 12 (mean $\pm$ SD) cm/sec, 10.4 ± 14.7 m, and $9.2 \pm 0.2^\circ\text{C}$, respectively. The fish showed sequential up-and-down movements near the thermocline during the twilight and during the daytime suggesting that the homing chum salmon allocated a proportion of their time to foraging. These data indicate that the homing chum salmon have navigation abilities in the homeward direction, and that salmon use ocean current transport to reduce energy expenditure during migration.

Lacustrine salmon populations offer an excellent model system for studying homing behaviors from open water to natal areas for reproduction. Homing migrations of mature lacustrine sockeye salmon, whose sensory cues were impaired by ablations, tracking from the center of the Lake Toya, a large caldera lake to their natal area using ultrasonic tracking system. Visual cues are critical to the straight homing of sockeye salmon, while magnetic cues do not appear to be necessary for successful return to the natal area (Ueda *et al.*, 1998). However, the Earth's magnetic forces during chum salmon homing migration in the Bering Sea was studied using magnetic tags that can record the magnetic force, and revealed that the homing migration route was approximately along the isoline of magnetic intensity (Azumaya *et al.*, 2016).

Spawning migration of lacustrine masu salmon attached with depth and temperature data loggers in Lake Toya clarifying that masu salmon can calculate the day length with an internal biological clock, because the diurnal movement after encounter with the river mouth might be usable to calculate the timing of upstream migration to their spawning river. To accurately home in open water, salmon must recognize exact locations (map) and compass direction (orientation) during migration, and must have an internal biological clock to calculate the duration from the feeding ground to the spawning ground (Ueda, 2016).

Neuroendocrinological Hormone Control Mechanisms of Imprinting and Homing Migration in Salmon

Salmon imprinting downstream migration in juveniles and homing upstream migration in adults are closely related to seawater adaptation and gonadal maturation, and are under the complex regulation of endocrine hormones mainly by the BPT axis and the BPG axis, respectively. The BPT axis involves thyrotropin-releasing hormone (TRH) in the brain, thyrotropin (TSH) in the pituitary gland, and thyroxine (T4) and triiodothyronine (T3) in the thyroid gland. The BPG axis involves salmon gonadotropin-releasing hormone (sGnRH) and chicken gonadotropin-releasing hormone-II (cGnRH-II) in the brain, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the pituitary gland, and estradiol- 17β (E2), testosterone (T), 11-keto testosterone (11 KT), and $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) in the ovary and testis.

It has been impossible to link hormonal control mechanisms to imprinting and homing migration of salmon because molecular markers that could permit the evaluation of memory formation and retrieval in the brain were lacking. Synaptic plasticity is thought to underlie the formation of memory. Long-term potentiation (LTP), which is a persistent strengthening of synaptic activity produced a long-lasting increase in signal transmission between two neurons, is widely considered one of the major cellular mechanisms that underlies memory formation and retrieval. The N-methyl-D-aspartate receptor (NMDAR) is a glutamate receptor channel subtype, mediates most of the fast-excitatory synaptic transmission in the central nervous system, and induces LTP. NMDARs are comprised of two subunits: the essential NR1 subunit and the differentially expressed NR2A-D subunit.

In the brain of hatchery-reared underyearling juvenile chum salmon, the gene expression of TRH increased immediately after release from a hatchery into the natal stream, and then the gene expression of NR1 increased during downstream migration. Gene expression of sGnRH and NR1 increased in the adult chum salmon brain during homing from the Bering Sea to the natal hatchery. Oral T4 administration in juvenile chum salmon enhanced NR1 gene activation. Juvenile imprinting downstream migration and adult homing upstream migration of chum salmon are controlled by endocrine hormones and could be clarified using NR1 as a molecular marker in the brain (Ueda *et al.*, 2016).

In Lake Shikotsu, a large caldera lake in Hokkaido, homing profiles of adult lacustrine sockeye salmon were different between males and females: 100% of males returned faster than females in early spawning season and 50% of males did not return to their natal spawning areas in late spawning season. In contrast, 70–90% of females returned over the spawning season. GnRH analog (GnRHa) treatment was highly efficient in shortening the homing duration of lacustrine sockeye salmon due to increases of serum DHP levels in both sexes (Sato *et al.*, 1997). Homing duration of male lacustrine sockeye salmon was significantly prolonged by NMDAR antagonists (APV and MK-801). These data suggest that GnRH and NMDAR are critically involved in salmon homing migration.

Neurobiological Olfactory Memory Formation and Retrieval of Natal Stream Odors in Salmon

Salmon olfactory organs respond only to limited dissolved chemical compounds, such as amino acids, steroids, bile acids, and prostaglandins. Electrophysiological responses of the olfactory nerve in masu salmon to artificial stream water (ASW) based on the composition of dissolved free amino acid (DFaa) were closely resembled to the corresponding natural stream water (Shoji *et al.*, 2000). DFaa concentration and composition of the Teshio River, Hokkaido, were analyzed during juvenile salmon downstream migration in spring and adult salmon upstream migration in autumn with a 4-year difference, and found that there were some

stable DFAA compositions between the spring and autumn samples within a 4-year span. Behavioral experiments of upstream selective movement in a 2-choice test tank (Y-maze) consisting of two water inlet arms and one pool revealed that four-year-old mature male chum salmon captured in the Teshio River showed significant preference for these stable DFAA compositions (Yamamoto *et al.*, 2013). The origin of DFAA in stream water was investigated focusing on biofilms that consists of various microorganisms embedding into a matrix of extracellular polymeric substances in the stream bed by incubation experiments in the laboratory. The DFAA concentration increased greatly in the biofilm incubation solution of the treatment group, but the DFAA composition showed little change after incubation, which was like stream water, suggesting that biofilms are a major source of DFAA in stream water. One-year-old sockeye salmon can imprint a single amino acid before and during smoltification, and that imprinting requires exposure for at least 14 days in the artificial rearing environment. These findings indicate that the long-term stability of the DFAA compositions in natal streams is crucial for olfactory imprinting and homing in Pacific salmon.

Behavior experiments were compared to test attractant effects on upstream selective movement among the four Pacific salmon species (pink, chum, sockeye, and masu salmon) using artificial natal stream water (ANW) prepared to the same composition and concentration of DFAA in their natural natal stream in the Y-maze. In both ANW and natural lake water, pink salmon showed the highest percentage of upstream movement among the four Pacific salmon species, but showed the least selectivity in the arm running ANW, whereas the other three Pacific salmon species (chum, sockeye, and masu salmon) showed significant selectivity in the arm running ANW. These results indicated that ANW had different attractive effects on upstream selective movement among four Pacific salmon. Pink salmon showed the highest upstream movement and the lowest selectivity to the artificial natal stream water (Ueda, 2016).

Impacts of Climate Changes on Migratory Fish

Climate changes concerning sea and lake levels, thermal structure, oceans currents, and acidification (seawater and freshwater) have caused many negative and a few positive impacts on important fisheries resources, habitats, and distributions of migratory fish. Migratory fish show different response to water temperature-dependent climate changes between cold-water species and warm-water species.

The stocks of Japanese chum salmon (a cold-water species) peaked in 1996 because of the successful improvement of propagation techniques and favorable habitats conditions in the North Pacific Ocean. However, numbers of adult return subsequently decreased rapidly, mainly due to high-water temperature along the coastal sea of northern Japan where adults make homing migration to their natal streams. In contrast, the stocks of Japanese amberjack or yellow tail (*Seriola quinqueradiata*: a warm-water species) increased steadily, mainly due to high-water temperature along their migratory routes in recent years. Their distribution areas move up to the north, and their catches increased in the northern Japan, especially in Hokkaido.

Physiological biotelemetry researches are valuable to reveal how migratory fish can navigate in open water using compass, map, and biological clock in response to new environments caused by climate changes. Molecular biological and biochemical endocrinological researches are necessary to reveal how and which endocrine systems involved in growth and reproductive migration of migratory fish respond to climate change-related new environments. Neurobiological researches are important to clarify which sensory systems involved in memory formation and retrieval of their spawning environments that might be affected by climate changes.

References

- Azumaya, T., Sato, S., Urawa, S., *et al.* (2016). Potential role of the magnetic field on homing chum salmon (*Oncorhynchus keta*) tracked from the open sea to coastal Japan. *North Pacific Anadromous Fish Commission Bulletin*, 6, 235–241.
- Hussey, N. E., Kessel, S. T., Aarestrup, K., *et al.* (2015). Aquatic animal telemetry: A panoramic window into the underwater world. *Science*, 348, 1255642.
- Kaeriyama, M., & Ueda, H. (1998). Life history strategy and migration pattern of juvenile sockeye (*Oncorhynchus nerka*) and chum salmon (*O. keta*) in Japan: A review. *North Pacific Anadromous Fish Commission Bulletin*, 1, 163–171.
- Murata, S., Takasaki, N., Saitoh, M., *et al.* (1996). Details of retropositional genome dynamics that provide a rationale for a generic division: The distinct branching of all the Pacific salmon and trout (*Oncorhynchus*) from the Atlantic salmon and trout (*Salmo*). *Genetics*, 142, 915–926.
- Naisbett-Jones, L. C., Putman, N. F., Stephenson, J. F., *et al.* (2017). A magnetic map leads juvenile European eels to the Gulf stream. *Current Biology*, 27, 1236–1240.
- Righton, D., Westerberg, H., Feunteun, E., *et al.* (2016). Empirical observations of the spawning migration of European eels: The long and dangerous road to the Sargasso Sea. *Science Advances*, 2, e1501694.
- Sato, A., Ueda, H., Fukaya, M., *et al.* (1997). Sexual differences in homing profiles and shortening of homing duration by gonadotropin-releasing hormone analog implantation in lacustrine sockeye salmon (*Oncorhynchus nerka*) in Lake Shikotsu. *Zoological Science*, 14, 1009–1014.
- Shoji, T., Ueda, H., Ohgami, T., *et al.* (2000). Amino acids dissolved in stream water as possible homestream odorants for masu salmon. *Chemical Senses*, 25, 533–540.
- Tanaka, H., Naito, Y., Davis, N. D., *et al.* (2005). Behavioral thermoregulation of chum salmon during homing migration in coastal waters. *Marine Ecology Progress Series*, 291, 307–312.
- Tsukamoto, K., Chow, S., Otake, T., *et al.* (2011). Oceanic spawning ecology of freshwater eels in the western North Pacific. *Nature Communications*, 2, 179.
- Tsukamoto, K., Nakai, I., & Tesch, W. V. (1998). Do all freshwater eels migrate? *Nature*, 396, 635–636.
- Ueda, H. (2016). Physiological mechanisms of imprinting and homing migration of Pacific salmon. *Aqua-Bioscience Monographs*, 9, 1–27.
- Ueda, H., Kaeriyama, M., Mukasa, K., *et al.* (1998). Lacustrine sockeye salmon return straight to their natal area from open water using both visual and olfactory cues. *Chemical Senses*, 23, 207–212.
- Ueda, H., Nakamura, S., Nakamura, T., *et al.* (2016). Involvement of hormones in olfactory imprinting and homing in chum salmon. *Scientific Reports*, 6, 21102.
- Yamamoto, Y., Shibata, H., & Ueda, H. (2013). Olfactory homing of chum salmon to stable compositions of amino acids in natal stream water. *Zoological Science*, 30, 607–612.

Migration and Navigation in Birds

Heather E Watts, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Migration is the movement of an animal between distinct habitats or home ranges; these movements are greater in scale than those that occur as part of the regular daily routines of animals (Dingle and Drake, 2007). Most commonly, migratory movements, including those of birds, take animals between a breeding location and non-breeding location or wintering ground. Although migration is itself an energetically demanding process, it allows animals to make use of resources that vary in space and time, such as food sources that are only available seasonally. For example, Gambel's white crowned sparrows (*Zonotrichia leucophrys gambelii*) breed at high latitudes such as in Alaska and northern Canada, where they can feed on insects that become abundant in the spring and summer. However, these high-latitude breeding sites become inhospitable in the winter. Therefore, the birds migrate to lower latitudes in the western US and Mexico for overwintering, where seeds, grasses and fruits are available to eat. In this way, migration allows birds to utilize resources that are available in a given area for only a limited period of time.

Types of Migration

Patterns of migratory behavior vary widely among species. The most well-known migrants are those species that make very predictable migrations that occur at the same times each year and move birds between the same locations each year. This type of migration is often called obligate, calendar, or to-and-fro migration. Most often birds move to breeding grounds in the spring and then to wintering grounds in the fall. Some species make an additional movement to a third location, such as a molt migration to a location for molting (i.e., replacing of the feathers). American redstarts, (*Setophaga ruticilla*), black-headed buntings (*Emberiza melanocephala*), Gambel's white-crowned sparrows, Northern wheatears (*Oenanthe oenanthe*), and red knots (*Calidris canutus*) are just a few well-studied examples of obligate migrants.

However, not all birds move so predictably. Migrations that occur less predictably in space and/or time are called facultative migrations. One form of facultative migration is nomadic migration. Nomadic migrants typically move unpredictably between different locations each year, such that they rarely return to the same location. Nomadic migrations also tend to vary more in their timing from year to year, and nomadic migrants may forgo migration in some years. Banded stilts (*Cladorhynchus leucocephalus*), gray teals (*Anas gracilis*), pine siskins (*Spinus pinus*), red crossbills (*Loxia curvirostra*), and Tengmalm's owls (*Aegolius funereus*) are examples of nomadic migrants.

It is also important to recognize that many species of birds (or, in some cases only certain populations within a species) do not migrate; instead they remain in the same location year-round. Such birds are referred to as permanent residents. For such a strategy to be successful, resources, particularly food, must be sufficiently available in the same location year-round. House finches (*Haemorrhous mexicanus*), spotted antbirds (*Hylophylax naevioides*), and gray partridges (*Perdix perdix*) are all examples of species that are permanent residents in at least some parts of their range. Furthermore, some populations are composed of a mixture of birds that migrate and birds that remain resident. Such populations are said to be partially migratory. In some cases, individual birds within a partially migratory population remain either migrant or resident for their entire lives. This is the case for European blackcaps (*Sylvia atricapilla*) where being migratory or resident is an inherited trait. In other species, such as tropical kingbirds (*Tyrannus melancholicus*) and skylarks (*Alda arvensis*), individuals can switch between being migrant and resident across years, depending on their body condition, social status, or prevailing environmental conditions.

In addition to nomadic migration, another form of facultative migration is fugitive or escape migration. Fugitive migrations are movements away from unpredictable and severe disruptions in the local environment, such as a severe storm or drought. They allow birds to escape these inhospitable conditions and return when the disruption subsides. Fugitive migrations can occur in any bird, regardless of their typical migratory pattern. Thus, obligate, nomadic, and partial migrants, as well as permanent residents, can all make fugitive migrations if necessary.

The Migratory Phenotype

Migration involves profound changes to the morphology, physiology and behavior of a bird. Prior to migration birds undergo a period of preparation. This includes an increase in feeding (known as hyperphagia) and, in some species, a shift in diet such as an increase in fruit intake. These changes in feeding behavior in turn support fat deposition, which increases dramatically in many species (Fig. 1). Ultimately, these fat reserves provide the fuel for migration (McWilliams et al., 2004). Birds also increase muscle mass, particularly through hypertrophy, an increase in size, of the pectoralis muscles, which power the wing downstroke and are critical to sustained flight (Fig. 1). Birds may also undergo changes that facilitate oxygen transport during the intense exercise involved in migration, such as increasing the proportion of red blood cells in the blood. The intensity of these preparations (e.g., extent of fat deposition) can vary seasonally (fall vs. spring migration) and across species, and migratory preparations have been suggested to vary with factors such as the length of the migratory journey and expected conditions *en route*. Moreover, in some

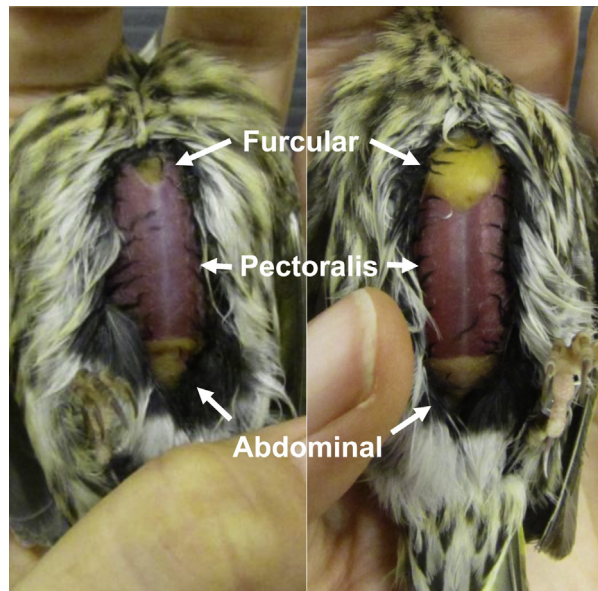


Fig. 1 Photos illustrating physiological preparations for migration. Subcutaneous fat deposition is visible in the furcular and abdominal regions as yellow tissue. The bird on the right has more extensive fat depots compared to the bird on the left. As premigratory fattening progresses, fat depots will even surpass those seen in the bird on the right. An increase in the size of the pectoralis muscles (muscle hypertrophy) can also be seen; on the right the muscles are bulging above the keel, this is not the case for the smaller muscles on the left. Photos are taken from pine siskins (*Spinus pinus*). Photos by H.E. Watts.

species, energy reserves that are deposited prior to migration or during stopovers may also provide energy for breeding efforts after arrival.

The preparatory period is followed by the expression of the migratory state. The immediate decision to begin a migratory flight is influenced by weather conditions such as precipitation, cloud cover, temperature and wind conditions, with warm weather, clear skies without rain, and no winds or tail-winds being most conducive to migration (Richardson, 1990). Time of day also influences when birds migrate. Many species of birds migrate at night, including numerous species that are otherwise diurnal in their activity patterns. Flying at night, rather than during the day, may occur because atmospheric conditions at night are more favorable for flight and/or for preventing dehydration and overheating (Berthold, 1996). Nocturnal flight may also be necessitated by the need to feed during the day. Despite these potential advantages of nocturnal migration, there are other species that migrate during the day.

In some species, such as bar-tailed godwits (*Limosa lapponica baueri*), migration occurs primarily as a single non-stop bout of flight, whereas in other species migratory flight is interrupted by frequent periods of stopover, during which birds refuel before resuming flight. Stopovers typically last a few days to a couple of weeks, during which time birds forage in order to replenish fat depots and prepare for further migratory flight. In selecting stopover sites, birds must select locations with sufficient food availability for refueling. The length of stay at a stopover site is influenced by the bird's condition when they arrive, as well as their ability to refuel at the site.

Once birds have arrived at their destination, migration terminates. This process of arrival and settlement is perhaps the least understood step in migration. In many species, arrival in the general geographic area of the destination is followed by a period of local movement as birds locate suitable habitats or sites before settling. Upon arrival birds may also maintain a degree of readiness to resume or continue moving if conditions are unsuitable or deteriorate (Ramenofsky and Wingfield, 2006).

Navigation

For to-and-fro migrants, migration involves travelling between the same breeding and wintering grounds each year. How these birds find their way during migration has been a question of great interest to scientists. Although we do not yet have a complete picture of how this is accomplished, we do understand several mechanisms that birds use to navigate. Birds have at least three biological compasses that they can use to orient themselves in space. Birds are able to use (1) the sun and patterns of polarized light, (2) earth's magnetic field, and (3) stars to orient (Berthold, 2001). Moreover, evidence suggests birds may often use a combination of these cues. For example, thrushes belonging to the genus *Catharus* appear to use cues from the sun at twilight to determine a migratory direction and then use a magnetic compass to maintain that direction during their nighttime flight (Cochran et al., 2004). Each biological compass requires a sensory mechanism to detect the cue, which is then processed by the brain. Recent research has focused on understanding how birds detect and process magnetic information for orientation, and there is now evidence that both the eye and beak contain magnetoreceptors that could function in the magnetic compass (Mouritsen and Ritz, 2005). Although birds can determine direction using one or more of the biological compasses, a compass alone does not inform a bird

about its relative location on the landscape. Although a number of potential mechanisms have been proposed by which birds might determine their location, few are well supported by empirical evidence. The best evidence for such a mechanism one is based on spatial cues that birds learn *en route* and at destinations in order to aid in navigation during subsequent migrations (Mettkke-Hofmann and Gwinner, 2003). While such cues are useful for adults, young birds lack such cues on their first migration.

The first time a bird migrates from the breeding grounds where it was born to its wintering grounds, it must travel a route it has never taken to a destination it has never seen. To do this, birds make use of a genetically inherited migratory direction. This migratory direction sets birds off on a trajectory towards their destination and is coupled with a programed duration of migration. Together this use of direction and duration of migration, which is referred to as vector navigation or clock-and-compass orientation, leads juvenile birds to their wintering grounds (but see Thorup *et al.* (2010) for a discussion of the limitations of this explanation). In flocking species, juveniles may make use of the knowledge of experienced adults by following them. But even species that typically rely on experienced guides may also be able to vector navigate, presumably as a backup mechanism (Mouritsen, 2003). Once a bird has completed this initial journey, it has had the opportunity to gather information on the breeding and wintering grounds that it may use on subsequent migrations. A bird will also have been able to gather information *en route*. Therefore, it is possible that birds could make the return journey to the breeding grounds by retracing their outbound route. However, in many species, birds take a different migratory return route. Consequently, vector navigation is likely used in combination with information that was previously learned about habitat, latitude, or landmarks on the breeding grounds or *en route*. Once birds have completed the entire outbound and inbound migratory journey the use of learned spatial cues seems to take precedence in navigation, though birds continue to possess the ability to vector navigate (Mouritsen, 2003).

Timing of Migration and Connections to Reproduction

In many birds, reproduction and migration are tightly linked in the annual cycle (Fig. 2). Preparation for breeding often begins with migration to the breeding grounds, and the termination of breeding can be closely tied to the migratory departure to the wintering grounds. In order to prepare for breeding, most birds undergo seasonal development of the reproductive system, a process coordinated by activation of the hypothalamic-pituitary-gonadal (HPG) axis. The HPG secretes reproductive hormones leading to the development of a gonad capable of producing gametes. Although some species, typically those that move only short distances, do not undergo the process of gonadal development until after they arrive on the breeding grounds, many species undergo gonadal development during migration and arrive on the breeding grounds in a state of near-readiness to breed. In Gambel's white crowned sparrows, for example, initial activation of the HPG axis begins on the wintering grounds. By the time birds arrive on the breeding grounds, males are producing mature spermatozoa in the testes and females have enlarged ovarian follicles, though they have not yet begun yolk synthesis or deposition (Ramenofsky and Wingfield, 2006).

Appropriately timing events like migration and reproduction is critical to the survival and reproductive success of individual birds, and ultimately to the persistence of bird populations. If birds arrive on the breeding grounds too early, conditions may still be inhospitable and their survival may be threatened. On the other hand, birds that arrive too late may not have enough time to complete breeding, or they may miss peak food availability which could compromise their reproductive success. The question of how birds time their migrations has been best studied in obligate migrants. Therefore, that is the focus here, but for greater

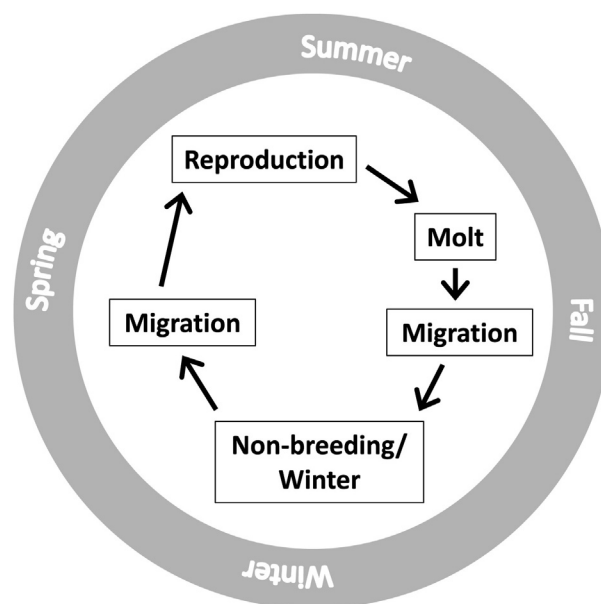


Fig. 2 Example of an annual cycle typical of many temperate zone birds.

discussion of other migratory types see [Watts et al. \(In press\)](#). Among obligate migrants increasing day length (photoperiod) in conjunction with an endogenous circannual rhythm are important mechanisms initiating spring migration ([Gwinner, 1996](#)), while other environmental conditions, such as temperature, can fine-tune the timing. Increasing photoperiod stimulates activation of the HPG axis, which is also important in preparation for breeding, and leads to increasing levels of circulating androgens, notably testosterone. In both males and females, increasing androgen levels are important in stimulating the behavioral and physiological changes associated with the transition to spring migration ([Wingfield et al., 1990](#)). Activation of thyroid hormone signaling pathways in the brain in response to increasing photoperiod is also critical in the spring migratory transition ([Pérez et al., 2016](#)).

The mechanisms triggering fall migration are not as well studied as those operating in the spring. As for spring migration, photoperiod (experienced in the fall as well as those experienced earlier in the year) and endogenous circannual rhythms likely play important roles. On the other hand, the endocrine mechanisms underlying the transition to a migratory state appear to differ from those in spring migration, though they are currently not well understood. The timing of fall migration tends to be more variable than spring migration, which likely reflects sensitivity to local environmental conditions (e.g., temperature, food availability) and differences among individuals in their progression through breeding and the feather molt usually needed before the migratory flight. For example, when breeding of mountain white-crowned sparrows is delayed due to heavy snow cover, birds subsequently delay the fall migratory departure ([Morton and Pereyra, 1994](#)).

Just as circumstances around breeding events can influence fall migration, so too can spring migration influence breeding. In many species, it has been found that birds arriving earlier on the breeding grounds enjoy greater reproductive success than later arriving birds. For example, in American redstarts (*Setophaga ruticilla*), which migrate from wintering sites in the Caribbean and Central and South America to breeding sites in the United States and Canada, earlier arrival corresponds to greater reproductive success in males. The potential advantages of earlier arrival for redstarts and other species include access to more and/or higher quality territories and mates, as well as the potential to lay more clutches during the breeding season. Interestingly, studies across a variety of species have revealed that it is those birds in the best physical condition that arrive earliest. Among American redstarts, males that winter in better quality (in this case, wetter) habitats are in better physical condition at the end of the winter, depart for spring migration earlier, and arrive on the breeding grounds earlier than males that winter in poorer habitats ([Marra et al., 1998](#)). Given the correlation between migratory timing and physical condition, it is difficult to disentangle whether the superior reproductive success of early arriving birds is a result of their earlier arrival or their superior condition, or a combination of the two.

Impacts of Climate Change on Migration

Changes in the earth's climate over the past century, including warming trends, are having considerable impacts on patterns of bird migration ([Carey, 2009](#)). Perhaps the most widely documented change has been in the timing of spring migration. In many species, birds are arriving on their breeding grounds earlier in the year. This may occur as birds adjust the timing of migration in response to conditions, such as warmer temperatures or advancing plant phenology, on their wintering grounds or *en route* (e.g., at stopover sites), though changes in the location of wintering grounds (see below) could also contribute ([Knudsen et al., 2011](#)). However, not all species or populations are showing such shifts in spring migratory timing.

The consequences of climatic shifts depend on the details of how conditions such as food availability are changing relative to changes in the timing of spring migration. In many species it has been found that the timing of arrival on the breeding grounds is becoming increasingly mismatched to the timing of food availability in the spring, a critical resource for successful reproduction. In some cases, this is occurring as species fail to advance their spring arrival. Pied flycatchers (*Ficedula hypoleuca*) in the Netherlands, for example, have advanced their spring breeding to some extent, but they are limited by arrival time, which has not advanced ([Both and Visser, 2001](#)). In other cases, birds are advancing the timing of their spring arrival but not enough to keep up with even greater advances in the timing of spring food availability. And in yet another scenario, birds are advancing their spring arrival but conditions on the breeding grounds have not advanced. For example, American robins (*Turdus migratorius*) in the Rocky Mountains have advanced their arrival on their high-altitude breeding grounds, presumably in response to conditions at their lower-altitude winter grounds. But the timing of snowmelt on the breeding grounds, which determines spring food availability, has not changed; the result is that robins must wait longer for food to become available ([Inouye et al., 2000](#)).

The effects of climate change on the timing of fall migration are not as well studied, but the effects seem to be even more varied than those on spring migration. Some species or populations are departing the breeding grounds earlier, while others are departing later, and yet others show no change. In addition to effects on the timing of migration, climate change is also altering the distances that birds migrate. The most common effect is for migratory distances to be shortened. Birds belonging to a number of species that breed in Europe have been documented overwintering at higher latitudes, closer to their breeding range, in recent decades compared to in the past. Furthermore, some populations are becoming increasingly sedentary, with fewer birds migrating and more birds remaining resident year-round ([Newton, 2010](#)).

Summary

Birds express a range of migratory patterns, from highly predictable obligate migration, to less predictable nomadic and fugitive migrations. Typically, spring migration moves birds from their wintering grounds to their breeding grounds, and fall migration returns birds to their wintering grounds. Frequently, migration is preceded by a period of physiological preparation, which can

include increases in fat deposition as an energy reserve for migratory flight and increases in the size of the flight muscles. Once preparations are complete, the initiation of migratory flight is often influenced by weather conditions such as precipitation and wind. Many birds migrate at night, even species that are typically diurnal. As the migratory journey progresses, birds may make stopovers to refuel along the way. The process by which migration terminates once birds reach their destination is one of the least understood aspects of migration. In order to find their way on their migratory journey, birds may make use of several biological compasses, as well as inherited migratory programs, and learned landmarks on the landscape. Birds time their migrations using cues in the environment, most notably day length, and an endogenous circannual clock. Events during migration and breeding are often closely linked such that the timing or success of one stage is influenced by the other. Changes in the earth's climate over the past century are having impacts on migratory timing and migratory patterns, as well as the extent to which the timing of migration matches the availability of key resources.

References

- Berthold, P. (1996). *Control of Bird Migration*. London: Chapman & Hall.
- Berthold, P. (2001). *Bird Migration: A General Survey* (2nd edn). Oxford: Oxford University Press.
- Both, C., & Visser, M. E. (2001). Adjustment to climate change is constrained by arrival date in a long-distance migrant bird. *Nature*, 411, 296–298.
- Carey, C. (2009). The impacts of climate change on the annual cycles of birds. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364, 3321.
- Cochran, W. W., Mouritsen, H., & Wikelski, M. (2004). Migrating songbirds recalibrate their magnetic compass daily from twilight cues. *Science*, 304, 405.
- Dingle, H., & Drake, V. A. (2007). What is migration? *Bioscience*, 57, 113–121.
- Gwinner, E. (1996). Circadian and circannual programmes in avian migration. *Journal of Experimental Biology*, 199, 39–48.
- Inouye, D. W., Barr, B., Armitage, K. B., & Inouye, B. D. (2000). Climate change is affecting altitudinal migrants and hibernating species. *Proceedings of the National Academy of Sciences*, 97, 1630–1633.
- Knudsen, E., Lindén, A., Both, C., et al. (2011). Challenging claims in the study of migratory birds and climate change. *Biological Reviews*, 86, 928–946.
- Marra, P. P., Hobson, K. A., & Holmes, R. T. (1998). Linking winter and summer events in a migratory bird by using stable-carbon isotopes. *Science*, 282, 1884–1886.
- McWilliams, S. R., Guglielmo, C. G., Pierce, B., & Klaassen, M. (2004). Flying, fasting, and feeding in birds during migration: A nutritional and physiological ecology perspective. *Journal of Avian Biology*, 35, 377–393.
- Mettke-Hofmann, C., & Gwinner, E. (2003). Long-term memory for a life on the move. *Proceedings of the National Academy of Sciences*, 100, 5863–5866.
- Morton, M. L., & Pereyra, M. E. (1994). Autumnal migration departure schedules in mountain white-crowned sparrows. *The Condor*, 96, 1020–1029.
- Mouritsen, H. (2003). Spatiotemporal orientation strategies of long-distance migrants. In P. Berthold, E. Gwinner, & E. Sonnenschein (Eds.), *Avian Migration* (pp. 493–513). Berlin: Springer-Verlag.
- Mouritsen, H., & Ritz, T. (2005). Magnetoreception and its use in bird navigation. *Current Opinion in Neurobiology*, 15, 406–414.
- Newton, I. (2010). *The Migration Ecology of Birds*. New York: Academic.
- Pérez, J. H., Furlow, J. D., Wingfield, J. C., & Ramenofsky, M. (2016). Regulation of vernal migration in Gambel's white-crowned sparrows: Role of thyroxine and triiodothyronine. *Hormones and Behavior*, 84, 50–56.
- Ramenofsky, M., & Wingfield, J. C. (2006). Behavioral and physiological conflicts in migration: The transition between migration and breeding. *Journal of Ornithology*, 147, 135–145.
- Richardson, W. J. (1990). Timing of bird migration in relation to weather: Updated review. In E. Gwinner (Ed.), *Bird Migration: Physiology and Ecophysiology* (pp. 78–101). Berlin: Springer.
- Thorup, K., Holland, R. A., Tøttrup, A. P., & Wikelski, M. (2010). Understanding the migratory orientation program of birds: Extending laboratory studies to study free-flying migrants in a natural setting. *Integrative and Comparative Biology*, 50, 315–322.
- Watts, H.E., Cornelius, J.M., Fudickar, A.M., Pérez, J., (In press). Understanding variation in migratory movements: A mechanistic approach. *General and Comparative Endocrinology*, doi: 10.1016/j.ygcen.2017.07.027.
- Wingfield, J. C., Schwabl, H., & Mattocks, P. W., Jr. (1990). Endocrine mechanisms of migration. In E. Gwinner (Ed.), *Bird Migration* (pp. 232–256). Berlin: Springer-Verlag.

Behaviour: Migration and Navigation (Sea Turtles)

Paolo Luschi, University of Pisa, Pisa, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

The seven extant species of marine turtles have complex life cycles that encompass long periods spent on the move, during which turtles often perform extended movements spanning over hundreds of km and lasting months or even years. In many cases, these movements have a clear migratory nature and are directed to individually-specific target sites, which frequently are of limited extension. Migrating turtles will then have to rely on efficient navigation systems to guide their movements, either when moving along coasts or in shallow water or while swimming in the vastness of the ocean, especially when heading to remote islands. This is particularly the case of adult individuals which, at every reproductive season, leave their residential foraging grounds and migrate towards their breeding/nesting sites: since these two places are typically located in different, often distant, regions, males and females face the problem of finding their way to reach them. The present chapter specifically focuses on the reproductive movements of adult turtles, that are more clearly configured as true migrations directed towards specific areas and, as such, surely involve navigational aspects. It is worth to recall however that important findings have also been obtained on non-breeding juveniles and newborn hatchlings, that have provided most relevant information on the spatial ecology and orientation abilities of these life stages (reviewed in [Luschi et al., 2003](#)).

The orientation and navigation mechanisms allowing mature turtles to migrate towards their distant destinations have been the subject of many conjectures and hypotheses, that are however difficult to be tested experimentally mainly because of the large technical and logistical difficulties encountered when studying migrating turtles ([Lohmann et al., 2008](#)). In spite of their origin from terrestrial ancestors, sea turtles are indeed genuine marine animals spending most part of their life submerged in the water, often in remote or inaccessible areas, and as such are very difficult subjects for any experimentation. Only with the advent of efficient tagging and tracking techniques, and in particular of satellite telemetry ([Godley et al., 2008](#)), substantial advances have been possible. Sea turtle movements can now be reconstructed with fair accuracy anywhere in the world and this allowed to collect empirical evidence on the migratory behaviour and navigational skills of migrating turtles. Also, such reliable tracking techniques have allowed researchers to perform experiments aimed at testing specific hypotheses on turtle navigation, for instance by altering the sensory abilities of migrating turtles or subjecting them to translocation (homing) experiments. In this way it has been possible to outline the main characteristics of turtle migrations in a satisfactory way and to document the actual navigational performances during these journeys, so to propose firm hypotheses on the orientation/navigation systems they rely on.

General Phenology of Sea Turtle Reproductive Migrations

With the notable exception of flatback turtles (*Natator depressus*), the other six species of marine turtles are migratory to some extent. In some species/populations the adults' reproductive migrations are limited to short-range movements between the nesting beach and nearby foraging areas, but in most cases adults undertake long-range migratory travels, that can involve prolonged open-sea legs or even transoceanic crossings. Even if the main features of adult migrations are now quite well known, the available information is largely biased towards the case of postnesting migrations of females, that are the only class of individuals that are easily accessed and captured during the egg-laying process on a beach. Conversely, quite scant data are available for the reproductive migrations of males and on the pre-breeding movements of both males and females ([Godley et al., 2008](#)).

Satellite findings have allowed to distinguish two main types of migrations in turtle females ([Luschi et al., 2003](#)). In most Chelonian (hard-shelled) species, females leave the nesting beach to swim directly towards a fixed feeding area in the neritic environment, usually of limited extension, where they then remain for long time. At the following breeding season, 1–4 years later, they will migrate back from this residential/foraging site to their nesting area, therefore performing regular, shuttling migrations between two well-defined terminals. The best examples of this kind of migrations are found in green (*Chelonia mydas*) and loggerhead (*Caretta caretta*) turtles ([Fig. 1](#)), but similar patterns are also known in hawksbills (*Eretmochelys imbricata*) and Kemp's Ridleys (*Lepidochelys kempi*). During these migrations turtles may follow coastal routes when moving along continental edges, but they will also have to cross oceanic areas when nesting and/or foraging in or around islands ([Fig. 1](#)). Tagging and satellite tracking data have shown that, after every breeding season, each turtle returns to a specific, individual foraging site, often following similar routes in successive migrations ([Fig. 1\(b\)](#)). In contrast, pelagic-dwelling turtles, like the Olive Ridley (*Lepidochelys olivacea*) and, especially, the soft-shelled leatherback (*Dermochelys coriacea*), do not have spatially-limited foraging sites and mostly move over offshore areas while searching for their pelagic prey. Their postnesting migrations are therefore undirected towards a fixed site and their migratory courses appear more complex and convoluted than those of the other species ([Fig. 1](#)), and are better described as long-range wandering movements.

Variations to this general pattern do however exist, and the most recent findings have clearly revealed the presence of a large behavioral variability in turtle migratory behaviour. This is especially true for loggerhead turtles, that can present some variants to the usual migration pattern described above ([Godley et al., 2008](#)). Loggerheads living in temperate zone, for instance, may have

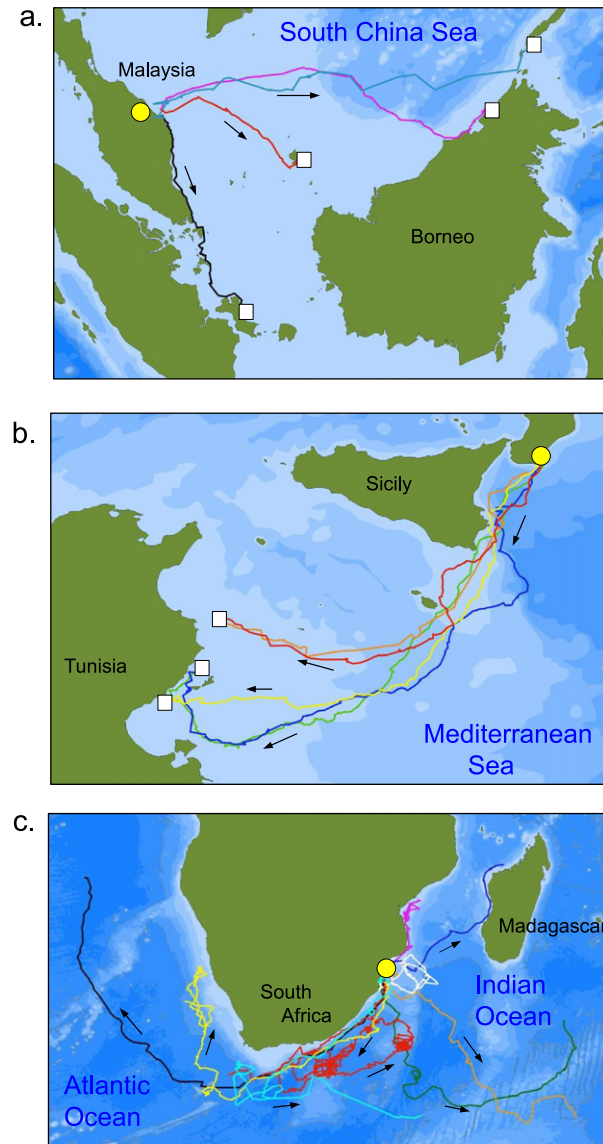


Fig. 1 Examples of postnesting migrations reconstructed by satellite tracking in (a) four green turtles nesting in peninsular Malaysia (data from Luschi, P., Papi, F., Liew, H.C., Chan, E.H., Bonadonna, F., 1996. Long-distance migration and homing after displacement in the green turtle (*Chelonia mydas*): A satellite tracking study. *Journal of Comparative Physiology* 178A, 447–452), (b) three loggerhead turtles nesting in Southern Italy (data from Mingozi, T., Mencacci, R., Cerritelli, G., Giunchi, D., Luschi, P., 2016. Living between widely separated areas: Long-term monitoring of Mediterranean loggerhead turtles sheds light on cryptic aspects of females spatial ecology. *Journal of Experimental Marine Biology and Ecology* 485, 8–17), (c) nine leatherback turtles nesting in KwaZulu-Natal, South Africa (data from Luschi, P., Lutjeharms, J.R.E., Lambardi, P., *et al.*, 2006. A review of migratory behaviour of sea turtles off southern Africa. *South African Journal of Science* 102, 51–58. Robinson, N.J., Morreale, S.J., Nel, R., Paladino, F.V., 2016. Coastal leatherback turtles reveal conservation hotspot. *Scientific Reports* 6, 37851). The yellow circle indicates nesting site location. In the first two cases, turtles migrated towards spatially-restricted neritic sites (identified by white squares), while leatherbacks moved over large oceanic areas. In panel (b), two successive migratory routes of the same female are shown, represented by orange and red, and by blue and green tracks.

more than one foraging site, and undertake seasonal migrations between distinct summer and winter areas at different latitudes. In other cases, postnesting loggerheads do not migrate to localized neritic sites, but rather frequent oceanic waters, where they display wandering movements similar to those of pelagic turtles. Interestingly, this alternative strategy likely involves a diet shift from benthic to epi-pelagic prey and is followed by only a part of the turtles nesting at a given site, with the remaining turtles following the usual pattern of migrating towards a neritic site. A similar dichotomy in migration strategy involving occupancy of oceanic waters have been also described in green turtles, an even more surprising finding since deep oceanic waters do not offer many foraging opportunities to these herbivorous turtles.

As mentioned above, similar detailed information is not available for males and for the pre-breeding migrations. Males spend virtually all their life in the water and so deployment of satellite tags is only possible upon their accidental or intentional capture.

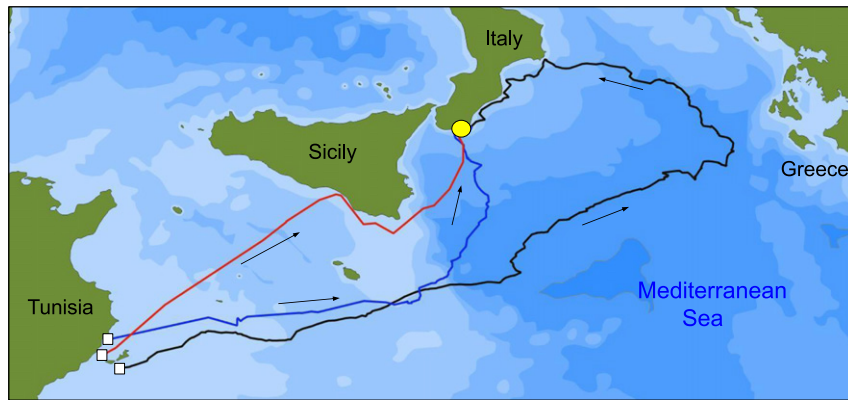


Fig. 2 Reconstructed routes of three loggerhead females migrating from their individual foraging sites along the Tunisian shelf to their breeding/nesting area in southern Italy. While two turtles took a rather direct route to their destination, the third one (black line) nearly reached the Greek coast before heading to her breeding grounds. Data from Mingozi, T., Mencacci, R., Cerritelli, G., Giunchi, D., Luschi, P., 2016. Living between widely separated areas: Long-term monitoring of Mediterranean loggerhead turtles sheds light on cryptic aspects of females spatial ecology. *Journal of Experimental Marine Biology and Ecology* 485, 8–17.

Satellite tracking results have shown that males display a migration pattern similar to that of postnesting females, with the difference (however not extensively documented) that they migrate every year to reach the mating areas, that are typically close to the females' nesting beaches. The pre-breeding migrations of either males or females have been tracked in only a few cases, as a result of tagging at the foraging site or, more commonly, after a prolonged tracking covering the entire 2–3 year inter-reproductive period (examples in Fig. 2). The reconstructed routes are usually quite direct towards the breeding site, but although long detours have been recorded in some cases (Fig. 2), whose biological significance is uncertain.

Migratory Navigation: Biological Compasses

Students of animal navigation usually distinguish two distinct stages in the process of returning towards a previously known site (homing behaviour): first, the direction to keep to get back home has to be determined during the so-called 'map step'; then, during the subsequent 'compass step', the navigator has to keep and maintain that direction leading home, typically by means of a biological compass. The turtles' reliance on compasses is quite well ascertained, thanks to laboratory experiments in which turtle orientation is tested under controlled conditions. For instance, tests in water tanks have shown that newborn and juvenile sea turtles can refer to the sun's position (sun compass) or to stimuli deriving from the Earth's magnetic field (magnetic compass) to assume a given direction in space (Lohmann *et al.*, 2008).

Indications on the usage of a biological compass in natural conditions are more difficult to be obtained, again given the heavy logistical constraints when working with large wild animals. The open-sea courses reconstructed in tracked turtles are often remarkably straight, leading to the conclusion that they rely on a compass of some kind, possibly based on magnetic cues since straight movements persist during the nights, even when moonless. However, the simple presence of straight segments does not necessarily mean that turtles maintained constant headings, since currents usually contribute substantially to the observed movements of marine animals and the straightness of tracked routes can largely derive from current drift. Recent studies with loggerheads equipped with special data loggers recording headings have provided convincing evidence of a compass orientation in the open sea, probably through a magnetic compass (Narazaki *et al.*, 2009), but these findings are unfortunately so far limited to a few turtles only.

On the whole, the evidence provided by various laboratory experiments fits well with the findings of field studies in suggesting that turtles have one or more biological compasses in their orientation toolkit. It is likely that a sun compass is used during daytime when travelling at or close to the sea surface, while during nocturnal and/or deep-water movements, correct orientation may be assured by a magnetic compass.

Migratory Navigation: Navigational Mechanisms

Much more debated, and interesting, is the problem of how turtles animals navigate, i.e. how they establish their position with respect to a given site when away from it (the 'map step' described above). A number of navigational mechanisms have been described in different animals, but only some of them will be available to turtles, especially for movements taking place in the open sea. At least to an human eye, the open sea seems essentially featureless, lacking landscape features or other visual cues (generally indicated as landmarks) that are known to be of great help for terrestrial navigators: for instance we humans basically rely on such information to find our way in our normal life – at least when not guided by the GPS in our smartphone. Without the

help of landmarks, reaching a specific distant target at the end of an oceanic journey, under the effects of waves and currents, may indeed be a very hard task, as was well known to human seafarers before the advent of GPS and radio-controlled maritime navigation.

Even if landmark-based navigation seems prohibitive in the open sea, turtles can still refer to some kind of signposts ('sea-marks') that may be present, undetectable to humans, in the apparently uniform oceans. In seabirds, it is thought that olfactory cues, and in particular dimethyl sulphide, a volatile odorous substance produced by phytoplankton, may constitute an odorous landscape by which birds recognize previously visited open-sea locations and perhaps navigate over oceanic areas (Nevitt and Bonadonna, 2005). It is unknown whether turtles too may rely on such a mechanism, but it is remarkable that a sensitivity to airborne dimethyl sulphide has been demonstrated in loggerhead turtles (Endres and Lohmann, 2012).

Also, it is likely that turtles do use landmarks or other cues of terrestrial origin when moving along the coast or even when in shallow waters (e.g. visual features from the sea bottom). Satellite tracking data have shown how migrating turtles often tend to follow the coastline as long as possible, even when this results in long detours with respect to the straight line courses that would lead towards the final destination crossing the open sea (Fig. 3). Indeed, in many cases, the females' postnesting migrations are best described by a biphasal process, in which turtles first aim at crossing the open sea to reach a coast with the shortest and quickest route and then hug the coast until getting to their final destinations (Fig. 3; Hays *et al.*, 2002).

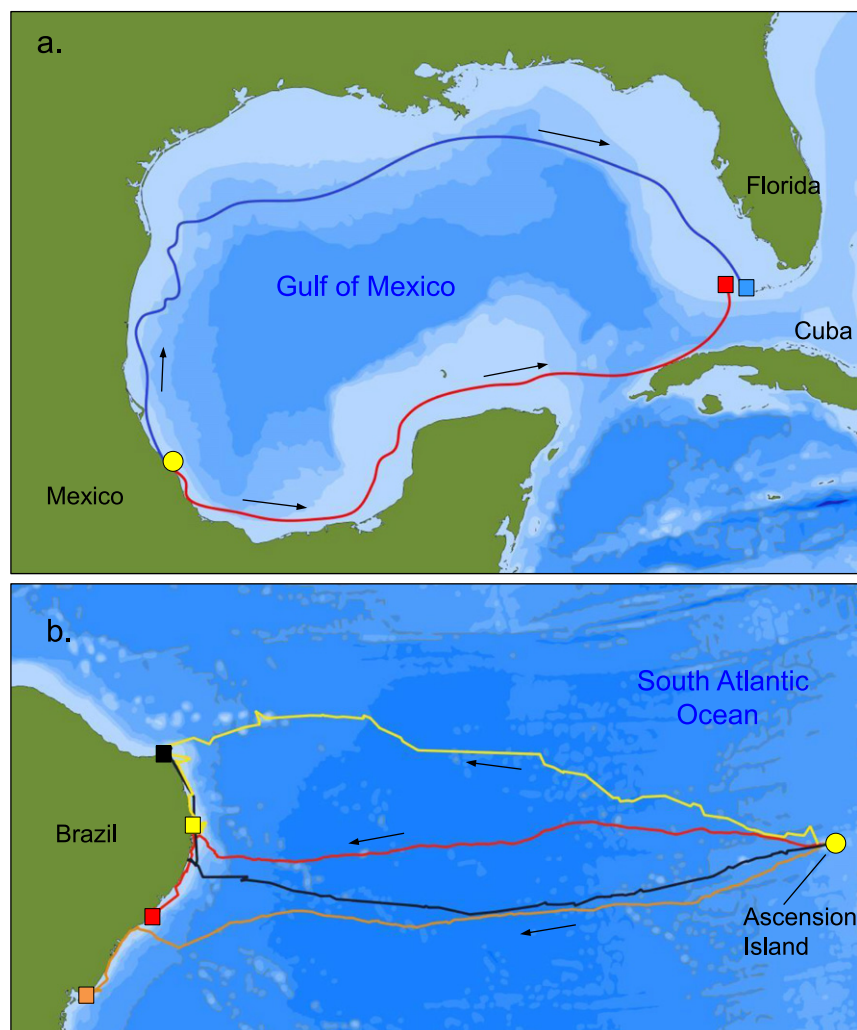


Fig. 3 Indirect courses taken by migrating turtles to reach a distant target. (a) Routes of two green turtles moving from their nesting site in Mexico (yellow dot) to their foraging sites (squares) in the Florida Keys. They followed a long, detoured route likely to remain in shallow/coastal waters and to avoid crossing the open sea. Data from Kinzel, M., 2001. Satellite tracking of green sea turtles in the Gulf of Mexico. *Argos Newsletter* 58, 5–7. (b) Routes of four green turtles migrating from breeding grounds at Ascension Island (yellow dot) towards their residential sites (squares) along the Brazilian coast. These turtles adopted a biphasal migratory strategy first covering the open sea stretch with the shortest route and moving along the coast until reaching to their destination. Data from Hays, G.C., Broderick, A.C., Godley, B.J., *et al.*, 2002. Biphasal long-distance migration in green turtles. *Animal Behaviour* 64, 895–898.

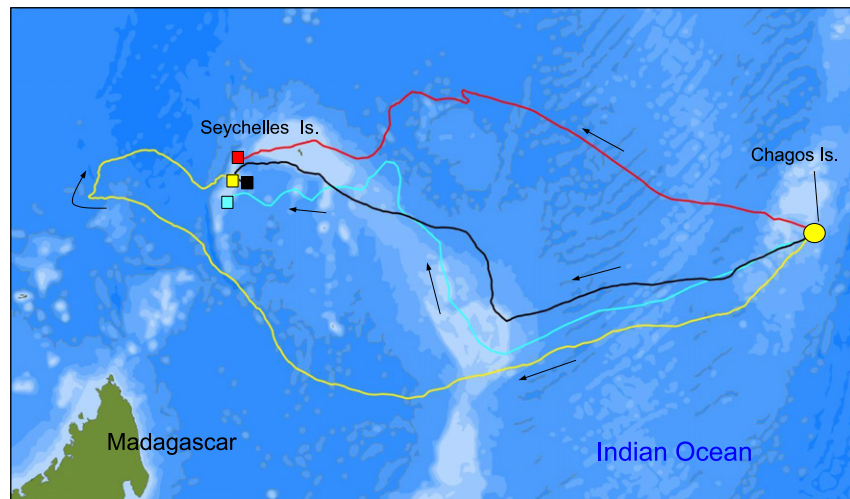


Fig. 4 Examples of migratory journeys directed towards an oceanic target: tracks of four green turtles that had nested on Chagos Islands in the Indian Ocean (yellow dot) and had their foraging sites (squares) in the shallow waters of the Amirante Banks, Seychelles Islands. Data from Hays, G.C., Mortimer, J.A., Ierodiaconou, D., Esteban, N. 2014. Use of long-distance migration patterns of an endangered species to inform conservation planning for the world's largest marine protected area. *Conservation Biology* 6, 1636–1644.

The situation is radically different for journeys taking place in the open sea, especially when directed to a specific target: how can a turtle find a small, remote island in the middle of the ocean after a trip of hundreds or thousands of km with no help of coastal/visual cues? The case is best epitomized by the green turtles nesting at Ascension Island, in the middle of the South Atlantic Ocean, that reach this tiny target migrating from the Brazilian coast, over 2000 km away. How can they do this is a unsolved question that is puzzling scientists from the time of Charles Darwin, who wondered about that when he visited the island on his voyage onboard the *Beagle*. Basically we do not yet have a clear answer to this question, and indeed we do not even know the modalities through which how ocean-moving turtles reach their specific targets, since not many females have so far been tracked while migrating towards foraging grounds located in oceanic islands (Fig. 4). Turtles have sometimes been found to arrive at their destination following curved, indirect paths or even after large detours (Hays *et al.*, 2014; Fig. 4).

Despite this paucity of empirical data, some hypotheses have been put forward to explain open-sea navigation in turtles, that however are corroborated by experimental indications in a few cases only. The most extreme hypothesis proposed is that turtles have no navigational information and move at random to find their target, more or less by chance. The directedness and efficiency of most turtle migratory journeys, makes this idea extremely unlikely, although the puzzling detours sometimes recorded (Figs. 2 and 4) as well as the responses of experimentally translocated turtles (see below) can be considered searching attempts for their targets. Such looping or convoluted routes could then be the result of a large-scale searching behaviour that may be not inefficient, since in most some cases turtles reach their destination after days or weeks of such convoluted journeys.

Another possible mechanism to explain oceanic navigation is the so-called vectorial navigation, by which an animal is informed about the distance and direction to travel to reach its target. For example, juvenile passerine birds are thought to possess an innate knowledge of the length and direction(s) of their migratory flight by which they guide their first journey towards their wintering sites: could an Ascension-bound turtle use a similar mechanism and find its target island by steering due East from Brazil and swimming for a certain amount of time? In theory, this is not impossible, but an Ascension-bound turtle only relying on such a simple information (that may be innate or acquired in some way), would then be totally dependent on possible inaccuracies in its steering mechanism and, especially, be exposed to passive translations due to the drifting effect of ocean currents (Lohmann, *et al.*, 2008). These factors cannot be corrected during movements taking place in the ocean, where the turtle orientation is not anchored in some way to stable cues like landmarks and where current drift play a major role. Detecting current drift while migrating would overcome this problem, as this would allow an immediate compensation for any shift from the due course, but such an apparently straightforward task is nearly impossible in the absence of stationary references and therefore is not a viable possibility for turtles travelling in the open sea - and for human seafarers as well. Recent studies have estimated the direction and speed of currents along the turtle routes and have showed that current drift effects on the oceanic movements are not negligible, and that turtles seem unable to detect the drift while swimming away from land (Galli *et al.*, 2012).

Reliance on vectorial information may still play a role in movements aiming at relatively large targets, like wide foraging areas or coastal sites, when any error can be corrected with the help of topographical features. For instance, in the biphasal postnesting migrations described before, turtles can correct navigational imprecisions during the oceanic leg of their migration after having reached the coast. Another way to correct for navigational errors may be to detect cues available in the relative vicinity of the goal, that may guide the final stages of target localization. Turtles translocated away from Ascension Island have been shown to return more easily when released downwind than upwind from the island, leading to hypothesize that the Southeast trade winds of the area may help turtles by generating a plume of wind-borne cues extending for tens of km in Northwest direction, which may act as

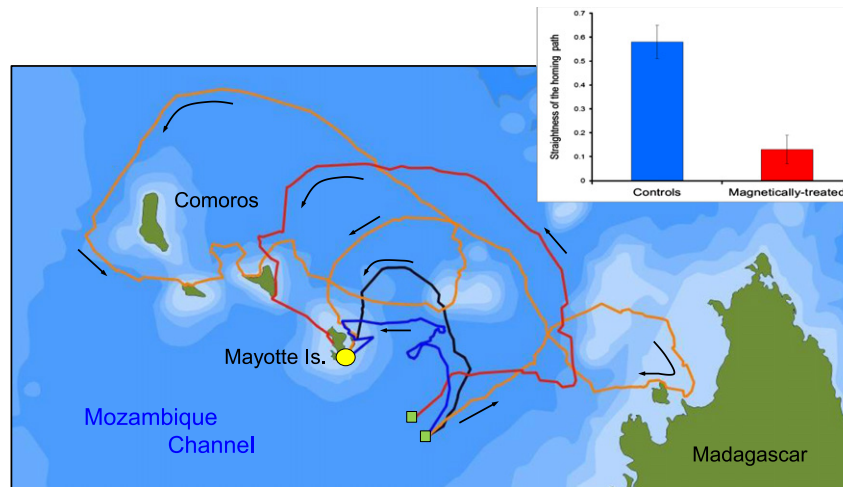


Fig. 5 Homing journeys of four turtles that had been translocated from their nesting site at Mayotte Is., in the Mozambique Channel (yellow dot), and released to open sea locations about 100 km away (green squares). Red and orange routes: turtles subjected to a magnetic treatment (application of a magnet to the head); blue and black route: control turtles. All turtles returned to the home island following indirect routes, but magnetically-treated individuals followed much longer, less efficient routes. As a result, the straightness of the homing paths resulted significantly lower in the magnetic group than in controls (insert). Data from Luschi, P., Benhamou, S., Girard, C., *et al.*, 2007. Marine turtles use geomagnetic cues during open-sea homing. *Current Biology* 17, 126–133.

an aerial beacon of airborne odors (Hays *et al.*, 2003). When inside such a plume, turtles would then be attracted by the island from some distance and could reach it simply by swimming against wind direction. Current-borne cues dissolved in the water can similarly exert such a beaconing effect, but no evidence for such a role has been ever been found in tracked turtles (Lohmann *et al.*, 2008).

The most efficient solution by which turtles could navigate is to rely on some kind of position-fixing mechanism through the development of large-scale maps. A turtle possessing such navigational maps would then be able to establish its position with respect to its target at any given location, irrespectively of whether it ended up in that position by its own motion and/or by the action of drifting factors - or because of experimental interventions. Reliance on such powerful mechanisms has been repeatedly proposed to explain the amazing performances of oceanic navigators, with several possible mechanism having been put forward (Lohmann *et al.*, 2008), but unfortunately the empirical evidence supporting them is limited. The most direct way to test reliance on position-fixing mechanisms is through displacement experiments, in which turtles are translocated away from a location where they reside (typically a nesting beach) and are then tracked to assess their ability to get back to the original home site. Performing such experiments is not an easy task, due to the enormous logistical problems involved in transporting nesting turtles tens of km away, but a few experiments of this kind have been made, also from oceanic islands, showing that displaced turtles are able to return home, although often by following circuitous routes (Fig. 5).

The sensory cues could be involved in such navigational maps have also been investigated. The best candidates for wide-ranging maps are geomagnetic cues, that are accessible everywhere and anytime and should indeed allow to establish one's position anywhere on Earth, although without high precision. A series of arena experiments with newborn loggerheads have shown that they can distinguish slightly different values of magnetic parameters (Lohmann *et al.*, 2008), and it is likely that this fine magnetic sensitivity is retained by juvenile and adult stages, that may develop a sort of geomagnetic map for large-scale navigation. In a milestone experiment, juvenile green turtles were tested in specially designed arenas while being exposed to magnetic fields matching those of locations about 300 km away from the test site, thus simulating a 'magnetic displacement' to these sites (Lohmann *et al.*, 2004). Tested turtles were faithful to the specific foraging areas where they had been captured and so the expectation was that they would orient towards home in the arena, just like they had been actually translocated to such locations. The turtle response was in accordance with this expectation, and the interpretation of these findings is unequivocal: tested turtles used geomagnetic cues to determine their (virtual) position with respect to home, and then oriented in such a way to correct the 'virtual magnetic displacement' they had been subjected to. This result, which unfortunately has never been replicated, provides the most clear-cut evidence so far available that the turtles can rely on large-scale magnetic maps to navigate.

The role of geomagnetic cues in oceanic navigation has also been investigated in natural conditions through different kinds of satellite tracking experiments. Green turtles migrating from Ascension Island to Brazil while being exposed to an artificial magnetic field created by mobile magnets around their body, were not affected by the treatment, showing that geomagnetic cues were not necessary at least to accomplish the migration towards continental Brazil (Papi *et al.*, 2000). In contrast, displacement experiments have highlighted the role of geomagnetic cues in green turtle navigation towards islands. In two successive experiments in the Indian Ocean (Luschi *et al.*, 2007; Benhamou *et al.*, 2011), turtles displaced to open-sea release sites were affected in their homing performances by the application of a mobile magnet glued to their head, being able to return to the home island only following longer and/or more or convoluted routes than controls (Fig. 5). These findings highlighted the role of geomagnetic information in

the homing process of freely-moving turtles, although it is not yet known whether the treatment specifically affected the turtles' magnetic map or another magnetically-mediated orientation process.

General Remarks

Satellite telemetry has provided a wealth of information on the migrations of adult marine turtles, by which various important aspects of the migratory behaviour of the different species have been documented, such as the kind and extent of the movements performed, the areas frequented during and after migrations, the behaviour during the journeys. Also, the reconstructed migratory courses revealed relevant navigational performances in ocean-moving turtles, that have often been found able to pinpoint a remote target following fairly efficient and accurate routes, even if not in all cases (examples in [Figs. 2–4](#)).

This relatively well-defined picture of migration phenology is not mirrored by our knowledge on the navigational aspects of the phenomenon: we still do not know how turtles find their way while migrating, and especially how they navigate to reach their final targets. On the whole, the best available results indicate that turtles may rely on a compass sense coupled with some kind of position-fixing mechanisms, possibly based on magnetic cues, to find their way during migrations. This would allow them to pinpoint their targets, even when distant and remote, overcoming the drifting effect of ocean currents. The recorded ability of turtles to compensate for actual or virtual displacements (although with some difficulties in freely-moving turtles), constitutes the best piece of evidence available for this idea, but these conclusions still derive from a few, sparse experiments, basically done on a single species, the green turtle, and with different experimental techniques. More empirical findings supporting this view should be collected before reaching a definitive conclusion. In any case, it is reasonable that migrating turtles would have other navigational options at their disposal when performing their long-distance migrations. Among these, reliance on short-range cues like wind-borne information or coastal topographical features available close to the targets, may facilitate the final stages of navigational processes.

It is therefore likely that the turtles' navigation system is actually composed by different mechanisms, active at different spatial scales, that act concurrently or sequentially during goal-directed navigation ([Bingman and Cheng, 2005](#)). Under this scenario, migrating turtles may rely on large-scale maps to get general positional indications, perhaps with a rather coarse accuracy, by which to determine the course towards destination and correct it during the journey. They could then integrate this information with other, more precise but not universally available, navigational cues to pinpoint the final target. We can try to 'translate' this hypothesis in practice by applying it to the concrete case of a turtle migrating in the ocean to return to its nesting beach after some years spent at its foraging sites. This could be a green or a loggerhead turtle moving from its coastal foraging area or a leatherback that has been wandering in pelagic waters for years before returning to its nesting beach. Initially, the turtle would determine the general migratory direction to keep by relying on its large-scale map (even if coarse in accuracy), and then start migrating maintaining this deduced heading with the help of its compass(es). During the oceanic leg, it may take advantage of map-like information even only intermittently, to avoid being displaced off route by current drift. This strategy should lead the turtle to arrive not far away from destination, where short-range mechanisms like those mediated by landmarks (for coastal sites) or wind-borne cues (for oceanic targets), may then enter into action and guide its final approach to the target. It is presently hard to tell whether this picture, still very speculative, is the correct one to explain sea turtle migratory navigation, and it can only be hoped that new empirical data will be collected in the near future to substantiate, or dismiss, this proposed view.

Acknowledgements

I wish to thank G.C. Hays, N.G. Robinson, M. Kinzell, and A. Mingozzi for providing the data used in [Figs. 1–4](#), R. Mencacci for help with the manuscript.

References

- Benhamou, S., Sudre, J., Bourjea, J., *et al.*, 2011. The role of geomagnetic cues in green turtle open sea navigation. *PLOS ONE* 6, e26672.
- Bingman, V.P., Cheng, K., 2005. Mechanisms of animal global navigation: Comparative perspectives and enduring challenges. *Ethology Ecology and Evolution* 17, 295–318.
- Endres, C.S., Lohmann, K.J., 2012. Perception of dimethyl sulfide (DMS) by loggerhead sea turtles: A possible mechanism for locating high-productivity oceanic regions for foraging. *Journal of Experimental Biology* 215, 3535–3538.
- Galli, S., Gaspar, P., Fossette, S., *et al.*, 2012. Orientation of migrating leatherback turtles in relation to ocean currents. *Animal Behaviour* 84, 1491–1500.
- Godley, B.J., Blumenthal, J.M., Broderick, A.C., *et al.*, 2008. Satellite tracking of sea turtles: Where have we been and where do we go next? *Endangered Species Research* 4, 3–22.
- Hays, G.C., Åkesson, S., Broderick, A.C., *et al.*, 2003. Island-finding ability of marine turtles. *Proceedings of the Royal Society of London B* 270 (Suppl. 1), 5–7.
- Hays, G.C., Broderick, A.C., Godley, B.J., *et al.*, 2002. Biphasal long-distance migration in green turtles. *Animal Behaviour* 64, 895–898.
- Hays, G.C., Mortimer, J.A., Ierodiaconou, D., Esteban, N., 2014. Use of long-distance migration patterns of an endangered species to inform conservation planning for the world's largest marine protected area. *Conservation Biology* 6, 1636–1644.
- Lohmann, K.J., Lohmann, C.M.F., Ehrhart, L.M., Bagley, D.A., Swing, T., 2004. Geomagnetic map used in sea-turtle navigation. *Nature* 428, 909–910.
- Lohmann, K.J., Luschi, P., Hays, G.C., 2008. Goal navigation and island-finding in sea turtles. *Journal of Experimental Marine Biology and Ecology* 356, 83–95.
- Luschi, P., Benhamou, S., Girard, C., *et al.*, 2007. Marine turtles use geomagnetic cues during open-sea homing. *Current Biology* 17, 126–133.

- Luschi, P., Hays, G.C., Papi, F., 2003. A review of long-distance movements by marine turtles, and the possible role of ocean currents. *Oikos* 103, 293–302.
- Narazaki, T., Sato, K., Abernathy, K.J., Marshall, G.J., Miyazaki, N., 2009. Sea turtles compensate deflection of heading at the sea surface during directional travel. *Journal of Experimental Biology* 212, 4019–4026.
- Nevitt, G.A., Bonadonna, F., 2005. Sensitivity to dimethyl sulphide suggests a mechanism for olfactory navigation by seabirds. *Biology Letters* 22, 303–305.
- Papi, F., Luschi, P., Åkesson, S., Capogrossi, S., Hays, G.C., 2000. Open-sea migration of magnetically disturbed sea turtles. *Journal of Experimental Biology* 203, 3435–3443.

Behaviour: Nesting, Brooding, Parental Care, Birds

Sophie C Edwards, Alice Bernard, and Susan D Healy, University of St Andrews, St Andrews, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

For all organisms, reproduction is the key to success. In birds, reproduction is a multi-stage process that is only part way through when a bird finds a partner and mates. The next stages are the choice of location for laying eggs, which may or may not be followed by building a nest, and then the laying of eggs. Eggs are incubated, chicks hatch and are fed, in or out of the nest, by parents, siblings or sometimes, unrelated individuals or may simply be guided to appropriate foraging locations. Although it is only the female that can lay the eggs, there may or may not be division of labour between the parents at all of the other stages. Here we will provide an overview of the progression from nest site location to successful fledging of the chicks with a flavour of the diversity that occurs across species at each stage.

Nest-Site Location to Nest Building

For some birds, choice of location is all-important because they lay their eggs on the ground. Because the Common Ringed Plover *Charadrius hiaticula*, for example, lays its eggs on dry riverbeds, the colour patterning of its eggs must be camouflaged against the background. Nesting female plovers may also attempt to redirect potential predators away from their nest by pretending to be injured, dragging a wing along the ground as they move away from the nest. Razorbills *Alca torda* and Common murre *Uria aalge*, also simply lay their eggs on the ground but because they choose locations on cliff ledges, their eggs and chicks are already less accessible to most predators.

Most birds, however, build some kind of nest to hold their eggs and chicks, to protect them from weather and predators while creating a suitable microclimate for development. Nest building can loosely be defined as the ability to organise bits of material into a certain spatial relationship. This is a loose definition because 'organising bits of material' ranges from excavation of burrows (e.g. burrowing owls *Athene cunicularia*), to scraping material into a pile (e.g. Australian brush turkeys *Alectura lathami*), from piling sticks up into a platform (e.g. storks *Ciconia* spp., bald eagles *Haliaeetus leucocephalus* and other raptors) to weaving (e.g. weaverbirds *Ploceus* spp.). While nests are predominately used to hold eggs and young, adults may also build nests for roosting in over night (as do chimpanzees, which build a new nest each night). And, although birds are the group of animals most commonly associated with nest building, it is taxonomically widespread and observed in insects, spiders, fish, and mammals (Collias and Collias, 1984; Hansell, 2000).

Mate Selection

For some birds, nest building occurs earlier in the reproductive cycle and plays an integral role in courtship behaviour. Courtship is the act of displaying to a potential mate, often of either one or more physical characteristics, such as the number of eye spots in a peacock train or the rate of singing by male great tits or by engaging in a particular behaviour, such as nest building (Davies *et al.*, 2012). Courtship displays allow a potential mate, usually the female, to assess an individual's potential quality, either as a genetic parent or for their ability to perform upcoming parental duties. Although uncommon, for some species, the male builds more than one nest and females appear to choose among them. In wrens *Troglodytes troglodytes*, the male then completes that nest and the female lays her eggs in it while Cape weaver *Ploceus capensis* males appear to complete several nests before a female chooses one in which to lay her eggs. In these species it is not clear what features the female uses to choose a nest but penduline tit *Remiz pendulinus* females select males that have constructed larger nests. This is perhaps because eggs that are laid in larger nests are better insulated from the environment, which should increase the probability that those eggs survive to become chicks. In Australian reed warblers the value of the nest is clearer. Males build two types of nests in their territory; type 1 is structurally capable of holding eggs and young, and type 2, which is used for display. The more type 2 nests a male builds the more food there is available on the male's territory so making it easier for the female to provision her young. In other species, the building itself seems to be a useful measure of future parental care. For example, male magpies *Pica pica* that bring more sticks to a nest also later bring more food to their young. Female magpies can then use building as a cue for male future investment.

Nest Site Selection

Most of the time birds choose the location in which to build their nest once they have a mate. Sometimes the male chooses, sometimes the female and sometimes both together. There are eight broad categories of nest site: (1) ground nests, where nest contents are either on a small cushion of nest material, usually vegetation or stones, or directly in contact with the ground;

(2) nests floating on the water; (3) ground hole/cavity nests those that are either excavated by one or both adults or are built in holes excavated by other species; (4) grass/reed nests are supported by vertical stems; (5) nests that are built on branches in trees or bushes at any height above the ground; (6) cavity nests are built in openings in branches and trunks, either naturally or caused by previous excavators; (7) nests that are attached to cliffs and buildings walls; and 8) nests that are built on ledges, which are similar to wall nests, but have support from below (Mainwaring *et al.*, 2014).

The category of nest a bird builds is usually species specific. For example, cliff swallows *Petrochelidon pyrrhonota* always build nests on walls, while blue tits always build in a cavity off the ground, usually in a tree hole, but they will also use nest boxes, which has made them readily accessible for research on avian reproduction. Nest site location is influenced by a combination of factors: (1) risk and experience of predation; (2) presence or absence of conspecifics and non-threatening species, their breeding success and territory competition; (3) local climatic conditions and (4) availability of suitable nest material. For example, orange-crowned warblers *Vermivora celata* will shift from building in trees or shrubs to building closer to the ground if there are any signs of predators in the area. Alternatively, by building under hawk nests, black-chinned hummingbirds *Archilochus alexandri* use predator proximity to protect their chicks from jays (hawks prey on jays).

Previous experience of predation will cause Pinjon jays *Gymnorhinus cyanocephalus* to change their choice of nest site. They usually build their nests on the outskirts of trees or shrubs, possibly to take advantage of the solar radiation. If the eggs, or chicks, are taken by a predator, the following year, the unlucky would-be parents will build their nest closer to the trunk of the tree and to the ground. The nest is then better protected from predators, but is also more shaded from the sun.

Choosing a location for a nest can also be dependent on conspecifics. Nearly all seabird species, for example, nest in colonies. There are a range of costs to group nesting such as increased competition for nest sites, nest materials and food. But colonies also enable earlier detection of predators and increased protection through mobbing. Some birds e.g. sociable weavers *Philetairus socius* build a permanent, communal nest. The majority of birds, however, nest alone. By doing so the nest is less conspicuous to predators.

As one of the main purposes of a nest is to create a suitable microclimate for development, nest site selection can also be influenced by the ambient climate. Optimal temperature for offspring development is usually higher than the ambient temperature, and nests in northern temperate regions are often built in sites that lose less heat than do sites selected at random. Horned larks *Eremophila alpestris* building in cool parts of North America build a nest with an entrance that protects the nest contents from prevailing winds while in warmer locations, the nest is built so that its contents are shaded for most of the day, protecting the young from overheating by solar radiation.

Nest site selection can also be influenced by the availability of nest material. Typically, birds will use local materials but nest material specialists can be influenced by the availability of appropriate material. For example, song thrushes *Turdus philomelos* build a nest of mud and rotten wood, which they can struggle to find in dry and/or urban environments, so do not often build a nest in these areas.

Nest Construction

Once a site has been selected, the building begins. Builder may be the male, the female or both. Nest structure is often very similar within species but varies a lot across species, ranging from woven nests with a roof, through cup shaped to platform or little more than a scrape on the ground (Breen *et al.*, 2016).

The materials used for building are also highly variable, both within and between species. Typically, it is plant material or sometimes mud (swallows) or stones (penguins) but there are increasing examples of birds using human materials (e.g. crows in Tokyo build nests with coat hangers and ospreys in Montana using brightly coloured plastic agricultural twine).

How builders use material may depend on local climatic conditions. Populations in colder areas put more feathers, animal and/or other insulatory materials into their nests than do populations in warmer areas. For example, northern populations of European blackbirds *Turdus merula* build bigger nests than do southern populations, the further north that European blue tits build, the more feathers they include while the Hawai'i 'amakihi *Chlorodrepanis virens* adds more lining and builds denser nest walls the higher the altitude. Bigger nests and nests with more insulatory material, are slower to cool, which is advantageous in colder environments. By cooling more slowly, egg temperature does not decline to dangerous temperatures when the parents leave the nest, and the parents do not have to exert as much energy to rewarm the nest contents on their return.

The actual availability of nest material can also affect what goes into a nest. As most birds use what is available to them, the surrounding flora and fauna can influence the nest composition dramatically. Dartford warblers *Sylvia undata* building in Suffolk, for example, typically use small heather and moss while warblers building in Hampshire will construct a nest from grass. The differences in materials would lead birders from either region to mis-identify the builder.

Some birds, like starlings, may include fresh green plant material containing hydrocarbons, apparently as a defence against pathogens and parasites. House sparrows *Passer domesticus* and house finches *Carpodacus mexicanus* in Mexico City, perhaps lacking access to appropriate plant material, use cigarette butts as a measure to repel parasites: the more cigarette butts, the fewer parasites in the nest. Material may also be used to reduce the risk of predation, the most obvious example being the use of material to camouflage the nest. There is, perhaps, surprisingly, little evidence that birds in the wild actually do this although zebra finches *Taeniopygia guttata* building nests in the laboratory will choose material of the colour that matches the nest surroundings.

Egg Laying

With the nest complete (if there is a nest), egg laying can begin. Egg laying can take anywhere between 1–12+ days, depending on the species and the number of eggs produced (clutch size). Eggs are usually laid at a rate of one per day, but this can vary with species. Some birds will begin to incubate as soon as they have laid the first egg, for example barn owls *Tyto alba*, but most species will wait until all, or nearly all, of the clutch has been laid. By waiting until all of the eggs have been laid before incubating, the parents can more readily synchronise the time at which the chicks hatch (Deeming and Reynolds, 2015).

The egg-laying period can be quite a dangerous time for the parents. When the parent is not on the nest, eggs are entirely defenceless. Nesting in colonies is one way to protect eggs and chicks from predation. For birds that build nests in the open, natural selection has driven many to camouflage their eggs. Egg colouration might match either the nest or the substrate surrounding the nest and ground-nesting birds typically have particularly well-camouflaged eggs.

Brood parasitism is also a risk for some species. A brood parasite manipulates its host (either a conspecific or, more often, a different species) to incubate and raise its young. A female brood parasite e.g. the European cuckoo *Cuculus canorus* or the brown-headed cowbird *Molothrus ater* will lay eggs in multiple host nests. Most brood parasites are specialists, parasitizing only one host, although both the European cuckoo and the brown-headed cowbird are able to parasitize a wide range of hosts (e.g. cuckoo hosts: dunnoek *Prunella modularis* and reed warbler *Acrocephalus scirpaceus*; cowbird hosts: Eastern phoebe *Sayornis phoebe* and American redstart *Setophaga ruticilla*). The choice of which species to parasitize is passed down the maternal line. Brood parasites have evolved a number of ways to avoid identification and/or eviction by hosts. For example, if the host builds an open-cup nest, the brood parasite egg is usually remarkably similar in coloration to that of the host eggs. The parasitic young also hatch earlier and develop more quickly than do the host chicks. Furthermore, once hatched, the parasite chick will often evict remaining host eggs and/or the chicks from the nest. If the host nestlings are not evicted they usually starve, as the larger parasite outcompetes them for food.

Incubation

Incubation is an aspect of parental care peculiar to birds whereby the parent sits on the eggs and nestlings to keep them at a temperature that is optimal for development. By providing the appropriate temperature for an appropriate duration, Incubation enables the cluster of cells on an egg yolk surface to differentiate and develop into a hatchling. The remaining yolk is used as a nutrient reserve by the developing chick.

Incubation usually occurs by either one or both of the parents warming the eggs via a brood patch. A brood patch is a highly vascularised area on a bird's breast, where all the feathers have either been removed or fallen out. The brood patch then warms the eggs by conduction. During incubation the eggs are turned and moved by the parent(s) to ensure heat is transferred equally through the yolk. Turning the egg also prevents the egg membrane from sticking to the shell.

Not all birds use their body to incubate. Megapodes (also known as incubator birds) like the Australian brushturkey *Alectura lathamii* scrape together mounds of combustible vegetation and one or more females lay eggs in the centre. The brushturkey male then attends to the mound as it composts, regulating the internal temperature to around 33–35°C by adding and/or removing vegetation. Variation in the temperature in the mound leads to skews in the sex ratio of the offspring produced: cooler temperatures lead to the production of more sons and warmer temperatures results in more daughters.

But the majority of birds use their bodies to regulate incubation temperature. While in cooler climes, the aim of incubation is typically to keep the eggs and chicks warm, in locations where the ambient temperature is greater than the optimal temperature, or when the eggs are not shaded from the sun, the parent(s) may use their bodies to cool the eggs. By positioning themselves over the nest contents, they can shade the eggs and limit heat transfer, or alternatively soak their breast feathers and drop water onto the eggs to aid cooling.

The task of incubation can fall to just the female, just the male or both parents. In about 50% of species both parents incubate, in some shape or form, but the female often does the brunt of the work. The male is not usually idle: he may defend the nest, eggs, his territory and his female(s). In some species, he will provision the female while she remains on the eggs. Indeed, in hornbills (Bucerotidae), the female is entirely dependent on her mate because she nests in a tree cavity, the entrance to which she begins to fill in. Once she has laid her eggs and begins to incubate, her mate will all but seal the entrance with mud leaving just a small hole through which he can feed her. The female remains on the nest until the eggs have hatched, when she breaks free, reseals the nest and assists the male with provisioning the young. In some species, such as rufous hummingbirds *Selasphorus rufus* however, the female incubates alone without help from her mate. He continues to display throughout the whole of nest building, egg laying and post-hatch care of the young.

As with other parts of reproduction, the length of incubation is species specific. It can take anywhere from 11 days (small songbirds) to 85 days (wandering albatross *Diomedea exulans*). But the actual length of incubation can be affected by a variety of factors. For example, the amount of time a parent incubates is often flexible and often changes in response to environmental conditions. During wet and/or cold periods the parent(s) will remain on the nest for longer and take fewer and shorter bouts off the nest. By remaining on the nest the incubating parent can maintain the eggs at an optimal temperature, and also protect them from the rain. Also by reducing their bouts off the nest the parents themselves will not return to the nest sodden and drench the eggs, which could be detrimental. In contrast, during periods of dry and/or warm weather the parent(s) may be away from the nest for

longer periods, using the time to find food. Unless, that is, the ambient temperature exceeds that of optimal incubation temperature. During these times the parent(s) remain on the eggs for longer, to protect them from the heat and control the nest temperature.

The risk of predation can also affect parental incubation behaviour. In areas of high predation risk, parents take fewer and longer bouts off the nest. This reduces the chance that the parents advertise the nest location to potential predators. The availability of food may also affect incubation behaviour. In experiments where incubating adults have been supplemented with food, the parents spend less time searching for food and more time incubating their eggs/chicks. Such supplementary food can even decrease the incubation period by a few days. By the same token, a limitation in food supply can lead to an increase the period of incubation.

Furthermore, the health and age of the parents can influence incubation. Healthier birds spend more of their time incubating, possibly because they do not have to spend as much time searching for food, which reduces the incubation period. In some species, for example the southern fulmar *Fulmaris glacialisoides*, older birds are better incubators (success almost doubles as birds age from around 7 years old to around 19 years old), which again can reduce the incubation period.

Parental Care

Parental care is the investment provided by the parent(s) to ensure development and survival of their offspring. It can include providing food, providing protection and/or thermoregulating the young. The degree to which chicks are developed when they hatch affects the kind of care their parents typically provide (Cockburn, 2006).

Altricial chicks, which include the young of songbirds, at hatch are blind, featherless and completely dependent on their parent(s). The parent(s) will either bring the chicks pieces of food, or regurgitate food into their offspring's mouth and continue to do so until the chicks fledge which could take several weeks, depending on body size. The chicks encourage the parent(s) to provision them with food by engaging in begging. During early development the parent(s) must also continue to incubate the chicks as they cannot thermoregulate. Parental time is divided between feeding, incubation and their own upkeep.

The offspring of some species are considered to be semialtricial, for example, owls (Strigiformes) and hawks (Accipitridae). The new hatchlings of these species are immobile, unable to thermoregulate and are completely dependent on their parents for food but their eyes are open when they hatch.

The next level of dependency is termed semiprecocial, which describes the state of development of gull and tern chicks when they hatch. Semiprecocial chicks are feathered and mobile just after hatching but remain in or close to the nest. The chicks are dependent on the parent(s) for feeding and protection.

Chicks that leave the nest just after hatching and remain with their parent(s) are considered to be subprecocial. Just after hatching, the chicks, for example grebes (Podicipediformes), are either fed away from the nest or shown by the parent(s) where food can be found and what can be consumed.

The most developed at hatch are the young of precocial species, which include the waterfowl e.g. ducks, geese and swans. Precocial chicks leave the nest very soon after hatching and follow their parent(s). The chicks are able to feed themselves almost immediately, but do rely on their parent(s) for protection.

All chicks are, dependent on some form of parental care, at least early after hatching. And most avian parents engage in parental care, but there are two exceptions. Brood parasites do not care for their young as they give that responsibility to their hosts. But their chicks do still require parental care, albeit from unrelated, unwitting foster parents. The chicks of megapodes, however, are completely independent from hatching and are not reliant on their parent(s).

In some instances, care of the young is not just carried out by the parents, but by family members. This is known as cooperative breeding. The common explanation for the existence of cooperative breeding is that it occurs due to environmental constraints, such as limited territory or food availability. When territory is limited, helpers are waiting for a territory to become vacant but when food is limited, parents may not be capable of finding enough food for their chicks without the help of others.

References

- Breen, A.J., Guillette, L.M., Healy, S.D., 2016. What can nest-building birds teach us? *Comparative Cognition & Behavior Reviews* 11, 83–102. doi:10.3819/CCBR.2016.110005.
- Cockburn, A., 2006. Prevalence of different modes of parental care in birds. *Proceedings of the Royal Society, B* 273, 1375–1383.
- Collias, N.E., Collias, E.C., 1984. *Nest Building and Bird Behaviour*. Princeton, NJ: Princeton University Press.
- Davies, N.B., Krebs, J.R., West, S.A., 2012. *An Introduction to Behavioural Ecology*, fourth ed. UK: Wiley-Blackwell.
- Deeming, D.C., Reynolds, S.J., 2015. *Nests, Eggs, and Incubation: New Ideas About Avian Reproduction*. Oxford: Oxford University Press.
- Hansell, M.H., 2000. *Bird Nests and Construction Behaviour*. Cambridge: Cambridge University Press.
- Mainwaring, M.C., Hartley, I.R., Lambrechts, M.M., Deeming, D.C., 2014. The design and function of birds' nests. *Ecology and Evolution* 4 (20), 3909–3928. doi:10.1002/ece3.1054.

Parental Behavior in Fish

Frieda Benun Sutton and Anthony B Wilson, Brooklyn College, Brooklyn, NY, United States; and The Graduate Center, City University of New York, New York, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Agnatha A superclass of 125 extant species representing jawless fishes, and includes hagfish and lampreys – the majority of the representatives of this group are extinct. Jawlessness, considered a primitive trait, is not found in any other vertebrate group.

Anisogamy A form of reproduction in which dissimilar gametes fuse during fertilization. Anisogamous gametes (typically large, energy-rich eggs and small, motile sperm) have been invoked as a driving force behind sexual selection.

Chondrichthyes A superclass of more than 1200 species representing the cartilaginous fishes and including sharks, rays, skates, and chimeras. Unlike the bony fishes (Osteichthyes), the chondrichthyan skeleton is made of cartilage. All members of this group have internal fertilization.

Fertilization The process by which a female egg and a male sperm fuse to form the first cell of a new offspring. It may be internal, in which the union of gametes occurs within a specialized reproductive organ, or external, in which parents release their gametes into the external environment, where fusion takes place.

Group spawning A form of mating in aquatic animals in which multiple males and females simultaneously release their gametes into the external environment.

Mating systems Describe the number of mates per individual. A mating system may entail pair bonding between a single male and female (monogamy), a single male and multiple females (polygyny), a single female and multiple males (polyandry), or multiple males and females (polygynandry). Promiscuity refers to a mating system in which no social bonding occurs, and males and females may mate with any number of partners.

Osteichthyes A taxonomic megaclass including close to 32,000 species representing the bony fishes, including the ray-finned Actinopterygii and the lobe-finned Sarcopterygii. Osteichthyans can be identified by their bony endoskeleton, plate-like scales, and air-filled swim bladder.

Pair spawning A form of mating in which a single male and female release their gametes into the water for fertilization.

Parental care Behavior by either parent that increases offspring survival after fertilization. Care includes internal gestation, egg burying, mouthbrooding, nest guarding and feeding.

Parental investment Parental energetic and time contributions toward both pre-fertilization gametes and post-fertilization offspring.

Phylogeny Traces the evolutionary history of organisms and the evolutionary relationships between groups.

Territoriality A behavior in which an individual occupies and defends a specific area. Territorial animals may defend breeding sites or access to resources such as food or mates.

Introduction

Fish are the most diverse group of vertebrates, and include more than 33,000 species exhibiting a remarkable diversity of parental care behaviors (Froese and Pauly, 2017). While exclusive paternal care is rare in birds and absent in mammals, more than a quarter of fish families exhibit male-only care (Fig. 1), a factor that has made this group a central model in the study of the ecology and evolution of parental care. For the purposes of this article, parental care is considered to be any parental behavior toward offspring that enhances offspring survival. While parental investment in gametes is thought to be a primary determinant of offspring success, parental care behaviors after mating are often essential for offspring growth and survival (Blumer, 1982).

Trends in Parental Care in Fish

Ecological Overview of Care

Over 95% of caregiving fish species are guarders, where one or both parents defend the nesting site (Gross and Sargent, 1985). Other common forms of care include nest maintenance during offspring development, substrate cleaning, in which the site of egg deposition is cleaned, and fanning, in which the parent waves its fins over developing offspring to provide aeration or to remove sediment. A number of groups of fishes employ mouthbrooding, in which one or both parents carry developing offspring in their oral cavity. While the male is the sole provider in the majority of fish species in which care has been documented (Fig. 1), internal gestation (retention of fertilized eggs within a brooding organ) is typically carried out by the female, with the notable exception of the male-pregnant syngnathids (seahorses, pipefish, and seadragons (Stolting and Wilson, 2007)). In most cases, parents care for fertilized eggs before hatching; less than six percent of care extends to fry and juveniles (Gross and Sargent, 1985).

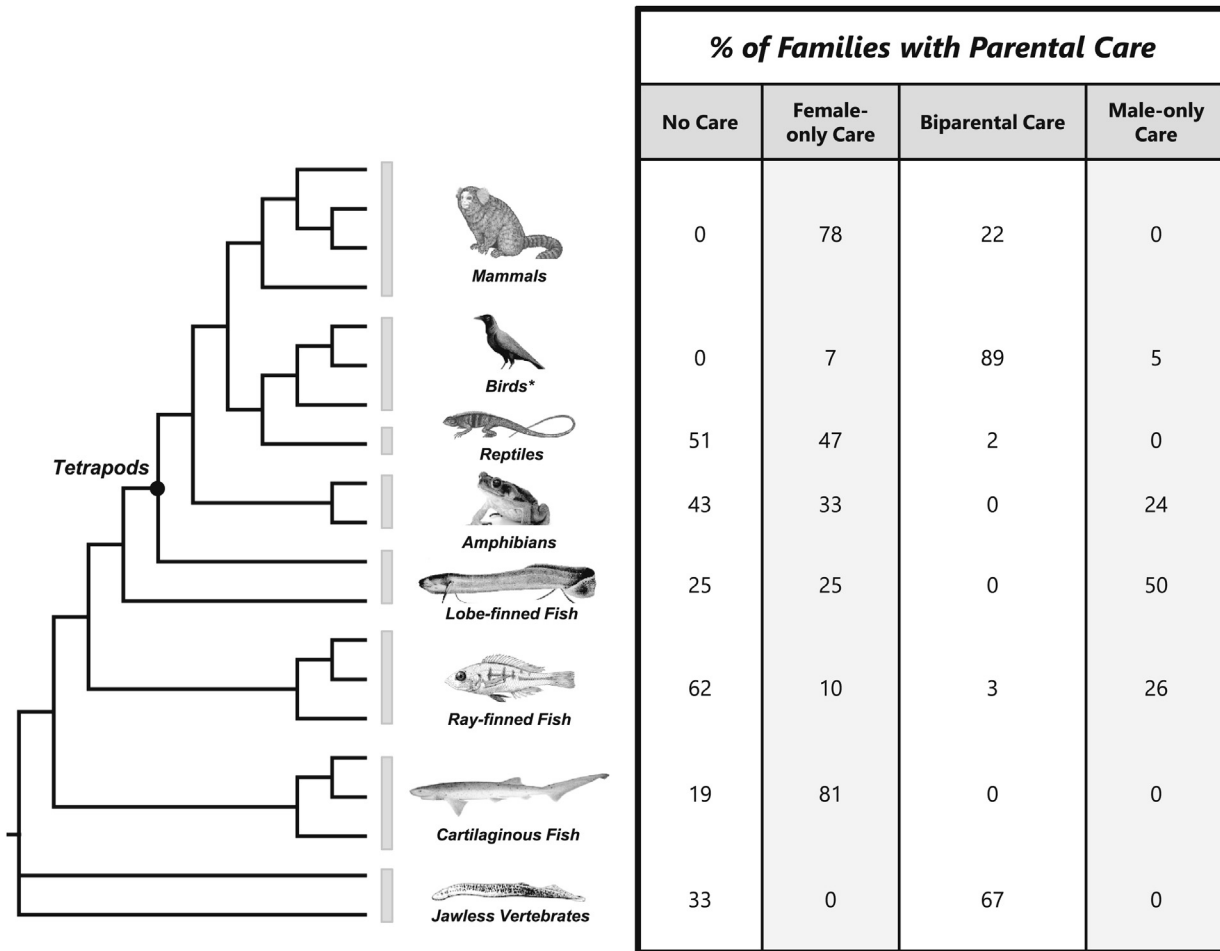


Fig. 1 Phylogenetic distribution of parental care in vertebrates. Phylogenetic tree showing genetic relationships among major vertebrate groups (After Amemiya, C.T., Alföldi, J., Lee, A.P., *et al.*, 2013. The African coelacanth genome provides insights into tetrapod evolution. *Nature* 496(7445), 311–316) and relative representation of care behaviors in each group, based on the dominant mode of care shown at the family level. *Note: Alternative forms of avian care (brood parasitism, cooperative breeding) *sensu* Cockburn (2006) not shown.

Interestingly, there is a major difference in the frequency of parental care in marine and freshwater environments, and while less than 20% of marine fish display care, parental care is found in more than 50% of freshwater species (Gross and Sargent, 1985). The widespread pelagic egg stage has been suggested to obviate the need for care in marine fishes, as the open sea is a more uniform environment with fewer egg predators and a higher egg survival rate relative to freshwater (Clutton-Brock, 1991). Guarding and nest building are thought to be more beneficial in freshwater environments, as favorable spawning sites are less abundant and unevenly distributed (Clutton-Brock, 1991). Care is especially uncommon in fish greater than 10 cm in length, possibly because larger fish tend to produce large numbers of pelagic eggs, whereas smaller fish produce fewer eggs that can be protected by small caves and cavities in the local environment (Clutton-Brock, 1991).

Phylogenetic Overview of Care

The role of evolutionary history is an important component in the study of any aspect of life history, including parental care, and incorporating phylogenetic relationships can help to clarify the major trends and drivers of caring behavior. Given their great diversity of care forms, bony fishes represent an ideal model in which to test evolutionary models of parental care. The last comprehensive review of parental care in fish was carried out by Blumer in 1982, and while subsequent studies have investigated evolutionary correlates of care in particular groups (e.g., Goodwin *et al.*, 1998; Ah-King *et al.*, 2005; Mank *et al.*, 2005; Kolm *et al.*, 2006), there has been no systematic attempt to update Blumer's analysis, despite the steady accumulation of studies detailing care behaviors in a wide variety of fish species. At the same time, the phylogeny of fish is better resolved than at any time in the past (e.g., Betancur-R *et al.*, 2013), due to the availability of genetic and genomic data for many species.

Here, we present the results of a systematic and comprehensive review of parental care in fish. Relying heavily on the public collection of life history data in FishBase (Froese and Pauly, 2017), we present data for 294 of 514 bony fish families (Fig. 2). While our updated review includes close to 60% of fish families, an examination of the data reveals the overrepresentation of

reproductively unique and commercially important groups (e.g., all but 3 of the 298 species of male-pregnant Syngnathidae have been investigated, and Cyprinidae, which includes highly-fished species such as carp, represent a large portion of parental care studies), and highlights the fact that unstudied groups may show cryptic diversity in care behaviors. For instance, recent work on Antarctic icefishes (Family Harpagiferidae) has revealed a wide range of parental behaviors in this group, including male, female, and biparental care (Detrich *et al.*, 2005), underscoring the need for continued research into the basic life history of many groups. Given the high taxonomic diversity of fish, we summarize data on an ordinal level (with the exceptions of Elopomorpha, Carangia,

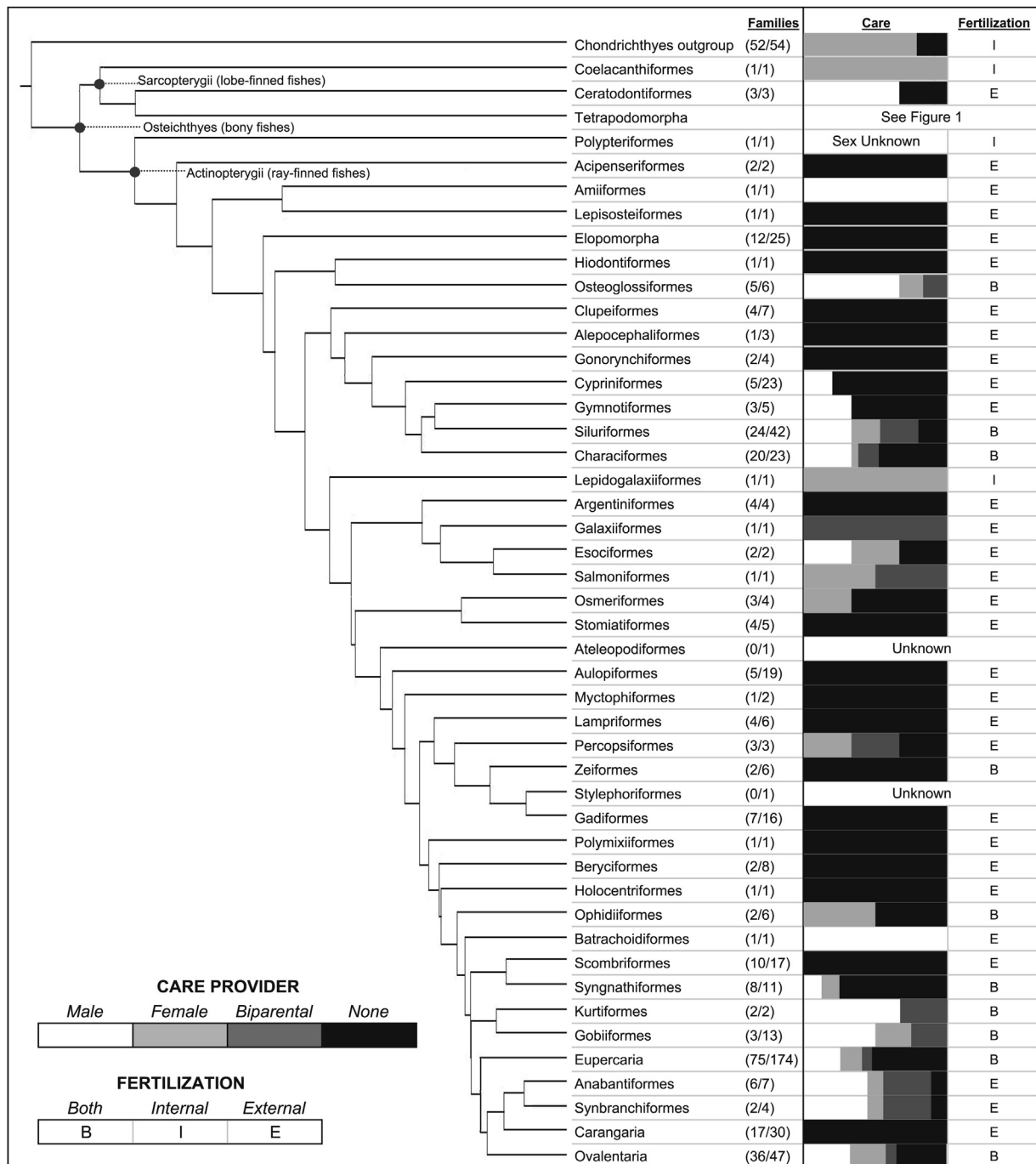


Fig. 2 Phylogenetic distribution of care in the bony fishes. Phylogeny of Osteichthyes showing higher-level patterns in the distribution of care and mode of fertilization. Groups are listed on the ordinal level except in cases of limited phylogenetic resolution (*sensu* Betancur-R. *et al.*, 2017). Shaded bars represent the relative frequency of care behaviors at the family-level within each group. The number of families for which care data are available is indicated, along with the total number of families per group. Phylogeny is based on Betancur-R. *et al.* (2017). Reproduced from Betancur-R R., Wiley, E.O., Arratia, G., 2017. Phylogenetic classification of bony fishes. BMC Evolutionary Biology 17(1), 162. doi: 10.1186/s12862-017-0958.-3.

Eupercaria, Ovalentaria, *sensu* Betancur-R *et al.* (2017)). A more detailed analysis of evolutionary trends in the parental care of fish will be published elsewhere.

The Evolution of Parental Care in Fish

Several explanations have been proposed to explain both the ecological and phylogenetic distribution of care behaviors in fish (e.g., Gross and Sargent, 1985; Wourms and Lombardi, 1992; Goodwin *et al.*, 1998; Ah-King *et al.*, 2005; Mank *et al.*, 2005). Here we review several prominent theories and their implications for understanding the evolution of parental care.

Anisogamy

Anisogamy, a reproductive system characterized by differentially-sized male and female gametes, has traditionally been invoked to explain sex differences in parental care and the widespread existence of maternal care across many animal species (Liker *et al.*, 2015). Whereas females typically invest large amounts of energy into egg production, sperm production by males is relatively inexpensive (Liker *et al.*, 2015). Trivers (1972) argued that the large initial investment by females in gamete production should encourage additional investment after fertilization. Despite its popularity, this argument has been criticized on both theoretical and empirical grounds (Dawkins and Carlisle, 1976; Kokko and Jennions, 2008; Liker *et al.*, 2015), as optimal decision making should be based on future costs and benefits, not past investment (Kokko and Jennions, 2008). Additionally, while the anisogamy argument predicts higher frequencies of maternal care (compared to paternal care) resulting from females' costly gametes, this prediction fails to explain the prevalence of male care in fish.

Anisogamy can, however, indirectly influence the distribution of care, as it generally creates a male-biased operational sex ratio in which males are nearly always available to mate, whereas females typically require more time to replenish their egg supply following fertilization (Kokko and Jennions, 2008). In a scenario in which females are scarce due to their reduced potential reproductive rate, the opportunity for additional matings by males may be limited. Under such conditions, recently mated males may enhance their reproductive success by caring for a current brood instead of seeking new mating partners (Kokko and Jennions, 2008).

Aquatic Environment and External Fertilization

One of the primary factors that sets fish reproduction apart from that of most other animals is the fact that it takes place in an aquatic environment. While aquatic species can release free-swimming gametes into the water column, terrestrial reproduction requires internal fertilization to ensure gamete motility (Gross and Sargent, 1985). Our analysis shows that external fertilization is the dominant mode of reproduction in fish (Fig. 2), and highlights a clear pattern of sexual dimorphism in care behaviors. External fertilization without care has independently given rise to male care at least 22 times within the ray-finned fishes, while males provide care in only three families in which fertilization is internal: Apogonidae, Pantodontidae, and Cottidae (included in Fig. 2 under Eupercaria, Kurtiformes and Osteoglossiformes, respectively) (Gross and Sargent, 1985). Conversely, maternal care has arisen only three times from external fertilization in the ray-finned fishes, but represents close to 90% of care in internal fertilizers (Gross and Shine, 1981; Mank *et al.*, 2005), highlighting a strong association between fertilization and parental care in this group. Interestingly, internal fertilization is found in all Chondrichthyans (sharks, skates and rays), and there are no instances of male parental care in this group (Wourms and Lombardi, 1992).

Recent phylogenetic work has rejected the hypothesis that biparental care serves as an evolutionary stepping stone from male-only to female-only care, a widely held notion in the latter half of the 20th century (e.g., Gittleman, 1981). Rather, data suggest two major evolutionary pathways from the basal state of external fertilization and no care: either male care evolves directly from this state, or internal fertilization evolves, followed by female care (Mank *et al.*, 2005).

Certainty of Paternity

Parental care is not expected to evolve under conditions in which the parent has a low likelihood of genetic relatedness to his or her brood (Kokko and Jennions, 2008). Both males and females, therefore, are expected to be more likely to invest in offspring when parentage is assured. The theory of reproductive optimization predicts that males should reduce their investment in a brood of doubtful paternity only if they can ensure a higher confidence of parentage in future broods (Kokko and Jennions, 2008). When paternity confidence varies across broods, fish can dynamically discriminate their reproductive investment based on perceived paternity. Research on bluegill sunfish indicates that males adjust their level of parental care based on olfactory and visual cues of cuckholders and fry (Neff, 2003).

When one looks more closely at externally fertilizing species, a striking pattern emerges. Paternal care is prevalent in pair spawners, in which a single male and female mate by releasing their gametes externally, but absent in group spawners, in which multiple males and females release gametes at the same time (Ah-King *et al.*, 2005). The simultaneous release of gametes in group spawners reduces confidence in paternity, and is thought to retard the evolution of parental care (Ah-King *et al.*, 2005). The potential relationship between parental investment and mating system is particularly intriguing, as the latter can vary widely across a single species and is linked to ecological parameters (Emlen and Oring, 1977), suggesting that environmental factors may indirectly influence care behaviors.

Territoriality

The anisogamy argument has also been used to explain the abundance of territorial males in nature (Kokko and Jennions, 2008). Females are reproductively limited by gamete production, whereas males are mate-limited. By controlling a prime breeding site, a male can attract multiple females and thus increase his mating success. When males actively defend territories, the guarding of eggs may not require significantly more energy or limit additional mating opportunities (Gross and Sargent, 1985), making it a cost-effective means of increasing offspring survival. Territoriality also increases a male's confidence in the paternity of his guarded eggs (Ah-King *et al.*, 2005), and while sneaker or satellite males may successfully fertilize a proportion of a territorial male's clutch in some species, the sneaker fertilization rate is typically much lower than that of the dominant male (DeWoody and Avise, 2001). If territorial males mate with multiple females, females are expected to be less likely to engage in care, due to their reduced relatedness to the genetically mixed clutch (see [Section Certainty of Paternity](#)).

Internal Fertilization

While internal fertilization is a derived state in fish and requires extensive specialization, it can provide greater reproductive security, especially in unpredictable habitats or fast-moving waters such as streams (Meyer and Lydeard, 1993). Under such conditions, the evolution of sperm localization toward potential mates, and ultimately internal fertilization, has clear adaptive benefits. Once internal fertilization has evolved, the progression to female care is achieved by extending the time that the female retains the eggs after fertilization (Rosen, 1962). Prolonged egg retention and viviparity (live birth) are thought to evolve when predation risk is high and resources for young are scarce, enhancing offspring survival at the life stage when mortality is highest (Stearns, 1976). Internal fertilization can also reduce energetic investment in both male and female gonadal development, as fewer gametes are needed to ensure successful fertilization when sperm are transferred directly to eggs (Buckland-Nicks and Scheltema, 1995). Maternal care through egg retention in its most primitive form is lecithotrophic, a form of care in which offspring are provisioned solely by the yolk. In more advanced forms of care, females may provide a supply of nutrients to the offspring from fertilization until their release (i.e., matrotrophy; Wourms, 1981; Wourms and Lombardi, 1992; Reznick *et al.*, 2002).

Maternal Care in Fish: The Exception Proves the Rule

Poeciliidae

While maternal care is relatively uncommon in most families of bony fish, it is the norm rather than the exception in the poeciliids, a group of close to 350 freshwater species (Froese and Pauly, 2017). Nearly all species in this family (which includes the well-known guppies and swordtails) are viviparous, and poeciliids have evolved a specialized placental organ that is similar in both structure and function to the well-characterized mammalian placenta (Reznick *et al.*, 2002). The high incidence of female care in poeciliids may represent a strategy to minimize reliance on a limited resource (i.e., spawning and nesting territory) through internal fertilization and gestation of young. Comparative work indicates that placentation in poeciliids is correlated with increased reproductive output during early life and shorter interbirth intervals, highlighting the potential benefits of maternal care in this group (Pires *et al.*, 2011).

Chondrichthyes

Viviparity (live bearing) has also evolved repeatedly in Chondrichthyes, the cartilaginous fishes, where over half of all species are live bearers, whereas the phenomenon occurs in less than 3% of Osteichthyes (Wourms and Lombardi, 1992). Chondrichthyan eggs are non-buoyant and thus unsuited for pelagic environments, and as many chondrichthyans are physiologically limited in the number of eggs they can produce, the transition to viviparity is not thought to be associated with a significant loss in fecundity (Wourms and Lombardi, 1992). Higher survival rates of live-borne offspring suggest a clear selective advantage for viviparity in this group. Internal egg development enables viviparous sharks and rays to colonize pelagic zones that are unavailable to egg-laying relatives (Wourms and Lombardi, 1992).

Proximate Mechanisms of Parental Care

While parental care has evolved independently multiple times in the bony fishes, caring species exhibit physiological similarities in the function and maintenance of care behaviors. Recent research has targeted hormonal, neural, and social control as three major proximate mechanisms underlying parental care in the group.

Hormonal Control

Prolactin

The hormone prolactin influences a wide variety of functions across vertebrates, and is particularly well known for its regulatory role in human pregnancy and milk production (Freeman *et al.*, 2000). In line with its regulatory activity in the mammalian placenta, prolactin is an important molecule in producing and maintaining the placenta-like brooding pouch in male seahorses. Male pregnancy in the seahorse is under the control of the prolactin-producing pituitary gland, and the detrimental reproductive effects induced by the removal of the pituitary are diminished following the experimental administration of prolactin. Similarly, female

pregnancy in *Gambusia poeciliids* appears to be under pituitary control; removal of the gland in early pregnancy causes almost complete offspring mortality, however no significant effects result from its removal in late pregnancy (Chambolle, 1964). Prolactin has also been shown to play a role the production of mucous, an essential component of offspring protection and nutrition in many types of piscine care (Whittington and Wilson, 2013). The multifunctional prolactin has been implicated in many other forms of fish parental care, including fanning, mouthbrooding, and guarding (reviewed in Whittington and Wilson (2013)).

Androgens

Androgen sex steroid hormones such as testosterone play an important role in the regulation of male reproductive traits. These hormones are often associated with male aggression and are thought to be antagonistic in regard to caring behavior (Scobell and MacKenzie, 2011). Indeed, 11-ketotestosterone decreases when males enter parental phases in many fish species (Páll *et al.*, 2002), and the hormone is not detectable in nesting plainfin midshipman males during offspring care (Knapp *et al.*, 1999). Similarly, male syngnathids (seahorses and pipefish) exhibit a decrease in androgen levels during brooding (Scobell and MacKenzie, 2011). In other species, however, manipulation of androgen levels does not appear to affect paternal care (Ros *et al.*, 2004; Dey *et al.*, 2010). The simultaneous maintenance of both high androgen levels and parental care in some species but not others suggests that endocrine pathways may be utilized differentially across species.

Neural Control

Evidence from cichlid fishes indicates that parents employ distinct visual and chemoreceptive cues to recognize their young depending on offspring life stage (Dulac *et al.*, 2014; Myrberg, 1966). While brain circuitry underlying parental behavior appears to involve conserved neural regions across vertebrates (Weitekamp and Hofmann, 2017), neural modulation of care in fish is still not well-characterized. Parental behavior is regulated in part by the preoptic area in the brain, a region that modulates the expression of the neuropeptides vasotocin and isotocin and has been implicated in care and bonding behavior in mammals (Bass and Grober, 2001). Many species of fish exhibit differential expression of preoptic vasotocin depending on reproductive status (Bass and Grober, 2001), and the disruption of preoptic isotocin receptors in male convict cichlids eliminates paternal care (O'Connell *et al.*, 2012). The mammalian homologues of vasotocin and isotocin (vasopressin and oxytocin, respectively) also play a key role in regulating parental care (Dulac *et al.*, 2014), highlighting the convergent neural pathways underlying parental behavior in vertebrates.

Social Control

Individuals of many species of fish show two or more reproductive phenotypes across their lives. Transformations of sex and dominance in such species can have significant implications for the control of parental care mechanisms, as these plastic identities are often associated with distinct forms of care (Bass and Grober, 2001), suggesting that care may be socially regulated. Male fish in many species start out as non-caring sneaker or satellite morphs, relying on huge gametic output to covertly fertilize eggs without the knowledge of the female or dominant parental male (Taborsky, 1994; see discussion above). Reproductive tactics in such species are often socially dependent, however, and the removal of the dominant male can create a cascade of sexual changes within a social group in which a subordinate individual, whether female or sneaker male, will quickly develop physically and behaviorally into the dominant male (Bass and Grober, 2001). The ability to transform a suite of reproductive characteristics including care based on social cues suggests that parental behavior in such species may be highly labile. Fish displaying alternative reproductive tactics are ideal candidates for exploring the mechanisms underlying parental care, owing to their context-dependent morphs that can be experimentally manipulated.

New Methodologies, New Perspectives: The Cichlid Model

Cichlids (Family Cichlidae) have proved to be a remarkably useful system in which to explore both the proximate (immediate biological) and ultimate (long-term evolutionary) causes of parental care. This family comprises over 1300 species and boasts a wide range of care types including nest-guarding, ectodermal feeding, and mouthbrooding and exhibits male, female, and biparental care (Blumer, 1982; Goodwin *et al.*, 1998). Social and reproductive behaviors vary broadly as well; care has been found in monogamous and polygamous species, and in single-pair breeders and cooperative groups (Goodwin *et al.*, 1998; Taborsky, 2016). Perhaps most significant, decades of scientific interest in cichlid evolution and behavior has resulted in a plethora of data and molecular tools that can be used to investigate key aspects of parental care in this group. Such studies have led to the characterization of behavior, ecology, and phylogenetic history in many cichlid species (e.g., Axelrod and Burgess, 1988; Brawand *et al.*, 2014; Brichard, 1989; Gashagaza, 1991), enabling the elucidation of drivers and consequences of parental care (Goodwin *et al.*, 1998; Kolm *et al.*, 2006). In a recent illustration of the power of a comparative approach, Kolm *et al.* (2006) mapped reproductive traits onto the cichlid phylogeny and found that contrary to model predictions, the duration of parental care in cichlids correlates not with egg size, but rather with the total number of offspring in a clutch.

Cichlids also show the highest frequency of cooperative breeding, a form of reproduction in which offspring are cared for by both parents and non-parental helpers (Taborsky, 2016). Parental and helper behaviors in lamprologine cichlids are ecologically

dependent and influenced by a range of factors such as predation threats and territory availability (Taborsky, 2016). Long-term field and laboratory studies and experimental manipulation in this system have provided a detailed understanding of intricate relationships such as task specialization and reciprocal trading among group members (Taborsky, 2016).

The recent sequencing of multiple cichlid genomes has significantly enhanced our understanding of the highly diversified East African cichlids, highlighting genome duplication and accelerated evolutionary rates as drivers of rapid adaptive radiations (Brawand *et al.*, 2014). Parental care has been cited as one of the components of cichlid biology conducive to rapid speciation (Henning and Meyer, 2014). Genomic tools are likely to be instrumental in establishing a more mechanistic understanding of reproductive behavior (i.e., Bendesky *et al.*, 2017), and the combination of detailed ecological data and genomic resources makes the cichlids an ideal candidate for such work.

In a particularly elegant recent study, Juntti *et al.* (2016) used a multipronged approach to demonstrate the biochemical, neural, and genetic pathways underlying reproductive behavior in *Astatotilapia burtoni*, a mouthbrooding cichlid. The authors experimentally demonstrated that injection of prostaglandin (a hormone-like lipid) induced spawning behavior in non-fertile females. Next, they identified the neural circuitry responsible for signal production and characterized how prostaglandin acts on the brain. Finally, they used CRISPR/Cas9 gene editing to delete the prostaglandin receptor gene, which prevented female spawning. This study illustrates the value of applying cutting-edge molecular tools to dissect reproductive behavior: by taking advantage of the cichlid's laboratory tractability, physiological data, and genomic resources, researchers characterized a pathway linking gene activity to reproductive behavior.

Conclusion

The cichlid model highlights potentially fruitful strategies for other groups of fishes. Recent work on the mechanisms of parental care in this group has combined genomic, hormonal, neural, and behavioral tools, illustrating the multimodal network of controls underlying complex reproductive behaviors (Fig. 3; e.g., O'Connell *et al.*, 2012; Juntti *et al.*, 2016). The combination of detailed studies of functional and physiological mechanisms of care in a small number of model systems, coupled with higher-level

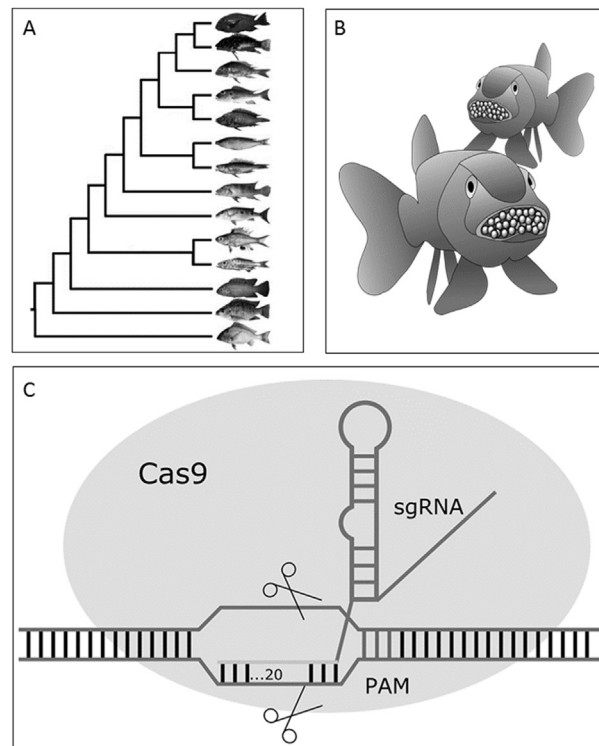


Fig. 3 The cichlid system as a model for the study of parental care in fish. We outline three key elements of the cichlid system that have been integral in establishing this group as a model for parental care. (A) A well-characterized phylogeny provides a solid foundation for the comparative analysis of life-history data (e.g., phylogeny of Tanganyikan cichlids after Meyer, B.S., Matschiner, M., Salzburger, W., 2015. A tribal level phylogeny of Lake Tanganyika cichlid fishes based on a genomic multi-marker approach. *Molecular Phylogenetics and Evolution* 83, 56–71). (B) Ecological diversity of care allows the precise characterization of the proximate and ultimate drivers of care (e.g., biparental mouthbrooders). (C) The application of new methods (e.g., genomics, CRISPR, neurobehavior) facilitates experimental manipulation, clarifying the underlying genetic mechanisms of care behaviors (e.g., the CRISPR/Cas9 method uses a naturally occurring form of immune protection in bacteria to precisely modify target sequences in the target genome; Reproduced from Nødvig, C.S., Nielsen, J.B., Kogle, M.E., Mortensen, U.H., 2015. A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLOS ONE* 10(7), e0133085. doi: 10.1371/journal.pone.0133085).

comparative analyses of life-history correlates of care, offer the opportunity to translate experimental results into an evolutionary context, and to link the proximate and ultimate drivers of parental care.

While parental care theory saw great progress in the 1970s and 1980s, new data are contributing to a more nuanced understanding of the evolution of care. We identify three major developments that are likely to play a central role in the future advancement of the field. First, general parental care theory will continue to mature through refinement of mathematical models and empirical testing (Kokko and Jennions, 2008), contributing to a theoretical foundation from which new models of care can be built. Second, sweeping efforts in genome research have begun to resolve contested phylogenetic clades, providing a solid evolutionary context in which to study the origin and diversification of parental care behaviors (e.g., Betancur-R *et al.*, 2013; Betancur-R *et al.*, 2017), and creating new experimental tools for the exploration of reproductive function (e.g., Juntti *et al.*, 2016). Finally, online databases such as FishBase are enhancing the dissemination of a wide range of biological data including information on reproduction and care, and are helping to identify understudied groups worthy of further attention. The continued integration of ecological, evolutionary and molecular data shows terrific promise for clarifying the evolution of parental care in fish and other reproductively diverse groups of vertebrates.

Acknowledgements

We would like to thank Ricardo Betancur-R., Scott Juntti and Michael Taborsky for discussions on the evolution of parental care, and for providing access to unpublished work.

References

- Ah-King, M., Kvarnemo, C., & Tullberg, B. S. (2005). The influence of territoriality and mating system on the evolution of male care: A phylogenetic study on fish. *Journal of Evolutionary Biology*, 18(2), 371–382.
- Axelrod, H. R., & Burgess, W. E. (1988). *African Cichlids of Lake Malawi and Tanganyika*. Neptune City, NJ: TFH Publications.
- Bass, A. H., & Grober, M. S. (2001). Social and neural modulation of sexual plasticity in teleost fish. *Brain, Behavior and Evolution*, 57(5), 293–300.
- Bendesky, A., Kwon, Y.-M., Lassance, J.-M., *et al.* (2017). The genetic basis of parental care evolution in monogamous mice. *Nature*, 544(7651), 434–439.
- Betancur-R, R., Broughton, R. E., Wiley, E. O., *et al.* (2013). The tree of life and a new classification of bony fishes. *PLOS Currents Tree of Life*. <https://doi.org/10.1371/currents.tol.53ba26640df0ccae75bb165c8c26288>.
- Betancur-R, R., Wiley, E. O., & Arratia, G. (2017). Phylogenetic classification of bony fishes. *BMC Evolutionary Biology*, 17(1), 162. <https://doi.org/10.1186/s12862-017-0958-3>.
- Blumer, L. S. (1982). A bibliography and categorization of bony fishes exhibiting parental care. *Zoological Journal of the Linnean Society*, 75(1), 1–22.
- Brawand, D., Wagner, C. E., Li, Y. I., *et al.* (2014). The genomic substrate for adaptive radiation in African cichlid fish. *Nature*, 513(7518), 375–381.
- Brichard, P. (1989). *Pierre Brichard's Book of Cichlids and all Other Fishes of Lake Tanganyika*. Neptune City, NJ: TFH Publications.
- Buckland-Nicks, J., & Scheltema, A. (1995). Was internal fertilization an innovation of early Bilateria? Evidence from sperm structure of a mollusc. *Proceedings of the Royal Society of London B: Biological Sciences*, 261(1360), 11–18.
- Chambolle, P. (1964). Influence de l'hypophysectomie sur la gestation de *Gambusia* sp. (Poisson téléostéen). *Comptes Rendus Hebdomadaires des Séances de l'Académie des Sciences*, 259, 3855–3857.
- Clutton-Brock, T. H. (1991). *The Evolution of Parental Care*. Princeton, NJ: Princeton University Press.
- Cockburn, A. (2006). Prevalence of different modes of parental care in birds. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1592), 1375–1383.
- Dawkins, R., & Carlisle, T. R. (1976). Parental investment, mate desertion and a fallacy. *Nature*, 262, 131–133.
- Detrich, H. W., Jones, C. D., Kim, S., *et al.* (2005). Nesting behavior of the icefish *Chaenocephalus aceratus* at Bouvetøya Island, Southern Ocean. *Polar Biology*, 28(11), 828–832.
- DeWoody, J. A., & Avise, J. C. (2001). Genetic perspectives on the natural history of fish mating systems. *Journal of Heredity*, 92(2), 167–172.
- Dey, C. J., O'Connor, C. M., Gilmour, K. M., Van Der Kraak, G., & Cooke, S. J. (2010). Behavioral and physiological responses of a wild teleost fish to cortisol and androgen manipulation during parental care. *Hormones and Behavior*, 58(4), 599–605.
- Dulac, C., O'Connell, L. A., & Wu, Z. (2014). Neural control of maternal and paternal behaviors. *Science*, 345(6198), 765–770.
- Emlen, S. T., & Oring, L. W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science*, 197(4300), 215–223.
- Freeman, M. E., Kanyicska, B., Lerant, A., & Nagy, G. (2000). Prolactin: Structure, function, and regulation of secretion. *Physiological Reviews*, 80, 1523–1631.
- Froese, R., Pauly, D. (Eds.). 2017. FishBase. Available at: <http://www.fishbase.org>.
- Gashagaza, M. (1991). Diversity of breeding habits in lamprologini cichlids in Lake Tanganyika. *Physiology and Ecology Japan*, 28, 29–65.
- Gittleman, J. L. (1981). The phylogeny of parental care in fishes. *Animal Behaviour*, 29(3), 941.
- Goodwin, N. B., Balshine-Earn, S., & Reynolds, J. D. (1998). Evolutionary transitions in parental care in cichlid fish. *Proceedings of the Royal Society of London B: Biological Sciences*, 265(1412), 2265–2272.
- Gross, M. R., & Sargent, R. C. (1985). The evolution of male and female parental care in fishes. *American Zoologist*, 25(3), 807–822.
- Gross, M. R., & Shine, R. (1981). Parental care and mode of fertilization in ectothermic vertebrates. *Evolution*, 775–793.
- Henning, F., & Meyer, A. (2014). The evolutionary genomics of cichlid fishes: Explosive speciation and adaptation in the postgenomic era. *Annual Review of Genomics and Human Genetics*, 15, 417–441.
- Juntti, S. A., Hilliard, A. T., Kent, K. R., *et al.* (2016). A neural basis for control of cichlid female reproductive behavior by prostaglandin F2 α . *Current Biology*, 26(7), 943–949.
- Knapp, R., Wingfield, J. C., & Bass, A. H. (1999). Steroid hormones and paternal care in the plainfin midshipman fish (*Porichthys notatus*). *Hormones and Behavior*, 35(1), 81–89.
- Kokko, H., & Jennions, M. D. (2008). Parental investment, sexual selection and sex ratios. *Journal of Evolutionary Biology*, 21(4), 919–948.
- Kolm, N., Goodwin, N. B., Balshine, S., & Reynolds, J. D. (2006). Life history evolution in cichlids 1: Revisiting the evolution of life histories in relation to parental care. *Journal of Evolutionary Biology*, 19(1), 66–75.
- Liker, A., Freckleton, R. P., Remeš, V., & Székely, T. (2015). Sex differences in parental care: Gametic investment, sexual selection, and social environment. *Evolution*, 69(11), 2862–2875.
- Mank, J. E., Promislow, D. E., & Avise, J. C. (2005). Phylogenetic perspectives in the evolution of parental care in ray-finned fishes. *Evolution*, 59(7), 1570–1578.
- Meyer, A., & Lydeard, C. (1993). The evolution of copulatory organs, internal fertilization, placenta and viviparity in killifishes (Cyprinodontiformes) inferred from a DNA phylogeny of the tyrosine kinase gene X-src. *Proceedings of the Royal Society of London B: Biological Sciences*, 254(1340), 153–162.
- Myrberg, A. A. (1966). Parental recognition of young in cichlid fishes. *Animal Behaviour*, 14(4), 565–571.
- Neff, B. D. (2003). Decisions about parental care in response to perceived paternity. *Nature*, 422(6933), 716–719.
- O'Connell, L. A., Matthews, B. J., & Hofmann, H. A. (2012). Isotocin regulates paternal care in a monogamous cichlid fish. *Hormones and Behavior*, 61(5), 725–733.

- Páll, M. K., Mayer, I., & Borg, B. (2002). Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus* L. – Changes in 11-ketotestosterone levels during the nesting cycle. *Hormones and Behavior*, 41(4), 377–383.
- Pires, M. N., Bassar, R. D., McBride, K. E., et al. (2011). Why do placentas evolve? An evaluation of the life-history facilitation hypothesis in the fish genus *Poeciliopsis*. *Functional Ecology*, 25(4), 757–768.
- Reznick, D. N., Mateos, M., & Springer, M. S. (2002). Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science*, 298(5595), 1018–1020.
- Ros, A. F., Bruintjes, R., Santos, R. S., Canario, A. V., & Oliveira, R. F. (2004). The role of androgens in the trade-off between territorial and parental behavior in the Azorean rock-pool blenny, *Parablennius parvicornis*. *Hormones and Behavior*, 46(4), 491–497.
- Rosen, D. E. (1962). Egg retention: Pattern in evolution. *Natural History*, 71(10), 46–53.
- Scobell, S. K., & MacKenzie, D. S. (2011). Reproductive endocrinology of Syngnathidae. *Journal of Fish Biology*, 78(6), 1662–1680.
- Stearns, S. C. (1976). Life-history tactics: A review of the ideas. *The Quarterly Review of Biology*, 51(1), 3–47.
- Stolting, K. N., & Wilson, A. B. (2007). Male pregnancy in seahorses and pipefish: Beyond the mammalian model. *BioEssays*, 29(9), 884–896.
- Taborsky, M. (1994). Sneakers, satellites, and helpers: Parasitic and cooperative behavior in fish reproduction. *Advances in the Study of Behavior*, 23, 100.
- Taborsky, M. (2016). Cichlid fishes: A model for the integrative study of social behavior. In W. D. Koenig, & J. L. Dickinson (Eds.), *Cooperative Breeding in Vertebrates* (pp. 272–293). Cambridge: Cambridge University Press.
- Trivers, R. L. (1972). Parental investment and sexual selection. In B. Campbell (Ed.), *Sexual Selection and the Descent of Man* (pp. 1871–1971). Chicago, IL: Aldine Publishing Company.
- Weitekamp, C. A., & Hofmann, H. A. (2017). 1.17 Brain systems underlying social behavior. In G. Striedter (Ed.), *Evolution of Nervous Systems* (pp. 327–334). Amsterdam, Netherlands: Elsevier.
- Whittington, C. M., & Wilson, A. B. (2013). The role of prolactin in fish reproduction. *General and Comparative Endocrinology*, 191, 123–136.
- Wourms, J. P. (1981). Viviparity: The maternal-fetal relationship in fishes. *American Zoologist*, 21(2), 473–515.
- Wourms, J. P., & Lombardi, J. (1992). Reflections on the evolution of piscine viviparity. *American Zoologist*, 32(2), 276–293.

Parental Behavior in Mammals

Toni E Ziegler, Wisconsin National Primate Research Center, Madison, WI, United States; and University of Wisconsin – Madison, Madison, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Offspring of mammalian species generally require considerable care since they are born vulnerable to the outside world. Parental investment varies considerably across mammalian species with primarily the main investment occurring from the mother. Only 5 to 10% of mammals show bi-parental care and many of these species are cooperative breeders (Kleiman and Malcolm, 1981). Evolutionarily, females are adapted for extensive parental care and it is much rarer that males care for their young in a direct way (Woodroffe and Vincent, 1994).

In mammals, mothers invest highly in their young since they are specialized to carry the offspring *in utero*, requiring an increased energy expenditure which then is further enhanced following parturition with nursing needs of the infant. The process of birth is especially designed to stimulate sensory stimuli and internal physiological changes in hormones; ensuring brain adaptation and enhancement of maternal – infant bonding. A special form of attachment, the mother – infant bond is where the infant's welfare depends upon maternal care throughout the period of lactation or well beyond this period in some species such as primates (Bowlby, 1969; Ziegler and Crockford, 2017). Primates are very slow to mature and require a heavy investment. In humans, the time to maturity involves an extended period of adolescence where support is still essential (Hrdy, 2009).

This review will discuss parenting in mammals with respect to the mother and mother parenting styles as these have a strong influence upon the offspring. Additionally, the review will extend to the different social structures influencing allocare, or mother's helpers in infant care, as in some species infant rearing requires help, and then on to paternal care behavior, or father's role in offspring development. Behavior is an essential component of any reproduction, especially in promoting successful outcomes of reproduction. One of the complexities of parental care occurs with the psychosocial aspect of offspring attachment and the severe issues that occur with negative parenting on successful growth towards adulthood and subsequent reproduction from the offspring.

Mother – Infant Bonds and Mothering Styles

Hormonal and neural-hormonal mechanisms have ensured that mothers are primed to form strong social bonds with their infants and thereby increase survival (Rilling and Young, 2014). Nurturing behavior from the mother is associated with attachment seeking behavior in the offspring, allowing for a successful mother-infant bond (Broad *et al.*, 2006). However, without the priming of the maternal brain with the hormones of pregnancy, most species of mammals do not show spontaneous maternal care (Keverne, 2005). Studies in rodents have shown that maternal behavior can be expressed in virgin females by repeated exposure to pups, thus sensitization can increase maternal motivation and behavior (Numan and Insel, 2003). Exposure to infants during the juvenile period is important for primates, as they learn about infants and parental care.

The maternal neuroendocrine system responds to the steroidal and peptide hormonal signals coming from the placenta to synchronize birth with maternal care behaviors and milk production for nurturing. Several of the hormones that have been shown to induce changes in the onset of maternal care are prolactin, oxytocin, glucocorticoids and estrogens. Changes in estrogen and its relationship with progesterone are important in mammalian onset of maternal care behaviors (González-Mariscal and Poindron, 2002). Estrogens stimulate the release of oxytocin and prolactin which are both involved in lactation and in mother – infant bonding, Fig. 1.

The maternal brain processes sensory signals from the offspring that stimulates neural activation initiating maternal care of infants. New mothers are attracted to their infant's odors (Fleming *et al.*, 1999). They also respond to their infant's vocalizations, thermal properties, and touch. The accumulation of these cues ensures mothers nurse and protect their offspring and provide them with the appropriate physical and stimulus environment in which to develop.

In primates, hormones may not be essential for primate maternal behavior but promote mother-infant bonding (Saltzman and Maestripieri, 2011). Oxytocin released from the brain coordinates the onset of maternal nurturing behavior at birth in rodents, sheep and primates (Ross and Young, 2009; Ziegler and Crockford, 2017). Since this peptide is conserved over species it is likely to serve the same function in all mammals as is the closely related arginine vasopressin (Ross and Young, 2009). Additionally, dopamine functions in recruiting cortical brain networks for supporting affiliation and is associated with increased dopamine responses supporting the formation of maternal – infant bonds, as dopamine activates the reward system of the brain (Feldman, 2017; Atzil *et al.*, 2017). Under normal environments, maternal care is very rewarding.

Mothering styles have been examined in a number of animal species but the rodent became the primary model for understanding the health and behavioral outcomes of offspring and epigenetics of parental effects (Kappeler and Meaney, 2010). In rodent mothers the different frequency of licking and grooming behaviors towards their pups in the first week of life plays an essential role in establishing behavioral phenotypes in their offspring (Francis *et al.*, 1999). High Frequency Licking and Grooming mothers stimulate and regulate their offspring's endocrine system in a positive manner; thereby influencing metabolism and somatic growth in the offspring (Champagne *et al.*, 2003). Pups from High Frequency Licking and Grooming mothers later show reduced fearfulness and less of a stress response on the output for glucocorticoids from the HPA axis (Hypothalamic-pituitary-adrenal). This is also found by handling a neonatal rat for 15 min daily (Meaney *et al.*, 1991). As adults, these rats

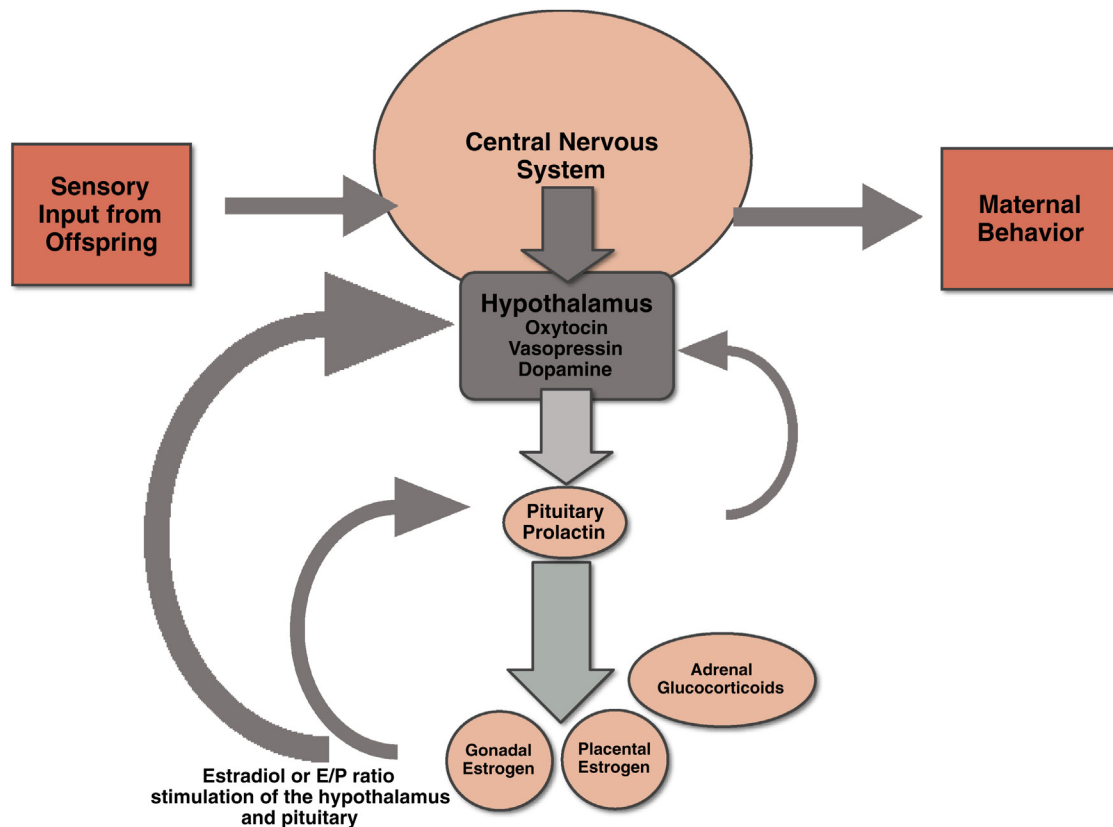


Fig. 1 Schematic of the relationships between the brain and the endocrine systems that regulate maternal behavior. Sensory input from the offspring on the mother acts simultaneous with the hormonally primed neural system to facilitate positive maternal behavior. Changes in the estradiol or the estradiol to progesterone (E/P ratio; decreasing progesterone) stimulate the onset of maternal behavior by activating oxytocin, prolactin, and dopamine. This diagram was adapted from Bridges, R.S., 2015. Neuroendocrine regulation of maternal behavior. *Front. Neuroendocrinol.* 36, 178–196 (10.1016/j.ynrne.2014.11.007).

have a tighter regulation of the secretion of glucocorticoids resulting in lower basal and post stress levels. These “stress hormones” bind to receptors in the hippocampus of the brain which plays a role in regulating the negative feedback regulation of the glucocorticoids release from the adrenal glands (Sapolsky, 2004). Early handling of pups results in adult rats that are more exploratory, less fearful, less reactive to stress and remain much more cognitively intact in their old age (Sapolsky, 2004). These maternal behaviors towards their offspring are translated by environmental effects, or epigenetic, and passed on to the adult pups such that pups from High Licking and Grooming mothers also become High Licking and Grooming mothers as they also pass this trait on to their offspring.

Maternal style also plays a large role in the adult personality of nonhuman primates. Long-term effects of early mothering style, in vervet monkeys (*Chlorocebus pygerythrus*) living in outdoor social groups, have been found on juvenile responsiveness to the external environment (Fairbanks and McGuire, 1988). These studies found that mothering style: either protective or rejecting of their offspring influenced the behavior of their offspring at two years of age. As juveniles, those who had more protective early mothering showed less interest in the external environment while those who had less protective mothers were more likely to explore their environment. The early environment appears to program some aspects of neurobiological development and, in turn, behavioral, emotional, cognitive and physiological development (Sánchez et al., 2001). Many maternal style studies have focused on the laboratory rhesus macaque (*Macaca mullatta*). Rhesus mothers exhibit marked individual differences in anxiety, and such differences are reflected in the differences of their maternal style (Saltzman and Maestripieri, 2011). This nonhuman primate species has been bred in captivity for many years with some of the earliest reports on the effects of social isolation on infants who have been removed from their mothers. Studies performed by Harlow and colleagues have provided actual data showing the need for mother-infant attachment (see Zhang, 2017). Primates need attachments with their mothers to acquire the necessary social skills and to develop normal psychologically and physically.

Cooperative Breeding – Allocare

Cooperative breeding systems occur when non-breeding individuals in the group provide care for the offspring of dominant group members (Lukas and Clutton-Brock, 2017). Very few animals incorporate this breeding system and it occurs only about 1% of the

time. It is associated with social monogamy and the production of multiple offspring per birth, such as twins and triplets. Those in a group that assist in caring for the young of the breeding female are called helpers. They assist in protecting and feeding the offspring. In many cooperative breeding species, few individuals disperse from their natal group or establish new breeding units so may remain in the natal group for extended time. Commonly, the helpers are prevented from breeding, either behaviorally or hormonally, by the dominant breeding female (Clutton-Brock, 2009). Genetic relatedness is high within cooperative breeders and therefore, the helpers are usually older offspring of the breeding couple.

Mammals that have a cooperative breeding form consists of 9 rodent genera (*Cryptomys*, *Heterocephalus*, *Microtus*, *Meriones*, *Rhabdomys*, *Castor*, *Atherurus*, *Peromyscus*), four carnivores (*Alopex*, *Canis*, *Lycaon*, and mongooses) and one in primates (Callitrichidae family, consisting of marmosets and tamarins). The rodents have different levels of cooperative breeding such that some species do communally nursing and mothers are taking care of more than their own offspring (König, 1993). Although house mice are known sometimes to live in monogamous pairs, more typically they occur in small polygynous breeding groups comprising a dominant male, a few females with their litters and occasional additional subordinate males (König, 1993). Canines demonstrate cooperative behavior within social groups ranging from hunting and food sharing to the provisioning of sick adults and dependent pups (Macdonald and Moehlman, 1983). The need for helpers is exhibited in the higher litter mass than with species that exhibit strictly maternal care (Moehlman and Hofer, 1997). Several species of canines have been reported to suppress reproduction in their helpers. The suppression occurs where the breeding female prevents by behavioral means or chemically via olfaction their female helpers in the group from reproducing. The reproductive female in the group needs the helpers to be there for her offspring. This has been demonstrated in captive gray wolves (*Canis lupus*), bush dogs (*Speothos venaticus*), red foxes (*Vulpes vulpes*) and arctic foxes (*Vulpes lagopus*; see Moehlman, and Hofer, 1997).

Among nonhuman primates, the marmosets and tamarins are the most notable for their cooperative care of infants, Fig. 2. However, species of owl (*Aotus sp.*) and titi monkeys (*Callicebus*) also show infant care by older offspring, and therefore, should be considered cooperative breeders (Wright, 1990). The cooperative system allows the species to offset the high costs of reproductive output by limiting maternal investment in each offspring and providing extra-maternal care for the young (Garber and Leigh, 1997). Both the common marmoset (*Callithrix jacchus*) and the cotton-top tamarin (*Saguinus oedipus*) have a postpartum ovulation that occurs as early as 10 and 13 days respectively, following birth (marmosets: Lunn and McNeilly, 1982; tamarins: Ziegler et al., 1990). Conception is high, occurring more than 80% of the time (Ziegler et al., 1987a,b). Therefore, mothers are lactating and pregnant at the same time. This high reproductive rate is energetically costly for mothers, requiring infant care support from the entire family. The ability to simultaneously lactate and conceive, as is seen for the callitrichid monkeys, is due to the lower frequency of suckling due to the lower frequency of nursing bouts allowed by mothers (Ziegler et al., 1990).

Cooperative breeders have delayed dispersal so that offspring of reproductive age forgo their own reproduction to remain in the family and care for younger siblings (Solomon and French, 1997). Cotton-top tamarin daughters are reproductively suppressed while living in such families with no signs of ovulation even though they have gone through puberty (Ziegler et al., 1987a,b). However, in the marmoset species there appears to be a flexibility of response to fertility control in which some families are associated with complete fertility suppression in daughters while in other families many eldest daughters display occasional ovarian cycles (for pygmy marmosets, *Cebuella pygmaea*, Carlson et al., 1997; for common marmosets, Abbott, 1984; Saltzman et al.,



Fig. 2 Family of common marmosets, *Callithrix jacchus*, at the Wisconsin National Primate Research Center. Older offspring remain with their families to learn parenting skills by taking care of younger siblings. Photo by Jordana Lennon, WNPRC.

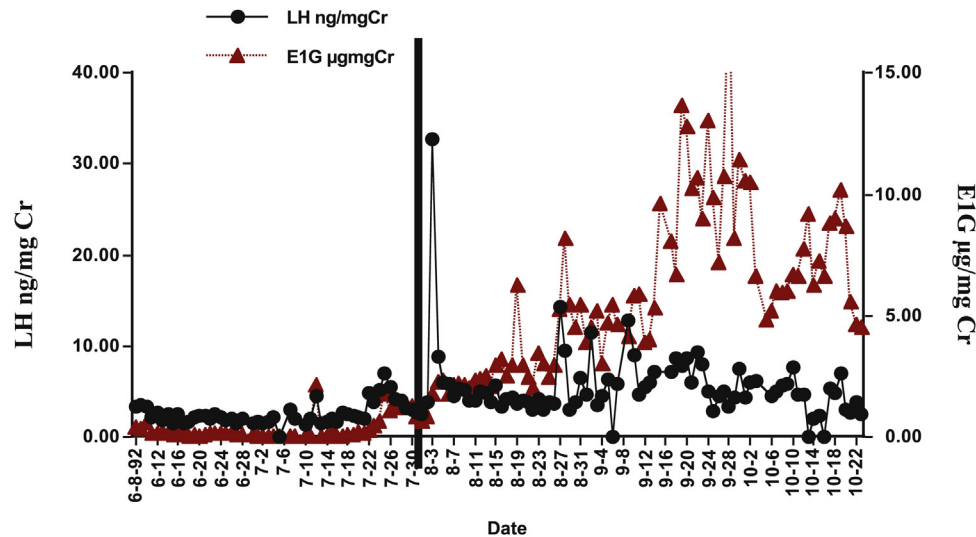


Fig. 3 Daily urinary hormonal values for a female cotton-top tamarin, *Saguinus oedipus*, demonstrating reproductive suppression while she lived with her family (left side of the graph). Both luteinizing hormone, LH, and estrone-glucuronide were low when living with the family. Once the female was removed and paired with a male (right side of the graph following the black line) she ovulated within 8 days and conceived, resulting in a pregnancy of twins.

1997; Ziegler and Sousa, 2002; for Wied's black tufted-ear, *Callithrix kuhli*, Smith *et al.*, 1997). This may be due to the age of the daughters where those well past puberty are less receptive to the suppressive signals from the mother (Ziegler and Sousa, 2002). While females show suppression of reproductive hormones, males do not. Cotton-top tamarin sons show pubertal hormonal changes while living in the family and even exhibit sexual behaviors but without an appropriate partner (Ginther *et al.*, 2001). A lack of suppression of reproductive hormones in subordinate males has been reported for other species: lion tamarins, *Leontopithecus rosalia* (French *et al.*, 1989) and black tufted-ear marmoset, (French and Schaffner, 1995) and the male offspring of the common marmoset may have a behavioral suppression for sexual behavior (Abbott, 1984) (Fig. 3).

Cooperative infant care for the cotton-top tamarin allows for a buffering of individual parenting styles. Female tamarins do not provide extensive care to their offspring. A female generally spends only around 20% of her time carrying infants in the first six weeks of their life and most of that time is spent nursing infants (Ziegler *et al.*, 1990). Fathers will provide for most of the carrying of infants but as the size of the family increases with more offspring, the amount of a father's carrying time decreases (Washabaugh *et al.*, 2002). Infants receive similar levels of care regardless of group size and parenting experience but the contribution from individual family members will vary with group size and parental experience.

Paternal Care

Parental care by fathers can be essential for the survival and normal development of offspring in biparental species (Saltzman *et al.*, 2017). Father care can involve such behaviors as warming, feeding, protecting, retrieving, and grooming infants. Paternal responsiveness may change across the course of the lifetime in individual males. Likewise, males of some rodent species undergo changes in their behavioral responses to offspring from nurturing to indifference and aggression (Saltzman *et al.*, 2017). In rodents, intra-uterine position during gestation can influence a males' behavioral response to his pups in adulthood (Clark *et al.*, 1998). Fathers who were between two females *in utero* make contact with their own pups significantly more than fathers who were between two males *in utero*. This may be due to different exposure to androgens and estrogens during the gestational period (Vom Saal *et al.*, 1983). Intrauterine position therefore, can modulate the steroids the male is exposed to in early development and shape his behavior towards his offspring.

The care a male receives during his postnatal development, can also have long-term effects on paternal behavior and contribute to stable differences among individuals within a species (Braun and Champagne, 2014). Males that are reared without a father present show less parental care behaviors than do males that are reared with a father present. This is exhibited in spending significantly less time licking and grooming their pups during the first days postpartum (Ahern *et al.*, 2011). Manipulating the levels of parental care received by young males can lead to long-term changes in the male's behavior toward their own offspring (Bester-Meredith and Marler, 2003). Cross-foster studies between parental species, such as the prairie vole and non-parental species such as the meadow vole indicate that male meadow voles raised by prairie voles receive higher levels of parental care and also provide parental care for their offspring (McGuire, 1988). Studies on males that are castrated or intact indicate that mice raised by a castrated father showed less parental care behaviors than mice that had been raised by intact fathers. Additionally, this was associated with changes in arginine vasopressin (AVP) signaling within the brain (Frazier *et al.*, 2006).

Paternal behaviors are associated with response to hormonal changes or behaviors of others can produce hormonal changes. In rodents and primates, there are many studies on the neural endocrine control of paternal behaviors (see [Saltzman and Ziegler, 2014](#)). The hormone prolactin has been associated in rodents and primates with parental care, rising during the mate's pregnancy and after infants are born. A causal relationship has not been established as it has been in birds. However, in the common marmoset it has been shown that prolactin performs as a metabolic hormone ensuring the male does not lose too much weight in his energetic output while caring for infants ([Ziegler et al., 2009](#)). This may be true for rodent fathers as well since in both humans and rodents prolactin is essential for energy homeostasis as it regulates adipogenesis in adipose tissues ([Carré and Binart, 2014](#)). Testosterone levels are typically reduced in fathers during parental care but this has not been a consistent find across all species of rodents and paternal care doesn't always decrease with castration of the father ([Saltzman et al., 2017](#)).

Neuropeptides are also involved in paternal behavior, especially arginine vasopressin, AVP, and oxytocin, OT. In rodents AVP is involved in the expression of paternal behavior and in aggression. It works within the brain to bind to areas such as the lateral septum and the amygdala ([Bales and Saltzman, 2016](#)). Injecting AVP directly into the brain promotes paternal behavior in two species of biparental rodents ([Wang et al., 1994](#); [Parker and Lee, 2001](#)). However, it is not essential for paternal care, indicating that other hormones are involved ([Lonstein and De Vries, 1999](#)). OT is known to facilitate maternal and allomaternal behavior in rodents ([Bridges, 2015](#); [Kenkel et al., 2013](#)). Blocking OT at its receptors does block parental behavior in adult male prairie voles. Additionally, exposure to androgens, estrogens and OT during the postnatal period is important in the expression of paternal behavior in adulthood ([Bales and Saltzman, 2016](#)). Vasopressinergic and possibly oxytocinergic signaling within the brain, is modulated by gonadal steroid hormones, and may represent mediating effects on development and experience that can vary between individuals in paternal care ([Saltzman et al., 2017](#)).

For primates, the mother-infant bond remains important in biparental and cooperative breeding monkeys, but with others to care for the infant, the bond may be strongest with the individual that provides the most care. In the bi-parental species of primates, such as in the titi monkey (*Callicebus moloch*) ([Mason and Mendoza, 1998](#)) and in the common marmoset ([Ziegler and Sosa, 2016](#)), paternal care may also influence infant survival. Extensive research in captivity has shown that a paternal-infant bond in common marmosets exists and the father's involvement plays an important part in the health and wellbeing of his offspring ([Ziegler et al., 2017](#)). Titi monkey males are the primary carrier of infants, with the exception that the mother primarily cares for them during lactation ([Mason, 1966](#); [Mendoza and Mason, 1986](#)). Interestingly, while titi monkey fathers care for infants, studies have shown they lack a selective bond towards their offspring; ([Mendoza and Mason, 1986](#)). Titi fathers show no behavioral or physiological separation distress in response to separation from their infants. In contrast, the infant shows increased vocalizations and plasma cortisol levels. This effect is seen even when the mother is present ([Hoffman et al., 1995](#); [Mendoza and Mason, 1986](#)). As with the titi monkey, father owl monkeys (*Aotus azarai*) contribute extensively to infant care and by the second week postpartum the infant is mainly transported by the father. Males also play with the infant and encourage weaning by food sharing ([Huck et al., 2014](#)). It is possible that the father-infant bond is stronger than the mother-infant bond in certain species.

During marmoset parenting and with other allocaregivers, prolactin is elevated during physical contact with infants ([Dixon and George, 1982](#); [Mota and Sousa, 2000](#)). In hypothalamic explants from the brain, experienced fathers' oxytocin and prolactin levels are significantly higher than levels found in non-fathers ([Woller et al., 2012](#)). Oxytocin stimulates the release of prolactin in the pituitary during reproductive events and there are oxytocin receptors on the lactotrophes that produce prolactin ([Grattan, 2015](#)). Increases in prolactin and oxytocin may promote parenting behavior by interacting with reward centers, and also other social interactions outside of parenting appear to be promoted through similar processes ([Snowdon and Ziegler, 2015](#)). In males, prolactin has important interactions with both estradiol and testosterone, particularly in the contexts of reproductive functioning and parental behavior ([Ziegler et al., 2009](#); [van Anders et al., 2012](#)). Prolactin and testosterone levels have a negative correlation in many animals, including birds, such that prolactin levels decrease and testosterone levels rise during the breeding season, and the reverse occurs during the birthing season ([Logan and Wingfield, 1995](#); [Gubernick and Nelson, 1989](#)). Prolactin's inverse relationship with testosterone levels during parenting has been demonstrated in captive marmosets and humans ([Ziegler et al., 2009](#); [van Anders et al., 2012](#)). When bi-parental males show maternal-like behaviors to their offspring, this coincides with decreased testosterone levels, such as in common marmosets, cotton-top tamarins *Saguinus oedipus*, and humans ([Saltzman and Ziegler, 2014](#)). It is suggested that changes in testosterone levels regulate the secretion of prolactin through a feedback mechanism that is conserved from rats to humans ([Gill-Sharma, 2009](#)). Estradiol enhances prolactin release from the pituitary and increased prolactin lowers testosterone. Dopamine is the main inhibitor of prolactin and is produced in the arcuate nucleus of the hypothalamus ([Freeman et al., 2000](#)). Estradiol has a direct effect on decreasing hypothalamic dopamine and thereby stimulates the release of prolactin, as was demonstrated in a study of marmoset hypothalamic explants from experienced fathers ([Woller et al., 2012](#)).

Since prolactin is involved in the regulation of weight in bi-parental male marmosets and tamarins, this hormone could be the link between affiliation and improved metabolism. Elevated prolactin postpartum, when males are actively caring for infants, may work to prevent excessive weight loss in males during their added energetic demands ([Ziegler et al., 2009](#)). Metabolic regulation of tissue such as the liver, pancreas, adipose tissue and brain all contain prolactin receptors and are regulated by prolactin ([Luque et al., 2016](#)). In pregnant females and during lactation, prolactin promotes fat deposition or mobilization to ensure optimal nutrition for the offspring ([Grattan, 2015](#)). Father marmosets have increased prolactin during body contact with their offspring. This may promote improved regulation of metabolic function during the energetically demanding period of carrying large and heavy infants. Further data is needed on prolactin's role in contact affiliation under different social conditions and between species that express different social patterns and relationships.

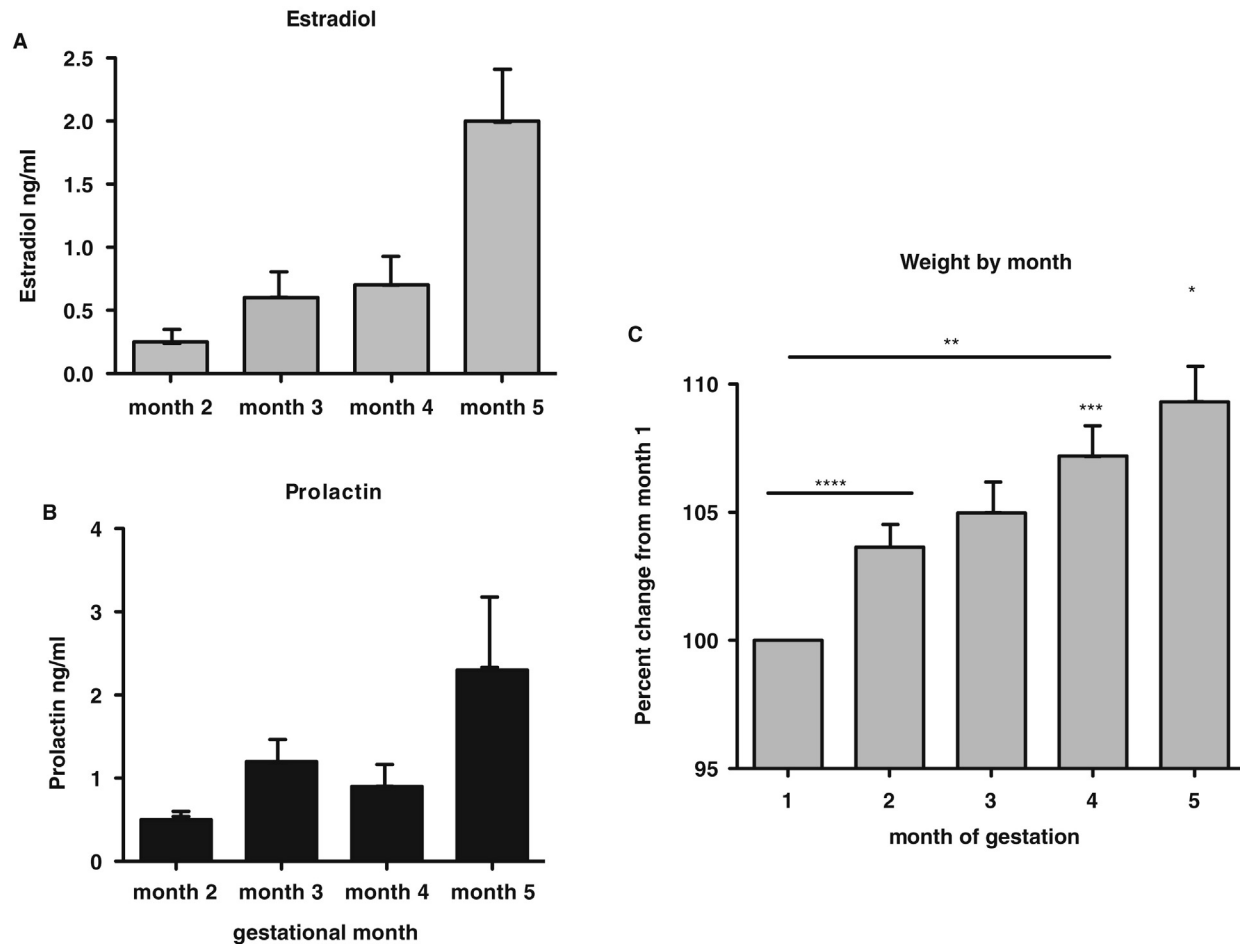


Fig. 4 Hormonal changes in (A) estradiol, (B) prolactin and (C) weight gain in male marmosets during their mates' pregnancy. Month 1 is the first month of gestation and 5 is the final month. These males were experienced fathers and all showed increased estradiol, prolactin and weight by the end of the gestation. Weight data has been published in Ziegler *et al.*, 2006. T.E. Ziegler, T.E., Prudom, S.L., Schultz-Darken, N.J., Kurian, A.V., Snowden, C.T., 2006. Pregnancy weight gain: Marmoset and tamarin dads show it too. *Biol. Lett.* 2, 181–183 (10.1098/rsbl.2005.0426).

Common marmosets have extensive physiological responses to the birth of their infants. Expectant fathers display hormonal changes and weight gain in preparation for their offspring's birth during their mate's gestation, [Fig. 4](#) ([Ziegler *et al.*, 2006](#)). Once the infants are born a different pattern of hormonal and weight changes affect the fathers.

Hormonal changes occur in callithricid fathers to help maintain the father's interest in the offspring and to motivate him to parent. The facilitation of parental care comes not only from the hormonal changes that occur in the expectant mother but also with the expectant father. Infants cause physiological changes in their fathers resulting in positive infant care and father – infant bonding ([Prudom *et al.*, 2008](#); [Ziegler *et al.*, 2011](#)). Fathers with dependent infants but isolated from their families, can respond hormonally to their infant's isolated scent secretion ([Prudom *et al.*, 2008](#)). Fathers tested with the scent of their infants show a significant decrease in testosterone levels while cortisol levels do not change ([Fig. 5](#), ([Prudom *et al.*, 2008](#))). When the fathers are compared with the response of naïve males, those who have never been fathers, it documents that this phenomenon is unique to fathers. Naïve males living with a non-pregnant female do not show a response to the infant scent for either testosterone or cortisol.

Further studies indicate that it is not just the reduction of testosterone that occurs to fathers following scent stimuli from their infants, but that simultaneously estrogens elevate ([Fig. 5](#) ([Ziegler *et al.*, 2011](#))). It is likely that a change in the aromatase enzyme that converts testosterone into estradiol is altering to reduce the testosterone in males. Less testosterone ensures a more tolerant parent plus increased estrogens work to increase maternal like behaviors. The influence offspring have on their father's hormones and consequently on their behavior does diminish after they are no longer dependent infants as the same infant scents collected at 3–4 months of age do not produce this effect on their fathers ([Ziegler *et al.*, 2011](#)). The reduced effect is likely due to the rapid reproduction that occurs in the common marmoset. By the time that infants are independent of constant carrying at 3 to 4 months of age, their mother is pregnant again and both mother and father are having hormonal changes preparing for the next set of infants.

Glucocorticoids in male primates appear to function in two main ways related to reproduction: they may facilitate paternal behavior in new fathers ([Nunes *et al.*, 2001](#)) and may promote communication of hormonal states within mated pairs

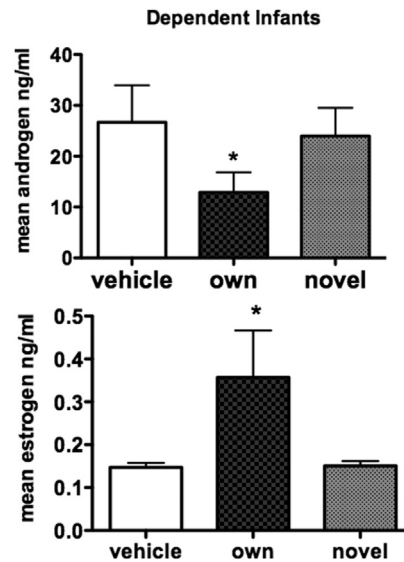


Fig. 5 Mean androgen and estrogen levels in male marmosets 30 min following smelling an isolated scent from their own approximately 2 week old infant or a novel infant of the same age. These are experienced fathers who show significant decreases in androgen and increases in estrogens in response to infant scent. Data published in Ziegler, T.E., Peterson, L.J., Sosa, M.E., Barnard, A.M., 2011. Differential endocrine responses to infant odors in common marmoset (*Callithrix jacchus*) fathers. *Horm. Behav.* 59, 265–270.

(Ziegler and Snowdon, 2000; Ziegler *et al.*, 2004). Nunes *et al.* (2001) found that male marmosets that frequently carried infants had lower cortisol levels than low carrying males. This result suggests that carrying infants is not extremely stressful but suggests that other stressors may impact both glucocorticoid levels and carrying frequency. Fathers raising their first litters, had higher cortisol levels than fathers raising their second consecutive litters, even without differences in carrying behavior, suggesting that glucocorticoids may help new fathers focus on the appropriate behavioral requirements of the new role (Storey and Ziegler, 2016).

Most reports on oxytocin in non-human primates have focused on pair bonding behaviors rather than on paternal care. However, in one study, intranasal oxytocin increased the tolerance of marmoset fathers when transferring food to weanling infants (Saito and Nakamura, 2011). Most of the work with oxytocin and vasopressin on non-human primates concerned how these hormones affect receptors in particular brain regions (e.g., Kozorovitskiy *et al.*, 2006). First-time and experienced marmoset fathers have a higher density of dendritic spines on pyramidal vasopressin neurons in prefrontal cortex compared to nonfathers (Kozorovitskiy *et al.*, 2006). These results provide evidence for structural reorganization in the prefrontal cortex and a parallel enhancement of vasopressin V1a receptors that indicate plasticity in the brain with parenting experience (Storey and Ziegler, 2016).

Summary

The behaviors of parenting can have a profound effect on the lives of their offspring. Offspring rely on their mothers for survival and for parenting styles they will pass on to their offspring. In the cooperative breeding and biparental care mammals, the care is shared and in biparental species both the mother and father can pass on their parenting styles to their offspring. There are individual differences in both mothers and fathers in parenting and these can influence the health and wellbeing of their offspring.

In both mothers and fathers, and even with alloparental care, hormones are driving parental care as well as responding to sensory signals from others such as the mate and the offspring. The brain and the endocrine act synergistically to create the homeostasis needed in all reproductive events and have been linked evolutionarily to behaviors to influence the next generation. With poor parenting, as in rodents who show lower levels of licking and grooming pups a pattern of poor parental behaviors are passed on can lead to other deficits such as those seen in the primates that can lead to lower social skills.

References

- Abbott, D. H. (1984). Behavioral and physiological suppression of fertility in subordinate marmoset monkeys. *Am. J. Primatol.*, 6, 169–186.
- Ahern, T. H., Hammock, E. A., & Young, L. J. (2011). Parental division of labor, coordination, and the effects of family structure on parenting in monogamous prairie voles (*Microtus ochrogaster*). *Dev. Psychobiol.*, 53, 118–131.
- Atzil, S., Touroutoglou, A., Rudy, T., et al. (2017). Dopamine in the medial amygdala network mediates human bonding. *Proc. Natl. Acad. Sci.*, 114, 2361–2366.
- Bales, K. L., & Saltzman, W. (2016). Fathering in rodents: Neurobiological substrates and consequences for offspring. *Horm. Behav.*, 77, 249–259. <https://doi.org/10.1016/j.yhbeh.2015.05.021>.

- Bester-Meredith, J. K., & Marler, C. A. (2003). Vasopressin and the transmission of paternal behavior across generations in mated, cross-fostered *Peromyscus* mice. *Behav. Neurosci.*, 117, 455.
- Bowlby, J., 1969. Attachment and Loss, Attachment. Basic Books.
- Braun, K., & Champagne, F. A. (2014). Paternal influences on offspring development: Behavioural and epigenetic pathways. *J. Neuroendocrinol.*, 26, 697–706.
- Bridges, R. S. (2015). Neuroendocrine regulation of maternal behavior. *Front. Neuroendocrinol.*, 36, 178–196. <https://doi.org/10.1016/j.ynme.2014.11.007>.
- Broad, K. D., Curley, J. P., & Keverne, E. B. (2006). Mother-infant bonding and the evolution of mammalian social relationships. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 361, 2199–2214. <https://doi.org/10.1098/rstb.2006.1940>.
- Carlson, A. A., Ziegler, T. E., & Snowdon, C. T. (1997). Ovarian function of pygmy marmoset daughters (*Cebuella pygmaea*) in intact and motherless families. *Am. J. Primatol.*, 43, 347–355. [https://doi.org/10.1002/\(SICI\)1098-2345\(1997\)43:4<347::AID-AJP6>3.0.CO;2-X](https://doi.org/10.1002/(SICI)1098-2345(1997)43:4<347::AID-AJP6>3.0.CO;2-X).
- Carré, N., & Binart, N. (2014). Prolactin and adipose tissue. *Biochimie*, 97, 16–21. <https://doi.org/10.1016/j.biochi.2013.09.023>.
- Champagne, F. A., Weaver, I. C. G., Diorio, J., Sharma, S., & Meaney, M. J. (2003). Natural variations in maternal care are associated with estrogen receptor alpha expression and estrogen sensitivity in the medial preoptic area. *Endocrinology*, 144, 4720–4724. <https://doi.org/10.1210/en.2003-0564>.
- Clark, M. M., Vonk, J. M., & Galef, B. G. (1998). Intrauterine position, parenting, and nest-site attachment in male Mongolian gerbils. *Dev. Psychobiol.*, 32, 177–181.
- Clutton-Brock, T. (2009). Structure and function in mammalian societies. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, 364, 3229–3242. <https://doi.org/10.1098/rstb.2009.0120>.
- Dixon, A. F., & George, L. (1982). Prolactin and parental behaviour in a male New World primate. *Nature*, 299, 551–553.
- Fairbanks, L. A., & McGuire, M. T. (1988). Long-term effects of early mothering behavior on responsiveness to the environment in vervet monkeys. *Dev. Psychobiol.*, 21, 711–724. <https://doi.org/10.1002/dev.420210708>.
- Feldman, R. (2017). The neurobiology of human attachments. *Trends Cogn. Sci.*, 21, 80–99.
- Fleming, A. S., O'Day, D. H., & Kraemer, G. W. (1999). Neurobiology of mother–infant interactions: Experience and central nervous system plasticity across development and generations. *Neurosci. Biobehav. Rev.*, 23, 673–685.
- Francis, D., Diorio, J., Liu, D., & Meaney, M. J. (1999). Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science*, 286, 1155–1158.
- Frazier, C. R., Trainor, B. C., Cravens, C. J., Whitney, T. K., & Marler, C. A. (2006). Paternal behavior influences development of aggression and vasopressin expression in male California mouse offspring. *Horm. Behav.*, 50, 699–707.
- Freeman, M. E., Kanyicska, B., Lerant, A., & Nagy, G. (2000). Prolactin: Structure, function, and regulation of secretion. *Physiol. Rev.*, 80, 1523–1631.
- French, J. A., Inglett, B. J., & Dethlefs, T. M. (1989). The reproductive status of nonbreeding group members in captive golden lion tamarin social groups. *Am. J. Primatol.*, 18, 73–86.
- French, J. A., & Schaffner, C. M. (1995). Social and developmental influences on urinary testosterone levels in male black tufted-ear marmosets (*Callithrix kuhli*). *Am. J. Primatol.*, 36, 123.
- Garber, P. A., & Leigh, S. R. (1997). Ontogenetic variation in small-bodied New World primates: Implications for patterns of reproduction and infant care. *Folia Primatol.*, 68, (Basel), 1–22.
- Gill-Sharma, M. K. (2009). Prolactin and male fertility: The long and short feedback regulation. *Int. J. Endocrinol.*, 2009.
- Ginther, A. J., Ziegler, T. E., & Snowdon, C. T. (2001). Reproductive biology of captive male cottontop tamarin monkeys as a function of social environment. *Anim. Behav.*, 61, 65–78. <https://doi.org/10.1006/anbe.2000.1587>.
- González-Mariscal, G., Poindron, P., 2002. Hormones, Brain and Behavior.
- Grattan, D. R. (2015). 60 years of neuroendocrinology: The hypothalamo-prolactin axis. *J. Endocrinol.*, 226, T101–T122.
- Gubernick, D. J., & Nelson, R. J. (1989). Prolactin and paternal behavior in the biparental California mouse, *Peromyscus californicus*. *Horm. Behav.*, 23, 203–210.
- Hoffman, K. A., Mendoza, S. P., Hennessy, M. B., & Mason, W. A. (1995). Responses of infant titi monkeys, *Callicebus moloch*, to removal of one or both parents: Evidence for paternal attachment. *Dev. Psychobiol.*, 28, 399–407.
- Hrdy, S. (2009). *Mothers and Others: The Evolutionary Origins of Mutual Understanding*. The Belknap Press of Harvard University Press.
- Huck, M., Fernandez-Duque, E., Babb, P., & Schurr, T. (2014). Correlates of genetic monogamy in socially monogamous mammals: Insights from Azara's owl monkeys. *Proc. R. Soc. Lond. B Biol. Sci.*, 281, 20140195.
- Kappeler, L., & Meaney, M. J. (2010). Epigenetics and parental effects. *BioEssays News Rev. Mol. Cell. Dev. Biol.*, 32, 818–827. <https://doi.org/10.1002/bies.201000015>.
- Kenkel, W. M., Paredes, J., Lewis, G. F., et al. (2013). Autonomic substrates of the response to pups in male prairie voles. *PLoS One*, 8, e69965.
- Keverne, E.B., 2005. Trophoblast regulation of maternal endocrine function and behaviour. In: Moffat, A. (Ed.), *Biology and Pathology of Trophoblast*. Cambridge, UK: Cambridge University Press, pp. 368–411.
- Kleiman, D.G., Malcolm, J., 1981. The evolution of male parental investment in mammals. In: Gubernick, D.J., Klopfer, P.H. (Eds.), *Parental Behavior in Mammals*. New York, NY: Plenum Press, pp. 347–387.
- König, B. (1993). Maternal investment of communally nursing female house mice (*Mus musculus domesticus*). *Behav. Processes*, 30, 61–73. [https://doi.org/10.1016/0376-6357\(93\)90012-G](https://doi.org/10.1016/0376-6357(93)90012-G).
- Kozorovitskiy, Y., Hughes, M., Lee, K., & Gould, E. (2006). Fatherhood affects dendritic spines and vasopressin V1a receptors in the primate prefrontal cortex. *Nat. Neurosci.*, 9, 1094–1095.
- Logan, C. A., & Wingfield, J. C. (1995). Hormonal correlates of breeding status, nest construction, and parental care in multiple-brooded northern mockingbirds, *Mimus polyglottos*. *Horm. Behav.*, 29, 12–30.
- Lonstein, J. S., & De Vries, G. J. (1999). Sex differences in the parental behaviour of adult virgin prairie voles: Independence from gonadal hormones and vasopressin. *J. Neuroendocrinol.*, 11, 441–450.
- Lukas, D., & Clutton-Brock, T. (2017). Climate and the distribution of cooperative breeding in mammals. *R. Soc. Open Sci.*, 4, 160897. <https://doi.org/10.1098/rsos.160897>.
- Lunn, S. F., & McNeilly, A. S. (1982). Failure of lactation to have a consistent effect on interbirth interval in the common marmoset, *Callithrix jacchus jacchus*. In *Folia Primatol. (Basel)*, 37 pp. 99–105.
- Luque, R. M., Cordoba-Chacon, J., Pozo-Salas, A. I., et al. (2016). Obesity and gender-dependent role of endogenous somatostatin and cortistatin in the regulation of endocrine and metabolic homeostasis in mice. *Sci. Rep.*, 6.
- Macdonald, D. W., & Moehlman, P. D. (1983). Cooperation, altruism, and restraint in the reproduction of carnivores. *Perspectives in Ethology* (pp. 433–467). New York: Plenum Press.
- Mason, W. A. (1966). Social organization of the South American monkey, *Callicebus moloch*: A preliminary report. *Tulane Stud. Zool.*, 13.
- Mason, W. A., & Mendoza, S. P. (1998). Generic aspects of primate attachments: parents, offspring and mates. *Psychoneuroendocrinology*, 23, 765–778.
- McGuire, B. (1988). Effects of cross-fostering on parental behavior of meadow voles (*Microtus pennsylvanicus*). *J. Mammal.*, 69, 332–341.
- Meaney, M. J., Mitchell, J. B., Aitken, D. H., et al. (1991). The effects of neonatal handling on the development of the adrenocortical response to stress: Implications for neuropathology and cognitive deficits in later life. *Psychoneuroendocrinology*, 16, 85–103.
- Mendoza, S. P., & Mason, W. A. (1986). Parental division of labour and differentiation of attachments in a monogamous primate (*Callicebus moloch*). *Anim. Behav.*, 34, 1336–1347.
- Moehlman, P.D., Hofer, H., 1997. Cooperative breeding, reproductive suppression and body size in canids.
- Mota, M. T., & Sousa, M. B. C. (2000). Prolactin levels of fathers and helpers related to alloparental care in common marmosets, *Callithrix jacchus*. In *Folia Primatol. (basel)*, 71 pp. 22–26.
- Numan, M., & Insel, T. R. (2003). Motivational models of the onset and maintenance of maternal behavior and maternal aggression. *Neurobiol. Parent. Behav.*, 69–106.

- Nunes, S., Fite, J. E., Patera, K. J., & French, J. A. (2001). Interactions among paternal behavior, steroid hormones, and parental experience in male marmosets (*Callithrix kuhlii*). *Horm. Behav.*, 39, 70–82.
- Parker, K. J., & Lee, T. M. (2001). Central vasopressin administration regulates the onset of facultative paternal behavior in *Microtus pennsylvanicus* (meadow voles). *Horm. Behav.*, 39, 285–294.
- Prudom, S. L., Broz, C. A., & Schultz-Darken, N. J. (2008). Exposure to infant scent lowers serum testosterone in father common marmosets (*Callithrix jacchus*). *Biol. Lett.*, 4, 603–605.
- Rilling, J. K., & Young, L. J. (2014). The biology of mammalian parenting and its effect on offspring social development. *Science*, 345, 771–776. <https://doi.org/10.1126/science.1252723>.
- Ross, H. E., & Young, L. J. (2009). Oxytocin and the neural mechanisms regulating social cognition and affiliative behavior. *Front. Neuroendocrinol.*, 30, 534–547.
- Saito, A., & Nakamura, K. (2011). Oxytocin changes primate paternal tolerance to offspring in food transfer. *J. Comp. Physiol. A*, 197, 329–337.
- Saltzman, W., Harris, B. N., De Jong, T. R., et al. (2017). Paternal Care in Biparental Rodents: Intra- and Inter-individual Variation. *Integr. Comp. Biol.*, 57, 589–602. <https://doi.org/10.1093/icb/ix047>.
- Saltzman, W., & Maestripietri, D. (2011). The neuroendocrinology of primate maternal behavior. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 35, 1192–1204. <https://doi.org/10.1016/j.pnpbp.2010.09.017>.
- Saltzman, W., Schultz-Darken, N. J., Severin, J. M., & Abbott, D. H. (1997). Escape from social suppression of sexual behavior and of ovulation in female common marmosets. *Ann. N. Y. Acad. Sci.*, 807, 567–570.
- Saltzman, W., & Ziegler, T. E. (2014). Functional significance of hormonal changes in mammalian fathers. *J. Neuroendocrinol.*, 26, 685–696. <https://doi.org/10.1111/jne.12176>.
- Sánchez, M. M., Ladd, C. O., & Plotsky, P. M. (2001). Early adverse experience as a developmental risk factor for later psychopathology: Evidence from rodent and primate models. *Dev. Psychopathol.*, 13, 419–449.
- Sapolsky, R. M. (2004). Mothering style and methylation. *Nat. Neurosci.*, 7, 791–792. <https://doi.org/10.1038/nn0804-791>.
- Smith, T. E., Schaffner, C. M., & French, J. A. (1997). Social and developmental influences on reproductive function in female Wied's black tufted-ear marmosets (*Callithrix kuhlii*). *Horm. Behav.*, 31, 159–168.
- Snowdon, C. T., & Ziegler, T. E. (2015). Variation in prolactin is related to variation in sexual behavior and contact affiliation. *PLoS One*, 10, e0120650.
- Solomon, N. G., & French, J. A. (1997). The study of mammalian cooperative breeding. *Coop. Breed. Mamm.*, 1–10.
- Storey, A. E., & Ziegler, T. E. (2016). Primate paternal care: interactions between biology and social experience. *Horm. Behav.*, 77, 260–271. <https://doi.org/10.1016/j.yhbeh.2015.07.024>.
- van Anders, S. M., Tolman, R. M., & Volling, B. L. (2012). Baby cries and nurturance affect testosterone in men. *Horm. Behav.*, 61, 31–36.
- Vom Saal, F. S., Grant, W. M., McMullen, C. W., & Laves, K. S. (1983). High fetal estrogen concentrations: correlation with increased adult sexual activity and decreased aggression in male mice. *Science*, 220, 1306–1309.
- Wang, Z., Ferris, C. F., & De Vries, G. J. (1994). Role of septal vasopressin innervation in paternal behavior in prairie voles (*Microtus ochrogaster*). *Proc. Natl. Acad. Sci. USA*, 91, 400–404.
- Washabaugh, K. F., Snowdon, C. T., & Ziegler, T. E. (2002). Variations in care for cottontop tamarin, *Saguinus oedipus*, infants as a function of parental experience and group size. *Anim. Behav.*, 63, 1163–1174.
- Woller, M. J., Sosa, M. E., Chiang, Y., et al. (2012). Differential hypothalamic secretion of neurocrines in male common marmosets: Parental experience effects? *J. Neuroendocrinol.*, 24, 413–421.
- Woodroffe, R., & Vincent, A. (1994). Mother's little helpers: Patterns of male care in mammals. *Trends Ecol. Evol.*, 9, 294–297. [https://doi.org/10.1016/0169-5347\(94\)90033-7](https://doi.org/10.1016/0169-5347(94)90033-7).
- Wright, P. C. (1990). Patterns of paternal care in primates. *Int. J. Primatol.*, 11, 89–102.
- Zhang, B. (2017). Consequences of early adverse rearing experience(EARE) on development: Insights from non-human primate studies. *Zool. Res.*, 38, 7–35. <https://doi.org/10.13918/j.issn.2095-8137.2017.002>.
- Ziegler, T. E., Bridson, W. E., Snowdon, C. T., & Eman, S. (1987a). Urinary gonadotropin and estrogen excretion during the postpartum estrus, conception, and pregnancy in the cotton-top tamarin (*Saguinus oedipus oedipus*). *Am. J. Primatol.*, 12, 127–140.
- Ziegler, T. E., & Crockford, C. (2017). Neuroendocrine control in social relationships in non-human primates: Field based evidence. *Horm. Behav.*, 91, 107–121. <https://doi.org/10.1016/j.yhbeh.2017.03.004>.
- Ziegler, T. E., Peterson, L. J., Sosa, M. E., & Barnard, A. M. (2011). Differential endocrine responses to infant odors in common marmoset (*Callithrix jacchus*) fathers. *Horm. Behav.*, 59, 265–270.
- Ziegler, T. E., Prudom, S. L., Schultz-Darken, N. J., Kurian, A. V., & Snowdon, C. T. (2006). Pregnancy weight gain: Marmoset and tamarin dads show it too. *Biol. Lett.*, 2, 181–183. <https://doi.org/10.1098/rsbl.2005.0426>.
- Ziegler, T. E., Prudom, S. L., Zahed, S. R., Parlow, A. F., & Wegner, F. (2009). Prolactin's mediative role in male parenting in parentally experienced marmosets (*Callithrix jacchus*). *Horm. Behav.*, 56, 436–443. <https://doi.org/10.1016/j.yhbeh.2009.07.012>.
- Ziegler, T. E., & Snowdon, C. T. (2000). Preparental hormone levels and parenting experience in male cotton-top tamarins, *Saguinus oedipus*. *Horm. Behav.*, 38, 159–167.
- Ziegler, T. E., & Sosa, M. E. (2016). Hormonal stimulation and paternal experience influence responsiveness to infant distress vocalizations by adult male common marmosets, *Callithrix jacchus*. *Horm. Behav.*, 78, 13–19.
- Ziegler, T. E., Sosa, M. E., & Colman, R. J. (2017). Fathering style influences health outcome in common marmoset (*Callithrix jacchus*) offspring. *PLoS One*, 12, e0185695. <https://doi.org/10.1371/journal.pone.0185695>.
- Ziegler, T. E., & Sousa, M. B. C. (2002). Parent–daughter relationships and social controls on fertility in female common marmosets, *Callithrix jacchus*. *Horm. Behav.*, 42, 356–367.
- Ziegler, T. E., Washabaugh, K. F., & Snowdon, C. T. (2004). Responsiveness of expectant male cotton-top tamarins, *Saguinus oedipus*, to mate's pregnancy. *Horm. Behav.*, 45, 84–92.
- Ziegler, T. E., Widowski, T. M., Larson, M. L., & Snowdon, C. T. (1990). Nursing does affect the duration of the post-partum to ovulation interval in cotton-top tamarins (*Saguinus oedipus*). *J. Reprod. Fertil.*, 90, 563–570.
- Ziegler, T. E., Savage, A., Scheffler, G., & Snowdon, C. T. (1987b). The endocrinology of puberty and reproductive functioning in female cotton-top tamarins (*Saguinus oedipus*) under varying social conditions. *Biol. Reprod.*, 37, 618–627.

DEVELOPMENT AND ANATOMY OF REPRODUCTIVE ORGANS

Comparative Sex Organs: Plants

Bharti Sharma and Elena M Kramer, California State Polytechnic University Pomona, Pomona, CA, United States; and Harvard University, Cambridge, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Terminology

Archegonium The female gametangium, a haploid structure that produces female gametes or eggs.

Antheridium The male gametangium, a haploid structure that produces numerous male gametes or sperm.

Embryophytes The lineage of green plants that retain a diploid embryo on the parent's body until it develops into a sporophyte.

Gamete A haploid cell that undergoes fusion to produce a zygote in the context of sexual reproduction.

Gametophyte The haploid multicellular stage of the land plant lifecycle.

Heterospory When the spores are not equivalent, with some spores giving rise to male gametophytes and others, to female gametophytes.

Homospory When the spores are all equivalent in potential and give rise to gametophytes that are hermaphroditic or could be of either gender.

Megaspore A spore that produces a female gametophyte.

Microspore A spore that produces a male gametophyte.

Spore An asexual haploid cell capable of developing into an adult without fusion with another cell.

Sporophyte The diploid multicellular stage of the land plant lifecycle.

Strobili A reproductive axis with specialized leaves bearing sporangia.

Introduction

The lineage “Plantae” includes glaucophyte algae, red algae, green algae and land plants. Representatives of the green algal lineage moved onto land around 470 million years ago and became the dominant inhabitants of the ecosystem (Delwiche and Cooper, 2015). The early land plants evolved a diverse set of characters ensuring successful reproduction. Key among these traits is the basis for their taxonomic designation as the Embryophytes, reflecting the evolution of a diploid multicellular stage of their lifecycle (Grotewold *et al.*, 2015). Land plants are characterized in three major groups: Non-vascular, vascular seedless, and vascular seed plants. This article focuses primarily on sexual reproduction in flowering plants, the largest clade of vascular seed plants which are also called the Angiosperms, but we will also highlight the major reproductive events in bryophytes, ferns and gymnosperms.

As already noted, a shared feature of all land plants is the presence of a multicellular diploid stage, the sporophyte, which is produced in alternation with a haploid multicellular stage, the gametophyte. These two stages comprise the “**alternation of generations**” life cycle (Haig and Wilczek, 2006). Critically, the length of gametophytic and sporophytic phases differs among land plant lineages. In non-vascular plants, the gametophytic phase is dominant while the sporophyte is transient, however, in vascular plants, the sporophyte is dominant and the gametophyte is short-lived. Regardless, the fundamental life cycle is the same: 1. The diploid sporophyte undergoes meiosis to produce unicellular haploid spores. 2. The spores divide mitotically to produce the haploid multicellular gametophyte. 3. The gametophyte use mitosis to produce haploid gametes, either sperm or retained eggs. 4. Male sperm travel to the egg-bearing structure and achieve fertilization. 5. The fusion of the haploid male and female gametes results in a diploid zygote. 5. The zygote undergoes mitosis and matures into the embryo, which gives rise to the multicellular diploid sporophyte (Fig. 1).

Plant reproduction is markedly different from animal reproduction. First, unlike animals, there is no sequestration of the germline during embryogenesis. Instead, the cells that will mature into gametes are recruited from the soma of the gametophyte. In non-seed plants with free-living gametophytes, the specialized gamete-producing structures are called antheridia (producing sperm) and archegonia (producing eggs). With the evolution of the seed, the antheridia are lost as the male gametophyte becomes severely reduced while the archegonium is present in gymnosperms but finally lost in angiosperms. Second, these gametes, being produced in haploid multicellular organisms, are the products of mitosis, not meiosis, which is instead used by the sporophyte to produce haploid spores in organs called sporangia.

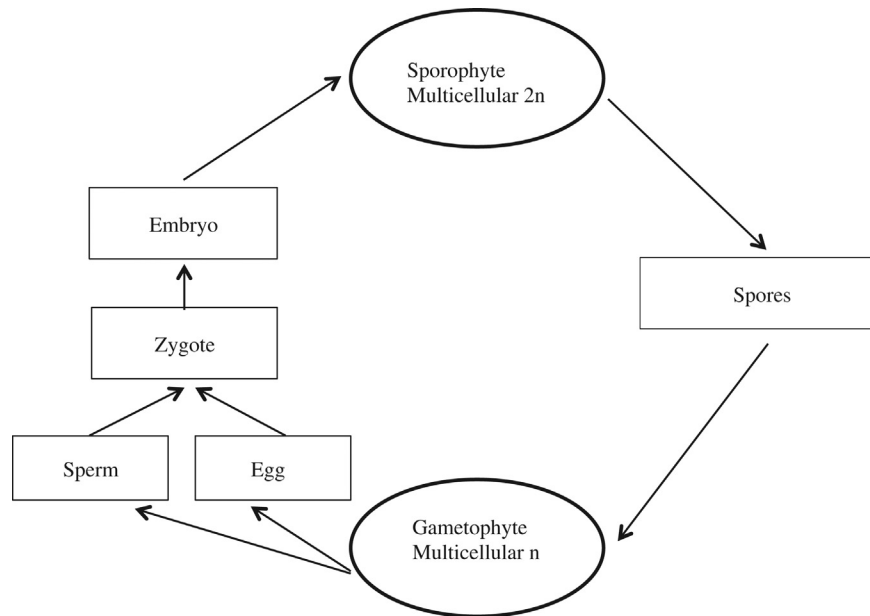


Fig. 1 A generalized depiction of alternation of generations in land plants.

Reproduction in Early Land Plants

The earliest diverging lineages of land plants, known collectively as the bryophytes or non-vascular plants, have three major lineages: The liverworts, mosses and hornworts (Shaw *et al.*, 2011). Due to their lack of vasculature and dependence on swimming sperm, these organisms require habitats with ample water. The lifecycle is gametophyte dominant in the bryophytes; the gametophyte is photosynthetic and persistent, providing nutrition to the sporophyte throughout its life. The gametophyte can have a leafy or thalloid structure and typically grows low on the substrate, which can be soil, rocks, or the surface of other plants. The non-vascular plants are homosporous, meaning that they have only one type of sporangium that makes spores of equivalent size. In many taxa, the gametophyte is bisexual and is capable of producing both egg and sperm. However, the liverworts actually have separate male and female gametophytes that are determined by the segregation of sex chromosomes at the time of meiosis in the sporangia. The male gametes are swimming sperm, which must travel to the egg via splashing rain and/or a continuous film of water. When the sperm reaches the egg, the two haploid cells fuse and undergo fertilization resulting in a diploid sporophyte embryo, which is enclosed in the archegonium. In bryophytes, the sporophyte is a relatively short-lived structure. The archegonium initially grows along with the expanding sporophyte but eventually splits into two parts, the upper calyptra, which protects the developing sporangium, and lower vaginula, which surrounds the foot of the sporophyte. The sporophyte itself is composed of three main regions: The foot, seta and capsule or sporangium. The foot is embedded in the gametophyte, arising from the original site of the egg, and mediates nutrient transfer between the sporophyte and parent gametophyte. Mitotic divisions in the stalk-like seta allow it to elongate, raising the distal sporangium into the air column. The sporangium or capsule is where meiosis occurs to produce haploid spores. The capsule eventually breaks open and the spores are released to be dispersed by the wind. The spores will germinate into a filamentous growth, or protonema, which differentiates into a variety of cell types, including chloronema, caulonema and rhizoids. From these filamentous strands, leafy buds will emerge that develop into the gametophores bearing antheridia or archegonia.

Vascular Seedless Plants – Ferns

The reproductive strategy of vascular seedless plants, here exemplified by ferns, is similar to that described for the bryophytes with the critical exception that the sporophyte phase of the lifecycle is dominant rather than the gametophyte stage. Fern gametophytes are very small, typically being photosynthetic and thalloid. On finding a suitable moist surface, haploid spores will germinate into a prothallus or young gametophyte. The gametophyte divides and expands in size into a notched or heart shaped mature structure with rhizoids on the lower surface (Schneider, 2013). Fern gametophytes can be unisexual or bisexual, depending on the light, temperature, and the density of other gametophytes. Male sperm are produced inside the male antheridium and female egg is produced in the archegonium. Upon maturation, the sperm are released and make their way to the egg by swimming. Fertilization occurs inside the archegonium and the zygote matures into a diploid sporophyte. Unlike the sporophyte of the bryophytes, however, this structure will be complex, photosynthetic, and completely independent from its parent at maturity. Fern sporophytes

produce lateral stems called rhizomes that bear clusters of upright leaves and positively gravitropic roots at regular nodes. Sporangia are arranged in clusters called sori, which are commonly positioned on the under or abaxial side of the leaves. In many species, the sori have an outer protective cap or jacket-like covering. Alternatively, sporangia may be grouped together on a common stem and arranged in a tightly packed spiral, termed a strobilus. Inside the sporangia, diploid sporocytes undergo meiosis to form spores. When the sporangium matures, it will crack open to release the multiple spores inside.

Heterospory

While all bryophytes are homosporous, several lineages of vascular seedless plants as well as the entire seed plant lineage have evolved heterospory (Bateman and DiMichele, 1994). There are three critical features of this reproductive system: 1) Heterosporous plants produce separate male and female spores, termed microspores and megaspores, respectively, which differ dramatically in size, consistent with their names; 2) microspores are specifically produced in microsporangia while megaspores are produced in megasporangia; 3) the resultant separate male and female gametophytes, termed microgametophytes and megagametophytes, respectively, are extremely reduced in size and grow to maturity inside the original spore wall, a phenomenon termed endospory. True endospory displays all of these features so, for instance, the sex determination described above for liverworts is not an example of heterospory because their male and female spores come from the same sporangia, are of equivalent size, and produce large, persistent gametophytes.

Seed Plants: Gymnosperms

The evolution of the seed marked a huge shift in the mechanisms of land plant sexual reproduction. The most dramatic changes are to the structures of the male and female gametophytes. The male gametophyte becomes reduced to only two main cell types: The vegetative cell, which is metabolically active, and the generative cell, which will give rise to just two sperm. Furthermore, the microgametophyte no longer releases free swimming sperm. Rather, it has become what we know as “pollen” – a simple cluster of cells that can travel through the air, delivering the sperm nuclei directly to the egg with no need for long distance swimming. The megagametophyte is also radically changed. In heterosporous ferns, the megasporangium releases megaspores that go out into the environment and develop into small, but still free-living, megagametophytes. In contrast, in seed plants, the megasporangia have become indehiscent. This means that the megaspore germinates inside the megasporangium and develops into the megagametophyte within this closed environment. This is the ovule, a modified megasporangium in which the megagametophyte will spend her entire life.

Arrangement of the microsporangia and microspore development: Microsporangia are typically borne on the surface of leaf-like structures called microsporophylls, which are then spirally arranged into a strobilus called a male cone. Inside each microsporangium, each microsporocyte cell divides by meiosis resulting in four haploid spores, which initially remain joined as a tetrad. As the microspores mature, they undergo a limited number of mitotic divisions – essentially germinating and becoming microgametophytes – before they are even released from the microsporangia. During microgametophyte maturation, they acquire a tough outer coating that is impermeable to water and impregnated with sporopollenin, which prevents desiccation and UV damage. These microgametophytes will be released as pollen.

Arrangement of the megasporangia and megaspore development: The megasporangia of gymnosperms are also typically borne on a specialized leaf-like structure termed a megaphyll. Sometimes these are borne singly, as in Ginkgo, but the most familiar gymnosperms arrange their megaphylls in strobili, such as the classic pine cone. In most species, the female cones are larger and longer-lived than the male cones. The megasporangia or ovules are borne on the upper surface of the megasporophylls, often in pairs. Inside each ovule, a single megaspore mother cell undergoes meiosis to produce four haploid cells, of which only one survives and develops into the megaspore. Since the ovule is indehiscent, the megaspore germinates inside the ovule, developing into the megagametophyte of ~1000 cells, including two archegonia.

Fertilization and seed development: Once the pollen has been released, it travels to the ovule, entering a hollow chamber created by leaf-like coverings called the integuments. This chamber, the micropyle, often secretes a drop of fluid, the pollination droplet, in order to better capture windborne pollen. When the pollination droplet evaporates, the pollen grains are drawn into the ovule itself, adjacent to the megagametophyte. In most gymnosperms, the pollen uses tip growth to produce a pollen tube that delivers the sperm nuclei directly to the eggs. However, in the early diverging Ginkgo and Cycad lineages, the pollen grain actually releases swimming sperm, although these only have to travel a tiny distance inside the ovule. Fusion of one of the sperm nuclei with one egg results in a diploid embryo. Critically, this embryo only develops for a short time before entering a dormant stage. During this embryo arrest, the seed develops a tough seed coat and prepares to be dispersed into the environment. On finding favorable conditions, the seed coat breaks and the young embryo emerges to reform a sporophyte with true leaves, roots, and a complex persistent body.

In general, the reproductive process is much slower in gymnosperms than in flowering plants. For example, in the case of pines, micro- and megaspore formation occurs during the first year, followed by pollen release in the second. The formation of the pollen tube, megagametophyte development, and eventual fertilization occurs over a period of months during the second year. Finally, seed maturation and release occurs during the third year.

Reproduction in Flowering Plants the Angiosperms

The diversification of flowering plants into more than 300,000 species approximately 140 million years ago is a fascinating evolutionary phenomenon (Crane *et al.*, 1995; Govaerts, 2001). While many traits are likely to have contributed to their success, the appearance of the hermaphroditic reproductive axes known as flowers, carpels enclosing the ovules, and double fertilization are all important factors. Flowers typically consist of four major organ types: The sterile perianth organs that comprise the sepals and petals, the microsporangia-bearing stamens, and the megasporangia bearing carpels. Similar to gymnosperms, all angiosperms are heterosporous, however, several aspects of their reproduction differ.

Pollen or male gametophyte production: The microsporophylls of angiosperms are known as stamens and consist of two parts, the anther and the filament. The anther contains diploid cells, known as microsporocytes, which divide by meiosis into four microspores. Each microspore then divides by mitosis into a vegetative cell, which eventually builds the pollen tube, and a generative cell, which produces the sperm nuclei. These together form the immature male gametophyte or pollen grain. The pollen is released upon anther dehiscence and is transported to the female gametophyte by animal pollinators or wind.

Embryo Sac or Female Gametophyte Production

As described for gymnosperms, the angiosperms bear indehiscent megasporangia called ovules. However, the ovules are further enclosed by a modified leaf, potentially derived from a megasporophyll, that is known as the carpel. Carpels have three main parts: The ovary containing the ovules, the style through which the pollen tubes grow, and the stigma on which the pollen grains germinate. Megaspore formation inside the ovules is essentially the same as described for gymnosperms, with a lone surviving haploid megaspore. When the megaspore germinates, however, it undergoes only three rounds of mitotic division, resulting in seven cells with eight nuclei. At one end of the megagametophyte, or egg sac, is a trio of cells termed the antipodals while at the other end are the two synergids nestled closely to the single egg cell. In between these cells is the large central cell, which contains two haploid nuclei (Endress, 2011; Friedman, 2006).

Double fertilization: Delivery of pollen onto the stigma can occur via abiotic (e.g., wind, water) or biotic (e.g., birds, bees, moths, bats) pollinators. Once deposited, the recognition of the pollen as belonging to same species may involve coordinated chemical signaling, both from the pollen and the sporophytic stigmatic tissue. In some angiosperm lineages, these interactions also allow the termination of self-pollen, thereby preventing self-fertilization. The pollen germinates by absorbing water from stigma and initiates a pollen tube, which will grow down through the style of the carpel. As the pollen tube reaches the ovary, it is attracted to a specific ovule by chemical signals from the synergids. The tube enters the micropyle of the ovule, triggering degeneration of the two synergids and allowing entry of the two sperm nuclei. Fertilization in angiosperms differs from that of gymnosperms in that it includes two distinct fusion events, known as double fertilization. One haploid sperm nucleus fuses with the haploid egg to produce the diploid zygote while the second sperm nucleus fuses with the two haploid nuclei of the central cell, resulting in a triploid cell. This triploid cell divides to become an angiosperm-specific, nutritive tissue known as endosperm, which will support the young sporophyte embryo in the seed when it is ready to germinate (Friedman, 1995).

From gametophyte to sporophyte: Following double fertilization, the zygote grows into the embryo and undergoes developmental arrest to allow for dispersal of the seed. At the same time, the endosperm matures and takes up nutritional resources from the surrounding sporophytic tissue of the ovule (Fig. 2). In some cases, the endosperm is transient and transfers these nutritional

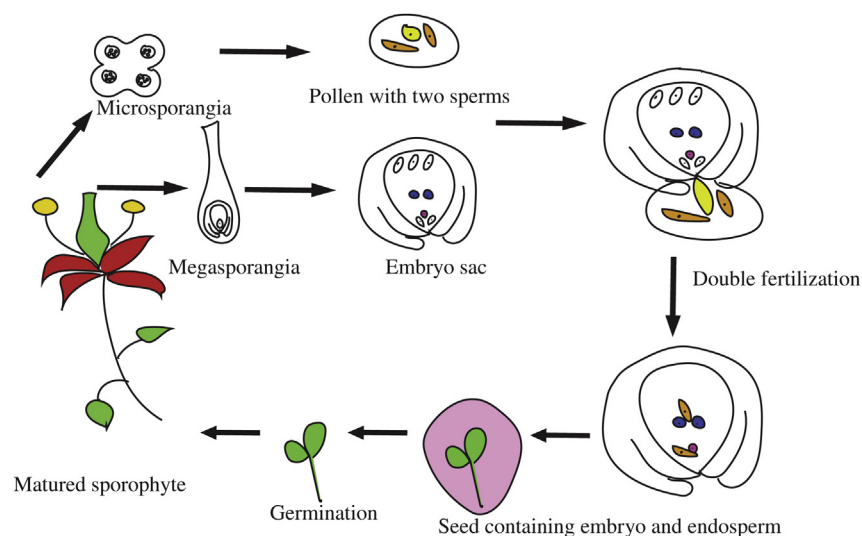


Fig. 2 Double fertilization in angiosperms.

reserves to the embryos (e.g., peanuts) while in others, the endosperm is persistent (e.g., rice). The seed coat matures into a protective layer to ensure the embryo is safe until it is ready for germination. The surrounding carpel also transforms itself into fruit, which may be fleshy (e.g., a berry), dry (e.g., a capsule) or woody (e.g., a nut). In some cases, the entire fruit is dispersed but in others, the fruit breaks open to disperse seeds individually. Upon germination, the embryo will grow to be the next diploid sporophyte generation.

Overview of Alternation of Generations in Seed Plants

1. Mature **diploid** sporophyte undergoes **meiosis** to produce haploid unicellular microspores and megaspores.
2. The microspore undergoes **mitotic** divisions to produce the male gametophyte, which is composed of a **haploid** vegetative cell and **haploid** generative cell.
3. The **haploid** generative cell will divide **mitotically** to form two **haploid** sperm nuclei.
4. The **haploid** megaspore undergoes three rounds of **mitotic** divisions that result in eight **haploid** nuclei contained in seven cells: Two synergids, one egg cell, a central cell with two nuclei, and three antipodals.
5. Double fertilization results in a **diploid** zygote and a **triploid** endosperm.
6. The **diploid** zygote grows **mitotically** into a next diploid sporophyte generation.

Summary of Sexual Reproduction in Plants

With their tremendous diversity in form, and as the dominant inhabitants of the land ecosystem, land plants have undergone many genetic, developmental and structural changes in reproductive structures over the course of their diversification. The transition from a water ecosystem for green algae to a land ecosystem posed many challenges, including desiccation of spores, secure transfer of male gametes to the female gametophyte, and nourishment and protection of embryo until it matures. Plants evolved the following characteristics to successfully reproduce.

1. A protective covering of sporopollenin around the spores to prevent desiccation.
2. Retention of the zygote in the female parent provided added protection and nutritional support to the young embryo.
3. Differentiation of the gametophyte into male or female. The multiple transitions from homospory to heterospory in early land plants to strictly heterospory in seed plants is associated with both reduced inbreeding and reduction of the gametophyte.
4. Evolution of the seed, which includes both the loss of swimming sperm in favor of a mobile male gametophyte and the evolution of indehiscent megasporangia, which protect the enclosed, reduced megagametophytes.
5. With the evolution of flower, we see the evolution of double fertilization as well as the carpel.

Diverse reproductive strategies allow plants to inhabit ecosystems where other organisms might not survive.

References

- Bateman, R. M., & DiMichele, W. A. (1994). Heterospory: The most iterative key innovation in the evolutionary history of the plant kingdom. *Biological Reviews*, 69, 345–417.
- Crane, P. R., Friis, E. M., & Pedersen, K. R. (1995). The origin and early diversification of angiosperms. *Nature*, 374, 27–33.
- Delwiche, C. F., & Cooper, E. D. (2015). The evolutionary origin of a terrestrial flora. *Current Biology: CB*, 25, R899–R910.
- Endress, P. K. (2011). Angiosperm ovules: Diversity, development, evolution. *Annals of Botany*, 107, 1465–1489.
- Friedman, W. E. (1995). Organismal duplication, inclusive fitness theory, and altruism: Understanding the evolution of endosperm and angiosperm reproductive syndrome. 1995. *Proceedings of the National Academy of Sciences*, 92, 3913–3917.
- Friedman, W. E. (2006). Embryological evidence for developmental lability during early angiosperm evolution. *Nature*, 441, 337–340.
- Govaerts, R. (2001). How many species of seed plants are there? *Taxon*, 50, 1085–1090.
- Grotewold, E., Chappell, J., & Kellogg, E. A. (2015). *Plant Genes, Genomes and Genetics*. John Wiley & Sons.
- Haig, D., & Wilczek, A. (2006). Sexual conflict and the alternation of haploid and diploid generations. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences*, 361, 335–343.
- Schneider, H. (2013). In *Annual Plant Reviews, Evolutionary Morphology of Ferns Ch4*. 45 pp. 115–140. John Wiley & Sons.
- Shaw, A. J., Szövényi, P., & Shaw, B. (2011). Bryophyte diversity and evolution: Windows into the early evolution of land plants. *American Journal of Botany*, 98, 352–369.

Comparative Placentation

Anthony M Carter, University of Southern Denmark, Odense, Denmark

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chorioallantoic placenta Placenta of which the fetal components are trophoblast and allantois.

Choriovitelline placenta Placenta of which the fetal components are trophoblast and yolk sac.

Hemotrophic nutrition Exchange of oxygen and nutrients between maternal and fetal blood.

Histotrophic nutrition Supply of nutrients as uterine secretions, cell debris or red cells taken up to the placenta by pinocytosis or endocytosis.

Lecithotrophy Nourishment of the embryo by egg yolk.

Matrotrophy Nourishment of the embryo by maternal sources other than egg yolk.

Oophagy Ingestion of eggs by the embryo.

Placentotrophy The supply of oxygen and nutrients through a placenta.

Viviparity Bearing of live young.

Introduction

Viviparity has evolved in many types of animal. The embryos may be retained in the reproductive tract or some form of breeding chamber, yet rely exclusively on the nutrients provided by the yolk (lecithotrophy). More often viviparity is accompanied by provision of nutrients to the embryo by one or both parents (matrotrophy) (Blackburn, 2015). Matrotrophy takes many forms, but supply of oxygen and nutrients through a placenta is the main focus of this article.

A recent survey found evidence of matrotrophy in 21 of 34 animal phyla (Ostrovsky *et al.*, 2016). It is common in flatworms, arthropods and bryozoans. In arthropods alone, matrotrophy is estimated to have evolved around 30 times. Arrangements for enhancing nutritional transport from parent to embryo take several forms. In velvet worms and arthropods they include placental analogs that mimic vertebrate placentation.

Even among chordates there are several variants of placentation, including the incubation chamber of salps described by Thomas Huxley (Lemaire and Piette, 2015). Salps are tubular animals found near the surface of oceans, where they swim by jet propulsion. The life cycle of salps involves alternation between asexual and sexual generations. The asexual phase (oozoid) develops within the jet chamber of the sexual phase (blastozoid). The placenta consists of two layers separating the embryonic and maternal circulations. Both layers are syncytial and maternal in origin.

Placentation in Anamniotes

Prior to evolution of the cleidoic egg, the yolk sac was the only fetal membrane, but non-amniotes have a long history of placentation. The males of some placoderms, the earliest jawed fishes, had clasper-like appendages, like those of modern sharks, suggesting they practiced intromission of sperm. From intromission, it is but a short step to matrotrophy and placentation. A remarkable fossil from Western Australia shows fetuses one-third of adult body size. This specimen (*Materpiscis attenboroughi*) was so well preserved that it was possible to identify a vitelline cord and show by high resolution computer tomography that it contained blood vessels. Thus viviparity and placentation in vertebrates dates back to the Devonian period, about 380 million years ago (Long *et al.*, 2008).

Cartilaginous Fish

All chondrichthyans, including the basal holocephalans, employ internal fertilization (Hamlett *et al.*, 1993). Many lay eggs but around 55% are viviparous; most of these practice some form of matrotrophy. Examples not involving placentation are oophagy and cannibalism of siblings, which occurs in mackerel sharks. Supplementation of yolk by uterine secretions or histotroph occurs in several species. Thus in sawfish histotroph is secreted by uterine villi. In stingrays, there are elaborate outgrowths from the uterine wall called trophonemata. They secrete lipid-rich histotroph and are also considered important for gas exchange.

There are instances where nutrition is essentially lecithotrophic yet maternal-fetal exchange of blood gases occurs across placenta-like structures. This can be difficult to assess in the absence of functional data. As an example, the embryos of viviparous dogfish derive their nutrition from yolk, but highly vascularized uterine villi act as a source of oxygen, and late embryos use buccal pumping to perfuse their gills and take up oxygen from the uterine fluid. However, modelling of gas exchange in dogfish (*Squalus*

spp.) has shown that oxygen diffusion across the uterine wall cannot provide sufficient oxygen to late stage embryos. Additional oxygen may be supplied through periodic flushing of the uterus with seawater (Tomita *et al.*, 2016).

True placentation occurs in requiem and hammerhead sharks (Carcharhiniformes), where gestation lasts 9–10 months. Initially, the embryo is supported by yolk. When this has been used up, the yolk sac persists and forms a placenta. In the sandbar shark, for example, there are well vascularized elevations of the uterine attachment site that interdigitate with the distal portion of the yolk sac. The uterine secretion products may be similar to yolk proteins.

Bony Fish

Viviparity has arisen in 13 clades of teleost fish including the coelacanth (*Latimeria chalumnae*), a relict of the lobe-finned fish. Studies in viviparous poeciliid and cichlid fish have shown that placentation can evolve quite rapidly.

Teleosts do not possess uteri or oviducts. Ovulation occurs into the ovarian lumen, which is connected to the exterior by a duct opening at the genital pore. Consequently, the embryos of viviparous fish develop either in the ovarian follicle or in the ovarian lumen (Schindler and Hamlett, 1993). In intraluminal gestation, the ovarian lining becomes highly vascularized and secretes histotroph to the lumen where the embryos develop. In goodeid fish appendages called trophotaenia, which protrude from the cloaca, serve as absorptive surfaces. Otherwise absorption of histotroph is through the embryonic gut. In viviparous brotulas (*Ogilbia*) projections from the ovarian lining protrude into the mouth of the embryo (ovarian nipples).

Where development is intrafollicular, the follicular epithelium answers for transfer of nutrients from the maternal circulation to the follicular fluid. Absorptive surfaces of the embryo may be closely apposed to this epithelium forming placenta-like structures. The embryonic component may be the yolk sac. However, in the four-eyed fish (*Anableps*), the pericardial trophoderm develops bulbs that interdigitate with pit-like depressions in the follicular epithelium (Knight *et al.*, 1985).

Viviparity in seahorses and pipefish involves incubation of the embryos in the brood pouch of the male. Analysis of the transcriptome of the brood pouch of the pot-bellied seahorse has identified the genes that are upregulated during pregnancy. Many of the same genes or their homologs are upregulated during pregnancy in mammals (Whittington *et al.*, 2015).

Amphibians

Viviparous species occur in all three orders of amphibians (Wake, 2015). Live-bearing in frogs enables them to abandon their amphibious lifestyle for a more terrestrial one. In the marsupial frogs from South and Central America (Hemiphysactidae), the embryo develops on the back of the mother in either a mucous-filled depression or a closed pouch (Duellman, 2014). The embryos may develop into tadpoles and be released to water-filled cavities in plants or skip the tadpole stage and develop directly into froglets. In embryos of the genus *Gastrotheca*, 1–2 pairs of external gills serve for respiratory gas exchange with maternal tissues in the brood pouch. The gills are shed around the time of birth. A similar strategy has been adopted by frogs in the Neotropical genus *Pipa* (Pipidae) where the eggs develop to tadpoles within individual chambers in the dorsal skin (Fernandes *et al.*, 2011). The chambers become highly vascularized suggesting that gas exchange could occur between mother and embryo. Recently, nutrient transfer from pouch to embryo has been shown to occur in *Gastrotheca* (Warne and Catenazzi, 2016).

Female frogs of the extinct genus *Rheobatrachus* ingested the fertilized eggs and incubated them in the stomach. The developing embryos secreted prostaglandin to inhibit gastric secretion. Male frogs of *Rhinoderma darwinii* ingest eggs and brood the embryos within their vocal sacs; there is evidence in support of nutrient transfer from the paternal circulation to the embryos. There are a few examples of frog larvae developing to froglets within the oviduct, although maternal provision of nutrients other than yolk has been documented only for the Mount Nimba viviparous toad (*Nimbaphrynoides occidentalis*).

Caecilians comprise an order of limbless amphibians adapted for a burrowing lifestyle. They embrace both oviparous and viviparous species. In the former there is extended parental care with the hatchlings feeding on the outer layer of the parent's skin. In viviparous caecilians, the fetuses have teeth and feed on the hypertrophied lining of the oviduct (Wilkinson *et al.*, 2008). A few species of salamander are viviparous. Embryos of the alpine salamander (*Salamandra atra*) ingest unfertilized eggs and feed on the hypertrophied epithelium of the oviduct. Placental analogs have not been described for caecilians or salamanders.

The Fetal Membranes of Amniotes

Whereas placentation in anamniotes often is based on the yolk sac, the cleidoic egg offers three more membranes that can be recruited for placentation (Mossman, 1987; Carter, 2016). These are the amnion, allantois and chorion (Fig. 1(A)). The fluid-filled amnion surrounds the embryo and offers protection from desiccation and mechanical shocks. The allantois is a pathway for gaseous exchange with the ambient air and its cavity is a receptacle for waste. Outermost is the chorion, which in birds and reptiles is situated beneath the egg shell.

Various combinations of these membranes form the basis of placentation in reptiles and mammals (Fig. 1(B)). The chorioallantoic placenta is formed by the chorion and allantois. It is the basis for placentation in viviparous lizards, some marsupials and extant eutherians (known misleadingly as “placental mammals”). The chorionic epithelium constitutes the trophoblast (in mammals the trophoblast). The chorionic mesoderm supplies the blood vessels. The choriovitelline placenta is formed by the chorion and yolk sac. There may be a substantial vasculature. This type of placenta is found in all marsupials. In addition,

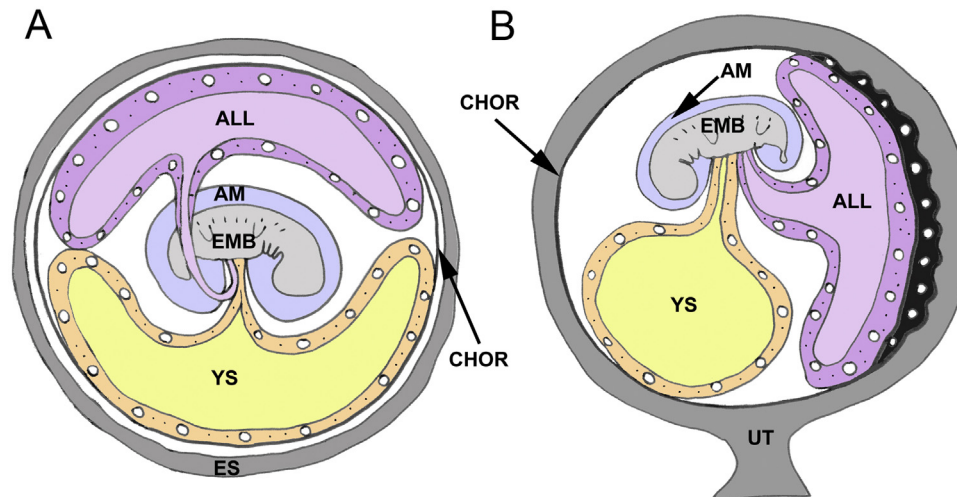


Fig. 1 Fetal membranes that contribute to placentation in reptiles and mammals. (A) The amniote egg as in birds, oviparous reptiles and monotremes. (B) Embryo and fetal membranes within the uterus of a generic mammal. The chorion (CHOR) is found beneath the egg shell (ES) in the former and facing the uterine wall (UT) in the latter. In birds and reptiles the other membranes enclose the amniotic sac (AM), allantoic vesicle (ALL) and yolk sac (YS). In mammals and viviparous reptiles, yolk sac and chorion can form a choriovitelline placenta and the allantois and chorion can form a chorioallantoic placenta. Reproduced with permission from Carter, A.M., Mess, A.M., 2014. Mammalian placentation: Implications for animal models. In: McManus, L., Mitchell, R. (Eds.), Pathobiology of Human Disease. San Diego, CA: Elsevier, pp. 2423–2442. © 2014 Elsevier.

non-vascular areas of the yolk sac can play a role in uptake of histotroph in marsupials, viviparous lizards and some eutherians. As described below, viviparous snakes have a third type of placenta derived from the allantochorion and a derivative of the yolk sac.

Placentation in Reptiles

Like birds, the non-avian dinosaurs generally had calcified eggs and fossil evidence of viviparity is restricted to the aquatic ichthyosaurs. Other extinct marine reptiles that were viviparous include archosaurs, mesosaurs and plesiosaurs. Among extant reptiles, neither crocodilians nor tortoises and turtles are viviparous, but live birth does occur in squamate reptiles, including several families of lizards and snakes (Blackburn, 2015; Stewart and Thompson, 2000).

Lizards

In oviparous and viviparous lizards the egg comprises two hemispheres with the chorioallantois covering the embryonic compartment and the yolk sac occupying the abembryonic compartment (Fig. 2). A yolk cleft separates most of the yolk from a smaller portion associated with the yolk sac ectoderm and endoderm (bilaminar omphalopleure). This membrane is a conduit for water even in oviparous species. In viviparous ones the oviduct epithelium facing the yolk sac may proliferate and perhaps secretes histotroph that can be taken up by the yolk sac.

However, in lizards with limited or extensive matrotrophy it is the allantochorion that develops into a placenta. Although four types of allantochorion are recognized it is by no means certain that Types I and II are true placentas (Stewart and Thompson, 2000). Type I occurs mainly in lecithotrophic species and the close association between the yolk sac and uterine epithelium may serve solely for respiratory gas exchange. In Type III, on the other hand, interdigitating folds of maternal and fetal tissues form a placentalome. This has been best described for a number of skinks, including the Italian three-toed skink (*Chalcides chalcides*) and the southern grass skink (*Pseudemoia entrecasteauxii*) from Australia. An even more complex placenta, designated Type IV, is found in a genus of long-tailed skinks (*Mabuya*) from South America (Fig. 3). Altogether there has been convergent evolution of placentotrophy in six clades of skinks. Ample evidence exists for maternal-fetal transfer of nutrients across Type III and IV placentas. In addition electron microscopy has indicated specializations for nutrient transfer even in lizards that are mainly lecithotrophic; these have been characterized as cases of incipient placentotrophy.

Snakes

Some 20% of snakes are viviparous, but since there is no net increase in dry weight from egg to neonate, placental transfer of nutrients may play a limited role in growth of the embryo. So far placentation has been described in just a handful of species.

As in lizards there are two distinct regions of placentation. The chorioallantois is similar to that of lizards. Recent work in water snakes (*Nerodia*) has established that it is specialized for respiratory gas exchange since both the chorioallantois and the apposing

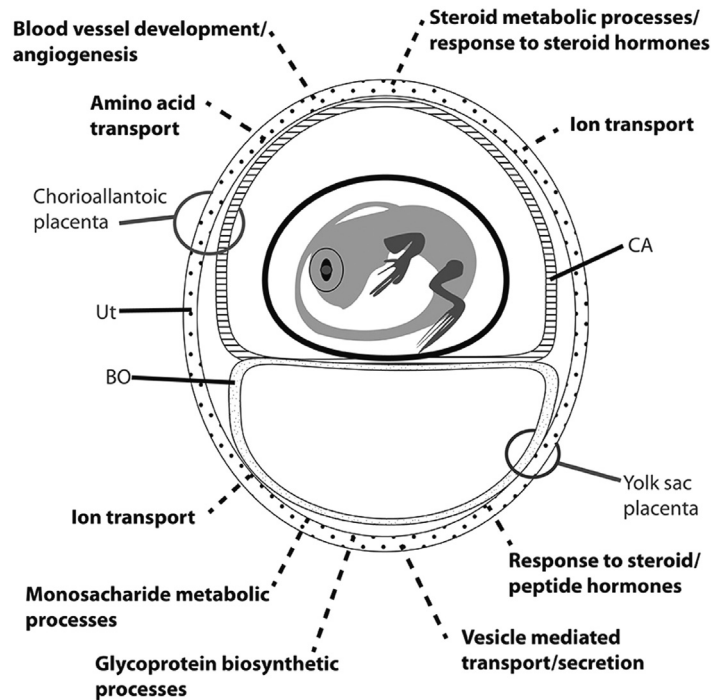


Fig. 2 Extraembryonic membranes of a viviparous lizard, the southern grass skink (*Pseudemoia entrecasteauxii*). The embryonic compartment is enclosed by the chorioallantois, which is apposed to hypertrophied uterine epithelium and the site of nutrient exchange. The bilaminar omphalopleure encloses the yolk. It also faces hypertrophied uterine epithelium and constitutes a yolk sac placenta. Putative sites of placental function are based on functional analysis of differentially expressed genes. Reproduced from Griffith, O.W., Brandley, M.C., Beloiv, K., Thompson, M.B., 2016. Reptile pregnancy is underpinned by complex changes in uterine gene expression: A comparative analysis of the uterine transcriptome in viviparous and oviparous lizards, *Genome Biology and Evolution* 8, 3226–3239. © The Author 2016.

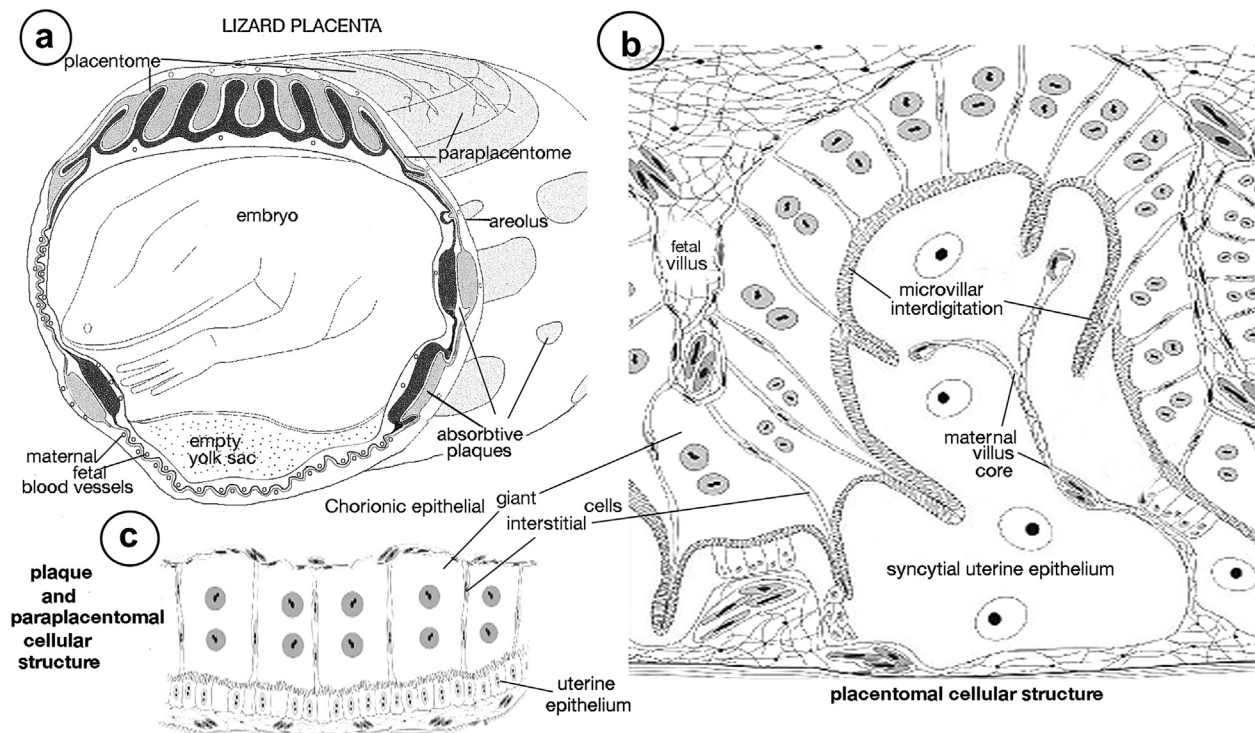


Fig. 3 Placentation in a South American long-tailed skink (*Mabuya* sp.). (a) Uterus with placentome (chorioallantoic placenta), paraplacenta, areolae and absorptive plaques. (b) Interface between the chorionic epithelium and syncytial uterine epithelium within the placentome. (c) Interface within the paraplacental area and plaques. Reproduced with permission from Wooding, F.B.P., Ramirez-Pinilla, M.P., Forhead, A.S., 2010. Functional studies of the placenta of the lizard *Mabuya* sp. (Scincidae) using immunocytochemistry. *Placenta* 31, 675–685. © 2010 Elsevier Ltd.

uterine epithelium are well vascularized (Blackburn *et al.*, 2017). The yolk sac placenta of snakes is unique in that the allantois enters the yolk sac cleft and expands to line the inner face of the omphalopleure. The resultant structure is known as an omphalallantoic placenta. It has some ultrastructural features consistent with a role in nutrient transfer.

Placentation in Monotremes and Marsupials

Monotremes are egg-laying mammals, yet two-thirds of embryonic development takes place in the uterus. The embryo is nourished partly by yolk and partly by endometrial secretions taken up through the egg shell membrane. This is porous and able to stretch so the embryo and its membranes increase in size before the egg is laid.

Marsupials are poorly developed at birth and most of their growth occurs as pouch young. The intrauterine phase of development is supported in all marsupials by a choriovitelline placenta and in some by an additional chorioallantoic one (Freyer and Renfree, 2003; Renfree, 2010). A shell membrane persists through the first two-thirds of gestation and the principal source of nutrition is uterine gland secretion. There are vascular and non-vascular parts to the yolk sac and both are able to absorb these secretions. Although exchange of blood gases occurs between maternal capillaries and those of the yolk sac, there is scant evidence of nutrient exchange by that route. A chorioallantoic placenta is found in the koala (*Phascolarctos cinereus*) and in bandicoots (e.g., *Perameles nasuta*). It is supplied by allantoic blood vessels and thought to function primarily as a respiratory organ. Interestingly, chorioallantoic placentation is not associated with greater maturity of the neonate; in bandicoots it may rather contribute to shortening of pregnancy duration. The marsupial placenta generally is considered to be epitheliochorial. However, invasive trophoblast has been described in several opossums (e.g., *Philander opossum*) and in the fat-tailed dunnart (*Sminthopsis crassicaudata*). The marsupial placenta secretes hormones, as evidenced by expression of growth hormone, prolactin and luteinizing hormone genes in the placenta of the tammar wallaby (*Macropus eugenii*) (Menzies *et al.*, 2011).

Placentation in Eutherian Mammals

Unlike in marsupials, the yolk sac plays a subsidiary role in eutherians, while all have a chorioallantoic placenta that supports the fetus to term (Carter and Enders, 2004; Mossman, 1987; Wooding and Burton, 2008). Moreover the trophoblast is invasive except in lower primates and three other orders (Carter *et al.*, 2015). Gestation generally is much longer in eutherians (although shorter in the mouse than the Eastern gray kangaroo). The newborn is better developed than in marsupials, but there is a striking difference between species like the mouse, which give birth to altricial young, and those like the guinea pig that have precocial young. As a rule mammals with precocial young have long gestations and small litters, sometimes just a single newborn, whilst the reverse applies to species with altricial young. There is a remarkable variation in the shape, structure and vascular arrangements of the placenta, but no consistent pattern in relation to gestation length or maturity of the young at birth. Humans are a special case in having a long pregnancy that results in a baby advanced in organ development yet entirely dependent on extended parental care. Portmann coined the term “secondary altriciality” to describe this unique state of affairs.

The remarkable variation in the shape and structure of the placenta is summarized by order in Table 1. As to shape, human placenta resembles a flattened cake; this type of discoid placenta is also found in rodents and several other orders. At the opposite extreme, the diffuse placenta of swine occupies most of the uterine horn and resembles a large sac. In between are the placentas of pecoran ruminants, with a variable number of small discs or placentomes, and the zonary placentas of carnivores.

The arrangement of fetal and maternal tissues in the placenta varies considerably. The most common pattern is a labyrinth. Broadly speaking, fetal capillaries run in one direction and maternal capillaries (or blood channels) in the other, an arrangement that promotes countercurrent exchange. An alternative design has interdigitation of fetal villi with maternal trabeculae that succeeds in minimizing the distance between fetal and maternal capillaries but without countercurrent flow. This is common in the diffuse type of placenta, as in dolphins, and in the placentomes of ruminants. In a small number of species, including catarrhine primates and armadillos, there is a villous tree with fetal capillaries that is suspended in an intervillous space perfused by maternal blood.

Traditionally most attention has been directed to the fine structure of the interhemal barrier and the number of tissue layers separating maternal and fetal blood (Fig. 4). In the epitheliochorial placenta, the uterine epithelium is intact and interdigitates with the trophoblast (Fig. 4(a)). In pecoran ruminants the overall pattern is similar, but a remarkable feature is fusion of binucleate trophoblast cells with uterine epithelial ones to form either a hybrid trinucleate cell (Fig. 4(b)) or syncytium. Some authors refer to this as synepitheliochorial placentation. In endotheliochorial placentas, the only maternal tissue remaining is the capillary epithelium, which frequently is hypertrophied (Fig. 4(c)). Finally, in hemochorial placentas, the trophoblast is directly in contact with maternal blood. The latter may flow in small channels in a labyrinth (Fig. 4(d) and (e)) or perfuse an intervillous space (Fig. 4(f)).

While exchange between maternal and fetal vessels is the principal function of the mammalian placenta, there may be additional supply of nutrients as uterine gland secretions, tissue debris or maternal red cells (Carter, 2012). This is particularly important where the interhemal barrier is epitheliochorial. Thus artiodactyls and perissodactyls have prominent areolae and strepsirrhine primates have chorionic vesicles. These structures contain columnar trophoblast cells arranged in proximity of the mouths of uterine glands and with the ability to phagocytose and process glandular secretions. There are also structures with a more specialized function that take up maternal red cells (hemophagous areas). Such modifications are not confined to epitheliochorial placentas; thus areolae are found in moles and hemophagous areas in tenrecs, both of which have hemochorial placentation.

Table 1 The orders of mammals and the principle characteristics of their placentation including the yolk sac at term

<i>Superordinal clade and order</i>	<i>Common names</i>	<i>Placental shape</i>	<i>Internal structure</i>	<i>Interhemal barrier</i>	<i>Yolk sac</i>
<i>Afrotheria</i>					
Hyracoidea	Hyraxes	Zonary	Labyrinth	Hemochorial	Absent
Proboscidea	Elephants	Zonary	Labyrinth	Endotheliochorial	Absent
Sirenia	Manatees, dugong	Zonary	Labyrinth	Endotheliochorial	Missing data
Macroscelidae	Elephant shrews	Discoid	Labyrinth	Hemochorial	Free
Afrosoricida	Tenrecs, golden moles	Discoid	Labyrinth	Hemochorial or endotheliochorial	Free (large in golden moles)
Tubulidentata	Aardvark	Zonary	Labyrinth	Endotheliochorial	Rudimentary
<i>Xenarthra</i>					
Cingulata	Armillos	Discoid	Intervillous space	Hemochorial	Completely inverted visceral yolk sac
Pilosa	Sloths, anteaters	Discoid or double discoid	Intervillous space (anteaters) or labyrinth (sloths)	Hemochorial (anteaters) or endotheliochorial (sloths)	Absent
<i>Euarchontoglires</i>					
Primates: Strepsirhini	Lemurs and lorises	Diffuse	Labyrinth	Epitheliochorial	Absent
Primates: Haplorhini	Tarsiers, monkeys and apes	Discoid or double discoid	Labyrinth in tarsiers; intervillous space	Hemochorial	Absent
Dermoptera	Colugos	Discoid	Labyrinth	Hemochorial	Free
Scandentia	Tree shrews	Discoid	Labyrinth	Endotheliochorial	Persistent choriovitelline placenta
Rodentia	Rodents	Discoid	Labyrinth	Hemochorial or endotheliochorial	Inverted visceral yolk sac
Lagomorpha	Rabbits, hares, pikas	Discoid	Labyrinth	Hemochorial	Inverted visceral yolk sac
<i>Laurasiatheria</i>					
Cetartiodactyla	Whales, dolphins, even-toed ungulates	Diffuse; cotyledonary in pecoran ruminants	Interdigitating villi	Epitheliochorial	Absent or free
Perissodactyla	Horses, tapirs	Diffuse	Interdigitating villi	Epitheliochorial	Absent or rudimentary
Chiroptera	Bats	Discoid or double discoid	Labyrinth	Hemochorial or endotheliochorial	Free or with inverted visceral yolk sac
Carnivora	Carnivores	Zonary	Labyrinth	Endotheliochorial or hemochorial (hyenas)	Persistent T-shaped visceral yolk sac
Pholidota	Pangolins	Diffuse	Interdigitating villi	Epitheliochorial	Small visceral yolk sac
Soricomorpha	Shrews, moles, solenodons	Discoid; zonary in some moles	Labyrinth	Hemochorial or endotheliochorial	Inverted visceral yolk sac
Erinaceomorpha	Hedgehogs, gymnures	Discoid	Labyrinth	Hemochorial	Inverted visceral yolk sac

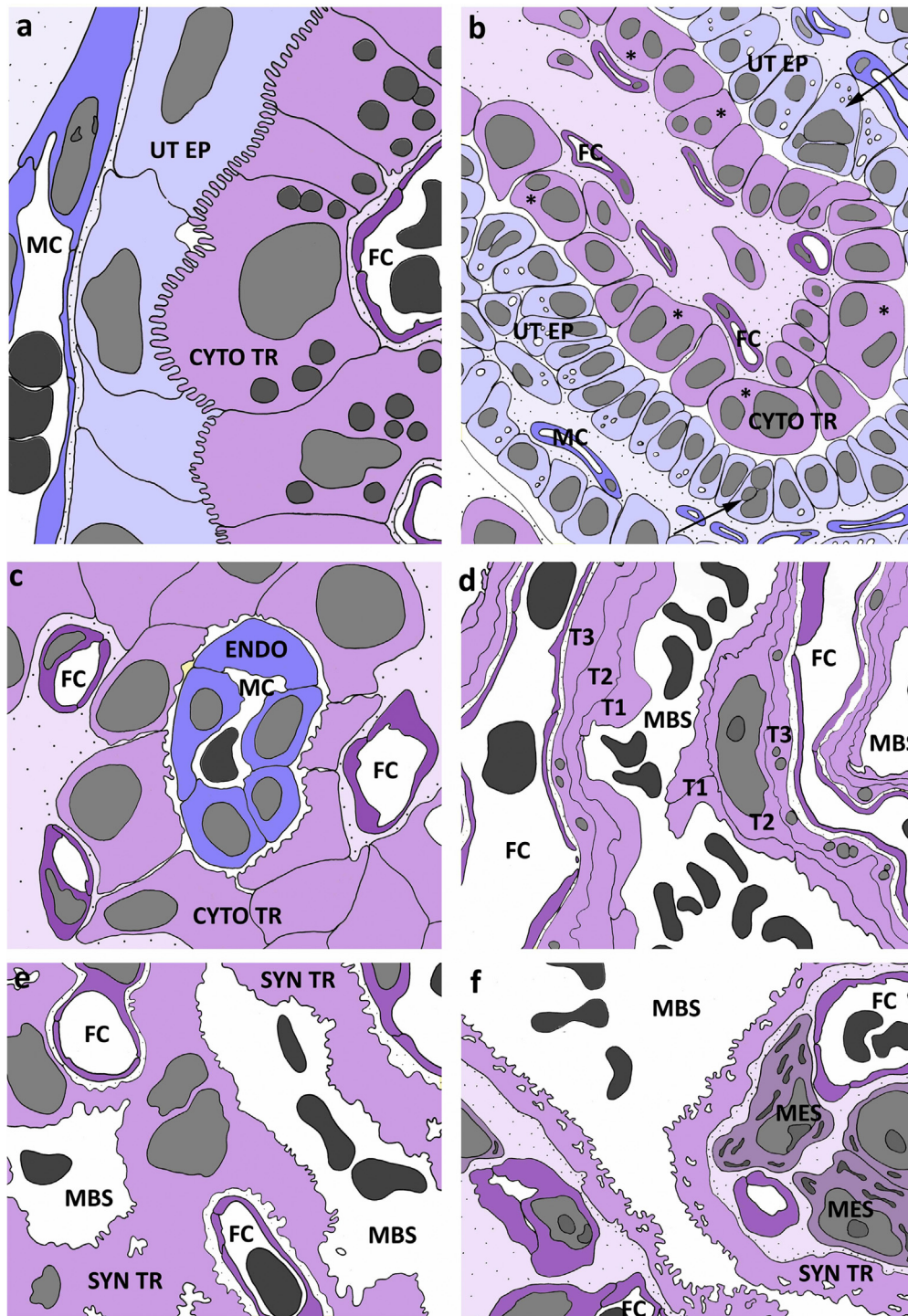


Fig. 4 Interhemal barrier of representative mammals. (a) Epitheliochorial placenta of the bush baby (*Otolemur crassicaudatus*). (b) Synepitheliochorial placenta of the cow (*Bos taurus*); note the binucleate trophoblast cells (asterisks) and trinucleate cells (arrows). (c) Endotheliochorial placenta of a bat (*Natalus* sp.); note the enlarged endothelial cells of the maternal capillary in contact with the trophoblast. (d) Hemotrichorial labyrinthine placenta of the mouse (*Mus musculus*). (e) Hemomonochorial labyrinthine placenta of the guinea pig (*Cavia porcellus*). (f) Hemomonochorial villous placenta of an armadillo (*Dasypus novemcinctus*). CYTO TR, cytotrophoblast; ENDO, Endothelium; FC, fetal capillary; MC, maternal capillary; MBS, maternal blood space; MES, enlarged mesenchymal cell; SYN TR, syncytial trophoblast; T1, trophoblast layer 1 (cellular); T2, trophoblast layer 2 (syncytial); T3, trophoblast layer 3 (syncytial); UT EP, uterine epithelium. Based on material kindly supplied by Dr. Allen C. Enders, University of California at Davis, United States. Reproduced with permission from Carter, A.M., Mess, A.M., 2014. Mammalian placentation: Implications for animal models. In: McManus, L., Mitchell, R. (Eds.), Pathobiology of Human Disease. San Diego, CA: Elsevier, pp. 2423–2442. © 2014 Elsevier.

There are no consistent patterns that can explain the variation of the chorioallantoic placenta in terms of parameters such as gestation length or developmental stage at birth. For example, the highly invasive nature of human placentation and the dilated vessels that supply the maternal side of the placenta seem designed to maximize the oxygen supply to the fetus and the fetal brain. But newborn whales and dolphins also have large brains, yet their development is supported by a non-invasive epitheliochorial placenta. It is striking that even-toed and odd-toed ungulates have small litters, long gestations and epitheliochorial placentation. A case can be made that evolution of this placental type conveyed an immunological advantage. On the other hand the African elephant gives birth to a single calf after 22 months, but has an endotheliochorial placenta.

There is further variation in the part played by other fetal membranes in various orders of eutherians. In particular, the visceral yolk sac may persist until term (Carter, 2016). As a rule there has been inversion of the germ layers at an early stage so that the yolk sac presents an absorptive epithelium, the yolk sac endoderm, to the uterine wall as in rodents, lagomorphs and some bats (inverted visceral yolk sac; Table 1). This may be a conduit for the transfer of maternal antibodies that confer passive immunity on the fetus. In many orders, however, the yolk sac is absent at term or is a small sac floating free in the exocoelom (Table 1). Passive immunity may then be conferred by antibodies crossing the chorioallantoic placenta. In orders with epitheliochorial placentation, on the other hand, passive immunity is conferred after birth by antibodies secreted to the colostrum. Finally, in many bats, the yolk sac assumes the morphological characteristics of a gland of unknown function.

References

- Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *Journal of Morphology*, 276, 961–990.
- Blackburn, D. G., Anderson, K. E., Lo, A. R., Marquez, E. C., & Callard, I. P. (2017). Placentation in watersnakes II: Placental ultrastructure in *Nerodia erythrogaster* (Colubridae: Natricinae). *Journal of Morphology*, 278, 675–688.
- Carter, A. M. (2012). Evolution of placental function in mammals: The molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. *Physiological Reviews*, 92, 1543–1576.
- Carter, A. M. (2016). IFPA senior award lecture: Mammalian fetal membranes. *Placenta*, 48(Suppl. 1), S21–S30.
- Carter, A. M., & Enders, A. C. (2004). Comparative aspects of trophoblast development and placentation. *Reproductive Biology and Endocrinology*, 2, 46.
- Carter, A. M., Enders, A. C., & Pijnenborg, R. (2015). The role of invasive trophoblast in implantation and placentation of primates. *Philosophical Transactions of the Royal Society B*, 370. <https://doi.org/10.1098/rstb.2014.0070>.
- Duellman, W. E. (2014). *Marsupial frogs: Gastrotheca and allied genera*. Baltimore, MD: Johns Hopkins University Press.
- Fernandes, T. L., Antoniazzi, M. M., Sasso-Cerri, E., et al. (2011). Carrying progeny on the back: Reproduction in the Brazilian aquatic frog *Pipa carvalhoi*. *South American Journal of Herpetology*, 6, 161–176.
- Freyer, C., & Renfree, M. B. (2003). The marsupial placenta: A phylogenetic analysis. *The Journal of Experimental Zoology A Comparative and Experimental Biology*, 299, 59–77.
- Hamlett, W. C., Eulitt, M. A., Jarrell, R. L., & Kelly, M. A. (1993). Uterogestation and placentation in elasmobranchs. *The Journal of Experimental Zoology*, 266, 347–367.
- Knight, F. M., Lombardi, J., Wourms, J. P., & Burns, J. R. (1985). Follicular placenta and embryonic growth of the viviparous four-eyed fish (*Anableps*). *Journal of Morphology*, 185, 131–142.
- Lemaire, P., & Piette, J. (2015). Tunicates: Exploring the sea shores and roaming the open ocean. A tribute to Thomas Huxley. *Open Biology*, 5, 150053.
- Long, J. A., Trinajtic, K., Young, G. C., & Senden, T. (2008). Live birth in the Devonian period. *Nature*, 453, 650–652.
- Menzies, B. R., Pask, A. J., & Renfree, M. B. (2011). Placental expression of pituitary hormones is an ancestral feature of therian mammals. *EvoDevo*, 2, 16.
- Mossman, H. W. (1987). *Vertebrate fetal membranes. Comparative ontogeny and morphology; evolution; phylogenetic significance; basic functions; research opportunities*. New Brunswick, NJ: Rutgers University Press.
- Ostrovsky, A. N., Lidgard, S., Gordon, D. P., et al. (2016). Matrotrophy and placentation in invertebrates: A new paradigm. *Biological Reviews of the Cambridge Philosophical Society*, 81, 673–711.
- Renfree, M. B. (2010). Marsupials: Placental mammals with a difference. *Placenta*, 31(Suppl. A), S21–S26.
- Schindler, J. F., & Hamlett, W. C. (1993). Maternal-embryonic relations in viviparous teleosts. *Journal of Experimental Zoology*, 266, 378–393.
- Stewart, J. R., & Thompson, M. B. (2000). Evolution of placentation among squamate reptiles: Recent research and future directions. *Comparative Biochemistry and Physiology Part A*, 127, 411–431.
- Tomita, T., Cotton, C. F., & Toda, M. (2016). Ultrasound and physical models shed light on the respiratory system of embryonic dogfishes. *Zoology (Jena)*, 119, 36–41.
- Wake, M. H. (2015). Fetal adaptations for viviparity in amphibians. *Journal of Morphology*, 276, 941–960.
- Warne, R. W., & Catenazzi, A. (2016). Pouch brooding marsupial frogs transfer nutrients to developing embryos. *Biology Letters*, 12, 20160673.
- Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B., & Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution*, 32, 3114–3131.
- Wilkinson, M., Kupfer, A., Marques-Porto, R., et al. (2008). One hundred million years of skin feeding? Extended parental care in a Neotropical caecilian (Amphibia: Gymnophonia). *Biology Letters*, 4, 358–361.
- Wooding, P., & Burton, G. (2008). *Comparative placentation. Structures, function and evolution*. Berlin: Springer-Verlag.

Insect Male Reproductive Glands and Their Products

Ben R Hopkins, University of Oxford, Oxford, United Kingdom; and Cornell University, Ithaca, NY, United States

Frank W Avila, University of Antioquia, Medellín, Colombia

Mariana F Wolfner, Cornell University, Ithaca, NY, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

The female insect is changed by mating. Behaviorally, physiologically, even anatomically, the mated female differs dramatically from her virgin self. In many cases, these changes are induced by proteins and other molecules carried in the male's ejaculate. Exactly how the post-mating phenotype differs from the pre-mating condition varies across taxa, being honed by selective forces that must strike a balance between the male-female cooperation necessary to achieve a successful fertilization, and the broader, often divergent, evolutionary interests of the sexes.

The male reproductive glands, particularly the accessory glands, are tasked with the production of the non-sperm component of the ejaculate (the seminal fluid) in insects. Divergent in number, structure, and developmental origin, they are functional analogues of mammalian glands such as the prostate and seminal vesicles. In this article, we begin by outlining the complexity of insect seminal fluid in terms of both its organization and its molecular composition. Following this, we briefly summarize the general functions of the secretions of male insect reproductive glands, which induce profound changes in female behavior, physiology, and anatomy. As much of our understanding of the activities of seminal fluid components comes from the fruit fly *Drosophila melanogaster*, we pay particular attention to two paradigmatic examples of its seminal fluid proteins, sex peptide (SP) and ovulin, before outlining what is known about another seminal fluid component, the male-transferred hormone 20-hydroxyecdysone, in the malaria vector mosquito *Anopheles gambiae*. We then review the considerable variation in the ultrastructure of reproductive glands across insects, including a detailed overview of *D. melanogaster* accessory glands and their two distinct secretory cell types, the main and secondary cells. Finally, we discuss forces that are thought to have influenced the evolution of accessory glands and their products. This evolutionary framework helps to explain some of the more surprising functions of accessory gland products, such as those that cause female harm.

The Complexity of Insect Seminal Fluid

The ejaculate is composed of sperm and seminal fluid, the latter encompassing a rich diversity of lipids, proteins, carbohydrates, nucleic acids, water, hormones, mucus, vitamins, vesicles, microbes and, in some species, glandular cells. In many insects, the protein component of the ejaculate, derived largely from the male accessory glands, is particularly diverse. To date, 121 accessory gland-expressed genes have been identified in the malaria vector *An. gambiae*, while nearly 100 seminal fluid proteins (SFPs) are transferred to females during mating in another mosquito, the yellow fever vector *Aedes aegypti*. In *D. melanogaster*, over 200 SFPs are known. These SFPs span a range of molecular classes from antioxidants and lectins to proteases and protease inhibitors, the latter two classes being particularly well-represented in both insect and mammalian seminal fluid. In *Drosophila* (and likely in other insects), SFPs' amino acid diversity is further supplemented by a variety of post-translational modifications, such as glycosylation, that may further influence their functions.

Insect ejaculates are generally of two types. First, sperm may be free-swimming within a seminal fluid medium, as in *D. melanogaster*. Alternatively, the ejaculate may be transferred as a spermatophore, as in butterflies and springtails, where both sperm and non-sperm components are encased within a proteinaceous capsule. Spermatophores may adopt complex, multi-layered conformations as in the rove beetle *Aleochara cutula*. In this species, the male secretes a 'tube-like structure' into the female sperm storage organ, which serves to guide the elongation of a secondary tube that will eventually inflate and burst to release sperm.

General Functions of Male Insect Reproductive Gland Products

More than just a vehicle for sperm, seminal fluid components have been implicated in the induction of diverse female post-mating phenotypes. SFPs have been shown to be particularly important in driving these changes, at least in *D. melanogaster*. Roles of SFPs in females can be broadly categorized into those affecting behavior, physiology, or anatomy. Some SFPs also play an important role within the male, by processing other seminal proteins as they pass through the male's reproductive tract *en route* to the female. Others promote activities of the sperm within the female reproductive tract, such as the successful release of sperm from storage.

Modulation of female behavior

After mating, females of many insect species show reduced receptivity to re-mating. This change may be short-term, e.g., just a few days in the Mediterranean fruit fly *Ceratitis capitata*, or permanent, as suggested by some studies of the dengue vector mosquito *Ae. aegypti*. In *D. melanogaster* females, a ~2-week reduction in receptivity post-mating, accompanied by a suite of specific rejection behaviors, is controlled by the SFP sex peptide (SP; see [Section Sex peptide of *Drosophila melanogaster*](#)).

Injection of accessory glands, or their extracts, into virgin females can elicit some of the same phenomena as mating. Many of these effects can also be induced by injecting purified SP into the circulation of virgin females, indicating that the peptide can also

access its targets from the hemolymph. Similarly, in *Ae. aegypti*, injection of accessory gland extracts into the thorax of virgin females or implantation of whole accessory glands prevents re-mating. In *An. gambiae*, injection of accessory gland extracts into virgin females makes them less likely to remate. Furthermore, *An. gambiae* females mated to spermless males (with fully functional accessory glands) exhibit typical changes in post-mating behavior, such as the induction of blood-feeding and mating refractoriness, indicating that sperm are not needed for these responses.

Mating often also causes a change in female feeding behavior. Nutritional geometry experiments in house crickets (*Gryllus bimaculatus*) have demonstrated a shift in dietary preference towards protein-rich food sources following mating. In *D. melanogaster*, SP also elevates female appetite and leads females to favor sodium- and protein-rich food sources.

Modulation of female physiology

In the mated female, seminal molecules affect egg production and ovulation. Consumption of a spermatophore in the two-spot ladybird (*Adalia bipunctata*) is associated with an increase in oviposition rate. This effect is independent of female diet, suggesting that it is driven by molecules from the spermatophore rather than through broader nutritional effects. Injection of male accessory gland extracts into virgin female cotton bollworms (*Helicoverpa armigera*) is also associated with earlier egg maturation and oviposition. In *Aedes* species, increased egg-production is induced by accessory gland extracts. In *D. melanogaster* the analogous effect has been shown to be due to the activity of specific SFPs, such as SP and ovulin, which act through the female's nervous or neuromuscular systems (see [Section Modulation of female anatomy](#), below). An increase in egg-laying has been shown to be a mating-induced effect that is independent of sperm in *An. gambiae*, again suggesting a role for seminal fluid components.

Transfer of SP to *D. melanogaster* females induces an increase in juvenile hormone synthesis. This in turn is associated with diminished female sex pheromone production, vitellogenic oocyte progression, and reduced resistance to systemic bacterial infection. The latter is suggested to reflect trade-offs between investment in reproduction and self-maintenance. Conversely, SP activates expression of genes encoding antimicrobial (AMP) peptides, via the Toll and Imd pathways, perhaps to protect against sexually transmitted microorganisms. SP also influences gut physiology and growth, leading to the production of more concentrated excreta.

Modulation of female anatomy

The SFP ovulin (see [Section Ovulin of *Drosophila melanogaster*](#)) induces visible physical changes in the conformation of the *D. melanogaster* female reproductive tract. By stimulating octopaminergic signalling, ovulin relaxes the musculature surrounding the oviduct. This relaxation leads to the uncurling of a tightly coiled loop in the upper oviduct within 90-min of mating, allowing for transit of an oocyte. Another *Drosophila* SFP, Acp36DE, relaxes a constriction in the upper uterus, facilitating the passage of sperm into storage.

Supporting the activities of sperm

Across insects, ejaculate components can form novel structures within the female reproductive tract. These 'mating plugs' provide a physical barrier to the entry of sperm from rival males, and have been implicated in reducing female receptivity to remating.

In some butterfly species, the mating plug includes an external component that covers the female copulatory opening. In contrast, in some other insects, including *D. melanogaster*, the mating plug sits within the posterior uterus at the distal end of the reproductive tract. The *D. melanogaster* mating plug is bipartite, combining a posterior portion composed predominantly of ejaculatory bulb derived proteins such as PEBme (a protein with some similarity to homopolymer-forming proteins in spider-silk), PEBII, and PEBIII, with an anterior section composed of accessory gland proteins such as Acp36DE. While the posterior component forms within the first 5 min of mating, the anterior section is formed by around 20 min after the start of mating.

Mating plugs also function in promoting sperm storage and retention. The seminal fluid of *An. gambiae* coagulates to form a mating plug in females and is required for the initial entry of sperm into the female storage organs. The *An. gambiae* accessory gland-derived SFP transglutaminase cross-links the SFP Pluglin to form the mating plug, which is slowly degraded in the female reproductive tract over the course of 1–2 days.

Individual SFPs have been identified that regulate the storage of sperm in females. For example, *D. melanogaster* Acp36DE is required for sperm accumulation into the storage organs by 'corralling' sperm and by altering uterine conformation, as noted in [Section Modulation of female anatomy](#). Once in storage, the SFP Acp29Ab, a C-type lectin, is required for the retention of sperm, while the SFPs SP, seminase, CG1652, CG1656, CG9997, and CG17575 are required for efficient release of sperm from storage. In the hymenopteran species *Apis mellifera* and *Atta colombica*, fertility may be promoted by the viability enhancing effect of accessory gland secretions on sperm. However, in polyandrous lineages, this promotion of viability is restricted to self-sperm and actively decreases the viability of rival sperm.

Seminal Fluid Proteins in Focus

Sex peptide of *Drosophila melanogaster*

Transplantation of accessory glands and injection of their extracts into virgin females each initially implicated their products in driving post-mating reduction in female sexual receptivity and elevation of egg-laying. These effects were traced to a 36-amino acid 'sex peptide' (SP) by fractionation experiments and injection of purified sex peptide into the female abdominal cavity, as well as by ectopic expression of SP in transgenic virgin females, and phenotypic characterization of the effects of

RNAi-knockdowns or SP-knockouts. The name ‘sex peptide’ derives from early chromatographic studies by Fox, who noted the presence of male specific peptides in the male body fluid.

SP is secreted by ‘main cells’ into the accessory gland lumen, where it may reach quantities of 3.1pMoles. During mating, mature males transfer between 30% and 50% of the gland’s SP, and are seemingly able to exercise some control over the quantity that they transfer (see [Section Phenotypic plasticity](#)). SP alters the transcriptome of the mated female, significantly changing the level of RNAs relating to immunity, egg development, behavior, early embryogenesis, nutrient sensing, and phototransduction. In females, SP also induces many of the behavioral, physiological, and anatomical effects noted in [Section General Functions Of Male Insect Reproductive Gland Products](#), as well as endocrine changes through activation of juvenile hormone synthesis ([Fig. 1](#)).

SP acts through a G-protein coupled receptor (sex peptide receptor; SPR) primarily in neurons that innervate the female reproductive tract to induce the post-mating change in receptivity. The involvement of additional receptors has also been suggested. While SP’s precise neural targets are not yet known, the ultimate target of its signalling pathway is likely the brain. Targets in the brain may also contribute to the mechanism by which SP reduces daytime ‘siesta’ sleep and elevates aggression towards other females.

While SP is able to induce physiological change in females on its own, its effects are extended in the presence of sperm. Studies in the 1960s noted that the female post-mating response could be separated into a short- and long-term component. The former occurs within the first 48 h of mating and is independent of sperm, while the latter can extend beyond 10 days post-mating but requires sperm (the ‘sperm effect’). It has since been shown that SP binds to both the head and tail of sperm via the N-terminus region of the peptide, a process that depends upon the action of an interdependent network of SFPs. SP’s C-terminus, which induces much of the female post-mating response, is gradually released from the tails of stored sperm by cleavage of SP at a trypsin site by an unknown protease. Stimulation of juvenile hormone synthesis, which is achieved by the peptide’s N-terminus, is thought to be carried out by free SP in the ejaculate, prior to sperm-binding.

Ovulin of *Drosophila melanogaster*

Ovulin, a 264-amino acid prohormone-like seminal protein, is one of the most rapidly-evolving proteins in *Drosophila*. Found in both secretory cell types in the male’s accessory gland, ovulin is transferred to females during mating, where it acts to increase ovulation rate. In particular, it induces the ovulation of mature oocytes that have accumulated in the unmated female, clearing the way for the increased oogenesis triggered by SP.

During mating, some ovulin enters the mated female’s circulation; the remainder stays within her reproductive tract, localizing primarily to the upper oviduct and the base of the ovaries. In the female reproductive tract, ovulin is cleaved by the seminal metalloprotease Semp1. During ejaculation, Semp1 is activated by the serine protease SFP seminase as it transits through the male reproductive tract. Semp1 then cleaves ovulin only after it is transferred to the female ([Fig. 2](#)). Full-length ovulin and two of its cleavage products stimulate ovulation.

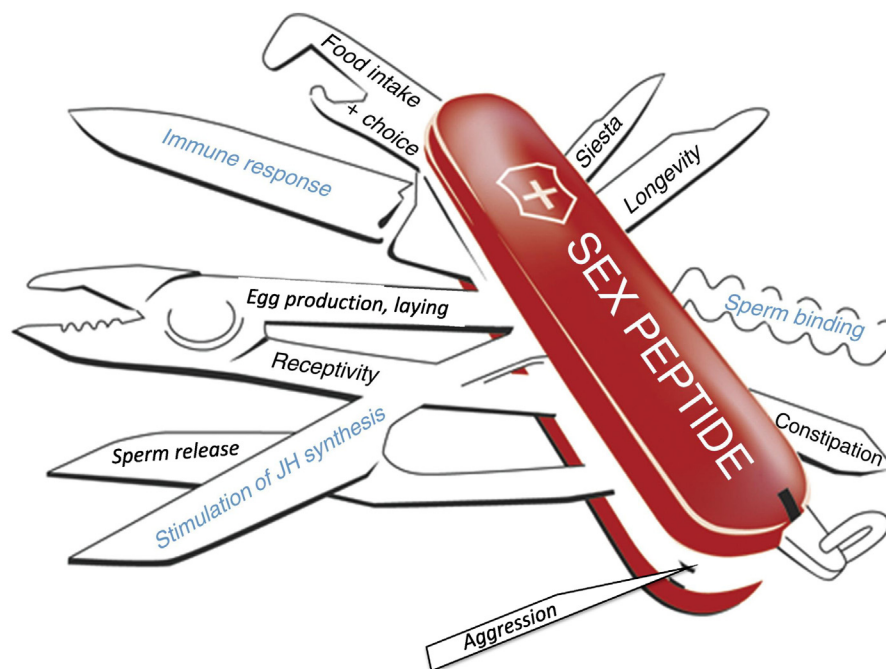


Fig. 1 The many effects induced by the seminal sex peptide, in mated female *Drosophila melanogaster*. Modified from Kubli, E., Bopp, D., 2012. *Curr. Biol.* 13, R520–R522.

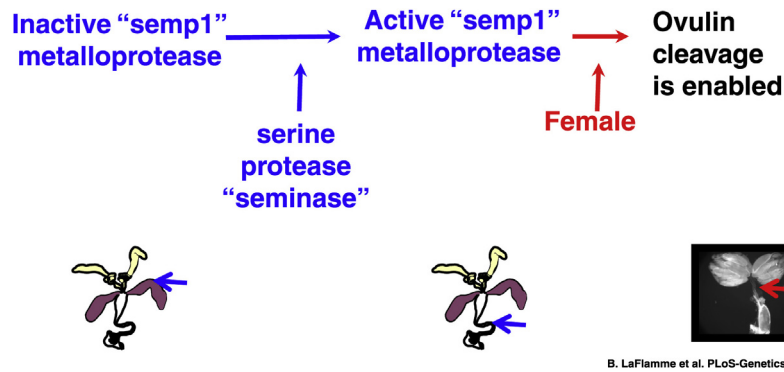


Fig. 2 The proteolytic pathway that cleaves ovulin. The seminal serine protease *seminase* cleaves the inactive precursor form of the seminal metalloprotease *Semp1* as the two move through the male during ejaculation. This cleavage activates *Semp1*, but the latter only cleaves its target, ovulin, once the proteins have entered the female. Arrows on the diagrams or photographs below the pathway show where the cleavages occur.

Ovulin increases octopaminergic signalling in the female's reproductive tract, although its site of action is unknown. As noted in **Section Modulation of female anatomy**, this leads to relaxation of the oviduct musculature, changing oviduct conformation, thus facilitating ovulation. Although ovulin is only detectable in the mated female for a few hours after mating, its action increases the number of boutons made by octopaminergic neurons on the oviduct musculature. Thus, ovulin's effects can in theory persist long after it has disappeared. However, its effects are only detectable on the first day post-mating; after that, other factors, such as SP activity, account for the increased egg production.

20-hydroxyecdysone of *Anopheles gambiae*

Although SFPs are the major mediator of female post-mating phenotypes in *Drosophila* and most insects that have been studied, in *An. gambiae* the steroid hormone 20-hydroxyecdysone (20E) acts as a major effector of post-mating changes. 20E is a common insect ecdysteroid that regulates molting in juvenile stages and multiple processes in adults, including controlling lifespan, learning, sleep, and social interactions.

An. gambiae males produce 20E in their accessory glands and pass it to the female reproductive tract during mating. Sexual transfer of 20E initiates a series of events that ends with the inhibition of female remating and the induction of oogenesis and oviposition. Furthermore, the mating-induced increase in oogenesis simultaneously decreases the efficiency of the *An. gambiae* immune system to kill the malaria-causing *Plasmodium* parasites.

Within the female *An. gambiae* reproductive tract, degradation of the mating plug occurs over a similar timeframe to the reduction in 20E level. Given that sexual transfer of 20E stimulates oviposition, that large titers of 20E are transferred to females within the mating plug, and the timing of the plug's degradation within the female, it may be that the plug acts as a store of 20E, gradually releasing the hormone following its transfer to females.

Interestingly, mating plug formation and 20E transfer may have coevolved in anopheline mosquitoes. Species that transfer high levels of 20E during mating produce a fully coagulated mating plug, and these species tend to be found in global regions where malaria transmission is high. Conversely, the species that have been studied from areas with low malaria transmission rates neither form a plug nor transfer 20E to females during mating. This has led to the suggestion that the divergent sexual transfer of 20E has influenced the ability of anopheline species to transmit malaria.

The Diversity of Insect Reproductive Glands

A typical insect male reproductive system consists of an ejaculatory duct, with an ejaculatory bulb at its base, and testes and accessory glands branching off from its proximal end. Products of these tissues meet during passage through the ejaculatory duct *en route* to the female.

Across insects, the accessory glands are the most studied contributor to the ejaculate. There is great morphological diversity among these glands (**Fig. 3**), ranging from *Drosophila*'s two lobes to the house cricket's (*Acheta domesticus*) tangled mass of several hundred tubules. Even within a single family, such as *Diptera*, there is considerable morphological diversity of accessory glands.

Species vary in accessory gland number. For example, male mealworm beetles (*Tenebrio molitor*) contain a pair of tubular accessory glands as well as a separate pair of bean-shaped accessory glands, whereas males of *Drosophila* species and the Colorado potato beetle (*Leptinotarsa decemlineata*) each have just a single pair of accessory glands. This morphological diversity is paralleled in the developmental origins of insect accessory glands, which may be ectodermal, mesodermal, or both. Indeed, in the Queensland fruit fly (*Bactrocera tryoni*), males have a pair of highly-innervated, sac-shaped mesoderm-derived glands as well as three or four pairs of spongy, distally bifurcated ectodermic glands.

The cellular complexity of accessory glands is also variable. For example, the bean-shaped glands of *T. molitor* contain seven distinct secretory cell types, whereas the accessory glands of *Drosophila* and *Leptinotarsa decemlineata* contain two and one visibly

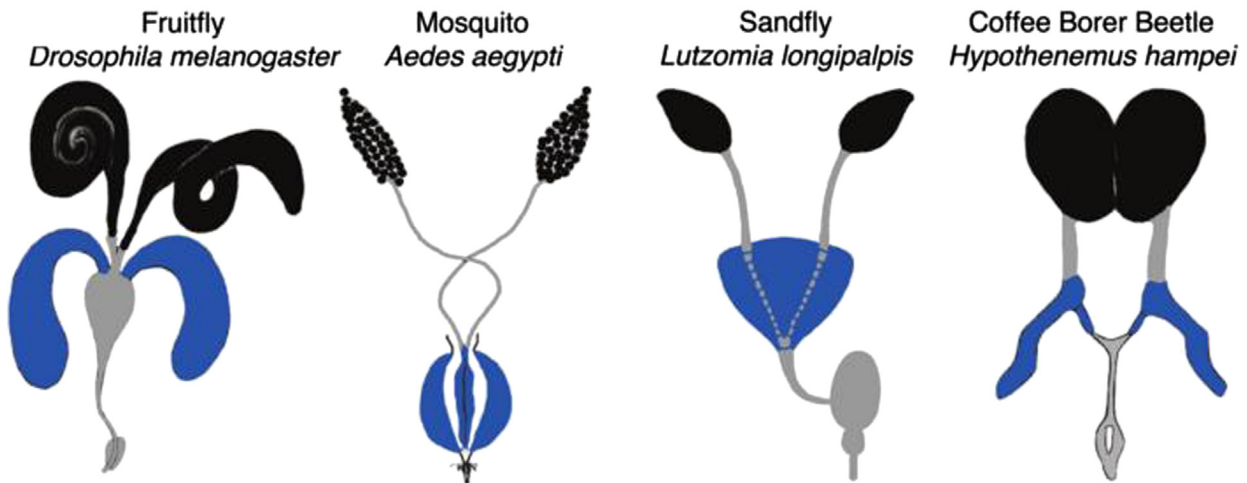


Fig. 3 Diagram of the male reproductive tracts of several insects, with reproductive glands shown in blue. Within-tract views are drawn to-scale. As noted in the text, male reproductive glands' developmental origins differ among insects so the blue tissues, although analogous in function, are not necessarily homologous. Drawing by S. Suarez is reprinted from McGraw, L.A., Suarez, S.S., Wolfner, M.F., *et al.*, 2015. *Bioessays* 37, 142–147 with permission.

different cell type respectively. The accessory glands of *Ae. aegypti* have anterior and posterior portions that differ in cell-type and density, as well as in the proteins – and potentially other secretory materials – they produce and contain. Transplantation experiments have demonstrated that the anterior accessory gland cells contain the active molecule(s) that elicit the post-mating female phenotypes of inhibited re-mating and increased egg-laying. Less is known about the posterior cells of the *Ae. aegypti* accessory gland, but they have been suggested to secrete a mucus-like substance that binds granules secreted from the anterior cells.

Different reproductive glands, as well as different cell types in these glands, work interdependently to produce the seminal fluid. For example, the *D. melanogaster* mating plug includes contributions from at least the ejaculatory bulb and accessory glands. In another case, spermatophores in beetles, crickets, and moths are composed of layers derived from the secretions of different reproductive glands. Among species within the suborder *Ensifera*, the 'rough glands' produce the larger spermatophylax component of the spermatophore, while the 'smooth glands' produce the ampulla, which bears the sperm. In the cabbage white butterfly (*Pieris rapae*), both the accessory glands and the distal section of the mating duct contribute to the soft inner matrix of the spermatophore, whereas the proximal region contributes most to the tough outer envelope.

The Accessory Glands of *Drosophila melanogaster*

Each lobe of the *D. melanogaster* male accessory gland is an epithelium that consists of a single layer of ~1000 binucleate secretory cells, encased by a muscular sheath (Fig. 4). The secretory cells belong to two morphologically and biochemically distinct types,

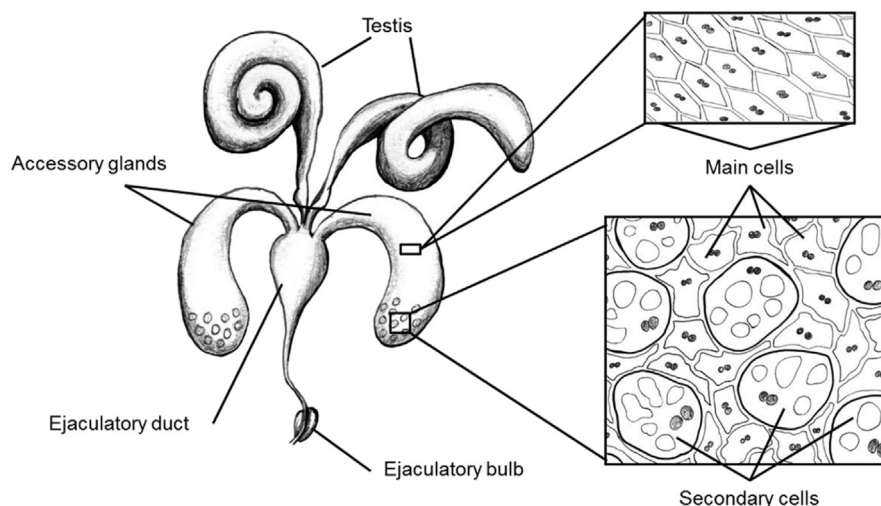


Fig. 4 The *Drosophila melanogaster* male reproductive tract. This drawing, approximately to scale, shows the male reproductive glands and testes. Insets show the position and appearance of the two secretory cell types of the male's accessory glands; nuclei in these binucleate cells are shaded. Drawing by J. Sitnik is reprinted from Gligorov, D., Sitnik, J.L., Maeda, R.K., *et al.*, 2013. *PLOS Genetics* e1003395, with permission.

whose development is differentially specified by the action of Hox, or other homeodomain, proteins. Small, flattened, and hexagonal in shape, the 'main cells' account for 96% of the secretory cells. Main cells produce SP, and several other SFPs including the proteases Semp1, seminase, and CG9997, as well as other proteins important for the binding of the sex peptide to sperm. At the distal tips of the accessory glands, ~40 secondary cells protrude into the lumen. These large, spherical cells contain numerous vacuoles as well as secretory granules and filamentous bodies that resemble structures found among sperm in the female storage organs. Secretion granule-like structures have also been observed in the accessory gland lumens of a number of insects, including species of leafhoppers, bees, and mosquitoes.

Secondary cells produce a cell-type specific set of SFPs including some of those required for the sperm-binding and storage of SP. Unlike main cells, secondary cells continue to grow throughout the life of the adult fly and, in older multiply-mated males, can delaminate from the apical surface of the epithelium, migrate towards the proximal end of the gland, and eventually can be transferred to females in the ejaculate. These processes are under the control of bone morphogenetic protein (BMP) signalling and seem to be accelerated by age (as well as mating frequency). The age-dependent growth and delamination has led to the suggestion that the *Drosophila* accessory gland, and its secondary cells in particular, can serve as a model for the human prostate.

BMP-signalling in the secondary cells also drives the secretion of small, ~30–100 nm diameter, extracellular vesicles (exosomes) and secretory organelles termed dense core granules. Each cell contains ~10 dense core granules, of which approximately 4 are lost during mating and are replenished within ~24 h. Blockage of BMP-mediated secretion in secondary cells impairs the male's ability to decrease remating by his mate, but does not affect his ability to stimulate her egg production. At least part of these effects have been suggested to depend on secondary cell exosomes, which have been reported to fuse with sperm once inside the female and to associate with female reproductive tract tissues. Such fusion could potentially introduce RNAs, proteins, or other molecules to the sperm or female tissues, thereby influencing the female's post-mating response. This model is based on studies in mammals, where the contents of exosomes secreted by malignant cells have been reported to prime nearby tissues for metastatic invasion, and where exosomes from male reproductive tissues have been shown to fuse with sperm and affect sperm activation, capacitation, and motility. These 'epididymosomes' and 'prostasomes' can deliver regulatory RNA species (including tRNA fragments, Y RNAs and microRNAs), as well as growth factors and cytokines, in combinations sensitive to the male's rearing environment and have been suggested to transmit information transgenerationally.

Evolution of Insect Reproductive Glands and Their Products

Several aspects of reproductive gland biology are commonly found among insects: the existence of accessory glands, along with other types of reproductive gland; the biochemical classes of the molecules that these glands produce; and the role of these products in inducing important post-mating responses such as increased egg laying, ovulation, immune gene expression, and feeding. Although many of the effects induced by seminal secretions appear to benefit both sexes in terms of their regulated fertility, some actions of seminal proteins can be deleterious to the female, decreasing her longevity or manipulating her behavior in ways that do not seem to serve her interests. To paraphrase Dobzhansky, in order to fully appreciate the biology of insect reproductive glands, we must understand the forces that have guided their evolution. These forces include:

Sexual selection

Due to their importance for reproductive success, male reproductive processes have been subject to strong and divergent selection. This concept of 'selection in relation to sex' was first laid out by Darwin 12 years after the publication of his theory of evolution by natural selection. 'Sickened' by the peacock's exaggerated plumage, Darwin sought to understand why such elaborate and seemingly deleterious traits can be maintained in populations. His solution came through focusing not on an individual's struggle for survival, but rather on its struggle for matings. Sexual selection theory is built on these conceptual foundations, positing that evolutionary change can be driven by (a) competition between individuals of one sex for access to individuals of the other (intrasexual selection), and (b) preferences held by members of one sex for certain features in the other (intersexual selection).

Post-copulatory sexual selection: Sperm competition

In 1970, Geoff Parker recognized that if a female mates multiply, as occurs in many insect species, a male's reproductive success depends on more than just his ability to compete for a mate. In such polyandrous mating systems, the opportunity for sexual selection continues after ejaculation through 'sperm competition', the competition between sperm from rival males for access to oocytes. In recent years, the development of *Drosophila* lines expressing green- or red-fluorescent proteins in sperm heads has facilitated the visualization of this process in these insects (Fig. 5).

Many traits promote the competitiveness of a male's ejaculate: changes to sperm morphology to promote faster swimming (even including the formation of motility-boosting aggregations), specialized genital morphologies that can physically displace a rival's sperm, or the physical guarding of a female from rival males. Products of the male's reproductive glands also contribute to these phenomena by affecting sperm storage and viability in females, potentially disabling the sperm of a rival, and by a chemical form of mate-guarding: rendering a female unreceptive to remating or stopping her production of 'calling' pheromones, thus preventing other males from being attracted to her.

Post-copulatory sexual selection: Cryptic female choice

The female, however, does not present a passive environment in which sperm competition plays out. Instead, she provides a partisan space in which the outcome of competition between rival males can be influenced in accordance with her own distinct evolutionary

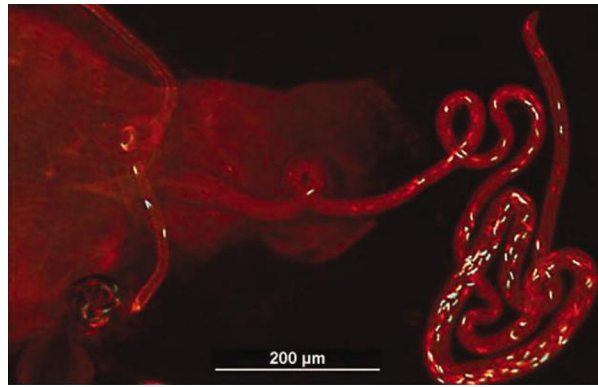


Fig. 5 Visualizing sperm competition in *Drosophila*. Sperm from two males are seen within the seminal receptacle of a doubly-mated female. The heads of the sperm from the first male are green (due to protamine-GFP); from the second male are red (protamine-RFP). Reprinted from Manier, M.K., Belote, J.M., Berden, K.S., *et al.*, 2010. *Science* 328, 354–357, with permission.

interests. This process, therefore, represents the post-copulatory analogue of inter-sexual selection and is named cryptic female choice. Males are expected to evolve mechanisms in response to female choice processes to boost the probability that their sperm will be preferentially retained for use in fertilizations. The roles of seminal fluid components in modulating female insect behaviors (such as sexual receptivity and sperm ejection), reproductive tract conformation, physiology, and immunity were likely shaped by their acting within a dynamic female environment.

Sexual conflict

Selection for traits that promote the success of a male in sperm competition can result in traits that cause collateral damage to females. This is thought to apply in the case of *Drosophila* SP, which limits female remating and thus sperm competition risk, but has toxic effects that shorten the female's lifespan. Additional SFPs, such as the *Drosophila* protease inhibitor Acp62F, have also been shown to be toxic to females.

Conflict can even arise between the interests of males and females long after mating. Individuals have a finite quantity of resources to allocate to reproduction. How a female should best partition these resources across successive breeding attempts depends on factors including mate quality, reproductive experience, and age. For example, her best strategy may be to withhold some resources in a given mating to allocate to her own immunocompetence, foraging ability, and/or somatic maintenance, thus boosting the probability that she survives to mate with a subsequent (possibly more fit) male. However, the optimal outcome for her mate is that she should invest at a high level in their brood, increasing the number or viability of his offspring. Thus, it is often not possible to simultaneously maximize the fitness of both male and female, giving rise to evolutionary tension.

These conflicts can lock males and females into co-evolutionary arms races, each responding over evolutionary time to the other's adaptations. For example, if males transfer seminal fluid components that lead to females over-investing in a current reproductive effort, selection for a counter-response could lead to reduced sensitivity of females to male molecules or mechanisms to neutralize the effects of those molecules. This, in turn, could drive renewed selection on males for new or stronger molecules to produce the male-beneficial effect. More complicated three-way evolutionary arms races arise when selection frees females from male control over their mating rate. This may intensify the degree of sperm competition, selecting for seminal fluid components in males that promote their sperm competitive success, but harm females in the process. These co-evolutionary processes are thought to contribute to rapid evolution in the primary sequence of a significant fraction of SFPs. The arms races may also help explain the diversity of seminal fluid products, evolving in response to female counter-evolution.

The conflicts described occur in the context of a process, reproduction, that also requires cooperation between the sexes: coordination is required between male and female cells and molecules, for egg-sperm binding, or for the processing of certain SFPs in *D. melanogaster* by molecules from both sexes. The extent to which conflict should manifest in reproductive processes will depend upon the degree of asymmetry in the fitness interests of interacting parties. In species with lifetime monogamy (where that monogamy is not enforced by one sex against the evolutionary interests of the other), male and female fitness interests will align closely. Moreover, conflict traits may not evolve even when male and female fitness interests diverge: the extent of the costs and benefits associated with antagonistic traits, as well as the opportunity for such traits to evolve (e.g., presence of sufficient genetic variation), can constrain the manifestation of conflict.

Phenotypic plasticity

Depletion of sperm and SFPs over successive matings constrains male fitness. In *D. melanogaster*, for example, SFP replenishment after mating can take ~3 days. Therefore, selection should favor the male's allocating these products in line with the reproductive context in which he finds himself. Consistent with this hypothesis, males have evolved to strategically allocate sperm and SFPs to females in response to factors such as female mating status, sexual novelty, and quality. In *D. melanogaster*, males transfer less ovulin when mating with recently mated females, but more sex peptide and ovulin when mating in the presence of rival males.

Concluding Remarks

Beyond the sperm and egg, a diversity of seminal fluid molecules are essential for the reproductive success of insects, as in many other animals. Seminal fluid components regulate processes that move sperm through the female reproductive tract and into storage, modify the reproductive tract to facilitate egg production and transit, and influence feeding and digestion to assist in high levels of egg production. Effects of seminal fluid components on females may be beneficial to both members of the mating pair, such as by coupling increased egg production to mating. Alternatively, they may primarily benefit the male, as is likely the case in their induction of female refractoriness to remating. Seminal fluid molecules are produced in male reproductive glands, which themselves vary across insects in number, structure, and cellular constitution. Ejaculate molecules fall into biochemically-conserved classes, but their sequences have come under strong selection to boost reproductive success under sperm competition situations or to co-evolve with reproductive processes in females. Nevertheless, the overlap in molecular and organellar types in the seminal fluid of insects and other taxa makes it increasingly apparent that the study of insect accessory glands provides significant and broad insights into mechanisms of fertility, has implications for our understanding of human male reproductive gland health and disease, and offers powerful opportunities for targeted control of insect disease vectors and pests.

Acknowledgements

We thank Dr. M. Skinner for the invitation to write this review and NIH grants R01-HD038921, R01-HD059060, and R01-AI095491 (to MFW, MFW & AG Clark, and MFW & LC Harrington, respectively), COLCIENCIAS, Max Planck Society and University of Antioquia Cooperation agreement grant 566-1 (to FWA), and an EMBO Short-Term Research Fellowship (to BRH) for support. We thank D. Chen, N. Gillies, and E. Bath for comments on the manuscript. We apologize to authors whose papers we could not cite directly due to the limited number of citations permitted.

Further Reading

- Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D., & Wolfner, M. F. (2011). Insect seminal fluid proteins: Identification and function. *Annual Review of Entomology*, 56(1), 21–40.
- Bath, E., Bowden, S., Peters, C., et al. (2017). Sperm and sex peptide stimulate aggression in female *Drosophila*. *Nature Ecology & Evolution*, 1, 154.
- Birkhead, T. R., & Pizzari, T. (2002). Postcopulatory sexual selection. *Nature Reviews Genetics*, 3(4), 262–273.
- Chen, P. S. (1984). The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annual Review of Entomology*, 29, 233–255.
- den Boer, S. P. A., Baer, B., & Boomsma, J. J. (2010). Seminal fluid mediates ejaculate competition in social insects. *Science*, 327(5972), 1506–1509.
- Eberhard, W. G. (1996). *Sexual Selection by Cryptic Female Choice*. Princeton University Press.
- Gillott, C. (2003). Male accessory gland secretions: Modulators of female reproductive physiology and behavior. *Annual Review of Entomology*, 48, 163–184.
- Hopkins, B. R., Sepil, I., & Wigby, S. (2017). Seminal fluid. *Current Biology*, 27(11), R404–R405.
- Manier, M. K., Belote, J. M., Berben, K. S., et al. (2010). Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science*, 328(5976), 354–357.
- Mattei, A. L., Riccio, M. L., Avila, F. W., & Wolfner, M. F. (2015). Integrated 3D view of postmating responses by the *Drosophila melanogaster* female reproductive tract, obtained by micro-computed tomography scanning. *Proceedings of the National Academy of Sciences of the United States of America*, 112(27), 8475–8480.
- McGraw, L. A., Suarez, S. S., & Wolfner, M. F. (2014). Insights & perspectives on a matter of seminal importance. *Bioessays*, 37(2), 142–147.
- Meslin, C., Cherwin, T. S., Plakke, M. S., et al. (2017). Structural complexity and molecular heterogeneity of a butterfly ejaculate reflect a complex history of selection. *Proceedings of the National Academy of Sciences of the United States of America*, 114(27), E5406–E5413.
- Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, 45(4), 525–567.
- Perry, J. C., Sirot, L., & Wigby, S. (2013). The seminal symphony: How to compose an ejaculate. *Trends in Ecology and Evolution*, 28(7), 414–422.
- Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioral Ecology and Sociobiology*, 60(3), 289–310.
- Rice, W. R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, 381(6579), 232–234.
- Rogers, D. W., Baldini, F., Battaglia, F., et al. (2009). Transglutaminase-mediated semen coagulation controls sperm storage in the malaria mosquito. *PLOS Biology*, 7(12), e1000272.
- Rubinstein, C. D., & Wolfner, M. F. (2013). *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 110(43), 17420–17425.
- Sirot, L. K., Wong, A., Chapman, T., & Wolfner, M. F. (2015). Sexual conflict and seminal fluid proteins: A dynamic landscape of sexual interactions. In W. Rice, & S. Gavrilets (Eds.), *The Genetics and Biology of Sexual Conflict* (pp. 49–72). Cold Spring Harbour Laboratory Press.
- Swanson, W. J., & Vacquier, V. D. (2002). The rapid evolution of reproductive proteins. *Nature Reviews Genetics*, 3(2), 137–144.
- Wedell, N., Gage, M. J. G., & Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *Trends in Ecology and Evolution*, 17(7), 313–320.
- Wilson, C., Leiblich, A., Goberdhan, D.C.I., Hamdy, F. 2017. The *Drosophila* accessory gland as a model for prostate cancer and other pathologies. *Current Topics in Developmental Biology* 121, 339–375.
- Yapici, N., Kim, Y.-J., Ribeiro, C., & Dickson, B. J. (2008). A receptor that mediates the post-mating switch in *Drosophila* reproductive behaviour. *Nature*, 451(7174), 33–37.

PRIMORDIAL GERM CELLS, SEX DETERMINATION AND DIFFERENTIATION

Primordial Germ Cells of *Drosophila melanogaster*

Leif Benner, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, United States; and Johns Hopkins University, Baltimore, MD, United States

Girish Deshpande, Princeton University, Princeton, NJ, United States

Dorothy A Lerit, Emory University School of Medicine, Atlanta, GA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chromatin DNA wrapped around Histone proteins, the modification of which contributes to gene regulation.

Embryo A fertilized egg.

Dynein Molecular motor that hydrolyzes ATP and transports cargo toward the minus-end of microtubules.

Germ plasm Maternally deposited mRNAs and proteins that confer the germline fate, also known as pole plasm.

Kinesin Molecular motor that hydrolyzes ATP and transports cargo toward the plus-end of microtubules.

Maternal-to-zygotic transition A program where the maternal contribution of gene expression ends and zygotic transcription begins.

Microtubules Polarized cytoskeleton polymer comprising α and β -Tubulin dimers.

Nuclear division Mitotic division without cytokinesis occurring within syncytial embryos.

Oocyte The developing egg.

Syncytium A single cell containing multiple nuclei.

Nomenclature

CTD C-terminal domain (of RNA Polymerase II)

Dsx Doublesex

Gcl Germ cell-less

Jak/STAT Janus kinase/Signal transducer and activator of transcription

Nos Nanos

Osk Oskar

Otu Ovarian tumor

Phf7 PHD finger protein 7

PGC Primordial germ cell

Pgc Polar granule component

RNAPI RNA Polymerase I

RNAPII RNA Polymerase II

RNP Ribonucleoprotein

Sxl Sex lethal

STAT92E Signal-transducer and activator of transcription protein at 92E

Tra Transformer

Tra2 Transformer 2

Upd Unpaired

Vas Vasa

Germ Plasm Assembly

Specification of the germline progenitor cells occurs during early embryogenesis. In *Drosophila*, the primordial germ cells (PGCs) are specified about an hour after fertilization through the inheritance of a specialized cytoplasm known as germ (or pole) plasm. Germ

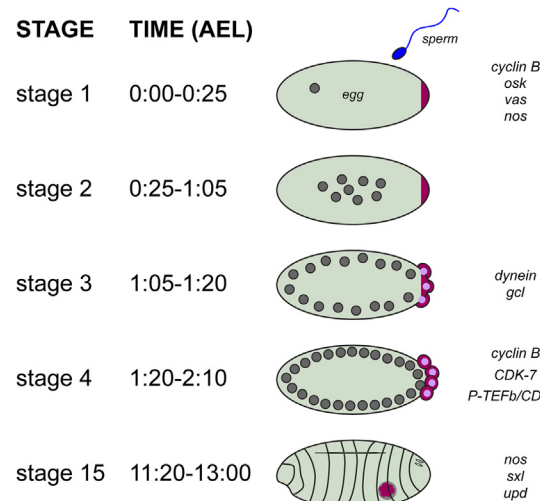


Fig. 1 Progression of PGC development in *Drosophila* embryos. Embryonic stages and approximate time after egg laying (AEL) are indicated. Several genes highlighted in the text as important for PGC development are listed. Embryogenesis commences upon fertilization of the oocyte/egg. At this time, germ plasm (purple) is already localized at the posterior pole. Once syncytial nuclei (grey) are proximal to the germ plasm (stage 3), dynein shuttles germ plasm to surround the posterior nuclei, which are encapsulated by pole buds. By stage 4, PGC cellularization is complete. Programs of transcriptional and mitotic quiescence ensue. Sex determination begins during stage 4, but is completed in late embryogenesis (stage 15 and beyond).

plasm is enriched in maternally-deposited mRNAs and proteins that specify the germline fate **Fig. 1**. The germ plasm is assembled prior to fertilization in the developing oocyte and commences with the localization of *oskar* (*osk*) mRNA, which is both necessary and sufficient to initiate germ plasm assembly (Mahowald, 2001). The mechanisms of *osk* localization are well-studied and entail the transport of *osk* together with associated RNA-binding proteins that function as RNA localization factors, such as Staufen, via kinesin motors. Due to the inherent polarization of the oocyte microtubule network, *osk* becomes enriched to the oocyte posterior pole. Once localized, *osk* and downstream germ plasm components are anchored in place to the posterior actin cortex, the organization of which requires several actin-binding proteins, including Tropomyosin, Moesin, Myosin V, Lasp, Mon2, and others (Tanaka *et al.*, 2011; Suyama *et al.*, 2009; Krauss *et al.*, 2009; Lantz *et al.*, 1999). Deregulation of actin organization is sufficient to impair *osk* localization and germ plasm assembly.

Localization of *osk* mRNA is required for the subsequent translation of Osk protein, which contributes to the anchoring of *osk* mRNA by a positive-feedback mechanism (Mahowald, 2001). Synthesis of Osk protein promotes the recruitment and assembly of other germ plasm components, including the RNA helicase, Vasa (Vas), which functions in piRNA synthesis and is also implicated in translation activation (Lasko, 2013). Subsequent recruitment of *nanos* (*nos*) is needed to silence soma-specific genetic programs, such as transcription, mitosis, and apoptosis to permit germline development (Mahowald, 2001; Hayashi *et al.*, 2004). Upon their localization to the posterior pole, germ plasm mRNAs and proteins are sorted and assembled into ribonucleoprotein (RNP) complexes. RNP assembly promotes the efficient segregation of germ plasm into nascent PGCs by permitting the simultaneous trafficking of multiple RNAs and proteins (Lerit and Gavis, 2011). Not all germ plasm components are trafficked into the PGCs, and proper sorting is essential for germline development (Little *et al.*, 2015). Dysfunction of *osk* localization, germ plasm assembly, or germ plasm passage into the PGCs are all sufficient to disrupt germline development. Historically, many of the original maternal effect sterile mutations (e.g., *osk*, *nos*, and *vas*) were identified by their grandchildless phenotype.

Primordial Germ Cell Formation

One conserved feature of PGCs is that they are typically set aside from the surrounding soma within the first hours of development. Because *Drosophila* embryos are syncytial for the first two hours of embryogenesis, normal development of both the soma and the PGCs requires the sequestration of the germ plasm from nearby somatic nuclei. As embryogenesis proceeds, the syncytial nuclei undergo repeated rounds of synchronous divisions. Concurrently, the nuclei migrate from the center of the embryo towards the cortex. Nuclear division and migration is coordinated by the centrosomes, which are microtubule-organizing centers. Ultimately, nearly 6000 nuclei surround the embryo cortex to form the syncytial blastoderm.

As microtubules propel the syncytial nuclei towards the cortex, a subset of nuclei will contact the germ plasm localized at the posterior pole and initiate the formation of the PGCs (Mahowald, 2001). The physical proximity of centrosomes associated with the migrating nuclei is sufficient to initiate a series of coordinated actin rearrangements that promote the budding of the posterior cortex (Warn *et al.*, 1985) and release of the anchored germ plasm RNPs (Lerit and Gavis, 2011; Lerit *et al.*, 2017). The proximal microtubules serve as tracks for the dynein-dependent active transport of germ plasm RNPs towards the centrosomes, resulting in the enrichment of germ plasm associated with each nucleus within the posterior pole region. This active transport

ensures the majority of the germ plasm is properly partitioned into the PGCs. The posterior nuclei complete their migration into the growing pole buds while continuously recruiting additional germ plasm RNPs until the formation of a contractile ring at the base of each bud completes their cellularization (Warn *et al.*, 1985). Formation of the PGCs occurs during nuclear division cycle 10, whereas the remaining somatic nuclei of the syncytial embryo continue to divide synchronously prior to cellularization at nuclear cycle 14 (Foe and Alberts, 1983).

Functional centrosomes are required for both the formation of the normal number of PGCs and ensuring that the correct quantity of germ plasm is inherited by each cell. The localization of germ plasm to the PGCs is important for two reasons. First, it prevents dilution of the germline determinants, which provide both instructive and permissive signals for germline development. Second, a critical level of germ plasm instructs cell fate. While ectopic germ plasm is sufficient to transform soma into germline, diminished germ plasm inheritance impairs PGC specification and produces cells with somatic characteristics (Lerit and Gavis, 2011).

Specification of Germ Cell Identity

Newly Formed PGCs Differ From the Soma

In addition to precocious cellularization, newly formed PGCs differ from the surrounding soma in several respects. The first difference is mitotic potential. During nuclear cycle 10, the newly formed PGCs undergo 0–2 rounds of asynchronous mitotic division prior to arresting in cell cycle phase G2. *Nos* functions to regulate Cyclin B activity in PGCs and halt their mitotic potential (Mahowald, 2001). In contrast, somatic nuclei of the syncytial embryo complete four additional rounds of synchronous divisions prior to their cellularization at nuclear cycle 14 (Foe and Alberts, 1983). A second difference is transcriptional activity. As in many organisms, early *Drosophila* development is supported by maternally-deposited mRNAs and proteins. The switch to zygotic transcription, the maternal-to-zygotic transition, commences in the soma beginning in nuclear cycles 10–11. In PGCs, however, transcription is attenuated (Nakamura and Seydoux, 2008). Evidence for a global downregulation of transcription in PGCs comes from studies of RNA Polymerase II (RNAPII) regulation (Deshpande *et al.*, 1999; Van Doren *et al.*, 1998). Although PGCs contain comparable levels of RNAPII relative to somatic cells, the RNAPII localized to PGCs lacks key activating post-translational modifications Fig. 2. The phosphorylation status of two serine (Ser) residues within the heptad repeats of the RNAPII C-terminal domain (CTD) serves as an indicator of RNAPII activity (Phatanani and Greenleaf, 2006). Phosphorylation of Ser5 is catalyzed by CDK-7 and likely mediates transcription initiation and promoter clearance. In contrast, phosphorylation of Ser2 is catalyzed by P-TEFb/CDK-9 and mediates transcription elongation. Increased levels of phosphorylated Ser2 and Ser5 in somatic nuclei mark the onset of zygotic transcription, whereas the RNAPII CTD remains largely unmodified in the PGCs. Further transcriptional repression is achieved specifically in PGCs via repressive chromatin marks. Germ cells in *Drosophila* and *C. elegans* lack methylated lysine 4 on Histone H3 (H3meK4), a chromatin mark associated with transcriptional activity (Schaner *et al.*, 2003). The global downregulation of RNAPII-dependent transcription is a conserved feature of embryonic germ cells in diverse organisms, including *C. elegans* and *M. musculus* (Saga, 2008; Seydoux and Dunn, 1997). As opposed to RNAPII-dependent mRNA synthesis, rRNA transcription controlled by RNAPI remains active in both germline and soma, rendering them translationally active (Van Doren *et al.*, 1998). Thus, PGCs exhibit delayed initiation of zygotic transcription and rely on maternally-provided mRNAs and proteins for their specification and development.

Protecting and Promoting PGC Identity

Special mechanisms protect PGCs from the signaling systems that engineer somatic development and differentiation. In the absence of such mechanisms, PGCs may lose their capacity to develop into germline stem cells (GSCs) and enter somatic differentiation. In *Drosophila*, at least two different mechanisms are believed to insulate PGCs from their environment and ensure their eventual progression to GSCs. One of these is the physical sequestration of PGCs from the soma as discussed above in *Primordial Germ Cell Formation*. Another mechanism is mediated by three maternally deposited, cell autonomous factors that impose transcriptional quiescence by downregulating RNAPII activity: *germ cell-less* (*gcl*), *polar granule component* (*pgc*), and *nos* (Leatherman *et al.*, 2002; Martinho *et al.*, 2004; Kobayashi *et al.*, 1996).

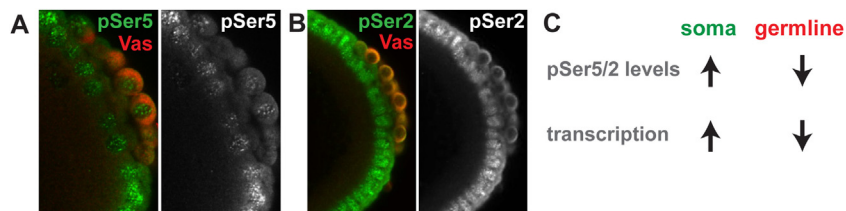


Fig. 2 RNAP II activity is downregulated in PGCs. Phosphorylation of Ser5 and Ser2 (pSer5 and pSer2) on the CTD of RNAP II promotes mRNA transcription. (A) Levels of pSer5 (green) are diminished in PGCs (Vas, red) relative to the proximal somatic cells. (B) Levels of pSer2 (green) are significantly reduced in PGCs (Vas, red), but abundant in somatic cells. (C) Table summarizes the relative enrichment of pSer5/2 and the predicted transcriptional output in soma versus germline cells.

To achieve establishment and/or maintenance of transcriptional silencing in PGCs, *gcl*, *pgc*, and *nos* employ distinct mechanisms. Namely, they act at different times during the early nuclear cycles and target an overlapping but distinct set of somatically active genes. For example, *gcl* acts between bud formation (nuclear cycle 10) and nuclear cycles 12–13. Thus, *Gcl* is involved in silencing genes such as *scute*, *sisterless a*, and *runt* that are selectively upregulated in the soma prior to the global onset of zygotic transcription (Leatherman *et al.*, 2002). In contrast, *pgc* and *nos* are required later after the onset of zygotic transcription to block the expression of somatic patterning genes. While *Pgc* encodes a short peptide that functions to inhibit CDK-9 and block transcriptional elongation of targets such as *zen* and *tailless* (Deshpande *et al.*, 2004), *Nos* functions cooperatively with Pumilio to prevent expression of targets such as *even-skipped* and *fushi-tarazu*, likely by repressing the translation of a key transcriptional regulator(s) (Deshpande *et al.*, 1999). Of the three transcriptional regulators, mutations in *nos* have the most profound effects both on transcription and PGC specification. In addition, *Nos* regulates numerous other targets to orchestrate subsequent steps in PGC development and viability.

The fact that *Drosophila* utilizes three distinct factors to inhibit RNAPII supports a model where PGC specification requires a global downregulation of mRNA transcription. Nonetheless, escaper PGCs can form, survive, proliferate, and give rise to a functional gonad in the absence of *gcl* or *pgc*. Although such animals are not completely sterile, they have low fecundity, indicating reduced fitness. On the contrary, germ cell-specific loss of *nos* activity is sufficient to induce sterility, demonstrating an essential role for *nos* in germline development (Deshpande *et al.*, 1999). Although PGCs normally repress RNAPII activity to attenuate mRNA transcription, they do maintain active transcription of rRNA mediated by RNAPI (Van Doren *et al.*, 1998; Seydoux and Dunn, 1997). Because rRNA is an essential component of ribosomes, these findings highlight the role of translational control of gene expression in PGC development.

Primordial Germ Cell Sex Determination

PGCs form far from the site of the future gonad. Upon gastrulation, *Drosophila* PGCs migrate dorsally along the surface of the embryo, invaginate into the midgut cavity, and then undergo trans-epithelial migration across the midgut to find their way through the mesoderm to populate the gonad (Mahowald, 2001). Here, PGCs are surrounded by somatic gonadal precursor cells that support gonad development. Within the gonad, PGCs develop into the germline that gives rise to sex-specific gametes. *Drosophila* sex determination is dependent on the karyotype of the sex chromosomes within somatic cells where XX flies are female and produce eggs while XY flies are male and produce sperm. The number of X chromosomes serves as the key determinant of sexual identity. In contrast, the Y chromosome has no role in sex determination but does function in sperm maturation. In order to give rise to their respective gametes, it is important that sex-specific genetic programs are established during PGC development.

Somatic Sex Determination

Somatic sexual identity is determined downstream of the sex chromosome karyotype and is specified during early embryogenesis prior to gonadogenesis. XX females activate the expression of *Sxl*, the master sex regulatory gene, from an early embryonic promoter, *Sxl-Pe*, at nuclear cycle 12, shortly after PGC formation. This is followed by *Sxl* expression from a late promoter, *Sxl-Pm*, in both sexes at nuclear cycle 14, after somatic cellularization and full activation of the maternal-to-zygotic transition. The presence of *Sxl* protein in XX females potentiates an autoregulatory loop where it binds to *Sxl* transcripts and causes skipping of an exon containing an early stop codon, thus allowing for the continued synthesis of functional *Sxl* protein. *Sxl* transcripts in XY males contain the early stop codon and, therefore, are unable to produce functional protein. A cascade of alternative splicing events involving *Tra*/*Tra2* downstream of *Sxl* eventually produce a female-specific isoform of the transcription factor *Dsx^F*, while the default isoform, *Dsx^M*, is male specific. These sex-specific *Dsx* isoforms instruct the majority of somatic sex differentiation (Oliver, 2002).

Germ Cell Sex Determination

The sexual identity of germ cells is specified by intrinsic signals based on their sex chromosomes, as well as extrinsic signals derived from the surrounding soma. We will first discuss autonomous factors determined by the karyotype, then we will describe the non-autonomous influence of the surrounding somatic cells.

Germ cells with two X chromosomes initiate an autonomous genetic program to promote female sexual identity. The determination of female sexual identity is downstream of a genetic hierarchy starting with the transcription factor *Ovo* (Oliver, 2002). Whereas XX germ cells show high levels of *ovo* expression, XY germ cells show little to no *ovo* expression. Consistent with this finding, *ovo* activity is necessary for XX germ cell viability but is dispensable in XY germ cells. An important downstream effector of *ovo* is the *otu* locus. In females, the high levels of *ovo* activity promotes the expression of *otu*, which in turn regulates the expression of *Sxl* through an unidentified mechanism. Thus, the genetic pathway necessary for specifying female sexual identity is defined by a cascade wherein *ovo* → *otu* → *Sxl* Fig. 3. Consistent with a role in promoting normal germline development, mutations in *ovo*, *otu*, or *Sxl* result in ovarian tumors and are female sterile.

Expression of *Sxl*, the key regulator of female germline identity, normally occurs at embryonic stage 9 in XX PGCs. Induction of female-specific *Sxl* in stage 9 XY PGCs transplanted into XX embryos is sufficient to switch the sexual identity of the PGCs. In contrast, ectopic expression of *Sxl* later during embryogenesis at stage 15 is insufficient to promote female identity in XY PGCs

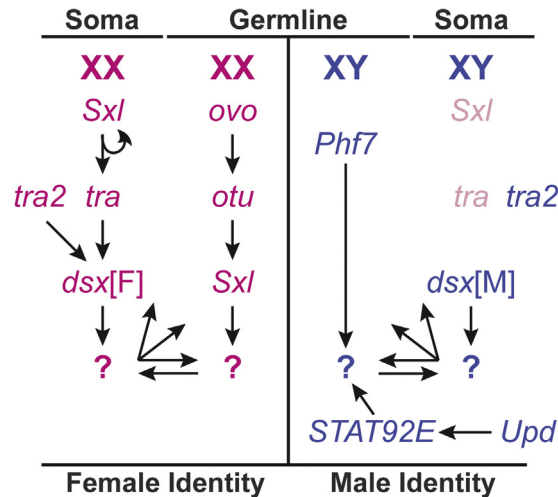


Fig. 3 PGC sex determination. PGC sexual identity is dependent on the sexual identity of both the PGC and surrounding somatic cells. During female (dark pink text) somatic sex determination, Sxl sets up an alternative splicing cascade eventually resulting in the presence of Dsx[F]. In female PGCs, Ovo promotes female identity and eventually results in the expression of Sxl at the bottom of the hierarchy. Downstream targets of Sxl have yet to be determined. In males (blue text), the absence of functional Sxl or tra (light pink text) results in the default Dsx[M] splicing, which carries out male somatic sex determination. The absence of Sxl in PGCs results in a male germline identity. Other factors, such as autonomous Phf7 and the non-autonomous Jak/STAT signaling cascade, promote male identity in PGCs as well. The interplay between autonomous and non-autonomous signaling has yet to be elucidated, and it is not determined where in the hierarchy these cascades influence each other in both female and male signaling. Arrows indicate signaling cascades, question marks (?) denote unidentified factors.

but results in germline tumors. Thus, the establishment of female PGC sexual identity likely occurs between embryonic stage 9 and 15, but this program cannot completely override the male sex determination pathway once it is established (Hashiyama *et al.*, 2011).

While Sxl promotes the female identity in PGCs during early embryogenesis, Phf7 is a factor active later in development that promotes male sexual identity. Phf7 is expressed around stage 17 of embryogenesis, after the establishment of sexual dimorphism in the germ cells of the coalesced gonad. Phf7 recognizes H3me2K4, a chromatin mark associated with transcriptional activity (Yang *et al.*, 2012).

Supporting the involvement of non-autonomous signaling in germ cell sex determination, aberrant PGC development ensues when they are transplanted into the somatic environment of the opposite sex. For example, XY germ cells surrounded by an XX soma form tumors filled with cells that are morphologically similar to early spermatocytes. By contrast, XX germ cells surrounded by an XY soma fail to develop. Alternative methods to transplantation, aimed at switching the somatic sex by targeting the canonical sex determination pathway at the level of Tra activity, generally mimic the results of transplantation studies (Oliver, 2002). Likewise, overexpression of Phf7 in XX PGCs in a female somatic environment leads to cell death, while expressing Phf7 in XX PGCs that are transplanted into a male soma is sufficient to induce a male sexual identity (Yang *et al.*, 2012).

One significant effector of the somatic sexual identification pathway is the dsx locus. Male and female somatic cells express sex-specific Dsx isoforms. There have been relatively few insights into the somatic factors downstream of sex-specific Dsx isoforms that constitute the extrinsic sex determination cues that influence PGC sexual identity. In males, Jak/STAT signaling promotes an increase in XY PGC cell number, one of the first sexually dimorphic characteristics. Stage 15 male somatic cells express the activating Jak/STAT ligand Unpaired (Upd), resulting in expression of the transcription factor STAT92E in the male germline. Loss of this cascade results in a reduction of XY germ cells. While Jak/STAT signaling is necessary for male PGC development, it is not sufficient (Wawersik *et al.*, 2005). Somatic induction of Upd in female somatic cells is able to activate STAT92E resulting in increased PGC number, however, female germ cells are still maintained and produce viable offspring. Thus, the ectopic somatic influence of Upd is not able to override the female PGC sex determination program. Taken together, these findings suggest that while PGCs are sensitive to sex-specific signals from their somatic environment, such signals alone are not necessarily sufficient to override or reset the cell autonomous sex determination program.

Presently, mechanisms that direct PGC sex determination are little understood. While Ovo activity is predominantly responsive to the germ cell karyotype, it is also influenced by the somatic environment. Somatic signals can also influence PGC sexual identity downstream of ovo. Therefore, it is still relatively ambiguous as to how and when somatic signaling influences germline sexual identity. Additional uncertainty underlies the mechanism of how Sxl defines female sexual identity in PGCs. While Sxl is necessary and sufficient to induce a female genetic program in PGCs beginning at embryonic stage 9, how this alternative splicing factor carries out this function remains unknown. Likewise, the male-specifying factor Phf7 induces a male sexual identity in PGCs later in development, although upstream and downstream regulatory factors remain to be identified. Other sex-specific signals downstream of the canonical sex determination pathway from the surrounding soma, such as STAT92E, also influence PGC sexual identity. But, again, precisely how Jak/STAT influences germline sexual identity is unclear. The interplay between these pathways is currently the

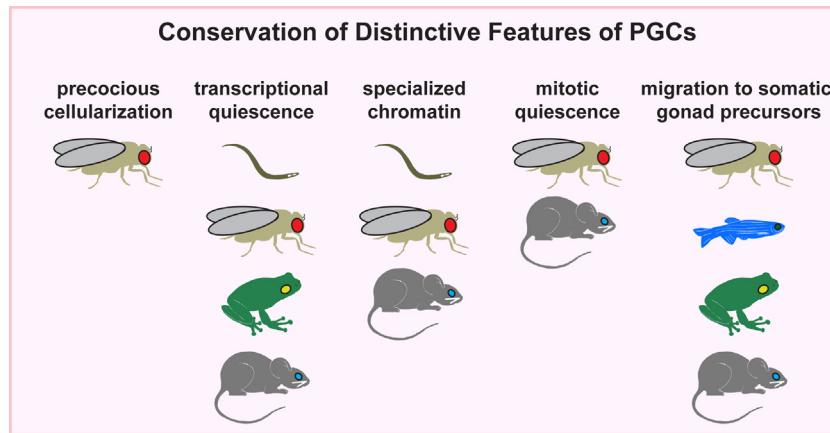


Fig. 4 Cartoon schematic showing the germline versus soma dichotomy. PGCs differentiate from the surrounding somatic cells through distinct regulation of genetic programs and signaling pathways. This table shows a few key differences that distinguish the PGCs from somatic cells (bold text in top row). Many of these differences are evolutionarily conserved among the species illustrated: *Drosophila* (fly), *C. elegans* (worm), *D. rerio* (zebrafish), *Xenopus* (frog), and *M. musculus* (mouse). Animals are not drawn to scale.

main focus of the field. Deciphering the inputs and crosstalk between these signaling cascades will likely elucidate a more comprehensive understanding of PGC sex determination.

Concluding Remarks

The germline-soma dichotomy is established early during embryogenesis of diverse organisms. Specification and isolation of the germline ensures proper determination of the PGCs and also protects the surrounding soma from cell fate transformation. One example of this paradigm is observed through the precocious cellularization of PGCs during the first hours of *Drosophila* embryogenesis. For many organisms, such as *Drosophila*, *C. elegans*, *Xenopus*, and *D. rerio*, the PGCs form following the inheritance of maternally-deposited germ plasm. Other organisms, including *M. musculus* and *Homo sapiens*, form PGCs by inductive cell-signaling cascades (Strome and Updike, 2015). It is interesting to note that despite these differences, many facets of the germline-soma dichotomy are conserved Fig. 4. For example, PGCs display transcriptional quiescence in *Drosophila*, *C. elegans*, *Xenopus*, and *M. musculus* through attenuation of RNAPII activity. Further global downregulation of transcription is achieved by regulating activating or repressive histone modifications, as described in *Drosophila*, *C. elegans*, and *M. musculus* (Lesch and Page, 2012). In addition, most organisms form their germline progenitor cells far from the site of the future gonad, and germline-soma interactions are often necessary to maintain PGCs. Remarkably, many of the genes involved in PGC specification and development are conserved from *Drosophila* to *Homo sapiens*, including *nos* and *vas*. In other cases, discrete protein domains are conserved in functional paralogs. For these reasons, investigation of the core conserved mechanisms of PGC development in tractable model organisms, such as *Drosophila*, continues to shape our understanding of germline formation and function.

Acknowledgements

The authors are grateful to Brian Oliver for critical reading of the manuscript and Mark Van Doren for insightful discussion. Leif Benner is supported by NIH grant 1ZIADK015600-21 awarded to Brian Oliver. This work is also supported by NIH grants R01GM110015-04 awarded to Girish Deshpande and Paul Schedl, as well as 1K22HL126922-01A1 awarded to Dorothy Lerit.

References

- Deshpande, G., Calhoun, G., & Schedl, P. (2004). Overlapping mechanisms function to establish transcriptional quiescence in the embryonic *Drosophila* germline. *Development*, 131(6), 1247–1257.
- Deshpande, G., Calhoun, G., Yanowitz, J. L., et al. (1999). Novel functions of nanos in downregulating mitosis and transcription during the development of the *Drosophila* germline. *Cell*, 99(3), 271–281.
- Foe, V. E., & Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.*, 61, 31–70.
- Hashiyama, K., Hayashi, Y., & Kobayashi, S. (2011). *Drosophila* sex lethal gene initiates female development in germline progenitors. *Science*, 333(6044), 885–888.
- Hayashi, Y., Hayashi, M., & Kobayashi, S. (2004). Nanos suppresses somatic cell fate in *Drosophila* germ line. *Proc. Natl. Acad. Sci. USA*, 101(28), 10338–10342.
- Kobayashi, S., Yamada, M., Asaka, M., et al. (1996). Essential role of the posterior morphogen nanos for germline development in *Drosophila*. *Nature*, 380(6576), 708–711.
- Krauss, J., Lopez de Quinto, S., Nusslein-Volhard, C., et al. (2009). Myosin-V regulates oskar mRNA localization in the *Drosophila* oocyte. *Curr. Biol.*, 19(12), 1058–1063.
- Lantz, V. A., Clemens, S. E., & Miller, K. G. (1999). The actin cytoskeleton is required for maintenance of posterior pole plasm components in the *Drosophila* embryo. *Mech. Dev.*, 85(1–2), 111–122.
- Lasko, P. (2013). The DEAD-box helicase Vasa: Evidence for a multiplicity of functions in RNA processes and developmental biology. *Biochim. Biophys. Acta*, 1829(8), 810–816.

- Leatherman, J. L., Levin, L., Boero, J., et al. (2002). *Germ cell-less* acts to repress transcription during the establishment of the *Drosophila* germ cell lineage. *Curr. Biol.*, 12(19), 1681–1685.
- Lerit, D. A., & Gavis, E. R. (2011). Transport of germ plasm on astral microtubules directs germ cell development in *Drosophila*. *Curr. Biol.*, 21(6), 439–448.
- Lerit, D. A., Shebelut, C. W., Lawlor, K. J., et al. (2017). Germ cell-less promotes centrosome segregation to induce germ cell formation. *Cell Rep.*, 18(4), 831–839.
- Lesch, B. J., & Page, D. C. (2012). Genetics of germ cell development. *Nat. Rev. Genet.*, 13(11), 781–794.
- Little, S. C., Sinsimer, K. S., Lee, J. J., et al. (2015). Independent and coordinate trafficking of single *Drosophila* germ plasm mRNAs. *Nat. Cell Biol.*, 17(5), 558–568.
- Mahowald, A. P. (2001). Assembly of the *Drosophila* germ plasm. *Int. Rev. Cytol.*, 203, 187–213.
- Martinho, R. G., Kunwar, P. S., Casanova, J., et al. (2004). A noncoding RNA is required for the repression of RNAPolIII-dependent transcription in primordial germ cells. *Curr. Biol.*, 14(2), 159–165.
- Nakamura, A., & Seydoux, G. (2008). Less is more: Specification of the germline by transcriptional repression. *Development*, 135(23), 3817–3827.
- Oliver, B. (2002). Genetic control of germline sexual dimorphism in *Drosophila*. *Int. Rev. Cytol.*, 219, 1–60.
- Phatnani, H. P., & Greenleaf, A. L. (2006). Phosphorylation and functions of the RNA polymerase II CTD. *Genes Dev.*, 20(21), 2922–2936.
- Saga, Y. (2008). Mouse germ cell development during embryogenesis. *Curr. Opin. Genet. Dev.*, 18(4), 337–341.
- Schaner, C. E., Deshpande, G., Schedl, P. D., et al. (2003). A conserved chromatin architecture marks and maintains the restricted germ cell lineage in worms and flies. *Dev. Cell.*, 5(5), 747–757.
- Seydoux, G., & Dunn, M. A. (1997). Transcriptionally repressed germ cells lack a subpopulation of phosphorylated RNA polymerase II in early embryos of *Caenorhabditis elegans* and *Drosophila melanogaster*. *Development*, 124(11), 2191–2201.
- Strome, S., & Updike, D. (2015). Specifying and protecting germ cell fate. *Nat. Rev. Mol. Cell Biol.*, 16(7), 406–416.
- Suyama, R., Jenny, A., Curado, S., et al. (2009). The actin-binding protein Lasp promotes oskar accumulation at the posterior pole of the *Drosophila* embryo. *Development*, 136(1), 95–105.
- Tanaka, T., Kato, Y., Matsuda, K., et al. (2011). *Drosophila* Mon2 couples oskar-induced endocytosis with actin remodeling for cortical anchorage of the germ plasm. *Development*, 138(12), 2523–2532.
- Van Doren, M., Williamson, A. L., & Lehmann, R. (1998). Regulation of zygotic gene expression in *Drosophila* primordial germ cells. *Curr. Biol.*, 8(4), 243–246.
- Warn, R. M., Smith, L., & Warn, A. (1985). Three distinct distributions of F-actin occur during the divisions of polar surface caps to produce pole cells in *Drosophila* embryos. *J. Cell Biol.*, 100(4), 1010–1015.
- Wawersik, M., Milutinovich, A., Casper, A. L., et al. (2005). Somatic control of germline sexual development is mediated by the JAK/STAT pathway. *Nature*, 436(7050), 563–567.
- Yang, S. Y., Baxter, E. M., & Van Doren, M. (2012). Phf7 controls male sex determination in the *Drosophila* germline. *Dev. Cell.*, 22(5), 1041–1051.

Epigenetic Programming of Germline, Nonmammalian Vertebrates

Jae Yong Han, Seoul National University, Seoul, South Korea

© 2018 Elsevier Inc. All rights reserved.

The Germline as a Transmitter of Genetic Code to the Next Generation

In multicellular organisms, the germline, from diploid primordial germ cells (PGCs) to haploid germ cells (sperms or oocytes), is a unique cell lineage that contains all of the genetic information of an individual and transmits it to the next generation. After production, gametes can be fertilized to form a new zygote conferring totipotency and establishing a new organism as progeny. Thus, the germline provides a permanent link between all generations.

Through development from the totipotent zygote to the pluripotent embryo, the embryonic cells differentiate into multiple lineages. Among them, PGCs, which constitute a small population in the early embryo and are clearly distinct from other somatic cells, are the specialized precursors of the germline. PGCs are unipotency, i.e., they contribute solely to produce mature gametes, in contrast to pluripotent stem cells that can contribute to all three germ layers. In this context, specification of PGCs at an early stage has been considered crucial because the germline is important not only for fundamental research, but also for the potential use of genetic resources. As such, when and how the germline first arose are essential questions in developmental and evolutionary biology.

In the animal kingdom, PGCs can be specified from two general modes: preformation and induction ([Strome and Updike, 2015](#)). In preformation mode, germ cells are uniquely determined by cytoplasmic organelles, generically referred to as germ granules or germ plasm. They contain maternally inherited mRNAs, proteins, and small RNAs, which play crucial roles in germ cell specification, including in roundworm, fruit fly, zebrafish, and frog. In contrast, in the inductive mode, which is observed in animals including mouse and human, the germline is initially found in proximal-posterior epiblasts and induced by bone morphogenic protein (BMP) signaling from neighboring extra-embryonic tissue during gastrulation.

After specification, PGCs originating from the extra-gonadal region migrate across the embryo into the developing genital ridge. On settling in the gonad, the germ cells increase rapidly in number and sexually differentiate into spermatogonia and oogonia via intrinsic and extrinsic factors. In males, germ cells undergo mitotic arrest until birth, and spermatogonial stem cells generate sperm through meiotic segregation during the process of spermatogenesis. In females, oogonia are arrested in prophase of the meiosis I cycle until puberty, and then exhibit maturation and the resumption of meiosis to form haploid oocytes in the metaphase of the meiosis II cycle during ovulation. Upon fertilization, the meiotic procedure of females is finally completed. In this way, the infinite cycle of the germline is maintained from generation to generation.

Epigenetics in Germline

Epigenetics is the mechanism underlying spatiotemporal control of gene expression during development without modification of the DNA sequence. The epigenetic status determines the characteristics of cells and tissues, and its alteration can cause the loss of cellular identity or cell fate. The most well-known molecular mechanisms of epigenetic regulation are DNA methylation, histone modification, and post-transcriptional regulation of small non-coding RNAs.

DNA methylation generally involves covalent modification, which occurs predominantly in CpG dinucleotides. Members of the DNA methyltransferase (DNMT) family convert cytosines to 5-methylcytosines (5mC). DNA methylation in the upstream promoter of a gene suppresses transcriptional activity directly by inhibiting the binding of specific transcription factors, or indirectly by recruiting methyl-CpG-binding proteins. Conversely, DNA demethylation takes place passively via DNA replication, or actively via ten-eleven translocation (TET), forming the hydroxylated metabolite of 5mC (5hmC). By DNA methylation reprogramming, mammalian PGCs establish the monoallelic expression of imprinting genes, maintain inactivated retrotransposons, inactivate one of the two X chromosomes, and suppress gene expression.

Histones undergo post-translational modifications that reorganize their interactions with DNA and nuclear proteins. In particular, the tails of histones H3 and H4 can be covalently modified at specific residues. In terms of the regulation of gene expression, the processes that have been investigated most intensively are methylation and acetylation in histone H3. In addition to DNA methylation, histone post-translational modifications also specify the cellular features of PGCs.

Small non-coding RNAs do not produce proteins, but act as post-transcriptional regulators. An example of these RNAs is microRNAs (miRNAs), which are 18–23 nucleotides in length, bind to the complementary sequences of a target mRNA at the 3' untranslated region (UTR), and silence it through translational repression or mRNA degradation. Another category is piwi-interacting RNAs (piRNAs, 23–30 nucleotides), which are longer than miRNAs, are mainly derived from repetitive elements in the genome, and have a specific role in retrotransposon suppression through DNA methylation in the germline. In terms of post-transcriptional regulation, small RNAs including miRNAs and piRNAs are required to maintain PGCs and germline development in mouse, zebrafish, and fruit fly.

The epigenetic status of the germline is regulated by such mechanisms acting on the expression of genes that determine germ-cell-specific characteristics. The germline establishes an epigenetic signature to specify its cellular features and induce the abolition of somatic lineage differentiation during embryogenesis. In addition, changes in epigenetic marks are required for germline development, the regulation of meiosis, and genome integrity. Epigenetic dynamics is critical for early embryonic development, and for the germline,

throughout the animal kingdom. In roundworm, the activation of germline genes and suppression of somatic genes by histone modifications are crucial for germ cell development (Strome and Updike, 2015). Moreover, during early embryonic development in mammals, the germline, from specification to gametogenesis, exhibits unique epigenetic dynamics such as genome-wide demethylation, X-chromosome reactivation, histone modification, and transposon silencing (Sasaki and Matsui, 2008).

Epigenetic programming and reprogramming have been extensively studied in mammals because dramatic differences in epigenetic marks were observed between somatic lineages and PGCs. In addition, excellent models, such as a transgenic mouse model, have been developed based on reproductive technologies. Therefore, we have obtained a clear overview of epigenetics during germline development in mammals, in terms of DNA methylation, histone modification, and small RNAs. Generally, after specification, genome-wide epigenetic reprogramming occurs, such as global DNA demethylation for the erasure of imprinting and X-chromosome reactivation, and histone modification in the germline, regardless of the established epigenetic status of somatic cells. During this erasure, however, suppressive histone markers such as histone H3 lysine 9 trimethylation (H3K9me3) are maintained, inhibiting the somatic program, and the expression of retrotransposons and repeat elements in the germline (Tang et al., 2016). Subsequently, reconstitution of imprints is initiated by DNA remethylation during sexual differentiation. The piRNA pathway also plays a critical role in the differentiated male germline through *de novo* DNA methylation and retrotransposon suppression (Sasaki and Matsui, 2008). Moreover, next-generation sequencing technology enables investigation of the specific mechanisms by which epigenetic regulation acts on gene expression. However, few studies of epigenetics in non-mammalian species have been performed. Hereafter, we present findings on the epigenetic programming that characterizes the germline in non-mammalian vertebrates, including birds, reptiles, amphibians, and fish.

Epigenetic Programming of the Germline in Birds

Historically, birds have been a valuable animal model in a broad range of scientific fields. Especially in embryology and reproductive biology, because the avian embryo develops in an egg before hatching, it is an excellent *in vitro*-like *in vivo* system; this has given rise to substantial research on the developmental events that occur during embryogenesis. Birds also exhibit a unique characteristic during germline development compared to other species, namely, the migration of PGCs into blood vessels through the circulatory system until they settle in the genital ridge (Nieuwkoop and Sutasurya, 1979) (Fig. 1(A)). This feature enables the production of germline chimeras or transgenic birds via the injection of germline cells, specifically PGCs, into the blood vessels of the recipient embryo. Furthermore, in a representative model of avian species, chicken PGCs can be easily isolated from the blood or gonads of embryos, and cultured *in vitro* for a long period without losing their cellular character. These factors together make the avian germline be important not only for germ cell biology, but also for biotechnology.

Chicken PGCs were first described in the germinal crescent in terms of their distinct morphological aspects compared with those of somatic cells. The central region of the blastoderm can only give rise to PGCs, probably by an inductive signal, because there is no evidence of germ plasm during the early stages in aves. However, after the discovery and tracing of chicken VASA homolog (CVH), the origin of chicken PGCs in early development was firstly observed (Tsunekawa et al., 2000). VASA homologs are members of the ATP-dependent RNA helicase of the DEAD-box family that are highly conserved across species including mouse, frog, zebrafish, fruit fly, and roundworm. VASA is also well-known to be involved in germline specification following the preformation mode, and plays an essential role in germ cell development. The localization of CVH protein was granulofibrillar structures surrounding the mitochondrial cloud and spectrin protein-enriched structure in oocytes, indicating the evidence of germ plasm in birds. Furthermore, CVH was found in cleavage furrows and restricted to only six to eight cells in the 300-cell-stage embryo, suggesting that germ cell specification in chicken might be determined by maternally inherited determinants and follow the preformation mode. In addition, the expression and tracing of deleted in azoospermia-like (DAZL), which is another germline-specific RNA-binding protein, were used to investigate the origin of PGCs and germplasm dynamics during early development of chickens (Lee et al., 2016). DAZL has been well-studied in vertebrate species, in terms of meiotic progression and the maintenance of pluripotency in germ cells. Using chicken DAZL as a germ plasm marker, the germ granule was found to exhibit asymmetric localization in oocytes, and a change from a subcellular to a diffused form at which zygotic genome activation (ZGA) occurs during early cleavage. These studies indicated that the germ cells of avian species could be predetermined by maternally derived germ plasm (Fig. 1(A)). However, there is still a need for additional investigations, such as on the specific functions of germ plasm components and confirmation of these findings in other birds, to clarify definitively the mode of germline specification in birds.

Comprehensive analysis of epigenetic status during germline development has not been conducted in birds. However, recent studies revealed the epigenetic signature, including DNA methylation, histone modification, and post-transcriptional regulation by noncoding small RNAs, in chicken germline (Fig. 1(B)). During the migration of chicken germ cells, blood PGCs (E2.5) showed lower global 5mC than gonadal PGCs (E4.5 and E6.5), indicating that genome-wide DNA reprogramming occurs at this stage (Rengaraj et al., 2011). Moreover, differentially methylated regions, including seven hypomethylated and five hypermethylated ones, were found in chicken PGCs and exclusively matched the differential expression of genes compared with their levels in somatic cells. This provides insight into the role of DNA methylation in gene regulation in chicken PGCs. In addition, regions showing differential methylation between male and female PGCs were detected in chicken (Jang et al., 2013).

In addition to methylated regions of DNA, the nuclear distributions of histone modifications and methylated cytosine were observed. Most histone modifications and 5hmC were similar to those in mammalian cells, except for one repressive histone tail, H3K9me3. Chicken PGCs exhibited H3K9me3 for heterochromatin formation, instead of the trimethylation of histone H3

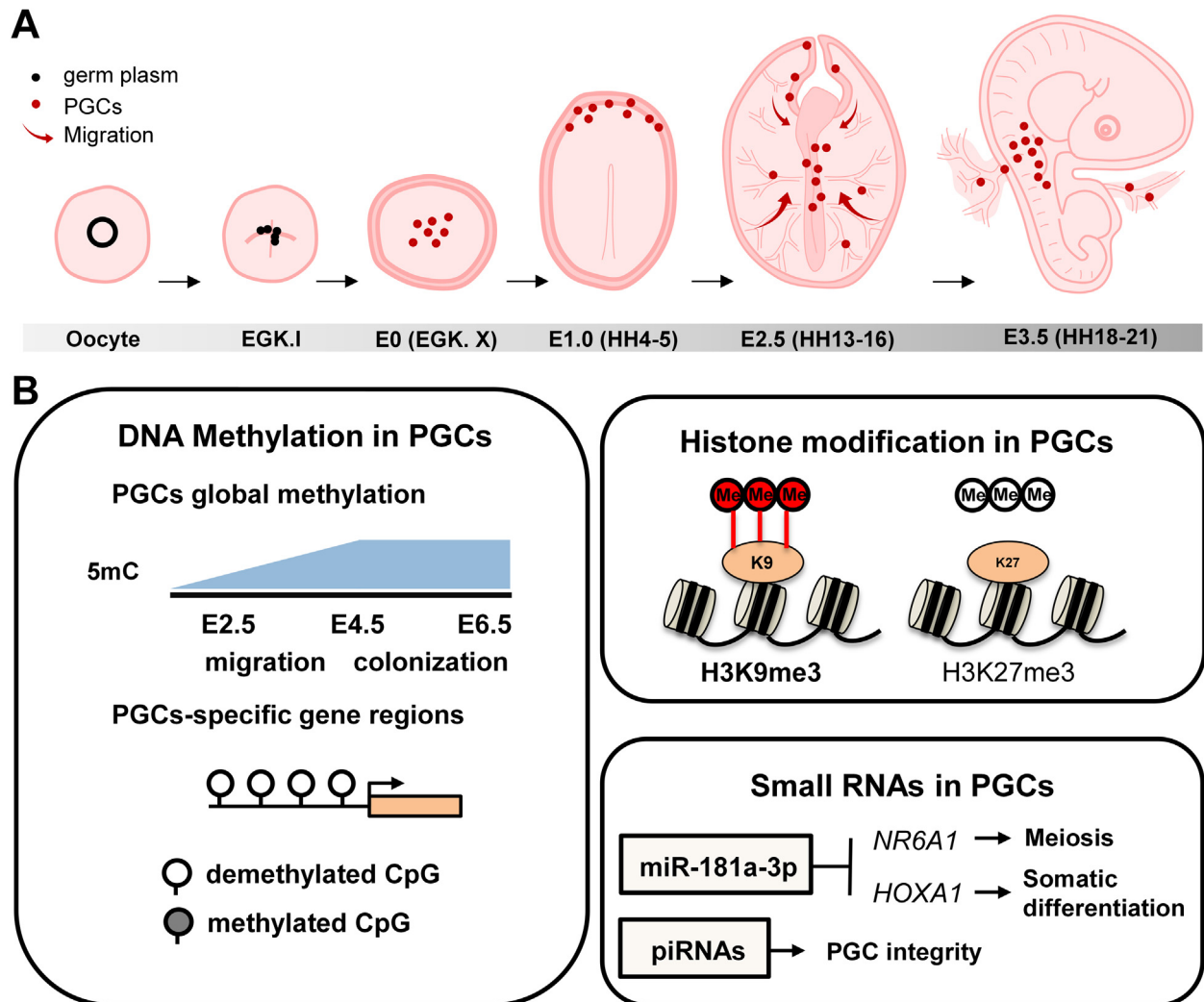


Fig. 1 Germline development (A) and epigenetic programming (B) in birds. (A) In chicken, germ plasm is expressed in germinal vesicles of oocytes. After the initiation of cell division at Eyal-giladi and Kochav stage I (EGK.I), germ plasm is localized in the cleavage furrow. During the formation of the primitive streak at Hamburger and Hamilton stage 4–5 (E1.0, HH4–5), primordial germ cells (PGCs) migrate to the germinal crescent. Subsequently, PGCs move into the bloodstream (E2.5, HH13–16) and migrate into the genital ridges (E3.5, HH18–21). Modified from Nieuwkoop, P.D., Sutasurya, L.A., 1979. *Primordial Germ Cells in the Chordates: Embryogenesis and Phylogenesis*. Cambridge University Press. (B) The reprogramming of DNA methylation in chicken PGCs seems to occur during migration. DNA methylation is also involved in germ cell-specific gene expression in chicken PGCs. Contrary to the findings for histone H3 lysine 27 tri-methylation (H3K27me3) in mammalian PGCs, histone H3 lysine 9 trimethylation (H3K9me3) is predominantly localized in heterochromatin in chicken PGCs. A chicken PGC-specific microRNA (miRNA), miR-181a-3p, has the dual functions of suppressing meiosis and somatic differentiation. Piwi-interacting RNAs (piRNAs) are also crucial small RNAs for PGC integrity.

on lysine 27 (H3K27me3) by polycomb group protein, as exhibited in mouse PGCs. This demonstrates that the avian-specific constitution of chromatin could differ from its epigenetic status in mammals (Kress *et al.*, 2016).

In the chicken germline, small RNAs have been shown to play very important roles, as revealed by the following studies. For example, after chicken PGC-specific miRNAs had been selected by a high-throughput microarray, miR-181a-3p was shown to have dual functions, namely, inhibition of somatic differentiation and prevention of meiotic progression, for the maintenance of undifferentiated characteristics of chicken PGCs. miR-181a-3p achieved this by suppressing the expression of genes related to the somatic lineage: homeobox A1, and a meiotic marker, nuclear receptor subfamily 6, group A, member 1 (Lee *et al.*, 2011). In addition to miRNAs, an abundance of germ-cell-specific small RNAs, piRNAs, which are derived from repetitive element sequences or protein-coding genes, was found in chicken PGCs using small RNA-seq. As in other species, piRNAs and piRNA pathway genes are required for the integrity of chicken PGCs (Rengaraj *et al.*, 2014). However, there is still a need for comprehensive research on epigenetic regulation in avian germline cells. In the future, the specific roles of epigenetic marks in the avian germline should be elucidated, by applying combined approaches including the use of the latest next-generation sequencing technology.

Epigenetic Programming of the Germline in Reptiles

In reptilian species, although there is still controversy about how PGC specification occurs, reptiles are believed to follow an inductive (epigenesis) mode, as also employed by mammals. Among reptiles, the germ plasm is considered to be absent in turtles, since *Dazl* and *Vasa* mRNAs were not detected in mature oocytes and PGCs were identified only after the early somite stage in turtle embryos (Bachvarova *et al.*, 2009); this indicates that an inductive mode of specification is employed in reptilian PGCs.

During the germline development of reptilian species, temperature-dependent sex determination is one of the major developmental events, which occurs by temperature-mediated epigenetic regulation. Since the expression of aromatase, *cytochrome P450 family 19 subfamily A member 1* (*cyp19a1*) gene, which is involved in the conversion of androgen into estrogen for female differentiation, is regulated in a temperature-dependent manner, DNA methylation of *cyp19a1* gene promoter is considered to be regulated by a temperature-induced DNMT or a temperature-induced signaling pathway (Navarro-Martin *et al.*, 2011). It is also found that upstream CpG islands near the gonad-specific TATA box are differently methylated during the period of temperature-dependent sex determination. In red-eared slider turtles, it is suggested that female-specific DNA hypomethylation and H3K4me3 modification in the promoter region of the *cyp19a1* aromatase gene are among the epigenetic regulatory mechanisms underlying sex determination in turtles in response to both temperature and genetic cues, allowing for the release of a transcriptional blockade, thus initiating a cascade resulting in ovarian differentiation (Matsumoto *et al.*, 2016).

Furthermore, a study conducted on sea turtles revealed a DNA methylation pattern that was sexually dimorphic between ovaries and testes. This depended on the incubation temperature, as the occurrence of DNA methylation or demethylation is temperature-dependent, since a difference in the DNA methylation signal has been correlated to the period of temperature-sensitive sex determination (Venegas *et al.*, 2016). More recently, in a study of bearded dragons, it was reported that temperature-induced sex-reversed female dragons preferentially retained an intron in the mature transcript of a jumonji chromatin modifier, *jumonji and AT-rich interaction domain containing 2* (*jarid2*) and *jumonji domain containing 3* (*jmjd3*) genes, which was not detected in normal female or male (Deveson *et al.*, 2017). This suggests that intron retention in *jarid2/jmjd3* can induce silencing and perturb the function of chromatin modifier genes, indicating that the epigenetic status of sex-reversed female changes dramatically compared with that of male, providing support for sex reversal and reptile temperature-dependent sex determination. These findings together suggest that DNA methylation events could drive differentiation of the bi-potential gonads of reptilian species into the male or female germline.

Epigenetic Programming of the Germline in Amphibians

Amphibian species are commonly used as vertebrate model systems for studying embryonic development, cell cycle, cancer and epigenetic mechanisms including miRNA function and chromatin structure. In particular, since the frog germline is established by localization of the maternally inherited components to the vegetal cytoplasm, called the germ plasm, studies on the molecular networks determining the germline have been intensively performed (Houston and King, 2000) (Fig. 2(A)).

In vertebrates, chromatin assembly mediated by histone modification and subsequent transcriptional regulation are critical steps for embryogenesis, as well as germline establishment, by regulating gene expression. However, in amphibian species, especially in frog PGCs, the histone modifications producing the chromatin structure are unlikely to involve germness-related gene expression. Previous research reported that there is no difference in histone modification between PGCs and somatic cells isolated from embryos at the maternal-to-zygotic transition stage. Instead, the suppression of zygotic gene expression of frog PGCs is due to the blocking of RNA polymerase II (RNAPII) activity by the failure to phosphorylate serine 2 in the carboxy-terminal domain (CTD) of the large subunit of RNAPII (Venkatarama *et al.*, 2010) (Fig. 2(B)).

Maintaining the germline characteristics, epigenetic regulation in the germline is also conserved in amphibian species. Indeed, large populations of mRNAs were already shown to be present in frog oocytes and fertilized eggs, and have been considered to play pivotal roles in embryonic development. In particular, miRNA-427, zebrafish ortholog miR-430, is abundant in early embryos and plays a role in maternal mRNA clearance. In contrast, the low expression of miR-427 in frog PGCs impedes efficient degradation of DEADSouth mRNA, which is localized in the germ plasm of fertilized eggs and encodes DEAD-box helicase 25 (DDX25), resulting in the maintenance of germness (Yamaguchi *et al.*, 2014). Similar to miR-427, miR-18 degrades *dnd1* mRNA in somatic cells, but not in the germline, indicating its regulatory roles in the expression of germness-related genes (Koebernick *et al.*, 2010) (Fig. 2(B)).

Besides the findings for miRNAs, translational regulation of small RNAs, including piRNAs, has been suggested in frog oocytes. High-throughput analysis of the frog female germline revealed that a number of small RNAs were specifically expressed in the female germline (Armisen *et al.*, 2009). Collectively, studies of the epigenetic programs in amphibian germlines showed their conserved roles and distinctive features in amphibian species compared with those in other species.

Epigenetic Programming of the Germline in Fish

During the first steps of embryo development, the parental somatic marks are completely erased in fish, like in other animal species. Subsequently, epigenetic marks are newly acquired in gametogenesis, allowing proper embryonic development. In mice, the PGCs migrating towards the embryonic genital ridge undergo a genome-wide erasure of methylation, which is known as the epigenetic ground stage (Sasaki and Matsui, 2008). However, the PGCs of fish arise from a few cells located in a matrix of mRNAs and proteins

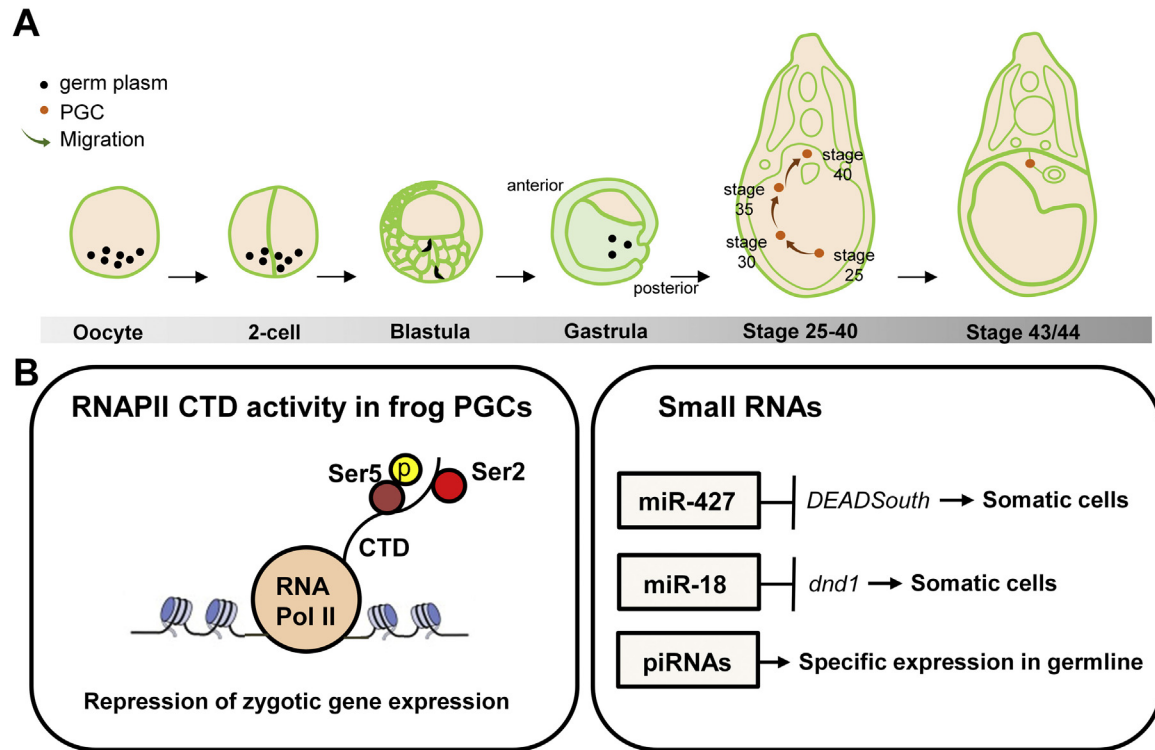


Fig. 2 Germline development and epigenetic programming in amphibians. (A) In frog, germ plasm is initially deposited in the oocytes. During the cleavage stages, germ plasm accumulates at the vegetal pole (2-cell). In the blastula stage, germ plasm still remains in the vegetal blastomeres. During gastrulation, germ plasm moves to the posterior endoderm, and PGCs leave the endoderm and migrate to the dorsal body wall through dorsal mesentery (Stage 25–44). Modified from Houston, D.W., King, M.L., 2000. Germ plasm and molecular determinants of germ cell fate. *Curr Top Dev Biol* 50, 155–181. (B) During epigenetic programming in frog PGCs, zygotic gene expression is suppressed by inactive RNA polymerase II (RNAPII) through blocking the phosphorylation of serine 2 in the carboxy-terminal domain (CTD). miR-427 and miR-18 are highly expressed in somatic cells and inhibit germline development. piRNAs in frog are also expressed exclusively in PGCs.

in oocytes, called the germ plasm (Raz, 2003). Because this event occurs much earlier than embryo development, whether there is any process for the erasure/rewriting of epigenetic events in fish, similar to that in gametogenesis in mammals, has not been elucidated. Fish PGCs are formed in the germ plasm where maternal mRNAs and proteins required for PGC specification, such as *vasa*, *nanos*, and *dnd*, are concentrated (Raz, 2003) (Fig. 3(A)). After specification, PGCs migrate to the embryonic gonad and differentiate into functional gametes.

Compared with PGCs, the epigenome of mature male gametes of fish has been relatively well studied, especially in terms of DNA methylation and histone marks. Promoter regions of genes related on developmental regulators, transcription factors and metabolism are hypo-methylated (Wu *et al.*, 2011), although highly methylated CpGs (over 90%) were found in zebrafish sperm (Potok *et al.*, 2013; Jiang *et al.*, 2013). Moderate enrichment of active histone marks, including histone H3 lysine 4 trimethylation (H3K4me3), H3K4me2, and histone H4 lysine 16 acetylation (H4K16ac), occurs in developmental loci, whereas they are prominently found in genes related to meiosis and spermatogenesis (Wu *et al.*, 2011) (Fig. 3(B)).

Female gametogenesis showed a different profile from that in the male germline. The nuclear DNA of fish oocytes is less methylated than that of sperm (Potok *et al.*, 2013). Also, during ZGA in sphere stage, it has a tendency to be hypomethylated in differentially methylated regions (DMRs) in oocyte related to germline and early development related genes, but hypermethylated in DMRs in sperm, related to later functions of embryo development (Potok *et al.*, 2013) (Fig. 3(B)). The *Piwi* homologs, *Ziwi* and *Zili*, were also found in zebrafish (Houwing *et al.*, 2007). In fish, piRNAs are prominently found in both testes and ovaries where *Ziwi* is expressed, and may be associated with the silencing of transposons in the zebrafish germline (Houwing *et al.*, 2007).

Future Perspectives of Epigenetic Programming

Pioneering studies have provided a solid foundation for work on germline origin and development throughout the vertebrate species. However, the molecular mechanisms that define the germline should be characterized in detail. Epigenetic programming is the main focus with respect to how gene regulation influences germline cells. From the perspective of developmental and evolutionary biology, epigenetic regulation of the germline is highly conserved across the animal kingdom. Studies reported to date have

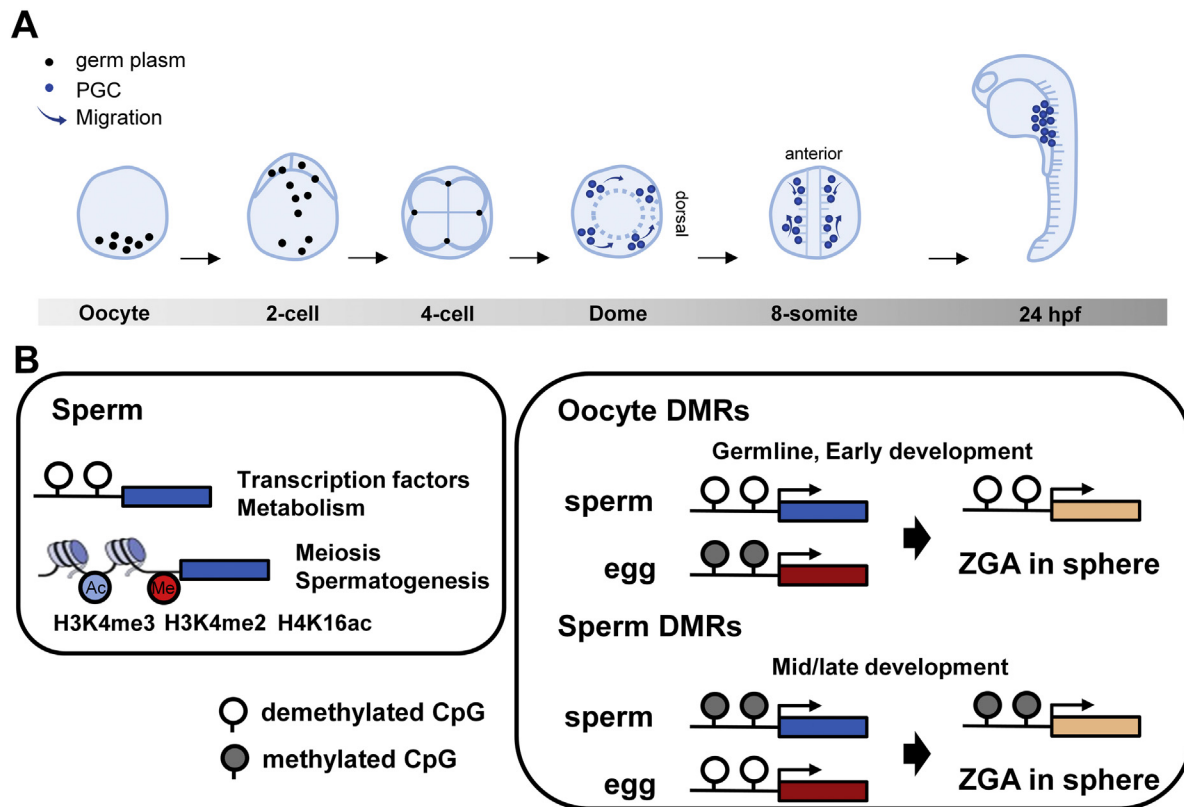


Fig. 3 Germline development and epigenetic programming in fish. (A) In zebrafish, germ plasm is first located in the oocytes. After fertilization, germ plasm is found in the cleavage planes (2-cell) and subsequently condensed into four clumps during 4-cell stage. These four clumps give rise to four PGC clusters (Dome). Then, PGCs migrate and accumulate in the region of the future gonad at 24 h post-fertilization. (24 hpf). Modified from Raz, E., 2003. Primordial germ-cell development: The zebrafish perspective. *Nat Rev Genet* 4, 690–700. (B) During epigenetic programming in zebrafish sperm and oocytes, sperm express the genes related to meiosis and spermatogenesis through active histone modifications. Differentially methylated regions (DMRs) involved in germline and early development in oocytes are going to be hypomethylated in sphere during ZGA.

indicated that there is an evolutionarily conserved regulatory mechanism involved in epigenetic programming in various vertebrates, including mammals, birds, reptilians, amphibians, and fish. Although core regulatory networks of epigenetic programs among vertebrates have been elucidated, there are also species-specific regulatory mechanisms that govern epigenetic regulation in response to their status and cellular environment. However, the specific mechanisms underlying the effects of each epigenetic mark are not fully understood, especially in non-mammalian species. Due to recent progress in genetic modification and genome editing technology using third-generation DNA scissors, clustered regularly interspaced short palindromic repeats (CRISPR)-associated (CRISPR/Cas9), there are now tools available for the genome editing of regions related to germline development, enabling the efficient production of model animals for germ cell research. This can be combined with reproductive technologies, such as those exploiting PGCs in birds and fish, as well as *in vitro* maturation and fertilization of embryos in amphibians. In addition, the advanced next-generation sequencing technologies facilitate epigenetic characterization via amplification of a small number of cells. Through these approaches, we can comprehensively monitor epigenetic dynamics from very small amounts of material from the early germline, such as PGCs, and even perform comparisons among single germline cells in individuals. These state-of-the-art techniques should provide us with a comprehensive understanding of epigenetic programming in the non-mammalian germline, and also contribute to fundamental research on epigenetics and the use of germ cells as genetic resources.

References

- Armisen, J., Gilchrist, M. J., Wilczynska, A., Standart, N., & Miska, E. A. (2009). Abundant and dynamically expressed miRNAs, piRNAs, and other small RNAs in the vertebrate *Xenopus tropicalis*. *Genome Res*, 19, 1766–1775.
- Bachvarova, R. F., Crother, B. I., Manova, K., et al. (2009). Expression of *Dazl* and *Vasa* in turtle embryos and ovaries: Evidence for inductive specification of germ cells. *Evol Dev*, 11, 525–534.
- Deveson, I. W., Holleley, C. E., Blackburn, J., et al. (2017). Differential intron retention in *Jumonji* chromatin modifier genes is implicated in reptile temperature-dependent sex determination. *Sci Adv*, 3, e1700731.
- Houston, D. W., & King, M. L. (2000). Germ plasm and molecular determinants of germ cell fate. *Curr Top Dev Biol*, 50, 155–181.
- Houwling, S., Kamminga, L. M., Berezikov, E., et al. (2007). A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in zebrafish. *Cell*, 129, 69–82.

- Jang, H. J., Seo, H. W., Lee, B. R., et al. (2013). Gene expression and DNA methylation status of chicken primordial germ cells. *Mol Biotechnol*, 54, 177–186.
- Jiang, L., Zhang, J., Wang, J. J., et al. (2013). Sperm, but not oocyte, DNA methylome is inherited by zebrafish early embryos. *Cell*, 153, 773–784.
- Koebernick, K., Loeber, J., Arthur, P. K., Tarbashevich, K., & Pieler, T. (2010). Elr-type proteins protect *Xenopus Dead end* mRNA from miR-18-mediated clearance in the soma. *Proc Natl Acad Sci USA*, 107, 16148–16153.
- Kress, C., Montillet, G., Jean, C., Fuet, A., & Pain, B. (2016). Chicken embryonic stem cells and primordial germ cells display different heterochromatic histone marks than their mammalian counterparts. *Epigenet Chromatin*, 9, 5.
- Lee, H. C., Choi, H. J., Lee, H. G., et al. (2016). DAZL expression explains origin and central formation of primordial germ cells in chickens. *Stem Cells Dev*, 25, 68–79.
- Lee, S. I., Lee, B. R., Hwang, Y. S., et al. (2011). MicroRNA-mediated posttranscriptional regulation is required for maintaining undifferentiated properties of blastoderm and primordial germ cells in chickens. *Proc Natl Acad Sci USA*, 108, 10426–10431.
- Matsumoto, Y., Hannigan, B., & Crews, D. (2016). Temperature shift alters DNA methylation and histone modification patterns in gonadal aromatase (*cyp19a1*) gene in species with temperature-dependent sex determination. *PLOS ONE*, 11, e0167362.
- Navarro-Martin, L., Vinas, J., Ribas, L., et al. (2011). DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLOS Genet*, 7, e1002447.
- Nieuwkoop, P. D., & Sutasurya, L. A. (1979). *Primordial Germ Cells in the Chordates: Embryogenesis and Phylogenesis*. Cambridge University Press.
- Potok, M. E., Nix, D. A., Parnell, T. J., & Cairns, B. R. (2013). Reprogramming the maternal zebrafish genome after fertilization to match the paternal methylation pattern. *Cell*, 153, 759–772.
- Raz, E. (2003). Primordial germ-cell development: The zebrafish perspective. *Nat Rev Genet*, 4, 690–700.
- Rengaraj, D., Lee, B. R., Lee, S. I., Seo, H. W., & Han, J. Y. (2011). Expression patterns and miRNA regulation of DNA methyltransferases in chicken primordial germ cells. *PLOS ONE*, 6, e19524.
- Rengaraj, D., Lee, S. I., Park, T. S., et al. (2014). Small non-coding RNA profiling and the role of piRNA pathway genes in the protection of chicken primordial germ cells. *BMC Genom*, 15, 757.
- Sasaki, H., & Matsui, Y. (2008). Epigenetic events in mammalian germ-cell development: Reprogramming and beyond. *Nat Rev Genet*, 9, 129–140.
- Strome, S., & Updike, D. (2015). Specifying and protecting germ cell fate. *Nat Rev Mol Cell Biol*, 16, 406–416.
- Tang, W. W., Kobayashi, T., Irie, N., Dietmann, S., & Surani, M. A. (2016). Specification and epigenetic programming of the human germ line. *Nat Rev Genet*, 17, 585–600.
- Tsunekawa, N., Naito, M., Sakai, Y., Nishida, T., & Noce, T. (2000). Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells. *Development*, 127, 2741–2750.
- Venegas, D., Marmolejo-Valencia, A., Valdes-Quezada, C., et al. (2016). Dimorphic DNA methylation during temperature-dependent sex determination in the sea turtle *Lepidochelys olivacea*. *Gen Comp Endocrinol*, 236, 35–41.
- Venkatarama, T., Lai, F., Luo, X., et al. (2010). Repression of zygotic gene expression in the *Xenopus* germline. *Development*, 137, 651–660.
- Wu, S. F., Zhang, H., & Cairns, B. R. (2011). Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. *Genome Res*, 21, 578–589.
- Yamaguchi, T., Kataoka, K., Watanabe, K., & Orii, H. (2014). Restriction of the *Xenopus DEADSouth* mRNA to the primordial germ cells is ensured by multiple mechanisms. *Mech Dev*, 131, 15–23.

Sex Determination in Vertebrates

Manfred Schartl, University of Wuerzburg, Wuerzburg, Germany; University Hospital Wuerzburg, Wuerzburg, Germany; and Texas A&M University, College Station, TX, United States

Amaury Herpin, Fish Physiology and Genomics Laboratory, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

AMH Anti-muellerian hormone

Amhy Anti-muellerian hormone on Y-chromosome

amhr2 Anti-muellerian hormone receptor type 2

amhr2-Y Anti-muellerian hormone receptor type 2 on Y-chromosome

Cbx2 Chromobox homolog 2

DGCR8 DiGeorges critical region 8 (double-cysteine-ligated heme protein)

DMRT1 Doublesex and mab-3 related transcription factor 1

ESD Environmental sex determination; when the sex of an individual is triggered by various external factors (*i.e.* temperature, PH, social interactions)

GSD Genetic sex determination; when the sex of an individual is triggered by its genotype only, including both mono or polygenic sex determination.

gsdf Gonadal soma-derived factor

gsdf-Y Gonadal soma-derived factor on Y-chromosome

HINTW Histidine triad binding protein, W-linked

HINTZ Histidine triad binding protein, Z-linked

HMG-box High mobility group DNA binding domain

nr5a1 Nuclear receptor 5a1 gene encoding the steroidogenic factor-1 (SF1)

rspo1 R-spondin 1

SD Sex determination system

sdY Sexual dimorphic on Y-chromosome (master sex-determining gene of salmonids)

Sox SRY-related HMG-box

sox3-Y SRY-related HMG-box factor 2 on Y-chromosome

SRY Sex determining region Y

TGF- β Transforming growth factor β (beta)

Wnt4 Wnt family member 4

XY System with X and Y sex chromosomes, females are XX (homogametic) and males XY (heterogametic)

ZZ/ZW System with Z and W sex chromosomes, males are ZZ (homogametic) and females WZ (heterogametic)

Mammals: RY all over since – and Forever?

Mammals are characterized by X and Y heteromorphic sex chromosomes (XY system). First discovered in the early 1990s the SRY gene (Koopman *et al.*, 1991) on the Y-chromosome in the sex-determining region has been shown to be the universal male master regulator. It initiates testis development in the bipotential gonad of most therian mammals (Waters *et al.*, 2007) (Fig. 1), with only a handful of exceptions known so far. In mice loss- and gain-of-function studies demonstrated that SRY is both necessary and sufficient for triggering testis development (Koopman *et al.*, 1991; Lovell-Badge and Robertson, 1990). In human, mutations of SRY lead to partial or complete gonadal dysgenesis, sex reversal or even in rare cases to hermaphroditism.

SRY acts as transcription factor. It has a single evolutionary conserved high mobility group DNA binding domain (HMG-box) which is flanked by more variable N- and C- terminal sequences. Being the prototype member of the SRY-box (Sox) gene family, SRY shares its protein architecture with other Sox factors. These include Sox3, Sox9 –a downstream target of SRY- and Sox10. These are able to substitute for SRY-driven activation of the male gonadal gene regulatory network when temporarily and topologically correctly expressed. Thus other Sox genes could, in principle, activate the male gonadal gene regulatory network. Intriguingly, the X-chromosomal Sox3-encoded protein is the Sox factor most similar to Sry (Stevanović *et al.*, 1993) sharing an overall amino acid identity of 67%, and up to 90% in the DNA binding domain (Table 1). Evolutionary studies integrating comparative molecular and cytogenetic data of mammalian sex chromosomes provided evidence that Sox3 is the likely ancestor of Sry. Sry most probably arose before the divergence of the therian lineage around 146–166 million years ago. The current model proposes that the proto-Y emerged after (i) a dominant mutation in the Y SOX3 allele attributed a new function and (ii) a consecutive

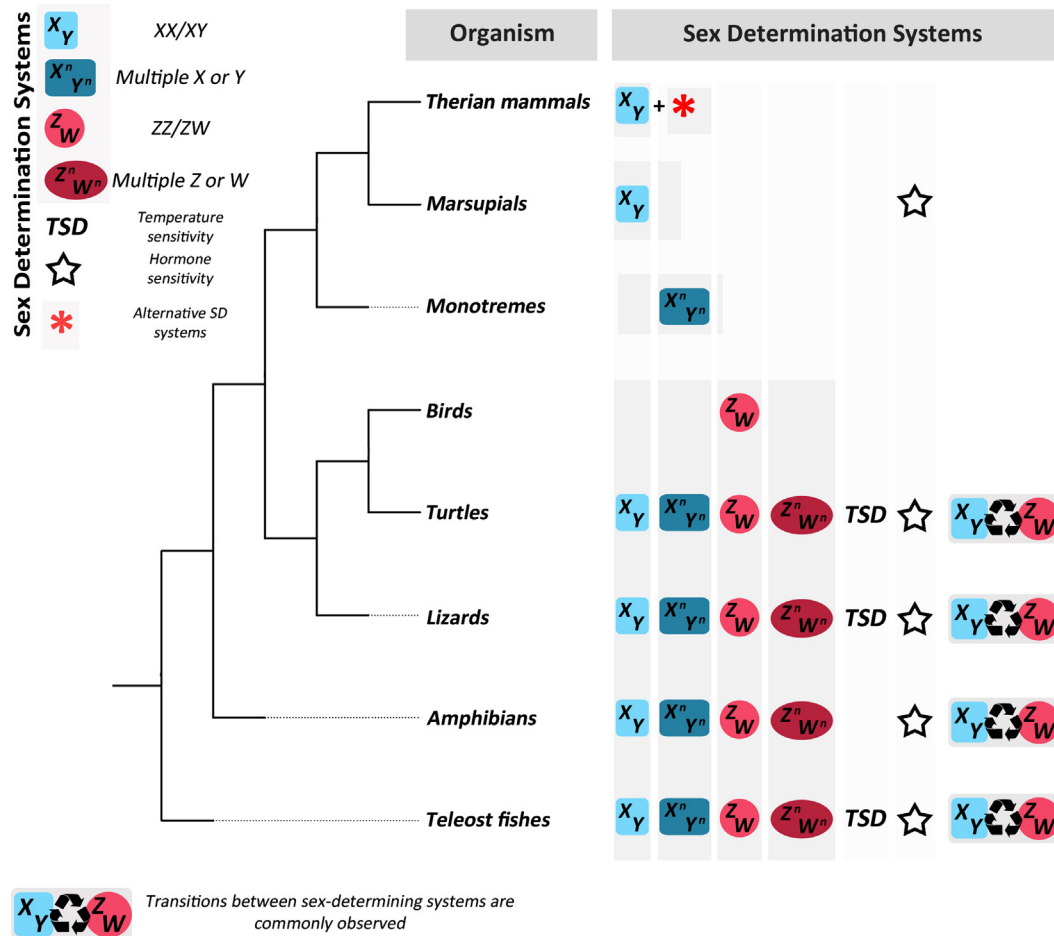


Fig. 1 Diversity and evolution of sex determination systems and master sex-determining genes in vertebrates. In contrast to therian mammals and birds, the other vertebrate groups including reptiles, amphibians and fish, exhibit plasticity of sex determination mechanisms (XX/XY, multiple X or Y, ZZ/ZW, multiple Z or W, autosomal modifiers and environmentally induced sex determination) and of master sex-determining genes.

Table 1 Diversity of sex determination systems in vertebrates

Organism	Master sex-determining gene	Sex-determination system	Sex-determining gene ancestor	Establishment
Therian mammals	SRY	XY	SOX3	Allelic Diversification
Birds	DMRT1	ZW	<i>Dmrt1</i>	Allelic Diversification
African clawed frog (<i>Xenopus laevis</i>)	<i>DM-W</i>	ZW	<i>dmrt1</i>	Gene Duplication
Medaka (<i>Oryzias latipes</i>)	<i>dmrt1bY</i>	XY	<i>dmrt1</i>	Gene Duplication
Luzon rice fish (<i>Oryzias luzonensis</i>)	<i>gsdf-Y</i>	XY	<i>gsdf</i>	Allelic diversification
Indian rice fish (<i>Oryzias dancena</i>)	<i>sox3-Y</i>	XY	<i>sox3</i>	Allelic Diversification
Rainbow trout (<i>Oncorhynchus mykiss</i>)	<i>sdY</i>	XY	<i>irf9</i>	Gene Duplication
Nile tilapia <i>Oreochromis niloticus</i>	<i>amhY</i>	XY	<i>amh</i>	Gene Duplication
Pejerrey (<i>Odontesthes hatcheri</i>)	<i>amhY</i>	XY	<i>amh</i>	Gene Duplication
Torafugu (<i>Takifugu rubripes</i>)	<i>amhr2-Y</i>	XY	<i>amhR2</i>	Allelic Diversification
Chinese tongue sole (<i>Cynoglossus semilaevis</i>)	<i>dmrt1</i>	ZW	<i>dmrt1</i>	Allelic Diversification
Sablefish (<i>Anoplopoma fimbria</i>)	<i>gsdf-Y</i>	XY	<i>gsdf</i>	Allelic Diversification
Turquoise Killifish (<i>Nothobranchius furzeri</i>)	<i>gdf6-Y</i>	XY	<i>gdf6</i>	Allelic Diversification

translocation-fusion-insertion of the mutated allele together with another gene (DGCR8) located on the X-chromosome (Sato et al., 2010) provided a novel expression pattern. The effective -though low- SOX3 mRNA levels in primordial gonads of frogs, fishes, chicken and mice together with the fact that overexpression of SOX3 leads to XX female to male sex reversal gene in mice and humans (Sutton et al., 2011) are consistent with this evolutionary theory. On the other hand, absence of SOX3 expression in

the developing gonads of marsupials (Pask *et al.*, 2000) and of any gonadal phenotype after loss-of-function mutations in mice and humans (Weiss *et al.*, 2003; Raverot *et al.*, 2005) nourished doubts about a comprehensive conserved role of SOX3.

Monotremes, Marsupials and the Origin of SRY

Studies on chromosome evolution in marsupials and monotremes provided important insight into the ancestral sex determination (SD) system of mammals around 180 million years ago, prior to the divergence of the therian mammals (Wallis *et al.*, 2008). Marsupials have a typical XY system, while monotremes display multiple different X and Y sex chromosomes (Fig. 1), which form a chain during meiosis.

Genes from the canonical SD pathway (*NR5A1*, *SOX9*, *RSPO1* or *WNT4*...) that were mapped on the platypus genome revealed that they are all located on autosomes (Grafodatskaya *et al.*, 2007); excluding them as legitimate candidates for master sex determining genes. Interestingly, *DMRT1*, the prime candidate sex determining gene in birds and the avowed master sex determining factor in several frog and fish species (Table 1 and Fig. 1), is located on X-chromosome #5 (El-Mogharbel *et al.*, 2007). This is intriguing, but means that it is present in two copies in females and only one in male. But all what we know so far about *DMRT1*, from human patients with haploinsufficiency of this gene and from sex determination in birds and several fish, is that double dosage is connected to male development, not female. Currently, the most promising candidate gene, identified by transcriptome analyses, encodes the anti-muellerian hormone (*AMH*). Further, its position within an ancient bloc of genes on Y-chromosome #5 is consistent with a function as sex-determining gene (Cortez *et al.*, 2014). Validation of this candidate awaits, however, functional characterization.

Marsupials, despite an apparently simpler genetic condition (XY system), with X-chromosomes homologous to a large part (more than 2/3rd) of the eutherian X-chromosomes and the *SRY* gene present on the Y-chromosome, display, however, major differences in sex differentiation compared to eutherians. Karyotyping of intersex marsupials revealed that a pouch-mammary/scrotum switch gene resides on the X-chromosome (Sharman *et al.*, 1970), pinpointing major genetic rewiring of the complete sex determining gene regulatory network between marsupials and eutherian mammals despite employing the same master sex determiner.

XY and XX Sex Reversals in Mammals: What's Next Beyond SRY?

In spite of proven robustness and apparent stability, the mammalian system involving heteromorphic XX/XY sex chromosomes and headed by *SRY* is nevertheless not totally locked and refractory to change. A handful of species – all rodents so far – have independently evolved viable alternative systems bypassing or surpassing *SRY* without significantly affecting their fertility (Table 2).

XY sex reversed mammals: Sry on the loose in evolution? XY sex-reversals have been reported in several mammalian species including: (i) the wood and arctic lemmings *Myopus schisticolor* and *Dicrostonyx torquatus*, respectively, (ii) nine species of the South American field mice of the genus *Akodon*, (iii) the African pygmy mouse *Mus minutoides* and (iv) Cabrera's vole *Microtus cabreræ*. Molecular and histological analyses confirmed the complete XY sex-reversed phenotypes. Cabrera's vole is so far the only mammalian species harboring non-functional copies of *Sry* on its X-chromosome. Although each time independently acquired, an evolutionary convergence underlies the genetics of XY sex reversals in all species. As common mechanism, the presence of a third sex chromosome, named *X**, which is derived from the X-chromosome, prevents the initiation of the Y-chromosome-driven male-specific program (Table 2). Thus three types of females are generated (XX, *XX**, *X*Y*) while males are "regular" XY. In essence this system behaves like a polygenic variation of the canonical mammalian scheme with segregation of two sex determining genes: *Sry* on the Y-chromosome and a dominant female determiner on the *X**-chromosome, which overrides *Sry* (see Parma *et al.* (2016) for review).

XX sex reversed mammals: Sry lost, who's next? Intriguingly, even SD systems without *SRY* on the Y or no Y-chromosome exist. As far as known this is still restricted to only four species of mole voles *Ellobius* (*tancrei*, *talpinus* and *alaicus*) and the spiny rats *Tokudaia*

Table 2 Alternative sex determination systems in therian mammals

Sex reversal	Species	Sex chromosomes	
		Female	Male
Female with Y-chromosome	Wood lemming (<i>Myopus schisticolor</i>)	XX, <i>XX*</i> , <i>X*Y</i>	XY
	Arctic lemming (<i>Dicrostonyx torquatus</i>)		
	South American field mice <i>Akodon</i> sp		
	African pygmy mouse (<i>Mus minutoides</i>)		
Male in absence of Y-chromosome	Mole voles (<i>Ellobius tancrei</i> ; <i>E. talpinus</i> ; <i>E. alaicus</i>)	XX	XX
	Mole vole (<i>Ellobius lutescens</i>)	X0	X0
	Spiny rats <i>Tokudaia</i> sp		

osimensis and *T. tokunoshimensis*. Lacking the complete Y-chromosome including *Sry*, both males and females are all XX or XO depending of the species (see [Table 2](#)). Although different hypothesis were followed up (Y-linked gene translocation, gene dosage of the *Cbx2* gene, segregating allelic variants...) the “new” testis determining factor of these species still remains to be identified (see [Parma et al. \(2016\)](#) for review).

The remarkable robustness of the mammalian system, headed by *SRY* on the Y-chromosome, has been explained by the propensity of sex chromosomes to hijack and selectively accumulate genes beneficial for the reproductive fitness of that sex once it was established ([Graves, 1995](#)). Indeed the mammalian Y-chromosome carries several genes involved in spermatogenesis. The more its specialization was reinforced the more its transmission in males became necessary and made it indispensable. Nevertheless, the examples from the “sex-reversed” mammalian species show that either ovarian or testis development with or without *Sry* (down to the complete absence of the Y-chromosome), respectively, are possible and viable.

Excitingly, much more than peculiar outliers, such „exceptions“ certainly represent invaluable cases of evolution in action. Hence, to challenge our hypotheses it will be interesting to find out what has happened to the male beneficial genes. Did they leave the Y-chromosome first and reside now elsewhere in the genome – thus making the Y vulnerable to full decay or loss? If they did leave, do they support, and to which degree, the emergence of a new sex determiner and sex chromosome in the vicinity of their new location?

Birds: DMRT1 all over since and forever?

Dmrt1 is a Dosage Sensitive Male Determiner on the Z-Chromosome

Akin to mammals, birds also evolved a stable pan-order system for determining sex ([Table 1](#) and [Fig. 1](#)). The ZZ/ZW female heterogametic sex chromosomes of birds are unrelated to those of mammals, and lack any homologue to the mammalian *Sry* gene.

Theoretically, for a female heterogametic system, three different mechanisms of SD can be considered: (i) a dominant female determiner located on the W-chromosome, (ii) Z-chromosome dosage of a male determining factor (2 copies in males, ZZ, and only one in females, ZW), or (iii) a combination of both eventually with different “strength” of the respective factors. Currently, although the fine-tuning of the mechanism(s) of avian SD remains to be resolved, the almost complete absence of Z-chromosome inactivation in birds favors a Z-dosage mechanism of a male determining factor. In that respect, *DMRT1* is the prime candidate. It is present on the Z but not on the W-chromosome in all birds including ratites (flightless emu, ostrich...) ([Nanda et al., 2002, 2008](#)). In chicken embryos *DMRT1* is always higher expressed in ZZ male primordial gonads. Once initiated above a certain threshold (two copies in ZZ *versus* one in ZW), a positive feedback loop amplifies *DMRT1* expression in ZZ gonads. Overexpression in female (ZW) primordial gonads drives male development, while suppression of gonadal *DMRT1* expression in males (ZZ) leads to feminization and sex reversal to female ([Smith et al., 2009](#)).

In summary, all evidence is compatible with *DMRT1* being the Z-linked dosage sensitive male determiner. Although less well studied than mammals for their master sex regulator, thus far no exception for such a mechanism has been reported from a bird species. Interestingly the *DMRT1* family of transcription factors is involved in SD or sexual development of a wide range of organisms from flies, worms, and corals to fish, frogs, birds and mammals.

The W Alternatives

In most avian Z- and W-chromosomes are highly heteromorphic. Similar to the X-chromosomes of mammals the chicken Z-chromosome displays a notable bias of sex-related genes. And, analogous to the mammalian Y, the chicken W-chromosome is basically the degraded counterpart of the Z-chromosome with only a handful of intact genes. Interestingly, in the W-specific region the *HINTW* gene is present in multiple copies and appears to have been subjected to positive selection ([Smith, 2007](#)). Being a truncated counterpart of the Z-linked *HINTZ* gene, its gene product might act as a dominant negative version, blocking a possible testis function of *HINTZ*. However, mis-expressing *HINTW* did not disturb male gonadal development. Further functional characterization of another W-linked candidate, *FET1* (female expressed transcript 1) was not more fruitful. Sequencing of the chicken W revealed 28 intact genes, but none of these was expressed exclusively in female-specific tissues ([Hirst et al., 2017](#)). This certainly does not close the book of a female determiner on the W, but - in conclusion - the counter-evidence is accumulating.

Reptiles: Exploiting all Systems

In contrast to mammals and birds with their relatively stable genetic systems, reptiles exhibit a continuum of SD ranging from male or female heterogamety (XX/XY and ZZ/ZW respectively) up to environmental mechanisms (mainly temperature sensitivity), where even closely related species can be different ([Fig. 1](#)). It has been suggested that temperature sensitivity is the ancient mechanism and sex chromosomes are derived, but this is not supported when sex-determining systems are plotted on the phylogenetic tree of reptiles ([Smith, 2007](#)). To date no gene that acts as master regulator in a sex chromosomal system has been identified in reptiles.

Systematic identification of cryptic sex chromosomes in some families of lizards and turtles revealed that changes between XX/XY, ZZ/ZW and temperature SD systems are common, suggesting frequent transitions ([Gamble et al., 2015](#)). The homomorphic, and molecularly lowly differentiated sex chromosomes in the vast majority of reptiles may not impose high evolutionary constraints for

transitions. But notably, even species with highly differentiated sex chromosomes (XX/XY or ZZ/ZW) are still exposed to temperature induced sex reversion. In some species of bearded dragons high temperatures cause ZZ male-to-female sex reversion, producing fertile females. Thus, constant high temperatures result in the total elimination of the W-chromosome within few generations. An analogous situation has been observed in the Eastern three-line skink where low temperatures induce sex reversal of XX females to males. In both cases the unidirectional sex reversal of only the homogametic, eliminates the disadvantage of producing possibly lethal or inferior WW or YY offspring and favours the emergence of a new system (Holleley *et al.*, 2016).

Amphibians: Transitions in Action

Amphibians appear to employ mainly GSD and all possible mechanisms (XY, ZW, multiple sex chromosomes, X0, W0) are reported based on cytogenetic findings (Fig. 1). The reports of environmental influence on SD all seem to be laboratory-borne and have no counterpart in nature. So far the genes triggering sexual development are known only in one frog species, and suspected in a second; and in both cases it is again *dmrt1*.

While *DMRT1* acts as a Z-linked dosage-dependent male determiner in birds, surprisingly in the African clawed frog *Xenopus laevis* a duplicated copy, named DM-W, this time on the W-chromosome, appears to fulfil its function as a dominant female determiner (Gamble *et al.*, 2015). Lacking the dimerization domain, it is proposed to affect the transcriptional activation of *Dmrt1* through a dominant-negative action. Thus in female frogs *Dmrt1* cannot exert its male determining function. Rather than initiating female development, the mutant version of *dmrt1* on the W in fact suppresses male development (Fig. 2).

In another frog, *Rana temporaria* nascent Y-chromosomes carry different alleles of *dmrt1* and a large collection of population genetic data and family analyses point to this gene as the most likely candidate for being the male sex determiner (Rodrigues *et al.*, 2017).

A large body of literature explains – theoretically – the ins and outs of sex chromosome evolution and transitions among sex chromosome systems. Nevertheless the opportunities to observe such evolution in action are more than sparse. Interestingly, several examples from SD in amphibians might offer that perspective. In the Western clawed frog, *Xenopus tropicalis*, it was reported that, in addition to the long known W and Z chromosomes, the presence of another male determining chromosome, a Y-chromosome, is present in some stocks derived from natural populations. Further on, overriding the feminizing effect of even two Z-chromosomes in triploids, the Y is much stronger than the Z, while the Z can determine maleness only in the absence of the W.

The Japanese wrinkled frog, *Rana rugosa*, is peculiar because populations from the North of Japan have female heterogamy and heteromorphic sex chromosomes, while the populations from the rest of Japan and Korea are XY with homo- or heteromorphic sex chromosomes (Uno *et al.*, 2008). Thus opposite SD systems appear to coexist within the same species. However, the question whether this situation is already reflecting allopatric divergence towards the formation of two separate species is open.

Fish Sex Determination: Variations on the Theme

For the most basal group of vertebrates, the agnathans, several reports (Johnson *et al.*, 2017) suggest environmental SD in lampreys. From the chondrichthyans (cartilaginous fish) there are reports from several ray species for heteromorphic chromosomes either in males or females and even multiple sex chromosome (Cruz *et al.*, 2011) and a X0 system (de Souza Valentim *et al.*, 2013). For the basal chondrichthyan group of chimaeras several cases of male heterogamy are reported (Maddock and Schwartz, 1996). For none of these basal vertebrates there is any clue what the molecular triggers behind their sexual development are.

From the ancient fish, gars, sturgeons, and bichirs, solid evidence for their SD mechanism is missing, because none of the so far studied species exhibited cytogenetic clues for the presence of sex chromosomes. The same is true for the sarcopterygian fish, the coelacanth *Latimeria chalumnae* and *L. menadoensis*, and the six lungfish species.

In the so-called modern fish, the teleosts, the diversity of vertebrate SD systems is particularly obvious (Fig. 1). Here, within groups of closely related species a wide spectrum of different systems can be found (Table 1). Interestingly, among each type a multitude of mechanisms, how to initiate either male or female gonadal fates, have been documented. Moreover, numerous variants and even combinations of the different systems (GSD and ESD) are commonly observed. Environmental cues are as diverse as density, growth rates, social relationships, water quality (O₂ levels, pH), or even visual cues. Of these the most prevalent environmental signal –assumed to be the most primitive mechanism of SD in vertebrates– is temperature (see Baroiller *et al.* (2009) for review).

In teleost species the “operating” genetic sex determination (GSD) systems range from “classical” male or female heterogamy and multiple sex chromosome, to autosomal modifiers acting on the sex-determining gene, and finally to true polygenic systems (Moore and Roberts, 2013; Volff and Schartl, 2001; Devlin and Nagahama, 2002). The coexistence of two or more of such systems is not rare and has even been reported within the same genus. For instance, species with XY and ZW GSD are found in ricefish, Tilapia or sticklebacks or even within the same species like in some populations of cichlids or platyfish (Volff and Schartl, 2001).

Interestingly, such a high plasticity regarding the terms and conditions of fish SD is also noticeable when both GSD and ESD coexist and modulate each other. At different latitudes, populations of Atlantic silverside (*Menidia menidia*) – a fish that exhibits a temperature-influence on SD – compensate for differences in thermal environment and seasonality by adjusting the response of sex ratio to temperature through altering the level of environmental control over a basic genetic control (Conover and Heins, 1987). Laboratory strains of zebrafish cope with a weak genetic polyfactorial system (Anderson *et al.*, 2012) involving four different

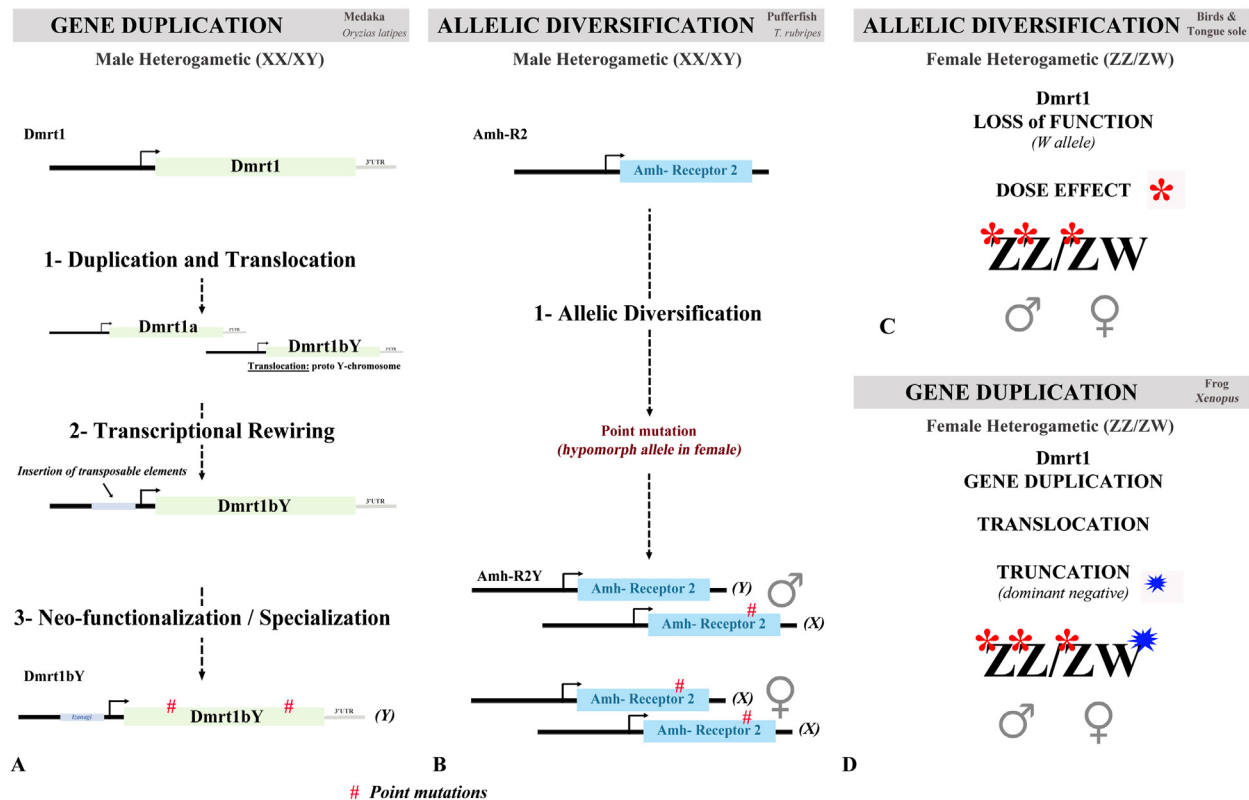


Fig. 2 Mechanisms accounting for the emergence of new master sex-determining genes after either gene duplication (examples medaka and frog) or allelic diversification (examples torafugu, birds and tongue sole). (A) The medaka *dmrt1* gene, together with neighboring genes (including *dmrt2* and 3), first underwent local gene duplication. This whole duplicated segment was then inserted into another chromosome, creating a proto Y-chromosome. Because of non-homology of the insert to the proto-X recombination is suppressed and this part of the proto Y segment degenerated. Transposable elements and repetitive sequences accumulated, resulting in transcriptional rewiring of the *dmrt1bY* gene and acquisition of an early gonadal expression pattern compatible with a master sex-determining function. Further on, point mutations accounted for neo-functionalization and specialization of the *dmrt1bY* gene (Reproduced from: Herpin and Scharl, 2008. Regulatory putsches create new ways of determining sexual development. EMBO Rep. 9, 966–968; Herpin and Scharl, 2009. Molecular mechanisms of sex determination and evolution of the Y-chromosome: Insights from the medakafish (*Oryzias latipes*). Mol. Cell. Endocrinol. 306, 51–58. and Herpin et al., 2010. Transcriptional rewiring of the sex determining *dmrt1* gene duplicate by transposable elements. PLOS Genet. 6, e1000844.). (B) In the pufferfish as a result of allelic diversification two versions of the *amh* receptor II exist differing only by one amino acid located in the kinase domain (H384D). This hypomorphic mutation conferring lower receptor activity is encoded on the X-chromosome. Quantitative variations in *Amh* signaling in females (homozygous for the hypomorphic *amhrll* allele) versus males (heterozygous for the wild type and hypomorphic alleles), account for male gonadal development (Reproduced from Kamiya et al., 2012. A trans-species missense SNP in *Amh2* is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (fugu) PLOS Genet. 8, e1002798). (C) Birds and the tongue sole and have a ZW female heterogametic system. A single loss of function event in the *dmrt1* gene occurred (W allele). This resulted in ZW individuals having only one copy of the *dmrt1* gene while ZZ individuals harbour two copies (red stars indicate functional *dmrt1* genes). Here a dosage effect leads to either female (ZW, 1 “dose” of *dmrt1*) or male (ZZ, 2 “doses” of *dmrt1*) gonadal development respectively (Smith et al., 2009. The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken Nature 461, 267–271; Chen et al., 2014. Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle Nat. Genet. 46, 253–260). (D) Sex determination in the frog *Xenopus laevis* is female heterogamety. Similar to medaka the frog *dmrt1* gene underwent gene duplication and translocation. Additionally truncation of the *dmrt1* gene occurred, generating a dominant negative version (DM-W). The DM-W protein product from the W-chromosome antagonizes the action of the wild type *dmrt1* gene on the Z-chromosome, leading to female gonadal development. ZZ individuals having two copies of the *dmrt1* gene (and no DM-W) develop as males (Yoshimoto et al., 2008). Red and blue stars indicate functional and dominant-negative version of the *dmrt1* gene respectively.

chromosomes (Dre 3, 4, 5, 16) and environmental modulators (hypoxia, high density, temperature, altered thermocycles, poor nutrition of even gamma rays,) for SD. Even in medaka, which has a strong Y-linked GSD, the system is still sensitive to thermal modulation: high temperature induces XX female to male sex reversal (Sato et al., 2005).

On the molecular level, different genes or pathways can be strong primary triggers (master sex-determining genes) for sexual development in fish (see Table 1 and (Herpin and Scharl, 2015)). The most classical ones, now referred as “usual suspects”, are genes that are derived from known actors of the sexual development regulatory network (Herpin and Scharl, 2015). First described in medaka (*Oryzias latipes*, XX/XY) *dmrt1bY* is probably the archetype of the transcription factor class of master sex determining genes (Table 1 and Fig. 2). This now also includes *dmrt1* in the Chinese tongue sole (*Cynoglossus semilaevis*, ZZ/ZW) (Table 1 and Fig. 2).

and *sox3-Y* in the Indian ricefish (*Oryzias dancena*, XX/XY). The other “usual suspects” come from the TGF- β signalling pathway (see **Table 1** and **Fig. 1**). The prototype members of this group are *amhy* and *amhr2-Y* in the Patagonian silverside (*Odontesthes hatcheri*) and the Japanese pufferfish (*Takifugu rubripes*) respectively (see **Table 1** and (Herpin and Schartl, 2015) for review). Other TGF- β factors that arose as SD triggers are *gsdf-Y* in the Luzon ricefish (*Oryzias luzonensis*) and the sablefish (*Anoplopoma fimbria*) derived from the fish specific gonadal soma derived factor gene, and *gdf6-Y* in the turquoise killifish (*Nothobranchius fuzeri*) (**Table 1** and **Fig. 1**). As exception so far, *sdY*, which is the master sex determining gene of rainbow trout (*Oncorhynchus mykiss*) and the whole salmonid family, has no homology to any known gene belonging to the SD pathways but is derived from an immune gene (*irf9*) (Yano *et al.*, 2012). The molecular mechanism(s) through which this “usurpator” triggers male gonadal development are still unknown.

An intriguing situation has been uncovered in the prejerrey, *Odontesthes bonariensis*, where sex is determined by temperature (Yamamoto *et al.*, 2014). Its congener *O. hatcheri*, has XY GSD with *amhy* as the dominant male determining gene on the Y. Surprisingly, the prejerrey also has *amhy*. At intermediate temperatures, which make about equal numbers of males and females, heterozygous presence of *amhy* determines males. At high and low temperature the action of *amhy* is overridden, but still may represent the interface between the environmental signal and the endogenous regulatory network.

The Commonality of Vertebrate Sex Determination: Evolutionary Principles for Variety

Generally speaking the development of the gonads in vertebrates is conceptually not different from other organs. During embryogenesis an uncommitted tissue is determined by a sequence of molecular events to become either a testis or an ovary. Once determined, differentiation genes establish the morphology and physiology of the respective organ. The preceding determination is initiated by expression of a master regulator that triggers a regulatory network, which finally activates the differentiation genes. The inherent program of the network is either to promote one sex or to prevent differentiation of the opposite sex or both.

The short survey in this article of the known SD mechanism and the genes which govern it already makes clear that SD in vertebrates is an evolutionary extremely plastic process – different from the majority of other developmental pathways, which display conservation of master regulators and effectors as well as their interaction. The non-conservation of the SD master regulators became obvious when *sry* was not detected outside mammals and was confirmed with the finding of *dmrt1bY* in medaka years later as a first instance of a non-Sry male determiner in vertebrates. Conversely, the major components of the downstream regulatory gene network governed by the different master genes were found in all species and led in conjunction with similar findings from flies and worms to the paradigm that “masters change, slaves remain”. More detailed studies revealed that the conservation of the members of the regulatory network is far from complete and that the genetic interactions and the resulting gene expression are also subject to change (Herpin *et al.*, 2013). An explanation for this extended plasticity is that the changes in the network are imposed by the change of the master SD gene. Most of the existing hypothesis of the evolutionary instability of SD systems in vertebrates aim at explaining the changes at the top and consequently would argue that the network changes are a consequence of emergence of a new genetic trigger for the system. These theories include the reasoning that any mutation, which creates a new sex determiner, will give a fitness advantage to its carrier if the new mutation is superior to the established mechanism. Over time this mutation will sweep through the population, and the previous SD gene disappears. The loss of the sex chromosome in several rodents and in the zebrafish laboratory strain supports another hypothesis, which posits that due to genetic degeneration or overriding external effects the heterogametic sex chromosome gets shed from the population and a new one with a new SD gene evolves from an autosome. The superiority of the new sex chromosome comes from its lower mutational load compared to the degenerated old one. Its establishment and persistence – at least for a while – is explained by one major theory that is based on linkage of the new SD gene to other genes that favor one sex and/or are detrimental to the other sex. The emerging SD gene, even when it is not a strong virtue to its carrier, benefits as a hitchhiker to the fitness advantage, which the sex beneficial or sex antagonistic gene in its vicinity brings with it. In any of these cases the new SD gene has to connect to a pre-existing genetic circuit and creating such an interface may also impact the conformation of further downstream interactions.

Besides these “top-down” explanations, which postulate positive selection for new SD genes there are also “bottom-up” (Wilkins, 1995) and “multi-hit” scenarios, which consider neutral or non-adaptive processes including random mutations, genetic drift and recombination as important drivers. Here the argument is that natural selection acts only on the final output of the SD process, namely the functionality of a testis and an ovary. As long as this is guaranteed changes of the upstream system can occur *ad libitum*. Those changes then can become fixed in a population by random genetic drift. This scenario allows the downstream SD network to vary in a similar or even more pronounced manner than the master SD genes.

The origin of the vertebrate SD genes basically follows two routes (**Table 1** and **Fig. 2**). (I) A duplication of a gene that is a member of the SD regulatory network (with the possible exception of *sdY* of salmonid fish) produces a second copy that is free to evolve, acquires some new features (neo-functionalization) and become the main trigger of the network. In all cases known so far this second copy was trans-located to another chromosome, which then became the Y or W. (II) Alternatively, allelic diversification at a locus leads to one of the alleles promoting either female or male SD. Again in vertebrates this happened as far as known at loci that are components of the regulatory network. In the ZW systems (birds, tongue sole) the non-sex promoting allele was non-functionalized and a dosage dependent male determiner, *dmrt1*, triggers testis development (**Fig. 2**). In the XY systems the allele on the proto-Y took over the new function, while it appears (although functional experimental evidence is missing) that the X-allele kept the ancestral function. In the allelic diversification cases the master SD genes did not change place in the genome.

How the environmental SD of fish and reptiles evolved and how SD works on the molecular level is barely understood, but there is accumulating evidence that the regulatory network is also in place. It is reasonable to assume that the environmental trigger, similar to a genetic trigger has an interface with the network, activating and/or deactivating the male or female program.

References

- Anderson, J. L., et al. (2012). Multiple sex-associated regions and a putative sex chromosome in zebrafish revealed by RAD mapping and population genomics. *PLoS One*, 7, e40701.
- Baroiller, J. F., D'Cotta, H., & Sailland, E. (2009). Environmental effects on fish sex determination and differentiation. *Sex. Dev. Genet. Mol. Biol. Evol. Endocrinol. Embryol. Pathol. Sex Determ. Differ.*, 3, 118–135.
- Chen, S., et al. (2014). Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nat. Genet.*, 46, 253–260.
- Conover, D. O., & Heins, S. W. (1987). Adaptive variation in environmental and genetic sex determination in a fish. *Nature*, 326, 496–498.
- Cortez, D., et al. (2014). Origins and functional evolution of Y chromosomes across mammals. *Nature*, 508, 488–493.
- Cruz, V. P., da, Shimabukuro-Dias, C. K., Oliveira, C., & Foresti, F. (2011). Karyotype description and evidence of multiple sex chromosome system X1X1X2X2/X1X2Y in *Potamotrygon aff. motoro* and *P. falkneri* (Chondrichthyes: Potamotrygonidae) in the upper Paraná River basin, Brazil. *Neotropical Ichthyol.*, 9, 201–208.
- de Souza Valentim, F. C., Porto, J. I. R., Bertollo, L. A. C., Gross, M. C., & Feldberg, E. (2013de). XX/XO, a rare sex chromosome system in *Potamotrygon* freshwater stingray from the Amazon Basin, Brazil. *Genetica*, 141, 381–387.
- Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191–364.
- El-Mogharbel, N., et al. (2007). DMRT gene cluster analysis in the platypus: New insights into genomic organization and regulatory regions. *Genomics*, 89, 10–21.
- Gamble, T., et al. (2015). Restriction site-Associated DNA sequencing (RAD-seq) Reveals an extraordinary number of transitions among Gecko sex-determining systems. *Mol. Biol. Evol.*, 32, 1296–1309.
- Grafodatskaya, D., et al. (2007). Search for the sex-determining switch in monotremes: Mapping WT1, SF1, LHX1, LHX2, FGF9, WNT4, RSP01 and GATA4 in platypus. *Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol.*, 15, 777–785.
- Graves, J. A. (1995). The evolution of mammalian sex chromosomes and the origin of sex determining genes. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, 350, 305–311 (discussion 311–312).
- Herpin, A., & Scharl, M. (2009). Molecular mechanisms of sex determination and evolution of the Y-chromosome: Insights from the medakafish (*Oryzias latipes*). *Mol. Cell. Endocrinol.*, 306, 51–58.
- Herpin, A., & Scharl, M. (2015). Plasticity of gene-regulatory networks controlling sex determination: Of masters, slaves, usual suspects, newcomers, and usurpators. *EMBO Rep.*, 16, 1260–1274.
- Herpin, A., et al. (2010). Transcriptional rewiring of the sex determining *dmrt1* gene duplicate by transposable elements. *PLoS Genet.*, 6, e1000844.
- Herpin, A., et al. (2013). Divergent expression regulation of gonad development genes in medaka shows incomplete conservation of the downstream regulatory network of vertebrate sex determination. *Mol. Biol. Evol.*, 30, 2328–2346.
- Herpin, A., & Scharl, M. (2008). Regulatory putches create new ways of determining sexual development. *EMBO Rep.*, 9, 966–968.
- Hirst, C. E., et al. (2017). Sex reversal and comparative data undermine the W chromosome and support Z-linked DMRT1 as the regulator of gonadal sex differentiation in birds. *Endocrinology*, 158, 2970–2987.
- Holley, C. E., Sarre, S. D., O'Meally, D., & Georges, A. (2016). Sex reversal in reptiles: Reproductive oddity or powerful driver of evolutionary change? *Sex. Dev. Genet. Mol. Biol. Evol. Endocrinol. Embryol. Pathol. Sex Determ. Differ.*, 10, 279–287.
- Johnson, N. S., Swink, W. D., & Brenden, T. O. (2017). Field study suggests that sex determination in sea lamprey is directly influenced by larval growth rate. *Proc. Biol. Sci.*, 284, 284.
- Kamiya, T., et al. (2012). A trans-species missense SNP in *Amhr2* is associated with sex determination in the tiger pufferfish, *Takifugu rubripes* (fugu). *PLoS Genet.*, 8, e1002798.
- Koopman, P., Gubbay, J., Vivian, N., Goodfellow, P., & Lovell-Badge, R. (1991). Male development of chromosomally female mice transgenic for *Sry*. *Nature*, 351, 117–121.
- Lovell-Badge, R., & Robertson, E. (1990). XY female mice resulting from a heritable mutation in the primary testis-determining gene. *Tdy. Dev. Camb. Engl.*, 109, 635–646.
- Maddock, M. B., & Schwartz, F. J. (1996). Elasmobranch cytogenetics: Methods and sex chromosomes. *Bull. Mar. Sci.*, 58, 147–155.
- Moore, E. C., & Roberts, R. B. (2013). Polygenic sex determination. *Curr. Biol. CB*, 23, R510–R512.
- Nanda, I., Haaf, T., Scharl, M., Schmid, M., & Burt, D. W. (2002). Comparative mapping of Z-orthologous genes in vertebrates: Implications for the evolution of avian sex chromosomes. *Cytogenet. Genome Res.*, 99, 178–184.
- Nanda, I., Schlegelmilch, K., Haaf, T., Scharl, M., & Schmid, M. (2008). Synteny conservation of the Z chromosome in 14 avian species (11 families) supports a role for Z dosage in avian sex determination. *Cytogenet. Genome Res.*, 122, 150–156.
- Parma, P., Veyrunes, F., & Pailhoux, E. (2016). Sex reversal in non-human placental mammals. *Sex. Dev.*, 10, 326–344.
- Pask, A. J., Harry, J. L., Renfree, M. B., & Marshall Graves, J. A. (2000). Absence of SOX3 in the developing marsupial gonad is not consistent with a conserved role in mammalian sex determination. *Genesis*, 27, 145–152.
- Raverot, G., Weiss, J., Park, S. Y., Hurley, L., & Jameson, J. L. (2005). Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. *Dev. Biol.*, 283, 215–225.
- Rodrigues, N., et al. (2017). Dmrt1 polymorphism and sex-chromosome differentiation in *Rana temporaria*. *Mol. Ecol.*, 26, 4897–4905. <https://doi.org/10.1111/mec.14222>.
- Sato, T., Endo, T., Yamahira, K., Hamaguchi, S., & Sakaizumi, M. (2005). Induction of female-to-male sex reversal by high temperature treatment in Medaka, *Oryzias latipes*. *Zoolog. Sci.*, 22, 985–988.
- Sato, Y., Shinka, T., Sakamoto, K., Ewis, A. A., & Nakahori, Y. (2010). The male-determining gene *SRY* is a hybrid of *DGCR8* and *SOX3*, and is regulated by the transcription factor CP2. *Mol. Cell. Biochem.*, 337, 267–275.
- Sharman, G. B., Robinson, E. S., Walton, S. M., & Berger, R. J. (1970). Sex chromosomes and reproductive anatomy of some intersexual marsupials. *J. Reprod. Fertil.*, 21, 57–68.
- Smith, C. A. (2007). Sex determination in birds: HINTs from the W sex chromosome? *Sex. Dev. Genet. Mol. Biol. Evol. Endocrinol. Embryol. Pathol. Sex Determ. Differ.*, 1, 279–285.
- Smith, C. A., et al. (2009). The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. *Nature*, 461, 267–271.
- Stevanović, M., Lovell-Badge, R., Collignon, J., & Goodfellow, P. N. (1993). *SOX3* is an X-linked gene related to *SRY*. *Hum. Mol. Genet.*, 2, 2013–2018.
- Sutton, E., et al. (2011). Identification of *SOX3* as an XX male sex reversal gene in mice and humans. *J. Clin. Invest.*, 121, 328–341.
- Uno, Y., et al. (2008). Comparative chromosome mapping of sex-linked genes and identification of sex chromosomal rearrangements in the Japanese wrinkled frog (*Rana rugosa*, Ranidae) with ZW and XY sex chromosome systems. *Chromosome Res. Int. J. Mol. Supramol. Evol. Asp. Chromosome Biol.*, 16, 637–647.
- Vollf, J. N., & Scharl, M. (2001). Variability of genetic sex determination in poeciliid fishes. *Genetica*, 111, 101–110.
- Wallis, M. C., Waters, P. D., & Graves, J. A. M. (2008). Sex determination in mammals – Before and after the evolution of *SRY*. *Cell. Mol. Life Sci. CMLS*, 65, 3182–3195.
- Waters, P. D., Wallis, M. C., & Marshall Graves, J. A. (2007). Mammalian sex – Origin and evolution of the Y chromosome and *SRY*. *Semin. Cell Dev. Biol.*, 18, 389–400.
- Weiss, J., et al. (2003). *Sox3* is required for gonadal function, but not sex determination, in males and females. *Mol. Cell. Biol.*, 23, 8084–8091.

- Wilkins, A. S. (1995). Moving up the hierarchy: A hypothesis on the evolution of a genetic sex determination pathway. *BioEssays News Rev. Mol. Cell. Dev. Biol.*, 17, 71–77.
- Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R. S., & Strüssmann, C. A. (2014). Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLoS One*, 9, e102574.
- Yano, A., et al. (2012). An immune-related gene evolved into the master sex-determining gene in rainbow trout, *Oncorhynchus mykiss*. *Curr. Biol.*, 22, 1423–1428.
- Yoshimoto, S., et al. (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proc. Natl. Acad. Sci. USA*, 105, 2469–2474.

Evolution of Sex Determining Genes in Fish

Qiaowei Pan, Yann Guiguen, and Amaury Herpin, INRA, Fish Physiology and Genomics laboratory, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Glossary

Amh Anti-mullerian hormone.

Amhr2 Anti-Mullerian hormone receptor type 2.

Allelic diversification A process that starts from a step-wise diversification of two alleles of a sex chromosome pair. One allele then becomes the sex determiner, while the other allele either retains a non-SD function or favors the development of the opposite sex.

DMRT1 Doublesex and mab-3 related transcription factors.

Environmental sex determination (ESD) When the sex of an individual is triggered by various external factors (i.e. temperature, pH, social interaction).

Gdf6 Growth differentiation factor 6.

Genetic sex determination (GSD) When the sex of an individual is triggered by its genotype only, including both mono or polygenic sex determination.

Gonochorism A state of having just one of at least two distinct sexes in any one individual organism.

Gsdf Gonadal soma derived growth factor.

Haploinsufficiency When a diploid organism has lost one copy of a gene and is left with a single functional copy of that gene and this single copy does not produce enough product to display the wild type's phenotypic characteristics.

Homomorphic/heteromorphic sex chromosomes If the two sex chromosomes in a pair can be distinguished cytologically by their differences in morphology, they are termed heteromorphic sex chromosomes. If the two sex chromosomes are morphologically identical, they are called homomorphic sex chromosomes (the degree of differentiation is usually correlated with the age of the sex chromosome pair).

Irf9 Interferon related factor 9.

Master sex determination gene A gene responsible for triggering the embryo towards either male or female development. It is not necessarily that this gene codes for a protein.

Neofunctionalization A gene changes its function or acquires a new function by altering the structure of its gene product and/or its expression pattern.

PGC Primordial germ cells, which are the precursors of the stem cells that will give rise to the germ cell lineage.

Recombination The process of forming new allelic combination in offspring by exchanges between parental genetic materials.

Sex chromosomes Chromosomes that usually harbour a master sex determination gene and are involved in the primary genetic sex determination.

Sex determination (SD) Primary mechanism leading to the expression of the phenotypic sex.

SDY Sexual dimorphic on the Y chromosome (Salmonids).

SNP Single nucleotide polymorphism.

SOX Sry-related high mobility group box.

SRY Sex determining region Y.

TE Transposable element.

TGF- β Transforming growth factor beta.

Therian mammal Non-egg-laying mammals, including placental mammals and marsupials.

Sex Determination and Sex Chromosomes

Sexual reproduction is a widespread phenomenon, which requires two sexes in a population. There are multiple ways to achieve this state, but the most common in vertebrates is gonochorism, where individuals acquire their sex during development and maintain their sexual fate throughout life. This sexual identity is triggered by a process called sex determination (SD), in which a “master switch” controls a downstream regulatory cascade leading to either male or female gonadal development. In contrast with other more conserved developmental programs, SD master switches can be highly variable with primary signals coming either from the genome (genetic sex determination or GSD), or from the environment (environmental sex determination or ESD). Genetic sex determination involves sex chromosomes that harbor inherited master SD genes (not necessarily coding for a protein), responsible for the initiation of the SD process (Herpin and Schartl, 2015). One species can have one master SD gene (monofactorial system) or multiple SD genes (polygenic system). In a monofactorial system, one sex is heterogametic with two different sex

chromosomes; the other sex is homogametic with two of the same type of sex chromosome. A male heterogametic system is termed XX-XY system (i.e., like in most mammals), where the Y chromosome carries the male master SD gene; a female heterogametic system is denominated ZZ-ZW system (i.e., like in birds), where the W chromosome carries the female SD gene (Heule *et al.*, 2014). Contrarily to monofactorial systems, polygenic systems (Moore and Roberts, 2013) involve more than two alleles: either one locus with multiple independently segregating alleles, or multiple loci interacting through epistasis. However, such systems are more complex and less well documented than monofactorial systems. Unlike most mammals and birds where one type of monofactorial system has been conserved for over 100 millions years, fish display a high diversity of sex determination systems including various types of monofactorial and polygenic systems. Moreover, sex determination systems in fish can differ between very closely related species, sometimes even among different populations of the same species. Furthermore, in some species, genetic factors can interact with environmental cues (most commonly temperature), generating intricate sex determination mechanisms. Such evolutionary dynamics makes fish an attractive group to study the evolution of sex determination systems. It is worth noting that studies from other taxa, such as amphibians and reptiles, have also revealed a comparable degree of diversity in sex determination systems (Kikuchi and Hamaguchi, 2013); however, to date, fish has provided the most abundant knowledge on sex determination and its evolution in vertebrates. While fish is a more generic term that can include several taxonomic groups, so far all the identified master SD genes were found in teleost fish. For this reason, we will be focusing on the evolution of sex determination genes in this subgroup.

Fish Sex Determining Genes

The high diversity of sex determination systems seen in teleost fish is linked to the high turnover rate of their sex chromosomes. As a result, most fish sex chromosomes are considered to be relatively young and are often described as being homomorphic, i.e., with little differentiation and no cytological difference. Sex chromosomes turnover is usually connected with the emergence of new master SD genes. Indeed, in therian mammals and birds, master SD genes are highly conserved with the quasi-exclusive usage of *SRY* for mammals and *DMRT1* for birds. But, a high diversity of SD genes (Fig. 1) has been found in the past two decades in many teleost fish and this has greatly expanded our understanding of the SD mechanisms and SD evolution. Despite this great diversity, most of these newly discovered fish master SD genes are classical “usual suspects” (Herpin and Scharl, 2015) that fall into three independent protein families implicated in the “canonical” vertebrate sex differentiation gene network, namely the Sry-related HMG box (SOX), the Doublesex and mab-3 related transcription factors (DMRT) and the transforming growth factors (TGF- β) family. Their recruitments as master SD genes do not follow any obvious phylogenetic relationship and are more likely to be independent and repeated events during teleost evolution (Fig. 1). Only one known exception to this “usual suspects” rule is the salmonid SD gene, *sdY* (sex determining region on the Y chromosome), which was derived from the duplication of an immune-related gene unsuspected to be involved in the sex differentiation pathway.

The birth of a new master SD genes can start with a duplication of an autosomal gene that will acquire a new SD function leading to new sex chromosomes. On the other hand, it can begin with allelic diversification from a step-wise diversification of two alleles of the future sex chromosome pair. One allele then becomes the sex determiner, while the other allele either retains a non-SD function or favors the development of the opposite sex. (Kikuchi and Hamaguchi, 2013). The gene duplication and allelic diversification mechanisms have both been found in teleost fish to generate new master SD genes and drive the turnover of sex chromosomes (Table 1).

In the following part of this article, we will review the cases of master sex determination genes in teleost fish and the key publications on their discoveries can all be found in Table 1.

DMRT1: A Multi-Context SD Gene

The *dmrt1* gene is a transcriptional factor belonging to the evolutionarily well-conserved DM (Doublesex and Mad-3) domain DMRT gene family, which is involved in sexual development in many phylogenetically distant groups from worms to mammals. In teleost fish, sexually dimorphic expression of *dmrt1* has been correlated with gonadal identity in both gonochoristic and hermaphroditic fish species (Herpin and Scharl, 2011). *dmy/dmrt1bY*, was the first non-mammalian SD gene found in vertebrates and it is a duplicated copy of autosomal *dmrt1/dmrt1a* gene found in two ricefish species of the genus *Oryzias*: the Japanese medaka (*O. latipes*) and the Malabar ricefish (*O. curvinotus*), both having an XX/XY sex determination system. *dmy/dmrt1bY* arose about 5–10 million years ago from a local duplication of an autosomal fragment encompassing the *dmrt1* gene. Consequently, this pair of X and Y chromosomes are very young in comparison with the chromosome pair found in therian mammals, and remain homomorphic.

The expression pattern of the medaka *dmrt1bY* is typical of a master SD gene with an early and transient expression during gonadal primordium differentiation, and low persistence in the adult gonads. Around the time of hatching *dmy/dmrt1bY* is first expressed in the nuclei of the somatic undifferentiated cells surrounding the primordial germ cells (PGC) and later on exclusively in the sertoli cells in males. In comparison, the autosomal *dmrt1/dmrt1a* is expressed much later at 20 days after hatching in males and shows a testis-specific expression pattern in adult males with 50 times higher expression than that of *dmy/dmrt1bY*.

Concomitantly to the acquisition of a dominant position within the sex-determining network, *dmy/dmrt1bY* was subjected to a profound rearrangement of its regulatory landscape brought about by exaptation of a “ready-to-use” cis-regulatory element

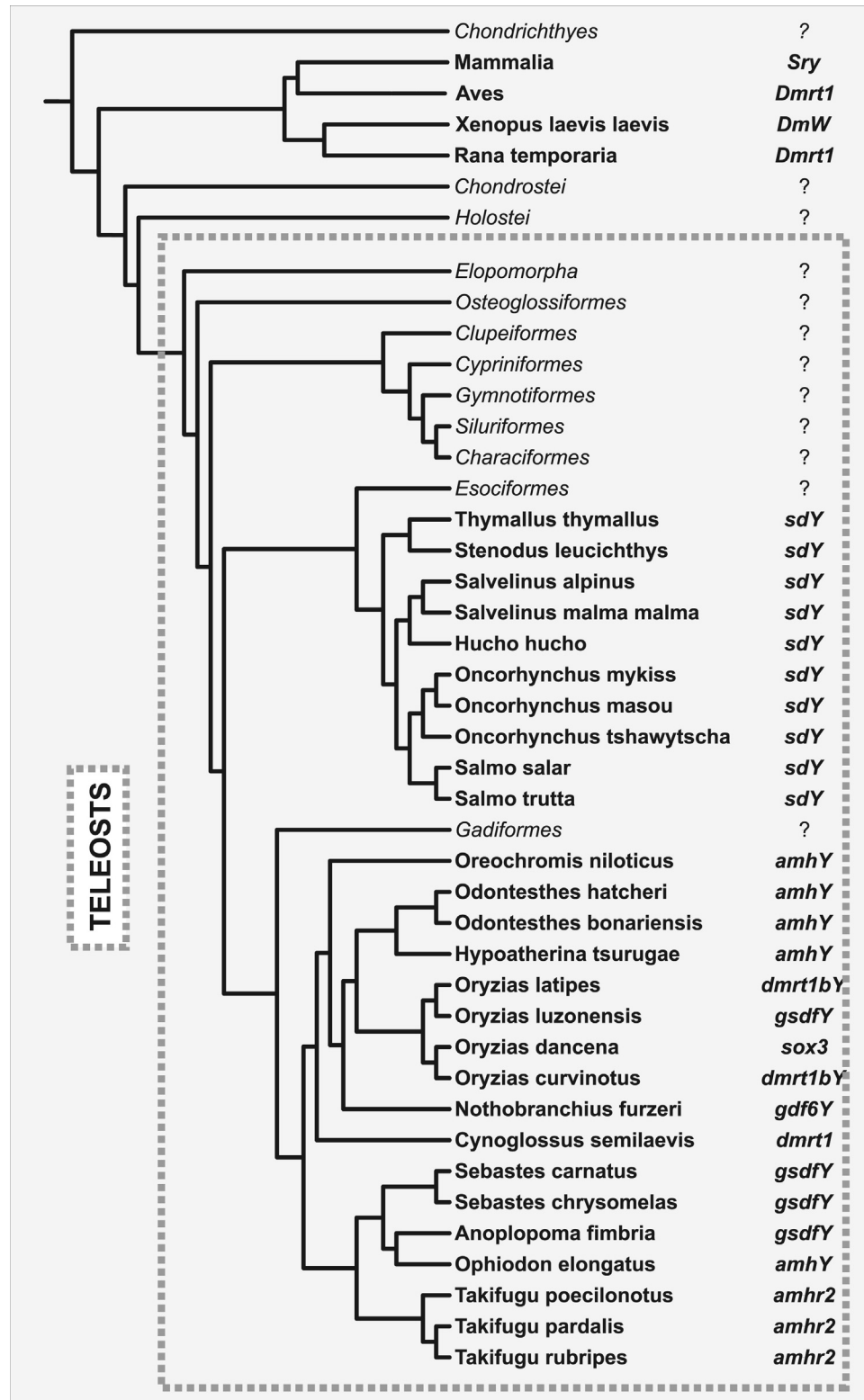


Fig. 1 Evolution and diversity of sex-determining genes in vertebrates with a focus on teleost fish. Species with a known or highly suspected SD gene are mentioned in bold type at each tip of the tree leaves along with the name of the SD gene. A few other taxa in which no SD gene is currently reported are also represented in normal type with a question mark.

Table 1 Currently known or suspected master sex determining genes in fish. Not all master SD genes found in teleost species have been shown to fulfill all classical SD requirements. Confidence in their sex determining role and function is given in validation column. References to species-specific publications are given in the publication column with a link to the DOI of the publication

Species	SD system	Master SD gene	SD gene ancestor	Gene group	Evolution mechanism	Validation	Key publications
Japanese medaka, <i>Oryzias latipes</i>	XY	<i>Dmy/dmrt1bY</i>	<i>Dmrt1</i>	Dmrt	Gene duplication	Expression Function Sex-linkage	Matsuda <i>et al.</i> , 2002 (doi:10.1038/nature751) Nanda <i>et al.</i> , 2002 (doi:10.1073/pnas.182314)
Malabar medaka, <i>Oryzias curvinotus</i>	XY	<i>Dmy/dmrt1bY</i>	<i>Dmrt1</i>	Dmrt	Gene duplication	Expression Function Sex-linkage	Matsuda <i>et al.</i> , 2003 (doi:10.2108/zsj.20.159)
Chinese tongue sole, <i>Cynoglossus semilaevis</i>	ZW	<i>Dmrt1</i>	<i>Dmrt1</i>	Dmrt	Allelic diversification	Expression Sex-linkage	Chen <i>et al.</i> , 2014 (doi:10.1038/ng.2890)
Indian medaka, <i>Oryzias dancena</i>	XY	<i>Sox3Y</i>	<i>Sox3</i>	Sox	Allelic diversification	Expression Function Sex-linkage	Takehana <i>et al.</i> , 2014 (doi:10.1038/ncomms5157)
Luzon ricefish, <i>Oryzias luzonensis</i>	XY	<i>GsdfY</i>	<i>Gsdf</i>	TGF- β	Allelic diversification	Expression Function Sex-linkage	Myosho <i>et al.</i> , 2012 (doi:10.1534/genetics.111.127497)
Sable fish, <i>Anoplopoma fimbria</i>	XY	<i>Gsdf^r</i>	<i>Gsdf</i>	TGF- β	Allelic diversification	Sex-linkage	Rondeau <i>et al.</i> , 2013 (doi:10.1186/1471-2164-14-452)
Rockfishes, <i>Sebastes carnatus</i> & <i>Sebastes chrysomelas</i>	XY	<i>Gsdf</i>	<i>Gsdf</i>	TGF- β	Allelic diversification	Sex-linkage	Flower and Buonaccorsi, 2016 (doi:10.1111/mec.13594)
Patagonia pejerrey, <i>Odontesthes hatcheri</i>	XY	<i>amhy</i>	<i>Amh</i>	TGF- β	Gene duplication	Expression Function Sex-linkage	Hattori <i>et al.</i> , 2012 (doi:10.1016/j.ygcen.2013.03.019)
Nile tilapia, <i>Oreochromis niloticus</i>	XY	<i>amhy</i>	<i>Amh</i>	TGF- β	Gene duplication	Expression Function Sex-linkage	Li <i>et al.</i> , 2015a (doi:10.1371/journal.pgen.1005678)
Lingcod, <i>Ophiodon elongates</i>	XY	<i>Amh</i>	<i>Amh</i>	TGF- β	Gene duplication	Sex-linkage	Rondeau <i>et al.</i> , 2016 (doi:10.1186/s13104-016-2030-6)
Fugu, <i>Takifugu rubripes</i>	XY	<i>Amhr2Y</i>	<i>Amhr2</i>	TGF- β	Allelic diversification	Expression Sex-linkage	Kamiya <i>et al.</i> , 2012 (doi:10.1371/journal.pgen.1002798)
Killyfish, <i>Nothobranchius furzeri</i>	XY	<i>Gdf6Y</i>	<i>Gdf6</i>	TGF- β	Allelic diversification	Expression Sex-linkage	Reichwald <i>et al.</i> , 2015 (doi:10.1016/j.cell.2015.10.071)
Rainbow trout, <i>Oncorhynchus mykiss</i>	XY	<i>sdY</i>	<i>irf9</i>	Interferon	Gene duplication	Expression Function Sex-linkage	Yano <i>et al.</i> , 2012 (doi:10.1016/j.cub.2012.05.045) Yano <i>et al.</i> , 2013 (doi:10.1111/eva/12032)

Note: References to species-specific publications are given in the publication column with a link to the DOI of the publication.

from a transposable element (TE). This element, called *Izanagi*, acts as a silencer to turn-off the *dmy/dmrt1bY* gene after it has fulfilled its male SD function (Herpin *et al.*, 2010).

In the medaka, the duplication/insertion of *dmrt1* led to a dominant *dmy/dmrt1bY* master SD on the Y chromosome, but interestingly *dmrt1* has also been found as the master SD gene in a fish species with ZZ/ZW sex determination system: the half-smooth tongue sole (*Cynoglossus semilaevis*). In this species *dmrt1* is only found on the Z chromosome while its homolog on the W chromosome is highly degenerated, probably a result of an old allelic diversification process. Even though homogametic sex is male in this species with two copies of Z chromosomes, SD operates through a Z-encoded mechanism that determines male development: the presence of two copies of *dmrt1* in ZZ animals leads to male development, while having only one functional copy (haploinsufficiency) in ZW individuals results in female development. *Dmrt1* is expressed specifically in male germ cells and pre-somatic cells of the undifferentiated gonad at the sex determination stage, fulfilling a key feature of male SD gene. Furthermore, functional knock-out of this gene resulted in male to female sex reversal, indicating its essential role of male SD gene in this species. The sex chromosome pair in tongue sole is thought to be around 30 millions years old, but whether closely related species of the tongue sole conserved *dmrt1* as a master SD gene is currently unknown. Interestingly, the sex chromosomes of the half-smooth tongue sole share the same ancestral vertebrate protochromosome as the W and Z chromosome in avians, which also have a Z dosage-dependent male sex determination system based on the *Dmrt1* gene, highlighting an evolutionary convergence around *Dmrt1*.

These two teleost cases show that *dmrt1* can acquire its position as a master SD gene through both allelic diversification and gene duplication mechanisms and its function as a SD gene can be triggered either by expression dosage effect or rewired transcription. Combined with its known SD roles in avians and some frogs (Yoshimoto *et al.*, 2008), *dmrt1* thus appears to be a highly versatile and flexible master SD gene.

Sox3 a Conserved Role in Vertebrate Sex Determination

SRY, the mammalian master SD gene on the Y chromosome is thought to be originated from an allelic diversification of the X-linked SOX3 gene. This is reminiscent of what has been found the Indian ricefish (*Oryzias latipes*), in which a novel sex chromosome recently evolved by co-option of this *Sox3* gene. In this species, the Y chromosome allele of *Sox3* (*Sox3^Y*) encodes the exact same amino-acid sequence as its X chromosome counterpart. However, a distant and specific *cis*-regulatory element of *Sox3* on the Y chromosome induces a gonadal specific expression of *Sox3* only in XY embryos, providing a SD signal in differentiating gonads. XX transgenic fish with the *Sox3^Y* regulatory segment developed as male and the loss of function mutation of *Sox3^Y* in XY fish lead to ovary-type gonads, supporting the SD role of *Sox3^Y* in that species. It was also demonstrated that the early testis specific expression of *Sox3^Y* initiates the expression of *gsdf*, a gene conserved in teleost testicular differentiation pathways, and induces testicular differentiation. However, the overexpression of *Sox3^Y* in the sister species *O. latipes* which has *dmy/dmrt1bY* as the master SD gene, could not induce testicular differentiation. This result indicates that co-evolved components in the downstream sex differentiation pathways are required for *Sox3^Y* to act as the master switch for male development (Herpin and Scharl, 2015).

The evolutionary parallel between *Sry* in therian mammals and *Sox3^Y* in Indian ricefish underlines the conserved importance of *sox3* in vertebrate sex determination.

The TGF- β Family: A SD Genes Jungle

A long-standing hypothesis, following the discovery of SRY in mammals, was that vertebrate SD genes should be transcription factors. The identification of *dmy/dmrt1bY* as the SD gene in *O. latipes* also supported this view. However, more recent evidences in teleosts have substantiated the idea that SD genes does not necessarily need to be transcriptional factors and members of the TGF- β family have emerged as master SD switches in teleost fish.

The first TGF- β related SD gene was identified in the Patagonian pejerrey (*Odontesthes hatcheri*). In this XX/XY species, a duplication of the anti-müllerian hormone (*amh*) was only found in males. *Amh* is a TGF- β hormone mainly known in mammals, birds and reptiles for its role in the regression of Müllerian ducts during male fetal development. Although teleosts lack the Müllerian ducts, they possess both *amh* and its type II receptor *amhr2* and this pathway has been implicated in the regulation of germ cell proliferation, spermatogenesis and regulation of gonadal sex-steroid synthesis. In the Patagonian pejerrey, this male-specific duplicated *amh*, called *amhy*, shares a high identity with its autosomal copy *amha* but has a Y-specific insertion in its third intron. Being very similar within the coding sequence to the autosomal *amha*, *amhy* is thought to fulfill its SD role through an earlier expression in the differentiating male gonads. This male-specific duplication of *amh* is also conserved in a closely sister species *O. bonariensis*, whose SD is known to be also modulated by temperature (Yamamoto *et al.*, 2014). In addition, in a more distant *Atheriniformes* species, a truncated duplicated copy of *amh* is suspected to be involved in male sex determination. However, due to greater sequence divergence, it has not been resolved whether this truncated duplicate of *amh* shares ancestry with the previous two cases or is an independent recruitment of the same SD gene. In the Nile tilapia (*Oreochromis niloticus*), a tandem duplication of *amh* has been found on the Y chromosome. This tandem duplication consists of a gene encoding a truncated protein lacking the TGF- β domain (*amh- Δ y*), and immediately downstream an *amhy* gene that is identical to the X-linked *amh* except for a single missense single nucleotide polymorphism (SNP). Both *amh- Δ y* and *amhy* are exclusively expressed in XY gonads during the onset of sex determination period in Nile tilapia. Similar expression profile of the X-linked *amh* and their receptor *amhr2* was found in both XX and XY gonads. However, only the knock-out of *amhy* lead to male to female sex reversal in XY individuals, while the knock-out of *amh- Δ y* alone had no effect. Additionally, overexpression of *amhy* caused female to male sex reversal in XX fish, but not the overexpression of the

X-linked *amh* or the truncated *amh-Δy*. Collectively, these results show that *amhy* is the master SD gene in Nile tilapia. More recently, another fish species, the lingcod (*Ophiodon elongatus*), has been characterized with a male-specific duplicate of *amh*. Although no expression nor functional data were available to consolidate its SD function, it potentially provides another independent case of recruitment of *amh* as a master SD gene in teleosts.

Interestingly the Amh type II specific receptor gene, *amhr2*, has also been identified as a SD gene in fish. In the pufferfish (*Takifugu rubripes*), a species with an XX/XY SD system, a missense SNP in the kinase domain of *amhr2* was found to be the only polymorphism strictly associated with phenotypic sex with males being heterozygous and females homozygous. Sex is then determined by the combination of these two X and Y *amhr2* alleles. This SNP leads to a single amino acid substitution and the X allele confers a decreased AMH signal transduction in XX females, while XY males with the wild-type *amhr2* Y allele would have a better Amh signaling, leading to testicular differentiation. This result is consistent with the mekade *hotei* mutant in which the impaired *amhr2* activity is linked with the feminization of the gonads. Surprisingly, this single missense SNP associated with male development is conserved over 10 million years of evolution, as it is present in two other Takifugu species (*T. pardalis* and *T. peocilonotus*).

The gonadal soma derived growth factor (*Gsdf*) is another vertebrate TGF- β ligand closely related to Bone Morphogenetic Proteins (BMPs), which has been lost in tetrapods. This *gsdf* gene was first characterized in rainbow trout as an inducer of germ cells proliferation, and has since been associated with testicular development due to its exclusive expression in the early differentiating testis of all teleosts examined so far (Herpin and Scharl, 2015). In *Oryzias luzonensis*, an XX-XY ricefish species closely related to the Japanese medaka *O. latipes*, X and Y alleles of *gsdf* have been identified, and *gsdf^Y*, the Y chromosome allelic form of *gsdf*, has been further characterized as a master SD gene. *gsdf^Y* and *gsdf^X* slightly differ in their sequence, but encode identical proteins. The testicular differentiation inducing role of *gsdf^Y* is triggered by a cis-regulatory region affecting a potential binding site for steroidogenic factor 1 (*sf1* or *nr5a1*) in the *gsdf^Y* promoter, conferring an elevated and earlier expression level of *gsdf^Y* during sexual differentiation than that of *gsdf^X* allele. Overexpression of the *gsdf^Y* locus in XX females is able to induce masculinization, corroborating its role as a male master SD gene in *O. luzonensis*. In addition, this overexpression of *gsdf^Y* is also able to induce masculinization of XX females in the sister species *O. latipes* which has *dmy/dmrt1bY* as the master SD gene, suggesting that *gsdf^Y* could functionally replace *dmy/dmrt1bY*. A few other fish species have been also described to rely on *gsdf* as a putative master SD gene, i.e., the sablefish, *Anaplopoma fimbria* and two rockfish species, *Sebastes carnatus* and *S. chrysomelas*.

Growth differentiation factor 6 (*Gdf6*) is a member of the TGF- β family known to be involved in vertebral segmentation and cell differentiation. While no gonadal function was reported previously for this gene, *gdf6Y* emerged recently as a putative SD gene in the turquoise killifish, *Nothobranchius furzeri*. In this species, the *gdf6* locus is sex-linked with specific X and Y chromosomal allelic variations that affect the Gdf6 protein function. So far, functional evidence of its role as a SD gene is not available, yet the Y chromosome allele, *gdf6Y*, exhibits a clear sex-linked expression during testicular differentiation, supporting its SD role in this species.

sdY: An Unusual and Conserved SD Gene in Salmonids

All of the above-described SD genes belong to the so-called ‘usual suspects’ category but the recent discovery of a new type of SD gene in rainbow trout (*Oncorhynchus mykiss*) demonstrated an unexpected flexibility in vertebrate SD. This gene, called *sdY*, came to the spotlight as it was found to be expressed only in embryonic testes and was also tightly linked to the sex-locus on the Y chromosome. It was further confirmed to be the trout SD gene based on functional proofs showing that *sdY* is by itself necessary and sufficient to induce testicular differentiation. One of the multiple unusual characteristics of *sdY* is that it evolved from the duplication of an immune related gene, *irf9* (interferon related factor 9) and compared to its ancestral protein, *sdY* lost the N-terminal DNA-binding domain, but preserved its C-terminal protein-protein interaction domain. Another interesting feature is that *sdY* is sex-linked in most salmonid species, indicating that it is a conserved SD gene in this teleost group. Yet, the SEX locus containing *sdY* is not located in the same chromosome in different salmonids species (Woram et al., 2003), suggesting a rapid turnover of sex chromosome while preserving what seems to be a jumping master SD gene (Faber-Hammond et al., 2015). How this unusual SD gene was recruited and the mechanism through which it regulates the downstream sex differentiation gene networks remains to be clarified. However, it presents a paradigmatic case that un-related genes outside the “usual suspects” are able to acquire *de novo* sex determining functions in teleosts.

What Lessons Can Be Learned From This Amazing Fish SD Gene Diversity?

The evolution of genetic sex determination mechanisms is closely linked to the evolution of sex chromosomes. The discovery of multiple new master SD genes in teleosts provided invaluable insights on the evolution of SD and the processes of the establishment of new sex chromosomes. Before the identification of the first fish master SD gene, models of sex chromosome evolution predicted that SD genes should evolve from a stepwise allelic diversification process starting from a single gene on an autosome. After fixation of a sex-specific allele in that newly formed SD gene, recombination reduction around that new sex locus in the proto-sex chromosome pair would give rise to classical heteromorphic sex chromosomes. Some newly described fish SD genes have evolved by this allelic diversification mechanism, for instance *sox3^Y* and *gsdfY* in some medaka species and *amhr2* in Takifugu. But this mechanism did not lead to strongly differentiated heteromorphic sex chromosomes in these teleost species. This phenomenon can probably be explained if we consider that these SD genes are very young in term of evolution. But the conservation of a single missense SNP in *amhr2* acting as the sole signal to initiate sex determination in at least three Takifugu species that diverged over 10 million

years ago, shows that recombination reduction around a new sex locus in the proto-sex chromosome is not necessary to fix a new SD gene. The characterization of new teleost SD genes also provided a novel mechanism for sex chromosome and SD gene evolution as many fish SD genes are the result of a duplication/insertion event. In these cases, a genomic area containing an ancestral gene is duplicated into a different chromosome that will initiate the formation of a new sex chromosome after subsequent functional and/or regulatory changes of the proto SD gene. This mechanism of gene duplication leading to sex chromosome formation first came in light for *dmr1/dmrt1bY* in Japanese medaka, and also been found to generate other vertebrate master SD genes, such as the female SD gene *DM-W* in the African clawed frog (*Xenopus laevis*) (Yoshimoto *et al.*, 2008).

In addition, thanks to their high turnover rate, teleost sex chromosomes also provide many different models to study the process of sex chromosome formation. One such case is from the study on half-smooth tongue sole in which a pair of relatively young Z and W chromosomes, comparing to those found in birds, have been sequenced and analyzed in details. This analysis showed that suppression of recombination, accumulation of transposable elements, fixation of sex specific genes, and gene dosage compensation are early events of sex chromosomes evolution. Furthermore, this half-smooth tongue sole W chromosome has already lost about two-thirds of its original protein coding genes and thus reached a roughly similar stage as much older mammalian and avian sex chromosomes within 30 million years. Combined with the finding that the gene-poor primate Y chromosome has been stable over the last 25 million years, they provide supports for the hypothesis that the decay of sex chromosome can happen rapidly in the early phase of sex chromosome formation then slow down and stabilize in its final stage, reaching a gene content equilibrium.

The 'limited options' hypothesis states that particular sex chromosomes or genes, especially those known to be involved in the SD gene network, might be preferentially recruited repeatedly in vertebrate lineages as new sex chromosomes or new master SD genes as they are 'good at doing the job' (Graves and Peichel, 2010). The multiple master SD genes recently found in teleost strongly endorse this idea as all of them with one single exception originate from three "usual suspect" gene family, namely *dmrt*, *sox* and *Tgf-β*. *Dmrt* and *sox* family members have also been also found in other vertebrate groups but several *Tgf-β* members have first emerged in teleosts as new 'usual suspects' and since then *Amh* has also been identified as the potential platypus master SD gene (Cortez *et al.*, 2014). Interestingly new knowledge on fish SD genes also provide an exception to this 'limited options' hypothesis as *sdy*, the salmonid master SD gene, is seemingly not related to any sex differentiation genes. However, it could not be excluded that this might be representing previously neglected factors in the SD gene networks (Herpin and Scharl, 2015), or that these unusual SD genes are underrepresented because they are just much more difficult to find.

Recently, a proposal with a developmental perspective has been put forward to view sex determination not as one hierarchical cascade with the master SD gene on the top, but as a complex network with different types of genetic and environmental factors influencing cell proliferation and hormone levels to push sexual development over either a male or female threshold (Heule *et al.*, 2014; Uller and Helanterä, 2011). Studies on the teleost SD provided supports to this view in which each node of the SD gene network has the potential of becoming the major-effect loci, not necessarily being on the top of the cascade. A prominent example of this hypothesis would be *amhr2* in Takifugu that modulate transduction efficiency of an upstream ligand signal, i.e., AMH acting on germ cell proliferation or regulating steroid synthesis activity (Kikuchi and Hamaguchi, 2013), to direct male or female development.

Many open questions still remain concerning teleost SD and probably the most important one is why the teleost fish use so many SD genes and SD systems with such a high sex chromosomes turnover rate? Rapid turnover of sex chromosomes is thought as a way to escape the decaying fate of sex chromosomes, assuming that the lack of recombination around the sex locus will lead to the accumulation of deleterious mutations. Alternatively, sexual antagonistic selection has also been put forward to explain the expansion of the sex locus and the accumulation of sex specific genes near it. Sex ratio selection provides a scenario that a new master SD gene could rise in frequency when it confers a more balanced sex ratio in the population. Other mechanisms such as random genetic drift and pleiotropic selection have also been used to explain the rise of new SD systems. Another hypothesis, explaining the lack of sex chromosome turnover in mammals and birds, proposes that these highly heteromorphic sex chromosomes might be acting as evolutionary traps that would stabilize SD genes for long span of evolutionary time (Bachtrog *et al.*, 2014). Interestingly, SD in many teleost species with a genetic SD system can still be influenced by environmental cues like for instance in the Nile tilapia and the half-smooth tongue sole. Transition stages between GSD and TSD are also well illustrated in the case of pejerrey species that conserves a SD gene (*amhY*) that is used alone in *O. hatcheri* and used in combination with temperature in the closely related species *O. bonariensis* (Yamamoto *et al.*, 2014). These transition stages could potentially facilitate the turnover of SD systems, leading to the emergence of new SD genes.

References

- Bachtrog, D., Mank, J. E., Peichel, C. L., et al. (2014). Sex determination: Why so many ways of doing it? *PLOS Biol.*, 12. <https://doi.org/10.1371/journal.pbio.1001899>.
- Cortez, D., Marin, R., Toledo-Flores, D., et al. (2014). Origins and functional evolution of Y chromosomes across mammals. *Nature*, 508, 488–493. <https://doi.org/10.1038/nature13151>.
- Faber-Hammond, J. J., Phillips, R. B., & Brown, K. H. (2015). Comparative analysis of the shared sex-determination region (SDR) among salmonid fishes. *Genome Biol. Evol.*, 7, 1972–1987. <https://doi.org/10.1093/gbe/ew123>.
- Graves, J. A. M., & Peichel, C. L. (2010). Are homologies in vertebrate sex determination due to shared ancestry or to limited options? *Genome Biol.*, 11, 205.
- Herpin, A., Braasch, I., Kraussling, M., et al. (2010). Transcriptional rewiring of the sex determining *dmrt1* gene duplicate by transposable elements. *PLOS Genet.*, 6. <https://doi.org/10.1371/journal.pgen.1000844>.
- Herpin, A., & Scharl, M. (2011). *Dmrt1* genes at the crossroads: A widespread and central class of sexual development factors in fish. *FEBS J.*, 278, 1010–1019. <https://doi.org/10.1111/j.1742-4658.2011.08030.x>.

- Herpin, A., & Scharf, M. (2015). Plasticity of gene-regulatory networks controlling sex determination: Of masters, slaves, usual suspects, newcomers, and usurpators. *EMBO Rep.*, 16, 1260–1274. <https://doi.org/10.15252/embr.201540667>.
- Heule, C., Salzburger, W., & Bohne, A. (2014). Genetics of sexual development: An evolutionary playground for fish. *Genetics*, 196, 579–591. <https://doi.org/10.1534/genetics.114.161158>.
- Kikuchi, K., & Hamaguchi, S. (2013). Novel sex-determining genes in fish and sex chromosome evolution: Novel sex-determining genes in fish. *Dev. Dyn.*, 242, 339–353. <https://doi.org/10.1002/dvdy.23927>.
- Moore, E. C., & Roberts, R. B. (2013). Polygenic sex determination. *Curr. Biol.*, 23, R510–R512. <https://doi.org/10.1016/j.cub.2013.04.004>.
- Uller, T., & Helander, H. (2011). From the origin of sex-determining factors to the evolution of sex-determining systems. *Q. Rev. Biol.*, 86, 163–180. <https://doi.org/10.1086/661118>.
- Woram, R. A., Gharbi, K., Sakamoto, T., et al. (2003). Comparative genome analysis of the primary sex-determining locus in salmonid fishes. *Genome Res.*, 13, 272–280. <https://doi.org/10.1101/gr.578503>.
- Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R. S., & Strüssmann, C. A. (2014). Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLOS ONE*, 9, e102574. <https://doi.org/10.1371/journal.pone.0102574>.
- Yoshimoto, S., Okada, E., Umemoto, H., et al. (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proc. Natl. Acad. Sci.*, 105, 2469–2474. <https://doi.org/10.1073/pnas.0712244105>.

Genetic & Environmental Sex Determination in Cold-blooded Vertebrates: Fishes, Amphibians, and Reptiles

J Adam Luckenbach, National Oceanic and Atmospheric Administration, Seattle, WA, United States

Yoji Yamamoto, Tokyo University of Marine Science and Technology, Minato-ku, Tokyo, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Anurans Amphibians of the order Anura comprising frogs, toads and tree frogs, all which lack a tail in the adult stage and possess long hind limbs for jumping and swimming.

Epigenetics Heritable traits or phenotypes that cannot be explained by differences in DNA sequences; examples are DNA methylation and histone modification.

Gonadal primordium The region of the body, consisting of bipotential germ and somatic cell lines, from which the gonads will ultimately develop.

Gonochorists Species in which individuals develop as either male or female and remain the same sex throughout life (i.e., non-sex changing species).

Heterogamety Refers to the sex of a species that possesses different sex chromosomes (e.g., XY in human males).

Homogamety Refers to the sex of a species that possesses the same sex chromosomes (e.g., XX in human females).

Temperature-dependent sex determination (TSD) A type of environmental sex determination in which the temperature to which an animal is exposed during embryonic or juvenile development determines its sex.

Thermosensitive period The developmental period during which the germ and somatic cells of the gonads are sensitive to and may be influenced by temperature.

Urodeles Amphibians of the order Caudata that have a tail throughout life.

Vertebrates Animals from the subphylum Vertebrata consisting of 7 living classes: Agnatha, (jawless fishes), Chondrichthyes (cartilaginous fishes), Osteichthyes (bony fishes), Amphibia (amphibians), Reptilia (reptiles), Aves (birds), and Mammalia (mammals).

Introduction

Sex determination is the process by which the sexual fate of an organism is set into motion. This pivotal event subsequently dictates numerous aspects of an organism's life history, potentially including its morphology, growth, behavior, and lifespan. In vertebrates, sex determination may be driven by the presence and activation of a single gene, combination of genes, exposure to an environmental cue(s), or a combination of both genetic and environmental cues. This triggers a relatively conserved molecular signaling cascade and cellular and anatomical changes leading to the formation of either ovaries or testes from an undifferentiated gonadal primordium.

Although sexual reproduction (mating of a female and male) itself is highly conserved through vertebrate evolution, the process of sex determination exhibits a higher degree of plasticity. This is especially true for cold-blood or poikilothermic vertebrates (fish, amphibians and reptiles), which make up over half of all living vertebrates. Species from these classes exhibit a dizzying array of sex determination systems that have traditionally been categorized as either having a genetic (genetic sex determination, GSD) or environmental (environmental sex determination, ESD) basis (Fig. 1). Nature defies these categories, however, as an ever-growing number of species previously thought to exhibit strict GSD or ESD demonstrate interplay between genetic and environmental cues leading to the ultimate female or male sexual phenotype.

The diversity of sex determination mechanisms in cold-blooded vertebrates has been driven by millions of years of radiation and adaptation to extreme aquatic and terrestrial environments. This exciting research area truly stands at the intersection where genetics meets environment and is important to our understanding of the evolution of sex determination mechanisms and sexual reproduction. The goal of this article is to provide a general summary of sex determination in cold-blooded vertebrates, including recent developments in the field and vital questions that remain.

Genetic Sex Determination

In nearly all mammals, including humans, sex is irreversibly determined at conception by the presence/absence of the sex-determining region of the Y-chromosome (*SRY*). If the *SRY* gene is paternally inherited and properly expressed, testicular development is triggered, whereas in the absence of *SRY*, ovarian development is initiated. This type of system is referred to as monogenic because it is controlled by a single sex-determining locus or 'master switch' that initiates the sex differentiation cascade. This

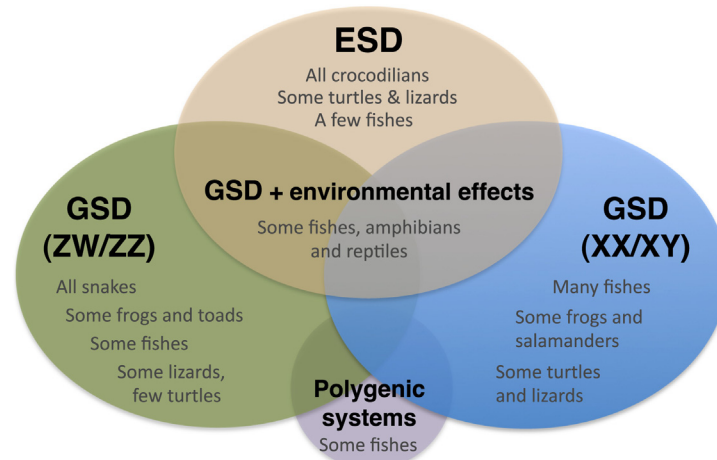


Fig. 1 The variety of sex determination systems in cold-blooded vertebrates. Environmental sex determination (ESD) is common in reptiles and observed in a few fishes. The major genetic sex determination (GSD) systems, ZW/ZZ and XX/XY, are found in all classes of cold-blooded vertebrates, although some species are sensitive to environmental factors and GSD can be influenced/overridden (GSD+environmental effects), at least under experimental conditions. Complex polygenic systems are observed in some fishes but considered rare.

relatively simple and highly conserved system in mammals is in stark contrast to what is seen in cold-blooded vertebrates. Indeed, these vertebrate classes exhibit a multitude of GSD systems ranging from monogenic to complex multi- and polyfactorial (polygenic) systems that are still not well understood (Fig. 1).

There are numerous examples of monogenic GSD in cold-blooded vertebrates. So-called XX/XY and ZW/ZZ systems are most common and appear to have evolved independently numerous times in some taxa. In XX/XY systems, like that of humans, males are the heterogametic sex (XY genotype) and females the homogametic sex (XX genotype). The opposite holds true for ZW/ZZ systems; females are heterogametic and males are homogametic. This basically means that sex is determined by the genetic contribution of the male parent in XX/XY species and the female parent in ZW/ZZ species. This does not mean however that the same gene or genes on the sex chromosomes determines sex for all XX/XY or ZW/ZZ species. In fact, in some taxa there appears to have been high turnover of master sex-determining genes as we discuss later.

GSD in Fishes

Many fish species are in the process of what is considered initial sex chromosome differentiation and possess 'homomorphic sex chromosomes,' which do not noticeably differ in appearance from autosomes (Penman and Piferrer, 2008). In contrast, some species have 'heteromorphic sex chromosomes,' which differ in cytological appearance. For example, the human Y-chromosome has undergone significant reduction in the number of genes it possesses and its overall size during evolution, making it easily distinguishable from autosomes by karyotype.

Systems of GSD have primarily been characterized in model fishes (e.g., medaka *Oryzias latipes*) and species of importance related to aquaculture (e.g., Nile tilapia *Oreochromis niloticus*; rainbow trout *Oncorhynchus mykiss*; pufferfish *Takifugu rubripes*). Identification of a species' sex determination system is often achieved through chromosome set manipulation (e.g., gynogenesis) and/or sex reversal and progeny testing (Devlin and Nagahama, 2002). Unlike mammals, which become refractory to sex steroid treatment, the processes of sex determination and gonadal differentiation in most cold-blooded vertebrates can be easily overridden by exogenous treatments during what is referred to as the labile or critical-sensitive period of development, which varies by species. Sex reversal typically involves immersion and/or dietary treatment of animals with either androgens, estrogens, or inhibitors of steroid synthesis (e.g., aromatase inhibitors (AIs); Fig. 2). These approaches can be used directly or indirectly to produce monosex stocks of fish for aquaculture, which is not only important from an applied perspective, for example, one sex exhibiting superior growth in aquaculture, but can also reveal the underlying GSD system (Fig. 2).

XX/XY and ZW/ZZ GSD systems are most common in fishes, with XX/XY being predominant (Fig. 1). Examples of species with a known XX/XY system include medaka, rainbow trout, Japanese flounder (*Paralichthys olivaceus*), Atlantic halibut (*Hippoglossus hippoglossus*), and sablefish (*Anoplopoma fimbria*). Examples of species with a ZW/ZZ system include yellowtail (*Seriola quinqueradiata*), blue tilapia (*Oreochromis aureus*), and half-smooth tongue sole (*Cynoglossus semilaevis*). There are also cases in which the system of sex determination differs among species of the same genera (e.g., within *Oryzias* and *Xiphophorus*) and even within a species (e.g., *Xiphophorus maculatus*; Devlin and Nagahama, 2002). Thus, there appears to be no strong genetic selection for male versus female heterogamety in fishes.

Variation on major GSD systems above has also been documented, as some fishes exhibit systems (XX/XO and ZW/ZO) in which one sex chromosome has been lost in males (Devlin and Nagahama, 2002; Penman and Piferrer, 2008). Females thus inherit two sex chromosomes (XX or ZW) while males inherit a single sex chromosome (X or Z). In these cases, "O" in the nomenclature

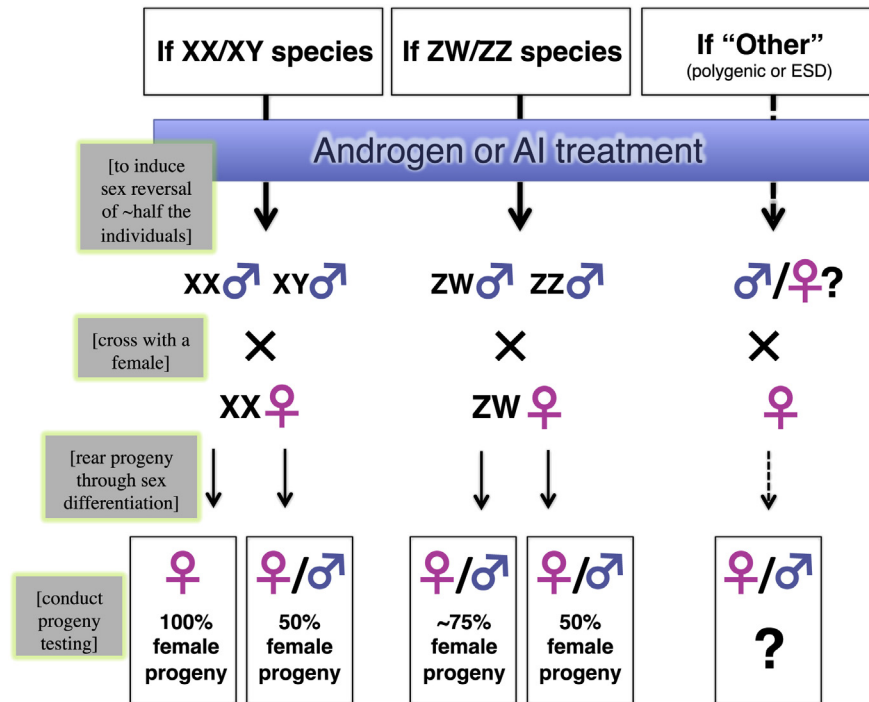


Fig. 2 Example of sex reversal techniques and progeny testing often used by researchers to identify the underlying sex determination system of cold-blooded vertebrate species. By exogenously treating a cohort of animals with an androgen or aromatase inhibitor (AI) during the sexually labile period, approximately half the individuals can be masculinized (e.g., XX male). When the treated animals are subsequently crossed with females, sex ratios of the progeny can be tested to potentially determine the mode of sex determination. For example, if 100% female progeny are obtained from half the crosses, it strongly suggests the species is XX/XY. Alternative sex ratios would suggest either a ZW/ZZ species or that other (polygenic GSD or ESD) mechanisms are involved. Estrogen treatments can alternatively be used to feminize cold-blooded vertebrates and similar breeding schemes performed.

denotes the lack of a second sex chromosome. Additionally, there are cases of multiple sex chromosome systems (e.g., XX/X_1X_2Y ; ZW_1W_2/ZZ) in which chromosomal fusions have given rise to females and males having a different diploid number of chromosomes.

Observation of sex ratios that do not fit any of the above patterns often indicates either a polygenic system or an influence of environmental factors, such as temperature (Fig. 2; see ESD section below). Polygenic systems are considerably more rare than monogenic ones and considered transient or intermediate states toward establishment of a monogenic system (Penman and Piferrer, 2008). Well-studied examples of polygenic systems are live-bearing species of platyfishes and swordtails (genus *Xiphophorus*) and the widely used model species, zebrafish (*Danio rerio*). In the case of zebrafish, sex determination differs among wild and domesticated strains and may involve four or more different chromosomes, as well as environmental factors.

GSD in Amphibians

Similar to fishes, GSD systems in amphibians have largely been identified through gynogenesis and/or sex reversal and progeny testing (Fig. 2). Research has shown that most amphibians have homomorphic sex chromosomes and either an XX/XY or ZW/ZZ system (Fig. 1). Most urodeles (salamanders) and anurans (frogs) of the genus *Rana* have an XX/XY system, whereas the well-known model frog species, *Xenopus laevis*, toad (*Bufo bufo*), and urodeles of the genus *Ambystoma* have a ZW/ZZ system (Nakamura, 2009).

Data indicate that the ancestral anuran (Leiopelmatidae) had a ZW/ZZ system, suggesting that ZW/ZZ may be ancestral to XX/XY systems in amphibians. However, evidence indicates that sex chromosomes have evolved numerous times in this lineage. Also as in fishes, within-species differences in the sex determination system have been documented. The pond frog (*Rana rugosa*) for instance exhibits either an XX/XY or ZW/ZZ system depending on its local population in Japan (Nakamura, 2009). Interestingly, the local pond frog populations even have different sex chromosome morphology. This variability within and among species again underscores the lack of selection for one particular GSD system in most cold-blooded vertebrates.

Sex ratios of some amphibians can also be affected by exposure to extreme low or high temperatures, outside of what the species would normally experience in nature ("thermal sex reversals"), but amphibians are still generally considered GSD species. It is worth noting however that temperature experiments have been conducted in very few amphibians and recent studies suggest that epigenetics may be important to sex determination in some species (e.g., European common frog, *Rana temporaria*).

GSD in Reptiles

Many reptile species exhibit ESD, however all snakes and some lizards and turtles exhibit GSD (Fig. 1; Janzen and Krenz, 2004). Consistent with fishes and amphibians, there are examples of both XX/XY and ZW/ZZ systems in reptiles. All snakes investigated to date are ZW/ZZ, while turtles and lizards with GSD have either a ZW/ZZ or XX/XY system (Fig. 1). Some examples of reptiles with a documented XX/XY system are the green iguana (*Iguana iguana*), tokay gecko (*Gekko gecko*), and musk turtles (genus *Staurotypus*). In addition to snakes, examples of ZW/ZZ species include racerunner lizards (genus *Eremias*), leaf-toed geckos (genus *Phyllodactylus*), and Komodo dragon (*Varanus komodoensis*).

Ancestral reptiles are generally thought to have exhibited ESD, but there is still debate regarding whether ESD or GSD came first. Based on phylogeny there appears to be no clear segregation of species into particular systems, aside from snakes (all ZW/ZZ, no ESD), iguanas (stable XX/XY), and crocodilians (all ESD). Another general observation is that ZW/ZZ systems are rare in turtles compared to lizards. That said, most genera of turtles exhibit ESD.

Environmental Sex Determination

Environmental sex determination is a form of sex determination in which non-genetic (environmental) cues serve as the master determinant of sex. ESD is generally considered a more primitive sex determination mechanism, which was largely replaced by the evolution of sex chromosomes and establishment of GSD. Yet, it has persisted in some reptiles and fishes and offers a high degree of plasticity that may hold fitness advantages. Prevailing theory proposed by Charnov and Bull (1977) indicates that ESD is advantageous when environmental conditions are patchy and different fitness levels exist for each sex (examples below).

Conventionally, GSD and ESD have been considered mutually exclusive systems. However, based recent discoveries, revealed in some cases via genomics technologies (e.g., next-generation sequencing), there are few if any cases of ESD that do not involve the genome or epigenome in some way. This new information is shifting the field's view of ESD, and whether classically defined ESD (i.e., that only influenced by non-genetic factors) exists.

In recent years there has also been accumulating evidence for what has been referred to as "thermal sex ratio distortions" or "anomalous GSD," which applies to cases where environmental factors influence sex determination and/or differentiation, and ultimate sexual phenotype, but effects may not be relevant in the natural environment (Conover, 2004; Ospina-Álvarez and Piferrer, 2008). An example of this would be exposure of a species to extremely low or high temperatures that result in skewed sex ratios (Fig. 3). It is generally accepted that amphibians only exhibit such thermal sex ratio distortions, not ESD (Nakamura, 2009). Therefore, the sub-sections below focus solely on fishes and reptiles.

ESD in Fishes

Environmental influences on sex determination/differentiation have now been documented in well over 50 species of fish (Devlin and Nagahama, 2002; Conover, 2004; Ospina-Álvarez and Piferrer, 2008). By far the most prevalent environmental factor

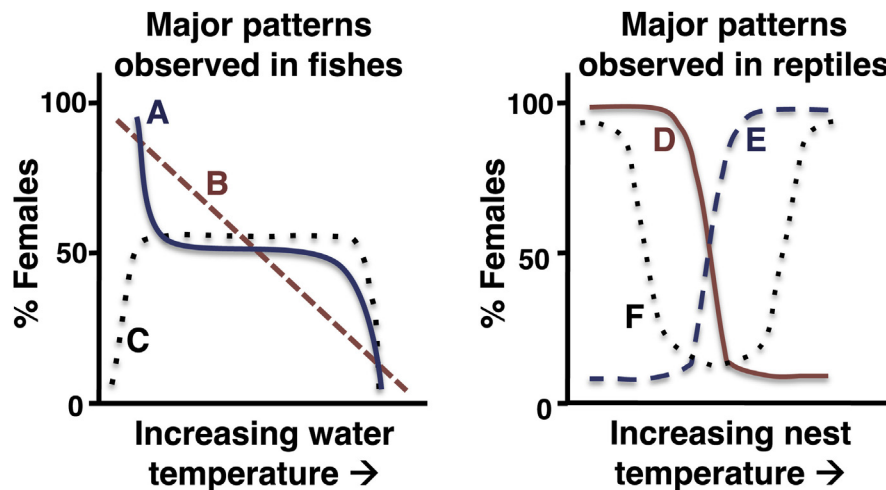


Fig. 3 Major observed patterns of temperature effects on sex determination in fishes and reptiles. Most commonly in fishes, increasing water temperatures significantly reduce female proportions (A–B). In some flatfishes, exposure to low or high temperatures reduce female and increase male proportions (C). It remains unclear whether patterns A and C represent environmentally relevant ESD or anomalous GSD, because sex ratios are stable at intermediate temperatures that might reflect the natural environment. In reptiles, low temperatures may induce high (D) or low female proportions (E) with the patterns flipping at higher temperatures. Other reptiles exhibit a female-male-female pattern (F) with high female proportions at low and high temperatures and high male proportions at intermediate temperatures. Based on Conover, D.O., 2004. Temperature-dependent sex determination in fishes. In: Valenzuela, N., Lance, V.A. (Eds.), *Temperature-Dependent Sex Determination in Vertebrates*, pp. 11–20. Washington: Smithsonian Books.

influencing sex is temperature (temperature-dependent sex determination; TSD). The first fish in which TSD was documented was the Atlantic silverside (*Menidia menidia*, family Atherinopsidae). In Atlantic silversides, exposure to different water temperatures during what is known as the thermosensitive period, occurring during early larval development, can affect sex determination. Atlantic silversides have an “annual” life cycle and attain maturity within 1 year. Sex ratios in this species are female-biased at colder temperatures at the beginning of the breeding season, however warmer temperatures later in the breeding season induce male-biased sex ratios (Fig. 3(B)). This is because reproductive fitness (e.g., body size, fecundity) is higher in females than males, and thus being born earlier and having a longer breeding season is more beneficial to females than males (Conover, 2004).

There is also geographical variation in the TSD response in Atlantic silversides (Conover, 2004; Duffy et al., 2015). Interestingly, no populations display pure TSD or GSD, but the level of TSD is uniformly high in their southern range and then declines rapidly approaching their northern range, where GSD dominates. The level of TSD then rebounds to a moderate level in northern-most populations. The length of the growing season and high gene flow among populations is what likely drives this latitudinal variation in the TSD response. As mentioned above, longer growing seasons are more beneficial to females than males. In lower latitude areas, cooler water temperatures during larval development signal a longer growing season compared to higher latitude areas. Following the Charnov and Bull model, female reproductive fitness would thus be enhanced in southern populations by TSD, whereas fitness is maximized for individuals born late in the season by developing as males, which have a better chance of reproducing at a smaller body size than do females.

Another Atherinid species, pejerrey (*Odontesthes bonariensis*), native to South America presents one of strongest known TSD responses in fishes (Strüssman et al., 1997). Monosex female or male stocks of pejerrey can consistently be produced when larvae from different broods are exposed to environmentally relevant temperatures of 17°C or 29°C, respectively. Recently, the master sex-determining gene, *Y-chromosome specific anti-Müllerian hormone (amhy)*, was discovered in *O. hatcheri*, a congeneric species that exhibits GSD. The homolog of *amhy* was then identified in pejerrey and it was demonstrated that its presence or absence favored male or female development, respectively, when fish were reared at a sexually neutral temperature (Yamamoto et al., 2014). Together, these results in Atherinid fishes indicate that despite the conventional view of ESD and GSD being autonomous mechanisms, the presence of ESD does not exclude the possibility of underlying GSD mechanisms.

In addition to temperature, several other environmental factors may influence fish sex ratios (Baroiller et al., 2009; Heule et al., 2014). An effect of pH on sex determination has been observed in swordtail and some Cichlid species. In the swordtail (*Xiphophorus helleri*), for instance, acidic water induces all-male populations, while a neutral pH produces mostly females. Fish density is also known as an environmental factor that can modulate sex ratios. In eels, both field and laboratory observations revealed that low densities tend to favor female development, while high densities favor males. Even the color of the rearing enclosure can influence sex. In southern flounder (*Paralichthys lethostigma*), rearing fry in blue-colored tanks can induce significantly male-biased sex ratios compared with black or gray tanks (Mankiewicz et al., 2013). This is in addition to documented temperature effects on sex ratios in this and other flatfish species (Luckenbach et al., 2009).

Clearly many environmental factors can influence sex determination/differentiation in fishes. However, the underlying mechanisms, interactions with GSD mechanisms, and potential fitness advantages among the sexes remain largely unknown. Indeed, a significant shortcoming in this field has been the dearth of field studies focusing on whether observations of environmental effects observed in captivity occur in wild populations. Of the 50+ species for which experimental temperature effects have been reported, ESD has only been confirmed in a few Atheriniform species and Nile tilapia (*Oreochromis niloticus*).

ESD in Reptiles

All crocodilian and many turtle species exhibit ESD (Fig. 1; Lang and Andrews, 1994). These egg-laying species bury their eggs below ground where the embryos develop until they hatch and depart the nest. Studies indicate that the depth and position of eggs within a nest can influence the temperature exposure (higher in the nest=warmer; lower=cooler) and determine the sex of individual offspring. Temperature exerts this affect during the thermosensitive period of embryogenesis, which is typically around the mid-trimester of development.

As in fishes, TSD responses in reptiles vary considerably by species. In crocodilians, a female-male-female pattern is ubiquitous (Fig. 3(F)). For example, in American alligator (*Alligator mississippiensis*), if embryos are incubated below 31.5°C or above 35°C during the thermosensitive period, monosex female populations can be obtained, while the percentage of males increase and reach 100% at 32.5–33.0°C and subsequently decline (Lang and Andrews, 1994). On the other hand, red-ear slider turtle (*Trachemys scripta*) exhibit a male-female pattern of TSD where incubation of embryos at 26 or 31°C produce monosex female or male populations, respectively (Fig. 3(E); Wibbels et al., 1991).

Another fascinating case that highlights the interplay between ESD and GSD mechanisms is the Australian central bearded dragon (*Pogona vitticeps*). This species has a ZW/ZZ system, however high temperature may override this GSD system and induce female sex reversal (i.e., production of ZZ females) under experimental conditions. It was recently reported that female sex reversal can also occur naturally in wild dragon populations and that sex-reversed females are viable and fertile (Holleley et al., 2015). Further, levels of GSD versus TSD were compared among progeny from ZW and sex-reversed ZZ mothers, in both cases mated with normal ZZ males. Progeny from normal ZW mothers were dominant in the range of 22–32°C (phenotypic sex ratio of 1:1), but temperature began to interact with and override GSD above 32°C. Conversely, in progeny from sex-reversed ZZ mothers, sex was determined solely by temperature, with no genetic influence observed. The female W-chromosome was thus eliminated from the lineage in just a single generation. This demonstrates a rapid transition under experimental conditions from

a predominantly GSD system (with heteromorphic sex chromosomes) to an ESD system (Holleley *et al.*, 2015). Although it has not been possible to clarify sex-specific fitness differences that could cause a transition from GSD to ESD, this rapid transition provides an alternative mechanism to the prevailing view that ESD evolves, or is maintained, in response to a sex-specific fitness advantage.

Mechanisms and Key Players

Recently Identified GSD and ESD Mechanisms

As discussed above, GSD is typically triggered by the expression of a paternally- or maternally- inherited master sex-determining gene(s) that may be species or even population specific. Advancement of genomics approaches has led to significant progress in the identification of master sex-determining genes in cold-blooded vertebrates, particularly in fishes (Heule *et al.*, 2014; Pan *et al.*, 2016). Interestingly, warm-blooded vertebrates, like mammals and birds, show a high degree of conservation in master sex-determining genes; nearly all mammals use *SRY* and most or all birds use a transcription factor, *DMRT1*. Fishes on the other hand appear to have experienced high turnover of sex-determining genes, with some exceptions. Just within the genus *Oryzias*, three unique sex-determining genes have recently been identified, two transcription factors (*dmrt1bY* and *sox3Y*) and one transforming growth factor (*gsdfY*). High gene turnover among these closely related species contrasts with conservation of *sdY*, a former immune-associated gene, as the master sex-determining gene in all salmonid fishes (genera *Salmo*, *Oncorhynchus*, *Salvelinus*). The reason for the evolution of such a high number of sex-determining genes in fishes and variability among genera is unknown, although with identification of more master genes, a pattern based on life history or phylogeny may ultimately arise.

With regard to amphibians, the GSD mechanism is best characterized in *Xenopus laevis*, which as mentioned above, has a ZW/ZZ system. The master sex-determining gene in this species is *DM-W*, a paralog of *Dmrt1*, which was identified on the female W-chromosome (Yoshimoto *et al.*, 2008). *Dmrt1* is a key upstream inducer of male development in *X. laevis* and other vertebrates. In ZW individuals, *DM-W* inhibits transcription of *Dmrt1*, which in turn inhibits the male pathway and induces female development. So far, *DM-W* is the only sex-determining gene identified in amphibians and only “W-linked,” female sex-determining gene identified in any animal.

For decades little has been known about how TSD occurs in reptiles; however, recent studies have led to several important discoveries. In the American alligator it was recently revealed that thermosensitive transient receptor potential (TRP) channels can act as molecular environmental sensors and are involved in the masculinization process during TSD (Yatsu *et al.*, 2015). Also, in red-eared slider turtle, it was demonstrated that *Dmrt1* may be a master gene associated with the TSD response (Ge *et al.*, 2017). Experiments showed that *Dmrt1* is both necessary and sufficient for masculinization of slider turtles, and importantly, that environmental temperature affects the DNA methylation pattern in a sex-specific manner in the promoter region of *Dmrt1*. This would be considered an epigenetic effect, as DNA methylation is one type of epigenetic mark that can influence gene transcription.

Another epigenetic mechanism is chromatin modification, which was highlighted in a new study with broad implications to reptile TSD. This study revealed that intron retention in *Jumonji* chromatin modifier genes (*JARID2/JMJD3*) is favored by extreme rearing temperatures in Australian central bearded dragon, affecting gene function and altering the epigenetic landscape (Deveson *et al.*, 2017). A cold-inducible RNA-binding protein (CIRBP) is thought to be responsible for intron retention during thermal stress.

Epigenetic regulation of genes involved in sex determination/differentiation has also been documented in fishes. For example, the promoter region of the *cyp19a1* gene (also known as aromatase), which encodes the rate-limiting enzyme responsible for estrogen production, is affected by high temperature exposure in European sea bass (*Dicentrarchus labrax*; Navarro-Martín *et al.*, 2011). This also occurs through differential methylation and leads to male development in genotypic female fish. In addition, differential DNA methylation has recently been documented in the *Dmrt1* gene of half-smooth tongue sole and linked to transgenerational sex effects (Shao *et al.*, 2014). These important recent discoveries in cold-blooded vertebrates provide evidence for genetic and epigenetic regulation of ESD, and plausible links between environmental factors and downstream processes associated with sex differentiation.

Conservation of Downstream Players

Regardless of the sex determination mechanism, downstream signaling associated with gonadal sex differentiation appears to be highly conserved among vertebrates and to reflect antagonistic pathways leading to either ovarian or testicular differentiation. In cold-blooded vertebrates, sex-specific downstream patterns of gene expression related to steroid biosynthesis and differences in steroid production are ubiquitous (Devlin and Nagahama, 2002; Trukhina *et al.*, 2013). A now classic example of this is *cyp19a1*/aromatase, which appears to have a highly conserved role in ovarian development and maintenance, and is thought to be major factor in TSD. This is supported in part by steroid, AI, and temperature-induced sex reversal in numerous species discussed above.

Conversely, androgen production appears to be an important component of testicular differentiation and development in many cold-blooded vertebrates. Treatment with androgens can generally masculinize genotypic female individuals (Fig. 2). However, interestingly, no studies to our knowledge have demonstrated a masculinizing effect of androgens in reptiles.

Environmental effects on sex ratios have also been linked to changes in production of gonadal steroids, such as estrogens and androgens. In addition, there is evidence that cortisol, the major glucocorticoid (stress hormone) produced by the adrenal gland, is a key player in the mechanism underlying ESD. In Japanese flounder (*Paralichthys olivaceus*), high temperature increases cortisol

production and causes masculinization of genotypic females by directly suppressing *cyp19a1* gene expression and estrogen production (Yamaguchi *et al.*, 2010). Elevated cortisol was also correlated with masculinizing effects of tank color in southern flounder. Furthermore, pejerrey larvae exposed to masculinizing temperatures display significantly elevated whole-body cortisol and 11-ketotestosterone (a potent androgen in fishes) levels, along with testis-like gene expression patterns (Fernandino *et al.*, 2013).

Conclusions and Future Considerations

Mechanisms of sex determination are highly variable in cold-blooded vertebrates; much more so than in warm-blooded vertebrates like mammals and birds. Two major systems of GSD (XX/XY and ZW/ZZ) are found in cold-blooded vertebrates and coexist with ESD in fishes and reptiles. In addition, there are examples of slight variation on these major systems, as well as complex, polygenic systems. Among cold-blooded vertebrates, GSD systems appear to show no clear phylogenetic segregation, suggesting there is little or no evolutionary pressure toward a unified system. This is further supported by the fact that the major GSD systems have independently evolved numerous times in closely related species, and in some cases even co-occur within a species.

ESD is generally considered a highly plastic, ancestral mechanism, yet this form of sex determination has stood the test of time. Reptilian species with ESD are numerous, whereas environmentally relevant ESD has only been ascribed to a few fishes and no amphibians. Still, there are numerous reports of environmental effects (e.g., temperature, pH, density) on sex ratios. Some of these cases are considered anomalous, thermal sex ratio distortions. However, more field-based experiments are needed to conclude on the prevalence of ESD. Mounting evidence also indicates that GSD and ESD are not mutually exclusive mechanisms, as once believed, and that even in classical ESD species, the genome and/or epigenome play important roles. As noted by Bull (2015), GSD and ESD mechanisms can each be highly functional and adaptive, and may, in theory, evolve back-and-forth along an evolutionary progression.

The high degree of variance in sex determination among cold-blooded vertebrates may leave one with the impression that there is little semblance of order in this biological event. However, downstream processes, such as those associated with steroid biosynthesis, appear to be highly conserved. This suggests that there are several paths toward the same outcome, and that upstream mechanisms can change/shift, as long as critical connections to factors that drive development of the two sexes are maintained.

We also touched on several cases of stability in sex determination in this article. A prime example of this is snakes, which all exhibit GSD and a ZW/ZZ system, despite the fact that their fellow reptilians have diverse sex determination mechanisms. While there are several theories as to why snakes display this remarkable stability (e.g., shorter lifespan), the answer remains unknown.

Given the intimate association of cold-blooded vertebrates with their immediate environment, especially with regard to temperature, it is not altogether surprising that they have maintained a higher level of plasticity in some physiological mechanisms relative to warm-blooded vertebrates. The fact that TSD has not been replaced in some extremely old vertebrate taxa (e.g., crocodilians) is intriguing. One might imagine that millions of years of environmental change and evolutionary pressure would have forced such change. Yet, TSD persists. One possibility is that natural environmental change typically occurs very slowly, allowing time for adaptation of ESD mechanisms. This is not the case, however, with climate change currently being driven by anthropogenic causes. The question now is whether species with TSD will be able to survive in this rapidly changing world, and if species with GSD may begin to display well-documented thermal sex ratio distortions, previously observed only in the laboratory. Skewed sex ratios resulting from these scenarios could put extreme pressure on survival of temperature-sensitive, cold-blooded vertebrates.

References

- Baroiller, J. F., D'Cotta, H., & Saillant, E. (2009). Environmental effects on fish sex determination and differentiation. *Sexual Development*, 3, 118–135.
- Bull, J. J. (2015). Reptile sex determination goes wild. *Nature*, 523, 43–44.
- Charnov, E. L., & Bull, J. J. (1977). When is sex environmentally determined? *Nature*, 266, 828–830.
- Conover, D. O. (2004). Temperature-dependent sex determination in fishes. In N. Valenzuela, & V. A. Lance (Eds.), *Temperature-Dependent Sex Determination in Vertebrates* (pp. 11–20). Washington: Smithsonian Books [Chapter 1].
- Deveson, I. W., Holleley, C. E., Blackburn, J., et al. (2017). Differential intron retention in *Jumonji* chromatin modifier genes is implicated in reptile temperature-dependent sex determination. *Scientific Advances*, 3, e1700731.
- Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological and environmental influences. *Aquaculture*, 208, 191–364.
- Duffy, T. A., Hice, L. A., & Conover, D. O. (2015). Pattern and scale of geographic variation in environmental sex determination in the Atlantic silverside, *Menidia menidia*. *Evolution*, 69, 2187–2195.
- Fernandino, J. I., Hattori, R. S., Moreno Acosta, O. D., Strüssman, C. A., & Somoza, G. M. (2013). Environmental stress-induced testis differentiation: Androgen as a by-product of cortisol inactivation. *General and Comparative Endocrinology*, 192, 36–44.
- Ge, C., Ye, J., Zhang, H., et al. (2017). *Dmrt1* induces the male pathway in a turtle species with temperature-dependent sex determination. *Development*, 144, 2222–2233.
- Heule, C., Salzburger, W., & Böhne, A. (2014). Genetics of sexual development: An evolutionary playground for fish. *Genetics*, 196, 579–591.
- Holleley, C. E., O'Meally, D., Sarre, S. D., et al. (2015). Sex reversal triggers the rapid transition from genetic to temperature-dependent sex. *Nature*, 523, 79–82.
- Janzen, F. J., & Krenz, J. G. (2004). Phylogenetics: Which was first, TSD or GSD? In N. Valenzuela, & V. A. Lance (Eds.), *Temperature-Dependent Sex Determination in Vertebrates* (pp. 121–130). Washington: Smithsonian Books [Chapter 13].
- Lang, J. W., & Andrews, H. V. (1994). Temperature-dependent sex determination in crocodilians. *The Journal of Experimental Zoology*, 270, 28–44.
- Luckenbach, J. A., Borski, R. J., Daniels, H. V., & Godwin, J. (2009). Sex determination in flatfishes: Mechanisms and environmental influences. *Seminars in Cell & Developmental Biology*, 20, 256–263.

- Mankiewicz, J. L., Godwin, J., Holler, B. L., et al. (2013). Masculinizing effect of background color and cortisol in a flatfish with environmental sex determination. *Integrative and Comparative Biology*, 53, 755–765.
- Nakamura, M. (2009). Sex determination in amphibians. *Seminars in Cell & Developmental Biology*, 20, 271–282.
- Navarro-Martín, L., Viñas, J., Ribas, L., et al. (2011). DNA methylation of the gonadal aromatase (*cyp19a*) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLOS Genetics*, 7, e1002447.
- Ospina-Alvarez, N., & Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLOS ONE*, 3, E2837.
- Pan, Q., Anderson, J., Bertho, S., et al. (2016). Vertebrate sex-determining genes play musical chairs. *Comptes Rendus Biologies*, 339, 258–262.
- Penman, D. J., & Piferrer, F. (2008). Fish gonadogenesis. Part I: Genetic and environmental mechanisms of sex determination. *Reviews in Fisheries Science*, 16(S1), 14–32.
- Shao, C., Li, Q., Chen, S., et al. (2014). Epigenetic modification and inheritance in sexual reversal of fish. *Genome Research*, 24, 604–615.
- Strüssman, C. A., Saito, T., Usui, M., Yamada, H., & Takashima, F. (1997). Thermal thresholds and critical period of thermolabile sex determination in two Atherinid fishes, *Odontesthes bonariensis* and *Patagonina hatcheri*. *Journal of Experimental Zoology*, 278, 167–177.
- Trukhina, A. V., Lukina, N. A., Wackerow-Kouzova, N. D., & Smirnov, A. F. (2013). The variety of vertebrate mechanisms of sex determination. *BioMed Research International*. Article ID 587460.
- Wibbels, T., Bull, J. J., & Crews, D. (1991). Chronology and morphology of temperature-dependent sex determination. *The Journal of Experimental Zoology*, 260, 371–381.
- Yamaguchi, T., Yoshinaga, N., Yazawa, T., Gen, K., & Kitano, T. (2010). Cortisol is involved in temperature-dependent sex determination in the Japanese flounder. *Endocrinology*, 151, 3900–3908.
- Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R. S., & Strüssmann, C. A. (2014). Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLOS ONE*, 9, e102574.
- Yatsu, R., Miyagawa, S., Kohno, S., et al. (2015). TRPV4 associates environmental temperature and sex determination in the American alligator. *Scientific Reports*, 5, 18581.
- Yoshimoto, S., Okada, E., Umemoto, H., et al. (2008). A W-linked DM-domain gene, DM-W, participates in primary ovary development in *Xenopus laevis*. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 2469–2474.

Sex Determination and Gonadal Development in Birds

Jason Ioannidis, Debiao Zhao, Michael J McGrew, and Michael Clinton, University of Edinburgh, Roslin, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

Sex determination initiates the process of sexual differentiation in developing embryos, generating the distinct sexual phenotypes that support the different systems of sexual reproduction observed across vertebrates. Sex determination mechanisms are varied; some are genetic (mammals, birds), some are environmental (crocodiles, primitive lizards) and some species adopt various combinations of both (fish, turtles; Barske and Capel, 2008). Mammals and birds both utilise genetic sex determination (GSD) mechanisms and while these share many molecular components with conserved functions, they have evolved independently.

The majority of information on mammalian sex determination derives from studies in the mouse, principally because the mouse is a relatively inexpensive, well-established model that is easily bred, manipulated and analysed. This knowledge has been complemented by observational studies of sex determination disorders in humans and by comparative genomic analyses in other mammalian species. The mammalian sex determining mechanism is one of Y-chromosome dominance, in which the Y-linked testis-determining gene, sex-determining region Y (SRY), is present and highly expressed in the gonads of heterogametic males (carrying XY) inducing a male gonadal phenotype, but that is absent in homogametic females (carrying XX), with the result that a female gonadal phenotype develops. In mammals, secondary sex determination (the appearance of secondary sexual characteristics) is principally regulated by gonadal hormones.

In contrast to mammals, female birds have heterogametic sex chromosomes (carrying ZW) while male birds are homogametic (carrying ZZ). In addition, efforts to identify an SRY gene in the avian genome, have been unsuccessful. Historically, the chick embryo has been used extensively as a model system for vertebrate embryonic development and has many advantages (Clinton *et al.*, 2016); not least are the accessibility of the developing embryo in the egg, the availability of large numbers of synchronised fertilised eggs, and the low cost of eggs. In recent years it has been considered an ideal comparative model system to study vertebrate sex determination and gonadal development. In the following sections we will present an overview of sex determination and gonadal differentiation in birds, principally focused on the chicken and including comparisons with the mammalian counterparts where appropriate.

Morphology of the Developing Avian Gonad

In general terms, the basic gonadal structure is well-conserved between avian and mammalian species, but there are many aspects of gonadogenesis that differ significantly between these classes of vertebrates. The genital ridges are the first visually distinct precursors of the gonads and in the chick these form from the intermediate mesoderm of the embryo on embryonic day three (E3) of development. The ridges form in symmetrical locations on either side of the developing gut, on the surface of the emerging mesonephroi (primitive kidneys). Over the following days, the precursors for steroidogenic cells, supporting cells, interstitial cells and primordial germ cells (PGCs) colonise the gonad, begin to proliferate and differentiate to form the characteristic structures of the male and female gonads. At this early stage in both male and female embryos, the surface epithelial layer of the left gonad is markedly thicker than that of the right gonad; in males this layer thins as development proceeds such that the gonads on both sides are equivalent. In the left female gonad, the surface epithelium further thickens into a structure known as the cortex, while the right female gonad ceases development. Sex cords form in the core region known as the medulla, and while these later degenerate in females, in males these develop into the seminiferous tubules. The PGCs, which are initially distributed throughout the gonads, are enclosed by the sex cords in both male gonads where they proliferate and will differentiate into spermatogonia. In females, the PGCs in the left gonad migrate into the cortex where they proliferate and eventually form oögonia.

In order to contribute to the gonad, the PGCs follow a complex migration route from their site of formation. These cells are thought to arise in the period immediately following fertilisation, when their fate is specified by maternal induction through a mechanism known as preformation. This specification is achieved through a maternally deposited cytoplasmic constituent termed germ-plasm, which contains VASA, a protein specific to PGCs. In contrast, mammalian PGCs are specified via a different process known as epigenesis where individual cells are induced via a bone morphogenetic protein (BMP) – dependent signalling pathway which occurs at a later stage of development (Table 1; Nakamura *et al.*, 2013). Following specification, PGCs are located in an area known as the zona pellucida in the centre of the early chick embryo, from whence they migrate cranially to the edge of the extra-embryonic region (E1) and subsequently into a region anterior to the embryonic head on known as the germinal crescent (E2). From this location, between E2 and E3, they enter the developing vascular system and migrate through the circulation and then the dorsal mesentery to enter the embryonic gonad around E3.5 – presumably under the influence of chemo-attractants. This migration route differs from that seen in mammalian species, where PGCs migrate from the proximal epiblast (where they are specified), through the allantois, the endoderm (future hindgut), then the mesonephros, to reach the gonad (Sekido and Lovell-Badge, 2007; Richardson and Lehmann, 2010).

At the time of PGC migration, somatic cells also migrate into the developing gonad. In both sexes, the precursors of the steroidogenic and other supporting cells migrate from the nephrogenic mesodermal mesenchyme into the developing gonad from

Table 1 Comparison of avian and mammalian gonadal development. A summary of morphological differences between avian and mammalian species during gonadal development

Process	Mammals	Birds	References
PGC specification	Epigenesis (BMP 4/8b)	Pre-formation (Germplasm/VASA)	Nakamura <i>et al.</i> (2013)
PGC migration	Allantois-hindgut-mesentery-mesonephros	Embryonic circulation-mesentery-mesonephros	Sekido and Lovell-Badge (2007)
Sertoli precursor migration	Coelomic epithelium	Nephrogenic mesenchyme	Sekido and Lovell-Badge (2007)
Primitive sex cord formation	None in XX females	Present in ZW females	Sekido and Lovell-Badge (2007)
Myoid cell differentiation	Detectable by E12.5 in males	Not present by E14 in males	Sekido and Lovell-Badge (2007)
Gonadal symmetry	Symmetrical Left=Right	Ovarian asymmetry Left>Right	Guioli <i>et al.</i> (2014)
Sexual phenotype	Hormone dependent	Cell autonomous (CASI)	Zhao <i>et al.</i> (2010)
Sex steroid influence	Decoupled from gonadal differentiation	Influences gonadal differentiation	Clinton <i>et al.</i> (2012)
Chromosome dosage compensation	X-chromosome inactivation	Partial, gene-by-gene basis	Mank and Ellegren (2009)

approximately E4 (Sekido and Lovell-Badge, 2007). In contrast, in mammals, the supporting cell precursors are known to migrate to the early gonad directly from the coelomic epithelium rather than the nephrogenic mesenchyme. These cells are the precursors of Sertoli cells in male and granulosa cells (GC) in females, and support PGC survival and differentiation. The steroidogenic cell precursors are also found within this migrating population and will give rise to Leydig cells in the male and theca cells in the female. In the chicken, non-steroidogenic interstitial cells migrate from the coelomic epithelium at the same time that laminin-1 expression ceases in the coelomic epithelium surrounding the gonads, and it is thought that this is representative of changes that allow the movement of cells across embryonic compartments (Sekido and Lovell-Badge, 2007).

For a short period after sex determination in chicks (E5-E6) the gonads of females and males remain indistinguishable with the medulla in both sexes developing sex cords (Sekido and Lovell-Badge, 2007). This stage of development in the mouse differs from the chick, in that the female mouse gonads do not acquire primitive sex cords.

In the medulla of the male chick by E7, tubules have formed that contain Sertoli cells surrounding PGCs. These tubules are surrounded by interstitial tissue, containing blood vessels, nerves, epithelial cells and Leydig cells and eventually join up to form the seminiferous ducts. In addition, the seminiferous tubules will be surrounded by fibroblast-like cells and myoid/peritubular cells (Rothwell and Tingari, 1973). In the mouse, the tubules are also lined with myoid cells which appear from E12.5. Myoid cells are not present in tubules in the chicken gonad even by E14, suggesting that these cells may appear later in development in avian species (Table 1; Sekido and Lovell-Badge, 2007). In both gonads in the male chick embryo, PGCs differentiate into spermatogonia from E13 onwards, and from approximately 10 weeks post-hatch, PGCs undergo further cell division and differentiation until the time of sexual maturity (~16 weeks) when spermatogenesis begins.

In female chick embryos, the structures equivalent to the male seminiferous tubules are called medullary cords, and form at the same developmental time (E7; Bellairs and Osmond, 2014). These primitive sex cords do not fuse into tubules/ducts but develop into epithelial-lined spaces known as medullary lacunae. The left female gonad develops a thickened cortex which contains PGCs and pre-granulosa cells that have migrated from the medulla, while the epithelium of the right gonad remains undifferentiated. Female PGCs begin differentiation earlier than male PGCs to form primary oocytes (E8) and enter meiosis I at E13, pausing at prophase I prior to hatching. Around the same time, these primary oocytes undergo a significant reduction in numbers by apoptosis, and then the survivors remain in meiotic arrest until shortly before ovulation. The germ cells proceed through meiosis I and those selected for ovulation enter meiosis II, and arrest at metaphase II until ovulation, when meiosis is completed.

Table 1 summarises the major morphological differences between avian and mammalian gonadal development.

The adult ovaries of birds and mammals are markedly different (Johnson, 2015). In mammals, pre-ovulatory follicles contain a fluid-filled compartment called the antrum which surrounds the oocyte-cumulus complex, and which increases in volume as the follicle grows. Avian follicles do not contain a fluid-filled compartment, and the growing yolk/oocyte is responsible for most of the follicular size. In mammals, follicular maturation is accompanied by the acquisition of granulosa cell (GC) layers to the follicular wall, and pre-ovulatory follicles typically have up to 6 layers of GCs. In contrast, at ovulation the chicken oocyte is surrounded by a single layer of GCs. In addition, avian GCs do not express the aromatase enzyme (Cytochrome P450 Family 19 Subfamily A Member 1 or CYP19A1), and consequently do not produce estrogens, while mammalian GCs do express aromatase and one of their primary functions is to convert theca-derived androgens into estrogen. In the chicken, the majority of aromatase is produced by the cells of the theca externa which comprises the outer layer of the growing follicle. Mammals and birds also differ in the nature of the follicular selection process: in mammals, follicles are selected for growth in cohorts of which one or more (depending on the species) will become dominant and ovulate while the remaining (subordinate) follicles will undergo atresia. In birds, individual

follicles are selected on a regular basis and growth is initiated. This generates a hierarchy of growing follicles which ovulate in sequence. This allows the laying of multiple eggs and the subsequent production of more offspring in a short period of time compared to mammals.

In the chicken, full ovarian development is restricted to the left gonad and by the midpoint of embryogenesis, the right ovary is significantly smaller than the left and lacks a thickened cortex. This is attributed to a failure of the paired-like homeodomain transcription factor 2 (PITX2) – steroidogenic factor (SF1) – cyclin D1 pathway to be activated in the right gonad, leading to a lack of estrogen sensitivity and a reduction in cell proliferation (Ishimaru *et al.*, 2008). Despite this, during the early stages of gonadal development, the right ovary is steroidogenically active. This left-right asymmetry in gonadal development is only one of a number of differences between bird and mammalian gonadal development (Table 1).

Mechanisms of Sex Determination in Birds

There is significant diversity in the sex determining mechanisms utilized by vertebrates (reviewed in Bachtrog *et al.*, 2014). The mechanism common to mammals and birds is described as GSD, and versions of GSD have also been described in amphibians, and some other reptiles and fish (Bachtrog *et al.*, 2014). This mechanism reflects the acquisition of different sex chromosomes in males and females, which are thought to have evolved gradually from autosomal chromosome pairs. In some cases there is a single sex-determining gene on a sex chromosome, however in other species there are multiple genes which drive sex determination (Barske and Capel, 2008). The vast diversity of the available sex-determining mechanisms reflects the speed with which these evolve and the high number of independent paths taken across vertebrate species.

The most well-known example of a heteromorphic sex-chromosome system is that of the mammals, where the male is heterogametic with one X and one Y chromosome, and the female is homogametic, with two X chromosomes. Although, the Y-chromosome is greatly reduced in size compared to the X and carries few functional genes, the sex determining mechanism is one of Y-dominance, where the presence of a Y chromosome is sufficient to induce testes and results in a male phenotype. This system was originally elucidated through analyses of individuals that carry XO chromosomes (who develop as females) and those that carry XXY chromosomes (who develop as males). As discussed in the next section, Y dominance is primarily established through a master sex (testis) determining gene, SRY.

Birds and a number of snakes and reptiles, where the female is the heterogametic sex, are described as possessing a ZW sex chromosome system. In birds, the male is the homogametic sex, with two Z chromosomes (ZZ) while the female is heterogametic, with one Z and one W chromosome (ZW). With the exception of ratite birds (flightless birds such as the ostrich and emu) the W chromosome is significantly smaller than the Z chromosome. Excluding the ratites, the sex chromosomes of different birds have evolved from a common pair of autosomes (Fridolfsson *et al.*, 1998). It is clear from the lack of homology between mammalian and avian sex chromosomes that these have evolved independently of each other.

One important factor that determines the effect of genetic sex is the mechanism that regulates the expression of sex-chromosome genes, but this is not conserved between species. In female mammals, which have two X sex chromosomes, the presence of two copies of the genes on the X chromosome is ‘compensated’ by a mechanism involving random inactivation of one X-chromosome in each cell. This is thought to be required to equalize the phenotypic effects of characteristics determined by genes on the sex chromosomes. Genes on the sex chromosomes are not conserved between birds and mammals, and until relatively recently, it was thought that avian sex chromosome genes were not subject to dosage compensation. However, we demonstrated that the levels of expression of some Z chromosome genes were equivalent in male and female chick embryos (i.e., compensated; McQueen *et al.*, 2001) and, since then, a large number of studies have confirmed and extended these initial findings. It is now generally accepted that a system of dosage compensation operates in avian tissues, but that this system is not chromosome-wide: many genes are either not compensated, or only partially compensated, and it has been proposed that dosage compensation in birds is regulated on a gene-by-gene basis (Mank and Ellegren, 2009).

Following the identification of Sry as the testis-determining gene in mammals, strenuous, but unsuccessful, efforts were made to identify an avian homologue to this master regulatory gene, and there are currently two proposed avian sex determining mechanisms that are generally considered credible. These systems are based on either a W-chromosome dominance or the dosage of a Z-chromosome gene product.

The theory of W-dominance suggests that a gene (or genes) located on the W (female-associated) chromosome would induce ovarian development and result in a female phenotype. The identification of phenotypically male ZO birds and/or phenotypically female ZZW birds would provide evidence of this mechanism and the existence of a W-chromosome ovary-determining gene. Unfortunately, there are no well-documented reports of aneuploid birds of this sort; the only ZZW birds reported were chromosomally triploid, infertile and developed ovotestes (Lin *et al.*, 1995). As such, there is currently no robust evidence for a female-dominant, W-linked sex determining gene in birds.

The alternative theory is based on the dosage of a Z-chromosome gene product, and depends on the fact that male cells carry two copies of the Z-chromosome while female cells carry only a single Z-chromosome. This hypothesis proposes that a certain amount of a specific gene product is required to trigger testis development, while lower levels lead to the development of an ovary. Here, testis development would be induced in males (ZZ) by elevated levels of expression of a Z-linked gene (or genes), while females (ZW) would fail to achieve the necessary testis-inducing expression level and ovarian development would proceed. As discussed

later, the Z-linked doublesex and mab-3 related transcription factor 1 (DMRT1) gene would be a suitable candidate for such a dosage-based system.

In mammals, while genetic elements determine the sexual fate of the developing gonads, it is generally accepted that the sexual phenotype of other tissues (secondary sexual characteristics) is principally dependent on the actions of gonadal hormones. In a recent demonstration of a major difference in the overall sex determining systems of birds and mammals, it was shown that the sexual phenotype of non-reproductive tissues in birds principally depends on the genetic make-up of the cells, i.e., whether they are genetically male or female, rather than the actions of gonadal hormones. It is interesting to note that, in mammals, hormones are not involved in primary sex-determining events, but are crucial to secondary sex determination, while in birds, hormones play a role in primary sex determination (blocking estrogen synthesis in female embryos leads to gonadal sex reversal), but have limited effects on secondary sex determination. The fact that the sexual phenotype of non-gonadal tissues is largely defined by its cellular genetic make-up rather than circulating sex steroids was demonstrated by the analysis of gynandromorph birds in which one side appeared male and one side appeared female. Studies revealed that these birds were naturally occurring chimeras composed of perfectly normal diploid male (ZZ) and female (ZW) cells, and that the side that appeared male contained predominately male cells and the side that appeared female contained predominately female cells. As both sides of these birds are exposed to the same levels of circulating hormones, the most obvious conclusion is that the different male and female phenotypes are due to the cellular composition of the tissues (Zhao *et al.*, 2010). This cell-autonomous sex identity (CASI) was confirmed by experiments where the pre-gonadal tissue from a donor embryo of one sex was transplanted to replace the pre-gonadal tissue of a host embryo of the opposite sex, and these grafted embryos re-incubated until the gonads had fully developed. In these experiments, the donor cells continued to differentiate and formed a gonad, however, this gonad was the type normally found in the donor embryo and not the type typical of the host (Zhao *et al.*, 2010). These findings were further strengthened by another study which induced the expression of aromatase in genetically male chicken embryos. The effects of the steroid hormones were secondary to the CASI of individual cells and tissues (Lambeth *et al.*, 2016).

Molecular Control of Sex Determination in Birds

DMRT1 and forkhead box L2 (FOXL2) are transcription factors which have been shown to play key roles in maintaining the adult gonadal phenotype in male and female mammals (reviewed in Huang *et al.*, 2017), and it may be that the molecular interactions and sex-specific roles of these transcription factors are conserved in birds during the embryonic phase of development. It has been proposed that both DMRT1 and FOXL2 may play key roles during primary sex determination. In keeping with this suggestion, DMRT1 and FOXL2 show sexually dimorphic expression in chick embryonic gonads at the onset of sex determination (E4.5; Ayers *et al.*, 2013), when there are a limited number of differences between males and females. Shortly afterwards (E6), there is

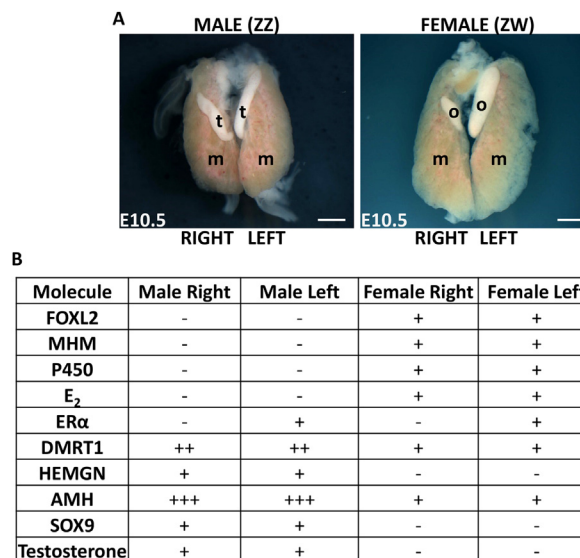


Fig. 1 Gonadal asymmetry and sexually dimorphic factors. (A) Light microscopy images of male and female chick embryo gonads at E10.5 of development. In males, right and left testes are of similar size, while in females the left ovary is markedly larger than the right ovary (m, mesonephros; t, testis; o, ovary). Size of bars=1 mm. (B) Summary of factors with sexually dimorphic/asymmetric distribution in developing gonads of male and female chicks ('-' indicates absence, while '+' indicates presence and relative level of individual factors). NB, While estrogen receptor is expressed in both males and females, expression is restricted to the left gonad. FOXL2, forkhead box L2; MHM, male hyper-methylated; P450, aromatase; E₂, estrogen; ER α , estrogen receptor alpha; DMRT1, doublesex and mab-3 related transcription factor 1; HEMGN, hemogen; AMH, anti-Müllerian hormone; SOX9, sex-determining region Y-box 9 protein.

a significant increase in the number of genes that differ in expression between male (656 male-biased genes) and female (347 female biased genes) embryos (see Fig. 1; Ayers *et al.*, 2015b).

In the chick embryo, the expression of DMRT1 is restricted to the gonads, PGCs and the Mullerian duct with higher levels detected in the males compared to females (Ayers *et al.*, 2015a). The timing, tissue-specificity and sexually dimorphic expression of DMRT1 makes it a good candidate for a dosage-based sex-determining gene in birds. This hypothesis is supported by experimental evidence that overexpression of DMRT1 in genetically female (ZW) chick embryos resulted in the suppression of female gonadal development. In these genetically female gonads, there were low numbers of medullary lacunae, and a thin ovarian cortex, coupled with increased expression of the male-enriched genes sex-determining region Y-box 9 protein (SOX9), Hemogen (HEMGN) and anti-Mullerian hormone (AMH), and reduced expression levels of the female-specific aromatase gene (Lambeth *et al.*, 2014). In the converse experiment, DMRT1 levels were reduced using a sh-RNAi approach in male chick gonads at E10, and this resulted in feminisation of the genetically male (ZZ) gonad. This was characterised by the development of a left-right asymmetry, an increase in the expression of the female-specific FOXL2 and aromatase genes, and a downregulation of the male-biased gene SOX9 (Smith *et al.*, 2009). These experiments suggest that DMRT1 is a significant driver of male gonadal differentiation and a regulator of many male-biased genes which are involved in sexual differentiation. Fig. 1 illustrates male and female gonadal morphology at E10.5 and list some of the molecular differences observed between the developing male and female gonads.

Currently, DMRT1 is thought to induce the expression of a number of male-biased genes following sex determination in the chicken, including HEMGN, SOX9 and AMH (see Fig. 2). Of these genes, HEMGN is the first to be expressed in the developing chick gonad, and is detectable as early as E5.5. Experiments where the expression levels of HEMGN are altered in genetically female (ZW) and male (ZZ) chicken embryos demonstrated that this gene plays a role in the male gonadal development pathway and lies upstream of SOX9 (Nakata *et al.*, 2013). The role of SOX9 in gonadal differentiation is thought to be conserved in both mammals and birds, with similar expression patterns observed in mouse, chicken, quail and duck (Fig. 2; Nakata *et al.*, 2013; Takada *et al.*, 2006a; Morais da Silva *et al.*, 1996). SOX9 is expressed in the male chicken gonad from E6.5 onwards, is responsible for induction of Sertoli cell differentiation and drives a positive feedback loop involving prostaglandin D2 synthase (PTGDS) and prostaglandin D2 (PGD2), which is also conserved in mammals (Moniot *et al.*, 2008; Wilhelm *et al.*, 2007).

In mammals, AMH expression is required to induce degeneration of the Mullerian duct in male embryos and is male-specific. In birds, although AMH is expressed in both male and female gonads, expression levels are much higher in males. In the male chick embryo, AMH is expressed from E6.5 onwards and that expression is primarily within the Sertoli cells of the seminiferous cords (Fig. 2; Oreal *et al.*, 1998). Interestingly, while AMH is directly induced by SOX9 in mammals, AMH expression in the chicken is SOX9-independent and is instead induced by SF1 (Takada *et al.*, 2006b). In knockdown-experiments in male embryos, loss of AMH led to reduced germ cell numbers in the testes, while over-expression of AMH in female embryos resulted in part-masculinised female gonads that showed increased DMRT1 expression and reduced FOXL2 expression. Both experimental scenarios

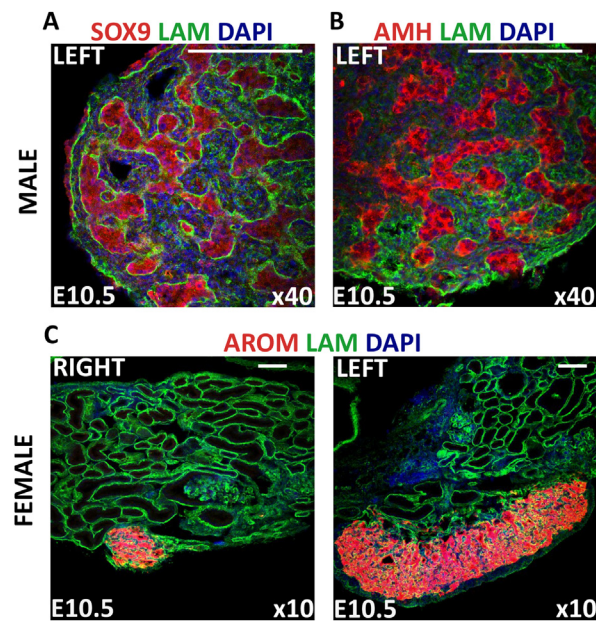


Fig. 2 Typical examples of sex-specific expression in male and female developing chick gonads. Immuno-staining of male and female chick embryo gonads on E10.5 of development for characteristic male-biased proteins SOX9 (A) and AMH (B), and female-specific aromatase protein (C). SOX9 and AMH expression in developing testes is restricted to the Sertoli cells within the sex cords bordered by laminin. Aromatase expression in the female gonads is restricted to the medulla. Laminin expression in the left female gonad defines the boundary of the cortex. There is no cortex present in the much smaller right female gonad, however, this organ is still steroidogenically active as demonstrated by aromatase expression. SOX9, sex-determining region Y-box 9 protein; LAM, laminin; AROM, P450 aromatase. Size of bars=100 μ m.

resulted in smaller gonads (Lambeth *et al.*, 2015, 2016). AMH has been shown to inhibit aromatase expression and the steroidogenic capacity was affected in both male and female gonads overexpressing AMH (Lambeth *et al.*, 2016; Bruggeman *et al.*, 2002). These studies demonstrate that AMH is involved in gonadal development in both sexes and it can partially induce testicular development in a genetically female (ZW) embryo.

FOXL2 is expressed in the female chick gonad at an earlier stage (E4.5) than other female-biased genes, and is often considered as the female counterpart of DMRT1, in the sense that it is thought to regulate ovarian development (Govoroun *et al.*, 2004). A sharp increase in the levels of FOXL2 in the gonad is observed at the beginning of sex determination in the female chicken, followed closely by an increase in the levels of aromatase (Fig. 2; Govoroun *et al.*, 2004). FOXL2 has been shown to regulate aromatase expression in the goat (Pannetier *et al.*, 2006) and it was thought to play a similar role in birds, however, it has recently been proposed that SF1 regulates expression of aromatase in the female chick gonad (Wang and Gong, 2017). During gonadal differentiation, FOXL2 is highly expressed in the female compared to male gonads (Govoroun *et al.*, 2004). Although there are no reports of direct FOXL2 manipulation in the chicken, FOXL2 has been shown to be essential for granulosa cell differentiation, oocyte differentiation and steroidogenesis in the mouse ovary, and ablation of FOXL2 in adult mice led to masculinisation, suggesting that its role in the adult mouse is to maintain sexual phenotype (Uda *et al.*, 2004; Uhlenhaut *et al.*, 2009).

In mammals, there is evidence for an alternative ovarian differentiation pathway that is independent of FOXL2. This pathway is the wingless-type MMTV integration site family (Wnt) canonical pathway, which includes r-spondin 1 (RSPO1) and wingless-type MMTV integration site family, member 4 (WNT4) and activates beta catenin (CATNNB; Tevosian, 2013). In the chicken, although this pathway is not well-characterised, elevated levels of RSPO1 are seen in female chick gonads from E4.5 onwards and coincide with both FOXL2 and aromatase expression (Fig. 2). The RSPO1 protein is localised to the cortex of the developing ovary and expression of RSPO1 was reduced by inhibiting aromatase expression, suggesting that estrogens affect RSPO1 similarly to FOXL2 in the chicken (Smith *et al.*, 2008). However, in goats, FOXL2 and RSPO1 are expressed in different ovarian compartments

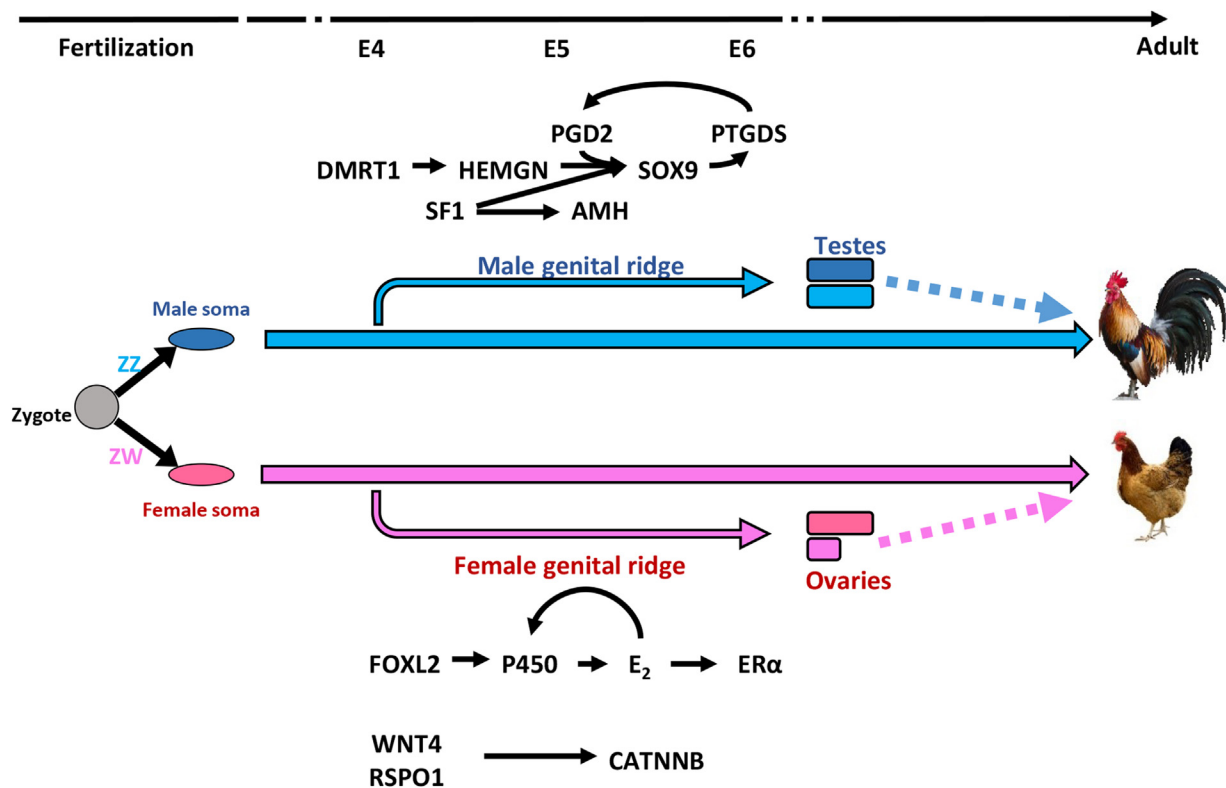


Fig. 3 Proposed scheme for regulation of sex determination in birds. Following fertilisation of Z-bearing or W-bearing oocyte, the major factor in defining the adult sexual phenotype, is the inherent sex identity (CASI) of individual cells and tissues. Male genital ridges develop into testes because they are composed of male (ZZ) cells, while female genital ridges initiate ovarian development because they are composed of female cells. Broken arrows indicate that gonadal factors have only a limited influence on the sexual phenotype of the adult bird. Factors regulating the differentiation of the genital ridge into either a testis or an ovary, are illustrated (see text for details). With minor exceptions, the majority of these factors are conserved between gonadal development in birds and mammals. A notable exception is estrogen (E₂) which plays a major role in ovarian development in birds. E4, embryonic day 4; DMRT1, doublesex and mab-3 related transcription factor 1; HEMGN, hemogen; SF1, steroidogenic factor 1; AMH, anti-Müllerian hormone; PGD2, prostaglandin D2; PTGDS, prostaglandin D2 synthase; SOX9, sex-determining region Y-box 9 protein; FOXL2, forkhead box L2; P450, aromatase; E₂, estrogen; ERα, estrogen receptor alpha; WNT4, wingless-type MMTV integration site family, member 4; CATNNB, beta catenin; RSPO1, r-spondin 1.

(FOXL2 in the medulla, RSPO1 in the cortex) and are therefore members of different pathways. Fig. 3 summarises our current understanding of the sex determination process in birds.

In contrast to mammals, chicken embryonic gonads are sensitive to sex steroids during development (reviewed in Bruggeman *et al.*, 2002), and both female gonads have the capacity to produce estrogen at the time of sex differentiation. The left gonad of both male and female embryos express estrogen receptor alpha (ER α) between E7 and E10 and are capable of responding to estrogen if this steroid is present. Gonadal sex reversal is possible through sex steroid manipulation in both sexes, but the resulting phenotypes are variable and mostly reversible (Bruggeman *et al.*, 2002), reinforcing the concept of cell autonomous sex identity. Inhibiting estrogen production in genetically female embryos leads to a decrease in FOXL2 levels, suggesting that FOXL2 can respond to estrogen abundance, putatively through a feedback loop (Hudson *et al.*, 2005). Steroid feminisation of genetically male embryos leads to gonadal asymmetry and decreased expression of male-biased genes, including DMRT1 (Lambeth *et al.*, 2013).

Another interesting female-biased, Z-linked region is male hyper-methylated (MHM), which contains multiple repeats of a long, non-coding RNA (lncRNA) that accumulates in the region of the DMRT1 promoter in ZW females. Hypothetically, MHM may be involved in the inhibition of DMRT1 in females, in a similar manner to X-inactive specific transcript (XIST) X-chromosome inactivation in mammals. Overexpression of MHM in male embryos did not induce sex reversal, however it affected male gonad development and led to a down-regulation of DMRT1 (Roeszler *et al.*, 2012). Further research suggests that MHM is associated with sex determination in both sexes (Yang *et al.*, 2016), but the exact association between MHM and the molecular pathways underlying sex determination in birds is not clear.

Conclusions

In summary, the sex determination mechanism in birds differs to that seen in mammals in that it is not obviously dependent on a single master regulatory gene and there is also a strong cell autonomous element. In mammals, primary and secondary sex determination are inextricably linked: genetic sex determines the type of gonad formed and this in turn determines the adult sexual phenotype. In birds, primary and secondary sex determination are less closely linked: genetic sex determines the type of gonad formed but the adult sexual phenotype is defined by cellular sex identity rather than gonadal hormone action. Despite this crucial difference, many aspects of the morphology and molecular control of sexual development is conserved in both classes.

At the morphological level, avian and mammalian gonadogenesis differ in terms of a left-right asymmetry and in the source of the gonadal cell precursors: avian PGCs migrate through the circulation to reach the developing gonad and avian Sertoli cells originate in the nephrogenic mesenchyme. At the molecular level, evidence suggests that DMRT1 is essential for testis development, and while estrogen influences gonadal fate, non-reproductive tissues are largely defined by the genetic make-up of the constituent cells.

Future investigation of the sex determining mechanisms of birds should delve into the complex regulatory interactions between the components of the pathways and clarify the influence of important genes such as DMRT1 and FOXL2 in the process. Advances in gene editing technology allow easy and cost-effective generation of transgenic organisms, which will undoubtedly assist with further characterisation of molecular components of the sex determining pathways of different species.

References

- Ayers, K. L., Cutting, A. D., Roeszler, K. N., Sinclair, A. H., & Smith, C. A. (2015a). DMRT1 is required for Mullerian duct formation in the chicken embryo. *Dev Biol*, 400, 224–236.
- Ayers, K. L., Davidson, N. M., Demiyah, D., et al. (2013). RNA sequencing reveals sexually dimorphic gene expression before gonadal differentiation in chicken and allows comprehensive annotation of the W-chromosome. *Genome Biology*, 14, R26.
- Ayers, K. L., Lambeth, L. S., Davidson, N. M., et al. (2015b). Identification of candidate gonadal sex differentiation genes in the chicken embryo using RNA-seq. *BMC Genomics*, 16, 704.
- Bachtrog, D., Mank, J. E., Peichel, C. L., et al. (2014). Sex determination: Why so many ways of doing it? *PLOS Biol*, 12, e1001899.
- Barske, L. A., & Capel, B. (2008). Blurring the edges in vertebrate sex determination. *Curr Opin Genet Dev*, 18, 499–505.
- Bellairs, R., & Osmond, M. (2014). *Atlas of Chick Development*. Oxford: Academic Press.
- Bruggeman, V., Van As, P., & Decuyper, E. (2002). Developmental endocrinology of the reproductive axis in the chicken embryo. *Comp Biochem Physiol A Mol Integr Physiol*, 131, 839–846.
- Clinton, M., Nandi, S., Zhao, D., et al. (2016). Real-time sexing of chicken embryos and compatibility with in ovo protocols. *Sex Dev*, 10, 210–216.
- Clinton, M., Zhao, D., Nandi, S., & McBride, D. (2012). Evidence for avian cell autonomous sex identity (CASI) and implications for the sex-determination process? *Chromosome Res*, 20, 177–190.
- Fridolfsson, A. K., Cheng, H., Copeland, N. G., et al. (1998). Evolution of the avian sex chromosomes from an ancestral pair of autosomes. *Proc Natl Acad Sci USA*, 95, 8147–8152.
- Govoroun, M. S., Pannetier, M., Pailhoux, E., et al. (2004). Isolation of chicken homolog of the FOXL2 gene and comparison of its expression patterns with those of aromatase during ovarian development. *Dev Dyn*, 231, 859–870.
- Guioli, S., Nandi, S., Zhao, D., et al. (2014). Gonadal asymmetry and sex determination in birds. *Sex Dev*, 8, 227–242.
- Huang, S., Ye, L., & Chen, H. (2017). Sex determination and maintenance: The role of DMRT1 and FOXL2. *Asian J Androl*.
- Hudson, Q. J., Smith, C. A., & Sinclair, A. H. (2005). Aromatase inhibition reduces expression of FOXL2 in the embryonic chicken ovary. *Dev Dyn*, 233, 1052–1055.
- Ishimaru, Y., Komatsu, T., Kasahara, M., et al. (2008). Mechanism of asymmetric ovarian development in chick embryos. *Development*, 135, 677–685.
- Johnson, A. L. (2015). Reproduction in the female A2 – Scanes. In G. Colin (Ed.), *Sturkie's Avian Physiology (sixth ed)*. San Diego: Academic Press (Chapter 28).
- Lambeth, L. S., Ayers, K., Cutting, A. D., et al. (2015). Anti-müllerian hormone is required for chicken embryonic urogenital system growth but not sexual differentiation. *Biology of Reproduction*, 93, 138.
- Lambeth, L. S., Cummins, D. M., Doran, T. J., Sinclair, A. H., & Smith, C. A. (2013). Overexpression of aromatase alone is sufficient for ovarian development in genetically male chicken embryos. *PLOS ONE*, 8, e68362.

- Lambeth, L. S., Morris, K. R., Wise, T. G., et al. (2016). Transgenic chickens overexpressing aromatase have high estrogen levels but maintain a predominantly male phenotype. *Endocrinology*, 157, 83–90.
- Lambeth, L. S., Raymond, C. S., Roeszler, K. N., et al. (2014). Over-expression of DMRT1 induces the male pathway in embryonic chicken gonads. *Dev Biol*, 389, 160–172.
- Lin, M., Thorne, M. H., Martin, I. C., Sheldon, B. L., & Jones, R. C. (1995). Development of the gonads in the triploid (ZZW and ZZZ) fowl, *Gallus domesticus*, and comparison with normal diploid males (ZZ) and females (ZW). *Reprod Fertil Dev*, 7, 1185–1197.
- Mank, J. E., & Ellegren, H. (2009). All dosage compensation is local: Gene-by-gene regulation of sex-biased expression on the chicken Z chromosome. *Heredity (Edinb)*, 102, 312–320.
- McQueen, H. A., McBride, D., Miele, G., Bird, A. P., & Clinton, M. (2001). Dosage compensation in birds. *Curr Biol*, 11, 253–257.
- Moniot, B., Boizet-Bonhoure, B., & Poulat, F. (2008). Male specific expression of lipocalin-type prostaglandin D synthase (cPTGDS) during chicken gonadal differentiation: Relationship with cSOX9. *Sex Dev*, 2, 96–103.
- Morais da Silva, S., Hacker, A., Harley, V., et al. (1996). Sox9 expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. *Nat Genet*, 14, 62–68.
- Nakamura, Y., Kagami, H., & Tagami, T. (2013). Development, differentiation and manipulation of chicken germ cells. *Dev Growth Differ*, 55, 20–40.
- Nakata, T., Ishiguro, M., Aduma, N., Izumi, H., & Kuroiwa, A. (2013). Chicken hemogen homolog is involved in the chicken-specific sex-determining mechanism. *Proc Natl Acad Sci USA*, 110, 3417–3422.
- Oreal, E., Pieau, C., Mattei, M. G., et al. (1998). Early expression of AMH in chicken embryonic gonads precedes testicular SOX9 expression. *Dev Dyn*, 212, 522–532.
- Pannetier, M., Fabre, S., Batista, F., et al. (2006). FOXL2 activates P450 aromatase gene transcription: Towards a better characterization of the early steps of mammalian ovarian development. *J Mol Endocrinol*, 36, 399–413.
- Richardson, B. E., & Lehmann, R. (2010). Mechanisms guiding primordial germ cell migration: Strategies from different organisms. *Nat Rev Mol Cell Biol*, 11, 37–49.
- Roeszler, K. N., Itman, C., Sinclair, A. H., & Smith, C. A. (2012). The long non-coding RNA, MHM, plays a role in chicken embryonic development, including gonadogenesis. *Dev Biol*, 366, 317–326.
- Rothwell, B., & Tingari, M. D. (1973). The ultrastructure of the boundary tissue of the seminiferous tubule in the testis of the domestic fowl (*Gallus domesticus*). *J Anat*, 114, 321–328.
- Sekido, R., & Lovell-Badge, R. (2007). Mechanisms of gonadal morphogenesis are not conserved between chick and mouse. *Dev Biol*, 302, 132–142.
- Smith, C. A., Roeszler, K. N., Ohnesorg, T., et al. (2009). The avian Z-linked gene DMRT1 is required for male sex determination in the chicken. *Nature*, 461, 267–271.
- Smith, C. A., Shoemaker, C. M., Roeszler, K. N., et al. (2008). Cloning and expression of R-Spondin1 in different vertebrates suggests a conserved role in ovarian development. *BMC Dev Biol*, 8, 72.
- Takada, S., Ota, J., Kansaku, N., et al. (2006a). Nucleotide sequence and embryonic expression of quail and duck Sox9 genes. *Gen Comp Endocrinol*, 145, 208–213.
- Takada, S., Wada, T., Kaneda, R., et al. (2006b). Evidence for activation of Amh gene expression by steroidogenic factor 1. *Mech Dev*, 123, 472–480.
- Tevosian, S. G. (2013). Genetic control of ovarian development. *Sex Dev*, 7, 33–45.
- Uda, M., Ottolenghi, C., Crisponi, L., et al. (2004). Foxl2 disruption causes mouse ovarian failure by pervasive blockage of follicle development. *Hum Mol Genet*, 13, 1171–1181.
- Uhlenhaut, N. H., Jakob, S., Anlag, K., et al. (2009). Somatic sex reprogramming of adult ovaries to testes by FOXL2 ablation. *Cell*, 139, 1130–1142.
- Wang, J., & Gong, Y. (2017). Transcription of CYP19A1 is directly regulated by SF-1 in the theca cells of ovary follicles in chicken. *Gen Comp Endocrinol*, 247, 1–7.
- Wilhelm, D., Hiramatsu, R., Mizusaki, H., et al. (2007). SOX9 regulates prostaglandin D synthase gene transcription in vivo to ensure testis development. *J Biol Chem*, 282, 10553–10560.
- Yang, X., Deng, J., Zheng, J., et al. (2016). A window of MHM demethylation correlates with key events in gonadal differentiation in the chicken. *Sex Dev*, 10, 152–158.
- Zhao, D., McBride, D., Nandi, S., et al. (2010). Somatic sex identity is cell autonomous in the chicken. *Nature*, 464, 237–242.

Sex Change in Fish

Jodi T Thomas, Hui Liu, Erica V Todd, and Neil J Gemmell, University of Otago, Dunedin, New Zealand

© 2018 Elsevier Inc. All rights reserved.

Glossary

Anterior pituitary The glandular, anterior lobe of the pituitary gland.

Bidirectional sex change A sequential hermaphrodite that can change from female to male, and male to female.

DNA methylation Physical changes to DNA that change gene expression without changing the underlying DNA sequence.

Epigenetic modifications Physical changes to DNA that change gene expression without changing the underlying DNA sequence.

Genome The entire set of genes of an individual.

Gonochoeristic An individual that functions as one sex its entire life (does not undergo sex change).

Lifetime reproductive success The total number of offspring an individual produces over its entire lifespan.

Monogamy One male and one female mate.

Ovotestis A bisexual gonad containing both ovarian and testicular tissue.

Polygyny One male mates with more than one female.

Protandry A sequential hermaphrodite that changes from male to female.

Protogyny A sequential hermaphrodite that changes from female to male.

Sequential hermaphrodite An individual that functions as both a female and male at different time points in their life.

Steroidogenesis The biological synthesis of steroid hormones.

Teleost A taxonomic grouping of fishes that make up approximately 96% of all fish, the ray-finned fishes.

Temperature dependent sex reversal An interaction between genetic sex determination and temperature sex determination in which the temperature experienced during development can reverse the chromosomal sex.

Tetrapod A taxonomic grouping of vertebrates, the four-limbed vertebrates.

Transcriptome The set of RNA molecules present in a cell or population of cells.

What is Sex Change?

The majority of organisms develop as one sex and remain that sex throughout their life. However, a range of plant and animal species are sequentially hermaphroditic, beginning life as one sex and changing to the opposite sex at a later point. The change associated with sequential hermaphroditism is commonly known as sex change and occurs as a natural part of the life cycle of many species. This is in contrast to artificially induced feminization or masculinization, e.g., by environmental pollutants. Teleost (bony) fishes are the only vertebrate lineage to contain species that undergo sex change as part of their natural life cycle. Sex change is widespread in teleosts, occurring in at least 27 families, spread across nine orders. Teleosts display three patterns of sequential hermaphroditism: protandry (male to female), protogyny (female to male), and bidirectional (serial sex change in both directions).

Why Change Sex?

The main theory explaining why sex change is beneficial is the size advantage model. This model states that sex change is adaptive when the reproductive success of each sex differs depending on the size of an individual, i.e., an individual has greater reproductive success as one sex when small but the opposite sex when large. The timing of sex change should occur to maximize the lifetime reproductive success of an individual (combined male and female lifetime reproductive success). The differential reproductive success of each sex is associated with a species' mating system and social structure. Therefore, the pattern and timing of sex change also depends on the species' mating system and social structure.

Protogyny

Protogynous (female to male) sex change is favored in a polygynous mating system, in which one or a few large males monopolize matings with females. In this system, males aggressively defend a mating territory for courtship with females. Therefore, male reproductive success strongly depends on body size, with smaller males being at a reproductive disadvantage and large males having higher reproductive success due to the ability to defend a mating territory and mate with multiple females. Reproduction as a female when small, followed by sex change to reproduce as a male when large, will increase lifetime reproductive success of an individual

despite the physiological cost of sex change. Highly social polygynous wrasses, such as the bluehead wrasse (*Thalassoma bifasciatum*) commonly display protogyny. In this species, dominant males defend spawning sites for courtship with females. Loss of a dominant male results in protogynous sex change of a large female.

Protandry

When mating is random, or is monogamous (one male mates with one female), protandry (male to female sex change) is favored. In these systems, female fertility increases more rapidly with size than male fertility and larger females usually have a higher reproductive success than males of the same size. Thus, it is beneficial for individuals to reproduce as males while small, and as females when older and larger. For example, monogamous clownfish (*Amphiprion* and *Premnas* sp.) show protandrous sex change. The small social group of clownfish consists of a single mating pair; a dominant female and small male, as well as several smaller, subordinate non-breeders. Loss of the dominant female results in protandrous sex change of the small male, and maturation of one of the subordinate non-breeders to replace the breeding male.

Bidirectional Sex Change

Bidirectional sex change allows an individual to mate with any other adult it encounters. Thus, bidirectional sex change is adaptive for species that occupy a patchy or highly heterogeneous habitat where there is a low probability of finding a new mate and searching for a new partner involves a high predation risk. For example, many monogamous coral gobies (e.g., *Gobiodon* and *Paragobiodon* sp.) are bidirectional sex changers. Coral gobies have limited mating opportunities, and predation risk is high when travelling between the spatially isolated coral colonies they inhabit. Bidirectional sex change allows any two fish to form a heterosexual breeding pair, increasing mating opportunities and decreasing the time and risk of finding a new partner.

What Happens During Sex Change?

Sex change consists of three stages; behavioral, gonadal and morphological sex change. These stages of sex change are clearly illustrated in the bluehead wrasse, a well-studied model of protogynous sex change. Within minutes to hours of removing a dominant male, the transitioning female starts showing male-specific behaviors such as aggression and courtship. Gonadal restructuring then takes place, in which the ovary regresses and is replaced by a testis in as little as eight to ten days. External morphological changes such as change in body shape and color are the last to be completed. In the bluehead wrasse, it can take up to three weeks for males to develop full coloration (Fig. 1).

Among sequentially hermaphroditic lineages there are differences in how gonadal sex change occurs. Some species show complete restructuring of the gonad. For example, in protogynous wrasses the ovary contains no testicular tissues prior to sex change, and the sex-changed male testis contains no ovarian tissue, with only a remnant ovarian lumen. As no male tissues are identifiable prior to sex change, it is unknown where the male cells originate from. In protogynous wrasses, it is likely that germ line cells found on the periphery of the ovarian lamellae are sources for testicular development. However, it has not been definitively established whether these cells are a dormant, but sexually differentiated population of germ cells (i.e. oogonia and spermatogonia co-existing in the gonad), or if they are primordial germ cells that are sexually bipotent and undifferentiated.

In other sequential hermaphrodites, both ovarian and testicular tissue is present in the gonad simultaneously, which is known as a bisexual gonad or ovotestis. Protandrous black porgy (*Acanthopagrus schlegelii*) are functionally male for the first two years of life, possessing an ovotestis dominated by testicular tissue that is separated by connective tissue from a few oogonia and primary oocytes. In the non-spawning season, before their third year of life, primary oocytes develop and ovarian tissue begins to dominate

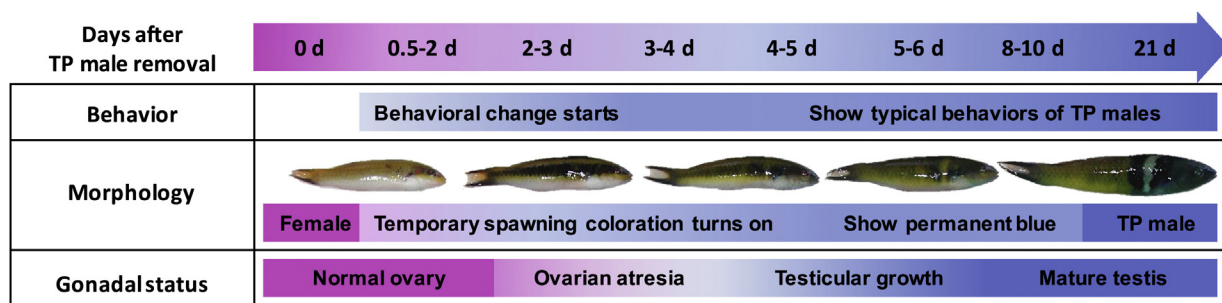


Fig. 1 Timeline of behavioral, gonadal and morphological changes across protogynous sex change in the bluehead wrasse. Reprinted with permission from Liu, H., Todd, E.V., Lokman, M.P., *et al.*, 2016. Sexual plasticity: A fishy tale. *Molecular Reproduction and Development* 84, 171–194. Copyright 2016, Wiley Periodicals, Inc.

the gonad as testicular tissue regresses. By three years of age, all fish are functionally female and the ovotestis is completely replaced by ovarian tissue. Bidirectionally sex changing gobies have an ovotestis throughout life. This allows rapid and serial switching of gonadal fate.

The time taken to complete sex change varies greatly between species, from as little as days to weeks in some species, to months in others. In temperate fishes that breed seasonally, sex change only occurs outside of the breeding season and occurs over a longer time-frame (months) than in tropical fishes that breed year-round and can change sex more rapidly and throughout the year.

How is Sex Change Regulated?

Fish use a variety of environmental or physiological cues to trigger sex change. One of the most common cues is the immediate social environment, e.g., an individual's size relative to others in the social group, the local sex ratio in a social group, the local density of fish in a social group, and the presence or absence of a dominant individual. Sex change may also be cued by reaching a critical age or size. For example, black porgy change sex from male to female between their second and third breeding seasons.

It is not fully understood how environmental cues are translated into a rapid behavioral response, and the ensuing physiological response that is sex change. However, it appears that neuroendocrine systems are key signaling pathways in controlling sex change, communicating information between the brain and gonad. There is likely cross-talk between multiple neuroendocrine systems to coordinate physiological changes at a whole-body level.

Regulation of Behavioral Sex Change

Neurochemical changes in the brain appear to drive behavioral sex change. However, how these neurochemical changes transduce the environmental cues into a rapid behavioral transformation is still poorly understood. Arginine vasotocin (AVT), and to a smaller extent isotocin, have been the focus of research for behavioral sex change. These neuropeptides are the teleost homologues of mammalian vasopressin and oxytocin, respectively. In mammals and other tetrapods, vasopressin mediates male social behaviors, such as aggression and territoriality, a role which appears to be conserved for AVT in teleosts. Experiments in the protogynous blue-head wrasse demonstrate that AVT likely plays a critical role in stimulating behavioral sex change. Oxytocin is well known for its role in female sociosexual behaviors in mammals, including parturition, lactation, maternal attachment, and pair bonding. In fishes, isotocin has also been associated with sociosexual behaviors, and gene expression and protein abundance of isotocin in the brain have been shown to change across protogynous sex change. However, opposing trends in isotocin expression are reported in different species, and the role of isotocin in sex change is not yet well understood.

Changes in other neurochemicals during early sex change may orchestrate behavioral sex change, and are also known to regulate the hypothalamic-pituitary-gonadal (HPG) axis that controls steroidogenesis in the gonad, thereby possibly acting as a link between behavioral and gonadal sex change. Norepinephrine appears to stimulate protogynous sex change, while dopamine and serotonin have inhibitory effects on protogynous sex change. Kisspeptin is well known for its role in stimulating the HPG axis, and it appears to also be involved in sex change, however its precise role remains unclear (Fig. 2).

Regulation of Gonadal Sex Change

Hypothalamic-Pituitary-Gonadal Axis

The HPG axis is a major signaling pathway controlling gonadal sex change in fish. Unlike other vertebrates, gonadotropin releasing hormone (GnRH) neurons located in the preoptic area of the hypothalamus directly innervate the anterior pituitary in fish. In the anterior pituitary, GnRH is released and stimulates the synthesis and release of the gonadotropins luteinizing hormone and follicle stimulating hormone into circulation. Gonadotropins act through their receptors in the gonad, either on follicle cells in the ovary or leydig cells in the testis, to regulate the production of sex hormones (including estrogens and androgens) (Fig. 2).

In most teleost fish, the main estrogen and androgen are 17 β -estradiol (E2) and 11-ketotestosterone (11-KT), respectively. The balance between these two sex steroids controls gonadal fate, with E2 promoting ovarian function, and 11-KT testicular function. Testosterone (T) acts as a prohormone in fish and is converted to E2 via the enzyme aromatase, and to 11-KT via the enzymes 11 β -hydroxylase (11 β H) and 11 β -hydroxysteroid dehydrogenase 2 (11 β HSD2) (Fig. 2). Therefore, relative functioning of these pathways in the gonad determines the balance of E2 and 11-KT, and subsequently gonadal fate.

Dramatic shifts in E2 and 11-KT blood serum levels occur across sex change in fish. In protogynous sex change, a rapid decrease in E2 is followed by a steady increase in 11-KT (Fig. 3). Treatment of adult females with either an aromatase inhibitor (AI) or 11-KT, can decrease E2 production and increase 11-KT plasma levels to induce complete sex change. It is thought that a decrease in E2 levels below a threshold will result in oocytes being unable to survive, while increasing 11-KT levels may accelerate ovarian atresia while also initiating proliferation of spermatogonia. The opposite pattern is seen in protandrous sex change: 11-KT levels drop before there is an increase in E2 production (Fig. 3). Similarly, AI administration to adult males can suppress natural sex change in protandrous species.

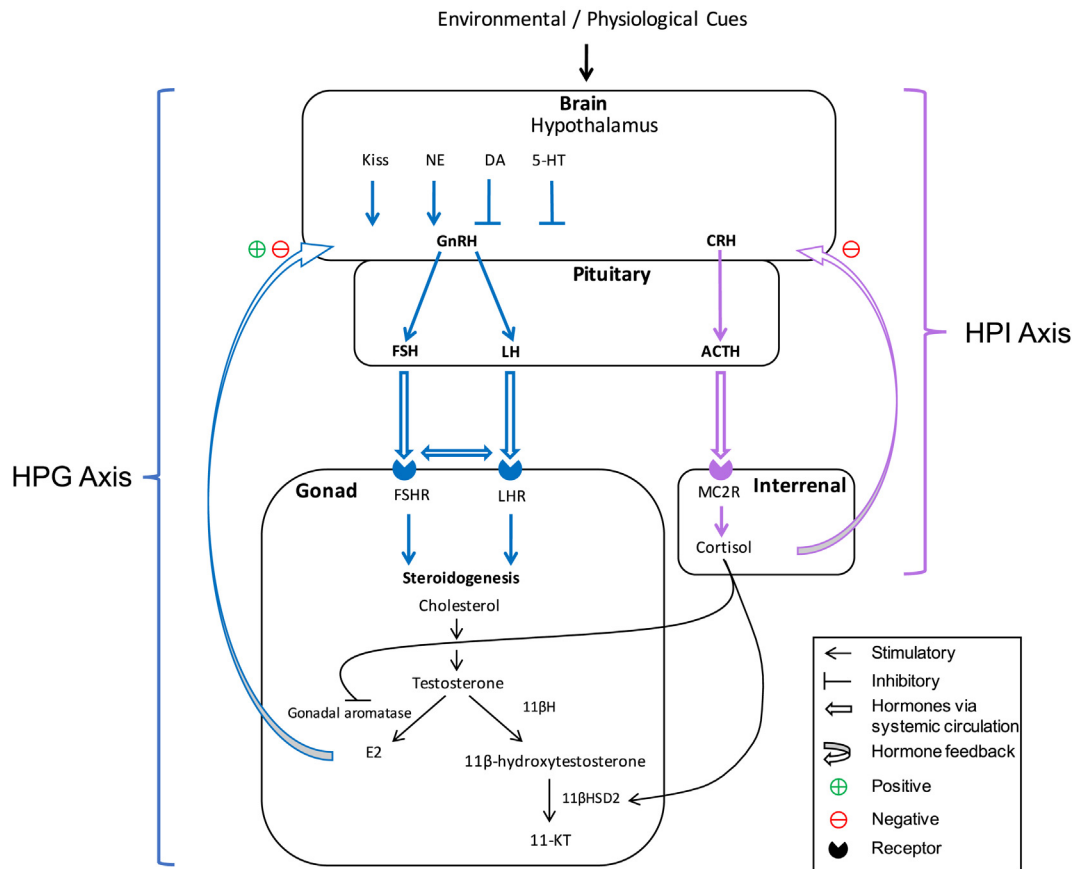


Fig. 2 Diagram of the HPG and HPI axes depicting the neuroendocrine regulation of steroidogenesis in fish, as well as the interaction of cortisol with the HPG axis. 5-HT, serotonin, ACTH, adrenocorticotropic hormone, CRH, corticotropin releasing hormone, DA, dopamine, FSH, follicle stimulating hormone, FSHR, follicle stimulating hormone receptor, GnRH, gonadotropin releasing hormone, kiss, kisspeptin, LH, luteinizing hormone, LHR, luteinizing hormone receptor, MC2R, melanocortin 2 receptor, NE, norepinephrine, 11 β H, 11 β -hydroxylase, 11 β HSD2, 11 β -hydroxysteroid dehydrogenase type 2. Figure adapted from Liu, H., Todd, E.V., Lokman, M.P., *et al.*, 2016. Sexual plasticity: A fishy tale. *Molecular Reproduction and Development* 84, 171–194.

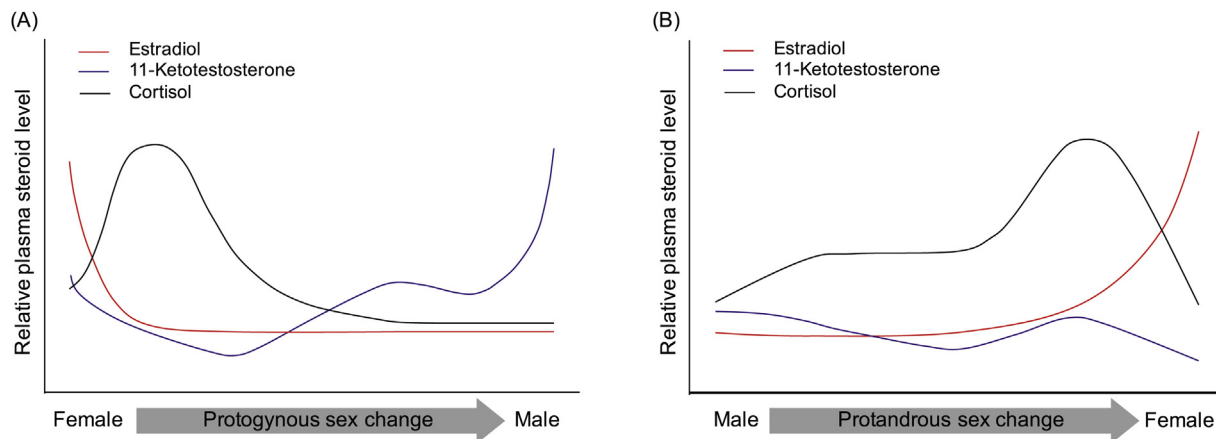


Fig. 3 Hormonal changes across protogynous (A) and protandrous sex change (B). Reprinted with permission from Todd, E., Liu, H., Muncaster, S., Gemmell, N., 2016. Bending genders: The biology of natural sex change in fish. *Sexual Development* 10, 229. Copyright 2016, S. Karger AG, Basel.

The importance of the HPG axis in controlling the E2–11-KT balance, and thus gonadal fate, has been demonstrated through several manipulative studies. For example, administration of GnRH and gonadotropins (or derivatives of these) in protogynous species results in a drop in E2 levels and an increase in 11-KT, inducing gonadal sex change. Thus, changes in gonadal sex steroid levels and subsequently gonadal sex change, can be controlled by the brain via the HPG axis.

Hypothalamic-Pituitary-Interrenal Axis

In fish, the hypothalamic-pituitary-interrenal (HPI) axis mediates the stress response and has also, more recently, been implicated in sex change. Corticotropin releasing hormone is secreted from the hypothalamus, stimulating the anterior pituitary to release adrenocorticotrophic hormone (ACTH) into the circulation. ACTH acts on melanocortin 2 receptors present on interrenal cells (dispersed throughout the head kidney and homologous to the mammalian adrenal glands), activating the biosynthesis and release of cortisol, the primary stress hormone in vertebrates (Fig. 2).

The HPI axis may interact with the HPG axis to promote sex change in fish. In a range of gonochoristic (non sex-changing) fish species, environmental stressors such as elevated temperature have been found to increase cortisol levels and cause gonadal masculinization. In sequential hermaphrodites, a transient peak in plasma cortisol levels has been observed during both protogynous and protandrous sex change (Fig. 3), and long-term cortisol treatment was shown to promote protogynous sex change in the three-spot wrasse (*Halichoeres trimaculatus*). Cortisol is thought to promote sex change via its actions on aromatase and 11-KT. Cortisol inhibits aromatase expression, halting the conversion of T to E2 (Fig. 2) and decreasing E2 production, which increases the availability of T to be converted into 11-KT. Furthermore, the expression of 11 β HSD2, the enzyme which catalyzes the production of 11-KT (this enzyme also catalyzes cortisol to the inactive form cortisone), is stimulated by cortisol, which may further increase 11-KT production (Fig. 2). Thus, the cortisol pathway may act as an interface between the HPI and HPG axes, tipping the E2–11-KT balance to promote sex change.

Molecular Regulation of Sex Change

Genetic Regulation

At a molecular level, primary sex-determination in the early embryo is no longer considered a permanent switch. Rather, there is a battle for dominance between female and male regulatory gene networks. Antagonistic feminizing (e.g., *foxl2*, *wnt4*, *cyp19a1a*) and masculinizing (e.g., *dmrt1*, *amh*, *sox9*) gene networks promote ovarian and testicular development respectively, while actively suppressing expression of the opposing sexual network. Activation of one sex-specific network, but also continual suppression of the opposing sex differentiation network, is required for maintenance of gonadal fate. This means that sexual fate can be manipulated by factors that interrupt the dominant sexual network and allow the opposite sexual network to overtake. In sex changing fish, it appears that this system has been exploited, with factors that interrupt the dominant sexual network having come under control of environmental cues.

Research shows that in both protandrous and protogynous fishes, a shift occurs from expression of one sex-specific gene network to the other. In the protandrous black porgy, males show high expression levels of male-related genes in the testis (e.g., *dmrt1*, *amh*, *amhr2*) and a decline in male-related gene expression is associated with testis regression. As protandrous sex change continues and the ovary develops, the gene expression profile becomes increasingly feminized (e.g., *cyp19a1a*, *foxl2*, *wnt4*) (Fig. 4). An opposite pattern is observed in the protogynous bluehead wrasse. Whole-transcriptome expression profiling in this species revealed a gradual decline in expression of female-pathway genes in the gonad (e.g., *dax1*, *cyp19a1a*, *figla*, *gdf9*, *hsd7b1*, and *hsd11b3*), followed by increased expression of male-related genes (e.g., *dmrt1*, *amh*, *amhr2*, *sox9a/b*, and *gsdf*) (Fig. 4).

The genetic mechanism that triggers this shift in sex-specific gene expression in sequentially hermaphroditic fish has yet to be determined in any species. However, a key component of the genetic mechanism that initiates protogynous sex change is the gene *cyp19a1a*, coding for the enzyme gonadal aromatase that converts T into E2. *Cyp19a1a* is currently the only gene shown to rapidly and completely shut down early in protogynous sex change, when the first signs of ovarian atresia are observed (Fig. 4). This rapid downregulation of *cyp19a1a* parallels the sharp decline in plasma E2 levels that occurs early in protogynous sex change (Fig. 3). Manipulative studies have shown that treatment of protogynous fish with an aromatase inhibitor decreases E2 levels and

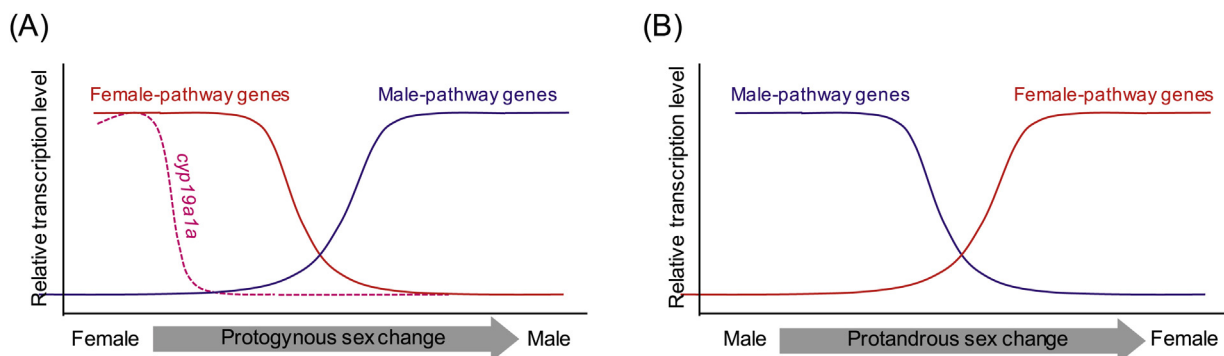


Fig. 4 Changes in sex-specific gene expression across protogynous (A) and protandrous sex change (B); a shift occurs from one sexual network to the opposing sexual network. Reprinted with permission from Todd, E., Liu, H., Muncaster, S., Gemmell, N., 2016. Bending genders: The biology of natural sex change in fish. Sexual Development 10, 229. Copyright 2016, S. Karger AG, Basel.

induces complete sex change. Thus, it appears that downregulation of *cyp19a1a* is a switch that initiates gonadal sex change in protogynous fish. Therefore, upstream factors that suppress *cyp19a1a* expression are potential components of a trigger mechanism.

Epigenetic Regulation

Epigenetic modifications, e.g., DNA methylation and histone acetylation, are physical changes to DNA that alter gene expression without changing the underlying genetic code. Such modifications affect gene expression by altering gene availability to transcription, and typically inhibit (via DNA methylation), or promote (via histone acetylation) transcription. These modifications occur naturally and are critical to development. Epigenetic machinery can respond to environmental influences, and so may be a means by which environmental cues are translated into changes in gene expression to elicit a phenotypic response.

As sequential hermaphroditism in many fishes relies on environmental cues, namely social cues, epigenetic modification of sex-pathway genes is a potential mechanism through which gene expression could be regulated, and sex change initiated and maintained. DNA methylation has been demonstrated as an upstream mechanism regulating *cyp19a1a* expression. In gonochoristic (unisexual) fish, *cyp19a1a* expression is higher in the ovary than the testis, and DNA methylation levels of the *cyp19a1a* promoter region are lower in the ovary than the testis. This suggests that DNA methylation of the *cyp19a1a* promoter region may suppress expression of *cyp19a1a* in the testis. DNA methylation changes to *cyp19a1a* may regulate temperature dependent sex reversal in fish, as well as sex change in sequentially hermaphroditic fish. In genetic females, high temperatures have been found to increase DNA methylation of the *cyp19a1a* promoter region, decrease *cyp19a1a* expression, and masculinize the female ovary. In protogynous species, DNA methylation levels of the *cyp19a1a* promoter region have been found to increase during sex change as *cyp19a1a* expression decreases. Thus, DNA methylation is an upstream factor that suppresses *cyp19a1a* expression and is a potential component of a trigger mechanism of sex change.

As well as DNA methylation influencing *cyp19a1a* expression, it is likely that DNA methylation occurs throughout the genome to regulate the shift that occurs across sex change from expression of one sex-specific gene network to the opposing gene network. In half-smooth tongue sole (*Cynoglossus semilaevis*), a species displaying temperature dependent sex reversal, sex pathway genes were differentially methylated depending on their level of expression in an ovary or testis, for example, female-pathway genes that were highly expressed in the ovary showed low levels of DNA methylation, while in the testis these female-pathway genes showed lower expression levels and higher levels of DNA methylation. The overall methylation pattern of pseudo-male (sex reversed females), and male testes was very similar suggesting that female genome-wide methylation patterns are altered to match those of normal males after sex change. Thus, temperature dependent sex reversal during development likely involves DNA methylation throughout the genome regulating the expression of one or the other sex-specific pathway. Implantation of protogynous fish with a DNA methylation inhibitor halted, or reversed, the protogynous sex change process, suggesting DNA methylation may function similarly in sequentially hermaphroditic fish that change sex in adulthood.

Appendix A Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/B978-0-12-809633-8.20555-4](https://doi.org/10.1016/B978-0-12-809633-8.20555-4).

Further Reading

- Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191–364.
- Frisch, A. (2004). Sex-change and gonadal steroids in sequentially-hermaphroditic teleost fish. *Reviews in Fish Biology and Fisheries*, 14(4), 481–499.
- Godwin, J. (2009). Social determination of sex in reef fishes. *Seminars in Cell and Developmental Biology*, 20, 264–270.
- Kazancıoğlu, E., & Alonzo, S. H. (2010). A comparative analysis of sex change in Labridae supports the size advantage hypothesis. *Evolution*, 64(8), 2254–2264.
- Lamm, M. S., Liu, H., Gemmell, N. J., & Godwin, J. R. (2015). The need for speed: Neuroendocrine regulation of socially-controlled sex change. *Integrative and Comparative Biology*, 55(2), 307–322.
- Liu, H., Todd, E. V., Lokman, M. P., et al. (2016). Sexual plasticity: A fishy tale. *Molecular Reproduction and Development*, 84, 171–194.
- Munday, P. L., Buston, P. M., & Warner, R. R. (2006). Diversity and flexibility of sex-change strategies in animals. *Trends in Ecology & Evolution*, 21(2), 89–95.
- Sadovy De Mitcheson, Y., & Liu, M. (2008). Functional hermaphroditism in teleosts. *Fish and Fisheries*, 9(1), 1–43.
- Todd, E., Liu, H., Muncaster, S., & Gemmell, N. J. (2016). Bending genders: The biology of natural sex change in fish. *Sexual Development*, 10, 223–241.

Sex Determination, Insects

Daisuke Kageyama, National Agriculture and Food Research Organization, Tsukuba, Japan

© 2018 Elsevier Inc. All rights reserved.

Sexual Dimorphism: Difference Between Males and Females

In insects, males and females have distinct structures, known as sexual dimorphism. Moreover, certain insect species remarkably exhibit this dimorphism. For instance, in stag beetles, only males have outstanding mandibles (Fig. 1(a)), whereas in scale insects, both males and females have distinct structures as well as sizes (Fig. 1(b)). Further, butterflies show differences in wing color between males and females (Fig. 1(c)). Hence, males and females have fundamentally distinct structures and functions in sexual behavior and reproduction. How do such sexual differences arise?

Sex determination refers to the developmental processes that lead to differentiation between males or females. Spontaneous occurrence of gynandromorphism (i.e., chimeric individuals of male and female tissues; See “gynandromorph and intersex”), albeit in low frequency, is considered as the ground for genetic sex determination during early embryogenesis. Moreover, it cannot be altered during later development (Narita *et al.*, 2010). Particularly, each cell comprising the insect body determines its sex independently (cell-autonomous sex determination) and is not affected by diffusible substances like sex hormones.

Gynandromorph and Intersex

A gynandromorph (synonymous with a sexual mosaic) is a genetically chimeric individual, whereas an intersex is a genetically uniform individual (Goldschmidt, 1934; Laugé, 1985; Fig. 2). Double fertilization of a binucleate egg, loss of a sex chromosome, or upregulation/downregulation of sex determining genes is considered developmental mechanisms for gynandromorph and intersex occurrence (Narita *et al.*, 2010).

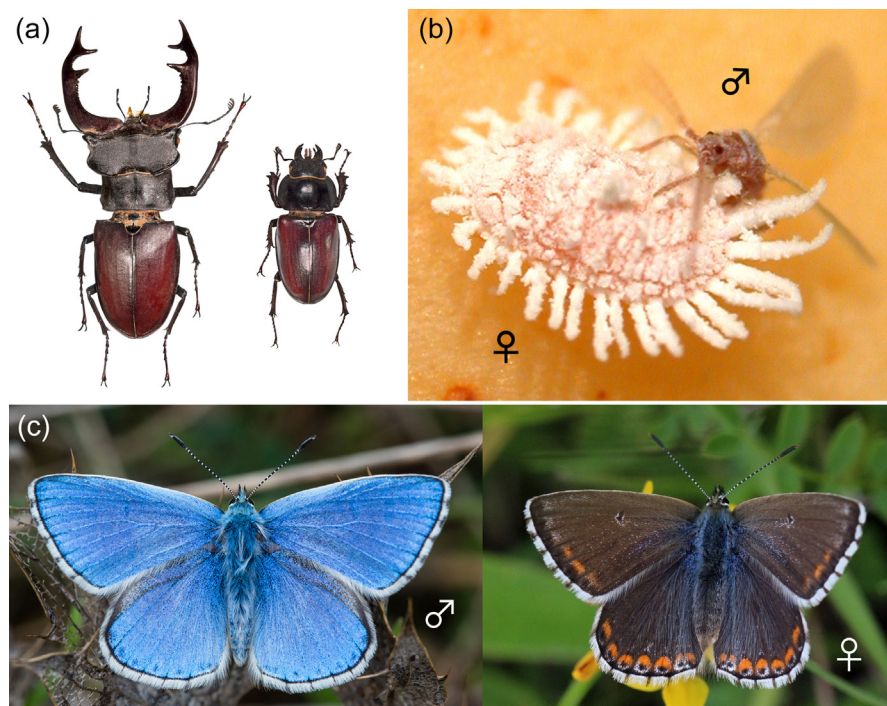


Fig. 1 Gynandromorphs. (a) A stag beetle, *Lucanus cervus*. Males (left) have outstandingly greater mandibles than females (right). (Source: Wikipedia. https://de.wikipedia.org/wiki/Sexualdimorphismus#/media/File:Ceif-volant_MHNT_male_et_femelle.jpg) (b) The pineapple mealybug, *Dysmicoccus brevipes*. Females are wingless and secrete a powdery wax layer to protect themselves, and hence, have a totally different outlook compared with males. (c) A lycaenid butterfly *Polyommatus bellargus* (Adonis blue). Left: male, Right: female. (Source: Wikipedia: https://en.wikipedia.org/wiki/Adonis_blue). (b) From Fig. 1 of Tabata, J., Ichiki, R.T., Tanaka, H., Kageyama, D., 2016. Sexual versus asexual reproduction: Distinct outcomes in relative abundance of parthenogenetic mealybugs following recent colonization. PLoS One 11, e0156587.

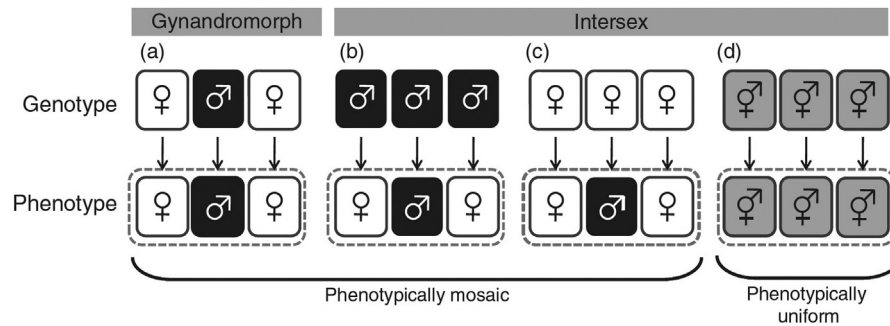


Fig. 2 Relationships between the genotype and phenotype of individual cells in gynandromorphs and intersexes. The squares indicate single cells (male: black; female: white; intermediate: gray). The dotted lines indicate single individuals. For simplicity, a single individual is assumed to comprise three cells. The genotype and phenotype are connected by arrows. (a) A gynandromorph (i.e., a sexual mosaic). (b) An intersex caused by partial feminization of a genetic male. (c) An intersex caused by partial masculinization of a genetic female. (d) An intersex with an intermediate genotype between male and female (e.g., a triploid intersex such as XXY). Reproduced from Narita, S., Pereira, R.A.S., Kjellberg, F., Kageyama, D., 2010. Gynandromorphs and intersexes: Potential to understand the mechanism of sex determination in arthropods. *Terrestrial Arthropod Reviews* 3, 63–96.

Sex Chromosomes and Sex Determination

In most insects, a chromosome pair (sex chromosomes) is distinctive between males and females. For example, in some flies and beetles, males are heterogametic (XY), whereas females are homogametic (XX), which is reversed in the case of butterflies and moths (i.e., ZZ males and WZ females). Moreover, in certain species, males have only one sex chromosome (X) without any pairing chromosome and are designated as X0, whereas females are XX. Conversely, in certain moths, females are Z0 and males are ZZ. Mendelian inheritance of sex chromosomes ensures 1:1 sex ratio in species having sex chromosomes (see “meiotic drive” for exceptions).

However, certain insects like wasps and bees, belonging to the family Hymenoptera, lack sex chromosomes. Instead, females have a genome sized twice as that of males, the reproductive system called haplodiploid, wherein unfertilized eggs develop as haploid males (n) and fertilized eggs develop as diploid females ($2n$). In some parasitic wasps like *Nasonia vitripennis*, which lay eggs in fly hosts wherein a limited number of wasps survive, females can control the proportion of eggs that get fertilized with the male sperm, thereby achieving most advantageous sex ratios that are theoretically deduced (cf. local mate competition).

Meiotic Drive

Meiotic drive is a violation of the Mendelian inheritance that results in a skewed ratio of functional X-bearing and Y-bearing sperms or W-bearing and Z-bearing eggs. In certain flies like *Drosophila*, some X chromosomes favor Y-bearing sperm inactivation, leading to all or nearly all females. Such X chromosomes are categorized as one of the selfish genetic elements that have a potential to spread in the population at the expense of the rest of the genome. Interestingly, autosomal and Y-linked suppressors against these X drive have been discovered, and some of them have reached near fixation in certain populations. Fixation of both drivers and suppressors results in a normal 1:1 sex ratio.

Molecular Mechanisms of Sex Determination

In insects, sex determination is governed by a cascade of several genes that can be well understood in a model insect i.e., the fruit fly *Drosophila melanogaster* (Fig. 3) (Sánchez, 2008). In *D. melanogaster*, the Y chromosome does not participate in sex determination, instead, certain specific genes located on the X chromosome activate the master sex determining gene *Sex-lethal* (*Sxl*) at a very early stage of embryogenesis. *Sxl* has two distinct “early” and “late” promoter regions. X-linked genes activate the “early” promoter in a dose-dependent manner. Further, *Sxl* turns ON in XX females and OFF in XY males. The positive regulatory function then allows continuous *Sxl* expression even without X chromosome signals. After the blastoderm stage, the “late” *Sxl* promoter begins to function in both sexes, and the *Sxl* mRNA undergoes splicing in distinctive manners in males and females. The male transcript gives rise to truncated, nonfunctional *Sxl* proteins (SXL^M). Functional *Sxl* proteins (SXL^F), are produced only in females and affect the splicing of another gene, *transformer* (*tra*), leading to a functional protein being produced (otherwise, *tra* produces a truncated nonfunctional protein TRA^M in males). The functional TRA protein (TRA^F) then affects another gene *doublesex* (*dsx*), which is to be spliced in specific manner to produce dsx^F (female-specific splicing product). Otherwise, *dsx* is spliced to produce dsx^M (male-specific splicing product). Their protein products (DSX^F and DSX^M) are both functional and induce somatic sexual differentiation. Behavioral differences among males and females such as mating behavior are governed by sex differences in the nervous systems. The neural sex differences in *Drosophila* are generated under the hierarchical control of two sex determining genes, *fruitless* (*fru*) and *dsx*. *dsx* also plays a key role in sex determination in soma, whereas *fru* exerts its sex determining function only in neural cells (Yamamoto and Koganezawa, 2013).

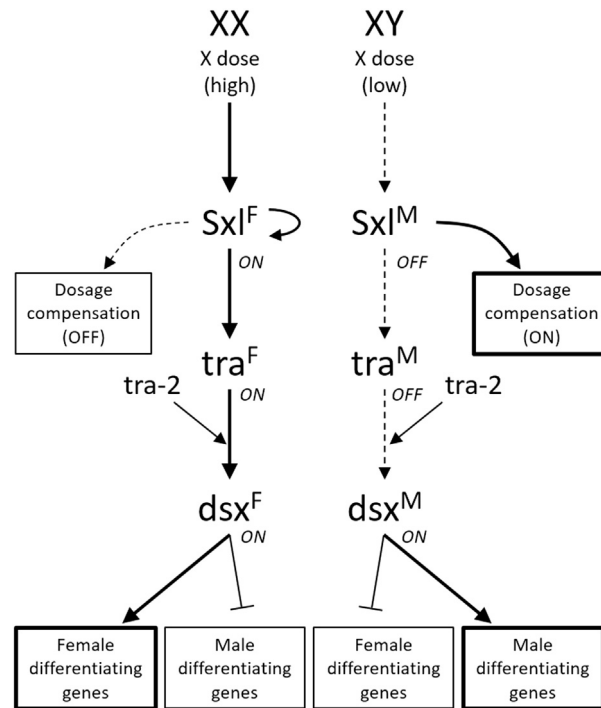


Fig. 3 Somatic sex determining gene cascade in *Drosophila melanogaster*.

Interestingly, the molecular mechanisms of sex determination in *D. melanogaster* cannot be simply extrapolated to other insects. Even in other flies (Diptera), *Sxl* does not play any role in somatic sex determination. Recently, it has become evident that the sex determining mechanism evolution is far more dynamic than that anticipated (Gempe and Beye, 2011). In insects, *dsx* is the only gene that is widely conserved structurally as well as functionally. Homologs of *dsx* (particularly, those sharing a DNA-binding motif called DM domain) can also be found in the soil-dwelling nematode *Caenorhabditis elegans* (*mab-3*) in mammals and birds (DMRT1). The above homolog functions as a sex determining gene for somatic sexual differentiation as well as germline differentiation. Conversely, *tra* is conserved between Diptera and Hymenoptera, but not in Lepidoptera. Further, *Sxl* is structurally conserved widely among insects and functions as a sex determiner only among closely related species of *D. melanogaster*. (Confusingly, *Sxl* functions as a sex determiner in *C. elegans* despite the long phylogenetic distance between the organisms.)

Furthermore, in the silkworm *Bombyx mori*, the presence of W chromosome has been recognized as important for female sex determination by the presence of variety of strains with chromosome aberrations. Critically, a recent molecular study revealed that a small RNA, particularly, a piwi-interacting RNA (piRNA) called *feminizer* (*fem*) is located on the W chromosome. Here, it acted as a female determiner by silencing the Z chromosome-located male determining gene *Masculinizer* (*Masc*) through the ping-pong cycle (Kiuchi et al., 2014). Independently, a P-element somatic inhibitor, expressed in both sexes in combination with sex-specifically spliced mRNA-binding protein (IMP), was shown to regulate *dsx* sex-specific splicing in *B. mori* (Suzuki et al., 2008, 2010).

In honeybees, which lack sex chromosomes, heterozygosity in *csd* locus leads to female sex determination (*complementary sex determination*). Therefore, haploid individuals are males whereas diploid individuals are usually females; however, under inbreeding conditions, diploid males appear as homozygous for *csd*. It is known that *csd* is homologous to *tra*, wherein *csd* regulates *dsx* splicing (Beye et al., 2003). More perplexingly, complementary sex determination is not the only sex determination mechanism in Hymenoptera. In the parasitic wasp *Nasonia vitripennis*, *tra* zygotic activity is autoregulated and dependent on a combination of maternal provisions of *tra* mRNA and a paternal genome set (Verhulst et al., 2010).

Is *dsx* A Master-Switch for Sex Determination?

dsx is widely conserved among insects as well as vertebrates (Fig. 3). Previously, *dsx* was regarded as the final master-switch gene in the sex determination cascade of all insect species. However, it was later found that *dsx* is not expressed in all cells, but rather forms an expression pattern mosaic in developing and adult individuals. Furthermore, homeotic genes (HOX genes) were noticed to regulate *dsx* expression. Growing evidence suggested that *dsx* regulation is based on sex-specific morphological differences (Fig. 4). Further, *dsx* is ordered to present this information in the case where sexual identity is required. Thus, *dsx* is not the final master-switch in the sex determination cascade, but is rather the central switch at the interface between sex determination and sexual differentiation (Verhulst and van de Zande, 2015).

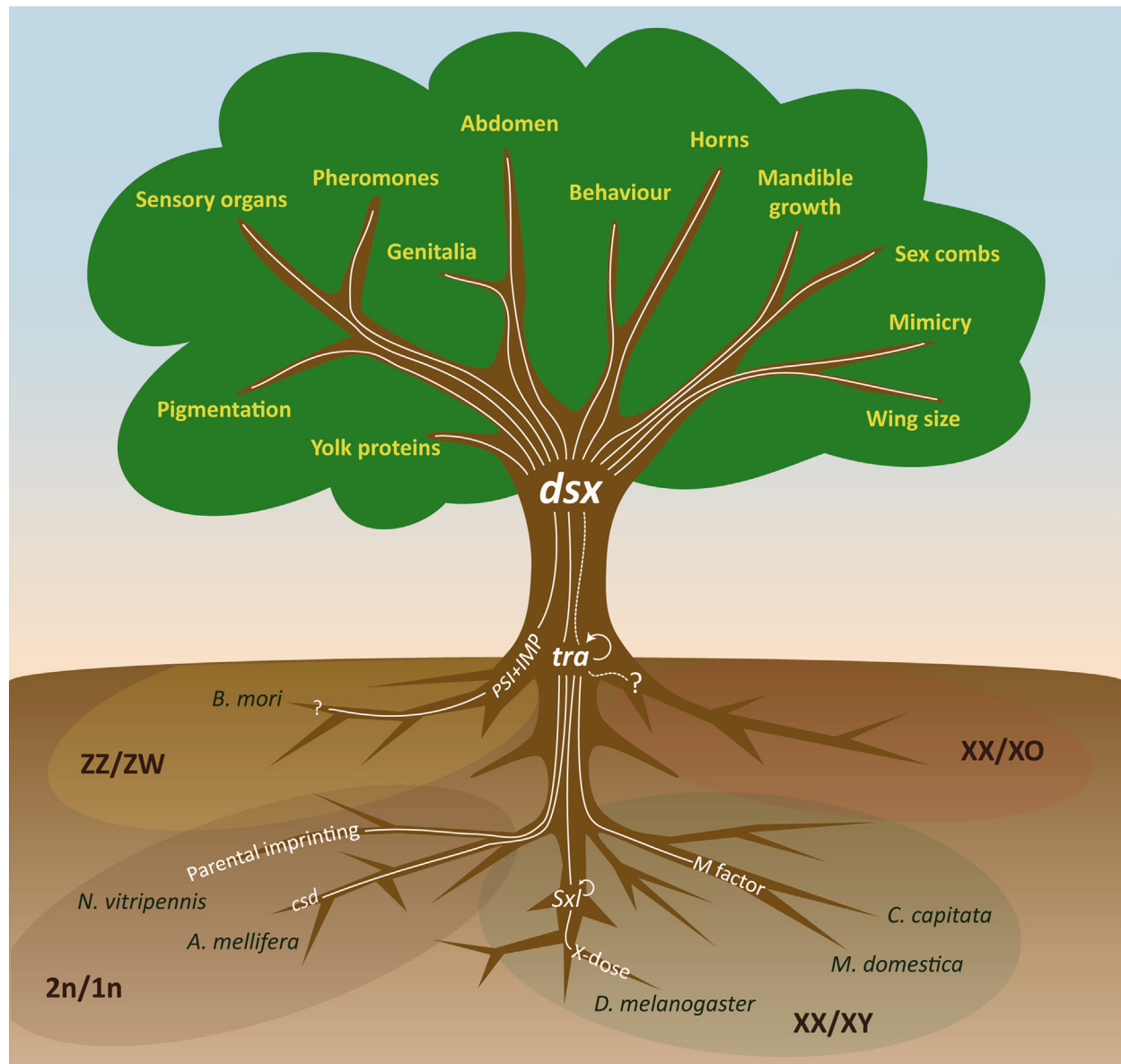


Fig. 4 *Doublesex (dsx)* as the interface of sex determination and sexual differentiation. Reproduced from Verhulst, E.C., van de Zande, L., 2015. Double nexus – Doublesex is the connecting element in sex determination. *Briefings in Functional Genomics* 14, 396–406.

Microorganisms as Sex Determiners

In insects, cytoplasmic elements (such as mitochondria and various microorganisms) are always transmitted from mothers to offspring. Therefore, a female-biased sex ratio is obviously advantageous for cytoplasmic elements (Burt and Trivers, 2006). Interestingly, some cytoplasmic elements could skew the sex ratio toward females in certain arthropod species (Kageyama *et al.*, 2012). In the woodlouse, *Armadillidium vulgare* (wherein females constitute WZ and males have ZZ sex chromosome), the presence of a maternally inherited microorganism (*Wolbachia* bacterium) results in the feminization of genetically male individuals (ZZ) into functional females. Interestingly, W chromosomes are lost in some *A. vulgare* populations (i.e., all individuals being ZZ) and the presence or absence of *Wolbachia* determines the sex. In such populations, *A. vulgare* could be regarded as an organism that lacks sex chromosomes (i.e., Z chromosomes behave like autosomes). In certain insects like *Drosophila*, ladybird beetles, butterflies, moths, planthoppers and lacewings, *Wolbachia* or other bacteria that reside in the insect body only kill males during embryonic or early larval development, leading to all female offsprings. In the moth *Ostrinia scapularis*, *Wolbachia*-infected ZZ males die during

embryonic development. Partial elimination of *Wolbachia* in mothers leads to the production of intersex individuals (genetically purely male, ZZ) comprising male-like as well as female-like tissues (Fig. 2(b)). Further, *Wolbachia* was considered to have a female determining function as the *dsx* splicing pattern for dying ZZ individuals was all female type. Z-linked gene expression levels revealed that the dosage compensation improperly functioned because of the sex alterations imposed by the bacterium (see below).

Several solitary wasp strains or species asexually reproduce, i.e., eggs laid by virgin females hatch and develop to all females. The lack of males in a considerable number of species, but not all, is caused by cytoplasmic bacteria such as *Wolbachia* and *Cardinium* (Rabeling and Kronauer, 2013).

Dosage Compensation

Not all sex chromosome genes (particularly, X and Z) have sex-specific functions. Particularly, equal gene product amounts are required between males and females for many sex chromosome genes. Thus, although chromosome numbers are different between males and females, X- or Z-linked gene products should be regulated to be comparable between males and females. Such regulatory mechanisms, called dosage compensation, widely exist in animals but with variable manners between the taxa. In *Drosophila*, single male X chromosome transcription increases by two-fold, thereby adjusting it with that of females (Fig. 5(a)). In mammals including humans and mice, the expression of one of the X chromosomes in an XX female is eliminated, so that both males and females express only one X chromosome. Either the maternal or paternal X chromosome is randomly inactivated in every cell of the female body. Hence, the above process is often called random X inactivation (Fig. 5(b)). The nematode *C. elegans* adopts an alternative way to equalize X-linked gene expression among males and females. In *C. elegans*, worms with two X chromosomes (XX) develop as hermaphrodites, whereas those with only one X chromosome (XO) develop as males. In this system, gene expression on both X chromosomes of the XX worms is downregulated by half, thereby equalizing the X-linked gene expression to that of XO males (Fig. 5(c)).

In *Drosophila*, the MSL (male-specific lethal) ribonucleoprotein complex comprising five proteins [*maleless* (*mle*), the *male-specific lethal* (*msl*) genes *msh-1*, *msh-2*, and *msh-3*, and *males absent on the first* (*mof*)] and two non-coding RNAs [*roX1* and *roX2*] is essential for dosage compensation in males by binding to the X chromosome in order to suppress X-linked gene expression. In females, dosage compensation (i.e., upregulation of X-lined genes) is prevented by *msh-2* expression repression by the SXL protein (Marín *et al.*, 2000).

As stated above, in the moth *O. scapularis*, the sex of ZZ offspring produced by *Wolbachia*-infected mothers are determined as female, and thus, genes on the Z chromosome are overexpressed by the dosage compensation mechanism, which is considered to be the cause of ZZ-specific deaths (Fukui *et al.*, 2015; Sugimoto *et al.*, 2015).

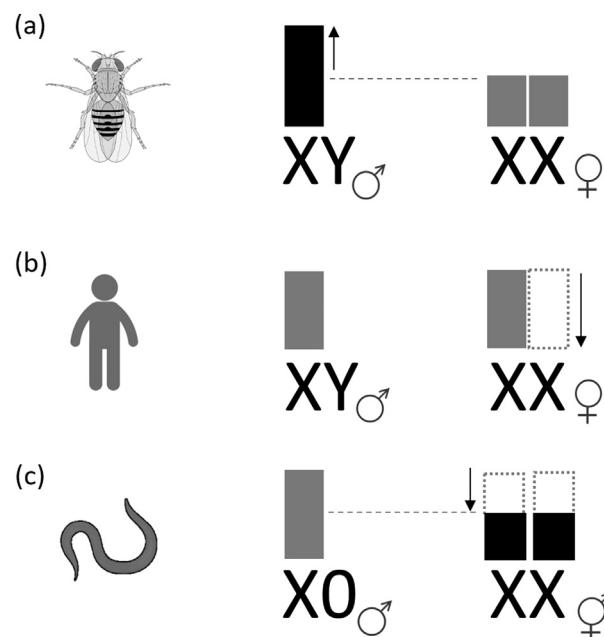


Fig. 5 Number of sex chromosomes (e.g., X chromosomes) differ between males and females. Dosage compensation occurs in order to equalize sex chromosomal gene dose between the sexes. (a) In *Drosophila*, X-linked gene expression is doubled only in males. (b) In mammals, X-linked gene expression derived from one of the parent is silenced (random X inactivation). (c) In nematodes (*Caenorhabditis elegans*), X-linked gene expression of hermaphrodites (XX) are halved as compared with that of XO males.

References

- Beye, M., Hasselmann, M., Fondrk, M. K., Page, R. E., & Omholt, S. W. (2003). The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell*, 114, 419–429.
- Burt, A., & Trivers, R. (2006). *Genes in Conflict: The Biology of Selfish Genetic Elements* (p. 632). Belknap Press.
- Fukui, T., Kawamoto, M., Shoji, K., et al. (2015). The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. *PLoS Pathogen*, 11, e1005048.
- Gempe, T., & Beye, M. (2011). Function and evolution of sex determination mechanisms, genes and pathways in insects. *Bioessays*, 33, 52–60.
- Goldschmidt, R. (1934). *Lymantria. Bibliographica Genetica*, 11, 1–185.
- Kageyama, D., Narita, S., & Watanabe, M. (2012). Insect sex determination manipulated by their endosymbionts: Incidences, mechanisms and implications. *Insects*, 3, 161–199.
- Kiuchi, T., Koga, H., Kawamoto, M., et al. (2014). A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature*, 509, 633–636.
- Laugé, G. (1985). Sex determination: Genetic and epigenetic factors. In G. A. Kerkut, & L. I. Gilbert (Eds.), *Embryogenesis and Reproduction: vol. 1. Comprehensive Insect Physiology, Biochemistry and Pharmacology* (pp. 295–318). Oxford: Pergamon Press.
- Marín, I., Siegal, M. L., & Baker, B. S. (2000). The evolution of dosage-compensation mechanisms. *Bioessays*, 22, 1106–1114.
- Narita, S., Pereira, R. A. S., Kjellberg, F., & Kageyama, D. (2010). Gynandromorphs and intersexes: Potential to understand the mechanism of sex determination in arthropods. *Terrestrial Arthropod Reviews*, 3, 63–96.
- Rabeling, C., & Kronauer, D. J. (2013). Thelytokous parthenogenesis in eusocial Hymenoptera. *Annual Review of Entomology*, 58, 273–292.
- Sánchez, L. (2008). Sex-determining mechanisms in insects. *International Journal of Developmental Biology*, 52, 837–856.
- Sugimoto, T. N., Kayukawa, T., Shinoda, T., Ishikawa, Y., & Tsuchida, T. (2015). Misdirection of dosage compensation underlies bidirectional sex-specific death in *Wolbachia*-infected *Ostrinia scapularis*. *Insect Biochemistry and Molecular Biology*, 66, 72–76.
- Suzuki, M. G., Imanishi, S., Dohmae, N., Asanuma, M., & Matsumoto, S. (2010). Identification of a male-specific RNA binding protein that regulates sex-specific splicing of *Bmdsx* by increasing RNA binding activity of BmPSI. *Molecular and Cellular Biology*, 30, 5776–5786.
- Suzuki, M. G., Imanishi, S., Dohmae, N., et al. (2008). Establishment of a novel in vivo sex-specific splicing assay system to identify a trans-acting factor that negatively regulates splicing of *Bombyx mori dsx* female exons. *Molecular and Cellular Biology*, 28, 333–343.
- Verhulst, E. C., Beukeboom, L. W., & van de Zande, L. (2010). Maternal control of haplodiploid sex determination in the wasp *Nasonia*. *Science*, 328, 620–623.
- Verhulst, E. C., & van de Zande, L. (2015). Double nexus – Doublesex is the connecting element in sex determination. *Briefings in Functional Genomics*, 14, 396–406.
- Yamamoto, D., & Koganezawa, M. (2013). Genes and circuits of courtship behaviour in *Drosophila* males. *Nature Reviews Neuroscience*, 14, 681–692.

GAMETE STRUCTURE

Gamete Structure: Egg, Comparative Vertebrate

Damien B Wilburn and Willie J Swanson, University of Washington, Seattle, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Sexual reproduction in vertebrates requires the fusion of egg and sperm to produce a viable embryo, yet the contributions of each gamete to the developing zygote are extraordinarily different. While sperm provide very little beyond genetic information to the developing organism, eggs (or ova) are highly specialized cells which possess a range of unique features that are essential for both sperm recognition and providing nutrients to the embryo. Between and within vertebrate classes, ova are highly variable in the size and composition of their subcellular compartments which are essential for the initiation (and often completion) of embryonic development. While most vertebrate somatic cells are $\sim 10\text{--}20\text{ }\mu\text{m}$, ova are much larger, often by orders of magnitude and even visible to the naked eye. The smallest eggs are found in the placental field vole (*Microtus agrestis*) at $\sim 50\text{ }\mu\text{m}$ in diameter, while the largest known ova are likely those of the frilled shark (*Chlamydoselachus auguineus*) which are $\sim 15\text{ cm}$ wide and $\sim 1.7\text{ L}$ in volume. Fish often have eggs with diameters on the order of $\sim 1\text{ mm}$, amphibians $\sim 1\text{--}10\text{ mm}$, birds and reptiles $\sim 10^1\text{--}10^2\text{ mm}$, and mammals the smallest with eggs of 10^{-1} mm . These sizes often reflect aspects of the animals' life histories, and more specific examples are included in Table 1 (Lombardi, 1998).

Table 1 Vertebrate egg sizes

Species	Approximate Diameter
<i>Agnatha</i>	
American brook lamprey (<i>Lethenteron appendix</i>)	1 mm
Atlantic hagfish (<i>Myxine glutinosa</i>)	20 mm
<i>Chondrichthyes</i>	
Whale shark (<i>Rhincodon typus</i>)	150 mm
Spadenose shark (<i>Scoliodon laticaudus</i>)	800 μm
<i>Osteichthyes</i>	
Zebrafish (<i>Danio rerio</i>)	800 μm
Threespined stickleback (<i>Gasterosteus aculeatus</i>)	1.5 mm
Western mosquitofish (<i>Gambusia affinis</i>)	1 mm
Shiner perch (<i>Cymatogaster aggregate</i>)	300 μm
<i>Amphibia</i>	
African clawed frog (<i>Xenopus laevis</i>)	1.2 mm
American bullfrog (<i>Rana catesbeiana</i>)	1.5 mm
Mexican axolotl (<i>Ambystoma mexicanum</i>)	2.3 mm
Eastern red-backed salamander (<i>Plethodon cinereus</i>)	3.5 mm
<i>Reptilia</i>	
Corn snake (<i>Pantherophis guttatus</i>)	30 mm
Carolina anole (<i>Anolis carolinensis</i>)	5 mm
Leatherback sea turtle (<i>Dermochelys coriacea</i>)	50 mm
<i>Aves</i>	
Chicken (<i>Gallus gallus</i>)	40 mm
Ostrich (<i>Struthio camelus</i>)	150 mm
Ruby-throated hummingbird (<i>Archilochus colubris</i>)	10 mm
Southern brown kiwi (<i>Apteryx australis</i>)	120 mm
<i>Mammalia</i>	
Human (<i>Homo sapiens</i>)	120 μm
House mouse (<i>Mus musculus</i>)	70 μm
Golden hamster (<i>Mesocricetus auratus</i>)	80 μm
Pig (<i>Sus scrofa domesticus</i>)	100 μm
Short-beaked echidna (<i>Tachyglossus aculeatus</i>)	4 mm
Platypus (<i>Ornithorhynchus anatinus</i>)	3 mm

Beyond the nucleus, the structure of vertebrate oocytes can be divided into three major layers: from inside to outside are the medulla, the cortex, and the accessory envelopes. Each of these regions can be further subdivided, and vary in size and molecular composition between species. Focusing initially on the medulla, it can also be referred to as the egg yolk, containing both the hyaloplasm (cytosol or the formative yolk) and the deutoplasm (nutritive yolk). The primary function of the deutoplasm is to provide additional nutrients for cell division and differentiation of the developing embryo, and contains granules that consist of vitamins, minerals, lipids, and proteins. In placental mammals – where developing offspring receive additional nutrients from the mother – the size of the deutoplasm is quite small (reflected in the decreased cell size of mammalian eggs), yet the opposite pattern is observed in birds and reptiles where gestation occurs entirely within the ovum. The distribution of the fatty, proteinaceous granules of the deutoplasm are also variable: ova with large quantities of yolk are termed *macrolecithal*, while those with little or no yolk are termed *microlecithal* and *alecithal*, respectively. Ova are further described by the distribution of the deutoplasm, such that those with highly polarized granules are termed *telolecithal* and those with central granules are termed *centrolecithal* (Lombardi, 1998). In non-eutherian vertebrates, the principal precursor of the lipoproteins found in deutoplasm is vitellogenin. Synthesized in the liver in response to elevated estrogen, multiple genes code for different isoforms of vitellogenin and vary by taxon: teleost fish have between 3 and 5 gene copies, African clawed frogs have 2 gene copies, and both chickens and platypuses have 3 gene copies (Babin, 2008). After hepatic secretion, vitellogenins circulate in the blood stream and are endocytosed by developing ova where they are proteolytically cleaved and lipidated. These modified vitellogenin products are packaged with phospholipids, neutral lipids, and small amounts of glycogen to produce the deutoplasm granules for nutritifying developing embryos (Kardong, 2006). In some fish, circulating plasma vitellogenin can serve additional functions, such as binding to bacteria and fungi to improve recognition and clearance by the immune system (Li *et al.*, 2008). The small eggs of eutherian (placental) mammals mostly lack a deutoplasm, as nutrition is primarily supplied by the mother during gestation. Coinciding with this transition from ovovivipary (egg-laying) to vivipary (internal gestation), the vitellogenin genes in eutherian mammals have been lost to pseudogenization, and instead the emergence of casein genes facilitates lactation (Brawand *et al.*, 2008).

Surrounding the medulla is the cortex, which contains the plasma membrane (oolemma) and the adjacent cytoplasm (cortical ooplasm). Compared to somatic plasma membranes, the oolemma contains multiple specialized proteins that confer unique features, such as rapid endocytosis, cell junctions with overlaying follicle cells, and recognition of sperm. In most vertebrate clades, binding of sperm causes a change in the ion content of the cortical ooplasm, altering the oolemma polarity and preventing multiple sperm from fusing with the oocyte (polyspermy) (Lombardi, 1998). While polyspermy is extraordinarily toxic to most oocytes, its notable that birds have no blocks to polyspermy, with the oocyte nucleus still fusing with only a single sperm nucleus (Ichikawa *et al.*, 2016). In fish, amphibians, mammals, and some reptiles, the cortical ooplasm contains large vesicles termed cortical granules that fuse with the oolemma upon fertilization, releasing proteins and mucopolysaccharides that likely serve in blocking polyspermy through unknown mechanisms (Guraya, 1989). The limited availability of material from cortical granules (picograms per oocyte) has impeded thorough characterization of its contents across diverse taxa, but several proteins have been identified in different mammalian species. For example, serine proteases and ovoperoxidase are released that result in remodeling of the zona pellucida (discussed in further detail below) that prevents recognition by sperm; calreticulin is a chaperone that also a lectin that may bind and block critical glycans involved in oocyte-recognition by sperm; similarly, N-acetylglucosaminidase may remove essential glycans from glycoproteins recognized by sperm on the oolemma or accessory envelopes (Liu, 2011). While few pairs of interacting egg and sperm proteins have been identified in any organism (Wilburn and Swanson, 2016), biochemical studies of laboratory mice have identified a likely common molecular basis of oolemma recognition in mammals. Folate receptor 4, renamed Juno after the Roman goddess of fertility marriage, is a GPI-anchored extracellular protein on the oolemma that binds to the sperm surface protein Izumo1, tethering the gametes to allow eventual plasma membrane fusion through unknown mechanisms (Fig. 1) (Bianchi *et al.*,

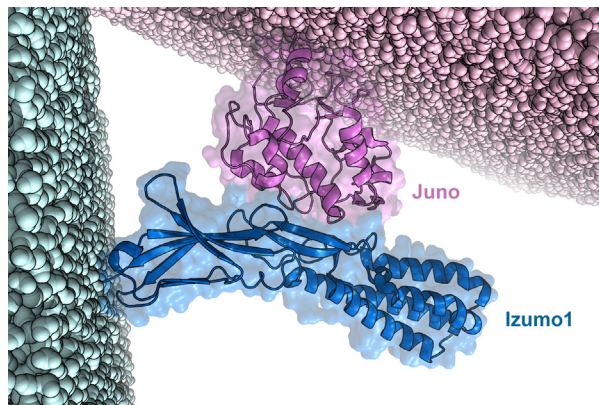


Fig. 1 Molecular basis of mammalian egg-sperm binding. The sperm surface protein Izumo1 (blue) binds the surface of the oolemma through the egg receptor Juno (pink). The interaction of Izumo1-Juno is species-specific, and following fertilization, Juno is shed from the oolemma by exocytosis to prevent polyspermy. The binding of Izumo1 and Juno only tethers mammalian gametes, and a second but currently unknown set of interactions induces fusion of egg and sperm.

2014; Aydin *et al.*, 2016). Izumo1 recognizes Juno in a species-specific manner, and may play a role in preventing hybridization. In mammals, following the change in oolemma polarity, vesicles containing Juno are exocytosed which prevents additional sperm from recognizing the oolemma (Bianchi *et al.*, 2014).

The outermost components of the oocyte are the accessory envelopes: barriers that surround the primary oocyte and can perform an array of functions in different taxa, including protection from pathogens, facilitate oxygen transfer, host beneficial microbes, promote sperm binding, and provide blocks to polyspermy (Shu *et al.*, 2015). Classically, accessory envelopes have been divided into three classes based on their site of origin: primary accessory envelopes are produced by the developing oocyte, secondary accessory envelopes are deposited by surrounding follicle cells, and tertiary accessory envelopes are derived from other parts of the female reproductive tract (Lombardi, 1998). However, as our biochemical understanding of the molecules that constitute accessory envelopes has become more sophisticated, these delineations break down as some envelopes contain molecules from multiple sources (Modig *et al.*, 2006). Between the accessory envelopes and oolemma is the fluid filled perivitelline space, which can widen as both the oocyte and surrounding follicle cells secrete material into it. At least one accessory envelope – the vitelline envelope (VE) – is common to all vertebrate oocytes, yet historically its name has varied between taxa. In fish, it is often referred to as the chorion; in amphibians, reptiles, and birds, it is the vitelline membrane; and in mammals, it is the zona pellucida. The glycoproteins that constitute the VE (described in further detail below) can be synthesized directly by the oocyte, the surrounding follicle cells, or by the liver and transported via the bloodstream, blurring its classification as a primary, secondary, or tertiary accessory envelope. The accessory envelopes that overlay the VE vary dramatically between different vertebrate classes (Fig. 2). In eutherian mammals, a group of follicle cells adhere to the VE and form the corona radiata, providing essential proteins to the developing ovum. In reptiles and birds, since embryogenesis occurs entirely inside the egg before hatching, the VE is surrounded by a thick layer of albumin which acts as a nutrient source during development. This albumin layer is then surrounded by a keratinous shell membrane. In reptiles, the shell may be composed of one or more separate keratin layers, and the outermost layer may also be impregnated with calcium carbonate. In birds, the albumin layer is significantly more heterogeneous and partially separated by chalazae: spirals of fibrous material that act as ligaments, holding the ovum in the center of the more fluid albumin layer. Additionally, the outer shells of bird eggs are always highly calcified. In amphibians, the VE is surrounded by up to eight concentric layers of neutral mucopolysaccharides and mucoproteins that often allow them to adhere to substrates. Given the diversity of habitats and life strategies for amphibians, these tertiary accessory coats often possess unique features: for terrestrially breeding species, the outer layers are highly permeable to water to provide a hydration reservoir for the egg; some species embed toxins to reduce risk of predation; and in some *Ambystoma* spp., the

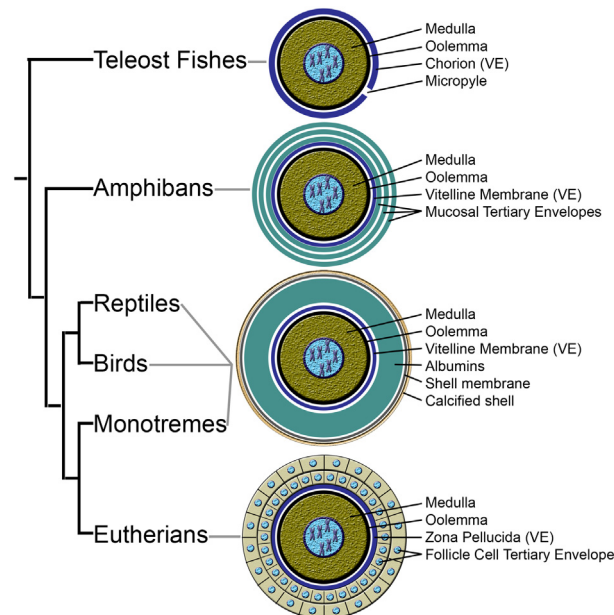


Fig. 2 Schematic of oocyte structure between vertebrate groups. All vertebrate oocytes share a common base structure of a nucleus with condensed nuclei, surrounded by nutrient rich cytoplasm (medulla) and plasma membrane (oolemma). Except for eutherian mammals, the medulla contains large quantities of vitellogenins that comprise the nutritive yolk. The vitelline envelope (VE) is a glycoprotein coat that encapsulates the oocyte, and goes by multiple names depending on the taxon. In teleost fishes, the VE contains a small hole (micropyle) which is the sole point of entry for sperm. Other groups have additional tertiary envelopes that surround the VE: in amphibians, these are concentric rings of mucoidal compounds that confer several properties; in reptiles, birds, and monotremes, there is a dense albumin layer that is a nutrient reservoir for the developing embryo contained with a keratinous shell membrane (and often a calcified shell); in mammals, follicle cells adhere to the VE and form a cellular tertiary envelope termed the corona radiata. Adapted from Lombardi, J., 1998. *Comparative Vertebrate Reproduction*, vol. 1, first ed., US: Springer.

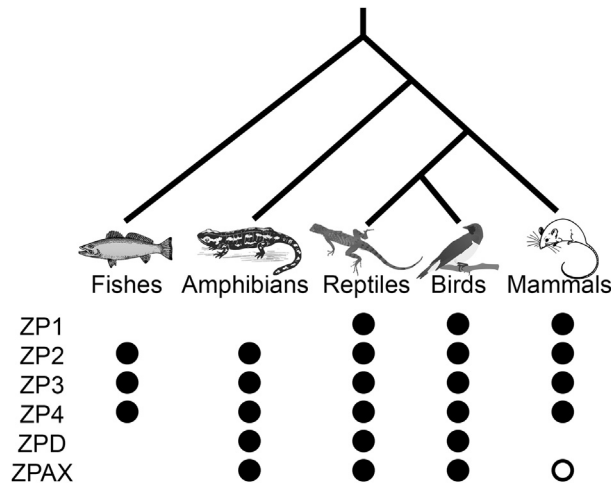


Fig. 3 Genetics of ZP proteins between vertebrate classes. Six major gene families of ZP proteins are found across vertebrate clades: ZP1, ZP2, ZP3, ZP4, ZPD, and ZPAX. Presence of genes and pseudogenes are denoted by filled and open circles, respectively. In many teleost fish species, ZP3 is highly duplicated and persists as multiple paralogs in each genome. In the branch leading to eutherian mammals, ZPD and ZPAX have both been lost, with ZPAX still being detectable as a pseudogene in many mammalian genomes. Critically, these data only reflect genomic presence and not protein abundance in vertebrate VEs (where there is limited data for most species).

accessory coats can host algae that increase available oxygen and remove nitrogenous wastes for the embryo. In contrast to the aforementioned groups, teleost fish lack additional accessory envelopes (Lombardi, 1998).

The VE is composed of multiple homologous glycoproteins often referred to as ZP proteins based on their original characterization in mammals. There are six major gene families that code for ZP proteins: ZP1, ZP2, ZP3, ZP4, ZPD, and ZPAX. Phylogenetically, ZP3 has been hypothesized to be the oldest ZP protein family (Claw and Swanson, 2012), but across different vertebrate clades, each of the six classes has exhibited varying degrees of gene duplication, pseudogenization, and gene loss (Fig. 3). For example, several fish species contain multiple paralogs of ZP3, yet it exists as a single copy in most tetrapods; similarly, while ZP4 is present in most mammalian species, it has been lost in rodents (Shu *et al.*, 2015). Despite many genomes containing multiple ZP protein coding genes, expression is unequal and the proteins account for different percentages of the VE proteome. In mammals, ZP2 and ZP3 are the major components, each comprising ~45% of the zona pellucida, with ZP1 and ZP4 only constituting ~5% each (in rodents, ZP1 is ~10%) (Moos *et al.*, 1995). In birds, the vitelline layer is principally composed of hepatic ZP1 and follicular ZP3, with minor amounts of ZP2, ZP4, and ZPD, and no ZPAX (Ichikawa *et al.*, 2016). Less is known about the composition of fish, amphibian, and reptilian VEs. A defining feature of ZP proteins is the presence of a ZP module: a structural motif consisting of adjacent ZP-N and ZP-C domains, two independent protein domains with predominantly β -sheet structures that can polymerize into filamentous aggregates and form the basic architecture of VE. While the ZP module was named based on its original identification in the mammalian VE, it is also found in other vertebrate proteins involved in kidney function, innate immunity, and cancer (Bokhove *et al.*, 2016). In biochemical experiments expressing each domain separately, ZP-C exists as a monomer while ZP-N homodimerizes, suggesting that it is the principal component that promotes VE polymerization (Monne *et al.*, 2008). ZP proteins are often elaborated with additional protein features, such as N- and O-linked glycosylation, additional ZP-N domains, or other protein domains (e.g., trefoil) (Fig. 4). Tandem repeats of ZP-N domains seem to be a common feature of ZP proteins, and appear to be gained and lost multiple times throughout evolutionary time, yet their precise functions are unknown (Callebaut *et al.*, 2007). At least in some marine invertebrates, such tandem arrays play a critical role in sperm recognition and possibly restricting polyspermy (Wilburn and Swanson, 2016).

While the specific molecules that mediate egg-sperm interactions are poorly characterized, the VE and other accessory envelopes play differing roles in mediating sperm binding and/or preventing polyspermy within each vertebrate group. For most taxa, the overarching cellular events have been characterized, with the precise molecular details yet to be determined. In teleost fishes where the VE is the only accessory envelope, the sole point of passage for sperm is a small hole in the VE surface termed the micropyle. It is hypothesized that sperm are attracted to the micropyle via chemoattraction, inevitably guiding them to the oolemma where fusion will occur. In amphibians, sperm penetrate the multilayered mucosal accessory envelopes through unknown mechanisms, yet in some species, this penetration is necessary to activate sperm for fusion with oolemma. In birds, sperm specifically bind the VE through glycans attached to the ZP proteins. As hepatically synthesized ZP1 is circulating in the female bloodstream, it is posttranslationally modified with ubiquitin: a common peptide tag which targets proteins for degradation. To penetrate the VE, proteases are secreted from the sperm acrosome that recognize this ubiquitin moiety, resulting in proteolysis that creates a hole through which sperm can pass. Bird oocytes have no blocks to polyspermy, and it is common for multiple sperm nuclei to coexist in the zygote. Through unknown mechanisms, the oocyte nucleus will select and fuse with a single sperm nucleus, the additional nuclei are degraded, and a normal diploid animal is produced. In mammals, to permeate the corona radiata, sperm release hyaluronidase from the acrosome to degrade glycosaminoglycans which bind the associated follicle cells to the VE. Next, sperm recognize the

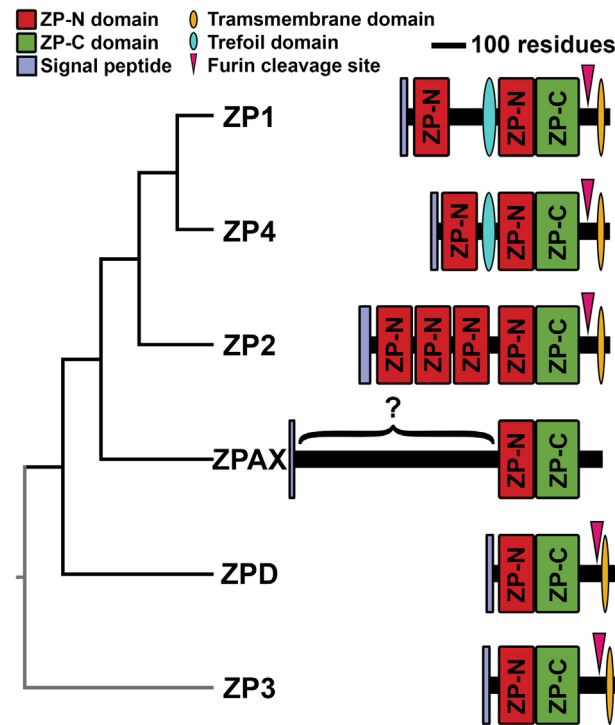


Fig. 4 Phylogeny and structure of ZP proteins. Across vertebrate evolution has emerged six major families of ZP proteins (ZP1, ZP2, ZP3, ZP4, ZPD, and ZPAX). Each ZP protein contains signal peptide which targets it to the extracellular matrix and a ZP module that consists of adjacent ZP-N and ZP-C structural domains. Beyond this conserved module, the additional ornamentalations in each protein vary, and include additional N-terminal ZP-N repeats and trefoil domains. Except for ZPAX, each ZP protein contains a C-terminal transmembrane domain that anchors it to the oolemma, and following proteolytic cleavage at the furin cleavage site, the ZP proteins are released into the VE. The large N-terminal region of ZPAX has not been characterized, and it is unknown if it contains additional domains. A tree denoting the phylogenetic relationship of the ZP proteins is included; while ZP3 is thought to be the ancestral gene, its exact position in the tree is unknown (denoted using a grey line). Adapted from Wilburn, D.B., Swanson, W.J., 2016. From molecules to mating: Rapid evolution and biochemical studies of reproductive proteins. *Journal of Proteomics* 135, 12–25 (schematics for ZP1, ZP2, ZP3, and ZP4 are based on the human homologs, with ZPD and ZPAX based on the homologs from *Xenopus tropicalis*).

VE in a species-specific manner via the first ZP-N domain of ZP2 (Avella *et al.*, 2014; Wilburn and Swanson, 2017). There is debate whether mammalian sperm penetrate the zona pellucida using proteases (like birds) or through non-enzymatic mechanisms (similar to marine invertebrates, (Lewis *et al.*, 1982)), but regardless of the mechanism, sperm create a hole in the VE that allows passage into the perivitelline space. Finally, fusion occurs after binding of Izumo1 and Juno at the gamete plasma membranes, completing fertilization. In addition to exocytosis of Juno, secretion of specific proteases and zinc ions from the oocyte causes hardening of the VE, preventing further sperm penetration and reducing polyspermy risk (Que *et al.*, 2017).

In summary, oocytes are a fascinatingly complex type of cell that vary dramatically between vertebrate clades: as part each organism's life history, a number of unique molecular and cytological phenotypes have emerged. The diameter of vertebrate oocytes spans four orders of magnitude, and common cellular features such as the VE are still extraordinarily variable at the genomic scale. The hepatic origin of both vitellogenins and some ZP proteins demonstrate complex inter-tissue dynamics, and the repeated expansion and loss of these genes over evolutionary time correspond to changes in life strategy. Perhaps the most essential and novel feature of ova is species-specific recognition and fusion with sperm: while birds uniquely have no blocks to polyspermy, distinct sets of accessory envelopes and surface proteins on the oolemma provide molecular barriers that permit precise regulation over fertilization rates. Despite more than 100 years of studying fertilization in diverse taxa, the molecular underpinnings of egg-sperm interactions remain to be elucidated, and represents a key research challenge in the future.

References

- Avella, M. A., Baibakov, B., & Dean, J. (2014). A single domain of the ZP2 zona pellucida protein mediates gamete recognition in mice and humans. *The Journal of Cell Biology*, 205(6), 801–809.
- Aydin, H., Sultana, A., Li, S., Thavalingam, A., & Lee, J. E. (2016). Molecular architecture of the human sperm IZUMO1 and egg JUNO fertilization complex. *Nature*, 534(7608), 562–565.
- Babin, P. J. (2008). Conservation of a vitellogenin gene cluster in oviparous vertebrates and identification of its traces in the platypus genome. *Gene*, 413(1–2), 76–82.
- Bianchi, E., Doe, B., Goulding, D., & Wright, G. J. (2014). Juno is the egg Izumo receptor and is essential for mammalian fertilization. *Nature*, 508(7497), 483–487.

- Bokhove, M., Nishimura, K., Brunati, M., et al. (2016). A structured interdomain linker directs self-polymerization of human uromodulin. *Proceedings of the National Academy of Sciences*, 113(6), 1552–1557.
- Brawand, D., Wahli, W., & Kaessmann, H. (2008). Loss of egg yolk genes in mammals and the origin of lactation and placentation. *PLOS Biology*, 6(3), e63.
- Callebaut, I., Mornon, J. P., & Monget, P. (2007). Isolated ZP-N domains constitute the N-terminal extensions of Zona Pellucida proteins. *Bioinformatics*, 23(15), 1871–1874.
- Claw, K. G., & Swanson, W. J. (2012). Evolution of the egg: New findings and challenges. *Annual Review of Genomics and Human Genetics*, 13, 109–125.
- Guraya, S. S. (1989). *Ovarian Follicles in Reptiles and Birds* (vol. 1). Berlin, Heidelberg: Springer-Verlag.
- Ichikawa, Y., Matsuzaki, M., Hiyama, G., Mizushima, S., & Sasanami, T. (2016). Sperm-egg interaction during fertilization in birds. *Journal of Poultry Science*, 53, 173–180.
- Kardong, K. V. (2006). *Vertebrates: Comparative Anatomy, Function, Evolution* (fourth ed.). McGraw-Hill.
- Lewis, C. A., Talbot, C. F., & Vacquier, V. D. (1982). A protein from abalone sperm dissolves the egg vitelline layer by a nonenzymatic mechanism. *Developmental Biology*, 92(1), 227–239.
- Liu, M. (2011). The biology and dynamics of mammalian cortical granules. *Reproductive Biology and Endocrinology: RB&E*, 9 [149-149].
- Li, Z., Zhang, S., & Liu, Q. (2008). Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. *PLOS ONE*, 3(4), e1940.
- Lombardi, J. (1998). *Comparative Vertebrate Reproduction* (first ed.) (first ed., vol. 1, first ed). US: Springer.
- Modig, C., Modesto, T., Canario, A., et al. (2006). Molecular characterization and expression pattern of zona pellucida proteins in gilthead seabream (*Sparus aurata*). *Biology of Reproduction*, 75(5), 717–725.
- Monne, M., Han, L., Schwend, T., Barendahl, S., & Jovine, L. (2008). Crystal structure of the ZP-N domain of ZP3 reveals the core fold of animal egg coats. *Nature*, 456(7222), 653–657.
- Moos, J., Faundes, D., Kopf, G. S., & Schurtz, R. M. (1995). Composition of the human zona pellucida and modifications following fertilization. *MHR: Basic Science of Reproductive Medicine*, 1(7), 319–323.
- Que, E. L., Duncan, F. E., Bayer, A. R., et al. (2017). Zinc sparks induce physiochemical changes in the egg zona pellucida that prevent polyspermy. *Integrative Biology*, 9, 135–144.
- Shu, L., Suter, M. J.-F., & Rasanen, K. (2015). Evolution of egg coats: Linking molecular biology and ecology. *Molecular Ecology*, 24, 4052–4073.
- Wilburn, D. B., & Swanson, W. J. (2016). From molecules to mating: Rapid evolution and biochemical studies of reproductive proteins. *Journal of Proteomics*, 135, 12–25.
- Wilburn, D. B., & Swanson, W. J. (2017). The ZP domain is not one, but likely two independent domains. *Molecular Reproduction and Development*, 84(4), 284–285.

Sperm: Comparative Vertebrate

Peter D Temple-Smith, Aravind Ravichandran, and Fabrizio E Horta Nunez, Monash University, Clayton, Australia

© 2018 Elsevier Inc. All rights reserved.

Introduction

The subphylum Vertebrata contains all the animals that develop a cartilaginous or bony vertebral column and a well-developed cranium (head) during embryogenesis. The group contains six groups: sharks and rays (Chondrichthyes), fishes (Osteichthyes), frogs and their relatives (Amphibia), birds (Aves) and the three orders of mammals – Prototheria/Monotremata (platypus and echidnas), Marsupials/Metatheria (the marsupials) and the Placentals (*Note: while the term placental mammals is used throughout the literature as a common name for the Eutheria, it is clear particularly since the 1970s that all marsupials also develop a functional placenta during their short period of gestation and are placental mammals.*)/Eutheria. The eutherians are the largest group of mammals containing all the species from mice to men.

The vertebrates display a broad range of reproductive characteristics that include external and internal fertilization, specialised egg (oocyte) structures, a wide variety of social structures and mating behaviours, pair bonding, mate guarding and sperm competition. These characteristics as they appear in various species and groups have influenced the evolution of sperm structure and function to ensure successful reproduction.

General Sperm Structure in Vertebrates

Throughout the animal kingdom, including the vertebrates, sperm structure is highly conserved and consists of a head containing the hereditary information in the form of DNA, arranged as chromosomes and neatly packaged into the nucleus, which is the main component of the head. In vertebrates, a neck or connecting piece usually attaches the head to the sperm tail or flagellum (**Fig. 1**). The sperm tail is usually subdivided into a midpiece and a principal piece and has the capacity to produce chemical energy in the form of adenosine triphosphate (ATP), from the power generators of the cell – the mitochondria. The mitochondria, which vary greatly in size, shape and number between species are usually associated with the proximal part of the tail, adjacent to the neck, to form the midpiece. The principal piece, which is usually the longest part of the spermatozoon, uses the energy from the ATP to provide the motive force through a structure called the axoneme or axial filament. The axoneme usually extends from the neck to the end of the tail and in most spermatozoa has a distinctive 9+2 microtubule structure which is also common to other

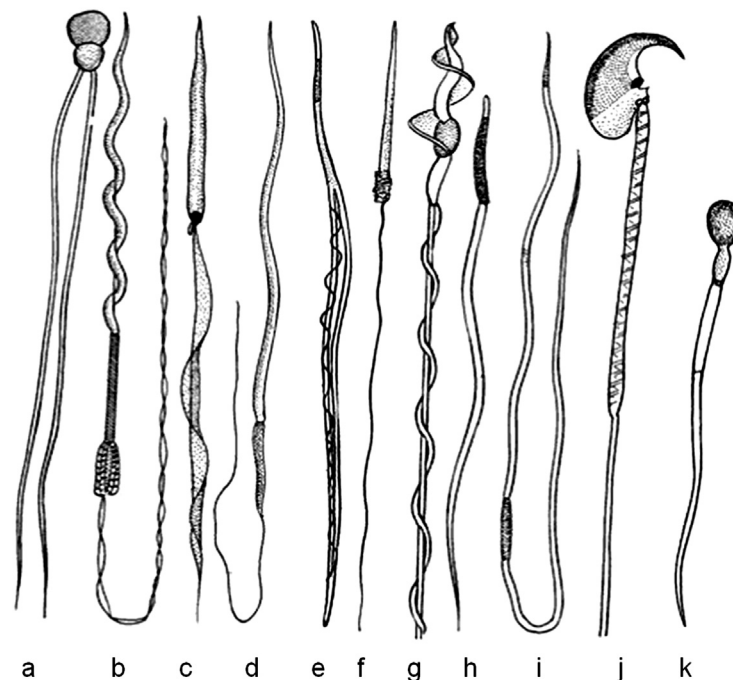


Fig. 1 Showing variations in sperm structure across different vertebrates. (a,b): Toad Fish, Elasmobranch (Fish); (c-e) Toad, Frog, Salamander (Amphibians) (f) Lizard; (g,h) Frigilla, Domestic fowl (Birds); (i) Monotreme (Echidna); (j,k): Mouse, Man (Eutheria) (Chordate Zoology by P.S.Verma).

motile organelles in the body, for example cilia. The critical function of the axoneme in all motile organelles and cells such as spermatozoa is to cleave a high energy phosphate molecule from the ATP and convert the chemical energy released from the ATP into mechanical energy that moves the tail and creates the forward motility that is required to reach and fertilise the oocyte.

Sperm Structure in Relation to Functions of Vertebrate Spermatozoa

In the previous section sperm structure in vertebrates has been described as highly conserved but there is also a close relationship between the evolution of structural and functional specialisations of vertebrate spermatozoa and the need to ensure successful fertilization of the oocyte (Bedford, 2004). Irrespective of whether spermatozoa are released externally in a cloud over a mass of released oocytes or ejaculated directly into the female and then required to swim through the female reproductive tract by movements of their tails to meet and fertilise the ovulated egg, sperm motility is needed to ensure that the spermatozoon makes contact with the oocyte during fertilization. In general, with external fertilization the main role of the tail is to move the spermatozoon to the surface of the oocyte. In species that practise internal fertilization the tail is required to move the spermatozoa from the site of deposition, usually in a cloaca, urogenital sinus or vagina but in some species directly into the uterus or part of the oviduct which receives the oocyte(s) at ovulation. This distance can vary from a few millimetres or centimetres in small species like small sharks, fish, reptiles, birds and mammals to a metre or more in some large species like elephants and whales. For these relatively long distances, forward progressive sperm motility is an absolute requirement, although smooth muscle contractions have also been shown to assist in sperm transport through the female tract. The most critical element in the process of fertilization is the successful penetration of the spermatozoa through the vestments around the oocyte, such as layers of follicle cells and a jelly coat or zona pellucida, to reach the surface of the oocyte and then fuse with the plasma membrane that encloses the oocyte. Fusion of sperm and egg membranes is a prerequisite for fertilization and for entry of the sperm nucleus into the cytoplasm of the oocyte. Usually for the spermatozoon, enzymes released from the acrosome, in addition to sperm motility, are required to assist the fertilizing spermatozoon to reach the oocyte and incorporate its nucleus into the oocyte cytoplasm. The acrosome covers the tip of the sperm head, and in some vertebrates, especially in the Class Mammalia, it may cover up to 2/3 rds of the anterior region of the head. The acrosome is a sac of enzymes that mostly breakdown proteins. When spermatozoa approach the oocyte the acrosome undergoes an acrosome reaction to release its contents which assists the spermatozoon, aided by the motility created by energy produced by the mitochondria and the motive force of the axial filament in the tail, to reach and enter the oocyte at fertilization.

Comparative Features of Vertebrate Spermatozoa in Relation to Their Phylogeny

Chondrichthyes

Chondrichthyes is a class that contains sharks, skates, rays and chimeras. They are jawed vertebrates, with skeletons made of cartilage instead of bone. The class can be divided into two subclasses; Elasmobranchii (sharks, rays, skates, and sawfish) and Holocephali (chimaeras). In terms of the spermatozoa present in this class, chondrichthyes appear as a simple and a homogeneous group. The chondrichthyan spermatozoa structure is generally similar to other vertebrates and consists of a head containing the nucleus and acrosome, a midpiece containing mitochondria and a tail showing an axoneme with microtubular arrangement in a typical 9+2 or 9+0 pattern (Fig. 2(a)).

The sperm head in Chondrichthyes is long (>30 µm) and helical in shape (Fig. 1(b)), with a moderately elongated conical acrosome present apically. The spermatozoa of some Chondrichthyes with helical head shape present different intranuclear fibres that join together during spermatogenesis. It has been suggested that these structures finally form the fibrillar nuclear sheets (Jamieson, 2001). The long midpiece of elasmobranch sperm consists of an axial rod around which the mitochondria are arranged (Fig. 1(b); Mattei, 1988). The axial rod is a structure present in some amphibians and unique to the class Chondrichthyes within fish. The axial rod forms nine coarse fibres at the centre of the sperm midpiece (Jamieson, 1991). The flagellum (or tail) is comprised of two key structures, the central axoneme and the longitudinal columns. In order to produce the sperm motility, the central axoneme rotates along the length of the flagellum. In contrast, the longitudinal columns remain fixed at doublet positions 3 and 8 (Fig. 2(b)). These characteristics allow the creation of a double helix structure (Jamieson, 1991).

Possible differences between the Elasmobranchii and Holocephali has been difficult to characterize. However, the Holocephali show a reduced longitudinal column at the position 8, a longer midpiece and absence of the proximal centriole compared to elasmobranchs (Fig. 2(c)). In spite of these findings, further studies are warranted to describe these possible differences with certainty. Interestingly, there are no spermatozoa features that separate sharks from rays. Nevertheless, it has been established that in terms of sperm metrics there is a species-specific variation among all Chondrichthyes (Jamieson, 1991).

Osteichthyes

Osteichthyes, commonly referred to as the bony fish, is the largest class of vertebrates in the animal kingdom. They are a very diverse group which includes 45 orders with more than 28,000 species currently identified (Betancur-R *et al.*, 2013). The group Osteichthyes is characterised by fish species that have skeletons primarily composed of bone and is divided into the ray-finned fish (Actinopterygii) and lobe-finned fish (Sarcopterygii).

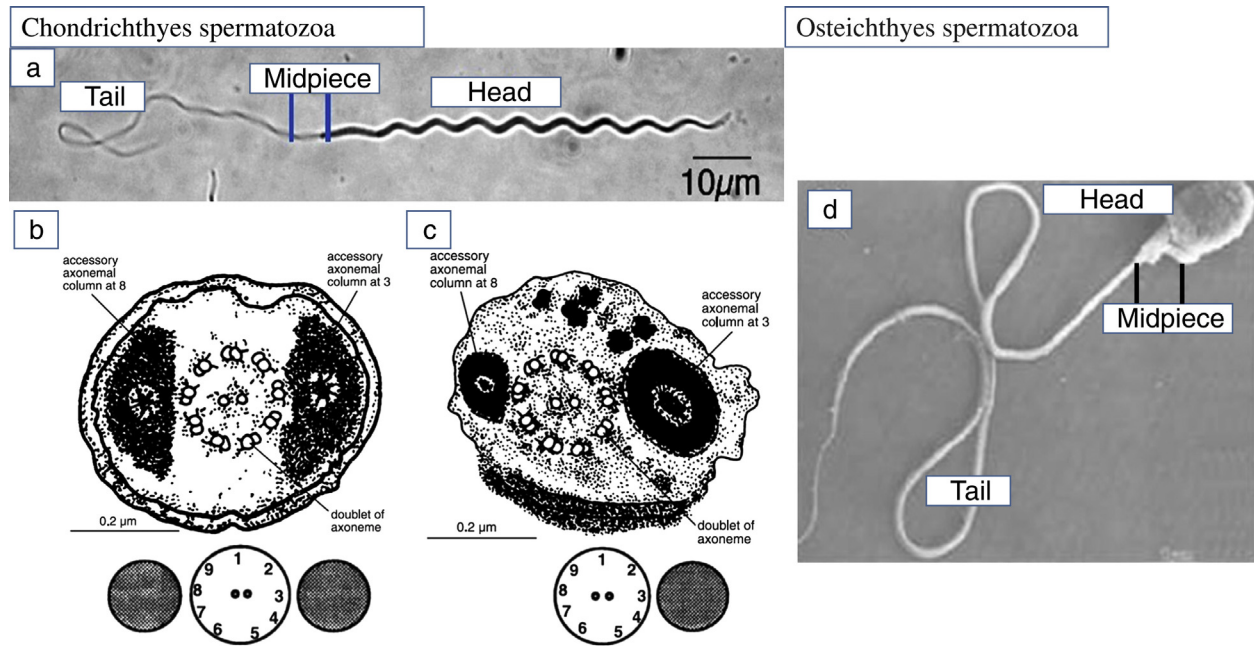


Fig. 2 Chondrichthyes, Osteichthyes and Amphibian spermatozoa. (a) Example of Chondrichthyes spermatozoa indicating head of helical shape, midpiece and tail. (b) Elasmobranchii spermatozoa flagellum in transversal sections, showing longitudinal columns in position 3 and 8. (c) Holocephali flagellum possibilities in transversal sections, showing a reduced longitudinal column at position 8 or absence of it. (d) Example of Osteichthyes spermatozoa. Reproduced from (a) Jamieson, B.G., 1991. *Fish Evolution and Systematics: Evidence from Spermatozoa: With a Survey of Lophophore, Echinoderm and Protochordate Sperm and an Account of Gamete Cryopreservation*, Cambridge: Cambridge University Press. xiv + 319pp. doi:10.1046/j.1420-9101.1992.5040721.x. (b) Jameison, B.G., 2005. In: Hamlett, W. (Ed.), *Reproductive Biology and Phylogeny of Chondrichthyes*. Science Publishers, pp. 201–236 (Chapter 7).

The structure of the spermatozoa, as in most vertebrates, consists of a head, midpiece and tail (**Fig. 2(d)**). The spermatid nucleus is elongated. The midpiece is very short; and contains two centrioles, mitochondria, and a dense body. However, specific features include the flagellar apparatus that contains an axoneme, with a 9+0 or 9+2 microtubular pattern and a flagellar membrane that presents two lateral extensions situated in line with the central tubules of the axoneme. These form two sidefins which are particularly developed in *Neoceratodus* and other fishes such as Actinopterygians. The periaxonemal cytoplasm is of average density and the tips of the sidefins in *Neoceratodus* contain an opaque substance (Mattei, 1988). The adaptive significance of the lateral fins and undulating membranes is unclear but presumed to enhance swimming capacity (Pitnick *et al.*, 2009). Recent studies exploring the significance of fins in spermatozoa have shown that the swimming capacities of these spermatozoa are enhanced by hydrodynamic characteristics (Gillies *et al.*, 2013).

Other examples include Chondrosteans, that possess similar characteristics but their membrane forms two short sidefins, and the Teleosts, that show a broad diversity of structure in their spermatozoa. Although approximately 95% of these fishes are external fertilizers, specific adaptations in sperm size are seen in those practising internal fertilization. Teleosts spermatozoa, which fertilise internally, are characterised by an elongated midpiece. Teleosts also differ from other vertebrates by lacking an acrosome. This adaptation reflects a mode of fertilization in which spermatozoa do not penetrate the zona pellucida, because they enter the egg through the micropyle, which is a small opening in the egg. A teleost example is the African butterfly fish (*Pantodon buchholzi*) which has an unusually long midpiece and reproduces by internal fertilization. Furthermore, sperm size is widely held to evolve as a consequence of postcopulatory sexual selection (Pitnick *et al.*, 2009). As most of fishes are external fertilizers, high levels of sperm competition can be present particularly in some promiscuous species to achieve fertilization and successful reproduction.

Amphibia

Amphibians are a special class of vertebrates that have demonstrated a variety of evolutionary tendencies in their reproductive biology compared to their aquatic counterparts, the fishes. The class includes three orders: Anura (comprising frogs and toads); Urodela (Salamanders) and Gymnophiona (Caecilians). The anurans predominantly practise external fertilization with a few exceptions like tailed frog (*Ascaphus truei*); the urodeles and caecilians embrace internal fertilization. This difference between the orders has specific implications in the evolution of their spermatozoa, with acquisition of unique morphological characteristics to optimise the reproductive process.

Unlike the primitive aquatic species which possess a basic sperm structure like a rounded head, small midpiece and simple, thin flagellum, the spermatozoa of amphibians, in general, are more filiform, showing an elongated head, tapering acrosome and

a perforatorium. The midpiece and tail are well developed and show specific morphological adaptations such as a rigid 'axial rod' that forms the main axis of the tail displacing the axoneme laterally and an undulating plasma membrane called 'lamellipodium' that traverses the length of the axoneme generating propulsive forces that bring about sperm motility.

The three orders differ in certain sperm characteristics that may be explained by the type of fertilization practised. In anurans that reproduce by external fertilization, where sperm motility is less significant, the spermatozoon is generally short (~40 µm). In primitive anurans, belonging to families Ascaphidae and Discoglossidae, the head possesses a conical acrosome, nucleus with poorly compacted chromatin, an ill-defined perforatorium and endonuclear canal (Fig. 3(a)). The axial rod is short and surrounded by mitochondria. The insertion of the tail is postnuclear in most of the primitive members except *Bombina orientalis* where it is prenuclear (Lee and Kwon, 1996) (Fig. 3(b)). In contrast, higher anurans in the Suborder Neobatrachia, show a structural evolution characterised by the disappearance of the perforatorium, endonuclear canal and axial rod. The urodeles are much closer to primitive anurans than the advanced group, but with certain atypical features. The spermatozoon is longer, measuring ~290 µm and the head consists of a clover-shaped acrosome, a prominent acrosomal barb and sub-acrosomal rod, a nuclear ridge, highly compacted chromatin, a discrete endonuclear canal and perforatorium (Fig. 3(c); Lee and Kwon, 1996). The axial rod is longer and stiffer with abundant mitochondria encircling the axial rod in most families, except Hynobiidae and Cryptobranchidae where it is perinuclear (Fig. 3(c)). These structures suggest a phylogenetic adaptation to support sperm kinetics that assists internal fertilization in urodeles. The caecilians resemble urodeles in structure, with spermatozoa measuring 75–105 µm in length. The acrosomal vesicle shows three distinct zones and a base plate. The flagellum consists of a 9+2 axoneme and an axial rod enclosed by a plasma membrane. Anteriorly, both structures lie close together in the midpiece but when traced posteriorly along the tail, they diverge and the leaves of the undulating membrane become separated by an increasing layer of abundant cytoplasm (Scheltinga *et al.*, 2003).

Reptilia

Reptiles are a spectacular class of ectothermic vertebrates that show impressive variations in physical and behavioural traits among its different members. Their particular physiological and morphological adaptations are important in understanding the evolution of reproduction in mammals. Class Reptilia includes four conspicuous orders namely, Chelonia (turtles and tortoises), Rhynchocephalia (tuatara), Squamata (lizards and snakes) and Crocodylia (crocodiles and alligators). In reptiles, internal fertilization is universal. Reptilian reproduction is characterised by multiple male mating (polyandry) that facilitates sperm selection and also decoupled insemination and fertilization mandating long term sperm storage.

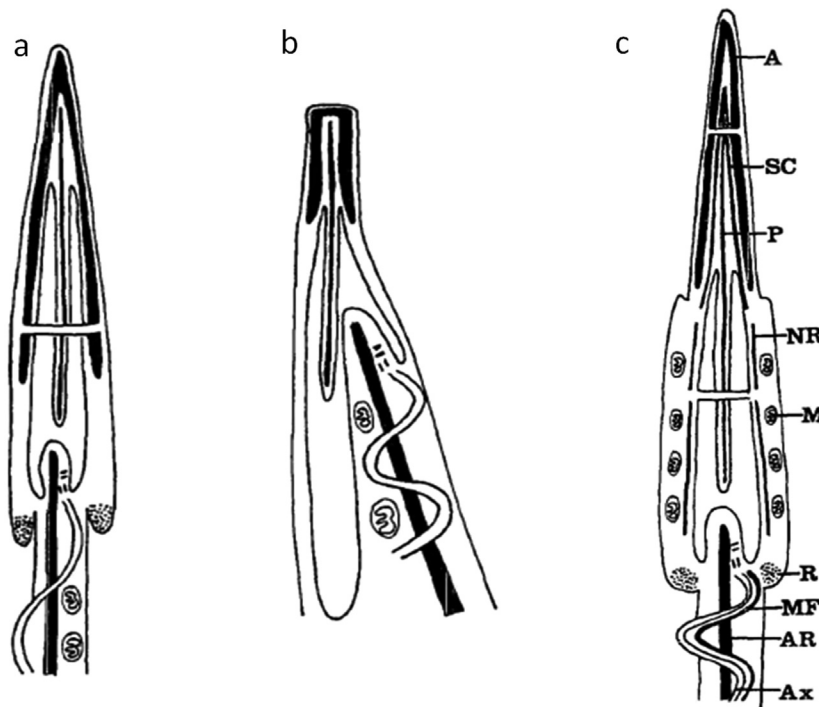


Fig. 3 Variations in sperm ultrastructure of Anurans (a & b) and Urodeles (c). (a) *Discoglossus pictus* (Anura) showing filiform head, inconspicuous neck and a principal piece showing post-nuclear axonemal insertion. (b) *Bombina orientalis* (Anura) with pre-nuclear insertion of axoneme and periaxonemal mitochondria. (c) *Hynobius leechii* (Urodele) showing a clover-shaped head, axial rod (AR) and perinuclear mitochondria (M). Adapted from Lee, Y.H., Kwon, A.S., 1996. Ultrastructure of spermatozoa in Urodela and Primitive Anura (Amphibia) with Phylogenetic considerations. *Anim. Syst. Evol. Divers.* 12(3), 253–264.

The reptilian spermatozoon is filiform and curved. It possesses a head, neck, midpiece with abundant mitochondria and a principal piece containing a 9+2 axoneme surrounded by heavily electron-dense fibrous sheaths resembling higher order mammals and variable in length among the four orders. The undulating membrane seen in amphibians is conspicuously absent, probably implying limited success of nature's experiment with amphibian spermatozoa. The chelonian spermatozoon is 50–55 μm long. The head consists of a perforatorial cap, cephalad to a compact nucleus containing deeply penetrating intranuclear tubes (Fig. 4). The distal centriole is peculiar with central microtubules running its entire length caudally and surrounded by 9 peripheral triplets. Laminated mitochondria are characteristic and are considered an adaptation to support sperm viability during protracted storage (Hess *et al.*, 1991). Storage even as long as three years has been noted. Sperm motility is also highest at low temperatures, perhaps pointing towards influence of copulatory milieu.

Sperm head from a tuatara shows a pointed acrosome, helical nucleus with tip constriction and paired endonuclear canals (Healy and Jamieson, 1994). A dense body lateral to the centriole is notable and shared by snakes and crocodiles. Most of the epididymal spermatozoa show huge cytoplasmic droplets suggesting a protracted maturation phase. Lizards and snakes produce spermatozoa that have a prominent neck cylinder separating the nucleus from midpiece. In lizards, the head shows a singular, para-crystalline perforatorium arising from the apex of the sub-acrosomal cone and an electrolucent zone around the nucleus (Scheltinga *et al.*, 2001). In snakes, mitochondria are abundant (around 14) and are concentrically arranged with an irregular distribution of unique intermitochondrial dense plaques (Hamilton and Fawcett, 1968). Long-term sperm storage is well-established in snakes, especially when egg production and mating seasons are separated. Crocodilians are similar to chelonians in basic morphology but certain features like an inconspicuous neck and presence of both linear and concentric mitochondrial cristae differentiate them from squamates. This mitochondrial specialisation possibly indicates progressive loss of concentric design with evolution.

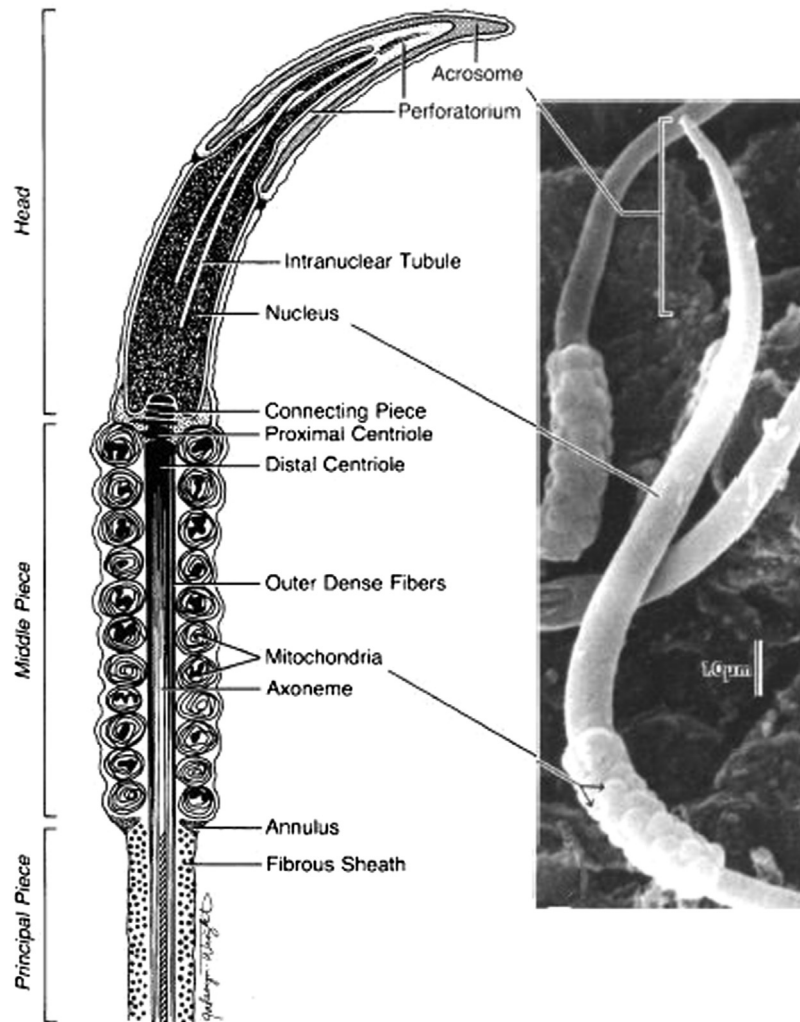


Fig. 4 Ultrastructure of spermatozoon in Turtle (*Chrysemys picta*), showing the various structural components. Reproduced from Hess, R.A., Thurston, R.J., Gist, D.H., 1991. Ultrastructure of the turtle spermatozoon. *Anat. Rec.* 229 (4), 473–481.

Aves

Birds (Aves) are homeothermic vertebrates that share features inherent to reptiles and mammals. Aves are divided into two Superorders (Palaeognathae and Neognathae) based on the palatal bone framework. Palaeognathae includes the 'flightless' birds (ratites) grouped under four orders: Apterygiformes (Kiwis), Casuariiformes (Emu, Cassowary), Rheiformes (Rheas) and Struthioniformes (Ostriches) and the 'flying' tinamous included under Order Tinamiformes. Superorder Neognathae includes 24 different orders representing all other birds like penguins, fowl, crows, parrots, pigeons and passerines. Reproductive biology in birds is unconventional in various aspects. Testes in birds are intra-abdominal and spermatogenesis occurs even at high temperatures compared with other vertebrates. Extragenital storage is less prominent and spermatozoa are usually released from the testes at ejaculation. Most avian males lack a phallus, with transfer of spermatozoa at insemination by the 'cloacal kiss'. Once ejaculated, the spermatozoa are stored in sperm-storage tubules (SST) of the female for up to two weeks prior to fertilization. In birds, unlike mammals, physiological polyspermy is common and does not impact egg survival.

Sperm morphology shows diversity among the various avian members (Fig. 5). Ratites share certain features with reptiles including a cylindrical sperm head with a pointed acrosome, perforatorium, an elongated nucleus with deep endonuclear canal, a prominent midpiece with abundant mitochondria encircling a long distal centriole and nine dense fibres in the principal piece. Unlike reptiles, the sub-acrosomal cone is absent in birds and the mitochondria have linear cristae instead of a concentric pattern (Jamieson, 2007). The phylogenetic significance of such features remains elusive. A glycogen sheath surrounding the principal piece is seen exclusively in tinamou. Most of the differences in sperm morphology have been postulated by classifying birds into non-passerines and passerines based on the anatomy of the toes that support perching. Non-passerine birds include ratites and certain members of Neognathae while all birds belonging to Order Passeriformes under Neognathae are labelled passerines (song birds). Non-passerine spermatozoa are filiform and closely resemble reptiles (Fig. 5(a)). They possess a perforatorium, two centrioles (proximal and distal), a short midpiece with prominent annulus and a flagellum producing a lashing movement. Passerines, in contrast, have a spirally twisted head resembling a 'corkscrew' with a shorter nucleus (Fig. 5(b)). They lack the perforatorium,

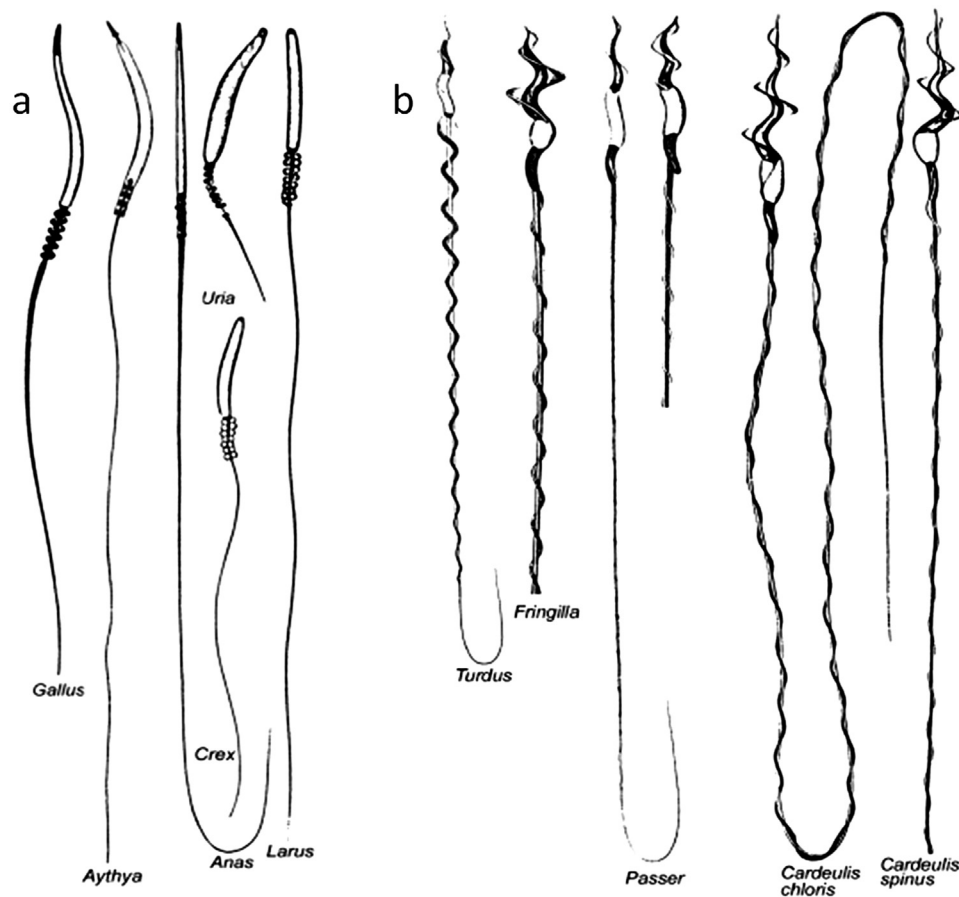


Fig. 5 Non-passerine (a) and Passerine (b) Spermatozoa. (a) Non-passerines: Filiform head resembling reptiles - Rooster (*Gallus gallus*); Tufted duck (*Aythya fuligula*); Domestic duck (*Anas platyrhynchos*); Common guillemot (*Uria aalge*); Corncrake (*Crex crex*); Lesser black-backed gull (*Larus fuscus*). (b) Passerines: Helical (Corkscrew-shaped) head - Song thrush (*Turdus philomelos*); Chaffinch (*Fringilla coelebs*); House sparrow (*Passer domesticus*); Greenfinch (*Carduelis chloris*); Siskin (*Carduelis spinus*). Reproduced from Jamieson, B.G.M., 2007. Reproductive Biology and Phylogeny of Birds, Science Publishers.

proximal centriole and annulus. The mitochondria are interestingly fused into a single helical structure surrounding the axoneme and referred to as the mitochondrial helix. This helicity is likely to contribute to the typical 'spin' witnessed as a part of the forward movement in passerine spermatozoa. The outer, dense fibrous sheath in the principal piece is a more prominent and uniform structure than that described in non-passerines and mammals respectively.

Mammalia

Each of the three infraclasses of mammals (Prototheria, Metatheria and Eutheria) in Class Mammalia have evolved sperm structures that are characteristic of, and specific to each group (Fig. 6). Within the marsupials and eutherians there are genera and families that have evolved new and special structural features that distinguish these groups.

Prototheria

The living order Monotremata in the infraclass Prototheria – platypus (*Ornithorhynchus anatinus*) and up to four species of echidnas (*Tachyglossus aculeatus* and *Zaglossus* spp.) – were derived from therapsid reptiles and are the earliest branching of the mammalian lineage. Monotremes diverged from the other two groups of mammals about 166 million years ago (Mya; Warren *et al.*, 2008) and display their reptilian/sauropsid ancestry in various characters including oviparity and sperm structure. Platypus and echidna spermatozoa are very similar to typical reptilia and avian spermatozoa (Fig. 6(a); Carrick and Hughes, 1982). They are characterised by an elongated, helical and filiform sperm head, consisting of 3–5 turns, about 40 µm in length in the platypus and 50 µm in echidnas and with a small rostrally-positioned acrosome, 5–7 µm in length, which occupies less than a sixth of the length of the sperm head. A characteristic reptile-like feature of monotreme spermatozoa is the circumferential and spiral pattern of condensation of the nuclear contents of spermatozoa that develops in the testis. The sperm tail inserts into the caudal end of the sperm head via a small neck and is divided by a thin fibrous annulus into a midpiece about 5 µm in length, containing the mitochondria, and a principal piece approximately 60 µm long (Fig. 6(a)). A standard 9+2 axoneme extends along the length

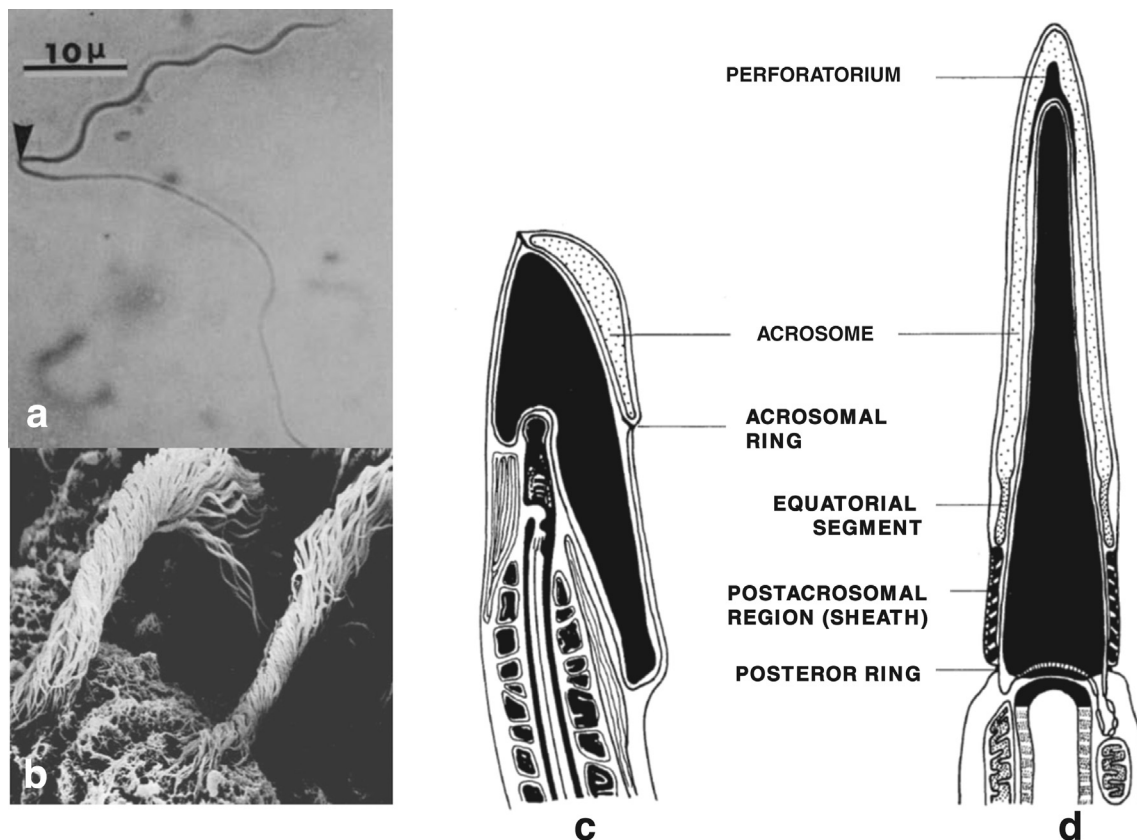


Fig. 6 (a) Monotreme spermatozoon (platypus) showing spiral nucleus, junction between the head and tail (arrow) and narrow flagellum. (b) Monotreme sperm bundles in the epididymis (c) & (d). Comparison of the structural specialisations of a generalised marsupial (c) and eutherian sperm head (d). Reproduced from (a) Carrick, F.N., Hughes, R.L., 1982. Aspects of the structure and development of monotreme spermatozoa and their relevance to the evolution of mammalian sperm morphology. *Cell Tissue Res.* 222, 127–141. (b) Gruzner, F., Nixon, B., Jones, R.C., 2008. Reproductive biology in egg-laying mammals. *Sex Dev.* 2, 115–127. (c & d) Temple-Smith, P.D., Comparative structure and function of marsupial spermatozoa. *Reprod. Fertil. Dev.* 6, 421–435.

of the flagellum and 9 very small hemispherical dense fibres are arranged around the axoneme for most of its length; one dense fibre is associated with each outer pair of microtubules in the axoneme. The principal piece features a cortical fibrous sheath in reptilian-like spirals beneath the sperm plasma membrane and lacking the elongated lateral columns that are found in the fibrous sheath in the principal piece of most marsupial and eutherian mammals. These structures are reputed to be present to stiffen the flagellum to improve the wave-pattern of flagellar movements and sperm motility. In all monotremes, the spermatozoa are released singly from the testis but during passage through the epididymis, aggregate into large bundles of often up to 100 spermatozoa. This, with the gradual caudal movement and loss of the residual cytoplasmic droplet, the acquisition of forward progressive motility and development of the fertilizing capacity, provides evidence of the complex role of the mammalian epididymis in post-testicular maturation of spermatozoa. Monotreme spermatozoa are released as sperm bundles at ejaculation (Fig. 6(b)) and are thought to only separate again into individual spermatozoa when they reach the site of fertilization in the oviduct (Gruzner *et al.*, 2008).

Metatheria

The marsupial branch of mammals in the Metatheria, have been separated from the eutherians for about 148 Mya. There are more than 250 living species distributed throughout the Americas and Australasia and characterised by birth, after a short period of gestation, of very early stage neonates that have long periods of development attached to a teat in a pouch or in the mammary area of some pouchless female marsupials. Marsupial spermatozoa vary in length depending on the family with the Australian honey possum and the Dasyuridae (small carnivorous marsupials) producing the largest spermatozoa in excess of 250 μm in length. Spermatozoa from the diminutive honey possum (*Tarsipes spenceri*) are recognised as the longest of any mammal, at about 360 μm . Spermatozoa of marsupials are unique in many aspects of their structure compared to monotremes and eutherians (Temple-Smith, 1994). In all species, with the exception of the American shrew opossums (Caenolestidae) and koala and the three closely related wombat species (Phascolarctidae), the flagellum connects via a long, straight or curved (Didelphidae) articulated connecting piece to a central indentation or implantation fossa in the sperm head (Figs. 6(c) and 7(a)). This articulation enables the sperm head to rotate on the flagellum which occurs as part of the process of sperm maturation during passage through the epididymis and in reverse during capacitation in the female reproductive tract prior to fertilization. Each marsupial family has a characteristic sperm

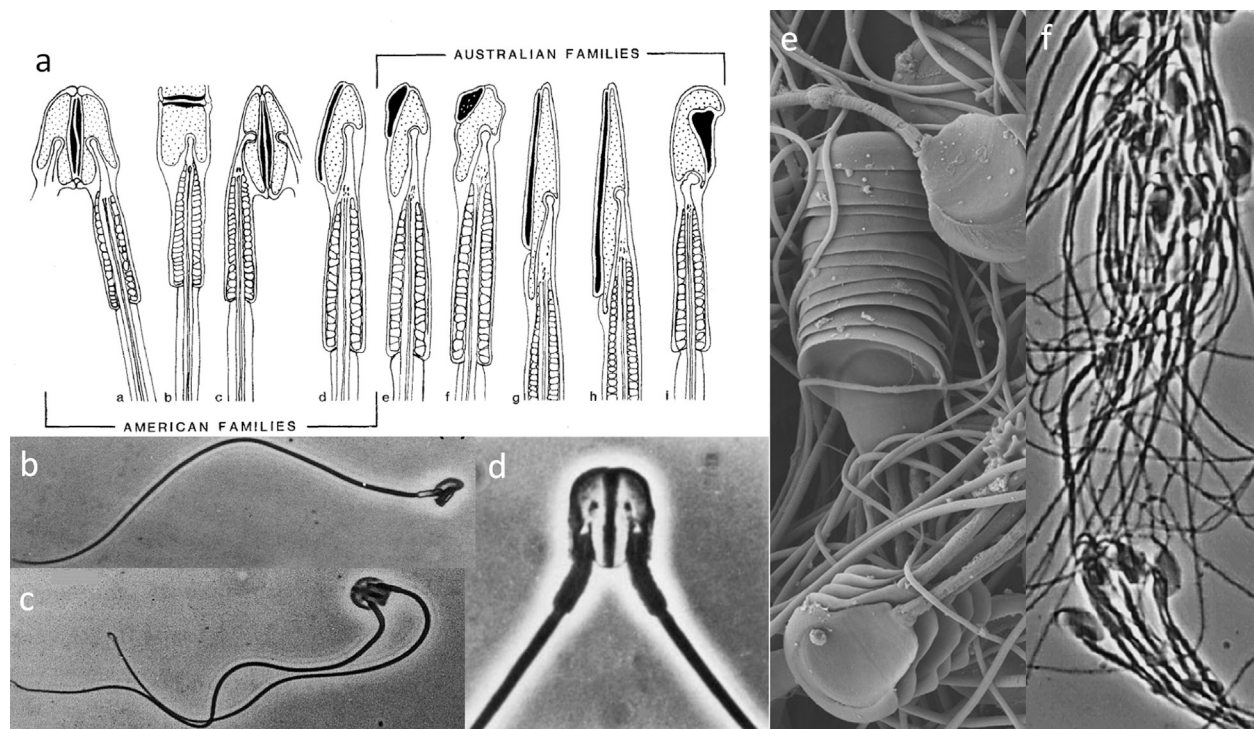


Fig. 7 (a) Comparison of the structural features of the head and midpiece of mature Australian and American marsupial spermatozoa. [Note that, except for the microbiotherid *Dromiciops glironia*, sperm pairing is restricted to the American marsupials and that each family has a distinct and specific nuclear shape. (a) Didelphidae (b) Caenolestidae (c) Caluromyidae (d) Microbiotheridae (e) Phalangeridae, Petauridae, Burramyidae, Macropodidae (f) Peramelidae (g) Dasyuridae (h) Tarsipedidae (i) Vombatidae.] (b) Unpaired marsupial spermatozoon. (c) Paired marsupial spermatozoa. (d) Paired marsupial spermatozoa showing the precise alignment of the sperm heads and tails, (e) guinea pig spermatozoa stacked in Rouleaux formation, and (f) common wood mouse sperm train showing the association of sperm head hooks with the sperm flagellum. (a) Temple-Smith, P.D., 1994. Comparative structure and function of Marsupial Spermatozoa. *Reprod. Fertil. Dev.* 6, 421–435. (b), (c) Moore, T., 2002. *Tree* 17, 112–118. (d) Temple-Smith, P.D., Bedford, J.M., 1980. Sperm maturation and the formation of sperm pairs in the epididymis of the opossum, *Didelphis virginiana*. *J. Exp. Zool.* 214, 161–171. (e) Leibniz Institute for Zoo and Wildlife Research (IZW). (f) Moore *et al.*, 2002. *Nature* 418, 174–177.

head shape (Fig. 7(a)) which is generally wedge-shaped and symmetrical with ventral gutter in all Australian marsupials, except the Phascolarctidae, and asymmetrical in all American marsupials with the exception of the microbiotherid *Dromiciops gliroides* – the Chilean monito del monte – which is more similar to sperm structure in many of the Australian marsupial families (Fig. 7(a)). Sperm head length varies from about 2 μm in the cylindrical, bullet shaped caenolestid spermatozoa to 14 μm in the honey possum and the acrosome varies in size and shape from a small button-shaped structure on the anterior aspect of the dorsal surface of the sperm head to a thin layer covering the entire dorsal surface. The koala and wombats show their close evolutionary relationship in having an unusual hook-shaped spermatozoa and a bent connecting piece with a distal insertion to the sperm head (Fig. 7(a) and (i)). They also have the most pleomorphic nuclear structure and shape of any known mammal except perhaps for human spermatozoa; the reason for this variability in sperm head shape is still not clear. The tail extends from the distal end of the connecting piece and is subdivided into midpiece, with 8 to in excess of 15 spirals (gyre) of densely packaged mitochondria depending on species, and principal piece by a very distinct annulus. In most species that have been examined except the Phascolarctidae there is a network of helical sub-membrane fibres that usually encircle the midpiece and extend to the annulus; their function is unknown but they may provide a stabilizing structure for the midpiece. As in some other vertebrate groups, there is a central axial filament consisting of 9+2 arrangement of microtubules and a set of nine outer dense fibres arranged in association with the 9 outer doublet microtubule pairs. The principal piece is the longest part of the tail and is characterised by a fibrous sheath that encloses the axial filament and its outer dense fibres. Unlike non-therian spermatozoa, marsupial and eutherian spermatozoa incorporate two of the dense fibres into the fibrous sheath to form two structural columns for most of the length of the principal piece. The fibrous sheath consists of a series of ribs that connect to the longitudinal columns and are thought to stabilize the sperm tail and direct the forward swimming movements of the spermatozoa. In cross-section the tail is essentially circular and symmetrical in most marsupial species but in those families with longer sperm tails, the cross-sectional shape is ovoid or flattened, a structural adaptation that may assist the unusual serpentine motility observed with extreme sperm tail lengths. One of the most intriguing aspects of marsupial spermatozoa is the structural and functional changes during passage through the epididymis conferring maturational changes in the spermatozoa which are characteristic of only mammalian vertebrates. These include extreme hardening of the dense fibres and fibrous sheath to improve motility, acquisition of the capacity for motility and fertilizing ability, rotational changes in the sperm head on the tail from a T-shape to a streamlined alignment with the tail (Temple-Smith and Bedford, 1976) and the intriguing precise head-to-head formation of sperm pairs in all the American, but none of the Australian, species except *Dromiciops gliroides* that resembles the Australian marsupial fauna and is thought to be phylogenetically the closest American species to Australian marsupials ((Fig. 7(a); Temple-Smith, 1994). The rotational changes of the head and sperm pair formation (Figure b, c and d) in the American marsupials are critical for effective progressive motility in the female tract. Studies show that the American marsupial sperm pairs separate in the oviduct prior to contact with the oocyte.

Eutheria

Of the three major living mammal groups the eutherians are the most abundant in species and diversity with more than 4000 species worldwide from tiny ~40 mm bumblebee bats weighing about 2 g to the more than 40 tons, 30 m long blue whale. Of the living families, the Rodentia is the most diverse consisting of more than 1500 species, and the least diverse is the Tubulidentata containing a single species, the aardvark; the primates contains the highest order of mammals including *Homo sapiens*, the most numerous species on earth. Species in the infraclass Eutheria have a broad range of sperm structures that reflect the functional needs of reproduction, especially sperm motility and fertilizing ability. As with each of the other two groups of mammals the general structure of eutherian spermatozoa is highly conserved but large variations in size, shape and structural specialisations have been reported which often demonstrate close evolutionary links between species, genera and families. The hooked sperm head in many murid rodent spermatozoa is an excellent example of an evolutionary relationship in sperm structure (Figs. 1(j) and 7(e)).

The eutherian spermatozoon structure follows the basic pattern described in the monotremes and marsupials, and also in most of the other vertebrate groups. It consists of the traditional sperm head with nucleus and acrosome, a connecting piece, and a tail divided into midpiece, principal piece and end piece. However some general structural differences set the eutherian spermatozoa apart from the spermatozoa of all other vertebrates including the other two extant mammalian infraclasses. In most eutherians the acrosome covers approximately the anterior 2/3 rds of the sperm head as a cap-like structure. It has obvious specialisations including a distinct equatorial segment, which forms a stable cuff around the middle of the spermatozoa during and after the acrosome reaction, and a postacrosomal region which fuses with the oocyte at fertilization (Fig. 6(d)). Acrosomal enzymes have often been suggested as critical for penetration of the zona pellucida surrounding the oocyte. However, the resilience of the eutherian zona to enzymatic digest suggests that this may not be as critical as originally proposed. More recently it has been suggested that the generally flattened shape of eutherian spermatozoa, the scythe-like perforatorium along the rostrum of the sperm head adjacent to the nucleus (Fig. 6(d)), and the hyperactive motility of the capacitated fertilizing spermatozoon all function to cut a slit-like path through the eutherian zona pellucida for the spermatozoon to gain access to the oocyte (Bedford, 2004). In eutherians, a complex maturation of spermatozoa occurs as they transit the epididymis. This includes the hardening of the sperm head, the outer shell of the mitochondria and the dense fibres and fibrous sheath in the sperm tail by disulphide crosslinking of the proteins in these structures. These specialisations are considered to have evolved to assist with sperm penetration through the eutherian zona pellucida, which is much thicker (6–16 μm , depending on species) than in any other vertebrate group including the marsupials (2–4 μm). This process allows the fertilizing spermatozoon to enter the perivitelline space inside the zona and make contact with the surface of the oocyte. The stable equatorial region of the sperm head is responsible for binding the spermatozoon to the membrane of the oocyte and activating the oocyte as a prelude to successful fertilization in eutherian mammals. The sperm membrane over the

postacrosomal region fuses with the oocyte membrane and release the contents of the nucleus into the cytoplasm of the oocyte to complete the process of fertilization. The dimensions and position of the acrosome is species-specific with some shrews having extremely large acrosomes compared to other eutherians (Bedford, 2004).

Spermatozoa in eutherians in general, display an inverse allometric relationship of sperm length to body size with the longest spermatozoa in rodents and the shortest in some sheep species, the collared peccary and hippopotamus; the Chinese hamster spermatozoa are currently regarded as the longest in the Eutheria with a total length of ~250 µm and midpiece and principal piece lengths of ~100 µm and ~140 µm respectively (Cummins and Woodall, 1985). In general those species with long spermatozoa tend to produce fewer spermatozoa whereas those with small spermatozoa produce large numbers of spermatozoa in their ejaculates. As in the monotremes and marsupials there are some examples of aggregations of spermatozoa in eutherians. In the guinea pig, the spermatozoa group together to form a rouleaux (Fig. 7(e)) during passage through the epididymis and in rodents it is common for them to associate into large aggregates by the hook-shaped head linking to the tail of adjacent spermatozoa after ejaculation (Fig. 7(f)). Like the sperm bundles in the monotremes, the aggregation of rodent spermatozoa appears to improve motility for more efficient passage through the female tract.

Commentary on Evolutionary Specialisations in Vertebrate Spermatozoa

Among vertebrates, polyandry has been observed as an interesting strategy to optimise reproductive success. By subjecting themselves to copulation attempts by multiple males, females have expanded the scope for selection of ideal male gametes for their offspring and at the same time established a competition among spermatozoa from different males to fertilise the ova (Birkhead and Møller, 1998). This has driven the evolution of structural and functional specialisations relating to sperm size, swimming speed, sperm production and energetics among the different classes of vertebrates (Parker and Pizzari, 2010). Evidence from many comparative studies have mostly supported the concept that longer spermatozoa swim faster. Three basic principles explaining this perception include: larger midpieces with abundant mitochondria powering flagellar kinetics; longer tails generating distinct modes of propulsion and an increased tail: head ratio offsetting the head drag that affects sperm velocity. Several morphological observations reported among the different vertebrates provides impetus to such principles. These observations include: helical head in elasmobranchs and holocephali; presence of side fins in sperm tails allowing enhanced kinetics in bony fish; an undulating membrane in amphibians; a larger mid-piece, well-developed long fibrous sheath and linear mitochondrial cristae in reptiles; helical head and mitochondrial helix in birds and various sperm bioenergetic pathways, sperm aggregation, a more complex maturation in the epididymis, and tail modifications that affect the motility in mammals, as seen in some marsupials. Nonetheless, the evidence that sperm competition is the main driving force is far from convincing because other factors like mode of fertilization, chemical characteristics of the seminal fluid, milieu of the female reproductive tract and sperm storage requirements also have potential implications on spermatozoal adaptations.

Conclusions

In this article we have tried to convey both the conservative structure of vertebrate spermatozoa and the amazing diversity of structural specialisation and functional requirements that exists within and between the various orders, infraclasses and classes to ensure successful transport of the sperm nucleus to its final rendezvous with the oocyte at fertilization. The improvements in light microscopy in the early to mid-1900s, followed by the evolution of scanning and transmission electron microscopy in the mid- to late 1900s have provided a detailed analysis of sperm structure and interpretation of the selective processes that have refined their structures, on which this article has been based. More recently the newer sciences of genomics, proteomics and metabolomics, for example, are expanding our knowledge of sperm structure and function and will shape our understanding in the future. This emphasises the need for further comparative research in identifying mechanisms that underlie the structural and functional trade-off reported in vertebrate spermatozoa.

References

- Bedford, J. M. (2004). Enigmas of mammalian gamete form and function. *Biol. Rev.*, 79, 429–460.
- Betancur-R, R., Broughton, R. E., Wiley, E. O., et al. (2013). The tree of life and a new classification of bony fishes. *PLOS Curr*, 5. <https://doi.org/10.1371/currents.tol.53ba26640df0ccae75bb165c8c26288>.
- Birkhead, T. R., & Møller, A. P. (1998). *Sperm Competition and Sexual Selection* (p. 783). New York, NY: Academic Press.
- Carrick, F. N., & Hughes, R. L. (1982). Aspects of the structure and development of monotreme spermatozoa and their relevance to the evolution of mammalian sperm morphology. *Cell Tissue Res.*, 222, 127–141.
- Cummins, J. M., & Woodall, P. F. (1985). On mammalian sperm dimensions. *J. Reprod. Fertil.*, 75(1), 153–175.
- Gillies, E. A., Bondarenko, V., Cosson, J., & Pacey, A. A. (2013). Fins improve the swimming performance of fish sperm: A hydrodynamic analysis of the Siberian sturgeon *Acipenser baerii*. *Cytoskeleton*, 70(2), 85–100.
- Gruzner, F., Nixon, B., & Jones, R. C. (2008). Reproductive biology in egg-laying mammals. *Sex Dev.*, 2, 115–127.
- Hamilton, D. W., & Fawcett, D. W. (1968). Unusual features of the neck and middle-piece of snake spermatozoa. *J. Ultrastruct. Res.*, 23(1), 81–97.
- Healy, J., & Jamieson, B. (1994). The ultrastructure of spermatogenesis and epididymal spermatozoa of the Tuatara *Sphenodon punctatus* (Sphenodontida, Amniota). *Philos. Trans. R. Soc. Lond. B: Biol. Sci.*, 344(1308), 187–199.

- Hess, R. A., Thurston, R. J., & Gist, D. H. (1991). Ultrastructure of the turtle spermatozoon. *Anat. Rec.*, 229(4), 473–481.
- Jamieson, B. G. (1991). *Fish Evolution and Systematics: Evidence from Spermatozoa: With a Survey of Lophophorate, Echinoderm and Protochordate Sperm and an Account of Gamete Cryopreservation* (p. 319). Cambridge: Cambridge University Press (Xiv).
- Jamieson, B. G. M. (2007). *Reproductive Biology and Phylogeny of Birds*. Science Publishers.
- Lee, Y. H., & Kwon, A. S. (1996). Ultrastructure of Spermatozoa in Urodela and Primitive Anura (Amphibia) with Phylogenetic considerations. *Anim. Syst., Evol. Divers.*, 12(3), 253–264.
- Mattei, X. (1988). The flagellar apparatus of spermatozoa in fish. Ultrastructure and evolution. *Biol. Cell*, 63(2), 151–158.
- Parker, G. A., & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biol. Rev. Camb. Philos. Soc.*, 85(4), 897–934.
- Pitnick, S., Hosken, D. J., & Birkhead, T. R. (2009). Sperm morphological diversity. *Sperm Biol.: Evolut. Perspect.*, 3, 69–149.
- Scheltinga, D. M., Jamieson, B. G., Espinoza, R. E., & Orrell, K. S. (2001). Descriptions of the mature spermatozoa of the lizards *Crotaphytus bicinctores*, *Gambelia wislizenii* (Crotaphytidae), and *Anolis carolinensis* (Polychrotidae) (Reptilia, Squamata, Iguania). *J. Morphol.*, 247(2), 160–171.
- Scheltinga, D. M., Wilkinson, M., Jamieson, B. G., & Oommen, O. V. (2003). Ultrastructure of the mature spermatozoa of caecilians (Amphibia: Gymnophiona). *J. Morphol.*, 258(2), 179–192.
- Temple-Smith, P. D. (1994). Comparative structure and function of marsupial spermatozoa. *Reprod. Fertil. Dev.*, 6, 421–435.
- Temple-Smith, P. D., & Bedford, J. M. (1976). The features of sperm maturation in the epididymis of a marsupial, the brushtailed possum *Trichosurus vulpecula*. *Am. J. Anat.*, 147, 471–500.
- Warren, W. C., Hillier, L. W., Marshall Graves, J. A., et al. (2008). Genome analysis of the platypus reveals unique signatures of evolution. *Nature*, 453(7192), 175–183.

Egg, Comparative Invertebrate

Amy L Moran and Kanoe Morishige, University of Hawai'i at Mānoa, Honolulu, HI, United States

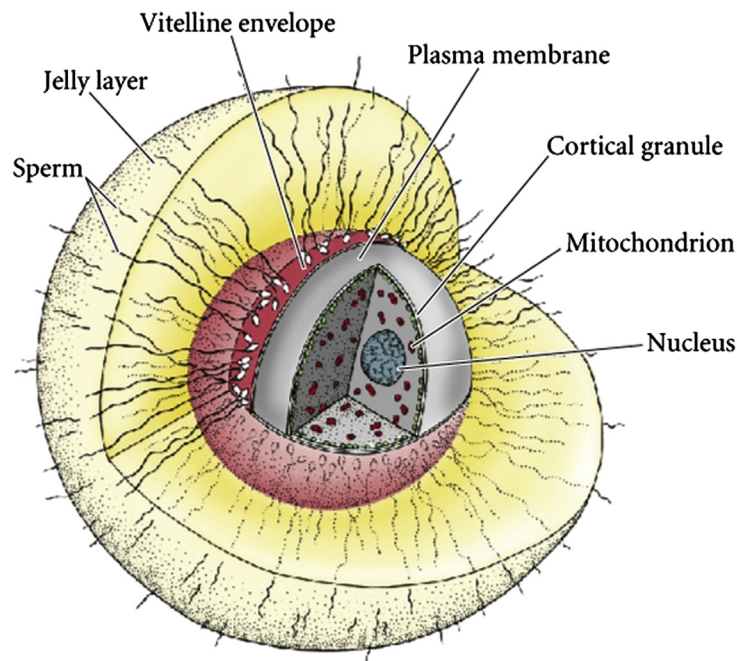
© 2018 Elsevier Inc. All rights reserved.

Introduction

With the exception of purely asexual animals, all metazoans pass through a single-celled stage called an 'egg' that is fertilized at the initiation of embryonic development. The diversity of egg structures among invertebrate metazoans is enormous – not surprisingly, since invertebrates comprise all non-chordate animals and more than 30 phyla, many of which diverged >500 million years ago – and detailed knowledge of egg structure, function, and ecology is known for a comparatively small number of taxa. Thus, a comprehensive review of invertebrate egg structure is impossible, and our goal is to summarize larger patterns in egg structure among invertebrates, while discussing how these are exemplified in different phyla. Along with the variation associated with the vast taxonomic breadth of invertebrates, describing egg structure in invertebrates is also complicated by the variety of objects that are called 'egg' both in scientific and common usage across the animal kingdom. Strictly speaking, it is perhaps most correct to reserve the word 'egg' for a haploid, female gamete; however, the word is also frequently applied to oocytes that are meiotically arrested until fertilization, and it is also very common to apply it to protective structures that contain developing embryos. We will discuss both types of eggs here, starting with the egg cell or ovum.

Structure of the Egg Cell

To describe the basic structure of a metazoan ovum we will turn to one of the most-studied examples, the sea urchin (phylum Echinodermata, class Echinoidea) egg, which is described by Gilbert (2000) (Fig. 1). DNA in the egg is contained within a nucleus, and the cytoplasm contains organelles (ribosomes, mitochondria, nucleus, etc.), mRNA, tRNA, and other morphogenetic factors which determine polarity and regulate development. The cytoplasm also contains energy reserves for the developing embryo in the form of glycogen, protein, and lipid (often collectively referred to as 'yolk'). The cytoplasm is enclosed by an egg membrane containing cortical granules and a vitelline envelope that lifts off the egg when the cortical granules discharge at fertilization. The vitelline envelope is surrounded by a jelly coat which, in sea urchins, may contain pigment granules or other inclusions (Fig. 2). This basic structure holds across invertebrate phyla although there are many variations (described in Gilbert and Raunio (1997)). In the egg cells of one sponge species, for example, long radiating collagen fibers extend through the vitelline envelope and are retracted into the egg after fertilization; eggs of most species of chitons (phylum Mollusca, class Polyplacophora) are covered with small



© 2000 Sinauer Associates, Inc.

Fig. 1 Structure of the sea urchin egg during fertilization. The drawing also shows the relative sizes of egg and sperm. Reprinted from Gilbert, S.F., 2000. *Developmental Biology*. Sutherland, MA: Sinauer Associates.

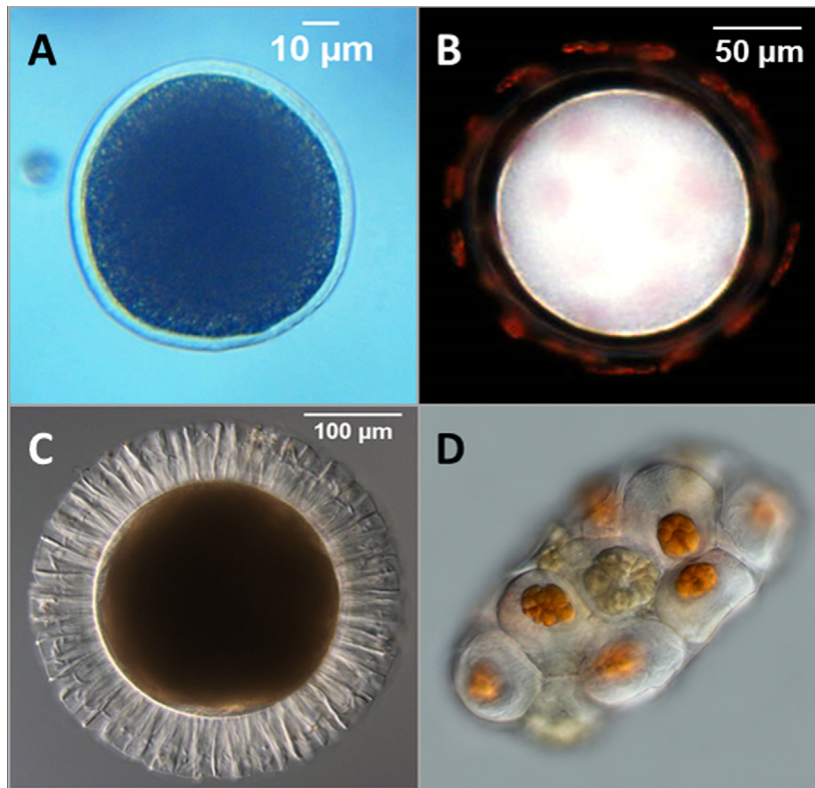


Fig. 2 Diverse egg forms of invertebrate taxa. (A) Sea urchin egg (Echinodermata, Echinoidea, *Colobocentrotus atratus*) showing surrounding vitelline layer (photo by K. Morishige) (Courtesy of Kanoe Morishige, University of Hawai'i at Mānoa). (B) Sea urchin egg (*Mellita quinquiesperforata*) showing jelly coat with pigmented granular inclusions (photo by A. Moran) (Courtesy of Amy Moran, University of Hawai'i at Mānoa). (C) Chiton (Mollusca, Polyplacophora) egg showing a covering of cupules (photo courtesy of J. Valley, University of Oregon) (Reproduced from Invertebrate Embryology Available at: <http://invert-embryo.blogspot.com/2012/06/chiton-development.html>). (D) Copepod (Arthropoda, Crustacea) egg mass (photo by R. Martin-Ledo; courtesy of R. Martin-Ledo; Reproduced from Marine Plankton. Available at: <https://marineplankton.net/2017/03/23/eggsac-of-a-copepod/>).

projections called cupules; and the eggs of many crustaceans, as well as many terrestrial invertebrates, are surrounded by a protective hull called a chorion or shell (Fig. 2).

Egg cells are produced through the process of oogenesis, during which cells develop and are provisioned in the adult body. Oogenesis varies considerably among invertebrates, as does the structure and composition of the resulting egg cells. During oogenesis egg cells are provisioned with some combination of yolk granules or yolk platelets (through the process of vitellogenesis), and this yolk fuels the early stages of development. Many invertebrates have nurse cells which directly transfer cytoplasm to the developing egg, and in some cases nurse cells are phagocytosed directly and their remnants can be seen inside mature eggs. Species can be categorized by their characteristic amount and location of yolk within the egg. Isolecithal eggs have a small amount of yolk that is more-or-less evenly spread throughout the cell; in telolecithal eggs, the yolk is found predominantly at one pole; and in centrolecithal eggs, yolk is concentrated in the center. The location and amount of yolk in an egg is associated with both cleavage patterns and the length and type of development in invertebrates, a topic we will cover in more detail below. The location of yolk is also related to egg polarity, a phenomenon in which the animal and vegetal poles are determined prior to fertilization. While not all taxa show pre-fertilization polarity (for example members of the phylum Nematoda), many do, including molluscs, annelids, echinoderms, nemertean, and arthropods. In these groups the vegetal pole, or the side of the egg which cleaves more slowly, generally contains more yolk and is appears to be determined by the region of the egg surface which provides the physical connection to the ovarian wall.

Along with yolk, egg cells also contain the information necessary for development. This information comes in many forms including DNA, maternally-derived mRNAs, tRNA, and other morphogenetic factors. When present these morphogenetic determinants specify egg polarity and many aspects of cell fate. The egg can also contain transcripts of its own genome, particularly those genes associated with meiosis and fertilization. In taxa that depend on endosymbiotic relationships with microorganisms for obtaining energy, such as corals (Cnidaria, Anthozoa) or deep sea hydrothermal vent clams (Mollusca, Bivalvia), endosymbionts can be packaged into the egg and thus passed on to offspring, a process known as vertical transmission. Finally, egg cells can also contain specific maternally-supplied factors that protect the developing embryo from environmental stress, such as heat shock proteins and UV protectant proteins.

Types of Eggs

Primary Oocytes, Secondary Oocytes, Ova

Sea urchins are unusual among invertebrates (and animals in general) in that they complete meiosis in the ovary prior to fertilization and spawning; this is the exception rather than the rule. Members of most other phyla have been shown to arrest in metaphase I or II as primary or secondary oocytes (Fig. 3). In many taxa, the sperm enters the egg when it is an oocyte rather than a haploid ovum; sperm entry then triggers the completion of meiosis and maturation to an ovum, followed by fusion of the egg and sperm pronucleus. Release from meiotic arrest can also be a two-part process with a hormonal or external signal causing release from prophase I, which permits germinal vesicle breakdown, progression to metaphase I, and fertilization. The mechanisms underlying this variation and its evolutionary significance are not well understood; we include this information to demonstrate that the word egg (when referencing a single cell) is applied not just to haploid female gametes, but to female gametes at a wide variety of stages of maturation.

Embryonated Eggs

Besides egg cells and their associated coverings, the second type of structure that is called an egg in invertebrate biology is an embryo that is protected during development by a surrounding, maternally-produced structure or casing (Figs. 2(d) and 4). Examples

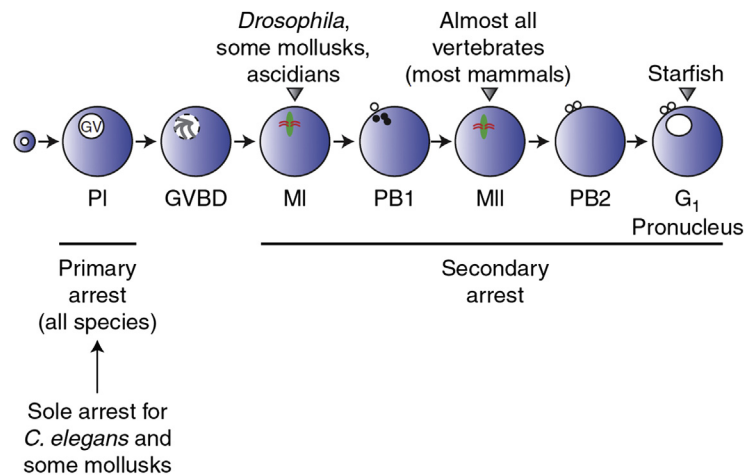


Fig. 3 Stage at which meiosis arrests prior to fertilization in different types of animals. Reprinted by permission from Fig. 1 of Sagata, N., 1996. Meiotic metaphase arrest in animal oocytes: Its mechanisms and biological significance. *Trends in Cell Biology* 6 (1), 22–28.

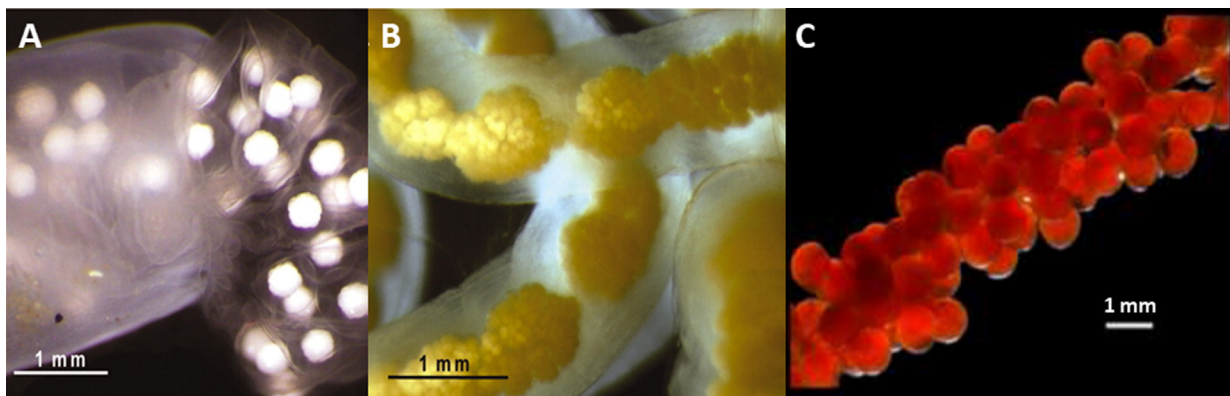


Fig. 4 Egg masses. (A) Egg mass of the nudibranch (Mollusca, Gastropoda) *Tritonia challengeriana* showing cleaving embryos that are contained singly in egg capsules; egg capsules are within a gelatinous string, which in turn is contained in a firm gelatinous outer casing (mass has been cut to release embryos from outer covering). (B) Egg mass of *Tritonia diomedea*, showing embryos (small yellow dots) contained within in egg capsules (clusters of yellow embryos). Egg capsules are embedded in a mucous sleeve. (A) and (B) reprinted by permission from Woods, H.A., Moran, A.L., 2008. Temperature–oxygen interactions in Antarctic nudibranch egg masses. *Journal of Experimental Biology* 211 (5), 798–804. **Fig. 1.** (C) Embryonated eggs of the deep-sea crab (Arthropoda, Crustacea) *Paromola cuvieri*. Reprinted by permission from Triay-Portella, R., González, J.A., Santana, J.I., *et al.*, 2014. Reproductive pattern and egg development of the deep-sea crab *Paromola cuvieri* (Brachyura, Homolidae) around the Canary Islands (NE Atlantic). *Deep Sea Research Part I: Oceanographic Research Papers* 85, 1–14.

include the embryonated eggs of many molluscs, arthropods, platyhelminths, and terrestrial insects; embryos develop in these structures until they hatch as larvae or juveniles. Embryos inside eggs can receive post-oogenetic maternal provisioning in various ways, including extraembryonic yolk, albumen, and nurse eggs (non-developing or unfertilized egg cells that are packaged with the embryo). When embryonated eggs are attached to the substrate and/or contain multiple embryos, they are often called egg capsules. Embryonated eggs that are clustered together and share a common external gelatinous matrix are generally termed 'egg masses', as in the egg masses of nudibranch molluscs (Fig. 4(a) and (b)) and many polychaete worms; the term egg mass is also applied to clusters of egg capsules or gelatinous masses produced by the same parent, for example the benthic attached egg capsules of buccinid gastropods or cephalopods (e.g., squid eggs) and the egg masses of many insects and crustaceans (Fig. 4(c)).

Ecological and Evolutionary Aspects of Egg Structure

The structure of eggs is driven by selection on a number of different pre- and post-zygotic forces, and is intertwined with the evolutionary history, ecology, and life histories of individual species and taxa. We cannot cover more than a scattering of examples here, and there are cases where species do not fall cleanly into the categories below – e.g., there is not a perfect correspondence between spawning mode and degree of parental care. However, we define some generalities below and discuss how some common life history traits are related to egg structure.

Broadcast Spawners

Eggs (whether ova or oocytes) of many marine taxa are released directly into the water by the parent; for these broadcast-spawning taxa, eggs must be provided by the parent with all the materials and structures needed for successful exit from the parent, fertilization, and early development. Broadcast spawning is widely distributed across the metazoan phylogeny, and occurs in at least some members of many major and minor phyla, including (but not limited to) Cnidaria, Ctenophora, Mollusca, Annelida, Nemertea, and Echinodermata.

Accessory coats

One prominent feature of the broadcast-spawned eggs of many taxa are accessory structures that surround the egg and are shed early in development, including jelly coats, textured fertilization envelopes, follicle cells, and hulls (Podolsky, 2002). These accessory structures vary in structure and function. Accessory structures can provide physical protection to the ovum and early embryo; they may act as a barrier to entry by microbial or viral pathogens, or, by increasing the effective size of the egg, provide protection from predators. In taxa that are vulnerable to polyspermy, accessory coats can also act as a physical barrier that limits sperm contact and slows sperm entry speed to facilitate monospermic fertilization. Eggs can also experience shear stress while passing through the oviduct and gonopore during spawning; work with echinoderms suggests that the extracellular coats that are found on the eggs of some free-spawning taxa may protect the ovum or developing embryo from damage due to these shear forces (Thomas and Bolton, 1999). Accessory structures such as jelly coats may also be important in maintaining egg and embryo integrity in the extraparental environment, particularly in environments with strong, turbulent water movement.

One additional important function of accessory structures is to enhance sperm-egg encounters and fertilization success in the water column, where environments can often be sperm-limited due to turbulence, currents, or low population density (Levitan, 2002). Accessory structures can enhance fertilization in a number of ways: by releasing chemical compounds that facilitate fertilization through altering sperm physiology and function; by increasing the effective target size of the egg, thus raising the probability of sperm-egg contact; by directing sperm to particular areas of the egg; and by slowing the sinking rate or altering the buoyancy of eggs to keep them suspended in the water column where they are more likely to encounter sperm (Podolsky, 2002). Because accessory coats are generally constructed of less energy-rich materials than the egg itself, they can alter the physical properties of eggs (e.g., size, density) at a considerably lower energetic cost than a similar change to the properties of the egg itself.

Egg composition

In addition to providing protection from environmental stresses for the egg and developing zygote, in free-spawning taxa the contents of the egg must also provide sufficient energy and materials for the zygote to develop to independent feeding. The extent to which embryos and larvae are dependent on materials in the egg varies tremendously across taxa and even among closely related species, and the variation in egg structure among taxa is thought to reflect these requirements. Among broadcast spawners, many taxa have larvae that must feed in the plankton to acquire energy for growth and metamorphosis (planktotrophy), while in others, the egg and/or egg coverings contain sufficient material and energy for offspring to develop without feeding on particulate material (lecithotrophy). Within planktotrophy, there is a continuum from species with tiny eggs and long feeding developmental periods, to species that have comparatively large eggs and are facultative feeders (meaning they can feed, but do not need to). Lecithotrophs overall have larger eggs than planktotrophs, at least within taxa. This pattern occurs in many phyla but to date has been most extensively studied in echinoderms, from which most conclusions about the relationships between egg size, larval ecology, evolution, and physiology have been drawn.

Egg size is a frequently-measured and reported life history character that is often used as a proxy for egg energetic content and per-offspring maternal investment. Larger eggs generally contain more energy than small eggs; however, egg size is not always a good

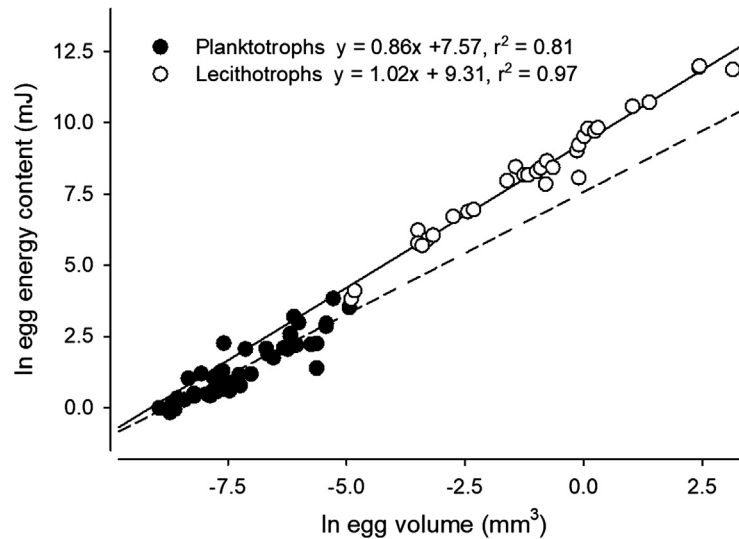


Fig. 5 Scaling of egg energy with mass for planktotrophic (filled circles, dotted line) and lecithotrophic (open circles) echinoderms, showing that eggs of lecithotrophs are larger and more energy-rich, and that the scaling of egg energy to volume differs between the two groups. Modified with permission from **Fig. 1** of Moran, A.L., McAlister, J.S., Whitehill, E.A.G., 2013. Eggs as energy: Revisiting the scaling of egg size and energetic content among echinoderms. *The Biological Bulletin* 224 (3), 184–191.

predictor of energy content, particularly within species. Assumptions about how egg energy scales with size are incorporated into most models of invertebrate life history evolution that invoke tradeoffs between size and number of offspring or between per-offspring quality and offspring fitness; thus, an understanding of the nature of this scaling is crucial. The most comprehensive attempts to describe the scaling of egg size and egg energy have focused on echinoderms. In this group, two patterns are evident; total energy increases with egg size, and lecithotrophic species have both larger and more energy-rich eggs than planktotrophs (Jaekle, 1995). However, when egg energy was regressed on egg size for planktotrophs and lecithotrophs separately, the scaling exponent of this relationship is lower for planktotrophs (slope = 0.86x) than for lecithotrophs (slope = 1.02x) (**Fig. 5**). Thus, among lecithotrophs, egg energy density does not change significantly as egg size increases; in contrast, in planktotrophic species, large eggs are proportionately more energy-poor than small eggs. The mechanism for this pattern is not known, but in one genus of planktotrophic tropical urchins (*Echinometra*), the proportional composition of protein, lipid, and carbohydrate does not change with egg size; this suggests that larger eggs contain a higher proportion of water or other non-organic materials (Moran *et al.*, 2013). Thus, egg size in this group may have evolved not in response to fecundity-time tradeoffs, but in a context of selection for some other attribute like target size for sperm.

In general, among echinoderms, eggs of lecithotrophic species are enriched in lipids compared to planktotrophs. This is likely because the egg of lecithotrophic species must contain sufficient energy to fuel not just larval development, but also metamorphosis and some post-metamorphic development as well. Comparing within the starfish family Asterinidae, Prowse *et al.* (2008) showed that eggs of lecithotrophic species had higher proportions of energetic lipids (largely triacylglycerols) than did planktotrophs, and inferred this was due to a prolongation of the lipogenetic phase of oogenesis. Also within this family, the eggs of lecithotrophic species varied in their buoyancy such that eggs with higher proportions of lipid were more buoyant. Increased buoyancy could be a strategy to enhance fertilization success if gametes concentrate at the air-water interface. In contrast, one lecithotrophic species in the genus with large eggs also makes eggs that are comparatively lipid-poor and protein rich; this species develops on the benthos, and the added protein may function as a weight belt to reduce egg and larval buoyancy, permitting development to occur on the bottom. Thus, while overall it seems to be the case that developmental mode, egg size, and egg composition are correlated across taxa (at least within echinoderms), egg composition can also evolve in different directions under specific selection regimes that are unique to a particular species.

Embryonated Eggs and Parental Care

Internal fertilization

While many invertebrates are free-spawners, there are also many that have internal fertilization, either through spermcasting (when sperm are broadcast-spawned but eggs are retained in the female) or via copulation or other mechanisms for active sperm transfer. The dynamics of internal fertilization are poorly understood for most invertebrates, even model systems like *Drosophila* (Loppin *et al.*, 2015). Logically, however, an egg is less likely to experience sperm limitation when fertilization is internal; indeed, many invertebrates have mechanisms for sperm storage in the female reproductive system, and the sperm are used in a highly efficient manner to fertilize eggs. When sperm-egg encounters are under the control of the female reproductive system, or when females

copulate with multiple males or have behaviors that bring eggs and sperm into close proximity, eggs are unlikely to experience strong selection for traits that increase the probability of sperm-egg contact in free-spawners, such as larger size, large accessory coats, or the production of strong sperm attractants.

Egg capsules and egg masses

When eggs are no longer freely spawned into the environment, many of the protective and nutritive functions of the oocytes of free-spawners can be taken over by some type of parental care (either internal or, more frequently, external). Many invertebrates produce structures that contain the developing embryo, creating an embryonated egg. When the structures contain a single zygote, they are often called eggs (as in the eggs of insects and crustaceans); when multiple zygotes share a single structure, or when these structures are clustered together, they are generally called egg capsules or egg masses (as in many gastropods, cephalopods, flatworms, and annelids). Egg capsules and masses are found in a wide variety of taxa and are often structurally complex, consisting of tough leathery capsules or complex gelatinous matrixes around the zygote (Fig. 5) (Thorson, 1950). In some cases, components of the egg itself facilitate egg mass formation; in most marine crustaceans, for example, the vitelline membrane adheres to the abdomen of the mother, creating an egg mass inside a facultative brood chamber (Subramoniam, 2016). The function of egg masses is generally thought to be protective; for marine invertebrates, embryos that develop in benthic capsules can avoid advection and predation in the plankton, and for marine and terrestrial embryos alike the capsules can contain chemical defenses or provide protection from predation, parasitism, desiccation, UV, and other environmental stressors (Pechenik, 1979). The nature and composition of capsule coverings varies tremendously depending on environment; for example, the coverings around insect embryos must walk a fine line between desiccation resistance and permeability to oxygen, two functions that are essential for development but are in opposition to each other (Kambysellis *et al.*, 1999). Similarly, the egg mass jelly that surrounds embryos of many gastropods and annelids must provide physical protection to developing embryos while allowing diffusion of oxygen into the mass, leading to the evolution of elaborate egg masses with high surface areas and short diffusion distances (Moran and Woods, 2007).

Extraembryonic nutrition

Another opportunity afforded by encapsulation of zygotes is the provisioning of the embryo with extra-embryonic nutrition (EEN). EEN is found in a wide variety of invertebrates, particularly molluscs and annelids, and takes many forms including protein-rich fluid (albumen), extraembryonic yolk, non-developing eggs (nurse eggs, trophic eggs, or nutritive eggs), or even sibling embryos (adelphophagy). Like internal fertilization, the provisioning of eggs with EEN changes the selective environment acting on egg size and structure, because the embryo does not need to be entirely built by or fueled from materials in the egg. Thus, while the oocytes of lecithotrophic free-spawning taxa tend to be large and rich in lipids, large offspring can be produced by species with encapsulated development regardless of whether the oocytes are large and energy-rich or small and energy-poor; these two strategies exist on a continuum and the functional and adaptive consequences of maternal provisioning of intra- vs. extraembryonic nutrition are not known (Marko *et al.*, 2014). One interesting and long-standing question about nurse eggs is how they are specified; in some cases, trophic eggs are unfertilized ova, but in others they appear to be ova whose development is arrested (Perry and Roitberg, 2006). Understanding the maternal mechanisms that regulate development in trophic eggs may lead to new discoveries about the evolution of egg structure and provisioning across many types of invertebrates.

Conclusion

Invertebrates comprise all of metazoan diversity and metazoans inhabit and produce eggs in almost every habitat on Earth; thus, the diversity of structure in egg cells and egg coverings in the invertebrates is far too great to adequately summarize in a single chapter. Above we have briefly described egg structure in the best-understood system, the sea urchin egg; reviewed the list of different types of structures that are called eggs in the scientific literature on invertebrates; and described some of the major ecological and evolutionary contexts that shape egg structure and function. However, egg structure has been described for only a very small percentage of the existing diversity, and detailed observations of ultrastructure, biochemistry, and physiology are even rarer. It is our hope that the advent of more comprehensive and accurate invertebrate phylogenies, along with the development of modern molecular techniques for understanding egg physiology and function, the factors that shape the adaptive structure and function of eggs will be better understood.

References

- Gilbert, S. F. (2000). *Developmental Biology*. Sutherland, MA: Sinauer Associates.
- Gilbert, S. F., & Raunio, A. M. (1997). *Embryology: Constructing the Organism*. Sutherland, MA: Sinauer Associates.
- Jaekle, W. B. (1995). Variation in size, energy content, and biochemical composition of invertebrate eggs: Correlates to the mode of larval development. In L. McEdward (Ed.), *Ecology of Marine Invertebrate Larvae* (pp. 44–77). Boca Raton, FL: CRC Press.
- Kambysellis, M. P., Margaritis, L., & Craddock, E. M. (1999). Egg coverings, insects. *Encyclopedia of Reproduction*, 1, 971–990.
- Levitani, D. R. (2002). Density-dependent selection on gamete traits in three congeneric sea urchins. *Ecology*, 83(2), 464–479.
- Loppin, B., Dubruielle, R., & Horard, B. (2015). The intimate genetics of *Drosophila* fertilization. *Open Biology*, 5(8), 150076.
- Marko, P. B., Moran, A., Kolotuchina, N. K., & Zaslavskaya, N. I. (2014). Phylogenetics of gastropod genus *Nucella* (Neogastropoda: muricidae): Species identities, timing of diversification and correlated patterns of life-history evolution. *Journal of Molluscan Studies*, 80(4), 341–353.

- Moran, A. L., McAlister, J. S., & Whitehill, E. A. G. (2013). Eggs as energy: Revisiting the scaling of egg size and energetic content among echinoderms. *The Biological Bulletin*, 224(3), 184–191.
- Moran, A. L., & Woods, H. A. (2007). Oxygen in egg masses: Interactive effects of temperature, age, and egg-mass morphology on oxygen supply to embryos. *Journal of Experimental Biology*, 210(4), 722–731.
- Pechenik, J. A. (1979). Role of encapsulation in invertebrate life histories. *The American Naturalist*, 114(6), 859–870.
- Perry, J. C., & D. Roitberg, B. (2006). Trophic egg laying: Hypotheses and tests. *Oikos*, 112(3), 706–714.
- Podolsky, R. D. (2002). Fertilization ecology of egg coats: Physical versus chemical contributions to fertilization success of free-spawned eggs. *Journal of Experimental Biology*, 205(11), 1657–1668.
- Prowse, T. A. A., Sewell, M. A., & Byrne, M. (2008). Fuels for development: Evolution of maternal provisioning in asterinid sea stars. *Marine Biology*, 153(3), 337–349.
- Subramoniam, T. (2016). *Sexual Biology and Reproduction in Crustaceans*. Netherlands: Academic Press.
- Thomas, F. I., & Bolton, T. F. (1999). Shear stress experienced by echinoderm eggs in the oviduct during spawning: Potential role in the evolution of egg properties. *Journal of Experimental Biology*, 202(22), 3111–3119.
- Thorson, G. (1950). Reproductive and larval ecology of marine bottom invertebrates. *Biological Reviews*, 25(1), 1–45.

Oogenesis and Ovulation

Oogenesis, Fish Amphibians

Joan Cerdà, Institute of Research and Technology in Food and Agriculture (IRTA), Barcelona, Spain

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

Bb Balbiani body
Bmp15 Bone morphogenetic factor 15;
Buc Bucky ball protein
cAMP Cyclic adenosine monophosphate
EGF Epidermal growth factor
EGFR EGF receptor
Erk Extracellular-related kinase
Fsh Follicle-stimulating hormone
G α s Stimulatory G protein α subunit
GPCR G protein-coupled receptor
GSCs Germline stem cells
Gdf9 Growth/differentiation factor 9
Gsdf Gonadal soma-derived growth factor
Igf-I Insulin-like growth factor-I
MAPK Mitogen activated protein kinase
PACAP The adenylate cyclase-activating polypeptide
PGRMC1 Progesterone receptor membrane component 1
piRNA Piwi-interacting RNA
PGC Primordial germ cells
Pgr Nuclear progesterin receptor
RA Retinoic acid
mRNP mRNA-protein aggregate
Tgf β Transforming growth factor- β
17,20 β P 17,20 β -dihydroxy-4-pregnen-3-one

Introduction

The term “oogenesis” or “ovogenesis” refers specifically to the transformation of oogonia into oocytes. In lower vertebrates, such as fish and amphibians, this process starts when oogonia enter into meiosis to become primary oocytes, and subsequently form a follicle when they are surrounded by somatic cells. Further growth of the oocyte mainly by the accumulation of large reserves of yolk (vitellogenesis), the hormone-induced meiosis resumption (oocyte maturation), and the release of the mature oocyte from the enveloping layer of somatic cells (ovulation), results in the production of a fertilizable egg. The main focus of this section, is on the early oogenic events including: the formation of oogonia, their transition into meiosis, and the first stages of oocyte development until the formation of the primary growth ovarian follicle.

Formation of Oogonia

Shortly after fertilization of the egg and during the early stage of embryogenesis, a small number of non-dividing primordial germ cells (PGCs) are produced, which will eventually divide through mitosis and colonize the genital ridge (Le Menn *et al.*, 2007). The PGCs colonizing the gonad stroma are relatively large, irregular in shape, with a rather large nucleus, and may exhibit temporary cytoplasm-filled projections of the cell (pseudopodia) indicative of their migratory status. The PGCs are bipotential cells capable of evolving into oogonia in females or into spermatogonia in males. The PGCs give rise to the recently discovered germline stem cells

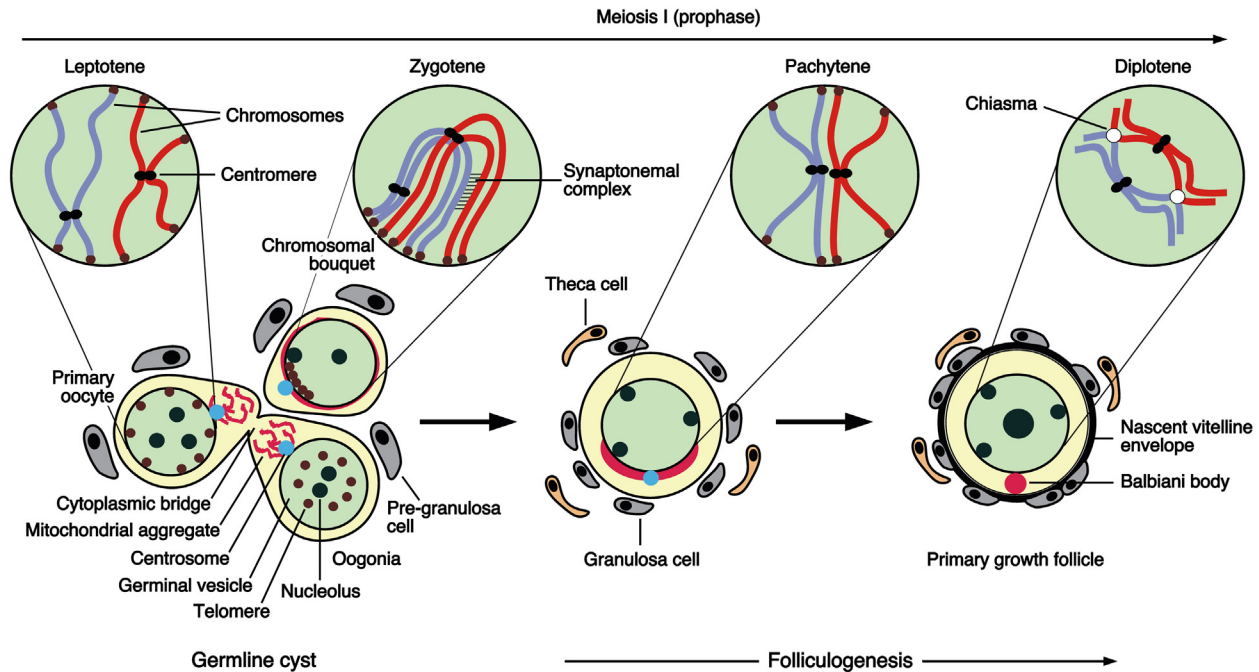


Fig. 1 Schematic representation of the stages of oogenesis from premeiotic oogonia through the formation of the primary growth follicle in amphibians and teleosts. The progressive phases of meiosis and the formation of the Balbiani body are shown.

(GSCs), which reside in a lateral, anterior-posterior band at the ovary periphery termed the germinal zone where meiotic oocytes are generated (Elkouby and Mullins, 2017). The mechanisms through which GSCs are born from PGCs, and how their proliferation, self-renewal, and generation of subsequent mitotic cells and differentiating oocytes are just beginning to be understood. For example, it has been shown that GSCs specifically express the RNA-binding proteins Nanos 2 and 3, required for their maintenance and competence to generate oocytes.

The mechanism through which the GSCs differentiate into mitotic oogonia, the precursor cells of meiotic oocytes, are yet to be determined. It has been established, however, that the transformation involves structural changes within the GSCs, including the progressive increase in nuclear and cell size, as well as of the number of nuclear pores and organelles in the cytoplasm, and the differentiation of the nucleolus (Le Menn et al., 2007). During this transition, the electron dense material called "ciment" or germinal dense bodies, which constitute mitochondrial aggregates, as well as the "nuage" or germ granules (see below), both also present in PGCs, increase in size and number. Once formed, the oogonia proliferate through a variable number of cell divisions, dependent upon the species, and form germline cysts within the germinal epithelium. Prior to the initiation of meiosis, the oogonia multiply rapidly, and remain interconnected by cytoplasmic bridges as a result of incomplete cytokinesis and abscission between daughter cells (Fig. 1). The cysts are separated by the stroma by a basement lamina secreted by somatic (pre-granulosa) cells. In some teleosts, however, oogonia may also become isolated and surrounded by a few pre-granulosa cells. This organization in germline cysts, in which oogonia are interconnected by cytoplasmic bridges, is thought to synchronize the development of individual oocytes (Elkouby and Mullins, 2017). In mammals, the transfer of cytoplasmic contents between cyst oocytes is thought to lead to the growth of one oocyte and subsequent death of the other, such that the majority of oocytes do not develop into primordial follicles. In the African clawed frog (*Xenopus laevis*) and the teleost medaka (*Oryzias latipes*), however, only a few cells in the mitotic cyst or its neighbour undergo apoptosis (Elkouby and Mullins, 2017).

The hormonal mechanisms controlling oogonial proliferation in fish and amphibians are poorly known, although early studies on hypophysectomized and unilaterally ovariectomized teleosts suggest that pituitary gonadotropins, especially follicle-stimulating hormone (Fsh), can increase oogonial proliferation (Lubzens et al., 2010). This mechanism is likely indirect through the local release of sex steroid hormones since studies on cyprinids and salmonids have shown that ovarian estrogens, such as 17β -estradiol, can induce the mitotic activity of oogonia. As observed in mammals, growth factors could also promote oogonia proliferation, such as the gonadal soma-derived growth factor (Gsdf), a cytokine belonging to the transforming growth factor- β (Tgf β) superfamily which has been involved in spermatogonial proliferation in the testes and is detected in the supporting cells surrounding oogonia and oocytes in several teleosts. The mechanisms regulating steroid and growth factor production in the fish and amphibian germinal epithelium are yet unknown, although the Fsh-mediated secretion of these factors by the pre-granulosa cells in close vicinity to oogonia might play a role.

Transformation of Oogonia into Primary Oocytes

The transition from oogonium to a primary oocyte is primarily characterized by the initiation of the first meiotic division, before leaving the germline cyst. The differentiation of oocytes is also accompanied by other key developmental events, such as the acquisition of cell polarity and the formation of the Balbiani body, and changes in cellular organization from cyst to follicles of individual oocytes.

Meiosis Initiation

Within the cyst, each oogonium is a diploid cell, with a nucleus containing two copies of each chromosome, called homologs, one from the father and one from the mother. During the interphase following the last mitotic division of a diploid oogonium and preceding the first meiotic division of the primary oocyte, each homolog is duplicated by DNA replication (pre-leptotene), giving rise to a pair of identical chromatids, known as a sister chromatid pair (Fig. 1). These homologous chromosomes pair and begin to condense (leptotene, zygotene), and subsequently shorten and thicken to form the synaptonemal complex (pachytene), when homologous recombination, including chromosomal crossover (crossing over), occurs. During the leptotene stage, chromosomes attach to the nuclear envelope via their telomeres, which then rotate on the envelope, tumbling entire chromosomes inside the nucleus, and enabling their homology searches for pairing (Elkouby and Mullins, 2017). This mechanism eventually clusters the telomeres to one side of the nuclear envelope, with the free looping ends of their chromosomes facing the opposite side, at the zygotene stage (Fig. 1). This polarized organization is called the zygotene chromosomal bouquet, thought to help stabilize chromosomal synapsis, which is regulated by centrosome-based microtubules in the cytoplasm that connect to the telomeres via Sun/KASH proteins spanning the two membranes of the nuclear envelope (Elkouby and Mullins, 2017). Finally, during the diplotene stage the chromosomes take on a 'lampbrush' configuration in which large chromatin loops extended laterally allowing the active transcription of heterogeneous RNA, which adds to the ribosomal RNA provided by the multiple nucleoli. Most of the former RNA is processed into poly(A)-containing RNA, the so-called "maternal RNA", as it moves from the germinal vesicle into the cytoplasm where it becomes asymmetrically distributed (see below). Arrested in late diplotene of the first meiotic prophase, the oocyte then begins an extensive period of growth concomitant with the differentiation of its follicular components. This is generally called the "perinucleolus stage of primary oocyte growth".

In teleosts and amphibians, the hormonal signals triggering meiosis in oogonia are not well known. In mammals, retinoic acid (RA) signaling has been established as a major meiosis inducer. In zebrafish (*Danio rerio*), both the RA-synthesizing enzyme aldehyde dehydrogenase 1 and RA-degradating enzyme Cyp26 Cytochrome P450 have been found to be expressed in gonadal somatic cells, suggesting that RA signaling might also play a role in the onset of meiosis in teleosts (Elkouby and Mullins, 2017). However, recent studies *in vivo* and *in vitro* using long-term explant culture systems in cyprinid and salmonid teleosts have implicated the progestin 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β P) in the entry into meiosis of oogonia, as well as of spermatogonia in the testis. The roles of 17,20 β P in teleosts and of progesterone in amphibians as the major hormonal signals for the resumption of meiosis during oocyte maturation are well established, and therefore these findings may indicate that 17,20 β P could control meiosis at different times during oocyte development in teleosts. However, the factors and mechanisms regulating steroid production at this time are unknown. In some teleosts, the onset of meiosis has been linked to the expression of insulin-like growth factor-I (Igf-I) in somatic cells and oocytes, but the mechanisms through which IGF-I could stimulate meiosis are not known.

Meiotic Arrest

As mentioned above, meiosis in early oocytes is arrested in the prophase of the first meiotic division, and prophase arrested oocytes proceed throughout the entire period of oocyte growth until the oocyte maturation stage, when oocytes resume meiosis and subsequently arrest again in metaphase of meiosis II. Arrest of meiosis I occurs in all vertebrates during oogenesis, and there is broad consensus that this process is maintained by high levels of cyclic adenosine monophosphate (cAMP) in the oocyte, which activates the enzyme protein kinase A.

In teleosts and amphibians, several mechanisms have been proposed to maintain high levels of cAMP in oocytes. One long-standing hypothesis is that cAMP produced in the surrounding follicular (granulosa) cells diffuses to the oocyte through gap junctions, which are aggregates of intercellular channels established between the oocyte and the granulosa cells that permit direct cell-cell transfer of ions and small molecules, such as the cAMP. This mechanism however might be more relevant in completely formed ovarian follicles than in primordial follicles in which the structures for cell-cell interaction between oocyte and granulosa cells are still in the course of being developed.

More recently the roles of a stimulatory G protein α subunit ($G_{\alpha s}$)-coupled G protein-coupled receptor (GPCR), such as GPR185 in amphibians, and the $G_{\alpha s}$ -coupled estrogen membrane receptor GPR30 in teleosts, have been proposed as mechanisms that maintain high levels of cAMP and oocyte meiotic arrest (Ríos-Cardona *et al.*, 2008; Thomas, 2017) (Fig. 2). Constitutive or estrogen activation of GPR185 and GPR30, respectively, leads to the stimulation of the adenylyl cyclase, which results in the increase of the intracellular concentration of cAMP. Activation of the GPR30 in teleost oocytes can also stimulate the $G\beta\gamma$ subunit signaling resulting in the activation of the Src family of protein tyrosine kinases followed by the up-regulation of matrix metalloproteinase (MMP), ultimately leading to release of heparan-bound epidermal growth factor (EGF) (Thomas, 2017). The EGF can then bind to the EGF receptor (EGFR) at the oocyte surface promoting mitogen activated protein kinases (MAPKs) signaling, phosphorylation of

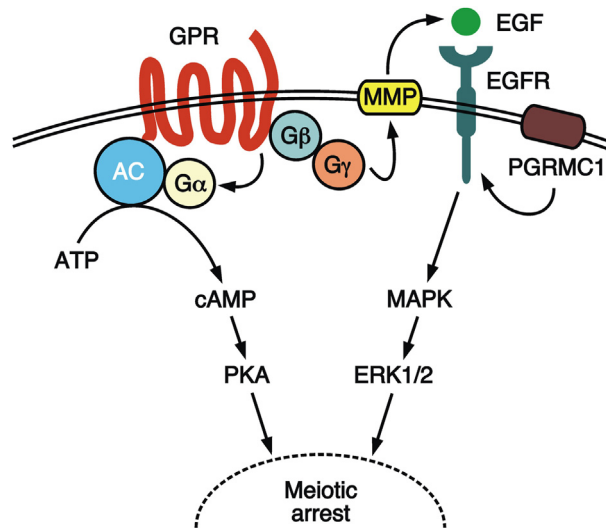


Fig. 2 Proposed model of the maintenance of oocyte meiotic arrest in amphibians and teleosts by constitutive or estrogen-activated G protein-coupled receptors (GPRs). In teleosts, the role of the epidermal growth factor receptor (EGFR) and its regulation by the matrix metalloproteinase (MMP) and the progesterone receptor membrane component 1 (PGRMC1) is also indicated. Modified from Thomas, P., 2017. Role of G-protein-coupled estrogen receptor (GPER/GPR30) in maintenance of meiotic arrest in fish oocytes. *Journal of Steroid Biochemistry and Molecular Biology* 167, 153–161.

extracellular-related kinase 1 and 2 (Erk1/2), and the maintenance of meiotic arrest. Recent studies also indicate that progesterone receptor membrane component 1 (PGRMC1) plays a crucial role in regulating the expression of the EGFR; that in turn might influence the inhibitory effect of estrogens on resumption of meiosis through GPER (Thomas, 2017). In teleosts, the adenylyl cyclase-activating polypeptide (PACAP) is another cAMP modulating paracrine system that might help to maintain meiotic arrest, although this mechanism has been less investigated.

The Balbiani Body and Oocyte Polarization

A rather prominent feature during the differentiation of the primary oocyte is the formation of the Balbiani body (Bb), also known as yolk nucleus/body/complex, vitelline nucleus/body, mitochondrial cloud/mass/aggregate, or sponge body (Kloc *et al.*, 2014). The Bb is an aggregate of mRNA-protein (mRNP) and mitochondria that initially forms in the cytoplasm adjacent to the nucleus of zygotene stage oocytes, and then localizes to the oocyte cortex, where it dissociates at the later stages of cortical alveoli and vitellogenesis. The movement of the Bb to the oocyte cortex, its disassembling and docking of its contents in the membrane defines the vegetal pole of the oocyte, and therefore the Bb first establishes the definitive animal-vegetal axis of the oocyte, which lays the foundation for the embryonic body plan (Elkouby and Mullins, 2017).

Inside or outside of the Bb, oocytes also accumulate granulo-fibrillar non-membrane-bound material, known as the nuage, which plays a role in the germ plasm pathway (Kloc *et al.*, 2014). The germ plasm is constituted by maternally inherited germ cell determinants in female germ cells which is required for the specification and maintenance of germ cell fate during embryonic development. Perinuclear nuage contains ribonucleoproteins implicated in diverse RNA metabolism, including components of a gonad-specific RNA silencing pathway, the piwi-interacting RNA (piRNA) pathway, which is involved in the protection of the genome against deleterious selfish genetic elements such as transposons. Nuage granules also contain many proteins that bear a Tud domain, a small 50–55 amino acid β -barrel module which forms an aromatic cage that interacts with methylated amino acids, lysines or arginines of target proteins, including Piwi family proteins (Gao and Arkov, 2013). In zebrafish, the Tud domain proteins have been shown to play crucial roles in germ granule assembly.

The Bb forms the pathway for the delivery of mRNPs granules to the vegetal pole of the oocyte, while other mRNAs are recruited later to the defined vegetal pole, which is known as the “late pathway” (Escobar-Aguirre *et al.*, 2017). In addition to the germ plasm components, the zebrafish Bb contains proteins involved in mRNAs and mRNPs aggregation, such as Bucky ball (Buc) (see below), and dorsal determinants and dorsalizing machinery mRNAs (Escobar-Aguirre *et al.*, 2017). In *X. laevis*, the mRNAs of the Bb and/or Bb-derived germ plasm include RNA binding proteins, mRNAs encoding proteins involved in primordial germ cell migration, mRNAs encoding proteins implicated in scaffolding and/or recruitment of germ plasm components, and non-coding RNAs (Kloc *et al.*, 2014). The proteins identified in *X. laevis* Bb and/or Bb-derived germ plasm can be classified into translational repressors, anti-transposon factors, germinal granule assembly and maintenance factors, cell cycle regulators and proteins involved in RNA transport and localization (Kloc *et al.*, 2014).

Very little is known about the molecular mechanisms controlling Bb formation, its cortical localization, and its disassembly and cortical tethering of its mRNP cargo. In zebrafish, two genes, *bucky ball* (*buc*), which encodes an intrinsically disordered protein, and microtubule actin cross-linking factor 1 (*macf1*), have been identified that can regulate some of these processes (Escobar-Aguirre

et al., 2017). Buc, and the *X. laevis* homolog Xvelo, are the only known proteins required for Bb formation, and it is believed that they form a scaffolding phase-separating lattice in Bb granules that aggregates mRNAs and mRNPs during Bb formation. The Macf1 protein is a member of the spectraplakins family, and may mediate microtubule and actin connections to localize the Bb to the oocyte cortex and mediate its disassembling.

Recent studies in zebrafish have revealed that the nuclear polarity resulting from the chromosomal bouquet at the zygote stage is also involved in Bb formation upstream to the Bucky ball protein (Elkouby and Mullins, 2017). In these studies, it has been shown that microtubules are concomitantly required to cluster the telomeres of the bouquet and localize the Bb mitochondrial precursors, demonstrating that these nuclear and cytoplasmic events are mechanistically coordinated. Therefore, the bouquet stage centrosome functions as a global cellular organizer that couples meiotic events with oocyte patterning, which has been termed the meiotic-vegetal center or centrosome organizing center (Elkouby and Mullins, 2017).

Ovarian Folliculogenesis and Primary Oocyte Growth

Before leaving the germinal ridge, a single layer of pre-granulosa cells initially surrounds the entire nest of early meiotic oocytes. As the cells in the nest cellularize, the cyst structure of the oocyte is gradually broken by the invagination of somatic cells and, finally, the oocyte becomes a single cell surrounded by a sheath of squamous granulosa cells, which is known as the primary growth follicle (Le Menn *et al.*, 2007). The granulosa cells will complete the secretion of the basement lamina from their basal membrane, outside of which multilayers of unorganized meso-epithelial cells will constitute the thecal layer, or theca, irrigated by numerous blood vessels. At the same time, the oolemma separates from some areas of the apical granulosa cell surface, and the intervening space is filled with an extra-oocyte matrix that will become the vitelline envelope (zona pellucida) separating the oocyte and the granulosa epithelium. Later in follicle development, the granulosa cells will gradually increase in height to become more cuboidal and separate from each other to form intercellular spaces.

During ovarian follicle formation, granulosa cells surrounding the oocyte are connected through gap junctions and by a number of typical junction complexes, such as desmosomes and tight (occluding) junctions. In addition, cytoplasmic projections from the granulosa cells and oocyte, which will become true microvilli that cross the vitelline envelope in later developmental stages, make focal contact, and these connections will be maintained throughout oocyte growth. Gap junctions are formed between the oocyte and granulosa cells, which allow chemical communication, as well as other cell contact structures, such as junction complexes resembling adhering junctions, which contain α - and β -catenin and E-cadherin-like molecules. Interestingly, β -catenin is also accumulated in the vegetal pole of amphibian oocytes, and both in teleosts and amphibians is a key effector of the Wnt/ β -catenin signaling regulating the transcription of genes required for the establishment of the overall body plan patterning during embryogenesis (St Amand and Klymkowsky, 2001; Hüsken and Carl, 2013).

Several oocyte-expressed growth factors, such as growth/differentiation factor 9 (Gdf9) and bone morphogenetic factor 15 (Bmp15), both of which play important roles in early ovarian follicle growth in mammals, have been implicated in primary ovarian follicle development in teleosts and amphibians. However, no experimental evidence exists on their functional role during early oogenesis. Only in zebrafish has it recently been shown that Bmp15 can regulate the expression of estrogen producing enzymes in the granulosa cells, thus contributing to female sex determination (Dranow *et al.*, 2016).

Control of Gene Expression in Primary Growth Oocytes

When the follicle starts to form, the synaptonemal organization of the oocyte nucleus is de-structured by disassembling the lateral protein axes of the ladder-like central core (Le Menn *et al.*, 2007). The lampbrush chromosome structure appears and each sister chromatid pair is highly active in RNA synthesis, due to the double amount of DNA, compared to somatic cells. At this stage, the nucleoli increase due to a huge amplification of the nucleolar organizer genes, and the nucleus is then generally characterized by a large central nucleolus and numerous smaller nucleoli.

In addition to the accumulation of maternal RNAs important for further embryo development mentioned above, the primary oocyte also initiates the transcription of several genes with important roles during follicle formation, oocyte growth and maturation, and in marine teleosts producing pelagic (floating) eggs, during oocyte hydration. These transcripts include proteins associated with vitellogenin (the major precursor of yolk proteins in the oocyte) and lipid uptake, such as the vitellogenin receptors, some of the zona pellucida glycoproteins that will form the nascent vitelline envelope, cysteine proteases such as cathepsins which will process vitellogenin-derived yolk proteins during oocyte growth and maturation, cell cycle proteins involved in meiosis resumption, molecular chaperone proteins such as heat shock protein 40, metal binding proteins such as ferritin, and water channel proteins (aquaporins) which will facilitate water uptake into the oocyte (hydration) during meiotic maturation prior to ovulation (Guzmán *et al.*, 2014; Cerdà *et al.*, 2017).

The endocrine pathways controlling gene expression in primary oocytes are largely unknown. However, studies on the transcriptional control of one of the oocyte aquaporins (Aqp1ab) in the marine teleost gilthead seabream (*Sparus aurata*) have revealed that this mechanism is dependent on the classical nuclear progesterone receptor (Pgr), which is expressed in the cytoplasm of oogonia and the nucleus of primary oocytes and can bind to the aquaporin gene promoter thus activating *aqp1ab* gene transcription (Cerdà *et al.*, 2017). In this model, the Pgr is activated by the progesterone 17,20 β P, produced by primordial granulosa cells associated to primary oocytes in response to Fsh, which upregulates the expression of steroidogenic enzymes involved in progesterone synthesis and

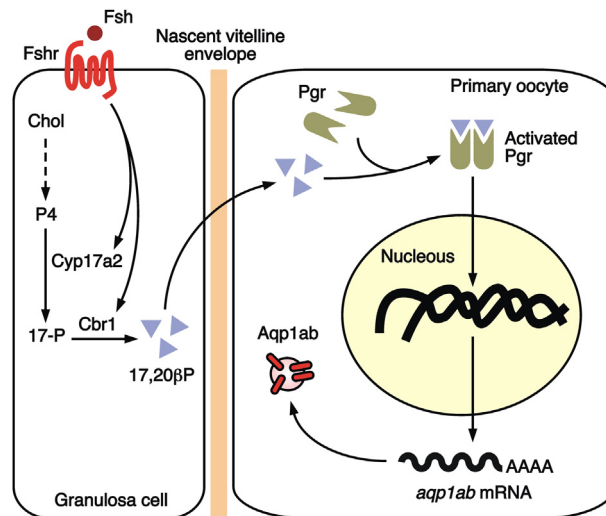


Fig. 3 Proposed model for the endocrine pathway involved in aquaporin-1ab (*aqp1ab*) transcriptional regulation in gilthead seabream primary growth oocytes. The scheme depicts the Fsh receptor (Fshr)-mediated progestin (17,20bP) synthesis and subsequent activation of *aqp1ab* transcription through the nuclear progestin receptor (Pgr). At the primary growth stage, activation of the Fshr in granulosa cells triggers 17,20bP production through the upregulation of the P450c17-II (Cyp17a2)/20b-hydroxysteroid dehydrogenase (Cbr1) steroidogenic pathway in granulosa cells. Binding of 17,20bP to Pgr activates the receptor, which promotes transcription likely through interaction with the *aqp1ab* proximal gene promoter. Modified from Zapater, C., Chauvigné, F., Tingaud-Sequeira, A., Finn, R.N., Cerdà, J., 2013. Primary oocyte transcriptional activation of *aqp1ab* by the nuclear progestin receptor determines the pelagic egg phenotype of marine teleosts. *Developmental Biology* 15, 377 (2):345–362.

downregulates the major enzymes responsible for estrogen synthesis, resulting in an enhanced production of 17,20bP in the granulosa cells (Fig. 3). These findings suggest that that Fsh/progestin/Pgr signaling pathway might also regulate the transcription of other genes in the primary oocyte of lower vertebrates, similarly to the action of the progesterone-activated PGR during primordial follicle assembly in mammals. However future studies are necessary to demonstrate this hypothesis.

References

- Cerdà, J., Chauvigné, F., & Finn, R. N. (2017). The physiological role and regulation of aquaporins in teleost germ cells. *Advances in Experimental Medicine and Biology*, 969, 149–171.
- Dranow, D. B., Hu, K., Bird, A. M., Lawry, S. T., Adams, M. T., Sanchez, A., Amatruda, J. F., & Draper, B. W. (2016). Bmp15 is an oocyte-produced signal required for maintenance of the adult female sexual phenotype in zebrafish. *PLOS Genetics*, 12(9), e1006323.
- Elkouby, Y. M., & Mullins, M. C. (2017). Coordination of cellular differentiation, polarity, mitosis and meiosis – New findings from early vertebrate oogenesis. *Developmental Biology* (pii: S0012-1606(16)[30613-3]).
- Escobar-Aguirre, M., Elkouby, Y. M., & Mullins, M. C. (2017). Localization in oogenesis of maternal regulators of embryonic development. *Advances in Experimental Medicine and Biology*, 953, 173–207.
- Gao, M., & Arkov, A. L. (2013). Next generation organelles: Structure and role of germ granules in the germline. *Molecular Reproduction and Development*, 80, 610–623.
- Guzmán, J. M., Luckenbach, J. A., Yamamoto, Y., & Swanson, P. (2014). Expression profiles of Fsh-regulated ovarian genes during oogenesis in coho salmon. *PLOS One*, 9(12), e114176.
- Hüsken, U., & Carl, M. (2013). The Wnt/beta-catenin signaling pathway establishes neuroanatomical asymmetries and their laterality. *Mechanisms of Development*, 130, 330–335.
- Kloc, M., Jedrzejowska, I., Tworzydło, W., & Bilinski, S. M. (2014). Balbiani body, nuage and sponge bodies – term plasm pathway players. *Arthropod Structure & Development*, 43, 341–348.
- Le Menn, F., Cerdà, J., & Babin, P. J. (2007). Ultrastructural aspects of the ontogeny and differentiation of ray-finned fish ovarian follicles. In P. J. Babin, J. Cerdà, & E. Lubzens (Eds.), *The Fish Oocyte: From Basic Studies to Biotechnological Applications* (pp. 1–37). Springer.
- Lubzens, E., Young, G., Bobe, J., & Cerdà, J. (2010). Oogenesis in teleosts: How eggs are formed. *General and Comparative Endocrinology*, 165, 367–389.
- Rios-Cardona, D., Ricardo-González, R. R., Chawla, A., & Ferrell, J. E., Jr. (2008). A role for GPRx, a novel GPR3/6/12-related G-protein coupled receptor, in the maintenance of meiotic arrest in *Xenopus laevis* oocytes. *Developmental Biology*, 317, 380–388.
- St Amand, A. L., & Klymkowsky, M. W. (2001). Cadherins and catenins, Wnts and SOXs: Embryonic patterning in *Xenopus*. *International Review of Cytology*, 203, 291–355.
- Thomas, P. (2017). Role of G-protein-coupled estrogen receptor (GPER/GPR30) in maintenance of meiotic arrest in fish oocytes. *Journal of Steroid Biochemistry and Molecular Biology*, 167, 153–161.

Oogenesis & Ovulation: Oocyte Maturation and Ovulation, Comparative

Aritro Sen and Ashley L Severance, Michigan State University, East Lansing, MI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Across species, the release of a mature healthy egg for fertilization is the center of the entire reproductive process. From the time of embryonic development till fertilization, the oocyte undergoes several stop-and-go periods which are tightly regulated throughout the reproductive cycle. Oocytes undergo first meiotic progression during embryonic development, and at the time of birth, they become arrested in the prophase stage. This meiotic arrest of oocytes is maintained until shortly before ovulation, when resumption of meiosis occurs and there is progression to metaphase II (MII), a process commonly termed *oocyte maturation*. Following resumption of meiosis I, there is organized disassembly of the nuclear envelope (germinal vesicle, GV) referred to as germinal vesicle breakdown (GVBD), followed by chromosome condensation, spindle formation and extrusion of the first polar body. Thereafter, the oocyte enters meiosis II and again gets arrested at metaphase II stage until fertilization. Upon fertilization, the oocyte resumes meiosis II extrudes the second polar body, thereby completing maturation (Dekel, 1995; Eppig *et al.*, 2004a,b). Oocyte maturation is one of the major steps for the oocyte to attain competence for successful fertilization and subsequent embryonic development. Acquisition of this developmental competence of the oocyte involves multiple factors regulated by different signaling pathways at various stages prior to fertilization (Jones, 2004; Mehlmann, 2005). While some crucial components of the signaling pathways involved in oocyte maturation are conserved from amphibians to mammals, there exists significant species-specific differences. This article highlights the differences in oocyte maturation among species and compares the various signaling pathways and underlying intracellular mechanisms involved in maintaining the meiotic arrest, regulation of oocyte maturation and ovulation across species.

Primary Meiotic Arrest

The segregation of the germ cell from the somatic lineage occurs very early during development in both invertebrates and vertebrates. Once the primordial germ cells (PGC) get committed to the germ cell lineage and enter meiosis, they progress to prophase I, where the first meiotic cycle is then arrested. This primary meiotic arrest occurs in all organisms considered in this article. The potential evolutionary rationale behind this highly conserved arrest (Mira, 1998) is based on three non-exclusive hypotheses. First, the “mutational hypothesis” suggests that the occurrence of prophase I arrest, early during meiosis to ensure that oocytes are made before division or prior to environmental perturbations causing DNA damage. This ensures that oocytes are as close to the “master-copy” as possible. The second “repair hypothesis” elucidates that by arresting at this stage when sister chromatids are present and held tightly together, the sister chromatid can be used as an ideal template to repair any DNA damage that arises. Lastly, an interesting “minimize conflict” hypothesis proposes that some type of inter-gamete competition may exist and arresting at prophase I ensures that all oocytes have four copies of each gene, discouraging the selection of oocytes based on genetic components solely useful to outcompete siblings instead of long-term offspring survival. Whether any or all of the above hypotheses are true or not is debatable, but the prophase I meiotic arrest is highly conserved from *C. elegans* and *Drosophila* to humans, and this arrest is critical to the oocyte growth and future embryonic development. However, the molecular means by which this arrest is maintained does differ somewhat (Sagata, 1998).

In 1935, Pincus and Enzmann were the first to show that the removal of prophase I arrested rabbit oocytes from their follicular environment resulted in the spontaneous resumption of meiosis (Pincus and Enzmann, 1935). This finding supported a model where the surrounding somatic cells regulated maintenance of the prophase I meiotic arrest. The prophase I meiotic arrest is maintained by several similar mechanisms across different species with some distinct differences. In general, in mouse, *Xenopus*, human, porcine, bovine, chicken, and *C. elegans* the primary meiotic arrest is maintained by synthesis of inhibitory factors in the somatic cells surrounding the oocyte and within the oocyte itself that result in CDK1/CDC2 inactivation in the oocyte (Parker and Piwnicka-Worms, 1992; Bilodeau *et al.*, 1993; Govindan *et al.*, 2006; Shimada, 2012; Cook, 2017). One of the primary factors involved in maintaining the oocyte in the prophase I meiotic arrest is cAMP. In mammals and *Xenopus*, cAMP is synthesized both in the oocyte and the surrounding cumulus cells where it is continuously transported into the oocyte by gap junctions (Shimada, 2012). In contrast, in the *C. elegans* oocyte, instead of direct transport of cAMP in the oocyte, the somatic gonadal sheath cells synthesize G_g/α and transport it into the developing oocytes, which activates adenylate cyclase and elevates cAMP levels (Govindan *et al.*, 2006, 2009). This differs slightly in mammalian oocytes where the constitutively active GS-linked receptors, GPR3 and GPR12, stimulate adenylate cyclase to make cAMP (Arur, 2017). The resulting high levels of cAMP in the oocytes activates the cAMP-dependent kinase, PKA, which then phosphorylates and activates Wee kinase family members, including the oocyte-specific Wee1B kinase (Han *et al.*, 2005). Wee kinase family members include Wee1 and Myt kinases which can phosphorylate and inactivate CDK1/CDC20 at two highly-conserved residues, Thr14 and Thr15 (Parker and Piwnicka-Worms, 1992; Fattaey and Booher, 1997). This CDK1/CDC20 inactivation prevents activation of the maturation promoting factor (MPF; a complex consisting of cyclin B and CDK1/CDC20) and continually holds the oocytes in prophase I arrest (Han *et al.*, 2005). In bovine, it appears that cAMP is degraded to AMP which activates adenosine monophosphate-activated protein kinase (AMPK), and although this is necessary for meiotic arrest, it is currently unknown how AMPK activity relates to low MPF which is also necessary to maintain the prophase I

arrest (Bilodeau-Goeseels, 2011). There is evidence in mouse, *Xenopus*, human, and porcine that activation of Wee kinases and inhibition of CDK1/CDC20 is a conserved mechanism, although whether the target kinase is Wee1 or Myt differs between organisms (Han *et al.*, 2005; Shimaoka *et al.*, 2009; Shimada, 2012). Despite being a powerful model organism, surprisingly little is known regarding mechanisms for maintenance of the prophase I arrest in *Drosophila*. It is clear that the *Drosophila* oocyte accumulates the CDK1-inhibitor, Dap, which keeps it in S-phase whereas the nurse cells have low levels of Dap and being multiple rounds of endoduplication and as a result, become highly polyploid (Hong *et al.*, 2003). An unique feature of the *Drosophila* arrest is that the surrounding somatic cells (nurse cells) are dispensable for maintenance of the prophase I arrest, which is quite interesting considering the important role nurse cells have in oocyte determination and growth and that they are also directly connected to the oocyte by cytoplasmic bridges (Bohrmann and Zimmermann, 2008). In summation, although the prophase I arrest is highly conserved across all organisms discussed, the mechanisms employed to maintain it are quite different across species including the importance of the surrounding somatic cells and specific downstream targets that modulate CDK and MPF activity.

Meiotic Resumption/Maturation

While the prophase I arrest is highly conserved, the resumption of meiosis in response to an extracellular stimulus varies across species. For example, in *C. elegans*, major sperm protein (MSP) has been identified as the hormonal stimulus that stimulates both meiotic resumption as well as somatic gonadal sheath cell contraction to move oocytes towards the proximal gonad (McCarter *et al.*, 1999; Miller *et al.*, 2001). *Xenopus* meiotic resumption occurs in response to progesterone which causes a rapid drop in intracellular cAMP levels in the oocyte (Maller *et al.*, 1979). In mammals and chickens, an endogenous luteinizing hormone (LH) surge stimulates meiotic resumption (Seibel *et al.*, 1982; Jaffe and Norris, 2010; Cook, 2017). In mouse oocytes, inactive MPF is present throughout ooplasm and the LH surge causes a rapid decrease in cellular cAMP levels which subsequently decreases PKA and Wee1b activity, and activates CDK1 (Eppig *et al.*, 2004a,b). Androgens (Hammes, 2004) have also been shown to induce meiotic resumption in *Xenopus* (Lutz *et al.*, 2001) and mouse oocytes (Gill *et al.*, 2004). Unlike rodents, bovine and porcine oocyte have very low levels of cyclin B throughout the ooplasm and require active translation of the MPF regulatory subunit to resume meiosis (Naito *et al.*, 1995; Levesque and Sirard, 1996). The signal that initiates meiotic resumption in *Drosophila* remains unknown, but MPF activation is also necessary (Von Stetina and Orr-Weaver, 2011).

After MPF activation and subsequent meiotic resumption, oocytes proceed through several physiological processes in preparation for fertilization. MPF is the master regulator during M phase, and active MPF phosphorylates multiple target proteins which mediate nuclear envelope breakdown (NEBD) also referred as germinal vesicle breakdown (GVBD), chromatin remodeling, and spindle formation (Sagata, 1998). In all species discussed in this article, oocytes do not resume transcription during meiotic maturation, highlighting the importance of maternal mRNA polyadenylation, stability, and degradation (De La Fuente *et al.*, 2004). MPF activates ERK1/2, which then phosphorylates and activates CPEB (Sha *et al.*, 2017), which is a sequence-specific RNA-binding protein that regulates polyadenylation-induced translation. Active CPEB polyadenylates specific mRNAs throughout the ooplasm for targeted translation even as global translation rates are decreasing during meiotic maturation (Schultz *et al.*, 1978; Sha *et al.*, 2017). Unlike mitosis, during meiosis, chromosomes remain tightly condensed as oocytes progress from meiosis I to meiosis II. As mammalian, *Xenopus*, and chicken oocytes progress through meiosis I, they give off a small polar body (PBI), and then continue to metaphase of meiosis II (Dumont, 1972; Mehlmann, 2005; Cook, 2017). Interestingly, in *C. elegans*, meiotic maturation first involves downregulation of MPK-1 (ERK) and later cellular levels rebound to ensure correct maturation of the oocyte, indicating MPK-1 has a dual purpose specific to *C. elegans* oocytes (Arur, 2017). In *Drosophila*, a protein called ENDOS, which is a member of a group of small proteins called α -Endosulfines with wide expression but largely of unknown functions is believed to be involved in meiotic maturation (Von Stetina and Orr-Weaver, 2011). ENDOS encodes a phosphoprotein that regulates all aspect of meiotic maturation, most likely through regulating MPF activity, although what controls activation of ENDOS remains unknown (Von Stetina *et al.*, 2008). Also unique to *Drosophila* is that there is no extrusion of first or second polar body during meiosis (Page and Orr-Weaver, 1997). Taken together, while the organisms discussed here have some distinct features, the critical role of MPF in regulating meiotic maturation is highly conserved.

Secondary Meiotic Arrest

With respect to the secondary meiotic arrest, *C. elegans* and *Drosophila* stand out as very different from the other animals discussed in this article. Unlike the other gonochoristic species in this article, the hermaphroditic *C. elegans* utilizes the major sperm protein (MSP) hormone as a signal for meiotic maturation, thereby ensuring the presence of sperm for fertilization and bypassing the need for a second meiotic arrest (Yamamoto *et al.*, 2006). *C. elegans* MSPs are the most abundant proteins in sperm, where they function as intracellular cytoskeletal proteins and secreted hormones. Secreted MSPs bind to multiple receptors on oocyte and ovarian sheath cell surfaces to activate MPK-1 and induce oocyte maturation and sheath contraction (Yang *et al.*, 2010). The coupling of meiotic resumption and meiotic maturation directly to sperm-sensing confers a selective advantage in *C. elegans* as they continually produce oocytes when sperm is present, and therefore are able to save energy by not producing the metabolically-costly oocytes when sperm is not available (Yamamoto *et al.*, 2006). In *Drosophila*, oocytes arrest at metaphase I and MPF is critically important in maintenance of the second meiotic arrest. For meiotic completion, Cyclin B must be degraded.

The completion of meiosis in *Drosophila* is sperm-independent and results from the mechanical pressure on the oocyte as it passes through the oviduct. It has been proposed that lack of coupling of fertilization to meiotic completion is a remnant from a time when *Drosophila* was able to asexually reproduce. Although most species of *Drosophila* do not support parthenogenic development, some species such as *Drosophila mercatorum* have viable progeny from parthenogenically-activated oocytes (Eisman and Kaufman, 2007; Von Stetina and Orr-Weaver, 2011).

In mammals, chickens, and *Xenopus*, the second meiotic arrest occurs at metaphase of meiosis II (MII) due to the cytostatic factor (CSF) (Nishiyama *et al.*, 2010; Nakamura *et al.*, 2013). CSF was first discovered in *Rana pipiens* where it was shown that injecting the cytoplasm from a MII oocyte halts cell division in a 2-cell blastomere, indicating some transferable cytoplasmic factor in MII oocytes that is responsible for the MII arrest (Masui, 1974). Ultimately, the Mos/MEK/MAPK/Rsk signaling cascade (MOS is a germ cell-specific Raf) has been identified as the CSF in *Xenopus* (Nishiyama *et al.*, 2010). The downstream effect of this cascade is activation of Emi-related protein 1 (ERP1), which is an inhibitor of the anaphase-promoting complex/cyclosome (APC/C), the result of which is maintaining the MII arrest until fertilization occurs (Schmidt *et al.*, 2005). In mice, the CSF also involves MOS and MAPK but not Rsk, although activation of ERP1 is still the end result (Nishiyama *et al.*, 2010). In bovine, porcine and human oocytes, MAPK is also required for the MII arrest, and although the downstream targets are likely similar to mouse or *Xenopus*, these have not been directly assayed (Sun *et al.*, 1999; Gordo *et al.*, 2001; Tatemoto and Muto, 2001). This MII arrest is carefully coupled to fertilization as sperm entry causes a Ca^{2+} wave throughout the oocyte, resulting in the destruction of ERP1, followed by destruction of cyclin B, activation of the APC/C, and finally initiation of zygotic development. Coupling the completion of meiosis with fertilization ensures that the maternal and paternal pronuclei form at the same time and will be in sync upon entry into S phase.

Ovulation

Ovulation is the release of the growing oocyte from the ovary into the reproductive tract for fertilization. Achievement of successful ovulation is carefully orchestrated by the mural granulosa cells responding to the LH surge by activation of their LH-receptor (Russell and Robker, 2007). In all animals discussed in this article the structure receiving the ovulated oocyte is called the oviduct, except in *C. elegans* where oocytes move into the spermatheca. The rate and efficiency of ovulation is a major factor in successful reproduction as it ensures that the oocyte is at the right time and place for successful fertilization. In *Xenopus*, mouse, bovine, and porcine normal ovulation is controlled by the release of luteinizing hormone and follicle-stimulating hormone from the pituitary gland. An important consideration when studying ovulation is whether the species of interest is a mono or poly-ovulator. The majority of the time, bovine and humans are both mono-ovulators whereas porcine and mice are poly-ovulators (Hunter *et al.*, 2004). Distinct from all other animals is *Xenopus* ovulation involving the expulsion of hundreds of mature oocytes into an aquatic environment for external fertilization.

In addition to the unique external fertilization in *Xenopus* discussed above, *C. elegans* and *Drosophila*, and chickens have some distinct ovulation features. In *C. elegans*, the oocyte ovulates into a specialized structure, the spermatheca, is rapidly fertilized and then moves into the uterus within 4 min of ovulation (Kim *et al.*, 2013). In *Drosophila*, surrounding somatic secretory cells in the reproductive tract secrete HR39 (a nuclear hormone receptor steroidogenic factor 1, SF1; NR5A1-related *Drosophila* nuclear hormone receptor), which stimulates ovulation, in addition to other proteins necessary for sperm storage in the reproductive tract after mating (Sun and Spradling, 2013). During *Drosophila* ovulation, only one of the many mature oocytes in the ovaries is released into the oviduct. Furthermore, in *Drosophila*, ovulation serves the dual-purpose of moving the oocyte forward and stimulating meiotic resumption (Von Stetina and Orr-Weaver, 2011). A common feature between *Drosophila*, *C. elegans*, and the chicken is that the female (or hermaphrodite in *C. elegans*) has the ability to store sperm for an extended period of time for fertilization of individually ovulated oocytes in an assembly-line fashion. After a single mating event in flies, females store the sperm and can remain fertile for up to two weeks (Schnakenberg *et al.*, 2012). Female *Drosophila* directly regulate fertilization as she controls the release of sperm from her storage seminal receptacle. *C. elegans* are hermaphroditic and therefore make their own sperm, but they only make a finite amount during a short period of development which is then stored in the spermathecal. Chickens possess specialized sperm storage tubules in their oviduct where they can store sperm until needed for fertilization (Sasanami *et al.*, 2013). The mechanisms by which viable sperm can be stored for an extended period of time is of great interest in other species where sperm storage would be financially beneficial, such as pigs.

Conclusion

This article summarizes the conserved and distinct features and the underlying mechanisms of four important stages of oogenesis and ovulation. In oogenesis, a critical feature of meiosis is the presence of primary and secondary meiotic arrests. While the primary meiotic arrest is conserved across species and organisms, *C. elegans* lack the secondary meiotic arrest. Intriguingly, in *Drosophila* the secondary meiotic arrest occurs in metaphase of meiosis I while in other organisms it takes place at metaphase of meiosis II (Fig. 1). Another important point of consideration while comparing oogenesis and ovulation across species and organisms are the various intra-cellular regulators involved at different stages of oogenesis and the conserved and distinct signaling pathways/underlying mechanisms, as shown in Table 1. Careful consideration of these patterns of conservation are critical when selecting the ideal animal model with the most similarity to humans for that particular process.

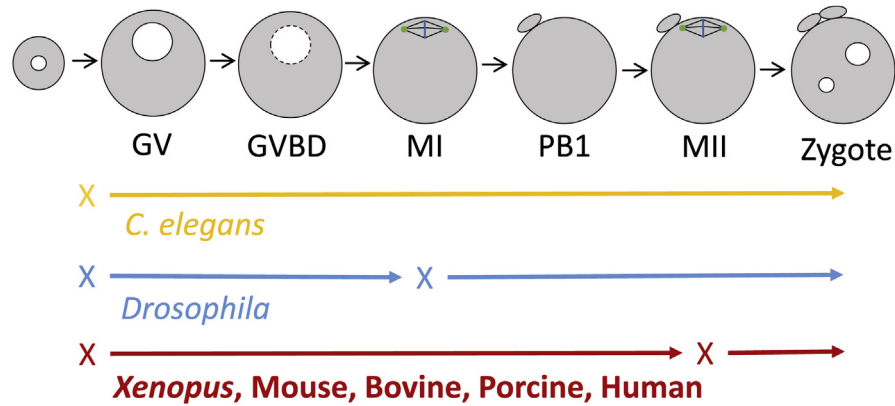


Fig. 1 Conserved primary and secondary meiotic arrests during meiosis. “X” represents points of meiotic arrest. The primary meiotic arrest is conserved across all organisms in this article. *C. elegans* does not have a secondary meiotic arrest. *Drosophila* has a unique secondary meiotic arrest that occurs at metaphase of meiosis I (MI). All other animals considered have a secondary meiotic arrest at metaphase of meiosis II (MII). Modified from Von Stetina, J.R., Orr-Weaver, T.L., 2011. Developmental control of oocyte maturation and egg activation in metazoan models. Cold Spring Harb Perspect Biol 3, a005553.

Table 1 Summary of conserved processes and regulators of meiosis across species

	Primary meiotic arrest	Meiotic		Secondary meiotic arrest	Ovulation	
		Resumption	Maturation		Mono or poly	Store sperm
Human	Prophase I	LH	MPF	MII	Mono	No
Bovine					Mono	Yes
Chicken					Poly	No
Mouse						
Porcine						
<i>Xenopus</i>		Progesterone				
<i>Drosophila</i>		Unknown		MI	Mono	Yes
<i>C. elegans</i>		MSP		None		

Acknowledgements

This work was supported in part by the Eunice Kennedy Shiver National Institute of Child Health and Human Development of the National Institutes of Health under the award numbers T32HD087166 (ALS) and RO1HD086062-01A1 (AS), by MSU AgBioResearch, and by Michigan State University (ALS and AS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

- Arur, S. (2017). Signaling-mediated regulation of meiotic prophase I and transition during oogenesis. *Results Probl Cell Differ*, 59, 101–123.
- Bilodeau-Goeseels, S. (2011). Cows are not mice: The role of cyclic AMP, phosphodiesterases, and adenosine monophosphate-activated protein kinase in the maintenance of meiotic arrest in bovine oocytes. *Mol Reprod Dev*, 78, 734–743.
- Bilodeau, S., Fortier, M. A., & Sirard, M. A. (1993). Effect of adenylate cyclase stimulation on meiotic resumption and cyclic AMP content of zona-free and cumulus-enclosed bovine oocytes in vitro. *J Reprod Fertil*, 97, 5–11.
- Bohrmann, J., & Zimmermann, J. (2008). Gap junctions in the ovary of *Drosophila melanogaster*: localization of innexins 1, 2, 3 and 4 and evidence for intercellular communication via innexin-2 containing channels. *BMC Dev Biol*, 8, 111.
- Cook, M.E., 2017. In: Scanes, C. (Ed.), *Sturkie's Avian Physiology*. Waltham, MA: Elsevier Inc., 1056 pp., ISBN 978-0-12-0407160-5. US \$120 hardback [2015. J Wildl Dis 53, 703–705].
- Dekel, N. (1995). Molecular control of meiosis. *Trends Endocrinol Metab*, 6, 165–169.
- De La Fuente, R., Viveiros, M. M., Burns, K. H., et al. (2004). Major chromatin remodeling in the germinal vesicle (GV) of mammalian oocytes is dispensable for global transcriptional silencing but required for centromeric heterochromatin function. *Dev Biol*, 275, 447–458.
- Dumont, J. N. (1972). Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol*, 136, 153–179.
- Eisman, R., & Kaufman, T. C. (2007). Cytological investigation of the mechanism of parthenogenesis in *Drosophila mercatorum*. *Fly (Austin)*, 1, 317–329.
- Eppig, J. J., Viveiros, M., Bivens, C., & De La Fuente, R. (2004). Regulation of mammalian oocyte maturation. In Peter C. K. Leung, & Eli Y. Adashi (Eds.), *The Ovary* (second ed., pp. 113–129) (Chapter 7).
- Eppig, J.J., Viveiros, M.M., Marin Bivens, C.L., De La Fuente, R., 2004b. Regulation of mammalian oocyte maturation. The Ovary.
- Fattaey, A., & Booher, R. N. (1997). Myt1: A Wee1-type kinase that phosphorylates Cdc2 on residue Thr14. *Prog Cell Cycle Res*, 3, 233–240.

- Gill, A., Jamnongjit, M., & Hammes, S. R. (2004). Androgens promote maturation and signaling in mouse oocytes independent of transcription: A release of inhibition model for mammalian oocyte meiosis. *Mol Endocrinol*, 18, 97–104.
- Gordo, A. C., He, C. L., Smith, S., & Fissore, R. A. (2001). Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. *Mol Reprod Dev*, 59, 106–114.
- Govindan, J. A., Cheng, H., Harris, J. E., & Greenstein, D. (2006). Galphao/i and Galphas signaling function in parallel with the MSP/Eph receptor to control meiotic diapause in *C. elegans*. *Curr Biol*, 16, 1257–1268.
- Govindan, J. A., Nadarajan, S., Kim, S., Starich, T. A., & Greenstein, D. (2009). Somatic cAMP signaling regulates MSP-dependent oocyte growth and meiotic maturation in *C. elegans*. *Development*, 136, 2211–2221.
- Hammes, S. R. (2004). Steroids and oocyte maturation – a new look at an old story. *Mol Endocrinol*, 18, 769–775.
- Han, S. J., Chen, R., Paronetto, M. P., & Conti, M. (2005). Wee1B is an oocyte-specific kinase involved in the control of meiotic arrest in the mouse. *Curr Biol*, 15, 1670–1676.
- Hong, A., Lee-Kong, S., Iida, T., Sugimura, I., & Lilly, M. A. (2003). The p27cip/kip ortholog dacapo maintains the *Drosophila* oocyte in prophase of meiosis I. *Development*, 130, 1235–1242.
- Hunter, M. G., Robinson, R. S., Mann, G. E., & Webb, R. (2004). Endocrine and paracrine control of follicular development and ovulation rate in farm species. *Anim Reprod Sci*, 82–83, 461–477.
- Jaffe, L., & Norris, R. (2010). Initiation of the meiotic prophase-to-metaphase transition in mammalian oocytes. *Oogenesis: The Universal Process*.
- Jones, K. T. (2004). Turning it on and off: M-phase promoting factor during meiotic maturation and fertilization. *Mol. Hum. Reprod.*, 10, 1–5.
- Kim, S., Spike, C., & Greenstein, D. (2013). Control of oocyte growth and meiotic maturation in *Caenorhabditis elegans*. *Adv Exp Med Biol*, 757, 277–320.
- Levesque, J. T., & Sirard, M. A. (1996). Resumption of meiosis is initiated by the accumulation of cyclin B in bovine oocytes. *Biol Reprod*, 55, 1427–1436.
- Lutz, L. B., Cole, L. M., Gupta, M. K., et al. (2001). Evidence that androgens are the primary steroids produced by *Xenopus laevis* ovaries and may signal through the classical androgen receptor to promote oocyte maturation. *Proc Natl Acad Sci USA*, 98, 13728–13733.
- Maller, J. L., Butcher, F. R., & Krebs, E. G. (1979). Early effect of progesterone on levels of cyclic adenosine 3':5'-monophosphate in *Xenopus* oocytes. *J Biol Chem*, 254, 579–582.
- Masui, Y. (1974). A cytosolic factor in amphibian oocytes: Its extraction and partial characterization. *J Exp Zool*, 187, 141–147.
- McCarter, J., Bartlett, B., Dang, T., & Schedl, T. (1999). On the control of oocyte meiotic maturation and ovulation in *Caenorhabditis elegans*. *Dev Biol*, 205, 111–128.
- Mehlmann, L. M. (2005). Stops and starts in mammalian oocytes: Recent advances in understanding the regulation of meiotic arrest and oocyte maturation. *Reproduction*, 130, 791–799.
- Miller, M. A., Nguyen, V. Q., Lee, M. H., et al. (2001). A sperm cytoskeletal protein that signals oocyte meiotic maturation and ovulation. *Science*, 291, 2144–2147.
- Mira, A. (1998). Why is meiosis arrested? *J Theor Biol*, 194, 275–287.
- Naito, K., Hawkins, C., Yamashita, M., et al. (1995). Association of p34cdc2 and cyclin B1 during meiotic maturation in porcine oocytes. *Dev Biol*, 168, 627–634.
- Nakamura, Y., Kagami, H., & Tagami, T. (2013). Development, differentiation and manipulation of chicken germ cells. *Dev Growth Differ*, 55, 20–40.
- Nishiyama, T., Tachibana, K., Kishimoto, T., 2010. Cytostatic arrest: Post-ovulation arrest until fertilization in metazoan oocytes. In: *Oogenesis: The Universal Process*. New York: Wiley.
- Page, A. W., & OrrWeaver, T. L. (1997). Activation of the meiotic divisions in *Drosophila* oocytes. *Dev Biol*, 183, 195–207.
- Parker, L. L., & Piwnicka-Worms, H. (1992). Inactivation of the p34cdc2-cyclin B complex by the human WEE1 tyrosine kinase. *Science*, 257, 1955–1957.
- Pincus, G., & Enzmann, E. V. (1935). The comparative behavior of mammalian eggs in vivo and in vitro: I. The activation of ovarian eggs. *J Exp Med*, 62, 665–675.
- Russell, D. L., & Robker, R. L. (2007). Molecular mechanisms of ovulation: Co-ordination through the cumulus complex. *Human Reproduction Update*, 13, 289–312.
- Sagata, N. (1998). Introduction: Meiotic maturation and arrest in animal oocytes. *Semin Cell Dev Biol*, 9, 535–537.
- Sasanami, T., Matsuzaki, M., Mizushima, S., & Hiyama, G. (2013). Sperm storage in the female reproductive tract in birds. *J Reprod Dev*, 59, 334–338.
- Schmidt, A., Duncan, P. I., Rauh, N. R., et al. (2005). *Xenopus* polo-like kinase Plx1 regulates XErp1, a novel inhibitor of APC/C activity. *Genes Dev*, 19, 502–513.
- Schnakenberg, S. L., Siegal, M. L., & Bloch Qazi, M. C. (2012). Oh, the places they'll go: female sperm storage and sperm precedence in *Drosophila melanogaster*. *Spermatogenesis*, 2, 224–235.
- Schultz, R. M., Lamarca, M. J., & Wassarman, P. M. (1978). Absolute rates of protein-synthesis during meiotic maturation of mammalian oocytes in vitro. *Proc Natl Acad Sci USA*, 75, 4160–4164.
- Seibel, M. M., Smith, D. M., Levesque, L., Borten, M., & Taymor, M. L. (1982). The temporal relationship between the luteinizing hormone surge and human oocyte maturation. *Am J Obstet Gynecol*, 142, 568–572.
- Sha, Q. Q., Dai, X. X., Dang, Y., et al. (2017). A MAPK cascade couples maternal mRNA translation and degradation to meiotic cell cycle progression in mouse oocytes. *Development*, 144, 452–463.
- Shimada, M. (2012). Regulation of oocyte meiotic maturation by somatic cells. *Reprod Med Biol*, 11, 177–184.
- Shimaoka, T., Nishimura, T., Kano, K., & Naito, K. (2009). Critical effect of pigWee1B on the regulation of meiotic resumption in porcine immature oocytes. *Cell Cycle*, 8, 2375–2384.
- Sun, J. J., & Spradling, A. C. (2013). Ovulation in *Drosophila* is controlled by secretory cells of the female reproductive tract. *Elife*, 2.
- Sun, Q. Y., Blumenfeld, Z., Rubinstein, S., et al. (1999). Mitogen-activated protein kinase in human eggs. *Zygote*, 7, 181–185.
- Tatemoto, H., & Muto, N. (2001). Mitogen-activated protein kinase regulates normal transition from metaphase to interphase following parthenogenetic activation in porcine oocytes. *Zygote*, 9, 15–23.
- Von Stetina, J. R., & Orr-Weaver, T. L. (2011). Developmental control of oocyte maturation and egg activation in metazoan models. *Cold Spring Harb Perspect Biol*, 3, a005553.
- Von Stetina, J. R., Tranguch, S., Dey, S. K., et al. (2008). alpha-Endosulfine is a conserved protein required for oocyte meiotic maturation in *Drosophila*. *Development*, 135, 3697–3706.
- Yamamoto, I., Kosinski, M. E., & Greenstein, D. (2006). Start me up: Cell signaling and the journey from oocyte to embryo in *C. elegans*. *Dev Dyn*, 235, 571–585.
- Yang, Y. F., Han, S. M., & Miller, M. A. (2010). MSP hormonal control of the oocyte MAP kinase cascade and reactive oxygen species signaling. *Dev Biol*, 342, 96–107.

Oogenesis, Final Oocyte Maturation and Ovulation, Insects

Yael Heifetz, The Hebrew University, Rehovot, Israel

Uyen Tram, The Ohio State University, Columbus, OH, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

More numerous than any other animal on Earth and found on every continent, insects are prolific and display diverse modes of reproduction (Thornhill and Alcock, 1983). A few reproduce asexually in true parthenogenetic fashion in which males are absent and unfertilized eggs develop into females. The vast majority reproduce sexually, with females either receiving sperm directly during copulation or indirectly collecting sperm packages males deposit in the environment. In some insects, fertilization determines whether the egg develops into males or females. In most Hymenopterans, eggs that are fertilized develop into females while unfertilized eggs develop into males. Female insects are capable of producing enormous numbers of progeny. Per day, a female fruitfly can lay 60 eggs, a honeybee queen 2000 eggs, and a termite queen 43,000 eggs (Klowden, 2013).

The process of producing and maturing an oocyte that is ready for to be fertilized and support embryonic development is composed of several sequential steps: oogenesis, ovulation, movement of the egg down the oviducts, fertilization (in the uterus), and finally deposition of the egg onto the food substratum. Each step is regulated and may feedback on steps preceding it in a regulatory feedback loop. *Drosophila melanogaster*, with its wealth of genetic and molecular tools, has served as a model organism for studying the various steps of this process (Bratu and McNeil, 2015; Hales et al., 2015). This article will focus on oogenesis, ovulation, and oviposition in *D. melanogaster* and highlight the insights provided by the myriad tools available in *Drosophila*.

Ovary Structure

Eggs are produced by the ovaries, which are composed of tubular units called ovarioles. The number of ovarioles per ovary varies from species to species and can be influenced by genotype and environmental conditions (Buning, 1994; Sarikaya et al., 2012). The number of ovarioles determines how many eggs the female is capable of producing. For example, *Drosophila* has 16–20 ovarioles/ovary, the honeybee queen 100–180 and the termite queen 1200. The ovarioles of the *Drosophila* ovary are held together by the peritoneal sheath (a vein-like network of muscle layer that surrounds each of the two ovaries) and converge basally where they attach to the lateral oviduct (King, 1970; Fig. 1). Ovariole development in *Drosophila* is dependent on formation of terminal filaments (TF) which are composed of stacks of 7–10 terminal filament cells (TFC) (Godt and Laski, 1995). Nutrition and rearing temperature during larval development influences the number of terminal filaments that develop and ultimately the number of ovarioles (Godt and Laski, 1995; Sahut-Barnola et al., 1995; Sarikaya et al., 2012). Larva that are provided with additional yeast in their food have more TFCs, TFs, and ovarioles while those on nutrient poor diets have fewer TFCs, TFs, and ovarioles. Rearing at temperatures below 21°C or above 25°C also results in ovaries with fewer ovarioles. At 18°C, the total number of TFCs is not affected but there are more TFCs per TF, thus fewer TF and fewer ovarioles.

Each ovariole is composed of three distinct regions: terminal filament at the anterior end; the germarium which houses the germline stem cells and differentiating germ cells, and produces the egg chambers; and the vitellarium which contains a series of consecutive developing egg chambers, each in a more advanced developmental stage than the one located anterior to it. There are two major types of ovarioles: panoistic in which maturation of the oocyte occurs without support of nurse cells; and meroistic in which the maturation is supported by associated nurse cells that synthesize and supplement the oocyte with organelles, RNA, and other macromolecules. Meroistic ovarioles can be further subclassified as telotrophic or polytrophic. In telotrophic-meroistic ovarioles (e.g., Hemiptera, Coleoptera), the nurse cells are restricted to the germarium but maintain a connection with the maturing oocyte by cytoplasmic processes called nutritive chords. In contrast, the nurse cells and oocyte in polytrophic-meroistic ovarioles stay connected and progress along the length of the ovariole as a unit (e.g., Hymenoptera, Trichoptera, Lepidoptera, and Diptera). The panoistic ovariole, characteristic of orders such as Odonata, Orthoptera and Dictyoptera, appears to be the ancestral type. Differences in ovariole structure do not limit the number of eggs a female can produce but can impact length of embryogenesis (Buning, 1994).

The *Drosophila* ovariole is polytrophic-meroistic. Each ovariole contains a series of consecutive developing egg chambers, each composed of a single oocyte and 15 nurse cells surrounded by a single layer of somatic cells called follicle cells. Consecutive egg chambers within the ovariole are connected by specialized interfollicular stalks (Fig. 1; King, 1970; Spradling, 1993; Deng and Bownes, 1998).

Oogenesis, *Drosophila* as a Model

Each oocyte develops within its own support unit, the egg chamber, which is formed in the germarium, a specialized structure at the anterior end of the ovariole (King, 1970; Spradling, 1993; Deng and Bownes, 1998; Matova and Cooley, 2001). The germarium is home to three different stem cell populations (germline stem cell, follicular stem cell, and escort stem cell) and the niche, a "regulatory microenvironment that controls stem cell self-renewal and differentiation" (Xie et al., 2008). The germline stem cell

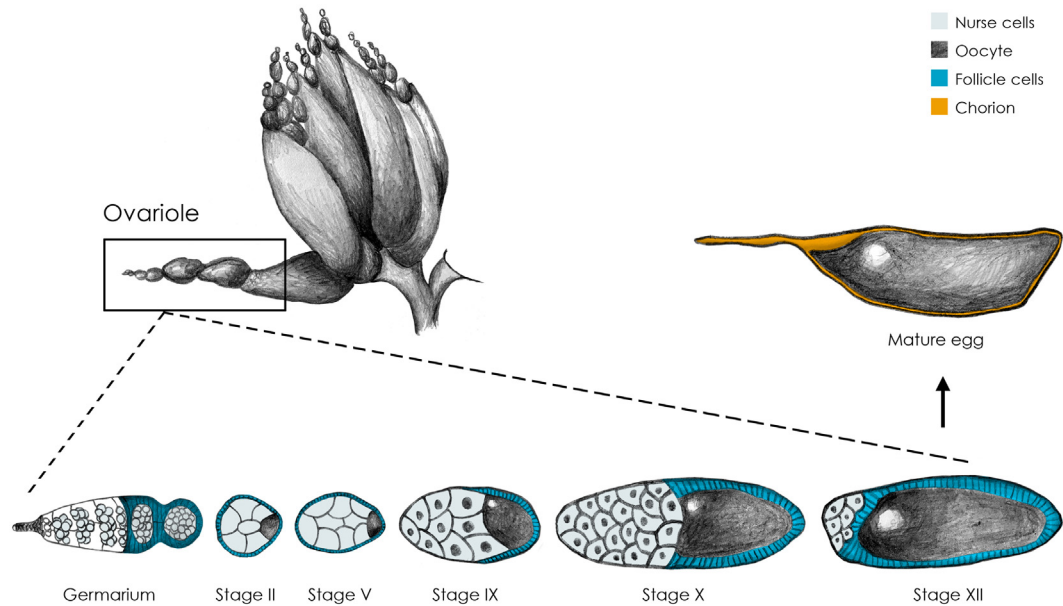


Fig. 1 The *Drosophila* ovary – an example of Polytrrophic Meroistic Ovaries. Schematic drawing of the *Drosophila* ovary. The *Drosophila* female has a pair of ovaries (only one ovary is shown here), each of which consists of 16-20 ovarioles (top left), each of which contains several egg chambers at different developmental stages. One ovariole has been teased out and the boxed area is shown in more detail below. At the tip of each ovariole is the germarium (bottom left), which contains germline and somatic stem cells which divide continuously and give rise to new egg chambers. Somatic follicle cells are shown in blue; nurse cells, light blue; oocyte, dark gray; oocyte nucleus, white. Different consecutive egg chambers (stage II-XII) and the mature oocyte (stage 14) are shown at the bottom. Vitellogenesis (yolk uptake) starts at stage VIII. In the mature egg, the nurse cells and the follicle cells have degenerated, and the oocyte nucleus is arrested at metaphase I. orange: chorion – the egg shell. See text for further details. Figure modified from Silva, D., Jemc, J.C., 2015. Sorting out identities: an educational primer for use with "novel tools for genetic manipulation of follicle stem cells in the *Drosophila* ovary reveal an integrin-dependent transition from quiescence to proliferation". *Genetics* 201,13 -22. Graphics by Zohar Nir-Amitin.

undergoes an asymmetric division, giving rise to a germline stem cell, which remains close to the terminal filament and cap cells of the germarium, and a cystoblast, which moves posteriorly. The cap cells maintain and support stem cell self-renewal. The cystoblast undergoes four rounds of synchronous mitotic divisions with incomplete cytokinesis, creating a cluster of 16 cells that remain connected by cytoplasmic bridges called ring canals. The cluster becomes enrobed by cellular extensions of escort cells which support their differentiation. The escort cells then hand off the cyst posteriorly where it becomes encapsulated by follicle cells which function in patterning the oocyte, physically shaping the egg chamber, synthesizing yolk proteins, secreting the egg shell, and creating specialized structures for sperm entry and respiration (Fig. 1).

Of the 16 cells in the egg chamber, one becomes the oocyte while the remaining 15 become nurse cells, which synthesize RNA, organelles, and macromolecules that supplement the developing oocyte, eventually dumping all of their cytoplasmic content into the oocyte (King, 1970; Spradling, 1993; Deng and Bownes, 1998; Matova and Cooley, 2001). The mitotic divisions that produce that 16-cell cyst are nonlinear and asymmetric resulting in 2 cells with 4 ring canals, and the remainder with 3, 2, or 1 ring canals. In addition to differences in the number of ring canals, the cells also differ in the amount of fusome, a cytoplasmic structure composed of vesicles and cytoskeletal components, inherited (Lin and Spradling, 1995). The fusome links all cells of the cyst together through the ring canals, synchronizing the cell cycle, and is required for specifying the future oocyte. The original fusome is inherited by the cystoblast from the germline stem cell. At each mitotic division, a new fusome forms in each ring canal, and one half of each new fusome fuses with the original fusome, resulting in one cell containing more fusome than its sisters. The cell with 4 ring canals and most fusomal material becomes the oocyte. A current model suggests that the fusome is an organizing matrix that directs oocyte determinants to a single cell. Once an oocyte is specified, the cystoblast becomes surrounded by follicle cells. The egg chamber then exits the germarium and enters the vitellarium where it completes development into a mature oocyte.

The follicle cells play an active role in the physical shaping and molecular patterning of the egg chamber and oocyte (Horne-Badovinac and Bilder, 2005). In the germarium, follicle cells envelope the 16-cell cyst, keeping each cyst separate. Polar cells, found at the anterior and posterior ends of the egg chamber, function in patterning follicle cells and the egg chamber; stalk cells connect consecutive egg chambers; and epithelial follicle cells envelope the entire egg chamber and undergo dramatic cell shape changes and movements that shape and pattern the egg chamber. In addition, the follicle cells secrete an extracellular matrix that encompasses the entire egg chamber and that may play a role in transforming the egg chamber from spherical to elliptical. As the egg chamber grows in size, the follicle cells proliferate, producing 1000 cells total.

Development of the egg chamber takes approximately 3 days and can be divided into 14 stages (Fig. 1). During this period, the egg chamber grows in volume 1000-fold, changing from a 20 μm sphere to an ovoid that is approximately 500 μm long and is

2.2–2.5 times longer than it is wide. Initially, the nurse cells and oocyte are approximately the same size (stages 1–6). As the egg chamber grows, the oocyte grows bigger than the nurse cells (stage 7–12), and ultimately occupies the whole egg chamber as the nurse cells shrink and undergo apoptosis (stage 13–14). In the early stages, mRNA and proteins made in the nurse cells are transported into the oocyte through the ring canals via the molecular motor dynein on microtubules and subsequently become localized in distinct regions within the oocyte. These mRNAs are essential for setting up the anterior-posterior and dorsal-ventral axes of the future embryo. Toward the end of this period, the egg chamber begins to grow more elongate along its anterior-posterior axis. Vitellogenesis begins at Stage 8 and the oocyte starts to uptake yolk proteins synthesized by the follicle cells and fat bodies, growing faster and becoming bigger than the nurse cells. During this period, the follicle cells undergo dramatic migrations and morphological changes. The majority of the follicle cells migrate toward the posterior end of the egg chamber where the oocyte is located while 50 remain over the nurse cells. At this time, there are 6–8 cells that do not participate in the migration. These are called border cells and they move in between the nurse cells and migrate toward the anterior end of the oocyte, coming to a rest across from the oocyte nucleus. The border cells create the pore in the micropyle, a specialized opening for sperm entry. In a third migration, follicle cells at the nurse cell-oocyte border, the so-called centripetal cells, move between the oocyte and nurse cells, covering the anterior end of the oocyte, and thus complete the encapsulation of the oocyte by follicle cells. The centripetal cells will form the operculum, a door through which the larva will exit the eggshell at hatching, and the cone structure that is the micropyle. The follicle cells covering the oocyte adopt a columnar shape while those of the nurse cells flatten (stretch cells). The columnar cells are secretory, and beginning at Stage 10A will secrete the components that make up the layers of the eggshell, the vitellum membrane and chorion, that protect the developing embryo from the environment. Beginning at Stage 10B, two patches of follicle cells located at the dorsoanterior of the oocyte are induced to create a pair of dorsal appendages, which are respiratory tubes for the embryo. During Stages 10B–14, the oocyte grows rapidly as the nurse cells transfer/dump their cytoplasm into the oocyte. By Stages 13 and 14, specialized eggshell structures (a pair of dorsal appendages, the micropyle, and the operculum) are visible, and the nurse cells and follicle cells undergo apoptosis (King, 1970; Spradling, 1993; Deng and Bownes, 1998; Matova and Cooley, 2001; Horne-Badovinac and Bilder, 2005).

The length of time to produce a mature egg from the germline stem cell stage depends on female genotype, age, and mating status, and environmental factors such as nutrition, degree of crowding, temperature, and humidity. Under optimal conditions, each ovariole is capable of producing on average two mature oocytes a day. The adult *D. melanogaster* female reaches her maximum daily output of egg production by day four and is able to maintain such a rate for two weeks.

The maturation of oocytes is regulated by several mechanisms (Drummond-Barbosa and Spradling, 2001; Soller *et al.*, 1999). Progression of oogenesis is negatively regulated by the presence of stage 14 oocytes in the ovary, which may exert mechanical pressure on the base of the ovary. In the ovaries of unmated females, two or three stage 14 oocytes are often present per ovariole, suggesting that oogenesis has ceased. After mating, the female ovulates the mature oocytes from the ovarioles and begins ovipositing, and maturation of additional oocytes commences. Thus, mating stimulates ovulation, which relieves the mechanical pressure on the ovary, which in turn stimulates oogenic progression via a feedback mechanism. It has been shown that the seminal fluid accessory gland proteins Acp70A (sex peptide) and Acp26Aa (ovulin) induces ovulation and stimulates oogenic progression. Sex peptide stimulates oogenic progression at a key control point of early vitellogenesis around stage 9. This control point regulates whether egg chambers will proceed with development or undergo apoptosis and is mediated by the balance of juvenile hormone and ecdysone (Soller *et al.*, 1999). In the sexually mature virgin female, when each ovariole contains 2 stage 14 oocytes, the production of new eggs is prevented by resorption of stage 9 oocytes. Oocyte resumption occurs because of high levels of 20-hydroxyecdysone (20E) in the hemolymph. Mating induces an increase in the level of juvenile hormone which relieves the 20E-mediated oocyte resorption. It has been shown that Acp70A (sex peptide) relieves this arrest, probably by increasing the level of juvenile hormone. Juvenile hormone stimulates progression of oocytes through the control point at stage 9, and it involves an increased uptake of yolk proteins from the hemolymph, as well as an increase of yolk protein synthesis in the ovary (Soller *et al.*, 1999).

Nutrient availability is another important regulator of oogenesis. Under starvation conditions, there is a decrease in proliferation of germline stem cells and follicle cells; cell death of cytotoblasts prior to encapsulation by follicle cells; accumulation of P bodies, cytoplasmic foci which act as a repository for mRNA storage or degradation, in previtellogenic egg chambers (Stage 6–8); and apoptosis of Stage 8 egg chambers (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; Burn *et al.*, 2015). Insulin signaling from the central nervous system and brain is required for mediating changes in proliferation rates and accumulation of P bodies in the response to changing nutrient levels, and these changes are reversible once high nutrient conditions are available again.

At the end of oogenesis, the mature oocyte is poised for fertilization and to begin embryogenesis. It contains maternal RNA, proteins and other molecules needed to initiate and support embryogenesis, and its nucleus is arrested at the first meiotic metaphase. The egg shell, composed of the chorion and vitelline membrane, is specialized to allow sperm binding and entry pre-fertilization and to protect the embryo during its development post-fertilization.

The Release of Oocytes From the Ovary – Ovulation

Ovulation, the release of an oocyte from the ovary, is a two-step process (Buning, 1994). First, the opening/rupturing of the oocyte follicle and of the innermost cells in the pedicel region at the base of the ovary releases the oocyte from the ovary. Then, contraction of the ovarioles, pedicels, and oviduct musculature moves the oocyte into the lateral oviduct. Ultrastructural studies of ovulation in the large aquatic beetle *Dysticus marginalis* (Coleoptera) and histological sections of whole ovaries in the mosquito *Culiseta inornata*

(Diptera) show that the pedicel is ruptured during ovulation and that the appearance of the first mature oocyte at the pedicel region triggers autolysis of the pedicel's innermost cells, opening the plug and releasing the mature oocyte from the ovariole (Buning, 1994, Fox and Brust, 1996).

For some insects, mating initiates ovulation (Grillot and Raabe, 1989; Raabe, 1986). In the blood-sucking hemipteran *Rhodnius prolixus*, for example, virgin females retain eggs in the ovary for many days while mated females deposit eggs soon after they are formed (Pratt and Davey, 1972). Ovulation in unmated *D. melanogaster* females, in contrast, occurs at a very low rate (1 egg/day) and becomes dramatically increased by mating (Heifetz *et al.*, 2000). Some insects, such as walking sticks, ovulate one oocyte at a time (*Clitumnus extradentatus*, Mesnier-Sabin, 1981; *Carausius morosus*, Thomas, 1979), while other insects, such as Orthoptera, ovulate oocytes from all ovarioles simultaneously. It is not known which mechanism operates in *D. melanogaster*.

Ovulation, *Drosophila* as a Model

Mature unmated *D. melanogaster* females spontaneously ovulate at a very low rate (~1 egg/day). Mating, however, dramatically and quickly increases the ovulation rate. Within 90 min post-mating, an increase in ovulation is evident and this increased rate is sustained for several days. A mated female can ovulate about 60 eggs a day for several days after mating. King (1970) indicated that degeneration of the innermost cells at the base of the ovariole, which plugs the entrance to the lateral oviduct, and massive muscle contractions at the base of the ovary and oviducts move the oocyte from the ovariole into and down the oviduct, to the uterus where fertilization takes place (Fig. 2). As occurs in mammals, the follicle cells remain in the ovary and form a "corpus luteum" which accumulate yellowish pigmentation and express genes encoding steroid hormone biosynthetic enzymes that are required for female fertility (Deady *et al.*, 2015).

In *Drosophila*, as in other insects that been studied thus far, rupturing of the follicle is a two-step process: 1) degradation of the posterior follicle cells of the mature oocyte and squeezing the remaining follicle cells toward the anterior, as the oocyte moves down into the lateral oviduct, and 2) rupturing of the follicle. Follicle rupturing, and oocyte release require the activity of proteolytic enzymes like matrix metalloproteinase 2 (Mmp2) which is expressed in the posterior follicle cells of stage-14 egg chambers. Reducing the expression level of *Mmp2*, specifically in stage 14 follicle cells, decreases ovulation and egg-laying rate. *Mmp2* activation is stimulated by octopamine. Octopamine binds to octopamine receptors in mushroom bodies (OAMB) located on mature follicle cells which leads to an increase in intracellular CA^{2+} that stimulates *Mmp2* activity. Furthermore, it was found that Shade, an enzyme involved in the last 4 stages of 20-hydroxyecdysone (20E) biosynthesis, is highly active in stage 14 follicle cells, converting ecdysone to the more active 20E. 20E signaling in stage 14 follicle cells is needed to stimulate *Mmp2* response to octopamine resulting in follicle rupture and ovulation (Lee *et al.*, 2003; Deady *et al.*, 2015; Knapp and Sun, 2017).

Octopamine signaling from the central nervous system coordinates an egg's release from the ovary and its passage into the oviduct. Octopamine binds G-protein coupled beta adrenergic-like octopamine receptors (*octβ2r*) and OAMB that are expressed in the oviduct epithelium. Octopamine binding stimulates a downstream cascade which may lead to relaxation of muscles and secretion of fluid for egg movement and possibly for egg activation. Octopamine might increase the amplitude of ovarian contraction and relax the oviduct musculature, allowing the oocyte to move into the oviduct more easily (Middleton *et al.*, 2006). Recently, Rubinstein and Wolfner (2013), showed that Ovulin relaxes the oviduct musculature, indirectly, by increasing the number of octopamine synaptic sites in the female reproductive tract after mating. In addition, Lim *et al.* (2014) found that *octβ2r* expressed in oviduct epithelial cells are essential for ovulation and movement of eggs along the reproductive tract. Homozygous *octβ2r* mutants are female sterile. Lim *et al.* (2014) suggests that mating activates octopamine neurons which innervate the oviduct. In addition, molecules released from secretory cells of *Drosophila* female reproductive glands, the spermathecal secretory cells and the female accessory glands (both connected by a thin stalk to the uterus), are required for efficient ovulation and egg movement along the female reproductive tract (Sun and Spradling, 2013; Schnakenberg *et al.*, 2011).

Activation of the egg to start development is triggered by fertilization in many animals (e.g., frogs, mice, other mammals), whereas in others, including *Drosophila*, activation is triggered by contact spawning media or squeezing of the egg and coming in contact with the oviduct environment. In *Drosophila*, ovulation initiates egg activation. Shortly after the onset of ovulation, while most of the egg is still in the ovary (Fig. 2), the egg starts to become impermeable to small molecules. The egg's impermeability increases progressively as the egg moves into the lateral oviduct. Once the egg is in the common oviduct, just shortly before the egg enters the uterus, cross-linking of the vitelline membrane proteins increases and meiosis resumes. Thus, ovulation causes changes in the egg that prepare it for fertilization in the uterus and subsequent embryogenesis. Another consequence of activation, resumption of translation, also initiates without fertilization in *Drosophila*.

Control of the Egg-Laying Process

Drosophila females begin to ovulate as early as 1.5 hrs after mating and by 6 hrs after mating has begun to deposit eggs at high rate. What regulates and sustains the high rate of egg-laying after the female is mated?

Neuro-hormonal regulation. The female reproductive tract is innervated by two pairs of nerves that branch separately from the abdominal median nerve trunk (AbNvTr) (Middleton *et al.*, 2006; Heifetz, 2004). One nerve projects to the junction of the ovaries and lateral oviduct, where it branches repeatedly. From here the nerves radiate anteriorly across the surface of the peritoneal sheath.

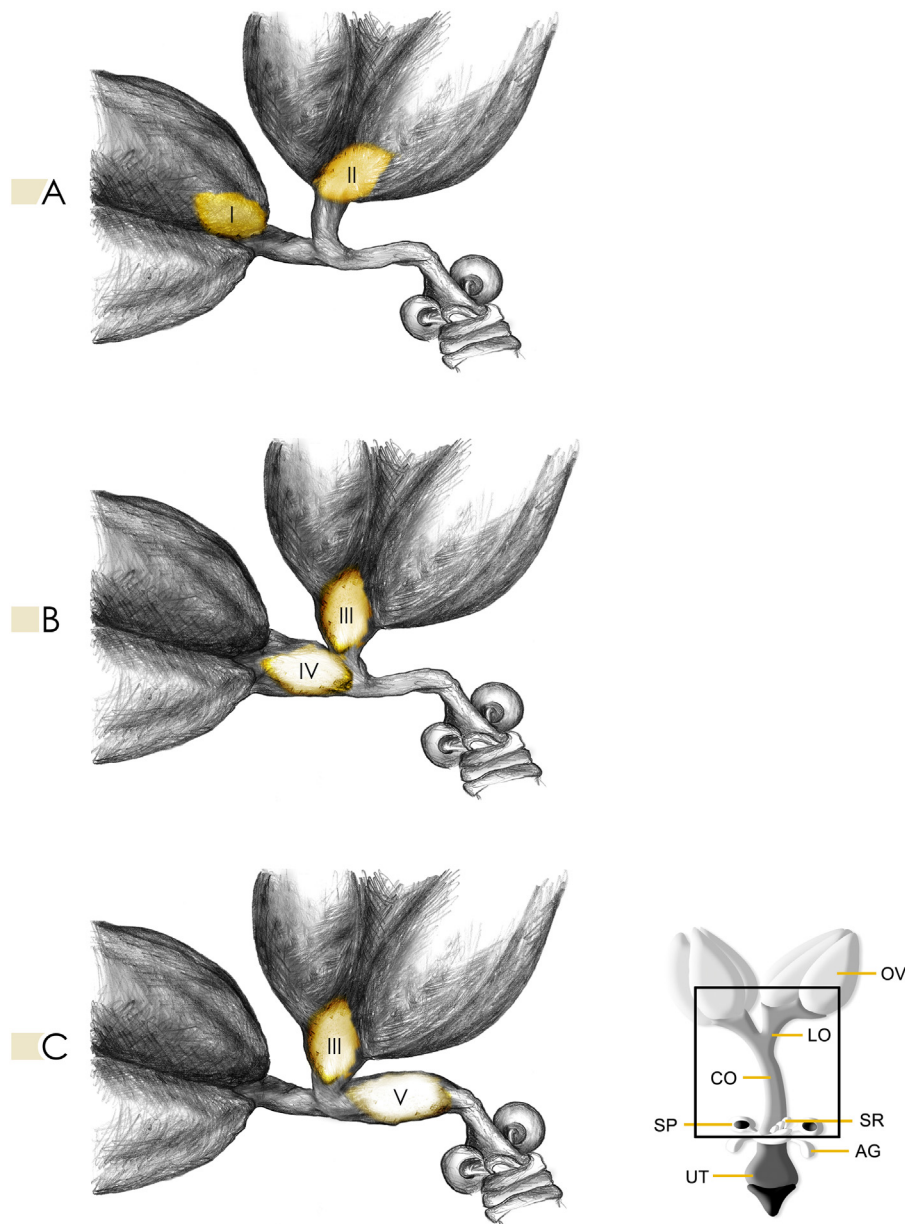


Fig. 2 Stages of ovulation in *Drosophila*. Schematic drawing of different stages of oocyte release from the ovary, based on Heifetz *et al.* (2001), which documented the positions of the eggs in the female oviduct at the time of dissection: (A) Eggs at positions I & II are mostly in the ovary and their posterior ends are at the anterior end of the lateral oviduct; (B) Eggs at position III are partly in the lateral oviduct and eggs at position IV have already reached the junction of the lateral and the common oviducts, but still remain partly in the ovary; (C) Eggs at position V are within the common oviduct and completely out of the ovary. As ovulation triggers egg activation, oocytes that have moved further in the lateral oviduct toward the common oviduct (i.e. stages III–V) are more likely to have been activated than those that are just at the anterior part of the lateral oviducts (i.e. stage I & II). Bottom right is a schematic drawing of the *Drosophila* female reproductive tract. The black box encloses the regions documented in (A)–(C). AG, accessory gland; CO, common oviduct; LO, lateral oviduct; OV, ovary; SP, spermatheca; SR, seminal receptacle; UT, uterus. Graphics by Zohar Nir-Amitin. Reproduced from Heifetz, Y., Yu, J., Wolfner, M.F., 2001. Ovulation triggers activation of *Drosophila* oocytes. *Dev. Biol.* 234, 416–424.

Studies of oviduct innervation in *Drosophila* reveal that the female oviduct is heavily innervated and receives input from aminergic, peptidergic (type II), and glutamatergic (type I) nerve terminals. Each of type of nerve terminal secretes specific neurohormones/neurotransmitters that elicits a specific response, such as muscle contraction or muscle relaxation. Aminergic nerves synthesize and release monoamines such as octopamine, serotonin, dopamine; peptidergic (type II) nerves synthesize and release peptides such dromyosuppression (DMS); and glutamatergic (type I) nerves synthesize and release glutamate which is the fast-excitatory neurotransmitter. In unmated females, octopamine, serotonin, and DMS immunoreactive nerve termini were found in all regions

of the reproductive tract and could serve neuromodulator functions in the female reproductive tract by regulating muscle contraction. Octopamine regulates ovulation rate in *Drosophila* and oviduct muscle contractions in locusts; serotonin regulates muscle contraction in locust oviducts; and DMS regulates contraction of *Drosophila* heart muscle and may regulate muscle contractions in the reproductive tract.

Octopamine, serotonin, and DMS often co-localized in the female reproductive tract, but each had a unique distribution throughout the tract. Furthermore, each region of the reproductive tract contains a unique, region specific combination of neuromodulators. These unique combinations of neuromodulators could potentially induce specific and different muscle modality (relaxation vs. contraction) in each specific region of the female reproductive tract (e.g., oviduct, uterus) that is needed to push the egg downward (Heifetz and Wolfner, 2004; Heifetz *et al.*, 2014).

Secretion. There are two major secretory glands in the female reproductive tract, the spermathecae, a sperm storage organ, and the female accessory glands. The spermatheca is surrounded by a layer of secretory glandular cells (spermathecal secretory cells) and a thick layer of fat body cells. Thus, two types of secretion, a glandular and a fat body-derived, may be produced by the spermathecae. When both the spermathecal secretory cells and female accessory glands were genetically ablated early in development, the female is completely sterile. Furthermore, reduction in the number of secretory cells in both glands during early development greatly decreased the ovulation rate. In addition, knocking down the nuclear hormone receptor - *hormone receptor-like in 39* (*hr39*) (required for normal development and function of the spermathecae and female accessory glands), in the secretory cells of both glands significantly increased the time that is required for ovulation. Mutations in *hr39* result in ovaries that are full of mature oocytes, but only few oocytes are released from the ovary (Sun and Spradling, 2013). Interestingly, genetic ablation of the spermathecal secretory cells later in development reduced female egg laying rate, mainly affecting the release of an egg from the uterus post fertilization. This completely changed the mode of reproduction to ovoviviparity, in which eggs remain in the uterus until they are ready to hatch (Schnakenberg *et al.*, 2011).

Mating components. Seminal fluid and sperm transferred to the female reproductive tract during mating have a central role in initiating and regulating the egg-laying process. Immediately after mating the reproductive tract supports the movement of sperm into the storage organs, but from 1.5 hrs after mating onwards the reproductive tract supports and regulates egg movement along the tract, in synchrony with moving sperm to the site of fertilization. Seminal fluid proteins and sperm are both required to stimulate oogenic progression and egg-laying. Seminal fluid proteins (e.g., ovulin) been also shown to initiate ovulation (Wolfner, 2009).

Different aspects of mating (sperm, seminal proteins, other seminal components, physical effects) also, independently, modulate changes in neuromodulator (octopamine, serotonin, octopamine, DMS) levels in the female reproductive tract. Interestingly, mating regulates the available combinations of neuromodulators in different regions of the *Drosophila* reproductive tract (i.e. induce or inhibit release of neuromodulators from the nerve termini). For example, at 90 min after the start of mating at ovulation onset, octopamine immunoreactivity decreases relative to unmated in the uterus. Serotonin, however, decreases in the ovary but increases in the common oviduct. DMS immunoreactivity decreases in the ovary and the common oviduct and increases in the uterus. This clearly indicates that mating changes region/time-specific balances among these neuromodulators, allowing the initiation of ovulation and movement of eggs (Heifetz *et al.*, 2014).

Conclusion

For many organisms, the female reproductive tract is a vital component in the process of internal fertilization. It facilitates the production and passage of gametes, gamete activation, and gamete fusion. Mechanisms that facilitate ovulation and egg-laying in many insects, including *Drosophila*, are still not fully understood. While insects display diverse modes of reproduction, the process of producing an egg has many parallels and show different degrees of conservation among insects, and beyond, highlighting many general principles of oogenesis, egg maturation and ovulation. Interestingly, recent studies on ovulation highlighted a degree of conservation between mammals and *Drosophila* in molecules that mediate the communication between the reproductive tract and gametes. More detailed studies about the cellular and molecular processes occurring in the female reproductive tract leading to oogenic progression, ovulation and egg activation in different insect from various reproductive modes is warranted. A comparative approach between insects and mammals could bring forth new insights into mammalian female reproduction and infertility, and in designing a new generation of pest control tools.

References

- Bratu, D. P., & McNeill, G. P. (Eds.). (2015). *Drosophila Oogenesis: Methods and Protocols*. New York: Humana Press.
- Buning, J. (1994). *The insect ovary: ultrastructure, previtellogenic growth and evolution*. London: Chapman and Hall.
- Burn, K. M., Shimada, Y., Ayers, K., et al. (2015). Somatic insulin signaling regulates a germline starvation response in *Drosophila* egg chambers. *Dev. Biol.*, 398, 206–217.
- Deady, L. D., Shen, W., Mosure, S. A., Spradling, A. C., & Sun, J. (2015). Matrix metalloproteinase 2 is required for ovulation and corpus luteum formation in *Drosophila*. *PLoS Genet.*, 11, e1004989.
- Deng, W. M., & Bownes, M. (1998). Patterning and morphogenesis of the follicle cell epithelium during *Drosophila* oogenesis. *Int. J. Dev. Biol.*, 42, 541–552.
- Drummond-Barbosa, D., & Spradling, A. C. (2001). Stem cells and their progeny respond to nutritional changes during *Drosophila* oogenesis. *Dev. Biol.*, 231, 265–278.
- Fox, A. S., & Brust, R. A. (1996). Parity diagnosis and ovulation in *Culiseta inornata* (Diptera: Culicidae). *J. Med. Entomol.*, 33, 402–412.

- Godt, D., & Laski, F. A. (1995). Mechanisms of cell rearrangement and cell recruitment in *Drosophila* ovary morphogenesis and the requirement of *bric a brac*. *Development*, 121, 173–187.
- Grillot, J.-P., & Raabe, M. (1989). Regulation of last reproduction steps in tsetse flies (Glossina: Diptera). *Invertebr. Repr. Dev.*, 16, 119–130.
- Hales, K. G., Korey, C. A., Larracuent, A. M., & Roberts, D. M. (2015). Genetics on the fly: A primer on the *Drosophila* model system. *Genetics*, 201, 815–842.
- Heifetz, Y., Lindner, M., Garini, Y., & Wolfner, M. F. (2014). Mating regulates neuromodulator ensembles at nerve termini innervating the *Drosophila* reproductive tract. *Curr. Biol.*, 24, 731–737.
- Heifetz, Y., Lung, O., Frongillo, E. A., & Wolfner, M. F. (2000). The *Drosophila* seminal fluid protein Acp26Aa stimulates release of oocytes by the ovary. *Curr. Biol.*, 10, 99–102.
- Heifetz, Y., & Wolfner, M. F. (2004). Mating, seminal fluid components, and sperm cause changes in vesicle release in the *Drosophila* female reproductive tract. *Proc. Natl. Acad. Sci. USA*, 101, 6261–6266.
- Heifetz, Y., Yu, J., & Wolfner, M. F. (2001). Ovulation triggers activation of *Drosophila* oocytes. *Dev. Biol.*, 234, 416–424.
- Horne-Badovinac, S., & Bilder, D. (2005). Mass transit: Epithelial morphogenesis in the *Drosophila* egg chamber. *Dev. Dyn.*, 232, 559–574.
- King, R. C. (1970). *Ovarian Development in Drosophila melanogaster*. New York: Academic Press.
- Klowden, M. J. (2013). *Physiological Systems in Insects* (Third ed.). San Diego: Academic Press.
- Knapp, E., & Sun, J. (2017). Steroid signaling in mature follicles is important for *Drosophila* ovulation. *Proc. Natl. Acad. Sci. USA*, 114, 699–704.
- LaFever, L., & Drummond-Barbosa, D. (2005). Direct control of germline stem cell division and cyst growth by neural insulin in *Drosophila*. *Science*, 308, 1071–1073.
- Lee, H.-U., Seong, C.-S., Kim, Y.-C., Davis, R. S., & Han, K.-A. (2003). Octopamine receptor OAMB is required for ovulation in *Drosophila melanogaster*. *Dev. Biol.*, 264, 179–190.
- Lim, J., Sabandal, P. R., Fernandez, A., et al. (2014). The octopamine receptor Octb2R regulates ovulation in *Drosophila melanogaster*. *PLOS ONE*, 9, e104441.
- Lin, H., & Spradling, A. C. (1995). The role of membrane skeletal proteins in germline stem cell division and oocyte determination during *Drosophila* oogenesis. *Mol. Biol. Cell.*, 6, 159a.
- Matova, N., & Cooley, L. (2001). Comparative aspects of animal oogenesis. *Dev. Biol.*, 231, 291–320.
- Mesnier-Sabin, M. (1981). Study of ovulation process in the stick insect, *Clitumnus extradentatus*. *J. Insect Physiol.*, 27, 425–433.
- Middleton, C. A., Nongthomba, U., Parry, K., et al. (2006). Neuromuscular organization and aminergic modulation of contractions in the *Drosophila* ovary. *BMC Biol.*, 4, 17.
- Raabe, M. (1986). Insect reproduction: Regulation of successive steps. In P. D. Evans, & V. B. Wigglesworth (Eds.), *Advances in Insect Physiology* (Vol. 19, pp. 30–154). London: Academic Press.
- Rubinstein, C. D., & Wolfner, M. F. (2013). *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. *Proc. Natl. Acad. Sci. USA*, 110, 17420–17425.
- Sahut-Barnola, I., Godt, D., Laski, F. A., & Couderc, J. L. (1995). *Drosophila* ovary morphogenesis: analysis of terminal filament formation and identification of a gene required for this process. *Dev. Biol.*, 170, 127–135.
- Sarikaya, A. A., Belay, A. A., Aisha, D., et al. (2012). The roles of cell size and cell number in determining ovariole number in *Drosophila*. *Dev. Biol.*, 363, 279–289.
- Schnakenberg, S. L., Matias, W. R., & Siegal, M. L. (2011). Sperm-storage defects and live birth in *Drosophila* females lacking spermathecal secretory cells. *PLOS Biol.*, 9, e1001192.
- Soller, M., Bowmes, M., & Kubli, E. (1999). Control of oocyte maturation in sexually mature *Drosophila* females. *Dev. Biol.*, 208, 337–351.
- Spradling, A. C. (1993). Developmental Genetics of Oogenesis. In M. Bate, & A. Martinez Arias (Eds.), *The Development of Drosophila melanogaster* (pp. 1–70). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
- Sun, J., & Spradling, A. C. (2013). Ovulation in *Drosophila* is controlled by secretory cells of the female reproductive tract. *eLife*, 2, e00415.
- Thomas, A. (1979). Nervous control of egg progression into the common oviduct and genital chamber of the stick-insect *Carausius morosus*. *J. Insect Physiol.*, 25, 811–821.
- Thornhill, R., & Alcock, J. (Eds.). (1983). *The Evolution of Insect Mating Systems*. Cambridge: Harvard University Press.
- Wolfner, M. F. (2009). Battle and ballet: Molecular interplay between the sexes in *Drosophila*. *Biol. Reprod.*, 81, 64.
- Xie, T., Song, X., Jin, Z., Pan, L., Weng, C., Chen, S., & Zhang, N. (2008). Interactions between stem cells and their niche in the *Drosophila* ovary. *Cold Spring Harbor Symposia on Quantitative Biology*, 73, 39–47.

Further Reading

- Bratu, D. P., & McNeil, G. P. (Eds.). (2015). *Drosophila Oogenesis: Methods and Protocols*. New York: Humana Press.
- Burn, K. M., Shimada, Y., Ayers, K., et al. (2015). Somatic insulin signaling regulates a germline starvation response in *Drosophila* egg chambers. *Dev. Biol.*, 398, 206–217.
- Cetera, M., Ramirez-San Juan, G. R., Oakes, P. W., et al. (2014). Epithelial rotation promotes the global alignment of contractile actin bundles during *Drosophila* egg chamber elongation. *Nat. Commun.*, 5, 5511.
- de Cuevas, M., Lilly, M. A., & Spradling, A. C. (1997). Germline cyst formation in *Drosophila*. *Annu. Rev. Genet.*, 31, 405–428.
- Klowden, M. J. (2013). *Physiological Systems in Insects* (Third ed.). San Diego: Academic Press.
- Huynh, J.-R., & St. Johnston, D. (2004). The origin of asymmetry: Early polarization of the *Drosophila* germline cyst and oocyte. *Curr. Biol.*, 14, R438–R449.
- Minkenberg, O. P. J. M., Tatar, M., & Rosenheim, J. A. (1992). Egg load as a major source of variability in insect foraging and oviposition behavior. *OIKOS*, 65, 134–142.
- Osterfield, M., Berg, C. A., & Shvartsman, S. Y. (2017). Epithelial patterning, morphogenesis, and evolution: *Drosophila* eggshell as a model. *Dev. Cell*, 41, 337–348.

Relevant Websites

- <http://flymove.uni-muenster.de/>. –FlyMove.
- <http://www.sdbonline.org/sites/fly/aimain/1aahome.htm>. –The Interactive Fly.

Oogenesis, Final Oocyte Maturation & Ovulation, Echinoderms

Masatoshi Mita, Tokyo Gakugei University, Tokyo, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cdk1 A highly conserved protein that functions as a serine/threonine kinase and is a key player in cell cycle regulation.

cyclic AMP A second messenger for intracellular signal transduction.

cyclin B A member of the cyclin family that is necessary for the progression of cells into and out of M phase of the cell cycle.

Follicle-stimulating hormone A gonadotropin, a glycoprotein polypeptide hormone, that is synthesized and secreted by gonadotropic cells of the anterior pituitary gland, and which regulates the development, growth, pubertal maturation, and reproductive processes of the body.

G protein-coupled receptors A large protein family of seven-transmembrane domain receptors that detect a ligand outside the cell and activate internal signal transduction pathways.

G proteins A family of proteins that act as molecular switches inside cells, and that are involved in transmitting signals from a variety of stimuli outside a cell to its interior.

Germinal vesicle The nucleus of an oocyte that is arrested in prophase of meiosis I.

Germinal vesicle breakdown The dissolution of the nucleus of an oocyte that is arrested in prophase of meiosis I.

Gonad index The growing state of gonads calculated as gonad weight/body weight $\times 100$.

Gonadotropin-releasing hormone A peptide hormone responsible for the release of follicle-stimulating hormone and luteinizing hormone from the anterior pituitary.

Greatwall kinase Gwl is originally described as a nuclear protein required for proper chromosome condensation and M phase progression.

Insulin-super family A group of evolutionarily related proteins that are characterized by having three disulfide bonds in a characteristic motif.

Luteinizing hormone A hormone produced by gonadotropic cells in the anterior pituitary gland. In females, this hormone is a trigger for ovulation and the development of the corpus luteum. In males, luteinizing hormone acts on Leydig cell to produce testosterone.

Maturation-inducing hormone A hormone that acts on oocytes to reinitiate meiotic arrest.

Maturation-promoting factor The complex of cyclin B and cyclin-dependent kinase 1 (cdk1) to stimulate the mitotic and meiotic phases of the cell cycle.

1-Methyladenine The maturation-inducing hormone of starfish.

Ovarian follicle cells Cells surrounding an oocyte in a single layer.

Relaxin A peptide hormone belonging to the insulin/insulin-like growth factor/relaxin superfamily that controls pregnancy and facilitates parturition in placental animals.

Relaxin-like gonad-stimulating peptide A hormone that acts on the gonads to induce gamete shedding by way of the production of maturation-inducing hormone in starfish.

Nomenclature

cAMP Cyclic AMP or 3',5'-cyclic adenosine monophosphate

cdk1 Cyclin-dependent kinase 1

FEED Follicular envelop breakdown

FSH Follicle-stimulating hormone

GI Gonad index

GnRH Gonadotropin-releasing hormone

GPCR G protein-coupled receptor

G proteins Guanine nucleotide-binding proteins

GSS Gonad-stimulating substance

Gwl Greatwall kinase

LH Luteinizing hormone

1-MeAde 1-Methyladenine

MPF Maturation (M phase)-promoting factor

RGP Relaxin-like gonad-stimulating peptide

Introduction

Echinoderms, which are exclusively marine animals, are divided into five classes, the Asteroidea (starfishes), Ophiuroidea (serpent-stars), Echinoidea (sea-urchins, heart-urchins, and sand-dollars), Holothuroidea (sea-cucumbers), and Crinoidea (sea-lilies and feather-stars). The male and female gametes, fertilized eggs, and embryos of echinoids have been one of the major materials for analyses of the mechanisms of gametes and in handling them in laboratories. Especially in sea urchins, both eggs and spermatozoa are readily used in fertilization experiments and can be obtained easily by injecting isotonic potassium chloride into the body cavity or by providing brief electrical stimulation. This is because sea urchin oocytes have already accomplished meiotic maturation within the ovary during the breeding season long before spawning occurs. However, because of the completion of meiosis within the ovary, sea urchin eggs are not suitable for analytical investigations on the mechanism of oocyte maturation. In contrast, in starfish, meiosis is arrested at the prophase of the first maturation division in fully grown oocytes just before spawning, and a ripe ovary contains a large number of such oocytes of almost equal size. Furthermore, in the starfish *Patiria pectinifera*, the time required for spawning and breakdown of germinal vesicles under the influence of some active substances is relatively short, less than 30 min, and the process can be observed using a microscope when oocytes are isolated in seawater. Thus, starfish is a suitable material for elucidating the mechanisms of oocyte maturation and spawning (ovulation). This article reviews the endocrine regulation of oocyte maturation and ovulation in starfish. Oocyte growth is also briefly described.

Oogenesis

Annual reproductive cycles are defined in echinoderms by estimation of breeding seasons from observations of spawning, histological examination of the ovaries, and calculation of the gonad index (GI) (gonad weight/body weight $\times 100$). The starfish *Patiria pectinifera* is commonly found in shallow waters of the Pacific and Japan Sea. The breeding season of this species varies greatly depending upon geographical locality, and occurs relatively earlier in southern Japan and later in northern Japan.

The growth of oocytes in starfish *Patiria pectinifera* is divided into five stages on the basis of their cytological appearance (diameter of oocytes) (Kanatani, 1979, 1985) (Fig. 1(A)). The primary oocytes at stage I are about 10 μm in diameter, with nuclei containing conspicuous strands of chromatin. These oocytes are still present in the gonial cysts and are not surrounded by follicle cells. Numerous synaptonemal complexes are seen throughout the nucleus. The oocytes at stage II (10–30 μm in diameter) are surrounded by a single layer of follicle cells. Some cortical granules first appear in the cytoplasm near the nucleus. In late stage II, some yolk granules begin to appear, often arranging themselves in groups. A marked increase in oocyte size begins with stage III (30–70 μm in diameter). The cytoplasm acquires an organization characteristic of a cell actively engaged in the synthesis and export of proteins. Lampbrush chromosomes are occasionally seen in some nuclei. There is a massive increase in the size of oocytes (70–150 μm in diameter) at stage IV, due in part to a further accumulation of yolk granules. Beginning at stage V, the vitelline envelope appears in the extracellular space between follicle cells and oocytes. The cortical granules in oocytes at this stage are present just beneath the oocyte surface. The oocyte changes little in size (up to ~ 160 μm in diameter) from the end of stage IV.

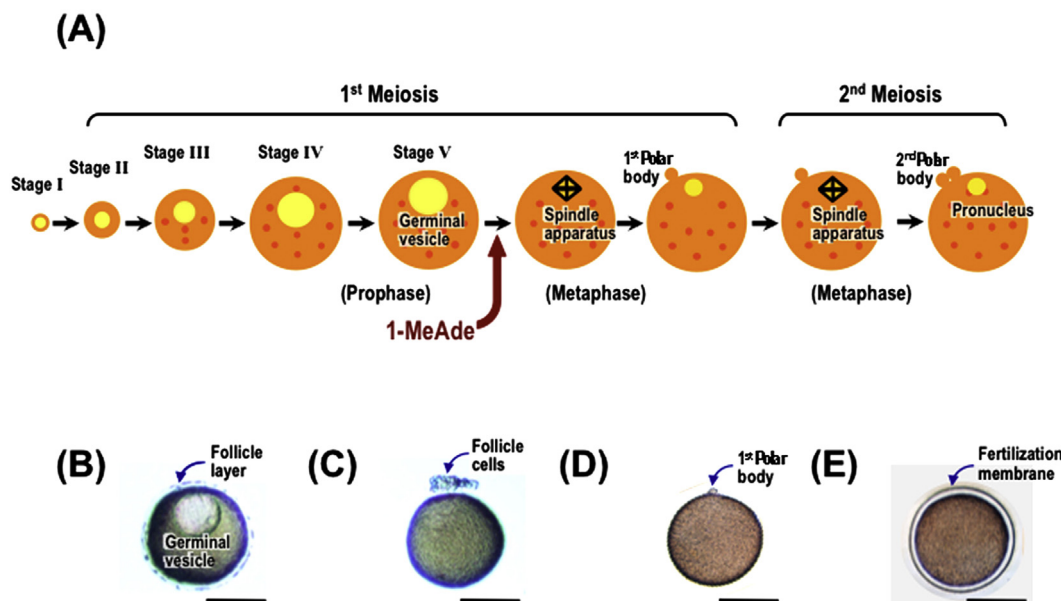


Fig. 1 (A) Meiotic stages in starfish oogenesis and maturation. The growth of oocytes of *Patiria pectinifera* is divided into five stages on the basis of their cytological appearance (diameter of the oocyte) as follows: stage I (ca. 10 μm), stage II (10–30 μm), stage III (30–70 μm), stage IV (70–150 μm), and stage V (>150 μm). (B) Immature oocyte of the starfish *Patiria pectinifera*. The oocyte possesses germinal vesicle, and is tightly surrounded by an envelope consisting of follicle cells. (C) Mature oocyte after 30 min of 1-MeAde treatment. The germinal vesicle and follicular envelope are breakdown. (D) Mature oocyte after 60 min of 1-MeAde treatment. The first polar body is discharged. (E) Fertilized egg. After insemination, fertilization membrane is formed. Bars show 100 μm .

Some vertebrate hormonal steroids, such as 17β -estradiol and progesterone, are present in starfish ovaries (Kanatani, 1985). These steroid hormones probably stimulate oocyte growth by activating vitellogenesis in Echinoderms.

Oocyte Maturation

The period of oocyte growth is followed by a process called oocyte maturation (the resumption of meiosis), which occurs prior to ovulation and is a prerequisite for successful fertilization. Gonadotropins play important regulatory roles in reproduction in vertebrates and invertebrates. In vertebrates, hypothalamic gonadotropin-releasing hormone (GnRH) acts on the pituitary gland to stimulate the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which then act synergistically to promote gamete maturation and gonadal function (Pierce and Parsons, 1981).

In starfish, a ripe ovary contains a large number of fully grown oocytes of almost equal size. Each oocyte possesses a large nucleus (a germinal vesicle), which remains arrested in late prophase of the first maturation division, and is surrounded by a single layer of follicle cells, which adhere firmly to each other and to the ovarian wall (Fig. 1(B)). These immature oocytes cannot be fertilized, and they still require maturation and ovulation processes that are induced by hormonal substances (Fig. 2(A)). Fertilization eventually occurs after these oocytes are spawned into the seawater (Fig. 1(E)), outside of the female's body, for most starfish.

Relaxin-Like Gonad-Stimulating Peptide

The first report of a gonadotropic substance in an invertebrate was the observation that extracts of radial nerve cords from the starfish *Asterias forbesi* induce shedding of gametes when injected in this species (Chaet and McConnaughy, 1959). The active substance was named gonad-stimulating substance (GSS) and was characterized biochemically as a peptide hormone (Kanatani, 1979). In 2009, GSS was purified from the radial nerve cords of starfish *Patiria pectinifera* (Mita et al., 2009). The purified hormone is a heterodimeric peptide with a molecular weight of 4740, comprising an A-chain of 24 amino acids and a B-chain of 19 amino acids, with disulfide cross-linkages (Fig. 2(B)). Furthermore, the A-chain contains a cysteine motif CCxxxCxxxxxxxxC, which is a signature sequence of the insulin/insulin-like growth factor/relaxin superfamily. More specifically, phylogenetic sequence analysis revealed that starfish GSS is a member of the relaxin-type peptide family (Mita et al., 2009; Mita, 2016). Therefore, the GSS identified in *Patiria pectinifera* has been designated as relaxin-like gonad-stimulating peptide or RGP.

Subsequently, orthologs of *Patiria pectinifera* RGP were identified in other starfish species (Mita, 2016). At least three kinds of RGP molecules are found in the class Asteroidea. The chemical structure of *Patiria pectinifera* RGP is mostly conserved among starfish of the order Valvatida beyond species including *Patiria miniata*, *Certanardoa semiregularis* and *Acanthaster planci*. In contrast, the chemical structures of RGP identified in *Asterias amurensis* and *Aphelasterias japonica* of the order Forcipulatida are quite different from that of *Patiria pectinifera* RGP. The amino acid identity levels between *Asterias amurensis* RGP and *Aphelasterias japonica* RGP were 84% for the A-chain and 90% for the B-chain. In contrast, the amino acid sequences of *Asterias amurensis* RGP and *Aphelasterias japonica* RGP were not quite as similar as that of *Patiria pectinifera* RGP, with homology levels of 58% and 58% for the A-chain, 73% and 68% for the B-chain, respectively.

Cross-experiments among different starfish revealed that RGP generally acts non-species-specifically, with some exceptions. Because the chemical structure of *Patiria pectinifera* RGP is almost the same as those of species among the order Valvatida, *Patiria pectinifera* RGP can induce oocyte maturation and ovulation in ovarian fragments from *Patiria miniata*, *Certanardoa semiregularis* and *Acanthaster planci*. Furthermore, *Patiria pectinifera* RGP was active in *Asterias amurensis* and *Aphelasterias japonica* ovaries. In contrast, neither *Asterias amurensis* RGP nor *Aphelasterias japonica* RGP induced spawning in the ovarian fragments of *Patiria pectinifera*, although *Asterias amurensis* RGP was active in *Aphelasterias japonica* ovaries. It may also be possible that, because of low specificity of the receptor proteins in ovarian follicle cells of *Asterias amurensis* and *Aphelasterias japonica*, *Patiria pectinifera* RGP can affect the ovaries of *Asterias amurensis* and *Aphelasterias japonica*.

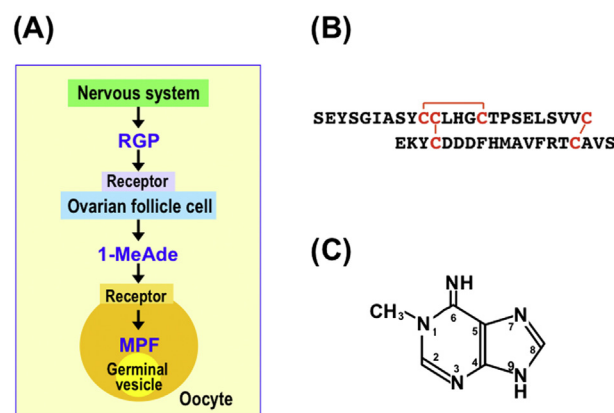


Fig. 2 (A) Hormonal mechanism of starfish oocyte maturation. (B) Starfish gonadotropin, relaxin-like gonad-stimulating peptide (RGP), identified from radial nerve cords of *Patiria pectinifera*. (C) Starfish maturation-inducing hormone, 1-methyladenine (1-MeAde).

Enzyme-linked immunosorbent assays using a specific antibody against *Patiria pectinifera* RGP revealed that high activity of RGP was present in the radial nerve cords and circumoral nerve rings, but RGP was undetectable in the gonads. RGP was localized predominantly in the neurosecretory granules on the supporting cell layer just beneath the outer cuticle of the radial nerve cords. On the basis of *in situ* hybridization, the mRNA of RGP was detected in the peripheral area of radial nerve cords proximal to the tube-feet, but not at the side of the haemal sinus. Positive immunoreactivities with the RGP antibody were also located in the cuticle layer of the outer surface of the radial nerve cords of *Patiria pectinifera* (Mita, 2016). Furthermore, RGP transcripts were abundantly expressed by cells in the arm tips of the starfish *Asterias rubens*. Thus, RGP is *de novo* synthesized in the cells as a prepro-RGP translated from its mRNA at first. After the formation of three disulfide cross-linkages between the A- and B-chains and within the A-chain, mature RGP is produced by elimination of the signal and C-peptides.

RGP was detected in the coelomic fluid only when starfishes were undergoing natural spawning (Kanatani, 1979, 1985). RGP was never found in the coelomic fluid in individuals that were not undergoing spawning. This is evidence that RGP is a hormone. On the other hand, RGP content in the radial nerve cords remains constant throughout the year, regardless of breeding and non-breeding season. In an *in vitro* experiment, RGP was capable of being released from the radial nerve cords in the presence of ionomycin. Perhaps, an increase in the level of intracellular Ca^{2+} is closely associated with RGP secretion. RGP seems to be transported from the ectoneural epithelium of the radial nerve cords to the radial haemal sinus. This sinus is connected to axial, visceral, and genital haemal sinuses of the haemal system. Presumably, RGP is transported from radial nerve cords to the gonads through the haemal system. It is also assumed that RGP, being secreted from the radial nerve cords, acts on ovarian follicle cells *via* coelomic fluid. At present, the secretion mechanism and transport pathway of RGP has not been elucidated.

Other than starfish RGP, a pentapeptide (NGIWamide), cubifrin, induces oocyte maturation and spawning behavior in the sea cucumber *Apostichopus japonicus* in a very similar manner to RGP of starfish (Kato *et al.*, 2009). However, cubifrin can induce maturation in follicle-enclosed oocytes only in the presence of the ovarian wall. Thus, the action of cubifrin resembles GnRH rather than gonadotropin.

Maturation-Inducing Hormone

Spawning behavior and subsequent spawning are observed in the females of *Patiria pectinifera* with fully grown ovaries upon injection of RGP into the body cavity. The starfish starts moving upward along the walls of the aquarium and then sheds its gametes within 30 min (Mita *et al.*, 2009). Although RGP is the primary mediator of oocyte maturation and spawning in starfish, its effect is indirect (Fig. 2(A)). Resumption of meiosis in immature oocytes and spawning from the ovary is induced by a second mediator, maturation-inducing hormone, identified as 1-methyladenine (1-MeAde) in starfish (Kanatani *et al.*, 1969; Kanatani, 1979, 1985) (Fig. 2(C)). RGP controls 1-MeAde production in ovarian follicle cells.

RGP bound specifically to a membrane preparation of ovarian follicles of *Patiria pectinifera*, and isolated follicle cells cultured with RGP showed a dose-related increase in cyclic AMP (cAMP) production, coinciding with an increase in 1-MeAde production. Thus, the action of RGP is mediated through the activation of its receptor, G-protein, and adenylyl cyclase in follicle cells. However, RGP failed to induce 1-MeAde and cAMP production in follicle cells of *Patiria pectinifera* ovaries during oogenesis (Mita, 2016). At the maturation stage, follicle cells acquire the potential to respond to RGP by producing 1-MeAde and cAMP. Adenylyl cyclase activity in follicle cells of fully grown ovaries is stimulated by RGP in the presence of GTP. Of note, the mRNA for the α subunit of the Gs-protein (*G α s*) was not detected in follicle cells of oogenesis-stage ovaries. The expression level of *G α s* in follicle cells also increased significantly as the size of the oocytes increased. This revealed that the potential of follicle cells to respond to RGP by producing 1-MeAde and cAMP occurs by *de novo* synthesis of the *G α s* protein.

Furthermore, 1-MeAde produced under the influence of RGP is newly synthesized, rather than being previously stored within ovarian follicle cells or a breakdown product of some 1-MeAde-containing substance, such as ribonucleic acid (Kanatani, 1985). The RGP-dependent 1-MeAde production also involves a methylation process. Because RGP causes a reduction in the intracellular level of ATP after 1-MeAde production, 1-MeAde is synthesized from ATP *via* the methylation process from S-adenosylmethionine in ovarian follicle cells of *Patiria pectinifera*.

Maturation (M-Phase)-Promoting Factor

In primary oocytes within ovaries, the cell cycle arrests generally at the prophase of the first meiosis. The release from prophase arrest in immature oocytes of *Patiria pectinifera* is induced by 1-MeAde, with no requirement for new protein synthesis (Kishimoto, 2015). On the other hand, the injection of 1-MeAde into fully grown starfish oocytes failed to induce maturation (Kanatani and Hiramoto, 1970). This revealed that 1-MeAde acts on the external oocyte surface, and that some hormonal message seems to be conveyed to the cytoplasm. Evidence for the activation of a third mediator, maturation-promoting factor (MPF), in the oocyte cytoplasm is that the cytoplasm from an oocyte of *Patiria pectinifera*, maturing after treatment with 1-MeAde, induces maturation when injected into an immature oocyte (Kishimoto and Kanatani, 1976). Starfish MPF, like that of *Xenopus* and fish, consist of two components: cyclin B and cyclin-dependent kinase 1 (Cdk1). MPF not only induces starfish oocyte maturation (Fig. 2(A)) but also regulates the G2/M-phase transition of mitosis from yeast to mammalian cells (Kishimoto, 2015).

Actually, 1-MeAde binds to an oocyte membrane receptor (Yoshikuni *et al.*, 1988). From the structure function relationship studied so far using many analogues of 1-MeAde, a lipophilic substitution on N1, an amino group at position 6, and a cationic charge are known to be pre-requisites for maturation-inducing activity (Kanatani, 1985). Any substitution in positions 7 or 9 or

a bulky 8-substituent is detrimental to the interaction between the hormone and its receptor. It was notable that the degree of biological activity for the induction of oocyte maturation decreases as the side chain at the N1-site is elongated, 1-MeAde > 1-ethyladenine > 1-propyladenine. This revealed that the molecular size at the N1-site is important for interaction with the receptor.

The receptor of 1-MeAde couples to the $\alpha\beta\gamma$ trimeric G protein (Chiba, 2000). After binding of 1-MeAde to its receptor, $G\beta\gamma$ subunits dissociate from the GTP-bound $G\alpha$ subunit, and the dissociated $G\beta\gamma$ stimulates phosphoinositide 3-kinase (PI3K), which brings about the activation of MPF (Chiba, 2000).

MPF is a universal regulator of M-phase in the eukaryotic cell cycle (Kishimoto, 2015). Its activation induces entry into M-phase and its subsequent inactivation is necessary for exit from M-phase. MPF consistently equates with the kinase cyclin B-Cdk1. 1-MeAde exerts its effects through the receptor, G-protein and PI3K. Subsequently, downstream of PI3K, the Akt switches the balance between the Wee1 family kinase, Myt1, and Cdc25 and causes the initial activation of cyclin B-Cdk1 at the meiotic G2/M-phase transition. In some condition, however, MPF is undetectable even though cyclin B-Cdk1 is fully active (Kishimoto, 2015). MPF was originally defined as a transferable activity that can induce the G2/M phase transition in recipient cells. According to classical microinjection assay results, MPF consists not only of cyclin B-Cdk1 but also Greatwall (Gwl) (Kishimoto, 2015). Involvement of Gwl in MPF can be explained by its contribution to the autoregulatory activation of cyclin B-Cdk1 and by its stabilization of phosphorylations on cyclin B-Cdk1 substrates, both of which are essential when MPF induces the G2/M phase transition in recipient cells.

Ovulation

Unlike sea urchins, neither electric stimulation nor contraction-inducing agents, such as potassium chloride and acetylcholine, induce spawning from intact starfish ovaries (Kanatani, 1979). In starfish, each immature oocyte within ovaries is tightly surrounded by an envelope consisting of follicle cells (Fig. 1(B)). Ovarian follicle cells are connected to the oocyte by slender cytoplasmic processes extending to the oocyte surface. These processes arise from the follicle cell cytoplasm, pass through the jelly coat and vitelline coat, and form characteristic desmosome-like junctions at their tips with the oocyte plasma membrane (Kishimoto *et al.*, 1984). After treatment with 1-MeAde, the follicular envelope of an isolated oocyte ruptures and forms a clump upon commencement of germinal vesicle breakdown by the oocyte (Fig. 1(C)). From this, the mature oocytes can move freely before their release from the ovaries (Kanatani, 1979, 1985; Kishimoto *et al.*, 1984). However, 1-MeAde does not induce contraction of ovarian wall. It is considered that acetylcholine secreted from the gonadal nerve cells after follicular envelop breakdown increases tension in the ovarian wall and leads to shedding of mature oocytes.

Therefore, starfish oocyte maturation and spawning (ovulation) induced by RGP can be understood as follows: RGP acts on ovarian follicle cells to produce 1-MeAde. 1-MeAde stimulates oocytes to activate intracellular MPF, which is a direct trigger for maturation and dissociation of the follicular envelope. After follicular envelop breakdown, the denuded mature oocytes become free within the ovary. Finally, acetylcholine-mediated contraction of the ovarian wall leads to spawning of the loose, mature oocytes.

References

- Chaet, A. B., & McConaughy, R. A. (1959). Physiologic activity of nerve extracts. *Biological Bulletin*, 117, 407–408.
- Chiba, K. (2000). Meiosis reinitiation in starfish oocyte. *Zoological Science*, 17, 413–417.
- Kanatani, H. (1979). Hormones in echinoderms. In E. Barrington (Ed.), *Hormones and Evolution* (vol. 1, pp. 273–307). London: Academic Press.
- Kanatani, H. (1985). Oocyte growth and maturation in starfish. In C. B. Metz, & A. Monroy (Eds.), *Biology of Fertilization* (vol. 1, pp. 119–140). New York, NY: Academic Press.
- Kanatani, H., & Hiramoto, Y. (1970). Site of action of 1-methyladenine in inducing oocyte maturation in starfish. *Experimental Cell Research*, 61, 280–284.
- Kanatani, H., Shirai, H., Nakanishi, K., & Kurokawa, T. (1969). Isolation and identification of meiosis-inducing substance in starfish *Asterias amurensis*. *Nature*, 221, 273–274.
- Kato, S., Tsurumaru, S., Taga, M., et al. (2009). Neuronal peptides induce oocyte maturation and gamete spawning of sea cucumber *Apostichopus japonicus*. *Developmental Biology*, 326, 169–176.
- Kishimoto, T. (2015). Entry into mitosis: A solution to the decades-long enigma of MPF. *Chromosoma*, 124, 417–428.
- Kishimoto, T., & Kanatani, H. (1976). Cytoplasmic factor responsible for germinal vesicle breakdown and meiotic maturation in starfish oocyte. *Nature*, 260, 321–322.
- Kishimoto, T., Usui, N., & Kanatani, H. (1984). Breakdown of starfish ovarian follicle induced by maturation-promoting factor. *Developmental Biology*, 101, 28–34.
- Mita, M. (2016). Starfish gonadotropic hormone: Relaxin-like gonad-stimulating peptides. *General and Comparative Endocrinology*, 230–231, 166–169.
- Mita, M., Yoshikuni, M., Ohno, K., et al. (2009). A relaxin-like peptide purified from radial nerves induces oocyte maturation and ovulation in the starfish, *Asterina pectinifera*. *Proceedings of National Academy of Science of the United States of America*, 106, 9507–9512.
- Pierce, J. G., & Parsons, T. F. (1981). Glycoprotein hormones: Structure and function. *Annual Review of Biochemistry*, 50, 465–495.
- Yoshikuni, M., Ishikawa, K., Isobe, M., Goto, T., & Nagahama, Y. (1988). Characterization of 1-methyladenine binding in starfish oocyte cortices. *Proceedings of National Academy of Science of the United States of America*, 85, 1874–1877.

MATERNAL RESERVES

Maternal RNAs, Fish & Amphibians

Caroline TY Cheung, Julien Bobe, and Violette Thermes, INRA, LPGP UR1037, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

In fish, maternal RNAs that are incorporated into the developing oocytes include ribosomal RNA (rRNA), transfer (tRNA), and messenger RNA (mRNA), the two former of which are components of the protein translation machinery and the latter of which is used as a blueprint to synthesize proteins, as well as microRNA (miRNA) and other non-coding RNA (ncRNA), which are not used to make proteins, but instead play regulatory roles (Pauli *et al.*, 2011; Pelegri, 2003). These factors are produced by the mother based on her genome and deposited into the developing egg at a very early stage of oogenesis. This article will focus on the localization, regulation, and function of coding and non-coding RNAs.

Much of the knowledge we have on maternal RNAs in fish have been obtained from teleost species (ray-finned fishes that make up 96% of all fishes) including zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and to a lesser extent goldfish (*Carassius auratus*). These are all small aquarium fishes used as experimental laboratory models due to their ease of reproduction in captivity and abundant egg production that are easily handled in culture. Findings were also obtained from wild and farmed fish species, such as rainbow trout, salmon, and tilapia just to name a few. The major differences between fish species and other vertebrates are that their eggs are fertilized and develop externally and can be produced year-round. In this article, we will introduce the localization, regulatory mechanisms and function of some protein-coding and non-coding transcripts that have previously been investigated in fish eggs.

Localization of Maternally-Inherited mRNAs in the Oocyte

Fish eggs immediately upon fertilization are spherical in shape with various components, including maternal mRNAs, numerous organelles, yolk inclusions, proteins, as well as lipid droplets and pigments intermingled in the egg cytoplasm (Lubzens *et al.*, 2010). Subsequently, a distinct blastodisc embodied as a thin region of yolk-free cell cytoplasm (animal pole) is separated from the yolk, which takes up most of the egg (vegetal pole). Only the cytoplasm of the blastodisc, but not the yolk, undergoes subsequent cleavages or cell divisions to become the full embryo which generates a mound of cells that sits on top of the yolk (Fig. 1(E)). The chorion membrane, consisting predominately of the glycoproteins *zona pellucida* (*zp*) 2 and *zp*3, surrounds the embryo and expands upon fertilization with an enlarged fluid-filled perivitellin space in-between.

At the earliest stage of oogenesis in zebrafish, most of the maternal mRNAs that have been investigated are observed throughout the cytoplasm of the immature oocyte. However, some transcripts in the developing oocyte appear to be quite mobile and change localization during the course of oogenesis. During oogenesis, the animal/vegetal polarity of the egg is established by the functions of genes including *bucky ball*, which regulate the distinct distribution of maternal RNAs and the asymmetrical location of the germinal vesicle (oocyte nucleus) and micropyle (a specialized structure for sperm entry) (Marlow and Mullins, 2008). In the unfertilized mature egg, while certain maternal transcripts remain ubiquitously distributed throughout the cytoplasm, other mRNAs follow specific distribution patterns, including localization to the animal pole, vegetal pole, and cortex (Fig. 1) (Howley and Ho, 2000). β -catenin is an example of an mRNA that is dispersed evenly throughout the cytoplasm of the oocyte during the entire oogenesis and remains spread out in mature and fertilized eggs. Others including *zorba*, *pou2*, and *cyclin B* are found specifically at the animal pole, possibly with roles in specification of this axis, while genes that are related to the future dorsal/ventral axis formation (*swirl*, *wnt8a*, and *grip2*) and germ-line specificity (*dazl* and *brul*) are found in the vegetal pole. In addition, *vasa* is observed to have a uniform cortical localization along the animal/vegetal axis in mature zebrafish eggs.

Upon fertilization, almost all of the maternal transcripts are pulled into the blastodisc through cytoplasmic streaming or ooplasmic segregation, a process based on cytoskeletal actin filaments and microtubules that ensures the orderly partition of the maternal factors to the animal pole and the yolk components to the vegetal pole, with the exception of *dazl* which remains at the vegetal pole until the 4-cell stage. The transport of maternal RNAs, especially those that are relevant for dorsal/ventral axis formation, is further dependent on the "cortical rotation" mechanism, whereby a vegetal pole-based microtubule system is used to pull RNAs from the vegetal pole to the animal pole, as well as a more general animal pole-oriented transport system that involves the mediolateral cortex. Notably, the same maternal RNAs (*bucky ball* and *kif5Ba*) that are involved in this process are also responsible for the regulation and establishment of the germ plasm, which forms at the distal ends of the cleavage furrows of 4-cell embryos. This important structure contains maternal mRNAs that are necessary for germ-line specificity, such as *vasa*, *nanos1*, *brul*, and *dazl*, which is finally released from the vegetal pole (Kosaka *et al.*, 2007).

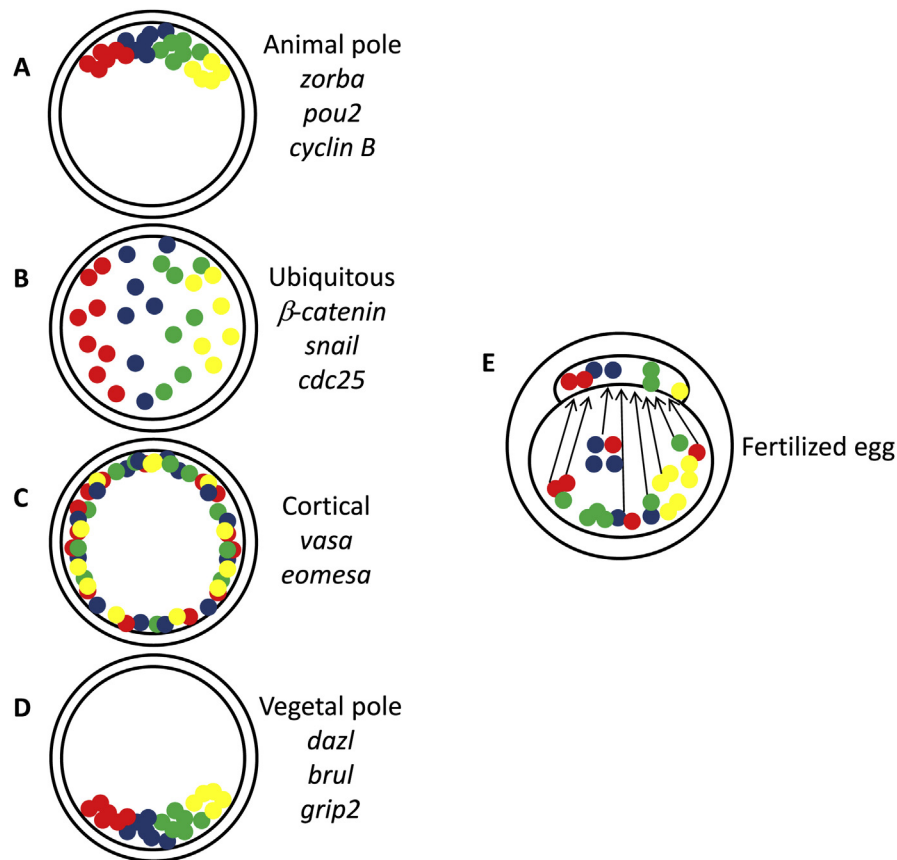


Fig. 1 Different distribution patterns of maternal transcripts in the mature fish egg. Maternally-accumulated mRNAs are localized to either the animal pole, evenly throughout the oocyte, cortically, or to the vegetal pole (A–D). However, there are some transcripts that are redistributed and change localization during oogenesis. (E) After fertilization, most of the transcripts are pulled into the animal cell cap by cytoplasmic streaming or cortical rotation.

Regulatory Mechanisms of Maternally-Inherited mRNA

RNAs are synthesized in the developing oocytes based on the maternal genome during oogenesis. The different RNA species that are stored in the egg are extremely important to the development of the embryos since they coordinate (1) early development, including cell division prior to the mid-blastula transition (MBT), (2) the expression of genes from the embryo's own genome, and (3) proper embryogenesis after MBT. The expression levels of transcripts differ depending on their function and maternal RNA levels decrease over time eventually being completely replaced by the embryonic RNAs, which are first expressed at MBT when zygotic genome activation (ZGA) occurs (Fig. 2). In fish, ZGA occurs at approximately the 1000-cell stage, in contrast to mammals where it begins earlier during the late 1-cell and early 2-cell stages. Most mRNAs such as *cytochrome P450 aromatase*, *zona pellucida*, and β -catenin are synthesized abundantly very early during oogenesis (stage I) and progressively decrease with still substantial amounts at fertilization, which are maintained until MBT (Howley and Ho, 2000). The quantity of some transcripts such as *star* and *apolipoprotein CI* are relatively small in very early oocytes (stage I), but they are progressively synthesized and accumulated during oogenesis peaking in later stage eggs and maintained until after fertilization. The amount of other mRNAs such as *bucky ball* is relatively constant throughout oogenesis and may increase in mature eggs until after fertilization.

After fertilization, translation of the maternal transcripts is under strict control to determine precisely their spatiotemporal synthesis, and the expression level of maternal RNAs is regulated by their stability and degradation since transcription does not occur before MBT. Many of the mRNAs are masked or repressed by binding to ribonucleoprotein particles, which inhibit the interaction of the transcripts with the translation machinery, until they are ready to be used. One clear example is the repression of transcripts for cell cycle regulatory proteins such as cyclins, which are activated in a very precise and timely manner to ensure proper cell division after fertilization. Another important mechanism for translational control of maternal transcripts is cytoplasmic polyadenylation driven by CPEB/zorba proteins, which bind to sequences in the 3'-untranslated regions of regulated mRNAs and mediate their storage, repression or translation. Notably, CPEB proteins are required for the activation of polyadenylation, which lengthen the poly(A) tail of transcripts, and subsequent translation of the stored maternal mRNA in the fertilized embryo. De-adenylation and shortening of the poly(A) tails, which lead to decapping of the 5' cap structure of transcripts, decrease their stability and subject them to degradation.

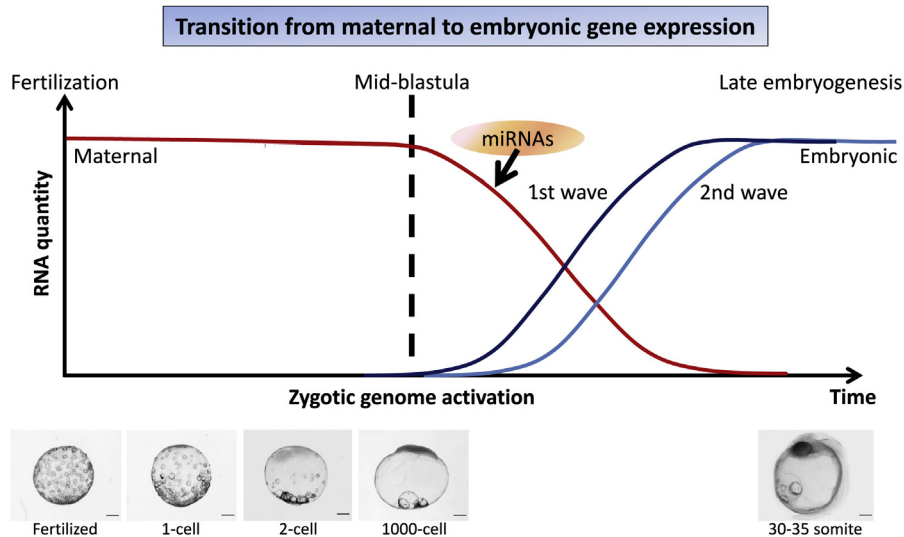


Fig. 2 Maternal and embryonic gene expression levels over time from fertilization to late embryogenesis. The total level of maternal RNAs starts to drastically decrease at the mid-blastula transition (MBT), at which point the “zygotic genome activation” occurs, and the level of embryonic gene expression begins to increase. There are two major waves of embryonic gene expression. Below the graph are images showing the medaka (*Oryzias latipes*) egg at various stages of embryogenesis: fertilization, 1-cell, 2-cell, 1000-cell, and 30–35-somite stages. Scale bar: 200 μ m.

Two major mechanisms have been described for the clearance and degradation of maternal RNAs in the developing embryo: one that is programmed and controlled by maternal factors and the other that is activated by embryonic factors (Mishima and Tomari, 2016). Both mechanisms depend on the shortened length of the poly(A) tail of the transcript based on the deadenylation status driven by the pan2-pan3 and ccr4-not deadenylase complexes. This leads to activation of decapping machineries involving the dcp1/2 complex which removes the 5' cap structure of transcripts and commits them to degradation by the xrn1 exonuclease. In addition, epigenetic changes such as methylation has been shown to mediate degradation; over one-third of zebrafish maternal mRNAs can undergo methylation at adenosines.

In general, *cis* elements in the transcripts in combination with sequence-specific RNA-binding factors that recruit degradation machineries are considered to contribute to both maternal- and embryonic-driven transcript decay. In contrast, a maternal factor that has been proposed to mediate maternal RNA decay involves codon identity or usage. Basically, there are uncommon synonymous codons within the open-reading frame of the maternal transcript that promotes its own degradation via the ccr4-not complex, which means that codon usage and amino acid composition influences RNA stability. The effect of codon usage also depends on the length of the 3'-untranslated region. Thus, the transcript not only conveys the genetic code which specifies the amino acid sequence, but also contains a conserved “codon optimality code” that shapes transcript stability and degradation (Bazzini *et al.*, 2016).

The most well-known embryonic factor that mediates maternal RNA clearance is the miR-430. The miR-430 is transcribed at the onset of the ZGA by maternally-accumulated transcription factors Nanog, Pou5f1 and SoxB1, and matured by the maternally-provided Dicer protein. Expression of miR-430 at the MBT significantly contributes to the clearance of many maternal mRNAs accumulated in the egg. The key role of this miRNA was first discovered by means of mutant embryos lacking maternal and embryonic Dicer activity (MZDicer^{-/-}) (Schier and Giraldez, 2006). In these mutants, synthesis of all embryonic miRNAs in the early embryo was impaired and gastrulation was drastically affected. Further, when the fertilized eggs were supplemented with exogenous miR-430, embryonic development could be partially restored. MiR-430 indeed promotes the deadenylation and decay of several hundreds of maternally-expressed mRNAs, and thus, contributes to the correct patterning of genes in the later embryo. These experiments clearly demonstrated the importance of early embryonic miRNAs, more specifically the miR-430, in the clearance of maternal mRNAs at the ZGA. However, it is believed that other miRNAs might also be involved in the mRNA turnover, but their identity still remains to be discovered.

Both the presence and absence of maternally-provided RNAs are important for proper embryogenesis; first, to coordinate early development of the embryo as well as promotion of ZGA, and then, their degradation is necessary for the progression of numerous subsequent processes, since it was shown that some maternal RNAs actually inhibit certain reactions after MBT. In the next section, we will introduce the functions of the maternally-inherited RNAs.

Function of Maternal mRNAs During Development

Despite the plethora of maternal transcripts (between 10,000 to over 30,000 in zebrafish) found in the fertilized egg, the number of and knowledge on maternal-effect genes, defined as those whose products, including mRNA, proteins, and other biomolecules, are

found predominately in the fertilized egg and are essential for the normal early development of the embryo before expression from the embryonic genome, remain low. Genetic manipulations to create mutants using model fish species have greatly expanded our understanding of maternal-effect genes. With this approach, it was observed that mothers with mutations in maternal-effect genes are phenotypically normal and viable, however, they suffer from subfertility or sterility due to the diminished quality of their eggs. The lack of or decreased expression of maternal-effect genes can result in decreased fertilization, as well as increased embryonic mortality and malformations.

Certain maternal transcripts that are present in the fertilized egg are essential for egg activation and the process of fertilization. Genetic screens showed that the maternal transcripts for *jump start*, *p11cv*, *clauastro*, *emulsion*, and *dp14nb* are critical for regulating changes in egg shape, ooplasmic segregation to the blastodisc, and chorion expansion, all of which are processes that occur during egg activation upon fertilization (Dösch *et al.*, 2004). Further, the *fue* mRNA is necessary for the congression of the sperm and oocyte pronuclei as well as chromosomal segregation during the subsequent cell divisions, thus, the lack of this transcript results in the failure to produce a proper diploid nucleus in the first cell of the embryo and subsequent cell divisions result in anucleate cells with local DNA accumulation in just one or two cells.

Many maternal RNAs have been found to be essential to the first cell cleavages or divisions following fertilization. It was found that lack of maternally-provided *aurora-A*, *cellular island/aurora-B*, *cellular atoll*, *acytokinesis*, *atomos*, *aura*, *barrette*, *cobblestone*, *indivisible*, *irreducible*, *nebel*, *weeble*, *bo peep*, *golden gate*, *kwai*, *screeching halt*, and *waldo* mRNA results in cell division failure due to defects in one or more of the following processes: centrosome duplication and assembly, formation of bipolar spindles, furrow formation, chromosome segregation, DNA stability, karyokinesis, and cytokinesis (Lindeman and Pelegri, 2010; Pelegri, 2003). For example, *aurora* kinases are pertinent regulators of mitosis since they play essential roles at various stages of mitosis, from mitotic entry to cytokinesis. It was found that *aurora-A* is essential for the intactness of the centrosome and establishment of the bipolar spindle assembly, and the lack of this transcript leads to the mitotic arrest of cells. On the other hand, *aurora-B* is not necessary for spindle formation, but instead is essential for furrow formation due to its regulation of cytoskeletal organization. Thus, in embryos lacking this mRNA, the cells do not divide properly and form a syncytium, following which the embryo subsequently dies.

From the onset of the first cleavage to the initiation of ZGA and even thereafter, various maternally-provided transcripts play critical roles in the regulation of early embryonic development such as axis polarity (dorsal/ventral and anterior/posterior), development of the germ plasm and subsequent germ line stability, and activation of the embryonic genome. The functions of the maternally-provided RNAs extend beyond ZGA and they are necessary for proper development of the later embryo, including body patterning. As mentioned above, mRNAs including the vegetally-positioned *swirl*, *unt8a*, and *grip2a* in addition to β -catenin, *dkk1*, and *tkk* among others have been found to be involved in the specification and establishment of the dorsal/ventral axis (Marlow, 2010). It has been shown that the Wnt signaling pathway is essential for dorsal axis determination, and a decrease in the mRNAs for members of this pathway, including *unt8a*, β -catenin, *dkk1*, and *tkk*, results in ventralization of the embryo demonstrated by the lack of notochord, neural tube, and trunk somites. In fact, the maternally-derived Wnt pathway is also essential for anterior/head formation, although only before MBT, since maternal Wnt transcripts present before MBT induces head formation, but blocks it afterwards. This was verified by embryos that lack maternal *headless* transcripts, which encodes a repressor of Wnt target genes and show complete loss of eyes, forebrain, and part of the midbrain with cranial-specific defects. Thus, it seems that although the *headless* mRNA is maternally-provided and accumulated in the egg during oogenesis, differential localization of it relative to the transcripts of the Wnt target genes enables activation of Wnt signaling before MBT and its repression afterwards. The *headless* transcript is widely distributed throughout the embryo, but excluded from the dorsolateral margin where the Wnt components are localized, during early development.

In addition to these, there are transcripts that are involved in the development of the endoderm and mesoderm layers, which are precursors to the major organs. It is well-known that factors belonging to the TGF- β superfamily play important roles in endoderm and mesoderm specification and patterning. Early nodal signaling relies on the maternally-provided *omesodermin-a* transcript, which subsequently activates embryonically-transcribed nodal genes that are necessary for initial mesodermal and endodermal fate specification. In addition, *activin* mRNA is also necessary for mesoderm induction and axis formation as demonstrated by mutants that have reduced maternal *activin* that show posteriorization of the head and diminished or delocalized notochord formation. There are also certain transcripts that are important for the differentiation of specialized cell types such as those in the eye, chromatophores (*gart* and *paics*) as well as primordial germ cells (*bucky ball*, *kif5Ba*, *vasa*, *nanos1*, *brul*, and *dazl*). A common characteristic in teleost fishes is that the primordial germ cells are frequently involved in ovary development and/or maintenance such that lack of maternal mRNAs for genes that are required for germ cell differentiation and maintenance undergo masculinization and become sterile males or have underdeveloped ovaries/oocytes as demonstrated by mutants that lack maternally-provided *vasa* or *nanos1*, respectively.

In addition, there are maternally-provided mRNAs that are involved in the activation of the embryonic genome, as evidenced by the function of *npm2b*. It was shown that *npm2b* transcript is stored in the egg in large amounts and is necessary for the activation of and subsequent transcription from the embryonic genome since its deficiency leads to arrest at the MBT and eventual death (Fig. 3) (Bouleau *et al.*, 2014). This may be due to the functions of Npm2b as a histone chaperone, which is involved in sperm DNA decondensation, and in the promotion of essential embryonic gene expression. Another important event at MBT that signals the transition is the clearance of maternal RNAs and, as mentioned above, miRNA-430 plays a large role in this as certain maternally-provided transcripts, such as those for *dicer* and *ago2*, which regulate the activity of miRNAs, were found to be important for the degradation of maternal RNAs and their inhibition leads to morphogenesis defects similar to the miR-430 mutants (Schier and Giraldez, 2006).

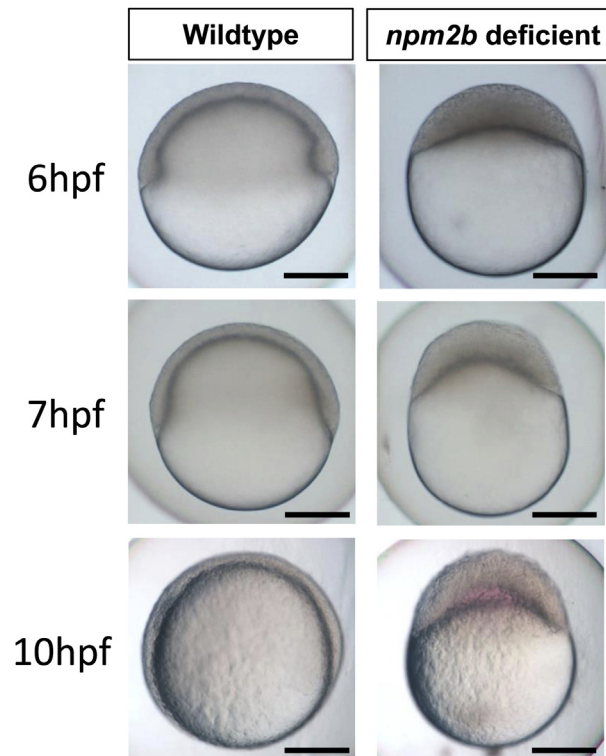


Fig. 3 Development at different stages of wildtype and *npm2b*-deficient zebrafish embryos. Representative images showing the developing zebrafish embryo at different stages of embryogenesis: 6, 7, and 10 h post-fertilization (hpf). Wildtype embryos were used to compare the embryos with *npm2b* inhibition, the latter of which demonstrated aberration in embryonic development whereby cell division was normal until 4 hpf, but was arrested thereafter until they eventually died between 12 and 24 hpf. Scale bar: 250 μ m.

There are some distinct differences between the reproduction of fish and tetrapods, such as the external development of the embryo and presence of yolk as primary nutrient, that necessitate the addition of different types of maternal transcripts in the egg. The externalized nature of the egg renders it more susceptible to damage at the structural level as well as at the DNA level, thus, mRNAs for *zp2* and *zp3* that encode structural proteins of the chorion membrane as well as those that encode components of the DNA repair machinery are found in the egg. Further, since the yolk is the primary source of nutrition for the embryo until they hatch and find food for themselves, many yolk-processing mRNAs (*cathepsins B, D, F, L*, and *LPL*), which are mostly enzymes that breakdown the yolk proteins to produce metabolically active substances, are also present (Kwon *et al.*, 2001). In addition, it has been revealed that the fertilized egg carries many immune system-related mRNAs such as those that encode members of the chemokine signaling/regulatory pathway, mhc class 2 chains, complement factors, T-cell receptor signaling pathway, and innate immunity.

Maternally-Inherited Small Non-Coding RNAs

As compared to mRNAs, small regulatory non-coding RNAs (ncRNA) have been much less extensively studied before MBT and only until very recently have researchers gained interest in their function in early fish embryos. Indeed, most studies focused on their role during oogenesis or in the maternal mRNA clearance at MBT. However, in recent years several studies using new generation sequencing revealed a large repertoire of miRNAs expressed in unfertilized eggs in rainbow trout or in zebrafish early embryo (Presslauer *et al.*, 2017), which may be stocked as maternal RNAs. At the present time, there is no information on the localization of miRNAs in the developing, mature or fertilized fish egg. However, maternally-inherited miRNAs appear to be rapidly down-regulated during the transition from maternal to embryonic gene expression, raising the possibility that they are subject to similar forms of regulation as maternally-inherited mRNAs for ZGA.

Regarding the role of these regulatory RNAs, little information has been obtained to date. A few functional studies were initiated in zebrafish to elucidate the role of single miRNAs during early development. The miR-34 is one of the first and a rare maternal miRNA for which a role has been identified. To this aim, the activity of miR-34 was inactivated in fertilized eggs, which led to defects in neural development and deregulation of the key Notch signaling pathway. This shed light on the importance of miR-34 in the regulation of embryonic genes necessary for neurogenesis after MBT. Another example is the miR-92a-3p, whereby its mature miRNA was inactivated in oocytes. It was observed that resulting embryos were arrested as early as the one-cell stage. Such a phenotype indicates that the maternally-inherited miR-92a plays a critical role during the first events that occur just after

fertilization. Reasons to explain this phenotype may be a failure in meiosis completion of the oocyte (oocyte activation), a lack of fertilization or a failure of the onset of the first cell cycle. In the future, identification and analysis of genes targeted by miR-34 and miR-92a will provide essential clues to identify cellular processes that are regulated.

Besides miRNAs, another class of small non-coding RNAs, called Piwi-interacting RNA (piRNA), was also detected in unfertilized eggs. Sequencing data revealed that, compared to miRNA, piRNA contribute to a high proportion of the total RNA, constituting about 80% of small RNAs present in the zebrafish unfertilized embryo (Presslauer *et al.*, 2017). However, despite the abundance of the maternally-inherited piRNAs, their role in the early embryo remains unknown. Nonetheless, piRNAs are known to be mainly involved in the regulation of transposon activity and in the genome protection, and to be essential for maintenance of germ cells and meiosis in zebrafish, which suggest that they may also function as maternal regulators of embryogenesis.

Conclusions

Thus, the maternally-provided RNAs in fish function to lay down the foundation for the body plan of the embryo, regulate the first few cell divisions, facilitate the development of the germ-line cells, coordinate the activation of the embryonic genome, degradation of its own RNAs, as well as proper embryogenesis after MBT, and provide primary defense and nutrition mechanisms to give the embryo a good head start in life. What has been described here is just the tip of the iceberg, and extensive studies are being carried out worldwide to understand in more detail the localization, regulation and function of more maternally-provided mRNAs as well as the roles of the miRNAs, piRNAs and other ncRNAs.

References

- Bazzini, A. A., del Viso, F., Moreno-Mateos, M. A., et al. (2016). Codon identity regulates mRNA stability and translation efficiency during the maternal-to-zygotic transition. *EMBO J.*, 35, 1721–1843. <https://doi.org/10.15252/embj.201694699>.
- Bouleau, A., Desvignes, T., Traverso, J. M., et al. (2014). Maternally inherited nrm2 mRNA is crucial for egg developmental competence in zebrafish. *Biol. Reprod.*, 91, 43.
- Dosch, R., Wagner, D. S., Mintzer, K. A., et al. (2004). Maternal control of vertebrate development before the midblastula transition: Mutants from the zebrafish I. *Dev. Cell*, 6, 771–780.
- Howley, C., & Ho, R. K. (2000). mRNA localization patterns in zebrafish oocytes. *Mech. Dev.*, 92, 305–309. [https://doi.org/10.1016/S0925-4773\(00\)00247-1](https://doi.org/10.1016/S0925-4773(00)00247-1).
- Kosaka, K., Kawakami, K., Sakamoto, H., & Inoue, K. (2007). Spatiotemporal localization of germ plasm RNAs during zebrafish oogenesis. *Mech. Dev.*, 124, 279–289. <https://doi.org/10.1016/j.mod.2007.01.003>.
- Kwon, J. Y., Prat, F., Randall, C., & Tyler, C. R. (2001). Molecular characterization of putative yolk processing enzymes and their expression during oogenesis and embryogenesis in rainbow trout (*Oncorhynchus mykiss*). *Biol. Reprod.*, 65, 1701–1709. <https://doi.org/10.1095/biolreprod65.6.1701>.
- Lindeman, R. E., & Pelegri, F. (2010). Vertebrate maternal-effect genes: Insights into fertilization, early cleavage divisions, and germ cell determinant localization from studies in the zebrafish. *Mol. Reprod. Dev.*, 77, 299–313.
- Lubzens, E., Young, G., Bobe, J., & Cerda, J. (2010). Oogenesis in teleosts: How eggs are formed. *Gen. Comp. Endocrinol.*, 165, 367–389. <https://doi.org/10.1016/j.ygcen.2009.05.022>.
- Marlow, F. L. (2010). Oogenesis: From germline stem cells to germline cysts 2004–2007. In *Maternal Control of Development in Vertebrates: My Mother Made Me Do It!*. San Rafael, CA: Morgan & Claypool Life Sciences.
- Marlow, F. L., & Mullins, M. C. (2008). Bucky ball functions in Balbiani body assembly and animal-vegetal polarity in the oocyte and follicle cell layer in zebrafish. *Dev. Biol.*, 321, 40–50. <https://doi.org/10.1016/j.ydbio.2008.05.557>.
- Mishima, Y., & Tomari, Y. (2016). Codon usage and 3' UTR length determine maternal mRNA stability in zebrafish. *Mol. Cell*, 61, 874–885. <https://doi.org/10.1016/j.molcel.2016.02.027>.
- Pauli, A., Rinn, J. L., & Schier, A. F. (2011). Non-coding RNAs as regulators of embryogenesis. *Nat. Rev. Genet.*, 12, 136–149. <https://doi.org/10.1038/nrg2904>.
- Pelegri, F. (2003). Maternal factors in zebrafish development. *Dev. Dyn.*, 228, 535–554.
- Presslauer, C., Tilahun Bizuayehu, T., Kopp, M., Fernandes, J. M. O., & Babiak, I. (2017). Dynamics of miRNA transcriptome during gonadal development of zebrafish. *Sci. Rep.*, 7. <https://doi.org/10.1038/srep43850>.
- Schier, A. F., & Giraldez, A. J. (2006). MicroRNA function and mechanism: Insights from zebra fish. *Cold Spring Harb. Symp. Quant. Biol.*, 71, 195–203. <https://doi.org/10.1101/sqb.2006.71.055>.

Maternal RNAs, *Drosophila*

Patricia Rojas-Ríos and Martine Simonelig, University of Montpellier, Montpellier, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

In most species, the newly formed zygote is transcriptionally silent, therefore the very first steps of embryogenesis depend on maternal reserves, namely mRNAs and proteins produced by the mother and loaded into the egg to kick-start embryonic development. The control of development then switches from the maternal to the zygotic genome during the maternal-to-zygotic transition, which includes the massive decay of maternal mRNAs and the transcriptional activation of the zygotic genome (Fig. 1; Laver *et al.*, 2015). In *Drosophila*, the oocyte is transcriptionally silent, and the maternal mRNAs are synthesized during oogenesis by specialized germ cells called the nurse cells. *Drosophila* ovaries are composed of about 16 ovarioles that are egg production units containing egg chambers of increasing maturity from anterior to posterior (Fig. 2(A)). Oogenesis is divided into 14 stages, stage 14 being the mature egg. It starts with the asymmetric division of a germline stem cell located at the anterior tip of an ovariole, to give rise to another stem cell and a daughter cell that undergoes differentiation. The daughter cell divides four times with incomplete cytokinesis to produce a cyst composed of 16 interconnected germ cells. One of these 16 cells adopts an oocyte fate, whereas the other 15 germ cells undergo polyploidy and become nurse cells (Fig. 2(A); Bastock and Johnston, 2008). Nurse cells produce large amounts of maternal mRNAs and proteins that are transported to, and towards the end of oogenesis, “dumped” into the oocyte -when nurse cells contract and pour their content into the oocyte-. This occurs through ring canals that connect nurse cells to the oocyte and to each other. RNA profiling in unfertilized eggs has shown that around 50% of the protein-coding genome is represented in maternal mRNAs (Thomsen *et al.*, 2010). Many of them undergo cytoplasmic controls that involve regulation of mRNA stability, localization and translation, three highly interconnected processes. In particular, 60% of maternal mRNAs present in the egg are degraded to some extent, at the maternal-to-zygotic transition (Thomsen *et al.*, 2010). An important mechanism underlying the regulation of maternal mRNAs involves the control of mRNA poly(A) tail lengths (Weill *et al.*, 2012). Virtually all mRNAs acquire a poly(A) tail at their 3' end through a nuclear cotranscriptional reaction called cleavage and polyadenylation. The poly(A) tail plays a major role in mRNA stability and translation and can be regulated in the cytoplasm: mRNA poly(A) tail shortening by deadenylation induces mRNA decay and/or translational repression. In oocytes, maternal mRNAs can be stored as dormant (untranslated) mRNAs with short poly(A) tails; their translation can then be activated at specific developmental stages by poly(A) tail lengthening through cytoplasmic polyadenylation (Barckmann and Simonelig, 2013). A recent study analyzing mRNA poly(A) tail lengths and translational efficiency at genome-wide level, using new technologies for poly(A) tail sequencing and ribosome-footprint profiling, confirmed that translation efficiency depends on mRNA poly(A) tail length in stage 14 oocyte and early embryo, up to gastrulation when this coupling disappears (Eichhorn *et al.*, 2016). This study also revealed that, although cytoplasmic polyadenylation is massive at these stages, the specific regulation of each mRNA is more dependent on deadenylation.

Specific maternal mRNAs are highly regulated and play essential roles in the development of the future embryo; their regulation has been the focus of many genetic studies and they can be used as model systems to illustrate a wide panel of mRNA regulation.

Maternal-to-Zygotic Transition (MZT) in the *Drosophila* embryo

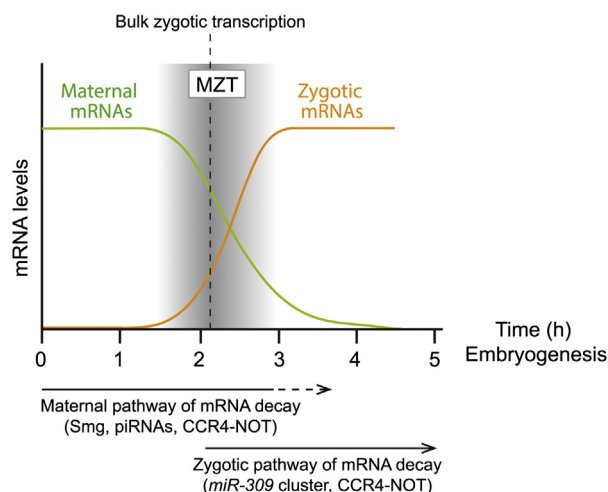


Fig. 1 Maternal-to-zygotic transition in the *Drosophila* embryo. Profiles of unstable maternal mRNAs (green) and of zygotic mRNAs (orange) are schematized. Smg, piRNAs and the CCR4-NOT complex are part of the maternal pathway of mRNA decay, whereas the *miR-309* cluster is part of the zygotic pathway. The role of CCR4-NOT in the zygotic pathway of mRNA decay is likely, but has not been formally addressed. The dashed vertical line indicates the time at which the bulk of zygotic transcription starts. Time is indicated in hours after egg deposition at 25°C.

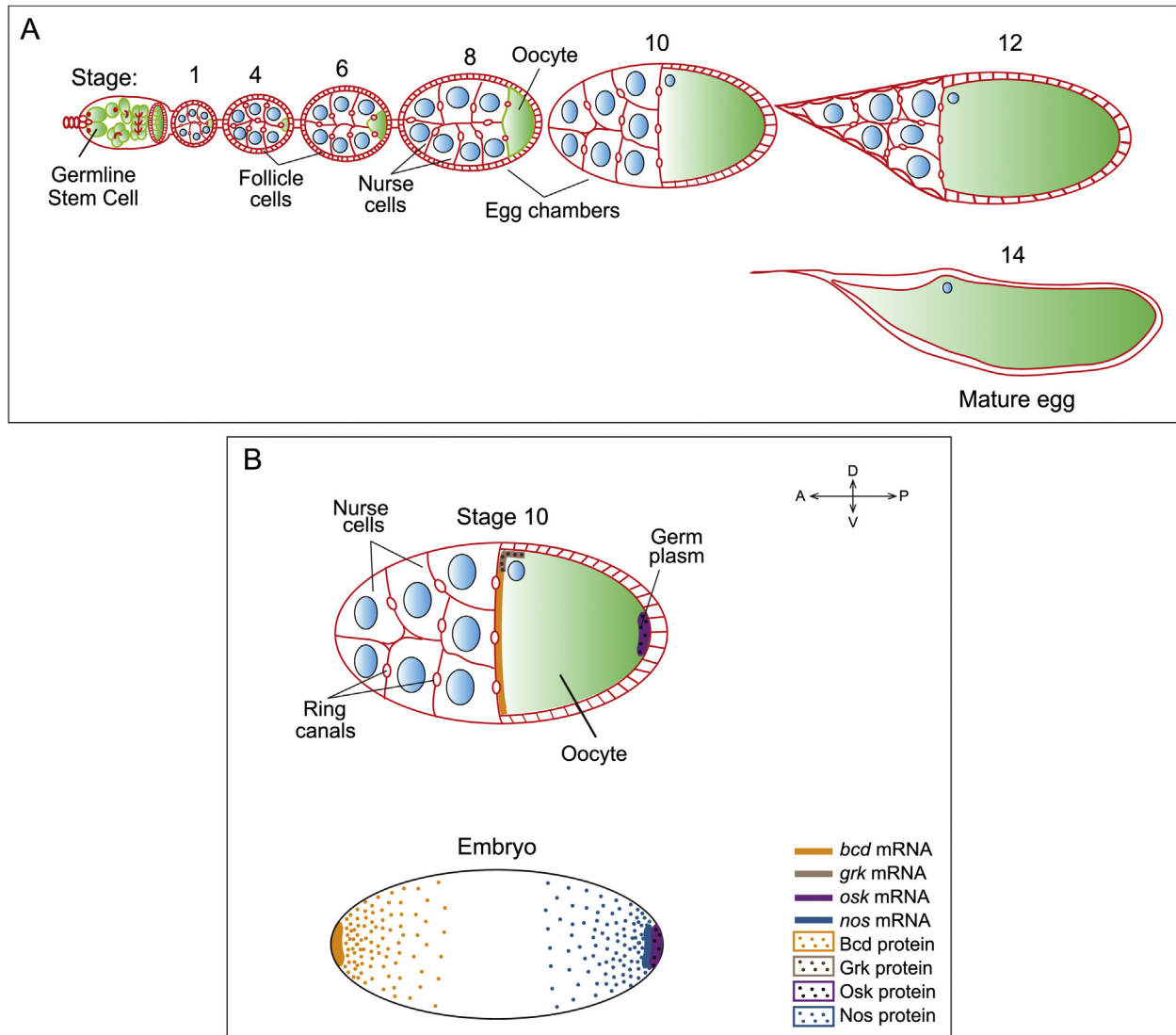


Fig. 2 *Drosophila* oogenesis and the role of maternal mRNAs in embryonic patterning. (A) Schematic representation of *Drosophila* oogenesis. An ovary is composed of about 16 ovarioles. An ovariole (A) contains egg chambers of increasing maturity from anterior (left) to posterior (right). The germarium is present in the anterior-most region of the ovariole. It contains two to three germline stem cells that are anteriorly localized. Each germline stem cell divides asymmetrically to produce a new stem cell and a daughter cell that undergoes four rounds of synchronous division with incomplete cytokinesis, producing 16 germ cells interconnected by ring canals. Egg chambers are composed of these groups of 16 germ cells – 15 nurse cells and one oocyte – surrounded by an epithelium of somatic follicle cells. Nuclei of nurse cells and of the oocyte are in blue. Egg chambers develop through 14 morphological stages (indicated above egg chambers) to produce mature eggs (stage 14). Throughout oogenesis, nurse cells provide maternal mRNAs and proteins to the oocyte through ring canals. These maternal components are required for the development of the oocyte and the future embryo. (B) Schematic representation of mRNA and protein localization for *bcd*, *grk*, *osk* and *nos* in stage 10 oocyte and early embryo. *bcd*, *grk* and *osk* mRNAs localize during mid-oogenesis, while *nos* mRNA localization occurs at later stages. mRNA localization is coupled to translational control such that protein synthesis is localized. The specific localization of *bcd* and *nos* mRNAs at the anterior and posterior poles, respectively, generates Bcd and Nos opposite protein gradients that shape the anterior-posterior axis of the embryo. Axis orientation is indicated at the top: A, anterior, P, posterior, D, dorsal, V, ventral.

Function of Maternal mRNAs: Establishment of the Oocyte and Embryonic Polarity

Maternal mRNAs and their post-transcriptional regulation play a key role in meiotic progression and regulation of cell cycle in the oocyte and early embryo. Oocyte maturation (meiotic progression from prophase to metaphase I) that takes place in stage 12–13 oocyte is accompanied by poly(A) tail lengthening of a large set of maternal mRNAs and large-scale changes in the proteome (Laver *et al.*, 2015).

Another major function of maternal mRNAs is to establish the polarity of the *Drosophila* embryo, which already takes place in the oocyte. Four maternal mRNAs, *oskar* (*osk*), *nanos* (*nos*), *bicoid* (*bcd*) and *gurken* (*grk*) play key roles in embryonic axis specification

(Lasko, 2012). The corresponding genes were identified through genetic screens: mothers mutant for these genes produce embryos lacking embryonic structures. These maternal mRNAs are transported along microtubules from the nurse cells to the oocyte, during early stages of oogenesis, through the ring canals. Localization of these mRNAs become highly asymmetric from mid-oogenesis (stage 9) and this localization coupled to translational regulation underlies axis specification (Fig. 2(B)). The anterior-posterior axis is established through the localization of *bcd* mRNA to the anterior pole of the oocyte and the localization of *osk* and *nos* mRNAs to the posterior pole. *osk* mRNA is translated when it reaches the posterior pole during mid-oogenesis. Osk protein is the primary determinant of the germ plasm, a specialized cytoplasm that is required for germ cell development at the posterior pole of the oocyte (Lehmann, 2016). Large RNA granules, so-called polar granules, containing mRNAs, small RNAs and proteins form in the germ plasm. Polar granules contain mRNAs encoding germ cell determinants and are essential for germline specification. Nos is one of those germ cell determinants, and is also required for abdominal development. Nos protein is expressed as a posterior to anterior gradient generated from *nos* mRNA localized in the germ plasm at the posterior pole. Osk is involved in the stabilization and translation of germ cell mRNAs, including *nos*, in the germ plasm. Osk is therefore essential for both posterior patterning and specification of the germline lineage in the embryo (Fig. 2(B)).

Although localized at the anterior pole of the oocyte during mid-oogenesis, *bcd* mRNA is translated only after egg activation -when the mature stage 14 oocyte passes through the oviduct, prior to fertilization-. Its translation depends on poly(A) tail elongation. Bcd protein is produced as a gradient from the anteriorly localized mRNA. This gradient of Bcd acting both as a transcription factor and a translational repressor, initiates a cascade of zygotic gene expression that directs anterior-posterior patterning of the embryo.

Grk is required in establishing both the anterior-posterior and dorsal-ventral axes during oogenesis. Grk is an epidermal growth factor receptor (EGFR) ligand secreted from the oocyte to locally activate EGFR in the adjacent somatic cells that surround the oocyte, the follicle cells. During early oogenesis, Grk plays a role in establishing anterior-posterior polarity by signaling to a posterior subpopulation of follicle cells to adopt a posterior fate. During mid-oogenesis, *grk* mRNA localizes to the antero-dorsal corner of the oocyte and produces localized protein that specifies the dorsal-ventral axis, by inducing a dorsal fate in the closest follicle cells (Fig. 2(B)).

Regulation of Maternal mRNAs in Oocytes

Localization of Maternal mRNAs in Oocytes

mRNA localization is a widespread process to generate cell asymmetry in diverse biological contexts, and it is instrumental for setting up axes in the *Drosophila* embryo. Several mechanisms are at play for localizing in the oocyte mRNAs that are required for axis formation, and the whole process is very complex and highly regulated. Extensive genetic and molecular studies of mRNA localization identified common principles: localization depends on sequence elements that are often present in mRNA 3'UTRs and on proteins that bind these elements; RNA localization elements often form stem-loop structures and are often repeated and redundant; distinct localization elements on the same mRNA can mediate distinct steps in localization (Martin and Ephrussi, 2009).

A common mechanism for mRNA localization in the oocyte corresponds to active transport along microtubules. The microtubule network in the nurse cells and oocyte is complex and reorganizes several times during oogenesis. Live-imaging studies have been essential to shed light on the mechanisms of mRNA transport and the cytoskeletal organization (Becalska and Gavis, 2009). An important tool for these studies, which was first developed in yeast and later adapted to *Drosophila*, is the MS2 system: an mRNA is GFP-tagged *via* the tethering of the bacteriophage MS2 coat protein fused to GFP, to MS2 coat protein binding sites inserted into the mRNA 3'UTR. The MS2 system allows visualization of mRNA movements (Becalska and Gavis, 2009; Bertrand *et al.*, 1998; Forrest and Gavis, 2003).

During early oogenesis, maternal mRNAs are transported from nurse cells to the oocyte through the ring canals, on microtubules that extend into nurse cells, from a microtubule-organizing center (MTOC) localized in the oocyte. mRNA transport proceeds towards microtubule minus ends driven by the dynein motor complex. Two interacting proteins Egalitarian and Bicaudal-D make the link between mRNAs, bound to Egalitarian, and the dynein complex interacting with Bicaudal-D through dynactin (Lasko, 2012) (Fig. 3(A)). At stage 7 of oogenesis, microtubules reorganize for a less organized network, but transport from nurse cells to oocyte remains dynein-dependent (Becalska and Gavis, 2009).

mRNAs localized to the posterior pole must travel long distances of more than 50 μm . mRNA live-imaging analyses have shown that, at mid-oogenesis (stages 9–10), the microtubule network within the oocyte is largely randomly organized, with a slight bias towards plus ends at the posterior pole. Posterior *osk* mRNA localization takes place through kinesin-directed transport towards plus ends of microtubules (Fig. 3(B)). Accumulation at the posterior pole is achieved through transport along this slightly biased microtubule network, over a period of several hours (Becalska and Gavis, 2009). Genetic studies have identified a number of proteins involved in *osk* mRNA posterior localization, including Staufen, Hrp48, and subunits of the exon-junction complex. This complex is recruited to the mRNA during splicing, thus revealing that nuclear events can play essential roles in mRNA cytoplasmic localization (Lasko, 2012; Lehmann, 2016; Fig. 3(B)).

The localization of *bcd* mRNA at the anterior pole of the oocyte proceeds through several steps, the first of which requires the Exuperantia protein that was proposed to select a subpopulation of microtubules polarized towards the anterior cortex for dynein-directed transport. At later stages (stage 12), *bcd* mRNA localization requires Swallow and the double strand RNA binding

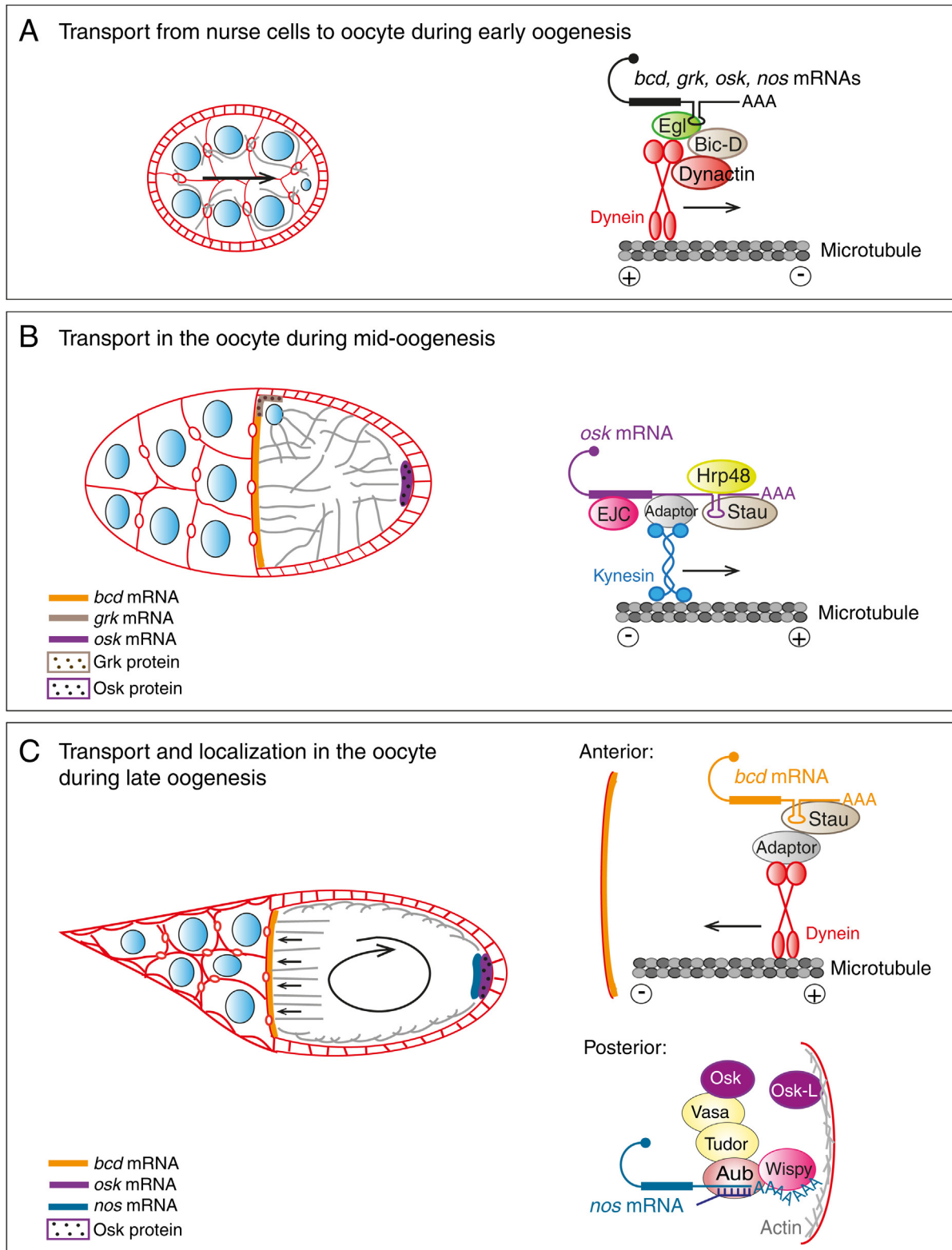


Fig. 3 Mechanisms of maternal mRNA transport during *Drosophila* oogenesis. Molecular mechanisms involved in the localization of *osk*, *bcd*, *grk* and *nos* mRNAs at different stages of oogenesis are schematized. For all panels, a scheme of an egg chamber is on the left; and a scheme showing the factors involved in localization is on the right. Nuclei of nurse cells and of the oocyte are in blue; microtubules and actin microfilaments are in grey; black arrows indicate mRNA movements. (A) Dynein-directed transport of mRNAs along microtubules in nurse cells during early oogenesis (stages 1–6). Egl and Bic-D proteins directly recruit mRNAs to the dynein-dynactin complex. (B) *osk* mRNA transport along microtubules in the oocyte during mid-oogenesis. *osk* is transported towards plus ends of microtubules with kinesin. Microtubules are largely randomly organized in the oocyte, with a slight bias for plus ends towards the posterior pole. RNA binding proteins and other components required for *osk* mRNA transport are schematized. The exon-junction complex (EJC) is deposited on the mRNA upon splicing in nurse cell nuclei. (C) *bcd* and *nos* mRNA localization in late stage oocytes. A stage 12 egg chamber is schematized. At this stage, nurse cells contract to dump their cytoplasm into the oocyte. *bcd*

protein Staufen, and depends on continuous dynein-mediated transport on microtubules organized from the anterior cortex of the oocyte (Fig. 3(C)). At late oogenesis (stage 14), *bcd* mRNA localization switches from continuous active transport to stable anchoring to the cortical actin cytoskeleton. This actin-dependent anchoring is maintained until egg activation, when *bcd* mRNA is dispersed (Becalska and Gavis, 2009).

In contrast to *bcd* and *osk*, *nos* mRNA localization does not involve transport along microtubules. *nos* mRNA localization to the posterior pole of the oocyte takes place during late oogenesis (stages 11–13), following the onset of nurse cell dumping. Cytoplasmic streaming (cytoplasmic flow into the oocyte based on microtubules parallel to the oocyte cortex) and nurse cell dumping produce a rotating flow of cytoplasm mixing nurse cell and oocyte contents. *nos* mRNA localization is achieved via posterior anchoring of the pool of mRNA that goes through the germ plasm during this cytoplasmic movement (Becalska and Gavis, 2009; Forrest and Gavis, 2003; Fig. 3(C)). Anchoring depends on actin cytoskeleton and its reorganization involving the longer of two Osk protein isoforms. This localization mechanism by diffusion and anchoring is used for posterior localization of a number of maternal mRNAs enriched in the germ plasm, and which encode proteins required for germ cell development. *osk* mRNA itself follows this localization process during late oogenesis. Novel quantitative imaging approaches to follow single mRNA molecules have shown that germ cell mRNAs (except *osk*) travel as single mRNA molecules in small particles, which then co-assemble in the germ plasm into large heterogeneous RNA granules containing different mRNAs: the polar granules (Lehmann, 2016; Little et al., 2015; Trcek et al., 2015). Recent studies identified Aubergine (Aub) protein as an important component of the posterior trapping complex, involved in direct interaction with germ cell mRNAs (Barckmann et al., 2015; Vourekas et al., 2016; Fig. 3(C)).

Translational Control of Maternal mRNAs in Oocytes

To achieve cell polarity through mRNA localization, an essential point is the coupling between localization and translational regulation: namely, translation must be repressed during transport, localization, or where localization is incomplete, and activated upon final localization. This coupling occurs by either of two mechanisms: (i) the presence of translational regulators together with localization factors on mRNAs during the localization process, or (ii) the dual role of some regulator proteins in both mRNA localization and translational control.

bcd mRNA translation is strictly repressed in oocytes and activated at egg activation. Cytoplasmic polyadenylation is involved in translation activation, however, *bcd* poly(A) tail length alone cannot explain the tight translational repression in the oocyte (Eichhorn et al., 2016). Thus, additional mechanisms should be involved, which have not yet been addressed.

osk mRNA translational control has been extensively studied. Osk protein is specifically synthesized at the posterior pole of the oocyte, starting during mid-oogenesis at stage 9, upon *osk* mRNA localization. An important component of the translational repressor complex is the Bruno protein that binds several elements in *osk* 3'UTR. Bruno represses *osk* mRNA translation by two mechanisms: (i) it induces the formation of large mRNA particles that are inaccessible to the translation machinery, and (ii) it recruits the Cup protein, which can directly affect translation through its interaction with the cap binding translation initiation factor eIF4E, thus preventing eIF4E-eIF4G interaction and translation initiation. Cup can also recruit the CCR4-NOT deadenylation complex (Lasko, 2012; Fig. 4(A)). CCR4-NOT is a conserved complex composed of eight proteins in *Drosophila*, including two deadenylases (Temme et al., 2014). Deadenylation is the first step of mRNA decay. However, in oocytes as opposed to most other cell types, deadenylation does not lead to immediate mRNA decay; instead deadenylated mRNAs can be maintained translationally repressed for re-activation at later developmental steps. Me31B is another translational repressor of *osk* mRNA, in complex with Cup and eIF4E. Additional translational repressors include PTB (polypyrimidine tract binding protein) and Hrp48 that is also involved in *osk* mRNA localization.

osk mRNA translation is locally activated at the posterior pole of the oocyte. Orb, the RNA binding protein homolog of CPEB (Cytoplasmic Polyadenylation Element Binding protein), plays a role in this process. Orb interacts with two poly(A) polymerases, PAP (poly(A) polymerase) and Wispy, the cytoplasmic poly(A) polymerase. Posterior translational activation of *osk* mRNA at mid-oogenesis depends on poly(A) tail elongation by PAP (Lasko, 2012; Fig. 4(A)). Wispy is required for polyadenylation of a large set of maternal mRNAs at late stages of oogenesis. Interestingly, Bruno, the translational repressor of *osk* mRNA during its transport, contributes to *osk* translational activation at the posterior pole, as does Staufen, a component of the *osk* mRNA transport machinery.

Regulation of Maternal mRNAs in Embryos

Maternal mRNA Decay During the Maternal-to-Zygotic Transition – Role of Small Non-Coding RNAs

In early embryos, maternal mRNAs undergo a massive decay during the maternal-to-zygotic transition (Fig. 1). Genome-wide transcriptome analyses have shown that 60% of maternal mRNAs are significantly degraded during this period. The comparison of maternal mRNA decay in fertilized embryos and activated unfertilized eggs, without zygotic transcription, revealed that maternal mRNA decay involves two pathways: an early pathway encoded maternally, and a later pathway encoded by the zygote, taking place

localization to the anterior involves continuous dynein-directed transport along microtubules (small black arrows), and the Staufen (Stau) RNA binding protein. Cytoplasmic streaming and nurse cell dumping induce a rotating flow of cytoplasm in the oocyte (circular black arrow) allowing diffusion in the oocyte of a pool of maternal mRNAs, including *nos*. These maternal mRNAs are anchored at the posterior pole via actin microfilaments, when they go through the germ plasm during their rotating movement with the cytoplasm. The long Osk protein isoform (Osk-L) is involved in actin remodeling. Aub binds maternal mRNAs, colocalizes with the Wispy poly(A) polymerase in the germ plasm and is present in polar granules together with Tudor, Vasa and Osk short isoform. The comb represents a piRNA, required for Aub binding to mRNA.

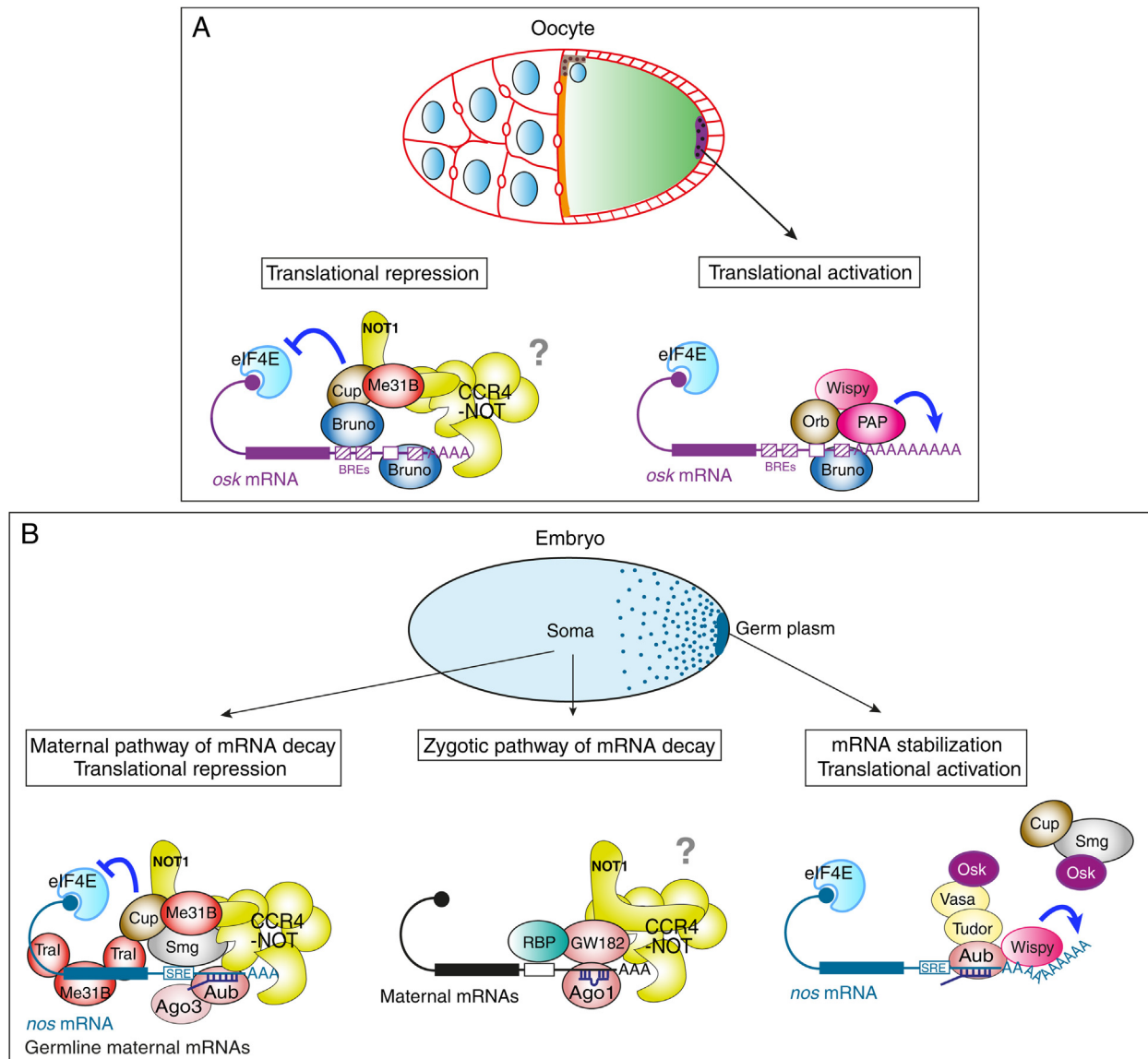


Fig. 4 Translational control coupled to mRNA localization in the oocyte and embryo. (A) Translational regulation of *osk* mRNA in the oocyte during mid-oogenesis. Bruno, Cup and Me31B are translational repressors of *osk* mRNA. The role of the CCR4-NOT complex in *osk* mRNA translational control has not been directly tested (indicated by a question mark). Its implication is suggested from Cup interaction with the NOT1 subunit of CCR4-NOT in *Drosophila* S2 cells, and the direct interaction between NOT1 and the human homolog of Me31B. Cup can repress translation through binding to eIF4E (blue inhibitory arrow). Bruno binds to several sites called Bruno response elements (BREs: striped boxes) in *osk* 3'UTR. The 3'-most BRE is involved in both translational repression and activation. The Orb RNA binding protein is in complex with both poly(A) polymerase (PAP) and Wispy in the ovary, but cytoplasmic polyadenylation depends on PAP during mid-oogenesis (blue arrow). Translation repression takes place during *osk* mRNA transport, whereas translation is activated at the posterior pole. *osk* mRNA is in purple; Osk protein is represented by black dots. (B) mRNA decay and selective stabilization coupled to translational control of *nos* mRNA in the embryo. *nos* mRNA localization by diffusion and anchoring is inefficient, leading to high amounts of *nos* mRNA (light blue) in the soma, in addition to its accumulation (dark blue) in the germ plasm. In pre-MZT embryos, Smg binds to stem loop structures in *nos* 3'UTR, the Smg recognition elements (SREs). Aub binds to *nos* 3'UTR through complementary piRNAs (comb). Smg, CCR4, Aub and Ago3 are found in complex in embryos and are involved in *nos* mRNA deadenylation in the somatic part of the embryo. Cup bound to Smg could prevent translation via its binding to eIF4E (blue inhibitory arrow). However *nos* translational repression is independent of the 5' cap and involves mRNA coating by Me31B and TraI. The zygotic machinery of maternal mRNA decay involves miRNAs (comb) from the *miR-309* cluster. The protein interactions are drawn from interactions shown in other contexts, but not yet validated in the *Drosophila* embryo (indicated by a question mark). Most mRNA targets of miRNAs also undergo decay by the maternal pathway, indicating that both pathways cooperate in mRNA decay. The maternal pathway can implicate Smg or other RNA binding proteins (RBP). In addition, RNA binding proteins such as Pum are also involved in the zygotic pathway of mRNA decay (not shown). In the germ plasm, Osk binds Smg and this prevents Smg binding to *nos* mRNA. CCR4 is depleted from the germ plasm. Aub bound to *nos* mRNA through piRNAs (comb) colocalizes and interacts with Wispy, allowing poly(A) tail elongation, localized stabilization and translation (blue arrow), which generates the Nos protein gradient (blue dots). Aub interacts with Tudor and is present in polar granules with Vasa and Osk.

after zygotic transcription. Maternal mRNAs can be degraded by either of these two pathways or by both pathways together, leading to different dynamics of mRNA decay (Fig. 1).

Maternal mRNA decay by the maternal machinery depends on deadenylation by the CCR4-NOT deadenylation complex. This complex can act unspecifically on all mRNAs, and specificity is achieved through its recruitment to sequence elements in mRNAs, by RNA binding proteins or small non-coding RNAs binding these elements. An important actor in this decay is the RNA binding protein Smaug (Smg) that binds about two-thirds of unstable maternal mRNAs and recruits CCR4-NOT (Barckmann and Simonelig, 2013; Fig. 4(B)). The timing of the decay is determined by Smg expression that peaks between 30 min and 3 h of embryonic development.

nos mRNA has been used as a paradigm to follow maternal mRNA decay during the maternal-to-zygotic transition. *nos* is posteriorly localized by the mechanism of diffusion and anchoring during late oogenesis. This mechanism is very inefficient and a huge fraction of *nos* mRNA (calculated to be 96%) remains unlocalized in the whole embryo. This pool of *nos* is deadenylated by the CCR4-NOT complex recruited to *nos* 3'UTR by Smg, which leads to its decay during the first hours of embryogenesis. Further analyses of *nos* mRNA deadenylation identified a specific class of small non-coding RNAs, the Piwi-interacting RNAs (piRNAs), as components of the maternal machinery of maternal mRNA decay. The piRNA pathway is an RNA silencing pathway that has been found in the germline of many species. piRNAs are non-coding RNAs of 23–30 nt, which like microRNAs (miRNAs), are loaded into specific Argonaute proteins, so-called PIWI proteins, and interact with their target mRNAs by complementarity to silence them. This can occur *via* endonucleolytic cleavage of the target mRNA by PIWI proteins (Senti and Brennecke, 2010; Weick and Miska, 2014). The piRNA pathway plays a crucial role in the repression of transposable elements in the germline and indeed, a large part of piRNAs are produced from transposable element sequences. piRNAs are produced in the nurse cells in the ovary and transferred to the oocyte while loaded into PIWI proteins. Therefore, piRNAs are present in the early embryo. Strikingly, a role of the piRNA pathway has been established in the decay of *nos* mRNA in the embryo. piRNAs produced from transposable elements target *nos* 3'UTR and guide interactions with the PIWI proteins Aub and Argonaute 3 (Ago3), which, together with Smg, contribute to recruitment of the CCR4-NOT deadenylation complex (Fig. 4(B)). This regulation of *nos* mRNA by piRNAs is required for embryonic patterning, thus identifying a biological function of transposable elements in *Drosophila* development (Barckmann and Simonelig, 2013). Aub was then shown to directly bind several hundred maternal mRNAs in the early embryo, and to be involved in the decay of a large proportion of them. Aub induces decay either through deadenylation by CCR4-NOT, or by direct cleavage through its endonucleolytic activity (Barckmann *et al.*, 2015).

Maternal miRNAs are also present in the embryo and are likely to be involved in maternal mRNA decay, since miRNA target sites are found in mRNAs degraded by the maternal pathway. However, to date, the role of miRNAs in the maternal pathway of maternal mRNA decay has not been formally demonstrated.

In contrast, miRNAs are a major component of the zygotic pathway of maternal mRNA decay. The *Drosophila* *miR-309* cluster which produces eight miRNAs is highly expressed at the onset of zygotic genome activation and induces the decay of hundreds of maternal mRNAs (Barckmann and Simonelig, 2013). How miRNAs induce maternal mRNA decay has not been addressed. However, in many biological contexts, RNA silencing by miRNAs involves deadenylation by CCR4-NOT and another general deadenylation complex, PAN2-PAN3. miRNAs are loaded into Argonaute proteins and these proteins directly interact with proteins of the GW182 family, which in turn can recruit both deadenylation complexes through direct protein interactions. These interactions, which are conserved from *Drosophila* to mammals, are likely to be involved in maternal mRNA decay by miRNAs (Fig. 4(B)). Maternal mRNA decay by Smg is indirectly involved in zygotic genome activation and expression of the *miR-309* cluster, thus reflecting an interplay between the maternal and zygotic pathways of maternal mRNA decay.

RNA binding proteins have also been identified in the zygotic machinery of maternal mRNA decay. Pumilio (Pum) is involved in the degradation of *bcd* mRNA that is degraded by the zygotic pathway. Brain tumor (Brat), another RNA binding protein, first described as a co-factor of Pum, targets maternal mRNAs *via* both the early and late pathways of mRNA decay. Both Pum and Brat are thought to act by directly recruiting the CCR4-NOT deadenylation complex (Laver *et al.*, 2015).

Localization of Maternal mRNAs in Embryos by Selective Stabilization

Strikingly, when genome-wide analyses of maternal mRNA decay in embryos are compared with expression patterns of the same mRNAs using large-scale RNA *in situ* hybridization, a link between maternal mRNA decay and localization to the posterior pole becomes apparent. This indicates that mRNA decay can be spatially regulated and result in posterior mRNA localization. As explained in the previous section, the mechanism of posterior localization by diffusion and anchoring, taking place during late oogenesis, is very inefficient. Posterior localization of these mRNAs is then reinforced in the embryo by decay and selective stabilization in the germ plasm. Studies of *nos* maternal mRNA in the embryo have shown that Osk is the major factor in mRNA stabilization in the germ plasm. Osk directly interacts with Smg and prevents the binding of Smg to *nos* mRNA. This precludes the recruitment of the CCR4-NOT deadenylation complex to *nos* mRNA and its deadenylation. This mechanism has the potential to apply to all Smg target mRNAs (Barckmann and Simonelig, 2013; Fig. 4(B)). Another important component in this spatial regulation of maternal mRNA decay is the PIWI protein Aub. Recent studies of Aub function revealed its dual role in maternal mRNA decay in the somatic part of the embryo and localization in the germ plasm. Aub binds germ cell mRNAs and is involved in their degradation in the somatic part of the embryo (Barckmann *et al.*, 2015). However, Aub accumulates in the germ plasm in the oocyte and embryo and was proposed to contribute to germ cell mRNAs anchoring at the posterior pole during late oogenesis (Vourekas *et al.*, 2016). In the embryo, Aub plays a role in selective stabilization of these mRNAs in the germ plasm,

by recruiting the Wispy poly(A) polymerase (Fig. 4(B)). Thus, Aub function switches in space from decay to stabilization through regulated interactions with the deadenylation and polyadenylation machineries (Dufourt *et al.*, 2017).

Another mechanism reduces mRNA decay at the posterior pole. The zygotic genome activation is delayed in the primordial germ cells, the first cells to form in the embryo, leading to a delay of mRNA decay by the zygotic pathway (Barckmann and Simonelig, 2013).

Translational Control of Maternal mRNAs in Embryos

Recent studies at genome scale have shown that in late stage oocytes and early embryos up to gastrulation, translation efficiency depends on poly(A) tail length, thus confirming the key role of this mode of translational control during early development (Eichhorn *et al.*, 2016). At these stages poly(A) tail lengths are determined by the balance between deadenylation and cytoplasmic polyadenylation occurring concomitantly on the same mRNAs.

nos mRNA proved to be also an excellent model to study translational control. *nos* mRNA localization by decay and selective stabilization, which is based on poly(A) tail length regulation, is highly coupled to translational control. The pool of *nos* mRNA present in the somatic part of the embryo is translationally repressed before its decay. This repression is instrumental for the development of anterior embryonic structures. In contrast, translation of the pool of mRNA localized and stabilized by polyadenylation in the germ plasm is activated. This generates the posterior to anterior gradient of Nos protein that is essential for the development of abdominal structures and germ cells. Smg was first identified as a translational repressor of *nos* mRNA. This Smg function is partially due to its role in deadenylation through the recruitment of the CCR4-NOT complex. Indeed, recent genome-wide studies revealed that translational repression by Smg strongly correlates with poly(A) tail shortening (Eichhorn *et al.*, 2016). Consistent with this, ectopic Nos protein accumulates in the somatic part of embryos mutant for the CCR4 deadenylase, showing the role of CCR4 in translational repression (Barckmann and Simonelig, 2013). Interestingly, the CCR4-NOT complex has been shown in other contexts to be involved in direct translational repression, in addition to its role in deadenylation. This CCR4-NOT function relies on a direct interaction between the translational repressor Me31B and the NOT1 subunit of the complex, described with human proteins (Temme *et al.*, 2014).

Smg interacts with Cup, which contributes to the recruitment of the CCR4-NOT complex. Cup can also directly inhibit translation through its binding to eIF4E and displacement of eIF4G. Recent *in vitro* analyses identifying the components of the *nos* mRNA translational repressor complex have shown that translational repression involves mRNA coating with Me31B and its co-factor Trailer hitch (Tral) (Gotze *et al.*, 2017; Fig. 4(B)).

Smg is involved in both *nos* mRNA decay and translational repression, therefore its interaction with Osk contributes to both localized stabilization and translational activation in the pole plasm. In addition, specific regulation through 3'UTR of germ cell mRNAs is involved in their temporal translational activation (Barckmann and Simonelig, 2013).

Conclusions

Drosophila has been a powerful model to establish the importance of maternal mRNAs in early embryogenesis, as well as to decipher the involved regulatory mechanisms. Early steps of development that take place before activation of the zygotic genome depend exclusively on maternal mRNAs and their regulation in oocytes and early embryos. This regulation can occur at a wide range of steps of the mRNA life in the cytoplasm, including transport, localization, stability and translation. The mechanisms underlying this regulation are also varied, and are determined by a combination of *cis* elements present in mRNAs and factors binding these elements. The widespread role of small non-coding RNAs in regulating numerous biological processes has emerged in the last decades. miRNAs play an important role in maternal mRNA decay in the embryo during the maternal-to-zygotic transition. The role of piRNAs was more surprising since these small RNAs were thought to specifically regulate transposable elements. piRNAs have an essential function in both the decay and posterior localization of maternal mRNAs in the embryo. Because a large part of these piRNAs are produced from transposable elements, this establishes transposable elements as important regulators of early developmental processes. piRNAs are produced in the ovary and provided maternally to the embryo, thus they are part of maternal reserves that play key roles in embryonic development.

Deciphering the mechanisms of maternal mRNA regulation has identified the central role of the CCR4-NOT complex that acts both as a deadenylating enzyme and a scaffold to recruit translational repressors. This complex can accommodate large sets of mRNAs through interaction with various RNA binding proteins, and is used at many developmental steps in different regulatory mechanisms.

Another aspect in the regulation of maternal mRNAs is the formation of granules containing mRNAs, proteins, as well as small RNAs in some cases. The assembly of maternal mRNAs as RNA granules is involved in several processes including mRNA transport and translational control. RNA granules are specifically important in the germ plasm. Polar granules are crucial for germ cell specification, and this function is conserved in several species (Voronina *et al.*, 2011). *Drosophila* polar granules contain Osk protein, germ cell mRNAs, translational repressors, as well as several components of the piRNA pathway, including Aub and the germ cell markers Vasa and Tudor. piRNAs are also present in polar granules where they have a major function in germ cell development and maintenance through mRNA regulation. Many questions regarding RNA granules and polar granules are open for future studies, including their relationships with translational repression and activation, or the recently recognized role of RNA binding protein phase transitions in granule formation (Uversky, 2017).

The *Drosophila* model should continue to be at the fore front to decipher mechanisms involved in maternal mRNA regulation, many of which are conserved in mammals.

Acknowledgements

We thank Elmar Wahle for comments on this article. We apologize to colleagues for being unable to cite primary publications due to space limitation. Work in the Simonelig lab is supported by UMR9002 CNRS-University of Montpellier, ANR (ANR-15-CE12-0019-01), FRM, Fondation ARC, the Labex EpiGenMed and AFM Telethon.

References

- Barckmann, B., Pierson, S., Dufourt, J., et al. (2015). Aubergine iCLIP reveals piRNA-dependent decay of mRNAs involved in germ cell development in the early embryo. *Cell Rep.*, 12, 1205–1216.
- Barckmann, B., & Simonelig, M. (2013). Control of maternal mRNA stability in germ cells and early embryos. *Biochim. Biophys. Acta*, 1829, 714–724.
- Bastock, R., & Johnston, D. S. T. (2008D.). *Drosophila* oogenesis. *Curr. Biol.*, 18, R1082–R1087.
- Becalska, A. N., & Gavis, E. R. (2009). Lighting up mRNA localization in *Drosophila* oogenesis. *Development*, 136, 2493–2503.
- Bertrand, E., Chartrand, P., Schaefer, M., et al. (1998). Localization of ASH1 mRNA particles in living yeast. *Mol Cell*, 2, 437–445.
- Dufourt, J., Bontonou, G., Chartier, A., et al. (2017). piRNAs and Aubergine cooperate with Wispy poly(A) polymerase to stabilize mRNAs in the germ plasm. *Nat. Commun.*
- Eichhorn, S. W., Subtelny, A. O., Kronja, I., et al. (2016). MRNA poly(A) - tail changes specified by deadenylation broadly reshape translation in *Drosophila* oocytes and early embryos. *Elife*, 5.
- Forrest, K. M., & Gavis, E. R. (2003). Live imaging of endogenous RNA reveals a diffusion and entrapment mechanism for nanos mRNA localization in *Drosophila*. *Curr. Biol.*, 13, 1159–1168.
- Gotze, M., Dufourt, J., Ihling, C., et al. (2017). Translational repression of the *Drosophila* nanos mRNA involves the RNA helicase Belle and RNA coating by Me31B and Trailer hitch. *RNA*, 23, 1552–1568.
- Lasko, P. (2012). mRNA localization and translational control in *Drosophila* oogenesis. *Cold Spring Harb. Perspect. Biol.*, 4.
- Laver, J. D., Marsolais, A. J., Smibert, C. A., & Lipshitz, H. D. (2015). Regulation and function of maternal gene products during the maternal-to-zygotic transition in *drosophila*. *Curr. Top. Dev. Biol.*, 113, 43–84.
- Lehmann, R. (2016). Germ plasm biogenesis – An Oskar-centric perspective. *Curr. Top. Dev. Biol.*, 116, 679–707.
- Little, S. C., Sinsimer, K. S., Lee, J. J., Wieschaus, E. F., & Gavis, E. R. (2015). Independent and coordinate trafficking of single *Drosophila* germ plasm mRNAs. *Nat. Cell Biol.*, 17, 558–568.
- Martin, K. C., & Ephrussi, A. (2009). mRNA localization: Gene expression in the spatial dimension. *Cell*, 136, 719–730.
- Senti, K. A., & Brennecke, J. (2010). The piRNA pathway: A fly's perspective on the guardian of the genome. *Trends Genet.*, 26, 499–509.
- Temme, C., Simonelig, M., & Wahle, E. (2014). Deadenylation of mRNA by the CCR4-NOT complex in *Drosophila*: Molecular and developmental aspects. *Front. Genet.*, 5, 143.
- Thomsen, S., Anders, S., Janga, S. C., Huber, W., & Alonso, C. R. (2010). Genome-wide analysis of mRNA decay patterns during early *Drosophila* development. *Genome Biol.*, 11, R93.
- Trcek, T., Grosch, M., York, A., et al. (2015). *Drosophila* germ granules are structured and contain homotypic mRNA clusters. *Nat. Commun.*, 6, 7962.
- Uversky, V. N. (2017). Intrinsically disordered proteins in overcrowded milieu: Membrane-less organelles, phase separation, and intrinsic disorder. *Curr. Opin. Struct. Biol.*, 44, 18–30.
- Voronina, E., Seydoux, G., Sassone-Corsi, P., & Nagamori, I. (2011). RNA granules in germ cells. *Cold Spring Harb. Perspect. Biol.*, 3.
- Vourekas, A., Alexiou, P., Vrettos, N., Maragkakis, M., & Mourelatos, Z. (2016). Sequence-dependent but not sequence-specific piRNA adhesion traps mRNAs to the germ plasm. *Nature*, 531, 390–394.
- Weick, E. M., & Miska, E. A. (2014). PiRNAs: From biogenesis to function. *Development*, 141, 3458–3471.
- Weill, L., Belloc, E., Bava, F. A., & Mendez, R. (2012). Translational control by changes in poly(A) tail length: Recycling mRNAs. *Nat. Struct. Mol. Biol.*, 19, 577–585.

Vitellogenesis and Yolk Proteins, Fish

Craig V Sullivan, Carolina AquaGyn, Raleigh, NC, United States
Ozlem Yilmaz, University of Bergen, Bergen, Norway

© 2018 Elsevier Inc. All rights reserved.

Glossary

Atresia The process by which ovarian follicles are degraded with resorption of yolk for recycling.

Egg A fully mature and ovulated female gamete that is competent to undergo fertilization and in which the embryo develops. After ovulation, oocytes become eggs. Also termed an ovum.

Follicle growth The period of time from when somatic prefollicle cells envelop the primary oocyte (folliculogenesis) to the end of vitellogenic oocyte growth.

Oocyte Female germ cell that arises from an oogonium after the onset of meiosis.

Oogenesis Process by which oogonia become mature eggs (or ova), the female gametes.

Ovarian follicle An oocyte surrounded by supporting somatic cells and acellular materials. These include a monolayer of granulosa cells overlain by a theca made up of connective tissue and vasculature covered by a surface epithelium. A vitelline envelope (zona pellucida) is formed between interleaved microvilli projecting from the oocyte and granulosa cells; this structure becomes the outer protective chorion of the egg and embryo. The follicle ruptures at ovulation to expel the oocyte, which is now termed an egg. Postovulatory follicles may persist and produce reproductive hormones for some time after ovulation.

Ovarian maturation The series of events following resumption of meiosis and initiation of cytoplasmic maturation by fully-grown (postvitellogenic) oocytes until ovulation of fertilizable eggs. The process also includes maturation of the follicle cells (theca, granulosa cells) and their steroidogenic activities and communication with one another and with the oocyte.

Vitellogenesis In common usage – the stage of oocyte growth characterized by the formation of egg yolk precursor proteins in the liver and their deposition in the ooplasm in the form of yolk proteins. By strict definition – the process by which estrogens induce the liver to produce and secrete the major precursors to yolk proteins, vitellogenins.

Vitellogenin receptors Oocyte surface proteins involved in uptake of vitellogenins (Vtgs) by growing oocytes. Two types of Vtg receptors (Vtgrs) have been described, the ‘classical’ Vtgr, which is a variant of the somatic very low-density lipoprotein receptor (Vldlr), and the low density lipoprotein (Ldl) receptor-related protein 13, termed Lrp13.

Vitellogenins Major precursors to the egg yolk proteins. Vitellogenins (Vtgs) are synthesized and secreted by the liver under estrogen stimulation, transported in the blood to the ovary, taken up by growing oocytes via receptor mediated endocytosis, and cleaved into their product yolk proteins, which are stored in the ooplasm. Several different types of fish Vtg have been described.

Yolk The content of the ooplasm of the egg, which provides essential nutrients and other materials required by the developing embryo. In addition to the major yolk proteins derived from vitellogenin, yolk is made up of diverse mélange of components of maternal or endogenous origin, including RNAs, regulatory proteins (e.g. growth and transcription factors), several classes of lipids, vitamins, minerals, and hormones.

Yolk proteins In common usage - any protein in the yolk. By strict definition - products derived from vitellogenins that are stored in the ooplasm. These include lipovitellin (Lv), phosvitin (Pv), β' -component, carboxy-terminal component (Ct), proteolytic derivatives of these 5 major yolk proteins, and complexes formed between them.

Abbreviations

Apo B Apolipoprotein B

β' -c β' -component yolk protein derived from vitellogenin

Ct Carboxy-terminal component yolk protein derived from vitellogenin

FAA Free amino acids

FFA Free fatty acids

Ldl/LDL Low density lipoprotein

Ldlr/LDLR Low density lipoprotein (Ldl) receptor

Lpl Lipoprotein lipase

LLTP Large lipid transfer protein

Lrp13 LDL receptor-related protein 13, vitellogenin receptor that preferentially binds VtgAa in acanthomorphs

LRX+1 Vitellogenin receptor and Lrp13 variant in salmonids

Lv Lipovitellin – the major yolk protein derived from vitellogenin that carries lipids to the ooplasm

LvH Lipovitellin heavy chain
LvL Lipovitellin light chain
MtTp Microsomal triglyceride transfer protein
Pv Phosvitin yolk protein, mainly comprised of polyserine and heavily phosphorylated
RNA Ribonucleic acid
TGL Triacylglycerol (triglyceride)
Vldl/VLDL Very low density lipoprotein
Vldlr/VLDLR Very low density lipoprotein (Vldl) receptor
Vtg Vitellogenin, major yolk protein precursor
VtgAa Acanthomorph A-type vitellogenin Aa
VtgAar Vitellogenin Aa receptor – also termed Lrp13
VtgAb Acanthomorph A-type vitellogenin Ab
VtgAbr Vitellogenin Ab receptor – also termed Vtgr
VtgC C-type vitellogenin that is phosvitinless in teleosts
Vtgr Vitellogenin receptor
YP Yolk protein

Introduction

Growth of ovarian follicles can be divided into primary growth and secondary growth stages (Fig. 1). During primary growth, the oocyte initiates meiosis (arresting at prophase I) and engages in intensive synthesis of RNAs that will direct further oogenesis and

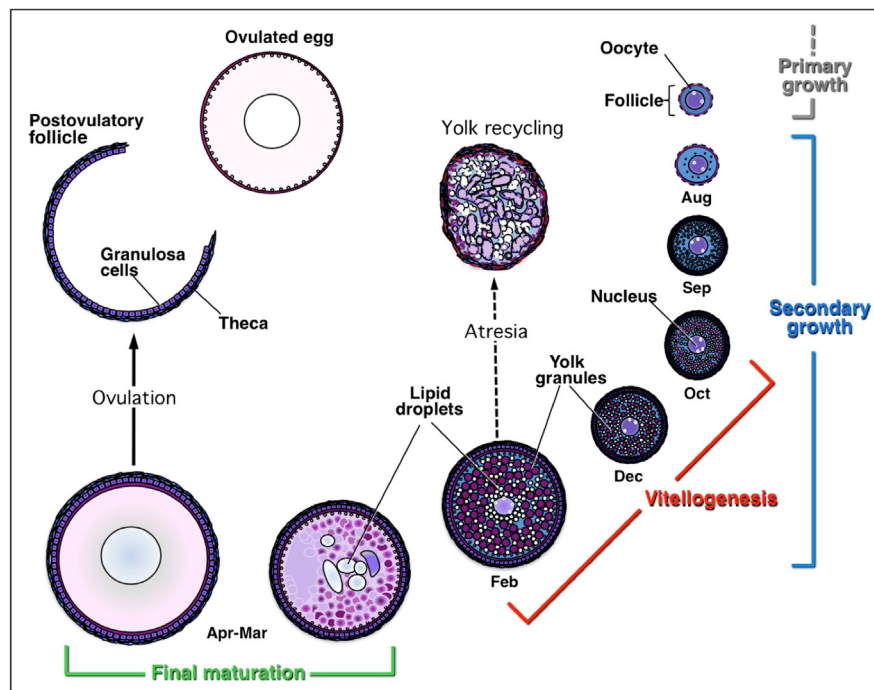


Fig. 1 Model depicting oogenesis in European sea bass, *Dicentrarchus labrax*. An oocyte enclosed by layers of supporting somatic tissues (granulosa cells and theca) is called a follicle. Primary growth oocytes enter 'secondary growth' and accumulate neutral lipids into droplets in their ooplasm. During vitellogenesis, lipid deposition continues as the oocyte accumulates yolk proteins, which are stored in the ooplasm as yolk granules. Fully-grown follicles undergo final maturation, which entails resumption of meiosis and cytoplasmic maturation of the oocyte, including fusion and dissolution of the yolk granules, coalescence of the lipid droplets, clearing of the ooplasm, and an increase in oocyte size due to uptake of water. Maturation is followed by ovulation of an egg capable of being fertilized. At any stage prior to ovulation, the follicle may undergo atresia (breakdown); the egg envelope is breached and granulosa cells enter the ooplasm to scavenge yolk proteins and lipids for recycling. Portions redrawn from Reading, B.J., Sullivan, C.V., 2011. Vitellogenesis in fishes. In: Ferrell, A.P. (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment. The Reproductive Organs and Processes. Reference Module in Life Sciences 2011 ed. Maryland Heights, MO: Elsevier, pp. 635–646. Available at: <https://doi.org/10.1016/B978-0-12-374553-8.00257-4>.

early embryo development. Secondary oocyte growth involves prodigious accumulation of materials in the ooplasm, accounting for most of the final mass of the ovulated egg (Lubzens *et al.*, 2017). These include neutral lipids, sometimes apparent as droplets in the ooplasm, and phospholipid-rich yolk protein (YP) precursors called vitellogenins (Vtgs). Vtgs are synthesized by the liver in response to estrogen, transported in the bloodstream to the ovary, taken up by growing oocytes, and processed into YPs, which are typically stored in yolk granules (also termed globules or platelets) but sometimes in an amorphous compartment ('fluid yolk'). Proliferation of neutral lipid droplets evident in the ooplasm may precede detection of yolk granules and is sometimes referred to as 'previtellogenic' growth. Postvitellogenic follicles containing oocytes filled with lipid droplets, where present, and yolk granules or other types of inclusions bearing YPs, proceed to final maturation, followed by ovulation. At any stage of oocyte growth or maturation prior to ovulation, the follicle may undergo atresia (breakdown) with resorption of the yolk contents (Fig. 1). Limited atresia is a normal process by which females adjust their fecundity, but massive preovulatory atresia entails reproductive failure. Images of ovarian follicles of European sea bass (*Dicentrarchus labrax*) in primary growth or undergoing secondary growth at 'previtellogenic' and vitellogenic stages are provided in Fig. 2.

Composition of Vitellogenins and Yolk Proteins

Vitellogenins are massive proteins, ~300–600 kDa by weight, comprised of homodimers made up of two identical polypeptides, each consisting of a linear array of YP domains bearing phosphate, carbohydrate and lipid components (Fig. 3(A)). Lipids comprise ~20% of Vtgs by weight, primarily phospholipids with lesser quantities of triacylglycerol (Tgl), cholesterol and wax- or steryl-esters (Fig. 3(B)). Vtgs are rich in polyunsaturated fatty acids (~50% of total) providing fluidity to cell membranes in developing embryos. In species spawning demersal (sinking) eggs or floating eggs lacking prominent oil droplets, Vtgs are major carriers of lipids into oocytes, polar lipids (e.g. phosphatidylcholine) predominate in the yolk (~70%–90% of total), and overall lipid levels are usually low (<5% of wet weight). In species spawning pelagic (floating) eggs with large oil droplets, Vtg makes only a minor contribution to yolk lipids, and neutral lipids including Tgl and wax- or steryl esters, predominate. Very low density lipoprotein (Vldl) delivers Tgl to the ovary, from which free fatty acids (FFA) are cleaved by lipoprotein lipase (Lpl) to enter the ooplasm where they are used to synthesize the neutral lipids found in the oil droplets (Hiramatsu *et al.*, 2015). In addition to phosphate, carbohydrate and lipids, Vtgs are important carriers of iron, calcium, magnesium and other minerals, fat-soluble vitamins and related compounds (e.g. vitamins A and E, other retinoids, carotenoids), and hormones, such as thyroid hormones and cortisol that are

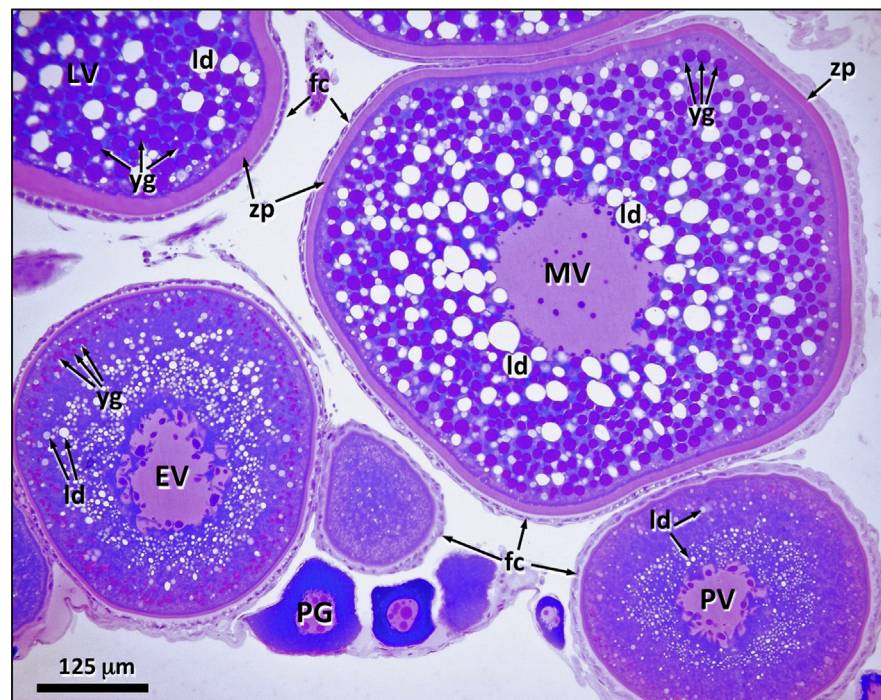


Fig. 2 Light micrograph of an ovary section from a vitellogenic European sea bass, *Dicentrarchus labrax*. Shown are a large mid-vitellogenic (MV) oocyte that is filled with yolk granules (yg) and lipid droplets (ld) and is enclosed in a well developed zona pellucida (zp) or egg envelope overlain by follicle cells (fc). Also shown are an early vitellogenic oocyte (EV) beginning to accumulate yolk granules peripherally, a pre-vitellogenic oocyte (PV) undergoing secondary growth and lacking definitive yolk granules, and several primary growth oocytes (PG). The sample was collected from a farmed female in late winter on the Southwest coast of Spain and subjected to routine histological procedures to obtain 3 μm paraffin sections stained with methylene blue-azure II to visualize the structures referenced above. Image from an unpublished study by Prat, F., Ibáñez, A.J., Yilmaz, O., with permission.

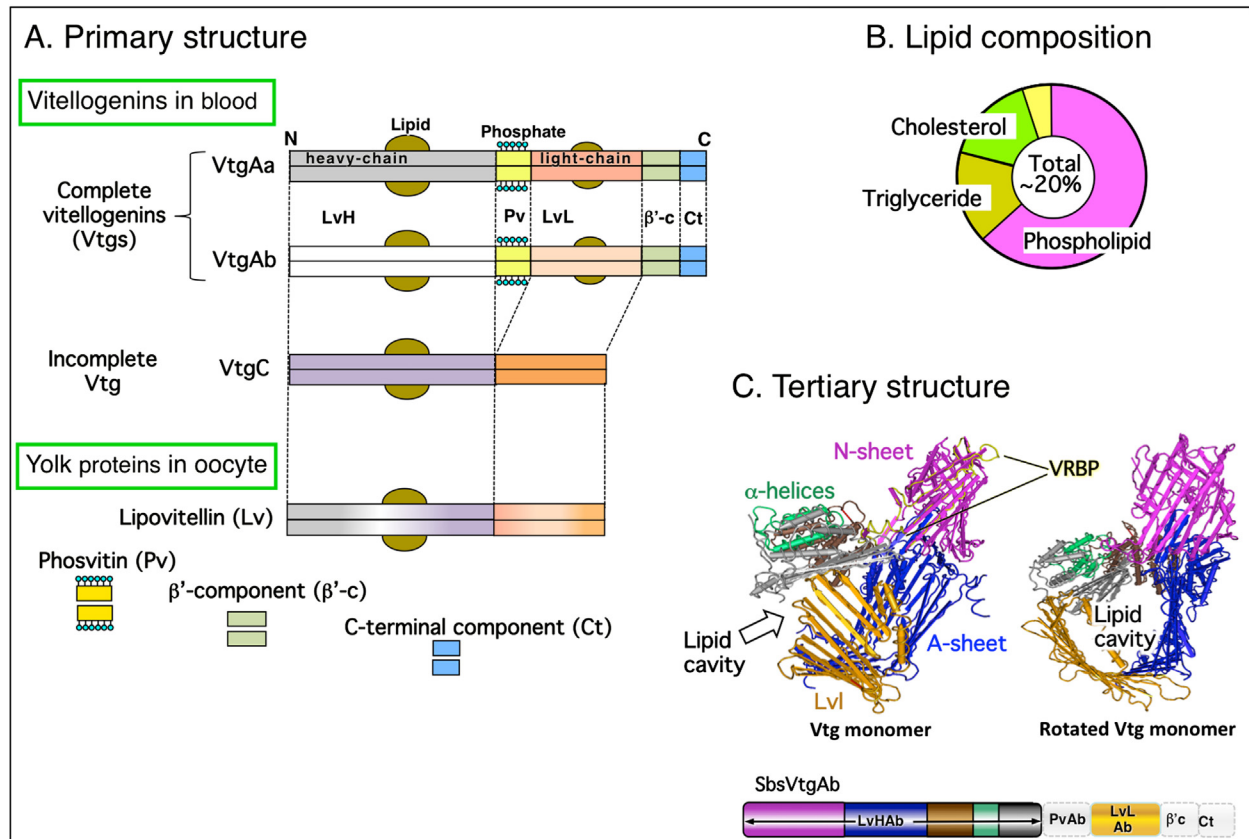


Fig. 3 Structural features of vitellogenins in higher teleosts. (A) Model depicting the linear subdomain structure of native (dimeric) complete vitellogenins (VtgAa and VtgAb) and incomplete vitellogenin (VtgC) and their yolk protein products. The yolk proteins include lipovitellin (Lv) heavy chain (LvH) and Lv light chain (LvL), which transport lipids (indicated by brown half ovals), phosvitin (Pv), which is mainly polyserine and is highly phosphorylated (indicated by blue dots), and two small domains toward the carboxy terminus, the β' -component (β' -c) and the C-terminal component (Ct). The incomplete VtgC is phosvitinless (Pv-less) and also missing β' -c and Ct domains. See text for details. Redrawn from Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C.V., Hara, A., 2006. Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. *Marine Biology* 149, 35–47. (B) Model of vitellogenin lipid composition. The approximate average percentage of lipid in teleost Vtgs by weight is shown in the central white circle; surrounding colored wedges indicate that this is mainly phospholipid with smaller amounts of triglyceride, cholesterol and other lipids. Redrawn from Reading, B.J., Sullivan, C.V., 2011. Vitellogenesis in fishes. In: Ferrell, A.P. (Ed.), *Encyclopedia of Fish Physiology: From Genome to Environment. The Reproductive Organs and Processes. Reference Module in Life Sciences*, 2011 ed. Maryland Heights, MO: Elsevier., pp. 635–646. Available at: <https://doi.org/10.1016/B978-0-12-374553-8.00257-4>. (C) Models of the tertiary structure of the European sea bass (Sbs) VtgAb polypeptide mapped to the 3-dimensional structure of a lamprey Lv, whose crystalline structure has been resolved. The major structural features of the rotated monomer are shown color-coded to the linear VtgAb sequence (below). The location of the 85-residue motif binding the VtgAb receptor, termed the Vtg receptor-binding peptide (VRBP) is indicated, as is the large cavity where lipids are transported. Modeled after Finn (2007). Images from an unpublished study by Yilmaz, O. and Sullivan, C.V., with permission.

soluble in their lipid components. Vtgs are the primary source of amino acids for developing embryos and larvae, and they are particularly rich in alanine, which usually comprises ~10%–12% of total amino acids. Alanine is a crucial substrate of intermediary carbohydrate metabolism, especially embryonic gluconeogenesis (Finn and Fyhn, 2010).

Structure of Vitellogenin and Yolk Proteins

Complete forms of Vtg are made up of a linear series of five YP domains, as follows: Amino terminus (N)-lipovitellin heavy chain (LvH), phosvitin (Pv), lipovitellin light chain (LvL), β' -component (β' -c), and C-terminal peptide (Ct)-carboxy terminus-(C) (Fig. 3(A)). LvH is the major component, with an average mass of ~115 kDa, as compared to ~25 kDa for LvL (Lubzens *et al.*, 2017). Once cleaved from the parent Vtg, LvH and LvL associate to form lipovitellin (Lv), which includes 65%–100% of the Vtg by mass. Lv is comprised of amphipathic structures that form a basket lined with hydrophobic amino acid residues to accommodate lipids (Fig. 3(C)). Lv bears a Vtg receptor (Vtgr)-binding peptide (VRBP), which has the special shape and surface charge distribution required to bind to Vtgs on the oocyte surface (Fig. 3(C)). Lv is the main source of the polar lipid and amino acid nutrients that serve as catabolic energy substrates, or in anabolic synthesis of membranes or proteins, during early development. Phosvitin is

a small (~5–20 kDa) YP made up mainly of long stretches of extensively phosphorylated serine residues, empowering Pv to bind calcium, magnesium, zinc, and iron via ionic interactions. In freshwater fishes, these ions delivered to the yolk by Pv may be crucial as they are largely unavailable for uptake from the environment. However, some incomplete forms of Vtg lack a Pv domain altogether. The remaining small YPs (β' -c and Ct) together contain 14 highly conserved cysteine residues known to form disulfide linkages thought to be required for the complicated folding of Vtg polypeptides. The β' -c also contains a CGxC motif implicated in formation of interchain disulfide linkages during Vtg dimerization, which is required for receptor-mediated endocytosis of the Vtgs by the oocyte. When released from Vtg, β' -c forms an ~16 kDa YP, but, with some exceptions, Ct (~13 kDa) may be degraded shortly after cleavage. Various Lv-Pv conjugates (LvH-Pv, Pv-LvI, LvH-Pv-LvI) have been detected in the yolk of several species. See Finn (2007) for further details on Vtg-derived YPs.

Vitellogenin Classification and Nomenclature

Current nomenclature for classification of piscine Vtgs (Fig. 4(A)) is based upon them having arisen via three rounds of whole genome duplication (WGD) and some lineage-specific gene duplications (Finn and Kristoffersen, 2007), as follows. The ancestral

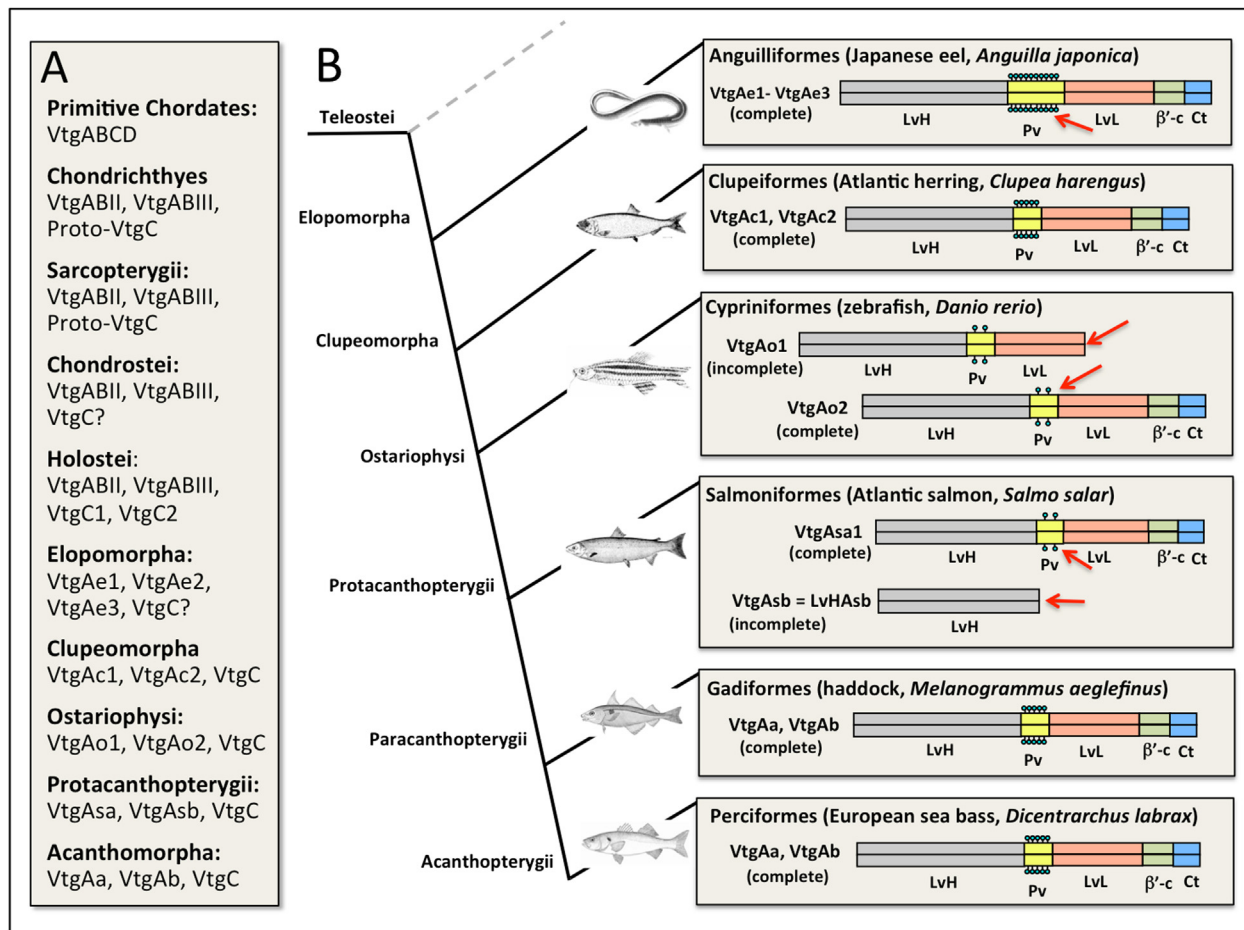


Fig. 4 Nomenclature and domain structure of fish vitellogenins. (A) Classification of multiple fish vitellogenins (Vtgs). VtgABCD, primitive chordate Vtg; VtgABII, chicken VtgII ortholog present in pre-teleost fishes; VtgABIII, chicken VtgIII ortholog present in pre-teleost fishes. Proto-VtgC, pre-teleostean VtgC containing a diminished phosvitin (Pv) domain; VtgC, chicken VtgI ortholog that contains Pv in pre-teleosts but is Pv-less in teleosts; VtgC?, indicates that the presence of VtgC in the taxon is unknown; VtgA, A-type teleostean Vtg; VtgAe, elopomorph VtgA; VtgAc, clupeomorph VtgA; VtgAo, ostariophysian VtgA; VtgAs, salmoniform VtgA. VtgAa, acanthopterygian ortholog of chicken VtgII; VtgAb, acanthopterygian ortholog of chicken VtgIII. Arabic numerals following a Vtg symbol identify duplicate forms of that Vtg. See text for details. (B) Major teleost taxa for which published information on Vtg structure is available are listed on the left side ordered by relationship. For each taxon, the corresponding linear subdomain structure of Vtg is shown for an example species of a representative order on the right side, with species images provided in the middle. Naming of yolk protein domains follows that shown in Fig. 3. Red arrows indicate special features of the respective Vtgs; see text for details. Portions redrawn from Reading, B.J., Sullivan, C.V., 2011. Vitellogenesis in fishes. In: Ferrell, A.P. (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment. The Reproductive Organs and Processes. Reference Module in Life Sciences, 2011 ed. Maryland Heights, MO: Elsevier., pp. 635–646. Available at: <https://doi.org/10.1016/B978-0-12-374553-8.00257-4>.

chordate VtgABCD, present in silver lamprey, arose after the first round of WGD and gave rise to VtgAB and VtgCD after the second round. The VtgCD subsequently gave rise to VtgC, present in most major fish taxa, and to VtgD, which is extinct. Duplicate forms of VtgAB arose and are present in holocephalans (Chondrichthyes), lobe-finned fishes (Sarcopterygii, e.g. coelacanths), chondrosteans (e.g. sturgeons), holosteans (e.g. gars) and tetrapods, including amphibians and birds (Fig. 4(A)). Synteny analyses place these three types of pre-teleost Vtg in a highly conserved Vtg gene cluster (VGC) that has existed for over ~450 million years (Andersen *et al.*, 2017; and references therein) and show that they are orthologs of chicken (*Gallus gallus domesticus*) VtgI (VtgABI, also called VtgC), VtgII (VtgABII) and VtgIII (VtgABIII). An ancestral VtgAB gave rise to the A-type Vtg universally present in teleosts, and to a B-type Vtg that is extinct. Multiple or dual A-type Vtgs are present, for example, in Elopomorpha (e.g. anguillid eel VtgAe1–3) and Ostariophysi (e.g. ostariophysan VtgAo1 and VtgAo2 in Cypriniformes) (Fig. 4(B)). After a late round of WGD, VtgA gave rise to two paralogs present in higher teleosts (Acanthomorpha) as VtgAa and VtgAb (Finn and Kristoffersen, 2007). Acanthomorph VtgC, VtgAa and VtgAb are also orthologs of chicken VtgI, VtgII and VtgIII, respectively.

The VtgC of teleost fishes is ‘incomplete’, without Pv, β' -c and Ct domains, consisting only of Lv (also termed ‘phosvitinless’ Vtg). The C-type Vtgs of the holocephalan elephant shark (*Callorhynchus milii*), a chimera (class Chondrichthys), and the West Indian coelacanth (*Latimeria chalumnae*), a sarcopterygian, both have shortened Pv domains and are referred to here as Proto-VtgCs (Fig. 4(A)). Pre-teleostean C-type Vtgs with complete Pv domains also exist, such as the tandem VtgC1 and VtgC12 in the spotted gar (*Lepisosteus oculatus*), a holostean. Other partial Vtg gene deletions are evident among teleosts (Fig. 4(B)). As examples, the ostariophysan VtgAo1 seen in zebrafish, *Danio rerio* (Cypriniformes) contains Pv but has lost its β' -C and Ct domains, and in the Atlantic salmon, *Salmo salar* (Salmoniformes), the VtgAsb has been reduced to just its LvH domain (Fig. 4(B)). An extreme example of Vtg duplication and deletion occurs in the rainbow trout, *Oncorhynchus mykiss* (Salmoniformes), where the locus of the gene encoding VtgAsa is duplicated in tandem 20 times and the gene encoding the corresponding VtgAsb has been deleted.

Regulation of Vitellogenesis

Major environmental cues regulating vitellogenesis include seasonal changes in water temperature and daylength, but almost anything predictive of successful reproduction can serve as a cue (Fig. 5). Examples include rainy weather (e.g. ‘monsoon season’), which may flood preferred spawning areas, and the presence of conspecific males. The converse is also applicable, as the perceived absence of positive cues is inhibitory. An example in some farmed fishes that spawn every few days is the response to removal of males from the tank, which causes females to cease reproductive processes, terminate vitellogenesis, and undergo massive pre-ovulatory atresia (Fig. 1). Internal cues signaling adequate nutritional status or fat reserves also influence

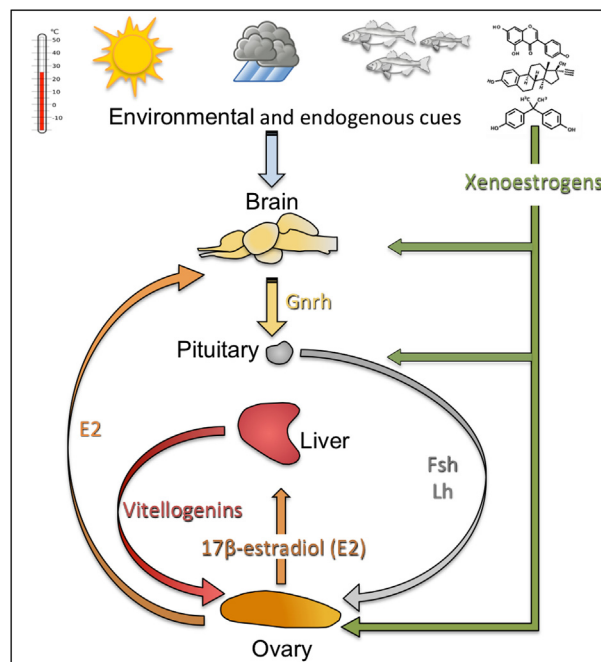


Fig. 5 Model for regulation of vitellogenesis in teleost fish via the brain-pituitary-gonad (BPG) axis. Environmental cues pictured at the top indicate (from left to right) water temperature, daylength, rainy weather, conspecific males and/or females, and xenoestrogens in the environment. Xenoestrogens are shown impacting the BPG axis at several levels. Fsh, follicle stimulating hormone; GnRh, gonadotropin-releasing hormone; Lh, luteinizing hormone; E2, 17 β -estradiol. See text for details.

vitellogenesis and may be especially important where seasonal cues are lacking. Information imparted by received cues is integrated in the brain, ultimately influencing the activity of neurons emanating from the preoptic area that course to the anterior lobe of the pituitary gland and terminate on the gonadotrophic cells. These neurons secrete a short peptide called gonadotropin releasing-hormone (Gnrh) that induces the gonadotrophs to synthesize and secrete gonadotropins (Fig. 5). Fish possess two gonadotropins similar to those in higher vertebrates, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh). Depending on species and reproductive status, one or both of these gonadotropins acts on responsive ovarian follicle cells to stimulate their synthesis and secretion of the estrogen, estradiol-17 β (E2). Circulating E2 then induces hepatocytes in the liver to synthesize and secrete Vtgs. E2 also feeds back to act on the brain and pituitary gland, providing homeostatic control of vitellogenesis and later maturational processes. Xenoestrogens present in the environment can 'short-circuit' the brain-pituitary-gonad axis and act directly on the liver to induce abnormal vitellogenesis in male and juvenile fish, sometimes resulting in the appearance of 'intersex' males bearing previtellogenic oocytes in their testes, and in reproductive failure (Fig. 5). Vitellogenesis is highly responsive to synthetic and natural estrogens, and fish Vtgs have been globally adopted as biomarkers of the presence of estrogenic chemical contaminants in aquatic environments, which is indicated by elevated Vtg levels in the blood or tissues of male and/or and juvenile fish (Dang, 2016; Adeel *et al.*, 2017).

Vitellogenin Synthesis and Secretion

Induction of vitellogenesis depends on interaction of estrogen with the classical nuclear estrogen receptor (Esr) (Babin *et al.*, 2007) (Fig. 6), of which there are two subtypes in teleosts, Esr α and Esr β . In most species examined, Esr α is more closely linked to activation of vitellogenesis but both Esr subtypes play a role in certain species. The form of Esr residing in the cell membrane that can rapidly activate transcription via non-genomic pathways is not involved in inducing vitellogenesis. Estrogen enters liver cells and binds to cytosolic Esr, inducing conformational changes that enable it to recruit several co-regulatory proteins required for activation of Vtg gene transcription. The occupied Esr is translocated to the cell nucleus where it dimerizes with another Esr, becoming an activated transcription factor that binds to specific palindromic DNA sequence motifs, named estrogen response elements (EREs), that are present in the promoter region of genes encoding Vtgs (Fig. 6). The bound hormone-receptor complex and interacting co-regulators alter the structure of chromatin, the complex of DNA and proteins forming the chromosomes, and

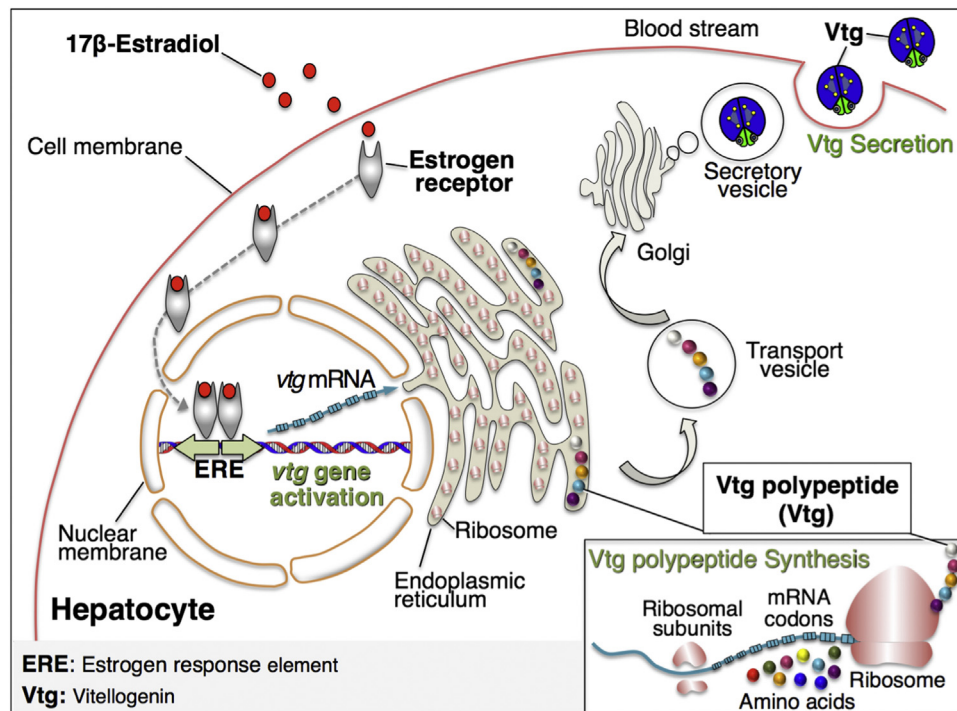


Fig. 6 Hepatic vitellogenesis regulated by estradiol. Estradiol (E2) passes from the bloodstream into the liver cell (hepatocyte) cytoplasm where it binds to estrogen receptors. The occupied receptors undergo conformational changes and are translocated to the nucleus where they dimerize and bind to estrogen response elements (EREs) in DNA sequences in the promoter region upstream of the target vitellogenin (Vtg) genes. Transcription of Vtg genes is initiated and resulting mRNAs transit to the rough endoplasmic reticulum where they provide a template for translation of Vtg polypeptides on the ribosomes. The nascent Vtg polypeptides are loaded with lipid and packed into transport vesicles for travel to the Golgi body where final post-translational modifications (glycosylation, phosphorylation) and dimerization are accomplished. Secretory vesicles carrying newly synthesized Vtg dimers bud off from the Golgi body and fuse with the peripheral cell membrane to discharge their contents. See text for details.

recruit onsite the machinery required to activate gene transcription (e.g. RNA polymerase II), leading to an increase in mRNA transcripts and to stabilization of the nascent mRNA. Estrogen-Esr complexes can also be tethered via protein-protein interactions to transcription factor complexes targeting binding sites distinct from EREs, and several transcription factors other than Esrs have binding sites located in the presumed promotor regions of genes encoding teleost Vtgs (Lubzens *et al.*, 2017). Ribosomes on the rough endoplasmic reticulum (RER) translate the mRNAs encoding Vtgs into their respective polypeptides (Fig. 6, inset) which are trimmed of their signal peptide, loaded with lipid by Mtp, partially folded and possibly partially glycosylated within the RER before being loaded into endosomes for transport to the Golgi apparatus. The Vtg polypeptides are then glycosylated, phosphorylated on their Pv domains, folded into final configuration, and dimerized. Once all post-translational modifications are complete, the finished Vtgs present in cisternae of the *trans*-Golgi are loaded into secretory vesicles that fuse with the cell membrane to release them into the circulation for transit to the ovary.

Vitellogenin Uptake and Processing Into Yolk Proteins

Vitellogenins exit fenestrated (porous) capillaries in the follicle theca and pass through the basement membrane and inter-cellular spaces among granulosa cells to reach the oocyte surface, where they bind to specific Vtg receptors (Vtgrs) (Fig. 7). Occupied Vtgrs cluster into clathrin-coated pits that invaginate and are endocytosed, becoming endocytotic vesicles from which the clathrin coat is later removed and recycled (not shown). Endocytosed Vtg dissociates from the Vtgrs due to slight vesicle acidification and the Vtgrs are recycled to the oocyte surface. The vesicles, now called endosomes, fuse with lysosomes containing inactive cathepsin D (CatD) proenzymes to form multivesicular bodies (MVBs). Vacuolar ATPases (i.e., proton pumps) located in the surface membrane further acidify the lumen of the MVBs and the decreased pH (~ 6.0) activates CatD, which cleaves Vtg at specific recognition sites into its product YPs (LvH, LvL, Pv, β -c, and Ct) (Fig. 7) (Reading *et al.*, 2009). In some cases, cleavage is imperfect and the Pv domain remains covalently linked to LvH, LvL, or both, generating LvH-Pv, Pv-LvL, or LvH-Pv-LvL conjugates, respectively. Smaller proteolytic fragments of LvH or other YPs may also be present and result from imperfect or nonspecific Vtg cleavage. LvH associates with LvL to form Lv dimers that, along with the remaining dimeric YPs, are stored in yolk granules, globules or platelets, or as fluid yolk.

It was recently discovered that there are two forms of Vtg receptor in acanthomorph fishes. A novel receptor termed Lrp13 that preferentially binds VtgAa was discovered in the white perch, *Morone americana* (Perciformes), where the 'classical' Vtgr

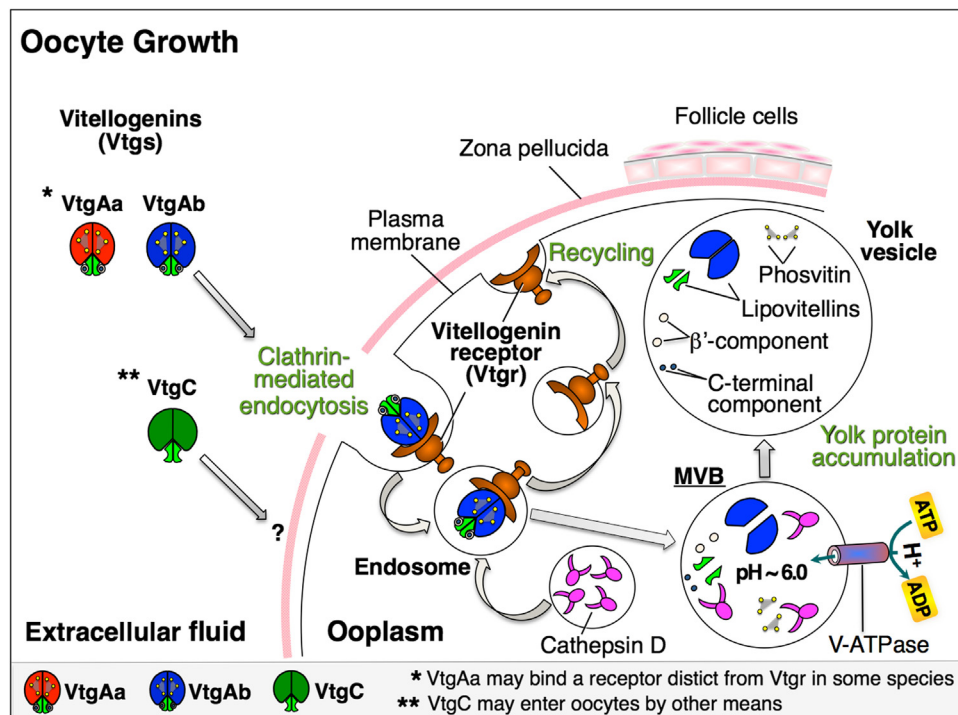


Fig. 7 Model of oocyte growth during vitellogenesis in an acanthomorph teleost. Complete forms of A-type vitellogenin (VtgAa and VtgAb) bind to the vitellogenin receptor (Vtgr) and are taken up into oocytes via clathrin-mediated endocytosis where they are cleaved by cathepsin D into product yolk proteins that are stored in the yolk vesicles. The C-type Vtg (VtgC) may enter the oocyte by other means. MVB, Multivesicular body. See text for details. Portions redrawn from Hiramatsu, N., Cheek, A.O., Sullivan, C.V., Matsubara, T., Hara, A., 2005. Vitellogenesis and endocrine disruption. In: Mommsen, T.P., Moon, T.W. (Eds.), *Biochemistry and Molecular Biology of Fishes*, vol. 6. Amsterdam: Elsevier, pp. 431–471.

preferentially binds VtgAb (Hiramatsu *et al.*, 2015). In the cutthroat trout, *Oncorhynchus clarki* (Salmoniformes), a paracanthopterygian fish, another version of Lrp13 bound both A-type Vtgs (VtgAsa and VtgAsb), as did the classical Vtgr. These findings suggest that a system of dual Vtg receptors with distinct affinities for the two forms of Vtg (VtgAa and VtgAb) evolved in acanthopterygian fishes, which would provide the capability to target them into different compartments in growing oocytes, where could be separately stored and independently mobilized for proteolysis during oocyte maturation (Reading and Sullivan, 2011; see below). Accordingly, the simple model for oocyte growth in acanthomorph fishes shown in Fig. 7 can be duplicated showing a different receptor for each form of A-type Vtg and possibly separate storage compartments (yolk vesicles) within the ooplasm. While proteins that bind to VtgC have been detected in several species, no specific VtgC receptor has been definitively identified and several lines of evidence suggest that VtgC may lack an oocyte receptor and enter oocytes via common endocytosis or in association with other proteins, possibly including Vtgs. See Reading *et al.* (2017) for further details on these Vtg receptors.

Cytoplasmic Maturation and Yolk Protein Proteolysis

Fully-grown, postvitellogenic follicles undergo final maturation, including resumption of meiosis and cytoplasmic maturation, which involves fusion and dissolution of the yolk granules, coalescence of the lipid droplets, ooplasm clarification, and oocyte hydration with an increase in oocyte size due to the uptake of water (Fig. 8, see also Fig. 1). In higher (acanthomorph) teleosts, a 'secondary proteolysis' of the YPs occurs at this time. The contents of the yolk vesicles are further acidified via activity of vacuolar ATPase (V-ATPase) and chloride channels on their outer membranes, activating cathepsins B or L (CatB/L), which are co-localized with YPs in the yolk vesicle during oocyte growth. These cathepsins accomplish proteolytic degradation of the YPs, generating a large pool of peptides and free amino acids (FAA). The FAA along with other osmolytes, including Pv-derived phosphate, ammonium (NH_4^+) from FAA metabolism, and potassium and chloride transported into the oocyte via the actions of Na^+ , K^+ -ATPase and pendrin, respectively, increase the osmotic pressure of the ooplasm, causing water to flow into the oocyte through aquaporin channels transiently expressed in the oocyte membrane (Fig. 8). The net results are hydration of the oocyte, adjusting its density to provide for proper egg buoyancy, provision of a pool of diffusible FAA nutrients, which are the preferred energy substrate of early embryos, and retention of remaining polypeptides and Lv fragments as nutrients for late-stage embryos and larvae, which have developed the

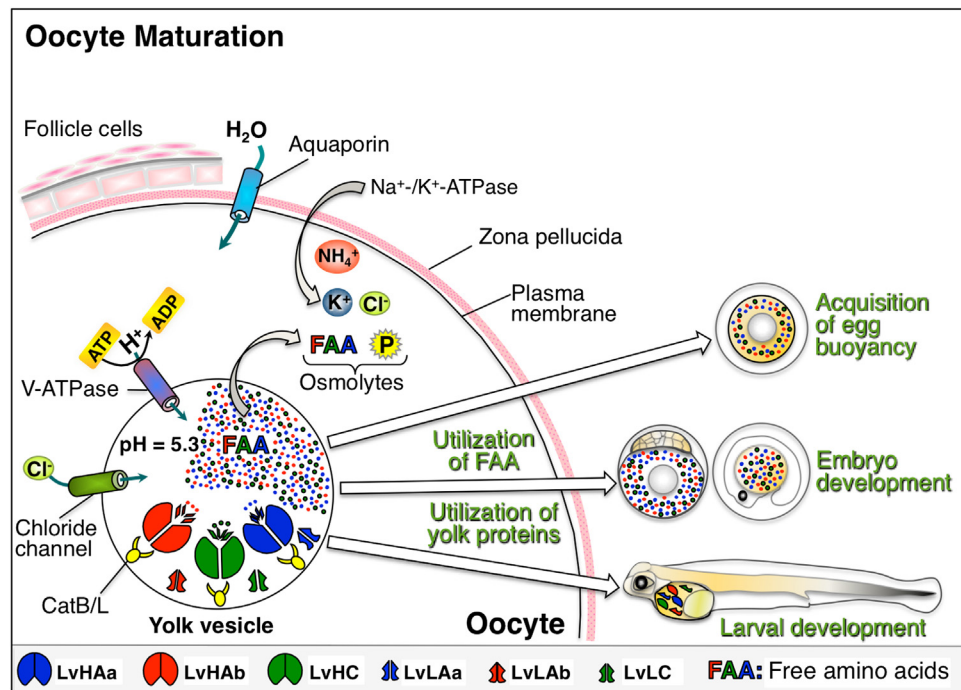


Fig. 8 Model of oocyte maturation and utilization of yolk proteins in European sea bass, *Dicentrarchus labrax*. Yolk proteins present in the yolk vesicles are cleaved into smaller polypeptides and free amino acids (FAA) by cathepsin (Cat) B and/or CatL activated by further acidification of the yolk vesicles mediated by V-ATPase and associated chloride (Cl^-) channels. The resulting pools of FAA and phosphate (P), together with potassium (K^+) and Cl^- ions pumped into the oocyte by sodium-potassium-ATPase (Na^+/K^+ -ATPase) and ammonium (NH_4^+) ions arising from FAA metabolism, increase osmotic pressure, leading to oocyte hydration. Hydration is mediated by the transient opening of water channels, termed aquaporins, embedded in the cell membrane, which allow water to flow into the ooplasm, causing the oocyte to swell in volume and ultimately resulting in the acquisition of proper egg buoyancy. The FAA are diffusible nutrients selectively utilized by early stage embryos. Late stage embryos and larvae are increasingly dependent on the larger polypeptides and lipoproteins for nutrition. Portions redrawn from Finn, R.N., Fyhn, H.J., 2010. Requirement for amino acids in ontogeny of fish. *Aquaculture Research* 41, 684–716.

machinery to process such macromolecules (e.g. the yolk syncytium). Aside from gas exchange, the egg is largely a closed system during early stages of embryogenesis, and the water taken up during final maturation is required for embryo metabolism, dilution of metabolic wastes (e.g. NH_4^+), and provision of adequate space for further development (Finn and Fyhn, 2010).

The model of oocyte maturation shown in Fig. 8 depicts the process as described for European sea bass (Yilmaz *et al.*, 2016) in which the Lvs derived from all three forms of Vtg undergo only limited proteolysis, with large portions of LvH and most of LvL remaining intact. Eggs of this species and other Moronidae family members possess a large oil droplet filled with low-density oil (e.g. wax esters) that provides for proper egg buoyancy. The requirement of oocyte hydration to achieve neutral buoyancy is, therefore, limited and the oocytes increase in volume only 2–3 fold during final maturation. In contrast, for oocytes of species such as barfin flounder (*Verasper moseri*), Atlantic halibut (*Hippoglossus hippoglossus*) and haddock (*Melanogrammus aeglefinus*), that spawn pelagic eggs lacking large oil droplets in seawater, the requirement for oocyte hydration is greater and the oocytes increase in volume 4–6 fold during final maturation (Yilmaz *et al.*, 2015). In these species, the YPs derived from VtgAa are almost entirely cleaved into FAAs, whereas proteolysis of the major YP derived from VtgAb (i.e., LvHAb) is quite limited. It is thought that the system of distinct receptors for the two forms of A-type Vtg in these species may allow them to be targeted into different compartments in the oocyte where maturational proteolysis of their product YPs could be separately regulated. Typically, the YPs derived from VtgC undergo very limited maturational proteolysis. It is possible that, in the absence of a VtgC receptor, these YPs may not be targeted into yolk vesicles or be subject to the proteolytic processes discussed above.

Novel Functions of Vitellogenins

In addition to their fundamental functions as primary precursors of YPs and as transporters of lipids and other nutritional resources into the egg yolk, teleost Vtgs have become objects of curiosity for their novel, non-nutritive functions. It is increasingly evident that Vtg is an immunocompetent factor capable of protecting the host against attack by microbes including bacteria and viruses (Sun and Zhang, 2015; Li and Zhang, 2017). Both serum Vtg and its product YPs, Lv and Pv, are immunologically active via their ability to recognize and bind to pathogen-associated molecular patterns (PAMPs). These properties of multivalent pattern recognition receptors were associated with the ability of Lv and Pv to bind Gram-positive and -negative bacteria through recognition of their conserved lipopolysaccharide, peptidoglycan, lipoteichoic acid and glucan components. Pv was shown to be an effector molecule capable of killing bacteria directly via cell lysis, while Lv was shown to act as an opsonin, facilitating phagocytosis of bacteria by macrophages. Serum Vtg has been additionally reported to bind and neutralize infectious pancreatic necrosis virus. The antimicrobial properties of certain conserved domains present in zebrafish VtgAo2 were verified by producing them as recombinant proteins and testing their bioactivity. These domains encompassed the carboxy-terminal third of LvH, most of LvL, and all of β' -c. All three recombinant domains functioned as pattern-recognition receptors, binding both Gram-positive and -negative bacteria and their isolated signature PAMPs, and those including portions of LvH or LvL also functioned as opsonins, promoting phagocytosis of *Escherichia coli* (–) and *Staphylococcus aureus* (+) by carp, *Cyprinus carpio*, macrophages. Native Lv was associated with immune defense of rosy barb, *Pethia conchonius*, embryos and larvae and Pv was shown to possess antimicrobial activity in zebrafish embryos and larvae. The protective immune functions of Vtg-derived YPs may extend through oocyte maturation and ovulation to embryonic and larval development, when the YPs have been degraded into smaller polypeptides. For example, three small polypeptides derived from the C-terminal 55 residues of a zebrafish Pv have been shown to have individual and enhanced collective activity against growth of *S. aureus* (+) and *Aeromonas hydrophila* (–) bacteria and, thus, may have protective activity in developing embryos. Vtg may also contribute to immune priming by carrying immunological memory from mother to offspring, as it does in insects.

Vitellogenins are also known to have antioxidant activity and the capability to suppress free-radical reactions in fish oocytes and to protect the host from oxidative stress. It has been demonstrated that hen egg yolk Pv exhibits strong antioxidant activity owing to its high serine and phosphorus content, which makes it a particularly strong iron-chelating agent. It has also been shown that zebrafish recombinant Pv (rPv) is an antioxidant capable of protecting against radical-mediated oxidation of cellular biomolecules (Sun and Zhang, 2015). Several studies have demonstrated that Vtg regulates honey bee aging by boosting antioxidant balance of the body via its antioxidative properties, thus slowing down physiological aging and inhibiting inflammation by reducing oxidative stress. It is intriguing to speculate that Vtg may have similar activities in fishes.

Future Directions and Perspectives on Vitellogenesis

The past 20 years have seen great strides in our understanding of vitellogenesis in fishes. However, much remains to be learned. Our first glimpses into molecular mechanisms for control of Vtg gene transcription in zebrafish have revealed much complexity and the likelihood that Vtg gene promoters are sites of considerable crosstalk between numerous types of transcription factors, just as physiology studies revealed that several hormones besides estrogen modulate vitellogenesis. Almost nothing is known about the ways in which different types of Vtg are regulated by these processes. This will be a rich and rewarding area for future research.

It is now understood that multiplicity of Vtgs is the norm in fishes and that parallel multiplicity of Vtg receptors may exist, particularly in higher (acanthomorph) teleosts. These fishes exhibit different ratios of abundance of VtgAa, VtgAb and VtgC in their bloodstream and these ratios change during the reproductive cycle and differ between blood plasma and oocytes. These differences

likely reflect variation in hepatic secretion of the different types of Vtg, and also differences in their rates of sequestration by oocytes via receptor-mediated endocytosis, with both processes modulated to optimize the mixture of Vtg-specific YP products deposited in growing oocytes. These hypotheses remain to be experimentally verified.

Maturation proteolysis of YPs in acanthomorph fishes spawning pelagic eggs in seawater appears to be regulated to optimize oocyte hydration, egg buoyancy and delivery of the preferred types of nutrients to early embryos and late stage larvae. In such species whose eggs lack prominent oil droplets, VtgAa is neofunctionalized for susceptibility proteolysis, and it is hypothesized that the newly discovered receptor that preferentially binds VtgAa (Lp13) may deliver it into a special compartment where it is fated for digestion during maturation, whereas the VtgAb is targeted by a different receptor (Vtgr) to a different compartment. The identity and functional details of these compartments need resolution.

The basic biology of the C-type Vtg is poorly understood. In higher teleosts, VtgC usually escapes significant proteolysis during final maturation, but it is uncertain how this is achieved. There is presently no definitive evidence that VtgC binds to a specific receptor that delivers it into a 'protected' compartment, nor is there evidence that VtgC even enters yolk vesicles where it would be subject to proteolysis by CatB/L during final maturation. It is known that dephosphorylated Vtg cannot bind to Vtg receptor(s), and VtgC, lacking a Pv domain, is constitutively 'dephosphorylated'. VtgC also lacks the vWfd domain (β' -c, Ct) thought to be important for dimerization and perhaps cell adhesion associated with receptor mediated endocytosis. In short, the molecular biology of VtgC is a mystery and 'low hanging fruit' for a scientist wishing to make an important contribution to the study of fish vitellogenesis.

References

- Adeel, M., Song, X., Wang, Y., Francis, D., & Yang, Y. (2017). Environmental impact of estrogens on human, animal and plant life: A critical review. *Environment International*, 99, 107–119.
- Andersen, Ø., Xu, C., Timmerhaus, G., Kirste, K. H., Naeve, I., et al. (2017). Resolving the complexity of vitellogenins and their receptors in the tetraploid Atlantic salmon (*Salmo salar*): Ancient origin of the phosvitin-less VtgC in chondrichthyan fishes. *Molecular Reproduction and Development*, 2017. <https://doi.org/10.1002/mrd.22881> [Epub ahead of print].
- Babin, P. J., Carnevali, O., Lubzens, E., & Schneider, W. J. (2007). Molecular aspects of oocyte vitellogenesis in fish. In P. J. Babin, J. Cerdà, & E. Lubzens (Eds.), *The Fish Oocyte: From Basic Studies to Biotechnological Applications* (pp. 39–76). New York, NY: Springer.
- Dang, Z. (2016). Interpretation of fish biomarker data for identification, classification, risk assessment and testing of endocrine disrupting chemicals. *Environment International*, 92–93, 422–441.
- Finn, R. N. (2007). Vertebrate yolk complexes and the functional implications of phosvitins and other subdomains in vitellogenins. *Biology of Reproduction*, 76, 926–935.
- Finn, R. N., & Fyhn, H. J. (2010). Requirement for amino acids in ontogeny of fish. *Aquaculture Research*, 41, 684–716.
- Finn, R. N., & Kristoffersen, B. A. (2007). Vertebrate vitellogenin gene duplication in relation to the "3R hypothesis": Correlation to the pelagic egg and the oceanic radiation of teleosts. *PLOS ONE*, 2(1), e169.
- Hiramatsu, N., Todo, T., Sullivan, C. V., et al. (2015). Ovarian yolk formation in fishes: Molecular mechanisms underlying formation of lipid droplets and vitellogenin-derived yolk proteins. *General and Comparative Endocrinology*, 221, 9–15.
- Li, H., & Zhang, S. (2017). Functions of vitellogenin in eggs. *Results and Problems in Cell Differentiation*, 63, 389–401.
- Lubzens, E., Bobe, J., Young, G., & Sullivan, C. V. (2017). Maternal investment in fish oocytes and eggs: The molecular cargo and its contributions to fertility and early development. *Aquaculture*, 472, 107–143.
- Reading, B. J., Hiramatsu, N., Sawaguchi, S., et al. (2009). Conserved and variant molecular and functional features of multiple vitellogenins in white perch (*Morone americana*) and other teleosts. *Marine Biotechnology*, 11, 169–187.
- Reading, B. J., & Sullivan, C. V. (2011). Vitellogenesis in fishes. In A. P. Ferrell (Ed.), *Encyclopedia of Fish Physiology: From Genome to Environment. The Reproductive Organs and Processes. Reference Module in Life Sciences* (2011 ed., pp. 635–646). Maryland Heights, MO: Elsevier. Available at: <https://doi.org/10.1016/B978-0-12-374553-8.00257-4>.
- Reading, B.J., Sullivan, C.V., Schilling, J., 2017. Vitellogenesis in fishes. In: Ferrell, A.P. (Ed.), Encyclopedia of Fish Physiology: From Genome to Environment. The Reproductive Organs and Processes. Reference Module in Life Sciences, 2017 ed. Elsevier, Maryland Heights, MO. Available at: <https://doi.org/10.1016/B978-0-12-809633-8.03076-4>.
- Sun, C., & Zhang, S. (2015). Immune-relevant and antioxidant activities of vitellogenin and yolk proteins in fish. *Nutrients*, 7, 8818–8829.
- Yilmaz, O., Prat, F., Ibanez, A. J., et al. (2015). Estrogen-induced yolk precursors in European sea bass, *Dicentrarchus labrax*: Status and perspectives on multiplicity and functioning of vitellogenins. *General and Comparative Endocrinology*, 221, 16–22.
- Yilmaz, O., Prat, F., Ibanez, A., et al. (2016). Multiple vitellogenins and product yolk proteins in European sea bass (*Dicentrarchus labrax*): Molecular characterization, quantification in plasma, liver and ovary, and maturational proteolysis. *Comparative Biochemistry and Physiology, Part B – Molecular Biology*, 194, 71–86.

Further Reading

- Carnevali, O., Cionna, C., Tosti, L., Lubzens, E., & Maradonna, F. (2006). Role of cathepsins in ovarian follicle growth and maturation. *General and Comparative Endocrinology*, 146, 195–203.
- Cerdà, J., Zapater, C., Chauvigné, F., & Finn, R. N. (2013). Water homeostasis in the fish oocyte: New insights into the role and molecular regulation of a teleost-specific aquaporin. *Fish Physiology and Biochemistry*, 39, 19–27.
- Grier, H. J., Uribe Aranzábal, M. C., & Patiño, R. (2009). The ovary, folliculogenesis, and oogenesis in teleosts. In B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes)* (pp. 26–84). Enfield, NH: Science Publishers.
- Hara, A., Hiramatsu, N., & Fujita, T. (2016). Vitellogenesis and choriogenesis in fishes. *Fisheries Science*, 82, 187–202.
- Hiramatsu, N., Matsubara, T., Fujita, T., Sullivan, C. V., & Hara, A. (2006). Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. *Marine Biology*, 149, 35–47.
- Johnson, R. B. (2009). Lipid deposition in oocytes of teleost fish during secondary oocyte growth. *Reviews in Fisheries Science*, 17, 78–99.
- Kagawa, H. (2013). Oogenesis in teleost fish. *Aqua-BioSciences Monographs*, 6, 99–127.
- Lubzens, E., Young, G., Bobe, J., & Cerdà, J. (2010). Oogenesis in teleosts: How fish eggs are formed. *General and Comparative Endocrinology*, 165, 367–389.
- Matsubara, T., Nagae, M., Ohkubo, N., et al. (2003). Multiple vitellogenins and their unique roles in marine teleosts. *Fish Physiology and Biochemistry*, 28, 295–299.

- Mommsen, T. P., & Korsgaard, B. (2008). Vitellogenesis. In M. J. Rocha, A. Arukwe, & B. G. Kapoor (Eds.), *Fish Reproduction* (pp. 113–169). Enfield, NH: Science Publishers.
- Patino, R., & Sullivan, C. V. (2002). Ovarian follicle growth, maturation, and ovulation in teleost fish. *Fish Physiology and Biochemistry*, 26, 57–70.
- Romano, M., Rosanova, P., Anteo, C., & Limatola, E. (2004). Vertebrate yolk proteins: A review. *Molecular Reproduction and Development*, 69, 109–116.
- Selman, K., & Wallace, R. A. (1989). Cellular aspects of oocyte growth in teleosts. *Zoological Science*, 6, 211–231.
- Wiegand, M. D. (1996). Composition, accumulation and utilization of yolk lipids in teleost fish. *Reviews in Fish Biology and Fisheries*, 6, 259–286.
- Yaron, Z., & Levavi-Sivan, B. (2006). Fish reproduction. In D. H. Evans, & J. B. Claiborne (Eds.), *The Physiology of Fishes* (third ed., pp. 345–388). Boca Raton, FL: CRC Press.

Vitellogenesis and Yolk Proteins, Birds

Sophie Réhault-Godbert and Nicolas Guyot, National Institute of Agronomic Research, Nouzilly, France

© 2018 Elsevier Inc. All rights reserved.

The formation of the avian egg relies on two distinct anatomical structures, the ovary and the oviduct, as part of the female reproductive tract. Initially, two ovaries are present during early embryogenesis but the right ovary will be progressively resorbed and only the left oviduct will finally develop at sexual maturity of the female bird. The ovary synthesizes sex steroids and is where the gametogenesis, the vitellogenesis and the inner layer of the vitelline membrane occur, whereas the outer layer of the vitelline membrane, the egg white, the eggshell membranes and the eggshell are successively deposited in the various segments of the oviduct around the mature yolk follicle, immediately after its ovulation in the infundibulum, the more proximal part of the oviduct (Fig. 1). In the domestic fowl (*Gallus gallus*), yolk accumulation into growing follicles is a 10–12 day process taking place in the ovary, where some oocytes are selected at daily intervals, among the several thousand oocytes present in the ovary at hatch. Sexual maturity coincides with the hormonal stimulation of hepatic synthesis of pre-existing proteins or synthesis of new proteins and lipids that are secreted in the blood as egg yolk precursors (very-low density lipoproteins, vitellogenins, etc.). Egg yolk precursors are then conveyed to the ovarian follicles, which each consists of an oocyte surrounded by layers of supportive tissues, the theca externa and the theca interna, containing embedded capillaries which are separated from the granulosa layer by a thick basal lamina (Fig. 2; Perry and Gilbert, 1979). Yolk precursors are progressively internalized in growing follicles by receptor-mediated endocytosis and further processed into functional proteins by endogenous yolk proteases. After oviposition and during incubation, yolk nutrients including proteins and lipids are subsequently assimilated by the embryo, via the yolk sac, which is a highly vascularized and specialized extra-embryonic tissue supporting the embryo growth during the entire phase of embryogenesis. It must be emphasized that most of the literature available on cellular and molecular mechanisms regulating vitellogenesis and follicle growth, are derived from domestic species (*Gallus gallus*, *Coturnix japonica*, *Meleagris gallopavo*).

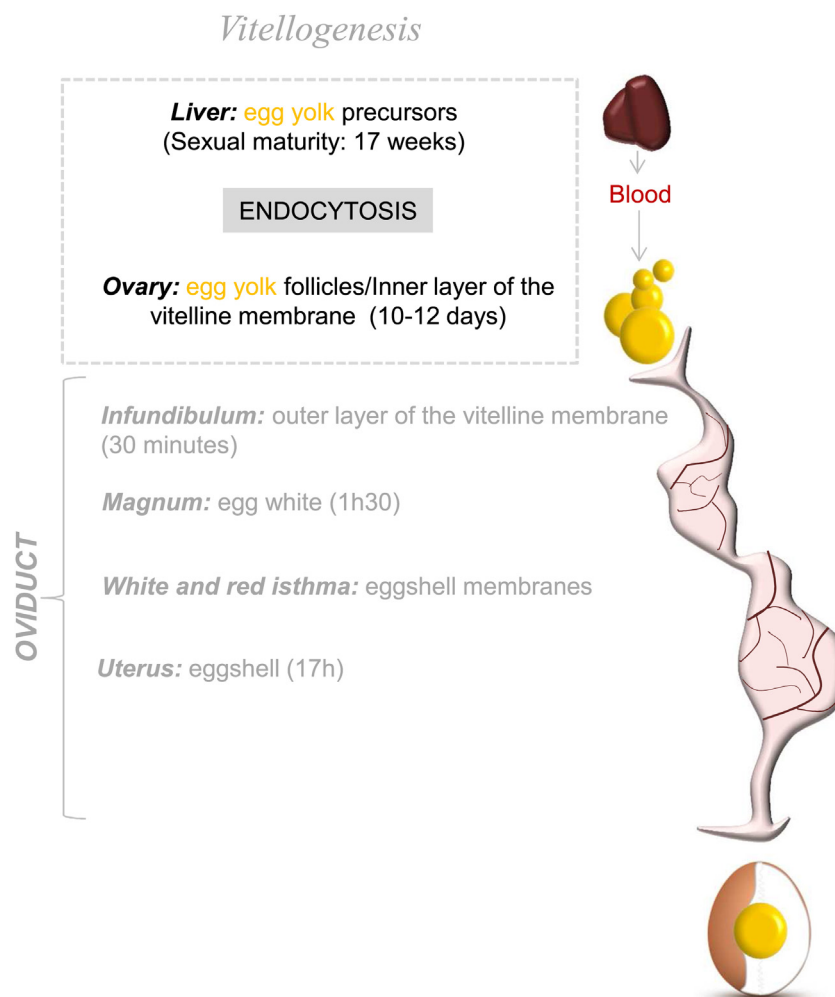


Fig. 1 Tissues involved in chicken egg formation.

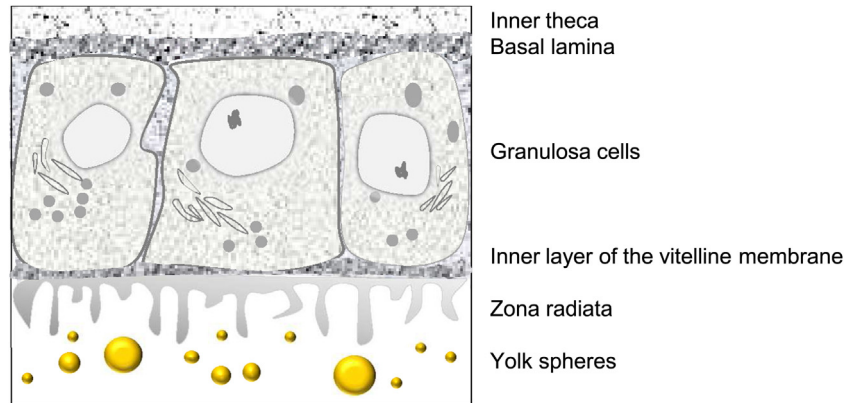


Fig. 2 Structure of the ovarian follicle of the domestic fowl (*Gallus gallus*). Drawn based on a micrograph from Perry, M.M., Gilbert, A.B., 1979. Yolk transport in the ovarian follicle of the hen (*Gallus domesticus*): Lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. *Journal of Cell Science* 39, 257–272.

Chronologia and Regulation of Vitellogenesis

Follicular Growth

The viability and the growth of follicles is controlled by endocrine factors (e.g., gonadotropins), paracrine and autocrine factors produced within the ovary, and neurochemical/neurohumoral factors locally expressed by the nervous system (Johnson and Woods, 2007). The process of follicle growth, from the primordial follicle to ovulation, is divided into three main phases (Fig. 3): early, slow and rapid growth phases. The early growth of primary follicles is assumed to take months to years. In contrast, the slow growth of prehierarchal (or previtellogenic) follicles lasts weeks to months. During this latter phase, follicles initially incorporate “white” yolk and can be referred to small white follicles (<2 mm) and large white follicles (2–5 mm). These latter then become small yellow follicles (5–9 mm) concomitantly with the uptake of “yellow” yolk. In comparison with “yellow” yolk, “white” yolk is characterized by a higher protein to lipid ratio and contains only 10–13% solid material (Gilbert, 1979). The rapid growth of preovulatory follicles is featured by the transfer of large amounts of “yellow” yolk and ends 2–3 h before ovulation. The duration of this phase varies according to avian species: 6 and 11 days in the domestic hen and barn owl, approximately 12 days in the Canada goose (*Branta canadensis*), up to 16 days in some penguins, 15–17 days in the Brown kiwi (*Apteryx mantelli*), but only 5–7 days in the Ring-necked dove (*Streptopelia capicola*) and Japanese quail (*Coturnix japonica*) (Gilbert, 1979). In domestic hens, the ovary generally contains five preovulatory follicles with increasing size (follicular hierarchy), named F1 to F5, F1 being the largest follicle.

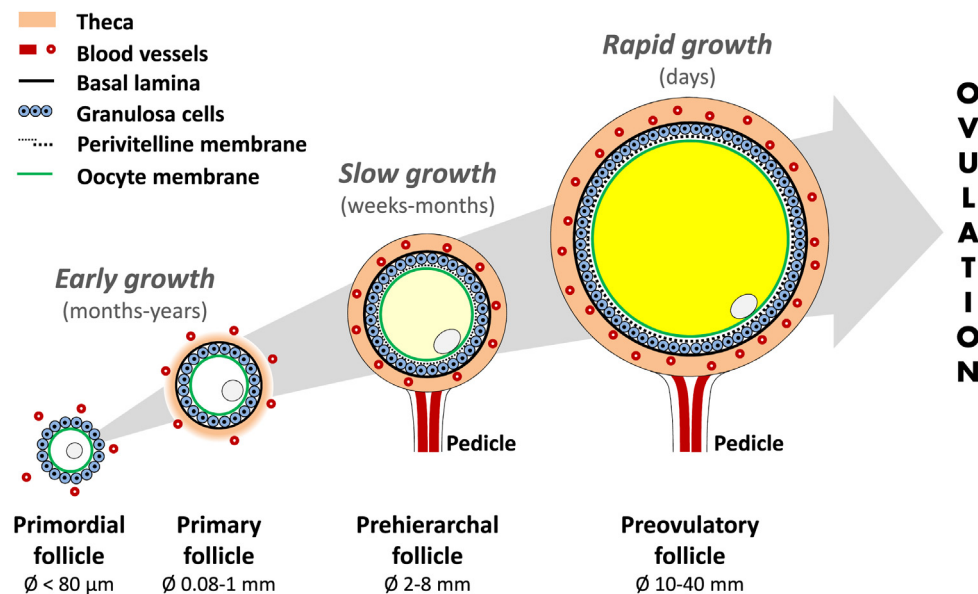


Fig. 3 Follicular growth in the domestic fowl (*Gallus gallus*). Drawn based on a picture from Johnson, A.L., Woods, D.C., 2007. Ovarian dynamics and follicle development. In: Jamieson B.G.M. (Ed.), *Reproductive Biology and Phylogeny of Birds (Part A)*, pp 243–277. Science Publishers.

Synthesis of Yolk Lipids and Proteins

Yolk lipids and protein precursors are synthesized in the liver and then secreted into the blood stream to reach the ovary where they are transferred into the oocyte. The synthesis of these yolk compounds, under the control of estrogen, is strongly induced at sexual maturity (about 16 weeks of age in domestic laying strains). A 15- and 20-fold increase in hepatic lipogenesis and lipemia can indeed be observed in mature hens compared with immature hens. Yolk lipids are produced and transported to the ovary as very low-density lipoproteins (VLDLs). VLDLs have a standard structure consisting of a core composed of triglycerides and cholesterol esters and surrounded by a surface layer of phospholipids, cholesterol and apoproteins (mainly apolipoprotein B and apovitellenin-1, also known as apolipoprotein VLDL- II) (Fig. 4). VLDL levels in blood plasma are lower than 1 g/L in immature hens and increase up to 10–20 g/L in mature hens. In mammals, VLDL undergoes a rapid lipolysis into low-density lipoprotein (LDL) by a lipoprotein lipase after being secreted by the liver; however, in hens, VLDLs are stable and remain under their native form until they are transferred into the follicle. This stability to lipolysis is likely to involve apovitellenin-1, a very potent lipase inhibitor (see [Section Lipoproteins](#)). Vitellogenins (VTG), the main protein precursors of yolk (see [Section Vitellogenins](#)), is not present in immature hens and its synthesis is stimulated by estrogen at sexual maturity. The gene transcription is induced by the association of estrogen with its nuclear receptor and subsequent interaction of this hormone-receptor complex with a specific palindromic DNA sequence (estrogen responsive element) located within the gene promoter region. An important fraction of egg yolk protein is constituted by maternal immunoglobulins IgY (mammalian equivalent of IgG), which provides protection against pathogens to the developing embryo and to newly hatched chicks. It is noteworthy that, unlike most yolk proteins, IgY is not synthesized by the liver but by plasma cells which themselves are derived from B lymphocytes, following exposure to pathogens.

Yolk Transfer into Growing Oocyte

Yolk precursors are transported to the follicular wall via the blood stream and are released near the basal lamina (Fig. 2) through highly fenestrated capillaries (Gilbert, 1979; Etches, 1996). The presence of gaps within the endothelial wall allows VLDL and VTG to exit the blood stream and to diffuse toward granulosa cell layer and the oocyte.

During the early growth of prehierarchal follicles, white yolk may cross the granulosa cell layer either by transcytosis or by a paracellular transport. Granulosa cells are tightly connected to each other's by tight junctions, which restrict the paracellular transport of yolk at this stage (Fig. 2). Occludin, a protein involved in tight junctions, is likely to have a major role in the regulation of yolk transport and follicular growth. Its expression is regulated by the stimulatory effects of Follicular Stimulating Hormone (FSH) and activin (Transforming Growth Factor β superfamily member). The level of occludin progressively decreases during follicle growth, thus allowing greater amounts of yolk (including the lipid-rich yellow yolk) to cross the granulosa cell layer toward the oocyte. The yellow color gained by the 5–9 mm prehierarchal follicles (small yellow follicles) results, at least in part, from this mechanism (Johnson and Woods, 2007).

VLDL and VTG are internalized into oocytes by receptor-mediated endocytosis. These two components bind to LR8 receptor present at high levels in oocyte plasma membrane (Nimph and Schneider, 1998). The binding of VLDL and VTG to the receptor and their internalization are mediated by apolipoprotein B and lipovitellin (a vitellogenin-derived product, see [Section Vitellogenins](#)), respectively. The association of VLDL and VTG to LR8 in the oocyte plasma membrane leads to the formation of a vesicle internalized by endocytosis. The vesicle internalization in the oocyte is facilitated by the presence of endophilin III, a member of a protein family (endophilins) involved in clathrin-mediated endocytosis and recycling. LR8 has the ability to bind not only VLDL and VTG, but also other moderate-abundant components. For instance, α 2-macroglobulin (α 2M), a protein able to bind covalently to and neutralize serum proteases in mammals, can also bind LR8. By this mechanism, α 2M might indirectly promote the transfer of blood proteases into the oocyte. A number of yolk proteins can also enter the oocyte either after interaction with LR8 ligands (especially VTG and VLDL) as it is the case for riboflavin-binding protein, which is able to associate with VTG, or possibly by passive internalization during the formation of vesicles. The crucial role of LR8 in the internalization of yolk components is

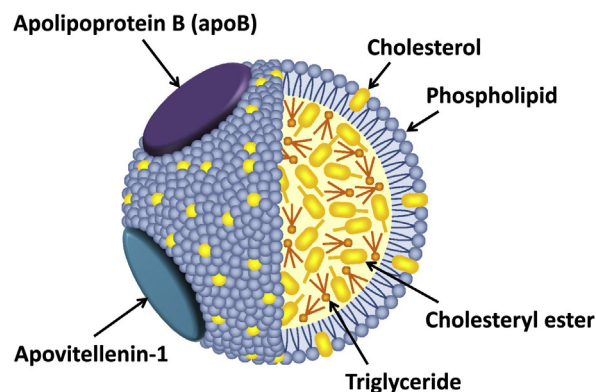


Fig. 4 Schematic representation of a very low-density lipoprotein (VLDL).

demonstrated in vivo in 'restricted ovulator' (R/O) hens, which carries a point mutation at the *Lr8* locus leading to functional inactivation of LR8 (Bujo *et al.*, 1995). R/O hens are indeed not capable of laying eggs due to the inability of oocytes to enter the rapid growth phase. LR8 is initially located within the oocyte cytoplasm in slow growing follicles, and the rapid yolk intake occurs only following its redistribution to the oocyte plasma membrane (Johnson and Woods, 2007). A recent proteomic study performed on small white yolk follicles demonstrated that the receptor is present in the cytoplasmic compartment while VTGs are mostly detected within the follicular wall and ovarian stromal cells, but not in the cytoplasm (Nepomuceno *et al.*, 2015).

Immunoglobulins IgY are transferred from the blood to the oocyte by an immunoglobulin receptor in the oocyte membrane called FcRY and belonging to the mannose receptor family. Up to 100 mg of IgY can be found in the egg yolk of one egg, which represents a total of 17–35 g of IgY transferred in egg yolk per hen, per year.

Egg Yolk Proteins

Egg yolk biological function in avian reproduction is to provide essential nutrients and bioactive components for the developing embryo. In this respect, egg yolk consists of approximately 48% water, 34% lipids, 16% proteins, 0.5% carbohydrates and 1.5% ash. It is also a rich source of vitamins (A, D, E, B1, B2, B3, B5, B6, B8, B12, with no vitamin C), minerals (phosphorus, calcium and potassium) but also trace elements (zinc, copper etc.). Yolk is a complex milieu containing 68% low-density lipoproteins, which structure relies on a core of triglycerides, and cholesteryl esters, surrounded by a monolayer of phospholipids and cholesterol in which apoproteins are embedded (Fig. 4), 16% high-density lipoproteins (HDLs), 10% livetins and other soluble proteins, and 4% phosvitins. These components are distributed between non-soluble protein aggregates (granules) and a clear yellow fluid (plasma) (Anton and Gandemer, 1997). Granules (19–23% of dry matter) account for about 50% of yolk proteins and 7% of yolk lipids, and include HDLs (70%) and phosvitins (16%). The yolk plasma (77–81% of dry matter) is composed of 85% LDLs (with apolipoprotein B and apovitellenin-1 as the major components) and 15% soluble proteins (serum albumin, immunoglobulins and ovalbumin...). It concentrates yolk lipids (and nearly all carotenoids) and 50% of yolk proteins.

Two proteomic approaches performed in parallel on yolks collected from freshly laid eggs revealed a total of 216 distinct proteins distributed between granules and the plasma fraction (Mann and Mann, 2008). The protein profile and their relative abundance are presented Fig. 5(a) and (b). In egg yolk, major proteins encompass serum albumin (Gene ID: 396197), 12kDa serum protein also known as "predicted beta-microseminoprotein-like" (Gene ID: 100858647), apovitellenin-1 (Gene ID:396476), vitellogenins 1, 2, and 3 (Gene ID: 424547, 424533 and 424534, respectively), ovalbumin (Gene ID: 396058), immunoglobulins Y (Gene ID: 416928), apolipoprotein B (Gene ID: 396535), similar to avidin (Gene ID: 426220), ovotransferrin (Gene ID: 396241), transthyretin (Gene ID: 396277), cystatin (Gene ID: 396497), similar to α 2-macroglobulin-like (Gene ID: 100858010) and apolipoprotein A-I (Gene ID: 396536) (Fig. 5). The next paragraphs will give an overview of the biological activities of some of these major proteins.

Lipoproteins

The most abundant proteins recovered in the yolk (essentially in granular fraction of the yolk) are apolipoprotein B and apovitellenin-1. The 520kDa apolipoprotein B functions as a recognition signal for the cellular binding and internalization of the very low-density lipoprotein (VLDL) particles from the blood by the chicken oocyte receptor, which is present at high levels in the oocyte membrane (Bujo *et al.*, 1997). Its role is to store and deliver lipid nutrients to the developing embryo. Apovitellenin is a 14kDa-homodimer integrated in the very low density lipoprotein (VLDL) of egg-laying females. It exhibits a potent lipase inhibiting activity, preventing the loss of triglycerides from VLDL, on their way from the liver to the growing oocytes. Its expression by the liver is 40-fold increased in hens at sexual maturity (Bourin *et al.*, 2012). Three other apoproteins of moderate or low abundance have also been detected by proteomic approaches: apolipoprotein A-I, apolipoprotein H and apolipoprotein C-III (Fig. 5(b); Mann and Mann, 2008).

Vitellogenins

Vitellogenin-1 and vitellogenin-2 (the most abundant of the three vitellogenins) are two high molecular weight glycoproteins (200kDa) containing 4 domains, lipovitellin-1, lipovitellin-2, phosvitin and a peptide of 40 amino-acids called YGP40, which are specifically released after intraoocytic proteolytic processing by cathepsin D, an endogenous aspartic protease (Retzek *et al.*, 1992). The genes coding these two proteins are overexpressed by more than 40 fold and 30 fold, respectively, in the liver of sexually mature hens (Bourin *et al.*, 2012). In contrast, vitellogenin 3 is a smaller glycoprotein (37kDa) lacking lipovitellins and YGP40 domains but containing phosvitin. Vitellogenin-derived phosvitins sequester and store calcium, iron and other cations from the bloodstream to relocate them to the yolk, to further provide minerals and trace elements for the developing embryo. Similarly, lipovitellins are lipid-bound proteins, which function is to fulfill lipid requirements for embryo growth.

Immunoglobulins

Immunoglobulin Y is the dominant immunoglobulin in serum and egg yolk, in birds. Compared to immunoglobulin G, its mammalian counterpart, its characteristics and functions are similar but it displays distinct structural differences containing three constant heavy domains (as opposed to two in mammals) and an absence of the hinge region between light and heavy chains, this

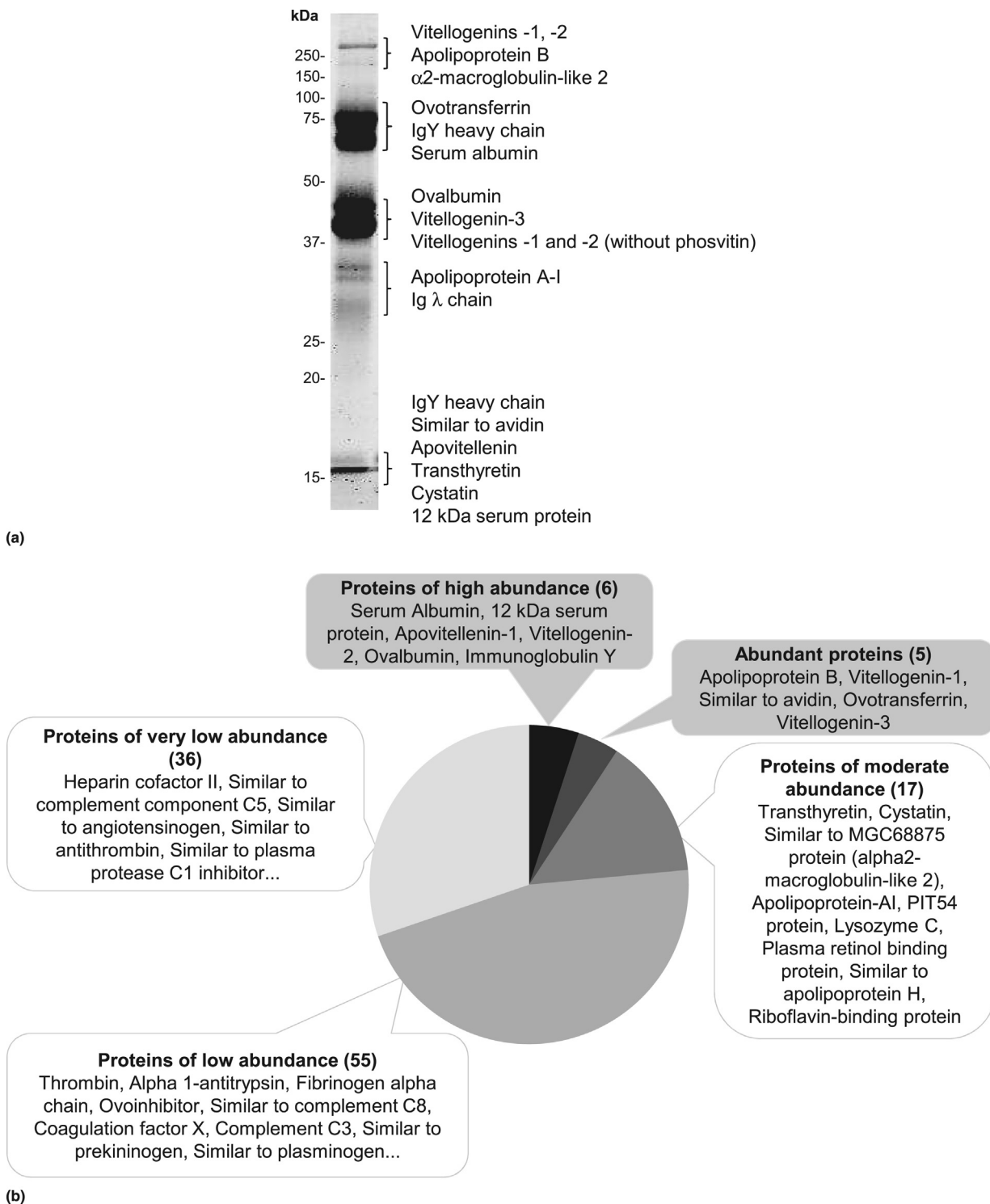


Fig. 5 Egg yolk proteins. (a) Protein profile of egg yolk (SDS-PAGE followed by Coomassie blue staining (Personal data)). (b) Relative abundance of egg yolk proteins: proteins of high abundance ($1650 < \text{emPAI} < 6840$), abundant proteins ($100 < \text{emPAI} < 400$), proteins of moderate abundance ($10 < \text{emPAI} < 100$), proteins of low abundance ($1 < \text{emPAI} < 10$), proteins of very low abundance ($\text{emPAI} < 1$). Adapted from Mann, K., Mann, M., 2008. The chicken egg yolk plasma and granule proteomes. *Proteomics* 8 (1), 178–191.

latter feature being associated with reduced flexibility compared to the immunoglobulin G. Immunoglobulin Y was initially associated with the gamma-livetin fraction of the yolk. Its concentration in egg yolk remains very variable from one individual to another and can reach up to 7–10mg/mL. These immunoglobulins are specific to the microbes encountered by the hen during its lifespan, and are captured from the bloodstream to be incorporated in ovarian growing follicles. These maternal

immunoglobulins ensure the protection of the embryo during incubation but more surely to the chick, after abdominal resorption of the residual yolk sac occurring just before hatching.

Vitamin and Iron-Binding Proteins

Yolk contains significant amounts of vitamin- and cofactor- binding proteins. Riboflavin-binding protein expression in the liver is stimulated by 68 fold in hens at sexual maturity (Bourin *et al.*, 2012). This highly abundant protein is presumed to provide riboflavin to the developing embryo since a lack of this protein in egg yolk is lethal for the 10-day old embryo. Some vitamin-binding proteins of moderate to low abundance have been detected in the yolk: "Similar to avidin," a biotin (vitamin B8)-chelating protein, plasma retinol (vitamin A)-binding protein and vitamin-D binding protein and two presumed cobalamin (vitamin B12)-binding proteins (Mann and Mann, 2008; Farinazzo *et al.*, 2009). All these vitamins are essential for the proper growth of the embryo.

Low Abundant Proteins

Besides, many proteins with low or very low abundance have been detected (Fig. 5(b)) (Mann and Mann, 2008) but their biological significance in the yolk remains uncertain. Indeed, most of these proteins are basically present in sera of pullets like hemostatic and fibrinolytic factors, carrier proteins and immune effectors (thrombin, alpha 1-antitrypsin, fibrinogen alpha chain, similar to complement C8, coagulation factor X, complement C3, similar to prekallikrein, similar to plasminogen, heparin cofactor II, similar to complement component C5, similar to angiotensinogen, similar to antithrombin, similar to plasma protease C1 inhibitor etc.) and no specific stimulation of their expression in hens at sexual maturity has been reported (Bourin *et al.*, 2012). The ongoing hypothesis is that they may be incorporated passively during vitellogenesis together with egg yolk specific proteins such as apovitellenin-1, apolipoprotein B, riboflavin-binding protein etc., which in contrast to these minor proteins, are specifically expressed or over-expressed in hens at sexual maturity (Bourin *et al.*, 2012). In such a context and with regards to their very low abundance in egg yolk, their active role during embryonic development remains controversial.

Evolution of Yolk Proteins During Embryogenesis

A recent proteomic analysis performed on egg yolk proteins contained in the plasma fraction, during the first half of incubation, reveals only few variations of their abundance: five proteins (vitronectin, α -fetoprotein, similar to thrombin, apolipoprotein B, and apovitellenin-1) showed a major increase in relative abundance, whereas 15 proteins showed a significant decrease in yolks of fertilized eggs (Rehault-Godbert *et al.*, 2014). The increase of abundance of some of these proteins might be explained by (1) secretion of proteins expressed by the embryo or its associated structures (such as α -fetoprotein, which is the embryonic form of serum albumin and that is present in a wide range of chicken embryonic tissues) or (2) progressive solubilization of some of these proteins from the fat-soluble fraction of yolk (granules) along with the decrease of yolk pH observed during incubation. On the other hand, proteins exhibiting a decreased abundance during this first half of incubation may be due to a selective use/assimilation of these proteins by the embryo at defined phases of its development. They include essentially immunoglobulins, hormone-binding proteins (transthyretin, which binds thyroid hormone) and the vitamin-binding proteins (retinol-binding protein 4 and riboflavin-binding protein). The progressive disappearance of these proteins is consistent with the development and the growth of the embryo as they all have been associated with many processes related to morphogenesis and organ development. Additionally, the fact that only 5 proteins have been reported to decrease during the first half of incubation confirms that up to day 11 of development, very small amounts of yolk nutrients are transferred to the embryo. This nutrient transfer relies on a very specialized structure namely the yolk sac, which develops at the very early days of incubation, from the developing gut of the embryo. It progressively spreads onto the vitelline membrane to totally surround the yolk by day 11 of development. In contrast to the first half of incubation, the second phase of incubation is characterized by prompt utilization of nutrients through the yolk sac, which is then fully matured, to support the rapid growth of the embryo. These yolk proteins serve as storage proteins for lipids, ions, iron, vitamins and hormones that are probably released locally in the embryo after absorption through the yolk sac. Moreover, these proteins probably undergo proteolytic degradation by digestive enzymes to provide free amino-acids for the embryo. It is also noteworthy that by the end of the 21-day incubation, the residual yolk sac is fully internalized into the abdominal cavity of the chick (followed by navel closing and healing). It is subsequently incorporated as part of the forming chick small intestine, and provides nutrients and protection (through remnant maternal immunoglobulins) to the newly hatched chick, until its own digestive tract and immune system become fully functional.

Conclusions

Vitellogenesis is under the control of estrogens and is temporally regulated in the ovary, the upper part of the reproductive tract. Yolk precursors are synthesized by the liver, secreted into the blood stream and incorporated into the forming yolks by receptor-mediated endocytosis. The largest yolk follicle will then ovulate in the oviduct and will be progressively surrounded by the other egg structures in very specialized segments of the oviduct. The resulting yolk consists of proteins bound or not to lipids that are all confined in a yolk container, delimited by the vitelline membrane, which in parallel anchors the egg yolk inside the egg. The progressive

assimilation of yolk components by the embryo initially relies on the vitelline membrane, an cellular protein layer bearing diverse functions: it triggers oocyte fertilization, it acts as a physical barrier against invading microbes and includes several antimicrobial proteins and peptides, and it drives the growth of blood vessels originating from the embryo to finally end in the so-called “yolk sac.” The function of the resulting “yolk sac” is to absorb, digest and transports nutrients composing the yolk toward the embryo (Yadgary *et al.*, 2014).

Egg yolk proteins are of major importance as some of them are specific to birds or oviparous species (vitellogenins, riboflavin-binding protein, ovalbumin). They have been shown to concomitantly accompany the evolution of animals from oviparity to viviparity and the transition of from egg-laying and yolk-dependent nourishment in birds and reptiles, toward the placentation and lactation in mammals (Tian *et al.*, 2010).

Altogether, recent proteomics on the egg yolk combined to bioinformatics analyses for functional protein annotation, and transcriptomics on the yolk sac at various time points of incubation, clearly brought new insights into (1) the identity and specificity of yolk proteins and, (2) the temporal regulation of the mechanisms by which yolk precursors are processed and absorbed via the yolk sac during embryogenesis. However, the role and the biological function of many egg yolk proteins and peptides with regards to the embryo development, remain fully opened.

References

- Anton, M., & Gandemer, G. (1997). Composition, solubility and emulsifying properties of granules and plasma of egg yolk. *Journal of Food Science*, 62(3), 484–487.
- Bourin, M., Gautron, J., Berges, M., et al. (2012). Transcriptomic profiling of proteases and antiproteases in the liver of sexually mature hens in relation to vitellogenesis. *BMC Genomics*, 13, 457.
- Bujo, H., Hermann, M., Lindstedt, K. A., Nimpf, J., & Schneider, W. J. (1997). Low density lipoprotein receptor gene family members mediate yolk deposition. *Journal of Nutrition*, 127(5 Suppl.), 801S–804S.
- Bujo, H., Yamamoto, T., Hayashi, K., et al. (1995). Mutant oocytic low density lipoprotein receptor gene family member causes atherosclerosis and female sterility. *Proceedings of the National Academy of Sciences of the United States of America*, 92(21), 9905–9909.
- Etches, R. J. (1996). *Reproduction in Poultry*. Wallingford: CAB International.
- Farinazzo, A., Restuccia, U., Bachi, A., et al. (2009). Chicken egg yolk cytoplasmic proteome, mined via combinatorial peptide ligand libraries. *Journal of Chromatography A*, 1216(8), 1241–1252.
- Gilbert, A. B. (1979). Female genital tract. In A. S. King, & J. McLelland (Eds.), *Form and Function in Birds* (pp. 237–360). London: Academic press.
- Johnson, A. L., & Woods, D. C. (2007). Ovarian dynamics and follicle development. In B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Birds (Part A)* (pp. 243–277). Science Publishers.
- Mann, K., & Mann, M. (2008). The chicken egg yolk plasma and granule proteomes. *Proteomics*, 8(1), 178–191.
- Nepomuceno, A. I., Muddiman, D. C., & Petite, J. N. (2015). Global proteomic analysis of functional compartments in immature avian follicles using laser microdissection coupled to LC-MS/MS. *Journal of Proteome Research*, 14(9), 3912–3923.
- Nimpf, J., & Schneider, W. J. (1998). The VLDL receptor: An LDL receptor relative with eight ligand binding repeats, LR8. *Atherosclerosis*, 141(2), 191–202.
- Perry, M. M., & Gilbert, A. B. (1979). Yolk transport in the ovarian follicle of the hen (*Gallus domesticus*): Lipoprotein-like particles at the periphery of the oocyte in the rapid growth phase. *Journal of Cell Science*, 39, 257–272.
- Rehault-Godbert, S., Mann, K., Bourin, M., Brionne, A., & Nys, Y. (2014). Effect of embryonic development on the chicken egg yolk plasma proteome after 12 days of incubation. *Journal of Agricultural and Food Chemistry*, 62(12), 2531–2540.
- Retzek, H., Steyrer, E., Sanders, E. J., Nimpf, J., & Schneider, W. J. (1992). Molecular cloning and functional characterization of chicken cathepsin D, a key enzyme for yolk formation. *DNA and Cell Biology*, 11(9), 661–672.
- Tian, X., Gautron, J., Monget, P., & Pascal, G. (2010). What makes an egg unique? Clues from evolutionary scenarios of egg-specific genes. *Biology of Reproduction*, 83(6), 893–900.
- Yadgary, L., Wong, E. A., & Uni, Z. (2014). Temporal transcriptome analysis of the chicken embryo yolk sac. *BMC Genomics*, 15, 690.

Vitellogenesis and Yolk Proteins in Insects

Muhammad Tufail, King Saud University, Riyadh, Saudi Arabia; and Kobe University, Nada, Kobe, Japan
Makio Takeda, Kobe University, Nada, Kobe, Japan

© 2018 Elsevier Inc. All rights reserved.

Introduction

All egg-laying species both vertebrates and invertebrates, including insects, provision their eggs with vitellogenin (Vg), a product of the Vg gene family, by a process known as vitellogenesis. During reproductive phase, the precursor Vg mRNA is expressed in large amounts in the female fat body, an organ functionally analogous to the vertebrate liver. It is then translated, secreted into the hemolymph and finally accumulated by the developing oocytes by membrane-bound receptors (VgRs) through receptor-mediated endocytosis (Tufail and Takeda, 2005, 2007, 2009a,b, 2012). In insects, Vg molecules undergo proteolytic cleavage, soon after their synthesis, and this post-transcriptional processing enables Vgs to carry with them the carbohydrates, lipids, and other nutrients to the oocytes (see for references therein a recent review by Tufail *et al.* (2014)). We have characterized six Vg molecules, both at biochemical and molecular basis including sequencing, from four insect species (*Periplaneta americana*, *Leucophaea maderae*, *Nilaparvata lugens* and *Lethocerus deyrollei*) and have shown by peptide mapping how these proteins undergo post-transcriptional proteolytic processing, and how quickly Vg subunits are sequestered by the oocyte when secreted into the hemolymph (Tufail *et al.*, 2000, 2001, 2005, 2007, 2010; Tufail and Takeda, 2002, 2004; Nagaba *et al.*, 2011). Once taken up by the competent oocytes, the Vgs are routed to the developing yolk body where they are crystallized and stored as vitellin (Vn), the major yolk protein, a nutritional source for the developing embryo.

The regulation of Vg genes is directly under the control of hormones at the transcriptional level. The sex- and tissue-associated, and hormone-mediated developmental specificities of Vg gene transcription have been reported for many insect species. The Vg gene is expressed in most, if not all, insect species in the female fat body, however, in some species, Vg mRNA has also been observed, although in smaller amounts, in larvae and males in the honeybee, and also that the distinct yolk proteins, YPs, were expressed in ovaries, in addition to the fat body, as was seen in *Cyclorrhapha* (see for references the recent review by Tufail *et al.* (2014)).

In insects, the YPs may be classified into four types: 1) The YPs (like Vgs of most insect species) that are synthesized in the fat body tissues in a sex-specific manner and taken up by the competent oocytes; 2) YPs that are produced both in the female fat body and ovarian follicle cells in a sex-specific manner and are incorporated into developing oocytes (YPs of *Cyclorrhapha*); 3) YPs that are synthesized in the ovarian follicle cells and incorporated into the developing oocytes in a sex-specific manner (egg-specific protein in *Bombyx mori*); and 4) Yps that are produced in the fat body cells in a sex non-specific manner and secreted into the hemolymph and internalized by the developing oocytes (such as the 30-kD protein of *B. mori*).

In this article, we provide our current understanding regarding vitellogenesis and yolk proteins in insects. We will briefly discuss the biochemical and molecular aspects of these (Vgs) important molecules in addition to the mechanisms that govern the regulation of Vg genes transcription. Subsequently, the uptake mechanism of Vg by the developing oocytes through its receptor (VgR) will be addressed.

Biochemical Traits of Insect Vgs

Insect Vgs are synthesized as a primary Vg gene product of ~200 kD, which is coded by a single Vg gene transcript of 6–7 kb (Tufail and Takeda, 2008). The primary Vg gene product undergoes post-translational proteolytic cleavage and splits into smaller polypeptides ranging from 50 to 180 kDa (see reviews: Tufail *et al.* (2005, 2014), Tufail and Takeda (2008, 2009c, 2012)). These cleaved Vg subunits are then assembled together, following extensive co- and post-translational modifications, and consequently secreted as big oligomeric phosphoglycolipoproteins (400–600 Da). Sometimes, Vgs are additionally cleaved in the ovary as being reported in the cockroach, *Leucophaea maderae* (Tufail and Takeda, 2002). The understanding that has been emerged from the recent molecular studies is that the Vgs molecules are cleaved in all insect species except Apocrita (higher Hymenoptera) where Vg gene product of ~180 kDa remains uncleaved (see reviews: Tufail and Takeda (2008, 2012)). However, the Vgs of some hemimetabolous insects (like cockroaches and the bean bug, *Riptortus clavatus*) are probably unique, cleaved into more than two subunit polypeptides (see reviews Tufail and Takeda (2008, 2009c)). In lieu, the YPs of *Cyclorrhapha* (higher Diptera) are quite different from the Vgs of other insects and also are not processed. These are much smaller (~45-kD) than those of other insects, and are also structurally similar to the mammalian triglycerol lipase gene family (see for references the reviews: Tufail and Takeda (2008, 2009c, 2012), Tufail *et al.* (2014)).

The comparison of protein primary structures has shown that insect Vgs are members of a greater superfamily of molecules known as large lipid transfer proteins, and are conserved among organisms as diverged as nematode and vertebrates (see for references the reviews: Tufail and Takeda (2008, 2012)) as has also been shown in our phylogenetic tree (Fig. 1). Our phylogenetic tree constructed based on 99 insect and non-insect Vg sequences reveals that insect Vgs are closer to arachnid and nematode Vgs as compared to vertebrate and crustaceans Vgs. Also, the crustacean Vgs represent the most diverged strongly supported cluster (with 100% bootstrap value) to all other Vgs (Fig. 1), and thus probably reflecting the divergence of the terrestrial and aquatic species. Furthermore, our phylogenetic analysis places all species in their respective taxa in a quite coherent manner, suggesting

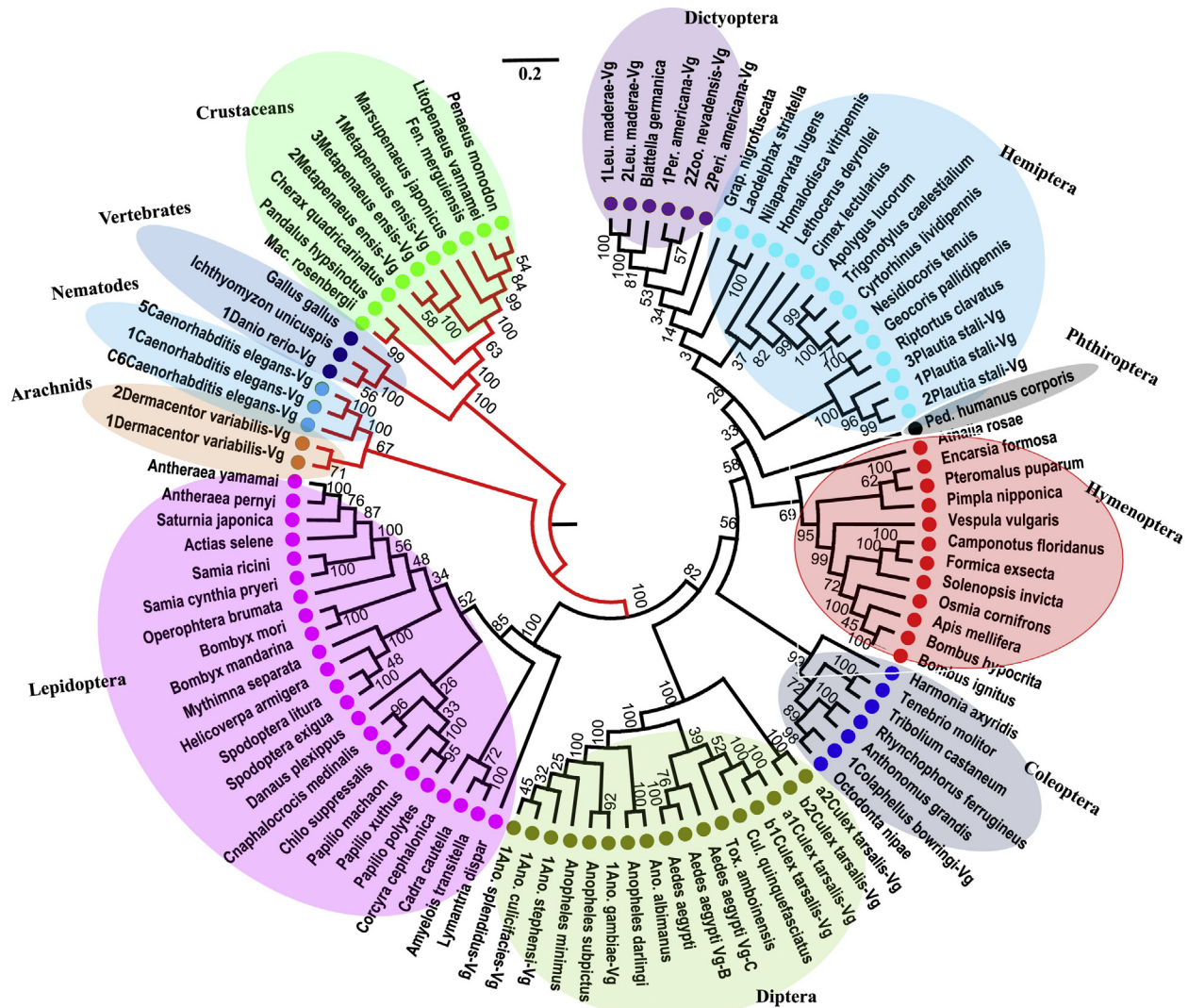


Fig. 1 Phylogenetic tree constructed based on 99 Vgs from both vertebrates and invertebrates. Protein sequences were aligned using the CLUSTAL W program and used as input for a neighbor-joining tree construction program (MEGA6). Bootstrap values (500 replications) are indicated at each node. The scale indicates the distance (number of amino acid substitutions per site). The tree branches with red and black color represent non-insect and insect Vgs, respectively. The Vg sequences used for phylogenetic study were from the following species (accession number are shown in parentheses): *Blattella germanica* (AJ005115), *Leucophaea maderae* (Vg1: BAB19327; Vg2: BAD72597), *Periplaneta americana* (Vg1: BAA86656; Vg2: BAB32673), *Zootermopsis nevadensis*-Vg2 (KDR08463), *Apolygus lucorum* (AFW97644), *Cimex lectularius* (BAU36889), *Cyrtorhinus lividipennis* (AJ143739), *Geocoris pallidipennis* (ALN70475), *Graptopsaltria nigrofuscata* (BAA85987), *Homalodisca vitripennis* (AAZ06771), *Laodelphax striatella* (AGJ26478), *Neosidicoris deyrollei* (BAG12118), *Nesidiocoris tenuis* (AGV05363), *Nilaparvata lugens* (BAF75351), *Plautia stali* (Vg1: BAA88075; Vg2: BAA88076; Vg3: BAA88077), *Riptortus clavatus* (AAB72001), *Trigonotylus caelestialium* (BAJ33507), *Pediculus humanus corporis* (EEB18660), *Apis mellifera* (CAD56944), *Athalia rosae* (BAA22791), *Bombus hypocrite* (ACU00433), *Bombus ignites* (ACM46019), *Camponotus floridanus* (EFN64902), *Encarsia formosa* (AAT48601), *Formica exsecta* (AII96912), *Osmia cornifrons* (AIY25493), *Pimpla nipponica* (AAC32024), *Pteromalus puparum* (ABO70318), *Solenopsis invicta* (AAP47155), *Vespula vulgaris* (AER70365), *Rhynchophorus ferrugineus* (ALN38803), *Anthonomus grandis* (AAA27740), *Colaphellus bowringi*-Vg1 (AMK38869), *Harmonia axyridis* (AMP18919), *Octodonta nipae* (AKR04341), *Tenebrio molitor* (AAU20328), *Tribolium castaneum* (EFA11425), *Aedes aegypti* (AAA18221; Vg-B: AAQ92367; Vg-C: AAQ92366), *Anopheles albimanus* (AAV31933), *Anopheles culicifacies*-Vg1 (AE051020), *Anopheles darlingi* (ETN67132), *Anopheles gambiae*-Vg1 (AAF82131), *Anopheles minimus* (AHN13887), *Anopheles splendidus*-Vg1 (AGZ02020), *Anopheles stephensi*-Vg1 (ABD97990), *Anopheles subpictus* (AHM10344), *Culex quinquefasciatus* (EDS34783), *Culex tarsalis* (Vg1a: ADH04224; Vg1b: ADH04225; Vg2a: ADH04226; Vg2b: ADH04227), *Toxorhynchites amboinensis* (AAV31932), *Actias selene* (ABP63663), *Amyelois transitella* (XP_013200083), *Antheraea pernyi* (BAB16412), *Antheraea yamamai* (BAF45319), *Bombyx mandarina* (BAB32642), *Bombyx mori* (BAA06397), *Cadra cautella* (ALN38805), *Chilo suppressalis* (AMD78107), *Cnaphalocrocis medinalis* (AEM75020), *Corcyra cephalonica* (AHZ89334), *Danaus plexippus* (EHJ67298), *Helicoverpa armigera* (AGL08685), *Lymantria dispar* (AAB03336), *Mythimna separate* (AHG29547), *Operophtera brumata* (KOB78233), *Papilio machaon* (KPJ19580), *Papilio polytes* (XP_013148766), *Papilio xuthus* (KPJ04900), *Samia cynthia pryeri* (BAD91196), *Samia ricini* (BAB32641), *Saturnia japonica* (BAD91195), *Spodoptera exigua* (ALR35190), *Spodoptera litura* (ABU68426), *Dermacentor variabilis* (Vg1: AAW78557; Vg2: ABW82681), *Caenorhabditis elegans* (Vg1: CCD67565; Vg5: CCD63027; Vg6C: CCD70605), *Danio rerio*-Vg1 (AAK94945), *Gallus gallus* (AAA49139), *Ichthyomyzon unicuspis* (AAA49327), *Cherax quadricarinatus* (AAG17936), *Fenneropenaeus merguensis* (ACV32381), *Litopenaeus vannamei* (AAP76571), *Macrobrachium rosenbergii* (BAB69831), *Marsupenaeus japonicus* (BAD98732), *Metapenaeus ensis* (Vg1: AAM48287; Vg2: AAN40700; Vg3: AAT01139), *Pandalus hypsinotus* (BAD11098), *Penaeus monodon* (ABB89953).

that Vgs are still phylogenetically bound, although there exists a divergence among them, and can be used as a molecular marker to identify the phylogenetic inferences.

Molecular Aspects of Insect Vgs

As far molecular traits of insect Vgs are concerned, the sequence comparison of 81 Vgs from 72 insect species that belong to 7 different taxonomic groups (Fig. 1) demonstrates that they can be aligned confidently along their entire lengths, and represent the usual five particular subdomains of high sequence similarity. Also, the hallmarks that remain conserved at the C-terminus of most insect Vgs like a highly conserved motif GL/ICG, the polycysteines conserved at their specific sites, and the DGXR motif that exists 17–19 residues upstream of the GL/ICG in most of the insect species, were found existed (data not shown). Furthermore, the comparison of the consensus cleavage site motif RXXR indicates that its position is particularly conserved near the N-terminus of most of the non-apocritan Vgs and is flanked by polyserine domains (see for details the reviews by the same authors: Tufail *et al.* (2005), Tufail and Takeda (2008, 2009c, 2012)).

Vg Genes in Insects

Because of the prime role of Vgs in egg development/reproduction, Vg genes and/or their transcripts have been sequenced and characterized from several insect species representing diverse taxa (see reviews: Tufail *et al.* (2005, 2014), Tufail and Takeda (2008)) (also see Fig. 1). In insects, Vgs are coded by a single transcript of 6–7 kb, however, the number of Vg genes differs (one to several) in different species (see review: Tufail and Takeda (2008)). We have cloned/sequenced two Vg gene transcripts from each of the two cockroach species, *P. americana* and *L. maderae* (Tufail *et al.*, 2000, 2001, 2005, 2007; Tufail and Takeda, 2007, 2008). Multiple Vg genes have also been reported from other insect species including *Locusta migratoria*, *Riptortus clavatus*, *Plautia stali* and *Aedes aegypti*. Also, the sequences of multiple Vg genes have been identified by the whole genome analysis in several mosquito species comprising *Anopheles albimanus*, *Aedes aegypti*, *A. polynesiensis*, *A. albopictus*, *Ochlerotatus atropalpus*, *Oc. triseriatus*, *Culex pipiens* and *Toxorhynchites amboinensis* (see for references the review by Tufail *et al.* (2005, 2014), Tufail and Takeda (2008)). Nonetheless, the physiological role of multiple Vg genes in some insect species remains unclear. We speculate that different Vg genes may play different physiological roles. The extra-reproductive function of Vgs is not ruled out. Our hypothesis is also supported by the fact that the Vg gene is expressed in male honey bees suggesting that Vg may have also some additional function than as a yolk. Moreover, in crayfish, the plasma clotting protein is structurally similar to insect Vgs but is responsible for clot formation in crustacean blood. Recently, it has been reported that Vg gene is involved in regulation of hormonal dynamics, and has multiple coordinating effects on social organization of worker honey bees (see for reference the review: Tufail and Takeda (2008, 2009c), Tufail *et al.* (2014)). Delineating the overall and precise physiological roles of multiple Vgs should prove their significance.

Regulation of Vg Genes in Insects

The regulation of Vg genes in insects is controlled by hormones. The sex- and tissue-specific, and hormone-mediated developmental profiles of Vg gene transcription have been described for many insect species. In insects, the hormones reported for Vg gene transcription are juvenile hormone (JH), ecdysone, and neuropeptides (see reviews: Tufail and Takeda (2008), Tufail *et al.* (2014)). Among them, JH is the best-known for transcription of Vg genes and the consequent control of Vg synthesis. The regulation of Vg gene transcription depends on binding of hormone-receptor complex at hormone responsive elements (HREs) generally present in upstream regulatory region of the genes (see for references the above reviews). Although, molecular mechanism of JH remains clear, yet, several efforts have been made to identify JH receptor by separating a protein that binds to radio-labeled JH or JH analogues. Currently, two candidates are proposed for the JH receptor: 1) USP (ultraspiracle), the nuclear receptor; and 2) MET (Methoprene-tolerant), the bHLH-PAS protein family member. There is also a suggestion that JH functions through insulin-like peptide signaling pathway to regulate Vg gene expression (see for references the above reviews). The future research will clarify the reality.

In general, insects can be divided, based on the mechanism of hormonal regulation of Vg gene transcription, into three major groups: 1) Insects where only JH directs the regulation of Vg gene transcription. In fact, this hormone (JH) is well recognized in vast majority of insects for its involvement in Vg gene transcription; 2) Insects where ecdysteroid, a product from ovaries, is required for regulation of Vg gene requires in addition to the JH (such as Dipteran: mosquitoes and *Drosophila melanogaster*); 3) Insects like lepidopterans that require JH, ecdysteroids and additional hormones to regulate their reproductive biology. For more detail and references see the reviews by the same authors (Tufail and Takeda, 2008; Tufail *et al.*, 2014).

Uptake of Vgs by the Oocytes

The accumulation of yolk proteins during oogenesis, a process known as vitellogenesis, is the major route for the supply of proteins and lipids in developing oocytes. The vitellogenesis is triggered, in many insect species, by the JH where other factors such as neural or endocrinological ones also play important roles. Recently, the effect of indolamines as neurohormones on the onset of

vitellogenesis has been identified, and proved that vitellogenesis is upregulated by the injection of tryptamine (Tn) and *N*-acetyl-tryptamine and that the action of Tn was both direct and indirect via JH regulation, evidence of cross-talk between monoamines and sesquiterpenoid routes (Kamruzzaman *et al.*, 2016). The uptake of Vgs by the competent oocytes, a typical phenomenon of all oviparous species including insects, is accomplished through receptor-mediated endocytosis (see reviews: Tufail and Takeda (2009a,b,c, 2012)). In this episode, follicle cells are in fact the key players to regulate Vg uptake by providing a pathway for oocytes to access Vg in a JH-dependent fashion during vitellogenesis (see for references the review: Tufail and Takeda (2012)). In insects, the accumulation of Vgs is facilitated by endocytotic machinery via VgRs of the low density lipoprotein receptor (LDLR) gene family (see above reviews) (Fig. 2). We have characterized VgRs, both at biochemical and molecular basis including sequencing, from two cockroach species (*P. americana* and *L. maderae*), and provided evidence that two distinct female-specific proteins Vgs (Vg1 and Vg2) are accumulated by their receptor (VgR) in the cockroach, *P. americana* (Tufail *et al.*, 2000, 2001, 2005; Tufail and Takeda, 2005, 2007). The VgRs primary proteins are highly conserved in organisms as diverged as insects, frogs and chickens, and this is probably because of their physiological role. The receptors (VgR) where Vg anchors are confined in coated pits on the surface of growth competent oocytes. After binding, the ligand/receptor (Vg/VgR) complexes concentrate in clathrin-coated pits that invaginate and pinch off to form intracellular coated vesicles. These vesicles then carry the Vg product to an endosome, which directs subsequent intracellular transportation of both the protein (Vg) and the receptor (VgR). The acidic environment of the endosome results in dissociation of Vg/VgR complexes, which results in recycling of the VgR back to the oocyte surface while Vg is transported to the mature yolk bodies, where it is processed and stored as Vn, the nutritional source for the future embryo (see for references and detail the reviews by the same authors: Tufail and Takeda (2009a,b,c, 2012)) (Fig. 2). The proteins accumulated by oocyte in addition to Vg include lipophorin, arylphorin, microvitellogenin and several other follicle specific proteins, however, the receptor specificity of these proteins

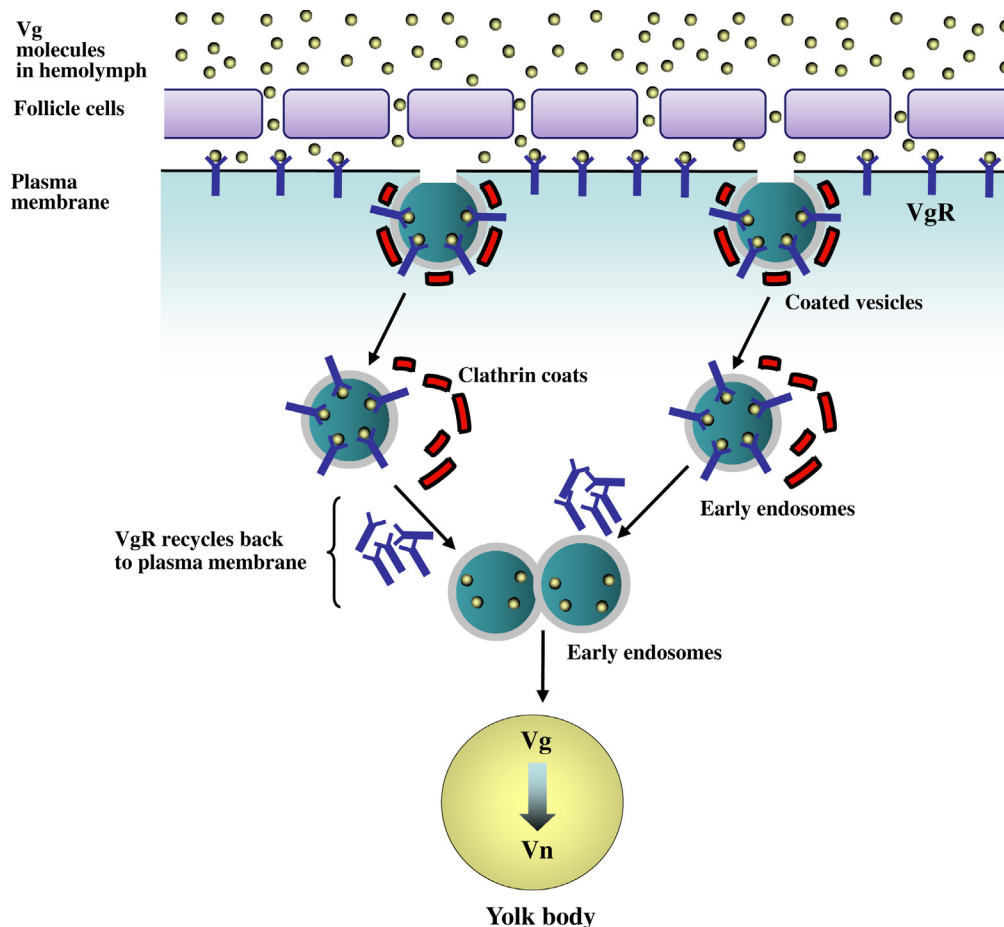


Fig. 2 A schematic representation of how the major yolk protein precursor Vg is endocytosed in the insect oocyte. The Vg molecules carried by the hemolymph pass through the spaces formed between follicular epithelial cells (patency) and bind to its counterpart, the VgR in the clathrin-coated pits, which are accumulated in coated vesicles and then, after losing clathrin coats, in early endosomes. These early endosomes then fuse and form the yolk bodies whereas VgR is recycled and goes back to oocyte membrane. Once Vg becomes a part of the egg yolk, it is called vitellin (Vn), a nutritional reserve for the future embryo. This diagram is produced based on Tufail, M., Takeda, M., 2012. Insect yolk proteins and their role in reproduction. In: Tufail, M., Takeda, M. (Eds.), *Hemolymph Proteins and Functional Peptides: Recent Advances in Insects and other Arthropods*, vol. 1. Dubai, UAE: Bentham Science Publishers, pp. 1–16.

remains unclear. Moreover, the oocyte cytoplasm also comprises a number of lipid droplets together with triglycerides, phospholipids and granules of glycogen in addition to some proteases taken up by the oocytes for yolk proteolysis during embryogenesis.

Immediately after vitellogenesis, the vitelline membrane, a thin protein sheet composed of several proteins (with molecular mass of 14–130 kD) and regulated by 20-hydroxyecdysone, is secreted at the inner surface of the follicle cells, and a chorion is synthesized. The chorion, a product of follicle cells composed of a number of sclerotized proteins, is placed on the egg, before fertilization, while the egg still exists in the ovary. As soon as, the chorion is secreted, this layer forms an impermeable covering except micropyle, a specialized opening through which a sperm enters. When emission of chorion is accomplished, the mature follicle cells are degraded and leave the chorion as the outermost covering or shell of the egg which is then fertilization in the median oviduct and oviposited (see for references and detail the reviews by the same authors: Tufail and Takeda (2009a,b,c, 2012)).

Concluding Remarks

Recent molecular studies have added immensely in our understanding about biochemical and structural aspects of Vgs, about their biosynthesis, processing and transcriptional regulation in addition to their uptake mechanism by the developing oocyte. Yet, many questions need to be answered. Vg structures appear to be conserved although in certain amino acid residues/motifs, in organisms as diverged as nematode and vertebrates. This structural maintenance in Vgs of varied taxa is, perhaps, because of their unique function as a yolk. However, why there exist multiple Vg genes in some insects and why insect species diverge in deploying different hormones to regulate their reproductive physiology remains unclear. We have proposed that different Vg genes may have different physiological roles (Tufail and Takeda, 2008, 2012). The physiological function of Vg other than yolk is not ruled out especially when Vg gene transcription has been detected in honey bee males; also connection of Vg gene in hormonal dynamics in addition to its multiple coordinating effects on social organization in worker bees has been reported. Moreover, antioxidant activities of Vgs and their role in extending lifespan of honey bee and *C. elegans* in addition to their involvement in promoting macrophage phagocytosis in fish have been reported. Also recently, Vg has been described to decrease the parasite-killing efficiency of the antiparasitic factor TEP1 (thioester-containing protein 1) in *Anopheles gambiae*, and has shown anti-bacterial activity in *Bombyx mori*, thus suggesting some link of this protein with immunity (see for references the reviews: Tufail and Takeda (2008, 2009c), Tufail et al. (2014)). Future studies must elucidate the extra-reproductive roles of Vgs in insects. Also, the molecular mechanism of hormonal regulation of Vg gene transcription, especially for JH, remains another hot issue for future research. Next, the precise mechanism of the endocytotic machinery for uptake of Vgs by the oocyte remains unclear.

References

- Kamruzzaman, A. S. M., Asano, H., Hiragaki, S., & Takeda, M. (2016). Indoleamines regulate vitellogenesis via cross-talks with allatotrophe in the American cockroach, *Periplaneta americana*. *International Journal of Advanced Research*, 4(7), 487–497.
- Nagaba, Y., Tufail, M., Inui, H., & Takeda, M. (2011). Hormonal regulation and effects of four environmental pollutants on vitellogenin gene transcription in the giant water bug, *Lethocerus deyrollei* (Heteroptera: Belostomatidae). *Journal of Insect Conservation*, 15(3), 421–431.
- Tufail, M., Bembenek, J., Elgendy, A. M., & Takeda, M. (2007). Evidence for two vitellogenin-related genes in *Leucophaea maderae*: The protein primary structure and its processing. *Archives of Insect Biochemistry and Physiology*, 66, 190–203.
- Tufail, M., Hatakeyama, M., & Takeda, M. (2001). Molecular evidence for two vitellogenin genes and processing of vitellogenins in the American cockroach, *Periplaneta americana*. *Archives of Insect Biochemistry and Physiology*, 48, 72–80.
- Tufail, M., Lee, J. M., Hatakeyama, M., Oishi, K., & Takeda, M. (2000). Cloning of vitellogenin cDNA of the American cockroach, *Periplaneta americana* (Dictyoptera), and its structural and expression analyses. *Archives of Insect Biochemistry and Physiology*, 45, 37–46.
- Tufail, M., Naeemullah, M., Elmogy, M., et al. (2010). Molecular cloning, transcriptional regulation, and differential expression profiling of vitellogenin in two wing-morphs of the brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae). *Insect Molecular Biology*, 19(6), 787–798.
- Tufail, M., Nagaba, Y., Elgendy, A. M., & Takeda, M. (2014). Regulation of vitellogenin genes in insects. *Entomological Science*, 17, 269–282.
- Tufail, M., Raikhel, A. S., & Takeda, M. (2005). Biosynthesis and processing of insect vitellogenins. In A. S. Raikhel, & T. W. Sappington (Eds.), *Progress in Vitellogenesis. Reproductive Biology of Invertebrates* (vol. XII, pp. 1–32). Enfield, UK; Plymouth, USA: Science Publishers, Inc. [Part B].
- Tufail, M., & Takeda, M. (2002). Vitellogenin of the cockroach, *Leucophaea maderae*: Nucleotide sequence, structure and analysis of processing in the fat body and oocytes. *Insect Biochemistry and Molecular Biology*, 32, 117–134.
- Tufail, M., Takeda, M. (2004). Molecular characterization of cockroach vitellogenins/vitellogenin receptor. In: Oishi, T., Tsutsui, K., Tanaka, S., Kikuyama, S. (Eds.), *Trends in Comparative Endocrinology, Proceedings of the 5th Congress of the AOSCE in Conjunction with the Annual Meeting of JSCE*, pp. 39–41. Nara, Japan: AOSCE and JSCE Societies.
- Tufail, M., & Takeda, M. (2005). Molecular cloning, characterization and regulation of the cockroach vitellogenin receptor during oogenesis. *Insect Molecular Biology*, 14, 389–401.
- Tufail, M., & Takeda, M. (2007). Molecular cloning and developmental expression pattern of the vitellogenin receptor from the cockroach, *Leucophaea maderae*. *Insect Biochemistry and Molecular Biology*, 37, 235–245.
- Tufail, M., & Takeda, M. (2008). Molecular characteristics of insect vitellogenins. *Journal of Insect Physiology*, 54, 1447–1458.
- Tufail, M., & Takeda, M. (2009a). Insect vitellogenin/lipophorin receptors: Molecular structures, role in oogenesis, and regulatory mechanisms. *Journal of Insect Physiology*, 55, 87–103.
- Tufail, M., & Takeda, M. (2009b). Molecular mechanisms of insect vitellogenin/lipophorin receptors. In R. Chandrasekar (Ed.), *Short Views on Insect Molecular Biology* (vol. 1, pp. 95–117). India: Insect Molecular Biology Unit, Bharathidasan University.
- Tufail, M., & Takeda, M. (2009c). Insect vitellogenins: Structural features, biosynthesis, and uptake mechanisms. In R. Chandrasekar (Ed.), *Short Views on Insect Molecular Biology* (vol. 1, pp. 69–94). Bharathidasan Uni, India: Insect Molecular Biology Unit.
- Tufail, M., & Takeda, M. (2012). Insect yolk proteins and their role in reproduction. In M. Tufail, & M. Takeda (Eds.), *Hemolymph Proteins and Functional Peptides: Recent Advances in Insects and other Arthropods* (vol. 1, pp. 1–16). Dubai, UAE: Bentham Science Publishers.

Vitellogenesis & Yolk Proteins, Crustaceans and Molluscs

Marcy N Wilder and Bong Jung Kang, Japan International Research Center for Agricultural Sciences (JIRCAS), Tsukuba, Japan
Junya Higano, National Research Institute of Fisheries and Environment of Inland Sea (FEIS), Fisheries Research Agency (FRA), Hatsukaichi, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Androgenic gland A male specific tissue associated with the sperm ducts (vas deferens); is an endocrine gland, and produces androgenic gland hormone, which regulates the sexual differentiation of the male reproductive system.

Consensus cleavage sites Cleavage sites that possess the amino acid sequence R-X-K/R-R that are targeted by enzymes of the subtilisin family, and are found within the crustacean vitellogenin molecule.

Ecdysteroids Collectively refers to substances related to 20-hydroxyecdysone, the molting hormone in insects and crustaceans; is also implicated to have a role in reproduction and embryogenesis.

Eyestalks The anatomical part of the decapod crustacean that supports the compound eye; harbors the X-organ/sinus gland complex, a neurosecretory tissue that produces numerous hormones that control molting, reproduction, and metabolism.

Farnesoic acid The precursor of methyl farnesoate in Crustacea. Thought to function in the regulation of molting and metamorphosis.

Follicles Ovarian cell layer of epithelium immediately surrounding an oocyte; regulates transport between blood/hemolymph and the oocyte. May also secrete certain components of the egg.

Gonadotropin-releasing hormone (GnRH) A neuro-hormone consisting of 10 amino acids that is produced in the arcuate nuclei of the hypothalamus. Stimulates the synthesis and secretion of the vertebrate gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Additionally implicated to have a reproductive role in molluscs.

Hemolymph The arthropod equivalent of blood; includes cells that are referred to as “hemocytes” and utilizes hemocyanin in most cases as the carrier of oxygen.

Hepatopancreas Serves as a digestive organ with pancreatic function in crustaceans and is also known as the “midgut gland”; established to be a major site of vitellogenin production in decapods.

Hermaphroditic Indicates an animal which can change from male to female (protandric) or from female to male (protogynous). In Crustacea, many protandric species exist.

Intersex An abnormal sexual phenotype; an individual having both male and female secondary sexual characteristics.

Mandibular organs Endocrine gland associated with the mandibular tendon; produces methyl farnesoate, and is negatively regulated by mandibular organ-inhibiting hormone (MOIH); located anteriorly above the Y-organs.

Perivitelline fluid Fluid that fills the space between the vitelline membrane and the zona pellucida. Appears in the ovum immediately following fertilization.

Sesquiterpenoids Chemical substances which consist of three isoprene (C_5H_8) units; examples include juvenile hormone, methyl farnesoate, and farnesoic acid.

Introduction

This entry discusses the current state of knowledge concerning yolk proteins in crustaceans and molluscs, covering structural facets, the hormonal control of their production, and their usage as a source of nutrients during embryogenesis. As detailed below, both animal groups include numerous species that are of economic importance and are consumed by humans; such species are the target of aquaculture and commercial fishing activity. From this perspective, it has been important to elucidate their reproductive mechanisms so that the life cycle can be closed and the animals reared efficiently in captivity; understanding the fundamentals of yolk proteins is requisite in this regard. Obviously, both groups are classified as invertebrates; Mollusca is a phylum unto itself, while Crustacea constitutes a sub-phylum within the Phylum Arthropoda. In addition, many crustacean species, while not a source of food for human consumption, are well-studied, and their reproductive ability serves as a means for assessing the state of the environment and the possible presence of endocrine disruptors. While much of the knowledge concerning yolk proteins in these animals is unified and cohesive, there exists a diversity of structure for the yolk proteins themselves. Nevertheless, structure often reflects taxonomic classification, which is especially apparent in the case of decapod crustaceans, discussion of which follows immediately below.

Crustacea

Yolk Protein Structure in Decapod Crustaceans

Decapod Crustacea constitute an order within the crustacean class Malacostraca, comprising over 10,000 species. Within this order are contained several infraorders, e.g., *Penaeidea* (marine shrimp), *Caridea* (marine, estuarine and freshwater shrimp), *Astacidea*

(lobsters, crayfish), *Palinura* (spiny lobsters), *Anomura* (mud-borrowing shrimp, hermit crabs, other) and *Brachyura* (marine, estuarine and freshwater crabs), among others. This order, therefore, comprises the majority of crustacean species that have economic value and are the targets of aquaculture and/or commercial fishing activities. As is well-known, shrimp culture is an established significant world-wide industry having a production scale of over 4 million tons per year, corresponding to a market volume of over 20 billion U.S. dollars (FAO, 2014). In this regard, much of the research on crustacean reproduction, and hence, crustacean yolk proteins, has focused on that of decapod species, especially for those where it is of importance to develop technology to close the life cycle in captivity, such as that for the whiteleg shrimp, *Litopenaeus vannamei* which constitutes over 80% of the world's aquaculture.

The structure, processing, and site of production of vitellogenin has been an important research topic in decapod crustaceans for nearly 45 years, and it is during approximately the past 15 years that this knowledge has come to be generally complete and unified. This body of research has shown that crustacean vitellogenins differ a great deal in terms of amino acid sequence and certain structural features, from those of other animal groups, even from insects, which are also included in the phylum Arthropoda. However, vitellogenesis in Crustacea follows the same basic process in which vitellogenin is produced in specific tissues as a large yolk protein precursor and is subsequently processed into smaller molecules. The end product is vitellin, which is accumulated in developing oocytes to serve as a source of nutrients for the developing embryo.

Initially, studies focusing on crustacean vitellogenins utilized electrophoretic or immunological techniques, yielding information on molecular weight and subunit composition. Many workers also characterized amino acid composition, as well as lipid and carbohydrate content. For example, in the giant freshwater prawn, *Macrobrachium rosenbergii*, various research groups found that vitellogenin was composed of three subunits, of molecular weights of around 200, 100 and 90 kDa, while vitellin consisted of two immunologically equivalent subunits of 90 kDa (Wilder *et al.*, 2010).

Later studies employing molecular biological techniques enabled workers to characterize vitellogenin cDNA and elucidate the primary structure for this molecule. The first report of a complete primary sequence in a crustacean species was accomplished by Tsutsui *et al.* (2000), in the kuruma prawn, *Marsupenaeus japonicus*. Tsutsui *et al.* (2000) used N-terminal amino acid sequence information, and the method of "library walking", to obtain a full-length cDNA encoding 2587 amino acids. This vitellogenin precursor contained two consensus cleavage sites R-X-K/R-R targeted by enzymes of the subtilisin family. Thus following, vitellogenin structure has been clarified in nearly 20 decapod species, including prawns/shrimps, crabs, crayfishes and burrowing shrimp. Thus far, all species have shown high levels of identity, the degree of which reflects phylogenetic classification. For example, members of the same family (in the case of Penaeidae) have identity levels of around 90% throughout the entire molecule. In less-related species, the degree of identity is especially high in the first part of the molecule, but somewhat lower in the middle and latter parts of the molecule. Fig. 1 shows a schematic diagram of the vitellogenin molecule and its confirmed/putative processing sites for three species elucidated by the authors of this entry and other co-workers. The percent of amino acid identity for *M. rosenbergii* and *M. japonicus* compared to *Pandalus hypsinotus* for the first and latter parts of the molecule are shown.

Of interest, crustacean vitellogenins (meaning decapod crustaceans) are lacking in polyserine domains that are found in most invertebrate and vertebrate vitellogenins. In the latter, such stretches of serine residues often flank processing sites targeted by subtilisin, possibly creating a beta-pleat arrangement that exposes the processing site to the enzyme (Chen *et al.*, 1997). Nevertheless, decapod vitellogenins do contain 2–3 consensus cleavage sites, e.g., R-X-K/R-R, and almost all studies covering nearly 20 decapod species have documented that actual cleavage occurs at the first of these sites, conserved around or after the 700th residue in the primary structure (for example, in *M. rosenbergii*, Arg⁷⁰⁷-Arg⁷¹⁰, where cleavage occurs between amino acid residues 710 and 711 (Okuno *et al.*, 2002)). It can now be said that the primary structure of decapod crustacean vitellogenin generally consists of around 2500 amino acids, and is processed initially at the hepatopancreas, and once more in the hemolymph, finally yielding three vitellins which are then found in the developing ovaries. As for the site of synthesis, vitellogenin mRNA expression is seen in only in the hepatopancreas in *M. rosenbergii*, *P. hypsinotus*, and similarly-classified species. In penaeid species, such expression occurs in both the hepatopancreas and the follicle cells of the ovaries. Processing mechanisms for vitellogenin synthesized at the ovaries is unclear, but there are no major differences in molecular structure from that synthesized at the hepatopancreas.

Vitellogenin, and more particularly, the final vitellin molecule is in general, are regarded to have various modifications, although full profiles have not been elucidated for the majority of species. These modifications may include glycosylation, metal binding, lipid conjugation, carotenoid conjugation, and hormone binding, particularly of ecdysteroids. The purpose of such modifications may be to assist in bringing necessary nutrients, in addition to vitellin itself, into the eggs for purposes of embryonic development, or ecdysteroids to help the embryo with molting until it can produce its own source of hormone. This topic is reviewed in Wilder *et al.* (2010), and the reader is referred to this source for further information. However, of note, Khalaila *et al.* (2004) elucidated fully the nature of carbohydrate moieties in vitellogenin in the crayfish, *Cherax quadricarinatus*. These authors employed mass spectrometry and other techniques and found, for example, that putative N-glycosylation sites, at Asn¹⁵², Asn¹⁶⁰, and Asn²⁴⁹³ were glycosylated with high-mannose glycans. Glycosylation of vitellogenin and vitellin is postulated to be important in processing and transport, in addition to its nutritional role.

Hormonal Control of Yolk Protein Synthesis in Decapod Crustaceans

Since the 1940s, eyestalk ablation has been known as a methodology for stimulating ovarian maturation and spawning in crustaceans. The rationale behind this methodology is the presence of inhibitory factor in the eyestalks, which are presently referred to as vitellogenesis-inhibiting hormone (VIH) (also gonad-inhibiting hormone; GIH). Thus, vitellogenin synthesis is controlled

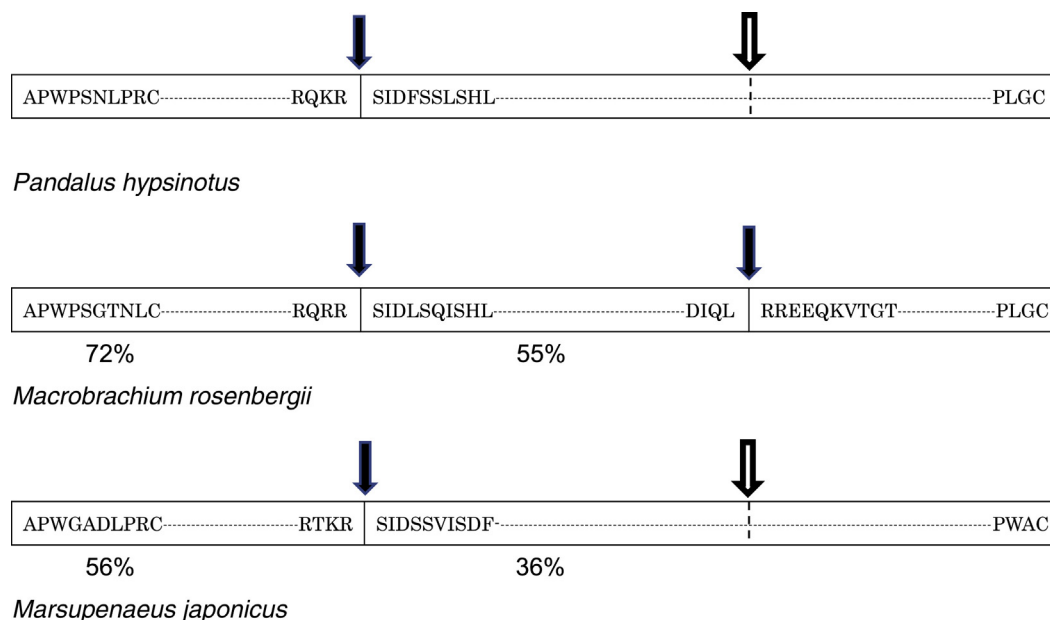


Fig. 1 Schematic diagram of the vitellogenin molecule and its confirmed/putative processing sites for several of the first decapod species that were elucidated. Percent (%) amino acid identity for *Macrobrachium rosenbergii* and *Marsupenaeus japonicus* compared to *Pandalus hypsinotus* are shown for the first and latter parts of the molecule. Confirmed processing sites are indicated by solid arrows; putative processing sites are shown by open arrows. Reproduced from Tsutsui, N., Kawazoe, I., Ohira, T., *et al.*, 2000. Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. Zool. Sci. 17, 651–660 and Wilder, M.N., Okumura, T., Tsutsui, N., 2010. Reproductive mechanisms in Crustacea focusing on selected prawn species: Vitellogenin structure, processing and synthetic control. Aqua-BioSci. Monogr. 3, 73–110.

negatively via the functioning of VIH. VIH is synthesized in the X-organs, and is then stored and/or secreted into the hemolymph from the sinus gland of the eyestalks. This tissue, a collection of neurosecretory cells, is known generally as the X-organ/sinus gland complex, and is responsible for the production of a host of important neuropeptides. Notably among these are members of the crustacean hyperglycemic hormone (CHH) family. CHH-family peptides also include VIH, molt-inhibiting hormone (MIH), and mandibular organ-inhibiting hormone (MOIH). The mature peptide of a typical CHH-family member is comprised of 72–78 amino acids with six conserved cysteine residues. These peptides are considered to have multiple functions and immunological cross-activity among themselves. For example, CHH exhibits hyperglycemic action, but also possesses inhibitory effects on molting and reproduction.

Eyestalk ablation is frequently used to induce ovarian maturation by removing the source of VIH, both in a laboratory situation, and in hatchery operations for commercially important species of shrimp. However, this procedure leads to potential hormonal imbalance, as it also removes the animal's means of producing other important hormones. Therefore, understanding of the physiological functioning of VIH is important for not only purposes of elucidating reproductive mechanisms more fully, but also for developing alternative methods to eyestalk ablation. The first report focusing on the dynamics of vitellogenin and VIH levels in the hemolymph in relation to molting was conducted in *L. vannamei* by Kang *et al.* (2014). It was seen that VIH levels in hemolymph fluctuated in adults and sub-adults. In adults, hemolymph VIH surged preceding the peak of vitellogenin levels that occurred around intermolt stage C (Fig. 2), although this pattern was not as clear in sub-adults. Although new information on VIH has accumulated in decapod crustaceans, the cues that actually trigger vitellogenesis, and how VIH prevents this in concert with other factors, remains to be elucidated. In this regard, the stimulating effects on vitellogenesis by putative vitellogenesis-stimulating hormone (VSH) which is thought to be secreted by the brain or/and thoracic ganglia, have been examined in several decapod species. It is possible that this VSH is either a protein or peptide hormone.

Hormonal control of vitellogenesis is also closely related with the molt cycle. As an additional candidate to VSH as a trigger of vitellogenesis, many workers have focused on methyl farnesoate (MF). MF, a sesquiterpenoid, and the precursor to juvenile hormone (JH) in insects, has been detected in Crustacea. Farnesoic acid (FA) is the precursor to MF in Crustacea, and is secreted from the mandibular organs which are controlled negatively by MOIH. MF is considered to act in developmental and reproductive roles in a similar fashion to JH in the case of insects, as JH has not been detected in the crustaceans. Regarding reproduction, the effects of MF have been tested in over 10 species of crustaceans both *in vivo* and *in vitro*. Stimulatory effects on ovarian maturation have been reported in seven species; nevertheless, the involvement of MF during vitellogenesis has not been confirmed for a full spectrum of species (see Subramoniam (2011) for review).

Neurotransmitters, such as serotonin (5-hydroxytryptamine; 5-HT), have also been reported to be involved in ovarian maturation in several decapod crustaceans. Ovarian maturation was induced after 5-HT injection in the Indian white prawn, *Fenneropenaeus*

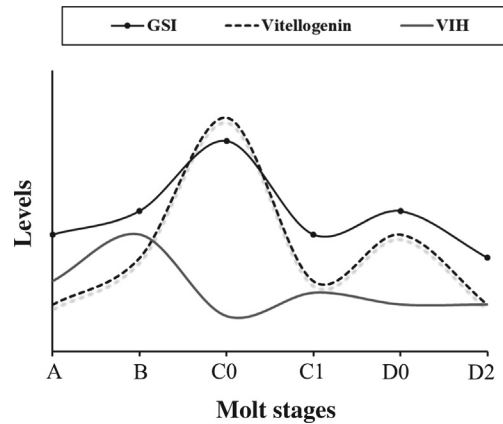


Fig. 2 Schematic diagram for the dynamics of important reproductive parameters in crustaceans based on data obtained for *Litopenaeus vannamei*. Relative levels for vitellogenin and vitellogenesis-inhibiting hormone (VIH) in the hemolymph are shown together with changes in gonado-somatic index (GSI). Changes in circulating vitellogenin levels are synchronized with those for GSI change, but not for those of VIH. When vitellogenin levels were seen to peak, VIH showed low concentrations in the hemolymph. Reproduced from Kang, B.J., Okutsu, T., Tsutsui, N., *et al.*, 2014. Dynamics of vitellogenin and vitellogenesis-inhibiting hormone levels in adult and subadult whiteleg shrimp, *Litopenaeus vannamei*: Relation to molting and eyestalk ablation. *Biol. Reprod.* 90 (1), 12, 1–10.

indicus, the banana shrimp, *Fenneropenaeus merguensis*, the lobster, *Homarus americanus*, the whiteleg shrimp, *L. vannamei*, the red swamp crayfish, *Procambarus clarkii*, the black tiger prawn, *Penaeus monodon*, and in the fiddler crab, *Uca pugilator*. It is considered that actions of 5-HT are to cause the release of VSH (from the thoracic ganglion and/or the brain) or vitellogenesis-stimulating ovarian hormone. Furthermore, this process may be inhibited/mediated by dopamine. Spiperone, which is a dopamine antagonist, has also been reported to induce ovarian maturation in crayfishes *C. quadricarinatus*, and *P. clarkii* and in the giant freshwater prawn, *M. rosenbergii* by *in vivo* treatment (Subramoniam, 2011).

Other factors that are also reported to be involved in ovarian maturation include androgenic gland hormone (AGH). AGH, which is produced in the androgenic glands, in some instances shows similar effects with VIH. In intersex males in *C. quadricarinatus*, vitellogenin syntheses can be induced by removal of the androgenic glands. In the hermaphroditic caridean prawn, *P. hypsinotus*, vitellogenic oocytes appear after degeneration of the androgenic glands (Subramoniam, 2011).

Uptake and Utilization of Yolk Proteins in Decapod Crustaceans

As detailed above, vitellogenin and vitellin have been extensively characterized in decapod crustaceans; in addition, there is a burgeoning literature on the crustacean vitellogenin receptor (VgR), although fewer species have been characterized. Vitellogenin enters the developing oocytes from the hemolymph via receptor-mediated endocytosis similarly to other oviparous animals, and once vitellogenin is deposited within the oocytes, VgR is re-circulated back to the oocyte membrane. The first molecular characterization of VgR in Crustacea was accomplished by Tiu *et al.* (2008) in the black tiger prawn, *P. monodon*. These authors obtained a 6.8 kb cDNA for VgR, with the deduced amino acid sequence being comprised of 1943 residues.

In the kuruma prawn, *M. japonicus*, a putative ovarian lipoprotein receptor was obtained having a full-length cDNA of 3598 bp, and the deduced amino acid sequence encoded 1120 amino acid residues (Mekuchi *et al.*, 2008). Although this molecule is much smaller than the VgR molecule in *P. monodon*, both sequences share common structural features characteristic of the LDL-receptor family, such as the ligand-binding domain, the epidermal growth factor (EGF) precursor domain, an EGF-like domain, and a trans-membrane region. Similarly, in terms of tissue-specific gene expression, both are expressed exclusively in the ovary, further underlining their respective roles in reproduction. More recently, Roth and Khalaila (2013) characterized the VgR molecule and its expression dynamics in *M. rosenbergii*, also illustrating the importance of its functioning during vitellogenesis in decapods.

Vitellin accumulated in the oocytes then serves as a source of nutrients during the ensuing process of embryogenesis, and is often referred to as “lipovitellin” at this point. Most decapod species including shrimps, crabs, and lobsters brood an egg mass which is held externally on the abdomen until the embryos hatch. This period may range from several days to several months, depending on species and habitat. Penaeid shrimp and prawns are the exception, and broadcast their eggs into the surrounding water, to generally hatch within 24 h. The egg also contains hydrolytic enzymes and proteases that break down the lipovitellin and make lipids, proteins, and amino acids available for usage.

This phenomenon has been closely studied in brine shrimp (*Artemia salina*), which belongs to the crustacean class Branchiopoda, and to a certain extent, in decapod crustaceans such as the freshwater shrimp, *Macrobrachium borelli* (Garcia *et al.*, 2008). *M. borelli* broods an egg mass for nearly 40 days, during which time embryogenesis occurs; lipovitellin which is firstly accumulated in the ovaries is also found in developing eggs. In the early embryonic stages, lipovitellin consists of the 112 kDa and 94 kDa subunits, but is degraded to smaller subunits of 70 kDa, and finally 43 kDa. It is likely that other *Macrobrachium* genus species engage in similar means of vitellin usage during the period while eggs are developing.

Yolk Protein Structure and Related Aspects in Other Crustacean Groups

In crustaceans other than in the Order Decapoda, yolk proteins encompassing both vitellogenin and vitellin have been purified and characterized in a variety of species, e.g., in the following: Class Maxillopoda, Subclass Copepoda (Orders Siphonostomatoida, Cyclopoida, Calanoida, Harpacticoida), Class Branchiopoda, and Class Malacostraca (Orders Mysida, Amphipoda). However, much fewer information has been reported in these crustacean groups compared to that available in decapod crustaceans.

In copepods, vitellogenin genes have been identified in four species, *Lepeophtheirus salmonis* (Siphonostomatoida), *Paracyclopina nana* (Cyclopoida), *Pseudodiaptomus annandalei* (Calanoida), *Tigriopus japonicus* (Harpacticoida). It was suggested that most copepods possess two copies of vitellogenin genes, because two vitellogenin genes were reported in all of the above species with the exception of *P. annandalei*. The major synthetic site for vitellogenin is in the hepatopancreas and/or the ovary in decapod crustaceans; however, extra-ovarian sites are reported as well in other crustacean groups. In the case of the salmon louse, *L. salmonis* (Siphonostomatoida), in which a hepatopancreas does not exist, vitellogenin is synthesized in the subcuticular tissue, but not in the ovaries. Circulating vitellogenin secreted into the hemolymph is thus accumulated into the oocytes. The cleavage of vitellogenin (225 kDa) in this species occurs for only one vitellogenin molecule, yielding two subunits (95 kDa and 120 kDa) of vitellin present in eggs, but not for the other vitellogenin (also of 225 kDa). The identity of the deduced amino acid sequences between two *L. salmonis* vitellogenin cDNAs is 23%, meaning that multiple vitellogenin genes (vitellogenin paralogues) may be duplicated in a species-specific manner (Dalvin *et al.*, 2011).

In viewing the structure of these vitellogenin cDNAs, it is observed that both an N-terminal “vitellogenin N domain” and a C-terminal “VWD domain” exist. The “vitellogenin N domain” is a lipoprotein amino terminal region which is thought to be involved in lipid transport, and the “VWD domain” is a von Willebrand factor type D domain. It is noted that the consensus R-X-K/R-R cleavage site of decapods is not present in the “vitellogenin N domain”. However, a GL/ICG motif characteristic of the “VWD domain” is sometimes found in decapod crustaceans with GLLG being substituted for GL/ICG.

Daphnia magna (Branchiopoda: Diplostraca) also possesses two vitellogenin genes: the cDNAs for both are around 6000 bp-long, and their identity is 91.5% at the level of deduced amino acid sequence. The genomic DNA for both genes have also been reported (Tokishita *et al.*, 2006). One prominent feature in these deduced amino acid sequences is the presence of a superoxide dismutase (SOD)-like domain in the N-terminal, which shows significant homology to that of Cu/Zn-type superoxide dismutase. As with copepods, these vitellogenin cDNAs also harbor an N-terminal “vitellogenin N domain” and a C-terminal “VWD” domain. In terms of phylogenetic analysis, *D. magna* vitellogenin is more closely related to insect vitellogenins; however, the SOD-like domain itself is closely related to viral and bacterial SODs. The *D. magna* vitellogenin molecule is approximately 220 kDa in eggs at the early stages, and is primarily cleaved into three subunits.

A high molecular weight vitellin has been reported in *Neomysis integer* (Malacostraca: Mysida), having a molecular mass of 700 kDa and being composed of six subunits (51, 55, 62, 66, 84 and 89 kDa). In particular, mysid crustaceans are often used in toxicity tests such as that for endocrine-disrupting substances, because their life cycles are well-understood, and how they grow and reproduce in context of environmental factors including temperature, salinity, food availability, and light, is widely documented. Nevertheless, the purification of vitellin has been carried out only in two species, e.g., *Americamysis bahia* and *N. integer* for purposes of developing an enzyme-linked immunosorbent assay (Ghekiere *et al.*, 2004). In this regard, several species of crustaceans have been used as model species for toxicity testing in context of the presence of environmental endocrine disruptors, which may alter the life cycle or bring about intersexuality. Many studies on protein levels or gene expression for vitellogenin in such crustaceans have therefore been conducted for purposes of determining how the exposure of animals to environmental disruptors affects such parameters in terms of their utility as biomarkers. However, the use of vitellogenin as a biomarker in crustaceans can often be difficult, because many aspects of their endocrine mechanisms remain unclear, compared with those of fish, for example. Therefore, it is not always easy to define the exact factors that cause endocrine disruption in crustaceans, even if sexual differentiation is disrupted. This is because the malleability of sexual differentiation occurs naturally in crustaceans, where many species exhibit intersexuality and hermaphroditism. Further basic research on reproductive mechanisms and the relationship between the life cycle and environmental influence is required in order to develop more effective usages of vitellogenin as a biomarker.

Mollusca

Yolk Protein Structure in Molluscs

The Phylum Mollusca is composed of seven classes, namely Aplacophora (no shell), Polyplacophora (chitons), Monoplacophora (single external shell), Gastropoda (snails and slugs), Cephalopoda (octopuses and squids), Bivalvia (clams, oysters, mussels, cockles, scallops, and other) and Scaphopoda (tusk shells) comprising over 80,000 species. The classes Gastropoda, Cephalopoda and Bivalvia harbor the majority of the economically important species that are the targets of aquaculture and/or commercial fishing activities. Total world-wide molluscan culture production has exceeded 16 million tons per year, corresponding to a market volume of over 19 billion U.S. dollars, while total capture production reached 7.7 million tons per year as of 2014 (FAO, 2014).

Until the early 1990s, there had been few reports detailing yolk formation or the characteristics of yolk proteins in molluscs. However, with the recent development and application of molecular biological tools to research in this field, certain advances have been made concerning the structure and characteristics of molluscan yolk proteins. Starting with gastropods, the lipid and protein composition of the perivitelline fluid of the eggs of the freshwater snail, *Pomacea canaliculate*, was investigated and two

lipoproteins (PV1 and PV2) and one lipoprotein fraction (PV3) were detected (Garin *et al.*, 1996). These represented 57.0%, 7.5%, and 35.5%, respectively, of this species' total egg proteins. PV1 was shown to be a glyco-carotene-protein complex having the characteristics of a very high-density lipoprotein (VHDL), containing 0.33% lipid comprised of mainly free sterols and phospholipids. Furthermore, this particle was observed to have a molecular weight of 300 kDa and be composed of three subunits of 35, 32, and 28 kDa. The PV2 particle was a VHDL of 400 kDa containing 3.75% lipid. Here, the major lipid classes consisted of free sterols and phospholipids, also having significant quantities of energy-providing triacylglycerides and free fatty acids. The subunits of PV2 corresponded to two apoproteins of 67 and 31 kDa. The PV3 particle in terms of density corresponded to high-density lipoprotein (HDL). It could be fractionated into two sub-fractions, which were referred to as "h" and "p". Fraction "h" contained 5.16% lipids, mainly free sterols, phospholipids, and free fatty acids, and two particles of 100 and 64 kDa. Fraction "p" was composed of a single particle of 26 kDa that contained 9.5% lipid, representing 30% of the total egg lipids. These three fractions are thought to serve as the major supply of lipids and amino acids to the developing embryo (Garin *et al.*, 1996).

Regarding the site of synthesis of molluscan lipoproteins, Dreon *et al.* (2002) investigated this for PV2; tissues (albumen gland, gonad-digestive gland complex, and muscle) from vitellogenic females were incubated *in vitro* with ^{14}C -leucine at 25°C for 12 h, after which soluble proteins from tissue homogenates and medium were analyzed for de novo protein synthesis. Using electrophoresis and high performance liquid chromatography (HPLC), radiolabeled proteins were quantified by liquid scintillation. Two radiolabeled proteins (67 and 31 kDa) originating from the albumen gland co-migrated with the subunits of PV2, and western blot analysis also confirmed the presence of PV2 only in the albumen gland. Other experimentation, such as *in vivo* injection of ^3H -leucine into adult females and immunochemical analysis of all tissues of the snail again confirmed the presence of PV2 only in the albumen gland. In this way, the above authors revealed that this tissue is the sole site of PV2 synthesis. The PV2 subunits were further characterized in terms of N-terminal amino acid sequence, but they showed no homology to other known proteins (Dreon *et al.*, 2002).

In bivalves, a female specific protein (FSP) was identified and purified from the ovary of the Japanese scallop, *Patinopecten yessoensis*, using a combination of immunological and chromatographical techniques including hydroxylapatite chromatography and gel filtration (Osada *et al.*, 1992). The molecular weight of FSP was determined to be approximately 450 kDa. Three other female specific proteins, two having the same molecular weight and the other being 700 kDa, showed differing electrophoretic mobility on native polyacrylamide gel electrophoresis, but did cross-react with antiserum against purified FSP. No FSP was immunologically detected in the extract of the digestive diverticula or in concentrated hemolymph. Extracts from the ovaries of Farrer's scallop, *Chlamys farrerii nipponensis*, and blue mussel, *Mytilus edulis*, cross-reacted with FSP antiserum, but a similar reaction was not seen for the Pacific oyster, *Crassostrea gigas*. The quantity of FSP in ovarian extract from the Japanese scallop increased with the advancement of ovarian development, and this protein itself was found localized in the oocyte cytoplasm. It is considered that the FSP is formed as a yolk protein complex in the ovary being possibly composed of four molecules synthesized from the same source. Furthermore, it has been shown that the antigenicity of FSP is partially conserved among bivalve species (Osada *et al.*, 1992). Regarding the above, the reader is also referred to a recent review on endocrine control in bivalve mollusks by Osada and Matsumoto (2016).

In cephalopods, oocyte growth has been studied in the cuttlefish, *Sepia officinalis*, and is characterized by a high level of yolk protein expression; these yolk proteins serve as a reserve of nutrients for use in embryonic development (Gaudron *et al.*, 2005). The combined techniques of SDS-PAGE and mass spectrometry allowed the identification of two yolk proteins referred to as vitellin 1, having a molecular weight of around 150 kDa, and vitellin 2, being greater than 250 kDa in molecular weight; these vitellins were detected in the ovarian follicles and the vitelline vesicles, and found to be a component of oocyte yolk. Vitellins 1 and 2 showed no sequence homology as demonstrated by MS/MS analysis of peptides fragments obtained by tryptic digestion. In addition, the anterior vitelline vesicle associated with the embryo contained a 100 kDa protein that shared a high degree of sequence homology with vitellin 2, suggesting that this protein undergoes post-translational processing to achieve its mature form during ovarian development.

Hormonal Control of Yolk Protein Synthesis in Molluscs

Gonadotropin-releasing hormone (GnRH) has been established to have an essential role in reproduction in molluscs, as it does in that of vertebrate species; this topic has been reviewed extensively by Osada and Treen (2013). In cephalopods and gastropods, the presence of a GnRH-like peptide was initially demonstrated using heterologous antibodies, and the thus-identified GnRH was thereafter suggested to be involved in both behavioral and reproductive aspects. A role for GnRH in molluscan reproduction has now been confirmed for both bivalves and cephalopods, and such findings are expected to provide useful insights into the evolution of reproductive endocrinology.

In gastropods, the first study to suggest that a native molluscan GnRH-like peptide could be expressed in the molluscan nervous systems was carried out using the gastropod *Hellisoma trivolvis* and heterologous GnRH antibodies (Goldberg *et al.*, 1993). These authors speculated that the *Hellisoma* nervous system showed characteristics consistent with the existence of a GnRH-like peptide functionally similar to human GnRH. At that time, GnRH was assumed to be an example of convergent evolution that had occurred in parallel with the evolution of the vertebrate hypothalamic-pituitary-gonadal axis; therefore, the discovery of its existence in a gastropod was unexpected. Thereafter, research into the existence of molluscan GnRH-like peptides in gastropods has yielded reasonable success. For example, it was shown that GnRH-like neurons are present in the nervous systems of the freshwater snails *Helisoma* and *Lymnaea*, with a possible role for reproduction being hypothesized. The commercially important gastropod, the Pacific abalone, *Haliotis discus hannai*, was also found to possess GnRH-like peptides in the nervous system as well as in the ovary.

In bivalves, the first research that involved the indirect use of mollusc GnRH was performed in the late 1980s where it was shown that extracts from the cerebral ganglion and hemolymph of the blue mussel *M. edulis* were capable of promoting the incorporation of radiolabelled thymidine into the DNA of isolated mantle cells (Mathieu *et al.*, 1988), thereby promoting mitosis. It was therefore suggested that molluscan GnRHs may have a conserved structure that is found in other animal groups, but is not recognized by the vertebrate receptor due to differences in evolutionary lineage. In this way, the term “gonadotropin-releasing hormone” may be a bit inappropriate when used to discuss protostomian GnRH peptides, as gonadotropins have not been identified in protostomes, and protostome GnRH may not necessary stimulate gonadotropin release in vertebrates.

In cephalopods, a major breakthrough in molluscan GnRH studies came about when the full coding sequence of a GnRH peptide in the common octopus, *Octopus vulgaris* was identified (Iwakoshi *et al.*, 2002). This substance was referred to as “oct-GnRH-like peptide”, and the amino acid sequence of the mature peptide was confirmed by techniques such as nanoflow electrospray ionization time-of-flight mass spectrometry (nano-ESI-TOF-MS) analysis. It was also shown that this peptide at concentrations of 10^{-5} M was capable of significantly inducing the release of luteinizing hormone from anterior pituitary cells in birds (quail); this provided the most convincing confirmation thus far that peptides which had been found in octopus and other molluscan species were likely to be true GnRH peptides.

References

- Chen, J. S., Sappington, T. W., & Raikhel, A. S. (1997). Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *J. Mol. Evol.*, 44, 440–451.
- Dalvin, S., Frost, P., Loeffen, P., et al. (2011). Characterisation of two vitellogenins in the salmon louse *Lepeophtheirus salmonis*: Molecular, functional and evolutionary analysis. *Dis. Aquat. Org.*, 94, 211–224.
- Dreon, M., Lavarias, S., Garin, C. F., Heras, H., & Pollero, R. J. (2002). Synthesis, distribution, and levels of an egg lipoprotein from the apple snail *Pomacea canaliculata* (Mollusca: gastropoda). *J. Exp. Zool.*, 292(3), 323–330.
- FAO, 2014. FAO Yearbook– Fishery and Aquaculture Statistics (2014). Available at: <http://ftp.fao.org/fi/stat/summary/default.htm>.
- Garcia, F., Cunningham, M. L., Garda, H., & Heras, H. (2008). Embryo lipoproteins and yolk lipovitellin consumption during embryogenesis in *Macrobrachium borelli* (Crustacea: palmonidae). *Comp. Biochem. Physiol.*, 151B, 317–322.
- Garin, C. L., Heras, H., & Pollero, R. J. (1996). Lipoproteins of the egg perivitelline fluid of *Pomacea canaliculata* snails (Mollusca: gastropoda). *J. Exp. Zool.*, 276, 307–314.
- Gaudron, S. M., Zanuttini, B., & Henry, J. (2005). Yolk protein in the cephalopod, *Sepia officinalis*: A strategy for structural characterization. *Invertebr. Reprod. Dev.*, 48, 129–135.
- Ghekiere, A., Verslycke, T., Smet, L. D., Beeumen, J. V., & Janssen, C. R. (2004). Purification and characterization of vitellin from the estuarine mysid *Neomysis integer* (Crustacea; Mysidacea). *Comp. Biochem. Physiol. A*, 138, 427–433.
- Goldberg, J. I., Garofalo, R., Price, C. J., & Chang, J. P. (1993). Presence and biological activity of a GnRH-like factor in the nervous system of *Helisoma trivolvis*. *J. Comp. Neurol.*, 336(4), 571–582.
- Iwakoshi, E., Takuwa-Kuroda, K., Fujisawa, Y., et al. (2002). Isolation and characterization of a GnRH-like peptide from *Octopus vulgaris*. *Biochem. Biophys. Res. Commun.*, 291(5), 1187–1193.
- Kang, B. J., Okutsu, T., Tsutsui, N., et al. (2014). Dynamics of vitellogenin and vitellogenesis-inhibiting hormone levels in adult and subadult whiteleg shrimp, *Litopenaeus vannamei*: Relation to molting and eyestalk ablation. *Biol. Reprod.*, 90(1), 12, 1–10.
- Khalaila, I., Peter-Katalinic, J., Tsang, C., et al. (2004). Structural characterization of the N-glycan moiety and site of glycosylation in vitellogenin from the decapod crustacean *Cherax quadricarinatus*. *Glycobiology*, 14, 767–774.
- Mathieu, M., Lenoir, F., & Robbins, I. (1988). A gonial mitosis-stimulating factor in cerebral ganglia and hemolymph of the marine mussel *Mytilus edulis* L. *Gen. Comp. Endocrinol.*, 72(2), 257–263.
- Mekuchi, M., Ohira, T., Kawazoe, I., et al. (2008). Characterization and expression of the putative ovarian lipoprotein receptor in the kuruma prawn, *Marsupenaeus japonicus*. *Zool. Sci.*, 25, 428–437.
- Okuno, A., Yang, W.-J., Jayasankar, V., et al. (2002). Deduced primary structure of vitellogenin in the giant freshwater prawn, *Macrobrachium rosenbergii*, and yolk processing during ovarian maturation. *J. Exp. Zool.*, 292, 417–429.
- Osada, M., & Matsumoto, T. (2016). Endocrine control of gametogenesis and spawning in bivalves. In S. Saleuddin, & S. Mukai (Eds.), *Physiology of Molluscs: A Collection of Selected Reviews* (pp. 179–220). Waretown, NJ: Apple Academic Press.
- Osada, M., & Treen, N. (2013). Molluscan GnRH associated with reproduction. *Gen. Comp. Endocrinol.*, 181, 254–258.
- Osada, M., Unuma, T., Mori, K., 1992. Purification and characterization of a yolk protein from the scallop ovary. *Nippon Suisan Gakkaishi* 58, 2283–2289. [In Japanese].
- Roth, Z., & Khalaila, I. (2013). Identification and characterization of the vitellogenin receptor in *Macrobrachium rosenbergii* and its expression during vitellogenesis. *Mol. Reprod. Develop.*, 79, 478–487.
- Subramoniam, T. (2011). Mechanisms and control of vitellogenesis in crustaceans. *Fish. Sci.*, 77, 1–21.
- Tiu, S. H. K., Benzie, J., & Chan, S.-M. (2008). From hepatopancreas to ovary: Molecular characterization of a shrimp vitellogenin receptor involved in the processing of vitellogenin. *Biol. Reprod.*, 79, 66–74.
- Tokishita, S.-I., Kato, Y., Kobayashi, T., et al. (2006). Organization and repression by juvenile hormone of a vitellogenin gene cluster in the crustacean, *Daphnia magna*. *Biochem. Biophys. Res. Commun.*, 345, 362–370.
- Tsutsui, N., Kawazoe, I., Ohira, T., et al. (2000). Molecular characterization of a cDNA encoding vitellogenin and its expression in the hepatopancreas and ovary during vitellogenesis in the kuruma prawn, *Penaeus japonicus*. *Zool. Sci.*, 17, 651–660.
- Wilder, M. N., Okumura, T., & Tsutsui, N. (2010). Reproductive mechanisms in Crustacea focusing on selected prawn species: Vitellogenin structure, processing and synthetic control. *Aqua-BioSci. Monogr.*, 3, 73–110.

SPERMATOGENESIS AND SPERMIOGENESIS

Spermatogenesis and Spermioogenesis, Fish

Rüdiger W Schulz, University of Utrecht, Utrecht, The Netherlands

François Chauvigné, Institute of Agrifood Research and Technology (IRTA)-Institute of Marine Sciences, Barcelona, Spain; and Spanish National Research Council (CSIC), Barcelona, Spain

© 2018 Elsevier Inc. All rights reserved.

Introduction

The vertebrate testis produces spermatozoa and signalling molecules, such as sex steroid hormones and growth factors. These two functions are interconnected, since efficient sperm production depends, amongst others, on these locally produced signalling molecules which, however, also can have functions outside the testis. The process of sperm production, spermatogenesis, takes place in the spermatogenic tubules of the testes. The tubules contain two cell types, germ cells (in different stages of development) and Sertoli cells, a somatic cell type that is of crucial importance for facilitating the survival and development of germ cells during spermatogenesis. Germ and Sertoli cells together constitute the germinal epithelium. The wall of the spermatogenic tubules is formed by extracellular matrix material, connective tissue elements, and contains on the outside a layer of smooth muscle cells known as peritubular myoid cells. Between the spermatogenic tubules, we find the interstitial tissue with blood vessels, connective tissue and nervous elements, immune cells and, last but not least, the Leydig cells. These cells are the main source of the male sex steroids, androgens like testosterone, but also produce non-steroidal factors. Therefore, Leydig cells and their products are of great relevance for male fertility.

The endocrine system in vertebrates has evolved as a master control system over reproduction. Regulated by the brain, the pituitary produces two gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) that jointly control pubertal maturation and adult functioning of the testis. Sertoli cells express the receptor for FSH (FSHR), which is of course important for regulating Sertoli cell functions. LH, on the other hand, controls Leydig cell activities via the LH receptor (LHCGR), such as the production of androgens. The latter regulate testis functions via the androgen receptor (AR), which is expressed by Sertoli, peritubular myoid and Leydig cells, cell types expressing many genes critical for spermatogenesis. It appears that FSH and androgens are the two most important hormones regulating Sertoli cell activities, such as providing structural and metabolic support and regulatory input for the developing germ cells. Receptors for FSH and, in many species, receptors for androgens are not expressed by germ cells. Therefore, the endocrine control of spermatogenesis first targets the FSHR- and AR-expressing somatic cells, which then relay the endocrine input in a paracrine manner or by direct cell-cell contacts to the germ cells, which then respond by adapting their proliferation and differentiation behaviour. During spermioogenesis in fish, however, steroid receptor expression in spermatids may play a role (see [Section Regulation of Spermioogenesis by Steroids and Sertoli Cells Factors](#)).

Despite an overall similarity among different vertebrate groups, evolution has taken a somewhat different path in fish with respect to the biological activities of the gonadotropic hormones. The main point is that in fish, FSH has gained a strong steroidogenic potency because Leydig cells not only express the receptor for LH, as in all higher vertebrates, but also the receptor for FSH. Therefore, FSH alone can fulfil the functions, for which in mammals FSH and LH are required. It is therefore not surprising that genetic experiments with zebrafish showed that loss of LH, or of the LH receptor, did not compromise male fertility ([Chu et al., 2015](#); [Zhang et al., 2015](#)). Also remarkable in fish is the observation that the receptor for LH is expressed by germ cells toward the end of spermatogenesis (see [Section Regulation of Spermioogenesis by Gonadotropins](#)).

The Main Steps of Spermatogenesis

A population of spermatogonial stem cells (SSCs) forms the basis of the germ cell population. When dividing, an SSC can undergo a self-renewal division resulting in two single SSCs, or it can undergo a differentiating division to produce two spermatogonia as the first step of the development toward spermatozoa. After differentiating divisions, germ cells do not separate completely when dividing but remain connected by a cytoplasmic bridge, forming first a pair and then an elongating chain of interconnected cells. This is a typical feature of differentiating germ cell divisions in all animals. The balance between self-renewal and differentiating divisions is tightly regulated: Too much self-renewal limits the number of differentiating cells, reduces sperm production and might lead to germ line stem cell tumours, while too much differentiation exhausts the SSC reservoir, eventually leading to infertility.

When SSCs undergo a differentiating division and form a pair of spermatogonia connected by a cytoplasmic bridge, they have entered the process of spermatogenesis, which consists of three main phases: the mitotic, the meiotic and the spermioogenic

phase. During the mitotic phase of spermatogenesis, germ cells proceed through a series of consecutive mitotic cell cycles as an interconnected cell clone of first 2, then 4, 8, 16, etc. differentiating spermatogonia derived from a single SSC. Via the intercellular bridges, the members of a given clone synchronize their development. The number of mitotic cell cycles a germ cell clone undergoes is genetically determined, and therefore fixed in a given species but can differ in different species. It ranges between 6 and 14 (often 8) in different fish species (Schulz and Nóbrega, 2011). After completing the mitotic expansion, germ cells proceed sequentially through two highly conserved processes. The first one is known as meiosis and involves two special cell cycles. First, in primary spermatocytes, the maternal and paternal genetic information is recombined (cross-overs) in preparation of the 1st meiotic division. The secondary spermatocytes emerging from this division quickly divide again in the second meiotic division that is not preceded by DNA duplication. The spermatids emerging from the second meiotic division therefore possess a haploid set of (recombined) genes. Germ cell development is completed during spermiogenesis (see **Section Spermiogenesis and Its Regulation**). During all phases, the germ cells are enveloped by Sertoli cells and depend on their support for survival and progression through the different steps constituting spermatogenesis.

The Sertoli - Germ Cell Relation in Cystic Spermatogenesis

The close relationship between Sertoli and germ cells is seen in all vertebrates, but there is an important difference in the Sertoli-germ cell relation between fish and amphibians (anamniote vertebrates) on the one hand and reptiles, birds and mammals (amniote vertebrates) on the other hand.

In amniote vertebrates, Sertoli cells proliferate until puberty. Then tight junctions are formed between neighboring Sertoli cells and their proliferation stops. The tight junctions create in the spermatogenic tubule a separation into a basal compartment, where we find the spermatogonia, and an adluminal compartment, in which meiosis and spermiogenesis take place (**Fig. 1(A)**). This also secludes meiotic and spermiogenic cells from the immune system, which is not familiar with the surface structure of these later developmental stages. Hence, during amniote spermatogenesis, germ cells move from the basal to the adluminal compartment along the lateral surface of the Sertoli cells. In this way, Sertoli cells are arranged as a single layer of cells residing on the tubular wall, where they constitute a permanently present, no longer proliferating cell layer interconnected by tight junctions. Germ cells form a number of layers of progressively differentiated germ cell types that move from the basal to the adluminal compartment during development. Since consecutive waves of spermatogenesis produce germ cells that pass along the Sertoli cells from the basal to the adluminal compartment, any given Sertoli cell is in contact with germ cells in different stages of development (**Fig. 1(A)**), implying that they belong to different clones, most likely derived from different SSCs.

In the fish testis, spermatogenesis of course also starts with a single SSC, which is enveloped by 1 or 2 Sertoli cells. This combination of an SSC and the associated 1 or 2 Sertoli cells is the basic functional unit of the germinal epithelium in anamniote vertebrates and is referred to as a spermatogenic cyst. When the SSC differentiates and forms a clone of 2, 4, 8, etc. spermatogonia, the growing germ cell number in the cyst requires that also the cyst-forming Sertoli cells proliferate, thereby providing the increasing space required by the growing germ cell clone (**Fig. 1(B)**). This highlights two important differences compared to the Sertoli germ cell relation in amniote vertebrates: First, Sertoli cell proliferation needs to accompany developing spermatogenic cysts, rendering Sertoli cell proliferation a characteristic of the postpubertal, spermatogenic testis. Secondly, a given Sertoli cell usually is in contact with only one or two germ cell clones at a time (**Fig. 1(B)**) and accompanies these clones through the different stages of development until the cysts opens to release of spermatozoa. Hence, while an amniote Sertoli cell is rather “busy” with taking care of several different stages of developing germ cells and therefore shows regional differentiation and polarity, an anamniote Sertoli cell fulfils the same tasks sequentially, usually taking care of only one or two stages of germ cell development at a given time (Leal *et al.*, 2009; Schulz *et al.*, 2010).

Stem Cell Niche in the Fish Testis

Prolonged male fertility depends on maintaining a population of SSC. Tissue-specific stem cells need a distinctive microenvironment to retain their stemness, which is referred to as the stem cell niche. In the testis, somatic cells provide and determine the niche environment. Sertoli cells are important contributors to the niche environment but other somatic cells, such as the peritubular myoid cells, are relevant as well for determining the SSC self-renewal/differentiation balance. Moreover, signals arriving via the blood, can reach SSCs and other spermatogonia and contribute to the niche environment in the testis. In mice, SSCs are motile cells and can leave the niche, thereby becoming exposed to conditions that do not allow maintaining their stemness, i.e., the cells will differentiate and hence contribute to sperm production. The niche place they vacated by moving out, on the other hand, becomes available for a newly formed SSC (Lord and Oatley, 2017).

It is not known if SSCs in fishes and amphibians are motile cells. However, being enveloped by Sertoli cells in the cystic type of spermatogenesis, an SSC is likely to become apoptotic when moving away from the Sertoli cell support, unless this SSC – when leaving its supporting Sertoli cell – can recruit a vacant or idle Sertoli cell. Interestingly, one of the earliest signs of pubertal or seasonal testis growth in salmonid fish is an increased proliferation activity of Sertoli cells, leading to an accumulation of vacant Sertoli cells (**Fig. 2**). Assuming that these vacant Sertoli cells produce signals tipping the balance in favour of self-renewal

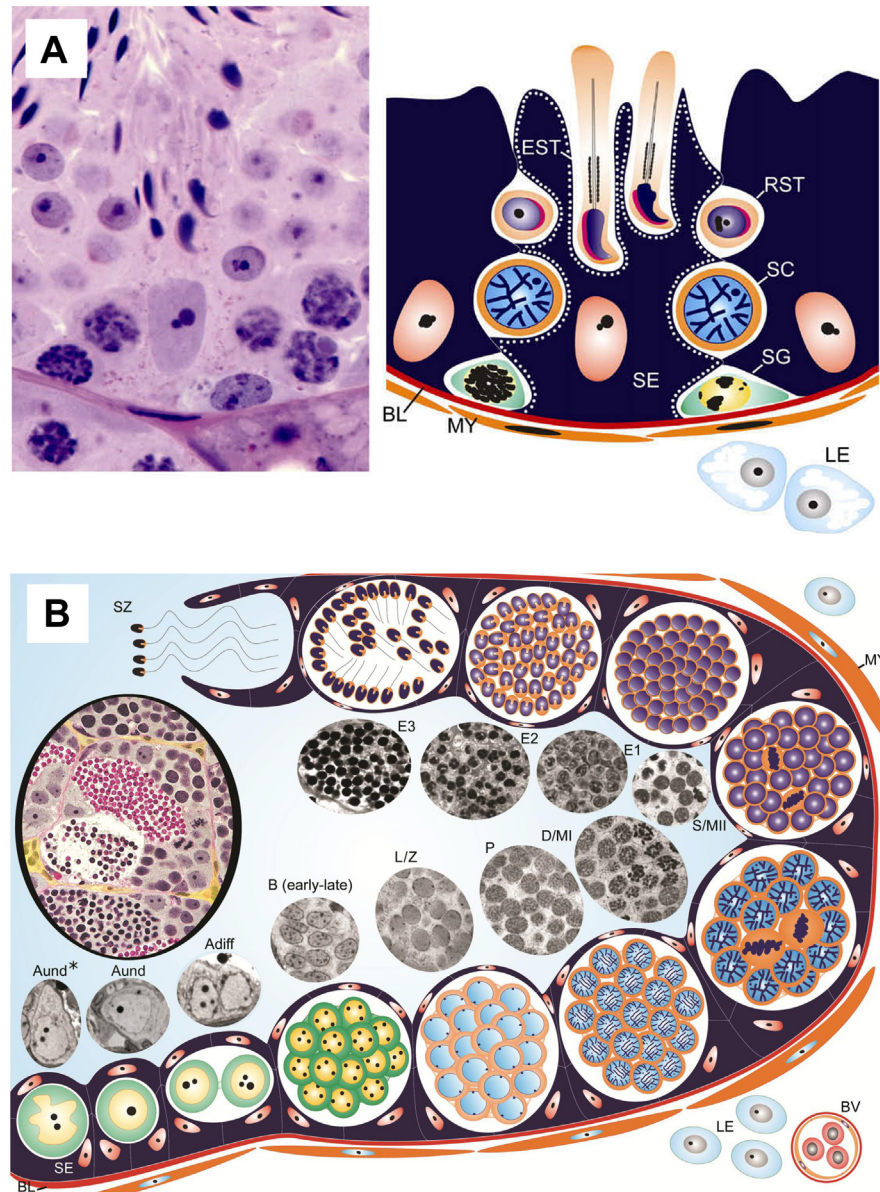


Fig. 1 Comparison of mammalian (A, mouse) and fish (B, zebrafish) testis. Segments of spermatogenic tubules are shown to illustrate the differences in Sertoli/germ cell relation between non-cystic (A) and cystic (B) spermatogenesis. The germinal epithelium contains Sertoli (SE) and germ cells, delineated by a basal lamina (BL) and peritubular myoid cells (MY). Also interstitial Leydig cells (LE) and blood vessels (BV) are shown. (A) spermatogonia (SG); spermatocyte (SC); round spermatid (RST); and elongated spermatid (EST). (B) Type A undifferentiated* spermatogonia (A_{und*}) (stem cell?); type A undifferentiated spermatogonia (A_{und}); type A differentiating spermatogonia (A_{diff}); type B spermatogonia (B (early-late)); leptotenic/zygotenic primary spermatocytes (L/Z); pachytenic primary spermatocytes (P); diplotenic spermatocytes/metaphase I (D/MI); secondary spermatocytes/metaphase II (S/MII); early (E1), intermediate (E2) and final spermatids (E3); spermatozoa (SZ). Reproduced from Schulz, R.W., de França, L.R., Lareyre, J.J., *et al.*, 2010. Spermatogenesis in fish. *General and Comparative Endocrinology* 165, 390–411.

divisions, newly formed SSCs would then associate with vacant Sertoli cells to form new spermatogenic cysts. This type of Sertoli cell proliferation seems the prerequisite to increase the SSC niche space in the testis, such that the testis becomes populated by the starting units of cystic spermatogenesis. In cystic spermatogenesis, each wave of spermatogenesis requires the production of new cysts and the associated Sertoli cell proliferation. The development of existing cysts also involves Sertoli cell proliferation. Therefore, a Sertoli cell progenitor population may be present in the testis of anamniote vertebrates but still needs to be demonstrated (França *et al.*, 2015).

In many species of fish (e.g., salmonid and cyprinid fish), these starting units of spermatogenesis (i.e., 1 or 2 Sertoli cells enveloping a single type A spermatogonium) are randomly distributed in the germinal epithelium of the testis, which is referred to as the unrestricted spermatogonial distribution. In fish species showing the restricted spermatogonial distribution, on the

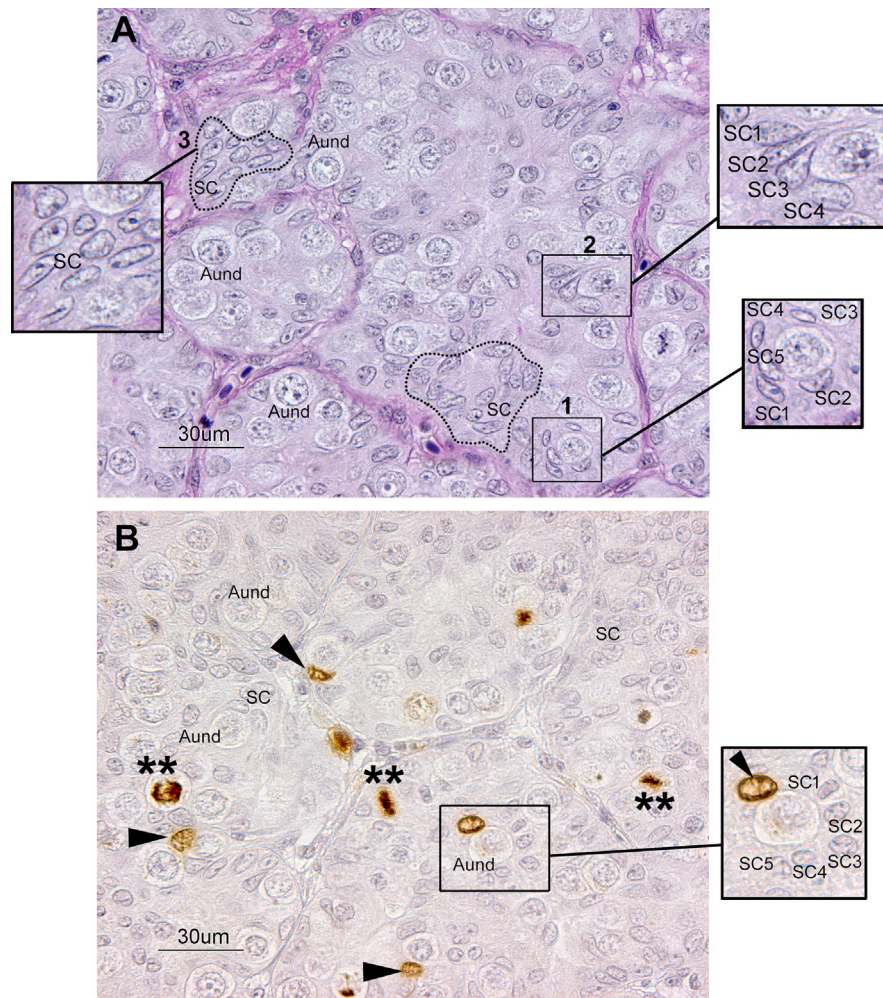


Fig. 2 Sertoli cell groups in Atlantic salmon testis at the beginning of the seasonal testis growth phase. (A) Squares (1,2) show several Sertoli cells, indicated by numbered Sertoli cell nuclei that are grouped around a single type A undifferentiated spermatogonium (A_{und}). Areas delineated by dashed lines (e.g., 3) show Sertoli cells apparently not (yet) in contact with germ cells (possibly containing Sertoli cell progenitor cells). Staining: Hematoxylin and eosin. (B) Immunocytochemical detection of a proliferation marker (phosphorylated histone H3 (pH3)) on another section of the same testis shown in A. pH3-positive Sertoli cell nuclei (arrowheads) indicate proliferation of Sertoli cells at the beginning of testis growth. The square highlights several Sertoli cells (SC1-SC5) that are already associated with a single spermatogonium type A_{und} . Sertoli cells continue proliferating, potentially providing niche space for new, single type A_{und} spermatogonia (cyst formation). Double asterisks indicate pH3-positive single type A_{und} spermatogonia. Reproduced from França, L.R., Nóbrega, R.H., Morais, R.D.V.S., de Castro Assis, L.H., Schulz, R.W., 2015. Sertoli cell structure and function in anamniote vertebrates. In: Griswold, M.D. (Ed.), *Sertoli Cell Biology*. Amsterdam: Elsevier, pp. 385–408.

other hand, the intratesticular location of stem cell cysts is non-random and becomes predictable. Stem cells are located in the periphery of the testis, close to the organ wall (tunica albuginea). Newly formed cysts that started differentiation are displaced along the spermatogenic tubule toward the central collecting duct. Hence, in these species, the developing cysts is moving centrally while progressing through spermatogenesis and then opens to release sperm into the collecting duct. This spatial arrangement is found in evolved taxonomic groups, such as silversides and toothcarps. Also an intermediate type exists, in which undifferentiated spermatogonia show a preferential but not exclusive localization close to the testicular periphery. This is found in some perch- or cod-like fish or in flatfish species.

Regulation of the Mitotic and Meiotic Phases

In this section, we will discuss aspects of the endocrine and paracrine regulation of the mitotic and meiotic phases. The overall dependency of spermatogenesis on regulatory input by the pituitary has been demonstrated already 30 years ago by surgical experiments removing the pituitary followed by gonadotropin replacement studies (Khan *et al.*, 1987). More recently, genetic experiments in zebrafish (Chu *et al.*, 2015; Zhang *et al.*, 2015) involving loss-of-function of the gonadotropins, FSH and LH, or of their receptors (FSHR and LHCGR), confirmed that spermatogenesis is not possible without gonadotropin signalling.

Incubating eel testis in a primary tissue culture system demonstrated that androgen treatment alone induced complete spermatogenesis and sperm production (Miura *et al.*, 1991). On the other hand, FSH was able to stimulate the differentiating proliferation of spermatogonia and their entry into meiosis in a steroid-independent manner in studies using zebrafish testis in a similar primary tissue culture system (Crespo *et al.*, 2016). This data suggests that FSH (or LH) exerts stimulatory effects on spermatogenesis involving sex steroid production but that FSH has in addition direct stimulatory effects not depending on a functioning steroidogenic system, probably mediated by the FSH receptor expressing somatic cells, which are in fish Sertoli and Leydig cells.

Steroid-regulated effects in fish involve oestrogen-mediated stimulation of SSC self-renewal, androgen-mediated effects on testicular gene expression, the latter identifying the importance of a growth factor belonging to the TGF β family, anti-Müllerian hormone (AMH) that blocks SSC differentiation and needs to be down-regulated (by androgen or FSH, depending on the fish species) to facilitate the initiation of spermatogenesis (Schulz *et al.*, 2010). Finally, also progesterone-mediated effects were identified in a number of species, stimulating progression through the later spermatogonial generations and entry into meiosis. Hence, all three types of sex steroids have specific effects on spermatogenesis in fish. Androgens have the widest range of effects since they stimulated the complete development from SSC differentiation to sperm production. On the other hand, recent genetic experiments in zebrafish show that spermatogenesis still proceeds normally when oestrogen production was blocked (Dranow *et al.*, 2016), or when nuclear progesterone receptors were not functional (Zhu *et al.*, 2015), suggesting that progesterone and oestrogen-mediated regulation is not of crucial relevance, at least for zebrafish spermatogenesis.

Fsh-induced, steroid-independent effects on testicular gene expression were examined in rainbow trout (Sambrovi *et al.*, 2013) and zebrafish (Crespo *et al.*, 2016). These studies identified a few hundred genes differentially expressed in response to FSH. Follow-up work on some of the most interesting candidate genes in zebrafish has shown that the stimulatory effects of FSH were mediated by down-regulating inhibitory factors (e.g., AMH) and by up-regulating stimulatory factors. The latter category contained growth factors produced by Leydig cells (insulin-like peptide 3, INSL3) or by Sertoli cells (insulin-like growth factor 3, IGF3) that then mediate the FSH effects to alter germ cell development. Future work will probably reveal the roles of more factors that have already been identified as regulated by reproductive hormones but where specific functions have not been characterized yet; many of these signalling systems are known from other physiological functions but also operate in the testis, such as the hedgehog and Wnt signalling systems best known for their roles in embryonic development and cancer.

What events in spermatogenesis are regulated by the different signalling systems mentioned so far? We have seen earlier that the number of mitotic cell cycles is genetically determined; also meiosis and spermiogenesis are conserved, genetically fixed processes. Therefore, modulating the quantity of sperm production seems limited to regulating how many starting units, i.e., cysts consisting of a single spermatogonium enveloped by one or two Sertoli cells, enter the process of spermatogenesis. Entering this differentiation process in fact reflects that a decision has been taken by such a starting unit, namely the decision regarding the question if the upcoming cell division should be a self-renewal, or a differentiating division. Before that, one other decision needs to be taken: should the starting unit show any proliferative activity at all or not, i.e., remain quiescent or become recruited into cell cycling? This is a question of the timing of proliferation activity.

Up until puberty, testis tissue in fish grows slowly in a so-called allometric manner (i.e., the growth speed of the testis is similar to the growth speed of the rest of the body). However, with puberty, a superallometric growth phase starts, reflecting the start of spermatogenic activity. This requires a change in the regulatory input, so that the slow self-renewal of SSCs in immature males is first changed to accelerated self-renewal and then to differentiation. In many seasonally reproducing species, testis tissue goes through a rapid growth phase, reaches the peak weight and then shows a certain weight loss toward the spawning season, since new cyst formation stops while existing cysts continue to develop through spermiogenesis to sperm, which is associated with a reduction in cellular mass and an overall weight loss (see **Section Spermiogenesis and Its Regulation**). After the spawning season, residual sperm is resorbed and testis weight decreases back to low starting levels. These repeated growth/shrinkage cycles in consecutive reproductive seasons require that first new spermatogenic cysts are formed, which then start differentiating while more cysts keep being formed. At a certain point in time, when maximum testis size has been reached, the production of new cysts ceases, and all cysts mature such that the fully mature testis contains only sperm and a few quiescent SSCs, representing the stem cell reserve for the next cycle. With respect to the production of new spermatogenic cysts, the conclusion is that their production is initiated at the beginning of puberty or a subsequent reproductive cycle and continues until the signals promoting cyst production cease. From then on, the existing cysts keep differentiating while no new ones are added, so that after some time, all cysts have spermiated and the testis is filled with sperm. Based on the present knowledge, we assume that FSH plays a decisive role in this regard, both with respect to the start as well as to the stop of cyst production. Environmental conditions (e.g., water temperature, photoperiod, food availability, behaviour of conspecifics etc., the significance of a specific set of parameter depending on the reproductive biology of the species in question) regulate changes in FSH release via the brain-pituitary axis, thereby timing the FSH release according to maximise the reproductive success of a given species.

The final point to consider here is apoptosis as factor modulating the germ cell production efficiency. Part of the germ cells undergoing normal spermatogenesis are lost to apoptosis (Shaha *et al.*, 2010). The apoptotic loss of 30%–70% of germ cells is the physiological range found in different species. It is important to realize that this loss is not reflecting toxicological insults or pathological conditions but simply the fact that spermatogenesis is a process producing very many cells in a short time (e.g., in humans ~1500 sperm cells per heartbeat). Under these conditions, it is inevitable that mistakes occur in some of the germ cells, often during DNA replication or DNA repair processes. If these errors cannot be repaired in time, apoptosis is activated in the defective cell at the next cell cycle check point. Also for this aspect, FSH and androgens are important to limit the apoptotic loss

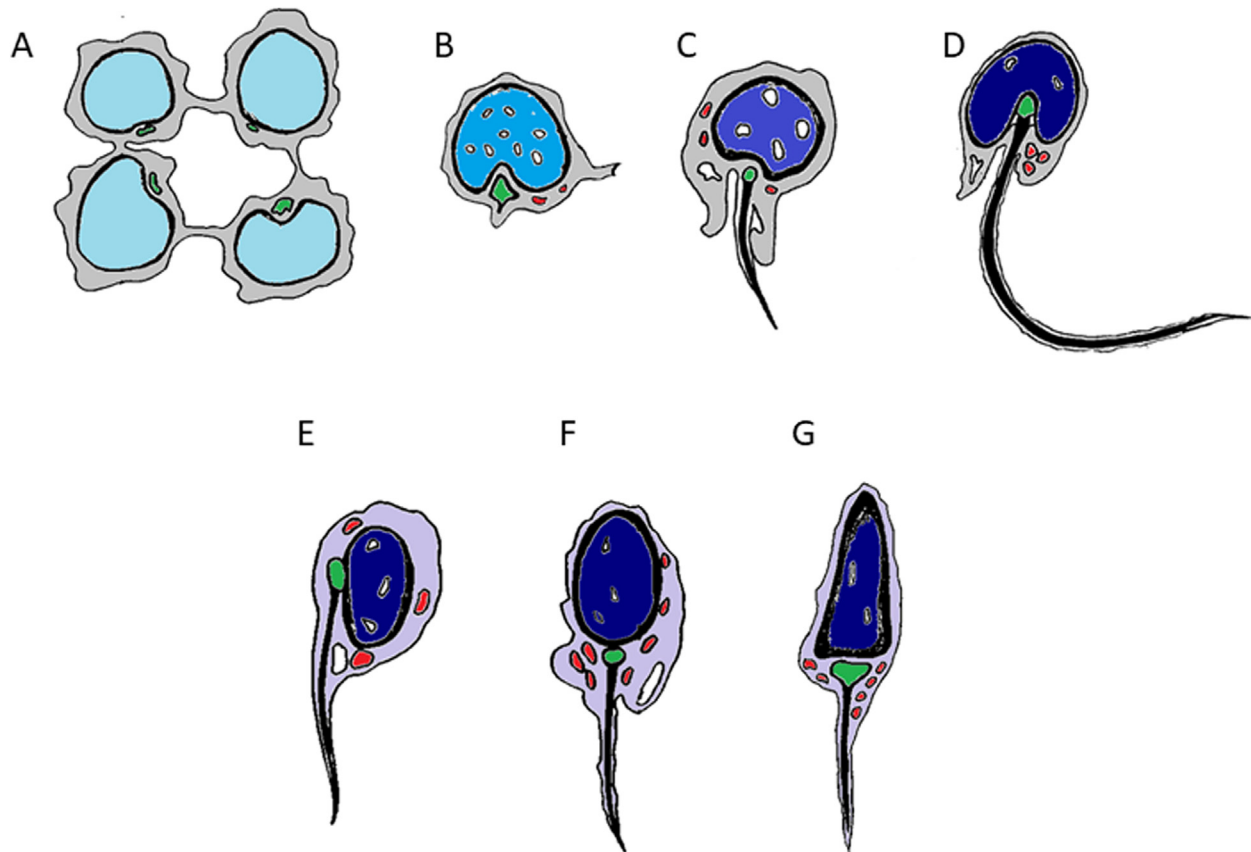


Fig. 3 A–D: Schematic drawing of steps of spermatid type I differentiation occurring within seminiferous tubule. (A) Early spermatid shows a spherical nucleus containing diffuse homogeneous chromatin (blue) and a forming centriolar complex medial to nucleus (green). Cytoplasmic bridges are linking two spermatids together. (B) Round spermatid is initiating chromatin condensation. The primordial nuclear fossa arise inside the nuclei where the forming centriolar complex is locating. The cytoplasm (grey), containing mitochondria (red) is reduced around the nucleus. (C) Intermediate spermatid shows advanced chromatin condensation and the perpendicular formation of the flagellum from the centriole. The cytoplasm is forming the middle piece along the developing flagellum and is reducing with the appearance of vacuoles (white). (D) Late spermatid shows highly condensed chromatin within the nucleus, a longer flagellum and a very reduced cytoplasm. Mitochondria moved to the mid piece, where they eventually fuse. E–G: Alternative types of spermatids. (E) In type II spermiogenesis, the flagellum develops parallel to the nucleus without nuclear rotation. (F) In type III spermiogenesis, the flagellum forms centrally without nuclear rotation. (G) In some teleost fish species, the nucleus is elongated at the final step of spermiogenesis.

of germ cells (O'Shaughnessy, 2014), by regulating Sertoli cell functions such that they can optimally support germ cells, thereby reducing apoptotic loss to the inevitable minimum.

Spermiogenesis and Its Regulation

Three types of spermiogenesis were described in fish and this classification is based on the orientation of the flagellum with respect to the nucleus (Wootton and Smith, 2014). Also, based on the progressive condensation of the nucleus, three stages of spermatid development were described (Fig. 3; Kunz-Ramsay, 2013). The process of spermiogenesis seems to be relatively short in teleost fish where it takes 2–7 days. However, the timing has been studied in not even a handful of species so far and more comparative data is required.

When spermatids have developed into fully differentiated spermatozoa, the cytoplasmic bridges that connected germ cells during their development are broken and the germ cells are individualized. Spermatozoa are released from the spermatogenic cyst into the lumen of the spermatogenic tubule by opening the junctional complex that connect neighboring Sertoli cells forming the wall of a spermatogenic cyst. This process is known as spermiation (O'Donnell *et al.*, 2011). Notably, in some teleost species (e.g., in certain flatfish species), spermatogenesis is semicystic, i.e., round spermatids from different germ cell clones are released into the lumen of spermatogenic tubules, where they complete spermiogenesis (Uribe *et al.*, 2014). In yet other species (e.g., in some live bearing tooth-carps), spermatozoa are not released into the lumen directly but Sertoli cells form a packaging capsule around spermatozoa, called spermatophores. For most teleost fish, spermiogenesis does not involve spermatid elongation, although it is common in the semicystic spermatogenesis (Fig. 3(G); Schulz *et al.*, 2010).

Molecular Regulation of Gene Expression in Spermatids

The molecular regulation of spermiogenesis in teleost fish is largely unknown. However, it appears that the nuclear condensation in spermatids involves acetylation and then displacement of histones from the nucleus and their replacement by transition proteins and protamines, similar to what has been described in mammals. In a context of extreme chromatin condensation, little transcription occurs in spermatids. Indeed, mRNAs transcribed earlier are stored often bound to RNA-binding proteins that also serve to distribute the required mRNAs via the intercellular cytoplasmic bridges. Nonetheless, the differentiation of round spermatids into spermatozoa requires the transcriptional activation of some post-meiotic genes, among them the genes encoding spermatid maturation factors and spermatozoa-specific proteins involved in flagellum formation. In mammals, this occurs in spermatids and depends on an intracellular cAMP rise and the expression of the cAMP responsive element modulator (CREM), that binds cAMP responsive *cis* elements in the promoter of target genes. However, the primary signals to activate this pathway in teleost germ cells is yet unknown.

Regulation of Spermiogenesis by Gonadotropins

The cellular differentiation steps occurring during spermiogenesis are regulated primarily by gonadotropins and/or sex steroids in mammals and fish (Uribe *et al.*, 2014). Although FSH does not appear to be necessary for spermatozoa formation itself, as neither disruption of FSH nor of the FSHR compromised fertility in male zebrafish, FSH is potentially an indirect signal to induce cAMP increases and the association of CREM with its activator (ACT) through a paracrine circuit operating between germ and Sertoli cells in mice. However, it is also possible that LH compensates the experimental lack of FSH in zebrafish, since in most teleost fish, high concentrations of LH can activate the FSHR. Indeed, loss of both, the FSHR and the LHCGR resulted in infertile zebrafish males, while loss of LH alone had no effect on fertility. In mammals, on the other hand, LH is crucial for fertility since in LHCGR (or LH) knockout mice, serum T levels are reduced and spermatogenesis is arrested at the round spermatid stage, leading to the total absence of spermatozoa. Also, LH-mediated T release by Leydig cells plays a crucial role in spermiogenesis since this androgen can fully restore spermiogenesis in LHCGR-null mice. However, this is not the case in humans, where T alone is insufficient to induce progression of spermatids through spermiogenesis *in vitro*. It is possible that the LH/LHCGR pathway plays an additional role in spermiogenesis independent of androgen production. This would require expression of the LHCGR by germ cells. Interestingly, in fish the LHCGR was found either in spermatocytes or round spermatids (Chauvigné *et al.*, 2014; Fig. 4). In Senegalese sole, treatment with LH promoted the differentiation of spermatids into spermatozoa *in vitro* after 3 days of incubation. LH also initiated the transcription of genes potentially involved in flagellum formation and protein ubiquitination through a cAMP/PKA-dependent signalling pathway. This corroborates the fact that in testis many genes belonging to the germ cell line are regulated, either indirectly or directly, by LH in trout or sole. However, whether LH directly activates gene transcription through a CREM-like factor in teleost spermatids or via one of the several other mediators found in different studies remains to be studied (Fig. 4).

Regulation of Spermiogenesis by Steroids and Sertoli Cells Factors

In Senegalese sole, androgens had no effect on the differentiation of spermatids *in vitro*. However, a role of these steroids during earlier stages of spermatid formation/differentiation cannot be ruled out because in this species, spermatocytes and spermatids

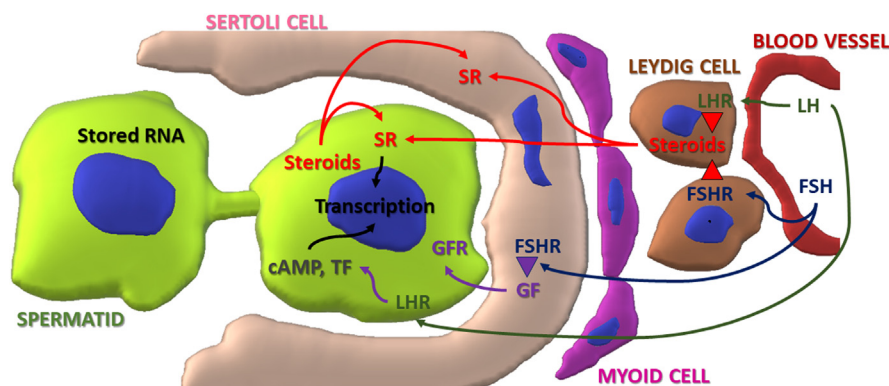


Fig. 4 Schematic drawing of potential endocrine regulation of spermiogenesis in teleost fish. Spermatids, enclosed in Sertoli cells, are subjected to different hormonal signals. Besides the store RNA, genes necessary for flagellum formation are transcribed *de novo* under the regulation of various transcription factors (TF), potentially dependent on cAMP pathway. LH from the circulation activates directly LHR in spermatid to drive spermiogenesis in a cAMP-dependent manner. Also, it activates steroidogenesis in Leydig cells. Steroids like androgens or progestins could activate their receptors (SR) in spermatid. Spermatid potentially can synthesize steroids also, which would create a regulatory loop. Finally, FSH activates its receptor in Sertoli that secrete growth factors (GF), that could activate their receptors (GFR) in spermatids to regulate spermiogenesis.

express androgen receptors (Fig. 4). In Sole and others species, the progesterone receptor was found in late germ cells and thus, progestins may have effects on spermiogenesis additionally to their well-known activation of spermiation. Also, in different fish species steroid-producing enzymes are present in germ cells which suggest that an autocrine regulation of spermatid differentiation could occur by steroids (Fig. 4). Finally, other factors may be necessary to support spermiogenesis, such as paracrine factors secreted by Sertoli cells such as insulin-like growth factors or trypsinogens (Fig. 4; Schulz *et al.*, 2010). The specific role of these endocrine mediators remains to be further investigated.

References

- Chauvigné, F., Zapater, C., Gasol, J. M., & Cerdà, J. (2014). Germ-line activation of the luteinizing hormone receptor directly drives spermiogenesis in a nonmammalian vertebrate. *Proceedings of the National Academy of Science of the United States of America*, 111, 1427–1432.
- Chu, L., Li, J., Liu, Y., & Cheng, C. H. (2015). Gonadotropin signaling in zebrafish ovary and testis development: Insights from gene knockout study. *Molecular Endocrinology*, 29, 1743–1758.
- Crespo, D., Assis, L. H., Furmanek, T., Bogerd, J., & Schulz, R. W. (2016). Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. *Molecular and Cellular Endocrinology*, 437, 237–251.
- Dranow, D. B., Hu, K., Bird, A. M., *et al.* (2016). Bmp15 is an oocyte-produced signal required for maintenance of the adult female sexual phenotype in zebrafish. *PLoS Genetics*, 12, e1006323.
- França, L. R., Nóbrega, R. H., Morais, R. D. V. S., de Castro Assis, L. H., & Schulz, R. W. (2015). Sertoli cell structure and function in anamniote vertebrates. In M. D. Griswold (Ed.), *Sertoli cell biology* (pp. 385–408). Amsterdam: Elsevier.
- Khan, I. A., Lopez, E., & Leloup-Hatey, J. (1987). Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotropin in intact and hypophysectomized immature european eel (*Anguilla anguilla* L.). *General and Comparative Endocrinology*, 68, 91–103.
- Kunz-Ramsay, Y. (2013). *Developmental Biology of Teleost Fishes* (pp. 131–147). Dordrecht: Springer Science & Business Media.
- Leal, M. C., Cardoso, E. R., Nóbrega, R. H., *et al.* (2009). Histological and stereological evaluation of zebrafish (*Danio rerio*) spermatogenesis with an emphasis on spermatogonial generations. *Biology of Reproduction*, 81, 177–187.
- Lord, T., & Oatley, J. M. (2017). A revised A(single) model to explain stem cell dynamics in the mouse male germline. *Reproduction*, 154, R55–R64.
- Miura, T., Yamauchi, K., Takahashi, H., & Nagahama, Y. (1991). Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*). *Proceedings of the National Academy of Science of the United States of America*, 88, 5774–5778.
- O'Donnell, L., Nicholls, P. K., O'Bryan, M. K., McLachlan, R. I., & Stanton, P. G. (2011). Spermiation: The process of sperm release. *Spermatogenesis*, 1, 14–35.
- O'Shaughnessy, P. J. (2014). Hormonal control of germ cell development and spermatogenesis. *Seminars in Cell and Developmental Biology*, 29, 55–65.
- Sambroni, E., Lareyre, J. J., & LeGac, F. (2013). Fsh controls gene expression in fish both independently of and through steroid mediation. *PLoS ONE*, 23, e76684.
- Schulz, R. W., de França, L. R., Lareyre, J. J., *et al.* (2010). Spermatogenesis in fish. *General and Comparative Endocrinology*, 165, 390–411.
- Schulz, R. W., & Nóbrega, R. H. (2011). Anatomy and histology of fish testis. In A. P. Farrell (Ed.), *Encyclopedia of Fish Physiology: From Genome to Environment* (vol. 1, pp. 616–626). San Diego, CA: Academic Press.
- Shaha, C., Tripathi, R., & Mishra, D. P. (2010). Male germ cell apoptosis: Regulation and biology. *Philosophical Transactions of the Royal Society London B Biological Sciences*, 365, 1501–1515.
- Uribe, M. C., Grier, H. J., & Mejia-Roa, V. (2014). Comparative testicular structure and spermatogenesis in bony fishes. *Spermatogenesis*, 4, e983400.
- Wootton, R. J., & Smith, C. (2014). *Reproductive Biology of Teleost Fishes* (pp. 74–100). Chichester: Wiley-Blackwell.
- Zhang, Z., Lau, S. W., Zhang, L., & Ge, W. (2015). Disruption of zebrafish follicle-stimulating hormone receptor (fshr) but not luteinizing hormone receptor (lhcr) gene by TALEN leads to failed follicle activation in females followed by sexual reversal to males. *Endocrinology*, 156, 3747–3762.
- Zhu, Y., Liu, D., Shaner, Z. C., *et al.* (2015). Nuclear progestin receptor (pgr) knockouts in zebrafish demonstrate role for pgr in ovulation but not in rapid non-genomic steroid mediated meiosis resumption. *Frontiers in Endocrinology (Lausanne)*, 6, 37.

Spermatogenesis and Spermiogenesis in Elasmobranchs, a Short Overview

Pascal Sourdaïne, Aude Gautier, and Laura Gribouval, Normandy University, UNICAEN, Caen, France; Sorbonne University, MNHN, UPMC Univ Paris 06, UA, CNRS, IRD, Caen, France; and Biology of Aquatic Organisms and Ecosystems Joint Unit, Caen, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

Elasmobranchs, comprising skates, rays and sharks, belong to chondrichthyans which include also the chimaeras (Holocephalans). Chondrichthyans are the sister group of bony vertebrates with a time of divergence estimated between 462 and 421 million years ago (MYA) and recent knowledge on fossilized chondrichthyans suggest that the last common ancestor of elasmobranchs and holocephalans lived about 370–380 MYA. Despite the phylogenetic interest of chondrichthyans and their high number of species (around 1200), they have been the subject of too few publications, notably concerning their reproduction. However, spermatogenesis in elasmobranchs is at the origin of old observations, particularly at the middle of the 19th century with the advent of microscopy, because of the advantages associated to the anatomical organization of their testis. In 1841, Lallemand observed that spermatozoa formation occurs into “spherical ampullae”, what we name presently spermatocysts, in rays and, in 1875, Semper gave a first description of the different stages of spermatogenesis in dogfish. [La Valette St George \(1878\)](#) was the first to describe the close association between germ cells and Sertoli cells to give, what he called “spermatogennum”, currently called “spermatoblast”. A century later, the stages of spermatogenesis were more precisely classified and quantified by works of [Mellinger \(1965\)](#), [Stanley \(1966\)](#) or [Holstein \(1969\)](#) in the lesser spotted dogfish *Scyliorhinus canicula* (Carcharhiniformes) and the spiny dogfish *Squalus acanthias* (Squaliformes). For all elasmobranchs, the unit structure of the testis is the spheroidal spermatocyst (cyst) (terms used by previous authors were ampulla or follicle), defining their testicular structure as polyspermatocystic type. Each cyst is made up of spermatoblasts in which one Sertoli cell is associated to a clone of synchronous developing germ cell, from 1 spermatogonium initially to 64 mature spermatids. Cysts originate from the germinative zone located at fixed sites on the dorsal or dorsal-lateral margins of the testis according to species. Because new cysts are being formed and added continually, older cysts are moved in sequential order leading to a zonate arrangement according to the spermatogenetic wave. Therefore, a cross section of the testis displays a dorsoventrally oriented succession of zones corresponding to spermatogonial, spermatocytes, early spermatids, late spermatids and degeneration zones.

The Male Genital Tract

Anatomically, the male genital tract of elasmobranchs is elaborated ([Fig. 1](#)). Two elongated testes have the particularity to be closely associated to the epigonal tissue, a lymphomyeloid tissue producing leukocytes, granulocytes and lymphocytes. Spermatozoa are collected through a paired duct system associated to each testis and made of intra-testicular tubules (branched and stem tubules),

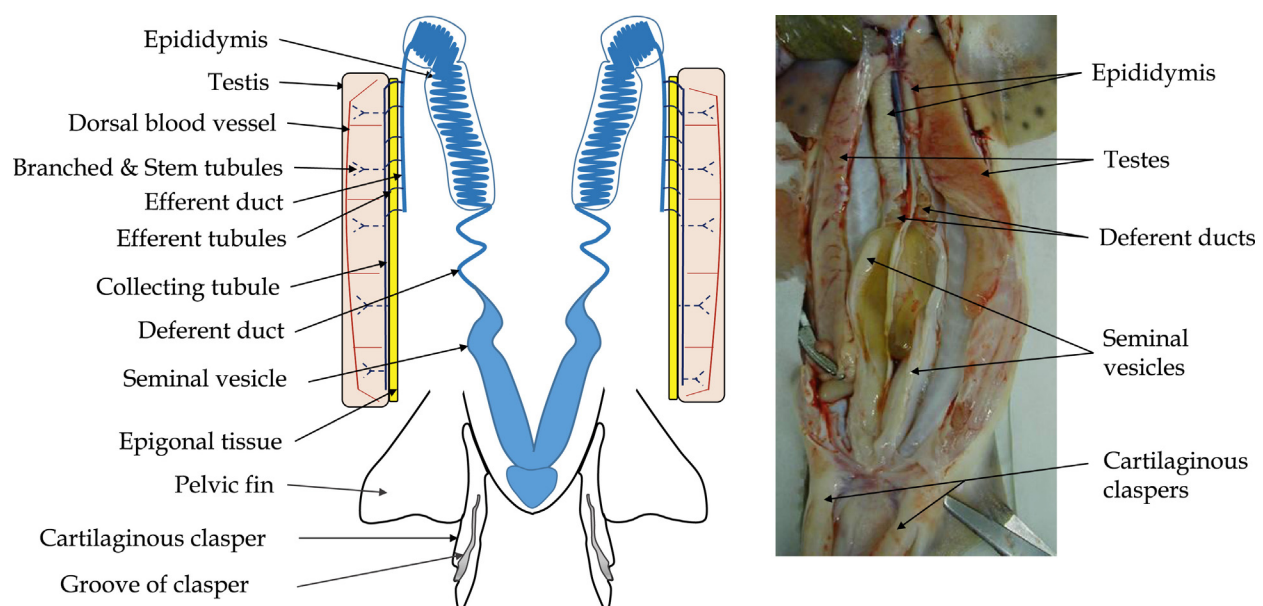


Fig. 1 Illustration of the male genital tract in *Scyliorhinus canicula*.

collecting tubules and from two to six efferent ducts (*rete testis* homolog) in sharks or one efferent duct in rays and skates. Efferent duct is followed by one epididymis composed of a proximal and distal segment, one deferent duct enlarged at its end into seminal vesicle. Seminal vesicle often expanded to form an ampulla used for sperm storage and the two seminal vesicles converge in their posterior region. In skate and rays, Marshall's alkaline glands (incorrectly named sperm sacs since they are devoid of sperm) arise as diverticula from this posterior region. Sperm passes from the urogenital sinus to the urogenital papilla which consists of the groove of pelvic clasper, the copulatory organs of chondrichthyans used for internal fertilization. Male elasmobranchs possess unique reproductive associated glands: the Leydig's gland and siphon sacs in sharks or clasper glands in skates and rays. Leydig's gland is closely associated and connected to the deferent duct and secretes part of the seminal fluid. Since their functions remain to be clarified, siphon sacs could either facilitate the transfer of sperm to the female (Gilbert and Heath, 1972) by inducing uterine contractions or assist sperm competition by washing female genital tract before ejaculation with a water flux. Most skates and rays have a clasper gland that may facilitate copulation by providing a lubricant and a fluid coagulating with sea water that could seal the groove of the clasper to form a tube (Wourms, 1977). A diversity of functions could be respectively attributed to siphon sacs and clasper glands but both, in terms, facilitate fertilization.

Anatomy of Testis

According to species and testicular type, germinative area is unique or multiple. In Squalomorph, Galeomorph and Carcharhinidae shark species, the germinative area consists of one band located between albuginea and the main testicular blood vessel which runs the dorsal length of the testis. This testicular structure has been classified as diametric-type testis (Pratt, 1988) (Fig. 2). Squaliformes as *Centroscyms coelolepes* and *Centrophorus squamosus* may present lobate testis and each lobe has a diametric structure (Girard et al., 2000). In Lamniformes, testis comprises seminiferous lobules (Radial-type testis). The germinative area is in the center of each seminiferous lobule and cyst development proceeds radially from the germinal area. In skates and rays, testis combines radial and diametric structure and was termed "compound" by Pratt. Their testis may comprise lobate divisions as observed for the Cownose Ray, *Rhinoptera bonasus* (Poulakis and Grier, 2014). Germinative areas originate from the germinal papilla that appears as a small projection at the dorsal surface of the testis and cyst development proceeds diametrically from each germinative area.

The Mitotic Proliferative Phase of Spermatogenesis

To describe precisely the different stages of spermatogenesis, the example of *Scyliorhinus canicula* will be detailed below (Loir et al., 1995) (Fig. 3). Stage I corresponds to large spermatogonia (10 µm in nuclear diameter) of the germinative zone, surrounded by elongated somatic cells which correspond to Sertoli cell precursors. In the different subparts of the germinative zone, from the area just behind the fibrous albuginea toward the blood vessel, spermatogonia are isolated, paired or in clusters of 4 and they can be qualified of A single (As), A paired (Apr) and A und4 undifferentiated spermatogonia, respectively. Their ultrastructure observed by transmission electron microscopy reveals a nucleus with chromatin of a mottled appearance, a nucleolus and a cytoplasm containing numerous mitochondria and a dense material, the germinative nuage. From the first mitotic division, all spermatogonia will be connected with intercellular bridges to form a germ cell syncytium (Fig. 4). While leaving the germinative area, both Sertoli cell precursors and spermatogonia proliferate and both types of cells regroup progressively to form spherical cysts with a lumen that progressively appears (stage II). Stage III corresponds to the end of the cyst formation with a single layer of Sertoli cell nuclei around the central lumen and a single layer (stage IIIa) or two layers (stage IIIb) of spermatogonia toward the basal lamina. Here, it is important to notice that the cyst formation (stage IIIa) coincides with the end of Sertoli cell divisions and to the further four mitoses of differentiated spermatogonia (stages IIIb to VI). Stage IV has four layers of spermatogonia and Sertoli cell nuclei remaining at the adluminal position. Stage V presents five layers of spermatogonia and the Sertoli cell nuclei are at an adluminal (majority) or at an intermediate position. Stage VI exhibits about six layers of spermatogonia (corresponding to pre-leptotene spermatocytes) and Sertoli cell nuclei are in an intermediate (majority) or basal position. The number of germ cells per spermatoblast is 4, 8 and 16 for stages IV, V and VI, respectively.

Considering the ratio number of 1 Sertoli for 1 spermatogonium at stage IIIa, and the number of Sertoli cell nuclei per cyst (reflecting also the number of spermatoblasts per cyst), it is possible to evaluate the number of cell mitotic divisions for undifferentiated spermatogonia. Holstein (1969) has determined 469 spermatoblasts per cysts in *Squalus acanthias* and this number is about 500 in *Scyliorhinus canicula* cysts (Stanley, 1966) and about 250 in *Torpedo marmorata* cysts (Wourms, 1977). In *S. canicula* and *S. acanthias*, this will correspond to 9 cell cycle divisions for undifferentiated spermatogonia (from As to Aund 512) and to 4 more divisions for differentiated spermatogonia (from Ad1 to PL spermatocytes), considering that no division occurs between the last stage of undifferentiated spermatogonia and the first stage of differentiated ones. Concerning *T. marmorata*, the total number of spermatogonial mitotic divisions is 12 instead of 13. This high proliferative phase allows to produce a large number of spermatozoa from a small number of germinal stem cells. Otherwise, the difference between the theoretical (512) and observed (469) number of spermatoblasts suggests an apoptosis control of testicular homeostasis as observed for all vertebrates and studied in the shark *S. acanthias* (McClusky, 2012) or in the ray *T. marmorata*.

This ability to observe these different generations of spermatogonia is also of interest for studying spermatogonial stem cells and their progeny by establishing a molecular signature for each of them using *in situ* hybridization and immunocytochemistry

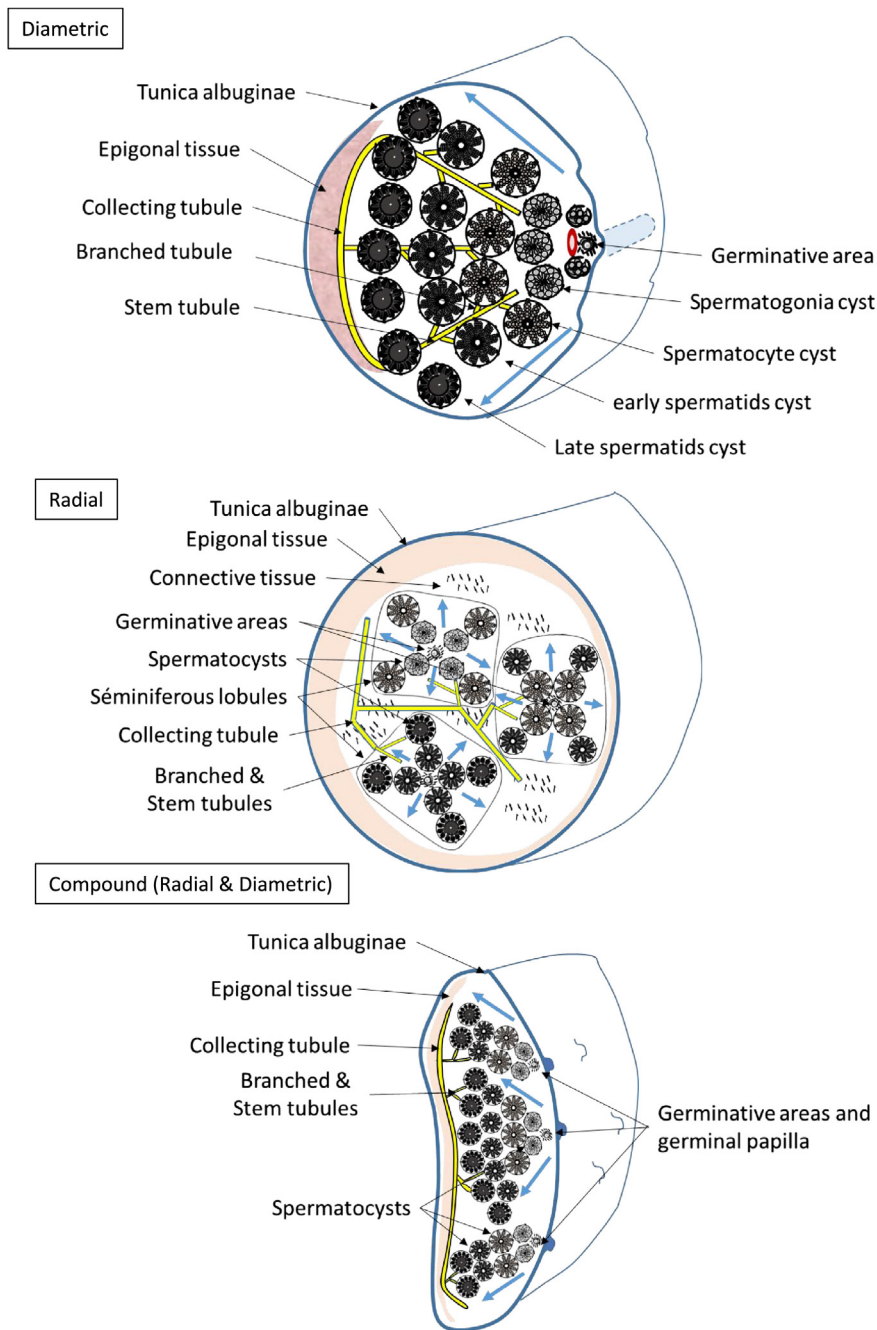


Fig. 2 Anatomy of testis. Adapted from Pratt, H.L., 1988. Elasmobranch gonad structure: A description and survey. Copeia 3, 719–729.

approaches. Such studies have shown, for example, that one of the most universal spermatogonial stem cell regulator, the GDNF family receptor GFRa1, is expressed in undifferentiated spermatogonia (Bosseboeuf *et al.*, 2014; Gautier *et al.*, 2014).

Meiosis

In the dogfish *S. canicula*, meiosis proceeds from stages VII to XI (Fig. 3). Stage VII corresponds to cysts containing the primary spermatocytes at the meiotic prophase and all the Sertoli cell nuclei are observed against the basal lamina. Stage VIII corresponds to the first meiotic division (metaphase, anaphase, telophase) which gives secondary spermatocytes (stages IX and X). At the end of meiosis, the number of early spermatids (or round spermatids) is of 64 per spermatoblast (stage XI).

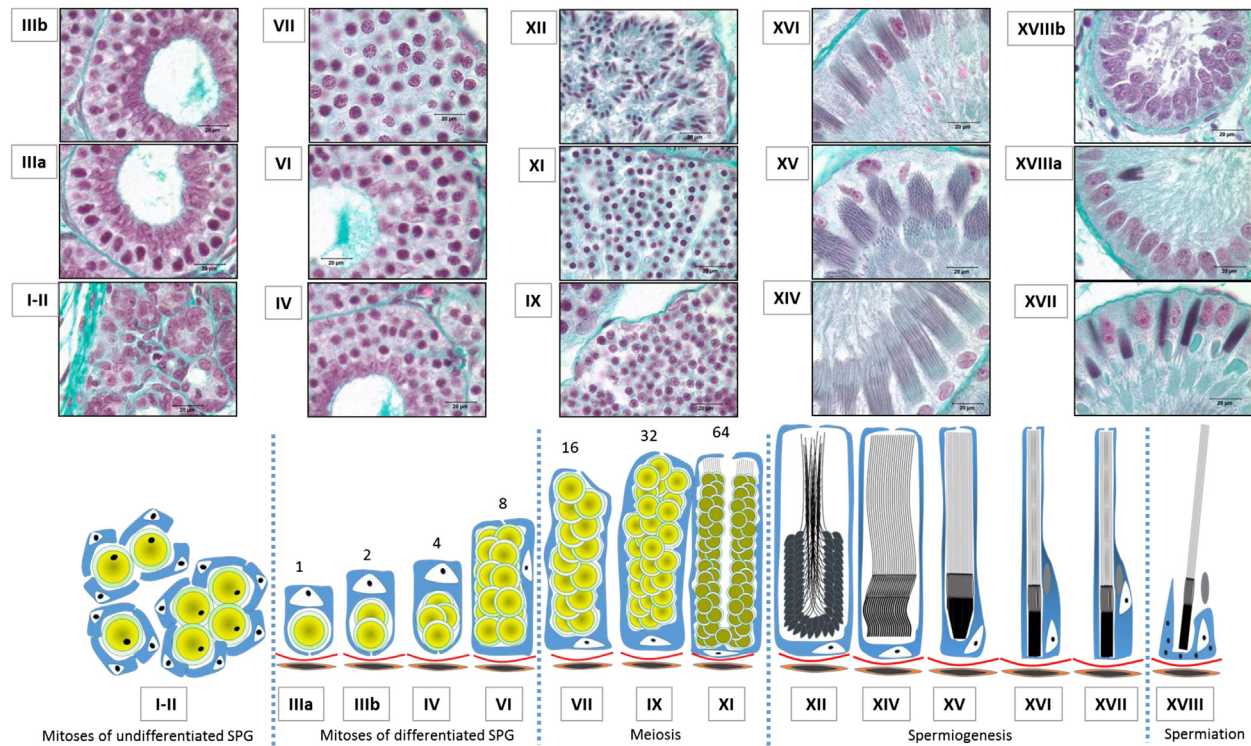


Fig. 3 Stages of spermatogenesis in *Scyliorhinus canicula*. Associations between Sertoli cells (in blue) and germ cells (in yellow or black) are illustrated during stages of spermatogenesis (I to XVIII). Schemes of the evolution of a single spermatoblast in cysts containing spermatogonia (IIIa to VI), spermatocytes (VII, IX), spermatids (XI–XVIII) are represented in the lower part of the figure. Corresponding histology is in the upper part of the figure. I–II: germinative and transition zones.

Spermiogenesis

Spermiogenesis is the development of spermatids into mature spermatozoa. During this process, the main cell transformations are the acrosome formation, the compaction of chromatin with the replacement of histones by transition proteins and then protamines, the flagella assembly and the cytoplasm reorganization with elimination of its major part and of its organelles to keep a cytoplasm mainly restricted to the midpiece containing mitochondria. In *S. canicula*, stage XI corresponds to cysts with young round spermatids arranged as “a festoon figure” with nuclei showing patches of dense chromatin and flagella which are noticeable (Fig. 3). In stage XII, condensation of the chromatin is well advanced and elongation of the spermatid head, a characteristic feature of chondrichthyan spermatozoa, occurs and is completed at stage XIV. At this stage spermatids are arranged in parallel bundles which are perpendicular to the basal lamina of the cyst. The helical twisting of the spermatid which includes the head, the midpiece and flagellar elements (the axoneme and/or microtubules according to species) has a function in spermatozoa motility achieved by revolving around their long axis. During stages XV to XVII, the nuclei of Sertoli cell migration towards the intermediate cytoplasm of the cell and spermatid bundles become more compact and close to the basal lamina. The midpiece of spermatid is also well noticeable at the back of the head. At the ultrastructure level, for the spiny dogfish, glycogen granules are present and dispersed between mitochondria, the acrosome forms an elongated cap over the nucleus of the spermatid and a short perforatorium develops between the nucleus and the acrosome (in the subacrosomal space) (Pudney, 1995). Classically, the function of the perforatorium is the penetration of the sperm through the egg chorion during fertilization. The flagellar apparatus of spermatozoa is also characterized by the presence of flagellar roots which form the axis of the midpiece and the existence of two longitudinal columns associated with the axoneme. The function of this longitudinal columns is unknown and could be associated to the revolving motility of spermatozoa by restringing lateral beating of the flagella.

Another characteristic feature during the stages of spermiogenesis in elasmobranchs, is the apparition of a large protein body in the intermediate cytoplasm of Sertoli cell (stage XVI), the Semper's body. Its function is unclear and it will be released with bundles of spermatozoa during spermiation. A similar body have been found in the semen of the spiny dogfish and named “Sertoli cell cytoplasm”. As it contains organelles (mitochondria, ribosomes, endoplasmic reticulum) and lipid droplets, some of them associated to steroid synthesis, Pudney and Callard hypothesized that it could contribute to the steroids found in the semen and necessary for maturation and maintenance of spermatozoa in the reproductive ducts (Pudney and Callard, 1986). Another hypothesis may be that the Semper's body is equivalent to the residual body observed for mammalian spermatids. Its function remains to be studied.

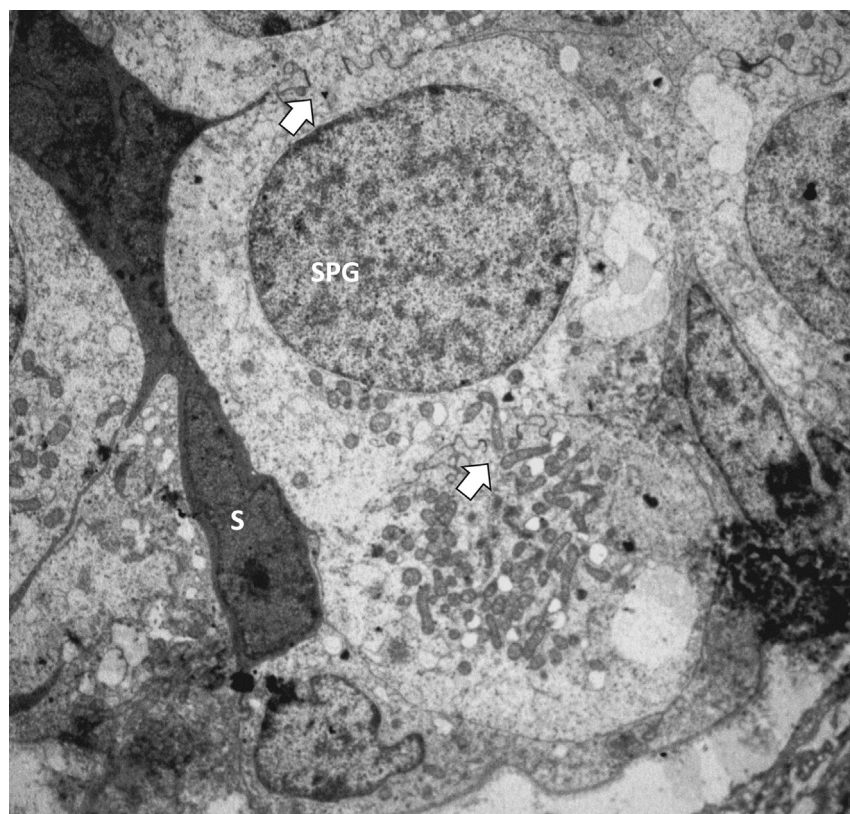


Fig. 4 Ultrastructure of spermatogonia associated to Sertoli cells at stage II. Intercellular bridges between spermatogonia (SPG) are indicated by arrows. S: Sertoli cell.

Stage XVIII corresponds to the spermiation which takes place by the detachment of the spermatid bundle which carries with him the adluminal part of the sertolien cytoplasm. Bundles of spermatozoa are then evacuated through the cystic collecting ductule (branched tubules), previously closed by a cellular plug. By taking into account the number of 500 spermatoblasts in *S. canicula* and of 250 in *T. marmorata*, it could be calculated that 32,000 and 16,000 spermatozoa will be produced per cyst, respectively for these species.

The post-spermiation cyst contains the remaining basal cytoplasm and nuclei of Sertoli cells and will be resorbed in the epigonal organ, underlying this characteristic feature of the close association between this lymphomyeloid tissue and the testis in elasmobranchs. Another interesting feature about this association was the inhibitory effect on DNA synthesis of premeiotic cysts by epigonal cytosolic extracts, suggesting a crosstalk between the two organs to regulate the spermatogenic cycle. Involvement of growth factors (GDNF, IGF1, bFGF...) and cytokines (IL1, G-CSF...) in the regulation of spermatogonial proliferation is now well documented in several species.

Blood-Testis Barrier

In amniotes, the blood-testis barrier (BTB) is essential for spermatogenesis and takes place during puberty when Sertoli cells stop mitosis. Classically, blood-tissue barriers restrict or control the entry of molecules present in circulation from entering a tissue or compartment. This barrier partially made of specialized junctions between Sertoli cells "protects" haploid cells in a specific micro-environment and allows also the production of the testicular fluid useful, in particular, for transporting spermatozoa. This blood-testis barrier can be visualized by exclusion of a high molecular weight (10 kDa) fluorescent dye. Using this approach, McClusky has shown that living post-meiotic cysts are impermeant, suggesting a functional blood-testis barrier in cysts containing spermatids in the spiny dogfish (McClusky, 2006). Furthermore, in spermatid cysts, tight junctions have been observed between Sertoli cells, reinforcing the presence of a BTB but the precise description of its ultrastructural components remains to be done.

Sperm Transport, Maturation and Storage

Structure of a branching intratesticular network of ducts has been well described in the starspotted smooth-hound *Mustelus manazo* (Park *et al.*, 2013). This system is composed of branched tubules, linked to cysts, which are connected via a secondary network of

stem tubules to the collecting tubules. Branched and stem ducts can be observed early in the cyst-forming area (stage II) and during the spermatogenic wave, cysts are still attached to branched tubules. This implies that both cyst and tubules will move together from the germinal to the mature zone of the testis, undoubtedly involving regulatory mechanisms not yet known. During spermiation, branched tubules open at their junction with the cyst allowing the flow of bundles of spermatozoa into the stem tubules. Transport of spermatozoa will be achieved thanks to the presence of ciliated epithelial cells lining the lumen of branched, stem and collecting ducts. Contraction of myoid cells surrounding the intratesticular ducts probably also contributes to the transport of spermatozoa towards the epididymis where maturation occurs. In mammals, spermatozoa acquire their motility and fertilizing properties during their epididymal transit. Epididymis has been poorly studied in elasmobranchs, which is surprising considering its size and its apparent similarity to that of mammals. Studies in the Port Jackson Shark, *Heterodontus portusjacksoni* and in the Clearnose Skate *Raja eglanteria* have shown that epididymis effectively participates in luminal fluid modifications, including sodium resorption, and that spermatozoa, immotile in the proximal segment, acquired their motility during their transit in the terminal segment of epididymis. In *Raja eglanteria*, the glycosylation activity of the Leydig's gland, connected to epididymis and to the deferent duct, is rather associated to spermatozoa aggregation. During their transport from the cyst, where they were aggregated into bundles, to the posterior segment of epididymis, bundles of spermatozoa are dissociated and spermatozoa reaggregate into the seminal vesicle where they are embedded in a mucoid matrix. Two basic types of spermatozoa aggregation are described for elasmobranchs: spermatozeugmata and spermatophores. In spermatophores, bundles of spermatozoa are embedded in a matrix with the heads inside and the flagella projected out the matrix. Spermatophores are found in Alopidae, Odontaspidae and Lamnidae. Spermatozeugmata are un-encapsulated masses of naked spermatozoa. Spermatozeugmata may consist of different architectures: simple aligned bundles of spermatozoa (*Squalus acanthias*, *Hydrolagus coliei*); radially aligned bundles of spermatozoa (*Charcharinus plumbeus*); several layers of sperm bundles (*Sphyrna lewini*).

Seasonality of Spermatogenesis

Spermatogenesis can show little or no seasonal changes in Elasmobranchs, as this is the case for *S. canicula* or the ray *T. marmorata* showing continuous spermatogenesis throughout the year. But in the spiny dogfish *S. acanthias*, caught along the United States Atlantic coast, the spermatogenic cycle is interrupted by germ cell apoptosis at the mitosis–meiosis transition in April–May, leading to a zone of degeneration. By studying this zone of degeneration, several information of interest can be found. Firstly, because this interruption is temporary, the resumption of the mitosis–meiosis transition will give new cysts that will lead to a displacement of the zone of degeneration (ZD) towards the mature side of the testis. So, the observation of this zone of degeneration along the time will give an idea of the duration of spermatogenesis. In the spiny dogfish, [McClusky \(2006\)](#) has thus established that the spermatogenic cycle spans about 2 years and that the duration from the Preleptotene stage to the more mature spermatid stage is of 9–10 months, underlining the long time required for spermatogenesis in that shark. In terms of sperm production, the displacement of the ZD at the mature side of the testis will lead to the absence of mature cysts and consequently the decrease of sperm production 9–10 months later, in February–March of the following year. Conversely, the higher number of cysts containing mature spermatids is observed between October and January and coincides with the matting period (December to February). Secondly, this zone of degeneration was experimentally reproduced in the dogfish *S. canicula* by partial hypophysectomy coupled with an increase of temperature starvation, suggesting that this mitotic–meiosis transition is a critical stage of spermatogenesis under gonadotrophin and environmental control. Consequently, seasonality of reproduction can be more marked to species that inhabit regions where environmental conditions vary widely with season.

A ZD has also been observed in the blue shark *Prionace glauca*, the Atlantic stingray *Hypanus sabinus*, in the skate *Raja erinacea* or in the ray *Urobatis halleri* for examples. Apart from the presence or absence of a ZD, it has been reported that the relative abundance of cysts at different stages of spermatogenesis varies seasonally according to species. As observed for *S. acanthias*, higher number of cysts containing mature spermatids or higher number of spermatozoa in seminal vesicle generally correlate with the mating period for sharks. In *S. canicula* (Carcharhiniformes, wide geographic range, Northeast Atlantic), mature spermatids containing cysts increase from April through to August and the greatest number of spermatozoa per seminal vesicle was observed from March through to May in species caught in the English Channel ([Garnier et al., 1999](#)). In Epaulette shark, *Hemiscyllium ocellatum* (Orectolobiformes, tropical, Western Pacific), higher amount of sperm storage occurs from August through to November, preceding mating period in December. In Starspotted smooth-hound *Mustelus manazo* and in Spotless smooth-hound *Mustelus griseus*, two Carcharhiniformes tropical species (Western Indian Ocean and Western Pacific), the highest number of cysts containing mature spermatids was observed in March and May, respectively, and accumulation of spermatozoa was greatest from December through to May. In both *Mustelus* species, it appears that spermatozoa production was at maximum before the June–August mating period. Among sharks, the Bonnethead *Sphyrna tiburo* (Carcharhiniformes, subtropical, Western Atlantic) appears as a special case since all stages of spermatogenesis are not present at all time of the year, a complete testicular regression and recrudescence is observed ([Parsons and Grier, 1992](#)). All the reports cited above concern sharks, what about rays? In the subtropical Myliobatiforms Atlantic stingray *Hypanus sabinus* (Western Atlantic) and round stingrays *Urobatis halleri* (Eastern Pacific), inactive phase of spermatogenesis was observed from March through to July and maximum production of mature cysts in winter (from August through to January in *H. sabinus*, December in *U. halleri*) preceding spring mating period when sperm stored in seminal vesicles will be used ([Tricas et al., 2000](#)). In lesser guitar fish *Rhinobatus annulatus* (Rhinopristiformes, subtropical, Southeast Atlantic), no spermatogenic

resting period was observed since all stages were found in the testes throughout the year. However, a peak in sperm production was observed from November through to January (Rossouw, 2014).

Control of Spermatogenesis

In Elasmobranchs, like in other vertebrates, the testicular function is hormonally controlled via a hypothalamus–pituitary–gonadal (HPG) axis involving the GnRH (Gonadotropin-releasing hormone) hypothalamic neuropeptide, pituitary gonadotropins LH (luteinizing hormone) and FSH (follicle stimulating hormone) and gonadal steroids. GnRH and gonadotropins acts by binding to their specific G-protein coupled-receptor (GnRHR, FSHR, LHR) expressed by their target cells. Three groups of GnRH (GnRH-I, GnRH-II, GnRH-III) have been identified in brain but it is classically considered that control of synthesis and release of gonadotropins is regulated by the GnRH-I localized in diencephalon. In Elasmobranchs, the transport of GnRH-I to pituitary occurs via the general circulation in absence of a direct vasculature such as the portal system found in mammals. Gonadotropins are heteromeric glycoproteins made of one common α subunit and of one specific β subunit (β -LH or β -FSH), the three subunits have been identified in *S. canicula* (Quérat *et al.*, 2001). However, presently, no FSH or LH receptors have been characterized in elasmobranchs, which underlines the work still to be undertaken in these species.

The Case of Leydig Cells

When testicular anatomy is presented, it is usual to present the seminiferous compartment in which spermatogenesis occurs and the interstitial compartment, which includes the Leydig cells. This pattern is probably true for amniotes but the presence of functional Leydig cells in elasmobranchs is still questioned. In amniotes, the Leydig cells are associated to androgen production and so what, they show an ultrastructure classically associated to steroid synthesis such as presence of mitochondria with tubular cristae, agranular endoplasmic reticulum and lipid droplets. In addition, they express key enzymes involved in the biosynthesis of steroids such as the 3β -hydroxysteroid dehydrogenase (3β -HSD) and 17β -hydroxysteroid dehydrogenase (17β -HSD). In elasmobranchs, Leydig cells have been described as absent or present according to authors and/or stages of development and/or seasonality. For example, they have been described as absent from the interstitial tissue of *Scyliorhinus stellaris* or rare in *Cetorhinus maximus* and *Scyliorhinus canicula* or present but undifferentiated in *Squalus acanthias*. Furthermore, Sertoli cells in these species show steroidal ultrastructure features and isolated cysts in *Squalus acanthias* or in *Scyliorhinus canicula* have the abilities to synthesize androgens. However, interesting studies conducted in the ray *T. marmorata* (Prisco *et al.*, 2008) has shed new light on the question. In fact, in this species, typical Leydig cells are observed in the interstitial compartment adjacent to cysts engaged in spermatogenesis but they have reduced cellular components when they are associated to cysts containing spermatogonia and, at the other end, when they are associated to cysts engaged in spermiation. Immunolocalization of 3β -HSD and 17β -HSD has confirmed a distinctive stage-related involvement of cells in steroid production: spermatogonia of the early stages, Sertoli cells of cysts containing spermatogonia, Leydig cells associated to meiotic stages and Sertoli cells in late spermatids stages.

Several studies, well reviewed by Awruch (2013), have analyzed steroid levels in relation with the seasonal reproductive cycle and spermatogenic stages. Briefly, androgens (mainly testosterone instead of 11-ketotestosterone) regulate development and maturation of cysts as well as the final stage of sperm maturation. Androgen receptor was primarily localized in premeiotic stages in the testis of *S. acanthias*, suggesting that androgens regulate the development of early stages of spermatogenesis (Cuevas and Callard, 1992). Among other steroids, 17α , 20β dihydroxyprogesterone (OHP) has been also identified and associated to cysts with late spermatids (Sourdaine and Garnier, 1993).

Conclusion

Elasmobranchs present a remarkable male genital tract that by some aspects, appears more related to the genital tract of amniotes than of anamniote species (epididymis for example). They have also characteristic features like the close association between the testis and the epigonal tissue or the diversity of testicular cells involved in steroidogenesis. What are the evolutionary meaning of these features? Endocrine and paracrine controls of spermatogenesis are still poorly understood while knowledge on the reproduction of elasmobranchs is still a challenge because of their vulnerability.

References

- Awruch, C. A. (2013). Reproductive endocrinology in chondrichthyan: The present and the future. In *General and Comparative Endocrinology*, 192 pp. 60–70). Elsevier Inc.
- Bosseboeuf, A., et al. (2014). Characterization of spermatogonial markers in the mature testis of the dogfish (*Scyliorhinus canicula* L.). *Reproduction*, 147(1), 125–139.
- Cuevas, M., & Callard, G. (1992). Androgen and progesterone receptors in shark (*Squalus*) testis: Characteristics and stage-related distribution. *Endocrinology*, 130(4), 2173–2182.
- Garnier, D. H., Sourdaine, P., & Jégou, B. (1999). Seasonal variations in sex steroids and male sexual characteristics in *Scyliorhinus canicula*. *General and Comparative Endocrinology*, 116(2), 281–290.

- Gautier, A., et al. (2014). Maintenance of potential spermatogonial stem cells in vitro by GDNF treatment in a chondrichthyan model (*Scyliorhinus canicula* L.). *Biology of Reproduction*, 91(4), 1–15.
- Gilbert, P. W., & Heath, G. W. (1972). The clasper-siphon sac mechanism in *Squalus acanthias* and *Mustelus canis*. *Comparative Biochemistry and Physiology – Part A: Physiology*, 42(A), 97–119.
- Girard, M., Rivalan, P., & Siquin, G. (2000). Testis and sperm morphology in two deep-water squaloid sharks, *Centroscymnus coelolepis* and *Centrophorus squamosus*. *Journal of Fish Biology*, 57, 1575–1589.
- Holstein, A. (1969). On the problem of the local control of the spermatogenesis of the spiny dogfish (*Squalus acanthias* L.). *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 93, 265–281.
- La Valette St George, A. (1878). *Dissertatio de spermatosomatum evolutione in plagiostomis. Bonnae: formis Caroli Georgi univ. typogr.*
- Lair, M., et al. (1995). Cell–Cell interactions in the testis of Teleosts and Elasmobranchs. *Microscopy Research and Technique*, 32, 533–552.
- McClusky, L. M. (2006). Stage-dependency of apoptosis and the blood–testis barrier in the dogfish shark (*Squalus acanthias*): Cadmium-induced changes as assessed by vital fluorescence techniques. *Cell and Tissue Research*, 325(3), 541–553.
- McClusky, L. M. (2012). Coordination of spermatogenic processes in the testis: Lessons from cystic spermatogenesis. *Cell and Tissue Research*, 349(3), 703–715.
- Mellinger, J. (1965). Stades de la spermatogénèse chez *Scyliorhinus caniculus* (L.): Description, données histochimiques, variations normales et expérimentales. *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, 67, 653–673.
- Park, J. C., et al. (2013). Structure of the intratesticular duct system for sperm emission in the star-spotted smooth-hound *Mustelus manazo*. *Fisheries Science*, 79(2), 203–211.
- Parsons, G. R., & Grier, H. J. (1992). Seasonal changes in shark testicular structure and spermatogenesis. *Journal of Experimental Zoology*, 261(2), 173–184.
- Poulakis, G. R., & Grier, H. J. (2014). Ontogenetic testicular development and spermatogenesis in rays: The Cow-nose Ray, *Rhinoptera bonasus*, as a model. *Environmental Biology of Fishes*, 97(9), 1013–1029.
- Pratt, H. L. (1988). Elasmobranch gonad structure: A description and survey. *Copeia*, 1988(3), 719–729.
- Prisco, M., et al. (2008). Immunolocalization of 3 β HSD and 17 β HSD in the testis of the spotted ray *Torpedo marmorata*. *General and Comparative Endocrinology*, 155(1), 157–163.
- Pudney, J. (1995). Spermatogenesis in nonmammalian vertebrates. *Microscopy Research and Technique*, 32, 459–497.
- Pudney, J., & Callard, G. V. (1986). Sertoli cell cytoplasts in the semen of the spiny dogfish *Squalus acanthias*. *Tissue and Cell*, 18(3), 375–382.
- Quérat, B., et al. (2001). Duality of gonadotropins in gnathostomes. *General and Comparative Endocrinology*, 124(3), 308–314.
- Rossouw, G. J. (2014). Maturity, spermatogenesis and seasonal reproductive cycle of male *Rhinobatos annulatus* (Müller & Henle, 1841) from Algoa Bay, South Africa, and a novel description for sperm release from the spermatocyst. *African Zoology*, 49(1), 128–139.
- Sourdaine, P., & Garnier, D. H. (1993). Stage-dependent modulation of Sertoli cell steroid production in dogfish (*Scyliorhinus canicula*). *Journal of Reproduction and Fertility*, 97(1), 133–142.
- Stanley, H. P. (1966). The structure and development of the seminiferous follicle in *Scyliorhinus caniculus* and *Torpedo marmorata* (Elasmobranchii). *Zeitschrift für Zellforschung*, 75, 453–468.
- Tricas, T. C., Maruska, K. P., & Rasmussen, L. E. (2000). Annual cycles of steroid hormone production, gonad development, and reproductive behavior in the Atlantic stingray. *General and Comparative Endocrinology*, 118(2), 209–225.
- Wourms, J. P. (1977). Reproduction and development in chondrichthyan fishes. *American Zoologist*, 17, 379–410.

Spermatogenesis and Spermiogenesis, Birds

Tom A Aire, St. George's University, Grenada, West Indies

© 2018 Elsevier Inc. All rights reserved.

Glossary

A 'cellular association' Comprises groups of spermatids, various spermatocytes and spermatogonia, each at a specific phase of development, and constantly associated with one another.

'Duration of spermatogenesis' For a given species each step of spermatogenesis "has a constant duration; thus, germ cell differentiation unfolds as if regulated by a rigid time-scaled program" (Clermont, 1972).

Spermatogenesis Is a phenomenon in which germ cells in the testis multiply, form haploid cells that differentiate to produce millions of spermatozoa.

Spermatogonia Are the most immature germ cells of the seminiferous epithelium that divide to produce other germ cells, which subsequently proliferate and differentiate into spermatozoa.

Spermiation Is the process by which Sertoli cells move mature spermatozoa toward, and into, the tubular lumen, as free cells.

Spermiogenesis Is the last phase of spermatogenesis in which the haploid, round spermatid is differentiated into a spermatozoon.

'Stage of the cycle of the seminiferous epithelium' When a 'cellular association' is found in a 'cycle of the seminiferous epithelium' it is known as a 'stage', e.g., stages I to X in the Japanese quail (Lin and Jones, 1990).

The 'cycle of the seminiferous epithelium' Is defined as a "series of changes occurring in a given area of the seminiferous epithelium between two successive appearances of the same cellular association." (Leblond and Clermont, 1952).

The acrosome Is a cap-like, membranous, organelle covering the proximal part of the nucleus, and containing enzymes involved in the process of fertilization of the ovum.

The Centriolar complex Comprises the proximal and distal centrioles, with the latter forming the foundation upon which the midpiece of the spermatozoon is built. The distal centriole produces the central pair of the axonemal microtubules, and it determines the length of the midpiece. The axoneme/flagellum has the typical 9+2 pattern of microtubules.

The Chromatoid body Is an irregular structure of dense, reticular material found lying close to the Golgi complex and acrosome in round spermatids, but close to the centriolar complex in elongated spermatids.

The Granular body/helix Is a round to oval or irregular retiform and granular structure that is seen to lie close to the nucleus and the Golgi complex in younger spermatids. It forms an elongated helix in the older spermatids and in spermatozoa.

The 'Helical membrane' Is a helical external structure of the spermatozoon irrespective of its composition (Jamieson, 2007). It contains three different components: a protrusion/an extension of the core of the acrosome, mitochondrial helix, and microtubular helix in the spermatid.

The Manchette Is a temporary sleeve of microtubules, circular or longitudinal, surrounding the nucleus, and thought to assist in shaping of the nucleus during spermiogenesis.

The Perforatorium Is a fibrous structure consisting of parallel bundles of filaments; it is embedded in the endonuclear canal and projects into the deep and ample subacrosomal space of the acrosome, in most non-passerine birds.

'Wave of the seminiferous epithelium' Clermont (1958) defined the wave of the seminiferous epithelium "as a complete series of the successive cell associations found along a seminiferous tubule."

Introduction

Spermatogenesis is similar in all vertebrates, with variations, which are species-specific. Spermatogenesis is rapid in birds, and therefore, spermatozoa are produced in large numbers over a short period of time. There are over 10,000 species of birds divided into passerine and non-passerine groups. Spermatogenesis has been poorly studied in birds although they constitute more than half of all vertebrates (see Aire (2007a, 2014)). However, spermatogenesis is the process by which diploid cells are transformed into haploid cells, which then differentiate during the next phase, spermiogenesis, into spermatozoa. During spermiogenesis, the spherical nucleus of the spermatid elongates and its chromatin undergoes transformations. Major events are the elaboration of the precursors, and subsequent development, of the acrosome, as well as nuclear morphological transformation into an elongated organelle with condensed chromatin. The centriolar complex is structurally similar to that of vertebrates generally, but differences exist not only between birds and other vertebrates, but also between groups of birds. Only concise, basic features of the various complex processes will be provided, here. For more advanced and detailed knowledge of spermatogenesis it is necessary to refer to selected references and reviews, some of which are provided, appropriately. For proper perspective, it is also necessary, first, to introduce very concisely the structure and nature of the avian male reproductive organs (Aire, 2007b). Jamieson (2007) has an excellent account of sperm structure in birds.

Avian Testis and Its Excurrent Ducts

Birds retain their testes within the abdomen, unlike in most mammals, but genetically controlled biochemical processes permit the testis to function efficiently in spite of the high core body temperature (Mezquita *et al.*, 1998). Avian testes are, however, generally similar to mammalian testes, in structure, composition, and color. Although there exists some variability in size between the bean- or oval-shaped left and right testis, yet each unit weight of the organ probably functions equally (see Aire (2007b)).

The main components of the testis, the seminiferous tubules, surrounded by the interstitium, are generally similar to those of mammals in composition and structure (Aire, 2007b). The columnar epithelium of the seminiferous tubule, usually branched in birds, contains fixed, somatic, non-germinal, Sertoli cells, which surround, support and nourish the series of germ cells. As in mammals, the germ cells move toward the tubular lumen, in a successive series of spermatogonia, primary and secondary spermatocytes, and spermatids; the latter differentiate into spermatozoa. Both the spermatid and spermatozoon are haploid. Spermatozoa leave the seminiferous epithelium and swim in the rather copious avian luminal testicular fluid (see Aire (2007b)) into, and through, the excurrent duct system.

The avian excurrent duct system comprises various ducts (the rete testis, efferent, connecting and epididymal ducts) within a small but complex epididymis, and a wavy ductus deferens (Aire, 2007b). These are, as in mammals, regulated by complex structural, endocrine, and paracrine, interactions (Skinner *et al.*, 1991; Hermo *et al.*, 2010).

Spermatogenesis in Birds

Within the seminiferous epithelium spermatogonia proliferate, the products of this undergo meiosis, and ultimately differentiate from round, non-motile cells into highly elongated, motile, male, gametes, the spermatozoa. This process, spermatogenesis, is 'midwived' by the Sertoli cells, as in mammals. The various cell types (spermatogonia, leptotene, zygotene, pachytene, diplotene primary spermatocytes and secondary spermatocytes, and spermatids) and the morphophysiological events taking place in the germ cells are, also, generally similar to those of mammals in which they have been best studied and elucidated (Clermont, 1972; Hermo *et al.*, 2010).

Spermatogonial Renewal and Proliferation

Spermatogenesis is a continuous, sustained process that must depend on regular renewal and availability of spermatogonia, which are the most primitive, progenitor, germ cells, from which other germ cell series originate. Spermatogonial renewal has been studied in numerous mammals (see Hermo *et al.* (2010)) but only two reports are available for birds. Clermont (1958) has demonstrated two types of spermatogonia, type A (stem cell; resting) and type B, with the latter differentiating into type C spermatogonia in the Mallard. In the Japanese quail (Lin and Jones, 1992), there is the dark type A (Ad) spermatogonium that replicates itself (stem cell renewal); one of them enters the proliferation phase of spermatogenesis by dividing into two variant spermatogonia, a pale type A (with Ap₁ and Ap₂ sub-variants) and type B (Fig. 1). There are fewer spermatogonial (mitotic) divisions in birds than in mammals. The type B spermatogonium proliferates through a definite series of primary and secondary spermatocytes. The secondary spermatocytes undergo meiosis, each producing two spermatids, which differentiate into spermatozoa (Fig. 2). Thus, the germ cells grow

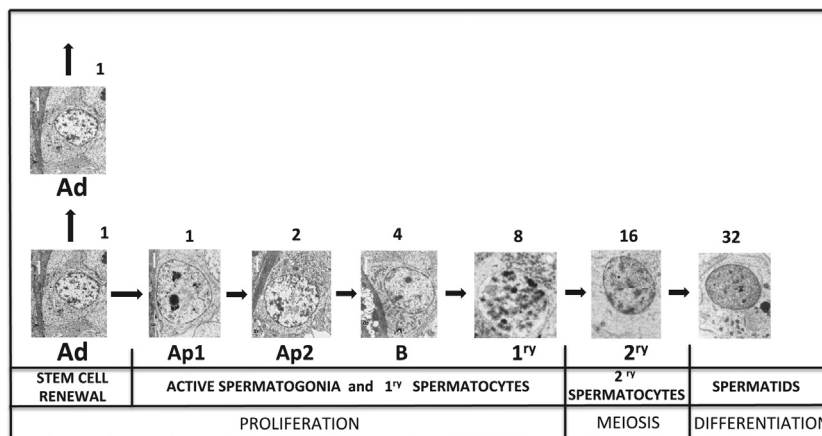


Fig. 1 A serial representation of stem cell renewal and spermatogonial proliferation in the Japanese quail. Ad=Dark type A spermatogonium; Ap1=pale type A1 spermatogonium; Ap2=pale type A2 spermatogonium; B=type B spermatogonium; 1^{IV}=primary spermatocytes; 2^{IV}=secondary spermatocytes. The Arabic numerals indicate the number of cells in each germ cell series. Fig. 2 provides the time of occurrence of each cell type and stage during the cycle of seminiferous epithelium. Modified from Lin, M., Jones, R.C., 1992. Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail. *Cell and Tissue Research* 267, 591–601, Figure 12.

11	11	11	12	12					
1	2	3	4	5	6	7	8	9	10
P	P	P	P	P	P	Dp	Dk	M-An	II
B	B	B	L	Z	P	P	P	P	P
Ap1	Ap1	Ap2	Ap2	Ap2	Ap2	B	B	B	B
Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ad	Ap1
Ad									
I	II	III	IV	V	VI	VII	VIII	IX	X

Stages of the cycle

Fig. 2 The cycle of the seminiferous epithelium in the Japanese quail showing association of germ cells in the 10 stages of the cycle. Ad, Dark type A spermatogonia; Ap1, pale type A1 spermatogonia; Ap2, pale type A2 spermatogonia; B type B spermatogonia; L, leptotene primary spermatocytes; Z, young primary spermatocytes in zygotene; P, pachytene primary spermatocytes; Dp, diplotene primary spermatocytes; An, anaphase primary spermatocytes; II, secondary spermatocytes; 1–12, Step 1 to Step 12 spermatids. From Lin, M., Jones, R.C., Blackshaw, A.W., 1990. The cycle of the seminiferous epithelium in the Japanese quail (*Coturnix coturnix japonica*) and estimation of its duration. *Journal of Reproduction and Fertility* 88, 481–490. Reproduced by permission of the Society for Reproduction and Fertility (1990).

older as they undergo transformation and move from the basement membrane of the seminiferous epithelium toward the tubular lumen. Spermatogenesis is a carefully, synchronized, orderly, precise, and unique biological event that, although generally similar in vertebrates, has unique elements for each species of animal. The evolution of spermatozoa from spherical undifferentiated cells is one of the most intriguing biological events, and knowledge of the numerous, complex, and intricate processes involved is necessary for better understanding of this phenomenon.

The Cycle of the Seminiferous Epithelium

The epithelium of a transverse section of a seminiferous tubule comprises germ cells and Sertoli cells, with the germ cells arranged in a concentric manner from the basement membrane, where the most primitive spermatogonia are situated, to the luminal border, where the most advanced germ cell types, that is the spermatids and spermatozoa, are situated. Thus, groups of spermatids at a given step of development are *constantly* associated with *certain specific* groups of spermatocytes and spermatogonia, each *at a specific phase of development* (Perey *et al.*, 1961), as well as with the same groups of nurse cells, the Sertoli cells (Skinner *et al.*, 1991). This is a specific, unique and constant ‘cellular association’ of successive generations of cells arising from the same spermatogonium, with each generation of cells being linked physiologically in a syncytium that ensures a synchronized process of development. It is therefore expected that there are several specific cellular associations, each occupying a small portion of the epithelium along the length of the seminiferous tubule (Fig. 2). For example, in a small space of the seminiferous epithelium, from the lumen to the basement membrane of the seminiferous epithelium in column III (‘cellular association’ or ‘stage’ III) (Fig. 2), there are always two spermatids at steps 3 and 11, which are always, specifically, and invariably associated with pachytene primary spermatocytes, type B spermatogonium, pale type Ap2 spermatogonium, and type Ad spermatogonium, while column X (Fig. 2) has step 10 spermatids, always associated with secondary spermatocytes, pachytene primary spermatocytes, type B spermatogonium, pale type Ap1 spermatogonium, and type Ad spermatogonium.

The seminiferous epithelium has 6 (numbered I to VI) cellular associations in the rooster (Courot *et al.*, 1970) and 10 (I to X) in the Japanese quail (Lin and Jones, 1990; Aire, 2007a). There are 14 in the rat (Leblond and Clermont, 1952). However, whereas only one cellular association occupies the epithelium of a transverse section of the seminiferous tubule in most mammals, there are *multiple, heterogeneous, or mosaic patterns* of cellular associations in a similar section in birds and primates (see Aire (2007a)). These species-specific numbers of cellular associations are arranged in an orderly, predictable succession, for example, I to X in the Japanese quail (Fig. 2) (Lin and Jones, 1990), to constitute a ‘cycle of the seminiferous epithelium’ within an area or a segment of the seminiferous tubule. Each cellular association within the cycle is known as a ‘stage of the cycle’.

The ‘wave of the seminiferous epithelium’ is regarded as the completion of a full series of cellular associations (e.g., I to X, for the Japanese quail) between two successive appearances of the same stage (e.g., I to X, followed by another cycle beginning with I) along the length of the seminiferous tubule (Perey *et al.*, 1961). Only a few reports on this phenomenon are available in birds (Lin and Jones, 1990), and in the human primate, apparently because they have heterogeneous cellular associations, unlike in most mammals (see Aire (2007a)).

A stage number, e.g., stage III of I to XIV stages of the cycle of the seminiferous epithelium in the rat, may be missing. This phenomenon, known as ‘modulation’ (Perey *et al.*, 1961), has not yet been reported in birds, apparently due to branching of the seminiferous tubules (Lin and Jones, 1990). The duration of spermatogenesis, i.e. from type Ad spermatogonial division to the release of spermatozoa from the seminiferous epithelium, is constant and independent of season (de Reviers, 1975). It is quite short in birds, being between 12 and 13 days (de Reviers, 1975; Marchand *et al.*, 1977; Lin and Jones, 1990, 1992; Aire, 2007a, 2014), unlike 25–65 days in several species of mammals (Clermont, 1972). In addition, although the number of spermatids produced per stem cell is much greater in mammals (128-fold), birds, however, invest heavily on sperm production. For example, the duration of spermatogenesis is short, and the gonosomatic index (% of testis weight relative to body weight) is much higher in birds than in

mammals (Clulow and Jones, 1982; Møller, 1991); it has been established that testis size and sperm production are highly and positively correlated.

Spermiogenesis in birds

Introduction

Spermiogenesis is the last phase of spermatogenesis in which the haploid, round spermatid is differentiated into a spermatozoon. The spermatids arising from the same secondary spermatocyte form a syncytium, and each of them has a spherical nucleus, containing finely granular and a few clumps of chromatin (Figs. 3 and 4). A large Golgi complex, surrounded by numerous mitochondria, lies close to the nucleus. Numerous long strands of rough endoplasmic reticulum, a few other mitochondria and lysosomes are scattered throughout the cell. The centriolar complex, comprising the proximal and distal centrioles, lies free in the cytoplasm, with the distal centriole making contact with the cell wall, from which junction the flagellum extends freely into the intercellular space. During spermiogenesis, certain organelles of the round spermatid are remodeled, completely or partially, some are newly differentiated organelles, and some are discarded. The round spermatid loses about 97% of its volume, with the nucleus losing about 96% of its own volume (see Aire (2007a)).

As for spermatogenesis, the nomenclature and terms used in mammalian spermiogenesis are generally adopted for birds. However, only a few reports on this phenomenon are available for birds, in general. A concise description of the main features of spermiogenesis, first, in the non-passerine, and then in the passerine, bird will be provided, below. The step-wise method will be employed in order to place emphasis on relative or spatiotemporal developmental changes in the spermatid. The major determinants of steps of spermiogenesis are acrosomal, nuclear, centriolar and mitochondrial developmental changes. In general, twelve steps of spermiogenesis have been described in a few birds, such as the turkey and Japanese quail (see Aire (2014)). Each step of spermiogenesis comprises specific, landmark, structural modifications in spermatid organelles.

Non-Passerine Spermiogenesis

Steps of spermiogenesis

In Step 1, the Golgi complex displays numerous small, round, granules, the proacrosomal granules (Fig. 3).

Step 2: Most of the proacrosomal granules fuse together to form a large acrosomal vesicle.

Step 3: Both the acrosomal vesicle and centriolar complex gradually approach the nucleus, and subsequently, the acrosomal vesicle attaches to the putative proximal pole of the nucleus.

Step 4: As the acrosomal vesicle develops a broad base on the nucleus and begins to elongate, it becomes the acrosome. The nucleus and elongating acrosome then move toward the cell membrane, with which the acrosome makes contact. Thus, the spermatid lies closer to the Sertoli cell for nourishment, support and possible modeling. The centriolar complex attaches to the nucleus, close to the acrosome. A few, scattered microtubules appear around the nucleus.

Step 5: The nucleus contains finely granular chromatin, and begins to elongate, while the cytoplasm gradually migrates posteriorly, away from the nucleus. The centriolar complex also commences migration toward the caudal pole of the nucleus, to which it attaches at a shallow implantation fossa. The caudal part of the distal centriole attaches to the cell membrane at a ring-like thickening, the annulus. An evagination from the acrosomal base into the nucleus forms the precursor of the perforatorium rod that projects into a corresponding invagination of the nuclear membrane, the fledgling endonuclear canal.

Step 6: The elongated and wavy nucleus contains moderately electron-dense, granulo-filamentous chromatin. The fibrous perforatorium rod inserts in the endonuclear canal, and both grow further. The perinuclear microtubules form a sleeve of circularly oriented microtubules, the circular manchette (CM), extending from the acrosome-nuclear junction to the centriolar complex, caudally. Close to this complex lies a granular, round body known as the chromatoid body whose function is unknown. Mitochondrial migration into the trailing cytoplasm continues.

Step 7: The acrosome elongates further, and nearly occupies the entire proximal surface of the elongated and narrow, vermiform, nucleus. Also, an excavation of the base of the acrosome, the subacrosomal space, develops and overlies the blunt proximal end of the nucleus. The mitochondria in the caudally trailing cytoplasm elongate significantly.

Step 8: The proximal end of the nucleus narrows into a pointed rostrum, which projects into the deepening and enlarging subacrosomal space. The nuclear chromatin forms dense, rod-shaped granules. Following considerable narrowing of the nucleus, the CM may transform rapidly into the longitudinal manchette (LM); elements of both sets of manchette may occur concurrently, during this re-arrangement process.

Step 9: The LM is fully established, extending farther caudally into the trailing cytoplasm than the CM does. The function of the manchette is not clearly understood, but it is thought that it is responsible for shaping the nucleus. However, the acrosome appears as an elongated cone, and the perforatorium rod extends more deeply into the rostral part of the subacrosomal space. The perforatorium, *inter alia*, is thought to assist in elongating and supporting the acrosome, during its development (Baccetti *et al.*, 1980), but not all non-passerine birds possess this structure.

Step 10: The nucleus elongates further, and contains electron-dense, compactly packed, spherical chromatin. Mitochondrial elongation continues.

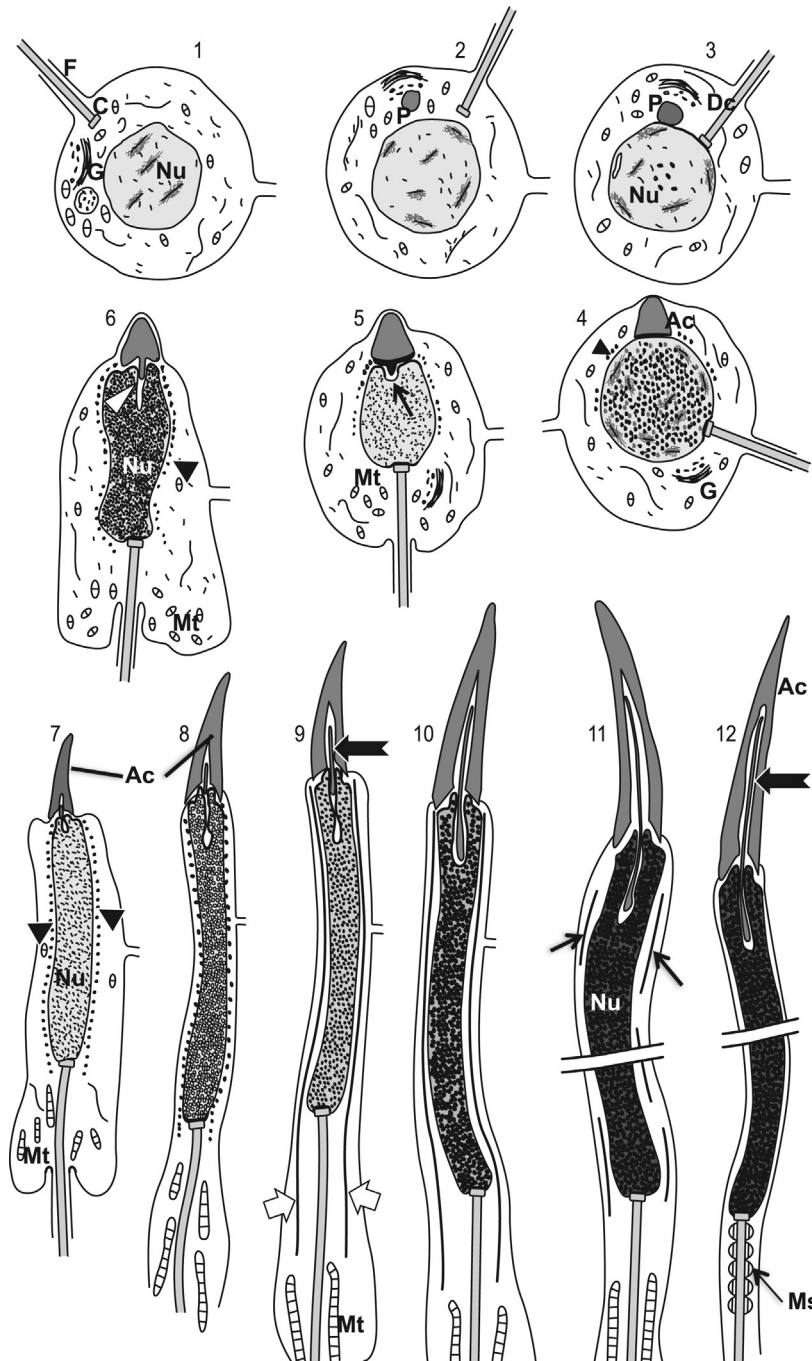


Fig. 3 A diagrammatic representation of the major changes that occur during spermiogenesis in non-passerine birds. The numerals represent the steps of spermiogenesis. Nu=nucleus, G=the Golgi complex, C=the centriolar complex, Mt=mitochondrion, F=flagellum, P=acrosomal granule, Dc=distal centriole, Ac=acrosome, dark arrowhead=microtubules of the circular manchette, white arrowhead=developing perforatorium and endo-nuclear canal, white broad arrows=longitudinal manchette, double dark arrows=disintegrating longitudinal manchette, Pp=principal piece of the flagellum, dark notched arrow=perforatorium rod, Ms=mitochondrial sheath. Not drawn to scale.

Step 11: The nuclear chromatin forms larger, compact, electron-dense granules. A major change in this step is the patchy break-up of the LM.

Step 12: During the last step of spermiogenesis the LM disappears completely, thus, allowing the elongated mitochondria to move toward the midpiece and form concentric rings around the distal centriole. The mitochondrial sheath, so formed, supplies energy for this motile cell. The spermatid becomes a mature spermatozoon, and ready for spermiation, which involves the movement of the mature spermatid toward the lumen of the seminiferous tubule, as the sleeves of cytoplasm of the Sertoli cell release

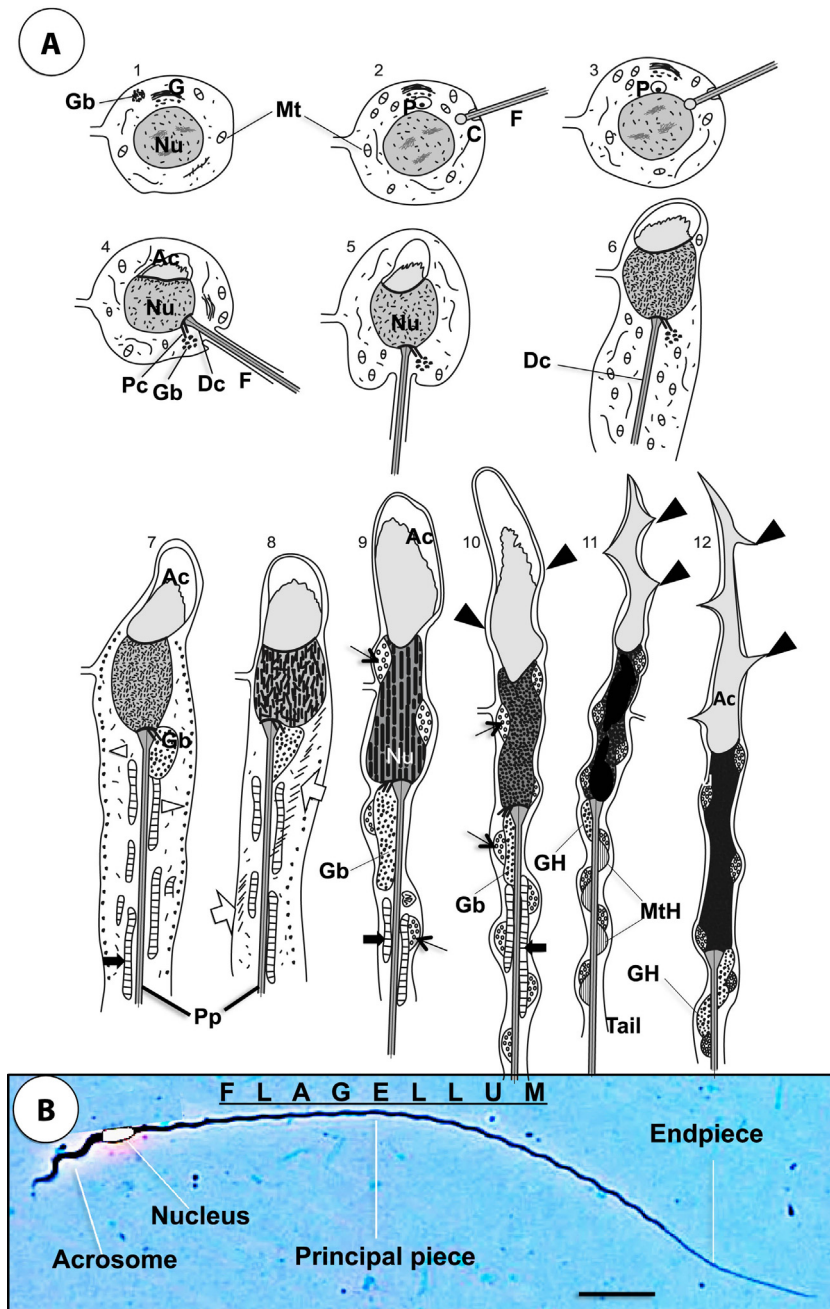


Fig. 4 (A). A diagrammatic representation of the major changes that occur during spermiogenesis in passerine birds. The numerals represent the steps of spermiogenesis. Nu=nucleus, G=the Golgi complex, Gb=granular body, Mt=mitochondrion, C=the centriolar complex, F=flagellum, P=acrosomal granule/vesicle, Ac=acrosome, Dc=distal centriole, Pc=proximal centriole, white arrowhead=cell membrane associated microtubules, block arrow=elongating mitochondria, Pp=principal piece of the flagellum, broad white arrow=microtubular bundles moving toward the axoneme, arrows=microtubular helix, dark arrowheads=developing keel of the acrosome, GH=granular helix, MTH=mitochondrial helix. (B). A phase-contrast view of a spiral-shaped spermatozoon of the passerine bird, *Quiscalus lugubris*, showing the various major parts. A is not drawn to scale. Bar scale in B is 10 μ m.

their hold on it. The redundant cytoplasm, containing unused organelles that include the Golgi complex and endoplasmic reticulum, becomes the *residual body*, which is phagocytized by Sertoli cells.

Passerine Spermiogenesis

There are several similarities as well as differences in the process of spermiogenesis between non-passerine and passerine birds. The organelle content and disposition of the round spermatid is similar between them, in most particulars, but a granular body, not

homologous to the chromatoid body of non-passerine birds, lies close to the Golgi and centriolar complexes in passerine birds. Both the proximal and distal centrioles are present in the spermatid, but the loss of the proximal centriole has been reported during spermiogenesis, in some passerine birds (Aire, 2014). The structure of the spermatozoon of the passerine bird has been reviewed recently (Aire *et al.*, 2017).

Steps of spermiogenesis

Step 1: The nucleus is round and contains fine granulofilamentous chromatin. A large Golgi complex, the centriolar complex, granular body, and mitochondria lie close together in the same region of the cytoplasm (Fig. 4). Numerous, small vesicles, proacrosomal vesicles, rather than granules, each with an encircled, smaller dense granular content proliferate from the trans-Golgi network zone. These are different from the homogeneous proacrosomal granules of non-passerine birds.

Step 2: Most of the proacrosomal vesicles coalesce to form a single, large, bipartite, acrosomal vesicle, comprising the wall or acrosomal crest, and its much smaller, dense granular content, the acrosomal core (Jamieson, 2007; Aire, 2014).

Step 3: The acrosomal vesicle, still linked with the Golgi complex, attaches to the nucleus, and the centriolar complex extends from its contact with the cell wall to the nucleus, attaching to it, close to the acrosomal vesicle. The annulus is very poorly developed or absent in passerine birds. The granular body lies close to or makes contact with the free end of the proximal centriole.

Step 4: The acrosome becomes conical in shape, with a broad dense acrosomo-nuclear junction. The centriolar complex, along with the granular body and Golgi complex, migrates from close to the rostral pole, toward the caudal pole, of the nucleus. The proximal and distal centrioles articulate at about 45° with each other, with the granular body attaching to the free end of the proximal centriole.

Step 5: The acrosome elongates and indents the euchromatic nucleus deeply. Concurrently, the cytoplasm, as in non-passerine birds, commences migration caudally, away from the nucleus.

Step 6: The acrosome displays signs of twisting in a spiral fashion. The nucleus, now shorter and goblet-shaped, contains electron-dense granular chromatin.

Step 7: The narrower nucleus elongates further. The mitochondria elongate considerably and align themselves, in four rows, alongside the axoneme. A set of obliquely aligned microtubules lie very close to the inner part of the cell wall; these microtubules are not homologous or analogous to the manchette of non-passerine birds. The axoneme, with the typical 9+2 microtubular configuration, develops fully. Unlike in non-passerine birds, there is no clear demarcation between the midpiece and principal piece, both of which have the dense outer fibers.

Step 8: The nucleus shortens, again, becoming dumb-bell shaped, and the chromatin forms variably arranged, dense, thick, rod-like strands. The granular body elongates alongside the distal centriole. Remarkably, the microtubules commence transformation into loose bundles that migrate toward the axoneme, in a helical fashion. The bundles extend from the acrosomo-nuclear junction into the caudally trailing cytoplasm. The acrosome is longer than the nucleus, and there is evidence of commencement of helical shaping.

Step 9: The helical formation of the acrosome increases, and the acrosomal core is, now, separated from the acrosomal crest, only rostrally. The acrosome also indents deeply a narrower and elongated nucleus containing thick strands of linearly arranged chromatin. The microtubular bundles form a compact microtubular helix around the nucleus, granular body, and the axoneme. The elongated strands of mitochondria join end-to-end, and fuse to form longer strands around the axoneme.

Step 10: The acrosome shows more pronounced alternating convexities and concavities as its spiraling process accentuates. The wall of the convexities is drawn out as a ridge, forming the 'helical membrane' or 'keel'. The acrosome is longer than the nucleus whose helical shape is also well established, as the chromatin forms denser, spherical, compactly packed granules.

Step 11: The acrosomal core completely fills the crest portion, including the keel, now sharply pointed. The chromatin of the helical nucleus is densely homogenous, unlike in most non-passerine birds. A single mitochondrial helix runs closely along with the microtubular helix. All other organelles, and possibly the proximal centriole, are in place and structurally definitive.

Step 12: This is the last step, and the organelles are refined and displayed as found in the spermatozoon; the spermatozoon is spirally shaped, the acrosome is longer than the nucleus, and the flagellum is extremely long, unlike in non-passerine birds. Spermatozoa undergo spermiation, as in non-passerine birds, but during this process some of them lose their microtubular helices, while others lose theirs thereafter, in the excurrent ducts of the testis.

General Remarks on Avian Spermatogenesis

Spermatogenesis is similar in vertebrates generally, with specific or unique differences between orders, families and even species. There are therefore differences in this phenomenon between the two main avian groups, passerine and non-passerine birds. Avian spermatogonial divisions are fewer, and therefore, fewer spermatozoa are produced per spermatogonium than in mammals, but spermatogenesis is much more rapid in birds. Acrosomogenesis produces a relatively short, conical, acrosomal complex comprising the acrosome and perforatorium in non-passerine birds, but a highly elongated, helical, bipartite, acrosome devoid of a perforatorium, and as long as, or longer than, the nucleus in passerine birds. The nucleus loses much of its volume, becomes highly elongated and narrow, and its chromatin ultimately condenses considerably in all birds. The distal centriole in non-passerine birds differentiates into a short rod in the mid-piece but it becomes extremely elongated in passerine birds, and not clearly demarcated from the considerably elongated principal piece of the flagellum. The proximal centriole is found in non-passerine birds generally, but

reported in spermatids and spermatozoa of only a few passerine birds. Passerine, but not non-passerine, spermatids develop five helically shaped organelles: acrosomal, nuclear, mitochondrial, granular, and microtubular helices, with the latter two structures being absent altogether in non-passerine birds.

References

- Aire, T. A. (2007a). Spermatogenesis and testicular cycles. In B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Birds*, 6A pp. 279–348). New Hampshire; Plymouth: Science Publishers, Inc (Phylogeny, morphology, hormones and fertilization).
- Aire, T. A. (2007b). Anatomy of the male reproductive tract and organs. In B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Birds*, 6A pp. 37–114). New Hampshire; Plymouth: Science Publishers, Inc (Phylogeny, morphology, hormones and fertilization).
- Aire, T. A. (2014). Spermiogenesis in birds. *Spermatogenesis*, 4(3), e959392. <https://doi.org/10.4161/21565554.2014.959392>.
- Aire, T. A., Du Plessis, L., Deokar, M. S., Rennie, E., & Gupta, S. K. (2017). Structural features of the spermatozoon of a passeridan bird, the Carib grackle, *Quiscalus lugubris*. *Tissue and Cell*, 49, 233–238.
- Baccetti, B., Bigliardi, E., & Burrini, A. G. (1980). The morphogenesis of vertebrate perforatorium. *Journal of Ultrastructural Research*, 71, 272–287.
- Clermont, Y. (1958). Structure de l'épithélium séminal et mode de renouvellement des spermatogonies chez le canard. *Archives d'Anatomie Microscopique et de Morphologie Experimentale*, 47, 47–66.
- Clermont, Y. (1972). Kinetics of spermatogenesis in mammals: Seminiferous epithelium cycle and spermatogonial renewal. *Physiological Reviews*, 52, 198–263.
- Culow, J., & Jones, R. C. (1982). Production, transport, maturation, storage and survival of spermatozoa in the male Japanese quail *Coturnix coturnix japonica*. *Journal of Reproduction and Fertility*, 64, 259–266.
- Courot, M., Hochereau-de Revers, M.-T., & Ortavant, R. (1970). Spermatogenesis. In A. D. Johnson, W. R. Gomes, & N. L. Vandemark (Eds.), *The Testis* (vol. I, pp. 339–432). New York, NY: Academic Press.
- de Revers, M., 1975. Sperm transport and survival in male birds. In: Hafez, E.S.E., Thibault, C.G. (Eds.), *The Biology of Spermatozoa*. In: INSERM International Symposium, Basel, Nouzilly: Karger, pp. 10–16.
- Hermo, L., Pelletier, R.-M., Cyr, D. G., & Smith, C. E. (2010). Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: Background to spermatogenesis, spermatogonia, and spermatocytes. *Microscopy Research and Techniques*, 73, 241–278.
- Jamieson, B. G. M. (2007). Avian spermatozoa: Structure and phylogeny. In B. G. M. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Birds*, 6A pp. 349–512). New Hampshire; Plymouth: Science Publishers, Inc (Phylogeny, morphology, hormones and fertilization).
- Leblond, C. P., & Clermont, Y. (1952). Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Annals of the New York Academy of Sciences*, 55, 548–573.
- Lin, M., & Jones, R. C. (1990). Spatial arrangement of the stages of the cycle of the seminiferous epithelium in the Japanese quail, *Coturnix coturnix japonica*. *Journal of Reproduction and Fertility*, 90, 361–367.
- Lin, M., & Jones, R. C. (1992). Renewal and proliferation of spermatogonia during spermatogenesis in the Japanese quail. *Cell and Tissue Research*, 267, 591–601.
- Marchand, C.-R., Gomot, L., & de Revers, M. (1977). Étude par autoradiographie et maaquage à la thymidine tritiée de la durée de la spermatogonèse du canard de barbarie (*Cairina moschata* L.). *Comptes Rendus des Seances, Societe de Biologie*, 21, 927–931.
- Mezquita, B., Mezquita, C., & Mezquita, J. (1998). Marked differences between avian and mammalian testicular cells in the heat shock induction and polyadenylation of Hsp70 and ubiquitin transcripts. *FEBS Letters*, 436, 382–386.
- Møller, A. P. (1991). Sperm competition, sperm depletion, paternal care, and relative testis size in birds. *American Naturalist*, 137, 882–906.
- Perey, B., Clermont, Y., & Leblond, C. P. (1961). The wave of the seminiferous epithelium in the rat. *American Journal of Anatomy*, 108, 47–77.
- Skinner, M. K., Norton, J. N., Mullaney, B. P., et al. (1991). Cell-cell interactions and the regulation of testis function. *Annals of the New York Academy of Sciences*, 637, 354–363.

The Insect Spermatozoon

Romano Dallai, University of Siena, Siena, Italy

© 2018 Elsevier Inc. All rights reserved.

Introduction

Insects are the largest and most diverse group of animals. Their richness is the result of several factors such as their relatively old origin (Pragian, 411–407 million years ago) (Whalley and Jarzembowski, 1981; Engel and Grimaldi, 2004), their morphological innovations making them highly adaptable to a variety of diets, the acquisition of wings, their capacity to tolerate variations of environments (Mayhew, 2007) and, more important, their impressive reproductive fitness allowing them to colonize suitable environments. Insects are characterized by a relatively short lifespan which facilitates the accumulation of mutations and a faster genetic divergence; this could explain their particular high speciation rate.

Insects display a great variability in sperm structure in comparison to other groups of animals. It was suggested that the great sperm diversification is the result of an intense sexual selection acting directly on the sperm form, even though we currently have only a rudimentary understanding of the adaptive significance of such variations (Sivinski, 1980; Pitnick *et al.*, 2009). A sperm competition between the ejaculates of different males at fertilization (polyandry) can also contribute to the sperm modifications (Parker, 1970, 1998), as well as after the sperm entry into the female genital tract, due to a sperm-female interaction (post-copulatory selection when polyandry is occurring), through which mechanisms enabling females to bias the outcome of sperm competition in favour of the sperm of certain males (cryptic female choice) (Eberhard, 1985, 1996) may be activated.

Insect Spermatogenesis

Insect testes contain several germ cyst cells each consisting of a certain number of diploid spermatogonia. After a variable number of mitosis, the cysts of primary spermatocytes are produced which move down along the testes and enter meiosis. At the end of the two meiotic divisions, haploid spermatids are formed, the number of which depends on the number of initial spermatocytes. After the first meiotic division the number of the secondary spermatocytes becomes double and the same occurs after the second meiotic division to produce spermatids. Thus, if there were 16 spermatocytes, at the end of the meiotic process we will obtain 64 spermatids. The number of spermatids, however, is reduced in those insects characterized by haplodiploidy and in those where parthenogenesis is a frequent mode of reproduction, with the males' haploid and meiotic process abolished. The same occurs in paternal genome elimination (PGE) (Dallai *et al.*, 1999, 2000; Normark, 2009). The number of spermatids may also depend on the size of germ cells with evident reduction when giant sperm are produced (Pitnick *et al.*, 2009). Contrary to the rule in other organisms, during insect spermatogenesis, the second meiotic division does not show centrosome (centriole+dense material surrounding it) duplication (González *et al.*, 1998; Callaini *et al.*, 1999) and the spindle exhibits only one centriole at each pole. Thus, at the end of meiosis, spermatids will receive only one centriole (Fig. 1(A) and (E)). The development of spermatids is syncytial, due to the presence of large intercellular bridges (ring canals) which allow the maintenance of synchronism among germ cells of a cyst. Spermatids only become individualized at the end of spermiogenesis. During the post-meiotic phase, transcriptional activity, at least in *Drosophila* males, is limited (Olivieri and Olivieri, 1965); thus, structural modifications occurring in spermiogenesis, depends on genes transcribed in primary spermatocytes.

Insect Spermiogenesis

The typical insect sperm is an elongated cell provided with an acrosome at the apical nuclear region, a long nucleus and a posterior long flagellum. The basal body, the centriole adjunct, the axoneme, the mitochondrial derivatives and the accessory bodies are present beneath the nucleus. All these sperm components are the results of the process of spermiogenesis which transforms the globular spermatids (Fig. 1(A)) into elongated sperm cells. The acrosome originates from the activity of the Golgi apparatus (Fig. 1(A)). It can be very elaborated and usually shows an inner "perforatorium" (Fig. 1(B)) of filamentous material containing actin, or it can be reduced to a simple apical vesicle (Fig. 1(C)) or completely missing. The nucleus, globular shaped at the beginning of spermiogenesis (Fig. 1(A)), becomes elongated, cylindrical shaped and with compact dense chromatin (Fig. 1(C)). During spermatid maturation, nuclear histones are replaced by basic structural proteins or protamines (Hecht, 1995) which neutralize the acidic charge of DNA, determining the chromatin compaction. The basal body derives from the centriole (Fig. 1(E)) after some modifications (Fig. 1(F)). It is involved in the production of the flagellar axoneme, which consists of a microtubular complex. As mentioned above, insect spermatids have only one centriole, but two or more centrioles can be found in some insects, due to a non-conventional spermatogenesis (Isoptera, Phthiraptera, Thysanoptera). The centriolar region usually exhibits a granular dense material surrounding the basal body, known as centriole adjunct (Fig. 1(F)), often extending along the flagellum in the shape of two dense structures, the accessory bodies, which are parallel to the axoneme. In the dipteran Diopsidae a prominent central dense band is present all along the flagellum. In the early spermatids, the centriole+dense material called pericentriolar material (PGM), constitute the centrosome. At the beginning of spermiogenesis, the centriole becomes the basal body and the proteins

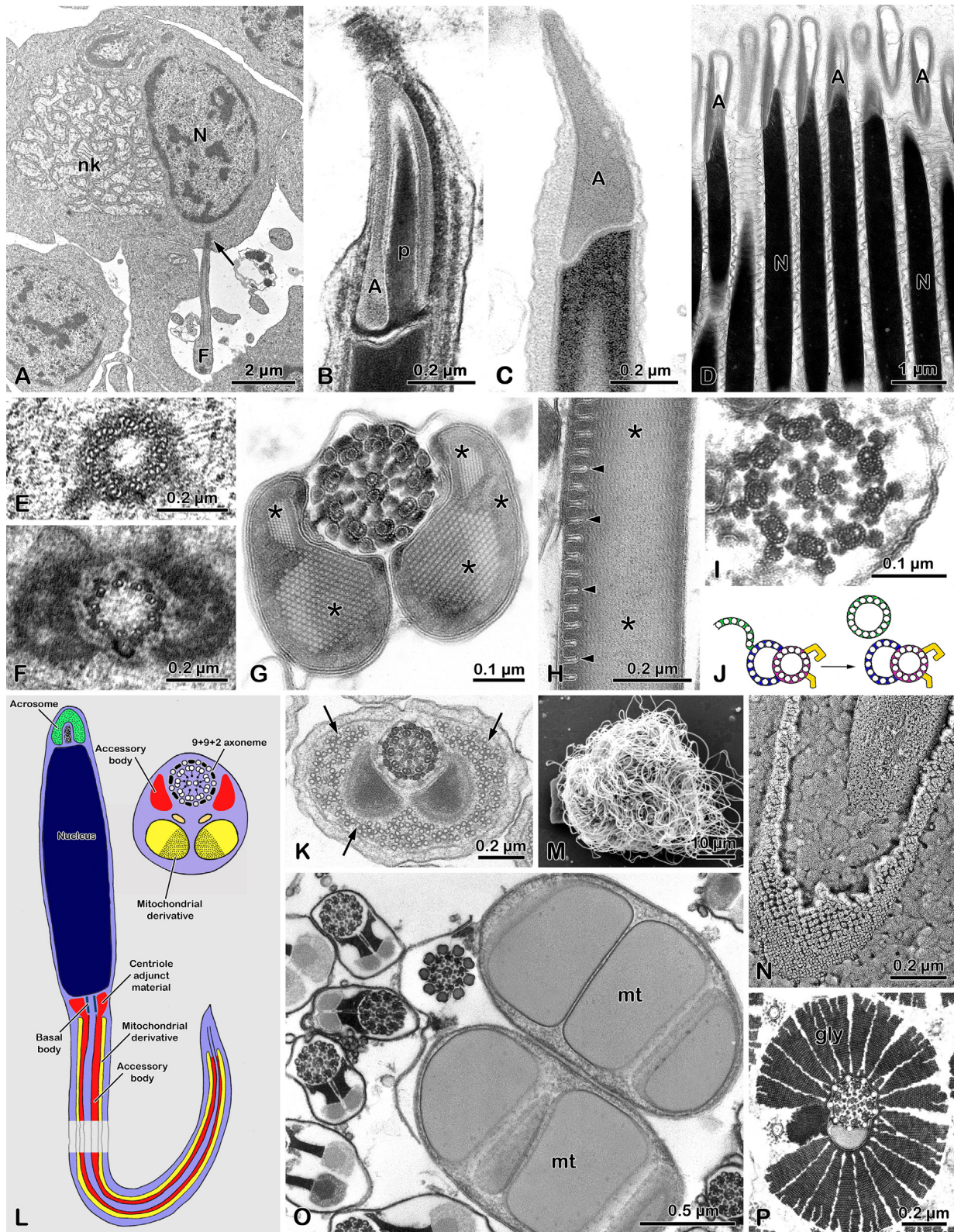


Fig. 1 (A) Spermatid of the phasmatodean *Timema poppensis* showing the “nebenkern” (nk), the nucleus (N), the basal body (arrow) producing the flagellum (F). (B) Acrosome of the coleopterian *Phyllobius pyri* with the acrosomal vesicle (A) and the perforatorium (p). (C) The simple acrosome (A) of the ephemeropteran *Cloeon dipterum*. (D) The sperm head of the neuropterid *Sialis lutaria* with the acrosome (A) and the nucleus (N). (E) The centriole in the phasmatodean *Timema poppensis* early spermatid. (F) The basal body, surrounded by the centriole adjunct material of the same species in the aged spermatid. (G) Cross section of the sperm flagellum of the heteropterian *Pyrrhocris apterus* with the axoneme 9+9+2 and the

of the PGM, γ -tubulin in particular, are involved in the production of numerous microtubules around the spermatid components for their elongation and shaping. During the spermiogenesis additional material is deposited beneath the nucleus to form the centriole adjunct (Dallai *et al.*, 2016a). The mitochondrial derivatives are the final result of the series of transformations, including aggregation and fusion, of spermatid mitochondria. After these processes, a “nebenkern” is formed (Fig. 1(A)) and, in turn, from this, two structures are produced which accompany the axoneme for most of its length (Fig. 1(G)). Transcripts encoding the proteins needed for mitochondrial morphogenesis are made in primary spermatocytes, but translation is delayed until the meiosis ends. Mitochondrial derivatives can be reduced to a single one or be greatly enlarged, as for instance in the zorapteran *Zorotypus impositus*, the neuropteran *Perlamantispa perla* (Fig. 1(P)), the coleopteran *Divaes bipustulatus*, and the dipterans *Diaemopsis* ssp (Kotrba *et al.* 2016). Mitochondrial derivatives have a dense matrix with a paracrystalline material (Fig. 1(G),(H) and (L)) containing the protein crystallomitin, characterized by a high content of proline and insoluble polypeptides with S-S disulphide cross-link. Differently from other animals, at fertilization, the whole sperm enters the egg, mitochondrial derivatives included, and these structures, with their paternal mt-DNA, are found almost intact after hatching and can be recovered in the faeces of the larva (Pitnick and Karr, 1998). The flagellar axoneme in the basal springtail Collembola, consists of a simple 9+2 (that is two central singlet microtubules, surrounded by a crown of 9 doublet microtubules) as an ancestral condition. This simple model is also present in some advanced orders, such as Mecoptera and Siphonaptera, as a derived condition acquired by reversal. Starting from the entognathan Diplura, through the ectognathan Archaeognatha and Zygentoma and up to all pterygote insects, the flagellar axoneme becomes more complex due to the presence of a set of outer accessory microtubules giving rise to a 9+9+2 axonemal model. The acquisition of this outer crown of tubules is an important synapomorphy of Insecta (=Ectognatha)+Diplura. The accessory tubules are formed during late spermiogenesis from the proliferation of the B-tubule of each microtubule doublet (Fig. 1(I) and (J)). The accessory tubules are more stable than the doublets due to the presence of associated proteins (MAPs) in their tubular walls. During the production of these tubules, a dense material is also deposited between two adjacent accessory tubules. As mentioned above, the elongation and shaping of the spermatid components is mediated by a series of cytoplasmic microtubules, which surround the different structures for all the spermiogenesis (Fig. 1(K)), but disappearing at the end of the process.

Insect Sperm Structure

The sperm is a specialized cell for reproduction. In insects, they usually are elongated cells, with an anterior head and a posterior tail (Fig. 1(L)). In *Drosophila bifurca* the sperm reaches up to 6 cm in length, 20 times the size of body specimen; in the deferent ducts the sperm rolled up to form filamentous balls (Fig. 1(M)). In the hymenopteran *Cotesia congregata*, the shortest sperm, only 7 μ m long, has been described so far. The sperm has a plasma membrane with a more or less complex glycocalyx. In some Orthoptera, it forms a dense thick layer with a complex inner organization, surrounding the sperm cell (Fig. 1(N)); in moths the glycocalyx is quite elaborated and consists of long laciniate appendages (Fig. 1(P)).

The sperm has an anterior acrosome the inner part of which, the perforatorium, can extend into the anterior axial nuclear region. The acrosome is rich in enzymes which can help the sperm entry the egg at fertilization. Moreover, in *Drosophila* ssp, the plasma membrane at the acrosomal level contains some glycosidases which serve for the sperm-egg interaction at fertilization. The α and β -acetylglucosaminidases are present on the sperm membrane of fertile males and the corresponding site of β -acetylglucosamine has been detected on the micropyle of the fruitfly egg (Perotti and Pasini, 1995). Glycosylation is retained of great importance for sperm-egg interaction.

The nucleus is elongated and contains compact chromatin material (Fig. 1(D)), unable to activate transcription. In some insects, such as Lepidoptera and the neuropteran *Perlamantispa perla*, characterized by heteromorphism, two types of sperm are present, the aberrant of which, is not involved in fertilization (Figs. 1(O) and 3(M); Jamieson *et al.*, 1999; Birkhead *et al.*, 2009).

Posterior to the nucleus, a centriolar region is adapted to host the basal body and the beginning of the flagellar components. The dense material of the centriole adjunct surrounds the basal body and the two mitochondrial derivatives. In a few examples, the material of the centriole adjunct is missing, but usually it extends along the flagellum on both sides of the axoneme adhering to

mitochondrial derivatives showing three crystallized areas (asterisks). (H) Longitudinal section of the mitochondrial derivative in the beetle *Tenebrio molitor* clearly showing the crystallization of matrix (asterisks) and the peripheral residual cristae (arrowheads). (I) The origin of the accessory tubules from a proliferation of the tubule B of each microtubule doublet in the axoneme of the dipluran *Campodea* sp. (J) Schematic drawing of the origin of the accessory tubules. (K) Cross section of aged spermatid in the heteropteran *Coptosoma scutellatum* showing the several flagellar components surrounded by many cytoplasmic microtubules (arrows). (L) Schematic drawing of the insect sperm in longitudinal and cross sections. (M) Scanning electron micrograph of the rolled long sperm of the dipteran *Drosophila bifurca*. (N) Freeze-drying preparation of the orthopteran *Pezzotettix giornai* sperm showing the elaborate glycocalyx. (O) Cross section of the two sperm types in the neuropterid *Perlamantispa perla*. Note the atypical giant sperm with large mitochondrial derivatives (mt). (P) The functional sperm of the moth *Apopestes spectrum* with the elaborate glycocalyx (gly) made up of long laciniate appendages. Parts (B)–(J), (N), (O) Reproduced from Dallai, R., 2014. Overview on spermatogenesis and sperm structure of Hexapoda. Arthropod Structure and Development 43, 257–290. (B) Dallai, R., *et al.*, 1988 Mus Reg Sci Nat Torino. (C) Dallai, R., *et al.*, 2007. Tissue and Cell 39. (D) Afzelius, B.A., Dallai, R., 1988. J Ultrastr Mol Struct 101. (E), (F) Gottardo, M., *et al.*, 2012. Zoomorphology 131. (G) Mercati, D., *et al.*, 2009. J Morphology 270. (L) Modified from Dallai, R., 2016. Ann Rev Entomol 61. (N) Lupetti, P., *et al.*, 2001. Ital J Anat Embryol 106 (Suppl.). (O) From Dallai, R., *et al.*, 2005. Tissue and Cell 37.

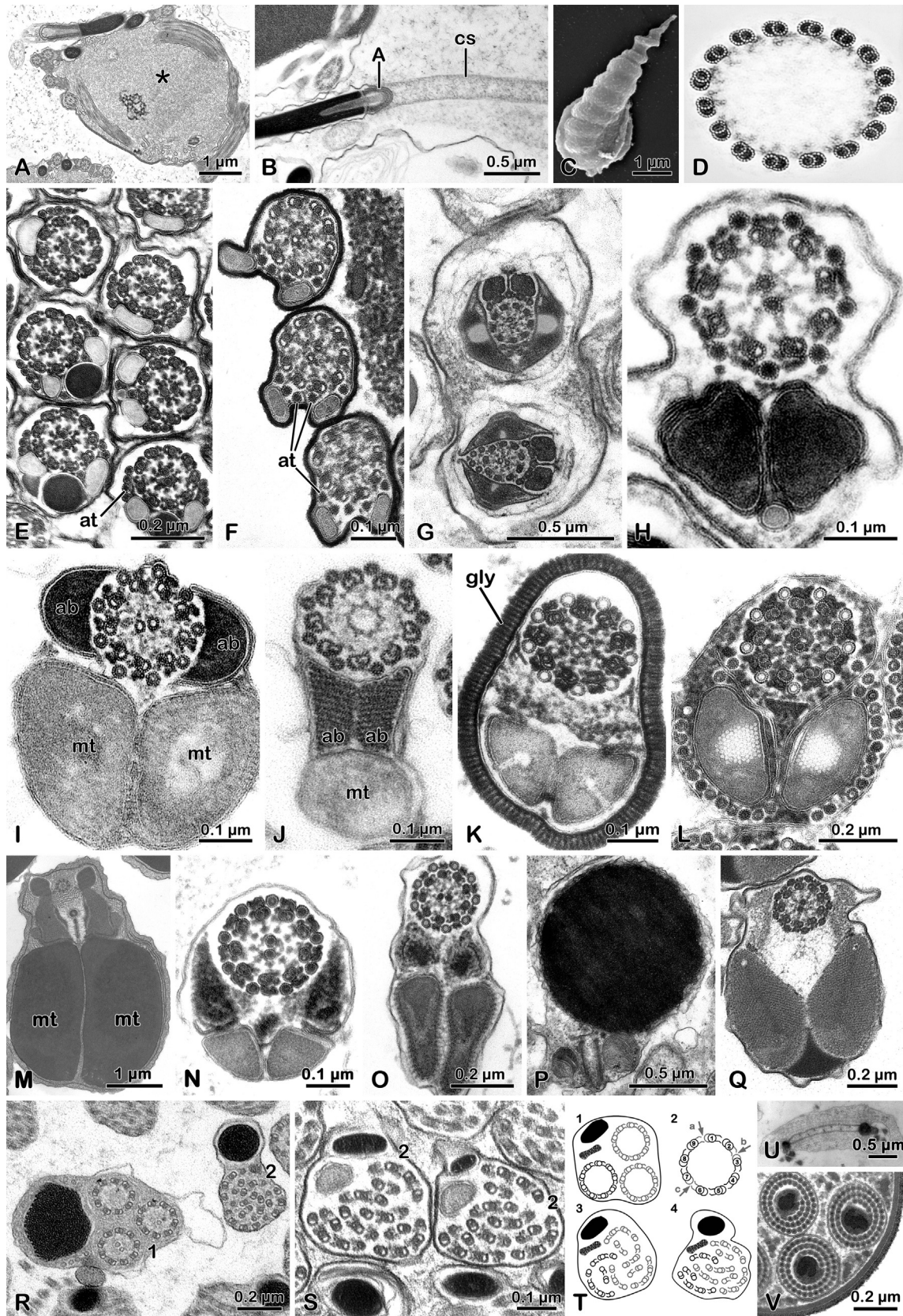


Fig. 2 (A) Cross section of the globular sperm of the collembolan *Allacma fusca*. Note the large extracellular cavity (asterisk) around which the sperm components are winding (B) Cross section of the apical long cylindrical structure (cs) adhering to the acrosome (A) of the collembolan *Bourletiella hortensis* sperm. (C) Scanning electron micrograph of the proturan *Acerentomon microrhinus* sperm. Note the spiral array of nucleus. (D) Electronic elaboration of the flagellar axoneme of the proturan *Acerentulus confinis* with 16 microtubule doublets provided with only dynein inner arms. (E)–(F) Cross sections of the flagellar axonemes of the diplurans *Campodea* sp. and *Japyx* sp., respectively, with the accessory tubules (at) on

the mitochondrial derivatives to constitute the accessory bodies (Fig. 1(L)). A direct connection of these structures to the centriole adjunct has been well ascertained (Dallai *et al.*, 2016a). The function of the centriole adjunct is not clear; it has been suggested that it provides reinforcement to the head-tail connection of the sperm or, together with the mitochondrial derivatives and the accessory bodies, it contributes to a better integration of the sperm components, making the sperm movement more uniform.

The flagellar axoneme of insects is the structure for the sperm motility. However, in several orders, there is the tendency towards a progressive degeneration up to the disappearance of the axoneme, with the consequent sperm immotility. This is evident in the basal apterygotan Protura, in the mayfly Ephemeroptera, in termites, among Hemiptera Sternorrhyncha, and within single families of Trichoptera (Hydroptilidae), of higher Coleoptera (Ptiliidae) and Diptera (Psychodidae and Cecidomyiidae). It has been suggested that the evolution of aflagellated and immotile sperm may be due to the removal of selective pressure generated by sperm competition (Morrow, 2004). The sperm movement is due to the interaction of the two arms present on the A-tubule of each microtubular doublet, with the B-tubules of the adjacent doublet. As known, such arms contain an adenosine triphosphatase (ATPase) that is responsible for the bending waves of live sperm tail (Satir, 1968; Summers and Gibbons, 1971). The binding and hydrolysis of ATP would cause a cyclic change in the angle of the arms which coordinated with the repeated making and braking of their attachment to successive binding site along the length of the B-tubule, would result in the sliding of one tubule along the other. Since the axonemal tubules are anchored within the cell body, the sliding is translated into bending of the flagellum. The loss of one dynein arm, as it occurs in some insect sperm, such as mayfly (Ephemeroptera) or caddisfly (Trichoptera) does not prevent the microtubule sliding and sperm movement which, instead, is lost when both arms are missing. In these cases, the subsequent developmental step is the loss of the whole flagellar axoneme with the sperm becoming immotile. A clear example of this process can be seen among the groups of the Hemiptera Sternorrhyncha (from Aphidoidea to Coccoidea). There are usually two mitochondrial derivatives of the same size and they accompany the axoneme for almost the whole its length (Fig. 1(L)); they end before the tail end. Sometimes they can be of different size or become giant structures as it occurs in the zorapteran *Zorotypus impolitus* (Fig. 2(M)), or in the not functional sperm of the neuropteran *Perlamantispa perla* (Fig. 1(O)), or to be missing altogether, as in the higher Phasmatodea. As mentioned above, the mitochondrial derivatives do not seem to be involved in some functional activities; they seem to play a passive role in sperm motility and after fertilization they maintain their structure apparently without modifications and are discharged almost intact with larval faeces. Their contribution could be to modulate the undulation of the axoneme. A similar function can also be suggested for the accessory bodies. In short sperm the accessory bodies are small in size or are lacking. According to Werner and Simmons (2008), sperm movement exhibits different levels of complexity: when the flagellum has a simple 9+2 axoneme, it shows a simple planar flagellar wave of low frequency; when sperm flagellum has a 9+9+2 axoneme, however, a double helical movement pattern is realized. This movement, which is the common pattern shared by insect sperm, consists of a small amplitude, high frequency wave which is superimposed on a high amplitude, low frequency wave (Werner and Simmons, 2008). In some insects a bidirectional movement has also been described, the sperm being able to perform forwards as well as backwards movement. Activation of sperm motility involves structural and metabolic changes to the sperm, or chemical stimuli leading to the initiation of motility. Such stimuli can be external agents which modify the flagellar membrane allowing seminal fluid components to entry the sperm cell (Osanai *et al.*, 1991), other than the pH, osmotic factors and the presence of oxygen (Werner and Simmons, 2008).

A synthetic overview on the sperm types exhibited by insects is extremely difficult as a great variation on the size and shape of their sperm components is quite common (Dallai, 2014; Dallai *et al.*, 2016b). In the basal hexapod Collembola (Springtails), a simple sperm with a flagellar axoneme of 9+2 type is present. The main characteristic of their sperm is realized at the end of spermiogenesis, when a flattened or globular sperm cell is produced, as the result of the nucleus and flagellum wrapping around an extracellular cavity filled with secretion. Moreover, a long extracellular cylindrical structure adheres to the acrosomal apex (Fig. 2(A) and (B)). An indirect sperm transfer by spermatophores, deposited by males on soil substrate and picked up by the female with its genital opening to transfer it into the spermatheca, is the normal mode of reproduction in this group. Protura

◀ a side of the 9+2 axoneme. (G) Cross section of the archaeognathan *Machilontus* sp. showing two sperm flagella within the same plasma membrane as a result of the flagellum bending. (H) Cross section of the zygentoman *Lepismodes inquilinus* sperm flagellum. (I) Cross section of the Odonata *Calopteryx* sp. sperm flagellum with two mitochondrial derivatives (mt) and two accessory bodies (ab). (J) Cross section of the mayfly *Cloeon dipterum* with a 9+9+0 axoneme sperm flagellum, two accessory crystallized bodies (ab), and a single mitochondrial derivative (mt). (K) Cross section of the orthopteran *Pezzotettix giornai* sperm flagellum with the elaborate glycocalyx (gly). (L) Cross section of the dermapteran *Forficula auricularia* sperm flagellum. (M) Cross section of the zorapteran *Zorotypus impolitus* giant sperm flagellum with the large mitochondrial derivatives (mt). (N) Cross section of the sperm flagellum in the Mantodea *Ameles spallanzanii*. (O) Cross section of the phasmatodean *Timema poppensis* sperm flagellum. (P) Cross section of the aflagellated sperm in the termite *Reticulitermes lucifugus*. (Q) Cross section of the blattodean *Cryptocercus* sp. sperm flagellum. (R)–(S) Cross sections of the sperm flagella in the thrips *Haplothrips simplex* and *Frankliniella occidentalis*, respectively. Note the presence of three axonemes in the early spermatids (1) and their amalgamation in the sperm (2). (T) Schematic reconstruction of the axoneme modifications occurring during thrips spermiogenesis (1–4). (U) Cross section of the flattened sperm flagellum in the Auchenorrhyncha *Psylla pyri*. (V) Cross section of the coccid *Planococcus* sp. (Sternorrhyncha) sperm flagellum with concentric rings of single microtubules surrounding the axial nucleus. Parts (A), (C)–(E), (G)–(I), (K), (M), (O), (R)–(T): Reproduced from Dallai, R., 2014. Overview on spermatogenesis and sperm structure of Hexapoda. Arthropod Structure and Development 43, 257–290. (H) Dallai, R., *et al.*, 1992. Acta Zool 73. (K) Lupetti, R., *et al.*, 2001. Ital J Anat Embryol 106 (Suppl.). (M) Dallai, R., *et al.*, 2012. Arthropod Structure and Development 41. (O) Gottardo, M., *et al.*, 2012. Zoomorphology 131. (R)–(T) Paccagnini *et al.*, 2007, Cell Motility 64. (U) Dallai, R., 1979. In: Fawcett, D.W., Bedford J.M. (eds.). The Spermatozoon. Baltimore: Urban and Schwarzenberg Inc., pp. 253–265. (V) Paoli *et al.*, 2015. Zool Anz 258. Reproduced from Paoli *et al.*, 2015. Zool Anz 258.

(Coneheads) have an aberrant motile sperm with a flagellar axoneme devoid of central tubules and with several peripheral microtubule doublets showing only inner dynein arms (Fig. 2(C) and (D)). Aflagellated immotile sperm are also present in some families. Diplura (Two-pronged bristletails), like all Ectognatha, have acquired 9 accessory outer microtubules external to the 9+2; these tubules, however, shift from their regular position close to the doublets to one side of the axoneme at the end of spermiogenesis (Fig. 2(E) and (F)). The accessory microtubules have also been acquired by Archaeognatha (Bristletails) (=Microcoryphia) and Zigentoma (Silverfish). The former group has a flagellum folded back at half its length, giving the wrong impression of a sperm with two axonemes in the same cytoplasm. Moreover the accessory microtubules, as in Diplura, are placed on two opposite sides of the 9+2 complex (Fig. 2(G)). Zygentoman sperm, to the contrary, is of a conventional type (Fig. 2(H)). The sperm are usually conjugated pairwise and in *Tricholepidion gertschi* fuse mature sperm form binucleate, biflagellate units. The Paleoptera, Odonata (Dragonflies and Damselflies) and Ephemeroptera (Mayflies), exhibit quite a different sperm structure, even though from phylogenetic perspective the two groups are closely related. Ephemeroptera sperm axonemes have lost central tubules and the microtubule doublets have only inner arms (Fig. 2(I)). Aflagellated immotile sperm have been described in the group. Odonata, to the contrary, have a typical insect sperm with a 9+9+2 axoneme flanked by two mitochondrial derivatives and two accessory bodies (Fig. 2(J)). The sperm structure is highly variable among Neoptera. Polyneoptera, the group comprising about one third of the pterygote orders, evolved sperm characters independently in each major lineage, possibly as a consequence of the extreme diversity in their ecophysiological features. Among this group, the large order Orthoptera (Grasshoppers, Locust, Katydid) (Fig. 2(K)), Plecoptera (Stoneflies), Embioptera (Webspinners), Dermaptera (Earwigs) (Fig. 2(L)), as well as the minor groups, such as Zoraptera (Groundlice) (Fig. 2(M)), Mantophasmatodea (Heelwalkers), and Grylloblattodea (Ice Crawlers) show an almost typical insect sperm. Mantodea (Praying Mantises) sperm are characterized by a flagellar axoneme with an extended intertubular material (Fig. 2(N)); Phasmatodea (Stick Insects), except for the basal *Timema poppensis*, which retains a typical insect sperm (Fig. 2(O)), have a flagellum without mitochondrial derivatives, substituted by two elongated, crystalline accessory bodies. Isoptera (Termites), which are a subordinate group of Blattodea, with the exception of the multiflagellated sperm of *Mastotermes darwiniensis*, exhibit aflagellated immotile sperm (Fig. 2(P)). Blattodea (Cockroaches) have a conventional insect sperm type (Fig. 2(Q)). Among Acercaria (=Paraneoptera), Thysanoptera (Thrips) have three centrioles at the beginning of spermiogenesis, which give rise to three axonemes (Fig. 2(R)). Later these axonemes fuse and amalgamate to form a single motile flagellum with a microtubular complex consisting of 27 units (Fig. 2(S) and (T)) consisting of doublet and singlet microtubules, without accessory tubules (Fig. 2(S) and (T)). Phthiraptera (Lice), and some species of Psocoptera (Barklice, Booklice) have two flagellar axonemes. Heteroptera (True Bugs), have a conventional insect sperm, often exhibiting a sperm heteromorphism with sperm of different length. Characteristic of their sperm flagellum is the presence of multiple points of crystallization in the matrix of the mitochondrial derivative and the lack of accessory bodies (Fig. 1(G)). Hemiptera, are one of the megadiverse insect lineages: Auchenorrhyncha (Cicadas, Leafhoppers, Spittlebugs, etc.) have a typical sperm pattern in many species, but modifications can occur; in Membracidae and Jassidae the posterior sperm region is flattened and microtubule doublets and accessory tubules are arranged in straight row (Fig. 2(U)). In some genera, the distal part of the flagellum divides into several branches. Sternorrhyncha (Plant Lice, Aphids, Jumping Plant Lice, Whiteflies, Mealybugs and Scale Insects) show an evolutive trend with a progressive involution of the flagellar axoneme, starting from Aphidoidea, provided with a conventional sperm, toward Coccoidea, which have lost the acrosome and the mitochondria, and the sperm flagellum has recovered motility due to the presence of a bundle of singlet microtubules (Fig. 2(V)). Among Holometabola (=Endopterygota), Hymenoptera (Sawflies, Wasps, Ants, Bees) is a large order thought to be the sister group of the rest of the Holometabola orders. (Beutel *et al.*, 2011; Peters *et al.*, 2014). Their sperm structure conforms to the typical pterygote model (Fig. 3(B)). The sperm leave testes in bundles (spermatodesms) as occurs in "Symphyta" (Fig. 3(A)) or may become free as in Apocrita. Sperm association has not been found in parasitic wasps and this character has been retained as an apomorphy, whereas the sperm association in bundle would represent the ancestral condition. Neuropteroida comprises two main groups, Coleoptera and Strepsiptera on one hand, and Neuroptera (Lace Wings), Megaloptera (Alderflies) and Raphidioptera (Camelneck Flies) on the other. Neuropteroida are related to Coleoptera based on the presence of a well-developed accessory bodies and the shape and extension of the intertubular material between accessory tubules in the axoneme (compare Fig. 2(C) and (D)). Strepsiptera sperm are almost typical, as are those of Coleoptera (Beetles), the largest insect order. The two groups are closely related. Within Coleoptera, some families such as Attelabidae and Rhynchitidae share the common flagellar axoneme of 9+9+0 type, while in some species of Ptiliidae the sperm are aflagellated and immotile. In the clerid *Divaes bipustulatus* the mitochondrial derivatives are greatly enlarged, with the sperm becoming giant (Mazzini, 1976). Also Neuroptera exhibit several sperm models. Coniopterygidae have a 9+9+3 axoneme and Mantispidae exhibit both conventional and giant sperm, these latter due to the great size of the mitochondrial derivatives and of the accessory tubules (Fig. 1(O)). Mecoptera (Scorpionflies) (Fig. 3(E)) and Siphonaptera (Fleas) (Fig. 3(F)) are closely related groups showing a simple 9+2 axoneme, as a clearly derived condition by reversal; in the mecopteran *Panorpodes* sp., in fact, the sperm axoneme is a typical 9+9+2. Diptera (Flies) is a large insect order with a great variety of sperm structures dealing in particular with the axoneme. Species of the suborder Nematocera show numerous aberrant models either with regard to the acrosome and the flagellar axoneme. Some basal Keroplatidae and Mycetophilidae have a typical insect sperm axoneme (Fig. 3(G)), but in related families, such as Culicidae, the axoneme has only one central tubule, thus becoming a 9+9+1 model, while in Bibionidae axoneme, the central tubule is missing. Among Psychodidae, Phlebotominae have flagellate motile sperm, but Psychodinae have lost the axoneme and the sperm has become immotile. The large Cecidomyiidae family have the largest

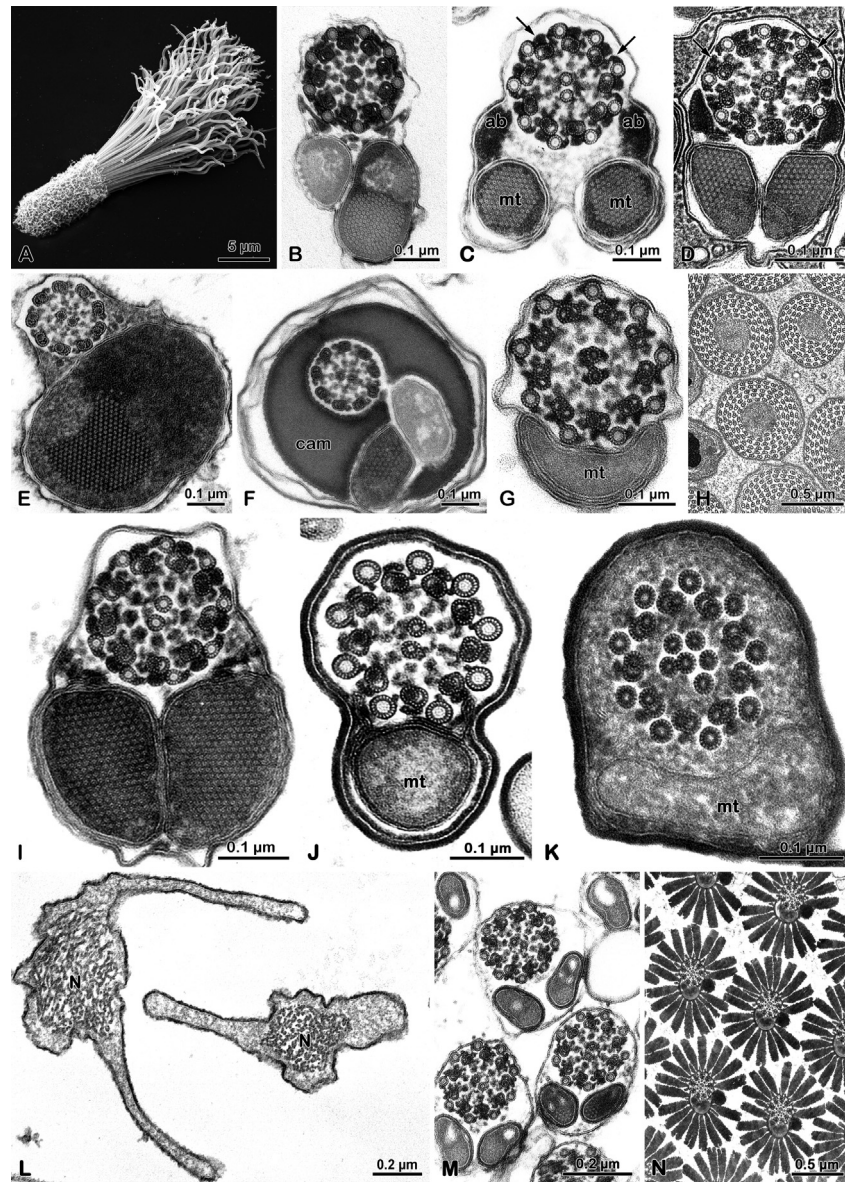


Fig. 3 (A) Scanning electron micrograph of the spermatodesm in the hymenopteran *Arge pagana*. (B) Cross section of the hymenopteran *Vespa crabro* sperm flagellum. (C) Cross section of the neuropterid *Libelloides* sp. sperm flagellum with evident two accessory bodies (ab) and two mitochondrial derivatives (mt). Intertubular material (arrows). (D) Cross section of the coleopteran *Ptilophorus dufouri* sperm flagellum. Note the similar shape of the intertubular material (arrows) between this and the previous species. (E) Cross section of the mecopteran *Panorpa* sp. sperm flagellum with a simple 9+2 axoneme. (F) Cross section of the flea *Ctenocephalides canis* sperm flagellum with a 9+2 axoneme surrounded by a prominent centriole adjunct material (cam). (G) Cross section of the nematoceran Diptera *Neoplatyura nigricauda* sperm flagellum with a single mitochondrial derivative (mt). (H) Cross section of the gall-midge fly (Diptera, Nematocera) *Bremia* sp. sperm flagella showing axonemes with many microtubule doublets, each provided with only outer arms. (I) Cross section of the brachyceran Diptera *Ceratitis capitata* sperm flagellum. (J) Cross section of the trichopteran Integripalpia *Stenophylax permistus* sperm flagellum with a single mitochondrial derivative (mt). (K) Cross section of the aberrant, flagellated but immotile sperm in the trichopteran Annulipalpia *Philopotamus montanus* due to the lack of dynein arms on the microtubule doublets. Mitochondrial derivative (mt). (L) Cross section of the aflagellated sperm in the trichopteran Annulipalpia Hydroptilidae *Stactobia caspersi*. Nucleus (N). (M)–(N) Cross sections of the moth sperm flagella in the *Gonopteryx* sp. and *Apopestes spectrum*, respectively. In the apyrenic (without nucleus), not functional sperm, of the former species the extended glycocalyx is lacking, while this is present in the second eupyrenic (with nucleus) functional species. Parts (A)–(D), (G), (I)–(N) Reproduced from Dallai, R., 2014. Overview on spermatogenesis and sperm structure of Hexapoda. Arthropod Structure and Development 43, 257–290. (A) Lino Neto *et al.*, 2008. Tissue and Cell 40. (B) Mancini, I., *et al.*, 2009. Arthropod Structure and Development 38. (D), (I)–(N) Jamieson, B.G.M., *et al.*, 1999. Insects, Their Spermatozoa and Phylogeny. Enfield, NH; Plymouth: Science Publishers, 555 pp. (E) Dallai, R., *et al.*, 2003. Zoomorphology 122. (G) Dallai, R., *et al.*, 1995. Zoomorphology 115. (H) Dallai, R., *et al.*, 1997. Acta Zool 78.

variety of flagellar axoneme described to date. Among some species of Micromyidi the axoneme has the peculiar 10+0 model, as also have the Stomatosematidi; in the Asphondylii and Cecidomyiidi the motile flagellar axoneme has a great number of microtubule doublets (Fig. 3(H)), up to two thousand, with the unique finding among insects to have only the outer dynein arm on each microtubular doublet. The sperm axoneme is reminiscent of one present in the related family Sciaridae. To the contrary, the species of the suborder Brachycera have an almost uniform sperm structure, but the sperm flagellum lacks accessory bodies (Fig. 3(I)). In the Diopsidae sperm, however, a central dense band extends all along the sperm tail. Among Brachycera, *Drosophila bifurca* has the longest sperm described so far. Within the group, a sperm heteromorphism has also been described. The same unexpected sperm difference, is present between the sperm structure of Trichoptera (Caddisflies) and Lepidoptera (Moths and Butterflies). In the former order, the Integripalpia exhibit a few modifications dealing with the presence of only the inner arm on the microtubular doublets and the presence of a single mitochondrial derivative (Fig. 3(J)); the Annulipalpia, instead, have several aberrant immotile sperm. In Polycentropodidae and Philopotamidae the doublets are always without arms and therefore the sperm are immotile (Fig. 3(K)). Hydropsychidae have a bizarre sperm provided with finger-like appendages extruding from the cell body and containing doublet microtubules. In the philopotamid *Chimarra florida* and some species of Hydroptilidae, the axoneme degenerate during spermiogenesis or it is absent and the sperm become immotile (Fig. 3(L)). To the contrary, Lepidoptera have a quite uniform sperm structure, with the main characteristic consisting in the presence of an expanded glycocalyx and the occurrence of two types of sperm: apyrenic and eupyrenic sperm, the former without the nucleus and evident glycocalyx (Fig. 3(M) and (N)). It has been suggested that apyrenic sperm may maximize the first male's fitness by delaying female remating behaviour (Snook, 1997).

A phylogenetic analysis of the whole group has been elaborated based on the several sperm characters described in the insect orders (Dallai *et al.*, 2016b; Gottardo *et al.*, 2016). Sperm characters strongly support the phylogenetic position of several major lineages, for instance Diptera and Insecta, Zygentoma and Pterygota, Psocoptera and Phthiraptera. To the contrary, some clearly related groups, such as Trichoptera and Lepidoptera exhibit a greatly different sperm structure. Some orders, have a very conservative sperm (Heteroptera and Odonata), while others show a highly variable sperm morphology (Diptera).

References

- Beutel, R. G., Friedrich, F., Hörschemeyer, T., *et al.* (2011). Morphological and molecular evidence converge upon a robust phylogeny of the megadiverse Holometabola. *Cladistics*, 27, 341–355.
- Birkhead, T. R., Hosken, D. J., & Pitnick, S. (2009). *Sperm Biology: An Evolutionary Perspective*. Oxford: Academic Press.
- Callaini, G., Riparbelli, M. G., & Dallai, R. (1999). Centrosome inheritance in insects: Fertilization and parthenogenesis. *Biology of the Cell*, 91, 355–366.
- Dallai, R. (2014). Overview on spermatogenesis and sperm structure of Hexapoda. *Arthropod Structure and Development*, 43, 257–290.
- Dallai, R., Fanciulli, P. P., & Frati, F. (1999). Chromosome elimination and sex determination in springtails (Insecta, Collembola). *Journal of Experimental Zoology. Part B, Molecular and Developmental Evolution*, 285, 215–225.
- Dallai, R., Fanciulli, P. P., & Frati, F. (2000). Aberrant spermatogenesis and the peculiar mechanism of sex determination in *Symphyleonan Collembola* (Insecta). *Journal of Heredity*, 91, 351–358.
- Dallai, R., Gottardo, M., & Beutel, R. G. (2016b). Structure and evolution of insect sperm: New interpretations in the age of phylogenomics. *Annual Review of Entomology*, 61, 1.1–1.23.
- Dallai, R., Paoli, F., Mercati, D., & Lupetti, P. (2016a). The centriole adjunct of insects: Need to update the definition. *Tissue and Cell*, 48, 104–113.
- Eberhard, W. G. (1985). *Sexual Selection and Animal Genitalia*. Cambridge, Mass: Harvard University Press.
- Eberhard, W. G. (1996). *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton, NJ: Princeton University Press.
- Engel, M. S., & Grimaldi, D. (2004). New light shed on the oldest insect. *Nature*, 427, 627–630.
- González, C., Tavosanis, G., & Mollinari, C. (1998). Centrosomes and microtubule organization during *Drosophila* development. *Journal of Cell Science*, 111, 2697–2706.
- Gottardo, M., Dallai, R., Mercati, D., Hörschemeyer, T., & Beutel, R. G. (2016). The evolution of insect sperm – An unusual character system in a megadiverse group. *Journal of Zoological Systematics and Evolutionary Research*, 54, 237–256.
- Hecht, N. B. (1995). The making of a spermatozoon: A molecular perspective. *Developmental Genetics*, 16, 95–103.
- Jamieson, B. G. M., Dallai, R., & Afzelius, B. A. (1999). *Insects. Their Spermatozoa and Phylogeny*. New Hampshire: Science Publisher.
- Kotrba, M., Heß, M., & Dallai, R. (2016). Giant spermatozoa of *Diasemopsis* (Diopsidae, Diptera)-Structural, ultrastructural and functional aspects. *Arthropod Structure and Development*, 45, 42–56.
- Mayhew, P. J. (2007). Why are there so many insect species? Perspectives from fossils and phylogenies. *Biological Reviews*, 82, 425–454.
- Mazzini, M. (1976). Giant spermatozoa in *Divaes bipustulatus* F. (Coleoptera, Cleridae). *International Journal of Insect Morphology and Embryology*, 5, 107–115.
- Morrow, E. H. (2004). How the sperm lost its tail: The evolution of aflagellated sperm. *Biological Reviews*, 79, 795–814.
- Normark, B. B. (2009). Unusual gametic and genetic systems. In T. R. Birkhead, D. J. Hosken, & S. Pitnick (Eds.), *Sperm Biology: An Evolutionary Perspective* (pp. 507–538). Oxford: Academic Press.
- Olivieri, G., & Olivieri, A. (1965). Autoradiography study of nucleic acid synthesis during spermatogenesis in *Drosophila melanogaster*. *Mutant Researches*, 2, 366–380.
- Osanai, M., Kasuga, H., & Aigaki, T. (1991). Motility-related ultrastructural changes in the flagellar membrane of apyrene spermatozoa of the silkworm *Bombyx mori* induced by Arg.-C endopeptidases. *Invertebrate Reproduction and Development*, 19, 193–202.
- Parker, G. A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological Review Cambridge Philosophical Society*, 45, 525–567.
- Parker, G. A. (1998). Sperm competition and the evolution of ejaculates: Towards a theory base. In T. R. Birkhead, & A. P. Møller (Eds.), *Sperm Competition and Sexual Selection* (pp. 3–54). San Diego: Academic Press.
- Perotti, M. E., & Pasini, M. E. (1995). Glycoconjugates of the surface of the spermatozoa of *Drosophila melanogaster*: A qualitative and quantitative study. *Journal of Experimental Zoology*, 271, 311–318.
- Peters, R. S., Meusemann, K., Petersen, M., *et al.* (2014). The evolutionary history of holometabolous insects inferred from transcriptome-based phylogeny and comprehensive morphological data. *BMC Evolutionary Biology*, 14(1) [art. no. 52].

- Pitnick, S., Hosken, D. J., & Birkhead, T. R. (2009). Sperm morphological diversity. In T. R. Birkhead, D. J. Hosken, & S. Pitnick (Eds.), *Sperm Biology: An Evolutionary Perspective* (pp. 69–149). Oxford: Academic Press.
- Pitnick, S., & Karr, T. L. (1998). Paternal products in *Drosophila* development. *Proceedings of the Royal Society of London, Series B, Biological Sciences*, 265, 821–826.
- Satir, P. (1968). Studies on cilia. III. Further studies on the cilium tip and a "sliding filament" model of ciliary motility. *Journal of Cell Biology*, 39, 77–94.
- Sivinski, J. (1980). Sexual selection and insect sperm. *Florida Entomologist*, 63, 99–111.
- Snook, R. R. (1997). Is the production of multiple sperm types adaptive? *Evolution*, 51, 797–808.
- Summers, K. E., & Gibbons, I. R. (1971). Adenosine triphosphate-induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. *Proceedings of the National Academy of Sciences*, 101, 12865–12869.
- Werner, M., & Simmons, L. W. (2008). Insect sperm motility. *Biological Review Cambridge Philosophical Society*, 83, 191–208.
- Whalley, P., & Jarzembowski, E. A. (1981). A new assessment of *Rhyniella*, the earliest known insect, from the Devonian of Rhynie, Scotland. *Nature*, 291, 317.

Spermatogenesis and Spermiogenesis in Crustaceans

Günter Vogt, University of Heidelberg, Heidelberg, Germany

© 2018 Elsevier Inc. All rights reserved.

Introduction

Crustaceans are a highly diverse animal group with respect to morphology, physiology, life history, and ecological adaptation. They comprise more than 67,000 species, including water fleas, copepods, ostracods, barnacles, amphipods, isopods, shrimps, lobsters, crayfish, and crabs. Their adult size ranges from 0.1 mm to almost 4 m (leg span) and their maximum age ranges from a few days to more than 70 years. All of the longer-lived crustaceans are iteroparous, i.e., they undergo multiple reproductive cycles over the course of their lifetime. Most species are free living but some are parasitic. Crustaceans occur in a broad variety of marine, limnic and terrestrial habitats from deep sea trenches to high mountain areas and from polar regions to the tropics. Accordingly diverse are their reproductive strategies, sperm types, and fertilization mechanisms (Vogt, 2016). In the following, germ line specification, spermatogenesis, spermiogenesis, sperm structure, and capacitation and activation of the sperm are described for selected taxa, highlighting some peculiarities unique to the Crustacea.

Sperm Types

Most higher taxa of the Crustacea have evolved aflagellate sperm types (Jamieson, 1991). Flagellate spermatozoa are found in a few “lower” crustacean groups (Entomostraca) including the basal Remipedia and the Cirripedia (Fig. 1(A)). The aflagellate spermatozoa are small rods (Fig. 1(B)) and discs of a few micrometer, larger globules with apical spikes or lateral arms (Fig. 1(C)), inverted umbrellas (Fig. 1(D)), or giant filiform tubes up to 1.2 cm in length (Fig. 1(E)). Some crustacean spermatozoa are cytologically very simple, consisting only of the nucleus and a small rim of cytoplasm like in Euphausiacea (krill). Others are highly complex including heterogeneous acrosomes (Fig. 2(E)) like in Decapoda (shrimps, lobsters, crayfish, and crabs) or unique cell organelles like in Ostracoda (mussel shrimps).

Germ Line and Testis Anlage

The primordial germ cells of crustaceans arise either by preformation or epigenesis (Extavour, 2005). Preformation is characterized by the asymmetric localization of maternal factors in the early cleavage stages, which determine the cell lineage that produces the primordial germ cells. Epigenesis describes the determination of germ cells via inductive signals from surrounding tissues later in development. Preformation is probably the plesiomorphic condition in crustaceans. It occurs in taxa associated with total cleavage such as the cladocerans, ostracods, copepods, cirripeds, and amphipods. Epigenesis is the derived condition and is typical of the superficially cleaving “higher” crustaceans (Malacostraca) including the isopods and decapods.

Development of the germ line and formation of the gonad anlage is rather well investigated in the amphipod *Parhyale hawaiiensis*, a crustacean model of development (Extavour, 2005). The germ line originates from a single blastomere of the 8-cell stage, the primordial germ cell (PGC). It is characterized by the RNA helicase Vasa, a general germ line marker of animals. Vasa protein is not required for the generation of the germ cells in the sand hopper but for their proliferation and maintenance. The PGCs multiply during the germ disc stage and are arranged in a row perpendicular to the longitudinal axis in the elongated germ band stage (Fig. 1(F)). At the beginning of limb development, the PGCs separate into right and left clusters and migrate then dorsolaterally. A few days before hatching they reach their final destination on either side of the heart primordium, which forms along the dorsal mid-line of the embryo. Finally, the PGC clusters elongate into rows of cells that extend over two thoracic segments. Together with surrounding soma cells they form the gonad anlage.

Freshwater crayfish are an example of epigenetic germ line determination. During early development, only the cell nuclei multiply and are homogeneously dispersed across the surface of the embryo. Cell membranes are formed from the 256-nuclei stage. It is not yet known when the first PGCs appear but in late embryos there is at least one larger PGC present on each body side underneath the dorsally located heart. Together with a thin layer of surrounding soma cells they form the paired gonad anlage (Taketomi et al., 1996). The gonad is specified as testis or ovary only after hatching. In genetic males, an insulin-like hormone from the androgenic gland induces development of the gonad anlage into a testis and transformation of the PGCs into primary spermatogonia. In the absence of the androgenic hormone, the gonad anlage develops into an ovary (Krol et al., 1992). During the early juvenile stages, the two testis anlagen fuse into a single Y-shaped organ that differentiates into testicular acini and collecting ducts. At the same time, the primary spermatogonia multiply. In the mid-period of juvenile development, spermatocytes and spermatids appear in the seminiferous epithelium of the testicular acini, and the testis becomes linked to the gonopores by paired vasa deferentia. In the final juvenile stages, the lumina of the acini get increasingly filled with spermatozoa.

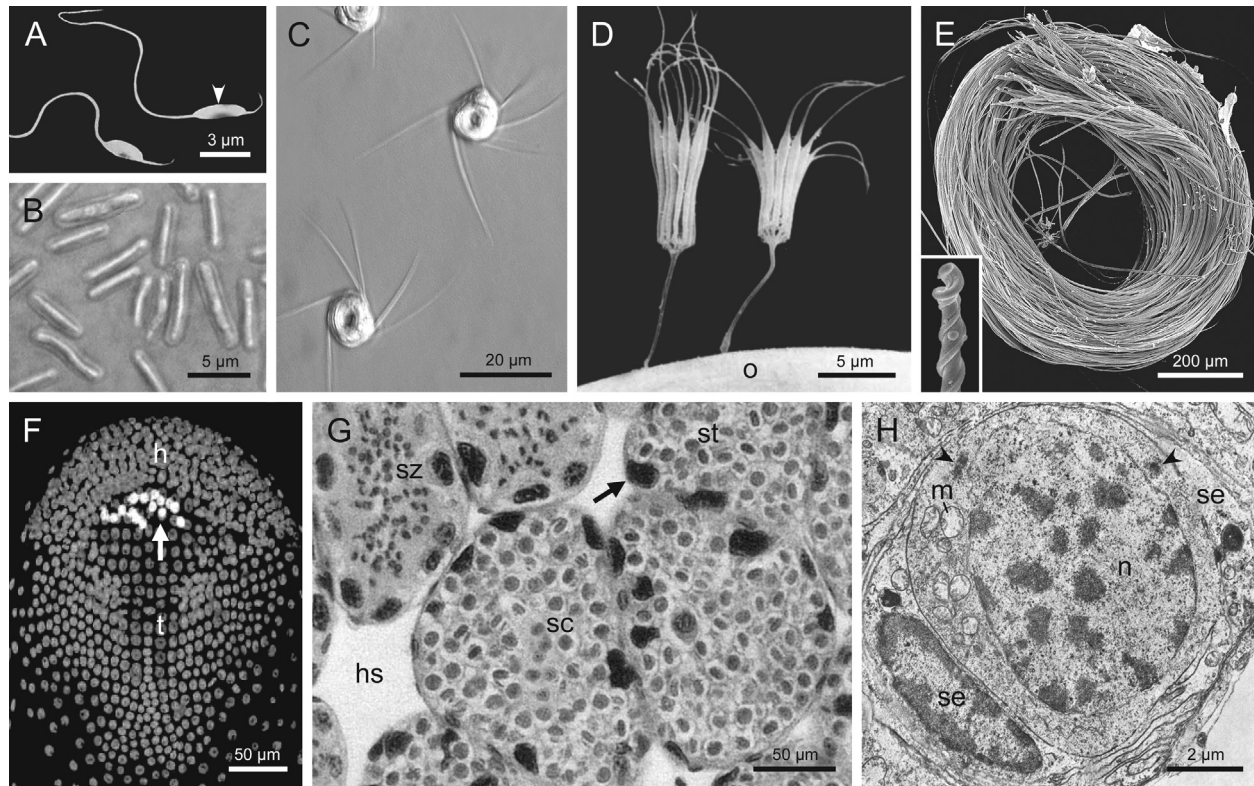


Fig. 1 Sperm types, primordial germ cells, and spermatogonia. (A) Flagellate spermatozoa of barnacle *Tetraclita japonica*. Arrowhead denotes large accessory body in anterior region (Reproduced with permission from Kubo *et al.*, 1979. Development, Growth and Differentiation 21, 445–456.). (B) Rod-like spermatozoa of water flea *Daphnia magna* (Reproduced with permission from Gómez, R., Van Damme, K., Gosálvez, J., Sánchez Morán, E. Colbourne, J.K., 2016. Male meiosis in Crustacea: Synapsis, recombination, epigenetics and fertility in *Daphnia magna*. Chromosoma 125, 769–787.). (C) Multistellate spermatozoa of crayfish *Austropotamobius italicus* consisting of compact cell body and radial arms (Reproduced with permission from Galeotti *et al.*, 2012. PLOS ONE 7, e43771.). (D) Inverted umbrella-like spermatozoa of shrimp *Rhynchocinetes typus* contacting an oocyte (o) with their anterior spike (Reproduced with permission from Barros *et al.*, 1986. Gamete Research 14, 171–180.). (E) Bundle of giant spermatozoa from male seminal vesicle of ostracod *Australocypris robusta*. Inset: corkscrew-like anterior tip of sperm of *Pseudocandona marchica* suitable for mechanical penetration of egg envelope (Reproduced with permission from Smith *et al.*, 2016. Acta Zoologica 97, 1–17.). (F) Embryo of amphipod *Parhyale hawaiiensis* showing group of Vasa-immunoreactive primordial germ cells (arrow) between head (h) and trunk (t) regions (Reproduced with permission from Extavour, C.G., 2005. The fate of isolated blastomeres with respect to germ cell formation in the amphipod crustacean *Parhyale hawaiiensis*. Developmental Biology 277, 387–402.). (G) Cross section of seminiferous tubules of crayfish *Procamburus clarkii* including spermatocytes (sc), spermatids (st), spermatozoa (sz), and Sertoli cells (arrow). hs, hemal space (Reproduced with permission from Krol, R.M., Hawkins, W.E., Overstreet, R.M., 1992. Reproductive components. In: Harrison, F.W., Humes, A.G. (Eds.), Microscopic Anatomy of Invertebrates. Volume 10: Decapod Crustacea. New York: Wiley-Liss, pp. 295–343.). (H) Electron micrograph of primary spermatogonium of crab *Rhithropanopeus harrisii* enveloped by Sertoli cell (se). Arrowheads, centrioles; m, mitochondria; n, nucleus (Reproduced with permission from Payen, 1977. Arch. Anat. Microsc. 66, 163–180.).

Spermatogonia and Spermatocytes

The male reproductive system of crustaceans is very variable with respect to shape and components (Krol *et al.*, 1992). However, there are always one or more testes in which the sperm is produced. The most variable parts are the efferent ducts, accessory glands, and copulatory organs. As in most other animals, the testis is subdivided into seminiferous acini or tubules that are lined by a seminiferous epithelium. This epithelium consists of germ cells and soma cells (Fig. 1(G)). The latter are sometimes called Sertoli cells. Like in mammals, the germ cells develop in close association with the Sertoli cells from spermatogonia through spermatocytes and spermatids to spermatozoa. In freshwater crayfish *Procamburus clarkii*, sperm development occurs synchronously within individual seminiferous acini but may differ among neighboring acini (Fig. 1(G)). In the crab *Portunus pelagicus*, each testicular tubule is longitudinally divided into germinal, transformation, and evacuation zones including successive stages of cellular differentiation (Stewart *et al.*, 2010).

The primary spermatogonia function as germ stem cells. They are capable of self-renewal and multiply by mitotic divisions during organogenesis and sperm production. In most longer lived crustaceans, the spermatogonia show alternating periods of activity and quiescence in an annual rhythm. The Sertoli cells obviously play a key role in activation and deactivation of the spermatogonia. In the quiescent testis of freshwater crayfish they surround the spermatogonia almost completely (Fig. 1(H)). In the

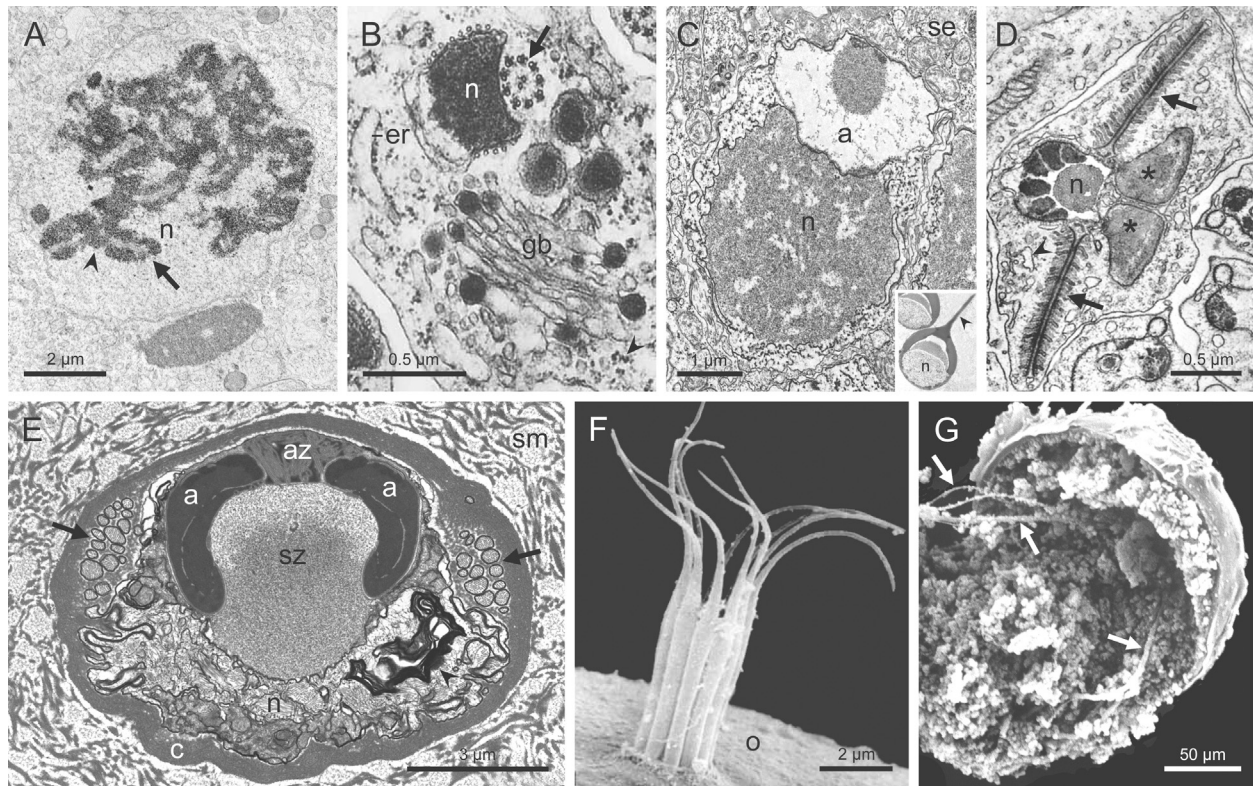


Fig. 2 Spermatocytes, spermatids, and spermatozoa. (A) Electron micrograph of diplotene spermatocyte of shrimp *Penaeus monodon* showing pairing of chromosomes (arrow) in the nucleus (n). Arrowhead denotes chiasma (Reproduced with permission from Krol, R.M., Hawkins, W.E., Overstreet, R.M., 1992. Reproductive components. In: Harrison, F.W., Humes, A.G. (Eds.), *Microscopic Anatomy of Invertebrates*. Volume 10: Decapod Crustacea. New York: Wiley-Liss, pp. 295–343.). (B) Mid-spermatid of barnacle *Amphibalanus amphitrite* showing nucleus, axial filament (arrow), endoplasmic reticulum (er), Golgi body (gb), and free ribosomes (arrowhead) (Reproduced with permission from Kubo *et al.*, 1979. *Development, Growth and Differentiation* 21, 445–456.). (C) Stage 4 spermatid of *Penaeus monodon* showing polarized organization and differentiating acrosome (a). se, Sertoli cell (Reproduced with permission from Krol, R.M., Hawkins, W.E., Overstreet, R.M., 1992. Reproductive components. In: Harrison, F.W., Humes, A.G. (Eds.), *Microscopic Anatomy of Invertebrates*. Volume 10: Decapod Crustacea. New York: Wiley-Liss, pp. 295–343.). Inset: mature sperm of *Litopenaeus vannamei* with acrosomal spike (arrowhead) (Reproduced with permission from Aungsuchawan *et al.*, 2011. *Aquaculture Research* 42, 188–195, 2011.). (D) Cross section of filiform spermatid of ostracod *Notodromas monacha* showing heterogeneous nucleus, mitochondrial derivatives (asterisks), feather-like organelles (arrows), and endoplasmic reticulum (arrowhead) (Reproduced with permission from Zissler, 1969. *Z. Zellforsch.* 96, 106–133.). (E) Spermatozoon of crayfish *Austropotamobius torrentium* in spermatophore. Arrows denote radial arms folded under the extracellular capsule (c). Arrowhead, multilamellar body; az, apical zone of acrosome; sm, spermatophore matrix; sz, subacrosomal zone (Reproduced with permission from Niksirat *et al.*, 2013. *Journal of Morphology* 274, 750–758). (F) Spermatozoon of shrimp *Rhynchocinetes typus* penetrating into oocyte (o) (Reproduced with permission from Barros *et al.*, 1986. *Gamete Research* 14, 171–180.). (G) Oocyte of ostracod *Mytilocypris praenuncia* broken up hours after oviposition. It includes a spermatozoon coiled in several spirals (arrows) (Reproduced with permission from Matzke-Karasch, 2005. *Journal of Experimental Zoology Part B* 304, 129–149.).

active testis, cytoplasmic processes of the Sertoli cells are often extremely attenuated, extending between the developing germ cells throughout much of spermatogenesis (Harvey *et al.*, 2001). They apparently have different functions. They provide a scaffold for the developing germ cells, phagocytose excess cytoplasm from the differentiating spermatids, and occlude the adluminal testicular epithelium preventing the spermatids and spermatozoa from contact with hemolymph-borne substances. In mammals, such a blood-testis barrier is more basally located between the spermatogonia. In crayfish, the Sertoli cells additionally secrete the material of the extracellular capsule that encloses each spermatozoon (Fig. 2(E)).

The spermatogonia give rise to spermatocytes that undergo meiosis and subsequently differentiate into spermatids. Depending on species, one or two spermatogonial stages and several spermatocyte and spermatid stages are discernible on the basis of morphological criteria. For example, in the shrimp *Penaeus monodon* two types of spermatogonia, five stages of spermatocytes, and six stages of spermatids have been distinguished (Feng *et al.*, 2017). Spermatogonia A are located on the basal lamina of the seminiferous tubules and give rise to smaller spermatogonia B with higher proportions of euchromatin. Spermatogonia B divide mitotically into primary spermatocytes that remain interconnected by cytoplasmic bridges and undergo meiosis. Therefore, the most conspicuous cytological alterations during spermatogenesis concern the nucleus and chromatin. The spermatocyte stages are subdivided according to the meiotic stages leptotene, zygotene, pachytene, diplotene (Fig. 2(A)), and diakinesis.

Meiosis is a key process in gamete production of all animals. It is a specialized cell division program that consists of a single round of DNA replication followed by two consecutive rounds of cell division thereby forming haploid gametes from diploid germ cells. Although meiosis is conserved across animals, there are substantial differences in timing and regulation of the various steps. Immunostaining of the testis of water flea *Daphnia magna* with antibodies against key proteins of meiosis revealed differences to other arthropods with respect to early recombination (Gómez *et al.*, 2016). There were also differences in epigenetic histone modifications throughout spermiogenesis. In water fleas taken from a natural population, chromosomal aberrations occurred in all meiotic stages amounting to 8.8%, 6.2%, 12.4% and 10.6% in metaphase I, telophase I, metaphase II and telophase II, respectively. In inbred lineages, the respective values were much higher.

Spermatids and Spermatophores

Spermiogenesis is characterized by the progressive differentiation of the germ cells. Some processes of spermiogenesis such as cytoplasm reduction and chromatin decondensation are shared by most crustaceans but others are taxon specific, depending on whether flagellae, acrosomes, or special cell organelles are produced. Mitochondria, endoplasmic reticulum, and Golgi bodies are present in spermatogonia (Fig. 1(G)), spermatocytes, and spermatids (Fig. 2(B) and (D)) but in the spermatozoa they are often lost. The degradation of these membranous cell organelles leads to the formation of multilamellar bodies (Fig. 2(E)). In flagellate sperm, formation of the axoneme dominates spermiogenesis. An example is the barnacle *Amphibalanus amphitrite* in which the axoneme is closely apposed to the nucleus. Early and mid-spermatids include numerous microtubules that form the axoneme and plenty of ribosomes, rough endoplasmic reticulum, and Golgi bodies (Fig. 2(B)) that are required to produce the proteins of the axoneme and the acrosome.

Acrosome formation is the most conspicuous feature in spermatids of decapods (Stewart *et al.*, 2010). An example is the shrimp *Penaeus monodon*, which shows six stages of spermiogenesis (Feng *et al.*, 2017). In stages 1–3, the acrosomal vesicle is formed and enlarged, and in stage 4, an electron-dense body appears in the anterior part of the acrosome (Fig. 2(C)). In stages 5 and 6, the nuclear envelope disintegrates and a spiky structure emerges from the acrosome. The following spermatozoal stage is characterized by a protruding apical acrosome, a subacrosomal chamber, and a basally located nucleus with decondensed chromatin (Fig. 2(C), inset).

The spermatids of barnacles and freshwater ostracods are characterized by the development of special organelles. Barnacle spermatids produce very large accessory bodies (Fig. 1(A)) of unknown function that disappear shortly before fertilization. Ostracod spermatids synthesize paired feather-like organelles and rod-like mitochondrial derivatives of considerable length (Fig. 2(D)). These organelles facilitate motility of the aflagellate sperm during fertilization. The paired mitochondrial derivatives originate from the fusion of small mitochondria at the beginning of spermiogenesis. The mitochondrial cristae are then reorganized around a central paracrystalline matrix. The feather-like organelles originate from membranes of the nucleus, the endoplasmic reticulum, and Golgi vesicles. As soon as the construction of the feather-like organelles is finished, the endoplasmic reticulum and the microtubules disappear.

The biochemistry and molecular biology of spermiogenesis in crustaceans is only poorly investigated. An example is the expression of a novel protein named EsSoxB2–1 in crab *Eriocheir sinensis*. This protein is essential for maturation of the nucleus in the spermatozoa as demonstrated with knockouts (Liu *et al.*, 2016). During fertilization it is delivered to the oocyte together with the chromatin and may act as a paternal transcription factor during early embryonic development. Another interesting example is the reduction of most histones in the decondensing nuclei of crab spermatids, with only small amounts of histones H2B and H3 remaining in the mature spermatozoa (Stewart *et al.*, 2010). Chromatin decondensation is shared by most decapods. This feature stands in sharp contrast to chromatin condensation in the mammalian spermatids, which is mediated by small basic proteins called protamines. An exception to the rule in crustaceans are the freshwater ostracods in which the nucleus becomes heterogeneous during spermiogenesis (Fig. 2(D)), including electron-dense clumps, fibrillar material, and an axial rod.

In most crustaceans, the spermatozoa are enclosed in spermatophores (Fig. 2(E)) that are produced and accumulated in the vas deferens. In freshwater crayfish, hundreds of spermatozoa are packaged in a spermatophore (Niksirat *et al.*, 2016). The different layers of the spermatophore wall are synthesized in different sectors of the vas deferens by secretory epithelial cells. In fish lice (Branchiura), the spermatophore material is produced in specific prostate glands and then delivered to the vas deferens. In the giant sperm producing freshwater ostracods, the spermatozoa are not enclosed in spermatophores. Their paired vasa deferentia include voluminous seminal vesicles and large, complicated sperm pumps (Matzke-Karasz *et al.*, 2017). The seminal vesicles serve for sperm accumulation and maturation and the sperm pumps for ejection of individual spermatozoa during copulation. They consist of flexible chitinous elements and interspersed musculature. In many decapods, ejaculation of sperm from the vas deferens can be artificially elicited by electrical stimuli, which is a convenient non-invasive method for sampling of sperm for experimentation.

Capacitation and Fertilization

Crustacean males do not release their sperm into the water as is the case in many other aquatic invertebrates. They rather transfer the sperm to the females for storage until oviposition. The sperm is usually transmitted by a penis or a secondary copulatory organ that

takes the sperm from the gonopores. It is either externally attached to the females as spermatophore clump or deposited within female sperm receptacles. Even the flagellate sperm of the sessile barnacles is transferred with a penis, which is unique in sedentary animals. As a result, cirriped penises are the longest copulatory organs relative to body length in the animal kingdom (Vogt, 2016).

In the female storage site the spermatozoa undergo further modification, which is called capacitation. In signal crayfish *Pacifastacus leniusculus*, these modifications include structural changes of the acrosome, the subacrosomal area and the chromatin, alterations of protein expression, and changes of the tyrosine phosphorylation pattern along the radial arms (Niksirat *et al.*, 2016). In penaeid shrimps, capacitation mainly concerns changes of the acrosome. In vitro assays with *Litopenaeus occidentalis* revealed that acrosome reactivity is significantly higher in sperm removed from the female storage site than in sperm taken from the male, indicating that post-testicular modifications are essential to obtain full fertility (Alfaro *et al.*, 2007). This information is important to develop in vitro fertilization techniques for shrimp aquaculture.

Fertilization of the eggs occurs either in sea water by the simultaneous release of eggs and stored sperm from the females (e.g., penaeid shrimps), in the female genital tract (e.g., brachyuran crabs) or in special transient compartments like the fertilization tent of freshwater crayfish. This unique gelatinous structure is produced at the underside of the females shortly before spawning and provides the special conditions required for activation of the spermatozoa and eggs (Vogt, 2016). The spermatozoa of most crustaceans are apparently activated by dissolution of the surrounding spermatophore material. In contrast, the giant sperm of ostracods are activated by disintegration of the outer sperm coat (Matzke-Karasz *et al.*, 2017). In the shrimp *Rhynchocinetes typus* unfolding of the stellate arms (Fig. 1(D)) is triggered by contact with sea water.

During fertilization the spermatozoa enter the ooplasm either partly or completely, depending on taxon. Examples of the former variant are the reptantian decapods, and examples of the latter variant are palaemonid shrimps (Fig. 2(F)) and ostracods (Fig. 2(G)). Curiously, the giant spermatozoa of freshwater ostracods enter the oocytes completely although they are several times longer than the eggs. The volume of the internalized sperm is about 0.5–1% of the volume of the corresponding egg, depending on species. The sperm constituents are dissolved before the 32-cell blastula.

The spermatozoa of some crustaceans, for example, palaemonid shrimps and freshwater ostracods, have no acrosome. Penetration of the egg envelope is therefore assumed to occur mainly mechanically (Fig. 2(F)). However, lytic enzymes bound to the apical cell membrane of the sperm may aid penetration. In contrast, in penaeid shrimps, invasion of the egg envelope is chemically mediated by a trypsin-like lysin and further enzymes from the acrosome (Chen *et al.*, 1994). A special case is the “explosion sperm” of reptantian decapods. Upon contact with the oocyte their large acrosome is abruptly everted, generating a forward directed mechanical force that “shoots” the sperm into the egg envelope (Talbot *et al.*, 1991). Concomitantly, the nucleus is torn from basal to apical facilitating fusion with the oolemma. Apparently, only the nucleus and a small acrosomal filament enter the ooplasm whilst the other parts of the sperm remain outside.

The duration of fertilization in crustaceans is variable. In the shrimp *Rhynchocinetes typus* it lasts about 45–60 min. Monospermy seems to be the rule but polyspermy may occur in some shrimps (Fig. 1(D)). In the crab *Maja squinado*, hyperpolarization of the egg membrane in response to fertilization was shown to constitute an electrical block to polyspermy.

Many crustacean females are promiscuous and store sperm from more than one male in their sperm receptacles, enhancing the genetic variability of the offspring (Vogt, 2016). The maximum number of fathers per clutch reported so far is 11 in the shrimp *Caridina ensifera*. In some brachyuran crabs, sperm can be stored and nourished for years in the female receptacles. This sperm largely retains viability and can be utilized for multiple fertilizations. For example, females of Tanner crab *Chionoecetes bairdi* isolated from males after copulation produced viable eggs by 100% in the mating year and 97% and 71% in the following two years. Long-term sperm storage enables posthumous paternity, the ongoing siring of offspring after the death of the sperm provider. This phenomenon may be of some relevance in male-based crab fisheries, in which the largest (i.e., oldest) specimens are regularly removed from the population.

References

- Alfaro, J., Ulate, K., & Vargas, M. (2007). Sperm maturation and capacitation in the open thelycum shrimp *Litopenaeus* (Crustacea: Decapoda: Penaeoidea). *Aquaculture*, 270, 436–442.
- Chen, T.-I., Green, J. D., & Clark, W. H., Jr. (1994). Sperm penetration of the vitelline envelope of *Sicyonia ingentis* eggs is mediated by a trypsin-like lysin of acrosomal vesicle origin. *Development, Growth and Differentiation*, 36, 259–273.
- Extavour, C. G. (2005). The fate of isolated blastomeres with respect to germ cell formation in the amphipod crustacean *Parhyale hawaiiensis*. *Developmental Biology*, 277, 387–402.
- Feng, T., Paterson, B., & Johnston, S. (2017). New insights into the spermatogenesis of the black tiger prawn *Penaeus monodon*. *Journal of Morphology*, 278, 689–703.
- Gómez, R., Van Damme, K., Gosálvez, J., Sánchez Morán, E., & Colbourne, J. K. (2016). Male meiosis in Crustacea: Synapsis, recombination, epigenetics and fertility in *Daphnia magna*. *Chromosoma*, 125, 769–787.
- Harvey, M. C., Hinsch, G. W., & Cameron, D. F. (2001). Sites of lanthanum occlusion in the testis of the crayfish *Procambarus paeninsulanus* (Crustacea: Cambaridae). *Tissue and Cell*, 33, 562–569.
- Jamieson, B. G. M. (1991). Ultrastructure and phylogeny of crustacean spermatozoa. *Memoirs of the Queensland Museum*, 31, 109–142.
- Krol, R. M., Hawkins, W. E., & Overstreet, R. M. (1992). Reproductive components. In F. W. Harrison, & A. G. Humes (Eds.), *Microscopic Anatomy of Invertebrates*. Volume 10: *Decapod Crustacea* (pp. 295–343). New York: Wiley-Liss.

- Liu, Z.-Q., Jiang, X.-H., Qi, H.-Y., Xiong, L.-W., & Qiu, G.-F. (2016). A novel *SoxB2* gene is required for maturation of sperm nucleus during spermiogenesis in the Chinese mitten crab, *Eriocheir sinensis*. *Scientific Reports*, 6, 32139.
- Matzke-Karas, R., Smith, R. J., & Heß, M. (2017). Removal of extracellular coat from giant sperm in female receptacle induces sperm motility in *Mytilocypris mytiloides* (Cyprididae, Ostracoda, Crustacea). *Cell and Tissue Research*, 368, 171–186.
- Niksirat, H., Vancová, M., Andersson, L., et al. (2016). Protein modification in the post-mating spermatophore of the signal crayfish *Pacifastacus leniusculus*: Insight into the tyrosine phosphorylation in a non-motile spermatozoon. *Animal Reproduction Science*, 172, 123–130.
- Stewart, M. J., Stewart, P., Soonklang, N., et al. (2010). Spermatogenesis in the blue swimming crab, *Portunus pelagicus*, and evidence for histones in mature sperm nuclei. *Tissue and Cell*, 42, 137–150.
- Taketomi, Y., Nishikawa, S., & Koga, S. (1996). Testis and androgenic gland during development of external sexual characteristics of the crayfish *Procambarus clarkii*. *Journal of Crustacean Biology*, 16, 24–34.
- Talbot, P., Poolsanguan, B., Al-Hajj, H., & Poolsanguan, W. (1991). Gamete interactions during *in vitro* fertilization of lobster (*Homarus americanus*) oocytes. *Journal of Structural Biology*, 106, 125–134.
- Vogt, G. (2016). Structural specialties, curiosities, and record-breaking features of crustacean reproduction. *Journal of Morphology*, 277, 1399–1422.

Spermatogenesis and Spermiogenesis in the Mollusca

Alan N Hodgson, Rhodes University, Grahamstown, South Africa

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome Membrane-bound vesicle in the head of a spermatozoon, usually anterior to the nucleus, that enables the sperm to penetrate the egg coat and enter the egg for fertilization.

Aquasperm Morphologically simple sperm that are released into the water column.

Axoneme The central structure of a cilium or flagellum composed of microtubules held together by cross-bridges. The microtubules are usually arranged as a ring of nine outer pairs (doublets) and a central pair of single microtubules.

Chromatin Material in the nucleus consisting of protein, DNA and RNA.

Cisternae Membranous plates or discs of some cell organelles. In the Golgi body they are arranged as a stack of membranous discs.

Cristae Infoldings of the inner membrane of the mitochondrion.

Euspermatozoon Fertilizing spermatozoon.

Heterochromatin A tightly packed form of chromatin. When viewed with a transmission electron microscope it is more densely stained.

Introsperm Morphologically more complex spermatozoa produced by animals with internal fertilization.

Mid-piece Region of a spermatozoon that includes the mitochondria and centrioles or centriolar complex from which the axoneme of the tail originates.

Paraspermatozoon Non-fertilizing cells produced in the testis.

Synaptonemal complex A large proteinaceous structure that assembles at the interface between aligned homologous chromosomes during meiotic prophase and holds the chromosomes together.

Taxon (plural taxa) Any unit (division) used in biological classification.

Introduction

The Mollusca is the second most diverse animal phylum with over 100,000 species. The phylum contains many well-known organisms including clams, mussels, snails, squids and octopuses. Molluscs are found in almost every ecosystem in the world from the Arctic to the Antarctic, as well as the deep sea, lakes, rivers, and mountain ranges. There are currently seven classes of mollusc recognized (Aplacophora, Monoplacophora, Polyplacophora, Bivalvia, Gastropoda, Scaphopoda, Cephalopoda) and nearly all species within these taxa reproduce sexually, a process that requires males to produce spermatozoa. The form (structure) of spermatozoa within the Mollusca is very diverse. In species where sperm and eggs are released into the water column for fertilization to occur (external fertilization), the spermatozoa (categorized as aquasperm) are relatively small, usually between 50 and 90 μm long, and simple in structure. By contrast, in species with internal fertilization (i.e., fertilization within the female reproductive tract) the spermatozoa (categorized as introsperm) are usually larger ($>130 \mu\text{m}$ long and up to 1.8 mm long in some pulmonate snails), are highly modified, and therefore structurally more complex. It is therefore not surprising that many of the structural changes that take place during spermatogenesis of these two categories of spermatozoa are different.

Spermatozoon formation is even more varied in some taxa with internal fertilization because males produce more than one type of spermatozoon in their testis. In addition to fertilizing spermatozoa (euspermatozoa), such males also produce non-fertilizing sperm (paraspermatozoa) (sperm terminology after [Healy and Jamieson \(1981\)](#)). The production of dimorphic or polymorphic spermatozoa has been reported for taxa within the class Gastropoda (many Caenogastropoda, Neritimorpha, and Skeneidae) and some species within the bivalve families Galeommatoidea, Lucinacea, Leptonacea and Corbiculacea. The diversity of spermatozoa and spermatogenesis has been used to provide characters in many taxonomic and phylogenetic studies.

Because there are important morphological and cytological differences during spermatogenesis of eusperm and parasperm, their formation will be considered separately.

Spermatogenesis – Euspermatozoa

The testis of molluscs is compartmentalized either as a series of lobular structures (acini) or tubes within which spermatogenesis occurs. The early stages of development are always located close to the wall of the compartments with progressively more developed stages closer to the lumen of each acinus or tube. Spermatogenesis can be divided into four developmental stages: (i) production of spermatogonia from germ cells; (ii) proliferation of spermatogonia by mitosis; (iii) formation of spermatocytes by meiosis; and (iv) spermiogenesis – the development of spermatids into spermatozoa. During cell division (stages ii and iii) cytoplasmic separation

(cytokinesis) is not complete. Therefore, throughout spermatogenesis groups of developing spermatozoa are connected as a syncytium by cytoplasmic bridges (**Fig. 1(C)**), the number of spermatozoa per group depending on how many mitotic divisions occurred during proliferation of the spermatogonia (stage i). For example, in some gastropods the original spermatogonium may divide to produce 32 primary spermatogonia that divide to form 64 secondary spermatogonia. After the two meiotic division of the spermatocytes, 256 haploid spermatids are produced. The cytoplasmic connections are eventually severed by cytokinesis late in spermiogenesis.

The structural features of spermatogonia, spermatocytes and spermatids, and morphological changes that occur during development have been described by light and electron microscopy for examples of all higher molluscan taxa. Up until spermiogenesis, the structural features of spermatogonia and spermatocytes, as seen with a transmission electron microscope, are very similar in all molluscs. Spermatogonia are roughly spherical to pyriform in shape with a central spherical nucleus (with patchy heterochromatin) containing one or two nucleoli surrounded by a relatively small amount of cytoplasm. The cytoplasm contains small rounded to oblong mitochondria (when seen in cross section), ribosomes and some endoplasmic reticula (**Fig. 1(A)**). Spermatocytes have a similar shape to spermatogonia but are usually larger owing to an increase in cytoplasmic volume. In primary spermatocytes the nucleolus disappears and the nucleus, which is often larger than that of spermatogonia, develops synaptonemal complexes during the zygotene and pachytene stages of meiosis (**Fig. 1(B)**). In the cytoplasm the mitochondria may increase in size and a Golgi body with several cisternae is conspicuous as well as two centrioles. After the first meiotic division the secondary spermatocytes and their nuclei are usually smaller, whereas the Golgi body may be more developed. In some species

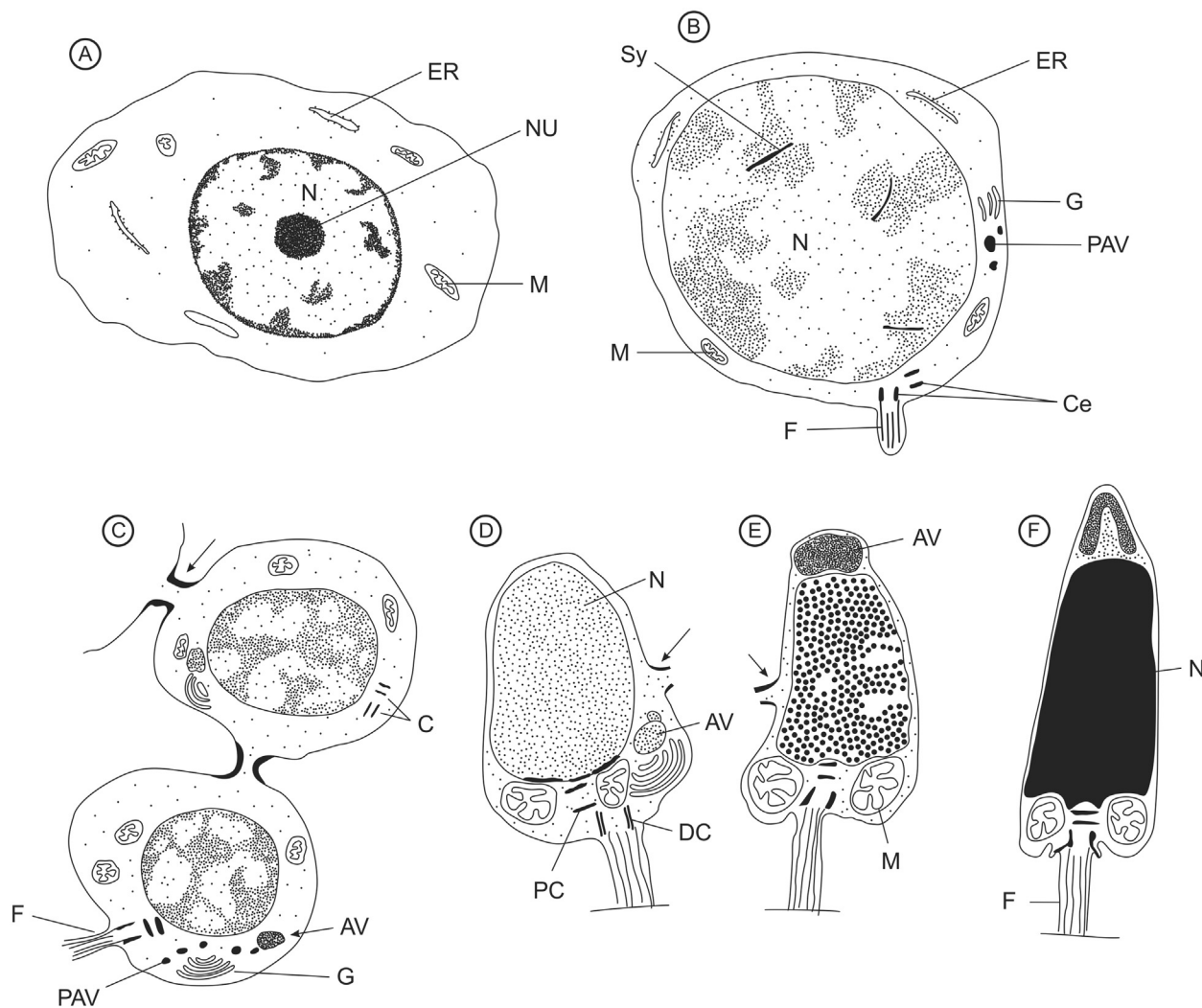


Fig. 1 Diagrammatic representation (interpreted from transmission electron microscope images) of spermatogenesis in molluscan aquasperm. A, Spermatogonium with spherical nucleus (N) containing a nucleolus (Nu). B, Spermatocyte with synaptonemal complexes (Sy) present in nucleus. Note also the presence of a short flagellum (F) and proacrosomal vesicles (PAV) indicating the first phase in acrosome formation. C, Early spermatids joined by cytoplasmic bridges (arrow). At this stage the nucleus of the spermatids is characterized by a patchwork of heterochromatin. D, Mid-spermatid showing fine granular phase of chromatin condensation. E, Late spermatid showing coarse granular phase of chromatin condensation and acrosomal vesicle (AV) positioned anteriorly. F, Spermatozoon in which the chromatin is completely condensed. AV, acrosomal vesicle; CE, centriole; DC, distal centriole; ER, endoplasmic reticulum; F, flagellum; G, Golgi body; M, mitochondrion; N, nucleus; PAV, proacrosomal vesicle; PC, proximal centriole. Arrow, cytoplasmic bridge. Not drawn exactly to scale.

the cytoplasm of spermatocytes develops small membrane-bound electron-dense vesicles. These vesicles, which appear to be produced by the Golgi body and possibly the rough endoplasmic reticulum, are the precursors of acrosomal vesicles. In addition, a small flagellum may be present (Fig. 1(B)) but this is resorbed prior to spermiogenesis.

The greatest changes, morphological and cytochemical, take place during spermiogenesis, the transformation of spermatids into spermatozoa in which three structural regions (head, mid-piece and tail) are recognized.

Spermiogenesis of Aquasperm

Aquasperm are produced by scaphopods, monoplacophorans, the chaetoderms (Aplacophora), and the majority of bivalves, chitons, patellogastropods and vetigastropods. The early spermatids of aquasperm have a relatively small spherical nucleus that contains a patchwork of heterochromatin (Fig. 1(C)). Early in spermiogenesis the chromatin becomes homogeneously distributed within the nucleus and takes on a fine granular appearance (Fig. 1(D)). The granules are about 20 nm in diameter and this change in chromatin appearance has signalled a transition in proteins associated with the DNA of the nucleus. Up until the early spermatid stage the DNA is associated with histones and these are gradually replaced with protamines that are sperm nuclear basic proteins (Chiva *et al.*, 2011). This change in nuclear proteins is necessary for chromatin condensation and a major change in shape of the nucleus. Within the cytoplasm of early spermatids, mitochondria surround the nucleus, a flagellum begins to form from one of the centrioles, and a Golgi body is present. In species where proacrosomal vesicles have not formed in spermatocytes, these now appear (Fig. 1(C)) although in some species a larger single proacrosomal vesicle develops from Golgi secretions. As the spermatid matures the chromatin in the nucleus further condenses because the granules become reorganized as coarse granules often about 60 nm in diameter (Fig. 1(E)). These larger granules are brought about by further substitution of histones by protamine (Chiva *et al.*, 2011). Therefore, the nucleus is now mainly composed of DNA and protamine. In the final stage of spermiogenesis the coarse granules usually coalesce into the fully condensed form of the nucleus of the mature spermatozoon (Fig. 1(F)). This process is brought about by the protamine being chemically dephosphorylated. In chitons, however, the chromatin becomes twisted into fibres before final condensation. The process of chromatin condensation usually results in a gradual change in shape of the nucleus. Whilst the nucleus may remain more or less spherical in some species, in others it becomes cylindrical, conical or bullet-shaped.

During spermiogenesis the acrosome develops from either the single proacrosomal vesicle, or more usually by fusion of the small proacrosomal vesicles into a single vesicle. An exception to this is found in the bivalve families Trigonoidea and Unionoidea where the vesicles do not fuse, the acrosomal complex of the mature spermatozoon consisting of several anteriorly positioned vesicles (Healy, 1989). In most species the acrosome forms in the presumptive posterior region of the spermatozoon. As the spermatid matures and changes shape, the developing acrosome migrates to the anterior pole of the nucleus (Fig. 1(E)) where it assumes its final form often becoming cap-shaped or conical (Fig. 1(F)). This can involve elongation and differentiation of the acrosomal contents, as well as the development of a posterior invagination that creates a subacrosomal space filled with fine granular or fibrillar material. An exception to where the acrosome develops is found in the bivalve group Anomalodesmata. In these bivalves the acrosome forms anteriorly then migrates to the posterior of the late spermatid to become located within the mid-piece (Healy *et al.*, 2008).

As the nucleus matures and changes shape, the amount of cytoplasm in the spermatid decreases and the mitochondria begin to fuse together. This results in an increase in their size and a decrease in their number to between four and seven. As this happens the fusing mitochondria along with the pair of centrioles migrate to the future posterior pole of the nucleus (Fig. 1(D)). Here the larger spherical to oval mitochondria, which have well developed cristae, surround the orthogonally arranged centrioles in tight proximity to the nucleus to form the developing mid-piece of the spermatid. One centriole (the proximal) becomes anchored in a small depression of the nucleus (posterior nuclear fossa), whilst the flagellum, which has the normal 9+2 arrangement of microtubules, continues to develop from the distal centriole to form the tail.

Spermiogenesis of Introsperm

Introsperm are produced by all cephalopods, the neomenioids (Aplacophora), a few bivalves, and many groups of gastropods including the caenogastropods, opisthobranchs and pulmonates. The introsperm of molluscs are extremely variable and more complex in structure with a variety of modifications to the head, mid-piece and tail regions. The morphological changes that occur during spermiogenesis, therefore, reflect this variability. Early spermatids, like those of aquasperm, tend to have a spherical nucleus with a patchwork of heterochromatin (Fig. 2(A)), and during this stage electron-dense material called plaques may form at the future posterior pole or anterior and posterior poles of the nuclear surface. The anterior plaque is usually composed of a layer of extranuclear material whereas the posterior plaque appears to be caused by thickening of the inner nuclear membrane. In early spermatids the cytoplasm has numerous mitochondria, often more than one Golgi body, well-developed endoplasmic reticulum, and one or two centrioles (some of these features shown in Fig. 2(A) and (B)). As the spermatid matures it elongates during which time the nucleus changes shape, and chromatin condensation proceeds.

In the decapod cephalopods (cuttlefish and squids) there is a granular phase of chromatin condensation (Fig. 2(B)) similar to that described for the early spermatids of aquasperm. The 20 nm granules become homogeneously distributed throughout the nucleus. As the spermatid matures, these chromatin granules are transformed structurally and biochemically. The granules are remodelled into thin fibres (about 35 nm diameter) with an antero-posterior orientation (Fig. 2(D)) that contain hyperacetylated histones and a protamine precursor (Chiva *et al.*, 2011). Fibre thickness then increases to about 50 nm in diameter with an increase in protamine precursor and a decrease in the hyperacetylated histone. Finally the thicker fibres coalesce that results in a uniformly electron-dense nucleus, and at this stage protamine is now associated with the DNA. In octopod cephalopods the nuclear protein

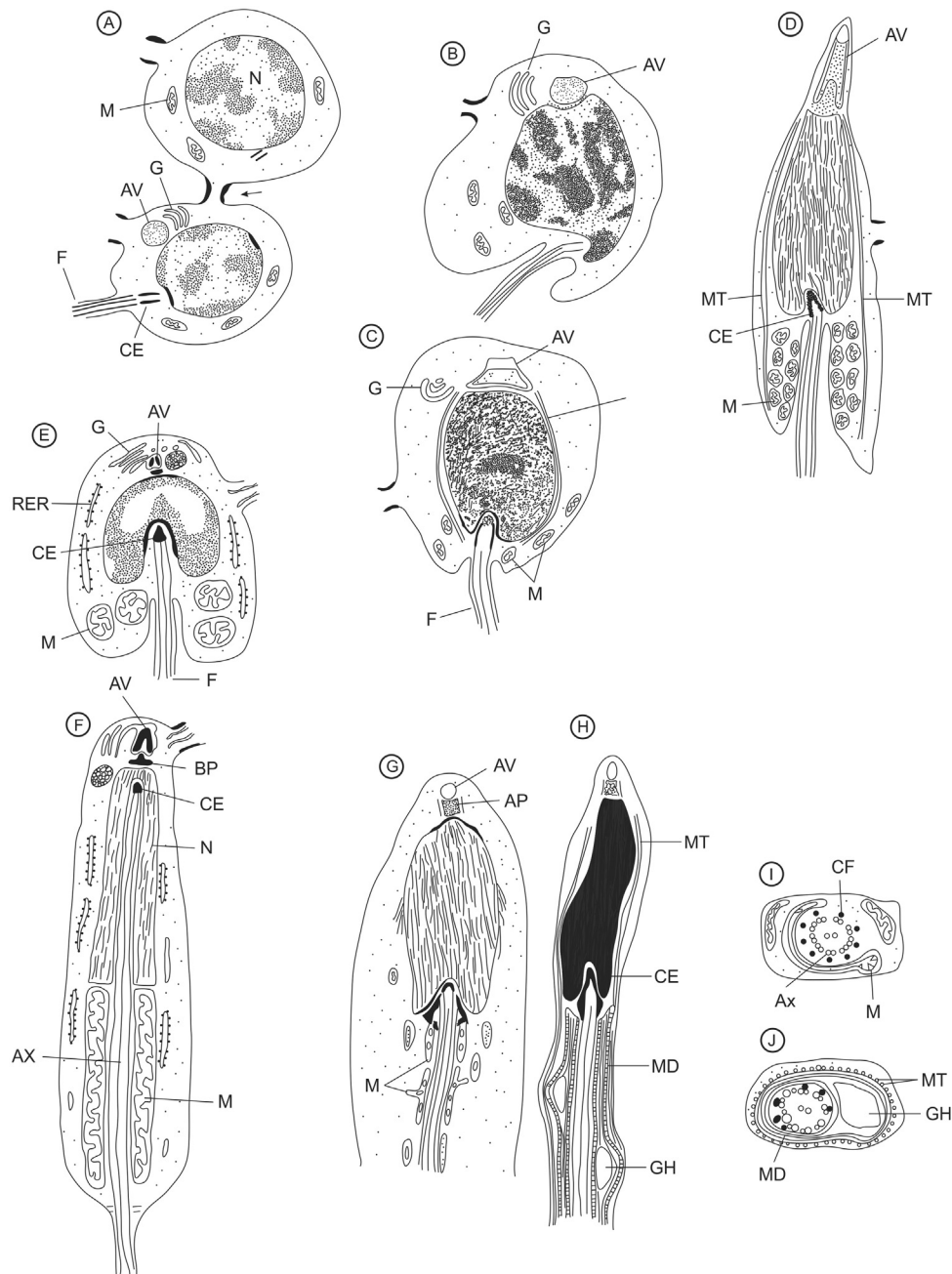


Fig. 2 Diagrammatic representation (interpreted from transmission electron microscope images) of some stages of introsperm spermiogenesis in some molluscan taxa. A, Early spermatids showing nuclei with patchwork of chromatin. B and C, Mid-spermatids in which chromatin is becoming granular. Note developing polarity of cell with the acrosomal vesicle (AV) positioned at the anterior pole. D, Late spermatid with fibrous chromatin in the nucleus. Note the microtubules (MT) surrounding the nucleus. E, Mid-spermatid with chromatin condensation beginning at the periphery of the nucleus. The nucleus has also invaginated posteriorly to form the posterior nucleus fossa that houses the centriolar complex (CE). F, Late spermatid with elongating nucleus and mid-piece. The chromatin is now fibrous in appearance and nucleus has invaginated completely to form a central canal that houses the centriolar complex (CE) and axoneme (AX). G and H, Longitudinal sections through late spermatids showing stages in the development of the mitochondrial derivative (MD). I, Transverse section through the mid-piece in the early phase of formation of the mitochondrial derivative. J, Transverse section through the mid-piece of a late phase of formation of the mitochondrial derivative. AP, acrosomal pedestal; AV, acrosomal vesicle; AX, axoneme; BP, basal plate; CE, centrioles of centriolar complex; CF, coarse fibre; F, flagellum; G, Golgi body; GH, glycogen helix; M, mitochondrion; MD, mitochondrial derivative; MT, microtubule; N, nucleus; RER, rough endoplasmic reticulum; arrow, cytoplasmic bridge. Not drawn exactly to scale. A–D: a decapod cephalopod (modified from Healy, J., 1990. Ultrastructure of spermatozoa and spermiogenesis in *Spirula spirula* (L.): Systematic importance and comparison with other cephalopods. *Helgoländer Meeresuntersuchungen* 44, 109–123); E and F: a caenogastropod (modified from Buckland-Nicks, J., Williams, D., Chia, F.-S., Fontaine, A., 1983. Studies on the polymorphic spermatozoa of a marine snail. 2. Genesis of the eupyrene sperm. *Gamete Research* 7, 19–37); G–J: a pulmonate gastropod (modified from Healy, J., 2001. Spermatogenesis and oogenesis. In: Barker, G.M. (ed.), *The Biology of Terrestrial Molluscs*. Oxon: CABI Publishing, pp. 357–382).

transition during spermiogenesis is similar to that of decapods, but chromatin condensation is somewhat different in that it begins at the polar regions (developing anterior and posterior of sperm) of the nucleus rather than simultaneously throughout. Chromatin condensation then progressively spreads out throughout the nucleus. In many caenogastropods, after the fine granular phase, chromatin condensation first occurs at the periphery of the nucleus (Fig. 2(E)), spreading inward, whereas in pulmonates, chromatin condensation occurs uniformly throughout the nucleus. This is then followed by the fibrillar phase (Fig. 2(F) and (G)) during which time the histones are gradually and continuously being transformed through a series of protamine precursors into protamines (Chiva *et al.*, 2011). The final phase of chromatin change involves a lamellar phase, the lamellae eventually coalescing to result in the uniformly electron-dense nucleus.

During chromatin condensation the change in nuclear shape of introsperm can be profound. Shape change first begins with the nucleus invaginating posteriorly to form what will become the implantation fossa for the centriole(s) or centriolar derivative (Fig. 2(C)–(H)). In some caenogastropods the elongating spermatid nucleus develops as a long tube because of the formation of a central intranuclear canal (Fig. 2(F)). This is brought about by the gradual deepening of the implantation fossa. As the fossa deepens the centriole(s) or centriolar derivative migrate into the canal along with the axoneme which therefore penetrates the length of the nucleus (Fig. 2(F)). In other gastropods such as the opisthobranchs, pulmonates, and some cephalopods, the nucleus of later spermatids undergoes some torsion (Fig. 2(H)), becoming helical or helically keeled.

The development of the acrosome begins in early spermatids often after polarity of the nucleus has been established. In the Neritimorpha (Gastropoda), acrosome formation begins with the production of several small membrane-bound, electron-dense proacrosomal vesicles by the basally located Golgi body. In most other taxa the Golgi complex or complexes secrete a single proacrosomal vesicle (Fig. 2(A)). As the spermatid matures, the vesicle migrates anteriorly and becomes positioned in the middle of the anterior nuclear plaque. In caenogastropods vesicle migration is often accompanied by the Golgi body which continues to produce material for the developing acrosome (Fig. 2(E) and (F)). The Golgi body may also be associated with endoplasmic reticulum. During migration of the acrosomal vesicle it usually acquires extravesicular material and structures that eventually form subvesicular material, a basal plate, or a pedestal between the acrosome and the nucleus (Fig. 2(E)–(H)).

The mid-piece of molluscan introsperm with its centriole, centrioles, or centriolar derivative and mitochondria varies in complexity and therefore structural changes during spermiogenesis are also highly variable. In cephalopods other than the Octopoda numerous small unmodified mitochondria congregate at the developing posterior end of the nucleus of mid to late spermatids to become located within a cell membrane spur or sheath that is adjacent to, or surrounds, the anterior section of the tail (Fig. 2(C) and (D)). In the mid-spermatids of octopods mitochondrial fusion occurs as the mitochondria become arranged around the axoneme at the base of the nucleus. In the mid to late spermatids of caenogastropods the mitochondria also begin to accumulate at the basal region of the nucleus (Fig. 2(E)) where they fuse (Fig. 2(F)) to form modified mitochondrial elements that vary in number, length and internal structural complexity among taxa. As the spermatid and its axoneme elongates, the mitochondrial elements surround and extend along the axoneme. In some species the mitochondrial elements of the mid-spermatid begin form a loose spiral around the axoneme and by the late spermatid stage they are arranged as continuous helical elements. As the mitochondria fuse the cristae may form a continuous membrane or become transformed into plate-like structures.

In higher gastropods, such as pulmonates and opisthobranchs, as spermiogenesis proceeds the axoneme continues to elongate and the small mitochondria migrate posteriorly where they cluster along the axoneme where they begin to fuse (Fig. 2(G) and (I)). At the same time, nine course fibres of unknown origin become associated with, and surround, the axoneme (Fig. 2(I) and (J)). As the spermatid matures the mitochondria continue to fuse and become wrapped around the axoneme as a sheath (Fig. 2(I) and (J)). As wrapping progresses the mitochondrial material is transformed and by the late spermatid is organized as circular parallel layers of paracrystalline matrix material into what is referred to as a mitochondrial derivative that spirals along the axoneme (Healy, 2001). During wrapping and transformation one or more tubular channels develop within the mitochondrial derivative. In the mature sperm these channels acquire glycogen and they are therefore called glycogen helices (Fig. 2(H) and (J)). Glycogen is a feature of the spermatozoa of many molluscs. This storage product usually appears late in spermiogenesis. It is often deposited intra-axonemally and in addition a section of the axoneme (often the posterior section) of the late spermatids of caenogastropods and opisthobranchs becomes surrounded by glycogen to form a region called the glycogen piece. To date the mechanism of uptake of glycogen into spermatids has not been explained.

A feature of late spermiogenesis of many molluscan taxa is elimination of excess cytoplasm from the spermatids nuclear, mid-piece or tail regions. The excess cytoplasm with organelles such as the Golgi body and endoplasmic reticulum may be discarded by cytoplasmic sloughing and/or reduced by autophagy and lysosomal activity. A further feature of late spermiogenesis of cephalopods, some caenogastropods and pulmonates is the development of a ring of microtubules (called a manchette) around the condensing nucleus and/or mid-piece of the spermatid (Fig. 2(C), (D), (H) and (J)). The precise role of the microtubules has not been established, although it has been suggested that in some species they play a role in helping to form the shape of the spermatozoon.

Spermatogenesis – Non-Fertilizing Spermatozoa (Paraspermatozoa)

Paraspermatozoa have a number of possible functions. In some species they are carriers for the fertilizing eusperm, whereas in other species they may have a nutritive role, stimulate motility of eusperm, or prevent the sperm from rival males being deposited into the female reproductive tract. Parasperms are extremely diverse in structure. Some have very little chromatin whereas others have no chromatin at all. Many are multiflagellate, whilst some lack flagella. Therefore, the morphological changes that occur during paraspermatogenesis reflect these profound structural differences.

Paraspermatogenesis usually occurs at the same time as euspermatogenesis either in the same or specific areas of the testis. Paraspermatogenesis, however, is characterized by atypical meiosis with the primary spermatocyte developing without a maturation division (see [Hodgson \(1997\)](#) for literature review). Paraspermatogonia ([Fig. 3\(A\)](#)) are similar in ultrastructure to the spermatogonia that produce fertilizing euspermatozoa although in some species the round to oval nucleus has distinctive heterochromatin distribution and the cytoplasm often contains well-developed granular endoplasmic reticulum swollen with secretions ([Buckland-Nicks and Tompkins, 2005](#)), and an abundance of mitochondria. Paraspermatocytes, groups of which are joined by cytoplasmic bridges, are readily identified by their relatively large lobulated nucleus and a cytoplasm with several Golgi bodies, numerous mitochondria, extensive granular endoplasmic reticulum, and some electron-dense bodies ([Fig. 3\(B\)](#)). A further feature of many paraspermatogonia and/or paraspermatocytes is the development of a centrosphere ([Fig. 3\(B\)](#)). This consists of a pair of parent centrioles around which satellite centrioles, termed procentrioles, develop. The number of procentrioles vary between and within species. During paraspermiogenesis the procentrioles migrate to the presumptive posterior pole of the developing paraspermatocyte and paraspermatid ([Fig. 3\(C\)](#)) where they become attached to the cell membrane by fibrogranular material. Each procentriole gives rise to an axoneme with a 9+2 arrangement of microtubules, a process known as flagellogenesis. The elongating axonemes may develop entirely within the body of the paraspermatid, or extend as free flagella in the form of a tail brush ([Fig. 3\(D\) and \(E\)](#)). In developing aflagellate parasperm, the centrioles (and therefore the centrosphere) are not present.

The greatest changes in cell morphology occur during paraspermiogenesis. In parasperm that lose most or all of their nuclear material, this often begins with the nucleus fragmenting into several smaller membrane-bound structures that have been termed caryomerites ([Fig. 3\(D\)](#)), each containing some genetic material. At this time lysosomes generated by the Golgi body and rough endoplasmic reticulum accumulate in the cytoplasm. As the paraspermatid matures and in many cases elongates, the chromatin material in each caryomerite first condenses before it degenerates to a mucus-like substance that may be discharged from the cell by exocytosis ([Fig. 3\(C\)–\(E\)](#)). In the lancet parasperm of the marine snail *Fusitriton oregonensis*, however, this material is retained. In other marine snail species (e.g. *Littoraria* spp. and *Cerithidea* spp.) the nucleus does not fragment but gradually decreases in size to leave a nuclear remnant that eventually forms a central core within the parasperm ([Fig. 3\(E\) and \(F\)](#); [Buckland-Nicks et al., 2000](#); [Buckland-Nicks and Hodgson, 2005](#)). The use of Hoechst's DNA stain has shown that the nuclear remnant retains fragments of DNA. The detailed study of [Buckland-Nicks and Tompkins \(2005\)](#) revealed that nuclear breakdown (partial or complete) involves programmed nuclear death (apoptosis) in which lysosomes secreted by Golgi bodies play an important role.

An unusual feature of the paraspermatids of the caenogastropod group Cerithioidea is the formation of what is thought to be an acrosome with a basal plate. These structures form in the presumptive posterior of the early paraspermatid next to the Golgi body, eventually migrating to the anterior of the nuclear remnant ([Fig. 3\(C\), \(E\) and \(F\)](#)). The function of this 'acrosome' in a cell that does not participate in fertilization is not known.

As previously mentioned, the centrioles of flagellated paraspermatids that are attached to the cell membrane function as basal bodies and are the sites of flagella formation ([Fig. 3\(C\)](#)). In parasperm that keep a nuclear core, the basal bodies break away from the cell membrane of the maturing paraspermatids and become attached to nuclear remnant ([Fig. 3\(E\)](#)). In parasperm that lose all the nuclear material the basal bodies may migrate through the cytoplasm as the paraspermatid elongates, ending up at the anterior of the cell where they usually become embedded in a cap-like structure ([Fig. 3\(D\)](#)). During paraspermatid elongation the mitochondria accumulate posteriorly between the axonemes ([Fig. 3\(C\)–\(E\)](#)).

A feature of many parasperm is the presence of numerous, often large, membrane-bound glycoprotein bodies in the cytoplasm as well as glycogen deposits ([Fig. 3\(F\)](#)). The glycoprotein bodies are produced during paraspermiogenesis, probably by the granular endoplasmic reticulum and Golgi bodies. They first appear as small vesicles with an irregular shape ([Fig. 3\(D\)](#)) but then coalesce into larger blocks that surround the axonemes of the flagella. In addition, glycogen is deposited in the final stage of cytodifferentiation, although its origin has not been established. In a few species other cytoplasmic structures form in the cytoplasm of paraspermatids. For example in *Littoraria* spp. (Caenogastropoda) rhomboid granules, which stain positively with RNA stains, arise within rough endoplasmic reticulum cisternae and eventually fuse to form a rod-like body ([Buckland-Nicks et al., 2000](#)).

Seasonality and Endogenous Control of Spermatogenesis

Whilst sperm production can be continuous in some molluscs living in lower latitudes, spermatogenesis in many molluscs can be highly seasonal, especially those living in environments that experience significant seasonal climatic changes. Thus at certain times of the year (e.g., winter in species living at high latitudes) the testis will lack any spermatogenic activity. A number of environmental factors are thought to affect and possibly stimulate spermatogenesis, including temperature change, rainfall or degree of moisture (especially for land snails living in arid environments), photoperiod, and food. Such exogenous factors must stimulate endogenous control of spermatogenesis. Relatively little, however, is known about the endocrine control of spermatogenesis. Indications are that hormones that either inhibit or promote spermatogenesis are produced by neurosecretory cells in the head tentacles and/or cerebral ganglia of some cephalopods and snails. For example removal of the optic glands in octopus resulted in shrinkage of the testis ([Wells and Wells, 1972](#)) and experiments by [Takayanagi and Takeda \(1985\)](#) showed that removal of the optic tentacles of the land snail *Euhadra peliomphala* inhibited spermatogenesis. In this snail injection of optic gland extract stimulated sperm production. Numerous studies have demonstrated the presence of vertebrate-type steroids such as oestradiol, progesterone, and testosterone (all of which play a role in vertebrate reproduction) in molluscan reproductive tissues, with hormonal increase often occurring at the commencement of sperm production. However, the source of these hormones (exogenous or endogenous) and their role is unclear.

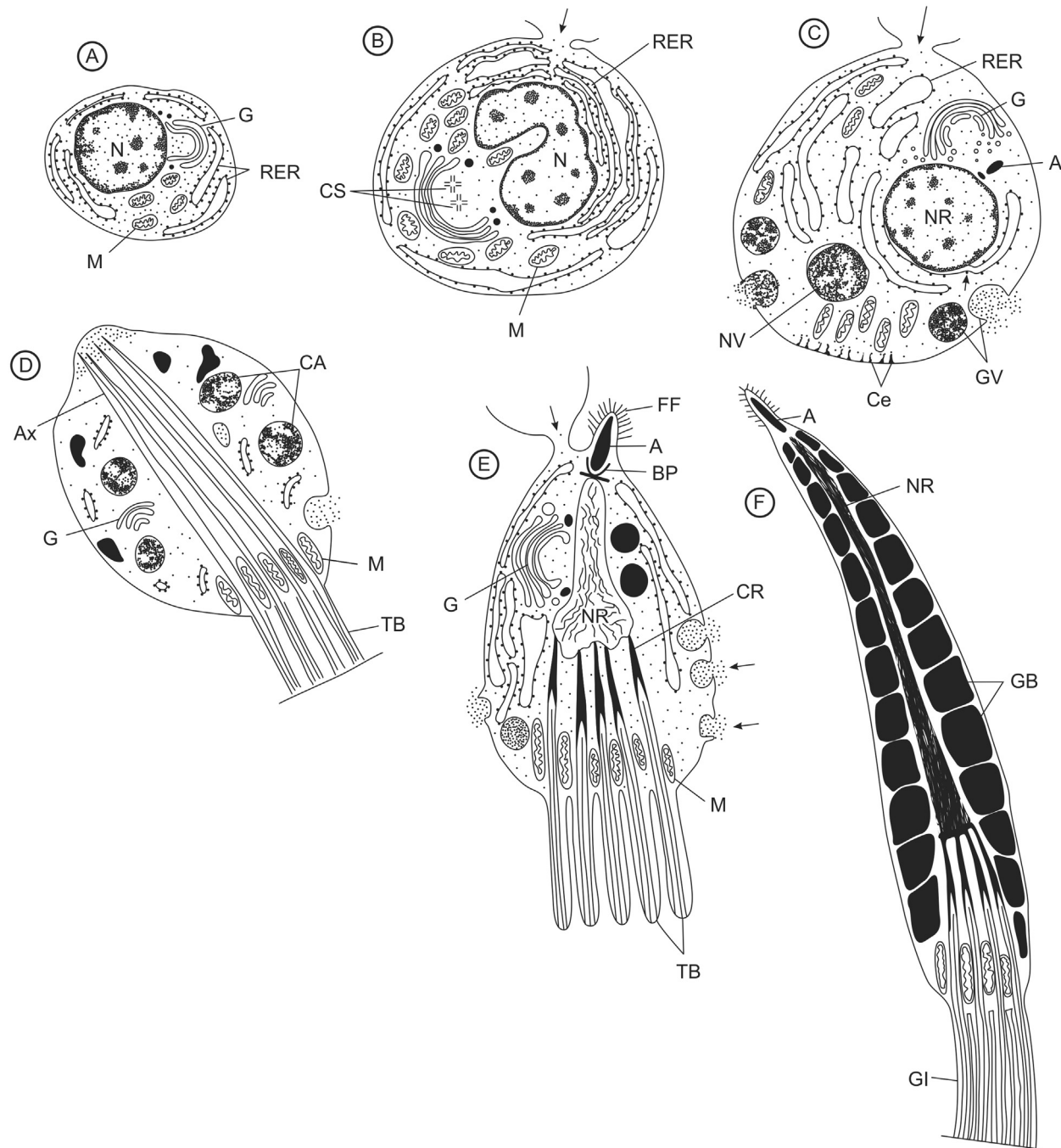


Fig. 3 Diagrammatic summary (interpreted from transmission electron microscope images) of some stages of paraspermatogenesis. A. Paraspermatogonium. B. Paraspermatocyte showing centrospheres (CS) and lobulated nucleus (N). Note also the cytoplasmic bridge (arrow). C. Early paraspermatid of a cerithioid snail with nuclear vesicle (NV) and nuclear remnant (NR), Golgi body (G) and adjacent developing acrosome (A). Granular vesicles (GV) are being exocytosed at the posterior of the cell and the centrioles (Ce) are now attached to the cell membrane. D. Mid-paraspermatid in which the nucleus has fragmented into caryomerites (CA) and the basal bodies have migrated through the cytoplasm to the anterior pole with the axonemes. Note the posterior position of the mitochondria (M) between the axonemes. E. Mid-paraspermatid of a cerithioid snail. Note the central nuclear remnant (NR) with ciliary rootlets (CR) attached posteriorly and exocytosis (arrows) of material posteriorly. The acrosome (A) with its basal plate (BP) is now at the developing anterior pole. Also present are fine filamentous (FF) material attached to the cell membrane. F. Mature paraspermatozoon with central nuclear remnant (NR) surrounded by glycogen bodies (GB). GL indicates the position of where glycogen is deposited between the axonemes of the tail brush. A, acrosome; AX, axoneme; BP, basal plate; CE, centriole; CR, ciliary rootlet; CS, centrosphere; G, Golgi body; M, mitochondrion; N, nucleus; NR nuclear remnant; NV, nuclear vesicle; RER, rough endoplasmic reticulum; TB, tail brush. Not drawn exactly to scale. (A),(C),(D)–(F): modified from Buckland-Nicks, J.A., Hodgson, A.N., 2005. Paraspermatogenesis of cerithioid snails: retention of an acrosome and nuclear remnant. *Journal of Morphology* 264, 314–326 and (D) from Buckland-Nicks, J., Williams, D., Chia, F-S., Fontaine, A., 1982. Studies on the polymorphic spermatozoa of a marine snail. 1. Genesis of the aupyrene sperm. *Biology of the Cell* 44, 305–314.

For example Scott (2012, 2013) not only questioned the evidence that such vertebrate-type sex steroids are synthesized by molluscs, but also the role that they play in molluscan reproduction.

Finally, recent studies have begun to identify many of the genes that are involved in euspermatogenesis and associated processes (e.g., see Llera-Herrera *et al.* (2013) and Song *et al.* (2017) for some literature). However, there are no published studies on the genes associated with paraspermatogenesis. Nevertheless, these gene studies will undoubtedly lead to a deeper understanding of the control of spermatogenesis.

References

- Buckland-Nicks, J. A., & Hodgson, A. N. (2005). Paraspermatogenesis of cerithioidean snails: Retention of an acrosome and nuclear remnant. *Journal of Morphology*, 264, 314–326.
- Buckland-Nicks, J., Healy, J., Jamieson, B. G. M., & Leary, S. (2000). Paraspermatogenesis in *Littoraria (Palustorina) articulata*, with reference to other Littorinidae (Littorinidea, Caenogastropoda). *Invertebrate Biology*, 119, 254–264.
- Buckland-Nicks, J., & Tompkins, G. (2005). Paraspermatogenesis in *Cerastostoma foliatum* (Neogastropoda): Confirmation of programmed nuclear death. *Journal of Experimental Zoology*, 303, 723–741.
- Chiva, M., Saperas, N., & Ribes, E. (2011). Complex chromatin condensation patterns and nuclear protein transitions during spermiogenesis: Examples from mollusks. *Tissue and Cell*, 43, 367–376.
- Healy, J. (2001). Spermatogenesis and oogenesis. In G. M. Barker (Ed.), *The Biology of Terrestrial Molluscs* (pp. 357–382). Oxon: CABI Publishing.
- Healy, J. M. (1989). Spermiogenesis and spermatozoa in the relict bivalve genus *Neotrigonia*: Relevance to trigonid relationships, particularly Unionoidea. *Marine Biology*, 103, 75–85.
- Healy, J. M., Bieler, R., & Mikkelsen, P. M. (2008). Spermatozoa of the Anomalodesmata (Bivalvia, Mollusca) with special reference to relationships within the group. *Acta Zoologica*, 89, 339–350.
- Healy, J. M., & Jamieson, B. G. M. (1981). An ultrastructural examination of developing and mature paraspermatozoa in *Pyrazus ebeninus* (Mollusca, Gastropoda, Potamididae). *Zoomorphology*, 98, 101–119.
- Hodgson, A. N. (1997). Paraspermatogenesis in gastropod molluscs. *Invertebrate Reproduction and Development*, 31, 31–38.
- Llera-Herrera, García-Gasca, A., Abreu-Goodger, C., Huvet, A., & Ibarra, A. M. (2013). Identification of male gametogenesis expressed genes from the Scallop *Nodipecten subnodosus* by suppressive subtraction hybridization and pyrosequencing. *PLOS ONE*, 8(9), e73176 (Available at: <https://doi.org/10.1371/journal.pone.0073176>).
- Scott, A. P. (2012). Do mollusks use vertebrate sex steroids as reproductive hormones? Part I: Critical appraisal of the evidence for the presence, biosynthesis and uptake of steroids. *Steroids*, 77, 1450–1468.
- Scott, A. P. (2013). Do mollusks use vertebrate sex steroids as reproductive hormones? Part II. Critical review of the evidence that steroids have biological effects. *Steroids*, 78, 268–281.
- Song, S., Yu, H., & Li, Q. (2017). Genome survey and characterization of reproduction-related genes in the Pacific Oyster. *Invertebrate Reproduction and Development*, 61, 97–109.
- Takayanagi, H., & Takeda, N. (1985). Hormonal control of gametogenesis in the terrestrial snail. *Euhadra pleiomphala*. *Development, Growth and Differentiation*, 27, 689–700.
- Wells, M. J., & Wells, J. (1972). Optic glands and the state of the testis in Octopus. *Marine Behaviour and Physiology*, 1, 71–83.

Fertilization, Comparative

Ryusaku Deguchi, Miyagi University of Education, Miyagi, Japan
Noritaka Hirohashi, Shimane University, Shimane, Japan

© 2018 Elsevier Inc. All rights reserved.

Introduction

In multicellular metazoan animals, two different types of gametes, eggs and spermatozoa, are unified by fertilization, and as the result fertilized eggs undergo mitotic cell divisions to develop into adult organisms. The processes of fertilization comprise multiple steps with highly sophisticated physical and chemical reactions within each gamete as well as between both gametes, ensuring normal fertilization. In recent years, research in this area has been carried out mainly using mammalian species including the laboratory model animals (e.g., mice and rats), domestic animals (e.g., pigs and cows), and humans. Thanks to that, a comprehensive view of fertilization has begun describing in terms of the cellular and molecular mechanisms. However, it is still worth studying with a wide range of animals to find the general principle as well as some features unique to particular groups or species. In this article, comparative aspects of the fertilization mechanisms in wide range of animal phyla of invertebrates and vertebrates, which could have evolved from a common ancestor ([Fig. 1](#)), are described.

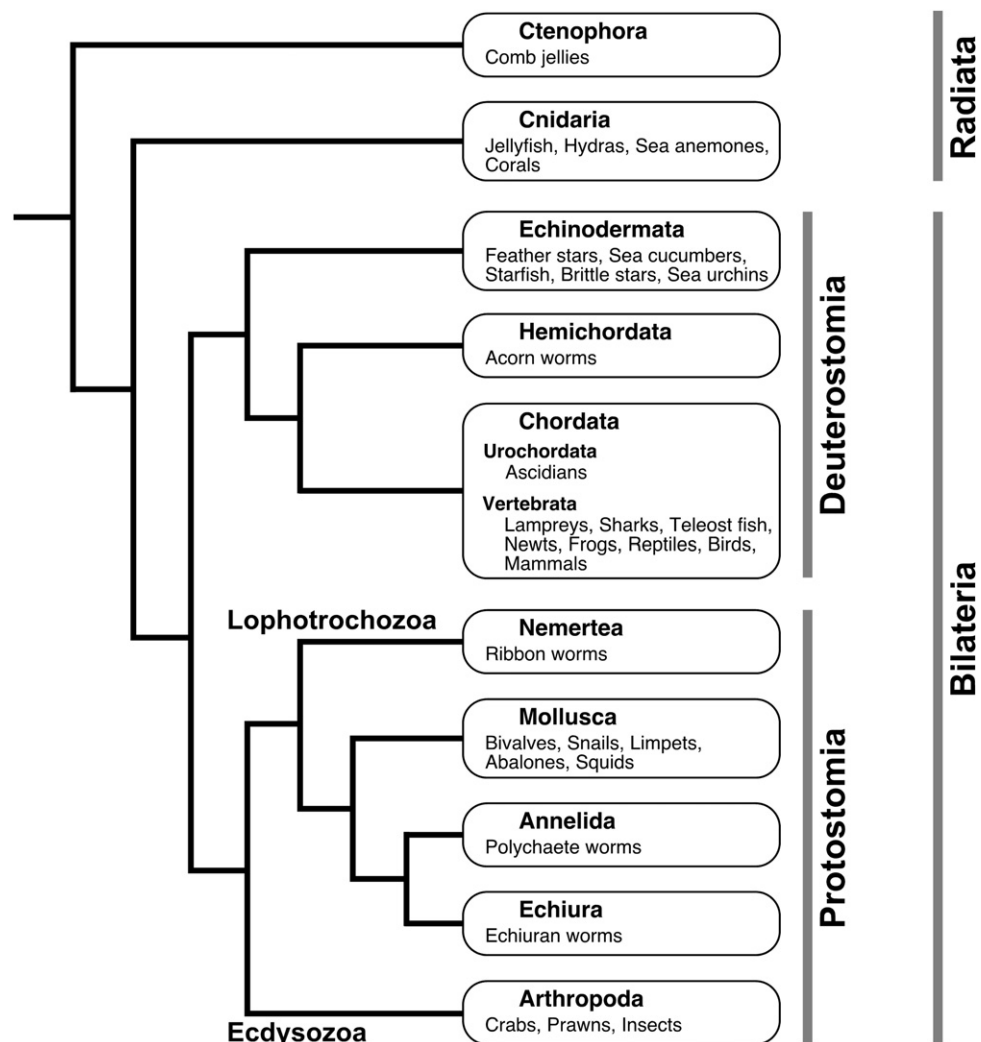


Fig. 1 Example of phylogenetic tree of the multicellular animals described in the text.

The Site of Fertilization

Fertilization occurs inside or outside the females. The site of fertilization is fundamental for determining the animal reproduction modes, oviparity (females lay unfertilized or developing eggs) and viviparity (females retain developing eggs inside their bodies and give birth to their offspring). Thus, all viviparous animals are internal fertilizers, whereas oviparous animals are either internal or external ones. Specifically, a mode in which females lay unfertilized eggs is called ovuliparity.

There are a number of thermic issues regarding the site of fertilization. In general, the site of fertilization does not coincide with the site of sperm deposition (insemination, ejaculation or spawning), therefore spermatozoa must travel in a certain distance to meet eggs. In internal fertilizers, especially in mammals, only a handful number of spermatozoa arrive in the upper (ampullae) part of the oviduct despite hundreds of those can reach the lower (isthmus) part of the oviduct. Several guidance mechanisms, such as thermotaxis, chemotaxis and rheotaxis, have proposed to explain how fertilizing spermatozoa are navigated to the site of fertilization. In the thermotaxis model, a temperature difference between the proximal ampulla and the distal isthmus regulates swimming direction of the ejaculated spermatozoa. Such a temperature difference was actually recorded 0.69°C in pigs and 0.8°C in rabbits. In the sperm chemotaxis model, spermatozoa can sense a gradient of progesterone derived from the cumulus cells surrounding the oocyte and swim toward the source of progesterone. In the rheotaxis model, spermatozoa swim against the fluid flow that is generated from oviduct to uterus after coitus, facilitating sperm guidance over long distances in the female reproductive tract (Miki and Clapham, 2013).

In external fertilizers, gametes are released thereafter diluted into aquatic environment so that timely (synchronous) spawning that increases frequency of sperm-egg encounter could have been developed. In addition, spermatozoa must find conspecific eggs to fertilize them. Hence, species-specificity in sperm-egg interactions, occurring at the level of sperm attraction to eggs, sperm acrosome reaction and sperm binding to the egg surface, are the central issues of research interests for last several decades. Occasionally, timing of sperm deposition (insemination) does not coincide with that of egg deposition (ovulation), therefore spermatozoa must await fertilization in the female reproductive tract, so that research addresses mechanisms of extended sperm survival at molecular, cellular and organismal levels. In many animals, females are promiscuous (polyandry; mating with multiple males), by which postcopulatory sexual selection, i.e., sperm competition and cryptic female choice, can favor fertilizations toward genetically more compatible males. This addresses evolutionary consequences of female reproductive systems and corresponding adaptive sperm traits (Birkhead and Pizzari, 2002).

Most, if not all, species thus far examined that employ external fertilization are cnidarians, echinoderms, ascidians, and teleost fish, whereas most species employing internal fertilization are insects, reptiles, birds, and mammals (both marsupials and eutherians). Mollusks, crustaceans, and amphibians are the groups that employ either internal or external depending on species. In teleost fish, synchronous spawning of gametes from both sexes into the same water column is a common strategy. Both gametes lose their fertilization competence shortly after the spawning, hence fertilization must be completed within a short period of time. Exceptionally, herring gametes, once released into ocean, are still capable of sustaining their fertilizing competence for days and spermatozoa remain quiescence until reached at the egg micropyle region where a sperm motility initiation factor activates spermatozoa and guides the sperm cells into the micropylar canal (Yanagimachi *et al.*, 2017). Sea urchins and starfish employ broadcast spawning where some millions to billions of gametes are released from single adults into sea. Spermatozoa encounter eggs coincidentally or with a chemical cue that guides spermatozoa to a conspecific egg, which is known for sperm chemotaxis. In some corals, spermatozoa and eggs are produced in the same individuals (hermaphrodite) and released together as bundles that drift to the ocean surface where fertilization takes place. Although broadcast spawners release massive amount gametes into sea, synchrony of gamete spawning from both sexes, presumably to gain fertilization opportunities, is a common feature. By contrast, there are some situations where gametes are released asynchronously. For example, in the mediterranean gobies, males deposit spermatozoa in the form of sperm trails laid on the nest surface before females start laying their eggs. To compensate the time-gap between sperm release by males and egg release by females, the sperm longevity (i.e., fertilizability) is guaranteed by either rendering their motility inactive or keeping them active for the extended duration. Several species in jellyfish, ascidians, and teleost fish are taking the former strategy; ejaculated spermatozoa remain quiescent until an egg factor activates sperm motility. Alternatively, spermatozoa are stored quiescently in the female storage organs, such as insect spermatheca, cephalopod or annelid seminal receptacle, bird sperm storage tubules (SSTs), and mammalian sperm reservoir. The average storing period of spermatozoa are varied from one group to another, and even from one species to another; hours to days in mammals, weeks in birds, months in reptiles and cephalopods, years to decades in bees and ants (Birkhead and Møller, 2008). Spermatozoa stored in the female storage organ are thereafter transferred to the site of fertilization. In mammals, fertilization occurs at the outermost portion of the fallopian tube, called the ampulla where ovulated oocytes are picked up by the infundibulum of the oviduct. In birds, spermatozoa in the SSTs at the utero-vaginal junction are transported to the infundibulum through the oviduct. In insects, spermatozoa are stored in spermatheca and fertilization occurs predominantly in the median oviduct. In the squid species, males implant sperm-containing capsules (spermatophores) to the external body surfaces or internal mantle cavities of the females. In some cases, the spermatophores themselves serve as sperm reservoirs, whereas in other cases, spermatozoa released from the spermatophores are stored in the female's seminal receptacles. Although fertilization may take place in the close vicinity of the implanted spermatophores or the seminal receptacles, direct observations are lacking.

Sperm Acrosome Reaction

Mature spermatozoa carry the acrosome, a Golgi-originated single large vesicle within the anterior portion of the sperm head. The acrosomal vesicle is discharged in response to a certain stimulus to release intra-vesicular contents as well as to expose inner surface

of the acrosomal vesicle (inside-out) during fertilization. The anterior tip of sperm head becomes elongated in some marine invertebrates, or sharpened (perforatorium) in mammals following breakdown of the acrosome. Such a series of morphological changes occurring in sperm head is called “the acrosome reaction”. The term “the acrosomal exocytosis” has been often used instead, particularly in mammals. The sperm acrosome reaction has been so far observed in wide range of animal groups such as mollusks (bivalves and sea snails), annelids (polychaete worms), arthropods (shrimps, crabs, and horseshoe crabs), echinoderms (feather stars, starfish, sea cucumbers, brittle stars, and sea urchins), cephalochordates (acorn worms), cyclostomes (hagfish and lampreys), and many other vertebrates including mammals. However, spermatozoa of some groups such as teleost fish lack the acrosome. It is postulated that as egg micropyle has evolved, the acrosome reaction has lost its significance. There are several distinct roles of the acrosome and acrosome reaction for fertilization. In marine invertebrates, the acrosome reaction occurs predominantly during sperm-egg interactions and acrosome-reacted spermatozoa can dissolve the layer covering the egg (egg coat) so that the acrosome reaction is believed to be prerequisite for penetrating the egg coat. The acrosome contains enzymatic activities such as proteases and glycosidases by which spermatozoa may be able to lyse the extracellular matrix of the egg. In sea snails and abalones, however, mode of action of sperm’s lytic activity is non-enzymatic. In sea urchins, the most abundant component of the acrosome is a ~30-kDa protein called bindin that plays the roles in species-specific sperm adhesion to egg surface and sperm-egg fusion. Upon the acrosome reaction, bindin becomes externalized and deposited on the surface of a finger-like protrusion (the acrosomal process) of the sperm head, enabling spermatozoa to attach on the egg vitelline layer (Vacquier, 2012). In mammals, the acrosome reaction is also required for tight adhesion of spermatozoa to the egg coat called zona pellucida and consequently penetration of bound spermatozoa through the zona matrix. Thus, the sperm acrosome reaction must occur at some points before penetrating the egg coat, however the exact location(s) remains unknown. The long-standing hypothesis proposed that acrosome-intact spermatozoa adhere the zona surface thereafter trigger the acrosome reaction. However, acrosome-reacted spermatozoa can also bind the zona and fertilize the egg (La Spina *et al.*, 2016).

In sea urchins, unfertilized eggs are surrounded by a gelatinous transparent layer called jelly coat where spermatozoa undergo the acrosome reaction. Jelly coat contains a sulfated fucose polymer (fucan) that induces the acrosome reaction. In all sea urchin species so far investigated except for one case (a sulfated galactan), egg jelly contains at least one form of sulfated fucans. A structural feature conserved among different sulfated fucans is that they are consisted of tandem repeat of a sulfated oligosaccharide unit (Vilela-Silva *et al.*, 2002). Differences are observed in the glycosidic linkage and positions of sulfation. Such the structural differences can explain the species-specificity in induction of the acrosome reaction. Spermatozoa recognize a conspecific sulfated fucan through a receptor for egg jelly (REJ) located on the flagellar membrane (Gunarathne *et al.*, 2007), which evokes concomitant changes in ionic permeability; the influx of Ca^{2+} and Na^{+} and the efflux of H^{+} and K^{+} . In particular, Ca^{2+} plays an essential role in fusion between the sperm plasma membrane and the outer acrosomal membrane (Gonzalez-Martinez *et al.*, 2001). This fusion event is highly regulated and conserved in the secretory pathway of other cell types (Belmonte *et al.*, 2016). During the acrosomal exocytosis, the membrane dynamics can be divided into several distinctive stages, i.e., priming, docking, fusion, fusion pore opening, vesiculation and shedding. In mammals, fusion pore opening occurs at multiple sites over the acrosome, resulting in massive loss of the plasma membrane overlying the acrosome (shedding of the acrosomal cap).

Sperm-Egg Binding and Fusion

In most animals, the egg plasma membrane is surrounded by a firm membranous acellular structure collectively called the egg coat, or more routinely called vitelline membrane, vitelline coat, vitelline envelope, vitelline layer, chorion, and zona pellucida for specific animal groups. In representative species so far examined, the egg coat is consist of several (glyco)proteins as the major components. The vitelline membrane seems to have essential roles for recognition of spermatozoa from conspecific species to avoid interspecific hybridization, and protection of eggs from microorganisms, mechanical damages and multiple-sperm entry. A fertilizing spermatozoon can penetrate the egg coat at any location in most animal species with a few exceptions such as birds.

In sea urchins, the vitelline layer is very thin and bound tightly to the egg plasma membrane before fertilization. Acrosome-reacted spermatozoa adhere to the vitelline layer in a species-preferential manner via the acrosomal process. Such the species-specific adhesion is mediated by interaction between the complementary molecules called sperm bindin and its receptor, a high molecular weight glycoprotein (approximately 350-kDa) on the egg vitelline layer (Hirohashi *et al.*, 2008). Cross-fertilizations, therefore hybrid embryos, were readily observed when the vitelline membrane is removed by protease treatment, hence the vitelline membrane serves the latest and most potent barrier against interspecific hybridization in sea urchins.

In ascidians, hermaphroditism is common and the level of self-sterility varied. For instance, all species of the families Asciidiidae and Corellidae, and many species of Styela and Molgula are self-fertile, whereas *Ciona intestinalis* and *Halocynthia roretzi* are self-sterile. Such the self-sterility system is accounted for operating at the level of sperm-egg coat interaction because naked eggs exhibit self-fertility. The vitelline membrane of ascidian eggs is present apart from the egg plasma membrane, implying the least involvement of eggs themselves in self/non-self recognition. Thus, the ascidian vitelline membrane is capable of discriminating the spermatozoa from not only foreign species but also same individuals. The molecules responsible for self/non-self recognition, which are easily removed from the vitelline membrane in acid seawater (such as pH 3), have been identified as v-Themis-A/B in *Ciona*. In the current model, it is proposed that when s-Themis-A/B on the sperm surface recognizes self v-Themis-A/B on the vitelline membrane, an intracellular Ca^{2+} rise takes place in the sperm, resulting in the detachment of spermatozoa from the vitelline membrane (Sawada *et al.*, 2014).

Mammalian eggs are surrounded by a thick egg coat called the zona pellucida that contains three (ZP1-3 in mouse) or four (ZP1-4 in human) glycoproteins. It seems that among the glycoproteins, ZP2 is necessary for the binding of spermatozoa to the zona pellucida and that this phenomenon is relatively, but not absolutely, species-specific. Recent analyses using gene-knockout mice have, however, shown that the spermatozoa lacking the zona-binding ability are also able to fertilize eggs in the presence of zona pellucida (Okabe, 2014).

In most species of molluscs including abalones, a vitelline membrane also acts as a barrier against interspecific fertilization, which is regulated by species-specific interaction between a sperm acrosome-derived cationic protein called lysin and its receptor glycoprotein called VERL (vitelline envelope receptor for lysin). Abalone spermatozoa (1 μm in diameter) can make a hole (3 μm in diameter) in the vitelline membrane and penetrate through it. Non-enzymatic action of abalone lysin is presumed to be responsible for such fine regulation. In contrast, it is thought that proteases are used to dissolve the egg coat in deuterostomes such as sea urchins, ascidians, and mammals. In ascidian *Halocynthia*, it is reported that sperm penetration through the egg coat is mediated by the extracellular ubiquitin/proteasome system (UBS), i.e., upon sperm activation, spermatozoa release proteasome, ubiquitin-conjugating enzyme, ubiquitin and ATP into the surrounding seawater. A vitelline coat 70-kDa glycoprotein, HrVC70, which serves as the receptor for sperm binding is subjected to extracellular ubiquitination followed by degradation by the sperm proteasome during fertilization (Sawada *et al.*, 2014). In mammals, extracellular UBS is also suggested to operate during zona lysis. In spite of energetic studies, the proteases responsible for the penetration of zona pellucida have not yet been determined. Since the mouse spermatozoa recovered from the perivitelline space can penetrate through the zona pellucida again (Okabe, 2014), the proteases, if any, are considered to persist on the sperm surface without diffusing after acrosome reaction.

In some animals, a tough and elastic membrane, called chorion, prevents sperm penetration even before fertilization. Alternatively, sperm entry is facilitated by the narrow canal produced in a certain region of chorion, micropyle. A single funnel-shaped micropyle is located near the animal pole in most teleost fish and cephalopod eggs, whereas 4–12 micropyles are present at the animal pole region in the paddlefish *Polyodon spathula*. The number and location of micropyles in insect eggs vary significantly among species. In the fruit fly *Drosophila melanogaster*, one micropyle is present in a pointed protrusion at the anterior tip of the egg. In the grasshopper *Eyprepocnemis plorans*, an average of 40 micropyles are arranged in a ring-like manner close to the posterior pole. Queens of the termite *Reticulitermes speratus* can produce offspring both sexually and asexually (parthenogenetically) by controlling proportion of micropyleless eggs over time (Yashiro and Matsuura, 2014).

Eggs of many cnidarians including hydras, hydrozoan jellyfish, sea anemones, and corals, generally lack the firm egg coat, and the plasma membrane is covered with jelly layer. Nevertheless, site of sperm adhesion and subsequently site of sperm fusion are restricted to the plasma membrane of the animal pole just above the female pronucleus. In some species such as *Hydra* and *Hydractinia*, site of sperm-egg fusion appears as an indentation called the fertilization pit.

Monospermy and Physiological Polyspermy

In general, the entry of more than two spermatozoa into the egg cytoplasm, referred to as polyspermy, causes aberrant effects on meiosis completion or embryo development and hence embryonic death, due mainly to excess male centrosomes delivered into the egg. Thus, most animals have evolved multiple mechanisms by which monospermic fertilization is ensured (known for polyspermy block).

Changes in the electric potential of the egg plasma membrane are thought to play a central role in the fast block to polyspermy in various animals (Gould and Stephano, 2003). In sea urchin eggs, a resting membrane potential of approximately -70 mV shifts to $+20$ mV within a few seconds after the contact of a fertilizing spermatozoon. When Na^+ concentration is lowered in the surrounding water, this sperm-induced jump-up in the membrane potential, i.e., depolarization, does not occur, resulting in polyspermy. In addition, holding the membrane potential of unfertilized eggs at either $> +5$ or < -20 mV prevents sperm entry, whereas holding them between -20 and $+5$ mV allows repeated sperm entries. Thus, it is thought that the fertilizing spermatozoa can enter the egg within the narrow window of time during which Na^+ -dependent depolarization takes place (or the egg becomes depolarized), ensuring monospermy at the initial phase of fertilization. However, it is a matter of debate whether or not the fast electrical polyspermy block is necessary under physiological conditions where not so many spermatozoa are assumed to reach the egg.

In many other marine invertebrates including ribbon worms, polychaete worms, echiuran worms, starfish, ascidians, bivalves, and gastropods, a similar depolarization from the resting negative potentials (between -80 and -10 mV) to the positive levels (between $+10$ and $+60$ mV) mainly due to Na^+ influx or Na^+ and Ca^{2+} influxes is also thought to play a role in the fast polyspermy block. In contrast, in eggs of the crab *Maja squinado*, fertilization induces K^+ efflux-mediated hyperpolarization (a decline of the membrane potential from -50 to -80 mV), which is accounted for polyspermy block. In eggs of freshwater lamprey and frog, fertilization evokes Cl^- efflux-mediated depolarization, resulting in polyspermy block.

In contrast to the aforementioned animals that show a fast electrical block to polyspermy, comb jellies, jellyfish, insects, teleost fish, newts, reptiles, birds, and mammals are regarded as devoid of such a system. Instead, in most teleost fish and various insects, the number of spermatozoa adhering to the egg in the initial phase of fertilization is limited in space and time by having a single or multiple micropyles on the chorion of an egg. Jellyfish eggs also ensure monospermic fertilization by restricting the site of sperm-egg fusion to the plasma membrane of the animal pole (5–10 μm in diameter).

After the successful entry of a spermatozoon, the fertilized egg generally exhibits a so-called late block to polyspermy, which includes physical and chemical modifications of the egg coat and the egg plasma membrane. In animals such as sea urchins, frogs, and mammals, secretion of the cortical granules results in the release of proteinous (e.g., proteases and ovoperoxidase) and chemical (e.g., H_2O_2) components to modify the egg coat (e.g., formation of the fertilization membrane in sea urchin eggs), which prevents the extra spermatozoa from adhering to or passing through it (Wessel and Wong, 2009). The cortical granule exocytosis in teleost fish eggs causes shrinkage of inner layer chorion, resulting in a decrease in the diameter of micropyle or the complete closure of this gate to abolish the following sperm entry (Murata, 2003). Late block to polyspermy at the level of plasma membrane independent of membrane potential has been reported in many animals including jellyfish, bivalves, and mammals. The loss of Juno (folate receptor 4), an egg receptor for the sperm ligand Izumo, is one of the reasons for the late plasma membrane block in mammalian eggs.

In contrast to monospermic fertilization that is absolutely necessary for eggs of many species to develop normally, there are some animals where polyspermy is mandatory for normal embryo development. This phenomenon, called physiological polyspermy, is found in vertebrates such as elasmobranchs (sharks and rays), urodeles (newts and salamanders), reptiles, and birds, as well as marine invertebrates such as comb jellies. In these animals, only a single sperm nucleus is selected to fuse with the egg nucleus, resulting in formation of a diploid zygotic nucleus. Consequently, other sperm nuclei and accompanying centrosomes are diminished without participating in the formation of fertilized egg (Iwao, 2012). Although monospermic fertilization is more common in insects, polyspermic fertilization is often seen in some species. Similarly, only a single male nucleus can unite with a female nucleus. This pattern is called compensable polyspermy (Snook *et al.*, 2011). Even in the species displaying physiological and compensable polyspermy, the time of fertilization is limited. For instance, fertilized avian eggs quickly lose their fertilizability due to the formation of chalaza-layer immediately after ovulation.

Egg Activation by Ca^{2+} Rise

Fertilized eggs exhibit a series of processes required for the release from cell cycle arrest, by which normal development can start, which is collectively called egg activation. The key event that leads to egg activation is a transient rise in the cytoplasmic Ca^{2+} concentration in fertilized eggs (from 100 nM to 2–3 μM in the case of sea urchins), which in turn modifies the activities of various Ca^{2+} -dependent proteins including protein kinases and phosphatases. Although it is known that eggs of all animal species investigated so far exhibit a Ca^{2+} rise at fertilization, there are considerably greater interspecific variations in the source of Ca^{2+} , the pattern of Ca^{2+} rises, and even the role of elevated Ca^{2+} (Kashir *et al.*, 2013).

In most animals, a fertilizing spermatozoon carries a substance(s) that triggers Ca^{2+} rise in the egg. In ribbon worms, ascidians, newts, birds, and mammals, it is thought that a sperm-borne soluble factor, called sperm factor, that evokes the Ca^{2+} rise is introduced into the egg cytoplasm after sperm-egg fusion. Recent analyses have shown that mammalian sperm factor corresponds to a sperm-specific phospholipase C (PLC) called PLC, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and diacylglycerol in eggs. In newts, delivery of citrate synthase from spermatozoa is the main pathway to trigger the Ca^{2+} rise probably through elevation of PLC activity in eggs. In contrast to the internally acting soluble sperm factor, several lines of evidence indicate that many other animals including echinurans and frogs use the system where sperm protein binds to an egg receptor that links to intracellular pathways leading to a Ca^{2+} rise. In some animals such as *Drosophila*, prawns, and zebrafish, a Ca^{2+} rise occurs at the time of ovulation without stimulation by spermatozoa. Up-regulation of egg surface mechanosensitive ion channels that occurs in response to squeezing or swelling the egg would be responsible for the Ca^{2+} rise in *Drosophila*. Induction of Ca^{2+} rise without sperm would lead the way that eggs can also reproduce via parthenogenesis, which is frequently seen in arthropods and other animals.

Both external medium that contains Ca^{2+} (such as seawater) and intracellular organelles that store Ca^{2+} (such as the endoplasmic reticulum, ER) are the potential sources from which the Ca^{2+} are mobilized to the egg cytoplasm. Typically, a Ca^{2+} wave starts at the site of sperm-egg fusion and thereafter propagates the antipode (Fig. 2(A)), which is observed at fertilizations of such animals as jellyfish, echinoderms, ascidians, amphibians, and mammals. Polyspermic fertilization in newt eggs is accompanied by multiple Ca^{2+} waves that originate from respective sperm-egg fusion sites. Since each Ca^{2+} wave in newt eggs only propagates to one-eighth to one-quarter of the egg, multiple sperm entries are required to generate a Ca^{2+} rise over the entire egg. The Ca^{2+} waves initiated from a single or multiple points are mainly mediated by ER membrane-located IP_3 receptors that promote the Ca^{2+} release from the ER lumen. In contrast, a centripetal Ca^{2+} wave initiated at the whole cortex (Fig. 2(B)), which is probably dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels on the plasma membrane, is detected in fertilized eggs of ribbon worms, bivalves, limpets, and echinurans.

In jellyfish, some bivalves, limpets, echinurans, sea urchins, starfish, fish, and frogs, a single Ca^{2+} rise lasts for a few minutes to several tens of minutes (Fig. 2(C)). Fertilized eggs of ribbon worms, some bivalves, polychaetes, ascidians, and mammals, in contrast, exhibit repetitive Ca^{2+} rises, called Ca^{2+} oscillations, that persist for several tens of minutes to several hours (Fig. 2(D)).

In spite of the difference in the pattern and source of Ca^{2+} , one of the universal roles of the Ca^{2+} rise in the entire egg is to trigger the resumption of cell cycle; unfertilized eggs are arrested at either the first prophase (e.g., some bivalves and echinurans), the first metaphase (e.g., some bivalves, limpets, insects, and ascidians), the second metaphase of meiosis (e.g., most vertebrates), or the G_1 stage after completion of meiosis (e.g., jellyfish and sea urchins). It is known that Ca^{2+} -dependent proteins such as Ca^{2+} /calmodulin-dependent protein kinase II and calcineurin are involved in the downstream effector molecules responsible for the resumption of cell cycle in insects, ascidians, frogs, and mammals. A Ca^{2+} rise also modifies the physical and chemical properties of egg coat and plasma membrane through cortical granule exocytosis or other mechanisms to protect embryos as well as to prevent polyspermy in the later

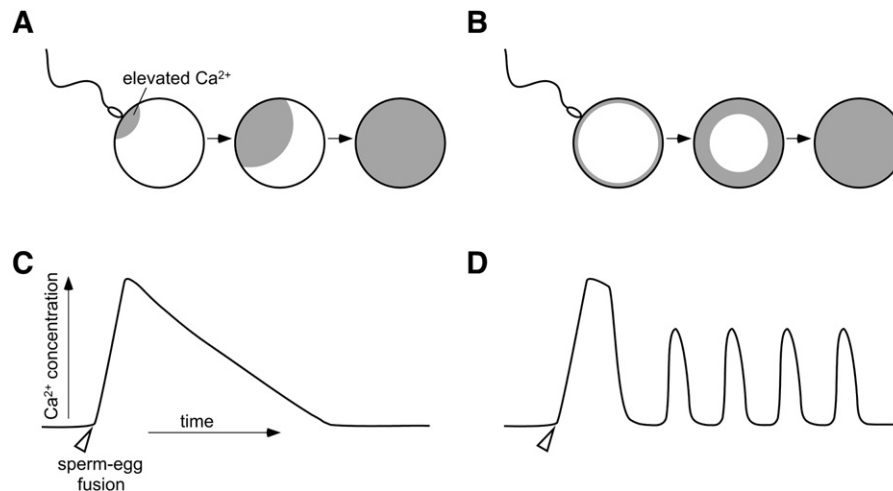


Fig. 2 Spatial and temporal patterns of intracellular Ca^{2+} rise in fertilized eggs. (A): Point-source Ca^{2+} wave initiated at the sperm-egg fusion site. (B): Centripetal Ca^{2+} wave initiated at the whole cortex. (C): Single Ca^{2+} rise. (D): Ca^{2+} oscillations.

phase. Ca^{2+} influx through voltage-dependent Ca^{2+} channels in many marine invertebrates and Cl^- efflux through Ca^{2+} -activated Cl^- channels in frogs contribute to the membrane depolarization that serves as fast electrical polyspermy block. It seems that the formation of cytoplasmic protrusion generated by a local Ca^{2+} rise helps the fertilizing spermatozoa on the vitelline envelope to enter the egg cytoplasm efficiently in some polychaetes, where unfertilized eggs have a wide perivitelline space (Nakano *et al.*, 2008). The pattern and amplitude of Ca^{2+} rise at fertilization also influence the later embryonic development (Whitaker, 2008). In ascidian and frog eggs, the direction of Ca^{2+} wave may specify the orientation of an embryonic axis. In mammals, abnormal patterns of Ca^{2+} oscillations are known to lower the developmental competence of blastocysts, resulting in fewer offspring.

References

- Belmonte, S.A., Mayorga, L.S., Tomes, C.N., 2016. The molecules of sperm exocytosis. *Advances in Anatomy, Embryology and Cell Biology* 220, 71–92.
- Birkhead, T.R., Møller, A.P., 2008. Sexual selection and the temporal separation of reproductive events: Sperm storage data from reptiles, birds and mammals. *Biological Journal of the Linnean Society* 50, 295–311.
- Birkhead, T.R., Pizzari, T., 2002. Postcopulatory sexual selection. *Nature Reviews Genetics* 3, 262–273.
- Gonzalez-Martinez, M.T., Galindo, B.E., de La Torre, L., *et al.*, 2001. A sustained increase in intracellular Ca^{2+} is required for the acrosome reaction in sea urchin sperm. *Developmental Biology* 236, 220–229.
- Gould, M.C., Stephano, J.L., 2003. Polyspermy prevention in marine invertebrates. *Microscopy Research and Technique* 61, 379–388.
- Gunaratne, H.J., Moy, G.W., Kinukawa, M., *et al.*, 2007. The 10 sea urchin receptor for egg jelly proteins (SpREJ) are members of the polycystic kidney disease-1 (PKD1) family. *BMC Genomics* 8, 235.
- Hirohashi, N., Kamei, N., Kubo, H., *et al.*, 2008. Egg and sperm recognition systems during fertilization. *Development, Growth & Differentiation* 50, S221–S238.
- Iwao, Y., 2012. Egg activation in physiological polyspermy. *Reproduction* 144, 11–22.
- Kashir, J., Deguchi, R., Jones, C., Coward, K., Stricker, S.A., 2013. Comparative biology of sperm factors and fertilization-induced calcium signals across the animal kingdom. *Molecular Reproduction and Development* 80, 787–815.
- La Spina, F.A., Puga Molina, L.C., Romarowski, A., *et al.*, 2016. Mouse sperm begin to undergo acrosomal exocytosis in the upper isthmus of the oviduct. *Developmental Biology* 411, 172–182.
- Miki, K., Clapham, D.E., 2013. Rheotaxis guides mammalian sperm. *Current Biology* 23, 443–452.
- Murata, K., 2003. Blocks to polyspermy in fish: A brief review. In: *Aquaculture and Pathobiology of Crustacean and Other Species, Proceedings of the 32nd UJNR Aquaculture Panel Symposium*, Davis and Santa Barbara, CA, pp. 1–15.
- Nakano, T., Kyoizuka, K., Deguchi, R., 2008. Novel two-step Ca^{2+} increase and its mechanisms and functions at fertilization in oocytes of the annelidan worm *Pseudopotamilla ocellata*. *Development, Growth & Differentiation* 50, 365–379.
- Okabe, M., 2014. Mechanism of fertilization: A modern view. *Experimental Animals* 63, 357–365.
- Sawada, H., Yamamoto, K., Otsuka, K., *et al.*, 2014. Allorecognition and lysin systems during ascidian fertilization. In: Sawada, H., Inoue, N., Iwano, M. (Eds.), *Sexual Reproduction in Animals and Plants*. Tokyo: Springer, pp. 231–244.
- Snook, R.R., Hosken, D.J., Karr, T.L., 2011. The biology and evolution of polyspermy: Insights from cellular and functional studies of sperm and centrosomal behavior in the fertilized egg. *Reproduction* 142, 779–792.
- Vacquier, V.D., 2012. The quest for the sea urchin egg receptor for sperm. *Biochemical Biophysical Research Communications* 425, 583–587.
- Vilela-Silva, A.C., Castro, M.O., Valente, A.P., Biermann, C.H., Mourao, P.A., 2002. Sulfated fucans from the egg jellies of the closely related sea urchins *Strongylocentrotus droebachiensis* and *Strongylocentrotus pallidus* ensure species-specific fertilization. *The Journal of Biological Chemistry* 277, 379–387.
- Wessel, G.M., Wong, J.L., 2009. Cell surface changes in the egg at fertilization. *Molecular Reproduction and Development* 76, 942–953.
- Whitaker, M., 2008. Calcium signalling in early embryos. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* 363, 1401–1418.
- Yanagimachi, R., Harumi, T., Matsubara, H., *et al.*, 2017. Chemical and physical guidance of fish spermatozoa into the egg through the micropyle, double dagger. *Biology of Reproduction* 96, 780–799.
- Yashiro, T., Matsuura, K., 2014. Termite queens close the sperm gates of eggs to switch from sexual to asexual reproduction. *Proceedings of the National Academy of Sciences of the United States of America* 111, 17212–17217.

Sperm Storage and Delayed Fertilization

Teri J. Orr, University of Utah, Salt Lake City, UT, United States

Patricia LR Brennan, Mount Holyoke College, South Hadley, MA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cryptic female choice The processes used by females to bias paternity towards certain males, a type of post-copulatory sexual selection.

Delayed fertilization The separation in time between mating (in particular insemination) and the fertilization of an ovum—used as a male-active and female-active synonym of ‘female sperm storage’.

Delayed ovulation The separation in time between mating and ovulation by a female, often used as a female-active synonym of ‘female sperm storage’ and ‘delayed fertilization’.

Female sperm storage The maintenance of viable sperm by females between insemination and fertilization.

Male sperm storage The maintenance of viable sperm by males between production (spermatogenesis) and use (insemination).

Spermatheca The sperm storage organ found in some invertebrate females.

Sperm competition The processes related to sperm biology including number, concentration, behavior, morphology, and related seminal fluids, that increase a male’s chances of fertilization when females mate with multiple males during a single reproductive bout.

Sperm longevity The duration of time sperm remain viable.

Sperm motility The activity patterns in particular flagellar (or similar) movement of spermatozoa.

Sperm senescence The process of aging in sperm that is associated with a decrease in function.

Sperm viability The ability of sperm to successfully fertilize an ovum.

Sperm Storage

The production of spermatozoa and their subsequent use by males (insemination) or by females (conception) may be separated by extended periods of time ranging from hours to days, months and even decades. Sperm storage is likely ubiquitous in most animals with internal fertilization because sperm are rarely, if ever, deposited directly in contact with the eggs. Both sexes are able to store sperm, and the numerous adaptations required for long-term storage by one sex, may provide pre-adaptations to sperm storage in the other sex, or become arenas of conflict. Here we deal primarily with female long-term sperm storage in mammals, and other vertebrates, though sperm storage is also common in invertebrates.

Sperm storage results in a temporal separation of events between spermatogenesis, mating and conception. Thus, sperm storage may be adaptive in a wide range of habitats, in species with diverse life histories, and it is likely to influence the evolution of mating systems, post-copulatory sexual selection such as cryptic female choice and sperm competition, as well as the degree of sexual conflict (reviewed in [Orr and Brennan, 2015](#)).

Male Sperm Storage

Male sperm storage (MSS) is the maintenance of mature viable spermatozoa for extended periods after spermatogenesis but before ejaculation. Male sperm storage refers to the temporal separation between sperm production and sperm transfer, and therefore MSS is likely found in nearly all animals as males are unlikely to produce sperm at the moment of insemination.

MSS can vary from a few hours or a few days (short-term), to several weeks or months (long-term). Long term MSS likely requires specializations to maintain the vitality of sperm and prevent the natural decline in motility and fertilization ability that is known to occur with aging sperm in many species including humans ([Racey and Entwistle, 2000](#)). These specializations however are poorly known. Long-term MSS is known in mammals primarily from bats where many species produce sperm in the spring or summer and these sperm are stored until their use in the late fall and winter ([Racey and Entwistle, 2000](#)). This phenomenon is also known to occur in some snakes ([Almeida-Santos *et al.*, 2004](#)).

In mammals, sperm are maintained in the epididymis until ejaculation, when contractions during copulation result in sperm transport along with secretions from the male reproductive tract (alkaline sugars and zinc in the prostate, enzymes, sugars, prostaglandins and other substances in the seminiferous vesicles). In other species, sperm may be maintained in the testes and spermatic cords (e.g., sharks), the caudal portion of the vas deferens (e.g., birds), or in spermatophores (e.g., salamanders).

Female Sperm Storage

Female sperm storage (FSS) is the extended maintenance of viable sperm received during mating until their use for conception. Female sperm storage (FSS) has been described in nematodes, annelids, squid, arthropods, and in all major groups of vertebrates including amphibians, cartilaginous fishes (sharks and rays), bony fish, squamates, reptiles birds, and mammals (Fig. 1). Female sperm storage can last from hours (mice) to days (dogs and horses), several years (turtles), and up to decades (leaf-cutter ants) (Suarez and Pacey, 2006; Orr and Zuk, 2013; Orr and Brennan, 2015).

Historically FSS has also been termed ‘delayed fertilization’. However, this term presents females as passive participants in the process of sperm storage by making it seem as if sperm themselves are simply awaiting their opportunity to fertilize an egg. The distinction between passive and active FSS is important, as selection on females to store sperm long-term is often confused with sperm survival inside the female that may result from selection on males to keep their sperm alive, even if prolonged survival of sperm inside the female reproductive tract runs counter to her reproductive interests. The term ‘sperm storage’ as currently implemented by some researchers may be referring to what may simply be ‘sperm longevity’ (sperm surviving inside the female but with no clear ‘female active’ role in storage) (see [Section Glossary](#)).

Similarly the term ‘delayed ovulation’ is used in the literature in place of long-term sperm storage. Delayed ovulation places the emphasis on just one aspect of female physiology that refers to the timing when ovulation takes place relative to insemination. Both delayed fertilization and delayed ovulation highlight only two aspects of the multivariate physiological processes that take place in order for fertilization to occur, and thus we focus on more neutral terminology.

The general lack of refined terminology and consistency of criteria for assigning FSS has been problematic. For example, several studies, have mistakenly described female sperm storage based on births occurring later than was expected (e.g., in skinks; [Sever and Hopkins, 2004](#)); a phenomenon that instead may have been caused by other types of reproductive delays (implantation or development) and not true FSS ([Birkhead and Møller, 1993](#); [Neubaum and Wolfner, 1999](#)). Similarly, several observations once believed to provide evidence of long-term FSS were later discovered to have resulted from parthenogenetic reproduction (‘virgin birth’, i.e., the use of unfertilized eggs to produce offspring) in a few vertebrates (e.g., some lizards, sharks, and snakes – see [Neubaum and Wolfner, 1999](#); [Holt and Lloyd, 2010](#)).

Thus, the need to clarify what constitutes FSS and the criteria to study its causes and consequences is apparent. Below we present criteria that can be used to study FSS in diverse taxa ([Orr and Brennan, 2015](#)), and review examples from major taxonomic groups. In all species, sperm will suffer from senescence and lose their fertilizing ability within some period of time after being made. Therefore, prolonged sperm storage by the female requires some mechanism to keep sperm viable, and often these mechanisms include specialized morphology, physiology, biochemistry and associated changes in sperm behavior.

Criteria Associated With Long-Term Female Sperm Storage

Anatomy

The presence of specialized sperm storage structures is strong evidence of long-term active FSS. Some of these structures are dedicated only to storing sperm (e.g., spermatheca, sperm storage tubules etc.), but others have been co-opted from pre-existing structures (e.g., folds in the lining of the uterus or cervix, the utero-tubule junction, uterine glands, etc.) as the site of sperm maintenance (Fig. 2). Therefore the absence of sperm storage organs per se is not evidence that long-term sperm storage has not evolved. Instead, in the absence of modified sperm storage structures, the location of sperm consistently in pre-existing locations (e.g., uterine glands or the utero-tubule junction), sperm interacting with epithelial cells of the female reproductive tract, for example, by having an ‘organized’ arrangement with sperm heads facing the epithelial lining of the female reproductive tract (e.g., bats, marsupials, dogs, humans), can serve as evidence of sperm storage ([Racey et al., 1987](#); [Suarez and Pacey, 2006](#); [Orr and Brennan, 2015](#)).

Females in some taxa have not only one but several types of sperm storage organs (e.g., both spiders and fruit flies – see examples below). The interpretation of such elaborate morphology for sperm storage in these groups might be interpreted as evidence of refined female cryptic control of sperm (i.e., [Eberhard, 1996](#)).

Additional structures associated with or actual placement inside of sperm storage structures include modified glands usually of secretory natures (e.g., in some marsupials [Kress and Morson, 2007](#)). Such glands may present the female with the capability to nourish sperm while in storage (see [Section Physiology](#)).

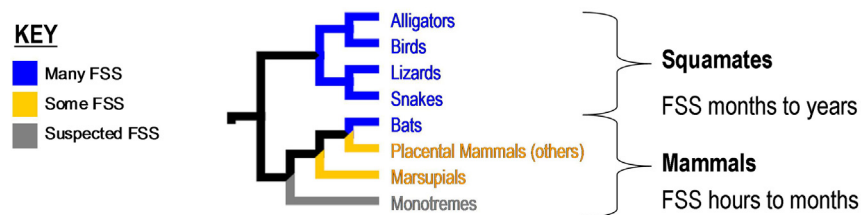


Fig. 1 A generalized phylogeny of key vertebrates discussed in this article known to exhibit long-term female sperm storage. Colors indicate general numbers of species known to have sperm storage (see key).

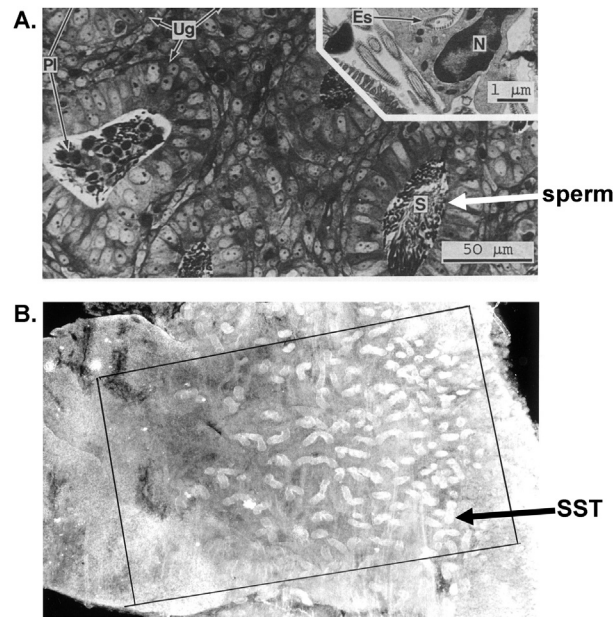


Fig. 2 (A) Photomicrograph of female sperm storage and associated structures (using toluidine blue stain) in the little brown bat (*Myotis lucifugus*). Indicated by Ug are uterine glands (Ug), sperm (S), polymorphonuclear leucocytes (PI). Inset is a thin section (TEM) of engulfed sperm interacting with polymorphonuclear leucocytes (PI). Illustration from Racey, P.A., Uchida, T.A., Mori, T., Avery, M.I., Fenton, M.B., 1987. Sperm-epithelium relationships in relation to the time of insemination in little brown bats (*Myotis lucifugus*). *Journal of Reproduction and Fertility* 80, 445–454, used with permission. (B) Photomicrograph of bird (zebra finch) sperm storage tubules (indicated by arrow). Photo used with permission from T. Birkhead.

Physiology

Sperm maintained within female humans, horses, pigs and dogs all are viable for a longer period of time than in similar conditions outside the female (Suarez and Pacey, 2006) suggesting that additional aspects of female biology may contribute to sperm storage.

Key aspects of female physiology are often also modified to enable long-term FSS. First, immune suppression is common, as a means to prevent the destruction and removal of stored sperm. Such immune suppression is taxon-specific (e.g., low leukocyte counts in mammals and plasmatocytes, crystal cells or lamellocytes in insects). Lowered immunity is a key cost of sperm storage such as in leaf-cutter ants (Baer *et al.*, 2006). However, a depressed immune response likely imposes selection on females to have a localized rather than systemic immune response to keep sperm from being destroyed by her immune system. Therefore the immune suppression that accompanies sperm storage is typically restricted such as in the oviduct of many mammals (Suarez and Pacey, 2006).

Females may also carefully regulate and maintain areas of specific pH and/or temperatures that are conducive to maintaining or even increasing sperm longevity (Holt, 2011; Suarez and Pacey, 2006; Suarez, 2008). Indeed, the pH of areas where sperm may be maintained for extended periods of time, for example, the oviduct in humans has a higher pH than the vagina that is more acidic, and this helps protect sperm in the oviduct (Suarez and Pacey, 2006).

Sperm have to come out of storage if they are to be successfully used for fertilization, and this event should coincide with ovulation. There is an interplay between male and female physiology for these events to coincide. When ovulation occurs, females may trigger release of sperm from storage areas/sites and sperm can become active in preparation for motion towards the site of conception during this period (Holt, 2011; Suarez and Pacey, 2006). Suspected mechanisms for this “re-activation”, i.e., hyperactivation and eventual capacitation to enable fertilization include changes in levels of cytoplasmic calcium (Suarez and Pacey, 2006). Similarly heparin and pH changes may change activity in bull sperm and may play an additional role in this process (Suarez and Pacey, 2006). However in birds, sperm behavior changes before sperm come out from storage. Sperm stop moving and therefore they flow out of the sperm storage tubules (see [Section Sperm storage in birds](#)). Whether this change in sperm behavior is mediated by the female or the male is unknown.

Females may nourish sperm while in storage (see [Sections Sperm storage in fruit flies](#) and [Sperm storage in bats](#)). However, demonstration of sperm nourishing by females remains debated – particularly in vertebrates.

Behavior

Upon ejaculation, sperm are typically propelled by muscular contractions of ejaculation in males, and are not actually swimming. However, when entering the female reproductive tract, sperm often begin to swim using their flagella to reach the female eggs or her sperm storage organs. Sperm movement has been shown to be oriented toward a flow gradient (rheotaxis), but female contractions and movement of cilia in the lining of her reproductive tract can facilitate sperm transport upstream (Suarez and Pacey, 2006).

In addition, sperm orient themselves through both chemical and temperature gradients (chemotaxis and thermotaxis). During storage sperm activity (movement) as mentioned above is usually limited (except in birds and fruit flies, see below). This lack of motion may allow sperm to conserve the limited energy stored in the mitochondria, so that this energy can be used to propel sperm towards the eggs as needed for fertilization.

Why Store Sperm?

Long-term FSS presents females many advantages including greater flexibility in timing of reproduction and in cryptic female choice. For example, females can separate the act of mating from the event of fertilization through FSS (Eberhard, 1996; Birkhead and Møller, 1993). If males and females have limited periods of interaction such as in migrating species – mating during limited periods of mate availability is extremely important – but so too is timing when offspring hatch or are born to coincide with abundant resources. Females thus, are limited by their ability to time mating, and parturition or egg-laying. By storing sperm, a female may be well-prepared to mate when needed (and store sperm) and still carefully time a variety of other reproductive events with advantageous biotic and abiotic conditions such as food availability, precipitation and temperature (see Sandell, 1990; Birkhead and Møller, 1993). Furthermore, females may receive indirect genetic benefits through increased offspring quality from storing sperm from multiple males, and deciding which exact sperm to use for fertilization. For example, some species with large home-ranges may have decreased likelihoods of encountering a mate. In such a system, females may accept sperm from less preferred mates as a form of “bet-hedging” or “fertilization insurance”, but if she finds a superior mate later in the season, she can preferentially use his sperm for fertilization (e.g., Eberhard, 1996).

In addition to allowing females increased opportunity for cryptic choice, long-term FSS raises the opportunity for sperm competition by increasing the likelihood a female may have sperm from multiple mates in her reproductive tract at the same time. Evidence has been found that males in bat species where females store sperm invest more in sperm production (Orr and Zuk, 2013), and thus may incur more sperm competition (Briskie and Montgomerie, 1993). Males then are expected to be under selection to modify their sperm and ejaculates, to bias paternity in their favor under those conditions. In addition to helping males outcompete the sperm of other males, these adaptations can include taking advantage of female resources or manipulating her physiology during storage, leading to sexual conflict.

Examples of Sperm Storage

Sperm storage in fruit flies

Perhaps the best example of sperm storage comes from insects, in particular fruit flies of the genus *Drosophila*. In this model system it has been repeatedly shown that females and males both play key roles in the successful storage of sperm, which from a single mating can last the lifetime of a female (~2 weeks) if sperm are not depleted prior to her death (reviewed in box 1 Orr and Brennan). Several female organs are important for sperm storage including a receptacle that maintains sperm for short-term storage, and paired spermatheca where sperm are stored long term. Female fruit flies have active control of sperm movement and thus, may have the ability to bias the use of sperm (cryptic female choice).

During ovulation sperm are released for fertilization. Genetic underpinnings of the molecular signal for release have clearly demonstrated a female role in this function (Wong *et al.*, 2008). However, male roles are also likely, but less well known. In most aspects of fruit-fly sperm storage both sexes produce key substances necessary for successful female sperm storage (males in the semen and females by associated glands (e.g., spermathecal secretory cells and parovaria)) (Wong *et al.*, 2008; reviewed Orr and Brennan, 2015). Genetic mutants and knockouts for these substances are no-longer able to store sperm. Sperm storage no-longer functions properly if the female and/or male production of relevant proteins is curtailed. Thus, at least in this one well-studied example we can see clear evidence of co-evolution between the sexes.

Sperm storage in birds

All birds examined to date have specialized female sperm storage organs (Fig. 2). Female turkeys may lay fertilized eggs 71 days after their last copulation, but shorter durations of sperm storage are more typical (Briskie and Montgomerie, 1993; Blesbois and Brillard, 2007). As in many other taxa, sperm are subjected to intense selection in the vaginal environment before being moved to storage, and to ‘survive’ this process the male relies at least partly on immunoglobulins found in his sperm flagella (Blesbois and Brillard, 2007).

Specialized sperm storage tubules (SSTs) are cylindrical blind-ended sacs that can house several sperm each. These sacs are found in the internal mucosa of the uterovaginal junction, and the lower portion of the infundibulum, closer to the fertilization site (Briskie and Montgomerie, 1993). Their number varies across species between 2000 in passerines and 20,000 in the Turkey.

Unlike sperm in many other taxa that remain immotile while stored, avian sperm are likely moving in the SSTs, and it is possible that exit from the tubules occurs when sperm stop moving and retrograde female-driven flow pulls them out of the tubule (Blesbois and Brillard, 2007). When females ovulate, the last sperm to enter these tubules can exit for conception to occur and thus sperm storage in birds leads to ‘last male advantage’. There is no evidence that females can selectively utilize sperm from particular males once sperm are in storage.

Not only do female birds have specialized anatomy for sperm storage, but their physiology also supports long-term sperm storage. It is possible that lipid components of the cells that make up the SSTs provide some nourishment to the sperm. The

prevention of oxidative damage to sperm during storage is likely achieved by polyunsaturated fatty acids present in the sperm membrane itself, and enzymes from the SST cells (Blesbois and Brillard, 2007).

Sperm storage in bats

Among mammals the longest periods of sperm storage are known from bats (Chiroptera). Female bats of some species can store sperm for upwards of a year (~300 days) (little brown bats, *Myotis lucifugus*, Racey *et al.*, 1987). Perhaps surprisingly, unlike many other species with long-term female sperm storage, bats do not usually have modified organs or structures, and instead use pre-existing structures such as folds in the lining of the uterus or the utero-tubal junction to store sperm (Fig. 2).

Several aspects of bat biology may explain the long duration of FSS. Males of most (if not all) temperate species undergo spermatogenesis in the spring/summer, and then store sperm (MSS) until copulation takes place in the fall/winter. Thus, modifications to spermatozoa for male sperm storage (anatomical, physiological or otherwise) may pre-adapt sperm for long-term female sperm storage (see Section Male Sperm Storage, Orr and Brennan, 2015). Furthermore, thermal biology (hibernation) may be a key aspect of sperm storage (Racey *et al.*, 1987; Orr and Zuk, 2013). Female bats of many sperm-storing species (particularly temperate ones) usually mate in the fall and store sperm throughout the winter when their core body temperature is extremely low (approximating ambient, i.e., while hibernating). This low body temperature may facilitate the maintenance of sperm by subsequently lowering the metabolic rate and thereby reducing possible oxidative damage. Although most sperm storing species are indeed found in temperate regions where females hibernate, a few tropical species also have long-term FSS, so hibernation is not a complete explanation for long-term sperm storage in bats.

Sperm storage in humans

Suarez and Pacey (2006) summarized what is known about potential sperm storage in humans, and we present the most relevant details here. After ejaculation, sperm are deposited in the anterior part of the vagina, and form a loose gel that may help keep sperm near the cervical entrance. Most sperm are lost as backflow and only a small fraction of the ejaculate (1%) ends up in the uterus. Changes in the hydration of the cervical mucus allow sperm to pass the cervix more easily, and there may be cervical crypts that could either entrap or store sperm. Sperm have been found still motile in the cervix up to 9 days after copulation. However, such observations may be due to sperm coming back down from the uterus, rather than actually stored in the cervix. Furthermore, it is unknown whether these sperm maintain any fertilizing potential. These could be argued to be only cases of sperm 'survival', i.e., male-driven sperm viability without an active female role in maintenance. In addition to the crypts, another potential site for sperm storage in humans may be the lining of the oviducts. In vitro experiments have shown that only healthy sperm are found attached to this lining, while abnormal sperm are found elsewhere (Ellington *et al.*, 1998). However, just as the case with 'storage' in cervical crypts, this could be evidence of sperm survival, rather than sperm storage. Therefore, evidence that humans have specialized sperm storage sites is lacking.

Nevertheless, human sperm seem to be protected against immune responses and phagocytosis, as the immune cells present in the female vagina and cervix preferentially attack potential microbes rather than sperm. Seminal plasma that coats spermatozoa also may protect them against leukocyte attack, but eventually all leftover sperm will be eliminated by the immune system. Sperm may also derive a benefit by attaching to the oviduct lining, as attached sperm survive longer than unattached, and female secretions may mediate this increased survival.

References

- Almeida-Santos, S. M., Laporta-Ferreira, I. L., Antoniazzi, M. M., & Jared, C. (2004). Sperm storage in males of the snake *Crotalus durissus terrificus* (Crotalinae: viperidae) in southeastern Brazil. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 139, 169–174.
- Baer, B., Armitage, S. A. O., & Boomsma, J. J. (2006). Sperm storage induces an immunity cost in ants. *Nature*, 441, 872–887.
- Birkhead, T. R., & Møller, A. P. (1993). Sexual selection and the temporal separation of reproductive events: Sperm storage data from reptiles, birds and mammals. *The Biological Journal of the Linnean Society*, 50, 295–311.
- Blesbois, E., & Brillard, J. P. (2007). Specific features of in vivo and in vitro sperm storage in birds. *Animal*, 1(10), 1472–1481.
- Briskie, J. V., & Montgomerie, R. (1993). Patterns of sperm storage in relation to sperm competition in passerine birds. *Condor*, 95(2), 442–454.
- Eberhard, W. G. (1996). *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton, NJ: Princeton University Press.
- Ellington, J. E., Jones, A. E., Davitt, C. M., et al. (1998). Human sperm function in co-culture with human, macaque or bovine oviduct epithelial cell monolayers. *Human Reproduction*, 13(10), 2797–2804.
- Holt, W. V. (2011). Mechanisms of sperm storage in the female reproductive tract: An interspecies comparison. *Reproduction in Domestic Animals*, 46, 68–74.
- Holt, W. V., & Lloyd, R. E. (2010). Sperm storage in the vertebrate female reproductive tract: How does it work so well? *Theriogenology*, 73, 713–722.
- Kress, A., & Morson, G. (2007). Changes in the oviducal epithelium during the estrous cycle in the marsupial *Monodelphis domestica*. *Journal of Anatomy*, 211, 503–517.
- Neubaum, D. M., & Wolfner, M. F. (1999). Wise, winsome, or weird? Mechanisms of sperm storage in female animals. *Current Topics in Developmental Biology*, 41, 67–97.
- Orr, T. J., & Brennan, P. L. (2015). Sperm storage: Distinguishing selective processes and evaluating criteria. *Trends in Ecology and Evolution*, 30, 261–272.
- Orr, T. J., & Zuk, M. (2013). Does delayed fertilization facilitate sperm competition in bats? *Behavioral Ecology and Sociobiology*, 67, 1903–1913.
- Racey, P. A., Uchida, T. A., Mori, T., Avery, M. I., & Fenton, M. B. (1987). Sperm-epithelium relationships in relation to the time of insemination in little brown bats (*Myotis lucifugus*). *Journal of Reproduction and Fertility*, 80, 445–454.
- Racey, P. A., & Entwistle, A. C. (2000). Life-history and reproductive strategies of bats. In E. G. Crichton, & P. H. Krutzsch (Eds.), *Reproductive Biology of Bats* (pp. 363–414). London: Academic Press.
- Sandell, M. (1990). The evolution of seasonal delayed implantation. *Quarterly Review of Biology*, 65, 23–42.
- Sever, D. M., & Hopkins, W. A. (2004). Oviductal sperm storage in the ground skink *Scincella laterale* Holbrook (Reptilia: scincidae). *Journal of Experimental Biology*, 301A, 599–611.

- Suarez, S. S. (2008). Regulation of sperm storage and movement in the mammalian oviduct. *Integrative Journal of Developmental Biology*, 52, 455–462.
- Suarez, S. S., & Pacey, A. A. (2006). Sperm transport in the female reproductive tract. *Human Reproduction Update*, 12, 23–37.
- Wong, A., et al. (2008). A role for Acp29AB, a predicted seminal fluid lectin, in female sperm storage in *Drosophila melanogaster*. *Genetics*, 180, 921–931.

Further Reading

- Crichton, E. G. (2000). Sperm storage and fertilization. In E. G. Crichton, & P. H. Kruttsch (Eds.), *Reproductive Biology of Bats* (pp. 295–320). New York, NY: Academic Press.
- López-Sepulcre, A., et al. (2013). Beyond lifetime reproductive success: The posthumous reproductive dynamics of male Trinidadian guppies. *The Philosophical Transactions of the Royal Society B*, 280, 20131116.
- Orr, T. J., & Brennan, P. L. (2015). Sperm storage: Distinguishing selective processes and evaluating criteria. *Trends in Ecology and Evolution*, 30, 261–272.
- Orr, T. J., & Zuk, M. (2013). Does delayed fertilization facilitate sperm competition in bats? *Behavioral Ecology and Sociobiology*, 67, 1903–1913.
- Wolfner, M. F. (2011). Precious essences: Female secretions promote sperm storage in *Drosophila*. *PLOS Biology*, 9, e1001191.

ENDOCRINE CONTROL OF REPRODUCTION

Pituitary Gland, Comparative Cellular Organization in Vertebrates

Olivier Kah, University of Rennes 1, Rennes, France

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

ACTH	Adrenocorticotropin hormone
AH	Adenohypophysis
dio2	Type II iodothyronine deiodinase
FS	Folliculostellate cells (FS)
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
GH	Growth hormone
GnIH	Gonadotrophin-inhibiting hormone
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
MSH	Melanocyte-stimulating hormone
NH	Neurohypophysis
NPY	Neuropeptide Y
PPD	Proximal pars distalis
PI	Pars intermedia
POMC	Proopiomelanocortin
PRL	Prolactin
PT	Pars tuberalis
RPD	Rostral pars distalis
T3	Triiodothyronine
TSH	Thyroid-stimulating hormone
SL	Somatolactin

The pituitary gland, also named the hypophysis, is a major endocrine gland located just below the hypothalamus to which it is attached by the pituitary stalk. It is present in all vertebrates and has an overall conserved organization and functions despite the fact that some significant variations can be observed between the different groups of vertebrates.

General Organization of the Pituitary in Tetrapods

In all vertebrates, the pituitary (also called hypophysis) consists of two parts, the adenohypophysis and the neurohypophysis. The adenohypophysis contains the different cells secreting the classical pituitary hormones and thus represents the glandular part of the pituitary. The neurohypophysis consists in an accumulation of neurosecretory fibers originating from different part of the brain and secreting various peptides in the vicinity of the pituitary cells (in fish only) or into the blood stream. In mammals (and other land vertebrates), the pituitary is often divided into three lobes (see Fig. 1): the anterior, intermediate and posterior lobes.

The Anterior Lobe

The anterior lobe contains the corticotrophs (ACTH cells), the mammatrophs (prolactin cells, PRL cells), the somatotrophs (growth hormone cells, GH cells), the thyrotrophs (TSH cells) and the gonadotrophs (LH/FSH cells). In tetrapods, the anterior lobe does not

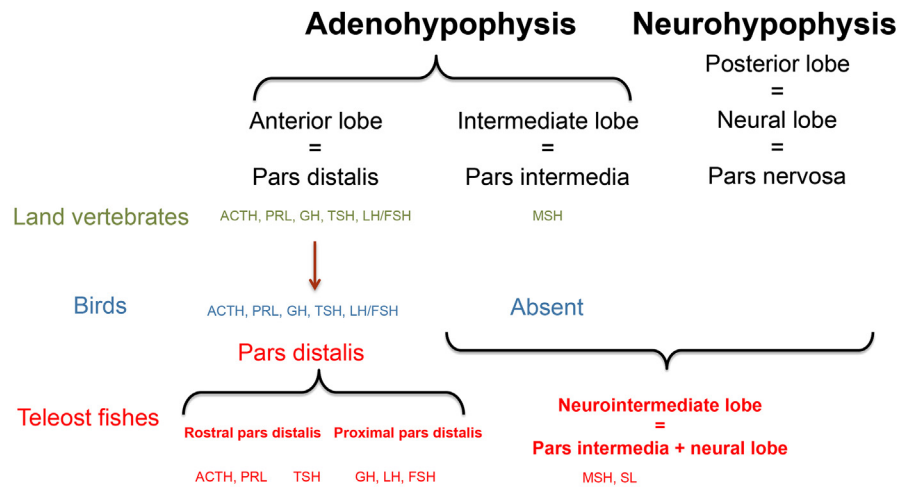


Fig. 1 The pituitary nomenclature is somewhat confusing. This figure shows the different synonyms for the different regions in land vertebrates and teleost fish.

receive a direct innervation due to the existence of a hypothalamo-pituitary portal system whose primary plexus of capillaries is located in the external zone of the median eminence. The median eminence is located in the floor of the hypothalamus and, at this level, the neurohormones secreted by the hypophysiotropic neurons are liberated in the vicinity of blood vessels and reach the anterior lobe via the blood stream to act on their target cells (Fig. 2).

The Intermediate Lobe

Between the anterior and nervous lobes is found the intermediate lobe (pars intermedia) which, except in fish, contains only one cell type, the melanotrophs (MSH cells). These cells secrete neuropeptides processed from the proopiomelanocortin gene. However, in contrast to the ACTH cells of the anterior lobe, ACTH is further processed through alternative splicing into α -MSH and CLIP. Beta-lipotrophin is further cut into γ -LPH, β -MSH and β -endorphin. The development of the pars intermedia varies considerably from species to species. While it is very well developed in teleost fish, it must be emphasized that in birds, the pars intermedia is lacking. In contrast to the anterior lobe, the pars intermedia receives a direct innervation from the hypothalamus. For example, in mammals, amphibians and teleosts, GABA, NPY and dopamine fibers have been reported (de Rijk *et al.*, 1990). In teleosts, the intermediate lobe harbours a cell type that is unique to fish, that produces a hormone belonging to the growth hormone/prolactin family, somatolactin.

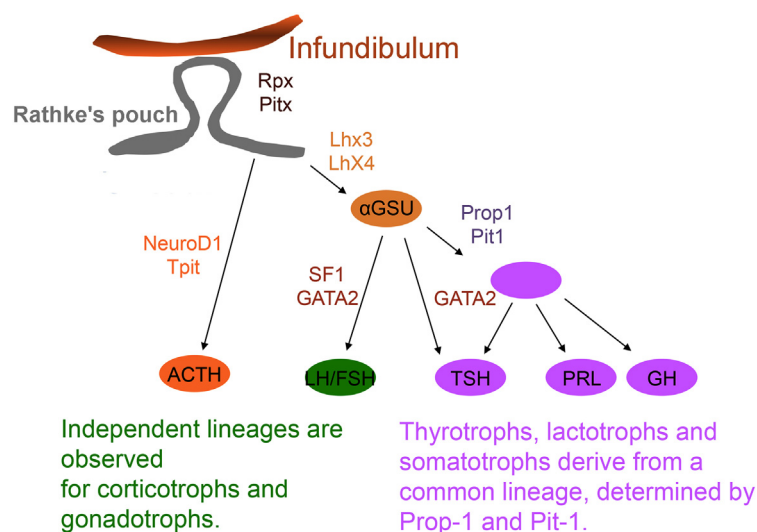


Fig. 2 Transcription factors implicated in the development of the anterior pituitary. Adapted from Cohen, L.E., Radovick, S., 2003. Molecular basis of combined pituitary hormone deficiencies. *Endocr. Rev.* 23 (4), pp. 431–442.

The Posterior Lobe

The posterior lobe (also called neural lobe) corresponds to the neurohypophysis and consists in an accumulation of nerve terminals which secrete various peptides, mainly arginine-vasopressin and oxytocin in mammals (related peptides in other vertebrates), directly into the blood stream. These nerve endings originate from the paraventricular and the supraoptic nuclei in mammals and from the magnocellular preoptic nucleus in teleost fishes.

The Pars Tuberalis

The pars tuberalis (PT) is located between the median eminence and the anterior lobe. It is well-developed in mammals where it contains both thyrotrophs and folliculostellate cells (see below). Although the function of the PT remained unknown for a long time, the PT recently surfaced as a major player in the regulation of seasonal reproduction. Surprisingly, cells in the PT express the highest density of melatonin receptor in mammals and birds and recent data have led to the assumption that the PT-specific thyrotroph feedbacks on the median eminence area to modulate DIO2 activity. In turn, T3 acts on GnIH and kisspeptin neurons to modulate GnRH release (review [Wood and Loudon, 2017](#)).

In lacertilian reptiles, it was shown that the PT develops from the Rathke's pouch, as it is the case of mammals, but during development cells of the PT gets progressively separated from the pars distalis and become completely incorporated to the lateral hypothalamus ([Bello et al., 1991](#)). In teleost fishes, there is no anatomically-distinct PT, but some studies have suggested that the saccus vasculosus (SV) would play in fish the role of the PT in mammals. The rationale for this assumption is that the coronet cells of the SV express, TSH β and DIO2 mRNAs in masu salmon. While this assumption may be true in some seasonal species like the salmon, it is unsure how this can be extended to all teleosts given that many seasonal fish do not exhibit such a structure (see below). Furthermore, studies in the rainbow trout failed to detect melatonin receptors in the SV.

The Folliculostellate Cells: The 7th Cell Type of the Anterior Lobe

In addition to the 6 classical secretory cell types, the anterior lobe also harbours the so-called folliculostellate cells (FS). These cells have a typical star-shape with long processes anchored by a basement membrane, hence their name ([Inoue et al., 1999](#)). FS do not contain secretory granules and they have been shown to release a number of cytokines and growth factors that participate in the regulation of anterior pituitary cell activity. FS cells are linked to each other by means of gap junction and form a meshwork that surrounds lobules containing secretory cells. It has been reported that FS cells provide large-scale communication system that allows rapid transfer of information between secretory cells by means of calcium waves that propagate through gap junctions ([Fauquier et al., 2001](#)). Such peculiarities seem to have been conserved invertebrates as it was shown in fishes that FS cells also form a network enabling communication from cell to cell ([Golan et al., 2016](#)).

FS cells express protein S100B, GFAP and vimentin and exhibit characteristics resembling astrocytes. Furthermore, recent data suggest that FS cells could act as stem cells within the pituitary. Recent results using S100B-GFP mice have shown that astrocytes and FS cells share the same differentiation capacity and are both capable to differentiate into skeletal muscle cells ([Osuna et al., 2012](#)). Interestingly, results obtained in rainbow trout, Japanese eel and zebrafish indicate that FS cells express the *cyp19a1b* gene that in the fish encodes the brain form of aromatase. In the eel, it was further demonstrated that SF cells can proliferate and may indeed behave as radial glial cells, which in teleost fishes carry many functions of astrocytes and are considered as neuronal stem cells ([Jeng et al., 2012](#)).

Altogether these results suggest that folliculostellate cells and astrocytes share a common origin and may represent stem cells in the pituitary and the brain, respectively.

Ontogeny of the Pituitary Gland

It is now clear that the origin of the pituitary gland is completely ectodermal. The anterior pituitary develops from an invagination of the roof of the stomodeum known as the Rathke's pouch, while the neurohypophysis originates from the infundibulum which consists of the floor of the hypothalamus. These two tissues come in contact with each other and it is admitted that interactions between the two are essential for the complete development of the gland. Cells in anterior part of the embryonic adenohypophysis proliferate more actively than cells of the posterior part, which causes the formation of two different regions, the future pars distalis, and the pars intermedia. The ontogeny of the pituitary has not been extensively studied in non-mammalian vertebrates but there is no reason to assume that the main mechanisms are different.

A number of homeodomain transcription factors are essential for defining the fate of pituitary lineage ([Fig. 2](#)). Among these factors, *Rpx* and *Pitx1* are present in the developing pituitary and in the adult somatotrophs, thyrotrophs and lactotrophs ([Fig. 2](#)). ACTH and LH/FSH cells develop from independent lineages under the influence of *NeuroD* and *Tpit* or *SF1* and *GATA2*, respectively ([Cohen and Radovick, 2002](#)).

The development of the PT differs from that of the pars distalis. It involves the bHLH transcription factor hairy enhancer of split (*HES1*).

The Pituitary is a Vertebrate Evolutive Novelty

It is usually accepted that the pituitary is a vertebrate evolutionary novelty. Indeed, there is no such homologous organ in invertebrates and although some groups have intended to identify the equivalent of the pituitary in urochordates, the sister group of vertebrates, no one has produced strong evidence for the existence of such an organ. Nevertheless, recent research suggests that cephalochordates may have a structure resembling the Rathke's pouch that expresses neuropeptides and possibly pituitary hormones (Li *et al.*, 2017). In fact, the anterior pituitary gland is supposed to be derived from the adenohypophyseal placode who could potentially have an equivalent in the cephalochordate. In this regard, it is important to keep in mind that the pituitary is derived from the adenohypophyseal neurogenic placode. Such placodes are regions of thickening of the embryonic head ectoderm layer that gives rise to neurons. It is usually considered that neurogenic placodes are found only in vertebrates.

Some authors have suggested that because of the genome duplications, early vertebrates have developed a number of new functions thanks to numerous extra genes and thus exhibit more complexity. At the same time, there is also an increase in size. Hence, while most of the physiological functions remain under the control of the brain, there has been a need for an amplification system that would allow a small number of neurons to control major physiological functions requiring fine-tuning. Thus, the hypophysis appears as a good manner to amplify neural signals in vertebrates in a way similar to the optic gland of cephalopods that also appears as an intermediate gland between the brain and peripheral organs, in particular the gonads.

Hence, in view of this information, it appears that the hagfish pituitary would represent the most ancient form of a "true" hypophysis.

The Pituitary of the Hagfish and Lampreys

Together with lampreys, hagfish (Mixini) represent jawless fish, the sister group of vertebrates. They have a skull but no vertebrate column and they are believed to be the first animals with a well-differentiated pituitary. Hagfish have been shown to have a hypothalamo-pituitary axis. The adenohypophysis takes the form of clusters of cells surrounded by connective tissue. The neurohypophysis is an evagination of nervous tissue that is located dorsal to the adenohypophysis with which it has no connection. Both adenohypophysis and neurohypophysis are wrapped in connective tissues. Hagfish have been shown to have GnRH system and possible three pituitary hormones, gonadotrophs, ACTH and GH (Nozaki, 2013). Recent data in the lamprey have highlighted the existence of two heterodimeric pituitary glycoprotein hormones, lamprey glycoprotein hormone (IGpH) and thyrostimulin. The corresponding receptors have also been characterized (Sower, 2017).

The Pituitary of Chondrosteans

The hypophysis of sturgeons, is flat and elongated in the rostral-caudal direction. It is composed of the adenohypophysis situated rostrally and the neurohypophysis located caudally in close vicinity to the transition zone between the infundibulum and a well developed saccus vasculosus (see below).

A comprehensive description of the hypothalamo-hypophyseal system was reported in the classical studies of Russian researchers. The adenohypophysis consists in a pars distalis and a pars intermedia, and the neurohypophysis is incorporated in a neurointermediate lobe that is made of tubular processes of the bottom of the infundibular wall that penetrate deeply between the cell cords of the pars intermedia. The distribution of the pituitary endocrine cells was studied by immunocytochemical techniques in the adenohypophysis of different sturgeon species and seven types of endocrine cells were identified: the adrenocorticotrophic, prolactin, growth hormone, gonadotrophic and thyroid stimulating hormone cells in the pars distalis, and, similar to teleosts, the melanocyte stimulating hormone and somatolactin cells in the pars intermedia.

In chondrosteans, such as the sturgeons, the hypothalamic floor located dorsal to the pars distalis of the adenohypophysis, resembles a typical median eminence named proximal neurosecretory contact region (Polenov and Garlov, 1973). In this region and the pars distalis there are blood vessels that form part of a hypothalamic-hypophyseal portal system where numerous neurosecretory fibers form synaptic like contacts, and, appear to release neurohormones into the portal circulation. For example, it has been shown that GnRH neurons located in the telencephalon and the preoptic region send projections to the area separating the hypothalamus from the pituitary but they do not enter into the pars distalis. The sturgeon pituitary exhibits the different subtypes found in other vertebrates and the organization of several neuroendocrine systems have been reported in details (Adrio-Fondevila and Kah, *in press*).

The Pituitary of Teleost Fishes

The pituitary of teleosts has been extensively studied in many species due to the fact that this group of fish includes close to 30,000 species and also because interest for commercial species varies from one region to another. Because the evolutive history of teleosts spans about 280 million years, there are significant variations in the organization of the pituitary gland.

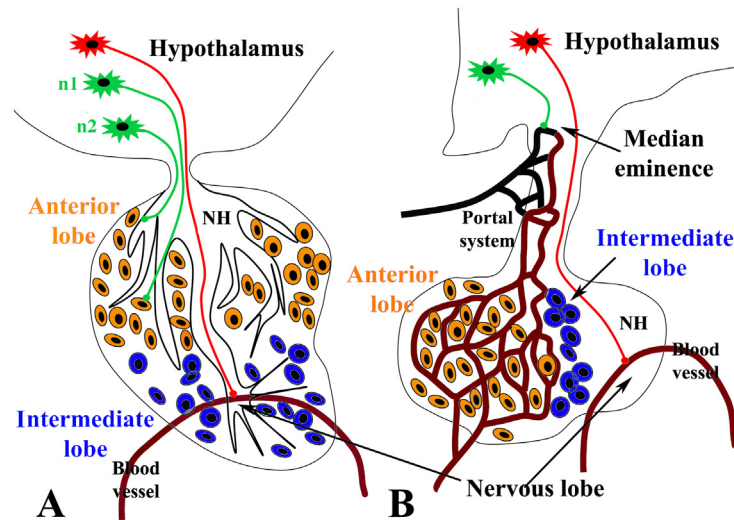


Fig. 3 Comparative organization of the hypothalamo-pituitary connections in teleost fishes (A) and mammals (B). In fish, unlike tetrapods, hypothysiotropic neurons send their projections directly into the anterior lobe. Nerve endings may connect directly with secretory cells of the anterior lobe (n1) or establish synaptic like contacts at a basal membrane (n2) that separates the neurohypophysis (NH) from the adenohypophysis. This innervation is homologous to that of the median eminence in tetrapods. In fish and mammals, hypothalamic neurons (ocytocin/isotocin and arginine vasopressin/vasotocin) send their projections to the neural lobe (NH) that in teleosts is intermingled with the pars intermedia forming the neurointermediate lobe. Adapted from Kah, O., Dufour, S., 2011. Conserved and divergent features of reproductive neuroendocrinology in fish. In: Norris, D.O., Lopez, K.H., (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 1: Fishes, San Diego, CA: Elsevier, pp. 15–42.

The Anterior Lobe in Teleost Fish

The anterior lobe of the teleost pituitary exhibits some striking features compared to that of land vertebrates. First, the cells corresponding to a given cell type are often found together in a specific part of the pars distalis. For this reason, the pars distalis is often divided into rostral region, rostral pars distalis, and a proximal region, the proximal pars distalis (Fig. 3). In general, the rostral pars distalis encompasses the ACTH cells and the prolactin cells which in some species, such as the eel of the salmonids, can be organized in follicles. In teleost fish, prolactin is a hormone dedicated mainly to osmoregulation. Prolactin cells exhibit considerable changes in synthesis and secretory activity depending on the similarity of the water. In most cases, ACTH cells are adjacent to the neurohypophyseal digitations that deeply penetrate the anterior lobe. The proximal pars distalis includes the growth hormone secreting cells and the gonadotrophs. In contrast to mammals in which there is a single type of gonadotroph that secretes both LH and FSH, the two gonadotrophins are clearly secreted by different cells in the teleosts (Nozaki *et al.*, 1990). FSH and LH cells differ in their aspect and localization according to the sex cycle. In many species, the LH cells invaded the pars intermedia or the rostral par distalis when the fish reach maturity. Similar to other vertebrates, FSH is involved in follicle growth and LH in ovulation.

The location of the pars intermedia of teleost varies from species to species. In eels, salmonids and most perciforms, the pars intermedia occupies the caudal portion of the pituitary gland, where as in cyprinids the pars intermedia is located ventral to the pars distalis. In all cases, the pars intermedia is deeply invaded by the nervous tissue corresponding to the nervous lobe of tetrapods. For this reason, this part of the gland is often referred to as the neurointermediate lobe (Fig. 1).

Another feature of the pars intermedia of teleost is the unique presence of cells that secrete hormone belonging to the prolactin growth hormone family. This hormone discovered in the flounder by Hirose Kawauchi in 1990, it has an unclear physiological functions that may vary from species to species. It has been involved in skin pigmentation, calcium regulation and/or reproductive functions.

The Pituitary Innervation in Teleost Fish

A major feature of the pituitary of teleost is the existence of a direct innervation of the anterior lobe. In all species, the neurohypophysis penetrates deeply into pars distalis from whose it is separated by a so-called double basement membrane. In many species, there is no direct contact between the neurosecretory fibers and secretory cells of the anterior lobe (Fig. 3). However, detailed studies have reported synaptic-like contacts exhibiting membrane thickening and accumulation of dense clear vesicles directly between nerve endings and secretory cells of the pars distalis. Such nerve endings were characterized and shown to contain dopamine, GABA, neuropeptide Y, GnRH, neurotensin, galanin, somatostatin and other neurohormones that act on the activity of the secretory cells. In species that do not exhibit direct contact between nerve endings and secretory cells, electron microscopy studies have indicated potential release of neurosecretory material close to the basement, which is in continuity with the intracellular spaces. Recently, it has been suggested in zebrafish that GnRH3 terminals could release their products in the vicinity of the gonadotrophs or in blood

vessels. However, in view of the lack of electron microscopy evidence to support these views, this hypothesis should be regarded with caution. In parallel with the rich direct innervation of the anterior lobe a major difference between teleosts and tetrapods is that the fish pituitary carries a plethora of receptors for different neuropeptides. For example, in the case of LH cells in fish, about 20 different factors have been reported to modulate LH secretion *in vitro*. This leads to redundancy and/or compensation and may explain why knocking out the GnRH or the kiss genes do not cause sterility in zebrafish.

The Saccus Vasculosus of Teleosts

Another unique feature of the pituitary teleost is the presence of a circumventricular organ referred to as the saccus vasculosus which is present in some but not all species of teleosts. For example, all salmonids exhibit a well-developed saccus vasculosus, the goldfish or the perciforms do not possess such a structure. Interestingly in the rainbow trout, the coronet cells of the SV exhibit strong immunoreactivity for aromatase B, the product of the *cyp19a1b* gene (Menuet *et al.*, 2003). As already mentioned above, it is believed that the saccus vasculosus is an integrator of photoperiodic cues in at least some teleosts.

Conclusions

In all vertebrates, the pituitary gland is a master gland, which under the control of the hypothalamus orchestrates the main physiological functions. Its overall organization is well conserved indicating that common ancestors to fish and land vertebrates already shared similar organization. The hagfish and lamprey pituitaries may provide an idea of what an ancestral pituitary looked like. Most pituitary hormones have conserved functions, with the exception of prolactin that is believed to serve more than 300 different functions in vertebrates. Prolactin provides a good example of how different animals make use of an existing gene to serve a variety of functions.

References

- Adrio-Fondevila, F., Kah, O., in press. Anatomy of the diencephalon of sturgeon. In: Williot, P., Nonnotte, G., Vizziano-Cantonnet, D., Chebanov, M. (Eds.). The Siberian Sturgeon. Springer, in press.
- Bello, A. R., Marti, E., Lancha, A., et al. (1991). Ontogeny and immunohistochemical differentiation of the pars tuberalis in the lizard *Gallotia galloti*. *J. Hirnforsch.*, 32(6), 755–760.
- Cohen, L. E., & Radovick, S. (2002). Molecular basis of combined pituitary hormone deficiencies. *Endocr. Rev.*, 23(4), 431–442.
- de Rijk, E. P., Jenks, B. G., Vaudry, H., & Roubos, E. W. (1990). GABA and neuropeptide Y co-exist in axons innervating the neurointermediate lobe of the pituitary of *Xenopus laevis* – An immunoelectron microscopic study. *Neuroscience*, 38(2), 495–502.
- Fauquier, T., Guérineau, N. C., McKinney, R. A., et al. (2001). Folliculostellate cell network: A route for long-distance communication in the anterior pituitary. *Proc. Natl. Acad. Sci. USA*, 98(15), 8891–8896.
- Golan, M., Hollander-Cohen, L., & Levavi-Sivan, B. (2016). Stellate cell networks in the teleost pituitary. *Sci. Rep.*, 6, 24426.
- Inoue, K., Couch, E. F., Takano, K., & Ogawa, S. (1999). The structure and function of folliculo-stellate cells in the anterior pituitary gland. *Arch. Histol. Cytol.*, 62, 205–218.
- Jeng, S. R., Yueh, W. S., Pen, Y. T., et al. (2012). Expression of aromatase in radial glial cells in the brain of the Japanese eel provides insight into the evolution of the *cyp19a* gene in Actinopterygians. *PLOS ONE*, 7(9), e44750.
- Li, M., Jiang, C., Zhang, Y., & Zhang, S. (2017). Activities of Amphioxus GH-Like Protein in Osmoregulation: Insight into origin of vertebrate GH family. *Int. J. Endocrinol.*, 2017, 9538685.
- Menuet, A., Anglade, I., Le Guevel, R., et al. (2003). Distribution of aromatase mRNA and protein in the brain and pituitary of female rainbow trout: Comparison with estrogen receptor alpha. *J. Comp. Neurol.*, 62(2), 180–193.
- Nozaki, M. (2013). Hypothalamic-pituitary-gonadal endocrine system in the hagfish. *Front. Endocrinol.*, 4, 200.
- Nozaki, M., Naito, N., Swanson, P., et al. (1990). Salmonid pituitary gonadotrophs. I. Distinct cellular distributions of two gonadotropins, GTH I and GTH II. *Gen. Comp. Endocrinol.*, 77(3), 348–357.
- Osuna, M., Sonobe, Y., Itakura, E., et al. (2012). Differentiation capacity of native pituitary folliculostellate cells and brain astrocytes. *J. Endocrinol.*, 213(3), 231–237.
- Polenov, A. L., & Garlov, P. E. (1973). The hypothalamo-hypophyseal system in Acipenseridae. 3. The neurohypophysis of *Acipenser güldenstädti* Brandt and *Acipenser stellatus* Pallas. *Z. Zellforsch. Mikrosk. Anat.*, 136(4), 461–477.
- Sower, S. A. (2017). Landmark discoveries in elucidating the origins of the hypothalamic-pituitary system from the perspective of a basal vertebrate, sea lamprey. *Gen. Comp. Endocrinol.* Available at: <https://doi.org/10.1016/j.ygcen.2017.10.016>.
- Wood, S., & Loudon, A. (2017). The pars tuberalis: The site of the circannual clock in mammals? *Gen. Comp. Endocrinol.* doi:20pii:S0016-6480(17)30350-7.

Endocrine Control of Reproduction, Fish

Jakob Biran, Agricultural Research Organization, Rishon Letziyon, Israel
Berta Levavi-Sivan, The Hebrew University of Jerusalem, Rehovot, Israel

© 2018 Elsevier Inc. All rights reserved.

Introduction

Fish comprise more than 40% of all vertebrate species and are extremely diverse. Nevertheless, the general endocrine components and pathways that regulate fish reproduction are highly conserved and as in other vertebrates, they are generated by the hypothalamus-pituitary-gonadal (HPG) axis (Levavi-Sivan *et al.*, 2010; Zohar *et al.*, 2010). Sensory information from the internal and external environments as well as social cues (such as presence of a potential mate, temperature, photoperiod, metabolism, energy stores, gonadal state etc.), are processed by the fish brain and integrated by the hypothalamus to initiate and regulate reproductive activity. The hypothalamus reacts to environmental and physiological conditions by synthesizing and releasing neuropeptides (primarily gonadotropin-releasing hormone; GnRH) and monoamines (mainly dopamine) which regulate the activity of endocrine cells in the anterior pituitary. That region of the pituitary produces the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH). Upon stimulation, gonadotrope cells located in the anterior pituitary produce and release their hormones into the blood (Levavi-Sivan *et al.*, 2010). Both gonadotropins reach the gonads, where they stimulate the synthesis of steroidal hormones that, in turn, regulate maturation of gametes, as well as convey feedback to the hypothalamus and pituitary.

In spite of the diversity of reproductive strategies among the approximately 29,000 recognized fish species, fish share several distinct endocrine characteristics: (i) The fish neurohypophysis is positioned dorsally to the adenohypophysis, in contrast to the anterior-posterior organization of the mammalian pituitary. Moreover, unlike the mammalian adenohypophysis, the teleostean adenohypophysis does not contain a cleft (a remnant of the mammalian Rathke's pouch), and so there is no clear morphological separation into a pars distalis and a pars intermedia as in mammals (Pogoda and Hammerschmidt, 2007); (ii) Fish lack both median eminence and a hypophyseal portal system. Instead, both neurohypophysis and adenohypophysis are directly innervated by hypothalamic nerve terminals (Ball, 1981) and apply a dual mode of gonadotrope regulation by GnRH, combining both neuroglandular and neurovascular components (Golan *et al.*, 2015); (iii) Cells producing distinct gonadotropin are well defined, spatially arranged, and grouped together in particular regions of the gland. In addition, while a single mammalian gonadotrope may produce both FSH and LH, fish gonadotropins are produced and secreted by distinct cell types (Levavi-Sivan *et al.*, 2010). LH cells exhibit close cell-to-cell contacts and form a continuous network throughout the gland while FSH cells are more loosely distributed, still maintaining some degree of cell-to-cell contact by cytoplasmic processes (Golan *et al.*, 2015); (iv) Due to events of whole-genome duplications that have occurred during fish evolution, as many as three GnRH-ligand and five GnRH-receptor (GnRH-R) genes have been identified in certain fish species. By contrast, only one or two such variants occur in mammals. The gene duplication in GnRH ligands has led to segregation in the expression pattern and central function of each ligand. A species-specific variant (seabream-type GnRH; GnRH1) that occurs in fish, is mainly located in the preoptic area, and plays a central role in regulating reproduction. A highly preserved variant (chicken-type cGnRH-II; GnRH2) in the midbrain may act as a neurotransmitter and/or neuromodulator. A salmon-type GnRH (sGnRH; GnRH3) that has been primarily identified in the terminal nerve ganglion and olfactory bulbs, is reported to have a neuromodulatory function (Zohar *et al.*, 2010). GnRH receptors are G-protein coupled receptors (GPCR). Piscine GnRH-Rs lack their C-terminal tail; therefore, no GnRH desensitization exists (Levavi-Sivan *et al.*, 2010).

Owing to their commercial value, the reproductive biology of the various fish species has been well studied. Thus, interest constantly increases in fish as model organisms for basic science, as well as for the agricultural aspects.

Reproductive Strategies

The high variability of fish and the ecological niches they inhabit has led to the adoption of various reproductive strategies including viviparity (*Poecilia*), sequential hermaphroditism (the protandrous seabream or the protogenous orange spotted grouper) and even simultaneous hermaphroditism (Black hamlet). Nevertheless, most fish species are gonochoristic and oviparous, meaning that the sexes are separate and fertilization and embryonal development are external (Yaron *et al.*, 2003). Fish may have synchronous ovaries and spawn only once in a season (carp) or even once in their lifetime (river eel and Atlantic salmon); or they can possess asynchronous (or group-synchronous) ovaries, producing several spawning events during the breeding season. During this period, their ovaries contain several generations of oocytes at various developmental stages. Spawning cycles of asynchronous fish may vary from 24 h in the gilthead seabream, up to one month in tilapia, (Yaron *et al.*, 2003).

Fish with synchronous ovaries undergo sharp metabolic changes during the vitellogenic period that differ from those occurring during final oocyte maturation and spawning. Endocrine changes that support each step reflect relatively simple and well-timed hormonal patterns. In contrast, simultaneous production and maintenance of several oocyte generations in asynchronous ovaries makes the sequential hormone release more complex. These endocrine differences are discussed in the following sections.

The Hypothalamus-Pituitary-Gonadal (HPG) Axis

The HPG axis consists of three major sites of endocrine regulation. A plethora of neuropeptides are secreted from specific neurons in the brain (primarily but not only in the hypothalamus) and form an interface between the central nervous system and the endocrine system. These neuropeptides may directly affect the gonadotropes in the pituitary, or may exert their action via the GnRH neurons. This action stimulates the production and release of two distinct but chemically related gonadotropins: FSH and LH. FSH and LH are released into the circulation, and stimulate the gonads to secrete sex-specific steroids that are responsible for gametogenesis, vitellogenesis and final oocyte maturation in females, as well as spermatogenesis and spermiogenesis in males (Fig. 1).

The Hypothalamus and its Neurohormones

The plethora of neuropeptides and monoamines released by the brain were shown to regulate piscine reproductive functions. These include: GnRH, pituitary adenylate cyclase-activating peptide (PACAP), kisspeptins, Neurokinin B (NKB), Neurokinin F (NKF), Neuropeptide Y (NPY), ghrelin, secretoneurin, leptin, gonadotropin-inhibitory hormone (GnIH), dopamine, serotonin, γ -aminobutyric acid (GABA), spexin, dynorphin, and more.

GnRH was first isolated from mammals, and later from other vertebrates (Sherwood *et al.*, 1983). To date, 30 structurally different forms of GnRH have been identified, while 12 structural variants of the GnRH molecule have been found in different fish species. The N-terminal amino acid sequence (pGlu-His-Trp-Ser) and carboxyl terminal amino acid sequence (Pro-Gly-NH₂) have been conserved over 400 million years of chordate evolution (Kochman, 2012). As indicated by its name, chicken GnRH II (GnRH-2) was first isolated from chicken brain and found to be the most ubiquitous form of GnRH molecule. The brain of most teleosts contains three forms of GnRH, encoded by three distinct genes and differ in their developmental origin, spatial distribution, and function. GnRH1 neurons are hypophysiotropic and are positioned in the preoptic area (POA); GnRH2 neurons reside in the midbrain tegmentum, and are suggested to be involved in food intake and reproductive behavior; and the GnRH3 neuronal population is localized in the terminal nerve and has no direct innervation to the pituitary (Zohar *et al.*, 2010). Some teleost species have only two GnRH forms: the salmon and zebrafish genomes do not contain the GnRH1 form, while catfish and eel lack the GnRH3. The lack of these forms is compensated by the axonal projections of the remaining forms (Abe and Oka, 2011). For example, in zebrafish, some of the GnRH3 neurons developing in the terminal nerve migrate into the hypothalamic POA and innervate the pituitary, suggesting that they acquire the role GnRH1 fulfills in “three-form” fish (Zohar *et al.*, 2010).

As in mammals, GnRH1 neurons in the fish POA display irregular and episodic spontaneous electrical activity with circadian fluctuations in the episodic firing frequency. These irregular activities are in contrast to those of the non-hypothalamic populations

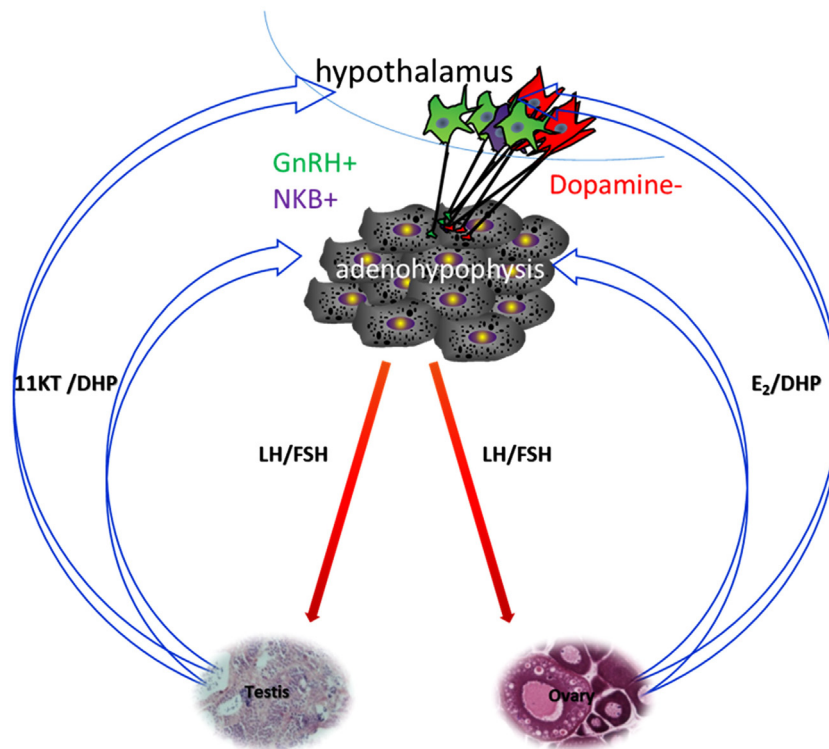


Fig. 1 Schematic diagram displaying HPG axis in fish. Schematic diagram showing potential regulatory signals conveyed by hypothalamic neurons to pituitary cells, leading to the gonads (HPG axis).

of GnRH neurons, i.e., GnRH2 and GnRH3, which display regular pacemaker activities, further supporting their functions other than – hypophysiotropic (Oka, 2010). In contrast to the similarities in the GnRH neurons' electrical activity between fish and mammals, a striking difference can be seen in the structure and function of the GnRH-R. In mammals, constant delivery of high GnRH doses leads to hypoactivity of the gonad and disrupted estrous cycles, due to desensitization of the GnRH-R. In contrast, chronic administration of high doses of GnRH in fish leads to increased gonadotropin release and even to spawning. (Zohar *et al.*, 2010).

While hypothalamic GnRH is a positive regulator of the HPG axis, neuroendocrine release of hypothalamic dopamine serves as a negative effector of HPG activity in many, albeit not all, fish species. Dopamine is a neurotransmitter, synthesized from tyrosine by tyrosine hydroxylase (the rate-limiting enzyme) and Dopa-decarboxylase. Although dopamine is known to have pleiotropic effects on both brain and pituitary functions, its involvement in the endocrine regulation of fish reproduction has been demonstrated in several fish species, including goldfish, zebrafish, carp, African catfish, tilapia, eel, grey mullet, sturgeon, and rainbow trout [reviewed by Levavi-Sivan *et al.* (2010); Zohar *et al.* (2010)]. Contrasting these effects, dopaminergic inhibition was not observed in several marine species, suggesting that its involvement in reproductive function is mainly important in freshwater fish. Dopamine receptors are GPCRs and are classically divided into two principal subtypes, according to their ability to activate (D1-like subtype) or inhibit (D2-like subtype) adenylyl cyclase, the key enzyme in the conversion of adenosine triphosphate to 3'5'-cyclic AMP. Various agonists and antagonists with high specificity to D1 or D2 receptors were employed to demonstrate that the inhibitory effects of dopamine on fish reproduction is due to specific activation of D2-like receptors. Fontaine and colleagues have demonstrated the expression of three D2-like receptors in LH cells in the adenohipophysis of the female zebrafish, and that the dopaminergic innervation of the adenohipophysis originates from the hypothalamic POA (Fontaine *et al.*, 2015). These findings provide neuroanatomical support for the existence of dopaminergic inhibition in piscine reproduction.

During recent years, a large surfeit of novel neuropeptides have been found to regulate the secretion of GnRH in mammals and other non-mammalian vertebrates. However, it was the identification of kisspeptin as an upstream regulator of the hypophysiotropic GnRH neurons that led to a significant change in the way that endocrinologists perceive neuroendocrine regulation of the HPG axis (Tena-Sempere *et al.*, 2012). The discovery that GnRH neurons have Kiss1-receptors (a.k.a. GPR54) was the first indication that the kisspeptin system is involved in the control of fish reproduction (Parhar *et al.*, 2004). Unlike most mammals, which possess only one kisspeptin system (KISS1), the majority of teleosts have two kisspeptin systems, comprised of Kiss1 and Kiss2; and two cognate receptors, Kiss1r and Kiss2r (Biran *et al.*, 2008). Nevertheless, certain fish species have only one kisspeptin gene and two Kiss1R genes; in others, two kisspeptin genes were identified with only one Kiss1R gene. This inconsistency can be explained by the fact that bony fish underwent two complete genome duplications during evolution, and many of these duplicated genes were subsequently lost (Mechaly *et al.*, 2013). As in the case of GnRH, chronic administration of kisspeptin was found to suppress reproductive activity in rats and monkeys (Seminara *et al.*, 2006; Thompson *et al.*, 2006), while chronic administration of kisspeptin in fish leads to increased reproductive functions (Nocillado *et al.*, 2013).

Deletion and site-directed mutagenesis of an estrogen-responsive element (ERE) from the kiss promoters of the protogynous orange-spotted grouper (*Epinephelus coioides*), indicated that Kiss1 is regulated by estradiol 17 β (E2), via the classical pathway utilized by Er β 1, as well as via an activator protein 1 (Ap1)-dependent, non-classical pathway utilized by Er β 2. Kiss2 was also regulated by E2 through the Creb transcription factor, as well as by Er β 1 and Er β 2 pathways. The effects of gonadal steroids on kisspeptin *in vivo* were also demonstrated. In the medaka, Kiss1 neurons drastically change their firing activity according to E2 levels, and in the European sea bass expression levels of Kiss2 were affected by circulating testosterone levels (Alvarado *et al.*, 2016). Taken together, accumulating data clearly support the assumption that E2 is involved in the feedback regulation of piscine kisspeptins via various estrogen receptors.

A relatively new neuropeptide found to be involved in fish reproduction is Neurokinin B (NKB, encoded by the gene *tac3*). NKB is a member of the tachykinin peptide family. The mammalian NKB is unique in that its prepro-hormone encodes a single mature peptide, while other prepro-tachykinins encode two mature peptides. Mammals also have one high affinity receptor for NKB (NKBR/NKR3). In contrast, fish have up to three NKB receptors and two genes encoding NKB. Importantly, prepro-tachykinin3 of fish matures into two peptides. Because the additional peptide appears only in fish and frogs, it was designated neurokinin F (NKF; Fig. 1) (Biran *et al.*, 2012). Moreover, while kisspeptin and NKB are co-expressed by the same neurons in the mammalian hypothalamus, the piscine kisspeptin and NKB are expressed by different neuronal subsets of the fish hypothalamus (Ogawa *et al.*, 2012). NKB-R are expressed in LH but not FSH cells, suggesting regulation of final oocyte maturation by NKB via LH surge (Biran *et al.*, 2014). *In vivo* administration of NKB/NKF peptides increased reproductive functions in zebrafish, tilapia, goldfish and striped bass (Biran *et al.*, 2012, 2014; Zmora *et al.*, 2017). However, *tac3* gene products in carp do not play a role in LH synthesis at the pituitary level, but may serve as novel stimulators for prolactin and somatolactin synthesis.

The Pituitary and its Gonadotropins

As in other vertebrates, the regulation of fish reproduction involves the synthesis and secretion of hormones from the adenohipophysis, including FSH (GTH-I), LH (GTH-II), growth hormone (GH), thyroid-stimulating hormone (TSH) and adrenocorticotrophic hormone (ACTH), with FSH and LH serving as the key regulators of gonadal development and function. FSH and LH are heterodimeric hormones with a distinct β subunit and a common α subunit, which is also common to TSH). Over the years, gonadotropin subunit genes were isolated in more than 56 fish species from at least 14 teleost orders, allowing a vast characterization of fish gonadotropin synthesis and release. Signals from hypothalamic neurohormones and gonadal steroids are integrated in

gonadotrope cells to regulate synthesis and release of both gonadotropins. Nonetheless, GnRH regulation of FSH synthesis and release is less clear than that of LH (Zohar *et al.*, 2010; Yaron *et al.*, 2003).

As previously described, there are two main modes of gonadal development in fish. Synchronous gonadal development is accompanied by a clear duality of gonadotropin secretion; while the adenohypophysis of immature salmon contains mainly FSH cells, the adenohypophysis of sexually mature rainbow trout contains numerous LH-producing cells. In addition, only FSH is released into the blood of immature coho salmon, with increased levels during the gonadal recruitment step, and reduced levels towards final oocyte maturation and spawning. While both gonadotropins induce E2 secretion, LH is more potent than FSH in raising 17 α ,20 β dihydroxy-4-pregnen-3-one (DHP) levels from post vitellogenic oocytes (Swanson, 1991). However, in carp, LH alone is sufficient to regulate both vitellogenesis and final oocyte maturation while FSH may have another, yet undefined role. In contrast, both FSH and LH are synthesized and released during all stages of reproduction in multi-spawning fish with asynchronous ovaries like the gilthead seabream, goldfish and tilapia (reviewed by Levavi-Sivan *et al.* (2010); Yaron *et al.* (2003)). These differences are probably due to the need to regulate the development of several oocyte generations in parallel. In this situation, the differential regulation of each oocyte generation may be attributed to differences in the expression of gonadotropin receptors.

The fish FSH and LH receptors are GPCRs belonging to the Class A of rhodopsin-like receptors. Mammalian FSHR and LHR show very high selectivity to their cognate ligands, leading to functional specificity. However, ligand-receptor promiscuity was shown in several fish species. Structural modeling combined with *in vitro* assays of gonadotropin receptor activation have demonstrated that in some fish species, FSHR may bind both FSH and LH, while in almost all fish species tested, LHR is highly specific for LH (Aizen *et al.*, 2012). Concomitant with their importance in induction of ovarian steroidogenesis, gonadotropin receptors are expressed by follicular cells of the ovary (theca and granulosa cells) and by interstitial and nurse cells of the testis (Leydig and Sertoli cells) (Yaron and Sivan, 2006).

Gonadal steroids transmit their feedback to regulate the synthesis and release of FSH and LH. This feedback can be of a positive or negative nature, depending on the reproductive state of the fish. Gonadectomy in sexually mature goldfish, catfish, Atlantic croaker, bass and salmonids results in increased LH secretion, while treatment with gonadal steroids may reverse this effect. Nevertheless, during earlier stages of gonadal development, gonadal steroids can induce LH β subunit expression and protein levels (Levavi-Sivan *et al.*, 2010; Yaron *et al.*, 2003). More anatomical work is required to understand whether these effects are direct or indirect.

LH acts on the ovarian follicle to produce DHP, the maturation-inducing Hormone, (MIH) in most fishes. The dramatic increase in the capacity of post vitellogenic follicles to produce DHP in response to LH is correlated with a decrease in P450c17 (*P450c17-I*) and P450 aromatase (*pP450arom*) mRNA and increase in the novel form of P450c17 (*P450c17-II*) and 20 β -hydroxysteroid dehydrogenase (*20 β -HSD*) mRNA.

The Gonads and Their Steroids

Most fish species used in research and aquaculture are gonochoristic. Therefore, a sexually mature fish would have either a functional testis (male) or a functional ovary (female). In most gonochoristic fish, FSH and LH stimulate the synthesis of three key sex steroids: E2 serves as the principal estrogen and stimulates germ-cell proliferation and growth and vitellogenesis; 11 ketotestosterone (11-KT) serves as the central androgen that regulates spermatogenesis and spermiogenesis; DHP regulates final oocyte maturation and ovulation in females as well as spermatozoa maturation and spermiation in males (Yaron, 1995). In addition, DHP is involved in the meiosis onset of ogonia in the ovary and of spermatogonia in the testis. Androgens and E2 (under FSH) are involved in the appearance of lipid droplets in previtellogenic oocytes. It is noteworthy that higher vertebrates utilize testosterone and not 11-KT as their main androgenic hormone. These pathways are regulated in parallel to the above-mentioned feedback regulation of gonadal steroids on reproductive functions of the hypothalamus and pituitary (Fig. 2).

Male

In spermiogenesis, non-functional sperm undergo the process of sperm maturation to become mature spermatozoa, fully capable of vigorous motility and fertilization. These processes are mainly controlled by sex steroid hormones. Spermatogonial renewal is controlled by E2 through the expression of platelet-derived endothelial cell growth factor. The proliferation of spermatogonia toward meiosis is initiated by 11-KT, which is produced by FSH stimulation. 11-KT prevents the expression of anti-Müllerian hormone (AMH), which functions to inhibit proliferation of spermatogonia and induce expression of activin B, which functions in the induction of spermatogonial proliferation. Meiosis is induced by DHP through the action of trypsin. DHP also regulates sperm maturation through the regulation of seminal plasma pH. Gonadotropic stimulation of Leydig cells in the testis induce the synthesis and release of 11-KT, which leads to the activation of Sertoli cells, resulting in stimulation of spermatogenesis. With the advancement of spermatogenesis, 11-KT levels start to decline and DHP levels rise, leading to induction of spermiogenesis. In some fish species, DHP was shown to further regulate sperm motility (Miura and Miura, 2011; Schulz *et al.*, 2010) (Fig. 2).

Female

In the case of female fish, the endocrine roles of gonadal steroids are more complex; alongside their roles in feedback regulation and gonadal development, gonadal estrogens induce the synthesis and release of vitellogenin (VTG), the primary storage protein in fish oocytes, by the female liver (Lubzens *et al.*, 2017). In fish with synchronous ovaries, theca cells of the follicle respond to FSH to produce testosterone, which is then aromatized into E2 by the granulosa cells. E2 stimulates the production of VTG and in parallel

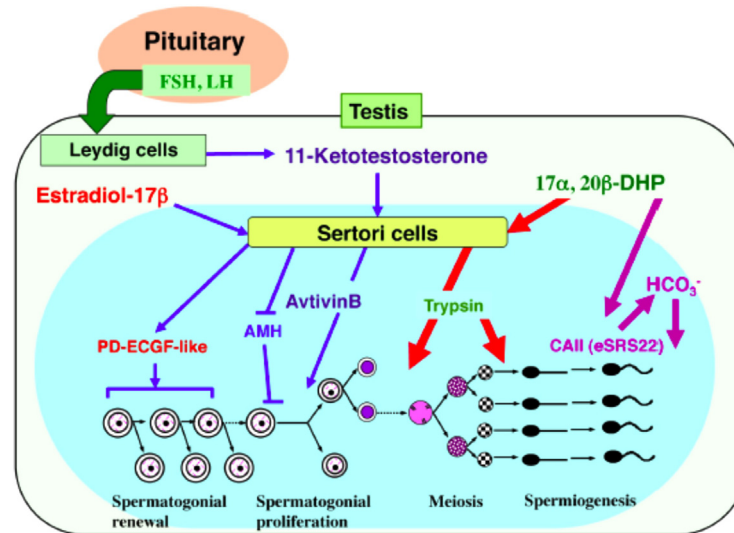


Fig. 2 Endocrine control of spermatogenesis A schematic diagram summarizing the possible control mechanisms of spermatogenesis in the Japanese eel. FSH, follicle-stimulating hormone; LH, luteinizing hormone; 17 α ,20 β -DHP, 17 α ,20 β -dihydroxy-4-pregnen-3-one; PD-ECGF, platelet-derived endothelial cell growth factor; AMH, anti-Müllerian hormone; CAII, carbonic anhydrase; eSRS, spermatogenesis related substances. Reproduced from Miura, C., Miura, T., 2011. Analysis of spermatogenesis using an eel model. *Aqua-Biosci. Monogr.* 4, 105–129.

regulates its accumulation in the yolk. Following the vitellogenic growth of the oocyte, FSH levels decline concurrently with the rise in LH levels. This leads to shifts in steroidogenesis that leads the ovary to produce mainly DHP (Yaron, 1995).

In addition to the classic genomic mechanism of steroid action mediated by activation of intracellular nuclear receptors, there is now extensive evidence that steroids also activate receptors on the cell surface to initiate rapid intracellular signaling and biological responses that are often non-genomic. Two recently discovered novel proteins with seven-transmembrane domains, GPCR30 (GPR30), and membrane progesterin receptors (mPRs), have the ligand binding and signaling characteristics of estrogen and progesterin membrane receptors, respectively. During the first phase – at the end of vitellogenic oocyte growth – the ovarian follicles produce large amounts of estrogens and minor quantities of progestins, resulting in activation of the inhibitory pathway regulating oocyte maturation, but not the stimulatory pathway. The estrogens act through GPR30 to activate a stimulatory G protein (Gs), resulting in stimulation of adenylyl cyclase activity and increases in cAMP production. The high levels of cAMP maintain meiotic arrest of fish oocytes, possibly through downstream signaling molecules such as protein kinase A. In addition, estrogens upregulate GPR30 expression to potentiate the inhibitory pathway and down-regulate mPR α expression to block the stimulatory one, through activation of GPR30 via unknown pathways. As a result, the oocytes remain in meiotic arrest. In the second phase, the steroidogenic pathway is switched to the production of progestins and estrogen production is declined. At the same time, progestins, through mPR α , cause down-regulation of GPR30 expression, which together with the reduction in estrogen levels, causes suppression of the inhibitory pathway. Progesterin binding to mPR α results in activation of an inhibitory G protein (Gi) decreasing adenylyl cyclase activity and cAMP levels, leading to inhibition of protein kinase A, thereby releasing the oocyte from meiotic arrest and allowing oocyte maturation to proceed (Thomas, 2012).

Genome Editing Era in Fish Reproduction

The emergence of the novel methods of transcription activator-like effector nucleases (TALEN) (Bedell *et al.*, 2012) and clustered regularly interspaced palindromic repeats-associated (CRISPR)/Cas9 (Gagnon *et al.*, 2014) systems, commenced a technological revolution in fish genome editing, allowing reverse genetics in fish. These methods are now applicable in several fish species of commercial importance, including Atlantic salmon (Edvardsen *et al.*, 2014), channel catfish (Khalil *et al.*, 2017), Nile tilapia (Wu *et al.*, 2016), sea lamprey (Square *et al.*, 2015) and model fish species zebrafish and medaka (Bedell *et al.*, 2012; Gagnon *et al.*, 2014; Luo *et al.*, 2015). These tools have already provided some exciting findings regarding the endocrine regulation of fish reproduction.

At the hypothalamic level in zebrafish, no phenotypes were found in fish carrying single or double mutations in genes encoding kisspeptin ligands or their receptors (Tang *et al.*, 2015). Similarly, no phenotypes were identified in GnRH3 mutant zebrafish, and even triple knockout fish for both kisspeptin ligands and GnRH3 resulted in normal reproductive development (Spicer *et al.*, 2016; Liu *et al.*, 2017). These findings strongly challenge the current dogma of neuroendocrine regulation of fish reproduction.

At the pituitary level, zebrafish carrying homozygous mutation in the gene encoding FSH β -subunit, display delayed gonadal maturation; and mutants in the gene encoding LH β -subunit show normal gonadal development but fail to spawn and hence are infertile (Zhang *et al.*, 2015). Double mutant zebrafish for both gonadotropins or both gonadotropin receptors develop an

infertile all-male population phenotype. However, while FSHR mutant females are infertile, FSHR mutant males show no significant phenotype (Chu *et al.*, 2015).

At the gonadal level, “loss of function” mutations in the zebrafish aromatase gene resulted in an all-male phenotype (Lau *et al.*, 2016); and mutating the nuclear progesterone receptor in zebrafish leads to female infertility due to ovulation failure with no reproductive phenotype in males (Tang *et al.*, 2016).

Taken together, these findings demonstrate the high importance and strength of genomic editing in fish. Nevertheless, it also highlights the need for additional knockout lines in other commercially important fish models.

Concluding Remarks

As in other vertebrates, in fish, endocrine regulation of reproduction is governed by the HPG axis. In the current article, we briefly reviewed the endocrine components of the axis with emphasis on the unique and different characteristics of these components. Taken together, the data presented show the high evolutionary importance of fish as model organisms for endocrine research in the reproductive field; they are an exciting model for studying vertebrate reproduction. Moreover, some features in the piscine HPG axis may provide explanations to phenomena that cannot be addressed in higher vertebrates. For example, the anatomical separation between FSH and LH enables the characterization of regulatory pathways for each gonadotropin, while their co-expression in mammals would hamper such efforts.

Finally, there is no doubt that novel genomic tools will significantly advance endocrine research in fish, which will lead to important breakthroughs. Nevertheless, adopting these methodologies should be advanced in concert with development of additional and more classical endocrine tools, such as specific hormone ELISAs, antibodies and anatomical research.

References

- Abe, H., & Oka, Y. (2011). Mechanisms of neuromodulation by a nonhypophysiotropic GnRH system controlling motivation of reproductive behavior in the teleost brain. *J. Reprod. Dev.*, 57(6), 665–674.
- Aizen, J., et al. (2012). Experimental and computational study of inter- And intra-Species specificity of gonadotropins for various gonadotropin receptors. *Mol. Cell. Endocrinol.*, 364(1–2), 89–100.
- Alvarado, M. V., et al. (2016). Actions of sex steroids on kisspeptin expression and other reproduction-related genes in the brain of the teleost fish European sea bass. *J. Exp. Biol.*, 219(Pt 21), 3353–3365.
- Ball, J. N. (1981). Hypothalamic control of the pars distalis in fishes, amphibians, and reptiles. *Gen. Comp. Endocrinol.*, 44(2), 135–170.
- Bedell, V. M., et al. (2012). In vivo genome editing using a high-efficiency TALEN system. *Nature*, 491(7422), 114–118.
- Biran, J., et al. (2012). Neurokinin B and neurokinin B receptor: A novel system involved in controlling fish reproduction. *Proc. Natl. Acad. Sci. USA*, 109(26), 10269–10274.
- Biran, J., et al. (2014). Direct regulation of gonadotropin release by Neurokinin B in Tilapia (*Oreochromis niloticus*). *Endocrinology*, 155(12), 4831–4842.
- Biran, J., Ben-Dor, S., & Levavi-Sivan, B. (2008). Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. *Biol. Reprod.*, 79(4), 776–786.
- Chu, L., et al. (2015). Gonadotropin signaling in zebrafish ovary and testis development: Insights from gene knockout study. *Mol. Endocrinol.*, 29(12), 1743–1758.
- Edvardsen, R. B., et al. (2014). Targeted mutagenesis in Atlantic salmon (*Salmo salar* L.) using the CRISPR/Cas9 system induces complete knockout individuals in the F0 generation. *PLOS ONE*, 9(9), e108622.
- Fontaine, R., et al. (2015). Dopaminergic neurons controlling anterior pituitary functions: Anatomy and ontogenesis in zebrafish. *Endocrinology*, 156(8), 2934–2948.
- Gagnon, J. A., et al. (2014). Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PLOS ONE*, 9(5), e98186.
- Golan, M., Zelinger, E., Zohar, Y., & Levavi-Sivan, B. (2015). Architecture of GnRH-gonadotrope-vasculature reveals a dual mode of gonadotropin regulation in fish. *Endocrinology*, 156(11), 4163–4173.
- Khalil, K., et al. (2017). Generation of myostatin gene-edited channel catfish (*Ictalurus punctatus*) via zygote injection of CRISPR/Cas9 system. *Sci. Rep.*, 7(1), 7301.
- Kochman, K. (2012). Evolution of gonadotropin-releasing hormone (GnRH) structure and its receptor. *J. Anim. Feed Sci.*, 21(1), 3–30.
- Lau, E. S.-W., et al. (2016). Knockout of zebrafish ovarian aromatase gene (*cyp19a1a*) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. *Sci. Rep.*, 6, 37357.
- Levavi-Sivan, B., et al. (2010). Perspectives on fish gonadotropins and their receptors. *Gen. Comp. Endocrinol.*, 165(3), 412–437.
- Liu, Y., et al. (2017). Genetic evidence for multifactorial control of the reproductive axis in zebrafish. *Endocrinology*, 158(3), 604–611.
- Lubzens, E., et al. (2017). Maternal investment in fish oocytes and eggs: The molecular cargo and its contributions to fertility and early development. *Aquaculture*, 472, 107–143.
- Luo, D., et al. (2015). Direct production of XY(DMY-) sex reversal female medaka (*Oryzias latipes*) by embryo microinjection of TALENs. *Sci. Rep.*, 5, 14057.
- Mechaly, A. S., Vinas, J., & Piferrer, F. (2013). The kisspeptin system genes in teleost fish, their structure and regulation, with particular attention to the situation in Pleuronectiformes. *Gen. Comp. Endocrinol.*, 188, 258–268.
- Miura, C., & Miura, T. (2011). Analysis of spermatogenesis using an eel model. *Aqua-Biosci. Monogr.*, 4, 105–129.
- Nocillado, J. N., et al. (2013). Chronic kisspeptin administration stimulated gonadal development in pre-pubertal male yellowtail kingfish (*Seriola lalandi*; Perciformes) during the breeding and non-breeding season. *Gen. Comp. Endocrinol.*, 191, 168–176.
- Ogawa, S., et al. (2012). Cloning and expression of tachykinins and their association with kisspeptins in the brains of zebrafish. *J. Comp. Neurol.*, 520(13), 2991–3012.
- Oka, Y. (2010). Electrophysiological characteristics of gonadotrophin-releasing hormone 1–3 neurones: Insights from a study of fish brains. *J. Neuroendocrinol.*, 22(7), 659–663.
- Parhar, I. S., Ogawa, S., & Sakuma, Y. (2004). Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. *Endocrinology*, 145(8), 3613–3618.
- Pogoda, H.-M., & Hammerschmidt, M. (2007). Molecular genetics of pituitary development in zebrafish. *Semin. Cell Dev. Biol.*, 18(4), 543–558.
- Schulz, R. W., et al. (2010). Spermatogenesis in fish. *Gen. Comp. Endocrinol.*, 165(3), 390–411.
- Seminara, S. B., et al. (2006). Continuous human metastin 45–54 infusion desensitizes G protein-coupled receptor 54-induced gonadotropin-releasing hormone release monitored indirectly in the juvenile male rhesus monkey (*Macaca mulatta*): A finding with therapeutic implications. *Endocrinology*, 147(5), 2122–2126.
- Sherwood, N., et al. (1983). Characterization of a teleost gonadotropin-releasing hormone. *Proc. Natl. Acad. Sci. USA*, 80(9), 2794–2798.
- Spicer, O. S., et al. (2016). Targeted mutagenesis of the hypophysiotropic GnRH3 in zebrafish (*Danio rerio*) reveals no effects on reproductive performance. *PLOS ONE*, 11(6), e0158141.

- Square, T., et al. (2015). CRISPR/Cas9-mediated mutagenesis in the sea lamprey *Petromyzon marinus*: A powerful tool for understanding ancestral gene functions in vertebrates. *Development*, 142(23), 4180–4187.
- Swanson, P., 1991. Salmon gonadotropins: Reconciling old and new ideas. In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe, M.S., (Eds.) Proceedings of the Fourth International Symposium on Reproductive Physiology of Fish, pp. 2–7. Norwich, United Kingdom.
- Tang, H., et al. (2015). The kiss/kissr systems are dispensable for zebrafish reproduction: Evidence from gene knockout studies. *Endocrinology*, 156(2), 589–599.
- Tang, H., et al. (2016). Gene knockout of nuclear progesterone receptor provides insights into the regulation of ovulation by LH signaling in zebrafish. *Sci. Rep.*, 6, 28545.
- Tena-Sempere, M., et al. (2012). Comparative insights of the kisspeptin/kisspeptin receptor system: Lessons from non-mammalian vertebrates. *Gen. Comp. Endocrinol.*, 175(2), 234–243.
- Thomas, P. (2012). Rapid steroid hormone actions initiated at the cell surface and the receptors that mediate them with an emphasis on recent progress in fish models. *Gen. Comp. Endocrinol.*, 175(3), 367–383.
- Thompson, E. L., et al. (2006). Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. *Am. J. Physiol. Endocrinol. Metab.*, 291(5), E1074–E1082.
- Wu, L., et al. (2016). R-spondin1 signaling pathway is required for both the ovarian and testicular development in a teleosts, Nile tilapia (*Oreochromis niloticus*). *Gen. Comp. Endocrinol.*, 230–231(Suppl. C), S177–S185.
- Yaron, Z. (1995). Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture*, 129(1–4), 49–73.
- Yaron, Z., et al. (2003). Regulation of fish gonadotropins. *Int. Rev. Cytol.*, 225, 131–185.
- Yaron, Z., & Sivan, B. (2006). Fish reproduction. In D. H. Evans, & J. B. Claiborne (Eds.), *Physiology of Fishes* (third ed., pp. 345–388). New York: CRC.
- Zhang, Z., Zhu, B., & Ge, W. (2015). Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. *Mol. Endocrinol.*, 29(1), 76–98.
- Zmora, N., et al. (2017). Neurokinin B regulates reproduction via inhibition of kisspeptin in a teleost, the striped bass. *J. Endocrinol.*, 233(2), 159–174.
- Zohar, Y., et al. (2010). Neuroendocrinology of reproduction in teleost fish. *Gen. Comp. Endocrinol.*, 165(3), 438–455.

Endocrine Control of Reproduction, Birds

Jenny Q Ouyang, University of Nevada, Reno, NV, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Birds *aves* are oviparous (egg-laying) vertebrates. Reproduction is an activity that consists of multiple stages in their life-history, which include maturing of the gonads, courtship and mating behaviors, nest building, female egg-laying, incubation, and feeding of the nestlings (Lack, 1968). Reproduction is followed by the next life-history stage, e.g., molt (the shedding and replacement of feathers), which require the regression of the reproductive organs. Therefore, the control of reproduction is an intricate and time-dependent process that needs to be responsive to the external environment. Hormones integrate internal and external conditions, mediating and controlling reproductive processes (Hau and Goymann, 2015).

Reproductive physiology and behavior of birds are governed by the hypothalamic-pituitary system (HPS), which sends signals to the rest of the body to initiate and end reproductive activities. Thus, this article will start with the role of the HPS and associated hormones, with a focus on two antagonistic neurohormones in the hypothalamus. Then it will highlight the endocrine control of ovulation, incubation, and parental care activities and conclude with a brief discussion of the endocrine systems associated with reproduction in response to seasonal and environmental change.

Hypothalamic-Pituitary System

The HPS controls reproductive activity in birds. Neurons in the hypothalamus integrate external (light, temperature, food availability) and internal (water and ion balance, nutrition, body condition) cues to help the animal make informed decisions about when to be reproductive (Fig. 1). Fig. 1 also summarizes the rest of the HPS with information about control and regulation. Gonadotropin-releasing hormone (GnRH), gonadotropin-inhibiting hormone (GnIH), hypothalamic vasoactive intestinal peptide (VIP), and arginine vasotocin (AVT) are neurohormones that play important roles in the brain. Neurohormones are transmitted through axons and released in the median eminence. They are then released directly into the anterior pituitary through the blood. AVT is transmitted through axons and released at the posterior pituitary (Oksche, 1978). Anterior pituitary hormones in birds include luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), thyrotropin (TSH), corticotrophin (ACTH), and growth hormone (GH).

GnRH and GnIH

GnRH regulate most reproductive behaviors of birds. There are two forms of this peptide. GnRH-I was first discovered in mammals and then isolated from chickens (Miyamoto *et al.*, 1982). Subsequently, this peptide was found in a wide array of species, including ducks, other galliformes, doves, and songbirds (Bentley *et al.*, 2006). The second form of GnRH is GnRH-II, which was first isolated

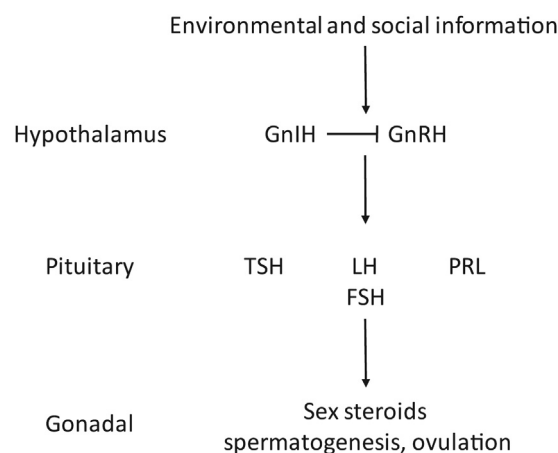


Fig. 1 A brief outline of the neuroendocrine mechanisms controlling reproduction in birds. Neurohormones in the hypothalamus integrate external cues, such as light or availability of mates, with internal cues, such as body condition. Gonadotropin-inhibiting hormone (GnIH) could be stimulated by melatonin and/or stress which directly inhibit reproductive activities. Gonadotropin-releasing hormones (GnRH) stimulates the pituitary to release thyrotropin (TSH), luteinizing hormone (LH), and follicle stimulating hormone (FSH), which all have actions on the gonads for regulating ovulation. Prolactin (PRL) can also stimulate and regulate a suite of reproductive behaviors. Finally, sex steroids are released from the gonads and regulate sexual and parental behaviors.

in the chicken (Miyamoto *et al.*, 1984). There is good evidence that GnRH-I directly stimulates LH and FSH release in vitro and in vivo (Hattori *et al.*, 1986) in chicken and in quail. GnRH-II's activity on LH and FSH is almost equal to that of GnRH-I, but is more noticeable on LH than FSH. GnRH is usually higher in ovulating birds because of its stimulating role on LH and the pre-ovulatory LH surge. GnRH-I's role is more direct on LH and FSH whereas GnRH-II seems to be more involved with sexual behaviors, such as aggression, courtship, and copulation. For example, GnRH-II enhances copulation when females are exposed to male song (Maney *et al.*, 1997).

GnIH may act as the antagonist of GnRH in the hypothalamus. GnIH was first found in the paraventricular nucleus in quail with neurons that project into the median eminence (Tsutsui *et al.*, 2010). Therefore, it can directly affect anterior pituitary hormone release. Since its discovery, GnIH has been found in the brains of many species, including songbirds. GnIH has been shown to inhibit gonadal development by decreasing FSH and LH (Ubuka *et al.*, 2006). This inhibition has direct effects on spermatogenesis and gonadal growth; specifically, LH in avian species stimulates the production of testosterone (T) in the Leydig cells. Injecting GnIH to mature birds can induce apoptosis of testicular tissue and regression of the gonads. Moreover, GnIH can inhibit copulatory behavior even if females are exposed to male song.

Ovulation, Incubation, and Parental Care

Ovulation

The ovaries of female birds contain developing follicles in a sequence with the largest follicle ovulated at regular intervals. FSH stimulates the maturation of these germ cells. Due to the regularity in follicle development, most birds lay one egg per day during the breeding season, although it is possible for females to pause laying or skip days in the laying sequence. Birds can also lay more than one group (clutch) of eggs during the breeding season, e.g., sparrows in the northern latitudes lay only one clutch but in southern latitudes can have up to three (Baker, 1995). Determinate layers are those that lay a fixed number of eggs (e.g., some raptors) and indeterminate layers are those that continue laying for a long time (e.g., domesticated chickens). Birds have one ovary and one oviduct so only one egg can be laid at one time.

Most of studies on ovulation and oviposition have been performed in the chicken. The initiation of ovulation begins with a LH surge, or a rise in LH levels pre-ovulation. FSH during this time remains relatively constant, and PRL levels are inversely related to LH. Estradiol (E₂) also peaks with the LH peak. Progesterone (P₄) triggers the LH surge and GnRH stimulates P₄ release (Hertelendy *et al.*, 1982). Ovulation then occurs approximately 7 hours after the LH surge. The egg spends the next 24 h in the oviduct before it is laid. The next ovulation can vary between 15 and 75 min after the previous egg was laid. This strict time sequence restricts the timing of ovulation and egg-laying. Light cues are important for ovulation, i.e., most songbirds lay their egg during the morning, especially around dawn. When chickens are placed in constant light, their rhythm of egg-laying becomes free-running because they are missing external light cues (Lewis *et al.*, 2001). In addition to light, abiotic cues, such as temperature, may also play a role (Payne *et al.*, 1965).

After ovulation, the egg travels to the oviduct, where fertilization and deposition of albumen occurs. The avian oviduct is an intricate structure with specific functions, such as deposition of melanin, pigments for coloration, albumen and coating. Albumen is laid down in layers with surrounding membranes. The uterus, also known as the shell gland in birds, coats the albumen and membranes with an egg shell that differs in pigment and coloration with large intra- and inter-species variation. For example, some species of ground nesters have eggs that camouflage with the environment (Cherry and Gosler, 2010).

Egg-Laying (Oviposition)

Oviposition (egg-laying) occurs through the vagina and cloaca. AVT, mesotocin (MST) and ovarian hormones are the main molecules responsible for oviposition. The mammalian homologs of AVT and MST are arginine vasopressin and oxytocin, respectively. In birds, AVT has a greater effect than MST on uterine contractions (Saito and Koike, 1992). AVT levels increase sharply to induce contractions and decrease within minutes after the egg is laid.

Incubation

After the last egg is laid in a clutch, a period of incubation, or warming the eggs, occurs so that the embryo can develop within the egg (Drent and Daan, 1980). Depending on the species, the male, female, or both sexes will develop a brood patch, a spot of featherless skin. However, not all species form a brood patch, e.g., male emperor penguins incubate eggs with a flap of skin and their feet. The presence of eggs will often influence brood patch formation. The brood pouch skin has an enlarged organic tissue due to cell proliferation and vascularization, which facilitates the transfer of heat from the parent to the egg. The process of brood patch development is regulated through a synergism between PRL and sex steroids. In females, estrogen and P₄ are the main regulators and in males, the main regulators are androgens.

VIP stimulates the release of PRL from the pituitary. PRL usually induces the bird to incubate and maintain incubation (Buntin, 1996). Incubation behavior is thought to be maintained through tactile stimuli from the nest or the eggs. In altricial species, or species that have young that are helpless and depend on their parents for food at hatch, parental PRL remains high during incubation and remains elevated throughout chick-rearing. In precocial species, or species that have independent young at hatch,

parental PRL are high during incubation and fall after chicks have hatched (Goldsmith, 1983). In species that produce crop milk to feed the young, PRL is also essential for producing the milk.

Parental Care

After the young hatch, especially if they are altricial, comes a period of parental care, in which parents deliver food to the offspring. Recent research on the hormonal regulation of parental care offer some promising avenues for identifying possible mechanisms (Vleck and Vleck, 2011). In general, PRL and P_4 are thought to increase paternal care whereas T and corticosterone are thought to decrease paternal care (Lynn, 2016). PRL and corticosterone, the steroid hormone that increases metabolism at baseline levels and decrease reproductive behaviors at higher concentrations, are suggested to be linked and mediate parental care activities (Angelier and Chastel, 2009). Studies show that increases in corticosterone may depress PRL levels. However, a direct mechanistic pathway has not been established. Studies have shown that experimentally elevated PRL in altricial species can cause an increase in parental care behaviors (Duckworth and Sockman, 2012), but there is evidence that experimentally elevated corticosterone concentrations at the baseline range also stimulate more reproductive behaviors (Love and Williams, 2008; Ouyang *et al.*, 2013). Moreover, high corticosterone concentrations are related to foraging and parental care activities in larger seabirds (Crossin *et al.*, 2012). The effects of corticosterone on parental care are not only concentration dependent but also context dependent, e.g., according to life-history stage. For example, high corticosterone concentrations before egg-laying but low corticosterone concentrations during chick-rearing correlate with high reproductive effort and thus the number of offspring produced (Fig. 2). In conclusion, there is an environmentally context-dependent role that both corticosterone and PRL play on parental care behaviors, but experimental evidence in other taxa are missing.

About 9% of all the species of birds have a form of cooperative breeding with alloparenting at the nest. Alloparenting consists of parental activities, e.g., food delivery or nest defense, by conspecifics that are not the parents. Whether parents are reproductively competent depends on the species. For example, in some birds, male helpers have equivalent T levels as those of breeding males but in other species, helper males have lower T and smaller testes than those of breeding males (Schoech *et al.*, 2004). Oftentimes, helpers at the nest will have elevated PRL levels as compared to non-helpers.

Obligate brood parasites are species that lay eggs and have no parental care behaviors after egg-laying. Brown-headed cowbirds display a rise in circulating PRL concentrations but no incubation behaviors, which suggests that PRL binding activity in the brain is decreased (Dufty *et al.*, 2002). Often, the offspring of parasitic species display more begging behavior than the host chicks, but eggs of the parasitic cuckoo have similar yolk T than the eggs of their host (Török *et al.*, 2004).

Seasonal Reproduction

Timing of reproduction is an active area of research in avian species as they have evolved their breeding to coincide with the maximal production of offspring. Seasonal breeding is controlled by proximate and ultimate factors. Proximate factors include individual condition, and is especially critical for capital breeders that need to store up enough energy for breeding. Ultimate factors include adequate food supply, predation pressures, or climatic conditions. However, the endocrine system is secreting hormones

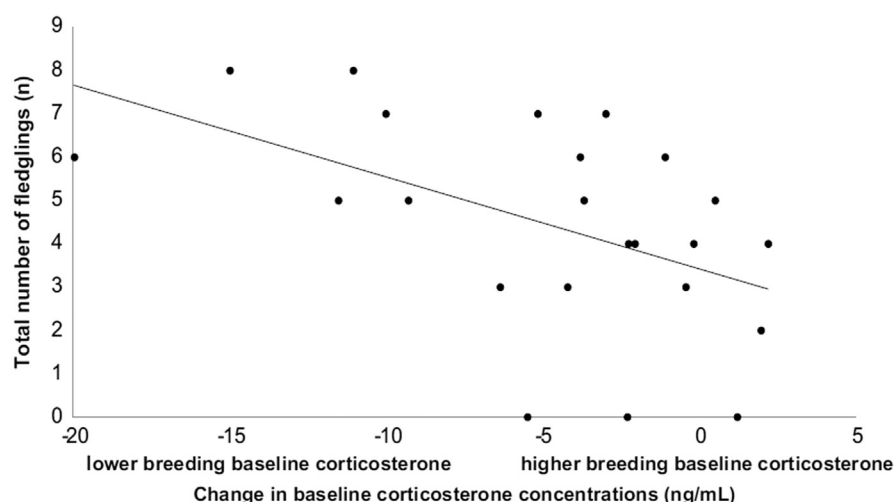


Fig. 2 The number of offspring (fledglings) produced by a small songbird during the breeding season was related to the change in corticosterone concentrations from March to May. March is the pre-laying period and May is the chick-rearing period. Adults with the highest March but lowest May concentrations raised the most offspring. Reproduced from Ouyang, J.Q., Sharp, P., Quetting, M., Hau, M., 2013. Endocrine phenotype, reproductive success and survival in the great tit, *Parus major*. *Journal of Evolutionary Biology* 26, 1988–1998.

to prime the individual for reproductive behaviors weeks ahead of when the chicks hatch, e.g., the time when these ultimate factors come into play. Therefore, other cues must be present.

In mid to high latitudes where photoperiod changes throughout the year, there is good evidence that photoperiod drives seasonal reproduction (Follett *et al.*, 1974). For these birds, photoperiod can be a proximate cue and provide a window that birds use to begin their gonadal growth. Even birds that live in tropical locations can respond to slight photoperiodic changes (Hau, 2001). The fine-tuning of subsequent reproductive activities can be the result of a combination of factors, such as male song inducing ovarian growth in females, ambient temperature, rainfall, or even budding on plants. Elegant experiments on temperature differences controlling the timing of reproduction show that timing is related to temperature but not absolute temperature. However, temperature shifts were not related to the circulating levels of hormones that were studied (Schaper *et al.*, 2012). For both temperate and tropical birds, the reproductive axis is probably set at ready due to photoperiodic cues and full functionality is then triggered by a combination of proximate cues that are species dependent. For example, opportunistic breeders, such as the zebra finch, are cued to rainfall and water availability (Zann *et al.*, 1995).

Photostimulation (increasing day length) triggers the growth of gonads and photorefractoriness (gonadal regression due to decreasing day length) occurs after breeding. Photorefractoriness may have evolved to minimize the cost of transporting more weight during migration or to inhibit reproduction when environmental conditions are not ideal (Wingfield *et al.*, 1995). Photostimulation causes an increase in FSH and LH, along with other gonadotropic hormones (Follett *et al.*, 1974). These hormones are still released even in castrated birds. During photorefractoriness, gonads are also not necessary. PRL may also be responsible for when birds regress their gonads after the breeding season. At the end of the breeding season, LH decreases and coincides with an increase in PRL. However, injection of PRL or blocking VIP does not cause photorefractoriness, or the non-reactiveness to differences in daylength (Dawson, 1998). PRL has been linked to molt and migration so may instead play an indirect role in life-history stage transition from breeding to molt or migration (Dawson *et al.*, 2001).

Changes in Hormones With Respect to Season and Daylength

Photoreceptors in the eye, the pineal, and the deep brain perceive light to help birds time reproductive activities. The avian eye acts as a photoreceptor but also contains a circadian clock and induces the production of melatonin (MEL). The pineal gland contains the circadian pacemaker and has photosensory cells, photopigments, and produces MEL. Lastly, deep brain photoreceptors can also perceive light information (Underwood *et al.*, 1984). GnRH and opsin, a group of light sensitive proteins, in the brain interact, suggesting direct communication between deep brain photoreceptors and GnRH neurons (Davies *et al.*, 2012).

The pineal gland of all vertebrates show daily rhythms that synthesize and produce MEL. In both diurnal and nocturnal birds, MEL is higher at night. The eyes contribute to photoperception and contribute to MEL in the blood. It has been thought that seasonal changes in MEL does not regulate reproductive effort in birds. For example, pinealectomy in birds results in individuals that are photosensitive in terms of stimulation and refractoriness (Wilson, 1991). However, increasing evidence shows that MEL is involved in some way. Injection of MEL stimulates LH and testicular size. It is hypothesized that MEL may be involved in the induction of GnIH expression, thus affecting reproductive activity (Ubuka *et al.*, 2005). Birds under constant light, sometimes due to artificial light at night, have different circadian profiles of MEL concentration and changes in reproductive activities, including advanced gonadal development (de Jong *et al.*, 2016; Dominoni *et al.*, 2013). These effects are not only dependent on light intensities but also on light spectra, possibly as a result of photoreceptor sensitivity.

Thyroidal hormones affect reproductive function but the mechanisms behind its function can be contradictory. For example, some studies find that thyroidectomy prevents gonadal regression but other studies find that photorefractoriness is accelerated (Dawson *et al.*, 2001). Thyroxine has to be present for responses to photoperiod to occur, and it could be possible that thyroid hormones affect GnRH synthesis (Bentley *et al.*, 2006). Genomic analyses reveal that gene expression of thyrotropin- β subunit peaked right after dawn, suggesting that thyroid hormones respond to changes in MEL signaling and thus are affected by photoperiod (Nakao *et al.*, 2008). Lastly, corticosterone concentrations also vary with respect to season, with higher concentrations during breeding than at other times of the year. It is possible that this elevation is due to the metabolically demanding reproductive activities (Romero, 2002).

Conclusion

Since Lack's seminar work on avian reproduction in five decades ago (Lack, 1968), we still have a relatively rudimentary understanding of the physiological mechanisms governing reproduction. Life-history traits, such as timing of breeding, egg size and number, and parental ability have received less integrated attention, especially with respect to understanding the role of hormones in regulating transitions between these life-history stages and control of these important life-history traits (Williams, 2012). Moreover, this article has focused on mechanistic and often causal effects of hormones on reproduction in birds. However, there is large, intrinsic variation among individuals, such that some females lay more eggs and some males raise more offspring. Identifying the physiological mechanisms that govern this individual variation will be key to understanding reproductive function in birds.

References

- Angelier, F., & Chastel, O. (2009). Stress, prolactin and parental investment in birds: A review. *General and Comparative Endocrinology*, 163, 142–148.
- Baker, M. (1995). Environmental component of latitudinal clutch-size variation in house sparrows (*Passer domesticus*). *Auk*, 112, 249–252.
- Bentley, G. E., Kriegsfeld, L. J., Osugi, T., et al. (2006). Interactions of gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH) in birds and mammals. *Journal of Experimental Zoology Part A: Comparative Experimental Biology*, 305A, 807–814.
- Buntin, J. D. (1996). Neural and hormonal control of parental behavior in birds. In J. S. Rosenblatt, & C. T. Snowdon (Eds.), *Advances in the Study of Behavior; Parental Care: Evolution, Mechanisms, and Adaptive Significance. Advances in the Study of Behavior*, 25 pp. 161–213. San Diego: Academic Press, Inc.; Academic Press Ltd.
- Cherry, M. I., & Gosler, A. G. (2010). Avian eggshell coloration: New perspectives on adaptive explanations. *Biological Journal of the Linnean Society*, 100, 753–762.
- Crossin, G. T., Trathan, P. N., Phillips, R. A., et al. (2012). Corticosterone predicts foraging behavior and parental care in Macaroni Penguins. *The American Naturalist*, 180, E31–E41.
- Davies, W. I., Turton, M., Peirson, S. N., et al. (2012). Vertebrate ancient opsin photopigment spectra and the avian photoperiodic response. *Biological Letters*, 8, 291–294.
- Dawson, A. (1998). Photoperiodic control of the termination of breeding and the induction of moult in house sparrows *Passer domesticus*. *Ibis*, 140, 35–40.
- Dawson, A., King, V. M., Bentley, G. E., & Ball, G. F. (2001). Photoperiodic Control of Seasonality in Birds. *Journal of Biological Rhythms*, 16, 365–380.
- de Jong, M., Jenina, L., Ouyang, J. Q., et al. (2016). Dose-dependent responses of avian daily rhythms to artificial light at night. *Physiology & Behavior*, 155, 172–179.
- Dominoni, D., Quetting, M., Partecke, J., 2013. Artificial light at night advances avian reproductive physiology, v. 280.
- Drent, R. H., & Daan, S. (1980). The prudent parent – Energetic adjustments in avian breeding. *Ardea*, 68, 225–252.
- Duckworth, R. A., & Sockman, K. W. (2012). Proximate mechanisms of behavioural inflexibility: Implications for the evolution of personality traits. *Functional Ecology*, 26, 559–566.
- Duffy, A. M., Clobert, J., & Møller, A. P. (2002). Hormones, developmental plasticity and adaptation. *Trends in Ecology & Evolution*, 17, 190–196.
- Follett, B. K., Mattocks, P. W., & Farner, D. S. (1974). Circadian function in the photoperiodic induction of gonadotropin secretion in the white-crowned sparrow, *Zonotrichia leucophrys gambelii*. *Proceedings of the National Academy of Sciences of the United States of America*, 71.
- Goldsmith, A. R. (1983). *Prolactin in avian reproductive cycles: Hormones and behaviour in higher vertebrates*. Berlin, Heidelberg: Springer-Verlag.
- Hattori, A., Ishii, S., & Wada, M. (1986). Effects of two kinds of chicken luteinizing hormone-releasing hormone (LH-RH), mammalian LH-RH and its analogs on the release of LH and FSH in Japanese quail and chicken. *General and Comparative Endocrinology*, 64, 446–455.
- Hau, M., 2001. Timing of breeding in variable environments: Tropical birds as model systems pp. 281–290.
- Hau, M., & Goymann, W. (2015). Endocrine mechanisms, behavioral phenotypes and plasticity: Known relationships and open questions. *Frontiers in Zoology*, 12, 1–15.
- Hertelendy, F., Lintner, F., Asem, E. K., & Raab, B. (1982). Synergistic effect of gonadotropin releasing hormone on LH-stimulated progesterone production in granulosa cells of the domestic fowl (*Gallus domesticus*). *General and Comparative Endocrinology*, 48, 117–122.
- Lack, D. (1968). *Ecological adaptations for breeding in birds*. Methuen & Co. Ltd.
- Lewis, P. D., Perry, G. C., Morris, T. R., & English, J. (2001). Supplementary dim light differentially influences sexual maturity, oviposition time, and melatonin rhythms in pullets. *Poult Science*, 80, 1723–1728.
- Love, O. P., & Williams, T. D. (2008). The adaptive value of stress-induced phenotypes: Effects of maternally derived corticosterone on sex-biased investment, cost of reproduction, and maternal fitness. *American Naturalist*, 172, E135–E149.
- Lynn, S. E. (2016). Endocrine and neuroendocrine regulation of fathering behavior in birds. *Hormones and Behavior*, 77, 237–248.
- Maney, D. L., Richardson, R. D., & Wingfield, J. C. (1997). Central administration of chicken gonadotropin-releasing hormone-ii enhances courtship behavior in a female sparrow. *Hormones and Behavior*, 32, 11–18.
- Miyamoto, K., Hasegawa, Y., Minegishi, T., et al. (1982). Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. *Biochemical and Biophysical Research Communications*, 107, 820–827.
- Miyamoto, K., Hasegawa, Y., Nomura, M., et al. (1984). Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proceedings of National Academy of Science of the United States of America*, 81, 3874–3878.
- Nakao, N., Ono, H., Yamamura, T., et al. (2008). Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature*, 452, 317–322.
- Oksche, A. (1978). Pattern of neuroendocrine cell complexes (Subunits) in hypothalamic nuclei: Neurobiological and phylogenetic concepts. In W. Bargmann, A. Oksche, A. L. Polenov, & B. Scharer (Eds.), *Neurosecretion and Neuroendocrine Activity: Evolution, Structure and Function* (pp. 64–71). Berlin, Heidelberg: Springer Berlin Heidelberg.
- Ouyang, J. Q., Muturi, M., Quetting, M., & Hau, M. (2013). Small increases in corticosterone before the breeding season increase parental investment but not fitness in a wild passerine bird. *Hormones and Behavior*, 63, 776–781.
- Payne, G. G., Lincoln, D. W., & Charles, D. R. (1965). The influence of constant and fluctuating environmental temperatures on time of oviposition under continuous lighting. *British Poultry Science*, 6, 93–95.
- Romero, L. M. (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *General and Comparative Endocrinology*, 128, 1–24.
- Saito, N., & Koike, T. I. (1992). Alterations in uterine contractility during the oviposition cycle in domestic hens. *British Poultry Science*, 33, 671–676.
- Schaper, S. V., Dawson, A., Sharp, P. J., Caro, S. P., & Visser, M. E. (2012). Individual variation in avian reproductive physiology does not reliably predict variation in laying date. *General and Comparative Endocrinology*, 179, 53–62.
- Schoech, S. J., Reynolds, S. J., & Boughton, R. K. (2004). Endocrinology. In W. D. Koenig, & J. L. Dickenson (Eds.), *Ecology and Evolution of Cooperative Breeding in Birds*. Cambridge: Cambridge University Press.
- Török, J., Moskát, C., Michl, G., & Péczely, P. (2004). Common cuckoos (*Cuculus canorus*) lay eggs with larger yolk but not more testosterone than their great reed warbler (*Acrocephalus arundinaceus*) hosts. *Ethology Ecology & Evolution*, 16, 271–277.
- Tsutsui, K., Bentley, G. E., Bedecarrats, G., et al. (2010). Gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. *Frontiers in Neuroendocrinology*, 31, 284–295.
- Ubuka, T., Bentley, G. E., Ukena, K., Wingfield, J. C., & Tsutsui, K. (2005). Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. *Proceedings of the National Academy of Sciences of the United States of America*, 102.
- Ubuka, T., Ukena, K., Sharp, P. J., Bentley, G. E., & Tsutsui, K. (2006). Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology*, 147, 1187–1194.
- Underwood, H., Binkley, S., Siopes, T., & Mosher, K. (1984). Melatonin rhythms in the eyes, pineal bodies, and blood of Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology*, 56, 70–81.
- Vleck, C. M., & Vleck, D. (2011). Hormones and regulation of parental behavior in birds: Hormones and reproduction of vertebrates. In *Birds*, 4.
- Williams, T. D. (2012). *Physiological Adaptations for Breeding in Birds*. Princeton, NJ: Princeton University Press.
- Wilson, F. E. (1991). Neither retinal nor pineal photoreceptors mediate photoperiodic control of seasonal reproduction in American tree sparrows (*Spizella arborea*). *Journal of Experimental Zoology*, 259.
- Wingfield, J. C., O'Reilly, K. M., & Astheimer, L. B. (1995). Modulation of the adrenocortical responses to acute stress in arctic birds – A possible ecological basis. *American Zoologist*, 35, 285–294.
- Zann, R. A., Morton, S. R., Jones, K. R., & Burley, N. T. (1995). The timing of breeding of zebra finches in relation to rainfall at Alice Springs, central Australia. *Emu*, 95, 208–222.

Hormones and Reproduction in Amphibians and Reptiles

David O Norris and Kristin H Lopez, University of Colorado, Boulder, CO, United States

© 2018 Elsevier Inc. All rights reserved.

The HPG Axis in Amphibians and Reptiles

Reproduction in amphibians and reptiles is tuned to environmental conditions acting through the nervous system to control the hypothalamo-pituitary-gonadal (HPG) axis. The HPG axes contain the elements described for mammals. Secretion of gonadotropins (follicle-stimulating hormone, FSH, and luteinizing hormone, LH) from the pituitary is controlled by hypothalamic gonadotropin-releasing hormone (GnRH). Release of GnRH is under the control of kisspeptins as in mammals (see [Pinilla et al. \(2012\)](#)). Gamete development is controlled by gonadotropins as is secretion of estrogenic, androgenic, and progestogenic hormones by the gonads. The latter hormones, sometimes cooperating with other hormones, are involved in development of sex accessory structures, mating behavior, and in some species gestation and parental care. Nonapeptide hormones (arginine vasotocin, AVT) and prolactin from the pituitary also affect reproduction (for detailed reviews, see [Norris and Carr \(2013\)](#) and [Norris and Lopez \(2011a,b\)](#)).

The HPG Axis of Amphibians

Reproductive endocrinology has been characterized primarily in anurans (frogs, toads) with some corroborative data from urodeles (salamanders, newts). Gymnophionid amphibians (caecilians or apodans) are similar to urodeles although the endocrine details are not well known.

The hypothalamus controls gonadotropin secretion through release of GnRH (see [Tsai \(2011\)](#)). Amphibian FSH stimulates spermatogenesis in males as well as follicle development and estrogen secretion in females. LH is responsible for androgen and progesterone synthesis by gonads as well as for stimulating oocyte maturation (see [Propper \(2011\)](#) and [Uribe Aranzabal \(2011\)](#)).

Secretion of corticosterone from the adrenals is elevated during breeding. Corticosterone can be converted to a potent androgen (5 α -dihydrotestosterone (DHT)). Very high levels of corticosterone, such as associated with stress, may inhibit reproduction.

The HPG Axis of Reptiles

Extant reptiles include squamates (lizards and snakes), turtles, crocodilians (crocodiles and alligators) and the tuatara. As in other vertebrates, reproduction is controlled by hormones: GnRH released by the hypothalamus regulates the secretion of gonadotropins by the pituitary gland. GnRH is probably released in a pulsatile manner as in mammals. The GnRH neurons of reptiles receive input from other regions of the brain that convey information about internal physiology (e.g., metabolic state) and the external environment (e.g., temperature, photoperiod, moisture, behavior of conspecifics) that influences GnRH release and thus can promote or inhibit reproductive functions. Several molecular forms of GnRH have been identified in reptiles.

Turtles and crocodilians have two distinct gonadotropins homologous to those found in other vertebrates: an FSH-like hormone that promotes gamete development and an LH-like hormone that controls gonadal steroidogenesis and gamete release. In contrast, squamates possess a single FSH-like form that performs both the gametogenic and steroidogenic functions (see [Lovem \(2011\)](#) and [Taylor and DeNardo \(2011\)](#)).

The gonadotropins bind to receptors in the gonads (testes and ovaries) of adults and promote synthesis of gonadal steroids – estrogens, androgens including testosterone, and progesterone. These steroids influence reproductive structures, functions and behaviors, and exert feedback to the hypothalamus and/or pituitary to regulate gonadotropin secretion. Most current knowledge of the neuroendocrine control of reproduction in reptiles comes from detailed studies of the green anole lizard, *Anolis carolinensis* (see [Johnson and Wade, \(2011\)](#)), but many gaps in our understanding remain.

Reproductive Cycles

A comprehensive assessment of reproductive cycles for amphibians and reptiles is available ([Norris and Lopez, 2011a,b](#)) and is summarized here. Most amphibians and reptiles become reproductively active only once a year but may produce multiple broods in a single breeding season. Reproductive cycles may occur in two forms with respect to hormonal patterns ([Fig. 1](#)). In the associative or prenuptial pattern, gonadal steroid levels are greatest during mating. Other species exhibit a dissociated or postnuptial pattern where gonadal steroids are lowest during mating.

Environmental factors operating through the brain and pituitary are important in regulating the timing of breeding. These factors include rainfall, temperature, and photoperiod. Additionally, social cues may influence hormone levels and fine-tune reproductive events.

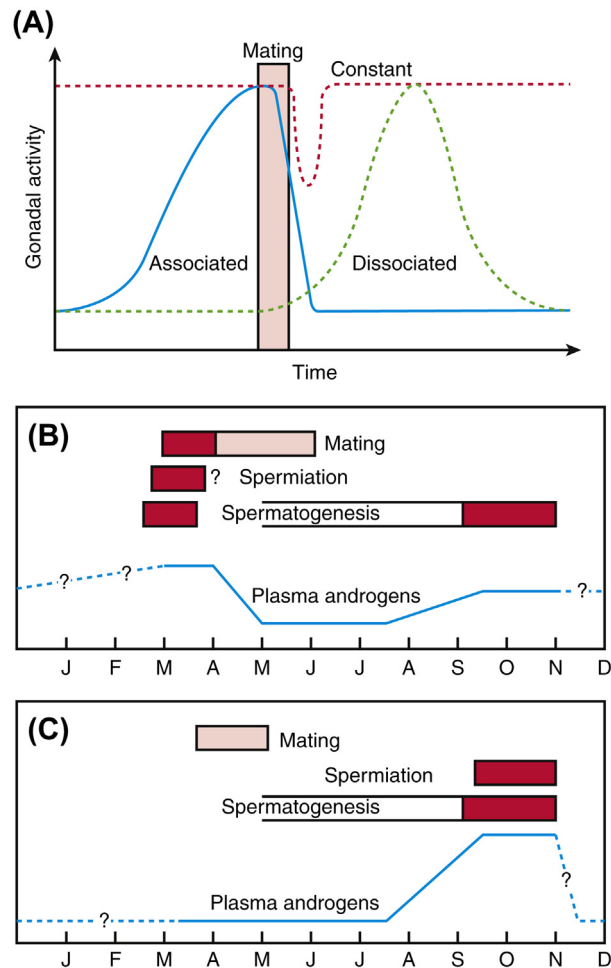


Fig. 1 (A) Associated (prenuptial) and dissociated (postnuptial) reproductive patterns. Mating occurs at the peak of gonadal activity in species exhibiting the associated pattern (B), whereas mating occurs when gonadal activity is low in the dissociated pattern (C). Reprinted with permission from Norris, D.O., Carr, J.A., 2013. *Vertebrate Endocrinology*, fifth ed. San Diego, CA: Academic Press (Elsevier).

Reproductive Cycles in Amphibians

Because of their moist skin, adult amphibians usually occur in aquatic habitats (rivers, lakes) or moist terrestrial habitats. A few species are adapted to deserts (e.g., spadefoot toads). Life history patterns are summarized in Fig. 2. Breeding may occur in spring or autumn, depending on the species. Terrestrial amphibians experience a “water drive” controlled by prolactin that causes mature adults to seek water where they mate and deposit their eggs.

Although the basic endocrinology of all amphibians is similar, there are major life history and reproductive differences among the three extant orders: anurans (see Rastogi *et al.* (2011)), urodeles, and gymnophionids (see Norris (2011)). Fertilization is internal in urodeles and gymnophionids whereas it is external in the vast majority of anurans. Typically, sexes are separate and hermaphroditism is extremely rare. Parthenogenesis (complete development of unfertilized eggs) has been described only in interbreeding ambystomid salamanders (Norris, 2011).

Steroids secreted by the gonads affect sex accessory structures (see Sever and Staub (2011)), reproductive behaviors, spermatogenesis, and oogenesis including secretion of the estrogen-dependent egg precursor protein, vitellogenin, by the liver.

Anuran cycles

Anurans mostly lay eggs in the water, and males grasp the females (amplexus) and fertilize eggs while they are being laid. Fertilized eggs hatch as a fish-like aquatic larval stage (tadpole) followed some time later by a dramatic anatomical and biochemical transformation (metamorphosis) to a terrestrial or semi-terrestrial adult. Metamorphosis is directed by thyroid (HPT) and adrenal (HPA) axes (Norris and Dent, 1989; Denver, 1996; Shi, 2000). A few species retain their eggs within or upon their bodies and give birth to metamorphosed juveniles (viviparous). Some lay eggs in water but metamorphosis occurs in the egg prior to hatching.

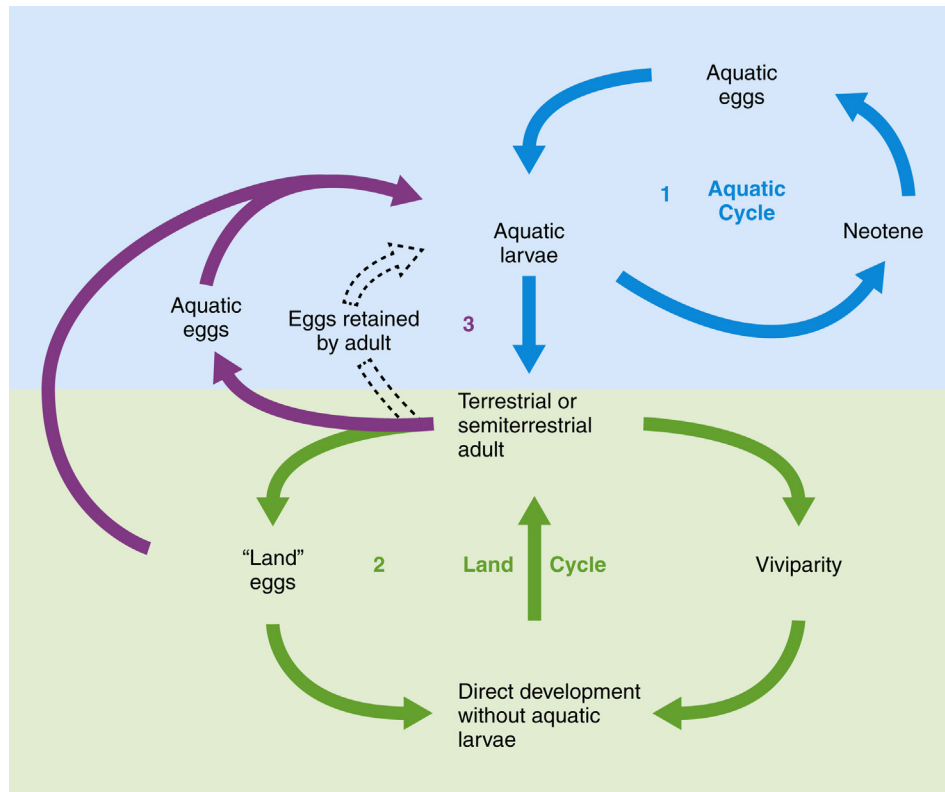


Fig. 2 Amphibian reproductive cycles. Life cycles vary from totally aquatic without metamorphosis (paedomorphic or neotenic) to production of terrestrial eggs or live-bearing (viviparity). In some cases metamorphosis is completed within the egg and a miniature adult rather than a larva appears at hatching. Reprinted with permission from Norris, D.O., Carr, J.A., 2013. *Vertebrate Endocrinology*, fifth ed. San Diego, CA: Academic Press (Elsevier).

Urodele cycles

Male salamanders and newts produce gelatinous structures called spermatophores with sperm deposited at their tips. A receptive female is induced to pass over the spermatophore and take the packet of sperm into her cloaca. Sperm are stored in the spermatheca associated with the oviduct until ovulation. The eggs are fertilized with stored sperm as they are shed, often in the absence of males. Urodeles generally produce aquatic larvae although they are more advanced in development at hatching than anurans (e.g., all 4 limbs emerge prior to hatching). Larvae are characterized by external gills for respiration. Typically, the larvae undergo metamorphosis to produce a semi-aquatic or terrestrial adult. In some species the aquatic larva doesn't undergo metamorphosis and becomes sexually mature, a condition referred to as paedomorphosis. Females of a few species (genus *Salamandra*) retain the fertilized eggs that develop into larvae or fully metamorphosed young within the oviducts.

Gymnophionid cycles

Adult gymnophionid amphibians are completely terrestrial. They are oviparous or live-bearing and exhibit internal fertilization accomplished by a male intromittant organ, the phallodeum. The fertilized eggs are either laid in burrows on land or develop within the oviducts of the females. Their reproductive endocrinology, however, is virtually unknown.

Reproductive Cycles in Reptiles

Although amphibians and reptiles share characteristics of their reproductive biology with other vertebrates, reptiles differ from amphibians in several regards. Reptilian reproduction is liberated from an aquatic environment. The amniotic egg appeared first in reptiles, allowing females to lay their eggs on land. Compared to the prodigious quantity of eggs produced by most amphibians, reptiles typically produce a few large eggs well-provisioned with yolk. Fertilization is internal, usually aided by a male copulatory organ. Because development of the fertilized egg begins within the maternal reproductive tract, the embryo can be retained *in utero* for variable periods of time. Squamate reptiles exhibit a wide spectrum of egg-retention strategies, from a period of development of the shelled egg in the uterus (oviparity) to complete gestation supported by a maternal placenta and birth of a fully-developed neonate (viviparity). Although sex is genetically determined in many reptiles, incubation temperature determines the sex of crocodilians and most turtles, and thus hatchling sex varies with nest temperature and egg position within the nest. Finally, all-female (parthenogenetic) species of some squamate reptiles reproduce without the genetic contribution of males.

The reproductive system of reptiles is responsive to endocrine and paracrine factors, and also is influenced by environmental cues including temperature, photoperiod, rainfall and the behaviors of conspecifics. Most reptiles exhibit a seasonal cycle of reproduction; even tropical species usually confine reproductive activity to a portion of the year. Individuals in continuously breeding populations may have asynchronous cycles. The annual female cycle involves one or more extended bouts of ovarian growth as oocytes accumulate yolk (vitellogenesis), after which females mate, ovulate, and lay their eggs. This cycle is extended in viviparous species as gravid females retain their developing embryos *in utero* and give birth to live young. The male reproductive cycle includes spermatogenesis (sperm formation), testicular secretion of testosterone and other sex steroids, and the expression of behaviors related to reproduction (territoriality, courtship, mating). In temperate species, testes regress and spermatogenetic activity ceases during the winter dormancy. Individual reptiles, especially females, may skip one or more reproductive cycles, so a given population may include both reproductively active and inactive adults.

Squamates

Current knowledge of squamate reproductive cycles is based largely on studies of temperate species despite the abundance of understudied tropical species. Most species of lizards and snakes exhibit a seasonal reproductive cycle (see [Lovern \(2011\)](#) and [Taylor and DeNardo \(2011\)](#)). In temperate zones, eggs are laid in the spring and the young hatch at a time when food resources are most abundant to support growth and survival. Temperate zone squamates usually display the prenuptial pattern of gametogenesis with maximal steroid hormone secretion preceding spring mating and ovulation. However, some viviparous lizards mate in the fall and give birth the following spring. Tropical squamates may time their reproductive periods to the wet or dry season, whereas some reproduce throughout the year, although no individuals breed continuously.

In females, estradiol is high during vitellogenesis and follicular growth. Progesterone is elevated between ovulation and oviposition, and in viviparous species progesterone remains high in gravid females. Testosterone is present in low concentrations in females but may be raised during the breeding season.

Testosterone is elevated in males during spermatogenesis and in most species during courtship and mating, regardless of whether mating activities coincide with spermatogenesis. Testosterone is low in nonbreeding males and during testicular regression in temperate species.

Turtles

Turtles exhibit an annual cycle of gametogenesis and breeding (see [Blanvillain et al. \(2011\)](#)). However, the frequency of reproductive bouts varies among species and individuals. For example, the painted turtle *Chrysemys picta* lays only one clutch per year, whereas the massive leatherback sea turtle (*Dermochelys coriacea*), which oviposits several clutches per reproductive season like other sea turtles, lays up to 10 clutches annually. However, individual female sea turtles reproduce every 1–4 years.

Terrestrial turtles in temperate zones exhibit an annual cycle of mating, nesting and gonadal activity. For example, the desert tortoise lays a single clutch in the spring or early summer. Follicular growth begins again in late summer and females enter winter hibernation with a full complement of yolked follicles that are ovulated the following spring. Testes also recrudescence in late summer and testosterone levels rise postnuptially. Mating occurs in the fall or after emergence from winter hibernation. Spermatozoa are stored in the epididymides during winter hibernation; sperm from fall matings may also be stored in the female reproductive tract. Male testosterone levels are low during the spring mating season. In females, progesterone levels rise during ovulation and may promote the secretion of albumin and shell around each ovulated egg prior to oviposition.

Sea turtles live and mate in the water but nest on land; their reproductive pattern is prenuptial. Reproduction is coupled with an extensive migration from feeding grounds to natal nesting beaches that may be thousands of miles away. In males, testosterone rises and spermatogenesis begins in the fall, before mating and migration. Follicular growth also starts in the fall, with rising estradiol levels stimulating a months-long period of vitellogenesis and ovarian growth. Testosterone in females rises in winter and remains elevated during migration and mating. These steroids decline during nesting. Progesterone peaks sharply at ovulation. In both sexes, gonadal steroids are lowest during the return migration. A unique feature of olive ridley and Kemp's ridley sea turtles is the aggregation of thousands of nesting females on a beach; this phenomenon is known as *the arribada*.

Crocodylians

Much of our knowledge of crocodylian reproductive endocrinology is based the American alligator, *Alligator mississippiensis* (see [Milnes \(2011\)](#)). Crocodylians inhabit temperate and tropical environments; all exhibit seasonal cycles with temperate species responding to photoperiod and temperature, and tropical species timing reproduction to rainfall and food availability ([Fig. 3](#)).

Development of the large yolky eggs takes several months. In female *A. mississippiensis*, vitellogenesis takes place in the fall when estradiol levels rise post-reproductively. After winter dormancy, a second phase of yolk deposition is accompanied by peak estrogen levels. Testosterone rises in females during the breeding season. Estradiol and testosterone then decline and progesterone rises at ovulation; progesterone later drops as females begin to lay their eggs within nests they have built of vegetation and mud near the water. In tropical species oocyte development takes place during the dry season immediately before breeding activities begin.

In males, after a period of quiescence in which the testes are spermatogenetically inactive, recrudescence occurs about four months before the mating season. Spermatogonia divide, enter meiosis and progress to produce mature spermatozoa by the beginning of mating activities. Testosterone remains high during spermatogenesis and the ensuing courtship and mating season, after which circulating levels decrease as the testes regress.

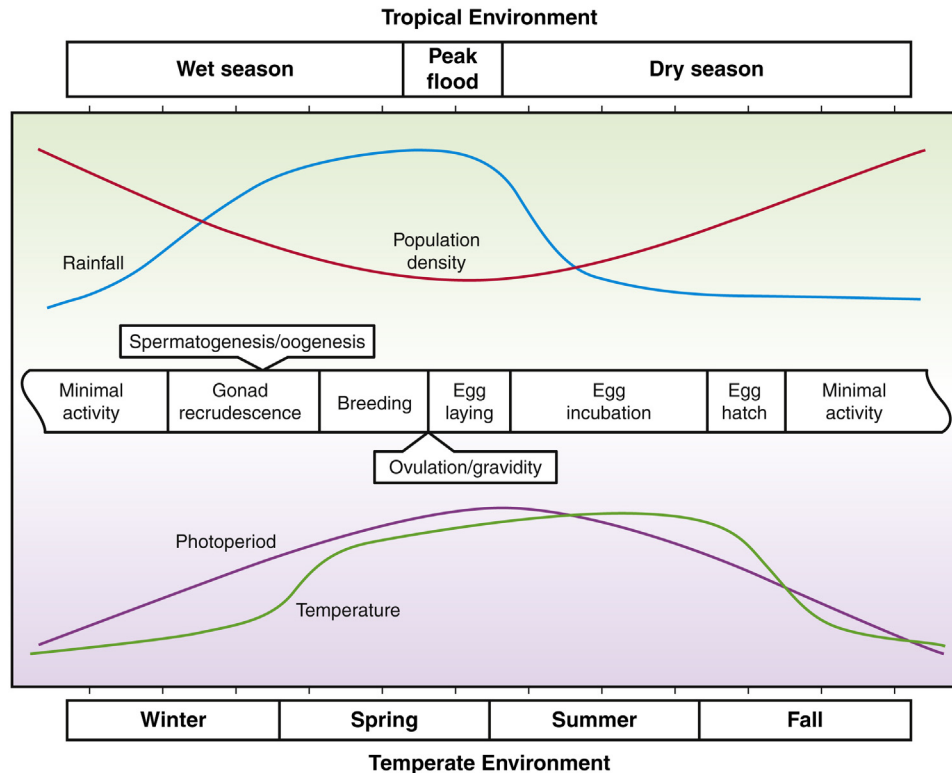


Fig. 3 Comparison of reproductive cycles of tropical and temperate crocodilians. Recrudescence refers to renewal of spermatogenesis/oogenesis following a previous reproductive period. Reprinted with permission from Norris, D.O., Carr, J.A., 2013. *Vertebrate Endocrinology*, fifth ed. San Diego, CA: Academic Press (Elsevier).

Gonadal Aspects of Reproduction

Details of spermatogenesis and testicular structure were reviewed recently for amphibians by [Propper \(2011\)](#) and for reptiles by [Kumar et al. \(2011\)](#). Ovarian and oviductal cycles were reviewed for amphibians by [Uribe Aranzabal \(2011\)](#) and for reptiles by [Jones \(2011\)](#).

Amphibian Gonads

The structure and function of amphibian testes and ovaries provided here is based on reviews by [Propper \(2011\)](#) and [Uribe Aranzabal \(2011\)](#).

Testes of amphibians

Spermatogenesis is of the cystic type in all amphibians as described for fishes, although the testes of anurans are sometimes described as consisting of seminiferous tubules. Urodele and gymnophionid testes are organized into distinct lobules, each containing several ampullae. In urodeles spermatogenesis proceeds synchronously within an ampulla, whereas it is asynchronous in gymnophionids.

Leydig cells (=lobule boundary cells) are located at the periphery of the ampullae and secrete testosterone and DHT in response to LH. These androgens stimulate development and secretion of the vasa deferentia and reach peak levels during sperm storage. They also are responsible for development of the nuptial pads used to hold females during copulation ([Fig. 4](#)).

Ovaries of amphibians

Oogenesis in amphibians is of the indeterminate type; that is, germ cells (oogonia) produce new oocytes annually. The nucleus of the oocyte begins meiosis but is arrested as a germinal vesicle until ovulation. Folliculogenesis begins with meiosis as each oocyte becomes surrounded by follicle cells that, under the stimulation of FSH, differentiate into thecal and granulosa cells. FSH also stimulates oocyte growth.

Granulosa cells are the major source of steroid hormones: androgens (testosterone, DHT, and androstenedione) and progesterone stimulated by LH, and estrogens (estradiol, estrone) stimulated by FSH. Ovaries also produce some deoxycorticosterone (DOC). High levels of circulating testosterone occur in some females ([Fig. 4](#)). Secretion of estrogens stimulates production of

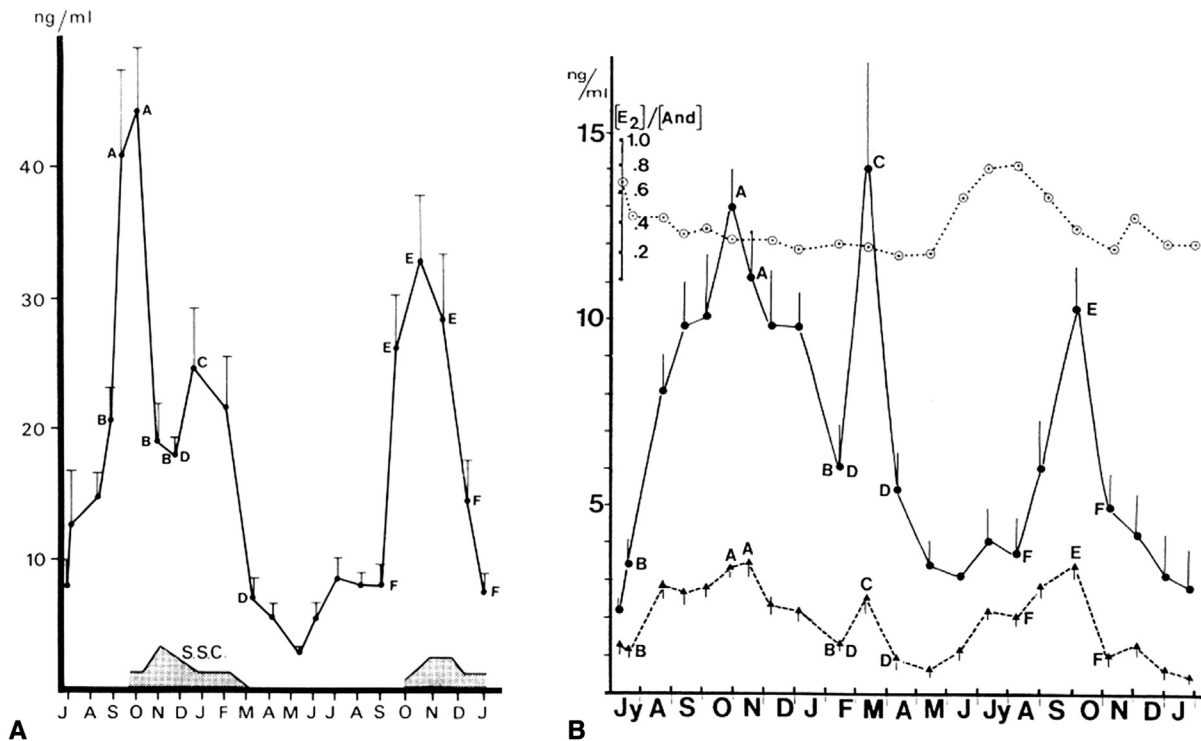


Fig. 4 Hormonal cycles in male and female salamanders, *Pleurodeles waltli*. (A) Seasonal changes in total androgens (testosterone, DHT, androstenedione) in captive males. SSC represents development of thumb pads, which are androgen-dependent secondary sex characters used in amplexus with females. Reprinted with permission from Garnier, D.H., 1985. Androgen and estrogen levels in the plasma of *Pleurodeles waltli* Michah. During the annual cycle I. Male cycle. Gen. Comp. Endocrinol. 58, 376–385 (Elsevier). (B) Total androgen (solid circles and solid line) are much higher than estradiol levels (triangles and dotted line) in females. Nevertheless, total androgens are much lower than in males (Fig. 4(A)). Ratio of estradiol to androgens is shown (open circles). Reprinted with permission from Garnier, D.H., 1985. Androgen and estrogen levels in the plasma of *Pleurodeles waltli* Michah. During the annual cycle II. Female cycle. Gen. Comp. Endocrinol. 60, 414–418 (Elsevier).

the yolk protein precursor vitellogenin by the liver. Vitellogenin travels through the blood to the ovary where it is used to make yolk proteins that are incorporated into the developing oocyte.

Ovulation is controlled by LH. Germinal vesicle breakdown is induced by LH acting through ovarian progesterone. Egg laying or birth of live young occurs in the spring whereas mating may occur in either fall or spring in different species.

Oviductal hypertrophy and secretion is stimulated by estrogens (Fig. 5). In oviparous species, the oviduct is responsible for secretion of the jelly coat that surrounds each egg. Progesterone-primed oviducts respond to AVT by contracting and expelling the coated eggs. In urodeles with internal fertilization, the eggs are fertilized prior to application of the jelly coat. In anurans with external fertilization, the eggs are fertilized prior to entering the water where the jelly coating swells and prevents entry of additional sperm.

Gonadal Aspects of Reproduction in Reptiles

The structure and function of reptilian testes and ovaries provided here is based on reviews by Kumar *et al.* (2011) and Jones (2011).

Testes of reptiles

The testes are paired oval organs that, as in other vertebrates, have two major functions: production of sperm (spermatogenesis) and synthesis of gonadal steroid hormones. Spermatogenesis occurs within seminiferous tubules that are tightly packed within the testes. Sertoli cells form the somatic epithelial component of the seminiferous tubules. They extend from the basal surface to the lumen of the tubule and maintain the blood-testis barrier. These “nurse cells” provide physical support and nourishment for the developing germ cells, which advance in stages of spermatogenesis from the basal to luminal surface.

The steroidogenic Leydig cells are located in the stroma between adjacent coils of seminiferous tubules. Androgens secreted by Leydig cells enter the peripheral circulation and act to develop and maintain sex accessory structures and promote mating behaviors. Testosterone is elevated during spermatogenesis in most species during the breeding season (Fig. 6), regardless of whether mating activities coincide with spermatogenesis. In temperate species, testosterone is low in nonbreeding males and during testicular regression. Sertoli cells also produce androgens that may act peripherally to induce reproductive behaviors as well as locally within the seminiferous tubules to promote spermatogenesis. These two androgen sources enable separate control of gamete production and reproductive behavior that may allow the uncoupling of spermatogenesis and mating in species with post-nuptial breeding. The

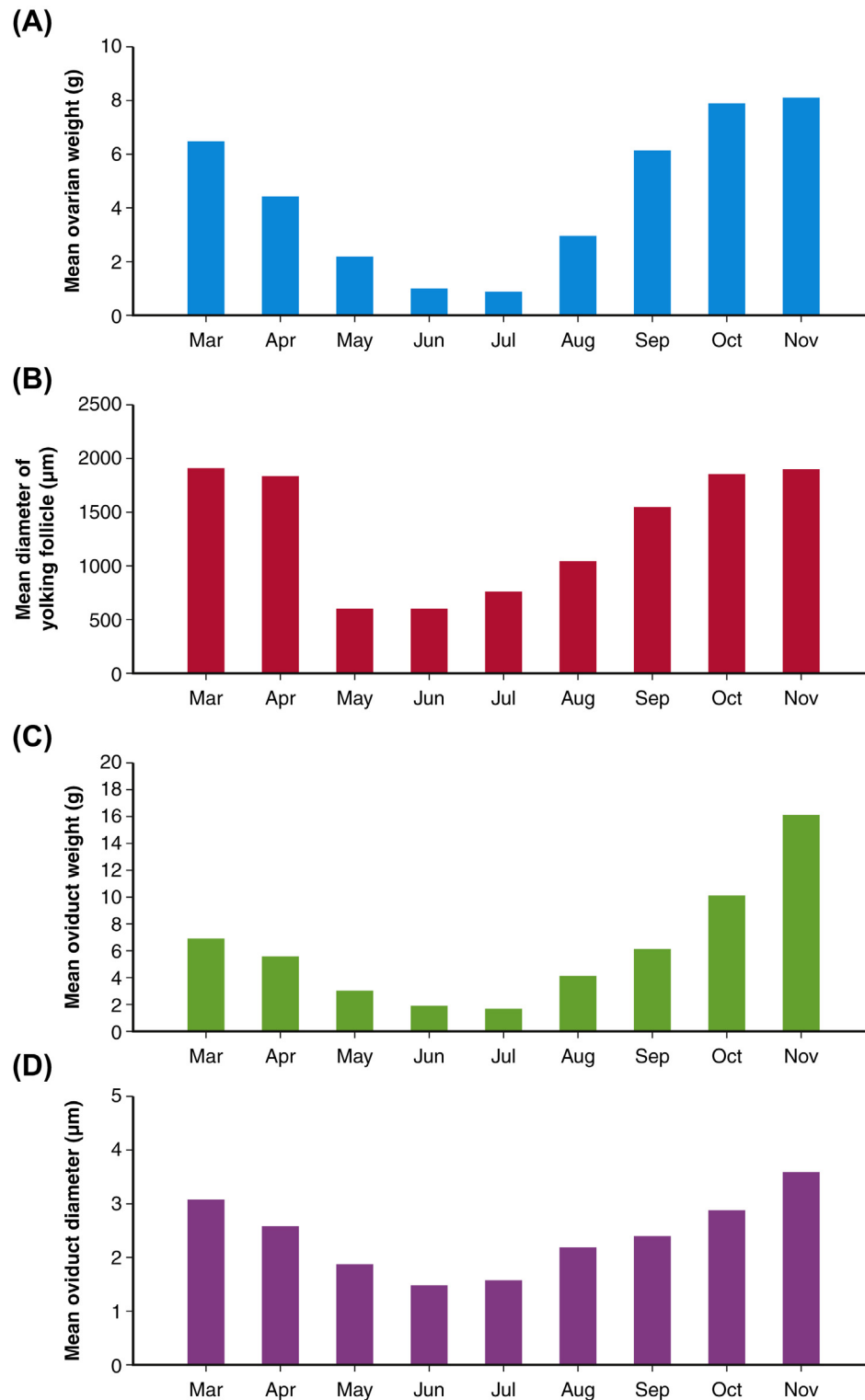


Fig. 5 Cycling of estrogen dependent characters in female tiger salamanders, *Ambystoma tigrinum*. (A) Mean ovarian weight. (B) Mean diameter of yolking follicles in ovary. (C) Mean oviduct weight. (D) Mean oviduct diameter. Reprinted with permission from Norris, D.O., Carr, J.A., 2013. Vertebrate Endocrinology, fifth ed. San Diego, CA: Academic Press (Elsevier).

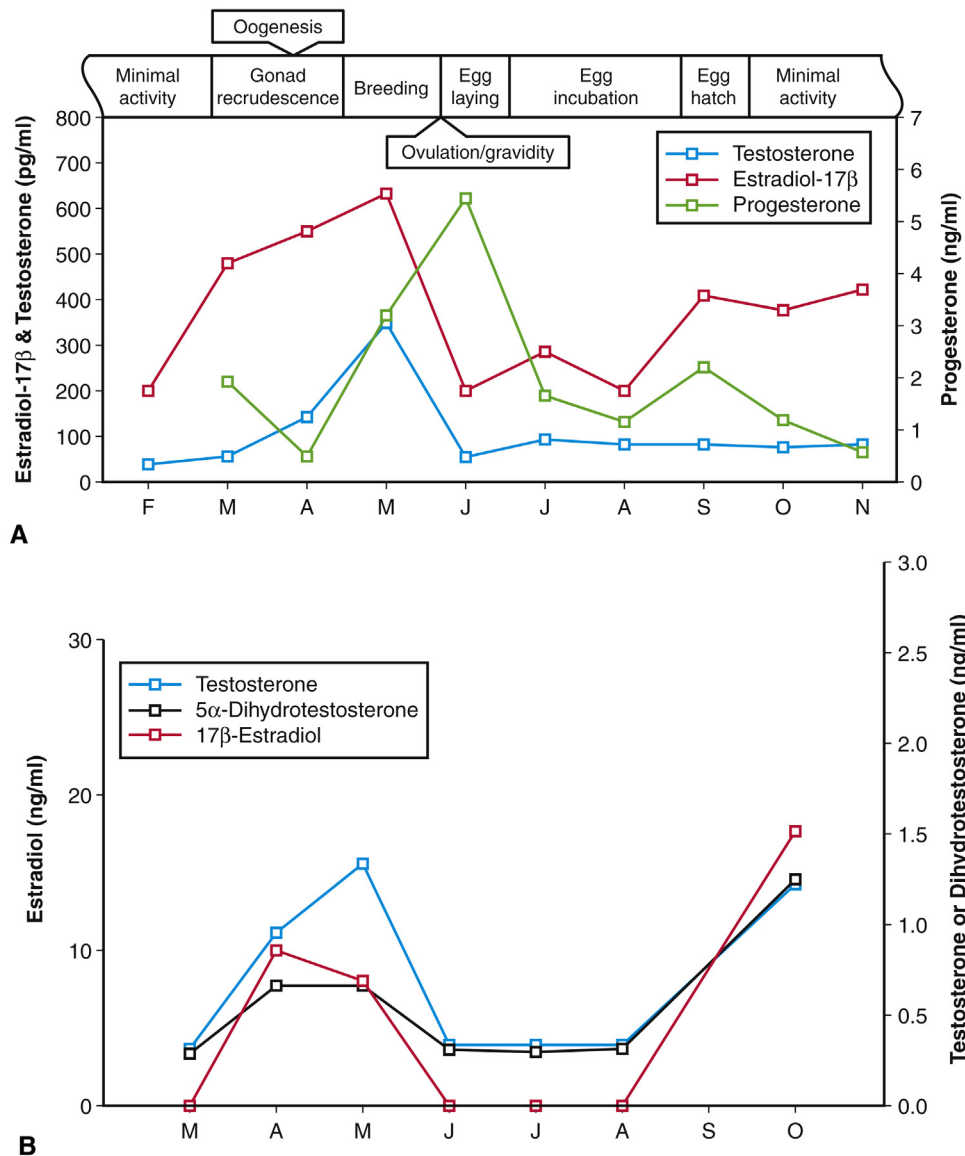


Fig. 6 Seasonal patterns of plasma steroid hormones in reptiles. (A) The American alligator, *Alligator mississippiensis*. (B) Northern Pacific rattlesnake, *Crotalus oreganus*. Reprinted with permission from Norris, D.O., Carr, J.A., 2013. Vertebrate Endocrinology, fifth ed. San Diego, CA: Academic Press (Elsevier).

testis is also the site of synthesis and action of estrogens. Estrogens are inhibitory to spermatogenesis in squamates and turtles, and thus may play a role in seasonal testicular quiescence.

Testicular function is regulated by gonadotropins. FSH promotes spermatogenesis, and LH regulates testicular steroidogenesis in turtles and crocodilians. In squamates, testicular functions are all regulated by a single FSH-like gonadotropin. Our understanding of the precise roles of reptilian gonadotropins is limited, as many studies are based on effects of mammalian gonadotropins, and data on plasma concentrations of reptilian gonadotropins are scarce. Stress-induced glucocorticoids inhibit plasma androgen levels as well as spermatogenesis in some reptiles.

Testicular size changes throughout the year in most male reptiles, particularly in temperate species. In response to environmental cues such as decreased temperature, shorter day-length and lower rainfall/food availability, spermatogenesis shuts down and the testes shrink. Inhibition of Leydig cell steroidogenesis may or may not accompany season testicular regression.

Sperm can remain viable for many months in the male reproductive system (epididymides) or female reproductive tract.

Ovaries of reptiles

Reptilian ovaries are paired, hollow oval structures (elongated in snakes) within the dorsomedial body cavity, loosely attached by a mesovarium to the dorsal body wall. Ovarian size varies greatly throughout the year depending on the number and size of ovarian

follicles present. Each follicle, the functional unit of the ovary, contains an oocyte surrounded by an enveloping layer of support cells. Very little connective tissue stroma is present.

Females can produce new oocytes from oogonial stem cells throughout their lives, similar to fishes and amphibians but unique among amniotic vertebrates. Mitotically dividing oogonia are clustered in a species-specific number of germinal beds located on the dorsal surface of the ovary. Newly-formed oocytes enter meiosis and become individually surrounded by granulosa cells. An external follicular layer, the theca, consists of an inner steroidogenic layer and an outer protective capsule. Under the influence of estradiol secreted from the developing follicles, the liver produces vitellogenin that serves as the precursor for yolk proteins. The telolethical oocytes accumulate large quantities of yolk during follicular growth. The ovaries secrete androgens in low amounts but in some species testosterone is significantly elevated during the mating season.

Ovulation of mature oocytes is likely under the influence of LH as in other vertebrates, with the exception of squamates. Granulosa cells of the postovulatory follicle transforms into a corpus luteum and secrete progesterone. Luteal progesterone peaks during the periovulatory period and remains elevated while females are gravid, dropping sharply at oviposition or birth of the young. Progesterone suppresses motility of the oviducts and probably inhibits follicular growth and vitellogenesis via feedback inhibition of gonadotropins. Corpora lutea have a species-specific life span, which is extended in viviparous squamates.

Endocrine Regulation of Reproductive Behavior

Studies of endocrine control of reproductive behaviors in amphibians and reptiles focus on two areas (see [Woodley \(2011\)](#), [Johnson and Wade \(2011\)](#), [Sinervo and Miles \(2011\)](#), and [Martín and López \(2011\)](#)). The first area involves courtship and mating behaviors. In amphibians and reptiles, auditory, visual and/or chemosensory (pheromonal) cues are important in courtship and mating, although reptiles mostly utilize visual and/or chemosensory cues.

The second area involves parental behavior. Although many species lay their eggs and never look back, some exhibit elaborate behaviors to protect their eggs and/or young and enhance their survival.

Reproductive Behavior of Amphibians

Reproductive behavior in amphibians involves a number of hormones in addition to those of the HPG axis. These include prolactin, AVT, and corticosterone. Pheromones have been identified in urodele reproduction, but anurans seem to rely more on visual, auditory, and tactile clues. Parental behavior has been described only in anurans.

Fertilization is external in most anurans but internal in urodeles and gymnophionids. Cloacal glands stimulated by androgens and prolactin are responsible for secretion of spermatophores in urodeles that are used in sperm transfer to the female. Gymnophionids employ a copulatory organ (phallodeum) for sperm transfer. Sperm may be stored in females in special structures called spermathecae associated with the oviducts, and the eggs may be fertilized at a later time.

Migratory behavior

Prior to breeding, some terrestrial amphibians migrate to aquatic breeding sites. This “water drive” behavior is triggered by prolactin. Endocrine control is not understood for desert spadefoot toads living in underground burrows that respond to the sound of heavy rains as a signal to leave their burrows and seek temporary ponds in which to lay their eggs.

Advertising

Once anurans reach the breeding site, males typically produce calls that attract reproductive females. Androgens appear to be responsible for development of the calling apparatus in males as well as the intensity of calling. Calling also is influenced by AVT although that role may vary by species. Additionally, plasma corticosterone levels are positively associated with calling in several species but negatively associated in others ([Carr, 2011](#); [Woodley, 2011](#)).

Clasping behavior (amplexus)

Male anurans characteristically grasp the back of a receptive female with the help of nuptial pads associated with forelimbs and release sperm as the female releases her eggs. This clasping behavior is termed amplexus and may trigger release of eggs from the female. Although androgens are responsible for secondary sex characteristics such as the nuptial pads, it is not clear what role androgens play in the initiation of amplexus. Administration of androgens can activate amplexus in male *Xenopus laevis* but are ineffective in *Rana pipiens*. A role for pheromones has yet to be demonstrated, but a peptide with female attracting properties has been isolated from the skin glands of one male anuran.

Amplexus in the newt, *Taricha granulosa*, is induced by AVT and antagonized by corticosterone. During amplexus, the male mounts from above and attempts to rub his chin on the female’s nasal region to transfer pheromones that will enhance his chances of spermatophore transfer. Pheromone roles are known for other urodeles as well.

Anuran parental behavior

Retention of eggs by females might be interpreted as prenatal care of young. Female marsupial frogs, for example, actively place fertilized eggs into an estrogen-dependent pouch that develops on their dorsal surface. Males of the midwife toad wrap the strings

of jelly containing the eggs around their legs and periodically return them to the water to moisten them and eventually to allow the hatching tadpoles access to water. Males of the terrestrial glass frogs of Central America carry moisture to their eggs that develop on land. They also protect the otherwise exposed developing young from predatory spiders until they hatch as miniature glass frogs. Other frog species produce elaborate foam nests where the young develop. The endocrine control of these behaviors has not been examined.

Reproductive Behaviors of Reptiles

At the onset of breeding, testosterone rises in male reptiles and is considered to be a key regulator of male reproductive behaviors. In many reptilian species, dominant males actively defend territories that contain females with whom they can mate. Defensive behaviors include aggressive attacks such as chasing and biting rival males, as well as dominance displays like head bobbing and push-ups as exhibited by many male lizards. Males lacking territories may still have mating opportunities; non-dominant “sneaker” males adopt the strategy of briefly entering a dominant male’s territory and copulating with females. Dominant males generally have higher androgen levels than their non-dominant conspecifics. Testosterone is also associated with the expression of male courtship behaviors in most species. Limited evidence demonstrating the presence of aromatase in the reptilian male brain suggests that testosterone may be converted to estradiol before stimulating male sexual behaviors.

The sexual behaviors of female reptiles may be influenced by estrogens, testosterone and/or progesterone. Mating usually takes place when estrogens released by the ovaries reaches a peak, and exogenous estradiol can activate receptive behaviors in females whose ovaries have been removed. Testosterone also is elevated in many female reptiles during courtship and mating, and may increase female receptivity. In the all-female lizard *Cnemidophorus uniparens* that reproduces by parthenogenesis, individuals engage in both male and female pseudosexual behaviors: before ovulation, they exhibit female-like receptive behaviors under the influence of high estradiol levels, and after ovulation, when progesterone levels are high, the behavior switches to male-like mounting (Fig. 7).

Pheromones secreted by cloacal glands of male and female reptiles also play important roles in reproductive behaviors. Pheromones can modulate male social dominance hierarchies, female mate choice, and mating behaviors (Martín and López, 2011). Stressors or corticosterone treatment inhibit male territorial, courtship and mating behaviors in some species, but female reproductive behaviors appear to be relatively unaffected by stress (Tokarz and Summers, 2011).

Although most reptiles abandon their eggs after oviposition or their young at birth, some species exhibit behaviors that increase the odds of survival of their young. These behaviors include nest site selection, nest-building, nest guarding and direct care of the

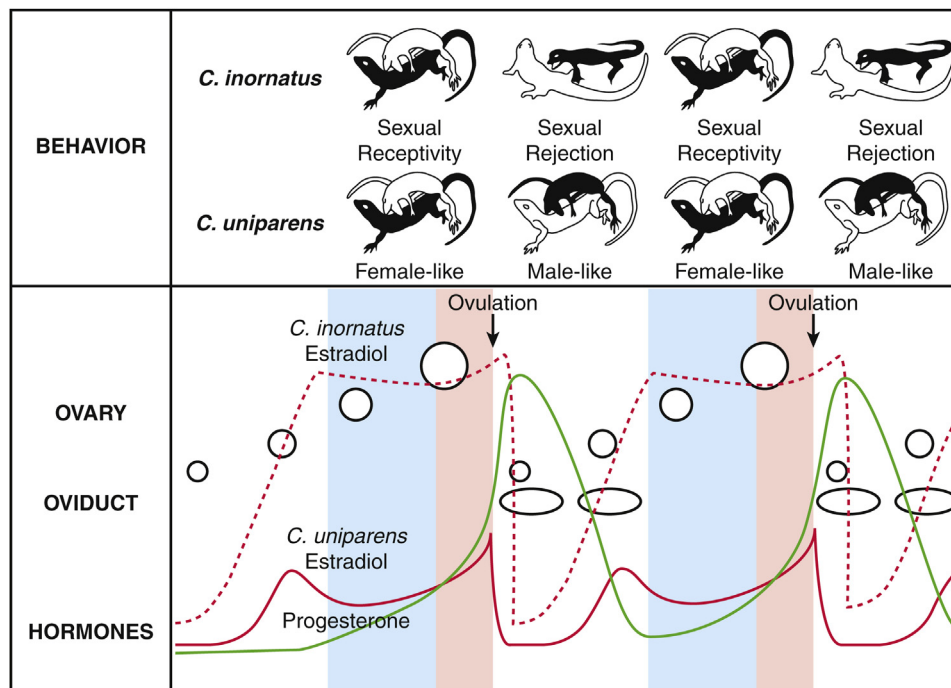


Fig. 7 Comparison of mating behavior in unisexual and bisexual lizards. Female-like and male-like behavior in the all female whiptail lizard (*Cnemidophorus uniparens*) is compared with female receptive behavior in a bisexual lizard, the little striped whiptail (*C. inornatus*). The differences in estradiol levels for the two species are illustrated (red lines). Female-like behavior in *C. uniparens* is elicited by a lower estrogen level and is followed by male-like copulatory behavior. The circles represent size of the ovarian follicles and the ovals indicate presence of eggs in the oviduct. Reprinted with permission from Norris, D.O., Carr, J.A., 2013. Vertebrate Endocrinology, fifth ed. San Diego, CA: Academic Press (Elsevier).

young, the latter observed in crocodilians. For example, female American alligators collect nesting materials to carefully create and shape their nests. While the eggs are incubating, females defend the nests against predators. The young begin to call even before hatching, and the female responds by excavating the terrestrial nest. She then carries the hatchlings in her mouth to the water. Males may also participate in care of the young to a lesser extent. The endocrine control of these parental behaviors is not well understood, but the possibility that prolactin may activate parental care in crocodilians as seen in other vertebrates is intriguing.

References

- Blanvillain, G., Owens, D. W., & Kuchling, G. (2011). Hormones and reproductive cycles in reptiles. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 277–303).
- Carr, J. A. (2011). Stress and reproduction in amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 99–116).
- Denver, R. J. (1996). Neuroendocrine control of amphibian metamorphosis. In L. I. Gilbert, J. R. Tata, & B. G. Atkinson (Eds.), *Metamorphosis: Postembryonic Reprogramming of Gene Expression in Amphibian and Insect Cells* (pp. 434–464). Academic Press.
- Johnson, M. A., & Wade, J. (2011). Neuroendocrinology of reptilian reproductive behavior. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 39–61).
- Jones, S. M. (2011). Hormonal regulation of ovarian function in reptiles. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 89–115).
- Kumar, S., Roy, B., & Rai, U. (2011). Hormonal regulation of testicular functions in reptiles. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 63–88).
- Lovern, M. B. (2011). Hormones and reproductive cycles in lizards. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 321–353).
- Martin, J., & López, P. (2011). Pheromones and reproduction in reptiles. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 141–167).
- Milnes, M. R. (2011). Hormones and reproductive cycles in crocodilians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 305–319).
- Norris, D. O. (2011). Hormones and reproductive patterns in urodele and gymnophionid amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 187–202).
- Norris, D. O., & Carr, J. A. (2013). *Vertebrate Endocrinology* (fifth ed.). San Diego, CA: Academic Press (Elsevier).
- Norris, D. O., & Dent, J. N. (1989). Neuroendocrine aspects of amphibian metamorphosis. In M. P. Schreibman, & C. G. Scanes (Eds.), *Development, Maturation and Senescence of Neuroendocrine Systems* (pp. 63–90). Academic Press.
- Norris, D. O., & Lopez, K. H. (2011a). *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles*. San Diego, CA: Academic Press (Elsevier).
- Norris, D. O., & Lopez, K. H. (2011b). *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians*. San Diego: Academic Press (Elsevier).
- Pinilla, L., Aguilar, E., Dieguez, C., Millar, R. P., & Tena-Sempere, M. (2012). Kisspeptins and reproduction: Physiological roles and regulatory mechanisms. *Physiol. Rev.*, 92, 1235–1316.
- Propper, C. R. (2011). Testicular structure and control of sperm development in amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 39–53).
- Rastogi, R. K., Oinelli, C., Polese, G., D'Aniello, B., & Chieffi-Baccari, G. (2011). Hormones and reproductive cycles in anuran amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 171–186).
- Sever, D. M., & Staub, N. L. (2011). Hormones, sex accessory structures, and secondary sexual characteristics in amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 83–98).
- Shi, Y.-B. (2000). *Amphibian Metamorphosis*. John Wiley and Sons.
- Sinervo, B., & Miles, D. B. (2011). Hormones and behavior of reptiles. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 215–246).
- Taylor, E. N., & DeNardo, D. F. (2011). Hormones and reproductive cycles in snakes. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 355–372).
- Tokarz, R. R., & Summers, C. H. (2011). Stress and reproduction in reptiles. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 3: *Reptiles* (pp. 169–213).
- Tsai, P.-S. (2011). Neuroendocrine control of reproduction in amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 21–37).
- Uribe Aranzabal, M. C. (2011). Hormones and the female reproductive system of amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 55–81).
- Woodley, S. K. (2011). Hormones and reproductive behavior in amphibians. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, vol. 2: *Amphibians* (pp. 143–169).

Endocrine Control of Reproduction, Insects[☆]

Roger R Huybrechts, Zoological Institute of KU Leuven, Leuven, Belgium

© 2018 Elsevier Inc. All rights reserved.

Glossary

AKH, or Adipokinetic hormone Peptide family, mainly produced and released from the glandular Corpora Cardiaca (CC), known regulators of the insect energy metabolism.

Corpora allata CA are the main Juvenile hormone synthesizing glands and part of the retrocerebral complex, present in both larvae and adults.

Dsx Doublesex gene a gender executer displaying a male and female specific splice variant.

E and 20-E, Ecdysteroids The typical insect steroids synthesized from dietary cholesterol. In larvae they initiate ecdysis. In adults the synthesized ecdysone mainly of gonad origin is enzymatically activated into 20-OH-ecdysone.

EcdR, Ecdysteroid receptor A cytoplasmic receptor family that, following ligand binding, heterodimerizes with a retinoid X co-receptor as ultraspiracle, and binds ecdysteroid responsive elements of target genes.

Fat body Metabolic center and energy storing organ.

Fruitless A gender executer gene displaying gender specific splice variants mainly regulating male accessory functioning, mating behavior and gender specific partner attraction via fruitless expressing neuronal plasticity.

Gynandromorph Insect with half the body cells displaying male phenotype and the other half displaying female phenotype. They result from X chromosome loss early in embryonic development in combination with the recessive X chromosome gender determination principle in insects.

ILP, insulin like hormones Neuropeptides involved in sensing and communicating the insects energy status. Some members are known ecdysiotropins. Insulin peptides regulate gene expression by sequestering FOXO, a known inhibiting transcription factor, from the nucleus.

JH, Juvenile hormones Sesquiterpenoid hormones mainly produced in CA but also released from male accessory glands. Most insects use JH III as functional hormone.

Met, methoprene tolerant receptor Binds JH, heterodimerizes with a steroid receptor type co-activator, as Taiman, prior to exerting transcription-regulating activity.

OEH Ovary ecdysteroidogenic hormone of mosquitoes, one of the few identified brain derived gonadotropins of insects displaying ecdysiotropic activity.

OMP Ovary maturing parsins of locusts, being ecdysiotropic.

Ovulin A male derived sperma peptide having receptors in the female oviducts.

Sex peptide A male derived sperm peptide affecting female reproduction.

TMOF Trypsin Modulating Oöstatic factor, an ovary derived factor that in the midgut translationally regulates Trypsin activity.

TOR Target of rapamycin, a cellular kinase pathway.

Vitellogenin Female specific yolk precursor protein, produced in the fat body and sequestered and accumulated as vitelline by the developing egg follicles.

YP Yolk polypeptides, dipteran female specific fat body derived yolk precursor polypeptides.

Introduction

The high reproductive capacity of insects, in combination with a terrestrial lifestyle of many species, represents a key element in explaining their evolutionary success and diversity. Insects reproduce sexually, a trait that most probably evolved in an attempt to cope with invading pathogens (De Loof *et al.*, 2016). Sexuality includes the integrated process of germ cell line formation, gonad differentiation, gametogenesis, yolk formation, partner attraction, courtship, copulation and deposition of fertilized chorionated, evaporation-resistant, eggs. Post-embryonic larval growth of insects is punctuated by molts, during which the animal produces a new skeleton (Krüger *et al.*, 2015). To acquire the necessary morphological and physiological characteristics for reproduction in the adult stage, insects undergo metamorphosis. Nymphs of hemimetabolous insects gradually acquire their adult phenotype. Internally, gonads develop and gametogenesis becomes apparent, starting in the second or third larval stage, as does the appearance of external sex-specific genitalia. Holometabolous larvae do not resemble the adult phenotype and, although gonadal imaginal discs develop, this is not yet visible in the differentiation of their external genitalia. However, the larval or pupal cuticle of holometabolous

[☆] A tribute to Arnold De Loof, Insect Physiologist and Visionary.



Fig. 1 Mature male *Locusta migratoria* guarding his female. During sexual maturation, the male head and some of parts of his body obtain a bright yellow colour.

insects can display either obvious or rather hidden sex-specific landmarks that allow gender determination. In contrast to spermatogenesis, being complete in most male insects at the start of their adult lifespan, many males display a lag period in their sexual maturation. This puberty process usually includes functionalization of the accessory glands and changes in behavior. Reaching sexual maturity can manifest itself externally, for example, by the cuticular yellowing in sexually mature male *Locusta migratoria* (Fig. 1). Whereas in Saturniid moths, the ovaries already develop to full maturity during metamorphosis, in most other species the ovarian follicles are still yolkless at the onset of the adult stage, necessitating a complex process of hormonally controlled vitellogenesis.

Sexual Differentiation: Combined Cell Autonomous and Hormonal Events. A Role for Sex Hormones in Insects

In most insects, as in other sexually reproducing organisms, gonadal sex (testis producing spermatozoa and ovaries producing eggs) is determined at fertilization, the moment of conception. This primary sex strictly depends upon the genotype of zygote-derived germ cells. In many insects, for example, in the fruit fly, *Drosophila melanogaster*, although having X and Y heteromorphic sex chromosomes, sex- and gender determination merely depends upon the ratio X chromosomes: autosomes, making XX: AA individuals female and both XY: AA and XO: AA males. Many other insects even do not have a morphological distinguishable Y chromosome, and sex is determined in XX: AA and XO: AA fashion. In Lepidoptera, like the silk moth *Bombyx mori*, as in birds, females represent the heterogametic sex (ZW), while males are homogametic (ZZ). Many insects, including social insects like bees and wasps, experience haploid-diploid sex determination. This means that females always derive from fertilized eggs whereas parthenogenetic development of unfertilized eggs gives rise to haploid males. Regarding the molecular mechanism of these seemingly different ways of sex determination, the generally accepted idea is that, governed by a cascade of differential transcriptional activation and inactivation steps, a female- and male splice variant of the gender executer gene, *doublesex* (*dsx*) is generated. Anyway, *primary sex differentiation* is said to be strictly genetic. Importantly, many scientists believe that also setting the *gender phenotype* in somatic cells of insects can only be strictly genetic, and has to follow the same principles as explained for gonadal differentiation. Such reasoning is in firm contrast to the established mechanism in vertebrates, in which upon differentiation, the primary gonads start secreting steroid sex hormones testosterone and 17 β -oestradiol that govern the development of the somatic- and behavioral sexual phenotype. The usual argumentation, supposed to confirm the “insects have no sex-hormones” hypothesis, is based upon the existence of insect gynandromorphs. One half of such individual has genotypic male cells (XO: AA) displaying the male phenotype, whereas in the other half the genotypic female cells (XX: AA) have female phenotype. Because both cell types are exposed to an identical hormonal blend of both ovary- and testis-derived hormones, it is thought that a role for sex hormones should be excluded. However, in some bird species, for example, chicken and zebra finch, comparable sexual gynandromorphs occur, though no one questions the existence of sex hormones in birds (review Clinton *et al.* (2012)). Thus, in insects too there should be a way out of the dilemma. Indeed, it is often overlooked that, as the result of developmental differentiation, both sides of a gynandromorph insect can have different receptors for the same hormones or that they do not as efficiently translate the hormonal input into downstream signals. That would explain why the same hormones can act differentially in different parts of the body. As will be further documented in this summary, genetic suppression of vitellogenin or yolk protein synthesis in previtellogenic female mosquitoes, is due to orphanizing the ecdysteroid receptor (EcdR). The mechanism involved is the selective recruitment of its intracellular USP binding partner toward an AHR38 receptor (Zhu *et al.*, 2000; Marquardt, 2005). Such mechanism may as well apply to male cells of the gynandromorph. Our reasoning gets extra support from experimental data obtained in *Drosophila* (Parisi *et al.*, 2010). They studied germ-line dependent gene expression in non-gonadal somatic tissues. They observed both germline-independent, cell-autonomous, sex-specific gene expression in germline-naïve animals, as well as germline-dependent gender-specific gene expression in germline-conditioned animals. These authors further observed the occurrence of a female-biased and

germline-dependent ecdysteroid titer, thereby confirming the existence of a hormonal axis between gonads and non-gonadal soma. Based upon research in female dipterans such as mosquitoes, fleshflies, fruitflies and houseflies, the importance of an ovary-fat body ecdysteroid axis indeed had long been evidenced. In Diptera, ecdysteroids are important for the female-specific stimulation of vitellogenin expression, a yolk protein precursor. Because injection of minute amounts of 20-OH ecdysone in adult male fleshflies induced the synthesis of yolk polypeptides by the fat body (Huybrechts and De Loof, 1977), these authors launched the idea that 20-OH ecdysone might very well be the female sex hormone of dipterans, and perhaps of all insects. They compared this induction of vitellogenin in male fleshflies with its counterpart in vertebrates, namely the induction of vitellogenins in the liver of roosters by injection of estrogens (De Loof and Huybrechts, 1998). Importantly, this hormonal overruling of repression of yolk protein synthesis in males was confirmed in *Drosophila*, in which gender executor DSX-responsive elements have later been identified within the promoter region of two of the yolk polypeptide encoding genes (Burtis *et al.*, 1991). Thus, combined action of cell-autonomous and hormonal steering is possible. In male *Bombyx mori* pupae, both ovary transplantation and injection of the vertebrate female sex steroid 17-beta estradiol stimulate the male fat body to synthesize vitellogenin. However, the situation in (some) Diptera and in the silk moth is not representative for all insects. Indeed, in the cockroach *Diploptera punctata* and in orthopterans as locusts the hormone that can overrule the genetic sex-specific expression turned out to be Juvenile Hormone, the other morphogenic hormone of insects (Mundall *et al.*, 1979; Hult *et al.*, 2015; Wang *et al.*, 2017). In the next paragraphs, we will elaborate on the role of either hormone in regulating reproduction-related activities in insects.

Re-use of Existing Hormonal Pathways in Regulating Female Reproduction

The silverfish *Lepisma saccharina*, and firebrat *Termobia domestica*, both members of the phylogenetically old, wingless insects display throughout their extended lifespan continued cycles of ecdysis, punctuated with cycles of vitellogenesis and egg deposition. The co-regulation of both cyclic processes suggests a role for the two classic developmental hormones, Juvenile Hormone (JH) and ecdysteroids in regulating both molting and reproduction. Combined, quantification of hormone titers and surgical removal or inactivation of endocrine glands indicated that vitellogenin synthesis in the firebrat happens under a condition of elevated JH and lowered ecdysteroids titers. In silk moths, both vitellogenin synthesis, starting in the cocoon spinning larvae, and yolk deposition occur under conditions allowing pupal-adult development. These implicate a lowered Juvenile Hormone titer in the haemolymph and elevated ecdysteroid concentrations (Swevers and Iatrou, 2003; Postlethwait and Giorgi, 1985). Combined, the apterygote and silk moth examples indicate the need for a correct balance regarding the concentration of both metamorphic hormones.

Juvenile Hormone and 20-OH-Ecdysone Acting in Concert

In almost all insects, copulation, fertilization and egg laying are restricted to the adult life phase, starting after the final molt in hemimetaboles, or after eclosion of the adult in holometaboles. These adults indeed continue to produce JH and ecdysteroids, both hormones that in the pre-adult stages are so important to drive developmental growth and metamorphosis. The corpora allata (CA), as part of the retrocerebral complex, continue to be the major site for synthesis of Juvenile Hormone, which, in most species, is JH III. Gonads and male accessory glands can be additional sources of active JH synthesis in the adult (Paroulek and Sláma, 2014). Only recently, the Methoprene-tolerant gene, Met, has been identified in different insects as an intracellular Juvenile Hormone receptor (Charles *et al.*, 2011). JH binding to its Met-receptor induces heterodimerization with a known steroid co-activator, Taiman, and this complex in the nucleus interacts directly with JH-responsive genes such as the *Locusta* vitellogenin gene (Wang *et al.*, 2017). Whether Met represents the only functional JH receptor is not known. De Loof *et al.* (2014) suggested that a sarcoplasmic reticulum SERCA-calcium pump harbors a binding pocket for sesquiterpenoids like JH that would activate the pump. In contrast to the CA, in most insects, but not in all, the prothoracic gland, the best documented larval site of ecdysteroid synthesis degenerates in adults. Nevertheless, all adult insects possess the ecdysteroid synthesizing Halloween genes and effectively synthesize ecdysteroids in their ovaries, testes and male accessory glands (see review by Lenaerts *et al.* (2016)). Noteworthy, ecdysteroids are usually synthesized and released as ecdysone, which later on can be converted into 20-OH ecdysone by peripheral organs such as Malpighian tubules. Ecdysteroids bind to their cognate cytoplasmic receptor, which, following heterodimerization with a retinoic acid or RXR-type receptor as Ultraspiracle, translocate to the nucleus. In the nucleus the complex, now being a transcription factor, interacts with ERE or ecdysteroid responsive elements that are present in the promoter region of target genes. In addition, a GPCR-type dopamine-ecdysteroid receptor (DopECR) has been described which regulates the non-canonical action of 20E affecting behavior, such as courtship, via CAMP signaling and neural plasticity (Abrieux *et al.*, 2013).

The extensive bibliographic data on the hormonal regulation of reproduction in insects, summarized in excellent reviews, report on results obtained in only a few dozen species, whereas the number of described insect species, in the United States alone, reaches over 100,000 as compared to the estimated number of some 30 million worldwide. Not every model insects turned out to be suitable for testing the effect of both JH and ecdysteroids. In the majority of models, the priming role of Juvenile hormone in making both fat body and ovaries competent to respond to additional hormonal instructions is generally accepted. Regarding vitellogenin synthesis, a select group of insects, including many orthopterans, cockroaches, coleopterans and hymenopterans was believed to function under JH dominancy (Group 1 insects according to Postlethwait and Giorgi (1985), Tufail *et al.* (2014), Tufail and Takeda, (2009)). In some of those species, for example, in *Leucophaea maderae* either no role for ecdysteroids (Don-Wheeler and Engelmann,

1997) or alternatively a repressor role for ecdysteroids was reported (Weaver *et al.*, 1984). Only in a few exceptions, aside the main role of JH, a stimulating role for ecdysteroids was evidenced or suggested for members of this group. The action of Ovary Maturing Parsin (OMP) upon vitellogenin synthesis in *Locusta migratoria* which, at least partially, might involve ecdysteroids represents a good example (Girardie *et al.*, 1998). In contrast to this mainstream, other insects are categorized as performing under ecdysteroid dominance. In the latter group, the Hagedorn model (Hagedorn, 1983) dedicated a primary role to the ovaries as major source of ecdysteroids, which in turn instruct the fat body to synthesize and secrete vitellogenin for uptake by the developing egg follicles (Group 2 insects according to Postlethwait and Giorgi (1985)). As in higher Diptera, yolk polypeptide synthesis by the fat body, in response to ecdysteroid stimulation, can occur independently of the presence of the ovaries in insects of Group 3. They have an additional, non-ovarian, site of ecdysteroid synthesis (Postlethwait and Handler, 1978; Huybrechts and De Loof, 1981).

Molecular Tools Evidence the Cross Talk Between JH- and Ecdysteroid-Signaling Pathways for Regulating Reproduction in Insects

In recent years, thanks to ever improving possibilities offered by the “molecular toolbox”, the role of JH and ecdysteroids became better understood in a few species by studying the expression and manipulation of enzymes and proteins involved in their synthesis, receptor signaling or metabolism. Data were collected under different physiological conditions and many times validated by quantification of their downstream gene products. In the cockroach, *Diploptera punctata*, gene profiling of EcdR and RXR/USP evidenced high ecdysteroid receptor expression not only in the ovary, but as well in nervous tissue and in the corpora allata. The combined RNAi knockdown of RXR/USP and EcdR, starting the first day of adult female development, demonstrated that ecdysteroids in this species come into action only toward the end of the vitellogenic cycle. At this particular moment, ecdysteroids initiate chorion gene expression. By their direct signaling at the level of the CA, they are assumed to negatively regulate JH, which is now no longer needed for vitellogenin synthesis (Hult *et al.*, 2015). In the hemipteran firebug, *Pyrrhocoris apterus*, allatotectomy abolishes egg development under reproduction-promoting long-day conditions. RNAi knockdown of either Met or its binding factor Taiman (Tai) both blocked vitellogenin synthesis in females. However, RNAi of Tai applied to last larval instars, blocked adult ecdysis as expected, but failed to introduce adult characteristics. In contrast, last instar adultoids, resulting from a similar RNAi using dsMet RNA, do display adult characteristics. As Tai represents also a known co-activator of EcdR, the question emerges to what extent the ecdysteroid pathway is affected by, or is affecting the clear-cut JH response in this species (Smykal *et al.*, 2013). Also in the locust *Schistocerca gregaria*, combined knockdown of both ecdysteroid receptors and their RXR heterodimer resulted in some surviving L5 instars that, although being unable to complete the final adult molt, already started vitellogenin synthesis. These data point toward a self-regulating feedback loop. Indeed, in the knockdown males an increase in total ecdysteroid titer was measured although the 20-E titer was not elevated. The observed start of vitellogenin uptake in the knockdown females further suggests a feedback interaction between the ecdysteroid- and the JH pathway. JH is believed to be indispensable for yolk accumulation by the developing follicles in insects, but authors did not yet challenge this possibility (Lenaerts *et al.*, 2016). Direct cross-talk between 20E and JH most probably also occurs via reciprocal interaction at the level of the EcdR and Met co-activators (Jindra *et al.* (2013) and cited refs) or via transcriptional interaction at the level of their effector genes (Jones *et al.*, 2011).

Feeding-Regulated Reproduction: AAs-TOR and Insulin as Main Mediators

In order to guarantee the production and deposition of chorionated, yolky eggs correct feeding conditions are required. Starvation usually inhibits or abolishes the progression of vitellogenesis. Hitherto, nutritional regulation of reproduction occurs in all insects (see review by Smykal and Raikhel (2015)). First, correct sensing of the nutritional status is essential, because without the necessary resources no yolky eggs can be produced. This nutritional sensing is of outermost importance for anautogenous species as blood-sucking mosquitoes and flesh flies, which need a protein meal to initiate vitellogenin synthesis. Amino acids sensing (AAs), mediated via the serine/threonine kinase TOR pathway represents the start for nutritional signaling along a parallel Insulin nutritional sensing and signaling mechanism (Badisco *et al.*, 2013). The increase in amino acids following digestion activates TOR that in turn activates S6K, and that inactivates 4EBP, allowing the GATA transcription factor being translated for transcriptional activation of vitellogenin synthesis. The conserved Insulin-signaling pathway starts by binding of Insulin-like peptides (ILPs), released upon feeding, to a tyrosine kinase transmembrane insulin receptor. It ultimately results in phosphorylation of FOXO and its sequestration from the nucleus allowing vitellogenin synthesis. Crosstalk between the insulin and AAs-TOR pathway exists via its main effector Akt/protein kinase B, a known suppressor of the negative regulators of TOR. Both TOR and Insulin-like peptides further connect the nutritional status to JH synthesis, which in JH-dominant insects is needed for vitellogenin synthesis and in ecdysteroid dominant insects it assures reproductive competence. In the latter, the importance of insulin-like peptides (ILPs) in the regulation of ovarian ecdysteroidogenesis is well documented (reviewed by Nässel and Vanden Broeck (2016)). In mosquitoes, blood feeding triggers both the release of brain-derived ILPs and ovary ecdysteroidogenic hormone (OEH). OEH binds to an ovarian receptor tyrosine kinase, and activates in this way protein kinase B/Akt of the insulin pathway in an insulin receptor-independent manner. This explains why either OEH or ILP3, in the presence of essential amino acids, can independently stimulate synthesis and release of ecdysteroids from mosquito ovaries to instruct the fat body for vitellogenin synthesis. Noteworthy, in insects as *Drosophila* and flesh flies, the follicle cells themselves produce yolk polypeptides under direct control of insulin, and independently of juvenile hormone

and ecdysteroids as well. All these data evidence a plethora of transactivation factors that converge at the level of the vitellogenin gene(s), which harbor(s) their cognate response elements in its promoter region.

Digestion itself is hormonally controlled. In cockroaches CCAP peptide and short neuropeptide-F (SNPF) both originating from neuro-endocrine cells and intestinal paraneurons, antagonistically regulate digestive activity. SNPF is a down regulator and CCAP is an up regulator (Mikani *et al.*, 2015). Also adipokinetic hormones (AKH), peptides known to be primarily involved in energy homeostasis and to act primarily on the fat body (Lorenz and Gäde, 2009), stimulate digestion in the firefly, *Pyrrhocoris apterus*, the cockroach, *Periplaneta americana* and the fleshfly, *Sarcophaga crassipalpis* (Bodláková *et al.*, 2016; Bil *et al.*, 2014). In the fleshfly, AKH receptor profiling attributed a switching function to AKH as it might favor the use of amino acids generated by digestion for vitellogenin synthesis in fed animals (Bil *et al.*, 2016). Noteworthy, the AKH receptor belongs to the GnRH receptor family. Finally, in *Drosophila*, the intestinal expression of allatostatin-A arrests food intake and digestion. It brings the fly in a post feeding energy-saving modus, again allowing the preferential use of digestion-derived amino acids (Chen *et al.*, 2016).

Brain Derived Gonadotropins and Gonadotropin Release Regulators

In contrast to the situation in vertebrates, the insect literature is confusing regarding the question which hormones are genuine gonadotropins, gonadotropin-releasing hormones, and sex hormones (=the gonadal executors). Both JH and ecdysteroids have often been described as gonadotropic hormones, erroneously in my opinion. Ecdysteroids, in the adult being mainly hormones synthesized by the gonads, are executors rather than gonadotropins. Accordingly ecdysteroids might better be named gonadal- or sex hormones as suggested earlier. Prothoracicotropic Hormone (PTTH), the ecdysiotropin that is synthesized by the brain, and that stimulates ecdysone synthesis in larval prothoracic glands has also been found in adults. Via interaction with its cognate receptor Torso, PTTH regulates ecdysteroid synthesis in male accessory glands (De Loof *et al.*, 2015). In adult female mosquitoes and in adult *Locusta migratoria* as well, ovary ecdysteroidogenic hormone (OEH) and ovary maturing parsin (OMP) respectively are identified gonadotropic ecdysiotropins. In other insect species, no such gonadotropins have been identified. Yet, their presence has been experimentally suggested in the fleshfly *Sarcophaga crassipalpis* (Bil and Huybrechts, 2016). The recent identification of the OEH hormone receptor in *Aedes aegypti* (Vogel *et al.*, 2015) might enhance the chances for identifying other members of this gonadotropin family in other species. This could be done via reverse pharmacology approaches, if the degree of structural conservation is substantial. Compared to ecdysteroids, the role of JH in adults is more complex. On one hand, JH produced by the CA is needed for the development of the ovaries and the male accessory glands (MAGs); thus, it functions as a true gonadotropin. Indeed, in many species allatectomy results in ovarian and MAG degeneration. On the other hand, in many insects it is as essential as ecdysteroids for control of the expression of the vitellogenin gene(s). Its binding to the Met JH receptor, upon heterodimerization, directly targets the vitellogenin gene expression, as does the ligand-activated EcdR-USp complex in some other insects. Brain-derived allatotropin (AT) and allatostatin (AST) neuropeptides, via their cognate receptors regulate JH synthesis by the CA in some species. By doing so, they function as genuine gonadotropins (see review by Verlinden *et al.* (2015)). However, from the literature, and especially from the tissue distribution profile of their receptors it is clear that the role of both AT and ATS is mainly situated beyond the brain-CA- ovary/fat body axis. This is illustrated by the antagonistic action of both ATs and ASTs on muscle contraction in the oviducts. Of the three types of identified allatostatins (Coast and Schooley, 2011) allatostatins B, or MIP-type allatostatins, are known prothoracicostatic peptides, but their role in downregulating adult ecdysteroid production remains to be clarified. In addition, Sex Peptide (see below) shares evolutionary relationship with B-type allatostatins, which are believed to be the ancestral ligands of its receptor. Allotostatins C, or PISCF AST, are also functionally pleiotropic. They have receptors on the reproductive organs. In addition to these ecdysteroids- and JH-regulating neural factors, numerous other neuropeptides exemplified by Nalasin, SIFamide and Corazonin affect reproduction and copulation-related behavior (Schoofs *et al.*, 2017). Finally, in the context of gonadotropins and anti-gonadotropins also the role of oostatic peptides produced by the ovaries themselves such as Ae-TMOF and Neb-TMOF downplay the synthesis of vitellogenins in the fat body. They act via translational inhibition of trypsin-type digestive enzymes (Hlaváček, 2013).

Mating Enables Males to Affect the Endocrine Regulation of the Female Reproduction Process

With the exception of facultative- or obligate parthenogenetic females, virgin females of most species, although completing oogenesis and vitellogenesis in some follicles, usually deposit a lesser number of eggs than mated ones. Their egg laying occurs less organized as well. Apart from the behavioral impact of mating, in many species the males transfer potent endocrine regulators to the females. The male accessory glands (MAGs) of *Anopheles gambiae* turned out to be major sites of 20E synthesis. Moreover, in this malaria-vectoring mosquito 20E is transferred during mating to the female in which this allohormone is suggested to prime vitellogenesis the same way as the endogenous female sex steroid 20E does (Pondeville *et al.*, 2008). Such mating-derived priming, if widely occurring, would explain the earlier mentioned initiation of yolk polypeptide synthesis in ovariectomized female flies. The *Anopheles* scenario may be an exception. Indeed, in most other dipterans adult males hardly produce any ecdysteroids except for the local synthesis in the testes were they even install local YP synthesis (Majewska *et al.*, 2014). The 20E titer is low in male insects, at least when compared to the values in females. A second hormone that may be transferred into females is JH. In some species, for example, in *H. cecropia*, MAGs synthesize and store substantial amounts of Juvenile hormone. Apart from these examples, it is

generally accepted that specific proteinaceous ejaculate components fulfill endocrine functions. This is important for assuring male evolutionary fitness. This can be achieved by, for example, reducing female receptiveness to other males, or by installing a copulation plug, or by directly stimulating oogenesis and egg deposition. In *Drosophila*, Sex peptide, a MAGs-derived peptide directly influences the neuroendocrine regulatory pathway in the female; it elicits both physiological and behavioral changes (Liu and Kubli, 2003). The seminal protein Ovulin mediates ovulation by interacting with the female octopamine-signaling pathway (Rubinstein and Wolfner, 2013). So far, both MAG-derived peptides, Sex peptide and Ovulin, have no proven orthologs in species outside the *Drosophila* genus, although several Mag components exert similar functions in most studied species.

Heterosexual and Homosexual Courtship Behavior in Males

Following stimulation by female sex pheromones, male flies start typical courtship behavior including chasing, singing and mounting. Accordingly, as does *dsx*, the *fruitless* gene and its sex-specific spliced transcript are considered executors of sexual differentiation in somatic cells, including behavior-regulating neurons. *Fruitless* gene mutant males obviously changed sexual preference as they started chaining with male flies when reared in co-habitation. Accordingly, changed sexual preference became a hallmark of *fruitless* mutants, as it was believed to install a male-directed neural circuitry in neurons expressing the mutant *fruitless* gene. In their recent review, Yamamoto and Kohatsu (2016) question this paradigm as they report that sexual preference in the *fruitless* mutants seems to be plastic. It has rather to be seen as an activity-induced or experience-dependent trait, installed by the experimental male group rearing. Indeed, male *fruitless* mutants that during the experiment have only contact with females will display some degree of chaining with their female partners, even if they do not belong to the same species. Complementing the experiments in fruitflies, which suggest an epigenetic interaction of *Fruitless* in the brain, in locusts, *fruitless* knockdown by RNAi indicates a direct involvement in male copulation behavior and fertility, the latter possibly due to an altered transfer of seminal fluid (Boerjan et al., 2011, 2012).

Endogenous Encoded miRNA, an Additional Level of Regulation

In conclusion, this summary on the combined cell-specific and hormonal regulation of reproduction in insects focuses more upon the role of hormones in females than in males. Fig. 2 summarizes the main actors in this process. It can be expected

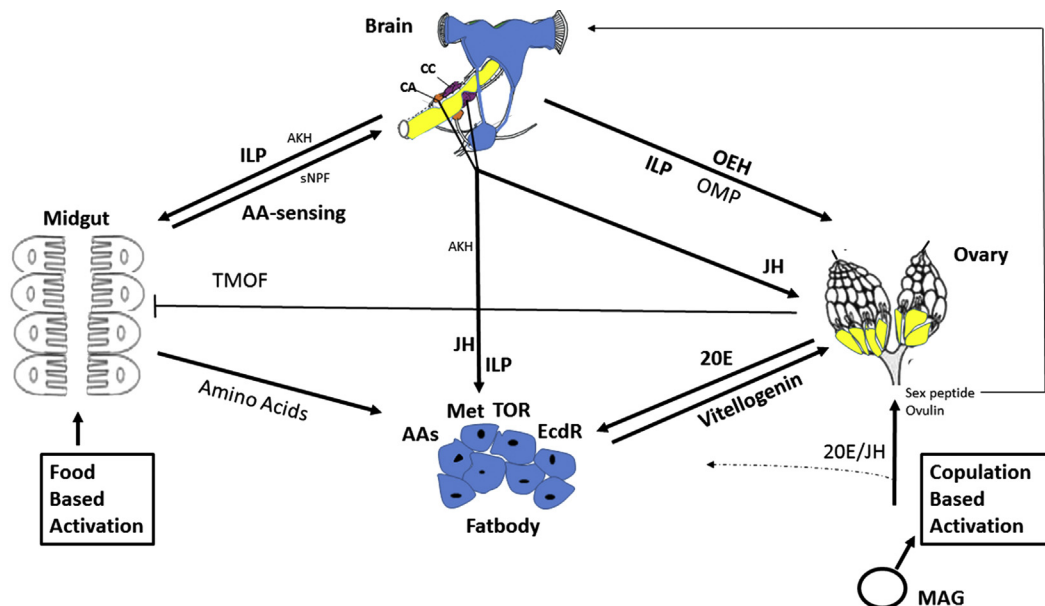


Fig. 2 Both feeding and copulation are important regulators of reproduction in insects. Insulin signaling, amino acid sensing and copulation derived factors, as Sex Peptide, communicate the energetic or mating status to the brain and initiate the reproduction process in the female. Brain derived gonadotrophins, mainly ecdysteroidogenic in nature instruct the ovaries. Allatotrophins and allatostatins control CA activity. In a species dependent fashion, Corpora allata derived JH or ovarian ecdysteroids in concert and mainly antagonistically regulate vitellogenin synthesis by the fat body. Within fat body cells multitudes of signals converge and turn the regulation of vitellogenin gene functioning into a complex process. 20E, 20 hydroxyecdysone; AAs, amino acids sensing; AKH, adipokinetic hormone; CA, corpora allata; CC, corpora cardiaca; EcdR, ecdysteroid receptor; ILP, insulin like peptide; JH, juvenile hormone; Met, methoprene tolerant JH receptor; OEH, ovary ecdysteroidogenic hormone; OMP, Ovary maturing parsin; sNPF, short neuropeptide F; TOR, target of rapamycin.

that in the near future small RNAs, which are encoded in the genome, will manifest themselves as an additional level of regulation of reproduction. Apparently, such small RNAs act via their intrinsic posttranscriptional gene-knockdown capacities (Wynant *et al.*, 2015).

References

- Abrieux, A., Debernard, S., Maria, A., *et al.* (2013). Involvement of the G-protein-coupled dopamine/ecdyseroid receptor DopEcR in the behavioral response to sex pheromone in an insect. *PLOS ONE*, 8, e72785.
- Badisco, L., Van Wielendaele, P., & Vanden Broeck, J. (2013). Eat to reproduce: A key role for the insulin signaling pathway in adult insects. *Frontiers in Physiology*, 4, 202.
- Bil, M., Broeckx, V., Landuyt, B., & Huybrechts, R. (2014). Differential peptidomics highlights adipokinetic hormone as key player in regulating digestion in anautogenous flesh fly, *Sarcophaga crassipalpis*. *General and Comparative Endocrinology*, 208, 49–56 [Erratum in: *Gen Comp Endocrinol.* 2015; 223:148–149].
- Bil, M., & Huybrechts, R. (2016). Pharmacological regulation of digestion in the anautogenous flesh fly, *Sarcophaga crassipalpis*, by simple injection of 6-hydroxydopamine. *Archives of Insect Biochemistry and Physiology*, 91, 137–151.
- Bil, M., Timmermans, I., Verlinden, H., & Huybrechts, R. (2016). Characterization of the adipokinetic hormone receptor of the anautogenous flesh fly, *Sarcophaga crassipalpis*. *Journal of Insect Physiology*, 89, 52–59.
- BodlÁková, K., Jedlička, P., & Kodrík, D. (2016). Adipokinetic hormones control amylase activity in the cockroach (*Periplaneta americana*) gut. *Insect Science*. <https://doi.org/10.1111/1744-7917.12314>.
- Boerjan, B., Tobback, J., De Loof, A., Schoofs, L., & Huybrechts, R. (2011). Fruitless RNAi knockdown in males interferes with copulation success in *Schistocerca gregaria*. *Insect Biochemistry and Molecular Biology*, 41, 340–347.
- Boerjan, B., Tobback, J., Vandersmissen, H. P., Huybrechts, R., & Schoofs, L. (2012). Fruitless RNAi knockdown in the desert locust, *Schistocerca gregaria*, influences male fertility. *Journal of Insect Physiology*, 58, 265–269.
- Burtis, K. C., Coschigano, K. T., Baker, B. S., & Wensink, P. C. (1991). The doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. *EMBO Journal*, 10, 2577–2582.
- Charles, J. P., Iwema, T., Epa, V. C., *et al.* (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proceedings of the National Academy of Sciences of the United States of America*, 108, 21128–21133.
- Chen, J., Reiher, W., Hermann-Luibl, C., *et al.* (2016). Correction: Allatostatin A signalling in *Drosophila* regulates feeding and sleep and is modulated by PDF. *PLOS Genetics*, 12, e1006492.
- Clinton, M., Zhao, D., Nandi, S., & McBride, D. (2012). Evidence for avian cell autonomous sex identity (CAS) and implications for the sex-determination process? *Chromosome Research*, 20, 177–190 [Review].
- Coast, G. M., & Schooley, D. A. (2011). Toward a consensus nomenclature for insect neuropeptides and peptide hormones. *Peptides*, 32, 620–631.
- De Loof, A., De Haes, W., Janssen, T., & Schoofs, L. (2014). The essence of insect metamorphosis and aging: Electrical rewiring of cells driven by the principles of juvenile hormone-dependent Ca(2+)-homeostasis. *General and Comparative Endocrinology*, 199, 70–85 [Review].
- De Loof, A., & Huybrechts, R. (1998). Insects do not have sex hormones: A myth? *General and Comparative Endocrinology*, 111, 245–260 [Review].
- De Loof, A., Schoofs, L., & Huybrechts, R. (2016). The endocrine system controlling sexual reproduction in animals: Part of the evolutionary ancient but well conserved immune system? *General and Comparative Endocrinology*, 226, 56–71.
- De Loof, A., Vandersmissen, T., Marchal, E., & Schoofs, L. (2015). Initiation of metamorphosis and control of ecdysteroid biosynthesis in insects: The interplay of absence of Juvenile hormone, PTTH, and Ca(2+)-homeostasis. *Peptides*, 68, 120–129.
- Don-Wheeler, G., & Engelmann, F. (1997). The biosynthesis and processing of vitellogenin in the fat bodies of females and males of the cockroach *Leucophaea maderae*. *Insect Biochemistry and Molecular Biology*, 27, 901–918.
- Girardie, J., Geoffre, S., Delbecq, J., & Girardie, A. (1998). Arguments for two distinct gonadotropic activities triggered by different domains of the ovary maturing parsin of *Locusta migratoria*. *Journal of Insect Physiology*, 44, 1063–1071.
- Hagedorn, H. H. (1983). The role of ecdysteroids in the adult insect. *Invertebrate Endocrinology*, 1, 271–304.
- Hlaváček, J. (2013). Oostatic peptides. *Amino Acids*, 44, 1095–1105.
- Hult, E. F., Huang, J., Marchal, E., Lam, J., & Tobe, S. S. (2015). RXR/USP and EcR are critical for the regulation of reproduction and the control of JH biosynthesis in *Diptera punctata*. *Journal of Insect Physiology*, 80, 48–60.
- Huybrechts, R., & De Loof, A. (1977). Induction of vitellogenin synthesis in male *Sarcophaga bullata*. *Journal of Insect Physiology*, 23, 1359–1362.
- Huybrechts, R., & De Loof, A. (1981). Effect of ecdysterone on vitellogenin concentration in hemolymph of male and female *Sarcophaga bullata*. *International Journal of Invertebrate Reproduction*, 3, 157–168.
- Jindra, M., Palli, S. R., & Riddiford, L. M. (2013). The juvenile hormone signaling pathway in insect development. *Annual Review of Entomology*, 58, 181–204.
- Jones, G., Jones, D., Fang, F., *et al.* (2011). Juvenile hormone action through a defined enhancer motif to modulate ecdysteroid-activation of natural core promoters. *Comparative Biochemistry and Physiology, B, Biochemistry and Molecular Biology*, 161, 219–225.
- Krüger, E., Mena, W., Lahr, E. C., Johnson, E. C., & Ewer, J. (2015). Genetic analysis of eclosion hormone action during *Drosophila* larval ecdysis. *Development*, 142, 4279–4287.
- Lenaerts, C., Van Wielendaele, P., Peeters, P., Vanden Broeck, J., & Marchal, E. (2016). Ecdysteroid signalling components in metamorphosis and development of the desert locust, *Schistocerca gregaria*. *Insect Biochemistry and Molecular Biology*, 75, 10–23.
- Liu, H., & Kubli, E. (2003). Sex-peptide is the molecular basis of the sperm effect in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 9929–9933.
- Lorenz, M. W., & Gäde, G. (2009). Hormonal regulation of energy metabolism in insects as a driving force for performance. *Integrated Comparative Biology*, 49, 380–392.
- Majewska, M. M., Suszczynska, A., Kotwica-Rolinska, J., *et al.* (2014). Yolk proteins in the male reproductive system of the fruit fly *Drosophila melanogaster*: Spatial and temporal patterns of expression. *Insect Biochemistry and Molecular Biology*, 47, 23–35.
- Marquardt, W. C. (2005). *Biology of Disease Vectors*. Burlington, MA: Elsevier Academic press.
- Mikani, A., Watari, Y., & Takeda, M. (2015). Brain-midgut cross-talk and autocrine metabolat via the sNPF/CCAP negative feed-back loop in the American cockroach, *Periplaneta americana*. *Cell and Tissue Research*, 362, 481–496.
- Mundall, E. C., Tobe, S. S., & Stay, B. (1979). Induction of vitellogenin and growth of implanted oocytes in male cockroaches. *Nature*, 282, 97–98.
- Nässel, D. R., & Vanden Broeck, J. (2016). Insulin/IGF signaling in *Drosophila* and other insects: Factors that regulate production, release and post-release action of the insulin-like peptides. *Cellular and Molecular Life Sciences*, 73, 271–290 [Review].
- Parisi, M. J., Gupta, V., Sturgill, D., *et al.* (2010). Germline-dependent gene expression in distant non-gonadal somatic tissues of *Drosophila*. *BMC Genomics*, 11, 11:346.
- Paroulek, M., & Sláma, K. (2014). Production of the sesquiterpenoid juvenile hormone (JH-I), and of vitamin E in the accessory sexual (colleterial) glands of adult male moths, *Hyalophoracacropia* (Linnaeus, 1758), (Lepidoptera: saturniidae). *Life: The Excitements of Biology*, 2, 102–124.
- Pondeville, E., Maria, A., Jacques, J. C., Bourgouin, C., & Dauphin-Villemant, C. (2008). Anopheles gambiae males produce and transfer the vitellogenic steroid hormone 20-hydroxyecdysone to females during mating. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 19631–19636.

- Postlethwait, J. H., & Giorgi, F. (1985). Vitellogenesis in Insects. In L. W. Browder (Ed.), *Developmental Biology. A Comprehensive Synthesis* (pp. 85–126). New York: Plenum Press.
- Postlethwait, J. H., & Handler, A. M. (1978). Non-vitellogenic female sterile mutants and the regulation of vitellogenesis in *Drosophila melanogaster*. *Developmental Biology*, 67, 202–213.
- Rubinstein, C. D., & Wolfner, M. F. (2013). *Drosophila* seminal protein ovulin mediates ovulation through female octopamine neuronal signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 17420–17425.
- Schoofs, L., De Loof, A., & Van Hiel, M. B. (2017). Neuropeptides as regulators of behavior in insects. *Annual Review of Entomology*, 62, 35–52.
- Smykal, V., Bajgar, A., Provaznik, J., et al. (2013). Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. *Insect Biochemistry and Molecular Biology*, 45, 69–76.
- Smykal, V., & Raikhel, A. S. (2015). Nutritional control of insect reproduction. *Current Opinions in Insect Sciences*, 11, 31–38.
- Swevers, L., & Iatrou, K. (2003). The ecdysone regulatory cascade and ovarian development in lepidopteran insects: Insights from the silkworm paradigm. *Insect Biochemistry and Molecular Biology*, 33, 1285–1297 [Review].
- Tufail, M., Nagaba, Y., Elgendy, A. M., & Takeda, M. (2014). Regulation of vitellogenin genes in insects. *Entomology Science*, 17, 269–282.
- Tufail, M., & Takeda, M. (2009). Insect vitellogenin/ lipophorin receptors: Molecular structures, role in oogenesis, and regulatory mechanisms. *Journal of Insect Physiology*, 55, 87–103.
- Verlinden, H., Gijbels, M., Lismont, E., et al. (2015). The pleiotropic allatoregulatory neuropeptides and their receptors: A mini-review. *Journal of Insect Physiology*, 80, 2–14 [Review].
- Vogel, K. J., Brown, M. R., & Strand, M. R. (2015). Ovary ecdysteroidogenic hormone requires a receptor tyrosine kinase to activate egg formation in the mosquito *Aedes aegypti*. *Proceedings of the National Academy of Sciences of the United States of America*, 112, 5057–5062.
- Wang, Z., Yang, L., Song, J., Kang, L., & Zhou, S. (2017). An isoform of Taiman that contains a PRD-repeat motif is indispensable for transducing the vitellogenic juvenile hormone signal in *Locusta migratoria*. *Insect Biochemistry and Molecular Biology*, 82, 31–40.
- Weaver, R. J., Strambi, A., & Strambi, C. (1984). The significance of free ecdysteroids in the haemolymph of adult cockroaches. *Journal of Insect Physiology*, 30, 705–711.
- Wynant, N., Santos, D., Subramanyam, S. H., Verlinden, H., & Vanden Broeck, J. (2015). Drosha, Dicer-1 and Argonaute-1 in the desert locust: Phylogenetic analyses, transcript profiling and regulation during phase transition and feeding. *Journal of Insect Physiology*, 75, 20–29.
- Yamamoto, D., & Kohatsu, S. (2016). What does the fruitless gene tell us about nature vs. nurture in the sex life of *Drosophila*? *Fly (Austin)*, 23, 1–9.
- Zhu, J., Miura, K., Chen, L., & Raikhel, A. S. (2000). AHR38, a homolog of NGFI-B, inhibits formation of the functional ecdysteroid receptor in the mosquito *Aedes aegypti*. *EMBO Journal*, 19, 253–262.

Endocrine Control of Reproduction, Crustaceans and Molluscs

Makoto Osada, Tohoku University, Sendai, Japan

© 2018 Elsevier Inc. All rights reserved.

Endocrine Control of Crustacean Reproduction

In oviparous animal except for mammals, vitellogenesis as a process of yolk formation is a crucial event in oogenesis, which is essential to normally advance embryonic and larval development. Crustaceans repeat molting to grow and their vitellogenesis undergoes between successive molt cycle. The repressive involvement of eyestalk in ovarian development of decapod crustaceans was demonstrated by eyestalk ablation first, which resulted in induction of ovarian maturation in the grass shrimp, *Leander serranus* (Panouse, 1943). Neurosecretory cells from the X-organ extend the axonal ends into the sinus gland and the X-organ-sinus gland (XOSG) is present in the eyestalk. The eyestalk ablation accelerates molting and maturation of ovary associated with secondary vitellogenesis, suggesting occurrence of hormones of molt and vitellogenesis inhibition in the XOSG distributed in eyestalk. Vitellogenesis-inhibiting hormone (VIH) was isolated from the XOSG and its primary structure was determined in American lobster, *Homarus americanus* (Soyez *et al.*, 1991). The VIH consisted of two isoforms and only one isoform showed the inhibiting effect on vitellogenesis. Around the same time, a family of multifunctional peptides was found from the XOSG in Mediterranean green crab, *Carcinus maenas*, which is called crustacean hyperglycemic hormone (CHH) peptides family (Keller, 1992). Sequence of CHH peptide was highly conserved among Crustacea and characterized by conservation of common structure to molt-inhibiting hormone (MIH) and VIH in the eyestalk, which are included in the same peptides family. The CHH regulated carbohydrate metabolism and somewhat inhibit vitellogenesis. MIH predominantly inhibited molting in the Mediterranean green crab, but MIH of blue crab, *Callinectes sapidus*, promoted vitellogenesis retaining molt-inhibiting activity (Zmora *et al.*, 2009). Mandibular organ-inhibiting hormone (MOIH) was also identified as a member of the CHH peptides family in the eyestalk, which has the inhibitory effect on vitellogenesis (Webster *et al.*, 2012). The VIH, CHH and MIH peptides regulate vitellogenesis directly from the XOSG, while the inhibitory function of MIH and MOIH on molting and vitellogenesis, respectively, are mediated through other organs. MIH inhibits synthesis of 20-hydroxyecdysone (20E) in the Y-organ and the 20E secreted from the Y-organ promotes both molting and vitellogenesis (Chang and Mykles, 2011). MOIH represses synthesis of farnesoic acid (FA) and methyl farnesoate (MF) in the mandibular organ and the FA and MF accelerate vitellogenesis associated with 20E (Mak *et al.*, 2005; Wainwright *et al.*, 1996; Fig. 1).

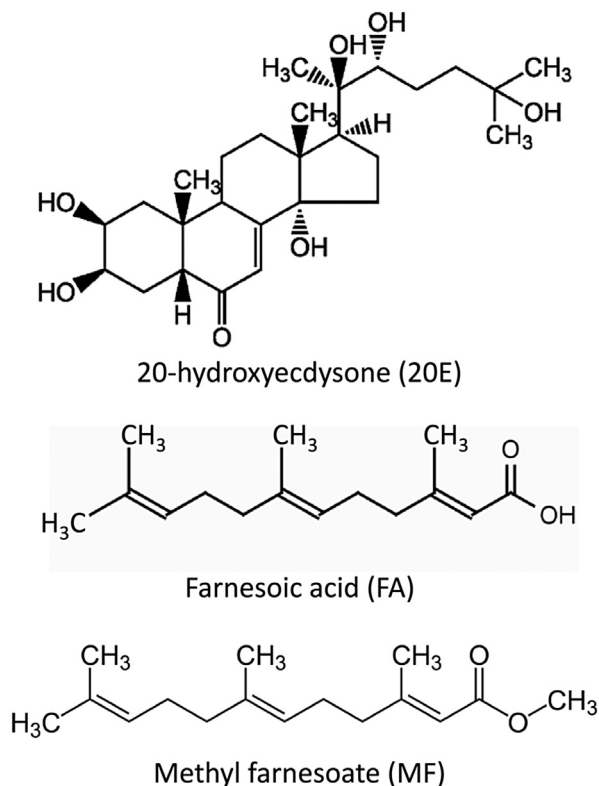


Fig. 1 Structural formula of 20-hydroxyecdysone (20E), farnesoic acid (FA) and methyl farnesoate (MF).

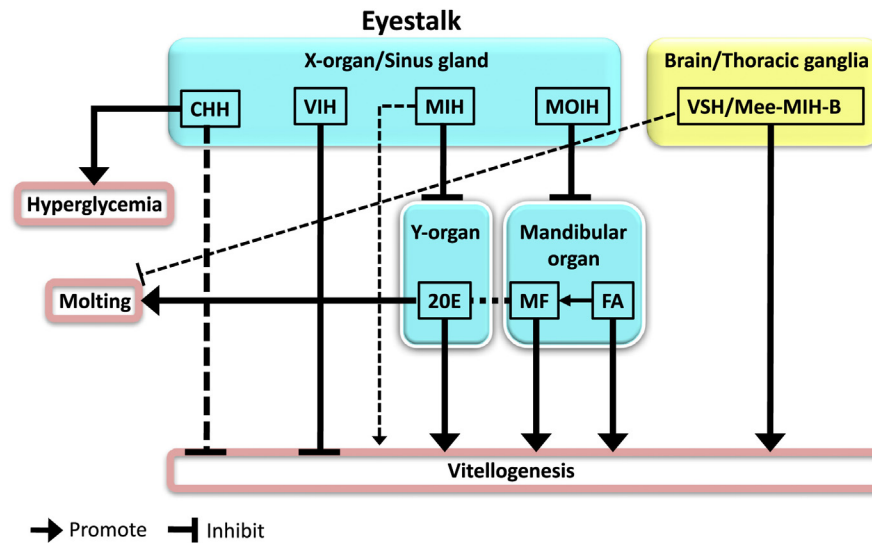


Fig. 2 A schematic diagram of hormonal control in crustacean vitellogenesis. CHH, crustacean hyperglycemic hormone; VIH, vitellogenesis-inhibiting hormone; MIH, molt-inhibiting hormone; MOIH, mandibular organ-inhibiting hormone; VSH, vitellogenesis-stimulating hormone; 20E, 20-hydroxyecdysone; MF, methyl farnesoate; FA, farnesoic acid.

The neurosecretory cells of brain and thoracic ganglia is known to secrete vitellogenesis-stimulating hormone (VSH) to promote ovarian maturation although the chemical nature is not adequately understood and its gonadotrophic function is only recognized by removal and implantation experiments (Yano, 1998). The vitellogenesis-stimulating neuropeptide, Mee-MIH-B, was found in the brain and thoracic ganglia in the shrimp, *Metapenaeus ensis*, and the Mee-MIH-B directly induced vitellogenin gene expression and increased vitellogenin protein in hemolymph retaining low molt-inhibiting activity (Tiu and Chan, 2007). Taken together, female maturation in crustaceans is under the control of the XOSG system directly or indirectly through Y-organ and mandibular organ, and also regulated by the brain and thoracic ganglia (Fig. 2). These peptides originated from the XOSG appeared to regulate spermatogenesis in decapods crustaceans like a function on female reproduction, but it is very little known (see Subramoniam (2017)).

Endocrine Control of Molluscan Reproduction

Bivalves

The mitotic division of gonial cells is a process to multiply germ cell at the early stage of gametogenesis in any kind of oviparous animals. Spermatogonia in bivalves is proposed to be classified into a spermatogonial stem cell and a series of development of differentiated spermatogonia during early spermatogenesis on the basis of proliferation potency of spermatogonia (Osada *et al.*, 2007). The central nervous system of bivalves generally consists of three ganglia; cerebral, pedal and visceral ganglia (Fig. 3). Mathieu *et al.* (1988) found occurrence of a neural factor named “gonial mitosis-stimulating factor” (GMSF) from the cerebral ganglion of the mussel *Mytilus edulis* first, which stimulated [³H] thymidine incorporation into dissociated germ cell suspensions of the mussel mantle tissue. Gonadotropin releasing hormone (GnRH) like factor found in the cerebral and pedal ganglia of scallop *Patinopecten yessoensis* stimulated mitosis of spermatogonia through receptor mechanism under a neuroendocrine control (Nakamura *et al.*, 2007). The GnRH superfamily, which includes GnRH, adipokinetic hormone (AKH), corazonin (Crz), and AKH/Crz-related peptides (ACP), is almost ubiquitous throughout the bilateral animals including molluscs (Roch *et al.*, 2011). Vertebrate GnRHs could also affect mitosis of germ cells in the mussel and the Pacific oyster and the GnRH-like neurons were identified in the central nervous system (CNS). And then two GnRH-like peptides were confirmed by mass spectrometry from the CNS in Pacific oyster and Japanese scallop, pQNYHFNSNGWQP-NH₂ (Cg-GnRH-a; amidated undecapeptide) and pQNYHFNSNGWQPG (Cg-GnRH-G; non-amidated doecapeptide) in Pacific oyster, and pQNFHYSNGWQP-NH₂ (py-GnRH11AA-NH₂) and pQNFHYSNGWQPG-OH (py-GnRH12AA-OH) in Japanese scallop (Bigot *et al.*, 2012; Nagasawa *et al.*, 2015a). The extra dipeptide insertion after N-terminal pyro-glutamate residue is recognized in both bivalve species in common with other molluscan species and characterized to be definitely different from vertebrate GnRHs. And then in vitro administration of the synthesized py-GnRH11AA-NH₂ induced spermatogonial proliferation in testis tissue of scallop mediated through estrogen whose synthesis could be induced by py-GnRH11AA-NH₂ (Osada and Treen, 2013). Interestingly, the synthetic py-GnRH11AA-NH₂ accelerated spermatogenesis associated with a significant increase in testis mass and conversely inhibited oocyte development associated with an apoptosis of oocyte and newly development of male germ cell, implying early phenotypic alteration to masculinization and GnRH involvement in sex differentiation in bivalve molluscs (Nagasawa *et al.*, 2015b).

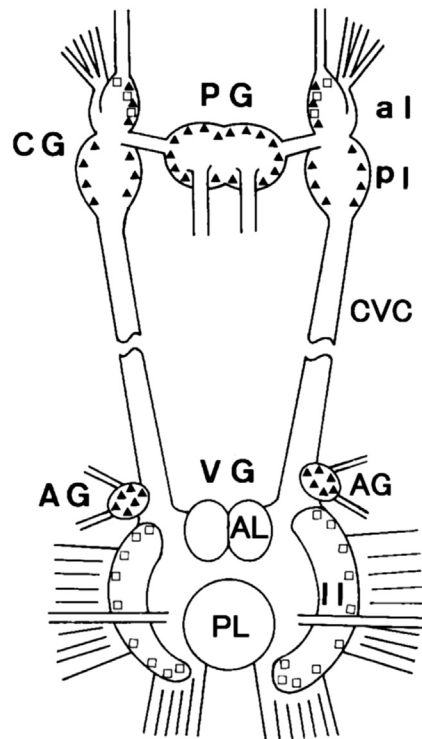


Fig. 3 Schematic drawing of the distribution of the monoamine perikarya in the central nervous system of *Patinopecten yessoensis* (Ventral view). AG, accessory ganglia; AL/al, anterior lobe; CG, cerebral ganglia; CVC, cerebrovisceral connective; II, lateral lobe; PG, pedal ganglia; PL/pl, posterior lobe; VG, visceral ganglia; ▲, serotonin perikarya; □, catecholamine perikarya. Modified from Matsutani, T., Nomura, T., 1986. Serotonin-like immunoreactivity in the central nervous system and gonad of the scallop, *Patinopecten yessoensis*. *Cell Tissue Res.*, 244, 515-517.

Steroid production and function on mollusc reproduction is well examined using vertebrate steroids; quantification of steroids by immunological method and HPLC, etc., identification of putative sites of steroid production, and induction of sex reversal and oogenesis have been demonstrated (see Lafont and Mathieu (2007)). In the developing or growing stage of the ovary, the oocyte rapidly accumulates vitellin (Vn) as a major yolk protein in oocytes. Vitellogenin (Vtg), precursor protein of Vn, is synthesized in and provided from the follicle like cells to the oocytes in bivalves (Matsumoto *et al.*, 2003; Osada *et al.*, 2004). The Vn accumulation into oocyte of scallop and oyster was experimentally induced with vertebrate estradiol-17 β (E₂) and a neural factor named "vitellogenesis promoting factor (VPF)" in vitro (Osada *et al.*, 2003). The estrogen signal is generally transduced into cells by estrogen receptor (ER), a member of the nuclear receptor (NR) superfamily, in vertebrates to regulate hormone-dependent transcription of target genes, such as Vtg. The bivalve ER ortholog was isolated in Pacific oyster and mussel and dominantly expressed in the gonad (Matsumoto *et al.*, 2007; Nagasawa *et al.*, 2015c). A significant increase in Vtg mRNA expression was observed in mussels exposed with vertebrate E₂ during the early stage of gametogenesis (Ciocan *et al.*, 2010), implying mediation of ER, although the question of what roles mollusc ERs have remains unanswered.

Spawning in bivalve mollusc is suggested to correlate with an environmental fluctuation, e.g. temperature, lunar age, illumination, salinity, abundance of phytoplankton, food availability, physical shock, tidal surge, drying and radical oxygen as a stimulus. These environmental cues are received by the CNS and converted into release of serotonin (5-hydroxytryptamine, 5-HT) of neurotransmitter as a trigger for spawning from the CNS. The primary role of 5-HT on spawning is an induction of meiosis reinitiation of prophase-arrested oocytes, oocyte maturation, as evidences by germinal vesicle breakdown (GVBD), and initiation of sperm motility (Tanabe *et al.*, 2006; Alavi *et al.*, 2014). The 5-HT signal transduction into oocyte and sperm is mediated through 5-HT receptor on the membrane with Ca²⁺ influx for oocyte, K⁺ efflux and Ca²⁺ & Na⁺ influx for sperm (Tanabe *et al.*, 2010; Alavi *et al.*, 2014). The 5-HT function is modulated with prostaglandin F₂ α (PGF₂ α) and PGE₂, which play a role as a suppressive and acceleratory modulators of 5-HT-induced egg release, respectively (Matsutani and Nomura, 1987). Vertebrate steroids, estrogen and testosterone, which are detected in several bivalves, led to rising sensitivity to 5-HT involved in spawning (Osada *et al.*, 1992; Wang and Croll, 2006). Peptidic or proteinous modulators for 5-HT-induced oocyte maturation and spawning were also found in bivalves. *Spisula* factor and oocyte membrane component originating from oocyte inhibited 5-HT function in the Atlantic surf clam *Spisula solidissima*, and a novel neural factor to arrest 5-HT-induced oocyte maturation, named "oocyte maturation arresting factor," OMAF was found in the CNS of the scallop (Yuan *et al.*, 2012). Intriguingly, neutralization of endogenous OMAF with antibody strongly amplified the 5-HT-induced release of egg and sperm. Taken all together, 5-HT is an essential neurohormone for crucial

events of oocyte maturation and sperm motility and following spawning of bivalves. The mode of action of 5-HT on activation of germ cell and gonoduct is regulated by OMAF, steroids and PGs.

Gastropods

The neuroendocrine control of egg-laying has been studied convergently in two kinds of gastropods, sea hare *Aplysia californica* and pond snail *Lymnaea stagnalis*. Egg-laying of these species involves in a series of reproductive process, ovulation, packaging the fertilized eggs into the egg string/egg mass and oviposition like in other gastropods (Mukai and Morishita, 2016).

The *Aplysia* CNS consists of pair of five ganglia, buccal, cerebral, pleural, pedal and abdominal ganglia as shown in Fig. 4. The bag cell neurons consisting of around 400 cells, in which an egg-laying stimulating function was found, are located adjacent the abdominal ganglion (Fig. 4). In fact injection of a homogenate of the bag cells into mature *Aplysia* induced egg-laying (Kupfermann, 1970). Other egg-laying stimulatory factors were identified in the atrial gland of an accessory sex organ as an exocrine organ which is located on the reproductive tract, and secreted from the atrial gland into the freshly deposited egg strings of *Aplysia*, which promoted egg-laying in conspecifics as a pheromone (Nagle *et al.*, 1985; Rothman *et al.*, 1986). The neuropeptide, egg-laying hormone (ELH), is secreted by depolarization of the bag cells in a Ca^{2+} -dependent manner. The ELH preprohormone with 242 amino acid residues was defined, which is cleaved to generate mature ELH, α , β , γ , δ -BCPs (bag cell peptides), califin (small) and acidic peptide (Table 1). The ELH is thought to be released from the bag cell in the space between the surface of the abdominal gland and the overlying connective tissue sheath and delivered to the abdominal ganglion to regulate the neural activities for egg-laying behavior. And then the ELH directly induced the release of oocytes from the isolated piece of ovotestis, implying a direct action on a series of reproductive process to deposit egg strings. The auto-excitatory BCPs originated from the bag cells induce depolarization of the bag cell membrane itself binding to the autoreceptors of the bag cell, which may involve in secretion of ELH from the bag cells. Other two peptides, califin and peptide A, identified in the atrial gland have an *in vivo* effect on induction of egg-laying, whereas the atrial gland is exocrine organ to secrete both califin and peptide A into the reproductive tract where egg strings pass, not endocrine organ to be able to directly affect the ovotestis (Table 1). ELH, BCPs and other peptides from the bag cell and atrial gland govern discharges of egg strings in *Aplysia*, but not gonadal maturation.

The structure of *Lymnaea* CNS is well studied as well as *Aplysia* CNS. The caudodorsal cells (CDCs) consisting of 50–100 cells are distributed on the dorsal side of pair of cerebral ganglia in *Lymnaea* and are functionally equivalent neurosecretory cells to the bag cell in *Aplysia* (Fig. 5). It was first evidence of occurrence of egg-laying activity in CDCs that ablation of the CDCs in adult snail arrested egg-laying while injection of crude extract of CDCs revived egg-laying process (Geraerts, 1976). The 259-amino acid-long preprohormone identified in the *Lymnaea* CDCs includes caudodorsal cell hormone (CDCH-I), α , β_1 , β_2 , β_3 -CDCPs (caudodorsal cell peptide) and calfluxin (Vreugdenhil *et al.*, 1988). The CDCH-I with 36 amino acid residues plays a key role in ovulation and egg laying behavior. Subsequently another CDCH (CDCH-II) with 44 amino acid residues encoded by different gene from CDCH-I was identified (Li *et al.*, 1992; Table 1). CDCPs show similar auto-excitation of CDCs to the bag cells in *Aplysia* which respond to BCPs via autoreceptor. Neuropeptides from CDCs, CDCH and CDCPs etc, may involve in coordinating from ovulation through oviposition.

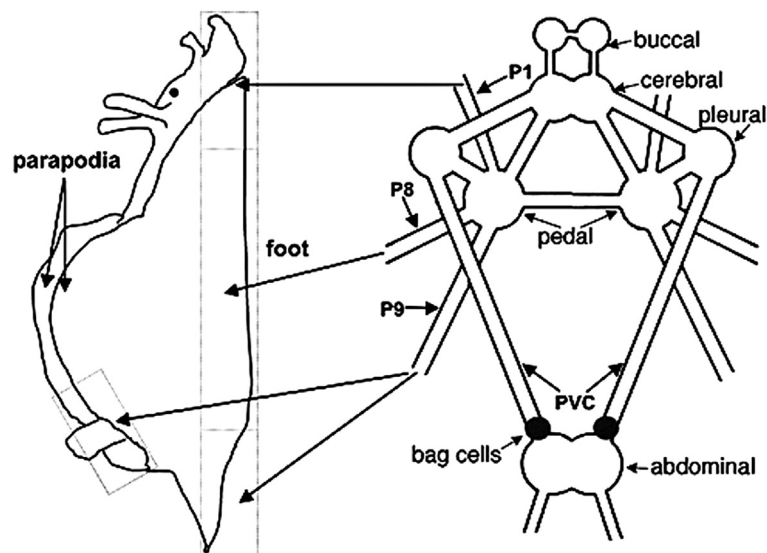


Fig. 4 Schematic drawing of the *Aplysia californica* ganglionic central nervous system. pvc, pleurovisceral connective nerve. From Sun, B., Tsai, P.-S., 2011. A gonadotropin-releasing hormone-like molecule modulates the activity of diverse central neurons in a gastropod mollusk, *Aplysia californica*. *Front. Endocrin.* 2, 36.

Table 1 Structures of egg-laying hormone and related peptides found on the ELH-related precursors in molluscs

Animal	Peptide name	Structure
Aplysia	ELH	ISINQDLKAITDMLLTEQIRERQRYLADLRQRLLLEKa
	Califin (large)	ISINQDLKAITDMLLTEQIQARRRCLDALRQRLLDLa
Lymnaea	CDCH-I	LSITNDLRAIADSYLYDQNKLRERQEENLRRRFLLELa
	CDCH-H	SITNDLRAIADSYLYDQHKLREQEENLRRRFFYELSLRPYPDNLa
Aplysia	Peptide A	IFVPNRAVKLSSDGNYPFDLSKEDGAQPYFMTPLRLRFYPI
	α -BCP	APRLRFYSL
	β -BCP	RLRFH
	γ -BCP	RLRFSD
	δ -BCP	DQDEGNFRFPPTNAVSMADENSFPDLSNEDGAVYQRDL
Lymnaea	α -CDCP	EPRLRFHDV
	β_1 -CDCP	RLRFH
	β_2 -CDCP	RLRAS
	β_3 -CDCP	RLRFN
Lymnaea	Calfluxin	RVDS – ADESNDGFD
Aplysia	Califin (small)	DSDVSLFNGDLLPNGRCS
	Acidic peptide	SSGVSLTTSNKDEEQRELLKAISNLLD

Source: Modified from Mukai, S., Morishita, F., 2016. Physiological functions of gastropod peptides and neurotransmitters. In: Saleuddin, S., Mukai, S. (Eds.), Physiology of Molluscs, vol. 2. Waretown, NJ: Apple Academic Press, pp. 379–476.

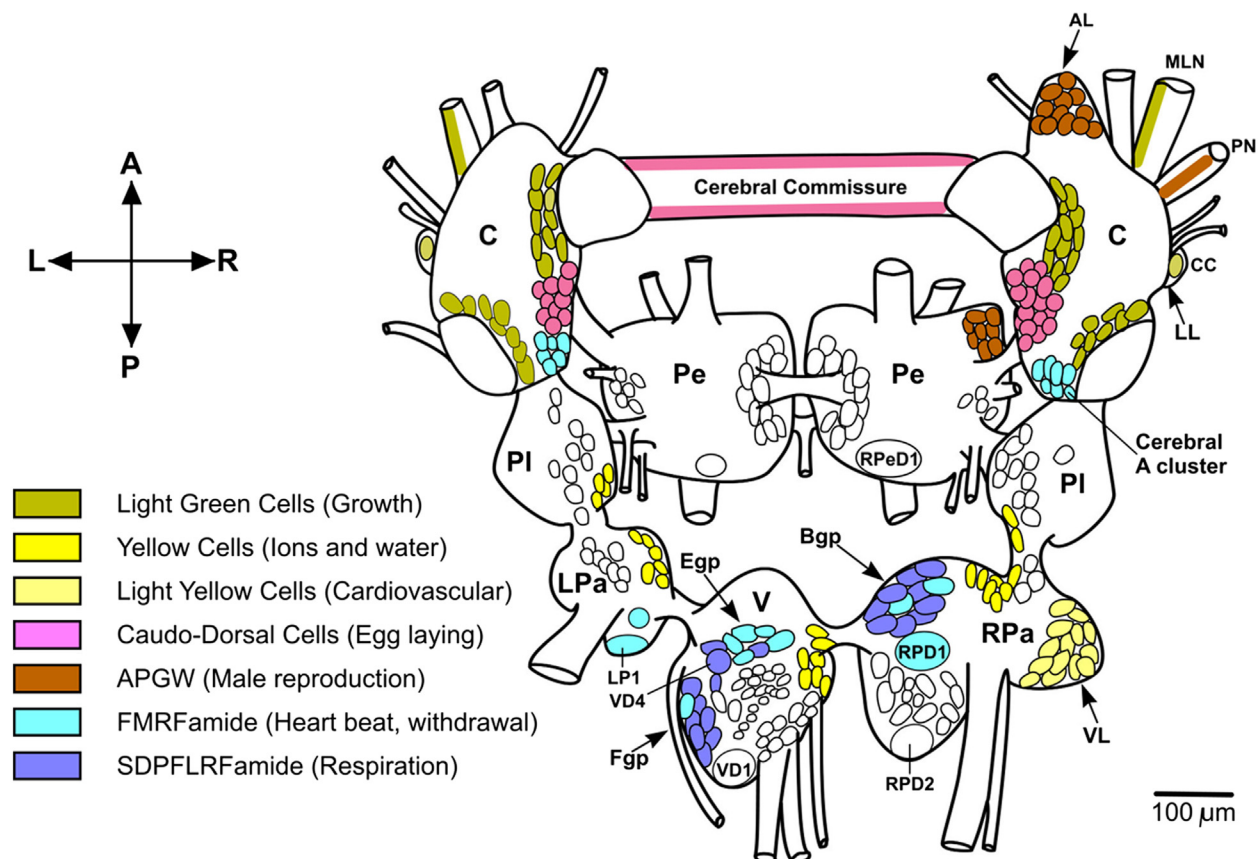


Fig. 5 Schematic drawing of the distribution of neurons in the ganglia of the central nervous system of *Lymnaea stagnalis*. The cerebral commissure is the neurohaemal organ of the CDCs. The anterior lobe (AL) APGW neurons project along the right penis nerve (PN) and innervate the penis complex. C, cerebral ganglion; CC, Canopy Cell; B/E gp, B/E group; LL, lateral lobe; LP1, left parietal 1; LPa, left parietal ganglion; Pe, pedal ganglion; PI, pleural ganglion; RPa, right parietal ganglion; RPD1/2, right parietal dorsal 1/2; RPeD1, right pedal dorsal 1; V, visceral ganglion; VD1, visceral dorsal 1; VD4, visceral dorsal 4; VL, ventral lobe; CDCs, caudo-dorsal cells. From Benjamin, P.R., Kemenes, I., 2013. *Lymnaea* neuropeptide genes. Scholarpedia 8(7), 11520.

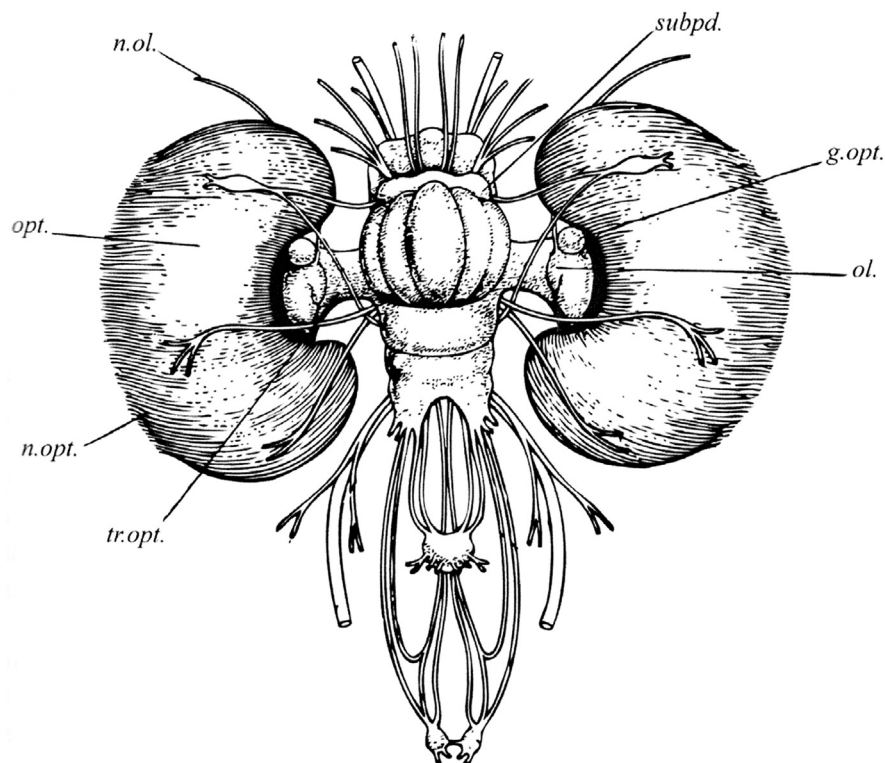


Fig. 6 Drawing of the central nervous system of *Octopus*. g. opt., optic gland; n. ol., olfactory nerve; n. opt., optic nerve; ol., olfactory lobe; opt., optic lobe; subpd., subpedunculate lobe; tr. Opt., optic tract. From Di Cristo, C., 2016. The physiology of reproduction in cephalopods. In: Saleuddin, S., Mukai, S. (Eds.), *Physiology of Molluscs*, vol. 2. Waretown, NJ: Apple Academic Press, pp. 220–270.

Cephalopods

In cephalopods, reproductive process is described as follows: for female vitellogenesis, egg laying, formation of protective egg coat by the oviduct gland and encapsulation of spawned egg mass by the nidamental gland, for male formation of spermatophore and ripening of reproductive tract. The involvement of CNS of cephalopods in hormonal control of reproduction was also expected as in other molluscan species (Fig. 6). Ablation and enlargement of the optic gland resulted in regression of testis and its duct, and enlargement of testis and its duct, respectively (Wells and Wells, 1972). In general gonad maturation of the cephalopods is controlled by photoperiod. Darkness activate development of gonad, whereas lightness inhibits the optic gland activity through activation of the subpedunculate lobe, implying that gonad maturation is regulated by the optic gland via the subpedunculate lobe receiving photoperiod signal through optic nerve. In addition to neural function, progesterone likely induces proliferation of follicle cell to synthesize yolk protein and results in vitellogenesis associated with the optic gland hormone (Di Cristo *et al.*, 2010). In the optic gland octopus GnRH (oct-GnRH) was identified, which is dodecapeptide with extra dipeptide insertion after N-terminal pyro-glutamate residue, pENYHFSNGWHPG-NH₂ (Iwakoshi *et al.*, 2002). The oct-GnRH neuron and nerve fiber distributed in the subpedunculate lobe and the posterior olfactory lobe, which innervate the optic gland (Iwakoshi-Ukena *et al.*, 2004). The oct-GnRH induces steroidogenesis of progesterone, testosterone and E₂ in the ovary where oct-GnRH receptor distributes (Kanda *et al.*, 2006). Oct-GnRH is suggested to be a key peptide in the subpedunculate/posterior olfactory lobes–optic gland–gonadal axis to control octopus reproduction.

References

- Alavi, S. M. H., Matsumura, N., Shiba, K., *et al.* (2014). Roles of extracellular ions and pH in serotonin-dependent initiation of sperm motility in marine bivalve mollusks. *Reproduction*, 147, 331–345.
- Bigot, L., Zatylny-Gaudin, C., Rodet, F., *et al.* (2012). Characterization of GnRH-related Peptides from the Pacific oyster *Crassostrea gigas*. *Peptides*, 34, 303–310.
- Chang, E. S., & Mykles, D. L. (2011). Regulation of crustacean molting: A review and our perspectives. *Gen. Comp. Endocrinol.*, 172, 323–330.
- Ciocan, C. M., Cubero-Leon, E., Puinean, A. M., *et al.* (2010). Effects of estrogen exposure in mussels, *Mytilus edulis*, at different stages of gametogenesis. *Environ. Pollut.*, 158, 2977–2984.
- Di, Cristo, Di Donato, P., Palumbo, A., *et al.* (2010). Steroidogenesis in the brain of *Sepia officinalis* and *Octopus vulgaris*. *Front. Biosci. (Elite Ed)*, 2, 673–683.
- Geraerts, W. P. M. (1976). The role of the Lateral Lobes in the control of growth and reproduction in the hermaphrodite freshwater snail *Lymnaea stagnalis*. *Gen. Comp. Endocrinol.*, 29, 97–108.

- Iwakoshi, E., Takuwa-Kuroda, K., Fujisawa, Y., et al. (2002). Isolation and characterization of a GnRH-like peptide from *Octopus vulgaris*. *Biochem. Biophys. Res. Commun.*, 291, 1187–1193.
- Iwakoshi-Ukena, E., Ukena, K., Takuwa-Kuroda, K., et al. (2004). Expression and distribution of octopus gonadotropin-releasing hormone in the central nervous system and peripheral organs of the octopus (*Octopus vulgaris*) by in situ hybridization and immunohistochemistry. *J. Comp. Neurol.*, 477, 310–323.
- Kanda, A., Takahashi, T., Satake, H., & Minakata, H. (2006). Molecular and functional characterization of a novel gonadotropin-releasing-hormone receptor isolated from the common octopus (*Octopus vulgaris*). *Biochem. J.*, 395, 125–135.
- Keller, R. (1992). Crustacean neuropeptides: Structures, functions and comparative aspects. *Experientia*, 48, 439–448.
- Kupfermann, I. (1970). Stimulation of egg laying by extracts of neuroendocrine cells (bag cells) of abdominal ganglion of *Aplysia*. *J. Neurophysiol.*, 33, 877–881.
- Lafont, R., & Mathieu, M. (2007). Steroids in Aquatic Invertebrates. *Ecotoxicology*, 16, 109–130.
- Li, K. W., Geraerts, W. P. M., & Joosse, J. (1992). Purification and chemical characterization of caudodorsal cell hormone-II from the egg-laying controlling caudodorsal cells of *Lymnaea stagnalis*. *Peptides*, 13, 215–220.
- Mak, A. S., Choi, C. L., Tiu, S. H., et al. (2005). Vitellogenesis in the red crab *Charybdis feriatus*: Hepatopancreas-specific expression and farnesic acid stimulation of vitellogenin gene expression. *Mol. Reprod. Dev.*, 70, 288–300.
- Mathieu, M., Lenoir, F., & Robbins, I. (1988). A gonial mitosis-stimulating factor in cerebral ganglia and hemolymph of the marine mussel *Mytilus edulis* L. *Gen. Comp. Endocrinol.*, 72, 257–263.
- Matsumoto, T., Nakamura, A. M., Mori, K., et al. (2007). Oyster estrogen receptor: Cdna Cloning and Immunolocalization. *Gen. Comp. Endocrinol.*, 151, 195–201.
- Matsumoto, T., Nakamura, A. M., Mori, K., & Kayano, T. (2003). Molecular Characterization of a cDNA Encoding Putative Vitellogenin from the Pacific Oyster *Crassostrea gigas*. *Zool. Sci.*, 20, 37–42.
- Matsutani, T., & Nomura, T. (1987). In vitro effects of serotonin and prostaglandins on release of eggs from the ovary of the scallop. *Patinopecten yessoensis*. *Gen. Comp. Endocrinol.*, 67, 111–118.
- Mukai, S., & Morishita, F. (2016). Physiological functions of gastropod peptides and neurotransmitters. In S. Saleuddin, & S. Mukai (Eds.), *Physiology of Molluscs vol., 2* pp. 379–476). Waretown, NJ: Apple Academic Press.
- Nagasawa, K., Osugi, T., Suzuki, I., et al. (2015a). Characterization of GnRH-like peptides from the nerve ganglia of Yesso scallop. *Patinopecten yessoensis*. *Peptides*, 71, 202–210.
- Nagasawa, K., Oouchi, H., Itoh, N., Takahashi, K. G., & Osada, M. (2015b). In vivo administration of scallop GnRH-like peptide influences on gonad development in the Yesso scallop, *Patinopecten yessoensis*. *PLoS ONE*, 10(6), e0129571.
- Nagasawa, K., Treen, N., Kondo, R., et al. (2015c). Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two *Mytilus* species. *Gene*, 564, 153–159.
- Nagle, G. T., Painter, S. D., Kelner, K. L., & Blankenship, J. E. (1985). Atrial gland cells synthesize a family of peptides that can induce egg laying in *Aplysia*. *J. Comp. Physiol. B*, 156, 43–55.
- Nakamura, S., Osada, M., & Kijima, A. (2007). Involvement of GnRH neuron in the spermatogonial proliferation of the scallop, *Patinopecten yessoensis*. *Mol. Rep. Dev.*, 74, 108–115.
- Osada, M., Harata, M., Kishida, M., & Kijima, A. (2004). Molecular Cloning and Expression Analysis of Vitellogenin in Scallop, *Patinopecten yessoensis* (Bivalvia, Mollusca). *Mol. Reprod. Dev.*, 67, 273–281.
- Osada, M., Mori, K., & Nomura, T. (1992). In vitro effects of estrogen and serotonin on release of eggs from the ovary of the scallop. *Nippon Suisan Gakkaishi*, 58, 223–227.
- Osada, M., Nakamura, S., & Kijima, A. (2007). Quantitative analysis of the pattern of gonial proliferation during sexual maturation in the Japanese scallop *Patinopecten yessoensis*. *Fish. Sci.*, 73, 1318–1324.
- Osada, M., Takamura, T., Sato, H., & Mori, K. (2003). Vitellogenin synthesis in the ovary of scallop, *Patinopecten yessoensis*: Control by estradiol-17 Beta and the central nervous system. *J. Exp. Zool.*, 299, 172–179.
- Osada, M., & Treen, N. (2013). Molluscan GnRH Associated with Reproduction. *Gen. Comp. Endocrinol.*, 181, 254–258.
- Panouse, J. B. (1943). Influence de l'ablation du pédoncle oculaire sur la croissance de l'ovaire chez la crevette *Leander serratus*. *C R Acad Sci Paris*, 217, 553–555.
- Roch, G. J., Busby, E. R., & Sherwood, N. M. (2011). Evolution of GnRH: Diving deeper. *Gen. Comp. Endocrinol.*, 171, 1–16.
- Rothman, B. S., Hawke, D. H., Brown, R. O., et al. (1986). Isolation and primary structure of the califins, three biologically active egg-laying hormone-like peptides from the atrial gland of *Aplysia californica*. *J. Biol. Chem.*, 261, 1616–1623.
- Soyez, D., Le Caer, J. P., Noel, P. Y., & Rossier, J. (1991). Primary structure of two isoforms of the vitellogenesis inhibiting hormone from the lobster *Homarus americanus*. *Neuropeptides*, 20, 25–32.
- Subramoniam, T. (2017). *Sexual Biology and Reproduction in Crustaceans*. London: Academic Press.
- Tanabe, T., Osada, M., Kyojuka, K., Inaba, K., & Kijima, A. (2006). A novel oocyte maturation arresting factor in the central nervous system of scallops inhibits serotonin-induced oocyte maturation and spawning of bivalve mollusks. *Gen. Comp. Endocrinol.*, 147, 352–361.
- Tanabe, T., Yuan, Y., Nakamura, S., et al. (2010). The role in spawning of a putative serotonin receptor isolated from the germ and ciliary cells of the gonoduct in the gonad of the Japanese scallop, *Patinopecten yessoensis*. *Gen. Comp. Endocrinol.*, 166, 620–627.
- Tiu, S. H., & Chan, S. M. (2007). The use of recombinant protein and RNA interference approaches to study the reproductive functions of a gonad-stimulating hormone from the shrimp *Metapenaeus ensis*. *FEBS J.*, 274, 4385–4395.
- Vreugdenhil, E., Jackson, J. F., Bouwmeester, T., et al. (1988). Isolation, characterization, and evolutionary aspects of a cDNA clone encoding multiple neuropeptides involved in the stereotyped egg-laying behavior of the freshwater snail *Lymnaea stagnalis*. *J. Neurosci.*, 8, 4184–4191.
- Wainwright, G., Webster, S. G., Wilkinson, M. C., Chung, J. S., & Rees, H. H. (1996). Structure and significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*. Involvement in multihormonal regulation of growth and reproduction. *J. Biol. Chem.*, 271, 12749–12754.
- Wang, C., & Croll, R. P. (2006). Effects of sex steroids on spawning in the sea scallop, *Placopecten magellanicus*. *Aquaculture*, 256, 423–432.
- Webster, S. G., Keller, R., & Heinrich Dirksen, H. (2012). The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. *Gen. Comp. Endocrinol.*, 175, 217–233.
- Wells, M. J., & Wells, J. (1972). Sexual displays and mating of *Octopus vulgaris* Cuvier and *O. cyanea* Gray and attempts to alter performance by manipulating the glandular condition of the animals. *Anim. Behav.*, 20, 293–308.
- Yano, I. (1998). Hormonal control of vitellogenesis in penaeid shrimp. In T. W. Flegel (Ed.), *Advances in Shrimp Biotechnology* (pp. 29–31). Bangkok: National Center for Genetic Engineering and Biotechnology.
- Yuan, Y., Tanabe, T., Maekawa, F., et al. (2012). Isolation and functional characterization for oocyte maturation and sperm motility of the oocyte maturation arresting factor from the Japanese scallop, *Patinopecten yessoensis*. *Gen. Comp. Endocrinol.*, 179, 350–357.
- Zmora, N., Trant, J., Zohar, Y., & Chung, J. S. (2009). Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 1: An ovarian stage dependent involvement. *Saline Systems*, 5, 7.

Photoperiodism in Fish

Jack Falcón, National Museum of Natural History, Paris, France

Yonathan Zohar, University of Maryland, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

BPG	Brain-pituitary-gonads axis	HPT	Hypothalamus
D	Darkness	Kiss	Kisspeptin
DA	Dopamine	L	Light
E2	Estradiol 17 β	LHβ	Luteinizing hormone subunit β
FSHβ	Follicle-stimulating hormone subunit β	MIS	Maturation inducing steroid
GnIH	Gonadotropin inhibiting hormone	NKb	NKf, neurokinins b and f
GnRH	Gonadotropin releasing hormone	POA	Preoptic area
GSI	Gonadosomatic index	SCN	Suprachiasmatic nuclei
		T	Testosterone

Glossary

Circadian Clock A molecular machinery that functions on a 24 ± 4 h base (from the Greek *circa*: approximately, and *dian*: day).

Circannual Clock A molecular machinery that functions on a seasonal base.

Photoperiod Alternation of light (L) and darkness (D) during the 24 h cycle.

Introduction

In fish, reproduction displays cyclic variations at all levels of control: the seasonal information relayed to the circadian (biological) clocks, allows the production of rhythmic signals, which modulate the activity of the brain-pituitary-gonadal (BPG) axis, gonadal development and maturation, and finally courtship behavior and spawning (Cowan *et al.*, 2017; Falcón *et al.*, 2010a,b; Migaud *et al.*, 2010; Zohar *et al.*, 2010). Photoperiod, the main factor influencing the timing of fish reproduction, acts at different timescales from daily to once in a life (Juntti and Fernald, 2016). Photoperiod determines if daily or annual spawning occurs in the morning, afternoon or (most common situation) at night (Claydon *et al.*, 2014; Oliveira and Sánchez-Vázquez, 2010). In equatorial areas, it may occur throughout the year (Claydon *et al.*, 2014; Oliveira and Sánchez-Vázquez, 2010). Seasonal fish display a restricted spawning window, allowing partitioning between the energetic needs of reproduction and growth, and providing optimal conditions for offspring survival. Both functions are interconnected and rhythmic (Migaud *et al.*, 2010). Months of preparation precede, and a phase of arrest or recrudescence follows, the spawning window. Evidence for the presence of a circannual clock controlling fish reproduction has been obtained from studies in the trout (Duston and Bromage, 1991), catfish (Aggarwal *et al.*, 2014) and seabass (Prat *et al.*, 1999), which exhibit self-sustained circannual rhythms of spawning under constant conditions. The timing and duration of the spawning window is species-dependent (Oliveira and Sánchez-Vázquez, 2010). Spawning rhythms are synchronized by monsoons, tides and rainfalls in the tropics (Oliveira and Sánchez-Vázquez, 2010). An extreme situation is observed in some salmon and eel species that reproduce only once in their life before dying (Juntti and Fernald, 2016).

In brief, the control of reproduction is highly dependent on a precise timing provided by the circadian system.

The Circadian System

Organization of the Circadian System

The circadian system is made of central clocks, of organs that provide input to the clocks and others that receive rhythmic input from the clocks. This system, which provides information on daily and calendar time to the rest of the organism, allows predicting and anticipating the cyclic variations in the environment. A single system controls a myriad of events (Fig. 1) (Ekström and Meissl, 2010; Falcón *et al.*, 2010a,b). Under constant conditions, the central clocks oscillate in a free running mode (24 ± 4 h). The underlying molecular mechanism involves the interaction of transcriptional activators and repressors that create two interlocking feedback loops, and virtually all fish cells possess such a machinery (Whitmore, 2010). Thus, multiple cellular clocks are present, organized in a network of more or less potent oscillatory units (Fig. 1). The 24 h light/dark (L/D) cycle is the main synchronizer of the clocks' activity, but other factors (temperature, food availability, water composition, social

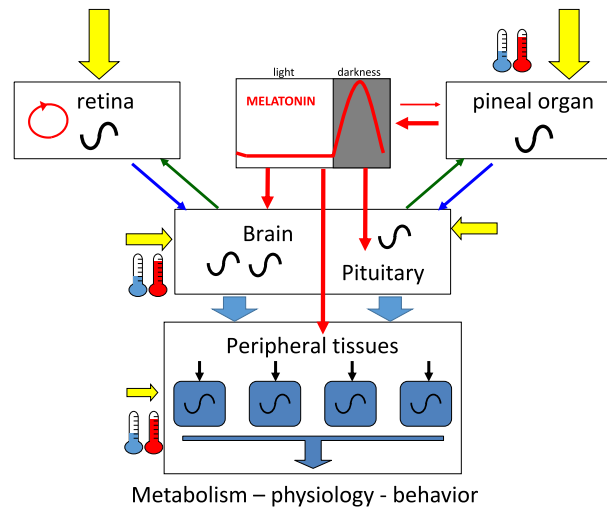


Fig. 1 Schematic view of the fish circadian system. In teleost fish the circadian system is made of more or less potent self-sustained units distributed in central and peripheral areas. The units are interconnected via nervous and/or hormonal pathways (arrows). They are directly and/or indirectly synchronized to the natural variations in photoperiod (yellow arrows) and temperature (thermometers) through photoreceptors and thermoreceptors located in the retina, pineal complex, deep diencephalon and skin. The pineal organ occupies a central position in the network through its rhythmic production of melatonin. Melatonin reaches multiple areas (red arrows), probably acting as a conductor/modulator of rhythms.

cues, rainfall, tides, and lunar cycles) may also provide input. Light is perceived through specific sensors, also located in different areas, including eyes, pineal gland, brain and skin (Baker *et al.*, 2015; Binder and McDonald, 2008; Chen *et al.*, 2014; Falcón *et al.*, 2010a,b).

How do all these different multi-oscillatory units get in tune? One hypothesis assumes the existence of one or several “master oscillators” that provide imprinting on “slave oscillators”. In this scheme, the pineal gland occupies a central position (Falcón *et al.*, 2010a,b). It is possible that this is by virtue of its rhythmic production of melatonin (Fig. 1).

The Pineal Gland and Melatonin

The pineal gland of fish contains functional photoreceptors, analogous to the retinal cones (Ekström and Meissl, 2010; Falcón *et al.*, 2010a,b). In most species, each pineal photoreceptor is a cellular circadian system by itself. At night, photoreceptors produce two kind of messages. One is an excitatory neurotransmitter, which stimulates the activity of second-order neurons (Ekström and Meissl, 2010). Light inhibits this release providing a highly sensitive indication on surrounding intensity. The neurons convey the nervous information to brain centers (Yáñez *et al.*, 2009) (Fig. 2). The other is melatonin produced from serotonin (Figs. 2 and 3) (Falcón *et al.*, 2010a,b). Light inhibits melatonin production, and the nocturnal rise is driven by intrinsic circadian clock machinery. Temperature controls the amplitude of the nocturnal surge in a species dependent manner (Figs. 2 and 3) (Falcón *et al.*, 2010a,b). It is worth mentioning that internal factors, including estradiol (E2), glucocorticoids, catecholamines, adenosine, GABA and melatonin itself, can also modulate this production (Falcón *et al.*, 2010a,b). Pineal melatonin is released into the blood and cerebrospinal fluid. The retina also produces melatonin however for autocrine/paracrine use only; it is catabolized *in situ*.

In some species, light perceived through the eyes contributes partially (seabass, cod) or totally (catfish, tilapia) to controlling pineal melatonin secretion (Bayarri *et al.*, 2003; Martínez-Chávez and Migaud, 2009), indicating a neural pathway connects the retina to the pineal. Finally, no circadian clock controls melatonin secretion in Salmonid fish, where melatonin production is just an on/off response to the LD information (Falcón *et al.*, 2010a,b).

In all cases, the pattern of the melatonin oscillations reflects the daily variations in light and temperature. Its secretion rhythm provides robust and reliable information on daily and calendar times. Melatonin is now recognized as the hormonal time-keeper of the organism (Falcón *et al.*, 2010a,b; Migaud *et al.*, 2010).

Melatonin Receptors

Melatonin acts through three subtypes of specific transmembrane receptors (MT1, MT2 and Mel1c), which may be duplicated, and displaying a wide distribution in nervous and peripheral organs (Falcón *et al.*, 2010a). Virtually all brain areas express one or another subtype (Herrera-Perez *et al.*, 2010; Mazurais *et al.*, 1999). The receptors are particularly abundant in the thalamus, optic tectum and cerebellum, in areas that generally integrate light signals from the retina and/or pineal neurons. It is interesting that the suprachiasmatic nuclei (SCN) and preoptic area (POA), two thalamic neuroendocrine centers, also receive pineal and retinal nerve endings (Yáñez *et al.*, 2009).

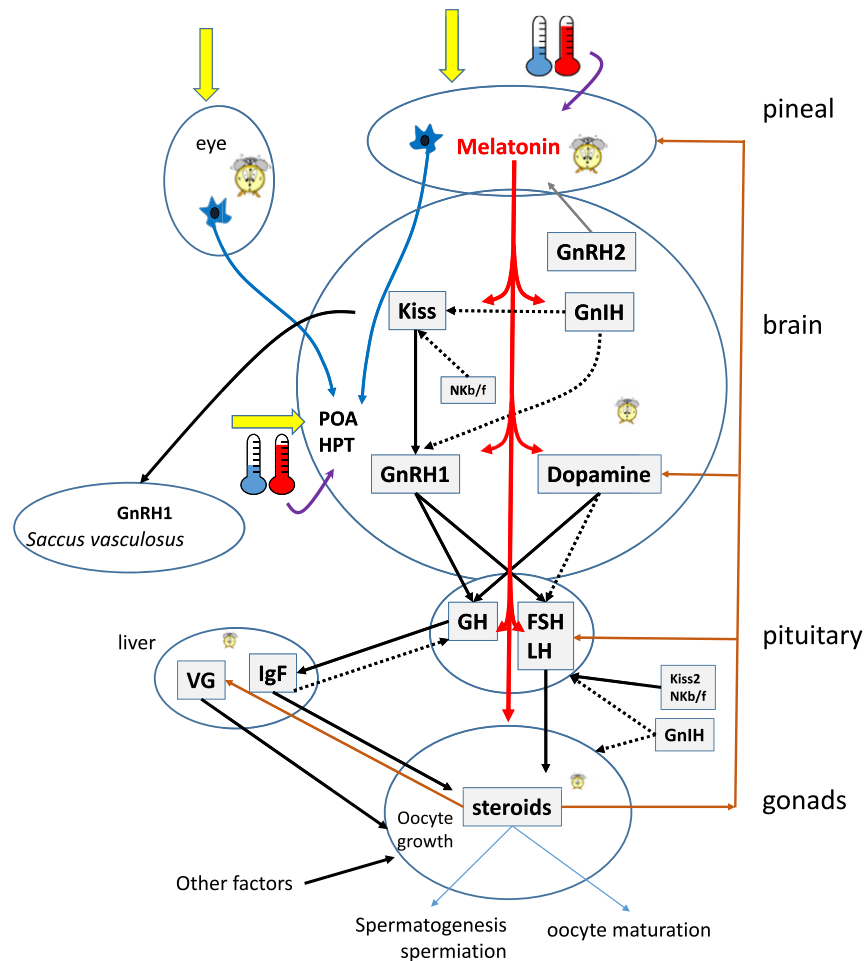


Fig. 2 Schematic view of the organization of the Brain/Pituitary/Gonads (BPG) axis in fish. The complex interactions at the different stages of the BPG axis are shown by the arrows (for details see text). Light and temperature inputs are shown by the yellow arrows and thermometers, respectively. Melatonin from the pineal gland (thick red arrows) acts at all levels of the axis. Neurons from the retina and pineal gland convey information to the hypothalamus (HPT) and preoptic area (POA). The sites of circadian clocks are shown by a clock. DA, dopamine; FSH, follicle-stimulating hormone; GnIH, gonadotropin inhibiting hormone; GnRH, gonadotropin releasing hormone; IgF, insulin like growth factor; Kiss, Kisspeptin; LH, luteinizing hormone; Vg, vitellogenin; NKb, NKf, neurokinins b and f.

The different melatonin receptor subtypes display daily variations (mRNA abundance, binding sites abundance or affinity for melatonin) (Bayarri *et al.*, 2010; Falcón *et al.*, 1996; Gaildrat *et al.*, 1998; Iigo *et al.*, 1994; Ikegami *et al.*, 2009) (Fig. 3). Binding sites abundance is generally higher during day than at night, but mRNA abundance may peak at different times of the LD cycle depending on the species and tissue investigated. These variations are maintained under constant, LL or DD, conditions in most but not all tissues, indicating a circadian clock control (Fig. 3) (Gaildrat *et al.*, 1998; Iigo *et al.*, 2003). Again, salmonid fish are an exception: no rhythm is detected under LL or DD (Amano *et al.*, 2006). Seasonal variations in abundance of melatonin mRNA or binding sites have also been described in the pituitary (Falcón *et al.*, unpublished), hypothalamus and optic tectum (Bayarri *et al.*, 2010) of seabass maintained under LD and LL, indicating control by a circannual clock. Finally, tropical species with lunar-cycles synchronized melatonin profiles, also display lunar-dependent expression of their melatonin receptors (Park *et al.*, 2014). In brief, studies on the effects of melatonin must consider the daily, lunar and/or seasonal variations of the hormone as well as of its receptors, which varies from a species to another and from an organ to another.

The Reproduction System

During the breeding season, levels of androgens rise in males, and those of estrogens and/or progestins rise in females, allowing gonad maturation and spawning behavior (Juntti and Fernald, 2016). This is under the control of the brain-pituitary-gonads (BPG) axis (Fig. 3). One main component of the BPG axis is the hypothalamic gonadotropin-releasing-hormone (GnRH), which

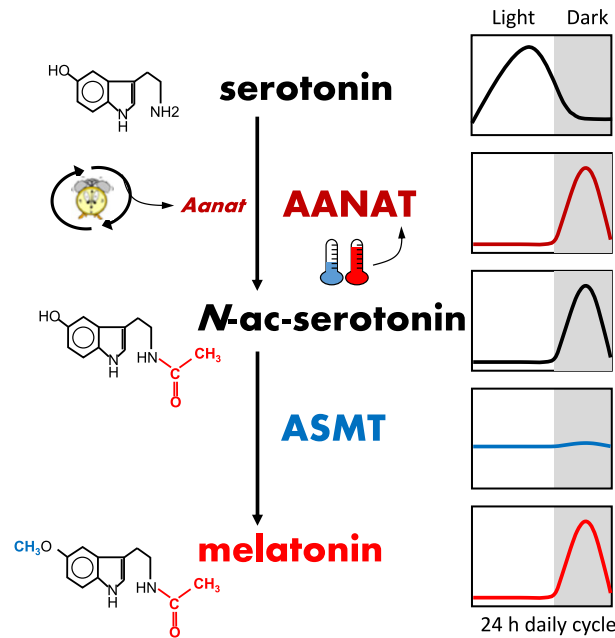


Fig. 3 Melatonin and its rhythmic biosynthesis from serotonin. Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesized from serotonin in two steps: (1) The aralkylamine *N*-acetyltransferase (AANAT) catalyzes the conversion of serotonin into *N*-acetylserotonin (*N*-ac-serotonin); AANAT gene expression (*Aanat*) is controlled by intrinsic molecular clock machinery (clock); light inhibits AANAT protein abundance and activity while temperature controls amplitude of AANAT nocturnal activity (thermometers); (2) *N*-ac-serotonin is then methylated through the action of the acetylserotonin-*O*-methyltransferase (ASMT). The nocturnal surge in melatonin secretion results from the cyclic activity of AANAT. The duration of the nocturnal surge reflects the photoperiod, while its amplitude reflects the temperature. ASMT remains more or less constant throughout the light/dark cycle. The right panel shows the light/dark variations of each component during a 24 h cycle.

stimulates the synthesis and release of gonadotropins: follicle-stimulating hormone (FSH β) and luteinizing hormone (LH β), which in turn regulate steroidogenesis. Sex steroids are necessary for gametogenesis, and final gamete maturation (Zohar *et al.*, 2010). Sex steroids in turn exert diverse positive/negative feedback effects on tissues throughout the body (Fig. 3) with the cumulative effect of preparing multiple tissues for reproduction (Juntti and Fernald, 2016).

In distant teleost (perciforms), three isoforms of *Gnrh* exist: the hypophysiotropic *Gnrh1* in the pre-optic area/hypothalamus, *Gnrh2* in the midbrain tegmentum, and *Gnrh3* in the terminal nerve/ventral telencephalon (Powell *et al.*, 1994; Gothilf *et al.*, 1996). In early teleost (salmonids and cyprinids) only *Gnrh2* and *Gnrh3* isoforms have been identified; *Gnrh3* is believed to assume the non-redundant roles of *Gnrh1* (Zohar *et al.*, 2010). GnRH stimulation of gonadotropins secretion may be opposed by dopamine (DA) inhibition in some, but not all, teleost fish (Dufour *et al.*, 2010). Both the GnRH hypophysiotropic and DA inhibitory neurons are located in the preoptic area (POA) (Dufour *et al.*, 2010; Zohar *et al.*, 2010).

Other neuropeptides are involved in the control of reproduction acting upstream or downstream GnRH1/3 and DA; amongst the most significant are the gonadotropin inhibiting hormone (GnIH), Kisspeptins (Kiss1, Kiss2) and Neurokinins (NKb and NKf) (Paullada-Salmerón *et al.*, 2016; Zohar *et al.*, 2010) (Fig. 2).

1. GnIH, which belongs to the RFamide peptide family, has been reported to either inhibit (Paullada-Salmerón *et al.*, 2016; Spicer *et al.*, 2017) or stimulate (Biran *et al.*, 2014) gonadotropins. The time of year and experimental procedure used might explain these discrepancies. GnIH peptides interact functionally with GnRH neurons (Peng *et al.*, 2016; Spicer *et al.*, 2017), and reduce GnRH1 mRNA levels (Paullada-Salmerón *et al.*, 2016). An inverse relationship characterizes GnIH and GnRH mRNA abundance during the reproductive cycle of the carp (Peng *et al.*, 2016). In this species, three GnIH receptor subtypes are detected at all stages of the BPG axis, which also display seasonal variations in mRNA abundance. In the zebrafish, LPXRFamide antagonizes the Kiss2 activation of Kiss receptors (Spicer *et al.*, 2017).
2. Kiss1 and Kiss2 genes are expressed, respectively, in the *habenula*/medio-basal hypothalamus, and hypothalamus (Alvarado *et al.*, 2016). In the striped-bass and seabass, Kiss2 acts on the hypothalamus (targeting GnRH neurons) and the pituitary, in a stage- and sex-dependent manner (Alvarado *et al.*, 2016; Zmora *et al.*, 2014, 2015). This results in the upregulation of GnRH1, FSH β and LH β expressions and secretion for the latter two corresponding peptides. Kiss and Kiss receptors are also present in the gonads but their roles remain enigmatic.
3. NKb and NKf stimulate FSH β and LH β levels in the tilapia, zebrafish and goldfish pituitaries as well as *fsh* and *lh* mRNA and LH β release in the striped-bass (Zmora *et al.*, 2017). It also inhibits *kiss2* expression and stimulate *Gnrh* expression (Zmora *et al.*, 2017).

Melatonin and Reproduction: A Rhythmic Adventure

Virtually all components of the BPG axis display daily and annual variations (Cowan *et al.*, 2017). How these variations are linked to those of the environmental cues remains largely unknown (Juntti and Fernald, 2016). As a time-keeper of the organism, melatonin has drawn attention. Early studies have shown that photoperiod manipulation, together with pinealectomy or melatonin administration, could induce pro- or anti-gonadal effects or have no effect at all in fish (Falcón *et al.*, 2010a). A confusing picture thus arose, which most certainly results from the use of different species and sex, at different reproductive states and seasons, with different doses and ways of melatonin administration. Although the modalities of action are not always well understood, evidence points in favor of a role of melatonin in fish, provided by studies on the localization of melatonin receptors and the reported effects of the hormone at multiple levels of the BPG axis.

The Pituitary

The fish pituitary expresses melatonin binding-sites as well as MT1, MT2 and/or Mel1c mRNA receptors (Chai *et al.*, 2013; Gaildrat and Falcón, 2000; Ikegami *et al.*, 2009). And, cyclic-AMP from cultured pituitaries varies after melatonin challenge in pike and trout (Falcón *et al.*, 2010a). In the sea bass, MT2 mRNA abundance displays seasonal variations in an inverse relationship with the FSH β and LH β mRNA annual variations and reproductive status (Falcón *et al.*, unpublished). This agrees with the idea that melatonin inhibits gonadotropins as first demonstrated in the Atlantic croaker (Khan and Thomas, 1996). Other *in vivo* studies later confirmed an inhibitory impact of melatonin on GnRH, LH β or FSH β mRNA (pituitary), or protein abundance (pituitary or plasma) in the eel (Dufour *et al.*, 2010), Pacific salmon (Amano *et al.*, 2004) and Mediterranean seabass (Alvarado *et al.*, 2015). A clear-cut mechanism of action is however difficult to highlight because of differences in the experimental protocols and species studied. Thus, in masu salmon, melatonin administration inhibited permanently (FSH β), while inhibition of LH β and GnRH was transient (Amano *et al.*, 2004). In contrast, melatonin implants impacted neither the annual rhythm of pituitary gonadotropins mRNA abundance, nor plasma levels of FSH in the Mediterranean seabass, while the February peak of plasma LH β was slightly decreased (Alvarado *et al.*, 2015). This contrasts with *in vitro* studies showing inhibition of gonadotropins mRNA abundance by melatonin or melatonin analogs (Falcón *et al.*, unpublished). The differences might result from a combination of factors, including fish strains and experimental conditions (e.g., *in vitro* the possible interactions with other brain factors are disrupted). In euryhaline fish, water salinity might also be of importance. In the Mediterranean seabass, the annual variations in gonadotropins mRNA abundance (Alvarado *et al.*, 2015) was not affected by water salinity in marked contrast with all other pituitary hormones (Falcón *et al.*, unpublished). However, the *in vitro* response to melatonin was dramatically modified by previous adaptation to sea or fresh water. The time of the year at which the experiments are done is also of importance.

The Brain (POA/Hypothalamus)

Melatonin injected into the 3rd ventricle of the Atlantic croaker inhibited LH β release by the pituitary glands (Khan and Thomas, 1996). This, and the identification of melatonin receptors in various brain areas, including the POA and hypothalamus (Falcón *et al.*, 2010a; Migaud *et al.*, 2010), paved the way to studies dealing with the effects of melatonin on the different regulators of the pituitary function, including kisspeptins, GnRH, GnIH, and DA. In female zebrafish, *in vivo* melatonin treatment elevated *kiss1*, *kiss2* and *gnrh3* gene expression in the brain and LH β in the pituitary (Carnevali *et al.*, 2011). The effect on GnRH was probably indirect. Indeed (1) in the seabass, where GnRH1 and GnRH3 display LD variations and respond to melatonin, melatonin receptors co-localize with Kiss1/Kiss2, not GnRH (Alvarado *et al.*, 2015; Servili *et al.*, 2013); and (2) in the orange-spotted grouper, LD variations in the expression of MT1 melatonin receptors, *kiss2* and *gnrh1*, are consistent with the idea that melatonin regulates day/night variations of *gnrh1* expression through Kiss2; a decrease in MT1 receptors expression would increase the expression of *gnrh1* by upregulating *kiss2* expression (Chai *et al.*, 2013).

In the eel, melatonin implants did not affect *gnRH* mRNA abundance, while decreasing *fsh β* and *lh β* mRNA and steroids plasma levels (Dufour *et al.*, 2010). In this species, melatonin stimulates DA synthesis in brain areas known to innervate the pituitary. Such a mechanism might also operate in other species where melatonin/DA interactions have been detected. Melatonin administration lowers DA levels in the brain and/or pituitary of rainbow trout (Hernandez-Rauda *et al.*, 2000), three-spot wrasse (Takemura *et al.*, 2010) and carp during the spawning period (Popek *et al.*, 2010a), while it increases DA release from brain explants of sapphire devil (Badruzzaman *et al.*, 2013). In the latter two species, DA and melatonin rhythms appear inversely correlated (Badruzzaman *et al.*, 2013; Popek *et al.*, 2010b).

Interactions of melatonin with GnIH have been demonstrated in two fish species, the cinnamon clownfish (Nikaido *et al.*, 2010) and zebrafish (Yumnamcha *et al.*, 2017). In the former, GnIH and MT1 melatonin receptors co-localize in the hypothalamus. In the latter melatonin induces a dose-dependent decrease in *gnih* transcription *in vitro*.

The Gonads

Melatonin binding sites or receptors have been identified in the gonads of teleost fish (Chai *et al.*, 2013; Chatteraj *et al.*, 2009a,b; Ikegami *et al.*, 2009; Molina-Borja *et al.*, 1994). The receptors display daily and annual variations in an inverse relationship with serum

melatonin levels (Chattoraj *et al.*, 2009). Steroids and corresponding receptors also display daily and annual variations in association with the reproductive cycle. Photoperiod and temperature synchronize these variations (Zohar and Billard, 1984), which might involve a circannual clock because testosterone (T) and E2 rhythms are maintained under constant conditions in the killifish (Shimizu, 2003). The levels of T, 11-ketotestosterone, E2, and 17 α ,20 β -dihydroxy-4-pregnen-3-one (also known as maturation inducing steroid or MIS), rise during the breeding season (Juntti and Fernald, 2016). It is the increases in FSH β and LH β levels that promote steroidogenesis in the gonads. Sexual steroids contribute then to the process of gonadal maturation and spawning.

Melatonin mediates, at least in part, the effects of photoperiod on gonadal steroids. *In vivo* melatonin administration (1) mimics the effects of short days on GSI, gonadotropins and T, without affecting spermiation of masu salmon (Amano *et al.*, 2000); and (2) advanced testicular maturation during the preparatory phase, but was inhibitory during the pre-spawning and spawning phases, and ineffective during the post-spawning phase in the Indian carp (Bhattacharya *et al.*, 2007). *In vitro* studies support evidence of such complex and direct effects of melatonin on the gonads: (1) steroidogenesis in cultured trout ovarian follicles was stimulated by melatonin at early stages of development and became inhibitory at later stages, with a bimodal dose-dependent effect at maximum follicular growth (Leatherland and Lin, 2001); (2) in carps, pre-incubation of oocytes with melatonin prior to addition of MIS resulted in an accelerated rate of germinal vesical breakdown (Chattoraj *et al.*, 2008); (3) in the zebrafish melatonin increased the expression of two membrane progesterone receptor isoforms (*mpr α* and *mpr β*) (Carnevali *et al.*, 2011).

A permanent communication between the brain/pituitary complex and the gonads allows the activity of the different components of the BPG axis to be synchronized at all stages of the life cycle (Zohar *et al.*, 2010). Thus, steroids released by the gonads exert a feed-back control at different levels of the brain-pituitary axis. They also feed-back on the liver where they control VTG production (Specker and Sullivan, 1994). In the brain, the two major points of gonadal feedback regulation are the pituitary and the DA neurons of the POA. Steroids also modulate *gnRH* expression and content but the effects are mediated through Kiss neurons (Alvarado *et al.*, 2016; Zohar *et al.*, 2010). The pineal gland is also a target: *in vitro* (1) trout pineal cells express a E2 receptors, which mediate complex effects on melatonin secretion (Falcón *et al.*, 2010a,b); and (2) in the catfish E2 and T inhibit one enzyme of the melatonin pathway and hence melatonin synthesis (Yanthan and Gupta, 2007).

Thyroid Hormones and the *Saccus Vasculosus*

Thyroid hormones, triiodothyronine (T3) and thyroxine (T4), control energy metabolism and growth, and regulate development and differentiation of tissues. The hypothalamic thyrotropin-releasing hormone (TRH) produced by cells in the POA stimulates the production of pituitary thyroid-stimulating-hormone (TSH β). In turn TSH stimulates the production of T4 and T3 by the thyroid follicles, but most of the T3 present in the circulation is obtained *via* the conversion of T4 to T3 by deiodinases (DIO) (Swapna and Senthilkumaran, 2007). Evidence linking thyroid hormones and reproduction is extensively documented elsewhere (Duarte-Guterman *et al.*, 2014; Flood *et al.*, 2013; Swapna and Senthilkumaran, 2007) and annual variations in T3/T4 production have been correlated with seasonal reproduction in several fish species (Cowan *et al.*, 2017). TSH and DIO have also been identified in the coronet cells of the SV of masu salmon where abundance of *tsh β* mRNA, *dio2* mRNA and/or the corresponding proteins display light dependent changes *in vivo* and *in vitro* (Nakane and Yoshimura, 2014). Coronet cells also express photopigment genes. Finally, removal of the SV abolished the short-photoperiod induced testicular growth in the fish. In the SV of the Atlantic salmon, both Kiss receptors and GnRH3 transcripts were detected in another cell type than the coronet cells, perhaps the cerebrospinal fluid contacting cells, albeit at lower levels than in the brain (Chi *et al.*, 2017). In addition, transcripts variations mirrored those in the brain in fish maintained under different photoperiodic conditions. Together, these data suggest that the SV contributes to regulating photoperiodic fish reproduction.

Photoperiodism in Fish Farming

The photoperiod control of gametogenesis, reproductive seasonality, and the subsequent interactions between reproduction and growth, have been implemented in broodstock management and performance optimization in commercial fish farming (Migaud *et al.*, 2010). Manipulated photoperiod and temperature regimes are routinely used in commercial broodstock operations to phase-shift the spawning season and obtain eggs all year round (Migaud *et al.*, 2010; Zohar *et al.*, 1995). Additionally, when fish in production systems enter gametogenesis and reproductive development, growth rate is significantly diminished due to a shift in utilization of energy sources toward gonadal growth instead of somatic growth (Good and Davidson, 2016; Taranger *et al.*, 2010). This represents a major obstacle in commercial aquaculture, as many species start gametogenesis before reaching market size. Long photoperiods have been implemented to prevent precocious sexual maturation in large-scale commercial systems, such as floating netpens or land-based tanks, in which submerged or overhead lights are used to extend the duration of the daylight hours (Porter *et al.*, 1999).

Conclusions

The control of reproduction involves a complex network of actors as summarized in Fig. 3. Most of the network components display daily and annual variations so that reproduction events occur at the right time of the day and year. Photoperiod and

temperature are the two main external synchronizers of the system that act through sensors located in different organs and tissues. The pineal gland is one of them. Evidence accumulates indicating that melatonin, its hormonal output, plays a major role in transducing photoperiodism to reproduction, acting at different levels of the network (Fig. 3). Melatonin might be considered a harmonizer or conductor of a concert of rhythms. No generalized model on the effects and roles of melatonin can be drawn; perhaps “several models” exist among the ~30,000 fish species listed. Differences between species have been highlighted and too few have been thoroughly investigated. Also, studies need to be exhaustive, considering sex and age of the fish investigated, time of day and year, water salinity and composition, etc. Indeed, melatonin appears to have multiple and sometimes complex effects on the reproductive system as well as on other neuroendocrine regulations more or less related to reproduction. To date such studies are lacking and attempts targeted mainly aquaculture fish, which do not necessarily behave and function like the wild counterparts. The necessary comparative studies will not only contribute to our understanding of fish biology and evolution, they should also help implementing conservation policies. This is indispensable in light of the ever-increasing anthropogenic pressures that fish are facing. The negative effects of intensive fisheries, physical and chemical pollution, reduction of wild natural spaces and global climate change (ocean acidification, temperature rise) are more than additive and put survival of species into challenge. A better knowledge of fish physiology and behavior will also allow a better management of fisheries policies and benefit the aquaculture industries. The large variability in regulatory systems, sensitivities and responses observed mean that species-specific regimes should be implemented in commercial settings to improve and standardize husbandry practices (e.g., use of light quality and intensity, temperature, etc.) in fish indoors and outdoors aquaculture.

References

- Aggarwal, N., Goswami, S.V., Khandelwal, P., Sehgal, N., 2014. Aromatase activity in brain and ovary: Seasonal variations correlated with circannual gonadal cycle in the catfish, *Heteropneustes fossilis*. *Indian Journal of Experimental Biology* 52, 527–537.
- Alvarado, M.V., Carrillo, M., Felipe, A., 2015. Melatonin-induced changes in kiss/gnrh gene expression patterns in the brain of male sea bass during spermatogenesis. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 185, 69–79.
- Alvarado, M.V., Servili, A., Molés, G., et al., 2016. Actions of sex steroids on kisspeptin expression and other reproduction-related genes in the brain of the teleost fish European sea bass. *Journal of Experimental Biology* 219, 3353–3365.
- Amano, M., Iigo, M., Ikuta, K., et al., 2004. Disturbance of plasma melatonin profile by high dose melatonin administration inhibits testicular maturation of precocious male masu salmon. *Zoological Science* 21, 79–85.
- Amano, M., Iigo, M., Ikuta, K., et al., 2000. Roles of melatonin in gonadal maturation of underyearling precocious male masu salmon. *General and Comparative Endocrinology* 120, 190–197.
- Amano, M., Iigo, M., Kitamura, S., Amiya, N., Yamamori, K., 2006. Changes in melatonin binding sites under artificial light–dark, constant light and constant dark conditions in the masu salmon brain. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 144, 509–513.
- Badruzzaman, M., Bapary, M.A., Takemura, A., 2013. Possible roles of photoperiod and melatonin in reproductive activity via changes in dopaminergic activity in the brain of a tropical damselfish, *Chrysiptera cyanea*. *General and Comparative Endocrinology* 194, 240–247.
- Baker, G.E., de Grip, W.J., Turlon, M., et al., 2015. Light sensitivity in a vertebrate mechanoreceptor? *Journal of Experimental Biology* 218, 2826–2829.
- Bayarri, M., Falcón, J., Zanuy, S., Carrillo, M., 2010. Continuous light and melatonin: Daily and seasonal variations of brain binding sites and plasma concentration during the first reproductive cycle of sea bass. *General and Comparative Endocrinology* 169, 58–64.
- Bayarri, M.J., Rol de Lama, M.A., Madrid, J.A., Sánchez-Vázquez, F.J., 2003. Both pineal and lateral eyes are needed to sustain daily circulating melatonin rhythms in sea bass. *Brain Research* 969, 175–182.
- Bhattacharya, S., Chattoraj, A., Maitra, S.K., 2007. Melatonin in the regulation of annual testicular events in carp *Catla catla*: Evidence from the studies on the effects of exogenous melatonin, continuous light, and continuous darkness. *Chronobiology International* 24, 629–650.
- Binder, T.R., McDonald, D.G., 2008. The role of dermal photoreceptors during the sea lamprey (*Petromyzon marinus*) spawning migration. *Journal of Comparative Physiology A – Neuroethology Sensory Neural and Behavioral Physiology* 194, 921–928.
- Biran, J., Golan, M., Mizrahi, N., et al., 2014. Direct regulation of gonadotropin release by neurokinin B in tilapia (*Oreochromis niloticus*). *Endocrinology* 155, 4831–4842.
- Carnevali, O., Giacchini, G., Maradonna, F., Olivetto, I., Migliarini, B., 2011. Melatonin induces follicle maturation in *Danio rerio*. *PLOS ONE* 6, e19978.
- Chai, K., Liu, X., Zhang, Y., Lin, H., 2013. Day-night and reproductive cycle profiles of melatonin receptor, kiss, and gnrh expression in orange-spotted grouper (*Epinephelus coioides*). *Molecular Reproduction and Development* 80, 535–548.
- Chattoraj, A., Seth, M., Basu, A., et al., 2009a. Temporal relationship between the circulating profiles of melatonin and ovarian steroids under natural photo-thermal conditions in an annual reproductive cycle in carp *Catla catla*. *Biological Rhythm Research* 40, 347–359.
- Chattoraj, A., Seth, M., Maitra, S.K., 2008. Influence of serotonin on the action of melatonin in MIH-induced meiotic resumption in the oocytes of carp *Catla catla*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 150, 301–306.
- Chattoraj, A., Seth, M., Maitra, S.K., 2009b. Localization and dynamics of Mel 1a melatonin receptor in the ovary of carp *Catla catla* in relation to serum melatonin levels. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 152, 327–333.
- Chen, S.C., Robertson, R.M., Hawryshyn, C.W., 2014. Ontogeny of melanophore photosensitivity in rainbow trout (*Oncorhynchus mykiss*). *Biology Open* 3, 1032–1036.
- Chi, L., Li, X., Liu, Q., Liu, Y., 2017. Photoperiod regulate gonad development via kisspeptin/kissr in hypothalamus and *saccus vasculosus* of Atlantic salmon (*Salmo salar*). *PLOS ONE* 12, e0169569.
- Claydon, J.A.B., McCormick, M.I., Jones, G.P., 2014. Multispecies spawning sites for fishes on a low-latitude coral reef: Spatial and temporal patterns. *Journal of Fish Biology* 84, 1136–1163.
- Cowan, M., Azpeleta, C., López-Olmeda, J.F., 2017. Rhythms in the endocrine system of fish: A review. *Journal of Comparative Physiology B – Biochemical Systemic and Environmental Physiology* 1–33.
- Duarte-Guterman, P., Navarro-Martín, L., Trudeau, V.L., 2014. Mechanisms of crosstalk between endocrine systems: Regulation of sex steroid hormone synthesis and action by thyroid hormones. *General and Comparative Endocrinology* 203, 69–85.
- Dufour, S., Sebert, M.E., Weltzien, F.A., Rousseau, K., Pasqualini, C., 2010. Neuroendocrine control by dopamine of teleost reproduction. *Journal of Fish Biology* 76, 129–160.
- Duston, J., Bromage, N., 1991. Circannual rhythms of gonadal maturation in female rainbow-trout (*Oncorhynchus mykiss*). *Journal of Biological Rhythms* 6, 49–53.
- Ekström, P., Meissl, H., 2010. Pineal photoreception and temporal physiology in fish. In: Kulczykowska, E., Popek, W., Kapoor, B.G. (Eds.), *Biological Clock in Fish*. Science Publishers, pp. 35–70.

- Falcón, J., Besseau, L., Manganou, E., *et al.*, 2010b. The pineal organ of fish. In: Kulczykowska, E., Popek, W., Kapoor, B.G., *et al.* (Eds.), *Biological Clock in Fish*. Boca Raton, FL: CRC Press.
- Falcón, J., Migaud, H., Muñoz-Cueto, J.A., Carrillo, M., 2010a. Current knowledge on the melatonin system in teleost fish. *General and Comparative Endocrinology* 165, 469–482.
- Falcón, J., Molina Borja, M., Collin, J.P., Oaknin, S., 1996. Age-related changes in 2-¹²⁵I-iodomelatonin binding sites in the brain of sea breams (*Sparus aurata*, L). *Fish Physiology and Biochemistry* 15, 401–411.
- Flood, D.E., Fernandino, J.I., Langlois, V.S., 2013. Thyroid hormones in male reproductive development: Evidence for direct crosstalk between the androgen and thyroid hormone axes. *General and Comparative Endocrinology* 192, 2–14.
- Gaildrat, P., Falcón, J., 2000. Melatonin receptors in the pituitary of a teleost fish: mRNA expression, 2-¹²⁵I-iodomelatonin binding, and cyclic AMP response. *Neuroendocrinology* 72, 57–66.
- Gaildrat, P., Ron, B., Falcón, J., 1998. Daily and circadian variations in 2-¹²⁵I-iodomelatonin binding sites in the pike brain (*Esox lucius*). *Journal of Neuroendocrinology* 10, 511–517.
- Good, C., Davidson, J.A., 2016. A review of factors influencing maturation of atlantic salmon, *salmo salar*, with focus on water recirculation aquaculture system environments. *Journal of the World Aquaculture Society* 47, 605–632.
- Gothilf, Y., Muñoz-Cueto, J.A., Sagrillo, C.A., *et al.*, 1996. Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): Complementary deoxyribonucleic acid characterization and brain localization. *Biology of Reproduction* 55, 636–645.
- Hernandez-Rauda, R., Miguez, J.M., Ruibal, C., Aldegunde, M., 2000. Effects of melatonin on dopamine metabolism in the hypothalamus and the pituitary of the rainbow trout, *Oncorhynchus mykiss*. *Journal of Experimental Zoology* 287, 440–444.
- Herrera-Perez, P., Del Carmen Rendon, M., Besseau, L., *et al.*, 2010. Melatonin receptors in the brain of the European sea bass: An in situ hybridization and autoradiographic study. *Journal of Comparative Neurology* 518, 3495–3511.
- Iigo, M., Furukawa, K., Tabata, M., Aida, K., 2003. Circadian variations of melatonin binding sites in the goldfish brain. *Neuroscience Letters* 347, 49–52.
- Iigo, M., Kobayashi, M., Ohtani-Kaneko, R., *et al.*, 1994. Characteristics, day-night changes, subcellular distribution and localization of melatonin binding sites in the goldfish brain. *Brain Research* 644, 213–220.
- Ikegami, T., Motohashi, E., Doi, H., Hattori, A., Ando, H., 2009. Synchronized diurnal and circadian expressions of four subtypes of melatonin receptor genes in the diencephalon of a puffer fish with lunar-related spawning cycles. *Neuroscience Letters* 462, 58–63.
- Juntti, S.A., Fernald, R.D., 2016. Timing reproduction in teleost fish: Cues and mechanisms. *Current Opinion in Neurobiology* 38, 57–62.
- Khan, I.A., Thomas, P., 1996. Melatonin influences gonadotropin II secretion in the Atlantic croaker (*Micropogonias undulatus*). *General and Comparative Endocrinology* 104, 231–242.
- Leatherland, J.F., Lin, L., 2001. Melatonin effects on steroid production by rainbow trout ovarian follicles in vitro: Influence of stage of follicle maturation. *Biological Rhythm Research* 32, 557–568.
- Martinez-Chávez, C.C., Migaud, H., 2009. Retinal light input is required to sustain plasma melatonin rhythms in Nile tilapia *Oreochromis niloticus niloticus*. *Brain Research* 1269, 61–67.
- Mazurais, D., Brierley, I., Anglade, I., *et al.*, 1999. Central melatonin receptors in the rainbow trout: Comparative distribution of ligand binding and gene expression. *Journal of Comparative Neurology* 409, 313–324.
- Migaud, H., Davie, A., Taylor, J.F., 2010. Current knowledge on the photoneuroendocrine regulation of reproduction in temperate fish species. *Journal of Fish Biology* 76, 27–68.
- Molina-Borja, M., Falcón, J., Urquiola, E., Oaknin, S., 1994. Characterization of 2-¹²⁵I-iodomelatonin binding sites in the brain, intestine and gonad of the gilthead sea bream (*Sparus aurata*). *Pflügers Arch* 427, R5.
- Nakane, Y., Yoshimura, T., 2014. Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Frontiers in Neuroscience* 8.
- Nikaido, Y., Aluru, N., McGuire, A., *et al.*, 2010. Effect of cortisol on melatonin production by the pineal organ of tilapia, *Oreochromis mossambicus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 155, 84–90.
- Oliveira, C., Sánchez-Vázquez, F.J., 2010. Reproduction rhythms in fish. In: Kulczykowska, E., Popek, W., Kapoor, B.G. (Eds.), *Biological Clock in Fish*. Enfield: Science Publishers, pp. 185–215.
- Park, Y.-J., Park, J.-G., Takeuchi, Y., *et al.*, 2014. Influence of moonlight on mRNA expression patterns of melatonin receptor subtypes in the pineal organ of a tropical fish. *Marine Genomics* 14, 67–70.
- Paullada-Salmerón, J.A., Cowan, M., Aliaga-Guerrero, M., *et al.*, 2016. Gonadotropin inhibitory hormone down-regulates the brain-pituitary reproductive axis of male European sea bass (*Dicentrarchus labrax*). *Biology of Reproduction* 94, 1–11.
- Peng, W., Cao, M., Chen, J., *et al.*, 2016. GnIH plays a negative role in regulating GtH expression in the common carp, *Cyprinus carpio* L. *General and Comparative Endocrinology* 235, 18–28.
- Popek, W., Drag-Kozak, E., Luszczek-Trojnar, E., 2010a. Influence of melatonin on hypothalamo-pituitary-ovarian axis in carp; mechanism and action. In: Kulczykowska, E., Popek, W., Kapoor, B.G. (Eds.), *Biological Clock in Fish*, pp. 217–234.
- Popek, W., Natane, H., Luszczek-Trojnar, E., 2010b. Circadian and seasonal rhythm in secretion of melatonin, dopamine and LH in carp. In: Kulczykowska, E., Popek, W., Kapoor, B.G. (Eds.), *Biological Clock in Fish*. Enfield, New Hampshire: Science Publishers, pp. 235–250.
- Porter, M., Duncan, N., Mitchell, D., Bromage, N., 1999. The use of cage lighting to reduce plasma melatonin in Atlantic salmon (*Salmo salar*) and its effects on the inhibition of grilising. *Aquaculture* 176, 237–244.
- Powell, J., Zohar, Y., Elizur, A., *et al.*, 1994. Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proceedings of the National Academy of Sciences* 91, 12081–12085.
- Prat, F., Zanuy, S., Bromage, N., Carrillo, M., 1999. Effects of constant short and long photoperiod regimes on the spawning performance and sex steroid levels of female and male sea bass. *Journal of Fish Biology* 54, 125–137.
- Servili, A., Herrera-Pérez, P., del Carmen Rendón, M., Muñoz-Cueto, J.A., 2013. Melatonin inhibits GnRH-1, GnRH-3 and GnRH receptor expression in the brain of the European sea bass, *Dicentrarchus labrax*. *International Journal of Molecular Sciences* 14, 7603–7616.
- Shimizu, A., 2003. Effect of photoperiod and temperature on gonadal activity and plasma steroid levels in a reared strain of the mummichog (*Fundulus heteroclitus*) during different phases of its annual reproductive cycle. *General and Comparative Endocrinology* 131, 310–324.
- Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: Status and perspectives. In: Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), *Perspectives in Comparative Endocrinology*. Ottawa, ON: The National Research Council of Canada, pp. 304–315.
- Spicer, O.S., Zmora, N., Wong, T.-T., *et al.*, 2017. The gonadotropin-inhibitory hormone (Lpxrfa) system's regulation of reproduction in the brain–pituitary axis of the zebrafish (*Danio rerio*). *Biology of Reproduction* 96, 1031–1042.
- Swapna, I., Senthikumar, B., 2007. Thyroid hormones modulate the hypothalamo–hypophyseal–gonadal axis in teleosts: Molecular insights. *Fish Physiology and Biochemistry* 33, 335–345.
- Takemura, A., Uchimura, M., Shibata, Y., 2010. Dopaminergic activity in the brain of a tropical wrasse in response to changes in light and hydrostatic pressure. *General and Comparative Endocrinology* 166, 513–519.
- Taranger, G.L., Carrillo, M., Schulz, R.W., *et al.*, 2010. Control of puberty in farmed fish. *General and Comparative Endocrinology* 165, 483–515.
- Whitmore, D., 2010. Cellular clocks and the importance of light in zebrafish. *Biological Clock in Fish*. Lebanon: Science Publisher, pp. 125–153.

- Yáñez, J., Busch, J., Anadón, R., Meissl, H., 2009. Pineal projections in the zebrafish (*Danio rerio*): Overlap with retinal and cerebellar projections. *Neuroscience* 164, 1712–1720.
- Yanthan, L., Gupta, B., 2007. In vitro effects of steroid hormones on arylalkylamine N-acetyltransferase (AA-NAT) activity in the pineal of fish, *Clarias gariepinus* (Burchell, 1822) during different phases of breeding cycle. In *IJEB* p. 12.
- Yumnamcha, T., Khan, Z.A., Rajiv, C., *et al.*, 2017. Interaction of melatonin and gonadotropin-inhibitory hormone on the zebrafish brain-pituitary-reproductive axis. *Molecular Reproduction and Development* 84, 389–400.
- Zmora, N., Stubblefield, J., Golan, M., *et al.*, 2014. The medio-basal hypothalamus as a dynamic and plastic reproduction-related kisspeptin-GnRH-pituitary center in fish. *Endocrinology* 155, 1874–1886.
- Zmora, N., Stubblefield, J.D., Wong, T.-T., *et al.*, 2015. Kisspeptin antagonists reveal kisspeptin 1 and kisspeptin 2 differential regulation of reproduction in the teleost, *Morone saxatilis*. *Biology of Reproduction* 93 (76), 71. 12.
- Zmora, N., Wong, T.-T., Stubblefield, J., Levavi-Sivan, B., Zohar, Y., 2017. Neurokinin B regulates reproduction via inhibition of kisspeptin in a teleost, the striped bass. *Journal of Endocrinology* 233, 159–174.
- Zohar, Y., Billard, R., 1984. Annual and daily changes in plasma gonadotropin and sex steroids in relation to teleost gonad cycles. *Transactions of the American Fisheries Society* 113, 444–451.
- Zohar, Y., Harel, M., Hassin, S., Tandler, A., 1995. Broodstock management and manipulation of spawning in the gilthead seabream. In: Bromage, N., Roberts, R.J. (Eds.), *Broodstock Management and Egg and Larval Quality*. Boston: Blackwell Science, pp. 94–117.
- Zohar, Y., Muñoz-Cueto, J.A., Elizur, A., Kah, O., 2010. Neuroendocrinology of reproduction in teleost fish. *General and Comparative Endocrinology* 165, 438–455.

Further Reading

- Berthelot, C., Brunet, F., Chalopin, D., *et al.*, 2014. The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nature Communications* 5.
- Bromage, N., Porter, M., Randall, C., 2001. The environmental regulation of maturation in farmed finfish with special reference to the role of photoperiod and melatonin. *Aquaculture* 197, 63–98.
- Falcón, J., 1999. Cellular circadian clocks in the pineal. *Progress in Neurobiology* 58, 121–162.
- Falcón, J., Besseau, L., Sauzet, S., Boeuf, G., 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. *Trends in Endocrinology and Metabolism* 18, 81–88.
- Iigo, M., Furukawa, K., Hattori, A., *et al.*, 1995. Effects of pinealectomy and constant light exposure on day-night changes of melatonin binding sites in the goldfish brain. *Neuroscience Letters* 197, 61–64.
- Lubzens, E., Young, G., Bobe, J., Cerdà, J., 2010. Oogenesis in teleosts: How fish eggs are formed. *General and Comparative Endocrinology* 165, 367–389.
- Oppedal, F., Taranger, G.L., Juell, J.-E., Fosseidengen, J.E., Hansen, T., 1997. Light intensity affects growth and sexual maturation of Atlantic salmon (*Salmo salar*) postsmolts in sea cages. *Aquatic Living Resources* 10, 351–357.
- Schulz, R.W., De França, L.R., Lareyre, J.-J., *et al.*, 2010. Spermatogenesis in fish. *General and Comparative Endocrinology* 165, 390–411.
- Tsutsui, K., Bentley, G., Kriegsfeld, L.J., *et al.*, 2010. Discovery and evolutionary history of gonadotrophin-inhibitory hormone and kisspeptin: New key neuropeptides controlling reproduction. *Journal of Neuroendocrinology* 22, 716–727.

Relevant Websites

- <http://www.fao.org/docrep/x5742e/x5742e00.htm#Contents>
FAO Corporate Document Repository.
- <http://www.fao.org/fishery/en>
Food and Agriculture Organization of the United Nations.

Seasonal Reproduction: Photoperiodism, Birds

Yusuke Nakane and Takashi Yoshimura, Nagoya University, Chikusa-ku, Nagoya, Japan

© 2018 Elsevier Inc. All rights reserved.

Photoperiodism and Seasonal Reproduction in Birds

In mid to high latitude regions, climatic conditions such as temperature, humidity, rainfall, and day-length, fluctuate dramatically throughout the year. Of these conditions, changes in day-length are precise and predictable. Therefore, animals inhabiting these regions exhibit sophisticated physiological and behavioral responses (molting, migration, etc.) to changing day-length (photoperiod), using it as a calendar, so to speak. This phenomenon is called "photoperiodism". In early spring, songbirds sing to attract females and declare territorial demarcations. In alpine regions, rock ptarmigans completely change the color of their feathers throughout the year. Several types of birds (e.g., the Arctic tern) are migratory and generally follow the same annual migratory routes. In addition, offspring of birds and other animals hatch or are born in spring through summer because of the abundance of food and mild climates. The time at which mating occurs in animals is dependent on their inherent period of egg incubation or gestation, where the precise timing of mating events accounts for the length of the incubation/gestational period (i.e., the longer the incubation/gestational period, the earlier the mating event). This reproductive strategy is known as "seasonal reproduction." Hamsters, medaka fish, etc. mate in the spring/summer and are called long-day seasonal breeders; while sheep, emu, masu salmon, etc. mate in fall/winter and are called short-day seasonal breeders. Generally, most non-tropical birds are long-day seasonal breeders owing to the length of the egg incubation period.

A Model Animal for Avian Photoperiodism

As previously described, most avian species are long-day seasonal breeders, and begin their reproductive activity when the photoperiod elongates. It is known that the photoperiodic responses of avian gonads are very robust and rapid. Avian species exhibit seasonal changes in their gonads, where the weight of the testes can increase or decrease more than one hundred-fold within a few weeks.

The first study on photoperiodism in vertebrates was reported in avian species by Rowan in 1925. Rowan demonstrated that gonadal development in the dark-eyed junco, a non-tropical bird, occurred in response to changes in photoperiod (Rowan, 1925). Japanese quails are ideal subjects for studying the mechanisms of seasonal reproduction because of their availability, ease of care, and drastic photoperiodic response. They rapidly increase plasma levels of hormones that stimulate the activity of the gonads (i.e., gonadotropins), such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in response to the changing length of the photoperiod. For instance, plasma LH concentration increases on the first day that there is a shift from short-day conditions to long-day conditions (Nakao *et al.*, 2008). Remarkably, after only two weeks of photoperiod elongation, the weight of their testes had reached full size.

It is thought that photoperiodism consists essentially of three systems, (1) photoreceptors which detect the light information of photoperiod, (2) circadian clocks which measure the length of photoperiod, and (3) endocrine system which transmit the light information to internal endocrine information (e.g., hormones). Japanese quails have been used in studies about these all systems owing to their most appropriate natures.

Relationship Between Mediobasal Hypothalamus and Photoperiodism

Series of studies using Japanese quails have revealed that the mediobasal hypothalamus (MBH) is the key region in the brain in the control of photoperiodic responses (Fig. 1). Lesions in the MBH have been shown to block the photoperiodic response under long-day conditions (Sharp and Follett, 1969). On the other hand, electric stimuli to the MBH enhanced plasma LH concentrations. The protein Fos, which is encoded by an immediate early gene *c-fos* and is often used as a marker for neuronal activity following stimulation, was reported to be induced in the MBH in response to a single long-day stimulus (Meddle and Follett, 1995). Therefore, the MBH has been considered to be the key region for photoperiodism in avian brain.

Local Activation of Thyroid Hormones in the Mediobasal Hypothalamus

Thyroid hormones are thought to regulate the plasticity and development of the central nervous system. The involvement of thyroid hormones in seasonal reproduction has been a focus of scientists for a long time, and it has been suggested that the responses of birds to prolonged photoperiod are related to changes in thyroid hormones. It has been reported that removing the thyroid gland prevented a seasonal reproductive response, while injecting thyroid hormones enhanced LH secretion and gonadal development in several species of vertebrates. Thyroxine (T₄), a prohormone of thyroid hormones, is catalyzed to the bioactive form, 3,5,3'-triiodothyronine (T₃) by type 2 deiodinase (DIO2), whereas T₃ is catalyzed to inactive diiodothyronine (T₂) by type 3 deiodinase

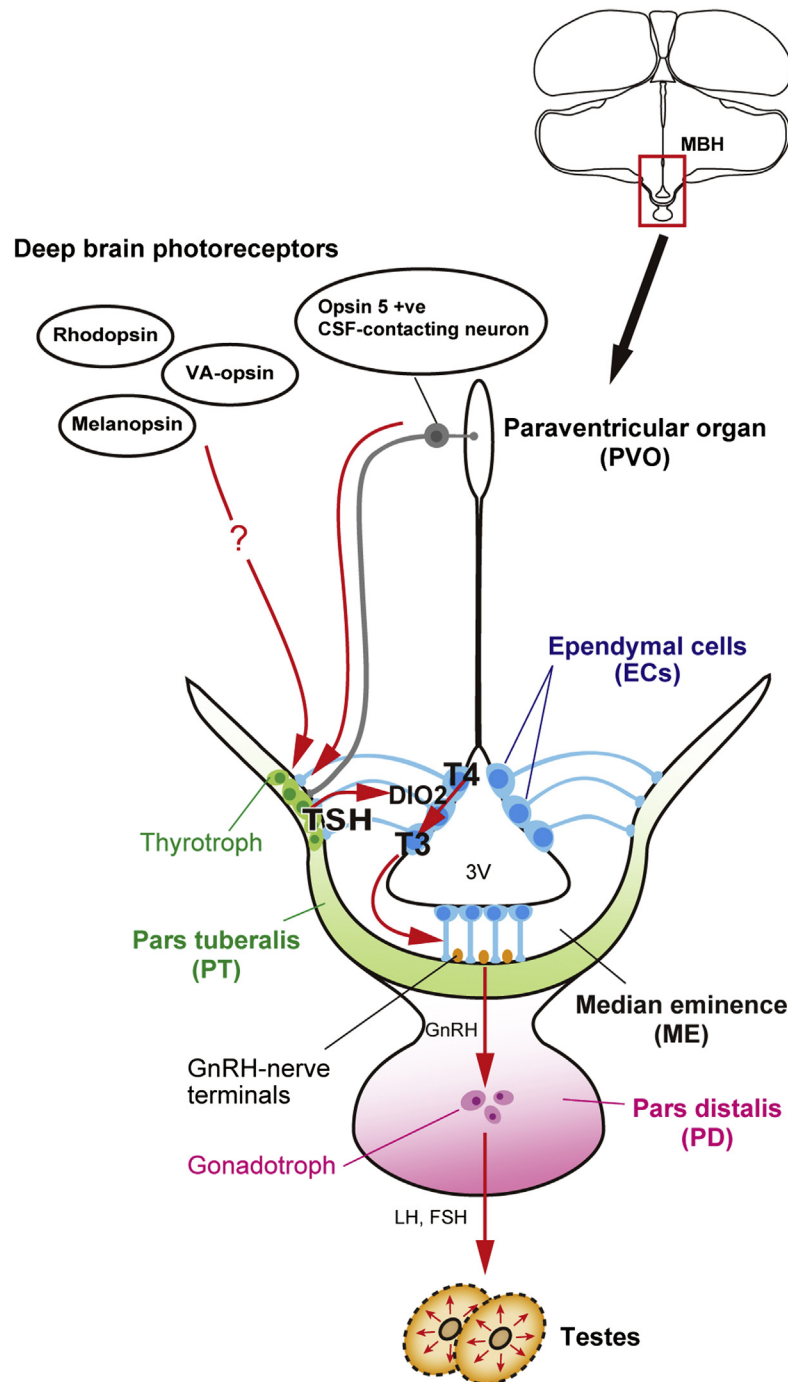


Fig. 1 In avian species, the mediobasal hypothalamus (MBH) is thought to be a key brain region in photoperiodism. Birds detect light information for photoperiod via deep brain photoreceptors where the photosensitive proteins, called opsins (rhodopsin, melanopsin, VA-opsin, and Opsin 5) are expressed. Light information is delivered to the pars tuberalis of the pituitary gland (PT) and induces the secretion of thyroid-stimulating hormone (TSH) to the MBH. TSH triggers an increase in DIO2 expression in the ependymal cells (ECs) and the local activation of thyroid hormones (T4 to T3 conversion). Activated thyroid hormone T3 alters a morphological change in GnRH-nerve terminals and glial cells, and photoperiodically control GnRH secretion to gonadotrophs in the pars distalis of the pituitary gland (PD). (Modified from Nakane, Y., Yoshimura, T., 2014. Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Front Neurosci.* 21(8), 115. doi: 10.3389/fnins.2014.00115. eCollection 2014.)

(DIO3). Interestingly, it has been found that *DIO2* mRNA increases in ependymal cells (ECs)-which form the epithelial lining of the ventricles in the brain and the central canal of the spinal cord-in the third ventricle of MBH, showing that T3 levels are light-induced within the MBH in quails under long-day conditions. In addition, thyroid hormone receptors, such as TR α and TR β are expressed in the MBH in quails (Yoshimura *et al.*, 2003).

In the median eminence (ME), (the part of the hypothalamus that is responsible for the release of regulatory gonadotropin-releasing hormone (GnRH)), within the MBH, there are abundant quantities of GnRH-nerve terminals projecting to the basal lamina (basement membrane) adjacent to the pituitary gland. It is thought that GnRH secretion is under the control of GnRH-nerve terminals rather than the somata of GnRH neurons. In quails, it has been observed that most GnRH-nerve terminals are encased in glial cells and do not approach the basal lamina under short-day conditions, while the nerve terminals directly contact the basal lamina under long-day conditions. It is also worth noting that the concentration of GnRH-nerve terminals does not appear to change between short-day conditions and long-day conditions. Interestingly, T3 implantation into the MBH of the quail under short-day conditions mimics the aforementioned morphological changes in GnRH-nerve terminals and glial cells (Yamamura *et al.*, 2006). It is thought that the local activation of thyroid hormone within the MBH causes GnRH-nerve terminals to approach to the pituitary gland, resulting in the photoperiod-dependent acceleration of GnRH secretion. The morphological plasticity observed in the GnRH-nerve terminals and glial cells of quails has also been observed during the reproductive cycle in rats and sheep. It is possible that the local elevation of active thyroid hormone in the MBH is a key event in seasonal reproduction in vertebrates (Fig. 1).

Thyroid-Stimulating Hormone Triggers Local Thyroid Hormone Activation

The analysis of wide range of genes expressed in the MBH have revealed that mRNA coding the beta subunit of thyroid-stimulating hormone (*TSHB*) is induced in the pars tuberalis (tuberal part) of the pituitary gland (PT) approximately 16 h after dawn in quails that have shifted from short-day to long-day conditions. After the temporal increase of *TSHB* mRNA, the expression of *DIO2* mRNA is also photoinduced in the ECs expressing the TSH receptor (TSHR). The intraventricular injection of TSH into quails under short-day conditions resulted in the induction of *DIO2* mRNA in the ECs (Nakao *et al.*, 2008). Thyrotrophs are cells that are responsible for the production, secretion of TSH, and they are distributed in the pars distalis (distal part) of the pituitary gland (PD), as well as in the PT. Thyrotrophs are thought to essentially be under the control of thyrotropin-releasing hormone (TRH), which is derived from part of the hypothalamus. Interestingly, the application of TRH or T4 can alter the expression of *TSHB* mRNA in the PD, but not in the PT, suggesting that TSH in the PT is under the control of changing the length of photoperiod, not of TRH. Therefore, at dusk of the first day of transfer to long-day conditions, TSH derived from the PT triggers the local activation of thyroid hormone through *DIO2* expression in the MBH in avian species. However, the transport system of PT- derived TSH to the ECs remains unclear (Fig. 1).

Detection Light Information Via Deep Brain Photoreceptors

In mammals, it is believed that the eye is the only photoreceptive organ, whereas non-mammalian vertebrates have extraretinal photoreceptors located in pineal organs and deep brain regions. It is thought that avian species detect light via deep brain photoreceptors. Historically, the existence of deep brain photoreceptors has been first indicated in the European minnow. The first study reporting deep brain photoreceptors in birds was performed by Benoit (1935), where ducks with black caps covering their heads did not exhibit photoperiodic responses in their gonads (Benoit, 1935). In addition, house sparrows that had black ink injected under their head skins also failed to exhibit any photoperiodic response in their gonads. Gonadal development takes place even in blinded and pinealectomized quails, and local illumination to several deep brain regions using light fibers and light-emitting beads elicited testicular development in sparrows and Japanese quails. It is worth noting that a broad range of wavelengths of light can penetrate the skin, and it has been suggested that light can also penetrate the skull and into the brains of various vertebrates including birds (Hartwig and van Veen, 1979). In fact, it has reported that various photosensitive proteins, called opsins, are expressed in deep brain regions of various avian species. The expression of rhodopsin, which is responsible for visual system in the retina, has been found in the area of forebrain, called lateral septum organ (LSO), in pigeons (Fig. 2(A)). In addition, the expression of rhodopsin in the brain has been reported in other avian species such as ring doves, ducks, and quails. Melanopsin, which is expressed in the intrinsically photosensitive retinal ganglion cells (ipRGCs) of the retina, is localized in the forebrain and the hypothalamus of chicken and turkey. Vertebrate ancient (VA)-opsin is also known to be expressed in the hypothalamus of chicken. Opsin 5 was reported to be localized in the cerebrospinal fluid (CSF)-contacting neurons of the paraventricular organ (PVO) in Japanese quails and chicken (Nakane *et al.*, 2010). The PVO is located within the MBH, where lesions around the PVO attenuate photoperiodic testicular development in Japanese quails (Sharp and Follett, 1969). CSF-contacting neurons, which are also present in the LSO, have been considered to be candidate deep brain photoreceptors

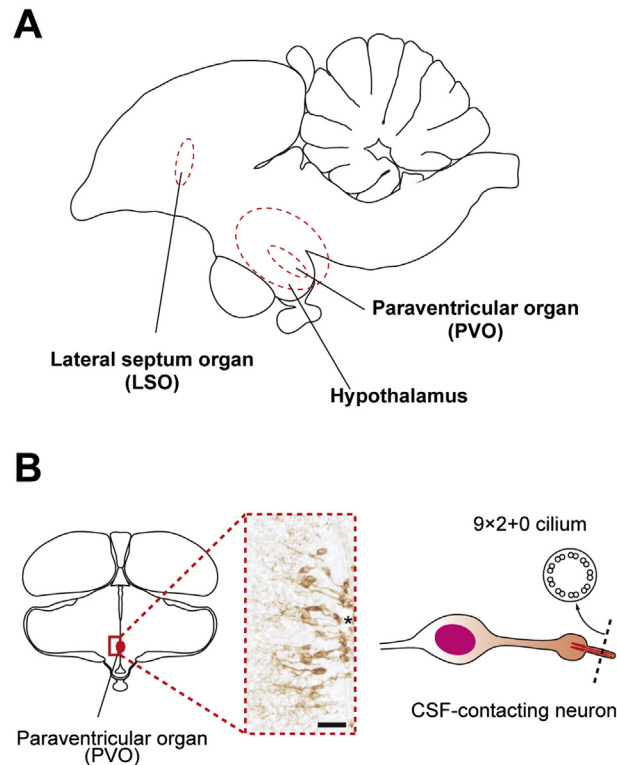


Fig. 2 (A) It has been reported that avian species have photoreceptors in several brain regions (red ellipses). Cerebrospinal fluid (CSF)-contacting neurons are distributed in the lateral septum organ (LSO) and paraventricular organ (PVO). (B) Representative image for Opn5-expressing CSF-contacting neurons and schematic image for CSF-contacting neurons in the paraventricular organ (PVO). A picture of cells stained brown shows where the Opn5 protein is expressed. They possess 9×2+0 cilium. Rod cells, cone cells, and photoreceptive cells in the pineal organ have the same cilium. Scale bars indicate 20 μm . (Modified from Nakane, Y., Yoshimura, T., 2014. Universality and diversity in the signal transduction pathway that regulates seasonal reproduction in vertebrates. *Front Neurosci.* 21(8), 115. doi: 10.3389/fnins.2014.00115. eCollection 2014.

because of their unique morphological nature. CSF-contacting neurons have knob-like terminals, and are thought to morphologically resemble photoreceptive cells in developing retina (Vigh-Teichmann *et al.*, 1980; Fig. 2(B)). In fact, it has been reported that Opn5-expressing CSF-contacting neurons have intrinsic electrophysiological photosensitivity (Nakane *et al.*, 2014).

Circadian Clocks and Photoperiodism

In Japanese quails, when the light period exceeds 12 h, gonads begin to develop. This threshold photoperiod is called the “critical photoperiod.” The critical photoperiod has also been observed in starlings. On the other hand, consecutive and long photoperiods are not necessarily required for gonadal development. Photostimuli in the afternoon are still able to promote gonadal development in finches under short-day conditions (Fig. 3; Hamner, 1963). This photosensitive time zone is called the “photoinducible phase”. White-crowned sparrows under constant darkness are still able to develop their gonads when exposed to a photostimulus early in the evening, where photoinducible phase still occurs in a 24-h cycle. This means that the emergence of the photoinducible phase is under the control of an endogenous circadian clock (also known as circadian rhythm). It is well known that the circadian clock occurs via negative autoregulatory feedback loops owing to the transcription/translation of clock genes (e.g., *CLOCK*, *BMAL1*, *PER*, *CRY*, etc.). Clock genes in vertebrates were first reported in mammalian species. In mammals, the suprachiasmatic nucleus (SCN), which located in the dorsal region of the optic chiasma, and is known to be the only master clock of circadian rhythm. In contrast, in avian species, it is thought the pineal organ and retina are the master clocks of circadian rhythm along with the SCN. These master clocks have been reported not to affect photoperiodism, which indicates the possibility that alternative clocks define the photoinducible phase. Although changing the length of photoperiod does not affect the expression of clock genes in the MBH, the expression of the clock gene *CRY1* in the PT is induced when photostimulus is exposed to photoinducible phase (Yasuo *et al.*, 2004). It is possible that clock genes expressed in this region trigger the emergence of photoinducible phase. Despite all we know, the mechanisms by which avian species, as well as the other vertebrates, measure the length of photoperiod still remain a huge mystery.

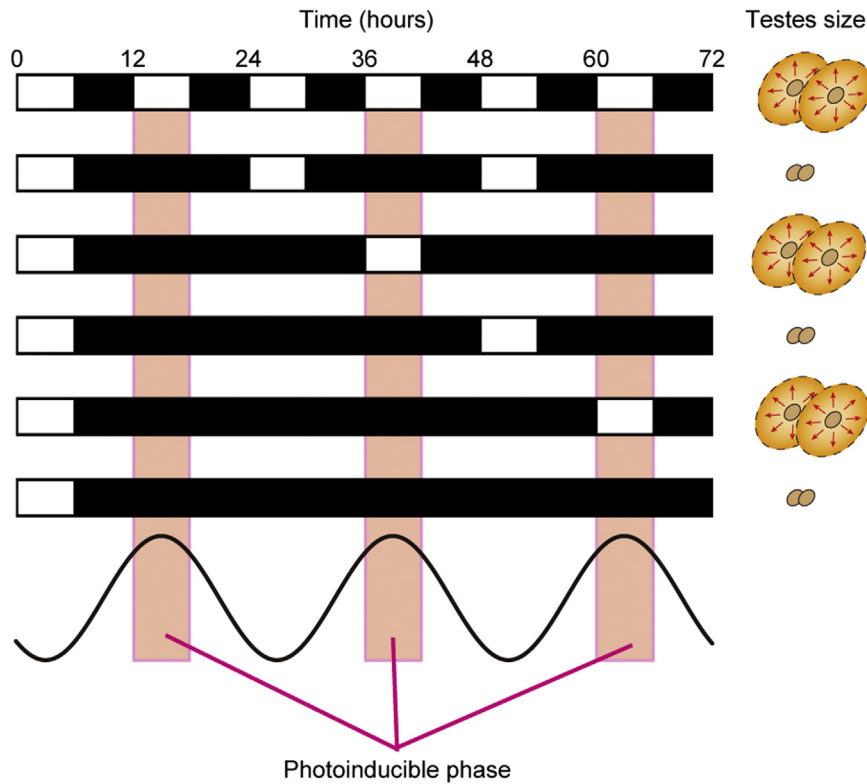


Fig. 3 Schematic drawing for photoinducible phase. A single white-colored box indicates 6-h light period, whereas black-colored box or bar means dark period. The effect of 6-h photostimulus exposed to house finch at various intervals after entry to darkness on testicular size (Hamner, 1963). Photoinducible phase where testes develop in response to the photostimulus emerges in approximately a 24-h cycle. Photoinducible phase is thought to be governed by an endogenous circadian clock. However, the detailed mechanism is yet unclear in any vertebrate species.

Seasonal Gonadal Regression

“Photorefractoriness” is the eventual regression of gonads as a result of a prolonged long-day conditions. At higher latitudes, where the breeding season is shorter, non-tropical species show remarkable photorefractoriness. In addition, a recent study indicated that short days and low temperature affect seasonal testicular regression in quails, where short-day stimuli lead to a reduction in testicular weight and low-temperature accelerates this regression. The more testicular growth decreased, the more plasma T3 concentration increased. Likewise, the daily peripheral administration of T3 to quails also caused testicular mass to decrease. These findings indicate that low temperature induces the increase in the plasma T3, as well as subsequent seasonal gonadal regression (Ikegami *et al.*, 2015).

Conclusion

In avian photoperiodism, long-day information detected via deep brain photoreceptors, (not eyes and pineal organs) stimulate the expression of TSH in the PT. Photoinduced TSH secreted from the PT has a role as a master regulator for local activation of thyroid hormone within the MBH. Activated thyroid hormone is thought to trigger the morphological change of GnRH-nerve terminals and control the seasonal change of GnRH secretion. However, mechanism involved in measuring the length of photoperiod remains a huge enigma not only in avian species but also in other vertebrates. Although seasonal reproduction is essentially under the control of photoperiod, seasonal regression in gonads seems to be involved in both photoperiod and temperature.

References

- Benoit, J. (1935). Le rôle des yeux dans l'action stimulante de la lumière sur le développement testiculaire chez le canard. *Comptes Rendus des Seances de la Societe de Physique et de ses Filiales*, 118, 669–671.
- Hartwig, H. G., & van Veen, T. (1979). Spectral characteristics of visible radiation penetrating into the brain and stimulating extraretinal photoreceptors. *Journal of Comparative Physiology*, 130, 277–282.
- Hamner, W. M. (1963). Diurnal rhythm and photoperiodism in testicular recrudescence of the house finch. *Science*, 142, 1294–1495.

- Ikegami, K., Atsumi, Y., Yorinaga, E., et al. (2015). Low temperature-induced circulating triiodothyronine accelerates seasonal testicular regression. *Endocrinology*, 156, 647–659.
- Meddle, S. L., & Follett, B. K. (1995). Photoperiodic activation of fos-like immunoreactive protein in neurones within the tuberal hypothalamus of Japanese quail. *Journal of Comparative Physiology A*, 176, 79–89.
- Nakane, Y., Ikegami, K., Ono, H., et al. (2010). A novel mammalian neural tissue opsin (Opsin5) is a deep brain photoreceptor in birds. *Proceedings of the National Academy of Sciences of the United States of America*, 107, 15264–15268.
- Nakane, Y., Shimmura, T., Abe, H., & Yoshimura, T. (2014). Intrinsic photosensitivity of a deep brain photoreceptor. *Current Biology*, 24, R596–R597.
- Nakao, N., Ono, H., Yamamura, T., et al. (2008). Thyrotrophin in the pars tuberalis triggers photoperiodic response. *Nature*, 452, 317–322.
- Rowan, W. (1925). Relation of light to bird migration and developmental changes. *Nature*, 115, 4944–4995.
- Sharp, P. J., & Follett, B. K. (1969). The effect of hypothalamic lesions on gonadotrophin release in Japanese quail (*Coturnix coturnix japonica*). *Neuroendocrinology*, 5, 205–218.
- Vigh-Teichmann, I., Röhlich, P., Vigh, B., & Aros, B. (1980). Comparison of the pineal complex, retina and cerebrospinal fluid contacting neurons by immunocytochemical anti-rhodopsin reaction. *Zeitschrift für mikroskopisch-anatomische Forschung*, 94, 623–640.
- Yamamura, T., Yasuo, S., Hirunagi, K., Ebihara, S., & Yoshimura, T. (2006). T₃ implantation mimics photoperiodically reduced encasement of nerve terminals by glial processes in the median eminence of Japanese quail. *Cell and Tissue Research*, 324, 175–179.
- Yasuo, S., Watanabe, M., Tsukada, A., et al. (2004). Photoinducible phase-specific light induction of *Cry1* gene in the pars tuberalis of Japanese quail. *Endocrinology*, 145, 1612–1616.
- Yoshimura, T., Yasuo, S., Watanabe, M., et al. (2003). Light-induced hormone conversion of T₄ to T₃ regulates photoperiodic response of gonads in birds. *Nature*, 426, 178–181.

Photoperiodism in Mammalian Reproduction

Kazuyoshi Tsutsui, Waseda University, Tokyo, Japan

Takayoshi Ubuka, Monash University Malaysia, Selangor, Malaysia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Arginine vasopressin (AVP) A form of a peptide, vasopressin, in which the eighth amino acid residue of the total nine amino acid residues is an arginine residue.

Arylalkylamine N-acetyltransferase (AANAT) An enzyme that is involved in the production of melatonin from serotonin.

Clock genes Circadian clock genes, including Period (Per) 1, 2, 3, Cryptochrome (Cry), Brain and muscle ARNT-like 1 (Bmal1), and Circadian locomotor output cycle kaput (Clock).

Cyclic adenosine monophosphate (cAMP) An intracellular signaling molecule important in many biological processes. cAMP is a derivative of adenosine triphosphate (ATP).

Dopamine A catecholamine that plays important roles in the brain such as reward and motor control.

Estradiol A steroid hormone that regulates estrous and menstrual cycles. Estradiol is produced in ovaries, but testosterone can be aromatized into estradiol.

Gonadotropin Glycoprotein polypeptide hormones, including follicle-stimulating hormone (FSH) and luteinizing hormone (LH), secreted by gonadotrope cells of the anterior pituitary gland.

Gonadotropin-inhibitory hormone (GnIH) A hypothalamic neuropeptide that suppresses gonadotropin synthesis and release from the anterior pituitary gland.

Gonadotropin-releasing hormone (GnRH) A hypothalamic neuropeptide that stimulates gonadotropin synthesis and release from the anterior pituitary gland.

Hypophyseal portal system Blood vessels that connects the median eminence in the hypothalamus with the anterior pituitary gland. Neuropeptides released in the median eminence are transported to the anterior pituitary gland through this system.

Hypothalamic-pituitary-gonadal (HPG) axis Hypothalamic neuropeptides, GnRH, GnIH and kisspeptin, control gonadotropin secretion from the anterior pituitary gland, and gonadotropin regulates estradiol and testosterone secretion as well as oogenesis and spermatogenesis in the ovary and testis, respectively.

Kisspeptin A hypothalamic neuropeptide that is involved in the stimulatory regulation of GnRH neurons.

Melanopsin A photopigment in retinal ganglion cells that communicate directly to the suprachiasmatic nucleus (SCN) in the brain.

Melatonin A hormone produced in the pineal gland and involved in the regulation of circadian rhythms, including sleep-wakefulness and seasonal reproduction.

Noradrenaline A catecholamine that plays important roles in the brain such as arousal and fight-or-flight response.

Pars tuberalis A part of the anterior pituitary covering the pituitary stalk.

Pineal gland An endocrine gland located in the epithalamus and produces melatonin.

Suprachiasmatic nuclei (SCN) A region in the hypothalamus above the optic chiasm. SCN generates circadian rhythms by circadian transcription of clock genes.

Testosterone A steroid hormone responsible for development of male reproductive organs and behavior.

Thyroid hormones Hormones produced by the thyroid gland, including triiodothyronine (T_3) and its prohormone thyroxine (T_4), responsible for regulation of metabolism.

Thyroid-stimulating hormone (TSH) An anterior pituitary hormone that stimulates the thyroid gland to produce T_4 and T_3 .

Type 2 deiodinase (Dio2) An enzyme that converts T_4 to T_3 .

Type 3 deiodinase (Dio3) An enzyme that inactivates T_4 and T_3 .

Vasoactive intestinal polypeptide (VIP) A vasoactive peptide hormone produced in many tissues including the gut, pancreas, and SCN of the hypothalamus.

Introduction

Photoperiodism is an organisms' ability to adjust their physiology and behavior to seasonal changes in the environment according to the length of day (photoperiod), because photoperiod is the most reliable information to detect the time of year. Many mammals that live in high latitude use photoperiod to time breeding so that mothers can raise their offspring when foods are available and the climate is favorable, which can maximize the survival and growth of the offspring. Long day (LD) breeders include relatively large animals such as horse and bear that have gestation period of about a year and relatively small animals such as hamsters that have

short gestation period of few weeks so that they can deliver their baby in spring or summer. On the other hand, short day (SD) breeders include medium size animals such as sheep and goats that have gestation periods of about half a year so that they can deliver their baby in spring.

Sunlight is detected in eyes and photic information is transmitted to the suprachiasmatic nuclei (SCN) in the brain through the retinohypothalamic tract (Fig. 1). The SCN contain the master biological clock and adjust the master clock according to photic information received. The SCN regulate the pineal gland through several synaptic pathways and enable nocturnal secretion of melatonin from the pineal gland. Reproductive activities are regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Fig. 1). In the hypothalamus, neuropeptides such as gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH) and kisspeptin play their roles to regulate gonadotropin secretion from the anterior pituitary, which regulate gonadal development and maintenance (Fig. 1). Photoperiodic reproductive activities depend on the ability of the HPG axis to read melatonin concentration that is a reliable endogenous indicator of day length. Here, we describe how model animals of photoperiodism, sheep (SD breeder) and hamsters (LD breeder), employ common and distinctive mechanisms to regulate reproduction according to season.

Retinohypothalamic Tract

The retinohypothalamic tract originates in retinal ganglion cells that contain melanopsin as the photopigment. The photic information sensed in the retinal ganglionic cells are monosynaptically transmitted to the SCN through the optic nerve (Fig. 1).

SCN

SCN neurons maintain their circadian rhythmicity by transcriptional auto-regulatory loop between the clock genes and their translated proteins. Per (Period 1, 2, 3), Cry (Cryptochrome), Bmal1 (Brain and muscle ARNT-like 1), and Clock (Circadian locomotor output cycle kaput) are the major clock genes that generate circadian rhythmicity in the SCN. Transcription of Per1 and Per2 is activated by photic signals and regulates the expression of Bmal1, which dimerizes with Clock to enhance circadian

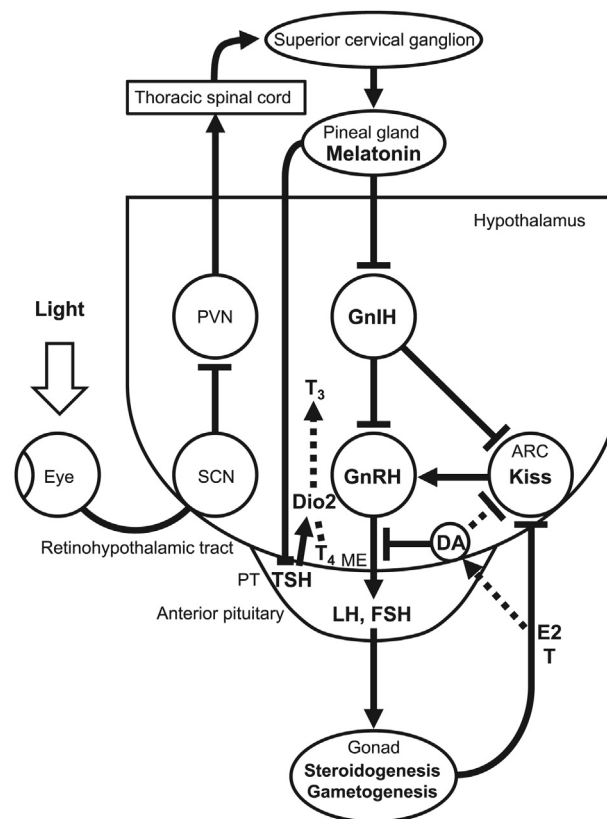


Fig. 1 Mechanism of how light-dark information is sensed in the eye and conveyed and translated in the retinohypothalamic tract, brain, thoracic spinal cord, superior cervical ganglion, pineal gland and pituitary to regulate gonadal activity according to season in photoperiodic mammals. Arc, arcuate nucleus; DA, dopamine; Dio2, type 2 deiodinase; E2, estradiol; FSH, follicle-stimulating hormone; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; Kiss, kisspeptin; LH, luteinizing hormone; ME, median eminence; PT, pars tuberalis; PVN, paraventricular nucleus; SCN, suprachiasmatic nuclei; T, testosterone; TSH, thyroid-stimulating hormone. Solid lines indicate direct regulation, whereas dotted lines indicate indirect regulation.

transcriptional activity of Per and Cry. Additionally, Per and Cry dimerize and repress Clock/Bmal1-induced transcriptional activity (Panda *et al.*, 2002).

The SCN can be divided into two major subdivisions ventrolateral (vl) SCN, the core, and the dorsomedial (dm) SCN, the shell. The vlSCN is the conductor of rhythmicity and transmits synchronizing cues to the dmSCN. vlSCN contains cell bodies producing a neuropeptide, vasoactive intestinal polypeptide (VIP), and dmSCN contains cell bodies producing another neuropeptide, arginine vasopressin (AVP). VIP neurons project to GnRH neurons in the preoptic area (POA), whereas AVP neurons project to kisspeptin neurons in the anteroventral periventricular nucleus (AVPV). Although direct innervation of hypothalamic neurons by SCN neurons are studied in terms of the regulation of female estrous or menstrual cycle, their role in photoperiodism is not understood.

Photoperiodic information processed in the SCN is transmitted to the pineal gland through a polysynaptic pathway, first to the paraventricular nucleus (PVN) then to the intermediolateral cell column of the thoracic spinal cord, the superior cervical ganglion and through the postganglionic noradrenergic fibers to the pineal gland (Fig. 1; Coomans *et al.*, 2015).

Pineal Gland and Melatonin

Noradrenaline is released from the fiber endings of the superior cervical ganglionic neurons during night to the pinealocytes in the pineal gland (Coomans *et al.*, 2015). Cyclic adenosine monophosphate (cAMP) level increases in the pinealocytes when $\beta 1$ and $\alpha 1$ adrenergic receptors are activated by noradrenalin. This elevated cAMP activates a key enzyme in melatonin synthetic pathway, arylalkylamine N-acetyltransferase (AANAT), resulting in rhythmic synthesis and secretion of melatonin during night (Klein *et al.*, 1970; Klein and Weller, 1970). Melatonin level is high during night in both diurnal and nocturnal animals. This melatonin rhythm conveys photoperiodic information to the hypothalamus and pituitary. Long duration of melatonin decodes short day length in fall and winter, whereas short duration of melatonin decodes long day length in spring and summer.

HPG Axis

Reproductive activities of mammals are ultimately regulated by the HPG axis. GnRH stimulates synthesis and release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), from the anterior pituitary gland. Gonadotropins act on the testis and ovary to stimulate steroidogenesis such as testosterone synthesis in male and estradiol (E2) synthesis in female and gametogenesis such as spermatogenesis and oogenesis. Testosterone or E2 not only feeds back to the hypothalamus and pituitary to adjust gonadotropin secretion but also facilitate reproductive behaviors by acting on the brain. GnIH and kisspeptin are relatively newly discovered hypothalamic neuropeptides that regulate the HPG axis (Fig. 1; Kriegsfeld *et al.*, 2015; Clarke and Arbabi, 2016; Ubuka *et al.*, 2016).

GnRH

Schally's and Guillemin's groups independently isolated and identified mammalian GnRH peptide from pig and sheep brains, respectively, in the early 1970s. Neuronal cell bodies producing GnRH exist in the preoptic area (POA) and send their axons to the median eminence (ME) (Fig. 1). GnRH is released at the ME into the hypophyseal portal system in a pulsatile manner and carried to the anterior pituitary gland. Gonadotropes that produce gonadotropins in the anterior pituitary gland express GnRH receptor and LH is released in a pulsatile manner following GnRH pulses (Levine *et al.*, 1982). GnRH pulse frequency is low in the non-breeding season and increases in the breeding season (Barrell *et al.*, 1992). GnRH pulse frequency in breeding season fluctuates according to estrous (menstrual) cycle in females but GnRH pulse amplitude in breeding season always exceeds that of non-breeding season (Barrell *et al.*, 1992).

GnIH

GnIH was discovered in Japanese quail as the first inhibitory hypothalamic neuropeptide of gonadotropin release (Tsutsui *et al.*, 2000). GnIH peptide was isolated and identified in various mammalian species including hamsters, monkeys and humans (Ubuka *et al.*, 2016). GnIH is also named RFamide-related peptide in mammals, because GnIH peptides have LPXRFamide (X = L or Q) amino acid sequence at their C-termini. GnIH neuronal cell bodies exist in the dorsomedial hypothalamic area (DMH) in mammals and terminate on GnRH and kisspeptin neurons that express GnIH receptors (Fig. 1). Expression of GnIH peptide and GnIH neuronal activities are regulated by natural and social environmental cues and stress. Therefore, one of the important roles of GnIH is to convey favorable and unfavorable environmental and social information to the HPG axis (Ubuka *et al.*, 2016).

Kisspeptin

Kisspeptin is also a relatively newly identified neuropeptide that is involved in the stimulatory regulation of GnRH neurons (Clarke and Arbabi, 2016). Two clusters of neuronal cell bodies that synthesize kisspeptin exist in the hypothalamus. In rodents, kisspeptin synthesizing neuronal cell bodies in the AVPV are involved in the regulation of GnRH/LH surge by positive feedback of E2 in cycling female. On the other hand, kisspeptin synthesizing neurons in the arcuate nucleus (ARC) are involved in the negative regulation of GnRH/LH pulse by testosterone or E2. Thus, kisspeptin plays an important role in the positive and negative regulation of GnRH/LH release by sex steroids. Kisspeptin neurons in the Arc play significant roles in seasonal regulation of the HPG axis (Fig. 1).

Photoperiodic Regulation of the HPG Axis in Sheep and Hamsters

Sheep: SD Breeders

Photoperiodism has been extensively studied in female sheep. Reproductive activity of adult female sheep is comprised of breeding season consisting of ovarian activity and ovulation in SD (fall and winter), and non-breeding anestrus season a period of ovarian quiescence and anovulation in LD (spring and summer) (Table 1; Weems *et al.*, 2015). This seasonal variation of ovarian activity is the result of changes in responsiveness of GnRH neurons to negative feedback actions of E2 and steroid-independent inhibition of GnRH neurons (Weems *et al.*, 2015). Sheep can become photorefractory if LD or SD was artificially prolonged and they show circannual changes in reproductive activity due to endogenous interaction of the HPG axis.

E2 gains the ability to suppress the frequency of GnRH/LH pulses during anestrus season even if E2 concentration is low in LD (Table 1). It is thought that E2 acts on estrogen receptor α expressing glutamate neurons in the ventromedial POA and retrochiasmatic area, and these neurons stimulate A15 dopamine neurons that directly act on GnRH neuronal terminals or indirectly on kisspeptin neurons in the Arc to inhibit GnRH pulse frequency. As the number of kisspeptin neurons in the Arc decreases due to stronger inhibition by E2 in anestrus season GnRH/LH pulse frequency decreases (Table 1; Weems *et al.*, 2015).

Pinealectomy of female sheep causes free running circannual rhythm of reproductive activity and inactivity and melatonin infusion for 3 months can synchronize reproductive rhythms suggesting that melatonin adjusts endogenous circannual rhythm of female sheep. Dardente *et al.* (2008) examined the expression pattern of GnIH in Soay sheep acclimated to LD or SD. GnIH was expressed widely in the hypothalamus and showed a generally moderate increase in LD. On the other hand, GnIH expression in the ependymal cells surrounding the base of the third ventricle was highly photoperiodic, which was undetectable in SD but consistently high under LD (Dardente *et al.*, 2008). These results suggest that long duration of melatonin secretion inhibits GnIH expression that allows higher GnRH neuronal activity in SD (Fig. 1; Table 1).

Melatonin receptor is most highly expressed in the pars tuberalis (PT) which is part of the anterior pituitary covering the pituitary stalk (Fig. 1; Klosen *et al.*, 2002). Thyroid hormones (T_3 and T_4) are thought to be critical for the transition from breeding to anestrus because thyroidectomy makes the sheep keep the breeding state but T_4 replacement can restore reproductive transition to anestrus. On the other hand, thyroid hormones are not required for the transition from anestrus to breeding state in sheep. Recent studies suggest that LD melatonin pattern increases the expression of a transcription factor, *eyes absent 3*, which stimulates thyroid stimulating hormone (TSH) synthesis in the PT. TSH diffuses in the nearby tissue and stimulates the expression of type 2 deiodinase (Dio2), an enzyme that converts T_4 to T_3 , and T_3 induces GnRH secretion. LD has an opposite effect on type 3 deiodinase (Dio3), an enzyme that inactivates T_4 and T_3 in sheep (Fig. 1; Table 1; Weems *et al.*, 2015).

Hamsters: LD Breeders

Hamsters are LD breeders that breed in spring and summer in the wild. However, if hamsters are artificially kept in constant SD and they will become photorefractory and they grow and maintain their high gonadal activity. Hamsters do not become photorefractory to artificial constant LD and they maintain their high gonadal activity.

The duration of melatonin secretion is the key signal that regulates the HPG axis in hamsters (Kriegsfeld *et al.*, 2015). DMH is the potential target to read melatonin signal for photoperiodic regulation of the HPG axis because it expresses both sex steroid and melatonin receptors and is essential for SD or melatonin induced gonadal regression. As GnIH neuronal cell bodies exist in the DMH, GnIH may convey melatonin signals to GnRH neurons (Fig. 1; Kriegsfeld *et al.*, 2015).

Ubuka *et al.* (2012) investigated how GnIH mRNA expression and GnIH neuronal activity are regulated by photoperiod and melatonin to investigate if GnIH conveys melatonin signals to GnRH neurons in Siberian hamsters. GnIH mRNA expression was higher in LD compared with SD. GnIH mRNA expression was also high in SD plus pinealectomized animals, and this

Table 1 Photoperiodic changes in neuroendocrine and endocrine signaling molecules in sheep, Syrian and Siberian hamsters

	Sheep		Syrian hamsters		Siberian hamsters	
	LD	SD	LD	SD	LD	SD
Photoperiod	LD	SD	LD	SD	LD	SD
Melatonin	↓	↑	↓	↑	↓	↑
TSH	↑	↓	↑	↓	↑	↓
Dio2	↑	↓	↑	↓	—	—
Dio3	↓	↑	—	—	↓	↑
T_3	↑	↓	↑	↓	↑	↓
GnIH	↑	↓	↑	↓	↑	↓
GnRH	↓	↑	↑	↓	↑	↓
Kisspeptin (Arc)	↓	↑	↑	↓	↓	↑
Dopamine	↑	↓	—	—	—	—
E2, T	↓	↑	↑	↓	↑	↓
Reproductive activity	↓	↑	↑	↓	↑	↓

high expression of GnIH mRNA is suppressed by melatonin. GnIH neuronal fiber density was also higher in LD and SD plus pinealectomized hamsters than LD plus melatonin or SD animals (Table 1). GnIH neuronal fibers extended to GnRH neurons and GnRH neurons expressed GnIH receptor. GnIH neuronal fiber terminals on GnRH neurons also increased in LD and decreased in SD. GnIH administration to the brain inhibited LH concentration in LD but increased LH concentration in SD. These results suggest that GnIH fine tunes LH levels through GnIH receptor expressed in GnRH neurons in an opposing fashion across seasons in Siberian hamsters (Ubuka *et al.*, 2012).

In transition from SD to LD, short duration of melatonin secretion may stimulate TSH synthesis in the PT, and TSH is transported to the hypothalamus to stimulate the conversion of T_4 to T_3 by inducing Dio2 expression in the hypothalamus of Syrian hamsters (Table 1). Although Dio3 is undetectable in both photoperiods in Syrian hamsters, changes in Dio3 was suggested to be important in Siberian hamsters (Table 1; Weems *et al.*, 2015).

Klosen *et al.* (2013) treated SD male Syrian and Siberian hamsters with a chronic central infusion of TSH. In both hamster species, TSH restored testicular activity. GnIH neuronal number was high in LD and TSH treated SD hamsters. Number of kisspeptin neurons in the Arc was significantly higher in LD than SD in Syrian hamsters but it was opposite in Siberian hamsters (Table 1). TSH was able to increase the number of kisspeptin neurons in the Arc in SD Syrian hamsters, whereas it reduced the numbers of kisspeptin neurons in the Arc to its LD level in SD Siberian hamsters (Table 1; Klosen *et al.*, 2013).

Conclusions

The mechanism to detect light-dark information in the eye and translate it into melatonin secretion from the pineal gland is the same among sheep, Syrian and Siberian hamsters (Fig. 1). Melatonin is secreted only during night either in diurnal and nocturnal animals or LD and SD breeders. Hypothalamic neuropeptides such as GnRH, GnIH and kisspeptin regulate gonadotropin secretion from the anterior pituitary gland, which regulate gonadal development and maintenance (Fig. 1). Therefore, photoperiodic reproductive activities depend on how GnRH, GnIH or kisspeptin neurons read melatonin concentration in the hypothalamus. There is also a mechanism of negative feedback of gonadal steroids to the hypothalamus to adjust gonadotropin secretion from the anterior pituitary gland. Sheep have developed an intricate mechanism to decrease GnRH pulse frequency by arcuate dopamine and kisspeptin neurons in non-breeding season (Fig. 1; Table 1). In sheep, photoperiodic information is conveyed to GnRH neurons by opposite changes in inhibitory GnIH expression and synchronized changes in stimulatory T_3 concentration according to duration of melatonin secretion (Table 1). Photoperiodic information is also conveyed to GnRH neurons by changes in GnIH expression and T_3 concentration in hamsters but changes in GnIH and GnRH neuronal activities synchronize in LD and SD (Table 1). This synchronization of GnIH and GnRH neuronal activities may be because GnIH has both inhibitory and stimulatory effects on gonadotropin secretion in hamsters and GnIH finetunes the activity of GnRH neurons according to season. In conclusion, changes in the direction of melatonin, TSH, T_3 , GnIH in LD and SD are the same among sheep, Syrian and Siberian hamsters, but GnRH, E2 or testosterone are activated in SD in sheep, short day breeder, but LD in hamsters, long day breeder. The primary roles of dopamine and kisspeptin neurons in the arcuate nucleus are to finetune pulsatile secretion of GnRH according to E2 or testosterone concentration instead of reading duration of melatonin secretion.

References

- Barrell, G. K., Moenter, S. M., Caraty, A., & Karsch, F. J. (1992). Seasonal changes of gonadotropin-releasing hormone secretion in the ewe. *Biology of Reproduction*, 46, 1130–1135.
- Clarke, I. J., & Arbabi, L. (2016). New concepts of the central control of reproduction, integrating influence of stress, metabolic state, and season. *Domestic Animal Endocrinology*, 56(Suppl.), S165–S179.
- Coomans, C. P., Ramkisoensing, A., & Meijer, J. H. (2015). The suprachiasmatic nuclei as a seasonal clock. *Frontiers in Neuroendocrinology*, 37, 29–42.
- Dardente, H., Birnie, M., Lincoln, G. A., & Hazlerigg, D. G. (2008). RFamide-related peptide and its cognate receptor in the sheep: cDNA cloning, mRNA distribution in the hypothalamus and the effect of photoperiod. *Journal of Neuroendocrinology*, 20, 1252–1259.
- Klein, D. C., Berg, G. R., & Weller, J. (1970). Melatonin synthesis: Adenosine 3',5'-monophosphate and norepinephrine stimulate N-acetyltransferase. *Science*, 168, 979–980.
- Klein, D. C., & Weller, J. L. (1970). Indole metabolism in the pineal gland: A circadian rhythm in N-acetyltransferase. *Science*, 169, 1093–1095.
- Klosen, P., Bienvu, C., Demarteau, O., et al. (2002). The mt1 melatonin receptor and RORbeta receptor are co-localized in specific TSH-immunoreactive cells in the pars tuberalis of the rat pituitary. *Journal of Histochemistry & Cytochemistry*, 50, 1647–1657.
- Klosen, P., Sébert, M. E., Rasri, K., Laran-Chich, M. P., & Simonneaux, V. (2013). TSH restores a summer phenotype in photoinhibited mammals via the RF-amides RFRP3 and kisspeptin. *FASEB Journal*, 27, 2677–2686.
- Kriegsfeld, L. J., Ubuka, T., Bentley, G. E., & Tsutsui, K. (2015). Seasonal control of gonadotropin-inhibitory hormone (GnIH) in birds and mammals. *Frontiers in Neuroendocrinology*, 37, 65–75.
- Levine, J. E., Pau, K. Y., Ramirez, V. D., & Jackson, G. L. (1982). Simultaneous measurement of luteinizing hormone-releasing hormone and luteinizing hormone release in unanesthetized, ovariectomized sheep. *Endocrinology*, 111, 1449–1455.
- Panda, S., Antoch, M. P., Miller, B. H., et al. (2002). Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell*, 109, 307–320.
- Tsutsui, K., Saigoh, E., Ukena, K., et al. (2000). A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochemical and Biophysical Research Communications*, 275, 661–667.
- Ubuka, T., Inoue, K., Fukuda, Y., et al. (2012). Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. *Endocrinology*, 153, 373–385.
- Ubuka, T., Son, Y. L., & Tsutsui, K. (2016). Molecular, cellular, morphological, physiological and behavioral aspects of gonadotropin-inhibitory hormone. *General and Comparative Endocrinology*, 227, 27–50.
- Weems, P. W., Goodman, R. L., & Lehman, M. N. (2015). Neural mechanisms controlling seasonal reproduction: Principles derived from the sheep model and its comparison with hamsters. *Frontiers in Neuroendocrinology*, 37, 43–51.

Acute Lymphocytic Leukemia: Diagnosis and Treatment[☆]

Katarzyna Szymańska, Science to the Point, Archamps Technopole, Archamps, France

Sophie Park, Grenoble-Alpes University, Grenoble, France; Institute for Advanced Biosciences, Grenoble, France

© 2018 Elsevier Inc. All rights reserved.

Definition and Classification	2
Presentation and Diagnosis	2
Epidemiology and Risk Factors	3
Burden	3
Etiology and Risk Factors	3
Pathology and Genetics	3
Management and Therapy	4
Further Reading	7

Abbreviations

6-MP	6-Mercaptopurine
ALL	Acute lymphocytic (lymphoblastic) leukemia
AYA	Adolescents and young adults
B-ALL	B-lymphoblastic leukemia
B-LBL	B-lymphoblastic lymphoma
CAR-T	Chimeric antigen receptor T-cells
CBC	Complete blood count
CD	Cluster of differentiation
CGH	Comparative genomic hybridization
CNS	Central nervous system
COG	The Children's Oncology Group
CR	Complete remission
CT	Computed tomography
DIC	Disseminated intravascular coagulation
FAB	French–American–British (histopathologic classification of acute leukemia)
FDA	US Food and Drug Administration
FISH	Fluorescence in situ hybridization
HSCT	Hematopoietic stem cell transplantation
ICD-O	International Classification of Diseases for Oncology
IGH	Immunoglobulin heavy locus
LDH	Lactate dehydrogenase
MRD	Minimal residual disease
NCCN	US National Comprehensive Cancer Network
NK	Natural killer
PET	Positron emission tomography
Ph	Philadelphia chromosome
RT-PCR	Reverse-transcriptase polymerase chain reaction
SNP	Single nucleotide polymorphism
T-ALL	T-lymphoblastic leukemia
TCR	T-cell receptor
TdT	Terminal deoxynucleotidyl transferase
TK	Tyrosine kinase
TKI	Tyrosine kinase inhibitor
TMPT	Thiopurine methyltransferase
WBC	White blood cells
WHO	World Health Organization

[☆]*Change History:* July 2018, Katarzyna Szymańska has updated the text and references.

Definition and Classification

Acute lymphocytic leukemia (also called acute lymphoblastic leukemia; ALL) is a neoplasm of immature B- or T-cells (lymphoblasts). In the most recent World Health Organization (WHO) classification of hematopoietic and lymphoid tumors, it is classified under “precursor B-cell lymphoblastic leukemia/lymphoma” and “precursor T-cell lymphoblastic leukemia/lymphoma.” This classification has replaced the French–American–British (FAB) histopathologic classification of acute leukemia, originally proposed in 1976, which classified ALL into three entities: adult, childhood, and Burkitt type ALL. The new WHO classification system takes into account not only morphological and cytochemical characteristics of the disease but also its cytogenetic and clinical diversity. All in all, the widely used term “ALL” encompasses several entities as classified by WHO, defined below.

B-lymphoblastic leukemia (B-ALL) not otherwise specified (NOS; ICD-O code: 9811/3) is a neoplasm of precursor lymphoid cells committed to the B-cell lineage, typically composed of small to medium-size blast cells with scant cytoplasm, moderately condensed to dispersed chromatin and inconspicuous nucleoli, involving bone marrow and blood. By convention, the term B-lymphoblastic lymphoma (B-LBL), which is included under the same ICD-O code, is used when a process is confined to a mass lesion with no or minimal evidence of blood and bone marrow involvement (generally defined as less than 20% of lymphoblasts in the marrow). The term B-ALL does not encompass Burkitt leukemia/lymphoma.

B-ALLs with specific recurrent genetic abnormalities associated with particular clinical and phenotypic features, and/or of prognostic significance are classified as separate entities. These are as follows:

B lymphoblastic leukemia with $t(9;22)(q34;q11.2)$; *BCR-ABL1* (ICD-O code: 9812/3)

B lymphoblastic leukemia with $t(v;11q23)$; *MLL* (also called *KMT2A* or *MLL1*) rearranged (9813/3)

B lymphoblastic leukemia with $t(12;21)(p13;q22)$; *TEL-AML1* (*ETV6-RUNX1*) (9814/3)

B lymphoblastic leukemia with hyperdiploidy (9815/3)

B lymphoblastic leukemia with hypodiploidy (Hypodiploid ALL; 9816/3)

B lymphoblastic leukemia with $t(5;14)(q31;q32)$; *IL3-IGH* (9817/3)

B lymphoblastic leukemia with $t(1;19)(q23;p13.3)$; *E2A-PBX1* (*TCF3-PBX1*) (9818/3)

B lymphoblastic leukemia, *BCR-ABL1*-like (9819/3)

Additionally, B lymphoblastic leukemia with *iAMP21* has been depicted as a provisional entity with no separate ICD-O code (it is coded under B-ALL, NOS; ICD-O code: 9811/3).

T-lymphoblastic leukemia (T-ALL) is a neoplasm of precursor lymphoid cells with the same cellular characteristics as B-ALL but concerning precursor cells committed to the T-cell lineage.

Presentation and Diagnosis

The symptoms of ALL are usually nonspecific and may appear only weeks or even days before diagnosis. Fatigue and weakness are among the most common nonspecific symptoms. Fever, with or without night sweats, and weight loss are frequent. The cause of fever is often not found, although granulocytopenia may lead to rapidly progressing and potentially life-threatening bacterial infections. Many patients present with thrombocytopenia and/or neutropenia, easy bruising and/or bleeding (bleeding gums, epistaxis, purplish patches in the skin or petechiae), and anemia as a result of bone marrow failure and disrupted hematopoiesis. Dyspnea, chest pain, and dizziness may also occur. Bone and joint pain due to bone marrow and periosteal infiltration is frequent in children in whom it may be the only presenting symptom. Headaches, vomiting, irritability, cranial nerve palsies, seizures, papilledema, and blurred vision are manifestations of the central nervous system (CNS) involvement and/or leukemic meningitis but initial CNS involvement is uncommon.

A sensation of abdominal fullness and/or discomfort may appear due to hepatomegaly and/or splenomegaly. Hepatomegaly, splenomegaly, and lymphadenopathy at physical examination are found in about 20% of all patients and in approximately 50% of adult ALL presentations. Extramedullary infiltration may also lead to leukemia cutis (a raised, nonpruritic rash). Oliguria may occur as a result of dehydration, uric acid nephropathy, or disseminated intravascular coagulation (DIC).

The initial workup includes complete blood count (CBC) with peripheral blood smear, blood chemistry profile, liver and kidney function tests, coagulation analysis (including measurement of prothrombin time, partial thromboplastin time, and fibrinogen), and a tumor lysis syndrome panel (including measurement of serum lactate dehydrogenase (LDH), potassium, uricemia, calcium, and phosphorus). Computed tomography (CT) scans of the neck, chest, abdomen, and pelvis are recommended for detecting possible organ involvement, lymphadenopathy, and organomegaly. If any extramedullary involvement is suspected, a positron emission tomography (PET)/CT is used for diagnosis.

The leukocyte count in ALL patients may be elevated (majority of cases), normal, or decreased. T-ALL patients typically present with a high leukocyte count and often with a large mediastinal mass or other tissue mass. Circulating blast cells are present in virtually all cases. However, the percentage may be very low in some patients.

ALL diagnosis is based on a hematopathological evaluation of bone marrow aspirate and biopsy material, with a demonstration of at least 20% of bone marrow lymphoblasts confirming a definitive ALL diagnosis. According to the guidelines of the US National Comprehensive Cancer Network (NCCN), the diagnostic workup should include a morphologic assessment of Wright/Giemsa-stained bone marrow aspirate smears and of hematoxylin-and-eosin-stained bone marrow core biopsy and clot sections, a comprehensive immunophenotyping using flow cytometry, and a baseline characterization of the leukemic clone(s) (see “Pathology and Genetics” section for more) to facilitate subsequent analysis of the minimal residual disease (MRD). For the

latter, cytogenetic testing by fluorescence in situ hybridization (FISH) with a panel of the acute leukemia probes and reverse-transcriptase polymerase chain reaction (RT-PCR) for *BCR-ABL1* fusions (and other fusions in case of *BCR-ABL1* negativity), including determining the transcript size, are commonly used. In cases of aneuploidy, array comparative genomic hybridization (CGH) may also be useful.

Epidemiology and Risk Factors

Burden

ALL is mainly a childhood disease, with 75% of cases diagnosed in children under 6 years of age. It is also the most common form of childhood leukemia, accounting for 75%–80% of all childhood leukemia cases, while it accounts for only 20% of cases in adults. The estimated annual incidence worldwide is 1–4.8 cases per 100,000 population. In the United States, the age-adjusted incidence rate of all ALL is 1.58 per 100,000 population per year, with approximately 5970 new cases and 1440 deaths estimated in 2017. The prevalence of specific B-ALL types and of T-ALL is summarized in Table 1.

Etiology and Risk Factors

The etiology of both B- and T-ALL is not well known. However, some environmental exposures have been associated with an increased risk. Radiation is a well-documented leukemogenic factor in humans, with a direct relationship between cumulative radiation dose and the incidence of some leukemia types. ALL may develop following exposure to ionizing radiation as well as radiotherapy. Also chemotherapy has been associated with an increased risk of developing the disease. Moreover, some chemicals, like benzene and toluene, contribute to the risk of acute leukemias, including ALL, likely by mutating or ablating the bone marrow stem cells. Acute leukemia usually develops 1–5 years after exposure and is often preceded by bone marrow hypoplasia, dysplasia, and pancytopenia. Age over 70 years is also a known risk factor for ALL.

Moreover, several hereditary conditions are associated with an increased risk of ALL, in particular Down syndrome but also less frequent disorders: neurofibromatosis type 1, Bloom syndrome, Klinefelter syndrome, Schwachman–Diamond syndrome, Fanconi anemia, and ataxia telangiectasia.

Genome-wide association studies have identified several single nucleotide polymorphisms (SNPs) associated with an increased risk of B-ALL, including in *GATA2*, *ARID5B*, *IKZF1*, *CEBPE*, and *CDKN2A*. In true familial ALL cases, even though rare, mutations in *PAX5*, *ETV6*, and *TP53* have been described.

Pathology and Genetics

In smear preparations, B-ALL lymphoblasts present variably, from small blasts with scant cytoplasm, condensed nuclear chromatin and indistinct nucleoli to larger cells with moderate amounts of light-blue to bluish-grey cytoplasm, sometimes vacuolated, dispersed chromatin and multiple variably prominent nucleoli. Nuclei are round or convoluted. Coarse azurophilic granules are present in some lymphoblasts in about 10% of cases. In bone marrow specimens, B-ALL lymphoblasts have a relatively uniform appearance, with round or oval, indented or convoluted nuclei. Nuclei range from inconspicuous to prominent, the chromatin is finely dispersed and the number of mitotic figures varies. Lymphoblasts in B-ALLs with specific genetic abnormalities usually do not have any specific morphological or cytochemical features that would allow a more specific diagnosis. Also T-ALL lymphoblasts are morphologically indistinguishable from B-ALL lymphoblasts.

Table 1 The prevalence of different acute lymphoblastic leukemia (ALL) entities

Entity	Epidemiology
B-ALL with t(9;22)(q34;q11.2); <i>BCR-ABL1</i>	More common in adults (25% of all adult ALLs) than in children (2%–4%)
B-ALL with t(1;11q23); <i>MLL</i> rearranged	The most common leukemia in infants under 1 year of age; less common in older children; incidence increases with age into adulthood
B-ALL with t(12;21)(p13;q22); <i>TEL-AML1 (ETV6-RUNX1)</i>	Common in children (25% of all B-ALLs in children) but not seen in infants; incidence decreases with age (this entity is uncommon in adults)
B-ALL with hyperdiploidy	Common in children (25% of all B-ALLs in children) but not seen in infants; incidence decreases with age; accounts for 7%–8% of adult B-ALL cases
B-ALL with hypodiploidy	1%–5% of cases, both adults and children (nearly-haploid ALL likely in children only)
B-ALL with t(5;14)(q31;q32); <i>IL3-IGH</i>	Less than 1% of all ALL cases, both adults and children
B-ALL with t(1;19)(q23;p13.3); <i>E2A-PBX1 (TCF3-PBX1)</i>	About 6% of childhood B-ALL cases, uncommon in adults
B-ALL, <i>BCR-ABL1</i> -like	10%–25% of all ALL cases; incidence increases with age
B-ALL with <i>iAMP21</i>	About 2% of ALL cases in children; no data on the incidence in adults
T-ALL	About 15% of childhood ALL cases (more common in adolescents than in younger children) and 25% of adult ALL cases; more common in males than in females

B-ALL, B-lymphoblastic leukemia; T-ALL, T-lymphoblastic leukemia.

B-ALL lymphoblasts are nearly always positive for the B-cell markers CD19, cCD79a, and cCD22. None of these molecules on its own is a specific diagnostic marker but their combination or high intensity strongly supports the B-ALL diagnosis. In most cases, the expression of CD10, surface CD22, CD24, PAX5, and terminal deoxynucleotidyl transferase (TdT) is also found. The myeloid-associated markers CD13 and CD33 may be expressed and this does not exclude the B-ALL diagnosis. The PAX5 expression is considered to be the most specific and sensitive marker for the B-cell lineage in tissue sections but it is also expressed in some variants of acute myeloid leukemia. The expression profile of cell surface markers within the B-cell lineage varies with the B-cell differentiation (maturation stage). The immunophenotypes characterizing B-ALLs with specific recurrent genetic aberrations are summarized in Table 2.

T-ALL lymphoblasts are usually positive for TdT and variably express CD1a, CD2–CD5, CD7, and CD8, with CD3 (cytoplasmic) and CD7 being positive most frequently. Only CD3, however, is specific for the T-lineage. CD4/CD8 coexpression is common but it is not specific to T-ALL. TdT expression combined with that of CD34, CD1a, and CD99 may help to establish the precursor nature of T-lymphoblasts. CD52 may be expressed in 30%–50% of adult T-ALL cases. One or both of the myeloid markers may also be expressed but, like for B-ALL, this does not exclude the T-ALL diagnosis. Moreover, many markers characteristic for premature T-cells (e.g., CD2 or CD7) may also be expressed by immature natural killer (NK) cells. Therefore, it may be very difficult to distinguish the true NK-cell ALL (which is rare) and T-ALL associated with the expression of immature T-cell markers.

Clonal rearrangements of the *immunoglobulin heavy* locus (*IGH*) gene and rearrangements of the *T-cell receptor* (*TCR*) gene are found in both B-ALL and T-ALL, albeit at different frequencies. In B-ALL, *IGH* clonal rearrangements are found in nearly all cases and *TCR* rearrangements in up to 70% of cases, whereas in T-ALL, clonal *TCR* rearrangements are seen in most cases and simultaneous *IGH* rearrangements in about 20%.

Moreover, cytogenetic abnormalities are seen in a majority of B-ALL cases, many of them defining specific entities with unique phenotypic and prognostic features (Table 2). In addition to those “classifying” aberrations, deletions of chromosome 6q, 9p, and 12p are frequent in B-ALL but they do not seem to impact prognosis. Some other abnormalities observed in B-ALL may emerge as having some clinical utility. For example, the rare $(17;19)(q22;p13.3)$ translocation is associated with a very poor prognosis but there are too few cases to validate this alteration as a prognostic marker and classify these B-ALL cases as a separate entity.

In T-ALL, abnormal karyotype is found in 50%–70% of cases. The most common recurrent cytogenetic abnormality involves the *alpha* and *delta* *TCR* loci (14q11.2) as well as the *beta* (7q34) and *gamma* locus (7p14.1), with a multitude of partner genes, most common of them being the *TLX1* (also called *HOX11*; 10q24.3) and *TLX3* (*HOX11L2*; 5q35.1) transcription factors. The most prevalent deletions are those of chromosome 9p, encompassing the locus of the *CDKN2A* gene.

Alterations at a gene level are also found in ALL. In particular, a large number of recurrent genetic alterations (copy number alterations and specific intragenic mutations) are found in B-ALL. Many of them, such as those in the *PAX5* gene, are found in most of the subtypes, suggesting that they play a fundamental role in leukemogenesis. Others, like those in *RAS* and *IKZF1*, tend to be associated with specific subtypes (Table 2). In T-ALL, the most frequently mutated gene is *NOTCH1* which encodes a protein critical for early T-cell development (mutated in 50% of cases). In 30% of cases, mutations in *FBXW7*, a negative regulator of *NOTCH1*, are found. According to data in the Cosmic database, the genes that are most frequently mutated in ALL are the following: *IKZF1* (35% of cases), *ABL1* (21%), *NRAS* (10%), *TP53* (10%), *CDKN2A*, *PAX5*, and *KMT2D* (9% each). However, the data do not distinguish between B-ALL and T-ALL, which—given remarkable differences between the subtypes—is a serious limitation.

Management and Therapy

The introduction of new therapies has resulted in a dramatic improvement of cure rates, in particular in B-ALL patients. ALL has a relatively good prognosis in children (5-year survival rates up to 89%). However, the prognosis for adults remains less favorable, especially for those of older age.

Risk assessment in ALL is an essential part of treatment planning and the criteria, widely debated, have been evolving. Patient age, initial white blood cells (WBC) count, disease subtype, presence of CNS disease, and rapidity of response to induction therapy are recognized as important factors in assessing prognosis. For years, clinicians used to stratify children, adolescents, and young adults (AYA) with ALL into two groups: standard risk and high risk, based mainly on age, WBC count, and disease subtype. The Children’s Oncology Group (COG) has refined risk assessment criteria, creating four groups: very high, high, standard, and low risk. The very high risk category comprises all B-ALL patients with the $t(9;22)$ translocation (Ph-positive ALL) and/or presence of the BCR-ABL1 fusion protein, B-ALL patients with hypodiploidy (defined as less than 44 chromosomes), BCR-ABL1-like, or *iAMP21* subtype, and those with *KMT2A* alterations or failure to achieve remission with induction therapy, regardless of age and WBC count. About 4% of nonadult B-ALL patients fall into this category. Risk stratification for T-ALL has been more difficult and this entity is frequently categorized as very high risk. However, newer treatments have resulted in improved outcomes also for T-ALL patients. According to the current NCCN guidelines, the initial risk stratification of all ALL patients should be based on the presence of the $t(9;22)$ chromosomal translocation and/or BCR-ABL fusion protein (Ph-positive versus Ph-negative). Further stratification may be based on patient age, WBC count, comorbidities, and the presence of minimal residual disease (MRD).

The treatment of ALL is extremely complex, with treatment regimens, selection of drugs, doses, and schedules which differ according to the age group of patients (children, AYA, or older adults), risk stratification, and ALL subtype. However, all treatment protocols follow the same basic principles, with three main treatment phases: induction, consolidation, and maintenance. CNS prophylactic and/or therapeutic treatment is also a mandatory element of ALL management.

Table 2 The genetic and immunophenotypic profiles of different B-lymphoblastic leukemia (B-ALL) entities as well as their clinical significance

Entity	Genetic profile	Immunophenotype	Prognosis and predictive factors
B-ALL with $t(9;22)(q34;q11.2)$; <i>BCR-ABL1</i>	The $t(9;22)$ translocation resulting from fusion of <i>BCR</i> at 22q11.2 and <i>ABL1</i> at 9q34.1, with production of a BCR-ABL1 fusion protein (p190 BCR-ABL1 in most childhood cases and in about half of adult cases); the resulting aberrant chromosome 22 harboring the fusion <i>BCR-ABL1</i> gene locus is called Philadelphia (Ph) chromosome	Typically positive for CD10, CD19, and TdT, and negative for CD117 (KIT); CD25 expression highly associated with this entity, especially in adults; myeloid-associated markers CD13 and CD33 frequently expressed; T-cell precursor phenotype in rare cases	The worst prognosis of all types, particularly in adults; patients potentially responsive to tyrosine kinase inhibitors (e.g. imatinib), so prognosis may change with the introduction of targeted therapies
B-ALL with $t(v;11q23)$; <i>MLL</i> rearranged	Fusions of the <i>KMT2A (MLL)</i> gene with multiple genes (over 100 identified fusion partners), the most common partner being <i>AFF1 (AFF4; 4q21)</i> , followed by <i>MLLT1</i> (19p13.3), and <i>MLLT3</i> (9p21.3); none of these fusions is specific to this entity; frequent overexpression of <i>FTL3</i> ; very few additional mutations in infants, usually in the RAS pathway	Typically positive for CD19 and CD15, and negative for CD10 and CD24; the NG2 homolog usually expressed and relatively specific	<i>KMT2A-FFA1</i> translocation associated with a very poor prognosis, particularly in infants under the age of 6 months
B-ALL with $t(12;21)(p13;q22)$; <i>TEL-AML1 (ETV6-RUNX1)</i>	The <i>ETV6-RUNX1</i> translocation with production of the fusion protein (a putative negative regulator of the transcription factor RUNX1), likely an early event in the leukemogenesis	Positive for CD10 and CD19, and usually also for CD34; characteristic nearly complete absence of CD9, CD20, and CD66c (relatively specific); myeloid-associated markers, especially CD13, frequently expressed	Very favorable prognosis (cure in >90% of childhood cases); relapses later than for other B-ALL types
B-ALL with hyperdiploidy	Increased chromosome number (usually without structural aberrations), most commonly extra copies of chromosome 21, X, 14, and 4; the least common: chromosome 2 and 3	Positive for CD10 and CD19, and other typical B-ALL markers; most cases positive for CD34 and negative for CD45	Very favorable prognosis in children (cure in >90% of cases); not enough data for adults; trisomies may have more significance as prognostic factors than the actual number of chromosomes; simultaneous trisomy of chromosome 4 and 10 being associated with the best prognosis
B-ALL with hypodiploidy	Loss of one or more chromosomes and nonspecific structural aberrations in the other chromosomes (rare in near-haploid cases)	Typically positive for CD10 and CD19 but no other distinct features	Poor prognosis (likely even without MRD); the worst prognosis for nearly-haploid cases suggested by some studies
B-ALL with $t(5;14)(q31;q32)$; <i>IL3-IGH</i>	A functional rearrangement between <i>IL3</i> (chromosome 5) and <i>IDH</i> (chromosome 14) leading to constitutional overexpression of IL3 and eosinophilia	Typically positive for CD10 and CD19; even small numbers of blasts expressing these two markers in a patient with eosinophilia strongly suggests this diagnosis	No data
B-ALL with $t(1;19)(q23;p13.3)$; <i>E2A-PBX1 (TCF3-PBX1)</i>	<i>TCF3-PBX</i> translocation with production of the fusion protein (oncogenic); the fusion gene is located on chromosome 19, may be associated with loss of chromosome 1 (unbalanced translocation); an alternative <i>TCF3</i> translocation involving the <i>HLF</i> gene (chromosome 17) in rare cases	Typically positive for CD10, CD19, and cytoplasmic mu heavy chain (pre-B phenotype); typical strong expression of CD9 with lack or very limited expression of CD34 (with these features, this diagnosis may be suspected even if cytoplasmic mu is undetermined)	The <i>TCF3</i> translocation associated with very poor prognosis; possibly an increased risk of CNS relapses in patients with this entity
B-ALL, <i>BCR-ABL1</i> -like	Various chromosomal rearrangements of multiple genes with various partners; <i>CRLF2</i> rearrangements in approximately half of the cases; the tyrosine kinase-type translocations often involve <i>ABL1</i> with partners other than <i>BCR</i> , as well as other kinases; additionally frequent deletions and point mutations in other genes, particularly <i>IKZF1</i> (not specific to this entity) and <i>CDKNA2A/B</i> ; mutations in <i>JAK1</i> or <i>JAK2</i> found in about a half of cases with <i>CRLF2</i> rearrangements	Typically positive for CD10 and CD19; very high levels of the translocated <i>CRLF2</i> gene product (detectable by flow cytometry) in a subset of cases with these translocations	Overall poor prognosis; this may be due to an increased risk of MRD (controversial); children resistant to induction therapy usually have a translocation involving <i>PDGFRB</i> (most often with <i>EBF1</i>) and are very responsive to ABL-class tyrosine kinase inhibitors (e.g. imatinib or dasatinib); patients with <i>JAK</i> mutations or translocations may be candidates for treatment with JAK inhibitors (to be tested)
B-ALL with <i>iAMP21</i>	Intrachromosomal amplification of chromosome 21 (<i>iAMP21</i>) detectable with FISH probes recognizing the <i>ETV6-RUNX1</i> translocation; multiple other chromosomal abnormalities (most commonly involving chromosome X and 7) in 80% of cases; deletions of <i>RB1</i> and <i>ETV6</i> as well as <i>CRLF2</i> rearrangements more frequent than in other ALLs	Unknown	Unclear

The goal of the induction therapy, the first phase of the ALL treatment, is to reduce tumor burden by clearing as many leukemic cells as possible from the bone marrow, that is to induce remission. Most inductions regimens are based on a combination of vincristine, anthracycline (e.g., daunorubicin or doxorubicin, in particular for higher risk patients), and a corticosteroid (e.g., dexamethasone or prednisone) with or without L-asparaginase and/or cyclophosphamide, given over the course of 4–6 weeks. An alternative is the so-called Hyper-CVAD regimen which alternates cycles of hyperfractionated cyclophosphamide, vincristine, doxorubicin (adriamycin), and dexamethasone with those of high-dose methotrexate and cytarabine.

Most children achieve complete remission (CR) within 4 weeks of the induction therapy. If CR is not achieved within 6 weeks, alternative treatments are started. In adults, a regimen based on a combination vincristine/prednisone/anthracycline is associated with 75% CR rates. Dexamethasone has been shown to give better outcomes compared to prednisone. It has a better penetration into the CNS, which explains its efficacy in preventing CNS relapse. However, it is associated with severe toxicities and its advantage for the overall survival has yet to be demonstrated.

CNS prophylaxis after induction chemotherapy aims at preventing CNS disease or early CNS relapse. The CNS is the initial site of ALL relapse in more than half of children unless prophylaxis is given and it is also a frequent site of relapse in adults. The goal of CNS prophylaxis and/or treatment is to clear leukemic cells within sites that cannot be easily accessed with systemic chemotherapy because of the blood–brain barrier. Different regimens of CNS-directed therapy exist. Many authorities recommend intrathecal methotrexate, often combined with cranial irradiation. Triple intrathecal therapy (methotrexate, hydrocortisone, cytarabine), with or without high-dose systemic chemotherapy (e.g., methotrexate or cytarabine), may substitute for cranial irradiation which can lead to late intellectual disabilities when used in children. For adults, prophylactic intrathecal chemotherapy alone is considered sufficient. CNS prophylaxis is typically administered to all patients throughout the entire course of ALL therapy.

The consolidation treatment aims at eliminating any leukemic cells remaining after induction therapy, further eradicating residual disease. The postremission induction phase of treatment may also be referred to as intensification therapy. It typically involves an intensive multidrug regimen. A variety of regimens are used in different centers and trials, although frequently containing similar drug combinations to those that were used during the induction treatment. High-dose methotrexate, cytarabine, 6-mercaptopurine (6-MP), cyclophosphamide, vincristine, corticosteroids, and L-asparaginase are frequently incorporated into consolidation/intensification regimens. For treatment of younger children, adjusted doses and frequencies are used.

Maintenance therapy aims to prevent disease relapse after postremission induction and consolidation therapy. Most regimens are based on daily 6-MP and weekly methotrexate, typically with a periodic addition of vincristine and corticosteroids, for 2–3 years. Of note, the efficacy of the maintenance therapy is determined by the metabolism of 6-MP. In particular, the efficacy of the active metabolite production is modified by polymorphisms in thiopurine methyltransferase (TMPT). Genotyping TMPT allows to anticipate decreased bioavailability or increased toxicity of 6-MP and adjust the dose. The optimal duration of the maintenance therapy is not clearly defined and the therapy is frequently stopped due to associated toxic effects.

The emergence of targeted drugs offers hope for more effective treatments of ALL. In particular, adding tyrosine kinase inhibitors (TKIs) to the induction treatment regimens of Ph-positive ALL patients has significantly improved their prognosis. Imatinib (also called imatinib mesylate; brand name: Gleevec®, Novartis Pharmaceuticals), an inhibitor of the BCR-ABL tyrosine kinase, has been approved by the US Food and Drug Administration (FDA) for treatment of relapsed or refractory Ph-positive ALL adult patients and of all untreated Ph-positive pediatric patients. Dasatinib, a second-generation TKI which inhibits both the BCR-ABL tyrosine kinase and kinases of the Src family, seems to be even more effective than imatinib due to its capacity to inhibit several TK-related signaling pathways. It has also been shown to maintain activity against cells harboring several (but not all) ABL domain mutations which confer resistance to imatinib. Furthermore, it has a better penetration of the blood–brain barrier and so it may prove useful in the CNS prophylaxis. Dasatinib (under the brand name of SPRYCEL, Bristol-Myers Squibb Co) has been approved by FDA for treatment of pediatric patients with Ph-positive chronic myeloid leukemia and it is being extensively trialed (phase II and III) for treatment of ALL. Single-agent TKI therapy in Ph-positive ALL patients has been shown to yield improved response to induction therapy as compared to chemotherapy. However, the remission-free periods are relatively short for both imatinib and dasatinib alone. TKIs have been shown to provide most benefit, with significantly improved disease-free and overall survival rates, when combined with corticosteroids. Patients with CD20-positive B-ALL benefit from the addition of rituximab, an anti-CD20 monoclonal antibody, to standard ALL chemotherapy regimens. All these agents may be incorporated as part of the induction, consolidation, and/or maintenance therapy in the initial treatment of ALL, or in regimens for treating relapsed and/or refractory disease.

Allogeneic hematopoietic stem cell transplantation (HSCT) used to be considered the standard of care in AYA and adults with Ph-positive ALL, and for relapsing ALL patients. However, with the advent of BCR-ABL-targeted TKIs, its role has been questioned. Still, it may offer some benefit to specific groups of Ph-positive patients who relapse after initial remission.

Treatment of relapsed ALL remains a challenge. Combining standard chemotherapy regimens with emerging targeted drugs offers much hope to this effect. In particular, mitoxantrone (Novantrone®, Immunex Corporation; an anthracycline derivative), cytarabine, fludarabine/cytarabine/granulocyte colony-stimulating factor/idarubicin (FLAG-IDA), methotrexate/asparaginase (CAPIZZI), and blinatumomab (a monoclonal antibody against CD19 and CD3) have been shown to provide benefit to patients with CD19-positive ALL, while inotuzumab ozagamicin (FDA-approved since August 2017) has been shown to be useful in patients with CD22-positive disease. Several first-, second-, and third-generation TKIs are being currently tested in clinical trials and their approval for treatment of relapsed/refractory ALL, and in particular for BCR-ABL1-like ALL, is likely just a matter of time. However, as of now, bone marrow transplant remains the only cure for relapsed/refractory ALL.

For patients who are not eligible for transplant, generation of chimeric antigen receptor T-cells (CAR-T) targeting specific molecules on the surface of cancer cells may be a solution. In August 2017, FDA approved the anti-CD19 CAR-T therapeutic

agent tisagenlecleucel (KYMRIA[®], Novartis Pharmaceuticals) for treatment of refractory or relapsed B-ALL patients (at least second relapse) below 25 years of age. However, CAR-T therapy is associated with severe potentially fatal toxicities (like cytokine release syndrome, B-cell aplasia, and cerebral edema) and its use should be preceded by a specific risk evaluation. FDA also requires a special certification for centers administering this therapy. For relapsed or refractory T-ALL, nelarabine, a purine nucleoside analog, is currently an approved treatment.

Assessing the response to treatment also remains a challenging issue. Complete remission (CR) is defined as the absence of circulating blasts and extramedullary disease. However, patients who achieved CR according to morphological assessment can potentially harbor leukemic cells in the bone marrow, giving rise to a low-level disease which is not detectable by conventional cytomorphological techniques (minimal residual disease, MRD). Many trials have confirmed that MRD is the strongest prognostic factor in both children and adults. Therefore, applying reliable sensitive methods to detect MRD is of utmost importance for further treatment planning and monitoring of treatment response at all stages following the induction therapy. MRD can be detected by multicolor flow cytometry or sensitive molecular techniques targeting characteristic genetic or chromosomal aberrations, like RT-PCR or next-generation sequencing.

Further Reading

Chmielewski B and Territo M (2017) *Manual of clinical oncology*, 8th edn. Philadelphia: Wolters Kluwer.

International Agency for Research on Cancer (2017) In: Swerdlow SH, Campo E, Harris NL, Jaffe ES, Pileri SA, Stein H, and Thiele J (eds.) *WHO classification of tumours of hematopoietic and lymphoid tissues*, Revised 4th edn, Lyon: International Agency for Research on Cancer.

Kotrova M and Brüggemann M (2017) Minimal residual disease in adult ALL: Technical aspects and implications for correct clinical interpretation. *Blood Advances* 1(25): 2456–2466.

National Comprehensive Cancer Network (NCCN) (2017) NCCN clinical practice guidelines in oncology: Acute lymphoblastic leukemia, Version 5.2017. October 27, 2017. https://www.nccn.org/professionals/physician_gls/default.aspx#site.

Relevant Website

<https://clinicaltrials.gov/>—Clinical trial information at the US National Library of Medicine, National Health Institutes.

Photoperiodism, Insects

Shin G Goto and Keiji Matsumoto, Osaka City University, Osaka, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Critical day length (or critical night length) The length of the light (or dark) fraction of the light-dark cycle that separates a strong long-day response from a strong short-day response in the photoperiodic response curve.

Diapause A state of hormonally- and genetically-regulated developmental or reproductive arrest, accompanied by a major shutdown in metabolic activity.

Diapause syndrome Traits tightly linked with diapause.

Photoperiodic response curve The response of a population to a range of photoperiods.

Photoperiodism Biological ability to respond to photoperiod.

Quiescence A state of dormancy directly imposed by adverse environmental factors.

Nomenclature

CA Corpora allata

JH Juvenile hormone

Insects and Photoperiodism

Insects belong to the class Insecta within the subphylum Hexapoda, the Phylum Arthropoda. Insects are invertebrates with three body parts (head, thorax, and abdomen) and three pairs of jointed legs, and are the most diverse group of animals on this planet. There are more than a million described species of insects, which represent more than half of all known living organisms. This high species richness is attributed to several factors, one of which is their small body size. Most insects are 1–10 mm in body length, allowing these small organisms to inhabit many more niches in any given environment than that by large organisms. Their small size, however, also makes them vulnerable. Small organisms possess larger surface area to volume ratios than large organisms, making them highly susceptible to environmental changes as a result of heat-transfer across their body surface. Ectotherms such as insects are the most severely affected by environmental temperature changes.

Although the axis of the Earth is tilted by only 23.4° from the plane of its orbit around the sun, this is enough to provide the dramatic contrast between the heat of summer and the cold of winter. Because insects are limited in their abilities to regulate their own body temperature, they are at the mercy of seasonal temperature changes. It is, therefore, a necessity that insects anticipate arrival of the seasons by reading environmental cues so as to prepare physiologically and/or behaviorally. Such cues should be informative and reliable, and should predate the arrival of unfavorable conditions, i.e., temperatures. A highly indicative cue is *day length*. Numerous organisms, including insects, anticipate seasonal changes by monitoring day length, and may exhibit a biological response, known as *photoperiodism*, to these photoperiodic variations. *Temperature* is also a useful cue for timing periodic behaviors and triggering physiological changes that affect reproductive success. Temperature, however, can be unreliable due to large daily fluctuations and inter-annual variation, particularly in terrestrial environments (see [Goto and Numata, 2014](#)).

Photoperiodism was first demonstrated in plants nearly a century ago. Shortly thereafter, photoperiodism was described in animals in the first published report involving an insect, the strawberry root aphid *Aphis forbesi*. Today, photoperiodic responses have been recorded in many insect species, and insects that lack a photoperiodic response are considered to be exceptions, at least in temperate zones.

This overview presents a collection of the current knowledge of insect photoperiodism. Please take note: certain entries relate to the endocrine control of reproduction in insects and other organisms in this encyclopedia, and readers are referred to these specific sections for more detailed information.

Diapause

The most well-known photoperiodic response in insects is the photoperiodic regulation of *diapause*. Diapause is a state in which development or reproduction is suppressed or arrested, and is accompanied by a major shutdown in metabolic activity. Insects that are destined to enter diapause generally accumulate additional energy reserves prior to the onset of diapause. Sufficient

energy reserves and low metabolic activity give rise to a high probability of survival during and after adverse seasons. Unlike a simple *quiescence*, i.e., an immediate response at any stage to an unfavorable environmental condition, diapause is a genetically and hormonally programmed response. Some insects enter diapause at a particular stage in each generation regardless of the environmental conditions; this is referred to as *obligatory diapause*. The entry into diapause can be triggered by a number of factors, but is most frequently determined by environmental factors, and usually involves day length. This environmentally governed type of diapause is referred to as *facultative diapause*. Insects that enter diapause for overwintering (*winter diapause*) generally react to the shortening of day lengths, short day lengths, or to a change from long to short day lengths. These species are referred to as “*long-day species*”, analogous with plants. *Summer diapause* is also widespread, is induced by a long photoperiod, and is an inverse of winter diapause in many ways. Species that enter diapause in response to long photoperiods are referred to as “*short-day species*” (see Tauber *et al.*, 1986).

A selection of photoperiodic response curves found in insects is seen in Fig. 1. Each response curve consists of a series of points that represent the percentage of diapause individuals in a sample population reared at a specific day length. *The critical day length*, i.e., the length of the light fraction of the light-dark cycles that separates a strong long-day response from a strong short-day response in the photoperiodic response curve, is the representative value of the photoperiodic response. It is also noteworthy that slope of the photoperiodic response curve between photoperiods that elicit a short-day response and those that elicit a long-day response is usually quite sharp. This indicates that insects can measure a change of as little as 10–15 min in the length of the daily light period and this change may result in a significant change in the proportion of the population entering diapause (Saunders, 2002).

Diapause occurs at a specific stage (or at two or more stages in some cases) in each species. *Embryonic diapause* (i.e., diapause in the egg stage) is common in Lepidoptera and Hemiptera and in some Diptera species. *Larval diapause* is especially common in the lepidopterans and frequently occurs at the final instar, but is also known to occur at early instars. *Pupal diapause* is particularly common in Lepidoptera and Diptera orders and frequently occurs at the true pupal stage. *Adult diapause* is widespread in the Coleoptera, Hemiptera, Hymenoptera, Orthoptera, Neuroptera, Diptera and Lepidoptera orders. Embryonic, larval and pupal diapauses are characterized by *developmental arrest*, whereas adult diapause is characterized by *reproductive arrest* (*reproductive diapause*), i.e., the suppression of reproductive organ development.

Photoperiod frequently regulates diapause induction, but also regulates the maintenance and termination of diapause. Individuals that enter diapause can remain locked in developmental or reproductive arrest, even if environmental conditions are suitable. For example, in the lacewing *Chrysoperla carnea*, reproductive diapause is deep and not easily terminated when induced in early autumn, thereby avoiding an unfavorable termination of diapause before the onset of winter. The diapause termination

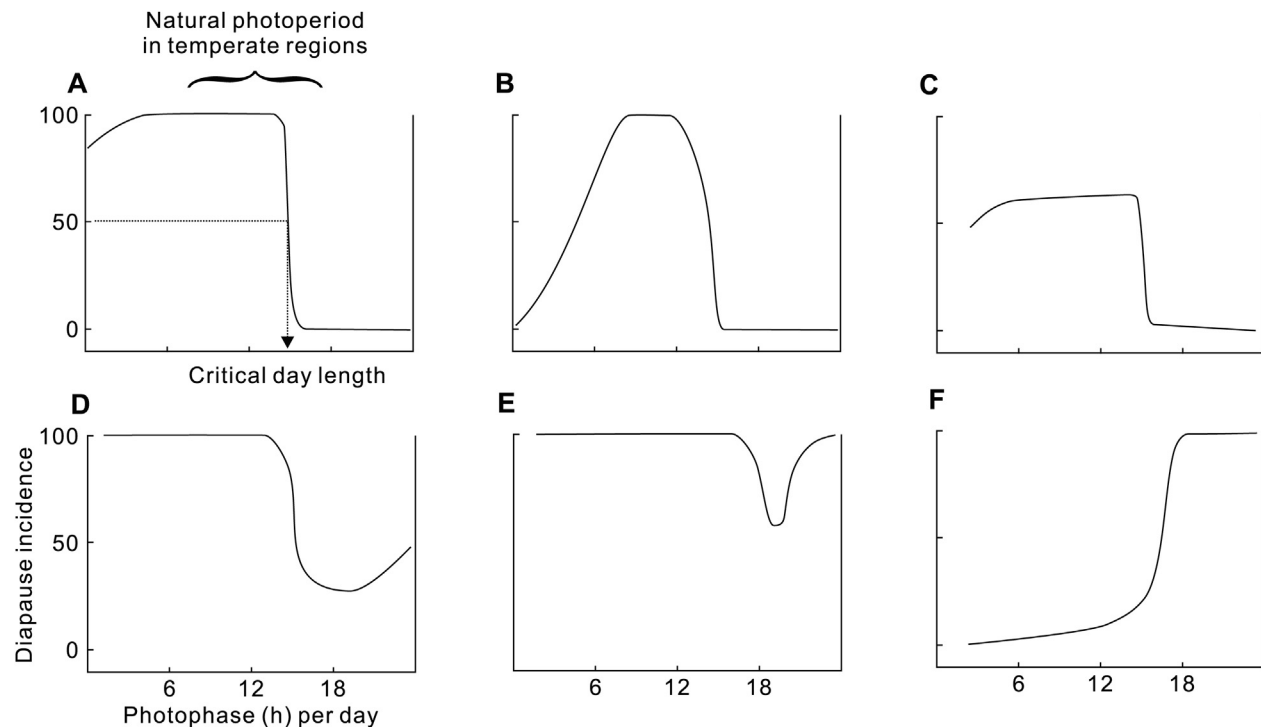


Fig. 1 A selection of photoperiodic response curves in long-day (A–D), intermediate (E) and short-day (F) species. Critical day length is also shown in (A). The responses under ultra-long and ultra-short photoperiods, which do not occur naturally, are generally quite variable among insect species.

process is regulated by photoperiod in some species. For example, reproductive diapause induced by short days is promptly terminated by exposure to long days in the bean bug *Riptortus pedestris*.

As a result of photoperiodic responses, insects are able to time their growth and reproduction to coincide with appropriate seasons, thereby influencing phenology, voltinism (the number of generations per year), and ultimately population growth (Denlinger, 2009).

Photoperiodic Regulation of Reproductive Traits

Reproductive diapause is the most common photoperiodic regulation of reproduction in insects. Both sexes enter diapause in many species. The ovaries of diapause females are small and the oocytes contain little or no yolk. The testes of males remain small during diapause in some species, but are well-developed and contain sperm in others. Development of the male accessory gland, i.e., the organ that produces spermatophores and factors responsible for sperm activation, is usually suppressed during diapause. Mating behavior is strongly suppressed during diapause. Mating takes place in spring after diapause termination in many species, but occurs in autumn before entering diapause or both in autumn and in spring in others. Sexual differences in the tendency to enter diapause have also been recorded, not only in adult diapause but also in diapause at other developmental stages. In some species, males are more likely to enter diapause than are females; however, the opposite tendency has also been observed. This sexual difference appears to reflect the energetic cost of diapause, wherein the overwintering sex has a significantly lower fecundity than its counterparts that developed directly. Extreme examples of sexual difference are found in mosquitoes, bees, and wasps. In these insects, only females enter diapause and overwinter, whereas males do not have the ability to enter diapause, and thus, die off before or during the unfavorable seasons.

The *photoperiod-sensitive stage* refers to the stage during which an insect receives the photoperiodic cues that trigger the photoperiodic response. In many species, the photoperiod-sensitive stage occurs far in advance of the actual diapause stage. The most extreme example of an “advanced” photoperiodic perception is when this sensitive stage occurs in one generation and the developmental arrest occurs in the next; i.e., *maternal effect*. This can be found in many parasitic wasps, higher Diptera, and some Lepidoptera species. The most classical example is seen in the silk moth *Bombyx mori*. Photoperiodic signals experienced by the embryos and larvae of one generation determine the developmental fate of the embryos of the next generation. In other cases, the mother influences the diapause fate of her progeny in their larval or pupal stages. Short day lengths influence female jewel wasps, *Nasonia vitripennis*, to produce eggs that are destined to enter diapause at the final larval instar. In this case, the progeny has no ability to respond to environmental factors to avoid diapause entry. In the blow fly *Lucilia sericata*, however, the progeny that is destined to enter diapause by maternal effect is still able to respond to environmental cues to avoid diapause entry. In this case, the progeny is able to integrate the information from the mother with the environmental information that they received so as to make a final decision to enter or avert diapause. Thus, mother insects are not only simply function in the production of offspring and in the embryonic development of her progeny, but are also able to initiate an effect that is expressed at a much later stage of development.

Seasonal parthenogenesis is found in aphids, in which there is a photoperiodic control of the mode of reproduction, namely, parthenogenetic and sexual reproduction. Most temperate species reproduce during the summer as a series of viviparous, parthenogenetic females (virginoparae), whereas they produce sexual forms (males and oviparae, i.e., females that are able to produce eggs) as the days become shorter in autumn. The fertilized oviparae deposit diapause eggs that overwinter. The viviparity and parthenogenetic reproduction of aphids promote rapid multiplication and allow for the maximum utilization of available resources during seasons that are suitable for population growth, whereas sexual reproduction allows for genetic recombination and production of a hibernating form of offspring, i.e., diapause progeny.

Photoperiodic Regulation of Other Traits

Photoperiod regulates not only reproductive traits but also other traits, such as stress tolerance, growth rate, seasonal morphs, behaviors, and migration, which can directly or indirectly contribute to reproductive success. Some of the traits are tightly linked with diapause, and thus, are components of diapause, i.e., the *diapause syndrome*, in some species, but not in others.

Insects that enter winter diapause require a degree of cold tolerance to survive severe winter conditions. In some cases, cold tolerance occurs as a diapause syndrome. In many insects, the rate of development is controlled by photoperiod. For example, the cricket *Modicogryllus siamensis* needs to molt 7 times over 45–55 days of long day lengths in order to emerge as an adult, whereas this species requires 10–13 molts over 65–145 days of short day lengths. This photoperiodic response prevents untimely adult emergence in an unfavorable season.

Seasonal morphs have been noted in Lepidoptera, Orthoptera, Hemiptera, and Thysanoptera, and in most cases photoperiod is the deciding environmental factor that is involved. One such good example has been observed in butterflies, whereby the long days of summer may favor the production of light-colored wings, which minimize overheating in the warm season, but, the short days of autumn may favor darkly pigmented wings, which increase the efficiency of the absorption of solar energy, and hence promote high activity and reproductive success. Color changes for camouflage are sometimes noted for diapause individuals. For example,

nondiapauses adults of the brown-winged green stink bug *Plautia stali* are green but turn brown when they enter diapause in autumn. When the bugs become reproductively active during the spring, they turn green again. Such color changes help the insect blend with the predominant colors of the environment. Photoperiodic regulation of alary dimorphism (dimorphism in wing form) and flight muscle degeneration are also widespread. Wing and flight muscles are particularly expensive to maintain, and thus, brachypterous morph and flight muscle degeneration can help save energy reserves that can otherwise be allocated to increase their chance of survival and reproductive success.

Although not mutually exclusive, migration and diapause are alternatives for insects to counteract changes in their environment. Insects may enter diapause in situ to withstand severe conditions or migrate to favorable areas; i.e., diapause entry is the temporal escape from the inimical environment, whereas migration is the spatial escape. The monarch butterfly, *Danaus plexippus*, is the classic model of the latter, and this species migrates to hibernation sites in autumn and returns to breeding and feeding areas in spring. Aphids also migrate from summer host plant species to winter one. Many of these migrations are components of diapause and are controlled by photoperiods.

Mechanisms Underlying Photoperiodic Responses

To show the photoperiodic response, insects must be equipped with a series of modules; i.e., photoreceptors, photoperiodic time measurement system, photoperiodic counter, and endocrine effectors (Fig. 2; Goto and Numata, 2014).

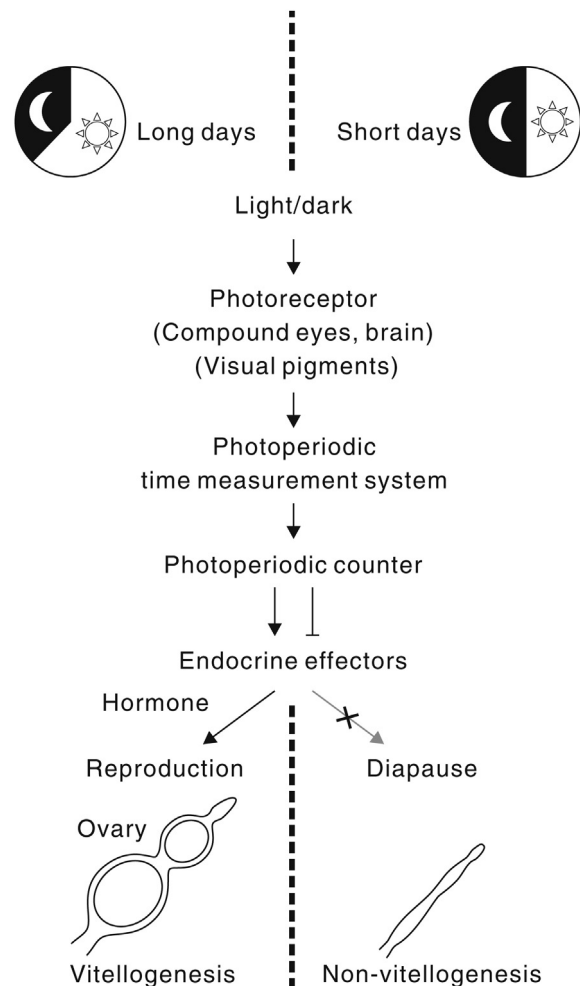


Fig. 2 Modules establishing photoperiodism, which regulate winter diapause at adult stage (reproductive diapause in the long-day species). Light/dark signals are received by photoreceptors for the photoperiodic time measurement system. The photoreceptors are retinal (the compound eyes) or extraretinal (the brain). The visual pigments are involved in this photoreception. The photoperiodic time measurement system measures the length of day or night and involves the circadian clock. The photoperiodic counter counts the number of photoperiodic cycles. When a hypothetical “diapause titer” exceeds an internal threshold, the activity of the corpora allata is suppressed and a short-day response (diapause) is induced. When the titer was at subthreshold level, the endocrine effectors actively synthesize and secrete hormone(s) for reproductive organ development. For further details, see in text discussions.

Photoreceptors

Insects possess three types of external photoreceptor organs, the *compound eyes*, *ocelli*, and *stemmata*. Compound eyes are primarily used for motion detection, ocelli are simple eyes found in immatures and adults, and stemmata are the visual receptors in larvae of many holometabolous insects. These organs follow the same basic structural plan: a lens to focus the light, light-sensitive cells, and axons from the photoreceptive cells projecting to the optic lobe of the brain. The usage of compound eyes and stemmata as photoperiodic photoreceptors has been reported in Hemiptera, Orthoptera, Lepidoptera, Coleoptera, and Diptera. Examples include *R. pedestris*, which uses the central part of the compound eyes for photoperiodic induction of adult diapause, and larvae of the carabid beetle *Leptocarabus kumagaii*, which use the stemmata for photoperiodic induction of larval diapause. Involvement of the ocelli in a photoperiodic response has not been demonstrated.

Involvement of the brain as the nonvisual extraretinal photoreceptor is also verified in Hemiptera, Orthoptera, Lepidoptera, Coleoptera, and Diptera. Notable examples include a photoperiodic induction of diapause in the hawkmoth *Manduca sexta*. In this species, short days in the larval stage induce pupal diapause, whereas diapause can be averted during long days. Brains attached with endocrine organs, corpora cardiaca and corpora allata (CA), were taken from the larvae and were cultured in vitro under long days or short days. The brains were implanted in recipient larvae that had been reared under a short day length regime. Brains that were cultured under long days altered the diapause program in some recipients, but the brains that were cultured under short days did not. These findings show that brains can receive photoperiodic information without retinal photoreceptors in vitro. The photoperiodic photoreceptor responsible for alteration of the reproductive modes in an aphid also lies in the brain. It is also important to note that retinal and extraretinal photoreception is not mutually exclusive. Usage of both types of photoreceptors has been demonstrated in adult diapause induction in *P. stali*.

Two types of photoreceptive molecules are known in animals: one is *visual pigments*, opsin proteins conjugated to vitamin A-based pigments retinal or 3-hydroxyretinal; and the other is *cryptochrome*, a cryptochrome protein conjugated to the vitamin B₂-based pigment flavin. Visual pigments are the major photoreceptive molecules in the retinal photoreceptors. Insect color vision is achieved with the presence of multiple types of visual pigments covering a broad range of wavelengths. Cryptochrome, which absorbs short wavelength light in the range from ultra-violet to blue, is well-known as a photoreceptor for resetting a circadian clock by phase delay or advance. The disruption of photoperiodic responses by the dietary vitamin A-deficient experiment in *B. mori* and other species, and by gene silencing of opsin expression in *M. siamensis* suggest an involvement of visual pigments in photoperiodic photoreception. The role of cryptochrome in photoperiodism has not been demonstrated.

Photoperiodic Time Measurement System

Although the term “day length,” i.e., the duration of the daily light phase, is commonly used to describe given photoperiodic conditions, most insects measure the *night length*, the duration of the daily dark phase, based on photoperiodic information acquired through photoreceptors. This information is processed by the *photoperiodic time measurement system*. Although time measurement is sometimes regarded as a simple qualitative or all-or-none mechanism, i.e., simple discrimination between short and long days, various experiments with many insect species have revealed that it is instead quantitative.

Erwin Bünning, a German chronobiologist, first proposed the involvement of a circadian clock in photoperiodic time measurement. In his hypothesis (the Bünning's hypothesis), a circadian clock with 24-h periodicity comprises two 12-h half-cycles (photophil and scotophil). Short-day effects are seen when light is restricted to the photophil, while long-day effects are produced when light penetrates the scotophil. Although this concept is too naïve to explain the range of photoperiodic responses in various insect species, the basic concept, i.e., involvement of a circadian clock in photoperiodic time measurement, is now widely accepted not only in insects but also in other organisms. A variety of photoperiodic time measurement models have been established by incorporating the data accrued under different experimental conditions; among these, the *external coincidence model* and *internal coincidence model* are highly influential. The external coincidence model is based on a circadian clock, which sets its phase and positions of the “photoinducible phase ((ϕ)i)” in the latter half of scotophase. Under short days, (ϕ)i is not illuminated by light, eliciting a short-day response; under long days, (ϕ)i is exposed to light and a long-day response is induced. The internal coincidence model proposes two oscillators entrained by dawn and dusk, respectively, and whose internal phase relationship changes with photophase length. Specific phases for each oscillator are active phases, and long-day responses occur when these overlap. The earliest attempts to model time measurement envisaged a non-repetitive *hourglass* timer that measures night length from the dusk signal of the daily cycle. This hourglass-timer concept is fascinating for insects that show little clear evidence for a role of the circadian system in photoperiodic time measurement.

Although for many years insect physiologists have focused on the time measurement system, functional elements involved in the system are still largely veiled. For further explanation on the photoperiodic time measurement models (see [Saunders, 2002](#)).

Photoperiodic Counter

Although a single inductive photoperiodic cycle is sufficient to promote a seasonal event in some species, such a case is quite rare. In most cases, a number of the inductive photoperiodic cycles are required to elicit a photoperiodic response. A *photoperiodic counter* counts the number of cycles during the photoperiod-sensitive period and compares the number (the induction sum) with an internal threshold, which triggers a physiological response mediated by endocrine effectors.

The experimental transfer of insects from long to short day cycles or vice versa can be used to determine the photoperiod-sensitive stage and also the number of inductive cycles required for photoperiodic induction (or termination). Both short days and long days can frequently be accumulated, but there are some examples of accumulation of either of them. In one model of photoperiodic summation (Gibb's model), insects accumulate or reduce (under short or long days, respectively) a hypothetical diapause titer in the counter system. The time measurement system may evaluate photoperiods quantitatively, and thus the accumulated diapause titer in each day may quantitatively differ among photoperiods. At the end of photoperiod-sensitive stage, insects compare the accumulated titer with the internal threshold. A short-day response is elicited upon exceeding the internal threshold, whereas a long-day response is induced at subthreshold values.

The counter model is largely conceptual at present, and therefore, biochemical and molecular biological dissection are eagerly awaited.

Endocrine Effectors

Various hormones such as the *juvenile hormones (JHs)*, *ecdysteroids*, *prothoracicotrophic hormone*, *insulin-like peptides*, and *diapause hormone* regulate induction, maintenance, and termination of diapause at each of developmental stages (Denlinger *et al.*, 2012). In this section, we focus only on hormones that regulate adult diapause.

The major role of JH, an acyclic sesquiterpenoid that is synthesized at and released from the CA, in adult diapause has been reported in a large number of insect species; high hemolymph titer of JH induces reproduction, whereas low JH titer induces adult diapause. The JH titer is regulated by both JH synthesis and the degradation of this hormone. Active suppression of the CA by the brain has been demonstrated during diapause in Coleoptera, Diptera, Hemiptera, and Orthoptera. Although the significance of the brain neurons which reside in pars lateralis and pars intercerebralis and that of neuropeptides in the brain and the corpora cardiaca for regulation of the CA are reported, specific neurons and molecules have not been identified in any insect. In the Colorado potato beetle, *Leptinotarsa decemlineata*, JH esterase, which degrades JH, plays a central role of regulating the JH titer in diapause-destined adults, while in the locusts there are no differences in JH esterase activity between long-day and short-day adults.

The role of ecdysteroids in the regulation of adult diapause has been mainly investigated in *Drosophila melanogaster*, although this arrest appears to be quiescence induced by low temperature, rather than photoperiod. Ecdysteroids are synthesized in the prothoracic gland in larval and pupal stages, and in the ovaries in the female adult stage. Ovaries produce low levels of ecdysteroids during this reproductive arrest, and injection of 20-hydroxyecdysone terminates the arrest in a dose-dependent manner. The significance of ecdysteroids in regulating adult diapause has been proposed in Diptera, Hemiptera, and Orthoptera.

The involvement of insulin signaling in adult diapause has recently received substantial attention, involving *D. melanogaster* and the mosquito, *Culex pipiens*. In both cases, reduced insulin signaling appears to be linked to adult diapause. In *C. pipiens*, the insulin signaling cascade appears to reside in upstream of the JH signaling pathway.

References

- Denlinger, D. L. (2009). Diapause. In V. H. Resh, & R. T. Cardé (Eds.), *Encyclopedia of Insects* (pp. 267–271). Amsterdam: Academic Press.
- Denlinger, D. L., Yocum, G. D., & Rinehart, J. P. (2012). Hormonal control of diapause. In L. I. Gilbert (Ed.), *Insect Endocrinology* (pp. 430–463). Amsterdam: Elsevier.
- Goto, S. G., & Numata, H. (2014). Insect photoperiodism. In K. H. Hoffmann (Ed.), *Insect Molecular Biology and Ecology* (pp. 217–244). Boca Raton, FL: CRC Press.
- Saunders, D. S. (2002). *Insect Clocks* (third ed.). Amsterdam: Elsevier.
- Tauber, M. J., Tauber, C. A., & Masaki, S. (1986). *Seasonal Adaptations of Insects*. New York: Oxford University Press.

PUBERTY AND METAMORPHOSIS RELATED TO REPRODUCTION

Puberty in Fish

Eva Andersson, Geir L Taranger, and Anna Wargelius, Institute of Marine Research, Bergen, Norway

Rüdiger W Schulz, Utrecht University, Utrecht, The Netherlands

© 2018 Elsevier Inc. All rights reserved.

Introduction

Puberty is the transition from an immature juvenile to a mature, adult stage when an individual becomes capable of reproducing sexually for the first time. This involves the functional competence of the endocrine system that governs maturation in vertebrates; the brain-pituitary-gonad (BPG) axis. This axis is composed of neuro-endocrine circuits in the brain that regulate the synthesis and release of the two gonadotropins in the pituitary, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh). The two gonadotropins regulate pubertal development and adult functioning of the gonads to ensure the production of hormones and growth factors required for the development and maturation of the germ cells. Gonadal hormones and growth factors also exert feedback effects on the brain/pituitary level, where the gonadal feedback is merged with information from other internal (e.g., nutritional or health status) and external (e.g., photoperiod, temperature, behavior of conspecifics) sources into an integrated output to control the synthesis and release of Fsh and Lh.

Among the regulatory systems operating in the brain to control the different aspects of reproduction there are a number of systems operating in parallel to regulate the release of Fsh and/or Lh. Examples include the kisspeptin-Gnrh system, the gonadotropin-inhibitory hormone (Gnih), the neurotransmitter dopamine, neuropeptide Y, or neurokinin. However, different from the situation in mammals, where the kisspeptin-Gnrh system clearly is the decisive gateway controlling the regulation of reproduction, this is not the case in fish (Liu *et al.*, 2017), where the above-mentioned set of parallel pathways apparently operate in a more balanced manner and can compensate for each other's activity.

The activities of these regulatory system converge onto the pituitary. Production, but in particular release, of Fsh and Lh is what determines pubertal development of the gonads. It is important to realize that different from land vertebrates, where the two gonadotropins are produced in a single cell type, while there are separate Fsh- and Lh-producing gonadotrope cells in fish. Here, we will focus on exogenous and endogenous factors that are integrated by the brain-pituitary system to modulate the pituitary gonadotropin release in such a way that puberty is either initiated or blocked, i.e., we will discuss conditions that influence the timing of puberty.

Puberty is a major lifecycle transition period, a prerequisite for vertebrate reproduction and thus the conservation of species. Studies of the physiological background determining the control of puberty in fish are highly relevant also from an applied point of view to provide efficient protocols for producing food for human consumption, as aquaculture accounts for around 50% of the global production of fish. The sustainability of aquaculture can be compromised by early (precocious) puberty, a major problem particularly in males in many farmed fish species. The compromise is largely due to negative effects on fish growth, health, and welfare. When puberty is reached before the fish reach harvestable size in aquaculture, the mobilization of energy and resources required for the development of the gonads, secondary sexual characters, and for reproduction-specific behaviors, drain energy for somatic tissues, thus impairing somatic growth and resulting in a higher feed demand to restore growth after completion of spawning. Many salmonids die after spawning, and hence are lost in terms of eatable product. Welfare issues are particularly relevant in several salmonid species that spend different periods of their lifecycle in either fresh or sea water, and where puberty is associated with a reduced capacity to survive in seawater, while the growth to harvest size takes place in sea water. Moreover, when fish escape from aquaculture facilities, the chance that they have a genetic impact on wild populations is much higher when the escapees are sexually mature. However, in other species, a delay or a complete failure to enter puberty can be a limiting factor for successful cultivation.

While important advances have been made regarding the control of final gonadal maturation, ovulation and spermiation under farming conditions, relatively few advances have been made regarding the control of puberty. Approaches to control puberty may imply techniques to delay/arrest, or to induce/advance puberty, according to the species-specific reproductive patterns shown under cultivation conditions.

Environmental Conditions Affect the Timing of Puberty

The reproductive cycle in those fish that exhibit a strong seasonality in their breeding activities is controlled by annual environmental cues related to climate and feed availability. Photoperiod, water temperature, rainfall, food availability, water quality and water level are environmental factors shown to synchronize the reproductive cycle to the optimal time of the season. In temperate regions photoperiod and/or temperature changes are the main cues controlling seasonal reproduction and thus the initiation and completion of puberty.

Pubertal development is particularly well studied in salmonids, such as rainbow trout, Coho salmon, and Atlantic salmon (Taranger *et al.*, 2010). As an example, the early puberty phenomenon in male Atlantic salmon can be observed at very different life stages; in freshwater parr (typically at 10–30 g), in post smolts after one summer in seawater (150–500 g), and after one sea winter (2–5 kg). The Atlantic salmon can also delay puberty into 2 or more years in seawater as fully-grown adults. Hence, male maturation shows considerable plasticity when it comes to age and size at puberty. This seems to be in particular affected by the somatic growth rate, but also by photoperiod and water temperature. These environmental conditions also affect females that usually mature at a larger body size than males, mainly because of the much higher metabolic demands of vitellogenesis/egg production, compared to spermatogenesis. Thus, size seems to matter in females more than in males. On the other hand, size also matters in males. Males that mature after one sea winter are larger than siblings that mature after two or three years in seawater, suggesting that the more rapidly growing individuals within a family will mature earlier than slower growing siblings.

Photoperiod manipulation can inhibit, or significantly delay, puberty in Atlantic salmon by using continuous light during the winter period, when initiated shortly after the winter solstice. An increasing photoperiod after the winter solstice usually triggers the decision to mature. However, only those individuals that meet the energetic and/or body mass threshold when experiencing an increasing day length, will initiate puberty. Those that do not meet these requirements will delay the decision to mature until the following spring (Fig. 1).

The same pattern seems to occur in several other fish species, where there is a strong relationship between somatic growth rates and age at puberty. Hence under farming conditions, high growth rates and/or high adiposity often result in precocious male puberty, compared to wild fish.

By advancing the seasonal perception of spring/summer via an artificially long photoperiod during the winter months, fewer fish are believed to have reached the required size and energy status when the “window” for puberty requires a decision and has been shifted back several months. Studies have also demonstrated that a delay of the normal increase in photoperiod during the winter months enable more fish to attain puberty in the upcoming spawning season. This principle seems to apply both in rainbow trout and Atlantic salmon, the species where most of photoperiod manipulation studies have been conducted. Based on such observations, photoperiod protocols have been developed to delay puberty in Atlantic salmon on-growing farms enabling improved growth performance, and typically delaying puberty for a full year. However, the precise effect of a specific photoperiod treatment will depend on the natural seasonal timing of spawning in a species or population. Photoperiod protocols that are effective in autumn spawning Atlantic salmon, for example, may not be effective in winter and spring spawning rainbow trout. Moreover, the outcome of the photoperiod treatment can also depend on the ambient water temperature cycle, e.g., in areas with higher sea water temperatures (i.e., conditions more favorable for rapid growth), the temperature/growth effect may override the photoperiodic cue for the timing of maturation.

Also depending on time in season and life-stage, a given photoperiod treatment can have opposite effects on age and size at puberty. For example, continuous light and elevated water temperature can induce early male maturation around the time of sea water transfer (Fjelldal *et al.*, 2011) at the post-smolt stage. In this case, high water temperatures and larger fish size increases the likelihood of males entering puberty early when exposed to a long photoperiod signal.

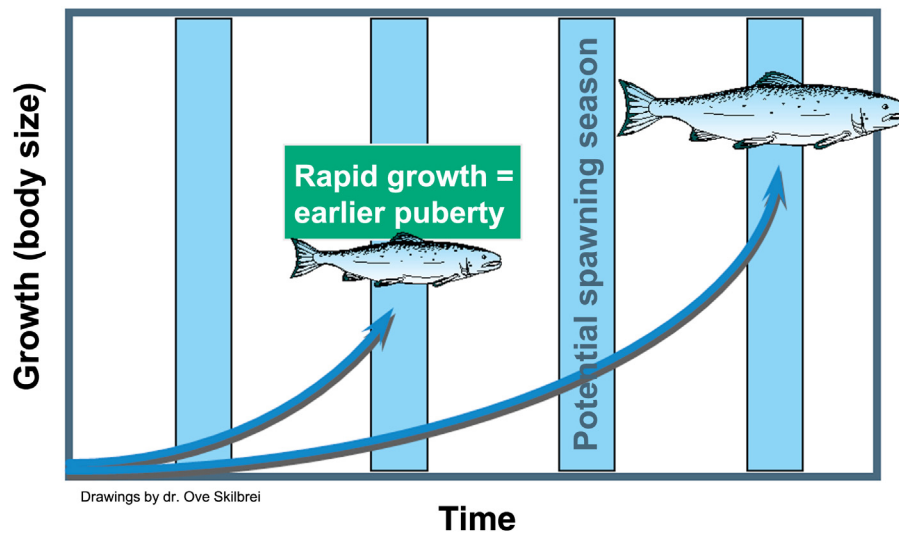


Fig. 1 Increased growth rate normally results in the fish reaching puberty at an earlier age. Such early puberty is often found under the rapid growing conditions normally found in aquaculture. In fish species living in temperate zones spawning is often confined to a short optimal period in the year, and a delay in puberty to later years will allow a prolonged growth period. This can in turn result in both a delay in age at maturity and larger body size at puberty, despite the overall slower growth rate. Figure modified from Taranger, G.L., Carillo, M., Sculz, R.W., *et al.*, 2010. Control of puberty in farmed fish. General and Comparative Endocrinology 165, 483–515.

Early puberty is also a common problem in aquaculture of other finfish species such as the European sea bass, the Atlantic cod, and Atlantic halibut. The problem is most commonly seen in males. As an example, male European sea bass normally reach puberty at two years of age, whereas females normally reach puberty at three years of age. By contrast, in aquaculture, some males reach puberty already at an age of one year. While the largest males are the ones that are likely to mature, their growth rate is hampered compared to immature males during the years to follow (Carrillo *et al.*, 2015). A similar situation is seen in the Atlantic halibut, where puberty in males typically occurs at an age of 4–5 years and at a size of around 2 kg in wild stocks. However, under farming conditions males typically mature at an age of 2–3 years at the same size as wild males. Upon reaching puberty, the male halibut typically show a much slower growth during the subsequent years compared to the still immature females. Hence, it is beneficial to use all-female populations in halibut farming. Also, in Atlantic cod puberty occurs earlier under farming condition than found in natural populations. Atlantic cod of the northeast arctic cod population usually enters puberty at 4 to 8 years, while the Norwegian coastal cod population does so starting at 3 years of age. This difference disappears under farming conditions as all tested stocks reach puberty at 2 years of age at 1.5–2 kg. Some cod mature even earlier at 1 year of age, and a mean body weight below 300 g creating major problem for cod farming sustainability.

Photoperiod treatments can delay puberty in farming of both sea bass and Atlantic cod, but the protocols are often found to be less effective when applied superimposed on the ambient light in sea cages as opposed to indoor tanks with full photoperiod control. Hence, more research is needed to refine photoperiod protocols for different species and strains for sea cage aquaculture.

The Endocrine System Mediates Environmental Signals to Regulate Gonad Maturation

One of the first tangible signs of puberty is an accelerated growth of the gonads in both sexes. Until puberty, gonadal and somatic growth show a similar pace (allometric growth), i.e., the gonado-somatic index (GSI, gonad weight expressed as per cent of the total body weight) is normally small and constant. At puberty, a superallometric growth phase of the gonad starts, the gonads grow faster than other organs/the rest of the body, and therefore the GSI increases.

The specific activation of gonadal growth is triggered by an increased release of gonadotropic hormone from the pituitary gland. The most detailed information on changes in blood hormone levels is available for salmonid species, like Atlantic and Pacific salmon and rainbow trout. In these species, the pituitary release of follicle-stimulating hormone (Fsh) increases when gonad growth starts; luteinizing hormone (Lh), the other gonadotropic hormone, is not secreted until much later close to the spawning season. Therefore, the initiation but also most of the gonadal growth period depends mainly on Fsh in these species.

The structure of the gonads, the cells that responds to the gonadotropic signal from the pituitary differs between the sexes. In the ovary, the follicular layer surrounds the oocytes, consisting of two somatic cell types, granulosa and theca cells. The receptor for Fsh is present in these somatic cells, mainly in the granulosa cells and to a lesser extent in the steroid producing cells of the theca layer (Miwa *et al.*, 1994; Andersson *et al.*, 2009). Upon the increased production and release of Fsh in the pituitary, Fsh stimulates growth of the ovary and the subsequent puberty onset, i.e., the entry into vitellogenesis, the process during which the oocytes accumulate yolk proteins to support embryogenesis after fertilization.

In the males, the receptor for Fsh is expressed by somatic cells in the testis. One of them are the Sertoli cells. These cells are in direct, close contact to developing germ cells during spermatogenesis and Sertoli cells regulate germ cell development via growth factors and cell-cell contacts. For example, Fsh increases Sertoli cell release of growth factors that promote spermatogenesis, such as insulin-like growth factor 3 (Igfb3), but Fsh also reduces the release of growth factors blocking spermatogenesis, such as anti-Müllerian hormone (Amh). Teleost fish are the only vertebrates, in which the interstitial Leydig cells, next to Sertoli cells, express the receptor for Fsh while in common with other vertebrates, these sex steroid producing cells in the interstitial area of the testis express the receptor for Lh (Schulz *et al.*, 2010). As mentioned above, however, this receptor usually is not stimulated by Lh until shortly before the spawning season when plasma Lh levels increase.

Hence, the gonadotropins, in particular Fsh, regulate sex steroid and growth factor production from somatic cells in the testis. The androgens produced by the Leydig cells are able to stimulate full spermatogenesis (Ohta *et al.*, 2007). Again, the respective androgen receptors are expressed by somatic cells, not by germ cells, so that androgens stimulate spermatogenesis indirectly by regulating Sertoli, myoid and Leydig cell gene expression. These somatic cells then modulate germ cell behavior by growth factor or direct somatic-germ cell signaling. In addition to steroid-dependent mechanisms, recent work in rainbow trout (Sambroni *et al.*, 2013) and zebrafish (Crespo *et al.*, 2016) has shown that Fsh changes the expression of hundreds of genes in the testis in a steroid-independent manner. The most prominent examples are again growth factors, such as the already mentioned Igfb3, but also the Leydig cell derived insulin-like peptide 3 (Insl3). In summary, for the initiation as well as completion of pubertal spermatogenesis, at least two parallel signaling systems are operating in the testis: androgens, which can stimulate full spermatogenesis in fish by yet ill defined mechanisms, and – in a sex-steroid independent manner – a set of growth factors that promote mainly the initial, spermatogonial phase of spermatogenesis.

Genetic Background of the Timing of Puberty

Since Atlantic salmon show a large variation for sea age at sexual maturity, it is a good model species to explore the genetic basis of the timing of puberty. It has also been known for a long time that there is a genetic component contributing to time

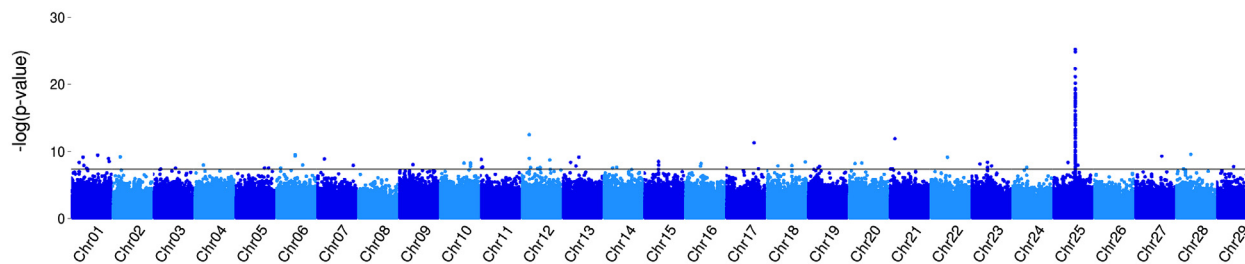


Fig. 2 Manhattan plot showing the region chromosome 25 under selection for time of maturity in Atlantic salmon. Picture modified from Ayllon, F., Kjærner-Semb, E., Furmanek, T., *et al.*, 2015. The *vgl3* locus controls age at maturity in wild and domesticated Atlantic salmon (*Salmo salar* L.) males. *PLOS Genetics* 11 (11), e1005628.

of maturity in salmonids (Gjerde *et al.*, 1984, 1994). Very recently, new biotechnological approaches served to find specific regions in the salmon genome significantly contributing to the timing of maturation, such as SNP platforms (Johnston *et al.*, 2014; Gutierrez *et al.*, 2015). Also, it has been possible to re-sequence entire genomes of individual wild salmon maturing either early or late and use the information for genome wide association studies (GWAS). This methodology has identified a region in the salmon genome (Fig. 2) explaining up to 36% of the time of maturity phenotype (Ayllon *et al.*, 2015; Barson *et al.*, 2015). The precision of this technology has made it possible to identify a single candidate gene controlling time of maturity in salmon, the *vestigial-like protein 3* (*vgl3*). This protein functions in the hippo pathway as a cofactor for transcription factors. *Vgl3* has previously been identified as an inhibitor of proliferation in gonads of the fruit fly and chicken, and inhibits adipocytes differentiation in mice. Also in humans, the *VGLL3* locus was linked to age at puberty. Changes in fat metabolism may cause changes in age at maturity, since increased adiposity has been linked to maturation in salmon. Further functional studies will show if *Vgl3* and/or associated proteins of the Hippo pathway are regulators of age at maturity and if this applies to fish in general.

References

- Andersson, E., Nijenhuis, W., Male, R., *et al.* (2009). Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (*Salmo salar* L.) ovaries. *General and Comparative Endocrinology*, 163, 329–339.
- Ayllon, F., Kjærner-Semb, E., Furmanek, T., *et al.* (2015). The *vgl3* locus controls age at maturity in wild and domesticated Atlantic salmon (*Salmo salar* L.) males. *PLOS Genetics*, 11(11), e1005628.
- Barson, N. J., Aykanat, T., Hindar, K., *et al.* (2015). Sex-dependent dominance at a single locus maintains variation in age at maturity in salmon. *Nature*, 528, 405–408.
- Carrillo, M., Espigares, F., Felip, A., *et al.* (2015). Updating control in male European sea bass: A holistic approach. *General and Comparative Endocrinology*, 221, 42–53.
- Crespo, D., Assis, L. H., Furmanek, T., Bogerd, J., & Schulz, R. W. (2016). Expression profiling identifies Sertoli and Leydig cell genes as Fsh targets in adult zebrafish testis. *Molecular and Cellular Endocrinology*, 437, 237–251.
- Fjelldal, P. G., Hansen, T., & Huang, T. (2011). Continuous light and elevated temperature can trigger maturation both during and immediately after smoltification in male Atlantic salmon (*Salmo salar*). *Aquaculture*, 321, 93–100.
- Gjerde, B. (1984). Response to individual selection for age at sexual maturity in Atlantic salmon. *Aquaculture*, 38, 229–240.
- Gjerde, B., Simianer, H., & Refstie, T. (1994). Estimates of genetic and phenotypic parameters for body-weight, growth-rate and sexual maturity in Atlantic salmon. *Livestock Production Science*, 38, 133–143.
- Gutierrez, A. P., Yanez, J. M., Fukui, S., *et al.* (2015). Genome-wide association study (GWAS) for growth rate and age at sexual maturation in Atlantic salmon (*Salmo salar*). *PLOS ONE*, 10, e0119730.
- Johnston, S. E., Orell, P., Pritchard, V. L., *et al.* (2014). Genome-wide SNP analysis reveals a genetic basis for sea-age variation in a wild population of Atlantic salmon (*Salmo salar*). *Molecular Ecology*, 23, 3452–3468.
- Liu, Y., Tang, H., Xie, R., *et al.* (2017). Genetic evidence for multifactorial control of the reproductive axis in Zebrafish. *Endocrinology*, 158, 604–611.
- Miwa, S., Yan, L., & Swanson, P. (1994). Localization of two gonadotropin receptors in the salmon gonad by in vitro ligand autoradiography. *Biology of Reproduction*, 50, 629–642.
- Ohta, T., Miyake, H., Miura, C., *et al.* (2007). Follicle-stimulating hormone induces spermatogenesis mediated by androgen production in Japanese eel, *Anguilla japonica*. *Biology of Reproduction*, 77, 970–977.
- Sambroni, E., Lareyre, J. J., & LeGac, F. (2013). Fsh controls gene expression in fish both independently of and through steroid mediation. *PLOS ONE*, 23, e76684.
- Schulz, R. W., de França, L. R., Lareyre, J. J., *et al.* (2010). Spermatogenesis in fish. *General and Comparative Endocrinology*, 165, 390–411.
- Taranger, G. L., Carrillo, M., Schulz, R. W., *et al.* (2010). Control of puberty in farmed fish. *General and Comparative Endocrinology*, 165, 483–515.

Puberty & Metamorphosis Related to Reproduction: Metamorphosis, Insects

Cynthia Lenaerts, Jozef Vanden Broeck, and Elisabeth Marchal, KU Leuven, Leuven, Belgium

© 2018 Elsevier Inc. All rights reserved.

Introduction

Although insects are often regarded as negative to our economy because of their phytophagous nature or their ability to act as vectors of diseases, mankind would have difficulties surviving without pollinator species, such as bumblebees and bees. Insects are the most abundant and diverse group of organisms on earth. Having maintained this position for over 400 million years, they have witnessed the rise and fall of the dinosaurs and survived planet-wide extinctions. Despite our best efforts at eradication of numerous pest insect populations, this very speciose group of animals continues to thrive. Insects share a unique combination of attributes that has enabled their unparalleled success. This includes a small body size, the ability to fly, high reproductive potential, the presence of an exoskeleton, their developmental plasticity and ability to undergo metamorphosis. The changes that take place during this process can be extreme, allowing juvenile and adult insects to take advantage of different food sources and adapt in an ever-changing environment.

Insect Life Cycles

Insects do not have an internal skeleton as vertebrates do, instead, they are surrounded by a rigid armor, called the exoskeleton. This exoskeleton or cuticle protects them from desiccation and mechanical injury, but also gives the insect shape and provides a base to which muscles are attached. Although the rigidity of this cuticle protects them, it makes the apparently 'simple' process of growing extremely complex. For the insect to increase in size, the cuticle must be shed periodically and a larger one must be synthesized. During this process of molting a new cuticle is synthesized under the old one, part of the old cuticle is digested and resorbed and finally the remaining old cuticle is shed in a behavioral process called ecdysis.

In this way, the life cycle of an insect is characterized by several successive stages, each separated by a molt. Most insects hatch from eggs into a first juvenile stage. When the immature insect grows, it will pass through a defined number of instar stages (exact number varies depending on the insect species), before completing metamorphosis into the (winged) adult reproductive stage. Three major modalities of metamorphosis can be found within the diversity of insects. These include no changes or ametaboly; partial or incomplete metamorphosis known as hemimetaboly; and complete metamorphosis or holometaboly.

Metamorphosis is a marked change in body form, through which the adult gains distinct features. Ametabolous insects, such as silverfish (order Thysanura), do not undergo metamorphosis. In this design, the immature stages resemble the adult stage, except from the presence of mature gonads and functional genitals. At each molt these insects increase in size and their gonads and genitals develop progressively, until the animal is able to reproduce. Unlike hemimetabolous or holometabolous insects, Ametabola can continue to molt and grow after reaching the reproductively competent stage. Hemimetabolous insects, such as locusts (Orthoptera), cockroaches (Dictyoptera) and true bugs (order Hemiptera), undergo incomplete metamorphosis. The adult morphology of these insects closely resembles that of the instar stages (also called nymphs), but only differs by the presence of functional wings, gonads and genitals. Throughout their life cycle these insects undergo a gradual transformation with a sharp discontinuity at the adult molt, undergoing metamorphosis during a single molt (Fig. 1(A)). Since they maintain a similar body plan throughout their life, juvenile and adult stages of most hemimetabolous insects live in the same habitat and use the same food. Consequently, competition may exist between the needs for growth and the needs for reproduction. Notable exceptions are the mayflies (Ephemeroptera) and dragonflies (Odonata), which evolved quite different nymphal and adult stages, thereby allowing each to exploit a different habitat. Holometabolous insects, such as butterflies (order Lepidoptera), beetles (order Coleoptera), wasps, ants and bees (order Hymenoptera) and flies (order Diptera), undergo complete metamorphosis. In this design, the body plan of the instars (also called larvae) differs drastically from that of adults. These animals are therefore able to populate different niches and separate the needs for growth from those for reproduction. To allow complete metamorphosis, Holometabola go through a non-feeding pupal stage (Fig. 1(B)). Upon hatching, the primordia of the adult organs (such as wings, legs, eyes, genitals, etc.) are already present within the larvae as so-called imaginal discs (Section Holometabola). These imaginal discs will only differentiate to adult organs during metamorphosis in the pupal stage. This innovation resulted in a rapid diversification of insects and the establishment of the holometabolous orders that are living today. According to the 'pronymph hypothesis' (Truman and Riddiford, 1999), the pupal stage in Holometabola corresponds to the nymphal stages in Hemimetabola, while the holometabolous larval stages correspond to the pronymphal stage in Hemimetabola. This pronymphal stage is the period during which hemimetabolous embryos are enveloped by a cuticle, which is shed upon hatching.

Control of Metamorphosis

Independent of the design of the life cycle, postembryonic development is highly timed and choreographed through the action of different factors. Here, we will focus on the action of the lipophilic insect hormones: juvenile hormones and ecdysteroids,

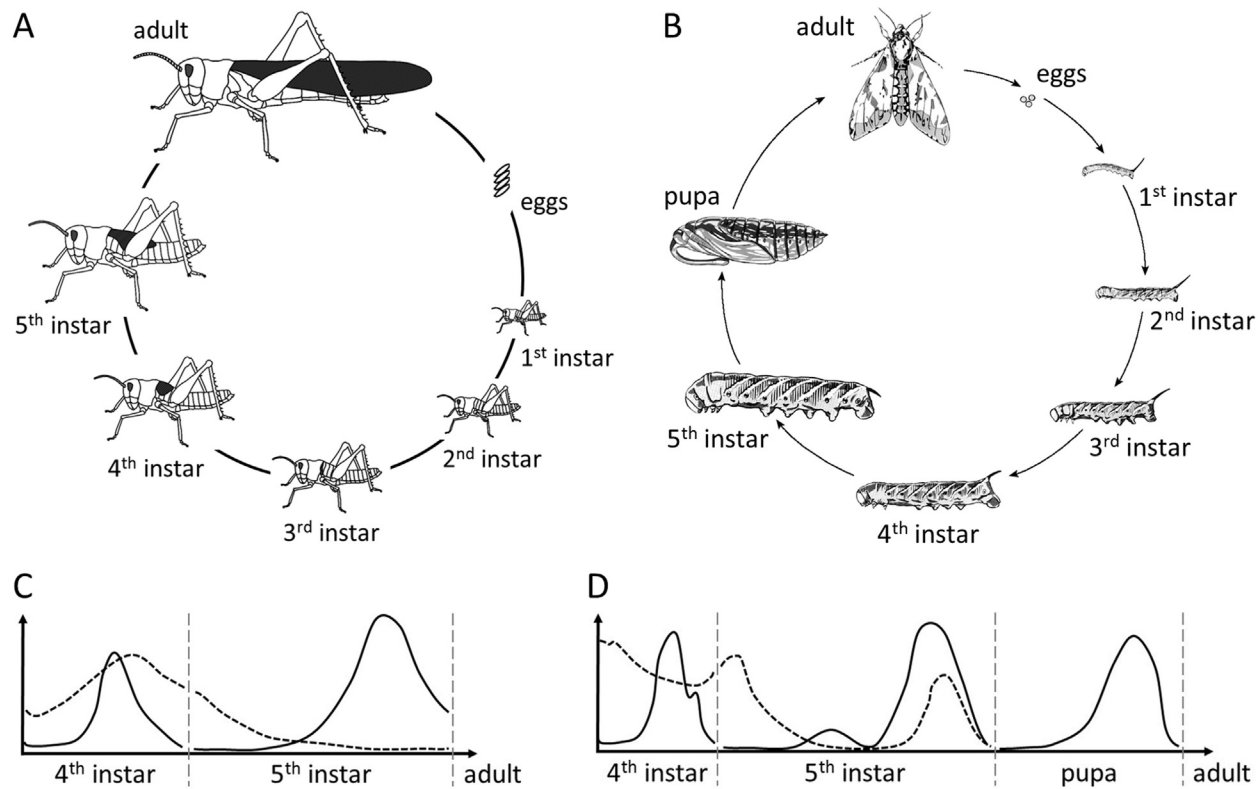


Fig. 1 Hemimetabolous and holometabolous life cycles and hormone titers. (A) Life cycle of the desert locust, *Schistocerca gregaria*, a typical hemimetabolous insect, containing 3 stages: egg, instars (or nymphs) and adult. (B) Life cycle of the tobacco hornworm, *Manduca sexta*, a typical holometabolous insect, containing 4 stages: egg, instars (or larvae), pupa and adult (moth) (Reproduced from Verlinden, H., Vleugels, R., Zels, S., et al., 2014. Chapter three – Receptors for neuronal or endocrine signalling molecules as potential targets for the control of insect pests. In: Cohen, E. (Ed.), Target Receptors in the Control of Insect Pests: Part II, Advances in Insect Physiology. Burlington: Academic Press, pp. 167–303. Available at: <https://doi.org/10.1016/B978-0-12-417010-0.00003-3>). (C–D) Changes in the juvenile hormone (dotted line) and ecdysteroid (full line) titers regulating molting and metamorphosis in (C) the desert locust and (D) the tobacco hornworm (Reproduced from Chapman, R.F., 2012. The Insects: Structure and Function, fifth ed. Cambridge: Cambridge University Press.).

neuropeptides and insulin-like peptides (ILP) during metamorphosis, a process that requires a strictly regulated balance between cell death of the juvenile tissues and proliferation of the adult ones.

Hormonal Control of Metamorphosis

Post-embryonic development of insects is highly dependent upon different types of hormones. The classic insect hormones are the lipophilic ecdysteroids and juvenile hormones (JHs). In several insect model species, such as the fly *Drosophila melanogaster* and the moth *Manduca sexta*, ecdysteroid biosynthesis is initiated through the action of prothoracicotropic hormone (PTTH). PTTH is secreted by a few neurosecretory cells in the brain that terminate in the corpora allata (CA), a pair of highly specialized neuroendocrine organs in the insect's head (see below). Other factors will also influence the ecdysteroidogenic activity of the PG and are discussed by Marchal et al. (2010). The secretion of PTTH is highly timed (Section Growth and Critical Weight) and occurs prior to each molt. PTTH will bind to its receptor Torso, which is present on the membrane of a specialized endocrine organ called the prothoracic gland (PG) (Rewitz et al., 2009). These glands express the so-called *Halloween* genes encoding cytochrome P450 enzymes responsible for ecdysone (E) biosynthesis. The release of E into the hemolymph surges prior to each molt. E will be converted to its active metabolite, the molting hormone 20-hydroxyecdysone (20E), in several non-endocrine tissues. 20E will bind to a nuclear receptor complex of ecdysone receptor (EcR) and ultraspiracle/retinoid-X-receptor (USP/RXR), thereby initiating transcription of different pathways and eventually triggering a strictly regulated cascade of neuropeptides, which induces the typical ecdysis behavioral sequence. For a detailed review on the neuropeptidergic regulation of ecdysis, the reader is referred to Žitňan and Adams (2012). The sesquiterpenoid JH is produced and secreted by the CA. The nature of the molt – larval to larval or larval to adult – is defined by the presence or absence of JH during this critical period. A surge in 20E titer in the presence of JH will ensure juvenile characteristics, resulting in a larval to larval molt. While a peak of 20E in the absence of JH results in a metamorphic molt. Because of this action, JH is also sometimes referred to as the 'status quo' hormone. JH exerts its function through methoprene-tolerant (Met), a basic-helix-loop-helix (bHLH)/Per-Arnt-Sim (PAS) protein. In several insect species, a steroid receptor co-activator (Taiman/SRC) has been identified as a functional partner of Met. JH binding to Met will ensure heterodimerization of Met with Taiman/SRC, allowing the complex to bind E box-like motifs present in the regulatory regions of downstream genes, such as the transcription factor Krüppel-homolog 1 (Nation, 2015).

Titers of these two critical hormones have been studied in a variety of hemimetabolous and holometabolous insect species. The changes in titers during metamorphosis of a representative hemimetabolous and holometabolous insect species will be discussed below.

To successfully fulfil their role in insect development, it is obvious that concentrations of both 20E and JH in the hemolymph must be strictly regulated and important cross talk exists. These concentrations are not only regulated by their biosynthesis, but also by their degradation and secretion. For a detailed review on the factors that regulate the ecdysteroid and JH titers in immature insect stages, the reader is referred to [Niwa and Niwa \(2016\)](#) and [Hiruma and Kaneko \(2013\)](#), respectively.

Hemimetabola

The JH and ecdysteroid titers throughout the final two instar stages of the desert locust, *Schistocerca gregaria*, are shown in [Fig. 1\(C\)](#). During the penultimate instar stage (4th instar) the JH titers are high during the critical period of molting. Consequently, the ecdysteroid peak precedes a nymphal to nymphal molt. On the other hand, in the last instar stage (5th instar) the lack of JH during the critical period results in a nymphal to adult molt. Experimental application of JH during this critical stage in last instars would lead to an extra nymphal stage.

Holometabola

Similar to Hemimetabola, high JH titers during the critical periods of molting result in a larval to larval molt ([Fig. 1\(D\)](#)). The larval-pupal transition in Holometabola is characterized by changes in hormone titers that have no counterpart in postembryonic development of hemimetabolous insects. In the penultimate instar stage (4th instar) of the tobacco hornworm, *M. sexta*, JH titers decline moderately prior to molting. However these titers are still high enough to produce a new larval cuticle and prevent the development of imaginal discs ([Section Holometabola below](#)). In the last larval stage, JH titers drop below detectable levels or reach trace levels right before a small peak in ecdysteroid titer. This first, small ecdysteroid peak in the absence of JH is called the commitment peak and is not sufficient to initiate molting, instead it results in an irreversible commitment to metamorphosis. Consequently, the insect will stop feeding and enters what is called the wandering stage, during which it will seek an appropriate place to pupate. It will also cause the epidermis to switch its genetic program so that it can no longer produce a larval cuticle, but only a pupal cuticle when exposed to the subsequent, 10 times higher, ecdysteroid peak. This second ecdysteroid peak eventually results in the secretion of the pupal cuticle and shedding of the larval one. Moreover, this second peak is accompanied by a peak in JH titer, which regulates the development of the imaginal discs. Due to the absence of the JH receptor in the epidermis at the time of this second ecdysteroid peak, a larval molt is prevented. When environmental conditions are suitable during the pupal stage, the adult molt will be initiated by a peak in ecdysteroid titer. As JH is absent during this surge in ecdysteroids, due to a rise in the level of JH esterase (which metabolizes JH), the adult cuticle can be produced. Application of JH during this ecdysteroid peak, would result in a pupal to pupal molt, indicating that the JH receptor is again present in the epidermis.

Growth and Critical Weight

Metamorphosis is a complex and tightly regulated process that concludes larval growth and results in the transformation to the adult stage. Since growth only occurs in immature stages, adult body size is determined by the size the larva attained when it stopped feeding and initiated metamorphosis. Control of size is therefore tightly connected to the mechanism that controls metamorphosis. Size determination has been well-studied in model insects, such as *M. sexta* and *D. melanogaster*. When the larvae of these holometabolous insects attain their critical weight, growth ceases and the above mentioned endocrine cascade and subsequent metamorphosis is initiated. Critical weight is defined as the weight of the insect at which initiation of metamorphosis can no longer be delayed by starvation, i.e., when last instar larvae have reached a certain body size after a period of feeding and intrinsic growth, the path to metamorphosis is inherently switched on. As long as the larva does not obtain its critical weight, its brain will be unable to release PTTH, the primary inducer of ecdysteroid biosynthesis in the PG ([Section Hormonal Control of Metamorphosis](#)). The attainment of critical weight provides a checkpoint that ensures that larvae have stored enough nutrients to make it through the non-feeding pupal stage, during which these nutritional stores will fuel the development of adult tissues and structures. Larval growth is therefore crucial for effective completion of metamorphosis and the success of reproduction in the adult stage. The mechanism that initiates PTTH and 20E secretion prior to the metamorphic molt, when JH is absent, will control the final body size and can be quite diverse in different insect species.

The attainment of critical weight is controlled by both environmental and genetic factors. The principal genetic regulators of growth are ILP and ecdysone, together with their receptors and signalling pathways. The biosynthesis and secretion of these hormones are in their turn regulated by complex interactions of other endocrine and molecular mechanisms, by environmental factors, such as nutrition, and by physiological mechanisms that sense body size. Also oxygen levels appear to be involved in critical weight sensing. For a detailed review, the reader is referred to [Nijhout \(2015\)](#).

Maturation of the Reproductive System in Immature Stages

The changes that take place during transformation of the juvenile to adult insect depend on the design of their life cycle, hemimetabolous or holometabolous. Nevertheless, molting of the insect to the adult stage inherently entails the development of functional gonads and genitals. Here, we will discuss changes that take place in hemimetabolous and holometabolous insects during metamorphosis with a focus on the reproductive organs. While only gradual changes take place in hemimetabolous insects, holometabolous insects go through a stage with very marked changes. Throughout post-embryonic development growth of internal

organs may result from an increase in cell size and/or cell number. In general, tissues that are destroyed upon metamorphosis undergo cell enlargement, while those that persist undergo cell multiplication.

Hemimetabola

Hemimetabola gradually develop adult features or instar features just resemble those of adults. Two examples of gradually developing traits are the wing buds and the genitalia. At each molt, the wing buds will increase in size, until they form functional wings in the adult stage (**Fig. 1(A)**, wing buds and wings in black). Similarly, the genitalia develop throughout post-embryonic development, but are only functional in the adult stage. Also the gonads are already present after hatching and gradually grow. In many insects, sperm production is already initiated during the last instar, although spermatogenesis continues in the adult stage. Also oogenesis can start in last instars, but vitellogenesis is a typical adult feature. On the other hand, the musculature of instars resembles that of adults, even the flight muscles are already present in instars. Although, these flight muscles still increase in size by an increase in both fiber size and the amount of fibers. Some muscles that are only used during ecdysis disappear in adults. Consequently, also the neuronal supply of these muscles dies. Other changes in the neuronal system are found in the sensory neuronal pathways, where some neurons change their role towards adult behavior.

Holometabola

In holometabolous insects the degree of modifications during metamorphosis depends on the resemblance between the larval and adult stage. Relatively small modifications take place in beetles (Coleoptera), while in flies (Diptera) and butterflies (Lepidoptera) tissues are almost completely rebuilt after hydrolysis and phagocytosis. The pupal stage allows the transition between the larval and adult form. During the pupal molt and subsequent pupal stage, all larval organs, except the central nervous system and reproductive organs, are broken down and major internal reconstruction takes place. In the pupal stage all adult features become recognizable, although they are not fully expanded to their adult form. As eclosion only takes place under the appropriate environmental conditions, metamorphosis may already be completed a few hours, days or weeks before eclosion.

Details on the developmental changes during metamorphosis vary between insect species and organs. In general there is an extensive reconstruction of the musculature and the central nervous system (CNS). For instance, flight muscles will be formed and muscles will be attached to the cuticle during the pupal stage. As the adult insect is characterized by a larger brain, compound eyes and larger antennae, the CNS is also thoroughly remodeled. Furthermore, the digestive system of most holometabolous insects undergoes drastic changes due to the different larval and adult diets. Larval stages often feed on leaves, while the adult stage often has a fluid diet. As some organs are remodeled, others are newly formed. These organs develop from imaginal discs, undifferentiated groups of ectodermal cells that will grow into adult tissues only under appropriate hormonal control during pupation. During the larval stages the cells of these imaginal discs only increase in cell number by mitotic division. Ecdysteroid secretion and falling JH levels in the final instar of *D. melanogaster* will signal the imaginal discs to undergo rapid differentiation into adult structures during the pupal stage. The best known and most studied imaginal discs are those of the wings, legs and genitalia. While the imaginal discs of wings and legs are paired, the genital disc is unpaired and bilaterally symmetrical. The genital disc is located in a medial position at the posterior end of the insect. Genital discs are sexually dimorphic, i.e., their morphology and growth are sex-dependent. Genital discs will give rise to the hindgut, analia and internal and external genitalia, but not to the gonads. These organs (testes and ovaries) are formed during the embryonic stage and will slowly develop throughout the larval stages (**Fig. 2(B)**, green structures). Also the genital discs are formed during the embryonic stage and will increase in cell number and differentiate into a male or female disc throughout the larval stages (**Fig. 2(A)**). However, it is only in the pupal stage that these genital discs will develop into the previously mentioned organs (**Fig. 2(B)**). The embryonic genital disc contains three regions, the male genital primordium (MGP), the female genital primordium (FGP) and the anal primordium (AP). During larval development the sex-determination genes and homeotic genes determine the proliferation of the different regions. In female larvae the MGP is brought into a 'repressed state', called the repressed male genital primordium (RMP). In male larvae the FGP will be repressed and is therefore called the repressed female genital primordium (RFP). For a detailed review on the genetic control of genital disc formation and differentiation, the reader is referred to [Estrada et al. \(2003\)](#). In the prepupa the external genitalia (vulva, vaginal plates of female; penis apparatus, clasper of male) start to develop. The genital ducts (oviducts, uterus, vagina, seminal vesicles, ejaculatory duct and sperm pump) and accessory structures (seminal receptacle, spermathecae, accessory glands and paragonia) on the other hand only start development in the early pupa. After development of these reproductive tissues, the testes will make contact with the seminal vesicles, while the ovaries fuse with the oviducts. The adult eventually emerges with a fully formed reproductive system.

Depending on the insect species the freshly emerged adult will be reproductively competent or it will have to go through a period of maturation before being able to reproduce. The time frame in which spermatogenesis occurs, is species dependent. In most insects spermatogenesis already starts in the larval stages and formation of spermatids, and subsequent spermiogenesis, is often delayed until the last larval or pupal stage. In species that do not feed as adults, the whole process of spermatogenesis (incl. spermiogenesis) is completed before adult eclosion. However, in most insect species spermatogenesis is only completed in the adult stage. Often the production of new sperm continues throughout adult life. For a long time, spermatogenesis in insects was not considered to be under hormonal control. However, it has now become clear that in a wide range of insect species, both Hemimetabola as well as Holometabola, ecdysteroids synthesized in the testes can stimulate spermatogenesis ([Nation, 2015](#)). Similarly to spermatogenesis, oogenesis is very species dependent. In general, oocytes get stuck in the prophase or metaphase of

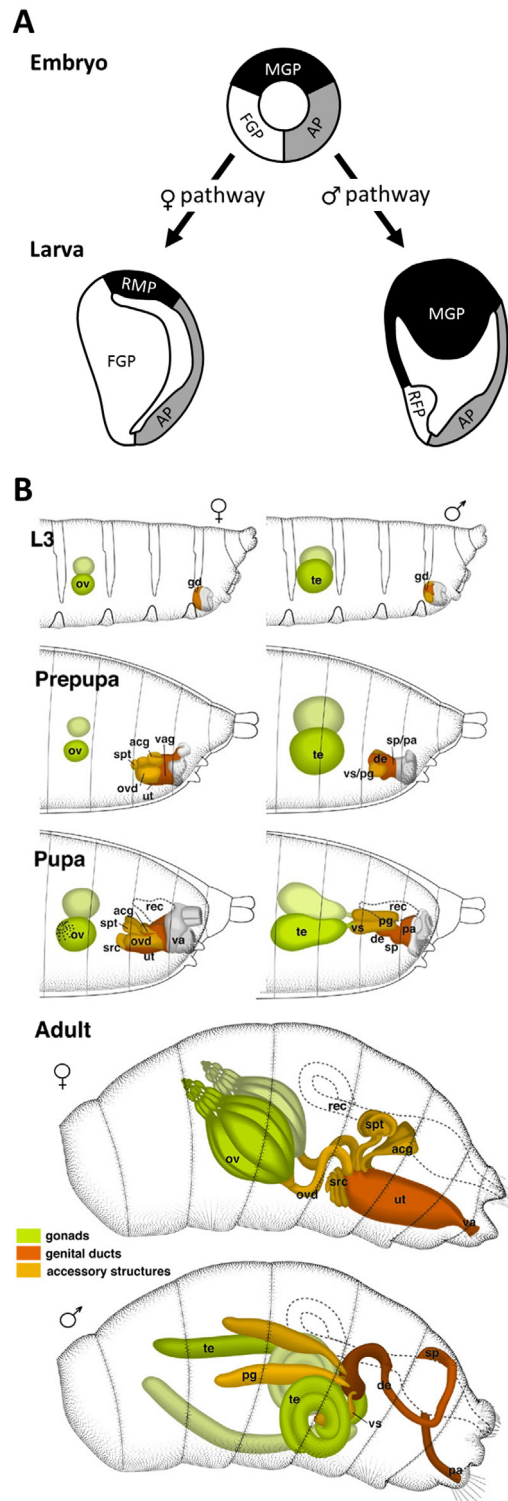


Fig. 2 Development of the reproductive organs in *Drosophila melanogaster*. (A) Schematic overview of the development of the genital discs in both sexes (from embryo to larva) (adapted from Sánchez, L., Guerrero, I., 2001. The development of the *Drosophila* genital disc. *BioEssays* 23, 698–707. doi:10.1002/bies.1099). (B) Schematic drawings of the development of the adult reproductive system from genital discs and gonads (ovaries or testes) (reprinted with permission from Atlas of *Drosophila* Development by Volker Hartenstein, Society for Developmental Biology's Web Server. Available at: <https://www.sdbonline.org/sites/fly/atlas/00atlas.htm>). acg, accessory glands; AP, anal primordium; de, ejaculatory duct; FGP, female genital primordium; gd, genital disc; L3, third instar larva; MGP, male genital primordium; ov, ovaries; ovd, oviducts; pa, penis apparatus; pg, paragonia (male accessory glands); rec, rectum; RFP, repressed female genital primordium; RMP, repressed male genital primordium; sp, sperm pump; spt, spermatheca; src, seminal receptacle; te, testes; ut, uterus; va, vagina; vs, seminal vesicles.

the first meiotic cycle, and a period of maturation in the adult stage is required before ovulation and resumption of meiosis take place. This maturation includes the incorporation of lipids and yolk into the growing oocyte. The yolk precursor proteins are produced by the fat body in a process called vitellogenesis. In most insects, vitellogenesis is considered an adult feature. However, in some Lepidoptera, which do not feed as adults, vitellogenesis is initiated during the last larval or pupal stage and yolk accumulation is completed prior to adult eclosion. Ovarian growth, vitellogenesis by the fat body and uptake of vitellogenin by the developing oocytes is under hormonal control of both JH and ecdysteroids, as well as additional hormonal factors including ILP and neuropeptides (Nation, 2015).

References

- Estrada, B., Casares, F., & Sánchez-Herrero, E. (2003). Development of the genitalia in *Drosophila melanogaster*. *Differentiation*, 71(6), 299–310.
- Hiruma, K., & Kaneko, Y. (2013). Hormonal regulation of insect metamorphosis with special reference to juvenile hormone biosynthesis. *Current Topics in Developmental Biology*, 103, 73–100.
- Marchal, E., Vandersmissen, H. P., Badisco, L., et al. (2010). Control of ecdysteroidogenesis in prothoracic glands of insects: A review. *Peptides*, 31, 506–519. <https://doi.org/10.1016/j.peptides.2009.08.020>.
- Nation, J. L. (2015). *Insect Physiology and Biochemistry* (third ed.). Boca Raton, FL: CRC Press Book.
- Nijhout, H. F. (2015). Big or fast: Two strategies in the developmental control of body size. *BMC Biology*, 13(1), 57.
- Niwa, Y. S., & Niwa, R. (2016). Transcriptional regulation of insect steroid hormone biosynthesis and its role in controlling timing of molting and metamorphosis. *Development, Growth & Differentiation*, 58(1), 94–105. <https://doi.org/10.1111/dgd.12248>.
- Rewitz, K. F., Yamanaka, N., Gilbert, L. I., & O'Connor, M. B. (2009). The insect neuropeptide PTTH activates receptor tyrosine kinase torso to initiate metamorphosis. *Science*, 326(5958), 1403–1405. <https://doi.org/10.1126/science.1176450>.
- Truman, J. W., & Riddiford, L. M. (1999). The origins of insect metamorphosis. *Nature*, 401(6752), 447–452.
- Žitňan, D., & Adams, M. E. (2012). Neuroendocrine regulation of ecdysis. In L. I. Gilbert (Ed.), *Insect Endocrinology* (pp. 253–309). Amsterdam: Elsevier.

Relevant Website

<https://www.sdbonline.org/sites/fly/atlas/00atlas.htm>. – Atlas of *Drosophila* Development by Volker Hartenstein, Society for Developmental Biology's Web Server.

PREGNANCY AND PARTURITION

Pregnancy and Parturition: Teleost Fishes and Elasmobranchs

Mari Kawaguchi, Sophia University, Tokyo, Japan

Keiichi Sato, Okinawa Churaumi Aquarium, Okinawa, Japan

© 2018 Elsevier Inc. All rights reserved.

Introduction

In this article, we focus on reproductive strategies with special reference to the modes of gestation, embryonic nutrition and their evolution in bony fishes (teleost fishes) and cartilaginous fishes (mainly elasmobranchs). According to [Lodé \(2012\)](#), reproductive strategies in these taxa can be classified into the following five types: (1) ovuliparity, where female fish deposit ova in the environment and these are fertilized externally; (2) oviparity, where fertilization occurs internally and embryo development occurs externally; (3) ovoviviparity, where fertilization and embryo development occur internally, but no nutritional derivatives are supplied through the parental body but through yolk reserves, called 'lecithotrophy' (yolk feeding); (4) histotrophic viviparity, where fertilization and embryogenesis occur internally while obtaining organic nutrients from the mother, called 'matrotrophy' (nourished by the mother); and (5) hemotrophic viviparity, where fertilization and embryogenesis occur internally with nourishment derived from the mother's bloodstream usually through a placental analogue. In teleostean fishes, the dominant strategy is ovuliparity. Some groups of fishes show ovoviviparity, histotrophic viviparity or hemotrophic viviparity (internal gestation). At present, only around 800 among more than 33,000 teleostean species are reported to show internal gestation ([Nelson et al., 2016](#)). They are distributed into 15 families or subfamilies as shown in [Table 1](#), suggesting that internal gestation emerged independently several times during the evolution of teleosts. Furthermore, Syngnathidae show a unique reproductive mode in that males can become pregnant. On the other hand, the dominant reproductive form of approximately 1000 cartilaginous fish species is known as internal gestation, and only some skates and sharks are known to be oviparous ([Table 2](#)). In this article, we will describe the manner(s) of pregnancy in (1) female teleostean fishes, (2) male syngnathid fishes, and (3) elasmobranchs.

Pregnancy in Female Teleostean Fishes

Manner of Gestation – From Fertilization to Parturition

The gestational manner of viviparous fishes differs from that of other vertebrates. Male fish often possess a characteristic structure called a 'gonopodium' or 'andropodium', a tubular structure protruding from the posterior of the anal fin, and transfer spermatozoa to the gonoduct of the female through this structure. The female gonoduct is not a derivative of the Müllerian duct, but is developed from the ovary and the peritoneum ([Wootton and Smith, 2015](#)). Therefore, internal fertilization occurs within the ovarian follicle in

Table 1 List of internally-gestating fishes together with their classification and type of matrotrophy

<i>Fish</i>	<i>Classification</i>	<i>Type of matrotrophy</i>
Coelacanth	Latimeriidae	Histotrophy, placentotrophy
Viviparous brotulas	Bythitidae	
Aphyonids	Aphyonidae	Placentotrophy
False brotulas	Parabrotulidae	
Baikal oilfishes	Comephorinae	Histotrophy
Eelpouts	Zoarcidae	
Eel-blennies	Ophiclininae	Histotrophy, placentotrophy
Kelp blennies	Clininae	Histotrophy
Llabrisomid blennies	Labrisomidae	Histotrophy, placentotrophy
Surfperches	Embiotocidae	
Halfbeaks	Zenarchopteridae	Histotrophy
Goodeids	Goodeinae	Histotrophy, placentotrophy
Four-eyed fish	Anablepinae	Histotrophy, placentotrophy
Livebearers	Poeciliinae	Histotrophy, placentotrophy
Rockfish	Sebastinae	Histotrophy

Sources: Reproduced from Blackburn, D.G., 2015. Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *Journal of Morphology* 276,961–990; Nelson, J.S., Grande, T.C., Wilson, M.V., 2016. *Fishes of the World*, fifth ed. Hoboken, NJ: John Wiley & Sons, Inc.

Table 2 List of elasmobranch orders together with their classification and type of reproduction

<i>Fish</i>	<i>Classification</i>	<i>Type of reproduction</i>
Frill and cow sharks	Hexanchiformes	Lecithotrophy, mucoid histotrophy
Bullhead sharks	Heterodontiformes	Oviparity
Nurse sharks	Orectolobiformes	Oviparity, lecithotrophy (<i>Rhincodon</i>), oophagy (<i>Nebrius</i>)
Mackerel sharks	Lamniformes	Oophagy, lipid histotrophy+oophagy (<i>Carcharodon</i>), adelphophagy+oophagy (<i>Carcharias</i>)
Requiem and cat sharks	Carcharhiniformes	Oviparity, histotrophy (mucoid or embryotrophy), placentotrophy, oophagy (<i>Pseudotriakis</i> and <i>Gollum</i>)
Dogfishes	Squaliformes	Lecithotrophy, mucoid histotrophy
Saw sharks	Pristiophoriformes	Lecithotrophy, (+histotrophy?)
Angel sharks	Squatiniformes	Lecithotrophy, (+histotrophy?)
Torpedo rays	Torpediniformes	Lecithotrophy, (+histotrophy?)
Shovelnose rays and sawfishes	Rhinopristiformes	Lecithotrophy, (+histotrophy?)
Skates	Rajiformes	Oviparity, lecithotrophy
Stingrays and eagle rays	Myliobatiformes	Lipid histotrophy

Source: Reproduced from Nelson, J.S., Grande, T.C., Wilson, M.V., 2016. Fishes of the World, fifth ed. Hoboken, NJ: John Wiley & Sons, Inc.

most of the ovoviparous and viviparous teleosts, and embryo development starts either within the ovarian follicle (intrafollicular) or ovarian lumen (intraluminal) in the ovary (Blackburn, 2015). Intraluminal gestation is found in most teleosts, while intrafollicular gestation is found in restricted species such as fishes of livebearers (Poeciliidae), four-eyed fish (Anablepidae), kelp blennies (Clinidae), and labrisomid blennies (Labrisomidae). In this type of reproduction, development of the embryo takes place within the follicle. Most of these fish species carry the same stages of embryos (one brood), while several species incubate different-staged embryos simultaneously as the result of different timing of fertilization (multi broods): two broods in labrisomid blennies (Labrisomidae), five broods in the blackstripe livebearer (*Poeciliopsis prolifica*), nine broods in *Heterandria formosa*, and up to 12 broods in *Clinus superciliosus*.

The gestation period also differs from species to species. For example, the gestation period of coelacanths is predicted to be 3 years, the longest in known vertebrates, while that of guppies is only 4 weeks. Parturition of the offspring occurs at the last stage of gestation.

The ovaries of internally-gestating fishes are well vascularized for oxygen supply to embryos and removal of metabolic waste. Hatching of the embryos occurs before parturition. In general, euteleostean embryos secrete two kinds of hatching enzymes – high choriolytic enzyme and low choriolytic enzyme – used to digest the egg envelope at the time of hatching. On the other hand, in some internally-gestating fishes such as rockfish and platyfish, hatching enzyme genes have been pseudogenized. Because these eggs are surrounded by thin and fragile envelopes, the embryos seem to be able to hatch without needing hatching enzymes. The role of egg envelopes in ovuliparous fishes is mainly protection from environmental (mechanical and chemical) stresses, while in some internally-gestating species, the envelope is thought to function in immunological defense.

Embryonic Nutrition

The fertilized eggs of internally-gestating fishes contain a large amount of yolk protein. In lecithotrophic species, development of the embryos depends only on yolk protein, especially in growth at an early stage. In some other viviparous fishes, the nutrient supply to embryos during development is performed from a different maternal body in addition to the yolk mass of the embryo (matrotrophy). Lecithotrophy and matrotrophy can be distinguished by changes in the weight of embryos during gestation: thus, the former embryos eventually lose their weight during development, and the latter types maintain or gain weight.

Matrotrophy is mainly categorized into the following two types of nutrition to embryos (Blackburn, 2015; Wootton and Smith, 2015): (1) histotrophy, where embryos absorb nutrients of the surrounding environment through mouth or body surface by eating the egg (oophagy), eating their siblings (embryophagy or adelphophagy), or eating maternal tissues (matrophagy); or (2) placentotrophy, where embryos absorb nutrients through placental analogues associated with the embryonic surface and maternal tissues. In placentotrophy, various types of organs of the maternal body connect to embryos for supplying nutrients, as shown in Table 1 (Wooding and Burton, 2008). Livebearer *Heterandria formosa* absorbs nutrients through the pericardial sac, which covers the entire head or neck strap (Fig. 1(A)). The sac is well vascularized to increase the efficiency of transferring organic materials. In the four-eyed fish *Anableps*, the pericardial sac expands ventrally, and adheres tightly to the maternal epithelium (Fig. 1(B)). The butterfly oodeid, *Ameca splendens*, possesses trophotaeniae (ribbon-like extensions from the hind gut epithelium) (Fig. 1(C)). The trophotaeniae and ovarian epithelium are not fused but are in contact, so that embryos can absorb organic materials from the maternal body. Different from fish species whose placental analogue extend from embryonic body as described above, the ovarian trophonemata of *Jennynsia* extends from the maternal epithelium into the mouth and buccal cavity of embryos (Fig. 1(D)).

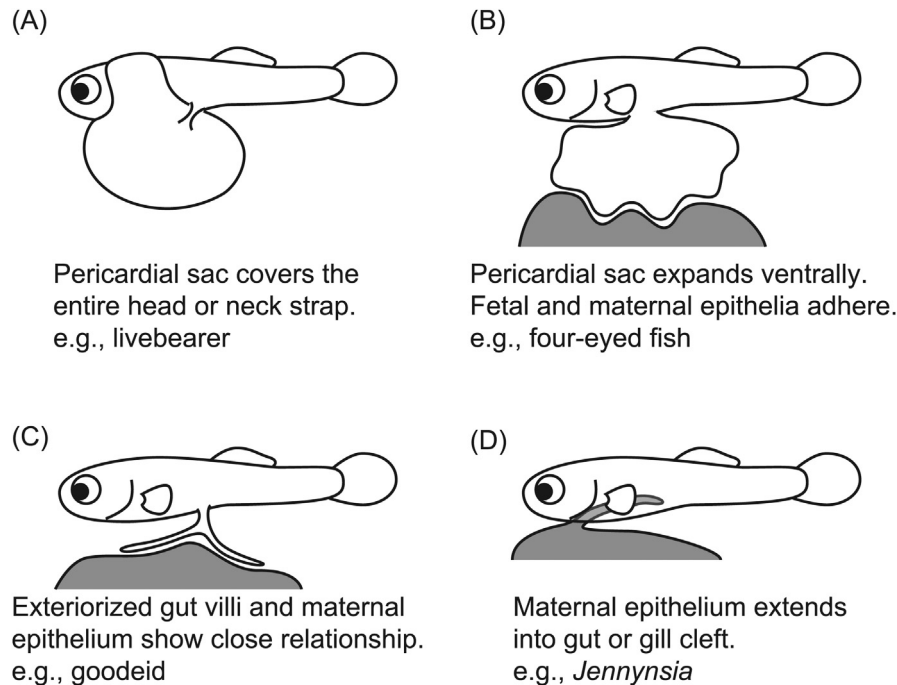


Fig. 1 Four types of placentotrophy. Modified from Wooding, P., Burton, G., 2008. Comparative Placentation. Structures, Functions and Evolution. Heidelberg: Springer.

Evolution of Viviparity

The genome sequence now decoded of one of the ovoviviparous teleosts, the platyfish *Xiphophorus maculatus*, provides a unique insight into the evolution of inner gestation (Schartl *et al.*, 2013). It has been considered that evolutionary transitions occur from ovuliparity and oviparity evolved to viviparity (Lodé, 2012). Interestingly, 13 homologs of mammalian genes related to placental development were found in platyfish, and three of them seemed to be under positive selection. These results suggest that the mammalian and teleostean placentas are convergent, not homologous, organs (Schartl *et al.*, 2013).

Male Pregnancy in Syngnathids

Manner of Male Pregnancy

Male seahorses possess an embryo-incubating area on their tail, called the brood pouch (Fig. 2). The female fish deposits eggs into this brood pouch, and fertilization occurs immediately. Within the pouch, the embryos are surrounded by a pseudoplacenta. During pregnancy, the brood pouch functions for gas exchange, waste removal, and osmoregulation: the functions are similar to those of the amniotic uterus. Interestingly, based on recent molecular studies, genes involved in these functions of brood pouch are commonly expressed in female internally-gestating fish and also in viviparous amniotes (Whittington *et al.*, 2015). For example, at the end of pregnancy in eutherian mammals, activation of the mitogen-activated protein kinase (MAPK) signaling pathway is suggested to be involved in parturition. In seahorses, genes associated with the MAPK signaling pathway are also upregulated at the post-parturition stage (Whittington *et al.*, 2015).

Embryonic Nutrition

Although viviparous female fish species supply nutrients to embryos during pregnancy, paternal nutrition transfer (patrotrophy) in seahorses is somewhat equivocal. According to Linton and Soloff (1964), this is not essential because embryos can develop even during *in vitro* incubation. Recent molecular analysis showed that Apolipoprotein M genes involved in lipid transport are expressed in the brood pouch during pregnancy. This evidence suggests that patrotrophy might occur in seahorses by providing histotroph (Whittington *et al.*, 2015).

Formation of Brood Pouch and Evolution

Formation of the brood pouch in the pot-bellied seahorse was reported as follows (Kawaguchi *et al.*, 2017). The pouch formation was initiated from linear projections of epithelia on both ventrolateral sides of the body. The projections elongated toward the

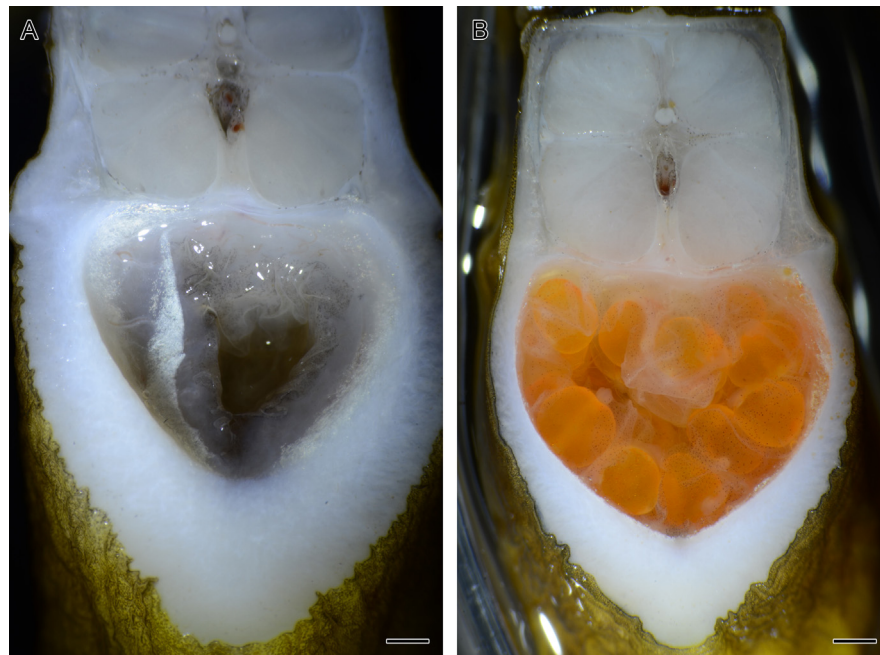


Fig. 2 Interior of the seahorse brood pouch. (A) Before the uptake of eggs and (B) after the uptake of eggs from female. Scale bar=1 mm.

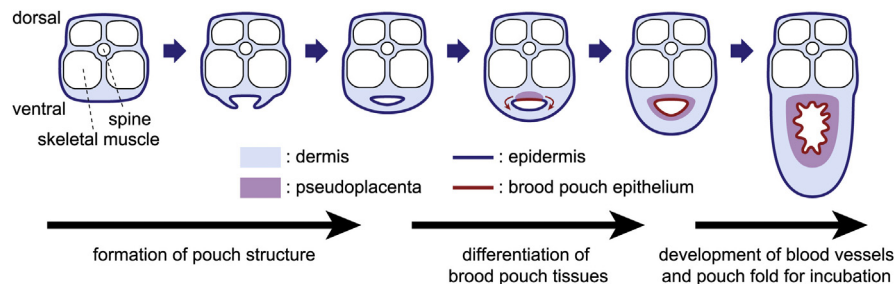


Fig. 3 A plausible pathway of brood-pouch formation in the male seahorse. The primordium of the brood pouch first appears as linear projections at both ventrolateral sides of the body. The projections become fused at the body midline. Differentiation of brood-pouch tissue begins, and the pseudoplacenta first appears on the dorsal side of the pouch. The pseudoplacenta entirely surrounds the pouch. Finally, the pouch folds are formed, ready to incubate embryos. Reproduced from Kawaguchi, M., Okubo, R., Harada, A., *et al.*, 2017. Morphology of brood-pouch formation in the pot-bellied seahorse *Hippocampus abdominalis*. *Zoological Letters* 3, 19.

ventral midline, and eventually fused together. Then, brood-pouch specific tissues such as pseudoplacenta differentiated, and finally, a pouch-fold ready to incubate embryos was developed in the fully formed brood pouch (**Fig. 3**).

Syngnathid fishes including seahorses, all of which possess the brooding area, commonly possess an elongated body with a series of bony rings and no pelvic fin (Nelson *et al.*, 2016). Syngnathids are mainly divided into two groups, Urophori and Gastrophori. The brooding structure of the Urophori is located on the tail, and that of the Gastrophori is on the abdomen. Interestingly, the brooding structure has diverged during evolution as summarized in **Fig. 4** (cited from Wilson *et al.*, 2003). (1) In the simplest case, the structure is such that eggs are attached to the surface of the ventral side of the male body either with only mucous or within membranous compartments of the male body. This type is found in straight-nosed pipefish (genus *Nerophis*), sea dragons (genus *Phyllopteryx*) and fragtail pipefish (genus *Doryrhamphus*). (2) In the intermediate case, the eggs attached onto the male body are further protected by skin elongated from both ventrolateral sides. The skin fold covers the eggs either partially, as found in the genus *Corythoichthys*, or completely, as found in some forms such as the deep-body pipefish (genus *Kaupus*) and pipefishes (genus *Syngnathus*). (3) In the most complicated case a baggy structure is used to incubate the eggs. This type is found in seahorses (genus *Hippocampus*). The brood pouch has an entrance to receive the eggs from female fish and for parturition.

These differences in brooding structures also affect the structures of eggs. The egg envelope of seahorses is thinner than that of the messmate pipefish (covered with pouch folds) or the alligator pipefish (simple form of the brooding structure). One of two kinds of hatching enzyme genes of seahorses has been pseudogenized, while the latter two species possess complete sets of hatching enzyme gene. Similar evolutionary events were observed in ovoviviparous fishes as mentioned above, suggesting the convergent evolution of hatching enzyme genes in pregnant female and male fishes.

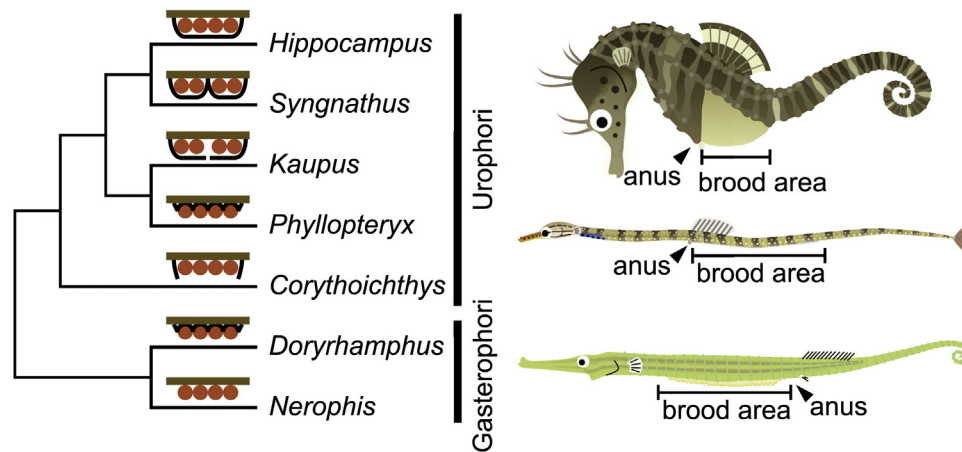


Fig. 4 Evolution of syngnathid fishes. Brood pouch types are shown with increasing complexity within the *Urophori* and *Gastrophori*. Modified from Wilson, A.B., Ahnesjö, I., Vincent, A.C., Meyer, A., 2003. The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). *Evolution* 57, 1374–1386.

Elasmobranchs

Manner of Elasmobranch Reproduction

The strategy of reproduction of elasmobranchs conventionally classified into oviparity and viviparity: viviparity is the major mode of reproduction, while oviparity is found only in the demersal groups, such as bullhead sharks (*Heterodontidae*), carpet sharks (*Orectolobidae*), catsharks (*Scyliorhinidae*), and skates (*Rajidae*). These species lay relatively large eggs encased with hard capsules of a species-specific shape, and the embryos consume nutrition only from the yolk (i.e., are lecithotrophic).

The cycles of reproduction in elasmobranchs are generally long and vary between species. The major cycles in viviparous species are annual, biennial, and 3-year cycles, and the cycle type is determined by the periods of vitellogenesis and gestation. Brood size differs for each species and for the body size of each individual: being generally small in the oophagous or lipid-rich histotrophic species. The species with the smallest brood size are eagle rays (*Mobulidae* and *Myliobatidae*), generally with only one embryo per uterus. The sand tiger shark has the smallest brood size among sharks; usually only one embryo is found in each uterus as a result of embryonic cannibalism. The brood sizes are generally large in placental species like hammerhead sharks (see Fig. 5). The largest brood size ever recorded was a whale shark landed in Taiwan, nurturing 304 embryos. In other groups, the blue sharks (80 embryos), tiger sharks (70 embryos), and probably the sleeper sharks also produce large broods (Castro, 2011).

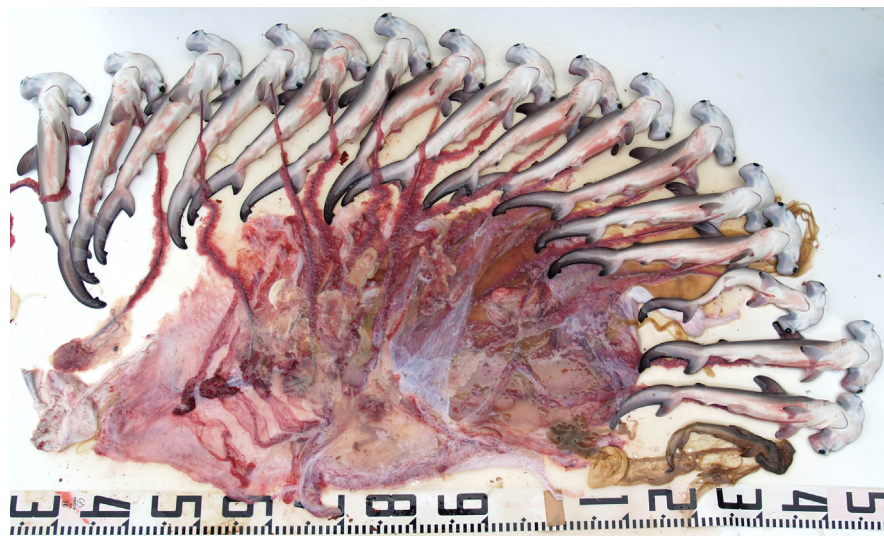


Fig. 5 A litter of embryos of the smooth hammerhead shark, *Sphyrna zygaena*. Each embryo has a placental connection to the mother's uterus via an umbilical cord.

Embryonic Nutrition

According to Wourms (1977), the internal gestation of elasmobranchs is classified into the following four types by the patterns of nutrient source to the embryos. (1) Lecithotrophy is the simplest and the most ancestral mode of nutrition among internally-gestating elasmobranchs, found in various lineages. The dry intrauterine embryo loses organic weight during gestation. Some spiny dogfishes (Squaliformes) and electric rays (Torpediniformes) are typical examples of this mode. (2) Placentotrophy is the most derived mode of reproduction in elasmobranchs, as it is only recognized in the carcharhiniform sharks. Like other groups, the embryos are initially dependent on nutrition from their own yolk sac. But later, the yolk sac is modified into a placenta to attach to the uterine wall, and the embryos are nourished by the maternal inputs that are shunted to them directly from maternal blood. Hence, this is termed a yolk-sac placenta (Fig. 6). Each intrauterine embryo is encased by a thin, membranous egg case inserted between the uterine wall and placenta. The reproductive cycle of this group is generally biennial with about 1-year gestation periods. (3) Histotrophy is a type of maternal contribution to produce larger neonates than simple lecithotrophy by reducing the number of offspring. It is difficult to distinguish this mode from lecithotrophy without definition of characteristics in the weight gain of the embryos compared with the initial fertilized ova. Musick and Ellis (2005) defined a matrotrophic contribution as a weight loss of less than 20%, or as weight gain during development. There are many categories of histotrophy in sharks and rays. The simplest mode is the mucoid (limited) form of histotrophy that is characterized by producing clear, relatively nutrient-limited fluid secreted from the maternal uterus. The tiger shark is one of the most obvious histotrophic sharks, and its embryos appear to reach gains of 1092% in dry weight during gestation. Lipid histotrophy by producing lipid-rich fluid (uterine milk) from highly developed 'trophonemata' in the uterus is found in the eagle rays (Myliobatiformes), and extreme weight gain occurs during gestation. Based on records of captive-born manta rays (*Mobula alfredi*) in the Okinawa Churaumi Aquarium, the embryo increases its weight to over 50 kg (in wet weight) after 1 year of gestation, and this appears to be the largest weight record of neonates in elasmobranch reproduction. Finally, oophagy (and adelphophagy) is one of the primary modes of embryonic nutrition in lamniform sharks (Wourms, 1977). The intrauterine embryos are initially nourished from their own small yolk sac, and subsequently ingest unfertilized ova ovulated from the maternal ovary into the uterus as their main nutrient source (Fig. 7). Such ova used for nutrition are generally smaller than fertilized ova, and are encased together with several other ova in the shell gland and transferred into the

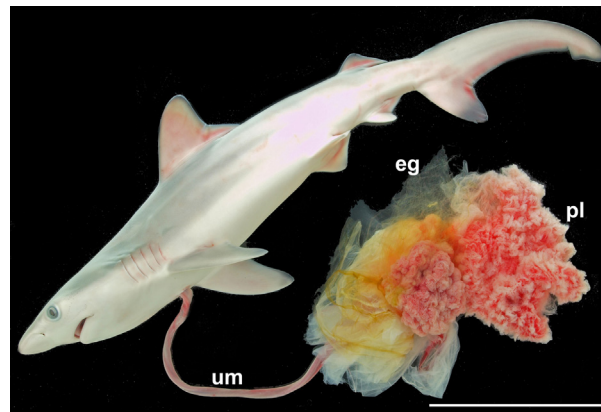


Fig. 6 The yolk-sac placenta (pl) and umbilical cord (um) of a term embryo taken from a pregnant blacktip shark, *Carcharhinus limbatus*. The thin, yellowish egg case (eg) is entwined with the surface of the placenta. Scale=10 cm.

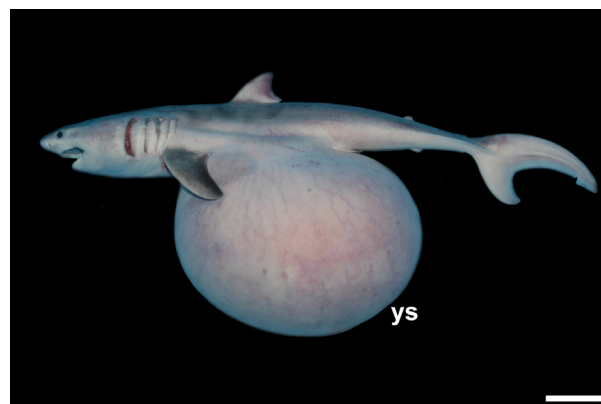


Fig. 7 A white shark embryo with a large yolk stomach (ys) full of nutrient ova. Scale=10 cm.

uterus. In the sand tiger shark (*Carcharias taurus*), the first embryo in the uterus feeds on its sibling embryos: a phenomenon known as adelphophagy (fraternal cannibalism). Oophagy is also found in carcharhiniformes (Genera *Pseudotriakis* and *Gollum*) and Orectolobiformes (Genus *Nebrius*), but this form is distinguished from lamnoid oophagy by the strategy of supplying nutrient ova and in terms of its evolutionary origins. According to Lodé (2012), oophagy is one types of histotrophy. The recent studies indicate that the nutrition of embryos of these type of sharks appears to be more complex than estimated previously as embryos probably rely on a changing source of nutrition over the course of their development. The embryos depend on at least three major sources of nutrition (Gilmore, 1993): yolk-sac lipids (lecithotrophy) in the initial phase; uterine milk (lipid histotrophy) in the second phase verified by Sato *et al.* (2016); and nutrient eggs in the third phase. Hence, the structure of the uterine epithelium in the histotrophic phase is completely different from that during late gestation after the oophagy phase as it needs to function for oxygen supply.

References

- Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *Journal of Morphology*, 276, 961–990.
- Castro, J. I. (2011). *The Sharks of North America*. New York, NY: Oxford University Press.
- Gilmore, R. G. (1993). Reproductive biology of lamnoid sharks. *Environmental Biology of Fishes*, 38, 95–114.
- Kawaguchi, M., Okubo, R., Harada, A., et al. (2017). Morphology of brood pouch formation in the pot-bellied seahorse *Hippocampus abdominalis*. *Zoological Letters*, 3, 19.
- Linton, J. R., & Soloff, B. L. (1964). The physiology of the brood pouch of the male sea horse *Hippocampus erectus*. *Bulletin of Marine Science*, 14, 45–61.
- Lodé, T. (2012). Oviparity or viviparity? That is the question. *Reproductive Biology*, 12, 259–264.
- Musick, J. A., & Ellis, J. K. (2005). *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids, and Chimaeras* (vol. 3, pp. 45–79). Enfield: CRC Press.
- Nelson, J. S., Grande, T. C., & Wilson, M. V. (2016). *Fishes of the World* (fifth ed.). Hoboken, NJ: John Wiley & Sons, Inc.
- Sato, K., Nakamura, M., Tomita, T., et al. (2016). How great white sharks nourish their embryos to a large size: Evidence of lipid histotrophy in lamnoid shark reproduction. *Biology Open*, 5, 1211–1215.
- Schartl, M., Walter, R. B., Shen, Y., et al. (2013). The genome of the platyfish, *Xiphophorus maculatus*, provides insights into evolutionary adaptation and several complex traits. *Nature Genetics*, 45, 567–572.
- Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B., & Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution*, 32, 3114–3131.
- Wilson, A. B., Ahnesjö, I., Vincent, A. C., & Meyer, A. (2003). The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). *Evolution*, 57, 1374–1386.
- Wooding, P., & Burton, G. (2008). *Comparative Placentation. Structures, Functions and Evolution*. Heidelberg: Springer.
- Wootton, R. J., & Smith, C. (2015). *Reproductive Biology of Teleost Fishes*. West Sussex: John Wiley & Sons, Ltd.
- Wourms, J. P. (1977). Reproduction and development in chondrichthyan fishes. *American Zoologist*, 17, 379–410.

Viviparity in Reptiles and Amphibians

Daniel G Blackburn, Trinity College, Hartford, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chorioallantois The vascular respiratory membrane that lines the eggshell in reptiles, birds, and mammals. It contributes to placental formation in viviparous reptiles and mammals.

Fetal membranes "Extraembryonic" tissues of reptiles that lie external to the embryo, such as the yolk sac and chorioallantois. They function in protection and gas exchange. In viviparous lizards and snakes, they contribute to placentas.

Lecithotrophy The developmental pattern in which yolk of the ovum provides nutrients for embryonic development. Lecithotrophy occurs in oviparous species and most viviparous species.

Matrotrophy The developmental pattern in which maternal nutrients are provided to the embryo by means other than yolk of the ovum (e.g., placental tissues, oviductal secretions, or sibling yolks).

Oviparity The reproductive mode in which females lay unfertilized or developing eggs that complete their development and hatch in the external environment.

Ovoviviparity An archaic term that was once applied to a variety of egg-laying and live-bearing reproductive modes. Among them are patterns now termed "lecithotrophic viviparity," "oviparous egg-retention," and "pseudoviviparity."

Placenta An organ formed through an intimate association of fetal and maternal tissues that functions in gas exchange and/or nutrient provision.

Pseudoviviparity An unusual form of oviparity in which eggs are fertilized externally and are brooded in some parental structure such as the stomach, vocal sacs, or skin pouches. This pattern occurs in a few frogs and fishes.

Viviparity The reproductive mode in which embryos develop to term inside the female reproductive tract and are born as larvae, altricial neonates, or well-developed offspring.

Yolk sac Extraembryonic tissues that surround and digest the yolk. The yolk sac contributes to placental arrangements in viviparous reptiles, therian mammals and some fishes.

Reproductive Patterns

Parity Modes and Embryonic Nutrition

Viviparity ("live-bearing reproduction") is a pattern in which pregnant females maintain developing embryos inside their reproductive tracts and give birth to their offspring. The alternative to viviparity is oviparity ("egg-laying reproduction"), in which females lay fertilized or unfertilized eggs. Viviparity in reptiles is of particular interest because it has evolved on more than 115 separate occasions, more than in all other vertebrates combined (Blackburn, 2015). Some viviparous reptiles and amphibians have evolved elaborate specializations (such as placentas) by which pregnant females provide nutrients to their developing embryos. Viviparity has been studied extensively to determine how it is accomplished and how and why it has evolved. Although most reptiles and amphibians lay eggs, viviparity occurs in many species of squamates (lizards and snakes) as well as in various caecilians (limbless amphibians) and a few frog and salamander species. Other extant reptiles (crocodilians, turtles, and the tuatara) and the great majority of living amphibians are oviparous. Viviparity also characterizes most mammals and is present in many fishes and invertebrates.

Oviparous embryos obtain nutrients from yolk of the ovum, a nutritional pattern called "lecithotrophy" ("lecithos" being Greek for "yolk"). Lecithotrophy also occurs in most lineages of viviparous vertebrates, including most live-bearing squamates and amphibians. Other viviparous species (like most mammals) have evolved "matrotrophy," in which the pregnant female provides nutrients to her embryos by some alternative means (e.g., uterine secretions, or a placenta [termed "placentotrophy"]). Lecithotrophy and matrotrophy are extremes of a continuum. Thus, in many viviparous species, most nutrients come from the yolk, but these nutrients are supplemented by matrotrophic sources. Likewise, in some viviparous species, nutrients for early development come from the yolk while later in development they are derived from other maternal sources. Viviparous species with a substantial degree of matrotrophy include only a few species of live-bearing lizards and amphibians.

In literature of the 19th century, some zoologists used the term "ovoviviparity" to distinguish the live-bearing patterns of reptiles and fishes from (the so-called) "true" viviparity of mammals. This distinction was based on the mistaken belief that live-bearing reproduction in reptiles is a simple phenomenon in which eggs are merely retained in the female oviduct and hatch during birth. An additional problem is that "ovoviviparity" was used in widely discrepant ways, through confusion between reproductive products (egg vs. neonate) versus embryonic nutritional modes. Accordingly, the word has been abandoned by most researchers in favor of such alternatives as "lecithotrophic viviparity," "oviparous egg retention" and "larviparity" (see below).

Functional and Evolutionary Issues

The widespread phylogenetic distribution of viviparity among animals has important functional and evolutionary implications. For embryos to develop in the maternal reproductive tract typically requires specializations for embryonic gas exchange. Likewise, matrotrophic viviparity requires specializations for nutrient transfer from the pregnant female to her embryos. Viviparity clearly has advantages as well as disadvantages that vary between animal groups. Studies of reptiles and amphibians have contributed markedly toward our understanding of how viviparity is accomplished and how it has evolved.

Viviparity in Reptiles

Phylogenetic Distribution

Among living reptiles, viviparity is confined to lizards and snakes, where it characterizes nearly 20% of the species. At least 13 families of lizards have viviparous species, including such families as skinks, chameleons, phrynosomatids, liolaemids, cordylids, geckoes, xantusiids, anguids, and lacertids (Stewart and Blackburn, 2015). Among snakes, viviparous habits occur in species of 14 families, including boids, colubrids, natricids, viperids, elapids, typhlopids, aniliids, and uropeltids (Blackburn and Stewart, 2011). Viviparous lizards and snakes are distributed worldwide and occupy every habitable continent, in latitudes that range from the equator to the Arctic Circle. They also occur in tropical, subtropical, temperate, and sub-arctic climes, as well as deserts, tropical rainforests, boreal forests, and even oceanic environments. Furthermore, among the viviparous squamates are forms that are terrestrial, arboreal, fossorial, and aquatic, and ones that range in size from small skinks of montane New Zealand to the enormous boas of the Brazilian rainforest. Fossil evidence indicates that viviparity also characterized several extinct reptilian groups, including ichthyosaurs, plesiosaurs, and the gigantic mosasauroid lizards (Blackburn and Sidor, 2014).

Viviparity in squamates is displayed at a variety of taxonomic levels. A few small families are entirely viviparous, such as the families Xantusiidae (night lizards) and Uropeltidae (shield-tailed snakes). However, viviparity usually is manifested at subgeneric levels. Recent reviews have documented 10 genera of snakes and 25 genera of lizards that include both oviparous and viviparous species (Blackburn and Stewart, 2011; Stewart and Blackburn, 2015). A few lizard species contain both oviparous and viviparous populations, such as the European lizard *Zootoca vivipara* and the Australian skink *Saiphos equalis*.

Reproductive Parameters

Gestation length varies widely among viviparous squamate species. In lizards and snakes, pregnancy commonly lasts 2–4 months, and (outside of the tropics) typically is timed to correspond with the warmer months of summer. Longer gestation periods occur among elapid seasnakes, in which pregnancy can last 6–8 months. Gestation lengths of about a year have been documented in a few lizards of cold climates (e.g., *Liolaemus magellanicus* of southern South America) and also occur in *Mabuya* skinks of tropical South America.

Litter sizes of viviparous squamates also vary widely. At one end of the range lie the New Zealand geckoes of the genera *Hoplodactylus* and *Naultinus*, which give birth to ≤ 2 offspring. At the other extreme lies the horned lizard *Phrynosoma douglasii* of North America, in which litter size averages 15 but can range up to 31 offspring. The African chameleon *Trioceros jacksonii* holds the record among viviparous lizards, with litters that range from 10 to 50 neonates. Viviparous snake species show a large range of litter sizes. Very small litters are exhibited by the sea snake *Lapemis curtus* (2–3 offspring) and the rattlesnake *Crotalus atrox* (which averages 4.5 offspring). The largest litters have been documented in various live-bearing vipers and elapid snakes, in which pregnancies have been reported to yield 80 to more than 100 offspring.

How Pregnancy is Accomplished

In viviparous squamates, eggs complete their development within the uterine portion of the maternal oviduct. Although the oviduct of oviparous reptiles secretes the eggshell, in viviparous squamates, only a thin, rudimentary shell membrane is deposited. Accordingly, the shell glands of live-bearing forms are reduced. Parturition (birth of the neonate) in viviparous squamates is functionally equivalent to oviposition of the egg.

Oviparous and viviparous squamates differ in the length of time eggs develop inside the oviduct. In oviparous forms, the time between fertilization and oviposition ranges from a couple to several weeks in duration, during which time the eggshell is deposited. As a result, about 25% of the entire developmental period (i.e., the time between fertilization and hatching) commonly precedes oviposition, and the embryo typically is in the limb-bud stage when the egg is laid (Shine, 1985; Blackburn, 1995). In a few oviparous lizards, the female retains the eggs for a longer proportion of the developmental period, and the embryo is more advanced at oviposition. This pattern of “oviparous egg-retention” may be an intermediate evolutionary stage toward viviparity.

Modest information is available about endocrinological control of pregnancy and parturition. Progesterone produced by the corpus luteum (the post-ovulatory follicle) has been implicated both in retention of eggs in oviparous species and in maintenance of gestation in live-bearing ones. However, viviparous species vary in progesterone profiles during gestation and differ according to whether the CL is essential for maintenance of pregnancy. This variation is evidence that viviparous reproduction has evolved via different mechanisms in unrelated lineages (Blackburn, 2006).

Fetal Maintenance and Placentation

For embryos of oviparous squamates, respiratory gases (oxygen and carbon dioxide) are exchanged with air that surrounds the egg, and water is taken up from the soil (Fig. 1). Nutrients for their embryonic development and growth are derived from the yolk, and calcium is provided via the yolk and the eggshell. In contrast, in viviparous squamates the embryo's physiological needs are met by maternal tissues. Accordingly, the uterine oviduct supplies oxygen and takes away carbon dioxide. It also supplies water and (given absence of an eggshell) provides calcium. Although most viviparous squamates are highly lecithotrophic, uterine tissues commonly supply at least small amounts of organic and inorganic nutrients (Stewart and Blackburn, 2015). Some lizard species are highly matrotrophic, with the oviduct providing nearly all of the nutrients for development (Blackburn, 1993).

In oviparous squamates, physiological exchange with the external environment is accomplished by two fetal membranes – the chorioallantois and yolk sac. As in other oviparous amniotes, the chorioallantois lines the eggshell in the dorsal hemisphere of the egg. With its rich supply of capillaries, it is well-suited for respiratory exchange. The yolk sac lines the ventral eggshell, an ideal location for a membrane involved in water uptake; however, this function has not been demonstrated definitively. Both fetal membranes can be involved in mobilization of eggshell calcium.

In viviparous squamate embryos, the chorioallantois and yolk sac are responsible for physiological exchange with maternal tissues, similar to how they function in oviparous species (Fig. 1). Because the eggshell is vestigial under conditions of viviparity, these two fetal membranes lie closely associated with the oviduct lining. The close association of fetal tissues with the oviduct meets classic criteria for placentation: “intimate apposition of fetal and maternal tissues for physiological exchange.” Thus, the chorioallantois and adjacent uterine lining forms the chorioallantoic placenta. The yolk sac and uterus form the yolk sac placenta.

Microscopic anatomy of the chorioallantoic placenta reveals how it can function so effectively in maternal – fetal gas exchange (Blackburn, 1993). Both the oviduct and the apposed chorioallantois are richly vascularized, and their epithelial coverings are very thin. As a result, the diffusion distance between fetal and maternal blood vessels measures only a few micrometers, enhancing the transfer of oxygen and carbon dioxide. In contrast, the definitive yolk sac placenta is structurally unsuited for gas exchange. Evidence from a few relatively-lecithotrophic species suggests that yolk sac placenta functions in transfer of water and small quantities of nutrients (Blackburn and Stewart, 2011; Stewart and Blackburn, 2015).

A few groups of viviparous skinks are highly matrotrophic. Their placentas are correspondingly specialized. For example, the Mediterranean skink *Chalcides chalcides* exhibits a “placentome,” a specialized region where secretory uterine tissue interdigitates with regions of chorioallantois lined with absorptive cell. In South American *Mabuia*, females ovulate tiny 1 mm eggs, and matrotrophy accounts for most of the nutrients for development. Lizards of this genus also have a placentome, a placenta region where greatly enlarged absorptive cells of the fetal chorion take up nutrients from the uterus (Fig. 2) (Ramírez-Pinilla, 2014). The African lizard *Trachylepis anchietae* is unique among viviparous squamates in that the uterine tissue becomes invaded by fetal cells. As a result the chorion lies in direct contact with the uterine blood vessels, yielding a highly effective means of nutrient transfer (Stewart and Blackburn, 2015). Such a degree of intimacy between fetal and maternal tissues is exceedingly rare among viviparous vertebrates; it is otherwise known only in certain eutherian mammals.

Viviparity in Amphibians

Phylogenetic Distribution and Characteristics

Although most amphibians lay eggs, viviparous habits occur in each of the three major lineages (Wake, 1993; Greven, 2011). Among anurans, viviparity is present in two toad genera of East Africa (*Nectophrynoides* and *Nimbaphrynoides*) as well as the

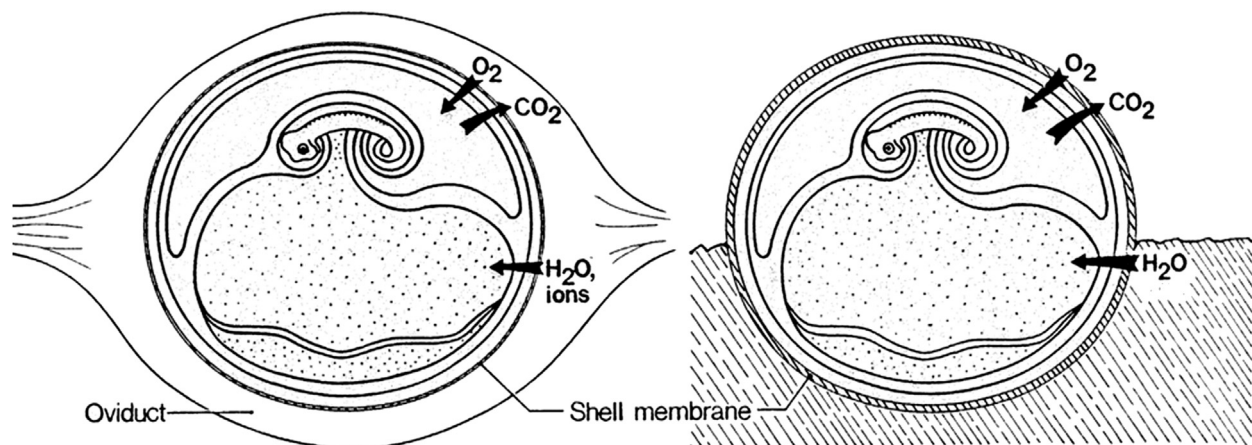


Fig. 1 Physiological relationship between a squamate egg and its environment under conditions of viviparity (left) and oviparity (right). In the viviparous condition, exchange occurs with the uterine oviduct, and in the oviparous condition, with the air and substrate. In each case, gas exchange occurs via the chorioallantois and water uptake, via the yolk sac. Apposition of the chorioallantois and yolk sac to the oviduct lining in the viviparous condition forms placentas.

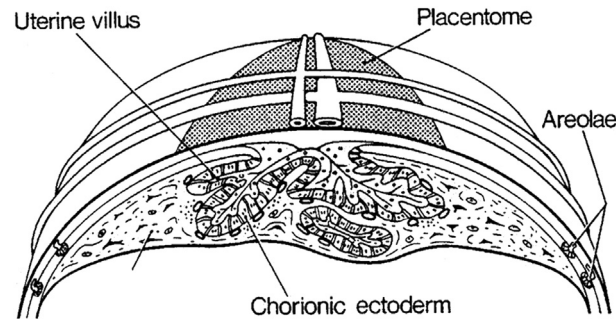


Fig. 2 Interface of uterine and chorioallantoic tissues in the placentome of the matrotrophic lizard genus *Mabuya* of South America. The uterine lining forms villi of secretory tissue, whereas the fetal chorion is absorptive. The placentome and areolae are sites of maternal – fetal nutrient transfer.

Indonesian fanged frog *Limnonectes larvaepartus*; it also characterized a now – extinct frog species of *Eleutherodactylus* from Puerto Rico. Among salamanders, viviparity is found in two closely-related European genera, *Salamandra* and *Lyciasalamandra* (Buckley *et al.*, 2007). Among the caecilians, viviparity occurs in species of four families (Dermophiidae, Scolecomorphidae, Typhlonectidae, and Indotyphlidae) (Blackburn, 2015).

Live-bearing amphibians differ according to state of development of the offspring at birth (Wake, 1993). For example, in *L. larvaepartus*, females give birth to young in the tadpole stage, a pattern known as “larviparity.” However, in *Nectophrynoides*, females give birth to metamorphosed young (“pueriparity”). Among viviparous salamandrids, populations vary according to whether the offspring at birth are larval or fully-metamorphosed (Buckley, 2012).

Although gestation lengths in live-bearing amphibians commonly are short, they vary according to habitat and degree of development of the newborn offspring. The 9 month gestation length in *Nimbaphrynoides occidentalis* is the longest known for anurans. However, gestation in the alpine salamander *S. atra* can last as much as 2 or 3 years, a record maximum for viviparous vertebrates.

Embryo Maintenance and Nutrient Provision

In all viviparous amphibians, fertilization is internal and the offspring develop inside the maternal oviducts. In several species, embryos are relatively lecithotrophic. Matrotrophy commonly is associated with pueriparity, since nutrients provided after fertilization facilitate the production of fully metamorphosed larvae.

Among amphibians with matrotrophic viviparity, oviductal embryos typically consume maternally - supplied nutrients (Wake, 1993, 2015). For example, in the east African frog *N. occidentalis*, embryos ingest oviductal secretions. Embryos of viviparous salamandrids feed upon unfertilized yolks, oviductal secretions, or developing siblings. Developing caecilian embryos commonly use specialized teeth to scrape and ingest nutrient-rich lining of the maternal oviduct. Viviparous *Typhlonectes* embryos combine this sort of pattern with ingestion of sibling yolks and embryos.

Oviparous Analogues to Live-Bearing Reproduction

Some oviparous anurans exhibit patterns that may be functionally analogous to live-bearing reproduction. These include species in which the male or female parent carries eggs in its vocal pouches, stomach, or specialized structures in the dorsal integument (Greven, 2011; Wake, 2015). Such patterns are equivalent to forms of parental care of eggs found in monotremes and fishes. These patterns should not be confused with viviparity, which always entails gestation in the female reproductive tract. The term “pseudoviviparity” can be applied to such patterns in recognition of their superficial similarity to true viviparity.

Evolution of Viviparity

Phylogenetic Origins

The widespread distribution of viviparity and matrotrophy among vertebrates clearly reveals that these patterns have evolved many times. A recent analysis recognized 115 evolutionary origins of squamate viviparity, or about 3/4ths of the 155 vertebrate origins of this pattern that can now be discerned (Table 1) (Blackburn, 2015). Viviparity usually is assumed to evolve irreversibly from oviparity. Several recent papers have found evidence for such a reversal to be both scarce and weak, with the possible exception of a few isolated cases.

The squamate origins of viviparity are widely distributed phylogenetically. Multiple origins have occurred in most families with live-bearing representatives, as well as in several genera (Shine, 1985). For example, viviparity has arisen at least 6 times in the family Phrynosomatidae, with 4 origins in the genus *Sceloporus* (fence lizards) and two in *Phrynosoma* (horned lizards). Families in which

Table 1 Evolutionary origins of viviparity and viviparous matrotrophy in reptiles, amphibians, and other vertebrates (modified from Blackburn, 2015). Figures represent conservative estimates based on recent phylogenetic analyses

Taxon	Minimum number of origins	
	Viviparity	Matrotrophy ^a
Squamate reptiles ^b	>115 ^c	6
Extinct reptiles	7	–
Lissamphibians	9	9
Mammals	1	1
"Osteichthyans" ^d	13	12
Chondrichthyans	9	5
Extinct placoderm fish	1	–
Total	≥155	33

^aViviparous matrotrophy in which non-ovum sources contribute most nutrients for development between fertilization and birth.

^bSquamate clades with extant representatives only. (Two Cretaceous lizard clades lie in the category of "extinct reptiles").

^cRecent analysis suggests that at least 120 to 130 squamate origins may be indicated.

^dBony fishes, excluding their tetrapod descendants.

viviparity has evolved even more often include colubrid snakes (≥ 15 origins) and scincid lizards (≥ 31 origins). Live-bearing habits also have arisen on multiple occasions among extinct reptiles. A recent phylogenetic analysis found that viviparity originated independently in 6 reptilian groups of the Mesozoic and Paleozoic, including mesosaurs, ichthyosaurs, choristoderans, mosasaurs, sauropterygians, and a Cretaceous lizard (Blackburn and Sidor, 2014). Evidence for a seventh such origin has recently been published in a basal archosauromorph. Among amphibians, viviparity has evolved at least 1–2 times in salamanders, 3 times in frogs, and 4 times in caecilians, for a total of 8 or 9 origins (Blackburn, 2015).

As for substantial matrotrophy, this fetal nutritional pattern has evolved in squamates only in the family Scincidae, where it has arisen 4–6 times. Among viviparous amphibians, matrotrophy has originated independently in the 4 caecilian families cited above, once among east African frogs, and at least 4 times among European salamandrids, for a total of 9 amphibian origins (Table 1).

Viviparity as a Reproductive Strategy

As compared to oviparous reproduction, viviparity entails both benefits and costs (Shine, 1985; Andrews and Mathies, 2000; Stewart and Blackburn, 2015). Among its benefits are that a pregnant female can protect her eggs from predators and environmental sources of mortality. She also can incubate the eggs at optimal temperatures for development (through behavioral thermoregulation) and can supplement yolk nutrients with matrotrophic sources. Potential costs include limits that viviparity can place on litter size, offspring size, and reproductive frequency, as well as detrimental effects on maternal ability to locomote, feed, and escape from predators. Viviparity commonly is viewed as a "reproductive strategy" that evolves in a particular lineage when its associated benefits exceed such costs.

How and Why has Viviparity Evolved?

Because viviparity entails egg development within the maternal reproductive tract, it can only evolve in clades with internal fertilization (Wake, 1993). This issue may help account for the frequency with which viviparity has arisen in squamates and caecilians, as well as its scarcity among anurans. However, despite the fact that all reptiles and most salamanders also have internal fertilization, oviparity predominates in these groups. Whether viviparity evolves in a particular group depends on a variety of factors, including selective pressures and constraints (Andrews and Mathies, 2000; Blackburn, 2006). Likewise, evolution of viviparity can require certain structural and functional specializations. For example, in squamates, viviparity is associated with reduction of the eggshell and oviductal glands that form it, as well as increases in vascularity of the uterus and fetal membranes.

The fact that oviparous squamates usually retain their developing eggs for $\geq 25\%$ of development and lay them in the limb bud stage may predispose them toward live-bearing reproduction. In a sense, squamates have already evolved part of the way toward viviparity. Squamates contrast with turtles, crocodilians, birds, and most salamanders, which deposit their fertilized eggs in a very early stage of development. Interestingly, the vast majority of squamates either oviposit eggs at the limb bud stage (typical oviparity) or give birth to their young. This dichotomous distribution is consistent with a punctuated equilibrium model for the evolution of viviparity (Blackburn, 1995). According to this model, the intermediate pattern of oviparous egg-retention (with deposition of eggs in an advanced stage of development) is an unstable strategy that is traversed quickly during evolution. The rationale is that retention of developing eggs in the oviducts requires a very thin eggshell for gas exchange to occur, but that such a thin eggshell would be unable to adequately protect the oviposited egg. Another feature of this model is that placentation and an incipient degree of placentotrophy (placental matrotrophy) evolve simultaneously with viviparity, rather than as subsequent steps, due to the close relationship between fetal and maternal tissues that viviparity involves (Fig. 3) (Blackburn, 2006). Substantial

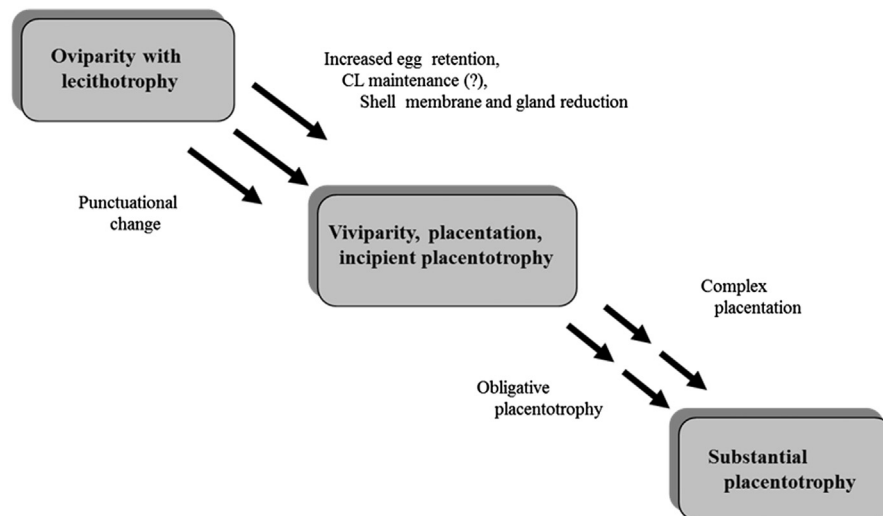


Fig. 3 Scenario for reproductive evolution in squamates. Viviparity has evolved simultaneously with placentation and incipient placentotrophy, rather than in three separate steps. Substantial placentotrophy and complex placentation have evolved in a few lizard clades (see Blackburn, 2006).

matrotrophy can evolve later (as in the skink genera *Mabuya* and *Chalcides*) as maternal nutrient provision becomes obligatory. The above scenario is consistent evidence that oxygen availability limits embryonic development of eggs held in the maternal oviduct (Parker and Andrews, 2006). In a recent modification of this model, maternal calcium provision to eggs was proposed to evolve prior to viviparity, to compensate for reduction of the eggshell during oviparous egg-retention (Stewart, 2013).

Viviparous squamates are disproportionately represented in cold climates of high altitudes and latitudes. This correlation led researchers to conclude that viviparity arose in cold conditions because pregnant lizards and snakes could thermoregulate their embryos at optimal temperatures, accelerating their development (Shine, 1985). In recent years, this “cold climate hypothesis” has been subsumed into a broader “maternal manipulation hypothesis.” According to this hypothesis, pregnant females can manipulate incubation conditions in ways that affect a variety of phenotypic traits to the benefit of the offspring and the mother (Shine, 2014). While this explanation seems to account for many of the origins of squamate viviparity, it may not apply in all cases, and some aspects remain controversial.

Factors affecting the evolution of viviparity in amphibians have received little attention. One plausible explanation is that viviparity has evolved as a means of protecting eggs from predation. Indirect evidence for predation as a selective pressure comes from the variety of mechanisms for egg protection that have arisen among oviparous amphibians, particularly frogs. As for amphibian matrotrophy, one major benefit in salamanders is that it allows females to produce offspring that are fully developed, bypassing the aquatic, pre-metamorphic larval stage (Buckley, 2012).

Viviparity in reptiles and amphibians is a fascinating phenomenon that continues to receive intensive attention from biologists. Such attention is sorely needed, since we lack data for most species on how pregnancy is accomplished and how embryos are maintained in the female oviduct. We also lack information on how and why matrotrophy has evolved. A broad range of approaches is now being applied, including morphological and physiological analyses, experimental approaches, and molecular studies of gene expression during pregnancy (Blackburn, 2006; Van Dyke *et al.*, 2014; Griffith *et al.*, 2016).

References

- Andrews, R. M., & Mathies, T. (2000). Natural history of reptilian development: Constraints on the evolution of viviparity. *Bioscience*, 50, 227–238.
- Blackburn, D. G. (1993). Chorioallantoic placentation in squamate reptiles: Structure, function, development, and evolution. *J. Exp. Biol.*, 266, 414–430.
- Blackburn, D. G. (1995). Saltationist and punctuated equilibrium models for the evolution of viviparity and placentation. *J. Theoret. Biol.*, 174, 199–216.
- Blackburn, D. G. (2006). Squamate reptiles as model organisms for the evolution of viviparity. *Herpetol. Monogr.*, 20, 131–146.
- Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *J. Morphol.*, 276, 961–990.
- Blackburn, D. G., & Sidor, C. A. (2014). Evolution of viviparous reproduction in Paleozoic and Mesozoic reptiles. *Int. J. Dev. Biol.*, 58, 935–948.
- Blackburn, D. G., & Stewart, J. R. (2011). Viviparity and placentation in snakes. In D. Sever, & R. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 119–181). Enfield, NH: CRC Press.
- Buckley, D., 2012. Evolution of viviparity in salamanders (Amphibia, Caudata). eLS. doi: 10.1002/9780470015902.a0022851.
- Buckley, D., Alcobendas, M., García-Paris, M., & Wake, M. H. (2007). Heterochrony, cannibalism, and the evolution of viviparity in *Salamandra salamandra*. *Evol. Dev.*, 9, 105–115.
- Greven, H. (2011). Maternal adaptations to reproductive modes in amphibians. In D. M. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates* (vol. 2). Burlington, MA: Academic Press.
- Griffith, O. W., Brandley, M. C., Below, K., & Thompson, M. B. (2016). Reptile pregnancy is underpinned by complex changes in uterine gene expression: A comparative analysis of the uterine transcriptome in viviparous and oviparous lizards. *Genome Biol. Evol.*, 8, 3226–3239.
- Parker, S. L., & Andrews, R. M. (2006). Evolution of viviparity in sceloporine lizards: In utero PO₂ as a developmental constraint during egg retention. *Physiol. Biochem. Zool.*, 79, 581–592.

- Ramírez-Pinilla, M. P. (2014). Biología reproductiva y placentotrofia en lagartijas del género *Mabuya*. *Rev. Acad. Colomb. Cienc.*, 38, 106–117.
- Shine, R. (1985). The evolution of viviparity in reptiles: An ecological analysis. In C. Gans, & F. Billet (Eds.), *Biology of the Reptilia*, 15 pp. 605–694. New York, NY: John Wiley.
- Shine, R. (2014). Evolution of an evolutionary hypothesis: A history of changing ideas about the adaptive significance of viviparity in reptiles. *J. Herpetol.*, 48, 147–161.
- Stewart, J. R. (2013). Fetal nutrition in lecithotrophic squamate reptiles: Toward a comprehensive model for evolution of viviparity and placentation. *J. Morphol.*, 274, 824–843.
- Stewart, J. R., & Blackburn, D. G. (2015). Viviparity and placentation in lizards. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 448–563). Boca Raton, FL: CRC Press.
- Van Dyke, J. U., Brandley, M. C., & Thompson, M. B. (2014). The evolution of viviparity: Molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. *Reproduction*, 147, R15–R26.
- Wake, M. H. (1993). Evolution of oviductal gestation in amphibians. *J. Exp. Zool.*, 266, 394–413.
- Wake, M. H. (2015). Fetal adaptations for viviparity in amphibians. *J. Morphol.*, 276, 941–960.

Pregnancy and Parturition, Mammals

Fuller W Bazer, Texas A&M University, College Station, TX, United States

Michael J Fields, University of Florida, Gainesville, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Adrenal gland The cortex or outer layer secretes steroid hormones, particularly cortisol, in response to ACTH secreted by the anterior pituitary gland.

Anterior pituitary Structure at the base of the hypothalamus that secretes luteinizing hormone (LH), follicle stimulating hormone (FSH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH) and growth hormone (GH) hormones in response to releasing hormones secreted by the hypothalamus, but PRL secretion is regulated by prolactin inhibitory factor which is dopamine.

Cervix A thick cartilaginous structure between the vagina and uterus that serves as a barrier protecting the developing conceptus within the uterus during pregnancy. During the periparturient period the cervix dilates and forms a birth canal through which the fetus and placenta pass to reach the external environment.

Conceptus The embryo/fetus and associated membranes.

Corpus luteum The endocrine gland(s) formed by the granulosa and theca cells of the ovarian follicle after ovulation that secretes progesterone which is necessary for the establishment and maintenance of pregnancy.

Ferguson reflex A neural response from stimulation of the vagina resulting in the release of oxytocin from the hypothalamus/posterior pituitary into maternal blood.

Hypothalamus The structure at the base of the brain that regulates many physiological functions through secretion of hormones.

Labor The process in which the mother's uterine contractions physically force the offspring out of the reproductive tract via the cervix and vagina into the external environment.

Luteolytic hormone Hormone, particularly prostaglandin $F_{2\alpha}$ that causes functional and structural demise of the corpus luteum.

Luteotrophic hormone A hormone such as luteinizing hormone from the anterior pituitary gland or chorionic gonadotropin from placenta that stimulates secretion of progesterone by the corpus luteum.

Parturition The process of giving birth to offspring.

Periparturient The time immediately preceding, during, and immediately following parturition.

Placenta Fetal membranes that include the chorion, allantois and amnion. The chorion and allantois fuse to form the chorioallantois which transfers nutrients, waste products and gases between maternal blood and the fetal blood. In many species, the chorion secretes progesterone.

Posterior pituitary Structure at the base of the hypothalamus that secretes oxytocin and vasopressin in response to neurological signals.

Progesterone block During pregnancy, progesterone from the corpus luteum and/or placenta sustains the pregnancy by inhibiting uterine contractions and other activities that would lead to premature delivery of the fetus and placenta.

Prostaglandin $F_{2\alpha}$ The luteolytic hormone that causes functional and structural demise of the corpus luteum.

Uterus The major component of the female reproductive tract in which the conceptus (embryo/fetus and placenta) develop. It has an outer muscular layer known as the myometrium and an inner secretory layer known as the endometrium.

Introduction

Pregnancy is established and maintained in subprimate mammals in response to a series of interactions between the conceptus (embryo/fetus and associated membranes), uterus and/or ovarian corpus luteum (CL) (Bazer and First, 1983; Bazer and Spencer, 2009; Bazer *et al.*, 2008; Bazer, 2015). These interactions prevent functional and structural regression of the CL, or luteolysis. During the peri-implantation period of pregnancy, pregnancy recognition signals from the conceptus to the maternal system are either antiluteolytic or luteotrophic. The functional life span of the CL is controlled by release of prostaglandin $F_{2\alpha}$ (PGF) from either the uterus (subprimate mammals) and/or ovaries (primates, including humans). Pregnancy recognition signals from the trophoblast act in a paracrine or endocrine manner to interrupt either uterine or intra-ovarian production of luteolytic PGF (antiluteolytic) or the hormone may act directly on the CL (luteotrophic).

Mammals have a placenta that surrounds the embryo/fetus and it is through these placental membranes that nutrients and other molecules from the maternal vascular system are transported to the vascular system of the conceptus to support its

growth and development. At some point in fetal development, depending on the species, there is maturation of the hypothalamic-pituitary-adrenal axis that is sufficient to induce parturition which involves expulsion of the fetus and its placenta from the uterus. Each species has a paradigm for producing live offspring so that the species is able to adapt to the external environment with maximum chances for survival of the neonate. This process of giving birth to offspring is called parturition (Latin, *parturio*, to be in labor). There are variations regarding factors controlling parturition; however, there is a common theme across species based on the roles of the fetal hypothalamic-pituitary-adrenal axis in inducing parturition. Parturition in sheep is the “classical” model because it has provided the preponderance of information that has advanced understanding of the endocrinology and physiology of parturition in subprimate mammals.

Pregnancy Maintenance in Ruminants

In ruminants, such as sheep and cows, an ovulatory surge of LH coincident with onset of estrus (Day 0) initiates events that culminate in ovulation about 30 h later. The antiluteolytic signal for pregnancy recognition in ruminants is interferon tau (IFNT) produced by mononuclear cells of conceptus trophoblast. IFNT exerts a paracrine, antiluteolytic effect on the endometrium to inhibit uterine secretion of luteolytic pulses of PGF. Other conceptus and/or uterine products secreted during early pregnancy, for example, prostaglandin E₂ (PGE) and platelet activating factor (PAF) may exert secondary luteal protective effects. The mechanism for pregnancy recognition is similar for sheep, cattle and goats.

Luteolysis is initiated as progesterone down-regulates expression of receptors for progesterone (PGR) in uterine epithelia 12–13 days after onset of estrus. With down-regulation of PGR there is an increase in expression of estrogen receptor alpha (ESR1) and then oxytocin receptors (OXTR) by the uterine epithelia. Oxytocin secreted by the corpus luteum (CL) and posterior pituitary binds OXTR and stimulates pulsatile secretion of PGF by uterine epithelia. IFNT inhibits expression of ESR1 and OXTR mRNAs in endometrial epithelia to abrogate oxytocin-induced release of luteolytic pulses of PGF that ensures maintenance of the CL and its production of progesterone, the hormone of pregnancy. Nevertheless, inter-estrous intervals of 30–35 days result when sheep conceptuses are removed from uteri of ewes on Day 16 or after intrauterine infusions of either highly purified native ovine IFNT or recombinant IFNT (roIFNT) between Days 11 and 15 after onset of estrus. Thus, a secondary endometrial luteolytic mechanism activated between 25 and 30 days post-estrus must be abrogated by the conceptus to allow maintenance of pregnancy. The secondary mechanism for maintenance of CL function has not been established, but neither placental lactogen (CSH1) nor placental growth hormone (GH1) were found to be the secondary “signal” from the conceptus that reinforces the antiluteolytic effects of IFNT.

Binucleated trophoblast cells in sheep conceptuses appear by Day 16 and secrete placental lactogen (CSH1). Placentae of other ruminant species secrete hormones structurally related to pituitary growth hormone (GH) and prolactin (PRL) and they are members of the CSH1 family. Both ovine GH and CSH1 have similar somatogenic activities; however, their circulating levels are regulated differently during the pregnancy. CSH1 is detected in blood of ewes by Day 50 of pregnancy and concentrations peak between 120 and 130 days of the 147 day period of gestation. Concentrations of CSH1 in fetal blood are less than in maternal blood and peak at mid-gestation after which time they may remain unchanged or decrease gradually to term. Concentrations of oGH are low in maternal serum throughout pregnancy and are not correlated with gestational age. Concentrations of oGH are greater in fetal than in maternal serum and greatest during mid-gestation. CSH1 may be critical to maintenance of pregnancy in sheep since lactogenic hormones influence steroidogenesis in CL and transport of water and other nutrients by placental membranes. Lactogenic hormones also stimulate proliferation of cells, expression of PGR, protein synthesis, exocrine secretion of PGF (pigs) by uterine epithelia, mammary growth and lactation. Ovine placental GH1 may have multiple roles in pregnancy.

Pregnancy Maintenance in Rodents

Gestation in rodents lasts 20–22 days, and functional CL must be maintained until Day 17. The transition in rodents, (i.e., rats, mice and hamsters) from recurring estrous cycles to pregnancy is dependent on maintenance of progesterone production by the CL, the main source of progesterone throughout pregnancy (Ben-Jonathan *et al.*, 2008; Soares *et al.*, 2007). Progesterone replacement alone is sufficient to maintain pregnancy in ovariectomized rats. In addition to lacking a true luteal phase during the estrous cycle, rodents do not exhibit a change in source of progesterone from the CL to the placenta. Thus, maternal recognition of pregnancy in rodents involves activation of nonfunctional CL of the cycle into functional CL of pregnancy.

Mating of rodents during estrus results in pseudopregnancy or pregnancy, and the activated CL secrete progesterone for 12–14 days. Extension of CL lifespan past Day 12 after onset of estrus depends on the presence of viable conceptuses within the uterus. A successful pregnancy in rats requires active secretion of progesterone from CL until at least Days 17–18. Therefore, establishment and maintenance of pregnancy in rodents requires two separate endocrine events. The first endocrine event initiated by mating results in diurnal and nocturnal surges of prolactin during the first 12 days of pregnancy or pseudopregnancy in rats. The prolactin is necessary for formation and maintenance of active CL and their secretion of progesterone. Therefore, the luteotrophic effects of PRL during early pregnancy are required to convert a nonfunctional CL of the estrous cycle into a functional CL of pregnancy or pseudopregnancy.

The second endocrine event required for the maintenance of pregnancy in rodents is dependent on implantation and development of normal conceptuses. In pregnant rodents, the placenta and stromal cells that differentiate to form the uterine decidua produce PRL-like hormones that have luteotrophic effects on the CL to ensure production of progesterone during the middle and late stages of pregnancy. The antimesometrial uterine decidual cells secrete numerous hormones, including PRL-like protein B (PLP-B) and decidual PRL-like protein (dPRP). The main luteotropic hormone of the decidua is a PRL-like protein, although uterine decidual cells also secrete PLP-B. Placental lactogens (CSHs) are found in a variety of subprimate mammals, including rodents. However, the only established physiological roles for CSHs are in rodents. In rats, seven members of the PRL gene family are expressed by trophoblast cells of the placenta: placental lactogen-I (CSH-I), CSH1-I variant (CSH1-Iv), CSH1-I mosaic (CSH1-Im), CSH1-II, PRL-like protein A (PLP-A), PLP-B and PLP-C. CSH1-I and CSH1-II have biological activities similar to those of pituitary PRL, including CL maintenance and growth of the mammary glands.

Factors regulating production of CSHs by trophoblast cells are not well known. The ontogeny of CSH1-I expression appears to be linked to differentiation of trophoblast cells during the peri-implantation period. In mice, expression of the CSH1-I gene appears to be regulated by the number of conceptuses and by the pituitary gland. Similarly, factors affecting expression of the CSH1-II gene include the number of conceptuses, genotype of the conceptuses, pituitary via growth hormone (GH), ovarian steroids and nutritional status of the mother. The shift from CSH1-I to CSH1-II is associated temporally with degeneration of the choriovitelline placenta between Days 13 and 14 of gestation.

In rats, PRL is essential for maintenance of progesterone secretion by CL throughout pseudopregnancy. However, in the pregnant rat, removal of the anterior pituitary after mid-gestation does not affect luteal function and pregnancy is maintained. Thus, pituitary PRL is not necessary after Day 6 of gestation in rodents. Given their PRL-like activity and their ontogeny during pregnancy, CSHs are likely luteotrophic factors from the placenta during mid- to late-pregnancy in rodents.

Pregnancy Maintenance in Swine

Estrogens produced by conceptuses between Days 11 and 12 of gestation provide the initial signal for maternal recognition of pregnancy in swine (Bazer, 2015). This signal results in a switch in the direction of secretion of PGF by uterine epithelia from an endocrine direction (i.e., into maternal blood) to an exocrine direction (i.e., into the uterine lumen). A second period of estrogen production occurs between Days 15 and 25–30 of pregnancy in pigs. Injection of exogenous estrogen (estradiol valerate, 5 mg/day) on Days 11 through 15 of the estrous cycle results in CL maintenance for a period equivalent to or slightly longer than pregnancy. This condition of estrogen-induced CL maintenance in non-pregnant pigs is known as pseudopregnancy and it persists for about 120 days. Thus, two phases of exogenous estradiol, similar to that produced by conceptuses on Days 11–13 and Days 15–25–30, are necessary for prolonged secretion of PGF into the uterine lumen. Estradiol may induce receptors for maternal hormones, for example, prolactin, or conceptus secretory proteins, which influence exocrine (into the uterine lumen) secretion of PGF. The first estrogen signal may induce receptors for PRL and the second estrogen signal may be required to replenish those receptors. Administration of estradiol on Day 9 advances the uterine secretory response in pregnant gilts, but leads to conceptus death by Day 16 due to degeneration of the extracellular matrix on the uterine luminal epithelium. An explanation for this “induced” conceptus death is not available, but it may result from asynchrony between the developing conceptus and uterine environment that adversely affects maintenance of the extracellular matrix.

Estrogen induces endometrial receptors for prolactin (PRLR) in pigs, and PRL acts on the endometrium to induce calcium cycling across the epithelium. In pigs, PRL interacts with estrogen and progesterone to increase total recoverable uteroferrin, glucose and PGF in uterine flushings. Available results strongly indicate that prolactin enhances uterine responsiveness to progesterone during periods critical for maintenance of pregnancy.

Pregnancy Maintenance in Horses

The uterine luteolytic hormone in mares is PGF and the conceptus produces an unidentified factor that inhibits uterine secretion of luteolytic pulses of PGF (see Bazer, 2015). In cycling mares, concentrations of PGF in uterine venous plasma and uterine flushings increase between Days 14 and 16 when luteolysis occurs and concentrations of progesterone decline in blood. The amount of PGF bound by luteal receptors is maximal on Day 14 of the estrous cycle and Day 18 of pregnancy. Since CL of mares can respond to circulating PGF during pregnancy, the conceptus must evoke an antiluteolytic mechanism. Pregnant mares have little PGF in uterine fluids, low concentrations of PGF in uterine venous plasma, and no pattern of pulsatile release of PGF (measured as the metabolite of PGF, PGFM) in peripheral plasma. In the presence of the conceptus, endometrial production of PGF in response to cervical stimulation and exogenous oxytocin is markedly reduced, indicating the absence or significant reduction in endometrial receptors for oxytocin in mares during early pregnancy.

The pregnancy recognition signal in mares is not known, but estradiol and/or proteins from the conceptus are likely candidates. There is also some evidence that PGE may play a role in pregnancy recognition signaling in mares. The equine conceptus migrates between the two uterine horns 12–14 times per day between Days 12–18 of pregnancy presumably to inhibit endometrial production of luteolytic PGF and protect the CL for production of P4. The equine conceptus also produces increasing amounts of estradiol

between Days 8 and 20 of gestation. A similar trend, but of greater magnitude, was found for estrone. Attempts to prolong CL lifespan in mares by injection of estrogens have been inconsistent.

Horse conceptuses secrete three major proteins between Days 12 and 14 of pregnancy with molecular weights of greater than 400,000, 50,000, and 65,000. However, the role(s) of those proteins is not known. Estrogens and/or conceptus secretory proteins may provide the maternal recognition of pregnancy signal in the mare by directly or indirectly inhibiting uterine production of luteolytic pulses of PGF.

Pregnancy Maintenance in Rabbits

Rabbits are induced ovulators and multiple ova are ovulated from each ovary approximately 10 h after mating. The oocytes remain fertilizable for about 6 h (Marcinkiewicz and Bahr, 1993). The fertilized ova arrive in the uterus 3 days post-ovulation and implantation occurs on Day 7 at the blastocyst stage of conceptus development. The rabbit placenta is not a significant source of progesterone and the CL are required for pregnancy to go to term. Following a sterile mating, CL form and persist for 14–16 days without support from conceptus products. For both pseudopregnant and pregnant does, progesterone begins to increase 2 days after mating to maximal levels of 12–20 ng/mL between Days 6–8 post-coitum. Between Days 8 and 10 post-mating progesterone profiles of pregnant and pseudopregnant does begin to diverge with concentrations of progesterone in blood declining rapidly after Day 12 to basal levels between Days 16 and 18 of pseudopregnancy. For pregnant does, concentrations of progesterone in blood do not begin to decline until 3–4 days prior to parturition (kindling).

Maternal recognition of pregnancy occurs after implantation between Days 10 and 12 of gestation. Estrogen and a placental luteotrophin interact to maintain progesterone production by the corpora lutea until term (28–35 days). Estrogen from developing follicles is required to stimulate progesterone production by CL for the first 10–12 days of pregnancy, but not to term. Luteal cells of corpora lutea of rabbits express LH receptors; however, LH does not stimulate progesterone production *in vivo*.

Production of a placental luteotrophin is necessary for production of progesterone by corpora lutea to term, but exogenous estrogen does not support progesterone production by corpora lutea of hysterectomized does during late pregnancy. Placentae of rabbits secrete a molecule having immunoreactive GnRH-like activity, but there is no evidence for it being transported from the uterus to the corpora lutea. A putative placental luteotrophic factor with a molecular weight greater than 6–8 kDa does enhance progesterone production by cultured luteal cells. Placental giant cells in rabbits also contain immunoreactive chorionic gonadotropin, and cytotrophoblast cells contain immunoreactive CSH1/PRL. However, the function(s) of those proteins has not been determined.

Pregnancy Maintenance in Cats

The cat, also an induced ovulator, ovulates 25–50 h post-mating (about 24–36 h after the LH peak) with frequent matings reducing the time to ovulation (Verstegen *et al.*, 1993; Brown, 2006). Fertilization takes place in the oviduct up to 48 h after ovulation. Feline embryos enter the uterus at the blastocyst stage, 4–6 days post-ovulation, blastocysts hatch from the zona pellucida on Day 11 and begin implanting by Days 12–13 of pregnancy. Following mating, concentrations of progesterone in plasma increase from about Day 3 to maximal levels (15–90 ng/mL) between Days 10 and 40 of pregnancy, or Days 13 and 30 of pseudopregnancy. Pseudopregnancy typically lasts 40 days and gestation ranges between 56 and 71 days, averaging 63–65 days. By Day 30, circulating levels of progesterone are significantly higher in pregnant than in pseudopregnant queens. The corpora lutea of queens are resistant to luteolytic effects of PGF until after Day 40 of pregnancy.

The placenta does not appear to be a significant source of progesterone during gestation in cats. Concentrations of prolactin in maternal blood increase after Day 40 of gestation in queens and it is considered the *be* luteotrophic. Inhibition of secretion of PRL in the last trimester of gestation causes abortion. Concentrations of PRL are greatest just prior to parturition (5–10 ng/mL) and remain elevated during lactation in response to suckling. Relaxin is produced by the fetal-placental unit, corpora lutea and uterus during pregnancy. Relaxin likely works in concert with progesterone to keep the uterus quiescent and to facilitate parturition by softening the connective tissues of the pelvis and cervix (Sherwood, 2004). Following parturition, queens experience a period of lactational anestrus and resume cycling 2–3 weeks after weaning kittens.

Pregnancy Maintenance in Dogs

Dogs are spontaneous ovulators and fertilization of oocytes occurs 2–5 days after ovulation in the bitch and embryos enter the uterus at the blastocyst stage around Day 10 (Concannon, 1993; Verstegen-Onclin and Verstegen, 2008). Embryos remain free-floating in the uterus until hatching and implantation around Day 16. The ovary is the primary source of progesterone and ovariectomy or hypophysectomy at any stage of pregnancy results in abortion. Since the lifespan of corpora lutea of pregnancy (64–66 days) and pseudopregnancy (60–63 days) are similar, there is no known requirement for signaling between the conceptus and maternal system for CL maintenance, pregnancy recognition or maintenance of pregnancy.

The corpora lutea of bitches are resistant to luteolytic effects of PGF until after Day 40 of pregnancy or pseudopregnancy. The secretion of prolactin increases after Days 30–40 of gestation and it is considered to be luteotrophic. Inhibition of secretion of PRL after about Day 30 of gestation causes abortion. The ovaries and pregnant uterus produce relaxin for uterine quiescence during pregnancy, as well as relaxation of the cervix and pelvic ligaments. Interestingly, it remains detectable in blood of bitches for up to 60 days after parturition.

Endocrine Cascade Leading to Parturition

Subprimate mammals have placentae that include the amnion and amniotic fluid therein that provides support for the embryo/fetus to exist somewhat like a marine mammal and develop symmetrically in a liquid environment. The other two membranes, chorion and allantois, fuse to form the chorioallantois that transports nutrients from the blood of the mother to the blood of the conceptus and allows for the exchange of gases (O_2 and CO_2) between blood of mother and conceptus. At some point in development of the conceptus, varying with species, the fetus and its placental membranes are expelled from the uterus into the external world. Each species has a unique paradigm for producing live offspring that includes development of the fetus in utero, the birth process, and adaptation of the newborn to the external environment so that survival of the offspring is maximized for propagation of the species. The physiological events leading to birth of subprimate mammals is known as parturition (Latin, *parturio*, to be in labor), is the focus of this section of this chapter (see Thorburn and Challis, 1979; Liggins, 1988; Currie *et al.*, 1988; Wood and Cudd, 1997; Challis *et al.*, 2005).

One of the oldest records depicting birth is a stone relief in the tomb of Ti (2450–2320 BCE) showing assistance being given to a cow by applying traction to the limbs of a partially protruding calf and another showing removal of fetal membranes. Around 2000 years later the Greek philosopher Hippocrates proposed that the baby determines the timing of birth. In the 1930s, Joseph Barcroft found that restriction of oxygen and other nutrients to the fetus due to the mother's inability to meet the increasing demands of a developing fetus for nutrients lead to birth (see Liggins, 1988). However, Liggins (1988) published seminal findings establishing that maturation of the fetal hypothalamic-pituitary-adrenal gland axis provides for endocrine regulation of parturition. From the first record over 4400 years ago through to today, the paradigm of pregnancy leading to parturition is that the fetus and placenta signal their presence to the mother, secrete hormones that establish and maintain the pregnancy, prepare the mother's mammary glands for lactation and determine the time of birth.

There are a multitude of variations on the theme of regulatory factors controlling reproduction in mammals and they are likely continuing to undergo evolutionary changes. Each species is exploring to find the “best fit” for maximizing survival of the species. The process of parturition also involves a multitude of variations in factors controlling parturition. However, there is a common theme across species in regulating the major events of reproduction including parturition. Most is known about the physiology and endocrinology of parturition in the ewe, which is the “classical” model that has provided the preponderance of information toward our understanding of parturition in subprimate mammals.

Parturition, once initiated, leads to rapid expulsion of the young from the reproductive tract of the mother to the external environment. The mother prepares for this throughout pregnancy with a gradual change in her endocrine environment to accommodate changes that range from alterations in cardiac function and respiratory functions to mammogenesis and lactogenesis. The placental membranes and the hypothalamic-pituitary-adrenal axis of the fetus mature with advancing gestation and once that maturation process reaches a critical point events leading to parturition are initiated. The key events leading to parturition were established based largely on results from research by Liggins and co-workers with sheep (see Fig. 1).

In fetal lambs the hypothalamus at the base of the brain begins to secrete corticotropin releasing hormone (CRH) into the local portal circulation around Day 100 of gestation. The CRH enters the hypothalamic portal system supplying the anterior pituitary gland. On about Day 125 of pregnancy, the fetal anterior pituitary gland begins to respond to CRH by producing and secreting adrenocorticotropin hormone (ACTH) into the systemic circulation where it can stimulate the adrenal cortex to release cortisol. Arginine vasopressin (AVP) secreted by the hypothalamus may also play a role in parturition by stimulating the secretion of ACTH from the anterior pituitary gland. Beginning around Day 135 of gestation the adrenal cortex of the fetus has matured sufficiently to begin responding to ACTH and secreting cortisol in increasing amounts so that concentrations in fetal blood are maximum 24–48 h prior to the onset of parturition in ewes on Day 147 of gestation. In sheep and in many other species including goats, cows, and pigs, the endocrine events in the fetus leading to production of cortisol and culminating in parturition and birth of the offspring are very similar.

The critical role of the fetal hypothalamic-pituitary-adrenal axis was revealed in an elegant series of studies by Liggins and co-workers. They removed the fetal anterior pituitary gland at the end of the second trimester of pregnancy and found that parturition was delayed significantly. But, when ACTH was administered to the fetus at the end of the second trimester of pregnancy parturition was induced. Likewise removal of the fetal adrenal gland and cortisol prevented parturition while administration of cortisol to the fetus induced parturition. In sheep and cows with deformities in the fetal brain resulting in the absence of hypothalamic secretion of CRH, parturition does not occur normally and fetuses must be removed by Caesarean section. This is because the fetus will continue to grow to an extremely large size and eventually compress the gastrointestinal system of the ewe or cow leading to her death. A similar result was reported for fetal calves with an abnormality of the adrenal gland that prevented secretion of cortisol.

During the last 3–5 days prior to parturition there are significant changes in the endocrinology of the pregnant female (see Table 1). First, concentrations of progesterone in maternal blood decrease rapidly. This is because the placenta stops producing

Parturition – Sheep Model

- Fetus
 - Hypothalamus
 - CRH
 - Anterior Pituitary
 - ACTH
 - Prolactin
 - Adrenal Gland
 - Secretion of Cortisol
 - Placenta
 - Progesterone decreasing, estrogen increasing due to activation of C-21 Steroid 17 alpha hydroxylase enzyme by cortisol
- Maternal
 - Corpus Luteum
 - Progesterone and Relaxin (some species)
 - Regresses due to PGF2-alpha from uterus in response to estrogen and oxytocin
 - Anterior Pituitary
 - Prolactin and Growth Hormone
 - Posterior Pituitary
 - Oxytocin released to stimulate secretion of PGF2-alpha and myometrial contractions
 - Uterus
 - PGF2-alpha secretion increasing
 - Gap junctions increase among myometrial cells and uterine contractions increase

Fig. 1 Parturition: The sheep model. The periparturient endocrine events that occur in the mature fetus initiate the endocrine cascade that leads to parturition. The fetal hypothalamus secretes corticotropin releasing hormone (CRH) that acts on the fetal anterior pituitary gland to induce production and secretion of adrenocorticotropin hormone (ACTH) into the general circulation of the fetus. The fetal adrenal gland responds to ACTH with secretion of cortisol primarily. Cortisol acts on cells of the placenta to activate C21-steroid 17 α -hydroxylase which allows progesterone to be metabolized to estrogens which are key to the initiation of parturition. The shift from progesterone dominance for maintaining pregnancy to estrogen dominance is integral to the cascade that leads to parturition. For example, estrogen activates enzymes that lead to synthesis of prostaglandin F_{2 α} (PGF2-alpha) that plays a major role in parturition, and estrogen increases in expression of receptors for oxytocin on uterine epithelial cells and smooth muscle cells of the uterine myometrium. The mother's hypothalamus secretes oxytocin which acts via its receptors on uterine epithelial cells induce pulsatile secretion of PGF2-alpha and both oxytocin and PGF2-alpha act on the myometrium to induce contractions of the myometrium. In addition to PGF2-alpha causing regression of the corpus luteum to further decrease circulating concentrations of progesterone, it also release relaxin (e.g., pigs) from the corpus luteum which is responsible for dilation of the cervix and relaxation of pelvic ligaments. In other species the placenta is the source of the relaxin (Sherwood, 2004). The decrease in concentrations of progesterone and increasing concentrations of estrogen also induce formation of gap junctions among smooth muscle cells of the uterine myometrium which is necessary for the coordination of contractions among smooth muscle cells to expel the fetus and placenta to complete the process of parturition.

progesterone and starts secreting estradiol and there is regression of the corpus luteum to eliminate that source of progesterone. The sheep placenta produces progesterone; however, cortisol activates the enzyme C21-steroid 17-alpha hydroxylase which converts progesterone to 17-alpha hydroxyprogesterone that can be metabolized further to androgens and estrogens. The fetal gonads in mares provide androgens for synthesis of estrogens by the placenta. The estradiol produced by the placenta increases phospholipase A2 activity and expression of oxytocin receptors in uterine epithelia and uterine myometrium. Phospholipase A liberates arachidonic acid from phospholipids in uterine epithelia for conversion by prostaglandin synthase 2 and prostaglandin F synthase to PGF. Oxytocin from the posterior pituitary gland is released in pulses as the fetal head pushes on the cervix during parturition

Table 1 Characteristics of periparturient endocrine events

Species	Days of gestation	Source of progesterone	Placental C21 Steroid 17 α - hydroxylase	Fetal adrenal hormone	Source of relaxin
Sheep	144–152	CL and Placenta	Yes	Cortisol	?
Goat	146–154	CL	Yes	Cortisol	?
Cow	270–300	CL and Placenta	Yes	Cortisol	CL
Pig	112–115	CL	Yes	Cortisol	CL
Horse	315–360	Placenta	No	Cortisol	Placenta
Dog	64–66	CL	Unknown	Unknown	CL and Placenta
Cat	64–66	Placenta	Unknown	Unknown	CL, Placenta and Uterus
Rat		CL	No	–	CL
Rabbit		CL	No	–	Placenta and Uterus
Primate		Placenta	No	Cortisol DHEA SO ₄	CL+Uterus

Abbreviations: CL, corpus luteum; DHEA-SO₄, dehydroepiandrosterone sulfate.

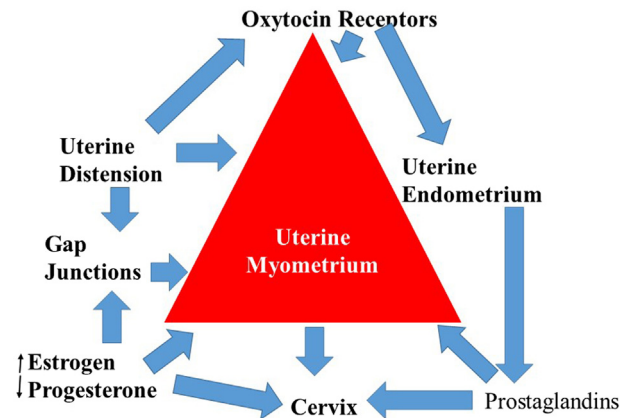


Fig. 2 The myometrium is composed of smooth muscle cells that are critical to providing the forces of contractions necessary for delivery of the fetus and placenta. A prerequisite for development of strong myometrial contractions is the increase in production of estrogens by the placenta and the decreased progesterone which leads to the formation of gap junctions among myometrial cells that allows them to contract in unison and with increasing force as oxytocin and prostaglandin $F_{2\alpha}$ act via their respective receptors to stimulate uterine contractions. Distension of the uterus and pressure of the fetus on the cervix induce release of oxytocin from the posterior pituitary gland of the mother in a pulsatile manner. The uterine endometrium is the primary source of the prostaglandin $F_{2\alpha}$ that acts on the myometrium and cervix to stimulate contractions. As noted in **Fig. 1**, relaxin softens and increases the distensibility of the pelvic ligaments and dilation of the cervix forming a passageway sufficient for delivery of the fetus. An increase in concentrations of oxytocin and prostaglandins in maternal blood, along with formation of gap junctions between smooth muscle cells of the uterine myometrium induce increasingly powerful contractions of the uterine myometrium that expel the fetus and placenta into the extra-uterine environment.

(Ferguson reflex) which causes pulsatile release of PGF from the uterine epithelia that is luteolytic and causes regression of the CL and their secretion of P4. So, cortisol changes the sex hormone ratio from high progesterone and low estradiol during pregnancy to high estradiol and low progesterone during parturition.

The decreasing concentrations of progesterone and increasing concentrations of estradiol in the ewe leads to the formation of gap junctions (known as connexin 43) between smooth muscle cells of the uterine myometrium (see **Fig. 2**). The gap junctions allow the smooth muscle cells to communicate with each other to contract and relax in unison. Visualize that early in parturition there are few gap junctions so contractions are weak and infrequent, but progressive increases in numbers of gap junctions allow strengthening of contractions at more frequent intervals as more and more smooth muscle cells contract and relax in unison. During this time in the peri-parturient period, oxytocin is being released in greater amounts with strength and frequency of contractions because the fetus is putting more pressure on the cervix (Ferguson reflex) at increasingly frequent intervals. Finally, uterine myometrial contractions are sufficient to expel the fetus and then the placenta from the uterus, through the cervix and vagina to the external environment. PGF is also acting with oxytocin to stimulate contractions of the uterine myometrium.

Relaxin is a hormone produced by large luteal cells of the corpus luteum of pigs, the placenta and corpus luteum of cow and placenta of the mare. Relaxin interacts with other hormones including estradiol, PGF and oxytocin to cause relaxation and dilation of the cervix and pelvic ligaments. Relaxin increases keratin sulfate and decreases dermatin sulfate in cervical collagen. With loss of dermatin sulfate there is a decrease in cross-linking of collagen fibers which increases distensibility of the cervical collagen and the diameter of the lumen of the cervix.

Epinephrine stimulates synthesis and cortisol induces secretion of surfactant by epithelial cells of the fetal lungs which is critical for maturation and function of the lungs to ensure respiration and survival of the offspring.

References

- Bazer, F. W. (2015). History of maternal recognition of pregnancy. In R. D. Geisert, & F. W. Bazer (Eds.), *Regulation of Implantation and Establishment of Pregnancy in Mammals: Tribute to 45 Year Anniversary of Roger V. Short's "Maternal Recognition of Pregnancy"* (pp. 1–25). Cham: Springer International Publishing AG.
- Bazer, F. W., & First, N. L. (1983). Pregnancy and parturition. *Journal of Animal Science*, 57(Suppl. 2), 425–460.
- Bazer, F. W., & Spencer, T. E. (2009). Hormones and pregnancy. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction in Vertebrates* (pp. 55–70). San Diego, CA: Academic Press.
- Bazer, F. W., Spencer, T. E., Ott, T. L., & Johnson, G. A. (2008). Mediators of maternal recognition of pregnancy. In J. D. Aplin, A. T. Fazleabas, S. R. Glasser, & L. C. Giudice (Eds.), *The Endometrium* (second ed., pp. 268–285). London: Informa Healthcare.
- Ben-Jonathan, N., LaPensee, C. R., & LaPensee, E. W. (2008). What can we learn from rodents about prolactin in humans? *Endocrine Reviews*, 29, 1–41.
- Brown, J. L. (2006). Comparative endocrinology of domestic and nondomestic felids. *Theriogenology*, 66, 25–36.
- Challis, J. R., Bloomfield, F. H., Bocking, A. D., et al. (2005). Fetal signals and parturition. *Journal of Obstetrical and Gynaecological Research*, 6, 492–499.
- Concannon, P. W. (1993). Biology of gonadotrophin secretion in adult and prepubertal female dogs. *Journal of Reproduction and Fertility*, 47(Suppl.), 3–27.
- Currie, W. B., Gorewit, R. C., & Michel, F. J. (1988). Endocrine changes, with special emphasis on oestradiol-17 beta, prolactin and oxytocin, before and during labour and delivery in goats. *Journal of Reproduction and Fertility*, 82, 299–308.

- Liggins, C. G. (1988). Endocrinology of parturition. In J. M. Novy, & J. A. Resko (Eds.), *Fetal Endocrinology* (pp. 211–237). New York, NY: Academic Press.
- Marcinkiewicz, J. L., & Bahr, J. M. (1993). Identification and preliminary characterization of luteotropic activity in the rabbit placenta. *Biology of Reproduction*, 48, 403–408.
- Sherwood, D. O. (2004). Relaxin's physiological roles and other diverse actions. *Endocrine Reviews*, 25, 205–234.
- Soares, M. J., Konno, T., & Khorshed Alam, S. K. M. (2007). The prolactin family: Effectors of pregnancy-dependent adaptations. *Trends in Endocrinology and Metabolism*, 18, 114–121.
- Thorburn, G. D., & Challis, J. R. G. (1979). Endocrine control of parturition. *Physiological Review*, 59, 863–919.
- Verstegen, J. P., Onclin, K., Silva, L. D. M., et al. (1993). Regulation of progesterone during pregnancy in the cat: Studies on the roles of corpora lutea, placentata and prolactin secretion. *Journal of Reproduction and Fertility Supplement*, 47, 165–173.
- Verstegen-Oncin, K., & Verstegen, J. (2008). Endocrinology of pregnancy in the dog: A review. *Theriogenology*, 70, 291–299.
- Wood, C. E., & Cudd, T. A. (1997). Development of the hypothalamus-pituitary-adrenal axis of the equine fetus: A comparative review. *Equine Veterinary Journal Supplement*, 24, 74–82.

OLFACTION AND REPRODUCTION

Pheromones, Fish

Peter C Hubbard, University of Algarve, Faro, Portugal

© 2018 Elsevier Inc. All rights reserved.

Glossary

Active space The active space is the calculated volume of water (in the case of fish) in which a given odorant, including pheromones, is above the concentration at which it can be detected by the olfactory system. It depends primarily on the release rate of, and the olfactory sensitivity to, that odorant; a potent odorant with a high release rate will have a correspondingly large active space. Clearly, in the natural environment, the active space will also depend on water movement (e.g., river or tidal flow), diffusion speed (depending on molecular weight and temperature) and degradation rate.

Bile acids and alcohols Bile salts are steroidal compounds produced by the liver, and secreted into the intestinal lumen, *via* the gall bladder, where they aid digestion by emulsifying lipids. Most are reabsorbed during intestinal transit, but a fraction is excreted with the feces. They are potent odorants in fish, but only in the sea lamprey has their role as pheromones been clearly established.

Chemical communication Broadly, chemical communication can be defined as the use of chemicals as signals or cues in intra-specific communication. It includes pheromones, wherein the response is innate (does not require learning), but also kin- or individual recognition, which may be learned.

Hormone Classically, a hormone is a chemical compound released by one tissue or organ within the body, and carried *via* the circulatory system to a distant 'target organ' where it has a physiological effect ('endocrine'). Some hormones also act locally ('paracrine') or even on the same cells that produce them ('autocrine'), without the involvement of the circulatory system. This is a functional biological definition; hormones are of many different chemical classes (e.g., steroids and polypeptides). Some hormones, and/or their metabolites (but far from all), may also act as pheromones; these are called 'hormonal pheromones'.

Odorant and odor An odorant is simply any substance that is detected by the olfactory system (the chemosensory system of cranial nerve I in vertebrates). It may – or may not – be a pheromone. An odor, however, is a complex mixture of odorants typical of a habitat, species or even an individual (a 'signature mixture' in this case).

Pheromone Pheromones are "...substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behavior or a developmental process". They require no previous experience or learning. This is a functional biological definition; it does delineate the types of compound a pheromone may be. In fact, many pheromones are mixtures of different compounds.

Prostaglandin Prostaglandins are lipid derivatives *via* arachidonic acid with 20 carbon atoms (including a 5-carbon ring) which act as hormones, particularly as paracrine or autocrine factors, in many tissues of vertebrates. Notably, some prostaglandins also act as pheromones in some species of fish (e.g., Fig. 1(A) (ii)).

Steroid A steroid is an organic compound with the four carbon ring (three cyclohexane rings and one cyclopentane ring) core structure (e.g., 17,20 β -P, Fig. 1(A) (i)). This is a structural chemical definition; many (but not all) steroids may be hormones, and some (but far from all) steroids are pheromones (in some species).

Introduction

Fish are aquatic, non-tetrapod vertebrates; not a strictly phylogenetic definition, true, but one with which most are familiar. As fish live in water, this has many consequences for almost every aspect of their physiology; their olfactory and chemical communication systems are no exception. Firstly, for a given compound to act as a potential odorant in fish it must be water-soluble; for a terrestrial vertebrate, it must be both water-soluble and volatile. This means that fish odorants are often different types of compounds from those of terrestrial animals; this is true for pheromones, too. Secondly, aquatic environments differ markedly in their physico-chemical characteristics from terrestrial environments. In a river, for example, the water flow renders chemical territorial marking, used by many mammals, ineffective. Conversely, this reliable unidirectional flow makes long-range migratory cues more viable (see [Section Sea Lamprey](#)). Aquatic environments may often be turbid, turbulent and/or dark. Therefore, chemical signals – such as pheromones – may be more reliable, or more easily discerned, than visual or auditory cues. However, whatever their reproductive strategy or habitat, most fish species do not rely on one particular mode of communication, but use a multi-modal mixture of visual (e.g., vivid color patterns), behavioral (e.g., courtship displays), auditory and chemical cues,

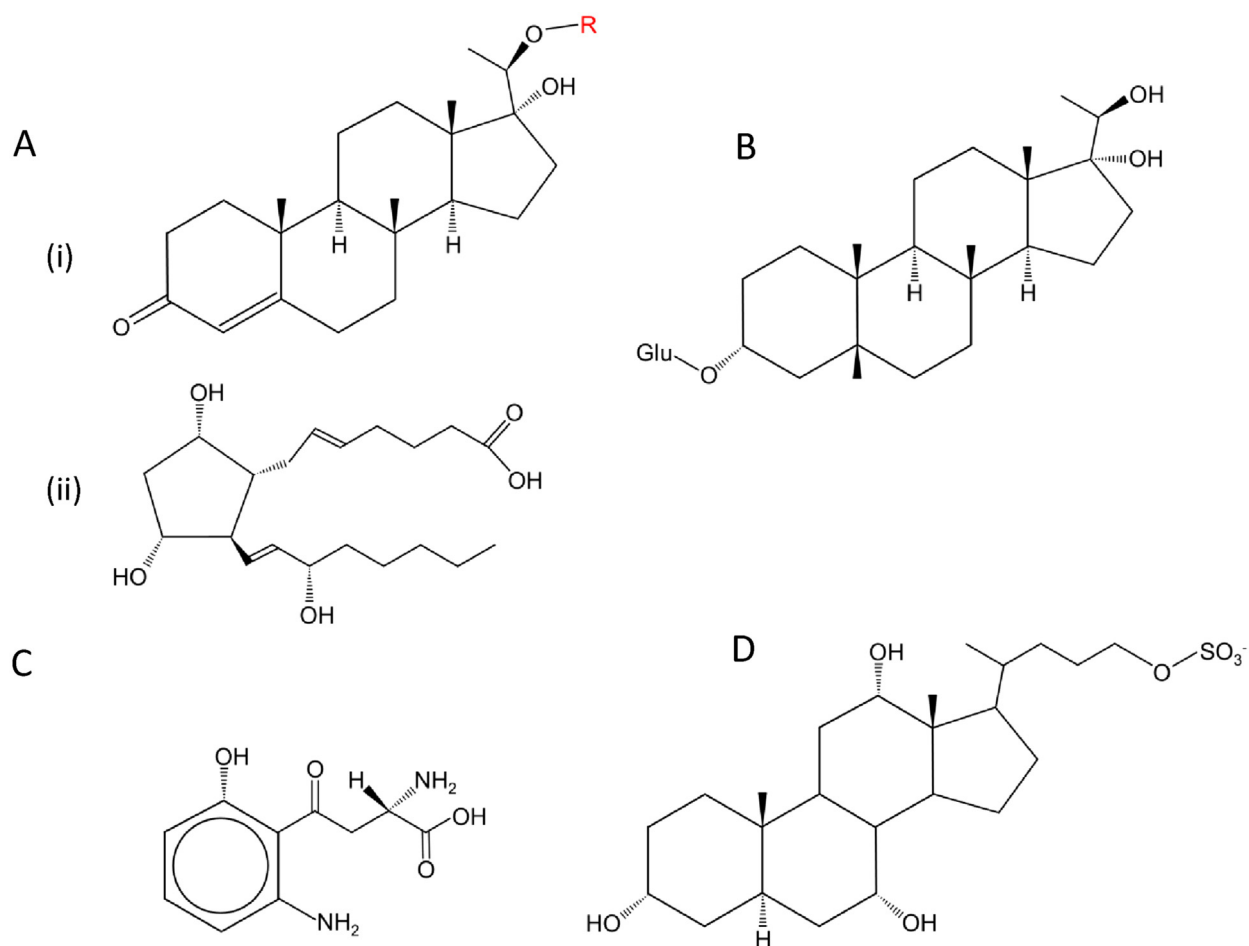


Fig. 1 Some examples of reproductive pheromones identified in teleosts. (A) Goldfish (*Carassius auratus*) female pre-ovulatory pheromone 17 α ,20 β -dihydroxy-4-pregnen-3-one (i), wherein the group at R can be -H, -glucuronate or -sulfate, and (ii) post-ovulatory pheromone prostaglandin F_{2 α} . (B) Mozambique tilapia (*Oreochromis mossambicus*) male urinary pheromone 5 β -pregnane-3 α ,17 α ,20 β -triol-3-glucuronate. (C) Masu salmon (*Oncorhynchus masou*) female urinary pheromone L-kynurenine (2-amino-4-(2-aminophenyl)-4-oxobutonic acid). (D) Sea lamprey (*Petromyzon marinus*) spermiating male pheromone 17 α ,12 α ,24-trihydroxy-5 α -cholan-3-one 24-sulfate (3-keto-petromyzonol sulfate or 3kPZS). In each case, it is likely that other components remain to be identified.

including pheromones, to communicate. Nevertheless, to almost exclusively audio-visual animals such as ourselves, the extent to which other animals communicate using odors and pheromones is not always obvious, either at a conceptual or sensory level. As a result, the importance of chemical communication is often sadly over-looked or under-estimated; this is as true for fish as other (non-human) animals.

Fish form the largest most diverse class of vertebrates and, as such, show a wide range of reproductive strategies in diverse habitats. However, most (but not all) are external fertilizers; hence, many mechanisms have evolved to ensure that eggs and sperm are released – and are ready to be released – at the same time to maximize fertilization rates and reproductive success. Pheromones, and other chemical cues, are an important – but not the only – part of this process. Such cues may also play important roles in related processes, such as mate-choice, individual recognition and migration.

Functions of Reproductive Pheromones in Fish

As most fish are external fertilizers, it is vital for reproductive success that sperm and eggs are released at the same time and place. Two key roles for reproductive pheromones are therefore, firstly, allowing males and females to find each-other (and recognize each-other as such) and, secondly, ensuring that they reach spawning readiness at the same time. The relative importance of each clearly depends on both reproductive strategy and habitat; schooling fish, for example, are continually surrounded by conspecifics, so finding each-other is not an issue. However, pheromones and other chemical cues may play a role in mate selection in such fish; females may recognize more dominant males through their pheromone release and choose to mate selectively with those. Synchrony can be ensured by either sex recognizing physiological indicators of maturity in the other. Those hormones responsible for stimulating gamete maturation, such as the maturation-inducing steroid (17 α ,20 β -dihydroxy-4-pregnen-3-one) and

prostaglandins in females, and androgens such as 11-ketotestosterone in males, are obvious chemical indicators, and this has given rise to the concept of 'hormonal pheromones'; the goldfish (see below) is a good example of this. However, this is far from universal; the female reproductive pheromone of masu salmon, for example, is an amino-acid derivative with no obvious role in reproductive physiology (see below).

Sites of Synthesis and Routes of Release

Reproductive steroid pheromones are mainly synthesized in the gonads; androgens by the cells of Leydig, and estrogens by the theca interna and granulosa cells of the ovaries. However, many hormonal steroids are physiologically 'deactivated' by conjugation; enzymatic addition of a glucuronate or sulfate group (usually to carbon 3 or 17 of the steroid core). This has three major effects: the conjugated steroid can no longer bind to its receptor (and is thereby unable to produce its physiological action); it cannot bind to its plasma binding protein (so can pass into the urine); and it is much more water-soluble. The latter two may promote its functionality as a pheromone, and many fish have evolved olfactory sensitivity to conjugated steroids, as well as – or even instead of – the free form. As the dynamics of release of these metabolites may be different from the parent steroid, they may also take on subtly different pheromonal roles (see [Section Goldfish](#)).

Fish are in intimate contact with their medium; water. Therefore, pheromones could potentially be released over any epithelium; across the gills, skin or *via* the urine or feces, or even with the gametes themselves. There is evidence that fish may use all of these. Free steroid pheromones may simply diffuse over the gills, or be actively excreted by specialized cells (e.g., sea lamprey). The skin mucus may also act as a route of release for pheromones, or other chemical cues involved in mate-choice or kin-recognition, such as the major histocompatibility complex (MHC), although the chemical types involved have not been identified. Urine, as in mammals, has been clearly identified as the vehicle of several reproductive pheromones (e.g., goldfish, tilapia and masu salmon). However, for osmoregulatory reasons, marine fish do not produce as much urine as their freshwater counterparts; in this case, feces may also act as a vehicle for pheromone release. Fish certainly have high olfactory sensitivity to feces! In some species, the ovarian and/or seminal fluids themselves may contain pheromones (e.g., the herring; [Carolsfeld et al., 1997](#)). Other fish may produce pheromones in specialized glands, such as the anal glands of blennies (e.g., [Serrano et al., 2008](#)), which are released directly to the water, or the testicular glands, seminal vesicles and blind pouches of many fish, where steroid conjugation is thought to occur ([Lambert and Resink, 1991](#)).

Detection of Pheromones: Olfaction

Fish live in water; one consequence of this is that a given compound only needs to be water soluble to have the potential to act as an odorant in fish whereas, in terrestrial animals (with some exceptions), it must be both water-soluble and volatile. Fish odorants are, therefore, often chemically distinct from tetrapod odorants; this includes pheromones. Unlike tetrapods, fish do not have an accessory olfactory system or vomero-nasal organ (VNO); pheromones are detected by the main (only) olfactory system. Fish have three types of olfactory receptor neurons (ORNs): ciliated, microvillous and crypt cells. Some circumstantial evidence suggests that pheromones are detected by crypt cells, but this remains controversial.

In vertebrates, fish included, olfactory receptors, including those for pheromones, are thought to be G protein coupled receptors (GPCRs). Recently, for example, a receptor for a female pheromone, prostaglandin $F_{2\alpha}$, was identified and cloned in zebrafish ([Yabuki et al., 2016](#)). However, for the majority of fish, the pairing of pheromone with its receptor is a field of active research.

Most reproductive pheromones are thought to exert their physiological effects *via* the hypothalamus-pituitary-gonad (HPG) axis, primarily through the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. The olfactory receptor neurons detecting pheromones converge on one, of few glomeruli in the olfactory bulb (the part of the central nervous system responsible for the first-order processing of olfactory input), and then second-order neurons send axons *via* the medial olfactory tracts to the hypothalamus; a clear case of 'hard wiring'. However, how these same pheromones exert their effects on behavior is less clear; possibly by involving the 'social brain network'. More research is needed to resolve this.

Model Species

Goldfish

The goldfish (*Carassius auratus*) is the best studied fish with respect to reproductive pheromones ([Dulka et al., 1987](#)). Goldfish belong to the family Cyprinidae which also includes the widespread research model species, the zebrafish (*Danio rerio*), although the chemical identities and biological roles of zebrafish reproductive pheromones are much less clear. Although Cyprinidae is the largest teleost family, it is exclusively fresh-water; most cyprinids, including the goldfish, are synchronous 'scramble competition' spawners wherein a post-ovulated female (i.e., a female ready to spawn) is pursued by several males. The males nudge the belly of the female, stimulating her to release her eggs onto underwater vegetation where they are fertilized by sperm released simultaneously by the males. Goldfish use steroids and prostaglandins as pheromones ([Dulka et al., 1987](#); [Sorensen et al., 1988](#)). However, the involvement of pheromones begins the night before; the ovaries produce a steroid hormone $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (' $17,20\beta$ -P'; [Fig. 1\(A\)](#)) that induces final maturation of the oocytes, ready for spawning

the next day. Somewhat unusually for a steroid hormone, $17,20\beta\text{-P}$ does not bind to a high affinity binding protein in the blood, but remains free to be released into the surrounding water, probably over the gills. It is then detected by the olfactory system of the males, at concentrations as low as 10^{-12} M, and this induces a release of luteinizing hormone (LH) from the males' pituitary gland which, in turn, stimulates the production of milt and sperm in the testes. Conjugated metabolites of $17,20\beta\text{-P}$, its sulfate and glucuronate forms (Fig. 1(A)) – hormonally inactive but pheromonally highly active, and released slightly later – are also detected by the males at pico-molar concentrations and have slightly different effects on male physiology and behavior (Poling *et al.*, 2001). The following morning, once the female has ovulated (i.e., the mature oocytes have been released from the ovaries into the ovarian ducts prior to release), the hormone responsible for this release, prostaglandin $F_{2\alpha}$ ($\text{PGF}_{2\alpha}$) and its metabolite $15\text{keto-PGF}_{2\alpha}$ are also released to the water, probably *via* the urine, where they stimulate the chasing and nudging reproductive behavior of the males and, ultimately, synchronous release of sperm and ova. Another steroid, androstenedione released by the males, increases aggression and competition among males while reducing their rivals' responsiveness to female pheromones (Sorensen *et al.*, 2005b).

Following these discoveries, many other cyprinids were found to both release, and have high olfactory sensitivity to, $17,20\beta\text{-P}$, $\text{PGF}_{2\alpha}$ and their metabolites. It is possible, likely even, that these compounds play similar pheromonal roles, given that most cyprinids have a similar reproductive strategy to the goldfish. However, this raises an intriguing and, as yet, unanswered question; how is species-specificity conferred to the pheromonal message? $17,20\beta\text{-P}$ or $\text{PGF}_{2\alpha}$ released by a carp or tench, for example (sympatric congeners of the goldfish), must smell the same as that released by a goldfish. The answer may lie in specific ratios of the compounds involved (e.g., as in insects), perhaps including other, unidentified, odorants. Conversely, the natural occurrence of many hybrids may suggest that pheromones are not completely species-specific, and that context, or other cues, are more important in conferring species-specificity. However, it will be clear from the following that other types of fish use very different chemicals as pheromones.

Masu Salmon

Although many salmonids spend significant parts of their lives in seawater, all spawn in freshwater. It is well known that many salmonids return to their natal stream to spawn, and that olfaction plays a vital role in this homing behavior. However interesting, this lies beyond the scope of a review of reproductive pheromones. Nevertheless, the masu salmon (*Oncorhynchus masou*) is one salmonid in which a female reproductive pheromone, an amino acid derivative, L-kynurenine (Fig. 1(C)) has been identified (Yambe *et al.*, 2006). Researchers noticed an intense blue fluorescent spot stained with ninhydrin reagent (which specifically stains amino acids) on TLC plates of ovulated female urine that was not present in immature female urine, or male urine. This was subsequently identified as 2-amino-4-(2-aminophenyl)-4-oxobutonic acid (christened 'L-kynurenine'), and was shown to attract mature males, but not immature males or females. Furthermore, the olfactory system of mature males was 1000 times more sensitive to L-kynurenine than immature males, and even more so than females, with a threshold of detection of 10^{-14} M. A calculated release rate of $0.4 \mu\text{moles h}^{-1} \text{ female}^{-1}$ therefore gives an active space of $40 \times 10^6 \text{ L h}^{-1} \text{ female}^{-1}$! This is more than sufficient to account for attraction in the mountain streams in which they spawn.

Although L-kynurenine is a major metabolite of L-tryptophan in vertebrates, and amino acids are potent odorants for fish, its link with reproduction is not as obvious as, for example, $17,20\beta\text{-P}$. The authors suggest that its metabolism is under the same hormonal regulation as oocyte maturation; however, urinary concentrations in other salmonids are much lower (around 1%). This therefore begs the question as to what other salmonids may be using as reproductive pheromones.

Tilapia

The Mozambique tilapia (*Oreochromis mossambicus*) and its close relative, the Nile tilapia (*O. niloticus*) are enormously important in aquaculture and, consequently, as invasive species world-wide. They are cichlids (family: Cichlidae), a scientifically outstanding taxon due to their complex social behaviors, including parental care, coloration and the bewilderingly rapid speciation seen in the African Rift Lakes cichlids. Despite, or perhaps because of, cichlids' bright coloration and behavioral displays, chemical communication has only been investigated in any depth in the Mozambique tilapia. Mozambique and Nile tilapia are maternal mouth-brooding, lek-breeding African cichlids. Males establish a hierarchy within the 'lek' (a term borrowed from ornithologists meaning a display area for courtship and breeding), wherein each male digs a simple nest in the sand or gravel bottom, with the more dominant males occupying the lek's center, and the submissive males towards the periphery. Females, when ready to spawn, approach the lek; the males express instantly recognizable courtship behavior towards the female, including characteristic black (Mozambique tilapia) or white (Nile tilapia) coloration, circling the female, 'grunting' (inaudibly, to humans at least) and trembling. Less obviously, Mozambique tilapia males also dramatically increase their urination rate (Fig. 2); dominant males, in particular, are able to store large volumes of urine in their bladder, which they release both during courtship and during aggressive displays against rival males. As the male circles the female, she is effectively surrounded by a cloud of male urine; this urine contains a steroid glucuronate, $5\beta\text{-pregnane-3}\alpha,17\alpha,20\beta\text{-triol-3}\alpha\text{-glucuronate}$ (and, to a lesser extent, its $20\alpha\text{-epimer}$; Keller-Costa *et al.*, 2014; Fig. 1(B)). This steroid pheromone, present at much higher concentrations in dominant male urine (up to 0.5 mM) than that of subordinates, dramatically increases the female's production of $17,20\beta\text{-P}$, the maturation inducing steroid (see Section Goldfish), thus insuring that a sub-set of her oocytes become mature at the correct moment (tilapia, unlike goldfish, are 'asynchronous'; not all oocytes mature at the same time). The female then chooses a male

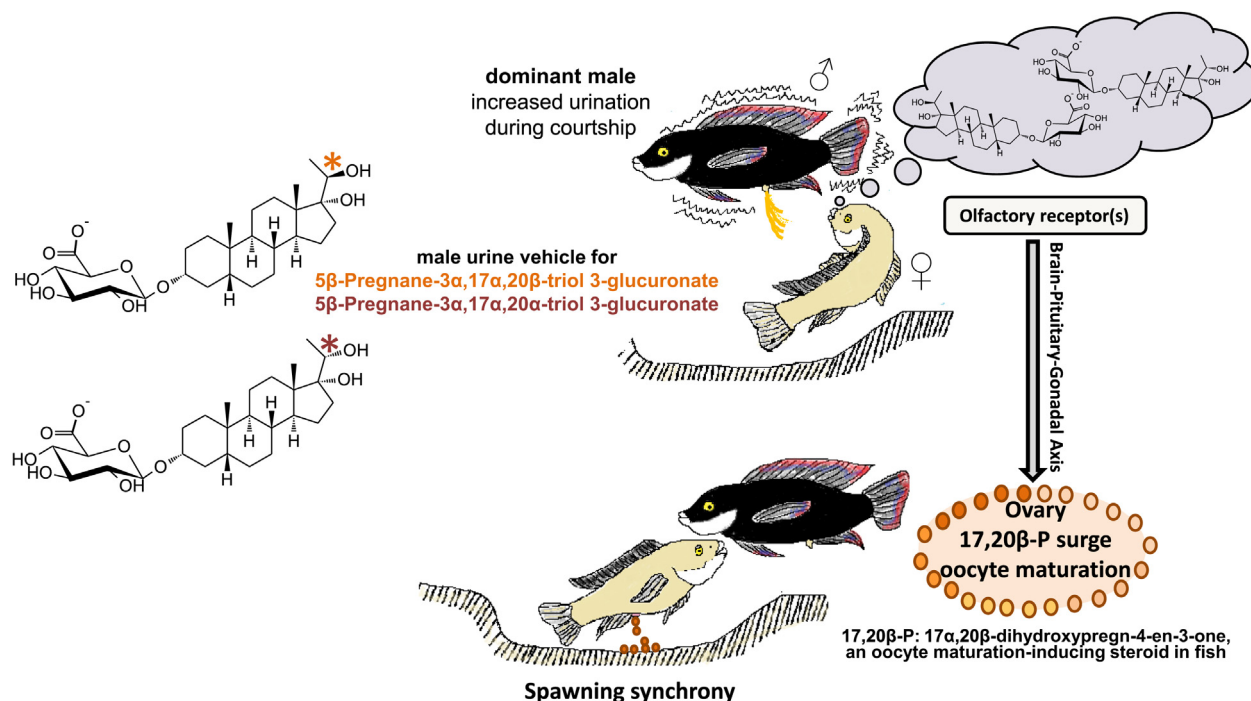


Fig. 2 The male reproductive pheromone of Mozambique tilapia. While performing his highly visible (and audible – at least to his intended audience) courtship display towards the female, the male releases many pulses of urine containing the pheromone 5β-pregnane-3α,17α,20β-triol-3-glucuronate (and its 20α epimer); dominant males have much higher urinary concentrations than subordinates. After smelling this, increased synthesis of the maturation-inducing steroid, 17,20β-P, is stimulated in the female. This ensures the final maturation of a cohort of ova, which she then releases to be fertilized by the male. Taken, with permission, from Keller-Costa, T., Canário, A.V.M., Hubbard, P.C., 2015. Chemical communication in cichlids: A mini-review. *General and Comparative Endocrinology* 221, 64–74.

with which to spawn; it is likely that this choice is made at least in part on the smell of his urine, including the perceived concentration of pheromone. After spawning, the female picks up the fertilized eggs in her mouth and moves away (often actively chased by the male) to brood them elsewhere. Although 17,20β-P acts as the maturation-inducing steroid in tilapia, as in goldfish, the tilapia cannot smell it, ruling out any pheromonal role for this steroid in this species. Nevertheless, males are still able to assess the reproductive status of the female through olfaction (possibly involving another steroid glucuronate, estradiol-3-glucuronate), and will only release urine towards a female who smells 'ready'. Although investigated in less detail, it seems likely that the Nile tilapia uses the same urinary steroids as reproductive pheromones, as they are present in male urine, and both sexes have similar olfactory sensitivity to them. Although the olfactory sensitivity of the tilapia to these steroids (threshold about 10^{-10} M) is much less than the examples from goldfish, masu salmon and sea lamprey, and the active space correspondingly lower, this is not a problem as the interactions take place at close quarters.

As previously mentioned, male Mozambique tilapia also use urine to signal to rival males during aggressive displays. These encounters are more likely to escalate to violent fights if the males are unable to smell one-another's urine, and thereby assess their relative ranking, suggesting the existence of a 'dominance' pheromone. However, the chemical identity of this pheromone has yet to be established.

Sea Lamprey

Unlike the other species discussed here, lampreys are not teleosts (bony fish), but agnathans; primitive jawless fish. Despite their name, sea lampreys (*Petromyzon marinus*) spawn in freshwater. Sadly threatened in their natural habitat (the north Atlantic Ocean and associated rivers), they have become a nuisance invasive species in the Great Lakes of North America, where they live their whole life-cycle in fresh-water, parasitizing many native species and thereby causing massive declines in their stocks. This has stimulated much research into possible methods of population control; one example is the use of pheromone traps, highly effective for pest insects. Obviously, this has necessitated the isolation and identification of the pheromones involved. Bile acids (a type of steroid synthesized by the liver and secreted into the intestine), principally petromyzonamine disulfate, from larval sea lamprey attract adult males when they are ready to spawn (Sorensen *et al.*, 2005a). It is likely that the presence of sufficient larvae to produce a detectable scent in the water entering the sea (or lake, in this case) is a reliable indicator that the stream is a suitable habitat for lamprey larvae. This is not 'homing' (as in the case of salmon), because the adults do not necessarily return to their natal stream, but

is a good example of a migratory pheromone. However, the true reproductive pheromone is a mixture of bile acids, including petromyzon sulfate (Fig. 1(D)), released by spermiating males. The males, lured by the larval migratory pheromone, arrive at the spawning sites before the females, to dig a crude nest in the gravel. The ripe females are then attracted by the mixture of bile acids released by the males (Li *et al.*, 2002), and join them in their nests where spawning occurs. However, in contrast to the other species discussed here, this pheromone is not released *via* the urine, but by the active secretion of specialized cells in the gills (Siefkes *et al.*, 2003). Also contrasting with the goldfish, at least, the gonads are an unlikely source of the bile acid pheromones; bile acids are synthesized by the liver. Although the sea lamprey is the only fish wherein bile acids have been shown to play pheromonal roles, fish in general have high and broad olfactory sensitivity to this class of compound. Thus, it is possible that bile salts also act as pheromones in other species; in the European eel, for example, the type of bile acid produced by the liver depends both on sex and sexual maturity.

Field tests with 'pheromone traps' baited with artificial mixtures of bile acids, based on these discoveries, have never been as effective as traps baited with the natural pheromone (i.e., spermiating males) in luring ripe female sea lamprey to their doom, suggesting that there are components to this pheromone yet to be discovered. However, such experiments at least provide 'proof of principal' for the use of pheromone traps for control of fish populations in a highly specific and environmentally harmless manner.

Conclusions

It should be clear from the above that the diversity of reproductive strategies in fish is matched by an equal diversity in their reproductive pheromonal systems. This is in marked contrast to the conservatism (evolutionarily speaking) of their internal regulatory mechanisms, the reproductive hormones, which have similar chemical structures and physiological functions across the vertebrates. Some fish, such as the goldfish, have expanded the roles of these internal regulators to that of reproductive pheromones, while others, such as the sea lamprey, have commandeered a class of compound – bile acids, with no apparent internal link to reproduction – as pheromones. However, what is also clear is that much remains to be discovered. How much 'overlap' is there in the types of chemicals used as pheromones? How are these related to phylogeny, or biological roles? How is species-specificity conferred to the pheromonal message? And how do marine fish use reproductive pheromones? These are all areas of active research.

References

- Carolsfeld, J., Tester, M., Kreiberg, H., & Sherwood, N. M. (1997). Pheromone-induced spawning of pacific herring I. Behavioral characterization. *Hormones and Behavior*, 31, 256–268.
- Dulka, J. G., Stacey, N. E., Sorensen, P. W., & Van Der Kraak, G. J. (1987). A steroid sex pheromone synchronizes male-female spawning readiness in goldfish. *Nature*, 325, 251–253.
- Keller-Costa, T., Hubbard, P. C., Paetz, C., et al. (2014). Identity of a tilapia pheromone released by dominant males that primes females for reproduction. *Current Biology*, 24, 2130–2135.
- Lambert, J. G. D., & Resink, J. W. (1991). Steroid glucuronides as male pheromones in the reproduction of the African catfish *Clarias gariepinus* – A brief review. *Journal of Steroid Biochemistry and Molecular Biology*, 40, 549–556.
- Li, W., Scott, A. P., Siefkes, M. J., et al. (2002). Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science*, 296, 139–141.
- Poling, K. R., Fraser, E. J., & Sorensen, P. W. (2001). The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comparative Biochemistry and Physiology B*, 129, 645–651.
- Serrano, R. M., Barata, E. N., Birkett, M. A., et al. (2008). Behavioural and olfactory responses of female *Salvia pavo* (Pisces: Blenniidae) to a putative multi-component male pheromone. *Journal of Chemical Ecology*, 34, 647–658.
- Siefkes, M. J., Scott, A. P., Zielinski, B., Yun, S.-S., & Li, W. (2003). Male sea lampreys, *Petromyzon marinus* L., excrete a sex pheromone from gill epithelia. *Biology of Reproduction*, 69, 125–132.
- Sorensen, P. W., Fine, J. M., Dvornikovs, V., et al. (2005a). Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. *Nature Chemical Biology*, 1, 324–328.
- Sorensen, P. W., Hara, T. J., Stacey, N. E., & Goetz, F. W. M. (1988). F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. *Biology of Reproduction*, 39, 1039–1050.
- Sorensen, P. W., Pinillos, M., & Scott, A. P. (2005b). Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. *General and Comparative Endocrinology*, 140, 164–175.
- Yabuki, Y., Koide, T., Miyasaka, N., et al. (2016). Olfactory receptor for prostaglandin F_{2α} mediates male fish courtship behavior. *Nature Neuroscience*, 19, 897–904.
- Yamabe, H., Kitamura, S., Kamio, M., et al. (2006). L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 15370–15374.

Further Reading

- Keller-Costa, T., Canário, A. V. M., & Hubbard, P. C. (2015). Chemical communication in cichlids: A mini-review. *General and Comparative Endocrinology*, 221, 64–74.
- Maynard Smith, J., & Harper, D. (2003). *Animal Signals*. Oxford: Oxford University Press.
- Sorensen, P. W., & Wisenden, B. D. (Eds.). (2015). *Fish Pheromones and Related Cues*. Ames: Wiley Blackwell.
- Stacey, N., & Sorensen, P. (2009). Hormonal pheromones in fish. In D. W. Pfaff, A. P. Arnold, Etgen, S. E. Fahrback, & R. T. Rubin (Eds.), *Hormones, Brain and Behavior* (vol. 1, pp. 639–681). San Diego, CA: Academic Press.

- Symonds, M. R. E., & Elgar, M. A. (2008). The evolution of pheromone diversity. *Trends in Ecology & Evolution*, 23, 220–228.
- Wyatt, T. D. (2010). Pheromones and signature mixtures: Defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology A*, 196, 685–700.
- Wyatt, T. D. (2014). *Pheromones and Animal Behavior*. Chemical Signals and Signatures (second ed.). Cambridge: Cambridge University Press.

Relevant Websites

- <https://en.wikipedia.org/wiki/Cichlid>. –Cichlid.
- <https://en.wikipedia.org/wiki/Cyprinidae>. –Cyprinidae.
- <https://en.wikipedia.org/wiki/Salmonidae>. –Salmonidae.
- https://en.wikipedia.org/wiki/Sea_lamprey. –Sea Lamprey.

Pheromones, Insects

Emmanuelle Jacquin-Joly, INRA, Institute of Ecology and Environmental Sciences of Paris, Versailles, France

Astrid T Groot, University of Amsterdam, Amsterdam, The Netherlands and Max Planck Institute for Chemical Ecology, Jena, Germany

© 2018 Elsevier Inc. All rights reserved.

Abbreviations

CHC	Cuticular hydrocarbon
GR	Gustatory receptor
MGC	Macroglomeruli complex
OR	Odorant receptor

ORN	Odorant receptor neuron
PKK	Pickpocket ion channel
PBAN	Pheromone biosynthesis activating neuropeptide
JH	Juvenile hormone

Glossary

Aggregation pheromone One sex produces the signal, and both sexes are attracted to the signal.

Anosmia Inability to detect or perceive a chemical compound.

Detection Identification of the presence of a signal.

Hormesis Dose-response phenomenon whereby a low dose (e.g. of insecticides) gives a high behavioral response, while at higher doses the response is reduced.

Natural selection Selection exerted by other species (biotic factors) or by abiotic factors (temperature, light).

Perception Detection of a signal including the interpretation of the signal after integration in the brain center.

Pheromone Infochemical that conveys information between individuals of the same species.

Pheromone component Chemical that has been behaviorally tested and confirmed to attract potential mating partners, in contrast to a pheromone compound, which has been chemically identified to be in the pheromone blend but not confirmed to be behaviorally important for attraction.

Plasticity Variation within individuals during development, sexual maturation, aging, or upon experience.

Reception Detection of a signal at the receptor level.

Sex pheromone Attractant between the sexes (one sex produces and emits the signal, and the other sex from the same species perceives and responds/is attracted to the signal).

Sexual selection Selection exerted by potential or rival mating partners within a species.

What are Insect Sex Pheromones?

Sex pheromones are ubiquitous in the animal kingdom and present in all insect orders (Gomez-Diaz and Benton, 2013). Since the first identification of the sex pheromone in the silkworm *Bombyx mori* in 1959, the sex pheromones of more than 1500 insect species have been identified (Pherobase.com). Generally, sex pheromones consist of a blend of at least two chemical components. Species or race specificity is ensured by the blend composition and the ratios of the components. The most famous example is the European corn borer, *Ostrinia nubilalis*, which consists of two pheromone races that have opposite ratios of the same two pheromone components.

There is enormous diversity in the chemical nature of sex pheromones, as they can consist of saturated or unsaturated hydrocarbon chain carrying an acetate, alcohol or aldehyde function (in most moths), polyenic hydrocarbons, methyl-branched hydrocarbons (e.g. in Lepidoptera and Coleoptera), epoxides, ketones (e.g. in some Coleoptera), terpenes (e.g. in beetles) and terpenoids (e.g. in some geometrids and pyralids), iridoids (e.g. in aphids), as well as alkaloids (e.g. in Scarabidae) (Howard and Blomquist, 2005; Hanks and Millar, 2016; Allison and Cardé, 2016; Jurenka, 2004; Dewhirst *et al.*, 2010; Yew and Chung, 2015). Representative structures of insect sex attractants are illustrated in Fig. 1, including those of moths, honeybee, cockroach, bug, and fruit fly.

Sex pheromones can be short(er) carbon chain volatiles that attract potential mating partners from a longer or shorter distance, or long(er) carbon chain cuticular hydrocarbons (CHCs) that are used to identify potential mating partners or rivals upon contact. Volatile and contact chemical cues may be integrated to reinforce or refine selection of the right mating partner. In species with additional cues, such as visual and acoustic cues, or species-specific movements, genitalia incompatibility, spatial and seasonal separation of populations, and diel separation of pheromone release, sex pheromones may consist of more ubiquitous compounds. The most striking example is found in aphids, where the sex pheromone of all species consist of nepetalactone and nepetalactol, and the species-specificity mostly comes from the combination of sex pheromone with other environmental cues (Dewhirst *et al.*, 2010). The amount of pheromone that is secreted from or is present in a pheromone-producing gland also varies enormously with species and, to some extent, behavioral function. Sex-attractant pheromones may be present in microgram, nanogram, and even picogram quantities per individual.

Silkmoth (*Bombyx mori*)

(E,Z)-10-12-hexadecadien-1-ol

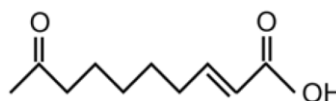
**Gypsy moth (*Lymantria dispar*)**

Disparlure (+)

(7R,8S)-cis-7,8-Epoxy-2-methyloctadecane

**Honeybee (*Apis mellifera*)**

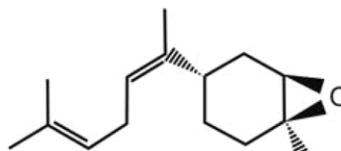
(E)-9-oxo-2-decenoic acid

**Green stink bug (*Nezara viridula*)**

(1S,4S,6R)-1-methyl-4-

((Z)-6-methylhepta-2,5-dien-2-yl)-

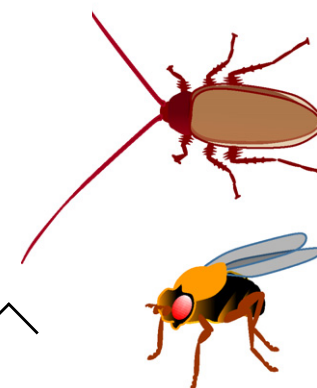
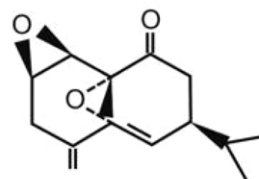
7-oxabicyclo[4.1.0]heptane

**Cockroach (*Periplaneta americana*)**

Periplanone B

(1Z,5E)-1,10(14)-diepoxy-4(15),

5-germacradien-9-one

**Fruit fly (*Drosophila melanogaster*)**

7 tricosene, non volatile



Fig. 1 Representative insect sex pheromone molecules illustrating the diversity of chemical structures.

Sex pheromones are not only diverse in terms of amounts, range or ratios of different compounds, but also in terms of enantiomers with opposing, inhibitory effects (Yew and Chung, 2015). For example, closely related species that occur sympatrically and have a similar sex pheromone may avoid hybridization by having different ratios of the same pheromone components or by having additional minor pheromone components that inhibits the attraction of the other species. One example for such behavioral inhibition is found in the gypsy moth (*Lymantria dispar*) and the nun moths (*L. monacha*) that share (+)-disparlure as attractant and live in sympatry in parts of Europe. In addition to (+)-disparlure, the female nun moth produces (–)-disparlure, that inhibits the attraction of male gypsy moths, thus ensuring species isolation. The use of such enantiomers or diastereoisomers in pheromone blends is quite common, especially in species where mate finding relies mostly on sex pheromone communication.

Who Produces Sex Pheromones?

Which sex is the signaler and which sex is the responder varies between insect orders, as well as on the range at which potential mating partners are attracted. The most typical example is found in moths: in most species females adopt a characteristic calling

behavior by which they extrude a pheromone gland for emission of a long-range sex pheromone in the air to attract males from a distance. In some lepidopteran species, such as the arctiid moth *Utetheisa ornatrix*, females group together for calling in chorus, which synchronizes and intensifies their signaling, thus favoring attraction. Receiver males fly upwind to the female with a stereotyped zig-zag behavior. Once in the vicinity of the female, males start courtship and in some species produce a close-range sex pheromone that may play a role in female choice. More rarely, as in the sugarcane borer *Eldana saccharina*, male moths emit a long distance pheromone from a gland/hair pencils on their abdomen, or from hair tufts on their wings, so-called coremata. In beetles, flies and several Hemiptera (true bugs), males attract females mostly at close range or upon contact. Tephritid flies display a lek mating system, where males aggregate and emit different visual, acoustic and chemical signals to attract females. Arriving female flies assess the multimodal calling behavior of a number of calling males before selecting a mate among the available ones.

Where are Sex Pheromones Produced?

The locations of the glands or tissues responsible for sex pheromone production are very diverse. Volatile sex pheromones are generally produced in specific glands, such as sex pheromone glands located at the tip of the abdomen in female moths, hair pencils/hair pockets (coremata) in male moths and butterflies, or epidermal glands located under the abdominal tergites of cockroaches. CHCs are produced in oenocytes, which are secretory cells inside the hemolymph (which can be close to epidermis or scattered throughout fat body). *Ips* beetles produce their sex pheromone in the hindgut, the signal being released with the frass. Tephritid flies produce pheromones in salivary and anal glands. In aphids, mature sexual females (oviparae) release a sex pheromone from scent plaques on their hind tibiae which attracts conspecific males (Dewhurst *et al.*, 2010).

Sex pheromones can be produced *de novo* or may be sequestered from plants (Jurenka, 2004). When produced *de novo*, the sex pheromones are generally derivatives from fatty acids (moth sex pheromones and CHCs). In some cases, microbes or symbionts participate in pheromone biosynthesis. For instance, gut microbiota may participate in the verbanone pheromone production in bark beetles. How aphids produce their sex pheromone components (nepetalactone and nepetalactol) is unknown, possibly nepetalactol is produced from a glycoside precursor, a proportion of which is sequentially oxidized to nepetalactone (Dewhurst *et al.*, 2010).

Some insects sequester or acquire host plant compounds and use them as sex pheromones or sex pheromone precursors. The most famous example is the arctiid butterfly *Utetheisa ornatrix* (Arctiidae), whose male courtship pheromone derives from pyrrolizidine alkaloids (PAs), which are ingested at the larval stage from the host plant *Crotalaria spectabilis*. Males also transfer PAs to females during mating as a nuptial gift, which females subsequently use to coat their oviposited eggs, which are then protected against predators (Eisner, 2003). In other arctiids, most Dancinae and Ithomiinae butterflies, males also obtain PAs from plants and use them as pheromone precursors. *Cysseps fulvicollis* (Arctiidae) males produce hydroxydanaidal from PAs of dead and damaged plants, and release it as a sex attractant. Host plant volatile emissions can also have an indirect impact on insect sex pheromone communication, for instance in *Helicoverpa* species where host plant volatiles induce female calling behavior. In some *Ips* beetles, exposure to plant volatile myrcene increases the amounts of ipsenol and ipsdienol pheromones in male hindgut tissues. On the receiver side, sex pheromone detection and processing has been also shown to be modulated by the presence of plant volatiles: chemicals from host plants often synergistically enhance the response of an insect to the sex pheromone, improving temporal resolution of pulsed pheromone signals, while some others can have inhibitory or repellent effects that interrupt the response.

When are Sex Pheromones Produced?

Upon Sexual Maturation

Pheromone emission and sensitivity usually coincide with sexual maturity. In most moth species, females and males start to become sexually active 1–2 days after emergence. In general, pheromone glands contain very low levels of pheromone on the first night after emergence, which then increases sharply in subsequent nights. When females get older or after mating, sex pheromone levels may decline again, at least for a short or a long period. In many moth species, female sex pheromone production follows a diel rhythm that is regulated by the nightly release of the pheromone biosynthesis activating neuropeptide (PBAN) from the subesophageal ganglion (Jurenka, 2004). In some moth species, previously synthesized fatty acids are stored as triglycerides in lipid droplets and mobilized by phosphorylation, while in other species, such as *Trichoplusia ni*, pheromone is continuously produced and not regulated by PBAN. In long-lived insects with multiple reproductive cycles, such as cockroaches, beetles and flies, pheromone production is regulated by juvenile hormone (JH) and ecdysone (Jurenka, 2004). For example, in beetles JH regulates pheromone production of both fatty acid and isoprenoid biosynthetic pathways, while JH in turn is regulated by environmental and physiological factors. In bark beetles, feeding on a new host tree elevates JH titers by stimulating activity of the corpus allatum, the endocrine gland that produces JH. In Diptera, ecdysone instead of JH stimulates egg maturation as well as sex pheromone production, most likely by regulating the fatty acyl-CoA elongases (Jurenka, 2004).

At Specific Times of the Day

Many moth species show specific daily activity rhythms in their sexual activities, some species being sexually active in the day, some early at night, while others are sexually active late at night. This differentiation has been suggested to have arisen to minimize

communication interference between closely related species, as co-occurring and closely related species with overlapping sex pheromone blends show a temporal differentiation in their daily sexual activities. For example, in the Mojave Desert of California *Hemileuca electra* calls from mid-morning to early afternoon; the closely related species *H. burnsi*, which shares pheromone components with *H. electra*, calls from midafternoon to dusk. Without exclusive times for mating activities, these species would cross-attract.

At Specific Times in the Year

Many insect species are sexually active only at specific times of the year, usually in the summer time. When the summer period is long (at lower latitudes), multiple generations may develop in one season, while at higher latitudes only one generation may develop. This is the case for example in the European corn borer, where populations near the Canadian border are univoltine, while more southern populations are bi- or even trivoltine, which genetically isolates these populations. Species may also be sexually active early or late in the season. In migrating species, such as the monarch butterfly *Danaus plexippus* and the noctuid moth *Helicoverpa armigera*, adults become sexually active only after arriving at their breeding site.

Variation and Plasticity in Insect Sex Pheromones

Geographic variation in sexual communication signals and responses has been found in many species, even when sex pheromones are mainly used as species-specific recognition cues, which inhibits variation and promotes retention of the species-specific blend (Allison and Cardé, 2016). This already indicates that environmental cues influence sexual communication; both the signal and response can be affected by both abiotic and biotic factors, such as photoperiod, temperature, the occurrence of closely related species that may interfere in the sexual communication when the sexual signals are very similar, and presence of predators and parasitoids that may home in on the sex pheromone to find their prey (Fig. 2). Since many female moths produce their sex pheromone *de novo* every night, females may adjust their pheromone blend depending on the current local environmental conditions.

The sex pheromone signal may vary in quantity as well as in quality. The quantity is mainly affected by host plant volatiles, nutrition and temperature, while the quality can be affected by immune defence responses and experience. For example, when *Heliothis subflexa* females emerged and remained in the odour of the closely related species *H. virescens* for three days, females contained significantly more of a sex pheromone component that not only attracts conspecific males but also inhibits the attraction of *H. virescens* males. Thus, early-adult experience of different chemical environments affects the sex pheromone

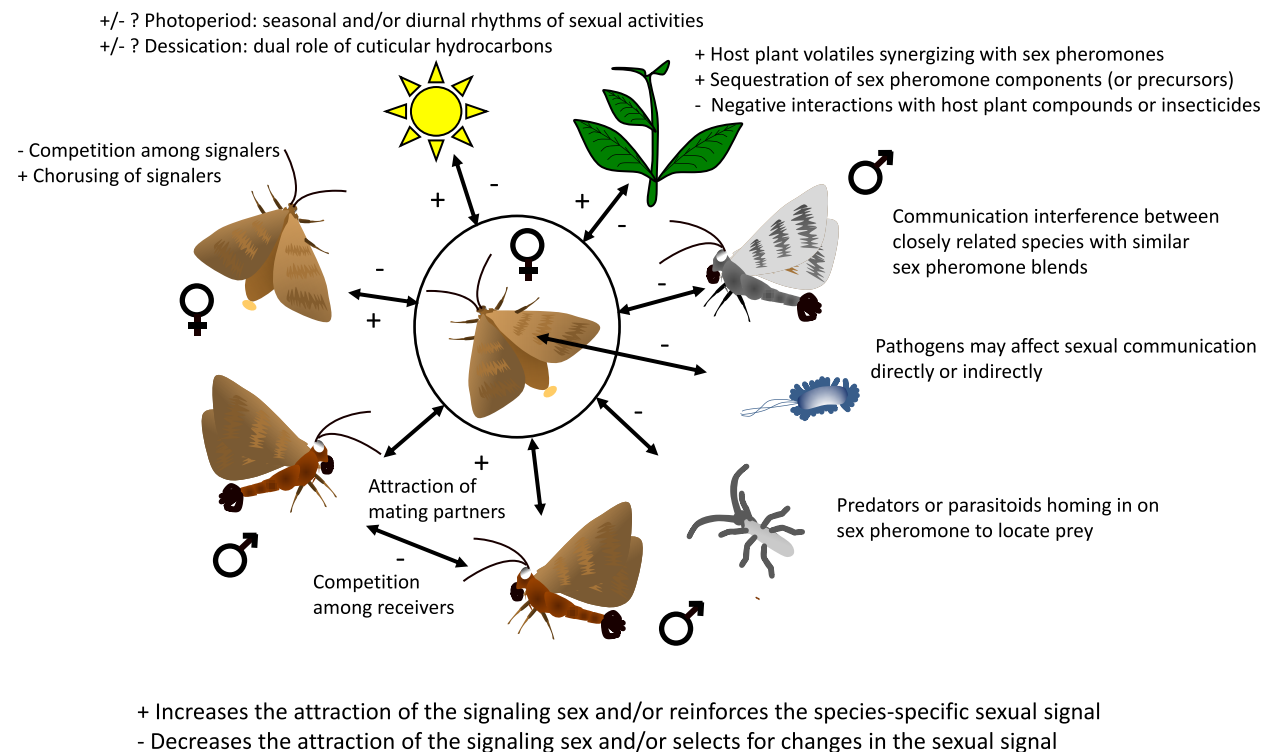


Fig. 2 Factors that may affect sex pheromone communication in moths.

composition in *H. subflexa* females. In aphids, the ratio between the two pheromone components nepalactone and nepelactol varies depending on the age of the females, increasing after maturation and then decreasing at later ages, which may be a symptom of senescence (Dewhurst *et al.*, 2010). In the cockroach *Nauphoeta cinerea*, the male sex pheromone varies depending on the carbohydrate intake, and a higher intake increases the male attractiveness, thus increasing their reproductive fitness. Changes in CHC composition have been found in the housefly *Musca domestica*, where the melting temperature of the surface lipids isolated from female houseflies decreased from 39°C to 35°C as the females attained sexual maturity and produced sex pheromone, whereas those prepared from males did not change with age.

The quality of the sex pheromone may also be affected by immune defence responses, as generally hypothesized by the Zahavi handicap principle (i.e., hypothesis proposed to explain how evolution may lead to “honest” or reliable signaling between animals which have an obvious motivation to bluff or deceive each other). The level of sexual attraction may signal the level and extent of health. However, if sexual attractiveness and immunity compete for the same resource pool, they may negatively affect each other. For example, the sex pheromone blend of *Heliothis virescens* females shifted towards an unattractive blend when females were infected with an entomopathogenic bacterium.

How are Sex Pheromones Perceived/Detected?

Mechanisms of Pheromone Detection in the Receiver

Volatile pheromones are detected by the olfactory organs of insects, the principal one being the antennae. These antennae harbour cuticular expansion called sensilla, many of which are dedicated to pheromone reception in the receiver sex (Fig. 3(A)). For instance, male silkmoth *Bombyx mori* antennae are covered by dizaines of thousands of pheromone sensitive sensilla. In addition, antennae may have a sophisticated shape, such as the pectinated antennae of the silkmoth that increase their surface to accommodate larger numbers of pheromone-sensitive sensilla. These sensilla usually house one or two olfactory receptor neurons (ORNs), which are bipolar cells that ensure the transformation of the chemical message into an electrical message that will be transmitted to the brain (Kaissling, 2014; Fig. 3(B)).

Pheromone-sensitive neurons usually respond to a single pheromone component, rarely two. This situation is clearly different from the responses of “generalist” ORNs that are usually activated by a family of related chemicals. The ORN dendrites extend in the sensillum lumen filled with the sensillum lymph, whereas their axons group together in antennal nerves that enter the antennal lobes (the primary processing center in the central nervous system) where they synapse with central neurons in glomerular structures. Axons of pheromone-sensitive neurons have a specific neural circuit as they project to a sexually dimorphic macroglomerular complex dedicated to pheromone signal integration. Pheromone information is relayed via antennal lobe

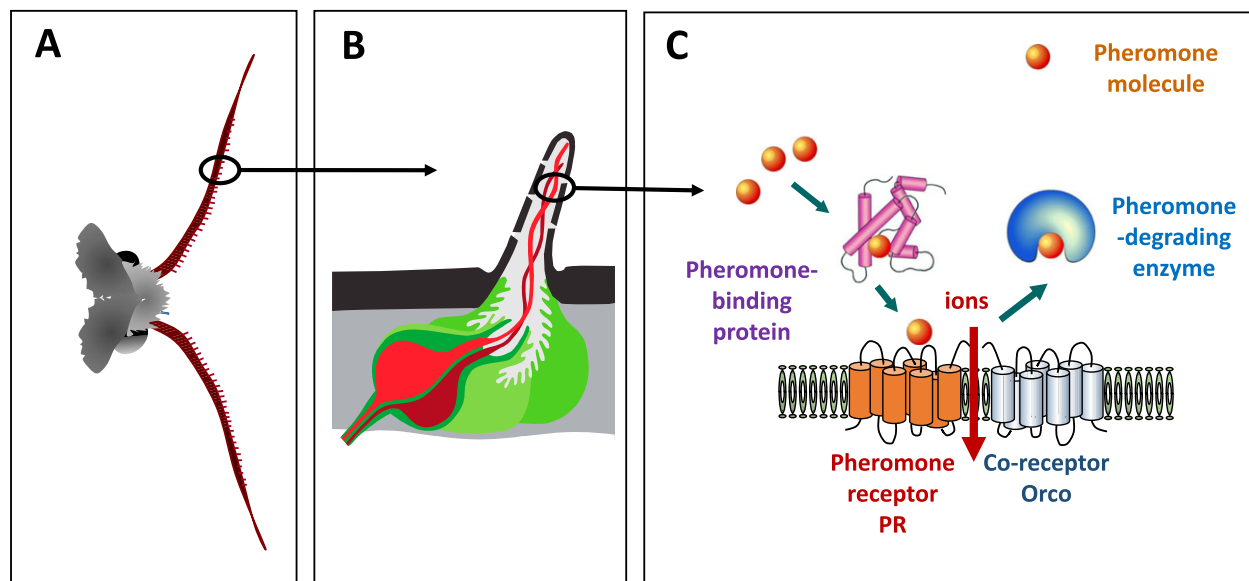


Fig. 3 Illustration of pheromone detection in moths. (A) Head of a moth with two antennae. Antennae carry cuticular expansions called sensilla, some of which are devoted to pheromone detection. (B) Pheromone sensitive sensilla usually house one to three olfactory receptor neurons (red) surrounded by accessory cells (green). The dendrite expands in the cuticular lymph while the axon goes to the central nervous system for signal integration. (C) Molecular mechanisms of pheromone reception. The pheromone molecule (orange) enters the sensillum via cuticular pores. It travels in the sensillum lymph bound by pheromone-binding proteins (PBPs) to the membrane bound pheromone receptor (PR). PR works together with a co-receptor (Orco) to transform the chemical signal into an electrical signal via ion channels opening. Modified from Kaissling, K.-E., 2004. Physiology of pheromone reception in insects (an example of moths). ANIR 6, 73–91.

projection neurons to both the mushroom bodies and the lateral protocerebrum. Understanding how the pheromone cues are converted *in fine* to the appropriate behavior is still a scientific challenge.

At the molecular level, the pheromone molecules enter the pheromone sensitive sensilla via cuticular pores. Most pheromones being hydrophobic, they are supposed to be solubilized by dedicated soluble proteins, the so-called pheromone-binding proteins, to cross the aqueous sensillum lymph and reach specific pheromone receptors (PRs) located in the dendritic membrane of the ORNs (Vogt, 2005; Fig. 3(C)). These PRs are part of the insect odorant receptor (OR) family, an unusual family of seven transmembrane domain ionotropic receptor proteins totally unrelated to mammal ORs. After pheromone interaction with its receptor, signal termination may be ensured by pheromone-degrading enzymes abundantly expressed in the sensillum lymph. A fast inactivation is indeed necessary for an efficient orientation behavior to the pheromone source. Although we still lack a consensus view on the exact function of each protein family, the occurrence of a diversity of members in each family accounts for their participation in the specificity of pheromone recognition. The combinatorial expression of these proteins within a sensillum may ensure the specificity, the sensitivity and the dynamic of the olfactory reception, defining the functional phenotypes of pheromone-sensitive neurons.

Most of the PRs functionally characterized to date – meaning their pheromone ligand is known – are from moths, and few others have been characterized in Diptera – mainly fruit fly–, Coleoptera – mainly Cerambycidae – and Hymenoptera. The general observation is that PRs tuned to the major pheromone components are usually very selective, like the ORNs that express them, while some others can recognize pheromone components from closely related species as well as behavioral antagonists (Montagné *et al.*, 2015; Zhang and Löfstedt, 2015).

Non-volatile close range pheromones are detected by contact chemodetection that involves taste structures such as the labellum or forelegs, for instance in the fruit fly. There, gustatory receptors (GRs) that are structurally related to ORs and/or two transmembrane domain Pickpocket (PKK) ion channels would ensure the signal recognition.

Who is the Receiver?

Because sex pheromone signaling is from one sex to the other, one would expect that only the opposite sex receives the emitted signal. Indeed, females of the silkmoth lack the specific receptor to the sex pheromone bombykol, they are thus anosmic to the pheromone they emit. However, females from other moth species express pheromone receptors as in males – although in a smaller amount – and autodetection by the signaler is frequently observed. The elicited behaviors are usually gender-specific: each sex exhibits a different behavior when exposed to the same cues, probably due to sexually dimorphic connectivity in higher-order neurons. In moths, while males would use the pheromone signal to detect an appropriate female, the female may detect its own pheromone to autoregulate its biosynthesis. Alternatively, detection of conspecific sex pheromone may allow evaluation of the local female density and thus the mating success probability. In the fruit fly, the male-specific sex pheromone cis-vaccenyl acetate (cVA) is detected by a pheromone receptor expressed in both male and female antennae. In males, cVA inhibits male–male courtship, while in the female it increases its sexual behavior.

Evolution of Pheromones and Their Role in Speciation

Insect sexual communication can be under different types of sexual and natural selection. Sexual selection may be exerted by the receiving sex when sex pheromones are used to choose among potential mating partners within a population (or species), or by the producing sex when mate competition occurs (Groot *et al.*, 2016). For example, in *Drosophila* flies and *Heliothis* moths, males perfume the females with their short-range sex pheromone, which inhibits the attraction of other, rival males. Natural selection can occur when other, predatory or parasitoid, species home in on the pheromone signal to locate their prey. A famous example is the bolas spider that can mimic the sex pheromone of different moth species. Natural selection can also occur when the pheromone has a dual function, which has been found to be the case in CHCs, as these also prevent insects from desiccation (Chung and Carroll, 2015). This has been illustrated in *Drosophila serrata* and *D. birchii* that co-occur in Australia and exhibit strong pre-mating isolation. *D. serrata* is a habitat generalist, with high amounts of methyl branched CHCs that prevent water loss and affect mating success, while *D. birchii* is a habitat specialist occurring in the humid rainforest, which is much less desiccation resistant, and produces only trace amounts of methyl branched CHCs that do not play a role in mating success.

When sex pheromones are subject to both sexual and natural selection, diversification of sex pheromones may be enhanced when both selection forces are in the same direction (as the example of CHCs). When sex pheromones are mainly used as species-recognition cues, i.e. to distinguish conspecific from closely related heterospecific individuals, and closely related species produce very similar sex pheromone blends that may only differ in the ratio of the different pheromone components, sex pheromones seem to be under stabilizing selection; For the signaler any deviation away from the mean lowers the probability of attracting the right (conspecific) mating partner. For the receiver, attraction to an off-blend can increase the chance of being attracted to another closely related species with a very similar sex pheromone. Counter-selection forces may occur when predators or parasitoids home in on the sex pheromone signals, which causes a trade-off between attraction to potential mating partners or to the enemy.

The use of sex pheromones in pest management may also cause selection towards alternatives sex pheromone blends (Witzgall *et al.*, 2008). For example, mating disruption is used in moth pest management: by saturating the air with synthetic sex pheromone lures, the males are unable to locate the females, which thus prevents matings. However, this may pose selection to both the

female signal and the male response to vary their sexual communication such that they will be able to find each other again. Such changes have for example been found in the summer fruit tortrix *Adoxophyes honmai*, where males did not respond to the common female pheromone blend anymore.

Pheromone Communication in a Changing World

Since pheromone signals and responses can be affected by many external biotic and abiotic factors (such as presence of plant volatile compounds, insect density, temperature, wind, humidity, pollution), global changes may also affect sex pheromone communication, either with direct effects on the insect physiology, or via modification of environmental factors that influence the pheromone communication. For example, as host plant volatile emissions have an impact on insect sex pheromone communication, one can expect temperature, CO₂ and ozone increases in the coming years to affect plant volatile emission and thus to impact the sex pheromone communication and by consequence the reproductive success of many insects.

Another interesting anthropomorphic impact is the effect of insecticides. Whereas the sex pheromone communication is generally altered when insects are exposed to insecticides, exposure to sublethal doses of some insecticides such as organochlorides, neonicotinoids or pyrethroids, has been shown to enhance reproduction abilities, a phenomenon called hormesis. Although neglected for long, insecticide-induced hormesis showing such a reversal of response between low and high doses of insecticides has been found in a number of species. The underlying mechanisms remain largely unknown, but the olfactory system appears to be affected and pheromone detection and associated behavior enhanced. For instance, chlordimeform treatment at low-dose induced a very high sensitivity to sex pheromone in the oriental fruit moth and the cabbage looper, and deltamethrin enhances the male response to pheromone in the cotton leafworm. Such phenomena may participate in pest insect resurgence.

References

- Allison, J.D., Cardé, R.T., 2016. *Pheromone Communication in Moths: evolution, Behavior, and Application*. Oakland, CA: University of California Press.
- Chung, H., Carroll, S.B., 2015. Wax, sex and the origin of species: Dual roles of insect cuticular hydrocarbons in adaptation and mating. *Bioessays* 37, 822–830.
- Dewhurst, S.Y., Pickett, J.A., Hardie, J., 2010. Aphid pheromones. *Vitamins And Hormones* 83, 551–574.
- Eisner, T., 2003. *For Love of Insects*. Cambridge, MA: The Belknap Press of Harvard University Press.
- Gomez-Diaz, C., Benton, R., 2013. The joy of sex pheromones. *EMBO Reports* 14, 874–883.
- Groot, A.T., Dekker, T., Heckel, D.G., 2016. The genetic basis of pheromone evolution in moths. *Annual Review of Entomology* 61, 99–117.
- Hanks, L.M., Millar, J.G., 2016. Sex and aggregation sex pheromones of cerambycid beetles: Basic science and practical applications. *Journal of Chemical Ecology* 42, 631–654.
- Howard, R.W., Blomquist, G.J., 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology* 50, 371–393.
- Jurenka, R., 2004. Insect pheromone biosynthesis. *Topics in Current Chemistry* 239, 97–132.
- Kaissling, K.-E., 2014. Pheromone reception in insects: the example of silk moths. In: Mucignat-Caretta, C. (Ed.), *Neurobiology of Chemical Communication*. Boca Raton, FL: CRC Press/Taylor & Francis, pp. 99–146.
- Montagné, N., de Fouchier, A., Newcomb, R.D., Jacquin-Joly, E., 2015. Advances in the identification and characterization of olfactory receptors in insects. *Progress in Molecular Biology and Translational Science* 130, 55–80.
- Vogt, R.G., 2005. Molecular basis of pheromone detection in insects. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), *Comprehensive Insect Physiology Biochemistry Pharmacology and Molecular Biology* 3. London: Elsevier, pp. 753–804.
- Witzgall, P., Stelinski, L., Gut, L., Thomson, D., 2008. Codling moth management and chemical ecology. *Annual Review of Entomology* 53, 503–522.
- Yew, J.Y., Chung, H., 2015. Insect pheromones: An overview of function, form, and discovery. *Progress in Lipid Research* 59, 88–105.
- Zhang, D.-D., Löfstedt, C., 2015. Moth pheromone receptors: Gene sequences, function and evolution. *Frontiers in Ecology and Evolution* 3, 105.

Relevant Website

<http://www.pherobase.net/>
Database of pheromones and semiochemicals.

Mammalian Pheromones

Peter A Brennan, University of Bristol, Bristol, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chemosignal A chemical signal.

Countermarking behavior Behavior of males to deposit a scent mark adjacent to, or on top of an existing scent mark.

Formylated Addition of the formyl functional group to a molecule.

Freezing response Behavior in which an animal is completely immobile so as not to attract attention from a possible predator.

Genome The genetic material of an organism.

Involatile Substances that do not vaporize in air.

Lipocalin Family of proteins that are soluble in water that bind and transport small hydrophobic molecules.

Lordosis Female sexual behavior that enables male mounting and intromission, often involving a characteristic arched back posture.

Major histocompatibility complex A family of cell surface proteins, in vertebrates, which are required for the immune system to recognize host cells and prevent them being rejected by the acquired immune system.

Odorant A single molecule that has an odor.

Primer pheromone A pheromone that causes a change in hormonal or developmental state.

Pseudogene A gene that has lost at least some functionality due to the accumulation of mutations.

Releaser pheromone A pheromone that induces an immediate behavioral response.

Signature mixture A mixture of chemosignals that provides information about an individual.

Sympathetic Part of the nervous system that is activated during arousal.

Unconditioned stimulus In classical conditioning this refers to a stimulus that is effective in initiating a response and when paired with another stimulus will cause that stimulus to become effective in eliciting the response.

Introduction

The term pheromones was first defined over 50 years ago by Karlson and Luscher as “substances secreted to the outside of an individual and received by a second individual of the same species in which they release a specific reaction, for example, a definite behavior or developmental process” (Karlson and Luscher, 1959). This original definition is not without its limitations, and there have been several attempts to refine the original definition, although none has received widespread acceptance. The result is that although the term pheromone is in widespread use, and everyone thinks that they know what it means, different users often imbue the term with subtly different meanings. For the concept to have much use it has to describe substances that elicit innate responses that do not require learning, to distinguish them from general odorants, which come to acquire meaning and elicit specific responses only following learning (Wyatt, 2010). It has also been argued that pheromones should refer to chemical signals that evolved to have a specific signaling role, rather than simply being metabolic by-products, excretions or secretions that are sensed by general sensory mechanisms. One thing that everyone can agree on is that pheromones refer to chemical signals conveyed between individuals of the same species, but this does not necessarily mean that those signals need to be exclusive to only one species. Perhaps the weakest part of the original definition is its reference to a specific reaction, as this is open to individual interpretation regarding what constitutes a definite behavioral or developmental response. Pheromonal responses are often more variable and context dependent in vertebrates, than invertebrates, and especially so in mammals. This has led to some debate about whether the term can be usefully applied to mammals and whether mammalian pheromones actually exist (Doty, 2003). Such skepticism has been very useful in highlighting problems with the original definition and weaknesses in the quality of much of the evidence for mammalian pheromones. However, recent application of molecular genetics techniques in mice, has led to the identification and the ability to experimentally manipulate both the pheromones and their sensory receptors, resulting in an ever-increasing list of examples of mammalian chemical signals that meet the original definition of a pheromone.

The Chemical Nature of Mammalian Pheromones

Pheromones have diverse chemical structures that reflect both their metabolic origin and the information that they convey (Fig. 1). For instance, sexual attractant pheromones and alarm pheromones need to act at a distance and therefore need to be small, volatile molecules that are carried by air (there are no known examples of mammalian pheromones being carried over distance by water). A good example of such pheromones are the boar sexual attractant pheromones 5 α -androst-16-en-3-ol and 5 α -androst-16-en-3-one.

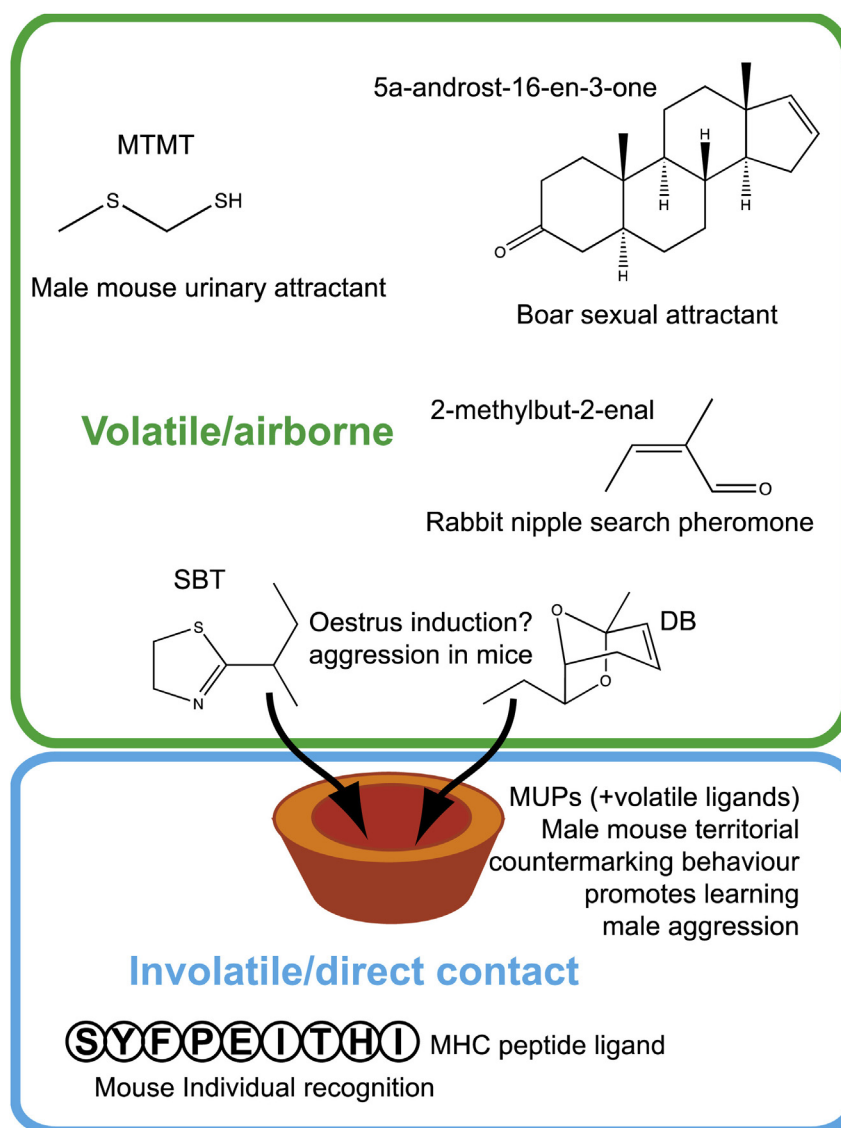


Fig. 1 Mammalian pheromones come in a wide variety of chemical structures related to their biological role of acting at a distance for volatile molecules or following direct physical contact for involatile peptides and proteins. Methylthiomethanethiol (MTMT) is found in male mouse urine and attracts females. 2-Methylbut-2-enal is found in rabbit milk and elicits nipple search behavior in neonates. 5 α -Androst-16-en-3-one is found in boar saliva and attracts and elicits mating behavior in sows. (S)-2-sec-butyl-4,5-dihydrothiazole (SBT) and (R, R)-3,4-dehydro-*exo*-brevicomin (DB) are found in male urine and induce estrus in female mice and aggression in other males. Major urinary proteins (MUPs) are found in male mouse urine and bind volatile ligands such as SBT and DB. They have numerous effects to convey individuality of males, elicit countermarking in males, elicit aggression in males, attract females and act as an unconditioned stimulus to induce learning in females. MHC peptides are found in mouse urine and convey information about individual identity.

These are derivatives of male androgen hormones with molecular masses around 272 g and are readily dispersed into the air from saliva. Another example is the mouse alarm pheromone 2-sec-butyl-4,5-dihydrothiazole (SBT), which has a molecular mass of 143 g. Both molecular mass and chemical structure affect the volatility of molecules, but in general molecules with molecular masses above about 400 g such as proteins and peptides would be expected to be relatively involatile.

Involatile substances can also function as pheromones and are ideally suited to situations where the pheromone has to be linked with a particular individual, such as the hamster vaginal protein aphrodisin, or place, such as mouse major urinary proteins (MUPs) that are used in territorial marking. Mouse MUPs are 19 kDa members of the lipocalin family of ligand binding proteins. As they are proteins, their structure is genetically determined and their relative stability across the lifespan of an individual make them ideal for conveying information about genetic identity (Sheehan *et al.*, 2016). Wild-derived mice produce around 5 of the 20 MUP variants and the profile of these variants is specific to the individual mouse that produced it. The MUP proteins themselves remain in the location in which they were deposited, which makes them ideal for a territorial marking function. Additionally, as they are also ligand binding proteins, MUPs act as a reservoir to slowly release volatile urinary ligands over a prolonged period. This not only

attracts investigation of other individuals towards the involatile MUPs, but the abundance of the volatiles being released also provides information regarding the freshness of the urine mark.

Sensory Systems for the Detection of Pheromones

The distinction between volatile and involatile pheromones has implications for the sensory systems that detect them (Fig. 2). The majority of mammalian species possess two main olfactory systems, the main olfactory system and the vomeronasal system. Species that do not possess a functional vomeronasal organ (VNO) include old-world primates including humans, fully aquatic mammals, such as cetaceans and manatees, and some species of bats. In the past, it was commonly believed that the main olfactory system detected general odorants and the vomeronasal system detected pheromones. However, this simple dichotomy has been revised in the light of more recent research and it is now accepted that in addition to its odor sensing role, the main olfactory system can also mediate certain pheromonal responses. Conversely, although many pheromonal responses are mediated via the vomeronasal system, it can also mediate non-pheromonal innate responses, such as avoidance and freezing responses to predator chemosignals. The distinguishing feature of the two systems is really in how the stimuli access the receptors. The main olfactory system is adapted to sense airborne stimuli, at a distance from the source, whereas the vomeronasal system is adapted to sense both volatile and involatile stimuli that are in the liquid phase, such as vaginal secretions, scent gland secretions or saliva, which require direct physical contact with the source. Moreover, the functions of the two systems are not as independent as originally thought. Substances acting as pheromones via the VNO are also able to stimulate sensory neurons in the main olfactory epithelium. There is also considerable convergence and integration of information from the two systems at central neural levels, such as the medial amygdala. Finally, sensory input via the main olfactory epithelium may be important to drive the direct investigation that is required for stimuli to gain access to the VNO.

The Main Olfactory System

There are a growing number examples of mammalian pheromonal responses that are mediated via the main olfactory system. For example, the sexual attractant effect of the boar mating pheromones androstenol and androstenone is not affected by blocking stimulus access to the VNO. This suggests that they are most likely to be mediated via the main olfactory system, which is also consistent with the airborne nature of the pheromones. Another example is the rabbit mammary pheromone 2-methyl-but-2-enal that elicits nipple search behavior in rabbit pups, which is vital in enabling them to suckle. Ablation of the main olfactory epithelium, but not the VNO, abolished both the nipple search behavior and general olfactory sensitivity. In mice, the ventrolateral region of the mouse MOB has even been termed the social fovea, as it predominantly responds to conspecific urinary chemosignals, such as the male urinary constituent (methylthio)methanethiol (MTMT) (Lin *et al.*, 2005). MTMT increases the attractiveness of male urine to female mice, but is inactive if presented in water. Thus, the attractant effect of MTMT depended on the context in which it was presented, which is often a feature of mammalian pheromones.

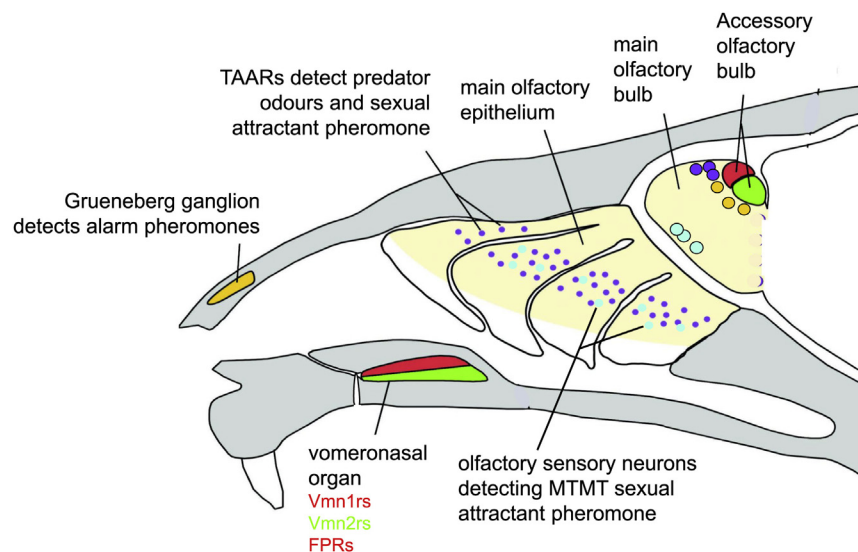


Fig. 2 Chemosensory systems for detecting pheromones in mice. Separate sensory organs, such as the Gruneberg ganglion and vomeronasal organ, detect pheromones in addition to the main olfactory system. Within the main olfactory system, separate subsystems detect pheromones and project to specific regions of the main olfactory bulb. Three main families of receptors detect pheromones in the vomeronasal organ and project to separate regions of the accessory olfactory bulb. FPRs, formyl peptide receptors; MTMT, (methylthio)methanethiol; TAARs, trace amine associated receptors.

General odors are identified by olfactory cortical regions that recognize the pattern of activity across olfactory sensory neurons that express a range of different olfactory receptor (OR) types. However, there are also olfactory subsystems within the main olfactory system, which project directly to neural systems that drive innate responses (Fig. 2). For example, neurons in the mouse MOB that receive input from OSNs expressing OR37 receptors, project directly to the medial amygdala and the paraventricular nucleus of the hypothalamus. OR37 stimuli, such as hexadecanal that is found in mouse feces, are therefore able to directly inhibit hypothalamic neurons regulating the endocrine stress response (Klein *et al.*, 2015).

A further olfactory subsystem has been identified as the subpopulation of OSNs that express members of the trace amine associated receptor (TAAR) family, which are evolutionarily unrelated to the classical family of olfactory receptors. Although some TAARs respond to non-pheromonal stimuli, such as the predator chemosignal phenylethylamine, TAAR5 has been shown to respond to trimethylamine, which is a male mouse urinary pheromone that is attractive to females (Ferrero *et al.*, 2013).

The Vomeronasal System

The VNO is both anatomically and functionally distinct from the main olfactory epithelium. It is a blind-ended tubular structure located in the nasal septum and connected to nasal cavity, and in some species also the oral cavity, by narrow ducts. The narrow lumen of the ducts means that stimulus access is limited to substances carried in mucus, which is pumped into the VNO by an active pumping mechanism. This enables the VNO to sense involatile substances that would not be conveyed in the airstream such as peptides and proteins. But the VNO is also capable of responding to smaller volatile substances that are soluble in mucus, such as SBT, which is transported in mouse urine bound to MUPs. Stimuli can be picked up from sources such as urine marks, scent marks, vaginal secretions or tear secretions, by direct nasal contact and licking of the nose, or directly from the oral cavity in species that possess a nasopalatine duct. There are two main mechanisms by which stimuli can be pumped into the VNO. In rodents, sympathetic nerve activity, in response to arousal and novelty, controls blood flow to a large blood vessel that runs along the medial side of the lumen. As the VNO is enclosed in a cartilaginous capsule, changes in blood flow in this vessel change the volume of the VNO lumen, pumping mucus carrying the vomeronasal stimuli into the organ. Carnivores and ungulates have an alternative mechanism linked to a characteristic behavior known as flehmen (Fig. 3). This behavior involved a baring of the teeth and a curling of the upper lip, which result in pressure changes that are thought to pump the stimulus-containing mucus into the VNO.

Like the main olfactory system, the vomeronasal system detects stimuli by means of G-protein coupled receptors on the surface of vomeronasal sensory neurons (VSNs). Three main classes of vomeronasal receptors have been identified, each of which only



Fig. 3 Flehmen behavior in a stallion. Note the curled upper lip and the barring of the teeth that sucks involatile stimuli into the vomeronasal organ following direct contact with the stimulus source.

distantly related to each other and to the main olfactory and TAAR receptor families. The Vmn1r class and the formyl peptide receptors (FPRs) are expressed by VSNs that are located apically in the vomeronasal epithelium. These are spatially segregated from the Vmn2r class that are both located in the basal region of the epithelium.

Vmn1rs Respond to Small Volatile Molecules

The analysis of a wide range of mammalian genomes has revealed dramatic variations in number of Vmn1rs (Young *et al.*, 2010). Around 240 intact Vmn1r genes have been identified in the mouse genome. Other species with a large number of functional Vmn1rs include the platypus, mouse lemur and rabbit, each with over 160 receptors. Other lagomorphs and rodents have around 60–110 functional Vmn1r genes, but most domestic species and companion animal species have far fewer: 40 in cows; 36 in horses; 28 in cats; and 9 in dogs. Numbers of functional Vmn1rs are low in old world monkeys and apes: 3 in baboons, gorillas and humans; 4 in chimpanzees; 5 in orangutans. Certain species appear to have no functional Vmn1rs at all including, little brown bats, macaques and cetaceans such as dolphins. In mice, Vmn1rs detect small volatile molecules, which have been shown to have pheromonal activity in mice, such as SBT, 3,4-dehydro-exo-brevicomin (DB), farnesenes and 2-heptanone (Leinders-Zufall *et al.*, 2000). These responses were found to be highly selective and also highly sensitive, with cells responding at concentrations as low as 10^{-11} M. This high level of sensitivity is a common feature of pheromone detecting systems that need to detect small amounts of signal that have been released into the environment. In addition to detecting these urinary volatiles, Vmn1rs have also been found to respond to sulphated steroids. Imaging of VSN responses in slices of VNO epithelium have revealed groups of receptors that specifically respond to derivatives of oestrogens, progestogens, androgens and corticosterone, suggesting the potential for detection of hormonal state of the producer (Isogai *et al.*, 2011), although the biological significance of this is currently unknown.

Vmn2rs Respond to Peptides and Proteins

Genomic analysis has identified around 120 intact Vmn2r genes in the mouse genome (Young and Trask, 2007). As is the case for Vmn1rs the Vmn2r repertoire varies dramatically across species, with rats and opossums possessing around 80–90 functional Vmn2rs. In contrast, several other mammalian species including cows, dogs, macaques, chimpanzees and humans appear to have no functional Vmn2rs, with only non-functional pseudogenes remaining in the genome (Young and Trask, 2007). In mice, Vmn2rs have been found to respond to involatile peptides and proteins, such as exocrine secretory protein 1 (ESP1). ESP1 is a 7 kDa peptide, that is produced by the tear glands of male mice. It selectively activates VSNs expressing the Vmn2r116 (V2Rp5) receptor (Haga *et al.*, 2010). Moreover, female mice, in which this receptor has been genetically deleted, show a reduced rate of female sexual behavior, known as lordosis, confirming the role of ESP1 as a sexually-dimorphic sex pheromone in mice. Other peptides that can be sensed by mice Vmn2rs include major histocompatibility complex (MHC) peptide ligands. These 9-amino acid peptides play a role in self/non-self recognition by the immune system, suggesting that they can also convey information about the genetic identity of the producer (Leinders-Zufall *et al.*, 2004). Vmn2rs are even more sensitive than Vmn1rs, with VSNs expressing Vmn2r26 (V2R1b) responding MHC peptides at a concentration of 10^{-14} M. This makes them one of the most sensitive chemical detection systems known.

Other Vmn2r stimuli include MUPs, which are members of the lipocalin family of ligand binding proteins. MUPs bind small volatile ligands, several of which are known pheromones that stimulate Vmn1rs. However, even when these volatiles have been displaced, MUPs still elicit aggression from male mice and lactating female mice when painted onto a normally unthreatening castrated male. This suggests that the MUPs can function as involatile pheromonal stimuli in their own right, via activation of Vmn2rs, in addition to carrying volatile pheromonal stimuli that act at Vmn1rs.

FPRs Respond to Infection-Related Peptides

The mouse genome contains seven functional FPRs of which five are expressed in the VNO. FPRs expressed in VSNs appear to respond to formylated bacterial peptides, such as lipopolysaccharide (LPS). They potentially have a similar role to FPRs in the immune system in detecting bacterial contamination and inflammatory responses. Evidence for this suggestion has come from mice that have been injected with LPS, or infected with natural pathogens. Other mice avoid both the diseased mice and their urine, and this avoidance behavior was not observed in mice with deficient vomeronasal function, strongly suggesting this pheromonal effect is mediated by vomeronasal FPRs (Boillat *et al.*, 2015).

Releaser Pheromonal Effects on Behavior

Releaser pheromones have immediate effects on the behavior of the receiver (Table 1). For example, the boar sexual attractant pheromones 5 α -androst-16-en-3-ol and 5 α -androst-16-en-3-one are accumulated in the boar's saliva by association with pheromone binding proteins. In the presence of a receptive sow, the boar salivates copiously, releasing the pheromones into the air. They cause the female to be attracted to the male and elicit female sexual behavior, known as standing, that enables the male to mate. A similar behavioral effect is elicited by the sex pheromone ESP1 in mice. The detection of this peptide in tear secretions, during investigation of males, more than doubles the rate of lordosis behavior of females (Haga *et al.*, 2010). Interestingly, males of the C57BL/6 inbred mouse strain naturally lack ESP1 production. This demonstrates that pheromonal deficiencies in certain

Table 1 Examples of mammalian pheromones, their nature, detection mechanism and effects

Pheromone	Nature	Species	Producer	Source	Receiver	Sensory system	Effects
2-Methylbut-2-enal	Volatile	Rabbit	Lactating female	Skin Milk	Neonates	MOS	Arousal Nipple search Learning
5 α -androst-16-en-3-one	Volatile	Pig	Adult male	Saliva	Female	MOS	Attraction Lordosis
(Methylthio)mentanethiol	Volatile	Mouse	Adult male	Urine	Female	MOS	Attraction
SBT+DB	Volatile	Mouse	Adult male	Urine	Female Male	VNS (Vmn1r)	Estrus induction Aggression
Sulphated steroids	Involatile	Mouse	Male and female	Urine	Male and female	VNS (Vmn1r)	Information on endocrine state
Formyl peptides	Involatile	Mouse	Male and female	Urine	Male and female	VNS (Vmn1r)	Information on infection state
ESP1	Involatile	Mouse	Adult male	Tears	Female	VNS (Vmn2r)	Lordosis
ESP22	Involatile	Mouse	Juvenile	Tears	Male	VNS (Vmn2r)	Inhibits sex behavior
MUP20 (darcin)	Involatile	Mouse	Adult male	Urine	Female Male	VNS (Vmn2r)	Attraction Learning Countermarking Aggression
Other MUPs	Involatile	Mouse	Adult male	Urine	Male and Female	VNS (Vmn2r)	Individual recognition
MHC peptide ligands	Involatile	Mouse	Adult male	Urine	Female	VNS (Vmn2r)	Individual recognition
Aphrodisin	Involatile	Hamster	Adult female	Vaginal secretions	Male	VNS (Vmn2r?)	Stimulates sex behavior

Abbreviations: DB, (R, R)-3,4-dehydro-exo-brevicomine; ESP, exocrine secretory peptide; MOS, main olfactory system; MHC, major histocompatibility complex; MUP, major urinary protein; SBT, (S)-2-sec-butyl-4,5-dihydrothiazole; VNS, vomeronasal system.

strains of mice, due to inbreeding, may not only affect their reproductive success, but also their usefulness in studies of natural reproductive behavior.

Another member of the ESP family of peptides, ESP22 is also produced by the tear glands but this time of juvenile mice of both sexes. Notably juveniles of the CBA and CH3 inbred strains lack ESP22 production and are subject to a high rate of inappropriate sexual behavior from adult males. However, if ESP22 is painted onto these juveniles then it reduces the level of male sexual behavior towards them to a low level typical of the other inbred strains that naturally produce the peptide (Ferreiro *et al.*, 2013). Therefore, ESP22 is acting as a releaser pheromone, via the vomeronasal system, to inhibit male sexual behavior. Such inhibitory releaser effects of pheromones can only be observed in the contexts of other behaviors and it is therefore not surprising that they have only recently been identified.

Yet another class of releaser sex pheromone is produced by female hamsters in their vaginal secretions. This pheromone is aphrodisin, a 17 kDa member of the lipocalin family of ligand binding proteins. When the protein containing fraction of vaginal fluid was painted onto an anaesthetized male hamster, it elicited mounting behavior in other males (Briand *et al.*, 2004). This effect is mediated by the vomeronasal system, but the vomeronasal system is only necessary to stimulate sexual behavior in sexually naïve males. Subsequent male sexual behavior can be elicited by female odor cues, sensed by the main olfactory system, which were learned during the first sexual experience. As these odor cues are eliciting a learned response, they would not be regarded as pheromones even though they elicit the same behavioral response as aphrodisin. Presumably this is an evolutionary adaptation to reinforce the appropriate behavioral response being elicited in a certain context.

Indeed, pheromones themselves can act as unconditioned stimuli to induce the learning of other chemosignals. One example of this is the rabbit mammary pheromone 2-methylbut-2-enal, which is produced by the skin around a lactating doe's nipples and is present in their milk. This pheromone is vital to the reproductive success of rabbits, as it elicits the stereotyped nipple search behavioral response in the neonates, enabling them to locate the nipples and suckle. However, when neonates were exposed to 2-methylbut-2-enal paired with an artificial odor, without suckling, the artificial odor alone was subsequently effective in eliciting nipple search behavior (Coureaud *et al.*, 2006). Another example of learning is the atypical male mouse MUP20, also known as darcin. This has a releaser pheromonal effect to attract female mice to urine marks. But it also acts as an unconditioned stimulus to induce the learning of the volatile odor signatures associated with the urine mark, as well as the context in which the urine mark occurs. These examples show that pheromonal stimuli can have dual roles of releaser pheromone and unconditioned stimulus and can be mediated by both the main olfactory and vomeronasal systems (Fig. 4). But when MUP 20 is sensed in the context of another individual, such as being painted on a castrated male, it has a different releaser pheromonal effect to elicit male aggression. This demonstrates the potential for pheromones to have multiple functions and that it is more useful to specify the pheromonal effects of a chemosignal rather than just identifying it as a pheromone. A further complexity is revealed by the ability of female mice to respond to MUP20 being dependent on their hormonal state. Increased progesterone during the non-receptive phase of their estrus cycle selectively silences activity of the VSNs that respond to MUP 20 without affecting other VSNs that respond to

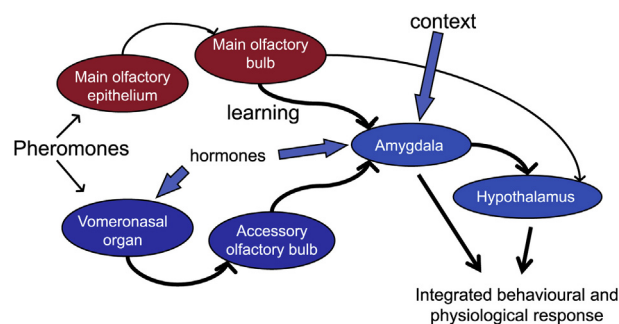


Fig. 4 Pheromones can be sensed by both the main olfactory system and the vomeronasal system, which converge in central brain areas such as the amygdala. Coordinated behavioral and physiological responses are driven by brain areas such as the hypothalamus. Identifying mammalian pheromones is complicated by learning, by which non-pheromonal odors sensed by the main olfactory system can drive pheromonal-like responses. A further complication is the context dependence of pheromonal responses, which may be influenced by other sensory cues as well as hormonal state.

predator chemosignals (Dey *et al.*, 2015). Once again this shows the context dependence of mammalian pheromonal effects that can lead to variability in the responses observed.

Primer Pheromonal Effects on Endocrine Responses

Primer pheromonal effects are longer-term effects on endocrine or developmental state (Table 1). For example, the Vandenberg effect is the acceleration of puberty in pre-pubertal female mice that are exposed to urine from adult males. This effect is mediated by stimulation of gonadotropin hormone releasing hormone neurons in the hypothalamus. It therefore has a similar neuroendocrine basis to the Whitten effect, which is the effect of adult male urine to induce and synchronize estrus in female mice. Conversely, exposure to female urine has the opposite effect of delaying puberty and lengthening estrus. Given their similar nature, the Vandenberg and Whitten effects have been proposed to be mediated by the same urinary pheromones. Previous reports had implicated androgen dependent urinary volatiles such as SBT, DB, 6-hydroxy-6-methyl-3-heptanone, α - and β -farnesene (Novotny *et al.*, 1999), which are bound by MUPs, and stimulate Vmn1rs (Leinders-Zufall *et al.*, 2000). But the effectiveness of these urinary volatiles has been disputed and the identity of these mouse primer pheromones underlying these pheromonal effects is unclear. A similar primer pheromonal effect, known as “male effect”, also occurs in sheep and goats. In these species, exposure of females to androgen-dependent pheromonal stimuli associated with head hair of males is sufficient to induce estrus in seasonally-anestrus females. Detailed analysis of the volatile compounds extracted from goat hair led to the identification of a single molecule, 4-ethyloctanal, which induces activity in the GnRH pulse generator (Murata *et al.*, 2014). However, 4-ethyloctanal has not yet been shown to be effective in inducing estrus in goats and it is likely that other components also contribute to the response.

Signature Mixtures Convey Information About Individuals

The pregnancy block effect, or Bruce effect, as it is known, is another primer pheromonal effect in mice. Exposure of a recently mated female mouse to male urine, during the first few days following mating, results in a reduction in progesterone levels, leading to implantation failure and return to estrus. However, pregnancy block does not occur in response to urine from the male that has mated with the female. This is because the female learns the chemosensory signature of their mate during a sensitive period following mating, which subsequently inhibits activation of the neuroendocrine pathway resulting in pregnancy block. The individuality of the mate’s chemosensory signals appears to be conveyed by MHC peptide ligands, as adding MHC peptide ligands typical of an unfamiliar strain to the mating male’s urine causes it to block the pregnancy of his mate (Leinders-Zufall *et al.*, 2004). These individuality signals would not classically be classed as pheromones, as their effects depend on learning. Instead they have been referred to as either signaler pheromones, or more recently as signature mixtures that provide information about individuals without directly eliciting a pheromonal effect (Wyatt, 2010).

Human Pheromones

As yet no pheromonal stimuli have been convincingly identified in humans in the same way that they have in other mammalian species. One reason for this might be the difficulty in looking for a definite behavioral response when much of human behavior is influenced by learning and societal expectations, which are difficult to control experimentally. But humans are a smelly species and become smellier after puberty. Therefore, it is likely that pheromones have existed in the recent evolutionary past. But whether they are still functional in modern society, or whether they have become non-functional, as have some pheromonal signals in different inbred mouse strains, remains to be determined. Past studies tend to have focused on effects on sexual preferences and indirect measures of sexual behavior. However, pheromonal effects may occur during more intimate sexual investigation, which is

particularly difficult to study in humans. More progress is likely to be made by in identifying possible pheromones involved in suckling behavior and possibly maternal bonding where contextual factors are more constant (Wyatt, 2015).

Conclusions

The recent application of molecular genetics techniques has revolutionized our understanding of the role of pheromones in mammalian behavior. And yet there are still many more receptors that have been identified than the pheromones that stimulate them and the effects that they mediate. Most of the recent advances in our understanding of mammalian pheromonal communication is based on studies of mice. But by their very nature, pheromonal signals have evolved species-specific roles, and a major challenge is to translate our understanding of pheromones in mice to the study of other species, in which genetic manipulation is not currently possible. What is becoming increasingly clear is the important role played by pheromonal signals in the reproductive biology of most mammals.

References

- Boillat, M., Challet, L., Rossier, D., et al. (2015). The vomeronasal system mediates sick conspecific avoidance. *Current Biology*, 25, 251–255.
- Briand, L., Trotier, D., & Pernollet, J.-C. (2004). Aphrodisin, an aphrodisiac lipocalin secreted in hamster vaginal secretions. *Peptides*, 25, 1545–1552.
- Coureaud, G., Moncomble, A. S., Montigny, D., et al. (2006). A pheromone that rapidly promotes learning in the newborn. *Current Biology*, 16, 1956–1961.
- Dey, S., Chamero, P., Pru, J. K., et al. (2015). Cyclic regulation of sensory perception by a female hormone alters behavior. *Cell*, 161, 1334–1344.
- Doty, R. L. (2003). Mammalian pheromones: Fact or fantasy? In R. L. Doty (Ed.), *Handbook of Olfaction and Gustation* (pp. 345–383). New York, NY: Marcel Dekker Inc.
- Ferrero, D. M., Moeller, L. M., Osakada, T., et al. (2013). A juvenile mouse pheromone inhibits sexual behaviour through the vomeronasal system. *Nature*, 502, 368–371.
- Haga, S., Hattori, T., Sato, T., et al. (2010). The male mouse pheromone ESP1 enhances female sexual receptive behaviour through a specific vomeronasal receptor. *Nature*, 466, 118–122.
- Isogai, Y., Si, S., Pont-Lezica, L., et al. (2011). Molecular organization of vomeronasal chemoreception. *Nature*, 478, 241–245.
- Karlson, P., & Lüscher, M. (1959). Pheromones: A new term for a class of biologically active substances. *Nature*, 183, 55–56.
- Klein, B., Bautze, V., Maier, A. M., et al. (2015). Activation of the mouse odorant receptor 37 subsystem coincides with a reduction of novel environment-induced activity within the paraventricular nucleus of the hypothalamus. *European Journal of Neuroscience*, 41, 793–801.
- Leinders-Zufall, T., Brennan, P., Widmayer, P., et al. (2004). MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science*, 306, 1033–1037.
- Leinders-Zufall, T., Lane, A. P., Puche, A. C., et al. (2000). Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature*, 405, 792–796.
- Lin, D. Y., Zhang, S.-Z., Block, E., & Katz, L. C. (2005). Encoding social signals in the mouse main olfactory bulb. *Nature*, 434, 470–477.
- Murata, K., Tamogami, S., Itou, M., et al. (2014). Identification of an olfactory signal molecule that activates the central regulator of reproduction in goats. *Current Biology*, 24, 681–686.
- Novotny, M. V., Weidong, M., Wiesler, D., & Zidek, L. (1999). Positive identification of the puberty-accelerating pheromone of the house mouse: The volatile ligands associating with the major urinary protein. *Proceedings of the Royal Society London B*, 266, 2017–2022.
- Sheehan, M. J., Lee, V., Corbett-Detig, R., et al. (2016). Selection on coding and regulatory variation maintains individuality in major urinary protein scent marks in wild mice. *PLOS Genetics*, 12, e1005891.
- Wyatt, T. D. (2010). Pheromones and signature mixtures: Defining species-wide signals and variable cues for identity in both invertebrates and vertebrates. *Journal of Comparative Physiology A*, 196, 685–700.
- Wyatt, T. D. (2015). The search for human pheromones: The lost decades and the necessity of returning to first principles. *Proceedings of the Royal Society B*, 282, 20142994.
- Young, J. M., Massa, H. F., Hsu, L., & Trask, B. J. (2010). Extreme variability among mammalian V1R gene families. *Genome Research*, 20, 10–18.
- Young, J. M., & Trask, B. J. (2007). V2R gene families degenerated in primates, dog and cow, but expanded in opossum. *Trends in Genetics*, 23, 212–215.

Olfaction and Reproduction: Olfactory Imprinting and Homing in Salmon

Chase Williams, University of Washington, Seattle, WA, United States

Andrew Dittman, Northwest Fisheries Science Center, NOAA Fisheries, Seattle, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

For many fish species, non-visual sensory systems play a critical role in survival and reproduction. In the aquatic environment, visual cues can be easily perturbed due to low light levels and increased turbidity, making recognition of distant objects nearly impossible. However, non-visual cues, such as auditory and odorant cues can travel long distances, and convey important information about a fish's surroundings independent of vision; making these sensory systems important in the aquatic environment. The olfactory system, in particular, plays a central role in many aspects of a fishes life cycle. Odorants in the water column can inform a fish of nearby food, predators, reproductive status of potential mates, conspecifics (same species), as well as favorable habitats for spawning and rearing (Hara, 1992).

For salmon, in particular, olfaction plays a critical role in all aspects of its life cycle. The life cycle of salmon plays out over large temporal and geographical scales, and in many different and dynamic environments. All salmon spawn in fresh water, and most salmon species have an *anadromous* life history, meaning they spend a portion of their life cycle in saltwater environments. The anadromous life history that salmon utilize affords them an enormous amount of rapid growth potential as well as the ability to support a disproportionately large population size produced from a relatively small freshwater systems due to the incredible abundance of food and space in the ocean environment. The anadromous life history does come at an expense however by increasing the risk of mortality as well as a greater demand on the ability to navigate over long distances, and achieve reproductive success. The latter is especially important as many salmon have a *semelparous* reproductive cycle, meaning that once they spawn they die soon after. The salmon olfactory system not only makes the anadromous life cycle possible, but also greatly increases the odds of survival and reproductive success.

A Brief Outline of the Salmon Life Cycle

Salmon start out life as an embryo residing in the interstitial spaces of a gravel mound known as a *redd*. A redd is the nest that the female salmon builds while depositing her eggs during mating. The embryos often incubate within the redd for up to several months before the tiny salmon hatch. At hatching, the juvenile salmon is known as an *alevin*, which survive off a large external yolk sac of nutrients. After their yolk sacs are depleted, the alevin migrate up and out of the gravel and emerge as *fry*. Fry must now feed on their own, and depending on the species, either migrate to the ocean immediately or stay and rear in the freshwater environment (Fig. 1).

Depending on the species, after rearing in the freshwater environment (for days to years), the juvenile salmon migrate to the ocean. During this migration downstream and upon entering estuaries, juvenile salmon undergo many physiological changes to

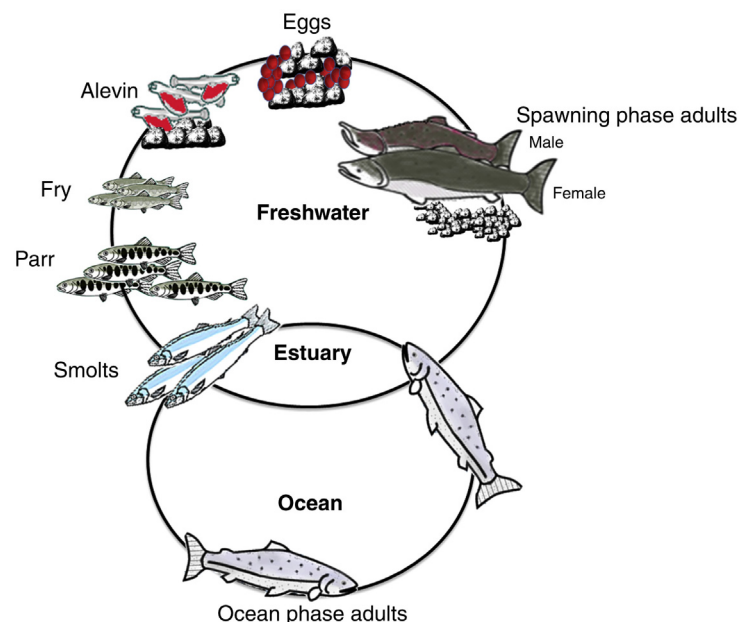


Fig. 1 Salmon lifecycle.

prepare them for survival in the marine environment. Their coloration changes from dark green to silver, their shape and behaviors change and many aspects of their physiology change including energy storage and osmoregulatory processes. During this transitional period between the fresh and saltwater environments, the juvenile salmon are known as *smolts*. Smolts will generally spend between days and months in the estuarine environment before making the final transition to the open ocean migration. Ocean phase salmon spend between 1 and 5 years at sea, depending on the species, before reaching sexual maturity and migrating back to freshwater to spawn in the natal stream they came from.

The Salmon Olfactory System

Morphology of the Olfactory Sensory System

The salmon olfactory system is a tightly coordinated and complex signaling system made up of several distinct neural centers (Fig. 2). The peripheral component of the olfactory system in salmon is composed of a pair of rosette shaped structures. Each rosette is located in a small pit on either side of the salmon's head. The olfactory pits have two openings that aid in directing water into and out of the pit to increase the water-sampling rate as the salmon swims through the water. The rosettes are made up of lamellae each covered in both non-sensory and sensory olfactory epithelia. The olfactory sensory epithelia is arranged in a pseudostratified layer comprised of several major olfactory cell types of both sensory and non-sensory function (Fig. 2). The cell types include sustentacular cells (SUS cells), which provide both structural support and a glial like role in supporting the mature olfactory sensory neurons (OSNs); goblet cells, that produce a protective mucosal layer; crypt cells, which are thought to detect odorant molecules; mature ciliated and microvillar OSNs, which detect odorant molecules; immature OSNs, their role is to replace the injured or senescing mature OSNs; and two classes of multipotent olfactory stem cell (OSC) populations, the globose basal cells (GBCs) and horizontal basal cells (HBCs), which can potentially generate and replace all of the cells that make up the olfactory sensory epithelium during natural cellular turnover, or following an injury (Døving, 2007). All of these cell populations work in coordination to form a functional peripheral olfactory system. As previously mentioned, it is the role of the ciliated and microvillar OSNs and crypt cells to detect odorants and send that signal to the central olfactory system in the brain. Initially, when an odorant binds to an odorant receptor (a G-protein coupled receptor, GPCR), located on the surface of cilia or microvilli of an OSN. This odorant binding initiates a cascade of signaling molecules, which ultimately open calcium and chloride ion channels that send a signal down the olfactory axonal bundle to the olfactory bulbs where the signal is decoded and a behavioral response is decided (DeMaria and Ngai, 2010; Whitlock, 2006).

Types of Odorant Receptors

The salmon olfactory system, and all vertebrates, are thought to employ the "one receptor-one neuron" rule, which means that each OSN only expresses one type of odorant receptor (Lewcock and Reed, 2004; Sato *et al.*, 2007; Serizawa *et al.*, 2003). Furthermore, the

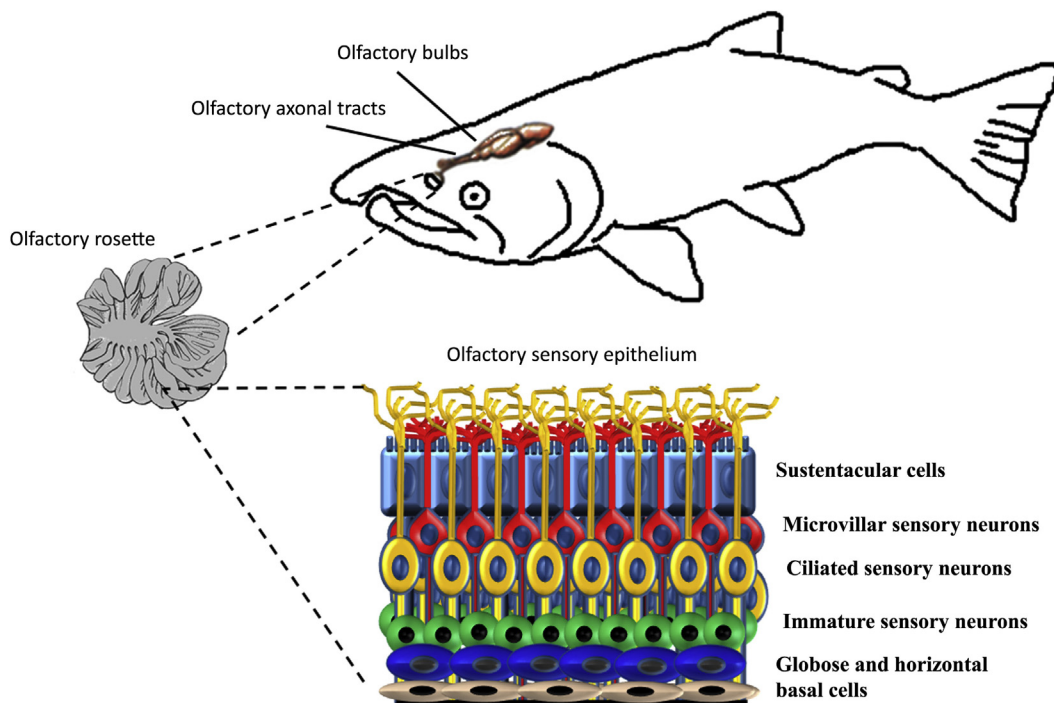


Fig. 2 Morphology of olfactory system.

salmon olfactory system utilizes between 100 and 200 unique putative odorant receptors (Johnstone *et al.*, 2012). The odorant receptors are divided into four main classes: the main olfactory receptors (mORs) and the trace-amine associated receptors (TAARs), which are located in ciliated OSNs and the vomeronasal type 2 receptor-like (Olfc) and vomeronasal type 1 receptor-like (ora), located in the microvillar OSNs. It is important to note that while salmon have odorant receptors that are characterized as vomeronasal receptors, they do not in fact have a distinct and separate vomeronasal sensory region like many other vertebrates. In fish, OSNs of all classes are arranged in a mosaic with no clear boundaries based on functional class between them. Functional characterization, i.e. odorant specificity, of the currently identified putative salmon odorant receptors within each of the major classes has not been completed; however, research in zebrafish and other vertebrate species has provided some insight into their possible specificities (Behrens *et al.*, 2014; Jiang *et al.*, 2015). For example, it is widely accepted that vomeronasal odorant receptors, associated with microvillous OSNs, play a large role in the detection of pheromone odorants and amino acids. Odorant receptors associated with ciliated OSNs likely also detect amino acids and other odorant cues not related to pheromones such as nucleotides released from food sources (Hansen *et al.*, 2003; Sato and Suzuki, 2001). In addition to the different classes of odorant receptors detecting different types of odorants, each major OSN class, i.e. ciliated and microvillous OSNs, further utilize distinct signaling machinery to relay specific odorant signals to the brain. The ciliated OSNs utilize a cAMP/adenylyl-cyclase III (ACIII) mediated pathway, whereas microvillar OSNs utilize an inositol triphosphate (IP3)/Phospholipase-C (PLC) mediated pathway. Each pathway utilizes calcium, chloride and sodium channels for signaling to propagate an odorant-induced signal to the olfactory bulb.

The salmon olfactory system may have up to 200 putative odorant receptors and is tasked with detecting many unique water-borne odorants (Johnstone *et al.*, 2012). To identify specific odorants, the brain uses a combinatorial signal integration method for odorant identification, wherein an single odorant may activate several different odorant receptors at the same time (Malnic *et al.*, 1999). Having one odorant activate a set of unique odorant receptors provides each odorant a unique identifier, a “fingerprint” that the brain can identify. This combinatorial activation method can potentially produce billions of unique odorant “fingerprints”, thus allowing the olfactory system to detect far more odorants than it has unique odorant receptors. The correct identification of odorants is important in allowing salmon to complete their life cycle, such as when adult salmon home back to their natal stream using olfactory cues associated with their home stream. Salmon learn these odor mixtures as juveniles prior to their seaward migrations through a process known as *imprinting*.

Salmon Life History and the Role of Olfaction

Downstream Migration and Imprinting

From the moment salmon emerge from the gravel, olfaction plays a critical role in their survival. Juvenile salmon use their sense of smell to locate food, detect predators and nearby kin. One critical function of the juvenile salmon olfactory system is imprinting on the odorant signature of their natal waters. It has been known for some time that adult salmon have an amazing ability to find their way back to their natal waters after being away for years. However, it was not until experiments by Arthur Hasler and his colleagues (Cooper *et al.*, 1976; Hasler and Scholz, 1983; Scholz *et al.*, 1976) that the link between the chemical make up of their rearing waters and their sense of smell were definitively tied to their successful homing abilities. In a classic series of experiments, they exposed groups of salmon from a hatchery to the natural water source at the hatchery or hatchery water plus artificial odorants, the chemical phenethyl alcohol (PEA) or morpholine (compounds that do not naturally occur in their rearing water). Following exposure to these different water chemistries, the fish were marked and transported and released into an unfamiliar lake. When these fish matured and began their spawning migrations, the researchers pumped the artificial odorants into unfamiliar streams and monitored the lake tributaries for returning salmon. Upon their return migration, over 90% of the salmon exposed to the artificial odorants were recovered in the stream scented with their exposure odorant while the control fish were scattered in tributaries throughout the lake. This landmark research showed that olfaction not only played a critical role in a salmonid’s ability to navigate back to their natal waters for spawning, but also that as juveniles they did indeed imprint on the scent of their rearing waters.

The parr-smolt transition (PST) is thought to be a particularly important period for olfactory imprinting in juvenile salmon (Dittman and Quinn, 1996). However, some salmon species, such as pink, chum and sockeye salmon, migrate away from their natal site very soon after emergence from the gravel. These salmon species would therefore not have much time to imprint on the scent of their natal waters after they emerge compared to other salmon species, which spend weeks to months in their natal waters. However, these species still possess the ability to home back to their natal waters with relatively high accuracy, indicating that odor imprinting must occur in earlier life-stages as well. Indeed, research has shown that to overcome this shortened residency time upon emergence, both pink and sockeye salmon have the ability to imprint on the scent of their natal waters during the alevin stage while they are still buried in the gravel of the redds (Tilson *et al.*, 1994; Bett *et al.*, 2016; Havey *et al.*, 2017).

During these windows of odorant imprinting, there is strong evidence that the release of hormones at these key moments may play a major role driving odorant imprinting (Nevitt and Dittman, 1998). It has been determined that when juvenile salmon experience novel stimuli, such as new water chemistries and environments, during rearing and during their downstream migration, they concomitantly experience surges in thyroxine (T4), later converted to triiodothyronine (T3) within the olfactory system (Lema and Nevitt, 2004). As previously mentioned, the PST represents a critical and sensitive time for odorant imprinting in the juvenile salmon olfactory system (Dittman and Quinn, 1996) and during the PST there is also a surge in circulating thyroxine (T4) levels within juvenile salmon. These hormonal surges are very important, as the conversion of T4 to T3 hormone within the olfactory system drives the proliferation of sensory cells that may be important in odorant imprinting (Lema and Nevitt, 2004).

A more complex and dynamic environment for young salmon aids in their ability to correctly imprint and thus navigate back for spawning. Indeed juvenile salmon imprinting on novel odorant stimuli in changing aquatic environments as they migrate downstream allows them to use these unique cues as navigational way-points later on as they migrate back upstream as adults to spawn (Quinn, 2011). Without unique cues and sequential imprinting on unique odorant mixtures, it would be extremely difficult to imagine a salmon being able to correctly navigate back to its natal waters. Returning adult salmon are unlikely to smell their natal waters at the mouth of a large river. This would be akin to trying to successfully navigate your car across the country with no road signs except for the last few miles of your journey. Using memorized waypoints allows them to overcome that hurdle and successfully migrate up the correct bodies of water.

Return Migration

When ocean-phase salmon begin to mature and are ready to make the return migration for spawning, the olfactory system again plays a central role in completing their life-cycle. The initiation of the home stream migration coincides with an elevation in gonadotropin releasing hormone (GnRH) levels in the brain (Takeshi *et al.*, 2010). In response, maturing salmon experience elevated levels of the gonadotropin follicle-stimulating hormone (FSH) which stimulates vitellogenesis and gonadal development. As salmon enter freshwater and approach their natal river, GnRH levels in other regions of the brain influence migratory behavior and olfactory function (Dittman *et al.*, unpublished; Ueda *et al.*, 2016). Finally, as salmon near their spawning grounds, GnRH induces surges in the gonadotropin luteinizing hormone (LH) that stimulate elevated plasma levels of the steroid $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) involved in final maturation.

GnRH and maturation appear to play an important role in migratory behavior during the spawning migration. Experiments in which salmon were displaced downstream after returning to their natal site, demonstrated that the time to return to the homesite declined as salmon neared final maturation suggesting a strong motivation to return as fish neared final maturation (Dittman *et al.*, unpublished, Ueda, 2011). In subsequent experiments, displaced salmon injected with GnRH implants returned more quickly than sham controls indicating that homing behavior is affected by maturation-associated hormones (Dittman *et al.*, unpublished, Ueda, 2011). Indeed, mature but not immature salmon were attracted to imprinted odors in studies with coho salmon (Hasler and Scholz, 1983; Dittman *et al.*, 1996) but whether this reflects an inability to recognize imprinted odors or a lack of motivation to respond to them is not clear. However, it does indicate that mature and immature salmon respond differently to odors learned as juveniles. It is important to note that homing and maturation are not inextricably linked. Many populations of salmon migrate back to their river of origin months before maturing (e.g., spring chinook salmon and summer steelhead). In these races, fish may home hundreds of kilometers upriver to the general vicinity of their natal stream, then hold in the main-stem river for several months before resuming their homing migration to their spawning site (Quinn *et al.*, 2016).

In addition to the behavioral effects of maturation on migratory behavior, the olfactory system itself is affected by the maturation process. For example, it has been demonstrated that olfactory sensitivity to reproductive pheromones is enhanced as fish mature. Several experiments have demonstrated that treatment with androgens induced increased olfactory sensitivity to reproductive pheromones in several cyprinid species (Cardwell *et al.*, 1995; Belanger *et al.*, 2010; Ghosal and Sorensen, 2016). Salmon are similarly responsive to reproductive pheromones during spawning (Yambe *et al.*, 2006) but a direct link between maturation and heightened olfactory sensitivity to these pheromones has not been demonstrated. However, electrophysiology, heart rate conditioning, and cellular studies of olfactory function have indicated that olfactory sensitivity to imprinted odorants increase during maturation (Hasler and Scholz, 1983; Dittman *et al.*, 1997). Experimental GnRH treatments of immature salmon or maturing salmon nearing their natal site increased their olfactory sensitivity to imprinted waters (Hasler and Scholz, 1983; Ueda *et al.*, 2016). One aspect of the imprinting process involves sensitization of the peripheral olfactory system (sensory neuron in the sensory epithelium of the olfactory rosette) to specific imprinted odorants (Nevitt *et al.*, 1994). Dittman *et al.*, (1997) demonstrated that sensitivity of signal transduction enzymes to imprinted odors in the sensory neurons of the olfactory epithelium increased only at the time of the homing migration as fish were maturing. This heightened sensitivity to imprinted odors may also be due to changes in expression of odor receptor proteins induced by maturation. Transcriptomic analysis comparing mRNA expression in the olfactory epithelium of immature and mature eels and salmon indicated that specific odorants receptors and olfactory signaling genes are unregulated in mature fish (Churcher *et al.*, 2015; Palstra *et al.*, 2015).

Conclusion

Salmon have an extraordinary life cycle, highlighted by their dramatic reproductive homing migrations which take them from oceanic feeding grounds back to their river of origin to spawn. At every stage in these migrations, there is a complex interplay between the reproductive endocrine axis and olfaction that allows a salmon to successfully return to their natal stream to spawn.

References

- Behrens, M., Frank, O., Rawel, H., *et al.* (2014). ORA1, a zebrafish olfactory receptor ancestral to all mammalian V1R genes, recognizes 4-hydroxyphenylacetic acid, a putative reproductive pheromone. *Journal of Biological Chemistry*, 289(28), 19778–19788.

- Belanger, R. M., Pachkowski, M. D., & Stacey, N. E. (2010). Methyltestosterone-induced changes in electro-olfactogram responses and courtship behaviors of cyprinids. *Chemical Senses*, 35, 65–74.
- Bett, N. N., Hinch, S. G., Dittman, A. H., & Yun, S. S. (2016). Evidence of olfactory imprinting at an early life stage in pink salmon (*Oncorhynchus gorbuscha*). *Scientific Reports*, 6, 36393.
- Cardwell, J. R., Stacey, N. E., Tan, E. S. P., McAdam, D. S. O., & Lang, S. L. C. (1995). Androgen increases olfactory receptor response to a vertebrate sex pheromone. *Journal of Comparative Physiology A*, 176, 55–61.
- Churcher, A. M., Hubbard, P. C., Marques, J. P., Canario, A. V. M., & Huerta, M. (2015). Deep sequencing of the olfactory epithelium reveals specific chemosensory receptors are expressed at sexual maturity in the European eel *Anguilla anguilla*. *Molecular Ecology*, 24, 822–834.
- Cooper, J. C., Scholz, A. T., Horrall, R. M., Hasler, A. D., & Madison, D. M. (1976). Experimental confirmation of the olfactory hypothesis with homing, artificially imprinted coho salmon (*Oncorhynchus kisutch*). *Journal of the Fisheries Board of Canada*, 33(4), 703–710.
- DeMaria, S., & Ngai, J. (2010). The cell biology of smell. *The Journal of Cell Biology*, 191(3), 443–452.
- Dittman, A., & Quinn, T. (1996). Homing in Pacific salmon: Mechanisms and ecological basis. *Journal of Experimental Biology*, 199(1), 83–91.
- Dittman, A. H., Quinn, T. P., & Nevitt, G. A. (1996). Timing of imprinting to natural and artificial odors by coho salmon (*Oncorhynchus kisutch*). *Canadian Journal of Fisheries and Aquatic Sciences*, 53, 434–442.
- Dittman, A. H., Quinn, T. P., Nevitt, G. A., Hacker, B., & Storm, D. R. (1997). Sensitization of olfactory guanylyl cyclase to a specific imprinted odorant in coho salmon. *Neuron*, 19, 381–389.
- Døving, K. B. (2007). The functional organization of the fish olfactory system. *Progress in Neurobiology*, 82(2), 80–86.
- Ghosal, R., & Sorensen, P. (2016). Male-typical courtship, spawning behavior, and olfactory sensitivity are induced to different extents by androgens in the goldfish suggesting they are controlled by different neuroendocrine mechanisms. *General and Comparative Endocrinology*, 232, 160–173.
- Hansen, A., Rølen, S. H., Anderson, K., et al. (2003). Correlation between olfactory receptor cell type and function in the channel catfish. *Journal of Neuroscience*, 23(28), 9328–9339.
- Hara, T. J. (1992). *Fish Chemoreception* (p. 180). London: Chapman and Hall.
- Hasler, A. D., & Scholz, A. T. (1983). *Olfactory Imprinting and Homing in Salmon*. Springer Science & Business Media.
- Havey, M. A., Dittman, A. H., Quinn, T. P., Lema, S. C., & May, D. (2017). Experimental evidence for olfactory imprinting by sockeye salmon at embryonic and smolt stages. *Transactions of the American Fisheries Society*, 146(1), 74–83.
- Jiang, Y., Gong, N. N., Hu, X. S., et al. (2015). Molecular profiling of activated olfactory neurons identifies odorant receptors for odors in vivo. *Nature Neuroscience*, 18(10), 1446–1454.
- Johnstone, K. A., Lubieniecki, K. P., Koop, B. F., & Davidson, W. S. (2012). Identification of olfactory receptor genes in Atlantic salmon *Salmo salar*. *Journal of Fish Biology*, 81(2), 559–575.
- Lema, S. C., & Nevitt, G. A. (2004). Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting. *Journal of Experimental Biology*, 207(19), 3317–3327.
- Lewcock, J. W., & Reed, R. R. (2004). A feedback mechanism regulates monoallelic odorant receptor expression. *Proceedings of the National Academy of Sciences of the United States of America*, 101(4), 1069–1074.
- Malnic, B., Hirono, J., Sato, T., & Buck, L. B. (1999). Combinatorial receptor codes for odors. *Cell*, 96(5), 713–723.
- Nevitt, G. A., Dittman, A. H., Quinn, T. P., & Moody, W. J. (1994). Evidence for a peripheral olfactory memory in imprinted salmon. *Proceedings of the National Academy of Sciences United States of America*, 91, 4288–4292.
- Nevitt, G., & Dittman, A. (1998). A new model for olfactory imprinting in salmon. *Integrative Biology Issues News and Reviews*, 1(6), 215–223.
- Palstra, A. P., Fukaya, K., Chiba, H., et al. (2015). The olfactory transcriptome and progression of sexual maturation in homing chum salmon *Oncorhynchus keta*. *PLOS ONE*, 10(9), e0137404.
- Quinn, T. P. (2011). *The Behavior and Ecology of Pacific Salmon and Trout*. Seattle: UW press.
- Quinn, T. P., McGinnity, P., & Reed, T. E. (2016). The paradox of “premature migration” by adult anadromous salmonid fishes: Patterns and hypotheses. *Canadian Journal of Fisheries and Aquatic Sciences*, 73, 1015–1030.
- Sato, Y., Miyasaka, N., & Yoshihara, Y. (2007). Hierarchical regulation of odorant receptor gene choice and subsequent axonal projection of olfactory sensory neurons in zebrafish. *The Journal of Neuroscience*.
- Sato, K., & Suzuki, N. (2001). Whole-cell response characteristics of ciliated and microvillous olfactory receptor neurons to amino acids, pheromone candidates and urine in rainbow trout. *Chemical Senses*, 26(9), 1145–1156.
- Scholz, A. T., Horrall, R. M., Cooper, J. C., & Hasler, A. D. (1976). Imprinting to chemical cues: The basis for home stream selection in salmon. *Science*, 192(4245), 1247–1249.
- Serizawa, S., Miyamichi, K., Nakatani, H., et al. (2003). Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. *Science*, 302(5653), 2088–2094.
- Takeshi, A., Makino, K., Ando, H., et al. (2010). Expression of GnRH genes is elevated in discrete brain loci of chum salmon before initiation of homing behavior and during spawning migration. *General and Comparative Endocrinology*, 168, 356–368.
- Tilson, M.B., Scholz, A.T., White, R.J., Galloway, H., Cheney, W., 1994. Thyroid-induced chemical imprinting in early life stages and assessment of smoltification in kokanee salmon: Implications for operating Lake Roosevelt kokanee salmon hatcheries. Bonneville Power Administration, US Department of Energy.
- Ueda, H. (2011). Physiological mechanism of homing migration in Pacific salmon from behavioral to molecular biological approaches. *General and Comparative Endocrinology*, 170(2), 222–232.
- Ueda, H., Nakamura, S., Nakamura, T., et al. (2016). Involvement of hormones in olfactory imprinting and homing in chum salmon. *Scientific Reports*, 6, 21102.
- Whitlock, K. E. (2006). The sense of scents: Olfactory behaviors in the zebrafish. *Zebrafish*, 3(2), 203–213.
- Yambe, H., Kitamura, S., Kamio, M., et al. (2006). L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *Proceedings of the National Academy of Sciences United States of America*, 103, 15370–15374.

REPRODUCTION, OVERVIEW BY PHYLOGENY

Sponge Reproduction

Alexander V Ereskovsky, Avignon University, Marseille, France; and Saint-Petersburg State University, St. Petersburg, Russia

© 2018 Elsevier Inc. All rights reserved.

Sponges (phylum Porifera) branch basally in the metazoan phylogenetic tree and comprise four distinct lineages in the rank of classes: Demospongiae, Hexactinellida, Calcarea and Homoscleromorpha. Recent Porifera number about 9000 species living in all aquatic environments at all depths. A sponge is traditionally defined as “a sedentary, filter-feeding metazoan.” They have no nerves, muscles, special digestive system or gonads. However, sponges and eumetazoans share the basic features of sexual reproduction such as oogenesis, spermatogenesis, gametes, morphogenesis etc.

The data on the reproduction must be accumulated and compared between the sponge lineages and eumetazoans in order to understand the early evolution of the animal reproduction and patterning in the relation to the genome evolution. Sponges are a key group to provide significant answers to these fundamental evolutionary questions.

Modes of Reproduction

Gonochorism and hermaphroditism as well as viviparity, oviparity, and ovoviviparity occur in sponges. The data on the sex phenotype have been obtained mainly from Demospongiae (Table 1). However, it was demonstrated that oviparous sponges are chiefly gonochoric, but viviparous (brooding) sponges chiefly are hermaphrodites.

Sequential or successive hermaphroditism (or sex reversal) is a type of the hermaphroditism that occurs when the individual changes sex at some period in its life. It can be change from a male to female (protandry), or from a female to male (protogyny). Successive hermaphroditism has been documented in only a few cases in demosponges and therefore the real extent of this phenomenon cannot be properly evaluated at the moment. This type of hermaphroditism has been indicated in: *Polymastia mammillaris*, *Suberites massa*, *Hymeniacion perlevis*, *H. heliophila*, *Chalinula ecbasis*, and *Spongilla lacustris*. A careful analysis of the sex cycle is also necessary to avoid confusion between hermaphrodite species with sexual phases far apart in time and gonochoric species.

Contemporaneous hermaphroditism is more common than successive and well known in many demosponges (order Poecilosclerida), some Homoscleromorpha and Calcarea. Some species could include mostly contemporaneous hermaphrodites in a population in co-existence with a few gonochoristic individuals and vice versa, populations consisting mainly of gonochoristic individuals have been showed to contain some contemporaneous-hermaphroditic individuals.

Both oviparity and viviparity (brooding) exist in sponges. In contrast to the sex phenotype, oviparity and viviparity are stable features of individuals and species. Viviparous sponges release larvae and the oviparous sponges release zygotes or unfertilized eggs.

Viviparity and oviparity are equally widespread reproductive modes in sponges. The embryonic development in the oviparous sponges is always external, leading to free-swimming larvae. Viviparous or ovoviviparous sponges are characterized by brooding of embryos in the mesohyl or inside the special temporary structure – follicles. Resulting free-swimming larvae release through the canals of the aquiferous system. The direct development without larval stage exists in some demosponges. In Demospongiae some orders are completely oviparous (Polymastiida, Clionaida, Tethyida, and Verongida), while other – have only viviparity (Spongillida, Dendroceratida, Dictyoceratida). However, some viviparous representatives can be found in the oviparous orders, for example, the viviparous genera *Alectona*, *Thoosa*, *Stylocordyla* (order Suberitida), genus *Halisarca* (order Chondrosida), genera *Haliclona*, *Xestospongia* (order Haplosclerida).

Viviparity in sponges often correlates with the hermaphroditism. For example, all investigated Hexactinellida, Homoscleromorpha and Calcarea are viviparous and hermaphrodites.

No sexual dimorphism exists in gonochorists or successive hermaphrodites.

Sexual Versus Asexual Reproduction

Asexual reproduction is an important reproductive strategy for sessile organisms such as sponges, and represents a significant evolutionary advantage that can compensate for the fact that mature adults are unable to move and search for mates. Sponge asexual reproduction may proceed by fragmentation, budding and gemmulogenesis.

The simplest mode of asexual reproduction is fragmentation. Fragmentation is characteristic for attached organisms with a low degree of integration and high regeneration capacities. It occurs accidentally, and the separation of the fragment from the parental sponge is not preceded by any changes in the cells composition. Fragmentation is widespread in Demospongiae and mostly occurs in the sponges whose body branches or bears projections. Fragmentation may be triggered by various factors: wave impact during storms, the activities of predators such as fishes and turtles, certain infections, ruptures and splits of the substrate and, in the case of encrusting sponges, growth of the sponge body.

Table 1 Sex phenotype, oviparity & viviparity, larval type, and asexual reproduction distribution in the phylum Porifera

<i>Taxa</i>	<i>Sex phenotype</i>	<i>Reproductive conditions</i>	<i>Larval type</i>	<i>Asexual reproduction</i>
Class Hexactinellida				
Order Hexactinosida	H	V	?	B
Order Lyssacinosida	H	V	Tr	No
Class Demospongiae				
Order Agelasida	G/H	O	?	No
<i>Fam Astroscleridae</i>	?	V	Pa	?
Order Axinellida	G	O	?	B
Order Biemnida	G	O	?	F
Order Bubarida	G	O	?	?
Order Chondrosida	G	O	Co	F
Order Chondrillida	G	O	Co	No
<i>Fam Halisarcidae</i>	G	V	Di	No
Order Clionaida	H	O	Co	G
Order Dendroceratida	G	V	Pa	B/No
Order Desmacellida	?	?	?	?
Order Dictyoceratida	G	V	Pa	B/No
Order Haplosclerida	G/H	V/O	Pa	F/G
Order Merliida	?	?	?	?
Order Poecilosclerida	H	V	Pa	F
Order Polymastiida	G	O	Co	B
Order Scopalinida	H	V	Pa	?
Order Sphaerocladina	?	?	?	?
Order Spongillida	G	V	Pa	G/F
Order Suberitida				
<i>Family Suberitidae</i>	G	O	?	G
<i>Family Halichondriidae</i>	H/G	V	Pa	F
<i>Family Stylocordylidae</i>	?	V/O	D	?
Order Tethyida	G	O	Pa	B
Order Tetractinellida	G	O	?	B/F
<i>Family Tetillidae</i>	G	V	D	B
<i>Family Thoosidae</i>	H	V	Ho	G
Order Trachycladida	?	?	?	?
Order Verongida	G	O	Co	B
Class	H	V	Ci	B
Homoscleromorpha				
Class Calcarea				
Subclass Calcaronea	H	V	Am	B
Subclass Calcinea	?	V	Ca	B

Abbreviations: Sex phenotype – G, gonochorisme; H, hermaphroditisme. Reproductive conditions – O, oviparity; V, viviparity. Cleavage pattern – Ch, chaotic; Ra, radial-like; In, incurvational; Po, polyaxial. Larval type – Am, amphiblastula; Ca, calciblastula; Ci, cinctoblastula; Co, coeloblastula; D, direct development Di, disphaerula; Ho, hoplitomella; Pa, parenchymella; Tr, trichimella. Asexual reproduction – B, Budding; F, fragmentation; G, gemmulogenesis; No, absence.

A general feature of budding in all investigated sponges, except Homoscleromorpha, is migration of the polypotent and some differentiated cells from the internal parts of the parental sponge to the ectosomal region, formation of the ectosomal cell aggregate, and development of a more or less dense bud. Budding in Homoscleromorpha does not involve the migration of cells from the mesohyl and their integration into the forming bud. In Homoscleromorpha bud development is based on the epithelial morphogenesis, i.e., the forming bud is simply an outgrowth of the parental sponge's body wall.

Gemmulogenesis was described only in demosponges. Gemmules develop from the polypotent archaeocytes. It usually occurs after completion of sexual reproduction or, more rarely, during embryogenesis. Gemmulogenesis starts with directed migration of the gemmule nurturing cells, archaeocytes and spongocytes into certain parts of the mesohyl, where they form dense aggregates. Archaeocytes in these aggregates actively phagocyte nurturing cells and symbiotic zoochlorellae. Gemmule envelope formation starts at one of the poles and gradually spreads to the other one, where a micropyle, a spicule-free area, is later formed. A fully-formed gemmule consists of a dense non-cellular envelope and the homogenous internal mass of thesocytes, originated from archaeocytes. The latter have numerous yolk plates or yolk granules. Gemmulogenesis is accompanied by a considerable or complete disorganization of the aquiferous system and the mesohyl of the parental sponge.

Asexual reproduction can occur occasionally. For instance, occasional budding is found in almost all sponge clades and in any type of habitat, but it is observed regularly among marine representatives of Porifera. Some cases of obligatory asexual reproduction have been recorded in many demosponges (orders Spongillida, Tethyida, Clionaida, Suberitida, Polymastiida and Tetractinellida). For instance, gemmulogenesis occurs in all families of Spongillida, except Lubomirskiidae, in the families Clionaidae (Clionaida) and Suberitidae (Suberitida). Obligatory budding has been recorded in the families Polymastiidae and Tethyidae.

When asexual reproduction exists in Demospongiae, the life cycle often presents a regular alternation of sexual and asexual phases. Potential explanations for this alternation rely on the "competition" for cells, especially for archaeocytes, which are likely polypotent cells, between these two reproduction modes.

Under unfavorable environmental conditions, some sponges become dormant and may remain in a dormant state for long periods. Such dormancy is often associated with the asexual reproduction.

Reproductive Strategies: Exogenous Influences

Factors associated with environmental conditions such as food availability, population density, light, habitat stability, and seasonal variation in temperature are perceived externally by individuals in a population and delivered to and interpreted by the internal effectors which direct the expression of asexual or sexual reproduction. Nevertheless, it seems that water temperature and food availability play the most important role in this process in sponges. Indeed, it has been showed, that in both tropical and temperate sponges temperature is one of the important factors for the regulation of sexual and asexual reproduction. An increase in the frequency of specimens with buds and increasing of buds per sponge occurs simultaneously with the rapid decrease in water temperature, as was shown for Mediterranean *Tethya citrina*, *T. aurantium*, *Mycale (Aegogropila) contarenii*, Arctic *Polymastia arctica*, and tropical species such as *Cinachyra australiensis* and *Cinachyrella cavernosa*. Sexual reproduction in deep-sea demosponges *Thenaea abyssorum* may be triggered by the vertical flux of particulate organic carbon to this food-limited environment. Development of embryos in *Chalinula ecbasis* is related to particulate food supply.

Numerous attempts have been made to correlate the reproductive activity with the environmental fluctuations. However, correlation does not necessarily indicate the direct causality, and there have been only few experimental studies of sponge reproduction. Clearly, experimental investigations are needed to confirm or refute the conclusions derived from the purely correlative studies.

Sexual Reproduction

The only common feature for all sponge species is the absence of organized gonads, though the spermatocysts, which presented in the choanosome, can be considered as a temporary dispersed gonad.

Male Reproduction

Spermatogenesis occurs in the spermatocysts (spermatocysts) in all sponges, except Calcarea. Spermatocysts are temporary spherical structures, which develop by the transformation of choanocytes of an entire choanocyte chamber (except of the carnivorous demosponges as they have no aquiferous system and choanocyte chambers). The spermatocysts are surrounded by the follicle cells deriving through the transformation of pinacocytes (pseudo-epithelial cells) or archaeocytes. In demosponges cell junctions between the follicle cells are simple (apposition) with no detectable membrane specialization. The follicular cells in the spermatocysts of Homoscleromorpha possess the specialized cell junctions and basement membrane.

According to species, the synchrony of spermatogenesis occurs at three levels: (1) population, as in *Neofibularia nolitangere*; (2) individual, as in *Aplysilla rosea*, *Myxilla incrustans*, *Iophon piceum*, in which all the choanocytes of the choanocyte chambers simultaneously transform into spermatogonia; (3) spermatocyst, as in *Suberites massa*, *Spongia officinalis*.

Except for species that reproduce all year round spermatogenesis appears to occur in a shorter period than oogenesis.

Female Reproduction

Sponge female gametes develop in the choanosome diffusely or, sometimes, in small clusters. This is true for both oviparous and viviparous sponges. No gonads are formed.

Gametogenesis, Fertilization and Early Development

A characteristic feature of gametogenesis in sponges is the origin of the gametes by the direct transformation from the somatic cells - choanocytes or archaeocytes. Otherwise, the stages and cytological features of the gamete development in sponges are similar to those in other animals.

Spermatogenesis

Development of male gametes within a cyst is usually synchronous. In Homoscleromorpha there is a gradient of male gamete maturation within a cyst, a feature they share with the Eumetazoa.

Two different types of somatic cells have been postulated as the source of spermatogonia. These cells are either archaeocytes or, more frequently, choanocytes (flagellated collar cells involved in the particle capture and sponge feeding).

Calcaronea have aberrant spermatozoa: spherical and non-flagellated. (The term “aberrant” is applied to spermatozoa that are very diverse as concerns their external morphology). Spermatozoa of most other sponges belong to the primitive type (ancestral-primitive type): they have a large cytoplasmic volume, several mitochondria and lack the acrosome. Homoscleromorpha and some demosponges from the order Poecilosclerida (*Crambe crambe*, *Crellomima imparidens*, *Asbestopluma hypogea*, and *Hymedesmia irregularis*) have “derived or typical” spermatozoa, characterized by the presence of acrosome, one or two big mitochondria and small volume of cytoplasm.

Sperm of most of the species, except Calcaronea, displays an ovoid or rounded conical head with nucleus. The nucleus could have a shallow recess (*Aplysilla*), kidney-like shape (*Spongia*), cylindrical shape (*Iophon piceum*, *Crambe crambe*, *Crellomima imparidens*), ring-like or helicoidal shape (*Halichondria panicea*), or subspheroidal shape (other species).

The number, size and shape of the mitochondria are very variable, according to the species: one long mitochondrion in *Halichondria*; one, two or three large mitochondria, located in the basal part of the spermatozoon, in *Crambe*, *Aplysilla*, or *Suberites*; and a few to several scattered mitochondria, located in the periphery of the head in the remaining, asymmetrical cytoplasm in *Spongia*, *Lubomirskia*, *Myxilla* or *Anhinoe*.

All primitive and typical poriferan spermatozoa are flagellate, always with a 9+2 axoneme. There are two centrioles.

During spermiogenesis, the Golgi cisternae disappear but in *Suberites*, *Spongia*, *Asbestopluma occidentalis*, and *Petrosia ficiformis* dense proacrosomal vesicles of Golgi origin persist near the nucleus and are considered to possibly represent a rudimentary acrosome.

In all Homoscleromorpha, a fully developed acrosome is presented. A large, conical acrosome has also been showed in the calcaronean *Sycon calcaravis*, and in some of poecilosclerid demosponges (*Crambe crambe*, *Crellomima imparidens*, *Asbestopluma hypogea*, and *Hymedesmia irregularis*).

Though organization of spermatozoa is considered to be a sound taxonomic and phylogenetic character, the presence of acrosome in spermatozoa of Poecilosclerida is at variance with this idea. For instance, *C. crambe*, *C. imparidens* and *H. irregularis* have an acrosome, but another sponge from the same order, *Myxilla*, *Iophon*, and *Anhinoe*, does not. The acrosome is likely to have originated several times in the course of the evolution. Its emergence is associated with the insemination and fertilization type, with the structure of the vitelline envelope and its accessibility for sperm penetration rather than with the taxonomic affiliation or phylogenetic position of the species.

Oogenesis

Two types of the oogenesis can be distinguished in animals – panoistic and meroistic. In panoistic oogenesis, all germ cells have the potential to become the egg cell. In meroistic oogenesis, only a few germ cells become egg cells, while the remaining cells differentiate into the nurse cells (trophocytes) that support the oocytes. Meroistic oogenesis is characterized by the formation of germ cell clusters, where the germ cells are interconnected by stable cytoplasmic bridges.

At the early stages of the sponge oogenesis oocytes have amoeboid-like shape and migrate through the mesohyl, incorporating diverse macromolecules and microorganisms. As the oocyte grows, it takes an oval form and proceeds to vitellogenesis. Usually, yolk-like inclusions are heterogeneous with electron-dense core and less electron-dense periphery. In other species oocyte can also include lipids, rests of digested microorganisms and fibrous or membrane structures. Vitellogenesis in sponges passes via autotynthesis, heterosynthesis or both processes simultaneously.

Autosynthesis is realized by the oocytes using basic precursors received through the oocyte membrane by endo-, pino-, or phagocytosis and followed by intense synthetic activity. Autosynthesis has been described for some oviparous demosponges, for example, *Suberites massa*, *Aplysina cavernicola*, *Tetilla serica*, *Stelletta grubii*, and *Raspaciona aculeata*.

Heterosynthesis involves participation of different types of somatic cells and is typical for most viviparous sponges. Somatic cells either elaborate yolk or its precursors in their cytoplasm, transporting and transferring all these materials to the growing oocytes, or whole cells or their fragments could be phagocytosed by oocytes. These somatic cells (choanocytes, different mesohyl cells) are often referred to as trophocytes or “nurse cells”. However, strictly speaking, sponges do not possess true trophocytes. First, trophocytes are cells developing from the germline in the course of oogenesis. Second, oocytes and trophocytes are connected by cytoplasmic bridges and surrounded by follicular epithelium, which is not characteristic for sponges. Thus, sponge oogenesis is panoistic.

In some demosponges (orders Haplosclerida and Spongillida), vitellogenesis is accompanied by the emergence of a special population of phagosome-rich nucleolated amoebocytes that migrate towards the oocyte and are phagocytosed by it. This is an indication of a higher specialization of oogenesis in these sponges as compared to other demosponge orders.

The combination of both autotrophic and heterotrophic pathways (a mixed pathway) allows the quick development of oocytes and has been described for many demosponges and some Calcareia.

Primitive features of oogenesis and oocyte structure in sponges are the lack of ooplasm segregation and, apparently, of cytoplasmic determinants. Consequently, sponges also lack mechanisms of determination of the axes of the future larva or, in

the case of direct development, the young sponge. In viviparous sponges, another primitive feature of oogenesis is the lack of specialized vitelline envelope. However, oviparous demosponges have a primitive vitelline envelope.

Gametes Resorption

The fate of waste gametes, unspawned or unfertilized oocytes has been investigated in sponges in several cases. Degeneration and resorption of unfertilized oocytes have been reported in some demosponges and calcareous sponges.

The spermatogonia shows phagocytic activity in *Spongia officinalis*. They form pseudopodia around mesohyl bacteria and have cytoplasmic vacuoles containing these bacteria. The follicle cells phagocytose defective elements in the cysts in *Suberites massa*. Somatic cells were found within the spermatogenic cysts of *Chondrilla australiensis*, *Raspaciona aculeate* and *Petrosia ficiformis*. These somatic cells are involved in both elimination of presumably aberrant spermatozoa during spermatogenesis and removal of unspawned sperm. Sperm phagocytosis by the follicle cells in *Asbestopluma occidentalis* may also be directed to achieve an appropriate cell number in spermatogenic cysts as occurs in many other animals.

Fertilization

It is generally accepted that in the oviparous sponges the fertilization is external and in viviparous sponges is internal. However, in some oviparous demosponges, for example, *Cliona celata*, *Chondrosia reniformis*, an internal fertilization occurs.

In the case of external fertilization, spermatozoa and eggs are realized into the surrounding water through the osculum. In some cases, as in the genus *Tetilla* and *Petrosia ficiformis*, after fertilization, a fertilization-like membrane appears externally to the mucous layer of eggs. In oviparous sponges, the extruded eggs or zygotes are often enclosed within a gelatinous material, which hold them together in a mass and binds them to the maternal sponge and adjacent substrate.

An internal fertilization is typical for viviparous sponges. In this case the spermatozoa enter egg-containing individual via the incurrent water system. Then they usually pass through one or more barriers to reach the eggs, which are often scattered in the mesohyl. Nevertheless, male sperm spawning in viviparous sponges has never been documented.

Internal fertilization has been well described only for calcareous sponges from subclass Calcaronea. Fertilization in the Calcaronea is carried out by the so-called carrier cells, which arise from a choanocyte, or more precisely, from an accessory complex cell, which acquired the capacity to capture a spermatozoon.

The carrier cell enlarges two or three-fold after capturing the sperm cell. In *Leucosolenia complicata*, accessory cells directly surrounds the carrier cell probably participating in the transformation of the latter; they actively accumulate inclusions and grow. Fertilization involves two carrier cells surrounding the sperm cell in *Sycon calcaravis*. Inside the carrier cell, the sperm undergoes certain changes and it is referred to as the spermicyst. Its structure is similar in different species. A dense protein capsule covers the spermicyst completely or partly. The nucleus has highly condensed chromatin. Electron-transparent vesicles are situated at the spermicyst's periphery. The spermicyst penetrates into the oocyte by means of one or two carrier cells, which push it into the ooplasm. Inside the oocyte the protein capsule is destroyed and the nucleus decondenses and swells.

Spermicyst penetration triggers the maturation divisions in the oocyte. Extrusion of polar bodies always occurs at the pole oriented towards the choanoderm, though not necessarily at the site of the spermicyst's penetration. After the chromatin in oocyte nucleus decondenses, both nuclei merge.

Embryogenesis and Postembryogenesis Overview

Embryonic development of ovoviviparous and viviparous sponges proceeds in the temporary brood chambers, often referred to as follicles. They are formed at the end of vitellogenesis from flattened cells originating from the choanocytes, amoebocytes, or endopinacocytes. The follicular epithelium later participates in the formation of the aquiferous system canals.

Four main cleavage patterns in sponges are: chaotic cleavage – Homoscleromorpha, most viviparous Demospongiae; radial-like cleavage – oviparous Demospongiae, Hexactinellida; polyaxial cleavage – Halisarcidae (Demospongiae), Calcinea (Calcarea); incurvational cleavage (table palintomy type) – Calcaronea (Calcarea). The spatial position of the blastomeres is usually unstable; divisions either become asynchronous very early or are not synchronous at all from the very beginning.

More complex animals possess embryogenesis comprising distinct stages – cleavage, blastulation, gastrulation, histogenesis and organogenesis. In sponges, these stages, except cleavage, are not so distinct. First, sponges lack the final phase of blastulation. In the end of cleavage, at the stage usually referred to as blastula or morula, the differentiation of the larval cells (external ciliated cells and sclerocytes) begins. Second, the fate of the specialized larval cells (cover cells, skeletogenic cells, etc.) may be different and it is not homologous to the fate of germ layers in Eumetazoa, since Porifera do not have body parts, organs and tissues homologous to those of other metazoans. Besides, there are no mechanisms of the early determination of the cells in the border and internal tissues of the adult sponge. Cleavage in sponges followed by the cell differentiation and migration resulting in a larva or young sponge (in the case of direct development) formation. In poriferan embryogenesis different types of epithelial and mesenchymal morphogeneses occur.

All sponges except the representatives of the demosponges genera *Tetilla* (Tetractinellida) and *Stylocordyla* (Suberitida) have indirect, i.e., larval development. Eight types of sponge larvae have been described: coeloblastula, calciblastula, cinctoblastula,

amphiblastula, disphaerula, hoplitomella, parenchymella and trichimella. In general, there are two principal larval constructions in sponges: hollow single-layered larvae (coeloblastula, calciblastula, cinctoblastula, amphiblastula) and two-layered larvae without cavity (parenchymella, hoplitomella, trichimella). Disphaerula occupies a morphologically intermediate position. All the larval types, except hoplitomella, are characterized by a distinct anterior-posterior polarity. Externally, it may be expressed in the body shape, the ciliation character at different poles and the unevenness of colour. At the histological level, the polarity is expressed in the distribution of various cell types, in orientation and position of spicules (parenchymellae and trichimella) and in unequal concentration of endosymbiotic bacteria (cinctoblastula).

Metamorphosis is a short stage of the post-embryonic development during which the larva undergoes radical morphological and physiological changes due to its transition to the attached adult stage. Some structures, usually the larval or provisional ones, disappear, the others, that were rudimentary, rapidly complete their development. Morphogeneses, accompanying sponge larvae metamorphosis could be epithelial type, epithelial-mesenchymal transitions or mesenchymal-epithelial transformations depending on the larval structure. The main feature of the metamorphosis of the sponge larvae is the acquisition of the sponge Bauplan, which is mainly represented by the aquiferous system. The first adult structure to be formed *de novo* is the exopinacoderm, which isolates the young sponge from the milieu. Later steps include organization of the choanocyte chambers and the water current canals, the opening of the ostia and osculum and the acquisition of the adult skeleton elements.

Larval metamorphosis usually results in a mono-ocular individual, whose aquiferous system is different from that of the adult sponge. In the Calcarea such a young individual has the aquiferous system of the asconoid type and is called the olynthus; in the Demospongiae and the Homoscleromorpha it has the aquiferous system of the leuconoid or syconoid type and is called the rhagon.

Sexual Behaviour

In sessile animals like the sponges, the field of phenomena related to behaviour, and consequently to the behaviour associated with reproduction, is very limited. The most studied aspects are those concerning the release of gametes.

In oviparous demosponges spermatozoa and eggs are shed into the excurrent canals and discharged through the oscula. The mass release of spermatozoa detected as a white 'smoke' is a simultaneous shedding of the spermatozoa by many individuals in a local population. This phenomenon suggests a regulation mechanism of spermatozoa release, which could be related to the diel light/dark regime. A synchronicity has been observed also for the egg release in oviparous demosponges. The synchronous release of eggs is highly correlated in seasonality and lunar phase from year to year and the oocytes release is often synchronous with that of sperm throughout local populations, as was shown for *Hemectyon ferox*.

In some sponges, living within relatively stable conditions, emission of spermatozoa is closely correlated with the lunar phase.

Acknowledgment

This work has been supported by grant no 17-14-01089 of the Russian Science Foundation.

Further Reading

- Boury-Esnault, N., & Jamiesson, B. (1999). Porifera. In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates. Progress in Male Gamete Ultrastructure and Phylogeny* (pp. 1–20). Chichester: John Wiley and Sons.
- Degnan, B. M., Adamska, M., Richards, G. S., Larroux, C., et al. (2015). Porifera. In A. Wanninger (Ed.), *Evolutionary Developmental Biology of Invertebrates*, 1 pp. 1–65. Wien: Springer-Verlag [Introduction, Non-Bilateria, Acoelomorpha, Xenoturbellida, Chaetognatha].
- Ereskovsky, A. V. (2010). *The Comparative Embryology of Sponges*. Dordrecht, Heidelberg, London, New York: Springer-Verlag.
- Ereskovsky, A. V., Renard, E., & Borchellini, C. (2013). Cellular and molecular processes leading to embryo formation in sponges: Evidences for high conservation of processes throughout animal evolution. *Development, Genes & Evolution*, 223, 5–22.
- Fell, P. E. (1983). Porifera. In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates* (pp. 1–29). Chichester: John Wiley & Sons Ltd.
- Fell, P. E. (1989). Porifera. In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates. IV, Part A. Fertilization, Development and Parental Care* (pp. 1–41). New York: John Wiley & Sons.
- Fell, P. E. (1993). Porifera. In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates. VI. Asexual Propagation and Reproductive Strategies* (pp. 1–44). New Delhi: Oxford & IBH Publishing Company.
- Lanna, E., & Klautau, M. (2010). Oogenesis and spermatogenesis in *Paraleucilla magna* (Porifera, Calcarea). *Zoomorphology*, 129, 249–261.
- Lanna, E., & Klautau, M. (2015). Some aspects of the oogenesis of three species of clathrinid sponges (Calcarea, Porifera). *Journal of the Marine Biological Association of the United Kingdom*, 96, 529–539.
- Maldonado, M., & Riesgo, A. (2008). Reproduction in the phylum Porifera: A synoptic overview. *Treballs de la SCB*, 59, 29–49.
- Rieswig, H. M. (1983). Porifera. In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates* (pp. 1–21). Chichester: Wiley and Sons.
- Riesgo, A. (2010). Phagocytosis of sperm by follicle cells of the carnivorous sponge *Asbestopluma occidentalis* (Porifera, Demospongiae). *Tissue and Cell*, 42, 198–201.
- Riesgo, A., Novo, M., Sharma, P. P., Peterson, M., Maldonado, M., & Giribet, G. (2014). Inferring the ancestral sexuality and reproductive condition in sponges (Porifera). *Zoologica Scripta*, 43, 101–117.
- Sarà, M. (1993). Porifera. In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates. V.5. Sexual Differentiation and Behaviour* (pp. 1–28). Chichester: John Wiley and Sons, Ltd.
- Simpson, T. L. (1984). *The Cell Biology of Sponges*. New York: Springer-Verlag.

Cnidaria

Shinya Shikina and Ching-Fong Chang, National Taiwan Ocean University, Keelung, Taiwan

© 2018 Elsevier Inc. All rights reserved.

Introduction

General Characteristics of Cnidarians

The Cnidaria phylum includes sea anemones, *Hydra* (sessile solitary species), corals, sea fans, sea whips (colonial species) and jellyfish (free-swimming species) that live in aquatic (mostly marine) environments (Ruppert *et al.*, 2003). Cnidarians appear to have diverse morphologies, but all members of this phylum universally possess stinging cells called cnidoblasts. Cnidoblasts are unique to the animals of this phylum and are used to define the phylum. The bodies of individuals in the Cnidaria phylum exhibit radial or biradial symmetry, and almost all tissues have a basic structure composed of two cellular layers, the epidermis (outside) and the gastrodermis (inside). A gelatinous-like substance called the mesoglea is present between the two layers of the tissues and maintains the integrity of the tissues and the body. In addition, cnidarian bodies are relatively simple, and well-organized organs or systems that are found in vertebrates are not present in cnidarians, such as vascular and central nervous systems. Cnidarians only have a passive transport system that depends on diffusion; a simple nerve net; some functionally defined tissues such as tentacles for predation, attack and defense mechanisms; and a sac-like gastrovascular cavity used for digestion and absorption.

Body Forms

Cnidarians are present as either polyps or medusae as adults and planulae as larvae. Sea anemones are examples of the polyp forms, while jellyfish are examples of the medusa forms. In the current classification, the Cnidaria phylum is divided into two major groups, Anthozoa and Medusozoa, based on the structure of polyps and the existence of a medusa stage in the life cycles (Fig. 1). Anthozoa is a class that consists of two subclasses, Hexacorallia (stony corals and sea anemones) and Octocorallia (soft corals and red corals). Medusozoa is a subphylum that consists of four classes, Hydrozoa (*Hydra*), Scyphozoa (true jellyfish), Cubozoa (box jellyfish), and Staurozoa (stalked jellyfish) (Technau and Steel, 2011).

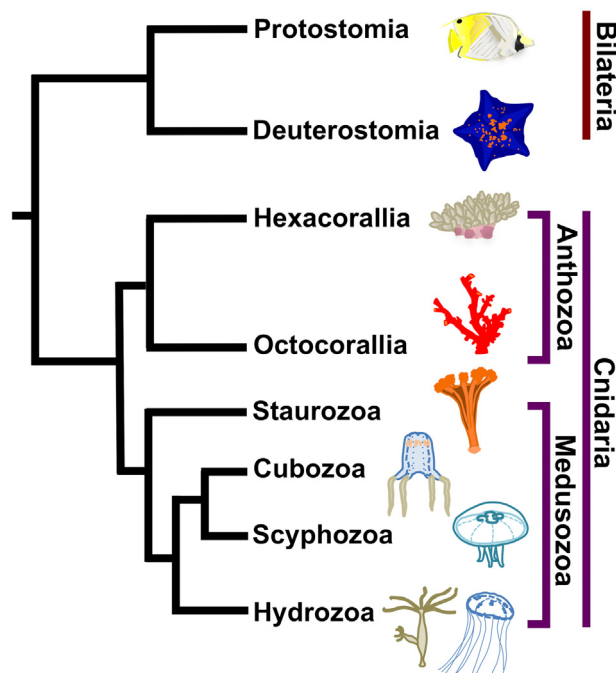


Fig. 1 Phylogenetic relationships of the classes in the phylum Cnidaria. The phylum Cnidaria is divided into two major groups, Anthozoa and Medusozoa. Anthozoa is a class that consists of two subclasses, Hexacorallia (stony corals and sea anemones) and Octocorallia (soft corals and red corals). Medusozoans is a subphylum that consists of four classes, Hydrozoa (freshwater *Hydra*), Scyphozoa (true jellyfish), Cubozoa (box jellyfish), and Staurozoa (stalked jellyfish). Modified from Technau, U., Steele, R.E., 2011. Evolutionary crossroads in developmental biology: Cnidaria. Development 138, 1447–1458.

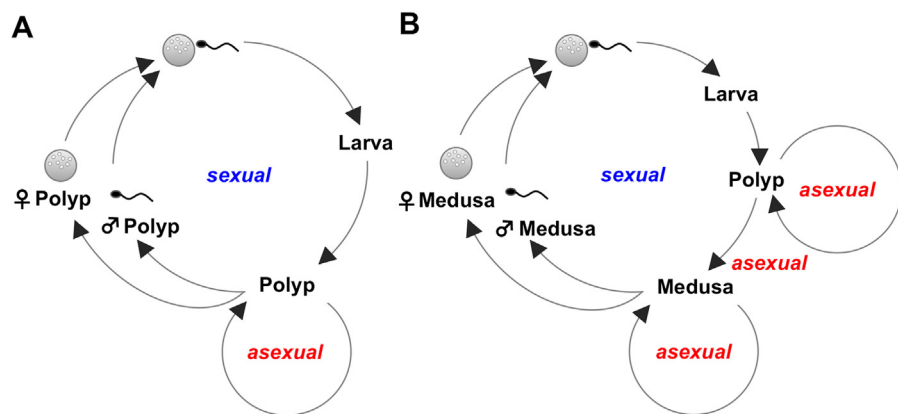


Fig. 2 Schematic representation of cnidarian life cycle. (A) A typical life cycle of anthozoan. (B) A typical life cycle of medusozoan.

Life Cycle

In anthozoans, the typical life cycle begins with the development of fertilized eggs into free-swimming larvae known as planula, which in turn transform into polyps that can undergo asexual reproduction to produce new polyps or form colonies. When the polyps (or colonies) reach certain ages or sizes, they begin to produce gametes for sexual reproduction under suitable environmental conditions (Fig. 2(A)). The medusa stage does not occur in the anthozoan life cycle. Many of the Medusozoa species alternate between the polyp and medusa stages during their life cycles. Similar to anthozoans, the typical life cycle of medusozoans begins with the development of fertilized eggs into planulae, which in turn transform into polyps. However, medusozoan polyps can produce medusae as well as polyps by asexual reproduction. Sexual reproduction generally occurs in the medusa stage (Fig. 2(B)). Considerable variations or modifications in the life cycle can be observed in many medusozoans.

Asexual Reproduction

Many cnidarians have the ability to create genetically identical individuals through asexual reproduction, as summarized in Table 1. Asexual reproduction allows cnidarians to increase the number of individuals in a population and form colonies in a relatively short period. Asexual reproduction mainly occurs in the polyp stage but sometimes occurs in various forms in the medusae and larval (planula) stages.

Table 1 Forms of asexual reproduction in cnidarians

Class	Production of polyps by polyps	Production of polyps by others	Production of medusae by polyps	Production of medusae by medusae	Production of polyps by medusae	Other phenomena related to asexual reproduction
Anthozoa	Budding Fission	Fragmentation Pedal laceration Autotomy Parthenogenesis	–	–	–	Polyp bail-out Polyp expulsion
Hydrozoa	Budding	Budding from stolon Podocysts Frustules Fragmentation Propagules	Budding	Budding Fission	Budding	Reverse development (medusa to polyp)
Scyphozoa	Budding Longitudinal fission	Podocysts Planulocysts Free-swimming propagules	Strobilation (transverse fission)	Unknown	Budding	Planula directly develops into ephyra Reverse development (ephyra to polyp)
Cubozoa	Budding	Regeneration from polyp fragments	Metamorphosis Monodisc strobilation	Unknown	Unknown	Unknown
Staurozoa	Budding	Planula budding Frustules	Metamorphosis (only apical part)	Unknown	Unknown	Unknown

Anthozoa

This class is composed of approximately 5000 marine species that include corals, sea anemones, sea pansies, and sea pens. Anthozoans are exclusively polyp species, and new polyps are principally produced by polyps or formed from a piece of polyp tissue (Table 1) (Harrison and Wallace, 1990; Bocharova and Kozevich, 2011). Budding, which is defined as the development of a new individual from an outgrowth (bud) of an existing parent tissue, may be the major form of asexual reproduction in anthozoans. In solitary species, new polyps are produced by the detachment of buds from the parent polyps. The buds may arise from the base of the parent polyp, body column, or oral disc depending on the species. In some colonial anthozoans possessing a coenosarc (a thin layer of tissue between the polyps in a colony), the buds (new polyps) may arise directly from and remain connected to the coenosarc. The successive budding results in the formation and enlargement of a colony. Fission is another major form of asexual reproduction that commonly occurs, which is defined as the division of an organism into two or more independent daughter organisms. Fission modes are divided into longitudinal fission and transverse fission based on the direction of division. Longitudinal fission is relatively common among anthozoans, but several species (e.g., *Nematostella vectensis*) undergo transverse fission.

Fragmentation is another important form of asexual reproduction that occurs in anthozoans and is defined as the reorganization and development of an entire organism from a fragmented piece of parental polyp tissue. In some sea anemones (e.g., *Exaiptasia pallida*), parts of the pedal disc (the opposite end of the mouth) break off, and each piece develops into a new individual (a phenomenon known as “pedal laceration”). The regeneration of an entire polyp from autotomized tentacles (e.g., the sea anemone *Bolocerooides mcmurrici* and the stony coral *Euphyllia glabrescens*) and tissue fragments (e.g., the soft coral *Dendronephthya hemprichi*) occurs in several anthozoans (Toh and Ng, 2016). The term “fragmentation” is also often used to describe the formation of a large colony from a fragmented piece of a coral colony.

A phenomenon known as “polyp bail-out” is also related to asexual reproduction (Kramarsky-Winter et al., 1997). In some colonial stony corals (e.g., *Pocillopora damicornis*), the polyps leave their skeleton when the corals become unhealthy under unfavorable environmental conditions. The polyp undergoes resettlement and calcification after reaching an appropriate location and forms a new colony by successive asexual reproduction (e.g., budding). A similar phenomenon called “polyp expulsion” occurs in the stony coral *Oculina patagonica*. The polyps leave their colony together with a part of the skeleton. Interestingly, this phenomenon occurs even in physiologically healthy corals in normal environmental conditions (Kramarsky-Winter et al., 1997). In addition, the production of offspring from unfertilized eggs, or parthenogenesis, has also been demonstrated in some anthozoans (Shikina and Chang, 2016).

Hydrozoa

This class is composed of approximately 3000 species and includes some freshwater species. Hydrozoans are generally thought to have a life cycle that alternates between the polyp and medusa stages, but modification of the life cycle occurs in many species in which the polyp or medusa stage is reduced or completely suppressed.

Asexual reproduction occurs in both the polyp and medusa stages with various patterns (Table 1) (Boero et al., 2002). Polyp budding is mainly responsible for the asexual production of new polyps and the growth of the colony. In solitary species (e.g., *Hydra magnipapillata*), buds (new polyps) may arise from the lateral part of the polyp. In some colonial species (e.g., *Hydractinia echinata*), buds may arise from the stolon, which is a creeping, root-like component of the colony that adheres to the substratum. In other species that form erect colonies of polyps (e.g., *Obelia* sp.), the buds may arise from the stolon and the stems of the polyps. In some hydrozoans, polyp budding can also produce podocysts (cellular aggregates containing the cellular and organic compounds required for the formation of new polyp) or frustules (small, simple pieces of tissue with planula-like structures) that can transform into polyps under favorable environmental conditions. In addition, the reformation of a colony from fragmented pieces of polyps (fragmentation) and the formation of propagules in the polyps also commonly occur in hydrozoans.

Hydrozoan polyps generally undergo budding to produce medusae. The buds (or gonophore buds) may develop into new medusae on the polyps and are released to begin the free-swimming medusa stage. However, in many hydrozoans, the medusa stage is no longer free-swimming. The medusae develop incompletely and remain attached to the polyp (Boero et al., 2002). The degree of medusa development (or reduction) on the polyp varies within hydrozoans. Medusae of some species have radial canals, subumbrellar cavities, and velum but do not have tentacles or mouths. Medusae of other species have no traces of medusae-like characteristics except for gonads.

Asexual reproduction of medusae also occurs in hydrozoans (Boero et al., 2002). In some hydrozoans, a single medusa can also directly produce many medusae by fission (e.g., *Cladonema* sp.) or budding (e.g., *Euphyssilla pyramidata*). Budding occurs at specific sites of medusae, including the bell margin, the marginal bulbs, the radial canals, and the gonads, and the budding sites may differ among species. In addition, some hydrozoans (e.g., *Zanclea* sp.) produce polyps directly on the body of the medusae by budding. The buds (new polyps) may arise either on the manubrium or on the radial canals where gonads normally develop. Some hydrozoans (e.g., *Hydra*) have a remarkable ability to regenerate. An entire animal can be regenerated from a small fragment of polyp tissues. *Turritopsis nutricula* is unique as it undergoes reverse development from an adult medusa to a polyp under unfavorable environmental conditions.

Scyphozoa

This class is composed of approximately 200 marine species. Scyphozoans generally have a life cycle that alternates between benthic polyp (known as scyphistoma) and planktonic medusa stages. Modification of the life cycle also occurs in some species.

Longitudinal fission and polyp budding are the main forms of asexual reproduction in scyphozoans (Table 1) (Arai, 1997; He *et al.*, 2015). The buds may arise either from the column of the polyp wall or a stolon, and become small polyps.

The polyps undergo transverse fission to form young medusae after they grow to a certain size under favorable environmental conditions (e.g., temperature, light, and chemical composition of water) at the appropriate time of year. This process, known as strobilation, is not only a mode of asexual reproduction but also a form of metamorphosis. The first sign of the strobilation is the appearance of transverse constrictions at the distal end of the polyp. As the number of constrictions on the polyp gradually increases, the disc-like medusae with eight tentacles become distinct, and the tentacles of the polyp regress. A polyp undergoing strobilation is called a strobila. Within a few days, the young medusae begin to actively contract on the distal part of the strobila and are released from the strobila by transverse fission. The young, free-swimming medusae are now called ephyrae. The number of ephyrae produced by strobilation varies among species and may be influenced by the environmental conditions. The number of ephyrae ranges from one (monodisc strobilation) to many (polydisc strobilation). Strobilation can be experimentally induced in some species (e.g., *Aurelia*) using treatments of iodine-containing compounds.

As another important means of asexual reproduction, some scyphozoan polyps (e.g., *Cyanea* spp.) can produce chitin-covered cysts (also called podocysts) that can directly develop into new polyps. Its formation can be induced under unfavorable environmental conditions (starvation, high temperature, etc.) and the podocysts can survive for several years. The podocysts hatch (excyst) under environmental conditions favorable for the survival of the polyp. The formation of podocysts is thought to be a way to maintain the population. The planulae of some scyphozoans can also produce cysts (called planulocysts) that are functionally similar to podocysts prior to the formation of polyps. In addition, some scyphozoan polyps can produce free-swimming propagules that can also develop into new polyps.

As a form of asexual reproduction of medusae, direct formation of polyps on living medusae has been observed in a species of *Aurelia* (He *et al.*, 2015). In addition, the direct development of ephyra from planula (without polyp formation and strobilation) occurs in some scyphozoans and represents an example of the modification of a typical life cycle. The formation of polyps from ephyrae (or “reverse development”) also occurs in some species (e.g., *Chrysaora hysoscella*, *Rhizostoma pulmo*, and *Aurelia aurita*) (He *et al.*, 2015).

Cubozoa

This class is composed of approximately 50 species. Cubozoans have a typical life cycle that alternates between the polyp (asexual) and medusa (sexual) stages. In asexual reproduction, polyps generally form buds to produce new small polyps (Table 1) (Toshino *et al.*, 2015). For medusa production, an entire polyp directly metamorphoses into a single medusa. However, in some species (e.g., *Morbakka virulenta* and *Carukia barnesi*), only the apical part of a single polyp is transformed into a single medusa and is detached from the polyp (called monodisc strobilation). In the species that undergo monodisc strobilation, the polyp left behind after the medusa detaches can also asexually reproduce by budding. The regeneration of a new polyp from a small portion of the polyp fragment (named residuum) left behind after the medusa detaches occurs in some species (e.g., *Carybdea marsupialis*).

Staurozoa

This class is composed of approximately 50 species. Staurozoans also have a life cycle with polyp (asexual) and medusa (sexual) stages similar to other medusozoans. However, the morphological differences between these two stages are much smaller than that observed in other medusozoan classes. A single medusa is produced by the metamorphosis of a polyp, but metamorphosis mainly occurs in the apical regions of the polyp, such as the formation of arms, gastric filaments, gonads, and adradial tentacular clusters (Miranda *et al.*, 2017). Because the polyp remains attached to the substrate even after metamorphosis into a medusa, staurozoans consequently have a mosaic body structure, in which the apical region has a medusa-like structure, whereas the basal region has a sessile polyp-like stalk during the medusa stage. Strobilation or budding does not occur when the polyps metamorphose into medusae.

In asexual reproduction, budding occurs in both the polyp and early larval stages (Table 1) (Miranda *et al.*, 2017). In the polyp stage, polyps form buds at the lateral region of the calyx (upper part of the polyp) or at the distal parts of some special tentacles of the polyp. The buds break off and are assumed to form frustules that develop into polyps under suitable conditions. In the early larval stages of some species (e.g., *Halicystus octoradiatus*), planulae that have attached to the substrate (microhydrula stage, hemispherically shaped planula) can also asexually produce protuberances (buds) that may become polyps.

Sexual Reproduction

As in other animals, cnidarians also have the ability to create sperm and eggs that contain all the materials required for the initiation of embryonic development and the maintenance of its physiological functions. Sexual reproduction allows cnidarians to not only foster genetic diversity but also facilitate species expansion into different habitats and create new populations. In the following section, the general aspects of cnidarian reproduction are outlined.

Sexuality

Gonochorism (sperm and egg production in separate individuals) is common in cnidarians. In many cases, there is no difference between the appearance of males and females. The type of gametes (sperm or eggs) created in the individual defines the sexuality. Hermaphroditism (simultaneously produce eggs and sperm in an individual) also occurs in cnidarians. For example, approximately 70% of stony corals (order Scleractinia) are hermaphroditic (Harrison and Wallace, 1990). In some anthozoans and hydrozoans, sex changes (or sex reversal) have been observed at different life stages (e.g., solitary mushroom stony corals and *Hydra*). Very little information is available regarding the mechanisms of sex determination in cnidarians. In *Hydra*, the sex of an individual is determined by the sex of the germline stem cells present in the body (Nishimiya-Fujisawa and Kobayashi, 2012).

Gonads

Cnidarians have specific sites for gamete production in their bodies. The sites are generally referred to as gonads (testis or ovary), although cnidarians are regarded to have no true organs. Gonads are very small, thin, and almost invisible when gametogenesis is not occurring, but they grow in size and can be observed to have distinct swelling (or evagination) during active gametogenesis. In many cnidarians, the appearance of mature ovaries and testes differs in size, shape, and color. The gonads of cnidarians generally develop in the gastrodermis, except in some hydrozoans in which the gonads develop in the epidermis of the polyps. The location of the gonads in the body varies between the classes.

Origin of the Gametes

The gametes of some hydrozoans, such as *Hydra* (e.g., *H. magnipapillata*) and colonial hydroids (e.g., *H. echinata*), originate from the interstitial cells (i-cells) that exist in the interstitial space of the polyp column epidermis (Nishimiya-Fujisawa and Kobayashi, 2012). In *Hydra*, the i-cell population primarily contains two types of stem cells, multipotent stem cells (MPSCs) and germline stem cells (GSCs). The MPSCs have the ability to both self-renew and differentiate into multiple somatic cell types (e.g., neurons, gland cells, and cnidocytes) and gametes. In contrast, the GSCs can self-renew but can only differentiate into gametes. Thus, *Hydra* gametes originate from these two types of stem cells. The origins of the gametes in other cnidarian classes have not been well demonstrated. However, cnidarian germ cells are assumed to originate in the endoderm and develop in the gastrodermis where many cnidarians have gonads. In some cnidarians, such as stony corals (order Scleractinia) and some scyphozoans, gametes are classically assumed to originate from i-cells as occurs in hydrozoans. Further studies are needed to demonstrate whether i-cells or cells functionally similar to i-cells also exist in other cnidarian classes.

Spermatogenesis

The detailed processes of spermatogenesis have been well documented in anthozoans, scyphozoans, and some hydrozoans using histological and ultrastructural analyses, and they appear to be similar to that of many other animals. The first recognizable constituent of spermatogenesis is the proliferation of spermatogonia (or interstitial cells) and the formation of spermatogonial clusters. In anthozoans and scyphozoans, the spermatogonial clusters have been observed to move into the mesoglea and form spermatogenic compartments in the mesoglea of gonads, which are called spermaries, cysts, or spermatic follicles (Arai, 1997; Shikina and Chang, 2016). Further spermatogenic processes, such as the successive proliferation of spermatogonia, meiotic differentiation, and spermiogenesis, occur within each of the spermatogenic compartments. During active spermatogenesis, the male germ cells at different developmental stages can be observed simultaneously in a single spermatogenic compartment. In some scyphozoans, male germ cells maintain contact with some testicular somatic cells, which may support the development of male germ cells.

Oogenesis

The processes of oogenesis are well documented in anthozoans, scyphozoans, and some hydrozoans. In anthozoans and scyphozoans, the first recognizable constituent of oogenesis is the proliferation of oogonia or the appearance of early oocytes in the gonads (Arai, 1997; Shikina and Chang, 2016). After oogonia differentiate into oocytes by entering meiosis, the early oocytes move into the mesoglea and develop there until maturation. Oocyte development is supported by specialized ovarian somatic cells (e.g., “trophocytes” observed in scyphozoans) that are in contact with or are adjacent to the oocytes. The somatic cells nourish the oocytes by providing nutritive materials such as yolk proteins. In some anthozoans (e.g., the stony coral *Euphyllia ancora*), several types of yolk proteins such as vitellogenin and egg proteins are produced by ovarian somatic cells and are then transported to and accumulate in the oocytes (Shikina and Chang, 2016). In contrast, the pattern of oocyte development is different in some hydrozoans from that described above. For example, in *Hydra*, oogenesis begins with the proliferation and migration of i-cells to form an ovary (egg patch) on the epidermis of the polyp column. Several thousand i-cells are observed in an egg patch, but only one of the i-cells grows as an oocyte, and the remaining i-cells differentiate into nurse cells. For oocyte growth, nurse cells transfer their cytoplasm to the developing oocytes, undergo apoptosis, and are finally phagocytosed by the oocyte. Hence, in *Hydra*, oocyte development is primarily supported by the fusion of germ-cell-derived nurse cells with oocytes but not by the ovarian somatic cells.

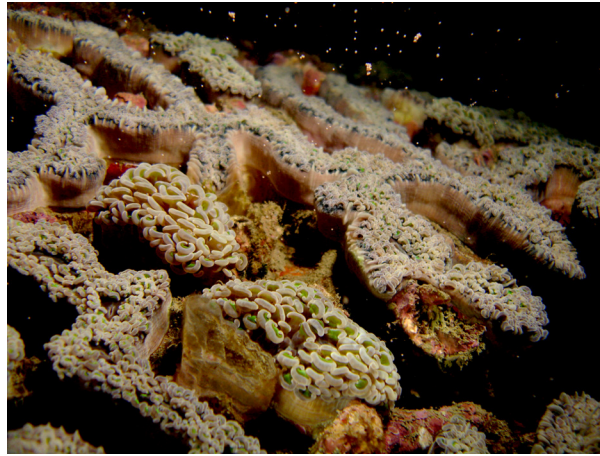


Fig. 3 Spawning of the stony coral *Euphyllia ancora* (Class Anthozoa, Order Scleractinia).

The processes of oocyte maturation are well documented in some hydrozoans. Germinal vesicle breakdown (GVB) and polar body formation occur in the oocytes prior to spawning. In cnidarians, the size of fully grown oocytes may range from 30 μm (e.g., the staurozoan *Halichystus octoradiatus*) to approximately 800 μm (e.g., the anthozoan *Acroporidae*) in diameter.

Spawning and Fertilization

In most cnidarians in which the gonads develop in the gastrodermis (such as anthozoans, scyphozoans, cubozoans, and staurozoans), mature gametes are released into the gastrovascular cavity and expelled through the mouth. In contrast, in some hydrozoans in which gonads develop in the epidermis, gametes are directly released to the surrounding water. External fertilization is typical in cnidarians, but internal fertilization also occurs in some cnidarians that brood their embryos. One of the best-known phenomena in cnidarian reproduction is stony coral spawning events (order Scleractinia) (Harrison and Wallace, 1990). Synchronized spawning of many stony corals (ranging from 10 to more than 100) over short periods (approximately 1 week) has been recorded in various locations in the Indo-Pacific and the Atlantic Ocean (Fig. 3). The presence of species-specific agents that attract sperm to spawned eggs has been suggested in some corals, but cross-fertilization appears to occur occasionally between closely related species. The timing of spawning at each location is becoming predictable with regard to the period (month) of the year, lunar day, and time of day based on previous spawning records.

Factors Affecting Gametogenesis and Spawning

Various environmental and intrinsic factors influence the timings of gametogenesis and spawning, and these factors may interact with each other. The environmental factors include light/dark period, light intensity, moonbeams, changes in seawater temperature, and food availability. For instance, in many cnidarians that spawn annually, the progress of gametogenesis is influenced by seasonal changes in sea surface temperature. The same species that inhabit different geographic regions may respond to different environmental factors, and this may be associated with the surrounding environmental conditions. In contrast, the intrinsic factors include age, body or colony sizes, and nutritional status. In addition, endocrine-like substances such as neuropeptides (LWamide family) and neurotransmitters (serotonin and dopamine) are also associated with the regulation of spawning in some cnidarians (Shikina and Chang, 2016). Little is known about the endocrine regulation of gametogenesis in cnidarians.

Embryonic and Larval Development

In a typical cnidarian life cycle, a fertilized egg develops into a planula, which in turn metamorphoses into a polyp. In embryonic development, the first cleavage is a meridional division that begins at the animal pole. Generally, as the first division is incomplete and the vegetal pole cytoplasm remains partially connected, a heart-shaped figure is formed. The zygote undergoes holoblastic cleavage to form a blastula. Later, gastrulation occurs at the animal pole to form the ectoderm and endoderm, which eventually become the epidermis and gastrodermis, respectively. The site of gastrulation (blastopore) becomes the mouth, and further embryonic development leads to a planula. Planula larvae are generally ciliated and swim with the aboral end (the opposite end of the mouth) in front. The planulae of staurozoans are not ciliated but can move by a series of extensions and reactions (creeping) (Miranda *et al.*, 2017). After locating an appropriate site to settle, the planula attaches its aboral end to the bottom and metamorphoses into a juvenile. In some anthozoans and hydrozoans, neuropeptides of the LWamide family are involved in the metamorphosis (Takahashi and Hatta, 2011). In addition, in some stony corals, metamorphosis requires extrinsic stimuli such as agents produced by bacteria that live in the ocean.

References

- Arai, M. N. (1997). *A Functional Biology of Scyphozoa*. Dordrecht: Springer.
- Bocharova, E. S., & Kozevich, I. A. (2011). Modes of reproduction in sea anemone (Cnidaria, Anthozoa). *Biology Bulletin*, 38, 849–860.
- Boero, F., Bouillon, J., Piraino, S., & Schmid, V. (2002). Asexual reproduction in the Hydrozoa (Cnidaria). In K. G. Adiyodi, & R. G. Adiyodi (Eds.), *Reproductive Biology of Invertebrates* (vol. XI, pp. 116–158). New Delhi: Oxford & IBH.
- Harrison, P. L., & Wallace, C. C. (1990). Reproduction, dispersal and recruitment of scleractinian corals. In Z. Dubinsky (Ed.), *Ecosystems of the World 25: Coral Reefs* (pp. 133–207). Amsterdam: Elsevier.
- He, J., Zheng, L., Zhang, W., & Lin, Y. (2015). Life cycle reversal in *Aurelia* sp.1 (Cnidaria, Scyphozoa). *PLOS ONE*, 10, e0145314.
- Kramarsky-Winter, E., Fine, M., & Loya, Y. (1997). Coral polyp expulsion. *Nature*, 387, 137.
- Miranda, L. S., Mills, C. E., Hirano, Y. M., Collins, A. G., & Marques, A. C. (2017). A review of the global diversity and natural history of stalked jellyfish (Cnidaria, Staurozoa). *Marine Biodiversity*. <https://doi.org/10.1007/s12526-017-0721-4>.
- Nishimiya-Fujisawa, C., & Kobayashi, S. (2012). Germline stem cells and sex determination in *Hydra*. *International Journal of Developmental Biology*, 56, 499–508.
- Ruppert, E. E., Fox, R. S., & Barnes, R. D. (2003). *Invertebrate zoology: A functional evolutionary approach* (seventh ed., pp. 111–180). Belmont, CA: Thomson-Brooks/Cole.
- Shikina, S., & Chang, C.-F. (2016). Sexual reproduction in stony corals and insight into the evolution of oogenesis in Cnidaria. In S. Goffredo, & Z. Dubinsky (Eds.), *The Cnidaria, Past, Present and Future* (pp. 249–268). Cham: Springer.
- Takahashi, T., & Hattai, M. (2011). The importance of GLWamide neuropeptides in cnidarian development and physiology. *Journal of Amino Acids*, 2011, 424501.
- Technau, U., & Steele, R. E. (2011). Evolutionary crossroads in developmental biology: Cnidaria. *Development*, 138, 1447–1458.
- Toh, T. C., & Ng, C. S. L. (2016). Tentacular autotomy and polyp regeneration in the scleractinian coral *Euphyllia glabrescens*. *Coral Reefs*, 35, 819.
- Toshino, S., Miyake, H., Ohtuka, S., et al. (2015). Monodisc strobilation in Japanese giant box jellyfish *Morbakka virulenta* (Kishinouye, 1910): A strong implication of phylogenetic similarity between Cubozoa and Scyphozoa. *Evolution and Development*, 17, 231–239.

Reproduction in the Platyhelminthes (Flatworms)

Graham Clive Kearn, University of East Anglia, Norwich, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Background

Platyhelminths (flatworms) have unsegmented, bilaterally symmetrical, soft bodies, with a triploblastic construction (comprising three layers of cells) and lacking a coelom (body cavity), skeleton and anus. The relatively large flatworms (e.g. planarians, tape-worms) are indeed dorsoventrally flattened but many of the small free-living platyhelminths are cylindrical.

Representatives of the main groups of the Phylum Platyhelminthes are illustrated in Fig. 1. Three of these groups, the Monogenea, Cestoda (tapeworms) and Digenea (flukes) are exclusively parasitic. Monogeneans have a single-host life cycle and are mostly external parasites (ectoparasites) on the skin and gills of fishes. Cestodes and digeneans are endoparasites (internal parasites) and have more complex life-cycles, each with two or three hosts, one of which is a vertebrate. Digeneans have an additional endo-parasitic stage in a mollusc.

Traditionally, all the flatworms that do not belong in the three parasitic groups are placed in the Turbellaria. This is not a valid taxon, but is widely used as a convenient term to accommodate largely free-living members such as acoels, rhabdocoels, triclads and polyclads.

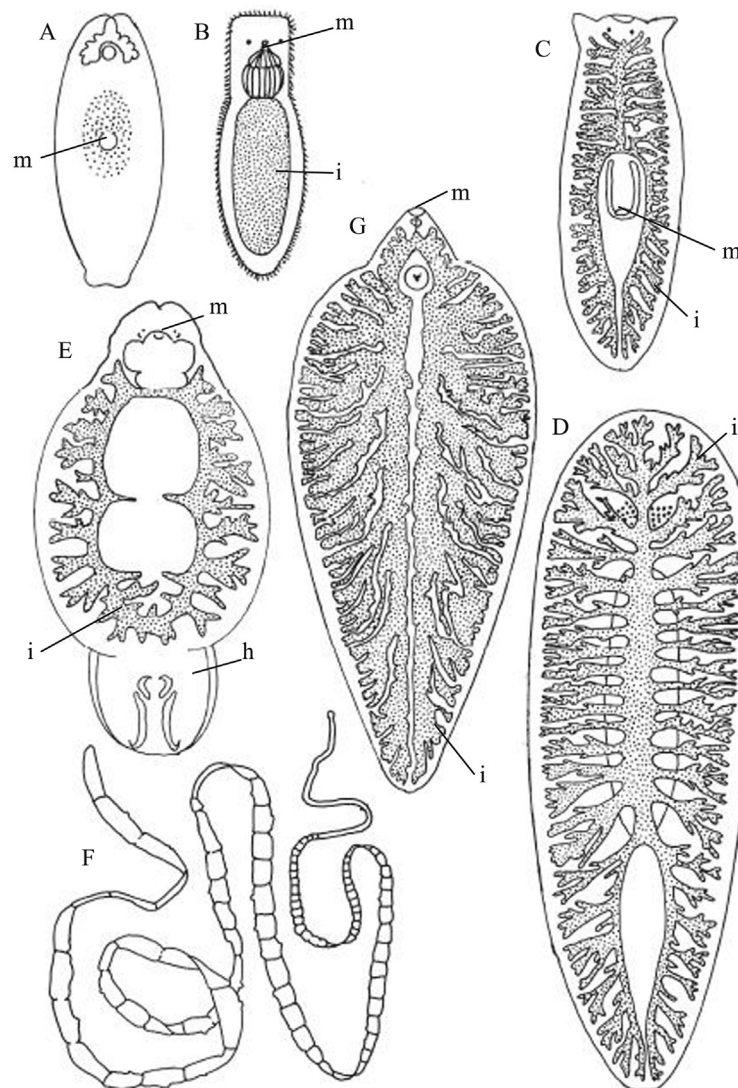


Fig. 1 The platyhelminth panorama. (A), an acoel, *Anaperus* sp.; (B), a rhabdocoel, *Paravortex* sp.; (C), a triclad, *Dendrocoelum lacteum*; (D), a polyclad, *Notoplana* sp.; (E), a monogenean, *Entobdella soleae*; (F), a cestode (tapeworm), *Taenia* sp.; (G), a digenean, *Fasciola hepatica*. Acoels lack a digestive cavity – the mouth opens into a syncytium (a mass of nucleated digestive cells). Cestodes lack a gut. h – haptor (attachment organ); i – intestine; m – mouth. Redrawn from Kearn, G.C., 1998. Parasitism and the Platyhelminths. London: Chapman & Hall.

Sexual Reproduction

In the cnidarians (anemones; jellyfish) the reproductive organs consist of nothing more than the gonads themselves, but in platyhelminths the reproductive organs have become remarkably complex. Fertilisation is internal in flatworms and a few have separate sexes (gonochorism), as, for example, turbellarians of the genus *Kronborgia* and schistosome digeneans (Kearn, 1998). However, most platyhelminths are hermaphrodite (male and female gonads occur in the same individual). They are also protandrous, i.e. the male system reaches maturity before the female system, with the exception of gyrodactylid monogeneans which are protogynous (female system is the first to reach maturity). The obvious advantage of the hermaphrodite condition compared with unisexual animals is that whenever two individuals meet bilateral cross-insemination is possible. There is potential too for unilateral insemination and self-insemination.

The Female Reproductive System

The Egg and the Eggshell

Platyhelminth embryos are protected in the outside world by a strong case, usually referred to as an eggshell when the case contains a single embryo, and a capsule when more than one embryo is enclosed, as in some planarians.

In the platyhelminths there has been an important shift in the source of provisions required for growth and development of the embryo or embryos, and for the assembly of the eggshell or capsule. In the primitive flatworms, so-called 'archoophorans', which include the acoel and polyclad turbellarians, the food (yolk) required by the growing embryo is included in the cytoplasm of the egg cell (endolecithal condition) and the shell material is partly derived from a shell gland and partly from droplets located near the surface of the egg cell. In the more advanced flatworms, the 'neophorans', which include most of the platyhelminths that are associated with other animals (symbiosis), an ectolecithal condition has arisen by subdivision of the ovary.

The production of egg cells destined to develop into new individuals is restricted to the compact region of the ovary known as the germarium (Fig. 2). However, the egg cells in the germarium no longer provide significant quantities of yolk. This is now provided by the other subdivision of the ovary called the vitellarium. This is a follicular organ spreading throughout the body of the animal and producing vitelline cells, which make no genetic contribution but contain abundant yolk. The vitelline cells also have the important role as the source material destined to become the eggshell or the wall of the capsule. The shell gland has become redundant.

The ducts from the vitelline follicles coalesce to form a vitelline duct, which joins the oviduct to form the ovovitelline duct (Fig. 2). The vitelline duct may expand to form a vitelline reservoir providing storage for vitelline cells until required. If there is a gut present (tapeworms have no gut), the vitelline follicles are closely associated with the intestine and its branches. This is a significant relationship since flatworms have no blood system and the proximity of gut and vitellarium ensures a supply of nutrients to the actively metabolising vitellarium with minimal transport costs.

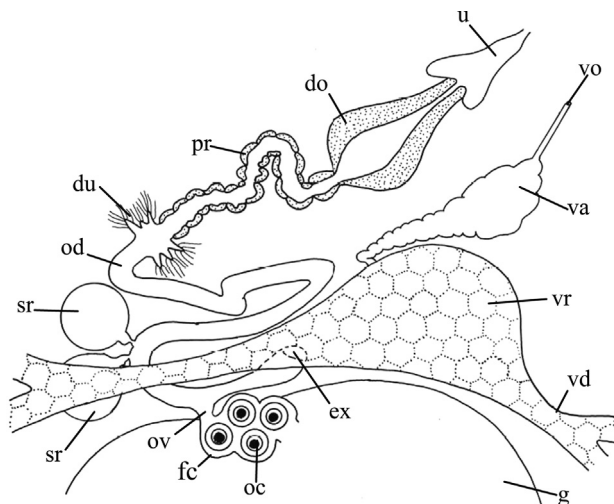


Fig. 2 The egg assembly apparatus of the monogenean *Entobdella soleae* in ventral view. do – distal tetrahedral chamber of oötype; du – ducts of oötype glands (Mehlis' gland?); ex – exit of common vitelline duct from the vitelline reservoir; fc – fertilisation chamber; g – germarium; oc – oöcyte; od – ovovitelline duct; ov – oviduct; pr – proximal tubular region of oötype (where the egg stalk is assembled); u – proximal region of uterus (distal region not shown); va – vagina; vd – transverse vitelline collecting duct; vo – vaginal opening; vr – vitelline reservoir; sr – seminal receptacle. Reproduced with permission from Kearn, G.C., 1985. Observations on egg production in the monogenean *Entobdella soleae*. International Journal for Parasitology 15, 187–194. Elsevier, © 1985.

The Oötype and Egg Assembly

Distally, the ovo-vitelline duct expands to form a muscular organ in which shelled eggs or capsules are assembled one at a time (Fig. 2; Kearn, 1998). In the umagillid turbellarians this organ is termed the “primary uterus”, followed in many umagillids by a “secondary uterus” (Shinn (1986) and references therein). However, in spite of using the same term “uterus” these two organs are functionally very different, the first being the site of egg/capsule assembly and the second acting as a store for shelled eggs or capsules (see below). Consequently, the terms “oötype” and “uterus” are preferred here (Fig. 2; Kearn, 1998), in place of “primary uterus” and “secondary uterus” respectively, as, for example, in Shinn (1986).

In the monogenean *Entobdella soleae* mature egg cells (oöcytes) are fertilised in a germarial fertilisation chamber (Fig. 2). One of these fertilised oöcytes is released and makes its way along the ovovitelline duct to the oötype, followed by a stream of vitelline cells from the vitelline reservoir. Vigorous contractions of the oötype are accompanied by release of vitelline droplets from the vitelline cells. These droplets coalesce around the contents of the oötype to form the eggshell. The oötype acts as a mould and presses the shelled egg into shape – a tetrahedron in *E. soleae*.

Quinone-Tanning

The eggshell/capsule wall is water-white when first made but undergoes a gradual change in colour from water-white to golden brown (Kearn, 1998). This colour change is the visual manifestation of a chemical reaction that leads to changes in the properties of the shell/capsule wall material from soft and pliable to hard and tough. Resistance develops to physical and chemical damage and, in particular, to attack by proteolytic enzymes (i.e. enzymes that digest proteins). These changes are attributable to the process of quinone-tanning or sclerotisation (see review by Waite (1990)). The chemistry of this process is not fully understood but basically it is as follows: catechols are oxidised by the enzyme catechol oxidase to quinone, which then combines with protein.

If the presence of catechols, catechol oxidase and protein indicate the presence of the quinone-tanning process, then the reaction is widespread throughout the Animal Kingdom. It occurs in the walls of spores, cysts, egg capsules and cocoons in protozoans, platyhelminths, nematodes, molluscs, annelids, arthropods and elasmobranch fishes and is prevalent in other structures such as cnidarian skeletons, byssus threads and shells of molluscs and arthropod cuticles.

Catechols, catechol oxidase and proteins are present together in peripheral droplets in the vitelline cells or in the cortical granules of archoophoran egg cells. Why these components do not interact prematurely is not known. Whatever the mechanism of quinone-tanning is, it renders eggshells/capsules physically strong and chemically resistant. Tanned shells/capsules provide sealed compartments to hold vitelline cells and egg cells in close proximity, preventing foreign microorganisms from gaining access to resources destined for the platyhelminth embryos. In fact, Macdonald (quoted by Kearn (1975)) demonstrated that shelled eggs of *Entobdella soleae*, fed experimentally to various crustacean predators, pass through the gut of the predator intact, and, provided that the eggshells are not punctured, are capable of continuing to develop and hatch. This provides experimental support for the suggestion of Llewellyn (1965) that the tanned shells/capsules of freely deposited platyhelminth eggs confer protection on embryos swallowed by predators.

Hatching

This raises the question as to how hatching takes place. A tanned flatworm egg, like that of *E. soleae*, has a single circular suture separating a lid or operculum from the rest of the eggshell. The larva (oncomiracidium) escapes by dissolution of the thin layer of cement holding the operculum in place. Observations by Kearn (1975) indicate that hatching in *E. soleae* is brought about by a proteolytic (protein-digesting) hatching fluid produced by two pairs of gland cells located ventrally in the head of the fully developed oncomiracidium. Hatching in *E. soleae* begins when the surface cilia on the body of the oncomiracidium begin to beat. This imparts rotation to the oncomiracidium about its longitudinal body axis. The head, which is located beneath the operculum, slowly rotates at a speed of about 6 s per revolution and during rotation the openings of the hatching glands are adjacent to the opercular discontinuity. Presumably the secretion that these glands produce slowly weakens the opercular cement until the seal finally breaks and the operculum becomes detached. The oncomiracidium squeezes through the opercular opening aided by traction generated by the continually beating cilia.

The tanned egg capsules of the parasitic turbellarian *Syndisyrinx franciscanus* usually contain 6–8 fertilised eggs (zygotes) (Shinn, 1983). Hatching takes place in a similar way to the shelled eggs of *E. soleae*. However, the turbellarian capsule has numerous sutures arranged in a reticulum. Shinn and Cloney (1986) observed that prior to hatching the sutures are severely weakened by the activities of the contained larvae and on hatching the capsule shatters into many pieces. Shinn and Cloney found evidence to suggest that the embryos secrete a hatching enzyme since intact capsules are unaffected by externally applied trypsin (a powerful protein-digesting enzyme) but when capsules are cut open and then incubated in trypsin, the sutures become weakened. Shinn and Cloney hypothesised that the ability of sutures to resist enzymatic attack from the outside but not from the inside, is the result of differences in the chemical properties of the cement in outer and inner parts of the sutures.

The digenean *Fasciola hepatica* (sheep liver fluke) has a tanned egg and contains a ciliated larva (miracidium) destined to infect a mollusc. Surprisingly, the hatching mechanism of *F. hepatica* has little in common with that of *Entobdella soleae*. They share the first step, which involves activation of the enclosed miracidium or oncomiracidium. Thereafter their hatching events are very different. As noted above *E. soleae* uses glands to dissolve the cement holding the operculum in place, while the miracidium of *Fasciola* is unable to enter the opercular end of the ovoid egg because it is occupied by a so-called viscous cushion (Fig. 3). After activation

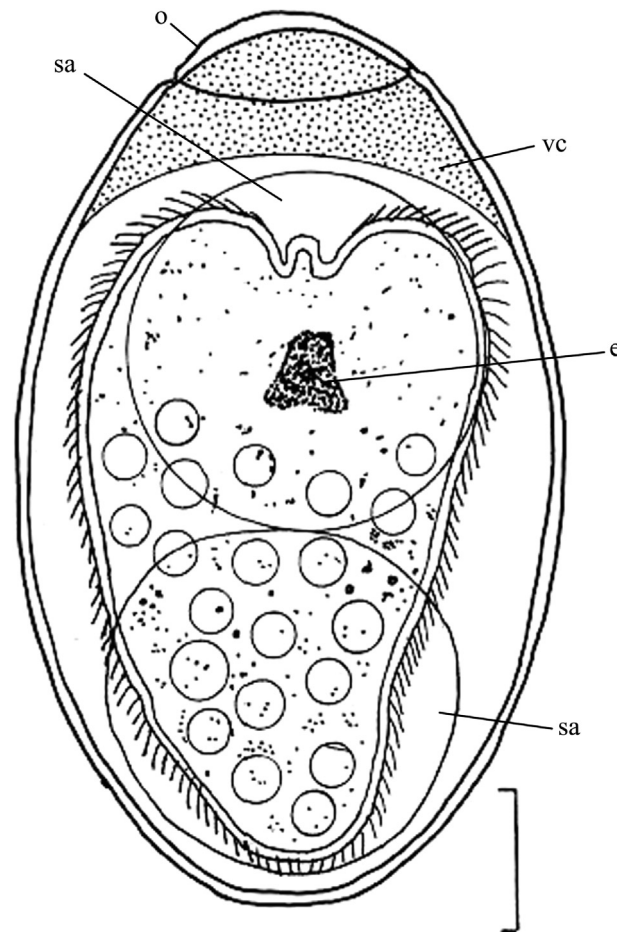


Fig. 3 Egg of the digenean *Fasciola hepatica* containing a fully developed larva (miracidium). e – eye; o – operculum; sa – sac; vc – viscous cushion. Scale bar: 25 μ m. Redrawn from Smyth, J.D., Halton, D.W., 1983. *The Physiology of Trematodes*, second ed. Cambridge: Cambridge University Press.

of the miracidium, the cushion and two separate thin-walled sacs expand, increasing the pressure progressively inside the shelled egg until the opercular seal ruptures (Wilson, 1968). Wilson offered arguments against the notion that a hatching enzyme weakens the opercular cement in *Fasciola*.

The Uterus

Many platyhelminths have no uterus, the oötype opening directly or via a short tube to the outside (Fig. 2). Tanning needs to be completed while the shelled egg or capsule is in the oötype, but the disadvantage of this is that, while the shelled egg/capsule occupies the oötype, further egg/capsule assembly cannot take place. The provision of a uterus (secondary uterus of Shinn (1986); see above) permits the shelled egg/capsule to leave the oötype and complete tanning of the shell or capsule wall, thereby making way for the assembly of the next shelled egg/capsule. Shinn (1986) found that the maximum rate of capsule production in the turbellarian *Wahlia pulchella* was 5–15 times greater than that of other umagillids lacking a uterus. Thus, acquisition of a uterus for the storage of shelled eggs/capsules opens up an evolutionary pathway that has been exploited in remarkable ways by many platyhelminths. This is well illustrated by the relationship between polystomatid monogeneans parasitising amphibious hosts (frogs and toads).

Amphibious vertebrates such as frogs and toads (anurans) present a formidable challenge for potential parasites because transmission cannot take place while the host is on land. The biology and ecology of the monogenean polystomatid parasite *Pseudodiploporchis americanus* from the urinary bladder of the North American desert toad (*Scaphiopus couchii*) has been studied in depth by Tinsley and co-workers over many years and provides some of the most exciting and interesting discoveries in Parasitology (see references in Kearn (1998)). *Scaphiopus couchii* is almost exclusively terrestrial, spending most of the year (9–10 months) buried in the desert sand (see references in Kearn (1998)). The toads emerge from the sand to spawn following torrential rain, which generally occurs on only 1–3 nights of the year, creating temporary pools in the desert. Remarkably, *P. americanus*, inhabiting the urinary bladders of these toads, is ready to take advantage of this very brief window of opportunity, since their uteri are packed with eggs containing fully developed oncomiracidia. As the toads enter the spawning assemblies in the pools, the larvae, each enclosed by

a thin sac possibly produced by the uterus, are released and hatch. The ciliated oncomiracidia attach themselves to half-submerged toads, crawl over the toad's skin above the water surface and enter the nostrils. They begin development in the respiratory sac then migrate through the gut to the urinary bladder. The parasites then mature and accumulate infective larvae in the uterus, so that they are ready one year hence for the next annual spawning event. This phenomenon is known as ovoviviparity.

Growth of the embryos in the uterus of *P. americanus* is substantial and the sac (shell?) enclosing each embryo stretches to accommodate the gradual increase in size. However, there are only two or three vitelline cells accompanying the early embryo and they are unlikely to make a significant contribution to the nutrition of the growing embryo. It seems more likely that food reaches the growing embryo via the wall of the uterus. A protective shell is no longer a requirement for eggs like those of *P. americanus* that hatch as soon as they leave the host. The parasite needs to accommodate as many eggs as possible in the uterus and this can be better achieved by having flexible 'shells' rather than rigid tanned shells.

The functions of the vitellarium in *P. americanus* have now been superseded and the organ is greatly reduced, the space being made available for the huge uterus. The germarium retains its anterior position with the uterus posterior to it (Tinsley, 1978, in [Kearn \(1998\)](#)). Another polystomatid, *Eupolystoma anterorchis*, from the urinary bladder of a South African toad (*Bufo pardalis*), is also ovoviviparous, storing shelled eggs in a large uterus while the toad is on land. However, *E. anterorchis* creates space for the large uterus in a different way from *Pseudodiplorchis* by posterior relocation of the germarium and lateral displacement of vitelline and testicular follicles. The implication is that ovoviviparity has evolved independently in *Pseudodiplorchis* and *Eupolystoma*.

Use of the uterus to store shelled eggs reaches unprecedented levels in the cyclophyllidean tapeworms. Their elongated bodies (strobilae) ([Fig. 1\(F\)](#)) are sub-divided into 'segments' termed proglottids each of which contains a complete set of reproductive organs including a uterus. The cyclophyllidean uterus is blind-ending, branched and contains thousands of fully developed eggs with complex untanned shells. Ultimately each gravid (egg-filled) proglottid becomes detached from the strobila, passes out in the host's faeces and bursts liberating and scattering the eggs. If eaten by the next host in the life cycle the untanned egg-shell is vulnerable and is digested releasing the living tapeworm larva.

In some cyclophyllideans profound changes affect the uterus as it fills with shelled eggs. New structures called paruterine organs may appear, into which the eggs are passed from the uterus. The function of these organs is unclear.

The Male Reproductive System

The male copulatory organs of monogeneans deserve special attention because of their remarkable diversity, greatly surpassing the rather conservative organs in the tapeworms (Cestoda) and the digeneans (Trematoda). The male gonad (testis) gives rise to a special gonoduct, the vas deferens, which transports mature sperm distally and often expands to form a seminal vesicle where sperm is stored.

Penis or Cirrus

The distal end of the vas deferens is modified to form an intromittent organ, which may be a penis or a cirrus. Of the two, penises encompass an enormous range of copulatory organs. In its simplest forms the penis may be a protrusible muscular organ or the sclerotised (hardened) tubular termination of the male gonoduct, but these organs are often accompanied by a remarkable range of accessory sclerites ([Fig. 4](#)) and accessory glands, the latter often misleadingly called 'prostate glands'. A cirrus has the ability to turn inside out as the organ is protruded, like the finger of a glove ([Fig. 5](#)).

The distal region of the vagina may be sclerotised, or specialized in other ways, in order to receive the male copulatory organ and to ensure that separation does not occur during mating.

Auto-Infection and Self-Insemination

The prolonged storage of shelled eggs in the uterus, as in *Eupolystoma* spp., is but a short step from hatching of some of these eggs and auto-infection (see [Kearn \(1998\)](#)).

Self-insemination has been reported in juveniles belonging to the monogenean genus *Benedeniella* (see [Kearn and Whittington \(1992\)](#)). Because of protandry the long male copulatory organ (cirrus) is able to enter the uterus and penetrate as far as the oötype, without meeting any obstructing female traffic in the opposite direction ([Fig. 6](#)). *Benedeniella* spp. also have a prominent vagina ([Fig. 6](#)), which has the potential to support self-insemination or cross-insemination when the female system comes on line and the uterine route can no longer be used. However, it has been shown experimentally that self-insemination does not occur in the related monogenean *Entobdella soleae* (see [Kearn and Whittington \(2015\)](#)).

Using a different experimental approach, [Dinh Hoai and Hutson \(2014\)](#) showed that self-insemination does occur in the monogenean *Neobenedenia* sp., a parasite of the commercially important fish barramundi (*Lates calcarifer*). This was achieved by infecting the uninfected hosts each with a single oncomiracidium. The isolated parasites reached maturity in 10 days and laid eggs that successfully infected specimens of *L. calcarifer* with second and third generation isolated parasites. The self-insemination route followed by this species of *Neobenedenia* is not known – there is no vagina – but the finding of the penis deflected into the parasite's own uterus in an individual of the related *Neobenedenia melleni* (see [Whittington and Horton \(1996\)](#)) suggests that selfing may occur via the uterine route in species of *Neobenedenia*.

Self-insemination was also observed by [Ogawa et al. \(2014\)](#) in *Neobenedeniagirellae* (see below).

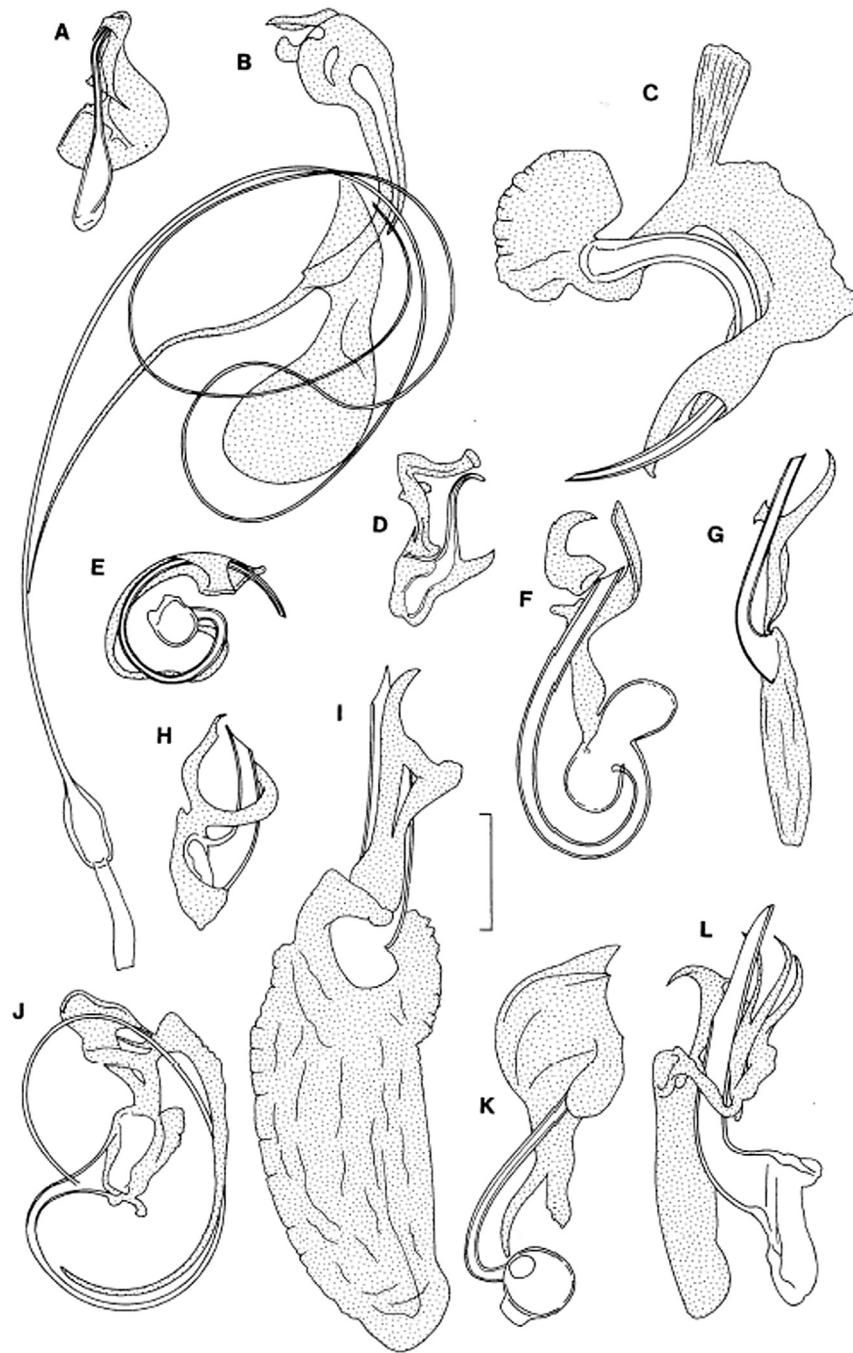


Fig. 4 Diversity of male copulatory sclerites in dactylogyrid monogeneans. All drawn to the same scale. Scale bar: 20 μ m. Selected and redrawn from Gusev, A.V., 1985. *Class Monogenea*. In: Bauer, O.N. (ed.). Key to Parasites of the Freshwater Fish Fauna of the USSR. vol. 2. Parasitic Metazoa. Leningrad: Leningrad Publishing House "Nauka". pp. 10–253 (In Russian). Reproduced with permission from Kearns, G.C., 1994. Evolutionary expansion of the Monogenea. *International Journal for Parasitology* 24, 1227–1271. Elsevier, © 1994.

Hypodermic/Transtegumental Insemination

Macdonald and Caley (1975) observed unilateral mating repeatedly in the monogenean *Diclidophora merlangi* but found no vagina. The muscular penis papilla armed with hooks is attached ventrolaterally to the mating partner, but this site does not correspond with the opening of the uterus. Macdonald and Caley came to the surprising conclusion that the tegument (surface covering) is breached in some way, either by the penis hooks or perhaps by a histolytic (tissue-digesting) glandular secretion, permitting sperm to enter the body. Macdonald and Caley showed that after penetration the sperms travel between the body cells (parenchyma) and, remarkably, navigate in some way to a storage organ, the seminal receptacle. No mutual (bilateral) insemination or self-insemination was observed.



Fig. 5 The partly everted cirrus of the monogenean *Trimusculotrema heronensis*, photographed alive in dorsal view. Scale bar: 250 μ m. Reproduced from Whittington, I.D., Kearns, G.C., 2008. *Trimusculotrema heronensis* sp. nov. (Monogenea, Capsalidae) from the skin of the pink whipray *Himantura fai* (Elasmobranchii, Dasyatidae) from Heron Island, Queensland, Australia. *Acta Parasitologica* 53, 251–257). With permission from De Gruyter.

More typical hypodermic impregnation may occur in the monogenean *Gastrocotyle trachuri* (see Llewellyn (1983)). The parasite has a tubular penis sclerite and no vagina. Llewellyn suggested that the penis passes through the tegument and deposits sperm in the parasite's tissues. Sperm was found inside the body but mating was not observed.

Spermatophores and Pseudospermatophores

The monogenean *Entobdella soleae* produces jelly-like spermatophores with a centrally located mass of sperm. The way in which the living animal transmits these spermatophores has been observed and recorded by Kearns (1970). Mutual exchange takes place and each spermatophore is attached externally to the co-copulant in the region of the relatively small ventral opening of the vagina. Contractions of the parasite's body in the region of the attached spermatophores serve to draw the sperm into the vagina, the rest of the spermatophore being discarded.

A novel method of impregnation by way of spermatophores has been described by Ogawa *et al.* (2014) in another monogenean *Neobenedenia girellae*. Each spermatophore consists of two components, a proximal portion that stains with the pink dye eosin and a distal, thin-walled portion containing sperms. Both portions are enclosed within a thin outer casing. There is no vagina. The spermatophores are implanted externally (dorsally), apparently at random sites, mainly on the posterior half of the body. Sperms were identified within the parenchyma (body tissues), in the vicinity of the attached spermatophores. Sperms gain access to these internal sites via damage to the surface layers (tegument), most likely inflicted by the penis. Ogawa *et al.* suggested that sperms reach the female tract via the vitelline ducts. Self-insemination was observed, as well as unilateral and mutual (bilateral) insemination.

Many monogeneans have elongated, encased structures which are assumed to be spermatophores (Fig. 7), but there is potential for confusion here because spermatophores and so-called pseudospermatophores are similar in appearance and lodged in the same site, the vaginal opening (Fig. 7). Pseudospermatophores contain no sperm and are put in place after the deposition of sperm in the vagina and withdrawal of the intromittent organ. It is assumed that, by blocking the vaginal opening, pseudospermatophores prevent leakage of freshly received sperm. Pseudospermatophores may also prevent sperm from a second challenging individual entering the vagina and competing with sperm already present from a previous mating.

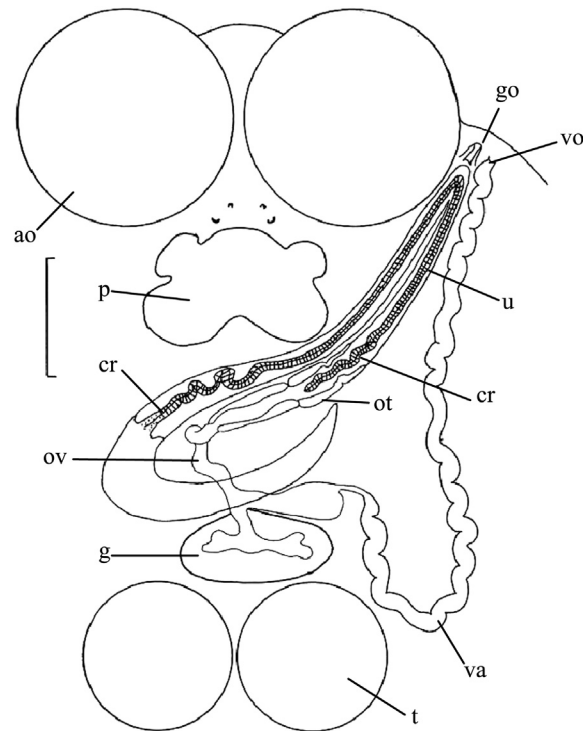


Fig. 6 Part of the reproductive system of a juvenile monogenean, *Benedeniella macrocolpa*, in ventral view, showing penetration of the uterus (u) by the cirrus (cr). ao – anterior attachment organ; g – germarium; go – common genital opening; ot – oötype; ov – ovovitelline duct; p – pharynx; t – testis; va – vagina; vo – vaginal opening. Scale bar: 250 μ m. Reproduced from Kearn, G.C., Whittington, I.D., 1992. Diversity of reproductive behaviour in platyhelminth parasites: Insemination in some benedeniine (capsalid) monogeneans. *Parasitology* 104, 489–496). With permission of Cambridge University Press.

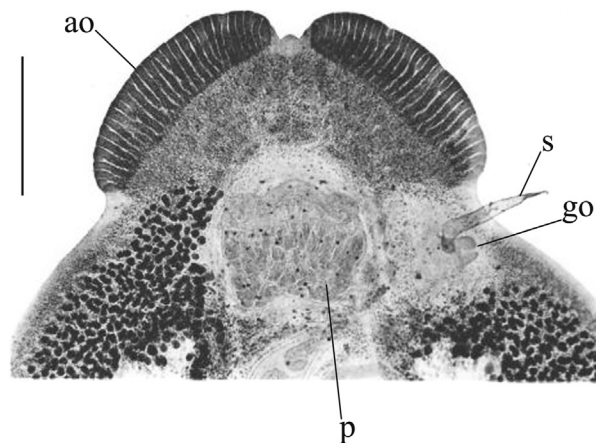


Fig. 7 The head region of the monogenean *Neoentobdella diadema* with a spermatophore (s) (or pseudospermatophore, see text) lodged in the vaginal opening. ao – anterior attachment organ; go – common genital opening; p – pharynx. Scale bar: 1 mm. Reproduced from Kearn, G.C., Whittington, I.D., 2015. Sperm transfer in monogenean (platyhelminth) parasites. *Acta Parasitologica* 60, 567–600. With permission from De Gruyter.

Permanent Cross-Insemination

The ultimate development in response to the need for cross-insemination occurs in diplozoid monogeneans. Permanent insemination is achieved by complete tissue fusion between two young parasites (**Fig. 8**). The reproductive ducts of the two fused individuals connect up, ensuring a potentially uninterrupted supply of sperm for each partner from the co-copulant and saving energy that would have to be expended on repeat matings. Curiously, the sperms of *Diplozoon* lack flagella and cannot swim, a condition which has come about perhaps because there is no longer a need for strong swimming to compete with other sperms to achieve fertilisation (see Justine, 1995, in Kearn (1998)).



Fig. 8 Two fused individuals of the monogenean *Diplozoon paradoxum*. Scale bar: 1 mm. Reproduced from Kearns, G.C., 2011. Monogeneans the ultimate fish parasites. *The Biologist* 58, 28–32. With permission of The Biologist.

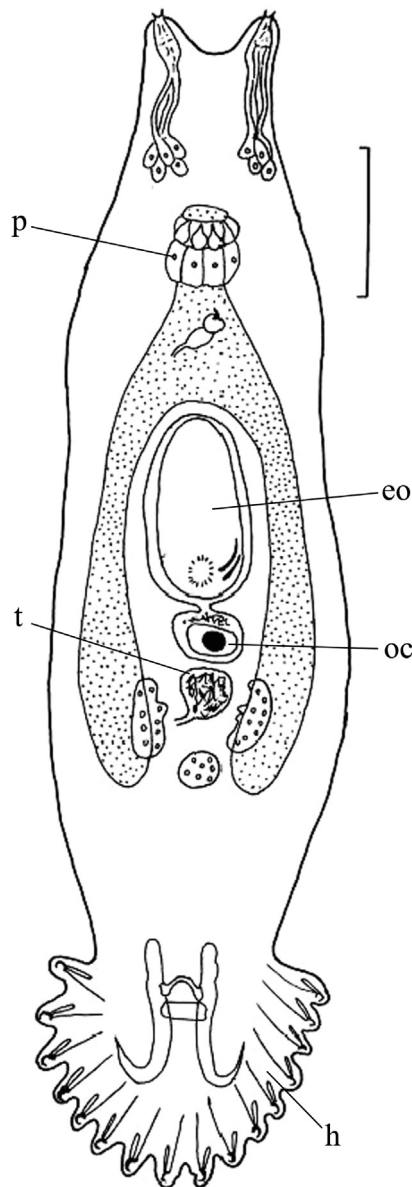


Fig. 9 A viviparous gyrodactylid monogenean, *Gyrodactylus pungitius*. eo – embryo in the uterus (or oötype?); oc – oöcyte; t – testis; Scale bar: 100 μ m. Redrawn from Malmberg, G., 1970. The excretory systems and the marginal hooks as a basis for the systematics of *Gyrodactylus* (Trematoda, Monogenea). *Archiv für Zoologi* 23, 1–235.

Asexual Reproduction

Cyrodactylid monogeneans are protogynous (female system matures first) and viviparous, giving birth to living young (Fig. 9). Some species of *Cyrodactylus* may reach ages and densities that favour sexual reproduction but others do not reach this level and are likely to reproduce asexually (references in [Kearn \(1998\)](#)).

A few families of tapeworms (cyclophyllideans) with two-host life cycles have acquired the ability to multiply asexually in the intermediate host by budding.

In digeneans the question is whether the sequential production of sporocysts, rediae and cercariae in the mollusc host involves an asexual process (polyembryony, budding) or is a modified sexual process (see [Kearn \(1998\)](#)). Also it is widely accepted that digenean reproduction involves the phenomenon of germinal segregation (germinal lineage), a process that appears to be unparalleled in other parasitic platyhelminths as far as is known (see references in [Kearn \(1998\)](#)). After fertilisation, the cell undergoes unequal cleavage, producing a somatic cell and a smaller propagatory cell. The former gives rise to the body of the miracidium and the latter persists as a germ cell. In other words a lineage of cells is established from the original fertilised cell and is conserved and maintained separately from the somatic cells throughout the life history phases (references in [Kearn \(1998\)](#)).

References

- Dinh Hoai, T., & Hutson, K. S. (2014). Reproductive strategies of the insidious fish ectoparasite, *Neobenedenia* sp. (Capsalidae: Monogenea). *PLOS ONE*, 9, 1–7.
- Kearn, G. C. (1970). The production, transfer and assimilation of spermatophores by *Entobdella soleae*, a monogenean skin parasite of the common sole. *Parasitology*, 60, 301–311.
- Kearn, G. C. (1975). The mode of hatching of the monogenean *Entobdella soleae*, a skin parasite of the common sole (*Solea solea*). *Parasitology*, 71, 419–431.
- Kearn, G. C. (1998). *Parasitism and the Platyhelminths*. London: Chapman & Hall.
- Kearn, G. C., & Whittington, I. D. (1992). Diversity of reproductive behaviour in platyhelminth parasites: Insemination in some benedeniine (capsalid) monogeneans. *Parasitology*, 104, 489–496.
- Kearn, G. C., & Whittington, I. D. (2015). Sperm transfer in monogenean (platyhelminth) parasites. *Acta Parasitologica*, 60, 567–600.
- Llewellyn, J., 1965. The evolution of parasitic platyhelminths. In Taylor, A. (ed.). *Evolution of Parasites*. Proceedings of the 3rd Symposium of the British Society for Parasitology. Oxford: Blackwell, pp. 47–78.
- Llewellyn, J. (1983). Sperm transfer in the monogenean gill parasite *Gastrocotyle trachuri*. *Proceedings of the Royal Society of London B*, 219, 439–446.
- Macdonald, S., & Caley, J. (1975). Sexual reproduction in the monogenean *Diclidophora merlangi*: Tissue penetration by sperms. *Zeitschrift für Parasitenkunde*, 45, 323–334.
- Ogawa, K., Shirakashi, S., & Ishitani, H. (2014). Insemination of the monogenean *Neobenedeniagirellae* (Capsalidae, Benedeniinae). *Parasitology International*, 63, 473–478.
- Shinn, G. L. (1983). The life history of *Syndisyrinx franciscanus*, a symbiotic turbellarian from the intestine of echinoids, with observations on the mechanism of hatching. *Ophelia*, 22, 57–79.
- Shinn, G. L. (1986). Life history and function of the secondary uterus of *Wahlia pulchella*, a umagillid turbellarian from the intestine of a Pacific sea cucumber (*Stichopus californicus*). *Ophelia*, 25, 59–74.
- Shinn, G. L., & Cloney, R. A. (1986). Egg capsules of a parasitic turbellarian flatworm: ultrastructure of hatching sutures. *Journal of Morphology*, 188, 15–28.
- Waite, J. H. (1990). The phylogeny and chemical diversity of quinone-tanned glues and varnishes. *Comparative Biochemistry and Physiology*, 97B, 19–29.
- Whittington, I. D., & Horton, M. A. (1996). A revision of *Neobenedenia* Yamaguti, 1963 (Monogenea: Capsalidae) including a redescription of *N. melleni* (MacCallum, 1927) Yamaguti, 1963. *Journal of Natural History*, 30, 1113–1156.
- Wilson, R. A. (1968). The hatching mechanism of the egg of *Fasciola hepatica* L. *Parasitology*, 58, 79–89.

Comparative Reproductive Biology in Nematodes

Beatriz Moreira-Pinto and Andre Pires-daSilva, University of Warwick, Coventry, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Introduction

Nematoda is one of the most diverse taxa, with nearly 26,000 described species. Nematodes live in almost any kind of environment, being on rotting organic matter, hot springs, desert, ocean, inside of animals (including humans) and plants, and even in deep mines 3 km down in the Earth. Free-living species feed mostly on bacteria, but also on fungi, algae and other nematodes. Their size vary from 100 μm to 14 m, and their reproductive output ranges from just a few eggs to up to two million eggs a day per individual. Some small (~ 1 mm long) nematodes, such as *Caenorhabditis elegans*, are very prolific because in addition of producing many progeny per individual by self- and cross-fertilization (up to 685 eggs in their lifetimes), they also have a short life cycle (3 days from egg to adult at 25°C). Thus, a single individual has the potential to give rise to many million descendants within a few weeks (Bird and Bird, 1991).

Most nematodes are sexually dimorphic, with sexes that differ in relative size, shape of the gonad, shape of the tail and presence/absence of a vulva (Fig. 1). Sexual dimorphism can be sometimes extreme, such as males of *Trichosomoides crassicauda*, which live inside the female uterus. Males use mechano- and chemosensory organs located in the head and tail to track the opposite sex and inseminate it by internal fertilization. Some nematode species might even have three sexual forms, the third being a self-fertilizing hermaphrodite. *Heterorhabditis bacteriophora* and *Rhabditis* sp. SB347, which have three sexual forms, reproduce by self-fertilization, crosses between hermaphrodites, as well as by crosses between males and females (Kanzaki et al., 2017).

The great diversity in the various aspects of nematode reproduction provides a great challenge to summarize in a short article. Here we focus on some recent studies in comparative biology of reproduction in nematodes. The phylogenetic relationships of nematodes covered in this article are depicted in Fig. 2.

Evolution of Modes of Reproduction

Nematodes have various types of modes of reproduction (Table 1). Dioecy is the most common, followed by parthenogenesis, androdioecy and trioecy. In some nematodes, reproductive modes can alternate between generations, so that a uniparental generation (hermaphrodite or parthenogenetic female) never meets the males of the biparental generation. In *Rhabdias*, for example, the parasitic generation is a hermaphrodite that lives inside the lung of amphibians and reptiles. The progeny of *Rhabdias* hermaphrodites develop into adults outside the host, becoming the male/female (dioecious) generation. Similarly, the mammal parasite *Strongyloides* facultatively alternates between a parasitic parthenogenetic generation and a free-living dioecious generation (Streit, 2008). Thus, in some species the three sexual morphs do not occur simultaneously in the same location. In trioecious species like *H. bacteriophora* and *Rhabditis* sp. SB347, however, hermaphrodites, females and males do occur simultaneously (Kanzaki et al., 2017), and males of those trioecious populations can facultatively cross with females or hermaphrodites.

The transition between reproductive modes has occurred multiple times during nematode evolution, across the nematode phylogenetic tree (Fig. 2). In the genus *Caenorhabditis*, the androdioecious species *C. elegans*, *C. briggsae* and *C. tropicalis* independently evolved the ability to self-fertilize from ancestral species that were dioecious (Fig. 2). The convergence in phenotype was achieved by distinct molecular mechanisms. The same direction of change, from dioecy to androdioecy, is also observed in

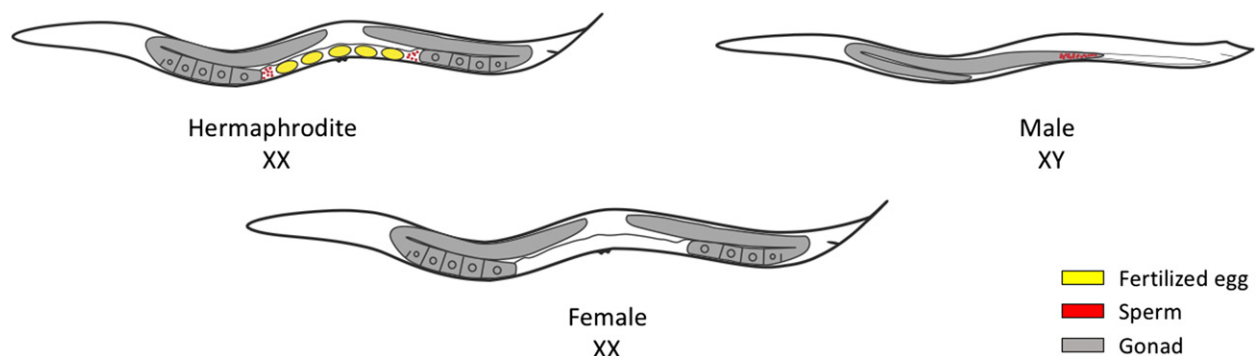


Fig. 1 Differences between sexual morphs in free-living nematodes. The hermaphrodite and female have two gonad arms, while the male gonad is composed of a single testis that runs the length of the nematode. The hermaphrodite produces and stores sperm for self-fertilization. The male tail is short and blunt and has mechanosensory and chemosensory neurons, while the female/hermaphrodite has a thin and long tail.

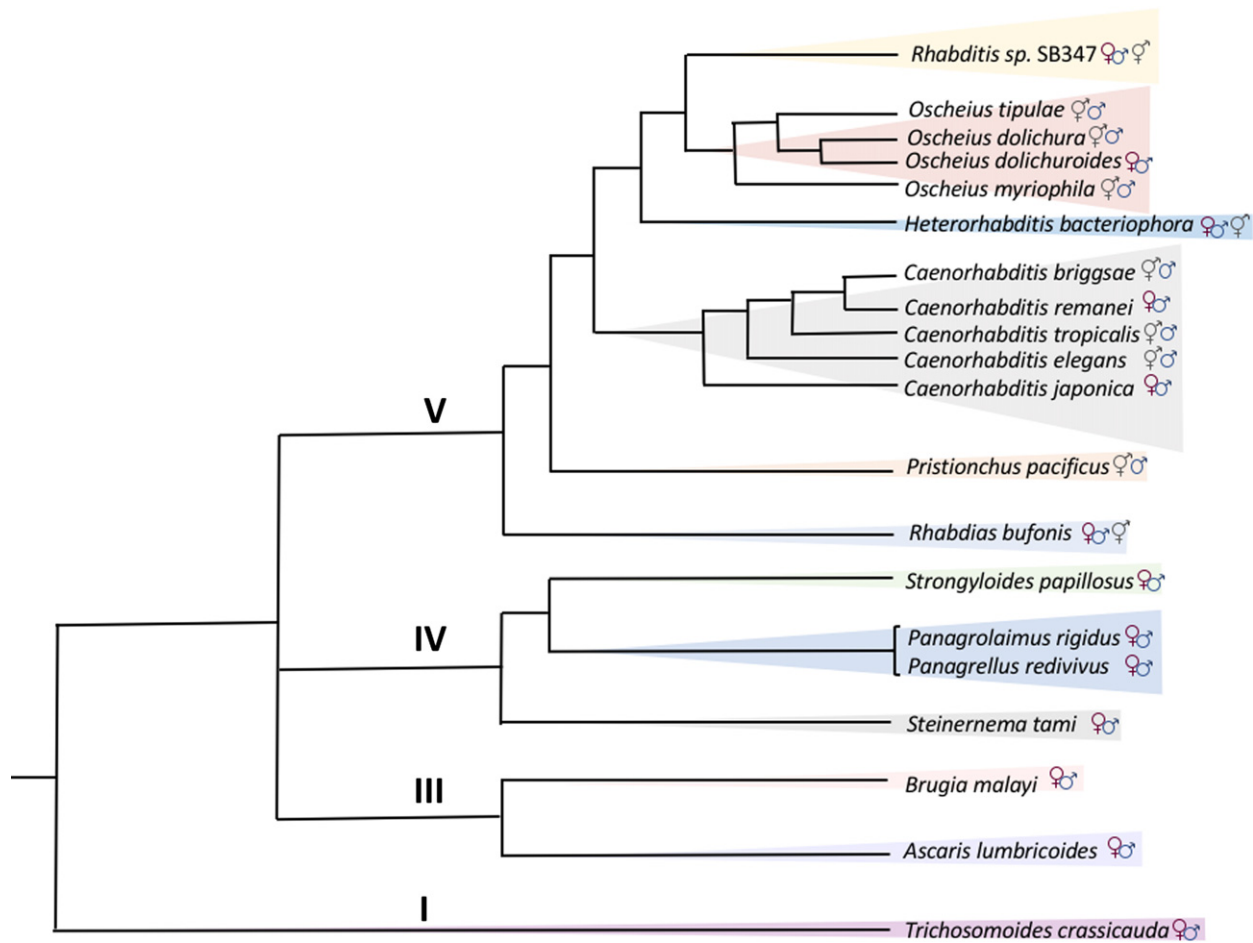


Fig. 2 Phylogenetic relationships and sexual morphs of nematodes covered in this article. The nematode phylogeny has five major clades, four of each are depicted here.

Table 1 Types of reproductive mode

Type	Definition
Dioecious	Sexual reproduction between males and females. In the nematode literature, is sometimes referred as 'gonochorism'.
Androdioecious	Sexual reproduction by self-fertilization of hermaphrodites, or by crossing between hermaphrodites and males.
Trioecious	Sexual reproduction by self-fertilization, or between males and females, or between males and hermaphrodites.
Parthenogenetic	Asexual reproduction only by females. Sometimes males are present, but do not contribute with genetic material to the next generation.

Pristionchus, but it can also occur in the reverse direction. *Oscheius dolichuroides*, for example, is a dioecious species derived from an androdioecious ancestor (Fig. 2) (Denver et al., 2011).

In principle, a single mutation that turns the sperm inviable in hermaphrodite, or that abolishes the production of sperm in hermaphrodites, is sufficient for the evolution of dioecy from androdioecy. In contrast, in the evolution androdioecy from dioecy, at least two mutations have to occur: (1) a mutation that briefly masculinizes the germline of the female for the production of sperm; (2) a mutation that activates the sperm in the female. Thus, the occurrence of these mutations in a population gives rise to the intermediate trioecious mating system. Trioecy is extremely rare in nematodes, and is known only in some undescribed free-living nematodes and in the insect parasite *H. bacteriophora*.

The evolution of self-fertilization facilitated the colonization of new environments by single individuals. Interestingly, *H. bacteriophora* and *Rhabditis* sp. SB347 independently evolved the coupling of dauer larva formation to the development of adult hermaphrodites (Kanzaki et al., 2017). Dauer is a facultative larval stage that resists to lack of food, detrimental environmental conditions, and has a series of adaptations for migration. By coupling dauer with hermaphroditism, these nematodes species further potentiated the ability to colonize new habitats.

Evolution of Sex Determination Systems

Sex determination is one of the fastest evolving developmental systems (Bachtrog *et al.*, 2014). This is reflected in the diversity of sex determining systems in the animal kingdom, which can be mediated by chromosomal and environmental factors. Predictions of sex determination in nematodes have been drawn from cytological studies, which indicate that most species perform chromosomal sex determination. However, the diminutive size of nematode chromosomes in many cases makes the karyotypic interpretation difficult, resulting in conflicting reports.

The most common type of chromosomal sex determination system in nematodes is in which the heterogametic sex is the male (Pires-daSilva *et al.*, 2007). In this case, sex chromosomes are denoted X and Y (e.g., in *Brugia malayi*). Thus, females are XX and males are XY. Several nematodes do not have a second sex chromosome, and in this case the sex determination system is denoted XX:XO. Heterogametic males, as the name implies, produce gametes with two kinds of gametes: XY males, produce equal proportions of sperm with an X chromosome and sperm with Y chromosome; XO males produce equal proportions of sperm with an X chromosome and the other half harboring only autosomes. The XX:XO system is thought to be derived from the XX:XY system, whereby the Y chromosome was lost during evolution.

In other groups of animals with a XX:XY system, sex is determined by either a dominant male-determining factor, or by the ratio of X chromosomes relative to autosomes (in this case, the Y chromosome has no role in sex determination). Unfortunately, the mechanisms that trigger the sex determination in nematodes with a XX:XY system are not known, probably because most of those species are parasitic and thus difficult to manipulate genetically.

The best characterized sex determination system is in *C. elegans*, which relies on an XX:XO system. The balance of X chromosomes to autosomes (X:A) determines sex in this nematode, with the X causing a feminizing effect and autosomes a masculinizing effect. Thus, *C. elegans* with two sets of autosomes and two of sex chromosomes (2A:2X) are hermaphrodites and 2A:1X animals are males. The XX:XO system is also present in other *Caenorhabditis* species (e.g., *C. remanei* and *C. briggsae*) (Stothard and Pilgrim, 2003). Genes in the X chromosomes and autosomes act in a dose-dependent manner to regulate the transcription of the masculinizing gene *xol-1* (XO lethal). The transcription of *xol-1*, which codes for a kinase, leads to a series of signal-transduction events that ultimately represses the transcription factor *tra-1* (*transformer-1*). In XX animals, *tra-1* promotes female development mainly by repressing expression of male-specific genes. Despite the rapid evolution of genes involved in sex determination, their function seems to be at large extent conserved in the soma.

The evolution of hermaphroditism, which independently occurred three times in *Caenorhabditis* (Kiontke *et al.*, 2004), required modifications of the sex determination in the germline of XX animals (Ellis and Lin, 2014). In these animals, there is a transient masculinization of the germline for the production of sperm, followed by the production of oocytes. The evolution of this trait in *C. elegans* and *C. briggsae* involved the recruitment of different sets of genes, independent recruitment of the same genes, and also the modification of the function of sex-determining genes (Baldi *et al.*, 2009).

Although *C. elegans* and *C. briggsae* hermaphrodites are XX and thus expected to produce only XX self-progeny, they may produce XO males in some rare (~0.1%) instances. This occurs because of X chromosome non-disjunction, in which abnormal chromosome pairing and synapsis during meiosis generates gametes containing only autosomes (nullo-gametes). Thus, a nullo-sperm, when fertilizing an X-bearing oocyte, generates a male. In other nematodes with XX:XO system, XX animals may generate male self-progeny by different mechanisms. In the *Rhabdias* genus, for example, XX hermaphrodites produce equal proportions of XX female and XO self-progeny. This occurs by a modification of the meiosis during hermaphrodite spermatogenesis, in which half of the gametes removes the X chromosome of the meiotic cell. In *Strongyloides papillosus*, the parthenogenetic XX female generates male progeny by a process called chromosome diminution. Interestingly, in this nematode the ancestral X chromosome became incorporated into an autosome (Nemetschke *et al.*, 2010). During meiosis of oocytes destined to become males, the region corresponding to the ancestral X is eliminated in one of the chromosomes, thus generating an XO animal.

Male heterogamy is the most common sex determination system in nematodes, and no examples for female heterogamy (ZZ/ZW system) are known. In haplo-diploids, which are known to occur only in oxyurid nematodes, unfertilized eggs develop into males and diploid fertilized eggs develop into females. In species with environmental sex determination, crowding, immune status of the host, and temperature are factors that regulate sex determination.

Evolution of Different Types of Gametes

The mitotic germline usually is located in the terminal part of the testis, followed by cells at more advanced meiotic stages as they become closer to the proximal part of the gonad (Chu and Shakes, 2013). The generation of a male gamete involves the meiotic division of a primary spermatocyte to generate two secondary spermatocytes, which divide to form four secondary spermatids (Fig. 3). Those spermatids undergo differentiation by shedding unnecessary contents for sperm function (e.g., microtubules, actin, endoplasmic reticulum, translation machinery). The activation of spermatids, which refers to the conversion of the spherical, quiescent spermatids into the mobile, mobile spermatozoa, is triggered by extracellular signals. Sperm activation results in ameboid-like cells, which form pseudopodia by the assembly and disassembly of a nematode-specific protein named Major Sperm Protein (MSP). Thus, contrary to sperm of insect and mammals, nematode sperm lack cilia or flagella.

Comparative studies on the evolution of sperm have been performed in the *Caenorhabditis* genus, in which sperm size ranges from 3 to 281 μm^2 (Vielle *et al.*, 2016). Interestingly, the size of the sperm correlates with their competitive advantage

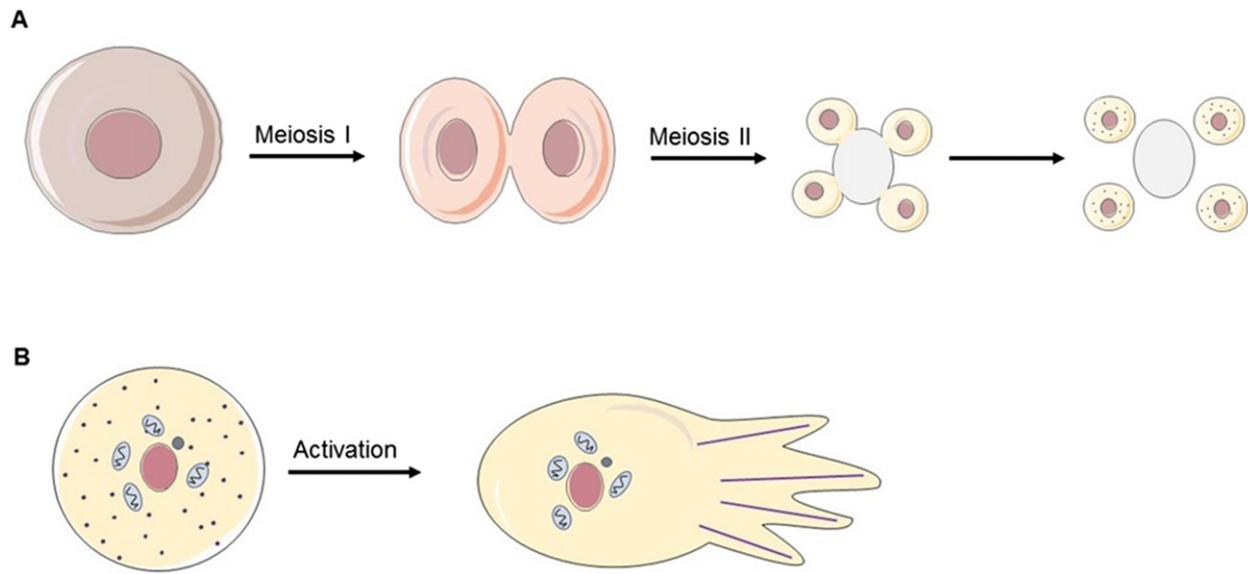


Fig. 3 Spermatogenesis. (A) The division of primary spermatocyte in meiosis I gives rise two secondary spermatocytes. After the division of secondary spermatocytes into four cells in meiosis II, unnecessary cytoplasmic components are discarded into a vesicle (grey circle). (B) After the activation of the spermatid, MSP (purple) polymerizes to form the pseudopods.

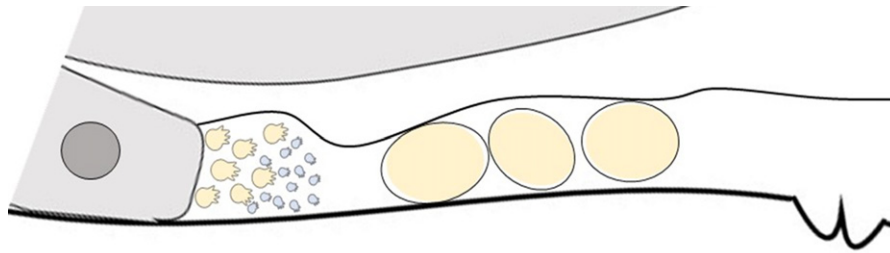


Fig. 4 Sperm competition in hermaphrodites. The larger male sperm (yellow) outcompetes the smaller hermaphrodite sperm (blue) to fertilized the oocyte. The color of the egg corresponds to the product of fertilization between a self and male sperm.

(LaMunyon and Ward, 1998). In androdioecious species, in which both sexes produce sperm, the male sperm is usually larger and outcompetes the hermaphrodite sperm (Fig. 4). However, size alone is probably not the only cause for the difference in competitiveness. It is still unclear whether this competitive edge of the larger sperm is due to their greater speed due to the larger pseudopod, greater adhesion to the wall of the hermaphrodite uterus, or greater capacity to dislodge the smaller hermaphrodite sperm.

Some species of nematodes produce more than one kind of sperm. Sperm with size dimorphism, for instance, have evolved independently in a few nematode lineages. In *Steinernema tami*, miniature sperm aggregate around giant sperm, which serve as transportation device in the female uterus (Yushin *et al.*, 2007). Males of *Rhabditis* sp. SB347 also produce two kinds of sperm, a functional X-bearing sperm and a non-functional nullo-X sperm (Shakes *et al.*, 2011). Thus, those XO males generate practically only XX progeny when crossing with the opposite sex. The production of two kinds of sperm in *Rhabditis* sp. SB347 is caused by a modification of meiosis and the differentiation of the spermatids. In the final spermatocyte division, the mitochondria and other cytoplasmic components necessary for sperm function (e.g., MSP) co-segregate with the X chromosome. Thus, unnecessary cytoplasmic components segregate to the nullo-X spermatid, generating a non-functional gamete.

How Sexes Find Each Other

Nematodes have mechanosensory and chemosensory organs that allow them to find the opposite sex in order to reproduce. Males and females of the free living *Panagrolaimus rigidus*, for example, when separated by a cellophane barrier, move towards individuals of the opposite sex but not to the ones of the same sex. These experiments suggest the release of sex-specific attractants, the sex pheromones. Some of these pheromones are small molecules containing a dideoxysugar connected to a fatty acid chain. The name of these pheromones, known as ascarosides, derives from *Ascaris lumbricoides*, a nematode in which these chemicals were first

characterized. The biological activities of ascarosides are determined by the length of the chain of side carbons, modifications of the dideoxysugar sugar, and the blend of the different ascarosides. Some of these blends elicit different behaviors, such as inducing dauer formation in early larvae.

In *Panagrellus redivivus*, both males and females produce mutually-attracting ascarosides. In the trioecious species *Rhabditis* sp. SB347, with a mating system thought to be intermediate between dioecy and androdioecy, females attract males more efficiently than hermaphrodites. The same pattern is observed in androdioecious species as *C. elegans* and *C. briggsae*, in which hermaphrodites are weaker in attracting males when compared to females of sister dioecious species (e.g., *C. remanei*). Interestingly, *C. remanei* males immobilize females by the secretion of paralyzing chemicals in the seminal fluid, facilitating copulation. Males of *C. remanei* or *C. elegans* cannot paralyze *C. elegans* hermaphrodites, indicating that hermaphrodites are refractory to these male chemicals and can escape from mating. These results suggest that hermaphrodites have evolved adaptations to avoid mating with males.

In *C. elegans*, all neurons have been mapped. Males and hermaphrodites share many of the head sensory neurons, but only males display mate searching behavior. Since *C. elegans* males and hermaphrodites share some of the same neurons, how is the sex-specific behavior triggered? The searching behavior is mediated by the expression of the neuropeptide PDF-1 in males, which is heightened when they are well-fed. Overexpression of PDF-1 in hermaphrodites, however, does not elicit a mate searching behavior in them, indicating that this neuropeptide acts through a sexually dimorphic circuit despite sharing the same neurons.

The male-attracting behavior is mediated by male-specific CEM neurons, as well as the core sensory olfactory neurons AWA, AWC and ASK. It is still not clear how many or which receptors are involved in the male attracting behavior, but a few such as the TRPV (transient receptor potential vanilloid) channel are starting to be discovered.

References

- Bachtrog, D., Mank, J.E., Peichel, C.L., *et al.*, 2014. Sex determination: Why so many ways of doing it? *PLoS Biol* 12, e1001899.
- Baldi, C., Cho, S., Ellis, R.E., 2009. Mutations in two independent pathways are sufficient to create hermaphroditic nematodes. *Science* 326, 1002–1005.
- Bird, A.F., Bird, J., 1991. Reproductive system. In: Bird, A.F., Bird, J. (Eds.), *The structure of nematodes*. San Diego, CA: Academic Press, pp. 230–273.
- Chu, D.S., Shakes, D.C., 2013. Spermatogenesis. *Adv Exp Med Biol* 757, 171–203.
- Denver, D.R., Clark, K.A., Raboin, M.J., 2011. Reproductive mode evolution in nematodes: Insights from molecular phylogenies and recently discovered species. *Mol Phylogenet Evol* 61, 584–592.
- Ellis, R.E., Lin, S.Y., 2014. The evolutionary origins and consequences of self-fertility in nematodes. *F1000Prime Rep* 6, 62.
- Kanzaki, N., Kiontke, K., Tanaka, R., *et al.*, 2017. Description of two three-gendered nematode species in the new genus *Auanema* (Rhabditina) that are models for reproductive mode evolution. *Sci Rep* 7, 11135.
- Kiontke, K., Gavin, N.P., Raynes, Y., *et al.*, 2004. *Caenorhabditis* phylogeny predicts convergence of hermaphroditism and extensive intron loss. *Proc Natl Acad Sci USA* 101, 9003–9008.
- LaMunyon, C.W., Ward, S., 1998. Larger sperm outcompete smaller sperm in the nematode *Caenorhabditis elegans*. *Proc Biol Sci* 265, 1997–2002.
- Nemetschke, L., Eberhardt, A.G., Hertzberg, H., Streit, A., 2010. Genetics, chromatin diminution, and sex chromosome evolution in the parasitic nematode genus *Strongyloides*. *Curr Biol* 20, 1687–1696.
- Pires-daSilva, A., 2007. Evolution of the control of sexual identity in nematodes. *Semin Cell Dev Biol* 18, 362–370.
- Shakes, D.C., Neva, B.J., Huynh, H., Chaudhuri, J., Pires-daSilva, A., 2011. Asymmetric spermatocyte division as a mechanism for controlling sex ratios. *Nat Commun* 2, 157.
- Stothard, P., Pilgrim, D., 2003. Sex-determination gene and pathway evolution in nematodes. *Bioessays* 25, 221–231.
- Streit, A., 2008. Reproduction in *Strongyloides* (Nematoda): A life between sex and parthenogenesis. *Parasitology* 135, 285–294.
- Vielle, A., Callemeyn-Torre, N., Gimond, C., *et al.*, 2016. Convergent evolution of sperm gigantism and the developmental origins of sperm size variability in *Caenorhabditis* nematodes. *Evolution* 70, 2485–2503.
- Yushin, V.V., Yoshida, M., Spiridonov, S.E., 2007. Riders on the sperm: Sperm dimorphism and spermatozuogmata in nematodes from the genus *Steinernema* (Rhabditida: steinernematidae). *Nematology* 9, 61–75.

Reproduction, Overview by Phylogeny: Rotifera

Manuel Serra, University of Valencia, Valencia, Spain

Terry W Snell, Georgia Institute of Technology, Atlanta, GA, United States

Robert L Wallace, Ripon College, Ripon, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acanthocephala A group of dioecious, obligatory sexual, parasitic worms that are evolutionarily related to rotifers.

Amictic (Synonymous: asexual; apomictic thelytoky) in the life cycle of monogonont rotifers, reproduction by diploid, ameiotic parthenogenesis. Female with this mode of reproduction. Phase of the life cycle of monogonont rotifers with this mode of reproduction.

Apomictic thelytoky See *amictic*.

Arrhenotokous parthenogenesis Parthenogenesis where unfertilized eggs develop into males.

Bdelloidea One of three classes of rotifers with a wormlike body, distinguished from the other two classes by reproducing exclusively asexually.

Cyclical parthenogenesis (Synonymous: heterogony) the alternation of generations between asexual and sexual facultative modes of reproduction.

Diapause A dormant period of arrested growth and development characterized by an encysted stage with greatly slowed metabolism.

Diapausing egg See *diapausing embryo*.

Diapausing embryo (Synonymous: resting egg; diapausing egg) a resistant (encysted), thick-walled, diploid embryo arrested in an early stage of development, with highly sculptured outer surfaces usually produced by sexual reproduction in monogonont rotifers.

Eutely (eutelic) A condition in metazoans in which there is a predetermined number of cells and typically no cell division after birth.

Haplodiploidy (haplodiploid) Sex-determination system in which males are haploid and develop from unfertilized eggs; related to *arrhenotokous parthenogenesis*.

Heterogony See *cyclical parthenogenesis*.

Intracytoplasmic lamina (ICL) A filamentous layer of protein embedded within the cells comprising the body wall of rotifers and acanthocephalans.

Micrometazoans Multicellular animals small enough to require a microscope to visualize.

Mictic (Synonymous: sexual) in the life cycle of monogonont rotifers, reproduction involving meiosis. Females reproducing mictically produce males from unfertilized eggs and diapausing eggs from fertilized eggs. Phase of the life cycle of monogonont rotifers with this mode of reproduction.

Mixis (Synonymous: sexual) the phase of the life cycle of monogonont rotifers where oogenesis occurs via meiosis, producing haploid ova and sperm and subsequent fertilization.

Monogononta One of three classes of rotifers, distinguished from the other two by having a single gonad and reproduction by *cyclical parthenogenesis*.

Oviparous Females that lay eggs that hatch outside the body. Reproduction with this characteristic.

Ovoviviparous Females that lay eggs that hatch within the body with subsequent birth of live offspring. Reproduction with this characteristic.

Parthenogenesis Reproduction evolved from sexual reproduction where fertilization is not involved in the embryo production.

Quorum sensing Responding to chemical signals related to population density, synchronizing activities like sexual reproduction, where individual fitness requires coordination with other individuals.

Resting egg See *diapausing embryo*.

Seisonidea One of three classes of rotifers, distinguished from the other two by reproducing only through sex.

Sexual induction signal (Synonymous: mixis induction signals) in the life cycle of monogonont rotifers, environmental signal inducing sexual reproduction in facultatively sexual individuals. E.g., a protein released into the environment by *Brachionus* females that triggers sexual reproduction.

Subitaneous egg In the life cycle of rotifers, a thin-walled, rapidly-developing egg produced by asexual females that immediately hatches into a young, diploid female that is either asexual or sexual.

Introduction

Description

Rotifers (Rotifera: wheel-bearers) includes >2000 species of minute (ca. 0.05–3 mm), short-lived, micrometazoans dwelling mostly in lakes, ponds, and streams and coastal marine habitats (Fig. 1). Three main features separate rotifers from other micrometazoans. (1) The apical end usually bears a ciliated region (corona) that may be embellished by various folds, lips, or ear-like extensions. (2) The ventral region of the pharynx (mastax) holds a complex series of articulating hard jaws comprised of chitin and scleroprotein. (3) Cells comprising the body wall form a syncytium that is variously thickened by a filamentous layer of protein termed the intracytoplasmic lamina (ICL). When the ICL is thin the body wall is flexible; in its thickest form it is rigid.

From the dawn of microscopy in 1600s, rotifers have been the object of intense study. Current knowledge on anatomy is well summarized by Fontaneto and De Smet (2015) who review modern work in imaging science, including the anatomical details revealed by the latest techniques in light and electron microscopy, and immunohistological staining. Wallace *et al.* (2015) also provide information on rotifer general biology, as well as their ecology and evolution.

Rotifers have direct development and are eutelic – there is no cell division after development has been completed – so females are born with their lifetime supply of oocytes. They are distinguished among other animals because the major clades (Seisonidea, Bdelloidea, and Monogononta) reproduce via obligate sexuality, obligate asexuality, and facultative sexuality, respectively (Fig. 2).

Phylogeny and Taxonomy

Phylogenetic relationships of rotifers with other groups and between the major rotifer clades are not yet resolved. Rotifera is still considered a phylum. However, based on morphological and molecular characteristics, Acanthocephala (a group of dioecious, obligatory sexual, parasitic worms) appears to be closely related. All share the unique feature of the ICL and there are similarities in the ultrastructure of the spermatozoa. Molecular analyses using mitochondrial 16S rRNA and nuclear 18S rRNA also argue for a close relationship. A possibility is that the phylogenetic branch including Bdelloidea and Monogononta is closer to

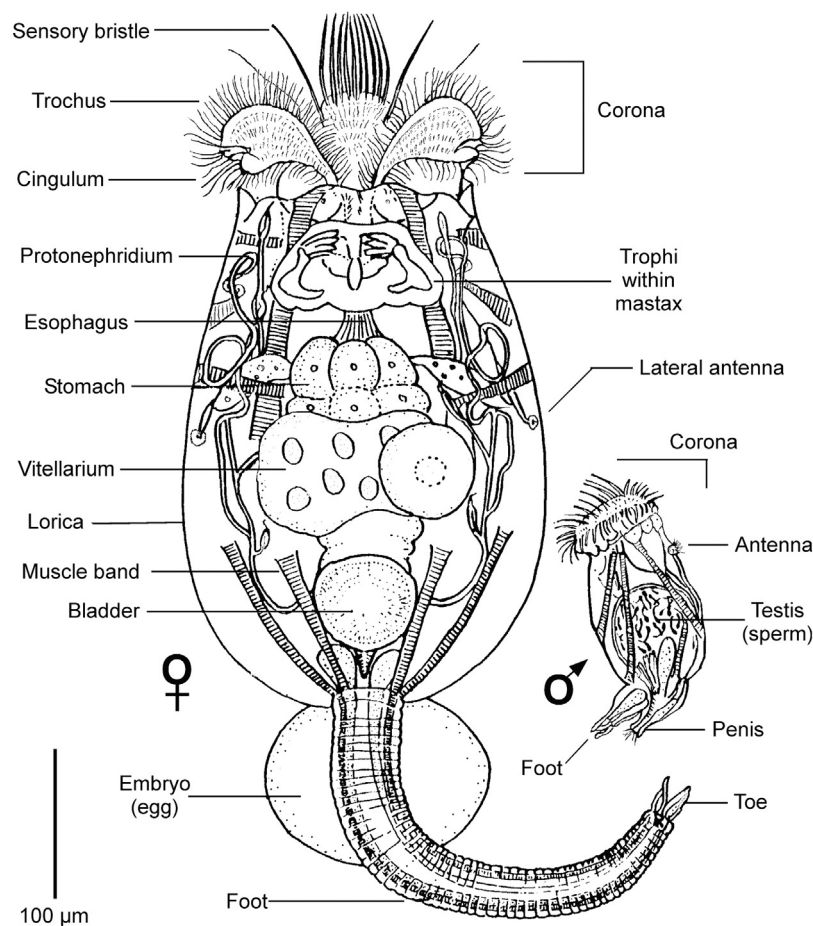


Fig. 1. Female and male *Brachionus* species. From Figure 13.2 in Wallace, R.L., Snell, T.W., Smith, H.A., 2015. Phylum rotifera. In: Thorp, J.H., Rogers, D.C. (Eds.), Thorp and Covich's Freshwater Invertebrates. Waltham, MA: Elsevier.

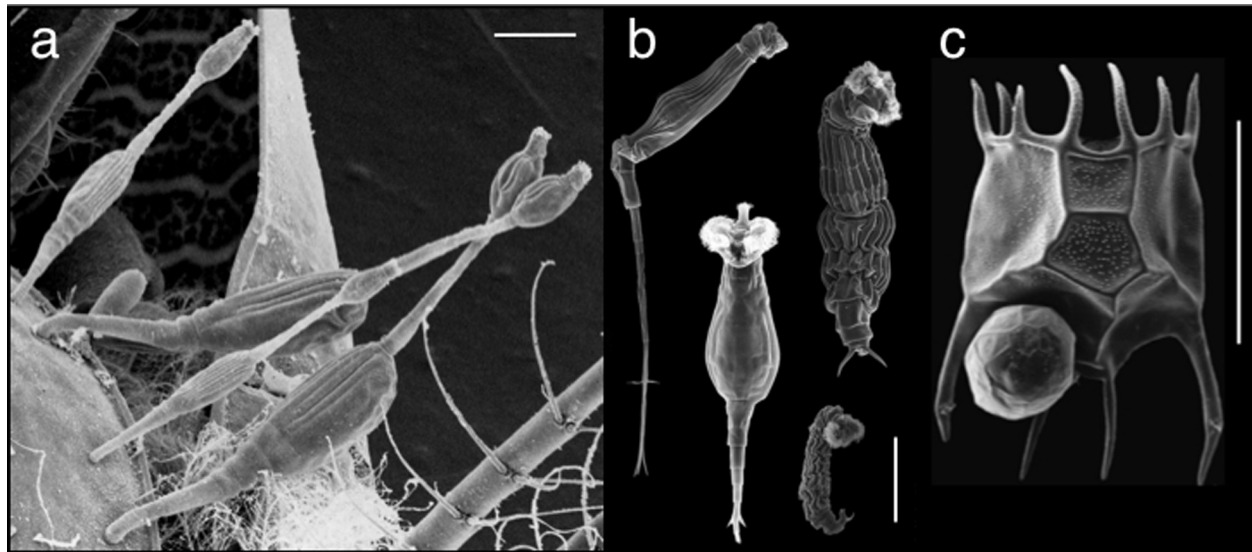


Fig. 2 The three major rotifer taxa. (a) Seisonidea: Females of *Seison nebaliae*. (b) Bdelloidea: Four bdelloid females. (c) Monogononta: Female of *Plationus patulus* with one embryo. Bars=200 μ m. SEM photomicrographs courtesy of Giulio Melone and Claudia Ricci (a), Giulio Melone and Diego Fontaneto, and licensed under the Creative Commons Attribution 2.5 Generic license (b), and Hendrik Segers (c).

Acanthocephala than to Seisonidea. If so, rotifers would not be a monophyletic group. Nevertheless, here we consider only the classical three rotifer clades, each of which being considered a class, although their taxonomical rank varies among authors.

Comprising a single family of only four species, seisonids are dioecious, diploid, wormlike animals that are larger than other rotifers (≤ 3 mm). The sexes are of similar size and morphology, but females have paired ovaries that lack vitellaria; males lack specialized copulatory organs.

Bdelloids possess a relatively uniform morphology characterized by a wormlike body and paired ovaries with vitellaria. They are smaller than seisonids (0.1–1.6 mm), but richer in species (~ 350 species). Males have never been reported in the class. This and their genomic architecture strongly support that they reproduce exclusively by parthenogenesis (Flot *et al.*, 2013).

Monogononts comprise the largest class of rotifers (~ 1600 species). Having a body size that ranges from 0.05–2.0 mm, their body form varies. Some are worm-like, some are ellipsoid-like, and others are dorso-ventrally or laterally flattened. While all monogononts are believed to be dioecious, males have never been observed in many species. The archetypal life cycle is characterized by relatively long periods of female parthenogenesis that occasionally are broken by spates of sexual reproduction (i.e., cyclic parthenogenesis). While females are diploids, males are haploids and usually are structurally reduced.

Ecology

Bdelloids usually are found in sediments, among plant debris, or crawling along surfaces. Some species inhabit the water films in soils or on mosses. Adults are capable of tolerating extreme environmental conditions, including freezing and desiccation (anhydrobiosis). Monogonont are benthic, free swimming, or sessile. Their occurrence is mainly restricted to pools, ponds, lakes, coastal lagoons and estuaries. Seisonids are marine rotifers that live as epibionts on small crustaceans. Rotifers are widely distributed geographically. Although small, they play important roles in aquatic habitats. They feed on a variety of bacteria, algae, and protists and then, in turn, are fed upon by protists, other rotifers, small crustaceans, and fishes. Rotifers possess high reproductive potential and may attain high population densities (occasionally > 5000 individuals per liter), thus making them important components in aquatic food webs.

Modes of Reproduction

Asexual Reproduction in Bdelloidea and Monogononta

Parthenogenetic production of daughters is the obligate mode of reproduction in bdelloids and the most common mode in monogononts (Fig. 3). During their embryological development, diploid females produce diploid oocytes by mitosis; two equational divisions produce two polar bodies and give rise to a diploid embryo. Thus, parthenogenetic production of daughters is believed to be ameiotic (i.e., apomictic thelytoky) and to lack crossing over. Thus, barring mutations, all offspring are females and genetic clones of their mothers. Oocytes are embedded in a tissue called the vitellarium that synthesizes yolk for the developing embryos and transfers it via a cytoplasmic bridge. Once embryos are fully loaded with yolk, they are deposited outside the female via an oviduct. Females of some species (e.g., *Brachionus*) carry their embryos until hatching attached by a slender thread. Others lay their

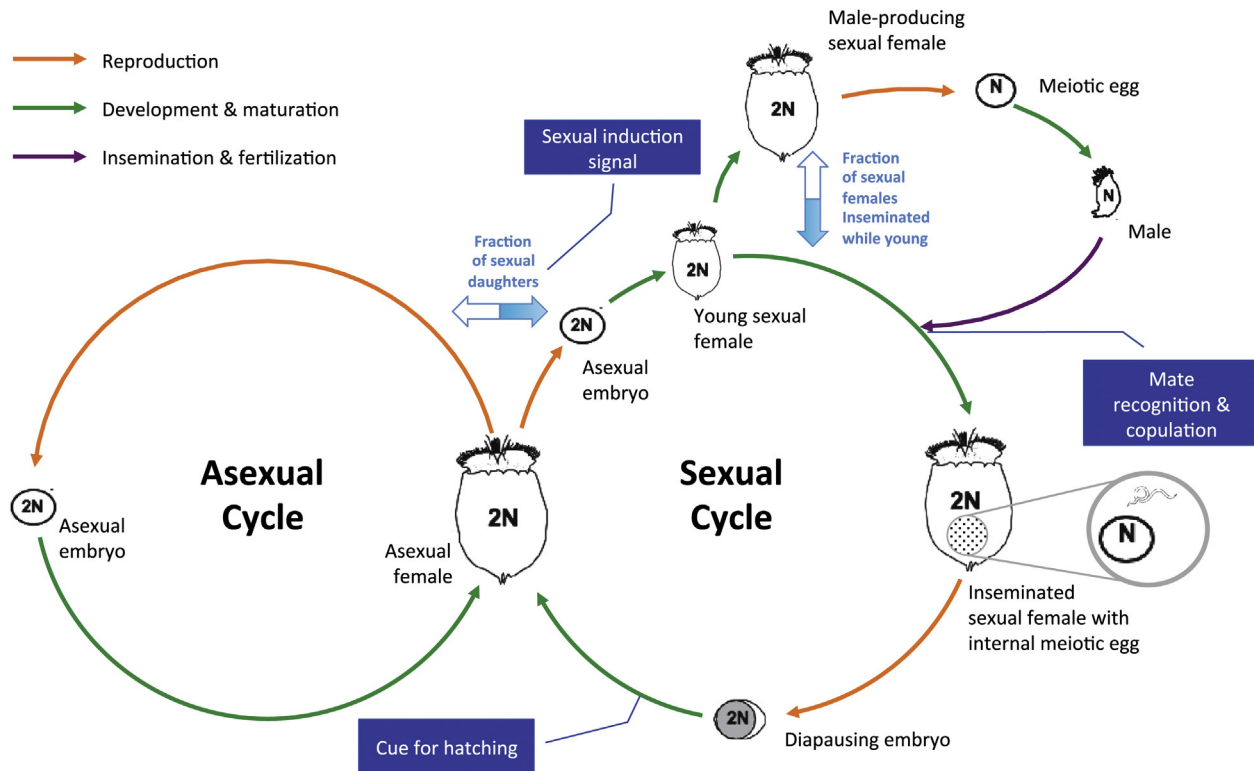


Fig. 3 Cyclical parthenogenesis of monogonont rotifer. Notice that (1) the fraction of sexual daughters is zero in absence of a sufficient sexual induction signal, so that a phase of exclusively asexual proliferation exists, and (2) some diapausing embryos may not respond to the hatching cues, they constituting a diapausing embryo bank. Fine line drawings courtesy of Africa Gómez and María José Carmona.

embryos on a substrate (e.g., *Philodina*) or simply drop them where they sink to the bottom (e.g., *Proales*). Embryos are typically deposited at the single cell stage before cleavage begins. In oviparous rotifer species, all embryological development takes place outside the mother. Some species (e.g., *Asplanchna*) are ovoviparous and give birth to fully formed live young. With a few exceptions reported, the asexual embryos are subitaneous (i.e., no dormancy). Both birth-flow and birth-pulse (i.e., those laying in clutches) species exist.

Cyclical Parthenogenesis in Monogononta

One of the most intriguing features of the life cycle of monogononts is their ability to engage in both asexual (amictic) and sexual (mictic) reproduction (Fig. 3). This life cycle is designated cyclical parthenogenesis (also, heterogony) because most reproduction is asexual (described above), with episodic bouts of sexual reproduction (mixis). Sexual reproduction starts with production of sexual females by asexual mothers. These two types of females are morphologically indistinguishable. Thus, the first visible sign of sexual reproduction in rotifer populations is the appearance of unfertilized sexual females who produce males (Fig. 4) (Wallace *et al.*, 2015), which occurs in response to specific environmental triggers. Researchers have investigated these environmental sexual induction signals for more than 50 years and examples of sexual reproduction induction by photoperiod, diet, or population density can be found in the literature. Many other intrinsic and extrinsic factors – such as age, genotype, salinity, temperature, and diet – influence the proportion of sexual daughters, but these usually are not regarded as key factors triggering mixis. The list of species responding to population density signals is growing and progress has been made over the last decade in identifying these chemicals. The process is understood as a case of quorum sensing (Kubaneck and Snell, 2008), widely known in bacteria, but its description in rotifers was the first for animals.

The general model for sexual reproduction in response to crowding is that rotifers excrete a chemical into the medium that accumulates to a threshold concentration, triggering a switch in oocyte maturation pathways to enable meiosis. Diploid mothers (i.e., sexual females) produce haploid embryos via meiosis that develop into haploid males if unfertilized (arrhenotokous parthenogenesis) or diploid diapausing embryos if they are fertilized. If a sexual female is inseminated when young, she will only produce diapausing embryos; otherwise, she will only produce males. Therefore, sex determination is haplodiploid.

Rotifers excrete a variety of compounds into their medium, producing 'conditioned medium', the chemical composition of which has been analyzed (Snell *et al.*, 2006). The signal triggering sexual reproduction was shown to be a protein in *B. manjavacas* and designated the mixis inducing protein (MIP) (Snell, 2017). It functions as a pheromone and *B. manjavacas* populations are exquisitely sensitive to the MIP signal, initiating sexual reproduction at population densities as low as 70 females L^{-1} .

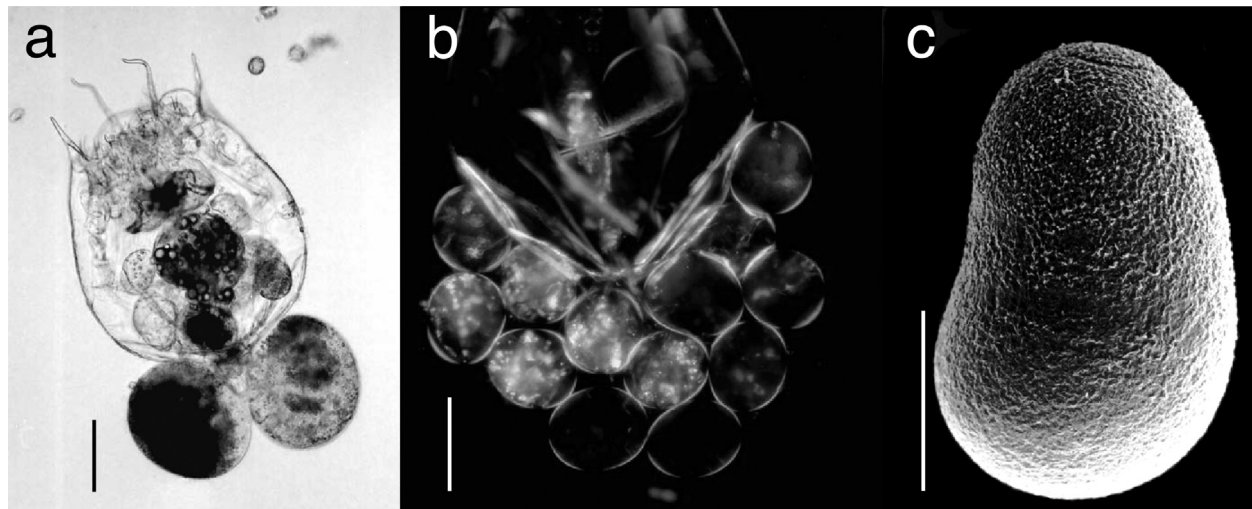


Fig. 4 Rotifer cyclical parthenogenesis in *Brachionus*. (a) Asexual female. (b) Male-producing sexual female carrying haploid eggs. (c) Diapausing embryo. Bars $\sim 50\ \mu\text{m}$. SEM photomicrograph (c) courtesy of Hendrik Segers.

The accumulation of the MIP to threshold concentrations triggers a cascade of events that results in oocyte maturation via meiosis rather than mitosis. The MIP pheromone is thought to bind to a receptor on the surface of females, initiating a signal transduction cascade that is regulated by hormones. These have not been characterized for rotifers, but the presence of steroid receptors for progesterone and estrogen-like molecules and presence of metabolic pathways for steroid synthesis suggest that steroids play an important role in these signaling pathways (Snell, 2011).

Male embryogenesis apparently parallels that of the female embryo to a certain point of organogenesis (e.g., in *Asplanchna*, formation of the cerebral-ganglion). At that point certain organs either degenerate (stomach) or fail to develop altogether (mastax). Spermatogenesis begins late in development of the unfertilized, haploid egg and includes, as its major features, cell elongation, flagellar differentiation, and development of the nuclear membranes. Developing along with the sperm are atypical germ cells that eventually degenerate. In recently matured males, sperm outnumber the atypical cell type by a ratio of 2:1. As they mature, the atypical cells extrude immobile, rod-like structures approximately $2 \times 10\text{--}15\ \mu\text{m}$ in size. These rods appear to facilitate passage of sperm through the female integument (see below). Except for a few species, most male monogononts have degenerate guts and do not feed; thus they are dependent on energy provided by their mother during oocyte development, and have shorter lifespan than females (Fig. 1).

Mating is another key step in sexual production (Fig. 5). Mature males cannot detect females from a distance and rely on random encounters to locate mates. Models of typical population densities and swimming speeds demonstrate that male encounters with fertile females during their short lifespans are sufficient to make mating probable. Upon encountering a conspecific young female, males of most monogonont species engage a stereotypical behavior called circling. They swim tight circles around females, skimming their body surface so that they maintain contact with their corona. Chemoreceptors on the male corona detect surface glycoproteins on females allowing conspecific identification. Brachionid males preferentially mate with young females. The chemical structure of this surface glycoprotein-mating signal has been identified and the underlying gene described. The role of this mate recognition protein in the development of rotifer reproductive isolation and speciation has been investigated (Gribble and Mark Welch, 2012).

Insemination usually is accomplished by hypodermic impregnation. Sperm are transferred by penetration of the female body wall by the male penis, but in a few species sperm are deposited directly into the cloaca. After introduction into the body cavity of females, they fuse with a mature oocyte in the ovary. The result of this fertilization is a diploid embryo that undergoes 5–6 rounds of cell division and then arrests its development at the 40–60 cell stage. The embryo, which is encased in a resistant shell and provisioned with yolk that enables dormancy, is a diapausing embryo (also called resting egg or diapausing egg). In this state the embryo is metabolically inactive and resistant to freezing and desiccation.

Diapausing embryos can remain dormant for decades, and are typically deposited in the sediments. Experimental work indicates that hatching from diapause is influenced by a variety of factors including temperature, light, salinity, and oxygen concentration. Hatching also is stimulated when diapausing embryos are exposed to UV light, dilute solutions of hydrogen peroxide, and certain prostaglandins. It has been hypothesized that active oxygen, either generated by UV photolysis or from the peroxide itself, oxidizes fatty acids within the dormant embryo. These fatty acids induce production of prostaglandins, and then initiate hatching.

Experimental studies on the monogonont life cycle are concentrated on a few model species. The remaining information has been recorded in more-or-less systematic field surveys, but that may be biased by variation in species abundance and distribution. Even though loss of sex is common in long-term laboratory populations, cyclical parthenogenesis is believed to be the ancestral state in monogononts. Out of a total of 30 monogonont families, males have been observed in 25 cases and diapausing embryos in 26. Only in two families (Birgeidae and Clariidaea) have none of these features been reported.

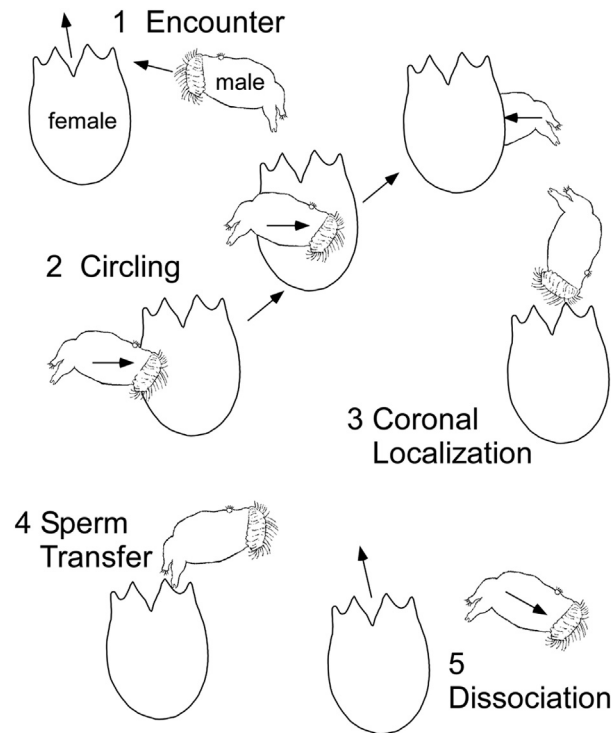


Fig. 5 Copulation in the monogonont rotifer as described in *Brachionus*. Other mating behaviors have been described in rotifers; these include male mate-guarding of unhatched mictic embryos and, apparently, cannibalism of their male consorts by newly mated mictic females.

Ancient Asexuality in Bdelloidea

Ancient asexuality in Bdelloidea was proposed a long-time ago because males had not been observed in either natural or laboratory populations, and meiosis has never been reported. Indeed the evidence against unobserved, rare sex is now much stronger and is supported by molecular signatures for lack of sexuality. Genomic analysis of the bdelloid *Adineta vaga* shows it is tetraploid and its genome comprises anciently duplicated segments and less divergent allelic regions, the latter sometimes being found on the same chromosome. Hence, meiotic pairing is not possible. Interestingly, transposable elements account only for 3% of the assembled genome sequence (Flot *et al.*, 2013).

It is now accepted that bdelloids have persisted for at least tens of millions of years and diversified into >300 species without engaging in sexual reproduction. This lack of the putative advantages of sex (i.e., mutation purge and/or adaptation to environmental change) is a unique case among animals and challenges evolutionary biologists to investigating the mechanisms and environments that make their evolutionary success possible.

Obligate Sexuality in Seisonidea

Seisonids reproduction is bisexual with a sex ratio of ~1:1. Gametogenesis takes place by ordinary meiosis with production of two polar bodies. Males and females are about the same size. The U-shaped, male genital apparatus consists of a pair of large, elongate testes and a large seminal vesicle where sperm receive additional processing. Two sperm are attached together, tightly rolled, and encysted with a secretion; the product is an encysted sperm pair (ESP) $\approx 1.5 \times 8 \mu\text{m}$. The ESP is moved through the vas deferens and into the cloaca. Mating in seisonids has never been observed, but workers suggest that the male transfers the ESP to a spatulate groove on the head and subsequently attaches it to the female's genital region. Unlike what is seen in the monogononts the embryo does not enter a dormant phase; i.e., it is subitaneous.

Reproductive Patterns and Their Adaptive Implications

Life-table experiments performed with bdelloids and monogononts show that life span, age at maturity and maximum age-specific fecundity are strongly affected by temperature, diet quality and quantity, salinity, pH, and toxicants. At 23–27°C, asexual offspring are produced by 2-day old mothers, potential lifetime fecundity ranges 10–40 offspring per female mostly concentrated in the early age classes, and lifespan is in the range of 5–40 d. In optimal laboratory conditions fecundity rate ranges from 0.2 to 1.2 offspring per female per day, and the populations can double their density every 1.5–2 d. In monogononts, age-specific fecundity is higher for

male-producing sexual females and lower for diapausing embryo-producing sexual females, as compared to asexual females. These differences correspond with allocation of resources per embryo. Lifetime production of diapausing embryos is only 5–40% that of asexual embryos. Generally reproduction is faster at higher temperatures over a range of 10–35°C.

Most rotifer habitats are suitable for population growth only temporarily. Rotifer populations in lakes are ecologically specialized so that they proliferate during specific seasons. Other rotifers inhabit temporary habitats such as rock pools and the water films of moist soils, mosses, and lichens. Rotifers are adapted to these recurrent, more or less predictable, adverse periods by producing resistant dormant stages with arrested metabolism. These resistant stages also function in dispersal. As expected from theoretical considerations, in both monogononts and bdelloids the resistant phases of the lifecycle are coupled with increases in genetic variation. In monogononts this is accomplished through recombination that occurs during sex. In bdelloids it arises from DNA breakage and repair during desiccation and results in large-scale horizontal gene transfer from a variety of prokaryotes and eukaryotes (Gladyshev *et al.*, 2008).

Many bdelloids have the ability to undergo anhydrobiosis: desiccate as an adult (xerosome) and as an embryo (xerooum), and to remain metabolically inactive, resuming their life cycle where they left off upon rehydration (Ricci, 2001). Few animals have the ability to engage in this amazing process. Desiccated individuals can survive for years in this state (Fig. 6). Not all adults survive desiccation, but bdelloid reproductive performance is not negatively affected by anhydrobiosis when metabolism is resumed. During desiccation, DNA breakage often occurs and bdelloids possess exceptional DNA repair mechanisms. This breakage is hypothesized to favor horizontal gene transfer, which accounts for about 8% genes in their genome unlike most other animal species (Gladyshev *et al.*, 2008). DNA repair is proposed to occur using collinearly paired chromosomes as homologous templates. These processes might compensate for their lack of recombination and enables adaptation and diversification similar to sexually reproducing animals (Boschetti *et al.*, 2012).

The growing season of a typical monogonont rotifer population starts when diapausing embryos hatch in response to warming temperatures and increased light level. The females hatched from diapausing embryos may have special features. For example, in *Polysartha* hatchlings lack paddle-like appendages that are usually present in females and are used to escape predators. In some *Brachionus* species, the females hatching from diapausing embryos are insensitive to mixis induction signals. This insensitivity persists, but is diminished, in the following generations, providing evidence for a transgenerational, epigenetic effect. Each female hatched from a diapausing embryo establishes a new clone (i.e., a lineage of genetically identical individuals, excluding mutations) via asexual reproduction. Such asexual reproduction results in fast population growth, an advantageous trait when growing in seasonally suitable or ephemeral habitats. When sexual reproduction is initiated, asexual females produce both sexual and asexual daughters. Thus, both modes of reproduction overlap. The diapausing embryos produced as a result of sexual reproduction fall to the sediment and where they can remain dormant for decades. Some sexual, diapausing embryos remain dormant only for a few days, whereas others will be dormant until the next growing season or for many years. The latter contributes to the diapausing embryo bank, a genetic and demographic buffer for the population. Mortality and hatching rates of diapausing embryos in the sediment are poorly known. In sediments chronologically structured, the density of viable embryos declines exponentially with depth. Long-term survival enables resurrection ecology of rotifers by hatching the diapausing embryos of past populations.

Sexual reproduction in monogononts has both benefits and costs (Stelzer, 2011). Regarding the later, (1) evidence suggests that the two-fold cost of sex is fully operative in rotifer populations. Theoretical evolutionary modeling predicts that half of the sexual females should be male-producers and half should be diapausing-embryo producers, the few empirical studies testing this being supportive of this prediction. As a result, two-fold more diapausing eggs could be produced if they could be produced asexually.

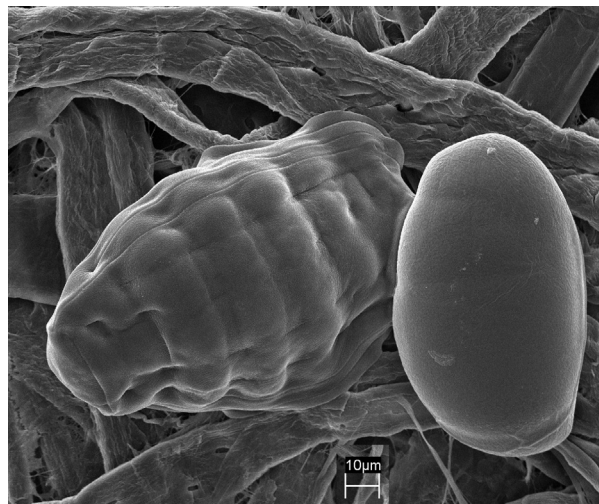


Fig. 6 Bdelloid adult (xerosome, left) and embryo (xerooum, right) in the anhydrobiotic state. Scanning photomicrograph courtesy of Giulio Melone.

(2) As male-female encounter occurs randomly, sex might be rare in low-density populations. (3) As populations composed of multiple clones, inbreeding is potentially important. (4) Resource allocation per unit is higher in diapausing embryos than in asexual embryos. (5) Metabolic arrest in diapausing embryos increases generation time. Thus, early sex might result in few male-female encounters and, by decreasing the rate of current population growth, could produce suboptimal exploitation of the environment. In contrast if delayed, sex could occur when the habitat is becoming unsuitable for sexual reproduction, diminishing diapausing embryo production. Consequently, the timing of sex in cyclically parthenogenetic life cycles is a critical component of fitness.

Three selective factors have been proposed to shape timing of sex in monogononts (Serra *et al.*, 2004): (1) habitat deterioration, (2) male-female encounter, and (3) resource demand. As a cue, population density might anticipate (#1), favor (#2), and occur in conditions favorable for reproduction (#3). Nevertheless, some diapausing embryos may hatch when population density is high. Proliferation of the resulting clones is much favored if they are temporarily insensitive to the mixis signal, as observed in *Brachionus*. Interestingly, laboratory populations evolve early sex in conditions mimicking ephemeral and fluctuating habitats (Smith and Snell, 2012). This last finding is consistent with the correlation between environmental unpredictability and propensity of sex found in natural populations. It is regarded as a conservative bet-hedging strategy, preventing the catastrophic effect of unexpectedly short growing seasons.

Besides enabling diapause, sex increases genetic variation, which is needed to drive natural selection and promote adaptation to novel conditions. Experimental monogonont populations in a novel environment evolve and can achieve an adaptive steady state in less than 2 months, which is equivalent to a few dozen generations (Becks and Agrawal, 2012). During adaptation they increase their rate of sex, but this rate declines to a plateau when the selection pressure ceases. Rotifer experimental systems where the diapause response is not a confounding factor (e.g., hatching rate of diapausing embryos is not dependent on the experimental treatment) offer an exceptional opportunity to explore the evolution of sex.

Applied Reproduction

Rotifers have proven useful in environmental toxicology and aquaculture. In environmental toxicology production of diapausing embryos by brachionids has enabled development of variety of toxicity tests that do not require culturing to supply test animals (Rico-Martinez *et al.*, 2013). These methods have allowed rotifer toxicity tests to be deployed widely without need to master the technical skills of culturing rotifers. Diapausing embryos of *B. plicatilis*, *B. manjavacas*, and *B. calyciflorus* now are commercially available and are readily shipped globally. All can be hatched in standard media within 24 h, yielding animals that are physiologically uniform for use in toxicity tests. Standard protocols have been developed using endpoints like survival, reproduction, swimming velocity, ingestion, and gene expression using well-characterized strains. Reproduction is especially important because it is one of the most sensitive endpoints and is a good estimator of chronic toxicity. The ecological significance of even small reductions in rotifer reproduction over many years has been demonstrated (Snell and Serra, 2000). As little as a 5% reduction in population growth rate can approximately double the probability of extinction for a population.

Hatchlings from diapausing embryos are all females who initiate asexual reproduction within 24 h of their birth. By 48 h, a typical female at 25°C will produce 5–10 daughters that can be counted and used to estimate the intrinsic growth rate (r), a statistic with considerable significance in ecology. Rapid asexual reproduction permits 2–3 generations of population growth to be quantified in one week, making the rotifer reproductive test one of the shortest for any animal. As a result of the convenience, speed, and sensitivity of rotifer reproductive tests, they have been widely employed to estimate chronic toxicity in fresh and marine waters.

A second industry that employs rotifers is aquaculture. Most marine fish larvae begin feeding on zooplankton of a size that is determined by the gape of their mouths. Rotifers have proven reliable and cost-effective as live feeds in the larviculture of many commercially important fish and crustacean species (Hagiwara and Yoshinaga, 2017). This is because marine *Brachionus* have a simple diet of microalgae, reproduce rapidly, are tolerant of a wide range of temperatures and salinities, and are tolerant of high population densities in mass cultures. Scientists in many countries have intensely investigated production by both marine and freshwater species of the genus *Brachionus*, how they can be manipulated to produce more rotifers per liter more efficiently. Most marine fish hatcheries worldwide have a small cadre of scientists and technicians dedicated to maintaining rotifer mass cultures. Aquaculture scientists have reported much of what is known about effects of diet, temperature, salinity, and pH on brachionid reproduction. Rotifer culture remains an active area of research, with investigations into how to enrich rotifers so that their nutritional quality closely matches the needs of fish larvae, identification of new rotifer species suitable for mass culture that are small enough to be ingested by young fish larvae, improving the stability of mass cultures to avoid crashes, and optimizing bio-processes of mixing and waste removal in mass cultures.

References

- Becks, L., & Agrawal, A. F. (2012). The evolution of sex is favoured during adaptation to new environments. *PLOS Biology*, 10, e1001317.
 Boschetti, C., Carr, A., Crisp, A., et al. (2012). Biochemical diversification through foreign gene expression in bdelloid rotifers. *PLoS Genetics*, 8, e1003035.
 Flot, J. F., Hespeels, B., Li, X., et al. (2013). Genomic evidence for ameiotic evolution in the bdelloid rotifer *Adineta vaga*. *Nature*, 500, 453–457.
 Fontaneto, D., & De Smet, W. (2015). Rotifera. In Schmidt-Rhaesa (Ed.), *Handbook of zoology, Gastrotricha and Gnathifera*. Berlin: De Gruyter.

- Gladyshev, E. A., Meselson, M., & Arkhipova, I. R. (2008). Massive horizontal gene transfer in bdelloid rotifers. *Science*, 320, 1210–1213.
- Gribble, K. E., & Mark Welch, D. B. (2012). The mate recognition protein gene mediates reproductive isolation and speciation in the *Brachionus plicatilis* cryptic species complex. *BMC Evolutionary Biology*, 12, 134.
- Hagiwara, A., & Yoshinaga, T. (Eds.). (2017). *Rotifers: Aquaculture, Ecology, Gerontology, and Ecotoxicology*. Tokyo: Springer.
- Kubaneck, J., & Snell, T. W. (2008). Quorum sensing in rotifers. In S. C. Winans, & B. B. Bassler (Eds.), *Chemical Communication Among Microbes*. Washington, DC: ASM Press.
- Ricci, C. (2001). Dormancy patterns in rotifers. *Hydrobiologia*, 446/447, 1–11.
- Rico-Martinez, R., Perez-Legaspi, I. A., Arias-Almeida, J. C., & Santos-Medrano, G. E. (2013). Rotifers in ecotoxicology. In J. F. Ferard, & C. Blaise (Eds.), *Encyclopedia of aquatic toxicology*. Berlin: Springer-Verlag.
- Serra, M., Snell, T. W., & King, C. E. (2004). The timing of sex in monogonont rotifers. In A. Moya, & E. Font (Eds.), *Evolution: From molecules to ecosystems*. Oxford: Oxford University Press.
- Smith, H. A., & Snell, T. W. (2012). Rapid evolution of sex frequency and dormancy as hydroperiod adaptations. *Journal Evolutionary Biology*, 25, 2501–2510.
- Snell, T. W. (2011). A review of the molecular mechanisms of rotifer reproduction. *Hydrobiologia*, 662, 89–97.
- Snell, T. W. (2017). Analysis of proteins in conditioned medium that trigger monogonont rotifer mictic reproduction. *Hydrobiologia*. <https://doi.org/10.1007/s10750-016-2936-y>.
- Snell, T. W., Kubaneck, J., Carter, W., et al. (2006). A protein signal triggers sexual reproduction in *Brachionus plicatilis* (Rotifera). *Marine Biology*, 149, 763–773.
- Snell, T. W., & Serra, M. (2000). Using probability of extinction to evaluate the ecological significance of toxicant effects. *Environmental Toxicology and Chemistry*, 19, 2357–2363.
- Stelzer, C. P. (2011). The cost of sex and competition between cyclical and obligate parthenogenetic rotifers. *The American Naturalist*, 177, E43–E53.
- Wallace, R. L., Snell, T. W., & Smith, H. A. (2015). Phylum Rotifera. In J. H. Thorp, & D. C. Rogers (Eds.), *Thorp and Covich's Freshwater Invertebrates*. Waltham, MA: Elsevier.

Relevant Websites

- https://www.youtube.com/watch?v=_LLRKYAiQYQ. – Brachionid (monogonont rotifer) Life Cycle (YouTube).
- <http://www.ucmp.berkeley.edu/phyla/rotifera/rotifera.html>. – Introduction to the Rotifera.
- <https://www.youtube.com/watch?v=44AhxpOaBml>. – Philodina (bdelloid rotifer) Life Cycle (YouTube).
- http://www.rotifera.hausdornatur.at/Rotifer_data/trophi/. – Rotifer Trophi Web Page.
- <http://www.ansp.org/research/systematics-evolution/collections/rotifera/>. – The Academy of Natural Sciences of Drexel University.
- <http://rotifera.hausdornatur.at/>. – The Rotifer World Catalog.
- <http://www.microscopy-uk.org.uk/mag/artnov99/rotih.html>. – Welcome to the Wonderfully Weird World of Rotifers.
- <http://jbpc.mbl.edu/wheelbase/>. – WheelBase.

Mollusca

Toshie Matsumoto, National Research Institute of Aquaculture, Mie, Japan

© 2018 Elsevier Inc. All rights reserved.

Mollusc is one of the most diverse groups in the animal kingdom and includes oysters, scallops, clams (Bivalvia), abalones, snails, slugs (Gastropoda), nautilus, squids and octopuses (Cephalopoda). Many bivalves, gastropods and cephalopods are economically valuable species and play an important role in the fisheries industries of many countries. Molluscs are mostly marine, but also include freshwater and terrestrial species. They burrow or crawl on rocky, sandy, and muddy substrata, become cemented to the surface, or swim. They have adapted to these environments and have developed diverse varieties of reproductive strategies. One of the primary reasons for the success of molluscs as a dominant phylum is the diversification of their reproductive systems. Although the reproductive strategies used by molluscs are indeed variable, some of the physiological mechanisms must be conserved in the phylum. Therefore, it is expected that the genes and proteins known to be involved in the fundamental processes of reproduction in other phyla are conserved and have physiological functions also in molluscs.

The genome sequencing and transcriptome analyses of various molluscan species including the sea hare *Aplysia californica*, the snail *Lymnaea stagnalis*, the slipper snail *Crepidula fornicata*, the snail *Ilyanassa obsoleta*, the owl limpet *Lottia gigantea*, the mussel *Mytilus galloprovincialis*, the Pacific oyster *Crassostrea gigas*, the scallop *Patinopecten yessoensis* and the clam *Meretrix meretrix*, have been performed for the survey of the conserved genes related to reproduction. Through bioinformatic analyses the reproduction-associated genes have been identified, and knowledge on the molecular mechanisms of reproduction in molluscs is gradually being accumulated. Similarly, draft genome of the pearl oyster *Pinctada fucata* was released and utilized to obtain structural information of genes and proteins involved in various reproductive processes of bivalves by screening reproduction-related genes reported from other molluscan species (Matsumoto *et al.*, 2013). In the article the reproduction-related genes of *P. fucata* were described based on 6 categories: germline differentiation; sex determination; gonad development; oocyte maturation and spawning; fertilization; others. Of these categories, this article focuses on *vasa* for germline differentiation, egg-laying hormone and caudo-dorsal cell hormone for spawning, and gonadotropin-releasing hormone for gonad development, because they are identified from several molluscan species. Other categories on molluscan reproduction are reviewed in another articles in this book (vitellogenesis, spermatogenesis, endocrine control of reproduction and genetics-oyster). More comprehensive reviews on the physiology of reproduction appear in Saleuddin and Mukai (2016).

Bivalvia

The majority of bivalves (clams, mussels, oysters, and scallops) are gonochoristic but several hermaphroditic species are known (e.g., the egg cockle *Fulvia mutica*, *Pecten albicans*). In bivalves, the gonad appears as a transient anatomical structure that develops during the reproductive season, when the body mass can more than double. The gonadic tissue consists of a series of connected tubules organized in sack-like structures, generally called genital tubules or acini, in which germ cells differentiate centripetally from the external border (the wall) to the center (the lumen), where mature gametes accumulate in the spawning season. The mature gametes are released from each gonad, and fertilization is external. After completion of spawning, unspawned gametes are reabsorbed and the gonad is disassembled. Consequently, the sex of the animal can be assessed only in the reproductive season, while it is not possible during the sexual rest, when there are no gametes. The gametogenic development of bivalves is usually classified into several stages such as undifferentiated, early developing, late developing, ripe, spawning and spent stages. At undifferentiated stage, the gonadal area is largely filled with the connective tissue cells, and genital tubules contain only undifferentiated germ cells (Fig. 1(a)). During developing stage, oögonia or spermatogonia develop from undifferentiated cells and divide actively, and gonadal area expands and invades the connective tissue (Fig. 1(b)).

Sexually reproducing metazoans generally segregate their germline away from their somatic tissue before they make gametes; in *Drosophila*, *Caenorhabditis elegans* and mouse, this occurs during embryogenesis. In these animals, maternal factors required for germline formation are localized in a posterior region of egg cytoplasm and are inherited into the primordial germ cells. Members of the *vasa* and *nanos* gene families are important for the specification and development of the germline in diverse animals. The *vasa* gene, firstly characterized in *Drosophila*, encodes a member of the DEAD (Asp-Glu-Ala-Asp) box family of ATP-dependent RNA helicases. In *Drosophila*, Vasa protein is detected in the germline throughout the development; it is incorporated in pole cells, expressed zygotically in migrating pole cells, found in the embryonic gonads and expressed in the germline during oogenesis. Pole cells of a *Drosophila* embryo contain maternal Nanos and start zygotic expression of *nanos* during their migration into the gonads. Vasa and Nanos are highly conserved among metazoans and many orthologues have been identified from various invertebrates and vertebrates.

In molluscs, *vasa* ortholog was first characterized in the Pacific oyster *Crassostrea gigas*, and isolation of *vasa* orthologs in other molluscs is continuing. The discovery of transcripts of a *C. gigas vasa* homolog led to the conclusion that *vasa* is present also in molluscs, and that it can be used as a germ line marker. A *vasa* homolog was also documented in *Mytilus galloprovincialis* and *Pinctada fucata*, at the transcript level, and in *Ruditapes philippinarum*, *Mya arenaria*, *Anadara kagoshimensis* and *C. gigas*, at the protein level (Milani *et al.*, 2017). Vasa expression during the gonad rebuilding of these bivalves was immunohistologically observed to verify its presence from undifferentiated germ cells to mature gametes using antibodies produced against *vasa* homolog of

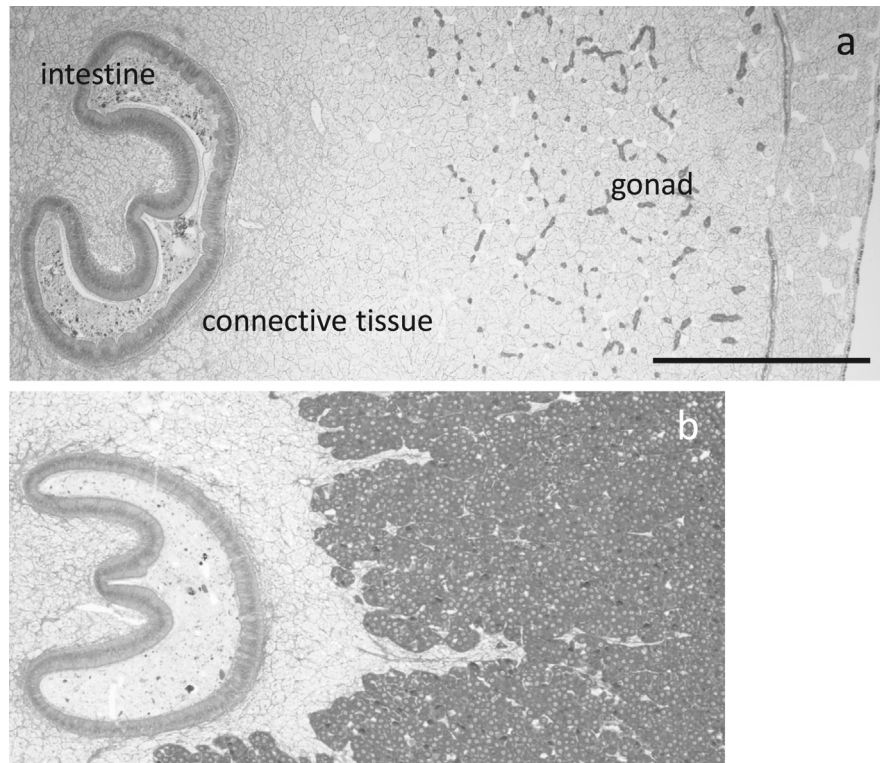


Fig. 1 Histological sections of the Pacific oyster *Crassostrea gigas*. (a) At undifferentiated stage the gonadal area is largely filled with the connective tissue cells. Genital tubules contain only undifferentiated cells. (b) At ripe stage genital tubules filled with full-grown oocytes enlarge and invade the surrounding connective tissue. Scale bar=1 mm.

R. philippinarum. In these species, the genital tubules are located close to the gut (intestine), extended inside the connective tissue. The gut epithelium consists of a monolayer of cells. In the basal side of the epithelium, vasa-stained cells are present. Stained cells with the same morphology are also dispersed in the connective tissue. At the beginning of gametogenesis, many stained cells are present in the connective tissue. During gamete maturation, acini are full of many differentiating germ cells and mature gametes. In female acini, the oocytes at the end of vitellogenesis show a faint vasa staining, while smaller oocytes show a stronger vasa staining. The male mature acini occupied by sperm show low or none of vasa staining. The immunohistological data obtained support a conserved mechanism of proliferation of “primordial stem cells” among the simple columnar epithelium of the intestine, as well as in the connective tissue, contributing to the seasonal gonad reconstitution. Vasa immunolabeled cells within the intestinal wall are commonly found across bivalves, the proliferation of vasa-stained cells in the intestinal epithelium is correlated to the stage of gonad maturation. The presence of germ cell precursors in the gut epithelium appears to be a feature in common with model organisms, such as mouse, fruit fly, and human.

In gastropods, *vasa* and *nanos* orthologs have been isolated from the snail *Ilyanassa obsoleta* and the abalone *Haliotis asinina*. In *I. obsoleta*, both *vasa* (mRNA) and *nanos* (mRNA and protein) are uniformly distributed during early cleavage and then become selectively expressed in the 4d lineage. In *H. asinina*, both *vasa* and *nanos* mRNA are detected in the mesodermal region of the larva. Furthermore, *nanos* is required to maintain the fate of the 4d lineage in *Ilyanassa*; knockdown of the *nanos* ortholog activity in the embryo clearly blocked normal development of the mesendodermal blast cell lineage derived from the 4d cell.

Gastropoda

All gastropods have a single gonad, and sexes are separate among most of members of prosobranchs but the majority of gastropods (opisthobranchs and pulmonates) are hermaphrodites. In these animals, both male and female gametes are produced in a single ovotestis and these are carried by a hermaphroditic duct to the carrefour/fertilization pouch where, following fertilization (self or cross), the eggs are coated by the perivitelline fluid secreted by albumen gland. They then pass through the female tract where the eggs are coated in a jelly mass and/or put in capsules. Most of the hermaphroditic species do reproduce by cross-fertilization. Since these animals have their male and female gonopores located on different parts of the body, mating chains or rings can form. In such a chain, the frontmost individual acts only as a female, the last individual only as a male, and each individual in between acts as male to the partner directly in front of it and female to the partner directly in its back. The role of neuropeptides in egg laying behavior has been extensively investigated in *Aplysia* and *Lymnaea*.

In the sea hare *Aplysia*, ovulation and spawning are triggered by a neuropeptide, the egg laying hormone (ELH), synthesized and released from the neuroendocrine bag cell neurons in the abdominal ganglia (Fig. 2(a)). Injection of an abdominal ganglion extract from *A. californica* induced egg laying and associated behaviors in several other *Aplysia* species. The bag cells are a pair of cell clusters, and each group contains about 400 cell neurons. When the animal is ready to lay eggs, a neural signal is initiated in the cerebral ganglia. This signal is relayed to the pleural ganglia, and then transmitted to the bag cell neurons. After the neural signal activates bag cell neurons, ELH is secreted. The large preprohormone of ELH is processed into mature ELH together with the shorter peptides, bag cell peptides (BCPs) which are also involved in the control of the egg laying. In addition, the exocrine atrial gland in the reproductive tract of *Aplysia* expresses multiple ELH-precursor-related genes, including one encoding peptide A and another encoding peptide B. Peptides A and B induce the electrical discharge of bag cells *in vitro*. Comparison of the structures of ELH precursor and the ones for the atrial gland peptide A and B (ELH related precursor A and B, ELHRa and b) revealed their close structural similarity. The ELHRs contain an ELH domain which is cleaved to produce ELH. The ELH and the ELHR genes form the egg-laying hormone gene family.

The egg-laying of *L. stagnalis* is regulated by neurosecretory system quite similar to the bag cell system of *A. californica*. The administration of cerebral ganglion extract from *L. stagnalis* also caused spawning of snails of the same genus. In *L. stagnalis* ovulation and spawning are controlled by a neuropeptide called caudo-dorsal cell hormone (CDCH) secreted from the caudo-dorsal cells (CDC) in the cerebral ganglia (Fig. 2(b)). These cells are located in two paired clusters of about 50 neurons each in the cerebral ganglia. Egg-laying consists of a sequence of behavioral events beginning with a rest period when the animal ceases to locomote, then a turning phase characterized by counterclockwise shell movements and high frequency rasping to clean the substrate, followed by oviposition and a final phase called inspection when the snail moves along the length of the egg-mass brushing it with lips and tentacles. Egg laying in *Lymnaea* is triggered by a discharge of spiking activity in CDCs, the resting phase. During this discharge the CDCs express several peptides such as CDCH and four kinds of caudo-dorsal cell peptides (CDCP), encoded on the CDCH-gene. ELH and CDCH, or BCP and CDCP, share 50%–70% similarity in amino acid sequence. CDCH also initiates the actual ovulation, which is followed by the fertilization and packaging of the released ripe eggs by the female accessory glands. During turning phase the animal starts locomoting again, turns its shell back and forth and rasps the surface. This process continues as long as the passage of eggs through the female tract is registered. The size of egg mass is independent of the injected dose of CDCH. Oviposition starts when the egg mass emerges from the female gonopore and is fixed to the substrate. This phase usually lasts about 10 min during which the animal continues rasping, stops shell turning and hardly locomotes. When the egg mass has been deposited, the animal crawls along the mass, without rasping or shell turning, before leaving it behind; this is referred to the inspection phase. When egg laying starts, ripe oocytes are ovulated from the ovotestis and fertilized in the fertilization pouch. Adult *L. stagnalis* can lay egg masses at a frequency of at least one mass per week, and such masses contain an average between 50 and 150 eggs depending on the individual's body size. *L. stagnalis* that are older than 300 days usually show a decline in egg production. Other factors that inhibit egg laying are starvation and dirty water. Spiking activity of the CDCs is not affected by dirty water. However, during starvation the CDCs become hyperpolarized, which elevates the threshold for egg laying.

In abalones *Haliotis rubra* and *Haliotis asinina*, using the same antibody, ELH-immunoreactivity was detected in neurons of the cerebral and pleuropedal ganglia, and in the ovary. The positive cells were numerous in order of the pleuropedal, cerebral and visceral ganglion. In addition, the intense reactivity was detected in the follicular and granular cells adjacent to mature oocytes in the ovary. In contrast, gastropods (*Aplysia* and *Lymnaea*) ELHs were also detected in the female reproductive tract (the oothecal gland, muciparous gland, pars contorta, and ovotestis, respectively) but none have been reported in the ovarian tissue.

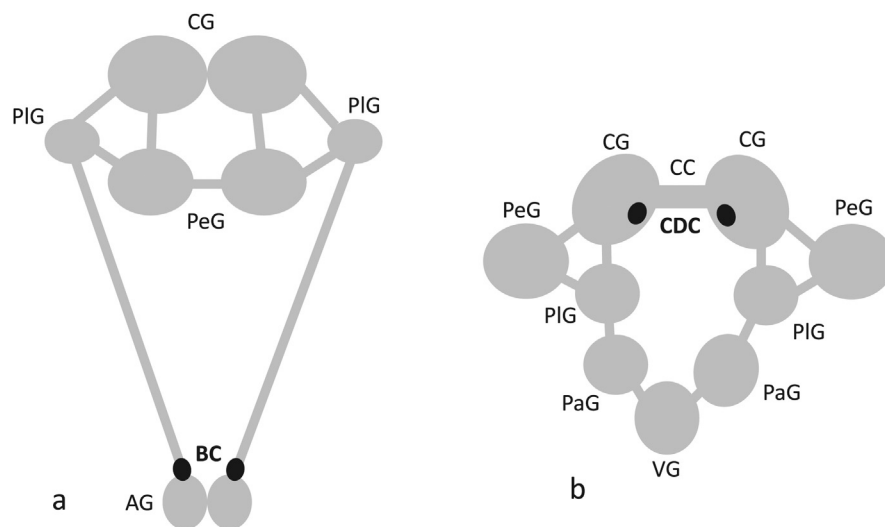


Fig. 2 Central nervous system of *Aplysia* (a) and *Lymnaea* (b). AG, abdominal ganglion; CG, cerebral ganglion; PeG, pedal ganglion; PIG, pleural ganglion; PaG, parietal ganglion; VG, visceral ganglion; CC, cerebral commissure; BC, bag cell; CDC, caudo-dorsal cell.

ELH and CDCH share relatively high structural similarity, and therefore CDCH is now often referred to as the ELH of *L. stagnalis*. In molluscs, presence of the ortholog genes of ELH have been reported from the abalone *H. rubra*, the limpet *Lottia gigantea*, the oyster *C. gigas* and the pearl oyster *P. fucata*. In *C. gigas* and *P. fucata*, ELH-related peptides are duplicated on the respective precursors. The amino acid sequence alignment of the two predicted ELH domains with the ELH domain of other ELH family peptides indicated structural similarity. But the overall structures of these ELH peptide precursor were different from those of *Aplysia* ELH, ELHR and *Lymanaea* CDCH. For instance, BCP or CDCP peptides are not found on the precursor proteins of *L. gigantea*, *C. gigas* and *P. fucata*. In the phylogenetic analysis, *L. gigantea*, *C. gigas* and *P. fucata* ELH form a separate clade to *Aplysia* and *Lymanaea* ELH (Stewart *et al.*, 2014).

Cephalopoda

Sexes are separate in cephalopods and sexual dimorphism is evident in almost all species. Males are smaller in a few species, possess hectocotylus arms that are used for the transfer of spermatophores into the female's body. Many cephalopod males exhibit an array of courtship behaviors during mating. Little is known about the life span of cephalopods. The majority of them are semelparous, in which death follows after the reproductive period in both sexes. The common octopus *Octopus vulgaris* normally breeds in its second year; the females lay eggs and care for them without feeding and then die shortly after the eggs hatch. Death occurs owing to a secretion from the optic gland, a pair of small bodies on the optic tracts connecting the optic lobes with the central regions of the brain. However, if the optic glands are removed from brooding females, the animals abandon their eggs, resume feeding, and the programmed death is delayed for a considerable period. Sexual maturity in *O. vulgaris* is always associated with enlargement of the optic gland. Implantation of the glands from sexually mature individuals to immature recipients induces precocious maturation. Optic gland implants between males and females are equally effective. Sexual maturation of the female octopus is hormonally controlled by a pair of optic glands. The gland regulates various reproductive events such as proliferation of gonadal cells and yolk-protein synthesis.

In vertebrates, gonad maturation is primarily regulated by the hypothalamus-pituitary-gonadal axis. Gonadotropin-releasing hormone (GnRH) is a neuropeptide originally identified to be synthesized in the hypothalamus of mammals. It is transported from nerve endings in the hypothalamus to the adenohypophysis where it stimulates the release of the gonadotropins, thus serves as a key regulator of reproduction of vertebrates. The vertebrate GnRH is a 10-mer peptide with N-terminal pyro-Glu and C-terminal Pro-Gly-NH₂ structure.

GnRH in cephalopods was initially demonstrated by immunohistochemistry with anti-chicken GnRH antibody; octopus GnRH-containing neurons were diffusely distributed in various lobes in the octopus brain, including the subpedunculate and the posterior olfactory lobes. Because GnRH-containing nerve process are found in the optic tract and optic gland, it is suggested that GnRH neurons stimulate optic gland activity, which results in gonadal maturation. The presence of molluscan ortholog of GnRH has been clarified in the octopus *O. vulgaris* which was followed by the identification of GnRH-like peptides in the swordtip squid *Loligo edulis*, the common cuttlefish *Sepia officinalis*, the sea hare *A. californica*, the limpet *L. gigantea*, the scallop *P. yessoensis*, the oyster *C. gigas* and the pearl oyster *P. fucata*. The precursor structure of vertebrate GnRHs is well conserved also in the structure of the molluscan counterparts deduced from cDNA information. Purified octopus GnRH-like peptide has two amino acid insertions at the N-terminal and C-terminal Pro-Gly-NH₂ structure that is critical to gonadotropin-releasing activity in vertebrates. Primary structures of the other two cephalopod GnRHs were predicted to be identical to octopus GnRH. Thus, cephalopod GnRHs are 12-mer peptides whereas for the other molluscan GnRHs, the C-terminal glycine residue is missing, and hence the deduced mature peptides are 11 amino acids long without C-terminal amidation. In *C. gigas*, two types of GnRH peptides (Cg-GnRH-a and Cg-GnRH-G) were characterized by mass spectrometry, and possessed an identical amino acid sequence but differed in the presence (Cg-GnRH-a) or absence (Cg-GnRH-G) of the amidation at the C-terminal. As for the physiological function of GnRH in molluscs, octopus GnRH-like peptide and scallop GnRH-like peptide are suggested to control the gonad development. In the chiton *Mopalia sp.*, exposure to dilutions of lamprey GnRH1 or tunicate GnRH-2 in sea water induced spawning which suggested pheromone-like function of GnRH in this species. In contrast, GnRH of the sea hare *A. californica* had no effects on the acute activation of various aspects of reproduction in this species.

References

- Matsumoto, T., Masaoka, T., Fujiwara, A., et al. (2013). Reproduction-related genes in the pearl oyster genome. *Zoological Science*, 30, 826–850.
- Milani, L., Pecci, A., Ghiselli, F., et al. (2017). VASA expression suggests shared germ line dynamics in bivalve molluscs. *Histochem Cell Biol*, 148, 157–171.
- Saleuddin, S., & Mukai, S. (Eds.). (2016). *Physiology of Molluscs*, 2. New Jersey: Apple Academic Press, Inc.
- Stewart, M. J., Favrel, P., Rotgans, B. A., et al. (2014). Neuropeptides encoded by the genomes of the Akoya pearl oyster *Pinctata fucata* and Pacific oyster *Crassostrea gigas*: A bioinformatic and peptidomic survey. *BMC Genomics*, 15, 840.

Reproduction in the Annelida

Susan D Hill, Michigan State University, East Lansing, MI, United States

Naim Saglam, Firat University, Elazig, Turkey

Daniel H Shain, Rutgers, The State University of New Jersey, Camden, NJ, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Albumenotrophy Yolk external to the embryo (e.g., in cocoon fluid) that is a food source during development.

Chaetae Bristle-like structures, also called setae.

Clitellum Glandular, annular swelling of the epidermis; secretes material to form a cocoon.

Cocoon Protective case, covering of an egg mass or immature stage.

Epididymis Coiled part of the vas deferens.

Epitoky Process by which an asexual individual (the atoke) is transformed into or gives rise to one or more sexual individual(s) called epitokes that produce eggs and sperm. Epitokes leave the bottom for the purpose of reproduction.

Errant (Errantia) Polychaetes are grouped into two large clades, Sedentaria and Errantia, originally based on their locomotory patterns.

Gonochoric Eggs and sperm produced in separate individuals.

Gonopore External opening of the male and female reproductive organs.

Hermaphrodite Individual with both male and female sexual systems.

Lecithotrophic Egg yolk provides all nourishment; larvae are non-feeding.

Metatrochophore Polychaete larva that displays segmentation. Feeding or non-feeding. Stage at which settling and metamorphosis occur in some species.

Nectochaete Polychaete pelagic larva that uses parapodia as locomotory organs. Feeding or non-feeding. Stage at which settling and metamorphosis occur in some species.

Oocyte Immature female gamete that undergoes meiosis, giving rise to an egg.

Oogonia Germ cells that give rise to oocytes by mitotic division.

Operculum A structure that seals or closes a tube; lid-like cover on the opening of a cocoon.

Parapodia Lateral, segmental outgrowths that usually bear chaetae.

Parthenogenesis The development of an individual from an unfertilized egg.

Planktotrophic Feeding on the plankton.

Predaceous Consuming other animals for nutrients.

Prostate gland An elaboration of the sperm canal that secretes a prostatic solution.

Protandry Type of hermaphroditic sex determination in which the male reproductive state precedes female. Individual produces sperm then, later in life, eggs.

Protogyny Type of hermaphroditic sex determination in which the female reproductive state precedes male. Individual produces eggs then, later in life, sperm.

Sanguivorous Consuming blood for nutrients.

Spermatheca A genital structure in which sperm is received and may or may not be stored.

Spermatophore A packet of sperm for transfer from male to female.

Spiral cleavage A type of cleavage in which blastomeres appear to be produced in a spiral pattern as opposed to a radial one. Spiral cleavage is the basic cleavage pattern of protostomes; radial cleavage is the basic pattern of deuterostomes.

Testis sac A membranous sac around the testis, seminal vesicle and the funnel to the vas deferens.

Trochophore Top-shaped swimming larva that is the common uniting factor of the trochozoan clade (part of the Lophotrochozoa clade). Usually with three bands of cilia, the prototroch, metatroch, and telotroch. May be feeding or non-feeding.

Tubicolous Living in a tube.

Vas deferens Sperm duct leading away from a testis, carries sperm to the male pore.

Vitellogenesis The production of yolk.

Introduction

Annelids are segmented worms that occupy marine, freshwater and terrestrial habitats. Historically, the Phylum Annelida was subdivided into the polychaetes (mostly marine worms), oligochaetes (e.g., earthworms) and hirudinids (leeches), but more recently the oligochaetes and hirudinids have been combined into the Superclass, Clitellata. Also, modern molecular phylogenies have reclassified a number of related phyla (i.e., Echiuroidea, Sipuncula, Siboglinidae (Pogonophora and Vestimentifera)) as derived polychaetes.

The general annelid body plan is relatively simple, comprising an anterior mouth and posterior anus separated by a variable number of segments. Polychaetes display segmental parapodia (lateral extensions which can be quite ornate) containing many bristle-like structures called chaetae, while clitellates (excluding leeches) have reduced, segmentally-iterated chaetae that occur in bundles. Leeches are unique in having a fixed number of 32 segments and specialized anterior and posterior suckers.

Reproduction in annelids ranges from fertilization in the open water to asexual fragmentation and parthenogenesis. Here we consider reproductive strategies in the two major annelid groups, the Polychaeta and the Clitellata.

Polychaeta

Polychaete worms, with few exceptions, are marine and estuarine. They are ubiquitous in the ocean, burrowing and hunting in the benthos, crawling on algal covered rocks, living in self-made tubes, or swimming in the water. Polychaetes are as diverse as any other major animal group and exhibit many variations in their reproductive modes. A few species are being developed as model organisms. *Capitella teleta* (Hill and Savage, 2009; Seaver, 2016), whose genome has been sequenced (see Relevant Websites section), *Platynereis dumerilii* (Fischer et al., 2010; Zantke et al., 2014; see Relevant Websites section) and members of the genus *Ophryotrocha* (Thornhill et al., 2009) are all rich sources of information. Bioinformatics programs allow many comparisons and data are accumulating. Since more than 10,000 polychaete species have been described, however, an obvious need exists for further investigation.

Polychaetes reproduce and disperse in many ways. Although sexual reproduction (fertilization of eggs by sperm) is most common, some display remarkable asexual reproductive capabilities.

Sexual Reproduction

Most polychaetes are gonochoric with sex determination usually being genetic, although not well-understood. In some species, sex is influenced by environmental conditions, bestowing a reproductive plasticity on these species. Perhaps the most unusual occurs in *Bonellia viridis*, an echiuroid now included within Polychaeta. Here, sex is determined by the settlement of the larva. A larva settling on substrate becomes female, but one settling on an adult female's yard-long proboscis or body wall migrates to the female's uterus, becoming a tiny parasitic male. In this species, material produced by the female has a masculinizing influence on the sex of the larva.

Production of eggs and sperm by specialized organs – ovaries and testes – is most common, but gametes also seem to arise directly from the linings of the coelomic cavity in some species. Ovaries and testes may be permanent or transient (meaning they are not visible when the adults are not in reproductive mode). Gonads may be present in nearly every segment or restricted to part of the body. In *C. teleta*, for example, ovaries are restricted to the first 20–25 abdominal segments. Gametes often mature in the ovaries (*C. teleta*) and testes, in the coelomic cavity (*P. dumerilii*), in other parts of the reproductive tract or in the seawater. Reproductive tracts may be elaborated with species-specific accessory structures, like seminal vesicles in males or seminal receptacles and uteri in females. Gametes reach the external environment via nephridia, specialized gonoducts or coelomoducts, or by rupture of the body wall.

Although males and females are usually separate, in many species hermaphroditism is common (Schroeder and Hermans, 1975). Some serpulids (*Spirorbis* spp.) and hesionids (*Microphthalamis* spp.) develop as simultaneous hermaphrodites, but in others gamete production is sequential. Among species that have been studied, protandry is more common than protogyny. *Ophryotrocha puerilis*, for example, is protandrous, developing first as male, then adopting a female morphology and reproductive mode. In *O. puerilis*, environmental factors (here, the presence of other worms) also play a role in sex determination. Similarly, *C. teleta* males predictably switch to egg-laying mode if the density of conspecifics is low. Whether such hermaphrodites are self-fertile or not is species-specific and often has not been determined.

Asexual Reproduction

Asexual reproduction by budding (seen most often in sessile filter feeders like sabellid and serpulid fanworms), fission or fragmentation is quite common. *Eurythoe* bristleworms, common inhabitants of saltwater tanks, reproduce readily by fragmentation. In many asexually reproducing worms, new anterior (eyes, palps) and posterior (growth zone) structures are well-formed before the individuals separate. Not surprisingly, animals that reproduce asexually are usually able to regenerate missing portions. Superficially, regeneration and asexual reproduction are similar; differences at the cellular/gene level exist and are being explored (Kosyuchenko et al., 2016).

Fertilization

Sexual reproduction involves the fusion of eggs and sperm, so gametes must come into contact. Polychaetes have solved this problem in a number of ways. In species where worms are in close proximity, fertilization is often internal and continues largely independent of environmental factors. Sperm transfer may occur by copulation, hypodermic impregnation or transfer of spermatophores from male to female. Often eggs that are fertilized internally are protected. They may be brooded in specially constructed tubes (*C. teleta*, *Polydora cornuta*), enclosed within the body (the operculum of many spirorbids), placed under the elytra of scale worms, attached to the parent's body (some syllids) or deposited in jelly masses that may or may not be guarded by the parents (*O. puerilis*).

In tube-dwelling polychaetes, broadcast spawning is common with one spawning event triggering other nearby individuals to release their gametes. In many errant polychaetes, massive spawning events are triggered by lunar, tidal and diurnal cycles. Sometimes, even short periods of quite dim light are adequate for synchronization of gametogenesis and spawning (some platynereids). The most famous of these synchronized sexual rendezvous is that of the Palolo worm (eunicids of the South Pacific and elsewhere) that spawn once a year around the seventh night after the full moon that follows the autumnal equinox, and whose gamete-filled bodies are considered a gourmet delicacy.

In preparation for these mating dances, an asexual individual may undergo a dramatic transformation known as epitoky, as breeding season approaches. *Platynereis*, for example, undergoes two types of metamorphic changes – first, from a five-segmented larva to a tubiculous feeding worm, and again when it reaches about 70 segments and begins to make gametes. At that point, it ceases to feed, the gut is resorbed, posterior parapodia change shape, becoming swimming paddles, eyes enlarge, the posterior fills with either eggs or sperm, and worms leave their tubes to become pelagic. Change involves almost every system in the body as an asexual form (the atoke) is transformed into a sexual individual (the epitoke) that is prepared for swimming and mating. Swarming is synchronized by light and the lunar cycle. The mature adults release gametes into the water and die.

In some polychaetes, a budding or splitting of the body occurs. In many nereids and eunicids, for example, the worm becomes divided into an anterior, asexual atoke and a posterior epitoke that produces gametes. At spawning, the epitoke detaches from the atoke and swims to the surface, usually leaving the atoke in the benthos to prepare for another reproductive round. In the Palolo worm (*Eunice viridis*), posterior segments of males and females lose most of their internal organs and become long chains of sperm-filled or egg-filled sacs. At swarming time after the autumnal equinox, these long, headless, strings (epitokes) break off from the parent and swim to the surface where the gametes are released. Spent epitokes drift to the bottom.

Some species use asexual reproduction to bud off entire new gonad-bearing individuals (epitokes), rather than just gamete-filled segments. In these cases, each budded individual develops eyes, palps and other anterior (as well as posterior) features. *Myrianida pachycera* forms a chain of individuals that grow and mature sexually as they are pushed posteriorly (Fig. 1). When gametes are mature, the individual drops off the posterior end.

Early Development

Polychaetes, like molluscs and several other lophotrochozoans, are spiralian. Most undergo typical spiral cleavage. In worms that have been investigated, stereotypical mosaic development is the norm so the fate of blastomeres is predictable. Experiments using *C. teleta* reveal many similarities with other spiralian but also differences, emphasizing the need for further investigations (Seaver, 2014). Like other protostomes, the mouth usually forms from or near the blastopore. The anus is usually terminal.



Fig. 1 *Myrianida pachycera*. Original asexual individual (atoke) budding off epitokes. Picture – Greg Rouse – Tree of Life (*Myrianida pachycera*), Available at: <http://tolweb.org/tolarchive/2486/20020807/Annelida.html>.

Larval Forms and Dispersal

For a reproductive event to be successful, offspring must survive to adulthood and themselves become reproductive. In marine organisms, this often involves a free-swimming, larval stage (Wilson, 1991). Some polychaetes are direct developers (many syllids, some sabellids) with no larval stage. More commonly, however, polychaetes have a swimming larval (dispersal) stage. Often the larva is also a feeding stage, especially if the egg from which it developed was small and non-yolky (Table 1). The classic trochophore – a larval form shared with some other lophotrochozoans (e.g., molluscs) – is a top-shaped free-swimmer that moves through the water propelled by characteristic bands of cilia and usually has a sensory apical tuft of cilia (Fig. 2). Prior to settlement, trochophore larvae undergo initial segmentation, becoming metatrochophores (*Riftia pachyptila*, *P. dumerilii*) or longer, more slender nectochaetes (Fig. 3) that use parapodia for swimming (*P. dumerilii*). Not all larvae go through these stages; *C. teleta*, for example, directly forms a segmented metatrochophore as its only larval stage, bypassing the trochophore and never forming a nectochaete. None are known to metamorphose prior to forming some segments.

Settling and Metamorphosis

For most polychaetes, the larva is an important dispersal stage, enabling the species to colonize new areas often distant from the parents. To accommodate their planktonic life style, larvae may use sense organs quite unlike those of the adult. Eyes (many larvae are phototropic), nuchal organs (putative chemoreceptive organs near the eyes), apical tufts or organs and other sensory cilia are all common. Larvae may remain in the water column, potentially feeding, depending on the species, for several days or weeks, until a suitable area for settlement is found. Even though such larvae are small, approximately 50–200 micrometers in length, their abilities to colonize distant, fragmented and discontinuous sites are remarkable. *Capitella teleta* inhabits disturbed areas of high organic content like the areas under fish farms, polluted harbors and whale falls that may be separated by many kilometers. When larvae in the water column sense suitable substrate, they settle and immediately metamorphose into tiny juvenile worms.

Hydrothermal vents are extremely ephemeral because of volcanic and seismic activities, and yet new vents are colonized quite rapidly even though they may be hundreds of kilometers from old vent sources. The vestimentiferan tubeworm, *Riftia pachyptila*, releases non-feeding trochophore larvae that survive at least 38 days in the water column. The metatrochophore larvae colonize new vent sites tens to hundreds of kilometers away from spawning sites!

Metamorphosis may be undramatic (*C. teleta*) because many adult features are already present, or may involve a major reorganization (*Owenia fusiformis*). At the completion of metamorphosis, young worms usually follow the life style of the adults. Growth, by the addition of new segments at a posterior growth zone in front of the pygidium, usually continues throughout life.

Clitellata

Clitellate annelids are distinguished from other worms by their characteristic reproductive structure, the clitellum, whose function is to secrete an egg capsule, or cocoon. The clitellum appears as a thickened sleeve or saddle a few segments in length within the

Table 1 Diversity of reproductive modes

Reproductive mode	Larval developmental mode	Percentage of > 300 species showing combination
Free-spawning	Planktotrophic	26
Free-spawning	Lecithotrophic	11
Free-spawning	Direct	3
Brooded in tubes	Planktotrophic	10
Brooded in tubes	Lecithotrophic	4
Brooded in tubes	Direct	18
Brooded on exterior of body	Planktotrophic	2
Brooded on exterior of body	Lecithotrophic	2
Brooded on exterior of body	Direct	5
Brooded internally	Planktotrophic	0
Brooded internally	Lecithotrophic	1
Brooded internally	Direct	6
Encapsulated in gelatinous mass	Planktotrophic	3
Encapsulated in gelatinous mass	Lecithotrophic	5
Encapsulated in gelatinous mass	Direct	4

Note: Table combines reproductive methods and type of larval development (feeding) modes. Planktotrophic – larvae feed on plankton; lecithotrophic – larvae are non-feeding and are provisioned with nutrients by yolky egg; direct – young develop directly as miniature adults with no larval form or metamorphosis.

Source: Modified from Wilson (1991. Sexual reproductive modes in polychaetes: Classification and diversity. Bulletin of Marine Science 48, 500–561). The journal is published by the Rosenstiel School of Marine & Atmospheric Science, University of Miami, 4600 Rickenbacker Causeway, Miami, FL 33149-1031, United States and based on characteristics of over 300 polychaete species belonging to 36 families illustrating diversity of reproductive modes.

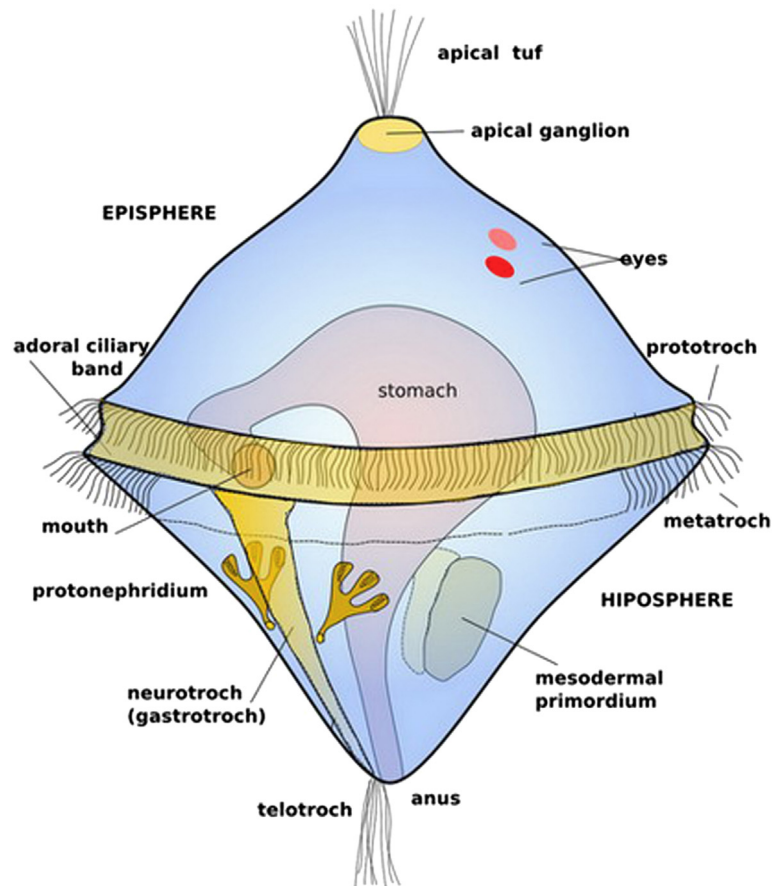


Fig. 2 Typical polychaete larva showing prototroch, metatroch and telotroch. Available at: https://images.search.yahoo.com/yhs/search?p=annelid+trochophore&fr=yhs-mozilla-003&hspart=mozilla&hsimp=yhs-003&imgurl=http%3A%2F%2Fupload.wikimedia.org%2Fwikipedia%2Fcommons%2Fthumb%2F2%2F24%2FPomatoceros_lamarckii_development.jpg%2F220px-Pomatoceros_lamarckii_development.jpg#id=6&iurl=http%3A%2F%2Fksuweb.kennesaw.edu%2F~jdinber%2FInvertZoo%2FLecMollusca%2FTroch.jpg&action=click.

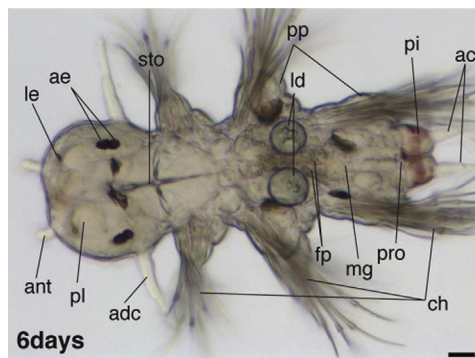


Fig. 3 Nectochaete larva of *P. dumerilii*. From Fischer, H.L., Thorsten, H., Arendt, D., 2010. The normal development of *Platynereis dumerilii* (Nereididae, Annelida). *Frontiers in Zoology* 7, 31. Available at: <https://doi.org/10.1186/1742-9994-7-31>. (photo also – <http://4dx.embl.de/platy/img/lab/4d.png>).

anterior third region of the worm. During periods of sexual maturity, it swells with thousands of glands that secrete proteinaceous material for building the cocoon (Coleman and Shain 2009; Rossi *et al.*, 2013).

Clitellates are hermaphrodites (i.e., individuals containing both male and female reproductive structures) and both male and female gonopores exit from the clitellum. Like polychaetes, most clitellates reproduce sexually but some proliferate rapidly by asexual fragmentation or budding, while others (e.g., Naididae) can alternate between sexual and asexual modes of reproduction depending upon environmental conditions (Parish, 1981).

Sexual Reproduction

Though some clitellates are capable of self-fertilization (e.g., *Tubifex tubifex*) or parthenogenesis, most cross fertilize by copulation. This involves physical contact between two individuals and the mutual exchange of sperm, usually via an intromittent organ or by the transfer of a sperm-filled sac called a spermatophore. Spermatophores are cemented to each other in the prostate bulb and transferred to the ventral body surface of the partner, typically in the clitellar region. Sperm exit the spermatophore, and by a mechanism not fully understood find their way via the coelomic sinuses to ovaries, where the eggs are fertilized internally.

Spermatogenesis occurs in paired testisacs and resulting spermatozoa pass through structures including the vas deferens and atrium before longer term storage in the epididymis (Fig. 4). Paired ovisacs give rise to oogonia and oocytes, which pass through oviducts that converge and lead to the female pore. Eggs may acquire yolk by a process called vitellogenesis and can be quite large (up to several mm in some Glossiphoniids), or yolk may be deposited into cocoon fluid as a nutritional source for developing embryos (albumenotrophy). Sperm from a partner may be stored in specialized compartments called spermathecae, and released during egg laying to fertilize eggs internally or in the cocoon.

Asexual Reproduction

Many clitellates, excluding leeches, can undergo asexual reproduction by fragmentation, paratomic division (forming a chain of zooids), or parthenogenesis. For naidids, fragmentation is the dominant form of reproduction and enables them to rapidly populate an environment under favorable conditions (Parish, 1981). Fragmenting worms are also a model system for studying regeneration (e.g., *Lumbricus variegatus*), since both anterior and posterior ends can fully regenerate from as little as two midbody segments!

Cocoon Production

In coordination with egg laying, clitellates secrete a proteinaceous cocoon that provides a microenvironment for embryonic development (Coleman and Shain, 2009). The process initiates by the proliferation of clitellum-specific granular cells that differentiate into two cell types, one of which makes fibrous protein that builds the cocoon wall and another that secretes a glue-like substance to seal the cocoon ends. Several million granules of each material type (i.e., cocoon wall, glue) are necessary to construct a single cocoon. Secreted wall granules initially self-assemble to form a sheath around the clitellum. Upon the release of eggs and cocoon fluid from the female pore into the sheath, the worm pulls its head through while simultaneously sealing both ends of the sheath with granulated structures called opercula (Sawyer, 1986). In some leeches (e.g., *Helobdella*), the cocoon is secreted from a ring of granular cells surrounding the female pore and forms a sac that is sealed with a single operculum. Typically only a few cocoons are secreted over the course of several hours, but up to ~50 have been reported in some worms (e.g., *Myzobdella lugubris*). The number of eggs deposited into each cocoon ranges from one to more than 100, depending on the species.

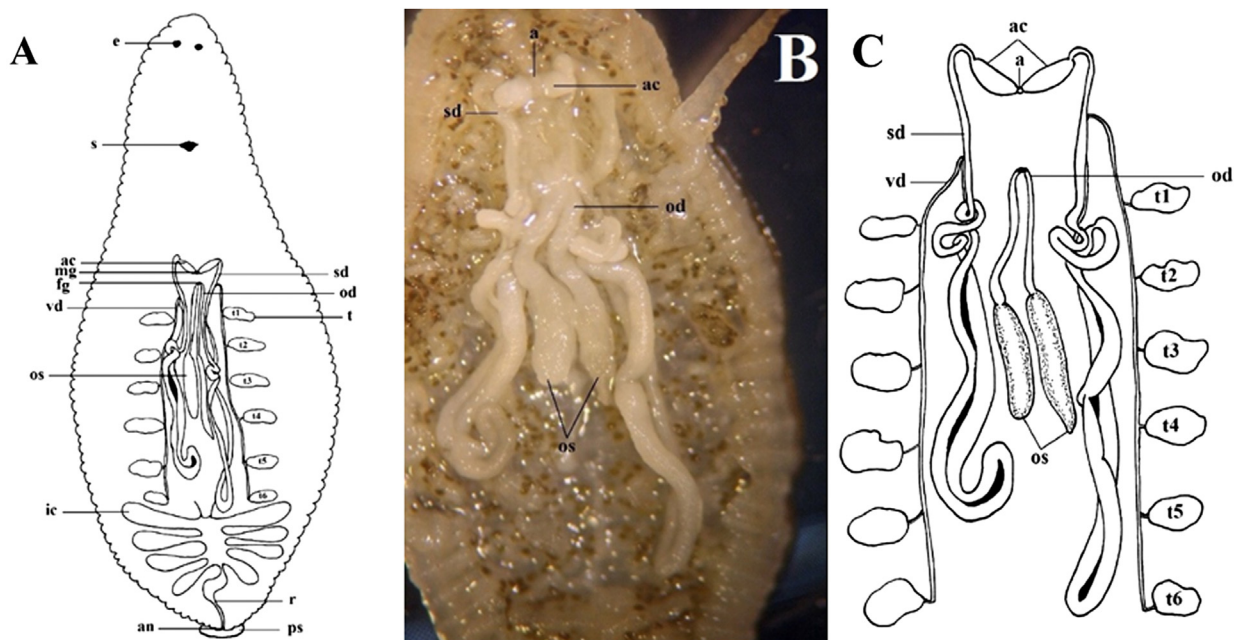


Fig. 4 Dorsal view of a clitellate (*Helobdella* sp.) reproductive system (A) Position of reproductive system in body. (B) Dorsal dissection. (C) Schematic reproductive organs. a, atrium; ac, atrial cornua; os, ovisac; od, oviduct; sd, sperm duct; t, testis; vd, deferens.

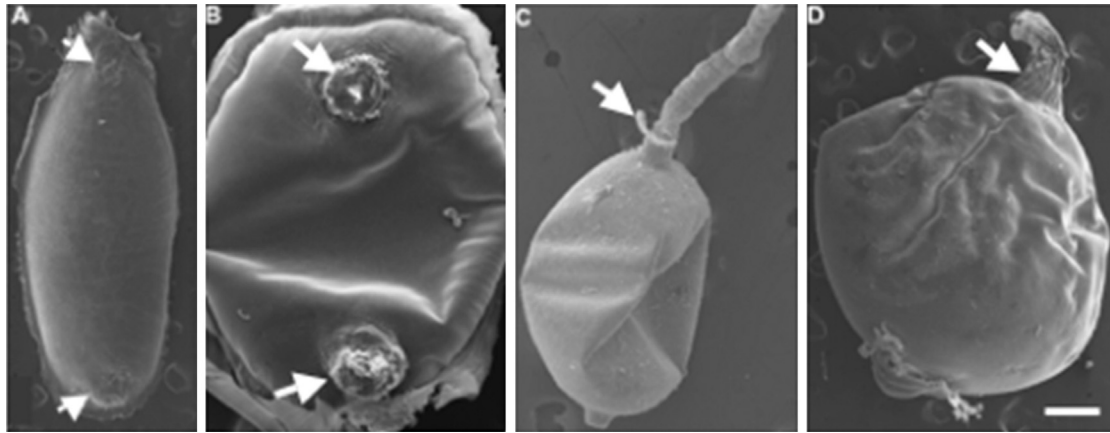


Fig. 5 Morphology of representative clitellate cocoons. (A) *Erpobdella punctata*. (B) *Theromyzon tessulatum* (C) *Tubifex tubifex*, with juvenile exiting. (D) *Eisenia fetida*. Arrows identify opercula. Scale bar=100 μ m.

Clitellate cocoons display a variety of morphologies (Fig. 5) but fall into three general categories: hard-shelled, membranous and gelatinous (Siddall and Bureson, 1996). These are distinguished mostly by the thickness of the fibrous cocoon wall. In some terrestrial taxa (e.g., *Hirudo*, *Haemopsis*), a spongy matrix surrounds the cocoon and appears to prevent desiccation by trapping water droplets. The components of clitellate cocoons display extraordinary physiochemical properties (e.g., thermal, chemical resilience), making them valuable resources as biomaterials and bioadhesives.

Parental Care

Annelids tend to show parental care of some form, ranging from brooding embryos in tubes to placing them in cocoons. Glossiphoniid leeches (e.g., *Haementeria*, *Helobdella*, *Theromyzon*) brood their cocoons until embryos hatch, at which time up to several hundred juveniles may attach to the venter of the parent. Throughout the care period, the parent provides protection from predators and ventilates the eggs/young to ensure that they receive sufficient oxygen. In the sanguivorous duck leech, *Theromyzon*, the parent brings juveniles to their first blood meal, a duck nose, before terminating its life cycle.

Some fish leeches (e.g., Piscicolidae) exhibit an adaptation that facilitates an early blood meal. Rather than abandoning their cocoons as most oligochaetes do, they instead cement them to the surface of crustaceans, which are later eaten by fishes. When the young leeches hatch, they readily attach to the fish host's buccal surfaces and migrate to the gills. In some Syllidae oligochaetes, eggs are not released but rather attach to the posterior end of the worm where they develop into juveniles before becoming independent.

References

- Coleman, J., & Shain, D. H. (2009). Clitellate cocoons and their secretion. In D. H. Shain (Ed.), *Annelids in Modern Biology* (pp. 328–346). Hoboken, NJ: John Wiley & Sons, Inc.
- Fischer, H. L., Thorsten, H., & Arendt, D. (2010). The normal development of *Platynereis dumerilii* (Nereididae, Annelida). *Frontiers in Zoology*, 2010(7), 31. Available at: <https://doi.org/10.1186/1742-9994-7-31>.
- Hill, S. D., & Savage, R. M. (2009). Evolution, development and ecology of *Capitella* sp. I: A waxing model for polychaete studies. In D. H. Shain (Ed.), *Annelids in Modern Biology* (pp. 88–115). John Wiley & Sons, Inc.
- Kostyuchenko, R. P., Kozin, V. V., & Kupriashova, E. E. (2016). Regeneration and asexual reproduction in annelids: Cells, genes and evolution. *Biological Bulletin*, 43, 185–194.
- Parish, J. (1981). Reproductive ecology of Naididae (Oligochaeta). *Hydrobiologia*, 83(1), 115–123.
- Rossi, A. M., Saidel, W. M., Marotta, R., Saglam, N., & Shain, D. H. (2013). Operculum ultrastructure in leech cocoons. *Journal of Morphology*, 274(8), 940–946.
- Sawyer, R. T. (1986). *Leech Biology and Behavior*. Oxford: Clarendon Press.
- Schroeder, P. C., & Hermans, C. O. (1975). Annelida: Polychaeta. In A. C. Giese, & J. S. Pearse (Eds.), *Reproduction of Marine Invertebrates* (vol. 3). Academic Press.
- Seaver, E. C. (2014). Variation in spiralian development: Insights from polychaetes. *International Journal of Developmental Biology*, 58, 457–467.
- Seaver, E. C. (2016). Annelid models I: *Capitella teleta*. *Current Opinion in Genetics & Development*, 39, 35–41.
- Siddall, M. E., & Bureson, E. M. (1996). Leeches (Oligochaeta?: Euhirudinea), their phylogeny and the evolution of life history strategies. *Hydrobiologia*, 334, 227–285.
- Thornhill, D. J., Dahlgren, T. G., & Halanych, K. M. (2009). Evolution and ecology of ophryotrocha (Dorvilleidae, Eunicida). In D. H. Shain (Ed.), *Annelids in Modern Biology* (pp. 242–256). Hoboken, NJ: John Wiley & Sons, Inc.
- Wilson, W. H. (1991). Sexual reproductive modes in polychaetes: Classification and diversity. *Bulletin of Marine Science*, 48, 500–561.
- Zantke, J., Bannister, S., Rajan, V. B. V., Raible, F., & Tessmar-Raible, K. (2014). Genetic and genomic tools for the marine annelid *Platynereis dumerilii*. *Genetics*, 197, 19–31. <https://doi.org/10.1534/genetics.112.148254> (PMCID: PMC4012478).

Relevant Websites

<http://4dx.embl.de/platy/>. – Platynereis resources.

<http://genome.jgi.doe.gov/Capca1/Capca1.home.html>. – US Department of Energy.

Echinodermata

Gary M Wessel, Brown University, Providence, RI, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Asteroids The echinoderm taxon consisting of sea stars.

Autonomy A common feature of sea stars and brittle stars in which the adult severs an arm, often to escape a predator.

Bilateral symmetry Symmetry along a midline of an organism. Both sides divided by the midline are roughly similar in morphology. For example, in a human, a line drawn from the forehead to the chin, going through the nose, separates a left and right side to the head, which superficially is the same.

Egg A female gamete capable of being fertilized. Different in development from an oocyte, which is not fertilization competent.

Gene regulatory network A mechanistic examination of how genes regulate expression of each other through their activation and repressive functions in transcription.

Gametes Eggs and sperm, the cells of sexual reproduction.

Germ line The lineage of cells from gametes to embryos, to gametic stem cells in the gonad that carries the genetic information used by an animal in sexual reproduction.

Meiosis The reduction division transforming the gametes from 4 copies of each chromosome, to one copy (haploid). Upon fertilization then, the number of copies of chromosomes is restored to two copies (diploid) by merger of haploid sperm genome with haploid egg genome.

Mutable connective tissue (MCT) Extracellular matrix that responds directly to nerve signals to change its structure, and thereby initiate movements within a section of the animal.

Oocyte A cell developing into an egg. The difference between the two is not in meiosis, or timing of fertilization but in a functional definition. It is whether the cell is capable of being fertilized (egg) or not (oocyte).

Pedicellariae Food catching appendages in the adult of many echinoderms, especially prevalent in sea urchins.

Pentameric symmetry Five-fold radial symmetry characteristic of adult body plan of echinoderms.

Tube feet Suction-cup like movable apparatus on echinoderm adults to attach to objects and/or pass food to the mouth. These devices are powered by the water vascular system.

Zygote Fertilized egg until the cell divides, when it is then referred to as an embryo.

Introduction

The image of echinoderms is prevalent in society. From sea star enhanced signs on restaurants to sand dollar key chains, most people have an inherent connection to echinoderms. Many people may not know though that they are a favorite of many for studying mechanisms of reproduction and development, and are more closely related to ourselves than all other invertebrates. Echinoderms represent an early branching phylum of deuterostomes, an evolutionary branch that resulted in vertebrates, with over 7000 living species identified worldwide. Echinoderms also have a rich fossil record enabling examination of speciation and evolutionary diversification of morphology. For general interest in reproduction, in development, in ecology and evolutionary biology, and in marine biology, echinoderms are an important group of animals to understand.

What Are Echinoderms?

The phylum Echinodermata is comprised of five taxa (classes) of organisms with overlapping, yet diverse, characteristics. The shared characters include radial symmetry – usually five-fold symmetry – in the adults as well as a body wall skeleton, a water vascular system used for motility in tube feet and food catching devices (pedicellariae), and mutable connective tissue (MCT). This latter character may be unique to echinoderms in that the extracellular matrix undergoes significant structural changes without the use of muscles. This character for many echinoderms likely participates in the process of mouth movement, and autonomy, a common feature of sea stars and brittle stars in which the adult severs an arm to escape a predator. In this process the MCT is likely stimulated to become highly flaccid and rupture by the tension of the arm.

The Five Classes of Echinoderms

Fig. 1 highlights the diverse groups of echinoderms (Cary and Hinman, 2017). Perhaps the most recognizable member of echinoderms are the sea stars (Asteroidea; historically many have called these animals starfish but because they are not fish, marine

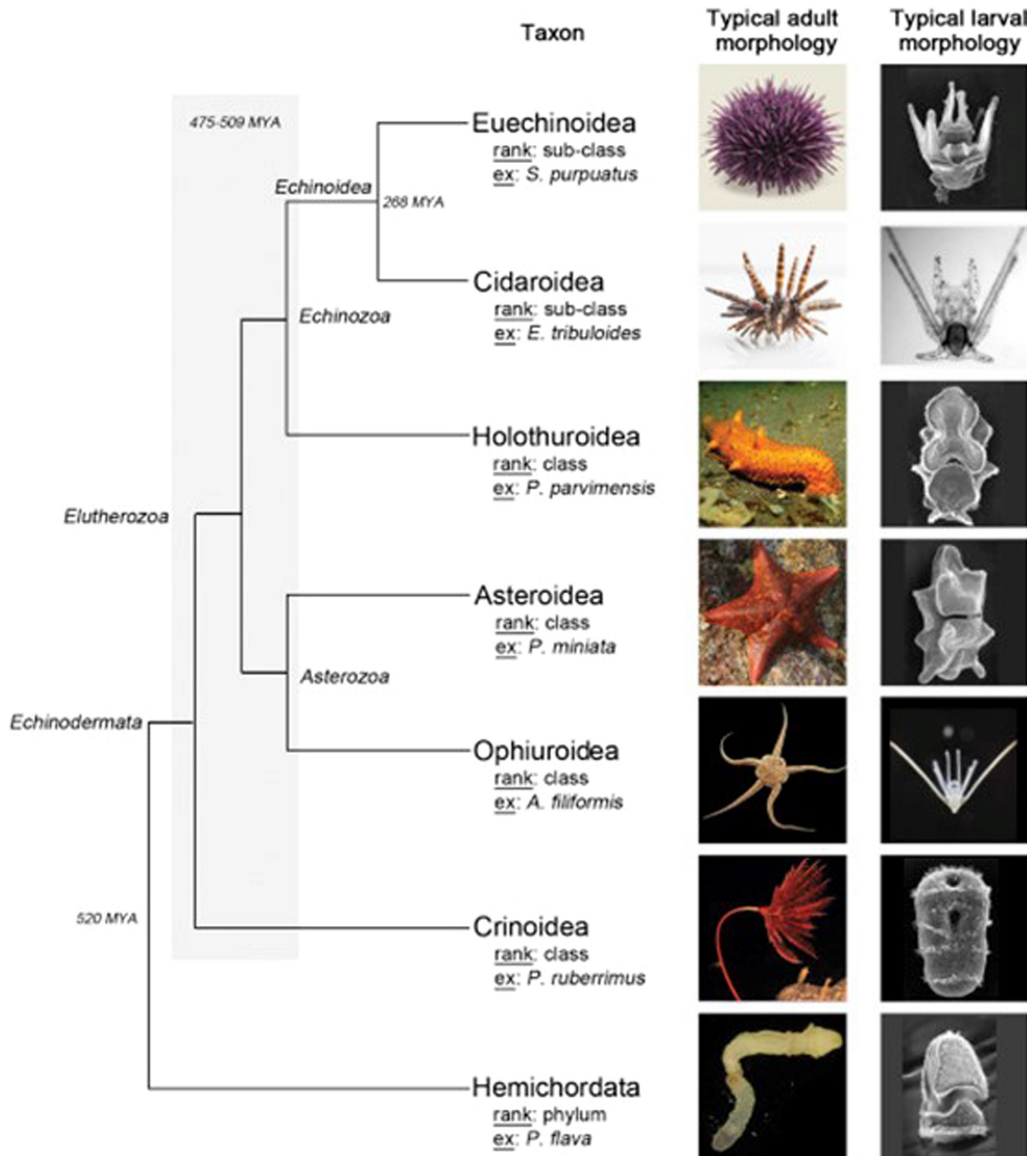


Fig. 1 Phylogenetic relationships and example adult and larval morphologies of echinoderms. Asterozoan topology, the consensus view of relationships within the echinodermata, is highlighted. Branch lengths are not drawn to scale. The images presented do not, in all cases, correspond to the example species cited. Photo credits: Adult Echinoidea and Cidaroid are © Ann Cutting, Caltech; Holothuroidea is © Richard Ling/ www.rling.com; Asteroidea is © Jerry Kirkhart, Los Osos, CA; Ophiuroidea is © Hans Hillewaert; Crinoidea is © NOAA Okeanos Explorer Program, INDEX-SATAL 2010; and Hemichordata is ©Moorea Biocode/calphotos.berkeley.edu. 4444 4444 0513 0997. Cidaroida larval image is adapted from Bennett, K.C., Young, C.M., Emlet, R.B., 2012. Larval development and metamorphosis of the deep-sea cidaroid urchin *Cidaris blakei*. Biol Bull 222 (2), 105–117, all other whole (SEM) images of echinoderm and tornaria larvae are © T.C. Lacalli and T.H.J. Gilmour (University of Saskatchewan).

biologists have preferred the term sea star to minimize any ambiguity of its taxonomic position, and for consistency with sea urchin, sea cucumber, etc.). These animals usually have five distinct arms, each connected to a central body disk that contains the mouth and integrated nervous system. Each of the arms has its own gonad (in the adult), nerve system, and digestive organ connected to the central digestive system. The mouth is on the bottom (ventral, oral surface) surface of the animal, the anal and five (pentaradial)

gonadal openings on the top (dorsal, aboral surface). Many sea stars (e.g. *Asterias*) are highly predatory and consume large numbers of animals even slower than they are e.g. molluscs. The main strategy in mollusk consumption for a sea star is to use their tube feet to attach to the shells of the mollusks and to apply separating tension over a long period of time. The slow movement muscles and water vascular system of the sea star eventually exhaust the mollusk, who eventually begins to open its shell. The sea star then everts its central digestive system through its mouth into the opening of the mollusk, and releases digestive enzymes to begin consuming and eventually overcoming the remaining mollusk tension. Other sea stars are capable of consuming benthic detritus for sustainability.

Similar in shape and design to the sea stars are brittle stars of the taxon, *Ophiuridea*. These animals have a distinct central disk with long slender arms emanating from five distinct sites. The movement is somewhat spider like, and their arms are densely packed with tube feet and graspers to capture and passage food to the mouth. The digestive system is much simpler than the sea star, with a stomach sack with openings in the mouth for intake and outflow. The gonads are located in the central disk with gonadopores equally spaced around the disk. A sub-group of *Ophiuridea* consist of the basket stars, containing highly elaborate branching arms that make the animal arms look more fern-like. These brittle star relatives are highly predatory in that they capture suspension zooplankton and pass the food along their convoluted arms to their mouth.

One of the more unusual groups of echinoderms are the sea cucumbers, *Holothuroidea*. As their common name implies, many of this group look remarkably like the cucumber vegetable, or pickles of the sea. These animals look very different than the pentaradially symmetric sea star and brittle star, with a strong anterior-posterior organization, dense and branched tentacles surrounding the mouth, a single gonad within the body, and a calcareous skeleton ring surrounding the mouth. The tentacles scoop sediment and debris into the oral opening, to be digested by a robust stomach system leading out of the animal in the posterior anal opening. When threatened, many of these animals will evert and autonomize their gut, but eventually regenerate this entire digestive structure.

Crinoidea (feather stars, sea lilies) are distinctive and unusual echinoderms. Highly arborized arms are joined on a central disk, or even long stalk that serves as a mechanism to be off the sea floor and maximize their filter feeding capabilities. The extensive arms on the stalked crinoids are capable of selecting suspension materials that they will then pass to the mouth. The digestive system leads to an anal opening near or on the stalk. Crinoids are thought to represent an early branching echinoderm and may represent an important and ancient diversification between protostomes (development in which the mouth is formed first and by the blastopore) and deuterostomes, in which the initial opening at gastrulation becomes the anal opening. Little is known about how these animals develop and function, but because of their highly calcified stalk system, the fossil record of these animals is rich.

Perhaps the best studied of echinoderms are the *Echinoidea*, the sea urchins and sand dollars. Their calcium carbonate test enables a dense fossil record, their prevalence in coastal areas and shallow-water marine environments has tremendous impact on the ecology of a system, and the many eggs spawned by the adults enable rapid and deep research into mechanisms as wide ranging as nutrient storage, fertilization and embryogenesis, cell shape changes and cell cycle regulation, gene regulatory networks, and ecotoxicology. The adults have characteristic spines that may extend only a few millimeters to many centimeters from the body wall. In some species these spines are thick and blunt as in the pencil urchins (*Eucidaris*), whereas in other species, the long spines are barbed like a fish hook and difficult to remove without further damage if the spines penetrates your skin (e.g. *Diadema*). The adult coelom is lined by five symmetric gonads and an asymmetric digestive system that loops around the body wall. The mouth has a distinct set of five teeth in the shape of Aristotle's lantern, leading to the digestive system, and the anal opening (on top, or the aboral surface) is surrounded by five openings, the gonadopores, through which eggs and sperm are released from each of the gonads.

Reproduction and Development

Overview of Reproduction in Echinoderms

As diverse as this phylum is in morphology, so too it is diverse in its reproduction. Most echinoderms reproduce sexually, making eggs and sperm, but some species also reproduce asexually, by splitting the adult body and regenerating the divided pieces. In some species, the larvae also reproduce by splitting off parts of the larval body in a process called larval cloning. Some adults are hermaphrodites (adults make both testis for sperm, and ovaries for eggs) whereas most species are single sexed. The vast majority of reproduction in echinoderms is by broadcast spawning, releasing eggs and sperm into the water column and favoring the strategy of making large numbers of eggs and sperm (Fig. 2), with the result that only a small percent of the gametes released will actually be successfully fertilized and develop. Yet an even smaller number will successful survive the time needed for development to another gravid adult. Yet in the generation of millions of eggs and trillions of sperm over an adult lifetime, growing a population of sea urchins is quite successful given appropriate nutrition.

Sexual Reproduction in Echinoderms

The reproductive anatomy of echinoderms generally follows their radial, usually five-fold, symmetry (Fig. 2). Adults of sea stars and sea urchins for example, have 5 openings on their aboral (the side opposite the mouth, or oral opening) through which are released the eggs or sperm. These pores of the gonad (gonadopores) are connected to each lobe of the gonad inside the body of the adult. Generally the structure of each gonad follows the same orientation for males and females (Fig. 3). The precursors of eggs and sperm

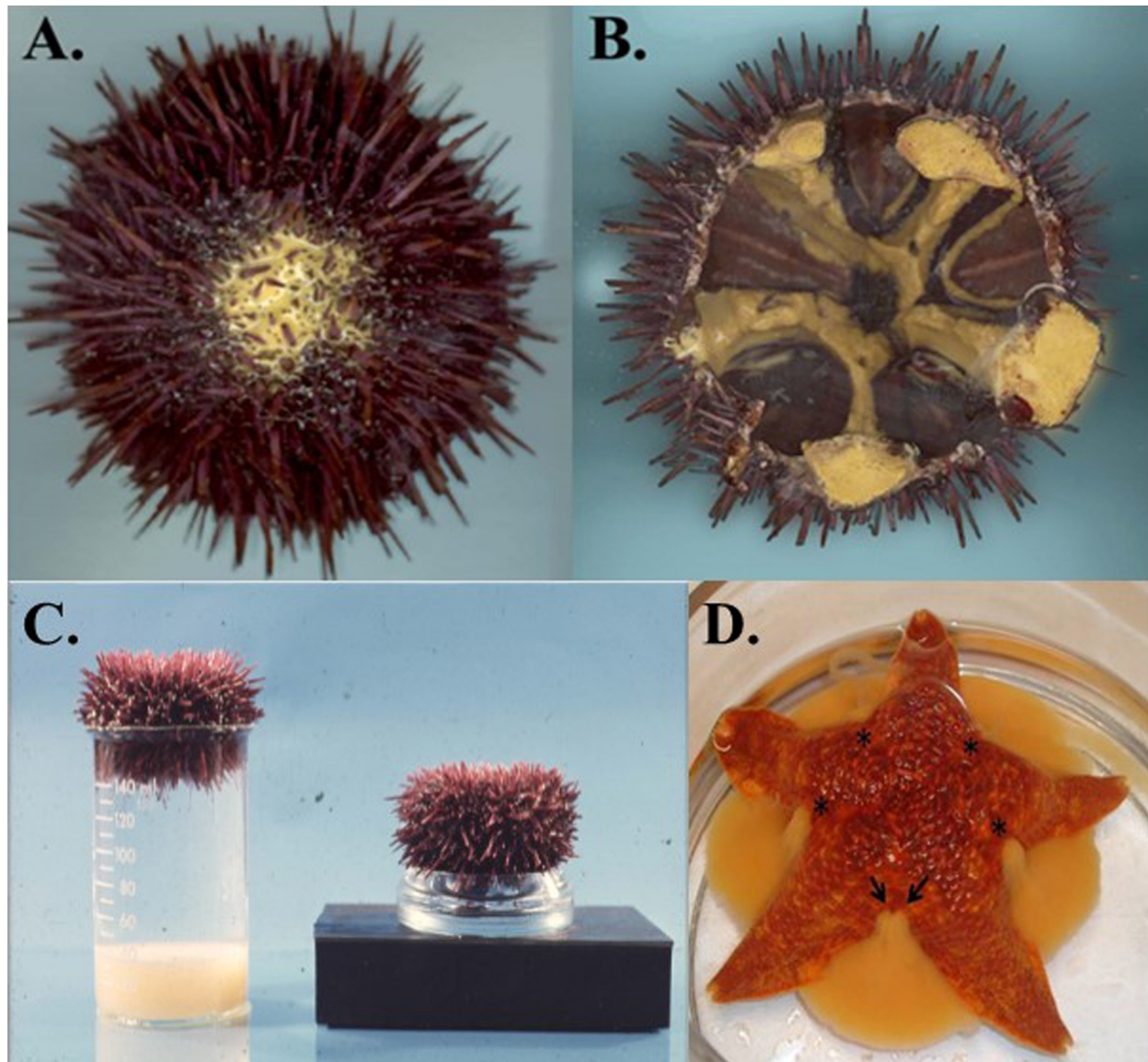


Fig. 2 (A) Spawning sea urchin (*Strongylocentrotus purpuratus*; native to the west coast of the United States), seen from the dorsal (aboral) surface of the adult. (B) When the animal is opened on the ventral aspect, the pentameric (five-fold) symmetry is obvious in the gonads – here a female with the yellowish, yolk laden ovaries seen prominently, each containing upwards of a million eggs. Photo courtesy of Jacqueline Brooks. (C) Female (left) and male (right) spawning in sea water and “dry” respectively. Fertilization is then accomplished by mixing the gametes in sea water. Photo courtesy of Richard Showman. (D) A spawning sea star (*Patiria miniata*). Arrows and asterisks (*) point to the eggs released through the gonadopores on the aboral side of the adult.

(oocytes, and spermatocytes, respectively) develop from stem cells along the periphery of the branched tubular structure (Fig. 3). Gametogenesis is apparently independent of age of the animal such that once juveniles reach a certain age/size, they are capable of making eggs or sperm for the rest of their life. Given that some species of echinoderms have been clocked at living over 200 years, we conclude that the reproductive stem cells are continually replicating and functional.

Most echinoderms are seasonal. That is, they make eggs and sperm only during parts of the year (Fig. 4). This cyclicity in gravidity is regulated by a variety of environmental conditions including nutrition, temperature, and light, and will fluctuate from gonads packed full of gametes to gonads only a shadow of their prime, with no detectable eggs or sperm present (Perez *et al.*, 2010). What happens to their stem cells in the gonad and why do they not make more sperm and eggs? This is not clear, but some reports suggest, at least in a sea star, that the stem cells accumulate in a specialized region of the body wall, near the gonadopore openings, and stop dividing. Presumably they re-ignite to replicate and make more precursors in the development of eggs and sperm following their migration, or re-proliferation to the branched tubular gonads. Recent research in germ line formation and function in a variety of animals suggests that at least some echinoderms may make germ line stem cells (the stem population of cells that give rise to eggs and/or sperm) in a variety of different developmental pathways, perhaps even using transitions in somatic (normal body) cells to make germ cells, dimming the normal divide of germ line and soma from classical studies.

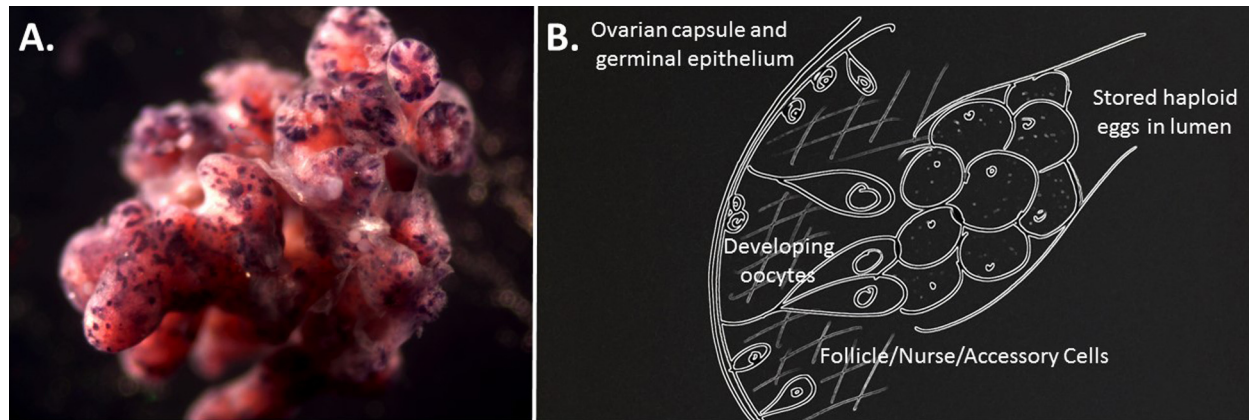


Fig. 3 Gonad structure and function. (A) In situ RNA hybridization of an mRNA for cortical granule contents (blue signal within a whole mount of a sea urchin ovary) showing the distribution peripherally of developing oocytes of many stages. (B) Diagrammatic representation of oocytes, eggs, and follicle (somatic) cells of a sea urchin ovary.

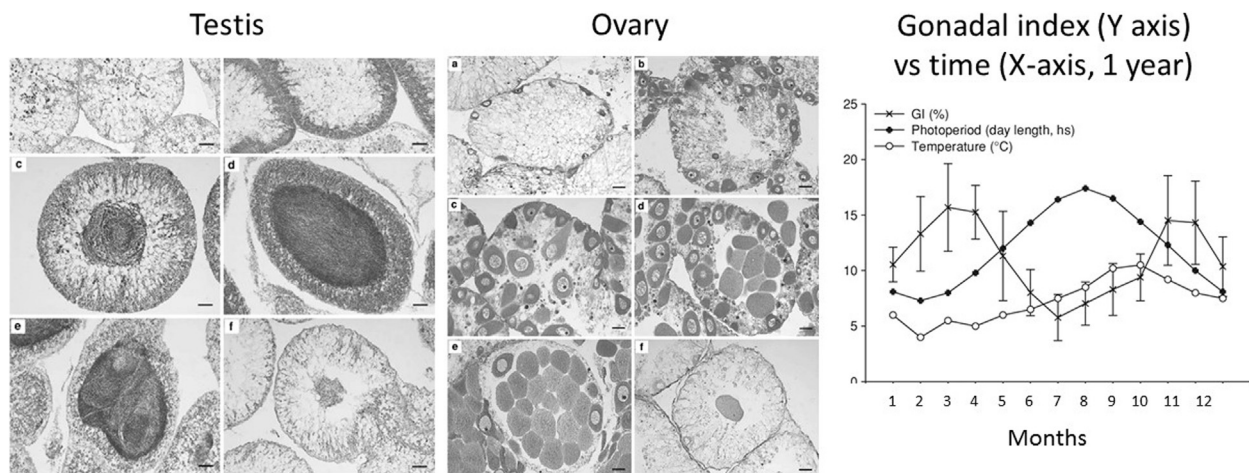


Fig. 4 Cyclic behavior of seasonal fecundity in a sea urchin. Photomicrographs of both testis and ovary are shown throughout a year, and graphically displayed showing how the gonad size varies with photoperiod and water temperature. Testis stages of *L. albus*: (a) immature, (b) growing, (c) premature I, (d) premature II, (e) mature, (f) spawned. Scale bar 50 m. Ovary: Photomicrographs of females gonadal stages of *L. albus*: (a) immature, (b) growing, (c) premature I, (d) premature II, (e) mature: free oocytes in the alveolar lumen, (f) spawned: immature oocytes attached to the alveolar wall and few free oocytes in the lumen. Scale bar 50 m. Great variation is seen in how animals respond to environmental conditions with both proportional and inversely proportional responses to light and temperature. Reproduced from Perez, A.F., *et al.*, 2010. Reproductive cycle and reproductive output of the sea urchin *Loxechinus albus* (Echinodermata: Echinoidea) from Beagle Channel, Tierra del Fuego, Argentina. *Polar Biol* 33 (3), 271–280.

Many investigators use echinoderms to study mechanisms of sperm-egg interaction, fertilization, and activation of development because of the many gametes available for study, that they can be fertilized outside of the adult and visualized under the microscope, and because they can be manipulated by microinjection of materials for experimentation. These are each valuable resources for scientists and educators alike. These investigators will stimulate the animal by gently shaking the adult, and/or by injection of KCl into the body cavity of the adult, stimulating contraction of the smooth muscles that surround the gonads causing expulsion of the gametes stored in each of the gonadal lumens out through the gonadopores (see especially sea star, Fig. 2). The mechanisms used normally by adults in the wild though are not at all understood.

A variety of mechanisms appear able to induce spawning in adult echinoderms. Stress from shaking (wild wave action), or rapid changes in environmental conditions (temperature, salinity, oxygenation) often induce the animals to spawn when gravid. Some species actually respond to the lunar cycle. For example, the sea urchin *Diadema* undergoes regular spawning at the full moon. Whether this is due to light from the moon (unlikely, since they still do so even with cloud cover or rain) or tidal conditions (more likely, but not clear how this would work since they may be many meters below the ocean surface), is not clear. Once induced to spawn though, they often release the vast majority of their gametes, and over the next 28 days of the lunar cycle, will synchronously develop a new cohort of eggs or sperm in time for the next full moon.

An adult echinoderm of one species that begins to spawn will induce nearby adults of the same species to also spawn. This leads to a large spawning event in the wild that brings eggs and sperm together at the same time and space, thereby increasing the chances that fertilization will occur. Some species can also induce spawning in other, closely related species.

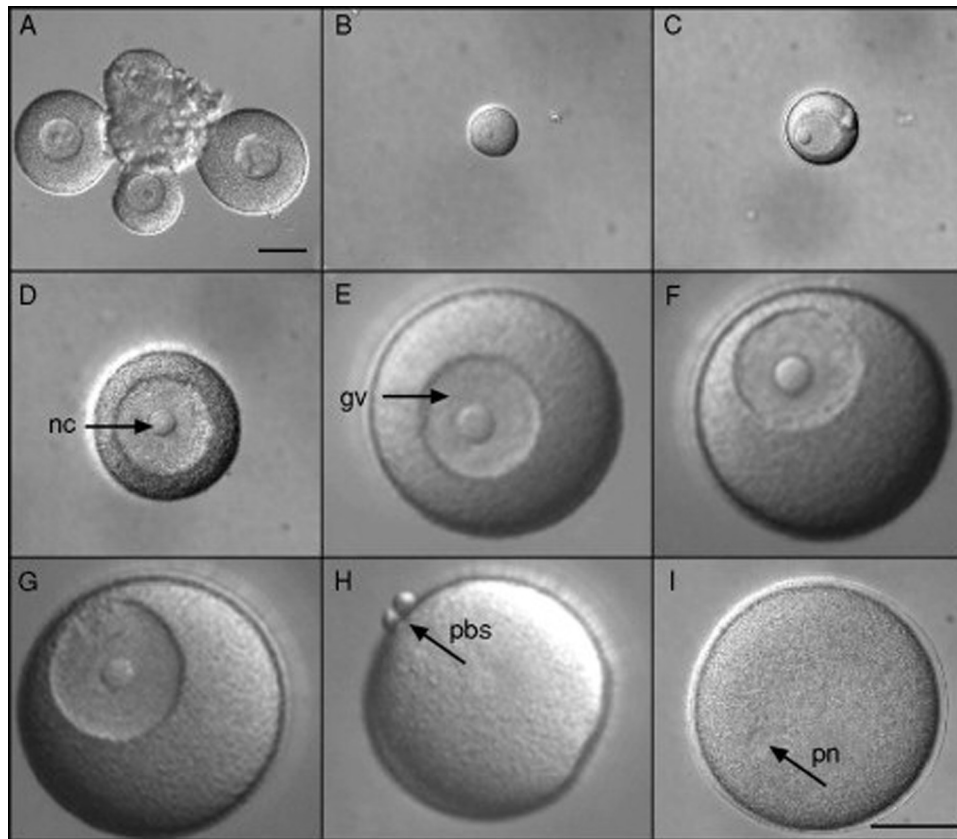


Fig. 5 Oogenesis and meiosis. These samples were shed and dissected an ovary of the sea urchin *L. variegatus* and viewed with a light microscope. (A) Oocytes attached to the somatic cells of the ovary. (B) Previtellogenic primary oocytes. (C–E) Vitellogenic growth phase of oogenesis. (E–I) Full-grown oocytes complete meiosis with the large germinal vesicle moving asymmetrically to the cell periphery, where it breaks down and extrudes two polar bodies to produce a haploid mature egg that is now ready for fertilization. Scale bar, 50 μ m; gv, germinal vesicle; nc, nucleolus; pbs, polar bodies; pn, pronucleus. Figure adapted from Brooks, J.M., Wessel, G.M., 2003. Selective transport and packaging of the major yolk protein in the sea urchin. *Dev Biol* 261 (2), 353-370, with permission.

Meiosis

Most eggs made by animals, including most echinoderms, are spawned and fertilized at some point during meiosis, the reduction division transformation the gametes from 4 copies of each chromosome, to one copy (haploid). Upon fertilization then, the number of copies of chromosomes is restored to two copies (diploid). Sea stars for example, store millions of oocytes in a germinal vesicle stage. Upon spawning, the follicle cells surrounding the oocyte release 1-methyladenine, which initiates resumption of meiosis. Fertilization can then be successful – prior to this, depending on the species, sperm may not bind, or the oocyte is incapable of blocking more than one sperm from entering the egg (block to polyspermy). Although the details differ from species to species, many other animals, including humans, have analogous events in the meiotic mechanisms and in fertilization. Thus, the many millions of oocytes stored by a sea star (see Figs. 2 and 5) enables more tractable study to the meiotic events in oocytes. Unusual in eggs of Echinoidea is that the eggs are released having completed meiosis. Thus, once the sperm fuses to the egg, two haploid cells are fusing to restore diploidy, independent of completion of meiotic processes. The only other eggs known that complete meiosis prior to fertilization are some cnidarian.

Fertilization Mechanisms in Echinoderms

The eggs and oocytes of echinoderms are wonderfully suited for observation of, and research into, meiosis (see above) and fertilization. Large numbers of eggs are readily observable and manipulatable in simple culture systems making them efficient for study. Further, many of the mechanisms of fertilization and activation of sperm and egg are highly conserved, even though the molecular details, or the molecules themselves, are diverse. A few of the more conserved features of fertilization include:

1. Eggs have an extracellular layer that activates sperm by a species specific mechanism. In sea urchins, this layer is the egg jelly that consists of sugar polymers and small peptides.
2. Activated sperm (acrosome reaction and metabolic increase) are able to penetrate the egg extracellular layer and bind to the egg species specifically.

3. Egg activation results in steps to block additional sperm from binding and fusing to the egg. This process is called the block to polyspermy.
4. Echinoderms and many other species have two mechanisms to block polyspermy, a) a change in the electrical potential of the plasma membrane which is rapid, but transient, and b) a slower, but permanent change in the egg extracellular matrix.
5. The slow block to polyspermy uses contents of the cortical granules, secretory vesicles in the periphery of the egg, to reconfigure the extracellular matrix into an impenetrable barrier to sperm. The embryo must subsequently hatch out of this encasement in order to feed (sea urchins) or implant (mammals).
6. Signal transduction pathways initiated by sperm fusion to the egg causes release of calcium from intracellular stores (endoplasmic reticulum) in the egg to stimulate secretion of the cortical granules and for activation of programs to initiate development of the embryo.

The abundance of eggs and sperm in echinoderms and their functionality in vitro has enabled a robust identification of many of the key features in egg activation. Some of these steps and the molecular players involved are diagrammed in Fig. 6. See also the

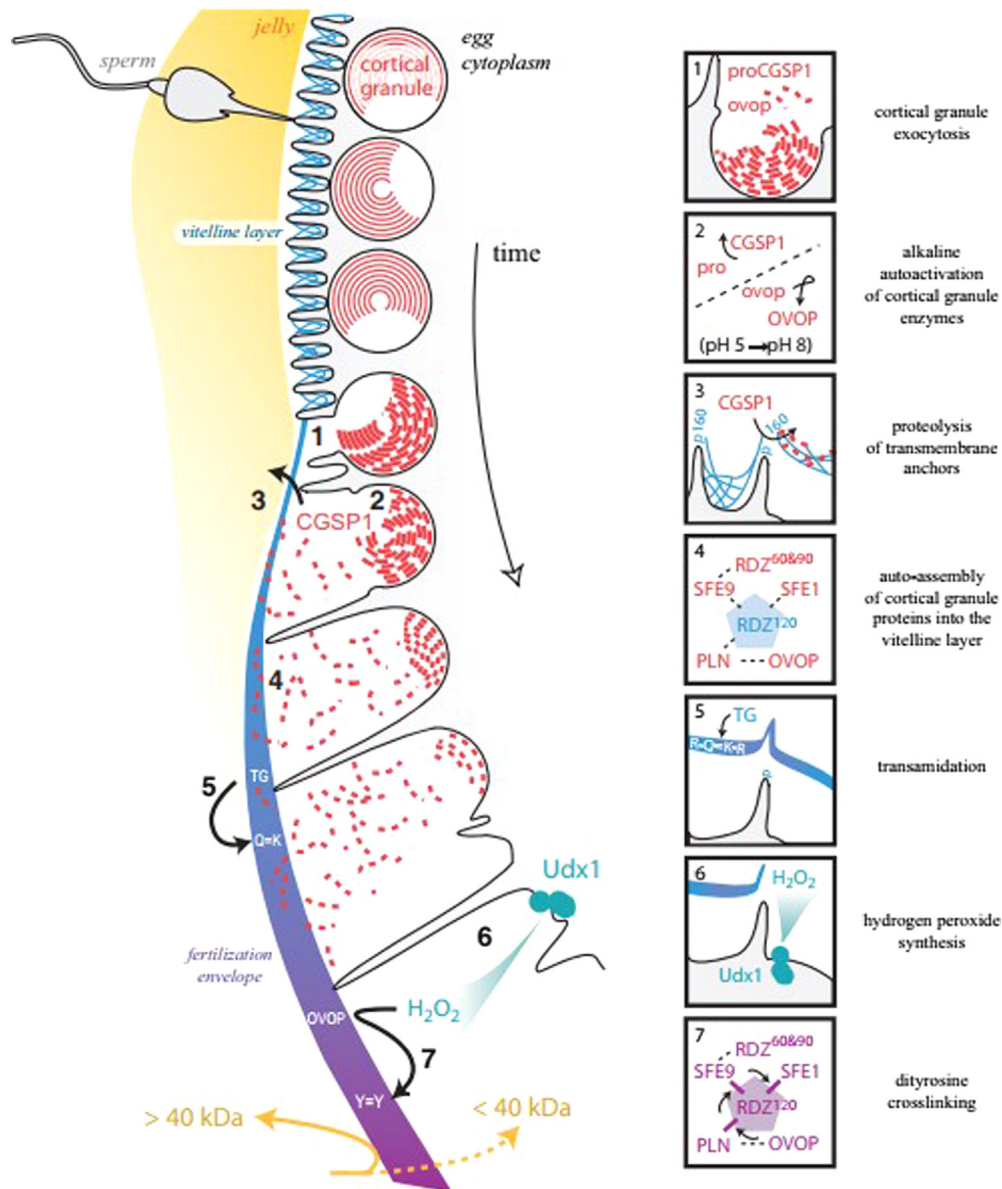


Fig. 6 Milestones of fertilization envelope formation. Diagram detailing the time-course of modification of the egg cortex and fertilization envelope assembly, starting from sperm fusion (top) to ovoperoxidase-dependent crosslinking (bottom). Milestones are highlighted, and are described in the text. In panel (7), arrows denote dityrosine crosslinks formed by active ovoperoxidase. CGSP1, cortical granule serine protease 1 (proCGSP1=zymogenic form); H₂O₂, hydrogen peroxide; OVOP, ovoperoxidase (ovop=zymogenic form); PLN, proteolisin; RDZ, rendezvin; "R-Y5Y-R", covalent dityrosine crosslink; "R-Q=K-R", covalent epsilon(gamma-glutamyl)lysine bonds; TG, transglutaminase; Udx1, urchin dual oxidase 1; 40 kDa, macromolecules of 40,000 daltons. Modified with permission from Wong, J.L., Wessel, G.M., 2008. Renovation of the egg extracellular matrix at fertilization. *Int J Dev Biol* 52 (5-6), 545-550.

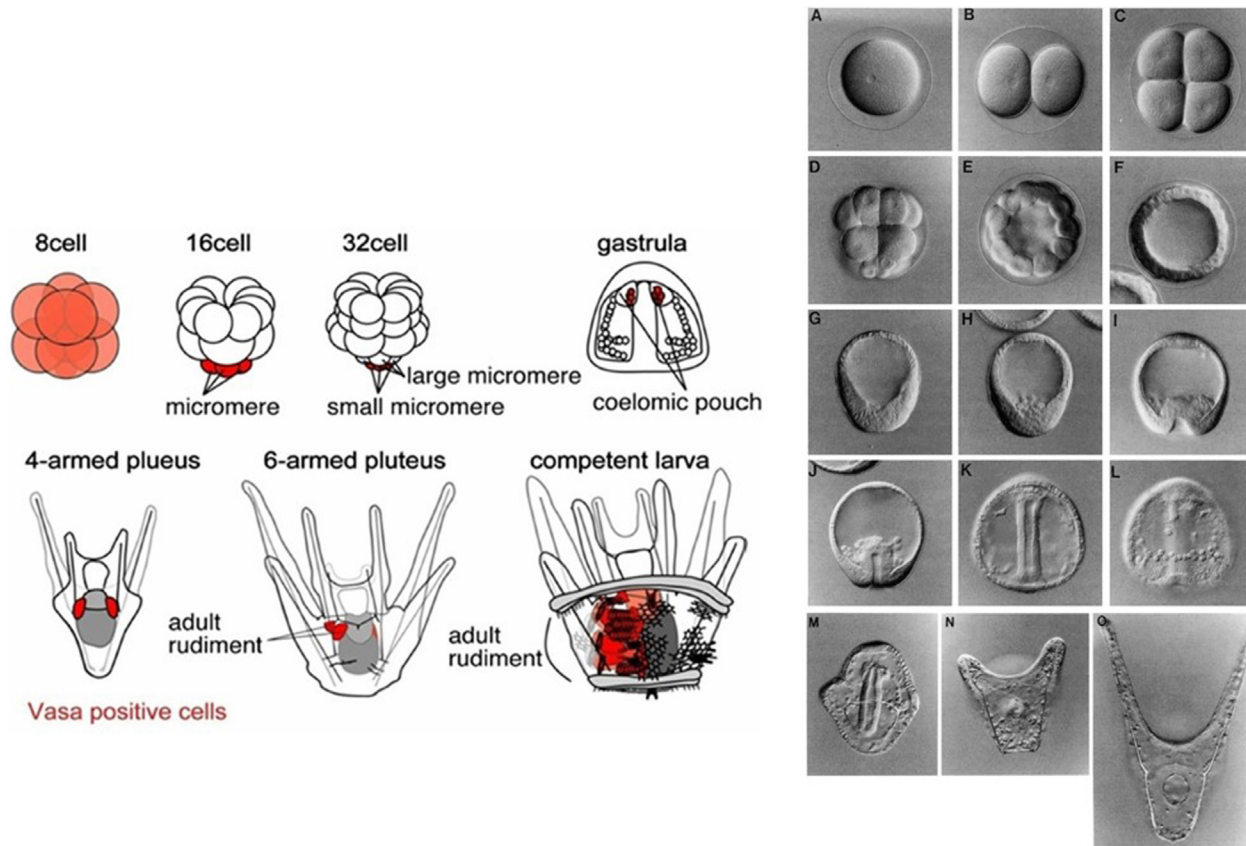


Fig. 7 Development in a sea urchin. Left, diagram of the development of a sea urchin. Early development yields Vasa-positive cells (shown in red, beginning with a uniform Vasa positive egg and early embryo). At the 32-cell stage, the sMics are uniquely Vasa-positive. These cells move into the coelom during gastrulation, segregate into the left and right coelomic pouches, and expand to contribute to the germ cells of the adult, and likely also to some somatic cells of the rudiment (Reproduced from Wessel, G.M., *et al.*, 2014. The biology of the germ line in echinoderms. *Mol Reprod Dev* 81 (8) 679–711.). On the right is a montage of key developmental transitions in the sea urchin *Lytechinus variegatus* (reproduced from Etensohn, C.A., Wessel, G.M., Wray, G.A., 2004. The invertebrate deuterostomes: An introduction to their phylogeny, reproduction, development, and genomics. *Methods Cell Biol* 74, 1–13, with permission). (A) fertilized egg with prominent fertilization envelope surrounding the cell; (B) two – cell stage; (C) four cells; (D) 16 cell stage showing the unequal cleavage at the bottom yielding 4 macromeres, and 4 micromeres; (E) morula; (F), Blastula; (G) late blastula becoming asymmetric from top (animal) to bottom (vegetal poles); (H) primary mesenchyme cell stage with invaginating primary mesenchyme cells; (I) early gastrula with invaginating endoderm; (J) mid-gastrula; (K) late gastrula in which the invaginating endoderm has reached the opposite pole to form the future mouth. The future anus is at the bottom, the site of invagination. (L) different focal plane of same embryo showing the primary mesenchymal cells. (M) prism stage – early larva. (N) larva with arms and skeleton extending. (O) larva with extensive growth in arms. For scale the egg is approximately 100 μm in diameter.

VirtualUrchin (see Relevant Websites section) as to how to visualize fertilization, adjust the microscope, and even ship adults and/or eggs and sperm for classroom or research utility. Echinoderm eggs and sperm are viable for several days out of the adults, and prior to fertilization, so shipping these cells is feasible for many applications.

Can eggs and sperm from different species cross fertilize? Generally not. Under experimental conditions, cross fertilization (hybridization) can occur, but success in the development of the embryos is usually low, and few, if any, reports of interspecies echinoderm adults propagating are known and validated.

What is the reason for eggs and sperm from different species not fertilizing each other? Both sperm and eggs have several mechanisms whereby molecular specificity is required for gamete interaction and successful activation of development. This starts with the jelly surrounding the egg that activates sperm in a species specific manner, to the rapid evolution in sperm and egg binding proteins on each gamete. This molecular lock and key between sperm and egg of the same species is just one mechanism required for a successful fertilization within the same species.

Embryonic Development

Development of echinoderm embryos is easy to observe (Etensohn *et al.*, 2004; Fig. 7) because the embryos normally develop outside of the adults in natural sea water. This condition is easily replicated in the lab or classroom and their size is amenable to manipulation without demands for advanced microscopy equipment.

Fertilization is readily apparent by formation of the fertilization envelop. The first few cell divisions are equal, the speed of which depends on the temperature and the species, but can be every 30 min to every few hours. For most echinoderm the cell divisions

continue equally, at least in size, but in sea urchins, the next two divisions have an asymmetric division. By definition the site of the egg where meiosis occurred is the animal pole, and the side opposite it is the vegetal pole. The small cells formed (micromeres) at the vegetal pole of the embryo of the fourth and fifth cell divisions have important differences in cell fate. One of the small cells becomes strictly limited to making skeleton in the larva, whereas the other cell (the smaller quartet of the two) contributes to the germ line, that lineage resulting in sperm or eggs in the adult. A blastocoel soon forms in the middle of the dividing cells and expands to form the blastocoel, a cell free space filled with extracellular matrix molecules. The embryo is now a single layered epithelium undergoing rapid cell divisions. Three primary germ layers form during gastrulation, the process of converting the hollow – balled blastula into a three layered structure; ectoderm (nerve cells and “skin cells” on the outside), endoderm (digestive tract in the inside) and mesoderm (muscle, immune cells, skeleton in between). These now distinct germ layers continue replicating their cells and become diverse in their structure and function. Once the mouth forms by fusion of the invaginating endoderm with the overlying ectoderm, the animal can begin feeding. In general, all indirect developing (see below) echinoderms have similar morphogenesis to this point. Eating is then associated with growth of the larva, which funnels food by elaborate cilia into the digestive track. The larvae become diverse and complex in their structure and function. Excellent coverage of these topics are found in e.g. [Byrne and O’Hara \(2017\)](#), [Giese et al. \(1991\)](#), [Smith \(1997\)](#) and from Relevant Websites section.

Direct Versus Indirect Development

Most of the embryos that develop in each of these taxa are indirect developers, that is, they develop into a motile, and bilaterally symmetric larva first that eventually metamorphoses into a juvenile. The subsequent adult stage will ultimately make eggs and sperm again to complete the cycle. There are, however, in each of the groups within the phylum, extreme variations in this strategy. Many species of echinoderms instead develop directly into an adult, without passing through a larval stage. These so call direct developers often have very distinct eggs laden with large amounts of yolk, oil droplets, and fatty acids to supply the developing embryo with the nutrition necessary to obviate a feeding larval stage until the juvenile is capable of feeding. In some cases these eggs have so much oil reserves that the eggs are buoyant. A great deal of research is invested in understanding the evolutionary transitions that may have occurred between indirect developers, likely the ancient strategy of development in echinoderm, and direct developers, a derived developmental fate in this phylum. For more detailed information and for more methodology on studying these major life history changes, see [Zigler and Raff \(2013\)](#) and [Israel et al. \(2016\)](#).

Fossil evidence suggests that the early echinoderms were bilaterally symmetric, and likely evolved from bilaterally symmetric precursors. The early evolving phylum then underwent a radical change in body structure within the adult, likely during metamorphosis within the rudiment of the adult developed by the larva. How this occurred is not known, but is of great interest in evolutionary developmental morphology. See [Zamora et al. \(2012\)](#) for details of the research leading to this conclusion.

Cell Cycle and Developmental Synchrony

Some echinoderms release millions of eggs and upon fertilization, will usually develop synchronously into larvae. Especially the early cell divisions are quite uniform in these animals, and this property enables a large diversity of research into developmental mechanisms since many embryos can be visualized at once, and molecular mechanisms can be tested more readily with the abundance of samples. This is very different to many other animals, especially e.g., humans, which release on average one egg every 28 days, and round worms (*C. elegans*), which make on average ~200 eggs. This property of echinoderms was leveraged to examine the cyclicity of specific protein synthesis and degradation, leading to the identification of the cell cycle regulators, the cyclins ([Evans et al., 1983](#)).

Gene Regulatory Network

Development of an embryo is a process of regulating gene expression in specific times and places. How genes are expressed, and then how they influence the expression of other genes, is referred to as the gene regulatory network. Echinoderms are an important model system in unraveling how this network functions, and how it evolves. By comparing closely related groups of echinoderms e.g. sea stars, sea urchins, one can begin to understand how interactions of certain groups of genes may change with time, or be retained unchanged to function in various processes. These investigators will document their findings by use of a “wiring diagram”-like graphic to indicate interactions between genes that are positive (activate transcription), or negative (repress transcription). See [Fig. 8](#) for an example from a sea urchin and the following references for more details on how the gene regulatory network is studied ([Cary and Hinman, 2017](#); [Hinman and Cary, 2017](#); [Hinman and Jarvela, 2014](#); [Peter and Davidson, 2015, 2016, 2017](#)).

Formation of the Germ Line in Development

The reproductive system and strategy of an organism is the most important mechanism for propagation and survival of the species. Within these mechanisms are the somatic components of the reproductive system and the germ line cells. Both of these lineages are essential for reproduction. The somatic cells are those cells that support the development, passage, and products of the germ line cells. These cells and tissues are not passed on to subsequent generations, but are essential for reproduction. The reproductive system is enormously diverse between various organisms, and depends on the reproductive niche of the organism. The germ line is that

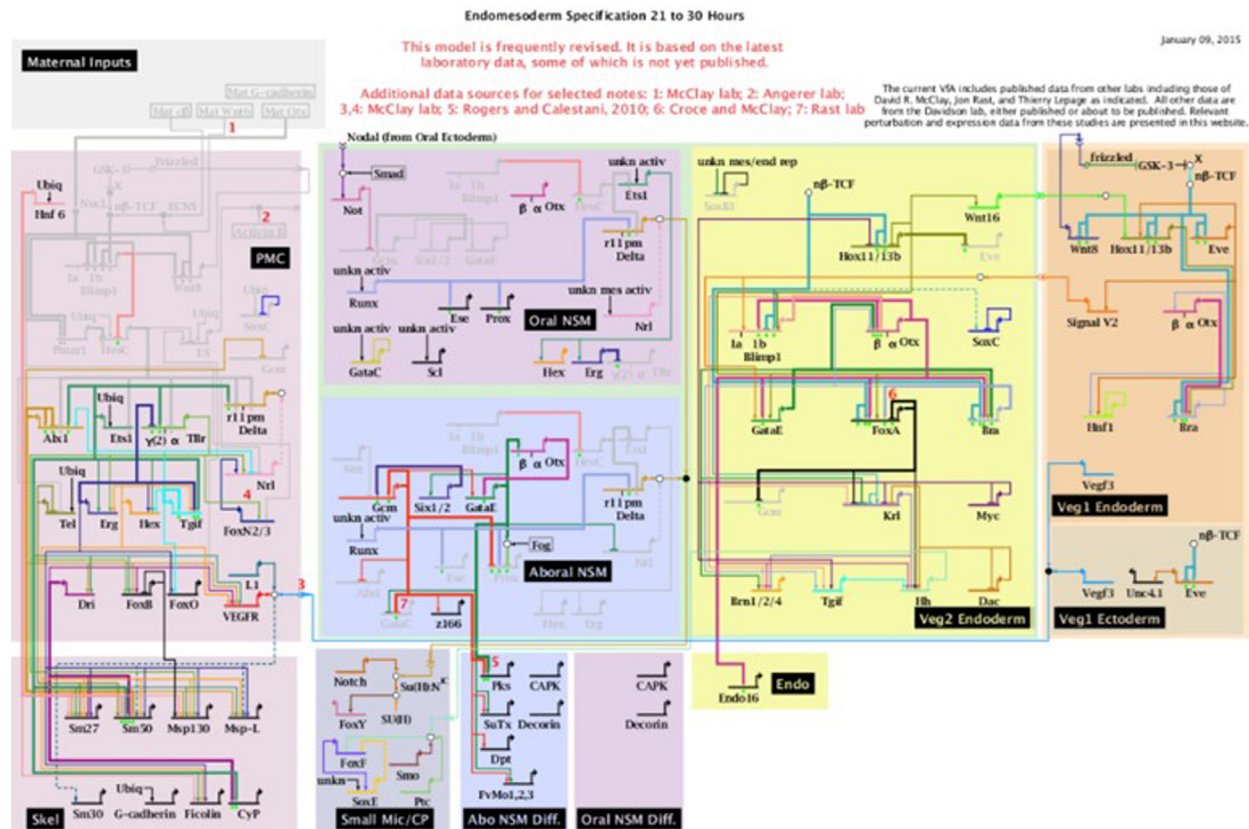


Fig. 8 Shown is a “wiring diagram” for gene regulatory networks in a region in the embryo of the sea urchin *Strongylocentrotus purpuratus*. Arrows indicate activation steps in the program whereas bars indicate repressive activities. Reproduced from Davidson Lab Gene Regulatory Networks. Available at: <http://www.spbase.org/endomes/>.

lineage which is committed to give rise to the gametes of the organisms, the sperm and eggs. The genome of these cells is potentially immortal in that they pass on their nuclei from generation to generation through the fertilization process. These cells too are enormously diverse and respond to, or modify the reproductive strategy, through which the organism is selected (Figs. 9 and 10).

How Do Animals Make a New Germ Line?

Two extremes are considered in understanding this process, though organisms are clearly graded in these mechanisms. One mechanism, originally found in the fly *Drosophila* and used by sea urchins, is to have molecular information stored in a specific region of the egg. As the embryo develops, cells that acquire the region with stored germ line information are directed to develop into the germ line of the organism. Many of the organisms used in laboratory research, such as zebrafish, the clawed toad, *Xenopus*, the round worm *C. elegans*, and the fly *Drosophila*, use this mechanism in germ line formation. Mammals instead use a distinct mechanism for germ line formation, and rely on cell–cell interactions to direct cell fate in the future germ line. This so called inductive mechanism appears to be the ancient, original, mechanism of how organisms selected their germ line cells, and many different species throughout phylogeny have evolved the derived strategy of germ factor acquisition instead. Embryos of sea stars, brittle stars, and sea cucumbers appear to have retain the ancient program of inductive germ line acquisition whereas sea urchins and sand dollars are thought to be the only taxon to have diverged from this strategy (Wessel *et al.*, 2014a,b).

Once the germ line is formed in the sea urchin, or in the sea star, the same group of molecules appear to be important in order for the germ line to become fated for the germ line, migrate to the somatic gonad, propagate into germ line stem cells, and eventually form functional gametes of eggs or sperm. These germ line factors are usually found in all organisms with a germ line, though it is becoming apparent that they are not expressed exclusively in the germ line. Many of these factors are now found in somatic cells, are over expressed in regenerative tissues, in cancers, and even in the nervous system.

Sea urchin orthologs identified that are generally involved in specifying the germ line in organisms widely throughout phylogeny are shown in Table 1, and the germ line cells in a sea urchin are shown in Fig. 7.

Brooding in Echinoderm Reproduction

Some echinoderm species have brooding pouches into which fertilized eggs are deposited. The embryos develop within these protected areas and only upon reaching more advanced stages are they released to populate the water column. While not the equivalent of a marsupial pouch, the pouch provides protection and may even supply nutrients to enhance the development of the young.



Fig. 9 The face page of echinobase.org the major resource for genomic datasets in echinoderms.

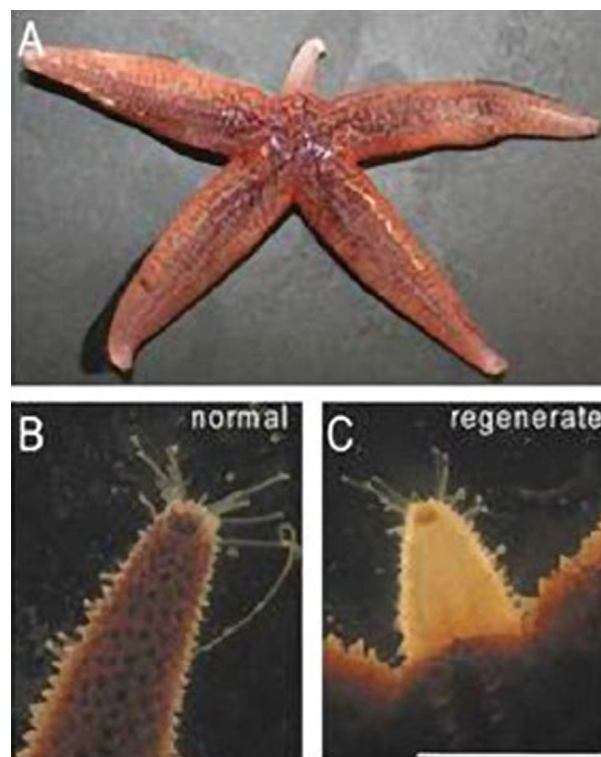


Fig. 10 The sea star *Asterias rubens* with an arm regenerating from the base (A–C). The early regenerate (C) is less pigmented than an intact arm of the same animal (B). Scale bar: 1 cm (B, C). Reproduced from Hernroth, B., *et al.*, 2010. Possibility of mixed progenitor cells in sea star arm regeneration. *J Exp Zool B Mol Dev Evol* 314 (6), 457–468.

Table 1 Listing of orthologs of germ line factors found in the sea urchin *S. purpuratus*

Gene	Genbank accession	Domains and function	Orthologs (BLAST score ^a)
<i>Sp-boule</i>	XP786845	RNA recognition motif (RRM) and DAZ repeats, translational regulator involved in germ cell development and meiosis	<i>S. oedipus</i> (1e–29) <i>H. sapiens</i> (4e–29)
<i>Sp-CPEB1</i>	XP786947	2 RRM domains, translational regulator during oogenesis (Chang <i>et al.</i> , 1999; Gebauer and Richter, 1996; Hake and Richter, 1994)	<i>M. glacialis</i> (2e–163) <i>H. sapiens</i> (1e–128)
<i>Sp-germ cell-less</i>	XP793954 XP784533	BTB (Broad-Complex, Tramtrack and Bric a brac) protein interaction domain, conserved nuclear pore-associated protein required for germ cell formation in <i>Drosophila</i> (Leatherman <i>et al.</i> , 2000)	<i>M. musculus</i> (5e–113) <i>D. rerio</i> (1e–112)
<i>Sp-DMRT</i>	XP790843	Doublesex DNA-binding motif (DM), transcription factor involved in sex determination	<i>P. troglodytes</i> (7e–33) <i>H. sapiens</i> (7e–32)
<i>Sp-mago nashi</i>	XP791004	No defined domains, required for localization of oskar mRNA in <i>Drosophila</i> germ plasm assembly (Newmark and Boswell, 1994)	<i>X. laevis</i> (6e–72) <i>H. sapiens</i> (8e–72)
<i>Sp-MORC</i>	XP790047	2 coiled-coil domains, nuclear protein required for spermatogenesis in mouse (Inoue <i>et al.</i> , 1999; Watson <i>et al.</i> , 1998)	<i>R. norvegicus</i> (1e–126) <i>M. musculus</i> (1e–126)
<i>Sp-MSY</i>	XP785816	CSP or Y-box binding domain, translational repressor during mouse spermatogenesis (Giorgini <i>et al.</i> , 2002)	<i>M. musculus</i> (6e–30) <i>H. sapiens</i> (1e–29)
<i>Sp-nanos2</i>	ABB89047	CCHC Zinc Fingers (2), translational regulator (with pumilio), conserved role in germ cell development	<i>N. vectensis</i> (7e–21) <i>H. sapiens</i> (2e–17)
<i>Sp-ovo</i>	XP788176	C2H2 Zinc Finger (4) transcription factor, plays a role in <i>Drosophila</i> oogenesis and mouse spermatogenesis (Dai <i>et al.</i> , 1998; Mével-Ninio <i>et al.</i> , 1991)	<i>H. sapiens</i> (6e–52) <i>D. melanogaster</i> (7e–52)
<i>Sp-pumilio</i>	XP794621	Pumilio-like repeats (3), translational regulator (with nanos), conserved role in germ cell development	<i>X. laevis</i> (0) <i>T. nigroviridis</i> (0)
<i>Sp-seawi</i>	AAG42534	PAZ (piwi, argonaute, zwiille) domain, required for maintenance of germ line stem cells	<i>H. sapiens</i> (0) <i>M. musculus</i> (0)
<i>Sp-SoxE</i>	XP786809	HMG box transcription factor involved in sex determination	<i>P. lividus</i> (0) <i>X. tropicalis</i> (3e–45)
<i>Sp-tudor</i>	XP780689	Tudor domains (5), required for <i>Drosophila</i> pole plasm assembly and is expressed in mouse spermatocytes (Chuma <i>et al.</i> , 2003; Thomson and Lasko, 2004)	<i>H. sapiens</i> (4e–45) <i>M. musculus</i> (8e–45)
<i>Sp-vasa</i>	XP781494	CCHC Zinc Fingers (3–4), DEAD box helicases, conserved marker of germ cells, function unknown	<i>C. savignyi</i> (0) <i>D. rerio</i> (5e–152)

^aThe first BLAST score listed for each gene is the best hit and the second BLAST score listed is the best hit amongst organisms whose germ lines have been intensively studied.

Regeneration

Sea stars are famous for their regenerative capabilities. An adult may lose one or multiple of its arms, but as long as it has its central disk, it is capable of regenerating into an adult indistinguishable from an undisturbed adult. The loss of an arm may occur from predation, or by autonomy. If by predation and the arm is severed not at the central disk, the arm first repairs its wound, and then grows out to normal size as the undisturbed arms. How this mechanism works is not clear, but it must include mechanisms of wound healing, transformation of cell fates since each major structure within the arm recovers i.e., gonad, neurons, and skeletal surface tissues.

More recently it was learned that the larvae of many echinoderms can also effectively regenerate. Bisecting a larvae along the oral/aboral axis, or cutting perpendicular to the oral/aboral axis, results in larval segments able to restore its major cell fates and proceed through development. An advantage of exploiting the larval regeneration mechanism may be in timing; instead of weeks to see are regeneration in an adult, one can examine larval regeneration in the matter of a few days, and by visualizing all the cell types through the transparency of the larval body. Some of the results of studying this process are now being released and may lead to a molecular mechanism for regeneration to link to existing models e.g. the Planarian flatworm.

Maybe even more intriguing is that the larvae of some species have been shown to clone. That is, larvae will bud off a new organism from the body wall of the larva, and begin a new life on its own. It is not clear how productive the clone is; does it eventually metamorphose and will it make eggs and sperm? For more details on this phenomenon see [Evaes and Palmer \(2003\)](#).

References

- Byrne, M., & O'Hara, T. (2017). *Australian Echinoderms: Biology, Ecology, and Evolution*. CSIRO Publishing.
- Cary, G. A., & Hinman, V. F. (2017). Echinoderm development and evolution in the post-genomic era. *Dev Biol*, 427(2), 203–211.
- Chang, J. S., Tan, L., & Schedl, P. (1999). The *Drosophila* CPEB homolog, orb, is required for oskar protein expression in oocytes. *Dev Biol*, 215(1), 91–106.
- Chuma, S., Hiyoshi, M., Yamamoto, A., et al. (2003). Mouse Tudor Repeat-1 (MTR-1) is a novel component of chromatoid bodies/nuages in male germ cells and forms a complex with snRNPs. *Mech Dev*, 120(9), 979–990.
- Dai, X., Schonbaum, C., Degenstein, L., et al. (1998). The ovo gene required for cuticle formation and oogenesis in flies is involved in hair formation and spermatogenesis in mice. *Genes Dev*, 12(21), 3452–3463.

- Ettensohn, C. A., Wessel, G. M., & Wray, G. A. (2004). The invertebrate deuterostomes: An introduction to their phylogeny, reproduction, development, and genomics. *Methods Cell Biol*, 74, 1–13.
- Evaes, A., & Palmer, A. (2003). Widespread cloning in echinoderm larvae. *Nature*, 425, 146.
- Evans, T., et al. (1983). Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*, 33(2), 389–396.
- Gebauer, F., & Richter, J. D. (1996). Mouse cytoplasmic polyadenylation element binding protein: An evolutionarily conserved protein that interacts with the cytoplasmic polyadenylation elements of c-mos mRNA. *Proc Natl Acad Sci USA*, 93(25), 14602–14607.
- Giese, J., Pearse, J., & Pearse, V. (1991). *Reproduction of Marine Invertebrates*. In J. In: Giese, J. Pearse, & V. Pearse (Eds.). Pacific Grove, CA: The Boxwood Press.
- Giorgini, F., Davies, H. G., & Braun, R. E. (2002). Translational repression by MSY4 inhibits spermatid differentiation in mice. *Development*, 129(15), 3669–3679.
- Hake, L. E., & Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell*, 79(4), 617–627.
- Hinman, V., & Cary, G. (2017). The evolution of gene regulation. *Elife*, 6.
- Hinman, V. F., & Jarvela, A. M. C. (2014). Developmental gene regulatory network evolution: Insights from comparative studies in echinoderms. *Genesis*, 52(3), 193–207.
- Inoue, N., Hess, K. D., Moreadith, R. W., et al. (1999). New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. *Hum Mol Genet*, 8(7), 1201–1207.
- Israel, J. W., et al. (2016). Comparative developmental transcriptomics reveals rewiring of a highly conserved gene regulatory network during a major life history switch in the sea urchin genus *Heliocidaris*. *PLOS Biol*, 14(3), e1002391.
- Leatherman, J. L., Kaestner, K. H., & Jongens, T. A. (2000). Identification of a mouse germ cell-less homologue with conserved activity in *Drosophila*. *Mech Dev*, 92(2), 145–153.
- Mével-Ninio, M., Terracol, R., & Kafatos, F. C. (1991). The ovo gene of *Drosophila* encodes a zinc finger protein required for female germ line development. *EMBO J*, 10(8), 2259–2266.
- Newmark, P. A., & Boswell, R. E. (1994). The mago nashi locus encodes an essential product required for germ plasm assembly in *Drosophila*. *Development*, 120(5), 1303–1313.
- Perez, A. F., et al. (2010). Reproductive cycle and reproductive output of the sea urchin *Loxechinus albus* (Echinodermata: Echinoidea) from Beagle Channel, Tierra del Fuego, Argentina. *Polar Biol*, 33(3), 271–280.
- Peter, I. S., & Davidson, E. H. (2015). *Genomic Control Process: Development and Evolution* (vol. xii, p. 448). London, UK; San Diego, CA: Academic Press.
- Peter, I. S., & Davidson, E. H. (2016). Implications of developmental gene regulatory networks inside and outside developmental biology. *Essays Dev Biol Part B*, 117, 237.
- Peter, I. S., & Davidson, E. H. (2017). Assessing regulatory information in developmental gene regulatory networks. *Proc Natl Acad Sci USA*, 114(23), 5862–5869.
- Smith, A. B. (1997). Echinoderm larvae and phylogeny. *Annu Rev Ecol Syst*, 28, 219–241.
- Thomson, T., & Lasko, P. (2004). *Drosophila* tudor is essential for polar granule assembly and pole cell specification, but not for posterior patterning. *Genesis*, 40(3), 164–170.
- Watson, M. L., Zinn, A. R., Inoue, N., et al. (1998). Identification of morc (microorchidia), a mutation that results in arrest of spermatogenesis at an early meiotic stage in the mouse. *Proc Natl Acad Sci USA*, 95(24), 14361–14366.
- Wessel, G. M., et al. (2014a). The biology of the germ line in echinoderms. *Mol Reprod Dev*, 81(8), 679–711.
- Wong, J. L., G.Wessel, G. M., et al. (2014b). Origin and development of the germ line in sea stars. *Genesis*, 52(5), 367–377.
- Zamora, S., Rahman, I. A., & Smith, A. B. (2012). Plated cambrian bilaterians reveal the earliest stages of echinoderm evolution. *PLOS ONE*, 7(6), e38296.
- Zigler, K. S., & Raff, R. A. (2013). A shift in germ layer allocation is correlated with large egg size and facultative planktotrophy in the echinoid *Clypeaster rosaceus*. *Biol Bull*, 224(3), 192–199.

Relevant Websites

<http://animaldiversity.org/accounts/Echinodermata/>. – Animal Diversity Web.

<http://www.asnailsodyssey.com/urchin.php>. – A Snail's Odyssey – Sea Urchin.

<http://www.echinobase.org/Echinobase/>. – EchinoBase.

<http://echinoblog.blogspot.com/>. – The Echinoblog.

<http://tolweb.org/tree/>. – Tree of Life Web Project.

<https://web.stanford.edu/group/inquiry2insight/cgi-bin/vu-r1a/vu.php>. – VirtualUrchin.

Reproduction in Tunicates

Fabio Gasparini and Loriano Ballarin, University of Padova, Padova, Italy

© 2018 Elsevier Inc. All rights reserved.

Glossary

Atrial (peribranchial) chamber The space between the pharynx and the body wall collecting the exhaust water exiting the pharynx through the pharyngeal slits and conveying it to the atrial siphon.

Endostyle Ventral pharyngeal glandular groove secreting the mucous net covering the pharynx internal surface and entrapping the particles suspended in the incurrent water.

Gonoduct The tube connecting the gonad with the exterior, through which gametes are released.

Hermaphroditism The capability of an organism to produce both male and female gametes.

Notochord The dorsal, axial flexible rod of chordate embryos, serving as the major support during development and as a source of signals that pattern surrounding tissues.

Pharynx Anterior part of the digestive system, provided with many slits and involved in both filtration and gaseous exchanges.

Siphon Tube-like anatomical structure in which water flows. Tunicates have both an oral and an atrial siphon for incoming and expelled water, respectively.

Stolon A horizontal branch extruding from the base of an organism.

Reproduction in Tunicates

Tunicates or urochordates (Gittenberger and Sanamyan, 2017) form a subphylum of the phylum Chordata and are considered the closest relatives of vertebrates (Delsuc *et al.*, 2006). They are benthic or pelagic filter-feeding marine animals with indirect development. Tunicate owe their name to the tunic, the peculiar covering in which the body is embedded, whereas the name urochordates comes from the notochord, the dorsal supporting rod characterizing chordates, here limited to the larval muscular tail. The pharynx is well developed in the adult and, generally, occupies most of the body volume. Even if the internal interrelationship among the tunicate taxa is controversial (Stach *et al.*, 2010), they traditionally include the three classes Ascidiacea, Thaliacea and Appendicularia (Fig. 1).

Almost all the tunicate species have a tadpole swimming larva that metamorphoses into a strongly derived and specialized juvenile, with a dramatic change of the body organization (Stolfi and Brown, 2015).

In term of reproductive capability, the subphylum is characterised by the presence, unique among chordates, of asexual reproduction: several species are colonial with complex life cycle, and new zooids appear by budding from the somatic tissues of a pre-existing one (Brown and Swalla, 2012).

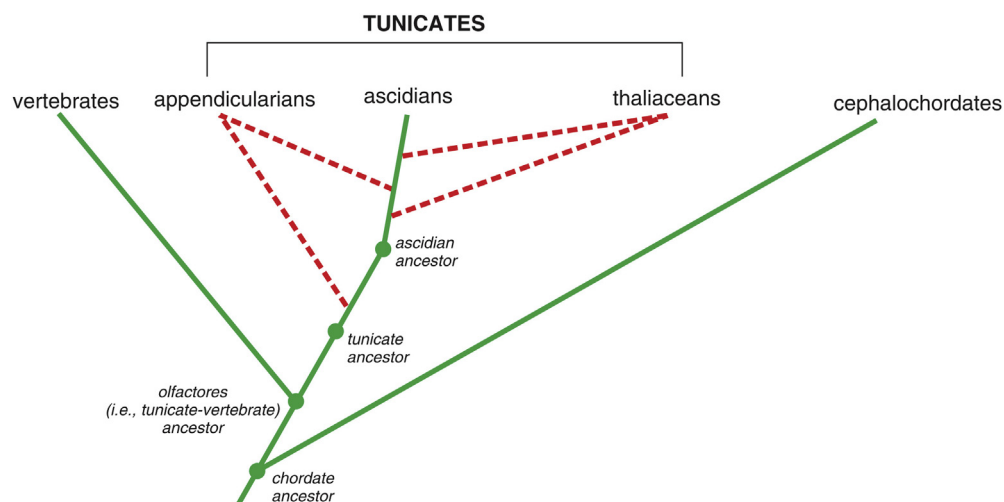


Fig. 1 Phylogenetic relationship among chordates. Red dashed lines represent possible relationships in uncertain branches.

Asciidiacea

Ascidians or sea-squirts (more than 5500 species) are sessile, marine invertebrates, including solitary and colonial species, diffused throughout the world, mainly in shallow tropical and temperate waters. At metamorphosis, the larval tail is resorbed, and the animals acquire a sac-like morphology. The body of the individuals or zooids ranges from 0.5 mm to more than 20 cm in length, that of colonial species being much smaller than solitary ones; it has a sac-like morphology mainly occupied by the fissured branchial chamber (the pharynx) and the flanking atrial (peribranchial) space, where the anus and the gonoducts open. The seawater flows from the oral siphon (the mouth), crosses the pharynx, enters the peribranchial chamber, and exits via the atrial siphon (Fig. 2).

In colonial ascidians, zooids can be either partially isolated and connected by stolons (deriving from the body wall and containing connective tissue and, sometimes, blood vessels) or partially or entirely embedded in the common tunic, frequently interconnected by a common circulation.

All ascidians are hermaphrodite, with simultaneous, protandric or protogynous hermaphroditism. Male and female gonads are usually distinct, although in some cases male and female tissues are present within the same, hermaphroditic gonad. Gonads may be unpaired, paired, or multiple and always have separate gonoducts that open into the atrial cavity. Their location varies and, even if this is a character no longer in use for the taxonomical subdivision of the group (Gittenberger and Sanamyan, 2010), ascidians may be distinguished on the basis of gonad position (Burighel and Cloney, 1997). Enterogona have gonads in close association with the intestinal loop, far from the atrial siphon, endowed with long gonoducts; in these species, sperms and eggs can be stored in the gonoducts before spawning. In Pleurogona, the gonads lie in the lateral body wall and are provided with short gonoducts (Fig. 3).

Ovaries are sac-like or tubular organs. Oocytes are surrounded by two layers (outer and inner) of follicle cells and an acellular vitelline coat; test cells are lodged between the plasma membrane and the vitelline coat.

Testes contain a system of branched tubules that end in blind, enlarged follicles (see Kawamura *et al.* (2011) for a detailed review of ascidian germline cell formation and differentiation). Sperms are uniflagellate, with small acrosome on the tip and a single mitochondrion, closely associated with the nucleus. In colonial ascidians, the sperm head is usually longer and more complex in structure, often with a coiled aspect (see Burighel and Martinucci (2000) for a detailed description of sperm ultrastructure). Sperm activation is accompanied by translocation of the mitochondrion towards the tail. Chemoattractants released by female gametes guide sperms to the eggs. Cross-fertilisation is the rule. Self-fertilisation, although reported in both solitary and colonial forms, is usually prevented by asynchronous maturation of male and female gametes. In some cases, gamete incompatibility exists.

External fertilisation takes place in solitary ascidians which, usually, have small eggs with little yolk. Inner follicular cells are greatly enlarged, highly vacuolated, and act as flotation devices. Spawning is usually triggered by light. Colonial species have internal fertilisation: sperms enter the peribranchial cavity through the atrial opening and reaches the oviducts. In this case, eggs are richer in yolk and, after fertilisation, embryos are brooded in the oviduct or in the peribranchial cavity. A few viviparous colonial species, with small alecithal eggs, have been described: in this case, parental zooids provide the nutrients required for embryonic development.

Cleavage is complete, of bilateral type. During gastrulation the blastopore, which marks the posterior part of the embryo, appears. Both the notochord, in the form of a solid rod, and the two muscular bands flanking the notochord, detach from the roof of the primitive intestine. A coelomic cavity never appears, and no mesoderm segmentation can be detected. The neural plate

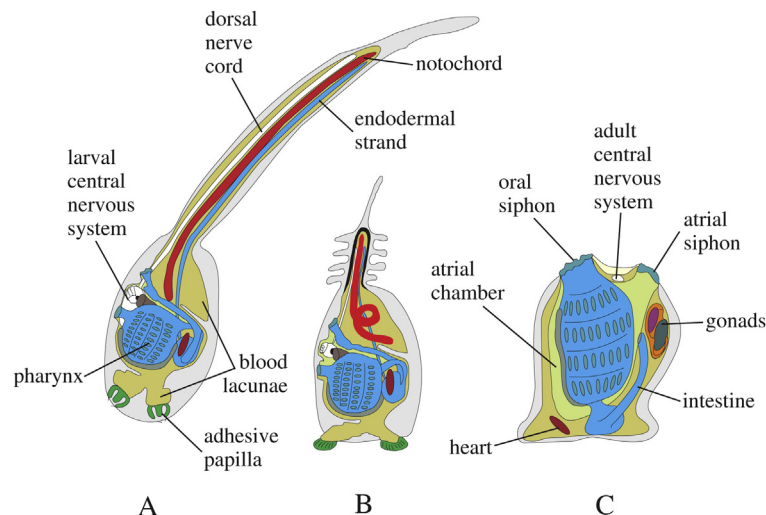


Fig. 2 Ascidian metamorphosis. A swimming tadpole larva (A) adheres to the substrate (B) and metamorphoses into a sessile oozoid (C). In (B) the tail is retracting and the axial organs are pushed into the head. Modified from Ballarin, L., Burighel, P., 2003. Tunicata and Cephalochordata. In: Minelli, A., Contrafatto, G. (Eds.), Biological Science Fundamentals and Systematics. Oxford: Eolss Publishers. Available at: <http://www.eolss.net>.

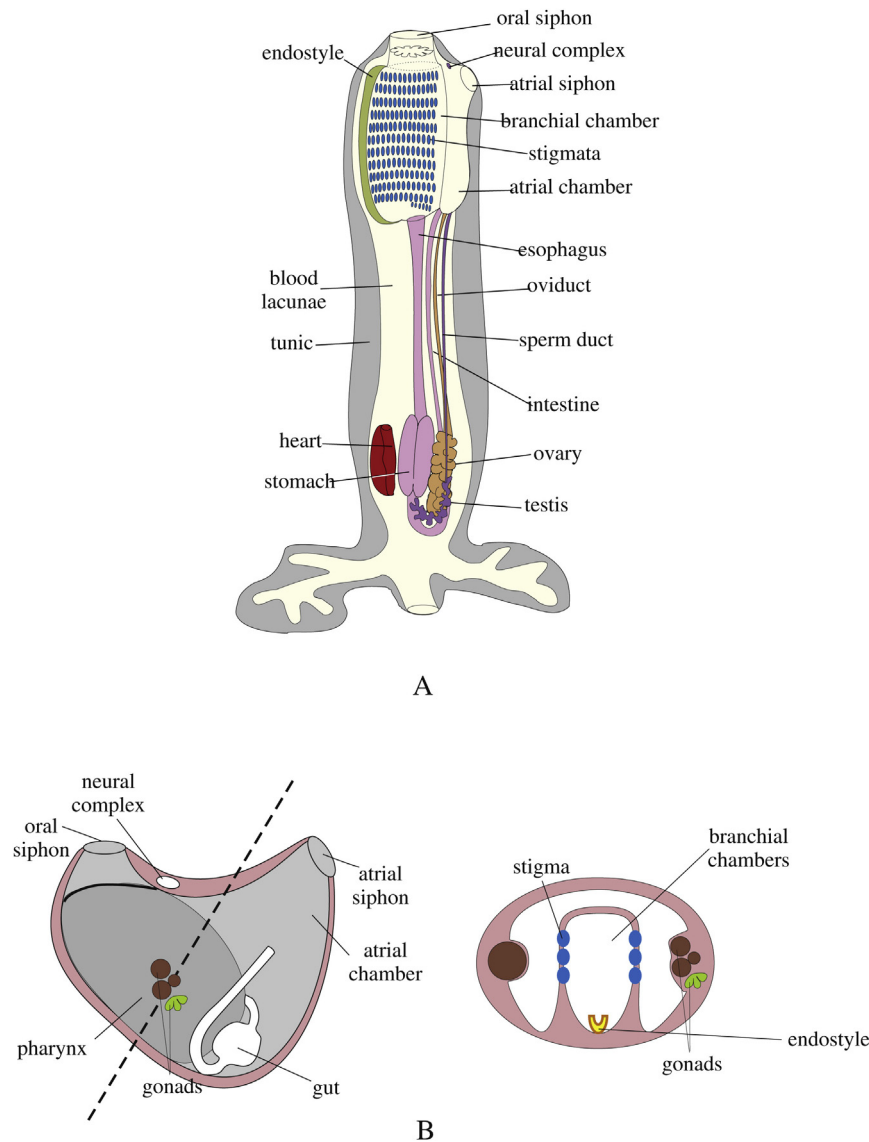


Fig. 3 General schematic morphology of *Clavelina lepadiformis* (A) and *Botryllus schlosseri* (B) as examples of the general morphology and gonads/gonoduct position in Enterogona and Pleurogona, respectively. (A) Modified from Ballarin, L., Burighel, P., 2003. Tunicata and Cephalochordata. In: Minelli, A., Contrafatto, G. (Eds.), Biological Science Fundamentals and Systematics. Oxford: Eolss Publishers. Available at: <http://www.eolss.net>. (B) Modified from Gasparini, F., Degasper, V., Shimeld, S.M., Burighel, P., Manni, L., 2013. Evolutionary conservation of the placodal transcriptional network during sexual and asexual development in chordates. Dev. Dyn. 242, 752–766.

sinks inwards and is covered by the ectoderm before it rolls up into a tube. The length of embryonic development is relatively short in solitary species (from some hours to a few days), whereas colonial species can brood their embryos for weeks or months. Free-swimming tadpole larvae hatch from the egg envelopes at the end of embryogenesis.

Although ascidians have high regenerating power, asexual reproduction is limited to colonial species. The oozoid, i.e., the first individual of a colony, deriving from metamorphosis of a tadpole larva, produce new zooids by budding. The resulting zooids are called blastozooids. Typically, a colony is a clone of genetically identical zooids and grows by continuous budding (Fig. 4).

Several modes of asexual reproduction have been described in different groups (Fig. 5) (see Brown and Swalla (2012) for a detailed review):

Stolonic budding, i.e., formation of buds from a branching stolon. Not common among ascidians; only found in Perophoridae.

Vascular budding, described in some botryllid ascidians. In this case, buds arise from aggregations of haemocytes (mainly lymphocyte-like cells) in tunic blood vessels.

Palleal or peribranchial budding, common in styelid ascidians. Buds derive from evagination of the mantle (i.e., the body wall, including the epidermis, the peribranchial epithelium and the mesodermal derivatives between them) at the sides of the body. In the genera *Botryllus* and *Botrylloides*, three blastogenetic generations are found in a colony: adult zooids with open siphons, buds and budlets, the latter coming from evagination of the bud body wall. At regular intervals, adult zooids close their siphons and are

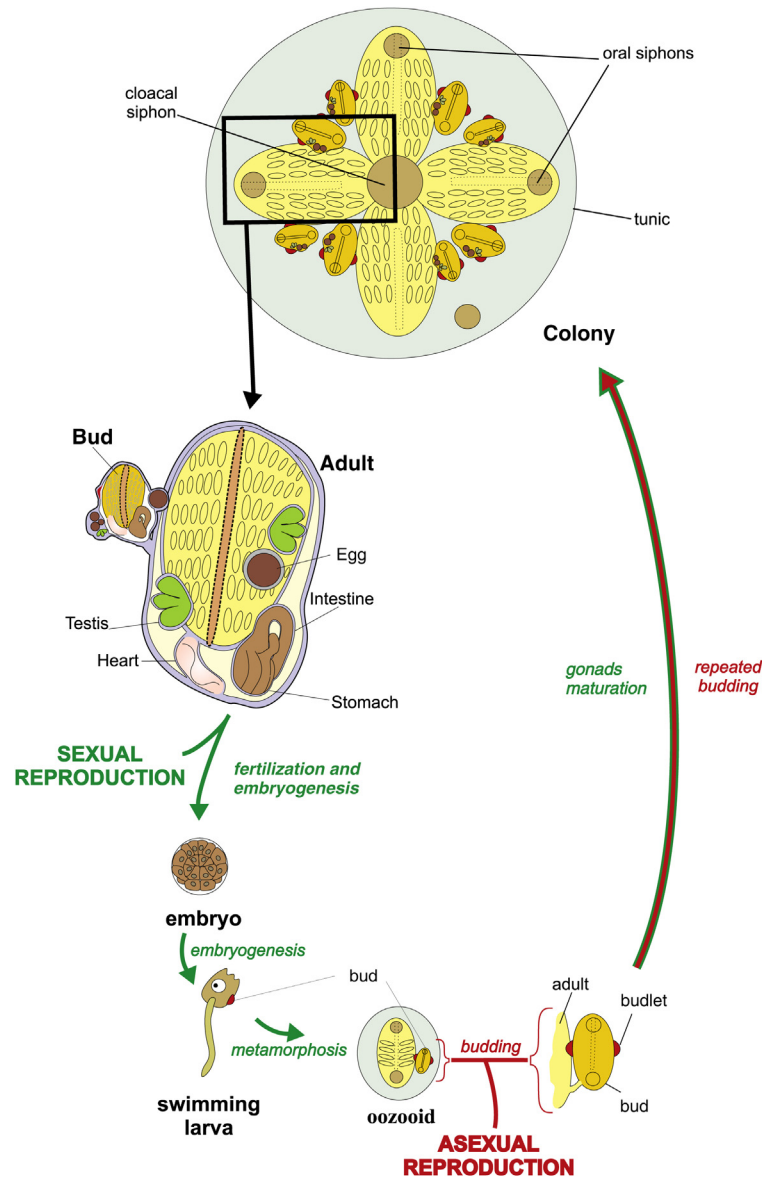


Fig. 4 Life cycle of *Botryllus schlosseri*, a colonial ascidian. Modified from Gasparini, F., Caicci, F., Rigon, F., Zaniolo, G., Manni, L., 2014. Testing an unusual in vivo vessel network model: A method to study angiogenesis in the colonial tunicate *Botryllus schlosseri*. Sci. Rep. 4, 6460.

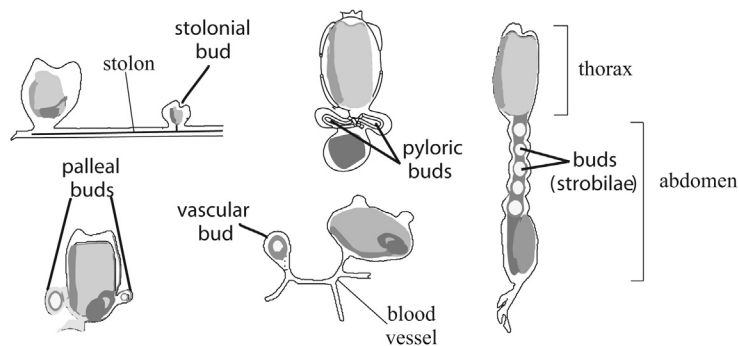


Fig. 5 Budding in ascidians. Modified from Nakauchi, M., 1982. Asexual development of ascidians: Its biological significance, diversity, and morphogenesis. Amer. Zool. 22, 753–763.

progressively resorbed in a process called takeover, while buds reach their functional maturity and open their siphons. Since a zooid gives rise to more than one bud, colonies progressively enlarge.

Budding by strobilation, widespread in the polyclinid and polycitorid ascidians. It involves fragmentation of the elongated body into two or more parts. Each part undergoes partial regression and dedifferentiation, then reorganises to form a complete individual.

Pyloric budding, typical of the didemnid ascidians. Two buds arise from oesophagus and abdomen, respectively. The oesophageal bud forms the new lower part of the body and the abdominal bud forms the new upper part. The two buds can either join together to form one zooid, or two zooids can form, each with an old and a new part.

Thaliacea

Thaliaceans are pelagic tunicates with complex life-cycle, alternating asexual and sexual generations (Deibel and Lowen, 2012). Their body is barrel-like, provided with wide oral and atrial openings at the opposite ends. The transparent zooids are embedded in a thin gelatinous tunic, so that circular bundles of muscles are easily visible. Members of this class include: Pyrosomatida, Doliolida (Cyclomyaria), and Salpida (Desmomyaria or Hemimyyaria).

Pyrosomatida

Pyrosomes, common in tropical and temperate seas, owe their name to luminescent organs which contain symbiotic bioluminescent bacteria. These bacteria enter the eggs and are transmitted to the new colonies. Colonies have the form of hollow cylinders with one blind end (Fig. 6).

Their size ranges from few centimetres to more than one metre in length. Zooids are located in the wall of the cylinder, embedded in the common tunic; the oral siphon of each zooid faces outwards, whereas the atrial aperture opens into the central cloacal cavity: water expelled through the cloacal aperture allows colonial locomotion by propulsion. The general structure of pyrosome zooids is quite similar to that of their ascidian counterparts: they are, hermaphroditic (an ovary and a testis lying ventrally, near the gut) and have a wide branchial basket occupying most of the body volume (Fig. 6).

Colonies grow by addition of new zooids that bud from a short stolon in the heart region of parental zooids. In a colony the oldest zooids are protandric and located near to the closed end; conversely, younger zooids are protogynous and located near the colonial opening, those in the middle are simultaneously hermaphroditic. Self-fertilisation is possible. Eggs, surrounded by both test and follicular cells, are provided with abundant yolk. Fertilisation is internal, and the zygote undergoes partial cleavage in a peribranchial pouch. A tadpole larva is missing and, at the completion of development, a goblet-like oozoid, named cyatozoid, is released from the atrial siphon. A stolon originates from its endostylar pharyngeal wall that buds four primary blastozooids. At this point the new colony reaches superficial waters and grows by repeated cycles of asexual reproduction.

Salpida

Salps live in all seas, from the surface down to 1500 m. About 200 species have been described; their size ranges from 1 to 20 cm in length. They are characterised by a metagenetic life cycle (i.e., they regularly alternate sexual and asexual generation). Similarly to pyrosomes, also salps lack a larval stage and the fertilised eggs develop directly into oozoids. The oozoids look like hollow

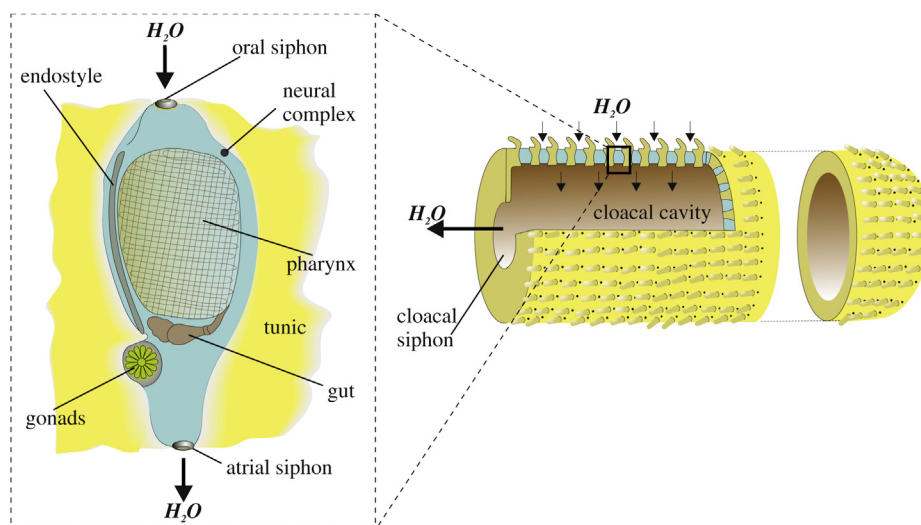


Fig. 6 General morphology of pyrosomes colony (right) and colonial zooid (left). Modified from Ballarin, L., Burighel, P., 2003. Tunicata and Cephalochordata. In: Minelli, A., Contrafatto, G. (Eds.), *Biological Science Fundamentals and Systematics*. Oxford: Eolss Publishers. Available at: <http://www.eolss.net>.

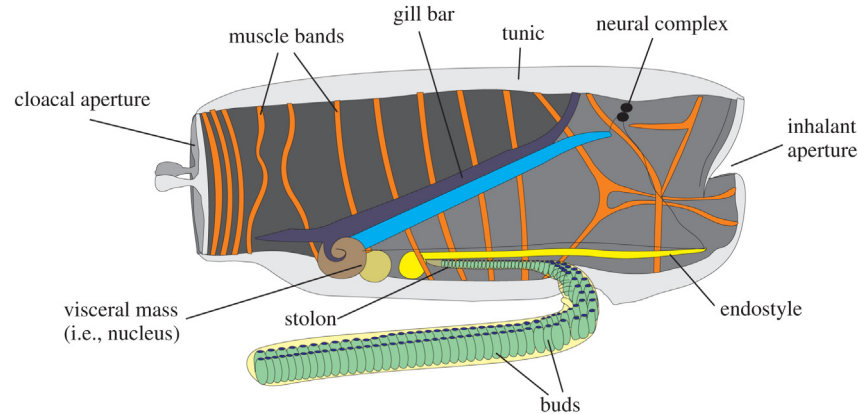


Fig. 7 General morphology of a salp oozoid. Modified from Ballarin, L., Burighel, P., 2003. Tunicata and Cephalochordata. In: Minelli, A., Contrafatto, G. (Eds.), *Biological Science Fundamentals and Systematics*. Oxford: Eolss Publishers. Available at: <http://www.eolss.net>.

cylinders, open on the opposite sides (Fig. 7). The anterior, inhalant, opening can be closed by two lateral folds (velum). The pharynx is separated from the atrial chamber by a gill bar which lies obliquely between the posterior end of the endostyle and the anterior dorsal part of the body. The branchial and atrial cavities communicate through two large gill slits that, differently from other tunicates, are not bordered by cilia. Indeed, water flow is created by the contraction of striated locomotory muscles. The latter run transversely in the form of 4–8, ventrally incomplete, bands.

At the posterior ventral side of the oozoid, close to the gill bar, a whole opaque visceral mass forms the so-called “nucleus”; anteriorly, a stolon emerges, consisting of an outer epidermal sheet around an intermediate mesodermal layer and an inner endodermal axis derived from the endostyle. The stolon, by strobilation, forms a series of sexual blastozoids. In some periods of the year, rapid budding is responsible for salp blooms. Groups of buds are progressively formed: in each group, blastozoids are of the same age. Each group is weakly connected by some abortive buds, so that chains of several zooids are progressively released. In young chains, the blastozoids remain connected while feeding and growing; older chains may break into single zooids. In blastozoids the stolon is absent, a testis lies next to the nucleus and matures after the ovary (protogynous hermaphroditism), located on the right posterior side of the body. Internal fertilisation occurs by sperms from older zooids penetrating the pharyngeal cavity with incoming water. In most species, they reach the egg through a solid oviduct. Cleavage of the alecithal eggs is holoblastic. A placenta is formed, which connects the embryo to maternal tissues, and development occurs within the maternal cloacal wall. In most cases, a single embryo is directly exposed to the cloacal cavity, where the young oozoid is finally released before being expelled outside.

Doliolida

Doliolida are small (0.5–5 cm in length) pelagic forms living in warm superficial waters. About 50 species have been described. Doliolids are unique among thaliaceans in having a free larval stage. Like other members of the class, doliolids alternate sexual and asexual generation, but their life-cycle is much more complex than in other thaliaceans, as various blastozoid bud types derive from oozoids (Fig. 8). The life cycle starts with a barrel-shaped oozoid which indirectly derives from the fertilised egg, after the metamorphosis of a free-swimming larva. A thin tunic covers the outer epidermis. Nine muscular bands (eight in blastozoids), easily visible through the transparent body, lie under the epidermis of the oozoid: the first and the last form the oral and atrial sphincters. Contraction of the muscle bands allows locomotion by jet propulsion. Both anterior and posterior apertures bear a row of flaps, provided with ciliated sensory cells, which can distally converge when the sphincters contract and close the openings. The internal cavity is divided by a transverse branchial septum into an anterior pharyngeal space, of endodermal origin, and a posterior, ectodermal atrial (cloacal) region. The gill is perforated by two rows of wide, ciliated slits. Beating of cilia bordering gill slits causes water to flow from the oral opening to the cloacal cavity through the branchial septum.

Oozoids bear a small ventral stolon and a dorsal, posterior appendix. As they age, most of the visceral organs (endostyle, branchial septum, gut) disappear, and the animals can no longer feed. At this stage, the ventral stolon continuously produces new buds by strobilation. Carried by special migrating cells, phorocytes, they travel around the right posterior side of the adult body to the dorsal appendix, and are arranged in three symmetrical rows in the dorsal surface of the appendix. The buds of the outer rows develop into gastrozooids or trophozooids, with enlarged pharyngeal region for nourishment of the whole colony, represented by the old oozoid and its growing blastozoids. Gastrozooids stick to the parental oozoid via a ventral mass of epidermal cells, called the placenta. The latter presumably ensures the exchanges of nutrients between gastrozooid and oozoid. Buds of the central row mature into phorozooids with ventral budlets. They can feed themselves and detach from the parental oozoid to form a free colony made up of the phorozooid and its buds. The latter grow to free-living gonozooids which represent the sexual generation. Gonozooids are protogynous hermaphrodites; their single ovary produces up to three eggs which, once fertilised, are released into the atrial cavity and then into the sea, before maturation of the testis. Fertilisation is followed by rapid embryonic development,

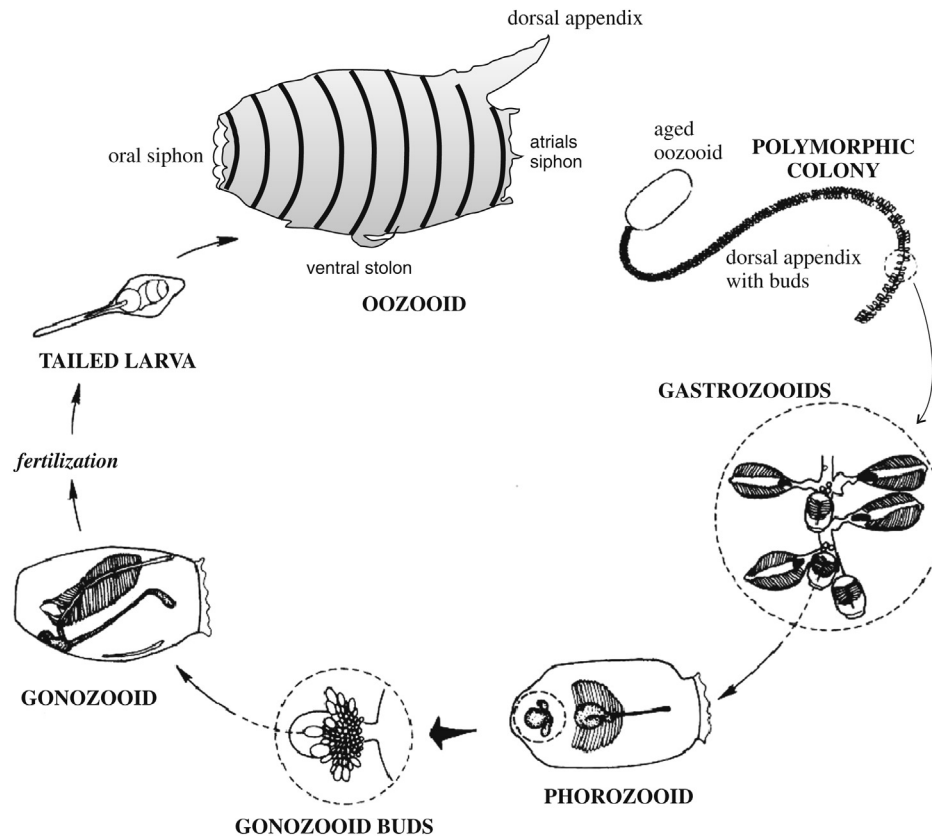


Fig. 8 General morphology of Doliolids. Modified from Alldredge, A.L., Madin, L.P., 1982. Pelagic tunicates: Unique herbivores in the marine plankton. *BioScience* 32, 655–663.

which leads to a free larva living in deep waters. Most doliolid larvae possess a muscular tail provided with a notochord. At metamorphosis, the larval trunk progressively assumes the barrel shape of the oozoid and the tail is resorbed. A new oozoid is thus formed and a new life-cycle starts.

Larvacea or Appendicularia

Larvacea or Appendicularia (about 70 species) are free-swimming, highly specialised, pelagic Tunicates found in all the oceans (Deibel and Lowen, 2012). The name Larvacea refers to the close similarity of adult Appendicularia with the ascidian tadpole larva, their body being formed of a “trunk”, 1–4 mm long, and a much longer tail (Fig. 9); in larger species, the animal may reach a total length of 6–8 cm. A remarkable feature of larvaceans is their constant cell number (euthely). They include three groups: Oikopleuridae (the most studied Appendicularians; Onuma *et al.*, 2017), Fritillariidae and Kowalewskidae.

The main feature of Appendicularia is the ‘house’, which is secreted and inhabited by the animal itself. This gelatinous structure is secreted by a specialised area of the epidermis of the trunk: the oikoplast, where large glandular cells are located. The house, mainly formed by mucopolysaccharides, is secreted in a condensed form as a gelatinous cap covering the anterior half of the trunk; it rapidly inflates (in a few seconds) due to animal movements and water absorption, thus increasing its volume about 300 times. In Oikopleuridae and Kowalewskidae, the whole body enters the house, whereas in Fritillariidae animals are external to the house, that resembles a large ball connected to the mouth. Houses act as filtering devices and traps for food. The tail acts as a muscular pump, sucking water into the house through two dorsal openings provided with coarse filtering grids to prevent oversized or harmful material from entering the house with incoming water currents. Water is driven into the posterior chamber and forced through two fine-meshed nets which trap and concentrate suspended particles, less than 5 µm in size. Outflowing water, leaving the posterior chamber, propels the whole house in such a way that the animal’s mouth, oriented towards the mucous traps, is directed posteriorly. A food-collecting tube connects the food filters with the mouth, and a weak water current, generated by the cilia lining the pharyngeal openings (spiracula), allows particles to reach the mouth and the pharynx, where they are re-captured by the mucous net secreted by the endostyle, before entering the oesophagus. When the filters clog, after some hours of filtration, houses are shed and replaced by new ones: up to five houses can be produced in one day.

Only sexual reproduction occurs in Larvacea, and all of them, apart from the only known tunicate gonocoric species *Oikopleura dioica*, are protandric hermaphrodites. The gonads lie in the posterior part of the trunk (Fig. 9): sperm is released through a dorsal

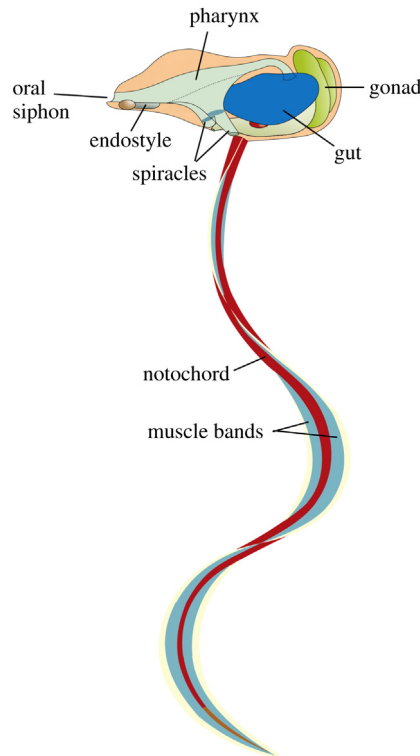


Fig. 9 Schematic representation of oikopleurid Appendicularia. Modified from Ballarín, L., Burighel, P., 2003. Tunicata and Cephalochordata. In: Minelli, A., Contratatto, G. (Eds.), *Biological Science Fundamentals and Systematics*. Oxford: Eolss Publishers. Available at: <http://www.eolss.net>.

spermiduct, whereas eggs are released by rupture of the trunk wall, which causes the death of the animal. External fertilisation occurs, and development gives rise to a tadpole larva which rapidly undergoes simple metamorphosis, involving a change in tail orientation and acquisition of competence for secreting 'houses'.

References

- Brown, F. D., & Swalla, B. J. (2012). Evolution and development of budding by stem cells: Ascidian coloniality as a case study. *Dev. Biol.*, 369, 151–162.
- Burighel, P., & Cloney, R. A. (1997). Urochordata: Ascidiacea. In F. W. Harrison, & E. E. Ruppert (Eds.), *Hemichordata, Chaetognatha, and the Invertebrate Chordates*, 15 pp. 221–347). New York, NY: Wiley-Liss, Inc.
- Burighel, P., Martinucci, G.B., 2000. Urochordata. In: Jamieson, B.G.M. (Ed.), *Progress in Male Gamete Ultrastructure and Phylogeny*. In: Rita, K.G., Adiyodi, G., *Reproductive Biology of Invertebrates Series*, vol. IX. New York, NY: John Wiley & Sons, pp. 261–298.
- Deibel, D., & Lowen, B. (2012). A review of the life cycles and life-history adaptations of pelagic tunicates to environmental conditions. *ICES J. Mar. Sci.*, 69, 358–369.
- Delsuc, F., Brinkmann, H., Chourrout, D., & Philippe, H. (2006). Tunicates and not cephalochordates are the closest living relatives of vertebrates. *Nature*, 439, 965–968.
- Gittenberger, A., Sanamyan, K., 2010. WoRMS – World Register of Marine Species. Available at: <http://www.marinespecies.org/aphia.php?P=taxdetails&id=154133>.
- Gittenberger, A., Sanamyan, K., 2017. Tunicata. In: Shenkar, N., Gittenberger, A., Lambert, G., *et al.* (Eds.), *Ascidiacea World Database*. Available at: <http://www.marinespecies.org/aphia.php?P=taxdetails&id=146420>.
- Kawamura, K., Tiozzo, S., Manni, L., *et al.* (2011). Germline cell formation and gonad regeneration in solitary and colonial ascidians. *Dev. Dyn.*, 240, 299–308.
- Onuma, T. A., Isobe, M., & Nishida, H. (2017). Internal and external morphology of adults of the appendicularian, *Oikopleura dioica*: An SEM study. *Cell Tissue Res.*, 367, 213–227.
- Stach, T., Braband, A., & Podsiadlowski, L. (2010). Erosion of phylogenetic signal in tunicate mitochondrial genomes on different levels of analysis. *Mol. Phylogenet. Evol.*, 55, 860–870.
- Stolfi, A., & Brown, F. D. (2015). Tunicata. In A. Wanninger (Ed.), *Evolutionary Developmental Biology of Invertebrates* (vol. 6, pp. 135–204). Wien: Springer.

Chondrichthyes (Sharks, Rays, Skates and Chimaeras)

Cynthia A Awruch, University of Tasmania, Tasmania, Australia; and Group of Ecophysiology Applied to the Management and Conservation of Wildlife, (CESIMAR) Center for the Study of Marine Systems, CENPAT (Patagonian National Centre) – CONICET, Chubut, Argentina

© 2018 Elsevier Inc. All rights reserved.

Introduction

Chondrichthyes are cartilaginous fish within the vertebrate lineage that are divided in two sub clusters: Elasmobranchs including sharks, rays and skates and Holocephali including the chimaeras. Chondrichthyes have survived and avoid mass extinction for over 400 million years, being one of the most successful and oldest group of vertebrates in terms of historical durability. This lasting success has largely depended on their diverse reproductive adaptations developed during their long evolutionary history. Why? Because the primary requirement for successful survival of any species is their ability to reproduce and give birth to fit newborns that will contribute to future generations. Understanding the process of reproduction requires knowledge of the chondrichthyan species' reproductive adaptations where the most effective adaptation is one that produces as many fit progeny as necessary to ensure species survival in any giving aquatic environment. Chondrichthyans developed different reproductive adaptations, and although generalizations of these reproductive strategies are difficult as many species uncover unique adaptations, shared grounds can be distinguished. The reproductive strategies are expressed through a combination of a wide range of reproductive modes and reproductive cycles. These reproductive modes are classified based on embryo development sites and embryo nourishment. According to embryo development sites, two categories can be distinguished: oviparity, where females lay eggs that typically develop and hatch outside the maternal body; and viviparity, where embryo development occurs inside the maternal body and females give birth to fully developed newborns. According to embryo nourishment throughout its development, these two reproductive categories are further divided as lecithotrophy, where the nutrients are supported solely by a yolk-sac with no maternal input; and matrotrophy, where at least part of the embryo nourishment are supplied by maternal input of nutrients.

The reproductive cycles denote the beginning to the end of each reproductive phase, combining: (1) the length of follicle development within the ovary that will be fertilized to form the embryos, (2) mating/fertilization, (3) the subsequent egg deposition/pregnancy period, and (4) a resting period preceding a new follicle development cycle (not all species have resting periods).

Finally, although out of the scope of this chapter, it is important to mention that the brain-pituitary-gonadal axis is a cascade system that triggers and regulates the entire reproductive process, promoting follicle production, ovulation, mating, fertilization, embryo development, and parturition.

Classification of Reproductive Modes

One of the most important reproductive physical adaptation in the evolution of chondrichthyans is the modification of the male pelvic fins into claspers (copulatory organs) allowing for internal fertilization. As a consequence, it is likely that chondrichthyans developed early in the evolution a viviparity reproductive mode, which has been confirmed by finding that multiple forms of viviparity are the ancestors of oviparity in both Elasmobranchii and Holocephali (Grogan and Lund, 2011).

Oviparity

Approximately 42% of Chondrichthyan species (including all Holocephali) are oviparous (Compagno, 1990; Dulvy and Reynolds, 1997). Oviparous species produce eggs that are enclosed in resilient egg cases for deposition on the seabed. The cases also contain the egg "jelly," a gelatinous material that serves as hydrodynamic support for the egg/embryo during early development. Embryo development occurs inside these egg cases and normally outside the maternal body (Fig. 1). Two forms of oviparity have been described, single (or extended) and multiple (or retained) oviparity. The majority of the oviparous species (39%) follow a single oviparity where one egg case is deposited at a time from each uterus with no embryonic development until the egg case is laid. Multiple oviparity has been found in nine elasmobranch species; females produce one or several eggs cases (each case contains one embryo) that, instead to be laid, are retained in the uterus where embryos develop to an advanced stage inside the maternal body. After egg cases deposition, embryo development is finalized in a relatively short time outside the maternal body.

According to embryo nutrition, all oviparous species are lecithotrophic, nutrients are supplied solely by the yolk-sac (Fig. 1). The yolk-sac is like a small balloon full of nutrients produced by the maternal liver and transfer to each follicle during its development. Once the follicles complete their development, follicles are ovulated and fertilized, the embryo starts to develop and the yolk remains as a sac attached to the embryo stomach transferring nutrients. As the development progresses yolk absorption is almost complete at hatching (Fig. 1).

Parental care is not evident in oviparous elasmobranchs (Jacoby *et al.*, 2012) therefore, embryo survival relies on the maternal selection of the appropriate environment for egg case deposition and posterior hatching.

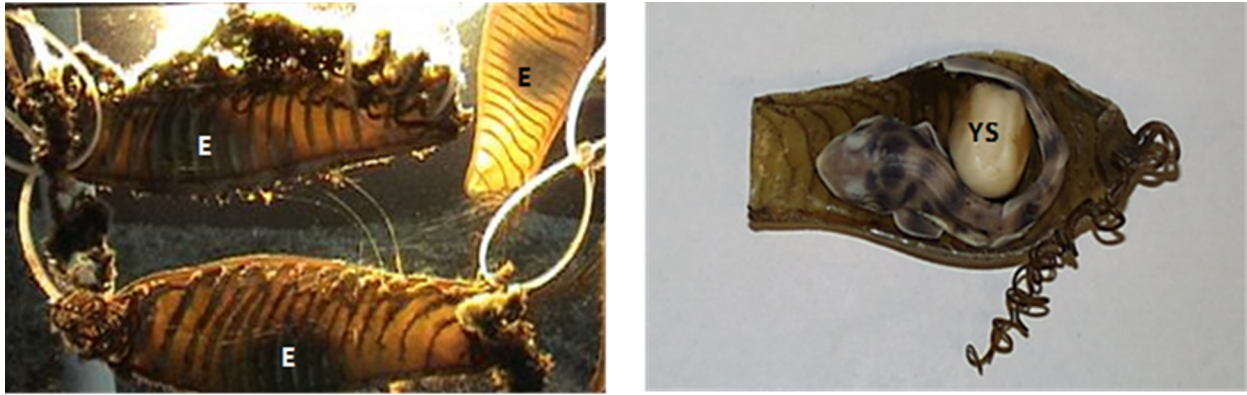


Fig. 1 Left Photo: Egg cases deposited from sharks. It can be seen inside the egg/embryo (E). Right Photo: Open egg case, late development embryo stage and yolk-sac (YS) visible. Photos by C.A. Awruch.

Viviparity

Viviparity is a highly successful reproductive mode and is the dominant form of reproduction found in approximately 58% of elasmobranchs (Compagno, 1990; Dulvy and Reynolds, 1997). The main characteristic of viviparous species is that embryo development occurs inside the maternal body. In live-bearing species, females invest and allocate higher energy resources to sustain larger size embryos than in oviparity. The evolutionary advantages of larger birth sizes are to improve embryo survival, with greater ability to prey and fewer predators at birth. As with oviparous species there is no evidence suggesting parental care of any sort following parturition.

According to embryo nutrition, viviparity can be divided into two main categories.

(1) *Lecithotrophic Yolk-Sac Viviparity*

Yolk-sac viviparity occurs in about 18% of living elasmobranch species (Hamlett and Koob, 1999). The embryo develops within the mother's uterus, providing the environment where oxygen supply, osmoregulation (water and inorganic ions exchange), and waste removal occurs. However, the maternal uterus does not provide nutrients. Thus, as with oviparous species, nutrients are supplied solely by the yolk-sac attached to the embryo (Fig. 2(A)).

The embryo development occurs in two stages. The initial gestation stages are called the pre-eclosion stage. Once ovulation and fertilization is completed, the egg/embryo is encapsulated in a "candle", an elongated, thin and translucent capsule (Fig. 2(B)). In

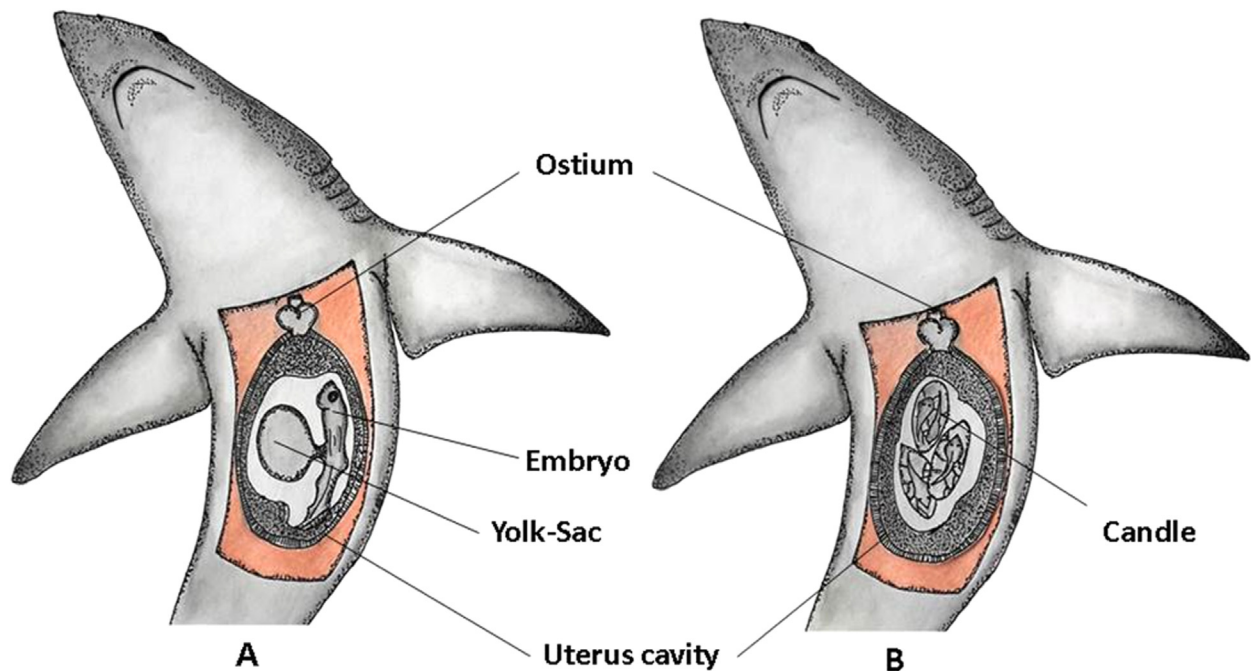


Fig. 2 Lecithotrophic yolk-sac viviparity. Illustrated by M.C. Sueiro.

general, the maternal females hold one candle per uterus, but the number of embryos that each “candle” can hold varies between species. For example, in the hidden angel shark, *Squatina occulta*, two to six embryos are enclosed in each candle (Sunye and Vooren, 1997), while, and very interestingly, a pregnant whale shark, *Rhinocodon typus*, was found to carry about 300 embryos many of which were still enclosed in individual cases (Joung *et al.*, 1996). Initial stages of embryo development occurs within the “candle”, after which, the “candle” breaks releasing the embryo freely into the maternal uterus to continue developing until parturition (Sunye and Vooren, 1997).

(2) Matrotrophic

This strategy occurs in around 40% of viviparous elasmobranchs. As with yolk-sac species, during the early stages of embryo development, nutrients are supplied by the yolk-sac, and once these are exhausted, the mother will supply nutrients through a variety of mechanisms: uterine secretions (histotrophy); unfertilized eggs (oophagy) or sibling (adelphophagy); and placental transfer (placentatroph).

The main advantage of matrotrophic reproduction is that embryos can gain significant weight and size during their development, in comparison with solely yolk-sac nutrition.

Histotrophy: The majority of the matrotrophic elasmobranchs follow this uterine secretion reproductive mode. The maternal uterus secretes into the intrauterine lumen nutritive organic fluid known as “uterine milk” or histotroph, which is consumed by the embryo by either ingestion or absorption across the external gill filaments.

Very recently a new possible type of reproductive mode according to embryo nutrition has been categorized, embryotrophy (Castro *et al.*, 2016). Up to date, this type of reproduction has only been observed in one species of sharks, the tiger shark *Galeocerdo cuvier*. In this type of nutrition, the egg case, a yellowish and thin membrane that surrounds the embryo throughout development, becomes filled early in the gestation with a large quantity (about one liter) of a clear yellowish fluid, embryotrophe. This embryotrophe is the maternal source of nutrition for the embryo after the yolk-sac has been consumed. However, although is likely that embryotrophe is secreted by the maternal uterus, more studies on the secretory function of the uterine epithelium needs to be done. Until then, this new reproductive mode cannot completely being confirmed.

Oophagy: This reproductive mode occurs only in sharks. Once the embryo depleted the yolk-sac reserves, the mother provides a constant supply of unfertilized and undeveloped eggs that will disintegrate providing exogenous yolk as a source of nutrition for the developing embryo.

Adelphophagy (also called embryophagy or intrauterine cannibalism): This reproductive strategy is a form of oophagy, where the mother produce few fertilized and unfertilized eggs as a source of nutrition (Fig. 3(A)). This type of reproduction has been, so far, identified in only one shark species, the grey nurse shark, *Carcharias taurus*. In this species, the gestation last 12 months, the mother produces fertilized eggs to form the embryos in the first 2.5 months, however only one embryo will develop teeth within the first 5–6 months which allows the embryo to consume the other siblings inside the maternal uterus, the embryophagous stage. Once the siblings are consumed, the embryo will use the yolk from the unfertilized eggs as a source of nutrients, the oophagous stage. A single embryo develops in each uterus. (Gilmore *et al.*, 2005).

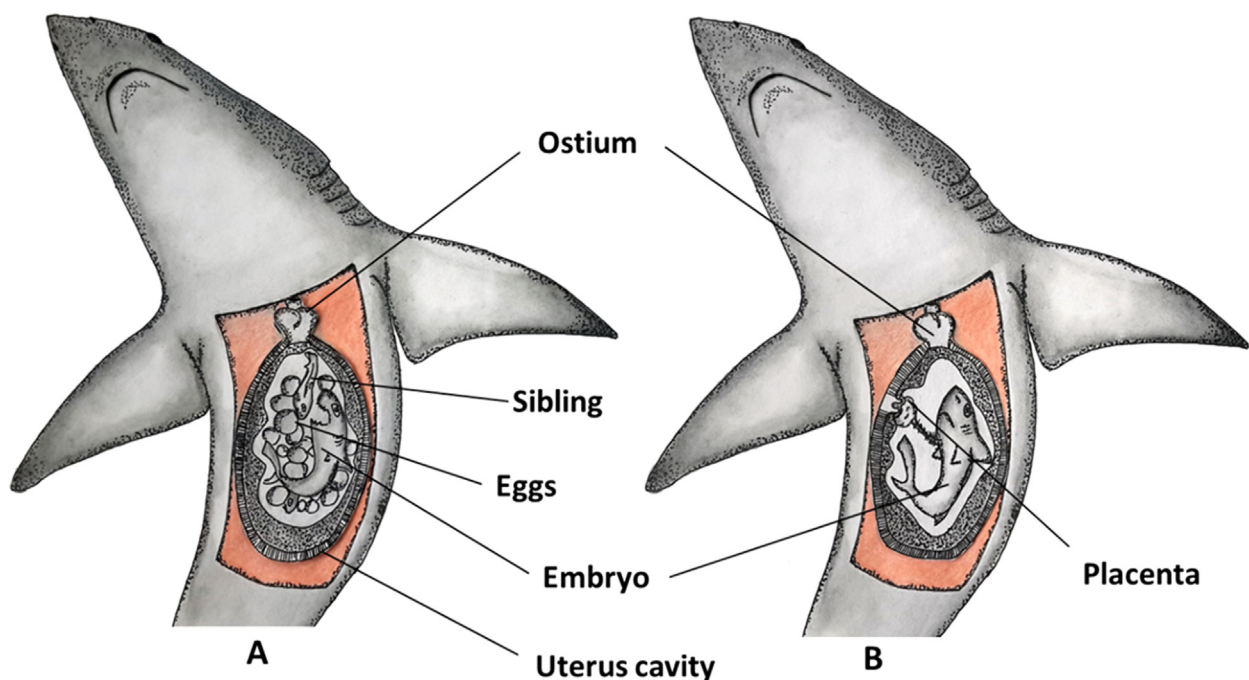


Fig. 3 Matrotrophic reproductive mode. (A) Adelphophagy. (B) Placentatroph. Illustrated by M.C. Sueiro.

Placentotrophy: This reproductive strategy occurs only in sharks. Generally involves a 12-month gestation period. During the first months of gestation, each embryo is enclosed in a separate thin and translucent capsule feeding the yolk-sac. Once the yolk is consumed, the embryo shift to an uterine milk (histotroph) source of nutrition. At the same time, the uterus compartmentalized surrounding each capsule/embryo while the sac (from the yolk-sac) modifies to form a placenta-like connection with the maternal uterus. The embryo will receive maternal nutrients until the end of the gestation through this placental structure (Fig. 3(B)).

Reproductive Cycles

A reproductive cycle is defined as a series of events that an organism must undergo in order to successfully reproduce. These events are regulated by physiological, genetics and environmental factors with the ultimately goal to produce a surviving progeny. In chondrichthyan females, these events are the ovarian cycle (follicle development for ovulation); mating; embryo gestation and parturition in viviparous species and egg case formation, retention and oviposition in oviparous species; and a possible final resting period preceding a new ovulatory cycle.

According with the timing of the reproductive cycles, chondrichthyan females are categorized in two main categories: (1) continuous breeders and (2) seasonal breeders.

Continuous Breeders: In this category, species can be: (i) reproductively active throughout the year or (ii) reproductively active throughout the year, but with seasonal periods where a greater proportion of reproductive activity occurs. Continuous breeders include both oviparous and viviparous species. For example, while the oviparous small-eyed catshark, *Apristurus microps*, lays eggs all year round (Ebert *et al.*, 2006), the draughtboard shark, *Cephalloscyllium laticeps*, lays eggs in pairs at monthly intervals throughout the year but shows a peak in egg deposition between January through June (Awruch *et al.*, 2009).

In viviparous continuous breeders, pregnancy lasts nearly a full year, with mating and the subsequent pregnancy ensuing soon after parturition. For example, the Pacific angel shark, *Squatina californica*, displays an annual reproductive cycle with a 9–12 months gestation period (Natanson and Cailliet, 1986), and while the spiny dogfish, *Squalus acanthias*, does not have an annual cycle, still display a continuous biannual cycle with a 22-month gestation period and mating occurring every two years (Chatzisprou and Megalofonou, 2005).

Seasonal breeders: As with continuous breeders, seasonal breeders comprise oviparous and viviparous species. In this type of reproductive cycle, species can be divided as: (1) species with a well-defined seasonal cycle, where animals are laying eggs/pregnant for only a portion of the year, and (2) species that are pregnant for approximately a full year, after which they spend a year or two non-pregnant. For example, in the oviparous epaulette shark, *Hemiscyllium ocellatum*, (Heupel *et al.*, 1999) the egg laying season occurs during a 6-month period of the year, while in the Port Jackson shark, *Heterodontus portusjacksoni*, the season only last 1–2 months of the year (Powter and Gladstone, 2008). In viviparous species such as the Atlantic stingray, *Dasyatis sabina*, the pregnancy last 5–6 months within a year cycle (Snelson *et al.*, 1997), while in the sandbar shark, *Carcharhinus plumbeus*, pregnancy last one year followed by one year of non-pregnancy, thus the reproductive cycle last two years (McAuley *et al.*, 2007).

Ovulatory cycles: The ovulatory cycle in chondrichthyans is defined as the period that involves follicle development (the accumulation of yolk by each follicle until reaching ovulation size), ovulation and a possible resting/dormant phase preceding the new follicle cycle.

In oviparous species, the events of ovulation, egg formation and encapsulation last 24–48 h. Thus, follicle development runs continuously throughout the year in oviparous species with a continuous reproductive cycle. While in oviparous seasonal species, follicle cycles occur between the egg production/laying periods with a possible resting/dormant phase in between. As these periods could last weeks or months, later stages of follicle development occurs in parallel with egg production during the laying period. After the last oviposition of the season, females can reach a dormant/resting phase that can last for weeks or several months preceding the new follicle development cycle (Fig. 4).

In viviparous continuous breeders, a significant proportion of follicle development for the subsequent pregnancy occurs concomitant with gestation, thereby allowing ovulation to occur soon after parturition. In species with seasonal cycles, follicle development is temporally separated from pregnancy and occurs well after parturition, allowing females to have a dormant/resting phase immediately after parturition before starting the new follicle development and gestation cycle.

Mating and parthenogenesis: The whole point of mating in chondrichthyan species is the successful deposition of semen by the clasper inside the female's reproductive tract producing offspring that share similar traits with their parents but are genetically diverse. This genetic diversity will increase the progeny fitness, and thus, the species survival throughout generations. After mating, sperm storage has been frequently reported in chondrichthyans. Female sperm storage is an important phase in the reproductive process enabling mating, ovulation, and fertilization to occur at different times. The time of sperm storage varies between species and can be as short as 5–6 weeks to, at least, more than two years (Pratt and Carrier, 2005).

In recent years, parthenogenesis, a form of asexual reproduction in which the production of offspring occurs in the absence of fertilization has been confirmed in increasing number of elasmobranchs. Females produce unfertilized eggs, with any male genetic contribution, that will develop into viable embryos. In the last 10 years, facultative parthenogenesis, the occurrence of asexual reproduction in otherwise sexually producing species, has been confirmed in six elasmobranch species. For example: the hammerhead shark *Sphyrna tiburo* (Chapman *et al.*, 2007) the whitetip reef shark *Triaenodon obesus* (Portnoy *et al.*, 2014) and very recently in the zebra shark *Stegostoma fasciatum* (Dudgeon *et al.*, 2017).

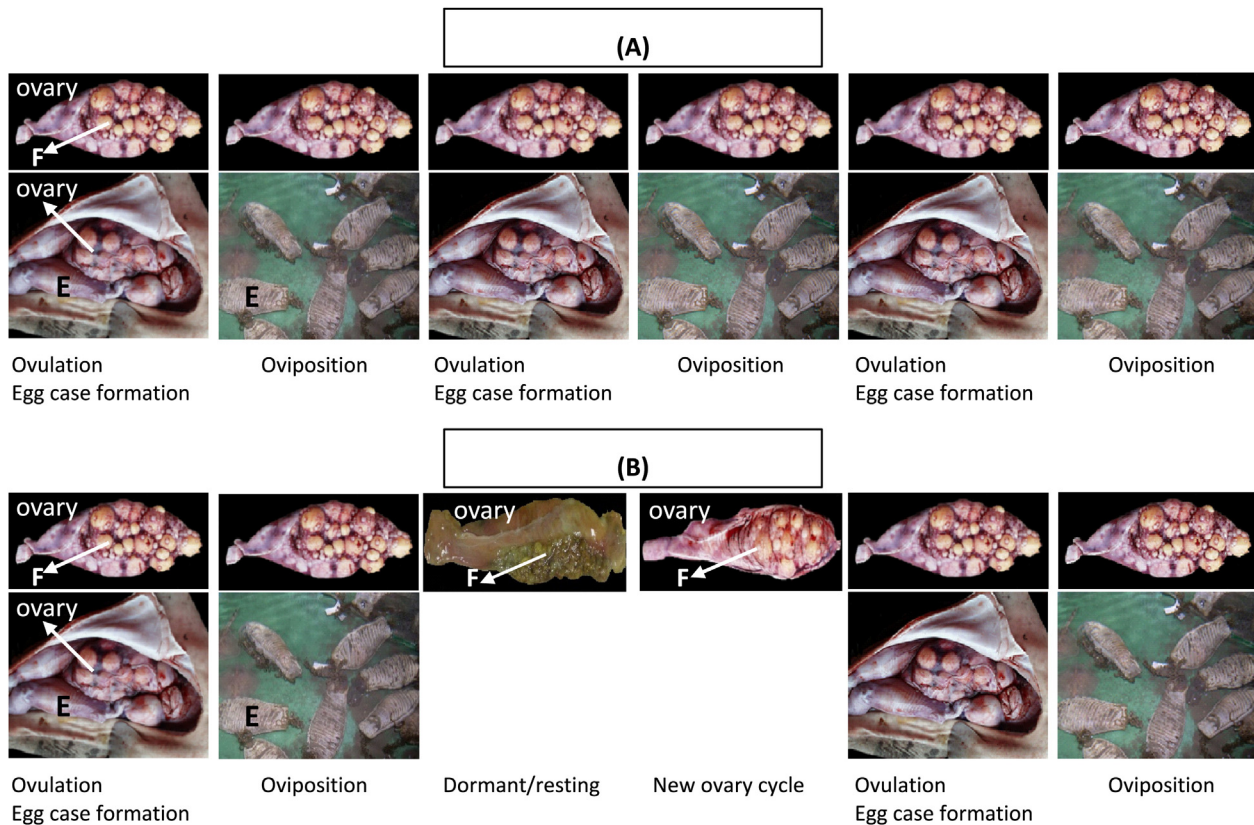


Fig. 4 Ovulatory cycle in oviparous chondrichthyans. (A) Continuous ovulatory cycle: Follicle development runs parallel with ovulation, capsule formation and oviposition. Follicles in all stage of development are present in the ovary. Follicles full of yolk ready to be ovulated are present throughout the year (top line photos). Recently formed egg cases can be distinguished inside the uterus (bottom line photos). (B) Seasonal ovulatory cycle: Follicle development runs in parallel with encapsulation only during the egg-laying period. The dormant/resting stage is characterized by the ovary carrying very small undeveloped follicles. E, Egg case; F, Follicle. Photos by C.A. Awruch.

While increasing cases of parthenogenesis have been reported in elasmobranchs, the benefits of this reproductive strategy remains unknown. Although, the ability for females to reproduce through facultative parthenogenesis would be selectively advantageous in situations where females have no other available options for passing on genes (e.g. absence of males), very little is known about the long-term viability of parthenogenesis where a reduction in genomic diversity could reduce the progeny fitness. A challenge for understanding the adaptive nature of facultative parthenogenesis is identifying the internal and external conditions that may trigger this, still quite unusual, reproductive strategy in elasmobranchs.

References

- Awruch, C. A., Pankhurst, N. W., Frusher, S. D., & Stevens, J. D. (2009). Reproductive seasonality and embryo development in the draughtboard shark *Cephaloscyllium laticeps*. *Marine and Freshwater Research*, 60(12), 1265–1272.
- Castro, J. I., Sato, K., & Bodine, A. B. (2016). A novel mode of embryonic nutrition in the tiger shark, *Galeocerdo cuvier*. *Marine Biology Research*, 12(2), 200–205.
- Chapman, D. D., Shivji, M. S., Louis, E., et al. (2007). Virgin birth in a hammerhead shark. *Biology Letters*, 3(4), 425–427.
- Chatzisprou, A., & Megalofonou, P. (2005). Sexual maturity, fecundity and embryonic development of the spiny dogfish, *Squalus acanthias*, in the eastern Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom*, 85(5), 1155–1161.
- Compagno, L. J. V. (1990). Alternative life-history styles of cartilaginous fishes in time and space. *Environmental Biology of Fishes*, 28, 33–75.
- Dudgeon, C. L., Coulton, L., Bone, R., Ovenden, J. R., & Thomas, S. (2017). Switch from sexual to parthenogenetic reproduction in a zebra shark. *Scientific Reports*, 7, 40537.
- Dulvy, N. K., & Reynolds, J. D. (1997). Evolutionary transition among egg-laying, live-bearing and maternal inputs in sharks and rays. *Proceedings of the Royal Society of London, B*, 264, 1309–1315.
- Ebert, D. A., Compagno, L. J. V., & Cowley, P. D. (2006). Reproductive biology of catsharks (Chondrichthyes: Scylliorhinidae) off the west coast of southern Africa. *ICES Journal of Marine Science*, 63(6), 1053–1065.
- Gilmore, R. G., Putz, O. J., & Dodrill, J. W. (2005). Oophagy, intrauterine cannibalism and reproductive strategy in lamnoid sharks. In W. Hamlett (Ed.), *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras* (pp. 435–462). Enfield (NH), USA: Science Publisher Inc.
- Grogan, E. D., & Lund, R. (2011). Superfoetate viviparity in a Carboniferous chondrichthyan and reproduction in early gnathostomes. *Zoological Journal of the Linnean Society*, 161(3), 587–594.
- Hamlett, W. C., & Koob, T. J. (1999). Female reproductive system. In W. Hamlett (Ed.), *Sharks, Skates, and Rays. The Biology of Elasmobranch Fishes* (pp. 398–443). Baltimore, London: The Johns Hopkins University press.

- Heupel, M. R., Whittier, J. M., & Bennett, M. B. (1999). Plasma steroid hormone profiles and reproductive biology of the Epauvette shark, *Hemiscyllium ocellatum*. *Journal of Experimental Zoology*, 284, 586–594.
- Jacoby, D. M. P., Croft, D. P., & Sims, D. W. (2012). Social behaviour in sharks and rays: Analysis, patterns and implications for conservation. *Fish and Fisheries*, 13(4), 399–417.
- Joung, S.-J., Chen, C.-T., Clark, E., Uchida, S., & Huang, W. P. (1996). The whale shark, *Rhincodon typus*, is a livebearer: 300 embryos found in one 'megamamma' supreme. *Environmental Biology of Fishes*, 46(3), 219–223.
- McAuley, R. B., Simpfendorfer, C. A., Hyndes, G. A., & Lenanton, R. C. J. (2007). Distribution and reproductive biology of the sandbar shark, *Carcharhinus plumbeus* (Nardo), in Western Australian waters. *Marine and Freshwater Research*, 58(1), 116–126.
- Natanson, L. J., & Cailliet, G. M. (1986). Reproduction and development of the Pacific angel shark, *Squatina californica*, off Santa Barbara, California. *Copeia*, 4, 987–994.
- Portnoy, D. S., Hollenbeck, C. M., Johnston, J. S., Casman, H. M., & Gold, J. R. (2014). Parthenogenesis in a whitetip reef shark *Triaenodon obesus* involves a reduction in ploidy. *Journal of Fish Biology*, 85(2), 502–508.
- Powter, D. M., & Gladstone, W. (2008). The reproductive biology and ecology of the Port Jackson shark *Heterodontus portusjacksoni* in the coastal waters of eastern Australia. *Journal of Fish Biology*, 72(10), 2615–2633.
- Pratt, H. L., & Carrier, J. C. (2005). Elasmobranch courtship and mating behavior. In W. C. Hamlett (Ed.), *Reproductive Biology and Phylogeny of Chondrichthyes* (pp. 129–170). Enfield NH: Science Publisher.
- Snelson, J. F. F., Johnson, M. R., Rasmussen, L. E. L., & Hess, D. L. (1997). Serum concentrations of steroids hormones during reproduction in the atlantic stingray, *Dasyatis sabina*. *General and Comparative Endocrinology*, 108, 67–79.
- Sunye, P. S., & Vooren, C. M. (1997). On cloacal gestation in angel sharks from southern Brazil. *Journal of Fish Biology*, 50, 86–94.

Further Reading

- Awruch, C. A. (2013). Reproductive Endocrinology in Chondrichthyes: The Present and the Future. *General and Comparative Endocrinology*, 192, 60–70.
- Awruch, C. A. (2015). Reproduction strategies. In R. E. Shadwick, A. P. Farrell, & C. J. Brauner (Eds.), *Physiology of Elasmobranch Fishes: Structure and Interaction with Environment*, 344 pp. 256–295. London: Elsevier.
- Gelsleichter, J., & Evans, A. N. (2012). Hormonal regulation of Elasmobranch physiology. In J. C. Carrier, J. A. Musick, & M. R. Heithaus (Eds.), *Biology of Sharks and their Relatives* (pp. 313–348). Boca Raton, FL: CTC Press Taylor and Francis Group.
- Hamlett, W. C. (Ed.). (2005). *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids and Chimaeras*. Enfield (NH), USA: Science Publisher Inc.
- Waltrick, D. S., Simpfendorfer, C., & Awruch, C. A. (2017). A review on the morphology of ovarian follicles in elasmobranchs: A case study in *Rhizoprionodon taylori*. *Journal of Morphology*, 278, 486–499.
- Wourms, J. P. (1981). Viviparity: The maternal-fetal relationship in fishes. *American Zoologist*, 21, 473–515.
- Wourms, J. P., et al. (1988). The maternal-embryonic relationship in viviparous fishes. In W. S. Hoar, & D. J. Randall (Eds.), *Fish Physiology*, XI pp. 1–134. United Kingdom: Academic Press Inc.
- Wyffels, J. T. (2009). Embryonic development of Chondrichthyan fishes – A review. In Y. W. Kunz, C. A. Luer, & B. G. Kapoor (Eds.), *Development of Non-Teleost Fishes* (pp. 1–103). Enfield: Science Publishers.

Relevant Websites

- <http://elasma.org/>. – American Elasmobranch Society.
- <http://www.eulasma.org/>. – European Elasmobranch Association.
- <http://www.oceanisharks.org.au/>. – Oceania Chondrichthyan Society.

Reproduction in Osteichthyes

Vance L. Trudeau, University of Ottawa, Ottawa, ON, Canada

© 2018 Elsevier Inc. All rights reserved.

Glossary

Gonoduct A pair of tubes that comprise the female reproductive tract that extends from their anterior, funnel-like openings to the cloaca.

Gonochoresis Having distinct female with ovaries and distinct male with testes in a given species.

Polygamy A mating system in which one sex mates with several partners.

Protandry When an individual starts as a sexually mature male, producing sperm, but at some time in its life it becomes a functional female.

Protogyny When an individual starts as a female, spawning eggs, but then later changes into a functional male, producing spermatozoa.

Sequential hermaphrodite A fish that first develops as one sex, but changes to the opposite sex at some point in life.

Introduction

The fishes are the most diverse group of vertebrates. Currently, >33,000 species are recognized in the online resource FishBase, which is close to 50% of all known vertebrate species. Of these, the Teleostei (teleosts), or the modern bony fishes are the majority and are represented by ~27,000 species. Osteichthyes is a taxonomic class of fish that includes the ray-finned fish (subclass or class Actinopterygii) and lobe-finned fish (subclass or class Sarcopterygii). Recent phylogenetic analysis is leading to reclassification of many fishes, and places the origin of Sarcopterygii and Actinopterygii in the Middle Silurian, with the sarcopterygian crown group evolving in the Early Devonian (~409 million years ago) and the actinopterygian crown group evolving at the Middle-Late boundary of the Devonian (~383 million years ago), both of which correspond to the Age of Fishes (Betancur *et al.*, 2017). The reader can consult the newest classification of bony fishes in Betancur *et al.* (2017) which is open-access and online (Fig. 1 is especially relevant). The living sarcopterygians are two species of coelacanth and six species of lungfish. There are clear evolutionary relationships with lungfishes and tetrapods, especially amphibians, but there remains considerable debate amongst evolutionary biologists as to the relationship relationships among lungfishes, coelacanth, and tetrapods. Actinopterygii can be broadly divided into the Neopterygii and the Chondrostei. According to Nelson (2006), the Neopterygii includes the typical bony fishes, the Teleostei as well as Amiiformes (bowfin) and Lepisosteiformes (gars). Given that the teleosts are the largest group, represented by many commercially important species, and that the vast majority of our information on fish reproduction comes from the study of these species, most of this article is focused on them. The Chondrostei include Acipenseriformes or the sturgeons, and Polypteriformes or the bichirs.

The Cyprinidae (carps and minnows) and the Gobiidae (many are bottom-dwelling gobies), are teleost and represent the largest vertebrate families in terms of number of species (Wootton and Smith, 2015). The vast array of colors and body forms, and reproductive strategies (Table 1) exhibited by fish attracts considerable attention from evolutionary biologists, zoologists, and those interested in fish culture (aquaculture and hobby aquaria). Sex determination is the process by which the sex of an individual is established early in development, be it genetically or environmentally. Many teleosts have genetic sex determination, meaning that female or male development is determined by a gene or a complex series of genes. Genetic sex determination is the typical condition in vertebrates, for example, the well-known X and Y chromosome system in most mammals, including humans. On the other hand, a good number of teleosts exhibit environmental sex determination, where factors such as temperature change promotes the development of one sex or another. This phenomenon, known as temperature-dependent sex determination, was first reported in *Menidia menidia* (Table 1), a species belonging to the family Atherinopsidae (Blázquez and Somoza, 2010). The pejerrey, *Odontesthes bonariensis*, is an Argentine silverside in the same family with strong temperature-dependent sex determination. In this species, sex ratios reach 100% female or 100% male at environmentally relevant temperatures of 17 and 29°C, respectively. Recently, the coexistence of genetic and temperature-dependent sex determination has been demonstrated in this pejerrey (Yamamoto *et al.*, 2014). This indicates that the environment can change the genotypic sex of the fish, leading to the existence of genotypic males but phenotypic females and, vice versa. However, the evolutionary significance of this is still under debate.

By far, the bony fishes exhibit the greatest diversity of reproductive patterns among the vertebrates (Denski, 1987; Devlin and Nagahama, 2002). Most species are gonochoristic meaning that they have the familiar distinct female with ovaries and male with testes (Fig. 1), forming monogamous pairs at spawning time. In one unusual example, the deep-sea anglerfish, the very small male attaches himself (fusion of his jaw to the female's body) to the large female who probably takes over control of his reproductive system (Denski, 1987). There are teleosts that are sequential hermaphrodites, starting off as one sex, and changing permanently to the other sex. In the case of protogyny, the animal is first female, then transforms into a fertile male. For protandry, the animal is first male and then transforms into a female. Although far less common than the previously mentioned modes of reproduction, there are also simultaneous hermaphrodites where individuals trade sperm and eggs during spawning, or where an individual can self-fertilize under natural breeding conditions. Given the dazzling variety of these fishes, it is impossible to cover all aspects.

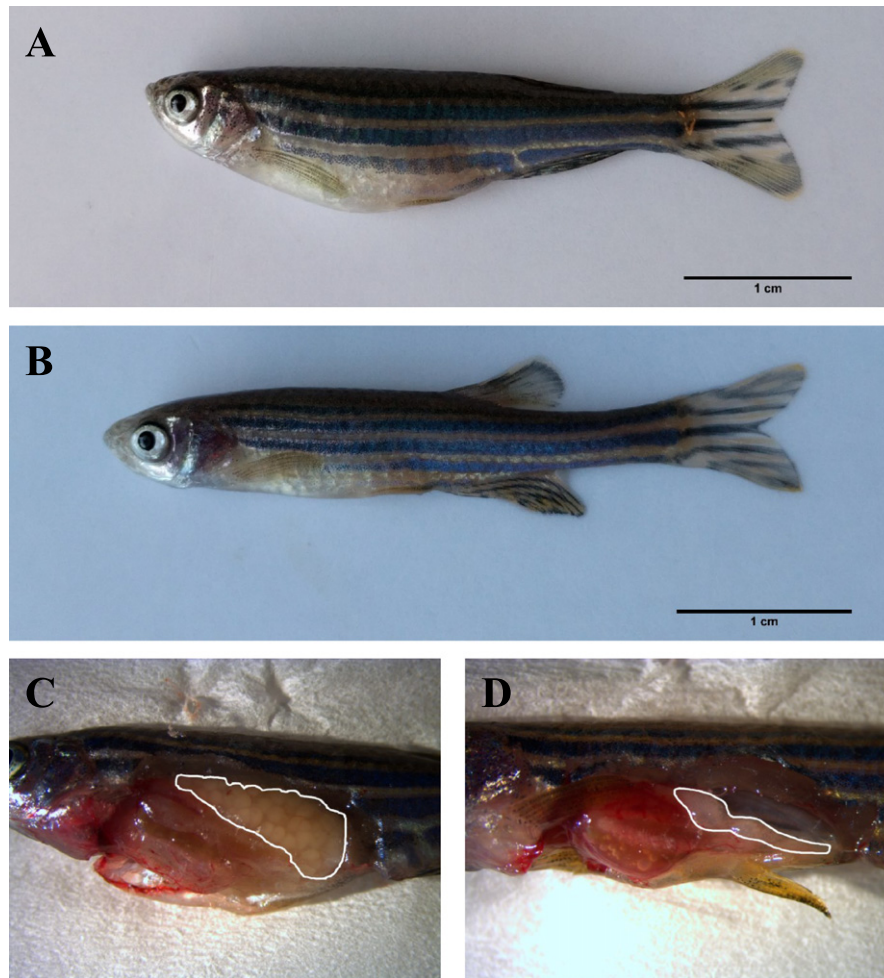


Fig. 1 The zebrafish *Danio rerio* (family *Cyprinidae*) as an example of a gonochoristic teleost. Zebrafish, also known as the zebra danio, originates from Pakistan, India, Bangladesh, Nepal and Myanmar. It is a common aquarium species, and is an important model species for scientific research on the mechanisms of development and reproduction in vertebrates. A: Sexually mature adult female. B: Sexually mature adult male. C: Dissection of the sexually mature adult female showing the large ovary outlined with the white line. D: Dissection of the sexually mature adult male showing the testis outlined with the white line. Photo credit: Mitchell K., Benson, P., Trudeau, V.L. (July 2017).

Therefore, the main objective of this article is to provide a brief overview of reproduction in bony fishes. The reader is also directed to Wootton and Smith's book titled *Reproductive Biology of Teleost Fishes* (2015), which is an excellent and comprehensive resource, one upon which this short overview is based.

Gender Systems

About 90% of Osteichthyes have a distinct female with ovaries and distinct male with testes. This is known as gonochorism. Following the early stages of sex determination, a gonochoristic fish develops either testes or ovaries, and after puberty they reach maturation. They have the ability to produce fertile sperm and eggs, and spawn to give rise to the next generations. The time to reach sexual maturation is highly variable amongst the various teleost species; for example, the fastest known is the goby *Schindleria praematura* from Okinawa, Japan, maturing in less than 2 months, which can be contrasted to European eels (*Anguilla anguilla*), which can be blocked in a pre-pubertal state for many years until environmental cues activate secretion of reproductive hormones, and then the eels migrate to their ocean spawning site (Dufour *et al.*, 2003). The white sturgeon *Acipenser transmontanus* from western North American can live to over 100 years old. They grow slowly and are not ready to spawn until the females are over 18 years of age and males are at least 14 years of age.

One usually associates the word hermaphrodite with ambiguous sex organs in humans, although nowadays a more acceptable term is the intersex person. Historically, the word hermaphrodite derives from the Latin: *hermaphroditus*, and from Ancient Greek *hermaphroditos* which itself comes from *Hermaphroditus*, the son of Hermes and Aphrodite in Greek mythology. According to the story *Hermaphroditus* fused with the nymph *Salmacis*, giving rise to one individual possessing physical traits of

Table 1 Sex and mating strategies in bony fishes

Sexuality or mating system	Example
1. Sex determination	
(a) Genetic	Most teleosts
(b) Environmental	Atlantic silverside, <i>Menidia menidia</i>
2. Gender systems	
(a) Gonochorism	Most teleosts
(b) Hermaphroditism	
(i) Simultaneous (synchronous)	
Self-fertilizing	Mangrove rivulus, <i>Kryptolebias marmoratus</i>
Outcrossing	Barred Seabass, <i>Serranus fasciatus</i>
(ii) Sequential	
Protogynous	Blue-headed wrasse, <i>Thalassoma bifasciatum</i>
Protandrous	Red and Black Anemonefish, <i>Amphiprion melanopus</i>
Serial (bidirectional)	Rippled Coral Goby, <i>Gobiodon rivulatus</i>
3. Modes of fertilization	
(a) External	Most teleosts
(b) Internal	Common guppy, <i>Poecilia reticulata</i>
(c) Buccal	Tilapia, <i>Oreochromis niloticus</i>
4. Mating systems	
(a) Promiscuity	Atlantic herring, <i>Clupea harengus</i>
(b) Polygamy	
(i) Polygyny	Tilapia, <i>Oreochromis niloticus</i>
(ii) Polyandry	Common guppy, <i>Poecilia reticulata</i>
(iii) Polygynandry	European bitterling, <i>Rhodeus amarus</i>
(c) Monogamy	Convict cichlid, <i>Amatitlania nigrofasciata</i>
5. Secondary sexual characteristics	
(a) Monomorphism	Atlantic herring, <i>Clupea harengus harengus</i>
(b) Sexual dimorphism	
(i) Permanent dimorphism	Siamese fighting fish, <i>Betta splendens</i>
(ii) Temporary dimorphism	Common goldfish, <i>Carassius auratus</i>
6. Spawning site preparation	
(a) No preparation	Eurasian minnow, <i>Phoxinus phoxinus</i>
(b) Site prepared, but not defended	Atlantic salmon, <i>Salmo salar</i>
(c) Site prepared, and defended	Three-spined stickleback, <i>Gasterosteus aculeatus</i>
7. Parental care	
(a) No care	Most teleosts
(b) Paternal care	Three-spined stickleback, <i>Gasterosteus aculeatus</i>
(c) Maternal care	
(i) Oviparity	Tilapia, <i>Oreochromis niloticus</i>
(ii) Viviparity	Common guppy, <i>Poecilia reticulata</i>
(d) Biparental care	Convict cichlid, <i>Amatitlania nigrofasciata</i>
(e) Juvenile helpers	Kambwimba or daffodil cichlid, <i>Neolamprologus pulcher</i>

Source: Modified from Wootton, R.J., Smith, C., 2015. Reproductive Biology of Teleost Fishes. Chichester: John Wiley & Sons. Common names are from either www.fishbase.org or <http://eol.org/>.

both a man and a woman. In a few fishes, the condition of hermaphroditism is a successful reproductive strategy. An unusual case is simultaneous (synchronous) hermaphroditism in the mangrove rivulus, *Kryptolebias marmoratus*, common along the coastal regions of Florida, the Caribbean and the eastern coasts of Central and South America. Mangrove rivulus exist primarily as hermaphrodites with preferential internal self-fertilization but the species exhibits environmental sex determination, with primary males developing from embryos incubated at low temperatures (18–20°C; Harrington, 1961).

Far more common are sequential hermaphrodites, especially in many coral reef fishes (Wootton and Smith, 2015). Some species with simultaneous hermaphroditism do outcross, mating with other individuals. Another unusual case is the barred seabass, *Serranus fasciatus* (Petersen, 1990). All individuals start life as simultaneous hermaphrodites, but the largest individuals in any location lose femaleness and change to become functional males. These males guard territories containing 1–8 hermaphrodites that they defend against other males. Barred seabass males pair-spawn with hermaphrodites within their harem (Petersen, 1990).

Sex change is documented in about 2% of teleost species spanning over 20 families (Liu et al., 2017). Such functional sex changes generally follow one of three patterns (Table 1): protogynous (female-to-male), protandrous (male-to-female), and sequentially bi-directional. Protogyny is when an individual starts as a female, spawning eggs, but then later changes into a functional male, producing spermatozoa. Protandry on the other hand describes the situation in which an individual starts as a sexually mature male, producing sperm, but at some time in its life it becomes a functional female.

A well-known example of a protogynous species is the blue-headed wrasse, *Thalassoma bifasciatum*, in the Gulf of Mexico and Caribbean regions. In this species, social conditions control the sexual transformation. In the blue-headed wrasse, one territorial terminal-phase male dominates a number of lower-ranking females. When the dominant male disappears (e.g., fishing, predation, etc.), one of the largest females in the area takes over and changes its sex to male. This is a rapid change in reproductive behavior, gonadal anatomy, and external body morphology. Please consult figure 1 in the research article by Liu *et al.* (2017), where this dramatic change is well-depicted and explained. In this wrasse, the first sign (minutes to hours) of the impending sex change is the temporary display of spawning colors (a bluish head and darkening of fin tips) and expression of terminal phase male-typical behaviors of aggression and courtship. Tissues of the ovary of this new dominant female start to disappear within 3 days, while development of the testes and external colors typical of sexually active males start by days 4–6. Sex changers can have functional testes filled with sperm in 8–10 days and the whole process is usually complete within 3 weeks (Liu *et al.*, 2017).

A recognizable protandrous fish is the Red and Black Anemonefish, *Amphiprion melanopus*, made famous in the 2003 Walt Disney film *Finding Nemo* (A. Stanton and L. Unkrich, directors). The anemonefishes form a specialized subfamily, the Amphiprioninae, within the damselfishes (Pomacentridae) and they are famous for their symbiotic relationship with large tropical sea anemones in the Western Pacific. This unusual association has produced a mating system unique among tropical reef fishes. Sex is controlled socially in *Amphiprion melanopus* (Godwin, 1994). Females are always the largest and the behaviorally dominant individuals in a group and disappearance or removal of the female allows sex change to proceed in her mate, a mature male that will become the new female. The dominant sub-adult individual in a group then matures and takes over the male breeding role. Detailed histological studies have shown that complete transition from male to female function in *Amphiprion melanopus* takes place in between 45 and 100 days. This seems to be true for the other species of *Amphiprion* (Godwin, 1994). In some serially changing species, the switch between male and female can take place more than once in a lifetime to favor reproductive success. The Rippled Coral Goby, *Gobiodon rivulatus*, from the Indo-West Pacific region is a good example. Males take care of eggs deposited on coral branches.

Modes of Fertilization

The most common mode of fertilization in teleost fishes is external. After courtship and sexual displays coordinated by environmental factors and sex pheromones, female and male release eggs and sperm for rapid fertilization. The biology of eggs and sperm is variable between fish species and the processes leading up to fertilization are often very different compared to mammals. For example, salmon and trout eggs are very large relative to sperm, and eggs are fertilized after the first sperm cell enters a single opening (the micropyle) in the egg membrane which is barely wide enough to allow entry of one sperm cell (Hoysak and Liley, 2001). Another notable characteristic of salmonid reproduction is the limited lifespan of the eggs and sperm. Hoysak and Liley (2001) studied this under experimental conditions in the sockeye salmon *Oncorhynchus nerka*, a well-known commercial fish species of the West coast of North America. In sockeye salmon, eggs had a very limited longevity after being immersed in water, with a large drop in fertility occurring after 20 s of exposure to water. Sperm had an even shorter period of fertility. They maintained high fertility for 10 s after being exposed to water but fertility dropped sharply after that. The fertilization of an egg by a sperm occurs very rapidly where 80% fertilization success was achieved with 5 s of sperm-egg mixing. In this example, the brief window of time during which eggs remain viable make position and timing critical for male reproductive success under the intense conditions of sperm competition in sockeye salmon (Hoysak and Liley, 2001) and other externally fertilizing fishes.

According to Wootton and Smith (2015) fertilization takes place internally in the female in about 500–600 teleost species. The male introduces the sperm into the gonoduct of the female, using a specialized intromittent organ. This is analogous to the mode of fertilization seen in the cartilaginous fishes, the Chondrichthyes, and in mammals and a few other vertebrates. In the guppy, *Poecilia reticulata* and the related poeciliid, the green swordtail *Xiphophorus hellerii*, common as aquarium fishes, the anal fin in males is shaped into a grooved, rod-shaped organ called a gonopodium used to deliver sperm to females (Van Tienhoven, 1983). The size and shape of the gonopodium reveals information about evolution and classification of the Poeciliidae. The coelacanth *Latimeria chalumnae* is ovoviviparous. There is internal fertilization, and the fetuses are retained within the mother until they have grown large enough to fend for themselves. There is no obvious copulatory organ in the male, so much remains to understand about reproduction in coelacanths. The gestation period has been estimated to be around 13 months.

The Mozambique tilapia, *Oreochromis mossambicus*, native to Africa, is a widely aquacultured species that is also highly invasive in some countries. Males construct nests in sparse vegetated areas of lakes where fertilization of the eggs takes place (Bruton and Bolt, 1975). Several different females may lay eggs in the nest. Males are generally aggressive and ritualistic during reproductive season. Once fertilized, the female takes the eggs into her mouth and broods them until hatching in about 3–5 days.

Diversity in Mating Systems

Promiscuity is common in Osteichthyes. The eggs of a female are potentially fertilized by several males, with minimal selection of partners. Alternatively, in some species males may potentially fertilize the eggs of several females. Breeding aggregations of promiscuous spawners can make some fish species particularly vulnerable to fishing. The pelagic Atlantic herring, *Clupea harengus harengus* is commercially important and one of the most abundant fish species on earth, gathering in large schools. The species migrates for feeding, spawning, and over-wintering purposes and are found on both sides of the Atlantic.

Polygamy generally refers to a mating system in which one sex mates with several con-specific partners, but with the same mate at each spawning. The Mozambique tilapia is an example of a polygynous species where males mate with several females, but each female spawns with only one male. Male tilapia perform site selection and nest building (Bruton and Bolt, 1975). They form dense aggregations of territories in which they dig and defend nest pits (Bruton and Bolt, 1975). Ripe females visit the breeding arenas and when male courtship is successful, enter the pits to spawn. The size of male spawning pits is positively correlated with male status (Oliveira and Canário, 2000), and may serve to attract desirable females for spawning. Female Mozambique tilapia may gain a direct benefit from choosing to spawn with a male with a larger pit, which could be associated with a reduced likelihood of interference and cannibalism (Oliveira and Canário, 2000).

Females in polyandrous species mate with several males, but each male spawns with only a single female. The guppy *Poecilia reticulata* is a highly polyandrous live-bearing fish in which males transfer sperm internally to females via consensual and forced mating. Guppies exhibit some of the highest recorded rates of multiple paternity in any vertebrate (Neff et al., 2008).

A form of promiscuity with the possibility of mate selection is polygynandry (Wootton and Smith, 2015). A male will spawn with several females and a female with several males over the course of a breeding season, but with mate choice operating. The European bitterling *Rhodeus sericeus* is a freshwater polygynandrous cyprinid with an unusual relationship with mussels (Smith et al., 2004). During the spawning season, males develop bright breeding colors and defend territories around mussels. Female bitterlings develop long ovipositors that they use to place their eggs onto the gills of a mussel through the mussel's exhalant siphon. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel, so that water filtered by the mussel carries the sperm to the eggs. Developing embryos live inside the mussel for 1 month as they develop into swimming larvae (Smith et al., 2004).

In monogamy, a single male and female form a mating pair and show some degree of bonding with the mate (Wootton and Smith, 2015). This type of mating system is not common in bony fishes but is associated with species in which parental care follows the fertilization of eggs. The pair bond may be long-lasting or more temporary, only persisting for a single breeding attempt. The convict cichlid, *Amatitlania nigrofasciata*, is a monogamous South American cichlid, perhaps not typical of many other cichlids that exhibit diverse breeding behaviors.

Secondary Sexual Characteristics

In many fishes, the sexes differ in appearance. This could be permanent or a sex hormone-dependent, temporary difference only evident at the breeding season. Female and male Atlantic herring resemble each other and are therefore called monomorphic. Atlantic herring are polygynandrous and aggregate into massive schools in the late summer and early fall. In the western Atlantic, they move into coastal waters at various locations in the Gulf of Maine and offshore banks of Nova Scotia to spawn. An example of permanent sexual dimorphic fish is the Siamese fighting fish, *Betta splendens*, a type of gourami. The male is very colorful, with long, elegant fins. He builds a bubble-nest which is aggressively guarded. The male *Betta* is well-known to aquarists because of the dramatic fin displays. The female is less colorful with smaller fins. Many fish only display sexual dimorphism in the breeding periods; for example, male cyprinids such as the common goldfish, *Carassius auratus*, develop breeding tubercles on the pectoral fins and the operculum as sex hormone (androgens) levels increase for spring spawning. Breeding tubercles may function to maintain body contact between the sexes during spawning; in the defense of nests and territories; in the stimulation of females in courtship; and in some forms, perhaps in sex and species recognition. Breeding tubercles are found in 15 families of 4 orders; Salmoniformes, Gonorhynchiformes, Cypriniformes, and Perciformes.

Spawning Site Preparation

Many fish do not exhibit any spawning site preparation. For example, in the Eurasian minnow, *Phoxinus phoxinus*, males and females form schools separately and arrive on the spawning site at different times. They spawn over clean gravel areas in flowing water or on wave-washed shores of lakes. Females lay sticky eggs deep into clean gravel (Kottelat and Freyhof, 2007). Atlantic salmon, *Salmo salar* are a commercially important species with a complex life cycle in the wild. They breed in freshwater but grow and live in the ocean. They return to home rivers, entering freshwater between April and November. In late fall the wild Atlantic salmon spawn. The female digs a 10–30 cm deep nest called a redd in the gravel bottom of the stream. Her eggs and the milt from an adult male are released into the redd, the eggs are fertilized, the gravel replaced with additional tail thrusts. The classic example of complex site preparation is nest-building and defense by the three-spined sticklebacks *Gasterosteus aculeatus* (Fig. 2). In the breeding season, the male with the bright red throat collects grasses and constructs a nest by gluing together the plant material with the specialized protein spiggin secreted from the kidney under the control of androgenic testicular hormones.

Parental Care

Most Osteichthyes do not exhibit parental care, and abandon fertilized eggs once spawning is completed. The African lungfish *Protopterus annectens* spawn in the swamps during the wet season; they build nests in which the eggs, white in color and about



Fig. 2 Classic illustration of three-spined stickleback breeding by A.F. Lyndon. This is taken from *British Fresh Water Fishes*, Volume 1, December 31, 1878 by Houghton, William, 1828–95, Lydon, Alexander Francis, 1836–1917 and is in the public domain.

4 mm diameter, are laid; the young are cared for by the males. The larvae hatch in 8 days, and leave the nest in twenty days. The male three-spined stickleback serves as a good example of paternal care of eggs in a nest (Fig. 2). In maternal care, the female takes care of the developing eggs. The female Mozambique tilapia, *Oreochromis mossambicus* takes eggs into her mouth and broods them until hatching, then continues to mouth-brood the fry until they are approximately 2–3 weeks old. A second form of maternal care is seen in viviparous species in which the eggs are fertilized and develop within the ovaries of the female. The classic examples of this are the viviparous live-bearing species in the family Poeciliidae. These are the tooth-carps, and include well-known aquarium fish such as the Common guppy, *Poecilia reticulata*, sailfin molly, *Poecilia latipinna*, and green swordtail *Xiphophorus hellerii*. Convict cichlid couples on the other hand, jointly care for their eggs, newly-hatched free embryos and free-swimming juveniles for four to six weeks (Wisenden, 1995). Eggs are laid on the ceiling of small caves which the pair have previously prepared by excavating sand from under large stones. Both parents guard the young, aggressively chasing away potential brood predators. The daffodil cichlid *Neolamprologus pulcher* is a cooperatively breeding cichlid endemic to Lake Tanganyika in East Africa. They live in social groups consisting of a dominant breeding pair and up to 14 related and unrelated helpers (Balshine *et al.*, 2001). Helpers clean and fan eggs in the breeding shelter and help to defend the territory and other group members against predators and intruding conspecifics.

Conclusions

Modes of reproduction are highly diverse in fish. Given the number of species it is difficult to classify reproductive patterns according to phylogeny. What is clear, however, is that both external and internal fertilization, complex sexual behaviors, and parental care have all evolved very early in the vertebrates, given the appearance of the first fishes approximately 400 million years ago. Much remains to be discovered in the majority of fish, and especially non-teleostean species.

Acknowledgements

Helpful comments and discussion with G. Somoza, K. Mitchell and F. Chapleau are acknowledged. The help of W. Zhang and P. Benson with tables and figures is acknowledged with appreciation.

References

- Balshine, S., Leach, B., Neat, F., *et al.*, 2001. Correlates of group size in a cooperatively breeding cichlid fish (*Neolamprologus pulcher*). *Behavioral Ecology and Sociobiology* 50, 134–140.
- Betancur, R.R., Wiley, E.O., Arratia, G., *et al.*, 2017. Phylogenetic classification of bony fishes. *BMC Evolutionary Biology* 6 (1), 162.
- Blázquez, M., Somoza, G., 2010. Fish with thermolabile sex determination (TSD) as models to study brain sex differentiation. *General and Comparative Endocrinology* 166, 470–477.
- Bruton, M.N., Bolt, R.E., 1975. Aspects of the biology of *Tilapia mossambica* Peters (Pisces: Cichlidae) in a natural freshwater lake (Lake Sibaya, South Africa). *Journal of Fish Biology* 7, 423–445.

- Demski, L.S., 1987. Diversity in reproductive patterns and behavior in teleosts. In: Crews, D. (Ed.), *Psychobiology of Reproductive Behavior: An Evolutionary Perspective*. Upper Saddle River, NJ: Prentice-Hall, pp. 1–27.
- Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological and environmental influences. *Aquaculture* 208, 191–364.
- Dufour, S., Burzawa-Gérard, E., Le Belle, N., Sbaili, M., Vidal, B., 2003. Reproductive endocrinology of the European Eel. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), *Anguilla anguilla*. Tokyo: Springer Verlag, pp. 373–383.
- Godwin, J., 1994. Historical aspects of protandrous sex change in the anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *Journal of Zoology* 232, 199–213.
- Harrington Jr., R.W., 1961. Oviparous hermaphroditic fish with internal self-fertilization. *Science* 134, 1749–1750.
- Hoysak, D.J., Liley, N.R., 2001. Fertilization dynamics in sockeye salmon and a comparison of sperm from alternative male phenotypes. *Journal of Fish Biology* 58, 1286–1300.
- Kottelat, M., Freyhof, J., 2007. In: Kottelat, M., Cornol, C.H., Freyhof, J. (Eds.), *Handbook of European Freshwater Fishes*. Berlin: Publications Kottelat, p. 646.
- Liu, H., Todd, E., Lokman, P., *et al.*, 2017. Sexual plasticity: A fishy tale. *Molecular Reproduction and Development* 84, 171–194.
- Neff, B., Pitcher, T., Ramnarine, I., 2008. Interpopulation variation in multiple paternity and reproductive skew in the guppy. *Molecular Ecology* 17, 2975–2984.
- Nelson, J.S., 2006. *Fishes of the World*, fourth ed. Hoboken, NJ: John Wiley & Sons, p. 601.
- Oliveira, R., Canário, A., 2000. Hormones and social behavior of cichlid fishes: A case study in the Mozambique tilapia. *Journal of Aquaculture and Aquatic Sciences, Cichlid Research: State of the Art*. 109–129.
- Petersen, C., 1990. Variation in reproductive success and gonadal allocation in the simultaneous hermaphrodite *Serranus fasciatus*. *Oecologia* 83, 62–67.
- Smith, C., Reichard, M., Jurajda, P., Przybylski, M., 2004. The reproductive ecology of the European bitterling (*Rhodeus sericeus*). *Journal of Zoology* 262, 107–124.
- Van Tienhoven, A., 1983. *Anatomy of the reproductive system, Reproductive Physiology of Vertebrates*, 2nd ed Ithaca, NY: Cornell University Press, pp. 96–136.
- Wisenden, B.D., 1995. Reproductive behaviour of free-ranging convict cichlids, *Cichlasoma nigrofasciatum*. *Environmental Biology of Fishes* 43, 121–134.
- Wootton, R.J., Smith, C., 2015. *Reproductive Biology of Teleost Fishes*. Chichester: John Wiley & Sons.
- Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R., Strüßmann, C., 2014. Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. *PLOS ONE* 9 (7), e102574.

Relevant Websites

- www.asf.ca/
Atlantic Salmon Federation.
- www.dfo-mpo.gc.ca/fm-gp/sustainable-durable/fisheries-peches/herring-hareng-eng.htm
Department of Fisheries and Oceans, Government of Canada.
- <http://eol.org/>
Encyclopedia of life.
- www.fishbase.org
FishBase.

Overview of Amphibian Reproduction

Sarah K Woodley, Duquesne University, Pittsburgh, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Some 360 million years ago, the ancestors of modern amphibians hauled themselves out of the water to colonize the land, escaping from aquatic threats and discovering new prospects. Early amphibians likely had a biphasic lifestyle: hatching and developing in water as a larval form with gills, but transforming into a terrestrial life form after undergoing a dramatic morphological and physiological reshaping called metamorphosis. The biphasic lifestyle is widespread in modern amphibians, but the innovative strategies used by amphibians to reproduce at the nexus of land and water are largely underappreciated. In fact, amphibians have the greatest diversity of reproductive strategies of all vertebrates, exploiting both aquatic and terrestrial niches to reproduce and pass on their genes. With these evolutionary innovations, amphibians are found in almost every habitat of the world.

To date, more than 7700 species of amphibians exist, distributed among anurans (frogs and toads, ~6800 species), urodeles (salamanders, ~700 species) and the secretive limbless gymnophionans (caecilians, ~200 species) (see Relevant Website section; Crump, 2009). Although anurans are found throughout the world, most salamanders live in temperate zones, with the exception of one large lineage of salamanders found in parts of Mexico and Central and South America (neotropics). In contrast, caecilians are mostly found in tropical areas. Members of the three orders of amphibians are united by a reliance on water. With their thin skin and shell-less eggs, they are prone to water loss and desiccation. As cool-blooded animals that prefer cool temperatures, amphibians have a low metabolic rate and assimilated energy supports growth and reproduction rather than thermoregulation. Despite their low metabolic rate, oxygen availability can be limiting and impair development.

The following overview of amphibian reproduction offers a taste of the rich diversity of modes while highlighting common themes. For more information on many of the themes and examples described in this overview, the reader is referred to the following reviews: Duellman and Trueb (1994), Crump (1996), Wake (1999), Jamieson and Exbrayat (2006), Wells (2007), Petranks (2010), Woodley (2010), Wake (2015), Woodley (2015). Reproduction encompasses the production of eggs and sperm, fertilization, and development, is shaped by physiological and environmental factors, and involves complex male-female and parent-offspring behavioral interactions. Despite their unique amphibian features, study of amphibians can teach us much about reproduction in other vertebrates, including humans, because of conserved foundational principles of vertebrate reproduction.

A discussion of amphibian reproduction is also relevant for conservation of biodiversity. Many fascinating reproductive modes are found in species that are dwindling or extinct. As an infamous example, in the now extinct gastric brooding frog (*Rheobatrachus*), the mother swallowed her fertilized eggs and incubated them in her stomach until they hopped from her mouth as froglets. Gastric brooding frogs are now extinct, likely due to habitat destruction or disease. Disease is particularly disturbing, impacting amphibians across the world, even those in relatively pristine habitats. The latest threat is the chytrid fungus (*Batrachochytrium salamandrivorans*), which is killing the charismatic fire salamanders of Europe and will likely reach North America in the near future, with similar consequences. Thus, an additional incentive to study amphibian reproduction is for captive breeding, as scientists desperately seek to preserve amphibians from extinction. Conversely, some amphibians like cane toads (*Rhinella marina*), after being introduced across the world to control pests, are now expanding their ranges and threatening native species. In this case, their reproduction is studied to control their expansion and to better understand properties of invasive species.

Proximate Control of Reproduction

Amphibian reproduction is shaped by the interplay of genetic and environmental factors. Environmental factors signal when it is suitable to reproduce. Moisture is necessary for hydration of adults and eggs, and temperatures must be sufficiently warm to support physiological processes like making sperm and eggs, sustaining reproductive behaviors, or feeding to gain energy for reproduction. Thus in temperate zones, warming temperatures and rainfall are key proximate signals of suitable conditions for reproduction. In species living in subtropical or tropical areas where temperatures are warm year round, rainfall and humidity are the primary cues. Additional important signals include food availability and social stimuli like the chorusing of male frogs at the breeding site.

Environmental cues are perceived by the nervous system which then activates the endocrine system, specifically the hypothalamic-pituitary-gonadal axis (Vu and Trudeau, 2016). As a conserved vertebrate mechanism, activation of the hypothalamic-pituitary-gonadal axis regulates sperm and egg production, and produces elevated levels of reproductive hormones like androgens and estrogens from the gonads. The reproductive hormones act throughout the body to ensure that sperm and egg production is synchronized with morphological and behavioral traits crucial for reproduction. As a few examples, in female red-bellied newts (*Cynops pyrrhogaster*), prolactin and estrogen stimulate the production of a pheromone which travels through the water to attract males for mating. In female marsupial frogs (*Gastrotheca riobambae*), gonadal hormones stimulate the seasonal development of specialized skin pouches that house developing offspring. In male anurans, androgens stimulate the enlargement

of horny thumb pads that help males grasp females during mating. Androgens also act on the vocal cords of male anurans to facilitate calling behavior that attracts females or warns off rival males. In the brain, reproductive hormones regulate expression of behaviors like searching for and finding potential mating partners, courtship, mating, and parental behaviors. The gonadal hormones tend to regulate behavior in a permissive manner, with additional hormones like prolactin, vasotocin, and corticosterone modulating more minute-to-minute changes in behavioral expression. Understanding of the proximate mechanisms underlying amphibian reproduction derives from a few common species of anurans and urodeles, typically found in temperate zones. Less is known about neuroendocrine mechanisms in neotropical or tropical amphibians, or in caecilians.

Reproductive Strategies and Seasonal Patterns

Amphibian strategies for maximizing their ability to produce offspring that survive to adulthood vary, and amphibians are quite flexible in their ability to reproduce given suitable conditions. Reproductive lifespan and fecundity varies across amphibian species. At one end of the spectrum, the aquatic European cave salamander (*Proteus anguinus*) lives an average of 60 years and breeds every 13 years laying less than 100 eggs at a time (Voituron *et al.*, 2011). At the other extreme, the gladiator treefrog (*Hysiboas rosenbergi*) in Panama reproduces in the rainy season following hatching, laying up to 6 clutches, each with thousands of eggs, and does not survive to the next rainy season. Most species have aquatic or terrestrial eggs/larvae, although some species, especially caecilians, are viviparous, whereby fertilized eggs develop inside the mother's oviducts, emerging as larvae or juveniles with the adult morphology.

The geography of an area sets the environmental parameters and thereby shapes the timing of reproduction. However, amphibians are excellent at coping with constrained environmental conditions, leading to many different reproductive modes in similar geographic zones. The following discussion describes amphibian reproduction according to terrestrial versus aquatic lifestyles, but note that the rich diversity of amphibian reproduction defies easy categorization.

Terrestrial Adults With Aquatic Breeding

The biphasic life cycle of terrestrial (including amphibians specialized for life in trees or burrows) adults that return to water to breed is quite successful. It was presumably the ancestral amphibian mode and is still a predominant life cycle in amphibians. Because the biphasic life cycle is less constrained by rainfall and humidity, especially in species that breed in permanent water, it has a wide geographic distribution.

In amphibians living in temperate zones, warm spring temperatures stimulate increases in gonad size and hormone levels, priming the system for reproduction when rainfall and humidity are also available. In many temperate zone amphibians, breeding occurs mostly in the spring and summer months wherever water is found, such as in ponds, streams, and wetlands. In some cases, reproduction is brief, concentrated in a few weeks of spring, when snow is melting and rain falls, creating temporary, predator-free sources of water for breeding. An example is the explosive breeding of spotted salamanders (*Ambystoma maculatum*) in eastern North America and Canada, where warm spring rains trigger the mass migration of salamanders from terrestrial burrows to vernal ponds for frenzied breeding over a few nights. Adults return to land to feed for the rest of the summer, leaving aquatic eggs to hatch into larvae that metamorphose into terrestrial juveniles a few weeks later before the pond dries up. Explosive breeding can also occur in desert-adapted amphibians, where late summer rainfall triggers the emergence of species like the desert spadefoot toad (*Scaphiopus*) from moist underground refuges to breed in ephemeral ponds or puddles. In cases where breeding areas remain wet throughout the summer, or in permanently aquatic habitats like streams, breeding can be prolonged, with multiple cycles of breeding. For example, wood frogs (*Lithobates sylvaticus*), an early spring breeder with aquatic eggs and tadpoles, will breed repeatedly if ponds and wetlands stay wet into the summer.

In warm and wet tropical rainforests where productivity and carrying capacity is high, frogs and caecilians are especially diverse. In the tropics, hormones appear to be continuously elevated and egg and sperm production is ongoing. Breeding can occur year-round in permanently aquatic areas or as a function of rainfall and humidity. In some species of caecilians, females lay eggs near water and hatchlings wriggle into adjacent water. Anurans of the humid Atlantic Forest of Brazil exemplify the diverse reproductive strategies of tropical amphibians, where terrestrial adults have adapted to breed in a wide range of watery sites including streams, ponds, puddles, nooks in plants, and even water filled basins or moist foam nests (Haddad and Prado, 2005).

Development requires oxygen, and in tropical areas with warm temperatures, oxygen content in the water decreases. Thus, many amphibians (in both tropical and temperate zones) lay eggs in well oxygenated streams, avoiding oxygen-poor sites like swamps. Other adaptations to ensure adequate oxygen include laying small eggs with a high surface to volume ratio to maximize oxygen diffusion, placing eggs on the surface of the water in partial contact with air, laying eggs in constructed foam nests or laying eggs out of water all together (see next section on terrestrial egg-laying).

Terrestrial Adults With Terrestrial Breeding

In the biphasic life cycle, reproduction is heavily constrained by availability of water because eggs and/or larvae are aquatic. Many amphibian species, however, have found ways to breed in the absence of water to avoid aquatic predators and competitors and to

exploit new niches. Although liberation from water provides new opportunities, the relatively dry, terrestrial environment can be harsh on developing young. Some traits associated with terrestrial development include larger egg size to minimize water loss, smaller clutch size, and parental care to keep terrestrial eggs or larvae hydrated and nourished. Terrestrial life styles are often found in warm humid tropics, but are found in more temperate climates as well.

A common terrestrial strategy is “direct development”, which is found in both temperate and tropical climates (Wake, 2003). Here, eggs are laid on land and development skips the free-living larval stage. At hatching, the offspring look like miniature adults, ready for a terrestrial lifestyle. With direct development, egg production is less constrained by annual cycles of rainfall, although moist conditions are still required to prevent desiccation of clutches and to allow adults to forage to gain energy for growth and reproduction. Virtually all species in the large family of eleutherodactylid frogs have direct development. An example is the coqui frog, (*Eleutherodactylus coqui*). Native to the tropical forests of Puerto Rico, the coqui frogs lay egg clutches of a few dozen eggs in plant fronds or in curled leaves, where they are cared for by the father. Within a few weeks, eggs hatch into froglets, which become sexually mature after a few months, with adults breeding every few weeks. Continuous reproduction combined with direct development has made coquis into a highly invasive species.

Direct development is also found in all neotropical plethodontid salamanders, the largest family of salamanders. Neotropical salamanders are found in cool, mid-elevation cloud forests that have wet and dry seasons. Study of the neotropical salamander (*Bolitoglossa rostrata*), found that adult males are capable of mating year-round, having sperm and pheromone-producing glands at all times. Females, however, lay eggs in moist refugia once a year at the beginning of dry season. Females attend to the clutch until the start of the wet season when the young hatch out fully formed, ready for a terrestrial life style (Houck, 1977). This illustrates that even in warm climates, reproduction may be highly seasonal.

Another strategy to reproduce with minimal water is to bear live young (viviparity) (Wake, 2003, 2015). In some subspecies of fire salamanders (*Salamandra salamandra*) living in cool montane habitats, fertilized eggs develop inside the mother's oviduct. Once the young's yolk is depleted, they feed on unfertilized eggs and oviductal secretions. After several months of gestation, fully metamorphosed juveniles are born.

Although live bearing is very rare in anurans and salamanders, it is a common strategy in caecilians. It has evolved independently four different times and is found in 75% of the caecilian species. Typically, the entire developmental process including fertilization, hatching of eggs, and metamorphosis occurs within the oviducts with fully-formed young emerging from the female. Interestingly, this form of viviparity (or live-bearing) is not restricted to species with terrestrial lifestyles; it also occurs in aquatic species of caecilians.

Aquatic Adults With Aquatic Breeding

Despite the diverse adaptations that emancipate amphibians from water, many amphibian species remain aquatic their entire lives. An example is the African clawed frog (*Xenopus laevis*), where tadpoles metamorphose into aquatic juvenile frogs. Another example is the South American caecilian, (*Typhlonectes*), which is aquatic/semi-aquatic, sheltering in submersed holes along muddy banks, giving birth to live young that are also aquatic. Aquatic lifestyles are also evident in the larger types of salamanders, like the proteids, sirenids, and cryptobranchids. Interestingly, aquatic lifestyles in salamanders are often associated with prolonged larval development. At its most extreme is the occurrence of paedomorphosis. Paedomorphosis occurs when gonads mature in the larval body form, and sexually reproducing adults retain larval traits like gills. The large blood-filled feathery gills found in adult paedomorphic mudpuppies, sirenids, and axolotls (*Ambystoma mexicanum*) are striking in appearance.

Reproductive Behaviors

For successful reproduction, males and females must find each other and mate. In addition, in some species, offspring survival requires parental care. A rich array of mating and parental care behaviors is displayed across the three orders of amphibians. The type of reproductive behaviors is somewhat related to the mode of fertilization and whether breeding is terrestrial or aquatic.

Mating

Anurans

In almost all anurans, fertilization occurs externally. While in a copulatory embrace called amplexus, the male releases sperm while the female lays her eggs, typically into water. This method of fertilization shapes male strategies for siring offspring. Male anurans typically congregate around breeding sites and advertise their presence by calling, using their vocal sacs for amplification. In species where mating occurs explosively over a short period of time, males directly compete with other males for access to females, often aggressively wrestling (scramble competition), and larger males sire the most offspring. In species where mating occurs over a more prolonged period, males may adopt alternative tactics for siring offspring. For example, within a species, some males may defend a territory while others patrol the periphery to find receptive females. In such systems, a female can choose the male who will fertilize her eggs. In some cases, females may choose a male based his ability to provide and defend resources like feeding or oviposition sites. In other cases, it is not clear what males might be contributing to the reproductive effort other than sperm.

In a very few species, males inseminate females and fertilization is internal. For example, male tailed frogs (*Ascaphus truei*) possess a cloacal extension, or “tail” that is inserted into the female’s cloaca, which is helpful for mating in fast-moving mountain streams.

Salamanders

In salamanders, fertilization in several families is external (Houck and Arnold, 2003). For example, hellbender (*Cryptobranchus alleganiensis*) males dig a shallow nest under rocks in clear streams. Females enter the male’s nest, oviposit and leave. Males release sperm over the eggs and then tend to the eggs and developing larvae for several weeks. The majority of salamander species, however, have internal fertilization. Salamanders lack intromittant organs and transfer sperm to females with a spermatophore. The spermatophore, a major evolutionary innovation, is a gelatinous mass, deposited on the substrate, with sperm on the top. A female will step over the spermatophore and collect the sperm into her cloaca. The sperm is stored in a special cloacal chamber until eggs are laid, at which point they are fertilized. The use of a spermatophore has important implications: mating and egg-laying can be separated in time, and females can mate with multiple males. In Ocoee salamanders (*Desmognathus ocoee*), females mate with multiple males from fall to spring and lay eggs during the summer months. This allows greater flexibility because environmental conditions suitable for mating are not always suitable for egg laying and development.

Salamanders do not vocalize, instead relying on chemical cues to find members of the opposite sex and coordinate mating interactions (Woodley, 2014). Furthermore, use of a spermatophore to transfer sperm involves complex courtship behaviors and pheromonal communication in multiple salamander families. All plethodontid salamanders express a tail-straddling behavior which has been evolutionarily static for millions of years and functions to coordinate male and females for successful sperm transfer. Similarly, male rough-skinned newts (*Taricha granulosa*) clasp females for hours both before and after spermatophore deposition and sperm transfer, presumably to shape female receptivity. During these courtship interactions, males secrete peptide and proteinaceous pheromones that induce females to mate more readily. In newts, the pheromones are simple peptides (sodefrin in red-bellied newts (*Cynops pyrrhogaster*)) while they are complex mixtures of different molecules and isoforms in plethodontid salamanders (e.g. red-legged salamanders (*Plethodon shermani*)).

Caecilians

Almost all caecilians have internal fertilization which they achieve with copulatory organ called a phallodeum which is inserted in the female’s cloaca during mating. They have well developed olfactory senses so chemical communication is likely important. Unfortunately, very little is known about their mating behavior (Jamieson and Exbrayat, 2006).

Parental Care

After mating, most amphibians lay their eggs and leave with no further care for offspring (Crump, 1996). However, parental care has evolved in multiple lineages in all three amphibian orders. Parental care is the investment in offspring after fertilization, and its main function is to protect and/or nourish offspring. Parental care is more common in species with terrestrial breeding as a way to enhance offspring survival in a potentially harsh terrestrial environment, but it is also found in aquatic habitats. Parental care includes nest construction; egg or larval attendance; egg or larval transport or brooding; larval feeding; and internal gestation in the female. Parental care can be exhibited by the mother, father, or both. External fertilization is often associated with male parental care, and internal fertilization with female parental care, although there are exceptions.

Anurans

Parental care is relatively rare in anurans, present in about 10% of species. Species may construct nests either on land or water. Foam nests are common and can protect aquatic eggs from dangerous water currents and terrestrial eggs from dehydration. In some species, parents dig out water-filled cavities or basins for eggs and larvae. In some terrestrial breeders, females lay eggs in leaves that are sealed into a protective shelter using oviductal secretions. Nests alone may not be sufficient to enhance offspring survival, and parents may attend eggs or larvae to protect them from predators, microbial infections, or desiccation. If eggs and larvae develop in nutrient poor sites such as leaf axils or tree holes, feeding is required. Mimic poison frogs (*Ranitomeya imitator*) from Peru breed in small pools in the folds of tree leaves, presumably to avoid predators. Males monitor the eggs, and females periodically deposit unfertilized eggs for the offspring feed on. Notably, the male-female pair is monogamous, one of the rare examples of monogamy in an amphibian (Brown *et al.*, 2008).

Another interesting form of parental care is carrying eggs/larvae to protect, nourish, or transport them. Varied adaptations support these functions. In aquatic pipid frogs, eggs develop while embedded in back of the female, either hatching into tadpoles or into froglets, depending on the species. In Darwin’s frog (*Rhinoderma darwinii*), the male attends the eggs until they are about to hatch at which point he laps them into his vocal sac. The young develop within the vocal sac, ingesting yolk or paternal secretions from the vocal sac lining. Finally, in the midwife toads of Europe and North America (*Alytes*), males carry strands of fertilized eggs entwined around their hind legs for about 1 month. At hatching, the clutch is deposited into a pool. Similar forms of parental care are found in fully terrestrial species, such as in the Australian pouched frog (*Assa darlingtoni*). Here, females attend eggs but males brood the tadpoles which slither into lateral pouches remaining there until metamorphosis, which may be several months later.

Finally, out of the more than 6800 species of anurans, only a few give birth to live young. In the African genera of *Nectrophrynoides* and *Nimbaphrynoides*, fertilized eggs develop within the oviduct and females give birth to froglets. In the fanged frog (*Limnonectes larvaepartus*) from Indonesia, females give birth to tadpoles in puddles along streams (Iskandar *et al.*, 2014). The rarity of viviparity in anurans reflects that only 17 anuran species have internal fertilization.

Salamanders

Salamander parental care is found in several families, but is less varied than in anurans. Usually restricted to egg attendance, its main functions are probably aeration of aquatic eggs, moistening of terrestrial eggs, and protection against egg predators and fungal infection. Male parental care is common in species with external fertilization. For example, the aquatic Japanese giant salamander (*Andrias japonicus*), like its relative the hellbender, cares for clutches of eggs. Behaviors include eating dead or infected eggs and tail-fanning. Asiatic salamanders in the hynobiid family have a biphasic life cycle where adults return to water to breed. Fertilization is external and males guard egg clutches.

Female parental care is very common in salamander species with direct development, such as those in the plethodontid family. Here, fertilization is internal, occurs at the time of oviposition, and usually occurs days to months after mating. Females stay with terrestrial eggs until they hatch. Behaviors include agitating the eggs, eating infected eggs, and moving through eggs. Egg attendance may function to inoculate clutches with antimicrobial secretions, bacteria, or bacterial metabolites from the mother's skin to protect eggs against fungal disease. Egg attendance can last several weeks to months, during which the mother does not eat. A rare case of alloparental care is found in the four-toed salamander (*Hemidactylium scutatum*), where multiple females may deposit eggs into the same nest, but only a single female attends the eggs.

Caecilians

Parental care is widespread in caecilians. In many egg-laying species, the mother coils around the eggs while they develop. An unusual behavior called maternal dermatophagy is found in several groups of caecilians where hatchlings quickly gain weight by using specialized fetal teeth to eat the outer layer of the mother's skin, which hypertrophies to become nutrient-rich (Wilkinson *et al.*, 2013). Maternal dermatophagy, with its associated specialized fetal teeth, may have been the antecedent to the evolution of caecilian viviparity, in which embryos survive within the oviduct by eating other eggs or by ingesting nutrients obtained by scraping the oviductal walls with specialized teeth.

Conclusions

Despite physiological constraints related to hydration, oxygen availability, and temperature, amphibians have evolved ingenious strategies for exploiting the water/land interface, especially in the tropics where suitable microniches are abundant and productivity is high. These innovations have occurred while adhering to conserved vertebrate mechanisms of gamete production and neuroendocrine control. Indeed, amphibians have the greatest diversity of reproductive strategies of the vertebrate classes. In addition to the marvelous species diversity, there is also substantial plasticity within species, providing additional opportunities for animals to breed and pass on their genes. Most work on the neuroendocrine control of amphibian reproduction is restricted to a few species, but new species are discovered every year offering new opportunities to test hypotheses about the physiological mechanisms and evolutionary forces that shape amphibian reproduction. However, the discovery of new species is tempered by the increasing loss of amphibian species, and this article may provide readers with a better appreciation for amphibian biodiversity and the importance of studying amphibian reproduction.

References

- Brown, J.L., Morales, V., Summers, K., 2008. Divergence in parental care, habitat selection and larval life history between two species of Peruvian poison frogs: An experimental analysis. *Journal of Evolutionary Biology* 21, 1534–1543.
- Crump, M., 1996. Parental care among the amphibian. *Advances in the Study of Behavior* 25, 109–139.
- Crump, M., 2009. Amphibian diversity and life history. In: Dodd, C.K. (Ed.), *Amphibian Ecology and Conservation, A Handbook of Techniques*. New York, NY: Oxford University Press, pp. 3–19.
- Duellman, W.E., Trueb, L., 1994. *The Biology of Amphibians*. Baltimore and London: Johns Hopkins University Press.
- Haddad, C., Prado, C., 2005. Reproductive modes in frogs and their unexpected diversity in the Atlantic forest of Brazil. *Bioscience* 55, 207–217.
- Houck, L.D., 1977. Reproductive biology of a neotropical salamander. *Bolitoglossa strata*. *Copeia* 1977, 70–83.
- Houck, L.D., Arnold, S.J., 2003. Courtship and mating. In: Sever, D.M. (Ed.), *Reproductive Biology and Phylogeny of Urodela (Amphibia)*. Enfield, NH: Science Publishers, pp. 383–424.
- Iskandar, D.T., Evans, B.J., McGuire, J.A., 2014. A novel reproductive mode in frogs: A new species of fanged frog with internal fertilization and birth of tadpoles. *PLOS ONE* 9, e115884.
- Jamieson, B.G.M., Exbrayat, J.M., 2006. *Reproductive biology and phylogeny of gymnophiona: Caecilians*. Enfield, NH: Science Publishers.
- Petranks, J.W., 2010. *Salamanders of the United States and Canada*. USA: Smithsonian Books.
- Voituron, Y., de Fraipont, M., Issartel, J., Guillaume, O., Clobert, J., 2011. Extreme lifespan of the human fish (*Proteus anguinus*): A challenge for ageing mechanisms. *Biology Letters* 7, 105–107.
- Vu, M., Trudeau, V.L., 2016. Neuroendocrine control of spawning in amphibians and its practical applications. *General and Comparative Endocrinology* 234, 28–39.
- Wake, M.H., 1999. Amphibian reproduction, overview. In: Knobil, E., Neill, J.D. (Eds.), *Encyclopedia of Reproduction*. San Diego: Academic Press, pp. 161–166.

- Wake, M.H., 2003. Reproductive modes, ontogenies, and the evolution of body form. *Animal Biology* 53, 209–223.
- Wake, M.H., 2015. Fetal adaptations for viviparity in amphibians. *Journal of Morphology* 276, 941–960.
- Wells, K.D., 2007. *Ecology and Behavior of Amphibians*. Chicago: University of Chicago Press.
- Wilkinson, M., Sherratt, E., Starace, F., Gower, D.J., 2013. A new species of skin-feeding caecilian and the first report of reproductive mode in *Microcaecilia* (amphibia: Gymnophiona: Siphonopidae). *PLOS ONE* 8, e57756.
- Woodley, S., 2015. Chemosignals, hormones, and amphibian reproduction. *Hormones and Behavior* 68, 3–13.
- Woodley, S.K., 2010. Hormones and reproductive behavior in amphibians. In: Norris, D.O., Lopez, K. (Eds.), *Hormones and Reproduction of Vertebrates*. San Diego, CA: Academic Press, pp. 143–169.
- Woodley, S.K., 2014. Chemical signaling in amphibians. In: Mucignat-Caretta, C. (Ed.), *Neurobiology of Chemical Communication, Frontiers in Neuroscience Series*. Boca Raton, FL: CRC Press/Taylor and Francis, pp. 251–280.

Relevant Website

<http://www.amphibiaweb.org/>
AmphibiaWeb.

Reproduction in Reptiles

Daniel G Blackburn, Trinity College, Hartford, CT, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Amniotic egg Terrestrial egg of reptiles, birds, mammals, and their extinct relatives.

Oviparity The reproductive pattern in which females lay eggs that complete their development and hatch in the external environment.

Parthenogenesis Asexual reproduction, in which females produce genetically similar female offspring without fertilization.

Sex determination pattern The mechanism affecting whether an embryo develops as a female or a male. Sex determination can be genetic or be affected by developmental temperature.

Squamates The scaly reptiles, i.e., lizards and snakes.

Viviparity The reproductive pattern in which embryos complete their development inside the female reproductive tract before being born.

Introduction

Living (non-avian) reptiles traditionally are classified in four orders: Squamata (lizards and snakes: with >10,000 species); Rhynchocephalia (the tuatara *Sphenodon punctatus*); Chelonia (turtles: 346 species); and Crocodilia (alligators, crocodiles, and gharials: 25 species). The tuatara is of special evolutionary interest as a lepidosaurian relict that shared a common Mesozoic ancestry with squamates. Extinct reptiles of the Paleozoic and Mesozoic include many other groups, such as the aquatic ichthyosaurs, plesiosaurs, and mesosaurs; winged pterosaurs; and dinosaurs (ornithischians and saurischians, including birds). Despite their enormous diversity, reptiles share several reproductive features inherited from their distant Paleozoic ancestors. Among these features are internal fertilization; a terrestrial “amniotic” egg; and production of fully-developed offspring. Reptiles contrast with most amphibians, which lay aquatic eggs that are fertilized externally, from which hatch aquatic larvae that must undergo metamorphosis to attain adult body form.

Despite their shared features, extant reptiles are highly diverse in terms of reproductive parameters. Any brief account can only touch upon the complexity and diversity of reptilian attributes. Readers can refer to the bibliography for more information.

Overview of Reptilian Reproduction

Overall structure of the male reproductive system is similar (but not identical) throughout reptiles (Pough *et al.*, 2016). Sperm are produced in the testes, transported by the ductus deferens, and either stored in the ductus or the epididymis. In male squamates, secretions of the kidney contribute to the seminal fluid; their functions are not well understood, although in anole lizards, such secretions may nurture the sperm. In crocodilians and turtles, the intromittent organ consists of a single vascular erectile body that protrudes from the cloaca. Squamates have paired hemipenes, each with a groove to carry sperm and a head ornamented with papillae and/or spines that fix it in place during copulation. An intromittent organ is lacking in tuatara (as in most birds) and copulation occurs through apposition of the male and female cloacae. A recent analysis of *Sphenodon* embryogeny revealed a phallic precursor, suggesting that absence of the intromittent organ represents an evolutionary loss (Sanger *et al.*, 2015).

In female reptiles, ovarian oocytes accumulate yolk, are ovulated, and eggs then are fertilized in the anterior oviduct (Ramírez-Pinilla *et al.*, 2015). Oviducts are paired structures in most squamates; however some snakes and lizards retain only one, as an adaptation to elongated body form. The oviduct consists of an infundibulum, where fertilization occurs; a posterior infundibulum which (in turtles and crocodilians) contains glands that secrete albumen; a uterus, which deposits the shell; and a posterior “non-glandular uterus” which expels the egg at oviposition (Blackburn, 1998; Cree, 2015). The oviductal epithelium and glands undergo changes in concert with reproductive cycles, under the influence of steroid hormones.

The extent of embryonic development that occurs before the egg is laid varies markedly by group (Pough *et al.*, 2016). In turtles, crocodilians, and the tuatara, eggs are laid in a very early developmental stage. In fact, in turtles, oviductal eggs undergo stasis prior to oviposition to prevent development beyond the gastrula stage. However, in oviparous squamates, eggs typically are laid in the limb bud stage. In viviparous (live-bearing) lizards and snakes, embryos complete their development in the uterine oviduct, and are born as fully-developed neonates.

Specializations of the amniotic egg (Table 1) have allowed reptiles to reproduce entirely on land (Pough *et al.*, 2016) and in fact, require terrestrial reproduction (except in viviparous forms). These specializations include a protective eggshell plus fetal (“extraembryonic”) membranes that sustain the developing embryo. Eggs of crocodilians and turtles (but not squamates or

Table 1 Features of the amniotic egg of reptiles.

Feature	Function
Eggshell	Mechanical & hydric protection; protection from microbes & predators
Chorioallantois	Respiratory gas exchange; calcium uptake from the eggshell
Amnion	Mechanical protection of the embryo
Albumen ^a	Water & protein provision
Large yolk	Production of fully-developed hatchlings
Yolk sac	Yolk storage & digestion
Fetal membranes ^b	Water uptake

^aAbsent in squamates and probably the tuatara.^bMechanism not known.

tuataras) also are supplied with albumen, which provides water and protein. Reptilian eggs can be buried in the ground, hidden in debris or in cavities in rocks or trees, or laid in decaying vegetation. Crocodilians lay eggs in above-ground nests. Once deposited, reptilian eggs exchange respiratory gases and (typically) absorb water across their eggshells. Although eggshells of most reptiles are flexible, many turtles and most gekkos have hard, calcareous shells that provide greater mechanical protection but are less permeable to water.

Reproductive Features

Sex Determination

Reptiles are highly diverse in mechanisms affecting sexual development of embryos (Pough *et al.*, 2016). In temperature – dependent sex determination (TSD), egg temperature during development affects whether the embryo develops as a male or female. TSD occurs in turtles, crocodilians, most lizards, and the tuatara. Species vary as to whether females result from eggs incubated at (a) relatively warmer temperatures (some turtles); (b) cooler temperatures (*Sphenodon*); (c) or at temperature extremes (crocodilians and some turtles). In each instance, males result from eggs incubated at the alternative temperatures. All three patterns are found among lizards.

In genetic sex determination (GSD), differentiation depends on sex chromosomes, and can show male- heterogamety (the XY system, as in mammals) or female heterogamety (the ZW system, as in birds) (Vitt and Caldwell, 2014). Snakes exhibit the ZW system; however, the Burmese python (*Python bivittatus*) and the Central American boa (*Boa imperator*) have an XX/XY system. Lizard species can have either form of GSD. However, in the Australian bearded dragon (*Pogona vitticeps*), chromosomes and incubation temperature affect phenotypic sex.

Much research has focused on the mechanisms, adaptive significance, and evolution of the patterns of sex determination. Theoretically, TSD should be favored evolutionarily when the optimal developmental temperature differs between males and females. The Australian agamid lizard *Amphibolurus muricatus* conforms to this prediction. Due to its TSD, reproduction in the spring produces proportionately more female offspring. These young lizards can grow and overwinter at larger body sizes than females from eggs laid later in the year. Because adult female size correlates with clutch size, TSD therefore has evolutionary advantages in this species. TSD appears to be ancestral for reptiles, but multiple evolutionary transformations between mechanisms clearly have occurred in reptilian history (Vitt and Caldwell, 2014). Currently, a major concern is that anthropogenic climate change puts many species with TSD at serious risk of extinction (Cree, 2014).

Clutch Sizes

In most reptile species, clutch size can vary widely between individuals, age classes, geographical location, and from year to year (Vitt and Caldwell, 2014; Mesquita *et al.*, 2016). Nevertheless, phylogenetic patterns are evident. In turtles, clutch size typically is about 1–6 eggs; however, clutches of up to 150 eggs have been reported in species with very large individuals. Clutch sizes in crocodilians commonly range from 6 to 60 eggs, with the largest figures being representative of the Nile crocodile. In tuataras, average clutch size varies by population from 9.4 eggs on Stephens Island to 5.9 eggs on some smaller islands. A few lizard species show invariant, small clutches. Among them are anole lizards (in which the female lays an egg every 7–10 days), and gekkos that oviposit clutches of 1–2 eggs. Some oviparous chameleons lie at the opposite extreme among lizards, with mean clutch sizes of 50 eggs, and clutches of up to nearly 100 eggs in individual cases. Oviparous snakes show a similar range, with average brood sizes that range from 3 (e.g., the crown snake, *Tantilla coronata*) to 46 (certain *Python*). Within and between squamate species, clutch size often correlates with female size. Clutch size also can vary in a species according to resource availability. In some species, such as the lizard *Lacerta agilis*, a tradeoff between clutch size and egg size has been documented. Such a tradeoff also is evident in comparisons of closely related species, and accounts for some of the broad differences apparent at higher taxonomic levels.

As for viviparous squamates, very small litters (1–3 offspring) characterize live-bearing gekkoes and cordylids, whereas the largest reported litters are in chameleons (up to 50 offspring) and vipers (80–100 offspring). Phylogenetic variation tends to obscure broad

differences that might be attributable to reproductive mode, although such differences sometimes are apparent in comparisons of closely related species (Stewart and Blackburn, 2015).

In lizards, measures of relative clutch mass ("RCM," the ratio of clutch to body mass) have been shown to relate to modes of foraging as well as predator escape (Vitt and Caldwell, 2014). The RCM in streamlined, active foragers tends to be much smaller than in lizards (such as horned lizards and chameleons) that "sit-and-wait" for their insect prey. Burden of the clutch has been shown to affect maternal locomotion. For example, in the side-blotched lizard *Uta stansburiana*, experimental reduction of the clutch by 30% produces higher levels of endurance and survival in gravid females. In the European lizard *Zootoca vivipara*, females with heavier litters weigh less after birth than other females. Thus, large litters can be costly to females in ways that may affect future reproduction.

Frequency of Reproduction; Life History Patterns

Reptilian species range from those that produce many clutches per year to those that reproduce only once every few years (Vitt and Caldwell, 2014; Mesquita *et al.*, 2016). For example, oviparous snakes commonly have one clutch annually; relatively few are reported to reproduce more than once per year. In contrast, oviparous lizards that live in warm and temperate environments often produce 5 or 6 clutches of eggs per year. As an extreme case, female leatherback sea turtles (*Dermochelys coriacea*) can yield 10 clutches with up to 90 eggs each in a reproductive season. Climate and reproductive seasonality can play a key role in affecting clutch frequency. Some turtles produce 4 clutches in the southern part of their range, but only 1 clutch annually at their northern geographical limit. In marked contrast to oviparous species that yield multiple clutches annually are those that do not reproduce each year. For example, female marine iguanas (*Amblyrhynchus cristatus*) of the Galapagos Islands reproduce on a biennial cycle. Likewise, some sea turtles breed once every 3 years. Tuataras reproduce no more frequently than once every two years, and on average, only once every 4–5 years (Cree, 2015).

Unlike typical oviparous lizards, viviparous squamates usually are restricted to a single litter annually (Blackburn and Stewart, 2011; Stewart and Blackburn, 2015). This restriction is readily explainable in seasonal environments, given the duration of pregnancy relative to the time available for reproduction. Nevertheless, a few live-bearing species (including certain chameleons and garter snakes) have been reported to accomplish more than one pregnancy in a single year. More common among viviparous species are reports of biennial and triennial reproduction. A number of live-bearing lizards and snakes from multiple families reproduce on a 2 or 3 year cycle. For example, various New Zealand geckoes reproduce biennially. In northern populations of the timber rattlesnake (*Crotalus horridus*), females reproduce only once every 3–4 years. The infrequency of reproduction reflects their extreme climatic conditions, coupled with the high costs of reproduction in such an environment. Snakes that skip reproductive years can replenish lipid stores before beginning another cycle.

Frequency of reproduction is best viewed in the context of overall life history patterns that also take into account clutch size, adult body size, and time to reach maturity (Mesquita *et al.*, 2016). Lizards have received considerable attention in these respects (Vitt and Caldwell, 2014). A classic analysis of oviparous lizards recognized three life history patterns: (a) production of one clutch annually, with delayed maturity (>1 year after hatching) coupled with large body size (as in the green iguana); (b) production of multiple clutches per year, in small bodied forms with early maturity (at less than 1 year of age) (as in anole lizards); and (c) production of multiple clutches by large bodied lizards with delayed maturity (as in basilisk lizards). Regarding the third pattern, large body size allows for larger clutches; meanwhile, delayed maturity can reflect resource limitations as well as the time needed to achieve large body size. Another analysis of lizards further divided the categories above, since some single-brood lizards yield small clutches, and some large-bodied forms exhibit early maturity. The lizard categories do not apply well to oviparous snakes, which commonly have no more than a single clutch annually.

Reproductive Cycles

Reptiles vary with respect to when mating occurs relative to gonadal growth and sex steroid secretion (Pough *et al.*, 2016). In the "associated gonadal cycle," gametogenesis and maximum hormone release occur shortly before mating, which is soon followed by fertilization. In the "disassociated gonadal cycle," mating and gonadal activity occur at different times of year. This pattern is made possible by sperm storage by the male or the female. The advantage of dissociated cycles is that gametogenesis and mating each can be scheduled when their energetic costs are minimal. Associated gonadal cycles are typical of crocodilians and most lizards, and occur in tropical/subtropical environments with long periods apt for reproduction. Disassociated cycles occur in both temperate and tropical climates, and often occur in turtles and snakes.

Reproductive cycles also can be characterized by duration and time of year (Mathies, 2011; Vitt and Caldwell, 2014; Méndez de la Cruz *et al.*, 2015; Pough *et al.*, 2016). In aseasonal cycles, reproductive activity occurs all year round, a common feature of reptiles in tropical and subtropical environments. Three forms of seasonal cycles have been distinguished. One is the "discontinuous" cycle, in which adult activity is restricted by seasonal changes, requiring that fertilization and embryonic development be accomplished during a few months of a single reproductive season. In this pattern, as in many temperate zone reptiles of North America, mating and fertilization occur in the spring and hatchlings or neonates emerge in the late summer. Discontinuous cycles also occur in tropical environments with seasonal patterns of rainfall. In such environments, reproduction can be timed to minimize reproductive costs to females or alternatively, to maximize survival of the offspring.

In a second type of seasonal cycle, the timing of egg production and hatching are uncoupled because embryos remain in the eggs through developmental arrest. For example, in some tropical turtles, fully-developed offspring remain in the eggshell during dry periods, and only hatch when conditions improve. A striking example is represented by the Madagascan chameleon *Furcifer labordi*. Eggs incubate for 8–9 months after oviposition, hatching synchronously at the beginning of the rainy season. The hatchlings reach sexual maturity in <2 months, reproduce, and die; thus, most of the life of individual chameleons is spent as an egg.

A third type of cycle is the “continuous” seasonal variety, in which reproduction takes most of the year to complete. This pattern is common in lizards of cool montane habitat and far southern distributions. Thus, in the North American lizard *Sceloporus jarrovi*, females mate in the fall, overwinter while pregnant, and give birth in the spring. This pattern maximizes the time neonates have to feed before the following cold period. Likewise, in one population of the New Zealand gecko *Hoplodactylus maculatus*, the female mates in the summer, stores sperm during the winter while oocytes are accumulating yolk, becomes pregnant in the spring, and gives birth a full 14 months later. Another example of a continuous seasonal pattern is the tuatara, in which eggs reside in the oviducts for 6–8 months, are oviposited, and then incubate for about a year before hatching (Cree, 2014).

Lipid Cycles

Reproductive activity in reptiles is partly fueled through lipid stored in the form of abdominal fat bodies. Consequently, lipid cycles (as measured through changes in total mass of the fat bodies) commonly reflect cyclic reproductive patterns, including vitellogenesis, spermatogenesis, and behaviors related to reproduction. In general, lipids are stored when resources are most available, and used during periods of energetic needs are maximal.

Lipid cycles can differ between males and females of a species when patterns of maximal energy use differ between the sexes. An example is provided by the viviparous skink *Mabuya heathi* of Brazil, a species that breeds cyclically in accord with wet – dry seasonality (Vitt and Caldwell, 2014; Stewart and Blackburn, 2015). This species is highly unusual in that females provide most of the nutrients for development by placental means, during the last few months of an 8–12 month gestation. In males of this species, mass of the fat bodies and the testes grow to a peak during the dry season (when resources are less abundant); both decline during the wet season, when mating occurs. In females, fat bodies decline during the dry season, during the time that maternal transfer of nutrients to the embryos is maximal. The timing of gestation ensures that offspring are born at the beginning of the wet season when nutrients are most abundant. Lipid cycles in females thus reflect embryonic and neonatal needs, and lipid cycles in males are timed to accommodate female readiness for mating.

Environmental and Hormonal Control

In reptiles that reproduce seasonally, environmental cues indicate optimal times of reproduction (Krohmer and Lutterschmidt, 2011; van Dyke, 2015). Species vary as to whether seasonal reproduction is under control of an endogenous cycle, or is influenced by exogenous cues, or both. Temperature is the most important environmental cue in many species, although photoperiod can play a secondary role. In some tropical species, seasonal rainfall stimulates reproductive activity. Detection of day length is mediated through the parietal-pineal complex, which in turn acts via melatonin secretion.

Neuroendocrine control of reproductive cycles in males and females has been studied intensively in some reptiles (Krohmer and Lutterschmidt, 2011). The hypothalamic-pituitary axis communicates environmental and social cues to the gonads. The gonads in turn stimulate accessory reproductive structures (including the oviduct and abdominal fat bodies) as well as behaviors, via the sex steroids. Given that gametogenesis and hormone production can either be associated or disassociated, with patterns that can differ between the sexes, environmental cues must act in a diversity of ways.

Specialized Aspects

Sperm Storage

In many reptilian species, females have the ability to store sperm in their reproductive tracts (Blackburn, 1998; Cree, 2015; Siegel *et al.*, 2015). This ability uncouples the timing of mating vs. ovulation/fertilization, such that each activity can be optimally timed for the benefit of the female and/or her offspring. In alligators, sperm are stored only within a given reproductive season; however, in turtles and squamates, sperm can be stored for several years. Male reptiles also can store mature sperm in their reproductive tracts (vas deferens or epididymis), a pattern that allows spermatogenesis and mating to be at different times of year. Many squamates store sperm crypts or tubules in the non-glandular uterus of the oviduct. In snakes and several lizard groups, storage structures for sperm are located in the posterior infundibulum of the oviduct. In snakes, these structures are sac-like receptacles associated with ciliated ducts. In garter snakes, cells of these receptacles provide nutrition for the sperm in the form of lipids and carbohydrates. In alligators and turtles, sperm storage tubules can be located in the posterior infundibulum as well as in the posterior uterus. The diversity of female structures for storage of sperm reflects multiple evolutionary origins.

Parental Care

Although most reptiles abandon their eggs following oviposition, various degrees of parental care have been documented in more than 100 species (Shine, 1988; Stahlschmidt and DeNardo, 2011). Parental care is extensive in crocodilians, in which one of the

parents (usually the female) guards the nest. Crocodiles and alligators not only construct the nest, but facilitate hatching, carry the young by mouth to the water, and remain with them to guard against predators. Although parental care is rare in chelonians, nest-attendance has been documented in an Asian tortoise species.

Despite the fact that parental care of eggs or offspring has been reported in less than 1% of the squamate species, it is broadly distributed and manifested in multiple ways (Shine, 1988; While *et al.*, 2015). Among lizards, egg-attendance (sometimes called “brooding”) is the most common form of parental care. It has been observed in species scattered among 12 families, including alligator lizards, skinks, varanids, and iguanids. Female skinks of the genus *Plestiodon* not only guard their eggs against predators, but remove eggs that become infected, and regulate hydric and thermal conditions of the eggs by coiling around them and by changing their positions in the nest. In two species of this genus, the mother facilitates hatching. Similarly, in some viviparous skinks, females help the newborns emerge from their fetal membranes at birth.

In snakes, maternal attendance of the eggs or young commonly is found in large or venomous species such as crotalids and elapids, but also occurs in some species of other families (Stahlschmidt and DeNardo, 2011). For example, in some *Psammophylax* of the family Colubridae, female snakes coil around their developing eggs for up to 6 weeks. Pythons of at least 3 genera bask in the sun, and return to the nest to transfer heat to the eggs. In at least a few python species, females warm their eggs by generating heat via muscle contraction.

Some squamates exhibit maternal care of the young after birth or hatching (While *et al.*, 2015). Such care ranges from tolerance of the offspring to their active defense. Various geckoes and skinks aggressively protect their young from conspecific predators. In certain viviparous pit vipers, the female remains with the young for several weeks after birth. In *Egernia* skinks (in which males and females can maintain long-term pair-bonds), juveniles can remain in the parental home range for years.

Parental care is hard to observe in nature, and probably is more broadly distributed among reptiles than we now recognize. Such care has a long evolutionary history; fossil evidence indicates that several species of extinct dinosaurs had parental care of the eggs and young. Thus, the well-known patterns of parental care in birds may have had their origins in their dinosaurian ancestors.

Viviparity

Although most reptiles lay eggs, viviparity (live-bearing reproduction) occurs in nearly 20% of squamates, including representatives of 27 families (Blackburn and Stewart, 2011; Stewart and Blackburn, 2015). Among its advantages, viviparity allows the pregnant female to incubate her embryos at optimal temperatures for development. In addition, viviparity helps protect eggs from predation and from environmental threats (such as temperature extremes and dehydration). Viviparity also allows species to inhabit environments unsuitable for egg-laying, such as aquatic and arboreal habitats. Much evidence supports the idea that viviparity allows pregnant females to manipulate conditions of gestation to promote neonatal quality and/or maternal survival. In addition, during gestation, the female provides oxygen, water, and calcium to the embryos via placental organs formed from apposition of fetal membranes to the oviduct lining. In some species, most of the nutrients for development are provided by the placentas, which are correspondingly specialized morphologically. Viviparity has been shown to have evolved among squamates in at least 115 separate clades. This pattern is a focus of considerable research with regard to how it is accomplished and how and why it has evolved (Blackburn, 2006).

Parthenogenesis

Some reptiles reproduce asexually via parthenogenesis. In this pattern, females produce female offspring without the need for fertilization (Pough *et al.*, 2016). This pattern occurs in more than 50 lizard species from 8 families (including geckoes, lacertids, agamids, and teiids). It also has been documented in the blind snake, *Indotyphlops braminus*. Among the best-studied forms are teiid lizards of the genera of *Cnemidophorus* and *Aspidoscelis* of the American continents. As in other parthenogenic squamates, the unisexual teiids have resulted from hybridization of two bisexual species. Unisexual *Aspidoscelis* engage in courtship and “pseudo-copulation” in which one female mounts another, a pattern that may enhance gonadal activity. Because reproduction involves clonal inheritance, parthenogenic offspring lack the genetic variation of sexual species. In the absence of genetic diversity, evolution by natural selection is inhibited; thus, such populations have poor prospects for long-term survival. In several species of snakes as well as monitor lizards, individual females have exhibited facultative parthenogenesis under conditions of captivity. This pattern occasionally has been documented in nature among snakes, a situation of current interest due to its potential evolutionary significance (Booth and Schuett, 2016).

References

- Blackburn, D. G. (1998). Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. *J. Exp. Zool.*, 282, 560–618.
- Blackburn, D. G. (2006). Squamate reptiles as model organisms for the evolution of viviparity. *Herpetol. Monogr.*, 20, 131–146.
- Blackburn, D. G., & Stewart, J. R. (2011). Viviparity and placentation in snakes. In D. Sever, & R. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 119–181). Enfield, NH: CRC Press.
- Booth, W., & Schuett, G. W. (2016). The emerging phylogenetic pattern of parthenogenesis in snakes. *Biol. J. Linn. Soc.*, 118, 172–186.
- Cree, A. (2014). *Tuatara: Biology and Conservation of a Venerable Survivor*. New Zealand: Canterbury University Press.

- Cree, A. (2015). Reproductive anatomy and cycles of tuatara (*Sphenodon punctatus*), an intriguing non-squamate lepidosaur. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 620–646). Boca Raton, FL: CRC Press.
- Krohmer, R. W., & Lutterschmidt, D. I. (2011). Environmental and neuroendocrine control of reproduction in snakes. In D. Sever, & R. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 289–346). Enfield, New Hampshire: CRC Press.
- Mathies, T. (2011). Reproductive cycles of tropical snakes. In D. Sever, & R. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 511–550). Enfield, New Hampshire: CRC Press.
- Méndez de la Cruz, F., Morán, M. L., Ríos, E. A., & Ibagüengoytia, N. (2015). Male reproductive cycles in lizards. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 302–339). Boca Raton, FL: CRC Press.
- Mesquita, D. O., Costa, G. C., Colli, G. R., et al. (2016). Life-history patterns of lizards of the world. *Am. Nat.*, 187, 689–705.
- Pough, F. H., Andrews, R. M., Crump, M. L., et al. (2016). Reproduction and life history of reptiles. *Herpetology* (fourth ed.). Sunderland, MA: Sinauer (Chapter 9).
- Ramírez-Pinilla, M. P., de Pérez, G. R., & Alvarado-Ramírez, C. (2015). Oogenesis and the ovarian cycle. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 213–252). Boca Raton, FL: CRC Press.
- Shine, R. (1988). Parental care in reptiles. In C. Gans, & R. B. Huey (Eds.), *Biology of the Reptilia*, 16 pp. 275–330). New York: Alan R. Liss Inc.
- Sanger, T. J., Gredler, M. L., & Cohn, M. J. (2015). Resurrecting embryos of the tuatara, *Sphenodon punctatus*, to resolve vertebrate phallus evolution. *Biol. Lett.*, 11(10), 20150694.
- Siegel, D. S., Miralles, A., Rheubert, J. L., & Sever, D. M. (2015). Female reproductive anatomy: Cloaca, oviduct and sperm storage. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 144–195). Boca Raton, FL: CRC Press.
- Stahlschmidt, Z. R., & DeNardo, D. F. (2011). Parental care in snakes. In D. Sever, & R. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 673–702). Enfield, New Hampshire: CRC Press.
- Stewart, J. R., & Blackburn, D. G. (2015). Viviparity and placentation in lizards. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 448–563). Boca Raton, FL: CRC Press.
- Van Dyke, J. U. (2015). Cues for reproduction in squamate reptiles. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 109–143). Boca Raton, FL: CRC Press.
- Vitt, L. J., & Caldwell, J. P. (2014). Chapter 4: Reproduction and life histories, and Chapter 5, Reproductive modes. *Herpetology* (fourth ed.). London: Academic Press.
- While, G. M., Halliwell, B., & Uller, T. (2015). The evolutionary ecology of parental care in lizards. In J. L. Rheubert, D. S. Siegel, & S. E. Trauth (Eds.), *Reproductive Biology and Phylogeny of Lizards and Tuatara* (pp. 590–619). Boca Raton, FL: CRC Press.

Bird Reproduction Overview

Elisabeth Blesbois, INRA (French National Institute of Agronomic Research), Paris, France

© 2018 Elsevier Inc. All rights reserved.

Introduction

Birds constitute a unique vertebrate animal class which physiology has evolved in order to permit flight and adaptation to highly different environments and climates. In addition to other specific physiological adaptations such as air bags and feathers, birds have developed an original reproductive process that allows an efficient transmission of the genome from one generation to the next in changing environments. The bird females produce telolecithal (large amount of vitellus at the vegetal pole) hard-shelled eggs, enabling the autonomous development of the embryo in a highly protected microenvironment. In most birds, only one oviduct is present in the adult female allowing a good management of the abdominal space that is expected to receive daily (in laying hens, common ducks and quails) a very big egg highly enriched in vitellus, and surrounded by white albumen and shell. The female is the heterogametic sex since the female chromosome pair is ZW while the male pair is ZZ. The presence of sperm storage tubules (SSTs) in the single avian oviduct dedicated to prolonged sperm storage provides “adaptive freedom” to the female in order to sustain fertilizing potential even in the absence of males. Most birds face annual reproductive cycles that vary with the climates. In mild climates the winter/spring growing photoperiods are the main factors acting to induce bird reproduction cycles while in desert climates, temperatures and water availability are important for the reproductive success. All the reproductive features observed in birds have a unique objective, ensure the highest viability of the progeny. In the migratory species, the young birds of the year need to be strong enough at the time of the migrations in order to support long flights and food privations. For that reason also, when eggs are destroyed, or removed for the nest, the bird female is able to lay an added egg. Humans have used this property to increase the egg production in domestic birds. Joined to an efficient system of artificial incubation of the embryo, the egg and chick production have consequently been considerably increased during the last centuries in species such as the chicken.

Based on this strategy (removing the eggs to stimulate added egg production, embryonic development through a “host” system), a mean of 15 bird species have been domesticated since more than 7000 years allowing a diversification in human diet. Egg and meat poultry production constitute nowadays one of the main animal protein source for human diet.

In this article, we will present the main specific features of the bird reproductive processes that contribute to the species evolution and are of huge importance for the management of genetic resources and biodiversity.

A Specific Female Reproductive Tract

The female bird reproductive system presents common features with reptiles by their oviparity and the long storage of sperm in specific places of the female tract. It shows also common features with mammalian reproduction by the chromosome determination of the sex and by the constant temperature needed for the oogenesis. But female bird reproduction presents specific features: the development in most bird species of a unique ovary and oviduct (with the exception of the some raptors or the kiwi), the structure in ovarian cluster of the ovary that makes precise the vitellus incorporation in the oocyte, the follicular hierarchy, the oocyte structure, the very high specialization of different parts of the oviduct.

An Ovarian Bunch With a Unique Follicular Hierarchy for the Production of Oocytes Enriched in Vitellus

The development of the ovary starts early in the embryonic life in the hen. Undifferentiated gonads are already visible at 3.5 days of incubation. The primordial germ cells (PGCs) that are in multiplication since the lay, migrate from the epiblast to the embryonic gonad via the blood circulation at approximately 5.5 days of incubation. Then the female PGCs follow their multiplication and start their differentiation in active oögonies that multiply under endocrine control (FSH, the sexual steroids estradiol and progesterone). In most cases, the right and left genital tracts start their development during the early embryogenesis but the right ovary and oviduct rapidly regress (day 8 of incubation in the hen) under the control of the AMH produced by the left ovary (Jacob and Bakst, 2007).

The entry in meiosis of the oögonies starts at approximately 14 days of incubation in the hen. The left ovary contains a mean of 500,000 primary oocytes (Stage prophase diplonema) at the time of hatching but only a small number of these oocytes is recruited at the puberty in order to undergo the next stages of maturation. The oocytes are in the cortical part of the ovary at hatching, and somatic cells (future follicular envelopes) rapidly surround them. The undifferentiated follicles (cortical follicles) grow up to the 3 months (low growing) and then stop up to the puberty (12–18 weeks old). The follicles, recruited under the FSH control after the puberty, undergo a new growing stage and accumulate proteins and a few lipids to form the white follicle. It then migrates at the ovary surface and undergoes great growing (hierarchical folliculogenesis, 5 days length in the hen) during which the meiosis maturation and the deposition of lipoproteic compounds of the vitellus occur. The follicles sequentially grows from the F6 stage (small yellow follicle) up to the F1 stage (ovulatory follicle, Figs. 1 and 2). They do not share an antral cavity as in mammals. The oocyte is surrounded in the follicle by granulosa cells (one layer) and by two theca (internal and external, of mesenchym origin). The theca produce the estradiol in birds, the granulosa produces mainly the progesterone. The external theca production of estradiol is already maximal at the early great growing follicular stage and then decreases from the F4 stage to disappear at

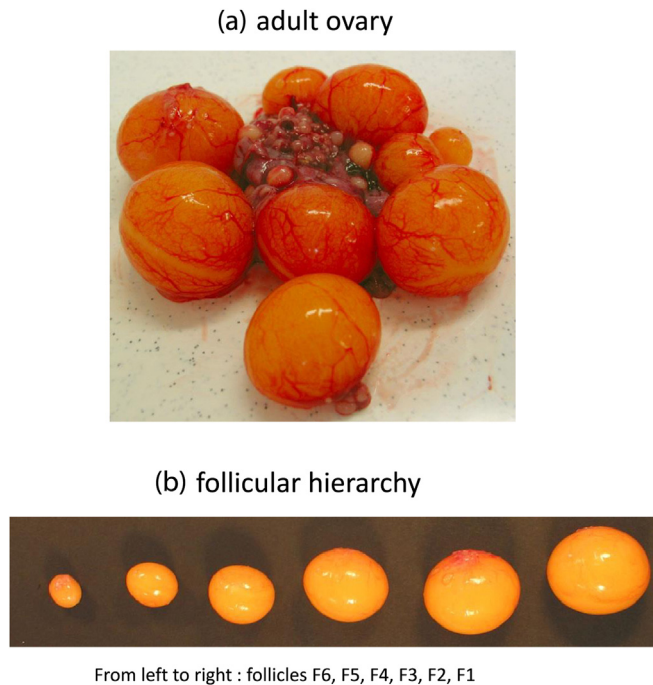


Fig. 1 Hen ovary and follicles (Photos Elis S., INRA). (a) The ovary includes in its central part small follicles named white follicles that have not yet started to accumulate vitellus. At the periphery of the ovary the yellow follicles are under rapid growing and accumulation of vitellus. The biggest follicle is the F1 follicle ready to be ovulated. (b) From the left to the right, the different stages of the follicular hierarchy, from F6 to F1. Reproduced from Blesbois, E., 2011. Gamètes et fécondation chez les oiseaux. Inra Productions Animales 24(3), 259–272.

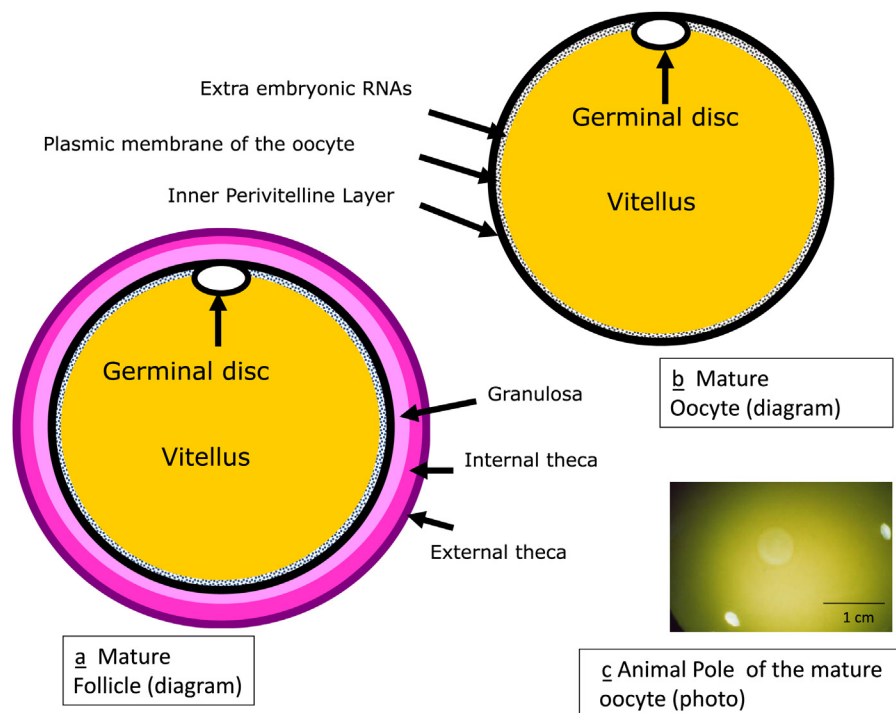


Fig. 2 Follicle and oocyte (Diagrams and photo Elis, S. INRA). (a) Diagram of F6 follicle just before the ovulation. The germinal disc contains the female pronucleus and is located in an area of the oocyte names “animal pole”. The opposite area that contains only vitellus and RNAs is called “vegetal pole”. (b) Diagram oocyte at the time of ovulation. The somatic cells of the follicle have been removed at ovulation. (c) Photo of the animal pole of the desmature oocyte. The central more dense area is the germinal disc. Reproduced from Blesbois, E., 2011. Gamètes et fécondation chez les oiseaux. Inra Productions Animales 24(3), 259–272.

the F1 stage while the granulosa production of progesterone increases, inducing at the end the ovulation under LH control. The mature oocyte is then captured by the infundibulum part of the oviduct (equivalent to the mammalian ampulla) by peristaltic movements. The mature ovulated oocyte is very different from the mammalian one. Its main diameter varies a lot with the species (8–10 cm in the ostrich, 3–5 cm in the hen, only 1 cm in some Passeriformes). The mature oocyte is mainly composed of vitellus (or yolk including lipoproteic fractions, proteins, steroids, thyroid hormones, growth factors, etc.) needed to ensure the diet and growing of the future embryo. A layer of “extra-embryonic” RNA with unknown role is present at the periphery of the vitellus. At the animal pole of the vitellus, the germinal disc contains most of the cellular organelles, a stock of “embryonic” RNA, different proteins, vitellus and the haploid female pronucleus containing, among others, the Z sexual chromosome (to make a male chick) or the small W (to make a female chick). A plasmatic membrane and an extracellular inner perivitelline layer (IPVL) surrounds the ovulatory oocyte. The IPVL is mainly composed of ZP proteins and is the initial point of contact between sperm and oocyte at fertilization. This IPVL is more or less the homologous of the fish chorion or the mammalian zona pellucida. However, contrary to the zona pellucida, the bird IPVL authorizes the transfer of 10–15 spermatozoa inside the oocyte and is not an interspecies barrier. This last property permits interspecies hybrids (i.e., mule ducks, pheasant-hen, quail-hen hybrids). Some IPVL ZP show original synthesis places since the liver synthesizes the ZP1, the granulosa synthesizes the ZP3 and the oocyte the ZPD (Elis *et al.*, 2008).

In case of fertilization, the oocytes RNA ensure alone the high number of zygote cells multiplication during the first 24 h of the embryo development in the oviduct.

A Multi-Functional Oviduct

The left oviduct develops in the young embryo from the left Muller canal while the right one regresses. At sexual maturity (20–25 weeks old in the hen), the oviduct is a long tube composed of five specialized areas (Fig. 3; Sauveur, 1988), suspended by two ligaments at the abdominal ring. The upper part of the oviduct is the infundibulum where the ovulated oocyte is captured. The infundibulum is the site of fertilization. It contains semen storage places (tubules or folds depending on the species) where the sperm that come from the SST of the utero-vaginal junction may wait for the oocyte. After the fertilization that occurs less

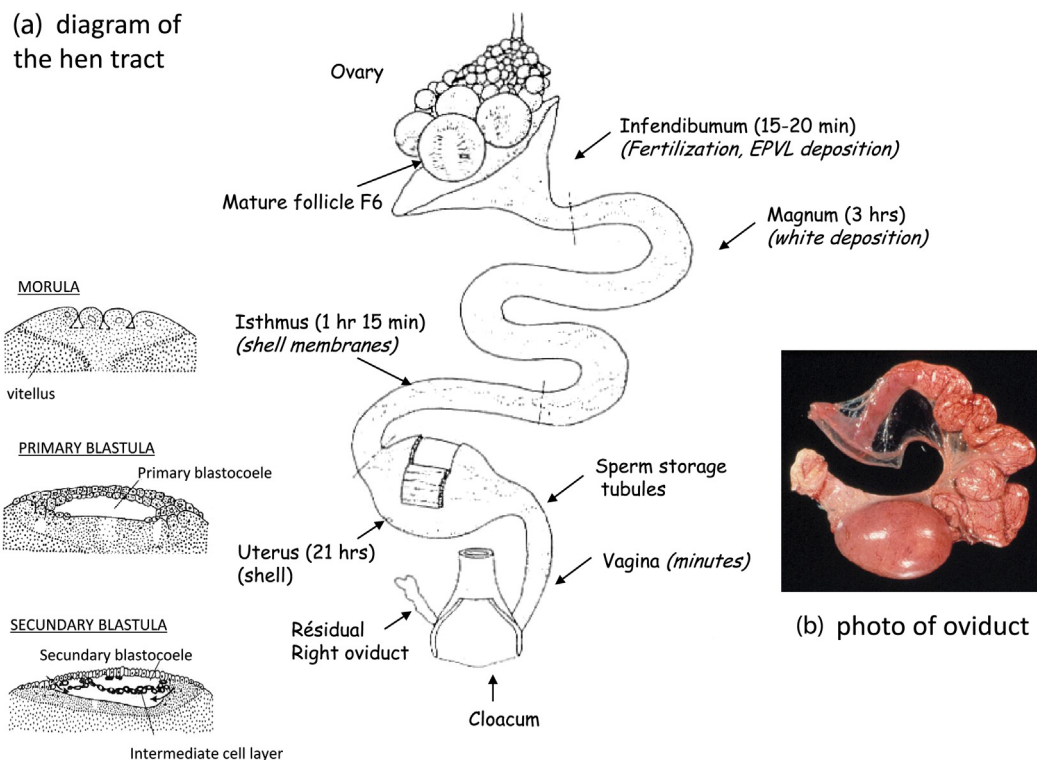


Fig. 3 The hen genital tract. (a) Diagram of the female tract (Reproduced from Sauveur, B., 1988. *Reproduction des volailles et production dœufs*. INRA Paris, ISBN 2-85340-961-9 p. 20, 231.). The central part of the figure represents all the segment of the tract, from the ovary in the upper part of the diagram to the opening of the vagina in the cloacum, in the lower part of the diagram. The fine lines are artificial representations of the delimitations of each part of the upper oviduct (infundibulum, magnum, isthmus). The time indicated near to the name of each compartment represents the mean time spent by the egg in each part of the oviduct. On the left part of the diagram, there are annex diagrams representing the stage of the embryo development in the corresponding part of the oviduct on its right. First divisions of the zygote in Morula in the isthmus (the four cells of the morula described here are in syncytium (opened cells)). Primary blastula in the upper uterus. Secondary blastula when the egg is oviposited (or layed). (b) Photo of a hen oviduct with an egg in the uterus (shell gland).

than 30 min after the ovulation, an external perivitelline layer (EPVL) is secreted in the infundibulum around the fertilized oocyte in order to increase the cohesion and strengthen of the egg. The EPVL contains many components with proteins such as lysozyme, ovomucin, ovomucoid, VMOI I. The fertilized oocyte, now more robust, spends then 3 h in the second part of the oviduct, the magnum that secretes the main proteins of the albumen or egg white (more than 12 different protein families, 50% of ovalbumin). During the stay in the magnum, at the germinal disc, the fusion of the male and female pronuclei occurs 3 h after the fertilization. The first divisions of the zygote takes place 1 h later in the isthmus giving a morula. The white egg hydration occurs also in the isthmus where the egg stays approximately 1.5 h and continue in the uterus (or shell gland) where it stays a mean of 20–22 h in the hen. The isthmus is also the place of secretion of the shell membranes and a first part of the shell, the rest being deposited in the uterus. The divisions of the new zygote continue at a very high rate during the stay of the embryo in the uterus. They are firstly perpendicular to the surface of the germinal disc, then parallel. The morula moves to a blastula and a subgerminal cavity, the blastocoele appears. After passage through the uterus, the whole shell surrounds the egg that rapidly cross the vagina with the help of peristaltic contractions before the oviposition (lay). The oviposition is also the time of the first activation of the young embryo genome. At this stage, the embryo (secondary blastula) contains 30,000–50,000 cells in the hen. At the mother endocrine level, a LH pic precedes the oviposition and has an action to stimulate the next ovulation that occurs approximately at the same time in the upper oviduct of the laying hen.

The embryo is then ready to follow its development under conditions of broodiness or artificial incubation, but it may also stay some weeks without development, waiting for the good conditions for its new history.

A Quite Simple Male Reproductive Tract

The avian male reproductive system is much simpler than the female (Fig. 4; De Reviers, 1988). The two internal testis, close to the kidneys, produce sperm in one-two weeks depending on the species (12 days in the chicken). The spermatogenesis occurs at the internal body temperature (41°C in most birds) at the opposite of mammals. After their liberation from the testis, the sperm undergo an “expeditious maturation” in the epididymis and deferent ducts that do not contain annex glands. The sperm are expelled at mating from the deferent duct to the female vagina, via genital papillae through a cloacal eversion of the two partners. A small number of bird species have a phallus (i.e., muscovy duck).

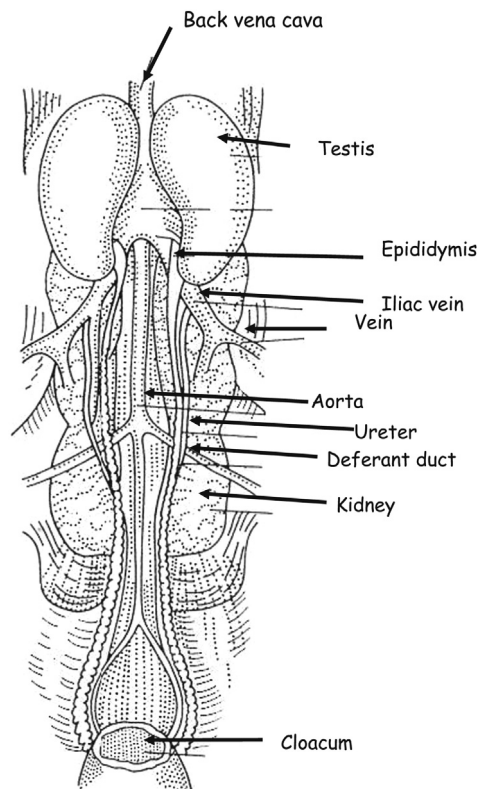


Fig. 4 Diagram of the internal male genital tract of a male fowl in reproduction. De Reviers, M., 1988. *Le Mâle en Reproduction*. Reproduction Des Volailles et Production d'œufs. Paris: Ed INRA, pp. 141–206.

The early differentiation of the gonad in testis occurs at 5.5 days of incubation in the chicken. The male PGCs that share the Z sexual chromosome migrate into the young gonad at this stage, and sertoli cells are placed around the PGCs, and seminiferous rings are rapidly visible. The embryonic mesonephros contributes to the differentiation of interstitial cells in leydig cells. The Wolf canal differentiates into epididymis and deferent canal as in mammals. The two male tracts follow their growing after hatching. Sertoli cells proliferate up to 7 weeks of age in the chicken and then differentiate. In the adult, the spermatogenesis occurs in seminiferous tubules that account for 90% of each testis volume. By contrast to mammals, the sperm that leave the testis already possess most of their fertilizing capacity and a part of their motility. During their transit in the epididymis and deferent duct, they acquire their full motility capacity in a rapid trip (24 h in the chicken). The principle of the sperm production seems to be, make the highest number of sperm (1–5 billion sperm/ejaculate in the chicken) as fast as possible.

The Hard Life of Bird Sperm

By opposite to the “expeditious” gamete production in the male, a difficult travel wait for the sperm that arrive in the female vagina. The competition is very hard and will be present even after the entry of many sperm inside the oocyte for the polyspermic fertilization.

The Sperm

Bird sperm are thin and long cells composed of a head (acrosome and nucleus), a midpiece and a flagellum (Fig. 5; reviewed by Blesbois, 2012). The acrosome is small and contains the acrosome vesicle that constitutes a calcium reservoir and contains the

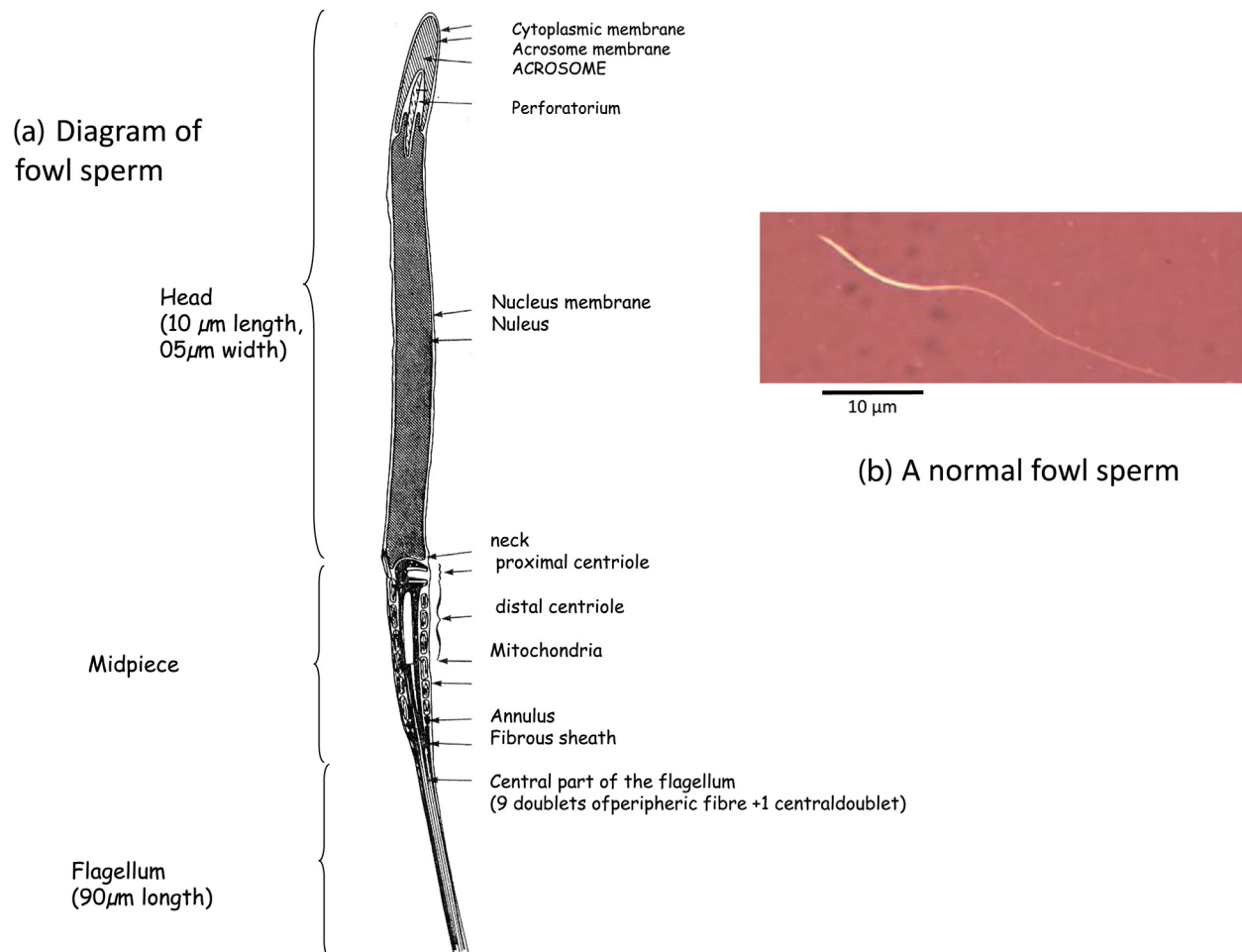


Fig. 5 The fowl sperm. (a) Diagram of the main parts of the sperm, from the acrosome in the upper part of the diagram to the start of the flagellum in the lower part of the diagram (Reproduced from De Reviers, M., 1988. *Reproduction des volailles et production d'œufs*. INRA Paris, ISBN 2-85340-961-9, p. 20, 151.). (b) A photo a fowl sperm colored with eosin-nigrosin (the viable sperm appears more or less white and the environment red) observed under phase contrast microscopy (photo I. Grasseau, INRA). Reproduced from Blesbois, E., 2011. *Gamètes et fécondation chez les oiseaux*. Inra Productions Animales 24(3), 259–272.

proteolytic enzymes needed to hydrolyze the IPVL and penetrate inside the oocyte at fertilization. A perforatorium (mainly actine) is present under the acrosome vesicle but its role is still unknown in birds. The nucleus is highly condensed for an optimal protection of the genes during the sperm travel. A helicoidally ring surrounds the sperm head only in specific species (Passeriformes). The mid-piece is composed of a proximal and a distal centriole for the motility need, and of a mitochondrial sheath that contributes to the energetic metabolism. Sperm motility is required for key actions such as reach the SSTs and initiate fertilization. Mitochondria are also present in a part of the flagellum of some species such as the quails. Finally, the number of sperm mitochondria varies a lot with the species: a mean of 30 in the chicken and the turkey, 1000 in the quail. The flagellum is the longest part of the sperm (70–90 μm in the chicken, 140 μm in some quails). It contains an axoneme that follows the distal centriole and contains one central doublet and nine distal doublets made of microtubules containing dynein dimers (close to mammalian sperm axoneme). In most birds (except Passeriformes), the axoneme is surrounded by a fibrous sheath in the longest part of the flagellum.

At the ejaculation time, sperm are surrounded by a seminal plasma that contains many compounds. Some of them are suspected to protect sperm to premature proteolytic attacks, other to sustain metabolism and other to be only by products of degradation (Blesbois, 2012; Labas *et al.*, 2015).

Conservation and Selection of Sperm in the Female Tract

When sperm arrive in the vagina, the seminal plasma is rapidly discarded and a hard selection starts. Only 1% of the sperm deposited in the vagina reach the SSTs, then 1% of them reach the fertilization site in the infundibulum. 99% of the male gametes are thus eliminated during their transit in the female tract that acts as a succession of filters. The selection to reach the SSTs depends on the motility capacity of each sperm and of different mechanisms including immune actions or vaginal peristalsis (reviewed by Blesbois and Brillard (2007), Stepinska and Bakst (2007), and Blesbois (2012)). The SSTs constitute one layer tubules glands (Fig. 6). Their number differ a lot with the species 150–20,000 depending, among others, on the length of storage of sperm in the SSTs (5 days in some Passeriformes, 3 months in Turkeys). In the hen, the length of fertile period after a single insemination or mating is close to 3 weeks with an optimal fertility (often near to 100%) in week one and then a gradual decrease. The sperm are released from the SSTs by mechanisms that are still discussed (i.e., quite passive; involving specific metabolic capacities, etc.) and cross the upper parts of the oviduct where they meet the previously fertilized egg that goes in the opposite way. In the uterus, the sperm face to an environment highly enriched in calcium (up to 15 mM) for the shell egg calcification, a factor that could be suspected to induce acrosome reaction (AR) of not well-protected sperm. Bird sperm do not undergo the so-called “capacitation” observed in mammals to prepare the gamete to fertilization by removing different protection systems. In birds, the acrosome reaction (RA) that allows sperm to release their enzymes contained in the acrosome vesicle and is the inaugural event of fertilization, occurs at the contact of the IPVL of the newly ovulated oocyte in the infundibulum. The RA needs extracellular calcium, is highly energy consuming and involves the activation of many signaling pathways (Lemoine *et al.*, 2009). Many sperm undergo the RA, and many of them

(a) Empty Sperm Storage tubules



10 μm

(b) Sperm Storage Tubules filled with sperm



10 μm

Fig. 6 Sections of sperm storage tubules in the hen (photos J.P. Brillard, INRA). (a) SSTs without sperm. The central lumen that is surrounded by the single SSTs cell layer is empty. (b) SSTs containing sperm. The central lumen is filled with sperm that appear in dark. Reproduced from Blesbois, E., 2011. Gamètes et fécondation chez les oiseaux. Inra Productions Animales 24(3), 259–272.

finally penetrate into the oocyte and differentiate in male pronuclei. The physiological polyspermy is denser at the animal pole of the oocyte. However only one male pronucleus merges with the female pronucleus in order to make a zygote. This access to the syngamy constitutes the last filter of selection of the male gamete.

Reproduction Cycles Adapted to Environmental Changes and/or Poultry Production Needs

Most of the wild species of birds show annual cycles of reproduction, the growing of the young birds occurring at the best moment of the year. In mild climates, spring is the best season for hatching, the reproduction is initiated by growing photoperiod and is maximum in long photoperiods. In tropical and equatorial areas where the variations of the day length are small, other outside stimuli such as available diets or water or suitable temperatures are more important. As an example, the sexual maturity of Australian Emus starts in (low) decreasing day length.

A “reproductive molt”, also called “premarital molt” (renewal of the feathers, often partial) indicates the entry in the annual reproduction period. Gonads grow at the same time. After this molt, the males often show a bright plumage that will support courtship rituals, and the females will prefer the most beautiful and robust males for mating! At the end of the reproductive period, another molt signs the removal of the reproductive plumage, the regression of the gonads and the stop of gamete production. Molt may be artificially induced in domestic birds in order to accelerate the renewal of the cycle.

In the domestic birds, the annual cycles are often less clear or even suppressed. The eggs freshly laid by the females are usually removed at each lay in order to stimulate new lays, if the photoperiod, diet and temperature conditions stay good. The fertilized eggs are incubated in order to produce chicks while the females continue to lay. This system has been the basement of the industrial production of poultry. Nowadays, the laying hens show a daily cycle of lay. At the same time, a hen lays one egg and an oocyte is ovulated in the infundibulum to be fertilized and laid the following day. In the poultry practice, the intervals between matings or artificial inseminations must take into account this daily cycle since the vaginal peristalsis that surrounds the lay, decreases the capacities of sperm to cross the vagina in order to reach the SSTs.

In the poultry breeding practice, an efficient genetic selection added to controlled photoperiods and diets adapted to the very high energetic level needed for an intense egg production, allowed the optimization of the lay. Laying hens, quails and common ducks may lay throughout the year with short “daily” cycles (up to more than 300 eggs produced per year in specific lines). It is not the case for the “meat” breeds. These breeds, selected mainly on their growing potential, often show poor reproduction capacities. They encounter problems of breeding, gamete production, mating, behaviors that depend on the species but must be taken into account in the selection programs of the 21st century.

Different reproductive biotechnologies have also emerged in birds: artificial incubation, artificial insemination, semen storage (reviewed by Blesbois, 2011), Primordial Germ cells methodology (reviewed by Whyte *et al.*, 2016) rely on the specific features of bird reproduction, and contribute to the growing of the poultry productions and also to conservation programs through the world.

Conclusion

Bird reproduction involves oviparity and a very complex system of internal fertilization. If the male shows highly visible external sexual attributes, its internal reproductive system turns toward an expeditious production of a maximum of gametes in a short time. By opposite, the female genital tract is very sophisticated, reaching a very high level of precision of the different steps leading to the egg production that has been the support of the development of the poultry productions. Modern laying hens export in their eggs more than 10 times their weight per year for the daily egg production, and the poultry productions constitute nowadays one of the first protein source for the human diet need.

References

- Blesbois, E. (2011). Freezing avian semen. *Avian Biology Research*, 4, 44–50.
- Blesbois, E. (2012). Biological features of the avian male gamete and their application to biotechnology of conservation. *Journal of Poultry Science*, 49, 141–149.
- Blesbois, E., & Brillard, J. P. (2007). Specific features of in vivo and in vitro sperm storage in birds. *Animal*, 1, 1472–1481.
- De Révières, M. (1988). Le Mâle en Reproduction. *Reproduction Des Volailles et Production d'oeufs* (pp. 141–206). Paris: Ed INRA.
- Elis, S., Batellier, F., Couty, I., et al. (2008). Search for the genes involved in oocyte maturation and early embryo development in the hen. *BMC Genomics*, 9(110), 1–20. Available at: <http://www.biomedcentral.com/content/pdf/1471-2164-9-110.pdf>.
- Jacob, M., Bakst, M.R., 2007. Development anatomy of the female reproductive tract. In: Jamieson, B.J.M., (Ed.), *Reproductive Biology and Phylogeny of Birds*, vol. 6A, 401–479. pp. 149–181.
- Labas, V., Grasseau, I., Cahier, K., et al. (2015). Qualitative and quantitative proteomic approaches to phenotyping chicken semen. *Journal of Proteomics*, 112, 313–335.
- Lemoine, M., Dupont, J., Guillory, V., Tesseraud, S., & Blesbois, E. (2009). Signaling pathways involved in the chicken acrosome reaction. *Biology of Reproduction*, 81, 657–665.
- Sauveur, B. (1988). *Reproduction Des Volailles et Production D'oeufs*. Paris: Ed INRA.
- Stepinska, U., Bakst, M.R., 2007. Fertilization. In: Jamieson, B.J.M., (Ed.), *Reproductive Biology and Phylogeny of Birds*, vol. 6A, 401–479, pp. 553–597.
- Whyte, J., Blesbois, E., & McGrew, M. (2016). Increased sustainability in poultry production: New tools and resources for genetic management. In E. Burton, J. Gatliffe, H. M. O Neil, & D. Scholey (Eds.), *Sustainable poultry production in Europe* (pp. 214–233). CABI Publishing (Chapter 12).

Chicken: Female Reproduction

Ramesh Ramachandran, Pennsylvania State University, State College, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Albumen Egg white, a mixture of proteins secreted by the oviduct.

Broiler A chicken selected for rapid growth and meat production.

Broiler breeder parent Parents of chicken selected for rapid growth and meat production.

Commercial egg layer Chickens bred for egg production.

Egg A hard shelled structure encasing albumen and yolk-filled oocyte.

Oviduct Female reproductive tract that produces albumen, shell membrane, and shell and lays around yolk-filled oocyte.

Oviposition Expulsion of egg from the female reproductive tract.

Ovulation Release of yolk-filled oocyte by rupturing the largest follicle.

Prehierarchical follicle Ovarian follicles that are 6–12 mm in size.

Preovulatory follicle Large ovarian follicles that are greater than 12 mm in size.

Small white follicle Small follicles that are less than 5 mm in size and appear white in color.

Sperm storage tubule Folds of mucus membrane at the uterovaginal junction that stores sperm.

Yolk Lipoproteins stored in the oocyte cytoplasm.

Bullet Points

- Commercial egg-type chickens are genetically selected to lay 280–300 eggs in 52 weeks.
- Appropriate stimulation of hypothalamic photoreceptors and activity of several groups of hypothalamic neurons are critical to the onset and sustenance of ovarian follicular development and maturation.
- Ovarian follicular selection, maturation and ovulation is a highly coordinated process regulated by pituitary gonadotropins and intra-ovarian growth factors.
- Different regions of oviduct secrete albumen, shell membrane, and shell components.
- Broiler breeder parent hens fed *ad libitum* develop excessive ovarian follicles and have poor egg production.
- Egg-type chickens develop epithelial ovarian carcinoma likely due to high rate of ovulation within 3 years of age.

Introduction

The modern day chicken (*Gallus gallus*) reared for table egg production and meat production are descendants of Red jungle fowl and have undergone intensive genetic selection for several economically important traits such as egg/meat production, feed conversion efficiency and livability. As a result, the meat-type or broiler chickens grow rapidly and reach market weight in approximately 42 days after hatch. During this period, broiler chickens voraciously eat 4.1 kg of feed to achieve a 40-fold increase in body weight. Egg-type chicken is a prolific egg layer and lay 280–300 eggs over a period of 52 weeks. Such a high level of egg production is initiated and sustained by coordination among several organ systems, most notably hypothalamus, anterior pituitary gland, ovary, liver, and medullary bones. This article will use egg-type hens and broiler breeder parent hens to describe the control of reproductive system. As in mammals, the reproductive system in chickens is driven by hormones secreted by hypothalamus, pituitary gland, and ovary in response to a variety of external and internal stimuli.

Hypothalamus

Reproduction in chickens is primarily driven by neuropeptides secreted from hypothalamus (reviewed in [Joseph et al. \(2013\)](#)). Gonadotropin releasing hormone-I (GnRH-I), a hypothalamic decapeptide, is the primary neuropeptide responsible for controlling reproduction. Immunoreactive GnRH neuronal cell bodies are found in several areas of the brain most notably in olfactory bulb, hypothalamic septal and preoptic areas. GnRH-I immunoreactive axonal fibers are distributed in hypothalamic septal, preoptic, and tuberal regions. Dense immunostaining of GnRH-I axons are noticeable in organum vasculosum lamina terminalis and in the external zone of the median eminence. The latter provides an access to the hypothalamo-hypophyseal portal blood vessel to transport GnRH-I to the anterior pituitary gland. Chickens also express another isoform of GnRH, GnRH-II, whose cell bodies are located in the mid-brain and the GnRH-II axons were not found in the median eminence or in other areas of the hypothalamus. Immunoneutralization of GnRH-I but not GnRH-II results in decreased plasma LH levels and regression of the reproductive system suggesting that only GnRH-I is involved in controlling LH secretion from the pituitary gland. GnRH-II is likely to be involved in reproductive behaviors.

Regulation of GnRH-I Neurons

Photoreception

The domestic chicken is a long-day breeder; photoperiod duration greater than 14 h per day stimulates the hypothalamic-pituitary-gonadal axis. Numerous studies have attempted to identify the sites of photoreception and found that photoreceptors in eyes, pineal gland, and neurons in the hypothalamic suprachiasmatic nucleus as found in mammalian system are not important for perceiving photoperiodic signals in avian species. On the contrary, deep brain photoreceptors located in four hypothalamic nuclei (lateral septal organ, paraventricular nucleus, premammillary nucleus, and paraventricular organ) are capable of light perception (Kuenzel *et al.*, 2015). Neurons in these hypothalamic nuclei were found to express classical photopigments such as melanopsin/opsin 4, neuropsin/opsin 5, and vertebrate ancient opsin. Communication between deep encephalic photoreceptor sites and GnRH-I neurons remains to be elucidated. Certain wavelengths of light within the visible light spectrum are more stimulative to the photoreceptors than others. Both sighted and blind chickens exposed to red light or a combination of red, green, and blue lights laid more eggs than chickens raised with green light alone suggesting that red light was necessary to maintain ovarian activity and egg production (Baxter *et al.*, 2014).

Neuropeptides and Neurotransmitters

Both norepinephrine acting via α_1 adrenergic receptors and neuropeptide Y stimulates GnRH-I release from the neurosecretory terminals. Inhibitory control of GnRH-I secretion is exerted by dopamine and β -endorphin. Gonadotropin inhibitory hormone (GnIH), an RF-amide peptide, is likely to affect GnRH secretion and to inhibit gonadotropin secretion from the pituitary gland. RF-amides share a common carboxyl-terminal motif comprised of RF-NH₂ (R=arginine, F=phenylalanine). GnIH-immunoreactive neuronal cell bodies are found exclusively in the hypothalamic paraventricular nucleus, whereas the GnIH-axons are distributed throughout the brain including the external zone of median eminence, a region that has access to vasculature for transporting of GnIH to anterior pituitary gland. Immunohistochemical studies suggest that GnIH-axonal terminals lie in close apposition to GnRH-axons. GnIH may potentially be modulating the effects of photostimulation in avians. Melatonin receptor, Mel1c, is expressed in GnIH neurons and administration of melatonin restores GnIH mRNA levels following pinealectomy in quails. Melatonin was also found to increase release of GnIH peptide from hypothalamic explants. Recent studies suggest that green light inhibits GnRH-I expression by activating GnIH and GnIH receptor (GnIHR) expression in chicken hypothalamus. Green light exposure was found to increase GnIH content and GnIH-immunoreactive area in magnocellular region of hypothalamic paraventricular nucleus. Furthermore, green light also increases plasma melatonin levels with a concomitant decrease in expression of GnRH-I in neurons within nucleus of the pallial commissure (Zhang *et al.*, 2017). GnRH-I neurons also co-localized with GnIH receptor (GnIHR) while GnIH-neurons coexpressed melatonin receptor subtype quinone reductase 2. Another hypothalamic neuropeptide, kisspeptin that plays an important role in the onset of puberty and linking metabolic status with reproduction, has not been found in chickens. Both *Kiss1* and *Kiss2* genes and its receptor GPR54 have been presumed to be lost from the avian genome. Other neuropeptides that are known to affect reproduction in mammals such as dynorphin have not been explored in chickens.

Anterior Pituitary Gland

Reproduction is controlled by follicle stimulating hormone (FSH) and luteinizing hormone (LH), secreted by the anterior pituitary gland. Both FSH and LH are glycoproteins and composed of two-subunits: an α subunit that is common to FSH, LH, and thyroid stimulating hormone (TSH) and a β subunit which is unique to FSH, LH, or TSH. Gonadotropes or gonadotropin-secreting cells are found in both cephalic and caudal lobes of the pituitary gland. FSH and LH are secreted by two distinct populations of gonadotropes. FSH-producing cells are found in the periphery of the pituitary gland. LH-producing cells are more numerous than FSH-producing cells. FSH influences maturation of prehierarchical follicles and steroidogenesis in smaller follicles while LH promotes progesterone secretion from ovarian preovulatory follicles and induce ovulation.

Pituitary GnRH Receptor

Two GnRH receptors (GnRHR) have been identified in chickens: (1) GnRHR-I localized in the brain, pituitary, and gonads; (2) GnRH-2, also termed as GnRHR-III, expressed only in the pituitary gland. Both GnRHR are G-protein coupled receptors (GPCR) and when stimulated activates signal transduction pathways involving both inositol triphosphate and cyclic adenosine monophosphate (cAMP) suggesting the existence of both G_q and G_s subtypes of GPCRs. GnRH-I stimulates LH secretion by activating a pituitary-specific GnRHR (GnRHR-III). GnRH-III-immunoreactive cells are distributed throughout the adenohypophysis.

Pituitary GnIH Receptors

GnIH signals through a transmembrane receptor (GnIHR) that belongs to the GPCR family. Activation of chicken GnIHR was found to abrogate forskolin-stimulated intracellular increase in cAMP abundance suggesting that GnIHR is coupled to G_{α_i} and potentially inhibits adenylyl cyclase activity. GnIHR activation does not seem to affect intracellular inositol triphosphate levels

(Bedecarrats *et al.*, 2009). GnIHR-expressing cells are found in the chicken pituitary gland cephalic and caudal lobes wherein they colocalize with LH β mRNA-, or FSH β mRNA-containing cells. GnIHR mRNA quantity was significantly higher in the pituitaries of sexually immature chickens relative to sexually mature chickens (Maddineni *et al.*, 2008). Pharmacological doses of estradiol or a combination of estradiol and progesterone decreases GnIHR mRNA quantity suggesting that GnIHR gene expression is possibly down regulated in response to a surge in circulating estradiol and progesterone levels as the chicken undergoes sexual maturation to allow gonadotrophin secretion. Treating chicken anterior pituitary gland explants with GnIH peptide was found to decrease LH secretion. Although the presence of GnIH peptide in hypothalamo-hypophyseal portal blood is unknown, evidences suggest that GnIH may act as a counteracting system to GnRH stimulation within the pituitary gland (Baxter *et al.*, 2014).

Role of Gonadal Hormones on LH Secretion

Estradiol plays an inhibitory role in the control of LH secretion. Estradiol stimulates expression of progesterone receptor (PRR) in the pituitary gland. Progesterone acting via PRR either stimulates or inhibits LH secretion depending on the daily reproductive cycle of the hen. Inhibin, a hormone secreted by the granulosa cells of the preovulatory follicles, inhibits FSH β -subunit expression.

Ovary

The female reproductive system consists of a single ovary and oviduct in the chicken. By around day 10 of incubation, the right ovary and oviduct undergoes atresia under the influence of anti-müllerian hormone. The ovarian stroma and follicles are richly supplied with blood through ovarian artery that arises from the left renolumbar artery or the dorsal aorta. Abdominal and pelvic plexi of the sympathetic nervous system as well the posterior continuation of the sympathetic trunk innervate the ovary. The ovarian stroma is embedded with thousands of primordial follicles measuring less than 80 μ m in diameter that mainly consists of oocyte surrounded by perivitelline membrane (PV) and granulosa cell precursors (Fig. 1 Johnson, 2015). Primordial follicles remain in dormant state for months to years until activated for further development. Primary follicles measuring 80 μ m to 2 mm develop from this pool of primordial follicle by formation of basal lamina separating the granulosa cell layer from theca cell layer. The primary follicles are connected the ovarian stroma by means of a stalk that carries blood vessels and nerves. From the pool of primary follicles, several small white follicles measuring 2–6 mm emerge following deposition of protein-rich yolk but some of them further mature to

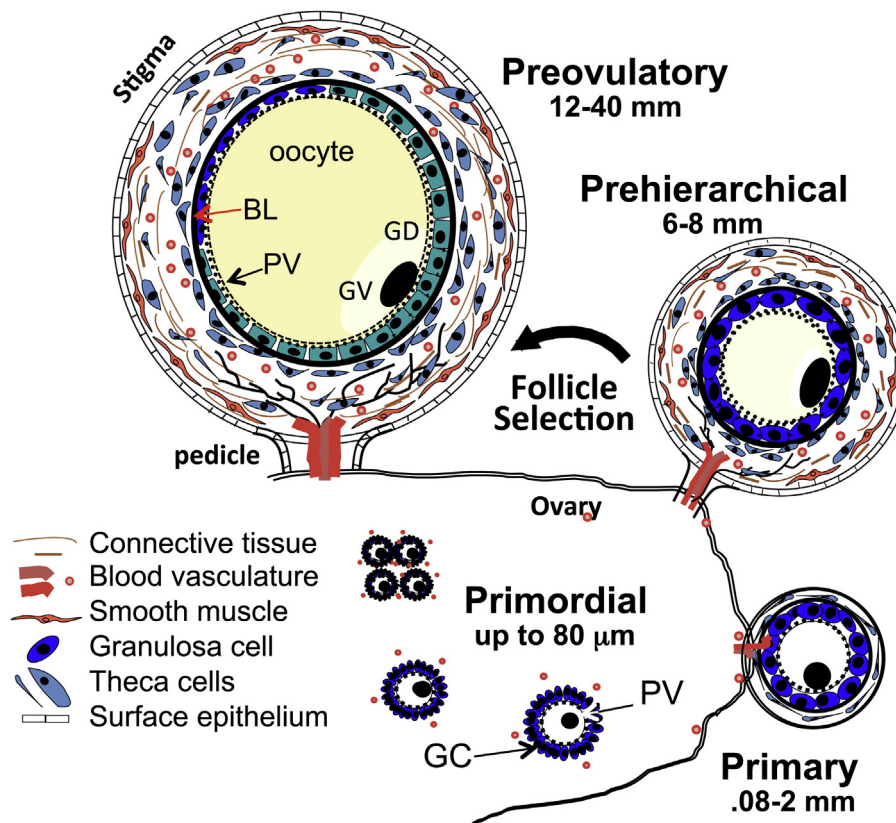


Fig. 1 Organization and structure of different types of ovarian follicles in a sexually mature hen. See Text for details. BL, basal lamina; GC, granulosa cell; GD, germinal disc; GV, germinal vesicle; PV, perivitelline membrane. Modified from Johnson, A.L., 2015. Reproduction in the female. In: Scanes, C.G. (Ed.), *Sturkie's Avian Physiology*, VI ed. Elsevier.

become prehierarchical follicles. Typically 8–12 prehierarchical follicles measuring 6–8 mm are present on the ovary and appear intense yellow due to the presence of carotenoids in the yolk. One of the prehierarchical follicles is selected almost every day in commercial egg-type chickens to undergo a rapid growth phase and join the preovulatory hierarchy. A typical preovulatory follicular hierarchy consists of 4–5 follicles termed as F1–F5 ranging in size from 12 to 36 mm in diameter (Fig. 2). F1 is the largest preovulatory follicle that will be ovulated next. A typical follicle consists of an oocyte whose cytoplasm is filled with vesicles of varying sizes each containing yolk lipoproteins (Fig. 3). The plasma membrane of the oocyte is surrounded by perivitelline membrane, a structure that is analogous to mammalian zona pellucida. Perivitelline membrane is composed of extracellular matrix and glycoproteins including zona pellucida proteins. A single layer of granulosa cells is found all around the perivitelline membrane. A basal lamina separates the granulosa cell layer and the theca layer. The theca layer is further divided into an internal and an external layer. While the theca cell layer is highly vascularized, the granulosa cell layer is avascular. Therefore, nutrients, hormones, and growth factors are transported by diffusion across basal lamina to reach granulosa cells and oocyte. Blood vessels are present throughout the surface epithelium of the preovulatory follicles except at stigma, the site at which the preovulatory follicle breaks open during ovulation to release the oocyte. The largest preovulatory follicle (F1) will undergo ovulation to release the oocyte. The post-ovulatory follicular membrane undergoes atresia over a 4–5 day period.

Follicular Selection and Maturation

Undifferentiated small white follicles (<1–3 mm) grow slowly under the influence of several autocrine or paracrine effects of growth factors and cytokines such as growth hormone, growth and differentiation factor-9 (GDF9), inhibins, activins, follistatin, and tumor necrosis factor- α (TNF- α). Rapid growth of multiple small white follicles is avoided by rendering both FSH receptor (FSHR) and vasoactive intestinal polypeptide receptor (VIPR) inactive by a mitogen-activated protein kinase activity. Removal of such inhibition and restoration of responsiveness to FSH and VIP leads to orderly follicle selection and entry of small white follicles into the pool of prehierarchical follicles (6–8 mm). A cascade of signal transduction events initiated by FSH signaling promotes transcription and translation of steroidogenic enzymes and steroid acute regulatory protein (STAR) leading to increased level of steroidogenesis. Members of the bone morphogenetic protein (BMP) plays a significant role in expression of FSHR and differentiation of granulosa cells in the 6–8 mm prehierarchical follicles. Granulosa cells are thought to be steroidogenically inactive

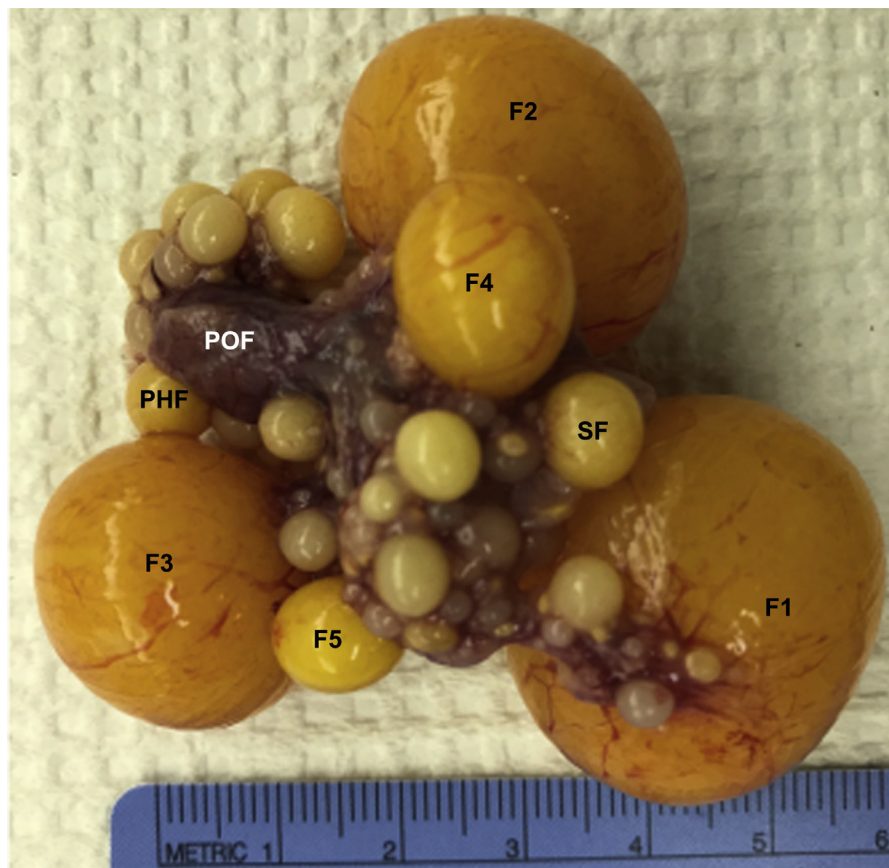


Fig. 2 Gross morphology of sexually mature chicken ovary. The ovary has 5 preovulatory follicles (F1–F5), one 9–12 mm follicle (SF) that is selected for further growth and future entry into the preovulatory hierarchy, several prehierarchical follicles (PHF, labeled on one), and post-ovulatory follicle (POF).

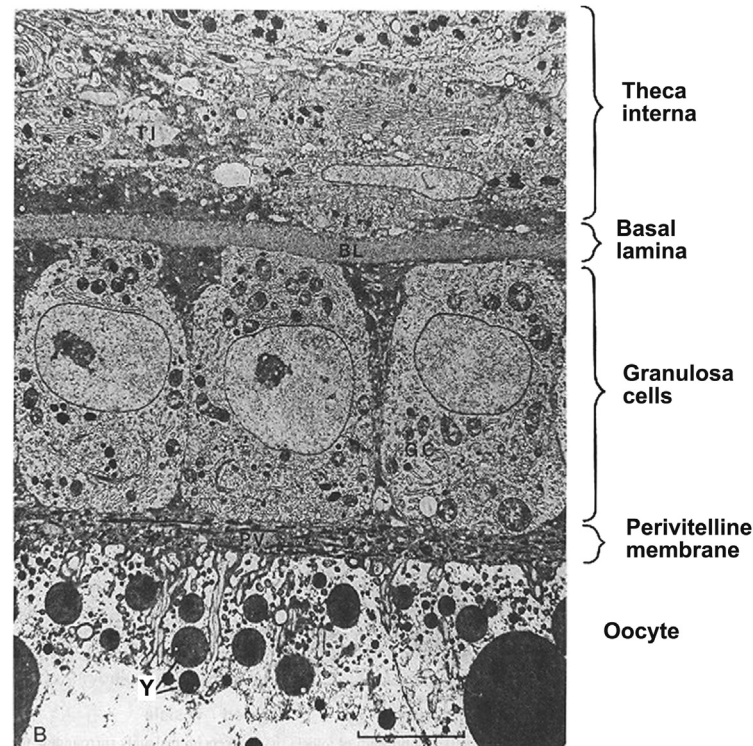


Fig. 3 Ultrastructure of a slow growing follicle. A part of the oocyte, perivitelline membrane (PV), a single layer of granulosa cells (GC), basal lamina (BL), and theca interna (TI) are shown. Note that oocyte has several yolk spherules (Y) and coated pits. Modified from Johnson, A.L., 2015. Reproduction in the female. In: Scanes, C.G. (Ed.), *Sturkie's Avian Physiology*, VI ed. Elsevier.

in follicles that are less than 10 mm in diameter while the cells located in the theca interna convert cholesterol to progestins and androgens. Androgens diffuse into the theca externa layer where they are converted to estrogens, a reaction catalyzed by aromatase. As the prehierarchical follicles enter the hierarchy of preovulatory follicles (>12 mm; also known as hierarchical follicles), they become more responsive to LH than FSH leading to heightened production of progesterone from granulosa cells. In addition, the granulosa cells produce inhibin A that inhibits FSH but not LH secretion from the anterior pituitary gland. Granulosa cells located in the germinal disc secrete BMP15 and GDF9 that control granulosa cell proliferation and progesterone production.

Role of Yolk Lipoproteins in Follicular Maturation

The precursors of yolk proteins (very low density lipoprotein and vitellogenins 1, 2, and 3) are synthesized in the liver under the influence of estradiol and gonadotropins and transported to the ovary through blood circulation. They diffuse out of the blood capillaries in the theca layer and pass through basal lamina and intercellular spaces between granulosa cells to reach the oocyte plasma membrane. Both very low density lipoprotein (VLDL) and vitellogenins (VTG) are transported into the oocyte by receptor-mediated endocytosis utilizing a 95 kDa VLDL receptor (VLDLR; reviewed in [Schneider \(2016\)](#)). Deposition of yolk lipoproteins is critical for further maturation and growth once a follicle enters the preovulatory hierarchy. Such requirement is evident from a mutant restricted ovulator (RO) line of the White Leghorn strain that carries a naturally occurring single nucleotide mutation in the VLDLR gene. Due to this mutation, RO hens fail to express a functional VLDLR protein on the oocyte membrane, which results in impaired uptake of circulating yolk precursor macromolecules. Consequently, VLDL and VTG accumulate in the blood, resulting in hypertriglyceridemia, hypercholesterolemia, and premature atherosclerosis. The RO hen ovary contains numerous large white follicles, small yellow follicles, and vitellogenic follicles, but most never reach full maturity, e.g., move from F4 to F1 in the follicular hierarchy.

Other Hormones Involved in Follicular Maturation

GnIH: A receptor for the hypothalamic GnIH was found to be expressed in both thecal and granulosa cell layers although existence of GnIH peptide in the circulation or in chicken ovaries has not been established. GnIHR mRNA quantity has been found to be lower in sexually mature hen ovaries compared with ovaries of sexually immature chickens. Treating sexually immature chickens with estradiol and/or progesterone decreases ovarian GnIHR mRNA abundance. Treatment of prehierarchical follicular granulosa cells with chicken GnIH peptide decreases basal but not FSH-stimulated cellular viability. Collectively, GnIHR may be involved in inhibition of ovarian follicular development. **Adipokines:** Leptin, an adipose tissue secreted hormone, affects energy balance and reproduction in mammals. Although mammalian leptin was discovered more than two decades ago, the authentic avian leptin

was discovered recently (Seroussi *et al.*, 2016). The avian leptin shares little homology to mammalian leptin and is expressed predominantly in the brain followed by ovaries. Considering the circulating levels of leptin are negligible, locally produced leptin in the ovary is likely to exert its effect through leptin receptor found both in granulosa and theca cells of preovulatory follicles. Adiponectin, an adipocytokine, is also involved in maintaining energy balance. Both adiponectin and two of its receptors, AdipoR1 and AdipoR2 are abundantly expressed in the ovarian granulosa and theca cells. Adiponectin is likely to affect gonadotropin or insulin-like growth factor-1 (IGF-1) induced progesterone production from preovulatory follicles.

Ovulation

Ovulation of the largest preovulatory follicle (F1) occurs by rupturing the follicular wall along the stigma with the help of proteolytic enzymes and vasoactive substances. The process is initiated by a surge in plasma LH levels 4–6 h prior to ovulation. An increase in plasma progesterone levels is required for initiating the preovulatory LH surge. Egg-type chickens ovulate every 24–28 h with a temporal rhythmicity that is regulated by expression of clock genes located in granulosa cells, liver, and hypothalamus. LH is thought to be the mechanistic link that integrates the hypothalamic circadian pacemaker with ovarian clock genes and therefore timing of ovulation.

Oviduct

Only the left oviduct is developed in the post-hatch chicken. Oviduct is a tubular organ (Fig. 4) consisting of infundibulum, magnum, isthmus, shell gland or uterus, and vagina. Oviduct is innervated by both sympathetic and parasympathetic nervous systems. The infundibular region starts as thin membranous funnel shaped organ that receives the oocyte following ovulation and retains it for 15–30 min. It is also the site where spermatozoa are stored and fertilization occurs. Infundibulum is followed by magnum, a long muscular and highly vascularized organ where the components of egg white (ovalbumin, lysozyme, conalbumin, and avidin) are secreted over a 3 h duration. Isthmus is a narrow tubular structure that is less muscular than magnum. This region secretes inner and outer shell membranes within a transit time of 1–2 h. The shell gland is a thick muscular organ where the soft-shelled egg is retained for 18–26 h at which time the shell gland transports 2.0–2.5 g of calcium from blood circulation to calcify one egg. Such large amounts of calcium are derived from two sources: (1) medullary bone (humerus, femur, and tibia) aided by parathyroid hormone and 1, 25 dihydroxyvitamin D₃; and (2) dietary calcium whose absorption is aided by 1, 25 dihydroxyvitamin D₃. Shell gland and vagina also secrete large quantities of mucin-rich fluid to coat the egg surface to prevent entry of pathogenic organisms into the egg.

Oviposition

Oviposition is the process of expulsion of egg from the shell gland by contracting the myometrium while relaxing abdominal muscles and the sphincter that separates shell gland and vagina. Arginine vasotocin acting via VT1 and VT3 receptors is likely to be involved in the contraction shell gland smooth muscle. The role of mesotocin, the avian homologue of oxytocin, in reproductive functions is unclear as the existence of a functional mesotocin receptor has not been identified. Prostaglandin F_{2α} and prostaglandin E and other factors secreted by the largest preovulatory follicle and/post-ovulatory follicular membrane may also aid in shell gland contraction during oviposition.

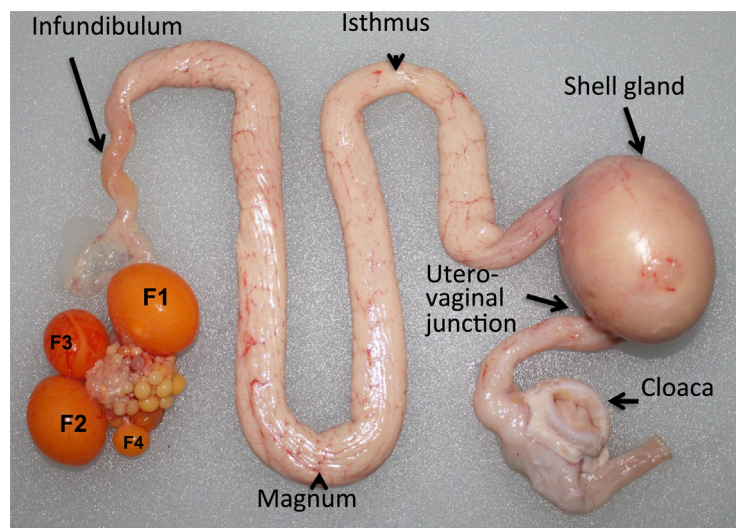


Fig. 4 Ovary and oviduct of sexually mature hen. F1–F4 Preovulatory follicles. F3 follicle has a prominent stigma. Reproduced from Johnson, A.L., 2015. Reproduction in the female. In: Scanes, C.G. (Ed.), *Sturkie's Avian Physiology*, VI ed. Elsevier.

Sperm Storage Tubules

Utero vaginal junction contains specialized sperm storage tubules that is lined by a mucosal surface to store spermatozoa for a period of 7–14 days. Upon insemination, the tubules are filled with sperm in a sequential fashion such that the spermatozoa from the latest insemination is released from the tubules first upon every oviposition. The sperm storage tubules lack innervation and smooth muscle for release of spermatozoa. However, hormonal factors such as progesterone may be involved in the release of spermatozoa from sperm storage tubule.

Reproductive Dysfunction

Excessive Follicular Recruitment in Broiler Breeder Hens

Ovarian follicular selection and maturation are extremely coordinated with ovulation occurring nearly every day in egg-type chickens. However, this process is deranged in the broiler breeder parent females leading to multiple follicles being selected simultaneously (Fig. 5). The desired economic traits in broiler chickens are body weight, growth rate, feed conversion efficiency, meat yield, breast muscle yield, livability, and leg strength. Most of the broiler selection traits are negatively correlated with egg production and fertility. Similar to their progenies, the broiler breeder parents are hyperphagic and become obese if provided unlimited access to feed. Consequently, broiler breeder hens lay only 160 eggs/hen during their economically productive life span compared with more than 280 eggs/hen laid by Leghorn hens. Excess feed intake appears to have direct deleterious effects on ovarian follicles. Therefore, broiler breeder hens are fed restricted amount of feed to prevent adiposity. Feeding broiler breeder hens with a diet that provides 30% more than the energy requirement leads to decreased egg production, follicular atresia, and early ovarian regression associated with increased levels of pro-inflammatory molecules such as interleukin 1 β and decreased amounts of phospho-Akt in the hierarchical follicles (Pan *et al.*, 2012). Feed restriction to broiler breeder pullets between 7 and 15 weeks-of-age followed by ad libitum feeding led to improved reproductive performance, although pituitary and plasma LH and FSH concentrations, and median eminence levels of GnRH-I, were not different compared with pullets fed ad libitum (Bruggeman *et al.*, 1998). This raises the possibility that FSH responsiveness in the pre-hierarchical follicles is increased in response to overfeeding in broiler breeder chickens. Inhibin, a hormone secreted predominantly by the granulosa cells of the ovarian follicle, acts as a negative feedback regulator of pituitary FSH secretion. Expression of the inhibin α -subunit and inhibin/activin β A and β B subunits as well the activin type II receptor have been documented in the developing follicles of broiler breeder hen ovaries suggesting a paracrine role for inhibin and activin within the ovary. Active immunization of broiler breeder hens against inhibin was found to increase cumulative number of eggs produced by 9.5% (Satterlee *et al.*, 2002).

Egg-Type Chickens as a Model for Ovarian Cancer

A high proportion (25–40%) of egg-type chicken develops ovarian cancer around 2–4 years of age. Although the cause of spontaneous ovarian cancer in chickens is unknown, incessant ovulation is likely to contribute to the high rate of ovarian cancer incidence in chickens since they ovulate nearly every day. Elevated levels of harmful mutagenic chemicals have been found in the surface epithelium of the preovulatory follicle. Some of the ovarian tumors in chickens are associated with mutations in the *p53* oncosuppressor gene. Ovarian tumor in chickens appears like a large cauliflower-like mass (Fig. 6). In advanced stages of the disease, numerous metastatic tumor nodules could be noticed on the walls of the intestine, peritoneum, and mesentery, and liver. Histological appearance of the chicken ovarian tumor resembles that of commonly found endometriod type of ovarian adenocarcinoma and/or anaplastic ovarian carcinoma in women (Fig. 6). As in women, advanced stages (stage III and IV) of ovarian cancer in chickens are accompanied by accumulation of large volume (0.5–1 L) of ascites. Ovarian cancer cells isolated from ascites

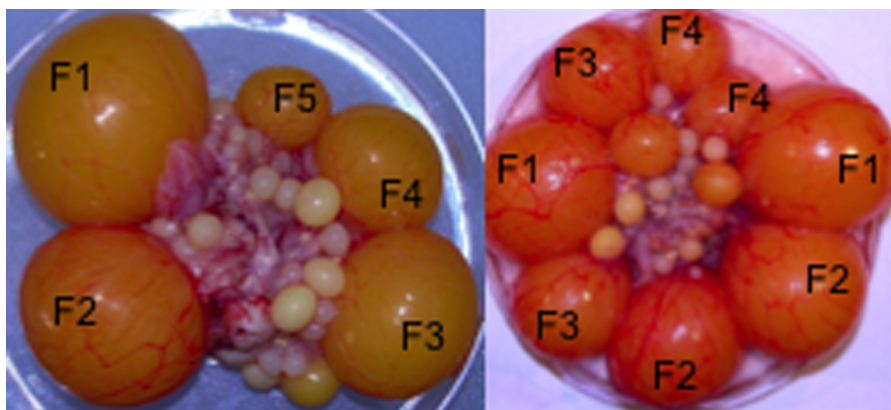


Fig. 5 Ovarian follicular hierarchy in leghorn (left) and broiler breeder hen (right). F1-F5 denote preovulatory follicles that will be ovulated sequentially almost one every day. Note double hierarchy of preovulatory follicles in broiler breeder hen ovary.

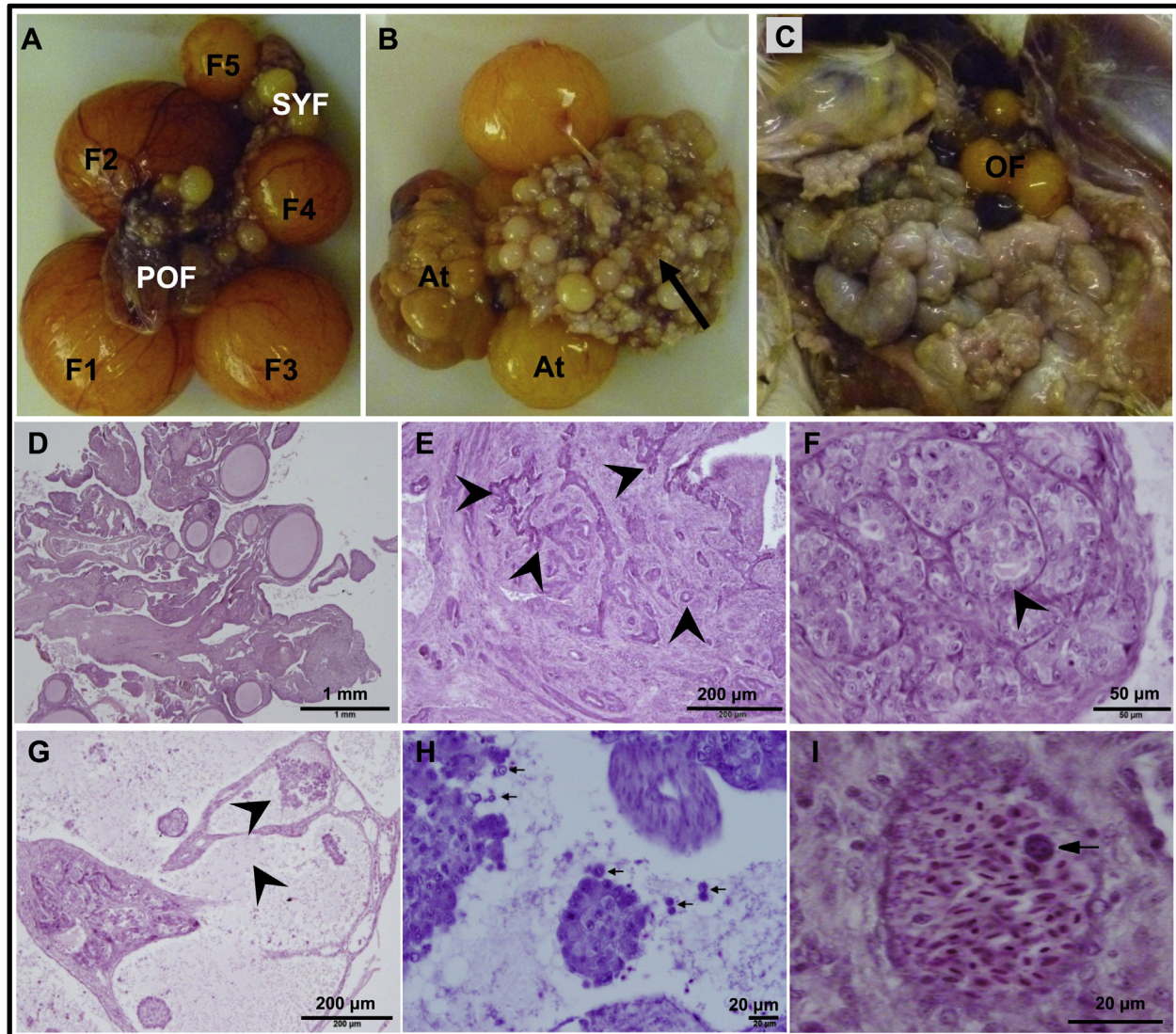


Fig. 6 Gross morphology and histology of normal and cancerous chicken ovaries. (A) Normal ovary containing a hierarchy of pre-ovulatory follicles (F1–5), prehierarchal follicles (SYF), and post-ovulatory follicle (POF). (B) Tumor mass (*arrow*) and a few atretic pre-ovulatory follicles (At). (C) Abdominal viscera showing tumors nodules on intestinal wall and peritoneum (*arrow heads*) and a few ovarian follicles (OF). (D–I) Photomicrographs of hematoxylin and eosin stained ovarian tissue sections. (D) Normal ovarian stroma containing several undifferentiated follicles. (E–I) Cancerous ovarian tissue section showing coarse fibrous stroma containing several acini and ducts (*arrows heads*; E), multiple cysts comprised of anaplastic cells (F), cystic spaces containing papillary projections of neoplastic cells (G), dyscohesive cells (*arrows*, H), and tumor-cell emboli in arterial lumen (I). Reproduced from Tiwari, A., *et al.*, 2013. Characterization of ascites-derived ovarian tumor cells from spontaneously occurring ovarian tumors of the chicken: Evidence for E-cadherin upregulation. PLOS ONE 8(2), e57582.

were found to be highly invasive in extracellular matrix proteins. In addition, the chicken ovarian cancer cells express several genes and proteins such as E-cadherin that are typically associated with ovarian cancer in women (Tiwari *et al.*, 2013). The chicken model of ovarian cancer is used as a preclinical model of human ovarian tumor (Johnson and Giles, 2013) to evaluate the effectiveness of ovarian cancer prevention strategies.

References

- Baxter, M., *et al.* (2014). Red light is necessary to activate the reproductive axis in chickens independently of the retina of the eye. *Poult. Sci.*, 93(5), 1289–1297.
- Bedecarrats, G. Y., *et al.* (2009). Gonadotropin-inhibitory hormone receptor signaling and its impact on reproduction in chickens. *Gen. Comp. Endocrinol.*, 163(1–2), 7–11.
- Bruggeman, V., *et al.* (1998). The effect of food intake from 2 to 24 weeks of age on LHRH-I content in the median eminence and gonadotrophin levels in pituitary and plasma in female broiler breeder chickens. *Gen. Comp. Endocrinol.*, 112(2), 200–209.
- Johnson, A. L. (2015). Reproduction in the female. In C. G. Scanes (Ed.), *Sturkie's Avian Physiology* (VI ed.). Elsevier.
- Johnson, P. A., & Giles, J. R. (2013). The hen as a model of ovarian cancer. *Nat. Rev. Cancer*, 13(6), 432–436.

- Joseph, N. T., et al. (2013). Reproductive neuropeptides: Prevalence of GnRH and KNDy neural signalling components in a model avian, *Gallus gallus*. *Gen. Comp. Endocrinol.*, 190, 134–143.
- Kuenzel, W. J., Kang, S. W., & Zhou, Z. J. (2015). Exploring avian deep-brain photoreceptors and their role in activating the neuroendocrine regulation of gonadal development. *Poult. Sci.*, 94(4), 786–798.
- Maddineni, S., et al. (2008). Gonadotrophin-inhibitory hormone receptor expression in the chicken pituitary gland: Potential influence of sexual maturation and ovarian steroids. *J. Neuroendocrinol.*, 20(9), 1078–1088.
- Pan, Y. E., et al. (2012). Ceramide accumulation and up-regulation of proinflammatory interleukin-1beta exemplify lipotoxicity to mediate declines of reproductive efficacy of broiler hens. *Domest. Anim. Endocrinol.*, 42(3), 183–194.
- Satterlee, D. G., Cadd, G. G., & Fioretti, W. C. (2002). Active immunization of broiler breeder hens with a recombinant chicken inhibin fusion protein enhances egg lay. *Poult. Sci.*, 81(4), 519–528.
- Schneider, W. J. (2016). Lipid transport to avian oocytes and to the developing embryo. *J. Biomed. Res.*, 30(3), 174–180.
- Seroussi, E., et al. (2016). Identification of the long-sought leptin in chicken and duck: Expression pattern of the highly GCrich avian leptin fits an autocrine/paracrine rather than endocrine function. *Endocrinology*, 157(2), 737–751.
- Tiwari, A., et al. (2013). Characterization of ascites-derived ovarian tumor cells from spontaneously occurring ovarian tumors of the chicken: Evidence for E-cadherin upregulation. *PLOS One*, 8(2), e57582.
- Zhang, L., et al. (2017). Green light inhibits GnRH-I expression by stimulating the melatonin-GnIH pathway in the chick brain. *J. Neuroendocrinol.*, 29(5).

Avian Reproduction – Overview (Wild Birds)

Tony D Williams, Simon Fraser University, Burnaby, BC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Domesticated species, especially the chicken, have been selected over centuries for continuous “aseasonal” reproduction with maximum rates of egg production (up to 300 eggs per year), minimum variation in egg size, and little or no post-hatching parental care. In contrast, the ~10,000 species of wild birds generally show seasonal breeding cycles with egg-laying and rearing of young restricted to a particular time of year (often spring and early summer) when environmental conditions (food, temperature) are optimal for successful reproduction. Among- and within-species there is large variation in egg size, clutch size and number of breeding attempts (1–4 broods per year). Furthermore, there is considerable variation in parental care (uniparental, bi-parental) including patterns of incubation behaviour, developmental maturity of chicks at hatching, and post-hatching care (brooding, guarding and/or feeding chicks to independence). Many birds breed first at 12 months of age, the year after they hatch, but some seabirds show long-deferred sexual maturity (up to 12 years).

Male Reproductive System

Male birds have internal, paired testes suspended from the dorsal wall of the body cavity at the top of the kidneys. Testis size varies seasonally with sexual activity, representing as little as 0.005% of body mass in immature or non-breeding males but increasing 500- to 1000-fold during breeding. For example, in the European starling (*Sturnus vulgaris*) testis volume increases gradually from <10 mm³ in late January to 500 mm³ in early April in response to increasing daylength (Fig. 1). Each mature testis consists of thousands of coiled seminiferous tubules containing germ cells, responsible for spermatogenesis, and Sertoli cells, which assist in sperm maturation. Interstitial or Leydig cells secrete steroid hormones (androgens) which control sexual behaviour (song and copulation) and development of some, but not all, secondary sexual characteristics (plumage and bill color). The seminiferous tubules join to form a long epididymis and deferent duct which carries mature sperm in seminal fluid to the cloaca. Sperm are generally short and relatively simple in non-passerines but longer and spiral-shaped in passerines (Jamieson, 2007). The distal end of the deferent duct forms the seminal glomus, a specialized sperm storage accessory structure. During the breeding season the male's cloaca swells forming a cloacal protuberance and in most species copulation and sperm transfer involves simple cloacal contact with the male everting his cloaca into the opening of the female's oviduct. Some ratites (e.g., ostrich) ducks, and geese have a more developed and erectile phallus or pseudopenis though this lacks an internal urethra.

Female Reproductive System

Two bilaterally symmetrical ovaries and oviducts develop in the avian embryo but in nearly all species only those on the left side are functional in adult life (birds of prey, Falconiformes, and the kiwi, *Apteryx* spp., retain two functional ovaries). The ovary is suspended from the dorsal wall of the body cavity near the top of the kidneys and consists of many oocytes embedded in a stroma of connective tissue. Oogenesis is terminated at hatching, at which time the ovary contains >450,000 primary oocytes although as few as 1–20 of these will mature and be laid as eggs depending on the species' breeding strategy and life span. Once oocytes

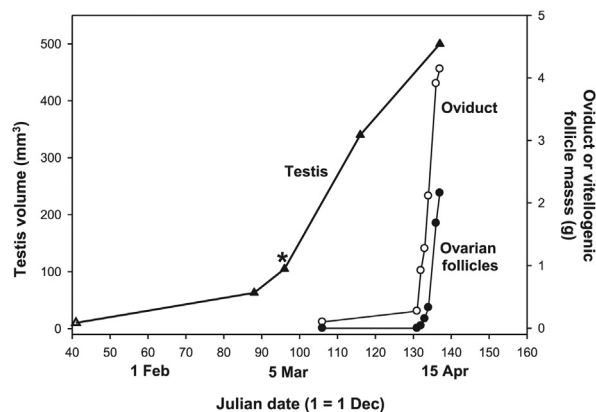


Fig. 1 Sex-specific differences in timing and rate of seasonal gonadal maturation in male (triangles) and female (circles) European starlings. Asterisk indicates when mature sperm are first produced. Based on data in Dawson, A., 2003. A comparison of the annual cycles in testicular size and moult in captive European starlings *Sturnus vulgaris* during their first and second years. *Journal of Avian Biology* 34, 119–123 and on unpublished data from T. D. Williams.

are selected into the hierarchy of developing follicles (Johnson, 2015) there is a variable phase of rapid yolk development (lasting 3–25 days in different species), with lipid- and protein-rich yolk precursors being taken up from the circulation to form the yolk. Follicle development is therefore characterised by estrogen-dependent hepatic synthesis of vitellogenin and yolk-targeted very low density lipoprotein (VLDL) the latter involving a major shift in the female's lipid metabolism (Williams, 2012). Granulosa and thecal cells proliferate and surround the follicle during oocyte maturation, synthesising and secreting hormones (progesterone, estradiol, growth factors) required for yolk development, oviduct function and control of female sexual behaviour. In contrast to mammals, the female is the heterogametic sex in birds (ZW) and thus determines offspring sex; there has been much interest in putative 'adaptive' sex ratio manipulation of offspring by mothers though the significance of this, and underlying mechanisms, remain poorly resolved (Rutkowska and Badyaev, 2008; Postma *et al.*, 2011).

At ovulation, the fully-developed yolk passes into the oviduct where albumen and shell are added to complete egg formation over about 24 h. The avian oviduct comprises five morphologically and functionally distinct regions. Fertilization occurs in the top section (infundibulum) shortly after ovulation before albumen deposition prevents sperm reaching the oocyte. Most albumen is added in the magnum, shell membranes in the isthmus, and shell and cuticle in the shell gland (over 18–20 h). Egg shells of wild birds can be highly, and variably, pigmented with porphyrins being associated with red and brown egg color and biliverdin giving blue and green coloration. The mechanism(s) determining patterning and egg coloration are still not known although it has been suggested that rotation of the egg in the shell gland might contribute to streaking.

Neuroendocrine Regulation of Gonadal Function

Seasonal cycles of gonadal maturation and regression in wild birds are regulated by the hypothalamic-pituitary-gonadal (HPG) axis (Dawson, 2008; Fig. 2), with the central nervous system and the hypothalamus receiving and integrating sensory information (both environmental and physiological), transducing this into humoral, hormonal signals which regulate gonadal function. Pituitary function is in turn regulated by the rate of secretion of chicken gonadotrophin-releasing hormone-I (cGnRH-I), and gonadotrophin-inhibiting hormone (GnIH). GnRH stimulates synthesis and release of the gonadotrophic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. LH is thought to primarily regulate gonadal steroidogenesis and ovulation, and FSH regulates spermatogenesis and follicle development. In addition to stimulating vitellogenesis and yolk uptake, ovarian steroids affect many other aspects of physiology and behaviors such as aggression, territoriality, nest building, courtship and copulation (Adkins-Regan, 2005) and development of secondary sexual characteristics.

This "top-down" model of control of gonadal function by the hypothalamus and pituitary appears to be appropriate for males, with a primary role for photoperiod, whereby gradually increasing day length in spring is associated with an early and gradual increase in testis size (Fig. 1). Artificial long photoperiods (>13 L) alone are sufficient to stimulate complete testicular maturation, steroidogenesis (albeit with somewhat lower testosterone titers) and spermatogenesis in males of wild birds in captivity, with photo-stimulation elevating plasma LH and FSH levels to those typical of free-living birds. Furthermore, testis development in captive males is very similar or identical to changes seen in free-living birds in response to gradually increasing day length in spring, and rate of testis growth is proportional to artificial photoperiod. In contrast females of most free-living species held in standard

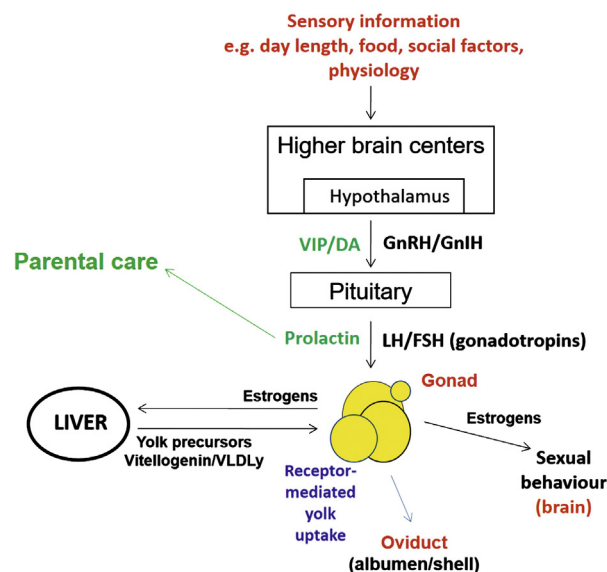


Fig. 2 Organization of the hypothalamic-pituitary-gonadal (HPG) axis, which integrates environmental information and regulates ovary, oviduct, and liver function for vitellogenesis, yolk, and egg formation, and parental care in birds. DA, dopamine; VIP, vasointestinal peptide (see text for other abbreviations).

captive conditions (small cages) will not undergo complete ovarian development: the ovary can develop to the pre-vitellogenic phase but onset of vitellogenesis, yolk formation and egg production is very unusual in captivity (except in large aviaries at low density). Although increasing day length in spring – or artificial long photoperiod in captivity – “switch on” the HPG axis at the hypothalamic and pituitary level in females, with increased GnRH and LH secretion, there is growing evidence for additional, (as yet unidentified) peripheral levels of control in the ovary and/or liver. For example, in female European starlings hypothalamic GnRH, GnIH, and pituitary FSH mRNA are already elevated in March before onset of ovarian development but LH and FSH receptor expression in the ovary does not increase until April with the onset of vitellogenesis (Perfito *et al.*, 2015). In free-living birds there is no gradual increase in ovarian or oviduct size with increasing day length (cf. male development; Fig. 1) and plasma estradiol levels remain low, until there is very rapid gonadal maturation 4–5 days before the first egg is laid (Williams *et al.*, 2004). The standard explanation for these sex-specific differences is that other non-photoc cues (e.g., temperature, social cues) contribute to regulation of ovarian development (see Section Environmental Regulation of Seasonal Reproduction) though the mechanism(s) by which this information is integrated with the HPG axis remain poorly understood (Visser *et al.*, 2010).

Photoperiod also allows most birds to predict in advance the time for termination of breeding, a process called photorefractoriness (Dawson, 2002). This is important since, if laying were to continue until food availability began to decline, young would be in the nest at a time when there is insufficient food to rear them successfully. However, again there are important (and unresolved) sex-specific differences in the timing or pattern of this process at least at the level of the testis and ovary. In males photorefractoriness is marked by rapid testicular regression at the very end of the breeding season and males can only respond to stimulatory long day lengths again after exposure to a period of short day lengths (<8 L) in fall. In females, ovarian and oviduct ‘regression’ (and follicle atresia) occur at the end of each bout of egg-laying, coincident with clutch completion typically early in the breeding season, and repeatedly in multi-brooded species. However, females are still capable of re-laying at this time, i.e., they are not photorefractory in the classic (male) sense.

Environmental Regulation of Seasonal Reproduction

In almost all wild birds breeding is restricted to a particular time of year coincident with a seasonal improvement in environmental conditions required for successful rearing of offspring, e.g., increased food availability, higher temperatures, or increased rainfall (Williams, 2012). Annual cycles of reproduction are most common, with birds in temperate and higher latitudes breeding in spring and early summer. In some tropical-breeding species individual birds can lay in any month of the year, although birds do not breed continuously and many species do have seasonal breeding cycles, albeit cycles not clearly associated with changes in day length but perhaps with cycles of rainfall or food availability (Beebe *et al.*, 2005).

In general, individuals that time breeding such that they have their chicks in the nest when food is most available will breed more successfully (Fig. 3), i.e., they will have higher fitness. Thus, food availability required for offspring growth sets a selective or evolutionary value on timing of breeding, and is the “ultimate factor” explaining *why* birds have evolved to breed when they do. However, before young hatch their parents must undergo extensive physiological and behavioral preparation for breeding, for example, maturation of the reproductive system, finding a nest site and mate, and laying and incubating eggs. These pre-laying events can take 6–13 weeks to complete and often must be initiated well in advance of any seasonal increase in food availability (Fig. 3) so birds must be able to *predict* the onset of their breeding season well in advance which they do using information from environmental cues or “proximate” factors. Proximate factors therefore explain *how* birds time their breeding season. The main proximate factor used by birds to time their breeding season, outside of the tropics, is the annual cycle of day length or photoperiod: since day length varies absolutely predictably each year, increasing day length in spring provides long-term or initial predictive information for timing of breeding. However, since there is usually small-scale, local variation in environmental conditions from year to year, other

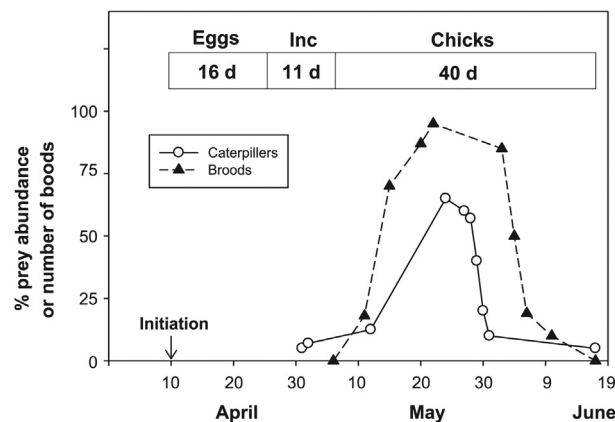


Fig. 3 Timing of breeding in blue tits in relation to availability of the bird's caterpillar prey showing that egg-laying is initiated well in advance of the seasonal increase in food availability. Based on data in Gibb, J.A., 1950. The breeding biology of the great and blue titmice. Ibis 92, 507–539.

non-photoc factors, such as temperature, food availability, and social interactions are thought to provide essential supplemental information allowing birds to fine-tune the onset of egg laying itself to local conditions. This overall conceptual framework for environmental control of seasonal reproduction is very long-standing (Murton and Westwood, 1977; Wingfield and Farner, 1993) and provides a reasonable explanation for control of reproduction in males. However, unequivocal experimental evidence for the role of non-photoc cues in free-living or non-domesticated birds is still lacking (e.g., temperature (Schaper *et al.*, 2012), social cues (Cornell *et al.*, 2017)) especially for females in relation to timing of egg-laying (Williams, 2012).

Variation in Breeding Seasonality, and Onset of Sexual Maturation

Most birds breed annually, though some exceptions include sooty terns (*Onychoprion fuscatus*) which breed sub-annually (with a mean period of 9.6 months, Reynolds *et al.*, 2014), king penguins (*Aptenodytes patagonicus*) which breed 2 out of every 3 years on average, and the wandering albatross (*Diomedea exulans*) which breeds biannually. Nevertheless, there is marked variation in age of first breeding. Most small-bodied, short-lived birds (e.g., passerines) breed for the first time in their first spring after hatching, that is, when they are about 1 year old. Other, typically larger, species delay breeding for 1 or more years after hatching and some long-lived species, such as albatrosses and penguins, show long deferred sexual maturation, breeding for the first time at 6–12 years of age. In contrast, a few species can show precocial sexual maturation, for example, the zebra finch (*Taeniopygia guttata*) can breed at around 3 months of age.

The mechanisms underlying timing of sexual maturation in wild birds remain poorly understood although nutritional status, body growth, or age per se do not appear to be important determining factors. In species which first breed at 1 year of age, there is evidence that timing of initial sexual maturation (puberty) is photoperiodically determined. Chicks hatch in a physiological state similar to adult photorefractoriness (termed juvenile refractoriness) ensuring they do not show an immediate response to the stimulatory effects of long day lengths with gonadal maturation in their first summer (as nestlings). Young birds remain photorefractory until the fall, when they become photosensitive due to the effect of short day lengths. Sexual maturation then occurs in response to long day lengths the following spring. In species with long-deferred sexual maturation there is some evidence that younger, pre-breeding birds are physiologically immature, with very low circulating sex steroid and gonadotropin levels even though these birds attend the breeding ground. However, the pituitary and gonads appear fully functional in one-year-old birds (e.g., responding appropriately to treatment with exogenous GnRH or LH). In males, and females of some species (e.g., macaroni penguin, *Eudyptes chrysolophus*), plasma levels of sex steroids and gonad size increase with age but older pre-breeding birds have plasma hormone profiles indistinguishable from those of adult birds one or more years before actual onset of breeding. In other species (e.g., wandering albatross) females appear to have different plasma steroid profiles even in their final pre-breeding year, compared to breeding females, with high progesterone and low estradiol levels and no evidence of follicular maturation. In addition to delayed physiological maturation in some species, there may also be a behavioral or social component to age of first breeding, with several years being required for birds to learn complex sexual displays or courtship rituals.

Eggs, Egg-Laying and Clutch Size

Free-living species show marked inter- and intraspecific variation in egg size and fecundity (clutch size). Egg mass scales with body mass across species, for example, Anna's hummingbird (*Calypte anna*) has a body mass of 4 g and egg mass of 0.5 g, compared with an ostrich (*Struthio camelus*) where body mass and egg mass are 100 and 1.4 kg respectively. Relative egg mass therefore scales negatively with body size (here, 12.5% and 1.4% respectively). Some species, such as blue tits, lay total clutch masses greater than their body mass. Egg size also varies markedly among individuals of the same species or population: the largest eggs laid by individual females within a population are generally at least 50% larger, and sometimes twice as large, as the smallest eggs laid by other individuals and approximately 70% of the total variation in egg size is due to variation between clutches, i.e., among individual females, not within clutches (Christians, 2002). Factors such as age, experience, or body mass, and 'environmental' factors (temperature, food) explain relatively little intraspecific variation in egg-size. There is much smaller, but systematic, variation in egg size within-clutches though this is smaller in precocial species compared with altricial species (0.68% vs. 3.91%), and in passerines is smaller in hole-nesting species compared with open nesting species (3.56% vs. 0.05%; Slagsvold *et al.*, 1984).

Many free-living passerines lay one egg per day and, in some species at least, daily timing of egg-laying is highly synchronous and occurs very soon after sunrise, i.e., there is a strong circadian pattern of oviposition (consistent with the "open period" model, Etches, 1996). Most other non-passerines, and some sub-oscine Passeriforms (e.g., Tyrannidae, Pipridae, Cotingidae) have laying intervals that are typically 2–4 days and the Ancient murrelet lays a 2-egg clutch with a 7-day laying interval. In some species at least it is clear that laying intervals >24 h are associated with longer intervals between initiation of yolk development (follicle recruitment) rather than differences in duration of development of successive yolks.

Despite the marked variation in egg size, there is relatively little evidence to support strong, positive, and especially long-lasting, effects of egg size on offspring growth and survival (Krist, 2011). Egg size is positively correlated with chick mass at hatching, and the primary benefit of larger eggs might be to increase offspring survival in the first few days after hatching. Positive, but weak, correlations between egg size and chick growth can persist for 7–10 days post-hatching but few studies have reported longer lasting effects and then only for certain morphological traits (e.g., wing length) not for body mass in general. Most studies have failed to find an effect of egg size on offspring growth or survival at fledging and, not surprisingly, larger egg size might only confer an advantage to

offspring in situations where resources are relatively low (poor quality habitats, late in the breeding season). In general, therefore, effects of egg size are largest at hatching (effect size, $r=0.4-0.6$) but are lower during the nestling and post-fledging stage ($r \leq 0.2$), and few post-fledging effects of egg size (e.g., on life-history or sexual traits) have been reported (Krist, 2011).

In addition to shell, yolk and albumen many substances, such as steroid, thyroid and protein hormones, antibodies (immunoglobulins), antioxidants and RNA, are transferred from the mother to the egg during egg formation in either the yolk or albumen. These 'minor' egg components have been viewed as potential mediators of "maternal effects": non-genetic contributions to offspring phenotype, by which mothers might "adaptively" adjust the development and the phenotype of their offspring to match prevailing environmental conditions increasing offspring and maternal fitness. Although many short-term effects of maternally-derived egg components on offspring phenotype have been documented (von Engelhardt and Groothuis, 2011) evidence for long-term fitness consequences in free-living birds remains equivocal (Williams and Groothuis (2015); but see Love and Williams (2008) and Duckworth *et al.* (2015)).

Some avian taxa have invariant clutch size, for example, among seabirds, all albatrosses and petrels lay a single-egg clutch, shorebirds (Charadriiformes) have an invariant clutch size of four eggs and most Larids (gulls) lay 3-egg clutches. In contrast marked inter-individual variation in clutch size exists in many species, for example, song sparrows (*Melospiza melodia*), European starlings, and lesser snow geese have modal clutch sizes of 4-eggs, 5-eggs and 4-eggs respectively, but clutch size varies from 2 to 5 eggs, 3–7 eggs, and 1–7 eggs respectively. In blue tits and mallard duck (*Anas platyrhynchos*) with larger modal clutch size of 10-eggs the range of individual clutch sizes is 4–17 eggs and 7–12 eggs respectively. Most species also show strong seasonal patterns of variation in clutch size. Single-brooded species, and some multiple-brooded species such as the Anatidae, Galliformes, and Ralliformes all show a linear, seasonal decline in clutch size (Fig. 4). In contrast, in many other multiple-brooded species clutch size first increases with laying date then declines (Fig. 4). This variation has been related to food availability, predation, and date-dependent reproductive value of offspring (Williams, 2012).

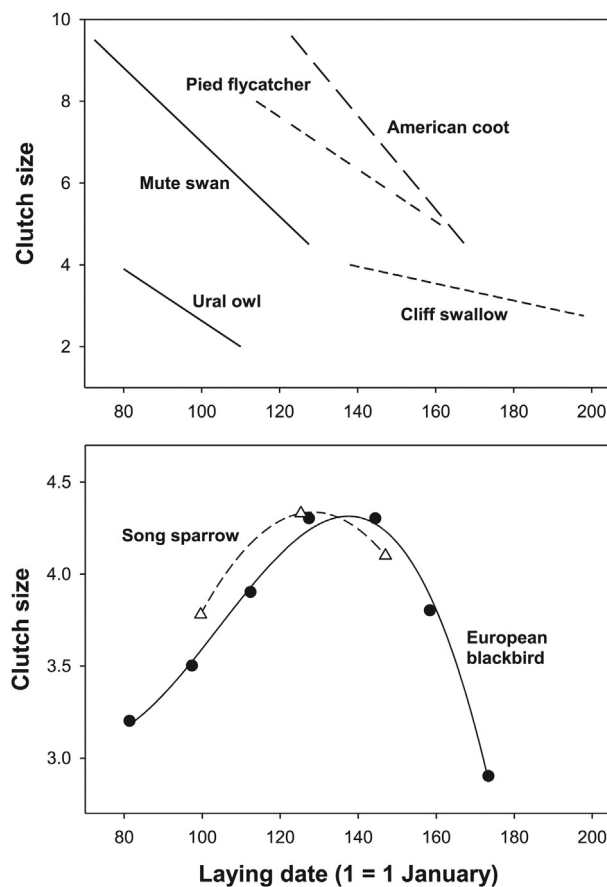


Fig. 4 Seasonal variation in clutch size in (a) single-brooded species (top panel; based on data in Meijer, T., Daan, S., Hall, M., 1990. Family planning in the Kestrel (*Falco tinnunculus*): The proximate control of covariation of laying date and clutch size. *Behavior* 114, 117–136; Brown, C.R., Brown, M.B., 1999. Fitness components associated with laying date in the Cliff Swallow. *Condor* 101, 230–245). (b) Multiple-brooded species (bottom panel; based on data in Snow, D.W., 1958. The breeding of the blackbird *Turdus merula* at Oxford. *Ibis* 100, 1–30; Travers, M., Clinchy, M.L., Boonstra, R., Zanette, L., Williams, T.D., 2010. Indirect predator effects on clutch size and the cost of egg production. *Ecology Letters* 13, 980–988).

Parental Care: Incubation and Chick-Rearing

All birds lay external eggs which must be incubated at temperatures $>37^{\circ}\text{C}$ to support embryo development, and most birds use their own body heat for incubation, sitting on eggs in specially constructed nests. In 'continuous incubators' both parents contribute equally to incubation, coordinating and alternating incubation bouts, with a high level ($>90\%$) of nest attendance and frequent, short, off-nest bouts for self-feeding (Deeming, 2002). Other species alternate longer feeding bouts with more prolonged incubation shifts which can range from 1 to 3 days up to 10–20 days, during which incubating birds must fast. Passeriformes and Apodiformes have lower levels ($<75\%$) of nest attentiveness (Deeming, 2002) and this is often associated with uniparental, female-only incubation, with incubating females having to leave the nest intermittently to self-feed. In many passerines with this 'intermittent-incubation' the female's physiological demands of incubation can be further reduced through incubating females being fed by their mates ('courtship feeding'). In contrast to passerines, some uniparental, female-only incubators (e.g., geese, eiders) have very high levels of nest attendance and, consequently, very short and infrequent nest reliefs for feeding, with no male courtship feeding. Incubation can then involve large decreases in body mass up to 30%–40% of initial body mass. Incubation periods last 11 days in small songbirds and up to 60–80 days in some penguins and albatrosses.

There is a continuum of developmental maturity of chicks at hatching, from altricial to precocial (Starck and Ricklefs, 1998). In altricial species, young hatch at a relatively early stage of development, often blind, relatively un-feathered, and unable to thermoregulate. This requires an obligate, but variable, post-hatching period of parental care with nest attendance and brooding of chicks. Chick-rearing periods average 9–20 days in small songbirds, 10–12 weeks in the bald eagle, and 12 months in the wandering albatross. Chicks of altricial species often leave the nest before they are capable of sustained flight and there is a variable period of post-fledging care (which can be longer than the nestling phase). In altricial species parents forage for food for offspring, returning frequently to the nest to feed chicks (central place foraging), as well as defending the nest and young from predators. In small-bodied birds, such as passerines, the number of nest visits made by parents provisioning chicks can be very high, and highly variable, from 300 to 1500 visits/nest/24 h blue tits, and from 0.6 to 4.00 visits/chick/h in European starlings. In contrast, many pelagic seabirds make long foraging trips at sea and return every 1–4 days to feed their chicks with single, very large meals. In precocial species chicks hatch fully-feathered, with a good ability to thermoregulate, they are capable of walking (though not flying), typically leave the nest shortly after hatching, and are often self-feeding. Parental care in precocial species thus mainly involves protecting chicks from predators, and leading them to high-quality foraging areas.

The physiological and hormonal basis of parental care remains poorly understood especially in free-living birds (Adkins-Regan, 2005). Prolactin is likely involved in development and maintenance of incubation behaviour, though perhaps not in clutch size determination (Ryan *et al.*, 2014) or in post-hatching care (Adkins-Regan, 2005). Testosterone has inhibitory effects on male parent care, and glucocorticoids might play a role in mediating foraging behaviour associated with parental care (Angelier and Chastel, 2009).

Costs of Reproduction, Trade-Offs and Carry-Over Effects

'Costs of reproduction' are associated with each breeding stage (egg production, incubation, and chick-rearing). These costs manifest in the short-term, through negative effects of reproductive effort on subsequent breeding stages in the same breeding attempt, in subsequent breeding attempts in the same year (e.g., second broods), or through long-term negative effects on future fecundity and survival many months, or even in the subsequent year, after reproduction (Williams, 2012). There can be direct temporal overlap generating trade-offs between reproduction and other life-history stages such as molt and migration. For example, 'costs' can be mediated by affects of timing of breeding on timing or rate of molt at the end of the breeding season, and resultant feather quality (Dawson *et al.*, 2000). Earlier stages of the annual cycle (wintering, migration, pre-breeding) can also significantly affect reproductive decisions via "carry-over effects" (Wilson *et al.*, 2007). Again, we still know very little about the specific physiological mechanisms underlying trade-offs or carry-over effects, including costs of reproduction (Harrison *et al.*, 2010; Williams, 2012).

References

- Adkins-Regan, E. (2005). *Hormones and Animal Social Behavior*. Princeton; Oxford: Princeton University press.
- Angelier, F., & Chastel, O. (2009). Stress, prolactin and parental investment in birds: A review. *General and Comparative Endocrinology*, 163, 142–148.
- Beebe, K., Bentley, G. E., & Hau, M. (2005). A seasonally breeding tropical bird lacks absolute photorefractoriness in the wild, despite high photoperiodic sensitivity. *Functional Ecology*, 19, 505–512.
- Christians, J. K. (2002). Avian egg size: Variation within species and inflexibility within individuals. *Biological Reviews*, 77, 1–26.
- Cornell, A., Hou, J. J., & Williams, T. D. (2017). Experimentally-increased pre-breeding male social behaviour has no effect on female breeding phenology and performance. *Animal Behavior*, 126, 243–251.
- Dawson, A. (2002). Photoperiodic control of the annual cycle in birds and comparison with mammals. *Ardea*, 90, 355–367.
- Dawson, A. (2008). Control of the annual cycle in birds: Endocrine constraints and plasticity in response to ecological variables. *Philosophical Transactions of the Royal Society B*, 363, 1621–1633.
- Dawson, A., Hinsley, S. A., Ferns, P. N., Bonser, R. H. C., & Eccleston, L. (2000). Rate of moult affects feather quality: A mechanism linking current reproductive effort to future survival. *Proceedings of the Royal Society of London B*, 267, 2093–2098.
- Deeming, D. C. (2002). *Avian Incubation*. Oxford: Oxford University Press.
- Duckworth, R. A., Belloni, V., & Anderson, S. R. (2015). Cycles of species replacement emerge from locally induced maternal effects on offspring behavior in a passerine bird. *Science*, 347, 875–877.

- Etches, R. J. (1996). *Reproduction in Poultry*. Wallingford: CAB International.
- Harrison, X. A., Blount, J. D., Inger, R., Norris, D. R., & Bearhop, S. (2010). Carry-over effects as drivers of fitness differences in animals. *Journal of Animal Ecology*, 80, 4–18.
- Jamieson, B. G. M. (2007). Avian spermatozoa: Structure and phylogeny. In B. G. M. E. Jamieson (Ed.), *Reproductive Biology and Phylogeny of Birds* (pp. 349–398). Enfield, NH: Science Publisher.
- Johnson, A. L. (2015). Ovarian follicle selection and granulosa cell differentiation. *Poultry Science*, 94, 781–785.
- Krist, M. (2011). Egg size and offspring quality: A meta-analysis in birds. *Biological Reviews*, 86, 692–716.
- Love, O. P., & Williams, T. D. (2008). The adaptive value of stress-induced phenotypes: Effects of maternally derived corticosterone on sex-biased investment, cost of reproduction, and maternal fitness. *The American Naturalist*, 172, E135–E149.
- Murton, R. K., & Westwood, N. J. (1977). *Avian Breeding Cycles*. Oxford: Clarendon Press.
- Perfito, N., Guardado, D., Williams, T. D., & Bentley, G. (2015). Social cues regulate reciprocal switching of hypothalamic Dio2/Dio3 and the transition into final follicle maturation in European starlings (*Sturnus vulgaris*). *Endocrinology*, 156, 694–706.
- Postma, E., Heinrich, F., Koller, U., et al. (2011). Disentangling the effect of genes, the environment and chance on sex ratio variation in a wild bird population. *Proceedings of the Royal Society B: Biological Sciences*, 278, 2996–3002.
- Reynolds, S. J., Martin, G. R., Dawson, A., Wearn, C. P., & Hughes, B. J. (2014). The sub-annual breeding cycle of a tropical seabird. *PLOS ONE*, 9, e93582.
- Rutkowska, J., & Badyaev, A. V. (2008). Meiotic drive and sex determination: Molecular and cytological mechanisms of sex ratio adjustment in birds. *Philosophical Transactions of the Royal Society B*, 363, 1675–1686.
- Ryan, C. P., Dawson, A., Sharp, P., Meddle, S. L., & Williams, T. D. (2014). Circulating breeding and pre-breeding prolactin and LH are not associated with clutch size in the Zebra Finch (*Taeniopygia guttata*). *General and Comparative Endocrinology*, 202, 26–34.
- Schaper, S. V., Dawson, A., Sharp, P. J., et al. (2012). Increasing temperature, not mean temperature, is a cue for avian timing of reproduction. *The American Naturalist*, 179, E55–E69.
- Slagsvold, T., Sandvik, J., Røstad, G., Lorentsen, O., & Husby, M. (1984). On the adaptive value of intraclutch egg size variation in birds. *Auk*, 101, 685–697.
- Starck, J. M., & Ricklefs, R. E. (1998). *Avian Growth and Development*. New York, NY: Oxford University Press.
- Visser, M. E., Caro, S. P., van Oers, K., Schaper, S. V., & Helm, B. (2010). Phenology, seasonal timing and circannual rhythms: Towards a unified framework. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 365, 3113–3127.
- von Engelhardt, N., & Groothuis, T. G. (2011). Maternal hormones in avian eggs. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates – Birds* (pp. 91–127). Amsterdam: Elsevier.
- Williams, T. D. (2012). *Physiological Adaptations for Breeding in Birds*. Princeton, NJ: Princeton University Press.
- Williams, T. D., & Groothuis, T. G. (2015). Egg quality, embryonic development and post-hatching phenotype: An integrated perspective. In D. Charles Deeming, & S. James Reynolds (Eds.), *Nests, Eggs and Incubation: New Ideas About Avian Reproduction* (pp. 114–126). Oxford: Oxford University Press.
- Williams, T. D., Kitaysky, A. S., & Vézina, F. (2004). Individual variation in plasma estradiol-17 β and androgen levels during egg formation in the European starling *Sturnus vulgaris*: Implications for regulation of yolk steroids. *General and Comparative Endocrinology*, 136, 346–352.
- Wilson, S., Norris, D. R., Wilson, A. G., & Arcese, P. (2007). Breeding experience and population density affect the ability of a songbird to respond to future climate variation. *Proceedings of the Royal Society of London B*, 274, 2669–2675.
- Wingfield, J. C., & Farner, D. S. (1993). Endocrinology of reproduction in wild species. In D. S. Farner, J. R. King, & K. C. Parkes (Eds.), *Avian Biology* (pp. 163–327). New York, NY: Academic Press.

Further Reading

- Lovette, I. J., & Fitzpatrick, J. P. (2016). *The Cornell Lab of Ornithology Handbook of Bird Biology* (third ed.). Chichester: John Wiley and Sons Ltd.
- Scanes, C. G. (2015). *Sturkie's Avian Physiology* (sixth ed.). Amsterdam: Elsevier.

Reproduction in Monotremes

Russell Campbell Jones and Brett Nixon, The University of Newcastle, Callaghan, Australia
Frank Grützner, The University of Adelaide, Adelaide, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Acrosome A membrane bound structure which caps spermatozoa. It contains digestive enzymes considered to assist spermatozoa penetrate the outer investments of an ovum.

Anti Mullerian hormone (AMH) A hormone secreted by the testes which inhibits the embryonic Mullerian ducts from developing into the adult female reproductive duct system.

Apocrine gland Sweat glands in the skin which develop from the epidermis or hair follicle.

Capacitation The last process in the maturation of mammalian spermatozoa which provides the capacity to fertilize an ovum.

Cloaca The posterior orifice of embryonic mammals and adults of other vertebrates which serves as the only opening for the digestive, reproductive, and urinary tracts.

Crural system Spurs and associated glands of platypus and echidna. A spur is located on the heel of each hind leg and its gland is located in the loin.

Disulphide bond Strong, covalent bond between adjacent cysteines (amino acids) in a protein. Disulphide bonds provide strength, rigidity and maintain the structure of a protein.

Ductuli efferentes The "efferent ducts" carry spermatozoa from the testis to the ductus epididymidis.

Endothermic Endothermic animals use their own metabolic processes to warm their body.

Epididymis A long, convoluted duct (ductus epididymidis) supported by connective tissue and which carries spermatozoa from the ductuli efferentes to the urethra. In mammals, it is involved in maturing and storing spermatozoa.

Homologous Structures which are considered to have a common genetic (evolutionary) origin.

Major histocompatibility complex (MHC) A group of proteins on the surface of vertebrate cells which recognize and display foreign molecules (eg. from pathogens) so that they can be processed by the appropriate cells (T-cells) of the immune system.

Meiotic chain An alignment of homologous pairs of chromosomes during meiosis.

Protease An enzyme which breaks down protein into amino acids.

Proteome The entire set of proteins expressed by a unit (cell, tissue, organ or organism) at a certain time.

Receptor A protein molecule that receives and responds to a chemical messenger such as a hormone.

Sauropsids Reptiles and birds.

Sex chromosomes Chromosomes involved in determining sex in animals that practice sexual reproduction. Sex chromosomes differ from one another whereas the other chromosomes in diploid cells (autosomes) appear as pairs with the same form.

Sex chromosome inactivation Involves one of the copies of the X chromosome in female mammals being prevented from expressing its code (transcribing). Inactivation of the X chromosome also occurs in the male, but is transient during spermatogenesis.

Sex determination The biological process involved in determining gender in an organism.

Testicond The condition in which testes are retained within the abdominal cavity in vertebrates and do not descend into a scrotum as in most mammals.

Tyrosine phosphorylation A key step in the transfer of a signal, such as a hormone, from the surface of a cell to a target molecule (enzyme). It is an enzyme catalysed process involving the addition of a phosphate group to the amino acid tyrosine on a protein.

Zona pellucida A glycoprotein layer surrounding and supporting a mammalian oocyte, and which is involved in recognition between spermatozoa and ova at fertilization.

Introduction

Reproduction defines monotremes as mammals as they have mammary glands that produce milk which solely supports their developing young until they are independent: at least 60% adult mass for platypus. They are also furred and endothermic. However, their sex chromosomes and method of sex determination are unlike eutherians and marsupials. Like non-mammalian vertebrates, they have a single orifice (the cloaca) for excretory and reproductive functions, their gametes most closely resemble the sauropsids (reptiles and birds), and they lay eggs which are incubated externally. The early work covering monotreme reproduction was reviewed by Griffiths (1978) and then more specifically by Temple-Smith and Grant (2001). Further, there has been a renewed interest in the subject (Grützner *et al.*, 2008) since the platypus genome was sequenced (Warren *et al.*, 2008).

Estimates of the time of divergence of monotremes from eutherian mammals range between 166 and 200 million years ago, making monotremes the oldest surviving mammalian lineage. Only three genera are extant and they are highly specialized animals. Platypus (*Ornithorhynchus anatinus*) are confined to rivers in eastern Australia, and Kangaroo Island, South Australia, where they have been introduced. Short-beaked echidna (*Tachyglossus aculeatus*) occur in almost all Australian habitats and the lowlands of New Guinea. Long-beaked echidnas (*Zaglossus*) are restricted to the highlands of New Guinea (three species have been described) and little is known of them as they are inaccessible to researchers. The extant monotremes have a normal body temperature of 32°C. Platypus are homothermic even in water at 5°C, whilst *Tachyglossus* lower their body temperature during torpor. Both species cope with high temperatures by avoidance behavior.

Male Reproduction

Testes and Sperm Production

The testes of monotremes develop between and caudal to the kidneys as in other vertebrates, but they do not descend into a scrotum as in most mammals. The testicond condition is probably because monotremes do not develop gubernaculum which in scrotal mammals are involved in testicular descent. Consistent with this interpretation is the finding that INSL3 (Insulin like factor 3), which regulates gubernaculum function in scrotal mammals, lacks specificity for its receptor (Lf20606-08-9780128118993: relaxin/insulin-like family peptide receptor 2) in monotremes. The specificity of INSL3 evolved in scrotal mammals by a single point mutation (Park *et al.*, 2008).

Spermatogenesis in monotremes is similar to other mammals. Associations of the stages of germ cell development (cellular associations) only occupy small areas of a seminiferous tubule as in humans and birds, and do not extend for mm along a tubule as in most mammals. According to Daniel Djakiew, the duration of spermatogenesis in *Tachyglossus* is within the range of other mammals: 62 days (4.5 cycles) and a daily sperm production of 6 ± 0.27 million sperm per gram of testis. However, monotremes have a different strategy of sperm production than most scrotal mammals: they produce spermatozoa at a greater rate per testis and are less dependent on storing them available for mating. Monotreme testes increase in size with age and become larger, relative to body mass, than could be comfortably carried in the scrotal position. Consequently, sperm production per testis in monotremes is greater than in scrotal mammals. For example, estimates of the ratio of testes mass to body mass are: 1.74%, 1.97%, 0.006%, 0.67%, 0.91% and 0.54% respectively for platypus, *Tachyglossus*, humans, rats, rams and tammar wallabies. The high rate of sperm production in monotremes is associated with the storage (in the cauda epididymidis) of fewer sperm available for mating: only 25% of extragonadal sperm compared to about 75% in scrotal mammals like the rat and ram.

Monotreme spermatozoa differ from other mammals in that their nuclei are vermiform and cylindrical like sauropsids and they are arranged in a helical shape. Michael Bedford recognized that they are less rigid than sperm from other mammals and related this to the relative thinness of the monotreme zona pellucida compared to the ova of other mammals. Monotreme sperm also have no post-acrosomal sheath over the nucleus as in eutherian sperm, and they have no striated columns in the neck region or coarse fibers supporting the principal piece. Also, the fibrous sheath supporting the principal piece of monotreme sperm is spiraled whereas in eutherian sperm it has longitudinally oriented connections between adjacent rings. Further, there is no disulphide bonding in the nucleus due to the absence of cysteine residues in the protamine-1 sequences of monotremes. The acrosome is small and mainly limited to the rostral surface of the nucleus and platypus lack the genome for testis-specific proteases which are considered to play important functions in degrading the zona pellucida of eutherians during fertilization. Nevertheless, orthologs of many of the sperm membrane proteins related to fertilization in other mammals are present in the platypus genome. They include the following genes for putative zona pellucida receptors and proteins implicated in sperm-oolemmal fusion in eutherian mammals: zona pellucida binding protein (*Zpbp*, *Sp38*), zona pellucida binding protein 2 (*Zpbp2*), zona pellucida sperm-binding protein 3 receptor (*Zp3r*; *Sp56*), hyaluronoglucosaminidase 5 (*Hyal5*; *Spam1*; *Ph20*), a disintegrin and metalloproteinase domain 1a (*Fertilin alpha*), sperm autoantigenic protein 17 (*Spa17*; *Sp17*), milk fat globule-EGF factor 8 protein (*Mfge8*; *Sed1*), UDP-Gal: betaGlcNAc beta 1,4-galactosyltransferase (*B4galt1*), fucosyltransferase 5(alpha (1,3) fucosyltransferase) (*Fut5*), mannosidase 2, alpha B2 (*Man2b2*) and izumo sperm-egg fusion 1 (*Izumo1*).

Excurrent Ducts

Sperm are transported from the testis by the ductuli efferentes which join the ductus epididymidis (epididymis) which then connects to the urethra (Fig. 1). The monotreme epididymis performs more functions than the epididymis of sauropsids. Relative to animal mass the duct is longer than sauropsids and as long as other mammals. Also, unlike the sauropsids, it is segmented, but only into an initial and terminal segment (caput and cauda epididymidis). Significantly, the epididymal epithelium of eutherians and marsupials is differentiated into about 6 segments indicating that their sperm are exposed to more opportunities for modification. The initial segment of *Tachyglossus* is more than 96% the length of the ductus epididymidis and the ultrastructure of the epithelium does not vary along the duct. It possesses features unique to the initial segment of the mammalian epididymis: epithelial structure and dependence on luminal fluid from the testis. This is significant as the initial segment plays an essential role in post-testicular sperm maturation in eutherians and marsupials (Jones *et al.*, 1987) and is also associated with post-testicular sperm maturation changes in monotremes: migration and loss of the cytoplasmic droplet along the middle piece and development of motility. The terminal segment of the epididymis partly compensates for its short length by possessing mucosa-forming folds, or villi, which

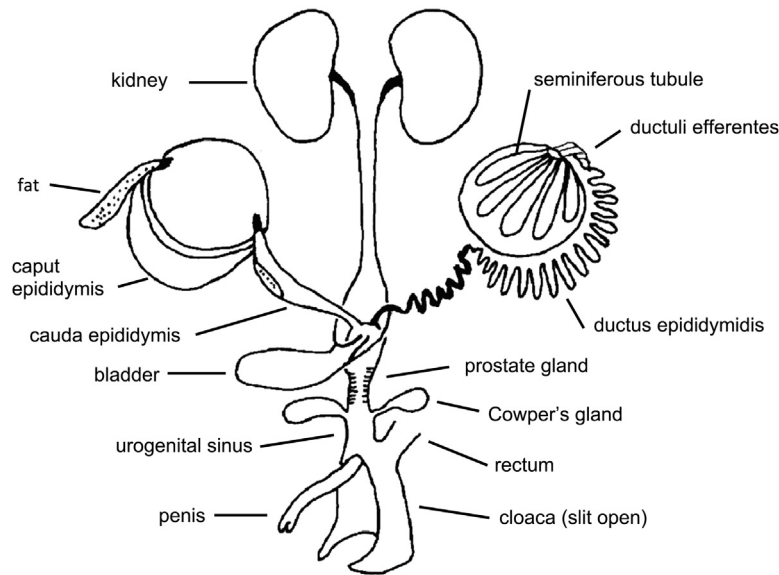


Fig. 1 Diagram of the male reproductive system of *Tachyglossus*. The gross anatomy of the testis and epididymis are shown on the left side and a diagrammatic representation of the arrangement of the sperm ducts is shown on the right side.

ensure that the relatively wide lumen is faced by a considerable area of epithelium. Djakiew estimated that it takes about 14 days for sperm to pass through the excurrent ducts of the testes of *Tachyglossus*: 29 min through the ductuli efferentes, and respectively 10 and 4 days to pass through the initial and terminal segments of the epididymis.

Work on the *Tachyglossus* showed that, as in other mammals, sperm are dilute when they leave the testes and that most of the accompanying fluid and protein is reabsorbed in the ductuli efferentes (74% and 46% respectively). The initial segment of the epididymis reabsorbs 83% of the fluid that enters and it secretes protein, the concentration of protein in the luminal fluid increasing from 4.9 to 58.8 $\mu\text{g}/\mu\text{l}$. The terminal segment also secretes protein, but the luminal concentration is only 39.8 $\mu\text{g}/\mu\text{l}$, a reduction due to either binding to sperm or reabsorption from the lumen. Jean-Louis Dacheux showed that hundreds of proteins are secreted into the initial segment and there is no variation along the segment in the composition of secreted proteins. Few of the epididymal proteins identified in eutherian mammals are present in the platypus epididymal proteome in which more than 50% of the total protein present consists of 3 proteins which have never been identified in epididymal secretions of any other mammal (PXN-FBPL, SPARC and E-OR20). However, SPARC (secreted protein, acidic and rich in cysteine or osteonectin) has been identified in mouse epididymal tissue (Sage *et al.*, 1989). Further, although E-OR20 (epididymal ornithorhynchus protein 20 kDa) is a new lipocalin, it is homologous with lipocalins identified in the epididymides of other mammals and a lizard (*Lacerta vivipara*).

The changes in protein concentration along the epididymis are associated with the formation of sperm bundles (Fig. 2) which are unlike any sperm association described in vertebrates. In histological sections the bundle formation is obvious as sperm leave the initial segment. One hundred sperm have been counted in transmission electron micrographs of *Tachyglossus* sperm bundles; the rostral ends of sperm heads are embedded in an electron dense material and more distally the heads are supported by less dense material. Brett Nixon and colleagues identified the supporting material as SPARC in sperm bundles of the platypus and *Tachyglossus*.

Sperm Capacitation

Platypus and *Tachyglossus* sperm bundles have a forward velocity about 3-times that of individual sperm when diluted and incubated *in vitro*. Sperm within bundles will not undergo an acrosome reaction or bind to the perivitelline membrane of domestic hen oocytes (which share homology with the platypus zona pellucida (see below)). Work on *Tachyglossus* showed that epididymal or ejaculated sperm could achieve these capacities, but only when they dispersed from the bundles after a period of incubation *in vitro* for about 1 h or more. In this respect, they conform to the original descriptions, by CR Austin and MC Chang, of sperm capacitation in mammals. However, the process is not the same as in eutherians and marsupials in which capacitation also involves significant membrane changes and activation of a cAMP mediated signaling pathway that promotes tyrosine phosphorylation of multiple sperm proteins. Consequently, it is interpreted that monotremes exhibit an early form of sperm capacitation.

Accessory Glands of Reproduction

Unlike sauropsids, monotremes have a prostate gland (like all mammals) and paired bulbourethral (Cowper's) glands, and there is evidence that these glands are androgen dependent in the platypus and *Tachyglossus*. The prostate glands are the disseminate type and not sufficiently large to be obvious macroscopically. Also, there is little epithelium in the Cowper's glands. Nevertheless,

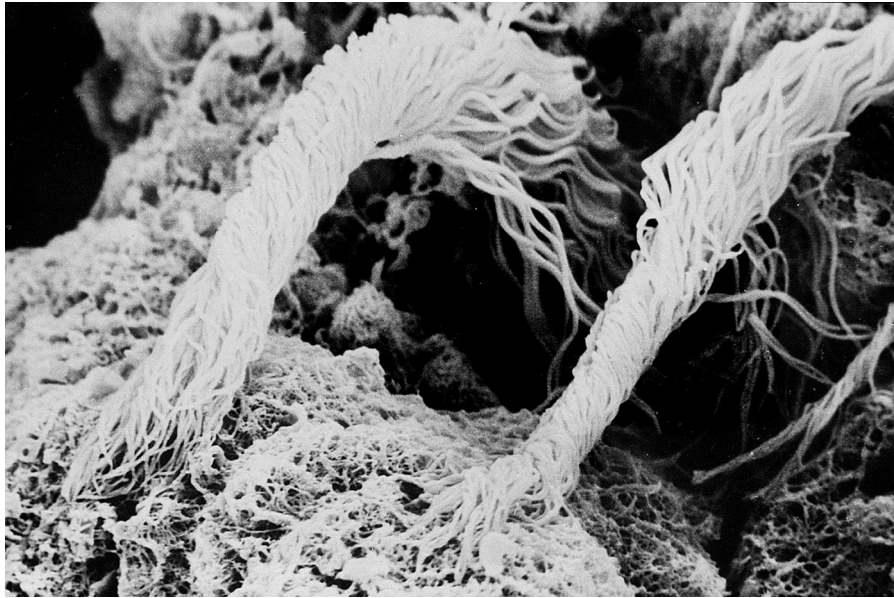


Fig. 2 Scanning electron micrograph of sperm bundles from the cauda epididymidis of *Tachyglossus*. Reproduced with permission from Djakiew, D., Jones, R.C., 1983. Sperm maturation, fluid transport and secretion and absorption of protein in the epididymis of the echidna, *Tachyglossus aculeatus*. *J. Reprod. Fertil.* 68, 445–456.

a domesticated *Tachyglossus* male trained for semen collection emitted a clear viscous fluid from the penis prior to emitting semen; it is unknown if there is mixing of these (presumably) accessory gland secretions and epididymal semen.

Penis

The penis of the platypus and *Tachyglossus* is normally retracted into a preputial sac in the ventral cloaca and when erect it protrudes through the cloaca. The urethra opens at the base of the penis so that only semen passes through the penis, while urine is normally excreted directly through the cloaca. The glans penis is bifid, as in marsupials, and the structure of the glands differs between species. Steve Johnson described an unusual ejaculatory mechanism in *Tachyglossus* in which alternate sides of the glans penis deliver successive ejaculations. While this appears unique among mammals, it is similar to the use of hemipenes in squamate reptiles.

Crural System

Male monotremes develop spurs on the heels of their back feet and each spur is connected to a secretory (crural) gland in the groin. In both platypus and *Tachyglossus* the glands are active during the breeding season, but they differ between genera in size and purpose. Platypus spurs are large in adults (25–30 mm long) and their glands produce a venom which can kill a small dog or cause humans excruciating pain when spurred. The venom contains a complex mixture of peptides (Wong *et al.*, 2013) including defensin-like peptides, C-type natriuretic peptides which can release histamine and effect calcium influx, hyaluronidase, amide oxidase, protease inhibitor, proteins associated with stress response and immune molecules. The spurs are probably involved in inter-male rivalry, either to establish territory or in competing for females. The venom is not lethal to platypus as they can degrade it.

Tachyglossus males have short spurs (about 5 mm long) and lack the ability to erect them. The transcriptome of the gland is quite different to the platypus and has functional terms associated with steroidal and fatty acid production. It is more likely involved in scent marking than overt aggressive behavior.

Female Reproduction

Reproductive Ducts

Both ovaries are functional in *Tachyglossus*, but only the left is functional in the platypus (Fig. 3) as in many sauropsids. The right platypus ovary remains embryonic retaining numerous primary follicles. As in sauropsids and virgin marsupials the female reproductive ducts of monotremes are paired and individually join the urogenital sinus. The latter communicates with the cloaca which also receives the rectum. Each duct is differentiated into an oviduct and uterus and, as in other mammals the junction between the uterus and oviduct contains numerous tubular glands. Peter Temple-Smith and Tom Grant consider that insemination is probably intrauterine.



Fig. 3 Female reproductive tract of the platypus showing that both uteri are well developed, but only the left ovary is developed. The inserts at the top of the figure show the ovaries dissected from the tract. Reproduced with permission from Grutzner, F, Nixon, B., Jones, R.C., 2008. Reproductive biology in egg-laying mammals. *Sexual Development* 2, 115–127.

Breeding and Early Development

Monotremes are solitary animals, and seasonal breeders with mating around July to August with some variation according to latitude. There have been no successful attempts to determine the endocrine control of the estrous cycle, although a study of seasonality suggested that the control resembles the avian rather than mammalian model.

At ovulation, the ovum is small (4 mm diameter) in monotremes compared to sauropsids. Unlike other mammals, monotreme ova contains yolk, but much less yolk than in sauropsid eggs. As at least 3 vitellogenin genes have been identified in the platypus genome, it is interpreted that monotreme yolk probably consists of glycoproteins derived from the vitellogenin precursor proteins, as in the sauropsids. The monotreme ovum is contained by the vitelline membrane within a very thin zona pellucida and a tunic of 2 layers of columnar, follicular cells. Oviduct secretions form additional layers. Two layers of albumen are formed, the upper two-thirds of the oviducts secreting the densest layer. A basal layer of the shell membrane is formed in the distal third of the oviduct and a second, denser layer, is formed in the proximal uterus. Uterine glands in the body of the uterus secrete a nutritive fluid which is absorbed by the egg. A third uterine secretion is the precursor of the protective layer of the shell which remains flexible and porous. The egg has a diameter of 14–15 mm when laid.

Fertilization occurs prior to or during the formation of the albumen layer and the second meiotic division is completed when the egg enters the uterus. Sperm-egg recognition probably involves the zona pellucida proteins as in other mammals. The 4 proteins of the human zona (Zp1, Zp2 (Zpa), Zp3 (Zpc), and ZP4/B) possess single orthologs encoded in the platypus with a high level of homology (approximately 40–50% identity on the amino acid level). They also display a high level of homology with proteins in the chicken inner perivitelline membrane which is analogous to the mammalian zona pellucida. However, whilst the avian membrane consists of 3 major components (homologs of the Zp1 and Zp3 families and a unique, phylogenetically distinct protein, ZPD) the ZPD is absent in the platypus and eutherian mammals.

Gestation, from mating to egg laying, is 15–20 days in platypus and 23 ± 3 days in *Tachyglossus*. In the uterus, the egg undergoes nuclear conjugation and incomplete meroblastic cleavage as in the sauropsids. When the egg is laid, the embryo has 19–20 pairs of somites within a parchment like shell. The platypus normally lays 2 eggs which it incubates in a sealed “birthing” burrow and *Tachyglossus* lays 1 egg which it retains in a “pouch” formed as a depression in the belly. The eggs are incubated for about 10 days and the young are born about 15 mm long. Platypus young are nursed within the birthing burrow until they emerge 121–126 days after hatching. *Tachyglossus* young are carried in the pouch for 45–55 days then kept in a burrow until weaning 170–195 days after hatching. Brian Green and colleagues showed that the rate of growth of *Tachyglossus* young is as fast as mammals like the rabbit. However, the duration of lactation of *Tachyglossus* varies between habitats. For example, even though the masses of *Tachyglossus* are much the same at weaning, Kangaroo Island young are about 60 days older than Tasmanian young (Morrow *et al.*, 2009).

Lactation

Mammary glands develop under the dermis on both sides of the abdomen, but there are no nipples in monotremes, the milk being ejected onto the surface of the hairy skin. Olav Oftedal suggested that the glands may have evolved from ancestral apocrine-like glands which provided moisture, and possibly other substances, to protect the porous eggs during incubation. Although the Hoxd genes involved in mammary gland development in eutherians and marsupials are present in the platypus genome, a sequence of the TgNCS39 transgene (named mammary bud regulatory element, MBRE) which is responsible for activating *Hoxd9* is conserved only in eutherians and marsupials and does not exist in the platypus (Schep *et al.*, 2016).

Mike Augée and collaborators found that *Tachyglossus* initially secretes dilute milk containing 1.3% fat, 7.9% protein and 2.9% carbohydrate whereas mature milk is very concentrated containing 31% fat, 12.4% protein and 2.8% carbohydrate. The fat concentration in adult milk is within the mammalian range, but platypus milk contains considerable polyunsaturated fatty acid whilst *Tachyglossus* milk contains little. The carbohydrate differs from other mammals: half the carbohydrate in platypus milk is difucosyllactose whilst *Tachyglossus* milk mainly contains sialyl- and fucosyllactose. Gene expression of platypus milk proteins is 50% β -lactoglobulin (whey protein) and 30% calcium binding casein; echidna milk contains a higher content of whey proteins than platypus milk. Casein genes are clustered tightly together in the platypus genome and contain recently duplicated β -casein matrix protein genes which are thought to have evolved by duplication of enamel matrix protein, either enamelin or ameloblastin.

Chromosomes and Sex Determination

Monotremes feature a remarkably complex sex chromosome system which is homologous to the sex chromosomes of birds rather than other mammals. The sex determination gene SRY of other mammals is absent in monotremes and Sox3 (from which Sry evolved) is located on platypus autosome 6 (Grützner *et al.*, 2004; Veyrunes *et al.*, 2008). A Y chromosome specific copy of the evolutionarily conserved Anti Mullerian Hormone Gene (AMH) has been discovered on platypus Y5 which is currently considered a candidate sex determination gene in monotremes (Cortez *et al.*, 2014).

Platypus have 5 pairs of X chromosomes in females and 5 X and 5 Y chromosomes in males (Grützner *et al.*, 2004; Murtagh, 1977). There is no homology between platypus sex chromosomes and the eutherian X which is largely homologous with the platypus autosome (Veyrunes *et al.*, 2008). The sex chromosomes of male platypus are segregated into sperm with either 5X or 5Y chromosomes. This segregation is mediated by the 10 sex chromosomes assembling as a chain at the first meiotic division via 9 pseudoautosomal regions. The order and timing of chromosome pairing is remarkably well regulated. In contrast to other mammals monotremes lack meiotic sex chromosome inactivation in male meiotic cells (Daish *et al.*, 2015) and X chromosome inactivation in female somatic cells (Julien *et al.*, 2012). The chromosomes are distributed non-randomly in the sperm nucleus as in other mammals and unlike the random distribution in avian sperm. Genes in the sex chromosomes differ from other mammals showing extensive homology with the chicken Z chromosomes as well as a number of autosomes (Grützner *et al.*, 2004; Veyrunes *et al.*, 2008).

Tachyglossus has 5 pairs of X chromosomes in females and 5 X and 4 Y chromosomes in males. The meiotic chain of the 9 sex chromosomes is similar to the platypus, and most chromosomes of both species are homologous. The only exceptions are platypus sex chromosomes Y3-X4 and parts of Y4 which show homology with *Tachyglossus* chromosome 27. The reason why *Tachyglossus* has one less Y chromosome than the platypus is because platypus Y5 has been trans-located to Y3 in *Tachyglossus*. Further, a demonstration of significant differences in the distribution of MHC genes (major histocompatibility complex) in the chromosomes of platypus and *Tachyglossus* indicates that the order of chromosomes in the meiotic chain differs between species (Rens *et al.*, 2007). This means that there have been ongoing changes to the meiotic chain after the monotremes diverged from the mammalian lineage.

Discussion

It is concluded that reproduction in monotremes involves characteristics of sauropsids, mammals and some unique characteristics. Whilst their sex chromosome system is homologous to the sex chromosomes of birds rather than other mammals, they have fully adopted lactation, a strategy of maternal care that characterizes mammals. Like marsupials, monotremes are dependent on lactation from an earlier stage of development than eutherian mammals and (their eggs) spend only a brief period *in utero*. However, the protection afforded by the monotreme egg shell precludes the apparent need of eutherians and marsupials for the ovum to have a thick zona pellucida to support early development. Consequently, according to Michael Bedford monotremes have retained sauropsid-like spermatozoa which lack the rigidity and forward motility that eutherian and marsupial sperm require to penetrate the thicker zona. On the other hand, the stiffened structure of eutherian and marsupial spermatozoa may be an adaptation in response to competition between males to achieve paternity (sperm competition). In this respect, sperm bundle formation by monotremes also increases the rate and strength of forward motility. It is also perhaps noteworthy that sperm bundle formation has the hallmarks of capacitation of eutherian and marsupial spermatozoa (see above). However, this association requires a better understanding of the biological significance of sperm capacitation.

References

- Cortez, D., Marin, R., Toledo-Flores, D., et al. (2014). Origins and functional evolution of Y chromosomes across mammals. *Nature*, *508*, 488–493.
- Daish, T. J., Casey, A. E., & Grützner, F. (2015). Lack of sex chromosome specific meiotic silencing in platypus reveals origin of MSCI in therian mammals. *BMC Biology*, *13*, 106.
- Griffiths, M. (1978). *The Biology of the Monotremes*. New York, San Francisco, London: Academic Press Inc.
- Grützner, F., Nixon, B., & Jones, R. C. (2008). Reproductive biology in egg-laying mammals. *Sexual Development*, *2*, 115–127.
- Grützner, F., Rens, W., Tsend-Ayush, E., et al. (2004). In the platypus a meiotic chain of ten sex chromosomes shares genes with the bird Z and mammal X chromosomes. *Nature*, *432*, 911–917.
- Jones, R. C., Clulow, J., Stone, G. M., & Setchell, B. P. (1987). The role of the Initial Segments of the Epididymis in Sperm Maturation in Mammals. In H. Mohri (Ed.), *New Horizons in Sperm Cell Research*. New York: Gordon & Breach Science Publishers.
- Julien, P., Brawand, D., Soumillon, M., et al. (2012). Mechanisms and evolutionary patterns of mammalian and avian dosage compensation. *PLOS Biology*, *10*, e1001328.
- Morrow, G., Andersen, N. A., & Nicol, S. (2009). Reproductive strategies of the short-beaked echidna – a review with new data from a long-term study on the Tasmanian subspecies (*Tachyglossus aculeatus setosus*). *Australian Journal of Zoology*, *57*, 275–282.
- Murtagh, C. A. (1977). A unique cytogenetics system in monotremes. *Chromosoma*, *65*, 37–57.
- Park, J. L., Semyonov, J., Chang, C. L., et al. (2008). Origin of INSL3 – mediated testicular descent in therian mammals. *Genome Research*, *18*, 974–985.
- Rens, W., O'Brien, P. C., Grützner, F., et al. (2007). The multiple sex chromosomes of platypus and echidna are not completely identical and several share homology with the avian Z. *Genome Biology*, *8*, R243.
- Sage, H., Vernon, R. B., Decker, J., Funk, S., & Iruela-Arispe, M. L. (1989). Distribution of the calcium-binding protein SPARC in tissues of embryonic and adult mice. *The Journal of Histochemistry and Cytochemistry*, *37*, 819–829.
- Schep, R., Necsulea, A., Rodríguez-Carballo, A. E., et al. (2016). Control of Hoxd gene transcription in the mammary bud by hijacking a preexisting regulatory landscape. *Proceedings of the National Academy of Sciences*, *113*, E7720–E7729.
- Temple-Smith, P., & Grant, T. (2001). Uncertain breeding: A short history of reproduction in monotremes. *Reproduction, Fertility and Development*, *13*, 487–497.
- Veyrunes, F., Waters, P. D., Miethke, P., et al. (2008). Bird-like sex chromosomes of platypus imply recent origin of mammal sex chromosomes. *Genome Research*, *18*, 965–973.
- Warren, W. C., Hillier, L. W., Marshall Graves, J. A., et al. (2008). Genome analysis of the platypus reveals unique signatures of evolution. *Nature (London)*, *453*, 175–183.
- Wong, E. S. W., Nicol, S., Warren, W. C., & Belov, K. (2013). Echidna venom gland transcriptome provides insights into the evolution of monotreme venom. *PLOS ONE*, *8*. <https://doi.org/10.1371/journal.pone.0079092>.

Comparative Mammalian Female Reproduction: Overview

Marilyn B Renfree and Geoffrey Shaw, The University of Melbourne, Parkville, VIC, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Altricial A condition describing young that are relatively underdeveloped at birth, although some features needed for survival may be more advanced.

Apomorphic A specialized (or derived) trait or character that is unique to a group or species.

Blastocyst An early stage of mammalian development where the embryo is a hollow ball of around 100 cells, reached before implantation or expansion.

Diapause A regulated transient delay in embryonic development, usually at the blastocyst stage.

Macropodid ‘big foot’ A member of the kangaroo and wallaby family of marsupials.

Nidifugous Leaving the nest shortly after hatching.

Nidicolous Remaining in the nest shortly for some time after hatching.

Oviparous Egg laying.

Plesiomorphic An evolutionary trait or character state that is homologous within a particular taxon but is not unique to members of that group.

Post partum oestrus The oestrus that occurs immediately after giving birth.

Semelparity A reproductive pattern in which the females normally only give birth once in their lifetime.

Synapomorphic A shared derived character or trait state that distinguishes a clade from other organisms.

Therian mammals Comprises the two viviparous groups of mammals, the marsupials and the eutherians.

Viviparous Live-bearing.

Introduction

The three groups of mammals – the monotremes, the marsupials and eutherians – primarily differ from one another by their reproduction and the anatomy of their reproductive tracts (Asdell, 1946; Tyndale-Biscoe and Renfree, 1987; Hayssen *et al.*, 1985). There are four species of monotreme, all of which are oviparous (Griffiths, 1978), whereas the 338 or more living species of marsupial all give birth to small, under-developed young after a short gestation supported by a placenta, and most species complete their development after birth within their mother's pouch during the extended lactational period (Tyndale-Biscoe and Renfree, 1987). There is considerable variation in the modes of reproduction amongst the 5000 or so eutherian mammals, with gestations ranging from 2 weeks to 2 years. However, the characteristic that unites all mammals is their ability to lactate and provide milk to support the growth and development of their young.

The reproductive processes of the three major groups of mammals have many similarities (Table 1). Fertilisation is internal, and even in the egg-laying monotremes there is placental exchange and development in utero. In the ovary, the Graafian follicles, functional corpora lutea have similar anatomy and functions. The early embryos begin as bilaminar blastocysts supported by uterine secretions and all have a yolk sac placenta, mammary glands, and lactate. Although monotremes retain some ancestral characters (e.g., egg laying), which show an early divergence from the stock leading to therian mammals, namely the marsupials and the eutherians. Marsupials and eutherians arose from a common ancestral group and are not a derivation of one from the other (Fig. 1). The basic mode of mammalian reproduction originated with the first small nocturnal insectivorous mammals in the Triassic and remained almost unchanged until the late Mesozoic era when mammals started to radiate to fill new habitats and ecological niches. Increasing body size imposed greater physiological and metabolic requirement that provided new selective pressures on reproductive strategies.

Reproductive Strategies

Reproduction in mammals is defined by lactation, which provides the young with nutrition following intra-uterine development. The durations of gestation and lactation are variable. The shortest gestation occurs in marsupials, with a 12.5 day pregnancy in the bandicoot, a peramelid marsupial, to the longest in the African and Asian elephants with 22 months. The monotremes, the echidnas and platypus, have a 22–23 day intra-uterine phase, with a 10 day incubation period within the egg before hatching. Lactation ranges from the ultra-short, 3–5 days as in the hooded seal, to the longest, as in years in whales, elephants and humans. The smallest mammal at birth is the marsupial honey possum, whose newborn young weigh less than 5 mg. The largest mammals at birth are the whales and elephants (2000 kg in the blue whale and around 100 kg in elephants) (Fig. 2).

Table 1 A comparison of reproductive characters amongst the three groups of living mammals

Character	Monotremes	Marsupials	Eutherians
1. Bulbo-urethral glands	Present	Present	Present
2. Prostate gland	Present, disseminate	Present, disseminate	Present
3. Seminal vesicles	Absent	Absent	Present
4. Glans penis	Bifid	Bifid or single	Single
5. Scrotum	Absent	Pre-penial	Pre- or post-penial
6. Testes	Abdominal	Inguinal or scrotal	Abdominal, inguinal, or scrotal
7. Testicular blood supply	Simple	Rete mirabile	Pampiniform plexus
8. Sperm head	Long, fusiform	Short	Short
9. Ureter's entry	Urogenital sinus, dorsal	Bladder, ventromedial	Bladder, ventrolateral
10. Endometrium	Secretory	Secretory	Secretory
11. Ovarian follicles	No antrum, liquor folliculi	Antral, liquor folliculi	Antral, liquor folliculi (no antrum in some species)
12. Corpora lutea	Secretory, autonomous	Secretory, autonomous	Secretory, pituitary or placenta dependent
13. Ovum	Large, yolk-filled	Small, yolk extrusion	Very small, no yolk
14. Cleavage	Meroblastic to blastocyst	Holoblastic to blastocyst	Holoblastic to morula (or blastocyst)
15. Bilaminar blastocyst with trophoblast	Present	Present	Present
16. Muroid coat	Present	Present	Present in few, absent in most
17. Shell coat	Present	Present	Absent
18. Shell	Present	Absent	Absent
19. Embryo formation	From outer layers	From outer layers	Inner cell mass in most
20. Vascularized yolk sac	Present in all	Present in all	Present in some
21. Vascularized chorioallantois	Present	Absent (except Peramelidae)	Present in all
22. Invasive villous placenta	Absent	Absent (except Peramelidae)	Present in all
23. Endocrine function of placenta	Unknown	Steroidogenesis slight; LH, PRL, RXN, GH, and prostaglandin, production	Multiple hormones
24. Immunoprotection of fetus	Unknown	Present	Present
25. Delivery of young	Altricial from egg	Altricial from uterus	Altricial to precocial from uterus
26. Hair and sweat glands	Present	Present	Present
27. Mammary gland, alveolar, and myoepithelial cells	Present	Present	Present
28. Mammary hairs	Present	Present	Absent
29. Mammary anlagen	Areola patches	Areola patches	Mammary lines
30. Teats	Present	Present	Present
31. Mammary glands in males	Absent	Absent	Present
32. Crural spurs and glands	Absent	Absent	Absent
33. Epipubic bones	Present	Present	Absent
34. Pouch	Present/absent	Present/absent	Absent
35. Lactation	Long duration	Long duration	Short duration
36. Milk composition	Major changes through lactation	Major changes through lactation	Minor changes through lactation

Source: Adapted and updated from Tyndale-Biscoe, C.H., Renfree, M.B., 1987. Reproductive Physiology of Marsupials. Cambridge University Press.

All mammals have a high level of parental investment in their young depending on the size and degree of maturation at birth (Derrickson, 1992; Langer, 2008). Some young are born immature and hairless, like most monotremes and marsupials in which the altricial (highly immature and relatively undeveloped) young are only around 0.10% of maternal body weight to many eutherian mammals in which the investment is 15% or more of maternal weight. Exceptions are bears, for example, the giant panda that delivers altricial young that are only 0.1% of maternal body weight and the brown bear whose young is only 0.4% of its mother's weight (Fig. 3). In higher primates including humans, despite their large size at birth, the young are secondarily altricial and need extended parental care extending well beyond lactation.

Comparative Anatomy of the Female Reproductive System

In all mammals the gonad originates as a thickening on the side of the mesonephros. The internal reproductive system develops from the paired Wolffian and Mullerian ducts in the early embryo. The Mullerian duct, is the precursor of the female duct system and regresses in males. In females, the Mullerian duct differentiates to form the oviduct, uterus, cervix and upper vagina, and the

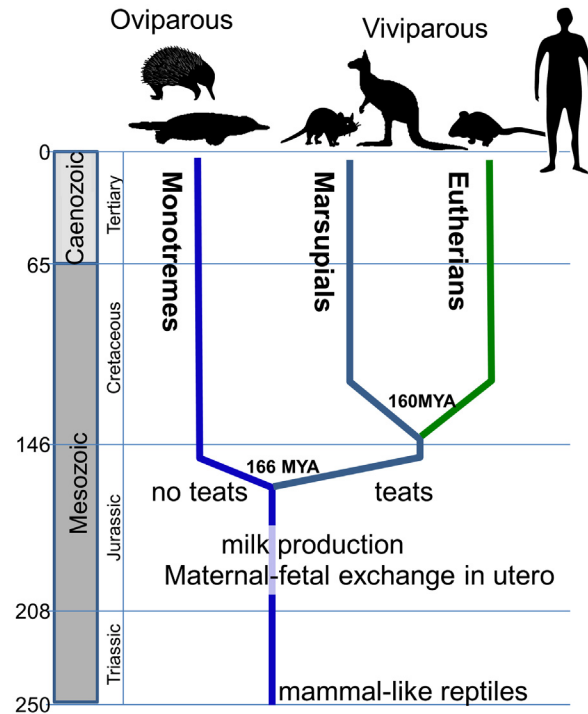


Fig. 1 Evolutionary timeline of the evolution of maternal-fetal exchange from the uterus, oviparity (monotremes) and viviparity (therians), lactation and teat development. Modified from Renfree, M.B., Hore, T.A., Shaw, G., Graves, J.A.M., Pask, A.J., 2009. Evolution of genomic imprinting: Insights from marsupials and monotremes. *Annual Review of Genomics and Human Genetics* 10, 241–262.

Wolffian duct regresses. Despite these common features, the three mammalian groups are also distinguished by their reproductive anatomy. In the monotremes, the ureters never make contact with the bladder, but instead urine reaches the bladder via the urogenital sinus for storage. In marsupials the ureters pass between the genital ducts; while in eutherians the ureters develop lateral to the genital ducts. This difference is established early in fetal development. In female eutherian mammals the paired embryonic Müllerian ducts fuse caudally to form a paired oviducts leading to a single vagina and a single uterus with single cervix (Fig. 4). This

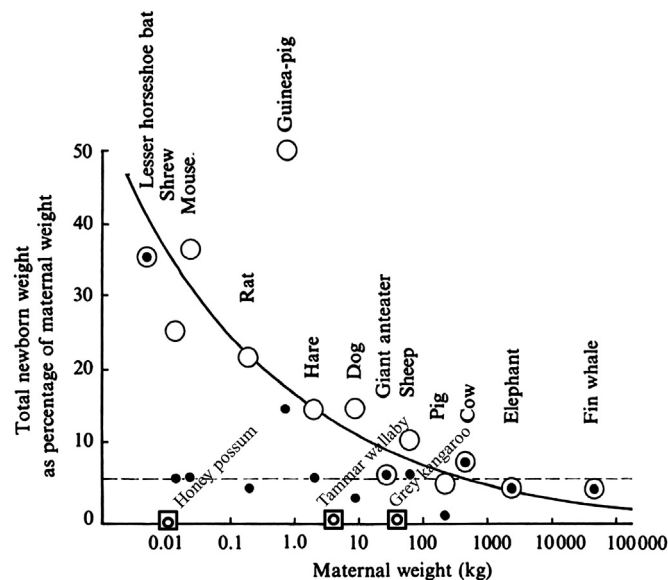


Fig. 2 Relationship between maternal weight and total newborn litter weight. The average birth weight of individual young (5% of maternal weight) is shown as broken line. Newborn individuals (● -eutherians; □): marsupials and litter weight (○) shown for a range of species. Modified from Land, R.B., 1984. *Genetics and reproduction*. second ed. In: Austin, C.R., Short, R.V., (Eds.), *Reproductive Fitness; Reproduction in Mammals*, vol. 4. CUP.

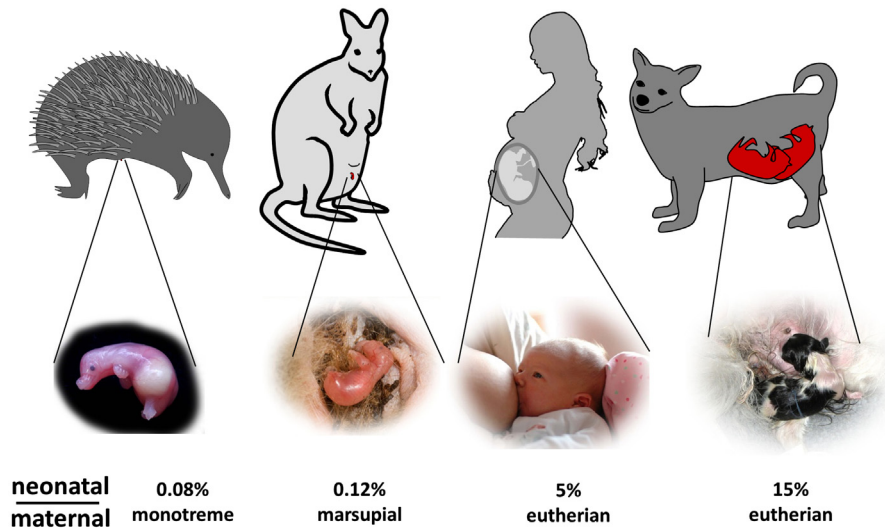


Fig. 3 Maternal investment between mammalian groups. The mass of marsupial and monotreme neonates relative to their mother (0.12% and 0.08% respectively) is much smaller than eutherian neonates (15%) (Hayssen *et al.*, 1985). Despite this, the total mass of monotreme litters at weaning relative to the mothers mass is around 50% (calculated from echidna data reported in Griffiths (1978)). This proportion is similar for marsupial (55%) and eutherian mammals (59%) of comparable size. Thus, marsupials and monotremes rely much more heavily upon lactation for nourishment of young than eutherians do. Modified from Renfree, M.B., Hore, T.A., Shaw, G., Graves, J.A.M., Pask, A.J., 2009. Evolution of genomic imprinting: Insights from marsupials and monotremes. *Annual Review of Genomics and Human Genetics* 10, 241–262.

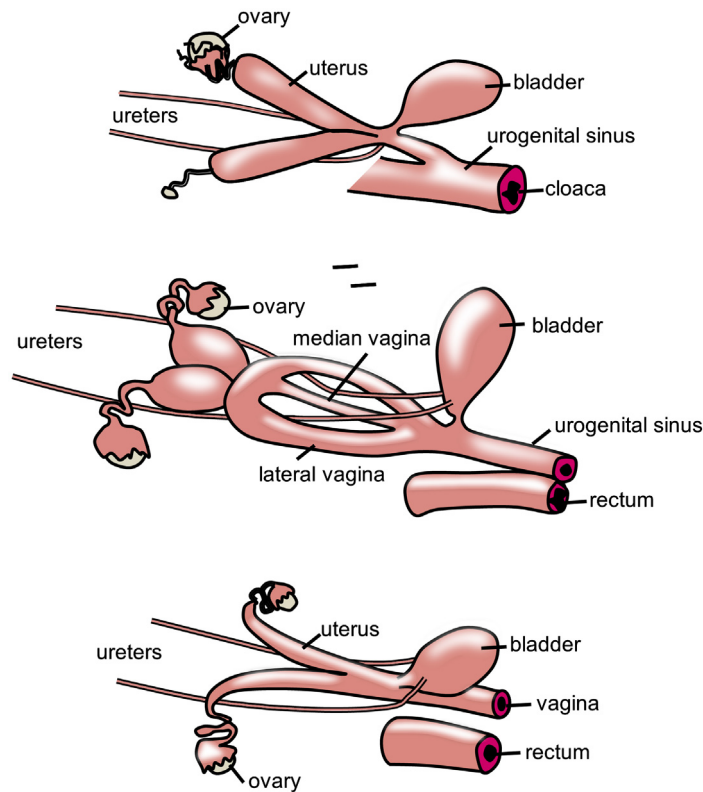


Fig. 4 The relationships of the excretory and genital ducts in marsupials (top), monotremes (center) and eutherians (bottom). The monotreme condition is a plesiomorphic amniotic condition. Note that in this example the right ovary is non-functional, as in the platypus. The ureters open into the urogenital sinus directly opposite the opening of the bladder, whereas in the synapomorphic therian condition the ureters, while taking either the medial (marsupial) or lateral (eutherian) route, open directly into the bladder. Modified from Renfree, M.B., 1993. Ontogeny, genetic control and phylogeny of female reproduction in monotreme and therian mammals. In: Szalay, F.S., Novacek, J.J., McKenna, M.C., (Eds.), *Mammal Phylogeny*. New York: Springer-Verlag, pp. 4–20.

cannot happen in marsupials because the ureters are in the way, so they form two lateral vaginae, and two completely separate uteri with separate cervixes. Whilst most mammals have paired ovaries connected via paired oviducts, in one monotreme, the platypus, only the left ovary is functional.

The mammalian ovary consists of somatic and germ cells. The primordial germ cells originate outside the embryo body and migrate to the gonadal ridges. During migration and in the ovary they proliferate mitotically, and eventually become enclosed in somatic cells to form primordial follicles. Female germ cells enter meiotic arrest before formation of primordial follicles and remain dormant until after puberty. After puberty individual germ cells reactivate over the reproductive life cycle. As reactivated follicles grow, they secrete increasing amounts of oestrogens which prime the reproductive tract and induce oestrus (mating behaviour). After ovulation the follicle transforms into a corpus luteum secreting progesterone, a hormone essential to maintain pregnancy. Thus female reproduction is characterised by cyclic hormonal changes caused by this ovarian cycle. In some species, such as humans, the follicular and luteal phases are well separated and last about 14 days each (Fig. 5). In others, such as sheep, follicular growth occurs in waves during the late luteal phase so that by the time the corpus luteum regresses after about 16–18 days follicles are nearly ready for ovulation so the follicular phase before ovulation is short – about 4 days. Mice and rats have ultra-short cycles of 4–5 days, with a truncated luteal phase unless the females mate, inducing a surge of prolactin that supports formation of progesterone secreting corpus luteum.

Towards the end of the follicular phase, elevated oestrogens act in the brain to induce oestrous behaviour, so that mating normally occurs only around the time of ovulation. A notable exception is the human and some other higher primates, where mating behaviour in females may occur at any stage of the cycle.

Ovulation in many species is spontaneous as a result of an LH surge induced by the elevated oestrogen levels produced by mature follicles. Some species, such as koalas, cats, rabbits, ferrets and camels, are induced ovulators. In these a neuroendocrine reflex induced by mating provides the trigger for the ovulation-inducing LH surge.

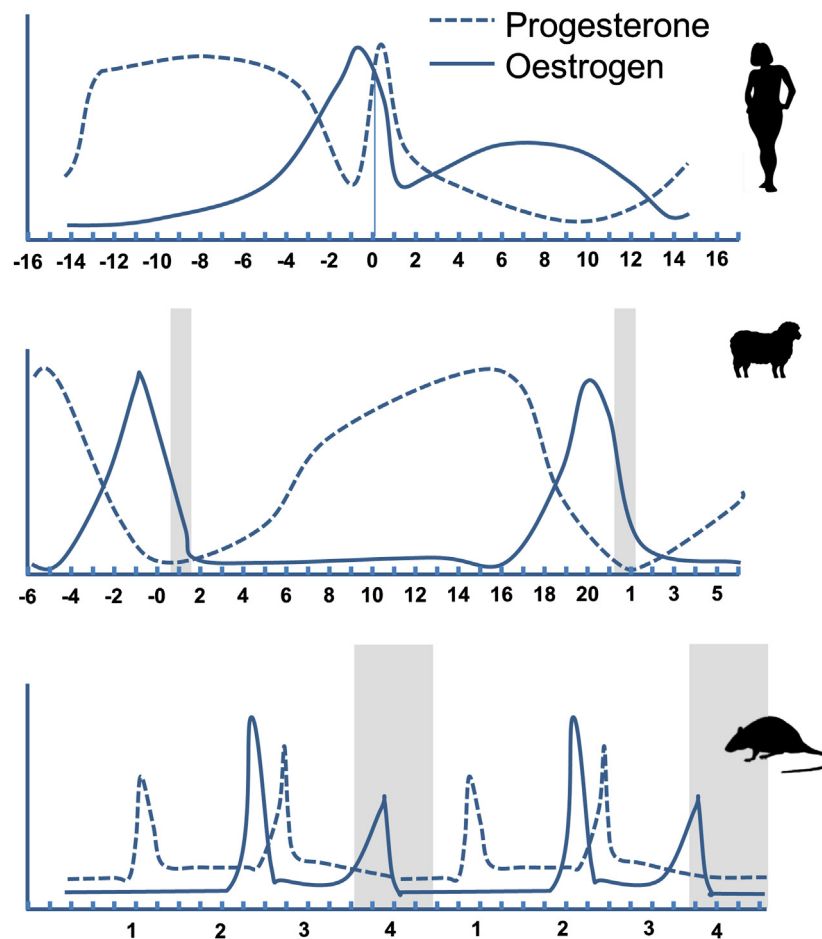


Fig. 5 The mammalian oestrous cycle. In humans, the follicular and luteal phases are well separated and last about 14 days each. The sheep oestrous cycle has a sharp peak of oestrogen during the short follicular phase of around 5 days which immediately precedes behavioural oestrus (green bars). This is followed by a longer luteal phase of about 20 days supported by the progesterone-secreting corpus luteum. Luteolysis occurs concomitantly with the rise in oestrogen for the follicles in the next cycle. Mice and rats have ultra-short cycles of 4–5 days, with a truncated luteal phase unless the females mate, inducing a surge of prolactin that supports formation of progesterone-secreting corpus luteum.

Pregnancy

After ovulation the released egg passes into the oviduct, where fertilisation may occur if mating has happened. After fertilisation the cleaving zygote passes into the uterus. Once there, an embryo-uterine dialogue begins. In many species the uterine lining reacts to the presence of the embryo by complex patterns of cell growth and differentiation mediated by inflammatory cytokines. This is a decidual reaction that establishes appropriate conditions for subsequent embryonic development. Other species, such as marsupials, establish pregnancy without a decidual reaction. As the embryo grows it establishes a placenta that mediates exchange of nutrients, gases and wastes, and regulates maternal physiology through specific hormone production. All mammals have a chorio-vitelline (yolk sac) placenta but in eutherians it is supplanted by a chorioallantoic (allantoic) placenta. In monotremes, the 3 mm fertilised egg undergoes significant intra-uterine development supported by choriovitelline and chorioallantoic transfer to about the 17 somite stage and a diameter of about 17 mm when the egg is laid, after which development is achieved entirely on resources in the egg until hatching and the start of suckling. Marsupials give birth to tiny young after a short gestation, supported by a choriovitelline placenta. Only a few marsupials, such as bandicoots, develop a short-lived chorioallantoic placenta in the last days of pregnancy. In most eutherians the choriovitelline placenta is functional before a chorioallantoic placenta develops and becomes the primary placental organ. In mice, the choriovitelline placenta becomes inverted and is the site of transfer of immunoglobulins to the fetus.

In eutherian mammals pregnancy is longer than the oestrous cycle. If pregnancy is established the life of the corpus luteum is extended to provide the progesterone needed to maintain a receptive and secretory endometrium essential for pregnancy. In marsupials the length of the oestrous cycle is around one month, and longer than gestation length, so extension of luteal life is not observed in pregnant females. Much less is known about the monotreme reproductive cycle. Reliable gestation periods of about 21 days have been recorded for the short beaked echidna. After oviposition, the egg is held in the pouch for about 10.5 days incubation before hatching occurs.

Pregnancy and lactation occupy very different relative periods of time during mammalian reproduction, depending on the reproductive strategy of each group (Fig. 6). In eutherian mammals, pregnancy is relatively long, and the young of most at delivery, whilst variable among species, are relatively mature in contrast to the marsupials and monotremes that deliver highly altricial young that complete their development in the pouch or nest, dependent of lactation (see below). Marsupials have effectively traded the umbilical cord for the teat.

Diapause

About 130 species of mammal have a suspension of embryonic development at the blastocyst stage known as embryonic diapause. Diapause allows synchronisation of reproduction with seasonal cycles or act as a species isolating mechanism in closely related species. It occurs in a wide range of species including members of rodents, bears, bats, marsupials (notably kangaroos, wallabies and small possums), armadillos, seals, sea lions, skunks, mink, martins, wolverine and other mustelids, and one species of deer

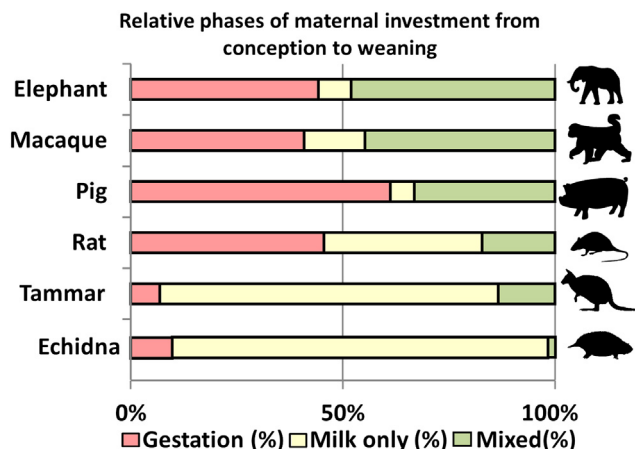


Fig. 6 Relative phases of maternal investment from conception to weaning. The percentage of time spent during gestation, lactation (milk-only period) and mixed feeding (milk and solids) for elephants, macaques, pigs, rats and tammar wallabies. There are 4 classes by which eutherians are grouped based on the characteristics of the young or litter at birth. Elephants represent group 4 and are described as precocial (open eyes and haired) and nidifugous (leave the nest shortly after hatching or birth). Macaques represent group 3 and are described as precocial and transported (young are supported or carried). Pigs represent group 2 and are described as precocial and nidicolous (dependent on parent for feeding, care and protection). Rats represent group 1 and are described as altricial (closed eyes and naked) and nidicolous. Tammars and echidna are classified as altricial, nidicolous and transported. Modified from Stringer, J.M., Pask, A.J., Shaw, G., Renfree, M.B., 2014. Post-natal imprinting: Evidence from marsupials. *Heredity* 113, 145–155.

(Tyndale-Biscoe and Renfree, 1987; Renfree and Shaw, 2000, 2014; Racey and Entwistle, 2000). Diapause may be controlled by the sucking stimulus of the young during lactation, or by seasonal factors such as photoperiod, or in some cases, such as in the tammar wallaby, by both.

Parturition

The physiology of parturition is a complex process in all mammals. Successful birth ultimately depends on a precise synchronization with the appropriate maternal and fetal physiology and behaviour. Parturition is fundamental to mammalian reproduction. There are many common mechanisms but the physiology of parturition in mammals is highly diverse and almost species specific. In all species examined to date prostaglandins and oxytocin/mesotocin are critically important as regulators of uterine contractions among other roles. A broad range of other hormones are also involved, but the details of these endocrine interactions vary widely between species.

Lactation and the Mammary Gland

Lactation is the defining characteristic of mammals (Fig. 6). Presumably in the ancestral mammal secretions from cutaneous glands must have enhanced the survival of eggs or hatchlings leading to the exceedingly sophisticated mammary gland and lactation of present day mammals.

Mammary glands comprise secretory alveoli connected by milk ducts to the teat. The number, shape and size of mammary glands varies between species, and in eutherian mammals they are present in both sexes. Mammary glands never develop in Australian male marsupials, but they are present in South American marsupial males. Mammary glands develop along the “mammary line” and may be thoracic, inguinal and/or abdominal. In all female mammals with the exception of monotremes there is a nipple or teat present. In monotremes the milk ducts empty directly onto the abdominal skin in a milk patch from which the young actively suck. Male monotremes, like male eutherians, possess mammary glands.

Development of this secretory tissue depends on an array of hormones that vary between species, but prolactin, insulin, cortisol, and progesterone are notable. In mammals the mammary gland only develops in the later stages of pregnancy and undergoes rapid regression after weaning. In humans the breast, which is a prominent and permanent characteristic of the female, develops around puberty, but this comprises mostly fatty tissue rather than secretory alveoli.

In all mammals, the first milk is colostrum, a low-fat high-protein fluid with free floating cells and with abundant immunoglobulin G- and A-like proteins that provide immunological protection to the suckled young. After a few days the milk composition changes to the mature milk. Composition of the milk is highly variable between species (Table 2), but in most eutherian species, once lactation is established, there is little variation in milk composition through lactation. In marsupials and monotremes, because of the immaturity of their young, lactation lasts for relatively longer than in most eutherian species. Unlike in eutherians, where there are relatively few changes in milk composition until weaning, in monotremes and marsupials milk composition changes profoundly to match the dramatic developmental changes of the young during pouch or nest life. The changing milk composition regulates the growth and development of the young.

Release of milk from the mammary gland is regulated by oxytocin or related hormones. These act on the contractile myoepithelial cells that surround the alveoli to increase intra-mammary pressure. Sensory stimuli such as sucking induce oxytocin release from the posterior pituitary and subsequent milk ejection (letdown). Milk synthesis is controlled by prolactin from the anterior pituitary together with other hormones.

Seasonality

Most mammals breed seasonally, with mating occurring at a time of year that leads to weaning of young at the optimum time for their survival (Gilmore and Cook, 1981). Seasonality may be controlled by many factors, though photoperiod and nutritional cues

Table 2 Energy content of mammalian milk

Component	Human	Elephant seal	Tammar early	Tammar late
Water	90	35	90	85
Protein	1.1	10	4.5	10
Carbohydrate	7.5	2	5.8	2
Lipid	4.2	55	2	10
Energy (MJ/100 mL)	5.4	65.3	8.0	22.9

Source: Adapted from Stringer, J.M., Pask, A.J., Shaw, G., Renfree, M.B., 2014. Post-natal imprinting: Evidence from marsupials. *Heredity* 113, 145–155.

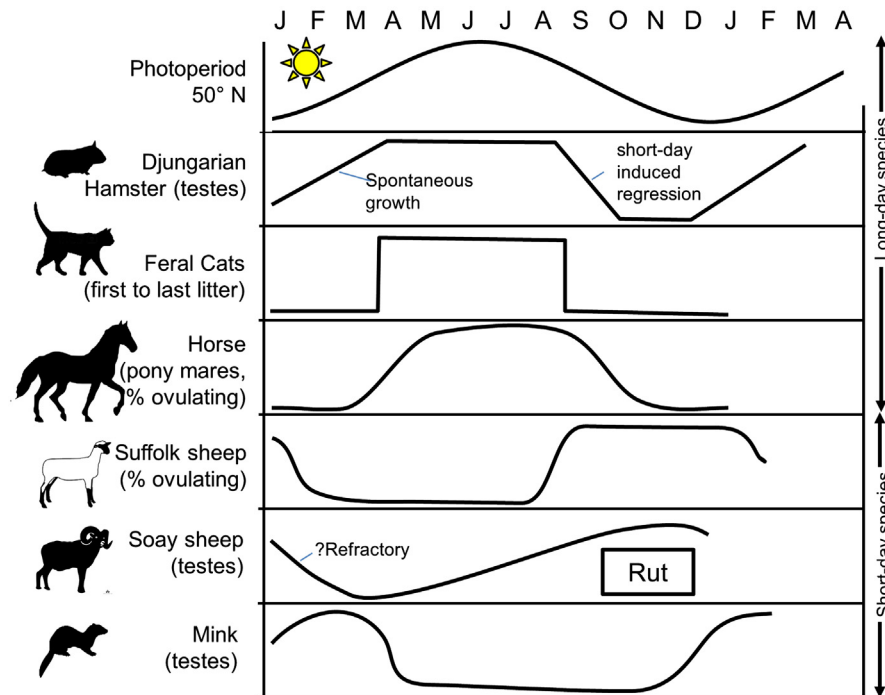


Fig. 7 Seasonal gonadal activity in a variety of photoperiodic mammals. Modified from Follett, B.K., 1984. The environment and reproduction. In: Austin, C.R., Short, R.V., Reproductive Fitness; Reproduction in Mammals. vol. 4, second ed., CUP.

are the most common. Photoperiod is the major environmental factor regulating seasonal reproduction (Fig. 7). When mammals are transferred from one hemisphere to the other, annual rhythms in season and photoperiod are shifted 6 months out of phase. Photoperiodic signals are mediated via circadian cycles of melatonin secretion from the pineal gland which regulates GnRH pulsatility in the hypothalamus that in turn regulates gonadotropin release from the pituitary. Similarly, nutritional or metabolic cues control seasonal cycles by modulating GnRH pulses in the hypothalamus. In species with long gestations or long lactations, the seasonal cue for onset of breeding is separated from the time of weaning by a considerable time period.

Seasonally breeding eutherian and marsupial species breed at various times of the year, depending on latitude, food availability and temperature. In some, the season lasts weeks or even months, as from early autumn to winter in the sheep, but in others, such as the tammar wallaby, the birth and mating season is highly synchronised to the photoperiod duration of the longest day. The platypus and echidna breed in the spring and wean their young 3.5–6 months later.

Conclusions

The approximately 5.5 thousand mammalian species have many reproductive features in common, but each species has its own unique reproductive pattern that adapts them to their own particular habitats and available resources. We know details about the reproduction of a mere handful of these species, yet their very survival depends on our ability to understand their needs. Comparative reproduction studies are important for this understanding.

References

- Asdell, S. A. (1946). *Patterns of Mammalian Reproduction*. Ithaca, New York: Comstock Publishing CO.
- Derrickson, E. M. (1992). Comparative reproductive strategies of altricial and precocial eutherian Mammals. *Functional Ecology*, 6, 57–65. <https://doi.org/10.2307/238977>.
- Gilmore, D., & Cook, B. (1981). *Environmental Factors in Mammalian Reproduction*. Springer.
- Griffiths, M. E. (1978). *The Biology of Monotremes*. New York, London: Academic Press.
- Hayssen, V., Lacy, R. C., & Parker, P. J. (1985). Metatherian reproduction: Transitional or transcending? *The American Naturalist*, 126, 617–632.
- Langer, P. (2008). The phases of maternal investment in eutherian mammals. *Zoology*, 111, 148–162.
- Racey, P. A., & Entwistle, A. C. (2000). Life history and reproductive strategies of bats. In E. G. Crichton, & P. H. Krutzsch (Eds.), *Reproductive Biology of Bats*. London San Diego: Academic Press.
- Renfree, M. B., & Shaw, G. (2000). Diapause. *Annual Review of Physiology*, 62, 353–375.
- Renfree, M. B., & Shaw, G. (2014). Embryo-endometrial interactions during early development after embryonic diapause in the marsupial tammar wallaby. *The International Journal of Developmental Biology*, 58, 175–181.
- Tyndale-Biscoe, C. H., & Renfree, M. B. (1987). *Reproductive Physiology of Marsupials*. Cambridge University Press.

Eutherians: Placental Mammals

Ulrich Zeller, Humboldt University of Berlin, Berlin, Germany

Kirsten Ferner, Leibniz-Institute for Evolution and Biodiversity Science, Berlin, Germany

Thomas Göttert and Nicole Starik, Humboldt University of Berlin, Berlin, Germany

© 2018 Elsevier Inc. All rights reserved.

Reproductive Organs

The eutherian ovary is located in the retroperitoneal space at the transition between abdominal and pelvic cavity. Egg cells (primary and secondary oocytes) develop in the ovary (oogenesis) and are generally yolk free. Primary oocytes derive from stem cells (primordial germ cells) and differentiate into secondary oocytes as a result of the first phase of meiotic division. Secondary oocytes remain arrested in the second phase of meiosis, which is only finished after fertilization. A fixed number of oocytes is determined around the time of birth (follicular pool). The formation of new oocytes after maturity (neo-oogenesis) is a rare phenomenon and has been reported to occur in *Mus musculus* and a number of primate species (e.g., *Galago senegalensis*, *Perodicticus potto* and *Daubentonia madagascariensis*). Follicle maturation and ovulation are regulated by hormones of the pituitary gland (hypophysis). A plasma membrane of the oocyte (oolemma) is surrounded by a Zona pellucida and a Corona radiata. Except for *Oryctolagus cuniculus*, tertiary egg membranes (muroid layer) are absent. According to the degree of paramesonephric duct fusion, the uterus can be completely paired (Uterus duplex: e.g. Lagomorpha, some Rodentia), partly fused (Uterus bipartitus: e.g. most Carnivora, some Chiroptera; Uterus bicornis: e.g. Pholidota, Eulipotyphla), or entirely fused (Uterus simplex: e.g. Primates, some Chiroptera and Xenarthra) (Fig. 1(a)). In contrast to marsupials, female eutherians are generally characterized by an unpaired vagina (monodelphy; diagnostic feature).

Testicles descend outside the abdominal cavity (Descensus testiculorum) and can be located either scrotal (e.g. Primates, Artiodactyla, Perissodactyla), or inguinal (e.g. Cetacea, Eulipotyphla, Pholidota). True testicondy (testicles not descended) occurs in Afrotheria and Xenarthra. In contrast to marsupials, eutherian males are characterized by a postpenial position of the scrotum (if present). In eutherians a variety of male accessory sex glands are present, which are important for the activation and transport of spermatozoa and other reproductive parameters (e.g., formation of a vaginal plug in some rodents and carnivores).

Sexual Cycle

After reaching puberty, females of most eutherian species possess an estrus cycle in terms of periodic physiologic changes of reproductive organs (and sexual receptivity) according to distinct phases: proestrus, estrus, metestrus, and anestrus. Sexual activity of females is strictly limited to a defined phase of sexual receptiveness (estrus phase), also as an adaptation to seasonal conditions. Seasonality of reproduction can also vary according to specific ecological conditions, even within one species. Depending on the cycle frequency, taxa can be grouped into monoestrous, diestrous, and polyestrous. In nonconceptive cycles, the endometrium is reabsorbed down to a basic layer. Domestication can lead to an increase in the cycle frequency and hyper-sexuality.

Within different groups of primates (Pongidae, Hominidae and Callitrichidae), an ovarian cycle, or a menstrual cycle, has evolved. In taxa with a true menstrual cycle, the endometrium regularly undergoes dissolution through menstruation (tissue shedding and bleeding), if conception does not occur.

Most eutherians ovulate periodically during a specific season (estrus). However, in several species and among various systematic groups, ovulation is triggered by external stimuli (induced ovulation; Carnivora, Lagomorpha, Rodentia, Eulipotyphla, Artiodactyla, Perissodactyla and Scandentia). In primates (and possibly other taxa), ovulation occurs spontaneously without mating-induced stimuli.

Females of some eutherian species communicate their reproductive status via scent glands and scents may even modulate reproductive physiology. Some callitrichids (Primates), for example, use olfactory clues (scents) to transmit information on reproductive status (e.g. identification of fertile phase of the ovarian cycle) and social status in order to adopt strategies depending on their socio-sexual role in the group (group cohesion). Other signals (e.g., visual, acoustic, and behavioral) are also important for the reproduction of eutherians.

Fertilization

In most eutherians, fertilization occurs in the uterine tubes (Tuba uterina). In some Eulipotyphla (Tenrecidae and the shrew *Blarina brevicauda*), the oocyte is fertilized in the ovary. Polyembryony, the development of a single fertilized oocyte into two or more genetically identical embryos, can occur in various eutherian taxa (including humans). Polyembryony is common in Dasypodidae; between two and 12 embryos develop from one fertilized oocyte. With the exception of some Macroscelididae, the number of ovulated oocytes resembles the number of offspring delivered.

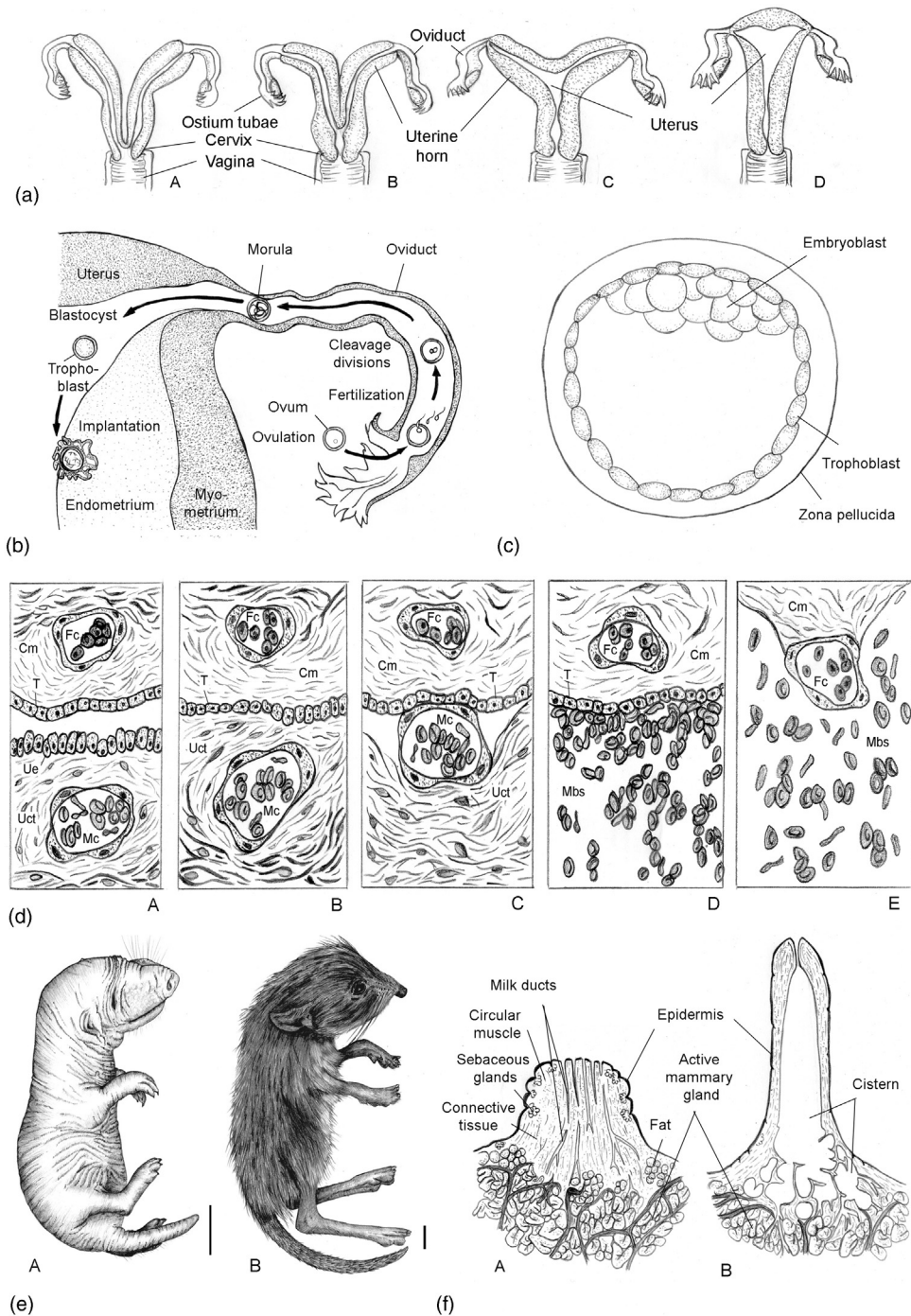


Fig. 1 (a) Female genital tracts, different formations: uterus duplex (A), uterus bipartitus (B), uterus bicornis (C), uterus simplex (D). Original drawing, based on information from the literature. (b) Early embryonic development in a human from ovulation to implantation of the trophoblast into the endometrium of the uterus. (c) Blastocyst of an eutherian mammal. (d) "Placental types" of eutherian mammals based on proximity of fetal and maternal circulations: epitheliochorial (A), syndesmochorial (B), endotheliochorial (C), hemochorial (D), and hemoendothelial (E). Ue, Uterus epithelium; T, Trophoblast; Cm, Chorion mesoderm; Uct, Uterine connective tissue; Fc, Fetal capillaries; Mc, Maternal capillaries; Mbs, Maternal blood sinus. (e) Neonates of an altricial (*Suncus murinus*), (A) and a precocial (*Macroscelides proboscideus*), (B) eutherian species; Scale bar: 0.5 cm. B taken from Ferner *et al.*, 2017. ©John Wiley & Sons. (f) Arrangement of mammary glands, milk ducts and associated tissue in a primate (A) and an artiodactyle (B). Parts (b) Redrawn from Vaughan, T.A., Ryan, J.M., Czaplewski, N.J., 2000. *Mammalogy*, fourth ed. Orlando: Saunders. ©2000 Elsevier Ltd. (c) Redrawn from Selwood, L., Johnson, M.H., 2006. Trophoblast and hypoblast in the monotreme, marsupial and eutherian mammal: Evolution and origins. *Bioessays* 28(2): 128–145. ©2006 Wiley Periodicals, Inc. (d) redrawn and modified from Roberts, R.M., Green, J.A., Schulze, L.C., 2016. The evolution of the placenta. *Reproduction* 152 (5), 179–189. ©2016 Bioscientifica. (f) Redrawn from Hildebrand, M., Goslow, 2004. *Vergleichende und funktionelle Anatomie der Wirbeltiere*. Berlin-Heidelberg: Springer. ©2004 Springer.

Oviduct Oocyte Transport

In contrast to monotremes and marsupials, cleavage in eutherians is equal and holoblastic. Coordinated oviduct musculature contractions, tubal fluids, and ciliary movement of the epithelium transport the cleaving oocyte through the uterine tube towards the uterus (**Fig. 1(b)**). A glycoprotein layer (Zona pellucida) prevents the fertilized oocyte (zygote) from implantation into the tubal epithelium. In eutherian taxa studied, the period of oviduct zygote transport varies between 2 days (*Sus scrofa* f. domestica) and 6 days (*Neovison vison*, *Equus przewalskii* f. caballus) but can be greatly prolonged in bat species possessing delayed fertilization.

Blastocyst

The eutherian blastocyst is characterized by an early differentiation into two distinct cell types: an outer layer of epithelial trophoblast cells and an aggregated inner cell layer (embryoblast) (**Fig. 1(c)**). The displacement of the embryoblast inside the germinal vesicle (trophoblast) is an apomorphic character of eutherians (entypy of the embryo). At an early stage, the blastocyst is surrounded by a glycoprotein layer (Zona pellucida). The embryoblast differentiates into two epithelial layers: epiblast (adjacent to the uterine wall) and hypoblast (adjacent to the blastocyst cavity).

Once arrived in the uterus, the Zona pellucida dissolves and releases the trophoblast. Mononuclear trophoblast cells begin to proliferate and differentiate into a multinuclear cell mass developing into a syncytiotrophoblast (outer layer) and a cytotrophoblast (inner layer). The cytotrophoblast produces lytic enzymes dissolving the extracellular matrix and allowing the active invasion of the syncytiotrophoblast into the endometrium. The syncytiotrophoblast forms the developing interface between fetus and maternal tissues or blood. A syncytiotrophoblast can also occur in marsupials, where it is regulated by retroviral genes, which have also been found in eutherians. Functionally, the syncytiotrophoblast seems more related to fetomaternal metabolism than immunotolerance. The syncytiotrophoblast evolved independently in marsupials and eutherians and even within eutherians (convergent evolution).

The invading (anti-genetically foreign) trophoblast is tolerated in the uterus; a phenomenon referred to as “immunological paradox of pregnancy”. The trophoblast escapes allorecognition and is protected from maternal immune system mediated by different immunotolerogenic mechanisms. A layer of fibrinoid can further protect the blastocyst.

Implantation

Fetal and maternal tissues are initially brought into close contact via trophoblast-uterine epithelial interactions. Implantation requires a state of “receptivity” of the endometrium (implantation window) and, in accordance with the degree of invasiveness of placentation, a certain degree of invasiveness of the trophoblast. Based on the degree of trophoblast-uterine epithelial interactions, different types of implantation can be distinguished (superficial vs. invasive). The least degree of invasiveness can be found among many Artiodactyla, Carnivora and Lagomorpha. Here, the blastocyst is located in the center of the uterine cavity (central implantation). In eutherian species with invasive implantations, the trophoblast uses different modes of penetrating the uterine epithelium. In species with hemochorial placentation, the fetal trophoblast invades maternal vasculature to form an intervillous space through which maternal nutrients pass to the growing fetus. Shortly after the initial invasion of the trophoblast, fetal blood vessels form fetal villi that are in direct contact with the maternal circulation.

In contrast to marsupials and as a consequence of a lack of an immune-protective shell coat, eutherians are characterized by a complex cascade of immunological pathways (antigen expression, regulated apoptosis, production of anti-inflammatory cytokines) leading to the immune-tolerance of the allogenic embryo by the maternal system during pregnancy.

Placentation

Eutherians are characterized by a chorioallantoic placenta, which forms the main organ of fetomaternal exchange. Chorioallantoic tissue (chorionic folds or villi) can deeply invade the maternal endometrium. An additional yolk sac placenta, which can be found in many eutherians at an early stage of gestation, loses its function (e.g., supply of iron-rich histotrophe to the embryo), when the definite chorioallantoic placenta has fully developed and provides iron supply, e.g. via hemophagous regions. Among eutherians, a vast variety of chorioallantoic “placental types” can be found. Various classifications schemes are in use, each focusing on different aspects of the complex continuum of eutherian placentation. It is, however, important to note that these schemes lead to artificial categories, which do not resemble phylogenetic associations among eutherians.

One classification approach refers to the placental shape and the degree of placental tissue concentration: In diffuse placentas (Placenta diffusa), the chorionic villi are distributed over the entire inner surface of the gestation chamber (Perissodactyla, Strepsirhini, Suidae). Cotyledone placentas (Placenta cotyledonaria) are characterized by small areas of placental tissue concentration in the form of so-called cotyledons or placentomes (most Artiodactyla). Zonary placentas (Placenta zonaria) show a restriction of chorionic villi to a circular zone (Carnivora, many Afrotheria). In discoidal placentas (Placenta discoidalis), the syncytium of chorionic villi forms a discoid area (Rodentia, most Primates, Afrosoricida, Chiroptera, Eulipotyphla).

Another widely used criterion to classify the different “placental types” refers to the composition of the interhemal barrier. According to the degree of placenta invasiveness and erosion of maternal tissue (superficial vs. invasive), the following “placental types” can be distinguished (**Fig. 1(d)**, **Table 1**):

- Epitheliochorial: chorionic villi attach to the intact uterine epithelium, the trophoblast does not erode the uterine epithelium (Cetacea, Perissodactyla, Lemniformes).

Table 1 Summary of data on reproduction and development for eutherian species

Order Species	Placentation (interhemal barrier)	Pregnancy (days)	Lactation (days)	Mamilla (location)	Litter size	Eye opening (days)	Furring (days)
Xenarthra							
<i>Bradypus variegatus</i>	hc	170	10	2 (p)	1	0	0
<i>Dasyus novemcinctus</i>	hc	120	42–49	2 (a)	4	12–18	–
Afrotheria							
Macroscelidea							
<i>Macroscelides proboscideus</i>	hc	61	7	6 (a)	1–2	0	0
Afrosoricida							
<i>Tenrec ecaudatus</i>	hc	57–63	35–42	24 (p, a)	1–31	4–5	5
Tubulidentata							
<i>Orycteropus afer</i>	enc	239–268	210	4 (a)	1	0	na
Hyracoidea							
<i>Procavia capensis</i>	hc	225	90	2 (p) 4(i)	3–4	0	0
Sirenia							
<i>Trichechus manatus</i>	enc	385–400	540	2 (p)	1	0	–
Proboscidea							
<i>Elephas maximus</i>	enc	628	365–730	2 (p)	1	0	0
Euarchontoglires							
Scandentia							
<i>Tupaia belangeri</i>	enc	45	38	4 (a)	2	21.5	4
Dermoptera							
<i>Cynocephalus volans</i>	hc	60	180	2 (a)	1	na	na
Primates							
<i>Daubentonia madagascariensis</i>	epc	158–176	280–350	2	1	0	0
<i>Macaca mulatta</i>	hc	167	210–300	2	1	0	0
<i>Homo s. sapiens</i>	hc	270		2	1	0	0
Rodentia							
<i>Mus musculus</i>	hc	19	28	10 (a)	6–8	12	8–10
<i>Mesocricetus auratus</i>	hc	16	21	10 (a)	6–8	12	10–11
<i>Cavia aperea</i>	hc	61	21	2 (a)	1	0	0
<i>Hystrix cristata</i>	hc	112	60	2 (a)	1	0	0
Lagomorpha							
<i>Lepus europaeus</i>	hc	42–44	14–21	4 (p) 6 (a)	1–6	0	0
<i>Oryctolagus cuniculus</i>	hc	30–37	20	4 (p) 6 (a)	4–15	10	3–7
Laurasiatheria							
Eulipotyphla							
<i>Talpa europaea</i>	enc	30	28–35	6 (a)	4–5	22	21
<i>Erinaceus europaeus</i>	hc	34–49	21–28	10 (a)	1–7	14	20
<i>Sorex araneus</i>	hc	21	23	10 (a)	5–7	19	
<i>Suncus murinus</i>	enc	30	19	6 (a)	1–3	9	4
Cetartiodactyla							
<i>Delphinus delphis</i>	epc	276	120	2 (a)	1	0	–
<i>Globicephala melaena</i>	epc	435	390–488	2 (a)	1	0	–
<i>Sus scrofa</i> f. domestica	epc	120	98–112	4(p) 8(a) 2(i)	3–5	0	0
<i>Hippopotamus amphibius</i>	epc	237	180	2 (i)	1	0	–
<i>Bos primigenius</i> f. taurus	sc	270	180	4 (i)	1–2	0	0
<i>Capra aegagrus</i> f. hircus	sc	150–160	120	2 (i)	1	0	0
<i>Ovis ammon</i> f. aries	sc	150	120	2 (i)	1–2	0	0
Perissodactyla							
<i>Diceros bicornis</i>	epc	475	420–480	2 (i)	1	0	0
<i>Tapirus bairdi</i>	epc	390–420	300	2 (i)	1	0	0
<i>Equus przewalskii</i>	epc	330–345	180–240	2 (i)	1	0	0
Pholidota							
<i>Manis tricuspis</i>	epc	139	90	2 (p)	1	0	–
Carnivora							
<i>Canis lupus</i> f. familiaris	enc	63	42–56	10 (a)	6	12–15	0
<i>Felis sylvestris</i> f. catus	enc	68	42–49	8 (a)	1–8	11	0
<i>Phoca vitulina</i>	enc	240	35–42	2 (a)	1	0	0
<i>Pagophilus groenlandicus</i>	enc	240	10–11	2 (a)	1	0	0
Chiroptera							

Table 1 Summary of data on reproduction and development for eutherian species—cont'd

Order Species	Placentation (interhemal barrier)	Pregnancy (days)	Lactation (days)	Mammilla (location)	Litter size	Eye opening (days)	Furring (days)
<i>Pipistrellus pipistrellus</i>	hc	44	60	2 (p)	2	5	7–14
<i>Rousettus leschenaulti</i>	enc	125	60	2 (p)	1	0	0

Abbreviations: hc, hemochorial; sc, syndesmochorial; enc-endotheliochorial; epc, epitheliochorial; p, pectoral; a, abdominal; i, inguinal; na, not available. Summarized info based on the following references: Roberts, R.M., Green, J.A., Schulze, L.C., 2016. The evolution of the placenta. *Reproduction* 152 (5), 179–189; Mess, A.M., Ferner, K., 2010. Evolution and development of gas exchange structures in Mammalia: The placenta and the lung. *Respiratory Physiology & Neurobiology* 173 (S), S74–S82; Ferner, K., Siniza, S., Zeller, U., 2014. The placentation of eulipotyphla – reconstructing a morphotype of the mammalian placenta. *Journal of Morphology* 275 (10), 1122–1144.

- Syndesmochorial: the trophoblast partly erodes the uterine epithelium, chorionic villi attach to the underlying tissue (most Artiodactyla).
- Endotheliochorial: the trophoblast erodes the uterine epithelium and underlying tissue and may directly attach to the maternal capillary endothelium (Carnivora, some Chiroptera and Eulipotyphla).
- Hemochorial: the trophoblast erodes all maternal tissue layers (including maternal capillary endothelium) and gets direct access to the maternal blood (Haplorhini, most Lipotyphla, some Chiroptera and Rodentia).
- Hemoendothelial: chorionic villi erode, the endothelium of the chorionic capillaries comes into direct contact with the maternal blood (*Oryctolagus cuniculus*, some Rodentia).

A different classification scheme refers to the placental fate at birth: in non-deciduous placentas (epitheliochorial type), chorionic villi easily detach from maternal tissue without causing maternal tissue damage during parturition. In deciduous placentas (all other placentas), a variable amount of maternal tissue is expelled at birth.

In contrast to the hypothesis, whereas superficial placentation modes represent a rather ancestral (plesiomorphic) state, recent findings on the placentation modes within Eulipotyphla lead to the assumption that invasive and compact placentation and the occurrence of altricial neonates represent the eutherian neonate morphotype.

Parturition

The parturition process underlies a complex interplay between fetal and maternal factors. Parturition is triggered by multiple fetal-maternal control mechanisms. With the onset of labour, the myometrium transforms from a quiescent state (important for fetal development during gestation) into a coordinately contracting organ; uterine contractions increase in frequency, intensity and duration. This transformation of the state of uterine musculature is associated with the dilatation of the uterine cervix in order to allow the passage of the fetus from the uterus. A variety of hormones of different origin is involved in the complex pathway of uterine contractility and parturition, including corticotropin releasing hormone, estrogens, progesterone, prostaglandins, oxytocin, and relaxin. The rearrangement of the extracellular matrix of the cervix (cervical ripening) is triggered by prostaglandines, relaxin, and inflammatory cell products. Progesterone plays a crucial role in the control of labour.

Neonates

In general, the length of the gestation period in eutherians is positively correlated with body weight and the degree of development of the newborn. As a general rule, the larger the species, the longer the gestation period (for mammals of equal weight, the species with the heaviest neonate has the longest gestation).

Eutherian mammals give birth to neonates of varying developmental degrees; generally categorized under the terms altricial and precocial (Fig. 1(e)). However, a wide spectrum of developmental degrees of neonates can be found along this spectrum. In contrast to marsupials, no permanent fixation to the nipples exists. However, in some desert rodents (*Baiomys spec.* and *Neotoma spec.*), a temporary fixation was described. Different aspects of development are discussed to define altriciality and precociality in eutherians: brain development, transitory closures, thermoregulatory, sensory, locomotory, and nutritional development.

Altricial neonates (Eulipotyphla, many Rodentia, some Chiroptera, Scandentia, many Carnivora, *Oryctolagus cuniculus*) are characterized by (1) closed eyes and ears, (2) hairless or sparsely haired, nearly translucent skin, (3) poor thermoregulation, (4) incomplete jaw development, (5) postnatal brain maturation, and (6) limited locomotion (neonates usually remain in a nest site).

Precocial neonates (Cetacea, Sirenia, Proboscidea, Hyracoidea, Perissodactyla, Artiodactyla, some Chiroptera, Macroscelidea, some Carnivora [Odobenidae, Phocidae, Otariidae], Rodentia [Hystricognathi], *Lepus europaeus*) are characterized by (1) open eyes and ears, (2) being completely furred (except Cetacea), (3) functioning thermoregulation, (4) complete jaw development, (5) prenatal brain maturation, (6) coordinated locomotion (no nest site). Precociality evolved on many occasions independently during eutherian evolution and can be found among almost all eutherian taxa.

Lactation

Lactation and parental care are fundamental adaptations concerning the reproduction of mammals. Lactation (and consequently the ability to suck) is a shared, derived (synapomorphic) character of mammals. In fact, the term Mammalia derives from the

mammary glands. These glands in females provide nourishment for the young during their postnatal period of rapid growth. Located along the paired mammary line, mammary glands are monoptyche (unilaminar layer of secreting cells), tubular, cutaneous glands with apocrine and eccrine secretion. They consist of a complex system of ducts that reach the surface of the skin through the mamilla. During late pregnancy, the growth and preparation of the mammary glands is stimulated by endocrine secretions (estrogens, progesterone, insulin, lactogen, and growth hormones). The epithelium of the ducts divides rapidly to produce secretory alveoli, which produce milk after birth. From the spacious alveoli (mammary gland), the milk passes via Ductus lactiferi into the cistern (Sinus lactiferous) and flows finally via milk ducts (Ductus papillares) into the mamilla (Fig. 6(f)). The production of milk is regulated by secretions from the pituitary (prolactin, oxytocin, and growth hormones). The milk flow is stimulated by nursing and emptying of milk from the mammary glands and continues until the stimulus ceases. The percentage composition of milk differs between eutherian species; however, the main components of the milk are the same (water, casein, whey proteins, carbohydrates, milk fat globules, and minerals). A major change in eutherian milk composition occurs after the first few days of lactation, during which colostrum (diluted liquid containing high levels of antibodies) is produced, followed by copious milk secretion of relatively constant composition.

The number and position of the mamillae is variable among eutherians and varies between 2 (e.g. most Primates, Proboscidea, Perissodactyla) and 24 (*Tenrec ecaudatus*, *Mastomys natalensis*) (Table 1). The location of the mammary glands corresponds to the paired mammary line; however, the exact position on the longitudinal axis is variable. Following positions can be distinguished:

- thoracic: Proboscidea, Sirenia, Chiroptera, Primates
- abdominal: Eulipotyphla, Rodentia, Carnivora, Suidae
- inguinal: many Artiodactyla, Cetacea, Perissodactyla

In contrast to marsupials, where the lactation period is always longer than gestation, the lactation period of eutherians can be shorter or longer than gestation. A high variability with regard to the duration of lactation exists among eutherians from about 7 days (or even less) in elephant shrews to 730 days in elephants (Table 1).

Ecology

In general, the eutherian embryonic and fetal development (the development between fertilization and parturition) is a continuous process. However, eutherians exhibit a pronounced plasticity in reproductive strategies towards ecological conditions. These adaptations refer to different stages of fetal and early postnatal development.

Delayed fertilization (delay of ovulation and fertilization until weeks or even months after copulation) is an adaptation towards periods of limited seasonal resource availability. It occurs in several species of mostly insectivorous and hibernating Chiroptera distributed over temperate ecosystems in the Palearctic and Nearctic region. In these species, copulation and insemination takes place before or during hibernation. The sperm is stored in the uterus or vagina for periods up to several months after copulation. During this time, secrets of the uterus keep the sperm viable. Directly after emergence from hibernation, ovulation, fertilization and implantation takes place in order to initiate gestation (and consequently parturition) at the earliest possible time of the respective season.

Phases of delayed embryonic development do also occur at the blastocyst stage (delayed implantation). While fertilization and early cleavage occur as usual, implantation does not take place immediately after the fertilized oocyte (blastocyst) reaches the uterus. Instead, the blastocyst maintains a state of minimal development and cleavage (dormancy) and remains free in the lumen of the uterus, without attachment to the uterine wall. Delayed implantation evolved independently in many occasions and occurs in various eutherian taxa. It can be found in Eulipotyphla (*Sorex araneus*, *Talpa altaica*), Chiroptera (e.g. *Lasionycteris noctivagans*, *Miniopterus schreibersii*, *Rhinolophus rouxii*), Xenarthra (*Dasyus hybridus*, *Dasyus novemcinctus*), Carnivora (Ursidae, Mustelidae, Mephitidae, Odobenidae, Phocidae, Otariidae), and Artiodactyla (*Capreolus capreolus*). The length of the delay is highly variable and can range from 30 days (*Neovison vison*) to 10.5 months (*Martes pennanti*).

In some taxa, delayed implantation only occurs under certain environmental conditions, including food deprivation and concurrent lactation (facultative diapause). Facultative diapauses can be observed in a number of species that show an estrus immediately after parturition (postpartum estrus). In these species, the delay is triggered by the suckling stimulus of the current litter in order to avoid the “double burden” of undergoing the stages of gestation and lactation at the same time. Other taxa exhibit an obligate diapause. In these taxa, delayed implantation is a regular part of the reproductive cycle, enabling an optimal timing of the birth of neonates in view of seasonal resource availability (seasonal embryonic diapause).

A less common type of reproductive delay refers to a period of slow or suppressed development of the embryo after implantation but prior to birth (delayed development). Delayed development has been found in a small number of bat species (*Macrotus californicus*, *Artibeus jamaicensis*, *Otopteropus cartilagonodus*, *Haplonycteris fischeri*, *Ptenochirus jagori*). In order to reduce metabolic demands of the embryo, delayed development occurs early after implantation. It enables a high degree of flexibility towards unfavorable environmental conditions and the synchronization of the timing of parturition in “nursery colonies”.

Another adaptation of reproductive strategies is the “pregnancy blocking phenomenon” or “Bruce-effect” observed under laboratory or captive conditions in a number of rodent species (e.g. *Mus musculus*, *Microtus pennsylvanicus*, *Microtus ochrogaster*, *Peromyscus maniculatus*), as well as in the domestic horse (*Equus przewalskii* f. *caballus*). In recently pregnant females, the exposure of the scent of an unfamiliar male can trigger pregnancy disruption during an early developmental stage of the embryo (before implantation). Direct evidence for the occurrence of a “Bruce-effect” under natural conditions is lacking and there is a debate,

whether this phenomenon might be an artefact. Recent data on observed pregnancy terminations and inter-birth intervals in free-ranging geladas (*Theropithecus gelada*) reveal arguments for the existence of pregnancy blocking strategies in primates under natural conditions. An early termination of pregnancy is also discussed as an adaptation strategy to avoid nonparental infanticide (see below).

Several eutherian taxa are known to directly affect the reproductive performance of conspecifics by committing nonparental infanticide. The killing of immature offspring by nonparental conspecifics (males and, or females) occurs in a wide variety of eutherian taxa (Primates, Carnivora, Rodentia, Artiodactyla, Perissodactyla, Lagomorpha, Chiroptera and Scandentia). Several theories exist to explain this behavior, including nutritional and resource-competitive aspects. Nonparental infanticide committed by males is also interpreted as a strategy to increase access over breeding females and optimize reproductive success.

In summary, eutherian mammals are characterized by a pronounced plasticity of reproductive strategies, enabling niche realization in virtually all existing habitats (aquatic, terrestrial, and aerial). Owing to the resulting ecological flexibility, eutherian mammals represent approximately 96% of all extant mammalian species.

Further Reading

- Bartoš, L., Bartošová, J., Pluháček, J., & Šindelářová, J. (2011). Promiscuous behaviour disrupts pregnancy block in domestic horse mares. *Behavioral Ecology and Sociobiology*, 65(8), 1567–1572.
- Belov, K., Deakin, J. E., Papenfuss, A. T., et al. (2006). Reconstructing an ancestral mammalian immune supercomplex from a marsupial major histocompatibility complex. *PLoS Biology*, 4(3), e46.
- Blair, F. (1941). Observations on the Life History of *Baiomys taylori subater*. *Journal of Mammalogy*, 22(4), 378–383.
- Bruce, H. M. (1959). An exteroceptive block to pregnancy in the mouse. *Nature*, 184(4680), 105.
- Clulow, F. V., & Langford, P. E. (1971). Pregnancy-block in the meadow vole, *Microtus pennsylvanicus*. *Journal of Reproduction and Fertility*, 24(2), 275–277.
- Cornelis, G., Vernochet, C., Carradec, Q., et al. (2015). Retroviral envelope gene captures and syncytin exaptation for placentation in marsupials. *Proceedings of the National Academy of Sciences*, 112(5), 487–496.
- Denker, H. W. (1993). Implantation: A cell biological paradox. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 266(6), 541–558.
- Denker, H. W., & Gerdess, H. J. (1979). The dynamic structure of rabbit blastocyst coverings. *Anatomy and Embryology*, 157(1), 15–34.
- Derrickson, E. M. (1992). Comparative reproductive strategies of altricial and precocial eutherian mammals. *Functional Ecology*, 6(1), 57–65.
- Ebensperger, L. A. (1998). Strategies and counterstrategies to infanticide in mammals. *Biological Reviews*, 73(3), 321–346.
- Eisenberg, J. F. (1981). *The Mammalian Radiations: An Analysis of Trends in Evolution, Adaptation, and Behavior*. Chicago: University of Chicago Press.
- Eleftheriou, B. E., Bronson, F. H., & Zarrow, M. X. (1962). Interaction of olfactory and other environmental stimuli on implantation in the deer mouse. *Science*, 137(3532), 764.
- Enders, R. K. (1952). Reproduction in the mink (*Mustela vison*). *Proceedings of the American Philosophical Society*, 96(6), 691–755.
- Epple, G., Belcher, A. M., Kunderling, I., et al. (1993). Making sense out of scents: Species differences in scent glands, scent marking behaviour, and scent-mark composition in the Callitrichidae. In A. B. Rylands (Ed.), *Marmosets and Tamarins – Systematics, Behaviour, and Ecology* (pp. 123–151). Oxford University Press.
- Ferner, K., Schulz, J., Zeller, U., 2017. Comparative anatomy of neonates of the three major mammalian groups (monotremes, marsupials, placentals) and implications for the ancestral mammalian neonate morphotype. *Journal of Anatomy*. doi: 10.1111/joa.12689.
- Frankenberg, S. R., de Barros, F. R. O., Rossant, J., & Renfree, M. B. (2016). The mammalian blastocyst. *Wiley Interdisciplinary Reviews: Developmental Biology*, 5, 210–232.
- Freyer, C., Zeller, U., & Renfree, M. B. (2003). The marsupial placenta: A phylogenetic analysis. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 299(1), 59–77.
- Garratt, M., Gaillard, J. M., Brooks, R. C., & Lemaître, J. F. (2013). Diversification of the eutherian placenta is associated with changes in the pace of life. *Proceedings of the National Academy of Sciences*, 110(19), 7760–7765.
- Giere, P., & Zeller, U. (2005). Small mammal diversity and reproduction along a transect in Namibia (BIOTA S 07). *African Biodiversity* (pp. 305–313). Boston, MA: Springer.
- Gobert, M., & Lafaille, J. J. (2012). Maternal-fetal immune tolerance, block by block. *Cell*, 150(1), 7–9.
- Greenfield, C., & Flaws, J. A. (2004). Renewed debate over postnatal oogenesis in the mammalian ovary. *Bioessays*, 26(8), 829–832.
- Grosser, O. (1927). *Frühentwicklung, Eihautbildung und Placentation des Menschen und der Säugetiere*. München: Bergmann.
- Gundling, W. E., & Wildman, D. E. (2015). A review of inter-and intraspecific variation in the eutherian placenta. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370(1663), 20140072.
- Herre, W., & Röhrs, M. (1990). *Haustiere – Zoologisch Gesehen*. Gustav Fischer Verlag.
- Heske, E. J., & Nelson, R. J. (1984). Pregnancy interruption in *Microtus ochrogaster*: Laboratory artifact or field phenomenon? *Biology of Reproduction*, 31(1), 97–103.
- Huggett, A. S. G., & Widdas, W. F. (1951). The relationship between mammalian foetal weight and conception age. *Journal of Physiology*, 114, 306–317.
- Hunter, R. H. F. (2012). Components of oviduct physiology in eutherian mammals. *Biological Reviews*, 87(1), 244–255.
- Huppertz, B., Berghold, V. M., Kawaguchi, R., & Gauster, M. (2012). A variety of opportunities for immune interactions during trophoblast development and invasion. *American Journal of Reproductive Immunology*, 67(5), 349–357.
- Ioannou, J. M. (1967). Oogenesis in adult prosimians. *Development*, 17(1), 139–145.
- Johnson, J., Bagley, J., Skaznik-Wikiel, M., et al. (2005). Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell*, 122(2), 303–315.
- Johnson, J., Canning, J., Kaneko, T., Pru, J. K., & Tilly, J. L. (2004). Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature*, 428(6979), 145–150.
- Kuhn, H. J., & Schwaier, A. (1973). Implantation, early placentation, and the chronology of embryogenesis in *Tupaia belangeri*. *Zeitschrift Für Anatomie und Entwicklungsgeschichte*, 142(3), 315–340.
- Lefevre, C. M., Sharp, J. A., & Nicholas, K. R. (2010). Evolution of Lactation: Ancient Origin and Extreme Adaptations of the Lactation System. *Annual Review of Genomics and Human Genetics*, 11, 219–238.
- Lewitus, E., & Soligo, C. (2011). Life-history correlates of placental structure in eutherian evolution. *Evolutionary Biology*, 38(3), 287–305.
- Macias, H., & Hinck, L. (2012). Mammary gland development. *Wiley Interdisciplinary Reviews: Developmental Biology*, 1, 533–557.
- Mess, A., Blackburn, D. G., & Zeller, U. (2003). Evolutionary transformations of fetal membranes and reproductive strategies. *Journal of Experimental Zoology*, 299(1), 3–12.
- Moffett, A., & Loke, C. (2006). Immunology of placentation in eutherian mammals. *Nature Reviews Immunology*, 6(8), 584–594.
- Morales-Prieto, D. M., Ospina-Prieto, S., Schmidt, A., Chaiwangyen, W., & Markert, U. R. (2014). Elsevier Trophoblast Research Award Lecture: Origin, evolution and future of placenta miRNAs. *Placenta*, 35, 39–45.
- Müller, F. (1972). Zur stammesgeschichtlichen Veränderung der Eutheria-Ontogenesen. *Revue Suisse de Zoologie*, 79, 1–97.
- Nicoll, M. E., & Racey, P. A. (1985). Follicular development, ovulation, fertilization and fetal development in tenrecs (*Tenrec ecaudatus*). *Journal of Reproduction and Fertility*, 74(1), 47–55.

- Oftedal, O. T. (2002). The mammary gland and its origin during synapsid evolution. *Journal of Mammary Gland Biology and Neoplasia*, 7, 225–252.
- Orr, T. J., & Zuk, M. (2014). Reproductive delays in mammals: An unexplored avenue for post-copulatory sexual selection. *Biological Reviews*, 89(4), 889–912.
- Parham, P., Norman, P. J., Abi-Rached, L., Hilton, H. G., & Guethlein, L. A. (2012). Review: Immunogenetics of human placentation. *Placenta*, 33, 71–80.
- Paulesu, L. (2013). Materno-fetal immunotolerance: An evolutionary view. *Journal of the Siena Academy of Sciences*, 5(1), 11–22.
- Pearson, O. P. (1944). Reproduction in the shrew (*Blarina brevicauda*). *Developmental Dynamics*, 75(1), 39–93.
- Pfeffer, P. L., & Pearton, D. J. (2012). Trophoblast development. *Reproduction*, 143(3), 231–246.
- Plak, G. E., Tacconi, E., Czernik, M., et al. (2012). Embryonic diapause is conserved across mammals. *PLoS One*, 7(3), e33027.
- Renfree, M. B. (2010). Review: Marsupials: Placental mammals with a difference. *Placenta*, 31, 21–26.
- Renfree, M. B., & Shaw, G. (2000). Diapause. *Annual Review of Physiology*, 62(1), 353–375.
- Roberts, E. K., Lu, A., Bergman, T. J., & Beehner, J. C. (2012). A Bruce effect in wild geladas. *Science*, 335(6073), 1222–1225.
- Saltzman, W., Tardif, S. D., & Rutherford, J. (2010). Hormones and reproductive cycles in primates. In D. O. Norris, & K. H. Lopez (Eds.), *Hormones and Reproduction of Vertebrates*, 1 pp. 291–327. USA: Academic Press.
- Schoepf, I., & Schradin, C. (2012). Better off alone! Reproductive competition and ecological constraints determine sociality in the African striped mouse (*Rhabdomys pumilio*). *Journal of Animal Ecology*, 81(3), 649–656.
- Schradin, C. (2005). When to live alone and when to live in groups: Ecological determinants of sociality in the African striped mouse (*Rhabdomys pumilio*, Sparman, 1784). *Belgian Journal of Zoology*, 135(Suppl. 1), 77–82.
- Siniza, S., Lupiáñez, D. G., Jiménez, R., & Zeller, U. (2012). Morphology and ultrastructure of the chorioallantoic placenta of the Iberian mole (*Talpa occidentalis*) with special reference to heterophagous areolas and the nature of interhaemal barrier. *Journal of Anatomy*, 221(2), 164–173.
- Skinner, J. D., & Chimimba, C. T. (2005). *The Mammals of the Southern African Sub-Region*. Cambridge University Press.
- Starck, D. (Ed.). 1995. *Lehrbuch der speziellen Zoologie. Band II: Wirbeltiere. 5. Teil: Säugetiere*. 1995, Jena/Stuttgart/New York: Gustav Fischer Verlag.
- Suarez, S. S. (2006). Gamete and zygote transport. In J. D. Neill (Ed.), *Knobil and Neill's Physiology of Reproduction* (3rd edition, 1 pp. 113–145). New York: Academic Press.
- Szdzuy, K., Dehnhard, M., Strauss, G., Eulenberger, K., & Hofer, H. (2006). Behavioural and endocrinological parameters of female African and Asian elephants *Loxodonta africana* and *Elephas maximus* in the peripartur period. *International Zoo Yearbook*, 40, 41–50.
- Szdzuy, K., & Zeller, U. (2009). Lung and metabolic development in mammals: Contribution to the reconstruction of the marsupial and eutherian morphotype. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 312(6), 555–578.
- Taglauer, E. S., Waldorf, K. M. A., & Petroff, M. G. (2010). The hidden maternal-fetal interface: Events involving the lymphoid organs in maternal-fetal tolerance. *The International Journal of Developmental Biology*, 54(2–3), 421–430.
- von Rango, U. (2008). Fetal tolerance in human pregnancy – a crucial balance between acceptance and limitation of trophoblast invasion. *Immunology Letters*, 115(1), 21–32.
- Wang, H., & Dey, S. K. (2006). Roadmap to embryo implantation: Clues from mouse models. *Nature Reviews Genetics*, 7(3), 185–199.
- Werdelin, L., & Nilsson, Å. (1999). The evolution of the scrotum and testicular descent in mammals: A phylogenetic view. *Journal of Theoretical Biology*, 196(1), 61–72.
- Wildman, D. E. (2011). Review: Toward an integrated evolutionary understanding of the mammalian placenta. *Placenta*, 32, 142–145.
- Zeller, U. (1999). Mammalian reproduction: Origin and evolutionary transformations. *Zoologischer Anzeiger*, 238(1), 117–130.
- Zeller, U., & Kuhn, H.-J. (1994). Postpartum erythrophagocytosis, iron storage and iron secretion in the endometrium of the tree shrew (*Tupaia*) during pregnancy. *Journal of Anatomy*, 184, 597–606.
- Zeller, U., Freyer, C., & Neumann, C. (2001). Mammalian reproduction: Origin and evolutionary transformations, with special reference to the K/T boundary. *Mitteilungen Aus Dem Museum für Naturkunde in Berlin, Zoologische Reihe*, 77(2), 245–246.

Reproduction, Overview by Phylogeny: Plant

Benjamin A Burrows and Andrew G McCubbin, Washington State University, Pullman, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Alternation of Generations A life cycle with alternating multicellular diploid and multicellular haploid stages.

Angiosperm The flowering plants, which comprise the majority of living plant species.

Dioecious Bearing male and female reproductive structures on separate individuals.

Diploid A cell with two compliments (copies) of chromosomes.

Gamete The male or female single celled haploid (1 n) units of sexual reproduction.

Gametophyte The multicellular, haploid (n) phase of the alternation of generations, which produces male and/or female gametes by mitosis.

Haploid A cell having only one compliment of chromosomes.

Heterosporous Producing two different sized spores, the smaller of which produce male gametophytes, the larger produce female gametophytes.

Homosporous Production of only one size of spore that may develop into a male, female, or bisexual gametophyte.

Monoecious Bearing male and female reproductive structures on the same individual.

Seed A diploid plant embryo encased in a protective coat.

Sporangium A receptacle in which asexual spores are formed.

Spore The single celled haploid product of meiosis by a sporophyte.

Sporophyll A leaf that bears sporangia.

Sporophyte The multicellular diploid (2 n) stage of the alternation of generations which produces spores by meiosis.

Strobili A collection of sporophylls that form a cone like shape.

Introduction

Kingdom Plantae is broadly composed of four evolutionarily related groups: bryophytes (mosses), (seedless vascular plants), gymnosperms (cone bearing seed plants), and angiosperms (flowering seed plants). These groups share features such as the production of embryos, photosynthetic chloroplasts, and cell walls primarily composed of cellulose. A variety of reproductive strategies can be found in plants, both sexual and asexual, often with more than one strategy utilized by a single species. While the aforementioned groups are primarily divided by differences in reproductive strategy, all land plants share a reproductive phenomenon named the *Alternation of Generations* (Fig. 1). This is characterized by a reproductive cycle that has both a multicellular *diploid* (2 n) as well as a multicellular *haploid* (1 n) stage. In this cycle the *sporophyte* (multicellular diploid) undergoes meiosis to produce haploid cells called *spores*. These spores then divide by mitosis to form the multicellular haploid *gametophyte*, which will in turn produce the male and female *gametes*. Unlike in animals, gametes are produced by mitosis in plants since the gametophyte is already haploid. These gametes fuse to produce a diploid *zygote*, which will then undergo mitotic divisions leading back to the sporophyte stage.

The ancestors of land plants were aquatic green algae and the transition to life in terrestrial environments presented a number of challenges not least in regard to reproduction. Indeed novel reproductive structures that enhance desiccation tolerance and remove the need for water in gamete transfer are predominant characteristics in the classification of these organisms. In addition, throughout the evolution of land plants the haploid gametophyte stage of the life cycle has been progressively reduced, from being the dominant life stage in bryophytes, to only 3–9 cells in *angiosperms* (flowering plants). This trend towards dominance of the diploid sporophyte and reduction of the haploid gametophyte life stages is often interpreted as being an adaptation to the substantially higher levels of mutagenic UV light in terrestrial than aquatic environments, favoring diploids which inherently possess a “back-up” set of genetic material.

Evolution of Land Plant Reproduction Overview

Estimates put evolution of the earliest land plants between 500 and 450 million years ago (MYA). As stated above, all land plants display Alternation of Generations, but the details by which they carry out this life cycle have changed drastically through evolutionary time. The earliest land plant fossils originated around 430 MYA and bear a strong similarity to the extant group of plants known as the Bryophytes, which includes liverworts, hornworts and mosses. In Bryophytes, the gametophyte (1 n) stage is the dominant life stage making up the bulk of the biomass. The gametophyte generates male and female reproductive structures known as antheridia and archegonia, respectively. Antheridia in these groups produce flagellated sperm cells that require water to swim to the archegonia and fuse with the egg cell contained within. The resulting embryo and sporophyte (2 n) is nutritionally dependent on the mother gametophyte and will grow out of the top of it. Ultimately, the sporophyte forms a single *sporangium*

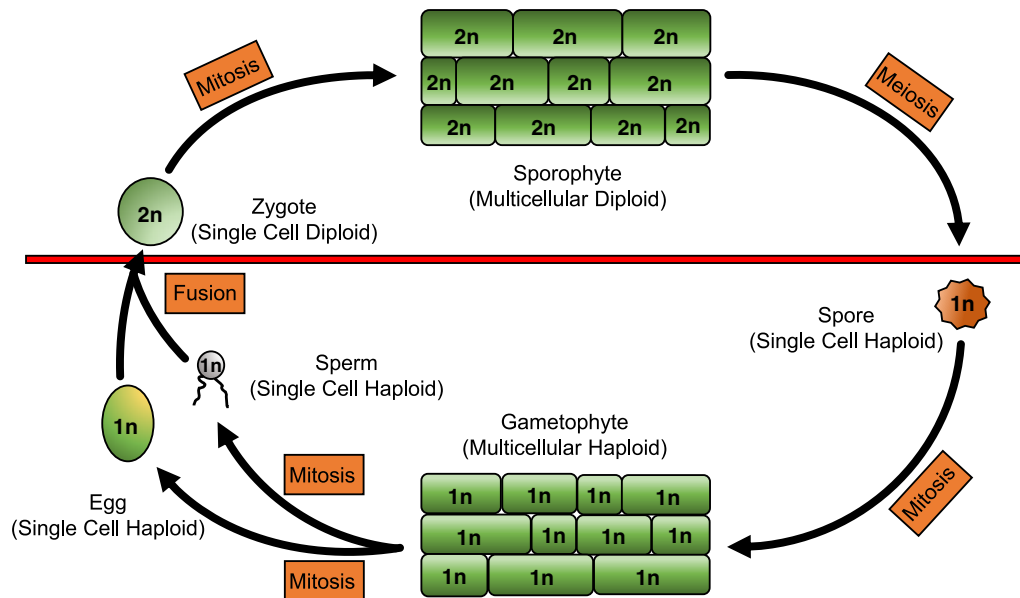


Fig. 1 The alternation of generations life cycle in land plants involves both a multicellular diploid ($2n$) stage, the sporophyte, and a multicellular haploid ($1n$) stage, the gametophyte. Unlike animals, meiosis in plants does not produce gametes but instead the sporophyte produces haploid single celled spores. A spore divides by mitosis to form a multicellular ($1n$) gametophyte. The gametophyte produces ($1n$) gametes by mitotic division. Fusion of the gametes yields a ($2n$) zygote, which will develop into a new sporophyte.

where $1n$ spores are produced by meiosis. These spores are the main dispersal structure of the bryophytes, and are primarily wind born. A thick wall of sporopollenin protects the spores from degradation and desiccation until ideal growth conditions are encountered.

The evolution of water and nutrient conducting vascular tissues, around 400 MYA, allowed plants to develop larger branched, body structures which had significant impacts on reproductive strategies. The first plants to utilize this are referred to as the seedless vascular plants. Though species diversity in this group was once extensive there are only two extant phyla, the Lycopodiophyta (club and spike mosses, and quillworts) and the Monilophyta (ferns and horsetails). Seedless vascular plants differ from bryophytes in that the sporophyte is the larger dominant stage of the life cycle, and is free-living and independent of the gametophyte at maturity. Spores are still the main dispersal structure, but the branching nature of the sporophyte allows more than one sporangium on an individual and hence greater spore production. In some species spore production occurs in specialized structures called *strobili* (cones), which form at the tips of branches. In ferns, however, sporangia are borne in large numbers on leaves known as *sporophylls*. While the majority of seedless vascular plants are *homosporous*, making only one type of spore, which develops into a gametophyte that can produce both antheridia and archegonia, many lycophytes and some ferns are *heterosporous*. In heterosporous plants two different types of spore are produced, *megaspores*, which develop into female gametophytes, and *microspores*, which become male gametophytes. These unisexual gametophytes are often highly reduced in size and complexity compared to the bisexual gametophytes of homosporous species. Heterospory is the norm in later plant groups and appears to have had multiple evolutionary origins in the vascular plants.

Evolution of the next key reproductive structure, the *seed*, provides the name of the next group, the gymnosperms (which translates to “naked seed”). A seed consists of a fertilized embryo packaged with stored food and encased in a protective seed coat. The seed represents a change in dispersal strategy, with spores no longer being the dispersal structure, and a dramatic shift in the alternation of generations life cycle. The gametophytes are highly reduced in size, with the female gametophyte no longer being free-living, instead being retained within the parental sporophytes megasporangium. Additionally, transfer of the sperm to the egg is no longer reliant on water. The male gametophytes (microgametophytes) develop into pollen grains, which facilitate transfer of sperm to the megagametophyte by alternative means, generally wind. As a consequence of the inefficiency of wind pollination, there is a shift to producing very large quantities of male gametophytes (pollen), which you may have observed as large clouds wafting off conifer trees. The gymnosperms consist of four extant phyla: Coniferophyta (conifers), Cycadophyta (cycads), Ginkgophyta (ginkgo), and Gnetophyta (gnetophytes). While the earliest seed-like structures found in the fossil record are found around 365 MYA, the origins of extant gymnosperms are more likely around 290 MYA.

The majority of extant plant species (> 300,000) are contained within the Angiosperms (flowering plants). The angiosperms are characterized by the evolution of key reproductive structures, flowers and *fruits*. The first angiosperm fossils date from around 130 MYA and they quite rapidly became the dominant plant group by 90 MYA. As can be observed in any florist shop, the flower has evolved a staggeringly diverse range of forms, shapes, colors, sizes, etc. This great diversity is directly related to the flower's role in enhancing the efficiency of pollination, frequently through relationships with animal pollinators, which often replace wind as pollen vectors. This structure is one of the primary reasons the angiosperms were able to dominate plant biodiversity in such a

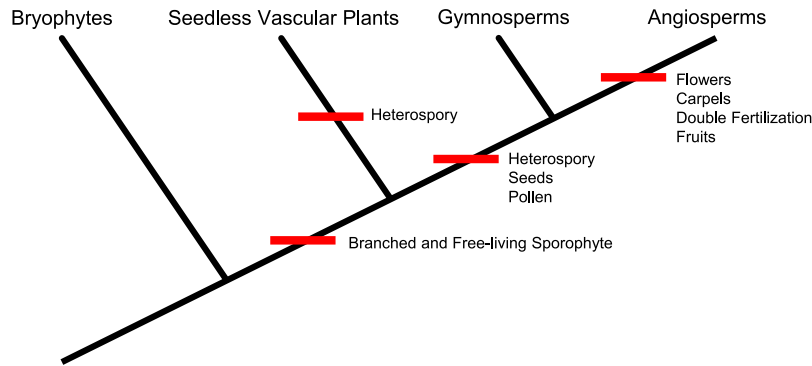


Fig. 2 A phylogenetic tree of land plant lineages. Key evolutionary traits are indicated by red lines, and are shared by all groups above their appearance. This figure is not to scale with evolutionary time.

short (geologically speaking) period of time. The evolution of fruit, which are formed from ovary tissue after fertilization, both protects the seeds as well as promoting dispersal by various biotic and abiotic means. Following the trend seen in the other groups, the gametophytes are even further reduced in the angiosperms, with the pollen containing only 3 nuclei and the female gametophyte (embryo sac) containing 7–9 nuclei. The fertilization process is also modified in the flowering plants and involves both sperm cells found within a pollen grain. One fuses with the egg cell to produce the zygote, the second fuses with the two haploid polar nuclei in the embryo sac forming the triploid (3 n) *endosperm*. This tissue is highly nutritive, aiding the developing zygote. This process is known as *double fertilization* (Fig. 2).

Details of Reproduction in Non-Seed Plants

Bryophytes

Sexual reproduction

The gametophyte is the dominant life phase in the Bryophytes. The gametophyte produces structures known as antheridia and archegonia, which produce the male and female gametes respectively. Collectively these structures are known as *gametangia*. While some bryophyte species have gametophytes that produce gametangia of both sexes on a single individual, others have separate male and female gametophytes. The antheridia produce many biflagellate sperm cells, which require liquid water to swim to the egg cell. Archegonia in contrast produce a single egg cell located within a chamber known as the *venter*. A canal to the outside forms within the neck of the venter and breakdown products of cells that degenerate to form this canal generate a signal that serves as a chemoattractant to the sperm cells. When a sperm cell swims down to the egg cell they fuse to form the diploid (2 n) zygote. This embryonic sporophyte will continue to develop in the archegonium and will continue to remain attached to, and nutritionally dependent on the parental gametophyte for the entirety of its life. For mosses the gametangia are often positioned at the top of the “leafy” gametophyte axis. The zygote and resulting sporophyte will develop and grow out of the archegonia on top of the gametophyte. When mature, a spore generating structure (sporangium), called a capsule, forms at the top of the sporophyte. Spores (1 n) are generated by meiosis and as the capsule matures it dries and finally pops open releasing the spores for dispersal. Given appropriate conditions, these spores will develop into new gametophytes. Positioning of gametangia and the resulting sporophytes differs in the different bryophyte lineages. The common liverwort *Marchantia* produces umbrella shaped structures that raise the gametangia above the main gametophyte body and the sporophytes develop on the underside of these structures. In the hornworts, the sporophyte structures provide the common name for this group as they rise out of the thalloid gametophyte like horns. The sporangia grow upward out of the apex of this sporophyte.

Asexual reproduction

Bryophytes often divide vegetatively with one individual breaking into several pieces that can become free-living individuals. Additionally, many bryophytes gametophytes produce structures known as *gemmae*, which are small balls of cells contained in cuplike structures known as splash cups. Falling water can splash gemmae out of the cups and when they reach soil they will divide to form a new gametophytes.

Seedless Vascular Plants

Sexual reproduction

This group was highly species rich and diverse from around 400–300 MYA, but many of those diverse lineages have gone extinct leaving a somewhat fragmented patchwork of reproductive mechanisms in the group when observed as a whole. The seedless vascular plants all exhibit a shift in the alternation of generations to a larger free-living sporophyte (2 n), and a general reduction

in size of the gametophyte ($1n$). The sperm produced are still flagellated and require water to swim to the egg in the archegonia. An important development that independently evolved several times in this group is heterospory. This is characterized by the production of two different types of spores, microspores that develop into unisexual male gametophytes, and megaspores which become unisexual female gametophytes. While unisexual gametophytes are found in many homosporous species there is no observable difference in the spores they arise from. In the living Lycopodiophyta species: spike mosses (Selaginellaceae) and quillworts (Isoetaceae) are heterosporous, while club mosses (Lycopodiaceae) are homosporous. Many club moss and spike moss species produce spores in specialized structures called strobili, which are a group of modified non-photosynthetic sporophylls (spore bearing leaves). The largest number of seedless vascular plant species is in phylum Monilophyta and the majority of these are ferns. Almost all are homosporous, with the water ferns being the only living heterosporous ferns. Fern sporangia are grouped into structures called *sori*, which are generally dot shaped in appearance, and are most often found on the underside or around the edges of leaves. Fern gametophytes are often small and heart shaped, and some bisexual gametophytes develop antheridia and archegonia at different times to increase the probability of cross-fertilization. After fertilization, the emerging sporophyte grows out of the gametophyte and obtains nutrients from it until the former is large enough for nutritional independence, at which point the gametophyte degrades. One familiar group of non-fern Monilophytes is the horsetails (*Equisetum*), which produce a sporophyte with a green photosynthetic stem with a large distinctive strobilus at the top of this axis. The homosporous spores from this can give rise to gametophytes that are either bisexual or exclusively male to promote cross-fertilization.

Asexual reproduction

There are very few seedless vascular plants with specialized asexual reproduction mechanisms. Due to the rhizomatous structures of many of the seedless vascular plants such as the lycophytes and *Equisetum*, roots form along the length of the rhizome and if the rhizome is split these continue to grow as separate individuals. There are also several fern species that grow exclusively as gametophytes, reproducing only asexually and never developing sporophytes.

Details of Reproduction in Seed Plants

Gymnosperms

Sexual reproduction

The gymnosperms present another shift of the alternation of generations, with a further major reduction in the size and complexity of the gametophyte ($1n$) as well as the use of seeds as the primary dispersal agents rather than spores. As the majority of extant gymnosperm species are conifers we will focus on sexual reproduction in this group (using pines as a common example) and later highlight significant differences in non-conifer gymnosperms. Conifer sporophytes are large, woody, shrubs or trees, which are heterosporous, producing megaspores and microspores in separate cones (strobili). In *monoecious* species, female and male cones are both present on a single individual, while in *dioecious* species female and male cones occur on different individuals (Williams, 2009).

Within the male cones the many microsporocytes undergo meiosis, each producing four microspores per meiotic event. These microspores then divide by mitosis each forming a male microgametophyte (*pollen grain*). In gymnosperms (and the later angiosperm lineage) the microgametophyte is reduced to a structure containing just a few cells. Pollination, the transfer of pollen to the megagametophyte, in gymnosperms is carried out by the wind and the pollen grains possess two air sacs to aid this process. Hence pollen provided a novel mechanism for transfer of the sperm to the egg cell, with free liquid water no longer being necessary. The gymnosperm pollen grain consists of four cells, the tube cell, the generative cell, and two prothallial cells. This microgametophyte is not fully mature and only becomes so after it has been transferred to the megagametophyte.

The female (ovulate) cones bear structures called ovuliferous scales, which each produce two ovules. The ovule, which after fertilization will become the seed, is initially made up only of parental sporophyte tissue, and consists of a tissue called the nucellus, surrounded by a layer called the integument that has an opening named the micropyle through which a pollen tube will enter. The ovule will only begin development of the female gametophyte (megagametophyte) after pollination has occurred. When the pollen grain has been transferred to the receptive ovule it germinates to form a pollen tube and the generative cell divides to form stalk and body cells, the latter will produce the sperm cells. The time between pollination and pollen germination varies between different conifer genera from almost immediately to several months after pollination. Once the pollen tube begins moving through the nuclear tissue it will stop and appear dormant for a period of time.

Only after this initial pollen germination has occurred will the female gametophyte begin to develop, although this can be delayed by several months. The megaspore mother cell divides by meiosis forming four megaspores, only one of which will survive and divide to form the megagametophyte. The female gametophyte is not free-living and is contained within the ovule, dependent on the parental sporophyte for nutrition. As the pollen tube makes its way toward the developing female gametophyte the body cell divides forming two sperm cells (male gametes). The megagametophyte forms several archegonia, which each produce egg cells (female gametes). Once a pollen tube reaches an archegonium the two sperm cells are released, but only one fuses with the egg cell. The time it takes for this reproductive process to reach completion varies, and in some species spans multiple years. After fertilization, the ovule develops into a seed, which consists of tissues from three generations, the parental sporophyte, the female gametophyte, and the new zygotic sporophyte. Unlike in previous plant lineages the seed is the dispersal structure. In some

conifers, such as yews and junipers the seed develops a fleshy seed coat called an *aril* (often called a berry, although it is not truly a fruit as it is formed from the integument of the ovule rather than ovary tissues). Gnetophytes like conifers have non-motile sperm, but cycads and *Ginkgo* still produce flagellated sperm, which are released by the pollen tube in the nucellus and swim to the archegonia in a pollination drop secreted by the ovule. Interestingly, many cycads are insect pollinated, representing the first instances of the use of animal pollinators in the evolutionary history of plants.

Asexual reproduction

Specialized asexual reproduction appears to be rare in gymnosperms. In the conifers, which are some of the best-studied gymnosperms due to their economic importance, some species can be propagated from cuttings, although there is little evidence to suggest that this occurs without human intervention. There is also at least one reported case of apomixis (formation of seeds asexually). Asexual reproduction in *Ginkgo*, cycads, and gnetophytes is possible but again does not appear to be common without human intervention.

Angiosperms

Sexual reproduction

The name Angiosperm translates to “vessel seed” which refers to ovules being enclosed in the female gynoecium (*carpel*). The carpel is one of the organs that make up what is arguably the defining feature of the angiosperms, the flower. Flowers are produced by Angiosperm sporophytes and composed of four types of organs arranged in whorls: sepals, petals, stamens and carpels (see Vol. 6 #22). In most angiosperms flowers are hermaphroditic, containing both male and female reproductive parts, but monoecious species with unisexual flowers are found, as well as dioecious species. Pollen is produced in the anthers of the stamen, while the ovules are produced in the ovary of the carpel. Microsporogenesis in the anther involves the meiotic division of pollen mother cells, which each produce four microspores. These then undergo mitosis to form pollen grains (microgametophytes), which at maturity consist of only three cells, the tube cell and two non-motile sperm cells. Each pollen grain is encased in a thick protective sporopollenin wall and dries to a desiccated state for dispersal. In many species the microgametophyte is “mature” only after pollen germination, as the pollen grains possess a single generative cell, which divides only after pollination and subsequent germination to form two sperm cells. On the female side, development of the megagametophyte proceeds slightly differently in various angiosperm lineages. Here we discuss the most common mechanism. In the ovary ovules are attached to an internal wall by placenta tissue. The megasporocyte within the ovule nucellus divides by meiosis forming four haploid cells, only one of which will survive. The nucleus of this megaspore divides mitotically to form the female gametophyte, the embryo sac, which has a total of eight nuclei that will form seven cells. Six of these, including the egg cell are haploid (1 n), while critically one cell (the central cell) contains the remaining two polar nuclei. While not truly diploid (2 n) this cell is critical in the development of the seed.

Pollination occurs when pollen is transferred to a receptive surface, known as the *stigma*, of the carpel. This pollen will then germinate and produce a pollen tube that grows down the style, which connects the stigma to the ovary, to the ovule(s). Pollen tubes follow chemical signals released by two cells located at the tip of the embryo sac known as *synergids*. When a pollen tube reaches a synergid cell, the tip bursts releasing the two sperm cells into the synergid. One of the sperm will then fuse with the egg cell to form the diploid (2 n) zygote, while the other will fuse with the two nuclei contained in the central cell to form a triploid (3 n) tissue called the endosperm. This process is known as double fertilization, and is unique to angiosperms. The endosperm tissue is important for two reasons. First this tissue contains large amounts of nutrients and is essentially the food reserve that will feed the developing embryo and young sporophyte. Secondly, in grass (monocotyledon) species including cereal crops, the endosperm contains most of the nutrients that humans extract from the seeds. Wheat flour, for example, is essentially powdered endosperm. In broad-leaved flowering plants (eudicotyledons), however, the endosperm functions only during seed development and the nutrients are transferred to the *cotyledons* (seed leaves) during seed maturation. The outer layers of the ovule develop into the seed coat, which protects the seed during dormancy. After fertilization, the ovary tissues of the flower also undergo changes that will lead to it becoming a fruit. Fruits are unique to angiosperms, and often function in protection of the seeds, as well as in aiding their dispersal.

The evolution of diverse pollination strategies has been one of the keys to angiosperm success by improving the efficiency of pollen transfer from anthers to stigmas, dramatically reducing the amount of pollen (and resources needed to make pollen) required for reproductive success. While some angiosperms rely on abiotic means for this (wind and water) like gymnosperms, many have evolved specialized relationships with animal pollinators that transport pollen from one flower to another with much greater efficiency than abiotic factors. Indeed co-evolution with a wide variety of animal pollinators has driven much of the incredible variation we see in floral diversity.

A consequence of the evolution of flowers is that most species male and female organs are in close proximity. Left unchecked this creates a situation favoring inbreeding, which leads to a loss of genetic variability and reduces the potential of species to adapt to environmental change (Holsinger, 2000). Though many species do inbreed, notably weedy species this is regarded as a short-term evolutionary strategy and most have evolved one of a variety of outbreeding mechanisms. These mechanisms may be temporal (male and female structure maturing at different times), spatial (single sex flowers), combining both temporal and spatial (positioning of stigma and/or anthers change as flowers age to promote cross-pollination), or biochemical. The latter are broadly classed as “self-incompatibility” systems, by which the stigma or style of a plant can recognize and inhibit self- or genetically related pollen as it grows through it, preventing fertilization. Such outbreeding mechanisms have been estimated to

exist in as many as 70% of wild Angiosperm species and are hypothesized to have been key to the diversification and success of this group (Richards, 1997).

Asexual reproduction

A wide variety of forms of asexual reproduction are found in the flowering plants. The majority of these involve vegetative structures that can lead to propagation by fragmentation, for example the bulbs of crocuses and daffodils, rhizomes of irises and orchids, and stolons (runners) of strawberries. There are, however, a number of forms of asexual reproduction in this group that utilize at least some of the aspect of sexual reproduction, in particular seeds. The term apomixis is commonly used in reference to flowering plants to refer to the asexual production of seeds (*agamospermy*). Apomixis has been the subject of considerable study as it is of economic interest in regard to being able to heritably “fix” the F1 hybrid vigor that occurs in many higher plant species and is widely utilized in agriculture and horticulture to increase yield.

Based on the phenomenology of apomixis in different lineages (it occurs in at least 33 families), there are many different mechanisms through which asexual seeds can be produced and it has arisen multiple times during angiosperm evolution. It is beyond the scope of this article to go into detail on the many forms that apomixis may take, but a general theme is that though seeds are produced, the embryos they contain are formed not from a fertilized egg. These embryos may form from maternal cells that would have become the gametophyte but failed to complete meiosis and as a result are still diploid, or from maternal sporophytic cells that never entered meiosis. In either case the cells are diploid and form embryos without the need for fertilization by a sperm cell. In some instances the second fertilization event to form the endosperm is still a requirement for seed development, but the embryo itself is a clone of the maternal plant. Notable commercial angiosperms with apomixis are *Citrus* sp. and *Allium* sp. as well as one of the more vigorous and problematic weedy species *Taraxicum officinale* (Dandelions).

References

- Holsinger, K.E., 2000. Reproductive systems and evolution in vascular plants. *PNAS* 97 (13), 7037–7042.
Richards, A.J., 1997. *Plant Breeding Systems*, second ed. London: Chapman and Hall.
Williams, C.G., 2009. *Conifer Reproductive Biology*. New York, NY: Springer.

REPRODUCTION, EXOTICS/ZOO ANIMALS/WILDLIFE

Penguins

Jack A Cerchiara, University of Washington, Seattle, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Penguins are sentinels of the marine environment, and by observing and studying them, researchers can learn about the rate and nature of changes occurring in the southern oceans. – P. Dee Boersma, PhD

Species & Distribution

Eighteen species of penguin breed south of the equator, and are broadly distributed across the southern hemisphere. Penguins breed in colonies that range in size from a few dozen to hundreds of thousands (Borboroglu and Boersma, 2013). The conservation status of Penguin species varies greatly. According to the IUCN, five species are endangered, five are vulnerable, three are near threatened and only five are considered least concern (IUCN, 2017). Understanding the breeding ecology of these species is vital to their conservation (Table 1).

Penguins breed in colonies of varying size across much of the southern hemisphere (Borboroglu and Boersma, 2013; IUCN, 2017). Penguins breed on both islands and on mainland Africa, South America, Antarctica, New Zealand and Australia (Fig. 1). Predation threats at these colonies vary considerably. For example, species breeding in Antarctica face considerable predation pressure from Leopard seals with little-to-no terrestrial threats, while Humboldt and Magellanic penguins face predation from fox and mountain lion species on land in S. America with few marine predators (Borboroglu and Boersma, 2013).

Penguin Reproduction

Penguins are unique among serially monogamous birds as they generally nest far from their food source, in colonies where they encounter complex social interactions and varied predators. When on land, many seabirds share parental time investment in chick rearing. During this time, they must exchange nest attendance, gaining metabolic resources to fast while incubating eggs and provisioning chicks.

Penguins in general are highly philopatric, returning to similar locations as their natal nest (Borboroglu and Boersma, 2013). A number of species migrate long distances during the non-breeding season, following food sources away from their breeding colony.

Table 1 Penguin species

Species name	Common name	Population trend	IUCN conservation status
<i>Aptenodytes forsteri</i>	Emperor Penguin	Unknown	Near Threatened
<i>Aptenodytes patagonicus</i>	King Penguin	Increasing	Least Concern
<i>Eudyptes chrysocome</i>	S. Rockhopper Penguin	Decreasing	Vulnerable
<i>Eudyptes chrysolophus</i>	Macaroni Penguin	Decreasing	Vulnerable
<i>Eudyptes moseleyi</i>	N. Rockhopper Penguin	Decreasing	Endangered
<i>Eudyptes pachyrhynchus</i>	Fiordland Penguin	Decreasing	Vulnerable
<i>Eudyptes robustus</i>	Snares Island Penguin	Stable	Vulnerable
<i>Eudyptes schlegeli</i>	Royal Penguin	Stable	Near Threatened
<i>Eudyptes sclateri</i>	Erect-crested Penguin	Decreasing	Endangered
<i>Eudyptula minor</i>	Little Penguin	Stable	Least Concern
<i>Megadyptes antipodes</i>	Yellow-eyed Penguin	Decreasing	Endangered
<i>Pygoscelis adeliae</i>	Adelie Penguin	Increasing	Least Concern
<i>Pygoscelis antarcticus</i>	Chinstrap Penguin	Decreasing	Least Concern
<i>Pygoscelis papua</i>	Gentoo Penguin	Stable	Least Concern
<i>Spheniscus demersus</i>	African Penguin	Decreasing	Endangered
<i>Spheniscus humboldti</i>	Humboldt Penguin	Unknown	Vulnerable
<i>Spheniscus magellanicus</i>	Magellanic Penguin	Decreasing	Near Threatened
<i>Spheniscus mendiculus</i>	Galapagos Penguin	Decreasing	Endangered

Penguin species name, common name, population trend and conservation status from IUCN, 2017. The IUCN Red List of Threatened Species. Version 2017-1. Available at: <http://www.iucnredlist.org>.

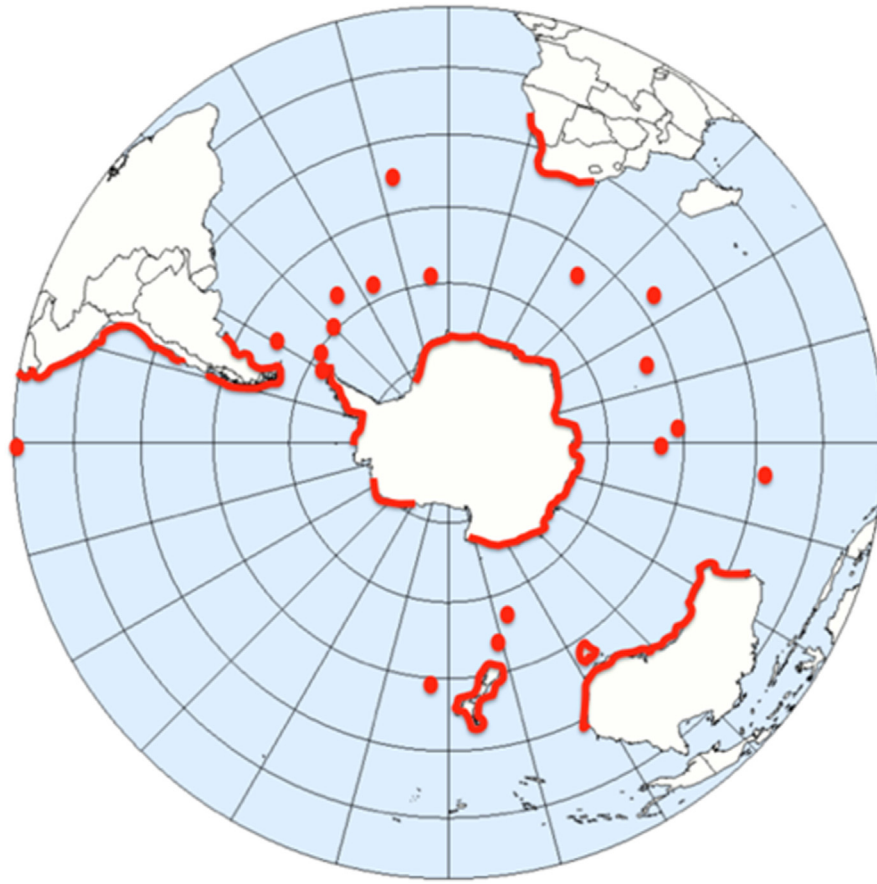


Fig. 1 Species Distribution. Penguins breed in both polar and temperate ecosystems and both on the islands and mainland of the Southern Hemisphere, species distribution from Borboroglu and Boersma. Data from: Borboroglu, P., Boersma, P.D. (Eds.), 2013. *Penguins: Natural History and Conservation*. Seattle & London: University of Washington Press. Base Map: Sean Baker – Free use, with attribution, under the CC-BY-2.0 license.

These distances vary greatly among species. Adelie penguins have the longest annual migration, some traveling distances over 8000 mi following prey assemblages, while Galapagos penguins remain at the archipelago (Borboroglu and Boersma, 2013). With climate change and migrating prey assemblages, young penguins will prospect and establish new colonies leading to a steady decline in previously large and stable populations (Borboroglu and Boersma, 2013).

There is variation in the seasonality of penguins breeding from highly constrained species like Magellanic, Adelie and Emperor penguins to Galapagos penguins that will breed and molt dependent on food resources. In general, penguins will return to breeding colonies from migrations where they spend weeks to months at sea. Many penguins will copulate at the colony after mates return from migration, though there is some evidence that penguins will copulate at sea and these copulation events can and do lead to successful fertilization. Upon returning to the colony, penguins undergo a settlement period where they secure and prepare nesting sites, court new mates and await return of former mates (Borboroglu and Boersma, 2013; Yorio *et al.*, 2001; Boersma *et al.*, 1990). Penguin nest types are generally defined by breeding habitat. Temperate species, on average, tend to nest in burrows or under bush cover. These nests provide both protection from terrestrial and avian predators, as well as favorable thermal conditions, helping to insulate young chicks from exposure (Fig. 2). Polar species, however, tend to nest on open ground. Some species, notably Chinstrap, Adelie and Gentoo penguins will gather material to elevate nests above ground level for incubation of eggs and chicks. Emperor penguins, however, will incubate eggs and chicks directly upon open ground and ice.

After settlement and copulation, fertilized eggs are laid. One or two eggs are laid depending on species, and for most penguin species, only one reproductive attempt is made yearly (Borboroglu and Boersma, 2013; Yorio *et al.*, 2001; Boersma *et al.*, 1990; Boersma and Rebstock, 2010). Some species, like Humboldt and Galapagos penguins, will lay secondary clutches in a year if eggs are lost before hatch or if reproductive conditions are optimal at the breeding colony (Borboroglu and Boersma, 2013).

Eggs range in size from Little blue penguin eggs measuring approximately 54×42 mm to Emperor penguin eggs at approximately 120×85 mm (Borboroglu and Boersma, 2013). Penguin eggs are also unusually thick among birds. Researchers believe this is due to the hard and rocky nesting conditions upon which penguins makes their nests (Boersma *et al.*, 2004). This shell thickness resists cracking during incubation and when adults exchange incubation duties. The shells of Magellanic penguins are particularly thick. Some are more than 56% thicker than eggs of birds of similar size (Borboroglu and Boersma, 2013; Boersma *et al.*, 2004). The food



Fig. 2 Magellanic penguins. Magellanic penguins during settlement at Punta Tombo, Argentina.

intake of most penguins however is not enough to supply the calcium needed to create these thick shells. To supplement this, female penguins appear to ingest small bivalves and mollusk shells to supply extra calcium to make these crack resistant eggs (Boersma *et al.*, 2004). In many species of penguin, first and second eggs vary in size, where the first egg is significantly larger than the second. The relationship however, is reversed in most *Eudyptes* penguins where the larger egg is laid second (Borboroglu and Boersma, 2013; Crossin *et al.*, 2012). In Macaroni penguins, there exists an extreme difference where the second egg is 60% larger than the first (Crossin *et al.*, 2012). In general, these larger eggs produce larger chicks and larger chicks tend to fledge at a significantly heavier weight and are more likely to survive and be seen as adults in the colony (Cerchiara *et al.*, 2017). This increased egg size also increases fledging success (Reid and Boersma, 1990). Penguin body condition can affect the size of these eggs as females that are better foragers, in better body condition, may have more resources to allocate to larger eggs.

Since penguins cannot feed on land, those individuals that arrived initially in poor body condition and must leave on foraging trips to self-provision in preparation for incubation bouts (Borboroglu and Boersma, 2013; Boersma, 2008). While mates are foraging, penguins incubate their egg(s) and protect offspring and nests from predators and conspecific competition (Borboroglu and Boersma, 2013; Yorio *et al.*, 2001; Boersma *et al.*, 1990; Boersma, 2008). Adult penguins will exchange incubation and brooding duties and these exchanges will occur more frequently as eggs mature and near hatching. In most species that lay more than one egg, viable eggs will hatch within a few hours to a few days of one another (Borboroglu and Boersma, 2013; Yorio *et al.*, 2001; Boersma *et al.*, 1990; Boersma and Rebstock, 2014). At this time it is critical that adults return from foraging with food ready to provision the chicks, as hatchling yolk sack may only provide nutrition to new chicks for a short time. Penguin chicks are termed semi-altricial. At hatch penguin chicks have limited mobility and, though downy, have limited thermoregulatory capacity (Borboroglu and Boersma, 2013; Yorio *et al.*, 2001; Boersma *et al.*, 1990; Boersma and Rebstock, 2014). It is during this period that adult penguins must incubate chicks, as they are under considerable risk of death from exposure, even in temperate penguin species. The frequency of storms can exacerbate this risk, flooding nests and soaking young chicks (Boersma and Rebstock, 2014). During the next few weeks, adults frequently attend nests during what is termed the *guard stage*. Young penguin chicks are under considerable mortality risk. During the guard stage, adults overlap between foraging trips to provision chicks, never leaving the chicks unattended. The metabolic needs of chicks, which are entirely dependent on their parents for food, increase with age, and movement of forage fish stocks affects the rate at which adults can deliver food to their chicks (Borboroglu and Boersma, 2013; Yorio *et al.*, 2001; Boersma, 2008). The amount, frequency and quality of food that the chicks receive depends on the quality of the parents, proximity of food stocks to the breeding colony, and food density. Unattended chicks are susceptible to increased risk of predation, exposure to temperature or severe weather, and also starvation. For chicks, primary mortality risk consists of thermoregulation and exposure, starvation and predation threat (Boersma and Rebstock, 2014). For many species the level of these risks vary throughout the breeding season.

During the guard stage, adults overlap between foraging trips to provision chicks, never leaving the chicks unattended. Due to the metabolic needs of the chicks, or movement of forage fish stocks, in some species the amount of time that adults overlap decreases until the point at which chicks are often left home alone in the nest for increasing amounts of time, a transition to the post-guard or *crèche stage*. After a few weeks of growth, penguin chicks are old enough that they are left home alone at the nest, during what is termed the post-guard or *crèche stage*. For small chicks, predation and thermoregulation may present the greatest risks, and starvation risk is diminished due to the low nutritional needs of very small offspring. At some point, however, these risks can shift. Older chicks may be large enough to limit their threat of predation through avoidance/evasion of predators and will benefit from a favorable surface area to volume ratio, diminishing their threat of exposure. However, larger chicks may have increased nutritional needs for metabolism and growth and therefore their risk of starvation may increase. It is during this time, penguin chicks will often *crèche*, or huddle together. Emperor penguins are well known to *crèche*, huddling together for warmth. Other penguins, including some temperate species, will minimize risk of predation through *crèche* behavior. As penguin chicks mature through *crèche stage*, they continue to gain mass and will begin to grow waterproofing juvenile feathers. It is during this stage that the foraging ability of adults can either mitigate or exacerbate starvation risk in larger penguin chicks.

Once feathering is complete, penguins have a defined juvenile plumage and are able to venture to the ocean. During the juvenile period, most penguins forage independent of their parents and are responsible for self-provisioning. Interestingly, Galapagos penguin parents have been recently observed feeding their juvenile offspring suggesting that post fledge provisioning may occur to support offspring in years of favorable food availability (Boersma *et al.*, 2017). Depending on the species, penguins will spend approximately one year in juvenile plumage after which they will haul out on land where they will lose all their juvenile feathers and grow feather of adult coloration (Borboroglu and Boersma, 2013).

Additional Threats to Penguin Chicks

In many colonies, starvation is the primary cause of mortality (Boersma and Rebstock, 2014). Overfishing, moving prey stocks, pollution, and climate change can all affect the ability of penguin adult to forage effectively to return to provision growing chicks. There are, however, additional environmental factors that can lead to chick mortality and result in a decrease in penguin fitness.

The second major cause of chick mortality is from predation. Penguin chicks are vulnerable to predation from both avian and terrestrial predators. Young chicks are under particular threat from avian predators. Other colonial seabirds, including species of gull, skua, and giant petrel, often breed among or near penguin colonies and will feed on small chicks and eggs. Cats, foxes, weasels and other mammals, as well as some reptiles (e.g. S. American armadillo) can predate larger penguin chicks while adults are away from the nest, even momentarily (Borboroglu and Boersma, 2013).

In some populations of penguins, diseases and parasites can infect and frequently kill penguin chicks. Blood, gastrointestinal and feather parasites are all known to affect penguin chicks of various species (Borboroglu and Boersma, 2013; Vanstreels *et al.*, 2016; Kleinertz *et al.*, 2014; Levin *et al.*, 2009). Blood parasites are among the most ubiquitous and significant parasites that infect penguin chicks. These parasites are spread primarily through invertebrate vectors and for this reason are more rare in wild colonies where these vectors are unlikely, like particularly cold and/or dry breeding sites (Vanstreels *et al.*, 2016). According to a review of blood parasites in penguins, only nine species had observed blood parasites, however, fifteen species were observed to be infected when in captivity or in rehabilitation centers (Vanstreels *et al.*, 2016). Gastrointestinal parasites and helminthes (worms) are ubiquitous among penguins, as well as the accumulation of alga toxins have also been observed in many breeding penguins (Kleinertz *et al.*, 2014; Shumway *et al.*, 2003). Algal blooms, commonly called red tides, are caused by dinoflagellates and are increasing in frequency across penguin habitat. Through bioaccumulation, toxins concentrate as alga are eaten by larger animals, eventually reaching harmful levels in prey eaten by penguins of all habitats (Borboroglu and Boersma, 2013; Shumway *et al.*, 2003). This can lead to the death of penguins and their chicks, and the frequency of these toxic blooms is increasing with climate change (Gobler *et al.*, 2017). Morbidity from these pathogens and parasites can directly impact the breeding success of penguins and risk large-scale mortality events in potentially struggling colonies.

One of the more interesting and puzzling conditions observed in growing penguin chicks is the appearance of featherless penguins. Initially observed at rehabilitation sites in S. Africa and breeding colonies in Argentina, featherless chick disorder appears in young Magellanic and African penguin chicks during the first few weeks of growth (Kane *et al.*, 2010) (Fig. 3). Penguins hatched normally feathered, however begin to lose feathers in patches until they become completely featherless. As they grow, chicks will begin growing adult feathers and can reach fledgling size. These chicks, however, have significantly slower growth curves and are smaller than feathered birds of the same age (Kane *et al.*, 2010). This suggests increased energetic needs of featherless chicks, likely due to thermoregulation (Kane *et al.*, 2010). Though the cause is unknown, scientists observed an increased occurrence in rehabilitation centers and believe a range of factors, from pathogens, to nutrient imbalance and pollution could cause the disorder (Kane *et al.*, 2010).



Fig. 3 Featherless Magellanic penguin chick. Olivia Kane, penguin researcher, holds featherless Magellanic penguin chick (Kane, O.J., *et al.*, 2010). Feather-Loss disorder in African and Magellanic penguins. *Waterbirds* 33 (3), 415–421). Photo: Jeffrey R. Smith.

Conservation

Thirteen species of penguin are in population decline or under threat of extinction (IUCN, 2017). Penguins are under threat of climate change, overfishing of prey-stocks, destruction of breeding habitat and pollution (Borboroglu and Boersma, 2013; Boersma, 2008; Boersma and Rebstock, 2014; Rebstock *et al.*, 2016; Boersma *et al.*, 2015). Penguins, especially breeding populations, act as sentinel species informing us about the health of their local environment and also the greater ocean ecosystem (Boersma, 2008, 1974). Five penguin species are endangered, five are vulnerable, three are near-threatened and only five are considered least concern. There are a number of conservation efforts to protect breeding penguins during the reproductive season – the period when both adults and chicks are at the greatest risk and with the greatest influence on fitness and population growth. Establishing no-take, or managed marine protected areas (MPA) that extend not only to the terrestrial breeding colony, but also the marine foraging grounds is critical to protecting penguins (Boersma *et al.*, 2015; Pichegru *et al.*, 2010; Costello and Ballantine, 2015).

For temperate species of penguins, particularly those in S. America and Africa, the establishment of marine protected areas has proved difficult and therefore few MPAs exist. Those that do exist were established as the result of years of long-term research and data collection and have seen success in impacting penguins foraging ability; notably a no-take fishing closure for marine areas surrounding African penguins in S. Africa and Namibia (Pichegru *et al.*, 2010). Penguins were tracked via time-depth recorders and after only 3 months of implementation, 70% of penguin dives were less than 20 km away, within the protected marine area (Pichegru *et al.*, 2010). Penguins also had to expend less effort searching for food and their search time decreased by 30%, reducing daily energy expenditure by 40% (Pichegru *et al.*, 2010). A similar MPA was recently developed in Argentina to protect the world's largest breeding colony of Magellanic penguins at Punta Tombo, Argentina – a population currently facing a near 30% decline in the last 30 yrs (Borboroglu and Boersma, 2013; Boersma *et al.*, 2015).

The Ross Sea Marine Protected area, off the coast of Antarctica, is the world's largest MPA and protects the breeding and foraging grounds of Adelie and Emperor penguins and supports a no-fishing zone exceeding 432,000 square miles included within the MPAs total 598,000 square miles (CCAMLR, 2017). The establishments of this MPA highlights not only the successes in unilateral protection of the world's oceans, but also the difficulty in developing marine protected areas on the foraging grounds for stable populations of breeding penguins. Scientists from the Commission for the Conservation of Antarctic Marine Living Resources (CCAMLR) initially proposed the MPA in 2011, after which years of scientific data helped refine the proposal. In 2016–2017, the two remaining hold-out countries (China and Russia) adopted the proposal, helping secure healthy breeding sites and prey assemblages for over 16,000 species that live in the Ross Sea, including polar penguins (CCAMLR, 2017).

Galapagos penguins are under particular threat of extinction; this species exists in only one population at the Galapagos Islands. These penguins are unique as they do not migrate and can breed year round, provided food resources are abundant enough to provision chicks (Borboroglu and Boersma, 2013; Boersma *et al.*, 2017; Boersma, 1974, 1978). Since these penguins do not follow food stocks, the success of their breeding and therefore the health of the species, depends directly on the health of oceans around the islands. The Galapagos Islands are uniquely placed, cold ocean currents, particularly the South Equatorial and Humboldt currents, jut out from the coast of S. America and bring nutrient rich waters to the islands (Borboroglu and Boersma, 2013; Boersma *et al.*, 2017; Boersma, 1974, 1978). In general, waters around the Galapagos are particularly rich with prey for penguins. For this reason, however, overfishing, as well as changes in ocean currents as a result of climate change, threatens the health of prey stocks (Borboroglu and Boersma, 2013; Boersma *et al.*, 2017; Boersma, 1974, 1978). To protect the prey that supports the penguins, and many other species of seabirds including the Blue-footed booby and Flightless cormorant, two no-take regions were developed to prevent fishing of the valuable food source (Borboroglu and Boersma, 2013). It is the hope that the establishment of these no-take zones, combined with efforts to expand usable breeding sites, will lead to increased reproduction and population growth for these endangered penguins.

References

- Boersma, P.D., 1974. The Galapagos Penguin: A study of adaptations for life in an unpredictable environment. PhD Thesis, Ohio State University, Columbus.
- Boersma, P. D. (1978). Breeding patterns of Galapagos Penguins as an indicator of oceanographic conditions. *Science*, 200, 1481–1483.
- Boersma, P. D. (2008). Penguins as marine sentinels. *BioScience*, 58(7), 597–607.
- Boersma, P. D., Cappelletto, C. D., & Merlen, G. (2017). First observations of post-fledging care in Galapagos penguins (*Spheniscus mendiculus*). *The Wilson Journal of Ornithology*, 129(1), 186–191.
- Boersma, P. D., & Rebstock, G. (2014). Climate change increases reproductive failure in Magellanic penguins. *PLOS One*, 9(1), e85602.
- Boersma, P. D., Rebstock, G., & Stokes, D. (2004). Why penguin egg shells are thick. *The Auk*, 121(1), 148–155.
- Boersma, P. D., & Rebstock, G. A. (2010). Calculating egg volume when shape differs: When are equations appropriate? *Journal of Field Ornithology*, 81(4), 442–448.
- Boersma, P. D., Rebstock, G. A., & García-Borboroglu, P. (2015). Marine protection is needed for Magellanic penguins in Argentina based on long-term data. *Biological Conservation*, 182, 197–204.
- Boersma, P. D., Stokes, D., & Yorio, P. (1990). Reproductive variability and historical change of magellanic penguins (*Spheniscus magellanicus*) at Punta Tombo, Argentina. In L. S. Davis, & J. T. Darby (Eds.), *Penguin Biology* (pp. 15–43). San Diego, CA: Academic Press.
- Borboroglu, P., & Boersma, P. D. (Eds.). (2013). *Penguins: Natural History and Conservation*. Seattle & London: University of Washington Press.
- CCAMLR, 2017. Commission for the Conservation of Antarctic Marine Living Resources. Available at: <https://www.ccamlr.org/node/92518>.
- Cerchiara, J., et al. (2017). Magellanic penguin telomeres do not shorten with age with increased reproductive effort, investment and basal corticosterone. *Ecology and Evolution*, 00, 1–10.
- Costello, M. J., & Ballantine, B. (2015). Biodiversity conservation should focus on no-take marine reserves: 94% of marine protected areas allow fishing. *Trends in Ecology and Evolution*, 30(9), 507–509.

- Crossin, G., Trathan, P., & Williams, T. D. (2012). Potential mode of clutch size determination and follicle development in Eudyptes penguins. *Polar Biology*, 35, 313–317.
- Gobler, C. J., et al. (2017). Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. *Proc Natl Acad Sci*, 114(19), 4975–4980.
- IUCN, 2017. The IUCN Red List of Threatened Species. Version 2017-1. Available at: <http://www.iucnredlist.org>.
- Kane, O. J., Smith, J. R., Boersma, P. D., et al. (2010). Feather-loss disorder in African and magellanic penguins. *Waterbirds*, 33(3), 415–421.
- Kleinertz, S., et al. (2014). Gastrointestinal parasite fauna of Emperor Penguins (*Aptenodytes forsteri*) at the Atka Bay, Antarctica. *Parasitology Research*, 113(11), 4133–4139.
- Levin, I. I., et al. (2009). Plasmodium blood parasite found in endangered Galapagos penguins (*Spheniscus mendiculus*). *Biological Conservation*, 2001–2005.
- Pichegru, L., et al. (2010). Marine no-take zone rapidly benefits endangered penguin. *Biology Letters*, 6(4), 498–501.
- Rebstock, G., Boersma, P. D., & Borboroglu, P. (2016). Changes in habitat use and nesting density in a declining seabird colony. *Population Biology*, 58, 105–119.
- Reid, W., & Boersma, P. D. (1990). Parental quality and selection on egg size in the Magellanic penguin. *Evolution*, 44(7), 1780–1786.
- Shumway, S. E., Allen, S. M., & Boersma, P. D. (2003). Marine birds and harmful algal blooms: Sporadic victims or under-reported events? *Harmful Algae*, 2(1), 1–17.
- Vanstreels, R. E., Braga, E. M., & Catao-Dias, J. L. (2016). Blood parasites of penguins: A critical review. *Parasitology*, 143(8), 931–956.
- Yorio, P., Borboroglu, P. G., & Boersma, P. D. (2001). Breeding biology of Magellanic penguins *Spheniscus magellanicus* at Golfo San Jorge, Patagonia, Argentina. *Marine Ornithology*, 25(2001), 75–79.

Hyenas

Christine M Drea, Duke University, Durham, NC, United States

Elizabeth M Coscia, Salesian College Preparatory, Richmond, CA, United States

Stephen E Glickman, University of California, Berkeley, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Androstenedione An androgenic steroid secreted by the ovaries and adrenals that is involved in the synthesis of testosterone.

Clitoris A small, sensitive erectile organ in the female corresponding to the male penis and located at the ventral end of the vulva.

Masculinize To cause an individual to develop the secondary sexual characteristics of a mature male, as by the natural exposure to or experimental administration of androgens.

Placenta A structure formed by fusion of the chorion with the wall of the uterus that serves to attach the embryo to the uterine wall and to exchange nutrients, wastes, and gases between the maternal blood and the embryonic blood.

Prohormone A precursor of a hormone.

Sexual differentiation The process by which the two sexes become different; whereby a bi-potential embryo develops along either male or female lines, as determined initially by the chromosomes and subsequently by hormones.

Spotted hyena A social carnivore of the family Hyenidae.

The Family Hyenidae

Hyenidae, a lineage that separated from viverrid ancestors 25–30 million years ago, comprises four extant species (**Fig. 1**): the aardwolf (*Proteles cristatus*), the striped hyena (*Hyaena hyaena*), the brown hyena (*Parahyaena brunnea*), and the spotted hyena (*Crocuta crocuta*). Although there is substantial variation in the details of their social and mating systems, gestation and development, parental care and feeding ecology (**Table 1**), the first three of these species present traditional, sexually dimorphic, external genitalia. In the fourth species, the spotted hyena, females exhibit remarkably ‘masculinized’ external genitalia. Because of the extraordinary similarity in *Crocuta* between male and female genital morphology, the common belief that these animals were hermaphrodites persisted well into the 19th century. More recently, it was discovered that female spotted hyenas also exhibit a suite of behavior patterns conventionally associated with a ‘masculine’ phenotype, being unusually aggressive, playing more roughly than males, scent marking in the same manner as males, and totally dominating adult males in social situations. This array of ‘masculine’ characteristics has directed attention to the process of sexual differentiation in the female spotted hyena: Either there are androgens circulating during fetal life that create the unusual external genitalia of these animals (and might enhance their ‘masculine’ behavior), or there is a hitherto unrecognized mechanism for the production of male-like genital morphology in a female mammal. The present article is, accordingly, focused on reproduction and sexual differentiation in the spotted hyena, although we begin with a brief outline of the major features of reproductive biology in the four extant hyenid species.

Reproduction in the Hyenidae – An Overview

Despite their phylogenetic divergence, all four hyenid species have commonalities in their reproductive biology and behavior (**Table 1**). For instance, all males lack a baculum. Both sexes of all species have anal scent glands contained within an anal pouch,



Fig. 1 Three of four living members of Hyenidae, including the (A) striped hyena, (B) brown hyena, and (C) spotted hyena. Photographs by C. M. Drea.

Table 1 Comparative reproductive biology and life histories of hyenids

Features	Hyenid species			
	<i>Aardwolf</i> (<i>Proteles cristatus</i>)	<i>Striped</i> (<i>Hyaena hyaena</i>)	<i>Brown</i> (<i>Parahyaena brunnea</i>)	<i>Spotted</i> (<i>Crocuta crocuta</i>)
Age at maturity (y)	M=1.8 F=1.8	M=2–3 F=2–3	M=1.8 F=1.5–2	M=2 F=3
Lifespan (y)	Wild: 7 Captive: 15	Wild: 15 Captive: 24	Wild: 12 Captive: 20	Wild: 20–25 Captive: >40
Size differentiation	Size monomorphism			Reverse size dimorphism
Adult mass (kg)	M=7.8–14 F=7.7–14	M=28.0–43.2 F=27.5–36.0	M=35–49.5 F=28–47.5	M=40–62.5 F=44–90.0
Sexual organs	Unspecialized sexual dimorphism No baculum			Specialized monomorphism
Secondary sexual characteristics	Anal glands and pouch Teats: ? pairs	Teats: 2–3 pairs	Teats: 2–6 pairs	Teats: 1 pair
Mating system	Monogamy (with cuckoldry)	Monogamy/ polyandry	Uni-male polygyny/ promiscuity ^a	Promiscuity ^a
Breeding	Seasonal	Aseasonal		
Estrous cycle	Monoestrous (+1)	Polyestrous		
Estrus duration	1–3 days	1 day	1 week	unknown
Mating behavior	Multiple mounting Thrusting	No thrusting	No thrusting	Thrusting
Multiple paternity	Unknown	Yes	Unknown	Yes
Placentation	Unknown			Hemochorial
Gestation (days)	90	90–91	90–96	110
Litter size	1–4 (x=3)	1–6 (x=2.4)	1–5 (x=2.3)	1–3 (x=2)
Neonatal mass	223 g	600–700 g	630–812 g	1.5 kg
Natal coat	No			Yes
Parental care	F suckles own cubs Biparental care No provisioning	F suckles own cubs Biparental care Provisioning by parents	F allosuckles Cooperative care Provisioning by clan	F suckles own cubs No paternal care Limited or no provisioning
Age at weaning (mo)	4	4–5	10–15	12–18
Interlitter interval (mo)	12	14	12–41	16–19

^aFemale brown hyenas mate with nongroup-living males, whereas female spotted hyenas mate with group-living males.

and engage in ‘pasting’ (and other forms of scent-marking behavior); the deposited chemical signals play a role in sexual and reproductive advertisement (Fig. 2). Lastly, although the physical structure varies depending on the environment, all species make use of natal dens to protect their young (Fig. 3). Regarding generalizations on reproduction, the similarities across species end here. Teat number varies individually and by species. Notably, in spotted hyenas, there is only a single pair of teats, a factor contributing to sibling rivalry in triplet litters. Also, only spotted hyenas show sexual size dimorphism favoring the female, and only female *Crocuta* display highly ‘masculinized’ sexual organs (see below). In all other hyenids, adults are sexually size monomorphic and the adult female sexual organs are not ‘masculinized.’



Fig. 2 Hyena olfactory communication. (A) A spotted hyena extrudes its anal pouch while straddling a blade of grass, depositing glandular secretions in a behavior termed ‘pasting.’ (B) The trademark, pasted scent mark of a brown hyena comprises chemically distinct ‘white’ and ‘brown’ components, deposited in close succession. (C) Spotted hyenas engaged in intense social sniffing of conspecific paste from a non-clan member. Photographs by C.M. Drea.



Fig. 3 Hyena dens. (A) Caves and crevices on the Namibian coast serve as a natal den for brown hyenas. (B) Bones accumulate at the den's entrance owing to the adult members of brown hyena clans provisioning cubs. (C) Abandoned aardvark burrows serve as a communal den for spotted hyenas in South Africa; the cub, which is still nursing, is seen transitioning from its black natal coat to its adult spotted coat. Photographs by C.M. Drea.

The Aardwolf

For such a small family, hyenids show fairly diverse reproductive behavior and mating strategies, from monogamy to promiscuity. The least typical member of Hyenidae, the aardwolf, is socially monogamous, forming pair bonds that can last anywhere from two to five years; however, both sexes are known to engage in extra-pair copulations. Aardwolves are the smallest, the shortest lived, and the only seasonal breeders among Hyenidae. Females generally have a single yearly estrous in July (although if not fertilized they will cycle a second time about two weeks later) and give birth in October to litters of one to four cubs. Because of this species' exclusive termite diet, cubs are entirely dependent on maternal milk for the first four months of life. Although male partners, even if cuckolded, guard and defend the young, they do not provide them with food.

Striped Hyenas and Brown Hyenas

Striped and brown hyenas are the most closely related and, consequently, the most similar in their reproductive biology. They are of roughly equal size and longevity, and have similar lifestyles in that both are solitary foragers and scavengers. In contrast to the aardwolf, these two species are aseasonal breeders, with females being polyestrous. Neonatal mass in striped and brown hyenas is at least threefold that in the aardwolf, but cubs of all three species are born with their eyes closed. Although striped and brown hyenas have multiple pairs of teats, only the two caudal pairs are functional.

The most prominent differences in reproduction between striped and brown hyenas are evidenced by adult behavior. Although behaviorally solitary, for breeding purposes striped hyenas form short-term, pair bonds or even polyandrous groups of up to three adult males per single adult female. Multiple paternity is likely and the resulting 'family' unit may endure for several years. There is biparental care as both sexes provision the young. The 'single family' approach of striped hyenas contrasts with the 'cooperative venture' of brown hyenas. The latter occur in small social groups in which members are related to each other. Females mate with nongroup-living, nomadic males; nevertheless, resident males provide care for the young they did not sire. Mothers are also cooperative, suckling the cubs of other females along with their own. All clan members participate in provisioning of the cubs (see Fig. 3(B)).

The Spotted Hyena

Unlike their relatives, spotted hyenas are pack hunters and live in the largest social groups of any carnivore. Within this complex social network, there is no extended pair-bonding of the sort seen in other hyenas: Mating is promiscuous and there is no paternal care. Spotted hyenas are aseasonal breeders, but there is little information on their estrous cycle. They are currently the only known member of Carnivora in which pregnant females develop hemochorial placentas. They have an exceptionally long gestation (of approximately 110 days) and their young are unusually precocial at birth (see below). Despite this 'head start,' spotted hyenas have longer lactation periods than do most other carnivores, which places a substantial energetic burden on mothers and by extension, puts lower-ranking females at a disadvantage. Not surprisingly, therefore, mothers generally suckle their own young exclusively. Details of spotted hyena reproductive biology are described below.

Reproduction in the Spotted Hyena

Morphology

In the female spotted hyena, the clitoris has hypertrophied, approximating the size and shape of the male's penis; it is fully erectile, allowing her to display erections similar to those of the male (Fig. 4(A) and (B)). Erections serve an important communicatory function and are used by both sexes in ritualized 'greeting' ceremonies (Fig. 4(C)). Uniquely, the elongated clitoris is traversed



Fig. 4 The erections of male and female spotted hyenas. The erect (A) penis and (B) clitoris are comparable in size and shape, and are used by both sexes, at all ages, in (C) species-specific, social 'greeting' ceremonies. Photographs by L.G. Frank, reprinted with permission.

by a central urogenital canal, through which the female urinates, copulates, and gives birth. There is no 'external' vaginal opening: The labia have fused to form a pseudoscrotum, marked by two pads of fibrous/fatty tissue that are outwardly visible and might be mistaken for testes. The internal reproductive system follows the standard mammalian pattern.

Careful visual examination of the external genitalia reveals sex differences in 'phallic' structure. Notably, the clitoris is shorter and thicker than the penis. The shape of the glans is also sexually dimorphic: The glans penis has an angular profile, whereas the glans clitoridis has a rounded contour (**Fig. 4(A) and (B)**). These visible differences in the shape of the glans are now used by field researchers to distinguish male from female spotted hyenas. Lastly, the central urogenital canal ends in an opening (the urogenital meatus) that is larger and more elastic in the female than in the male. Internally, based on histological examination, the female urogenital canal is pleated and unconstrained by erectile or connective tissue, allowing for significant expansion that would not be afforded the male. This elasticity is prerequisite for mating and birth, and is facilitated in adulthood by the actions of estrogens and the peptide hormone relaxin.

These structural differences emerge much earlier in development, however (**Fig. 5**). Although there are no sex differences in either the internal or external morphology of male and female genital tubercles at 30–34 days of fetal life, major differences in internal phallic structure are identifiable at 45–50 days of gestation when the fetal gonads are morphologically differentiated. As gestation proceeds, a thick, fibrous tunica completely envelops the corpus spongiosum and urethra of the developing male and prevents expansion of the urethra (**Fig. 5(A)**). In the developing female, however, a tunica envelops only the corporal body (**Fig. 5(B)**), thus later permitting expansion of the urogenital sinus during mating and parturition. These functionally significant sex differences, noted previously in adult spotted hyenas, are dependent upon testicular androgens in males, and are present at birth.

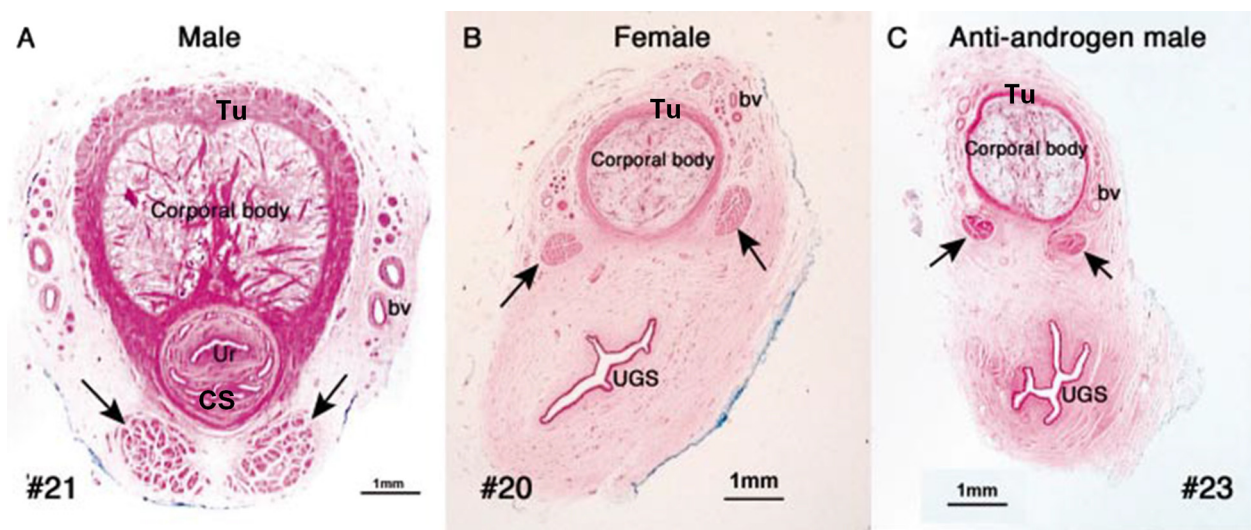


Fig. 5 Sexual dimorphism and feminization of the internal 'phallic' structures of neonatal spotted hyenas. Mid-shaft sections through the phallus of a (A) normal male, (B) normal female, and (C) a male treated with anti-androgens. Specimens #21 and #20 (A and B) were obtained at 95 days of gestation and specimen #23 (C) was obtained at 105 days. In A and B, respectively, the sexually differentiated structures include the urethra (Ur) vs. the urogenital sinus (UGS), the presence vs. absence of the corpus spongiosum (CS), the size and expanse of the tunica (Tu), and the position of the retractor muscles (denoted by black arrows). Prenatal anti-androgen treatment of males caused dramatic feminization of these structures (C). Photographs by G.R. Cunha, modified with permission.

Mating Behavior

Mating occurs in the social context of the hyena clan. Unlike the social canids, in which breeding is commonly restricted to the dominant female, all female spotted hyenas mate and give birth; nevertheless, their social standing within the clan influences their success at rearing offspring to independence. Females typically achieve sexual maturity at 24–36 months of age and reproduce in the clan of their birth. Males generally disperse at the time of puberty (at 18–24 months) and mate only after joining a new clan. At present, little is known of the spotted hyena estrous or ovulatory cycle (if, in fact, there is a cycle). The presence of spines on the glans penis of spotted hyenas is characteristic of other species that are induced ovulators, suggesting the possibility of induced ovulation in spotted hyenas. Although there are seasonal peaks in the frequency of births, associated with rainy seasons in sub-Saharan Africa, there is no sharply defined breeding season and mating occurs throughout the year.

Because of their unusual external anatomy, the act of mating requires patience and cooperation on the part of the female, as well as some temerity and agility on the part of the male (Fig. 6). During the sexual act, the female retracts her clitoris into the abdomen and, standing exceptionally still, assumes a head-down posture. The male mounts the female and stands on his hind legs with his forepaws on her flanks (Fig. 6(A)). He then ‘flips’ his erect penis against her lower abdomen (Fig. 6(B)), searching for the clitoral opening (Fig. 6(C)), which is well anterior to the position normally occupied by the external vagina (Fig. 6(D)). To achieve entry, the male must squat down on his haunches, move forward, and reposition his pelvis (Fig. 6(E)). After achieving entry there is an interval of pelvic thrusting followed by ejaculation. During the minutes following ejaculation, the male maintains insertion and often assumes a posture in which he leans forward resting his head on the back of the female. A number of additional ejaculatory sequences may follow, with the female taking an active role in soliciting the male’s attention.

Gestation and Parturition

Relative to the other hyenids, gestation is prolonged in the spotted hyena. Concentrations of plasma progesterone increase within the first two weeks and, as pregnancy proceeds, plasma concentrations of estrogen, testosterone, and relaxin also show a substantial rise from pre-pregnancy values. A marked increase in the size of the nipples is often the first external indicator of pregnancy, particularly in primiparous animals. In nature, as the female proceeds toward term, she locates a site removed from the communal den of the clan and gives birth at this ‘natal den’ (see Fig. 3). The young will be nursed and maintained at this den for a variable period (2–4 weeks) before being carried by the mother to the communal den and introduced to the clan.

Costs of Clitoral Delivery

Giving birth to a large (1–2 kg) fetus through the clitoris is a mechanically challenging feat (Fig. 7(A)). The urogenital meatus, although highly elastic, is not adequately large to permit passage of the fetus and must tear before an initial birth can occur (Fig. 7(B)). The process is further complicated by the presence of a short umbilical cord and a rather tortuous route from the point of placental attachment in a uterine horn. The fetus must travel through the uterus and upper vagina before finally reaching the clitoris. If delivery of the fetus does not proceed in a timely fashion, the infant will be stillborn (presumably due to anoxia, consequent to detachment of the placenta from the uterine wall). In captivity, approximately 60% of first births result in stillborn offspring due to the time required for passage of the fetus. Moreover, in some captive, primiparous females, the fetus became lodged in the birth canal and, without veterinary intervention, would have taken the life of the mother. Similar complications may explain the loss of primiparous female hyenas in nature. In subsequent pregnancies, after a fetus has successfully passed through and torn the clitoris, infant mortality (and indeed maternal mortality) is much lower. Because there are substantial costs to clitoral delivery in this species, it is probably not surprising that this process has evolved only once.

Behavior of Infants

The twins that generally constitute a hyena litter are quite precocial. At birth, their eyes are open, they have substantial locomotor skills, and they possess an impressive array of needle-like teeth (Fig. 7(C)). In addition, within minutes of the birth of the second cub, the twins begin to fight. The fighting between siblings is intense and, if uninterrupted, wounds result from the vigorous biting. At a minimum, fighting during the first days of life establishes a dominance relationship between the siblings that lasts minimally for several years. In nature, if the dominant sibling can keep its young twin sequestered in a burrow and prevent it from emerging to nurse, death will result from a combination of starvation, wounding, and infection. When siblicide occurs, it has been found to be based on resource availability, as in other species displaying this behavior. If both infants survive, however, the intense aggression is largely replaced by playful, prosocial interactions. The development of prosocial behavior is also on a precocial timeline, emerging in the second week of life and increasing over subsequent months. This schedule ensures that both aggressive and prosocial behavior patterns will be established within the infant’s repertoire when it joins the clan. As outlined below, the exposure of all fetuses to high concentrations of androgens during fetal life might be expected to facilitate the aggression observed at birth in this species.

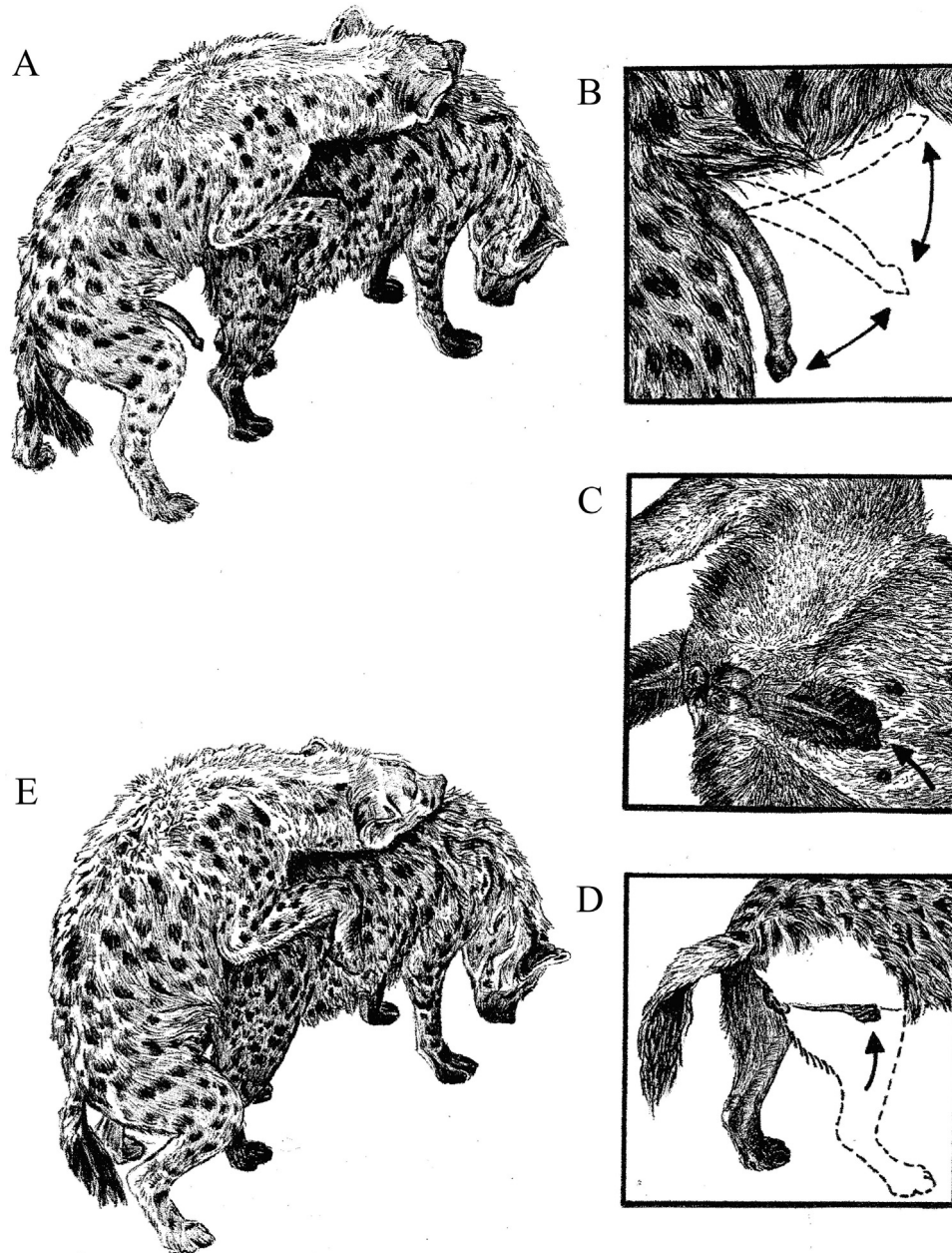


Fig. 6 The mating sequence in spotted hyenas. (A) The male mounts the female, who stands still, with her head lowered and her clitoris retracted into her abdomen. (B) The male shows penile 'flips,' as indicated by arrows. (C) Underside view and (D) lateral view of a female spotted hyena, with arrows showing the site of the clitoral opening, which is located far anterior to the point of entry in other female mammals. (E) Once the male locates the opening, he squats down and scoots forward to enable intromission, which is followed by pelvic thrusting. Illustrations by C.M. Drea, reprinted with permission.



Fig. 7 Spotted hyena clitoral delivery of precocious neonates. (A) The clitoris of a primiparous female becomes engorged with a full-term fetus. (B) Delivery cannot progress until the clitoris tears, the delay often leading to fetal hypoxia and stillbirth. (C) Live cubs are precocial at birth. Photographs by S.E. Glickman, and L.G. Frank, reprinted with permission.

Sexual Differentiation in the Spotted Hyena

The Search for Androgens in Adult Female Hyenas

Contemporary understanding of sexual differentiation requires the secretion of androgens, by the fetal testes, for the production of masculine external genitalia from embryological precursors. Such androgens are also known to affect the developing nervous system, modifying hypothalamic mechanisms responsible for sex-typical profiles of hormone secretion, as well as structures modulating sexually dimorphic behavior patterns. Given the exceptionally 'masculine' external genitalia of the female spotted hyena observed at birth, it is not surprising that early research was directed toward a search for raised testosterone concentrations in females. The size, aggressiveness, and dominance of these animals also suggested that substantial concentrations of this androgen might be found in adult females. It soon became clear, however, that non-pregnant, adult female hyenas have relatively low plasma concentrations of testosterone. By contrast, androstenedione, primarily of ovarian origin, is the primary androgen found in high concentrations in female spotted hyenas. Androstenedione is an interesting steroid. It is known to bind with low affinity, if at all, to conventional estrogen or androgen receptors, but it is readily converted to an active estrogen or to testosterone in the presence of appropriate enzymes. Following conversion, androstenedione could exert powerful hormonal effects on a developing fetus or on the postnatal nervous system.

The Search for Androgens in Fetal Hyenas

In all mammals studied to date, the fetal testes secrete androgens during embryonic life that result in the formation of male genitalia; fetal ovaries are typically found to be inactive during fetal life and, in the absence of androgens, a female genital phenotype emerges. In the spotted hyena, both the fetal testes and fetal ovaries are active during the embryonic period, and are capable of secreting androgens; however, based on anatomical studies, it is clear that both the penis and the penile clitoris are fully formed at an early stage (before 36 days) of gestation, prior to maturation of the fetal gonads. The fetal testes are capable of androgenic secretion before the fetal ovaries, and such earlier secretions presumably account for the major differences in internal and external penile/clitoral morphology present at birth.

In addition to the fetal gonads, it appears that the placenta of the spotted hyena is a significant source of androgen from an early stage of gestation. A clue as to potential sources of fetal testosterone was provided by the high concentrations of this steroid observed in the circulation of pregnant hyenas. It seemed possible that the gradual increment in maternal testosterone concentration during pregnancy reflected two processes: the metabolic conversion of maternal ovarian androstenedione to testosterone by the placenta, and; the absolute growth in size of this structure during the course of gestation. Subsequent studies of placental activity supported this hypothesis and verified that maternal androstenedione, converted to testosterone in the placenta, was transferred to the developing fetus via the umbilical circulation (and the maternal circulation via the uterine venous drainage of the placenta).

A mechanism was thus in place that could account for masculinization of the external genitalia in this unique species. This possibility was reinforced by the appearance of several human cases in which genetic female infants were born with masculinized genitalia due to a defect in aromatase activity, i.e., a defect in which an abnormal human placenta acted, metabolically, like a normal hyena placenta; however, recent studies suggest that the essential penile/clitoral phenotype is formed before placental androgen production could be involved in formation of these organs.

Evidence for Significant Non-Androgenic Processes During Prenatal and Postnatal Life

The possibility that non-androgenic mechanisms account for formation of the penis/clitoris in the spotted hyena was tested by interfering with the putative action of gonadal or placental testosterone on the developing fetus of both sexes. Anti-androgens administered to pregnant female dogs during appropriate stages of pregnancy result in their male offspring having a very small penis, a urethral opening near the base rather than the tip of the penis (i.e., hypospadias), and a blind vaginal pouch in the midline of the scrotal area. By contrast, anti-androgens (flutamide and finasteride) administered to pregnant female spotted hyenas had a rather different phenotypic effect. Male offspring exposed to these compounds as early as day 12 of gestation were indeed 'feminized,' but their genitalia now approximated those of a normal female spotted hyena, rather than those of a generic female mammal. Such males had a short, thick penis with a large urogenital meatus and the glans was rounded like the glans clitoridis (as in Fig. 4(B)), rather than angular like a normal glans penis (as in Fig. 4(A)). Moreover, the size and shape of the urethra (or urogenital sinus) in the absence of a corpus spongiosum, the size and expanse of the tunica, and the position of the retractor muscles in these males (Fig. 5(C)) corresponded to features of normal female spotted hyenas (Fig. 5(B)). Female offspring exposed to anti-androgens prenatally were still more 'feminized,' with an exceptionally short, thick clitoris and a still larger urogenital sinus. In sum, the data from anti-androgen experiments suggest that androgens normally modulate fetal development in both male and female spotted hyenas. However, the robust development of a penis or penile clitoris from the genital tubercle, and a fully formed scrotum from the genital swellings, in the absence of androgenic activity, suggests that a non-androgenic mechanism may be involved in the initial formation of these structures.

Recent studies, involving the administration of letrozole to pregnant female spotted hyenas, suggest a role for estrogens in formation of the penile and clitoral shaft that encloses the urethra in both sexes. Letrozole interferes with the aromatase enzyme required for production of estrogens. Embryos collected from letrozole-treated pregnant females, as well as infants born to

letrozole-treated mothers, displayed incomplete fusion of epithelial tissues on the ventral surface of the penis or clitoris, as required for the urethra to be a fully enclosed structure.

Further evidence for the existence of androgen-independent mechanisms regulating the growth of the clitoris and the penis is provided by examining the effects of pre-pubertal gonadectomy. In other mammalian species, there is typically a period of accelerated growth of the genitalia accompanying puberty. Moreover, castration or ovariectomy prior to puberty inhibits such growth. Both in male and female spotted hyenas, the normal growth curve actually decelerates during puberty, with more rapid development both of genitalia and overall body size occurring prior to the pubertal period. Pre-pubertal gonadectomy causes a major reduction in adult concentrations of androstenedione and testosterone, suggesting that the ovaries, not the adrenal glands, are the primary source of androstenedione in female spotted hyenas; however, there are modest effects of such surgery on growth of the clitoris or penis (aside from some changes in the elasticity and thickness of the clitoris, which are normally induced by the presence of estrogens in females).

Summary – Mechanisms and Implications of Sexual Differentiation in the Spotted Hyena

Sexual Differentiation and Development: Integration of Androgenic and Androgen-Independent Mechanisms

It appears that both androgenic and non-androgenic mechanisms are involved in the development of the penile clitoris of the female spotted hyena. The basic formation of the clitoris, including enclosure of the urethra within the genital tubercle, can apparently proceed without the intervention of androgens; however, gonadal and placental androgens play a role in the early development of the clitoris and penis *in utero*. Paradoxically, the changes produced in the normal hyena clitoris by naturally circulating testosterone (i.e., a smaller urogenital sinus and opening and a thinner clitoris) serve to make a very difficult initial parturition still more costly.

General Implications From an Unusual Animal

Despite the very unusual events that underlie formation of the clitoris and scrotum in the spotted hyena, the contemporary theory of sexual differentiation survives intact. That theory was always an explanation of how one arrives at a male phenotype instead of a female phenotype for each species. Presumably, the sex differences in genital morphology observed in spotted hyenas at birth are the result of both placental and testicular testosterone acting on the male embryo, while only placental testosterone is circulating in the female embryo. Further separation of male and female genital morphology is produced by the different hormonal environments that are present postnatally, particularly the activity of estrogens in the female, but not in the male. Consideration of the spotted hyena calls attention to the absence of a substantive theory of feminine development. There is wide variation in mammalian clitoral morphology. Such species as lemurs and European moles also display varying degrees of clitoral 'masculinization.' To date, development of female genitalia often has been treated as a passive process. This situation must change in the future.

Studies of the spotted hyena also highlight the potential significance of androstenedione as a significant prohormone in all female (and male) mammals. Often dismissed in the contemporary literature as a 'weak androgen,' studies of the spotted hyena and other species (e.g., the redwing blackbird and meerkat) demonstrate the capacity of androstenedione to act as a very powerful steroid following conversion to estrogen or testosterone.

Finally, the role of placental metabolism is typically ignored in accounts of sexual differentiation. After all, within a species, the effects of that metabolism are assumed to be the same for male and female fetuses. This oversight may be rectified in the future, as the recent accounts of human pseudohermaphroditism, produced by a defect in placental aromatase activity, direct attention to the impact of placental metabolism on development. Studies of the hyena also emphasize the role of species differences in placental metabolism in modulating structural development in embryos of both sexes. Examination of 'experiments-of-nature,' such as the spotted hyena, reveal common processes in other, less exotic, species.

Further Reading

- Browne, P., Place, N. J., Vidal, J. D., et al. (2006). Ontogeny of endocrine differentiation of fetal ovaries and testes of the spotted hyena (*Crocuta crocuta*): Timing of androgen-independent versus androgen-driven genital morphogenesis and development. *Reproduction*, 132, 649–659.
- Conley, A. J., Corbin, C. J., Browne, P., et al. (2006). Placental expression and molecular characterization of aromatase cytochrome P450 in the spotted hyena (*Crocuta crocuta*). *Placenta*, 28, 668–675.
- Conte, A. F., Grumbach, R. M., Ito, Y., Fisher, C. R., & Simpson, E. R. (1994). A syndrome of female pseudohermaphroditism, hypergonadotropic hypogonadism, and multicystic ovaries associated with missense mutations in the gene encoding aromatase (P450arom). *J. Clin. Endocrinol. Metabol.*, 78, 1287–1292.
- Cunha, G. R., Risbridger, G., Wang, H., et al. (2014). Development of the external genitalia: Perspectives from the spotted hyena (*Crocuta crocuta*). *Differentiation*, 87, 4–22.
- Drea, C. M., Weldele, M. L., Forger, N. G., et al. (1998). Androgens and masculinization of the genitalia in the spotted hyena (*Crocuta crocuta*). 2. Effects of prenatal anti-androgens. *J. Reprod. Fertil.*, 113, 118–128.
- Forger, N. G., Frank, L. G., Breedlove, S. M., & Glickman, S. E. (1996). Sexual dimorphism of perineal muscles and motoneurons in spotted hyenas. *J. Comp. Neurol.*, 375(2), 333–343.
- Frank, L. G. (1997). Evolution of genital masculinization: Why do female hyenas have such a large 'penis'? *Trends Ecol. Evol.*, 12, 58–62.
- Frank, L. G., Holekamp, K. E., & Smale, L. (1995). Dominance, demography, and reproductive success of female spotted hyenas. In A. R. E. Sinclair, & P. Arcese (Eds.), *Serengeti II: Dynamics, Management, and Conservation of an Ecosystem* (pp. 364–384). Chicago, IL: University of Chicago Press.

- Glickman, S. E., Coscia, E. M., Frank, L. G., Weldele, M. L., & Drea, C. M. (1998). Androgens and masculinization of the genitalia in the spotted hyaena (*Crocuta crocuta*). 3. Effects of juvenile gonadectomy. *J. Reprod. Fertil.*, 113, 129–135.
- Hammond, G. L., Miguel-Queral, S., Yalcinkaya, T., et al. (2012). Phylogenetic comparisons implicate sex-hormone binding globulin in "masculinization" of the female spotted hyena (*Crocuta crocuta*). *Endocrinology*, 153, 1435–1443.
- Kruuk, H. (1972). *The Spotted Hyena: A Study of Predation and Social Behavior*. Chicago, IL: University of Chicago Press.
- Licht, P., Hayes, T., Tsai, P.-S., et al. (1998). Androgens and masculinization of the genitalia in the spotted hyaena (*Crocuta crocuta*). 1. Fetal urogenital anatomy and placental metabolism. *J. Reprod. Fertil.*, 113, 106–117.
- Marneweck, D., Cameron, E. Z., Ganswindt, A., & Dalerum, F. (2015). Behavioural and endocrine correlates to the aardwolf mating system. *Mammalian Biology-Zeitschrift für Säugetierkunde*, 80(1), 31–38.
- Mills, M. G. L. (1990). *Kalahari Hyenas: Comparative Behavioural Ecology of Two Species*. London: Unwin-Hyman.
- Owens, D., & Owens, M. (1979). Notes on social organization and behavior in brown hyenas (*Hyaena brunnea*). *J. Mammal.*, 60, 405–408.
- Szykman, M., Van Horn, R. C., Engh, A. L., Boydston, E. E., & Holekamp, K. E. (2007). Courtship and mating in free-living spotted hyenas. *Behaviour*, 144(7), 815–846.
- Steinetz, B., Lasano, S., de Haas van Dorsser, F., et al. (2009). Relaxin concentrations in serum and urine of endangered and crazy mixed-up species: New methods, uses and findings. *Annal. N.Y. Acad. Sci.*, 1160, 179–185.
- Wagner, A. P., Creel, S., Frank, L. G., & Kalinowski, S. T. (2007). Patterns of relatedness and parentage in an asocial, polyandrous striped hyena population. *Mol. Ecol.*, 16(20), 4356–4369.
- Yalcinkaya, T. M., Siiteri, P. K., Vigne, J.-L., et al. (1993). A mechanism for virilization of female spotted hyenas in utero. *Science*, 260, 1929–1931.

Reproductive Biology of Crocodilians

Matthew D Hale, Savannah River Ecology Laboratory, Aiken, SC, United States; and University of Georgia, Athens, GA, United States

Jessica A Cloy-McCoy, College of Charleston, Charleston, SC, United States

Brenna M Doheny and Benjamin B Parrott, Savannah River Ecology Laboratory, Aiken, SC, United States; and University of Georgia, Athens, GA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Anamniotic Egg-laying vertebrates that do not produce an amniotic egg; fish and amphibians.

Archosaurian Belonging to the amniote lineage Archosauria, which includes dinosaurs, crocodilians, and birds; contrasts with Lepidosauria, which includes non-crocodilian reptiles (squamates).

Folliculogenesis The process of development, in which a female germ cell matures into a large haploid oocyte, surrounded by supporting granulosa and thecal cell layers.

Genital ridge Embryonic structure, precursor to the gonad.

Granulosa cell Female somatic support cell that immediately surround oocytes, responsible for estrogen synthesis and promotion of early ovarian development.

Intromittent organ Phallus or penis, used for sperm delivery in species with internal fertilization.

Lacunae Large spaces or cavities in the medullary region of the ovary of birds, crocodilians, and other reptiles.

Leydig cell Male somatic support cell in the testis, responsible for androgen synthesis.

Multiple paternity Phenomenon of a single clutch of eggs that are fertilized by sperm from more than one father.

Neonate Hatchling crocodilian.

Oocyte Female germ cell, egg.

Pod or crèche Group of hatchlings and/or young crocodilians.

Primordial germ cell (PGC) Germ cells that will migrate into the genital ridge and become either oocytes or spermatogonia.

Sertoli cell Male somatic support cell in the testis, responsible for controlling spermatogonia development and promotion of early testis development.

Spermatogonial stem cells Germ cells in males that give rise to sperm cells.

Steroidogenic Capable of producing steroid hormone(s).

Testis cord, medullary sex cord Embryonic structure in the testis that will develop into seminiferous tubules.

Theca cell Female somatic support cell that also surround oocytes, derived from granulosa cells, responsible for androgen synthesis.

Thermosensitive period Window of development during which the embryonic gonad is sensitive to temperature in sex determination; temperature cues outside this window do not effect sex.

Thermosensitivity Sensitivity to temperature in the context of sex determination during embryogenesis.

TSD Temperature-dependent sex determination, a mode of environmental sex determination where the temperature experienced during a window of embryogenesis determines sex of offspring.

Natural History of Crocodilian Reproduction

Crocodilians are globally distributed in tropical and subtropical areas, found on every continent excluding Europe and Antarctica. The International Union for Conservation of Nature recognizes 24 extant species of crocodilians within the order Crocodylia, with seven of these species listed as endangered or critically endangered. Crocodilians are spread across three families, including: Alligatoridae, which encompasses alligators and caiman; Crocodylidae, which includes all crocodiles; and Gavialidae, which includes only a single species, the gharial (*Gavialis gangeticus*) (although some lines of evidence suggest that a member of Crocodylidae, *Tomistoma schlegelii*, or the false gharial, is more closely related to the gharial than to crocodiles). Members of family Alligatoridae are found predominantly in the Americas, with the single exception being the Chinese alligator (*Alligator sinensis*), which is found exclusively in China. In contrast, members of Crocodylidae are found across the Americas, Asia, and Africa, while the single member of Gavialidae is found only on the Indian subcontinent. Despite their global distribution, all crocodilians are characterized by a shared reproductive biology and ecology that includes hierarchical social structures, complex reproductive behaviors, environmental sex determination, and Archosaurian reproductive traits. In this article we review this shared biology, discussing aspects of crocodilian nesting ecology, sex determination, and the reproductive tract. In the following section, we begin with a review of selected characteristics of crocodilian reproductive and nesting ecology, describing (1)

crocodilian mating systems and courtship and (2) nesting ecology, focusing on types of nests built by crocodilians and parental behavior during and after nesting.

Mating Dynamics

Hierarchies and male competition

Mating systems of crocodilians are characterized as polygamous male-dominated hierarchies, in which sexually mature males will compete for and establish territories, permitting access to receptive females. In this system, dominant males will typically mate with multiple females in a single year. In most crocodilians, environmental cues serve as triggers of breeding behavior. In subtropical species, increasing spring temperatures can cue reproduction, while equatorial species rely more heavily on hydrologic cycle periodicity (rainy/dry season transitions). The age and size at sexual maturity for male crocodilians varies by species, and the time required to reach maturity ranges from 10–18 years, although it can be lesser for species that reach maturity at smaller sizes. However, intrasexual competition among males promotes systems where only the largest, dominant individuals will successfully breed each year, leaving smaller males, while technically sexually mature, with few or no breeding opportunities. For example, male American alligators (*Alligator mississippiensis*) in some habitats reach maturity at 1.8 m (approximately 6 ft), but only males >2.7 m (approximately 9 ft) are expected to breed in a given reproductive season (Lance, 1989). In these competitive interactions, dominant males establish territories and preclude other males from entering and mating with females in that area. Males of some species will also actively patrol their territory during breeding season to engage competitors.

In contrast to males, female crocodilians typically reach sexual maturity at smaller sizes and most do not engage in the same intrasexual competition that would preclude younger females from breeding. Although, captive female American alligators have been reported to aggressively defend their future nest sites against other females, and captive female American crocodiles (*Crocodylus acutus*) can form female hierarchies, similar to males. Female American alligators reach sexual maturity at approximately 1.8 m, however, young females generally produce smaller clutches than older individuals. So, while females might become sexually mature at 10–16 years of age, their maximum reproductive potential may not be reached until >20 years (Lance, 1989). Additionally, not every mature female (or male) will breed each year. While the likelihood of nesting in a year increases with body size, estimates of the annual proportion of nesting alligators in Florida range from 16%–58% of adult females in a population (Lance, 1989), and females produce only a single clutch of eggs each year that they breed. However, the reproductive life span of an individual female can be quite lengthy, with long term data from American alligators suggesting that females can successfully reproduce for 30–40 years after reaching maturity (Wilkinson *et al.*, 2016).

Courtship behavior

Courtship in crocodilians involves a complex series of behaviors that can include vocal displays and responses and coordinated physical exchanges. Generally, a male will use a series of vocal displays (bellowing, head slapping, roaring) to attract receptive females to his territory, to which females may respond with their own vocalizations. Once in close physical proximity, male and female pairs engage in snout-snout and snout-neck contact and make a series of coughing exhalations. These initial displays are usually followed by a series of guided, circling swims lead by either sex, riding, additional snout contact, and bubble-blowing. If courtship is successful and neither party disengages, copulation will usually follow (Garrick and Lang, 1977). The physical act of copulation can last minutes and entails the insertion of the male penis-like intromittent organ into the cloaca of the female, permitting gamete transfer and fertilization. Crocodilians also possess two pairs of secretory glands (the gular glands on the lower jaw and the paracloacal glands in the cloaca) that produce secretions that are thought to be involved in reproduction, but the exact role of these glands and their secretions is not well understood in the context of courtship behavior, copulation, or nesting.

Multiple paternity

Despite territorial dominance and aggressive intrasexual competition among males, a mounting body of evidence supports the polygamous nature of crocodilian mating systems. Genetic analyses have uncovered clutches of eggs fertilized by multiple males (as many as four) in a number of crocodilian species, indicating the possibility that female crocodilians are also promiscuous. This multiple paternity could be driven by either 1) females mating with more than one male in a single season and using sperm from multiple mates to fertilize a single clutch of eggs; 2) females mating with a single male each season, but storing sperm from previous year(s) and using sperm from multiple years to fertilize a single clutch; or 3) both. Multiple paternity is likely a widespread phenomenon, and has been documented in the American alligator, Chinese alligator, estuarine (or saltwater) crocodile (*Crocodylus porosus*), Morelet's crocodile (*Crocodylus moreletii*), broad-snouted caiman (*Caiman latirostris*), Orinoco crocodile (*Crocodylus intermedius*), black caiman (*Melanosuchus niger*), and spectacled caiman (*Caiman crocodilus*).

Nesting Ecology

Nesting strategy

Approximately 3 weeks to one month following copulation, females lay a clutch of 20–40 eggs, although clutch size is strongly influenced by individual body size and age, as well as species. Average clutch size in American alligator in some populations can range from 40–60 eggs, with the largest clutch ever recorded containing 91 eggs (although this “single” clutch could have been

produced by two females sharing a single nest). In contrast, the African dwarf crocodile (*Osteolemus tetrapsis*) regularly produces clutches of 10 eggs or fewer. Egg size also varies with species; female gharial produce the largest eggs of any crocodilian (~160 g), while the Chinese alligator produces much smaller eggs (~50 g).

Crocodilian nesting strategy can be classified as either mound-nesters or cavity-nesters, and the choice in strategy seems to be influenced by habitat type. Species inhabiting forested riparian zones or wetlands regularly build nest mounds consisting of vegetation and soil, while species inhabiting riverine or marine systems deposit eggs in cavities excavated on sandy beaches. All species, however, choose nesting sites within close proximity to water. Mound-nesting species include the American and Chinese alligators, all caiman species, and several crocodiles, including the estuarine, New Guinea (*Crocodylus novaguineae*), and Orinoco crocodile, the false gharial and, in certain habitat types, the American and Cuban crocodile (*Crocodylus rhombifer*) (these species can build both types of nests). Hole-nesting species include the gharial, the freshwater crocodile (*Crocodylus johnstoni*) and the Nile crocodile (*Crocodylus niloticus*). Generally, members of family Crocodylidae display greater diversity in nesting strategy, as both mound and cavity nesting is found in crocodiles. In comparison, all members of Alligatoridae are mound nesters and the single member of Gavialidae, the gharial, is a hole nester.

Temperature and nesting

The temperature eggs experience during incubation is critically important to crocodilians, as all species studied to date exhibit temperature-dependent sex determination (discussed in greater detail in [Section The Crocodilian Sex Determination System](#)). In this system, sex of the developing embryo is determined by temperature, rather than by genetic factors or sex chromosomes. Both cool and warm temperatures drive offspring to develop as females, while intermediate temperatures promote the development of males. The exact range of temperature promoting development of the different sexes varies subtly from species to species, and exact thermal ranges are not known for all crocodilians. Temperature also influences the time required for eggs to hatch, as warmer nests will promote faster growth and development of embryos. Eggs typically require anywhere from 60–70 days to hatch once laid, although this varies among species and may require as long as 90–115 days.

Adult behavior during nesting

During the incubation period, females of many crocodilian species will remain in close proximity to the nest, both guarding it from predators and waiting to excavate hatchlings. Female American alligators will frequently remain in nearby ponds or holes and advance upon intruders in defensive displays that include open-mouthed charging and hissing vocalizations. Some female crocodilians will also regularly attend the nest, climbing on top and crossing over it several times and will repair the nest if damaged or disturbed by predators. Nest defense and attendance varies within and among species, and the purpose of the latter (beyond protection from predators and excavation of young) is not fully understood. Despite nest guarding, predation of nests is still a common occurrence in most crocodilian species, and both mammals and reptiles represent a persistent threat to nesting success. Flooding presents an additional natural threat to crocodilian nests, however habitat loss or destruction due to anthropogenic activity likely presents a much greater existential threat to crocodilian nesting than flooding or predators.

At the conclusion of the incubation period and, in response to collective vocalizations from hatchlings, females of most species will excavate nests with their mouths and forelimbs to assist hatchlings in exiting the nest. And, in some cases, the maternal female will grasp unhatched or partially hatched eggs in her jaws and assist in hatching by gently manipulating and cracking eggs with her teeth. In addition, the female will often carry hatchlings in her mouth to nearby water. Like nest defense and attendance, these behaviors vary within and among species, but some form of nest attendance or care is likely uniform to all crocodilians. Once hatching is complete, neonates will frequently remain in groups, termed pods, near the nest site for one year or more, staying in close proximity to maternal female for protection. Adult care for neonates is most commonly exhibited by the laying female, however, adult male Gharial will frequently guard large groups (100–1000s), called creches, of mixed-clutch and mixed-year neonates and juveniles, guarding them for many months after hatching occurs. This behavior is not frequently observed in adult males of other species, however, and cannibalism of hatchlings and juveniles by adults is common.

The Crocodilian Sex Determination System

All crocodilian species studied to date display a version of environmental sex determination that is regulated by the temperature that embryos experience during development. The key aspects of the crocodilian temperature dependent sex determination (TSD) system are reviewed below and include: (1) developmental timing of thermosensitivity, (2) the crocodilian TSD molecular network and (3) the influence of hormones on TSD ([Fig. 1](#)).

The Developmental Timing of Thermosensitivity

Ferguson and Joanen first described TSD in crocodilians in 1982 and demonstrated the adjustable nature of the alligator sex determination system ([Ferguson and Joanen, 1982](#)). This study along with others established the pattern of TSD in the American alligator (*Alligator mississippiensis*) along a gradient of increasing temperatures as female-male-female, with females being produced at extremes of the viable temperature range (below 31°C and above 34°C) and males produced at intermediate temperatures (32°C) ([Fig. 2\(A\)](#)). Mixed sex ratios are expected from temperatures between the male and female producing temperatures. Variation in



Fig. 1 Comparison of mound and hole nesting in *A. mississippiensis* and *C. acutus*. Representative images of an unopened (A) and opened (C) mound nests of *A. mississippiensis* in coastal South Carolina (United States). Nests are built from surrounding vegetation and soil, and eggs are deposited in the center of the mound, covered, and left to develop. For comparison, images of an unopened (covered in a mesh screen; B) and opened (D) hole nests of *C. acutus* from the Tempisque Basin (Costa Rica), where the maternal female excavates a cavity in the soil, deposits, and then covers her eggs. Photos (B, D) Courtesy of Chris Murray (Tennessee Technological University), (C) courtesy of Thomas Rainwater (Clemson University).

the sex ratios amongst different clutches incubated at identical non-extreme temperatures indicates that relative clutch effects also contribute to the equation that dictates how temperature impacts crocodilian sex determination (Lang and Andrews, 1994). The specific period of thermosensitivity in all reptiles displaying TSD is thought to encompass the embryonic stages during which the developmental trajectory of the bipotential gonad splits resulting in either a testis or an ovary. After the sexual differentiation of the gonad progresses beyond a specific developmental threshold, the sex of that individual is irreversibly established and the thermosensitive window is permanently closed. The specific period and associated developmental stages that characterize the opening of the thermosensitive window are not as clearly defined, likely a result of the practical variation in experimental techniques and the criteria used to define the window of sensitivity to incubation temperatures.

Knowledge regarding the temporal and developmental features of thermosensitivity originates primarily from detailed studies in the American alligator and involves thermal shift experimental designs. These design schemes incorporate the incubation of embryos at male- and female- producing temperatures (MPT and FPT, respectively). Single shift designs involve moving eggs from one temperature to the other at different developmental stages and are often used to broadly identify the pattern (FME, MF, etc.) and the temperature sensitive period (TSP). Double shift designs allow for examination of how sex ratios are affected by temporary pulses of a MPT against the background of a lower FPT and vice versa during different developmental periods. In 1994, Lang and Andrews applied both single- and double-temperature shift experiments to refine the period of thermosensitivity in *A. mississippiensis* and demonstrated that irrespective of incubation temperature, a discrete thermosensitive period that occurs from embryonic stage 21–24 (approximately 30–45 days of incubation) and encompasses the period of gonadal differentiation (Lang and Andrews, 1994). The period of development between embryonic stages 21 and 24 has served as the canonical period

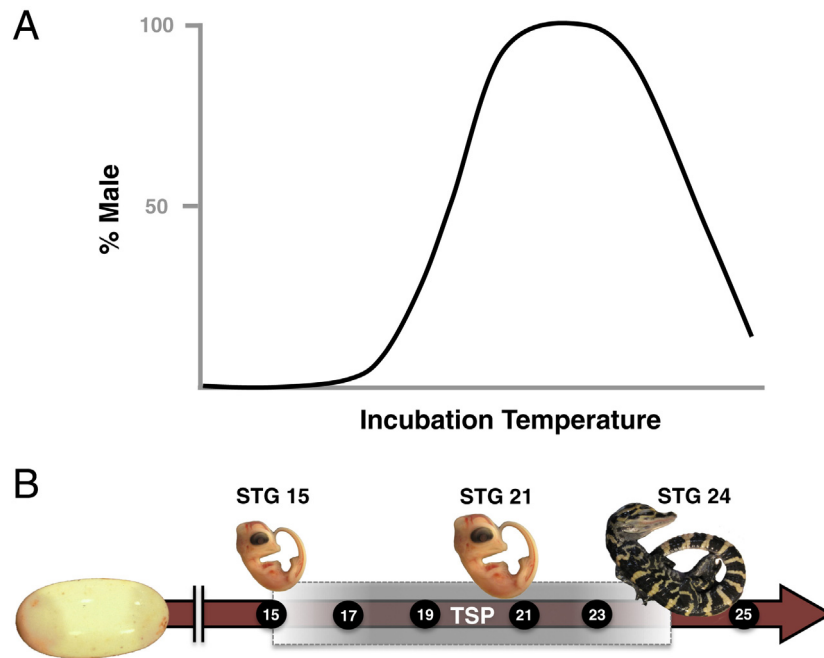


Fig. 2 The thermosensitive period of sex determination in crocodilians. (A) Several crocodilian species display the female-male-female (FMF) pattern of sex determination. Extreme temperatures (below 32°C and above 34°C) produce primarily females and intermediate temperatures (between 32 and 34°C) produce primarily males. (B) The thermosensitive window of sex determination in crocodilians initiates as early as stage 15 of embryonic development and persists until the period of gonadal differentiation (stage 24).

of thermosensitivity in the American alligator and numerous studies have incorporated this specific period into designs aimed at characterizing the origins and mechanisms of temperature dependent sex determination. Subsequent thermal shift experiments, however, have challenged the discrete nature of this window and show that the thermosensitive period for the American alligator initiates by embryonic stage 15 of development, six stages prior than previously reported (McCoy *et al.*, 2015) (Fig. 2(B)). This finding further highlights the context dependent nature of the temperature dependent sex determination system in crocodilians.

The Crocodilian TSD Molecular Network

The morphological features of the adult testis and ovary display a remarkable degree of conservation across amniotes. This observed morphological conservation has prompted targeted investigations aimed at elucidating the molecular cascade that translates temperature into sex. Many of the key regulatory molecules involved in mammalian sex determination also appear to regulate sex determination in crocodilians. Sex determining region Y (SRY)-box 9 (SOX9) mRNA is expressed in alligator embryos and its abundance increases in gonads incubated at the MPT. SOX9 is a conserved transcription factor that promotes Sertoli cell differentiation and testis development. In mammals, SOX9 is directly downstream of the male determining factor, SRY. Reptiles and other non-mammals, however, lack SRY and the precise mechanisms by which SOX9 is regulated in these species remain elusive. Temperature-induced alternative splicing is involved in Indian mugger (*Crocodilus palustris*) gonadal sex determination and results in a female version of the Sox9 transcript that is not translated into a functional protein, thereby limiting its ability to induce testis development (Agrawal *et al.*, 2009). Further investigations are needed to determine how prevalent this mechanism is across other crocodilian species. Anti-Müllerian hormone (AMH), another key regulator of testis differentiation, is also expressed in alligator during the thermosensitive period. The regulatory mechanisms that govern the expression of AMH during testis development remain unclear for American alligator *in ovo*, although SOX9 can induce AMH transcription *in vitro*.

Conserved genes involved in ovarian differentiation have also been identified in crocodilians including aromatase (*CYP19A1*), forkhead box L2 (*FOXL2*), and R-spondin 1 (*RSPO1*). The sequencing and assembly of the American alligator genome has helped to overcome many of the technical challenges associated with genetic studies of non-traditional model organisms and will provide additional insights into the puzzle of temperature dependent sex determination.

The Influence of Hormones on Crocodilian TSD

It is evident that hormones also play central roles in promoting ovarian differentiation in TSD. In cases where estradiol is applied exogenously to eggs incubated at MPT, the process of testis differentiation is completely overridden and females are

produced. In the American alligator, estrogen activation of estrogen receptor- α (ESR1) appears to be sufficient, but not necessary for instigating the most upstream steps of ovarian development based in part on the expression profile of aromatase, the catalytic enzyme responsible for estrogen production, which is not detected until after the first morphological signs of gonadal differentiation occur (Parrott *et al.*, 2014). Many persistent contaminants found in the environment are categorized as endocrine disrupting chemicals (EDCs) due to their ability to impact the endogenous endocrine system. Exposure to EDCs during hormone-sensitive developmental windows can promote permanent alterations in reproductive system function. The nature of the crocodilian sex determination system makes it inherently sensitive to environmental factors (temperature, humidity, contaminants, etc.). This feature, along with other key aspects of crocodilian life history, warrant these species as exceptional wildlife sentinels for environmental health.

The Crocodilian Reproductive System

Gonadogenesis

As in other vertebrates, the gonads of crocodilians originate as a genital ridge, develop into bipotential gonads, and after sex determination differentiate into testes in males and ovaries in females. Primordial germ cell (PGC) specification and migration has not yet been observed in crocodilians due to experimental difficulties, but based on their shared Archosaurian lineage they are expected to follow the patterns seen in birds; namely, PGC specification follows the predetermined mode and the cells migrate through the bloodstream to arrive in the genital ridge at Ferguson stage 2 of development (Kohn *et al.*, 2014).

In *Alligator mississippiensis*, gonads develop in complex with the adrenal gland and the embryonic kidney (gonadal-adrenal-mesonephros complex, or GAM). The germinal epithelium and underlying medullary sex cords are observable by stage 12, and by stage 20 the bipotential gonads exhibit a distinct cortex containing PGCs. The earliest signs of gonadal sexual differentiation occur during the thermosensitive window of TSD, with observable presumptive Sertoli cells at stage 21 in the gonads of embryos incubated at the MPT, and germ cell proliferation in the gonadal cortex at stage 22 in embryos incubated at the FPT (Smith and Joss, 1993).

Sexually dimorphic gonadal differentiation continues throughout the rest of embryonic development. In the testes, the germinal epithelium diminishes and germ cells disperse into the medulla, where seminiferous cords become more organized, with well-defined Sertoli cells (Smith and Joss, 1993). In contrast, the ovarian cortex becomes more defined, characterized by proliferating germ cells, while lacunae form in the fragmenting medulla (Smith and Joss, 1993). Oocytes develop in the cortex, enclosed by granulosa cells and steroidogenic theca cells. The caudal portion of the ovary, known as the medullary rest, does not follow this pattern but instead exhibits the dense arrangement of medullary sex cords seen in the testis (Forbes, 1940).

Adult Gonadal Structure and Function

Full maturation of the gonads of crocodilians, as with birds, continues post-hatching. Unlike birds, however, crocodilian ovaries maintain a reserve of cortical oogonial nests rather than converting all oogonia into primary oocytes during gonadal maturation, and thus can produce new follicles throughout their lifetime (Kohn *et al.*, 2014). The lacunae that develop in the medullary stroma are characterized by a framework of collagen and smooth muscle, and may function to facilitate follicular expansion. The signaling processes guiding folliculogenesis in crocodilians have not yet been fully elucidated, but developmental exposure to environmental estrogens disrupts the process, resulting in multi-oocytic follicles (Guillette *et al.*, 1994).

The adult crocodilian testis exhibits the tubular morphology characteristic of amniotes, with highly convoluted seminiferous tubules lined with an epithelium of Sertoli cells. The testosterone-generating Leydig cells of the testis localize around cord structures and mature sperm are typically replenished throughout reproductive life due to the persistence of mitotic spermatogonial stem cells. In *A. mississippiensis*, spermatogenesis begins in early spring, and germ cells mature in a single cohort to produce one continuous spermiation event during the breeding season from May-June, which differs from the consistent spatial developmental pattern of spermatogenesis observed in other amniotes. This temporal pattern is reminiscent of spermatogenesis in anamniotic amphibians, but was also documented in two other seasonally breeding reptile species, the slider turtle (*Trachemys scripta*) and the European wall lizard (*Podarcis muralis*), suggesting that the testes of crocodilians and these other reptiles are transitional between the anamniotic and amniotic testis (Gribbins *et al.*, 2006).

Adult Gonadal Structure and Function

Full maturation of the gonads of crocodilians, as with birds, continues post-hatching. Unlike birds, however, crocodilian ovaries maintain a reserve of cortical oogonial nests rather than converting all oogonia into primary oocytes during gonadal maturation, and thus can produce new follicles throughout their lifetime (Kohn *et al.*, 2014). The lacunae that develop in the medullary stroma are characterized by a framework of collagen and smooth muscle, and may function to facilitate follicular expansion. The signaling processes guiding folliculogenesis in crocodilians have not yet been fully elucidated, but developmental exposure to environmental estrogens disrupts the process, resulting in multi-oocytic follicles (Guillette *et al.*, 1994).

The adult crocodilian testis exhibits the tubular morphology characteristic of amniotes, with highly convoluted seminiferous tubules lined with an epithelium of Sertoli cells. The testosterone-generating Leydig cells of the testis localize around cord structures

and mature sperm are typically replenished throughout reproductive life due to the persistence of spermatogonial stem cells. In *A. mississippiensis*, spermatogenesis begins in early spring, and germ cells mature in a single cohort to produce one continuous spermiation event during the breeding season from May–June, which differs from the consistent spatial developmental pattern of spermatogenesis observed in other amniotes, including birds. This temporal pattern is reminiscent of spermatogenesis in anamniotic amphibians, but was also documented in two other seasonally breeding reptile species, the slider turtle (*Trachemys scripta*) and the European wall lizard (*Podarcis muralis*), suggesting that the testes of crocodilians and these other reptiles are transitional between the anamniotic and amniotic testis (Gribbins *et al.*, 2006).

Reproductive Tract Structure and Function

The crocodilian female reproductive tract, or oviduct, is highly functionally and structurally regionalized. Whereas the oviducts of other reptiles produce both the fibrous proteins that form the eggshell membrane and the outer calcareous layer of the eggshell in one uterine region sequentially, crocodilians and other Archosaurs have two spatially distinct regions, complete with distinct glandular structures (Palmer and Guillette, 1992). Thus crocodilian eggs can be packaged in an “assembly line” fashion, an evolutionary adaptation that has been seen as an innovation allowing for flight in birds, although birds process eggs through their oviducts one at a time, whereas crocodilians process an entire clutch at once.

When reproductively active, the oviduct is highly responsive to estrogen stimulation, undergoing dramatic increases in size and extensive glandular development for producing the necessary secretory products for proper egg packaging (Palmer and Guillette, 1992). As in other vertebrates, the oviducts originate from the embryonic Müllerian ducts. After gonadal sex determination, Müllerian ducts regress in males due to the influence of AMH, but they are fully retained in females, and unlike in birds, both mature into functional adult oviducts.

In contrast to female reproductive structures and function, the male reproductive tract has not been well studied in crocodilians. However, male extra-gonadal reproductive structures are generally similar to mammals and birds, with the ductus deferens linking the testes to the phallus for gamete transfer (although the crocodilian phallus is distinct from both mammalian and avian structures). The crocodilian ductus deferens serves to collect, store, and deliver sperm from the epididymis and seminiferous tubules within the testes during breeding season. Crocodilians appear to lack a dedicated accessory secretory gland for production of seminal fluid. However, evidence is beginning to emerge that multiple regions of the male reproductive tract may contribute to seminal secretions. In spectacled caiman, the morphological and histochemical properties of the epididymis and ductus deferens suggest its involvement in the production and modification of seminal fluid, and structural characteristics at the gross morphological, cellular, and ultrastructural levels are highly similar to birds, more so than to other reptiles, supporting the Archosaurian lineage of crocodilians. (Guerrero *et al.*, 2004).

These findings in caiman have been corroborated by recent work in the American alligator that have reported the presence of exocrine glands in the male phallus that likely contribute secretions during reproduction to form seminal fluid. The crocodilian phallus is itself a highly specialized and complex structure, characterized by three distinct morphological regions: the tip, cuff, and base, and an open groove, termed the sulcus or penile groove, for sperm delivery. During copulation, it is likely that increased blood flow to vascular regions surrounding the sulcus close it into a complete tube, while coordinated smooth muscle contractions promote sperm movement into the female reproductive tract (Moore *et al.*, 2012). Unlike mammalian or avian penile structures, most of the crocodilian phallus is rigid, containing dense collagenous regions, and only a small region is erectile. The phallus is also retained within the cloaca until copulation occurs, when it is everted for intromission into the female (Moore and Kelly, 2015).

References

- Agrawal, R., Wessely, O., Anand, A., Singh, L., & Aggarwal, R. K. (2009). Male-specific expression of Sox9 during gonad development of crocodile and mouse is mediated by alternative splicing of its proline-glutamine-alanine rich domain. *The FEBS Journal*, 276, 4184–4196.
- Ferguson, M. W. J., & Joanen, T. (1982). Temperature of egg incubation determines sex in *Alligator mississippiensis*. *Nature*, 296, 850–852.
- Forbes, T. R. (1940). Studies on the reproductive system of the alligator. VI. Further observations on heterosexual structures in the female alligator. *Anatomical Record*, 77, 343–365.
- Garrick, L. D., & Lang, J. W. (1977). Social signals and behaviors in adult alligators and crocodiles. *American Zoologist*, 17, 225–239.
- Gribbins, K. M., *et al.* (2006). Cytological evaluation of the germ cell development strategy within the testis of the American alligator, *Alligator mississippiensis*. *Acta Zoologica*, 87(1), 59–69.
- Guerrero, S. M., *et al.* (2004). Morphology of the male reproductive duct system of *Caiman crocodilus* (Crocodylia, Alligatoridae). *Annals of Anatomy*, 186(3), 235–245.
- Guillette, L. J., Gross, T. S., JR, Masson, G. R., *et al.* (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environmental Health Perspectives*, 102, 680–688.
- Kohn, S., Parrott, B. B., Yatsu, R., *et al.* (2014). Gonadal differentiation in reptiles exhibiting environmental sex determination. *Sexual Development*, 8, 208–226.
- Lance, V. A. (1989). Reproductive cycle of the American alligator. *American Zoologist*, 29(3), 999–1018.
- Lang, J. W., & Andrews, H. V. (1994). Temperature-dependent sex determination in crocodilians. *Journal of Experimental Zoology*, 270, 28–44.
- Mccoy, J. A., Parrott, B. B., Rainwater, T. R., Wilkinson, P. M., & Guillette, L. J., JR (2015). Incubation history prior to the canonical thermosensitive period determines sex in the American alligator. *Reproduction*, 150, 279–287.
- Moore, B. C., & Kelly, D. A. (2015). Histological investigation of the adult alligator phallic sulcus. *South American Journal of Herpetology*, 10(1), 32–40. Available at: <http://www.bioone.org/doi/10.2994/SAJH-D-14-00037.1>.

- Moore, B. C., Mathavan, K., & Guillette, L. J. (2012). Morphology and histochemistry of juvenile male American alligator (*Alligator mississippiensis*) phallus. *Anatomical Record*, 295(2), 328–337.
- Palmer, B. D., & Guillette, L. J., JR (1992). Alligators provide evidence for the evolution of an archosaurian mode of oviparity. *Biology of Reproduction*, 46, 39–47.
- Parrott, B. B., Kohno, S., Cloy-McCoy, J. A., & Guillette, L. J., JR (2014). Differential incubation temperatures result in dimorphic DNA methylation patterning of the SOX9 and aromatase promoters in gonads of alligator (*Alligator mississippiensis*) embryos. *Biology of Reproduction*, 90, 2.
- Smith, C. A., & Joss, J. M. P. (1993). Gonadal sex differentiation in *Alligator mississippiensis*, a species with temperature-dependent sex determination. *Cell Tissue Research*, 273, 149–162.
- Wilkinson, P. M., et al. (2016). Determinate growth and reproductive lifespan in the American alligator (*Alligator mississippiensis*): Evidence from long-term recaptures. *Copeia*, 104(4), 843–852. Available at: <http://www.bioone.org/doi/10.1643/CH-16-430>.

Elephants

Janine L Brown, Smithsonian Conservation Biology Institute, Front Royal, VA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Elephants are important ambassadors for global conservation: they are keystone species that have a major impact on ecosystems; they are umbrella species that indirectly protect other species in its habitat; and as iconic flagship species, they raise awareness for action and funding of broader conservation efforts. Despite their importance, however, wild elephants are threatened (African) or endangered (Asian) throughout their natural ranges because of habitat destruction, human-elephant conflict, and poaching for ivory, skin and other products. The future of both species ultimately depends on whether humans and elephants can learn to coexist peacefully in the same environment, although effective solutions have so far remained elusive. As wild elephant numbers decline, captive breeding may be viewed as a means to maintain important populations as “insurance” against environmental or anthropomorphic catastrophe. So long as they are self-sustaining, and not replenished by wild capture, *ex situ* populations also offer opportunities to study elephant biology in ways that are not possible in the wild, and provide information that can aid *in situ* conservation.

Several aspects of female elephant reproduction differ from other mammals, including luteal steroidogenic activity, pituitary gonadotropin secretion, fetal development and reproductive tract morphology, while males exhibit the unique phenomenon of musth and an unusual reproductive anatomy (internal testes, ampullary semen storage). The finding of notable species differences in biological mechanisms (e.g., prolactin secretion, gestational steroid patterns, sperm cryosensitivity) and problems associated with uterine pathologies and ovarian acyclicity means that inferences from one species to another should be made cautiously.

Female Reproduction

General Reproductive Biology

Elephants are polyestrous, and exhibit the longest spontaneous cycle of any mammal: 13–17 weeks in duration, with a 4- to 7-week follicular phase, and an 8- to 10-week luteal phase (see review, Brown (2014), Fig. 1). Unlike most mammals, the major

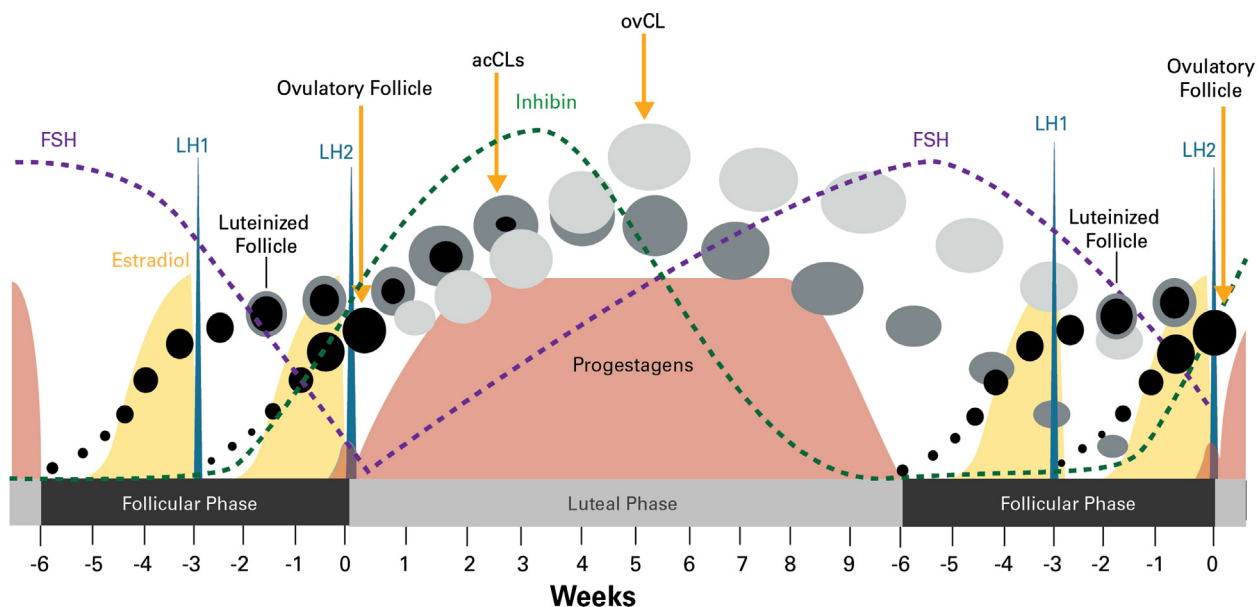


Fig. 1 Model of the elephant ovarian cycle, showing relationships among the secretion of estradiol, progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin and inhibin, and development of follicles and corpora lutea (CL), both ovulatory (ovCL) and accessory (acCL). Adapted from Brown, J.L., 2000. Reproductive endocrine monitoring of elephants: An essential tool for assisting captive management. *Zoo Biol* 19, 347–367; Brown, J.L., 2014. Comparative reproductive biology of elephants. In: Holt, W.V., Brown, J.L., Comizzoli, P. (Eds), *Reproductive Sciences in Animal Conservation – Progress and Prospects*. Advances in Experimental Medicine and Biology. New York, NY: Springer Science and Business Media, pp. 135–169; Hildebrandt, T.B., Göritz, F., Hermes, R., *et al.*, 2006. Aspects of the reproductive biology and breeding management of Asian and African elephants. *Int Zoo Yrbk* 40, 20–40; Lueders, I., Taya, K., Watanabe, G., *et al.*, 2011. Role of the double luteinizing hormone peak, luteinizing follicles, and the secretion of inhibin for dominant follicle selection in Asian elephants (*Elephas maximus*). *Biol Reprod* 85, 714–720; Lueders, I., Niemüller, C., Rich, P., *et al.*, 2012. Gestation for 22 months: Luteal development and pregnancy maintenance in elephants. *Proc Royal Soc B* 279, 3687–3696; Lueders, I., Hildebrandt, T.B., Gray, C., *et al.*, 2014. Suppression of testicular function in a male Asian elephant (*Elephas maximus*) treated with gonadotropin-releasing hormone vaccines. *J Zoo Wildl Med* 43, 611–619.

steroid produced by the corpus luteum (CL) is not progesterone, but 5α -reduced pregnanes (e.g., 5α -pregnane-3,20-dione, 5α -pregnane-3-ol-20-one, 17α -hydroxyprogesterone), herein referred to as 'progestagens.' Estradiol is low in circulation due to rapid conjugation and urinary excretion (Czekala *et al.*, 2003), and thus is of limited use for characterizing follicular cycles. While elephants are easily trained for blood collection to track ovarian activity, some aspects also can be assessed noninvasively; for example, analysis of progestagens in saliva, feces and urine, and estrogen conjugates in urine (Wasser *et al.*, 1996; Czekala *et al.*, 2003). Females reach puberty between 10 and 14 years of age in the wild, but can mature earlier in captivity (Sukumar, 2003; Glaeser *et al.*, 2012), perhaps because of improved nutrition. It is not uncommon for Asian elephants to begin cycling as young as 5 years of age, with the first pubertal rise in progestagens being quantitatively and qualitatively similar to subsequent cycles (Glaeser *et al.*, 2012; Fig. 2).

Elephants also have the longest gestation period of any mammal, lasting 20–23 months on average (Figs. 2 and 3), and typically give birth to a single offspring. Calves weight 90–120 kg at birth, with males generally being heavier. Lactational anestrus lasts for 8–12 months, and the inter-birth interval ranges between 4 and 6 years, depending upon resource availability. Calves nurse for up to 5 years, so females can be pregnant and lactating at the same time. Elephants are not obligatory seasonal breeders; however, conception rates often are higher during or shortly after peak rainfall in seasonal regions, and can be negatively affected by drought (Sukumar, 2003; Wittemyer *et al.*, 2007). Elephants are capable of reproducing into their 50's, although conception rates decline with age and the inter-birth intervals lengthen. There is no evidence that elephants experience a true 'menopause' like that in humans (Lahdenperä *et al.*, 2014).

Anatomy

The elephant has the longest reproductive tract of any land mammal, approximately 3 m from vulva to ovary, and has been well-described (Hildebrandt *et al.*, 2000, 2003, 2006, 2011, 2012a; Fig. 4). Unusual features of the female urogenital tract are: (1) a long vestibule that averages 1.3 m in length and opens ventrally between the hind legs; (2) a permanent hymenal membrane in nulliparous females with a small vaginal os (4 mm×2 mm in diameter) that ruptures only during parturition; and (3) two blind pouches of similar size on either side of the vaginal opening. The vagina is up to 50 m in length, consists of longitudinal folds, and produces mucus during estrus. The mix of excreted urine and mucus contains pheromones that attract bulls to estrous females. The cervix is about 10 cm long and has a prominent cone-shaped opening. Semen is deposited into the vagina through the vaginal os, often resulting in retrograde semen loss after mating. After 8 weeks of gestation, a thick vaginal mucus plug is formed that provides

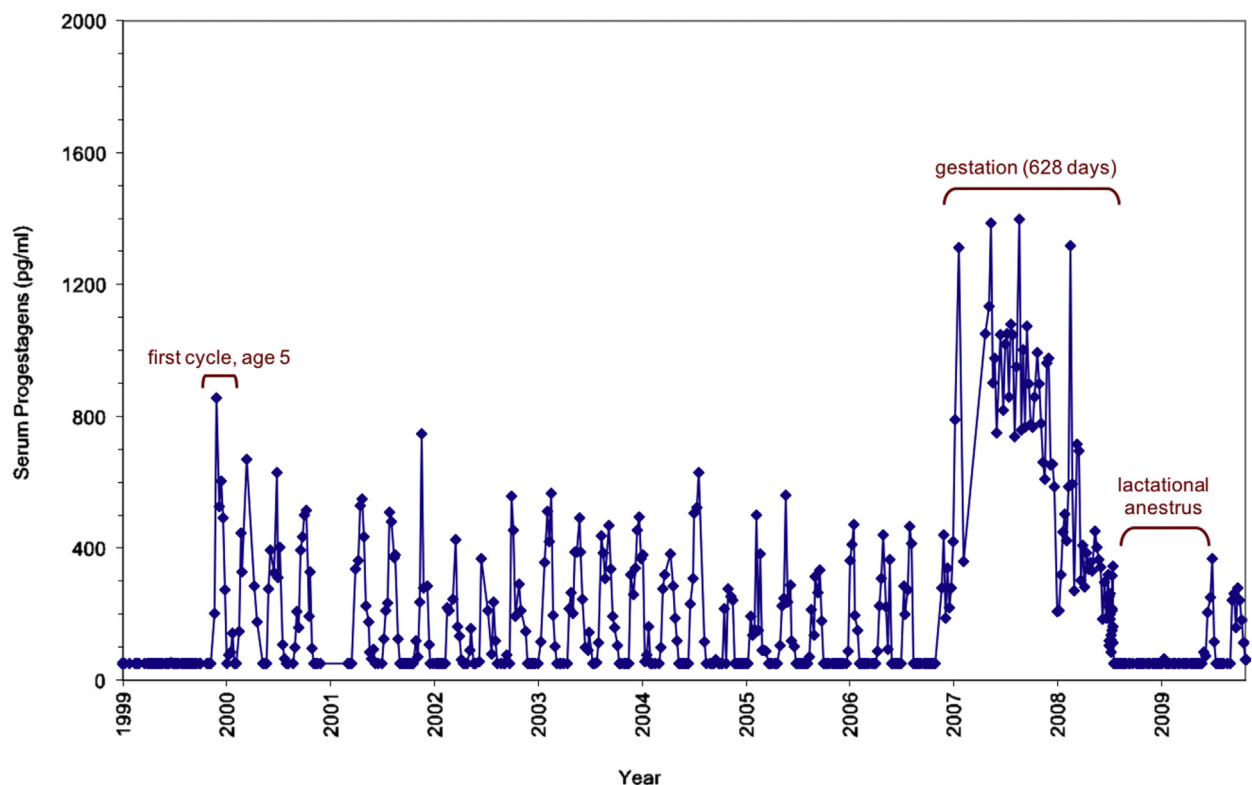


Fig. 2 Longitudinal progestagen profile in a female Asian elephant, demonstrating initiation of puberty, continuous ovarian cyclicity, gestation and lactational anestrus. Adapted from Glaeser, S.S., Martin, M.S., Hunt, K.E., Finnegan, M., Brown, J.L., 2012. Investigation of individual and group variability in estrous cycle characteristics in female Asian elephants (*Elephas maximus*) at the Oregon Zoo. *Theriogenology* 78, 285–296 with permission.

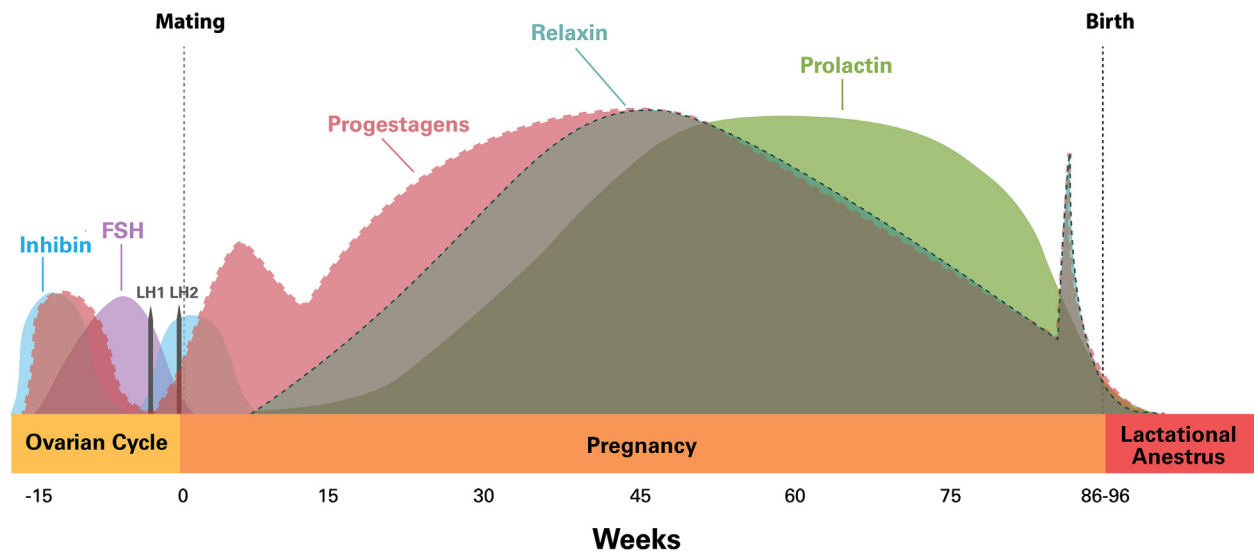


Fig. 3 Model of the relationships among the secretion of progesterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin, relaxin, and inhibin during gestation and a prior estrous cycle in the female elephant. Adapted from Meyer, J.M., Walker, S.L., Freeman, E.W., Steinetz, B.G., Brown, J.L., 2004. Species and fetal gender effects on the endocrinology of pregnancy in elephants. *Gen Comp Endocrinol* 138, 263–270; Yamamoto, Y., Yuto, N., Yamamoto, T., *et al.*, 2012a. Secretory pattern of inhibin during the estrous cycle and pregnancy in African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants. *Zoo Biol* 31, 511–522; and Lueders, I., Niemuller, C., Rich, P., *et al.*, 2012. Gestation for 22 months: Luteal development and pregnancy maintenance in elephants. *Proc Royal Soc B* 279, 3687–3696.

a protective barrier against infective agents. The uterus is bicornuate, with a short body (<10 cm) and long uterine horns (>60 cm). Implantation takes place in one of the uterine horns. On the rare occasion of twins, fetuses are found in each horn. The oviducts are ~10 cm long. Paired ovaries, located behind the kidneys, are proportionally small (5.5–6.5 cm).

Ovarian Cycle

Most of our knowledge about the female elephant ovarian cycle is based on hormonal and ultrasound assessments (Lueders *et al.*, 2010, 2011; Brown, 2014). A model of the ovarian cycle is presented in Fig. 1. A distinguishing feature is the “double LH surge” that is observed in both species: two precisely timed luteinizing hormone (LH) surges associated with follicular waves that occur 3 weeks (18–22 days) apart during the follicular phase, referred to as LH1 (anovulatory) and LH2 (ovulatory) (African: Kapustin *et al.*, 1996; Asian: Brown *et al.*, 1999). The surges are quantitatively and qualitatively similar, and generally are more distinct in Asian elephants. During the first wave, multiple follicles develop, but none ovulate (Hermes *et al.*, 2000). Instead they luteinize and become accessory CL (acCL) (Lueders *et al.*, 2010). During the second wave, a new group of follicles develop, but only one becomes dominant, ovulates and forms a single, ovulatory CL (ovCL). All CLs are present throughout the follicular phase, with some of the larger ones remaining in subsequent luteal periods (Lueders *et al.*, 2010). Formation of multiple CLs in addition to the ovulatory CL may be a mechanism to ensure there is sufficient luteal capacity for sustaining a long gestation should conception occur.

Inhibin follows a distinct pattern in elephants (Brown *et al.*, 1991; Kaewmanee *et al.*, 2011a; Lueders *et al.*, 2011; Yamamoto *et al.*, 2012a). In other species, both small and large estrogenic follicles are significant sources of inhibin. However, in the elephant, circulating inhibin does not increase until after follicle luteinization post LH1, about 9 days before LH2 (Fig. 1), suggesting it is the cells of luteinized follicles and acCLs that are the main source of inhibin. Inhibin also is increased during the luteal phase; immunohistochemical staining has localized inhibin subunits to the lutein cells of CLs, similar to that in primates (Yamamoto *et al.*, 1991, 1992). Follicle-stimulating hormone (FSH) secretion is inversely related to inhibin throughout the cycle in both elephant species (Brown *et al.*, 1991, 1999; Kaewmanee *et al.*, 2011a; Fig. 1). It is highest toward the end of the luteal phase and throughout the first follicular wave when inhibin is low, then decreases to nadir concentrations before LH2 as inhibin is rising. The FSH pattern in elephants, albeit more protracted, is similar to that of the horse, with high concentrations at the end of the luteal phase that decline progressively towards ovulation (Brown *et al.*, 1991, 1999; Kaewmanee *et al.*, 2011a). This prolonged FSH stimulation may be necessary to induce the two successive follicular waves. In the face of declining FSH, the transition of one follicle from an FSH-dependent to an independent state likely is needed for dominant follicle selection, as described in other species.

Prolactin is folliculogenic in many species (Freeman *et al.*, 2000; Frasier and Gibori, 2003), and in African elephants is elevated during the follicular phase of the cycle (Yamamoto *et al.*, 2010; Dow and Brown, 2012; Fig. 5). Interestingly, no secretory prolactin pattern is observed across the estrous cycle in Asian elephants (Brown *et al.*, 2004a). As discussed later, this species difference may explain why ovarian cycle problems related to abnormal prolactin secretion are common in African, but not Asian elephants. Recently, cortisol has been shown to exhibit a distinct pattern in both species, with concentrations being higher during the follicular phase (Fanson *et al.*, 2014), the importance of which is unknown.

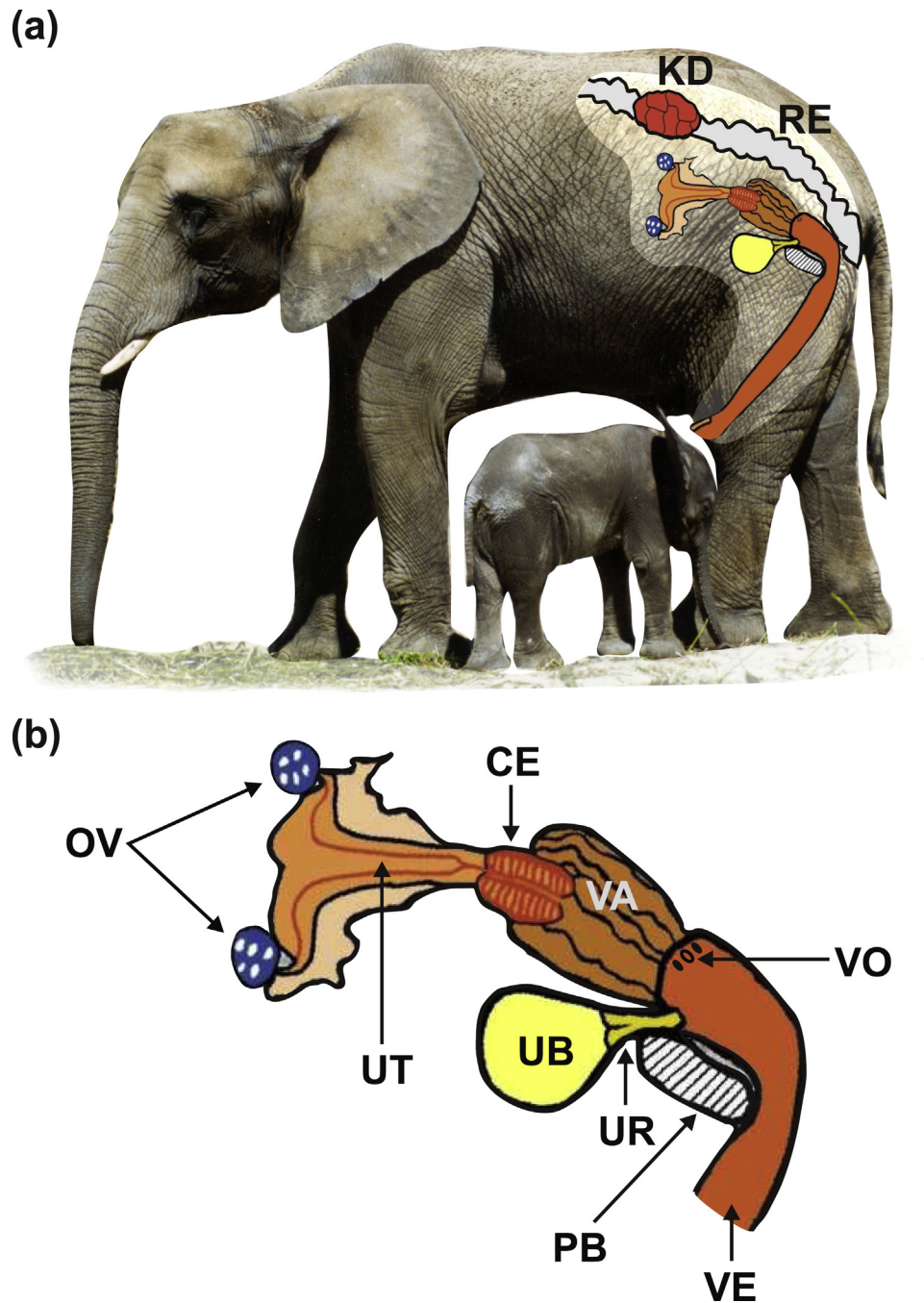


Fig. 4 Schematic diagram of the reproductive tract of the female elephant: (a) KD, lobulated kidney; RE, rectum. (b) OV, ovaries; UT, uterus bicornis; CE, cervix; VA, vagina; VO, vaginal os with two bilateral blind pouches; VE, vestibule; UB, urinary bladder; UR, urethra; PB, pelvic bone. Reprinted with permission from Hildebrandt, T.B., Göritz, F., Hermes, R., *et al.*, 2006. Aspects of the reproductive biology and breeding management of Asian and African elephants. *Int Zoo Yrbk* 40, 20–40.

Both species advertise impending fertility, albeit through different chemical signals. Female Asian elephants excrete a urinary signal, (Z)-7-dodecenyl acetate (Z7-12: Ac) that stimulates male breeding behavior, the concentrations of which increase during the follicular phase and peak just before ovulation (Rasmussen *et al.*, 1997; Rasmussen, 2001). African elephants do not produce Z7-12: Ac, but excrete frontalin, exo-brevicomin, endo-brevicomin, (E,E)-alpha-farnesene and (E)-beta-farnesene in urine leading up to and during estrus (Goodwin *et al.*, 2006). Considering the long distances bulls travel in search of estrous females in the wild, a prolonged pattern of pheromonal signaling would be a beneficial strategy for species survival.

Pregnancy

Endocrinology

In the elephant, acCLs are produced throughout the follicular phase of preceding cycles (Lueders *et al.*, 2010, 2011), but there are no additional CLs produced during gestation (Lueders *et al.*, 2012). Based on histology and analysis of luteal progestagens, the corpus

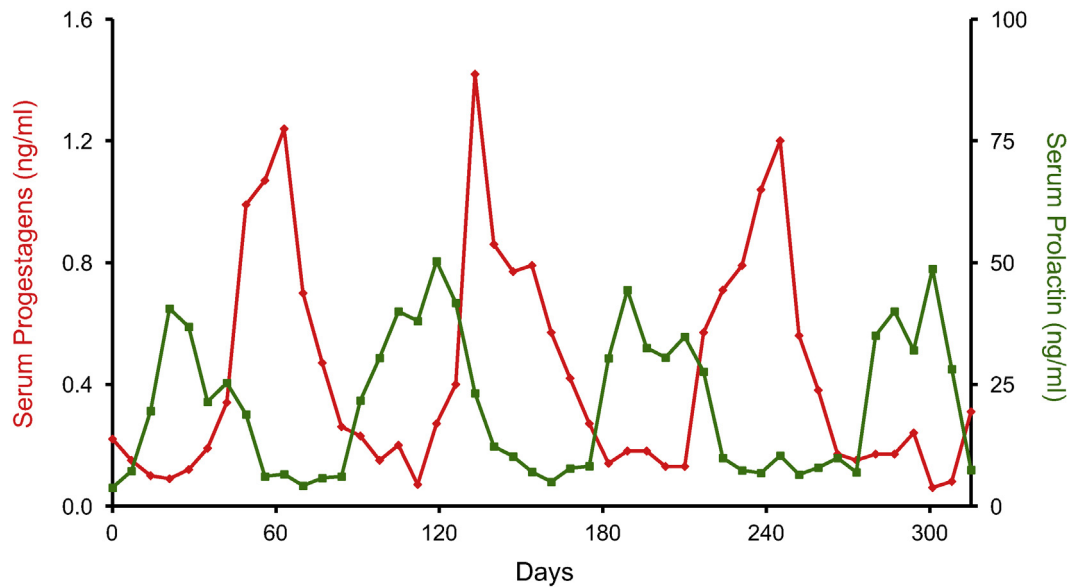


Fig. 5 Longitudinal profiles showing the relationship between progesterone and prolactin secretion in a normal cycling female African elephant.

luteum of pregnancy appears to be the most steroidogenically active between 3 and 15 months of gestation. This period is characterized by progesterone concentrations that on average are higher than those during the non-pregnant luteal phase (Meyer *et al.*, 2004; Lueders *et al.*, 2012; Figs. 2 and 3). In both species, acCLs and the ovCL begin to regress about 5–6 weeks after conception (the normal luteal phase lifespan). They then rebound and grow significantly larger than CL during a non-conceptive luteal phase, in conjunction with a marked secondary rise in progesterone concentrations and presumed implantation (Meyer *et al.*, 2004; Lueders *et al.*, 2012). Inhibin also is elevated during the first 8 weeks, as in a normal luteal phase. But in contrast to progesterone, which rebound to even higher concentrations, inhibin declines and remains at baseline throughout gestation (Yamamoto *et al.*, 2012a; Fig. 3). This pattern suggests a shift in CL function between cycling and pregnant elephants, and that a role for inhibin in pregnancy is unlikely. After 5–7 months, both relaxin and prolactin markedly increase (Meyer *et al.*, 2004; Yamamoto *et al.*, 2012b; Fig. 3). Prolactin remains elevated throughout, whereas the relaxin profile is biphasic and concentrations start to decline after mid-gestation, with a secondary surge at parturition (Meyer *et al.*, 2004). To date, no evidence of any gestational gonadotropin-like activity in serum or placental extracts has been found.

There are some notable species differences in gestational hormone patterns. Progesterone concentrations are higher in African elephants during the first half of gestation, but then decline to levels below those observed in Asian elephants (Meyer *et al.*, 2004). There also is a fetal gender effect on progesterone patterns, but only in Asian elephants; females carrying male calves produce more progesterone as compared to those carrying females (Meyer *et al.*, 2004). It is possible to sex the fetus after about a year of gestation with near 100% accuracy in Asian elephants by measuring circulating maternal testosterone, which presumably is of testicular origin and higher in females carrying a male calf (Duer *et al.*, 2002; Brown *et al.*, 2004b). This fetal-sexing technique is less accurate in African elephants. Parturition can be predicted on the basis of a rapid fall in progesterone about 2–5 days before birth (Brown and Lehnhardt, 1995; Carden *et al.*, 1998). Surges in cortisol often are observed in the last 2 weeks of gestation, with a significant surge at parturition (Meyer *et al.*, 2004).

Placentation and implantation

The placenta is chorioallantoic, zonary endotheliochorial with some hemochorial regions (see reviews, Allen (2006); Hildebrandt *et al.* (2006)). Implantation is central and superficial, with a mesometrial orientation of the yolk sac, and antimesometrial orientation of the embryo, and occurs during the second to third month (Drews *et al.*, 2008). Longitudinal transrectal ultrasound has identified an initial period of comparatively slow embryonic development in both elephant species (Hildebrandt *et al.*, 2006; Drews *et al.*, 2008). The embryonic vesicle is visible at ~8 weeks post-conception, which is smaller (~10 mm) than that of other species at that stage (cattle, 40 mm; sheep, 70 mm; horse, 40 mm). Likewise, time of implantation is estimated to be ≤ 20 days in human, dog and sheep, but ≥ 50 days in the elephant. The fetus then doubles in size between the fourth and fifth month, and at 8 months drops into the abdominal cavity and no longer is visible by transrectal ultrasound. Permanent uterine scars are formed by the invasive attachment of the placenta, which allows quantifying the number of pregnancies by ultrasound or at necropsy.

Parturition and lactational anestrus

Hermes *et al.* (2008) has reviewed the current knowledge of elephant obstetrics. A week or more before parturition, the female may begin showing signs of discomfort, while passage of the vaginal mucus plug or mucoid discharge occurs in the days leading up to parturition. Once labor begins, the female becomes restless and changes positions often. Abdominal contractions increase in frequency, but may not always be visually evident. The cervical canal becomes dilated by pressure from the allantoic sac during labor, followed by partial emergence of fetal legs entering the vaginal cavity. A large bulge appears under the tail, initially consisting

of the fluid-containing amniotic sac, but eventually the calf crests the caudal rim of the pelvis and slips out between the elephant's hind legs. Active labor and delivery is relatively short: 2–4 h. The majority of births are in the posterior position. Dystocia is not uncommon in captive elephants, and is related to excessive body weight, advanced age, lack of exercise, urogenital track pathologies, anterior presentation of the calf, and large calf size. In zoos, nulliparous females are generally considered post-reproductive after ~30 years of age because of a greater risk of dystocia and stillbirth. If labor stalls, oxytocin can be administered, but only after confirming the cervix is sufficiently relaxed and the calf is in the birth canal.

The length of the post-partum anestrus period generally lasts 12–24 months (e.g., Fig. 2). Problems with retained placenta, reduced milk production, death of a calf, or premature weaning can reduce the post-partum period to as short as 8 weeks (Brown, 2000). The first luteal phase increase in progestagens after the lactational anestrus period, when cycles resume, is similar to subsequent ones (Glaeser *et al.*, 2012; Fig. 2).

Reproductive Problems

Ovarian acyclicity

Reproductive problems of captive elephants are well documented, and for Africans in particular, a high rate of ovarian acyclicity across the age categories thwarts population sustainability (Brown, 2014). In North America, 52% of African elephant females exhibit abnormal cycles, with 38% not cycling at all (Brown *et al.*, 2016). Comparatively, the acyclicity rate in Asian elephants is lower (<20%) and affects mostly older females (Dow *et al.*, 2011a; Brown *et al.*, 2016). Several conditions known to be associated with infertility in other species are not related to ovarian cycle problems in African elephants, such as hyperandrogenism, hyperestrogenism, thyroid derangements, pituitary dysfunction and elevated cortisol (Brown, 2014). The finding of no differences between cycling and acyclic elephants in anti-müllerian hormone (AMH) concentrations suggests the problem is not due to a lack of functional follicles (Dow *et al.*, 2011b). One association with ovarian acyclicity in African elephants is hyperprolactinemia (Brown *et al.*, 2004a; Yamamoto *et al.*, 2010; Dow and Brown, 2012), which is the most common disorder of the hypothalamic-pituitary axis related to infertility in women (Serri *et al.*, 2003; Wang *et al.*, 2012). Over half of noncycling elephants exhibit levels of prolactin that are 5- to 30-fold higher than those in normal cycling females. As in other species, pituitary prolactin secretion in elephants is under inhibitory control by dopamine (Brown, 2014). The dopamine agonist, cabergoline, effectively suppresses prolactin secretion in hyperprolactinemic elephants, but has yet to induce a resumption of ovarian cyclicity even after a year of treatment (Morfeld *et al.*, 2014a). No underlying physiological cause of hyperprolactinemia has yet been found, but it does not appear to be related to abnormal production of cortisol, estradiol or thyroid hormones (TSH, T₃, T₄) (Brown *et al.*, 2004a,b). Asian elephant females rarely present with hyperprolactinemia (Brown *et al.*, 2004a,b), representing a significant species difference.

Several studies in the United States and Europe have applied epidemiological methods to identify factors in the zoo environment that are associated with reproductive success, and noted several differences between Asian and African elephants (Harris *et al.*, 2008; Brown *et al.*, 2016). For both species, the chance a female will cycle is greater when they are housed in a mixed-sex herd, and decreases with age. For African elephants, social stability and compatibility, and enrichment are important for promoting normal pituitary-ovarian function, whereas social isolation has a negative effect. Behaviorally, dominant females identified as herd peacekeepers often are more likely to be acyclic (Freeman *et al.*, 2004). There also appears to be a social aspect to hyperprolactinemia in African elephants, which is more prevalent in females exposed to many social groups throughout the day, and perhaps related to social instability (Brown *et al.*, 2016). Another finding is that the majority of zoo Asian and African elephants are overweight (Harris *et al.*, 2008; Morfeld and Brown, 2014b), and in Africans, a high body mass index and body condition score has been related to acyclicity, metabolic hormone (insulin and the glucose to insulin ratio) derangements, and elevated leptin (Morfeld and Brown, 2014b). Interestingly, ovarian cycle activity in Asian elephants seems to be less affected by obesity, despite the fact that over three-quarters are considered overweight.

Reproductive tract pathologies

As reviewed by Hildebrandt *et al.* (2006), older nulliparous females, or those that have not calved within ~10 years often develop urogenital tract pathologies. The most common are cysts and tumors, with some species differences. Endometrial hyperplasia and vestibular cysts are found in both species, whereas uterine leiomyomas are present only in Asian, and vestibular polyps are observed only in African elephants. Vaginal cysts and neoplastic formations may be extensive and fill the lumen, blocking semen flow and causing discomfort during estrus and mating. These pathologies may be a consequence of continuous ovarian cyclicity and hormone exposure in non-bred females. In the wild, most elephants are either pregnant or lactating and thus experience comparatively few reproductive cycles in their lifetime. Consequently, rates of urogenital tract pathologies are higher in captive elephants (20–80% in Asians and 10–33% in Africans), and relatively rare in wild elephants (<5%). Use of GnRH vaccines to inhibit pituitary FSH and LH release reduces ovarian steroidogenic activity, and have been effective in resolving uterine cysts and tumors in older Asian elephants (Boedeker *et al.*, 2012).

Male Reproduction

General Reproductive Biology

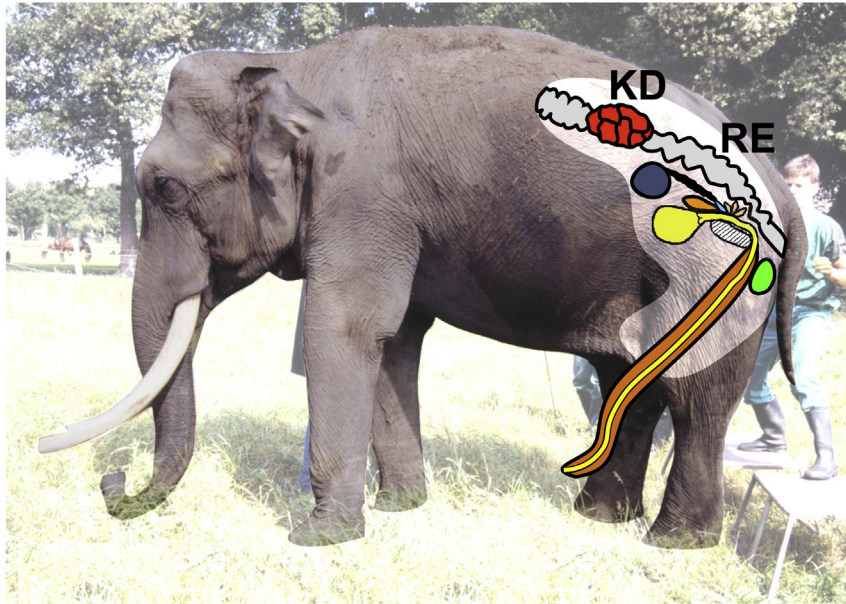
Similar to females, there is a shift in the onset of sexual maturity between captive and wild elephant bulls, particularly for Asians. Successful matings have occurred in captive males as young as 6 years of age, whereas wild bulls generally do not breed until they are

25 years of age or older. Elephant society is matriarchal, and bulls generally are ousted from the herd at puberty. A misconception is that bull elephants are solitary in the wild, when in fact they often travel in small, bachelor groups in search of estrous females. Both captive and wild bulls form social hierarchies that are influenced by age, size, and musth status.

Anatomy

There are a number of notable anatomical differences between elephants and other terrestrial mammals, in part related to the intra-abdominal testes (Short *et al.*, 1967; Hildebrandt *et al.*, 1998, 2000; Fig. 6). These hang from the dorsal wall of the abdominal cavity medial to the kidneys, and can weigh up to 2 kg each. The bull elephant has no inguinal canal, cremaster muscle, or

(a)



(b)

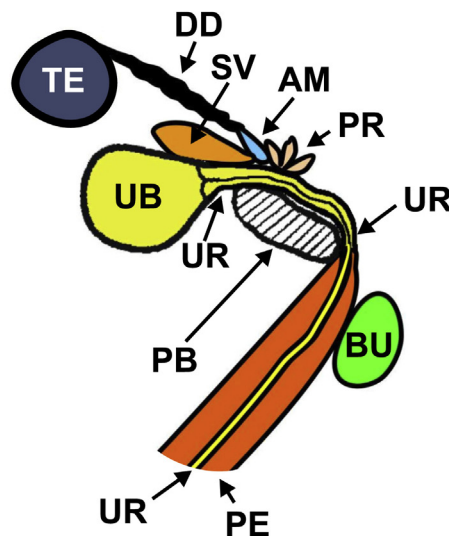


Fig. 6 Schematic diagram of the reproductive tract of the bull elephant: (a) KD, lobulated kidney; RE, rectum. (b) TE, testis; DD, ductus deferens; AM, ampulla; SV, seminal vesicle; PR, prostate; BU, bulbourethral gland; UB, urinary bladder; UR, urethra; PE, penis; PB, pelvic bone. Reprinted by permission Hildebrandt, T.B., Göritz, F., Hermes, R., *et al.*, 2006. Aspects of the reproductive biology and breeding management of Asian and African elephants. *Int Zoo Yrbk* 40, 20–40.

pampiniform plexus. The efferent ducts are highly convoluted and radiate dorsally from the extragonadal rete testis on the anterior aspects of the testes. The epithelial lining the efferent ducts is a low pseudo-stratified columnar type composed of ciliated and stereociliated cells and some basal and halo cells. The epididymis runs posteriorly along the testis within the mesenteric fold, and is comparatively less developed than that of scrotal mammals. Structurally, it can be divided into proximal, isthmus and distal regions, although these are not as distinct as those in other mammals. The epididymis is lined by pseudostratified stereociliated, columnar epithelium composed primarily of principal and basal cells. Motile sperm are present in the distal epididymis, but most sperm storage occurs in cone-shaped ampullae, widened terminal portions of the vas deferens and large glandular structures that open into the ducts of the corresponding seminal vesicles. The prostate glands are lobulated and located on the dorsal wall of the urethra, immediately posterior to the seminal vesicles. In Asian elephants, the three lobes of the prostate fuse into a globe-shaped structure, whereas in the African elephant there are clearly distinguished lobes with separate, irregularly shaped internal cavities. The seminal vesicles are the largest accessory glands, characterized by a single cavity, and produce a fructose-rich fluid. Paired bulbourethral glands are located directly below the anus on both sides of the urethra and penis, and produce a clear, sticky secretion containing sialic and citric acids. The penis is muscular and vascularized, about 1.5–2 m in length, and weighs up to 27 kg. It has a well-defined corpus cavernosum and large paired muscles on the dorsal side that allow for considerable voluntary control during mating. The shape of the penis is sigmoidal when erect, which facilitates entry into the vestibule that opens between the hind legs of the female.

Semen Characteristics and Cryopreservation

Semen can be collected by electroejaculation, artificial vagina or rectal manual message. Determining ampullae fullness by transrectal ultrasound indicates the probability of successful collection. Ejaculates normally range between 50–200 mL in volume, and are white in color, although yellow, orange and brown color variants occur. Low motility, sometimes accompanied by agglutination, is observed in many semen samples, often in samples contaminated with urine during the collection process (Schmitt and Hildebrandt, 1998; Kiso *et al.*, 2011). In Asian elephants, lactotransferrin, a potential fertility marker in human semen (Milardi *et al.*, 2012), has been shown to be abundant in ejaculates with good motility, and primarily absent in those with poor motility (Kiso *et al.*, 2013). Semen stored at 35°C exhibits a rapid decline in motility after a few hours in both species, whereas spermatozoa held at 22°C and 4°C can maintain at least partial motility for 12–24 h (Kiso *et al.*, 2011; O'Brien *et al.*, 2013). DNA damage and morphological degeneration is a contributor to poor semen quality (Imrat *et al.*, 2012; O'Brien *et al.*, 2013). Asian elephant spermatozoa appear to be particularly susceptible to DNA damage, more so than that of African elephants and other mammalian species (Imrat *et al.*, 2012). Addition of semen extenders increases spermatozoal longevity at chilled temperatures, with some species differences: for example, Asian elephant spermatozoa are best maintained in extenders containing egg yolk or skim milk, whereas spermatozoa from African elephants have no such requirement (Graham *et al.*, 2004; Saragusty *et al.*, 2005; Hermes *et al.*, 2009; Saragusty *et al.*, 2009; Kiso *et al.*, 2011). Cryoprotectants (i.e., dimethyl sulfoxide, glycerol, ethylene glycol and propylene glycol) added before cooling and freezing enhance cryosurvival, with glycerol appearing to be the most effective (Thongtip *et al.* 2004; Saragusty *et al.* 2009; Buranaamnuay *et al.*, 2013). For cryopreservation, most extenders for elephant semen cryopreservation contain a minimum of 15–20% egg yolk (Graham *et al.*, 2004).

Musth

In both species, musth is a period of heightened aggressive and sexual behavior associated with temporal gland secretions (TGS), urine dribbling (UD) and elevated androgen (e.g., testosterone, dihydrotestosterone, androstenedione) production (Jainudeen *et al.*, 1971, 1972; Ganswindt *et al.*, 2002, 2005; Brown *et al.*, 2007). It has been compared to rut in other ungulates; however, unlike rut, musth is not strictly seasonal. Wild bulls generally exhibit musth once a year, which can last a few weeks to several months, and is related to age, nutrition, and social status (Jainudeen *et al.*, 1972; Cooper *et al.*, 1990; Lincoln and Ratnasooriya, 1996). Testosterone concentrations increase 10- to 50-fold during musth compared to non-musth. In the wild, musth occurs in sexually mature bulls, generally those over 25 years of age. However, in captivity, musth signs have been observed in bulls as young as 7 years old (Cooper *et al.*, 1990). In many zoo elephants, musth-like changes often are irregular and can occur several times a year (Brown *et al.*, 2007). These patterns may not reflect true musth, however, because they often are not accompanied by UD. By definition, musth refers to the competitive state in sexually active male elephants, with the presence of UD being a defining physical signal (Ganswindt *et al.*, 2005). Analogous to females, captive African bulls appear to mature slower than Asian males based on hormonal analyses and musth signs (Brown *et al.*, 2007).

Bull elephants advertise their musth status through a variety of chemicals exuded in TGS and urine. In young Asian bulls <15 years of age, TGS consists of sweet odors: acetates, an alcohol (3-hexen-2-ol) that smells of leaves, and pleasant smelling ketones (acetophenone and 2-heptanone) (Rasmussen *et al.*, 2002; Riddle *et al.*, 2006). This is referred to as honey or moda musth, and is associated with behaviors that are more erratic and unpredictable. It also is of a shorter duration and associated with comparatively lower androgen levels. Moda musth has not been described for juvenile African elephant bulls, however, which may reflect a species difference. In Asian elephants, the compounds of moda musth are transitionally replaced by more malodorous compounds, like carboxylic acids, and a chemical signal found in insects – frontalin. In older Asian and African elephant bulls, musth urine contains higher levels of alkan-2-ones, alkan- 2-ols, and some aromatic compounds compared to urine of females and non-musth males (Riddle *et al.*, 2006; Goodwin *et al.*, 2012). Elephants have well-developed primary and secondary (vomeronasal) olfactory systems

(Lazar *et al.*, 2004), and both males and females respond to these chemicals. Estrous females seek out musth bulls, while sub-adult and non-musth males exhibit avoidance behavior when exposed to musth semiochemicals (Riddle *et al.*, 2006; Goodwin *et al.*, 2012). Ultimately, the state of musth confers an advantage to adult bulls, which gain more access to estrous females and experience a higher paternity success (Hollister-Smith *et al.*, 2007).

Function of the hypothalamo-pituitary-testicular axis in elephants appears to be similar to that in other species. Pulses of testosterone closely follow those of LH (~1 pulse/3 h), and GnRH administration results in LH-stimulated testosterone release (Niemuller and Liptrap, 1991; Brown *et al.*, 1993). There is little difference in LH pulse rate between musth and non-musth Asian bulls; however, testosterone patterns indicate testes become hyper responsive to LH, both pulsatile and GnRH-induced, during musth (Brown *et al.*, 1993; Lincoln and Ratnasooriya, 1996). Increased numbers of LH receptors in testicular tissue of musth bulls are related to this hyper testosterone secretion.

A schematic of hormonal relationships associated with musth is presented in Fig. 7). Circulating cortisol increases during musth, at least in captive bulls, and is positively correlated with serum testosterone (Brown *et al.*, 2007; Yon *et al.*, 2007). Serum gonadotropin patterns are not always consistent, but in many bulls, LH is elevated for several weeks about a month before musth begins, while FSH concentrations are higher during the pre-musth period and decline during musth (Kaewmanee *et al.*, 2011b). FSH in elephant bulls is inversely related to inhibin, which in turn correlates positively with testosterone (Kaewmanee *et al.*, 2011b). In other species, and presumably elephants, inhibin is produced by Sertoli cells in response to FSH, which then controls pituitary FSH secretion through a negative feedback loop. Another hormone produced by Sertoli cells under FSH control is AMH, which can be used as a marker of FSH and testicular function. AMH is higher in pre- versus postpubertal elephant bulls; however, there are no differences in concentrations between musth and nonmusth males (Dow *et al.*, 2011b). AMH concentrations are higher than in female elephants (over 100-fold), regardless of age or gonadal status, which has been found other species.

Due to the dangers associated with managing bulls, there is considerable interest in finding treatments to mitigate musth behaviors. Castration is not a practical option given the abdominal location of the testes, although it has been done. Musth is affected by nutritional status, so a common management strategy is to reduce water and food. Musth bulls also are often isolated, and in Asia they are kept on short chains away from other elephants and people. Both of these strategies raise animal welfare concerns, so alternative therapies that decrease androgen secretion or action are being sought. GnRH agonists, like leuprolide acetate, can reduce testosterone during musth, but need regular injections (Brown *et al.*, 1993; de Oliveira *et al.*, 2004). Cyproterone acetate (an antiandrogen) has been given to Asian elephants, but with little effect on aggressive behaviors (Niemuller *et al.*, 1998). More recently, GnRH vaccines have shown promise as a means to reduce testosterone production (De Nys *et al.*, 2010; Somgird *et al.*, 2016). Not all bulls respond with a reduction in musth symptoms, however, which may be a learned behavior. Long-term treatment with GnRH vaccines also can result in permanent immune-castration, so should be used cautiously (Lueders *et al.*, 2014).

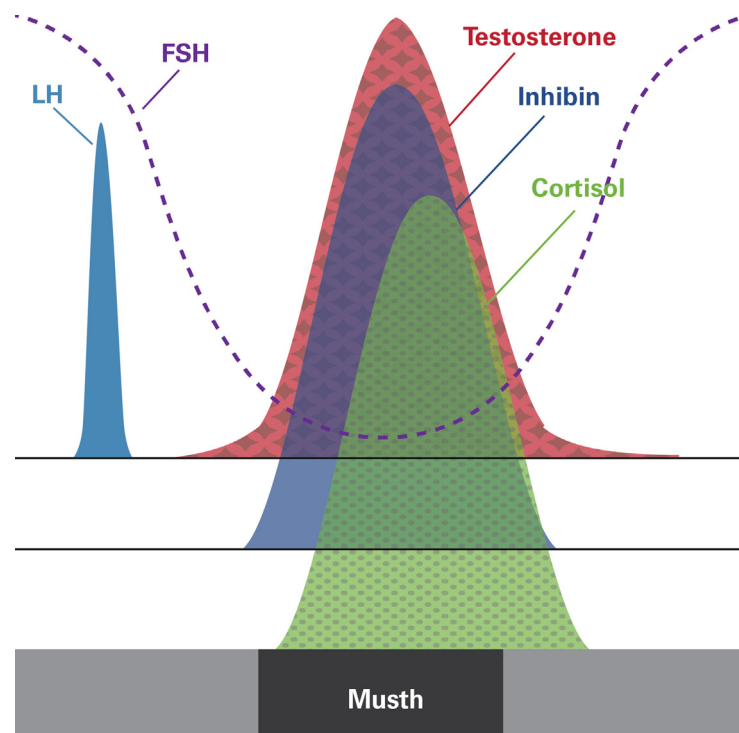


Fig. 7 Schematic of the hormonal relationships among testosterone, luteinizing hormone (LH), cortisol, and inhibin, in association with musth in bull elephants. Adapted from Brown, J.L., Somerville, M., Riddle, H., *et al.*, 2007. Comparative endocrinology of testicular, adrenal and thyroid function in Asian and African elephant bulls. *Gen Comp Endocrinol* 151, 153–162 and Kaewmanee, S., Watanabe, G., Keio, M., *et al.*, 2011b. A surge-like increase in luteinizing hormone preceding musth in a captive bull African elephant (*Loxodonta africana*). *J Vet Med Sci* 73, 379–383.

Assisted Reproduction

One of the most significant advances in elephant reproductive management has been the development of an artificial insemination (AI) technique in the 1990s (Hildebrandt *et al.*, 1999; Brown *et al.*, 2004b; Thongtip *et al.*, 2009). The non-surgical procedure involves a custom-made balloon catheter (2.5-cm diameter 140 cm length), lubricated with nonspermicidal sterile gel, inserted into the vestibule to slightly distend the tract for optimal visualization and to allow passage of a flexible 3.0-m endoscope containing a disposable semen catheter. Both endoscopic and transrectal ultrasonographic visualizations are used to guide the insemination catheter to the distal vagina in front of the cervical opening or intracervically; proper semen deposition is then confirmed ultrasonographically. The timing of AI is based on identifying LH1 in daily blood samples during the follicular phase of the cycle, and then conducting 2–3 inseminations ~18–21 days later to coincide with LH2. Monitoring LH in elephants is only possible with blood serum or plasma, as no immunoreactivity has been detected in urine (Brown *et al.*, 2010). The first elephant AI birth was in 1999; since then >50 births have resulted worldwide. Continuing challenges in the successful use of AI in elephants include: (1) the inability to consistently obtain good quality ejaculates; (2) poor spermatozoa survival after liquid storage and transport; and (3) low post-thaw motility of cryopreserved sperm. Calves have been produced successfully by AI using cryopreserved sperm in African elephants, including with imported frozen semen from a wild African bull (Hildebrandt *et al.*, 2012b). However, most AIs are done using fresh or chilled semen, which limits AI effectiveness if good quality ejaculates are not collected on peak fertility days. Thus, while AI is clearly an important management tool for breeding elephants, maximal impact will not be achieved until good quality samples can be reliably collected and cryopreserved.

The benefit of AI also would be greatly enhanced by the ability to preferentially produce female offspring using sex-sorted semen (Johnson *et al.*, 1987). As more zoos become involved in breeding elephants by both AI and natural mating, the number of males will continue to increase. A high proportion of male elephants in captive collections is not needed for sustainability nor is it desired. The successful separation of elephant sperm based on the DNA difference between X and Y chromosomes using flow cytometric methods suggests this is a possibility (Hermes *et al.*, 2009). Ultimately the goal is to establish genome resource banks for elephants, which if they include sex-sorted cryopreserved sperm from captive and wild bulls, would greatly enhance the genetic management of elephants under human care.

Conservation and Management

Through advances in reproductive monitoring, and the ability to collect longitudinal biological data, many unique aspects of elephant biology have been identified, and today the two species are considered relatively well-studied compared to many other wildlife. The knowledge gained has contributed to improved management of *ex situ* elephants, and a better understanding of factors affecting the survival of elephants *in situ*. Today, extensive conservation efforts are underway to protect the remaining wild elephant populations, with varied success. African elephants are declining at an alarming rate, around 8% annually, mostly due to poaching. There are <400,000 wild African elephants today, down from several million in the early 20th century. Asian elephants are even more endangered, with <50,000 distributed across 13 range countries. Population declines in Asia are due not only to poaching, but to deforestation, habitat loss, population fragmentation, and human-elephant conflict. Many Asian range states now have <1000 elephants left in the wild, often fragmented, and so those populations may not be sustainable in the long term. In captivity, there are perhaps 1500 African elephants held in zoological collections, whereas there are as many as 15,000 Asian elephants currently used for tourism, logging, religious activities and poaching patrols. Unfortunately, because of poor breeding success, replenishment of captive stocks has involved capturing of wild elephants, which further depletes fragile populations and raises ethical and welfare concerns. In Asia, wild capture is now banned, although not always enforced.

We need to understand why reproductive rates are low in many elephant populations under human care, and develop ways to mitigate roadblocks to achieving reproductive success and sustainability. New approaches for managing social groups, meeting nutritional needs, improving enrichment and exercise regimens, and finding ways to promote normal behaviors might avoid some of the problems associated with cohort incompatibility, asymmetric reproductive aging, gonadal dysfunction and poor pregnancy outcomes. It is unlikely that any one factor is responsible, especially given the diversity of ways elephants are managed worldwide, so it will be key to ascertain if problems are of physical or behavioral origin, and identify the best approaches to ameliorate them. Ironically, left alone, elephants reproduce well in the wild – so much so that in some highly-protected areas, population control methods, such as culling or immuno-contraception (e.g., GnRH or porcine zona pellucida vaccines), are necessary.

Last, while elephant conservation has centered on saving habitat and, by extension, the species therein, the magnitude of population declines now means that all options must be considered, including management of captive collections in zoos, breeding centers, and sanctuaries. Today, many wild elephant populations are intensively managed and protected, so in a sense they also are ‘under human care,’ just on a much larger scale. Ultimately, the long-term preservation of elephants will depend on the use of a wide range of tools, low and high tech, often modified from traditional animal models and adapted to meet species-specific needs.

References

- Allen, W. R. (2006). Ovulation, pregnancy, placentation and husbandry in the African elephant (*Loxodonta africana*). *Phil Trans R Soc B*, 361, 821–834.
- Boedeker, N. C., Hayek, L. C., Murray, S., de Avila, D. M., & Brown, J. L. (2012). Effects of a gonadotropin-releasing hormone vaccine on ovarian cyclicity and uterine morphology of an Asian elephant (*Elephas maximus*). *J Zoo Wild Med*, 43, 603–614.

- Brown, J. L. (2000). Reproductive endocrine monitoring of elephants: An essential tool for assisting captive management. *Zoo Biol*, 19, 347–367.
- Brown, J. L. (2014). Comparative reproductive biology of elephants. In W. V. Holt, J. L. Brown, & P. Comizzoli (Eds.), *Reproductive Sciences in Animal Conservation – Progress and Prospects. Advances in Experimental Medicine and Biology* (pp. 135–169). New York: Springer Science and Business Media.
- Brown, J. L., Bush, M., Wildt, D. E., et al. (1993). Effects of GnRH analogues on pituitary-testicular function in free-ranging African elephants (*Loxodonta africana*). *J Reprod Fertil*, 99, 627–634.
- Brown, J. L., Citino, S. B., Bush, M., Lehnhardt, J., & Phillips, L. G. (1991). Cyclic patterns of luteinizing hormone, follicle stimulating hormone, inhibin, and progesterone in the Asian elephant (*Elephas maximus*). *J Zoo Wildl Med*, 22, 49–57.
- Brown, J. L., Goritz, F., Pratt-Hawkes, N., et al. (2004b). Successful artificial insemination of an Asian elephant at the National Zoological Park. *Zoo Biol*, 23, 45–63.
- Brown, J. L., Kersey, D. C., & Walker, S. L. (2010). Assessment of luteinizing hormone and prolactin immunoactivity in Asian and African elephant urine using assays validated for serum. *Gen Comp Endocrinol*, 169, 138–143.
- Brown, J. L., & Lehnhardt, J. (1995). Serum and urinary hormones during pregnancy and the peri- and postpartum period in an Asian elephant (*Elephas maximus*). *Zoo Biol*, 14, 555–564.
- Brown, J. L., Paris, S., Prado-Oviedo, N. A., et al. (2016). Reproductive health assessment of female elephants in North American zoos and association of husbandry practices with reproductive dysfunction in African elephants (*Loxodonta africana*). *PLOS ONE*, pone.0145673.
- Brown, J. L., Schmitt, D. L., Bellem, A., Graham, L. H., & Lehnhardt, J. (1999). Hormone secretion in the Asian elephant (*Elephas maximus*): Characterization of ovulatory and anovulatory luteinizing hormone surges. *Biol Reprod*, 61, 1294–1299.
- Brown, J. L., Somerville, M., Riddle, H., et al. (2007). Comparative endocrinology of testicular, adrenal and thyroid function in Asian and African elephant bulls. *Gen Comp Endocrinol*, 151, 153–162.
- Brown, J. L., Walker, S. L., & Moeller, T. (2004a). Comparative endocrinology of cycling and noncycling Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. *Gen Comp Endocrinol*, 136, 360–370.
- Buranaumnay, K., Mahasawangkul, S., & Saikhun, K. (2013). The in vitro quality of frozen-thawed Asian elephant (*Elephas maximus*) spermatozoa in semen supplemented with Equex STM paste and oxytocin during and after cryopreservation. *Reprod Biol*, 13, 169–171.
- Carden, M., Schmitt, D., Tomasi, T., et al. (1998). Utility of serum progesterone and prolactin analysis for assessing reproductive status in the Asian elephant (*Elephas maximus*). *Anim Reprod Sci*, 53, 133–142.
- Cooper, K. A., Harder, J. D., Clawson, D. H., et al. (1990). Serum testosterone and musth in captive male African and Asian elephants. *Zoo Biol*, 9, 297–306.
- Czekala, N. M., MacDonald, E. A., Steinman, K., et al. (2003). Estrogen and LH dynamics during the follicular phase of the estrous cycle in the Asian elephant. *Zoo Biol*, 22, 443–454.
- De Nys, H. M., Bertschinger, H., Turkstra, J. A., et al. (2010). Vaccination against GnRH may suppress aggressive behaviour and musth in African elephant (*Loxodonta africana*) bulls – A pilot study. *J S Afr Vet Assoc*, 81, 8–15.
- de Oliveira, C. A., West, G. D., Houck, R., & Leblanc, M. (2004). Control of musth in an Asian elephant bull (*Elephas maximus*) using leuprolide acetate. *J Zoo Wildl Med*, 35, 70–76.
- Dow, T. L., & Brown, J. L. (2012). Evidence that hyperprolactinemia is associated with ovarian acyclicity in female zoo African elephants (*Loxodonta africana*). *Reprod Fertil Dev*, 24, 1019–1027.
- Dow, T. L., Holaskova, I., & Brown, J. L. (2011a). Results of the third reproductive assessment survey of North American Asian (*Elephas maximus*) and African (*Loxodonta africana*) female elephants. *Zoo Biol*, 30, 699–711.
- Dow, T. L., Roudebush, W., Parker, F. N., & Brown, J. L. (2011b). Influence of age and gender on secretion of anti-müllerian hormone in Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants. *Theriogenology*, 75, 620–627.
- Drewe, B., Hermes, R., Göritz, F., et al. (2008). Early embryo development in the elephant assessed by serial ultrasound examinations. *Theriogenology*, 69, 1120–1128.
- Duer, C., Carden, M., Schmitt, D., & Tomasi, T. (2002). Utility of maternal serum testosterone analysis for fetal gender determination in Asian elephants (*Elephas maximus*). *Anim Reprod Sci*, 69, 47–52.
- Fanson, K. V., Keeley, T., & Fanson, B. G. (2014). Cyclic changes in cortisol across the estrous cycle in parous and nulliparous Asian elephants. *Endocr Connect*, 3, 57–66.
- Frasor, J., & Gibori, G. (2003). Prolactin regulation of estrogen receptor expression. *Trends Endocrinol Metab*, 14, 118–123.
- Freeman, E. W., Weiss, E., & Brown, J. L. (2004). Examination of the impact of physical and behavioral factors on ovarian cyclicity status in captive Asian and African elephants. *Zoo Biol*, 23, 431–448.
- Freeman, M. E., Kanyicska, B., Lerant, A., & Nagy, G. (2000). Prolactin: Structure, function and regulation of secretion. *Physiol Rev*, 80, 1524–1631.
- Ganswindt, A., Heistermann, M., Borrigan, S., & Hodges, J. K. (2002). Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. *Zoo Biol*, 21, 27–36.
- Ganswindt, A., Rasmussen, H. B., Heistermann, M., & Hodges, J. K. (2005). The sexually active states of free-ranging male African elephants (*Loxodonta africana*): Defining musth and non-musth using endocrinology, physical signals, and behavior. *Horm Behav*, 47, 83–91.
- Glaeser, S. S., Martin, M. S., Hunt, K. E., Finnegan, M., & Brown, J. L. (2012). Investigation of individual and group variability in estrous cycle characteristics in female Asian elephants (*Elephas maximus*) at the Oregon Zoo. *Theriogenology*, 78, 285–296.
- Goodwin, T. E., Broederdorf, L. J., Burkert, B. A., et al. (2012). Chemical signals of elephant musth: Temporal aspects of microbially-mediated modifications. *J Chem Ecol*, 38, 81–87.
- Goodwin, T. E., Eggert, M. S., House, S. J., et al. (2006). Insect pheromones and precursors in female African elephant urine. *J Chem Ecol*, 32, 1849–1853.
- Graham, L. H., Bando, J., Gray, C., & Buhr, M. M. (2004). Liquid storage of Asian elephant (*Elephas maximus*) sperm at 4°C. *Anim Reprod Sci*, 80, 329–340.
- Harris, M., Sherwin, C., Harris, S., 2008. The welfare, housing and husbandry of elephants in UK zoos. Final report, University of Bristol, United Kingdom.
- Hermes, R., Behr, B., Hildebrandt, T. B., et al. (2009). Sperm sex-sorting in the Asian elephant (*Elephas maximus*). *Anim Reprod Sci*, 112, 390–396.
- Hermes, R., Olson, D., Göritz, F., et al. (2000). Ultrasonography of the estrous cycle of female African elephants (*Loxodonta africana*). *Zoo Biol*, 19, 369–382.
- Hermes, R., Saragusty, J., Schaftenaar, W., et al. (2008). Obstetrics in elephants. *Theriogenology*, 70, 131–144.
- Hildebrandt, T. B., Brown, J. L., Hermes, R., & Göritz, F. (2003). Ultrasound for the analysis of reproductive function in wildlife. In D. E. Wildt, W. V. Holt, A. R. Pickard, & J. C. Roger (Eds.), *Reproduction and Integrated Conservation Science* (pp. 166–182). Zoological Society of London.
- Hildebrandt, T. B., Göritz, F., Hermes, R., et al. (2006). Aspects of the reproductive biology and breeding management of Asian and African elephants. *Int Zoo Yrbk*, 40, 20–40.
- Hildebrandt, T. B., Göritz, F., Hermes, R., et al., 1999. Artificial insemination of African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants. In: Proceedings of the American Association of Zoo Veterinarians Conference, Omaha, NE, pp. 83–86.
- Hildebrandt T.B., Hermes R., Pratt N.C., et al., 2000. Ultrasonography of the urogenital tract in elephants (*Loxodonta africana* and *Elephas maximus*): An important tool for assessing male reproductive function, *Zoo Biol* 19, 333–345.
- Hildebrandt, T. B., Hermes, R., Saragusty, J., et al. (2012b). Enriching the captive elephant population genetic pool through artificial insemination with frozen-thawed semen collected in the wild. *Theriogenology*, 78, 1398–1404.
- Hildebrandt, T. B., Lueders, I., Hermes, R., Göritz, F., & Saragusty, J. (2011). Reproductive cycle of the elephant. *Anim Reprod Sci*, 124, 176–183.
- Hildebrandt, T. B., Lueders, I., Hermes, R., Göritz, F., & Saragusty, J. (2012a). Anatomical, physiological, behavioral and pathological aspects of the estrous cycle in elephants. *Jpn J Zoo Wildl Med*, 17, 97–109.
- Hollister-Smith, J. A., Poole, J. H., Archie, D. A., et al. (2007). Age, musth and paternity success in wild male African elephants, *Loxodonta africana*. *Anim Behav*, 74, 287–296.
- Imrat, P., Hernandez, M., Ritem, S., et al. (2012). The dynamics of sperm DNA stability in Asian elephant (*Elephas maximus*) spermatozoa before and after cryopreservation. *Theriogenology*, 77, 998–1007.

- Jainudeen, M. R., Kartongole, C. B., & Short, R. V. (1972). Plasma testosterone levels in relation to musth and sexual activity in the male Asiatic elephants, (*Elephas maximus*). *J Reprod Fertil*, 29, 99–103.
- Jainudeen, M. R., Mackay, G. M., & Eisenberg, J. F. (1971). Observation on musth in the domesticated Asiatic elephants. *Mammalia*, 36, 247–261.
- Johnson, L. A., Flook, J. P., Look, M. V., & Pinkel, D. (1987). Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. *Gamete Res*, 16, 1–9.
- Kaewmanee, S., Watanabe, G., Keio, M., et al. (2011b). A surge-like increase in luteinizing hormone preceding musth in a captive bull African elephant (*Loxodonta africana*). *J Vet Med Sci*, 73, 379–383.
- Kaewmanee, S., Watanabe, G., Kishimoto, M., et al. (2011a). Secretion of inhibin during the estrous cycle in the female Asian elephant (*Elephas maximus*). *J Vet Med Sci*, 73, 77–82.
- Kapustin, N., Critser, J. K., Olson, D., & Malven, P. V. (1996). Nonluteal estrous cycles of 3-week duration are initiated by anovulatory luteinizing hormone peaks in African elephants. *Biol Reprod*, 55, 1147–1154.
- Kiso, W., Brown, J. L., Siewerdt, et al. (2011). Liquid semen storage in elephants (*Elephas maximus* and *Loxodonta africana*): Species differences and storage optimization. *J Androl*, 21, 420–431.
- Kiso, W. K., Selvaraj, V., Nagashima, J., et al. (2013). Lactotransferrin in Asian elephant (*Elephas maximus*) seminal plasma correlates with semen quality. *PLOS ONE*, 8, e71033.
- Lahdenperä, M., U Mar, K., & Lummaa, V. (2014). Reproductive cessation and post-reproductive lifespan in Asian elephants and pre-industrial humans. *Front Zool*, 11, 54–68.
- Lazar, J., Rasmussen, L. E. L., Greenwood, D. R., Bang, I. S., & Prestwich, G. D. (2004). Elephant albumin: A multipurpose pheromone shuttle. *Chem Biol*, 11, 1093–1100.
- Lincoln, G. A., & Rathnasooriya, W. D. (1996). Testosterone secretion, musth behavior and social dominance in captive male Asian elephants living near the equator. *Reprod Fertil*, 108, 107–113.
- Lueders, I., Hildebrandt, T. B., Gray, C., et al. (2014). Suppression of testicular function in a male Asian elephant (*Elephas maximus*) treated with gonadotropin-releasing hormone vaccines. *J Zoo Wildl Med*, 43, 611–619.
- Lueders, I., Niemuller, C., Gray, C., Rich, P., & Hildebrandt, T. B. (2010). Luteogenesis during the estrous cycle in Asian elephants (*Elephas maximus*). *Reproduction*, 140, 777–786.
- Lueders, I., Niemuller, C., Rich, P., et al. (2012). Gestation for 22 months: Luteal development and pregnancy maintenance in elephants. *Proc Royal Soc B*, 279, 3687–3696.
- Lueders, I., Taya, K., Watanabe, G., et al. (2011). Role of the double luteinizing hormone peak, luteinizing follicles, and the secretion of inhibin for dominant follicle selection in Asian elephants (*Elephas maximus*). *Biol Reprod*, 85, 714–720.
- Meyer, J. M., Walker, S. L., Freeman, E. W., Steinetz, B. G., & Brown, J. L. (2004). Species and fetal gender effects on the endocrinology of pregnancy in elephants. *Gen Comp Endocrinol*, 138, 263–270.
- Milardi, D., Grande, G., Vincenzoni, F., et al. (2012). Proteomic approach in the identification of fertility pattern in seminal plasma of fertile men. *Fertil Steril*, 97, 67–73.
- Morfeld, K. A., Ball, R. L., & Brown, J. L. (2014a). Recurrence of hyperprolactinemia and continuation of ovarian acyclicity in captive African elephants (*Loxodonta africana*) treated with cabergoline. *J Zoo Wildl Med*, 45, 569–576.
- Morfeld, K. A., & Brown, J. L. (2014b). High body condition scores and elevated insulin and leptin are associated with ovarian acyclicity in zoo African elephants (*Loxodonta africana*). *Reprod Fertil Dev*, 28, 640–647.
- Niemuller, C., Brown, J. L., & Hodges, J. K. (1998). Reproduction in elephants. In E. Knobil, & J. Neill (Eds.), *Encyclopedia of Reproduction* (vol. 1, pp. 1018–1029). New York, NY: Academic Press.
- Niemuller, C. A., & Liptrap, R. M. (1991). Altered androstenedione to testosterone ratios and LH concentrations during musth in captive male Asian elephants (*Elephas maximus*). *J Reprod Fertil*, 91, 139–146.
- O'Brien, J. K., Steinman, K. J., Montano, G. A., Love, C. C., & Robeck, T. R. (2013). Sperm DNA fragmentation and morphological degeneration in chilled elephant (*Elephas maximus* and *Loxodonta africana*) semen collected by transrectal massage. *Andrology*, 1, 387–400.
- Rasmussen, L. E. L. (2001). Source and cyclic release pattern of (Z)-7-dodecenyl acetate, the pre-ovulatory pheromone of the female Asian elephant. *Chem Senses*, 26, 611–623.
- Rasmussen, L. E. L., Lee, T. D., Zhang, A., Roelofs, W. L., & Daves, G. D., Jr. (1997). Purification, identification, concentration and bioactivity of (Z)-7-dodecen-1-yl acetate: sex pheromone of the female Asian elephant, *Elephas maximus*. *Chem Senses*, 22, 417–437.
- Rasmussen, L. E. L., Riddle, H. S., & Krishnamurthy, V. (2002). Mellifluous matures to malodorous in musth. *Nature*, 415, 975–976.
- Riddle, H. S., Greenwood, D. R., & Rasmussen, L. E. L. (2006). The shifting chemical signals of musth. *Gajah*, 24, 39–44.
- Saragusty, J., Hildebrandt, T. B., Behr, B., et al. (2009). Successful cryopreservation of Asian elephant (*Elephas maximus*) spermatozoa. *Anim Reprod Sci*, 115, 255–266.
- Saragusty, J., Hildebrandt, T. B., Natan, Y., et al. (2005). Effect of egg-phosphatidylcholine on the chilling sensitivity and lipid phase transition of Asian elephant (*Elephas maximus*) spermatozoa. *Zoo Biol*, 24, 233–245.
- Schmitt, D. L., & Hildebrandt, T. B. (1998). Manual collection and characterization of semen from Asian elephants (*Elephas maximus*). *Anim Reprod Sci*, 53, 309–314.
- Serri, O., Chik, C. L., Ur, E., & Ezzat, S. (2003). Diagnosis and management of hyperprolactinemia. *Canadian Med Assoc J*, 169, 575–581.
- Short, R. V., Mann, T., & Hay, M. F. (1967). Male reproductive organs of the African elephant, *Loxodonta africana*. *J Reprod Fertil*, 13, 517–536.
- Somgird, C., Homkong, P., Sripiroon, S., et al. (2016). Potential of a gonadotropin-releasing hormone vaccine to suppress musth in captive male Asian elephants (*Elephas maximus*). *Anim Reprod Sci*, 164, 111–120.
- Sukumar, R. (Ed.). (2003). *The living elephants: Evolution, ecology, behavior and conservation*. Oxford: Oxford University Press.
- Thongtip, N., Mahasawangkul, S., Thitaram, C., et al. (2009). Successful artificial insemination in the Asian elephant (*Elephas maximus*) using chilled and frozen-thawed semen. *Reprod Biol Endocrinol*, 7, 75–81.
- Thongtip, N., Saikhun, J., Damyang, M., et al. (2004). Evaluation of post-thaw Asian elephant (*Elephas maximus*) spermatozoa of extender and cryoprotectant. *Theriogenology*, 62, 748–760.
- Wang, A. T., Mullan, R. J., Lane, M. A., et al. (2012). Treatment of hyperprolactinemia: A systematic review and meta-analysis. *Syst Rev*. <https://doi.org/10.1186/2046-4053-1-33>.
- Wasser, S. L., Papageorge, S., Foley, C., & Brown, J. L. (1996). Excretory fate of estradiol and progesterone in the African elephant (*Loxodonta africana*) and pattern of fecal steroid concentrations throughout the estrous cycle. *Gen Comp Endocrinol*, 102, 255–262.
- Wittemyer, G., Ganswindt, A., & Hodges, K. (2007). The impact of ecological variability on the reproductive endocrinology of wild female African elephants. *Horm Behav*, 51a, 346–354.
- Yamamoto, Y., Yamamoto, T., Watanabe, G., et al. (2010). Prolactin secretion and ovarian function in cycling and non-cycling African female elephants (*Loxodonta africana*). *J Vet Med Sci*, 72, 845–852.
- Yamamoto, Y., Yamamoto, T., Yuto, N., et al. (2012b). The secretory pattern and source of immunoreactive prolactin in pregnant African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants. *J Reprod Dev*, 58, 105–111.
- Yamamoto, Y., Yuto, N., Yamamoto, T., et al. (2012a). Secretory pattern of inhibin during the estrous cycle and pregnancy in African (*Loxodonta africana*) and Asian (*Elephas maximus*) elephants. *Zoo Biol*, 31, 511–522.
- Yamato, M., Minami, S., & Nakano, R. (1991). Immunohistochemical localization of inhibin subunits in human corpora lutea during menstrual cycle and pregnancy. *J Clin Endocrinol Metab*, 73, 470–477.
- Yamato, M., Minami, S., Nakano, R., & Kobayashi, M. (1992). Immunolocalization of inhibin/activin subunits in human ovarian follicles during the menstrual cycle. *J Clin Endocrinol Metab*, 74, 989–993.
- Yon, L., Kanchanapangka, S., Chaiyabutr, N., et al. (2007). A longitudinal study of LH, gonadal and adrenal steroids in four intact Asian bull elephants (*Elephas maximus*) and one castrate African bull (*Loxodonta africana*) during musth and non-musth periods. *Gen Comp Endocrinol*, 151, 241–245.

Snakes

Amanda M Sparkman, Westmont College, Santa Barbara, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Snakes are amniotic vertebrates with internal fertilization and genetic sex determination that share much in common with other amniotes with regard to reproductive anatomy, physiology, behaviour and life history. However, numerous distinctive features of snakes, including a limbless, elongate body form, secretive lifestyle, diversity in reproductive modes, ectothermy, indeterminate growth, diverse life-history strategies and flexible responses to environmental variables make them an excellent model for the study of reproduction, in mechanistic, ecological, and evolutionary dimensions.

Basic Reproductive Anatomy

Like other reptiles, male snakes have paired testes which are housed internally (**Fig. 1(A)**). Testes are displaced within the narrow body cavity, with the right testes more anterior than the left, so as to prevent overlap. Sperm is released via paired hemipenes, male intromittent organs present in all squamate reptiles (i.e., snakes and lizards). Hemipenes are maintained in an inverted position at the anterior end of the tail (**Fig. 1(B)**), but are everted from the vent (cloaca) during copulation. A single hemipene is inserted into the female, but males may alternate left and right hemipenes during subsequent matings. Male snake also have region of the kidney called the sexual segment of the kidney, which is involved in the production of copulatory plugs (discussed below).

Female snakes have paired ovaries, also displaced from one another, with follicles from each ovary arranged in a roughly linear fashion (**Fig. 2(A)**). After vitellogenesis, eggs are ovulated into a single oviduct prior to fertilization. Within the oviduct, eggs tend to be arranged in a linear sequence (**Fig. 2(B)**), filling the maternal abdomen from just below the gall bladder to just above the vent, though overlap among eggs may occur, especially in large snakes. Both oviparity and viviparity are broadly distributed across the snake phylogeny ([Seigel and Ford, 1987](#)). Developing embryos in oviparous species rely primarily on nutrient stores within yolk deposited during vitellogenesis. Developing embryos in viviparous species (**Fig. 2(C)**) will also primarily rely on yolk for nutrients, but can additionally derive some level of nutrients and/or water via placental connection with their mother ([Stahlschmidt and DeNardo, 2011](#)).

Reproductive Cycles

Annual timing of reproduction is a critical ecological decision that should minimize the costs to breeders and maximize the chances of offspring success in a given environment. The most basic requirement is that both sexes must be reproductively active at the same time for fertilization to occur. Furthermore, environmental temperature must be at a level that allows the movement required to find mates and avoid predators during courtship and mating activities ([Krohmer and Lutterschmidt, 2011](#)). Offspring development and hatching/birth should also occur under favorable thermal conditions, and the stage of juvenile development at which individuals are prepared to forage independently should coincide with peaks in resource availability. Natural selection favors responsiveness to environmental cues that help maximize reproductive success given different environmental challenges. Across vertebrates,

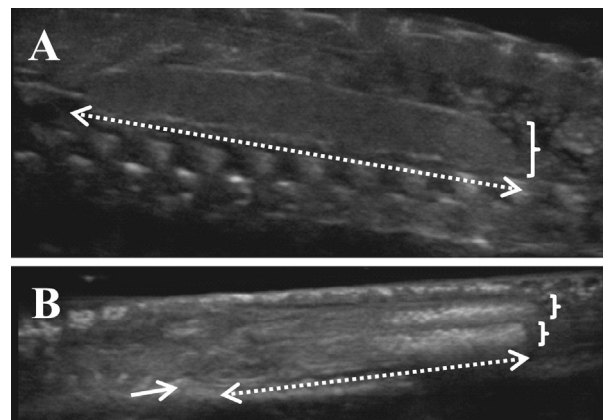


Fig. 1 Ultrasound images of male sexorgans in common kingsnakes from southern California. (A) The right testes. (B) Paired hemipenes (small arrow indicates location of vent.).

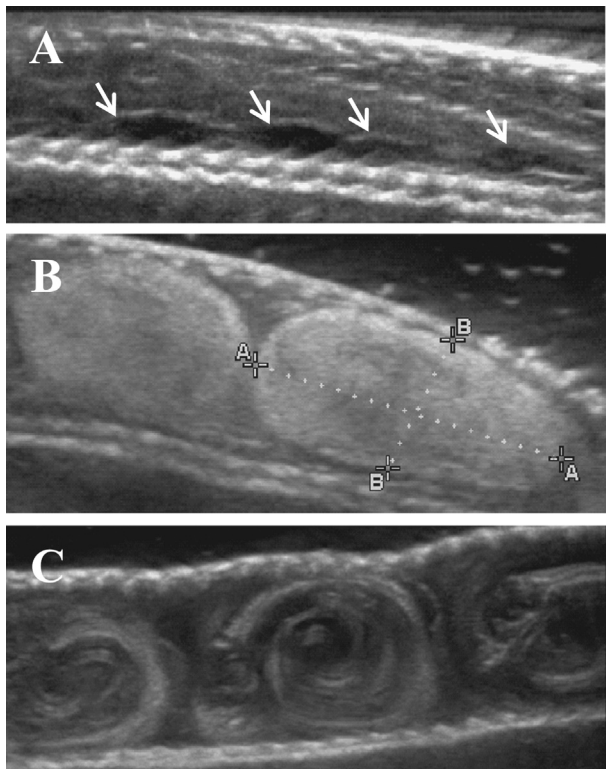


Fig. 2 Ultrasound images of the abdominal cavity of viviparous female common garter snakes from northern California. (A) Inactive ovary; arrows indicate individual follicles. (B) Two postovulatory eggs, with A/B arrows demonstrating the full dimensions of a single egg. (C) Three late-stage embryos, with little yolk remaining, and the skull and coiled thoracic vertebrae of the middle embryo clearly evident.

environmental cues for the commencement of reproductive activity include changes in photoperiod, temperature, rainfall, resource availability, and social activity.

Evidence suggests that environmental temperature is a major cue for the commencement of the breeding season for snakes in temperate regions, where seasonal changes in temperature and resource availability are pronounced. This may be a consequence of the fact that snakes living in these regions may overwinter underground in hibernacula, where other cues such as photoperiod and rainfall may not penetrate, but surface warming can result in detectable changes in ground temperature (Krohmer and Lutterschmidt, 2011). In tropical regions, where temperatures can remain high year-round, environmental cues for breeding are less well understood. In these areas, reproductive schedules may be more determined by cycles in predators that prey on eggs or hatchlings, or by cycles of resource availability. There is also evidence that reproductive schedules in tropical snakes may be timed to coincide with annual reductions in rainfall, which reduces the danger of eggs becoming waterlogged (Brown and Shine, 2006). In both temperate and tropical regions, snakes can also respond to more immediate cues, such as pheromones from receptive females (discussed below), that trigger mating behaviour.

General trends in reproductive cycles for both males and females have been most clearly elucidated for temperate species (Seigel and Ford, 1987; Fig. 3). Spermatogenesis in temperate regions has been categorized into two general patterns: prenuptial and

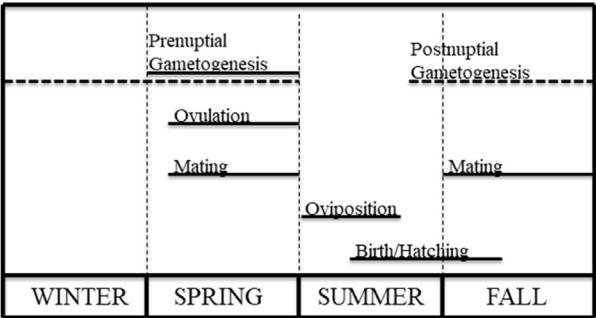


Fig. 3 A general timeline for annual reproductive events in temperate snakes.

postnuptial. Prenuptial spermatogenesis (also called the associated pattern of reproduction) is the more intuitive and widespread pattern, where sex steroids and testicular activity peak in the spring in preparation for spring mating. In contrast, for postnuptial spermatogenesis (also called the dissociated pattern of reproduction), the peak in sex steroids and testicular activity occurs in late summer and early fall, and sperm produced during this time is stored over the winter months, to be used during breeding the following spring.

In temperate regions, timing of vitellogenesis may also occur pre- or post-nuptially. For prenuptial vitellogenesis, most follicular growth occurs in the spring immediately prior to mating. Postnuptial vitellogenesis, in contrast, begins at the end of summer, and is completed post-hibernation. In some species, mating can also occur in the fall, and sperm is stored by the female in the oviduct over the winter months. Ovulation and fertilization generally occur mid to late spring, oviposition at the beginning of summer, and hatching/birth in late summer and early fall. Many species may undergo vitellogenesis and ovulation within the same year, though some species may take years for eggs to reach ovulation. Mating typically occurs after ovulation, and fertilization occurs in the oviduct; however, there is evidence that in some species, mating itself may induce ovulation (Seigel and Ford, 1987).

The vast majority of snakes in both temperate and tropical regions exhibit seasonal reproductive cycles, though reproduction may be more asynchronous among individuals and occur over a more extended season in tropical species. Even so, there is some evidence that spermatogenesis can occur continuously in some tropical species, even when mating is seasonal (Mathies, 2011). However, in spite of numerous studies in the past few decades, particularly in South America and Australia, reproductive cycles of tropical snakes have been difficult to elucidate. This is likely due to the fact that individual cycles are less synchronous and patterns in individual cycles are still largely unknown, making general population and species-specific patterns more difficult to dissect.

Courtship and Mating

Only one snake, the flowerpot snake is known to be entirely parthenogenetic and reproduce clonally (Gillingham, 1987). While facultative parthenogenesis (involving occasional clonal reproduction by unmated females) has been reported in diverse snake clades, the vast majority of snake species reproduce sexually (Booth and Schuett, 2016). Thus a major component of reproduction in snakes is locating a mate and successfully initiating copulation. Snakes in high latitudes can hibernate in large aggregations and are able to engage in courtship activities almost immediately after emergence. In many species, however, it is more of a challenge to locate a mate, especially when population density is low, as snakes do not vocalize, and visual signals are effective only over short distances.

One potential means for males to locate females is via pheromone trails deposited by females as they move across the landscape (Parker and Mason, 2011). Pheromones are chemicals that can provide information-bearing signals to receivers. In snakes, pheromones can be detected via tongue-flicking, which collects pheromones from a substrate and delivers them to the vomeronasal organ in the roof of the mouth. Experimental studies show that males can recognize the direction of female movement given a pheromone trail. The extent to which pheromone trailing is used in the wild is unknown; however, when it occurs, it is likely complex, especially when numerous males are trailing a single female, disrupting her pheromone trail.

Whatever the means, the ability to locate females is likely a major factor in reproductive success, particularly when females are sparsely distributed. Body size also appears to be an important factor, with larger males in some species tending to obtain more matings. In European adders, for instance, the ability of larger males to obtain more matings may be due at least in part to an advantage during ritualized male-male combat (Madsen *et al.*, 1993). Smaller male adders, however, may employ alternative mating tactics, avoiding combat altogether and achieving some success in mating with females as they are leaving the territories of larger males.

Unlike other terrestrial vertebrates, snake courtship is primarily a tactile and chemosensory affair, with limited visual and auditory components. Their elongated, limbless morphology allows a high level of body contact between mating individuals. With the exception of some species that mate regularly in highly visible aggregations, reports of snake mating behaviour in the wild are largely anecdotal in nature, with a limited number of mating events completely described for any given species. However, there is enough information to suggest that courtship can be classified into three general phases: (1) tactile-chase, (2) tactile-alignment, and (3) intromission and coitus (Gillingham, 1987).

The tactile-chase phase (1) involves initial contact and preliminary courtship. During this period, males tongue-flick rapidly, evaluating pheromones from the female's skin that can encode information about species, body condition, and reproductive status. During this time males may also make visual assessments of females, and evidence suggests that males tend to prefer larger females. Since body size and body condition can be strongly positively correlated to fecundity, this suggests that both of these visual and chemosensory signals may act as honest signals of female quality. Interestingly, however, male common garter snakes can also produce "female" pheromones upon emergence from communal hibernacula (Shine, 2003). These so-called "she-males" can induce other males in large snake aggregations to attempt to mate with them. This form of female mimicry is currently thought to attract other males that can confer thermoregulatory advantages for cold snakes that are just emerging, by allowing heat transfer between individuals and reducing exposure. Furthermore, attracting other males may help shield female mimics from predators, which can be numerous at den sites. Since species such as this form mating balls that may involve numerous males attempting to mate with a putative female, both thermoregulatory and antipredator benefits could be substantial. Whatever the case, this appears to be a short-term strategy, as female pheromones tend to dissipate from males soon after they emerge, and males do show preference for true females over female mimics.

After preliminary contact, a male will attempt to mount the female. If the female flees, the male may pursue her and attempt to mount again. If mounting is successful, the male will engage in precopulatory behaviours such as chin rubbing on the female dorsum, undulation, caudocephalic and/or cephalocaudal waves (waves of contraction running from head to tail, or vice versa), tail whip, head shake, and courtship biting, with some clades more likely to exhibit certain behaviours than others. Male pythons or boas, for instance, use small vestigial hindlimbs called pelvic spurs to “spur” or stroke the female back. Chin-rubbing on the female’s dorsum and head/body jerking appears to have evolved ancestrally in the Colubroidea and is widespread throughout that clade (Senter *et al.*, 2014).

The tactile-alignment phase (2) begins with the first copulation attempt, and involves tail searching behaviour, wherein the male attempts to align his cloaca with that of the female. This is the most consistent courtship behaviour across taxa. If the search is successful, and female gaping of the cloaca occurs, there is hemipene intromission and coitus (3). Copulation time varies considerably among species, with some requiring only a few minutes, and others remaining joined for over 24 h. Short copulatory times are hypothesized to be favored due to heightened predation risk during mating; however, sexual selection may favour longer copulatory times in some species as it may allow a male to prevent competitors from mating with the same female.

There appears to be little direct post-mating interaction between males and females. In some species, particularly those that engage in male-male combat, males may occasionally guard females after mating. In other species, a male may leave the female with a gelatinous copulatory plug, which derives from secretions from the sexual segment of the kidney and is deposited during ejaculation. It was originally proposed that this plug primarily functioned as a form of mate guarding, effectively preventing mating by other males in the short-term. However, recent work suggests that this may not be the only (or a particularly effective) function of copulatory plugs, at least in common garter snakes (Friesen *et al.*, 2013). Rather, it may also prevent the sperm leakage and loss immediately after copulation and act as a spermatophore, slowly releasing sperm as the plug degrades. There is some evidence that male garter snakes may deposit an inhibitory pheromone into the female that reduces her receptivity and attractiveness to other males; however, controversy remains as to whether an antiaphrodisiac pheromone such as this exists, or whether female willingness to mate again is driven by other factors, such as cryptic female choice based on quantity and quality of sperm received from her previous mate (Friesen *et al.*, 2014).

Mating systems among snakes tend to be polygynandrous, with both males and females mating multiple times. Sperm from each mating is stored in sperm storage tubules within the female urogenital tract, and multiple paternity is widespread throughout the snake phylogeny (Jellen and Aldridge, 2011). There is some evidence that multiple paternity can increase female fitness. However, little is currently known regarding the dynamics of cryptic female choice in snakes, whereby a female’s reproductive tract gives fertilization preference to some sperm over others. Studies using molecular methods to investigate paternity dynamics in the wild suggest that in some cases sperm from larger males, or males that are last to mate may fertilize the most eggs; other work, however, shows no clear relationship between male body size and precedence and fertilization success (Clark *et al.*, 2014).

Parental Care

Parental care can be defined as any non-genetic contribution by a parent that increases the fitness of offspring, and can occur before or after laying or birth (Stahlschmidt and DeNardo, 2011). In snakes, females are the primary parents providing care for offspring. Female energetic investment and thermoregulatory choices constitute most basic forms of prenatal parental care. On an energetic level, this may include the quantity and type of fats and proteins deposited by a female into her eggs during vitellogenesis. Female snakes are also known to transfer minerals, immunoglobins, antioxidants, and defensive toxins into their eggs, though the adaptive significance of such provisioning is still unclear. The amount of investment in eggs can contribute to individual differences in growth rate and offspring survival.

Behavioral thermoregulation by females is another critical component of prenatal parental care, wherein gravid females alternately bask and seek shade to modulate their body temperature, sometimes in aggregations with other gravid females. Even oviparous squamates may complete at least a quarter of embryonic development prior to oviposition, such that embryos in both oviparous and viviparous species are dependent on maternal thermoregulatory behaviour for optimal developmental conditions. In fact, one of the major hypotheses for the evolution of viviparity in squamates, the Cold Climate Hypothesis, suggests that viviparity has evolved primarily in cold climates due to increased maternal control of temperature in developing embryos (Stahlschmidt and DeNardo, 2011). Viviparous females can also be involved in hydoregulation of embryos during development, transferring water via placental connection. A more generalized hypothesis for the evolution of viviparity, the Maternal Manipulation Hypothesis, suggests that females retain embryos in order to better protect them from a range of ecological challenges, ranging from excessive temperatures to dehydration risk to predation. Recent work suggests that the evolution of viviparity itself may have introduced novel selective pressures on embryos competing for water within their mother’s body, particularly in arid or marine environments where fresh water is limited (Bonnet *et al.*, 2017).

For oviparous species, nest-site selection is an important facet of parental care. Snakes commonly oviposit in areas such as rock crevices, rodent burrows, and rotting logs. In some cases, female may excavate sites with soft substrate. Some may choose sites with eggshell remnants indicating successful incubation in the past, and/or return to a site where they themselves have previously nested. Females must select nest sites in areas with appropriate magnitude and variability in environmental temperature for embryonic development within the egg, and appropriate levels of humidity and substrate water content (neither too dry nor too wet) to aid in hydoregulation. Another critical factor in nest-site selection is minimization of predation risk, as a variety of predators,

ranging from birds to other snakes, can pose a risk to egg survival. Some species are well known to nest communally. Though the significance of such behaviour is unknown, potential explanations range from defense from predators, to thermoregulatory and hydoregulatory benefits of clustering, to hatching synchrony via communication among embryos. Recent experimental research in water snakes suggests that embryos incubated without contact with siblings are generally less sociable after birth, indicating that there may be communication, possibly via heartbeats or odours, among eggs laid in the same nest that influences social bonding (Aubret *et al.*, 2016).

Parental care after birth or oviposition has been documented only occasionally or not at all in most snake families. Reports generally involve females remaining with eggs or neonates. Benefits of maternal attendance could involve thermoregulation, hydoregulation, active defense from nest predators, and/or cryptic colouration of the mother concealing white eggs from predators. At this point, it is unclear whether reports of this type of parental care are sporadic because they are uncommon, or because snakes tend to be secretive, allowing limited opportunities for observation of their interactions with offspring. That said, postnatal care has been well-documented in two major clades: pit vipers and pythons (Stahlschmidt and DeNardo, 2011). In temperate pit vipers, such as rattlesnakes, neonate attendance is widespread, and generally lasts until the first shedding (ecdysis) of neonates. In pythons, egg attendance may involve the female coiling around her clutch and engaging in shivering thermogenesis, generating heat to warm the eggs. Coiling around eggs may also prevent both heat and water loss.

Life History Variation

Snakes exhibit remarkable diversity in life-history traits, ranging from age and size at maturity, to reproductive lifespan, to size and quantity of young. For instance, while many snakes may have clutch sizes ranging from two to two dozen, there are reports of individuals of some species having clutch sizes of over 50, or even more than 100 offspring. One of the major factors affecting life history variation is maternal body size, with larger females tending to have more offspring due to a greater capacity within the abdominal cavity to accommodate eggs. Larger females also tend to have larger offspring, though this relationship does not appear to be as strong (Seigel and Ford, 1987).

The relationship between maternal size and clutch size is not perfect, as number of offspring may also be related to phylogenetic constraints (i.e., the constraints on reproduction within a particular lineage), size of individual offspring, and ecological context. Size of offspring may trade off with number for any given amount of energy available for reproduction, resulting in a choice of whether to make a few large young or many small (Seigel and Ford, 1987). Furthermore, the ecological context, which includes factors such as availability of prey and predation risk, can generate both plastic (i.e., environmentally induced) and genetic variation in life-history strategy.

Intraspecific geographic variation in life-history strategy among snakes has been described across latitudinal and altitudinal gradients, between island and mainland populations, as well as across habitat types within the same region. Well-characterized populations of western terrestrial garter snake in California, United States, provide a prime example of geographic variation in life-history strategy across habitat types (Bronikowski and Arnold, 1999). These populations are distributed across two major habitat types within the same vicinity: the rocky lakeshore of a large natural lake, and nearby grassy montane meadows. Differences among habitats are thought to be major factors in the evolution of two distinct life-history ecotypes. Lakeshore snakes exhibit rapid growth to large body sizes, early maturation, large litters, and short lifespan, whereas meadow snakes exhibit slow growth to smaller body sizes, delayed maturation, small litters, but more extended lifespans (Table 1). Evidence suggests that higher resource availability as well as higher predation pressure have selected for a faster life-history strategy in lakeshore populations, which may have generated a trade-off between fast growth/high reproduction and lifespan, as theory would predict. Interestingly, while classic life-history theory predicts that fast-living organisms should age more rapidly, lakeshore snakes do not show evidence of faster reproductive senescence than meadow snakes (Sparkman *et al.*, 2007). Rather, lakeshore snakes continue to increase their reproductive output as they get older and larger. This indeterminate growth has been proposed as a means of evading some of the trade-offs expected in determinately growing species. Though the two ecotypes differ according to a variety of physiological metrics, such as metabolic rate, DNA damage and repair, innate immunity, and corticosterone levels, the physiological mechanisms underlying differences in reproduction per se between the two habitat types have not been fully elucidated (Schwartz and Bronikowski, 2011). However, there is some evidence that ecotype-differences in circulating insulin-like growth factor-1 in the wild may be related to differences in litter size.

Table 1 Differences in body size (snout-vent length) and reproductive variables between two garter snake life-history ecotypes residing in meadow and lakeshore habitats in the vicinity of Eagle Lake, California, United States

	<i>Meadow ecotype</i>	<i>Lakeshore ecotype</i>
Mean adult body size	538 mm (range: 370–598)	660 mm (range: 395–876)
Female maturation size/age	400 mm/5–7 years	425 mm/3 years
Reproductive rate	Biennial or even less frequent	Annual
Mean litter size	4.3 (range: 1–6)	8.8 (range: 1–21)
Newborn mass	2.85 g (range: 1.8–3.5 g)	3.27 g (range: 2.5–4.2 g)

In general, reproductive life history in snakes, as in other species, is a negotiation between the fitness costs and benefits involved in any given reproductive event. In snakes, costs to gravid or pregnant females include not only the energetic costs of egg production, but also reduced mobility and increased predation risk. Furthermore, frequency of reproduction in snakes can be a function of both past and current resource availability, with some species relying more on energetic stores from the previous years (capital breeders), and others relying more on current foraging (income breeders). In contrast to lizards and turtles, which commonly reproduce annually with multiple clutches, snake species in temperate regions appear to be restricted to a single litter or clutch per year in the wild, likely due to both seasonal and energetic constraints. However, there have been some recent reports of multiple clutches per year in tropical species (Mathies, 2011). Furthermore, frequency of reproduction across years may differ phylogenetically and geographically, or even among individuals within a population, with females breeding every year in some cases, or every other year (or even less frequently) in others.

Conclusion

While much valuable knowledge has been gained thus far regarding the physiological, ecological, and evolutionary dynamics of reproduction in snakes, much remains to be learned. Studies have become increasingly sophisticated over recent years, but the use of advanced techniques, such as molecular methods for studying mating systems and sexual selection, has only just begun. Similarly, ultrasonography has yet to be used to its full potential to discern patterns and test hypotheses on topics ranging from the physiology of maternal-fetal interactions to phenological changes in development in response to global climate change. Furthermore, as technologies to track and monitor individuals in the wild continue to advance, and more long-term studies are conducted in a broader array of species, much-needed data on factors involved in annual cycling, mate choice, nesting, parental care, and reproductive effort will continue to fill in our understanding of the ways in which reproductive physiology, behaviour and life history vary within and across species, and across ecological contexts.

References

- Aubret, F., Bignon, F., Kok, P. J. R., & Blanvillain, G. (2016). Only child syndrome in snakes: Eggs incubated alone produce asocial individuals. *Scientific Reports*, 6, 35752.
- Bonnet, X., Naulleau, G., & Shine, R. (2017). The evolutionary economics of embryonic-sac fluids in squamate reptiles. *The American Naturalist*, 189, 333–344.
- Booth, W., & Schuett, G. W. (2016). The emerging phylogenetic pattern of parthenogenesis in snakes. *Biological Journal of the Linnean Society*, 118, 172–186.
- Bronikowski, A. M., & Arnold, S. J. (1999). The evolutionary ecology of life history variation in the garter snake *Thamnophis elegans*. *Ecology*, 80, 2314–2325.
- Brown, G., & Shine, R. (2006). Why do most tropical animals reproduce seasonally? Testing hypotheses on an Australian snake. *Ecology*, 87, 133–143.
- Clark, R. W., Schuett, G. W., Repp, R. A., et al. (2014). Mating systems, reproductive success, and sexual selection in secretive species: A case study of the western diamond-backed rattlesnake, *Crotalus atrox*. *PLOS ONE*, 9, e90616.
- Friesen, C. R., Shine, R., Krohmer, R. W., & Mason, R. T. (2013). Not just a chastity belt: The functional significance of mating plugs in garter snakes, revisited. *Biological Journal of the Linnean Society*, 109, 893–907.
- Friesen, C. R., Uhrig, E. J., & Mason, R. T. (2014). Females remate more frequently when mated with sperm-deficient males. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 321, 603–609.
- Gillingham, J. (1987). Social behavior. In J. T. Collins, R. A. Seigel, & S. S. Novak (Eds.), *Snakes: Ecology and Evolutionary Biology* (pp. 184–209). New York, NY: Macmillan.
- Jellen, B., & Aldridge, R. (2011). Paternity patterns. In D. M. Sever, & R. D. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 619–644). Enfield, NH: Science Publishers.
- Krohmer, R. W., & Lutterschmidt, D. I. (2011). Environmental and neuroendocrine control of reproduction in snakes. In D. M. Sever, & R. D. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 289–346). Enfield, NH: Science Publishers.
- Madsen, T., Shine, R., Loman, J., & Håkansson, T. (1993). Determinants of mating success in male adders *Vipera berus*. *Animal Behaviour*, 45, 491–499.
- Mathies, T. (2011). Reproductive cycles of tropical snakes. In D. M. Sever, & R. D. Aldridge, (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 511–550). Enfield, NH: Science Publishers.
- Parker, M., & Mason, R. (2011). Pheromones in snakes: History, patterns and future research directions. In D. M. Sever, & R. D. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 551–572). Enfield, NH: Science Publishers.
- Schwartz, T., & Bronikowski, A. (2011). Molecular stress pathways and the evolution of life histories in reptiles. In T. Flatt, & A. Heyland (Eds.), *Molecular Mechanisms of Life History Evolution: The Genetics and Physiology of Life History Traits and Trade-Offs* (pp. 193–209). Oxford, England: Oxford University Press.
- Seigel, R., & Ford, N. (1987). Reproductive ecology. In J. T. Collins, R. A. Seigel, & S. S. Novak (Eds.), *Snakes: Ecology and Evolutionary Biology* (pp. 210–252). New York, NY: Macmillan.
- Senter, P., Harris, S. M., & Kent, D. L. (2014). Phylogeny of courtship and male-male combat behavior in snakes. *PLOS ONE*, 9, e107528.
- Shine, R. (2003). Reproductive strategies in snakes. *Proceedings of the Royal Society of London B: Biological Sciences*, 270, 995–1004.
- Sparkman, A. M., Arnold, S. J., & Bronikowski, A. M. (2007). An empirical test of evolutionary theories for reproductive senescence and reproductive effort in the garter snake *Thamnophis elegans*. *Proceedings of the Royal Society of London B: Biological Sciences*, 274, 943–950.
- Stahlschmidt, Z., & DeNardo, D. (2011). Parental care in snakes. In D. M. Sever, & R. D. Aldridge (Eds.), *Reproductive Biology and Phylogeny of Snakes* (pp. 673–702). Enfield, NH: Science Publishers.

Reproduction in Nonhuman Primates

David H Abbott, University of Wisconsin, Madison, WI, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Today, almost 400 species of nonhuman primates live across five continents in forests, savannas, grasslands, semi-deserts, snowy mountains and even cosmopolitan cities. While some are mostly solitary, many are social, ranging from breeding pairs and small family groups to large gregarious troops comprising multiple breeding males and females and their offspring. “The nonhuman primate” is therefore a misnomer, even though the term is widely used, and usually means a select few species of macaques and baboons (Strier, 1994). Nonhuman primate diversity is rich, highly evolved and comprises five “natural groups”: Malagasy (Madagascar) lemurs, the loris group, tarsiers, New World monkeys (NWP), and most well-known, Old World monkeys, apes and humans (OWP) (Martin, 2007). NWP and OWP are collectively known as anthropoid primates. Most nonhuman primates live in the tropics and sub-tropics, so seasonality in breeding is regulated by reliable changes in climate, such as dry or rainy seasons, e.g., New World cotton top tamarins (*Saguinus oedipus*), in contrast to those in more temperate climes in which seasonal breeding is regulated by changes in day length, e.g. Old World Japanese macaques (*Macaca fuscata*) or “snow monkeys”. These five “natural groups” nicely delineate female reproductive differences, while in contrast, different types of mating systems better delineate male differences (Table 1).

So, how to address diverse nonhuman primate reproductive variation? We will examine selected aspects of reproductive biology for both males and females, illustrating reproductive specialization together with how sex-typical characteristics differ. In doing so, it will become clear how much we know about reproductive biology in a few, biomedically relevant nonhuman species, and how little we know about reproduction in most others. This skew in knowledge is becoming more pronounced as we realize the potential for genetic engineering of nonhuman primate oocytes, spermatozoa, and even mature adult somatic cells, as the bases for eradicating genetically-based diseases in ourselves (Sasaki, 2015).

Table 1 Distinct aspects of nonhuman primate reproduction

Males

- Permanent descent of the testes into a scrotum behind the base of the penis, typically around the time of birth
- In certain species, investment in individual offspring is considerable partly because age at sexual maturity is attained relatively late
- Multi-male and dispersed, small group mating systems: notable sexual dimorphism, relatively large testis size, voluminous ejaculates with high concentrations of sperm containing large, mitochondria-containing midpiece, complex penile morphology, multiple or prolonged intromissions prior to ejaculation, multiple ejaculations in relatively short period of time do not deplete sperm concentrations
- Single male and female mating systems: less extreme sexual dimorphism, less frequent and shorter duration, single intromission prior to ejaculation, multiple ejaculations in relatively short period of time deplete sperm concentrations

Females

- Complete separation of urinary and reproductive tracts
- Give birth to well-developed (precocial) neonates after comparatively long gestation
- Most species have just a single infant at a time
- Considerable investment in individual offspring, partly because age at sexual maturity is attained relatively late
- Placentation of lemurs and lorises is non-invasive, epitheliochorial
- Placentation of tarsiers, New and Old World primates is invasive, haemochorial
- Embryonic chorionic gonadotropin (CG) provides pregnancy recognition signal in New and Old World primates
- Estrous cycles: lemurs, lorises, tarsiers
- Ovarian cycles: certain New World primates
- Menstrual cycles: most New and all Old World primates

General

- Yolk-sac eliminated after mid-gestation
- Relative to body size, fetal and postnatal growth are slow
- Sexual maturity attained relatively late and life-spans are long
- New World primates exhibit transcriptional repression and decoy cytoplasmic binding with consequent elevation in circulating levels of steroid hormones
- New World primates replace pituitary LH with CG, and their LH/CG receptor only binds CG
- Old World primates retain pituitary LH and an LH/CG receptor that binds both LH and CG

Source: Adapted from Anderson M.J. and Dixon A.F., 2002. Sperm competition: Motility and the midpiece in primates, *Nature* 416, 496; Martin R.D., 2012. Primates, *Curr. Biol.* 22, R785–R790; and Strier K.B., 1994. Myth of the typical primate, *Yearb. Phys. Anthropol.* 37, 233–271.

New World Exceptionalism #1: Neuroendocrine Regulation

Typical of mammals and many vertebrates, nonhuman primate reproductive physiology is governed by the brain, in particular gonadotropin-releasing hormone (GnRH) producing neurons in the neuroendocrine hypothalamus. GnRH neurons migrate from the embryonic nose to the hypothalamus during early fetal life, and are triggered into action during puberty when they increase production and action-potential gated release of GnRH into surrounding extracellular fluid. Such synchronized bursts of GnRH release ensure rapid diffusion of high concentrations of the peptide into a network of tiny blood vessels flowing only to the anterior pituitary. At anterior pituitary gonadotrope cells, GnRH stimulates synthesis and release of the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the systemic circulation. NWP, however, differ from all other nonhuman primates and non-primate mammals, with regard to pituitary LH secretion and its action.

To put this neuroendocrine difference into perspective we need to appreciate primate phylogeny. From fossil, isotope and molecular dating, primates originated in the late Cretaceous about 80–90 million years ago. The lemur and loris lineages split off from those of the tarsiers and anthropoid primates by 65–70 million years ago. The first gene duplication of the *LHβ* gene into separate *CGβ* and *LHβ* genes occurred only in anthropoid primates, with molecular dating placing that event after the divergence of tarsiers from anthropoid primates about 45 million years ago (Maston and Ruvolo, 2002). Continental drift separated South America from Africa about 35 million years ago, thus preventing further influx of nonhuman primates to a now isolated, multi-island South America, causing the New and Old World split among anthropoid primates. Due to intense environmental selection pressure after continental separation, exaggerated expression of an otherwise rare variant of the 12-exon gene coding for primate LH/chorionic gonadotropin (CG) receptor type 1 (*LHCGR type 1*) occurred among NWP, alone. This genetic variant expresses an *LHCGR* that omits exon 10, designated *LHCGR type 2*, and binds CG, alone, and not LH. Not surprisingly in these circumstances NWP, unlike their OWP counterparts, retained pituitary gonadotrope transcriptional responsiveness to GnRH for the β subunit of CG, alone, instead of LH. OWP, in contrast, silenced pituitary transcription for the β subunit of CG (Troppmann et al., 2013).

Females in both anthropoid primate groups, however, retained CG expression in the embryonic trophoblast and fetal placenta as the “pregnancy recognition” signal to the mother. CG from the embryo binds to *LHCGR* on the maternal ovarian corpus luteum (CL), slowing the CL’s natural demise, preventing menses and prolonging the CL’s copious production of progestins, estrogens and relaxin required to support a nurturing uterine endometrial environment until the luteo-placental shift, when the maturing fetal placenta becomes self-sufficient in hormone production. NWP placentae employ a different gene promoter sequence for CG transcription than found in pituitary gonadotropes, reflecting differing transcriptional control for testis-ovary versus placental function. As clearance of CG from the circulation is prolonged in comparison to LH, episodic bursts of GnRH-stimulated CG release are relatively indistinct in terms of increased CG levels in the blood and so do not emulate LH reliability as a peripheral biomarker for frequency and amplitude of GnRH release. CG action in NWP, nevertheless, closely emulates that of LH in terms of stimulating testicular and ovarian function.

New World Exceptionalism #2: Steroid Hormone Synthesis and Action

Circulating levels of NWP steroid hormones, including testicular and ovarian progestins, estrogens and androgens, are commonly 5–10 times those found in OWP and other nonhuman primates, again reflecting NWP adaptation to environmental selection pressure following continental drift separation from OWP about 35 million years ago. Exaggerated expression of intra-cellular chaperone molecules diminish steroid hormone-mediated transcriptional action, and at least for progesterone (P_4), compensatory consequences led to the silencing of liver production of the circulating steroid hormone binding protein, cortisol-binding globulin (CBG), resulting in ~100% unbound and bioavailable circulating P_4 . Testosterone (T) and estradiol (E_2) prove exceptions to absent circulating binding globulin, since sex hormone binding globulin (SHBG) remains expressed, enabling anthropoid-typical bioavailability of unbound androgens and estrogens at usually <5% of total circulating concentration for each hormone (Kraynak et al., 2018).

In specific consideration of P_4 and T, exaggerated intracellular expression of the heat shock protein 90-bound immunophilin, FKBP51, diminishes high affinity intracellular binding of these steroid hormones and subsequent formation of transcription complexes by respective cytoplasmic or nuclear P_4 (PR) or androgen (AR) receptors. Increased expression of heat shock protein (hsp) in OWP usually only occurs when cells are exposed to altered protein folding, such as during heat exposure or malignancy, and buffers the cells from succumbing to apoptosis, oxidative stress or a variety of cell death mechanisms triggered by immune responses. Accompanying diminished expression of FKBP52, a molecular chaperone enabling high-affinity receptor binding and transcription complex formation, reinforces this intracellular block. Since FKBP51 expression, at least, is regulated by both P_4 and T, these contrasting differences between NWP and OWP molecular engagement of P_4 and T suggest accompanying contrasting differences in rapid, cell-specific ultrashort feedback loops regulating PR- and AR-mediated signaling.

Diminished NWP E_2 -mediated gene transcription, however, is accomplished by different, but analogous, intracellular mechanisms. Exaggerated expression of hsp27, also called HspB1, diminishes high affinity binding of E_2 and formation of transcription complexes by estrogen receptors (ER) ERalpha and ERbeta. In addition, over-expression of another intracellular binding protein, estrogen response element-binding protein (ERE-BP), also called heterogeneous nuclear riboprotein C-like 1 (hnRNP C-like 1), an RNA splicing repressor binding microRNAs, further contributes E_2 insensitivity. ERE-BP competes with classic, E_2 -bound ERalpha and ERbeta assembled transcription complexes for ERE-DNA binding sites around or within target genes. Such transcriptional

repression and decoy cytoplasmic binding are both overcome with elevated circulating levels of E_2 . E_2 -ER displacement of ERE-BP from DNA, however, liberates the RNA splice repressor to alter RNA splicing that may include its binding to microRNA and its interactions with intra-cellular proteins.

These complex intracellular molecular differences between NWP and OWP are still not fully understood in terms of steroid hormone action, and are particularly of interest as they also play key roles in cellular responses to cancer. Both OWP and NWP, however, differ from many non-primates in using the delta-5 steroidogenic pathway to derive androgens. In other words, anthropoid primate dehydroepiandrosterone (DHEA) is the major precursor for androstenedione (A_4) and T, not 17-hydroxyprogesterone. This becomes important when treating aspects of infertility and hormone abnormalities in primates.

Male Reproductive Physiology

Pituitary LH (all except NWP) or CG (NWP) acts on testicular Leydig cells to metabolize cholesterol into T, while FSH acts on Sertoli cells located within testicular seminiferous tubules, enabling their responsiveness to T and their metabolism of T into E_2 crucial for nurturing male germ cells into spermatozoa (sperm) and for their transport to the epididymis for maturation into motile, fertile germ cells. Epididymal sperm are ejaculated with prostatic and seminal fluids. Sperm maturation cycles in nonhuman primate testes, however, are not organized into synchronized tubule segments, in contrast to mice and rats.

In addition to its actions within the testes, T is released into the blood to differentiate and support species-typical male body structures and functions, including behavior, and to provide negative feedback control on hypothalamic GnRH and pituitary LH or CG to assure optimal (homeostatic) circulating levels of T. As AR and ER are both involved in mediating testicular T negative feedback on GnRH release in male nonhuman primates, and GnRH neurons do not express AR or ER, local hypothalamic conversion of T to E_2 completes feedback action through neighboring kisspeptin and neurokinin B neurons that synapse on to GnRH neurons. In adult males, kisspeptin and neurokinin B neurons do not exhibit integrated regulation of GnRH, in contrast to females (Garcia *et al.*, 2017). The testes, alone, produce insufficient circulating levels of E_2 to fully complete negative feedback control without contributions from testicular T and hypothalamically-derived E_2 .

While GnRH also stimulates FSH synthesis and release from pituitary gonadotropes, and thus negative feedback regulation of GnRH contributes to FSH release, testicular Sertoli cells synthesize and release inhibin B that specifically engages the majority of FSH negative feedback regulation at pituitary gonadotropes, alone. Inhibin B does not cross the blood-brain barrier and does not regulate GnRH. This additional negative feedback on FSH is required to ensure optimal FSH stimulation of spermatogenesis-nurturing Sertoli cells.

From experiments with fetal rhesus macaques, *Macaca mulatta*, negative feedback control in males develops by mid-gestation, in contrast to sometime after birth in females. Rising fetal T levels, released from the fetal testes, program male hypothalamus to respond only to ~5 times the concentrations of E_2 that are required in females to engage negative feedback control. This ensures considerably higher circulating levels of T compared to females because of the need to differentiate and support male phenotype. When female rhesus macaque hypothalamus are re-programmed to a male-like hypothalamus by experimental administration of T before mid-gestation, higher than normal levels of GnRH and LH become the norm because ovarian hormones, alone, are insufficient to fully control re-programmed negative feedback. As adults, ovaries of such gestational T-exposed females produce and release too much T, partly because of increased circulating levels of LH (Barnett and Abbott, 2003).

Positive feedback, a response that drives ovulation in females, is retained in the hypothalamus of male anthropoid primates. This is in complete contrast to male non-primate mammals in which T conversion to E_2 in the fetal male hypothalamus during sexual differentiation eradicates any positive feedback response in addition to programming for male-like behavior. In fetal anthropoid primates, androgens perform these roles. Male nonhuman primates, such as New World common marmosets (*Callithrix jacchus*) or Old World rhesus macaques, release a surge of LH in response to rapidly increasing concentrations of E_2 in the blood. Such blood E_2 dynamics are normally only produced by dominant, pre-ovulatory ovarian follicles in order to trigger a GnRH-induced LH surge that will ovulate one or more oocytes from within pre-ovulatory follicle(s). Consequently, when adult male rhesus macaques receive ovarian transplants, they spontaneously exhibit ovulatory cycles, confirming the absence of an otherwise typical sexual dimorphism in mammalian reproduction.

Male Mating Systems Determine Male Reproductive Attributes

In most, but not all, nonhuman primate species, adult males are larger than adult females. At one extreme is the flamboyantly colored male mandrill, *Mandrillus sphinx*, from west Africa weighing in at 30–35 kg, over three times the size of mandrill females, while at the other is the diminutive, monochromatic male common marmoset, *Callithrix jacchus*, from northeastern Brazil weighing in at 0.3–0.4 kg, equaling that of marmoset females. Robust sexual selection is responsible for the standout appearance of male mandrills, in contrast to its meagre presence in marmosets (Dixon, 2012). Mandrills live in multi-male troops or male-only groups, in which multiple males mate with a female when she is most fertile, around the time of ovulation, and exhibit seasonally-heightened, intense competition for access to breeding females. Male marmosets, on the other, usually live in extended, single family groups, in which male competition for breeding females is considerably less, probably due to the need for breeding males to contribute substantially to infant care, including carrying paternal twins, the usual birth quotient, from within a few minutes of birth.

Socially dominant, male nonhuman primates living in multi-male mating systems or in dispersed, small group mating systems exhibit T-dependent increased body size that can include pronounced body or scrotal coloration, body appendages such as facial phalanges, relatively large testicular sizes for their body weight, and large numbers of highly motile sperm (powered by enlarged mitochondria-containing midpieces) in voluminous ejaculates that plug up a female's vagina (Anderson and Dixson, 2002). The ability to repeatedly produce such ejaculates with high concentrations of motile sperm enable a male to continue competing against its rivals from within the mated female's reproductive tract and gain first access to an ovulated oocyte, and thus male reproductive success. Complex penile morphology and copulatory patterns, including multiple intromissions or prolonged intromission prior to ejaculation, also contribute to competitive strategies when more than one male mates the same female in quick succession.

Relatively meek, socially subordinate males in multi-male mating systems have developed 'sneaky' strategies to mate fertile females, including befriending females, i.e., low-ranking male rhesus macaques, or forceful mating ('raping') females, i.e., low-ranking male orangutans (*Pongo* spp.). Subordinate males diminish attracting the wrath of high-ranking males by minimally developing species-specific male attributes, such as color (mandrills) or facial phalanges (orangutans) (Setchell and Dixson, 2001). From genetic testing of free-ranging populations of nonhuman primates with multiple male mating systems, however, both dominant/overt and subordinate/sneaky male mating strategies garner reproductive success for both types of males.

Family-dwelling, breeding male marmosets, at the opposite end of this male attribute spectrum, ejaculate less frequently and usually only with their breeding female around the time of ovulation. Conception occurs, nevertheless. Single, relatively short duration penile intromission is typical of sexual intercourse among these more monogamous, single breeding male and female mating systems when considerably less male competition for females is the norm.

Male muriqui (*Brachyteles* spp.), living in multi-male and female groups in the Atlantic coastal forests of Brazil, bring a fascinating variation to the multi-male mating system (Strier, 1994). Male muriqui usually do not migrate between groups, so most males within a group are related. Within group males commonly embrace one another as a means of strengthening their egalitarian relationships. Male muriqui mate with multiple females, have relatively large testes, but do not physically compete for females. Male competition appears limited to sperm interactions within a female's reproductive tract.

Typical of male mammals, pre-optic, anterior and ventromedial hypothalamic areas of the brain are crucial for male sexual interest and sexual behavior, at least in NWP marmosets and OWP rhesus monkeys. Brain lesions in these areas eliminate male marmoset sexual interest in and behavior with female partners, while their reproductive physiology is otherwise normal.

Female Reproductive Physiology

At the beginning of each ovulatory cycle, pituitary LH (all except NWP) or CG (NWP alone) acts on theca cells in sufficiently mature ovarian follicles to metabolize cholesterol into androstenedione (A_4), an androgen with little bioactivity. The ovary, unlike the testis, favors production and release of a non-potent androgen to convert into estrogen, with little risk of developing male-like characteristics. A_4 is converted into E_2 within the same follicles by the neighboring inner layer of granulosa cells that nurture a single oocyte. During the early-to-mid follicular phase, FSH alone acts on granulosa cells stimulating them to release inhibin B in addition to E_2 . A few (all, except OWP and most NWP), or one (OWP and most NWP) of these follicles, undergo selection into a dominant follicle(s). A dominant follicle expresses LH receptors on its granulosa cells in addition to those for FSH, enabling its continued growth and maturation. Remaining non-dominant follicles become atretic due to inhibin-driven suppression of FSH. Onset of granulosa cell LH receptor expression also initiates gradual luteinization, a phenotypic change of theca and granulosa cells into cells emphasizing production of P_4 over E_2 . Dominant follicles contribute to the clamp on FSH release through an LH- (or CG-) mediated switch to inhibin A production. Rapidly rising circulating E_2 levels from dominant follicles trigger a positive feedback response, instigating greatly elevated release of LH (or CG) that triggers ovulation of an oocyte and its surrounding layer of specialized granulosa cells, cumulus cells, from each dominant follicle, and completes the conversion of theca and granulosa cells into the luteal cells of a corpus luteum (CL), predominantly secreting P_4 . Nonhuman primate CLs, unlike their non-primate counterparts, recover their ability to produce and release A_4 (luteinized theca cells) and E_2 (luteinized granulosa cells). They also continue to secrete inhibin A, clamping FSH at low levels. There are no uterine derived sources of luteolysis. Nonhuman primate CLs have endogenous biological clocks that progressively disengage luteal cell responsiveness to pituitary LH (or CG). High levels of embryo-produced CG are needed to 'rescue' the CL to support early pregnancy until the fetal placenta eventually assumes this role (Table 1).

Hypothalamic GnRH, in an analogous fashion to males, governs female nonhuman primate reproductive physiology. In contrast to males, negative feedback regulation engages after birth and is responsive to lower circulating levels of steroid hormones, particularly E_2 and P_4 , and positive feedback engages at puberty to regulate each ovulation. In contrast to non-primate female mammals, GnRH and surrounding neurons in the medial basal hypothalamus, alone, mediate feedback regulation in nonhuman primate females, and anterior pituitary gonadotrope cells exhibit E_2 -induced positive feedback LH surges without requiring a preceding GnRH surge. Negative feedback includes integrated hypothalamic kisspeptin and neurokinin B neuronal regulation, again in contrast to nonhuman primate males. Interestingly, ovarian and extra-ovarian (likely hypothalamic) production of E_2 provide separate, additive contributions to negative feedback (Kraynak et al., 2018). Production and release of E_2 from the hypothalamus is even more crucial for positive feedback, as blockade of E_2 synthesis at the site of hypothalamic GnRH release prevents rapidly rising circulating E_2 levels from eliciting an ovulation-inducing LH surge (Kenealy et al., 2017). Such distinct neuroendocrine regulation of female nonhuman primate reproduction in anthropoid

primates may have accompanied a degree of emancipation of reproductive physiology and behavior from the dictat of neuro-endocrine determination in non-anthropoid primate and non-primate females.

Female Phylogeny Aligns With Estrus, Ovarian and Menstrual Cycles

Estrous cycles, typical of the more ancient form of female reproductive cycle common to many non-primate mammals, predominate in lemurs, lorises and tarsiers. Elevated ovarian E_2 from one or more dominant follicles immediately preceding ovulation dictates 4–5 day opening of the vagina to accompany onset of female sexual behavior and receptivity to males, or estrus (Bogart *et al.*, 1977). Female sexual behavior and patent vagina only occur during this limited, E_2 -determined period of time, but there are species exceptions to vaginal closure outside estrus, e.g., ringtailed lemur, *Lemur catta*. Estrus is usually accompanied by changes in female genital scent and color, lasts ~1–8 days, and all aspects are attractive to males. Lemurs, lorises and tarsiers have well-developed vomeronasal organs in both sexes, and thus employ a substantial secondary sense of smell, particularly for non-volatile molecules, that directly alters behavior and physiology without engaging cognitive processing. Mating with a male, however, is not necessary for ovulation to occur. Photoperiod-entrained, annual synchrony of estrus is best demonstrated by Malagasy lemurs: all conceptions occur within one, 4–8 week period each year (Pereira, 1991). Progesterone secreted by the CL inhibits female estrous behavior. Estrus cycles are relatively lengthy at 33–40 days, reflecting a prolonged pre-estrous phase, without obvious elevations in circulating E_2 , and there is a distinct post-ovulatory P_4 -dominated luteal phase. It is unclear how female lemurs, lorises and tarsiers physiologically recognize conceptive cycles.

Ovarian cycles, in contrast to estrous cycles, do not rigidly constrain ovarian and extra-ovarian E_2 -supported vaginal opening or periods of female sexual behavior to periods of estrus. Ovarian cycles are exhibited by small-sized NWP, including marmosets and tamarins. Marmosets and tamarins typically ovulate two pre-ovulatory follicles per cycle, resulting in dizygotic or paternal twin gestations that share a placental circulation and the twins are hemopoietic chimeras. Non-mensing, female anthropoid primates do not require the substantial, highly vascular uterine endometrium of mensing female anthropoids because their placentae invade the uterine endometrium less aggressively to establish maternal-embryo/fetal nutrient, fluid and gas exchange. Following an internal clock-driven demise of CL(s) at the end of each luteal phase, an E_2 -dominated follicular phase comprises FSH-driven follicular recruitment that induces a growth spurt in ~6–20 non-dominant, medium-sized antral follicles per ovary. Selection of a species typical number of dominant follicles occurs around mid-follicular phase from the largest follicle(s) at that time in both ovaries combined. The consequent post-ovulatory formation of one or more CLs reflects the number of E_2 and LH/CG-driven ovulations for that cycle. In common marmosets at least, non-conceptive luteal phases (no pronounced rise in circulating levels of CG or ultrasonographic visualization of embryo sacs) can be lengthy in duration, lasting up to 29 days, approximately double that more commonly found among anthropoid female primates. As common marmosets frequently ovulate with 2–3 weeks of giving birth, and there is no lacion-induced anovulatory period typical of anthropoid primate females, marmosets carry two pregnancies each year. CL demise may therefore be less tightly controlled. Environmental and kin selection pressure on marmosets in their semi-arid, changeable and highly predated habitat of northeastern coastal Brazil may well have driven development of cooperative breeding and unusual high fecundity (Kraynak *et al.*, 2018).

Many NWP and OWP exhibit menstrual cycles, during which the end of luteal phase is marked by shedding of previously highly vascularized endometrial tissue through the vagina. The first day of menstrual bleed denotes the first day of the menstrual cycle. The endocrine dynamics of the follicular and luteal phases, and peri-ovulatory period, closely resemble those observed in ovarian cycles, and selection of a single dominant follicle is the norm. Peri-ovulatory changes in genital scent (marmosets), color (rhesus macaques) or swelling (chimpanzee spp.), or changes in chest color (Gelada baboons, *Theropithecus gelada*), attract attention and mating from male conspecifics, but females can copulate throughout the menstrual cycle.

Typical of female mammals, pre-optic, anterior and ventromedial hypothalamic areas in the brain are crucial for female nonhuman primate sexual solicitation of and receptivity towards males. Lesions in these brain areas eliminate female marmoset sexual solicitation (pre-optic) and receptivity (ventromedial).

Ovarian Androgen Excess as a Beneficial Female Phenotype

A variety of female nonhuman primates employ ovarian androgen excess during female fetal development to epigenetically reprogram (“organize”) multiple organ systems enabling better survival, fertility and fecundity. These naturally occurring attributes are further enforced (“activated”) by continuing ovarian androgen excess in adulthood. Unlike non-primates such as spotted hyenas, *Crocuta crocuta*, however, androgenized female nonhuman primates only comprise portions of a female population, not all. Lemur species commonly employ this high T female polymorphism (genetically determined) or polyethism (epigenetically determined) to dominate males, develop enhanced body size, vigorous play, and male-like, but vagina patent, external genitalia (Petty and Drea, 2015).

Studies of female macaques, however, suggest that elevated T in female nonhuman primates may have limits to its benefits. Fetal female rhesus macaques, whose mothers were exposed to T injected during early-to-mid gestation, frequently exhibit as adults ovarian hyperandrogenism, infrequent or absent menstrual cycles, impaired fertility, abnormal hypothalamic-pituitary regulation of ovarian function and metabolic dysfunction, including increased risk for type 2 diabetes mellitus (T2DM). Interestingly, these

traits emulate those found in women with polycystic ovary syndrome (PCOS). About 15% of women exhibit PCOS and usually have T levels that exceed the 95th percentile for female values. Origins of PCOS in women is unknown and there is no cure. In animal models, however, including nonhuman primates, PCOS-like traits are reliably epigenetically programmed by gestational exposure of females to higher-than-normal T levels. Consistent with a developmental origin hypothesis for PCOS, captive adult female rhesus macaques with naturally occurring circulating T levels above the 95th percentile mostly fail to get pregnant (Abbott *et al.*, 2017). These females also emulate neuroendocrine and ovarian abnormalities found in PCOS women, including a positive correlation between circulating androgen levels and anogenital distance, a biomarker for fetal T exposure. Improved understanding of high T in nonhuman female primate reproduction is providing novel insight into PCOS-like origins and its molecular pathogenesis (Barnett and Abbott, 2003).

Knowledge of Nonhuman Primate Reproduction Translates into Benefits for Conservation and for Humans

Increasingly non-invasive sampling, e.g., body hair, feces and urine, as well as sensitive and specific analytical techniques, e.g., tandem mass spectrometry, are enabling detailed understanding of reproductive function in both free living and captive nonhuman primates. Such accurate information is transforming efforts to better manage threatened and endangered populations in the wild, together with improved captive breeding program success (Jurke *et al.*, 1997).

The many shared similarities in genetics, physiology, development and aging between nonhuman primates and humans have also contributed to an impetus to better understand nonhuman primate reproduction and its potential application to human health. Researchers have developed intracytoplasmic sperm injection (ICSI) in a variety of nonhuman primates, including marmosets, rhesus macaques and yellow baboons (*Papio anubis*), in combination with assisted reproductive technologies (ART) to generate multiple pre-ovulatory follicles, enabling aspiration of high quality oocytes, and successful *in vitro* fertilization and embryo transfer resulting in term deliveries of healthy infants. Genome editing technologies, such as CRISPR-Cas9, are being used to create point mutations in marmoset embryos, such as in the LRRK2 gene associated with familial inheritance of Parkinson's disease. Such gene changes are permanently maintained, and infants carrying the desired mutation are being utilized for developmental studies aimed at developing treatments and therapies that can be translated into clinical practice (Sasaki, 2015).

References

- Abbott, D. H., Rayome, B. H., Dumesic, D. A., et al. (2017). Clustering of PCOS-like traits in naturally hyperandrogenic female rhesus monkeys. *Hum. Reprod.*, 32, 923–936.
- Anderson, M. J., & Dixon, A. F. (2002). Sperm competition: Motility and the midpiece in primates. *Nature*, 416, 496.
- Barnett, D. K., & Abbott, D. H. (2003). Reproductive adaptations to a large-brained fetus open a vulnerability to anovulation similar to polycystic ovary syndrome. *Am. J. Hum. Biol.*, 15, 296–319.
- Bogart, M. H., Kumamoto, A. T., & Lasley, B. L. (1977). A comparison of the reproductive cycle of three species of Lemur. *Folia Primatol. (Basel)*, 28, 134–143.
- Dixon, A. F. (2012). *Primate Sexuality: Comparative Studies of the Prosimians, Monkeys, Apes, and Humans* (second ed.). Oxford: Oxford University Press.
- Garcia, J. P., Guerriero, K. A., Keen, K. L., et al. (2017). Kisspeptin and neurokinin B signaling network underlies the pubertal increase in GnRH release in female rhesus monkeys. *Endocrinology*, 158, 3269–3280.
- Jurke, M. H., Czekala, N. M., & Fitch-Snyder, H. (1997). Non-invasive detection and monitoring of estrus, pregnancy and the postpartum period in pygmy loris (*Nycticebus pygmaeus*) using fecal estrogen metabolites. *Am. J. Primatol.*, 41, 103–115.
- Kenealy, B. P., Keen, K. L., Garcia, J. P., Kohlenberg, L. K., & Terasawa, E. (2017). Obligatory role of hypothalamic neuroestradiol during the estrogen-induced LH surge in female ovariectomized rhesus monkeys. *Proc. Natl. Acad. Sci. USA*, 114, 13804–13809.
- Kraynak, M., Levine, J. E., & Abbott, D. H. (2018). Insight gained from marmoset endocrine research. In R. Marini, L. Wachtman, S. Tardif, & J. Fox (Eds.), *The Common Marmoset in Captivity and Biomedical Research*. Amsterdam: Elsevier Press.
- Martin, R. D. (2007). The evolution of human reproduction: A primatological perspective. *Am. J. Phys. Anthropol.*, (Suppl. 45), 59–84.
- Maston, G. A., & Ruvolo, M. (2002). Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. *Mol. Biol. Evol.*, 19, 320–335.
- Pereira, M. E. (1991). Asynchrony within estrous synchrony among ringtailed lemurs (primates: Lemnidae). *Physiol. Behav.*, 49, 47–52.
- Petty, J. M., & Drea, C. M. (2015). Female rule in lemurs is ancestral and hormonally mediated. *Sci. Rep.*, 5, 9631.
- Sasaki, E. (2015). Prospects for genetically modified non-human primate models, including the common marmoset. *Neurosci. Res.*, 93, 110–115.
- Setchell, J. M., & Dixon, A. F. (2001). Arrested development of secondary sexual adornments in subordinate adult male mandrills (*Mandrillus sphinx*). *Am. J. Phys. Anthropol.*, 115, 245–252.
- Srier, K. B. (1994). Myth of the typical primate. *Yearb. Phys. Anthropol.*, 37, 233–271.
- Troppmann, B., Kleinau, G., Krause, G., & Gromoll, J. (2013). Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. *Hum. Reprod. Update*, 19, 583–602.

Deer Reproduction

Geoffrey W Asher, AgResearch Ltd., Mosgiel, New Zealand

© 2018 Elsevier Inc. All rights reserved.

Distribution and Diversity of Cervids

While the taxonomy of cervids is often debated (particularly at the species/sub-species level), according to [Whitehead \(1993\)](#) deer are represented by 43 species and 206 subspecies distributed widely across Europe, Asia, North America, South America and northern Africa. Their evolution and geographical radiation over the last 20–40 million years has resulted in remarkable diversity in morphology, physiology, and ecology. In particular, reproductive strategies amongst cervids vary widely within and between regions. As such, no one species can be considered to represent a ‘typical’ deer in terms of reproductive function. For example, some cervids exhibit highly seasonal patterns of birth in cool temperate climes, while others are aseasonal in equatorial regions. Also, while many species are strictly monovular and bear single offspring annually, others are normally polyovular and bear multiple offspring. Even embryonic development and placentation vary amongst species ([Asher et al., 1999a,b](#)). [Harrington \(1985\)](#) expressed this variation in terms of ‘life-history strategies’ in which cervids can be classified into the two extremes; one maximises the reproductive capacity of individuals and the potential growth rate of the population (r), and the other maximises the competitive ability of individuals and the stability of the population (K). Unstable or early-successional habitats favour the r -strategy, while stable or late-successional (climax) habitats favour the K -strategy ([Harper et al., 1961](#)). The r -strategists are identified by their greater reproductive potential, exhibiting multiple births, early sexual maturation (<1 year) and short breeding life (<10 years). They are predominantly territorial browser- or concentrate-feeders adapted to rapid colonisation of early stage successional habitats; including species such as roe deer (*Capreolus capreolus*), whitetailed deer (*Odocoileus virginianus*), moose (*Alces alces*) and numerous species of tropical, forest-dwelling deer. K -strategists have lesser reproductive potential that includes lower fecundity (i.e., singleton births) and later sexual maturation (>1 year) and are generally mixed grazer/browsers with marked gregariousness and social/behavioural plasticity. They tend to occupy climax habitats and include nearly all taxa within *Cervus* (red deer, wapiti, sika deer, sambar deer, etc.) and *Rangifer* (reindeer, caribou). Because of their ability to adapt to human management regimens, it is the K -strategists that are predominantly represented in the research literature. It is therefore important to note that while there was an increased understanding of reproductive function in cervids over the last few decades, this relates mainly to those species of importance to agriculture (i.e., deer farming) and a few endangered species undergoing ex situ conservation efforts. In general, the research has been largely directed toward successful application of assisted reproductive technologies to manipulate the genetic composition of populations (e.g., selection for improved agricultural productivity and avoidance of inbreeding depression). The studies to date are clearly biased toward a few key species, particularly the larger-bodied, monovulatory species with greater environmental and behavioural plasticity (K -strategists) that makes them suitable candidates for captive propagation. The smaller-bodied, forest-dwelling and solitary species (r -strategists) are clearly under-represented in such research. This latter group includes numerous species whose populations are under threat or are endangered (see [Asher et al., 1999a](#)).

Seasonality Verses Aseasonality

The necessity for most cervid species to give birth at an appropriate time of year for optimal survival and growth of offspring has exerted considerable influence on their reproductive physiology ([Lincoln and Short, 1980](#)). Species of northern temperate or arctic origins typically conceive in autumn and calve in summer. In contrast, species of tropical origin often exhibit limited seasonality or are completely aseasonal ([Lincoln, 1985](#)). The endogenous mechanisms governing seasonal reproductive patterns in temperate species are robust, being manifest rigorously when animals are transferred between localities despite subtle variations in seasonal feed supply. The transference of temperate species across the equator results in an exact 6-month phase change ([Marshall, 1937](#)), even though the relationship between season and feed production differs considerably between continental northern hemisphere and insular southern hemisphere environments ([Asher et al., 1993a](#)). Tropical species transferred from equatorial zones exhibit either pronounced ‘reverse seasonality’ (spring conceptions and autumn/winter calving; e.g., rusa deer, *Cervus timorensis*, in southern Australia), wide birth seasons (e.g., chital deer, *Axis axis*, in Australia) or complete aseasonality (e.g., Reeve’s muntjac, *Muntiacus reevesii*, in United Kingdom) ([Chapman et al., 1984](#); [Loudon and Curlew, 1988](#); [Monfort et al., 1991](#); [Mylrea, 1992](#)). It is noteworthy that there is a general paucity of information on the birth season of tropical species in their native tropical environment, although there are well-documented examples of species that exhibit strictly seasonal patterns of birth.

For temperate and arctic species it is generally accepted that entrainment of seasonal reproductive cycles is effected by endogenous recognition of photoperiod changes, with the majority of species initiating mating activity during decreasing day-length of late summer and autumn ([Lincoln and Short, 1980](#)). Variations in the actual mating season between species of up to 8 weeks usually offsets differences in the duration of gestation, such that parturition generally occurs in mid-summer ([Lincoln, 1985](#)). A notable exception amongst temperate species is the Pere David’s deer (*Elaphurus davidianus*), which initiates mating activity in early summer and calves in late spring despite an unusually long gestation of ~280 days ([Wemmer et al., 1989](#)).

There is increasing evidence that some tropical species also perceive, and respond to, changes in photoperiod, although in a different manner than temperate species. Rusa deer living in the tropics exhibit asynchronous antler cycles and birth timings. However, once translocated to temperate, high-latitude zones they appear to establish an evolutionary-based 'long-day' reproductive rhythm (Van Mourik and Stelmasiak, 1990). Brow-antlered Eld's deer (*Cervus eldi thamin*) exhibit strong reproductive seasonality in their sub-tropical native range. This pattern persists after translocation to high latitudes (48°N), even though this strategy results in the birth of offspring during harsh winter months (Monfort *et al.*, 1991). It is hypothesised that Eld's deer respond to the relatively subtle low-amplitude photoperiodic oscillations experienced in their native subtropical latitudes. Higher latitudes simply serve to reinforce or strengthen seasonal rhythms because the direction of photoperiodic change is similar, just of greater amplitude. Chital deer appear to exhibit only limited seasonality within their native tropics and following translocation to temperate zones. However, exogenous melatonin treatment has been shown to hasten and synchronise antler casting amongst stags (Mylrea, 1992), indicating possible photoperiodic responsiveness in a species that, in other respects, appears unresponsive. In contrast, tropical sambar deer (*Cervus unicolor*) acclimatised to temperate zones in New Zealand exhibit a loose pattern of 'reverse seasonality' in calving but fail to show any discernible seasonal patterns in prolactin secretion (Asher *et al.*, 1997a).

Because tropical cervid species are not exposed to strong circannual photoperiodic rhythms, reproductive patterns may have evolved in response to a variety of factors including seasonal fluctuations in the availability of specific food items, local resource competition amongst sympatric species (species that occupy about the same area of land but do not interbreed), or predation pressure; and many of these factors may be directly related to rainfall patterns. For example, it has been asserted that conceptions are timed in white-tailed deer living in Columbia (5°N) and the Everglades National Park (25°N) so that births occur during the dry season (Blouch, 1987; Smith *et al.*, 1996). The fawning season of hog deer (*Axis porcinus*) in Nepal (27°N) coincides with new vegetative growth that occurs after seasonal grass fires caused by lightning strikes or agricultural burning, believed to have been occurring for thousands of years (Mishra and Wemmer, 1987). However, how these species synchronise their season of conceptions to achieve birth synchrony remains to be resolved. Even species like sambar deer and chital deer sharing the same habitat in Nepal fawn 6 months out-of-phase to one another (Mishra and Wemmer, 1987).

It is this lack of reproductive uniformity amongst sympatric species in the tropics and subtropics from which can infer differing adaptations to the environment based on a variety of regulatory mechanisms. In Nepal's Royal Chitawan National Park four cervid species co-exist, including two browsers (sambar deer and muntjac), one grazer (hog deer) and one mixed feeder (chital deer). Each of these species differs in their annual reproductive cycle, habitat selection and feeding ecology (Mishra and Wemmer, 1987). Although photo-responsiveness in tropical cervid species may persist as an evolutionary relic, the tight linkage between photoperiod and seasonal reproduction observed in temperate species may not completely explain the existence of reproductive seasonality in many tropical species.

Cervid species occupying wide latitudinal and longitudinal ranges have generally developed discrete populations that exhibit pronounced phenotypic and genotypic differences. For example the red deer/wapiti species (*Cervus elaphus*) includes at least 23 recognised subspecies in a west/east cline from Western Europe to North America (Whitehead, 1993). Climatic variation between subspecies zones may be associated with subtle variation in reproductive seasonality of the various subspecies. Studies have indicated that Western European (Scottish) red deer (*C.e. scoticus*) and Eastern European red deer (*C.e. hippelaphus*) farmed in New Zealand exhibit a 2–3 week difference in rutting activity and subsequent mean calving dates (Scott *et al.*, 2006). These differences occur at the same latitudes in both Europe and New Zealand (and indeed within sympatric populations on New Zealand farms), indicating subtle genetic differences in subspecies responses to the prevailing photoperiodic regimen.

Female Reproductive Cycles

With few exceptions, female cervids are polyoestrous, and non-pregnant animals are capable of exhibiting continuously repeated oestrous cycles (some tropical species) or, more commonly, alternating periods of oestrous cyclicity and anoestrus. Oestrous cycles have been characterised for a number of species from studies on oestrous behaviour and/or luteal secretion of progesterone. In non-pregnant females of temperate species, such as red deer and fallow deer, the onset and termination of oestrous cyclicity occur in autumn and spring respectively, with 5–8 cycles expressed. Anoestrus is characterised by low peripheral plasma concentrations of progesterone indicative of complete ovulatory arrest, and may persist for 4–6 months from spring to early autumn. In marked contrast, some tropical species, such as Brow-antlered Eld's deer and rusa deer, translocated to higher, temperate latitudes exhibit 'reverse seasonality', initiating oestrous cycles in spring and entering anoestrus in the following autumn. This 6 month phase shift in tropical species within temperate climes possibly indicates retention of photoperiodism from a temperate ancestral form. Some tropical species, such as chital deer appear to have become completely aseasonal, with females exhibiting free-running oestrous cycles in the absence of pregnancy (Mylrea, 1992). There are indications for some tropical species within temperate latitudes, such as sambar in New Zealand, that responsiveness to photoperiod has been lost, as indicated by an inability to detect measurable levels of plasma prolactin in sambar hinds during the year (Asher *et al.*, 1997a).

The transition into the female breeding season is characterised by 'silent ovulations' (ovulations not preceded by overt oestrus) and short-lived (8–10 days) corpora lutea in most cervid species studied. In fallow deer (*Dama dama*), multiple successive silent ovulations leading up to the start of the breeding season have been observed (Asher, 1985). The transient nature of the preliminary corpora lutea may actually serve to promote within-herd synchrony of first overt oestrus of the season.

Subsequent oestrous cycles are generally of normal duration, as determined genetically for each species, although occasional 'long cycles' have been observed in some Pere David's deer and Eld's deer (Curler *et al.*, 1988; Monfort *et al.*, 1991). Average

duration of the normal oestrous cycle ranges from 17 days in sambar and axis deer, 18–20 days in red deer and Eld's deer, 21–23 days in fallow deer, and 24–27 days in moose and blacktailed deer (*Odocoileus hemionus*). The mean duration of the oestrous cycle tends to increase progressively during the breeding season in most of these species (Guinness *et al.*, 1971; Asher, 1985; Curlewis *et al.*, 1988; Monfort *et al.*, 1991; Chapple *et al.*, 1993; Schwartz and Hundertmark, 1993; Wong and Parker, 1993).

Overt oestrous behaviour has been observed in a number of cervid species but the duration of mating receptivity at a single oestrus has been difficult to determine. In some species at least, including red deer and fallow deer, overt oestrus is generally terminated following successful copulation and may only persist for a matter of minutes. In the absence of copulation, oestrous behaviour in these species has been observed to occur for up to 24 h (Asher *et al.*, 1993b). Early studies on both of these species have shown that the onset of oestrus coincides with the onset of the pre-ovulatory LH surge, which itself peaks about 24 h before ovulation (Berg *et al.*, 1994, see Asher *et al.*, 1999a).

Luteal events during the cervid oestrous cycle are similar between species. Luteinisation of post-ovulatory follicles is associated with increased secretion of progesterone, with maximal concentrations in peripheral blood occurring between days 10 and 16 of the oestrous cycle (Day 0=oestrus). Although absolute plasma concentrations vary between species, the relative changes from Day 0 are quite similar between species (Asher *et al.*, 1999a). Luteal regression, which for fallow deer involves the classic interaction between oxytocin and prostaglandin F2a (Asher *et al.*, 1988a), occurs rapidly about 1–3 days before the return to basal progesterone concentrations and return oestrus.

Ovarian follicular dynamics during the oestrous cycle have been described from ultrasonographic studies on red deer, fallow deer and, more recently, North American Wapiti (*C.e. manitobensis*) (Asher *et al.*, 1997b, 1999b; McCorkell *et al.*, 2006). These studies, all of which involve monovulatory species, clearly demonstrated discrete, non-random patterns of antral follicular growth and regression during the oestrous cycle, with individual cycles characterised by 2–4 dominant follicle waves, similar to that observed in cattle.

Relatively little research has been dedicated to pregnancy establishment and maintenance in cervids. In the seasonally breeding red deer hind, conception rates following natural mating during the autumn rut appear to be high (>85% per mated oestrus), and early embryonic mortality (i.e., within 20 days of fertilisation) appears to be low (i.e., <1%; Berg *et al.*, 1994). Given that hinds are polyoestrus, under circumstances where nutrition and disease are not limiting, adult female red deer generally exhibit greater annual fertility (i.e., >96% pregnancy rates), albeit with lesser (i.e., <1% of pregnancies) twinning (Asher and Pearse, 2002). This overall pattern of greater annual fertility appears to be a common feature of many cervids, although actual fecundity varies between species. As expected for the greater fecund species, such as white-tailed deer and roe deer, the actual fecundity (i.e., incidence of multiple-foetus gestations) in a given year and locality is strongly influenced by habitat and nutritional quality variables.

Oocyte maturation, fertilisation and early embryonic development have recently been studied *in vivo* and *in vitro* for red deer (Berg *et al.*, 2008). These studies have shown that these events in red deer are similar to those occurring in other domestic ruminants, with the exception that the red deer embryo enters the uterus 2–3 days later at the blastocyst stage, 5–6.5 days after ovulation. Embryonic implantation in red deer occurs shortly before day 27 from ovulation (McMahon *et al.*, 1997).

Luteal support of pregnancy in red deer persist through the term of gestation, with peripheral blood progesterone concentrations remaining elevated to within 1–2 days of parturition. The contributory role of placental progesterone production in pregnancy maintenance is unknown. Studies on red deer and white-tailed deer clearly show that the presence of a functional corpus luteum is a requisite for a viable pregnancy (Asher *et al.*, 1996; Plotka *et al.*, 1982). However, ovariectomy of reindeer in late pregnancy was not always associated with abortion, indicating a possible contributing role of the fetoplacental unit in progesterone secretion and pregnancy maintenance in this species (Sjaastad *et al.*, 1990).

Importantly, these studies may not be representative of cervids in general. Placentation systems vary across cervid species (McMahon *et al.*, 1997), and in the case of one species, the roe deer, a form of embryonic diapause/delayed implantation occurs to lengthen the interval between mating and births in order to ensure optimal birth seasonality (Short and Hay, 1966). This is the only ruminant species known to exhibit delayed implantation.

Genetic and Environmental Control of Gestation Length

Cervid species have gestation lengths ranging from 200 to 280 days, loosely correlated with species mature live weight. There is a general underlying acceptance that gestation length for any given species is genetically programmed and relatively robust in the face of various environmental conditions. Recognition is given to the influence of foetal genotype as being the single most important determinant of gestation length: as well as determining interspecific differences in gestation length, it also accounts for subspecies and breed differences (Kenneth and Ritchie, 1953; Racey, 1981). Indeed, this has been demonstrated for the red deer species (*Cervus elaphus*) in which there is considerable subspecies variation in observed mating-birth intervals ranging from 233 days for Scottish red deer (*C.e. scoticus*) to 247 days for North American Wapiti (*C.e. nelsoni*, *roosevelti*, *manitobensis*) (Guinness *et al.*, 1971; Haigh, 2001). Red deer hinds gestating F1 crossbred red×Wapiti foetuses show a mating-calving interval of ~239 days (Asher *et al.*, 2005b). Similarly, although Pere David's deer, with a reported gestation length of ~280 days, are classified within a different genus, hybridisation with red deer hinds can result in fertile offspring. Hinds gestating F1 hybrid red×Pere David foetuses exhibit a wide range in mating-calving intervals from 262 to 274 days (Asher *et al.*, 1988b).

However, it has become apparent that gestation length within red deer subspecies is much more variable than previously thought (Asher, 2007). Guinness *et al.* (1978) noted a 5-day difference in mean gestation length between Scottish red deer hinds managed under differing nutritional regimens. More recently, Asher *et al.* (2005a) observed marked variation in gestation length

between hinds that were fed either greater or lesser amounts of nutrients during the third trimester of pregnancy. In that study gestation length was negatively correlated with change in hind live weight and the authors considered that under conditions of modest feed imbalance that appeared to influence foetal growth trajectory, a gestation length compensatory mechanism ensured optimal birth weight. In effect, the hypothesis argued that gestation length matched foetal growth rate to ensure optimum birth weight (possibly at the expense of optimal birth date).

Since the study of Asher *et al.* (2005a), other research has demonstrated a statistically robust association between conception date and gestation length for red deer subspecies. Garcia *et al.* (2006) observed that Spanish red deer hinds (*C.e. hispanicus*) that were artificially induced to conceive up to 68 days before the standard mating date exhibited longer gestation lengths than those conceiving later in the natural breeding season. They postulated that hinds were able to modify gestation length to counteract out-of-season breeding to optimise calving date. However, these previous data were confounded by the fact that early conceiving hinds exhibited lower live weight gains over pregnancy, thereby adding some support to the hypothesis of Asher *et al.* (2005a) that nutrition was a driver of gestation length variation. Most recently, Scott *et al.* (2008) investigated the relationship between conception date and gestation length in Scottish and Eastern European subspecies of red deer (*C.e. scoticus* and *C.e. hippelaphus*), using data for 393 naturally-mated hinds across two herds, as well as 91 hinds in which oestrus/conception were artificially synchronised across a 4-week range of dates spanning the natural rut. In all cases there were highly significant negative slopes for the regression of gestation length against conception date. Effects for hind age, hind live weight, calf gender, birth weight, sire genotype and year were not significant. The data supported a hypothesis that within-herd synchrony of red deer births is facilitated by a 'push/pull' control over gestation length, such that hinds conceiving early and late in the breeding season have longer and shorter gestation periods, respectively. The individual data sets indicate that for every 10 days difference in conception date there was a change in gestation length of 1.9–4.9 days. Recent studies by Scott *et al.* (2015) provide support for the hypothesis that variation in foetal age during the latter stages of pregnancy, when feed quality and voluntary feed intake cycles of dams are in a state of flux, drives differential growth trajectories for early and late conceiving foetuses, leading to nutritional control over foetal maturation and induction of parturition.

The aforementioned studies on red deer subspecies indicate a hitherto unexpected environmental control over gestation length in, at least some, cervids. Recent information indicates that this may also be the case for reindeer (Rowell and Shipka, 2009) and sika deer (*Cervus nippon*) (Matsuura *et al.*, 2004).

Environmental and Genetic Influences on Female Puberty

Female puberty, defined as the age of first ovulation, normally occurs between 1 and 3 years of age in most cervid species, although this is strongly influenced by climatic and nutritional factors. Such influences are mediated through body mass, as demonstrated for red deer hinds (Hamilton and Blaxter, 1980; Clutton-Brock *et al.*, 1982). Red deer hinds on New Zealand deer farms are expected to attain puberty at 16 months of age, and produce their first calf at 24 months of age. However, high incidences of reproductive failure within populations of rising-two-year old hinds (i.e., 10–60% of hinds not attaining pregnancy) have been common (Asher and Pearse, 2002), prompting research into factors controlling entry into puberty. Studies involving observations of over 20,000 young hinds have clearly shown a strong relationship between live-weight at 16 months of age and the ability to enter puberty (Asher and Cox, 2013), with hinds that fail to attain about 70% of the expected mature body-mass of their genotype (sub-species) by 16 months of age delaying puberty by at least one year. This has been termed as the 'permissive body mass threshold for entry into puberty' (Asher and Cox, 2013). While this has provided producers with live-weight targets to ensure a high proportion of the younger hinds attain puberty at 16 months of age, complications have arisen in the deer farming industry due to widespread and indiscriminate introgression of various sub-species that exhibit widely differing mature hind body mass phenotypes. The most extreme case is that involving the cross-breeding of Western European red deer (mainly *Cervus elaphus scoticus*), with a mature hind weight of 100–110 kg, with North American wapiti (mainly *C.e. nelsoni*, *roosevelti* and *manitobensis*), with a mature cow weight of 180–220 kg, to produce fertile cross-breds with an expected mature weight of 170–190 kg. The difficulties experienced with attaining high rates of puberty amongst the larger cross-bred hinds has been due to failure of the animals to reach the crucial body mass threshold required at 16 months of age (Asher *et al.*, 2005c). A similar issue has been observed in the introgression of Western European red deer with sub-species with less extreme mature body-mass differences, such as Eastern European red deer (*C.e. hippelaphus*), but, in general the '70% rule' applies to cross-bred genotypes. However, recent studies have provided support for the hypothesis that the nutritional environment in early-life (birth to 3 months of age) influences the permissive body mass threshold for puberty at 16 months of age. Detailed analysis of a multi-year dataset, in which the relationship between live-weight and predicted pregnancy rate was modelled, demonstrated that hinds under nutritional constraints early in life had a higher permissive body mass threshold for puberty for their genotype, raising intriguing possibilities self-regulatory mechanisms to control reproductive processes in the face of variable environmental (e.g., climatic and nutritional) conditions (Asher and Cox, 2013).

Male Reproductive Cycles

Circannual variation in testicular function is evident in most deer species, reflecting changes in fertility, gross morphometry and behaviour. Of the temperate species, these changes have been best described for red deer and fallow deer (see Asher *et al.*,

1999a,b). Episodic and pulsatile secretion of LH and testosterone change dramatically through the seasons, being maximal during 'rutting' (hyper-sexual activity by males around the short mating period in autumn). Similar patterns of secretion have been observed for Eld's deer, a tropical but seasonally breeding species (Monfort *et al.*, 1993).

Testosterone mediated changes in secondary sexual characteristics are very pronounced in both temperate and tropical cervid species. The antler cycle is linked closely to the testis cycle, with antlers cast annually when testes regress to their minimal dimensions. Casting is in response to a marked decline in testosterone secretion and can be induced by castration (Goss, 1983). New antler growth occurs during the quiescent phase of the testes, in the relative absence of testosterone secretion. Mineralisation (calcium phosphate deposition) of antlers coincides with increasing testicular activity and increasing testosterone secretion, and hard antlers are generally retained while testicular tissue is actively secreting modest to high amounts of testosterone. This relationship holds for most antlered species studied (Goss, 1983), although the seasonal patterns vary between species and the antler-testis cycles may not be synchronised amongst individuals of some tropical species (Loudon and Curlew, 1988).

Circannual patterns of testicular regression and recrudescence occurring in temperate cervid species relate to annual cycles of sperm production. Unlike most seasonally breeding domestic ruminants, which show moderate fluctuations in testicular activity (e.g., sheep), temperate cervids exhibit alternating periods of sperm production and complete spermatogenic arrest that reflect about fivefold changes in seasonal testicular volume (Lincoln, 1971). Although tropical cervid species often exhibit circannual cycles of reproductive development, either in a synchronous (e.g., Eld's deer) or non-synchronous manner (e.g., chital deer), males appear to retain a degree of fertility through-out the year (Loudon and Curlew, 1988; Chapman and Harris, 1991; Mylrea, 1992; Monfort *et al.*, 1993).

Conclusion

Clearly, there is no one 'model' species that defines typical cervid reproductive cycles, attesting to the complex array of life histories and reproductive strategies of the cervids in general. Our current understanding of reproductive biology largely centres around large-bodied, temperate (and highly seasonal) species that have a close association with man. In contrast, reproductive cycles of the smaller-bodied, tropical (aseasonal) species are generally poorly understood, with this group represented by many taxa that are at serious risk of extinction.

References

- Asher, G. W. (1985). Oestrous cycle and breeding season of farmed fallow deer *Dama dama*. *Journal of Reproduction and Fertility*, 75, 521–529.
- Asher, G. W. (2007). Gestation length in red deer: Genetically determined or environmentally controlled? In J. L. Juengel, J. F. Murray, & M. F. Smith (Eds.), *Reproduction in Domestic Ruminants VI* (pp. 255–260). Nottingham: Nottingham University Press.
- Asher, G. W., Adam, J. L., Otway, W., et al. (1988b). Hybridisation of Pere David's deer (*Elaphurus davidianus*) and red deer (*Cervus elaphus*) by artificial insemination. *Journal of Zoology (London)*, 215, 197–203.
- Asher, G. W., Archer, J. A., Scott, I. C., et al. (2005c). Reproductive performance of pubertal red deer (*Cervus elaphus*) hinds: Effects of genetic introgression of wapiti subspecies on pregnancy rates at 18 months of age. *Animal Reproduction Science*, 90, 287–306.
- Asher, G. W., & Cox, N. (2013). The relationship between body-mass and puberty in young red deer (*Cervus elaphus*) hinds: Evidence of early-life effects on permissive live-weight thresholds. *Animal Reproduction Science*, 143, 79–84.
- Asher, G. W., Fisher, M. W., Berg, D. K., Waldrup, K. A., & Pearce, A. J. (1996). Luteal support of pregnancy in red deer (*Cervus elaphus*): Effect of cloprostenol, ovariectomy and lutectomy on the viability of the post-implantation embryo. *Animal Reproduction Science*, 41, 141–151.
- Asher, G. W., Fisher, M. W., Fennessy, P. F., et al. (1993b). Oestrous synchronization, semen collection and artificial insemination of farmed red deer (*Cervus elaphus*) and fallow deer (*Dama dama*). *Animal Reproduction Science*, 33, 241–265.
- Asher, G. W., Fisher, M. W., Fennessy, P. F., Suttie, J. M., & Webster, J. R. (1993a). Manipulation of reproductive seasonality of farmed red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) by strategic administration of exogenous melatonin. *Animal Reproduction Science*, 33, 267–287.
- Asher, G. W., Monfort, S. L., & Wemmer, C. (1999a). Comparative reproductive function in cervids: Implications for management of farm and zoo populations. *Journal of Reproduction and Fertility*, 54, 143–156.
- Asher, G. W., Muir, P. D., Semiadi, G., et al. (1997a). Seasonal patterns of luteal cyclicity in young red deer (*Cervus elaphus*) and sambar deer (*Cervus unicolor*). *Reproduction Fertility and Development*, 9, 587–596.
- Asher, G. W., Mulley, R. C., O'Neill, K. T., et al. (2005a). Influence of level of nutrition during late pregnancy on reproductive productivity of red deer (1) adult and primiparous hinds gestating red deer calves. *Animal Reproduction Science*, 86, 261–283.
- Asher, G. W., Pearce, A. J., 2002. Managing reproductive performance of farmed deer: The key to productivity. In: Proceedings of the Third World Deer Farming Congress, Austin, TX, pp. 99–112.
- Asher, G. W., Peterson, A. J., & Watkins, W. B. (1988a). Hormonal changes during luteal regression in farmed fallow deer, *Dama dama*. *Journal of Reproduction and Fertility*, 84, 379–386.
- Asher, G. W., Scott, I. C., Mockett, B. G., O'Neill, K. T., Diverio, S., Inskeep, E. K., Townsend, E. C., 1999b. Ultrasonographic monitoring of ovarian function during the oestrous cycle of fallow deer (*Dama dama*). In: Proceedings of the Fourth International Congress on the Biology of Deer, Kaposvar, Hungary, pp. 148–152.
- Asher, G. W., Scott, I. C., O'Neill, K. T., & Littlejohn, R. P. (2005b). Influence of nutrition during late pregnancy on reproductive productivity of red deer (2) adult hinds gestating wapiti×red deer crossbred calves. *Animal Reproduction Science*, 86, 285–296.
- Asher, G. W., Scott, I. C., O'Neill, K. T., et al. (1997b). Ultrasonographic monitoring of antral follicle development in red deer (*Cervus elaphus*). *Journal of Reproduction and Fertility*, 111, 91–99.
- Berg, D. K., Thompson, J. G., Peterson, A. J., Asher, G. W., 1994. Morphology and chronology of red deer (*Cervus elaphus*) pre-implantation embryonic development. Recent developments in deer biology. In: Proceedings of the Third International Congress on the Biology of Deer, Edinburgh, pp. 179–180.
- Berg, D. K., Thompson, J. G., Peterson, A. J., & Asher, G. W. (2008). The temporal relationship between oocyte maturation and early fertilization events in relation to the pre-ovulatory LH peak and pre-implantation embryo development in red deer (*Cervus elaphus*). *Animal Reproduction Science*, 105, 332–343.
- Blouch, R. A. (1987). Reproductive seasonality of the white-tailed deer on the Colombian Llanos. In C. M. Wemmer (Ed.), *Biology and Management of the Cervidae* (pp. 339–343). Washington: Smithsonian Institution Press.

- Chapman, D. I., Chapman, N. G., & Dansie, O. (1984). The periods of conception and parturition in feral Reeves' muntjac (*Muntiacus reevesi*) in southern England based upon age of juvenile animals. *Journal of Zoology (London)*, 204, 575–578.
- Chapman, N.G., Harris, S., 1991. Evidence that the seasonal antler cycle of adult Reeves' muntjac (*Muntiacus reevesi*) is not associated with reproductive quiescence. *Journal of Reproduction and Fertility* 92, 361–369.
- Chapple, R. S., English, A. W., & Mulley, R. C. (1993). Characteristics of the oestrous cycle and duration of gestation in chital hinds (*Axis axis*). *Journal of Reproduction and Fertility*, 98, 23–26.
- Clutton-Brock, T. H., Guinness, F. E., & Albon, S. D. (1982). *Red Deer: Behaviour and Ecology of Two Sexes*. Chicago: University of Chicago Press.
- Curlew, J. D., Loudon, A. S. I., & Coleman, A. M. P. (1988). Oestrous cycles and the breeding season of the Père David's deer hind (*Elaphurus davidianus*). *Journal of Reproduction and Fertility*, 82, 119–126.
- García, A. J., Landete-Castillejos, T., Carrion, D., Gaspar-Lopez, E., & Gallego, L. (2006). Compensatory extension of gestation length with advance of conception in red deer (*Cervus elaphus*). *Journal of Experimental Zoology*, 305A, 55–61.
- Goss, R.J., 1983. *Deer Antlers: Regeneration, Function and Evolution*. New York: Academic Press.
- Guinness, F. E., Gibson, R. M., & Clutton-Brock, T. H. (1978). Calving times of red deer (*Cervus elaphus*) on Rhum. *Journal of Zoology (London)*, 185, 105–114.
- Guinness, F. E., Lincoln, G. A., & Short, R. V. (1971). The reproductive cycle of the female red deer, *Cervus elaphus*. *Journal of Reproduction and Fertility*, 27, 427–438.
- Haigh, J. C. (2001). The gestation length of wapiti (*Cervus elaphus*) revisited. *Animal Reproduction Science*, 65, 89–93.
- Hamilton, W. J., & Blaxter, K. L. (1980). Reproduction in farmed red deer. I. Hind and stag fertility. *Journal of Agricultural Science*, 95, 261–273.
- Harper, J. L., Clatworthy, J. N., McNaughton, I. H., & Sagar, G. R. (1961). The evolution and ecology of closely related species living in the same area. *Evolution*, 15, 209–227.
- Harrington, R. (1985). Evolution and distribution of the Cervidae. In P. F. Fennessy, & K. R. Drew (Eds.), *Biology of Deer Production* (pp. 3–11). Wellington, New Zealand: The Royal Society of New Zealand [Bulletin 22].
- Kenneth, J.H., Ritchie, G.R., 1953. Gestation periods, a table and bibliography. Technical Communications of the Commonwealth Bureau of Animal Breeding and Genetics no. 5.
- Lincoln, G.A., 1971. The seasonal reproductive changes in the red deer stag (*Cervus elaphus*). *Journal of Zoology* 163, 105–125.
- Lincoln, G. A. (1985). Seasonal breeding in deer. In P. F. Fennessy, & K. R. Drew (Eds.), *Biology of Deer Production* (pp. 165–179). Wellington: Royal Society of New Zealand [Bulletin No. 22].
- Lincoln, G. A., & Short, R. V. (1980). Seasonal breeding: Nature's contraceptive. *Recent Progress in Hormone Research*, 36, 1–52.
- Loudon, A. S. I., & Curlew, J. D. (1988). Cycles of antler and testicular growth in an aseasonal tropical deer (*Axis axis*). *Journal of Reproduction and Fertility*, 83, 729–738.
- Marshall, F. H. A. (1937). On the change-over in the oestrous cycle in animals after transference across the equator, with further observations on the incidence of the breeding seasons and the factors controlling sexual periodicity. *Proceedings of the Royal Society of London, Series B*, 122, 413–428.
- Matsuura, Y., Sato, K., Suzuki, M., & Ohtsishi, N. (2004). The effects of age, body weight and reproductive status on conception dates and gestation periods in captive sika deer. *Mammal Study*, 29, 15–20.
- McCorkell, R. B., Woodbury, M. R., & Adams, G. P. (2006). Ovarian follicular and luteal dynamics in wapiti during the estrous cycle. *Theriogenology*, 65, 40–56.
- McMahon, C. D., Fisher, M. W., Mockett, B. G., & Littlejohn, R. P. (1997). Embryo development and placentome formation during pregnancy in red deer. *Reproduction, Fertility and Development*, 9, 723–730.
- Mishra, H. R., & Wemmer, C. (1987). The comparative breeding ecology of four cervids in Royal Chitwan National Park. In C. M. Wemmer (Ed.), *Biology and Management of the Cervidae* (pp. 259–271). Washington, USA: Smithsonian Institution Press.
- Monfort, S.L., Brown, J.L., Bush, M., et al., 1993. Circannual inter-relationships among reproductive hormones, gross morphometry, behaviour, ejaculate characteristics and testicular histology in Eld's deer stags (*Cervus eldi thamin*). *Journal of Reproduction and Fertility* 98, 471–480.
- Monfort, S. L., Wemmer, C., Kepler, T. H., et al. (1991). Monitoring ovarian function and pregnancy in Eld's deer (*Cervus eldi thamin*) by evaluating urinary steroid metabolite excretion. *Journal of Reproduction and Fertility*, 88, 271–281.
- Mylrea, G.E., 1992. Natural and artificial breeding of farmed chital deer (*Axis axis*) in Australia. PhD Thesis, University of Sydney, NSW.
- Plotka, E. D., Seal, U. S., Verme, L. J., & Ozoga, J. J. (1982). Reproductive steroids in white-tailed deer. IV. Origin of progesterone during pregnancy. *Biology of Reproduction*, 26, 258–262.
- Racey, P. A. (1981). Environmental factors affecting the length of gestation in mammals. In B. Cook, & D. F. Gilmore (Eds.), *Environmental Factors in Mammal Reproduction* (pp. 199–213). London: Macmillan.
- Rowell, J. E., & Shipka, M. P. (2009). Variation in gestation length among captive reindeer (*Rangifer tarandus tarandus*). *Theriogenology*, 72, 190–197.
- Schwartz, C. C., & Hundertmark, K. J. (1993). Reproductive characteristics of Alaskan moose. *Journal of Wildlife Management*, 57, 454–468.
- Scott, I. C., Asher, G. W., Archer, J. A., & Littlejohn, R. P. (2008). The effect of conception date on gestation length of red deer (*Cervus elaphus*). *Animal Reproduction Science*, 109, 206–217.
- Scott, I. C., Asher, G. W., Jopson, N., et al. (2015). Effect of conception date and hind nutrition on fetal growth trajectory and gestation length of red deer (*Cervus elaphus*). *Animal Production Science*, 55, 1064–1074.
- Scott, I. C., Asher, G. W., Lach, J. E., & Littlejohn, R. P. (2006). The influence of red deer genotype on conception pattern: Eastern vs. Western subspecies. *Proceedings of the New Zealand Society of Animal Production*, 66, 270–273.
- Short, R.V., Hay, M.F., 1966. Delayed implantation in the roe deer, *Capreolus capreolus*. In: Symposium of the Zoological Society of London, vol. 15, pp. 173–194.
- Sjaastad, O. V., Blom, A. K., Austad, R., & Oen, E. O. (1990). Plasma progesterone in reindeer in relation to ovariectomy and hysterectomy. *Acta Veterinaria Scandinavica*, 31, 45–51.
- Smith, T. R., Hunter, C. G., Eisenberg, J. F., & Sunquist, M. E. (1996). Ecology of white-tailed deer in eastern Everglades National Park. *Florida Museum Natural History*, 39, 141–172.
- Van Mourik, S. A., & Stelmasiak, T. (1990). Endocrine mechanisms and antler cycles in rusa deer, *Cervus (Rusa) timorensis*. In G. A. Bubenik, & A. B. Bubenik (Eds.), *Horns, Pronghorns and Antlers* (pp. 265–297). New York, NY: Springer-Verlag.
- Wemmer, C., Halverson, T., Rodden, M., & Portillo, T. (1989). The reproductive biology of female Père David's deer (*Elaphurus davidianus*). *Zoo Biology*, 8, 49–55.
- Whitehead, G. K. (1993). *The Whitehead Encyclopaedia of Deer*. Shrewsbury: Swan Hill Press.
- Wong, B., & Parker, K. L. (1993). Estrus in black-tailed deer. *Journal of Mammalogy*, 69, 168–171.

REPRODUCTION GENERAL, LAB ANIMALS

Caenorhabditis elegans Reproduction

Muhan Hu, Shara Legg, Hala Zein-Sabatto, and Michael A Miller, University of Alabama at Birmingham, Birmingham, AL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cloaca Cavity in the male tail that is used to release waste, sperm, and seminal fluid.

Cilia Hair-like cellular structures that extend into the extracellular environment.

Dauer An alternative life cycle stage that is migratory and resistant to harsh conditions.

Free-living Refers to nematodes that do not require a host for development and reproduction.

Insulin Growth factor ligand secreted from cells.

Notch Cell surface receptor for Delta ligands.

Oocyte meiotic maturation Process by which the oocyte prepares for fertilization.

Outcross Refers to progeny produced by mating to a male.

Polyunsaturated fatty acid Fatty acid chain containing 2 or more double bonds.

Prostaglandin Secreted lipid signaling molecule containing a 5-carbon ring structure.

Spermatheca Sac-like structure containing sperm where fertilization occurs.

Spermiogenesis Process by which sessile spermatids are converted into motile spermatozoa.

Steroid Lipid signaling molecule derived from cholesterol.

TGF- β Growth factor ligand of the transforming growth factor β superfamily.

Vas Deferens Male duct connecting the gonad to cloaca.

Vulva Hermaphrodite opening where eggs are laid and sperm are inseminated.

Introduction

Sydney Brenner introduced the nematode *C. elegans* to the biomedical research community more than 40 years ago (Brenner, 1974). Its simplicity in cell types, transparent epidermis, rapid generation time, and prodigious reproductive capacity makes this species well suited for studying the molecular genetics of animal development and behavior. Today, labs across the world conduct research on *C. elegans* to address fundamental questions related to developmental biology, reproductive biology, neurobiology, immunology, evolution, and human disease. The *C. elegans* genome is sequenced and annotated, its cell lineage and neural connectivity are described, and an arsenal of techniques is available to investigate gene function and regulation. These and other features have made *C. elegans* a popular choice for scientists and teachers.

C. elegans is a small (<1 mm in length), free-living roundworm found in soil and rotting vegetation, where it feeds on bacteria and other microorganisms (Frezal and Felix, 2015). In the lab, *C. elegans* is grown on agar plates seeded with *E. coli* (Corsi et al., 2015). Its life cycle consists of embryonic development, four larval stages called L1, L2, L3, and L4, and reproducing adults. To develop from a one-celled embryo into an adult takes 3–4 days, depending on the temperature. In the absence of food or in crowded conditions, *C. elegans* larva enter the dauer stage, an alternative life cycle where reproductive development stalls. Dauer larvae can survive harsh environmental conditions for several months.

C. elegans has two sexes, hermaphrodites and males (Fig. 1). During the L4 stage, the hermaphrodite gonad produces approximately 300 spermatids before switching to oocyte production. These spermatids form motile spermatozoa (termed sperm) that fertilize maturing oocytes during the first few days of adulthood until all sperm are consumed. Sperm are stored in the spermatheca, the site of fertilization. Embryonic development initiates in the uterus before eggs are laid through the vulva into the external environment (Fig. 1(A)). Hermaphrodites reproduce by self-fertilization (i.e., selfing) using sperm and oocyte from the same gonad.

Males are rare in the lab and nature, resulting from X chromosome nondisjunction during meiosis. Hermaphrodite selfing produces over 99.8% hermaphrodite progeny, which contain two X chromosomes. When a male and hermaphrodite mate, nearly all progeny will be outcross (i.e., fathered by the male) and approximately half will be male. The switch from self-fertilization to cross-fertilization is due to the competitive superiority of male-derived sperm. During mating, males use their tail (Fig. 1(B)) to

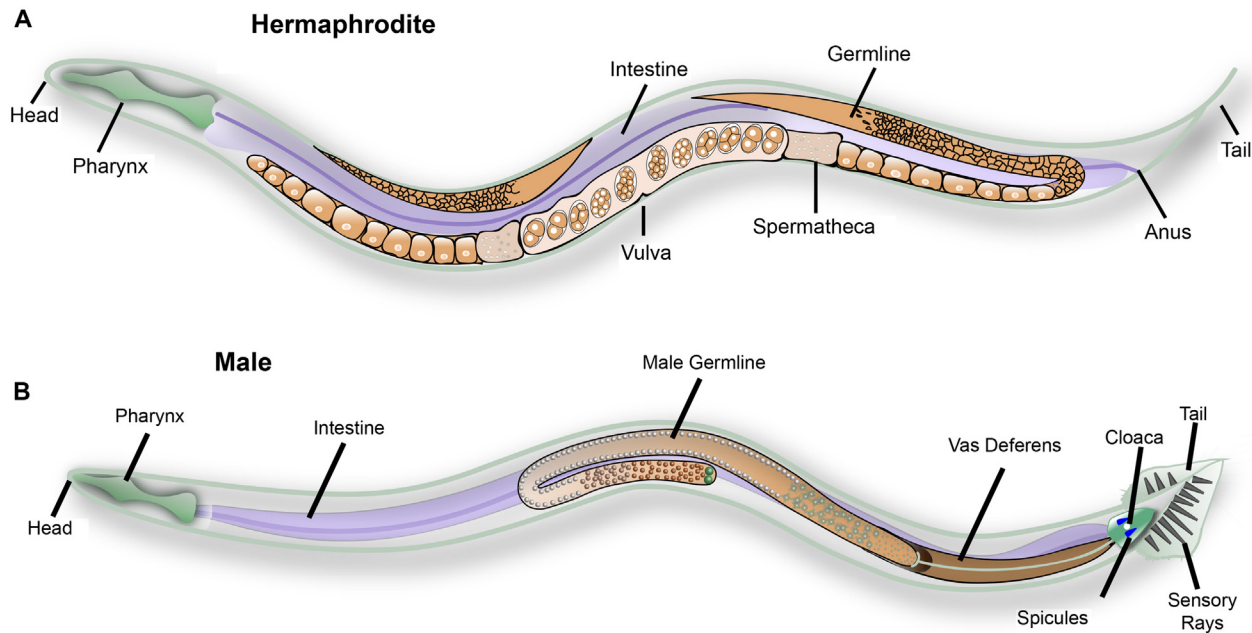


Fig. 1 Cartoon diagram of adult *C. elegans* hermaphrodite (A) and male (B) anatomy. Major tissues are indicated.

deposit sessile spermatids along with seminal fluid into the hermaphrodite uterus, where spermiogenesis occurs. Spermiogenesis triggers formation of the sperm pseudopod, a structure comprised of major sperm protein (MSP) filaments that drive locomotion. Motile sperm migrate over fertilized eggs and uterine cell membranes to the spermatheca (Fig. 1(A)). Sperm and oocyte secrete chemical signaling molecules that stimulate oocyte meiotic maturation and sperm chemotaxis, respectively. Below, we discuss important features of hermaphrodite and male reproductive development, mating, and fertilization in more detail.

Hermaphrodite Reproductive Development

The hermaphrodite gonad develops post-embryonically from a four-celled primordium, reaching sexual maturity about two days after hatching (Fig. 2) (Hubbard and Greenstein, 2000). The gonad primordium consists of two germ cells called Z2 and Z3 surrounded by two somatic gonadal precursor cells called Z1 and Z4. The germ line is formed during early embryonic development (Wang and Seydoux, 2013). Food is essential for gonad development, which also requires chemical signals from germ cells, somatic gonadal cells, and the nervous system. Gonadal cells start proliferating during the L1 stage, forming a tubular structure with a single distal tip cell (DTC) at each end. The DTCs promote gonad elongation during larval development, as well as promote germ cell proliferative fate. Ultimately, the hermaphrodite gonad forms into two mirrored U-shaped arms, one anterior and one posterior, that connect to a common uterus (Fig. 2). At the distal end of each arm lies a germline stem cell population that gives rise to differentiating gametes. Germ cells form a syncytium of shared cytoplasm called the rachis. As germ cells are displaced from distal to proximal positions, they enter meiosis and differentiate. The Notch signaling pathway controls germ cell fate. The Notch ligands LAG-2 and APX-1 are expressed in the DTCs, whereas the GLP-1 receptor is expressed in the germ cells. Disrupting Notch signaling causes all germ cells to enter meiosis. In addition to the DTC, Z1 and Z4 descendants form the gonadal sheath cells, spermatheca, uterus, and anchor cell, which is necessary for vulva induction.

Spermatogenesis starts during the L4 stage (Fig. 2) (L'hernault, 2006; Ellis and Stanfield, 2014). The first 30–40 most proximal germ cells in each gonad arm enter the spermatogenesis pathway. Primary spermatocytes bud off the rachis and enter meiosis I. The resulting secondary spermatocytes enter meiosis II to produce four haploid spermatids. Spermatids bud from an anucleate residual body that contains unneeded materials. In L4 stage and young adult hermaphrodites, spermatids are located in the proximal gonad arm. When the first oocyte undergoes meiotic maturation and ovulation, spermatids are swept into the spermatheca, where they rapidly undergo spermiogenesis. In hermaphrodites, spermiogenesis is regulated by the *spe-8* pathway, which is thought to respond to zinc signals.

Hermaphrodite gonads switch to oogenesis after spermatogenesis (Fig. 2). Germ cells enter meiosis and start progressing through prophase I. Not all meiotic prophase I germ cells will become oocytes. Some germ cells become nurse cells that help produce materials for the oocyte and embryo. Oocyte differentiation begins as germ cells progress from pachytene to diplotene stages of prophase I, near the bend in the gonad arm. Oocytes rapidly grow in size from provision of materials through the rachis and uptake of yolk, which is synthesized in the intestine. Oocytes are arranged in an assembly line in the proximal gonad with the most proximal oocyte adjacent to the spermatheca (Fig. 2). A valve prevents the oocyte from entering the spermatheca. Sperm

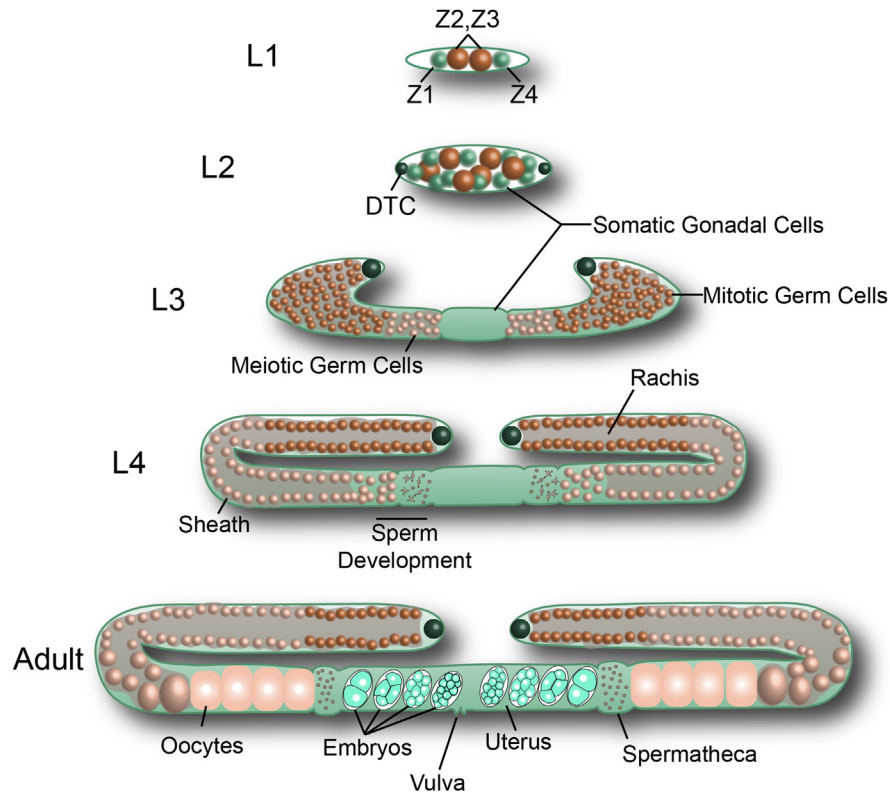


Fig. 2 Cartoon diagram of postembryonic gonad development during larval (L1–L4) and adult stages. The gonad primordia of freshly hatched L1 larvae contain Z2 and Z3 germline precursors that give rise to mitotic germ cells. During L3 and L4 stages, these cells enter meiosis and initiate spermatogenesis. Afterwards, all meiotic germ cells commit to oogenesis. The somatic gonad precursors Z1 and Z4 give rise to the distal tip cell (DTC), gonadal sheath cells, the spermatheca, and the uterus. See text for additional details. Gonad sizes are not drawn to scale.

secrete MSPs into the spermatheca lumen and proximal gonad. MSPs bind to receptors on oocyte and surrounding sheath cell surfaces, triggering oocyte meiotic maturation, sheath cell contraction, and ovulation. The most proximal oocyte ovulates into the spermatheca every ~25 min, while the next one back assumes the most proximal position. The gonadal sheath cells surround the developing germ cells and oocytes. Sheath cells are critical for germ cell proliferation, differentiation, meiotic progression, and ovulation.

The uterine lumen connects to the external environment through the vulva, a specialized opening composed of epithelial, neuronal, and muscle cells (Figs. 1(A) and 2). The vulva is required for egg laying and for entry of male sperm during mating. During larval development, the vulva is organized by the anchor cell, a descendant of Z1 and Z4. The anchor cell also patterns the uterine cells to form the connection with the vulva. Several evolutionarily conserved signaling cascades, such as WNT, epidermal growth factor (EGF), and Notch help pattern the vulva.

Male Reproductive Development

Morphologically, males have thinner bodies than hermaphrodites and a fan-shaped tail used for mating (Fig. 1). The male gonad consists of a single J-shaped tube that connects to a ventral opening in the tail called the cloaca (Hubbard and Greenstein, 2000; Emmons, 2005). Similar to the hermaphrodite gonad, the male gonad derives from a four-celled gonad primordium that divides during larval stages. Although males have two DTCs, they do not migrate. Instead, a specialized cell called the linker cell is responsible for gonad elongation. A germline stem cell population gives rise to meiotic germ cells that form spermatocytes, which detach from the rachis and complete meiosis. The resulting spermatids are stored in the seminal vesicle. The vas deferens connects the seminal vesicle to the cloaca (Fig. 1(B)). The TRY-5 trypsin-like protease is secreted into the seminal fluid, where it triggers spermiogenesis upon mixing with sperm during ejaculation.

Males also develop male-specific muscles and neurons for mating. During the L3 stage, six mesoderm cells divide to form 41 new muscle cells not found in hermaphrodites. Fifteen of these muscles run between the ventral and mid-lateral body wall and are used to curl the tail during mating. The male tail develops around the L4 stage. The anterior tail tip cells detach from the larval cuticle and retract, leaving behind a fluid-filled cavity. Nine rays then fan out into the cavity. These sensory rays house two sensory neurons each (Fig. 1(B)). The cloaca joins the intestine and gonad to the external environment via the cloacal opening. Two prong-like structures called spicules, the sensory rays, the hook, and the post-cloacal sensilla are all sensory organs that form in the male tail.

Mating

The majority of the sensory and motor behaviors associated with copulation are performed by the male. The male nervous system contains 383 neurons, 81 more than the hermaphrodite nervous system. Most male-specific neurons are in the posterior region and are thought to be involved in mating behaviors. Male mating behavior is complex and involves a series of steps (O'hagan *et al.*, 2014).

Prior to mating, the adult hermaphrodite secretes pheromones called ascarosides that cause an increase in male reversal frequency (Ludewig and Schroeder, 2013). This mating behavior requires chemosensory cilia formation in males, suggesting that males recognize ascarosides via chemoreceptors on sensory cilia. During mating, the male likely responds to both chemosensory and mechanosensory signals. When the sensory neurons in the male's tail contact a hermaphrodite, male response behavior is initiated. The response involves ceasing forward motion, placing the ventral side of his tail flat against the hermaphrodite's body, and moving backwards to scan for the vulva. It is mediated by the 9 pairs of rays in the male's tail. Each ray is made up of 1 structural cell and 2 sensory neurons. The dendritic processes of the 2 sensory neuron bodies span the length of each ray and terminate in ciliated sensory endings. Mutant males that are defective in sensory cilia formation have defects in mating response behavior, locating the vulva, and ejaculation. Males with mutations in the polycystin-1 homolog *lov-1* and polycystin-2 homolog *pkd-2* are defective in response behavior. Studies of *lov-1* and *pkd-2* were instrumental in establishing the association between human polycystic kidney disease and abnormal cilia function (O'hagan *et al.*, 2014).

If the male initially contacts the hermaphrodite on the dorsal side, he turns at the hermaphrodite's head or tail. This turning behavior requires sensory input from the ray neurons and is mediated by the male specific CP ventral cord motor neurons and EF interneurons. Male specific diagonal muscles, which are responsible for moving the tail, are innervated by the CP neurons. The neurotransmitters serotonin and dopamine are also required for turning behaviors. Exogenous application of serotonin causes ventral tail curling, comparable to that seen during mating. Dopamine or neuropeptide deficiency causes abnormal turning behavior.

The male locates the vulva on the ventral side of the hermaphrodite's body (Fig. 1(A)). Once the male contacts the vulva, he stops moving backwards, places his tail directly over the vulva, inserts his spicules, and ejaculates spermatids and seminal fluid into the hermaphrodite uterus. The hook sensillum mediates stopping, whereas the post-cloacal sensilla and spicules mediate tail positioning. The hook sensillum consists of a mechanosensory neuron, a chemosensory neuron, and two support cells. The post-cloacal sensilla are arranged as a bilateral pair with each sensillum composed of three neurons and three support cells.

Spicule insertion behavior begins when the male cloaca contacts the hermaphrodite vulva. Male-specific neurons and sex muscles located in the male tail coordinate this behavior. Spicules exhibit bilateral organization and individually contain portions of two sheath cells, four socket cells, and two sensory neurons wrapped in a sclerotized cuticle. Protractor and retractor muscles are associated with each spicule. Shortening of the protractors causes the spicules to stick out from the tail, and shortening of the retractors draws them back. The hook and post-cloacal sensilla trigger the protractors to begin periodic contractions. The spicule tips prod the vulva until they partially penetrate it, causing the protractors to contract completely so that the spicules extend through the vulva. After penetration, spicule movements stop until ejaculation is completed about 4 s later. The SPV neurons play a role in coordinating spicule penetration and ejaculation. Males that have undergone laser ablation of SPV neurons prematurely ejaculate during prodding behavior.

Gamete Interactions and Fertilization

For sperm and oocyte to successfully fuse, sperm must be in the spermatheca when an oocyte undergoes meiotic maturation and ovulation. Coordinating these events requires multiple intercellular signaling cascades (Han *et al.*, 2010; Ellis and Stanfield, 2014; Geldziler *et al.*, 2011). Developing oocytes located next to the spermatheca secrete lipid signaling molecules called prostaglandins that guide migrating sperm toward them. Sperm that enter the spermatheca are prevented from prematurely contacting immature oocytes by the distal spermathecal valve. Sperm compete for position next to the valve to be the first to contact the maturing oocyte during ovulation. Prostaglandins stimulate an increase in sperm migration velocity and prevent reversals during their journey through the uterus to the spermatheca. Once in the spermatheca, sperm are often pushed back out into the uterus by fertilized eggs passing through the spermatheca-uterine junction. Prostaglandins are thought to direct both hermaphrodite-derived and male-derived sperm, but the migration response by the latter is better understood. Prostaglandin synthesis requires 20-carbon polyunsaturated fatty acid (PUFA) transport from the intestine to the oocytes in yolk lipoprotein complexes. Oocytes secrete a heterogeneous mixture of F-series prostaglandins derived from multiple PUFA precursors. Prostaglandins are thought to form a concentration gradient within the reproductive tract.

Sperm alert oocytes to their presence through secretion of protein hormones called MSPs. These 126 amino acid polypeptides fold into an evolutionarily conserved immunoglobulin-like domain that has a conserved extracellular signaling function (Han *et al.*, 2010). MSPs bind to receptors on the oocyte and surrounding gonadal sheath cell membranes to induce oocyte meiotic maturation and sheath contraction. *C. elegans* oocytes, like those of most animals, arrest in meiotic prophase. Meiotic maturation is characterized by progression from prophase (or more specifically, diakinesis) to metaphase of meiosis I. It involves nuclear envelope breakdown, rearrangement of the cortical cytoskeleton, and meiotic spindle assembly (Greenstein, 2005). MSP binding activates heteromeric G-protein pathways in sheath cells and the mitogen-activated protein kinase cascade in oocytes, resulting in meiotic

maturation. The maturing oocyte secretes an EGF homolog called LIN-3 to induce distal spermatheca valve dilation. Meiotic maturation occurs about every 25 min in adult hermaphrodite gonads containing sperm, as oocytes initiate this process sequentially. MSPs have another major function as the cytoskeleton in the sperm pseudopod. The mechanism by which MSPs are secreted involves the budding of novel membrane-bound vesicles.

During ovulation, the spermathecal valve dilates and the oocyte enters the spermatheca. The sperm and oocyte then bind and fuse. SPE-9, a transmembrane protein containing an extracellular domain with 10 EGF-like repeats, is required in sperm for fertilization (Ellis and Stanfield, 2014; Geldziler *et al.*, 2011). *spe-9* null mutant sperm have normal morphology and motility, trigger oocyte meiotic maturation, and contact ovulating oocytes. SPE-9 is hypothesized to function as a ligand for an unidentified oocyte receptor. Consistent with this model, SPE-9 localizes to the pseudopod plasma membrane, the first structure to contact the oocyte plasma membrane. Mutations in several other genes have been characterized that give phenotypes similar to *spe-9* mutants. These genes include *spe-41/trp-3*, which encodes a TRP family calcium channel expressed on the sperm plasma membrane, and *spe-45*, which encodes a transmembrane protein with a single immunoglobulin-like domain. SPE-45 is similar in structure and function to Izumo1, a protein expressed in mouse sperm that is required for fertilization.

C. elegans oocytes were thought to lack a zona pellucida, a glycoprotein layer surrounding the plasma membrane involved in sperm binding. However, recent studies suggest that oocytes contain a thin basal lamina that may be analogous to a zona pellucida. Fertilization triggers a rise in oocyte intracellular calcium, completion of the meiotic divisions, extrusion of polar bodies, a polyspermy block, and egg shell formation. These processes are collectively referred to as egg activation. SPE-11 is a novel protein that is expressed in sperm and transferred to the oocyte during fertilization. *spe-11* loss does not impact fertilization, but it does impair egg activation events. In addition to a haploid nucleus, sperm also provide a pair of centrioles that form a centrosome. Oocytes lack a centrosome, which is required for mitotic spindle assembly. The sperm also specifies the posterior axis of the embryo via the Rho GTPase-activating protein CYK-4. Several genes have been identified that are expressed in oocyte cortex, where they regulate various aspects of egg activation. These genes encode proteins involved in protein phosphorylation, redistribution, and degradation. Thus, both paternally supplied and maternally supplied proteins help convert the oocyte into a dividing and differentiating embryo.

Embryogenesis can be broken into two major stages: a proliferation stage and an organogenesis/morphogenesis stage. The proliferation stage occurs during the first half of development until about 330 min post-fertilization and is further divided into two phases. The first phase covers the time from zygote formation to the production of embryonic founder cells. The second phase spans the majority of cell divisions and gastrulation. The embryo is laid into the external environment around gastrulation, which initiates at the ~26 cell stage. Gastrulation involves cell migrations, an increase in cell number, and cycles of concomitant stem cell divisions (Nance *et al.*, 2005). Gastrulation results in a spheroid-shaped embryo that is organized into three germ layers. The organogenesis/morphogenesis stage involves terminal differentiation of cells without additional cell divisions, threefold embryo elongation, and formation of fully differentiated tissues and organs. Following embryogenesis, the main body plan of the worm is fixed and does not change much during postembryonic development.

Environmental Regulation

C. elegans development and reproduction hinge on three environmental parameters: population density sensed through ascaroside pheromones, food supply, and temperature (Corsi *et al.*, 2015; Frezal and Felix, 2015). Environmental cues are detected through ciliated sensory neurons in the worm's nose and tail (Bargmann, 2006). Cilia extend into the external environment through pores and contain chemosensory G-protein coupled receptors that integrate specific signals into neuroendocrine pathways, such as TGF- β and insulin pathways. Temperature is sensed by the AFD neuron, which is also ciliated, and other thermosensory neurons.

In nutrient replete environments, the embryo hatches out of a virtually impermeable eggshell, and begins postembryonic development through the four larval stages. At the end of each stage, the animal molts and a new cuticle is made during the lethargus. Gonad development progresses as described above. However, when nutrients are scarce or pheromone levels are high, the larvae undergo a metabolic shift after the second molt and transition to the dauer stage (Hu, 2007). Dauers produce a specialized cuticle containing alae, block their mouth with an internal plug, stop pharyngeal pumping, and shift their metabolism toward the glyoxylate cycle to generate carbohydrates from lipid stores. Gonad development arrests, preventing reproduction. Dauer is a migratory stage that is resistant to harsh conditions and can survive for several months without food. Nutrient availability triggers the resumption of larval development and gonadogenesis, resulting in a fertile adult.

Genetic screens have led to the identification of genes that contribute to dauer entry and exit. These genes are divided into dauer-constitutive (Daf-c) and dauer-defective (Daf-d) classes based on their mutant phenotypes. There are four broad pathways that contribute to dauer.

1. The guanylyl cyclase pathway. *daf-11* is a Daf-c gene that encodes a transmembrane guanylyl cyclase expressed in amphid chemosensory neurons such as ASI, ASJ, and ASK. cGMP ions produced by *daf-11* modulate ion channels involved in thermosensation and chemosensation, including ascaroside detection.
2. The TGF- β pathway. *daf-7* is a Daf-c gene that encodes a TGF- β homolog expressed in ASI sensory neurons. DAF-7 expression is regulated by food and ascaroside levels. DAF-7 transmits signals through the DAF-1 type I and DAF-4 type II TGF- β receptors, which are broadly expressed. Further downstream are multiple SMAD class transcription factors.

3. The insulin pathway. *daf-2* is a Daf-c gene that encodes an insulin receptor homolog. DAF-2 transmits signals in the nervous system through a canonical pathway that includes the AGE-1 phosphoinositide 3-kinase catalytic subunit, PDK-1 and AKT-1/AKT-2 kinases, and the DAF-16 forkhead box O class transcription factor.
4. The steroid hormone pathway. The Daf-c gene *daf-9* and Daf-d gene *daf-12* encode proteins with similarity to cytochrome P450 class steroid hydroxylases and nuclear hormone receptors, respectively. DAF-9 helps synthesize steroid ligands called dafachronic acids, which bind with high affinity to DAF-12. The *daf-9/daf-12* pathway acts downstream of TGF- β and insulin pathways.

In addition to dauer, DAF-7 TGF- β and DAF-2 insulin receptor pathways also impact germline stem cell dynamics, oogenesis, and fertilization in late larval and adult stages (Hubbard *et al.*, 2013; Mcknight *et al.*, 2014; Lopez *et al.*, 2013). These mechanisms are thought to fine tune reproduction to nutrient levels. The DAF-7 pathway transduces signals in at least two gonadal cell types, the DTCs and germ cells. DAF-7 signaling to the DTCs negatively regulates DAF-3 Co-SMAD and DAF-5 Sno-Ski transcription factors, resulting in expansion of the germ cell progenitor pool. In adults, DAF-7 transmits signals through the nervous system and germ line to promote oocyte prostaglandin synthesis. As discussed above, prostaglandins provide sperm with positional information, ensuring that sperm target the spermatheca efficiently. Thus, increases in DAF-7 expression induced by food perception promotes gamete production and sperm function critical for fertilization. When nutrient levels are low or ascaroside concentration is high, reduced DAF-7 expression down-regulates these pathways, thereby reducing reproductive investment and output.

The DAF-2 pathway acts on oogenesis and prostaglandin synthesis (Lopez *et al.*, 2013; Han *et al.*, 2010). In the presence of food, DAF-2 activates the mitogen-activated protein kinase cascade to drive oocyte meiotic prophase I progression and oogenesis. DAF-2 signaling via the DAF-16 transcription factor promotes oocyte prostaglandin production. Ultimately, DAF-2 inhibits DAF-16 activity in the intestine and germ line, causing an increase in prostaglandin precursor flux into oocytes. Reduced DAF-2 signaling stalls oogenesis and inhibits sperm motility. Neurons are thought to secrete insulin ligands that regulate DAF-2 activity. The bacterial diet, temperature, oxygen tension, and other environmental factors also influence hermaphrodite and male fertility, but the mechanisms are less well understood or recently emerging. In summary, *C. elegans* uses multiple sensory circuits to integrate various environmental cues into developmental pathways governing gonadogenesis, gametogenesis, and fertilization.

References

- Bargmann, C. I. (2006). Chemosensation in *C. elegans*. *Worm Book*, 1–29.
- Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77, 71–94.
- Corsi, A. K., Wightman, B., & Chalfie, M. (2015). A transparent window into biology: A primer on *Caenorhabditis elegans*. *Worm Book*, 1–31.
- Ellis, R. E., & Stanfield, G. M. (2014). The regulation of spermatogenesis and sperm function in nematodes. *Semin Cell Dev Biol*, 29, 17–30.
- Emmons, S. W. (2005). Male development. *Worm Book*, 1–22.
- Frezal, L., & Felix, M. A. (2015). *C. elegans* outside the Petri dish. *Elife*, 4.
- Geldziler, B. D., Marcello, M. R., Shakes, D. C., & Singson, A. (2011). The genetics and cell biology of fertilization. *Methods Cell Biol*, 106, 343–375.
- Greenstein, D. (2005). Control of oocyte meiotic maturation and fertilization. *Worm Book*, 1–12.
- Han, S. M., Cottee, P. A., & Miller, M. A. (2010). Sperm and oocyte communication mechanisms controlling *C. elegans* fertility. *Dev Dyn*, 239, 1265–1281.
- Hubbard, E. J., & Greenstein, D. (2000). The *Caenorhabditis elegans* gonad: A test tube for cell and developmental biology. *Dev Dyn*, 218, 2–22.
- Hubbard, E. J., Korta, D. Z., & Delfo, D. (2013). Physiological control of germline development. *Adv Exp Med Biol*, 757, 101–131.
- Hu, P. J. (2007). Dauer. *Worm Book: The Online Review of C. Elegans Biology*, 1–19.
- L'hernault, S. W. (2006). Spermatogenesis. *Worm Book*, 1–14.
- Lopez, A. L., 3rd, Chen, J., Joo, H. J., et al. (2013). DAF-2 and ERK couple nutrient availability to meiotic progression during *Caenorhabditis elegans* oogenesis. *Dev Cell*, 27, 227–240.
- Ludewig, A. H., & Schroeder, F. C. (2013). Ascaroside signaling in *C. elegans*. *Worm Book*, 1–22.
- Mcknight, K., Hoang, H. D., Prasain, J. K., et al. (2014). Neurosensory perception of environmental cues modulates sperm motility critical for fertilization. *Science*, 344, 754–757.
- Nance, J., Lee, J. Y., & Goldstein, B. (2005). Gastrulation in *C. elegans*. *Worm Book*, 1–13.
- O'hagan, R., Wang, J., & Barr, M. M. (2014). Mating behavior, male sensory cilia, and polycystins in *Caenorhabditis elegans*. *Semin Cell Dev Biol*, 33, 25–33.
- Wang, J. T., & Seydoux, G. (2013). Germ cell specification. *Adv Exp Med Biol*, 757, 17–39.

Reproduction General, Laboratory Animals: *Drosophila*, Tobacco Hornworm, Cockroaches

Lynn M Riddiford, University of Washington, Friday Harbor, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Chorion The insect eggshell.

Choriogenesis The synthesis and secretion of the chorion by the ovarian follicle cells.

Corpora allata Paired endocrine glands in the insect head that produce juvenile hormone.

Diapause A suspended development phase which is accompanied by a lowered metabolism.

Ecdysis The process of shedding the unresorbed, old cuticle.

Eclosion The ecdysis of the adult from the pupal stage.

Fat body The combined liver and adipose tissue of the insect.

Molt Synthesis of a new cuticle followed by resorption of most of the old cuticle, then ecdysis.

Ootheca Egg case of cockroaches.

Oviposition Egg laying.

Vitellogenin Yolk protein.

Vitellogenesis Includes both the synthesis of vitellogenin and its uptake into the oocyte.

Introduction

In insects reproductive maturation usually begins at metamorphosis in response to the steroid hormone ecdysone in the absence of the sesquiterpenoid juvenile hormone (JH). The immature state has been maintained by JH through the growth phase which is punctuated by periodic molts (production of a new, larger cuticle or exoskeleton followed by shedding of the old cuticle) initiated and orchestrated by ecdysone. In the lower insects such as cockroaches which molt directly from the last stage nymph to the adult, reproductive maturation occurs in the young adult. In the higher insects that have complete metamorphosis such as the tobacco hornworm and the fruit fly, some of the maturation occurs in the pupa, whereas the final steps occur in the adult. In both cases, at least in the females, JH is again secreted in the adult and regulates some aspects of reproduction which differ depending on the species being studied. Ecdysone from the ovaries may also be involved, again in different roles depending on the species.

This chapter will describe reproduction in four species commonly used in the laboratory: the fruit fly *Drosophila melanogaster*, the tobacco hornworm *Manduca sexta*, and two cockroach species *Blattella germanica* and *Diploptera punctata*. Both reproductive maturation and behavior and their regulation will be summarized.

Fruit Fly (*Drosophila melanogaster*)

At 25°C, the eggs hatch in 1 day, followed by 3 larval stages during which the larvae feed and grow, then wander out of the food and pupariate about 4 days after hatching. The puparium is the old larval cuticle which forms a protective covering for the formation of the pupa during the next 10–12 h. The adult emerges (ecloses) from the puparium 4 days later. The female is not receptive to the male until about 12–24 h after eclosion.

Male Reproduction

In *Drosophila* primary spermatocytes are first seen in the second instar larval testis, but meiosis and subsequent spermatogenesis does not begin until the prepupal period (Fuller, 1993). Thereafter, they continue throughout the male's life time. All stages of sperm differentiation are seen in squashes of adult testis. The stem cell niche at the apex of the testis contains both germline stem cells and cyst stem cells that support the differentiation of the germline stem cells into sperm. These stem cells require 20-hydroxyecdysone (20E) for their maintenance (Lie *et al.*, 2014). The source of the steroid hormone ecdysone in the adult male is not yet clear although the enzyme that converts ecdysone to 20E is expressed in the testis.

The male accessory glands produce peptides and proteins that are transferred to the female upon mating and diffuse into her hemolymph (blood) to play various roles in reproduction (Ravi Ram and Wolfner, 2007). A major player is the "sex peptide". This peptide binds to the sperm tails in the seminal fluid so first goes to the uterus, then is stored with the sperm in the seminal

receptacle and the spermatheca from which it is gradually released. It has two major functions: 1) to turn off female receptivity and thus mating behavior; 2) to stimulate the rate of JH production by the corpora allata. To turn off receptivity, sex peptide in the uterus decreases the activity of specific sensory neurons that are tonically active in the virgin females and activate specific abdominal neurons that relay the signal to the brain (Feng *et al.*, 2014). Loss of this signal turns off female receptivity. Sex peptide also directly activates JH release from the corpora allata; the role of JH will be discussed below in the section on Female Reproduction. In addition, sex peptide stimulates feeding activity by the mated female. Ovulin, another accessory gland protein, stimulates release of mature eggs from the ovary. Other accessory gland proteins are involved in sperm storage and maintenance.

Female Reproduction

At the time of metamorphosis, 16–20 ovarioles differentiate in each ovary, each of which has an apical germarium containing germline stem cells (Fig. 1). Oogenesis begins during adult development inside the pupa and continues throughout adult life. During oogenesis the germline stem cell divides asymmetrically to produce another germline stem cell and a daughter cell (Spradling, 1993). The daughter cell divides 4 times but the cells retain cytoplasmic connections and form a cyst of 16 cells that are interconnected through these “ring canals”. Two of these cells have connections with four other cells, and one of these becomes the oocyte. The others become the nurse cells that produce nutrients and cytoplasmic components such as mitochondria and ribosomes for the oocyte. The oocyte nucleus begins meiosis which subsequently is arrested in Prophase I during most of oogenesis. The cyst becomes surrounded by a single follicular cell layer. This follicle, usually called the egg chamber, separates from the germarium, and the oocyte matures into the egg through 14 stages. Yolk protein (vitellogenin) synthesis begins in stage 8, both in the follicle cells and in the fat body (the combined liver and adipose tissue of the insect), but is not taken up into the oocyte until stage 10. Vitellogenesis is completed by stage 11. In stage 11 the nurse cells dump their contents into the oocyte and the oocyte progresses to meiosis Metaphase I arrest. Chorion (egg shell) deposition occurs in stages 13 and 14 at the end of which ovulation occurs and meiosis is completed. The eggs are then fertilized as they are laid (oviposited).

At the time of adult eclosion, the female has partially mature ovaries in which oogenesis of the terminal oocyte in each ovariole has proceeded up to previtellogenic stage 8. Vitellogenin synthesis begins after emergence. Mature eggs are found on day 2, and maturation continues asynchronously throughout her lifetime as long as the female mates and feeds.

Both ecdysone and JH are involved in regulation of reproductive maturation in the adult female (Fig. 2). In the ovary, ecdysone is produced by the mature follicle cells at low levels beginning at stage 8 and is involved in several aspects of normal oogenesis (Bownes, 2005; Belles and Piulachs, 2015; Fig. 2). It is necessary both for continued proliferation of the germline stem cells and for their maintenance within the germarium. Ecdysone is also necessary for the onset of meiosis in the oocyte nucleus and for the initial follicle cell formation that occurs in the germarium. Later in stage 9 it causes the initiation of border cell migration between the nurse cells to form a boundary between the nurse cells and the oocyte. These border cells later contribute to the formation of the micropyle in the chorion which allows sperm entry. In stage 10, ecdysone is required to promote lipid uptake and accumulation in the oocyte. Finally it is required for choriogenesis (Papantonis *et al.*, 2015) and ovulation of the mature egg.

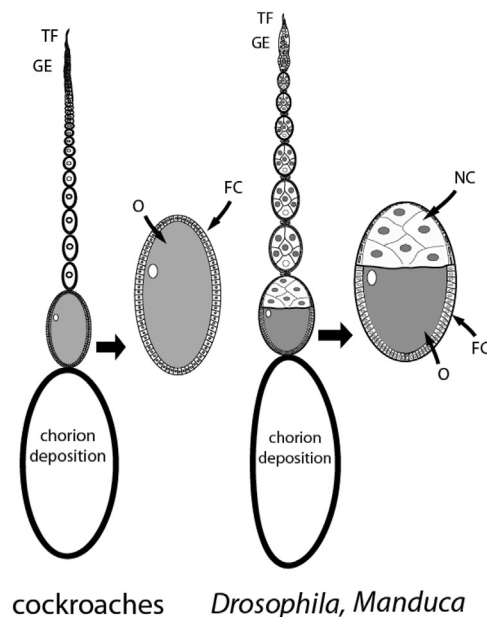


Fig. 1 Diagram of oocyte maturation within an ovariole of the panoistic ovary of the cockroaches *Blattella germanica* and *Diploptera punctata* and of the meroistic ovary of *Drosophila* and *Manduca*. In the panoistic ovary, only the terminal oocyte in each ovariole matures in each reproductive cycle. In the meroistic ovary, there is progressive maturation of the egg chambers (oocytes and associated nurse cells). FC, follicle cells; GE, germarium; NC, nurse cells; O, oocyte; TF, terminal filament.

		<i>Blattella</i>	<i>Diploptera</i>	<i>Manduca</i>	<i>Drosophila</i>
Juvenile Hormone	Vitellogenin synthesis	X	X	0	initiation
	Vitellogenin uptake	X	X	↑ rate	X
	Accessory gland secretions	X	X	?	?
	Receptivity for mating	X	0	0	X
	Sex pheromone synthesis	X	?	0	X
	Midgut remodeling	NA	NA	?	X
Ecdysone	Germline stem cell proliferation & maintenance	?	?	?	X
	Meiosis onset	?	?	?	X
	Lipid uptake	?	?	?	X
	Border cell migration	NA	NA	?	X
	Choriogenesis	X	X	?	X
	Ovulation	?	X	?	X

Fig. 2 Summary of the roles of juvenile hormone and ecdysone in female reproduction in the 4 species being discussed in this chapter. See text for details. X, hormone required; 0, no effect; ?, not studied; NA, not applicable.

Feeding on a protein-rich diet causes the release of insulin-like peptides from brain neurosecretory cells which directly stimulate stem cell proliferation and indirectly germ cell maintenance through effects on the ovarian stem cell niche (Das and Arur, 2017). Insulin action is independent of ecdysone action on these stem cells. If the females are starved or not given any protein, the oocytes at stage 9 begin to be resorbed rather than continuing their maturation (Bownes, 2005; Belles and Piulachs, 2015). This resorption was found to be mediated by increased ecdysone synthesis and accumulation in the ovary. When the females were fed after a period of starvation, the ovarian ecdysteroid level rapidly declined and vitellogenesis proceeded.

The synthesis and release of JH is activated at eclosion and continues thereafter (Bownes, 2005). JH is required for the rapid uptake of vitellogenin by endocytosis during stage 10 of oogenesis (Fig. 2). It is thought that JH stimulates the initiation of yolk protein synthesis in the fat body; but once this synthesis is turned on, its continuation does not require further hormonal stimulation.

JH also mediates the normal timing of mating which usually occurs one to two days after eclosion. In the absence of JH, both female receptivity to male courtship and the production of two female-specific sex attractants are delayed by at least two days (Bilen et al., 2013; Fig. 2). The finding that they are not prevented but only delayed indicates that other, yet unknown factors are involved in regulation of these events. After mating the sex peptide from the male turns off female receptivity as described above and increases both JH production and feeding in the female.

The increase of JH after mating causes remodeling of the midgut (Reiff et al., 2015; Fig. 2). The JH causes proliferation of the midgut cells followed by their differentiation so that the midgut becomes larger. JH also increases fatty acid synthesis and accumulation in the cells of the posterior midgut. These changes in turn lead to increased egg production.

Tobacco Hornworm (*Manduca sexta*)

In the wild tobacco hornworm larvae feed on the leaves of tobacco, tomato and other solanaceous plants, and the resulting moths feed on the nectar of these same plants. In the laboratory the larvae feed on an artificial diet that contains no plant material, but the moths require the plant for oviposition (egg laying). Virgin females begin “calling” (the protrusion of the ovipositor exposing the sex pheromone glands near the tip), thus releasing their pheromone, about two hours after lights-off in a long day photoperiod (16 h light: 8 h dark) (16L:8D) (Sasaki and Riddiford, 1984). At this time the males are flying; when they enter the pheromone plume, they fly upwind to find the female and mate. Mating occurs during the next few hours during the night and usually lasts about 3–4 h at 24°C. No plants are necessary for this phase. Eggs are not laid until the following night beginning at the time of lights off and continuing through the night. The females lay their eggs on the underside of the plant leaves. At 25°C, the eggs hatch four days later.

Male Reproduction

Male reproductive maturation in *Manduca* begins in the prepupal period. Primary spermatocytes can be seen in the penultimate larval testis, but maturation into eupyrene (nucleate) sperm starting with meiosis only begins in the prepupal period and is initiated by the rise of ecdysteroid that causes the pupal molt (Friedlander and Reynolds, 1988). The final maturation of the spermatids

begins in the early pupa and continues through the lifetime of the male. Lepidoptera such as *Manduca* also have apyrene (non-nucleated) sperm. These do not begin their development until early in the pupa. They are transferred along with the eupyrene sperm to the female during mating. Although apyrene sperm have been found necessary for male fertility in the silk moth, their precise role is still unknown (see [Friedlander \(2007\)](#) for a review of the various theories).

When *Manduca* larvae are reared under a short day photoperiod of 12L:12D, the resultant pupae undergo a pupal diapause (a suspended development phase accompanied by a low metabolic rate). Importantly, maturation of the eupyrene sperm begins as described above and proceeds up into early spermatid differentiation at which point continued development is blocked and these spermatids subsequently die ([Friedlander and Reynolds, 1992](#)). This process continues throughout diapause. In contrast, maturation does not begin in the apyrene spermatocytes which appear abnormal and subsequently die. Only when diapause is terminated by the secretion of ecdysone to cause adult development is normal spermatogenesis resumed in both eupyrene and apyrene sperm.

Female Reproduction

Egg maturation begins in the developing adult inside the pupa so that by the time that the adult emerges, she has partially mature eggs that have some yolk in the oocyte but retain the nurse cells ([Nijhout and Riddiford, 1974; Fig. 1](#)). Vitellogenin is synthesized by the fat body and first appears in the hemolymph (blood) 3 days before the adult emerges. Upon eclosion the corpora allata begin to produce JH which mediates full maturation of about 150 eggs in an unfed virgin female over the next week. The first mature eggs are found at 24 h after eclosion. Sucrose feeding increases the number of eggs matured to about 400 by 9 days, apparently by maintaining or increasing the JH secretion by the corpora allata. Neither the release of sex pheromone nor mating is dependent on JH ([Sasaki and Riddiford, 1984; Fig. 2](#)).

Mating increases the production of JH. The stretching of the bursa copulatrix by the spermatophore from the male that contains the male accessory gland secretions and the sperm sends a nervous signal to the brain ([Stringer et al., 1985](#)). This signal causes release of an allatotropin from several pairs of neurosecretory cells in the brain which in turn activates JH secretion. Consequently, a mated female that has access to a continued food supply can mature and lay up to 1400 eggs over her lifetime of two to three weeks.

The molecular basis of the action of JH in mediating egg maturation in *Manduca* has not been studied. In the absence of JH in the adult, the terminal oocyte in each ovariole (the female has 8 ovarioles) grows to about half of its final length and contains some yolk, then begins to resorb the yolk and die whereupon the next one in line matures to this critical stage ([Nijhout and Riddiford, 1974](#)). When JH is present, the oocytes take up yolk protein at a faster rate ([Fig. 2](#)); once sufficient vitellogenin is present, the oocyte hydrates to its final size and the chorion (egg shell) is deposited. How JH increases the rate of vitellogenin uptake is not known.

The role of ecdysone in *Manduca* female reproductive maturation has not been studied ([Fig. 2](#)).

Cockroaches

Two favorite laboratory species of cockroach in which reproduction is studied are *Blattella germanica* (the German cockroach) and *Diploptera punctata* (the Pacific beetle cockroach). In both cases, JH is the main regulator of reproductive maturation in the female ([Fig. 2](#)); male reproductive development begins at metamorphosis and appears to be largely unregulated in the adult. Each will be considered separately.

Blattella germanica

Blattella germanica are usually reared at 27°–30°C. on either dog chow or rodent chow. There are usually 6 nymphal stages followed by metamorphosis to the adult.

Male Reproduction

In *Blattella*, the male accessory glands that contribute to the spermatophore are at least partially regulated by JH since in its absence their protein content was at least 50% reduced ([Schal et al., 1997](#)). The onset of male readiness to mate is also delayed in the absence of JH.

Female Reproduction

At the time of adult ecdysis, the terminal oocyte in each of the 20 ovarioles per ovary is previtellogenic ([Schal et al., 1997; Fig. 1](#)). The female begins feeding and on day 2 at 27°C, terminal oocyte maturation begins. During the next few days, the female becomes sexually receptive and produces and releases her sex pheromone, and mating ensues. The egg case (ootheca) containing 40 eggs

is extruded partially from the female on day 9 but not released until 21 days later when the nymphs hatch. This 21 day period is called gestation or pregnancy. Maturation of the new terminal oocytes does not begin until the ootheca is released. The next ootheca is extruded 7 days later. These cycles repeat throughout the lifetime of the female which lives about 180 days.

In this cockroach, feeding after adult ecdysis is prerequisite for egg maturation since it is necessary for the onset of JH biosynthesis. Juvenile hormone is required for vitellogenic oocyte maturation as it stimulates vitellogenin production by the fat body and likely vitellogenin uptake by the oocytes (Fig. 2). In a fed female, JH synthesis and release begins on day one after adult emergence and continues throughout vitellogenesis, then declines during the final stage of choriogenesis to undetectable levels by the time of extrusion of the ootheca. No JH is produced during the time that the embryos develop; its synthesis and release begins again when the ootheca is released. In addition, JH stimulates the female accessory glands to produce the proteins that are necessary for production of the ootheca. The cessation of JH production during the gestation period is due to nervous inhibition from the brain but exact mechanisms are not known.

JH also is necessary for female sex pheromone production and for the female to become receptive to the male (Fig. 2). Mating causes the immediate cessation of both female receptivity and pheromone release due to presence of the spermatophore in the bursa copulatrix that sends a nervous signal to the brain; continued inhibition of both is due to sperm in the spermatheca.

Social interactions with other females accelerate reproductive maturation by increasing the rate of JH production. Under these conditions, there is a synchronization of oothecal extrusion by the females. However, overcrowding prevents this phenomenon.

Ecdysteroids are also involved in oogenesis in this species. Ecdysone from the follicle cells of the ovary induces choriogenesis once vitellogenesis is complete (Belles and Piulachs, 2015; Fig. 2).

Diploptera punctata

In the laboratory *Diploptera* are reared on lab chow at 27°C. Males have three nymphal stages while females have 4. The females mate immediately after adult ecdysis whereas the males usually do not court and mate until they are about a week old. The females are viviparous and retain their embryos in a brood sac until they hatch.

Male Reproduction

Diploptera males mature more slowly and normally do not show courtship or mating behavior for at least a week after adult ecdysis. The corpora allata produce a small amount of JH but accessory glands, spermatophore production and sexual behavior are normal in its absence (Tobe *et al.*, 1979). Further studies on the regulation of male reproduction have not been done in this species.

Female Reproduction

In virgin females, nervous inhibition prevents the synthesis and secretion of JH from the corpora allata (Marchal *et al.*, 2013). Upon mating, the presence of the spermatophore in the bursa copulatrix acts via the brain to relieve this inhibition and to activate JH production by the corpora allata. Mating also stimulates oocyte growth. There are 6 ovarioles in each ovary and only the terminal oocyte matures in each reproductive cycle. Vitellogenesis begins on day 2 with vitellogenin synthesis and secretion by the fat body and uptake into the terminal oocyte and continues until day 6. Then choriogenesis occurs, so by day 7, the eggs are mature and ovulated and fertilized, then covered by an incomplete ootheca. Instead of being extruded as in *Blattella*, the ootheca is retracted into the brood sac where embryonic development occurs. During this 63 day period, the brood sac produces “milk proteins” and carbohydrates for nourishment of the embryos which they drink during later embryonic development.

Juvenile hormone is necessary for both vitellogenin production by the fat body and its uptake by the oocyte (Fig. 2). As vitellogenesis nears its end, the corpora allata become sensitive to allatostatin from brain neurosecretory cells through an increase of allatostatin receptors and JH synthesis declines. During pregnancy no JH is synthesized. Synthesis begins again after the embryos are born, and a second cycle begins. The female accessory glands also require JH for the production of the ootheca.

Studies of the effects of the depletion of ecdysone receptors in female *Diploptera* have indicated that ecdysone is necessary for chorion formation and for ovulation of the eggs (Hult *et al.*, 2015; Fig. 2). Ecdysone is produced by the follicle cells during the later phases of oocyte maturation. It also seems to be involved in the shutting down of JH synthesis by the corpora allata at the end of vitellogenesis.

Acknowledgements

I thank Dr. James Truman for drawing Fig. 1 and comments on the manuscript.

References

- Belles, X., & Piulachs, M.-D. (2015). Ecdysone signaling and ovarian development in insects: From stem cells to ovarian follicle formation. *Biochimica et Biophysica Acta*, 1849, 181–186.
- Bilen, J., Atallah, J., Azanchi, R., Levine, J. D., & Riddiford, L. M. (2013). Regulation of onset of female mating and sex pheromone production by juvenile hormone in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 18321–18326.
- Bownes, M. (2005). The regulation of yolk protein gene expression and vitellogenesis in higher Diptera. *Reproductive Biology of Invertebrates*, 12, 95–128.
- Das, D., & Arur, S. (2017). Conserved insulin signaling in the regulation of oocyte growth, development, and maturation. *Molecular Reproduction and Development*, 84, 444–459.
- Feng, K., Palfreyman, M. T., Hässemeyer, M., Talsma, A., & Dickson, B. J. (2014). Ascending SAG neurons control sexual receptivity of *Drosophila* females. *Neuron*, 83, 135–148.
- Friedlander, M. (2007). Control of the eupyrene-apyrene sperm dimorphism in Lepidoptera. *Journal of Insect Physiology*, 43, 1085–1092.
- Friedlander, M., & Reynolds, S. E. (1988). Meiotic metaphases are induced by 20-hydroxyecdysone during spermatogenesis of the tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology*, 34, 1013–1019.
- Friedlander, M., & Reynolds, S. E. (1992). Intratesticular ecdysteroid titres and the arrest of sperm production during pupal diapause in the tobacco hornworm, *Manduca sexta*. *Journal of Insect Physiology*, 38, 693–703.
- Fuller, M. T. (1993). Spermatogenesis. In M. Bate, & A. Martinez Arias (Eds.), *The Development of Drosophila melanogaster* (vol. 1, pp. 71–147). Plainview, New York: Cold Spring Harbor Laboratory Press.
- Hult, E. F., Huang, J., Marchal, E., Lam, J., & Tobe, S. S. (2015). RXR/USP and EcR are critical for the regulation of reproduction and the control of JH biosynthesis in *Diploptera punctata*. *Journal of Insect Physiology*, 80, 48–60.
- Lie, Y., Ma, Q., Cherry, C. M., & Matunis, E. L. (2014). Steroid signaling promotes stem cell maintenance in the *Drosophila* testis. *Developmental Biology*, 394, 129–141.
- Marchal, E., Hult, E. F., Huang, J., Stay, B., & Tobe, S. S. (2013). *Diploptera punctata* as a model for studying the endocrinology of arthropod reproduction and development. *General and Comparative Endocrinology*, 188, 85–93.
- Nijhout, M. M., & Riddiford, L. M. (1974). The control of egg maturation by juvenile hormone in the tobacco hornworm moth, *Manduca sexta*. *Biological Bulletin*, 146, 377–382.
- Papantonis, A., Swevers, L., & Iatrou, K. (2015). Chorion genes: a landscape of their evolution, structure, and regulation. *Annual Review of Entomology*, 60, 177–194.
- Ravi Ram, K., & Wolfner, M. F. (2007). Seminal influences: *Drosophila* ACPs and the molecular interplay between males and females during reproduction. *Integrative and Comparative Biology*, 47, 427–445.
- Reiff, T., Jacobson, J., Cognigni, P., et al. (2015). Endocrine remodeling of the adult intestine sustains reproduction in *Drosophila*. *eLife*, 4, e06930.
- Sasaki, M., & Riddiford, L. M. (1984). Regulation of reproductive behavior and egg maturation in the tobacco hawk moth, *Manduca sexta*. *Physiological Entomology*, 9, 315–327.
- Schal, C., Holbrook, G. L., Bachmann, J. A. S., & Sevala, V. L. (1997). Reproductive biology of the German cockroach, *Blattella germanica*: Juvenile hormone as a pleiotropic master regulator. *Archives of Insect Biochemistry and Physiology*, 35, 405–426.
- Spradling, A. (1993). Developmental genetics of oogenesis. In M. Bate, & A. Martinez Arias (Eds.), *The Development of Drosophila melanogaster* (vol. 1, pp. 1–70). Plainview, New York: Cold Spring Harbor Laboratory Press.
- Stringer, I. A. N., Giebeltoewicz, J., & Riddiford, L. M. (1985). Role of the bursa copulatrix in egg maturation and reproductive behavior of the tobacco hawk moth, *Manduca sexta*. *International Journal of Invertebrate Reproduction and Development*, 8, 83–91.
- Tobe, S. S., Musters, A., & Stay, B. (1979). Corpus allatum function during sexual maturation of male *Diploptera punctata*. *Physiological Entomology*, 4, 79–86.

Sea Urchins as Lab Animals for Reproductive and Developmental Biology

Amro Hamdoun, Catherine S Schrankel, Katherine T Nesbit, and Jose A Espinoza, University of California San Diego, San Diego, CA, United States

© 2018 Elsevier Inc. All rights reserved.

Early History of the Sea Urchin Embryo as a Lab Animal

The sea urchin has a rich history as a model organism, dating back to the mid 1800s (Table 1; reviewed in Ernst, 2011). Given the ease of gamete collection and embryo observation, the sea urchin became an important experimental model for 19th century scientists exploring the fundamental questions of development: What is the role of the nucleus in development (as sperm and egg each appear to have one)? What information exchange occurs at the union of egg and sperm? How does this enable the fertilized egg to blossom (or “differentiate”) into an adult organism that exhibits the same body plan, cell types and organs as the parents? Early studies in sea urchins overturned long-held theories of preformation – that a miniature individual is already encapsulated in totem within a sperm (the “homunculus”) – and demonstrated that the individual is formed by the fusion of nuclear material from both sperm and egg.

A Single Sperm for Every Egg

The sea urchin was used to study fertilization as early as the 1840s, by Derbs and colleagues (reviewed in Briggs and Wessel, 2006). In the later 1800s, using sea star and sea urchin eggs, Hermann Fol and Oscar Hertwig were the first scientists to observe that a single sperm penetrates the egg membrane during fertilization (Hertwig, 1877). Fol also observed that an envelope immediately forms around the egg, presumably to prevent additional sperm from fusing (known as “polyspermy”) (Fol, 1877). Fol and Hertwig also documented that the nucleus of a successfully penetrated sperm will fuse with the nucleus of the egg.

Table 1 Milestones and major discoveries in sea urchin embryology ~1875–2000s

Fusion of sperm and egg nuclei at fertilization; fusion of a single sperm with the egg at fertilization	Hertwig (1877), Fol (1877)
Demonstration of phagocytosis and foundation of the cellular theory of immunology (Nobel Prize 1908, Elias Metchnikoff)	Metchnikoff (1893)
Chromosomes in nucleus are determinants for development; individual chromosomes possess different qualities	Boveri (1902)
DNA is found in chromosomes and RNA is found in cytoplasm of all cells; first correlation of RNA and protein synthesis	Brachet (1945)
Isolation of the mitotic apparatus during cleavage	Mazia and Dan (1952)
Discovery of long-lived maternal mRNAs and polyribosomes	Gross and Cousineau (1963), Monroy and Tyler (1963)
First characterization of hybridization kinetics of single and double-stranded DNA; gene-battery model of eukaryotic transcription proposed	Britten and Kohne (1968), Britten and Davidson (1969)
Cloning of the first eukaryotic gene (histone)	Kedes <i>et al.</i> (1975b)
Na ⁺ /H ⁺ exchanger identified; first description of amino acid transporter activation at fertilization; first isolation of intact cortical granules	Vacquier (1975), Epel (1975)
First demonstration of the electrical block to polyspermy in an animal egg	Jaffee (1976)
Isolation of bindin, the first protein shown to bind sperm to eggs	Vacquier and Moy (1977)
Demonstration of the first cyclin (2001 Nobel Prize to Tim Hunt)	Evans <i>et al.</i> (1983)
First demonstration of chemotaxis of sperm to an egg-derived peptide (resact)	Ward <i>et al.</i> (1985)
Discovery of the Ca ²⁺ messenger, cyclic ADP ribose	Lee <i>et al.</i> (1989)
Cloning of the first membrane receptor guanylyl cyclase, involved in sperm binding	Chinkers <i>et al.</i> (1989)
Accumulation of beta-catenin specifies the animal/vegetal axis of sea urchins	Wikramanayake <i>et al.</i> (1998); Logan <i>et al.</i> (1998)
Sea urchin endomesoderm GRN: the first and most extensive analysis of the specification of a germ layer lineage in any organism	Davidson <i>et al.</i> (2002)
Isolation of the bindin receptor EBR1 from sea urchin eggs	Kamei and Glabe (2003)
<i>S. purpuratus</i> genome sequenced: Expansion of innate immunity, defensome, and select TF gene families	Sodergren <i>et al.</i> (2006); special issue of Developmental Biology (Davidson <i>et al.</i> , 2006)
Identification of the primordial germ cell lineage	Juliano <i>et al.</i> (2006, 2010)
Demonstration of the activation of xenobiotic transporters at fertilization, and the xenobiotic metabolism pathways of the embryos; first complete description of embryonic and larval immune system of the purple sea urchin	Hamdoun <i>et al.</i> (2004), Shipp and Hamdoun (2012), Ho <i>et al.</i> (2016)
First demonstration of CRISPR/Cas9 editing in the sea urchin embryo	Lin and Su (2016)
Deuterostome origins of the Spemann organizer	Lapraz <i>et al.</i> (2015)

The Nuclear Basis of Inheritance

Fol and Hertwig's work on pronuclear fusion illuminated that the zygote contains nuclear substance derived from both parents. Biologist Theodor Boveri subsequently demonstrated that nuclei were necessary for proper development of the fertilized egg. Boveri also characterized that a normal set of chromosomes in each cell is required for early development (Boveri, 1902). By watching chromosomes separate during mitosis of urchin zygotes, Boveri was the first person to make the giant conceptual leap to state that Mendel's factors of inheritance must be on the chromosomes. Around the turn of the 20th century, Hans Driesch also used sea urchin embryos to define the equipotent potential of early quadrants of the embryo ("blastomeres"). He showed that one blastomere isolated from a 2 or 4-cell stage sea urchin embryo can develop into a smaller, yet complete larva. These experiments were refined by Sven Horstadius in the 1930s who clarified the role of localized determinates in the egg (molecules that define future body axes) by splitting or stretching individual blastomeres or embryos along different axes and assessing developmental outcomes (reviewed in Ernst, 2011).

Sea Urchins Used in the Lab

Major Species Used and Their Experimental Advantages

Sea urchins are ancient marine invertebrates of Class Echinodea within the phylum Echinodermata. There are approximately 900 extant species of sea urchins distributed throughout the world's coastal oceans. Of these a handful have been used as lab animals, primarily *Strongylocentrotus purpuratus*, *Lytechinus variegatus*, *Arbacia punctulata*, *Hemicentrotus pulcherrimus* and *Paracentrotus lividus* (Table 2) which are native to the Pacific, Atlantic and Mediterranean Oceans. Several of these species have fully sequenced and publically available genomes (www.echinobase.org). Two other species, including *Lytechinus pictus* and *Temnopleurus hardwicki* are also useful as lab models, due their relatively small adult size and shorter generation times.

Table 2 Summary of species information and breeding season for select sea urchin lab species

Species name	Common name	Habitat range and temperature	Breeding season ^a	Egg diameter	Key developmental stages ^a (time after insemination)	
<i>Strongylocentrotus purpuratus</i>	Purple urchin	Alaska-Baja California; 12–17°C	November–March	80 µm	Blastula	16 h
					Gastrulation	30 h
					Pluteus larva	4 Days
					Metamorphosis	40–80 Days
<i>Lytechinus variegatus</i>	Green urchin	North Carolina-Bahamas; 20–25°C	May–September	120 µm	Blastula	6 h
					Gastrulation	11 h
					Pluteus larva	20–24 h
					Metamorphosis	30–40 Days
<i>Lytechinus pictus</i>	Painted urchin	Southern California; 18–22°C	May–September	120 µm	Blastula	6 h
					Gastrulation	24 h
					Pluteus larva	48 h
					Metamorphosis	30–40 Days
<i>Temnopleurus</i> species (<i>T. toreumaticus</i> and <i>T. reevesii</i>)	Japanese urchin	Western Pacific; Japan and China Seas; 22–26°C	July–January	100 µm	Blastula	8–10 h
					Gastrulation	11–12 h
					Pluteus larva	28–30 h
					Metamorphosis	30–33 Days
<i>Paracentrotus lividus</i>	Mediterranean urchin	Mediterranean Sea-Eastern Atlantic; 18–25°C	May–September	75 µm	Blastula	4 h
					Gastrulation	5 h
					Pluteus larva	24 h
					Metamorphosis	20–30 Days

^aReproductive cycles and time to metamorphosis may fluctuate, based on local environmental conditions; the earliest pluteus larval stage (2–4 arm) is indicated.

Sources: Reproduced from Strathmann, R.R., 1987. Larval feeding. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (Eds.), Reproduction of Marine Invertebrates. Volume IX: General Aspects: Seeking Unity in Diversity. Pacific Grove, CA: Blackwell Scientific Publications, Palo Alto/Boxwood Press, pp. 465–550 and Pearse, J., Cameron, R.A., 1991.

Echinodermata: Echinoidea. In: Giese, A.C., Pearse, J.S., Pearse, V.B. (Eds.), Reproduction of Marine Invertebrates. vol. VI: Echinoderms and Lophophorates. Pacific Grove, CA: Boxwood Press, pp. 514–662.

The purple sea urchin, *S. purpuratus*, was the first echinoderm species to have its genome sequenced, and is the most widely used in the lab. The genome of *S. purpuratus* is approximately 814 million bases in length and encodes 23,300 genes (Sodergren *et al.*, 2006). As basal deuterostomes, the branch of evolution leading to vertebrates, sea urchins share many genes with humans. Complete developmental transcriptomes from multiple stages of *S. purpuratus* development are publically available at www.echinobase.org. Roughly one third of urchin genes encode proteins that are found in humans. These include many genes that are responsible for human genetic diseases. Remarkably, sea urchins also have a diverse repertoire of genes involved in hearing and vision, the latter genes functioning in photoreception of the adult tube feet. They also exhibit a significantly expanded innate immune gene repertoire compared to vertebrates.

The defining feature of sea urchins as lab animals is their extreme fecundity. Ripe animals can be easily induced to spawn by gentle shaking, injection of potassium chloride (KCl) into the coelomic cavity, or by mild electric shock. In the case of potassium chloride, the potassium ions cause muscle contractions that expel gametes. During spawning, eggs or sperm are released through five gonopores located at the top (aboral) surface of the animal, encircling the anus. A gravid female purple urchin yields 5–10 mL of packed eggs, while males produce approximately 0.5–1 mL of “dry” (i.e., prior to dilution of seawater) semen. “Dry” *S. purpuratus* semen is consistently 4×10^{10} cells per mL. Eggs with jelly coats removed are 2.8×10^6 eggs per mL. Egg sizes vary from 70–150 μm in diameter depending on species (Table 2). Eggs can be maintained for several days in sea water containing antibiotic(s) such as ampicillin, sulfamethoxazole and/or trimethoprim to prevent microbial growth. Concentrated sea urchin sperm collected directly from the surface of the animal are immotile until diluted into sea water. If collected directly from the males, sperm can be stored for several days at 4°C.

Adult Anatomy and Anatomical Structure of Gonads

Sea urchins typically range in size from 6 to 12 cm. The largest species can reach up to 36 cm in diameter. Adult sea urchins have radial symmetry: the body is roughly spherical and is composed of five equally sized parts radiating out from a central axis. The inner shell, called a test, is made up of interlocking plates of calcium carbonate material and is covered by a thin skin and rows of spines (the classification “Echinoderm” derives from the Greek words for “spiny skin”). Spines vary in size and diameter according to species. An internal water vasculature system pumps water through the spines and tube feet, which are flexible tube-like structures that terminate with miniature suction cups. The internal organs are enclosed within the test, and the nervous system consists of a small nerve net encircling the mouth, from which five nerves enervate the rest of the animal. The mouth is found on the bottom, or oral face, of the animal. The sea urchin “jaw” consists of five bony teeth and tongue-like protrusion, collectively known as Aristotle’s lantern.

Sea urchins are benthic creatures and eat plant and animal matter, largely preferring kelp, algae, and sponges in their rocky habitats, as well as decaying matter that settle down from the water column. Sea urchins have a simple digestive tract encircled within the test. The anus is located at the aboral surface of the animal. The body cavity has an open circulatory system and is filled with coelomocytes. Coelomocytes perform essential clotting, scavenging and phagocytosis functions. Passive gas exchange in the coelomic fluid occurs through gill-like structures located around the mouth.

The gonads also reside within the body cavity and comprise a much as 30% of the body mass. The gonad of the sea urchin is the major reproductive and nutrient storage organ for the adult. The reproductive cycle is unique to each species (see Table 2), and is closely linked to photoperiod, water temperature, and food availability (reviewed in Pearse and Cameron, 1991). The gonad is made up of reproductive cells (oogonium, oocyte and ovum stages for females; spermatogonium, spermatocyte, spermatid and spermatozoon for males) as well as storage cells, also known as nutritive phagocytes (NPs). Various proteins, carbohydrates and lipids that are needed for gametogenesis and emergency metabolism are stored within the NPs. The percentage of NPs vs reproductive cells contributes significantly to the size and quality of the gonad, and varies throughout the reproductive cycle.

Reproductive Cycles

Despite the differences in spawning seasons for individual sea urchin species (Table 2), sea urchin reproductive cycles can be divided broadly into four stages (reviewed in Walker *et al.*, 2007; Pearse and Cameron, 1991). Stage I occurs immediately post-spawning (egg or sperm release) and lasts for about 3 months. Reproductive cells remain sparse at the beginning of Stage I, as NPs phagocytose unused egg and sperm precursors. The number of NP cells increases during this stage, yet reproductive cells eventually reemerge around the periphery of the gonads by the end of Stage I. In Stage II, which lasts 3–4 months, NP renewal continues and reproductive cells increase in number and size at the gonad periphery. By Stage III, the reproductive cells migrate into the gonad and continue to proliferate. This stage can last several months depending on the species, and the ratio of reproductive cells to NPs increases markedly during this time. At stage IV, gonads are packed full of differentiated gametes: eggs or sperm, whereas NP numbers are reduced or absent. Spawning occurs towards the end of Stage IV, in which most or all of the gametes are released. In the lab, this can be stimulated by 0.5 M KCl injection or by gentle shaking.

Fertilization and Early Cleavages of the Sea Urchin Embryo

Fertilization of Sea Urchin Eggs

The sea urchin is an excellent organism for live demonstration of fertilization and early development in lab or classroom settings (see Relevant Websites section). *In vitro* fertilization is easily conducted by mixing diluted eggs and sperm of the same species in sea

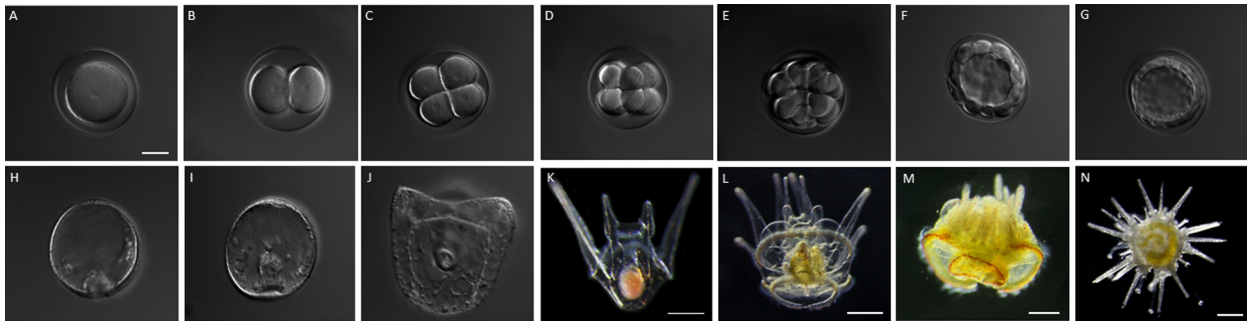


Fig. 1 Larval development of the sea urchin, *Lytechinus pictus*. Sea urchins were raised at room temperature. (A) Newly fertilized zygote; (B) 2-cell stage, 1.5 h post fertilization (hpf); (C) 4-cell stage, 2.5 hpf; (D) 8-cell composite image of two focal planes within an embryo, 3.5 hpf; (E) 16-cell stage, 4hpf; (F) 60-cell stage, 5hpf; (G) Early blastula before hatching from the fertilization envelope, 6.5 hpf; (H) Early gastrula, 24hpf; (I) Mid-stage gastrula, 26 hpf; (J) Prism larvae, 43.5hpf; (K) 4-arm pluteus larvae; (L) 8-arm stage; in a competent larvae, the rudiment that becomes the juvenile body begins to form around the region of the gut (yellow mass in the center of the embryo); in (M) the rudiment grows and the juvenile emerges from the larval body during metamorphosis; (N) Newly settled juvenile urchins are approximately 1 mm in diameter and form mouth parts to begin feeding on benthic diatoms and biofilms after 3 days. Later, their diet incorporates kelp and other macroalgae. Settlement of the juvenile takes approximately 4–5 weeks from fertilization. Scale bar = 50 μ m for (A)–(J). Scale bar = 0.25 mm for panels (K)–(M), and 0.5 mm for panel (N).

water. Given that sea urchin spawn externally, mechanisms exist to ensure species fidelity of the sperm and egg interaction. Sea urchin sperm contain a molecule known as bindin that is released during the acrosome reaction, and binds to vitelline layer of conspecific eggs. Evolution of this molecule is essential for the reproductive isolation of different species of sea urchin (Vacquier and Moy, 1977).

In the lab, when mature gametes are used at appropriate concentrations, successful fertilization routinely exceeds 95% of all eggs. Embryos develop through gastrulation within 2–3 days of fertilization, requiring nothing more than temperature-controlled sea water (Fig. 1). The feeding larval stage of the sea urchin, termed the pluteus larva, begins several days after fertilization and lasts from several weeks to months depending on species (Table 2). The rudiment, or juvenile sea urchin, develops indirectly within this larva and sheds the larval body upon settlement onto the substratum. The larvae and newly settled urchin juveniles feed on phytoplankton including microalgae and diatoms such as *Rhodomonas* and *Nitzschia*. Juveniles become gravid between 6 months and 2 years post-settlement, depending on the species.

Egg Activation and Surface Reorganization

Eggs of sea urchins are mature at release, having completed meiosis and arrested at the G1 phase of mitosis. Eggs are quiescent at spawning, but undergo a massive increase in metabolic activity at fertilization (reviewed in Pearse and Cameron, 1991). The initial signal for activation is a wave of calcium ions that sweeps across the egg, from the point of sperm fusion. Calcium release from intracellular stores is mediated by canonical second messengers, including the molecule cyclic ADP ribose (cADPR) which was discovered in sea urchins (Lee et al., 1989). These initial signaling events lead to a rapid change in membrane voltage from -70 to $+20$ mV, which acts as a fast block to fusion of other sperm with the egg (Jaffee, 1976). This is termed the fast block to polyspermy.

Following fertilization, dramatic cell surface reorganization involving sequential waves of vesicles insertion and membrane retrieval transforms the surface of the egg from one adapted to bind and fuse sperm to one adapted to promote development (reviewed in Vacquier, 1981). A conspicuous consequence of this reorganization is elevation of the fertilization envelope (Fig. 1(A)), which occurs within minutes of fertilization and is mediated by the exocytic release of 16,000 (in *S. purpuratus*) cortical granules docked at the egg surface. This envelope is formed by elevation of a glycoprotein meshwork under the plasma membrane termed the egg vitelline layer as proteins are released from the cortical granules (Vacquier et al., 1973). The resulting envelope is hardened by enzymes present in the cortical granules. This envelope acts as the slow block to polyspermy, and remains around the embryo until hatching thus likely also acting to protect against physical damage of the embryo.

The surface changes in the egg also include the activation of a several types of membrane transport activity including a Na^+/H^+ exchanger, amino acid transporters, and xenobiotic transporters. The exchanger alkalizes and activates the egg, while amino acid transporters concentrate nutrients from sea water into the egg (Epel, 1975). A notable surface change is an 80–100 fold increase in multidrug transporters activity by insertion of vesicles containing a homolog of mammalian P-glycoprotein (P-gp), the protein responsible for chemotherapeutic resistance of several types of cancer (Hamdoun et al., 2004). In early development, P-gp is responsible for effluxing environmental toxins and metabolic by-products from the embryo.

Early Cleavage Stages

An important feature of sea urchin embryonic development is the synchrony of development across embryos. As such, the early cleavage stages of sea urchins have been favorite models for the study of cell division. The mitotic apparatus was first isolated as

a structure in the sea urchin (Mazia and Dan, 1952), as were cyclins, which are cell cycle regulators that oscillate in abundance during the cell cycle and thereby regulate mitotic progression (Evans *et al.*, 1983). The duplication of the centrosome, which gives rise to the two poles of the mitotic apparatus and is the heart of the division apparatus of all eukaryotic cells, was first isolated from sea urchin zygotes (Thompson-Coffe *et al.*, 1996).

The first three cleavages of sea urchins are synchronous and symmetric. By the fourth cell cycle, cells at the vegetal pole of the embryo undergo two critical asymmetric cleavage events. The fourth cleavage produces four cells at the vegetal pole termed micromeres that eventually contribute to the larval skeleton. At the fifth cleavage, the micromeres divide once more to form four daughter cells termed the small micromeres. These small micromeres express canonical molecular markers of the germline (*vasa*, *nanos* and *piwi*) and are the presumptive primordial germ cells (PGCs), the embryonic precursors to the germline (Juliano *et al.*, 2006, 2010). Ablation of these cells results in formation of adults that are morphologically normal but lack egg or sperm (Yajima and Wessel, 2012).

Segregation of the Primordial Germ Cell Lineage

The germline lineage contributes to gametogenesis (the development of egg and sperm) in adult organisms. The location and developmental stage of PGC specification differs among species. For many animals, PGCs are specified by an inherited mechanism, in which determinants within the oocyte get partitioned into cells that subsequently acquire the germline fate. In contrast, an inductive signaling mechanism generates the germline in other animals. Sea urchins have a distinct embryonic segregation of PGCs. They appear to utilize an inherited mechanism of germline formation, whereby germline genes present in the egg are selectively retained only in the PGCs, and degraded elsewhere in the embryo (reviewed in Wessel *et al.*, 2014).

Sea urchin embryos form four PGCs (also termed small micromeres), at the vegetal pole at the fifth cleavage. The cells divide only once more in early development to form eight cells. The eight PGCs are motile cells capable of migration shortly after their formation, although they remain relatively stationary on the developing gut tube during gastrulation (Campanale *et al.*, 2014). At the end of gastrulation these cells home to the left and right coelomic pouches in a characteristic 5-left/3-right pattern (Pehrson and Cohen, 1986) (Fig. 2). The cells on the left seed the germline of the juvenile, while those on the right pouch undergo apoptosis. The evolutionary significance of this asymmetric germ cell segregation, and subsequent programmed death of the right fated cells, remains unclear. An intriguing hypothesis is that this serves as some PGC quality control mechanism.

The motility mechanisms for left-right segregation to the coelomic pouches are not yet well understood. It is hypothesized that the extended filopodia sense migratory cues, in the form of a morphogen or signaling gradient. In other animals, germline chemoattractants are derived from rhodopsin, CXCR4, or phospholipid signaling pathways. In the sea urchin system, the exact signals that home the PGCs have yet to be determined, although several transcription factors that operate in the left coelomic pouches (Materna *et al.*, 2013) have been implicated upstream of the homing signal (Martik and McClay, 2015).

An important aspect of PGC biology in all animals is that specification and migration occurs during a vulnerable window of inductive intercellular signaling and (re)programming. This requires a system that protects the cell from mutagens, as these could directly harm the next generation. Unexpectedly the protective transporters that are activated at fertilization are selectively reduced in the sea urchin primordial germ cells (Campanale and Hamdoun, 2012), suggesting a potential trade-off between signaling and protection pathways. The significance of this down-regulation is unclear, however it could be required for the detection of developmental signaling molecules. This phenomenon has been exploited to load sea urchin primordial germ cells with fluorescent dyes and isolate them to purity using flow cytometry (Swartz *et al.*, 2014).

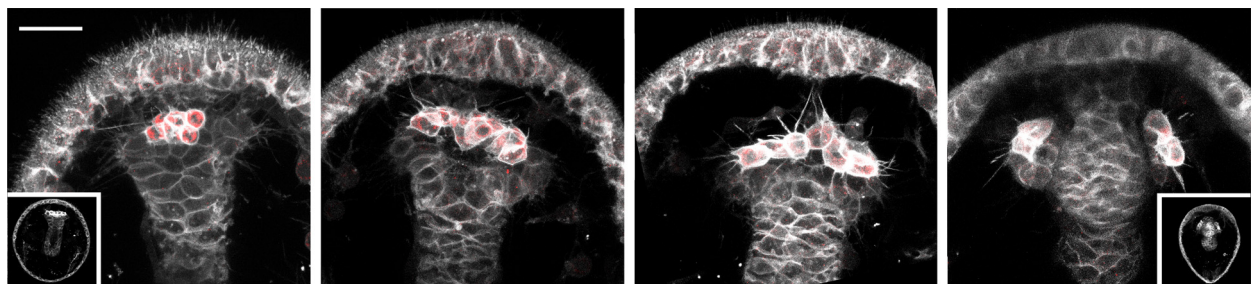


Fig. 2 Migration of sea urchin primordial germ cells (PGCs) in *S. purpuratus*. The 8 PGCs (red) are located in a cluster at the tip of the developing gut tube (the archenteron) at the end of gastrulation (left inset). The PGCs at this stage are rounded, and maintain membrane (grey) contact with neighboring PGCs. Once migration is initiated the PGCs adopt an amoeboid shape and have short filopodia. In the final stage of migration the PGCs produce long filopodia and segregate into coelomic pouches on the left and right side of the archenteron (right most panel, right inset). Scale bar = 20 μ m.

Genetic and Cellular Control of Embryonic Development in Sea Urchins

Polarity and Establishment of the Major Body Axes

Both cellular and developmental manifestations of polarity are apparent in sea urchin eggs and early embryos. Purple sea urchin eggs have a polarized distribution of molecules that regulate axis formation along the animal-vegetal axis (A/V; i.e., top/bottom or future neural vs endomesoderm territories). In one species of urchin, *Paracentrotus lividus*, a reddish band of pigment granules is concentrated in the vegetal pole of embryo. In the purple sea urchin, the establishment of the A/V axis is initiated through β -catenin accumulation in the vegetal half of the egg (Wikramanayake *et al.*, 1998). Nuclearization of β -catenin and other maternal factors in the vegetal half of the developing embryo drives Delta/Notch signaling transduction. This initiates an endomesodermal program (future endoderm and mesoderm) in a ring of vegetal cells of the early embryo (Logan *et al.*, 1998; Davidson *et al.*, 2002). These include proteins in the *wnt* pathway, which are signaling pathways that turn on additional gene networks responsible for A/V axis formation. The secondary body axis, the oral-aboral (OA) axis (analogous to the dorsal-ventral axis), is established during cleavage stage by a maternal distribution of mitochondria (Coffman and Davidson, 2001). Downstream nodal signaling in the presumptive oral ectoderm further reinforces the OA axis (Lapraz *et al.*, 2015).

Cellular polarity, defined here as the segregation of membrane compartments to apical (outward facing) and basal (inward facing) compartments, is evident as early as the two-cell stage in sea urchins (Alford *et al.*, 2009). This polarity is also characterized by the segregation of thousands of membrane projections, known as microvilli, to the outward facing surfaces of the embryo. These microvilli are dynamic and grow and shrink with fertilization and the progression of the cell cycle (Schroeder, 1978). They appear to function in control of vesicle trafficking events and to increase the surface area of the embryo for absorption of nutrients from sea water. The individual cells of the embryo do not collectively form an epithelium until later in development when the blastocoel forms. At this stage (blastula) the embryo resembles a hollow ball of cells (Fig. 1(F)), with tight junctional complexes between cells and polarized distribution of membrane proteins to apical and basal surfaces.

Gene Regulatory Networks of Development

For scientists studying the flow of information from the nucleus, the sea urchin has long been an attractive model organism. Major advances in understanding or describing gene regulation of development, often for the first time, were pioneered in sea urchin embryos (reviewed in Ernst, 2011). Such discoveries include differential localization of DNA and RNA in the cell, maternal long-lived mRNAs present in the oocyte, functional separation of transcription and translation, and cytoplasmic polyadenylation of mRNAs (Table 1 and references therein). Indeed, the first eukaryotic genes to be cloned were histone genes from the sea urchin (Kedes *et al.*, 1975).

Studies in sea urchin embryos have generated exceptionally detailed descriptions of embryonic gene regulation and function. Reports in the 1970s and 1980s marked the birth of linking gene regulation definitively to developmental differentiation. By the 1990s and 2000s a “systems biology” approach to development – involving the generation of gene regulatory network (GRN) models – was established using the sea urchin (Davidson *et al.*, 2002). GRNs model hierarchical cell fate decisions by describing the interactions between transcription factors (TFs) and the promoters of their target genes. These cascades in gene regulation result in several layers of differentiation, from the initial patterning of major axes and body part plans, to the establishment of terminal cell-type specific differentiation batteries (reviewed in Davidson, 2010). The ability to selectively identify and perturb TF expression within the sea urchin embryo enabled the characterization of one of the most detailed gene expression analyses of any embryonic event described to date: endomesoderm specification (Davidson *et al.*, 2002). GRNs for other major developmental processes in the sea urchin have been characterized as well, including epithelial to mesenchymal transitions (EMT), biomineralization, coelomic pouch development and PGC homing, and muscle and immunocyte specification.

An active area of research in the sea urchin is devoted to linking the information encoded in the GRN to cellular events occurring during development. One example is EMT, a complex mix of changes in cell adhesion and migration that accompanies a cell's transition from an epithelial to mesenchymal phenotype. These events have been well-characterized at the GRN level in sea urchin primary mesenchyme (Saunders and McClay, 2014). In this context, distinct regulatory sub-circuits that involve at least thirteen different TFs work in parallel to initiate various EMT processes. These include remodeling of the basement membrane, apical constriction, epithelial de-adhesion, directed motility, loss of apical-basal polarity, and acquisition of mesenchymal adhesion and polarity.

Protection of the Sea Urchin Embryo From Pathogens and Environmental Stressors

Sea urchin embryos and larvae develop in the marine environment without maternal protection from environmental stressors. An integral aspect of development is protection of the germline and developing embryo from external mutagens or pathogens. Immune competence during development was first characterized in Echinoderm embryos in the late 1800s. Comparative embryologist Élie Metchnikoff observed wound healing and phagocytic behaviors in mesenchymal cells of sea star embryos. He documented the functional parallels between the Echinoderm phagocytes and vertebrate macrophages (Metchnikoff, 1893). In 1908, he was awarded the Nobel Prize in Physiology or Medicine for defining the “universality of phagocytosis”, a fundamental contribution to the field of

cellular immunology. Nearly a hundred years later, molecular biology has allowed for the characterization of immunocytes in the sea urchin larvae (and analogous adult coelomocytes) at the genetic level. The sequencing and assembly of the *S. purpuratus* genome identified an expanded innate gene repertoire (over 10-fold of that found in vertebrates), and illuminated the shared regulatory heritage of sea urchin and vertebrate immune systems (Hibino *et al.*, 2006; Rast *et al.*, 2006). These genes and TFs have now been mapped across adult and larval immune cell types and in responses to various pathogens (reviewed in Buckley and Rast, 2017).

Sea urchin embryos also express several hundred genes associated with xenobiotic metabolism (or the “chemical defensome”) in the first several days of development (Goldstone *et al.*, 2006). These include anti-oxidants, heat shock proteins, xenobiotic transporters and transcription factors that sense and respond to small molecules. The best studied of these defense pathways is the xenobiotic transporter system, including members of the ATP Binding Cassette (ABC) transporter and Solute Carrier (SLC) family. These transporters translocate a wide range of hydrophobic small molecules across membranes including xenobiotics and even signaling molecules. Sea urchin embryos have homologs of several human transporters responsible for chemotherapeutic resistance of cancer cells including ABCB1 (aka P-gp), ABCC1 (aka MRP1) and ABCG2 (aka BCRP). These genes are selectively regulated throughout embryonic and larval development, for either broad protective mechanisms, or for discrete functions in developmental signaling or morphogenesis (Shipp and Hamdoun, 2012).

Genome Manipulation and the Future of Sea Urchin Research

The unique biology and experimental advantages of the sea urchin mean that this animal will likely continue to serve an important role in developmental and reproductive biology research. Recent technological advances in gene editing are opening the door to the possibility of stable genetic manipulation as seen in fruit flies, mice and zebrafish. The ability to efficiently deliver molecules into the sea urchin embryo by microinjection has made it relatively easy to apply CRISPR-CAS gene editing systems to knockout genes in this embryo (Lin and Su, 2016). It is anticipated that these technologies will soon enable researchers to not only describe reproduction and development in this animal, but also to manipulate the genome across generations. This will prove valuable for the modeling of human disease pathways in the sea urchin, or for the development of new technologies to understand how the environment influences development.

References

- Alford, L. M., Ng, M. M., & Burgess, D. R. (2009). Cell polarity emerges at first cleavage in sea urchin embryos. *Developmental Biology*, 330(1), 12–20.
- Boveri, T., 1902. On multipolar mitosis as a means of analysis of the cell nucleus. *Neue Folge* 35, 67–90. (S. Gluecksohn-Waelsch, Trans.). In: Willier, B., Oppenheimer, J. (Eds.), *Foundations of Experimental Embryology*. New York, NY: Hafner Press, pp. 74–97.
- Brachet, J. (1945). *Chemical embryology* (Barth, L.G., 1950, Trans.). New York: Interscience Publishers, Inc.
- Briggs, E., & Wessel, G. M. (2006). In the beginning. Animal fertilization and sea urchin development. *Developmental Biology*, 300(1), 15–26.
- Britten, R. J., & Davidson, E. H. (1969). Gene regulation for higher cells: A theory. *Science*, 165, 349–357.
- Britten, R. J., & Kohne, D. E. (1968). Repeated sequences in DNA. *Science*, 161, 529–540.
- Buckley, K. M., & Rast, J. P. (2017). An organismal model for gene regulatory networks in the gut-associated immune response. *Frontiers in Immunology*, 8, 1297.
- Campanale, J. P., *et al.* (2014). Migration of sea urchin primordial germ cells. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 243(7), 917–927.
- Campanale, J. P., & Hamdoun, A. (2012). Programmed reduction of ABC transporter activity in sea urchin germline progenitors. *Development*, 139(4), 783–792.
- Chinkers, M., Garbers, D. L., Chang, M. S., *et al.* (1989). A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature*, 338, 78–83.
- Coffman, J. A., & Davidson, E. H. (2001). Oral-aboral axis specification in the sea urchin embryo. I. Axis entrainment by respiratory asymmetry. *Developmental Biology*, 230(1), 18–28.
- Davidson, E. H., *et al.* (2002). A genomic regulatory network for development. *Science*, 295(2002), 1669–1678.
- Davidson, E. H. (2010). Emerging properties of animal gene regulatory networks. *Nature*, 468(7326), 911–920.
- Davidson, E., Hynes, R., & McClay, D. (Eds.). (2006). Sea Urchin Genome: Implications and Insights. *Developmental Biology*, 300(1), 1–496 (1 December 2006).
- Epel, D. (1975). The program of and mechanisms of fertilization in the echinoderm egg. *American Zoologist*, 15(3), 507–522.
- Ernst, S. G. (2011). Offerings from an urchin. *Developmental Biology*, 358(2), 285–294.
- Evans, T., *et al.* (1983). Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell*, 33(2), 389–396.
- Fol, H. (1877). Sur le commencement de l'henogenie divers animaux. *Archives de Zoologie Expérimentale et Générale*, 6, 145–169.
- Goldstone, J. V., *et al.* (2006). The chemical defensome: Environmental sensing and response genes in the *Strongylocentrotus purpuratus* genome. *Developmental Biology*, 300(1), 366–384.
- Gross, P. R., & Cousineau, G. H. (1963). Effects of actinomycin D on macromolecule synthesis and early development in sea urchin eggs. *Biochemical and Biophysical Research Communications*, 10, 321–326.
- Hamdoun, A. M., *et al.* (2004). Activation of multidrug efflux transporter activity at fertilization in sea urchin embryos (*Strongylocentrotus purpuratus*). *Developmental Biology*, 276(2), 452–462.
- Hertwig, O. (1877). Beitrage zur kenntnis der bildung, befruchtung und theilung des thierischen eies. *Morphol. Jahr*. 3, 1–86. In J. Farley (Ed.), *Gametes and Spores: Ideas About Sexual Reproduction 1750–1914* (p. 161). Baltimore, MD: Johns Hopkins University Press.
- Hibino, T., *et al.* (2006). The immune gene repertoire encoded in the purple sea urchin genome. *Developmental Biology*, 300, 349–365.
- Ho, E. C. H., Buckley, K. M., Schrankel, C. S., *et al.* (2016). Perturbation of gut bacteria induces a coordinated cellular immune response in the purple sea urchin larva. *Immunology and Cell Biology*, 94, 861–874.
- Jaffee, L. A. (1976). Fast block to polyspermy in sea urchin eggs is electrically mediated. *Nature*, 261(5555), 68–71.
- Juliano, C. E., Voronina, E., Stack, C., *et al.* (2006). Germ line determinants are not localized early in sea urchin development, but do accumulate in the small micromere lineage. *Developmental Biology*, 300(1), 406–415.

- Juliano, C. E., Yajima, M., & Wessel, G. M. (2010). Nanos functions to maintain the fate of the small micromere lineage in the sea urchin embryo. *Developmental Biology*, 337(2), 220–232.
- Kamei, N., & Glabe, C. G. (2003). The species-specific egg receptor for sea urchin sperm adhesion is EBR1, a novel ADAMTS protein. *Genes and Development*, 17, 2502–2507.
- Kedes, L., Chang, A., Houseman, D., & Cohen, S. (1975b). Isolation of histone genes from unfractionated sea urchin DNA by subculture cloning in *E. coli*. *Nature*, 225, 533–538.
- Kedes, L. H., et al. (1975a). The organization of sea urchin histone genes. *Cell*, 6(3), 359–369.
- Lapraz, F., Haillot, E., & Lepage, T. (2015). A deuterostome origin of the Spemann organiser suggested by Nodal and ADMPs functions in Echinoderms. *Nature communications*, 6, 8434.
- Lee, H. C., et al. (1989). Structural determination of a cyclic metabolite of NAD⁺ with intracellular Ca²⁺-mobilizing activity. *The Journal of biological chemistry*, 264(3), 1608–1615.
- Lin, C.-Y., & Su, Y.-H. (2016). Genome editing in sea urchin embryos by using a CRISPR/Cas9 system. *Developmental Biology*, 409, 420–428.
- Logan, C. Y., Miller, J. R., Ferkowicz, M. J., & McClay, D. R. (1998). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development*, 126(2), 345–357.
- Martik, M. L., & McClay, D. R. (2015). Deployment of a retinal determination gene network drives directed cell migration in the sea urchin embryo. *eLife*, 4.
- Materna, S. C., Swartz, S. Z., & Smith, J. (2013). Notch and Nodal control forkhead factor expression in the specification of multipotent progenitors in sea urchin. *Development*, 140(8), 1796–1806.
- Mazia, D., & Dan, K. (1952). The isolation and biochemical characterization of the mitotic apparatus of dividing cells. *Proceedings of the National Academy of Sciences of the United States of America*, 38(9), 826–838.
- Metchnikoff, E., 1893. Lectures on the Comparative Pathology of Inflammation: Delivered at the Pasteur Institute in 1891, first ed. (Starling, F.A., Starling, E.H., Trans.). London, UK: Kegan Paul, Trench, Trubner & Co. Ltd.
- Monroy, A., & Tyler, A. (1963). Formation of active ribosomal aggregates (polyribosomes) upon fertilization and development of sea urchin eggs. *Archives of Biochemistry and Biophysics*, 103, 431–435.
- Pearse, J., & Cameron, R. A. (1991). Echinodermata: Echinoidea. In A. C. Giese, J. S. Pearse, & V. B. Pearse (Eds.), *Reproduction of Marine Invertebrates. vol. VI: Echinoderms and Lophophorates* (pp. 514–662). Pacific Grove, CA: Boxwood Press.
- Pehrson, J. R., & Cohen, L. H. (1986). The fate of the small micromeres in sea urchin development. *Developmental Biology*, 113(2), 522–526.
- Rast, J. P., et al. (2006). Genomic insights into the immune system of the sea urchin. *Science*, 314(5801), 952–956.
- Saunders, L. R., & McClay, D. R. (2014). Sub-circuits of a gene regulatory network control a developmental epithelial-mesenchymal transition. *Development*, 141, 1503–1513.
- Schroeder, T. E. (1978). Microvilli on sea urchin eggs: A second burst of elongation. *Developmental Biology*, 64(2), 342–346.
- Shipp, L. E., & Hamdoun, A. (2012). ATP-binding cassette (ABC) transporter expression and localization in sea urchin development. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 241(6), 1111–1124.
- Sodergren, E., et al. (2006). The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science*, 314(5801), 941–952.
- Sodergren, E., Weinstock, G. M., Davidson, E. H., et al. (2006). The genome of the sea urchin *Strongylocentrotus purpuratus*. *Science*, 314, 941–952.
- Swartz, S. Z., et al. (2014). Deadenylase depletion protects inherited mRNAs in primordial germ cells. *Development*, 141(16), 3134–3142.
- Thompson-Coffe, C., et al. (1996). Cold-treated centrosome: Isolation of centrosomes from mitotic sea urchin eggs, production of an antacentrosomal antibody, and novel ultrastructural imaging. *Cell Motility and the Cytoskeleton*, 33(3), 197–207.
- Vacquier, V. D. (1975). The isolation of intact cortical granules from sea urchin eggs: Calcium ions trigger granule discharge. *Developmental Biology*, 43, 62–74.
- Vacquier, V. D. (1981). Dynamic changes of the egg cortex. *Developmental Biology*, 84(1), 1–26.
- Vacquier, V. D., & Moy, G. W. (1977). Isolation of bindin: The protein responsible for adhesion of sperm to sea urchin eggs. *Proceedings of the National Academy of Sciences of the United States of America*, 74(6), 2456–2460.
- Vacquier, V. D., Tegner, M. J., & Epel, D. (1973). Protease released from sea urchin eggs at fertilization alters the vitelline layer and aids in preventing polyspermy. *Experimental Cell Research*, 80(1), 111–119.
- Walker, C., Unuma, T., & Lesser, M. (2007). Reproduction of sea urchins. In J. Lawrence (Ed.), *Edible Sea Urchins: Biology and Ecology* (pp. 11–33). Amsterdam: Elsevier.
- Ward, G. E., Brokaw, C. J., Garbers, D. L., & Vacquier, V. D. (1985). Chemotaxis of *Arbacia punctulata* spermatozoa to resact, a peptide from the egg jelly layer. *Journal of Cell Biology*, 101, 2324–2329.
- Wessel, G. M., et al. (2014). The biology of the germ line in echinoderms. *Molecular Reproduction and Development*, 81(8), 679–711.
- Wikramanayake, A. H., Huang, L., & Klein, W. H. (1998). beta-Catenin is essential for patterning the maternally specified animal-vegetal axis in the sea urchin embryo. *Proceedings of the National Academy of Sciences of the United States of America*, 95(16), 9343–9348.
- Yajima, M., & Wessel, G. M. (2012). Autonomy in specification of primordial germ cells and their passive translocation in the sea urchin. *Development*, 139, 3786–3794.

Relevant Websites

www.echinobase.org. — EchinoBase.
virtualurchin.stanford.edu. — VirtualUrchin.

Zebrafish

Wei Ge, University of Macau, Taipa, China

© 2018 Elsevier Inc. All rights reserved.

Nomenclature

Bmp15	Bone morphogenetic protein 15	hpf	Hour post-fertilization
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/CRISPR-associated protein-9 nuclease	HPG	Hypothalamic-pituitary-gonad
DHP	17 α , 20 β -dihydroxy-4-pregnen-3-one	LH	Luteinizing hormone
dpf	Day post-fertilization	LV	Late vitellogenic
EV	Early vitellogenic	mpf	Month post-fertilization
FG	Full-grown	MV	Mid-vitellogenic
FSH	Follicle-stimulating hormone	PG	Primary growth
Gdf9	Growth differentiation factor 9	PGC	Primordial germ cell
GnRH	Gonadotropin-releasing hormone	PV	Pre-vitellogenic
		SG	Secondary growth
		TALLEN	Transcription activator-like effector nuclease

Glossary

Apoptosis A process that kills a cell by genetic death program.

Cortical alveoli Special membrane-bound secretory structures in developing eggs that are located in egg cortex and involved in the cortical reaction during fertilization.

Fecundity Capability of reproduction indicated by number of eggs produced.

Fertilization The union of egg and sperm to form fertilized egg.

Genital ridge The structure in the embryo that develops into ovary or testis.

Germ cells Any cells that develop into eggs or sperm.

Gonochorist A species whose individuals have either ovary or testis.

Granulosa cell The somatic cells that surround oocytes or eggs to support their development.

Hermaphrodite An organism that has both ovary and testis in its lifespan.

Leydig cell The endocrine cells in the testis that are responsible for steroid production.

Oviposition The process of laying eggs.

Ovulation The process of releasing mature eggs from the follicles.

Pheromone Chemical signals from one individual that trigger responses in other individuals of the same species.

Primordial germ cells A special group of cells in early development that will develop into eggs or sperm.

Protogynous Change sex at certain time of life cycle from female to male.

Puberty The period during life cycle when an individual becomes sexually mature.

Sertoli cell The somatic cells that are closely associated with sperm cells to support their development.

Sex reversal Change of sex to the opposite one after sex differentiation.

Somatic cells Any cells of an organism that are not part of the germ cell lineage.

Spermatogonium Undifferentiated germ cells in the testis before entering meiosis.

Theca cell The endocrine cells of the ovarian follicle that are responsible for steroid production.

Toxicology Study of adverse effects of chemicals on living organisms and their mechanisms.

Vitellogenin A precursor protein that forms egg yolk.

Introduction

The zebrafish, *Danio rerio* (formerly *Brachydanio rerio*), is a small freshwater fish (<4 cm in standard body length from snout to the origin of the tail fin) native to South Asia including India, Bangladesh, Pakistan, Nepal and Myanmar, where it inhabits various waters, preferably shallow and still ponds, slow-moving streams, and even rice fields (Parichy, 2015; Spence *et al.*, 2008). Zebrafish was first recorded by Francis Hamilton in 1822, and it biologically belongs to the Family Cyprinidae of the Order Cypriniformes. The fish gets its name for its similarity to the zebra by having a few horizontal stripes on each side of the body. In its natural habitats, zebrafish prefers areas with overhanging vegetation and, as an omnivorous species, it feeds on a wide range of diet such as zooplankton, small insects, algae and other plant materials (Arunachalam *et al.*, 2013; Spence *et al.*, 2008). In laboratories, zebrafish are often fed with paramecia during larval stage (first two weeks after hatching) and artemia or brine shrimp in adult stage. Depending on conditions, the lifespan of zebrafish ranges from 3 to 5 years.

In the late 1960s, George Streisinger, who is considered the founding father of zebrafish research, first introduced this tiny slender fish as a vertebrate model for research on genetics and development at the University of Oregon. In the past 20 years, the zebrafish has gradually risen as one of the top model organisms for biological and biomedical research due to its biological advantages such as short generation time (2–3 months), high fecundity (~200 eggs/clutch), in vitro development, transparent embryos, and easy manipulation and maintenance. In addition, the susceptibility to many state-of-the-art cell and molecular biology techniques and amenability to chemical and genetic manipulations have contributed to its increasing popularity. Although the zebrafish was first developed as a model for developmental genetics, its utility has been quickly extended to many other fields of biological and biomedical sciences, including physiology, toxicology, disease modelling and drug development.

The zebrafish research has gained further momentum in recent years due to two major technological breakthroughs: (1) completion of its genome sequencing (Howe *et al.*, 2013) and (2) development of genome-editing technology (Gaj *et al.*, 2013). According to the genome sequencing data, the zebrafish shares 70% genes with humans and more than 84% of genes that cause human genetic diseases are present in the zebrafish, making it an excellent model for studying human gene functions, especially those that cause various human genetic diseases.

Zebrafish is well known to be highly prolific. In its natural habitats, zebrafish does exhibit clear seasonality in reproduction with the spawning season beginning right before the onset of monsoon (Spence *et al.*, 2008). However, under constant laboratory conditions, the domesticated zebrafish has lost such seasonality and it reproduces year-round every day or every few days. Each female zebrafish can produce hundreds of eggs per week. Such a high fecundity has made zebrafish an excellent model for studying reproduction as well as reproductive toxicology (Lawrence, 2011). This article reviews basic aspects of zebrafish reproduction and the major applications of this model in both basic and applied science.

Zebrafish Reproduction in Natural Habitats and Laboratories

Typically, the zebrafish becomes sexually mature around 3 months post-fertilization (mpf); however, this is highly variable, ranging from 2 to 6 mpf, depending on environmental condition, nutritional state and genetic background. It is not difficult to distinguish females and males in sexually mature zebrafish based on external appearance and morphology. The male zebrafish exhibit a slim shape with brownish coloration whereas the females display a more rounded body shape with silverish coloration. However, in juvenile zebrafish, it is very hard to identify males and females without dissection (Spence *et al.*, 2008). Studies have shown that the timing of sexual maturity is more related to body size rather than age (Chen and Ge, 2013). As a daily spawner, each female zebrafish can lay about 200 eggs on average per spawning, but the number can go up to 1000 eggs (our lab record is 800 per day). In its natural habitats, the zebrafish spawns in groups in early morning, and its spawning sites are normally shallow areas near the edge of waters, preferably with heavy vegetation (Laale, 1977). In laboratory settings, zebrafish spawn mostly within one hour after light is on in the morning. At 28°C, the embryonic development completes within 48–72 h post-fertilization (hpf). The newly hatched zebrafish larvae rely on the remaining yolk reserve for nutritional support, and they start to feed on zooplankton such as paramecia about 4 dpf.

Sex Determination and Differentiation

Like most other vertebrates and fish species, zebrafish is also gonochoristic in adults with individuals being either male or female. However, different from most other gonochoristic fish species such as medaka and salmonids whose gonads develop directly into ovaries or testes from undifferentiated state (differentiated gonochorists), the gonadal development of all zebrafish juveniles undergoes a special pseudo-female phase with non-functional ovary containing oocyte-like germ cells, which starts around 10–15 days post-fertilization (dpf). This phenomenon was first reported by Takahashi as “juvenile hermaphrodite” (Takahashi, 1977), and the fish is therefore considered a undifferentiated gonochorist. Sex determination and differentiation in the zebrafish occur later around 25 dpf with some individuals continuing female development pathway to become true functional females whereas others undergoing a process of sex reversal to become males (Maack and Segner, 2003). During sex reversal, the oocyte-like germ cells die through a process of programmed cell death or apoptosis (Uchida *et al.*, 2002). This gives way to the development of male germ cells from the germline stem cells. Because of this, zebrafish is also considered “juvenile protogynous hermaphrodite” (Fig. 1).

Similar to those of other animals, the germ cells originate at a distant location in the zebrafish embryos. To form the gonads, these early germ cells (primordial germ cells, PGCs) have to migrate a long distance, guided by both attractive and repulsive signals, to reach their final destination (genital ridges) at the dorsal side of the body cavity (coelom), where they will develop into either ovary or testis together with other somatic cells (Paksa and Raz, 2015). Unlike most gonochoristic fish species whose PGCs differentiate directly into either eggs or sperm in females and males respectively without sex reversal, zebrafish undergoes a special period of juvenile females followed by further differentiation into males and females. Unlike mammals and some fish species such as medaka and tilapia with sex-determining genes, the mechanism that determines the direction of gonadal development (ovary or testis) remains entirely unknown in the zebrafish. It is generally accepted that instead of having single sex-determining gene, the sex of zebrafish is determined by multiple genes (polygenic hypothesis) (Liew *et al.*, 2012).

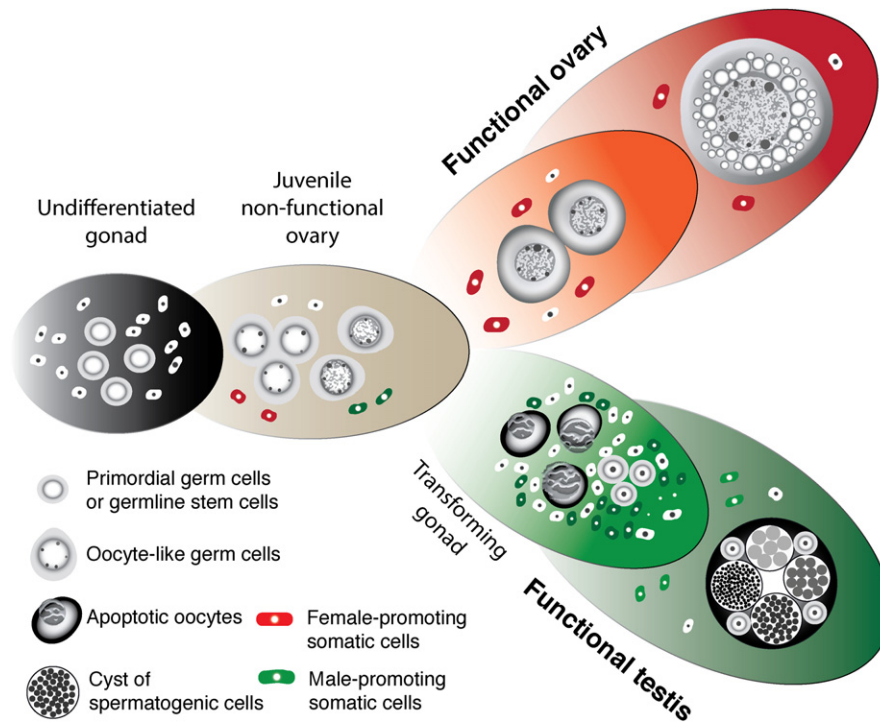


Fig. 1 Schematic illustration of zebrafish gonadal differentiation. All juveniles of zebrafish undergo a special pseudo-female phase with non-functional ovaries. In about half of individuals, the oocyte-like germ cells continue to grow to become functional oocytes in the ovary of females; whereas in the other half individuals, the germ cells die through a special process called apoptosis while the stromal cells proliferate to fill up the space between the dying oocytes. Among the stromal cells are spermatogonial stem cells that will develop into spermatogenic cells of different stages in a cystic manner.

Control of Zebrafish Puberty Onset

Once after sex differentiation is completed, the zebrafish will stay in immature juvenile stage for a short while before becoming sexually mature at puberty, which is a critical period when an animal becomes sexually mature and attains reproductive capacity. In female zebrafish, the initiation of puberty or puberty onset is marked by the ovarian follicles that start to accumulate a special structure called cortical vesicles/alveoli in the oocytes (Chen and Ge, 2013; Taranger *et al.*, 2010). These follicles are staged as pre-vitellogenic (PV) or stage II. Interestingly, the timing of puberty in the zebrafish seems related to the body size rather than age, and the body weight and standard length of female zebrafish are 100 mg and 1.8 cm respectively when puberty starts around 45 dpf under optimal environmental conditions (Chen and Ge, 2013). After puberty onset, the ovarian follicles undergo fast vitellogenic growth to reach sexual maturity, and the fish may start spawning as early as 60 dpf (our laboratory), when the standard length reaches about 2.3 cm (Lawrence, 2011; Spence *et al.*, 2008).

Although the mechanism by which body growth influences zebrafish reproduction remains largely unknown, it has been generally believed that the puberty onset in the zebrafish is controlled by neuroendocrine signals from the hypothalamus (brain), in particular the kisspeptin-gonadotropin-releasing hormone (GnRH) pathway (Servili *et al.*, 2011). This pathway is well known in mammals to be responsible for initiating puberty, and numerous studies in fish models including the zebrafish have also pointed to the involvement of this pathway in puberty onset. However, recent studies using the state-of-the-art genome-editing technology do not support this concept in the zebrafish (see below).

Regulation of Zebrafish Reproduction

After puberty onset, both males and females will embark on a continual process of gamete production, viz. oogenesis/folliculogenesis in females and spermatogenesis in males. The maturation and function of the zebrafish reproductive system are controlled by the hypothalamic-pituitary-gonad (HPG) axis in both males and females. Similar to that in other vertebrates, the zebrafish HPG axis is believed to be governed by the master neuropeptide from the hypothalamus, i.e., GnRH, which acts at the pituitary to control the production and secretion of gonadotropins, viz. follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Both FSH and LH are released into the blood and circulated to the gonads, ovary in females and testis in males, where they control all aspects of gonadal functions, including production of gametes (eggs and sperm) and sex steroid hormones.

Recent genetic studies using gene knockout approach have shown that FSH is essential in controlling early stage of gonadal development in both males and females whereas LH is responsible for the final maturation and release of eggs in females (Zhang *et al.*, 2015b). Like their counterparts in mammals, zebrafish FSH and LH also signal through specific receptors on the membrane of gonadal cells. However, in addition to its cognate receptor Lhcgr, zebrafish LH can also activate FSH receptor Fshr to some extent (So *et al.*, 2005). The unique LH-Fshr pathway has recently been demonstrated to play an essential role in rescuing the phenotype of FSH loss (Zhang *et al.*, 2015b). In addition to pituitary gonadotropins and gonadal steroids, the regulation of reproduction also involves various peptide growth factors that work mostly as paracrine factors at different levels of the axis (Ge, 2005).

Female Reproduction

Like that in other animals, ovary plays a central role in female zebrafish reproduction. Different from other organ systems, the ovary in the zebrafish or any fish in general is the most dynamic organ that undergoes dramatic changes in both size and function during the reproductive lifespan. The basic structural and functional units in the ovary are follicles. Based on the size and morphology, the growing zebrafish follicles can be divided into two major phases: primary growth (PG, also called stage I; < 150 μm) and secondary growth (SG). The SG phase can be further divided into several stages: pre-vitellogenic (PV, or stage II; ~ 250 μm), early vitellogenic (EV, or early stage III; ~ 350 μm), mid-vitellogenic (MV, or mid-stage III; ~ 450 μm), late vitellogenic (LV, or late stage III; ~ 550 μm), and full-grown (FG; ~ 650 μm) (Selman *et al.*, 1993; Zhou *et al.*, 2011). During SG phase or vitellogenic growth, the oocytes absorb large amount of yolk proteins (vitellogenin) produced in the liver, and these proteins are converted and deposited in the oocytes as yolk granules, which serve as the nutritional reserve to support embryonic development after fertilization.

Each follicle consists of a centrally-located oocyte and a surrounding layer of somatic follicle cells. There are two types of cells that form the follicle layer, the granulosa and theca cells. The granulosa cells form the avascular inner layer that has direct contact with the oocyte whereas the theca cells are located outside with rich blood supply. These two types of cells are physically separated by a layer of amorphous basement membrane, and they perform distinct functions during follicle growth or folliculogenesis (Ge, 2010). Different from their counterparts in mammals where the follicles consist of multiple layers of granulosa and theca cells, there is only one monolayer of granulosa and theca cells each in the zebrafish follicle. The granulosa cells have direct contact with the oocyte to support and regulate its development whereas the theca cells are the major steroidogenic cells that produce steroid hormones. Evidence has been increasing that the oocyte and outside somatic cells (granulosa and theca) work closely in the ovary to drive follicle development. It is now generally believed that the somatic follicle cells are the site where various external endocrine hormones work to regulate follicle development, e.g., pituitary FSH and LH. However, the germ cell oocyte also plays an important role by releasing various biologically active factors, mostly peptide growth factors, to influence follicle cell functions, such as growth differentiation factor 9 (Gdf9) and bone morphogenetic protein 15 (Bmp15). Various regulatory factors from the oocyte and follicle cells therefore form an intrinsic network of communication within the follicle to orchestrate follicle development (Ge, 2005).

Upon completion of follicle growth, the full-grown (FG) follicles will undergo a process of final maturation followed by release of mature eggs (ovulation) for fertilization. Numerous factors have been reported to participate in controlling this critical final event, including pituitary gonadotropin LH, steroid hormone progesterone (17α , 20β -dihydroxy-4-pregnen-3-one; DHP) and local growth factors such as activin, epidermal growth factor (Egf), and insulin-like growth factor 3 (Igf3). Recent studies using state-of-the-art genome editing technologies have provided unequivocal evidence for the importance of some of these factors in regulating final oocyte maturation and ovulation.

Male Reproduction

Like the ovary in females, the testis is the central player in male reproduction in the zebrafish. Similar to those of other vertebrates, the zebrafish testis also consists of seminiferous tubules where sperm development or spermatogenesis occurs. In addition to the germ cells, testis also contains two types of somatic cells that are essential for male function; they are the Sertoli cells located within the tubular compartment together with the germ cells and the Leydig cells located outside the tubules or interstitial space. Similar to situation in the ovary, the two types of somatic cells are separated by a basement membrane (Schulz *et al.*, 2010). The Sertoli and Leydig cells in the testis are functionally equivalent to the granulosa and theca cells in the ovary respectively in that the former are in physical contact with the developing sperm cells without blood supply and the latter are richly supplied by blood and are responsible for production of male sex steroid hormones.

Like other fish species, the zebrafish testis belongs to the cystic type. In the tubular compartment, the germinal epithelium contains numerous cysts of different sizes and developmental stages, and each cyst consists of a clone of germ cells surrounded by the Sertoli cell extensions. Each clone originates from a single spermatogonium and the development of all cells within a clone or cyst is synchronous. The cystic type of spermatogenesis makes identification of different developmental stages a lot easier as compared to the non-cystic type seen in higher vertebrates. In the zebrafish testis, the production of sperm or spermatogenesis is divided into three stages: the mitotic stage of spermatogonial proliferation, the meiotic stage of spermatocytes (primary and secondary), and the spermatogenic stage of final maturation into motile spermatozoa. Like folliculogenesis in the ovary, the

spermatogenesis in the testis is also tightly controlled by both endocrine hormones such as FSH and LH from the pituitary, local regulatory factors such as anti-Müllerian hormone (Amh) and Igf3, as well as steroid hormones (Schulz *et al.*, 2010).

Reproductive Behavior

After production of gametes (eggs in females and sperm in males), the successful reproduction depends on appropriate spawning behavior to ensure fertilization. In laboratory condition, the zebrafish spawns continuously on daily basis or at intervals of a few days. The trigger for spawning behavior is well known to be lighting after darkness. Upon light-on, the male and female zebrafish perform spawning behavior that results in release of mature eggs and sperm for fertilization in water. The process of spawning normally lasts for about one hour after light on. In its natural habitats, zebrafish spawning occurs at dawn, also depending on photoperiod.

During spawning, the males display an aggressive chasing behavior to pursue females with an attempt to lead the females to the spawning sites. In both laboratory condition and natural habitats, zebrafish prefer shallow areas with gravel substrate, preferably with vegetation. At spawning sites, the male would position itself closely to the side of the female so that their genital pores are well aligned. This behavior triggers the release of eggs by the female (oviposition) and sperm by the male for fertilization (Spence *et al.*, 2008).

The courtship behavior displayed by males is thought to be induced by pheromones (olfactory cues) released by females. Waterborne pheromones are perceived by olfaction and important for aquatic animals to communicate for individual recognition and synchronization of reproductive behavior. Exposure to pheromones from one sex may trigger responses of the endocrine system in the opposite sex, which influences sexual maturity and reproductive success. Studies have shown that male zebrafish can release chemical cues to stimulate female ovulation (van Den Hurk *et al.*, 1987) whereas pheromones from females activate males while suppress the reproductive performance of other females (Gerlach, 2006). The exact chemical identities of zebrafish pheromones remain unclear, and early studies have implicated potential roles for steroid glucuronides in pheromonal communication in both male and female zebrafish (van den Hurk and Lambert, 1983; van Den Hurk *et al.*, 1987).

Zebrafish as a Model for Reproductive Endocrine Disruptors

In the past decade, the zebrafish has not only emerged as a model species of choice for basic study on fish reproduction, but also become popular as a model for studying reproductive toxicity of environmental pollutants, especially those with endocrine-disrupting activities. Endocrine-disrupting chemicals (EDCs) refer to the compounds, either natural or synthetic, that interfere with the function of the endocrine system in wildlife and humans. The zebrafish has been used for screening compounds, evaluating effects, and understanding action mechanisms (Segner, 2009). The polygenic sex determination and high plasticity of sex differentiation make zebrafish particularly susceptible to external and internal conditions or factors that may influence reproduction, therefore providing a unique model for assessing materials that may disrupt reproductive development and function. Our knowledge of zebrafish reproduction has provided a foundation for establishing endpoints of assays, identification of biomarkers, experimental design and data interpretation.

As a vertebrate species, the zebrafish is close to humans in many aspects of body functions. The endocrine system of the zebrafish is very similar to that of humans. Therefore, the information obtained from the zebrafish model on EDCs will be more relevant to human health than invertebrate models. The high fecundity (~200 eggs per spawning), in vitro and fast embryonic development (72 h), and short generation time (~3 months) all make zebrafish a superior model as compared to the rodents. Furthermore, numerous mutant zebrafish lines have been established, and these mutants will be valuable tools for dissecting and understanding the action mechanisms of various EDCs (Segner, 2009).

Several approaches can be used with the zebrafish model to assess EDCs. First, a specific marker molecule that responds to a particular endocrine pathway can be used to monitor the effects of EDCs on specific endocrine axes, e.g., vitellogenin expression in the liver in response to exposure to estrogenic substances. Second, without targeting any particular pathways, a more global approach, i.e., transcriptomics, will provide comprehensive information on which endocrine pathways are affected. Third, since gonads are the ultimate organs of the reproductive system, histopathological analysis of gonadal development and maturation as compared with the control will reveal the impacts of EDCs on the overall functionality of the HPG axis. Forth, transgenic zebrafish lines that carry reporter genes such as green fluorescent protein (GFP) whose expression is controlled by specific endocrine-responsive promoters can be useful for live monitoring of EDCs in water.

Zebrafish as a Genetic Model for Vertebrate Reproduction

Although zebrafish has been a popular model for developmental biology using reverse genetics approach, the major tool used was morpholino-mediated gene knockdown. Since gene knockdown does not involve gene deletion from the genome and it works only for a short period of time (<5 dpf), the study of zebrafish reproduction by reverse genetics had been hindered. However, this situation has quickly changed in recent years with the new generation of gene knockout tools available to non-rodent species, in

particular TALEN and CRISPR/Cas9. Using these tools, a variety of functional genes in the zebrafish HPG axis have been disrupted for functional assessment. Phenotype analysis of these mutant zebrafish lines has confirmed some well-accepted concepts regarding fish reproductive axis and its control, but also revealed novel aspects of gene functions that would be difficult to demonstrate without using these genome-editing technologies. In this wave of technological revolution, zebrafish has become one of the top models for testing and evaluating the efficacy of various protocols.

It is generally believed that the HPG axis in fish is similar to that of mammals in terms of organization and function. The hypothalamus produces and releases a master neuropeptide, GnRH, which controls biosynthesis and secretion of gonadotropins (FSH and LH) from the pituitary. GnRH in turn is subject to regulation by another neuropeptide kisspeptin in the hypothalamus. The gonads, in response to FSH and LH, produce sex steroids including estrogens in females, which are produced by the action of aromatase, an enzyme that converts androgens to estrogens. Using gene targeting technology, these important functional genes in the axis have been evaluated for their roles and importance in the zebrafish.

As expected, the deletion of the gene coding for FSH β (*fshb*) significantly delayed puberty onset in both males and females although the mutant fish was fertile in the end because of the rescue of FSH function by LH, which can activate FSH receptor (*Fshr/fshr*). On the other hand, the loss of LH by deleting LH β gene (*lhb*) had no effect on gonadal growth in both males and females, but the female mutant was infertile due to failed oocyte maturation and ovulation (Zhang *et al.*, 2015b). Interestingly, disruption of *fshr* gene resulted in severe retardation of ovarian development and sex reversal to males whereas the loss of LH receptor (*Lhcgr/lhcgr*) displayed no phenotype (Zhang *et al.*, 2015a). This discovery would be extremely difficult to make without the genome-editing technology. Estrogens have long been believed to play important roles in gonadal differentiation in fish. This view is fully supported by deleting the ovarian aromatase gene (*cyp19a1a*) in the zebrafish. As expected, the loss of *cyp19a1a* resulted in an all-male phenotype with no females in the mutants (Lau *et al.*, 2016). A surprising discovery concerns the role of hypothalamic kisspeptin-GnRH pathway in zebrafish reproduction. Deleting kisspeptin genes (*kiss1* and *kiss2*) and its receptors (*kissr1* and *kissr2*) in the zebrafish caused no abnormality in reproduction for both males and females (Tang *et al.*, 2015), and this is supported by a separate study demonstrating that the loss of GnRH gene (*gnrh3*) also had no effect on reproduction in both males and females (Spicer *et al.*, 2016).

The powerful genome-editing technology has provided an unprecedented tool for scientists to explore the regulatory network of the reproductive axis. Although many results support the traditional views about the roles and importance of different functional genes in the axis, some results have provided information totally beyond the current concepts. With its technical and biological advantages, especially amenability to genetic manipulation, it is conceivable that zebrafish will be a dominant model for genetic analysis of reproductive functions in fish.

Summary

In the past 20 years, there has been a substantial growth of studies on zebrafish reproduction in both males and females. Our understanding of zebrafish reproduction now covers a wide range of topics including primordial germ cell development, sex/gonadal differentiation, folliculogenesis/oogenesis in the ovary, spermatogenesis in the testis, and spawning behavior and reproductive success. This knowledge has increased the popularity of zebrafish as a model in other areas including environmental science for evaluating man-made and natural compounds that may interfere with normal reproduction. Recent emergence of the genome-editing technology and its successful application in the zebrafish will further strengthen the status of this tiny fish as a model organism for studying fish as well as vertebrate reproduction (Fig. 1).

References

- Arunachalam, M., Raja, M., Vijayakumar, C., Malaïammal, P., Mayden, R.L., 2013. Natural history of zebrafish (*Danio rerio*) in India. *Zebrafish* 10, 1–14.
- Chen, W., Ge, W., 2013. Gonad differentiation and puberty onset in the zebrafish: Evidence for the dependence of puberty onset on body growth but not age in females. *Mol. Reprod. Dev.* 80, 384–392.
- Gaj, T., Gersbach, C.A., Barbas 3rd, C.F., 2013. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol.* 31, 397–405.
- Ge, W., 2005. Intrafollicular paracrine communication in the zebrafish ovary: The state of the art of an emerging model for the study of vertebrate folliculogenesis. *Mol. Cell. Endocrinol.* 237, 1–10.
- Ge, W., 2010. Paracrine control of fish ovarian follicle development and function. In: Garcia-Ayala, A., Penalver, J.M., Chaves-Pozo, E. (Eds.), *Recent Advances in Fish Reproduction Biology*. Kerala: Research Signpost, pp. 141–173.
- Gerlach, G., 2006. Pheromonal regulation of reproductive success in female zebrafish: Female suppression and male enhancement. *Anim. Behav.* 72, 1119–1124.
- Howe, K., Clark, M.D., Torroja, C.F., *et al.*, 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496, 498–503.
- Laale, H.W., 1977. The biology and use of zebrafish, *Brachydanio rerio* in fisheries research. *J. Fish Biol.* 10, 121–173.
- Lau, E.S., Zhang, Z., Qin, M., Ge, W., 2016. Knockout of zebrafish ovarian aromatase gene (*cyp19a1a*) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. *Sci. Rep.* 6, 37357.
- Lawrence, C., 2011. The reproductive biology and spawning of zebrafish in laboratory settings. In: McGrath, P. (Ed.), *Zebrafish: Methods for Assessing Drug Safety and Toxicity*. Hoboken, NJ: John Wiley & Sons, Inc, pp. 1–13.
- Liew, W.C., Bartfai, R., Lim, Z., *et al.*, 2012. Polygenic sex determination system in zebrafish. *PLOS ONE* 7, e34397.
- Maack, G., Segner, H., 2003. Morphological development of the gonads in zebrafish. *J. Fish Biol.* 62, 895–906.
- Paksa, A., Raz, E., 2015. Zebrafish germ cells: Motility and guided migration. *Curr. Opin. Cell Biol.* 36, 80–85.
- Parichy, D.M., 2015. Advancing biology through a deeper understanding of zebrafish ecology and evolution. *Elife* 4.

- Schulz, R.D.W., De França, L.R., Lareyre, J.-J., *et al.*, 2010. Spermatogenesis in fish. *Gen. Comp. Endocrinol.* 165, 390–411.
- Segner, H., 2009. Zebrafish (*Danio rerio*) as a model organism for investigating endocrine disruption. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 149, 187–195.
- Selman, K., Wallace, R.A., Sarka, A., Qi, X.P., 1993. Stages of oocyte development in the zebrafish, *Brachydanio rerio*. *J. Morphol.* 218, 203–224.
- Servili, A., Le Page, Y., Leprince, Y., *et al.*, 2011. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. *Endocrinology* 152, 1527–1540.
- So, W.K., Kwok, H.F., Ge, W., 2005. Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits – Their spatial-temporal expression patterns and receptor specificity. *Biol. Reprod.* 72, 1382–1396.
- Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, *Danio rerio*. *Biol. Rev. Camb. Philos. Soc.* 83, 13–34.
- Spicer, O.S., Wong, T.T., Zmora, N., Zohar, Y., 2016. Targeted mutagenesis of the hypophysiotropic GnRH3 in zebrafish (*Danio rerio*) reveals no effects on reproductive performance. *PLOS ONE* 11, e0158141.
- Takahashi, H., 1977. Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*. *Bull. Fac. Fish Hokkaido Univ.* 28, 57–65.
- Tang, H., Liu, Y., Luo, D., *et al.*, 2015. The kiss/kissr systems are dispensable for zebrafish reproduction: Evidence from gene knockout studies. *Endocrinology* 156, 589–599.
- Taranger, G.L., Carrillo, M., Schulz, R.D.W., *et al.*, 2010. Control of puberty in farmed fish. *Gen. Comp. Endocrinol.* 165, 483–515.
- Uchida, D., Yamashita, M., Kitano, T., Iguchi, T., 2002. Oocyte apoptosis during the transition from ovary-like tissue to testes during sex differentiation of juvenile zebrafish. *J. Exp. Biol.* 205, 711–718.
- van den Hurk, R., Lambert, J.G.D., 1983. Ovarian steroid glucuronides function as sex pheromones for male zebrafish *Brachydanio rerio*. *Can. J. Zool.* 61, 2381–2387.
- van den Hurk, R., Schoonen, W.G., Van Zoelen, G.A., Lambert, J.G., 1987. The biosynthesis of steroid glucuronides in the testis of the zebrafish, *Brachydanio rerio*, and their pheromonal function as ovulation inducers. *Gen. Comp. Endocrinol.* 68, 179–188.
- Zhang, Z., Lau, S.W., Zhang, L., Ge, W., 2015a. Disruption of zebrafish follicle-stimulating hormone receptor (*fshr*) but not luteinizing hormone receptor (*lhcg*) gene by TALEN leads to failed follicle activation in females followed by sexual reversal to males. *Endocrinology* 156, 3747–3762.
- Zhang, Z., Zhu, B., Ge, W., 2015b. Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption. *Mol. Endocrinol.* 29, 76–98.
- Zhou, R., Tsang, A.H., Lau, S.W., Ge, W., 2011. Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors in the zebrafish ovary: Evidence for potentially dual roles of PACAP in controlling final oocyte maturation. *Biol. Reprod.* 85, 615–625.

Relevant Websites

- <https://zfin.org>
The Zebrafish Information Network (ZFIN).
- <https://en.wikipedia.org/wiki/Zebrafish>
Zebrafish.
- https://en.wikibooks.org/wiki/The_Zebrafish_in_Toxicology/Ovary_staging
Zebrafish Gonadal Staging.
- <http://zebrafish.org/home/guide.php>
Zebrafish International Resource Center (ZIRC).

Overview of the Reproduction of Laboratory Mice and Rats

Marcia Brower, Boston Scientific Corporation, St. Paul, MN, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Blastocyst The blastocyst is a structure formed in the early development of mammals after fertilization of an ovulated egg. There is an inner cell mass which forms the embryo (and is the source of embryonic stem cells). The outer layer of the blastocyst consists of cells called the trophoblast. This layer surrounds the inner cell mass and a fluid-filled cavity known as the blastocoele. The trophoblast gives rise to the placenta.

Chimerism or chimera (also spelled chimaera) A mouse or rat composed of cells from different zygotes. Animal chimeras are produced by coupling multiple fertilized eggs in one embryo which can result in two blood types, male and female organs in the same animal or other variations.

Hemodichorial placenta Approximately two layers of tissue separating the fetal and maternal blood flow.

Hemotrichorial placenta: simplistic

definition Approximately three layers of tissue separating the fetal and maternal blood flow.

Intramuscular Referring to injection of material directly into the muscle layers.

Mosaicism Inclusion of two different sets of genes in one individual.

Pheromones Chemicals produced by an organism that transmits a message to other members of the same species.

Photoperiod The number of hours of light to the number of hours of darkness.

Puberty The onset of sexual maturity and ability to bear young. Puberty occurs before full body size and weight are obtained and is not an ideal time to breed for the first time.

Subcutaneous Referring to injection of material into the space between the skin and underlying muscle layers.

Transgenic animals Strains of animals produced by genetic manipulation – includes “knock-out” animals (a strand of DNA deleted) or “knock-in” animals (a strand of DNA added). These strains are developed then stabilized by strategic breeding strategies.

Triploidy Chromosome abnormalities in which an extra set of chromosomes is included into the fetus genetic material.

Wild type animals Wild type animals have had no genetic manipulation and may be considered equivalent to “out-bred” or “random source” animals.

Introduction

This article is to present an overview of laboratory rat (*Rattus rattus*) and mouse (*Mus musculus*) reproduction. Mice have 40 chromosomes, while the rat has 42. Rats and mice do not breed naturally with each other and attempts to merge rat and mouse DNA into eggs (to develop chimeric animals) has not been successful. When working with both species, appreciating the similarity and differences between these two species can be very helpful.

Because of the many genetic strains of mice and rats that are available, the reproductive data such as length of gestation, age at puberty or number of pups/litter is variable. Average numbers are used in this article but successful breeding is always dependent on knowledge of the reproductive tendencies of the strain of interest. For example, androgenic knock-out male mice show decreased reproductive performance due to anatomical changes in the penis anatomy compared to wild type animals (Yang *et al.*, 2010). Some strains of hairless rats tend to be poor mothers as they produce little to no milk (Sharp and Vilano, 2013). New transgenic lines always need to be monitored carefully for reproductive abilities since the many genes which drive reproductive success can be altered in unexpected ways by targeted genetic alterations.

Anatomy

Reproductive anatomy of rats and mice is basically similar to all mammals and in depth discussions are easily found (Jacoby *et al.*, 1984; Kohn and Clifford, 1984). A few significant points are discussed below (Table 1).

Female Rats and Mice

Cervical Anatomy

The literature often describes rats and mice as possessing two cervixes which is incorrect. Rats have two separate cervixes, one in each uterine horn, which unite at the vagina. The mouse uterine horns unite into an externally undivided uterine body with cranial and caudal internal parts. Internally the uterine body has two lumen separated by a midline septum which are sometimes labelled as paired cervixes. However, the two lumens join in into one narrowed, tapered canal which forms the uterine neck or cervix of the

Table 1 Similarities/Differences between rat and mouse reproductive anatomy

<i>Anatomy</i>	<i>Rat</i>	<i>Mouse</i>
Mammary glands	Six pairs	Five pairs
Cervix	Two cervix	One cervix
Clitoral glands	Connect to urethra	Variable connection to urethra depending on strain
Ovaries, Oviducts, Uterine Horns	Two	Two
Neonate vaginal anatomy	Vaginal plate	No vaginal plate
Male accessory glands	Seminal vesicles, prostate gland of which the anterior lobe is considered the coagulating gland, bulbourethral (Cowper's) glands, ampullary glands, and preputial glands	The same as the rat

mouse which then projects into the vagina ([Leppi, 1964](#); [Hamilton, 1947](#)). Technically, the mouse has one cervix while rats have two.

Exocrine Glands

Clitoral exocrine glands are present in both species which secrete sebaceous pheromones, very important to the sex life of mice (apparently not such a big deal for rats!). The clitoris of rats connects directly to the urethra while the mouse clitoris may be completely separate from the urethra or may be partially connected, depending on the strain of the mouse ([Yang et al., 2010](#)).

Mammary Tissue

Female rats have six pairs of mammary glands, while mice have five pairs. Mammary gland tissue is extensive, extending from the nipples up along the sides and flanks of the animal towards the back and the sides of the neck. Older rats and mice can develop mammary tumors in unusual areas, such as the flanks or neck.

Male Rats and Mice

Inguinal Canal

The inguinal canals remain open throughout the animal's life and testicles may be retracted into the abdomen. A large fat pad exists which tends to prevent inguinal hernias secondary to castration, however it is recommended to close the inguinal canal when performing castration.

Accessory Glands

Rats and mice possess seminal vesicles, coagulating glands (which may be part of the prostate), prostate glands, ampullary glands and preputial glands. Secretions from the coagulating gland and seminal vesicles make up most of the plug that is deposited after coitus. The preputial and ampullary glands are not found in humans and are a source of pheromones.

Determining Gender

Male Rats and Mice

Male adult animals show distinct scrotums with visible penises. Sometimes the testes may be retracted into the inguinal canal. Hold the animal vertically (head up) to allow the testes to slide into the scrotum and become visible.

Female Rats and Mice

Female mice and rats show a shorter anal – genital distance than males but sometimes the genital opening can be confused with a penis, especially in very young animals. Prominent rows of nipples start to become visible at 8–9 days of age in females of both species.

Pre-Pubertal Animals

To determine gender it is easiest to compare ano-genital distances of new born animals. Males tend to be about 1.5–2X greater distance than females. In male mice there may be a dark pigmentation visible in the pre-scrotal region. Pale undescended testes

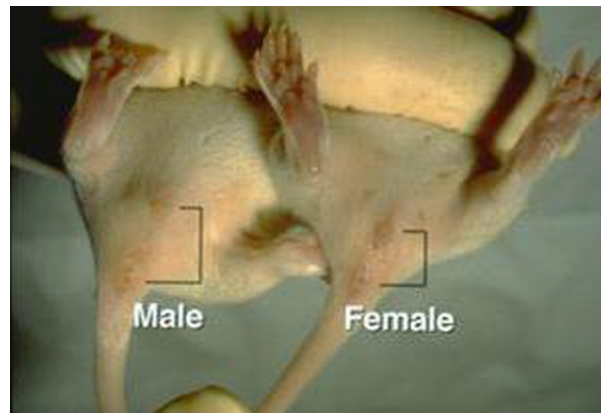


Fig. 1 Gender identification of neonatal mice.

may be visualized through the abdominal wall of mice. As neonates age, it can become harder to differentiate gender until 8–10 days of age when nipples appear on females. At 18–28 days (weaning), the male penis is more recognizable ([Fig. 1](#)).

Puberty

Puberty begins as Follicle Stimulating Hormone (FSH) promotes egg, sperm and hormone production leading to the animals becoming capable of reproducing. Age of onset of puberty varies between strains. For example, the C3H/HeOJ mouse begin successful mating at about 6 weeks old, where BALB/cJ mice wait until about 8 weeks of age ([Silver, 2008](#)). But puberty can begin as early as 30 days and genders should be separated to prevent uncontrolled mating. It is best to allow the animal to mature before allowing breeding.

Males

Mature sperm has been reported as early as 30–35 days of age ([Sharp and Vilano, 2013](#)) with most sources reporting best fertility beginning about 5010 days. If a male is still a virgin at 2.5–3 months, he will not plug well (create a vaginal plug after mating) and is less likely to be a prolific breeder ([Burds Connor, 2007](#)).

Females

Though some female rats and mice may ovulate as early as 28–30 days of age, most animals first ovulate naturally around 6–8 weeks of age. Both species require the vagina to open before they can be bred. Female rats have a vaginal plate which must degenerate prior to vaginal opening. (For more details, see [Kohn and Clifford \(1984\)](#) or [Del Vecchio \(1992\)](#)). In mice, exposure to adult males or their urine can bring on earlier ovulation; exposure to mature females or their urine may delay the initial ovulations. A female will produce best over her lifetime if she is allowed to obtain most of her body growth before breeding. Once paired with a mate, a litter should be successfully produced in less than 2 months. The number of litters per female mouse can vary per strain – for example, the average number of litters is 2.2 per AKR/J females compared to 4.8 in FVB/N strains ([Burds Connor, 2007](#)).

Reproductive Cycles

Estrous

Both rats and mice are continuously polyestrous and ovulate spontaneously. The entire cycle of four stages (proestrus, estrus, metestrus, diestrus) is approximately 4–5 days long in both species ([Marcondes et al., 2002](#)). Knowing where a female animal is in her cycle enables coordination of pup production or embryo transfer. The stages are identified by characteristic cellular changes in microscopic exams of vaginal smears and are well described (see [Fig. 2](#)). Rats tend to ovulate later in their cycle than mice ([Table 2](#)).

Cycle regularity is generally stable in adult animals but may be disrupted. Rats exposed to 24 h light tend to develop persistent estrus and cystic ovaries ([Sharp and Vilano, 2013](#)) while chronic low light exposure during dark cycles causes earlier vaginal opening and ovarian atrophy ([Fox and Laird, 1970](#)). Exposure to estrogenic substances in feeds or plastic housing may affect cycling regularity. The cycles become more irregular in older animals. Pheromones play an important role, more in mice than in rats, and can be manipulated for timed breeding.

Table 2 General characteristics of reproductive cycles in mice and rats

	Mouse ^a (unless marked)	Rat ^b (unless marked)
Sexual maturity	45–65 days ^a	50–75 days ^a
Puberty	28–49 days	Variation reported – 50–72 days males Females as early as 35 days ^a
Vaginal Opening (days)	24–28	28–60
Recommended first mating	6–8 wks ^a	65–110 d ^d
First mating wt male (gm)	20–30 ^d	300 ^d
First mating wt female (gm)	20–30 ^d	250 ^d
Estrus length	6–8 h	20 h
Post partum Estrus (Hours)	14–24, fertile	3–18 h, fertile
Gestation (d)	19–21 ^a	21–23 ^a
Duration of pup delivery	2 h ^a	15–30 min ^a
Litter Size (pups)	6–12 ^a	3–18 ^a
Birth weight	0.75–2.0 gm	5–6 gm
Eyes open (d)	12–14	10–12
Able to hear	10 ^c	9 ^c
Weaning (d)	21–28d	20–21
Eating solid food (d)	14–16 ^c	11–14 ^c
Breeding lifespan	210–270 days ^a	350–440d ^a

^aMay vary with strain.^bSharp, P., Vilano, J., 2013. The Laboratory Rat, second ed. A volume in the Laboratory Animal Pocket Reference Series. Boca Raton, FL: CRC Press Taylor and Francis Group. pp. 12–13, 26–29.^cHrapkiewicz, K., Medina, L., 2007. Clinical Laboratory Animal Medicine, An Introduction, third ed. Ames, IA: Blackwell Publishing. pp. 42–44, 81–83.^dHarkness, J.E., Wagner, J.E., 1995. The Biology and Medicine of Rabbits and Rodents, fourth ed. Media, PA: Williams and Wilkins.^eJacoby, R.D., Fox, J.G., Davisson, M., 1984. Biology and diseases of mice. In: Fox, J.G., Anderson, L.C., Loew, F.M., Quimby, F.W. (Eds.), Laboratory Animal Medicine, American College of Laboratory Animal Medicine Series, second ed. San Diego, CA: Academic Press, pp. 40–52.

Timing of Ovulation

Ovulation in mice usually occurs during the dark period about 2–3 h after the onset of estrus (though there may be cycles without ovulation). Rats tend to ovulate during the metestrus period (Sharp and Vilano, 2013)] about 8–11 h after the onset of estrus and usually between midnight and 2 am (Peluso, 1992). Ova remain viable about 10–12 h after ovulation (Fox and Laird, 1970). Rats and mice both have fertile post-partum estrus.

Physical Changes Associated With the Cycle

In female mice, the opening of the vaginal orifice becomes more evident and the vulvar tissue swells during proestrus and estrus. The changes can be visible to the experienced eye (see Fig. 2). Female rats in estrus will exhibit characteristic behavior: ear quivering when the back or head are stroked and lordosis (a “sway-back” posture) when the pelvic area is stimulated. Rat vulvar tissue may swell and the vaginal wall appears dry (due to thickening of the tissues). The rat vaginal wall area is moist during metestrus and diestrus.

Determining Stage of Cycle

Vaginal cytology is used to determine the stage of an animal’s cycle. Methods to obtain cells for analysis are well described (Bronson *et al.*, 1996; Soleberg, 2004) and multiple videos are available on Youtube. Vaginal wall cells can be gently collected and then microscopically examined. Presence and proportions of epithelial cells with nuclei, cells with no nuclei (“cornified”), leukocytes and bacteria can be evaluated to identify the cycle stage. (see Fig. 3).

As shown by Koto *et al.* (1987), measurement of electrical impedance of the vagina may also be used to determine the stage of the cycle in female rats. The correlation of the changes with hormonal changes is not exactly the same for every strain but is consistent within strains – correlations must be established for each strain for the procedure to be predictive.

Pseudopregnancy

Pseudopregnancy, a condition resembling pregnancy that is marked by a persistent corpus luteum and no pups, is purposefully induced during embryo transfer procedures to produce recipient mice (see below). Vaginal stimulation of an animal near or in estrus or mating with an infertile (exhausted, vasectomized or poor breeder) male will induce pseudopregnancy. The condition may also be induced by manipulating pheromones in mice. Pseudopregnancy may last 13 days or longer in some strains of mice and rats.

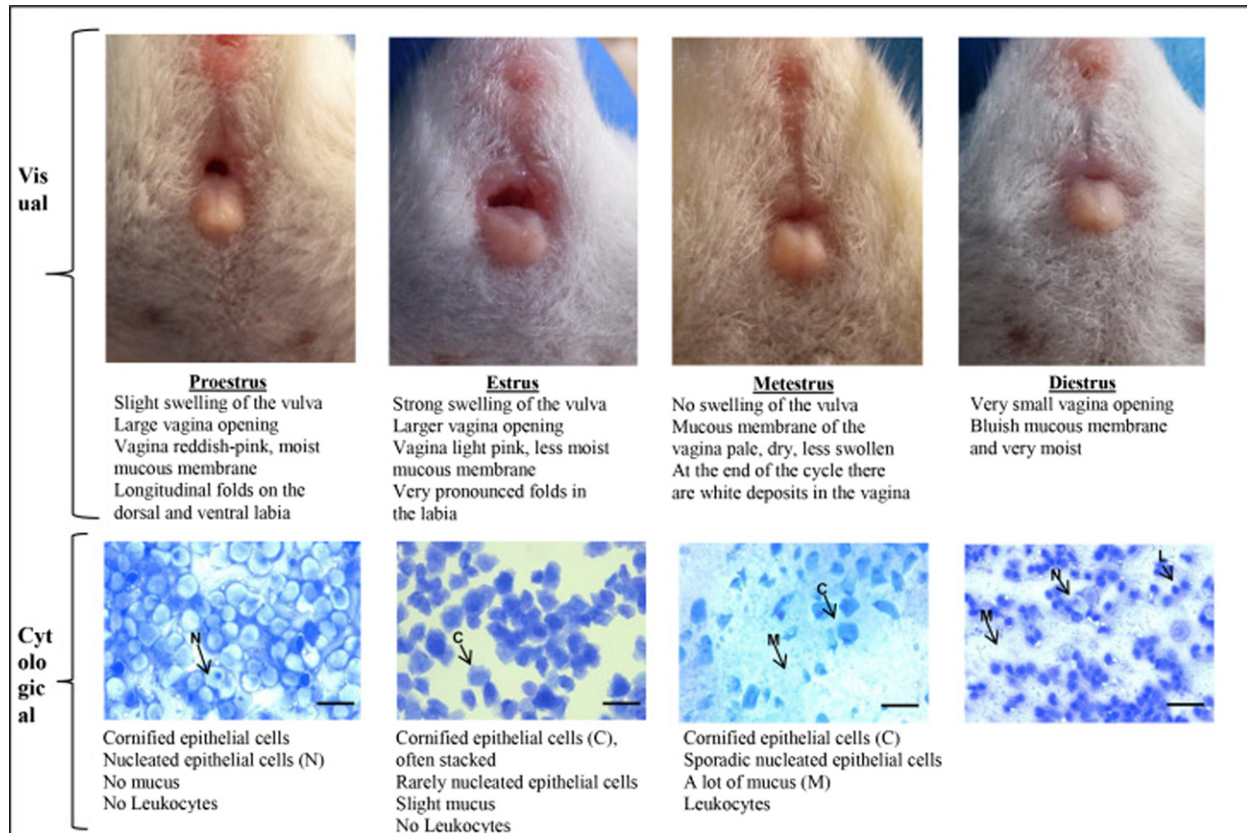


Fig. 2 Characteristics of vaginal smears and external changes. Reproduced from Heykants, M., Mahabir, E., 2016. Estrous cycle staging before mating led to increased efficiency in the production of pseudopregnant recipients without negatively affecting embryo transfer in mice. *Theriogenology* 85 (5), 813–821.

Breeding

Consistent husbandry of breeding animals and knowledge of the strain's reproductive tendencies are important in any successful breeding system. Consistent photoperiod is crucial to regular cycling (as discussed above). Caloric restriction may cause total cessation of estrous cycles and delay sexual maturity. High ambient temperatures (above 26.6 °C) can result in male infertility, causing irreversible degeneration of seminiferous epithelium (Pucak *et al.*, 1977) while cooler temperatures may lead to chilling and loss of pups.

Cycling can be irregular in young females but tends to stabilize at about 50–60 days of age. Aging female rats are considered “reproductively senescent” by about 10–12 months (Sharp and Vilano, 2013). Mice are usually retired from 6 months to one year of age depending on strain (Fox *et al.*, 1984). As animals age, their eggs show higher frequencies of genetic abnormalities such as triploidy and mosaicism in blastocytes recovered after superovulation (Shaver and Martin-DeLeon, 1975; Del Vecchio, 1992).

Copulation and Fertilization

Rats and mice perform coitus during the dark phase of the photoperiod. If the light cycle is reversed, the behavior will reverse as well (Jacoby *et al.*, 1984). Male activity is described by Dewsbury (1970). Again, activity is strain dependent. For example, recovery of libido occurs in about one hour in DBA mice but may take up to four days in B6 mice (Silver, 1995).

Plugging

Mating is confirmed when a visible vaginal plug is observed in the vagina or on the cage floor. The plug is formed by secretions of the vesicular and coagulating glands of the male following copulation, and extends from the vulva to the cervix. Pregnancy is rare if the plug is not observed, but the presence of the plug does not guarantee pregnancy.

When a plug is observed, the next day is considered day #1 of pregnancy (if the mating was fertile). To observe a plug, the animal is gently lifted by the tail and the vagina inspected. The plug is deeper in the mouse and often the vulvar lips need to be

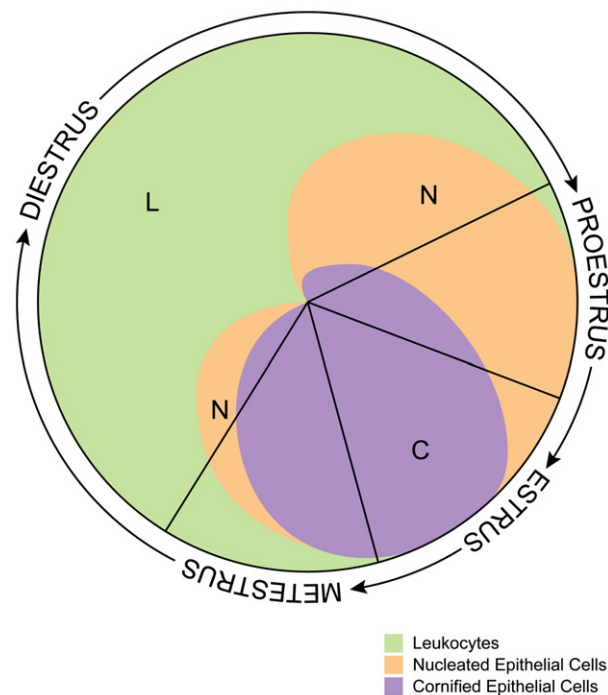


Fig. 3 Mouse Estrous Cycle Identification Tool. Reproduced from Byers, S.L., Wiles, M.V., Dunn, S.L., Taf, R.A., 2012. Mouse Estrous Cycle Identification Tool and Images. Open access article: <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0035538> PLoS One 7 (4): e35538.

gently spread with a cotton swab or a dull probe to facilitate viewing. Plugs tend to dislodge after 12–24 h so it is best to look for them right away in the morning. The combination of plug observation and vaginal cytology will determine where an animal is in the reproductive cycle.

Fertilization

Fertilization usually occurs in the ampulla or upper portion of the oviduct in mice. The ova can be fertilized up to 10–12 h after ovulation. The blastocyst implants into the endometrium within 4–7 days after post-coitus. For a detailed description of implantation, apposition, attachment and invagination processes, see [Lee and DeMayo \(2004\)](#).

Implantation

Mice and rats experience delays in implantation. This delay appears to be at least partially controlled by quiescent uterine stromal cells which cause the embryos in the blastocyst state to remain dormant ([Lee and DeMayo, 2004](#)). Pregnancies which occur during the post-partum estrus in a lactating female are often extended by delayed implantation 7–16 days beyond the normal 19 or 21 days of gestation ([Bronson et al., 1996](#)).

Manipulation of Breeding Cycle

Mice, more than rats, are strongly affected by pheromones which are chemical substances secreted from the body to elicit a specific behavioral reaction in the recipient.

Mice show three well-documented pheromone-driven responses which are used to help synchronize estrus and breeding.

Lee-Boot Effect

A group of females together housed together will tend to go into anestrus (or pseudopregnancy) and stop cycling for a period of time. Even smelling a male mouse will counter-act this effect, so the animals must be truly removed from male mice.

Whitten Effect

Introducing a male mouse to a group of non-cycling females will cause 40%–50% of those females to show estrus within 3 days and a second estrus in about 11 days. This can be used to synchronize ovulation in mice that would be

super-ovulated as ova/embryo donators, used as embryo recipient mice or to control breeding intervals to synchronize pup arrival.

Bruce Effect

Pheromones from an unfamiliar (different strain) male mouse may cause delayed implantation, pseudopregnancy or abortion in female mice bred within 24 h to 4 days of introduction (depending on strain). This effect is not seen if the new male is the same inbred strain as the female (Silver, 2008). Females pregnant over five days will not be affected by a new male introduced to the cage, so when pairing new couples, it is prudent to introduce the new male after the fifth day of gestation. The male is more likely to accept the pups as his own if they are born while he is in the cage (as opposed to being introduced to a new female after her litter is born).

Some of strains of rats may show mild Whitten effects but the literature is divided about this. It is generally agreed that rats are not susceptible to the Bruce effect. Rats, in general, do not seem to be as sensitive to pheromone effects as mice.

Post-Partum Estrus

Post parturition estrus occurs usually within the first 12–24 h after birthing. Approximately 50% of the time the post-partum estrus is fertile. This can make it possible for a new litter to be born to a mother who has not fully weaned her previous one. Optimal pup survival depends on close attention to weaning on-time when the post-partum estrus is used. It is important to have a system to track breeding and expected weaning dates!

Determination of Pregnancy

Rats' gestation period is 21–23 days with conception rates of 85% or better for outbred animals, slightly lower for inbred animals. At about 10 days, experienced palpation of the rat's abdomen can detect fetuses (easier to feel after 12 days). Mammary glands and nipple development become evident at 12–14 days.

Mouse gestation is about 19–21 days. An increased rate of weight gain may be noted during daily weight measurements at about 13 days into gestation. Fetuses may be very gently palpable mid to late gestation. Mammary structures develop beginning about 9 days and are pronounced by day 14. The abdomen is noticeably enlarged by day 14 as well.

Breeding Systems

Monogamous

Monogamous systems (1 male to 1 female) are used in both the rat and mouse species depending on the strain and aggression tendencies of the parents. Monogamous systems require more male animals and more labor to be sure litters are removed prior to the next parturition. Monogamous systems maximize the numbers of litters per female but litters may be smaller with slower growth rates over the lifetime of the female.

Colony Mating

In a colony mating scheme, one male and 2–6 females may be housed together. The young are removed at weaning. This system is efficient for space and labor, as the post-partum estrus is utilized, however tracking individual female production and specific maternal parentage of offspring can be challenging. Pups will be allowed to nurse off several females, especially if pups are close to the same age. Accurate record keeping of breeding and birth dates enables prompt removal of weanlings prior to the arrival of the next litter.

Polygamous or Harem Mating

In this scheme, one male is housed with 2–6 females but the females are moved to separate cages before parturition. The post-partum estrus is lost as a breeding opportunity; however females tend to produce larger young with more animals weaned per litter. There are a lowered number of total litters per the life of the female rat and there is an increased labor cost with more individual cages.

Some females can become territorial, so it is best practice to place the female animal in the male's cage. Once bred, females will not accept another male until the pregnancy terminates.

Generating and Maintenance of Genetic Lines

Generating and maintaining specific desirable genetic lines of animals is done both by general pet breeders as well as scientists. In the past, spontaneous mutations were recognized and bred – resulting in many inbred strains of interest. Now scientists can genetically engineer animals, where, simply stated, a specific gene can be inserted into or removed from an animal's gene pool and the effects studied. A very short list of genetic alterations includes animals which are prone to obesity or diabetes, rats that become spontaneously hypertensive, animals that lack specific parts of their immune systems, and animals that develop signs consistent with Parkinson's disease or Alzheimer's disease, animals with genetic modifications that include a genetic "switch" to turn the modification on and off.

Sophisticated techniques of superovulation, genetic manipulation and embryo transfer are used in labs to generate new strains of mice and rats (transgenic and chimeric animals). Once the genetic modification is established, careful breeding for about 20 generations stabilizes the mutation into a recognized strain of animal. Once that strain is stabilized, careful out breeding must occur to decrease inbreeding and maintain hybrid diversity. Breeding to establish and maintain stable genetic lines is a skill!

Superovulation

Superovulation is used to generate embryos for collection and transfer to living recipients or freezing (cryopreservation) for storage of desirable strains. Female animals are chemically induced to ovulate a greater number of eggs than normal at a predictable time. After treatment with hormones, the animals are mated (by a fertile or infertile male depending on desired outcome) to stimulate egg release into the oviduct. Eggs may be surgically collected from the oviduct for in-vitro fertilization (if not already fertilized), development into blastocytes and freezing or implant (see below). For reviews of technique see [Silver \(2008\)](#) or [Luo et al. \(2011\)](#).

Embryo transfer

Embryo transfer serves at least three purposes: to support a genetic line which has difficulty reproducing, to develop disease free animals or to manipulate genetics. Embryo transfer is performed by transplanting embryos from a donor animal into a pseudopregnant recipient. It is necessary to generate a number of recipients at the same time by bringing multiple females into estrus at the same time.

Most strains of mice can be brought into synchrony relatively easily using the Lee-Boot and Whitten pheromone effects discussed above. Rats are not as susceptible to pheromone manipulation and require chemical synchrony ([Rouleau et al., 1993](#); [Borjeson et al., 2014](#)). Not all females will cycle as predicted but usually over 50% can be brought into a state close enough to estrus to be tested with a vasectomized male. For technique review, see [Silver \(2008\)](#).

Parturition

Rats and mice have a hemotrichorial placenta with multiple layers separating the fetal and maternal blood flow (humans have hemodichorial) ([Benirschke, 2011](#)). Average litter sizes are 6–12 pups. Nursing is usually allowed only after all the pups are delivered. Rodent mothers become better mothers with age behaviorally but "fetal wastage" (a measure of loss of fetuses as animals age) increases with age due to implantation failure and post-implantation mortality. Stress and strenuous maternal exercise can lead to increased fetal wastage as well ([Mottola et al., 1993](#)). Dystocia is rare but may occur. Cannibalism is not frequent and is a sign of maternal stress.

Mice

Mice should be supplied sufficient bedding material to build nests. Nest building behavior is an important external indication of the animal's general health. First litters tend to be the smallest with production in mice improving after the 2nd litter. There are strain differences in gestation length and size of litters produced between strains (for example the average litter size of BALB/CJ mice is about 5, where the average litter size of FVB/N mice is about 9 ([Silver, 2008](#))).

Mother mice will retrieve scattered pups and return them to the nest. She will lay over the top of the pups to nurse. In cages with more than one litter, the young may suckle several lactating dams.

Rats

Many rat strains will build shallow nests beginning about 5 days prior to birth of the litter. About 1.5–4 h prior to the first pup there may be a clear mucoid fluid noted from the vagina. The female will move about the cage, stretching and then lie on the floor with the rear legs extended off the cage floor. As the pups come, she will consume the placenta. The entire process takes approximately 1.5–3.5 h depending on litter size.

Dystocia

Since most births occur during the dark period of the photoperiod, an animal found laboring in the morning should be suspected to be in dystocia. Causes of dystocia may include uterine inertia, malposition of the pups or obstruction of the birth canal. Consult a veterinarian. To treat dystocia, the state of the mother should be assessed. If she is exhausted, dehydrated, quiet or lethargic, the wisdom of trying to treat the dystocia vs surgical delivery of the pups should be considered. The birth canal should be examined and if a pup is found, it should be gently delivered if possible.

If chemical treatment of dystocia is elected, the following actions could be considered. Support the mother with subcutaneous fluids (such as LRS with 2.5% dextrose – about 1 cc per 10 g body weight), extra warmth and perhaps antibiotic if considered necessary. Oxytocin (0.3–4 IU/Kg) may be given intramuscularly or subcutaneously. For mice, one can dilute 20 IU Oxytocin in 1 cc of LRS and give approximately 0.1 cc subcutaneously or IM. Give the animal approximately 30–60 min. If there are no more pups produced, surgical extraction is recommended.

Post-Partum

Pups are altricial (born in an undeveloped state) and require feeding and care from their mothers. The pups are blind, hairless and their ear canals are closed. The larger the litter, the smaller the pups will be. Retina's take about a month to mature; even though the animals' eyes open prior to that, they are not fully visual. Maternal antibody is transferred across the yolk sac in utero and from colostrum in the milk. It is recommended that newly born pups be left undisturbed for the first 2 days if possible to allow full bonding with the mother; however some early quiet careful observation of pups may be prudent, especially when developing a new genetic strain (Wells, 2016). When changing cages for the first time after parturition, moving the nest and some dirty bedding into the new cage helps smooth the transition.

Rats

The external ear opens at about 2.5–3.5 days and pups appear to be able to hear at about 9 days. Incisors erupt about 6–8 days of age. Pups are generally fully haired by about 7–10 days of age. Eyes open at about 14–17 days of age.

Mice

Pups are called “pinkies”. When they are weaned, they are called “hoppers”. These terms are used by breeders (and reptile feeders) to identify the size of mouse. Mouse pups are mildly transparent and the white “milk spot” can be observed in their stomachs after they have nursed. They are fully haired by about 10 days of age and their eyes open at about 12 days of age. They can begin eating solid food and drink water at about 14 days of age.

Weaning

Weaning is generally done at about 21–28 days of age. Rats can be weaned as early as 17 days but 20–21 days (40–50 g body weight) is standard. Inbred strains may need to be more time before weaning. One indication of readiness for weaning is when pups remain very active when the cage cover is removed. If they just sit still, they are still too young and should not be weaned (Burd Connor, 2007). Recently weaned pups should be kept together by gender at least one week to help maintain body temperature.

If mothers are not bred on the post-partum estrus, most females will resume cycling about 2–4 days after weaning. If the post-partum estrus was used, wean the litter before a new litter is produced. Competition between the older animals and the newborns can cause injury to the newborns and the mother may not have enough milk for all the offspring.

Orphans

Wikipedia features a nice article about how to raise an orphan mouse (see Relevant Websites section). See that article for more specific details.

The best way to raise an orphan less than 10 days old is to try to foster it onto a mother of similar aged pups (within 1–2 days). Rub orphans with scented material from the “natural” nest (perhaps moist dirty bedding, feces and birth fluids if the litter is that young), then mix the new pup(s) in with the original pups in the nest. Without disturbing the cage, recheck in several hours. If the mother has gathered the babies together, she will most likely accept them.

If the pup is over 10 days old, the pup may be able to be hand raised but this is difficult to do successfully. Kitten Milk Replacer can support baby rodents. Baby rats and mice cannot urinate or defecate on their own, so their genitals should be gently stimulated with a soft moist q-tip or cotton after feeding. Do not allow them to get chilled!

Trouble Shooting Breeding Problems

If there are problems with breeding of strains, it is important to look at the husbandry and environment of the animals as well as the specific animals themselves. Temperatures in the wrong range, presence of vibrations or disturbing ultrasound noise, multiple disturbance of the cages (explosive noise, people opening and closing the cages), absence of nesting materials, dietary fat content (too much or too little) all can have an effect. It may take some investigation to determine the cause of breeding issues. For a checklist of questions to consider, see [Perret-Gentil \(2015\)](#).

References

- Benirschke, K., 2011. Comparative placentation. <http://placentation.ucsd.edu/index.html>.
- Borjeson, T.M., Pang, J., Fox, J.G., Garcia, A., 2014. Administration of luteinizing hormone releasing hormone agonist for synchronization of estrus and generation of pseudopregnancy for embryo transfer in rats. *J. Am. Assoc. Lab. Anim. Sci.* 53 (3), 232–237.
- Bronson, F.H., Dagg, C.P., Snell, G.D., 1996. Reproduction. In: Green, E.L. (Ed.), *Biology of the Laboratory Mouse*, second ed. New York: McGraw Hill, pp. 187–204.
- Burds Connor, A., 2007. Aurora's Guide to Mouse Colony Management at MIT. Available at: <https://ki.mit.edu/files/ki/cfile/sbc/escell/mouseManagement.pdf>.
- Del Vecchio, F.R., 1992. In: Mohr, U., Dungworth, D.L., Capen, C.C. (Eds.), *Pathobiology of the Aging Rat*. Washington DC: ILSI Press, pp. 331–349. (vaginal plate).
- Dewsbury, D.A., 1970. Copulatory Behavior. In: Hafez, E.S.E. (Ed.), *Reproduction and Breeding Techniques for Laboratory Animals*. Philadelphia: Lea and Febiger, pp. 123–136.
- Fox, J.G., Anderson, L.C., Loew, F.M., Quimby, F.W. (Eds.), 1984. *Laboratory Animal Medicine*, American College of Laboratory Animal Medicine Series, second ed. San Diego, CA: Academic Press.
- Fox, R.R., Laird, C.W., 1970. Sexual Cycles. In: Hafez, E.S.E. (Ed.), *Reproduction and Breeding Techniques for Laboratory Animals*. Philadelphia: Lea and Febiger, pp. 107–122.
- Hamilton, C.E., 1947. The cervix uteri of the rat. *Anat. Rec.* 97 (1), 47–62.
- Jacoby, R.D., Fox, J.G., Davisson, M., 1984. Biology and diseases of mice. In: Fox, J.G., Anderson, L.C., Loew, F.M., Quimby, F.W. (Eds.), *Laboratory Animal Medicine*, American College of Laboratory Animal Medicine Series, second ed. 1984 San Diego, CA: Academic Press, pp. 40–52.
- Kohn, D.F., Clifford, C.B., 1984. Biology and diseases of rats. In: Fox, J.G., Anderson, L.C., Loew, F.M., Quimby, F.W. (Eds.), *Laboratory Animal Medicine*, American College of Laboratory Animal Medicine Series, second ed. San Diego, CA: Academic Press, pp. 127–134.
- Koto, M., Miwa, M., Tsuji, K., Okamoto, M., Adachi, J., 1987. Change in the electrical impedance caused by cornification of the epithelial cell layer of the vaginal mucosa in the rat. *Jikken Dotsu* 36, 151–156.
- Lee, Kevin Y., DeMayo, F.J., 2004. Animal models of implantation. *Reproduction* 128, 679–695. Available at: www.reproduction-online.org/content/128/6/679.full.
- Leppi, J.T., 1964. A study of the uterine cervix of the mouse. *Anat. Rec.* 150 (1), 51–66.
- Luo, C., Zúñiga, J., Edison, E., et al., 2011. Superovulation strategies for 6 commonly used mouse strains. *J. Am. Assoc. Lab. Anim. Sci.* 50 (4), 471–478.
- Mottola, M.F., Mezzapelle, J., Schachter, C.L., McKenzie, K., 1993. Training effects on maternal and fetal glucose uptake following acute exercise in the rat. *Med. Sci. Sports Exerc.* 25, 841–856.
- Marcondes, F.K., Bianchi, F.J., Tanno, A.P., 2002. Determination of the estrous cycle phases of rats: Some helpful considerations. *Braz. J. Biol.* 62/4a (4a). Available at: <https://doi.org/10.1590/S1519-69842002000400008>.
- Peluso, J.J., 1992. Morphologic and physiologic features of the ovary. In: Mohr, U., Dungworth, D.L., Capen, C.C. (Eds.), *Pathobiology of the Aging Rat* vol. 1. Washington, DC: ILSI Press, pp. 337–349.
- Perret-Gentil, M., 2015. Breeding Troubleshooting Questionnaire. SanAntonio, TX. Available at: <http://research.utsa.edu/wp-content/uploads/2015/02/Breeding-Troubleshooting-Questionnaire-Updated-2-21-15.pdf>.
- Pucak, G.J., Lee, C.S., Zaino, A.S., 1977. Effects of prolonged high temperature on testicular development and fertility in the male rat. *Lab. Anim. Sci.* 27, 76–77.
- Rouleau, A.M., Kovacs, P.R., Junz, H.W., Armstrong, D.T., 1993. Decontamination of rat embryos and transfer to specific pathogen – free recipients for the production of a breeding colony. *Lab. Anim. Sci.* 43, 611–614.
- Sharp, P., Vilano, J., 2013. *The Laboratory Rat*, A Volume in the Laboratory Animal Pocket Reference Series, second ed. Boca Raton, FL: CRC Press Taylor and Francis Group, pp. 12–13, 26–29.
- Shaver, E.L., Martin-DeLeon, P.A., 1975. Effects of aging of sperm in the female and male reproductive tracts before fertilization on the chromosome complement of the blastocysts. In: Blandau, R.J. (Ed.), *Aging Gametes, Their Biology and Pathology*. Washington. Basel: Karger, pp. 151–165.
- Silver, L.M., 1995, revised 2008. *Mouse Genetics Concepts and Applications*, Oxford University Press Adapted for the Web by Mouse Genome Informatics Maine. The Jackson laboratory. Available at: <http://www.informatics.jax.org/silver/index.shtml>.
- Soleberg, P., 2004. Examination of vaginal smears in the rat. Available at: <https://norecopa.no/education-training/other-teaching-materials/rat-oestrus>.
- Wells, S., 2016. Harwell, MRC: It starts at the very beginning – the importance of neonatal assessment. Available at: <https://www.nc3rs.org.uk/it-starts-very-beginning-importance-neonate-assessment>.
- Yang, J.H., Menshenina, J., Cunha, G.R., Place, N., Baskin, S.L., 2010. Morphology of mouse external genitalia: Implications for a role of estrogen in sexual dimorphism of the mouse genital tubercle. *J. Urol.* 184 (4 Suppl), 1604–1609. Available at: www.ncbi.nlm.nih.gov/pmc/articles/PMC3428050/.

Further Reading

- Bronson, F.H., Dagg, C.P., Snell, G.D., 1996. Reproduction. In: Green, E.L. (Ed.), *Biology of the Laboratory Mouse*, second ed. New York: McGraw Hill, pp. 187–204.
- Hafez, E.S.E. (Ed.), 1970. *Reproduction and Breeding Techniques for Laboratory Animals*. Philadelphia: Lea and Febiger.
- Jackson, I.J., Abbott, C.M. (Eds.), 2000. *Mouse Genetics and Transgenics: A Practical Approach*. New York: Oxford University Press.
- Jackson Laboratory Staff. *Biology of the Laboratory Mouse*, second ed. Blakiston Division of McGraw-Hill.
- Pinkert, C.A. (Ed.), 2014. *Transgenic Animal Technology A Laboratory Handbook*. Boston, MA: Elsevier.
- Silver, Lee M., 1995, revised 2008. *Mouse Genetics Concepts and Applications*, Oxford University Press Adapted for the Web by Mouse Genome Informatics Maine. The Jackson laboratory, <http://www.informatics.jax.org/silver/index.shtml>.
- Suckow, M., Weisbroth, S., Franklin, C., 2005. *The Laboratory Rat*, second ed. Toronto: Academic Press, (American College of Laboratory Animal Medicine).

Relevant Websites

<https://ki.mit.edu/files/ki/cfile/sbc/escell/mouseManagement.pdf>

Burds Connor, A., 2007. Aurora's Guide to Mouse Colony Management at MIT.

http://research.utsa.edu/wp-content/uploads/2015/02/tips_for_successfully_breeding_your_mice.pdf

Good info on mouse breeding, University of Texas, San Antonio.

<http://placentation.ucsd.edu/indexs.html>

In depth discussion of placentation of various species, Benirschke, K., Comparative placentation.

<http://www.afirma.org/>

Mouse Genome Information – Jackson Labs website: www.informatics.jax.org. A wealth of information about rodent strains and breeding techniques. Dr. Silver's Mouse Genetics and Applications publication is available here. American Fancy Rat and Mouse Association.

<http://research.utsa.edu/research-funding/laboratory-animal-resources-center/training/>

Scroll through this site, much rodent information with specific breeding information at the bottom of the page, University of Texas, San Antonio.

<https://norecopa.no/education-training/other-teaching-materials/rat-oestrus>

Soleberg, P., 2004. Examination of vaginal smears in the rat.

<http://www.med.umich.edu/tamc/tgoutline.html>

Transgenic reproduction mice and rats, University of Michigan.

<https://www.nc3rs.org.uk>

Under 3Rs resource tab scroll down to Hubs and microsites – will find several involving rodents including one focused on Genetically altered mice. National Centre for the Replacement Refinement and Reduction of Animals in Research (NC3Rs).

<http://www.wikihow.com/Care-for-Baby-Mice>

wikiHow to care for baby mice.

Honey Bees, Royal Jelly, Epigenetics

Benjamin P Oldroyd, Rebecca J Reid, Alyson Ashe, and Emily J Remnant, University of Sydney, Sydney, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Caste

Bee, ant and wasp societies are characterized by their 'castes'. Colonies typically contain a single reproductive queen and hundreds or thousands of workers that rarely if ever lay eggs. Queens and workers are morphologically distinct; in many species greatly so (Fig. 1, Snodgrass, 1956). In honey bees, for example, queens lack the pollen baskets, honey stomach and the elongated tongue of workers, so they are incapable of foraging. Workers, on the other hand, have all the specialized equipment for foraging, but lack a spermatheca (sperm storage organ) so they cannot mate and have far fewer ovarioles (typically 10 across both ovaries) than do queens (typically 500–600). There are physiological differences too; queens typically live one or 2 years and up to 5 years, whereas workers live about a month.

Queens and workers arise from genetically similar eggs, and so the different morphologies of queens and workers are entirely environmental in origin. In honey bees the developmental differentiation between workers and queens is mediated by one factor only: larval diet. That such fundamental differences in morphology, physiology and behavior can be caused by environmental cues is unusual in the animal kingdom.

How Queens and Workers are Made

When a queen honey bee lays a fertilized egg the egg is 'totipotent', that is it can be reared either as a queen or a worker. (This fact is the basis of the queen bee production industry – by creating the right environment for larval development, a beekeeper can produce almost limitless numbers of queens.) Fertilized eggs are laid by queens in either of two kinds of brood cells – the large, peanut shell-shaped queen cells that hang down off the normal brood comb, or the much smaller horizontal cells that comprise the worker brood comb (Fig. 2). (Queens also lay eggs in intermediate-sized drone cells, but these develop as male drones, and we shall not consider them further here). Whatever kind of cell an egg is laid in, eggs hatch after 3 days producing a tiny larva, about the size and shape of a plump letter 'c' in an 8-point typeface. In both queen cells and worker cells the newly-hatched larvae are fed by nurse workers – young bees. The food is a mixture of secretions from the salivary glands, and in the case of workers, regurgitated stomach contents (Shuel and Dixon, 1960). Both queen larvae and worker larvae are fed similar amounts of food for the first 2 days of life and are always provided with more food than they can eat (Nelson *et al.*, 1924). After 2 days, however, the diets change. Queen-destined larvae are fed a hyper-abundance of royal jelly. Queen larvae 'float' (actually hang) in a glob of about one milliliter of royal jelly, and it is remarkable that both jelly and larva do not fall out of the cell. Nurse workers continuously top up the royal jelly in queen cells, so there is always far more than the larva can eat. In contrast, worker larvae that are more than 2 days of age are 'progressively' provisioned. Worker larvae are only 30 min or so from starvation.

By the time a larva is 3.5 days old it loses its totipotency. If 3 day old larvae are transferred from worker cells to queen cells containing royal jelly, they do not develop as queens but will either die or develop as a worker or inter-caste (Weaver, 1957). Similarly, if a larva is transferred from a queen cell to a worker cell at 1 day of age it will develop as a worker, whereas larvae transferred after more than 1 day in a queen cell fail to develop in worker cells (Weaver, 1957).



Fig. 1 A queen bee (centre and marked with a disk numbered 74) is surrounded by her workers. The workers are much smaller than the queen. Photo B. Oldroyd.



Fig. 2 Peanut shaped queen-cells hang down from a comb constructed of worker cells in a dwarf honey bee *Apis florea* colony. Photo M. Rickets.

Royal Jelly is Responsible for the Differential Development

It is unequivocal that royal jelly is the key environmental determinant of whether a larva develops as a queen or a worker, and not the cell size or cell orientation. Queens can be reared in the lab in the horizontal orientation, provided that the diet contains substantial amounts of fresh natural royal jelly (Kamakura, 2011). It is mostly the greater size of the queen cell, and not its orientation, that causes nurse workers to feed queen larvae and worker larvae differentially (Shi *et al.*, 2011), though there is some evidence that worker larvae ‘beg’ for food (Beekman *et al.*, 2000). Thus the orientation and size of the brood cell is only important in determining what and how much the larva is fed by nurse workers, and is unimportant to how the larva develops.

Royal jelly is a thick, cream-coloured jelly. It contains proteins, amino acids, carbohydrates, fatty acids, vitamins and minerals (Townsend and Lucas, 1940). ‘Worker’ jelly fed to young worker-destined larvae differs from ‘royal jelly’ fed to queen-destined larvae in that royal jelly contains more carbohydrate (about 33% on a dry weight basis) than worker jelly (12%). However the proportion of sugar in worker jelly increases to 47% as worker larvae age (Shuel and Dixon, 1959). It seems likely that the carbohydrates (mostly glucose and fructose) that are present in the jelly fed to older workers are derived from honey regurgitated by nurse workers.

About 90% of the proteins in royal jelly belong to a class of proteins known as the Major Royal Jelly Proteins (MRJPs) (Schmitzova *et al.*, 1998). There are nine MRJPs that are coded by a group of clustered genes on chromosome 11. The *MRJP* genes are related to the *yellow* gene family, a common class of genes in insects, and the cluster of *MRJP* genes is flanked by five *yellow* genes (Drapeau *et al.*, 2006). This kind of arrangement suggests that the *MRJP* genes were derived from *yellow-e3* by gene duplication. Interestingly, it seems likely that *yellows* are bacterial in origin, and were horizontally transferred to insects early in insect evolution (Drapeau *et al.*, 2006). *Yellow* proteins are highly conserved and have multiple roles in insects including pigmentation, sex determination and behavior (Drapeau *et al.*, 2006).

All nine MRJPs are found in royal jelly (Schmitzova *et al.*, 1998), and presumably have a nutritive role. But they are also expressed in diverse tissues including ovaries, testes, head and brain suggesting additional functions (Drapeau *et al.*, 2006).

Three Hypotheses for the Trigger for Queen Development Contained in Royal Jelly

The mechanism by which RJ causes queen development is controversial and far from being resolved. Resolution is hampered by wildly different estimates of the constituents. It is unclear whether this is due to analytical inconsistencies or because the constituents are indeed highly variable.

Below we list three plausible hypotheses for the caste-determining property(ies) of royal jelly, noting that (1) and (2) are mutually exclusive, but (1) and (3) are not. In other words, there may be caste-determining factors in royal jelly, but they must be consumed in large amounts to make a queen.

- (1) There is some specific factor(s) in royal jelly, absent from worker jelly, which converts the developmental path toward the queen phenotype.
- (2) There is some specific factor(s) in worker jelly, absent from royal jelly, which converts the developmental path toward the worker phenotype.
- (3) After 2–3 days of age, queen-destined larvae are fed a super-abundance of food, and continue to feed on mass-provisioned royal jelly during the last larval instar after the brood cell is sealed. Worker-destined larvae are progressively provisioned and do not feed after the brood cell is sealed (Beetsma, 1979).

Royalactin, a Candidate Queen-Determining Protein From Royal Jelly

In 2011 Masaki Kamakura published a paper in *Nature* suggesting that the most abundant protein in royal jelly, *Major Royal Jelly Protein 1* (MRJP-1) is necessary and sufficient to cause the development of the queen phenotype (Kamakura, 2011). MRJP-1, which Kamakura called ‘royalactin’ is heat-unstable, and completely degrades after storage at 40°C for a month (Kamakura, 2011). Royal jelly stored in this way cannot induce the queen phenotype in lab-raised larvae, whereas fresh royal jelly can. Addition of purified royalactin to stored royal jelly rescues its queen-producing properties (Kamakura, 2011). This is good evidence that royalactin is the key queen-determining factor, though of course one might argue that its an open question as to whether royalactin is causative agent of queen development, or contributes indirectly to the development of queenliness (e.g., by acting as an appetite booster), without causing it. Kamakura proposed that royalactin directly interacts with the mTOR gene network, which regulates cell proliferation and is known to regulate development in insects. In particular, Kamakura showed that royalactin alters the expression of the gene *epidermal growth factor receptor* (*Egfr*), which is part of the mTOR pathway.

Unfortunately, the role of royalactin in queen development is far from resolved. Kamakura’s study was criticized, and more prosaic functions for royalactin were proposed by Buttstedt *et al.* (2016), who were unable to replicate Kamakura’s study and suggested that the real function of royalactin is to act as the thickener that stops royal jelly from running out of vertical queen cells. Its also difficult to see how a protein that forms a heteromer of four individual royalactin proteins bridged by the sugar apismin (Mandacaro *et al.*, 2017) could cross the gut wall intact and directly alter gene expression.

Nutritional Castration

As we have seen, worker-destined larvae are fed less than queen-destined larvae, and there is less sugar in the jelly fed to worker-destined larvae. Asencot and Lensky (1985) argued that it is the additional carbohydrates, particularly fructose, that are present in royal jelly in greater abundance relative to worker jelly, that cause larvae to consume more food, going so far as to suggest that the physical engorgement of the intestinal tract triggers increased juvenile hormone production and the development of queenliness. More recently it has been shown that genes in the insulin-signaling pathway, which respond to carbohydrates in the diet, also influence caste development (Wang *et al.*, 2013). Asencot and Lensky (1988) were able to rescue the queen phenotype by adding carbohydrate, particularly fructose, to worker jelly, which increased consumption. This is not to discount the likely role of the amount of food a larva has available to consume, as well as the amount of sugar that is in it, in caste development. In summary, there is good evidence that meager amounts of brood food that are low in fructose increase the likelihood that a larva will develop as a worker.

p-Coumaric Acid in Pollen and Nectar May Inhibit Queen Development

Mao *et al.* (2015) have argued that it’s not so much what’s in royal jelly, but what it lacks, that may be the key caste-determining factor. Almost all pollens contain phenolics that affect gene expression in insects. Thus, inclusion of pollen in the diet of older larvae may enhance worker development over queen development because it lacks plant phenolics. However, to us, this seems an unlikely basis for caste differentiation. Even though all pollens may contain at least some phenolics, the amount and kind is extremely variable, so this seems an unreliable way to determine caste. Further, there are many social insects (termites, ants and wasps) that do not consume pollen, yet still have caste differentiation.

Epigenetics and Caste Determination

The development of an organism is dependent in part on gene regulation. To overly simplify a complex process, in order for a cell in your big toe to do its job properly it needs to express different genes in different networks, and the tissue that contains it needs to develop in a different way from the cells in your liver. Major contributors to the regulation of gene expression in eukaryotes include histone acetylation and methylation, mechanisms that change RNA translation post-transcription, and DNA methylation (Box 1).

The information contained in a DNA molecule resides in the sequence of four bases, cytosine, guanine, thymine and adenosine that make up much of the molecule. However the genomic information contained in the sequence of bases is flexibly modified as part of the context-specific gene regulation process. DNA is packed tightly around proteins known as histones. Histones are subject to chemical modifications that affect the level of expression of associated genes. Further, the DNA itself can be modified by the addition of a methyl group to cytosines. Cytosine methylation generally reduces transcription, and a gene that is highly methylated will have reduced expression. Cytosine methylation is mediated by DNA methyltransferases (Dnmt 1 and 3), whereas removal of methyl groups from cytosines is mediated by TET dioxygenases (TET 1–3) that convert 5-methylcytosine to 5-hydroxymethylcytosine (Box 1).

One of the most important discoveries of the honey bee genome project (Weinstock *et al.*, 2006) was that the honey bee has a full set of DNA methyltransferase enzymes (Wang *et al.*, 2006). This was a surprising finding because the only other insects that

Box 1: Epigenetic mechanisms

Epigenetics refers to a range of modifications that alter gene expression and phenotype, without altering the DNA nucleotide sequence. In this way, epigenetic mechanisms change how the genome is regulated to facilitate cellular differentiation, developmental processes and to respond to changes in the environment. Epigenetic modifications are implicated in diverse regulatory activities across the genome, ranging from gene regulation and alternate splicing, to the suppression of transposable elements, chromosome inactivation and genomic imprinting (Paulsen and Ferguson-Smith, 2001; Jaenisch and Bird, 2003; Glastad *et al.*, 2011; Drewell *et al.*, 2012).

Epigenetic mechanisms can involve modifications to chromatin structure via histones (such as histone acetylation and methylation), modifications to cytosine residues via DNA methylation, or post transcriptional regulation of genes by noncoding RNA molecules such as small RNAs.

DNA methylation is one of the most important and well understood mechanisms of epigenetic modification. The process of DNA methylation involves the covalent bonding of a methyl group to a DNA cytosine residue. In animal genomes, methylation usually occurs at CpG dinucleotides. DNA methyltransferase (DNMT) enzymes catalyse the methylation of cytosine nucleotides. DNMT1s are maintenance enzymes that copy existing methylation patterns onto newly synthesized DNA strands during DNA replication. DNMT3s target unmethylated cytosine residues to establish new methylation patterns (Schaefer and Lyko, 2007).

Methylation in mammals and plants occurs widely throughout the genome, including in promoter regions, transposable elements, and the introns and exons of genes (gene bodies) (Yan *et al.*, 2015). Mammals have high levels of DNA methylation; 60%–90% of CpG dinucleotides are methylated (Lister *et al.*, 2009). In mammals promoter region methylation acts to repress gene transcription (Bird, 2002).

Insect methylation profiles are quite different from those of mammals and angiosperms. In insects, DNA methylation is restricted to gene bodies, and occurs at a much lower frequency than in mammals; just 0.2%–8% of CpG dinucleotides genome-wide are methylated (Feng *et al.*, 2010; Zemach *et al.*, 2010). Methylation within gene bodies is thought to regulate alternative splicing (Suzuki and Bird, 2008).

had been sequenced at the time, fruitfly and mosquito, did not have a functional DNA methylation system. Soon it was discovered that indeed, many honey bee cytosines are methylated, and that the methylation patterns seemed to differ between castes, suggesting that methylation helps mediate cast differentiation (Lyko *et al.*, 2010). This observation provided a clue to the mechanism by which larval diet might affect gene expression and change the course of development in worker and queen-destined larvae; epigenetic modification of the genome via DNA methylation.

DNA Methylation and Caste Determination

The corpora allata sits beneath the brain of insects, where it produces Juvenile Hormone (JH). JH has profound effects on insect growth and development. In honey bees, JH is involved in caste differentiation (Asencot and Lensky, 1984; Mutti *et al.*, 2011). The corpora allata is nearly twice the size in queen-destined larvae than it is in worker-destined larvae as a result of polyploidization (duplication of chromosomes) (Kucharski *et al.*, 2008). DNA methyltransferase 3 (DNMT-3) is the key enzyme that mediates de-novo methylation of DNA cytosines. DNMT-3 is highly expressed in the corpora allata of honey bee larvae, hinting that methylation of genes in the corpora allata may be central to the process of cast differentiation. Honey bees respond well to RNA interference technology, and this allowed Kucharski *et al.* (2008) to knockout expression of DNMT-3 in young larvae. Larvae so treated developed as queens, suggesting that indeed methylation of key genes prevents development of the (default) queen phenotype, steering the developing larva toward a worker.

While the specific genes that orchestrate caste polymorphism as a result of differential methylation are unknown, there is at least one strong candidate. *Dynactin p62* is expressed in the corpora allata, where it is involved in the regulation of juvenile hormone levels. This gene has higher levels of methylation in worker-destined larvae than in queen-destined larvae. Experimental knockdown of DNMT-3 expression results in the queen pattern of methylation of *dynactin p62* (Kucharski *et al.*, 2008), and the queen phenotype. No doubt there are other genes and processes involved, but this is an extremely exciting finding.

Other Epigenetic Factors Potentially Involved in Caste Determination

One of the components of royal jelly, phenyl butyrate, acts as a histone deacetylase inhibitor (Lyko *et al.*, 2010) (see Box 1 for a description of histones and chromatin in gene regulation). Histone deacetylases may act to suppress transcription by removing acetyl groups from histones and compacting chromatin (Kouzarides, 2007). They are known to interact with DNMTs (Jaenisch and Bird, 2003).

Small RNAs, including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and short interfering RNAs (siRNAs) are small noncoding regulatory molecules that are necessary for many cellular processes, including epigenetic regulation (Mattick and Makunin, 2006; Carthew and Sontheimer, 2009). They can control chromatin structure and mediate intergenerational epigenetic inheritance (Ashe *et al.*, 2012).

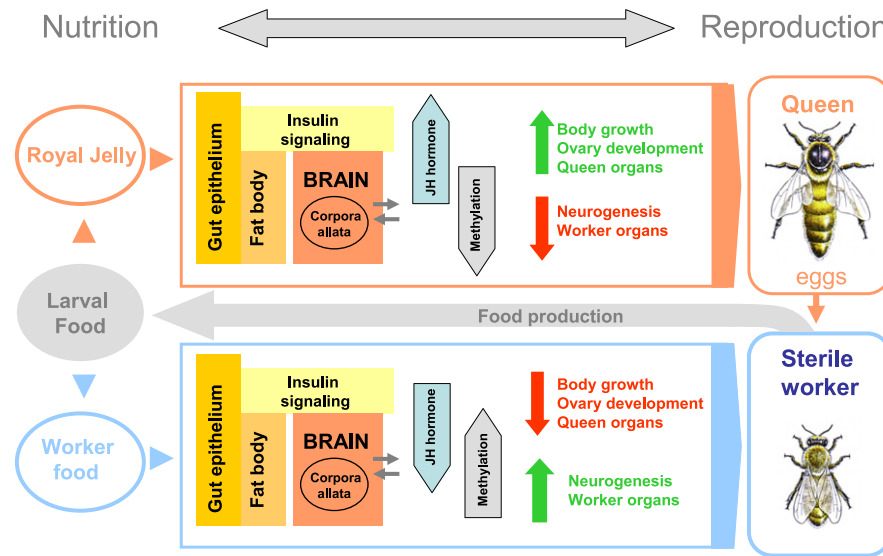


Fig. 3 Model showing some of the main factors affecting differential caste development in the honey bee. Reproduced from Maleszka, R., 2008. Epigenetic integration of environmental and genomic signals in honey bees. *Epigenetics* 3, 1–5 with permission.

A variety of small RNAs are found in the jelly provisioned to developing honey bee larvae. Worker jelly is enriched with miRNAs compared to royal jelly, with miRNAs of greater abundance and complexity suggesting that miRNA secretions by nurse bees may contribute to the regulation of caste development (Guo *et al.*, 2013). Queen and worker bees differ in miRNA expression profiles (Ashby *et al.*, 2016) and queens also express higher levels of piRNAs compared to workers (Wang *et al.*, 2017). piRNA expression may be related to germline suppression of transposable elements in reproductive queens compared to sterile workers, but may also have developmental roles in caste-specific phenotypes.

Putting it All Together

Thus far in this article we have learned that larval nutrition is the key factor in determining caste development and that epigenetic modification of gene networks arises as a consequence of differential nutrition of queen-destined and worker-destined larvae. But what connects the two? We summarize the likely associations in Fig. 3. We emphasize that this is a working model, and much of the detail remains to be elucidated.

Nutritional signals, including the amount of dietary carbohydrate and royal jelly protein available to and consumed by larvae, interact with the insulin signaling and mTOR gene networks within the fat body of the developing larva. In queen-destined larva these nutrition-sensitive gene networks cause changes in the polyploidization of the cells of the corpora allata, while simultaneously changing and generally decreasing methylation patterns genome wide. Increased JH production in the expanded corpora allata, and methylation changes, alter gene expression resulting in the queen phenotype (Fig. 3).

Conclusions

Although cast differentiation in social insects is one of the most extreme examples of phenotypic plasticity known in animals, and has been subject to considerable research over many decades, the actual mechanisms by which royal jelly changes the course of development is not understood. The role of royal jelly is beyond dispute, but the special ingredient(s) that cause the path to royalty remain to be elucidated.

References

- Asencot, M., Lensky, Y., 1984. Juvenile hormone induction of 'queenliness' on female honey bee (*Apis mellifera* L.) larvae reared on worker jelly and on stored royal jelly. *Comparative Biochemistry and Physiology B* 78, 109–117.
- Asencot, M., Lensky, Y., 1985. The phagostimulatory effects of sugars on the induction of "queenliness" in female honey bee (*Apis mellifera* L.) larvae. *Comparative Biochemistry and Physiology A – Molecular and Integrative Physiology* 81, 203–208.
- Asencot, M., Lensky, Y., 1988. The effect of soluble sugars in stored royal jelly on the differentiation of female honeybee (*Apis mellifera* L.) larvae to queens. *Insect Biochemistry* 18, 127–133.
- Ashby, R., Forêt, S., Searle, I., Maleszka, J., 2016. MicroRNAs in honey bee caste determination. *Science Reports* 6, 18794.

- Ashe, A., Sapetschnig, A., Weick, E.-M., *et al.*, 2012. piRNAs can trigger a multigenerational epigenetic memory in the germline of *C. elegans*. *Cell* 150, 88–99.
- Beekman, M., Calis, J.N.M., Boot, W.J., 2000. Parasitic honeybees get royal treatment. *Nature* 404, 723.
- Beetsma, J., 1979. Process of queen-worker differentiation in the honeybee. *Bee World* 60, 24–39.
- Bird, A., 2002. DNA methylation patterns and epigenetic memory. *Genes Devel* 16, 6–21.
- Buttstedt, A., Ihling, C.H., Pietzsch, M., Moritz, R.F.A., 2016. Royalactin is not a royal making of a queen. *Nature* 537, E10–E12.
- Carthew, R.W., Sontheimer, E.J., 2009. Origins and mechanisms of miRNAs and siRNAs. *Cell* 136, 642–655.
- Drapeau, M.D., Albert, S., Kucharski, R., Prusko, C., Maleszka, R., 2006. Evolution of the Yellow/Major Royal Jelly Protein family and the emergence of social behavior in honey bees. *Gen. Res.* 16, 1385–1394.
- Drewell, R.A., Lo, N., Oxley, P.R., Oldroyd, B.P., 2012. Kin conflict in insect societies: A new epigenetic perspective. *Trends Ecol. Evol.* 27, 367–373.
- Feng, S., Cokus, S.J., Zhang, X., *et al.*, 2010. Conservation and divergence of methylation patterning in plants and animals. *Proceedings of the National Academy of Sciences* 107, 8689–8694.
- Glastad, K.M., Hunt, B.G., Yi, S.V., 2011. DNA methylation in insects: On the brink of the epigenomic era. *Ins. Mol. Biol.* 20, 553–565.
- Guo, X., Su, S., Skogerbee, G., *et al.*, 2013. Recipe for a busy bee: MicroRNAs in honey bee caste determination. *PLOS ONE* 8, e81661.
- Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nature Genetics* 33, 245–254.
- Kamakura, M., 2011. Royalactin induces queen differentiation in honeybees. *Nature* 473, 478–483.
- Kouzarides, T., 2007. Chromatin modifications and their function. *Cell* 128, 693–705.
- Kucharski, R., Maleszka, J., Foret, S., Maleszka, R., 2008. Nutritional control of reproductive status in honeybees via DNA methylation. *Science* 319, 1827–1830.
- Lister, R., Pelizzola, M., Dowen, R.H., *et al.*, 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462, 315–322.
- Lyko, F., Foret, S., Kucharski, R., *et al.*, 2010. The honey bee epigenomes: Differential methylation of brain DNA in queens and workers. *PLOS Biology* 8, e1000506.
- Mandacaro, S.C., do Vale, L.H.F., Vahidi, S., *et al.*, 2017. Characterizing the structure and oligomerization of Major Royal Jelly Protein 1 (MRJP1) by mass spectrometry and complementary biophysical tools. *Biochemistry* 56, 1645–1655.
- Mao, W., Schuler, M.A., Berenbaum, M.R., 2015. A dietary phytochemical alters caste-associated gene expression in honey bees. *Science Advances* 1, e1500795.
- Mattick, J.S., Makunin, I.V., 2006. Non coding RNA. *Human Molecular Genetics* 15, R17–R29.
- Mutti, N.S., Dolezal, A.G., Wolschin, F., *et al.*, 2011. IRS and TOR nutrient-signaling pathways act *via* juvenile hormone to influence honey bee caste fate. *J. Exp. Biol.* 214, 3977–3984.
- Nelson, J.A., Sturtevant, P., Lineburg, B., 1924. The growth rate of the honey bee larva. *Bulletin of the US Department of Agriculture* 1222, 1–24.
- Paulsen, M., Ferguson-Smith, A.C., 2001. DNA methylation in genomic imprinting, development and disease. *Journal of Pathology* 195, 97–110.
- Schaefer, M., Lyko, F., 2007. DNA methylation with a sting: An active DNA methylation system in the honeybee. *Bioessays* 29, 208–211.
- Schmitzova, J., Kloudiny, J., Albert, S., *et al.*, 1998. A family of major royal jelly proteins of the honeybee *Apis mellifera* L. *Cellular and Molecular Life Sciences* 54, 1020–1030.
- Shi, Y.Y., Huang, Z.Y., Zeng, Z.J., *et al.*, 2011. Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, Apidae). *PLOS ONE* 6.
- Shuel, R.W., Dixon, S.E., 1959. Studies on the mode of action of royal jelly in honey bee development. II Respiration of newly emerged larvae on various substrates. *Can. J. Zool.* 37, 803–813.
- Shuel, R.W., Dixon, S.E., 1960. The early establishment of dimorphism in the female honeybee *Apis mellifera*. *Ins. Soc.* 7, 265–282.
- Snodgrass, R.E., 1956. The anatomy of the honey bee. Ithaca: Comstock.
- Suzuki, M.M., Bird, A., 2008. DNA methylation landscapes: Provocative insights from epigenomics. *Nature Rev. Genet.* 9, 465–476.
- Townsend, G.F., Lucas, C.C., 1940. The chemical nature of royal jelly. *Biochemical Journal* 34, 1155–1162.
- Wang, W.-W., Ashby, R., Ying, H., Maleszka, R., Forêt, S., 2017. Contrasting sex-and caste-dependent piRNA profiles in the transposon depleted haplodiploid honeybee *Apis mellifera*. *Genome Biol. Evol.* [In press].
- Wang, Y., Azevedo, S.V., Hartfelder, K., Amdam, G.V., 2013. Insulin-like peptides (AmILP1 and AmILP2) differentially affect female caste development in the honey bee (*Apis mellifera* L.). *J. Exp. Biol.* 216, 4347–4357.
- Wang, Y., Jorda, M., Jones, P.L., *et al.*, 2006. Functional CpG methylation system in a social insect. *Science* 314, 645–647.
- Weaver, N., 1957. Effects of larval age on dimorphic differentiation of the female honey bee. *Ann. Ent. Soc. Am* 50, 283–294.
- Weinstock, G.M., Robinson, G.E., Worley, K.C., *et al.*, 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature*, 443, 931–949.
- Yan, H., Bonasio, R., Simola, D.F., *et al.*, 2015. DNA methylation in social insects: How epigenetics can control behavior and longevity. *Ann. Rev. Ent* 60, 435–452.
- Zemach, A., McDaniel, I.E., Silva, P., Ziberman, D., 2010. Genome-wide evolutionary analysis of eukaryotic DNA methylation. *Science* 328, 916–919.

Further Reading

Cridge, A., Harrop, T., Lovegrove, M., Remnant, E., Dearden, P., 2017. Nutrition and epigenetic change in insects: Evidence and implications. *Advances in Insect Physiology*.

The Genetics and Epigenetics of Life History and Reproduction: Fish

Matthew C Hale, Texas Christian University, Fort Worth, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Many aspects of fish biology such as their rapid generation time, their high fecundity, and, for many species, their relative ease of housing in a controlled environment lend themselves to genetic and epigenetic studies. Additional feature, such as the wide array of mating systems used makes fishes an interesting taxonomic group coupled with variation in how sex is determined. Unlike in birds and mammals, sex determination in fishes is notoriously labile (Devlin and Nagahama, 2002; chapter 31 this issue), and closely related species can vary in the placement of the master sex-determining gene, the identity of the master sex-determining gene, or even if genetic sex determination is used at all. This diversity in sex determining mechanisms and mating systems coupled with a large array of adaptive phenotypes and life histories makes the fishes an ideal group to study adaptive biology and evolution. In this article I will discuss the genetic and epigenetic basis for the development of a few of the amazing adaptive phenotypes exhibited by fishes. These phenotypes will be discussed through the lenses of adaptation to maximize the fitness of the individual and how different forces of selection promote the development of different life histories in males and females. Specifically, I will focus on several reproductive behaviors; the evolution of anadromy and migration in the salmonids (salmon, trout, and char), the evolution of warm-water adaptation in Winter Skate, and the ability of the environment to alter sex in several species of fishes (Environmental Sex Reversal: ESR).

The recent development of next-generation sequencing methods has ushered in the “omics” era. These advancements have had an impact on genetic research in non-model organisms, allowing the collection of genome-wide and transcriptome-wide data, to answer evolutionary and ecologically important questions. Recently, these same methods have been adapted to studying the epigenome (modifications to the structure of DNA that are heritable but do not include mutations to the DNA sequence). Although the epigenome is more complex than the genome, and relatively few epigenetic marks have been investigated in detail, these new developments are extremely exciting for evolutionary biologists as more and more experiments suggest that the epigenome is important in the development of adaptive phenotypes. This will inevitably lead to many more discoveries that link changes in the epigenome to the development of adaptive traits.

The Genetic and Epigenetic Control of Life History Development: Anadromy in Salmonids

The salmonids (salmon, trout, and char) are a charismatic and economically important group of fishes native to high latitudes in the northern hemisphere. Their importance in native communities, sport fishing, and aquaculture has caused them to be well characterized at the genome level, resulting in sequenced genomes for both Rainbow Trout (*Oncorhynchus mykiss*; Berthelot *et al.*, 2014) and Atlantic Salmon (*Salmo salar*; Lien *et al.*, 2016). These complete genomes serve as an essential resource for further genetic research, as newly generated sequences can be aligned to these genomes to determine, amongst other things, the location of differentially expressed genes and the location of genomic variation (Single Nucleotide Polymorphisms or SNPs) associated with the development of adaptive traits. In sum, the large amount of genetic data available for salmon and trout makes them an excellent system for asking questions about the genetic basis of ecologically and evolutionarily important traits.

The salmonids are perhaps best known for their spectacular long-distance migrations that in some cases can take an individual from Canada to Japan. Migration is a fascinating behavior found in many taxonomic groups including birds, insects, fish, and mammals. The main reason individuals migrate is to take advantage of temporary resources that are either not present or are only present seasonally in their natal areas (e.g., food resources in the open ocean, or the summer emergence of winged insects in the Boreal forest; Dingle, 2006). Multiple studies on Rainbow Trout have found that many migratory related traits have considerable additive genetic variance, suggesting that migratory traits are heritable and are passed on from parents to their offspring (e.g., Hecht *et al.* (2015)). Despite this, very few studies have determined the genes associated with migratory related traits in any species (excellently reviewed in Liedvogel *et al.* (2011)). There are two reasons for this: (1) most migratory species do not have sequenced genomes and (2) it can be difficult to distinguish migratory individuals from resident individuals. This last point is especially important, as in many species where populations exhibit partial migration (i.e., where some individuals remain resident and others migrate) it is very difficult to visually distinguish migrants from residents. Salmon and trout represent an excellent system for studying the genetic basis of migration as not only are there two completed genome sequences available, but migrants and residents develop clearly differentiated phenotypes that allow for easy identification of an individual's migratory status (see Fig. 1).

All salmon and trout hatch and spend a portion of their early lives in freshwater. At some point in development (variable depending on species and population) migratory individuals must prepare for leaving their natal streams (see Fig. 2 for the life cycle of anadromous salmon, char, and trout). Salmonids display variation in life history tactics depending on the population and/or the species. Some species (such as Pink (*O. gorbuscha*) and Chum Salmon (*O. keta*)) are obligate migrants in their native areas, whereas most other species are partial migrants in at least part of their range. Migration can take different forms. Some populations, such as Brook Trout (*Salvelinus fontinalis*) in the North American Great Lakes regions, are potadromous (migrating from streams to large bodies of fresh water) whereas other populations are anadromous and migrate from freshwater streams to the ocean. Regardless of the type of migration, all migratory individuals undergo a physiological process called smoltification that prepares migrants for leaving their natal streams. The end-result of smoltification is to change juvenile salmonids from dark-colored, freshwater



Fig. 1 Phenotypic differences between resident and smolt *Oncorhynchus mykiss* juveniles after smoltification. Both photographs were taken on the same date and both fish are of the same age. The photograph clearly shows the difference in coloration between residents and smolts and the change in body shape. Both features are typical of smoltification and are required before a smolt can migrate to the ocean. Figure taken from Baerwald, M.R., Meek, M.H., Stephens, M.R., *et al.* 2016. Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. *Molecular Ecology* 25, 1785–1800, see references for a complete citation. Permission was obtained from Wiley.

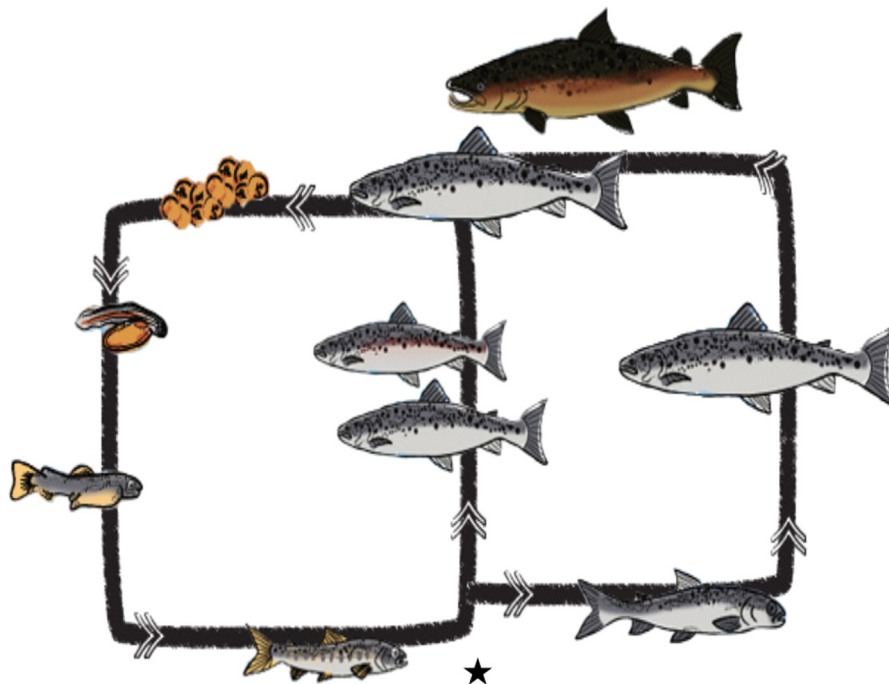


Fig. 2 Life history development in salmonids that show partial migration, such as Rainbow Trout, Sockeye Salmon, Chinook Salmon, Brook Trout, Dolly Varden, and (rarely) Atlantic Salmon. All salmonids hatch in freshwater. They then spend a portion of their lives in freshwater (from a couple of weeks in Pink Salmon, to several years in Chinook and Sockeye Salmon). At the point in development marked by ★ individuals either stay in freshwater (In species or populations that show partial migration) or undergo the smoltification process and move to a pelagic environment for a variable portion of time before returning to their natal streams to spawn. Figure kindly provided by Krista Nichols.

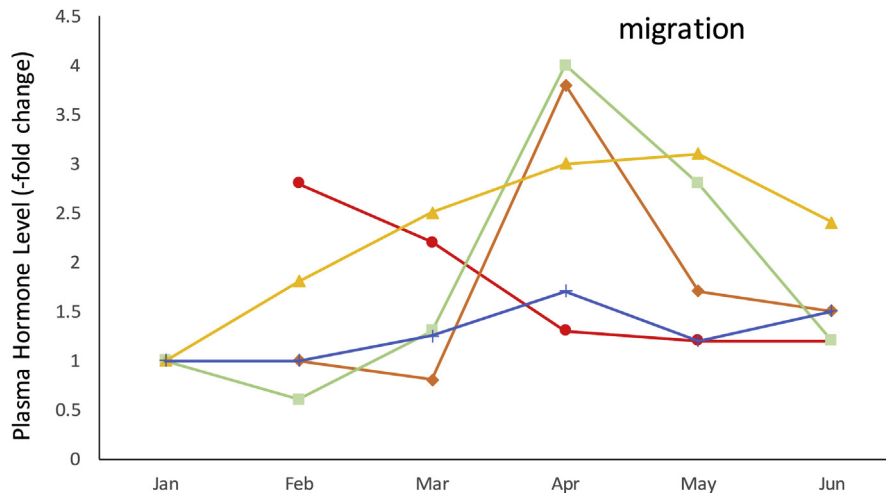


Fig. 3 Changes in plasma hormone levels during smoltification in Atlantic Salmon. Data for plasma hormonal levels are normalized to the January time point. Note, similar patterns of hormonal change have been noted in other salmonids in the lead up to smoltification, especially in Chinook Salmon and Rainbow Trout. Hormones are: Prolactin in red, IGF-1 (insulin growth factor-1) in yellow, Growth hormone in orange, Thyroxine (T₄) in blue, and cortisol in green. Modified from McCormick, S.D., 2013. Smolt Physiology and Endocrinology in Fish Physiology, pp. 199–251.

adapted parr into streamlined, pelagic adapted silvery smolts (see Fig. 1). The process of smoltification is characterized by increases in lipid metabolism, protein synthesis, changes in behavior, and, in anadromous individuals, hypo-osmoregulatory ability (Hoar, 1976; Dickhoff *et al.*, 1997). These changes require upregulation of several hormones including, growth hormone (GH), thyroid hormones (especially T₄), and cortisol (See Fig. 3: McCormick, 2009). These changes are especially demanding of anadromous individuals, as the physiological demands of moving from freshwater to seawater are stronger than moving from rivers to lakes. As a result, most research on smoltification has been dedicated to anadromous populations of Atlantic Salmon and Rainbow Trout. After spending a variable portion of time utilizing the extensive resources in the pelagic environment, migratory individuals begin returning to their natal streams. On their way back home, migratory individuals undergo sexual maturation in preparation for spawning, and in so doing, begin the next generation.

Timing of the smoltification process is critical, as the length of time that smolts have to complete smoltification and migrate to the ocean is limited (the so called “smolt window”). Key to the timing of this process is the coordinated upregulation of several hormones shown in Fig. 3. Indeed, experimental modifications of these hormones results in a reduction in the number of individuals that undergo smoltification, and, for individuals that do smolt, reduced survival in seawater. Several of these hormones (such as growth hormone and prolactin) are peptide hormones that are synthesized via the processes of transcription and translation. Others, such as thyroid hormones and cortisol are either synthesized directly by the endocrine system, or are modified from steroids. Regardless of the chemistry of the hormone receptors are required for hormones to initiate their desired effect and these receptors are transcribed and translated from the genome. Therefore, there is a link between the genome and the action of hormones, and this link allows for adaptation at the genome level.

Due in part to these large-scale physiological changes migration is a costly process. Not only do these changes incur a significant energetic cost, but migratory individuals also suffer from increased predation and disease compared to residents. Therefore, migration must present sufficient benefit to overcome these costs. The underlying driving force behind migration is access to nutrient rich foods that are absent, or in limited supply, in many natal freshwater streams. The result of increased access to nutrients is abundantly clear when migratory salmonids return to spawn. Migratory individuals are larger and more fecund than residents and are better able to compete for access to breeding sites (redds). However, this size discrepancy between residents and migrants serves to vary the benefit of migrating between the sexes.

Sexual Maturation and the Decision to Migrate

In salmon and trout, undertaking a migratory life style requires individuals to delay their sexual maturation until they return from the pelagic environment. As all salmon and trout breed in freshwater, sexual maturation precludes migration (although note that there are examples of male salmonids (jacks) undergoing maturation in freshwater and then migrate to the ocean). The nutrient availability in the pelagic environment leads to sex bias in the propensity to migrate, with multiple studies reporting a strong female-bias in both the proportion of out-migrating smolts and in the proportion of returning spawning adults. Body size has a stronger influence on fecundity for female salmonids than it does for males. Larger females are able to devote more energy to producing eggs than are smaller (resident) females, whereas it is the number of matings a male can obtain that determines his reproductive success (Quinn *et al.*, 2011; Fleming, 1998). Therefore, in partially migrating populations, females receive more benefit to migrating than males. Further supporting this hypothesis, Ohms *et al.* (2014) found a consistent female-bias in out-migrating

Rainbow Trout from nine river catchments along the Pacific coast of the United States and Canada, with an average female proportion of out-migrating smolts of 63%. Similar results have been found in other partial migrant salmonids including Brook Trout (*Salvelinus fontinalis*; Theriault *et al.*, 2007), and Dolly Varden (*S. malma*; Koizumi *et al.*, 2006), suggesting this is a feature common to partially migratory salmonid populations. Interestingly, multiple studies in Rainbow Trout have not reported a significant male-bias in resident populations both above and below barriers (Sloat and Osterback (2013), Ohms *et al.* (2014); but see Rundio *et al.* (2012)). It is possible that many of the populations reported in Ohms *et al.* (2014) favored residency over anadromy, as populations with lots of anadromous individuals typically report a high proportion of male residency. It is important to note that different populations display partial migration differently, as the occurrence of such a process is influenced by population genetics (discussed below), environmental variance, and differences in demography.

Tied into the differences in the sex ratio of migrants and residents are the energetic demands of the process of sexual maturation. In most species, the timing of sexual maturation is not uniform (Taranger *et al.*, 2010). Sexual maturation is governed by the release of hormones from the brain that stimulate cellular development in the gonads. In salmonids sexual maturation occurs once individuals have reached a certain body size and have the energy resources necessary for reproduction, and, although multiple studies have documented the energetic requirements for sexual maturation, the exact size, lipid concentration, and physiological status that are required for the initiation of sexual maturation are, for the most part, unknown (Thorpe *et al.*, 1998).

The Genetic Basis of Migration

The cost-benefit tradeoff to migrating is a key reason why many salmonid populations exhibit partial migration. In these populations, whether an individual undergoes migration or remains resident depends on the interplay between the environment and genetics (Dodson *et al.*, 2013). Studies in Rainbow Trout have shown that the propensity to migrate is highly heritable (Thrower *et al.*, 2004; Hecht *et al.*, 2015). Although resident fish can produce migrants and migratory fish can produce residents, there is evidence to suggest that migrants produced by residents have lower seawater survival than migrants with migrant parents. Research attempting to find the genes associated with the development of the migratory phenotype suggests that (1) migration is a complex trait that involves polymorphisms across the genome (e.g., Hale *et al.* (2013); See Fig. 4), and (2) there appears to be population specific genetic effects. However, migration in salmonids is also influenced by environmental factors such as food availability in natal streams, density of conspecifics, elevation, water flow, and the latitude at which a given population is located (Narum *et al.*, 2011). Most high latitude streams have limited resources, causing an increased proportion of offspring to undergo smoltification and migrate; as seen in Atlantic Salmon (Valiente *et al.*, 2005) and Arctic Char (Jorgensen *et al.*, 2013). Clearly, the development of the migratory phenotype in salmonids is a classic example of a complex quantitative trait, with influences both from genetics and the environment.

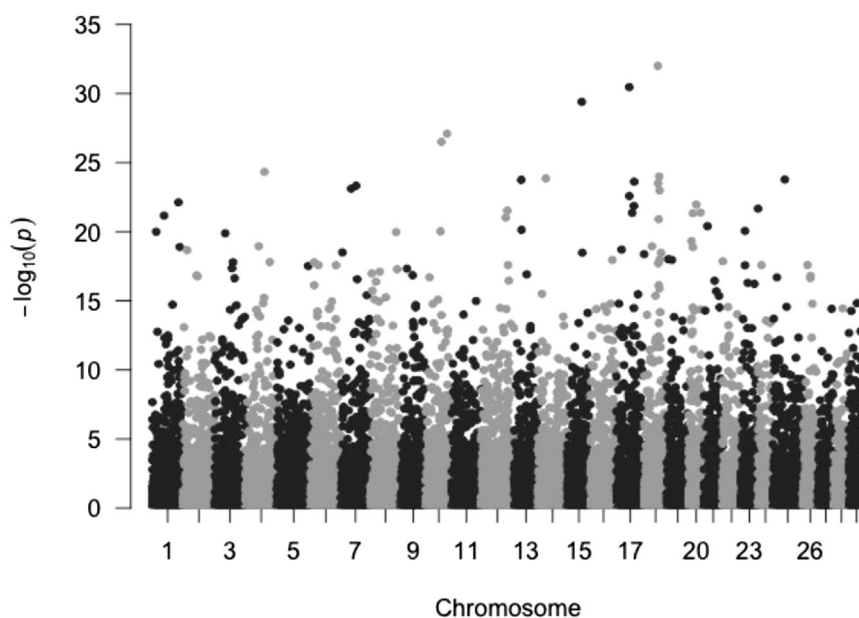


Fig. 4 GWAS plot showing association between migratory phenotype (resident or returning migrant) and 9990 polymorphisms scattered throughout the genome. Samples consist of 100 residents from Sashin Lake Alaska sampled in 2010 (56.365°N and -134.679°W) and 105 returning sexually mature adults sampled between 2006 and 2010 from Sashin Creek Alaska (56.379°N and -134.653°W). The Y-axis shows the $-\log_{10}$ of the P value (so higher values=smaller P-value). Chromosomes are alternatively colored to aid in comparisons. Modified from Hale, M.C., Thrower, F.P., Berntson, E.A., Miller, M.R., Nichols, K. M. 2013. Evaluating adaptive divergence between migratory and nonmigratory ecotypes of a Salmonid fish, *Oncorhynchus mykiss*. *G3-Genes Genomes Genetics* 3,1273–1285. Note that this figure was built by aligning sequences in Hale *et al.* (2013) to new version of the rainbow trout genome (as of June 2017).

This interplay between genotype and the environment can lead to extreme changes in the development of the different life histories from one year to the next. In some years environmental conditions may select the development of residents over migrants (e.g., Nichols *et al.*, 2008; Hale *et al.*, 2013). Such years would be characterized by abundant freshwater resources and limited competition from other fishes. On the other hand, the environment in these northern streams is often fickle, and in the following year the situation may reverse and there are limited resources in the same streams, and therefore many more migrants produced. While migration is known to be heritable, it appears that the underlying genetic basis for the development of migratory related traits is complex, and caused by either many genes of small effect or by other genetic changes. This last point is important for understanding the inheritance of migration, and potentially in using that information for conservation as many populations of anadromous salmonids in the lower 48 United States are declining. More fully understanding the genetic basis of migration could be a great boon to fisheries management in these regions as scientists could preferentially select individuals that are more likely to successfully migrate than individuals that have alleles associated with residency.

The Epigenetic Control of Migration in Rainbow Trout

Phenotypic plasticity describes the process that allows individuals to alter their phenotype in response to environmental change alter their phenotype due to new environments. Altering the regulation of gene expression is one mechanism that allows organisms to change the amount of mRNA produced in direct response to environmental variance. This change in gene expression allows for phenotypic plasticity, as changes in mRNA expression have a direct link to protein abundance. This ability to respond to environmental stimuli necessitates a molecular mechanism that permits RNA polymerase to change the amount of mRNA produced in the targets gene or genes. There are many different molecular mechanisms that can induce changes in gene expression, including histone modification, changes in DNA methylation, and changes in the production of noncoding RNAs. All of these changes can be grouped together as epigenetic modification, i.e., modifications that are passed on through successive generations but do not involve changes in the DNA sequence. Until recently, relatively little information was available regarding the effects of modifications on the epigenome and their potential influence on the development of different life history tactics. Baerwald *et al.* (2016) have investigated patterns of DNA methylation in both resident and migrant Rainbow Trout finding 57 regions of the genome with altered methylation patterns (so called differential methylated regions: DMRs) between the two phenotypes. The presence of these regions suggests that epigenetic changes in rainbow trout are associated with the development of different migratory phenotypes (Fig. 5).

However, it is important to note that not all individuals showed methylation patterns that were consistent with their migratory status. Three samples deviated from expected; two residents had methylation patterns characteristic of smolts, and one smolt possessed methylation patterns characteristics of residents. This suggests that there is epigenetic plasticity in the development of the migratory phenotype, which is not surprising given how variable patterns of gene expression are between migrants and residents (McKinney *et al.*, 2015; Hale *et al.*, 2016). More than 50% of the differentially methylated regions identified by Baerwald *et al.* were found within or next to CpG islands. CpG islands are regions of the genome rich in cytosine and guanine bases, are often found in or adjacent to promoter regions in the 5 prime untranslated region (UTR). These regions are extremely important in RNA polymerase binding and transcription initiation. Hypermethylation of cytosines in CpG islands is often associated with a decrease in the expression of downstream genes, as adding methyl groups to cytosine restricts access of the promoter region to RNA polymerase thereby decreasing transcription. Alternatively, hypomethylation of cytosine in CpG islands is more commonly associated with increased expression of down stream genes, as fewer cytosines are methylated. Baerwald *et al.* (2016) found evidence for hypermethylation of several genes in resident fish, including two genes connected to circadian rhythm signaling: aryl hydrocarbon receptor 2 alpha (*AHR2A*) and *RAS1*. Circadian rhythm pathways help organisms anticipate and prepare for changes in photoperiod, both with respect to daily changes in the light-dark cycle and to annual changes in photoperiod. Previous research has suggested that several circadian rhythm genes are connected to the smoltification process, including *CLOCK*, *BMAL*, and several Cryptochromes (McKinney *et al.*, 2015). Several of these genes are more expressed in the brains of migrants than they are in residents; Baerwald *et al.* found evidence for increased methylation in the promoter regions of *AHR2A* and *RAS1* in residents compared to migrants, providing a mechanism for increased circadian rhythm gene expression in migrants. The expression of circadian rhythm genes is important in the timing of outmigration, as many studies in salmonids have found that the window of time for leaving natal areas is narrow. As Baerwald *et al.* (2016) studied out migrating individuals, it is possible that these methylation changes provide a mechanism that allow migrants to be more sensitive to the time of year than residents.

Warm Water Adaptation in the Winter Skate

A commonly held viewpoint in evolutionary biology is an extension of Fisher's original evolutionary genetic theory: i.e., that in order for populations to be able to adapt to changes in the environment, the population in question must have considerable genetic variation. A natural consequence of this is that populations or species with reduced genetic variation will be less able to adapt to environmental change. This is especially concerning for species of conservation concern, as such species will typically have reduced genetic heterozygosity (due to population bottlenecks and low effective population sizes) and therefore, in theory, will be less able to adapt to changing environments. However, this does not mean that such populations or species are doomed to extirpation or extinction. Recent research has shown that changes in gene expression (i.e., being phenotypically plastic) can allow for rapid adaptation to novel environments. For example, studies in the Winter Skate (*Leucoraja ocellata*) have shown a link between changes in

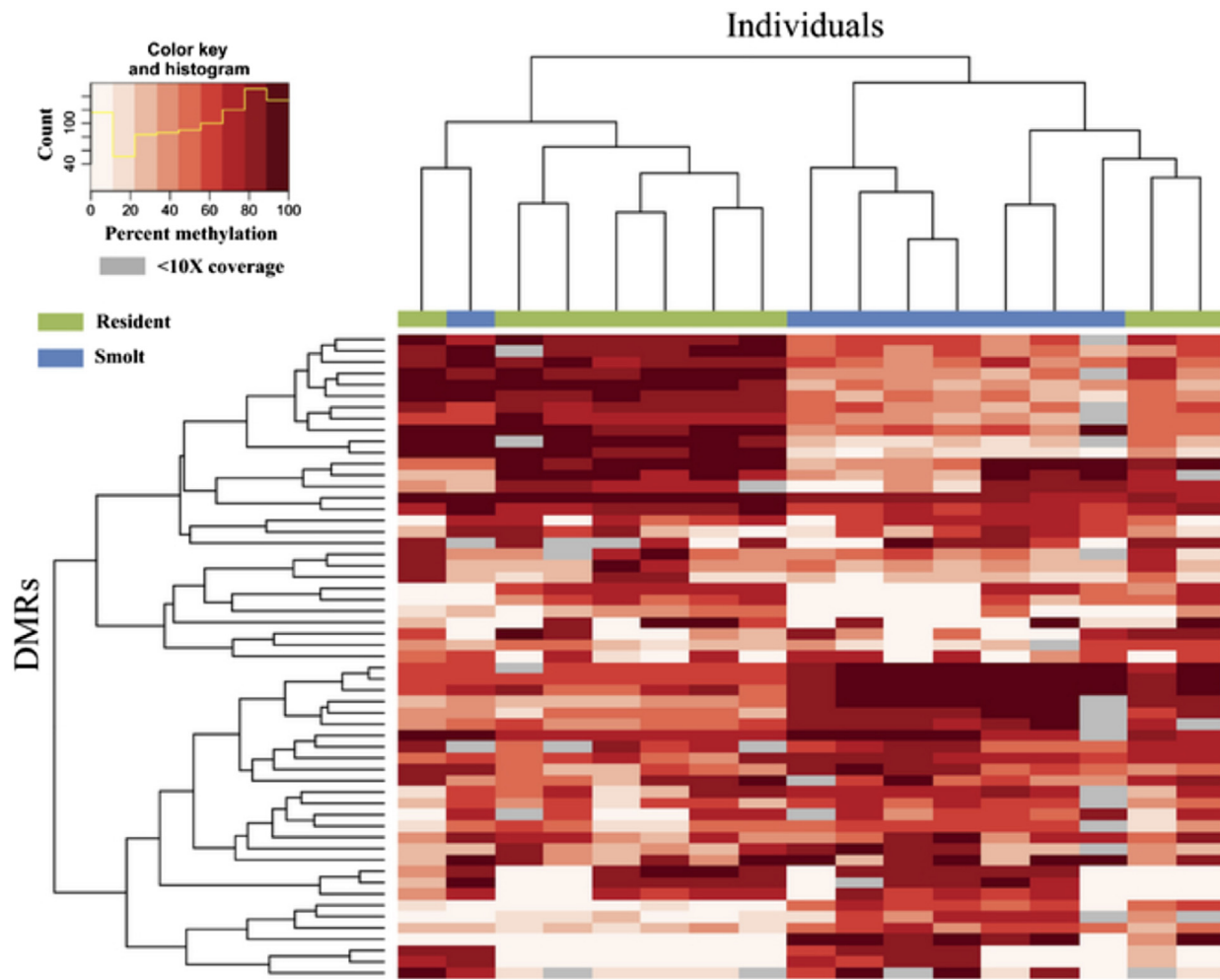


Fig. 5 Heatmap showing clustering of differentially methylated regions (DMRs) between smolts and residents. Each column represents an individual two-year old rainbow trout: Green columns represent residents, blue columns represent smolts. Each row represents a DMR that was statistically significant between phenotypes, the darker the red, the more methylated that individual is for that DMR. DMRs below a minimum read depth of 10 were excluded and are represented as grey boxes. Figure taken from Baerwald, M.R., Meek, M.H., Stephens, M.R., *et al.* 2015. Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. *Mol. Ecol.* 25, 1785–1800; see reference list for full citation). Permission granted for use was obtained from Wiley.

gene expression and exposure to increased water temperatures (10°C warmer than most of the species range), proving that some Winter Skate have been able to adapt to novel environments. Lighten *et al.* (2016) used RNA-seq technology in a comparative framework to determine patterns of gene expression between warm and cold water Winter Skates. They found substantial changes in gene expression between these two forms of Skate, confirming that changes in gene expression allow rapid adaptation to new environments. Although varying patterns of gene expression are well known to be linked to adaptation to novel (and in some cases, very different) environments in several species of fish (for example in stickleback and killifish), in very few cases have those changes in gene expression been linked to an underlying mechanism. While technological limitations have long made studying epigenetic changes difficult, recent advances in Next Generation Sequencing techniques are allowing researchers an increased understanding of the epigenome, and its involvement in adaptation and evolution.

Epigenetic Control of Sex Reversal in Fishes

As mentioned in the beginning of this article, one aspect of fish biology that sets them apart from other vertebrates is the multitude of sex determination systems that they employ. Broadly speaking, evolutionary biologists split these mechanisms into two groups: genetic sex determination (GSD) and environmental sex determination (ESD). However, a key feature of sex determination in fishes is the ability for some species to undergo environmental sex reversal (ESR). ESR describes the ability of the environment to override the individual's karyotype resulting in the development of the opposite sex. In other words, ESR produces XY females in systems

with male heterogamety and ZW males in systems with female heterogamety. The Half-smooth Tongue Sole (*Cynoglossus semilaevis*) is a species with female heterogamety, however, environmental effects such as temperature cause about 14% of genetic females (i.e., ZW) to be functional males. Evidence from other species of fish, such as the Sea Bass (*Dicentrarchus labrax*), also reports similar findings, with temperature fluctuations causing ESR.

While many species of fish are known to exhibit ESR, the mechanisms responsible for these sex reversals are poorly known. Research on Sea Bass strongly suggests that epigenetic modifications are at least partially connected to the process of ESR. More specifically, Navarro-Martin *et al.* (2011) found methylation differences in the promoter regions of *cyp19a1a*, a gene strongly connected to female development in fishes, leading to decreased in *cyp19a1a* expression and male development. Thus, epigenetic change, induced by environmental variance, could modify the expression of important genes in the molecular pathways connected to sexual development and result in the development of the opposite sex than encoded by the karyotype. Research on the Half-smooth Tongue Sole found similar results, with high water temperature (>22°C) decreasing methylation of *dmrt1* (doublesex and Mab3 related transcription factor; a well-known candidate gene in vertebrate sex determination), increasing its expression and causing the development of functional males with the ZW karyotype (pseudomales: Shao *et al.*, 2014). The importance of *dmrt1* (and its homologs) in male development has already been discussed. However, research from both Half-smooth Tongue Sole, and Medaka suggest that environmental conditions can override its methylation, modifying its expression, and resulting in ESR (Shao *et al.*, 2014; Matsuda *et al.*, 2002).

That environmental variance can cause epigenetic change has far-reaching implications for the survivability of a population and ultimately, a species. The long-term effects of ESR could be problematic for the persistence of a population if ESR biases the sex-ratio in maladaptive ways (Ospina-Alvarez and Piferrer, 2008). The effects of global climate change for species with either ESD or ESR will require animals to either adapt quickly to these new environmental conditions, or move to new areas that have not (yet) felt the consequences of climate change. This potentially puts such populations under considerable pressure. Evolutionary, ESR can also result in problems as it necessitates that pseudomales (or pseudofemales in a XY system) develop methods to combat the expression of genes that promote the development of the phenotype encoded by the karyotype, (i.e., female development in ZW systems or male development in XY systems). Such genes would presumably be of detriment for the homogametic sex, and therefore their expression would need to be controlled. ESR clearly shows how changes in epigenetics caused by variation in the environment can have a large effect on the phenotype of the affected individual. Like the previous examples in the Half-smooth Tongue Sole and Rainbow Trout, it is clear that epigenetic modifications can cause the development of different phenotypes. These phenotypes may not always be adaptive, and in such circumstances the longevity of a population or species might be in jeopardy.

Conclusions

Both genetic and epigenetic modifications are clearly important in the development of many life history characteristics in fishes. It has been known for some time that development of adaptive phenotypes is caused by changes in the genome (e.g., freshwater adaptation in sticklebacks: Colosimo *et al.*, 2005; Hohenlohe *et al.*, 2010). More recently, microarray and RNA-seq studies have reported that changes in gene expression are also linked to the control of many adaptive phenotypes. However, such studies (usually) do not provide a mechanism that allows for gene expression to change. Epigenetic modification directly interacts with the structure of the DNA, altering the configuration of the DNA around target genes, and therefore changing the ability of the gene to be transcribed. Here we provide some examples of phenotypic plasticity enabled by modifications of the epigenome that allow adaptive phenotypes to develop. However, we are just beginning to understand how important epigenetic modifications are in the development of adaptive traits. Recent technological advancement will facilitate the collection of both genome and epigenome data that can be used to look for links with the development of adaptive phenotypes. Certainly, the fishes offer an excellent opportunity for asking these questions making this an exciting time to be studying evolutionary biology in fishes.

References

- Baerwald, M. R., Meek, M. H., Stephens, M. R., *et al.* (2016). Migration-related phenotypic divergence is associated with epigenetic modifications in rainbow trout. *Molecular Ecology*, 25, 1785–1800.
- Berthelot, C., Brunet, F., Chalopin, D., *et al.* (2014). The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nature Communications*, 5.
- Colosimo, P. F., Hosemann, K. E., Balabhadra, S., *et al.* (2005). Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. *Science*, 307, 1928–1933.
- Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture*, 208, 191–364.
- Dickhoff, W. W., Beckman, B. R., Larsen, D. A., Duan, C., & Moriyama, S. (1997). The role of growth in endocrine regulation of salmon smoltification. *Fish Physiology and Biochemistry*, 17, 231–236.
- Dingle, H. (2006). Animal migration: Is there a common migratory syndrome? *Journal of Ornithology*, 147, 212–220.
- Dodson, J. J., Aubin-Horth, N., Theriault, V., & Paez, D. J. (2013). The evolutionary ecology of alternative migratory tactics in salmonid fishes. *Biological Reviews*, 88, 602–625.
- Fleming, I. A. (1998). Pattern and variability in the breeding system of Atlantic salmon (*Salmo salar*), with comparisons to other salmonids. *Canadian Journal of Fisheries and Aquatic Sciences*, 55, 59–76.
- Hale, M. C., McKinney, G. J., Thrower, F. P., & Nichols, K. M. (2016). RNA-seq reveals differential gene expression in the brains of juvenile resident and migratory smolt rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology D-Genomics & Proteomics*, 20, 136–150.

- Hale, M. C., Thrower, F. P., Berntson, E. A., Miller, M. R., & Nichols, K. M. (2013). Evaluating adaptive divergence between migratory and nonmigratory ecotypes of a salmonid fish, *Oncorhynchus mykiss*. *G3-Genes Genomes Genetics*, 3, 1273–1285.
- Hecht, B. C., Hard, J. J., Thrower, F. P., & Nichols, K. M. (2015). Quantitative genetics of migration-related traits in rainbow and steelhead trout. *G3-Genes Genomes Genetics*, 5, 873–889.
- Hoar, W. S. (1976). Smolt transformation – evolution, behavior, and physiology. *Journal of the Fisheries Research Board of Canada*, 33, 1233–1252.
- Hohenlohe, P. A., Bassham, S., Etter, P. D., et al. (2010). Population genomics of parallel adaptation in threespine stickleback using sequenced RAD tags. *Plos Genetics*, 6.
- Jorgensen, E. H., Martinsen, M., Strom, V., et al. (2013). Long-term fasting in the anadromous Arctic charr is associated with downregulation of metabolic enzyme activity and upregulation of leptin A1 and SOCS expression in the liver. *Journal of Experimental Biology*, 216, 3222–3230.
- Koizumi, I., Yamamoto, S., & Maekawa, K. (2006). Female-biased migration of stream-dwelling Dolly Varden in the Shiisorapuchi River, Hokkaido, Japan. *Journal of Fish Biology*, 68, 1513–1529.
- Liedvogel, M., Akesson, S., & Bensch, S. (2011). The genetics of migration on the move. *Trends in Ecology & Evolution*, 26, 561–569.
- Lien, S., Koop, B. F., Sandve, S. R., et al. (2016). The Atlantic salmon genome provides insights into rediploidization. *Nature*, 533, 200.
- Lighten, J., Incarnato, D., Ward, B. J., et al. (2016). Adaptive phenotypic response to climate enabled by epigenetics in a K-strategy species, the fish *Leucoraja ocellata* (Rajidae). *Royal Society Open Science*, 3.
- Matsuda, M., Nagahama, Y., Shinomiya, A., et al. (2002). DMY is a Y-specific DM-domain gene required for male development in the medaka fish. *Nature*, 417, 559–563.
- McCormick, S. D. (2009). Evolution of the hormonal control of animal performance: Insights from the seaward migration of salmon. *Integrative and Comparative Biology*, 49, 408–422.
- McKinney, G. J., Hale, M. C., Goetz, G., et al. (2015). Ontogenetic changes in embryonic and brain gene expression in progeny produced from migratory and resident *Oncorhynchus mykiss*. *Molecular Ecology*, 24, 1792–1809.
- Narum, S. R., Zedler, J. S., Frederiksen, C., et al. (2011). Candidate genetic markers associated with anadromy in *Oncorhynchus mykiss* of the Klickitat river. *Transactions of the American Fisheries Society*, 140, 843–854.
- Navarro-Martin, L., Vinas, J., Ribas, L., et al. (2011). DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the european sea bass. *Plos Genetics*, 7.
- Nichols, K. M., Edo, A. F., Wheeler, P. A., & Thorgaard, G. H. (2008). The genetic basis of smoltification-related traits in *Oncorhynchus mykiss*. *Genetics*, 179, 1559–1575.
- Ohms, H. A., Sloat, M. R., Reeves, G. H., Jordan, C. E., & Dunham, J. B. (2014). Influence of sex, migration distance, and latitude on life history expression in steelhead and rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences*, 71, 70–80.
- Ospina-Alvarez, N., & Piferrer, F. (2008). Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. *Plos One*, 3.
- Quinn, T. P., Seamons, T. R., Vollestad, L. A., & Duffy, E. (2011). Effects of growth and reproductive history on the egg size-fecundity trade-off in steelhead. *Transactions of the American Fisheries Society*, 140, 45–51.
- Rundio, D. E., Williams, T. H., Pearse, D. E., & Lindley, S. T. (2012). Male-biased sex ratio of nonanadromous *Oncorhynchus mykiss* in a partially migratory population in California. *Ecology of Freshwater Fish*, 21, 293–299.
- Shao, C. W., Li, Q. Y., Chen, S. L., et al. (2014). Epigenetic modification and inheritance in sexual reversal of fish. *Genome Research*, 24, 604–615.
- Sloat, M. R., & Osterback, A. M. K. (2013). Maximum stream temperature and the occurrence, abundance, and behavior of steelhead trout (*Oncorhynchus mykiss*) in a southern California stream. *Canadian Journal of Fisheries and Aquatic Sciences*, 70, 64–73.
- Taranger, G. L., Carrillo, M., Schulz, R. W., et al. (2010). Control of puberty in farmed fish. *General and Comparative Endocrinology*, 165, 483–515.
- Theriault, V., Bernatchez, L., & Dodson, J. J. (2007). Mating system and individual reproductive success of sympatric anadromous and resident brook charr, *Salvelinus fontinalis*, under natural conditions. *Behavioral Ecology and Sociobiology*, 62, 51–65.
- Thorpe, J. E., Mangel, M., Metcalfe, N. B., & Huntingford, F. A. (1998). Modelling the proximate basis of salmonid life-history variation, with application to Atlantic salmon, *Salmo salar* L. *Evolutionary Ecology*, 12, 581–599.
- Thrower, F. P., Hard, J. J., & Joyce, J. E. (2004). Genetic architecture of growth and early life-history transitions in anadromous and derived freshwater populations of steelhead. *Journal of Fish Biology*, 65, 286–307.
- Valiente, A. G., Juanes, F., & Garcia-Vazquez, E. (2005). Reproductive strategies explain genetic diversity in Atlantic salmon, *Salmo salar*. *Environmental Biology of Fishes*, 74, 323–334.

Genetics & Epigenetics in Life History and Reproduction: Oysters

Mackenzie Gavery and Steven Roberts, University of Washington, Seattle, WA, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Oysters represent a cosmopolitan molluscan taxa with a rich diversity of reproductive strategies that makes them a unique study system from a comparative physiology perspective. Oyster sex is greatly influenced by the environment and most species demonstrate varying degrees of hermaphroditism. In addition to having fascinating reproductive biology, oysters are ecologically and economically important marine invertebrates. They serve a critical ecological niche, serving as habitat and, as benthic filter feeders, contributing to the removal of excess organics, nutrients, and particulates. Additionally, several oysters species have gained culinary prominence, supporting fisheries and aquaculture industries. Commercial and recreational harvest of wild populations contributes to local economies and provides an important source of protein and nutrients. Aquaculture of bivalve species such as oysters has ancient origins, but has increased in recent decades. Today, shellfish aquaculture accounts for 76% of marine aquaculture production worldwide (FAO, 2012). The oyster that makes up the majority of the production and harvest worldwide is *Crassostrea gigas*, regionally referred to as the Pacific oyster (Fig. 1(a) and (d)). In addition to *Crassostrea*, there are several other families in the order *Ostreidae*, considered 'true oysters', including *Ostrea* and *Saccostrea*. *Pteroida* is another oyster order that represents the pearl oysters such as those in the genus *Pinctada*.

This article will cover what is known regarding sex determination and gametogenesis in oysters from a genetics and epigenetics perspective, with a focus on *Ostreidae*. Most of what is known comes from studies in *C. gigas*, as this species has received significant scientific attention given its high commodity value. This includes a genome sequence (Zhang *et al.*, 2012) that has contributed to understanding the molecular underpinnings of reproductive biology. Much of what is known about reproduction in oysters relates

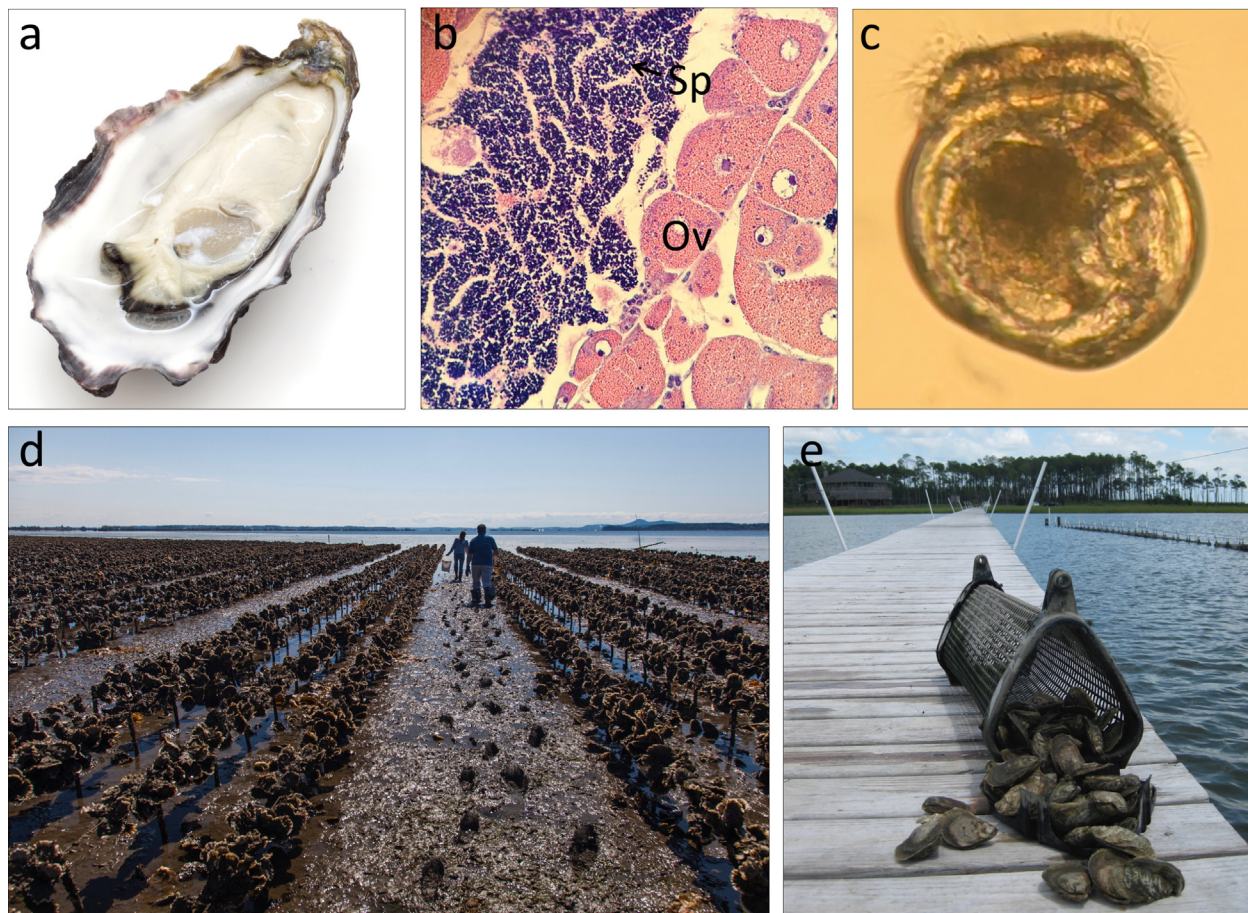


Fig. 1 Images of oyster life-stages and farming practices (a) adult *C. gigas*, (b) histological slide of a hermaphroditic *O. lurida* developing both male and female gametes, (c) *O. lurida* veliger larvae (planktonic stage), (d) *C. gigas* aquaculture in Washington, United States using long-lines, (e) *C. virginica* aquaculture in Mississippi, United States using off-bottom floating bags.

to aquaculture and the desire to control gonad development. Before getting into molecular aspects, we will first provide a survey of life history strategies, paying particular attention to reproduction. This article concludes with perspectives on the role of epigenetic processes in oyster reproduction and future directions of research that could provide a mechanistic connection between environmental conditions and reproduction.

Life History Strategies

As juveniles and adults, oysters are sessile, benthic organisms inhabiting intertidal and subtidal marine and estuarine environments. Oysters, like many marine invertebrates, exhibit an r-selected reproductive strategy, meaning they produce a high number of 'cheap' offspring, but relatively few survive to maturity. While juvenile and adult oysters are sessile (Fig. 1(a)), early developmental stages are planktonic (Fig. 1(c)). Interestingly, from a comparative physiology perspective, various oyster genera within the order *Ostreidae* take different approaches to larval investment.

Oysters of the genera *Crassostrea* and *Saccostrea* are gonochoristic, alternate hermaphrodites with low but consistent frequency of simultaneous hermaphroditism (Coe, 1936). Some species, such as *C. gigas*, are protandric, meaning they mature first as males then as females in subsequent seasons. However, recent studies have reported that *C. gigas* is capable of changing sex within a single spawning season (Yasuoka and Yusa, 2016). Oysters of these genera are broadcast spawners. Broadcast spawners release male and female gametes into the water column where external fertilization occurs. The larvae that develop from external fertilization are consequently free-living, and remain in the water column consuming primarily phytoplankton for a number of weeks, depending on the species and environmental conditions.

Alternatively, flat oysters of the genus *Ostrea* are viviparous and female oysters of this genus brood their larvae. Oysters in the genus *Ostrea* exhibit rhythmic consecutive hermaphroditism, where they can flip back and forth maturing as males then females and back again during a single spawning season (Coe, 1943). As may be expected with this strategy, histological observations of the gonad in *Ostrea* species tend to show both male and female stages (Fig. 1(b)) though they spawn only as a single sex at a time. Mature male *Ostrea* species will release sperm balls into the seawater enabling uptake into the mantle cavity of the female where fertilization occurs. Females will brood larvae in the mantle cavity for approximately 1–2 weeks depending on the species and environmental conditions, then release the shelled larvae into the water column where they will be planktonic for an additional 1–2 weeks. Compared to the broadcast spawners, brooding oysters produce relatively larger, but fewer number of eggs and sperm. Regardless of genus, larval oysters will end their planktonic stage once they are competent to settle on suitable substrate (such as the shells of conspecifics) and will metamorphose into juvenile oysters, sometimes referred to as 'spat', at which time they begin a sessile existence.

Sex Determination

Sex determination remains poorly understood in oysters as in other bivalves. The mechanisms underlying the high plasticity of sex in oysters, particularly in *Ostrea* species that alternate sexuality within a single season, remain a topic for future work. Nevertheless, there is evidence in *Crassostrea* species to support a combination of genetic sex determination (GSD) where sex is established at fertilization, and environmental sex determination (ESD) where sex is determined after fertilization in response to environmental cues. The following sections will describe what is known about genetic and environmental influences on sex determination in oysters, primarily in *C. gigas*.

Genetic Sex Determination

Heteromorphic sex chromosomes have not been identified in any oyster species examined to date. A vast majority of the evidence for a genetic basis for sex determination in oysters come from sex ratio studies, where the sex ratios of offspring from known parents is measured. The first evidence for GSD in oysters came from a study with controlled crosses in *C. virginica*, where significant differences in sex ratios were observed between families sired by the same male (Haley, 1977). Subsequently, Guo *et al.* (1998) extended these findings by evaluating sex ratios in pair-mated *C. gigas* families and also reported significant family and parental effects on sex ratio. Based on these results, Guo *et al.* (1998) proposed a single-locus model of sex determination in *C. gigas* with a dominant male allele (M) and a protandric female allele (F), such that FM oysters are true males and FF individuals are protandric females that have the potential to sex-change. This model was not sufficient to explain the heterogeneity in sex among families generated by a single male and different female parents, thus Hedrick and Hedgecock (2010) proposed a 3-genotype model with fixed females (FF) fixed males (MM) and protandric females (FM), and included additional parameter 'f' in the model, which is the probability that FM individuals will mature as females.

The models of sex determination in *C. gigas* were derived with no knowledge of the sex determining genes involved with this process. Support for a single major sex determining locus comes from a genetic analysis (i.e., quantitative trait loci, or QTL analysis) that identified a single locus moderately associated with sex in *C. gigas* (Hubert and Hedgecock, 2004). However, additional studies would be needed to rule out the possibility that other loci were also involved. Studies investigating genes involved in sex-determination are limited in bivalves, but have been recently facilitated by technological advances in the genomics field. In

general, the approach has been to identify and characterize orthologs of canonical sex determining genes in oysters to determine if the gene expression profiles are consistent with a role in sex determination. Initial studies were successful in identifying the presence of canonical sex determining genes in oysters including *Dmrt1* an important upstream regulator of male sex determination in mammals and *FoxL2* a conserved gene in female sex determination (Dheilly *et al.*, 2012; Teaniniuraitemoana *et al.*, 2014). However the expression patterns of these genes were not consistent with sex determining functions in oysters, meaning they were either expressed in the gonad of both males and females or in somatic tissues. The most comprehensive study to date comes from Zhang *et al.* (2014), who used a comparative approach to identify *C. gigas* homologs of major genes in sex determining pathway from both mammals and model invertebrates (*Drosophila melanogaster* and *Caenorhabditis elegans*). Here, they expanded the search of homologs in *C. gigas* to larger families of genes than had been previously investigated. They identified *Sox30* in *C. gigas*, a homolog to the mammalian sex determining *Sry* gene, which had a pattern of expression consistent with a sex determining function. They also identified *Dsx* (a transcription factor similar to *Dmrt1* that plays a role in male sex determination and differentiation mammals) and *FoxL2* (a transcription factor that plays a key role in female sex differentiation). Based on their findings, the authors proposed a model where *Sox30* is the major sex determining gene that is directly or indirectly linked with *Dsx* and *FoxL2*. This is the first time an *Sry*-like gene has been associated with sex determination outside of the vertebrates. Based on their findings (Zhang *et al.*, 2014), concluded oyster sex determination pathways are more similar to mammalian systems than invertebrates such as *D. melanogaster* and *C. elegans*. Additional work would be needed to follow up on this premise and also to determine how environmental factors influence sex determination.

Environmental Sex Determination

While there is clearly a genetic component of sex determination, genetics alone does not control sex in oysters, as various exogenous factors have been reported to impact sex ratios of oyster populations. Temperature has a significant effect on sex ratios in oysters. Typically high temperatures are associated with femaleness (Fabieux *et al.*, 2005; Lango-Reynoso *et al.*, 2006), but for some species, such as *C. corteziensis*, males are predominant at high temperature (Chávez-Villalba *et al.*, 2008). Food availability also has the ability to skew sex ratios in many bivalve species including *C. gigas*, where it has been shown that high food availability promotes the development of females (Lango-Reynoso, 1999). Exogenous steroids, such as 17beta-estradiol, have also been reported to skew sex ratios toward females in *C. gigas* when administered early (but not late) in development (Mori and Nakamura, 1969). Many of these studies examine the influence of exogenous factors on adult oyster sex ratios, but very few have studied the effect of environmental exposure at initial gametogenesis. An exception is a controlled laboratory study in *C. gigas* spat (i.e., recently metamorphosed juveniles) where sex-ratios switched from female to male-biased between 18 and 28°C (Santerre *et al.*, 2013). Interestingly, although *C. gigas* are reported to be protandrous (maturing as males first) more than half of the oysters in this study first matured as females under normal rearing temperatures, suggesting other factors, such as food abundance, may also be important for sex determination.

Gametogenesis

Just as with sex determination, environmental conditions influence gametogenesis in oysters. Temperature is a primary factor associated with gametogenesis but food availability and photoperiod are also important. Because temperature has such a strong influence on gametogenesis, it is not surprising that there is a large amount of variation in the timing and extent of reproductive output across geographic areas, and specifically latitudes (Berthelin *et al.*, 2000; Enríquez-Díaz *et al.*, 2009). Although there is variation among oyster species and even among populations within the same species, it is common for initiation of gametogenesis to occur in winter followed by an active phase of gametogenesis in spring when water temperatures increase. Maturation and spawning typically occur in summer followed by a resorption and resting phase. However, in addition to environmental factors, various aspects of oyster reproduction including reproductive timing and output have been shown to be under genetic control (e.g., Lannan *et al.*, 1980). Although a genetic effect has been identified, the role of genes and hormones continue to be explored. The remainder of this section will provide an overview of gonad development, followed by insight into gene and steroid hormone control of gametogenesis in oysters.

The gonad of oysters is a transient tissue. In *C. gigas*, the germline is specified by maternal cytoplasmic determinants (preformation) through the 4d cell lineage and larvae possess putative primordial germ cells (Fabieux *et al.*, 2004). Primordial germ cells will eventually give rise to mature male or female gametes, and when it comes to oysters, frequently both within the same individual. At the initiation of gametogenesis, small clusters of stem cells are scattered in conjunctive tissue. Then germ cells divide by mitosis and gonial proliferation induces the expansion of tubules that invaginate the area surrounding the digestive system. These developing gonadal tubules grow at the expense of storage tissue and at the final stage of maturation, prior to spawning, gonads completely fill the gonadal tubules. Gametogenesis is considered a period of negative energy balance in oysters due to the high metabolic cost of gamete production and a concomitant loss of up to 60% body weight (Mathieu and Lubet, 1993). As early as 1936, it was suggested that a connection exists between metabolism and reproduction in oysters (Orton, 1936). It is likely that the variation observed in the timing and extent of gametogenesis in oysters is influenced by the inherent ability of the oyster to store and metabolize glycogen. Molecular approaches have given insight into the role of energy storage and mobilization in the reproductive cycle in oysters. For example, expression of genes related to glycogen metabolism show seasonal variation in association with the gametogenic cycle

(Bacca *et al.*, 2005). Samain *et al.* (2007) reported significant differences in expression of glucose metabolism genes in oyster strains that vary in the extent of reproductive investment.

A seminal study by Dheilly *et al.* (2012) that examined transcriptome variation throughout reproductive development in *C. gigas* found over 2000 genes differentially expressed over the course of maturation in males and females, shedding light on genes involved both in early sex differentiation as well as gametogenesis. Using a microarray platform to study the gonadal maturation process in males (spermatogenesis) and females (oogenesis), Dheilly *et al.* (2012) identified a suite of genes indicative of these respective processes. A number of candidate genes for sex differentiation were identified in early stage gonad samples including *dpy1*, a histone methyltransferase essential for hermaphroditism in *C. elegans* (Hsu *et al.*, 1995) in early male gonads and *FoxL2* a highly conserved female specific transcription factor in early female gonads. In mature male gonads, a number of genes involved in ubiquitination and proteasomal degradation of ubiquitinated proteins were uniquely expressed. In females, there was evidence of elevated cell cycle activity in maturing gonads and interestingly *Methyl-CpG binding domain protein 2* (*MBD2*) was found to be highly expressed in mature female gonads. *MBD2* is involved in binding methylated DNA and thus presumably involved in gene regulatory activity. Dheilly *et al.* (2012) suggested the high expression of *MBD2* in developing oocytes may indicate an epigenetic transfer of information.

In vertebrates, sex steroid hormones are a critical part of reproductive physiology and regulate a variety of reproductive activities including sex differentiation, maturation and estrous cycles. However, unlike vertebrates, the role of sex steroids in sex differentiation is controversial in bivalves. Although findings can be contradictory, a majority of the evidence suggests that sex steroids are important regulators of bivalve reproduction involved in gametogenesis, vitellogenesis and spawning. All of the major sex steroid groups including estrogens, androgens and progestins have been identified in bivalves. Sex steroids may also play a role in regulating the metabolism of glycogen, proteins and lipids in bivalves. For example, injections of estradiol have been shown to stimulate glycogenolysis by regulating activities of glucose-6-phosphate dehydrogenase in *C. gigas* (e.g., Mori *et al.*, 1972). Vitellogenesis, an essential part of oocyte maturation in oysters, can also be regulated by estradiol (Matsumoto *et al.*, 1997). These results indicate the steroids may accelerate the metabolic rate in the gonad providing more energy to the maturing gametes.

Triploidy in Oysters for Aquaculture

In bivalves, as in most animals, nutrients and energy are required for two major physiological processes, reproduction and growth. By reducing the development of the gonad, more resources can be used for somatic growth. This has been exploited in oyster aquaculture through the use of non-reproductive triploid oysters. Triploidy is the state of an organism or cell having three sets of chromosomes in each nucleus, and differs from the natural diploid (two sets of chromosomes) state found in most animal cells. Triploid individuals are commonly sterile because homologous chromosomes cannot pair during meiosis. The benefits of triploidy in oysters is that reproduction is often accompanied by the deterioration in taste and quality of the flesh as glycogen stores are used up, thus triploid oysters can be harvested year round as quality does not decline during summer months when oysters would normally be sexually mature. It has also been shown that triploid oysters grow faster than their diploid counterparts (Allen and Downing, 1986).

Triploidy was first induced in oyster aquaculture in the early 1980s using chemical manipulation of fertilized eggs (Stanley *et al.*, 1981). Specifically, the compound cytochalasin B was used to chemically inhibit extrusion of the second polar body in fertilized eggs, resulting in the retention of two sets of maternal chromosomes and one set of paternal chromosomes. Currently, triploid oysters are produced through crossing tetraploid and diploid oysters. In this process haploid eggs from diploid females are fertilized by diploid sperm from tetraploid males. The first tetraploid oysters were generated in *C. gigas* and were obtained by inhibiting the first meiotic division of eggs from triploid females. Although a majority of triploid oysters are sterile, some individuals are capable of producing gametes that, under tight control, can be used to produce tetraploids. Triploid induction does not guarantee sterility and there are numerous reports of slow growing gonads and abnormal gametogenesis or gonads that are actually fertile. Temperature is an important factor here as well, as triploid oysters show higher rates of maturation under higher temperatures (Normand *et al.*, 2008).

Epigenetics in Oysters

The plasticity of functional sexual morphology coupled with the clear relationship between environmental conditions and gametogenesis suggests an intriguing role of epigenetics in oyster sexual differentiation and reproductive development. Epigenetics refers to heritable processes that alter gene activity without changes to the underlying DNA sequence (Jablonka and Lamb, 2002). In this respect, heritable could be used in a mitotic sense, such as heritable between cell divisions, and in some cases also in a meiotic sense meaning being passed to the next generation through the germ cells. Common epigenetic marks include DNA methylation, histone modifications and non-coding RNAs. All of these marks have been shown to have important roles in mediating processes involved in reproductive biology, though primarily in mammalian systems. One interesting exception is the work of Navarro-Martín *et al.* (2011) where they demonstrated DNA methylation of the aromatase promoter is mechanistically involved in temperature dependent sex determination in European sea bass. Specifically, elevated temperature was associated with increased methylation of the aromatase promoter, which silenced the expression of this key enzyme responsible for conversion of testosterone

to estrogen, thus resulting in masculinization. It has also been shown in the half-smooth tongue sole (*Cynoglossus semilaevis*), a fish that exhibits both GSD and ESD, that exposure to high temperature early in development induces a 'pseudomale' phenotype (sex reversed genetic female) that can be inherited at a high frequency in F1 offspring (Chen *et al.*, 2014). This phenotype is associated with an inherited pattern of DNA methylation on the sex chromosome derived from the pseudomale parent, implying a role for DNA methylation in maintaining this phenotype across generations (Shao *et al.*, 2014). These examples highlight ways in which DNA methylation may be playing a role in facilitating environmental responses to sex determination in species that, similar to oysters, exhibit a combination of GSD and ESD. The presumption remains that epigenetic processes are integral in the link between the environment and biological traits in oysters (Fig. 2), however, caution must be taken when extrapolating possible mechanisms to oysters as DNA methylation landscapes are strikingly different between vertebrates and invertebrates and thus functional roles could differ.

DNA methylation in oysters, similar to other invertebrates, is much lower than vertebrates. Whereas approximately 70%–80% of CG dinucleotides are methylated in vertebrate genomes (Bird and Taggart, 1980), only about 15% are methylated in oysters, where it is further limited in the genome to primarily gene bodies (Gavery and Roberts, 2013). Even considering this dramatic difference in pattern between vertebrates and invertebrates characterization of DNA methylation patterns in *C. gigas* indicate that this epigenetic mark has an important role in regulating gene expression (Gavery and Roberts, 2013). There have not been any studies carried out to look specifically at the regulatory role of DNA methylation in oyster reproduction, but some studies have assessed DNA methylation pattern in gametes. A study by Olson and Roberts (2015) showed that DNA methylation patterns in sperm between related individuals were more similar than unrelated individuals. A recent study by Riviere *et al.* (2017), found dynamic temporal DNA methylation patterns across development from oocytes to spat.

While most of the studies examining epigenetics in oysters have targeted DNA methylation, there have been efforts to describe miRNA and histone modifications, though none have been specifically designed to determine their role in sex determination or differentiation. Certainly one of the most intriguing aspects of environmentally induced epigenetic changes is the potential for epigenetic information to be transferred meiotically from parent to offspring via gametes. One example of how this may be accomplished is through the transmission of histone modifications in mature gametes. It has been shown in both mammals and zebrafish that during spermatogenesis, when most histones are replaced with protamines, certain modified histones are non-randomly retained and tend to mark important early embryonic development genes, suggesting that these marks may have a role transferring epigenetic information to the embryo (Brykczynska *et al.*, 2010; Wu *et al.*, 2011). Histones are not replaced with protamines in bivalve sperm as they are in mammals (Eirín-López and Ausió, 2009). Rather, canonical histones are replaced with various sperm nuclear basic proteins that can be classified as either protamine-like type, histone-type or protamine-type (Ausio, 1986; Eirín-López and Ausió, 2009). It will require additional work to determine to what extent canonical histones and variants are retained in bivalve sperm, but this avenue of research could be very valuable toward a greater understanding of the potential transmission of epigenetic information from parent to offspring in bivalves. Histone modifications themselves remain largely understudied in shellfish, but it has been reported that bulk histone methylation levels and the expression of histone demethylases were responsive to temperature during development, suggesting a role for histone modifications in mediating the physiological responses of oysters to temperature (Fellous *et al.*, 2015). Because of the importance of temperature in sex

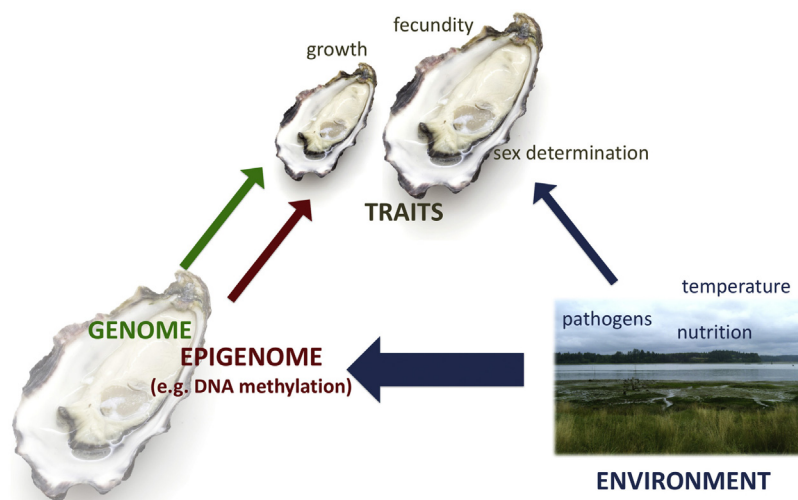


Fig. 2 An organism's genotype and the environment it encounters contribute to traits (phenotypes). Increasingly it's been shown that an organism's epigenetic information also contributes to phenotypes. Unlike the genome, the epigenome can be directly impacted by the environment, providing mechanisms to allow an organism to respond or adapt to external changes, and perhaps even transmit these induced traits to offspring.

determination and gametogenesis in oysters, additional studies on the role of histone modifications during these processes will be important.

Another important class of epigenetic marks that remain wholly uncharacterized in bivalve molluscs are regulatory microRNAs (miRNAs), though genes for miRNA biogenesis have been detected in available bivalves genomes (Rosani *et al.*, 2016). It has been shown that miRNAs exhibit sex-biased expression in the reproductive axis of vertebrates such as chicken (Bannister *et al.*, 2009) and Atlantic alibut (Bizuyeyu *et al.*, 2012). Furthermore, miRNAs have been shown to play an important role during gonadogenesis in insects (e.g., Jin and Xie, 2007). Given the increasing evidence for the essential role of miRNAs in reproduction, it is likely that characterization of miRNA expression patterns during gametogenesis in oysters will provide insight into regulatory mechanisms of this process.

An often underappreciated taxa, oysters offer a unique system to explore the intersection of genetics and the environment and their respective roles in reproductive biology. As described here, there are still significant gaps in knowledge with regard to mechanistic underpinnings of sex determination and gonad development. Given that oysters are found worldwide in susceptible ecosystems, coupled with an expanding suite of genomic resources, there will likely be a focus on the oyster to help define the role of epigenetic phenomena in mediating genotype by environment interactions.

References

- Allen, S. K., & Downing, S. L. (1986). Performance of triploid Pacific oysters, *Crassostrea gigas* (Thunberg). I. Survival, growth, glycogen content, and sexual maturation in yearlings. *J. Exp. Mar. Bio. Ecol.*, 102, 197–208.
- Ausio, J. (1986). Structural variability and compositional homology of the protamine-like components of the sperm from the bivalve molluscs. *Comp. Biochem. Physiol. B: Comp. Biochem.*, 85, 439–449.
- Bacca, H., Huvet, A., Fabioux, C., et al. (2005). Molecular cloning and seasonal expression of oyster glycogen phosphorylase and glycogen synthase genes. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, 140, 635–646.
- Bannister, S. C., Tizard, M. L. V., Doran, T. J., Sinclair, A. H., & Smith, C. A. (2009). Sexually dimorphic microRNA expression during chicken embryonic gonadal development. *Biol. Reprod.*, 81, 165–176.
- Berthelin, C., Kellner, K., & Mathieu, M. (2000). Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (West Coast of France). *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.*, 125, 359–369.
- Bird, A. P., & Taggart, M. H. (1980). Variable patterns of total DNA and rDNA methylation in animals. *Nucleic Acids Res.*, 8, 1485–1497.
- Bizuyeyu, T. T., Babiak, J., Norberg, B., et al. (2012). Sex-biased miRNA expression in Atlantic halibut (*Hippoglossus hippoglossus*) brain and gonads. *Sex Dev.*, 6, 257–266.
- Brykczynska, U., et al. (2010). Repressive and active histone methylation mark distinct promoters in human and mouse spermatozoa. *Nat. Struct. Mol. Biol.*, 17, 679–687.
- Chávez-Villalba, J., Hernández-Ibarra, A., López-Tapia, M. R., & Mazón-Suástegui, J. M. (2008). Prospective Culture of the Cortez Oyster *Crassostrea corteziensis* from Northwestern Mexico: Growth, gametogenic activity, and condition index. *J. Shellfish Res.*, 27, 711–720.
- Chen, S., et al. (2014). Whole-genome sequence of a flatfish provides insights into ZW sex chromosome evolution and adaptation to a benthic lifestyle. *Nat. Genet.*, 46, 253–260.
- Coe, W. R. (1936). Environment and sex in the oviparous oyster *ostrea virginica*. *Biol. Bull.*, 71, 353–359.
- Coe, W. R. (1943). Sexual Differentiation in Mollusks. I. Pelecypods. *Q. Rev. Biol.*, 18, 154–164.
- Dheilly, N. M., et al. (2012). Gametogenesis in the Pacific oyster *Crassostrea gigas*: A microarrays-based analysis identifies sex and stage specific genes. *PLOS ONE*, 7, e36353.
- Eirín-López, J. M., & Ausió, J. (2009). Origin and evolution of chromosomal sperm proteins. *Bioessays*, 31, 1062–1070.
- Enriquez-Díaz, M., Pouvreau, S., Chávez-Villalba, J., & Le Pennec, M. (2009). Gametogenesis, reproductive investment, and spawning behavior of the Pacific giant oyster *Crassostrea gigas*: Evidence of an environment-dependent strategy. *Aquac. Int.*, 17, 491.
- Fabioux, C., et al. (2004). Oyster vasa-like gene as a marker of the germline cell development in *Crassostrea gigas*. *Biochem. Biophys. Res. Commun.*, 320, 592–598.
- Fabioux, C., Huvet, A., Le Souchu, P., Le Pennec, M., & Pouvreau, S. (2005). Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. *Aquaculture*, 250, 458–470.
- FAO – Food and Agriculture Organization of the United Nations, Fisheries and Aquaculture Department, 2012. The state of world fisheries and aquaculture, Rome.
- Fellous, A., Favrel, P., & Riviere, G. (2015). Temperature influences histone methylation and mRNA expression of the JmJ-C histone-demethylase orthologues during the early development of the oyster *Crassostrea gigas*. *Mar. Genomics*, 19, 23–30.
- Gavery, M. R., & Roberts, S. B. (2013). Predominant intragenic methylation is associated with gene expression characteristics in a bivalve mollusc. *PeerJ*, 1, e215.
- Guo, X., Hedgecock, D., Hershberger, W. K., Cooper, K., & Allen, S. K. (1998). Genetic determinants of protandric sex in the Pacific oyster, *Crassostrea gigas* Thunberg. *Evolution*, 52, 394–402.
- Haley, L. E. (1977). Sex determination in the American oyster. *J. Hered.*, 68, 114–116.
- Hedrick, P. W., & Hedgecock, D. (2010). Sex determination: Genetic models for oysters. *J. Hered.*, 101, 602–611.
- Hsu, D. R., Chuang, P. T., & Meyer, B. J. (1995). DPY-30, a nuclear protein essential early in embryogenesis for *Caenorhabditis elegans* dosage compensation. *Development*, 121, 3323–3334.
- Hubert, S., & Hedgecock, D. (2004). Linkage maps of microsatellite DNA markers for the Pacific oyster *Crassostrea gigas*. *Genetics*, 168, 351–362.
- Jablonka, E., & Lamb, M. J. (2002). The changing concept of epigenetics. *Ann. N. Y. Acad. Sci.*, 981, 82–96.
- Jin, Z., & Xie, T. (2007). Dcr-1 maintains *Drosophila* ovarian stem cells. *Curr. Biol.*, 17, 539–544.
- Lannan, J. E., Robinson, A., & Breese, W. P. (1980). Broodstock management of *Crassostrea gigas*. *Aquaculture*, 21, 337–345.
- Lango-Reynoso, F., Chávez-Villalba, J., & Le Pennec, M. (2006). Reproductive patterns of the Pacific oyster *Crassostrea gigas* in France. *Invertebr. Reprod. Dev.*, 49, 41–50.
- Lango-Reynoso, F., Devauchelle, N., Le Pennec, M., & Hatt, P.-J. (1999). Elements of reproductive strategy in oysters, *Crassostrea gigas*, from the 'Rade de Brest', France. *Invertebr. Reprod. Dev.*, 36, 141–144.
- Mathieu, M., & Lubet, P. (1993). Storage tissue metabolism and reproduction in marine bivalves – A brief review. *Invertebr. Reprod. Dev.*, 23, 123–129.
- Matsumoto, T., Osada, M., Osawa, Y., & Mori, K. (1997). Gonadal estrogen profile and immunohistochemical localization of steroidogenic enzymes in the oyster and scallop during sexual maturation. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, 118, 811–817.
- Mori, K., et al. (1972). Effects of steroids on oyster: V. Acceleration of glycogenolysis in female *Crassostrea gigas* by estradiol-17h injection under natural conditions. *Bull. Jpn. Soc. Sci. Fish.*, 38, 1185–1189.
- Mori, K., Muramatsu, T., & Nakamura, Y. (1969). Effect of steroid on oyster III: Sex reversal from male to female in *Crassostrea gigas* by estradiol-17β. *Bull. Jap. Soc. Sci. Fish.*, 35, 1072–1076.
- Navarro-Martin, L., et al. (2011). DNA methylation of the gonadal aromatase (cyp19a) promoter is involved in temperature-dependent sex ratio shifts in the European sea bass. *PLOS Genet.*, 7, e1002447.

- Normand, J., Le Pennec, M., & Boudry, P. (2008). Comparative histological study of gametogenesis in diploid and triploid Pacific oysters (*Crassostrea gigas*) reared in an estuarine farming site in France during the 2003 heatwave. *Aquaculture*, 282, 124–129.
- Olson, C. E., & Roberts, S. B. (2015). Indication of family-specific DNA methylation patterns in developing oysters. *BioRxiv*, 012831. <https://doi.org/10.1101/012831>.
- Orton, J. H. (1936). Observations and experiments on sex-change in the European oyster, *Ostrea edulis* L. Part V. A simultaneous study of spawning in 1927 in two distinct geographical localities. *Mém. Mus. r. his. nat. Belg. Series 2*, 3, 997–1056.
- Riviere, G., et al. (2017). Dynamics of DNA methylomes underlie oyster development. *PLOS Genet.*, 13, e1006807.
- Rosani, U., Pallavicini, A., & Venier, P. (2016). The miRNA biogenesis in marine bivalves. *PeerJ*, 4, e1763.
- Samain, J. F., Dégremont, L., Soletchnik, P., et al. (2007). Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. *Aquaculture*, 268, 227–243.
- Santerre, C., et al. (2013). Oyster sex determination is influenced by temperature – First clues in spat during first gonadic differentiation and gametogenesis. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.*, 165, 61–69.
- Shao, C., et al. (2014). Epigenetic modification and inheritance in sexual reversal of fish. *Genome Res.*, 24, 604–615.
- Stanley, J. G., Allen, S. K., & Hidu, H. (1981). Polyploidy induced in the American oyster, *Crassostrea virginica*, with cytochalasin B. *Aquaculture*, 23, 1–10.
- Teaniniuraitemoana, V., et al. (2014). Gonad transcriptome analysis of pearl oyster *Pinctada margaritifera*: Identification of potential sex differentiation and sex determining genes. *BMC Genomics*, 15, 491.
- Wu, S.-F., Zhang, H., & Cairns, B. R. (2011). Genes for embryo development are packaged in blocks of multivalent chromatin in zebrafish sperm. *Genome Res.*, 21, 578–589.
- Yasuoka, N., & Yusa, Y. (2016). Effects of size and gregariousness on individual sex in a natural population of the Pacific oyster *Crassostrea gigas*. *J. Molluscan Stud.*, 82, 485–491.
- Zhang, G., et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature*, 490, 49–54.
- Zhang, N., Xu, F., & Guo, X. (2014). Genomic analysis of the Pacific oyster (*Crassostrea gigas*) reveals possible conservation of vertebrate sex determination in a mollusc. *G3*, 4, 2207–2217.

Nutrition and Reproduction in Fish

Helene Volkoff and Sydney London, Memorial University of Newfoundland, St. John's, NL, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Nutrition is crucial to the reproductive performance of all vertebrates, including fish. Successful reproduction requires adequate resources in order to sustain the high-energy demands for the production of gametes and reproductive behaviors. In most cases, negative energy balance and low food consumption inhibit the reproductive axis.

Fish are the largest and most diverse vertebrate group, yet very little is known about the interactions between nutrition and reproduction in fish and only a small number of studies have evaluated the effects of feeding on early and adult reproductive performance. Although species-specific variations occur, decreased food availability or nutrient-deficient diets in fish generally cause gonad regression, a decrease in spawning, and a decrease in the number of eggs produced by a female. In contrast, increased food availability promotes growth and larger body sizes, and causes earlier maturation and production of a higher number of eggs.

Life Stages in Fish Reproduction and Energy Demands

The fish reproductive cycle consists of a series of stages with the final purpose to obtain a progeny through the fertilization of eggs by spermatozoa (Fig. 1). The success of reproduction thus depends on the successful progression through each stage of the reproductive cycle, nutrition being crucial for some of these stages.

When immature (sexually quiescent) fish reach a given size, they undergo puberty. As they mature, they will begin producing gametes and exhibiting sexual behavior. In both males and females, the reproductive cycle involves two major phases; the phase of gonadal growth and development (gametogenesis), and the phase of maturation, which involves gamete release and spawning. After spawning, gonadal recrudescence (resting phase) may occur in some species.

External fertilization is common in fish. Most fish are oviparous, meaning that females will release their eggs into the environment to be fertilized. However, there are some species that are viviparous, where internal fertilization takes place, and developing eggs/embryos are retained in the maternal reproductive tract to produce live young.

Once mature, many fish exhibit seasonal changes in their reproductive cycle, which are dependent on annual variations in environmental factors. Spawning/mating usually occurs at specific periods of the year that coincide with optimal environmental conditions for the growth of the offspring. Depending on the species, these conditions can include food availability, water temperature and photoperiod among others.

During the maturation phase of the adult reproductive cycle, gonads develop until they reach a maximum size during the spawning season and produce gametes. Both the production and the release of gametes are under hormonal control (see below). During egg formation, females invest considerable energy to provide the eggs with nutrient stores (i.e. the yolk/vitellogenin). Some species, known as capital breeders, store energy prior to breeding, drawing on it later during reproduction, therefore reducing the extent to which reproductive success is dependent on environmental conditions at the time of breeding. Other species, known as income breeders, will feed during spawning, using the energy of food directly for reproduction. Salmonids, for example, are extreme capital breeders as they stop feeding before their upstream migration to the breeding grounds.

Whereas females seem to invest considerable energy in gametogenesis, males might use more energy in reproductive behavior. In several species, the courtship of females by the male is a complex and energetically costly behavior, involving swimming, parading, and fighting. Although most oviparous fish leave their eggs after spawning, some species invest energy in parental care by preparing nests and guarding the eggs, or by not feeding while carrying the eggs in their mouth (e.g. cichlids, some catfish). Viviparous species display parental care by offering protection, and in rare cases provide maternal nutrients (some poeciliids, some sharks), which are an energy investment for the female.

A variety of methods are available to assess reproductive condition in fishes, including examination of the gonads, measurement of sex steroid levels, and gonadal indices. The gonadosomatic index (GSI) is the relative size of the gonads as a proportion of total body mass. It is widely used as an indicator of reproductive stage as it increases with the maturation of the fish, being maximal during the peak period of maturity and declining shortly after spawning. Fecundity (number of eggs produced by fish expressed either in terms of eggs/spawn or eggs/body weight) is also used as a measure of the reproductive capacity of female fish.

Endocrine Control of Reproduction/Dual Endocrine Control of Reproduction and Metabolism/Feeding

In fish, as in other vertebrates, reproduction is coordinated by the hypothalamus-pituitary-gonadal (HPG) axis. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which regulates the synthesis and release of pituitary gonadotropins, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins act on the gonads to stimulate

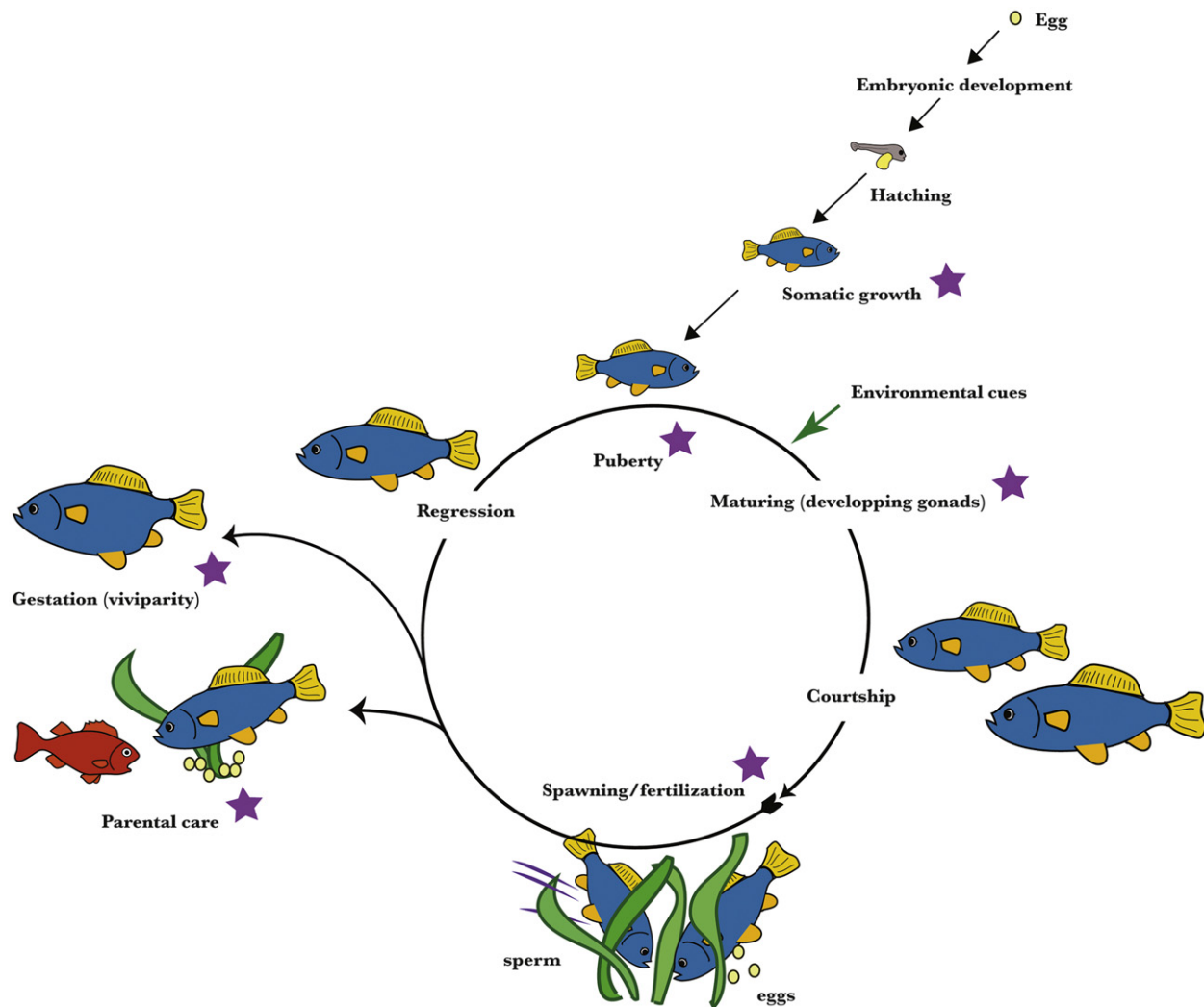


Fig. 1 Schematic representation of the life cycle of fish, and the major steps affected by nutrition (stars).

gonadal development through the secretion of sex steroid hormones. These steroids, in turn, feed back to the brain and the pituitary (Fig. 2). Feeding and reproduction are regulated by many endocrine factors produced by both the brain and peripheral organs (e.g. gastrointestinal tract).

The endocrine system plays an important role in regulating internal responses based on changes in the external environment. It also acts as a mediator between physiological systems, including feeding and reproduction. Animals often modulate their reproductive physiology according to available energy stocks. For example, food limitation, which depletes energy stocks, inhibits the HPG axis in order to save energy supplies for vital functions. In both mammals and fish, a number of peptides have been identified that regulate both feeding and reproduction. These include brain factors such as neuropeptide Y (NPY) and orexin, as well as peripheral factors such as leptin and ghrelin.

Effect of Nutrition on Fish Reproduction

Fish are often faced with reductions in food availability (and thus the ability to store energy) during their natural life cycle, in particular during seasonal cycles and spawning migrations. The sensory and endocrine systems of fish recognize when external and internal conditions, such as size, age, and storage of macromolecules (sugars, amino acids, and lipids), are optimal for reproduction. Reproductive development can be completed if conditions are optimal, but may be delayed/terminated under non-optimal conditions. In cases of "malnutrition", females often exhibit more serious reproductive problems (inhibition of vitellogenesis, oocyte maturation and spawning) than males (decreased sperm volume and diminished milt fluidity, which can affect negatively the success of egg fertilization). However, the mechanisms involved are not yet clear.

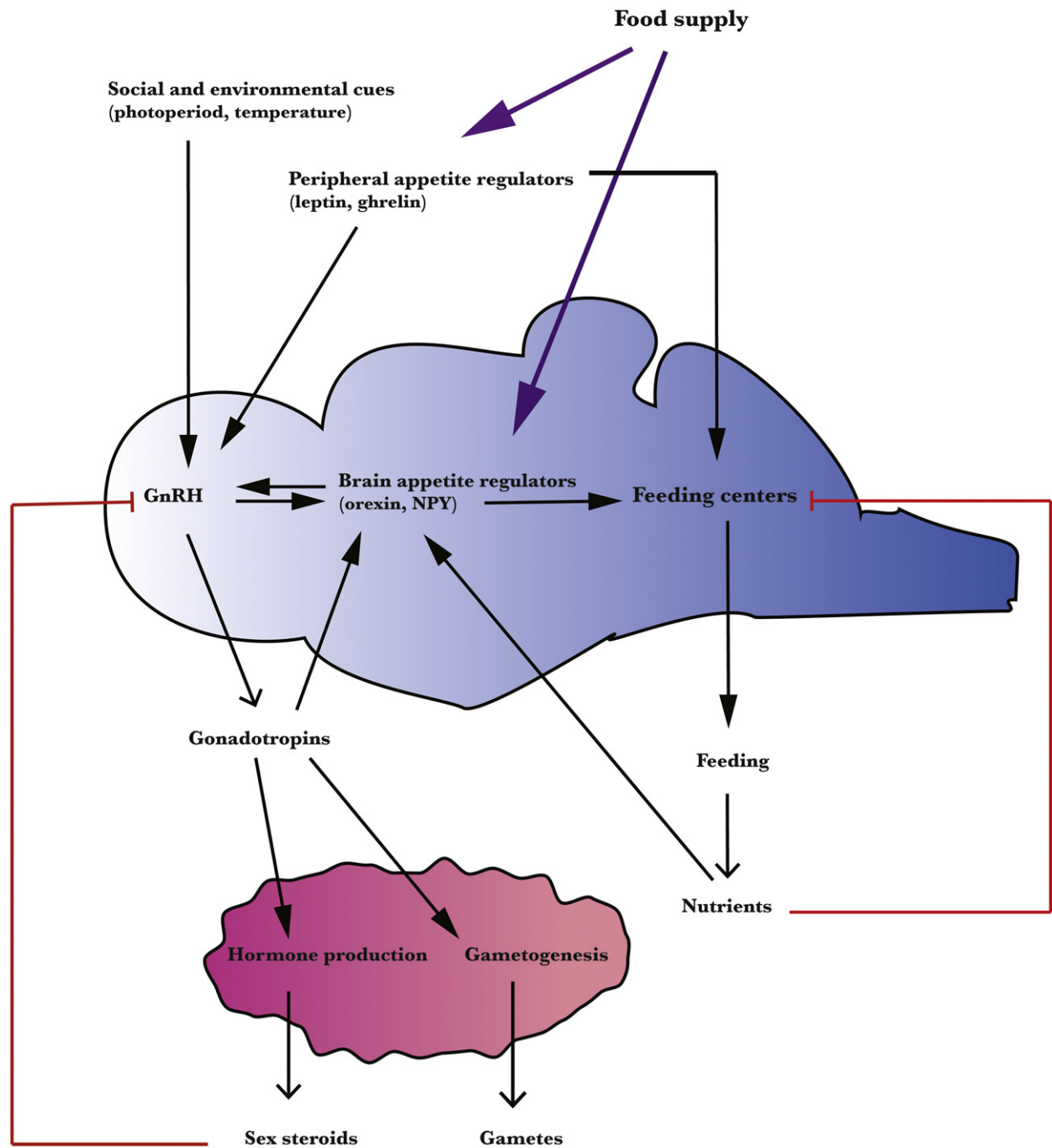


Fig. 2 Major interactions between the endocrine regulators of appetite and reproduction in fish, and influences of nutrients. Red lines indicate inhibition.

Effects of Food Restriction

There is a high variability in the reported effects of fasting on fish reproduction. This may be due to the large variation in both age and size at puberty in different fish species, and between males and females of the same species, with the growth in males typically slowing at a smaller size and age compared to females (e.g. sea bass, halibut). As in all vertebrates, puberty in fish occurs when individuals have reached optimal age and size, having accumulated sufficient energy reserves to meet the nutritional and energetic requirements of maturation. It has been suggested that the onset of puberty in fish is in part linked to absolute levels or rates of accumulation of lipid stores. Increased plasma sex steroid levels also usually accompany puberty.

The onset of puberty can initially have positive effects on appetite and somatic (body) growth, with larger individuals in a population undergoing puberty earlier than smaller individuals. However, as puberty progresses, energy is reallocated from

somatic growth to gametogenesis and reproduction-related behaviors, such as migration and/or sexual behavior. Appetite is often reduced just prior to and during large parts of the spawning season. Studies show that, in some species, restricted feeding reduces energy stores and adiposity, and can reduce the percentage of fish that complete maturation, thus delaying puberty.

In many species, reducing the feeding rates of mature fish during the reproductive cycle decreases growth, GSI, gonadal maturation, and spawning frequency (or spawning duration) during the reproductive season. There are also gender specific responses to food restriction, with females being more sensitive. Food-restricted females often experience decreases in final oocyte maturation and egg quality, and produce smaller eggs/hatched larvae compared to those fed full rations. In some species, fasting in females decreases brain GnRH and plasma estradiol levels. In immature female coho salmon, for example, prolonged fasting impairs endocrine function by reducing growth-related hormones (IGF-1), gonadotropins, and sex steroid levels. Therefore, it is evident that fasting inhibits the HPG axis, showing a clear relationship between food intake and reproduction.

Effect of Food Quality on Fish Reproduction

Lipids, proteins, and carbohydrates are nutrients metabolized by the body to produce the energy needed for various physiological processes and physical activities. In fish, there are considerable inter-species differences in the requirements of these nutrients and the ability to use them. These differences are associated with the natural feeding habits of fish, which can be classified as carnivorous (meat eaters), herbivorous (plant eaters), or omnivorous (those who eat both plants and meat). Carnivorous species use dietary protein and lipids very efficiently for energy but are not efficient in using dietary carbohydrates. Conversely, herbivores and omnivores can use carbohydrates and require less dietary protein than some carnivorous species. Vitamins (A, E, and C) and minerals are also important nutritional factors for fish (Table 1), and fish living in different environments have different dietary requirement (marine vs. freshwater fish). Regardless, nutrition is related to growth and can interfere directly or indirectly with several aspects of fish reproduction, including gonadal development, reproductive performance, spawning rate, fecundity, the quality and quantity of egg/sperm, as well as progeny quality.

Lipids

Lipids are hydrophobic compounds that include triglycerides (“fats”), phospholipids, and sterols. They are indispensable to growth and the maintenance of structural and cellular functions. Triglycerides are composed of fatty acids and are the primary means of energy storage in animals. Phospholipids, which also contain fatty acids, are the main constituents of cellular membranes, and sterols, such as cholesterol, are precursors for sex steroid hormones, which are responsible of the development of the gonads. In addition, fatty acids are precursors for prostaglandins, which stimulate gametogenesis and sexual behavior. Lipids are also essential for the intestinal absorption of liposoluble vitamins. Dietary lipids consist mainly of triglycerides and essential fatty acids (EFA), which cannot be synthesized by the body. In fish, lipids are mostly stored in the muscle and liver, and are the main source of energy for growth and reproduction. They are mobilized during gametogenesis and, in females, processed into a yolk precursor (vitellogenin) in the liver, and transferred to the ovaries to be incorporated as nutritive material in the egg/yolk, serving as the main source of food for the future embryo. Low levels of dietary lipids and fatty acids have been shown to negatively affect reproduction and offspring success in fish. Females fed a diet high in fatty acids usually have higher quality and number of viable eggs, leading to higher fertilization, hatching, and offspring survival rates. In males, high fat diets increase the production and release of sperm with higher motility and increase the levels of male sex steroid hormones. However, an excess of dietary lipids has also been reported to cause inadequate protein intake and suppress growth in some fish.

Table 1 Overall effects of nutrition-related treatments on reproductive parameters in fish

<i>Treatment</i>	<i>Puberty</i>	<i>Maturation of gonads/fertility</i>	<i>Gamete quality</i>	<i>Spawning</i>	<i>Offspring survival</i>
Fasting	↓	↓	↓	↓	↓
Lipid deficiency	↓	↓	↓	↓	↓
Protein deficiency	?	↓	↓	?	↓
Carbohydrate deficiency	✖	✖	✖	✖	✖
Vitamin E deficiency	↓	↓	↓	?	↓
Vitamin C deficiency	?	↓	↓	?	↓
Vitamin A deficiency	?	↓	↓	?	↓
Phosphorus deficiency	?	?	↓	?	↓
Calcium deficiency	?	?	↓	?	↓
Probiotics supplementation	?	↑	↑	?	↑

Note: ↓, negative effects; ↑, positive effects; ✖, no known effects; ?, effects uncertain or not examined.

Proteins

Although lipids are the primary source of energy in fish, dietary protein provides essential amino acids, which are critical for growth and development, as they are necessary for enzymatic function and the formation of muscle tissue and sexual products.

Well-balanced proteins diets usually increase the mean total weight and the number of eggs produced/released by females. Conversely, low protein diets have been shown to alter GnRH and gonadotropin release, leading to an increase in maturation time, and reductions in reproductive performance, oocyte maturation, ovulation, and in the number of eggs produced and their viability. These effects are particularly pronounced in carnivorous fish, such as seabream, seabass and catfish. However, in some species, high protein diets can induce low reproductive performance, fertilization and hatching rates. Herbivore species, like pacu and rohu, are particularly sensitive to high levels of proteins. In addition, in all fish, providing excessive levels of dietary protein can result in increases the excretion of nitrogenous wastes, leading to high levels of ammonia in the surrounding water, which can negatively affect food intake, growth, and therefore reproduction.

Carbohydrates

Carbohydrates (CHO) are composed of simple sugars (i.e. glucose, fructose, galactose) and are found mainly in plants (usually stored as starches or cellulose). Though starch can be enzymatically broken down, cellulose is indigestible to most animals, including fish. The ability of fish to utilize dietary carbohydrates for energy varies considerably, and most species have limited ability to metabolize them. Some carbohydrates are deposited in the form of glycogen in tissues such as liver and muscle, where they are accessible as a source of energy. Some dietary carbohydrates are converted to lipid and stored in the body for energy. Omnivorous and herbivorous fish can metabolize high quantities of carbohydrates whereas carnivorous species use it less efficiently. Overall, fish do not appear to have a specific dietary requirement for carbohydrates. Consequently, very low carbohydrate levels generally do not compromise reproductive performance, but reproduction can be affected as carbohydrate levels increase proportionally in the diet. Complications only arise when carbohydrate levels are high, but tolerability depends on the species' feeding habits with herbivores (e.g. pacu) tolerating the highest levels, followed by omnivores and carnivores (e.g. seabass).

Vitamins

Vitamins are necessary for the normal growth, metabolism, health, and reproduction of fish, though in small amounts. Important vitamins include water-soluble vitamins such as vitamins B and C, as well as fat-soluble vitamins such as vitamins A, D, and E. Vitamins are often not synthesized by fish, and deficiencies may result in reduced growth and impaired reproduction. There are considerable differences in vitamin requirements among fish species, which depend in particular on their diet and the morphology of their gastrointestinal tract. Within a species, demands can also vary with age, size, and the physiological state of the fish (e.g. physical health, growth rate, stress level). All these factors may interfere with the ability of the fish to absorb, transport, and metabolize dietary vitamins. The most commonly studied vitamins in fish are A, E, and C.

Studies show that vitamin E influences the quality of the gonads, fecundity, egg quality, embryonic development, percentage of fertilization, hatching, and survival of larvae in both herbivores/ omnivores and carnivores. Its deficiency results in immature gonads, low fecundity and fertility, and reductions in hatching rates and fry survival. Conversely, higher levels of dietary vitamin E increases the development of gonads, GSI, gonadal maturation, egg quality and viability, hatching rates, and percentage of normal larvae.

Vitamin C (ascorbic acid) affects ovarian development, steroidogenesis, vitellogenesis, and embryogenesis. Low dietary vitamin C levels induce decreases in the reproductive performance of females, by reducing egg numbers, inhibiting hatchability, and increasing the number of deformed larvae and their mortality. However, it appears that males are less sensitive as sperm motility is unaffected by low vitamin C levels. Embryos and juvenile fish, on the other hand, seem more affected by vitamin C deficiency than adults, likely because this vitamin is necessary for the synthesis of collagen during embryonic development and growth. In females, higher vitamin C concentrations in the diet improve egg quality and development, with the contents of the maternal diet reflected in the contents of the egg.

During oxidation reactions, which occur during normal essential metabolic processes in the body, toxic free radicals can be produced. These molecules are highly reactive and can cause significant cellular damage (through a process called oxidative stress). Vitamin C and E act as antioxidant agents by inhibiting oxidation and therefore reducing the number of free radicals. Dietary antioxidant requirements increase during reproduction, as free radicals are produced in steroid hormone biosynthesis. Accumulations of free radicals can deteriorate membranes and the membrane integrity of both sperm and eggs. The antioxidant function of vitamins C and E provide an important protection for the gamete cells. The synergistic effect of the supplementation of vitamins C and E in fish feed is seen through higher egg and semen quality.

Inclusion of vitamin A (or its precursors, the carotenoids) in fish diets increases gonadal development, fertility rate, fecundity, egg size, and egg quality in many species, although high levels may induce mortality at some embryonic stages. Vitamin A is important for embryo and larval development due to its important role in bone development, retina formation, and differentiation of immune cells. Most vitamin A is stored in the liver with increased concentrations being observed during gonad maturation, suggesting a higher requirement of vitamin A during gametogenesis.

There is little information available on the effects of vitamins B on fish reproduction. Thiamin (vitamin B₁) is important for embryo survival and larval development, pyridoxine (vitamin B₆) is important in the synthesis of steroid hormones, and folic acid (vitamin B₉) has a role in hatchability of eggs.

Minerals

Minerals are inorganic elements necessary for normal body functions. They can be divided into macro-minerals, which are present at larger levels in the body and required in larger amounts in the diet (e.g. sodium, chloride, potassium and phosphorous), and micro-minerals (trace minerals), which are required in small amounts as components of enzyme and hormone systems (e.g. copper, iron, chromium, iodine, zinc and selenium). Fish require the same minerals as terrestrial animals but they can absorb many minerals directly from the water through their gills and skin, allowing them to compensate to some extent for mineral deficiencies in their diet. Minerals required in the formation of embryos, such as phosphates and calcium, are obtained through the vitellogenin in yolk.

Phosphorus is the most critical macro-mineral in fish diets because there is little of it in water and its absorption is limited. Phosphorus is a major constituent of hard tissues such as bone and scales, and is also required for various biochemical processes. Impaired growth and feeding efficiency, as well as reduced tissue mineralization and impaired skeletal formation in juvenile fish are common symptoms of a phosphorous deficiency. Phosphorus-deficient females can experience reduced fecundity, and their progeny might display low egg hatchability and high larval deformity rates.

Calcium is important in cell signalling, including the activation of eggs. In order for the eggs to begin development, they must be activated by the sperm. Once “activated”, the membrane of the eggs will harden upon contact with water. It has been shown that both the water-hardening and activation processes are calcium-dependent.

Micro-mineral deficiencies in the diet apparently do not affect growth or reproductive processes in fish, but may have a negative effect if the deficiency continues for an extended period of time.

Probiotics

Probiotics are live microorganisms that, when ingested in adequate amounts, exert a health benefit on the host. In fish, a wide range of microalgae, yeasts, and bacteria have been shown to act as probiotics, mostly with benefits in digestion, growth, and immunity. However, in some fish species, positive effects of probiotics on reproduction have been demonstrated as well.

In zebrafish, probiotics induce earlier maturation in juveniles, and increase larval growth, bone calcification and development of the gonads. In a number of species, feeding mature females with probiotics increase gonadal growth (GSI), fecundity, production and viability of fry, as well as embryo survival and hatching rate of larvae. These effects might be mediated by changes in expression of genes such those encoding GnRH, leptin, and vitellogenin.

Summary and Conclusion

Fish are an incredibly diverse group, making it difficult to generalize the effects of nutrition on reproduction. However, a clear relationship between the two has been established and it appears that overall, both the quantity and the quality of food are crucial for a successful reproductive cycle in fish.

Further Reading

Bone, Q., Moore, R.H., 2008. *Biology of Fishes*. New York: Taylor & Francis.

Izquierdo, M.S., Fernandez-Palacios, H., Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. *Aquaculture* 197, 25–42.

Luquet, P., Watanabe, T., 1986. Interaction nutrition-reproduction in fish. *Fish Physiology and Biochemistry* 2, 121–129.

Nutrition and Reproduction, Birds

Tony D Williams, Simon Fraser University, Burnaby, BC, Canada

© 2018 Elsevier Inc. All rights reserved.

Introduction

Small birds must find and eat enough food each day to satisfy their nutritional requirements and to maintain energy balance, though larger birds might balance energy and nutrient requirements over several days. In theory, reproduction should impose very different demands in terms of energy and nutrient balance compared with the non-breeding period: parents must find not only more, and potentially different, food to meet their own elevated metabolic demands from courtship, mating, incubation and parental care but in many (altricial) species, they also have to obtain large amounts of additional food to feed their growing offspring. Food availability, and potentially diet quality, are therefore widely assumed to be critical determinants of variation in breeding phenology, i.e., when birds breed, reproductive investment (e.g., the number and size of eggs produced), and overall breeding success (the number and quality of chicks produced). In addition, nutrition might play a role in carry-over effects whereby food intake or diet quality on the wintering grounds, or during pre-breeding migration, can impact reproductive decisions after arrival on the breeding grounds. In fact, data supporting each of these 'intuitive' ideas are surprisingly equivocal.

Generalists, Specialists and Seasonal Variation in Diet

In relation to foraging and diet selection, a long-standing dichotomy has been between "generalists", that eat a diverse array of foods, and "specialists" that eat only a narrow range of available foods – though these represent a continuum (Sherry, 2016). It is also now recognised that Individuals within the same species can often have different dietary niche preferences even in the same environment, i.e., they can show individual diet specialization (IDS) due either to genetic differences (e.g., sexually-dimorphic morphological feeding adaptations) or because of variation in learned behaviors (Bolnick *et al.*, 2003). Most birds are generalists and omnivores, eating a variety of animal and plant matter, although some species are extreme diet specialists, for example, snail Kites (*Rostrhamus sociabilis*) feed almost exclusively on *Pomacea* snails, and flamingos (Phoenicopteridae) rely on filter feeding on brine shrimp and blue-green algae while breeding.

At temperate and higher latitudes, invertebrate prey abundance can be relatively low in winter, so birds rely more on plant diets during these non-breeding periods. However, diet varies seasonally in most species since reproduction imposes different demands in terms of energy and nutrient balance. Insect diets therefore predominate in spring and summer, during breeding, to provide chicks with energy- or protein-rich food to support growth and development (Fig. 1). Thus, although adult birds can utilise a wide variety of food types during the annual cycle, young birds (chicks) are almost invariably reared on protein-rich, animal diets, for example, in the granivorous house sparrow, *Passer domesticus*, adults consume 97% plant matter but nestlings consumed 31% plant matter and 68% animal matter (Welty, 1975). Only a few species do not show diet shifts, with increased use of insect prey, associated with reproduction. In the house finch (*Haemorrhous mexicanus*) 97% of the diet consists of seeds, buds, flowers, leaves, and fruits throughout the year, and American and European goldfinches feed their chicks almost exclusively on thistle seeds breeding relatively late, in mid-summer, when these seeds ripen. Similarly, zebra finches (*Taeniopygia guttata*) are granivorous year-round, although they feed their chicks sprouting seed, made available by seasonal onset of rainfall, which have specific amino acid composition suitable for egg-production (similar to an insect diet; Allen and Hume, 1997).

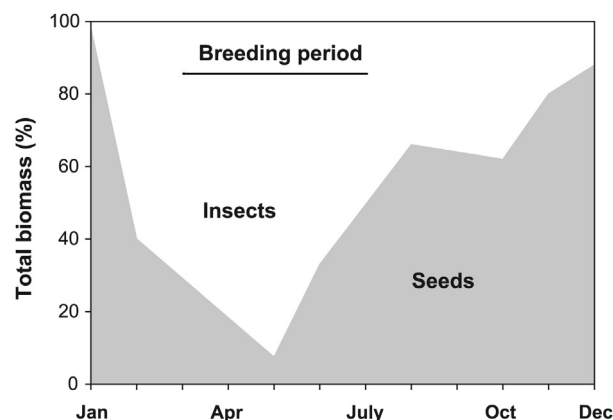


Fig. 1 Seasonal variation in the percentage of seeds and insects in the diet of Horned Larks (*Eremophila alpestris*) in relation to breeding. Redrawn from Rotenberry, J.T., 1980. Dietary relationships among shrubsteppe passerine birds: Competition or opportunism in a variable environment? Ecological Monographs 50, 93–110, with permission from John Wiley and Sons, Inc.

Few birds rear chicks exclusively with frugivory (i.e., a fruit diet), in part because few plants fruit in spring and early summer in temperate and higher latitudes when most birds breed. Year-round availability of fruit is more common in the tropics, but tropical frugivorous birds can also switch to insect diets to feed chicks, due to the low nutritional quality of many fruits (Poulin *et al.*, 1992). Some tropical species select specific fruit diet to meet the mineral and energetic demands of breeding, starting with calcium-rich fruits during egg-laying, use of lipid- and iron-rich fruits through breeding, and protein-rich fruits supplemented with invertebrates (up to 25%–30% of diet) for protein to feed young (Lamperti *et al.*, 2014). Again, a very few tropical species rely on plant diets year-round including when feeding their chicks, for example, oilbirds (*Steatornis caripensis*).

Some species show unique adaptations to avoid problems of variation in natural food availability, or even no food availability! Many species cache or store food to survive periods of low food availability, though typically food is stored in summer and used during the winter. However, grey jays (*Perisoreus canadensis*) rely on cached food during the reproductive period and use this stored food to nest much earlier than other birds (Sechley *et al.*, 2014), laying eggs between late February–March when little natural food is available (and often incubating eggs in temperatures below -20°F). Pigeons (Columbidae) and Emperor penguins (*Aptenodytes forsteri*) both feed their newly hatched chicks “crop milk” a secretion from the lining of the crop or oesophagus that is regurgitated to young birds. Male emperor penguins feed their newly-hatched chicks this protein-rich “curd” after fasting for 110 days, until their female returns to the nest with food.

Specific Nutritional Requirements for Reproduction

Adult birds must find and eat enough food each day, or over several days, to meet their own nutritional requirements for self-maintenance, and during reproduction parents in altricial species must also find enough food for their chicks, to support growth and development. All birds therefore require variable amounts of energy, which they can obtain from carbohydrates, lipids, or protein, as well as vitamins and minerals. Certain “essential” nutrients must also be obtained from the diet because birds cannot synthesise these in sufficient quantities to meet their requirements. Although nutrient requirements have been estimated from controlled, laboratory studies of captive birds nutritional requirements of free-living birds are poorly known, especially during breeding, with the majority of studies focusing on total energy requirements (reviewed in Williams, 2012).

Seasonal variation in diet (described above) is assumed to reflect the need to provide chicks with energy- or protein-rich food to support growth and development. However, specific amino acid requirements are unknown for the majority of wild birds. Certain essential amino acids such as phenylalanine and tyrosine, and the sulfur-rich amino acids (SAA) methionine and cysteine are thought to be important for synthesizing egg proteins during egg formation, and lysine and methionine are the first limiting amino acids for growth. However, Murphy (1994) suggested that most birds could probably meet these specific amino acid requirements without selective foraging as long as they consume enough total protein in their daily diet.

Birds require similar essential micronutrients to mammals, including vitamins (e.g., vitamins A, B, D, E and K) and minerals (calcium, sodium, magnesium). Tropical avian frugivores and nectarivores are thought to have particular difficulty in meeting their mineral needs since many fruits are quite deficient in individual minerals. Calcium is probably the most limiting micronutrient in egg-laying birds (Burley and Vadehra, 1989) required to produce the calcium-rich egg shell. In passerines, production of a single clutch of eggs requires more calcium than is in the bird's entire skeleton. Small birds do not store much calcium for reproduction (Pahl *et al.*, 1997) and rely on daily intake of calcium-rich food (particularly snails). Many species also feed their chicks bones from small mammals or fish or egg shells. Larger species, such as geese and shorebirds, store calcium for eggshell formation in medullary bone, a non-structural osseous tissue that serves as a temporary storage site for calcium (Squire *et al.*, 2017). Reproductive impairment, such as egg shell thinning, has been reported in birds breeding in areas with depleted soil calcium, for example, associated with acid rain (Graveland and Drent, 1997). Most birds consume grit and other hard particles which assist in grinding and digestion of hard food items in the stomach and gizzard (especially seeds or insects), and this might also contribute to mineral balance. In many birds the bright plumages (reds, yellows, and oranges) used in sexual displays and mate choice during breeding require carotenoid pigments that are also acquired solely from the diet; stored carotenoids do not appear to be an important source of feather pigments. In some species birds which ingest food with a higher concentration of carotenoid pigments have brighter ornamental plumage or integument (Simons *et al.*, 2014). However, there appears to be little direct evidence that lack of specific nutrients in bird's diets (e.g., vitamins, minerals) routinely cause reproductive failure, with the possible exception of calcium. Nevertheless, insect prey selected by birds and occurring at high frequency in diets have higher crude protein, total fat, and mineral concentrations than insect taxa not commonly taken as prey (e.g., Razeng and Watson, 2015), suggesting birds do select prey of high nutritional quality in general.

Experimental Studies of Nutrition and Reproduction

A seemingly straightforward way to test if food availability, or specific nutrition, limits reproduction is to provide free-living birds with supplemental food before or during egg-laying, or throughout breeding. If food is a limiting factor the prediction is that food supplementation will lead to earlier laying, larger egg or clutch size, or improved breeding success because birds will be released from some nutritional or energetic constraint. Results of a very large number of such ‘food supplementation’ experiments are surprisingly mixed (Nager, 2006): 61% of 57 studies reported earlier egg-laying in supplementary fed birds compared to controls.

However, only 38% of studies also found an increase in clutch size of supplemented birds and this effect was independent of the advance in laying date in only 14 studies (28%). Finally, only 42% of studies reported larger egg size of food-supplemented females compared with controls. A meta-analysis of all such studies (Ruffino *et al.*, 2014) indicated that the mean effect size of food supplementation was significantly positive for four reproductive parameters: laying date, clutch size, chick body mass and breeding success, but that food supplementation had no detectable effect on egg size, hatching success or brood size (Fig. 2).

Where supplemental food does have a positive effect on reproductive traits the magnitude of this effect is often quite small, and food effects are modulated by environmental factors, i.e., food supplementation has little effect on bird reproduction when the background level of food abundance in the environment is high. Food supplementation advances laying date in single-brooded species by ≤ 5 days but has a greater effect on multi-brooded species (c. 15 days; Drent, 2006). Similarly, increases in clutch size (mean 0.7 eggs) and egg size (+8.1%) are small relative to natural among-female variation in these traits, and food supplementation does not reduce the marked inter-individual variation in egg or clutch size, i.e., “small-egg” females do not lay large eggs simply with access to more or better food. Rather, traits such as egg and clutch size appear to be phenotypic components of individual ‘quality’ that are relatively unaffected by food availability. Thus, while the overall positive response to food supplementation supports the idea that birds might be constrained by food for some components of reproduction the relatively modest response to food suggests there must be other factors involved (see Williams, 2012; for more discussion).

The often contradictory, and limited, outcomes of supplemental feeding experiments could be due to several factors. First, experimental diets are chosen for experimenter convenience and do not necessarily reflect the nutritional quality or composition of the bird’s natural diets. Second, it has been suggested that *specific* resources or nutrients rather than gross energy or nutrient availability might be limiting. However, providing birds with specific foods, for example, protein- lipid- or calcium-rich food, also has inconsistent effects on reproduction. In the majority of supplemental feeding experiments birds are provided with additional food weeks, and sometimes months, before the start of egg-laying and in most studies an “enormous” amount of food is supplemented (Meijer and Drent, 1999). Long-term food supplementation could provide birds with a predictive cue that might actually lead to inappropriate or maladaptive reproductive decisions. For example, urban birds have access to greater food availability than rural conspecifics, due to human-provided food, and generally lay earlier but on average clutch size, nestling mass and productivity per nesting attempt are lower in urban landscapes (Chamberlain *et al.*, 2009). Similarly, chronic supplemental feeding of blue tits advanced laying date but *decreased* clutch size and breeding success (Harrison *et al.*, 2010). This suggests that although birds can be “tricked” (Verhulst and Nilsson, 2008) into laying earlier, the response to food supplementation might be maladaptive and does not reflect ecological, evolutionary or physiologically-relevant modulation of endogenous response mechanisms.

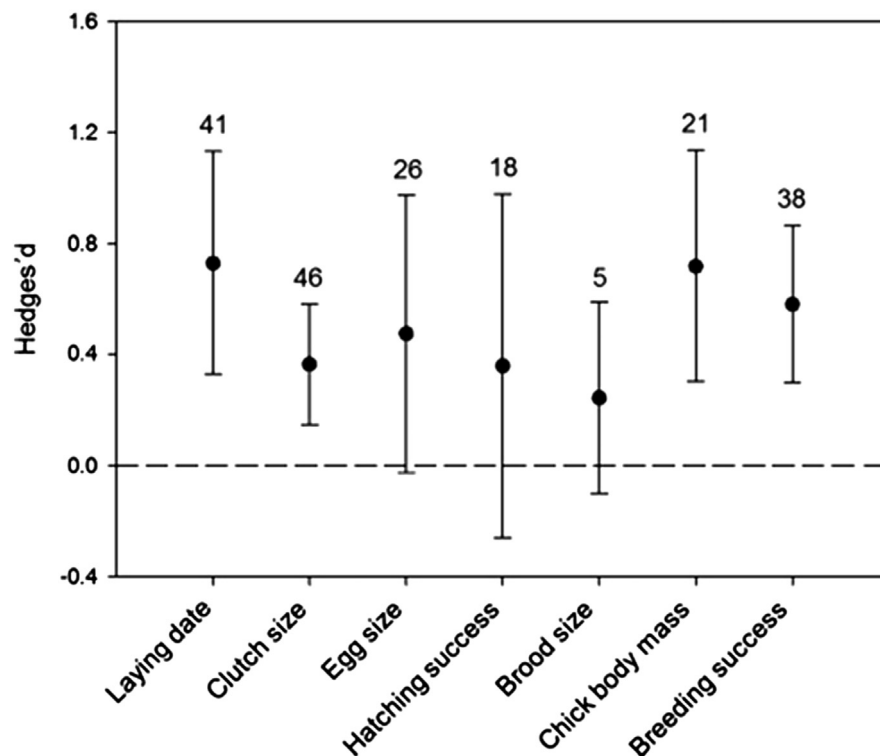


Fig. 2 Overall effects of food supplementation on reproductive parameters of birds. Mean effect sizes (Hedges' d) and 95% confidence intervals are presented, with sample size (number of experiments) above bars. Reproduced from Ruffino, L., Salo, P., Koivisto, E., Banks, P.B., Korpimäki, E., 2014. Reproductive responses of birds to experimental food supplementation: A meta-analysis. *Frontiers in Zoology* 11, 80, with permission from Springer International Publishing.

Direct supplemental feeding of chicks can be used to test the specific hypothesis that nutrition or food availability limits chick growth and development. In fact, in many studies food-supplemented chicks do not gain more mass than control chicks (except in naturally-poor foraging conditions). This is, most likely, because supplemental feeding decreases chick begging and parents reduce their own provisioning to food-supplemented chicks. In European starlings, supplemental feeding does increase body mass prior to fledging but food-supplemented chicks show a greater pre-fledging mass recession and fledged at the same mass and body size as control chicks (Fig. 3). Furthermore, supplemental feeding does not advance developmental maturity for physiological traits (haematocrit and haemoglobin) even though chicks fledge in an immature physiological state relative to adult values (Cornell *et al.*, 2017). The long-term effects of neonatal (chick) nutrition on adult reproduction have been little studied, especially in long-lived species. In general, chick mass at fledging is positively related to survival and recruitment in birds (Schwagmeyer and Mock, 2008). However, supplemental-fed kittiwake (*Rissa tridactyla*) chicks actually had lower recruitment than control chicks, and supplemental food had no clear effect on subsequent lifetime reproductive success of fed chicks (Vincenzi *et al.*, 2015).

Nutrition and Timing of Reproduction

In almost all species breeding is restricted to a particular time of year coincident with a seasonal increase in food availability required to support demands of egg formation, incubation, and growth and development of chicks (Williams, 2012). In general, individuals that time breeding such that they have their chicks in the nest when food is most available will breed more successfully, i.e., they will have higher fitness (Perrins, 1970). Many temperate-breeding species time their breeding season to coincide with seasonal increases in abundance of specific insect species, for example, great tits (*Parus major*) feeding on winter moth caterpillars (*Operophtera brumata*) or European starlings feeding on *Tipulid* larvae (Fig. 4). Given that birds which have their chicks in the nest when food is most available will have the highest breeding success or fitness, food availability sets a selective or evolutionary value on timing of breeding, i.e., food is the “ultimate factor” explaining *why* birds have evolved to breed when they do. However, breeding requires extensive physiological and behavioral preparation before onset of egg-laying itself, which can take 6–12 weeks (at least in males). Traditionally it has therefore been assumed that food cannot act as a proximate factor, which birds would use to predict timing of breeding weeks in advance (cf. day length) since seasonal increases in food do not start early enough. However, if ovarian/oviduct development does occur only a few days before onset of egg-laying (Fig. 3; Williams, 2012) this opens up the possibility that females do respond directly to cues associated with food availability. This would be consistent with Perrins (1970, 1996) suggestion that an individual female initiates egg-laying on the date on which she is able to find enough food to form eggs “without risk to herself” (Perrins, 1970). Thus, seasonal increases in food availability might lift a constraint allowing earlier timing of reproduction in individual females sufficient to support the demands of ovary and oviduct development and egg production. Nevertheless, the physiological mechanisms linking food availability, food intake and nutritional state to the reproductive axis remain virtually unknown (see below).

Climate change has caused well-documented, but complex, shifts in timing or phenology which can vary widely among different trophic levels (e.g., plants, insects, birds). Some birds are shifting their timing of breeding at different rates (faster or slower) than

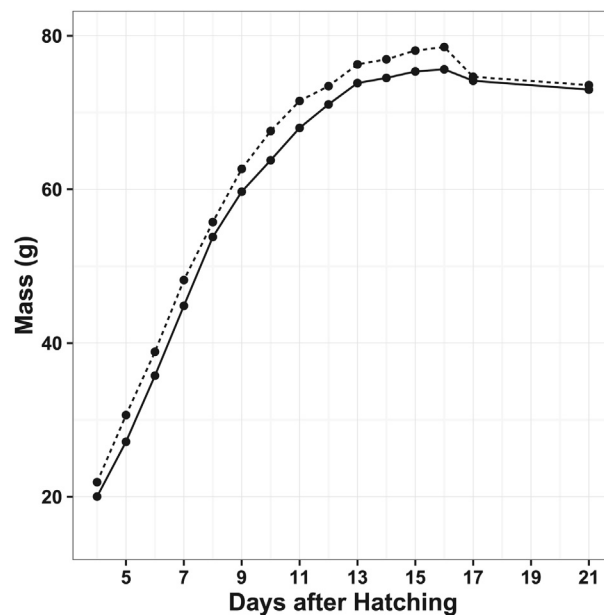


Fig. 3 Effects of food supplementation on change in body mass throughout the nestling period in European starling chicks. Chicks were not measured days 18–20. Data from Allison Cornell Pers. Comm.

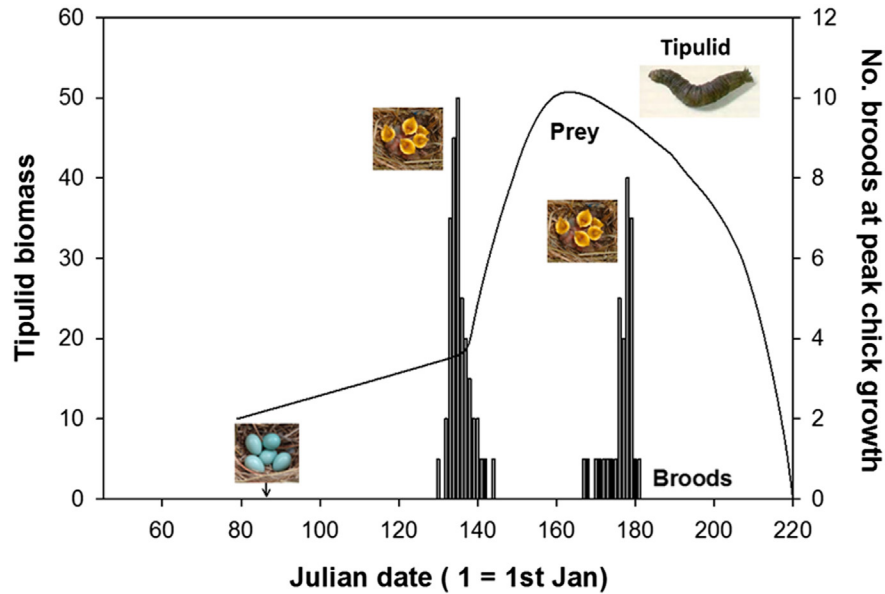


Fig. 4 Timing of egg-laying, first and second broods, in relation to predicted prey (Tipulid) availability in European starlings. Prey data based on Dunnet, G.M., 1955. The breeding of the Starling *Sturnus vulgaris* in relation to its food supply. Ibis 97, 619–661.

other species in their food chain (their prey or their predators). Where advances in timing of breeding in birds lag behind that of their insect prey this will lead to phenological mismatch, i.e., breeding will no longer coincide with peak food availability, which could affect fitness and population growth (Visser *et al.*, 2012; Fig. 5). The timing and duration of seasonal increases in natural food availability also selects for variation in the number of nesting attempts each breeding season, for example, European starlings can produce two but not three broods in a year (Fig. 3). In great tits, when the first clutch is laid early relative to peak caterpillar abundance then a greater proportion of birds produce second broods (Husby *et al.*, 2009). However, neither natural variation in prey biomass or chronic food supplementation throughout breeding affects probability of second broods. Thus, food might provide a cue for breeding decisions but data are equivocal in supporting the idea of nutritional constraints on the timing of breeding or the number of breeding attempts birds make.

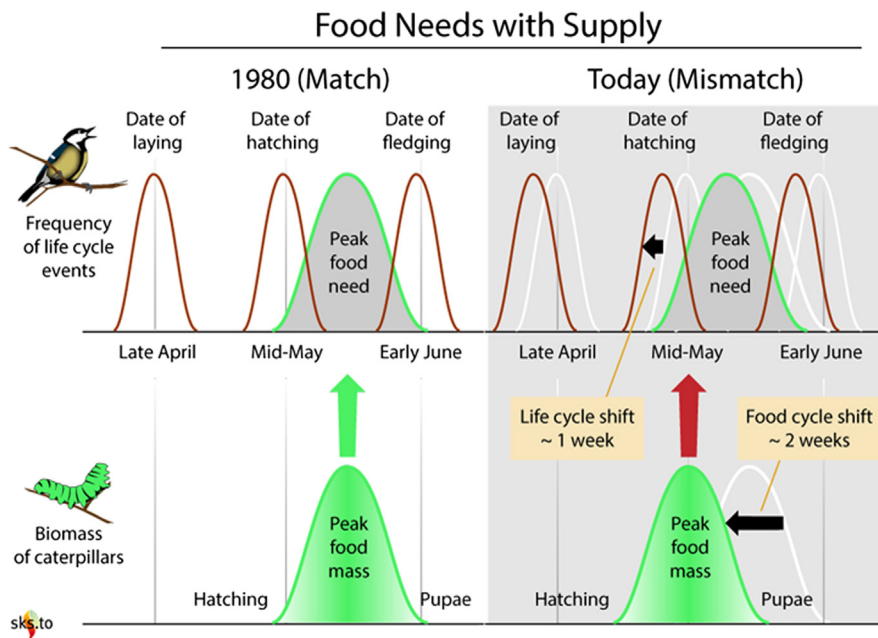


Fig. 5 Schematic illustration of match and mismatch between female great tits and their insect (caterpillar) prey. Image provided by Dr. Marcel Visser.

Nutrition and Reproductive Investment

It seems axiomatic that reproduction will require more resources than during non-breeding periods. Parents should need to find and consume more food to meet the elevated demands of their own reproductive activities (incubating eggs, foraging to obtain food for chicks) as well as finding additional food to provision chicks to support their growth and development. In general, birds might use different strategies to obtain resources for egg production and incubation. ‘Capital breeders’ rely on stored (endogenous) reserves accumulated prior to egg-laying and ‘income breeders’ rely on daily food intake (exogenous resources). It used to be assumed that “classic” capital breeders included migratory, Arctic-nesting shorebirds, ducks and geese, due to the short Arctic summer and limited availability of food early in the season when birds first arrive on their breeding grounds. However, even in Arctic-nesting common eider (*Somateria mollissima*) and snow geese (*Chen caerulescens*) up to 70%–80% of yolk proteins can be derived from exogenous resources, although endogenous fat reserves are more important for production of yolk lipids (~55%–80%; [Sénéchal et al., 2011](#); [Hobson et al., 2011](#)). Many smaller-bodied Arctic-nesting shorebirds also rely on exogenous resources, obtained via daily food intake, for egg production ([Klaassen et al., 2001](#)). In these species, endogenous stored reserves might be more important in allowing females to sustain incubation when there can be limited feeding opportunities. ‘Capital’ and ‘income’ therefore represent extremes on a continuum and most species likely use a mixed strategy – a balance of exogenous and endogenous resources – to meet demands of reproduction, depending on their life-history and ecological context. Most smaller-bodied birds (e.g., Passerines) and many larger-bodied birds (e.g., raptors, ducks) rely primarily on daily food intake to meet costs of egg production and incubation. During chick-rearing parents have fewer constraints on self-feeding, while finding food for their offspring, though how parents balance, and regulate, foraging for self- versus offspring remains largely unknown.

Larger-bodied breeders which rely at least to some extent on stored, endogenous reserves often produce large clutches of energy-rich eggs (e.g., waterfowl) and it has been suggested that food availability or nutrient reserves could be important in determining clutch size in ([Lack, 1967, 1968](#)). However, even for these species there is little unequivocal evidence for nutritional constraints on variation in egg or clutch size. Pre-breeding body mass or condition can be related to reproductive success in some capital-breeders, and some species (e.g., snow geese) will delay onset of egg-laying after arrival to accumulate more somatic reserves even at the expense of survival rate of juveniles ([Gauthier et al., 2003](#)). In altricial or income-breeding species, neither the number nor quality of eggs are directly related to the energetics or nutrition of the laying female. Indeed, there are good theoretical reasons why clutch size itself should *not* be nutrient-limited in species relying on daily food intake ([Arnold and Rohwer, 1991](#)). Rather, clutch size is ultimately determined by the average number of chicks for which parents can find food ([Lack, 1967, 1968](#)). Thus, in many birds, egg, clutch and brood size are likely determined not by food availability per se, but rather by the parent’s ability to forage to obtain food, and/or their physiological efficiency in converting food intake into eggs or chicks (see below).

Nutrition and Carry-Over Effects

Reproduction does not occur in isolation of other stages of bird’s life-cycles and several studies have linked feeding and nutrition on wintering areas or during pre-breeding migration to subsequent reproduction, i.e., there might be nutritional “carry-over” effects. Neo-tropical migrants using higher quality habitats in winter have higher body condition, earlier spring departure and earlier timing of arrival on their temperate-zone breeding grounds and it has been suggested this has positive effects on subsequent breeding performance. In migratory passerines and geese body condition (fat stores) during migration or at arrival on the breeding grounds can be correlated with timing of egg-laying, size and number of eggs laid, or overall reproductive success. Similarly, in seabirds earlier laying dates are correlated with pre-breeding diet quality or efficiency of foraging behaviour during the winter prior to laying ([Daunt et al., 2006](#); [Sorensen et al., 2009](#)). Nevertheless, studies where birds receive supplemental food in winter – perhaps mimicking the general public feeding garden birds – have produced mixed results, with positive, negative or no effects on subsequent reproduction (reviewed in [Crates et al., 2016](#)). A simple explanation for these results is that use of higher quality wintering or migratory habitat allows some individuals to maintain higher nutrient reserve levels which then contribute directly to reproduction. In other words, carry-over effects would reflect simple cycles of nutrient acquisition (during the non-breeding period) and utilisation (during breeding). However, few studies have directly confirmed that resource-acquisition, food availability or diet quality per se prior to breeding is what improves reproductive performance. Rather ‘high quality’ individuals might use higher quality wintering sites, occupy higher quality breeding sites, and also have higher breeding performance just by virtue of being ‘higher quality’.

Physiological and Hormonal Regulation of Food Intake and Feeding Behaviour

Mammalian studies have identified a wide range of hormonal signals that contribute to regulation of food intake, meal size, and feeding frequency, and that might explain concepts such as hunger and satiety. These include many “brain-gut” peptides, for example, ghrelin, glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) released by enteroendocrine cells throughout the gastrointestinal tract, and other peripheral hormones secreted by adipocytes (leptin, adiponectin) and the pancreas (insulin, glucagon). These signals are relayed to the hypothalamus or brain stem, either humorally or via paracrine effects on vagal nerve stimulation, and integrated by anorexigenic (appetite-inhibiting) and orexigenic (appetite-stimulating) neurons in the arcuate nucleus of the hypothalamus – the melanocortin system – to regulate food intake and energy balance. In contrast to mammals, very little work

has been conducted on any of these potential regulatory mechanisms, especially in non-domesticated, free-living birds, and in relation to reproduction. Most brain-gut peptides identified in mammals have been identified in birds and most have similar inhibitory or stimulatory effects on food intake based on experimental studies, though our knowledge of this comes almost entirely from work with poultry (reviewed in [Denbow and Cline, 2015](#)). Interestingly, some hormones appear to have opposite effects, for example, ghrelin is orexigenic in mammals but anorexigenic in birds. The neuroanatomical architecture underlying central regulation of food intake and energy expenditure, and in particular the hypothalamic melanocortin system, is also well conserved between birds and mammals, although much less is known about how this system functions in birds ([Denbow and Cline, 2015](#)). There have been virtually no studies of peripheral and central hormone signalling in the context of potential changes in food intake, nutrient and energy balance during seasonal gonadal maturation, egg production, and chick-rearing in free-living birds. Plasma leptin, which might function as a “lipostat” indicating nutrient or energy status, systematically varies in free-living birds in relation to egg-production ([Kordonowy et al., 2010](#)) and chick-rearing ([Quillfeldt et al., 2009](#)). However, to date other studies of brain-gut hormones that might be involved in regulation of food intake have been restricted to other energy-demanding phases of avian life-cycles, for example, migration ([Goymann et al., 2017](#)). It is important to note that chick-rearing requires that parents find more food, to feed their chicks, but this might not represent a period of elevated food intake or hyperphagia for the parent’s themselves. Nevertheless, hormonal, metabolic and physiological regulation of food intake and energy balance during reproduction in free-living birds represents a rich field for future research.

References

- Allen, L. R., & Hume, I. D. (1997). The importance of green seed in the nitrogen nutrition of the Zebra Finch *Taeniopygia guttata*. *Australian Journal of Ecology*, 22, 412–418.
- Arnold, T. W., & Rohwer, F. C. (1991). Do egg formation costs limit clutch size in waterfowl? A skeptical view. *Condor*, 93, 1032–1038.
- Bolnick, D. I., Svanback, R., Fordyce, J. A., et al. (2003). The ecology of individuals: Incidence and implications of individual specialization. *American Naturalist*, 161, 1–28.
- Burley, R. W., & Vadehra, D. V. (1989). *The Avian Egg: Chemistry and Biology*. New York, NY: John Wiley & Sons.
- Chamberlain, D. E., Cannon, A. R., Toms, M. P., et al. (2009). Avian productivity in urban landscapes: A review and meta-analysis. *Ibis*, 151, 1–18.
- Cornell, A., Gibson, K., & Williams, T. D. (2017). Physiological maturity at a critical life-history transition and post-fledging flight ability. *Functional Ecology*, 31, 662–670.
- Crates, R. A., Firth, J. A., Farine, D. R., et al. (2016). Individual variation in winter supplementary food consumption and its consequences for reproduction in wild birds. *Journal of Avian Biology*, 47, 678–689.
- Daunt, F., Afanasayev, V., Silk, J. R. D., & Wanless, S. (2006). Extrinsic and intrinsic determinants of winter foraging and breeding phenology in a temperate seabird. *Behavioral Ecology and Sociobiology*, 59, 381–388.
- Denbow, D. M., & Cline, M. A. (2015). Food intake regulation. In C. G. Scanes (Ed.), *Sturkie's Avian Physiology* (pp. 469–485). Amsterdam: Elsevier.
- Drent, R. (2006). The timing of birds' breeding seasons: The Perrin's hypothesis revisited especially for migrants. *Ardea*, 94, 305–322.
- Gauthier, G., Bety, J., & Hobson, K. A. (2003). Are greater snow geese capital breeders? New evidence from a stable-isotope model. *Ecology*, 84, 3250–3264.
- Goymann, W., Lupi, S., Kaiya, H., Cardinale, M., & Fusani, L. (2017). Ghrelin affects stopover decisions and food intake in a long-distance migrant. *Proceedings of the National Academy of Sciences*, 114, 1946–1951.
- Graveland, J., & Drent, R. H. (1997). Calcium availability limits breeding success of passerines on poor soils. *The Journal of Animal Ecology*, 66, 279–288.
- Harrison, T. J. E., Smith, J. A., Martin, G. R., et al. (2010). Does food supplementation really enhance productivity of breeding birds? *Oecologia*, 164, 311–320.
- Hobson, K. A., Sharp, C. M., Jefferies, R. L., Rockwell, R. F., & Abraham, K. F. (2011). Nutrient allocation strategies to eggs by lesser snow geese (*Chen caerulescens*) at a sub-arctic colony. *The Auk*, 128, 156–165.
- Husby, A., Kruuk, L. E. B., & Visser, M. E. (2009). Decline in the frequency and benefits of multiple brooding in great tits as a consequence of a changing environment. *Proceedings of the Royal Society B: Biological Sciences*, 276, 1845–1854.
- Klaassen, M., Lindstrom, A., Møtøfte, H., & Piersma, T. (2001). Arctic waders are not capital breeders. *Nature*, 413, 794.
- Kordonowy, L. L., McMurtry, J. P., & Williams, T. D. (2010). Variation in plasma leptin-like immunoreactivity in free-living European starlings (*Sturnus vulgaris*). *General & Comparative Endocrinology*, 166, 47–53.
- Lack, D. (1967). The significance of clutch size in waterfowl. *Wildfowl*, 18, 125–128.
- Lack, D. (1968). *Ecological Adaptations for Breeding in Birds*. London: Methuen & Co Ltd.
- Lampert, A. M., French, A. R., Dierenfeld, E. S., et al. (2014). Diet selection is related to breeding status in two frugivorous hornbill species of Central Africa. *Journal of Tropical Ecology*, 30, 273–290.
- Meijer, T., & Drent, R. (1999). Re-examination of the capital and income dichotomy in breeding birds. *Ibis*, 141, 399–414.
- Murphy, M. E. (1994). Amino acid compositions of avian eggs and tissues: Nutritional implications. *Journal of Avian Biology*, 25, 27–38.
- Nager, R. G. (2006). The challenges of making eggs. *Ardea*, 94, 323–346.
- Pahl, R., Winkler, D. W., Graveland, J., & Batterman, B. W. (1997). Songbirds do not create long-term stores of calcium in their legs prior to laying: Results from high-resolution radiography. *Proceedings of the Royal Society of London, Series B*, 264, 239–244.
- Perrins, C. M. (1970). The timing of bird's breeding seasons. *Ibis*, 112, 242–255.
- Perrins, C. M. (1996). Eggs, egg formation and the timing of breeding. *Ibis*, 138, 2–15.
- Poulin, B., Lefebvre, G., & McNeil, R. (1992). Tropical avian phenology in relation to abundance and exploitation of food resources. *Ecology*, 73, 2295–2309.
- Quillfeldt, P., Everaert, N., Buyse, J., Masello, J. F., & Dridi, S. (2009). Relationship between plasma leptin-like protein levels, begging and provisioning in nestling thin-billed prions *Pachyptila belcheri*. *General and Comparative Endocrinology*, 161, 171–178.
- Razeng, E., & Watson, D. M. (2015). Nutritional composition of the preferred prey of insectivorous birds: Popularity reflects quality. *Journal of Avian Biology*, 46, 89–96.
- Ruffino, L., Salo, P., Koivisto, E., Banks, P. B., & Korpimäki, E. (2014). Reproductive responses of birds to experimental food supplementation: A meta-analysis. *Frontiers in Zoology*, 11, 80.
- Schwagmeyer, P. L., & Mock, D. W. (2008). Parental provisioning and offspring fitness: Size matters. *Animal Behaviour*, 75, 291–298.
- Sechley, T. H., Strickland, D., & Norris, D. R. (2014). Causes and consequences of pre-laying weight gain in a food-caching bird that breeds in late winter. *Journal of Avian Biology*, 45, 85–93.
- Sénéchal, É., Bety, J., Gilchrist, H., Hobson, K., & Jamieson, S. (2011). Do purely capital layers exist among flying birds? Evidence of exogenous contribution to arctic-nesting common eider eggs. *Oecologia*, 165, 593–604.
- Sherry, T. W. (2016). Avian food and foraging. In I. J. Lovette, & J. W. Fitzpatrick (Eds.), *Handbook of Bird Biology* (pp. 265–310). Chichester: John Wiley & Sons Ltd.

- Simons, M. J. P., Briga, M., Leenknegt, B., & Verhulst, S. (2014). Context-dependent effects of carotenoid supplementation on reproduction in zebra finches. *Behavioral Ecology*, 25, 945–950.
- Sorensen, M. C., Hipfner, J. M., Kyser, T. K., & Norris, D. R. (2009). Carry-over effects in a Pacific seabird: Stable isotope evidence that pre-breeding diet quality influences reproductive success. *Journal of Animal Ecology*, 78, 460–467.
- Squire, M. E., Veglia, M. K., Drucker, K. A., et al. (2017). Estrogen levels influence medullary bone quantity and density in female house finches and pine siskins. *General and Comparative Endocrinology*, 246, 249–257.
- Verhulst, S., & Nilsson, J.-A. (2008). The timing of birds' breeding seasons: A review of experiments that manipulated timing of breeding. *Philosophical Transactions of the Royal Society London B*, 363, 399–410.
- Vincenzi, S., Hatch, S., Merkling, T., & Kitaysky, A. S. (2015). Carry-over effects of food supplementation on recruitment and breeding performance of long-lived seabirds. *282*(1812).
- Visser, M. E., te Marvelde, L., & Lof, M. E. (2012). Adaptive phenological mismatches of birds and their food in a warming world. *Journal of Ornithology*, 153(Suppl. 1), 75–84.
- Welty, J. C. (1975). *The Life of Birds* (second ed.). Philadelphia, PA: Saunders.
- Williams, T. D. (2012). *Physiological Adaptations for Breeding in Birds*. Princeton: Princeton University Press.

Further Reading

- Lovette, I. J., & Fitzpatrick, J. P. (2016). *The Cornell Lab of Ornithology Handbook of Bird Biology* (third ed.). Chichester: John Wiley and Sons Ltd.
- Scanes, C. G. (2015). *Sturkie's Avian Physiology* (sixth ed.). Amsterdam: Elsevier.

Torpor During Reproduction in Mammals and Birds: Balancing Energy Expenditure for Survival

BM McAllan, The University of Sydney, Sydney, NSW, Australia; and University of New England, Armidale, NSW, Australia
Fritz Geiser, University of New England, Armidale, NSW, Australia

© 2018 Elsevier Inc. All rights reserved.

Glossary

Heterothermy Condition where birds and mammals can change their body temperatures in a regulated manner.

Homeothermy Condition where birds and mammals maintain a single body temperature in a regulated manner.

Nomenclature

T_b Body temperature

Introduction

Mammalian reproduction and expression of torpor are often viewed as mutually incompatible processes. Reproduction typically requires acquisition of nutrients and high energy expenditure for mating behaviour and the production of young (Tyndale-Biscoe and Renfree, 1987), whereas torpor is characterised by a substantial reduction of energy expenditure by up to 90%–99% and also a fall body temperature (T_b) to between 0 and 25°C, depending on the species. Animals usually remain torpid between a few hours up to about 6 weeks (Ruf and Geiser, 2015). Birds and mammals that use torpor or hibernation and change T_b over time are called ‘heterotherms’ in contrast to ‘homeothermic’ species that maintain a constant high T_b .

To avoid the energetic incompatibility many species use a sequence of torpor followed by reproduction during the yearly cycle. In typical heterothermic rodents from the Northern Hemisphere torpor is expressed from autumn to early spring, after which reproduction commences in spring and summer (Geiser *et al.*, 2008). This pattern is especially observed in cricetid (hamsters) and sciurid (ground squirrel family) rodents (Fig. 1), which live in strongly seasonal environments with highly productive summers. These species refuse to express torpor during the reproduction season, reduce their gonads during the torpor season and even may remain homeothermic when reproductive hormones are administered.

Interestingly, not all species conform to this dogma. Torpor during the reproductive season has been observed during both pregnancy and lactation in many mammals, and also during incubation and brooding in birds (Brigham *et al.*, 2012; McKechnie and Mzilikazi, 2011; McAllan and Geiser, 2014; Geiser *et al.*, 2017). This is especially true for small mammals from the Australian arid zone, who seem to not only use torpor during reproduction, but to enable reproduction on limited resources (McAllan and Geiser, 2014). We aim to summarise available information about those mammals and birds that enter torpor during reproduction and examine the taxonomic diversity of the phenomenon.

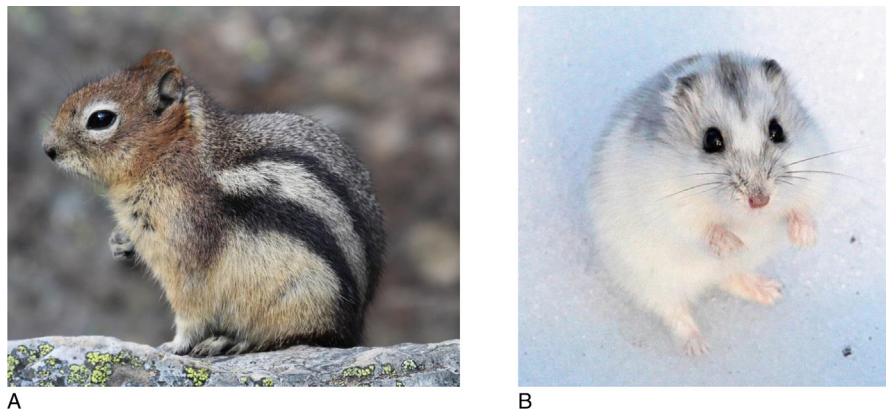


Fig. 1 Examples of mammals that use torpor or hibernation, however the golden-mantled ground squirrel and the Djungarian hamster do not express torpor during the reproductive season. (A) Golden-mantled ground-squirrel *Callospermophilus lateralis* (Sciuridae); (B) The Djungarian hamster *Phodopus sungorus* (Cricetidae). Photos are by Fritz Geiser and cannot be reproduced without permission of Fritz Geiser.

Torpor and Reproduction in Heterothermic Birds

Birds from at least nine orders are known to be heterothermic (McKechnie and Mzilikazi, 2011; Brigham *et al.*, 2012). These include barbet relatives, kingfishers, mousebirds, swifts, hummingbirds (Fig. 2(A)), nightjar relatives, owls, pigeons and songbirds. Although heterothermy is less common in non-reproductive birds than for similarly-sized mammals (Ruf and Geiser, 2015), some use torpor while incubating eggs. For example Andean broad-tailed hummingbirds, *Selasphorus platycercus*, incubate eggs with a T_b as low as 6.5°C and in this species torpor may be as pronounced as in non-reproductive birds.

There is only one known avian hibernator, the poorwill, *Phalaenoptilus nuttallii* (Brigham *et al.*, 2012). Like many mammalian hibernators it can enter torpor for several days and drop their T_b to 5°C (Brigham *et al.*, 2012). When reproductive, few individuals reluctantly become torpid and with a cost to reproductive output (Brigham *et al.*, 2012).

Torpor and Reproduction in the Monotremes (Egg-Laying Mammals)

Echidnas (*Tachyglossus aculeatus*, Fig. 2(B)) will hibernate when non-reproductive in many areas of Australia (Morrow and Nicol, 2009). The echidnas, although having a distinct seasonal biology, also have overlapping hibernation and reproductive seasons (Morrow and Nicol, 2009). In Tasmania reproductively active males hibernate from mid-February to mid-June, while reproductively active females hibernate from early March until mid-July. Reproductive males have been found with torpid females, or with females that re-entered hibernation after mating (Morrow and Nicol, 2009). Pregnant, torpid, recently mated females also have been observed in the wild (Morrow and Nicol, 2009). Unlike other hibernators, male echidnas have enlarged testes during hibernation but not all males mate each year, and those that are not involved in reproduction continue to hibernate (Morrow and Nicol, 2009).

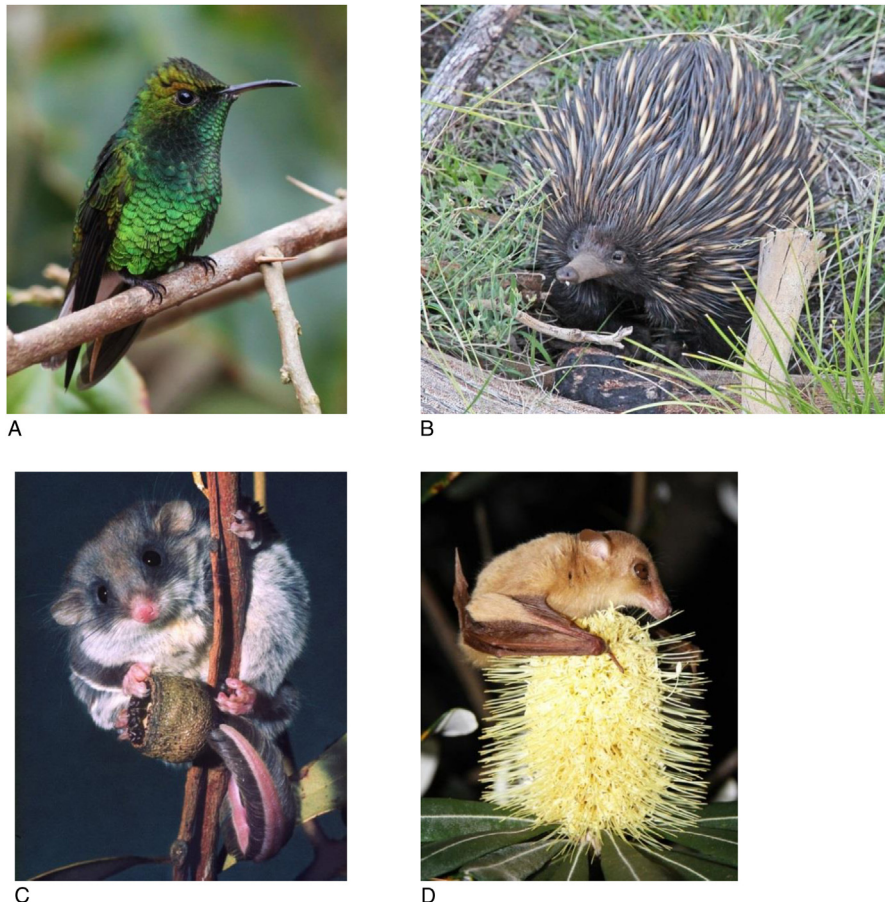


Fig. 2 Examples of birds and mammals that use torpor or hibernation. The blossom bat, hummingbird, feathertailed glider and echidna are all capable of using torpor or hibernation during reproduction. (A) Volcano hummingbird (*Selasphorus flammula*, Apodiformes Trochilidae). (B) Echidna *Tachyglossus aculeatus* (Monotremata). (C) Feathertailed glider *Acrobates pygmaeus* (Acrobatidae). (D) The Australian blossom bat *Syconycteris australis* (Pteropodidae). Photos are by Fritz Geiser and cannot be reproduced without permission of Fritz Geiser.

Torpor and Reproduction in Marsupials

Daily torpor and hibernation are known to occur in a number of marsupial families. Many dasyurids (carnivorous marsupials) have been observed to use torpor for most of the year, including the reproductive season. Fat-tailed dunnarts (*Sminthopsis crassicaudata*) enter daily torpor throughout the year and do so during lactation for females, and in males when testes are large and they are reproductive. Unlike many rodents, torpor patterns are similar in fat-tailed dunnarts no matter their reproductive condition (McAllan and Geiser, 2014). Pregnant stripe-faced dunnarts (*S. macroura*), can use torpor, but only in the first half of the short pregnancy. Males used torpor when reproductive hormones were present or absent, however torpor traits such as torpor bout duration, were affected by presence of testosterone in males. In females the effects of reproductive hormones on patterns of torpor use are mixed, with only the pregnancy hormone progesterone really disrupting torpor use. This contrasts to data from heterothermic rodents, where presence of testosterone abolishes the use of torpor in males, and oestrogen inhibits torpor use in females (McAllan and Geiser, 2014).

Another carnivorous marsupial the Mulgara [*Dasyurus cristicauda (blythi)*] uses daily torpor with minimum T_b around 14°C and reproduce in winter (Körtner *et al.*, 2008). Reproductively active males and pregnant females use torpor under both winter field and even mild laboratory conditions (Körtner *et al.*, 2008). Females can increase their body mass by 35% during pregnancy by frequently using torpor to conserve energy, but because neonates weigh less than 1% of the mother's body mass, it seems that females try to enhance fat storage for the more energetically demanding period of lactation, which is the main time of energy and nutrient transfer to growing young in marsupials (McAllan and Geiser, 2014). In contrast to most other mammals for which there are data, the use of torpor in mulgaras appeared to increase during pregnancy in comparison to non-reproductive individuals. However, free-ranging males displayed only shallow and brief torpor during the mating season in early winter, but after mating daily torpor was pronounced (Körtner *et al.*, 2008).

Torpor during the reproductive season also occurs in kowaris (*Dasyuroides byrnei*). Similar to the mulgaras, reproduction also influenced thermoregulation in females in the field. Pregnant female kowaries can enter torpor, but maintain high normothermic T_b 's throughout lactation in McAllan and Geiser (2014). Moreover, pregnant antechinus (*Antechinus flavipes*) enter daily torpor in captivity (Stawski and Rojas, 2016).

Torpor during reproduction also occurs in possums (diprotodont marsupials). In contrast to the dasyurids, which predominantly express torpor during pregnancy, possums appear to use torpor mainly during lactation, perhaps because their neonates are substantially larger than those of dasyurids. Feathertail gliders (*Acrobates pygmaeus*, Fig. 2(C)) undergo both short and prolonged torpor when non-reproductive with T_b falling as low as 2°C and torpor bouts lasting for up to a week in Geiser *et al.* (2008). Torpor has been observed in lactating females with pouch young in the reproductive season (Austral Spring). Similarly, free-ranging female sugar gliders (*Petaurus breviceps*), displayed daily torpor during lactation, when the pouch young were 19–34 days of age (Geiser *et al.*, 2008). During pregnancy wild female sugar gliders maintained a high and rather constant T_b , males only use torpor occasionally.

Torpor and Reproduction in the Afrotheria

The Afrotherians are often considered as basal clade in eutherian phylogeny and include elephants, sea cows, hyraxes tenrecs, golden moles, sengis, and armadillos (McKechnie and Mzilikazi, 2011). Torpor use has been described in many tenrecs. Generally these species typically have a hibernation season over the winter and can show prolonged torpor bouts from 2–4 days although more frequently the bouts are shorter are less than 24 h duration (McKechnie and Mzilikazi, 2011), whereas others may remain in shallow torpor for weeks (Lovegrove *et al.*, 2014). There is a large variability in gestation periods within each tenrec species, which is perplexing until it is noted that this phenomenon may be linked to the opportunistic use of torpor during the reproductive season, similar to marsupials. The T_b s of torpid shrew-tenrecs (*Microgale dobsoni* and *Microgale talazaci*) and large-eared tenrecs (*Geogale aurita*) are higher when females are pregnant or lactating than when they are non-pregnant and torpid (see McKechnie and Mzilikazi, 2011). Torpor was not observed during pregnancy in other tenrec species (*Hemicentetes semispinosus*, *H. nigriceps*, *Echinops telfairi*, *Setifer setosus*; see McKechnie and Mzilikazi, 2011; McAllan and Geiser, 2014). Torpor use in pregnant females appears to lengthen the gestation period (see McKechnie and Mzilikazi, 2011; McAllan and Geiser, 2014). Sengis (elephant shrews, Macroscelidea), express daily torpor or short-term hibernation when non-reproductive and exhibit seasonal torpor patterns (McKechnie and Mzilikazi, 2011). Their torpor use is adaptive to the weather conditions, as they can respond to changing conditions in spring, by returning to occasional torpor use if the weather is poor. For the sengis, the interaction between torpor use and reproduction is not clear (Lovegrove *et al.*, 2014).

Torpor and Reproduction in the Xenarthra (Anteaters, Armadillos, and Sloths)

Torpor is used by the pichi (*Zaedyus pichii*) and these temperate zone armadillos appear to have distinct non-overlapping hibernation and reproductive seasons in Ruf and Geiser (2015). However, there is one record of "hypothermia" in a pregnant three-toed sloth, (*Bradypus griseus griseus*, Morrison 1945 in McAllan and Geiser (2014)).

Torpor and Reproduction in the Eulipotyphla (Shrews, Moles and Hedgehogs)

In contrast to reports from the past, torpor has been recorded in many shrews *Crocidura russula*, *C. leucodon*, *C. suaveolens* and in *Suncus etruscus* (McAllan and Geiser, 2014). Data are scant because the very small shrews are difficult to keep in captivity and there are technological limitations for measuring their thermal physiology in the field. Data on thermoregulation of shrews during reproduction are even sparser. Hedgehogs readily use torpor both in captivity and in the wild with long torpor bouts lasting up to 27 days in some species, although 2–4 days are more usual (Dmi'el and Schwartz, 1984). In all hedgehogs studied torpor could be induced at any time of year by cool weather (Dmi'el and Schwartz, 1984). However, only one study has specifically examined the use of torpor by reproductive animals (Fowler, 1988). Pregnant European hedgehogs (*Erinaceus europaeus*) demonstrated the same daily fluctuations in T_b as non-pregnant animals, and defended their normothermic T_b only during late pregnancy (Fowler, 1988).

Torpor and Reproduction in Primates (Lemurs and Bushbabies)

Several species of primates belonging to the Cheirogaleidae (Malagasy lemurs) from five genera and in the South African lesser bushbaby (*Galago moholi*, Dausmann, 2014) all express torpor. Some of the dwarf lemurs hibernate with a distinct hibernation season, separated temporally from the reproductive season (Dausmann, 2014) and if food is scarce or the habitat is poor animals will choose not to reproduce, instead using torpor during the reproductive season (Dausmann, 2014).

In contrast the mouse-lemurs (*Microcebus* spp.) are very flexible in their use of heterothermy with some species using only daily torpor occasionally (e.g. *Microcebus berthae*, in Dausmann, 2014), whereas others showing an extended regular pattern of torpor, depending fat reserves accumulated during the active season (*M. griseorufus*, *M. murinus* in Dausmann, 2014). For the most part torpor use has not been seen in pregnant or lactating females, with most observations of torpor occurring in the “winter” (dry) period (Dausmann, 2014). The exception to this was found in *M. murinus*, in which severe food restriction in the laboratory induced torpor use in both pregnant and lactating females (Dausmann, 2014). While the experiment may seem divorced from the animals’ experiences in nature, the unpredictable environment and pronounced changes in water availability in their natural environment may require torpor use during pregnancy and lactation in wild mouse-lemurs (Dausmann, 2014). In contrast to the Madagascan lemurs, the African lesser bushbaby, *Galago moholi*, uses torpor only rarely as an emergency mechanism for energy savings, suggesting that torpor and reproduction in bushbabies are likely to be mutually exclusive (Dausmann, 2014).

Torpor and Reproduction in Bats

Many insectivorous bats (Microchiroptera) typically exhibit prolonged torpor in winter, but also short bouts of torpor during the summer reproductive season. There are many examples of bats using torpor during pregnancy and lactation (McAllan and Geiser, 2014; Stawski et al., 2014). Torpor use in reproductive bats can cause a delay in gestation in pregnant females, and a possible reduction in milk production. The main physiological disadvantage for female bats and their offspring could be that delays in parturition and embryonic and postnatal growth could result in insufficient pre-winter fattening to fuel the energy requirements during the long hibernation season in winter (Ruf and Geiser, 2015). The physiological advantage is that any transient energy shortfalls are managed such that both mother and offspring remain viable, although reproductive activities are delayed (Willis et al., 2006).

Many vespertilionid bats mate in late summer and early autumn (Stawski et al., 2014; Ruf and Geiser, 2015). After mating, sperm is stored in the oviduct and following the hibernation season, pregnancy starts immediately after fertilization. The demanding energetic costs of pregnancy and lactation continue to increase until weaning thus food consumption increases for lactating females, however female *M. lucifugus* will use torpor during pregnancy and lactation, although the bouts are shallower and shorter than in non-reproductive individuals (Stawski et al., 2014).

Torpor extends the length of pregnancy of many bats (mouse-eared bat *M. myotis*, mouse-eared bat *M. blythii*, pipistrelle *Pipistrellus pipistrellus*). Food availability coupled with cooler temperatures seems to be the main trigger for torpor use during pregnancy and lactation in these bats. In the wild this results in variable gestation lengths in animals between roost sites and over several years in the lump-nosed bat, *Corynorhinus* (= *Plecotus*) *rafinesquei* (Pearson et al., 1952). Similar to other mammals, torpor bout duration and depth was reduced in reproductive females (Stawski et al., 2014). Miniopertid bats also mate in autumn or winter, but females enter hibernation in a pregnant condition, embryonic development is delayed, and births do not occur until the following summer (Stawski et al., 2014). In the desert-dwelling Hemprich’s long-eared bats (*Otonycteris hemprichii*) torpor use occurred in pregnant female bats up to the third trimester of pregnancy, when T_b could be still lower than during normothermia, but torpor was shallower than in non-reproductive bats (Stawski et al., 2014). Shallow torpor was also expressed in lactating females, although the incidence of torpor use was reduced. In the big brown bat (*Eptesicus fuscus*), torpor has been observed during both pregnancy and lactation (Audet and Fenton, 1988; Stawski et al., 2014). Deep torpor (ie. a skin temperature drop by more than 10°C) was regularly observed during pregnancy and also during lactation.

Male *Miniopterus* also show seasonal changes in reproduction, with spermatogenesis and fertility unimpaired by hibernation use (Pearson et al., 1952). Similar to *M. schreibersii*, the vespertilionid male Daubenton’s bats (*Myotis daubentonii*) and male Bechstein’s bats (*M. bechsteinii*) also use torpor during the reproductive periods in summer, although to a lesser extent than other times of year

(Stawski *et al.*, 2014). Male big brown bats regularly used torpor throughout the year, but show a seasonal cycle in torpor use (Audet and Fenton, 1988).

The differences in torpor use between many small insectivorous bats may be because of their different roost selections. Roosting alone and in exposed roosts incurs much higher costs for maintenance of homeothermy when compared to roosting in maternity colonies in well-insulated roosts (Willis *et al.*, 2006). The importance of roost selection or thermal condition for torpor use by reproductive bats is supported by laboratory work where captive pregnant and lactating female and male long-eared bats (*Nyctophilus geoffroyi* and *N. gouldi*) studied under identical thermal conditions, showed indistinguishable thermal physiology (McAllan and Geiser, 2014). The reluctance by reproductive females to enter torpor in the field may be because of ecological rather than physiological differences, and may explain why many females roost together whereas male bats roost alone (Willis *et al.*, 2006).

Subtropical insectivorous bats also use torpor. Long-eared non-reproductive male bats (*N. bifax*) continue to use torpor no matter what the season suggesting that torpor use is beneficial throughout the year even in the subtropics (McAllan and Geiser, 2014; Ruf and Geiser, 2015). However, non-reproductive males used torpor more frequently than pregnant females during spring, similarly to male big brown bats and male Daubenton's bats (McAllan and Geiser, 2014; Stawski *et al.*, 2014). It appears that occasionally pregnant females may have been energetically constrained and therefore needed to enter torpor for short periods in McAllan and Geiser (2014).

We know less about torpor use in the Megachiroptera. The nectarivorous blossom-bat (*Syconycteris australis* Fig. 2(D)) displays daily torpor in captivity (Stawski *et al.*, 2014; Ruf and Geiser, 2015). Torpor in non-reproductive *S. australis* is used by all individuals and lasts for up to 12 h, metabolic rate falls to about 15% of resting animals and T_b drops to a minimum of about 18°C. Torpor has been observed in one of three pregnant bats, but the torpor bout of this female was shorter and the reduction of the metabolic rate was less pronounced than in most non-reproductive individuals (Stawski *et al.*, 2014). Interestingly, pregnant *R. aegyptiacus* depress their metabolism by 19% of resting metabolic rates of non-pregnant females, perhaps enabling them to spend more time on foraging or to allocate more energy to fat storage or milk production (Stawski *et al.*, 2014).

Torpor and Reproduction in Other Rodents (Dormice)

Torpor use has also been observed in several rodent families besides the sciurids and cricetids. Many species of dormice (Gliridae) use torpor (Ruf and Geiser, 2015) and use is linked to food availability, with complex relationships observed between torpor, seasonal food availability and reproduction. The hibernation cycle of the edible dormouse (*Glis glis*) is closely tied to the seasonal change, but successful reproduction is associated with seed production by beech trees (Mast years), and females may not reproduce every year (Lebl *et al.*, 2011). Because of the secretive nature of this dormouse it is unclear whether the females use torpor while in reproductive condition in the wild although being in good physical condition does not preclude their use of summer torpor (Lebl *et al.*, 2011).

Food scarcity also promotes torpor use in any season in garden dormice (*Eliomys quercinus*, in Ruf and Geiser, 2015). Torpor regularly occurs in summer in African woodland dormice (*Graphiurus murinus*, in Ruf and Geiser, 2015). Both spontaneous torpor (with food) and induced torpor (food removed) was observed in this species, although it was not stated whether the animals were reproductive, it was certainly during their reproductive season. Wild common dormice (*Muscardinus avellanarius*) hibernate for prolonged periods in winter but torpor was also observed in reproductive males and females, pregnant females, females with litters and in nestlings during the breeding season (Juškaitis, 2005). Similar to some bats, torpor bouts for pregnant females were not as deep as those found in females at other times of year (Juškaitis, 2005).

Torpor and Reproduction in the Carnivora (Bears and Badgers)

Torpor use is restricted to bears (Ursidae) and some badgers (Mustelidae). Hibernating black bears (*Ursus americanus*) and brown bears (*Ursus arctos*) show long periods of dormancy in winter and actually give birth during their hibernation period. All animals remain aware and capable of moving throughout hibernation (Tøien *et al.*, 2011). Bears do not eat, drink, defecate, or urinate during the 3–6 month hibernation period, and unlike many small hibernators, which often allow they T_b to fall near 0°C (Ruf and Geiser, 2015), their core T_b decreases to only about 30°C. Even so, because metabolic rate is very low, considerable energy savings are made over winter. Pregnancy and birth are only weakly associated with activity patterns in the brown bear (Tøien *et al.*, 2011), and circulating sex steroids have been found to be independent of torpor activity in black bears and in polar bears in McAllan and Geiser (2014).

Badgers have a hibernation season that appears independent of the reproductive season for males, and delayed embryonic implantation occurs in females, with post-implantation gestation occurring during the height of winter conditions. The European badger (*Meles meles*) will remain heterothermic when delayed implantation occurs, although in one female T_b was raised somewhat from 28.4°C before implantation to between 32.1 and 34.7°C during the remainder of the gestation period (Fowler and Racey, 1988). In males increased circulating testosterone concentrations are seasonal and begin to rise in winter, when hibernation is still common (Fowler and Racey, 1988). Similar patterns of heterothermy are seen in the American badger (*Taxidea taxus*), although T_b s have not been measured for pregnant females (McAllan and Geiser, 2014).

Discussion

Torpor use during reproduction in birds and mammals is widespread, occurring in many taxa (e.g., Monotremata, Marsupialia, Afrotheria, Eulipotyphla, Chiroptera). However, we lack information on torpor and reproduction in other mammalian groups. We clearly need more information about thermoregulatory capacities of species from several orders.

Most monotremes and marsupials have short pregnancies and the mass of an echidna egg and that of single neonates or neonate litters of marsupials is less than 0.3% of the mothers' masses (Tyndale-Biscoe and Renfree, 1987; McAllan and Geiser, 2014). Energy expenditure/day for gestation in both groups and lactation in marsupials is relatively low even over the long lactational time that they have compared to eutherians (Nicoll and Thompson, 1987). Small rodents, such as the scurids and circetids who do not have an overlap of reproduction and torpor use, have a relatively short gestation periods, heavy neonate litters (10%–65% of maternal weight) and fast development after birth and both gestation and lactation are thus energetically expensive (Nicoll and Thompson, 1987). Bats have one large neonate (13%–40% of maternal weight), but growth of fetal and young bats, as in marsupials and monotremes, is slow and energy expenditure/day during gestation is much lower than in small rodents (Nicoll and Thompson, 1987). In contrast carnivores have small altricial young with fast neonatal growth rates, but because of their size heterothermic carnivores have vastly different energetic demands than most other heterotherms.

Similarly for the birds the nightjar relatives (Caprimulgiformes) and hummingbirds (Trochiliformes) have relatively small clutch sizes and relatively long rearing periods and the additional energy expenditure required during reproduction should be small. We can conclude that mammals and birds that produce few offspring or spread the reproductive effort and the associated metabolic costs over a long time, may display torpor during the period of reproduction. The rate of development of young may be slower, but the chance of survival in offspring is unaffected because a small delay in growth within the long period of development has minimal impact.

Many of the species that use torpor during reproduction are animals from unpredictable environments, where seasonal availability of food can be cut short by abrupt weather changes. For some species the adverse weather conditions or the food shortages must be severe for induction of heterothermy (Dausmann, 2014), for others, such as bats heterothermy during pregnancy is part of the yearly cycle. For bears and badgers heterothermy during pregnancy seems part of the yearly cycle in a predictable environment, but their body size also plays an important role for carnivores. For these mammals the small extension of pregnancy or lactation may be an important strategy to optimise reproductive outcomes later in spring and summer.

Besides reproductive strategies another explanation for the differences between thermoregulatory patterns during reproduction in different mammalian groups and birds, could be diet. Abundance of most insects (and other arthropods) and nectar strongly fluctuate with season and use of torpor during reproduction may be linked to their availability. If food supply is predictable food supplies and a species can reproduce during a period of high primary productivity then strict homeothermy during reproduction will be an advantage, whereas species with relatively unpredictable food supplies may be better off using torpor when the cost is only a small extension of the reproductive period. The use of heterothermy during the reproductive season appears to be quite widespread and is a strategy for small birds and mammals to weather temporary unpredictable energetic adversities, while continuing to invest in reproduction.

Acknowledgments

This work was support by grants from the Australian Research Council (DP130101589 to BMM, and DP130101506 to FG).

References

- Audet, D., & Fenton, M. B. (1988). Heterothermy and the use of torpor by the bat *Eptesicus fuscus* (Chiroptera: vespertilionidae): A field study. *Physiol. Zool.*, *61*, 197–204.
- Brigham, R.M., McKechnie, A.E., Doucette, L.I., Geiser, F., 2012. Heterothermy in caprimulgid birds: A review of inter- and intraspecific variation in free-ranging populations. In: Ruf, T., Bieber, C., Arnold, W. and Milleli, E. (Eds.), *Living in a Seasonal World: Thermoregulatory and Metabolic Adaptations*, pp. 175–187.
- Dausmann, K. H. (2014). Flexible patterns in energy savings: Heterothermy in primates. *J. Zool.*, *292*, 101–111.
- Dmi'el, R., & Schwartz, M. (1984). Hibernation patterns and energy expenditure in hedgehogs from semi-arid and temperate habitats. *J. Comp. Physiol. B*, *155*(117), 123.
- Fowler, P. A. (1988). Thermoregulation in the female hedgehog, *Erinaceus europaeus*, during the breeding season. *J. Reprod. Fertility*, *82*, 285–292.
- Fowler, P. A., & Racey, P. A. (1988). Overwintering strategies of the badger, *Meles meles*, at 57° N. *J. Zool., Lond.*, *214*, 635–651.
- Geiser F., Christian N., Cooper C.E., *et al.*, 2008. Torpor in marsupials: Recent advances. In: Lovegrove, B.G., McKechnie, A.E. (Eds), *Hypometabolism in Animals: Torpor, Hibernation and Cryobiology*. Proceedings of the 13th International Hibernation Symposium. University of KwaZulu-Natal, Pietermaritzburg. pp 297–306.
- Geiser, F., Stawski, C., Wacker, C. B., & Nowack, J. (2017). Phoenix from the ashes: Fire, torpor and the evolution of endothermy. *Front. Physiol.*, *8*, 842. <https://doi.org/10.3389/fphys.2017.00842>.
- Juškaitis, R. (2005). Daily torpor in free-ranging common dormice (*Muscardinus avellanarius*) in Lithuania. *Mamm. Biol.*, *70*, 242–249.
- Körtner, G., Pavey, C. R., & Geiser, F. (2008). Thermal biology, torpor and activity in free-living mulgaras in arid zone Australia during the winter reproductive season. *Physiol. Biochem. Zool.*, *81*, 442–451.
- Lebl, K., Rotter, B., Kürbis, K., Bieber, C., & Ruf, T. (2011). Local environmental factors affect reproductive investment in female edible dormice. *Journal of Mammalogy*, *92*, 926–933.
- Lovegrove, B. G., Lobban, K. D., & Levesque, D. L. (2014). Mammal survival at the Cretaceous-Paleogene boundary: Metabolic homeostasis in prolonged tropical hibernation in tenrecs. *Proc R Soc B*, *281*, 20141303.
- McAllan, B. M., & Geiser, F. (2014). Torpor during Reproduction in Mammals and Birds: Dealing with an Energetic Conundrum. *Integr. Comparative Biol.*, *54*(3), 516–532.

- McKechnie, A. E., & Mzilikazi, N. (2011). Heterothermy in afrotropical mammals and birds: A review. *Integrative and Comparative Biology*, 51, 349–363.
- Morrow, G., & Nicol, S. C. (2009). Cool sex? Hibernation and reproduction overlap in the echidna. *PLOS ONE*, 4(6), e6070. <https://doi.org/10.1371/journal.pone.0006070>.
- Nicol, M. E., & Thompson, S. D. (1987). Basal metabolic rates and energetics of reproduction in therian mammals: Marsupials and placentals compared. *Symp. zool. Soc. Lond.*, 57, 7–27.
- Pearson, O. P., Koford, M. R., & Pearson, A. K. (1952). Reproduction of the lump-nosed bat *Corynorhinus rafinesquei* in California. *J. Mammal.*, 33, 273–320.
- Ruf, T., & Geiser, F. (2015). Daily torpor and hibernation in birds and mammals. *Biol. Rev.*, 90, 891–926.
- Stawski, C., & Rojas, A. D. (2016). Thermal physiology of a reproductive female marsupial, *Antechinus flavipes*. *Mammal Res.*, 61, 417–421.
- Stawski, C., Willis, C. K. R., & Geiser, F. (2014). The importance of temporal heterothermy in bats. *J. Zool.*, 292, 86–100.
- Tøien, Ø., Blake, J., Edgar, D. M., et al. (2011). Hibernation in black bears: Independence of metabolic suppression from body temperature. *Science*, 331(6019), 906–909.
- Tyndale-Biscoe, H., & Renfree, M. B. (1987). *Reproductive Physiology of Marsupials*. Cambridge: Cambridge University Press.
- Willis, C. K. R., Brigham, R. M., & Geiser, F. (2006). Deep, prolonged torpor by pregnant, free-ranging bats. *Naturwissenschaften*, 93, 80–83.

REPRODUCTIVE TECHNOLOGY (NON-HUMAN/NON-PRIMATE)

Germ Cell Transplantation in Fish

Goro Yoshizaki, Tokyo University of Marine Science and Technology, Tokyo, Japan

© 2018 Elsevier Inc. All rights reserved.

Glossary

Dead end gene A type of RNA-binding protein that is specifically expressed by germ cells and crucial for primordial germ cell survival.

Donor An individual that supply germ cells used for transplantation into another individual.

Germ-line stem cells Stem cells that generate gametes.

Primordial germ cells Sexually undifferentiated germ cells that emerge in the extragonadal area and subsequently migrate to gonadal anlagen using pseudopodia.

Recipient An individual that received foreign germ cells. After germ cell transplantation, the recipient produce gametes derived from the transplanted germ cells.

Spermatogonial stem cells Stem cells that generate sperm.

Triploid Individuals carrying three sets of chromosomes; two maternal and one paternal set of chromosomes. Usually triploid fish develop normally but do not produce functional gametes due to abnormal meiosis.

Introduction

Germ cell transplantation is a method that converts immature germ cells into gametes capable of producing individual fish following fertilization. In other words, manipulation of fish germ cells has the same significance to manipulation of adult fish, making this technology useful for the fish bioengineering field (Fig. 1).

Cryopreservation of fish eggs or embryos is not possible due to their large size (approximately 0.3–8 mm in diameter) and high lipid and yolk content. Therefore, stable and reproducible methods to preserve their genetic resources were not available until very recently. The combination of cryopreservation of immature or undifferentiated germ cells (primordial germ cells (PGCs) or germ-line stem cells) and their subsequent transplantation into recipient fish is a powerful alternative to cryopreservation of fish eggs or embryos, since the transplanted germ cells undergo gametogenesis in the recipient fish gonads and eventually become functional eggs or sperm (Fig. 1(a)).

Further, germ cell transplantation can simplify the procedure to produce fish seedlings. Seedling production of large fish species, such as bluefin tuna or giant grouper requires space and is both labor-intensive and expensive. However, if their germ cells are transplanted into closely related small-bodied fish recipients, the resulting recipients, called surrogate broodstock, are able to produce gametes derived from the donor species. By doing so, seedling production of the donor-species can be simplified and performed in small facilities at lower costs and is less labor-intensive (Fig. 1(b)).

If an in vitro cultured method for donor germ cells is established, genetic modification including transgenesis and gene knockout can be easily applied to the cultured germ cells. By transplanting the resulting genetically modified germ cells into recipients, transgenic fish or knockout fish can be effectively produced in the next generation (Fig. 1(c)).

In general, donor germ cells are transplanted into sterile recipients that do not produce their own gametes. PGCs harvested from sexually undifferentiated embryos or germ-line stem cells harvested from either testes or ovaries of juveniles or adults are used as donor cells for transplantation. Unlike mammals, the ovaries of most adult fish species still carry germ-line stem cells.

Embryonic Recipients

Recipient fish used for germ cell transplantation need to be capable of nursing the donor-derived germ cells, without having their own germ cells. Otherwise, the recipient fish will produce a mixture of donor-derived and recipient-derived gametes, following transplantation. Several methods have been applied to sterilize the recipients.

First, triploidization is one of the simpler and easier methods (Okutsu *et al.*, 2007). In lower vertebrates, including fish, triploids can be easily produced by applying temperature or pressure shock to fertilized eggs. These treatments suppress the extrusion of the second polar body and produce embryos carrying three sets of chromosomes (paternal and maternal nucleus with second polar body). Noticeably, triploid fishes are viable and develop normally, although they do not produce functional gametes due to

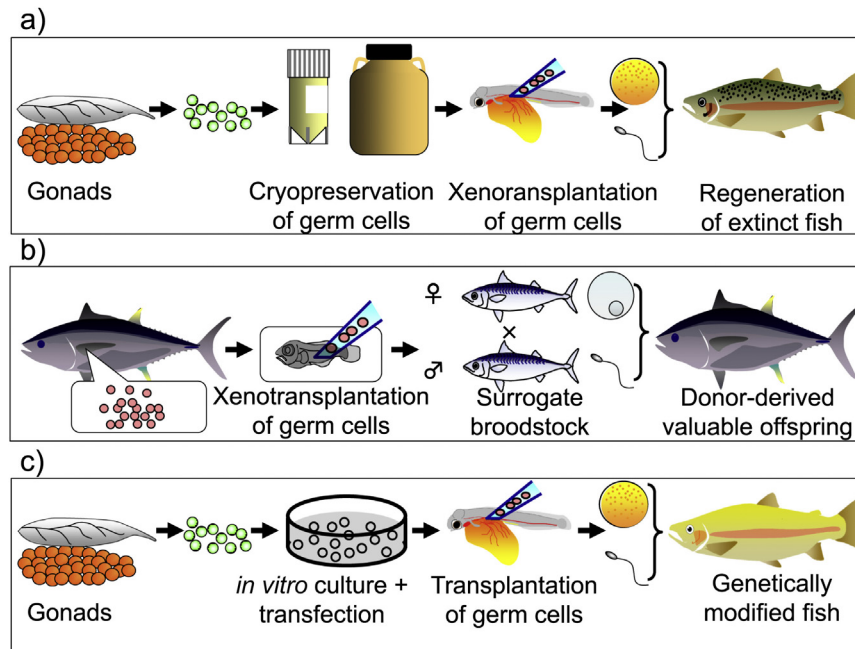


Fig. 1 Potential applications of germ cell transplantation in the field of fish bioengineering. (a) Long-term preservation of genetic resources of endangered species. The combination of cryopreservation and xenotransplantation of germ cells from endangered species make it possible to regenerate them whenever needed. (b) Production of surrogate broodstock that can produce gametes of valuable species. Gametes of the target species can be obtained using surrogate broodstock by transplanting the germ cells of the species into xenogenic recipients. By using small-bodied and short-generation species that are easily reared, gametes of the target species can be obtained in a small facility in a short period. (c) Germ cell-mediated gene modification. If we can apply transgenesis or genome editing to the in vitro-cultured germ cells, transgenic or genome-edited fish can be produced via germ cell transplantation.

abnormal disjunction during meiosis. Triploidization efficiency in salmonid species is nearly 100% and as good quality eggs are used, survival rates are not sacrificed. Further, triploidization is easy to apply to large-scale production. However, it is difficult to produce 100% triploid populations in most pelagic fish species that produce small eggs. Although it is easy to perform triploidization treatment, especially with temperature shock, one theoretical problem is that triploid gonads have relatively large numbers of mitotic germ cells (prior to entering meiosis) to occupy the germ cell niche (see below).

A second option is the knockdown of genes that are essential for survival or for normal migration of PGCs (Saito *et al.*, 2008; Yoshizaki *et al.*, 2016). In the case of fish, gene knockdown can be effectively performed by microinjection of antisense morpholino oligonucleotides against the DNA region around the start codon of the target gene into fertilized eggs prior to the first cleavage. Among the many genes that are related to germ cell development, *dead end* (*dnd*) is widely used since its effects are specific to germ cells and the resulting knockdown fish do not show any malformation other than their gonads lack endogenous germ cells. Without endogenous germ cells, the *dnd* knockdown recipients avoid competition between endogenous and donor-derived (transplanted) germ cells for the germ cell niche. This is an advantage over using triploidization. Although delivering antisense morpholino oligonucleotides into individual eggs is both labor intensive and time consuming.

Recently, germ cell-free fish were produced by knockout of the *dnd* gene using artificial nucleases such as CRISPR/Cas9 (Wargelius *et al.*, 2016). This strategy requires producing founder mutants by microinjection into their fertilized eggs. This mutant strain can be maintained as heterozygous mutants (since homozygous mutants are sterile) by mating heterozygous males and females. One fourth of the resulting offspring are homozygous mutants, namely germ cell-free fish, making this an attractive option to produce recipients for germ cell transplantation.

Transplantation of PGCs into Embryos or Hatchlings

In any animal species, including fishes, PGCs emerge in the extra-gonadal area and then migrate to the gonads, partly through chemotaxis. The gonadal anlagen secrete a chemokine, CXCL12, known as stromal cell-derived factor 1 (SDF-1) and PGCs express its receptor, CXCR4. The PGCs migrate by the guidance of CXCL12. If migrating-stage PGCs are isolated and transplanted into the blastodisc of blastula embryos, they migrate to the recipient gonads where they begin gametogenesis (Giraldez *et al.*, 2005; Fig. 2(A)). Alternatively, PGCs can be isolated from the gonadal anlagen immediately after they have completed migration, and transplanted into the body cavity of newly hatched embryos (Fig. 2(B)). The transplanted PGCs migrate to the recipient gonads and resume gametogenesis (Takeuchi *et al.*, 2003). A micromanipulator under a binocular dissection microscope can be used for such transplantations. In either case, if the recipient is male the donor-derived PGCs differentiate into functional sperm and into

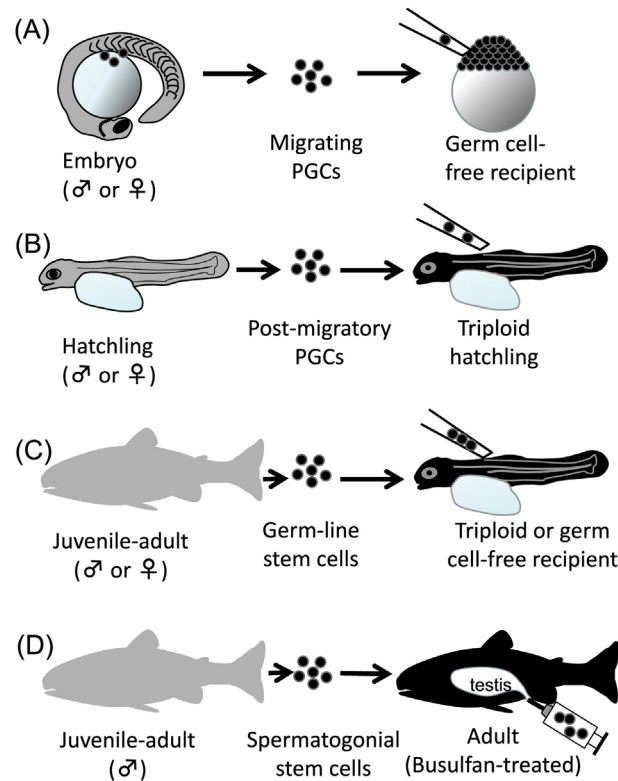


Fig. 2 Methods to transplant germ cells into recipients. (A) Transplantation of migrating PGCs into blastulae embryos. Germ cell-free recipients produced by knock down of the *dead end* gene are used. (B) Transplantation of post-migratory PGCs into the body cavity of hatchlings. Triploid recipients are used. (C) Transplantation of germ-line stem cells into the body cavity of hatchlings. Triploid or germ cell-free recipients produced by knock down of the *dead end* gene are used. (D) Transplantation of spermatogonial stem cells into the testes of adult recipients. Recipients with depleted numbers of germ cells following busulfan-treatment are used.

eggs if the recipient is female, regardless of the sex of donor PGCs. Although obtaining large numbers of PGCs is difficult, only a few PGCs (from one to ten) are needed for each recipient. Noticeably, in zebrafish even single PGC transplantation results in recipients able to produce donor-derived gametes (Saito *et al.*, 2008). It is notable that migrating PGCs isolated from early embryos cannot be used for intraperitoneal transplantation. In addition, gonadal (post-migratory) PGCs from gonadal anlagen cannot be used for transplantation into blastula recipients. In both cases, the donor-derived PGCs are unable to migrate to the recipient gonads appropriately.

This type of PGC transplantation (transplantation into blastula embryos and hatchlings) can be applied to interspecies transplantation (xenotransplantation) (Takeuchi *et al.*, 2004; Saito *et al.*, 2008). The resulting gametes produced by the xenogeneic recipients are functional with viable offspring. It has been reported that pearl danio (*Danio albolineatus*) eggs and sperm, were successfully produced using germ cell-free zebrafish (*Danio rerio*) recipients through knockdown of the *dnd* gene. On the other hand, the recipient zebrafish could produce sperm of goldfish (*Carassius auratus*) and loach (*Misgurnus anguillicaudatus*), but not their eggs (Saito *et al.*, 2008). Thus, production of donor-derived eggs is more difficult than that of donor-derived sperm and requires closely related recipient species. The transplanted allogeneic or xenogeneic germ cells were not immune rejected since the donor-derived germ cells were transplanted into recipient embryos before they acquired a mature immune system. The recipient may have acquired immune tolerance following transplantation of certain amounts of foreign cells.

It has been reported that gonadal anlagen of rainbow trout right after the PGCS completed migration and zebrafish embryos carrying migrating stage PGCs can be cryopreserved by slow-freezing (Kobayashi *et al.*, 2007) or vitrification (Higaki *et al.*, 2010) respectively. Further, these PGCs can be used for the transplantation methods discussed above. These methodologies are powerful techniques for the long-term storage of fish genetic resources, since the resulting recipients produced functional egg and sperm.

Germ-Line Stem Cell Transplantation into Hatchlings

As is the case with PGC transplantation, mitotic germ cells retrieved from juvenile or adult testes or ovaries, can be used for transplantation. When whole testicular or ovarian cell suspensions are transplanted into body cavities of newly hatched embryos, some of the mitotic germ cells within the gonadal cell suspension migrated towards the recipient genital ridges where they eventually incorporated. The transplanted germ cells began spermatogenesis or oogenesis when they were transplanted into

male or female recipients respectively. The resulting recipients produced functional eggs or sperm, depending on the sex of the recipients (Okutsu *et al.*, 2006; Yoshizaki *et al.*, 2010; Fig. 2(C)). Thus, the sex of the germ cells, are plastic, controlled mainly by somatic environments, rather than germ cell autonomy. Further, the recipient fish can produce large numbers of donor-derived gametes (rainbow trout recipients produce approximately one billion sperm in a spawning season) for several spawning seasons (Okutsu *et al.*, 2006). Thus, the donor-derived germ cells incorporated into recipient gonads can produce large numbers of gametes for several spawning seasons, suggesting they can both self-renew and differentiate. Thus, only germ-line stem cells (either spermatogonial stem cells or oogonial stem cells) that were included in the gonadal cell suspension incorporated into recipient gonads and resumed gametogenesis. This transplantation method was first established in Salmonidae species and recently applied to many other species, including Carangidae, Scianidae, Tetraodontidae, Cichlidae, Cyprinidae and Adrianichthyidae and it was confirmed that they all produced donor-derived gametes.

Xenotransplantation is also possible using germ-line stem cells as donor cells (although whole gonadal cell suspensions are usually used as donor cells, this transplantation is called germ-line stem cell transplantation hereinafter). To date, triploid masu salmon (*Oncorhynchus masou*) broodstock producing only donor-derived rainbow trout (*Oncorhynchus mykiss*) eggs or sperm have been produced through germ-line stem cell transplantation (Okutsu *et al.*, 2007). Further, by mating male and female recipient salmon, all donor-derived rainbow trout offspring were produced for at least three consecutive spawning seasons, using masu salmon as parents. In addition, viable tiger puffer (*Takifugu rubripes*) offspring were produced using surrogate triploid grass puffer (*Takifugu niphobles*) parents. In this case, the donor tiger puffer required at least two and three years to mature into adult males and females respectively. However, the recipient grass puffer produced tiger puffer gametes in 11 months and two years in males and females, respectively. Thus, germ-line stem cell transplantation can shorten the generation time and accelerate breeding processes that usually require several generations.

Cryopreservation of Germ-Line Stem Cells

Whilst cryopreservation of germ-line stem cells is possible, cryopreservation of whole testes or ovaries without dissociation is more frequently used for practical purposes, since the handling of whole gonads is easier than dissociated gonadal cell suspensions (Lee *et al.*, 2013, 2016). The germ-line stem cells retrieved from cryopreserved testes or ovaries can be used for intraperitoneal transplantation using the same method that was developed using freshly isolated germ-line stem cells. The resulting female and male recipients successfully produced functional eggs and sperm. Using this method, gametes can be produced from germ-line stem cells retrieved from cryopreserved testes or ovaries; easily harvested from juvenile to adult fish. When considering practical applications, this is the advantage of this method compared with using PGCs, since PGC cryopreservation always requires embryos with migrating PGCs or gonadal anlagen containing post-migratory PGCs. Therefore, when long-term preservation of farmed fish (their whole life-cycle is managed in captivity) is needed, both cryopreservation of PGCs or germ-line stem cells are options. However, in the case of endangered species, cryopreservation of the germ-line stem cells is the only realistic option, since isolating embryos from endangered fish species would be difficult. Since cryopreservation of fish eggs or embryos are still not possible, this combination of germ cell cryopreservation and their transplantation is a powerful tool to preserve genetic resources of endangered fish species. It is advisable to use testes, as the donor material for germ-line stem cell transplantation, since they contain more transplantable germ-line stem cells compared with ovaries. Approximately 500–10,000 type-A spermatogonia are needed for one recipient individual, resulting in 20%–80% of recipients producing donor-derived gametes, although this varies dependant on species. In the case of rainbow trout, juvenile trout (approximately 10 cm in body length) can supply approximately one million of type-A spermatogonia and this is not a limiting step.

Germ Cell Transplantation into Adult Recipients

In the previous sections, donor-germ cells are transplanted into embryos or hatchlings whose immune systems are still immature, although spermatogonial transplantation into adult gonads is also possible (Lacerda *et al.*, 2010; Majhi *et al.*, 2009; Fig. 2(D)). In these studies the endogenous germ cells of recipients was removed by treatment with busulfan, a type of alkylating agent. The donor testicular cell suspension, which contained spermatogonial stem cells, was transplanted into recipient gonads via the urogenital pore or a surgical procedure. As in the case of germ-line stem cell transplantation mentioned above, it has been predicted that only the spermatogonial stem cells can colonize the recipient gonads and undergo gametogenesis. This method does not require micromanipulation and seedling production processes. Further, the time period required to obtain donor-derived gametes is relatively short. However, it requires large numbers of donor testicular cells (10^6 – 10^7 , dependant on the target species). When considering real applications to conservation of endangered species, the supply of donor cells will be a limitation. Recently, protocols to propagate in vitro spermatogonial stem cells from several species have been reported. This is a solution to generate large numbers of donor cells from small donor individuals. Theoretically this technology cannot be applied to species whose ovary lacks an ovarian capsule, called gymnovarian or semi-gymnovarian. To date, this type of germ cell transplantation has been performed in tilapia (*Oreochromis niloticus*), pejerrey (*Odontesthes bonariensis*) and zebrafish. However, donor-derived eggs were only obtained in the pejerrey. In addition, transplantation of cryo-preserved spermatogonial stem cells produced functional sperm (Lacerda *et al.*, 2010).

As mentioned above, each method has both advantages and disadvantages. Therefore, selection of the right method for each purpose is important. Although several potential applications of these technologies are introduced in this article, there are more applications that can be applied to fish bioengineering field.

References

- Giraldez, A. J., Cinalli, R. M., Glasner, M. E., et al. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. *Science*, 308, 833–838.
- Higaki, S., Eto, Y., Kawakami, Y., et al. (2010). Production of fertile zebrafish (*Danio rerio*) possessing germ cells (gametes) originated from primordial germ cells recovered from vitrified embryos. *Reproduction*, 139, 733–740.
- Kobayashi, T., Takeuchi, Y., Takeuchi, T., & Yoshizaki, G. (2007). Generation of viable fish from cryopreserved primordial germ cells. *Mol Reprod Dev*, 74, 207–213.
- Lacerda, S. M., Batlouni, S. R., Costa, G. M., et al. (2010). A new and fast technique to generate offspring after germ cells transplantation in adult fish: The Nile tilapia (*Oreochromis niloticus*) model. *PLOS ONE*, 5, e10740.
- Lee, S., Iwasaki, Y., Shikina, S., & Yoshizaki, G. (2013). Generation of functional eggs and sperm from cryopreserved whole testes. *Proc Natl Acad Sci USA*, 110, 1640–1645.
- Lee, S., Katayama, N., & Yoshizaki, G. (2016). Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. *Biochem Biophys Res Commun*, 478, 1478–1483.
- Majhi, S. K., Hattori, R. S., Yokota, M., Watanabe, S., & Strüssmann, C. A. (2009). Germ cell transplantation using sexually competent fish: An approach for rapid propagation of endangered and valuable germ lines. *PLOS ONE*, 4, e6132.
- Okutsu, T., Shikina, S., Kanno, M., Takeuchi, Y., & Yoshizaki, G. (2007). Production of trout offspring from triploid salmon parents. *Science*, 317, 1517.
- Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., & Yoshizaki, G. (2006). Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. *Proc Natl Acad Sci USA*, 103, 2725–2729.
- Saito, T., Goto-Kazeto, R., Arai, K., & Yamaha, E. (2008). Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. *Biol Reprod*, 78, 159–166.
- Takeuchi, Y., Yoshizaki, G., & Takeuchi, T. (2003). Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. *Biol Reprod*, 69, 1142–1149.
- Takeuchi, Y., Yoshizaki, G., & Takeuchi, T. (2004). Biotechnology: Surrogate broodstock produces salmonids. *Nature*, 430, 629–630.
- Wargelius, A., Leininger, S., Skafnesmo, K. O., et al. (2016). Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Sci Rep*, 6, 21284.
- Yoshizaki, G., Ichikawa, M., Hayashi, M., et al. (2010). Sexual plasticity of ovarian germ cells in rainbow trout. *Development*, 137, 1227–1230.
- Yoshizaki, G., Takashiba, K., Shimamori, S., et al. (2016). Production of germ cell-deficient salmonids by dead end gene knockdown, and their use as recipients for germ cell transplantation. *Mol Reprod Dev*, 83, 298–311.

Gamete Preservation

Estefania Paredes, University of Vigo, Vigo, Spain

© 2018 Elsevier Inc. All rights reserved.

Glossary

Cryoprotecting agents or CPAs Cryoprotecting agents or CPAs are chemical compounds which need to have some specific properties as high solubility in water even at low temperatures, present low toxicity, and depress the freezing point of a solution (Mazur, 1970, 1977, 1980, 2004; Muldrew *et al.*, 2004). CPAs can be classified according to their molecular weight as permeating cryoprotectants – those with low molecular weight, which can permeate through biological membranes-, and non-permeating cryoprotectants – those with high molecular weight, which, therefore, cannot permeate through the membranes. The most used CPAs are: dimethyl sulfoxide (Me2SO), ethylene glycol (EG), propylene glycol (PG), trehalose (TRE), sucrose (SUC) and polyvinyl pyrrolidone (PVP).

Introduction

The term Cryobiology was originally coined by Sir Alan Parkes, defined as the study of “frosty life” in 1964. Cryopreservation consists on freezing, banking and thawing living organisms, cells or tissues in the presence of cryoprotecting agents (CPAs) (Mazur, 2004). This is a well-developed and worldwide recognized technique for achieving long-term preservation and storage of biological material at low temperatures. The preservation of biological material in a stabilized stage over time is an essential need for both basic and applied sciences in different research areas (Mazur, 2004; Pegg, 2007).

Despite the studies in the field it was not until 1972 when Mazur, Leibo and Whittingham (independently and simultaneously with Wilmut and Rowson) reported a scientific landmark event: the first ever live mouse pups born from cryopreserved embryos making the startpoint for the development of cryopreservation protocols for gametes of livestock for commercial purposes. The development of the science soon broaden with applications in human reproduction, conservation or aquaculture. The first reported livestock animal born from a cryopreservation procedure was a calf at Wilmut and Rowson laboratory in 1973. Sperm cryopreservation in fish was first reported more than 50 years ago (Blaxter, 1953) and the first marine invertebrate gametes cryopreserved were sea urchin sperm by Lanan (1971) on oyster sperm and Asahina and Takahashi (1977) (Figs. 1 and 2).

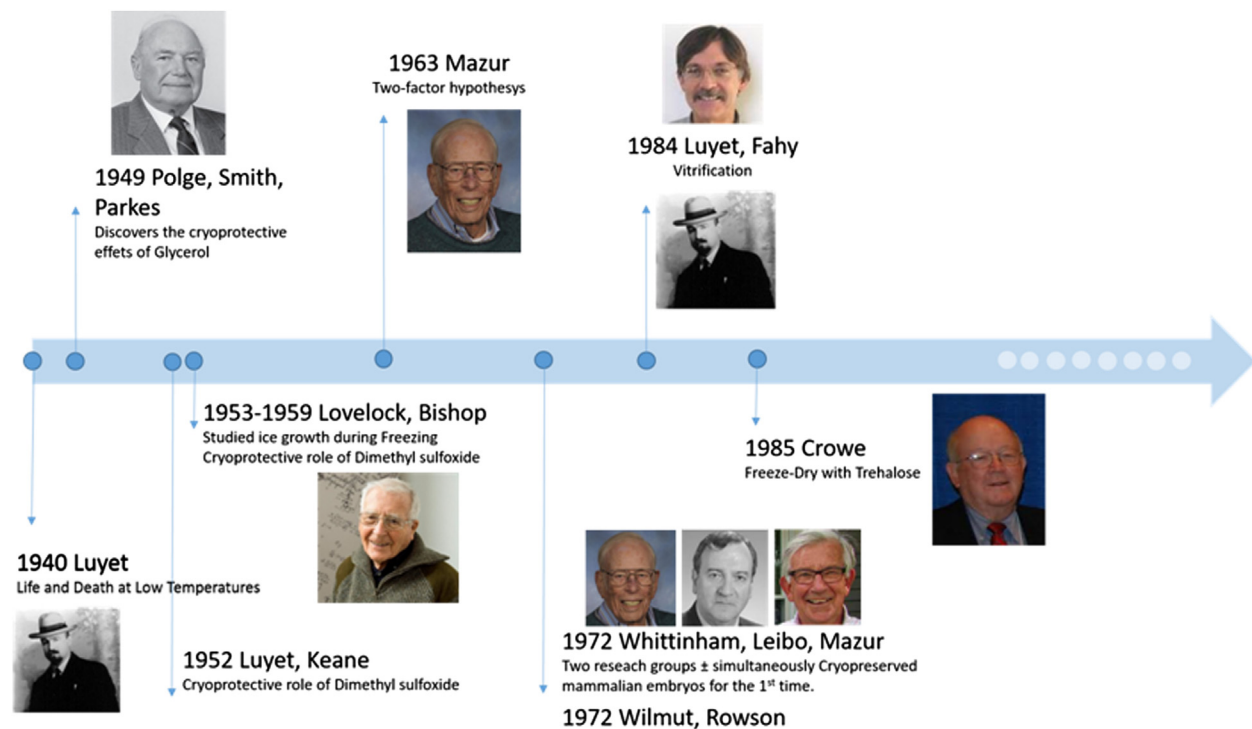


Fig. 1 Summary of important core events in the history of Cryobiology. References for the main papers related to those event are listed in the bibliography (Crowe *et al.*, 1985; Fahy *et al.*, 1984; Lovelock, 1953, 1954; Lovelock and Bishop, 1959; Luyet and Keane, 1952; Luyet and Geherio, 1940; Mazur, 1963; Polge *et al.*, 1949; Swanson *et al.*, 2007; Toledo *et al.*, 1989; Whittingham *et al.*, 1972; Wilmut, 1972; Wilmut and Rowson, 1973).

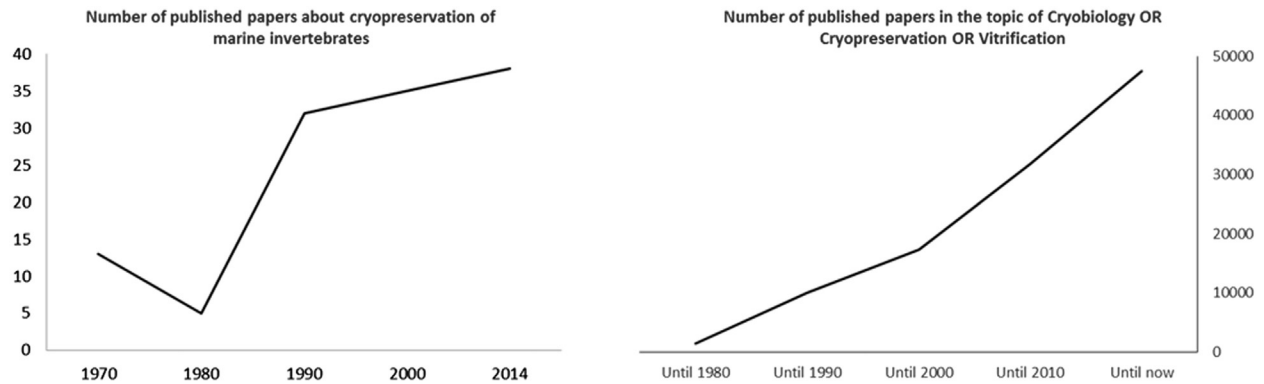


Fig. 2 Numbers of published papers per decade in the topic of Cryopreservation as a scientific area has grown steadily over the years (right), a focussed view on a smaller area of cryobiology as marine invertebrate cryopreservation, shows the same trend, the number of published papers (right) shows also an increase. Data obtained from PubMed And Scopus.

The aims of cryopreservation are very clear: to develop methods to preserve biological material structurally and functionally as intact as possible for future use. The range of uses is very wide, medical, species conservation and stock management,

Preservation of fundamental model organisms germplasm like mice (Critser and Mobraaten, 2000; Nakagata, 2000) and zebrafish (Hagedorn *et al.*, 1998; Zhang and Rawson, 1996; Khosla *et al.*, 2017) that will help maintain the different transgenic breeds securely whilst reducing the costs of time, space and cost of life breeding of all these lines along time.

Cryopreservation can be a very powerful biotechnological tool for aquaculture production, i.e. for fisheries conservation and wild stock enhancement (Zhang, 2004), for the production of all year-round spat supply without the need to condition broodstock (Adams *et al.*, 2011a,b) and for the improvement of selective breeding programs management (Adams *et al.*, 2011a,b; Zhang, 2004).

Principles of Cryopreservation

When cells are exposed to a solution containing a CPA, the differences in the osmotic pressure of the solution inside and outside the cell will cause an initial diffusion of water from the cell to equilibrate the osmotic pressures. At the same time, the higher concentration of the CPA in the external solution will cause the diffusion of the CPA into the cell. The process will stop once the concentration of the CPA equalize outside and inside the cell (Pegg, 2007) (Fig. 3).

The freezing point of the external solution is dependent on the solution media and the CPAs present. Once the cooling starts the external media will freeze before the cells and water inside will be under its thermodynamic freezing point. Water below its freezing point is defined as supercooled and has higher vapour pressure at a given temperature than the ice outside the cell (Dennison *et al.*, 2000; Mazur, 2004). As long as this difference in potential remains, water will slowly leave the cell and freeze externally and the cell will dehydrate. The dehydration will cause the concentration of solutes inside the cell to increase and the potential is reduced to reach the thermodynamic equilibrium and the cell will freeze without the formation of internal ice crystals which would be lethal (Fig. 2).

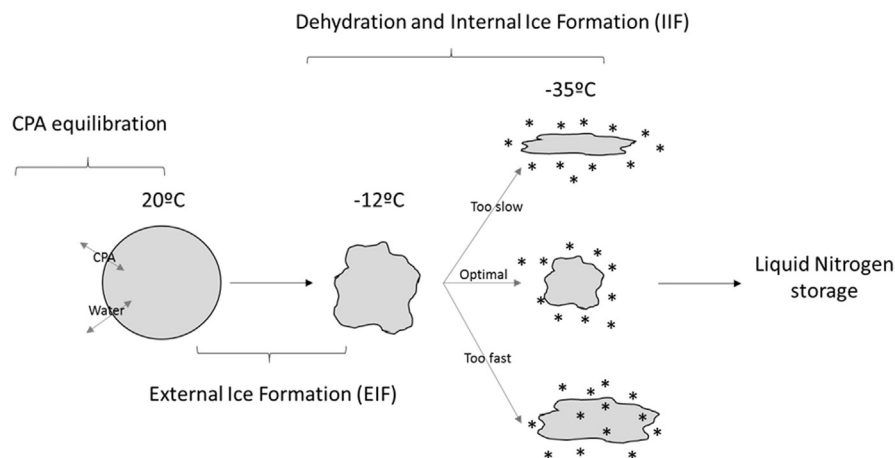


Fig. 3 Schematic representation of the most important physical-chemical processes during slow cooling cryopreservation. Modified from: Figure 1. Mechanisms of cryoinjury in living cells. Doyong Gao, Critser, J.K., 2000. ILAR Journal 41 (4), 187–196.

When cooling, the cell will slowly dehydrate due to the differences in water vapour pressure inside and outside the cell. If cells are cooled too slow, the cell would dehydrate too much, the concentration of internal solutes would increase and the whole process would cause irreparable damage to the cell that would not recover cell structure and functions afterwards. On the contrary, if cells are cooled too fast, water does not leave the cell becoming increasingly supercooled and when the Intracellular nucleation temperature is reached, the water will freeze internally. Optimal cooling rates are cell and species specific. Factors like membrane permeability, cell size or cell shape will determine the optimal cooling rate.

The cryopreserved cells or embryos will need to be stored in liquid nitrogen (-196°C) to ensure the conservation for as long as necessary (Leung, 1991; Leibo and Songssassen, 2002). At this low temperature no chemical reactions take place and cellular metabolism is on hold. Once cells are stored in liquid nitrogen their viability would only be affected by background radiation- which at normal level will take 2000 years to become a hazard to stored cells (Glenister *et al.*, 1984; Fig. 4).

Sperm Cryopreservation

Since Polge *et al.* (1949) discovered quite by accident the cryoprotective role of glycerol while working of the cryopreservation of sperm, sperm cryopreservation protocols have been developed more or less successfully for many organisms like Fish (Kopeika *et al.*, 2007; Cabrita *et al.*, 2010), mice (Jin and Mazur, 2015), marine invertebrates (Tiersch and Mazik, 2011; Adams *et al.*, 2004; Liu *et al.*, 2014), livestock (Curry, 2000), endangered wild animals (Fickel *et al.*, 2007). Sperm in general is easy to cryopreserve by slow cooling with the minimum handling possible because it is very sensitive to factors like handling, osmotic changes and drastic changes in temperature (Walters *et al.*, 2009). Sperm cryopreservation usually leads to reduced motility and therefore fertilization capability, in most cases the solution for increasing fertility is to increase the egg: sperm ratio carefully. Cryopreserved sperm can show reduced binding capacity and/or morphological changes that will also affect fertility, in these cases methodologies like Intracytoplasmic Sperm Injection (ICSI) might be the solution (Fig. 5).

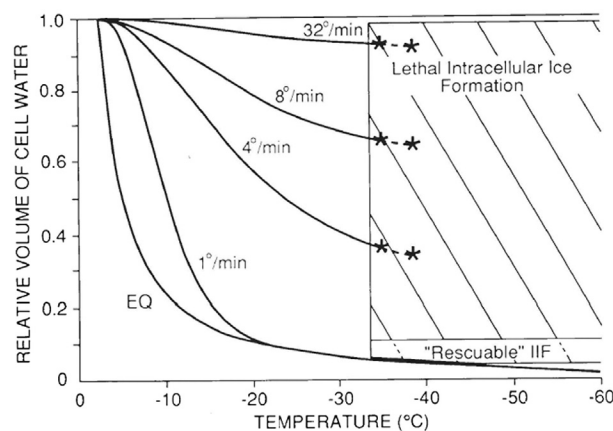


Fig. 4 The cooling rate is one of the factors that will determine if the cell can dehydrate at the right pace, following equilibrium (EQ). If the cooling rate is too fast, the volume of cell water will be too high and Lethal Internal Ice Formation (IIF) will take place. Cell size and shape, the volume of intracellular water, membrane composition and permeability are other factors which play a part in the movement of water and cryoprotectants in and out of the cell during cryopreservation. Figure from Mazur, P., Seki, S. Pinn, I.L., Kleinhans, F.K., Edashigue, K., 2005. Extra and Intra-cellular ice formation in mouse oocytes. *Cryobiology* 51(1), 29–63.

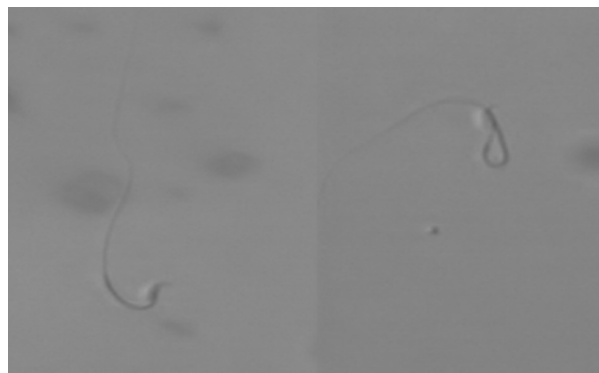


Fig. 5 ICR Mice sperm showing irreversible altered morphologies and membrane fusion (either curved sperm, which can be considered as a light distortion or sperm with a knot/hairpin shape, which is an extreme distortion) due to dehydration by exposure to 2M Sucrose for 2 min. Mice sperm total length is around 122 μm .

Developed sperm cryopreservation for model organisms: mice, primates, freshwater and marine fish, marine invertebrates.

Oocyte Cryopreservation

Oocyte cryopreservation had been a complicated task. In general, less work has been carried out with oocytes due to their chilling-sensitivity and their large yolk content. There are no published protocols with more than a symbolic survival described for fish oocytes or marine invertebrate oocytes for example. Mammals are the exception. The reason for these differences in survival between species to cryopreservation can be explained by the study of several factors: oocyte size, membrane composition, membrane permeability and % of yolk and water. Lipid content of oocytes and embryos is an important parameter linked to both quality and cryotolerance. Physical changes of lipids submitted to freezing temperatures are among the major causes of cellular cryodamages (Pereira and Marques, 2008).

Developed oocyte cryopreservation for model organisms: mice, primates.

Embryo and Larvae Cryopreservation

Cryopreservation of embryos and larvae as an alternative to oocyte cryopreservation have given more promising results in many cases, in special within the marine environment (Asahina and Takahashi, 1979; Olive and Wang, 1997; Toledo *et al.*, 1989; Grout and McFadden, 1991; Lin *et al.*, 1999; Wang *et al.*, 2011; Paredes *et al.*, 2015) but the cryopreservation of more developed stages brings also new challenges. Both oocytes and embryos show high sensitivity to sub-zero temperatures, sensitivity to CPA exposure and lower permeability than sperm, embryos and larvae present sometimes very complex membrane systems and compartments that complicate CPA and water transportation (Fig. 6).

Developed embryo/larvae cryopreservation for model organisms: mice, primates, drosophila (Mazur *et al.*, 1992), marine invertebrates.

Future Lines of Research

Fundamental and hypothesis driven research will continue to increase in cryopreservation helping us understand all the steps and crucial points of the process. More research is needed in order to optimize and standardize cryopreservation procedures for many organism's gametes. The answers about lethal and sub lethal cryoinjuries related to cryopreservation would probably require a molecular point of view. The understanding of how metabolism standby happens and, more importantly, what happens after thawing at the molecular level, will help us to understand organogenesis and cryoinjuries that could be responsible for the damage of those cells that could not survive after cryopreservation or show non-lethal cryoinjuries.

We still lack of basic information about membrane permeability and osmotic tolerances of different cells and species. There will be a movement from classical cryoprotectants like Me₂SO or Gly towards the use of antifreeze proteins from freezing tolerant organisms or engineered CPAs with less toxicity and higher membrane permeability, this line of research would be specifically interesting for organism's cell types/species which manifest high sensitivity to CPA exposure.

Sperm cryopreservation still has to address the issue of post-thawing sperm damage, both the causes and possible minimization that will increase sperm performance and therefore fewer spermatozoa will be needed per fertilization round. Oocytes have proven to be more difficult to cryopreserve than other cellular types, and despite numerous trials and promising results, the oocyte cryopreservation of several organisms will continue to be a very active research line, specifically due to their chilling sensitivity. Embryos and larvae will continue be studied focusing attention on cryoinjuries, like growth and morphology modifications, activity post thawing and low larval development in order to increase long term survival.

In special, vitrification will be profusely addressed for all species and cell types within the next decade, and vitrification procedures and mechanisms will continue to develop and increase survival, this will be very important for chilling-sensitive cells as oocytes and embryos.

Vitrification involves converting water into a non-crystalline or amorphous glass. To achieve this conversion, the initial view has been that cells have to be immersed in solutions containing a very high concentration of glass-inducing solutes (typically ≥ 6 molal) which was highly toxic and in general, they have to be cooled very rapidly (typically $\geq 50,000^\circ\text{C}/\text{min}$). The belief that rapid cooling was essential meant that the volumes of cell suspensions being cooled had to be very small and the carriers or containers had to have very small masses (Kuwayama, 2005). Fahy, 1987, Boutron and Mehl, 1990, later the Mazur's group (Seki and Mazur, 2008 and, 2009) or even more recently (Papatheodorou *et al.*, 2013; Panagiotidis *et al.*, 2013; Schiewe *et al.*, 2015) that studied and compared survivals using open and closed vitrification devices, reported that the high survivals reported were due to the rapid warming of those samples and not the fast cooling.

The especially high sensitivity to warming rate found, strongly suggests that the lethality of slow warming is either a consequence of the crystallization of intracellular glassy water during warming or the growth of small intracellular crystals formed during cooling by their recrystallization during slow warming (Mazur and Seki, 2011).

Recent work in vitrification and fast rewarming had led to the reporting of interesting advances in the cryopreservation of important model species. Seki *et al.* (2014) and Jin and Mazur (2015) have reported that when the vitrification solution contains only the non-permeating solute (sucrose), survivals after vitrification and laser-induced ultra-rapid warming are also about 90% for

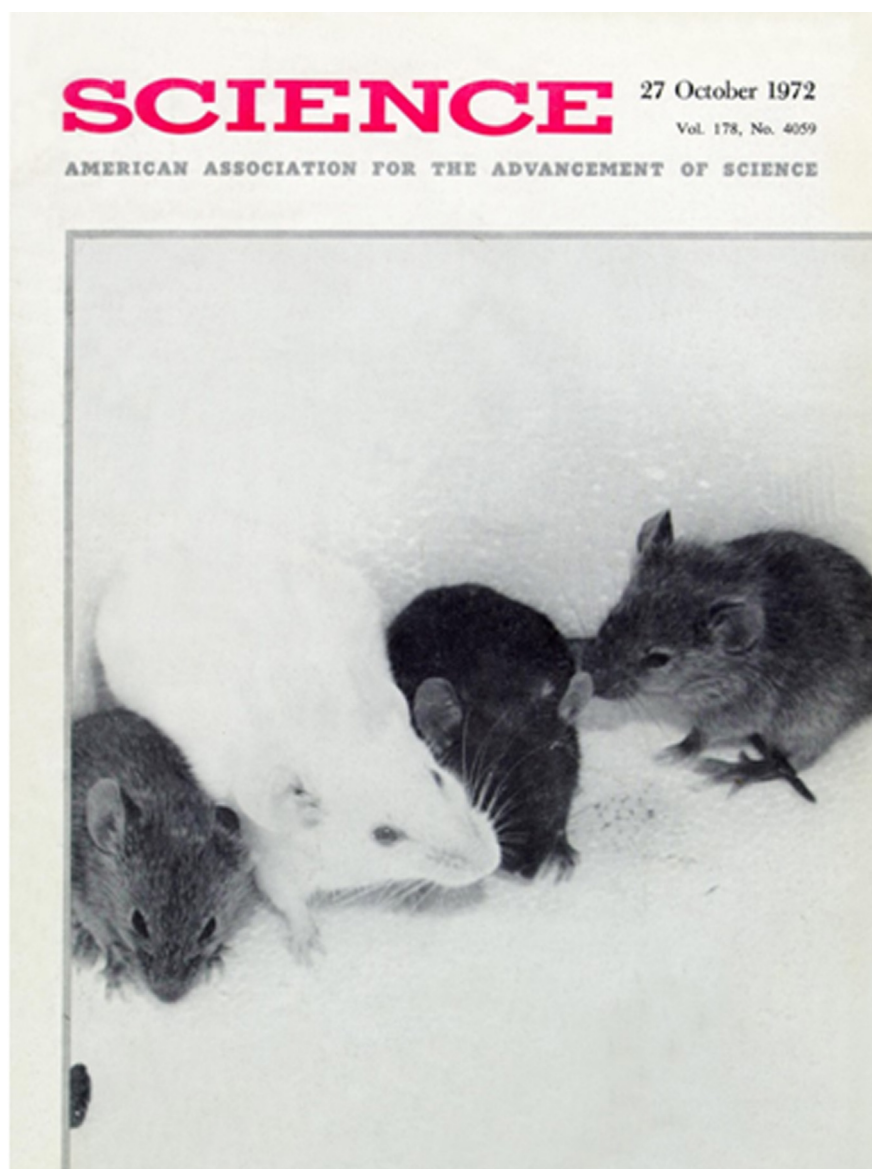


Fig. 6 Cover of Science from October 1972 reporting on the cryopreservation of mice embryos for the First time by Whittingham, Leibo and Mazur. The cryopreserved and thawed embryos were later implanted and in the picture you can see the recipient mother and the pups born from the cryopreserved embryos.

mice embryos and oocytes. Paredes (2016) reported the high survivals of yeast also using the same methodology. Khosla *et al.* (2017) reported the vitrification and fast rewarming of Zebrafish embryos that survived up to 5 days post-vitrification using gold nanoparticles heated by a laser in order to achieve high warming temperatures. More advances will be produced by the use of these methods in the following years.

References

- Adams, S. L., Smith, J. F., Roberts, R. D., et al. (2004). Cryopreservation of sperm of the Pacific oyster (*Crassostrea gigas*): development of a practical method for commercial spat production. *Aquaculture*, 242, 271–282.
- Adams, S. L., Smith, J. F., Tervit, H. R., et al. (2011b). Cryopreservation of molluscan sperm: Oyster (*Crassostrea gigas*, thunberg), mussel (*Perna canaliculus*) and abalone (*Haliotis iris*). In T. R. Tiersch, & C. C. Green (Eds.), *Cryopreservation in Aquatic Species* (second ed., pp. 562–573). Baton Rouge, LA: World Aquaculture Society.
- Adams, S. L., Tervit, H. R., Salinas-Flores, L., et al. (2011a). Cryopreservation of Pacific oyster oocytes. In T. R. Tiersch, & C. C. Green (Eds.), *Cryopreservation in Aquatic Species* (second ed., pp. 616–623). Baton Rouge, LA: World Aquaculture Society.
- Asahina, E., & Takahashi, T. (1977). Survival of sea urchin spermatozoa and embryos at very low temperatures. *Cryobiology*, 14(1977), 703.
- Asahina, E., & Takahashi, T. (1979). Cryopreservation of sea urchin embryos and sperm. *Development, Growth & Differentiation*, 21, 423–430.
- Blaxter, J. (1953). Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature*, 172, 1189–1190.
- Boutron, P., & Mehl, P. (1990). Theoretical prediction of devitrification tendency: Determination of critical warming rates without using finite expansions. *Cryobiology*, 27, 359–377.

- Cabrita, E., Sarasquete, C., Martínez-Paramo, S., et al. (2010). Cryopreservation of fish sperm: Applications and perspectives. *Journal of Applied Ichthyology*, 26, 623–635.
- Critser, J. K., & Mobraaten, L. E. (2000). Cryopreservation of murine spermatozoa. *ILAR J.*, 41(4), 197–206.
- Crowe, L. M., Crowe, J. H., Rudolph, A., Womersley, C., & Appel, L. (1985). Preservation of freeze-dried liposomes by trehalose. *Archives Biochemistry Biophysics*, 242–1, 240–247.
- Curry, M. R. (2000). Cryopreservation of semen from domestic livestock. *Oxford Reviews of Reproductive Biology*, 5(1), 46–52.
- Dennison, R. S., Michelet, S., & Godke, R. A. (2000). Principles of cryopreservation. In T. R. Tierch, & P. M. Mazik (Eds.), *Cryopreservation in Aquatic species* (pp. 59–74). World Aquaculture Society.
- Fickel, J., Wagener, A., & Ludwig, A. (2007). Semen cryopreservation and the conservation of endangered species. *European Journal Wildlife Research*, 53, 81–89.
- Fahy, G. M. (1987). Biological effects of vitrification and devitrification. In D. E. Pegg, & A. M. Karow, Jr. (Eds.), *The Biophysics of Organ Cryopreservation* (pp. 265–297). New York: Plenum Press.
- Fahy, G. M., McFarlane, D. R., Angell, C. A., & Meryman, H. T. (1984). Vitrification as an approach to cryopreservation. *Cryobiology*, 21, 407–426.
- Glenister, P. H., Whittingham, D. G., & Lyon, M. F. (1984). Further studies on the effect of radiation during the storage of frozen 8-cell mouse embryos at -196°C . *Journal of Reproduction and Fertility*, 70, 229–234.
- Grout, B.W.W., McFadzen, I.R.B., 1991. Biological cryopreservation. Patent application PCT/GB90/01267.
- Hagedorn, M., Kleinhans, F. W., Artemov, D., & Pilatus, U. (1998). Characterization of a major permeability barrier in the zebrafish embryo. *Biology of Reproduction*, 59, 1240–1250.
- Jin, B., & Mazur, P. (2015). High survival of mouse oocytes/embryos after vitrification without permeating cryoprotectants followed by ultra-rapid warming with an IR laser pulse. *Scientific Reports*, 5, 9271.
- Khosla, K., Wang, Y., Hagedorn, M., Qin, Z., & Bischof, J. (2017). Gold nanorod induced warming of embryos from the cryogenic state enhances viability. *ACS Nano*. Available at: <https://doi.org/10.1021/acsnano.7b02216>.
- Kopeika, E., Kopeika, J., & Zhang, T. (2007). Cryopreservation of fish sperm. *Methods Molecular Biology*, 368, 203–217.
- Kuwayama, M. (2005). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reproductive BioMedicine*, 11 v5, 608–614.
- Lanan, J. E. (1971). Experimental self-fertilization of the Pacific oyster *Crassostrea gigas*, using cryopreserved sperm. *Genetics*, 68, 599–601.
- Leung, L. K. P. (1991). Principles of biological cryopreservation. In L. K. P. Leung, & B. G. M. Jamieson (Eds.), *Fish evolution and systematics: Evidence from spermatozoa* (pp. 231–244). New York: Cambridge University Press.
- Leibo, S. P., & Songsassen, N. (2002). Cryopreservation of gametes and embryos of non-domestic species. *Theriogenology*, 57, 303–326.
- Lin, T. T., Chao, N. H., & Tung, N. H. (1999). Factors affecting survival of cryopreserved oyster (*Crassostrea gigas*) embryos. *Cryobiology*, 39, 192–196.
- Liu, Y., Xu, T., Robinson, N., Qin, J., & Li, X. (2014). Cryopreservation of sperm in farmed Australian greenlip abalone *Haliotis laevigata*. *Cryobiology*, 68, 185–193.
- Lovelock, J. (1953). The haemolysis of human red blood cells by freezing and thawing. *Biochimica et Biophysica Acta*, 10, 414–426.
- Lovelock, J. (1954). The protective action of neutral solutes against haemolysis by freezing and thawing. *Biochemical Journal*, 56, 265–270.
- Lovelock, J., & Bishop, M. (1959). Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature*, 183, 1394–1395.
- Luyet, B., & Keane, J. (1952). Comparative efficiency of ethylene glycol, glucose and sodium chloride in protecting tissues against freezing injury. *Biodynamica*, 7, 119–131.
- Luyet, B.J., Geherio, M.P., 1940. Life and Death at low temperatures. No 1, Biodynamica, Normandy, Missouri.
- Mazur, P. (1963). Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *Journal of General Physiology*, 47, 347–369.
- Mazur, P. (1970). Cryobiology: The freezing of biological systems. *Science*, 168, 939–949.
- Mazur, P. (1977). The role of intracellular freezing in the death of cells cooled at supraoptimal rates. *Cryobiology*, 14, 251–272.
- Mazur, P. (1980). Limits to life at low temperatures and at reduced water contents and water activities. *Origins of Life*, 10, 137–159.
- Mazur, P. (2004). Principles of Cryobiology. In B. J. Fuller, N. Lane, & E. E. Benson (Eds.), *Life in the Frozen State* (pp. 3–65). CRC Press.
- Mazur, P., Cole, K., Hall, J., Schreuders, P., & Mahowald, A. (1992). Cryobiological preservation of *Drosophila* embryos. *Science*, 258(5090), 1932–1935.
- Mazur, P., & Seki, S. (2011). Survival of mouse oocytes after being cooled in a vitrification solution to -196°C at 95° to $70,000^{\circ}\text{C}/\text{min}$ and warmed at 610° to $118,000^{\circ}\text{C}/\text{min}$: a new paradigm for cryopreservation by vitrification. *Cryobiology*, 62, 1–7.
- Muldrew, R. K., Acker, J. P., Elliot, J. A. W., & McGann, L. E. (2004). The water to ice transition: implications for living cells. In B. J. Fuller, N. Lane, & E. E. Benson (Eds.), *Life in the frozen state* (pp. 67–108). CRC Press.
- Nakagata, N. (2000). Cryopreservation of mouse spermatozoa. *Mammalian Genome*, 11, 572–576.
- Olive, P. J. W., & Wang, W. B. (1997). Cryopreservation of *Nereis virens* (Polychaeta, Annelida) Larvae: The Mechanism of Cryopreservation of a Differentiated Metazoan. *Cryobiology*, 34, 284–294.
- Panagiotidis, Y., Vanderzwalmen, P., Prapas, Y., et al. (2013). Open versus closed vitrification of blastocysts from an oocyte-donation programme: a prospective randomized study. *Reproductive BioMedicine Online*, 26, 470–476.
- Papatheodorou, A., Vanderzwalmen, P., Panagiotidis, Y., et al. (2013). Open versus closed oocyte vitrification system: A prospective randomized sibling-oocyte study. *Reproductive BioMedicine Online*, 26, 595–602.
- Paredes, E. (2016). Vitrification and ultra-rapid laser warming of yeast *Saccharomyces cerevisiae*. *Cryobiology*, 73–3, 421–422.
- Paredes, E., Bellas, J., & Costas, D. (2015). Sea urchin (*Paracentrotus lividus*) larval rearing – Culture from cryopreserved embryos. *Aquaculture*, 43–7, 366–369.
- Pegg, D.E., 2007. Principles of cryopreservation. In: Day, J.G., Stacey, G.N. (Eds.), Cryopreservation and freeze-drying protocols. In: Methods in Molecular Biology, vol. 368, pp. 39–57.
- Pereira, R. M., & Marques, C. C. (2008). Animal oocyte and embryo cryopreservation. *Cell Tissue Bank*, 9–4, 267–277.
- Polge, C., Smith, A. U., & Parkes, A. S. (1949). Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*, 164(4172), 666.
- Schiewe, M. C., Zozula, S., Anderson, R. E., & Fahy, G. M. (2015). Validation of microSecure vitrification (IS-VTF) for the effective cryopreservation of human embryos and oocytes. *Cryobiology*, 71 v2, 264–272.
- Seki, S., Jin, B., & Mazur, P. (2014). Extreme rapid warming yields high functional survivals of vitrified 8-cell mouse embryos even when suspended in a half-strength vitrification solution and cooled at moderate rates to -196°C . *Cryobiology*, 68, 71–78.
- Seki, S., & Mazur, P. (2008). Effect of warming rate on the survival of vitrified mouse oocytes and on the recrystallization of intracellular ice. *Biology of Reproduction*, 79(2008), 727–737.
- Seki, S., & Mazur, P. (2009). The dominance of warming rate over cooling rate in the survival of mouse oocytes subjected to a vitrification procedure. *Cryobiology*, 59, 75–82.
- Swanson, W. F., Magarey, G. M., & Herrick, J. R. (2007). Sperm cryopreservation in endangered felids: developing linkage of in situ-ex situ populations. *Society of Reproduction Fertility Supply*, 65, 417–432.
- Tiersch, T. R., & Mazik, P. M. (2011). *Cryopreservation in Aquatic Species*. In T. R. Tiersch, & C. C. Green (Eds.) (second ed.). Baton Rouge, LA: World Aquaculture Society.
- Toledo, J. D., Kurokura, H., & Kasahara, S. (1989). Preliminary studies on the cryopreservation of the blue mussel embryos. *Nippon Suisan Gakkaishi*, 1661.
- Walters, E. M., Benson, J. D., Woods, E. J., & Critser, J. K. (2009). The history of sperm cryopreservation. In A. A. Pacey, & M. J. Tomlinson (Eds.), *Sperm banking: theory and practice*. Cambridge University Press.
- Wang, H., Li, X., Wang, M., et al. (2011). Effects of larval cryopreservation on subsequent development of the blue mussels, *Mytilus galloprovincialis* Lamarck. *Aquaculture Research*, 42, 1816–1823.
- Whittingham, D. G., Leibo, S. P., & Mazur, P. (1972). Survival of mouse embryos frozen to -196°C and 269°C . *Science*, 178, 411–414.

- Wilmut, T. (1972). Effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mice embryos during cooling and thawing. *Life Sciences*, 11(part 2), 1071–1079.
- Wilmut, T., & Rowson, L. E. A. (1973). Experiments on the low temperature preservation of cow embryos. *Veterinary Research*, 92, 686–690.
- Zhang, T. (2004). Cryopreservation of gametes and embryos of aquatic species. In B. J. Fuller, N. Lane, & E. E. Benson (Eds.), *Life in the Frozen State* (pp. 415–436). CRC Press.
- Zhang, T., & Rawson, D. M. (1996). Feasibility studies on vitrification of intact zebrafish (*Brachydanio rerio*) embryos. *Cryobiology*, 33, 1–13.

Further Reading

- Fuller, B. J., Lane, N., & Benson, E. E. (Eds.). (2004). *Life in the Frozen State*. CRC Press.
- Day, J. G., & Stacey, G. N. (2007). *Cryopreservation and Freeze-Drying Protocols*. Humana Press Springer Science & Business Media.
- Tiersch, T. R., & Green, C. C. (Eds.). (2011). *Cryopreservation in Aquatic Species* (second ed.). Baton Rouge, LA: World Aquaculture Society.

Relevant Websites

- <https://www.journals.elsevier.com/cryobiology>. – Cryobiology Journal.
- <http://www.cryoletters.org/>. – Cryoletters Journal.
- <http://www.societyforcryobiology.org/>. – Society for cryobiology.
- <http://www.slbtb.info/>. – Society for Low temperature Biology.

Reproductive Cloning

Mark Westhusin and Charles Long, College of Veterinary Medicine and Biomedical Sciences, College Station, TX, United States

© 2018 Elsevier Inc. All rights reserved.

Reproductive Cloning

The goal of reproductive cloning is to produce one or more genetically identical animals that carry the same genetic makeup (genotype). Many examples of genetically identical offspring can be found in nature and range from monozygotic (identical) twins in humans to the seven-banded armadillo that can produce up to 15 genetically identical pups in a single litter. These identical animals are formed from the division of a single embryo into two or more parts, each of which can develop into a fully formed individual. Although these animals are genetically identical, they are technically not considered clones as they result from an embryo produced sexually (the combination of the genetic material of a sperm and egg) that somehow splits into several embryos during development. Reproductive cloning involves the production of genetically identical animals by employing a specific technology developed by scientists, nuclear transplantation.

In its simplest form, nuclear transplantation refers to the transfer of the nucleus (chromosomes/DNA) of one cell into another. The utilization of this technique to produce cloned animals involves transferring the nucleus of a single cell derived from the animal being cloned (cell donor), into an unfertilized oocyte collected from a female (preferably from the same animal species), and that has had its own DNA removed (enucleated oocyte). When this happens, components contained within the cytoplasm of the enucleated oocyte have the ability to reprogram the donated nucleus such that it resets the developmental clock back to the one-cell stage of development, similar to an egg just after fertilization by a sperm cell, prior to the first cell division. If successful, the reconstructed embryo derived from this process will begin to develop and can be transferred into the reproductive tract of a surrogate mother where it will develop to term and result in an animal with the same genetic traits as the cell donor (Figs. 1 and 2.)

Historical Overview

The idea of developing methods to produce, and reproduce genetically identical animals, has been around since the early 1900s. Hans Spemann, often referred to as the “father of cloning”, performed extraordinary studies involving nuclear transplantation using cells derived from developing salamander embryos, resulting in the production of genetically identical offspring. He later referred to a “fantastical experiment” in his book, *Embryonic Development and Induction* (1938), where he outlined the process of nuclear transplantation, and described the basic methodology still used today to clone animals (Speman, 1938).

At the time Spemann described this procedure, the methodology and equipment needed to perform such a difficult task were not available. Therefore, it was not until the early 1950s that Robert Briggs and Thomas King were able to perform such experiments. Using a small glass pipet they removed the DNA from a *Rana pipiens* egg, replaced it with the nucleus from a blastula stage embryo, and demonstrated that cloned frogs could be produced by transferring nuclei obtained from early stage embryos into enucleated oocytes (Briggs and King, 1952).

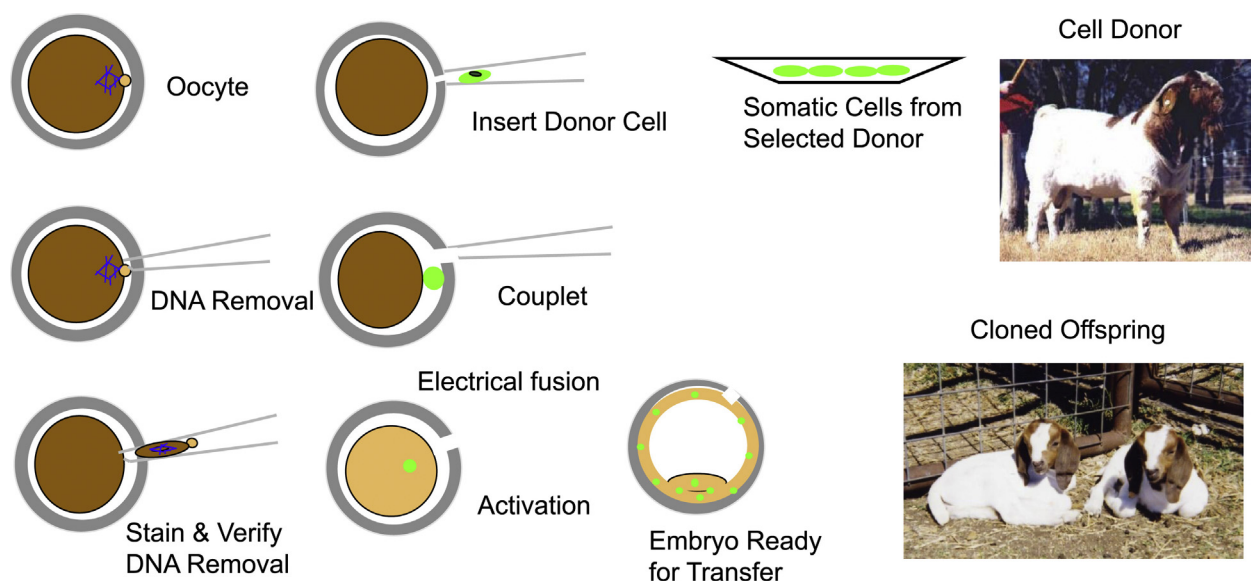


Fig. 1 Reproductive cloning by nuclear transplantation.

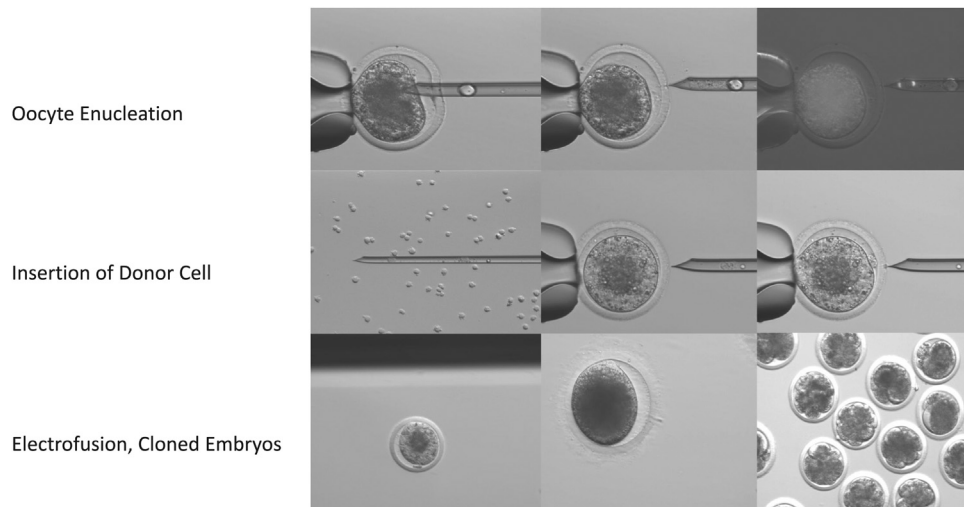


Fig. 2 Reproductive cloning by nuclear transplantation.

It is important to note that cells utilized for cloning in these early experiments were derived from growing embryos rather than adult animals. The dogma at that time was embryonic cells could be used for cloning because the cells had not yet differentiated into a specific cell type and thus remained totipotent and capable of developing into any tissue in the body. However, a major breakthrough, supporting the idea that nuclear transplantation could potentially be utilized to clone adult animals using cells that were more differentiated came in 1962 when John Gurdon reported the utilization of nuclear transfer to produce cloned frogs using cells obtained from the gut of feeding tadpoles. The transferred nucleus in these experiments had originated from cells that had clearly differentiated into a somatic cell type and thus dispelled the prevailing theory that nuclei were incapable of directing development after differentiation. The importance of this work was recognized by Gurdon being awarded a Nobel Prize in 2012 ([Gurdon, 1962](#)).

While work with amphibians was being carried out in the early and mid-1900s, it was not until the late 1970s that any significant work involving the utilization of nuclear transfer to try and clone mammals was performed. This was likely due to the much smaller size of mammalian eggs compared to amphibians, limitations in the equipment and difficulties with adapting the technology to other species. Procedures had to be developed that would allow micromanipulation of individual cells, ova and embryos in addition to the expertise needed to transfer cloned embryos into the reproductive tract of the appropriate surrogate mother. Moreover, simply obtaining large numbers of oocytes from mammals that could be utilized for experiments involving nuclear transfer was in itself a challenge.

The great majority of early work with nuclear transfer in mammals involved the manipulation of ova and embryos derived from mice. Peter Hoppe and Karl Illmensee reported the successful production of cloned mice by transferring cells derived from the inner cell mass of a developing embryo into enucleated zygotes, then transferring the cloned embryos into recipient females ([Illmensee and Hoppe, 1981](#)). However, many attempts by other laboratory groups failed to repeat this work; and in 1984 Jim McGrath and Davor Solter published the results of their research stating that “cloning mammals by simple nuclear transfer was biologically impossible” ([McGrath and Solter, 1984](#)). Solter, McGrath and other scientists working with mice at that time, again thought the problem was related to embryonic differentiation. In spite of the experiments performed previously by Gurdon that demonstrated differentiated cells could be used for cloning frogs. Researchers working with mice hypothesized that in mammals, as embryonic cells became more differentiated, they could not be used for cloning. McGrath and Solter were successful at producing cloned mice by nuclear transplantation when 2-cell embryos were used as nucleus donors, but not when nuclei were derived from the 4-cell stage or beyond. In mice, the 2-cell stage of development represents the time at which the embryonic genome is activated; in other words, the first time in mouse development when cells actually begin to use their own genes to transcribe mRNA, produce new protein and differentiate into unique cell types.

Early work in other mammalian species continued to support the idea that differentiated cells sequentially lose the ability to regain totipotency following nuclear transfer. Steen Willadsen reported the successful cloning of sheep, and then cattle, using 8–16 cell embryos as nuclei donors ([Willadsen, 1986](#)). In terms of development, this is similar to mice, as maternal/zygotic transition, the time point in development when an embryo starts producing its own mRNA and protein (embryonic genome activation), begins at the 2-cell stage in mice and the 8–16 cell stage in sheep and cattle.

Willadsen’s work with cattle, a species with substantial economic value, no doubt played the major role in efforts to move cloning from the research laboratory to commercial application. The initial business model was based on cloning to propagate (replicate) cattle representing the top 5%–10% in terms of phenotypic performance. If cloning could be used to increase the number of animals representing genetics of the top producers, large increases could be made in the efficiency of milk and meat production.

In the late 1980s, Willadsen moved from England to the United States to work for Granada Genetics Inc., and commercialize cloning in cattle. His efforts proved extremely successful, however, he left Granada after only about a year and took his talents

to Canada where he started a similar program focused on cloning cattle for Alta Genetics. Granada, Alta, and shortly thereafter American Breeders Services (ABS) represented the first 3 commercial entities in the world to offer commercial cloning to the cattle industry.

Early on, this approach proved to be viable, and a large number of cloned cattle were produced for various cattle producers. However, a number of unanticipated problems also occurred, and the dream of producing large numbers of cloned livestock representing the top individuals of beef or dairy cattle and utilized for the production of food and fibre never materialized. A major problem that interfered with the large scale production of clones was founded in the inefficiency of nuclear transfer when measured by the actual number of live offspring produced. Regardless of the countless experiments that were conducted to try and improve the process, the great majority of embryos produced by nuclear transfer failed to result in a viable pregnancy and/or a normal calf. This problem could be overcome by simply producing and transferring more cloned embryos. However, it soon became clear that in most cases, the costs far outweighed the benefits. Only 10%–20% of embryos produced by nuclear transplantation resulted in a viable pregnancy. In addition, another serious and unexpected problem was that a significant number (approximately 30%) of the offspring born as a result of cloning exhibited abnormal development, most notably, large birth weights associated with placental anomalies. It was not uncommon to see calves born weighing almost 2 X normal, which caused significant health problems at birth for both the calf and surrogate mothers (Wilson *et al.*, 1993). As a result, all recipient cows carrying cloned pregnancies had to be carefully monitored and many needed assistance at birth. This was simply unacceptable to the cattle industry. A final issue that also clearly played a major role in the failure to adopt cloning to produce large numbers of high-producing cattle was the unexpected observation that even though the animals were genetically identical, clones could exhibit remarkable differences in their phenotypes. As mentioned above, this first became obvious when looking at birth weights and the enormous variation that resulted. However, as more cloned animals were born, including not only cattle but sheep, goats, pigs, horses, etc., it was clear that while the genotypes were identical, the phenotypes were not. The cause of this was not clear and remains uncertain even today. However, obvious culprits include the environment in which fetal development occurs and unexplained differences in nuclear reprogramming within the oocyte needed to support normal development. In addition, since cloned animals were derived using enucleated oocytes collected from other animals, the possibility existed that the mitochondria which are exclusively inherited through the cytoplasm of the ova were also having an effect on the final phenotype.

Although various problems with producing cloned livestock using cells derived from early stage embryos prevented the large scale utilization of this technology for production agriculture, other potential applications continued to drive the research and development of cloning. The primary interest changed from using the technology to produce large numbers of genetically identical animals to producing a few, or even “one” animal representing for example, a genotype that was extremely rare or had been lost (i.e., unique animals of extremely high value that had died or were close to death, endangered and extinct animal species). Although clones did not always exhibit similar phenotypes, their genotypes were identical, therefore clones were extremely useful for breeding, thus a powerful tool that could be used for the conservation and/or propagation of a particular genotype. The other driving force was founded in the utilization of cloning as a potential tool for producing genetically modified animals. However, both of these applications required methods that would allow the utilization of genetically identical cells that had differentiated beyond embryonic, and which could be expanded in culture to obtain large numbers of cells.

One of the first key experiments which provided strong support for addressing this challenge and test the hypothesis that differentiated cells could be reprogrammed by nuclear transplantation and used to produce cloned animals was reported by Sims and First in 1994 and again by Campbell *et al.* (1996). Sims and First derived cell lines from the inner cell mass of a blastocyst stage bovine embryo and cultured these cells using methods and conditions designed to expand the cell population while at the same time maintain pluripotency (Sims and First, 1994). Campbell *et al.* utilized the embryonic disc of a sheep embryo that during culture differentiated to an epithelial type cell (Campbell *et al.*, 1996). In both cases, nuclear transfer to an enucleated oocyte produced live offspring, proving that differentiation in vitro during cell culture did not prevent reprogramming to a totipotent state when the nucleus of these cells was transferred into an enucleated oocyte. In short, differentiated cells growing in culture could be successfully utilized for reproductive cloning of mammals, a situation similar to that demonstrated by Jon Gurdon when using cells derived from frogs, over 30 years earlier.

From a historical point of view these initial experiments involving cells growing in culture predicted that reproductive cloning with cells derived from a living adult animal would soon follow; and in 1997, the scientific community, indeed the entire world was shocked by the announcement that a living mammal had been cloned (Wilmut *et al.*, 1997). The birth of a cloned sheep derived by employing nuclear transplantation and a single mammary epithelial cell obtained from an adult animal was reported. Being a female (ewe) and given the cell type utilized for cloning, she was named “Dolly” (Fig. 3). The birth of Dolly, as with many great scientific breakthroughs represented a serendipitous surprise. Keith Campbell and Ian Wilmut were using fetal cells (fibroblasts) growing in culture for nuclear transfer, with the primary objective being to genetically modify the cells prior to nuclear transfer so to produce transgenic sheep. In one series of experiments, adult cells derived from mammary epithelia were utilized as a control treatment and not expected to develop to term, but resulted in the birth of Dolly.

It is clear that the birth of Dolly was a complete surprise to everyone involved. The overwhelming response from the general public and the world as a whole was also surprising given the long history of cloning animals and having previously produced cloned sheep and cattle from cells growing in culture. The major difference of course was that Dolly was derived from a cell obtained from an adult animal and as such it revived all sorts of scary scenarios about the possibility of cloning humans. The idea of being able to clone human beings was now too close to reality, sending a shock-wave throughout society. This resulted in an avalanche of controversy and ethical debates all over the world, not to mention legal actions quickly being taken at all levels of government to

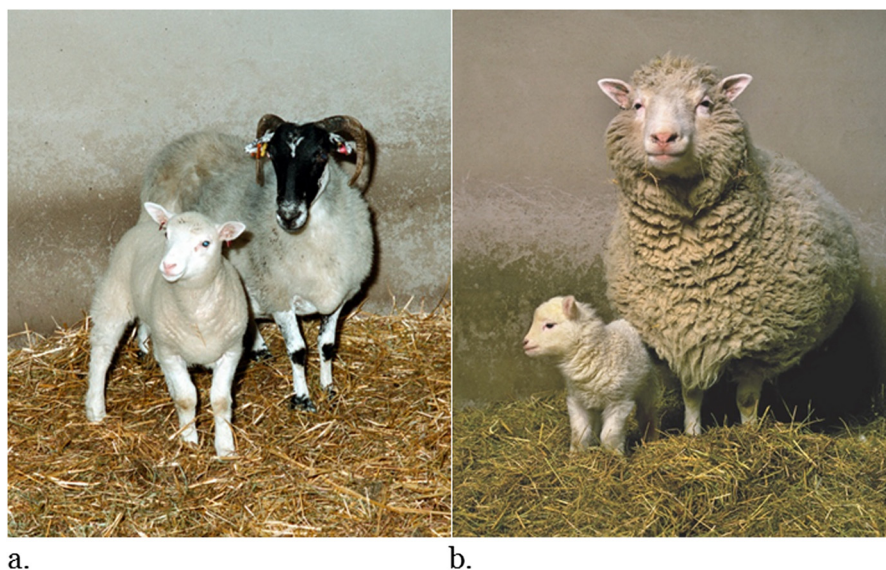


Fig. 3 (a) World's first cloned mammal, a sheep named Dolly derived from a mammary epithelial cell collected from an adult animal. The black-faced sheep is her surrogate mother (Wilmut *et al.*, 1997). (b) Dolly as an adult and her first lamb. Photo courtesy of The Roslin Institute, The University of Edinburgh and in the Acknowledgments "Photo courtesy of The Roslin Institute, The University of Edinburgh, Roslin, Scotland, United Kingdom."

ban human reproductive cloning. Even now, legislation to regulate the application of reproductive cloning is in place and enforced throughout the world, with some countries even banning reproductive cloning of animals. The birth of Dolly resulted in an immediate and enormous increase in research conducted all over the world, focused on the utilization of reproductive cloning to produce genetically identical animals. Dozens of laboratory groups launched efforts to adapt methods involving nuclear transplantation in attempts to clone a wide variety of different animal species. Cloned animals representing more than 20 different species have now been produced by reproductive cloning, providing strong evidence for the robustness of nuclear transplantation as a tool for producing genetically identical animals.

Current State of the Art

Most reproductive cloning conducted today is focused on applications to livestock species and companion animals. Advancements in the technology have made it possible to produce cloned animals using almost any cell type that can be collected from an adult animal, however, the most common is fibroblasts derived by employing standard methods for tissue culture to a skin biopsy. Also, as indicated above, the goal in the vast majority of cases is not to produce large numbers of genetically identical animals, rather to obtain just a few, even one, of a very unique animal with a highly valued genotype. Examples of this include cloning prize winning steer to obtain a bull(s) that can then be used for breeding, cloning deceased pets, and even cloning animals that are deceased but which had cells cryopreserved for storage years prior to the time cloning was even thought possible (Westhusin *et al.*, 2007). In at least one case, cloned mice have been reported that were derived from cells obtained from whole carcasses that were stored in a normal freezer (Wakayama *et al.*, 2008).

The efficiency of cloning animals by nuclear transplantation remains low, with on average only 5%–10% of the cloned embryos transferred into surrogate mothers resulting in live offspring. The outcome also remains variable and uncertain. However, over time small changes have been introduced into the protocols to control cell cycle or changes in chromatin structure of the donor cells that have, at minimum, reduced the variability of outcomes and decreased the incidence of abnormal development including large birth weights. Early on there were concerns that cloned animals would age more quickly due to shortening of telomeres as a result of using cells derived from adult animals (and older animals) for nuclear transplantation. Experiments in our own laboratory and others demonstrated this not to be the case. CC, the world's first cloned cat is now almost 16 years old (Fig. 4), and the world's first cloned white-tailed deer (also derived from work in our laboratory) is 15 years old, well beyond the average age these animal species normally live.

Although 5%–10% of cloned embryos will develop into normal offspring, it is important to point out that the efficiency of nuclear transplantation is oftentimes misunderstood and seemingly much more robust than it truly is. The process involves several steps and losses occur at each of these steps. For example, one may need to begin with 1000 oocytes to obtain 500 that are usable. Enucleation may leave only 200 which survive the procedure to serve as recipients for donor cells, 100 may survive transfer of the donor cell into the enucleated oocyte, and only 10 develop into a normal cloned embryo. Following the transfer of these 10 cloned



Fig. 4 The world's first cloned cat, CC. (a) Cat from which donor cells were collected. (b) CC and her surrogate mother. Note that the cell donor is a calico but CC does not exhibit this coat color pattern, indicative that cloned animals do not always exhibit the same phenotype as the cell donor. It is also noteworthy that failure of the orange allele to express may indicate the inactive X chromosome was not completely reprogrammed by nuclear transplantation. Taken from Shin, T., Kraemer, D., Pryor, J., *et al.*, 2002. A cat cloned by nuclear transplantation. *Nature* 415, 859.

embryos one could expect to get on average only 1 cloned offspring. Again, this is extremely variable, but the example is not unusual and explains the considerable amount of time, effort and expense that is still required to produce animals by reproductive cloning.

This example also exposes the enormous challenges that can arise when attempting to apply reproductive cloning to certain animals. In dogs for example, surgical procedures must be employed to obtain viable oocytes from the oviducts following natural ovulation. Dogs do not respond to hormone treatments that are used in other species for superovulation and estrus synchronization, and they only exhibit estrus every 6 months – 1 year. As a result, considerable time and effort is required simply to produce a few cloned embryos which have to be surgically implanted into the surrogate female. Other species present even greater challenges, most of which have nothing to do with the process of nuclear transplantation itself, but the logistics of all the other steps required for “reproductive cloning”. In recent years, a few scientists have suggested the possibility of cloning long extinct species. Simple logistics and common sense make this highly unlikely. Where would one obtain oocytes to produce cloned embryos? What would serve as the surrogate mother? How would embryos ever be transferred, where and when? It is not unreasonable to expect reproductive cloning could potentially be used to re-derive some animal species that have become extinct, but the list would likely be pretty short.

At present, the overall inefficiency of reproductive cloning keeps the costs high, so the technology remains primarily a tool for the reproduction of elite commercial genotypes, i.e., animals with a specific/special purpose. Market demand has been estimated at approximately 100 horses per year, <500 cattle, and a few hundred pets (dogs and cats, **Fig. 4**). There is also a smaller but real demand for cloning in pigs, sheep and goats. The cost of cloning varies but ranges from 10 s of thousands of dollars to over \$100,000 depending on the animal and specific circumstances. Besides the application of cloning to reproduce specific genotypes, it is also important to point out that reproductive cloning continues to play a significant role in the production of genetically modified (GM) animals, in particular livestock (**Wheeler, 2007**). In fact, a common method currently employed to produce GM livestock is to first produce cells growing in culture that have the desired genetic modification, then utilize these as donor cells for nuclear transplantation (reproductive cloning).

Today there are literally hundreds if not thousands of scientific publications reporting various success rates with cloning animals by nuclear transplantation. Regardless of how the data are analysed and interpreted, reproductive cloning in animals remains a very inefficient process. Although numerous experiments have been carried out to identify a variety of different culprits potentially responsible for the inefficiency of cloning, such as abnormal epigenetic regulation of gene expression or problems with cell cycle synchrony; the true mystery that remains to be explained is how it works at all. It is unremarkable that embryos produced by nuclear transplantation fail to develop. The true “miracle” is that it actually sometimes works, ultimately resulting in a normal animal.

References

- Briggs, R., & King, T. J. (1952). Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. *Proc Natl Acad Sci USA*, 38, 455–463.
- Campbell, K. H. S., McWhir, J., Ritchie, W. A., & Wilmut, I. (1996). Sheep cloned by nuclear transfer from a cultured cell line. *Nature*, 380, 64–66.
- Gurdon, J. B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. *J Embryol Exp Morphol*, 10, 622–640.
- Illmensee, K., & Hoppe, P. C. (1981). Nuclear transplantation in *Mus musculus*: Developmental potential of nuclei from preimplantation embryos. *Cell*, 23, 9–18.
- McGrath, J., & Solter, D. (1984). Inability of mouse blastomere nuclei transferred to enucleated zygotes to support development in vitro. *Science*, 226, 1317–1319.
- Sims, M., & First, N. L. (1994). Production of calves by transfer of nuclei from cultured inner cell mass cells. *Proc Natl Acad Sci USA*, 91, 6143–6147.

- Speman, H. (1938). *Embryonic Development and Induction*. LWW.
- Wakayama, S., Ohta, H., Hikichi, T., et al. (2008). Production of healthy cloned mice from bodies frozen at -20 degrees C for 16 years. *Proc Natl Acad Sci USA*, 105, 17318–17322.
- Westhusin, M. E., Shin, T., Templeton, J. W., Burghardt, R. C., & Adams, L. G. (2007). Rescuing valuable genomes by animal cloning: A case for natural disease resistance in cattle. *J Anim Sci*, 85, 138–142.
- Wheeler, M. B. (2007). Agricultural applications for transgenic livestock. *Trends Biotechnol*, 25, 204–210.
- Willadsen, S. M. (1986). Nuclear transplantation in sheep embryos. *Nature*, 320, 63–65.
- Wilmot, I., Schnieke, A. E., McWhir, J., Kind, A. J., & Campbell, K. H. S. (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature*, 385, 810–813.
- Wilson, J. M., Williams, J. D., Bondioli, K. R., et al. (1993). Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. *Anim Reprod Sci*, 38, 73–83.

Captive Wildlife Breeding: Mammals & Birds

William F Swanson, Center for Conservation and Research of Endangered Wildlife (CREW), Cincinnati, OH, United States
Linda M Penfold, South-East Zoo Alliance for Reproduction & Conservation (SEZARC), Yulee, FL, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Artificial insemination Manual deposition of semen into the female's reproductive tract.

Cryopreservation Method for ultralow temperature storage of sperm, oocytes and embryos.

Electroejaculation Application of electrical stimuli by a rectal or penile probe to induce semen recovery.

Embryo transfer Manual placement of pre-implantation embryos into the female's reproductive tract.

Fecal hormone metabolite analysis Endocrine method used to assess reproductive hormone byproducts in voided fecal samples.

In vitro fertilization Insemination of recovered oocytes to produce embryos in culture.

Laparoscopy Examination of the contents of the abdominal cavity using a fiberoptic telescope.

Introduction

The Earth is currently experiencing the sixth major extinction crisis in its four and one half billion year history, with population declines documented across most major wildlife taxa over the past 50 years. Of the ~5500 mammal and ~10,000 bird species in the world, 20% and 12%, respectively, have been classified under some degree of extinction threat. Causes for this ongoing extinction crisis are multiple and complex, but one overriding factor has been the exponential growth in human populations and related acceleration in resource consumption, predominantly habitat loss. Reversal of wildlife species decline will require concerted efforts addressing global socioeconomic issues and disparities coupled with immediate implementation of remedial actions to slow species loss. For many species, assurance populations may be needed to bridge this critical time period and ensure that these endangered taxa survive long enough to once again have the opportunity to thrive in nature.

Accredited zoological parks are working collaboratively on a regional, and, increasingly, global level to maintain and manage genetically and demographically viable wildlife populations under human care. The evolution of zoos and aquariums from menageries to conservation organizations is ongoing and essential if many of these species are to remain extant for the next century. The 231 institutions accredited by the US-based Association of Zoos and Aquariums (AZA) currently manage 217 mammal and 181 bird species, respectively, within Species Survival Plans (SSPs). SSPs use pedigree analyses to estimate the genetic relatedness of individuals in the managed population and make breeding recommendations to maximize genetic diversity (based on mean kinship values). Despite intensive management efforts, few of these populations (<10%) in AZA-accredited zoos are considered sustainable (>90% gene diversity) over the next 100 years – the genetic benchmark targeted by zoos. In the 356 facilities accredited by the European Association of Zoos & Aquariums (EAZA), similar breeding management programs (termed European Endangered species Programs, or EEPs) oversee 146 mammal species and 49 bird species with a comparable lack of genetic sustainability for most taxa projected over time.

The crux of the issue for most species is the availability of too few exhibit spaces within zoos for populations established initially with too few genetic founders, confounded by poor breeding success, leading to inbreeding and further decreases in genetic heterozygosity. The reversal of species decline within zoos, as in nature, requires concerted efforts globally, but, at least in zoos, this goal may prove attainable for some taxa with the judicious application of reproductive sciences. In particular, management challenges related to low breeding success, limited exhibit space, small founder number, and restricted gene flow may be addressed via reproductive science and technology.

Reproductive sciences encompass multiple disciplines including gamete biology, endocrinology, cryobiology and ultrasonography, which, in turn, are the fields most essential to wildlife propagation. These scientific disciplines fostered the reproductive technologies that generate the most media interest (whether for actual births or speculation about future offspring) but also have the greatest potential to produce meaningful and long-lasting benefits for species management and conservation. For wildlife species, the most relevant of these reproductive technologies comprise semen collection, hormonal and ultrasonographic analyses, artificial insemination (AI), in vitro fertilization (IVF), embryo transfer (ET), and semen/embryo cryopreservation. These technologies are considered almost rudimentary to many reproductive biologists studying laboratory rodents or agricultural species, in which high pregnancy percentages have been routinely achieved with frozen semen AI or frozen embryo transfer for decades. For most wildlife species, information of basic reproductive biology is extremely limited or non-existent and application of any assisted reproductive technology is unlikely to succeed until that deficit is addressed. The keys for effective wildlife propagation are to first establish that fundamental knowledge base and then build from that foundation to explore the appropriateness, feasibility and application of assisted reproduction for species management.

Understanding Basic Reproductive Physiology

Semen Collection and Analysis

Evaluation of semen production and quality is an essential component for basic characterization of reproductive traits in wild mammals and birds. For these analyses, effective and safe methods for semen collection must be available and suitable for use across diverse taxa. For some mammal species (e.g., elephants, gorillas, chimpanzees, cetaceans), voluntary collection using prostatic massage, artificial vaginas, or direct masturbation of non-sedated individuals has proven feasible (O'Brien and Robeck, 2010). However, these approaches can require time-intensive operant conditioning and training, limiting their usefulness on a population level. For most wild, intractable mammals, males must be anesthetized for semen collection, requiring proven drug regimens that provide adequate anesthetic depth while minimizing patient risk. The most common collection approach for mammal species involves electroejaculation, using a rectal or penile probe coupled with a controlled voltage electrostimulator to promote ejaculation of semen from anesthetized or sedated individuals. This approach is most commonly used with felids, canids, primates, and ungulate species (Herrick *et al.*, 2010; Pukazhenthi, 2016). For some mammal species, such as felids and bears, an alternative approach using urethral catheterization in combination with alpha 2 agonist drugs permits recovery of highly concentrated semen. High quality sperm samples also may be recovered from many mammals post-mortem by flushing the vas deferens and caudal epididymides (up to 24 h after death if tissues are kept chilled). For bird species, although semen collection has been attempted by electroejaculation, the preferred method is massage of the vent to directly express semen, or manual stimulation of the male to induce voluntary eversion of the cloaca and ejaculation (Malecki *et al.*, 2008). This technique has been effective in multiple avian species, from several raptor and pheasant species to birds of paradise. In some cases, imprinted birds have been trained to ejaculate onto inanimate objects such as a dummy bird model (houbara bustard), a hat (raptor species) or even a shoe (African penguin, California condor). Training and conditioning of birds for handling and semen collection is necessary for some species such as cranes and raptors, but less essential for other species, such as ducks and songbirds (Gee *et al.*, 2004).

Semen analysis methods are similar across mammal and bird species, comprised of assessment of seminal volume, sperm concentration, progressive motility (percentage of motile and rate of forward progression), morphology, and other characteristics, including acrosome and plasma membrane status (Herrick *et al.*, 2010; Gee *et al.*, 2004; Pukazhenthi, 2016). Although many of these traits may be evaluated subjectively, objective assessment methods using flow cytometry, videography and computer-assisted analyses for motility and morphology are becoming more routine with wildlife species. More advanced analyses including measurement of sperm metabolism, chromatin fragmentation, and sperm functionality (acrosome reactivity, heterologous *in vitro* fertilization) have been used with some felid and hoof stock species. For species such as primates, macropods and armadillos, semen recovery and analyses is confounded by presence of coagulum that may require thermal and/or enzyme-induced liquification. Seminal analyses in highly seasonal mammal and bird species may reveal poor sperm quality parameters initially, but these can rapidly improve as the season progresses. Seminal quality also may be partially dependent on the mating system, with higher motile sperm numbers and normal morphology observed in large herd species, and greater sperm numbers and larger midpieces in primates with polygamous mating systems.

Semen analyses provide basal information on the reproductive status of the individual male, including potential fertility, and normative values for the species as a whole (*ex situ*). Comparative seminal data from wild (*in situ*) populations are lacking for most mammals, with the exception of some felids (Florida panther, cheetah, lion, jaguar, Pallas' cat, black-footed cat), ungulates (giraffe, gerenuk) and African elephants (Hildebrandt *et al.*, 2012). As zoos increasingly collect and bank semen *in situ* from wild males as a potential source of new founders, more comparative data are expected to be generated for wild populations.

Reproductive Endocrinology

Endocrinology represents a vital scientific discipline for defining normative reproductive characteristics and basic hormonal patterns in wildlife species. The development of radioimmunoassays (RIAs) and, subsequently, enzyme immunoassays (EIAs) with broad cross-species applicability has allowed assessment of reproductive hormonal patterns in more than 100 wild mammal and bird species over the past 30 years (Brown *et al.*, 1994; Kersey and Dehnhard, 2014; Stoops *et al.*, 2016). Coupled with the ability to measure hormonal metabolites in excretory products (saliva, urine, feces) collected non-invasively, these immunoassays have provided our first detailed insights into the basal endocrine function that forms the basis for successful wildlife propagation. As steroid molecules are highly conserved and metabolism is similar across species, the same assays, using a limited number of group-specific antibodies, have been validated and applied across evolutionary-diverse taxa. The increasing importance of this wildlife-focused field over the past few years is reflected in the founding of a dedicated scientific organization, the International Society of Wildlife Endocrinologists (see Relevant Website section).

For many mammalian species, studies of basic reproductive biology include hormonal assessment of ovarian cyclicity, ovulatory mechanisms, seasonality, and pregnancy and pseudopregnancy. In birds, reproductive behavior and courtship studies have been more prevalent. Frequently, adrenocortical hormones also are measured to evaluate the effect of putative management stressors (exhibit size and complexity, noise, crowding, etc.) on reproductive function. These reproductive characteristics are primarily assessed by monitoring steroid metabolites (testosterone, estrogen, progesterone) in urine or fecal samples collected longitudinally from the individual/species of interest. Evaluation of feces and urine offers the added advantage that measured values represent the pooling of hormonal levels over several hours to days, with less fluctuation than observed with blood. For each species, the respective assays must be first validated by assessing hormone recovery during sample extraction, parallelism of serially diluted

samples with a standard curve, and relationship of assay results to physiological events, such as observed breeding, pregnancy and parturition. Depending on species, certain hormones/metabolites may not be adequately detected with standard assays. For example, fecal estrogens are readily measured in felids, but the commonly-used estrogen assays work poorly in otters, bears, and most rhino species. These variations likely occur due to differences in hormone metabolism prior to excretion, alternative excretory pathways (feces vs. urine) and poor cross-reactivity to the assay antibody. In many species, the main hormone metabolites are unknown, but can be determined using liquid or gas chromatography/mass spectrometry methods if identification of a more specific antibody/assay is required (Kersey and Dehnhard, 2014).

The reproductive profiles deduced from hormone monitoring provide valuable metrics for defining optimal endocrine characteristics for success reproduction and managing breeding pairs (e.g., determining time or season for pairing), but also assessing potential causes of infertility or sub-fertility in individual animals that fail to reproduce (Fig. 1). Similarly, normative profiles of estrus, non-pregnant luteal phases and pregnancy provide a benchmark for evaluating the effectiveness of ovarian synchronization/stimulation protocols for inducing appropriate hormonal responses, and determining pregnancy following natural breeding, AI or ET. For pregnancy diagnosis, longitudinal monitoring of fecal or urinary progesterone can detect temporal changes most consistent with either a non-pregnant luteal phase, pseudopregnancy or an ongoing pregnancy (Fig. 2). For some

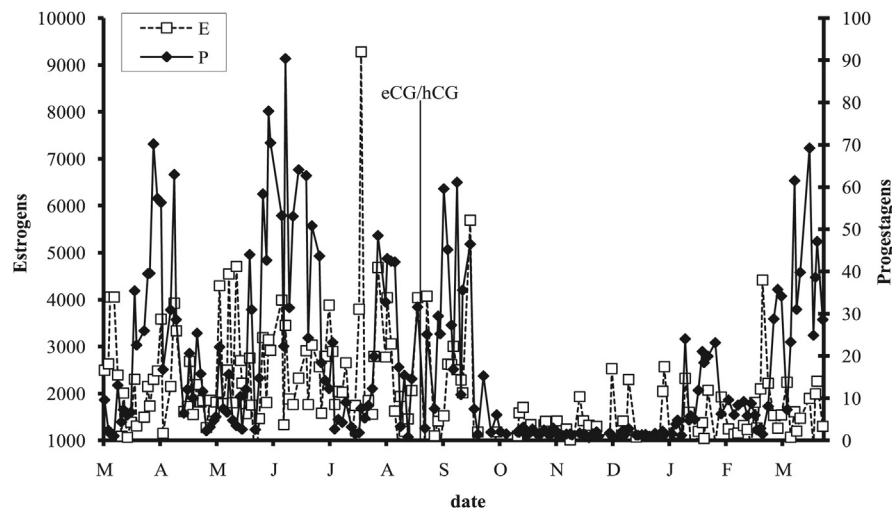


Fig. 1 Fecal estrogen and progestagen metabolite profile in a black-footed cat, showing multiple non-pregnant luteal phases following spontaneous ovulation and an induced luteal phase in response to eCG/hCG treatment. Reprinted from Herrick J.R., Bond J.B., Campbell M., *et al.*, 2010. Fecal endocrine profiles and ejaculate traits in black-footed cats (*Felis nigripes*) and sand cats (*Felis margarita*). *Gen. Comp. Endo.* 165, 204–214, with permission from Elsevier Science

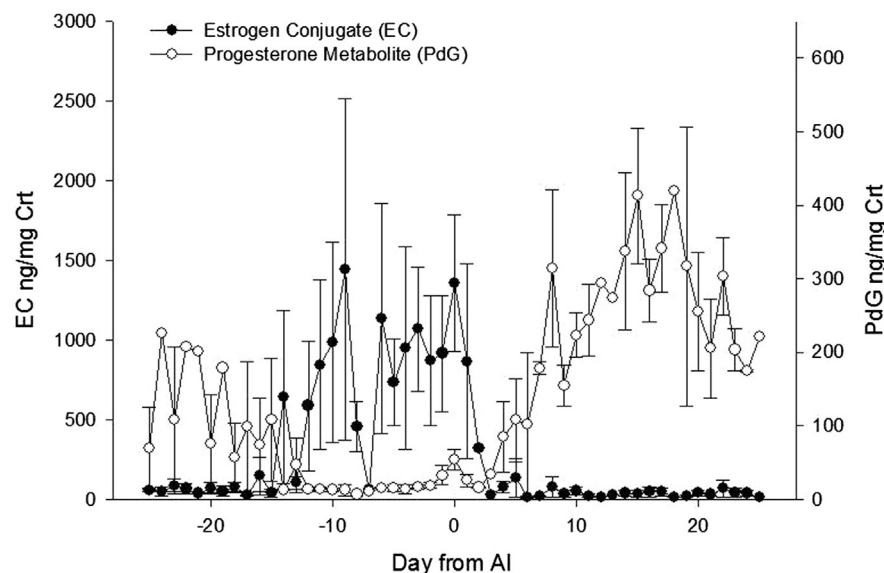


Fig. 2 Urinary estrogen and progesterone metabolite profiles in four Indian rhino pregnancies produced via transcervical AI with frozen semen during natural estrous cycles. Reprinted from Stoops M.A., Campbell M.K., DeChant C.J., *et al.*, 2016. Enhancing captive Indian rhinoceros genetics via artificial insemination of cryopreserved sperm. *Anim. Reprod. Sci.* 172, 60–75, with permission of Elsevier Science.

species, other fecal or urinary pregnancy biomarkers (e.g., PGFM, relaxin, chorionic gonadotropins) may provide more definitive diagnoses (Kersey and Dehnhard, 2014). In tractable or easily restrained species, transabdominal or rectal ultrasonography can provide invaluable correlative data for hormonal assays, and allow real-time monitoring of ovarian function and pregnancy (O'Brien and Robeck, 2010; Stoops *et al.*, 2016).

Assisted Reproduction

Artificial Insemination

Artificial insemination (AI) represents the most direct approach to propagating wildlife – essentially simulating natural breeding without requiring the direct participation of the male (Pukazhenthil and Wildt, 2004). For some wildlife species, AI can be conducted vaginally or transcervically in a non-sedated, naturally-estrous female with minimal restraint, but for numerous other species, the AI process is much more complex. For many species, the availability of a domesticated reproductive model has been instrumental in making progress, most notably with domestic cats/wild felids, domestic hoof stock/wild ungulates and domestic poultry/wild birds. From an applied perspective, AIs (combined with semen cryopreservation) are conducted in zoos for four primary reasons, specifically to: (1) propagate behaviorally or physically incompatible pairs, (2) create genetic exchange between individuals housed in distant zoos, (3) introduce new founders from the wild into captive populations, and (4) allow long-term maintenance of genetic diversity within liquid nitrogen tanks (Pukazhenthil and Wildt, 2004; Swanson, 2006; Howard and Wildt, 2009). Prior to initiating AI studies, it is important to address its relevance for species management, and determine if AI is really the most appropriate strategy. Despite its relative simplicity compared to newer reproductive technologies, AI has been only moderately successful for propagating wildlife species in zoos, especially if the ultimate goal is production of viable, genetically-valuable offspring for population management. Of the world's >5000 wild mammal species, a total of 58 species (~1%) have been propagated with AI, with 34 of these species reproduced using frozen semen, the gold standard for management purposes (Table 1). Of the ~10,000 bird species, AI has proven successful in producing offspring in ~37 species, with 12 of these from frozen semen AI (Table 2). Relatively few laboratories globally are dedicated to studying the reproduction of wildlife species, which hampers progress in developing effective AI approaches and reflects the sparse availability of funding for these conservation-based research activities.

Considerations for successful AI in wild mammals and birds include (1) estrus detection and ovarian synchronization, (2) timing of AI, and (3) AI technique. For many species, the preference is to conduct AI during a natural estrus period, either by monitoring endogenous ovarian cyclicity over time or by employing pharmaceutical methods to induce estrus within a defined (fixed) time period. The benefit to conducting AI on a natural estrus cycle is potentially higher pregnancy percentages, but longitudinal monitoring of individuals for real-time detection of natural estrus can be challenging and costly, limiting its applicability to a few high profile species. Two of these species, the giant panda and black-footed ferret, are highly seasonal breeders, allowing intensive estrus monitoring to be restricted to a short time period in the spring months. By assessing vaginal cytology (ferrets) or urinary hormones (pandas) daily, the onset of natural estrus can be detected and AI timed coincidentally with spontaneous or induced ovulation, resulting in high fertility (Pukazhenthil and Wildt, 2004; Howard and Wildt, 2009). Similarly, AIs in elephants are conducted exclusively during natural estrus cycles, based on frequent blood sampling and monitoring of serum LH concentrations. Because elephants exhibit biphasic peaks in LH at three week intervals prior to ovulation, the detection of the first LH peak provides adequate lead time for AI scheduling (Hildebrandt *et al.*, 2012). Within zoos, these three species represent the prime examples of AI being used as a population management tool, with more than 25 offspring produced in each by AI (Table 1). AI procedures in cetaceans and rhinos depend primarily on using rectal/transabdominal ultrasonography and urinary hormone assays to monitor ovarian function and detect natural estrus phases for AI, also resulting in multiple pregnancies and offspring with positive population genetic impact (O'Brien and Robeck, 2010; Stoops *et al.*, 2016). In other species, primarily ungulates, ovarian activity is synchronized by inducing luteal regression (using prostaglandin injections) and/or ovarian suppression (using synthetic progestin implants or intravaginal devices) with females typically coming into natural estrus within a set time frame post-treatment for AI (Morrow *et al.*, 2009; Pukazhenthil, 2016). In contrast, felids are usually treated with exogenous gonadotropins (eCG or pFSH; hCG or pLH) to induce ovarian follicular growth and ovulation, but, in most cat species, response is confounded by endogenous ovarian activity and occasional spontaneous ovulations. Greatest AI success has been reported in the cheetah, with ~15 pregnancies reported following eCG/hCG treatment, presumably aided by intrinsically low levels of basal ovarian cyclicity (Howard and Wildt, 2009). Recent advances with felids include the use of oral progestin to suppress ovarian activity prior to exogenous gonadotropins (eCG/pLH) and fixed-time AI. In birds, AI usually is conducted during the species breeding season, which often is preceded by observation of specific reproductive behaviors. These displays may involve stereotypic rituals including courtship dances and collection of nesting materials (Gee *et al.*, 2004).

The timing of AI relative to spontaneous or induced ovulation also varies among species, but, for most mammals, insemination is conducted just prior to ovulation so the spermatozoa arrive at the site of fertilization prior to the ovulated oocytes. In some species, such as felids, AI is typically conducted post-ovulation because of concerns that anesthesia (required for most felid AIs) may interfere with the ovulatory process. In more tractable species (e.g., cetaceans, elephants), AI can be conducted both prior and after the expected time of ovulation. In birds, hormonal treatment is not used to induce estrus or egg laying, but timing of AI is less critical, due to the presence of oviductal sperm storage glands that allow several eggs to be fertilized with a single AI. In species that lay multiple eggs, production of the first egg is the most obvious cue to initiate AI to fertilize subsequent ova. For species that lay only single eggs, AIs should be conducted at least once weekly.

Table 1 Successful artificial insemination in wild mammal species^a

Mammals (order)	Common name	Genus/species	Approach	Semen status
Carnivora	Tiger	<i>Panthera tigris</i>	V/LU/LO	F
	Puma	<i>Puma concolor</i>	SU/LU	F
	Snow leopard	<i>Uncia uncia</i>	LU	F
	Clouded leopard	<i>Neofelis nebulosa</i>	LU/LO	F/FT
	Cheetah	<i>Acinonyx jubatus</i>	LU	F/FT
	Leopard	<i>Panthera pardus</i>	TC	F
	Ocelot	<i>Leopardus pardalis</i>	LU/LO	F/FT
	Tigrina	<i>Leopardus tigrinus</i>	LU	F
	Fishing cat	<i>Prionailurus viverrinus</i>	LO	F
	Pallas' cat	<i>Otocolobus manul</i>	LO	F/FT
	Leopard cat	<i>Prionailurus bengalensis</i>	LU	F/FT
	Asian golden cat	<i>Catopuma temminckii</i>	TC	F
	Gray wolf	<i>Canis lupus</i>	V/TC	F/FT
	Red wolf	<i>Canis rufus</i>	TC	F
	Red fox	<i>Vulpes vulpes</i>	TC	F/FT
	Blue Fox	<i>Alopex lagopus</i>	TC	F/FT
	Giant Panda	<i>Ailuropoda melandeuca</i>	TC	F/FT
	Black-footed ferret	<i>Mustela nigripes</i>	LU	F/FT
	American Mink	<i>Neovison vison</i>	TC	FT
Primates	Chimpanzee	<i>Pan troglodytes</i>	TC	F/FT
	Spider monkey	<i>Ateles geoffroyi</i>	V	F
	Cynomolgus monkey	<i>Macaca fascicularis</i>	UTU	FT
	Rhesus monkey	<i>Macaca mulatta</i>	TC	F/FT
	Lion-tailed macaque	<i>Macaca silenus</i>	TC	F
Squirrel	Common marmoset	<i>Callithrix jacchus</i>	V/TC	F/FT
	monkey	<i>Saimiri sciureus</i>	V	F
Artiodactyla	Bactrian camel	<i>Camelus bactrianus</i>	TC	F/FT
	Barbary sheep	<i>Ammotragus lervia</i>	LU	F
	Spanish ibex	<i>Capra pyrenaica</i>	LU	FT
	Bison	<i>Bison bison</i>	TC	F/FT
	Anoa	<i>Bos javanicus</i>	TC	F
	Banteng	<i>Bubalus depressicornis</i>	TC	F
	Addax	<i>Addax nasomaculatus</i>	TC	FT
	Scimitar-horned oryx	<i>Oryx dammah</i>	TC	FT
	Blackbuck	<i>Antilope cervicapra</i>	TC/LU	F/FT
	Gerenuk	<i>Litocranius walleria</i>	TC	FT
	Mhorr gazelle	<i>Nanger dama</i>	LU	FT
	Speke's gazelle	<i>Gazella spekei</i>	V	F
	Eld's deer	<i>Cervus eldi</i>	LU	FT
	Axis deer	<i>Axis axis</i>	TC/LU	F/FT
	White-tailed deer	<i>Odocoileus virginianus</i>	TC/LU	F/FT
	Fallow deer	<i>Dama dama</i>	TC/LU	F/FT
	Red deer	<i>Cervus elaphus</i>	TC/LU	FT
	Reindeer	<i>Rangifer tarrandus</i>	TC	F
	Giraffe	<i>Giraffa camelopardalis</i>	TC	F
Perissodactyla	Przewalski horse	<i>Equus ferus</i>	TC	F
	Persian onager	<i>Equus hemionus</i>	TC	F
	Indian rhino	<i>Rhinoceros unicornis</i>	TC	FT
	White rhino	<i>Ceratotherium simum</i>	TC	FT
Cetacea	Killer whale	<i>Orcinus orca</i>	TC	F/FT
	Beluga whale	<i>Delphinapterus leucas</i>	TC	F/FT
	Bottlenose dolphin	<i>Tursiops truncatus</i>	TC	F/FT
	Pacific white-sided dolphin	<i>Lagenorhynchus obliquidius</i>	TC	FT
	Indo-Pacific bottlenose dolphin	<i>Tursiops aduncus</i>	TC	F
Proboscidea	African elephant	<i>Loxodonta africana</i>	TC	F/FT
	Asian elephant	<i>Elaphas maximus</i>	TC	F
Diprotodontia	Koala	<i>Phascolarctos cinereus</i>	UGS	F
	Tammar wallaby	<i>Macropus eugenii</i>	SU	F

^aBirth of full-term offspring.

Abbreviations: Approach – LO, laparoscopic oviductal; LU, laparoscopic uterine; SU, surgical uterine; TC, transcervical; UGS, urogenital sinus; UTU, ultrasonographic transabdominal uterine; V, vaginal. Semen status – F, fresh (or chilled); FT, frozen-thawed.

Table 2 Successful artificial insemination in wild bird species^b

Birds (order)	Common name	Genus/species	Approach	Semen status
Anseriformes	Northern pintail	<i>Anas acuta</i>	V	F
	Mallard	<i>Anas platyrhynchos</i>	O	F
	Aleutian Canada goose	<i>Branta canadensis leucopareia</i>	V	FT
Columbiformes	Blue rock pigeon	<i>Columba livia</i>	V	F
Falconiformes	American goshawk	<i>Accipiter gentilis</i>	C	F
	Prairie falcon	<i>Falco mexicanus</i>	V	F
	Peregrine falcon	<i>Falco peregrinus</i>	V	FT
	American kestrel	<i>Falco sparverius</i>	V	F/FT
	Golden eagle	<i>Aquila crysaetos</i>	M	FT
	Lady Amherst's pheasant	<i>Chrysolosis amhestiae</i>	V	FT
Galliformes	Berlioz's pheasant	<i>Lophura berliozii</i>	V	FT
	Cabot's tragopan	<i>Tragopan caboti</i>	V	FT
	Chinese pheasant	<i>Chrysolophus pictus</i>	V	F/FT
	Mikado pheasant	<i>Symaticus mikado</i>	V	F
	Japanese quail	<i>Coturnix coturnix japonica</i>	V	F
	Swinhoe's pheasant	<i>Lophura swinhoii</i>	V	F
	Blyth's tragopan	<i>Tragopan blythii</i>	V	F
	Cabot's tragopan	<i>Tragopan cabotii</i>	V	F
	Temminck's tragopan	<i>Tragopan temminckii</i>	V	F
	Himalayan monal	<i>Lophophorus impeyanus</i>	V	F
	Common piping guam	<i>Pipile pipile</i>	CU	F
Gruiformes	Blue crane	<i>Grus antigone</i>	V	F
	Whooping crane	<i>Grus americana</i>	V	F/FT
	Sandhill crane	<i>Grus canadensis</i>	V	F/FT
	Siberian crane	<i>Grus leucogeranus</i>	V ^a	F
	Red crowned crane	<i>Grus japonensis</i>	V ^a	F
	White naped crane	<i>Grus vipio</i>	V ^a	F
	Brolga crane	<i>Grus rubicanda</i>	V ^a	F
	Hooded crane	<i>Grus monacha</i>	V ^a	F
	Blue crane	<i>Anthropoides paradisea</i>	V ^a	F
	Wattled crane	<i>Buggeranus carunculatus</i>	V	F
Otididae	Houbara bustard	<i>Chlamydotis undulata</i>	V	F/FT
Psittaciformes	Red-vented cockatoo	<i>Cacatua haematurpygia</i>	O	F
	Eclectus parrot	<i>Eclectus roratus</i>	O	F
Spheniscidae	Magellanic penguin	<i>Spheniscus magellanicus</i>	C	F/FT
Struthioniformes	Cassowary	<i>Casaurius casaurius</i>	V	F
	Ostrich	<i>Struthio camelus</i>	V	F

^aAssumed AI method based on previous studies.^bHatching of offspring.

Abbreviations: Approach – C, cloacal; CU, cloacal urodeum; M, magnum; O, oviductal; V, vaginal. Semen status – F, fresh (or chilled); FT, frozen-thawed.

The AI technique also is often species-specific, depending on anatomical constraints and tractability of the individual. For many larger ungulates, use of drop chute restraint systems with conditioned animals has allowed transcervical AI to become the standard insemination method, whereas with small ungulates (such as deer), females are anesthetized for laparoscopic intra-uterine AI (Morrow *et al.*, 2009). In tractable species, such as elephants, rhinos and cetaceans, transcervical AI may be performed with minimal restraint (sometimes with standing sedation), whereas in carnivores, anesthesia is almost always required, with possible consequences for fertility. Vaginal and transcervical AI have been minimally successful in felids, necessitating development of laparoscopic uterine, and most recently, oviductal AI methods for improved conception (Howard and Wildt, 2009; Swanson, 2012). However, in other carnivores such as giant pandas and some canids, anesthesia of naturally-estrous females for transcervical AI has generated high pregnancy percentages. In birds, only the left ovary and oviduct are functional and semen is typically deposited in the vagina, oviduct or magna using manual restraint. Smaller birds can be handled and restrained by one individual while a second person inseminates, but AI in larger species such as cranes usually requires three people. For crane, raptor and ratite species, prior operant conditioning and training is necessary for AI procedures (Gee *et al.*, 2004; Malecki *et al.*, 2008).

The number of motile spermatozoa needed for successful AI varies by species and is partly dependent on the total sperm numbers typically recovered from the males. For some larger species (elephants, rhinos, cetaceans), sperm recovery numbers in the billions, allowing the use of very high inseminate doses (100–500 million) for intrauterine AI (O'Brien and Robeck, 2010; Hildebrandt *et al.*, 2012; Stoops *et al.*, 2016). In smaller species, such as felids, total semen recovery may average just 10–20 million sperm per ejaculate, limiting its availability for even a single AI attempt. Deposition of semen directly into the oviduct or distal uterus can substantially reduce sperm requirements, and allow the use of semen compromised by cryopreservation or suboptimal

morphology/motility (Swanson, 2012). In birds, deep insemination close to the sperm storage tubules usually produces superior fertility. For crane species, AI with ≥ 16 million sperm two to three times each week is recommended, but in other species, a single AI can fertilize multiple eggs. AI with excess sperm numbers should be avoided in birds since polyspermia and early embryo death may occur.

For wildlife population management, AI has been applied mainly to address issues of behavioral or physical incompatibilities, using freshly collected or chilled semen from resident males or males housed at nearby institutions. Although valuable for managing some populations, the dependence on freshly collected semen negates one of the primary advantages of AI – the ability to incorporate cryopreserved semen into species management. As with other aspects of wildlife reproduction, protocols for processing and cryopreserving semen from wild mammals and birds must be investigated on a species by species basis, requiring multi-factorial evaluations of each step in the cryopreservation process (Pukazhenthi and Wildt, 2004). The effectiveness of cryopreservation methods are often judged based on assessment of post-thaw motility, viability and acrosome status over time, less frequently with in vitro function tests, and rarely with AI trials. Although >30 wild mammal species have been propagated using AI with frozen semen (Table 1), technique efficacy is relatively low for most, and frozen semen AI is not a significant contributor to population management of wild mammal species. In birds, ~12 wild species have been reproduced using frozen semen, but as with mammals, this approach is rarely used for management purposes.

Embryo Transfer

Another assisted reproductive technology, embryo transfer (ET), has received considerable interest for propagation of wild mammal species. ET adds another layer of complexity, requiring either flushing of in vivo-produced embryos (produced by natural breeding or AI) from the female or generation of embryos in vitro following recovery and insemination of viable oocytes. ET provides a more direct approach for managing and conserving the genetic potential of females and, with IVM/IVF, facilitate the genetic salvage of deceased females. For individuals or species that produce minimal or poor quality sperm, IVF/ET also may represent the only feasible avenue for assisted propagation. As with AI, many ET protocols for wildlife are derived from those developed initially in domestic cattle, sheep, and cats. To date, ET has been used to produce term offspring in at least 30 wild mammal species (Table 3).

Table 3 Successful embryo transfer in wild mammal species^a

Mammals (order)	Common name	Genus/species	Embryo source	Transfer method	Embryo status
Carnivora	Tiger	<i>Panthera tigris</i>	IVF	SO	F
	Ocelot	<i>Leopardus pardalis</i>	IVF	LO	FT
	Fishing cat	<i>Prionailurus viverrinus</i>	IVF	SU	F
	Caracal	<i>Caracal caracal</i>	IVF	SU	F/FT
	Serval	<i>Leptailurus serval</i>	IVF	SU	F
	Black-footed cat	<i>Felis nigripes</i>	IVF	LO/LOi	FT
	Sand cat	<i>Felis margarita</i>	IVF	LO	F
	Wild cat	<i>Felis silvestris</i>	IVF	SUi	F/FT
	Black bear	<i>Ursus americanus</i>	IV	SU	F
	European mink	<i>Mustela lutreola</i>	IV	SU/SUi	F
	European polecat	<i>Mustela putorius</i>	IV	SU	FT
	Silver fox	<i>Vulpes vulpes</i>	IV	SU	F
Primates	Gorilla	<i>Gorilla gorilla</i>	IVF	TC	F
	Baboon	<i>Papio cynocephalus</i>	IV/IVF	TC/LO	F/FT
	Rhesus monkey	<i>Macaca mulatta</i>	IV/IVF	TC/SO	F/FT
	Cynomolgus monkey	<i>Macaca fascicularis</i>	IVF	SO	F/FT
	Common marmoset	<i>Callithrix jacchus</i>	IV/IVF	SU lu	F/FT
Artiodactyla	Mouflon sheep	<i>Ovis musimon</i>	IV	SO/SUi	F
	Bison	<i>Bison bison</i>	IV	TC	F
	Scimitar-horned oryx	<i>Oryx dammah</i>	IV	TC	F
	Eland	<i>Taurotragus oryx</i>	IV	TC	F/FT
	Gaur	<i>Bos gaurus</i>	IV/IVF	SUi/TCi	F
	Bongo	<i>Trafelephus euryceros</i>	IV	TCi/TC	F
	Suni antelope	<i>Neotragus moschatus</i>	IV	LU	F
	Eld's deer	<i>Cervus eldi</i>	IVM/IVF	SO	F
	White-tailed deer	<i>Odocoileus virginianus</i>	IV	SU	FT
	Sika deer	<i>Cervus nippon</i>	IV	SU	F/FT
	Red deer	<i>Cervus elaphus</i>	IV	TC/SU	F/FT
	Fallow deer	<i>Dama dama</i>	IV	SU/SO/LU	F/FT
	Przewalski's horse	<i>Equus przewalski</i>	IV	SUi	F
	Grant's zebra	<i>Equus burchelli</i>	IV	TCi/SUi	F

^aBirth of full-term offspring.

Abbreviations: Embryo source— IV, in vivo; IVF, in vitro fertilization; IVM, in vitro maturation. Transfer method— i, interspecies; LO, laparoscopic oviductal;

LU, laparoscopic uterine; SO, surgical oviductal; SU, surgical uterine; TC, transcervical. Embryo status— F, fresh; FT, frozen-thawed.

The primary difficulty for many species is recovery of viable oocytes or embryos in a minimally-invasive manner. For some wild ungulates, transvaginal oocyte aspiration has been possible, but for other species (elephants, rhinos), oocyte collection is challenging, confounded by lack of established ovarian hyperstimulation and IVF methods. In cats, protocols for ovarian stimulation, laparoscopic oocyte collection and IVF have been applied to ~15 species, with transfer of IVF-derived embryos resulting in offspring in eight species (Pope *et al.*, 2006; Swanson, 2006). In primates and ungulates, transcervical flushing of embryos produced from natural mating has facilitated successful ET in the past, but this approach is being supplanted by IVF-based embryo production methods.

As with AI, ET success requires knowledge of the basal reproductive physiology of each target species, with protocols adapted to their unique physiology and anatomy. Ovarian synchronization may rely on natural estrus phases or induced cycles using similar strategies employed for AI. ET techniques also are similar to those used for AI, with transcervical transfers preferred in ungulates and some primates, and laparoscopic oviductal methods favored for felids. One advantage to oviductal ET is the ability to transfer early-stage embryos, reducing the detrimental impact of extended in vitro culture on embryo/fetal health (Swanson, 2012). For transfer of freshly-collected or produced embryos, synchronized females must be readily available to serve as recipients. Interspecies ET (to domestic cows or ewes) was evaluated as one potential solution in ungulates, with the birth of multiple offspring in decades past, but efficacy was poor and this approach is rarely used now. Embryo cryopreservation presents another possible option for the shortage of recipients, allowing long-term storage prior to ET. Wildlife embryo cryopreservation has received much less research attention than semen freezing, with just 14 wild mammal species propagated to date via transfer of frozen-thawed embryos (Table 3). ET with fresh or frozen embryos does not contribute meaningfully to genetic management programs for any wild mammal species.

Emerging Reproductive Technologies

New reproductive technologies continually emerge from research institutions working with laboratory rodents or agricultural species, and, in many instances, are investigated for potential applicability to wildlife propagation. Early examples include interspecies embryo transfer, embryo micromanipulation (e.g., splitting, ICM transfer), and intracytoplasmic sperm injection (ICSI), and more recently, somatic cell nuclear transfer (SCNT), induced pluripotent stem cells (iPSC) for gamete derivation, semen freeze-drying, and sperm (sex) sorting. Some of these new technologies are valuable as investigational tools with wildlife, providing comparative insights into species-specific physiology and reproductive mechanisms, and a few have proven useful in limited applications for producing offspring. For example, AI with sex-sorted sperm in dolphins allows preferential production of offspring of the desired gender for management purposes (O'Brien and Robeck, 2010). Similarly, in birds, sexing of eggs via genetic analysis of blood collected from the developing embryo permits gender selection prior to hatching. However, extrapolation of novel approaches to wildlife propagation frequently results in failure, primarily due to lack of basal reproductive knowledge and awareness of species differences. When viable offspring are produced, these births may receive extensive media attention, but often are one-time events due to poor technique efficacy, funding barriers and lack of a defined applied purpose (creating a science-conservation disconnect). The still limited applicability of AI and ET for wildlife breeding management, after decades of research, exemplifies the challenges associated with applying even relatively basic reproductive technologies to propagation of non-domesticated species.

Future Priorities

Reproductive technologies play an important role in captive breeding management of wild mammal and bird species, notably for generating basal knowledge of species biology and improving natural breeding. Its true potential, however, has not been realized. The sustainability of >90% of managed populations in zoos cannot be assured for the next century through natural breeding alone. AI and, to a lesser extent, ET, in concert with sperm and embryo cryopreservation, can help to change that trajectory, but only if adequate resources (animals, research staff, infrastructure, funding) are available to advance these techniques beyond proof of feasibility to achieve adequate efficacy for applied use. Animal managers and zoo veterinarians also must embrace the science and receive adequate training to apply these new tools for species propagation. Once that benchmark is reached, these technologies will be poised to substantially benefit wildlife management programs, provided that zoos and society remain committed to supporting the broader implementation of these applied reproductive sciences.

References

- Brown, J. L., Wasser, S. K., Wildt, D. E., & Graham, L. H. (1994). Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biol. Reprod.*, 51, 776–786.
- Gee, G. F., Bertschinger, H., Donoghue, A. M., Blanco, J., & Soley, J. (2004). Reproduction in nondomestic birds: Physiology, semen collection, artificial insemination and cryopreservation. *Avian Poult. Biol. Rev.*, 15, 47–101.
- Herrick, J. R., Bond, J. B., Campbell, M., et al. (2010). Fecal endocrine profiles and ejaculate traits in black-footed cats (*Felis nigripes*) and sand cats (*Felis margarita*). *Gen. Comp. Endo.*, 165, 204–214.
- Hildebrandt, T. B., Hermes, R., Saragusty, J., et al. (2012). Enriching the captive elephant genetic pool through artificial insemination with frozen-thawed semen collected in the wild. *Theriogenology*, 78, 1398–1404.
- Howard, J. G., & Wildt, D. E. (2009). Approaches and efficacy of artificial insemination in felids and mustelids. *Theriogenology*, 71, 130–148.

- Kersey, D. C., & Dehnhard, M. (2014). The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. *Gen. Comp. Endo.*, 203, 296–306.
- Malecki, I. A., Rybnik, P. K., & Martin, G. B. (2008). Artificial insemination technology for ratites: A review. *Aust. J. Exp. Agri.*, 48, 1284–1292.
- Morrow, C. J., Penfold, L. M., & Wolfe, B. A. (2009). Artificial insemination in deer and non-domestic bovids. *Theriogenology*, 71, 149–165.
- O'Brien, J. K., & Robeck, T. R. (2010). The value of ex situ cetacean populations in understanding reproductive physiology and developing assisted reproductive technology for ex situ and in situ species management and conservation efforts. *Inter. J. Comp. Psych.*, 23, 227–248.
- Pope, C. E., Gomez, M. C., & Dresser, B. L. (2006). In vitro production and transfer of cat embryos in the 21st century. *Theriogenology*, 66, 59–71.
- Pukazhenthi, B. S. (2016). Saving wild ungulate diversity through enhanced management and sperm cryopreservation. *Reprod. Fertil. Dev.*, 28, 1133–1144.
- Pukazhenthi, B. S., & Wildt, D. E. (2004). Which reproductive technologies are most relevant to studying, managing and conserving wildlife? *Reprod. Fertil. Dev.*, 16, 33–46.
- Stoops, M. A., Campbell, M. K., DeChant, C. J., et al. (2016). Enhancing captive Indian rhinoceros genetics via artificial insemination of cryopreserved sperm. *Anim. Reprod. Sci.*, 172, 60–75.
- Swanson, W. F. (2006). Application of assisted reproduction for population management in felids: The potential and reality for conservation of small cats. *Theriogenology*, 66, 49–58.
- Swanson, W. F. (2012). Laparoscopic oviductal embryo transfer and artificial insemination in felids: Challenges, strategies and successes. *Reprod. Dom. Anim.*, 47(Suppl. 6), 136–140.

Relevant Website

<http://www.iswe.org>. – International Society of Wildlife Endocrinology.

Captive Breeding Wildlife: Fish, Amphibians and Reptiles

Paul D'Ortona and Scott McRobert, Saint Joseph's University, Philadelphia, PA, United States

© 2018 Elsevier Inc. All rights reserved.

Glossary

Amplexus The mating position for frogs and toads in which the male grasps the female around the waist or under the arms, from behind.

Anadromous A system in which ocean-living fish migrate into fresh water to spawn.

Anurans Frogs and toads.

Carapace The upper portion of a turtle's shell.

Catadromous A system in which fish living in fresh water migrate down rivers to the ocean to spawn.

Cloaca The common cavity into which the intestinal, urinary, and reproductive canals open in birds, reptiles, amphibians, many fishes, and certain mammals.

External fertilization Situations in which sperm and egg come into contact outside the bodies of the male and female.

Female Choice A mating system in which females choose their mates.

Husbandry The care and breeding of animals.

Intromission The process of inserting a male sexual organ into a female sexual organ during copulation.

Metamorphosis The process of transformation from an immature form to an adult form in two or more distinct stages.

Model Species Organisms that are ideal for scientific studies.

Morphology The form and structure of an animal.

Oviparity Producing eggs that mature and hatch after being expelled from the body.

Ovoviviparity Producing eggs that are hatched within the body, so that the young are born alive but without placental attachment.

Parthenogenesis The production of offspring via asexual reproduction.

Pedomorphosis The retention of juvenile features in the adult animal.

Phenotype The appearance of an organism resulting from the interaction of genotype and the environment.

Plastron The lower portion of a turtle's shell.

Protandry Form of hermaphroditism in which a male transforms into a female.

Protogyny Form of hermaphroditism in which a female transforms into a male.

Sequential hermaphroditism A reproductive system in which animals change sex.

Spawning The release of gametes (sperm and egg) directly into the water by aquatic organisms.

Squamata The largest recent order of reptiles that include lizards and snakes.

Temperature-Dependent Sex Determination A situation in which the temperatures experienced during embryonic/larval development determine the sex of the offspring.

Ventral Region The underside of an animal.

Viviparity The process of giving birth to living young rather than eggs.

Introduction

Reproductive activity is common, obviously, to every biological species on the planet. We often think of Darwinian natural selection, as 'survival of the fittest,' but in truth, a better abbreviation of Darwin's work would be, 'survival, and reproduction, of the fittest.' Because, indeed, survival alone will not address evolutionary success. Organisms must also reproduce in order to pass their genes on to the next generation.

But while reproduction is an elementary component in the life history of every species, the job of getting organisms to reproduce in captivity can be quite challenging. And yet, from many different perspectives, this task is being met. Scientists, conservationists, and entrepreneurs all find themselves in situations where breeding animals in captivity is necessary. From a scientific perspective, one of the major requirements for animals known as "model species," (species that are ideal for use in scientific studies), is the ability to easily create scenarios in which these organisms will reproduce in the laboratory. A classic example being the often misnamed "fruit fly" *Drosophila melanogaster*, whose glorious history as a model organism for the study of genetics was promoted by the ease with which these animals will prodigiously reproduce under the artificial conditions of the laboratory.

In this article, we will address the idea of captive reproduction in groups of animals that are loosely referred to as wildlife: Fish, Amphibians and Reptiles. In truth, these groups are so large, and so disparate, that a single article would be scarcely sufficient to cover any one of them alone. So, we will provide a brief overview of the major categories within each group, and examine the challenges and successes of captive breeding, focusing on a few model species, as well as species that would be considered quite exotic within a scientific laboratory. This will, in no way, provide exhaustive coverage on breeding behavior in these groups, but will offer a general overview.

Fish

Determining the total number of species, of any group, is a very difficult task, but fish, indeed, represent a huge collection of organisms. Estimates suggest there are somewhere between 30,000 and 40,000 different species of fish. However, again, this is only a rough estimate, and new species of fish seem to be discovered every year. The vast numbers of fish occupy a number of subgroups, including the bony fishes (Osteichthyes), whose skeletons are composed primarily of bones (example: tuna), and the cartilaginous fishes (Chondrichthyes), whose skeletons are composed of cartilage (example: great white shark). In general, a basic definition of a fish would be a streamlined aquatic organism that utilizes gills to extract oxygen from water.

As fish are such a large, diverse group, there are many ways to separate these animals into different categories. One common scheme is to divide this group into freshwater fish, which live in streams, rivers, ponds, and lakes, and salt-water, or marine, fish, which live in the oceans, coral reefs, and estuaries. The multitudes of species in each of these groups are specifically adapted to the salinity conditions of the places they live. And while most fish fall into one of these two categories, some fish actually spend their lives in both types of aquatic ecosystems. Anadromous fish, like salmon, are born in fresh water (typically the head waters of a river), swim downstream and spend their lives in the ocean, then return to the river to reproduce. Catadromous fish, like the American eel, are born in salt water, then migrate to freshwater rivers where they spend their lives before returning to the ocean to spawn.

Another way to divide fish is to identify different species by their mode of reproduction. Some fish are egg-layers. Some are live-bearers. And, within both groups there are species who provide parental care to their offspring and species that don't. There are even species that demonstrate sequential hermaphroditism, in which sex change is possible, and comes in two forms: protandry, where males change into females (i.e., clownfish), and protogyny, where females change into males (i.e., parrotfish).

In terms of captive reproduction, every fish species requires its own set of conditions. It is critical to work with animals that can be maintained in optimal health in captivity before breeding can even be considered. One well-known example is the zebrafish (*Danio rerio*). This species has become important in scientific research, owing largely to the fact that its eggs and offspring are transparent, allowing researchers to visualize the developmental process and test the effects of mutations on cell division and differentiation. And since zebrafish are vertebrates, they are more closely related to humans than other model organisms (like *Drosophila*). About 70% of the zebrafish genome is similar to that of humans, and this species has become a model system to understand the effects of human diseases like muscular dystrophy and many forms of cancer.

With respect to breeding, zebrafish are egg-layers, and their reproductive biology can easily be adapted to the laboratory (see [Westerfield \(2007\)](#)). Males and females are sexually dimorphic, with males being more streamlined and colorful, while females are plump and less colorful. Spawning is stimulated by light cues at daybreak and a common method for breeding includes housing a single male and female overnight in a small tank with a divider down the middle. At daybreak the divider is removed, allowing the fish to be together. Spawning involves the male and female simply releasing their gametes (sperm and eggs) into the water. Fertilization occurs outside the body and the eggs sink to the bottom. After spawning, the researchers can simply collect the hundreds of eggs produced in each spawning event.

An example of an egg-laying fish that is popular in both scientific laboratories and the pet trade is the convict cichlid (*Amatitlania nigrofasciata*), native to Central America. Their common name derives from the dark stripes that run along their bodies, which are reminiscent of uniforms worn by convicts in many prison systems.

Breeding convict cichlids requires little more than housing a small group of them in a large freshwater tank, preferably with lots of complex features (rocks, driftwood, plants, etc.). These fish are relatively aggressive, so care must be taken not to crowd them, nor to house them with smaller fish (which will get eaten), or with other aggressive species (such as other cichlids), as fights will occur. The mating system is female-choice, and the process will begin when a female picks a male and they pair off together in the tank (see [Noonan \(1983\)](#), [Leese \(2012\)](#), [Cleveland-Roberts and Itzkowitz \(2009\)](#)). However, if the owner wishes to exercise more control on this process by setting up specific male/female pairs, the sexes are relatively easy to identify, with females being more colorful, with darker stripes and bright orange, yellow and red pigmentation on their vents.

Breeding in *A. nigrofasciata* begins with the female choosing and preparing a site on which to adhere her sticky eggs. This will always be a hard surface. An ideal spawning location is a clay flower pot, laid on its side at the bottom of the tank. Flower pots not only provide a hard surface on which the female can lay her eggs, but also provides a shelter for the newly hatched young. Once the eggs have been laid, the male releases sperm into the water to fertilize them. This spawning system, like that seen in zebrafish, involves external fertilization.

Convict cichlids are highly involved parents, displaying brooding behavior. Both male and female parents guard their eggs and offspring, aggressively chasing other fish away from the spawning site. This can actually be quite stressful, and possibly dangerous, to other fish in a tank with a brooding pair of convict cichlids. They are, again, quite aggressive and almost any movement on the part of other fish may be interpreted as a threat to the young, eliciting an attack by the parents.

Amphibians

Reproductive behavior is one of the elements that helps define most amphibian species. These animals, which include Anura (frogs and toads), Urodela (salamanders), and Apoda (caecilians), typically begin life as aquatic larvae with gills, then undergo metamorphosis, transforming into a distinctly different adult form, which often live on land and respire through lungs. Amphibians are distinctly tied to freshwater (there are no salt water amphibians) as their eggs, typically laid in water, need to remain moist.

The fertilization of eggs is achieved in a number of ways by amphibians. For Anurans, fertilization is almost exclusively external. The male grasps the female around the waist or under the arms, from behind, and literally squeezes the eggs out of her, which he then covers with sperm. In the Urodela, fertilization is typically internal, but does not involve intromission. Instead, the male deposits a packet of sperm, which the female then picks up with her cloaca. And in the Apoda, fertilization is internal, and does involve intromission, with the male inserting his copulatory organ into the female's cloaca.

Frogs

Amphibians are well represented in both the pet trade and the laboratory, and thus captive breeding can be accomplished for a number of species. In the South American poison frog, *Epipedobates anthonyi*, males are territorial and court females by singing their high-pitched trill from their home bases. Singing is a common male courtship behavior amongst Anurans. The females, attracted by the song, enter a male's territory where he grasps them around their waist or neck, and holds the female until she releases around 15–40 eggs (in a clear gelatin), onto a leaf. The male fertilizes the eggs, and then guards them. Upon hatching, the male nestles into the egg mass and allows the tadpoles to wriggle onto his back. He then carries them to a small body of water and releases them. They will then complete their development and metamorphosis on their own.

Salamanders

Salamanders are characterized by four short limbs, rounded snouts, long/slender bodies, and the presence of a tail in both larval and adult stages. Most salamanders undergo metamorphosis, starting life as an aquatic form and becoming terrestrial as adults. However, some variation exists, such as in *Ambystoma mexicanum*, which exhibits pedomorphosis, and *Notophthalmus viridescens*, which experiences a life cycle that includes an aquatic larva, red eft (juvenile terrestrial stage), and fully aquatic adult, ultimately undergoing metamorphosis twice (Davis and Grayson, 2007). Salamanders also vary in their reproductive methods, exhibiting oviparity (Jockusch, 2004), with species that lay eggs in both aquatic and terrestrial environments, as well as ovoviviparity and viviparity (Velo-Antón *et al.*, 2012). Fertilization also varies, with most species exhibiting internal fertilization and some exhibiting external fertilization.

Among salamanders kept in captivity, fire salamanders (*Salamanca salamandra*), native to Europe, seem to be the easiest to breed in captivity. Mating takes place on land, with males depositing spermatophores for females to pick up, leading to internal fertilization. Depending on subspecies, fire salamanders can be either ovoviviparous or viviparous. Ovoviviparous subspecies will internally incubate eggs until giving birth to aquatic larvae, while viviparous subspecies will give birth to fully developed terrestrial juveniles. In addition to proper husbandry, captive breeding can be accomplished by housing a mature male and female together and by artificially creating seasonal changes in the enclosure. A gradual decrease in temperature down to mid-40°F should take place for about one month, and then gradually brought back to 67–75°F over a two-week period. Towards the end of the cooling period, an ovoviviparous female will deposit her offspring in water, and larvae can then be housed communally in a simple aquarium with a gentle filtration system and fed a diet of live blackworms, daphnia, and commercial salamander food. Once aquatic larvae develop all four legs, they should be moved to a shallow enclosure so they can easily exit the water.

Reptiles

Reptiles mark the next evolutionary step, when amphibians moved further from their reliance on freshwater for living conditions and reproduction. The reptilians have colonized just about every possible habitat, including the land, standing freshwater ponds, swamps and lakes, flowing freshwater rivers and streams, and even the open seas. Reptiles are represented by the lizards, crocodilians, snakes, and turtles, and are commonly found in captivity in the pet trade, laboratories, and zoos.

Lizards

Lizards belong to the order Squamata and consist of more than 6000 species. Diverse in their colors, patterns, and sizes, lizards like leopard geckos (*Eublepharis macularius*), crested geckos (*Correlophus ciliatus*), and bearded dragons (*Pogona vitticeps*) in particular, have become popular in the pet trade. Lizards have also become popular in research due to their unique qualities, behaviors, and relative ease of captive breeding. Examples of scientific research conducted on lizards includes temperature-dependent sex determination (Viets *et al.*, 1993) and embryonic development (Wise *et al.*, 2009) in *Eublepharis macularius*, toe pad adhesion in *Gekko gecko* (Autumn *et al.*, 2000), eggshell characteristics in *Correlophus ciliatus* (Andrews, 2017), and reproductive morphology and behavior in *Anolis carolinensis* (Lovern *et al.*, 2004).

Breeding lizards in captivity can be accomplished rather easily when species-specific needs are met. Crested geckos, native to New Caledonia, are an excellent example of a species that can be easily bred in captivity when maintained properly. Breeding crested geckos in captivity is as straightforward as introducing a healthy adult male into a healthy adult female's enclosure. Males usually become sexually mature around 9 months of age and females typically around 1-year old. Copulation usually occurs soon after introduction, with the male performing head bobs and chirping sounds, and then holding onto the female's neck with his mouth during copulation. Approximately 30–45 days later the female will lay 2 eggs in moist soil or moss (Hamper, 2005). Eggs should be removed from the enclosure as soon as possible and into an incubator (78–82°F) in damp vermiculite. Eggs typically hatch in 60–75 days.

Snakes

Snakes, also belonging to the order Squamata, consist of more than 3000 species, and are characterized by the lack of limbs and extra skull joints, allowing for a highly moveable jaw. Reproductive modes vary across species (Shine, 2003), with recent documentations of reproduction in snakes via oviparity (Brown and Shine, 2004), ovoviviparity (Birchard *et al.*, 1984), viviparity (Bertona and Chiaraviglio, 2003), and/or facultative parthenogenesis (Booth *et al.*, 2012). Similar to lizards, snakes have also become popular in the pet trade, with ball pythons (*Python regius*), cornsnakes (*Pantherophis guttatus*) and kingsnakes/milksnakes (*Lampropeltis* spp.) being very popular pets.

Captive reproduction in ball pythons starts with selecting sexually mature females weighing at least 1000 g, and males weighing at least 650 g. A cooling period (75–82°F) and feeding cessation for 2–3 months, followed by a return to normal temperatures (86–95°F), will stimulate copulation (De Vosjoli, 2003). Females will ovulate 6–30 days after copulation and will lay a clutch of 1–11 eggs. Eggs should be incubated (86–91°F) in damp vermiculite and will hatch in 53–60 days.

Turtles

Turtles are reptiles of the order Testudines (formerly Chelonia), best known for their bony protective shell consisting of an upper portion (the carapace), lower portion (plastron), and an interconnecting region (known as the bridge). Turtles are considered one of the oldest groups within the Reptilia, and today there are approximately 250 species. These animals have occupied almost every ecosystem on Earth, including freshwater rivers, lakes, ponds and swamps, brackish water estuaries and marshes, the ocean, and on land in forests, grasslands and deserts.

Turtles have long been part of the pet trade, although, in truth, turtles are difficult animals to keep in captivity. Turtles are very long-lived animals (lifespans of 60–80 years are not uncommon), and many people who purchase pet turtles simply grow tired of them. What's more, the lifestyle of most turtles requires both an aquatic zone for swimming and feeding, and a land area for basking. Such complex requirements are often hard to create in standard home fish tanks. However, despite concerns over longevity and aquarium care, turtles have been popular pets for decades.

Breeding turtles in captivity is not common for a number of reasons. Turtles often require many years (possibly 5–50) to reach sexual maturity. Owners must also have, obviously, a male and female of the same species for breeding to occur. And, finally, for successful breeding, the housing must include a location that will enable the female to dig a nest in which to lay her eggs.

Breeding in turtles is nearly the same for all species. Males approach females and typically perform some type of courtship display behavior. Females may initially retreat from males, who are persistent, following the females and continuing to preform courtship. If the courtship behavior is successful, the female will stop trying to escape the male, who will mount her from behind, allowing his tail to wrap around hers, such that his cloaca is lined up with hers. The cloaca is an orifice on the underside of the tail that serves as an opening for the digestive, urinary, and reproductive tracts. Typically, in turtles, female cloacae are located at the base of the tail, while male cloacae are located further down the tail, which enables males to press their cloaca up against the female's, allowing intromission to occur. Sperm is expelled from the male cloaca directly into the female, who is then able to store the sperm for fertilization of her eggs (see Pearse and Avise (2001)).

Probably the most well-known captive turtle is the red-eared slider (*Trachemys scripta elegans*). This animal, whose natural habitat is streams, ponds, and lakes in the southeastern United States, has been a mainstay of the pet trade and scientific research for decades. Unfortunately, as people all over the world have grown tired of their pets, this species has been released into the wild and is now found, as an invasive species, in practically every region of the world whose natural environment is capable of supporting turtles.

Reproductive behavior in *T. scripta elegans* begins with a fascinating male courtship display. When a sexually mature male encounters a sexually mature female he attempts to place himself in front of her, so he and the female are face-to-face. He will then extend both front feet and vibrate them rapidly. During this behavior, the males toe-nails (which are much longer than the female's, and are a good way to determine sex in these species), brush against the female's face. This behavior may cause the female to bite at the male's toe-nails, or simply flee. The persistent male will give chase, repeatedly trying to get in front of the female and vibrate his nails in her face. If he succeeds in stimulating the female sufficiently, she will stop trying to escape and simply pull her head into her shell. This allows the male to swim behind her, mount her, and use his cloaca to deliver sperm into her cloaca.

Following mating, in *T. scripta elegans*, or practically any other captive turtle, it is important to provide the female with a tank that includes an egg-laying region. At this point the male's work is done, and since he has no further role in reproduction, it is best to separate him from the female. The female will eventually begin searching for a location to lay her eggs. Female turtles are notoriously picky about the place they deposit their eggs, and so the keeper needs to create a suitable setting. For *T. scripta elegans*, a proper

nesting location typically consists of a plastic box containing a substrate such as soil and sand (although keepers may also try peat moss or hardwood mulch in place of the soil). The nesting substrate must be deep enough for the female to dig her nest. If the substrate is too shallow and she hits the bottom of the box before digging a deep nest, she will abandon the nesting attempt. What's more, the substrate needs to be kept moist, not wet. If these conditions are met, the female will dig a nest and deposit her eggs (typically 5–20 eggs for *T. scripta elegans*). She will then cover the nest very carefully and she is then done. Again, there is no paternal care in turtles.

Under captive conditions it is usually best for the keeper to now remove the eggs. Incubating the eggs in the female's tank may not be successful, and if it is, the keeper runs the risk of having the eggs hatch and the baby turtles entering the water with large adult turtles where they may drown or be eaten. So, removing the eggs is standard operating procedure. To do this, the keeper excavates the nest, taking care not to damage the fragile eggs. Once the eggs are uncovered they are often marked in pencil with a small 'x' to denote the upper region of the eggs. It is critical that the eggs are not rotated at this point, as rotation will kill the developing embryo. As each egg is marked and carefully exhumed, it can be placed into a suitable tray containing a suitable substrate, such as vermiculite or perlite. The tray of eggs can then be placed into an incubator. The temperature of the incubator is then an important consideration. In the turtles that have been studied, sex is determined by incubation temperature, with one range of temperatures causing the embryos to develop into females and another range of temperatures leading to male development (see Gilbert and Barresi (2016), Ewert and Nelson (1991)). In *T. scripta elegans*, lower temperatures (around 26°C) primarily produce males, while higher temperatures (around 31°C) primarily produce females.

References

- Andrews, R. N. (2017). Novel eggshell of the New Caledonian Diplodactylid Gecko Species *Correlophus ciliatus* (= *Rhacodactylus ciliatus*). *J. Herpetol.*, 51(2), 173–177.
- Autumn, K., Liang, Y. A., Hsieh, S. T., et al. (2000). Adhesive force of a single gecko foot-hair. *Nature.*, 405, 681–685.
- Bertona, M., & Chiaraviglio, M. (2003). Reproductive biology, mating aggregations, and sexual dimorphism of the Argentine Boa constrictor (*Boa constrictor occidentalis*). *J. Herpetol.*, 37(3), 510–516.
- Birchard, G. F., Black, C. P., Schuett, G. W., & Black, V. (1984). Foetal-maternal blood respiratory properties of an ovoviparous snake the cottonmouth, *Agkistrodon piscivorus*. *J. Exp. Biol.*, 100(1), 247–255.
- Booth, W., Smith, C. F., Eskridge, P. H., et al. (2012). Facultative parthenogenesis discovered in wild vertebrates. *Bio Lett.*, 8(6), 983–985.
- Brown, G. P., & Shine, R. (2004). Maternal nest-site choice and offspring fitness in a tropical snake (*Tropidonophis mairii*, Colubridae). *Ecology*, 85(6), 1627–1634.
- Cleveland-Roberts, A., & Itzkowitz, M. (2009). The role of sex ratios and resource availability on the pre-mating behavior of a monogamous fish. *Behav. Proc.*, 80, 46–50.
- Davis, A. K., & Grayson, K. L. (2007). Improving natural history research with image analysis: The relationship between skin color, sex, size and stage in adult red-spotted newts. *Herp. Conserv. Biol.*, 2(1), 65.
- De Vosjoli, P. (2003). *The Ball Python Manual*. Irvine, CA: I5 Publishing.
- Ewert, M. A., & Nelson, C. E. (1991). Sex determination in turtles: Diverse patterns and some possible adaptive values. *Copeia*, 1991(1), 50–69.
- Gilbert, S. F., & Barresi, M. J. F. (2016). *Developmental Biology* (eleventh ed.). Oxford, U.K.: Sinauer Associates.
- Hamper, R. (2005). *Crested Geckos in Captivity*. Rodeo, NM: ECO Herpetological Publishing and Distribution.
- Jockusch, E. L. (2004). Techniques for obtaining and raising plethodontid salamander eggs. *Int. J. of Dev. Biol.*, 40(4), 911–912.
- Leese, J. M. (2012). Sex differences in the function of pair bonding in the monogamous convict cichlid. *Anim. Behav.*, 83, 1187–1193.
- Lovern, M. B., Holmes, M. M., & Wade, J. (2004). The Green Anole (*Anolis carolinensis*): A reptilian model for laboratory studies of reproductive morphology and behavior. *ILAR Journal.*, 45(1), 54–64.
- Noonan, K. (1983). Female mate choice in the cichlid fish *Cichlasoma nigrofasciatum*. *Anim. Behav.*, 31, 1005–1010.
- Pearse, D. E., & Avise, J. C. (2001). Turtle mating systems: Behavior, sperm storage and genetic paternity. *J. Hered.*, 92, 206–211.
- Shine, R. (2003). Reproductive strategies in snakes. *Proc. R. Soc. Lond. B: Biol. Sci.*, 270(1519), 995–1004.
- Velo-Antón, G., Zamudio, K. R., & Cordero-Rivera, A. (2012). Genetic drift and rapid evolution of viviparity in insular fire salamanders (*Salamandra salamandra*). *Heredity.*, 108(4), 410.
- Viets, B. E., Tousignant, A., Ewert, M. A., Nelson, C. E., & Crews, D. (1993). Temperature-dependent sex determination in the Leopard Gecko, *Eublepharis macularius*. *J. Exp. Zool.*, 265, 679–683.
- Westerfield, M. (2007). *The Zebrafish book. A Guide for the Laboratory use of Zebrafish (Danio rerio)* (fifth ed.). Eugene, OR: University of Oregon Press.
- Wise, P. A. D., Vickaryous, M. K., & Russel, A. P. (2009). An embryonic staging table for *In Ovo* Development of *Eublepharis macularius*, the Leopard Gecko. *Anat. Rec.*, 292, 1198–1212.

Reproductive Technology (Non-Human/Non-Primate): Sex Control and Sterilization in Fish

Ten-Tsao Wong and Yonathan Zohar, University of Maryland, Baltimore, MD, United States

© 2018 Elsevier Inc. All rights reserved.

Introduction

Fish, the most diversified group among vertebrates, exhibit prolific and variable reproductive strategies, which reveal the complexity of sex control in nature. Unlike mammals, no common mechanism of sexual development can be applied to all fishes. The gonads of vertebrates generally develop in the dorsal lateral lining of the peritoneal cavity and possess double origins, whereas the more laterally located cortical portion generates the ovary and the medullary portion gives rise to the testis. In teleost fish, only a single primordium (cortex) appears to be involved in the ontogeny of both ovary and testis. This may contribute to the greater plasticity of gonadal development in fish, in contrast with the more stable patterns found in higher vertebrates. This sexual plasticity offers a great opportunity for studying the evolution and mechanisms of sex determination and differentiation in nature, as well as practical applications of sex manipulation in fish.

Sex changes in hermaphroditic species and sexual dimorphisms in growth, size, appearance (ornamental fish) and maturation are vital to the practices of aquaculture broodstock management, seed production, and grow-out performance in commercial operations. In addition, gonadal development and sexual maturation compromise fish health, reduce production output and jeopardize the protection of wild stocks and ecosystems. As such, sex control and sterility induction are two key reproductive technologies that provide significant solutions to enhance environmentally-friendly and cost-effective aquaculture practices through (1) promoting controlled reproduction and constant and voluminous seed production; (2) offering monosex populations; (3) providing reproductively sterile populations to ensure genetic containment; and (4) preventing precocious maturation to enhance fish growth, health and market value.

Sex Control in Fish

The ability to manipulate sexual development in fish provides broad economic gains for aquaculture. Optimal sex ratio maximizes seed production in hatcheries. Hermaphroditic sex change creates instability of broodstock management of both sexes, which impairs the seed supply. The shortages of a specific sex in the protandrous gilthead seabream (Zohar *et al.*, 1995) or in the protogynous groupers (Tan and Tan, 1974), which change sex depending on their size and/or age, have been a major challenge in the development of seabream and grouper aquaculture. In some commercially valuable groupers, it may take 7 or 9 years to establish male populations (Chauvet, 1988; Tan and Tan, 1974). Sex control technologies also enable the monosex culture that helps achieve higher growth rates and reduces size variation and aggressive territorial or sexual behavior, as well as prevents undesirable precocious maturation and undesired reproduction in culture systems. All-female trout and all-male tilapia culture are two common practices in monosex culture to enhance economic return in the aquaculture industry (Devlin and Nagahama, 2002).

Hormonal Sex Control Approaches

Hormonal manipulation is the most common approach for sex control in fish. Desired sex is attained by exposing sexually undifferentiated individuals to female sex steroids (estrogens) or male sex steroids (androgens), with the aim of forcing the course of sex differentiation towards the favored sex. Identification of the responsive window, optimal dose, and duration of hormone treatment are principal steps towards the successful sex control of the undifferentiated gonad and the prevention of deformation and contradictory outcomes. The most widely used and effective masculinization hormone is 17 α -methyltestosterone (MT), which can be used to produce males directly or females indirectly (Fig. 1(A), also see genetic sex control approaches). In addition to the use of the more metabolically stable MT, aromatase inhibitors, such as Fadrozole, have been utilized to block the activity of aromatase, a key enzyme that catalyzes the formation of estrogen from androgen (Fig. 1(A)), which resulted in the reduction of estrogens and the acceleration of the female-to-male sex reversal process in grouper (Bhandari *et al.*, 2004). In contrast, the most commonly used feminization hormone is 17 β -estradiol (E₂), which can be utilized to produce females directly or males indirectly (Fig. 1(B), also see genetic sex control approaches). Although these approaches have been used in the industry, they are not widely accepted by regulatory agencies due to the potential of exposure of workers to the steroids or residues in the fish, water and/or culture systems (Rothbard *et al.*, 1990).

Temperature Sex Control Approaches

In teleosts, the process of sexual development, sex determination and differentiation, is controlled by genetic, environmental factors, and genotype–environment interactions during a critical phase of early development. Temperature is the most common environmental

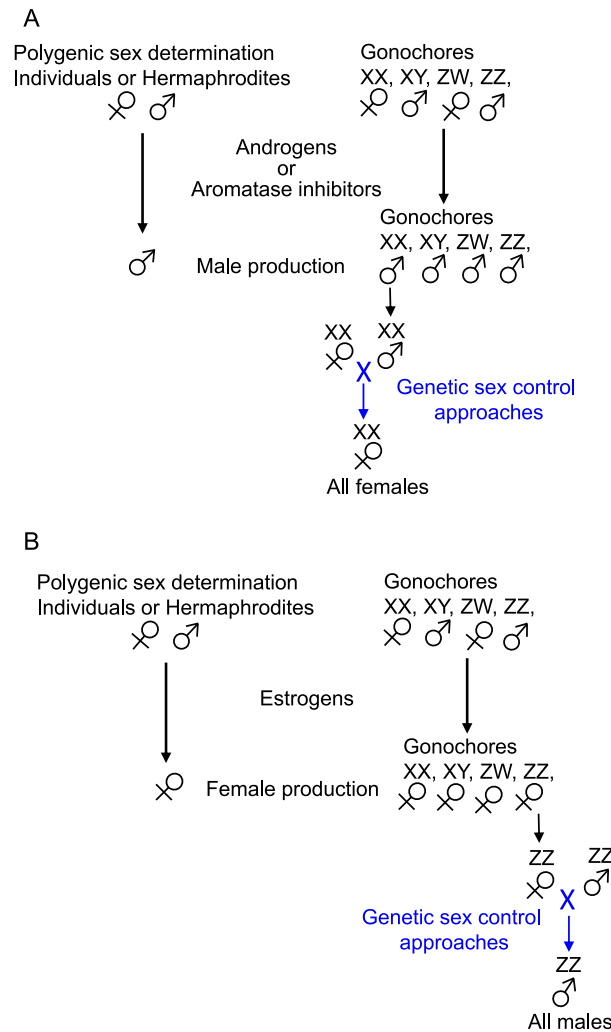


Fig. 1 Hormonal sex control approaches. (A) Androgens or aromatase inhibitors are used for direct male production or indirect female production that involves genetic sex control approaches. (B) Estrogens are used for direct female production or indirect male production that involves genetic sex control strategies.

factor affecting sex determination. Since the first description of temperature-dependent sex determination (TSD) in Atlantic silverside, *Menidia menidia* (Conover and Kynard, 1981), about 60 different fish species have been reported to possess TSD, in which male-leaning sex ratios are linked to either high, low or extreme (both high and low) temperatures (Ospina-Alvarez and Piferrer, 2008). The actions of thermal cues require their interactions with genes that are involved in regulating sexual development, such as doublesex and mab-3 related transcription factor 1 (*dmrt1*), anti-Müllerian hormone (*amh*) and transcription factor SRY box 9 (*sox9*) related to male-development, as well as cytochrome P450 aromatase (*cyp19a1a*) and forkhead box protein L2 (*foxl2*) linked to female-development. Changes in expression levels of these candidate genes have been shown to be associated with temperature-induced sex differentiation, which triggers the thermosensitive and undifferentiated gonads to follow the testis- or ovary-developmental pathway. Furthermore, epigenetic-, cortisol- and hypoxia-mediated pathways have also been reported to mediate the effects of high temperature by inducing methylation of the *cyp19a1a* promoter, suppressing germ cell proliferation and *fshr* expression or increasing the testosterone/estradiol ratio, respectively, to promote male-skewed sex ratio (Shen and Wang, 2014).

Knowledge of TSD offers environmentally- and consumer-friendly approaches for sex control in fish. It can be utilized to control sex ratio in broodstock management by selectively breeding one sex over the other and producing sex-skewed or mono-sex populations in aquaculture applications. High temperature regimes (34–35°C) have been used to produce high male ratios (95% to 100%) of Nile tilapia (Desprez and Méléard, 1998) (Fig. 2).

Genetic Sex Control Approaches

One of most commonly used genetic sex control approaches to produce monosex populations for commercial aquaculture operations is the indirect utilization of hormone to first produce neo-males (XX) or neo-females (ZZ) that can be used to cross

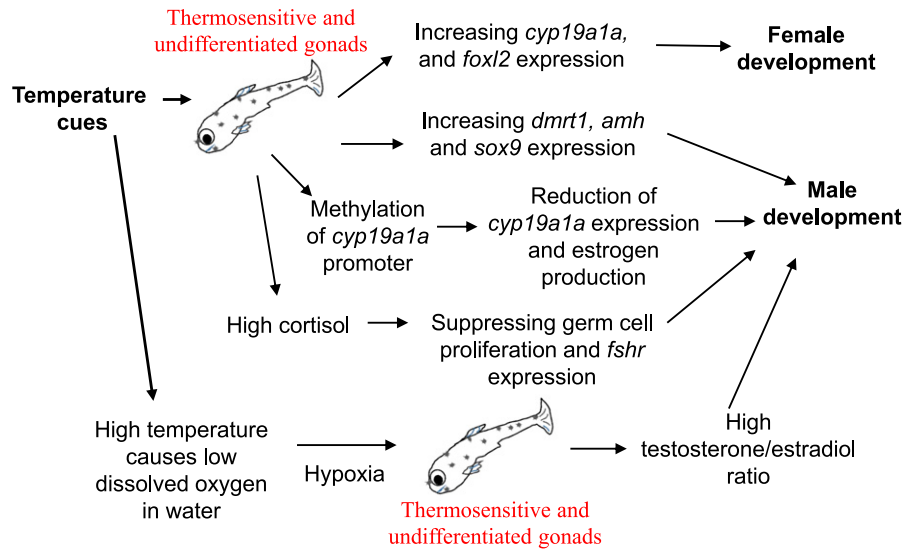


Fig. 2 Signal pathways and molecular players involved in temperature-dependent sex determination.

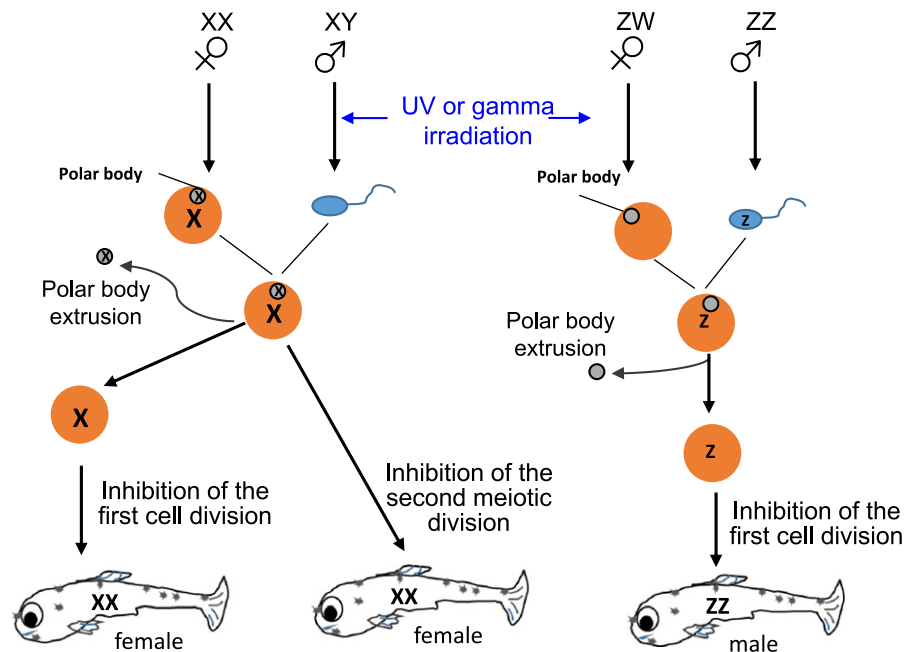


Fig. 3 Gynogenesis or androgenesis to produce monosex populations.

with normal females (XX) or males (ZZ), respectively, to produce all-female (XX) or all-male (ZZ) populations (Fig. 1(A and B)). Other genetic approaches that have applied uniparental or parthenogenetic reproduction by gynogenesis or androgenesis to produce homozygous (double haploid) monosex populations have also been described. In these approaches, the genetic material of one gamete (either sperm or egg) is destroyed by UV or gamma irradiation, followed by fertilization with an untreated egg or sperm to form a haploid embryo. Later in development, a pressure, thermal or chemical shock is applied to inhibit either the second meiotic division (extrusion of the polar body) or the first cell division. When the first cell division is blocked, the resulting individual is a double haploid homozygous individual that inherits a duplicated set of chromosomes from the untreated gamete (Komen and Thorgaard, 2007), which can be also used to produce all-female (XX) or all-male populations (Fig. 3). In aquaculture practices, gynogenetic approaches have been successfully applied to produce all-female fish in several species including carp and flounders (Mei and Gui, 2015).

Another genetic sex control approach is to employ interspecies hybridization to produce all-male or all-female fish. In some closely related species, the strength of male- and female-determining genes located on autosomes or sex-chromosomes are different among different species, which may enable one species to override the others on sex determination to allow the

development of a skewed sex ratio or monosex population by interspecies hybridization. This approach has been successfully used to produce monosex populations in tilapia, bass and cyprinodons. For example, all-male tilapia populations were obtained by crossing female *O. mossambicus* (XX) with male *O. hornorum* (ZZ), and all-female bass can be achieved by crossing female striped bass with male yellow bass (Devlin and Nagahama, 2002; Mei and Gui, 2015).

In recent years, more sophisticated techniques including quantitative trait locus (QTL), single nucleotide polymorphism (SNP), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSR), RNA-Seq and transcriptomic approaches were utilized to successfully identify and locate sex traits, sex- or sex-chromosome-specific markers, temperature-dependent sex reversal markers and sex-biased gene expression (Mei and Gui, 2015; Shen and Wang, 2014). These advances provide a strong foundation to accelerate the genetic improvement of sex control in commercial hatcheries through selective breeding programs. Ultimately, these efforts will assist in the production of monosex populations and maintenance of expected broodstock sex ratios in aquaculture operations.

Sterility Induction in Fish

Sterility carries environmental significance, as the escaped infertile fish are not able to propagate and/or interbreed with wild stocks. In addition, many farmed fish attain sexual maturity before reaching market size. Sexual maturation is associated with a substantial decrease in somatic growth due to the diversion of energy toward the development of the gonads. The period of intensive gonadal growth during sexual maturation also results in deterioration of flesh quality and an increase in susceptibility to stress and disease. Sterilization minimizes energy input toward gonadal growth while enhancing muscle (flesh) development and promoting fish health and overall performance (Manzoor Ali and Satyanarayana Rao, 1989; Zohar, 1989). Sterile fish are also a prominent tool for biological control of non-native fish. The sterile-male-release technique (Bergstedt *et al.*, 2003; Twohey *et al.*, 2003), similar to the sterile-insect-technique (Krafsur, 1998), can be used to control the populations of non-native species by competitively interrupting reproduction. Furthermore, sterility is a means for producers to protect their valuable, genetically selected strains from unauthorized propagation. Methods used to induce sterility are (1) manipulation of chromosome set number by triploidization or interspecies hybridization; (2) genetically-engineered germ cell ablation via expression of a specific transgene or knockout of an essential gene that disrupts germ cell development or causes germ cell death; (3) transient gene silencing to knockdown germ cell essential genes to disrupt germ cell development.

Chromosome Set Manipulation

The most common methods used to induce sterility in the aquaculture industry are chromosome manipulations to cause triploidization or interspecies hybridization, which disrupts the normal pairing of homologous chromosomes during gametogenesis and results in sterility. The triploidization method involves pressure or thermal shock of the fertilized eggs. To directly produce triploids (3N), shock is applied shortly after fertilization to inhibit the second meiotic division and retain the additional maternal 1N chromosome in the first polar body resulting in embryos possessing 2N from the eggs and 1N from the sperm. Indirectly, triploids can also be created from crossing tetraploids (4N) with diploids (2N) gametes. To produce tetraploids, shock is applied to block the first cell division (mitosis), resulting in individuals possessing 2N from the eggs and 2N from the sperm (Arai, 2001; Donaldson and Benfey, 1987). Hybridization is the crossing of genetically differentiated but closely related individuals or groups that are within a species (different line or strain crossing) or between separate species. This technique is used to produce fish with desired characteristics, which includes transferring other desirable traits from one line/species to another, combining valuable traits into a single group, or producing sterile individuals (Chevassus, 1983).

Chromosome set manipulation does not always result in 100% sterility of the treated fish. Studies done on triploid rainbow trout, Atlantic salmon and Atlantic cod found that male fish were seldom completely sterile and many of the treated Atlantic salmon females continued to possess a small number of oocytes that could be fertilized. Another disadvantage of chromosome set manipulation is that it requires that specific methods be developed for each species of fish being treated (Arai, 2001; Wong and Zohar, 2015b). Additionally, triploidy often results in compromised performances (Benfey, 1999; Maxime, 2008; Piferrer *et al.*, 2009), which prevents the implementation of this strategy in the industry.

Germ Cell Depletion Technology

The recently updated strategies that have been used to produce sterile fish are genetically-engineered germ cell depletion methods that disrupt germ cell development or cause germ cell death (Wong and Zohar, 2015b). Among these, one approach is to disrupt primordial germ cell (PGC) development. In fish, PGCs, the precursor cells of gametes, are specified outside of gonads during early development, and then migrate to the developing gonad, guided by a gradient of the chemokine, stromal-derived growth factor 1a (Sdf1a). Therefore, an inducible over-expression of Sdf1a was used to mask the Sdf1a gradient and to saturate the Sdf1a receptor, Cxcr4b, on PGCs, which prevented PGCs from responding to the endogenous Sdf1a signal and from migrating to the developing gonads. As a result, the treated fish developed into sterile individuals with severely under-developed gonads that lacked germ cells (Wong and Collodi, 2013). Another approach is to express *E. coli* nitroreductase specifically in the germ cells. When the fish were

treated with metronidazole, the nitroreductase catalyzed production of a cytotoxin that killed the germ cells. A third method is a maternal sterility approach that targets the elimination of PGCs, using a maternal deposit mRNA that encodes a pro-apoptotic protein B-cell lymphoma 2 associated X protein (Bax). Expression of pro-apoptotic Bax protein subsequently activates caspases and DNases, which leads to apoptosis and the elimination of PGCs. The latest approach is to knock out *deadend* (*dnd*), an essential gene for early germ cell development in fish. *Dnd* is an evolutionarily conserved RNA-binding protein that is specifically expressed in PGCs and plays a critical role in their development during embryogenesis (Weidinger *et al.*, 2003). In Atlantic salmon, homozygous *dnd* knockout effectively ablated the germ cells, which resulted in the production of sterile fish (Wargelius *et al.*, 2016). Results obtained from these transgenic strategies indicate that transgenic ablation of the germ cells to produce reproductively sterile fish is achievable. However, a major downside of these approaches is that it requires the generation of genetically-engineered fish in each species, thus introducing genetic modification into fish and leading to greater ecological risk, long delays for regulatory approval and potential consumer resistance.

Transient Gene Silencing to Disrupt Germ Cell Development

Due to negative public perception, genetically modified foodfish may face long-term resistance from consumers. To overcome the serious disadvantages and hurdles of previous sterilization methods, non-transgenic approaches to produce reproductively sterile fish are desperately needed to reduce food safety concerns and move aquaculture operations toward more environmentally responsible production. As such, a bath-immersion approach to efficiently deplete germ cells and produce sterile fish without introducing genetic modification into fish was developed. In fact, a transient gene-silencing method has been used in the laboratory to produce small-numbers of sterile fish in several species (Wong and Zohar, 2015b). This approach involved microinjecting embryos with a “gene silencing” Morpholino oligomer (MO) that transiently blocked *Dnd* protein expression (*dnd*-MO), which disrupted PGC development, and ultimately resulted in the production of reproductively sterile fish. This finding, together with the production of sterile fish by *dnd* knockout in Atlantic salmon (Wargelius *et al.*, 2016), indicates that blocking *Dnd* function may induce widespread reproductive sterility in fish. One important consideration, however, is that, *dnd*-MO is a relatively large molecule (7–8 kDa) that would not typically be able to cross the egg chorion, a thick acellular multi-

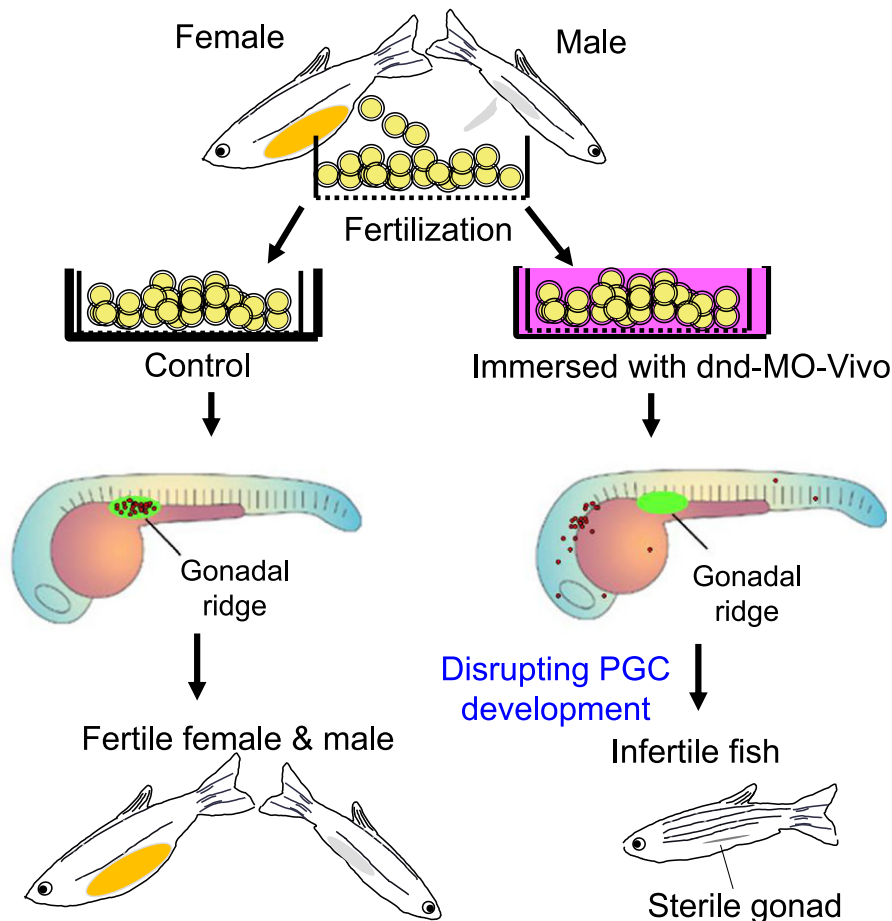


Fig. 4 Vivo conjugated Morpholino oligomer (MO) against *deadend* (*dnd*-MO-Vivo) induced sterility in fish.

layer envelope, and reach the embryos without using microinjection. To overcome this hurdle, a bath-immersion technology was developed to administer the *dnd*-MO into fish embryos using a molecular transporter, also known as Vivo, comprised of a dendrimeric oligoguanidine with a triazine core. Vivo can effectively carry *dnd*-MO across the chorion, enter the embryo and reach the target PGCs. Indeed, immersion of zebrafish embryos in the Vivo-conjugated MO against zebrafish *dnd* effectively disrupted PGC development (Fig. 4). The failure of normal PGC migration and development led to 100% production of reproductively sterile fish (Wong and Zohar, 2015a). Currently, this technology is being transferred from zebrafish, the experimental model, to rainbow trout, Atlantic salmon, tilapia and sablefish. Since the egg chorion differs between species, the immersion conditions will need to be optimized for each species. The successful development of this technology in multiple aquaculture species could have profound implications for eliminating reproductive threats, as well as enhancing fish health and performance. It thus alleviates public concerns associated with food safety and an adverse environmental impact of aquaculture.

Concluding Remarks

During the past two decades, the rapid progression of new biotechnologies, bioinformatics and molecular tools has prominently advanced our knowledge of the genes involved in regulating gonadal development, sex determination and differentiation, and the mechanisms by which environmental factors interact with these genes to alter the outcomes of sexual development. Therefore, novel biotechnologies that incorporate current and expanding knowledge will indeed lead to further successful advances in sex control and sterility induction of fish. The commercial implementation of these superior and reliable technologies will effectively promote more environmentally-friendly and cost-effective aquaculture production practices.

References

- Arai, K., 2001. Genetic improvement of aquaculture finfish species by chromosome manipulation techniques in Japan. *Aquaculture* 197, 205–228.
- Benfey, T.J., 1999. The physiology and behavior of triploid fishes. *Reviews in Fisheries Science* 7, 39–67.
- Bergstedt, R.A., McDonald, R.B., Twohey, M.B., et al., 2003. Reduction in sea lamprey hatching success due to release of sterilized males. *Journal of Great Lakes Research* 29 (Supplement 1), 435–444.
- Bhandari, R.K., Higa, M., Nakamura, S., Nakamura, M., 2004. Aromatase inhibitor induces complete sex change in the protogynous honeycomb grouper (*Epinephelus merra*). *Molecular Reproduction and Development* 67, 303–307.
- Chauvet, C., 1988. Etude de la croissance du mérout *Epinephelus guaza* (Linné, 1758) des côtes tunisiennes. *Aquatic Living Resources* 1, 277–288.
- Chevassus, B., 1983. Hybridization in fish. *Aquaculture* 33, 245–262.
- Conover, D.O., Kynard, B.E., 1981. Environmental sex determination: Interaction of temperature and genotype in a fish. *Science* 213, 577–579.
- Desprez, D., Mèlard, C., 1998. Effect of ambient water temperature on sex determinism in the blue tilapia *Oreochromis aureus*. *Aquaculture* 162, 79–84.
- Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: An overview of genetic, physiological, and environmental influences. *Aquaculture* 208, 191–364.
- Donaldson, E.M., Benfey, T.J., 1987. Current status of induced sex manipulation. In: Idler, D.R., Crim, L.W., Walsh, J.M. (Eds.), *Proceedings of the Third International Symposium on Reproductive Physiology of Fish*, pp. 108–119. Saint John's, Canada: Memorial University of Newfoundland.
- Komen, H., Thorgaard, G.H., 2007. Androgenesis, gynogenesis and the production of clones in fishes: A review. *Aquaculture* 269, 150–173.
- Krafsur, E.S., 1998. Sterile insect technique for suppressing and eradicating insect population: 55 years and counting. *Journal of Agricultural Entomology* 15, 303–317.
- Manzoor Ali, P.K.M., Satyanarayana Rao, G.P., 1989. Growth improvement in carp, *Cyprinus carpio* (Linnaeus), sterilized with 17[alpha]-methyltestosterone. *Aquaculture* 76, 157–167.
- Maxime, V., 2008. The physiology of triploid fish: Current knowledge and comparisons with diploid fish. *Fish and Fisheries* 9, 67–78.
- Mei, J., Gui, J.-F., 2015. Genetic basis and biotechnological manipulation of sexual dimorphism and sex determination in fish. *Science China Life Sciences* 58, 124–136.
- Ospina-Alvarez, N., Piferrer, F., 2008. Temperature-dependent sex determination in fish revisited: Prevalence, a single sex ratio response pattern, and possible effects of climate change. *PLOS ONE* 3, e2837.
- Piferrer, F., Beaumont, A., Falguière, J.-C., et al., 2009. Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture* 293, 125–156.
- Rothbard, S., Zohar, Y., Zmora, N., et al., 1990. Clearance of 17 α -ethynyltestosterone from muscle of sex-inversed tilapia hybrids treated for growth enhancement with two doses of the androgen. *Aquaculture* 89, 365–376.
- Shen, Z.G., Wang, H.P., 2014. Molecular players involved in temperature-dependent sex determination and sex differentiation in Teleost fish. *Genetic Selection Evolution* 46, 26.
- Tan, S., Tan, K., 1974. Biology of the tropical grouper, *Epinephelus tauvina* I. A preliminary study on hermaphroditism in *E. tauvina*. *Singapore Journal of Primary Industries* 2 (2), 123–133.
- Twohey, M.B., Heinrich, J.W., Seelye, J.G., et al., 2003. The sterile-male-release technique in Great Lakes sea lamprey management. *Journal of Great Lakes Research* 29 (Supplement 1), 410–423.
- Wargelius, A., Leininger, S., Skafnesmo, K.O., et al., 2016. Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *Scientific Reports* 6, 21284.
- Weidinger, G., Stebler, J., Slanchev, K., et al., 2003. dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. *Current Biology* 13, 1429–1434.
- Wong, T.T., Collodi, P., 2013. Inducible sterilization of zebrafish by disruption of primordial germ cell migration. *PLOS ONE* 8, e68455.
- Wong, T.-T., Zohar, Y., 2015a. Production of reproductively sterile fish by a non-transgenic gene silencing technology. *Scientific Reports* 5, 15822.
- Wong, T.T., Zohar, Y., 2015b. Production of reproductively sterile fish: A mini-review of germ cell elimination technologies. *General & Comparative Endocrinology* 221, 3–8.
- Zohar, Y., 1989. Endocrinology and fish farming: Aspects in reproduction, growth, and smoltification. *Fish Physiology & Biochemistry* 7, 395–405.
- Zohar, Y., Harel, M., Hassin, S., Tandler, A., 1995. Broodstock management and manipulation of spawning in the gilthead seabream. In: Bromage, N., Roberts, R.J. (Eds.), *Broodstock Management and Egg and Larval Quality*. London: Blackwell Science Inc, Publisher, pp. 94–117.

Methods of Sterilization and Contraception in Mammals

Valerie A Ferro, University of Strathclyde, Glasgow, United Kingdom

Joanna H Sliwowska, Poznan University of Life Sciences, Poznan, Poland

Mohammed Al-Qaraghuli and Manal Alsaadi, University of Strathclyde, Glasgow, United Kingdom

© 2018 Elsevier Inc. All rights reserved.

Glossary

Adenogenesis The development of a gland.

Anogenital Relating to the anus and genitals.

Blood-brain barrier (BBB) Protects the brain from most pathogens.

Castrati singers Male singers in the 18th century castrated in boyhood so as to retain a soprano or alto voice.

Endometrial adenogenesis Development of new blood vessels in the endometrium (the mucous membrane lining the uterus, which thickens during the menstrual cycle in preparation for possible implantation of an embryo).

Eunuch a man who has been castrated, especially (in the past) one employed to guard the women's living areas at an oriental court.

Fallopian Tubes Tubes through which an egg travels from the ovary to the uterus.

Human chorionic gonadotrophin A hormone secreted in the female reproductive tract upon conception, in order to aid implantation of the fertilized egg to the endometrial lining.

Hypothalamic hypogonadism A condition characterized by alterations or lack of sex steroid production by gonads due to a problem with the pituitary gland or the hypothalamus.

Hysteroscopy A procedure in which a device called a hysteroscope is inserted into the uterus through the cervix to view the inside of the uterus or perform surgery.

Laparoscopy A surgical procedure in which an instrument called a laparoscope is inserted into the pelvic cavity through a small incision. The laparoscope is used to view the pelvic organs. Other instruments can be used with it to perform surgery.

Microphallus A small penis.

Minilaparotomy A small abdominal incision used for a sterilization procedure in which the fallopian tubes are closed off.

Orthologs Genes in different species that have evolved from a common ancestral gene by speciation and that normally retain the same function in the course of evolution.

Perinatal Relating to the time, usually a number of weeks, immediately before and after birth.

RF-amide superfamily Characterized by the possession of an Arg-Phe-NH₂ motif at their C-terminal extremities.

Sterilization In fertility, to eliminate the ability of a person or animal to produce offspring, as by altering or removing the reproductive organs.

Tachykinins Family of peptides, which cause hypotension, smooth muscle contraction (e.g., contraction of gut and bladder), and secretion of saliva.

Zona pellucida A complex glycoprotein matrix surrounding the mammalian oocyte and early conceptus.

Sterilization

Definition and Context of Sterilization

In reproductive terms, sterilization is a term used to describe the elimination of the ability of animals, including humans, to reproduce by removing or altering the function of the reproductive organs. In the past this involved permanent solutions, such as irreversible surgical intervention, but increasingly, the trend is towards developing methods that are reversible should the need or desire to reproduce arise. Fertility control in humans, companion animals, farm animals, zoo animals and wild animals (including feral and pest species) have different criteria, requirements and hurdles that need to be overcome when it comes to development of effective sterilization methods ([Garside et al., 2014](#)). Some of these are considered below, but this is by no means a comprehensive list.

Surgical Sterilization Methods

Human: Female tubal ligation

In women, the surgical sterilization procedure is known as tubal occlusion. This involves closing the fallopian tubes to prevent the egg from moving to the uterus and prevents the sperm and egg from meeting. It is a highly effective method, and less than 1% of women become pregnant within 1 year of having the procedure. After 10 years, the pregnancy rate can rise to 4% depending on the

method used. The three main methods are minilaparotomy, laparoscopy or hysteroscopy. A tubal ligation reversal is a procedure to restore fertility after a woman has had tubal ligation, during which the blocked segments of the fallopian tubes are reconnected to the remainder of the fallopian tubes. Success rates depend on the initial extent of surgery and age of the patient; pregnancy success rates range between 45–69% and are lower in the first year after reversal (van Seeters *et al.*, 2017).

Human: Male vasectomy

Historically, human males have been castrated since ancient times, to provide eunuchs as guardians of harems in the Middle or Far East or to produce castrati singers. Surgical procedures involved severing the spermatic chords or crushing the testis with the fingers (Koutsiaris *et al.*, 2014). Today in men, the surgical sterilization procedure is known as vasectomy and is mainly used for contraceptive purposes. In a vasectomy, the vas deferens are tied, cut, clipped or blocked to prevent the release of sperm, which in turn prevents an egg from being fertilized. This procedure is slightly more effective than tubal ligation in women, after 1 year. Around 3–6% of men opt for surgical reversal due to changes in circumstances, indicating that there is a need for reversible sterilization methods (Patel and Smith, 2016).

Animal: Female ovariectomy

Tubal ligation and vasectomy are not commonly carried out in companion or farm animals. Instead, gonadectomy is performed. In females, this is known as spaying (or ovariectomy) where the uterus and ovaries are removed (DeTora and McCarthy, 2011). This is commonly carried out in cats and dogs, but is avoided in larger animals such as horses and cattle. Similarly, this is not a method applied to wild and pest animals for economical and logistical reasons.

Animal: Male neutering

In males, in a procedure known as neutering or castration, each testis with attached epididymis is removed. In dogs, both gonads are usually removed through a single incision made just anterior to the scrotum and the incision is sutured closed. Typically in cats, an incision is made into each side of the scrotum and left open to heal. This method is also used for small farm animals such as piglets. In larger farm animals, gonadectomy is performed, but does not utilize surgical procedures (also known as bloodless or closed techniques, see section below).

In males, the reason for castration varies from reducing aggressive behaviour, improving management in the presence of female animals, to prevent unwanted off-spring, to promote weight-gain in meat animals and to treat injury to the testes or to correct scrotal hernia.

Non-Surgical Sterilization Methods

Due to the expense, inconvenience of surgery and risk of infection, a range of non-surgical sterilization methods have been developed. In the following sections, we will consider some of the alternatives, some of which are in mainstream use, while others are likely to become mainstream in the future.

Closed methods of castration

A range of methods is available, mainly used for castrating male farm animals (see Relevant Websites section). The main advantage of these methods over surgical gonadectomy is there is no need for anaesthesia; however, some of these procedures are considered to be inhumane.

- 1 The Burdizzo® clamp involves crushing the spermatic cords in such a way as to prevent blood from reaching the testicles. Once the blood supply ceases, necrosis occurs and the testicles shrink and eventually deteriorate. The cords are clamped one at a time and at different levels in order to allow enough blood to the scrotal sac to prevent gangrene. This procedure is commonly used in farm animals such as goats, sheep and calves.
- 2 Elastic rubber rings are used in ram lambs, calves and kid goats, with certain age limitations. The rubber ring is placed around the scrotum, above the testicles.
- 3 The short scrotum method also involves a rubber ring, but this time placed below the testicles to push them closer to the body wall to place them at body temperature (to encourage infertility). The disadvantage of this method is that the sex hormones are still produced, so this does not prevent aggressive behaviour.

Synthetic steroids and non-steroidal compounds

There has been a drive to develop chemical methods to enable reversible sterilization. These are designed to have wider application for situations where controlled fertility is required. In humans, companion animals or zoo animals, steroids and non-steroidal implants or injections are commonly used.

Products used in humans

In humans there are a wide range of oral pills, injections, implants and vaginal rings containing steroid and non-steroidal compounds. Up to date and reliable information can be found on government health websites, such as the National Institutes of Health website. In brief, the NHS has established a comprehensive contraception guide that has recommended the utilization of

contraceptive injections (Medroxyprogesterone acetate, MPA: Depo-Provera[®]; Norethisterone: Noristerat[®]), combined pills (Monophasic: Microgynon[®], Brevinor[®] and Cilest[®]; Phasic: Binovum[®] and Logynon[®]; Every Day: Microgynon ED[®] and Logynon ED[®]), progesterone-only pill (Desogestrel: Cerazette[®] and Cerelle[®]; Norethisterone: Micronor[®] and Noriday[®]; levonorgestrel: Norgeston[®]), vaginal rings, intrauterine devices and systems, and contraceptive caps, implants, patches, and condoms. Reviewed by [Garside et al. \(2012\)](#).

Products used in animals

Typically, in companion animals, progestin contraceptives are used in various countries in the form of injections or oral contraceptives. Synthetic progestins and their brandnames, include megestrol acetate (MA or MGA; Megecat, Ovaban[®]), MPA (Depo-Provera[®]), and proligestone (Covinan[®]). Some of these are also used in zoo animals. Over population of some pest animals (e.g., deer) can also be treated using steroids used to regulate estrus in cattle and there are various symposia that describe these and other contraceptive developments on the United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) website.

In male companion animals, a number of products have been developed, including Zeuterin[™] or EsterilSol[™] which is an intratesticular injection (which may no longer be on the market, but still has FDA approval), gonadotrophin releasing hormone (GnRH) agonists such as Suprelorin[™] which is a slow release subcutaneous implant of a GnRH analog (deslorelin) used in male dogs to suppress libido and fertility for 6 or 12 months and the progestin contraceptives used in various countries in the form of injections or oral contraceptives, typically used in female companion animals, but with some use in male animals as well.

Upto date information for companion animals can be found in the Alliance of Contraception in Cats and Dogs website (ACC&D) or for zoo animals information is available from the Saint Louis Zoo website. In some wild animals (e.g., cheetah and leopard), deslorelin (Suprelorin GnRH agonist implants) has been used to disrupt fertility. The AfriCat website also describes other species (monkey, wild dog, baboon, mandrill, tiger) for which this product works, but larger animals such as the elephant require immunocastration.

Some companies, such as Senes Tech, a US Biotechnology company are developing products that chemically delete oocytes in female rodents, using a chemical solution of 4-vinylcyclohexene diepoxide (VCD) in bait stations (see Relevant Websites section). The chemical is rapidly metabolized by the animals, and so does not affect the food chain. The company is also developing products for use in other pest species; taking into account sensitivity to the sterilant and the method of delivery.

Natural compounds: Potential antifertility activity

There are a vast number of natural compounds identified as having antifertility activity, which have been published by [Kumar et al. \(2012\)](#). Natural products usually require chemical synthesis in order to produce sufficient quantity of the active, or use with suitable delivery systems to ensure the compounds are efficiently administered. The original birth control pill originated from the compound diosgenin, isolated from Wild Yam (*Dioscorea villosa*) – this story exemplifies the timelines and challenges associated with natural product development ([Ferro and Garside, 2011](#)).

Immunocastration

In the case of wild animals and pests, logistics of trapping and administering long-term sterilization have driven many of methods described in this section, while others have been developed for contraceptive as well as anti-cancer approaches in humans and for animal welfare reasons in farm animals. Many of these have been developed for male animals. The first vaccines were developed in the 1970s and research in the field reached a height in the 1990s. The concept was that immunocastration would involve harnessing the immune system to target key components of the reproductive system (hormones and gametes). The main focus has been on gonadotrophin releasing hormone (GnRH), follicle stimulating hormone (FSH) and luteinising hormone (LH) in both males and females, as well as human chorionic gonadotrophin (hCG) in females. However recent fertility controlling hormones have been discovered, that has expanded potential targets to include kisspeptin (KP) ([Greives et al., 2008](#)). While the discovery of gonadotrophin inhibitory hormone (GnIH) has extended research into the role of both KP and GnIH in surgical castration and immunocastration in males, and differences thereof, indicating that this field is still in its infancy and we have much to learn ([Han et al., 2017](#)). In addition, immunocastration vaccines have been developed to disrupt sperm-egg interactions, by targeting key components of the gametes, with some success in female animals. [Fig. 1](#) shows some of the targets in the female system.

Established hormonal targets: GnRH, hCG, LH and FSH

Anti-GnRH vaccines have been in development for several decades, and there are different products used in male farm, wild and pest animals ([Garside et al., 2014](#)). Due to the complexity of GnRH control over female fertility, and the discovery of several isoforms of GnRH and receptors has restricted the development of these vaccines in humans (particularly women), whereas in other mammals vaccine development has advanced to products on the market. The most advanced anti-GnRH vaccine used for controlling fertility in wild animals is GonaCon[™]. This consists of a GnRH-hemocyanin conjugate, which was developed by the National Wildlife Research Center in the US (the research arm of the USDA APHIS) to induce antibodies against GnRH and to remove circulating GnRH from the body ([Benka and Levy, 2015](#)). The US Environmental Protection Agency (EPA) has also approved its use in female white-tailed deer, with GonaCon[™]-Equine being used in female wild horses and burros. This vaccine is also thought to be suitable for female felines.

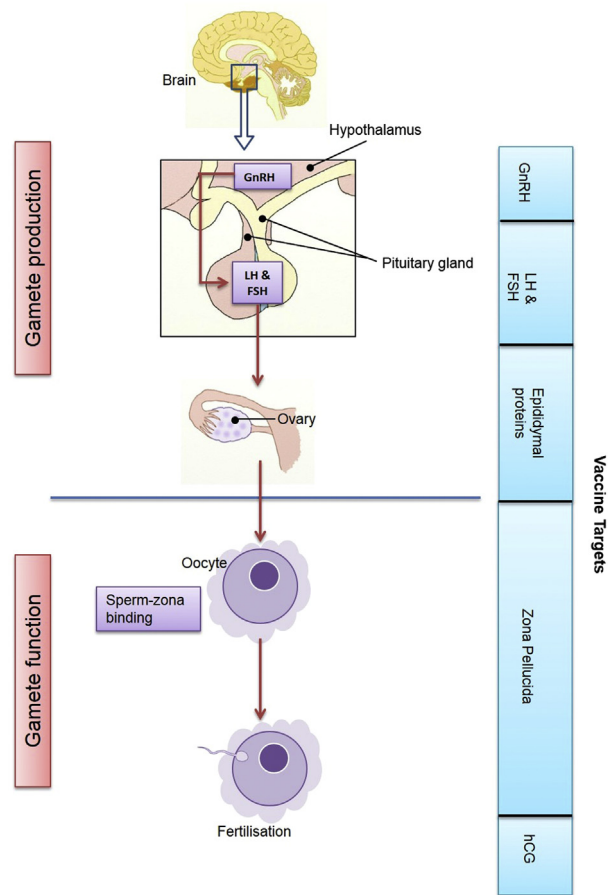


Fig. 1 Vaccine targets in the female reproductive system.

Another hormone that has had a long history in immunocontraceptive development is hCG. Vaccination against hCG targets the egg at implantation, resulting in a localized vaccine that would only take effect in the event of conception, minimizing systemic side-effects. Problems with achieving suitable levels of neutralizing antibodies, and maintaining the vaccine response for a sufficient length of time limited the use of this vaccine as a contraceptive.

Some early studies in the 1980s were carried out on immunization against LH in female sheep. Later studies were carried out on immunization against the LH receptor, in cats and dogs, whereby corpus luteal activity was reversibly suppressed. However, these targets have not progressed to the market. On the other hand FSH immunization has been examined in humans, but only as a potential male immunocontraceptive.

Recent hormonal discoveries: KP and GnIH

In terms of KP, there is some evidence that the *KISS 1 gene* is being investigated in DNA immunocastration vaccine in ram lambs (Han *et al.*, 2015), but as yet information regarding this approach in female animals has not been published. KP is involved in various physiological pathways as described below, indicating that the complexity of the role of KP (not only in fertility) warrants that an immunocastration approach should proceed with caution. Understanding the role of these hormones will help identify future applications in immunocastration.

Role of KP and GnIH in regulation of the reproductive axis

As this is a relatively new field, the role of these hormones is described in some detail here. The HPG axis, which governs reproduction, is regulated by a complex network of stimulatory and inhibitory factors (Greives *et al.*, 2008). The dynamic and cyclic nature of the female reproductive function and the potency of stimulatory signals of the HPG axis, necessitates the existence of counterbalance inhibitory mechanisms, in order to keep stimulation at check and allow adaptation of reproductive maturation and function to different endogenous and environmental conditions. One of the main stimulatory factors in the initiation of the onset of puberty and control of adult fertility is KP (Yang and Dhillon, 2016). This peptide is encoded by the *KISS1 gene* and stimulates GnRH secretion from the hypothalamus via the KISS1 receptor (KISS1R, previously known as GPR54). In 2003 two independent research groups showed that loss of KP signaling causes hypothalamic hypogonadism (HH) in humans and mice (see review Lippincott *et al.*, 2013).

In 2000 a novel peptide of the RF-amide superfamily, named GnIH was identified in birds. Later, putative orthologs of GnIH named RFamide-related peptides (RFRPs) were identified in mammals including humans, with the capacity of inhibiting gonadotropin release. Of interest, it was shown that GnIH may not directly inhibit KP signaling in GnRH neurons (Leon and Tena-Sempere, 2016).

KP as a gatekeeper of the fertility

Numerous examples of genetic dysfunctions of KP signaling implicate the crucial role of this system in regulation of fertility (Sills and Walsh, 2008). In humans, inactivating GPR54 mutations cause normosmic HH, while activation of GPR54 signaling is associated with premature puberty. Moreover, patients with mutations that inactivate KP signaling are infertile. Similarly, mice with mutations in KP and the KP receptor, *Kiss1*(−/−) and *Kiss1r*(−/−), respectively have abnormal sexual maturation and infertility. Additionally males show defects in spermatogenesis with impaired production of spermatozoa, with these being more pronounced in *Kiss1r*(−/−) than in *Kiss1*(−/−) mice (Mei *et al.*, 2011). In a mouse model with deletion of the *Kiss1r* gene only in the GnRH neuron, it was found that male knockout animals had a microphallus and decreased anogenital distance compared with wild-type controls. Since both processes are androgen specific, it was suggested that disruption to the kisspeptin receptors on the GnRH neuron is specifically capable of altering the production of sex steroids. Moreover, delay in pubertal onset was observed both in male and female knockout mice, and circulating gonadotrophins were lower than in wild-type mice. Additionally, fertility was affected with no pregnancies observed when knockout males were mated with female mice previously shown capable of becoming pregnant. The above findings indicate a role for KP at the level of the GnRH neuron on pubertal onset and reproductive health (Novaira *et al.*, 2014). Furthermore, in genetically modified mice, peripheral KP signaling is indispensable for endometrial adenogenesis and function, essential aspects of reproductive competence.

KP does not act alone: Role of KNDy neurons

However, KP does not act alone and a mechanism underlying pulsatile GnRH release in rodents is believed to be the synchronized activity of a subset of arcuate nucleus (ARC) neurons in the part of the brain called the hypothalamus, termed KNDy neurons, expressing neuropeptides: KP, neurokinin B (NKB) and dynorphin (Dyn). It was proposed that pulse generation is initiated in the KNDy neuronal network by a reciprocating interplay of stimulatory NKB signals and inhibitory Dyn inputs. In rodents another population of KP neurons, in the anterior periventricular (AVPV) was found. While, the ARC neurons seem to be important for the initiation of the puberty process, normal pulsatile LH release and estrous cyclicity; the KP neurons in the AVPV control the LH surge in females. In males, KP neurons play a role in activation of neurons during the perinatal period, and that the subsequent GnRH surge generates testosterone necessary for ensuring sexual differentiation of the brain (Lehman *et al.*, 2010).

In humans, KP expression has also been found within the hypothalamus in the AVPV and the infundibular region (analog of the rodent's ARC). However, unlike in rodents and sheep, the homologous KP and NKB neurons rarely express Dyn – but often exhibit Substance P (SP) immunoreactivity, indicating remarkable species differences in the neurochemical phenotype of these neurons.

KP is a potent activator of the reproductive axis

Proper KP/GPR54 signaling is viewed as the principal trigger for activation of GnRH neurons and subsequent ovulation. Thus, elucidation of how this pathway is modulated is likely to bring novel pharmacologic strategies for fertility treatment and contraception. In animal models KP affect gonadotrophin release in females as well as males. KP induces the LH surge necessary for ovulation and oocyte maturation and in transgenic mice null for KP or its receptor, lack the LH surge and subsequent failure of ovulation is observed. KP administration has been found to induce ovulation in several mammals, including sheep, musk shrews and rats (Clarkson *et al.*, 2008; Caraty *et al.*, 2007; Matsui *et al.*, 2004).

In healthy human volunteers, the acute intravenous (icv) administration of KP increases plasma LH, FSH and testosterone (T) levels without side effects in both males and in females, particularly in the preovulatory phase of the menstrual cycle. Acute KP stimulates gonadotropin secretion in a wide range of species of non-human mammals, including rats, mice, hamsters, sheep, pigs, goats, cows, horses, and monkeys. Importantly, administration of KP by either the subcutaneous (sc) or icv route stimulates gonadotrophin release through influencing GnRH secretion responses in human subjects with reproduction disorders. Different lengths of KP are found, of 54, 14, 13 or 10 amino acids (aa), which share a common C-terminal 10 aa sequence. While both KP-54 and KP-10 have been widely used to stimulate the reproductive axis, it was found that KP-54 was able to cross the blood-brain barrier (BBB) and had a longer half-life in a bloodstream (d'Anglemont de Tassigny *et al.*, 2017).

Role of KP in placental invasion, fertilization, and reproductive disorders

Besides the above described functions of KP, it is also abundantly expressed in the placenta, where it plays role in placental invasion and its level in plasma rises dramatically during normal pregnancy. The urinary measurement of KP may offer a non-invasive and simple method of screening for pregnancy and obstetric complications. Increased plasma KP is associated with reduced miscarriage risk, even after adjusting for age, body mass index, gestational age, smoking, and blood pressure.

KP-54 is also a promising approach to effectively and safely trigger oocyte maturation in women undergoing in vitro fertilization (IVF) as a fertility treatment in women with subfertility (d'Anglemont de Tassigny *et al.*, 2017). KP is a potent stimulus of the reproductive axis in patients with idiopathic HH (IHH), which results from defective synthesis, secretion, or action of GnRH, as well as in women with the hypothalamic amenorrhea (HA), which is the one of the most common causes of period loss in women of reproductive age.

Moreover, alterations of the *Kiss1* gene were found in patients with polycystic ovary syndrome (PCOS), which is a highly prevalent heterogeneous disease characterized by ovulatory dysfunction, hyperandrogenism, and metabolic alterations, and the leading cause of anovulatory infertility. Women with PCOS have dysregulated gonadotropin secretion with higher LH pulsatility and perturbed LH-FSH ratios, which likely contributes to the ovarian phenotype and might be indicative of disrupted GnRH secretory activity. Animal models of PCOS mimic some of the above dysfunctions of the reproductive system. It has also been found that in a rat model of PCOS, prenatal exposure to androgens may result in higher KP and NKB levels in the ARC in rodents (Osuka *et al.*, 2017; Paixão *et al.*, 2017), and hypertrophy of KNDy cells in the ARC of sheep, resembles that seen in KP cells of postmenopausal women (Cernea *et al.*, 2015).

Beyond the well-recognized functions at hypothalamic levels, data strongly support direct production and activity of KP in the testis and its involvement in the control of Leydig cells, germ cell progression and sperm functions (reviewed by Chianese *et al.* (2016)). In mice, *Kiss1* and *Kiss1r* genes have been detected in Leydig cells, seminiferous tubules, and spermatids, but KP has not been detected in spermatids, while KISS1R has been found expressed in the acrosomal region of spermatids and mature spermatozoa. It has been suggested that KP might regulate sperm function as similarly to human sperm, KP triggers an increase in intracellular Ca^{2+} in mouse sperm. Moreover, in vitro fertilization rates decreased after sperm were treated with the KISS1R antagonist peptide 234 (Mei *et al.*, 2013; Hsu *et al.*, 2014).

It has also been shown that transgenic male mice with only a 5% level of *Kiss1* transcripts are fertile and give rise to normal-sized litters, however they have smaller testes and lower FSH levels than normal mice (Popa *et al.*, 2013). Based on above findings it has been suggested that KP could regulate mammalian fertilization (see review Bhattacharya and Babwah, 2015).

KP and GnIR/RFRP as potential links between reproductive and metabolic homeostasis

Reproductive function is tightly regulated by nutritional status and undernutrition or obesity can lead to subfertility or infertility (for reviews see: Seminara, 2014; Gawałek and Sliwowska, 2015). Female mice lacking the KP receptor gained more weight compared to control animals, and this weight gain was caused not by increased food consumption, but by an overall decrease in energy and metabolism. Additionally, *Kiss1r* KO females had higher leptin levels, adiposity and impaired glucose tolerance. In contrast, male *Kiss1r* KO mice had normal body weight and glucose regulation (Tolson *et al.*, 2014). There is also a link between GnIR/RFRP and metabolism regulation – icv administration of RFRP-3 stimulated food intake in adult male rats. Evidence has recently been presented supporting a role for KP and GnIH/RFRP as potential links between reproductive and metabolic homeostasis.

Possible clinical applications of KP, NKB, Dyn and GnIH/RFRP in treatment of fertility disorders

Future research is likely to involve investigation of novel KP and GnIH/RFRP analogs and further exploration of the role of NKB, Dyn and other tachykinins in the regulation of the HPG axis (see reviews Bhattacharya and Babwah, 2015; Chianese, *et al.*, 2016; d'Anglemont de Tassigny *et al.*, 2017; Yang and Dhillon, 2016). KP stimulates gonadotrophin secretion less potently when compared with GnRH; however, KP may stimulate gonadotrophins in a more physiological manner when compared with current therapies. Thus, both KP and GnIH/RFRP based strategies may represent potential therapeutic targets in the treatment of fertility disorders and contraception in human and domestic and wild animals.

Sperm-egg interactions

Disappointing results in human trials against hormone targets led to examination of gamete targets. Sperm proteins are attractive vaccine targets as they are highly immunogenic and are often sperm-specific and hence do not affect other biological processes. Proteins have been chosen based on their sperm-specificity, surface expression, involvement in fertility and ability to raise long-lasting antibodies capable of inhibiting fertility.

Sperm proteins

To date, numerous sperm proteins have been investigated for their immunocontraceptive potential driven in part by the knowledge that males and females with autoantibodies against some of these result in infertility (Adoyo and Koyama, 2003; Naz, 2011). Contraceptive vaccines can trigger the immune system to block indispensable step in the process of gamete interaction during fertilization. These include sperm capacitation, sperm-cumulus interaction, sperm-zona binding, the acrosome reaction, zona penetration and gamete fusion. The challenge is to use sperm antigens that are not found in somatic cells. The ideal candidate should be sperm-specific, be accessible on the surface of the spermatozoa, and have a key role in fertilization. Therefore, targets involved in ZP interaction have received most interest. Individually, the proteins have not been 100% effective, and it is recognized that more than one protein is important, therefore multi-antigen vaccines are now being developed. Some targets are detailed below and their general location on spermatozoa shown in Fig. 2. This is not a comprehensive list as many targets have been investigated, but not pursued (reviewed in Garside *et al.*, 2015).

Izumo

Izumo is found on the surface of mouse sperm, and is essential for egg-sperm fusion. Immune-infertile female sera have circulating isoantibodies against Izumo, which suggests that this protein could have a role in contraceptive vaccine development. Female mice immunized against B-cell epitopes have shown infertility.

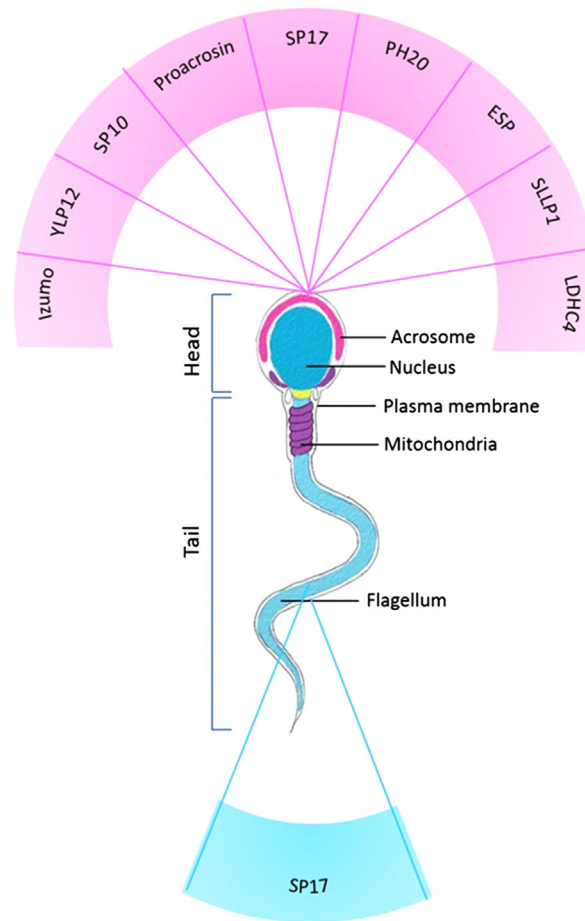


Fig. 2 Sperm antigen targets and localization on the sperm.

YLP12

YLP12 is a target arising from research into human sperm-ZP interactions, a dodecamer sequence. The peptide sequence is localized on the acrosomal region of human sperm and is involved in recognition and binding to the ZP. Immunization of female mice has shown reversible infertility.

SP10

One of the early sperm specific proteins investigated as a possible contraceptive target was SP10, a human intra-acrosomal protein. Functional studies have shown that SP10 is involved in sperm-oolemma binding, but not sperm-ZP binding during fertilization in the human.

Posterior head 20(PH20)

PH20 is a sperm surface hyaluronidase, found in a wide number of species. In human spermatozoa it is located on the outer surface of the membrane overlying the acrosome, where it enables sperm passage through the cumulus mass to the ZP.

Sperm protein 17(Sp17)

Spermatozoa express Sp17 to mediate the interaction with oocytes after sperm-ZP contact is initiated (Adoyo *et al.*, 1997, McLeskey *et al.*, 1998). This protein was identified on the apical surface of the head of spermatozoa and along the flagellar tails of mature spermatozoa. This makes an interesting target as it is found across the whole spermatozoa. Structurally, the 151 aa of Sp17 construct a three-domain structure composed of the N-terminal, Central, and C-terminal domains (Fig. 3). The highly conserved N-terminal domain matches 45% to the human type II α regulatory subunit (RII α) of protein kinase A (PKA) that is encoded on Exon 2. This domain can bind specifically to a group of A-kinase anchor proteins (AKAP), including AKAP-3 in sperm flagella. The middle heparin-binding domain of Sp17, which is encoded by aa 76–104 (exon 4), represents the less conserved heparin binding domain. The C-terminal Ca²⁺/calmodulin (CaM) binding domain extends over aa 105–151 (exon 5). CaM transmits the Ca²⁺s messenger response in numerous signal transduction pathways (Gaines, 2006). Sp17 has been sequenced and cloned from human, baboon, mice, and rat sperm; and full length and partial Sp17 sequences of different other species have been deposited into the National Center for Biotechnology Information (NCBI) GenBank records.

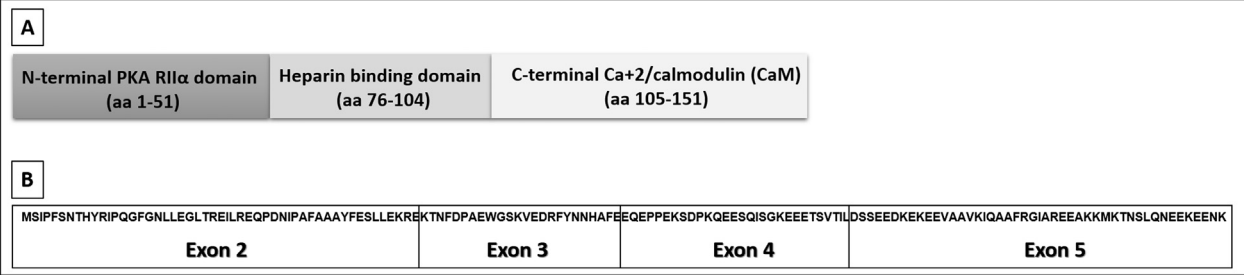


Fig. 3 (A) Structural and (B) amino acid sequence alignment of human SP17.

Both human and mouse recombinant Sp17 have been synthesised, and immunization of mice with the sequences has shown significant decrease in fertility. Mouse Sp17 is not available to bind antibody on the surface of live, acrosome-intact spermatozoa, but it is presented on the equatorial surface of live, acrosome-reacted spermatozoa (**Fig. 4(B)**). Likewise, both human Sp17 and synthetic peptides derivatives have resulted in the production of both oviductal and serum antibody titers following immunization of female cynomolgous macaques. **Fig. 4(C)** shows how sperm-egg interactions may be disrupted by antibodies raised against a particular target (such as Sp17).

Equatorial segment protein (ESP)

Sperm-specific human ESP (hESP) protein is localized on the equatorial segment of the acrosome. It has been shown to be involved in acrosome biogenesis and is able to cross react with sera from patients (both male and female). Immunization of female mice with N-terminal mouse ESP significantly decreases fertility and litter size.

SLLP1 (also known as SPRASA)

The presence of this non-bacteriolytic, lysozyme-like protein, was discovered in 2003 in the acrosome of human sperm. This was also found in mouse spermatozoa following the acrosome reaction and has a role in sperm-egg binding and fertilization. Further research in the female, confirmed an oocyte specific membrane metalloproteinase, Sperm Acrosomal SLLP1 Binding (SAS1B), that was found to bind SLLP1. SAS1B knockout female mice showed a 34% reduction in fertility, indicating that SAS1B-SLLP1 had been

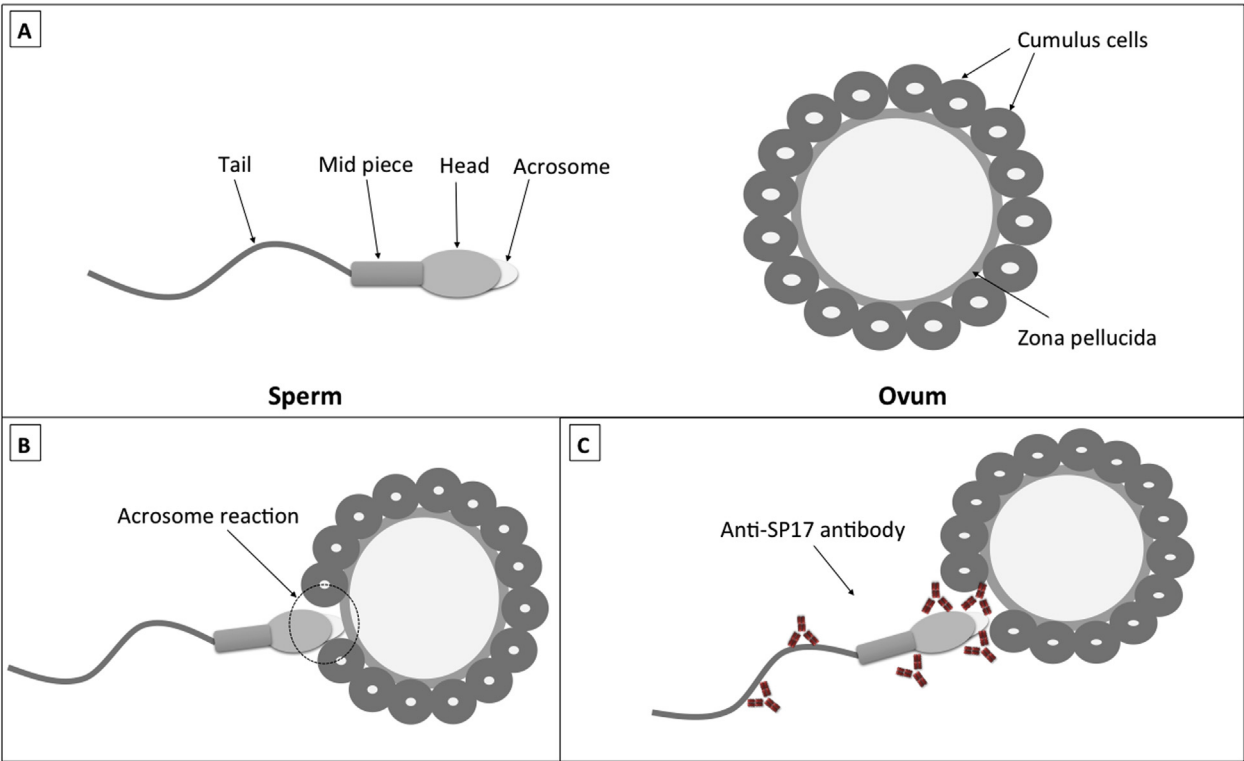


Fig. 4 Structural component of the mammalian gametes. (A) Gamete components. (B) Acrosome reaction between the two gametes. (C) Fertilization inhibition by anti-SP17 antibody.

identified as a pair of novel sperm-egg binding partners, involving the oolemma and intra-acrosomal compartment during fertilization. SPRASA is found in both sperm and oocytes, so could be used effectively as a male and female vaccine. It is highly conserved in different animal species, including farm animals. Female mice immunised with SPRASA have shown significant decrease in fertility (Wagner *et al.*, 2015).

LDH-C4

This is one of the most studied sperm proteins. In male animals orchitis has been observed, but in female animals there are no side-effects. The protein has been tested in mice, rabbits, baboons, but full infertility has not been achieved.

Zona pellucida

The ZP plays an essential role in the fusion of the oocyte and spermatozoon during mammalian fertilization. The ZP is also involved in the induction of the acrosome reaction, the prevention of polyspermy, and protection of the early embryo. Immunocastration with porcine ZP has been commonly used in pest and zoo species. However, long-term ovarian dysfunction remains an issue (Joonè *et al.*, 2017). Taken together with a lack of understanding for why some species are susceptible to ovarian dysfunction while others are not, this vaccine is not used in humans. However, it may have a continuing role in effective immunocastration in some situations.

References

- Adoyo, P. A., & Koyama, K. (2003). Prospects for sperm immunocontraceptive vaccines. *Reproductive Immunology and Biology*, 18, 7–18.
- Adoyo, P. A., Lea, I. A., Richardson, R. T., Widgren, E. E., & O'Rand, M. G. (1997). Sequence and characterization of the sperm protein Sp17 from the baboon. *Molecular Reproduction and Development*, 47, 66–71.
- Benka, V. A., & Levy, J. K. (2015). Vaccines for feline contraception: Gonacon GnRH-hemocyanin conjugate immunocontraceptive. *Journal of Feline Medicine and Surgery*, 17, 758–765.
- Bhattacharya, M., & Babwah, A. V. (2015). Kisspeptin: Beyond the brain. *Endocrinology*, 156, 1218–1227.
- Caraty, A., Smith, T., Lomet, D., et al. (2007). Kisspeptin synchronizes preovulatory surges in cyclical ewes and causes ovulation in seasonally acyclic ewes. *Endocrinology*, 148, 5258–5267.
- Cerneia, M., Padmanabhan, V., Goodman, R. L., Coolen, L. M., & Lehman, M. N. (2015). Prenatal testosterone treatment leads to changes in the morphology of KNDy neurons, their inputs, and projections to GnRH cells in female sheep. *Endocrinology*, 156, 3277–3291.
- Chianese, R., Cobellis, G., Chioccarelli, T., et al. (2016). Kisspeptins, estrogens and male fertility. *Current Medicinal Chemistry*, 23, 4070–4091.
- Clarkson, J., d'Anglemont de Tassigny, X., Moreno, A., Colledge, W., & Herbison, A. (2008). Kisspeptin-GPR54 signaling is essential for preovulatory gonadotrophin-releasing neuron activation and the luteinising hormone surge. *Journal of Neuroscience*, 29, 3920–3929.
- d'Anglemont de Tassigny, X., Jayasena, C., et al. (2017dAnglemont). Mechanistic insights into the more potent effect of KP-54 compared to KP-10 in vivo. *PLOS ONE*, 12(5).
- DeTora, M., & McCarthy, R. J. (2011). Ovariohysterectomy versus ovariectomy for elective sterilization of female dogs and cats: Is removal of the uterus necessary? *Journal of the American Veterinary Medical Association*, 239, 1409–1412.
- Ferro, V. A., & Garside, D. A. (2011). Reproductive component vaccine developments for contraceptive and non-contraceptive uses. *Expert Opinion on Therapeutic Patents*, 21, 1473–1482 [Epub 2011 Jun 15].
- Gaines, J.P., 2006. The role of sperm protein 17 (Sp17) in somatic cells. PhD Thesis, University of Alabama, Birmingham.
- Garside, D.A., Acevedo, R., Alsaadi, M., et al., 2015. Vaccines against non-infectious, non-cancer novel targets. In: Singh, M., Salnikova, M. (Eds.), *Novel Approaches and Strategies for Biologics, Vaccines and Cancer Therapies*, first ed. pp. 221–250.
- Garside, D., Gebriel, A., Alsaadi, M., & Ferro, V. A. (2014). Fertility control in wildlife: Review of current status, including novel and future technologies. *Advances in Experimental Medicine and Biology*, 53, 467–488.
- Garside, D., Gebriel, A., Nimmo, N., et al. (2012). An update on developments in female hormonal contraception. *Current Women's Health Reviews*, 8, 276–288.
- Gawalek, M., & Sliwowska, J. H. (2015). Neuronal basis of reproductive dysfunctions associated with diet and alcohol: From the womb to adulthood. *Reproductive Biology*, 15, 69–78.
- Greives, T. J., Kriegsfeld, L. J., Bentley, G. E., Tsutsui, K., & Demas, G. E. (2008). Recent advances in reproductive neuroendocrinology: A role for RFamide peptides in seasonal reproduction? *Proceedings of the Royal Society of London B*. <https://doi.org/10.1098/rspb.2008.0433>.
- Han, Y., Liu, G., Jiang, X., et al. (2015). KISS1 can be used as a novel target for developing a DNA immunocastration vaccine in ram lambs. *Vaccine*, 33, 777–782.
- Han, X., Zhou, Y., Zeng, Y., et al. (2017). Effects of active immunization against GnRH versus surgical castration on hypothalamic-pituitary function in boars. *Theriogenology*, 97, 89–97.
- Hsu, M. C., Wang, J. Y., Lee, Y. J., et al. (2014). Kisspeptin modulates fertilization capacity of mouse spermatozoa. *Reproduction*, 147, 835–845.
- Joonè, C. J., Schulman, M. L., & Bertschinger, H. J. (2017). Ovarian dysfunction associated with zona pellucida-based immunocontraceptive vaccines. *Theriogenology*, 89, 329–337.
- Koutsouris, E. A., Alamanis, C., Eftychiadis, A., & Zervas, A. (2014). Castrati singers: Surgery for religion and art. *Italian Journal of Anatomy and Embryology*, 119, 106–110.
- Kumar, D., Kumar, A., & Prakash, O. (2012). Potential antifertility agents from plants: A comprehensive review. *Journal of Ethnopharmacology*, 140, 1–32.
- Lehman, M. N., Coolen, L. M., & Goodman, R. L. (2010). Minireview: Kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: A central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology*, 151, 3479–3489.
- Leon, S., & Tena-Sempere, M. (2016). Dissecting the roles of gonadotropin-inhibitory hormone in mammals: Studies using pharmacological tools and genetically modified mouse models. *Frontiers in Endocrinology (Lausanne)*, 6, 189.
- Lippincott, M. F., True, C., & Seminara, S. B. (2013). Use of genetic models of idiopathic hypogonadotropic hypogonadism in mice and men to understand the mechanisms of disease. *Experimental Physiology*, 98, 1522–1527.
- Matsui, H., Takatsu, Y., Kumano, S., Matsumoto, H., & Ohtaki, T. (2004). Peripheral administration of metastatin induces marked gonadotropin release and ovulation in the rat. *Biochemical and Biophysical Research Communications*, 320, 383–388.
- McLeskey, S. B., Dowds, C., Carballada, R., White, R. R., & Saling, P. M. (1998). Molecules involved in mammalian sperm-egg interaction. *International Review of Cytology*, 177, 57–113.
- Mei, H., Doran, J., Kyle, V., Yeo, S. H., & Colledge, W. H. (2013). Does kisspeptin signaling have a role in the testes? *Front Endocrinol (Lausanne)*, 4, 198.
- Mei, H., Walters, C., Carter, R., & Colledge, W. H. (2011). Gpr54K/K mice show more pronounced defects in spermatogenesis than Kiss1K/K mice and improved spermatogenesis with age when exposed to dietary phytoestrogens. *Reproduction*, 141, 357–366.

- Naz, R. K. (2011). Antisperm contraceptive vaccines: Where we are and where we are going? *American Journal of Reproductive Immunology*, 66, 5–12.
- Novaira, H., Sonko, M., Hoffman, G., et al. (2014). Disrupted kisspeptin signalling in GnRH neurons leads to hypogonadotropic hypogonadism. *Molecular Endocrinology*, 28, 225–238.
- Osuka, S., Iwase, A., Nakahara, T., et al. (2017). Kisspeptin in the hypothalamus of 2 rat models of polycystic ovary syndrome. *Endocrinology*, 158(2), 367–377.
- Paixão, L., Ramos, R. B., Lavarda, A., Morsh, D. M., & Spritzer, P. M. (2017). Animal models of hyperandrogenism and ovarian morphology changes as features of polycystic ovary syndrome: A systematic review. *Reproductive Biology and Endocrinology*, 15(1), 12.
- Patel, A. P., & Smith, R. P. (2016). Vasectomy reversal: A clinical update. *Asian Journal of Andrology*, 18, 365–371.
- Popa, S. M., Moriyama, R. M., Caligioni, C. S., et al. (2013). Redundancy in *Kiss1* expression safeguards reproduction in the mouse. *Endocrinology*, 154, 2784–2794.
- Seminara, S. B. (2014). Fatness and fertility: Which direction? *Journal of Clinical Investigation*, 124, 2853–2854.
- Sills, E. S., & Walsh, A. P. (2008). The GPR54-kisspeptin complex in reproductive biology: Neuroendocrine significance and implications for ovulation induction and contraception. *Neuroendocrinology Letters*, 29, 846–851.
- Tolson, K. P., Garcia, C., Yen, S., et al. (2014). Impaired kisspeptin signaling decreases metabolism and promotes glucose intolerance and obesity. *Journal of Clinical Investigation*, 124(7), 3075–3079.
- van Seeters, J. A. H., Chua, S. J., Mol, B. W. J., & Koks, C. A. M. (2017). Tubal anastomosis after previous sterilization: A systematic review. *Human Reproduction Update*, 23, 358–370.
- Wagner, A., Holland, O. J., Tong, M., Shelling, A. N., & Chamley, L. W. (2015). The role of SPRASA in female fertility. *Reproductive Sciences*, 22, 452–461.
- Yang, L., & Dhillon, W. (2016). Kisspeptin as a therapeutic target in reproduction. *Expert Opinion on Therapeutic Targets*, 20, 567–575.

Further Reading

- Gordon, I. R. (Ed.). (2004). *Reproductive Technologies in Farm Animals* (second ed.). CABI.
- Habenicht, U.-F., & Aitken, R. J. (Eds.). (2010). *Fertility Control*. Springer.
- Krause, W. K. H., & Naz, R. K. (Eds.). (2017). *Immune Infertility: Impact of Immune Reactions on Human Fertility* (second ed.). Springer.
- Liechty, E. R., Bergin, I. L., & Bell, J. D. (2015). Animal models of contraception: Utility and limitations. *Open Access Journal of Contraception*, 6. <https://doi.org/10.2147/OAJC.S58754>.
- Massei, G., & Miller, L. A. (2013). Nonsurgical fertility control for managing free-roaming dog populations: A review of products and criteria for field applications. *Theriogenology*, 80, 829–838.
- Miller, L. A., Fagerstone, K. A., & Eckery, D. C. (2013). Twenty years of immunocontraceptive research: Lessons learned. *Journal of Zoo and Wildlife Medicine*, 44, S84–S96.

Relevant Websites

- <http://acc-d.org/available-products>. – ACC&D Website.
- <http://www.africat.org/contraception-in-wildlife>. – AfriCat Website.
- https://www.aphis.usda.gov/wildlife_damage/nwrc/symposia/contraception_symposium/kesler.pdf. – APHIS USDA Downloads.
- https://www.aphis.usda.gov/wildlife_damage/nwrc/symposia/invasive_symposium/content/Mauldin434_444_MVIS.pdf. – APHIS USDA Downloads.
- <http://www.nadis.org.uk/bulletins/castration-of-calves.aspx>. – National Animal Disease Information Service.
- <http://www.ncbi.nlm.nih.gov>. – National Center for Biotechnology Information (NCBI) GenBank records.
- <https://www.nichd.nih.gov/health/topics/contraception/conditioninfo/Pages/types.aspx#hormonal>. – National Institutes of Health.
- <https://www.stlzoo.org/animals/scienceresearch/reproductivemanagementcenter/>. – Saint Louis Zoo Website.
- <http://senestech.com/>. – Senestech.